PUBLIC VERSION

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UNITED STATES INTERNATIONAL TRADE COMMISSION
WASHINGTON, D.C.

Before the Honorable Clark S. Cheney
Chief Administrative Law Judge

In the Matter of **CERTAIN DRUG PRODUCTS CONTAINING C-TYPE NATRIURETIC PEPTIDE VARIANTS, AND COMPONENTS THEREOF**	**Inv. No. 337-TA-1447**

RESPONDENTS' OPENING CLAIM CONSTRUCTION BRIEF

BIOMARIN EXHIBIT 2007
Ascendis Pharma A/S v. BioMarin Pharmaceutical Inc.
PGR2026-00013

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Ex. No	Description
1	U.S. Patent No. RE48,267 (the "RE'267 patent")
2	Curriculum Vitae
3	Carey and Sundberg, *Advanced Organic Chemistry*, 3rd Edition, Vol. A (Plenum Press, New York 1992) (Carey Ch. 4)
4	Carey and Sundberg, *Advanced Organic Chemistry*, 3rd Edition, Vol. B (Plenum Press, New York 1992) (Carey Ch. 8)
5	Francesco M. Veronese & Anna Mero, *The Impact of PEGylation on Biological Therapies*, 22 Biodrugs 320, 323, 326 (2008) (Veronese)
6	*The Facts On File Dictionary Of Organic Chemistry*, Ed. Daintith (2004) 6 (Facts on File Dictionary)
7	*Glossary of Terms Used in Physical Organic Chemistry* (IUPAC Recommendations 1994) International Union of Pure and Applied Chemistry, Organic Chemistry Division, Commission on Physical Organic Chemistry, Hydrolysis: "Solvolysis by water" (1123); Solvolysis (IUPAC Glossary)
8	*Hawley's Condensed Chemical Dictionary*, 15th Edition, Ed. Lewis (2007) (Hawley's)
9	Wenmao Huang *et al*., *Maleimide-Thiol Adducts Stabilized Through Stretching*, 11 Nat. Chem. 311, Figure 1a (2019) (Huang)
10	Zhi Wang *et al*., *Hot Water-Promoted Ring-Opening of Epoxides and Aziridines by Water and Other Nucleopliles*, 73 J. Org. Chem. 2270 (2008) (Wang)
11	Hayato Yuzawa *et al*., *Anti-Markovnikov Hydration of Alkenes Over Platinum-Loaded Titanium Oxide Photocatalyst*, 3 Catal. Sci. Technol.1743 (2013) (Yuzawa)
12	NOF Corporation Catalogue Ver. 8, (NOF Catalogue)
13	Meyer, M., et al., "56: Natriuretic Peptides," *Acute Heart Failure*, Springer London (2008) (Meyer)
14	Waschek, J. A., "*Developmental actions of natriuretic peptides in the brain and skeleton*," Cellular and Molecular Life Sciences, 61 (2004) (Waschek)
15	Itoh, H., et al., "*Chapter 7: Cellular and Molecular Aspects of C-Type Natriuretic Peptide (CNP)*," Natriuretic Peptides In Health and Disease, Humana Press (1997) (Itoh)
16	Furuya, M., et al., "*Structural Requirements of C-Type Natriuretic Peptide for Elevation of Cyclic GMP In Cultured Vascular Smooth Muscle Cells*," Biochemical and Biophysical Research Communications, 183(3) (1992) (Furuya 1992)
17	Potter, L., et al., "*Natriuretic Peptides, Their Receptors, and Cyclic Guanosine Monophosphate-Dependent Signaling Functions*," Endocrine Reviews, 27(1) (2006) (Potter 2006)
18	July 10, 2019 Remarks (Excerpt of File History of U.S. Patent No. RE48,267)

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Ex. No	Description
19	July 3, 2019, Applicant-Initiated Interview Summary (Excerpt of File History of U.S. Patent No. RE48,267)
20	November 26, 2019, Nonfinal Rejection (Excerpt of File History of U.S. Patent No. RE48,267)
21	Yasoda, A., et al., "*Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway*," Nature Medicine, Fig. 6 (2004) (Yasoda)
22	July 11, 2017, Preliminary Amendment (Excerpt of File History of U.S. Patent No. RE48,267)
23	May 10, 2019, Office Action (Excerpt of File History of U.S. Patent No. RE48,267)
24	UPC Notice with BM Infringement Action
25	U.S. Patent No. 8,598,121 ('121 patent)
26	Cambridge Dictionary of American English, 179 (2d ed. 2008)
27	Concise Oxford English Dictionary, 307 (11th ed. 2008)

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I. INTRODUCTION

U.S. reissue patent 48,267 (the "RE'267 patent") claims macromolecules, formulations, and methods of treatment involving a peptide called CNP (C-type natriuretic peptide). CNP's therapeutic potential for treating achondroplasia, a form of dwarfism, has long been known. The problem with administering CNP to treat achondroplasia is that CNP has a short *in vivo* half-life (the length of time CNP remains active in the body)—too short to be useful as a treatment. Three mechanisms were known to contribute to CNP's short half-life: degradation by an enzyme called NEP (neprilysin, also known as neutral endopeptidase), clearance by a receptor called NPR-C (natriuretic peptide receptor C), and elimination by the kidneys.

Years ago, Complainant BioMarin contended that it had found a solution to the problem. It developed a number of so-called "CNP variants" that were more resistant to NEP degradation and that retained at least some activity when tested *in vitro*. BioMarin filed a patent application claiming many such CNP variants, which ultimately issued as U.S. Patent Nos. 8,198,242 and 8,598,121. BioMarin formulated one of these CNP variants into a daily injectable drug that treats achondroplasia, which BioMarin sells under the brand name Voxzogo®. Although Voxzogo has resistance to NEP, it is still subject to clearance by NPR-C and elimination by the kidneys. That is why it must be injected every day. Despite its shortcomings, Voxzogo is currently the only FDA-approved therapy for achondroplasia. BioMarin has enjoyed this monopoly for nearly a decade.

Meanwhile, Respondent Ascendis has dedicated enormous resources to developing a different kind of treatment for achondroplasia. The accused product in this case, TransCon CNP, is the result of nearly 20 years of independent research and innovation. The inventors at Ascendis started with a different CNP sequence than any of the ones claimed in BioMarin's '242 or '121 patents. Ascendis also developed a sophisticated proprietary linker (not described in BioMarin's

[redacted]

patents) and used it to attach this CNP peptide to a carrier (also not described in BioMarin's patents) containing four polyethylene glycol ("PEG") polymers to form an entirely new product—a CNP prodrug.

Ascendis's prodrug is not an active drug. It is an *inactive* substance that produces functional molecules only after being broken down in the body. In TransCon CNP, the carrier not only shields the CNP peptide from degradation and clearance but also prevents the peptide from acting on the body. After administration, the prodrug undergoes a chemical transformation that gradually releases active CNP over time. As a result, unlike the daily injections required with Voxzogo, TransCon CNP can be administered once weekly, subjecting children to significantly fewer needle sticks. Ascendis's prodrug approach has other functional benefits as well. For example, the side effects associated with administering Voxzogo's active CNP variant include low blood pressure and injection-site swelling, which the inactive TransCon CNP prodrug does not cause.

Upon learning of Ascendis's prospective product, BioMarin returned to the Patent Office nearly 10 years after its patents issued and manipulated the reissue process to radically rewrite its claims in an attempt to capture TransCon CNP. In doing so, BioMarin repurposed words that the specification used to describe unrelated features and introduced strategic ambiguities into the claims. Under any reasonable construction, however, the claims either fail to capture Ascendis's product or they lack written description, enablement, and patentability over the prior art.

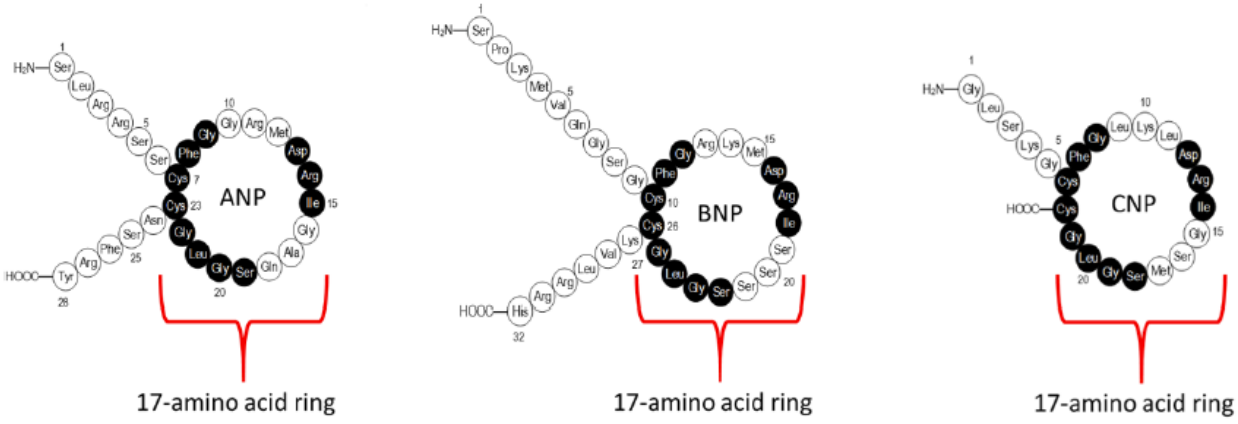
II. OVERVIEW OF THE TECHNOLOGY

Natriuretic peptides (NPs) are a family of cyclic peptide hormones. NPs play important roles in maintaining heart and kidney function by regulating fluid volume, blood pressure, and sodium concentration, promoting growth, and regulating neural development. *See* Meyer, M., *et al.*, "56: Natriuretic Peptides," *Acute Heart Failure*, Springer London, 608 (2008), Exhibit 13

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(Meyer). The NP family comprises three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). *See* Waschek, J. A., "Developmental actions of natriuretic peptides in the brain and skeleton," *Cellular and Molecular Life Sciences*, 61:2332 (2004), Exhibit 14 (Waschek). NPs contain a ring of 17 amino acids. CNP has one tail extending from the ring. ANP and BNP have two tails extending from the ring. *See* Itoh, H., *et al*., "*Chapter 7: Cellular and Molecular Aspects of C-Type Natriuretic Peptide (CNP)*," Natriuretic Peptides In Health and Disease, Humana Press, Fig. 1 (1997), Exhibit 15 (Itoh).

As shown in the figure below, the NPs display extensive identity among the amino acids inside the ring (see darkened circles). *See id*. at 108, Fig. 1.



CNP works in the body by stimulating the production of a messenger molecule called cyclic guanosine monophosphate (cGMP). The ring portion of the CNP must be intact and unobstructed to stimulate cGMP. *See* Furuya, M., et al., "*Structural Requirements of C-Type Natriuretic Peptide for Elevation of Cyclic GMP In Cultured Vascular Smooth Muscle Cells*," Biochemical and Biophysical Research Communications, 183(3) (1992), Exhibit 16 (Furuya 1992). CNP promotes bone growth by stimulating the production of cGMP in specialized cells called chondrocytes. *See* Potter, L., et al., "Natriuretic Peptides, Their Receptors, and Cyclic Guanosine

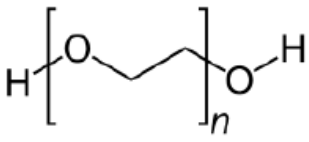
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Monophosphate-Dependent Signaling Functions," Endocrine Reviews, 27(1) at Fig. 9 (2006), Exhibit 17 (Potter 2006).

CNPs are generally identified by appending a number to the acronym "CNP" indicating the number of amino acids in the peptide. Thus, CNP53 refers to a naturally occurring human CNP peptide having 53 amino acids in a specific sequence. CNP22 refers to a shorter peptide (which is also naturally occurring and is shown on the right of the figure above) containing the last 22 amino acids found in CNP53, including the 17-amino-acid ring.

The half-life in the body of wild-type (unmodified) CNP22 was reported to be only 2.6 minutes. The clinical applications of CNPs were therefore believed to be "limited because of their short biological half-life." Nesher 2008 at Abstract.

One known way to extend the half-life of a NP was to lengthen it to a size greater than 3 kDa (kilodaltons) or the equivalent of about 27 amino acids, which reduces the degradation of the peptides by the enzyme NEP. The increase in size could be done by adding more amino acids to the CNP tail, and/or by attaching a polymer to the CNP. Polymers are compounds made of repeat units called monomers. For example, PEG, discussed above in connection with Ascendis's carrier, is a synthetic polymer made of repeated units of ethylene glycol.



or H-[O-CH_2-CH_2]$_n$-O-H

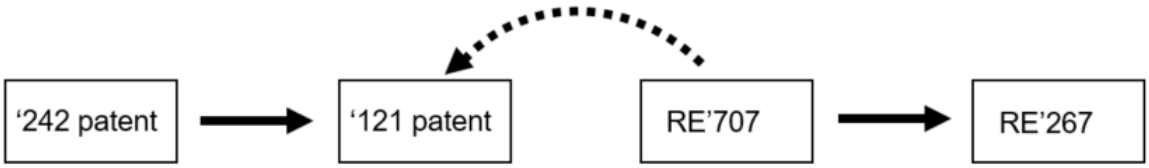
The brackets show the unit that is repeated, and "*n*" represents the number of repeat units in the polymer. PEGs with a very large *n* can be very large molecules. Attaching a PEG to a peptide — a process called "PEGylation"—was a well-known technique as of 2009. *See, e.g.*, Exhibit 5 (Veronese), 320, 323, 326.

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The specification for the patent in suit shows that the named inventors were trying to develop a CNP variant that exhibited increased resistance to NEP while retaining biological activity—that is, they were trying to make *active* drugs with longer *in vivo* half-lives than wild-type CNP. They were not trying to make prodrugs. We know this because the named inventors performed two assays on candidate CNP variants, one for NEP resistance and the other for cGMP stimulation. *See* RE'267 Tables 1-4. The CNP variants that the named inventors deemed successful were all large molecules (over 3 kDa including any PEGylation) that are both resistant to NEP degradation and functionally active at the same time. In the words of the patent, what BioMarin claimed to have invented is "a successful strategy for making CNP resistant to NEP while retaining its functionality." RE'267 patent at 4:33–35.

III. OVERVIEW OF THE ASSERTED PATENT

The RE'267 patent is a continuation of RE46,707, which is a reissue of U.S. Pat. No. 8,598,121, which claims priority to U.S. Pat. No. 8,198,242.



The original '242 patent is listed in the FDA's Orange Book as covering BioMarin's CNP product Voxzogo. The application that issued to become RE'267 was filed after BioMarin learned of Ascendis's forthcoming prodrug. All of the original claims were canceled and strategically replaced. The claims asserted here recite a macromolecule or a sustained-release formulation that comprises a CNP variant selected from a Markush group. The domestic industry claims recite processes in which CNP variants from that same Markush group contact a cell. The Markush group lists five variants of CNP27, including PEGylated variants. It lists seven variants of CNP37, including Pro-Gly-CNP37 (i.e., CNP 37 with the amino acids proline and glycine

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added to one end of the peptide), which is the active ingredient used in BioMarin's commercial product, Voxzogo. It also lists wild-type CNPs 30 through 40, including CNP38, the peptide incorporated within Ascendis's prodrug product.

The asserted claims also have newly drafted preambles that add structural features described nowhere in the specification. Specifically, independent claim 15 recites "[a] macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein…" RE'267 patent at 269:64–66. The specification discloses that PEG is a synthetic polymeric group, *id*. at 7:25–28, but does not describe a "macromolecule" or "releasing a CNP variant". The disclosure related to macromolecules in the specification refers to large biological molecules, such as *poly*peptides (a longer and more complex chain of amino acids than a peptide), for example, modifying a polypeptide in a site-specific fashion "based on wild-type macromolecule reactivity-directed derivatization." *Id*. at 79:46–51, 38:66-39:18, 85:44-50. That disclosure has nothing to do with releasing a CNP variant.

Independent claim 18 recites "[a] sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein…" RE'267 patent at 271:4–6. The specification does not provide any examples of sustained-release formulations of CNP, but it notes that sustained-released drug-delivery systems are known in the art and "can take the form of, e.g., microparticles, microspheres or liposomes." *Id*. at 89:1–6. The specification also discusses Alzet pumps and stents covered with porous membranes. *Id.* at 91:8-38. All of these systems contain pharmaceutical compositions and use physical forces (rather than chemical reactions) to diffuse them into the bloodstream steadily over time. Claim 18 recites the same synthetic polymeric group coupled to the CNP variant

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through a hydrolysable linkage as claim 15, but without referencing any macromolecule. The Markush group lists the same CNP variants as claim 15.

IV. PERSON OF ORDINARY SKILL IN THE ART

A person of ordinary skill in the art of developing PEGylated CNP peptides typically would have had a Ph.D. in polymer, organic, protein, bioconjugation, or biological chemistry and also typically be a chemist with experience or training in the preparation, development, and use of both cleavable and non-cleavable linkers and their application to the creation of conjugates containing bioactive moieties.

A POSA may also have worked as part of a multi-disciplinary team and drawn upon not only his or her own skills, but also taken advantage of certain specialized skills of others in the team that might lie outside the POSA's primary training, to solve a given problem. For example, such a team may be comprised of a physician with knowledge of cartilage and bone biology or a biologist specializing in natriuretic peptide structure and function, a protein biochemist specializing in chemical peptide synthesis, and a pharmacologist.

V. LEGAL STANDARDS

Claim construction is a question of law. *Markman v. Westview Instruments*, *Inc.*, 517 U.S. 370, 391 (1996). To determine a claim's meaning, one first looks to the intrinsic evidence. *Phillips v. AWH Corp.*, 415 F.3d 1303, 1315–17 (Fed. Cir. 2005) (*en banc*). Intrinsic evidence includes the "words of the claims themselves [both asserted and nonasserted] to define the scope of the patented invention." *Id.* at 1312 (internal citations omitted). One then turns to the specification, which may serve as the "single best guide to the meaning of a disputed term." *Id.* at 1321. Statements made during prosecution also constitute intrinsic evidence. *Comput. Docking Station Corp. v. Dell, Inc.*, 519 F.3d 1366, 1374 (Fed. Cir. 2008).

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Finally, "it is important to construe claim language through the 'viewing glass' of a person skilled in the art." *Budde v. Harley-Davidson, Inc*., 250 F.3d 1369, 1380 (Fed. Cir. 2001). Extrinsic evidence, such as expert opinions and pertinent dictionary definitions, may be helpful in determining the ordinary meaning that a term would have had to a skilled artisan at the relevant time. *CCS Fitness, Inc. v. Brunswick Corp.*, 288 F.3d 1359, 1367 (Fed. Cir. 2002).

VI. DISPUTED CLAIM CONSTRUCTIONS

A. The preambles of the asserted claims are limitations.

The parties all agree that the preambles for claims 21 and 22 (which are not asserted as infringed, but which BioMarin claims to practice as part of its domestic industry requirement) are limiting. BioMarin argues that the preambles for claims 15 and 18, however, are not limiting at all. Staff argues that certain terms in the preambles for claims 15 and 18 are limiting to the extent they provide antecedent basis for terms in the body of the claims. As discussed below, Respondents' position is that all of the preambles are limitations and should be construed accordingly.

BioMarin argued to the Patent Office that it was entitled to seek broadened reissue claims based on the subject matter recited in the preambles. *See* July 10, 2019 Remarks, Exhibit 18; July 3, 2019 Applicant-Initiated Interview Summary, Exhibit 19. BioMarin cannot rely on the scope of the language in the preambles to secure allowance of the claims, and then disavow that those preambles have any limiting effect when it comes time to assert its patent. *See Southwall Techs., Inc. v. Cardinal IG Co*., 54 F.3d 1570, 1583 (Fed. Cir. 1995); *see also Bell Commc'ns Rsch., Inc. v. Vitalink Commc'ns Corp.*, 55 F.3d 615, 620 (Fed. Cir. 1995) ("[W]hen the claim drafter chooses to use both the preamble and the body to define the subject matter of the claimed invention, the invention so defined, and not some other, is the one the patent protects.").

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Furthermore, the examiner expressly treated the entire preambles as limiting in examining and mapping the reissue claims to the cited prior art. *See, e.g.*, November 26, 2019 Nonfinal Rejection at 7 (comparing the claimed "peptides' properties which are recited in the claim preambles" of claims 15, 18, 21, 22 and 23 to prior-art references "Potter, Yeung, Golembo, Nakao '425"), Exhibit 20. BioMarin cannot receive the benefit of the preambles serving as limitations during prosecution, and then be free of those limitations in asserting the claims in litigation. *See Storage Tech. Corp. v. Cisco Sys., Inc.*, 329 F.3d 823, 831 (Fed. Cir. 2003).

Finally, it is not appropriate to give some terms in a preamble limiting weight and other terms not. The terms of the preambles form operative phrases that describe the scope of the invention as a whole. *Bell*, 55 F.3d at 620. That is how the examiner treated them in prosecution. The Markush group of claims 15 and 18 provides additional restrictions for one preamble term (CNP variant). Claim 15's "wherein" clause provides additional restrictions for another preamble term (hydrolysable linkage). These terms are entwined with the rest of the preamble. *See Bio-Rad Lab'ys, Inc. v. 10X Genomics Inc.*, 967 F.3d 1353, 1371 (Fed. Cir. 2020) ("[W]e disagree that these limiting terms can be read separately from the remainder of the preamble. The language relied upon for antecedent basis in the preamble at issue is intertwined with the rest of the preamble."). Accordingly, all of the words in all of the preambles of the asserted claims should be construed as limitations.

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B. "CNP Variant" (claims 15–22, 31–49, 54)

Complainant's Construction	Staff's Construction	Respondents' Construction
a variant of C-type natriuretic peptide (CNP).	a version of C-type natriuretic peptide that has reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleave by NEP and/or clearance by NPR-C, but which has substantially similar or better functionality than wild-type CNP22	C-type natriuretic peptide comprising the cyclic Cys_6 to Cys_{22} of CNP-22 along with any modifications, substitutions, or conjugations, that is capable of binding to and activating NPR-B, and thereby inducing cGMP production

Independent claim 15 recites "a macromolecule capable of releasing a *CNP variant*," RE'267 patent at 269:64–67 (emphasis added), and claim 18 recites "a sustained release *CNP variant* formulation." *Id*. at 271:4 (emphasis added). The term "CNP variant" does not have a plain or established meaning in the art. It is important to determine what exactly a "CNP variant" is, so as to know whether a given macromolecule is capable of releasing one, or whether a sustained release formulation contains one.

The RE'267 patent defines CNP variants as containing the characteristic cyclic Cys_6 to Cys_{22} of CNP22–*i.e.*, the ring structure formed by the amino acids between the cystine at position 6 and the cystine at position 22–along with modifications that improve resistance to degradation. *Id*. at 3:60–67. These modifications may add, delete, or substitute amino acids, or conjugate the peptide to polymers like PEG. *Id*. at 3:63–4:31, 4:39–5:21. The specification describes many different types of molecules as CNP variants, including naturally occurring CNPs, mutated CNPs, and PEGylated CNPs:

[redacted]

In one embodiment, the disclosure encompasses CNP variants represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:139), or

(x)-Gly_1Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_8-$Glyl_{19}$-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:140), wherein:

(x) and (z) each independently are a natural polymer (e.g., a peptide sequence containing at least one amino acid) and/or a synthetic polymer (e.g., PEG) as described herein,

RE'267 patent at 47:7–17 (annotated); *see also* 7:9–39 (providing CNP variant formula). An attached PEG or other natural or synthetic polymer is therefore part of the general formula for a CNP variant. As discussed in Section VI.D., below, PEGs can be attached to peptides through chemical linkages. The claim itself is also clear that PEGylated CNPs themselves are a type of CNP variant, because the Markush group lists three PEGylated CNPs as CNP variants from which to select. *Id*. at 260:1–63. Accordingly, the term "CNP variant" includes the peptide sequence as well as any linkage, and any attached synthetic polymer.

As noted above, the stated purpose of the invention is "making CNP resistant to NEP while retaining its functionality." *Id*. at 4:33–35. Accordingly, "CNP variants of the disclosure advantageously retain CNP activity and exhibit increased serum half-life." *Id*. at 24: 23-24. The main way that CNP variants work on the body to treat achondroplasia is by binding to NPR-B (also called GC-B) receptors, which stimulates cGMP production. *See* Yasoda, A., et al., "Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway," Nature Medicine, Fig. 6 (2004), Exhibit 21 (Yasoda). BioMarin tested several CNP variants for NEP resistance and biological activity (as measured by an *in vitro* cGMP assay). RE'267 patent at Table 3 and Table 4. Only those variants that exhibited both NEP resistance and

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biological activity progressed on to *ex vivo* and *in vivo* experiments.[1] RE'267 patent at 6:13–35, 9:25–46, 10:11–25, 10:33–43, 46:56–47:6, 60:40–43, 68:13–30, 115:27–63, Examples 3, 4, 5, 8A, 10, and 12–19.

The specification emphasizes that "[i]n any of the embodiments disclosed herein, the CNP variants may have substantially the same or better biological activity than wild-type CNP22" (which requires the capability of binding to and activating NPR-B). RE'267 patent at 22:56-58. By way of example, the embodiments and selection criteria described at 6:13–35, 9:25–46, 10:11–25, 10:33–43, 46:56–47:6, 60:40–43, 68:13–30, 115:27–63, and in Examples 3, 4, 5, 8A, 10, and 12–19 provide functionally active CNP variants. Precisely to retain CNP activity via binding to NPR-B receptors, the specification advises avoiding PEGylating within the ring-portion of CNP and recommends using small PEGs (from about 0.2 kDa up to 5 kDa). *See*, *e.g., Id.* at 10:33-43. Hence, in the context of the patent, CNP variants are modified versions of CNP that retain activity and thus must have the capability to bind to and activate NPR-B receptors.

Complainant's proposed construction merely provides what the letters "CNP" stand for and reuses the word "variant." Parroting the claim language itself does not engage with the claim-construction process. *See Verinata Health, Inc. v. Ariosa Diagnostics, Inc.*, 2014 WL 7205108, at *5 (N.D. Cal. Dec. 17, 2014) (rejecting proposed construction that "merely parrots the language of the claim, without explaining it"). A proper claim construction should confirm that a CNP variant has the defining 17 amino-acid ring that is essential for activity along with whatever modifications were made to improve its NEP resistance, including PEGylation.

[1] Indeed, the sole construct with a large PEG that was tested in the cGMP in vitro assay, CNP22-PEG20K (i.e., CNP22 with a large polydispersed PEG having an average molecular weight of 20 kDa attached at one end) exhibited little to no cGMP response and was not selected for further testing. *See* Fig. 22.

[redacted]

Staff's proposed construction focuses only on functional attributes and does not clarify the structure of CNP variants. It also fails to account for the defining structural formula disclosed in the specification or the fact that three PEGylated peptide sequences appear in the Markush group. Both confirm that "CNP variant" includes CNPs coupled to polymers through linkages. Moreover, although the inventors may have desired to achieve improved resistance to NEP degradation and NPR-C clearance, they succeeded only with respect to NEP degradation. The patent does not even suggest, let alone demonstrate, that any of the variants it identifies exhibit improved resistance to NPR-C clearance.

C. "hydrolysis" (claims 15–17 and 31–39)

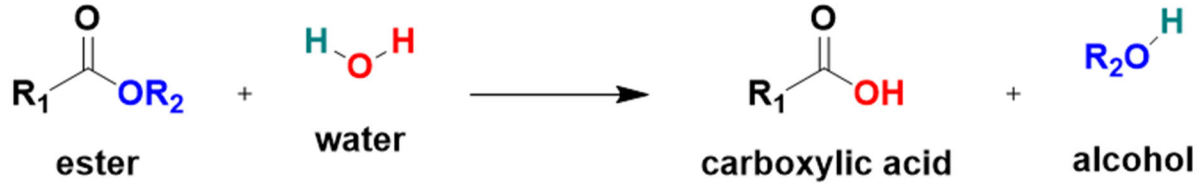
Complainant's Construction	Staff's Construction	Respondents' Construction
chemical reaction in which water reacts with another substance to form two or more new substances	chemical reaction in which water reacts with another substance to form two or more new substances	reaction between a compound and water in which a bond in the compound is cleaved and the water components (H+ and OH-) are added where the bond was broken

Claims 15–17 and 31–39 recite various "macromolecules" comprising "a CNP variant" and "a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage." RE'267 patent at 274:21–276:53, 279:51–281:13. The claims further require that "*hydrolysis* of the hydrolysable linkage releases the CNP variant." *Id.* (emphasis added). The specification does not define or describe hydrolysis, but provides that the "[d]efinition of standard chemistry terms may be found in reference works, including Carey and Sundberg, Advanced Organic Chemistry, 3rd Edition, Vols. A and B (Plenum Press, New York 1992), Exhibits 3 and 4." RE'267 patent at 34:57-60. Hydrolysis is a standard chemistry term. Sessler Decl. ¶ 26. Carey does not purport to formally define hydrolysis, but uses the term extensively and provides several examples of reactions that it identifies as "hydrolysis." *See, e.g.*, Exhibits 3 and 4 (Carey Ch. 4) 226-227, 238;

[redacted]

(Carey Ch. 8) 465-70, 473-75. The term "hydrolysis" also has an ordinary and customary meaning in the art, which is consistent with how the term is used in Carey, and with Respondents' proposed construction. Sessler Decl. ¶ 26.

Hydrolysis comes from the Greek constituents *hydro*, meaning water, and *lysis*, meaning to cut. Hydrolysis is a chemical reaction between water and some other compound, in which water is a reactant. Sessler Decl. ¶ 27. A water molecule cleaves a chemical bond in the target compound by attacking and inserting itself into the compound at the site of the bond. *Id.* A classic illustration is shown below:



In this reaction, an ester reacts with water to form two new products, but it does so in a particular way: the water reacts to break the bond between an oxygen atom (blue) and a carbon atom in the ester, with OH from the water (red) ending up on one product and H from the water (green) ending up on the other product at precisely the spots where the bond was broken. *Id.* This insertion of water components (H^+ and OH^-) where the bond was broken is ordinarily what skilled artisans mean when they use the term hydrolysis. Sessler Decl. ¶ 28.

Claim 15 of the RE'267 patent recites a hydrolysis reaction solely in the context of a hydrolysable linkage releasing a CNP variant from a synthetic polymeric group. While the specification never explains what "hydrolysis" means in this context and provides no example or description of detecting a *release of a CNP variant* via hydrolysis, hydrolysis reactions in the context of PEGylation and reversible PEG-assisted peptide delivery were well understood as of May 2009. *See* Sessler Decl. ¶ 26. One of skill in the art would understand that these reactions

[redacted]

proceed when a water molecule cleaves the bond between the PEG and the peptide, and the water components (H^+ and OH^-) are added where the bond was broken. Sessler Decl. ¶ 29.

Examples of hydrolysis and hydrolysable linkages described in the specification are consistent with Respondents' construction. *See, e.g.*, RE'267 patent at 10:37–38, 60:67–61:1, 62:17–19, 101:25–27, 113:42, Table 4, Table 16 at col. 138, fn 1. These examples include ester and amide bonds. When these bonds undergo hydrolysis, they do so similarly to the example reaction illustrated above, whereby a water molecule is added to the target compound at the site of the cleaved bond. Sessler Decl. ¶ 43. Crucially, the specification contains no example or description that suggests any other kind of reaction might qualify as "hydrolysis."

The prosecution history also supports Respondents' construction. During prosecution, the examiner stated: "Long-chain peptides contain amino acids linked via peptide bonds, a type of amide bond, that can be broken by amide hydrolysis, wherein the bonds are cleaved with the addition of a water molecule." (5/10/2019 Office Action, 9). BioMarin did not disagree. Skilled artisans would understand the amide hydrolysis described in this comment to refer to a water molecule cleaving a peptide bond by being added at the site of the cleaved bond. Sessler Decl. ¶¶ 44, 47.

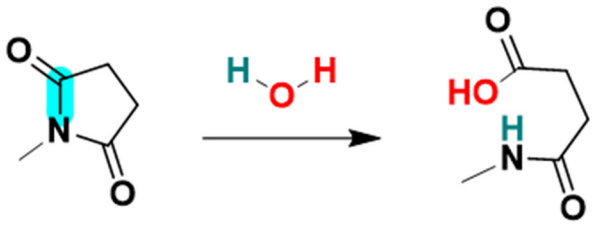
A number of other "reference works" also demonstrate that Respondents' proposed construction reflects the ordinary and customary meaning that "hydrolysis" would have had to a skilled artisan at the relevant time. RE'267 patent at 34:57-60. For example, the leading authority on the meaning of hydrolysis is found in the *Glossary of Terms Used in Physical Organic Chemistry* (IUPAC Recommendations 1994), International Union of Pure and Applied Chemistry, Organic Chemistry Division, Commission on Physical Organic Chemistry. Sessler Decl. ¶ 30. Exhibit 7 at 1123, it provides: Hydrolysis: "Solvolysis by water." The corresponding entry for solvolysis, on page 1165, provides: Solvolysis: "Generally, reaction with a solvent, or

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with a lyonium ion or lyate ion, involving the rupture of one or more bonds in the reacting solute. More specifically the term is used for substitution, elimination and fragmentation reactions in which a solvent species is the nucleophile ('alcoholysis' if the solvent is an alcohol, etc.)." For hydrolysis, the "solvent" is water. The nucleophile (the atom or ion that donates a pair of electrons to form a new chemical bond) is OH and comes from the water molecule.

A simpler definition, but also informative, is found in *The Facts On File Dictionary Of Organic Chemistry*, Ed. Daintith (2004). Exhibit 6 at 115. That dictionary provides "[a] reaction between a compound and water. An example is the hydrolysis of an ESTER to give a carboxylic acid and an alcohol: $CH_3COOC_2H_5 + H_2O \rightleftharpoons CH_3COOH + C_2H_5OH$." This exemplary reaction is the one depicted above. This definition is also consistent with Respondents' construction. Sessler Decl. ¶ 30.

Complainant and Staff propose defining hydrolysis as a "chemical reaction in which water reacts with another substance to form two or more new substances." There are two problems with this construction. First, a hydrolysable bond may be cleaved by hydrolysis without forming two or more new substances. Sessler Decl. ¶¶ 32-34. For instance, a molecule may have a ring structure, and hydrolysis of one of the bonds may open the ring to create a linear structure. *See* Exhibit 9 (Huang) 311, Figure 1a. In such a reaction, only one new substance may be formed. To illustrate:

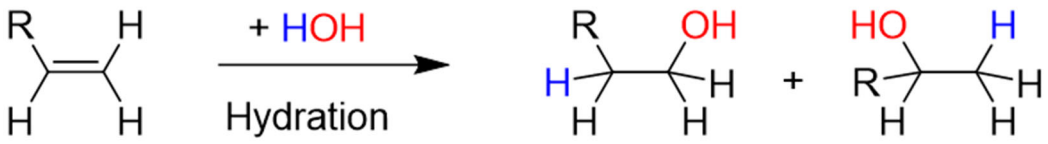


The left side shows a type of compound called a succinimide, which is hydrolysed at an amide bond (blue) to form a succinamic acid on the right. Sessler Decl. ¶¶ 34-35. Here, the addition of

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water cleaves a bond, adding an OH to one side and H to the other at the sites where the bond was broken, but only one new substance is formed. This is nonetheless a hydrolysis reaction, as persons of skill in the art would understand. Sessler Decl. ¶ 35. Recognizing this type of reaction as hydrolysis is fully consistent with Respondents' proposed construction and with the usage of the term in the art. *See, e.g.*, Exhibit 9 (Huang) (Figure 1a); *see also* Sessler Decl. ¶ 36 (discussing epoxide hydrolysis). Complainant's and Staff's construction, which excludes these reactions, cannot be correct.

Second, there are well-known reactions that are not hydrolysis, that would nonetheless fall within Complainant's proposed definition. Sessler Decl. ¶ 37. One example would be acid-catalyzed hydration of alkenes to produce alcohols. An illustrative example is shown below.



See Sessler Decl. ¶ 37. This is a chemical reaction in which water reacts with another substance to form two or more new substances, but any POSA would agree that acid-catalyzed hydration is not hydrolysis. *Id.; see also id.* at ¶ 38 (discussing catalyzed alkane hydration). Complainant's construction is therefore under-inclusive in some ways, and over-inclusive in others.

Respondents' construction avoids these problems because it specifies that hydrolysis cleaves a bond and the components of the water molecule bind at the sites of the cleaved bond. That is a defining feature of hydrolysis. *See* Sessler Decl. ¶ 39. If the water molecule's constituent H and OH bind to the products of the reaction somewhere other than where the bond was broken, then that is an indication that the water molecule itself did not effectuate the cleavage. *Id*. Hypothetically, components of a water molecule may bind somewhere else on a compound as part of some subsequent reaction that involves a bond being broken at some other

[redacted]

point. *Id.* But if the water molecule itself (*hydro*) did not cut that bond (*lysis*), then that bond was not cleaved by hydrolysis. *Id.*

Accordingly, Respondents' construction is most faithful to the plain meaning of hydrolysis in the context of the specification, including the "reference work" that the specification identifies as "defining[g] standard chemistry terms." RE'267 patent at 34:57-60; Sessler Decl. ¶ 40. It also best resolves the actual dispute among the parties. It should be adopted.

D. "coupled to the CNP variant through a hydrolysable linkage" (claims 15–20, 31–48)

Complainant's Construction	Staff's Construction	Respondents' Construction
connected to the CNP variant through a connection capable of chemical reaction in which water reacts with another substance to form two or more new substances	connected to the CNP variant through a connection capable of chemical reaction in which water reacts with another substance to form two or more new substances	connected to the CNP variant through a chemical bond cleaved by the addition of a water molecule

A hydrolysable linkage that couples the CNP variant is a chemical bond that is susceptible to hydrolysis. That is the meaning that one of ordinary skill in the art would understand this term to have in the context of the RE'267 specification. *See* Sessler Decl. ¶ 41.

The specification does not define a hydrolysable linkage. To the extent that the specification lists any hydrolysable linkages, such as ester, amide, aminal and imine *bonds*, all of those are chemical bonds that are cleaved by a reaction with water, in which the bond is cleaved and the water components (H+ and OH-) are added where the bond was broken (i.e., hydrolysis). *See, e.g.*, RE'267 patent at 10:37–38, 60:67–61:1, 62:17–19, 101:25–27, 113:42, Table 4, Table 16 at col. 138, fn 1; Sessler Decl. ¶¶ 42-43. While the patent does not label any exemplified linkages as "hydrolysable," Table 5 lists commercially-available PEG reagents, including "Sunbright ME-200CS," "Sunbright ME-50CS," and "Sunbright ME-20CS," which the vendor

[redacted]

describes as "possess[ing] esters." *See*, Exhibit 12 (NOF Catalogue) at 10. Such ester bonds are susceptible to hydrolysis through the addition of the water components at the site where the bond is cleaved. *See,* Exhibit 8 (Carey Ch. 8) at 465; Sessler Decl. ¶ 43. No other kind of hydrolysable linkage in the context of a PEGylated compound is described in the specification, nor is it suggested that other kinds of linkages exist.

The prosecution history also supports Respondents' construction. *See, e.g.*, July 17, 2017 Preliminary Amendment at 28, Exhibit 22. As the Examiner stated: "Long-chain peptides contain amino acids linked via peptide bonds, a type of amide bond, that can be broken by amide hydrolysis, wherein the bonds are cleaved with the addition of a water molecule." *See also* May 10, 2019 Office Action at 9, Exhibit 23. Thus the Examiner expressly interpreted a hydrolysable linkage as a bond that can be cleaved by the addition of a water molecule in ultimately allowing the patent to issue. Sessler Decl. ¶ 44. That phrasing from the prosecution history is the source of Respondents' proposed construction here.

Complainant's and Staff's construction has three problems. *First*, it incorporates Complainant's flawed construction of hydrolysis, which is problematic for the reasons discussed above. *Second*, it uses the imprecise phrase "connected through a connection," which implies that a linkage can be something other than a chemical bond. Sessler Decl. ¶ 46. Every instance of a linkage identified in the specification is a chemical bond:

> The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG molecules) that may vary in type (. . .), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa).

RE'267 patent at 10:33-43. As noted above, amide, imine, aminal, and ester bonds can be hydrolyzed. Additionally, the specification explains that a synthetic polymer conjugated to a CNP

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peptide "in general has the formula: CH_3—[—O—CH_2CH_2—]$_n$—C(=O)—NHR, where *n* is the number of ethylene oxide units and R denotes the rest of the peptide." *Id*. at 43:29-56 (yellow added). This formula confirms that the PEG is coupled to the peptide through a chemical bond (yellow). Sessler Decl. ¶ 47.

During prosecution, the named inventors purported to identify written-description support for the claimed "hydrolysable linkage" by pointing to this portion of the specification *See* Exhibit 22. Likewise, in the parallel European proceeding, BioMarin pointed to an amide bond in the accused product (not the linker molecule containing the amide bond) as allegedly satisfying the hydrolysable linkage limitation. *See* UPC Notice with BM Infringement Action, ¶59, Exhibit 24. There is no evidentiary support that a "linkage" could be anything other than a chemical bond. Complainant and Staff thus erroneously broaden the term "linkage," which the intrinsic evidence consistently uses to describe bonds, as a synonym for "linker"—a more complicated structure connecting different parts of a molecule.

Third, Complainant and Staff erroneously broaden the claim even further by saying that any part of the "connection" need only be "capable" of undergoing a "chemical reaction in which water reacts with another substance to form two or more new substances"—regardless of the conditions that would be required to make this chemical reaction happen. If a linkage is more than a bond, and it need be merely "capable" of undergoing hydrolysis under some undefined, unspecified conditions, then the claim would sweep up countless structures that bear no relation to releasing a CNP variant via hydrolysis of the bond linking the CNP variant to the rest of the molecule. Under Complainant's theory, the CNP variant could be released by a different reaction entirely, yet the product would nevertheless fall within the scope of the claims so long as Complainant could point to some part of the molecule that could potentially react with water under some set of conditions to form two or more new substances, no matter how extreme or

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outlandish those conditions may be. The specification, however, does not support such a broad construction. *United States v. Adams*, 383 U.S. 39, 49 (1966) ("it is fundamental that claims are to be construed in the light of the specification and both are to be read with a view to *ascertaining the invention*.") (emphasis added); *In re Prater*, 415 F.2d 1393, 1395-96 (C.C.P.A. 1969) (observing that "giving broader coverage than is justified" undermines the notice function of patent claims). Respondents' construction should be adopted.

E. "formulation" (claims 18–20, 40–48)

Complainant's Construction	Staff's Construction	Respondents' Construction
a mixture containing a system for maintaining a relatively constant level of CNP variant over a desired time period	a pharmaceutical composition of a CNP variant and one or more pharmaceutically acceptable excipients, carriers and/or diluents	a pharmaceutical composition of a CNP variant and one or more pharmaceutically acceptable excipients, carriers and/or diluents

Respondents and Staff agree that the preamble is limiting. Respondents and Staff also agree that the ordinary and customary meaning of "formulation" denotes an active pharmaceutical ingredient (API) and one or more pharmaceutically acceptable excipients, carriers and/or diluents.

The specification does not expressly define formulation, but the use of that term throughout the specification supports Respondents' and Staff's plain-meaning construction. RE'267 patent at 86:11–18 (describing liquid formulations as expected for an injectable); 85:62–65 ("The use of excipients, diluents and carriers in the formulation of pharmaceutical compositions is known in the art"); 88:1–8 (describing lyophilized (i.e., freeze-dried) formulations); 87:39–41 (describing flavoring, coloring, or sweeteners for formulations); 87:43–65 (describing formulations for oral or parenteral administration); 88:15–20 (describing pharmaceutically acceptable injectable or ingestible formulations); 89:1–7 (describing systems for slow release, controlled release, and sustained release formulations).

[redacted]

Complainant's construction reads on any mixture (pharmaceutically acceptable or otherwise) "containing a system for maintaining a relatively constant level of CNP variant over a desired time period." The first problem with this construction is that the word "formulation" indicates to one of skill in this art something appropriate to administer to a subject. It is therefore an API plus whatever excipients, carriers and/or diluents will make the API pharmaceutically acceptable for administration. Not just any mixture is a "formulation." RE'267 patent at 85:62–65.

The second and more glaring problem is that Complainant proposes the exact same construction for "sustained release CNP variant formulation" (see below) that they propose for the word "formulation" alone. That simply cannot be. Complainant is asking either for the words "sustained release CNP variant" to be rendered superfluous, or for the term "formulation" to be meaningless. Both options are highly disfavored. *See Intel Corp. v. Qualcomm Inc.*, 21 F.4th 801, 810 (Fed. Cir. 2021) ("[i]t is highly disfavored to construe terms in a way that renders them void, meaningless, or superfluous"). That alone precludes adoption of Complainant's construction.

Furthermore, not every drug formulation is a sustained-release formulation. *Compare* 87:43–65 *and* 88:15–20 *with* 89:1–7. Indeed, there are well-known formulations in the art that provide, for example, rapid release or delayed release rather than sustained release. *See* Remington's Pharmaceutical Sciences, 18th Edition, pp. 1435–1712, Mack Publishing Co. (Easton, Pa. (1990)) cited at RE'267 patent at 85:62–65. For Complainant to suggest that the word "formulation" necessarily "contain[s] a system for maintaining a relatively constant level of CNP variant over a desired time period" reads in excessive limitations from the specification without justification.

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F. "sustained release CNP variant formulation" (claims 18–20, 40–48)

Complainant's Construction	Staff's Construction	Respondents' Construction
a mixture containing a system for maintaining a relatively constant level of CNP variant over a desired time period	a CNP variant formulation that is formulated as a system for maintaining a relatively constant level of dosage over a desired time period	a pharmaceutical composition, which includes a CNP variant coupled to synthetic polymeric group through a hydrolysable linkage, that is formulated in a system known in the art for maintaining a relatively constant level of dosage over a desired time period

The term "sustained release CNP variant formulation" recites an essential feature of the claim and should be construed as limiting. *See Glaxo Wellcome v. Impax Labs. Inc.*, 356 F.3d 1348, 1353 (Fed.Cir.2004) ("The 'sustained release tablet' phrase recited in the preamble gives life and meaning to the claims, because sustained release is an essential feature of the invention."). Here, a "sustained release CNP variant formulation" denotes a pharmaceutical composition (formulation) that includes the molecular structure recited in the claim (a CNP variant coupled to a synthetic polymeric group through a hydrolysable linkage) formulated according to how the specification describes "sustained release formulations." RE'267 patent at 88:63–89:7. Indeed, the specification provides the meaning of this term:

> In addition, *pharmaceutical compositions* comprising a CNP variant can be formulated as a slow release, controlled release or sustained release *system for maintaining a relatively constant level of dosage over a desired time period*. … Slow release, controlled release and sustained release formulations can be prepared using, e.g., biodegradable polymeric systems {which can comprise, e.g., hydrophilic polymers [e.g., polylactide, polyglycolide, poly(lactide- glycolide)]}, and can take the form of, e.g., microparticles, microspheres or liposomes, *as is known in the art*.

RE'267 patent at 88:63–89:7 (emphases added). Respondents' construction most closely tracks this disclosure.

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All three parties' constructions include the language "a system [known in the art] for maintaining a relatively constant level of dosage over a desired time period," indicating that all three parties are using the above passage from the specification to provide the meaning of this term. The passage makes clear, however, that it is the delivery system (the microparticles, microspheres, liposomes) that imparts the sustained release property to the pharmaceutical composition, rather than some other aspect of the CNP variant formulation. *Id.*

Complainant's construction deviates from the specification in two problematic ways. *First*, it introduces the term "mixture" which does not appear in the specification in the context of describing formulations and does not have a clear meaning in the context of this claim. The specification describes that sustained release formulations are pharmaceutical compositions, but a "mixture" could be anything. *See, e.g.*, RE'267 patent at 104:10–12 (describing a manufacturing titration "mixture"); 104:20–21 (describing a "mixture" of different CNP variants); 105:20–25 (describing an *in vitro* experiment "mixture"). In fact, every time the word "mixture" is used in the specification, it describes an experiment or manufacturing step, and *not* a formulation.

Second, Complainant's construction provides that the mixture contains a system for maintaining a constant level of dosage, but does not indicate what the system is or what it contains. Under Complainant's overbroad construction, the system could be the delivery systems disclosed in the specification (microparticles, microspheres, or liposomes containing a CNP variant) or the "system" could be the CNP variant itself. All Complainant's construction requires is that the system maintains a relatively constant level of CNP over a desired time and be in the undefined "mixture." This is problematic because nowhere does the specification identify any CNP variant or CNP variant coupled to anything having any kind of sustained-release property. Despite discussing modifications like PEGylation (which slows the degradation of CNP)

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extensively throughout the specification, PEGs or PEGylated CNP variants are *never* referred to as a sustained-release system.

Contrastingly, Respondents' construction specifies that the CNP variant and coupled synthetic polymeric group are formulated *in* the system. The recited CNP-polymer conjugate is not itself the system because the system contains the API—as microparticles, microspheres, and liposomes do.

Staff's construction does not use the problematic word "mixture," and is closer to tracking the description provided in the specification. Staff proposes that the pharmaceutical composition is formulated *as* a system, rather than *in* a system (as Respondents propose). It is therefore unclear whether Staff intends to construe the term as reciting a CNP variant and coupled polymer contained in a sustained release system like a microparticle, microsphere, or liposome, or if Staff intends to mean that the CNP variant and attached polymer *are* the system for maintaining a constant level dosage (in which case it is hard to see how the single macromolecule qualifies as a sustained-release "formulation"). The former is consistent with the specification as it would be understood by one of skill in the art familiar with sustained release systems known in the art; the latter has the same problems as Complainant's construction discussed above.

In sum, the term "sustained release" appears only in the passage quoted above and only in the context of pharmaceutical compositions formulated in systems known in the art to provide slow and constant delivery of any API. The term "sustained release CNP variant formulation" should be construed accordingly.

[redacted]

G. "macromolecule" (claims 15–17, 31–39)

Complainant's Construction	Staff's Construction	Respondents' Construction
molecule with a molecular mass greater than a few thousand daltons.	molecule with a molecular mass greater than a few thousand daltons	a functionally active molecule with a molecular mass greater than a few thousand daltons

Claim 15 recites "a *macromolecule* capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage." RE'267 patent at 269:64–67 (emphasis added). By the plain language of the claims, the recited macromolecule must include (1) a synthetic polymeric group, (2) a CNP variant, and (3) a hydrolysable linkage connecting the synthetic polymeric group to the CNP variant. After the Markush group listing the CNP variants, claim 15 goes on to recite, "wherein hydrolysis of the hydrolysable linkage releases the CNP variant." RE'267 patent 270:64–65.

The claim language itself thus requires that the macromolecule be something more than its three itemized parts, and that it have a functionality beyond its ability to release the CNP variant (which is recited elsewhere in the claim). Otherwise, the language "a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage" without the word "macromolecule" would have exactly the same effect. The word "macromolecule" would be superfluous if it did not confer additional requirements. *See Intel*, 21 F.4th at 810 ("[i]t is highly disfavored to construe terms in a way that renders them void, meaningless, or superfluous"). Respondents' construction gives meaning to the word "macromolecule" as used in this claim, in view of what a person of skill in the art would understand to be the claimed invention: "a successful strategy for making CNP resistant to NEP while retaining its functionality." RE'267 patent at 4:33–35 (distinguishing the invention from the sum total of prior art).

As a caveat, to the extent that "macromolecule" appears in the RE'267 specification, that disclosure has nothing to do with the macromolecule recited in claim 15. For example, the term

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appears in a section titled "chemically modified CNP variants." The discussion notes that "[c]hemical modification of polypeptides can be performed in a non-specific fashion (leading to mixtures of derivatized species) or in a site-specific fashion (e.g., based on wild-type *macromolecule* reactivity-directed derivatization. . ." RE'267 at 79:46–51 (emphasis added). For another example, it appears in a discussion related to isolation[2] of "the compounds of the disclosure … with respect to the macromolecular starting materials used in their production." *Id*. at 39:16-19. The specification therefore provides no direct insight into the meaning of the term other than functionality being an important feature of macromolecules in the context of the invention described and claimed.

During prosecution of the reissue, when challenged to provide support for the "macromolecule" limitation of claim 15, the named inventors stated the following: "[the] macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage is described at col. 10, lines 9–16 of U.S. Patent No. 8,598,121, Exhibit 25 ('121 patent); Exhibit 22. That cited portion of the specification—which does not use the word "macromolecule"—reads as follows:

> The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG molecules) that may vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP.

Exhibit 25 at 10:9–16. Thus, according to the prosecution history, this passage defines the "macromolecule." It is the product of any of a variety of hydrophilic or water soluble polymers

[2] To the extent that the specification uses "isolation" in connection with CNP variants, it refers to the production of CNP variants, e.g. "[t]he expressed fusion polypeptide can be isolated from the host cell or culture medium, and the isolated fusion polypeptide can be contacted with a cleaving agent to release the CNP variant." RE'267 at 71:50-53.

[redacted]

conjugated to a CNP outside the cyclized region. The specification also talks about a PEGylated CNP variant that is "further conjugated" to "a polymeric group." RE'267 at 9:9–20. The specification also makes clear that the reason why this passage describes the polymer as attached to the peptide at the ends and preferably not in the cyclized region (i.e., the 17-amino-acid ring) is to maintain functionality when the polymer is attached. *Id*. at 80: 19-22.

In the context of claim 15, the macromolecule therefore includes a CNP variant listed in the Markush group (including any PEGylation or mutation as listed) and an *additional* conjugated synthetic polymer *that does not interfere with CNP activity*.

It is a black-letter principle of claim construction that, when a specification contains a number of examples of a claim term, "this can be a good indication" that the term is limited to "additional items of the same kind." *Power Integrations, Inc. v. Fairchild Semiconductor Int'l, Inc.*, 711 F.3d 1348, 1362 (Fed. Cir. 2013); *see also AstraZeneca AB v. Mutual Pharm. Co.*, 384 F.3d 1333, 1341 (Fed. Cir. 2004). Where the specification contains no indication that there are any other "items of the same kind," the phrase may be limited to the listed examples only. *See, e.g.*, *Sinorgchem Co., Shandong v. Int'l Trade Comm'n*, 511 F.3d 1132, 1136-39 (Fed. Cir. 2007) (finding that phrase after "e.g." provided a definition of the disputed term); *see also Intellectual Ventures II LLC v. FedEx Corp.*, 2017 WL 5896180, at *18 (E.D. Tex. Nov. 29, 2017) (construing "entity" to mean "an individual, an automated device, or a robot," where specification recited "a system and method for determining the identity of an entity, (e.g., an individual or an automated device)" because specification required that the "entity" be an "active" device).

That principle applies here. As previously noted, the named inventors tested several examples for NEP resistance and biological activity (as measured by an *in vitro* cGMP assay). RE'267 at Table 3 and Table 4. Only those variants that exhibited both NEP resistance and

[redacted]

biological activity progressed on to *in vitro and in vivo* experiments. For instance, the embodiments and selection criteria described in the RE'267 patent at 6:13–35, 9:25–46, 10:11–25, 10:33–43, 46:56–47:6, 60:40–43, 68:13–30, 115:27–63, and in Examples 3, 4, 5, 8A, 10, and 12–19 provide functionally active macromolecules.

Complainant's and Staff's constructions give no meaning to the word macromolecule, and contend that it is not limiting at all. Both construe the word "macromolecule" in a vacuum, to mean a molecule with a molecular mass greater than a few thousand Daltons. But the word "macromolecule" is not being used in a vacuum here. There is important context in the claims and prosecution history that cannot be ignored. As noted above, the stated purpose of the invention is "making CNP resistant to NEP while retaining its functionality." RE'267 at 4:33–35. Only candidate molecules that satisfied both of these criteria were considered viable for further experimentation, which ultimately led to the development of BioMarin's Voxzogo product. The term "macromolecule" should therefore be construed to mean "a functionally active molecule with a molecular mass greater than a few thousand Daltons." That construction is the only way to give the term a meaning consistent with the specification as a whole.

H. "synthetic polymeric group" (claims 15–20, 31–48)

Complainant's Construction	Staff's Construction	Respondents' Construction
a man-made large molecule formed by the polymerization of molecules of low molecular mass	a man-made large molecule formed by the polymerization of molecules of low molecular mass	a man-made large molecule formed by the polymerization of molecules of low molecular mass that, when coupled to CNP, confers NEP resistance to CNP while retaining CNP's functionality

A *polymer* is a large molecule formed by the linking of smaller unit molecules. In chemical parlance, a "mer" is a building block. "Poly" means many. Accordingly, a "polymer" is a molecule made from many repeated building blocks. A *synthetic* polymer is man-made as

[redacted]

opposed to naturally occurring. As discussed above, PEG is the synthetic polymer discussed in the RE'267 patent specification.[3]

A *group* in chemical parlance is a collection of atoms in a molecule that has some functional identity and acts as a unit. *See*, Exhibit 6 (Facts On File Dictionary) at 97 (defining "functional group"). For example, an amino acid has a carboxyl group (-COOH) and an amino group (-NH_2). When amino acids are linked together to form a peptide, the carboxyl group of one amino acid reacts to form a bond with the amino group of the adjacent amino acid. (These bonds are, incidentally, the so-called peptide bonds that the Examiner described during prosecution as "a type of amide bond, that can be broken by amide hydrolysis, wherein the bonds are cleaved with the addition of a water molecule." (5/10/2019 Office Action, 9).

Every time PEG or synthetic polymeric groups are disclosed in the specification, their function is to confer NEP resistance to the coupled CNP while retaining the CNP's ability to stimulate cGMP. *See, e.g.*, RE'267 at 7:9–39, 9:9–9:46, 10:33-43, 115:38–43, 61:48–57; Examples 1–19 (including FIGs. and Tables), Table 4. The specification explains that the contemplated length of the PEG is from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa—large enough to inhibit NEP degradation but small enough to preserve CNP functionality. RE'267 at 10:42-43. As discussed above, when the specification contains a number of examples of a claim term that all have a common property, and no examples that lack this property, the term is properly construed as limited to items of the same kind. *See Power Integrations*, 711 F.3d at 1362; *AstraZeneca*, 384 F.3d at 1341; *Sinorgchem*, 511 F.3d at 1136-39; *Intellectual Ventures*, 2017 WL 5896180, at *18.

[3] The specification does not disclose any example of a synthetic polymeric group that is not a PEG, which the specification defines as having a particular formula. *See* RE'267 at 43:29-56 (disclosing "CH_3—[—O—CH_2CH_2—]$_n$—C(=O)—NHR, where *n* is the number of ethylene oxide units and R denotes the rest of the peptide").

[redacted]

A *synthetic polymeric group*, therefore, denotes a man-made large group of atoms formed by the polymerization of molecules of low molecular mass that has a specific function and acts as a functional unit. Respondents' construction therefore gives the term its ordinary and customary meaning, while also giving meaning to all of the words of the term in the context of the claimed invention.

Complainant's and Staff's construction correctly reflects the plain meaning of synthetic polymer, but reads out the word "group" and does not reflect the express function of the synthetic polymeric group as described in the specification. There is no support in the specification for a synthetic polymeric group in the context of the invention that has any function other than the function reflected in Respondents' construction.

I. "contacting the/a cell" (claims 21, 22, 49, and 54)

Complainant's Construction	Staff's Construction	Respondents' Construction
contacting the/a cell, including NPR-B	touching the/a cell itself	touching the/a cell itself

The phrase "contacting the/a cell" is a broad but straightforward term that, in the context of molecular biology and pharmacology, refers to touching the cell itself. Staff and Respondents' construction—"touching the/a cell itself"—reflects this plain meaning.

Meanwhile, Complainant's construction is self-contradictory. Complainant states that the term needs no construction, but then imports an additional limitation, namely, "including NPR-B." The named inventors made a deliberate decision to use the broad term "contacting the cell" instead of a more specific term such as "binding to NPR-B." The named inventors were aware of what binding to NPR-B meant, as the specification is replete with reference to substrates binding to NPR-B. *See e.g.*, RE'267 at 2:14-15 ("Both CNP-53 and CNP-22 bind similarly to NPR-B").

[redacted]

In fact, the specification uses the term "bind" or "binding" dozens of times, but the claims do not use the term even once.

By choosing to broaden the claim to encompass contact with any part of the cell, the named inventors risked having their claims invalidated for lack of sufficient written description and enablement. These risks cannot be mitigated through claim construction. *See Elekta Instrument S.A. v. O.U.R. Sci. Int'l, Inc.*, 214 F.3d 1302, 1309 (Fed. Cir. 2000) (declining to "construe the claim differently from its plain meaning in order to preserve its validity").

The term "contacting" is common and easily understood. A POSITA would understand that "contacting" does not require binding, activation, internalization, or a receptor-specific interaction. Rather, as with a lay person's understanding, "contacting" denotes making contact with, *i.e.*, touching, the cell itself.

The intrinsic evidence aligns with Respondents' and Staff's proposed construction of "contacting." The term appears only a few times in the specification, and never in reference to NPR-B. Instead, "contacting" is used in the context of general exposure. For instance, the specification describes contacting a fusion polypeptide with a cleaving agent and minimizing contact between a CNP variant and water. *See* RE'267 at 27:65–67, 28:27–29, 71:51–53 and 139:40–42. These examples support that "contacting" carries its plain and ordinary meaning—referring to a physical interaction, such as touching.

The extrinsic evidence further aligns with Respondents' and Staff's proposed construction. Dictionaries at the time of the priority date of the RE'267 patent define "contacting" to mean "touching." *See* Cambridge Dictionary of American English, 179 (2d ed. 2008), Exhibit 26 (defining "contact" as "the touching of two objects or surfaces"); *see also* Concise Oxford English Dictionary, 307 (11th ed. 2008) (defining "contact" as "the state or condition of physical touching"), Exhibit 27. Complainant has neither argued nor can reasonably

[redacted]

argue otherwise; its proposed construction, which merely repeats the claim language regarding "contacting," underscores this point.

Complainant's insertion of the phrase "including NPR-B" into its proposed construction is also inconsistent with the plain and ordinary meaning of the term "cell." Complainant's construction redefines the meaning of "cell" to include "NPR-B." But the intrinsic evidence shows that cells and NPR-B are distinct concepts. *E.g.*, RE'267 at col. 21 (reciting "in a cell expressing natriuretic peptide receptor B (NPR-B)," "contacting the cell expressing NPR-B"); 2:65-3:1 (describing NPR-B being expressed only by certain cells). The inappropriateness of Complainant's limiting construction is especially evident in claims 22 and 54, neither of which are limited to cells that even express NPR-B.

If the named inventors had wanted to limit the meaning of "cell" it should have made a clear and express statement evincing its intent to act as its own lexicographer, but did not. *Thorner v. Sony Comput. Entm't Am. LLC*, 669 F.3d 1362, 1365 (Fed. Cir. 2012) ("To act as its own lexicographer, a patentee must clearly set forth a definition of the disputed claim term other than its plain and ordinary meaning."). Absent such a definition, the relevant claims provide that "cell" is distinct from "NPR-B." If the named inventors wanted to specifically claim the CNP variant contacting NPR-B, it could have done so. The specification provides many examples indicating that the named inventors plainly knew how to phrase such a claim. *E.g.*, RE'267 at 11:22-28, 13:55-60, 22:27-33, 22:33-37, 23:17-21, 46:63-47:6 (all describing a CNP variant binding to NPR-B).

Respondents' and Staff's construction—"touching the/a cell itself"—provides a clear and enforceable boundary for the scope of the claims. It is consistent with the intrinsic evidence, reflects the ordinary meaning of the term, and avoids ambiguity. Complainant's construction

[redacted]

should be rejected because it improperly imports a limitation, equates "cell" and "NPR-B," and lacks support in the intrinsic evidence.

VII. CONCLUSION

The CALJ should adopt Respondents' proposed constructions of the disputed clam terms.

Certificate of Word Count

Pursuant to Ground Rule 7.2, counsel for Respondents certify that this Opening Claim Construction Brief contains 9,865 words. The word count function of Microsoft Word was used to prepare this Objection.

Dated: October 8, 2025

Respectfully submitted,

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CERTAIN DRUG PRODUCTS CONTAINING C-TYPE NATRIURETIC PEPTIDE VARIANTS, AND COMPONENTS THEREOF	**Inv. No. 337-TA-1447**

CERTIFICATE OF SERVICE

I, hereby certify that on this day, October 8, 2025, a true and correct copy of the foregoing **RESPONDENTS' OPENING CLAIM CONSTRUCTION BRIEF** was served upon the following as indicated:

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UNITED STATES INTERNATIONAL TRADE COMMISSION
WASHINGTON, D.C.

Before The Honorable Clark S. Cheney
Chief Administrative Law Judge

In the Matter of CERTAIN DRUG PRODUCTS CONTAINING C-TYPE NATRIURETIC PEPTIDE VARIANTS, AND COMPONENTS THEREOF	Inv. No. 337-TA-1447

DECLARATION OF JONATHAN L. SESSLER, PH.D.
IN SUPPORT OF RESPONDENTS' OPENING CLAIM CONSTRUCTION BRIEF

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I, Jonathan Sessler, hereby declare as follows.

I. INTRODUCTION

1. I have been retained as an expert witness on behalf of the Respondents for the above-captioned proceeding. I am being compensated for my time in connection with this ITC proceeding at my standard consulting rate, which is $750 per hour.

2. I understand that this declaration accompanies a claim-construction brief defining certain terms of the U.S. Patent No. RE48,267 (Exhibit 1, "the RE'267 patent"). I understand that the Complainant, BioMarin, asserts that RE'267 patent has a priority date of May 20, 2009.

3. In preparing this Declaration, I have reviewed the RE'267 patent and each of the documents cited herein, in light of general knowledge in the art before May 20, 2009. In formulating my opinions, I have relied upon my experience, education, and knowledge in the relevant art. In formulating my opinions, I have also considered the viewpoint of a person of ordinary skill in the art (POSA) (as defined further below in Section VI) prior to May 20, 2009.

II. MY BACKGROUND AND QUALIFICATIONS

4. I am the Doherty-Welch Chair in Chemistry at The University of Texas at Austin. I have been a member of the faculty of this department since 1984. I received my B.S. in Chemistry with the Highest Honors from the University of California, Berkeley in 1977. I received my Ph.D. in Organic Chemistry from Stanford University in 1982.

5. Following the completion of my doctorate, I worked as an NSF-CNRS and NSF-NATO Postdoctoral Fellow at L'Université Louis Pasteur de Strasbourg, France. After a brief stay in Japan, I joined the faculty at The University of Texas at Austin in 1984 as an Assistant Professor and was subsequently promoted to a full professorship with tenure in 1992.

6. Over the past several decades, I have taught and conducted research in organic chemistry. My areas of research have included the study of redox-active compounds for the

design and synthesis of new chemical entities of biological interest, as well as the application of inorganic chemistry, biochemistry, spectroscopy, and synthetic organic chemistry to synthesize molecules with specific desirable properties, such as pharmaceutical lead compounds.

7. I have authored or co-authored hundreds of research publications on chemical methodology, synthetic organic chemistry, and pharmaceutical drug design in high-impact, peer-reviewed journals, with over 200 in the Journal of the American Chemical Society alone. My work has been cited more than 42,000 times not counting self-citations. My h-index (Web of Science) is 113.

8. I previously served as the Associate Editor for *ChemComm* and am currently, or have previously been, a member of the editorial advisory boards of peer-reviewed journals including *Inorganic Chemistry*, the *Journal of Porphyrins and Phthalocyanines*, the *Journal of Organic Chemistry*, the *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *Chem*, and *Accounts of Chemical Research*. In addition to authoring several books, I am also the inventor of record on more than 75 U.S. patents and have presented around 700 invited lectures at scientific meetings, educational institutions, and pharmaceutical companies. In 2021, I was elected to the U.S. National Academy of Sciences.

9. In addition to my decades of research and work in academia, I was also the co-founder of Pharmacyclics, Inc., a publicly traded company later acquired by AbbVie dedicated to developing new cancer therapies. As part of my duties at Pharmacyclics, I oversaw work on conjugate development, including those based on both cleavable and non-cleavable linkers. More recently, I co-founded InnovoTEX, Inc., a company whose lead agent is a cleavable Pt(IV) conjugate. I have continued my research and development efforts involving conjugates as an academic and have published extensively on the topic. I am qualified to opine on the subject of non-cleavable and cleavable linkages, including hydrolysable linkages.

10. Accordingly, I am an expert in the creation of conjugates containing bioactive moieties linked through both cleavable and non-cleavable linkages, including cleavable linkers developed in my laboratory. I am familiar with PEGylation chemistry and have used it in the context of my own drug discovery and development work both as an academic and through my entrepreneurial activities. For that reason, I am qualified to provide an opinion as to what a person of ordinary skill in the art would have understood, known, or concluded as of the dates referenced herein. As an expert, I offer opinions throughout this declaration from the perspective of a POSA.

11. A copy of my Curriculum Vitae is attached to this declaration as Exhibit 2.

III. RELEVANT LEGAL PRINCIPLES

12. I understand from counsel that the claim terms are given the ordinary and customary meaning that they would have to a POSA, in light of the specification and prosecution history, as of the filing date of the patent. I understand that dictionary definitions are considered extrinsic evidence, which is secondary to the intrinsic evidence of the claims, specification, and prosecution history. But dictionaries—especially specialized dictionaries used by those in the art—may shed light on the ordinary and customary meaning that terms would have to a person of ordinary skill in the art.

IV. SUMMARY OF OPINIONS

13. Based on my extensive experience in chemistry and my familiarity with the terms "hydrolysis" and "hydrolysable linkage," after reviewing the RE'267 patent's specification and prosecution history, it is my opinion that a POSA as of May 20, 2009, would have understood that these terms are being used consistently with their formal and precise definition in the art. Similarly, "hydrolysis" refers to a reaction between a compound and water in which a bond linking two components in the compound is cleaved ("lysed") and the water ("hydro")

components (H^+ and OH^-) are added where the bond was broken. Likewise, a "hydrolysable linkage" refers to a chemical bond that is cleaved by the addition of a water molecule. These ordinary and customary meanings are best captured in Respondents' proposed claim constructions for these terms.

V. BACKGROUND AND CONTEXT

14. The RE'267 patent issued from U.S. Appl. No. 15/646,822 ("the '822 application"), filed July 11, 2017. The '822 application is a continuation of U.S. Appl. No. 14/604,262 (now RE46,707 (RE'707), filed on January 23, 2015 as a reissue application of U.S. Patent No. 8,598,121 (the '121 patent). The '121 patent issued from U.S. Appl. No. 13/466,672 (the '672 application), filed on May 8, 2012 as a continuation of U.S. Application No. 12/784,117 (the '117 application), filed on May 20, 2010. The '117 application issued as U.S. Patent No. 8,198,242 (the '242 patent) and claims the priority benefit of U.S. Provisional Appl. Nos. 61/180,112 (the '112 application), filed May 20, 2009, and 61/252,563 (the '563 application), filed October 23, 2009.

15. The RE'267 patent discloses a strategy for increasing the serum half-life of CNP peptides by—among other things—modifying the peptides by adding, e.g., "hydrophilic or water-soluble polymers," such as polyethylene glycol ("PEG") to "wtCNP22 or a variant thereof." RE'267 patent, 4:41−46, 6:13−17; 6:20−25; 9:9-25. The RE'267 patent states that, PEGylated CNPs have "increased serum half-life" as a result of "reduced ability to bind to neutral endopeptidase (NEP), greater resistance to proteolysis by NEP and/or reduced affinity to the clearance natriuretic peptide receptor C(NPRC), while retaining the functionality of CNP." RE'267 patent, 4:42-46.

16. The RE'267 patent does not specify a particular type of PEG for conjugation, stating that: "homopolymer or copolymer; random, alternating or block copolymer; linear or

branched; monodispersed or polydispersed." RE'267 patent, 10:35-37. The RE'267 patent also does not indicate the nature of the linkage, stating: "e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond." RE'267 patent, 10:37-38; *see also* 60:63-61:19. This is the only context in which the specification mentions a "hydrolysable linkage."

17. Claims 15-20 and 31-48 recite macromolecules comprising "a CNP variant" and "a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage." RE'267 patent, 274:21-276:53, 279:51-281:13. The claims require that "hydrolysis of the hydrolysable linkage releases the CNP variant." RE'267 patent, claims 15-17, 23-26, 31-39. Specifically, independent claim 15 recites:

> A macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of [15 listed "CNP variants"] and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

RE'267 patent, 269:64-270:65.

18. Claims 16, 17, and 31-39 of the RE'267 patent depend directly or indirectly from claim 15 and specify additional limitations.

19. Independent claim 18 recites:

> A sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of [15 listed "CNP variants"].

20. RE'267 patent, 271:4-272:5. Claims 19, 20, and 40-48 of the RE'267 patent depend directly or indirectly from claim 18 and specify additional limitations.

VI. PERSON OF ORDINARY SKILL IN THE ART

21. I understand that a POSA is a hypothetical person who is presumed to be aware of all pertinent art, thinks along conventional wisdom in the art, and is a person of ordinary creativity.

22. I believe that a POSA developing PEGylated CNP peptides typically would have had a Ph.D. in polymer, organic, protein, bioconjugation, or biological chemistry and also typically be a chemist with experience or training in the preparation, development, and use of both cleavable and non-cleavable linkers and their application to the creation of conjugates containing bioactive moieties. I believe I am at least a POSA in this field as of 2009.

23. A POSA may also have worked as part of a multi-disciplinary team and drawn upon not only his or her own skills, but also taken advantage of certain specialized skills of others in the team that might lie outside the POSA's primary training, to solve a given problem. For example, such a team may be comprised of a physician with knowledge of cartilage and bone biology or a biologist specializing in natriuretic peptide structure and function, a protein biochemist specializing in chemical peptide synthesis, or a pharmacologist.

24. I base this definition of a POSA based on review of the RE'267 patent and its prosecution. I also base it on my experience in organic and inorganic chemistry and having taught many students who would also fit the definition supplied above.

VII. CLAIM CONSTRUCTION DISPUTE

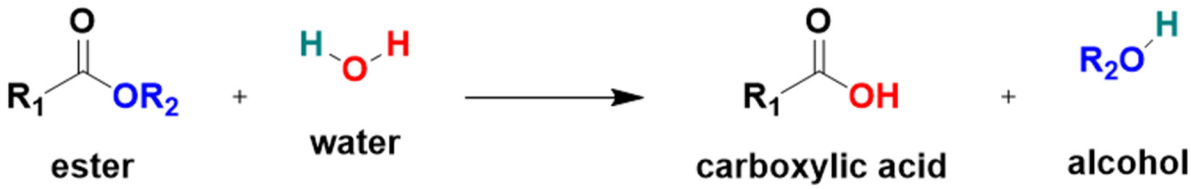
25. I understand that the parties dispute the meanings of the terms "hydrolysis" and "coupled to the CNP variant through a hydrolysable linkage" that appear in claims 15-20 and 31-48. I understand that the parties have proposed competing constructions for these terms.

A. Hydrolysis

26. The RE'267 patent claims recite "hydrolysis" in the context of a "hydrolysable linkage" attaching a "synthetic polymeric group" (e.g. PEG) to a peptide (CNP). The specification does not define hydrolysis. Nor does it discuss any hydrolysis reactions. It provides that "[d]efinition of standard chemistry terms may be found in reference works, including Carey and Sundberg, *Advanced Organic Chemistry*, 3rd Edition, Vols. A and B (Plenum Press, New

York 1992).” RE’267 patent at 34:57-60. Hydrolysis is a standard chemistry term. Carey does not purport to formally define hydrolysis, but uses the term extensively and provides several examples of reactions that it identifies as “hydrolysis.” *See, e.g.*, Exhibits 3 and 4 (Carey Ch. 4 and Ch. 8), Chapter 4: 226-227, 238; Chapter 8: 465-70, 473-75. Even without a definition, a POSA would understand the meaning of “hydrolysis” because hydrolysis reactions were well understood in the art as of 2009 and had a well-defined plain and ordinary meaning. Hydrolysis in the context of PEGylation and reversible PEG-assisted peptide delivery was also well understood as of May 2009. *See, e.g.*, Francesco M. Veronese & Anna Mero, *The Impact of PEGylation on Biological Therapies*, 22 Biodrugs 320, 323, 326 (2008), Exhibit 5 (Veronese).

27. Specifically, hydrolysis is a chemical reaction involving the cleavage of a chemical bond that serves to link two portions of a molecule together, and this cleavage occurs with the formal addition of a water molecule at the site of the broken bond. Water acts as a reactant in this reaction. A classic illustration is shown below:



In this illustration, the balanced chemical equation shows that an ester molecule reacts with a water molecule, resulting in cleavage of the ester bond that holds together the two portions of the molecule and splitting of water. Specifically, the water molecule attacks the carbonyl (a C=O bond) of the ester. When the water molecule is added to the carbonyl, a new C-O bond is formed between the carbonyl carbon and the oxygen atom of water, and the C-OR_2 bond is broken. The final products of this reaction are obtained after proton transfers, often assisted by the solvent. These final products are a carboxylic acid, which incorporates an OH (red) from water into its new structure, and an alcohol, which incorporates an H (green) from water into its new structure.

28. It is important to note that the H^+ and OH^- components of the water molecule are added across the cleaved bond. This means they are bound to the newly formed product(s) of the reaction at the location where the original bond linking two components is broken. Generally, when a bond between atoms is cleaved in a hydrolysis reaction, the pair of electrons making up the bond goes to one atom where the bond was broken, creating an incipient minus charge on one side of the broken bond, and an incipient plus charge on the other side. To balance this charge imbalance, the water molecule donates a proton (H^+) to the atom with the extra pair of electrons (minus charge), and a pair of electrons (in the form of OH^-) directly to the atom with the plus charge on the other side.

29. Accordingly, the plain meaning of "hydrolysis" is a "reaction between a compound and water in which a bond in the compound broken ("lysed") and the compound itself is cleaved and the water ("hydro") components (H^+ and OH^-) are added where the bond was broken." As discussed further below, this construction reflects how a POSA would have understood the term hydrolysis in the context of the RE'267 patent. I understand that this is the construction that Respondents propose.

30. I note that two additional dictionaries used in this art also support Respondent's proposed construction, to the extent dictionaries are informative here:

- *The Facts On File Dictionary Of Organic Chemistry*, Ed. Daintith (2004) ("A reaction between a compound and water. An example is the hydrolysis of an ESTER to give a carboxylic acid and an alcohol: $CH_3COOC_2H_5 + H_2O \rightleftharpoons CH_3COOH + C_2H_5OH$.") (115), Exhibit 6 (Facts on File Dictionary);
- *Glossary of Terms Used in Physical Organic Chemistry* (IUPAC Recommendations 1994), International Union of Pure and Applied Chemistry, Organic Chemistry Division, Commission on Physical Organic Chemistry, Hydrolysis: "Solvolysis by water" (1123); Solvolysis: ("Generally, reaction with a solvent, or with a lyonium ion or lyate ion, involving the rupture of one or more bonds in the reacting solute. More specifically the term is used for substitution, elimination and fragmentation reactions in which a solvent species is the nucleophile ('alcoholysis' if the solvent is an alcohol, etc.).") (1165), Exhibit 7

(IUPAC Glossary).

I have reviewed the definitions in these dictionaries and can confirm that they are consistent with and reinforce my opinions as to the ordinary and customary meaning that the term "hydrolysis" would have had to a POSA at the relevant time, in the context of reviewing the RE'267 patent.

31. I understand that BioMarin and the Office of Unfair Import Investigations (OUII) contend that the plain and ordinary meaning of "hydrolysis," is "chemical reaction in which water reacts with another substance to form two or more new substances." This construction is problematic for at least three reasons.

32. First, Complainant's proposed construction appears to be an excerpt taken out of context from the definition of "hydrolysis" provided in *Hawley's Condensed Chemical Dictionary*, 15th Edition, Ed. Lewis (2007). Hawley's defines hydrolysis as:

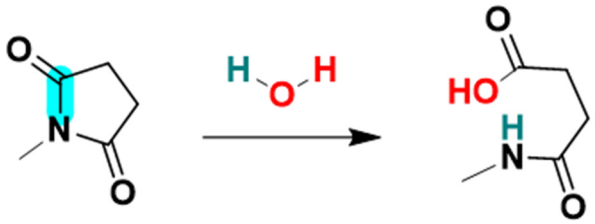
> A chemical reaction in which water reacts with another substance to form two or more new substances. This involves ionization of the water molecule as well as splitting of the compound hydrolyzed, e.g., $CH_3COOC_2H_5 + H_2O \rightarrow CH_3COOH + CH_3OH$. Examples are conversion of starch to glucose by water in the presence of suitable catalysts; conversion of sucrose (cane sugar) to glucose and fructose by reaction with water in the presence of an enzyme or acid catalyst; conversion of natural fats into fatty acids and glycerol by reaction with water in one process of soap manufacture; and reaction of the ions of a dissolved salt to form various products, such as acids, complex ions, etc." Exhibit 8 (Hawley's)

Id. at 688. Complainant's construction reproduces only the first sentence of this definition.

33. As a preliminary matter, the first sentence of this definition in isolation does not provide a full or precise definition of hydrolysis because it does not acknowledge the ionization of water or splitting of the compound being hydrolyzed. That is part of what defines a hydrolysis reaction. That deficiency alone makes Complainant's proposal a misleading and unhelpful claim construction, because it does not provide a complete understanding.

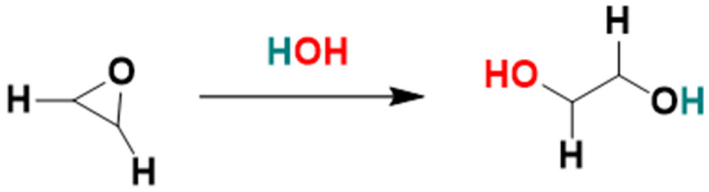
34. But the bigger problem is an imprecision in the first sentence of Hawley's definition itself. Specifically, a bond may be cleaved by hydrolysis *without* forming "two or

more new substances," as the Complainant's proposal requires. For instance, a molecule may have a ring structure, and hydrolysis of one of the bonds may open the ring to create a linear structure. In that reaction, only one new substance is formed. To illustrate, a well-known reaction that I teach in my organic chemistry classes, and which a POSA would know of, is that of succinimide ring-opening hydrolysis, shown below.

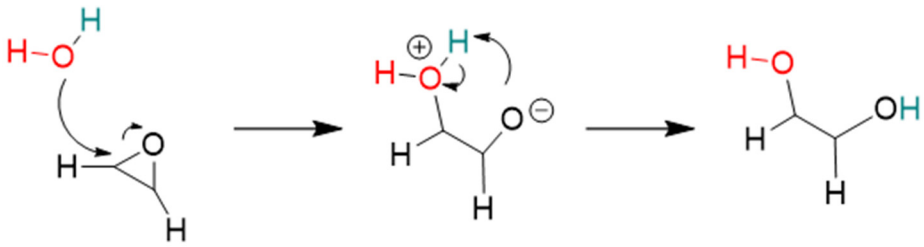


35. The left side shows a succinimide, which is hydrolyzed at its amide-like bond (blue) to form 4-(methylamino)-4-oxobutanoic acid on the right. Here, the formal addition of water cleaves the bond, adding an OH^- (in red) to one side and H^+ (in green) to the other at the sites where the bond was broken, but only one new substance is formed. This is nonetheless a hydrolysis reaction, and a person of ordinary skill in this art would have understood it as such. Yet, Complainant's proposed claim construction for "hydrolysis" would improperly exclude this example. A recent example of this type of chemistry is described as a "ring-opening hydrolysis reaction," as described in Wenmao Huang *et al*., *Maleimide-Thiol Adducts Stabilized Through Stretching*, 11 Nat. Chem. 311, Figure 1a (2019), *see* Exhibit 9 (Huang).

36. Another such example is that of epoxide hydrolysis. This is another example of a basic organic chemistry reaction that I teach in my organic chemistry classes and of which a POSA would be aware. An example of epoxide hydrolysis is shown below.



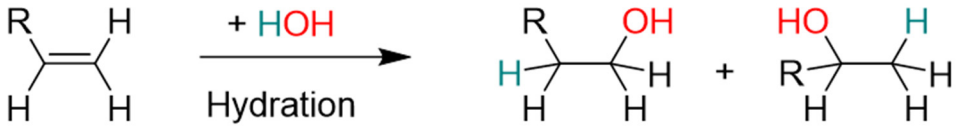
Here, water cleaves an O-C bond, such that OH^- is added formally to one side of the cleaved bond at the C, with H^+ added to the other side of the cleaved bond at the O released as the result of ring opening. Once again, only one new substance is formed. This is, again, a hydrolysis reaction, despite the fact that only one new substance is formed, and a POSA would recognize it as such. A simplified mechanism showing this ring-opening hydrolysis is shown below, with water cleaving the O-C bond and OH^- (red) being added to one side of the site where the bond was broken, and H^+ (green) being added to the other site where the bond was broken. Complainant's proposed claim construction for "hydrolysis" would improperly exclude this example of epoxide ring-opening hydrolysis.



An example of this type of ring-opening epoxide hydrolysis from just before the time of the patent is described by Zhi Wang *et al*., *Hot Water-Promoted Ring-Opening of Epoxides and Aziridines by Water and Other Nucleopliles*, 73 J. Org. Chem. 2270 (2008), *see* Exhibit 10 (Wang).

37. Conversely, there are well known reactions that are not hydrolysis, that would nonetheless fall within Complainant's proposed definition. One example would be acid-catalyzed hydration of alkenes to produce alcohols. A generalized example, representative of what I teach in my organic chemistry classes, and of which a POSA would be aware, is shown below. It is well-known in the art that hydration of terminal alkenes is known to produce two regioisomers, called Markovnikov (left) and anti-Markovnikov (right) products. A POSA would understand that stereoisomers might be formed in these types of reactions, increasing the

possible number of products. While certain hydration conditions may favor one regioisomer or the other, both products are frequently formed in alkene hydration reactions. As such, this illustrative example shows a well-known reaction, the hydration of a terminal alkene, that falls within Complainant's proposed definition (as it forms at least two products) but nonetheless is not hydrolysis; rather, it is hydration, and a POSA would recognize it as such.[1]



38. A literature example of such a reaction is described by Hayato Yuzawa *et al*., *Anti-Markovnikov Hydration of Alkenes Over Platinum-Loaded Titanium Oxide Photocatalyst*, 3 Catal. Sci. Technol.1743 (2013). Here, Yuzawa shows an example of alkene hydration via a metal catalyst that results in both the Markovnikov and anti-Markovnikov products in a single reaction. A portion of the abstract from this report is reproduced below. *See* Exhibit 11 (Yuzawa).



39. Further, in the specific context of the RE'267 patent and the parties' dispute here, there is another problem with Complainant's and OUII's construction. The definition does not specify that the components of the water molecule bind to one or both products of the reaction at the sites of the cleaved bond, i.e. where the bond and connection between two components

[1] In the *Glossary of Terms Used in Physical Organic Chemistry*, "hydration" is defined as: "Addition of water or of the elements of water (i.e. H and OH) to a *molecular entity*. For example, hydration of ethene: …" (1122) (emphasis original), Exhibit 7 (IUPAC Glossary). A POSA would understand that the term "hydrolysis" refers to a different concept than "hydration."

making up the original molecule was broken. That is required for a reaction to be hydrolysis. In the context of large molecules like PEGylated polypeptides, that may undergo multi-step complex chain reactions, it is important to understand that if the constituent H^+ or OH^- of the water molecule bind to the products of the reaction at a site other than the cleaved bond, then that is an indication that the water molecule itself did not effectuate the cleavage. The bond may have been cleaved by some other mechanism.

40. Accordingly, Respondents' construction for "hydrolysis" is most faithful to the plain meaning of hydrolysis in the context of the specification.

B. Coupled to the CNP variant through a hydrolysable linkage

41. The phrase "coupled to the CNP variant through a hydrolysable linkage" means "connected to the CNP variant through a chemical bond cleaved by the addition of a water molecule." This construction is consistent with the definition of hydrolysis that I provide above. It also clarifies further that a hydrolysable linkage is a chemical bond tethering two components of the original molecule that is broken by hydrolysis in the presence of water. That is the meaning that one of ordinary skill in the art would understand this term to have in the context of the specification. I understand that is the construction that Respondents' propose.

42. The specification does not ever define what it means by a hydrolysable linkage, but hydrolysable linkages in the context of PEGylation and reversible PEG-assisted peptide delivery were well understood as of May 2009. *See, e.g.*, Exhibit 5 (Veronese), 320, 323, 326. A POSA would have understood the plain and ordinary meaning of "hydrolysable linkage" to be a chemical bond cleaved by the addition of a water molecule, which is fully consistent with the meaning of the term "hydrolysis" that I explained above. This definition is also consistent with the specification.

43. Specifically, in discussing "hydrolysable or stable linkage[s]," the specification lists "amide, imine, aminal, alkylene, or ester bond." RE'267 patent, 10:37-39 and 60:63-61:19. To the extent these linkages can undergo hydrolysis,[2] the hydrolysis proceeds as I detail above: the linkage is cleaved, and a water molecule is added to the products across the cleaved bond. The specification also provides an example of a hydrolysable linkage, Sunbright CS, which a POSA would have understood to possess an ester bond that is also cleaved by addition of a water molecule across the bond—i.e., by hydrolysis. *See*, NOF Corporation Catalogue Ver. 8, Exhibit 12 (NOF Catalogue); *see also* Exhibit 4 (Carey Ch. 8) at 465. Thus, all "hydrolysable linkages" described in the RE'267 patent are cleaved by the addition of a water molecule, which is consistent with the plane and ordinary meaning of "hydrolysable linkage."

44. The prosecution history also supports Respondents' construction for the term "coupled to the CNP variant through a hydrolysable linkage." *See, e.g.*, RE'267 patent, 10:33-43 (corresponding to col. 10, lines 9-16 of U.S. Patent No. 8,598,121 cited in 7/11/17 Preliminary Amendment, 28). During prosecution of the RE'267 patent, the Examiner stated: "Long-chain peptides contain amino acids linked via peptide bonds, a type of amide bond, that can be broken by amide *hydrolysis*, wherein the bonds are cleaved *with the addition of a water molecule*." (5/10/2019 Office Action, 9, emphasis added).

45. I understand that BioMarin and OUII contend that the plain and ordinary meaning of "coupled to the CNP variant through a hydrolysable linkage," is "connected to the CNP variant through a connection capable of chemical reaction in which water reacts with another substance to form two or more new substances."

[2] Not all linkages are hydrolysable (e.g., alkylene is not) and most hydrolysable linkages only hydrolyze under certain conditions.

46. This construction repeats the problem of using a portion of Hawley's definition, discussed above, with the additional problem of using the phrase "connected . . . though a connection" which is unclear and implies a connection other than a chemical bond. A hydrolysable linkage is a chemical bond that is cleaved by the addition of a water molecule.

47. Indeed, while the patent does not define "linkage," when the patent provides examples of a "hydrolysable or stable linkage," the patent equates "linkage" to a "bond." RE'267 patent, 10:37-38 ("linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond"); *see also* 60:63-61:19. Additionally, the specification explains that a synthetic polymer conjugated to a CNP peptide "in general has the formula: CH_3—[—O—CH_2CH_2—]$_n$—C(=O)—NHR, where *n* is the number of ethylene oxide units and R denotes the rest of the peptide." RE'267 patent, 43:29-56 (yellow added). This formula confirms that the PEG is coupled to the peptide through a chemical bond (yellow). That is what a person of skill in this art would understand to be meant by the word "linkage" in the context of the RE'267 patent.

48. For that reason, and the other reasons discussed above, Respondents' construction is most faithful to the way a POSA would understand molecular structures to be coupled through a hydrolysable linkage in the context of the specification.

VIII. CONCLUSION

49. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

Executed on this 7th day of October, in Austin, TX.

JONATHAN L. SESSLER, PH.D.

Exhibit 1

US00RE48267E

(19) **United States**

(12) **Reissued Patent**

Wendt et al.

(10) **Patent Number: US RE48,267 E**

(45) **Date of Reissued Patent: *Oct. 20, 2020**

(54) **VARIANTS OF C-TYPE NATRIURETIC PEPTIDE**

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(*) Notice: This patent is subject to a terminal disclaimer.

(21) Appl. No.: **15/646,822**

(22) Filed: **Jul. 11, 2017**

Related U.S. Patent Documents

Reissue of:

(64) Patent No.: **8,598,121**
Issued: **Dec. 3, 2013**
Appl. No.: **13/466,672**
Filed: **May 8, 2012**

U.S. Applications:

(63) Continuation of application No. 14/604,262, filed on Jan. 23, 2015, now Pat. No. Re. 46,707, which is an application for the reissue of Pat. No. 8,598,121, which is a continuation of application No. 12/784,117, filed on May 20, 2010, now Pat. No. 8,198,242.

(60) Provisional application No. 61/254,563, filed on Oct. 23, 2009, provisional application No. 61/180,112, filed on May 20, 2009.

(51) **Int. Cl.**

A61K 38/22	(2006.01)
A61K 38/00	(2006.01)
A61K 38/16	(2006.01)
C12P 21/04	(2006.01)
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A61K 47/60	(2017.01)
A61K 9/08	(2006.01)
A61K 47/10	(2017.01)
A61K 47/20	(2006.01)
A61K 47/22	(2006.01)
A61K 47/26	(2006.01)

(52) **U.S. Cl.**

CPC ***C07K 14/58*** (2013.01); ***A61K 9/08*** (2013.01); ***A61K 38/22*** (2013.01); ***A61K 38/2242*** (2013.01); ***A61K 47/10*** (2013.01); ***A61K 47/20*** (2013.01); ***A61K 47/22*** (2013.01); ***A61K 47/26*** (2013.01); ***A61K 47/60*** (2017.08); *A61K 38/00* (2013.01); *G01N 2333/58* (2013.01); *G01N 2800/10* (2013.01); *G01N 2800/105* (2013.01); *G01N 2800/385* (2013.01); *G01N 2800/52* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — Bruce R Campell

(74) *Attorney, Agent, or Firm* — Marshall Gerstein & Borun LLP

(57) **ABSTRACT**

The present disclosure provides variants of C-type natriuretic peptide (CNP), pharmaceutical compositions comprising CNP variants, and methods of making CNP variants. The CNP variants are useful as therapeutic agents for the treatment of diseases responsive to CNP, including but not limited to bone-related disorders, such as skeletal dysplasias (e.g., achondroplasia), and vascular smooth muscle disorders (e.g., restenosis and arteriosclerosis).

62 Claims, 61 Drawing Sheets

Specification includes a Sequence Listing.

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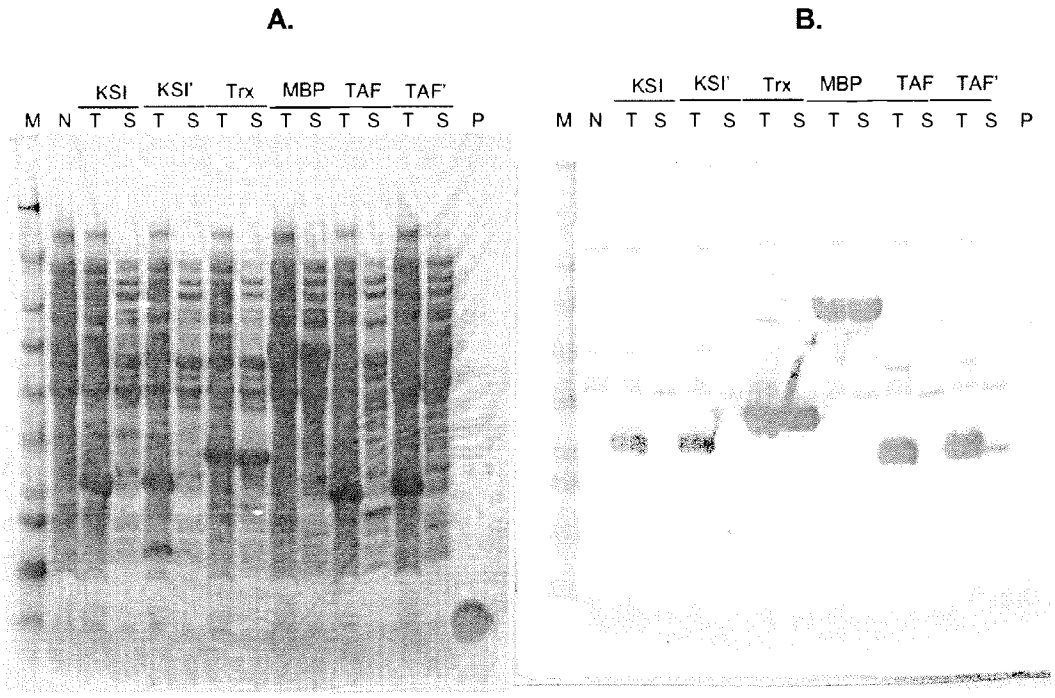
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FIGURE 1



Coomassie blue stain

Western blot with anti-CNP antibody

FIGURE 2

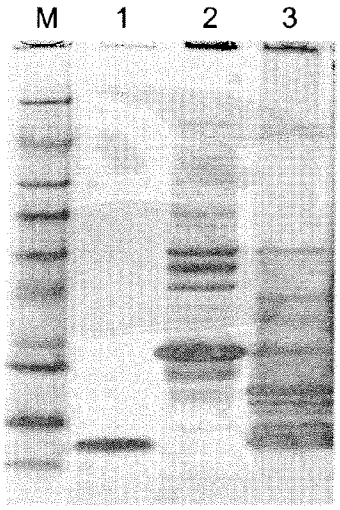


FIGURE 3

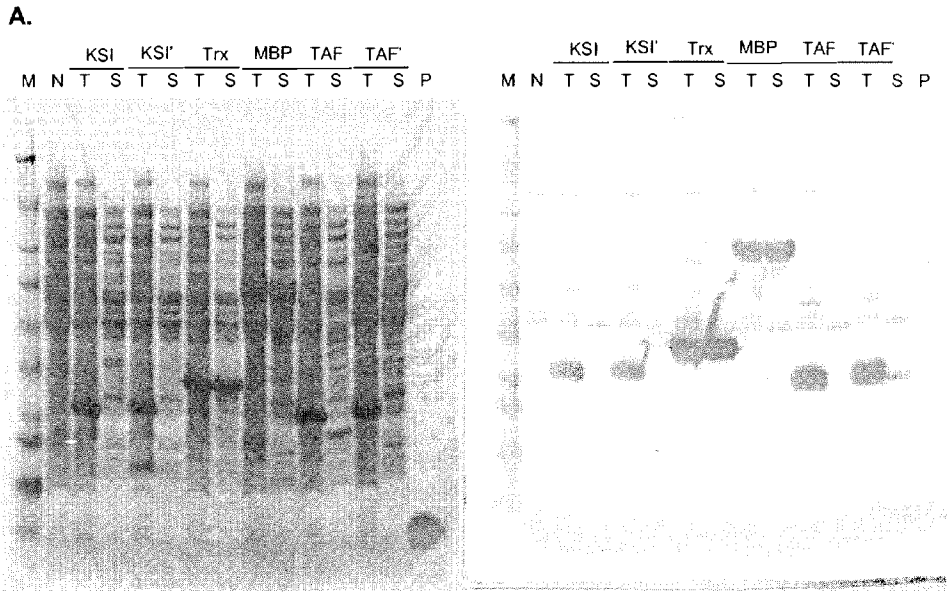

A.
KSI KSI' Trx MBP TAF TAF'
M N T S T S T S T S T S T S P
KSI KSI' Trx MBP TAF TAF'
M N T S T S T S T S T S T S P
Coomassie blue stain
Western blot with anti-CNP antibody
B.
BMP-Pro-CNP37 TAF-Pro-CNP37
M C Tu T S P Tu T S P
BMP-Pro-CNP37 TAF-Pro-CNP37
M C Tu T S P Tu T S P
Coomassie blue stain
Western blot with anti-CNP antibody

FIGURE 3 continued

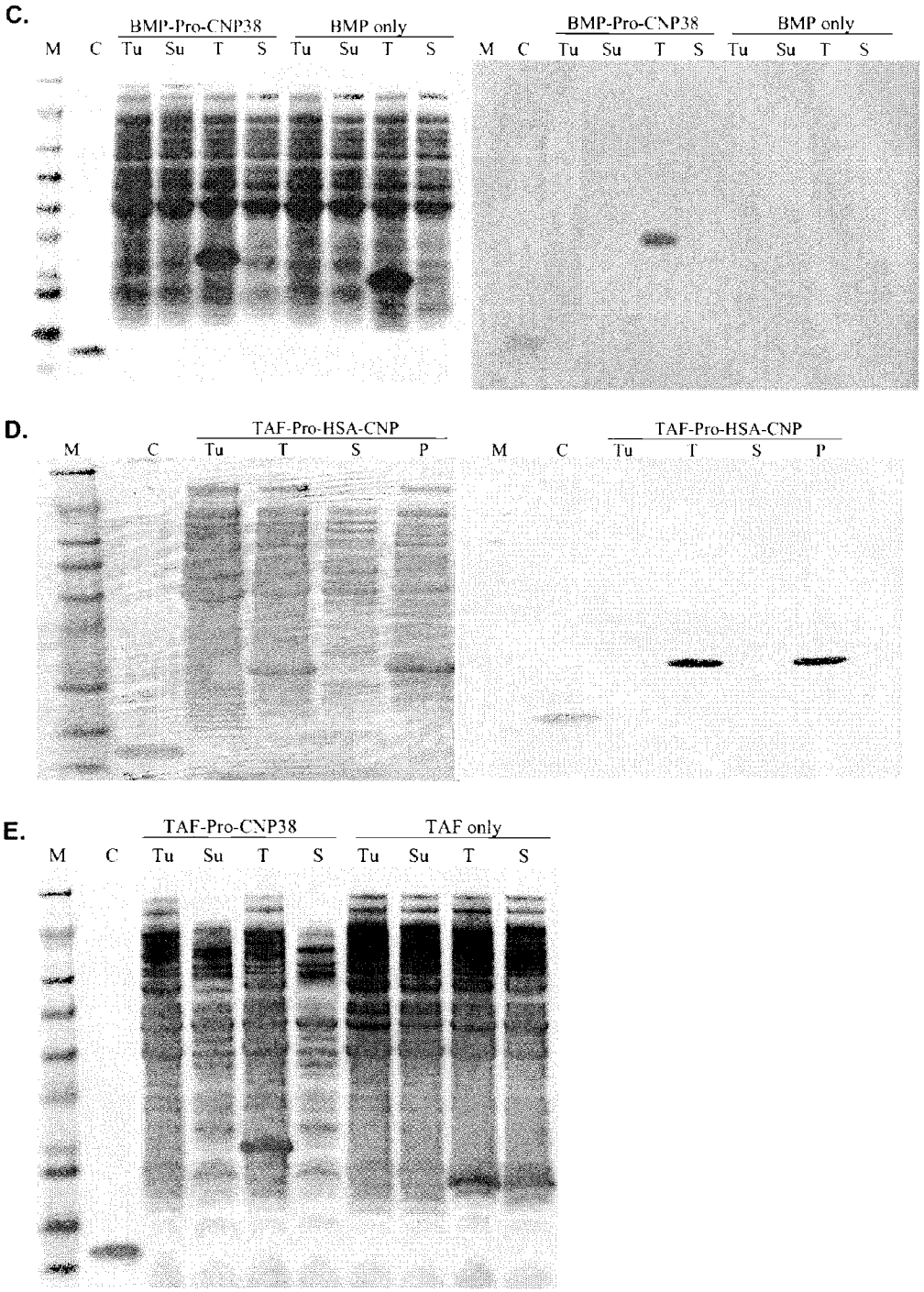


FIGURE 4

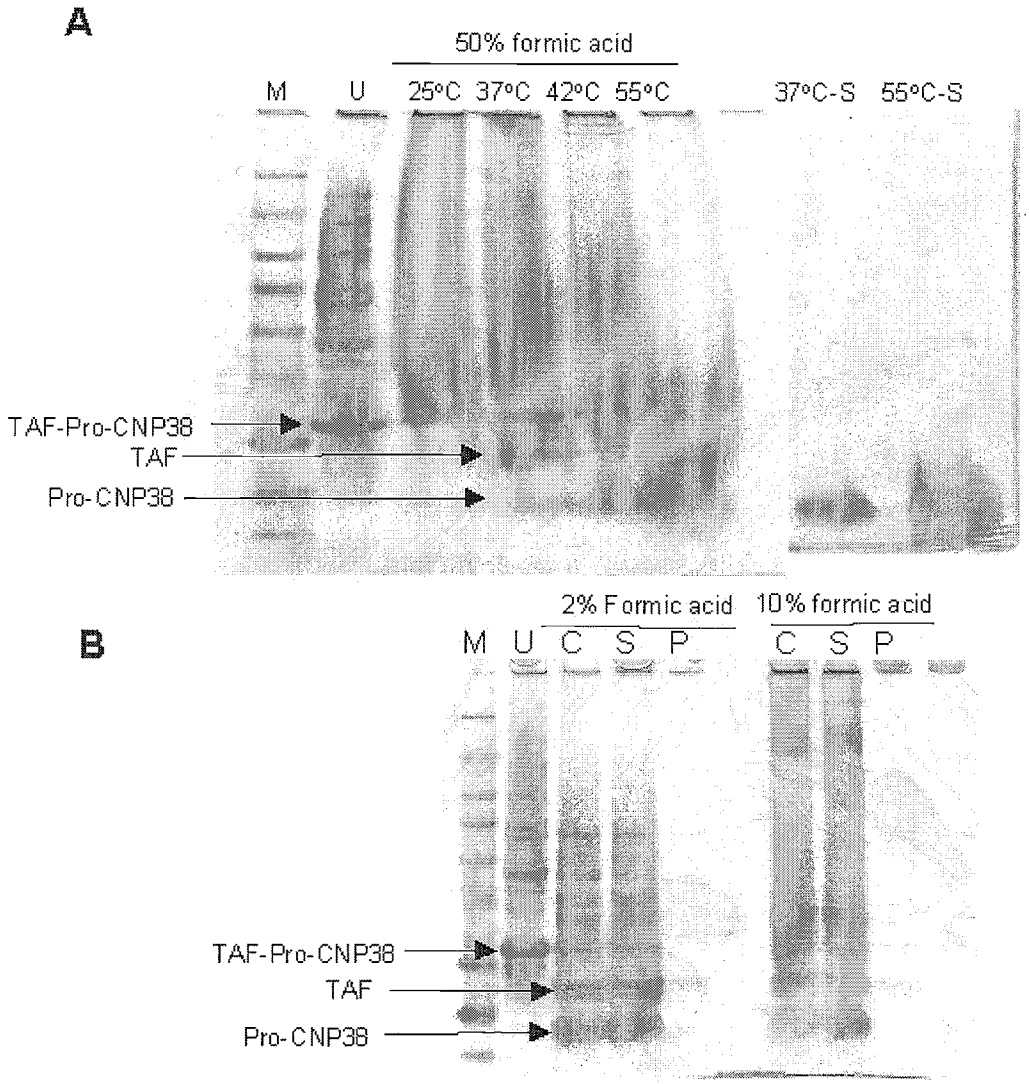

A
50% formic acid
M
U
25°C
37°C
42°C
55°C
37°C-S
55°C-S
TAF-Pro-CNP38
TAF
Pro-CNP38
B
2% Formic acid
10% formic acid
M
U
C
S
P
C
S
P
TAF-Pro-CNP38
TAF
Pro-CNP38

FIGURE 4C

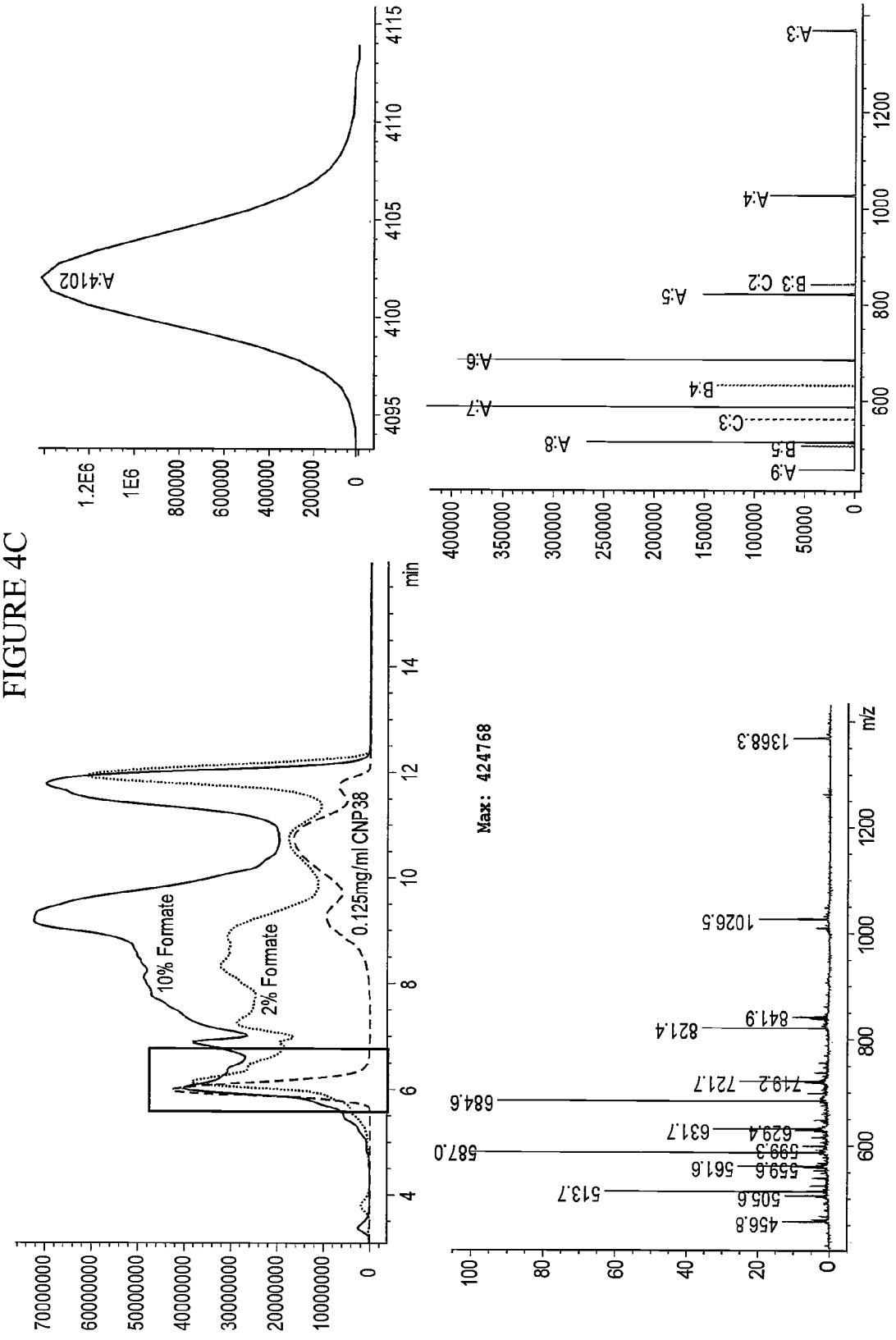

10% Formate
2% Formate
0.125mg/ml CNP38
min
A:4102
Max: 424768
m/z
A:3
A:4
B:3 C:2
A:5
A:6
B:4
A:7
C:3
A:8
B:5
A:9

FIGURE 5

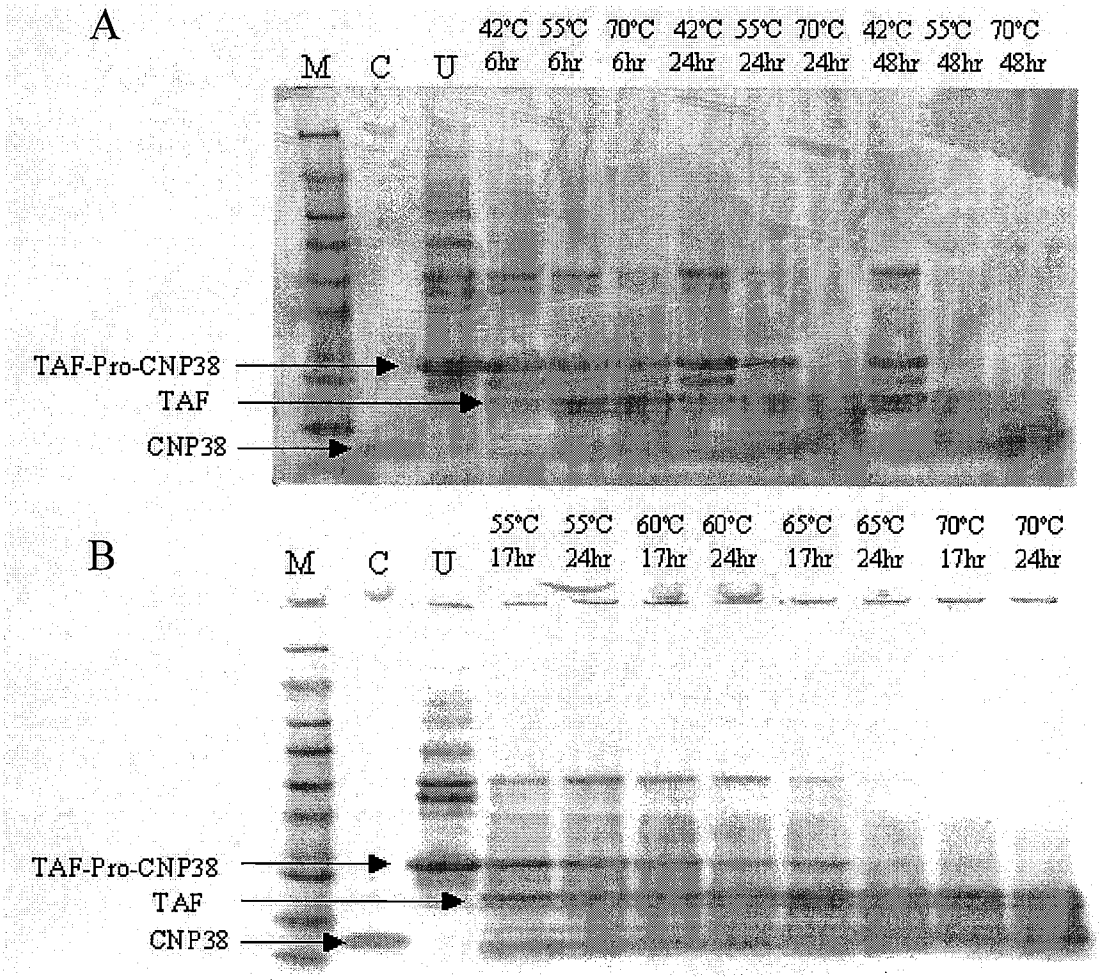

A
M
C
U
42°C 6hr
55°C 6hr
70°C 6hr
42°C 24hr
55°C 24hr
70°C 24hr
42°C 48hr
55°C 48hr
70°C 48hr
TAF-Pro-CNP38
TAF
CNP38
B
M
C
U
55°C 17hr
55°C 24hr
60°C 17hr
60°C 24hr
65°C 17hr
65°C 24hr
70°C 17hr
70°C 24hr
TAF-Pro-CNP38
TAF
CNP38

FIGURE 5C

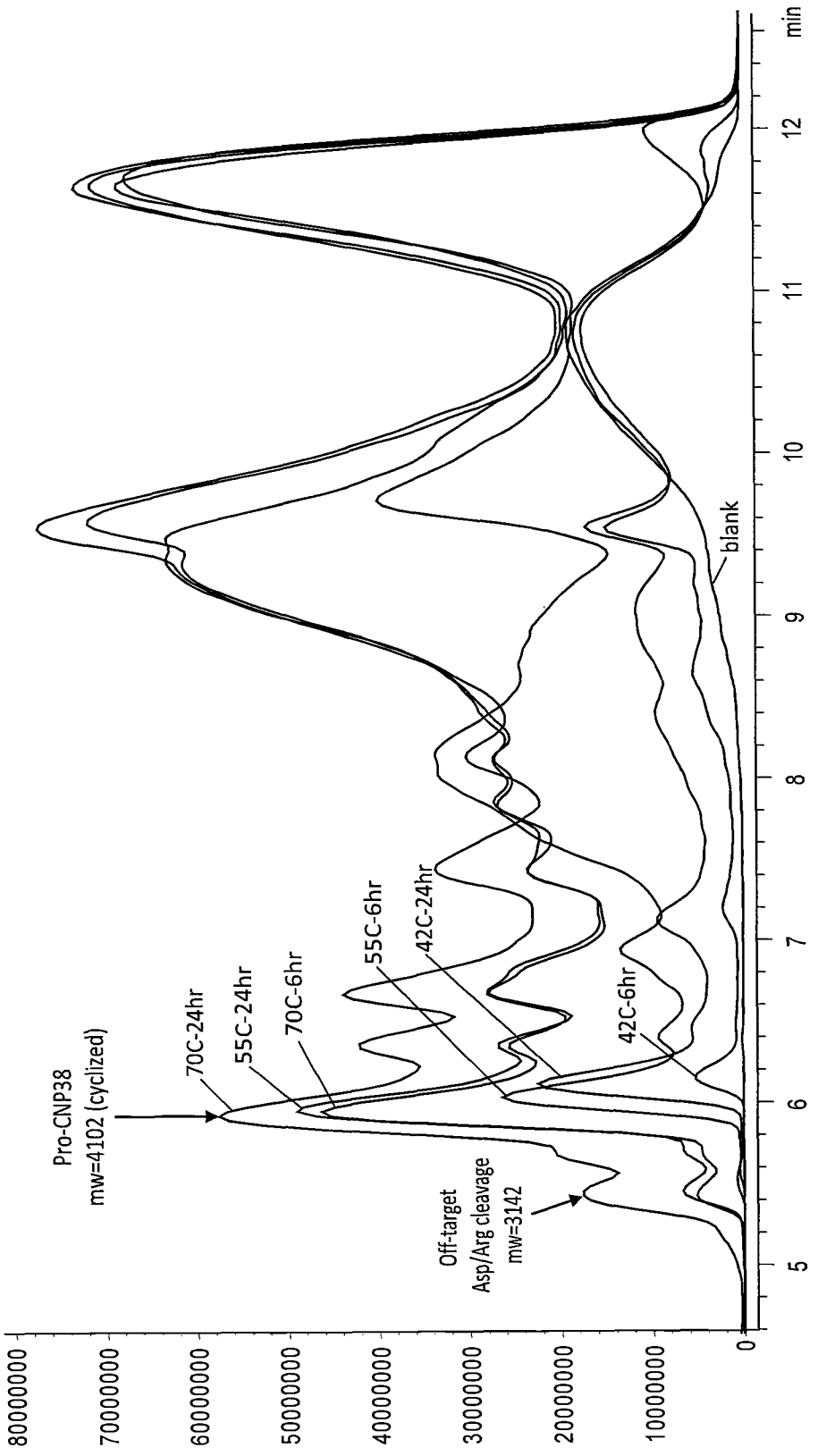

Pro-CNP38
mw=4102 (cyclized)
Off-target
Asp/Arg cleavage
mw=3142
70C-24hr
55C-24hr
70C-6hr
55C-6hr
42C-24hr
42C-6hr
blank
min
80000000
70000000
60000000
50000000
40000000
30000000
20000000
10000000
0
5
6
7
8
9
10
11
12

FIGURE 6

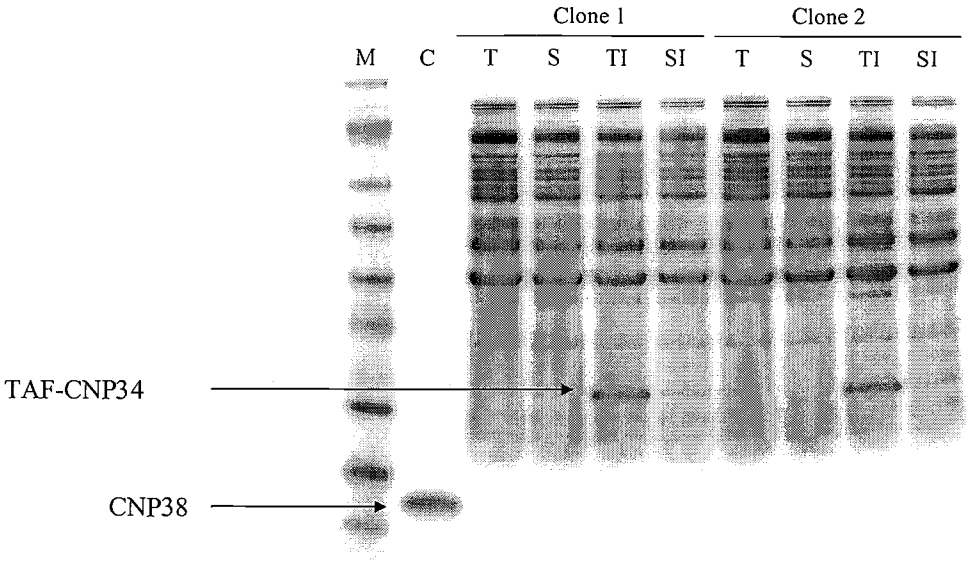


FIGURE 7

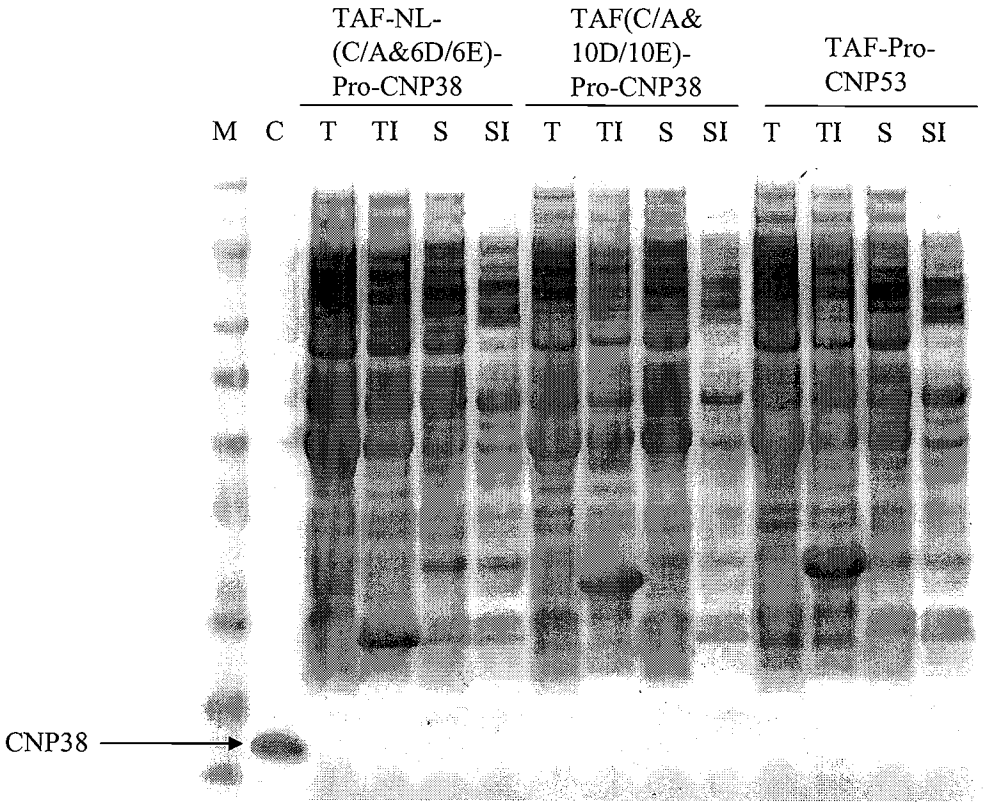


FIGURE 8

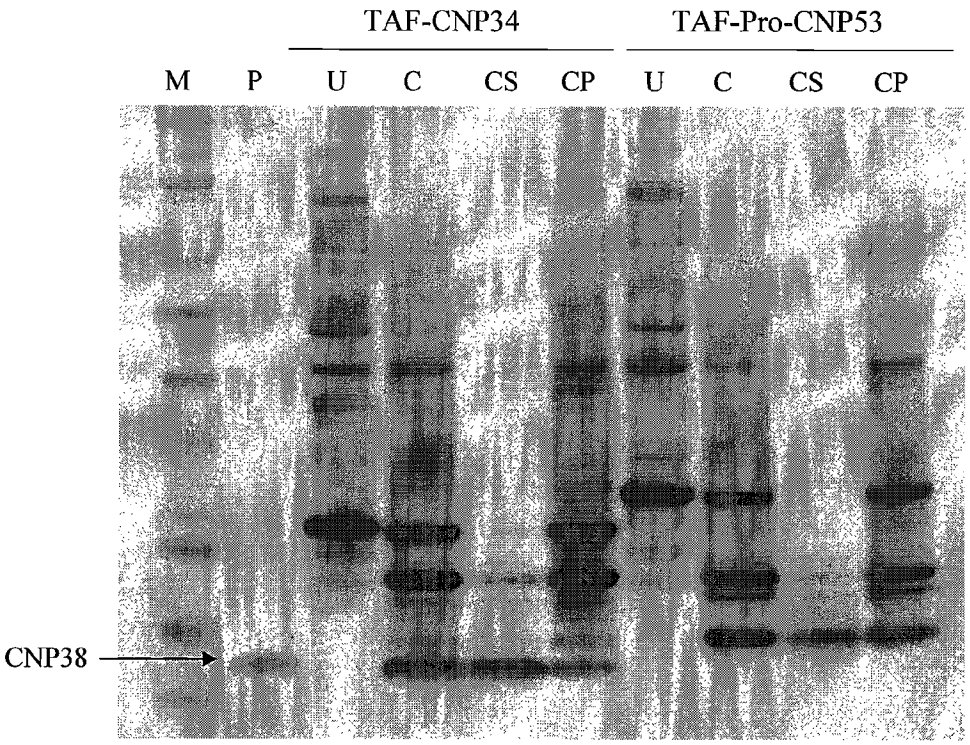


FIGURE 9

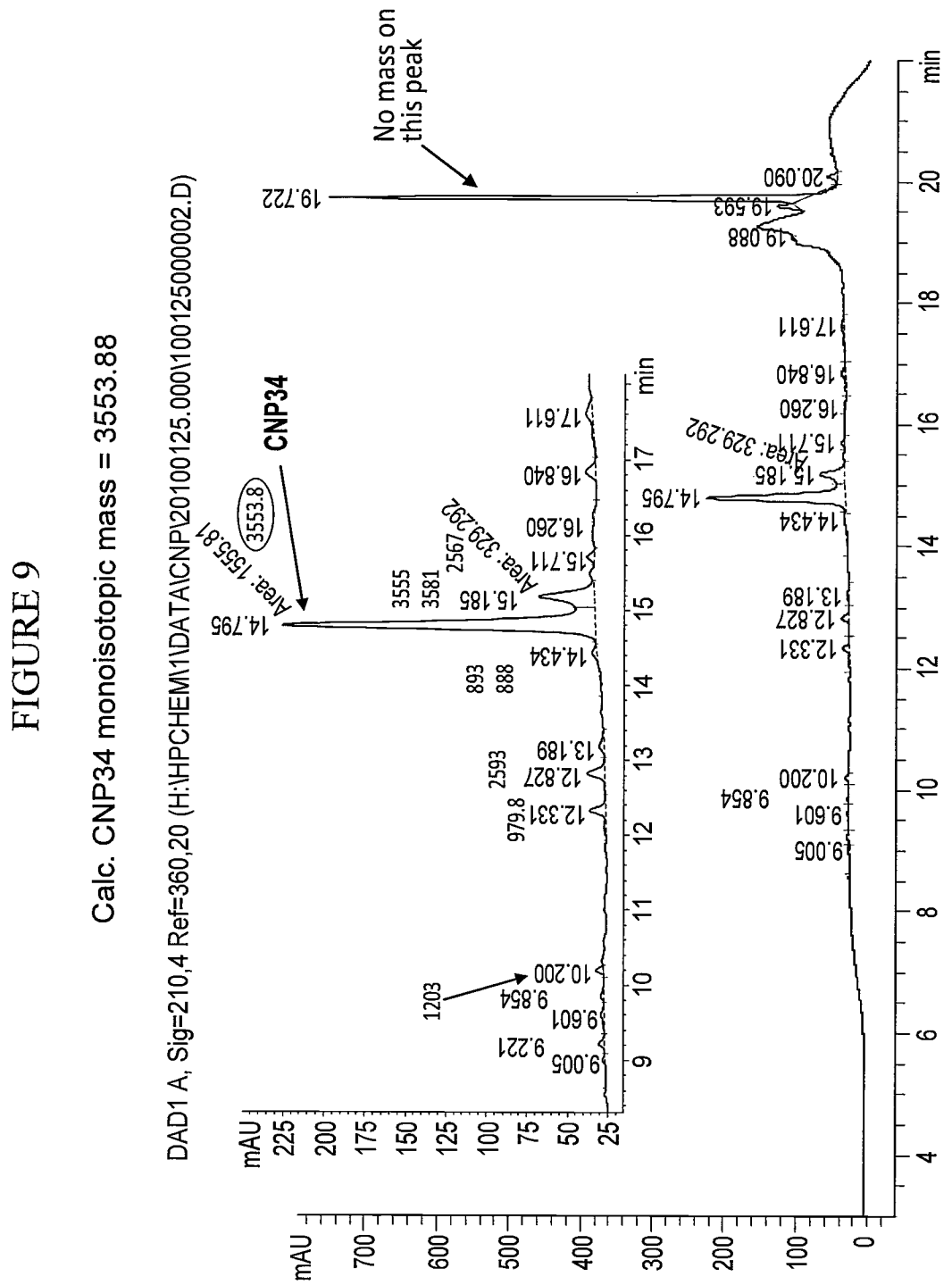

Calc. CNP34 monoisotopic mass = 3553.88
DAD1 A, Sig=210,4 Ref=360,20 (H:\HPCHEM\1\DATA\CNP\20100125.000100125000002.D)
CNP34
3553.8
No mass on this peak
mAU
min

FIGURE 10

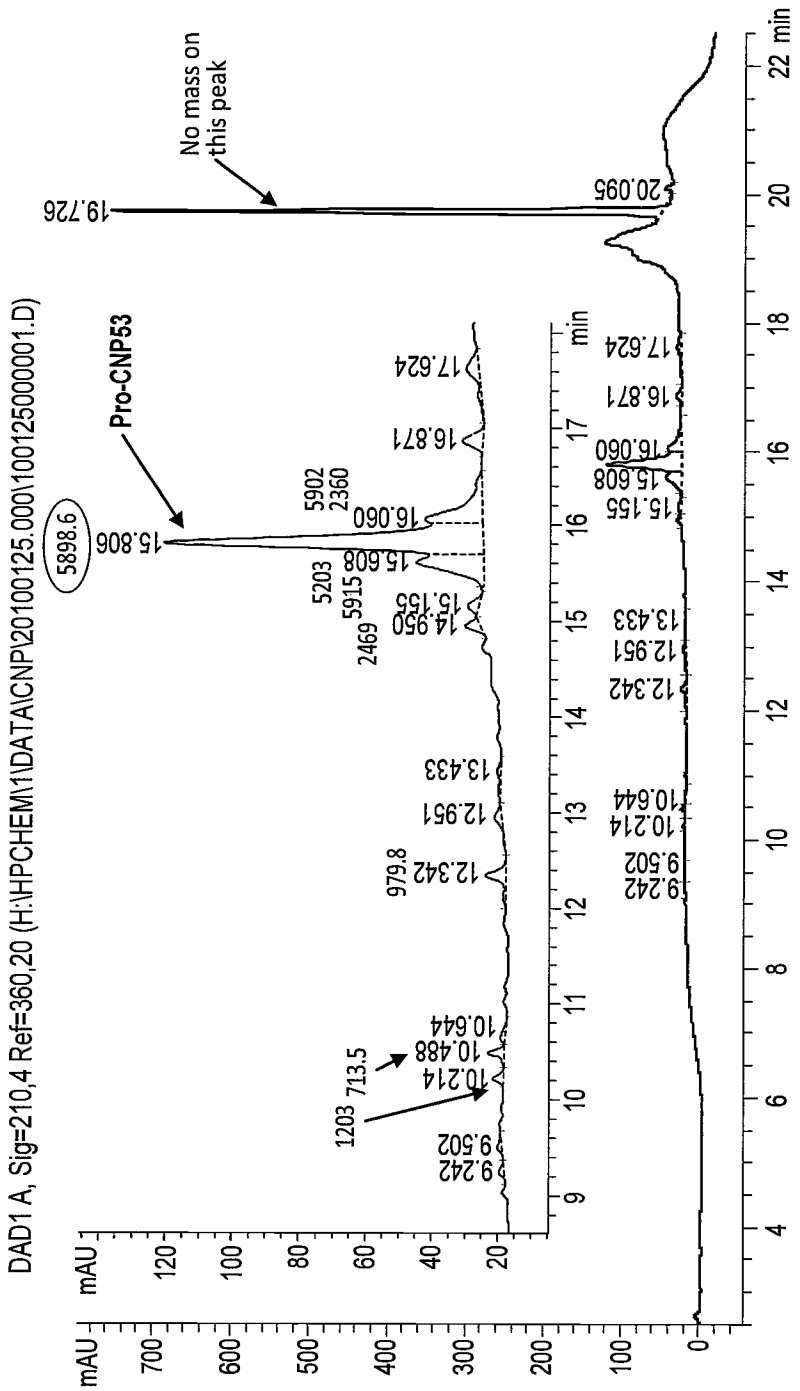

Calc. Pro-CNP53 monoisotopic mass = 5898.14
DAD1 A, Sig=210,4 Ref=360,20 (H:\HPCHEM\1\DATA\CNP\20100125.000\100125000001.D)
Pro-CNP53
5898.6
No mass on this peak
mAU
min
22 min

FIGURE 11

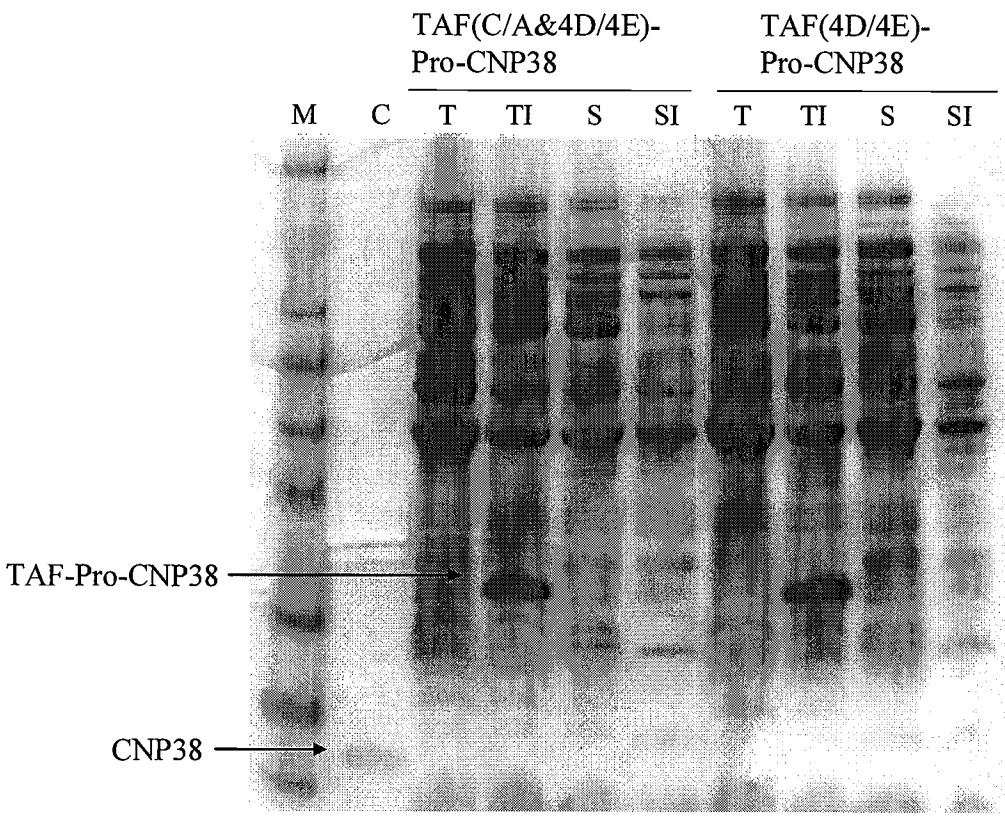

TAF(C/A&4D/4E)-Pro-CNP38
TAF(4D/4E)-Pro-CNP38
M
C
T
TI
S
SI
T
TI
S
SI
TAF-Pro-CNP38
CNP38

FIGURE 12

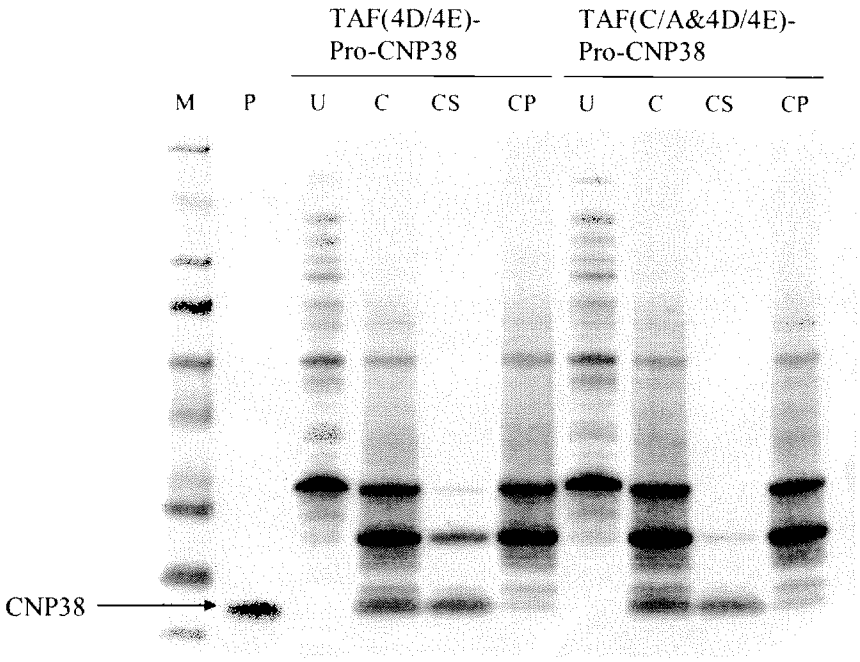


FIGURE 13

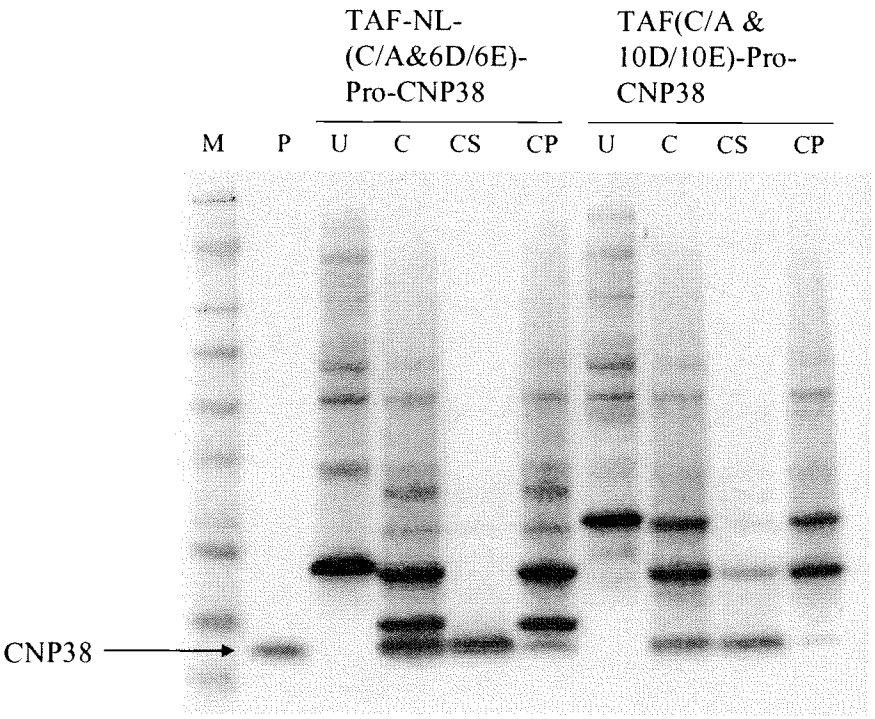


FIGURE 14

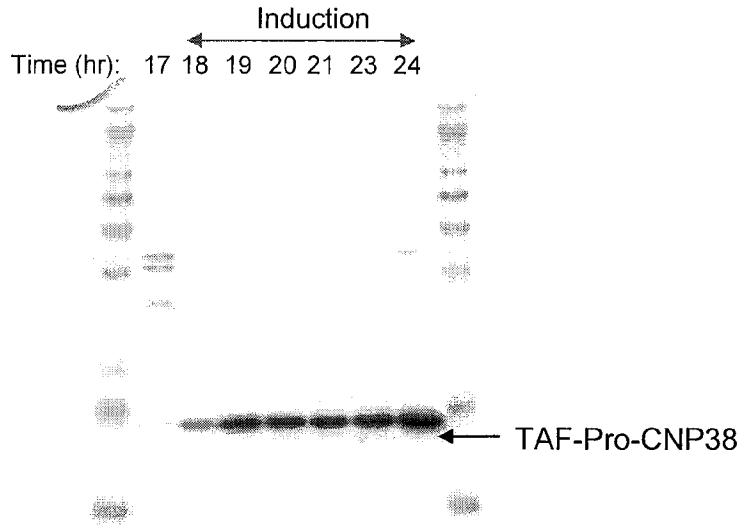


FIGURE 15

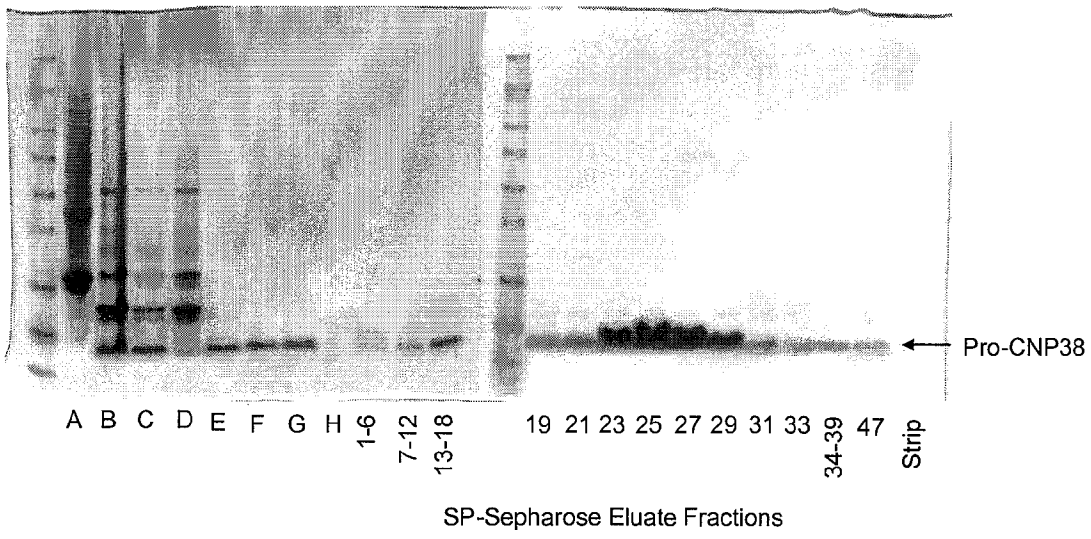


FIGURE 16

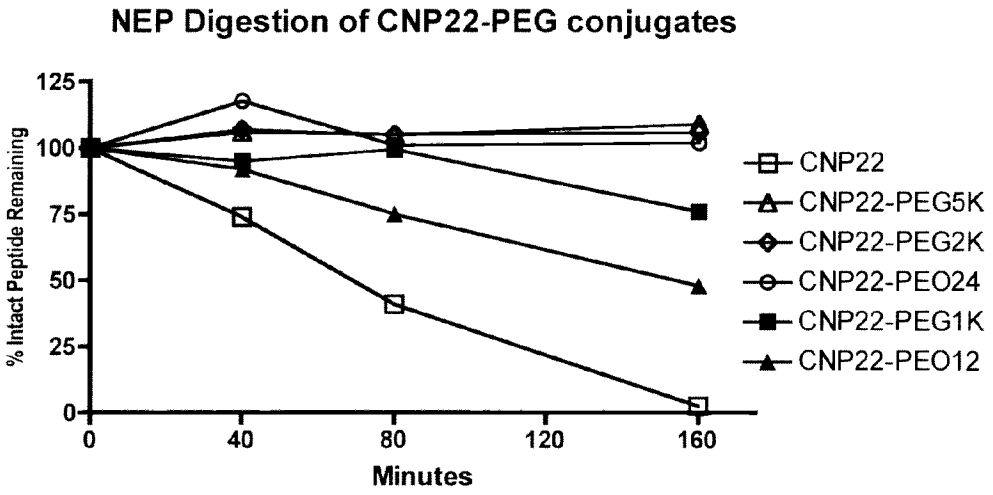


FIGURE 17

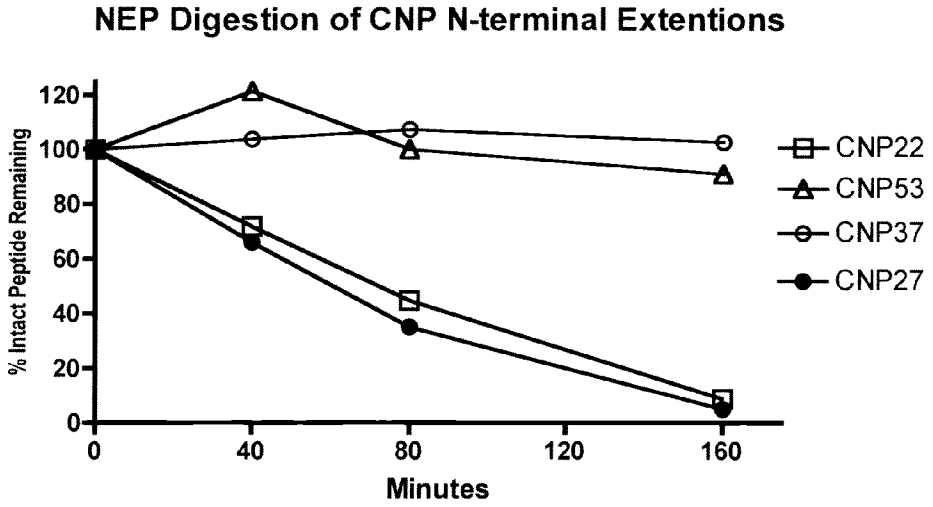


FIGURE 18

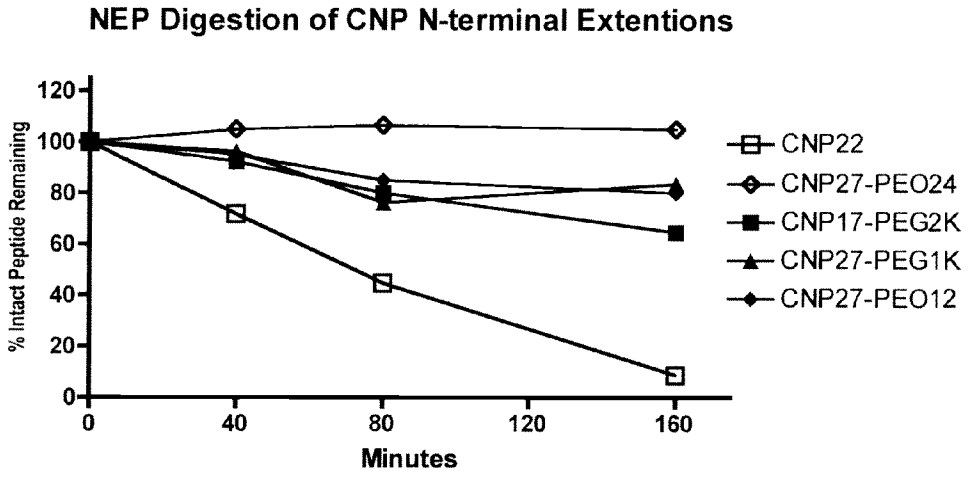


FIGURE 19

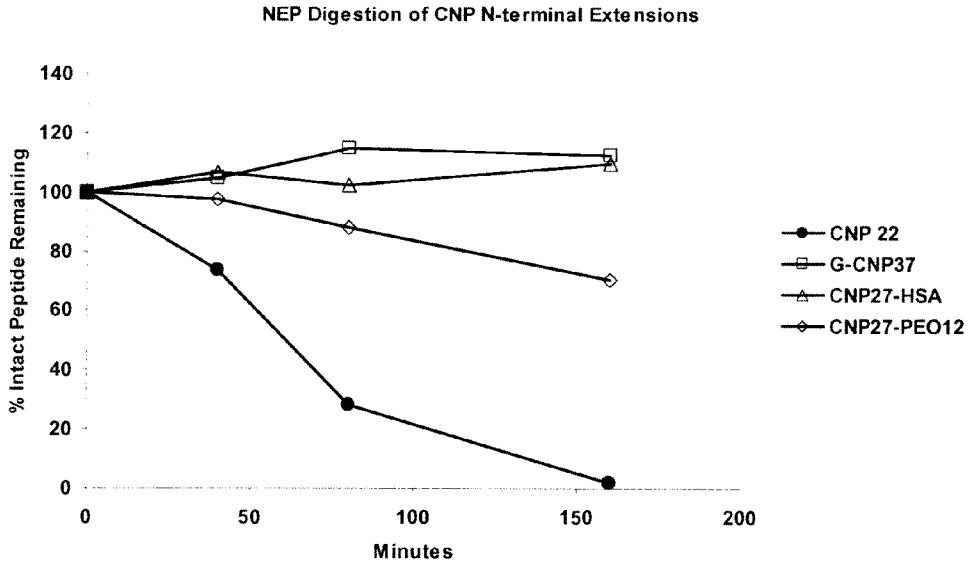


FIGURE 20

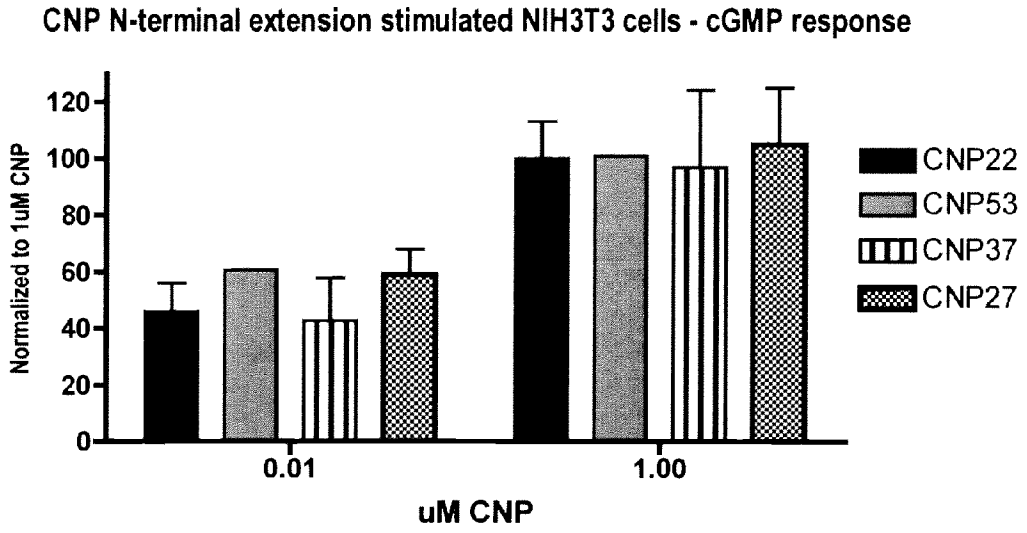


FIGURE 21

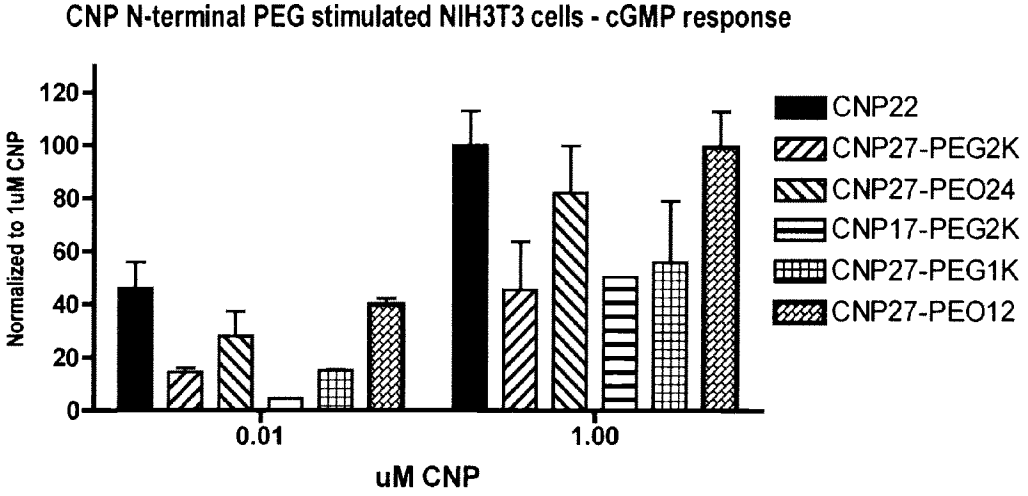


FIGURE 22

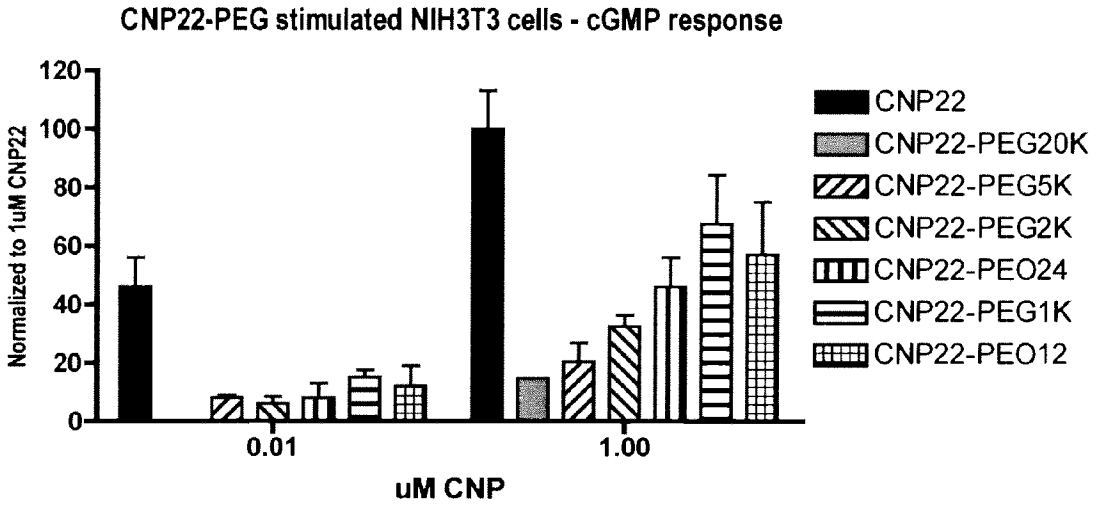


FIGURE 23

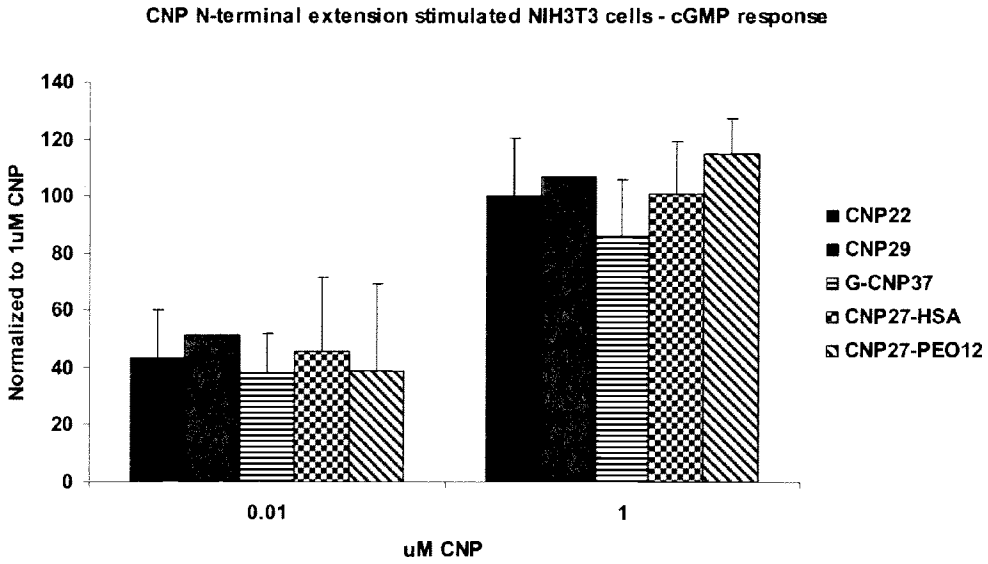
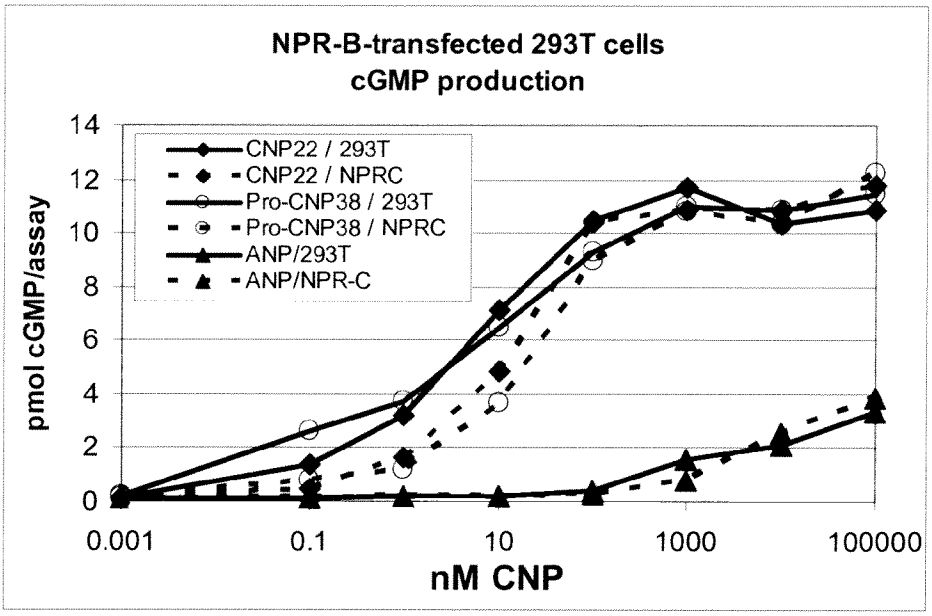


FIGURE 24

A.


NPR-B-transfected 293T cells
cGMP production
pmol cGMP/assay
nM CNP
CNP22 / 293T
CNP22 / NPRC
Pro-CNP38 / 293T
Pro-CNP38 / NPRC
ANP/293T
ANP/NPR-C
0
2
4
6
8
10
12
14
0.001
0.1
10
1000
100000

B.

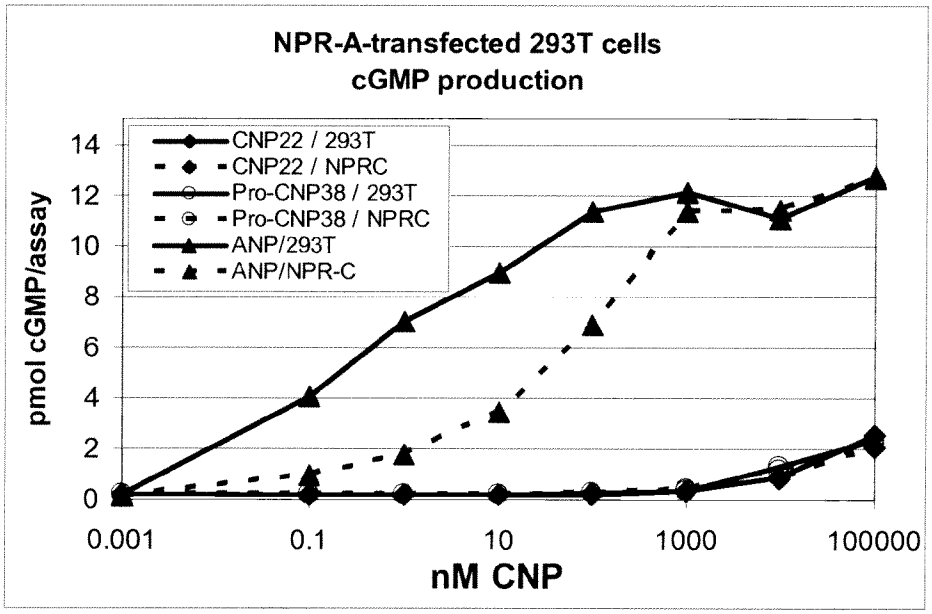
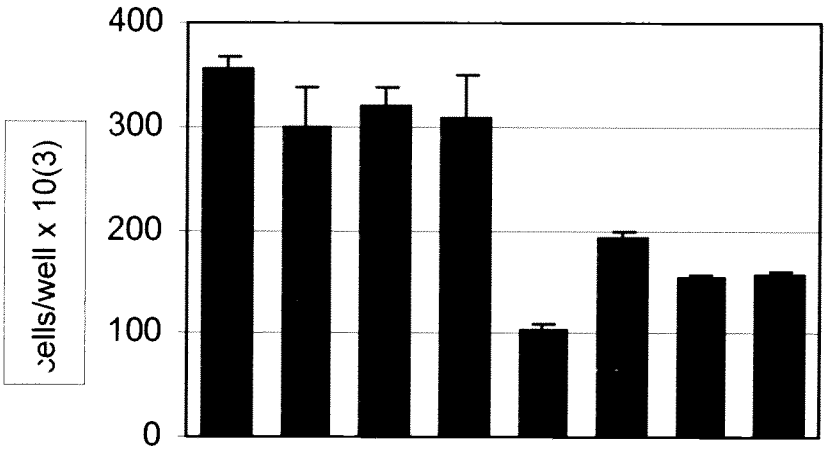

NPR-A-transfected 293T cells
cGMP production
pmol cGMP/assay
nM CNP
CNP22 / 293T
CNP22 / NPRC
Pro-CNP38 / 293T
Pro-CNP38 / NPRC
ANP/293T
ANP/NPR-C
0
2
4
6
8
10
12
14
0.001
0.1
10
1000
100000

FIGURE 25



Bars from left to right:

1 control (no CNP22, no FGF2)
2 CNP22 (0.2 uM) continuous
3 CNP22 (0.2 uM) 1 hr once daily
4 CNP22 (0.2 uM) 2 hr once daily
5 FGF2 (5 ng/mL) continuous
6 FGF2 + CNP22 (0.2 uM) continuous
7 FGF2 + CNP22 (0.2 uM) 1 hr daily
8 FGF2 + CNP22 (0.2 uM) 2 hr daily

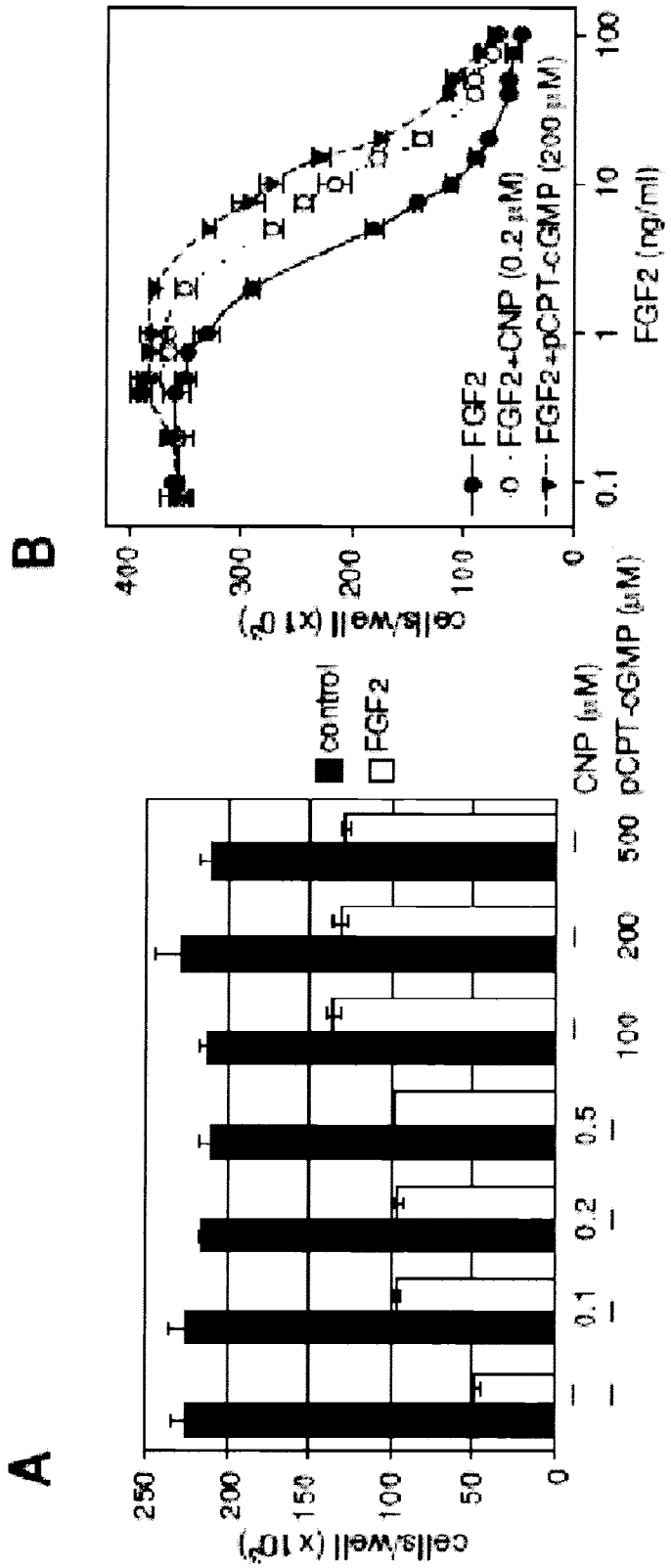

A
cells/well (x 10⁵)
250
200
150
100
50
0
control
FGF2
—
0.1
0.2
0.5
100
200
500
CNP (μM)
pCPT-cGMP (μM)
B
cells/well (x 10⁵)
400
300
200
100
0
FGF2
FGF2+CNP (0.2 μM)
FGF2+pCPT-cGMP (200 μM)
0.1
1
10
100
FGF2 (ng/ml)

FIGURE 26

FIGURE 27

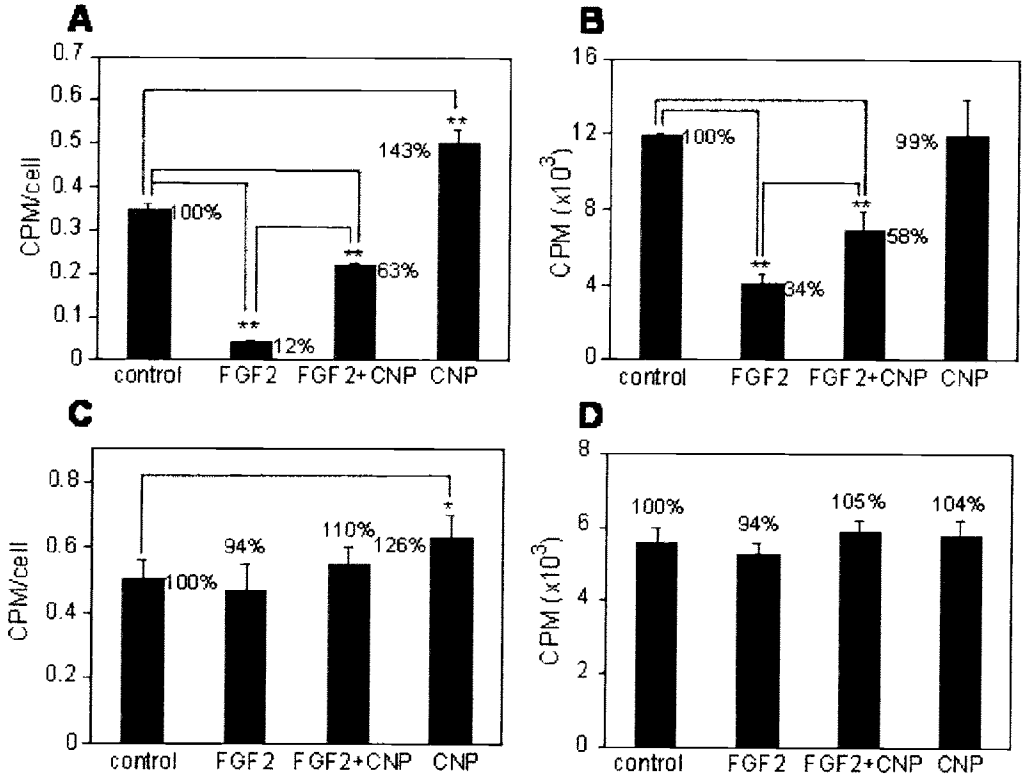

A
CPM/cell
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100%
12%
63%
143%
**
control
FGF2
FGF2+CNP
CNP
B
CPM (x10^3)
16
12
8
4
0
100%
94%
58%
99%
control
FGF2
FGF2+CNP
CNP
C
CPM/cell
0.8
0.6
0.4
0.2
0
100%
94%
110%
126%
*
control
FGF2
FGF2+CNP
CNP
D
CPM (x10^3)
8
6
4
2
0
100%
94%
105%
104%
control
FGF2
FGF2+CNP
CNP

FIGURE 28

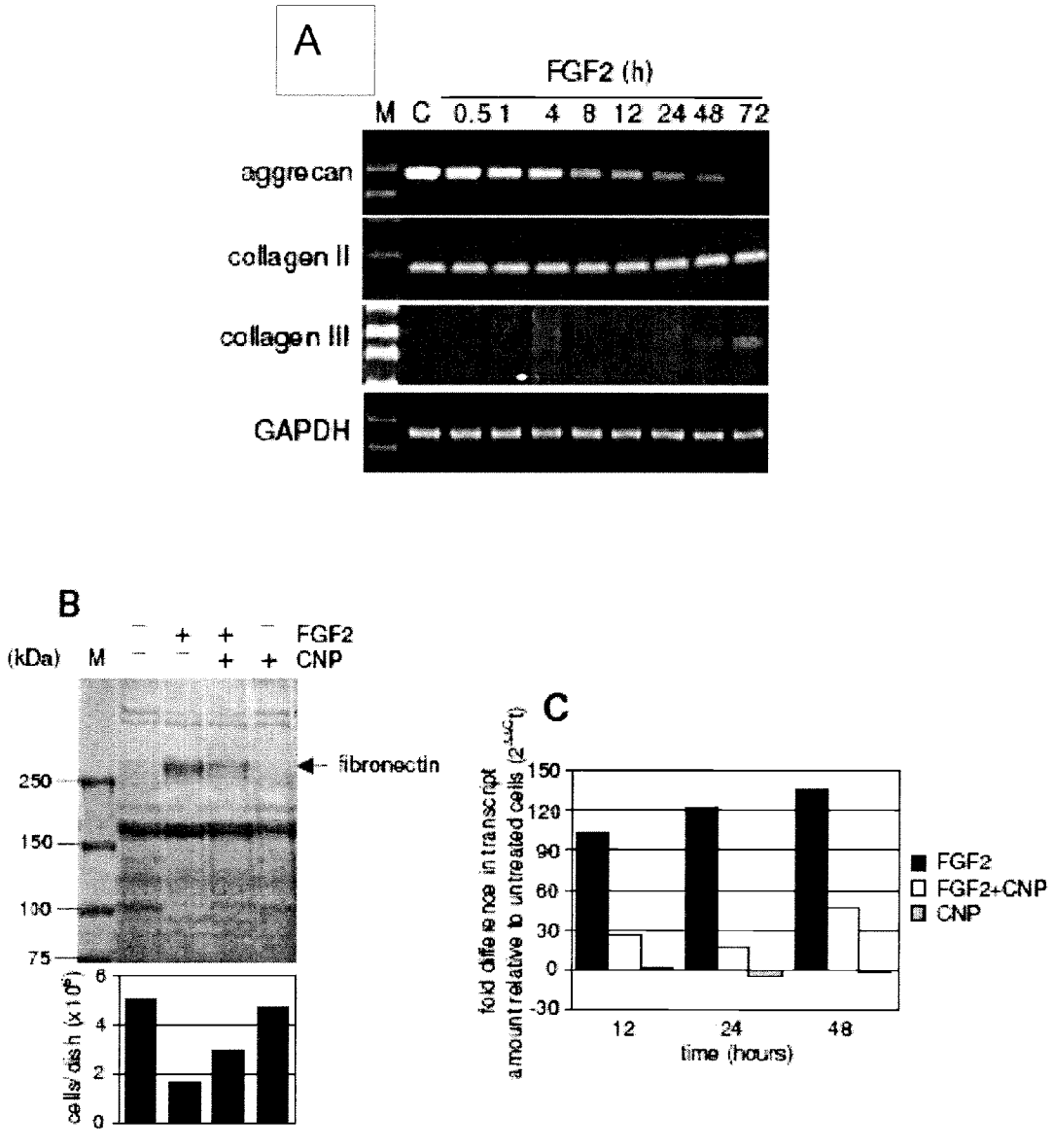

A
FGF2 (h)
M C 0.5 1 4 8 12 24 48 72
aggrecan
collagen II
collagen III
GAPDH
B
(kDa) M
FGF2
CNP
fibronectin
250
150
100
75
cells/ dish (x10⁴)
C
fold difference in transcript amount relative to untreated cells
time (hours)
FGF2
FGF2+CNP
CNP

FIGURE 29

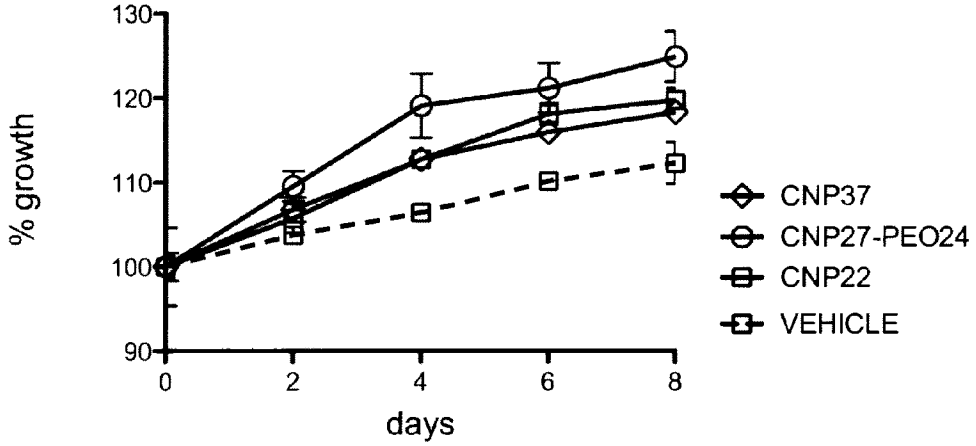


FIGURE 30

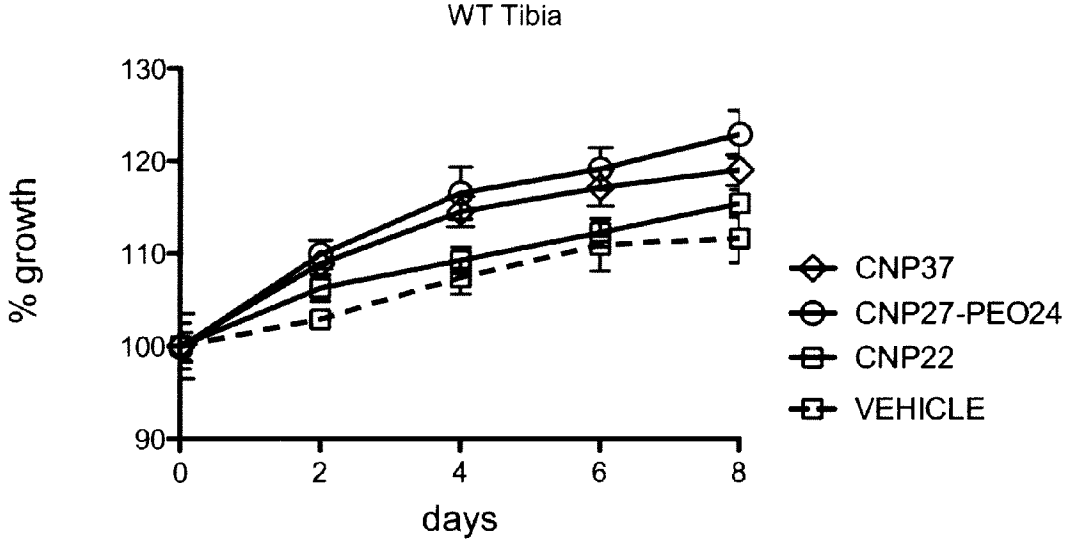


FIGURE 31

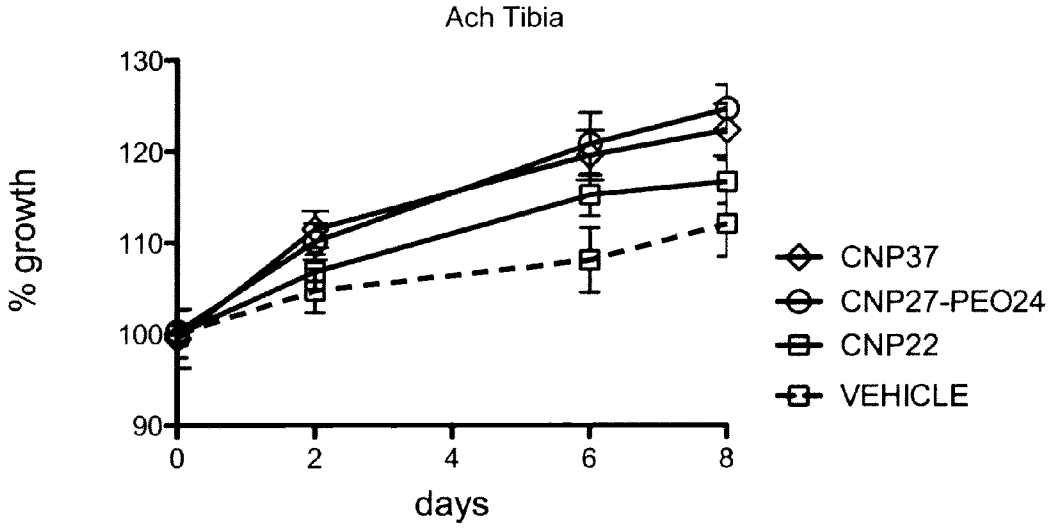


FIGURE 32

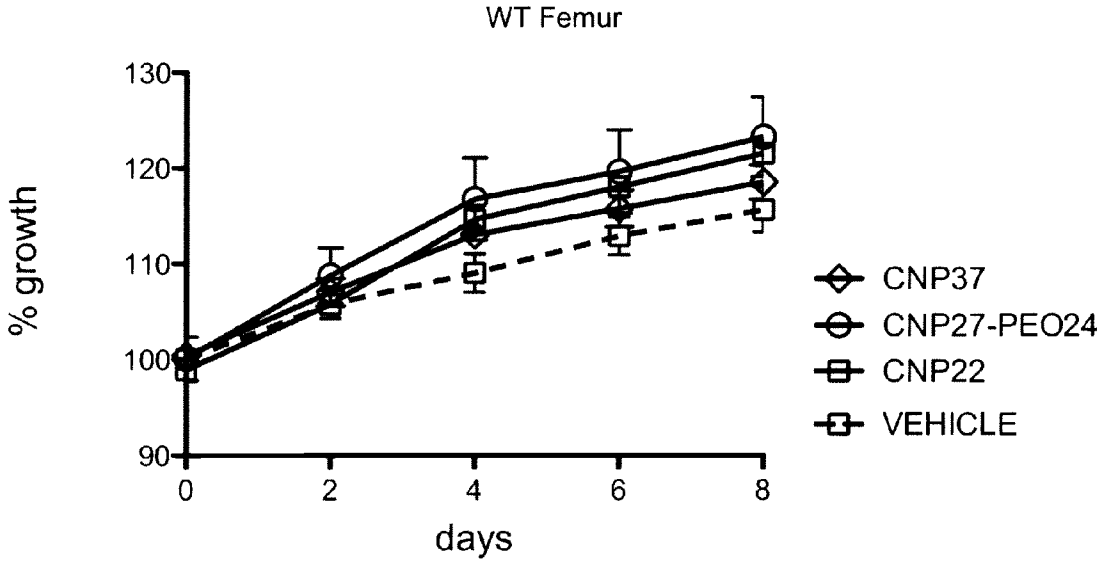


FIGURE 33

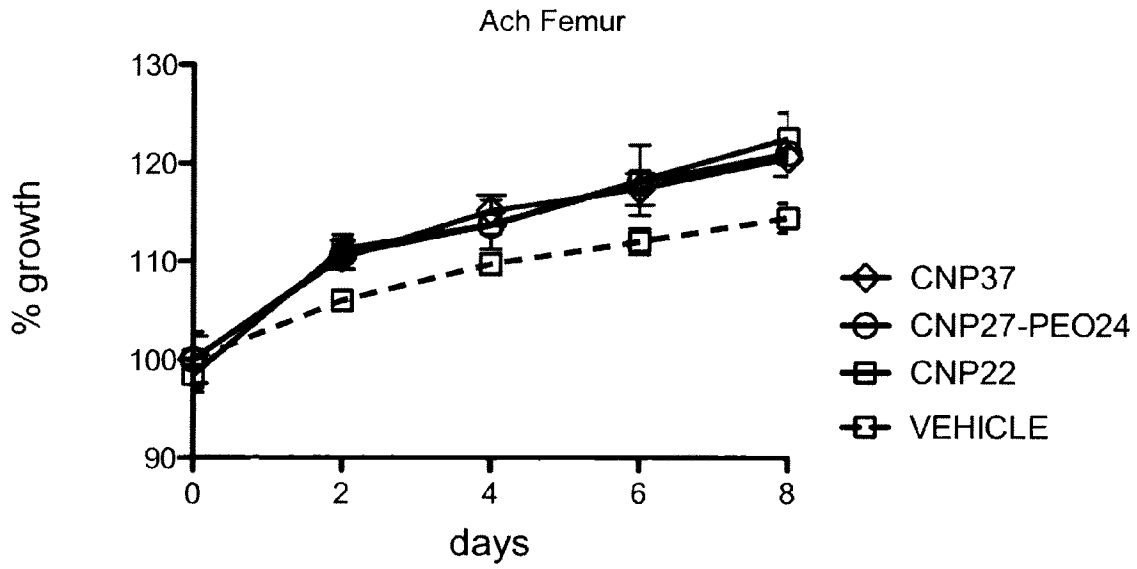

Ach Femur
% growth
130
120
110
100
90
0
2
4
6
8
days
CNP37
CNP27-PEO24
CNP22
VEHICLE

FIGURE 34

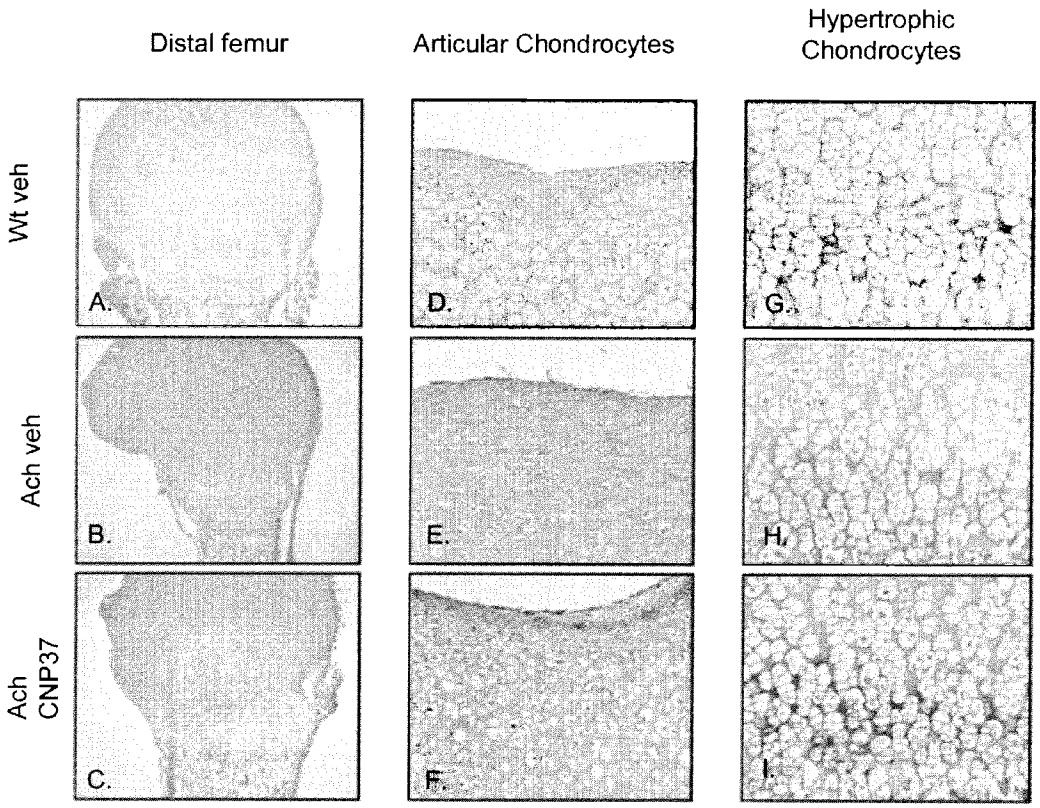

Distal femur
Articular Chondrocytes
Hypertrophic Chondrocytes
Wt veh
Ach veh
Ach CNP37
A.
B.
C.
D.
E.
F.
G.
H.
I.

FIGURE 35

Cellularity of Proliferating Columns

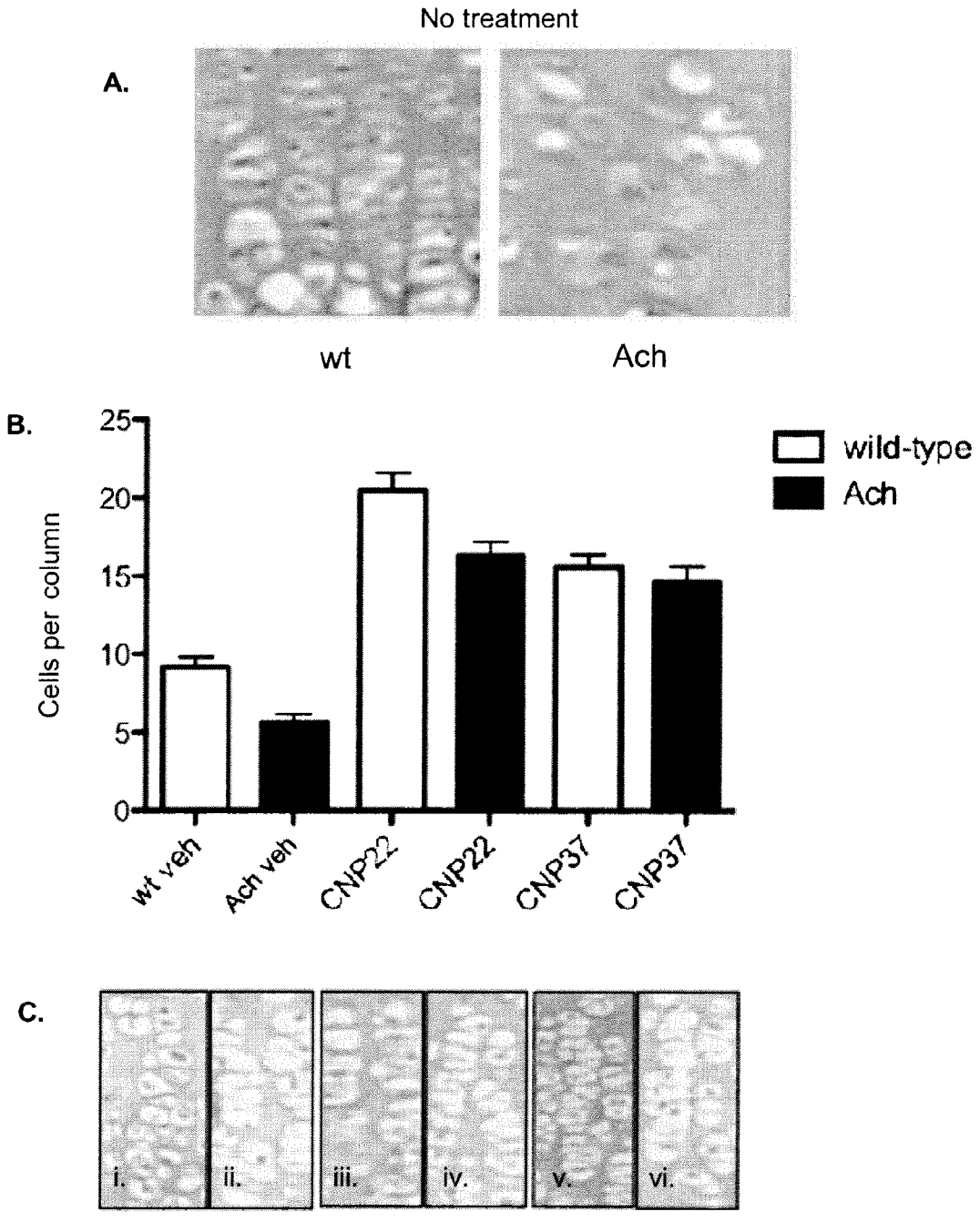


FIGURE 36

Chondrocyte Hypertrophy

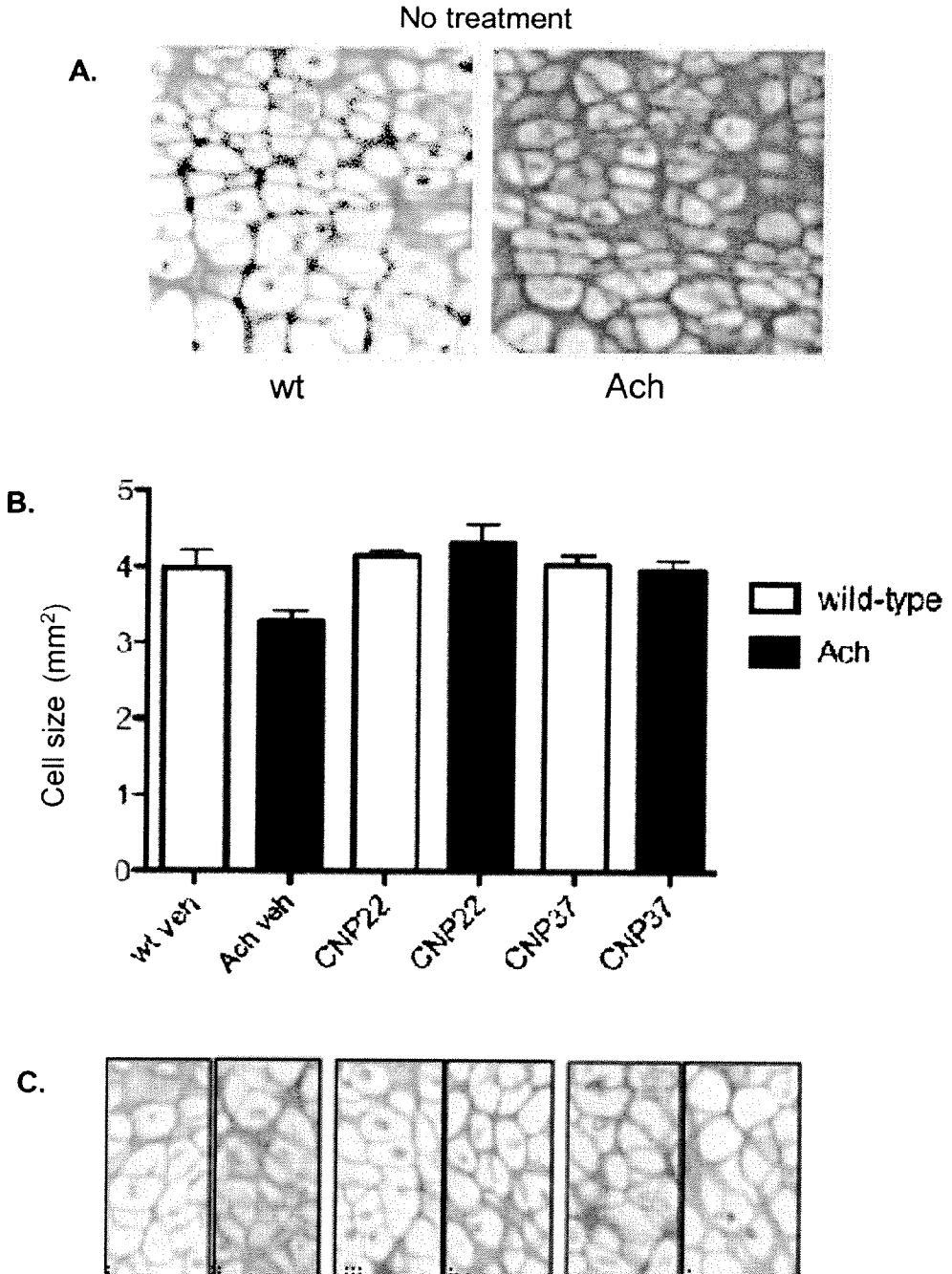


FIGURE 37

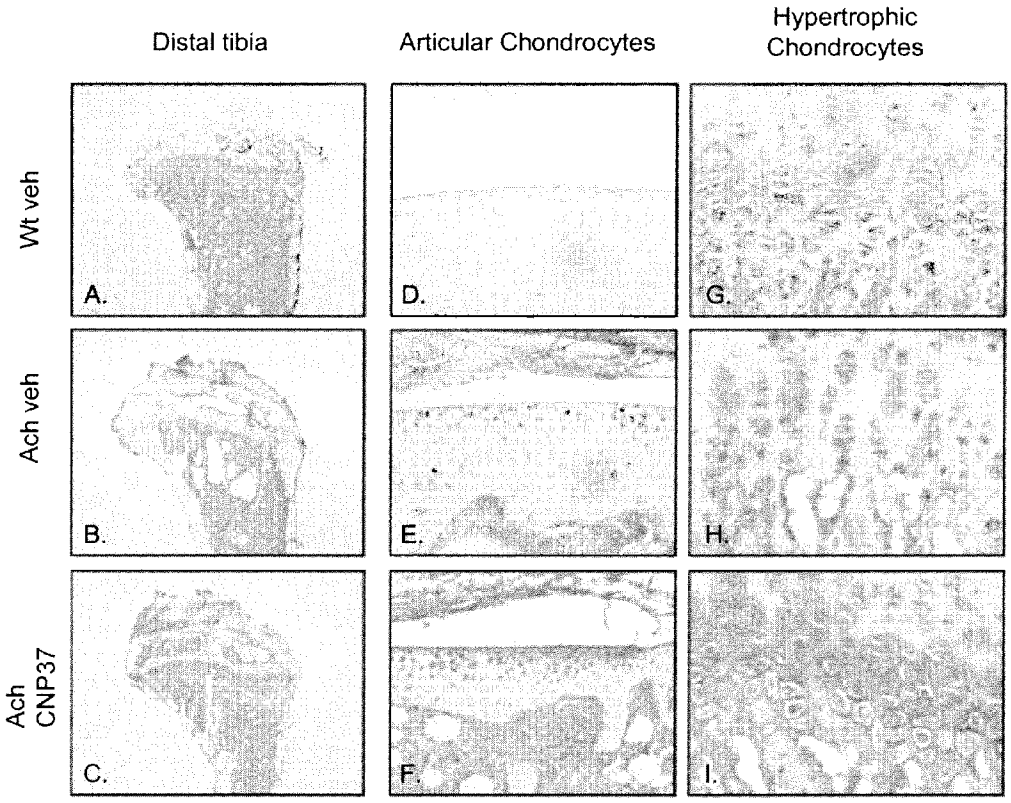
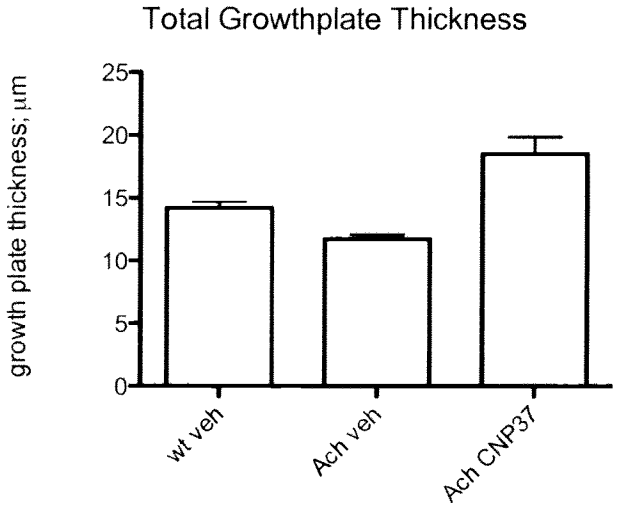
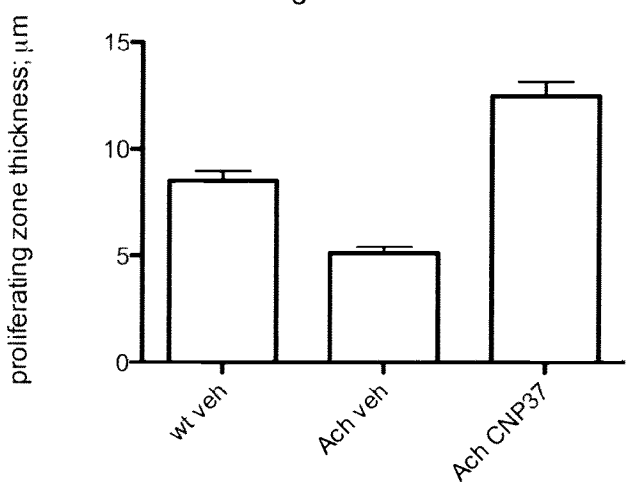

Distal tibia
Articular Chondrocytes
Hypertrophic Chondrocytes
Wt veh
Ach veh
Ach CNP37
A.
B.
C.
D.
E.
F.
G.
H.
I.

FIGURE 38

A.



B.



C.

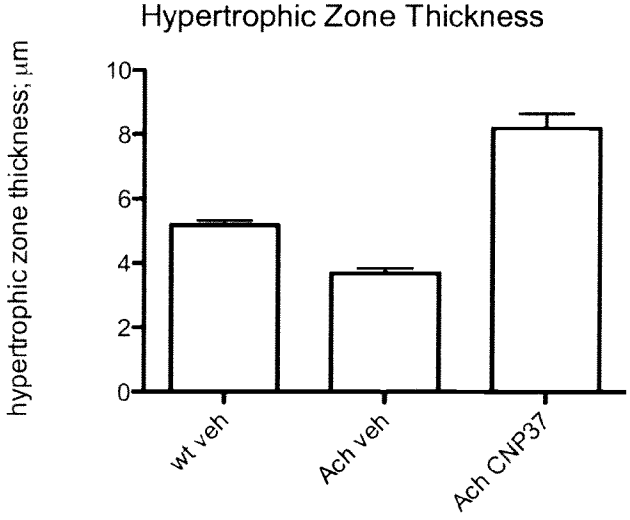


FIGURE 39

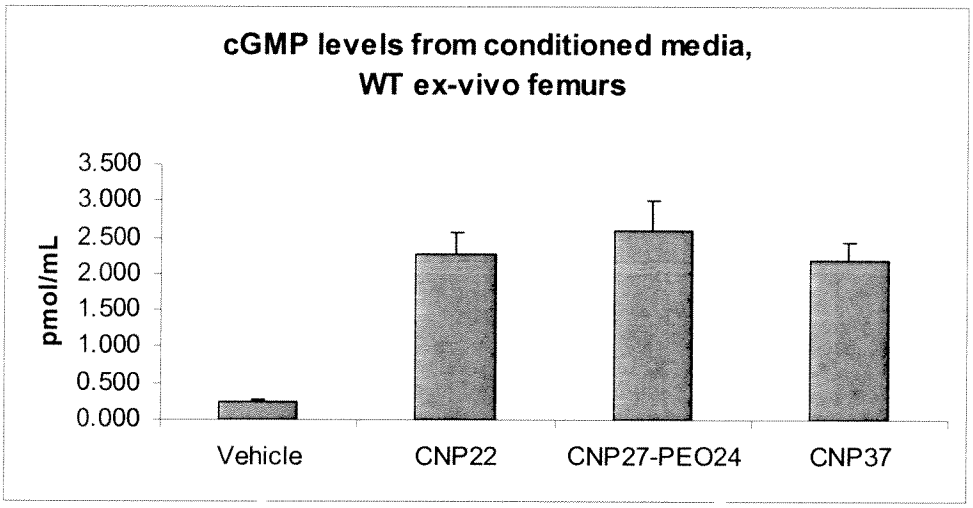


FIGURE 40

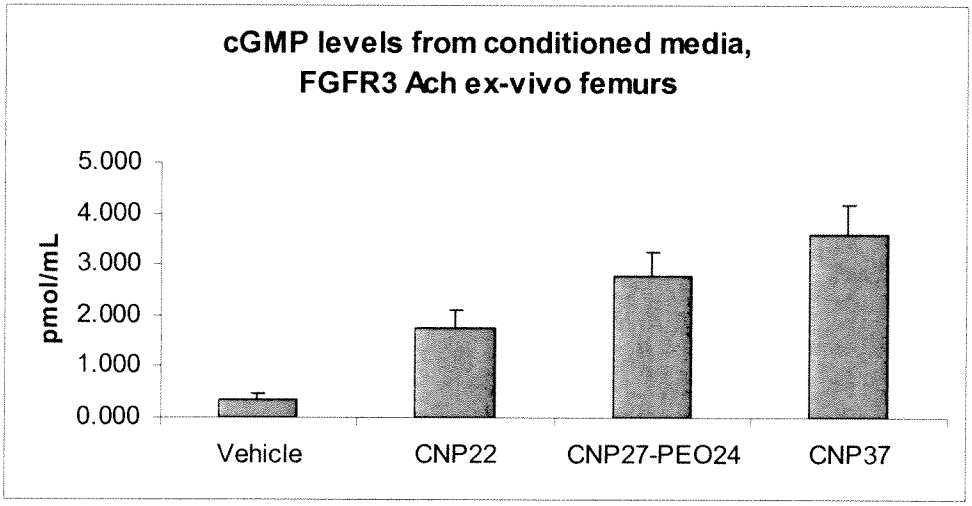


FIGURE 41

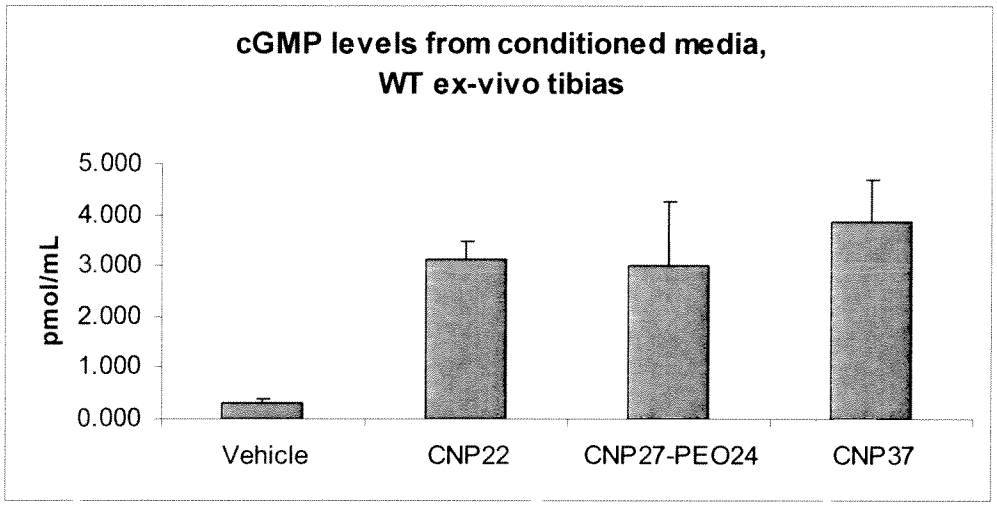


FIGURE 42

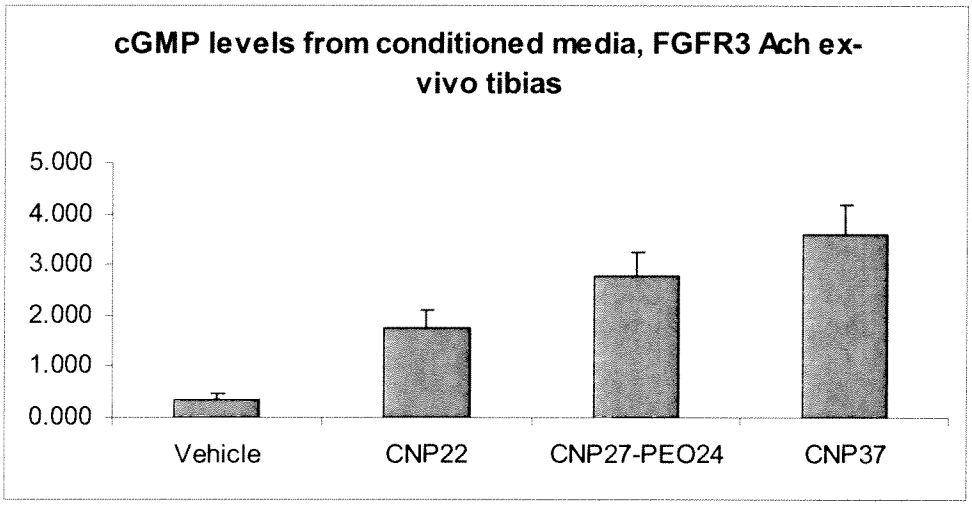


FIGURE 43

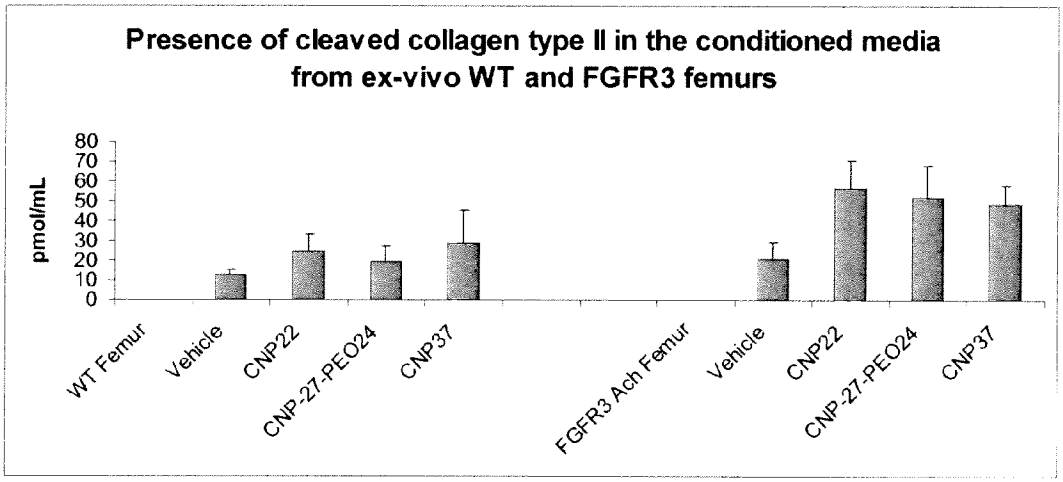


FIGURE 44

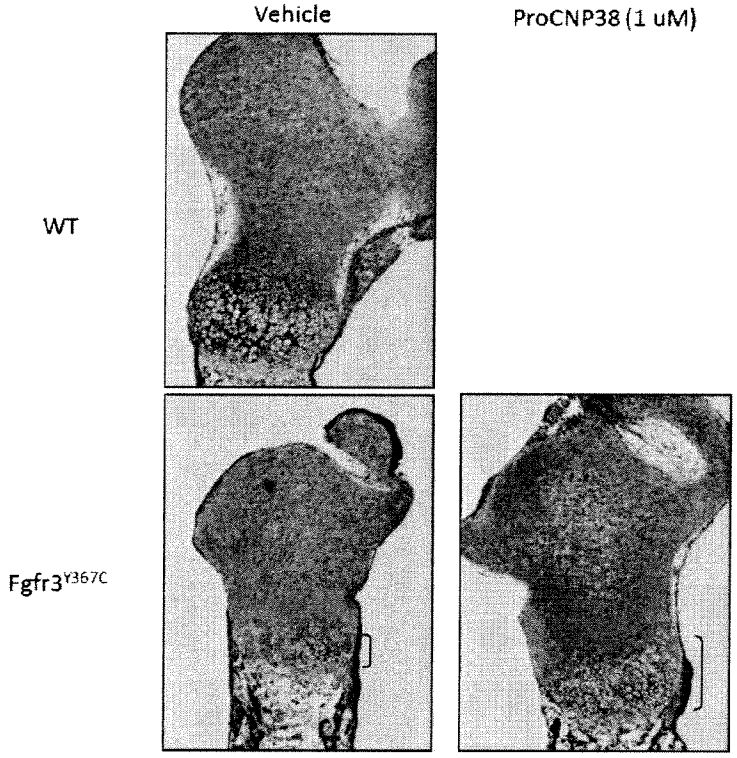


FIGURE 45

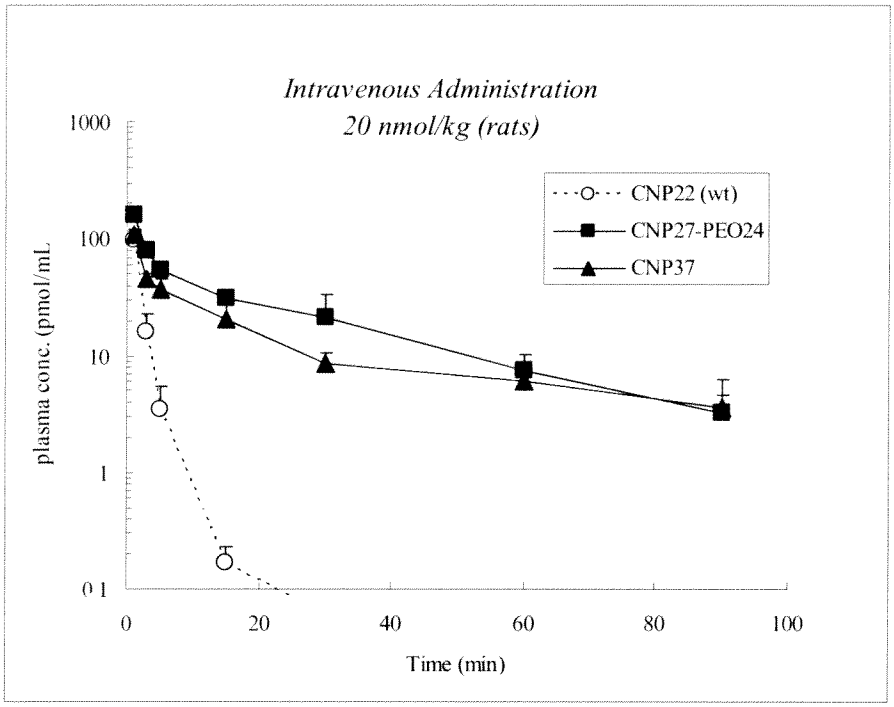


FIGURE 46

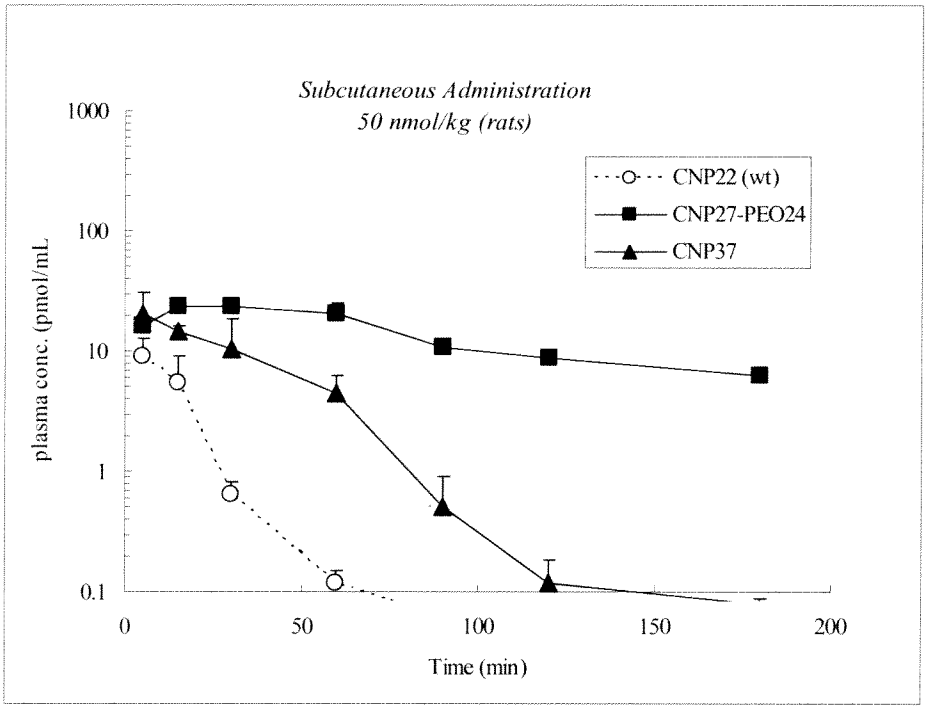


FIGURE 47

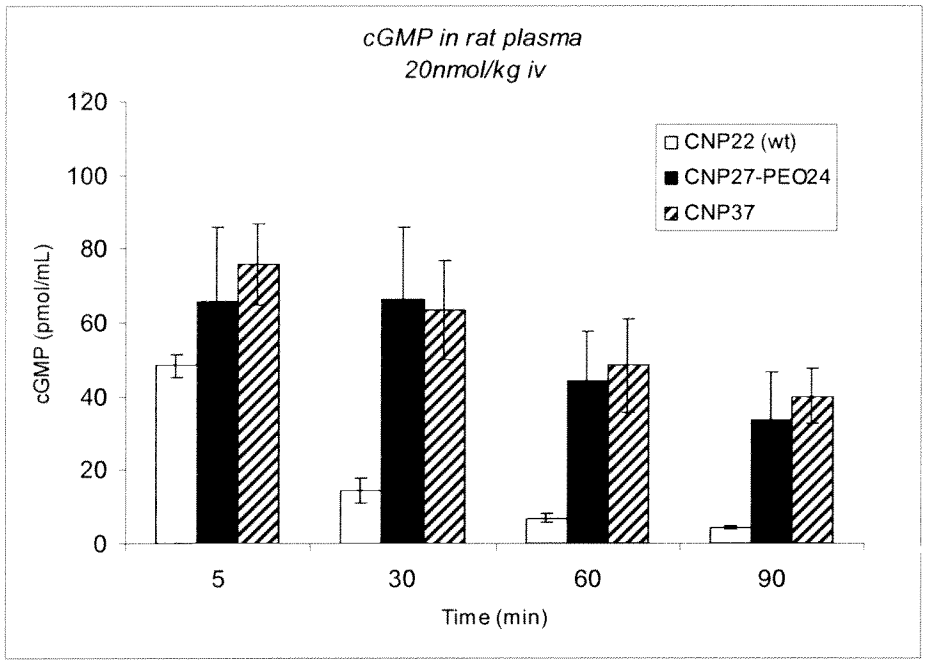


FIGURE 48

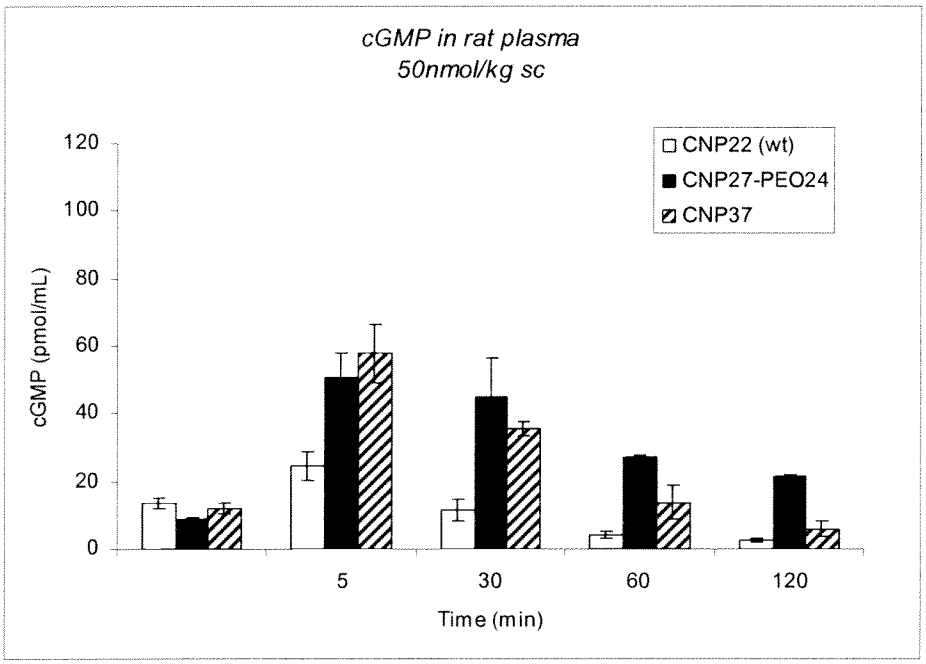


FIGURE 49

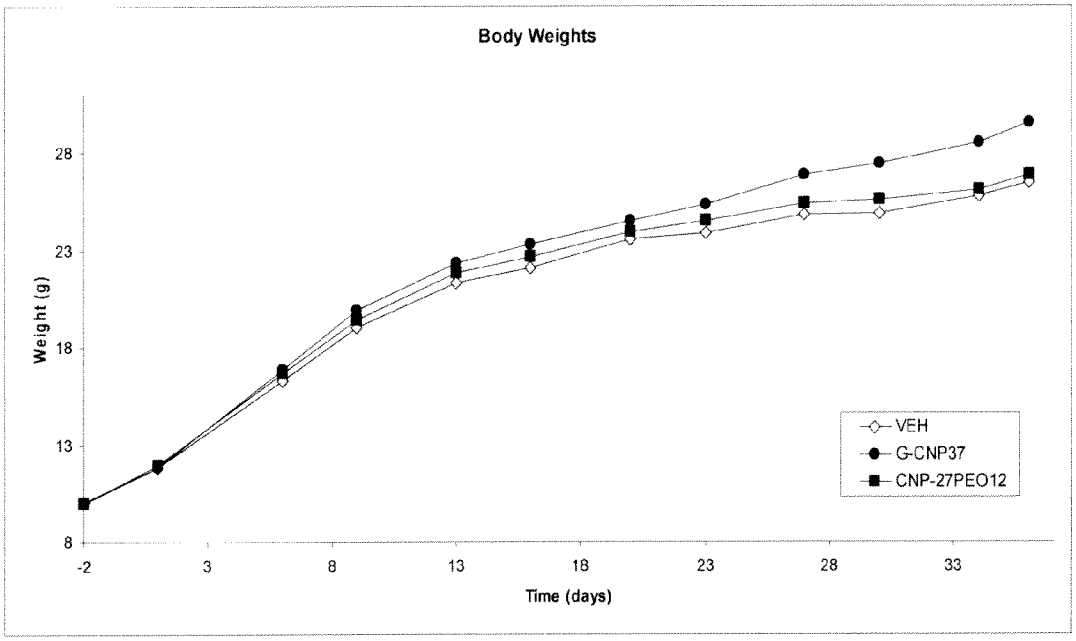


FIGURE 50

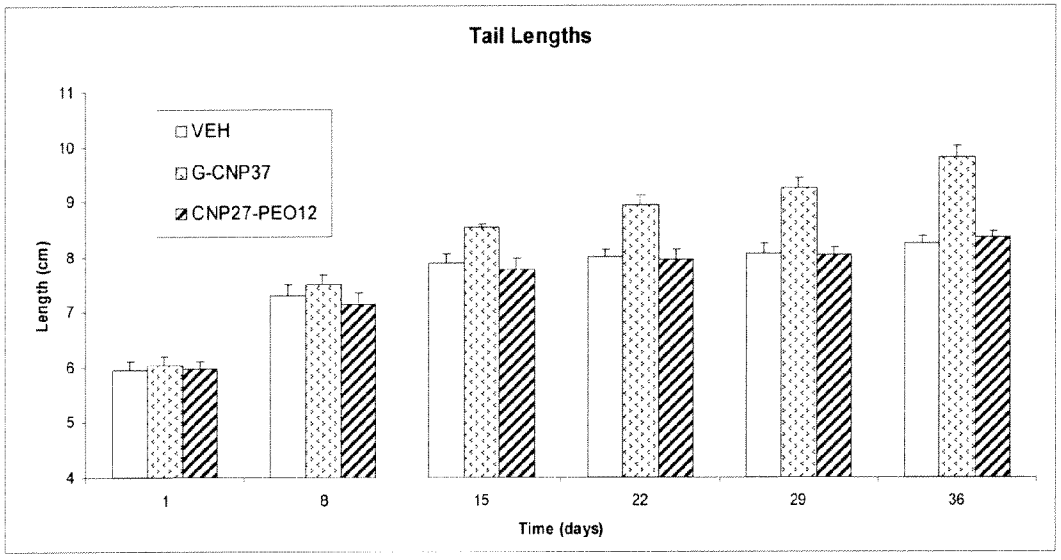


FIGURE 51

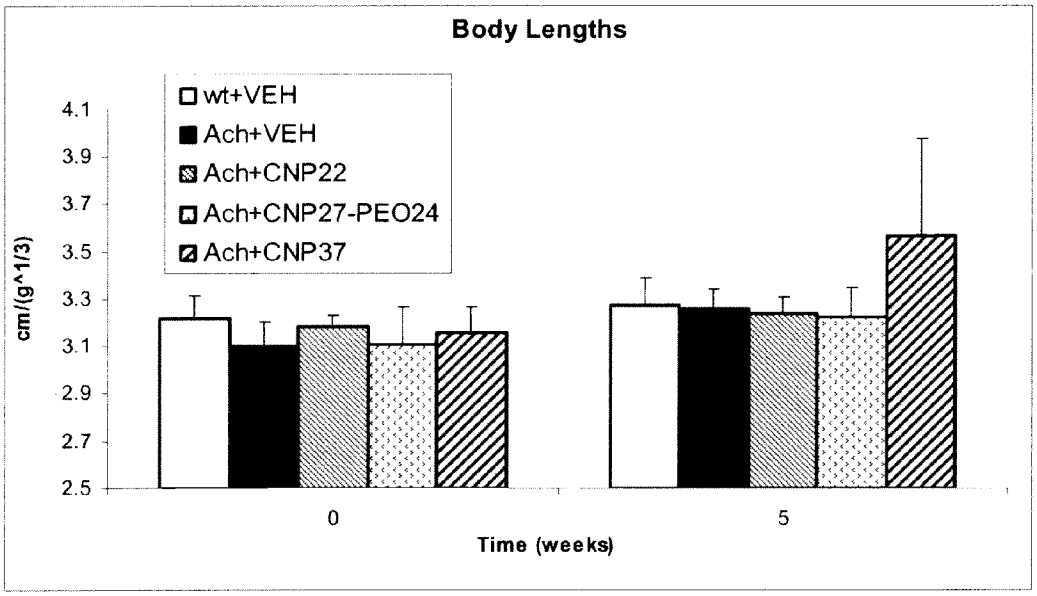


FIGURE 52

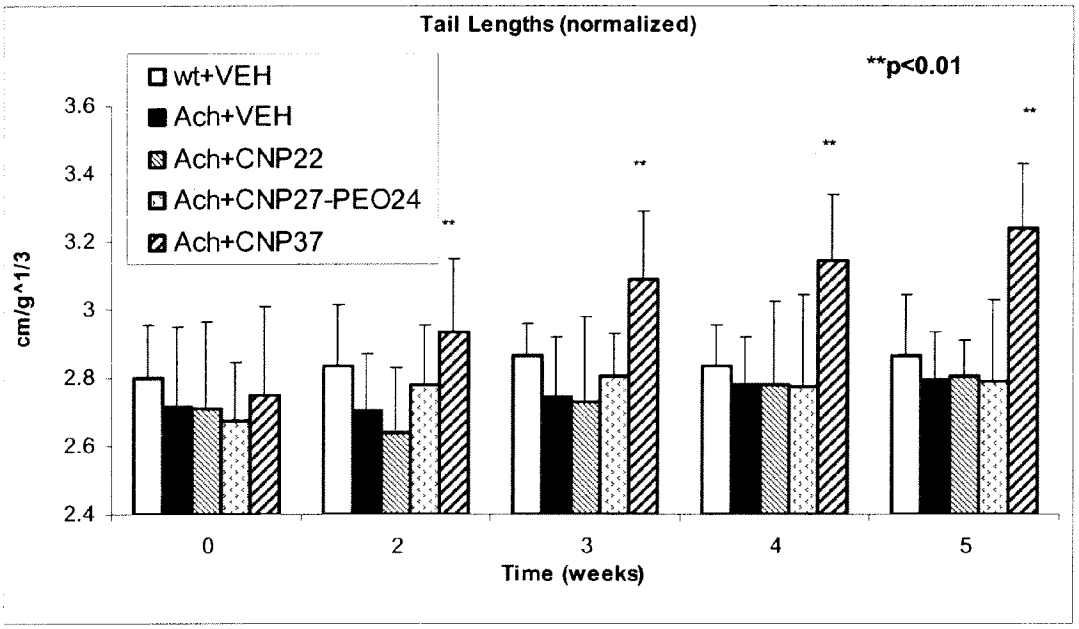
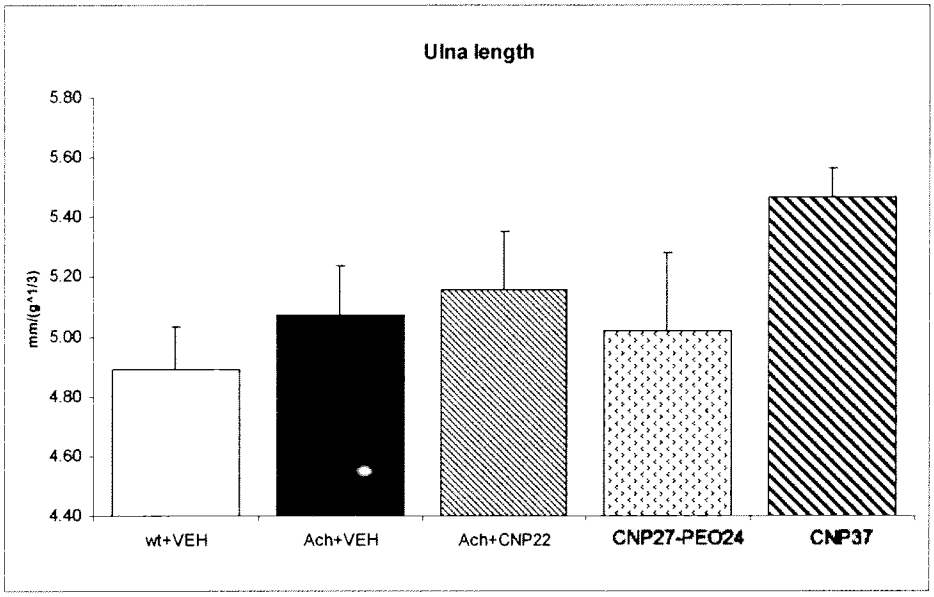


FIGURE 53

A.


Ulna length
mm/(g^1/3)
5.80
5.60
5.40
5.20
5.00
4.80
4.60
4.40
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

B.

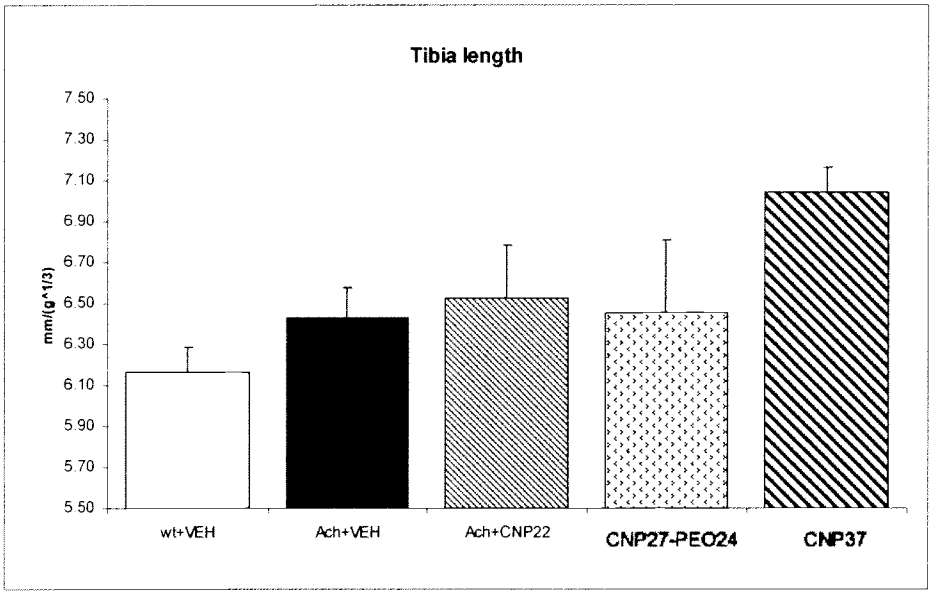
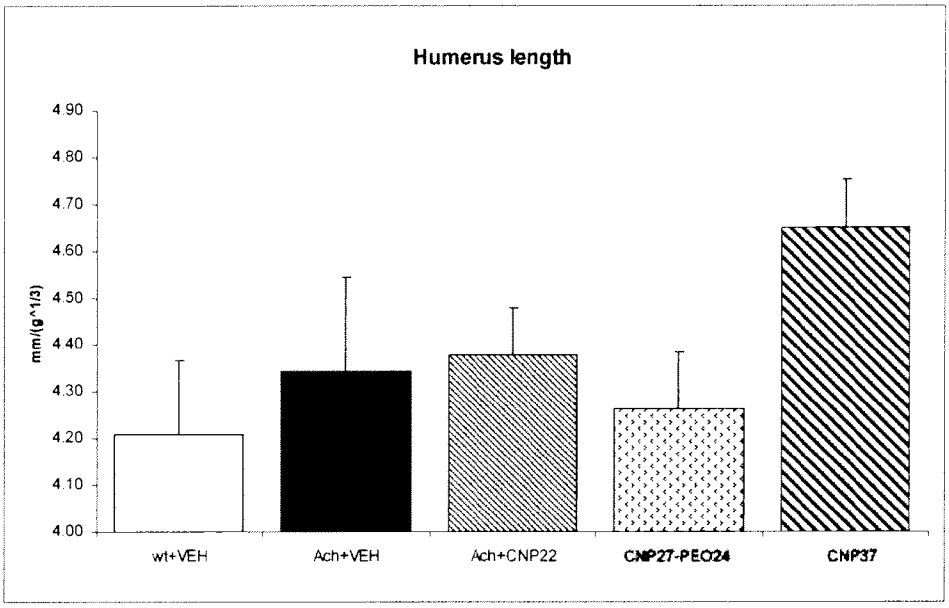

Tibia length
mm/(g^1/3)
7.50
7.30
7.10
6.90
6.70
6.50
6.30
6.10
5.90
5.70
5.50
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

FIGURE 54

A.


Humerus length
mm/(g^1/3)
4.90
4.80
4.70
4.60
4.50
4.40
4.30
4.20
4.10
4.00
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

B.

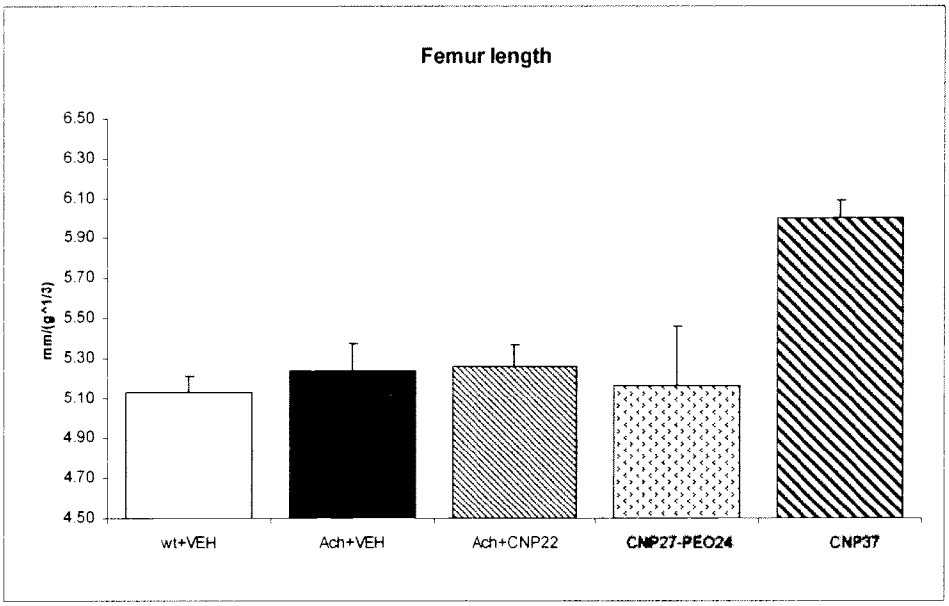

Femur length
mm/(g^1/3)
6.50
6.30
6.10
5.90
5.70
5.50
5.30
5.10
4.90
4.70
4.50
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

FIGURE 55

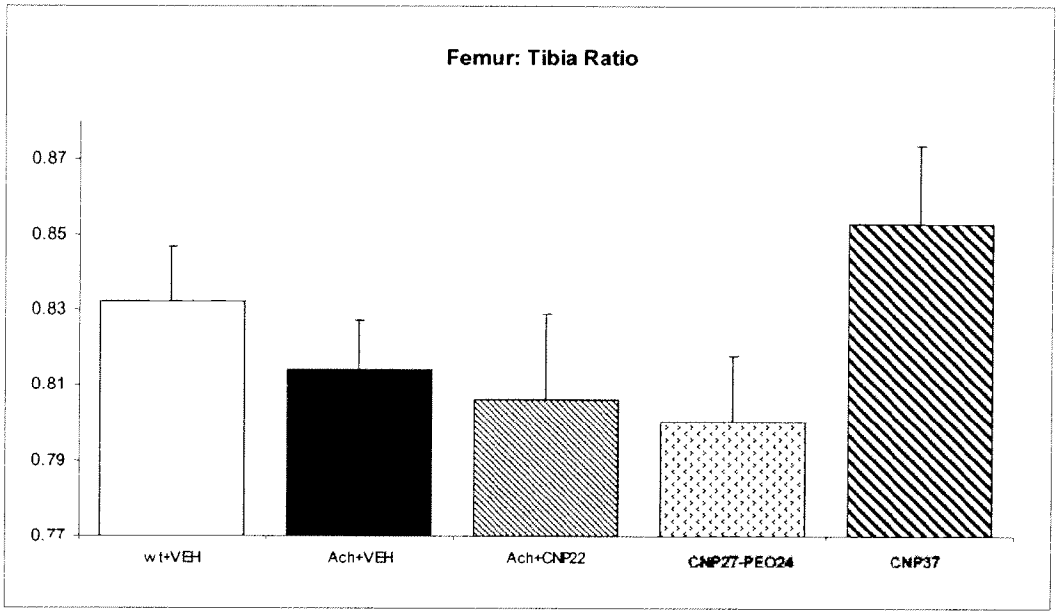


FIGURE 56

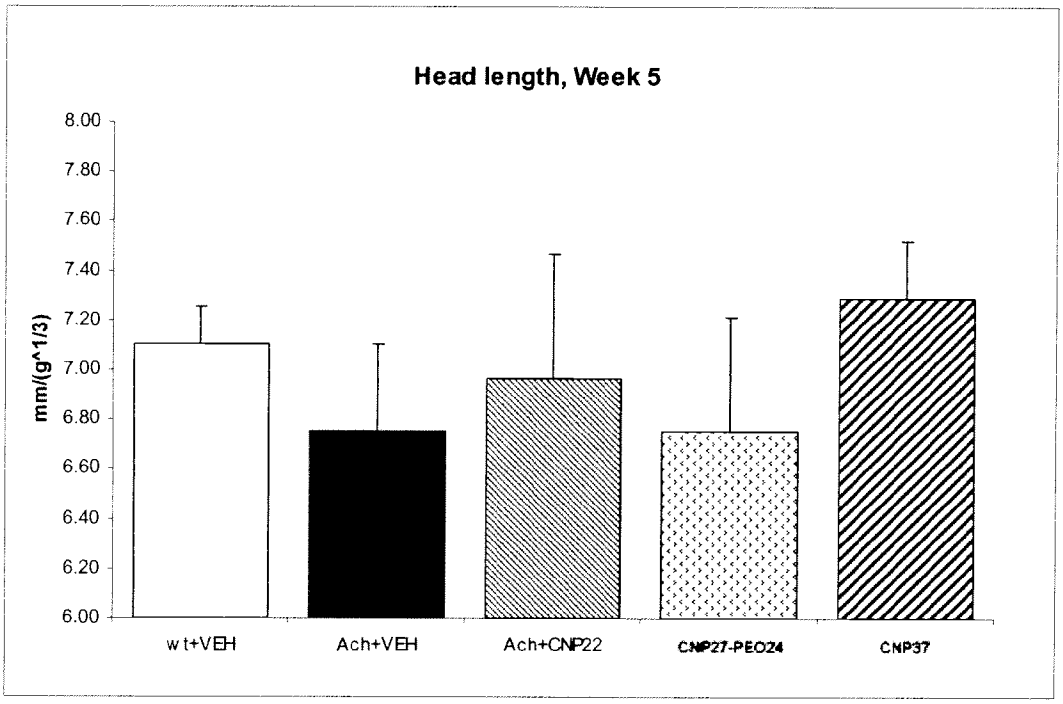


FIGURE 57

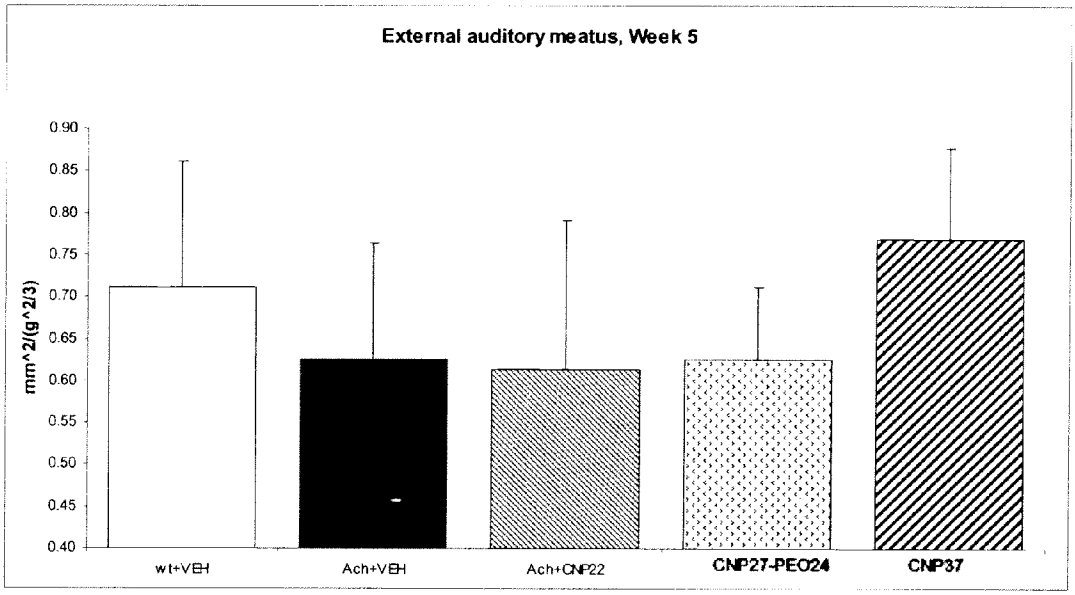


FIGURE 58

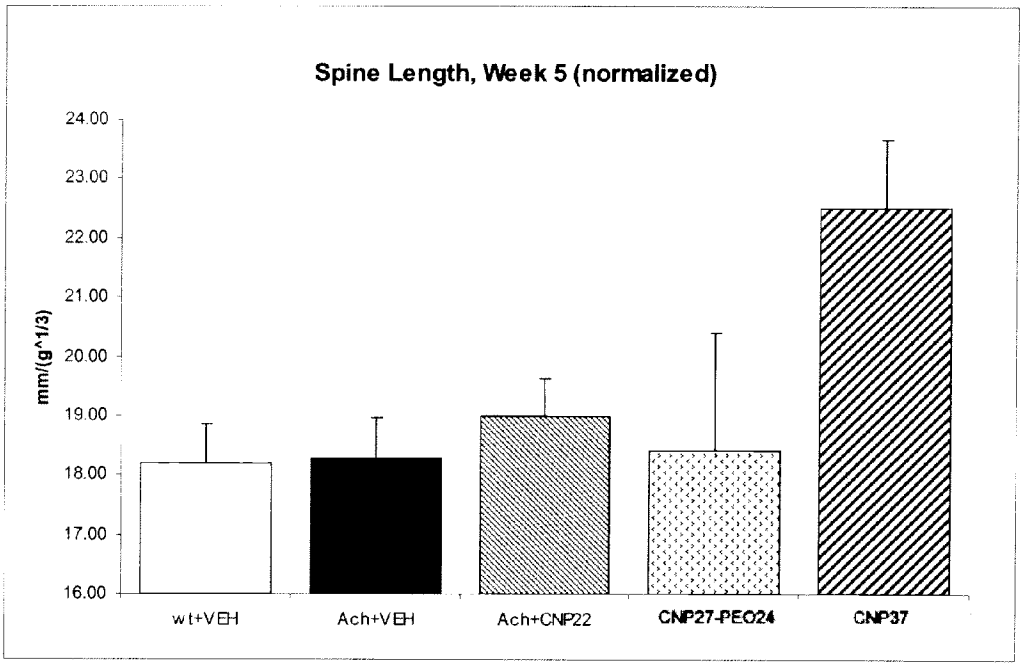


FIGURE 59

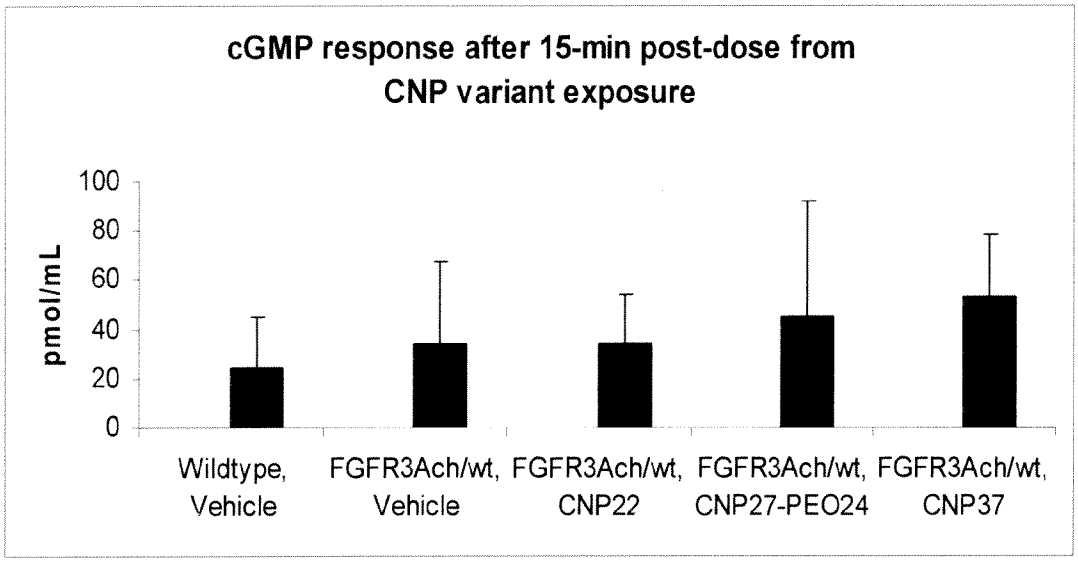


FIGURE 60

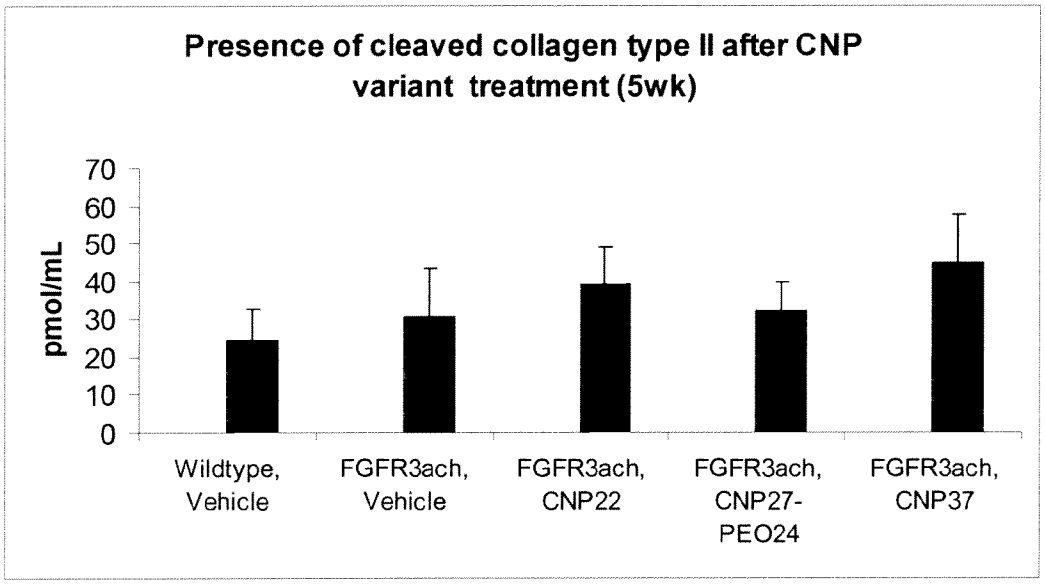


FIGURE 61

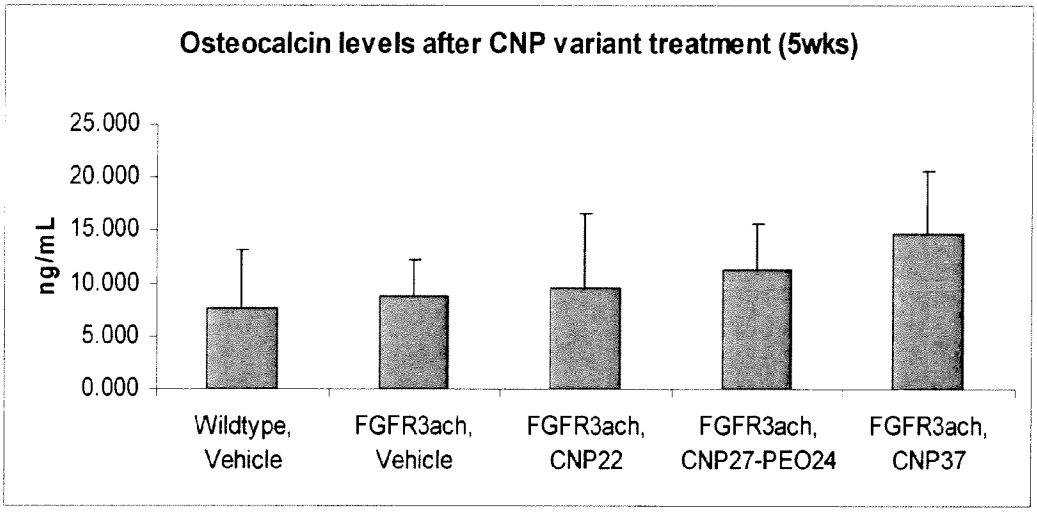


FIGURE 62

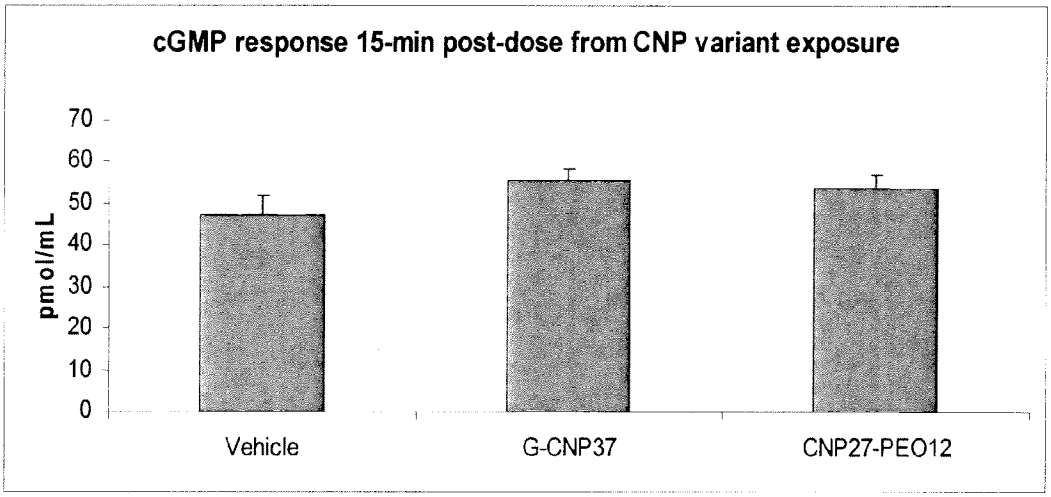


FIGURE 63

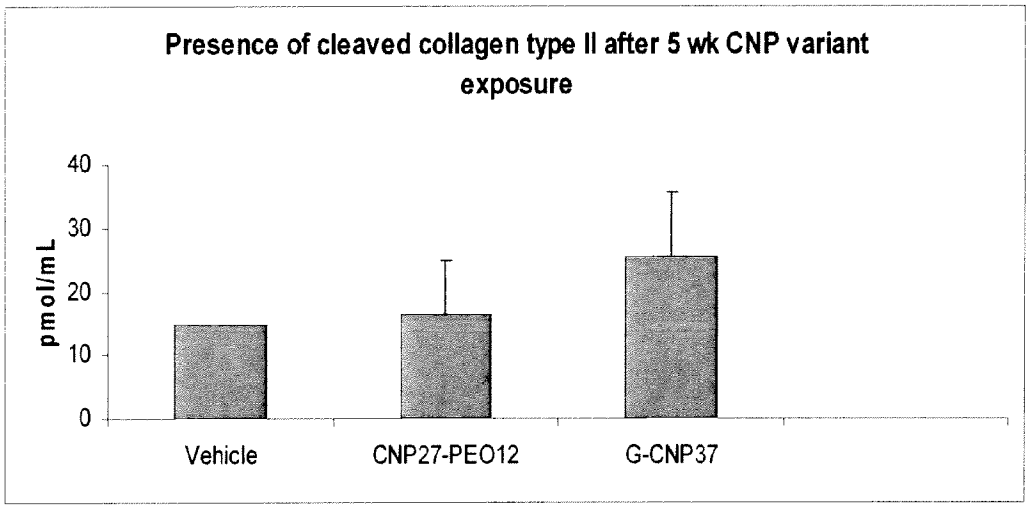
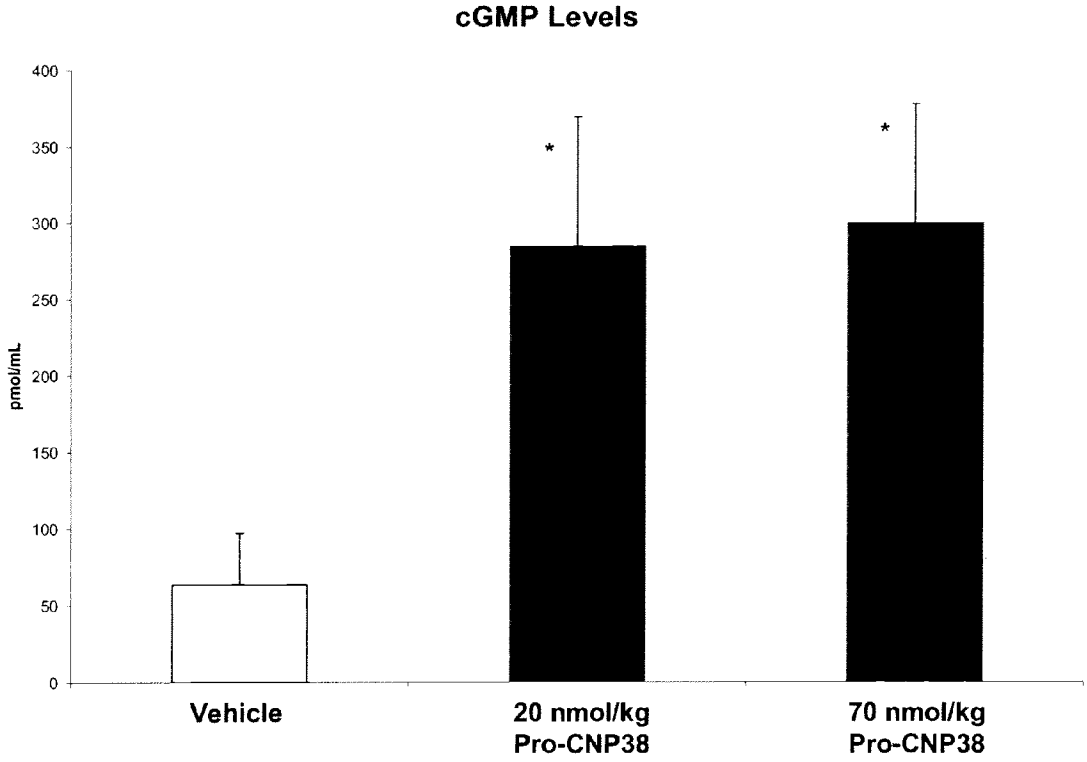


FIGURE 64



* $p < 0.05$

FIGURE 65

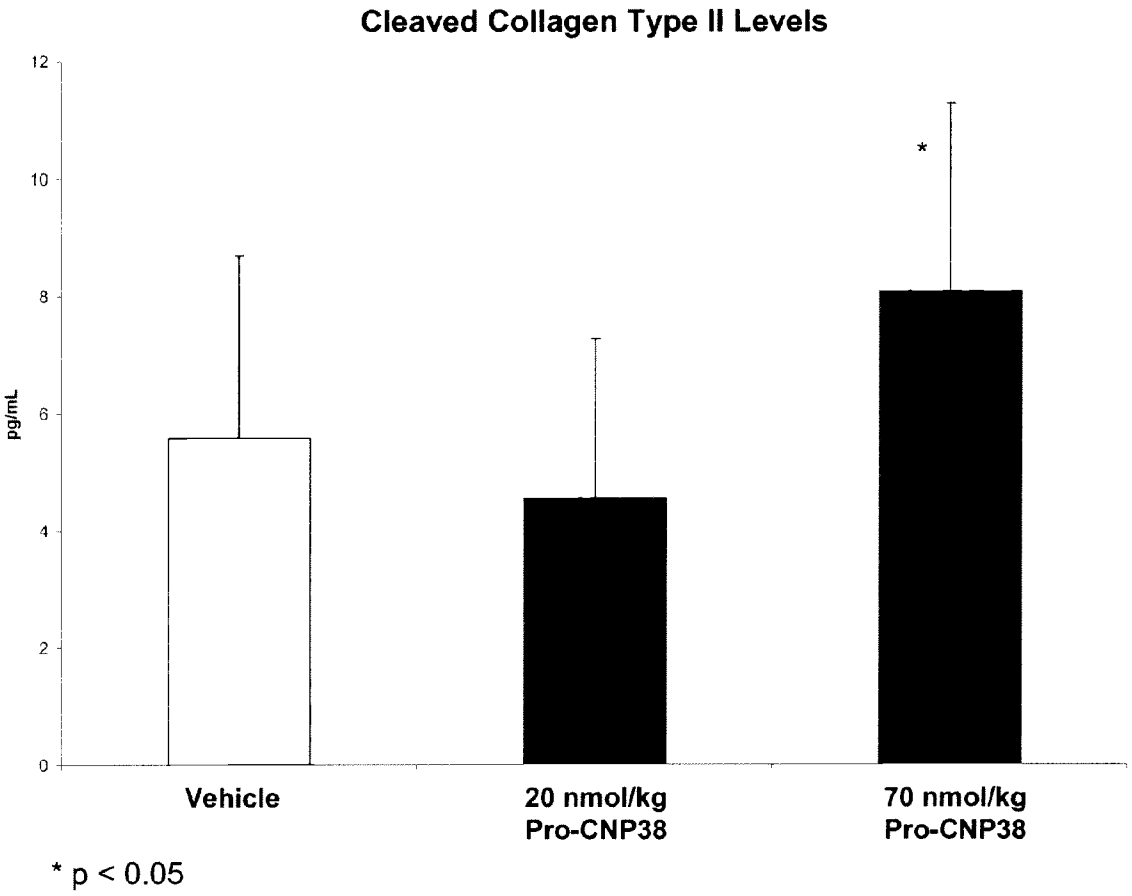

Cleaved Collagen Type II Levels
pg/mL
12
10
8
6
4
2
0
*
Vehicle
20 nmol/kg Pro-CNP38
70 nmol/kg Pro-CNP38
* p < 0.05

FIGURE 66

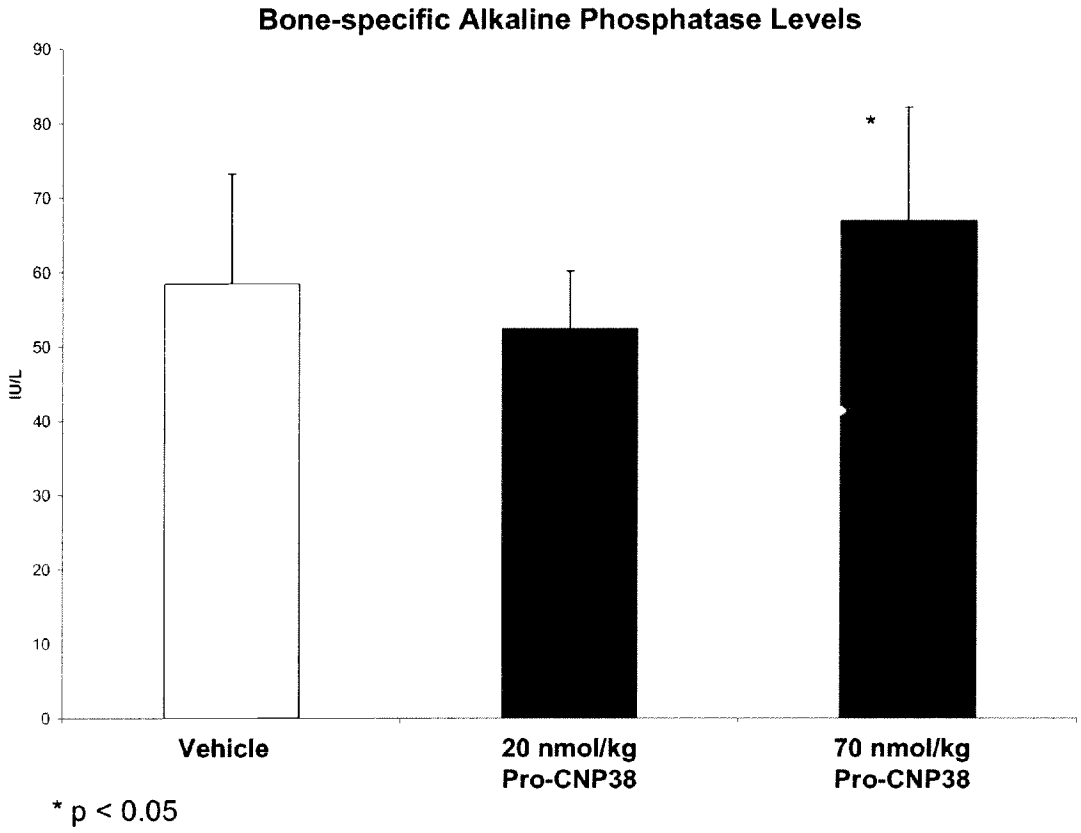

Bone-specific Alkaline Phosphatase Levels
IU/L
90
80
70
60
50
40
30
20
10
0
*
Vehicle
20 nmol/kg Pro-CNP38
70 nmol/kg Pro-CNP38
* p < 0.05

FIGURE 67

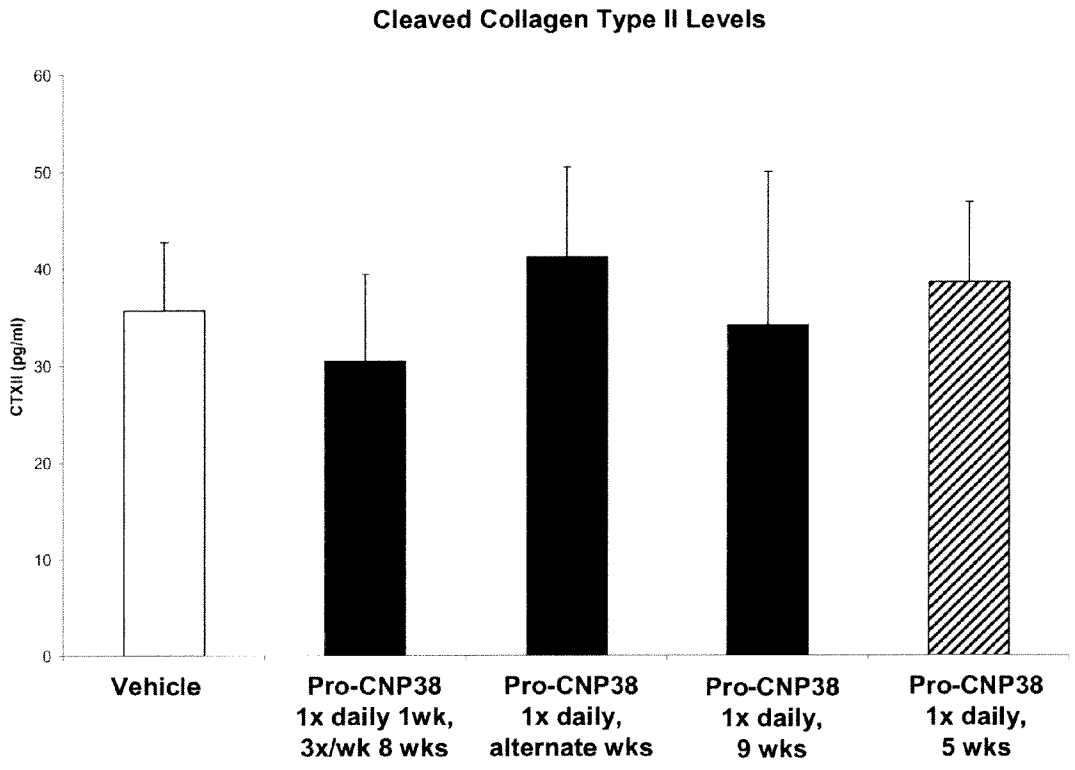

Cleaved Collagen Type II Levels
CTXII (pg/ml)
60
50
40
30
20
10
0
Vehicle
Pro-CNP38 1x daily 1wk, 3x/wk 8 wks
Pro-CNP38 1x daily, alternate wks
Pro-CNP38 1x daily, 9 wks
Pro-CNP38 1x daily, 5 wks

FIGURE 68

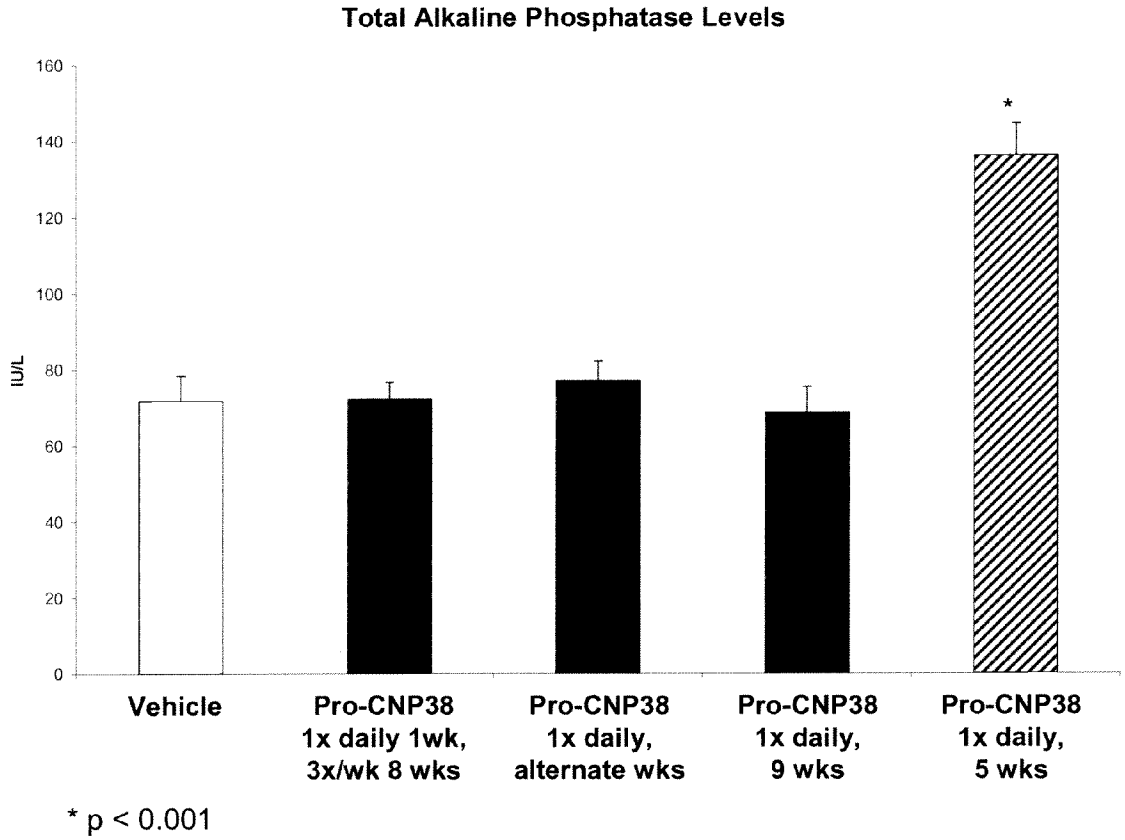

Total Alkaline Phosphatase Levels
160
140
120
100
80
60
40
20
0
IU/L
*
Vehicle
Pro-CNP38 1x daily 1wk, 3x/wk 8 wks
Pro-CNP38 1x daily, alternate wks
Pro-CNP38 1x daily, 9 wks
Pro-CNP38 1x daily, 5 wks
* p < 0.001

FIGURE 69

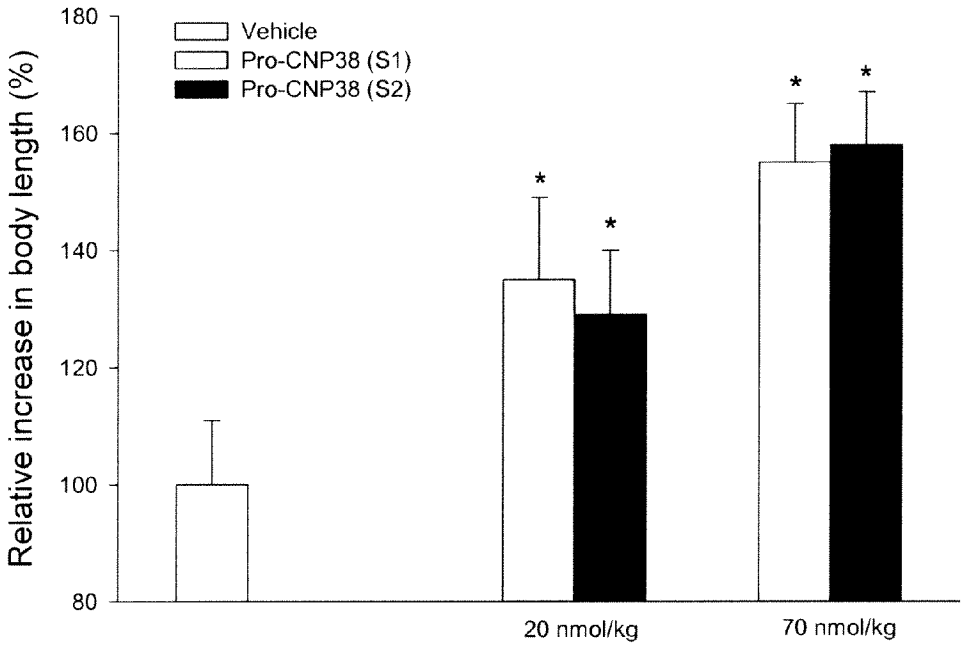
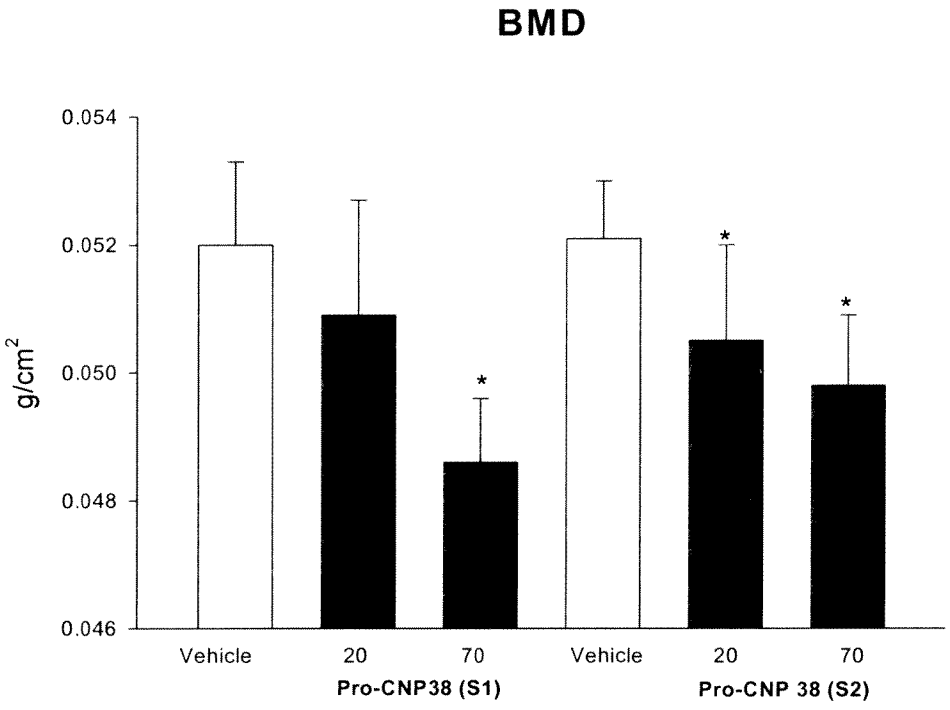

Vehicle
Pro-CNP38 (S1)
Pro-CNP38 (S2)
Relative increase in body length (%)
180
160
140
120
100
80
*
*
*
*
20 nmol/kg
70 nmol/kg

FIGURE 70

A.


BMD
0.054
0.052
0.050
0.048
0.046
g/cm2
*
*
*
Vehicle
20
70
Vehicle
20
70
Pro-CNP38 (S1)
Pro-CNP 38 (S2)

B.

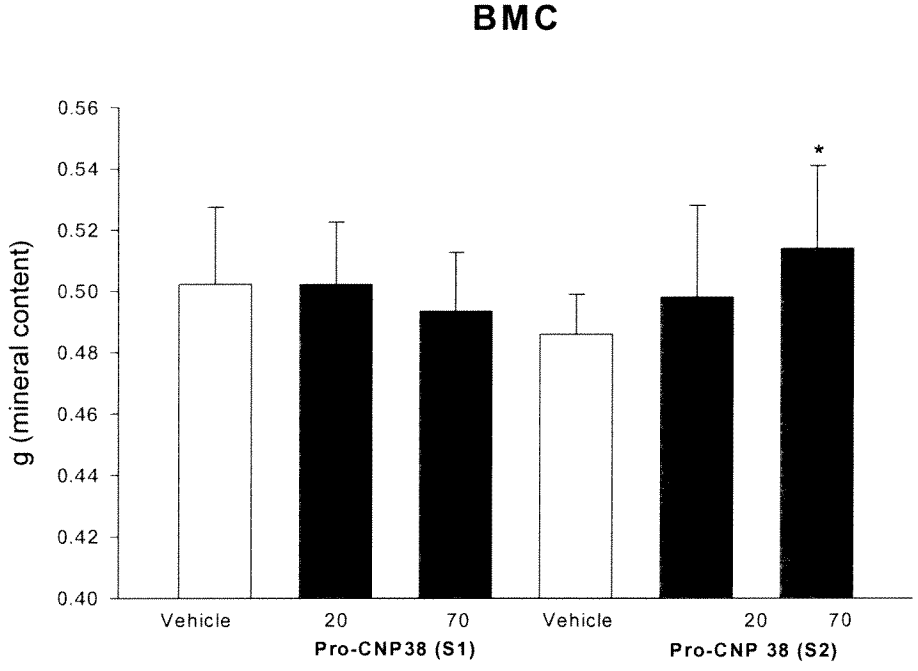

BMC
0.56
0.54
0.52
0.50
0.48
0.46
0.44
0.42
0.40
g (mineral content)
*
Vehicle
20
70
Vehicle
20
70
Pro-CNP38 (S1)
Pro-CNP 38 (S2)

FIGURE 71

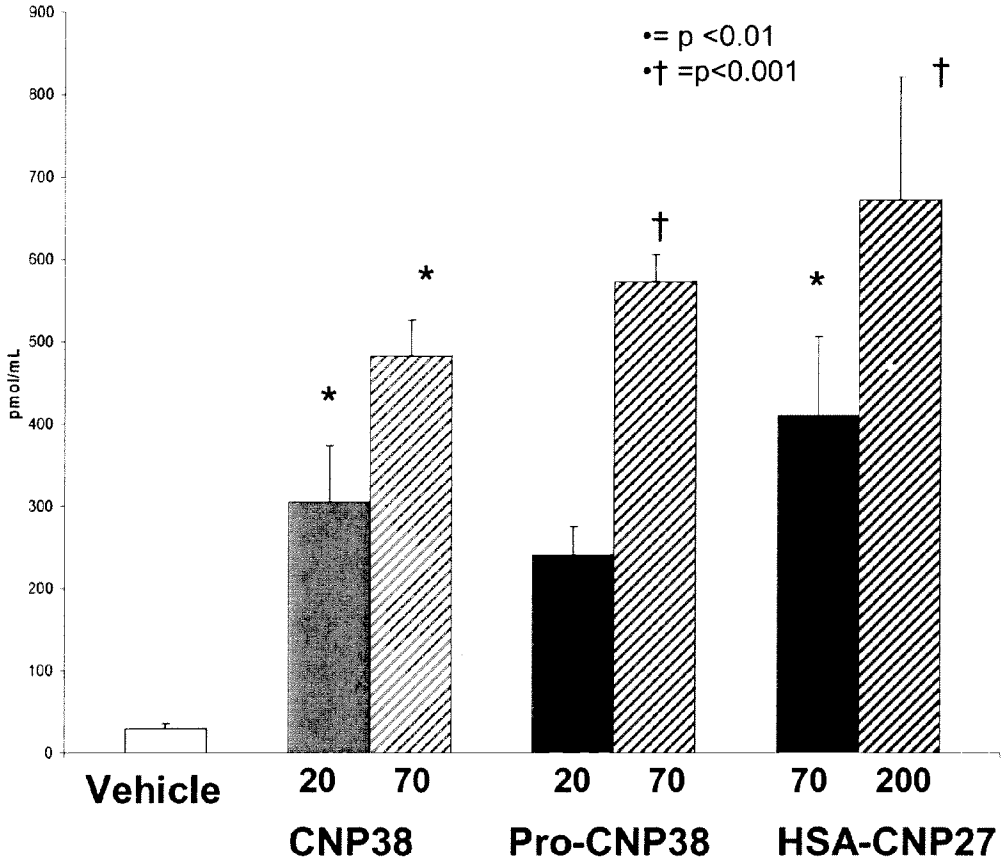

•= p <0.01
•† =p<0.001
pmol/mL
900
800
700
600
500
400
300
200
100
0
*
*
*
†
†
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 72

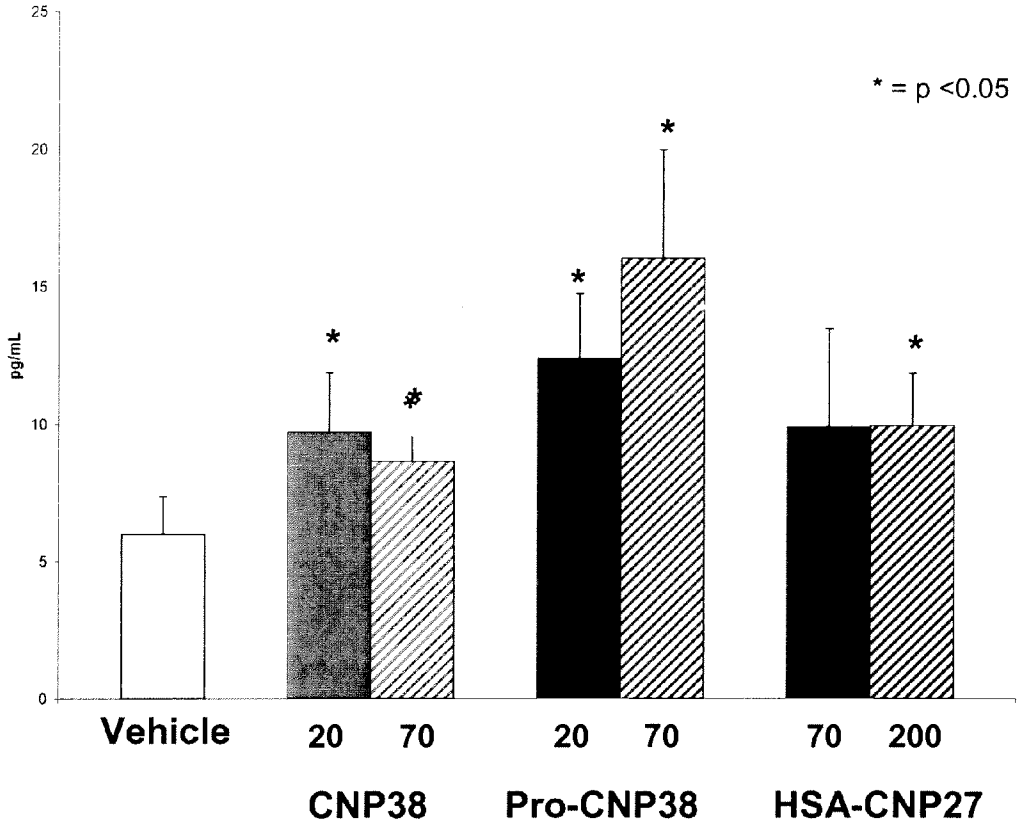

25
20
15
10
5
0
pg/mL
* = p <0.05
*
*
*
*
*
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 73

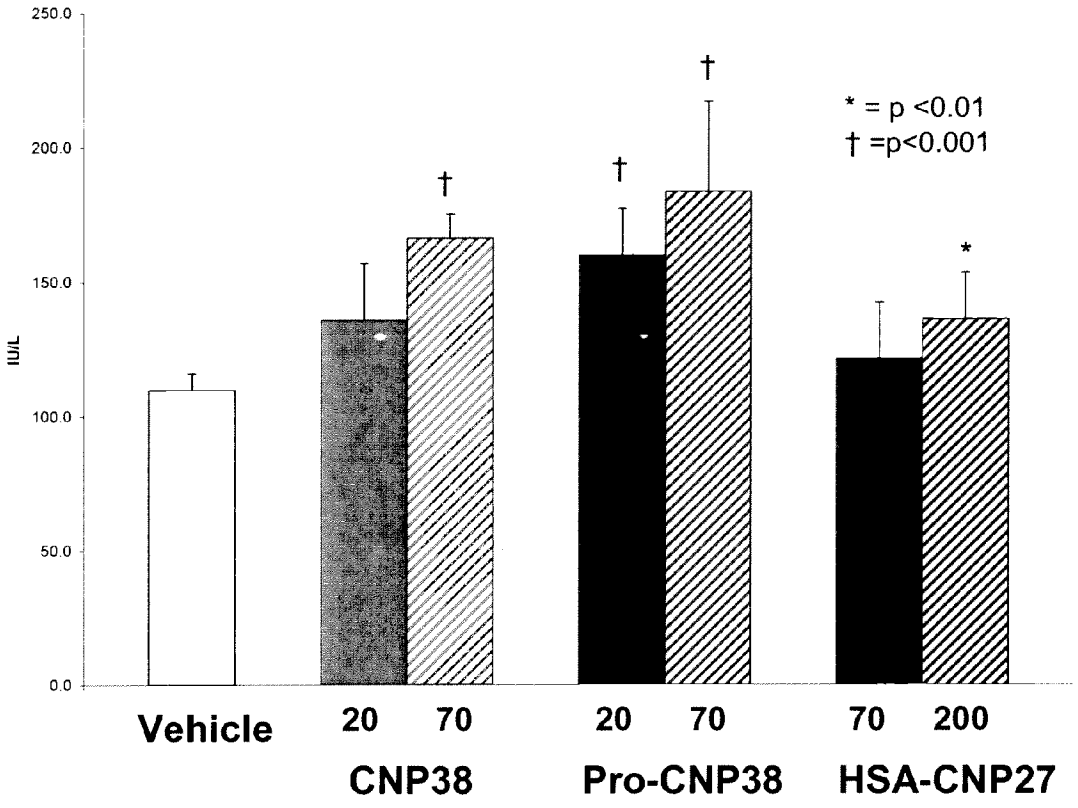
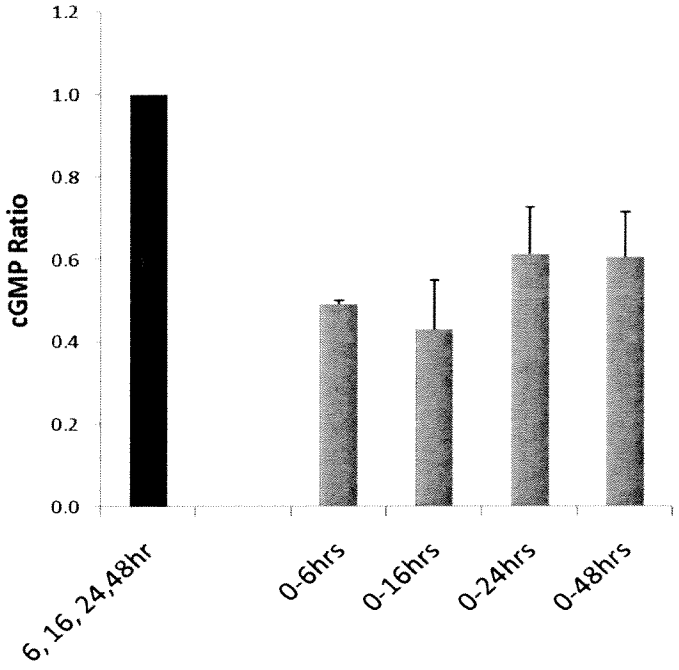

250.0
200.0
150.0
100.0
50.0
0.0
IU/L
* = p <0.01
† =p<0.001
†
†
†
*
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 74

A.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
cGMP Ratio
6, 16, 24, 48hr
0-6hrs
0-16hrs
0-24hrs
0-48hrs

B.

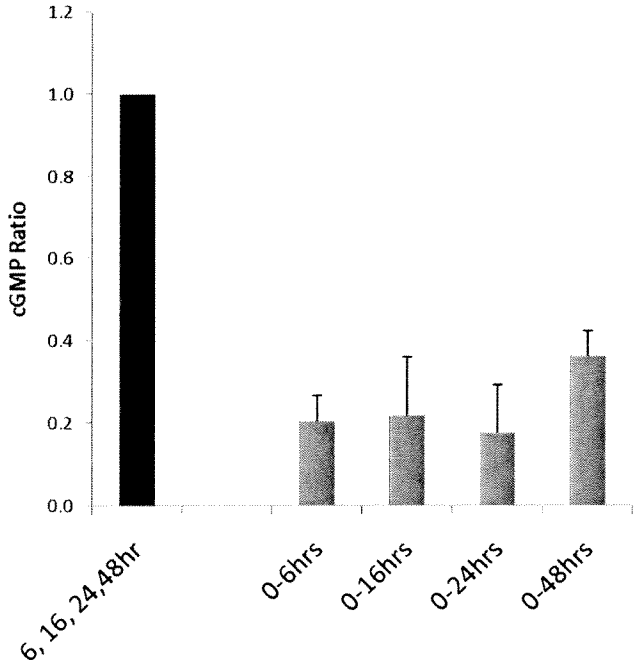

1.2
1.0
0.8
0.6
0.4
0.2
0.0
cGMP Ratio
6, 16, 24, 48hr
0-6hrs
0-16hrs
0-24hrs
0-48hrs

FIGURE 75

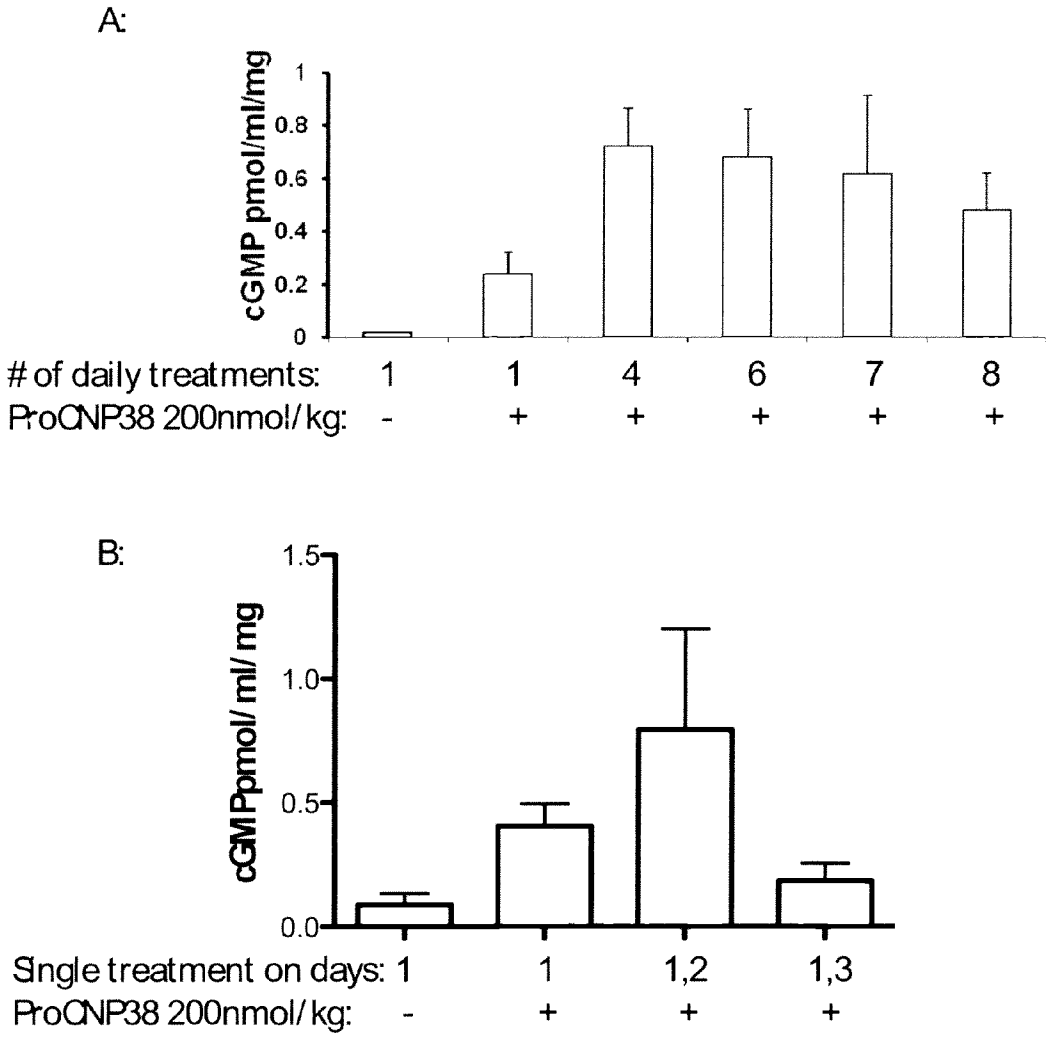

A:
cGMP pmol/ml/mg
1
0.8
0.6
0.4
0.2
0
of daily treatments: 1 1 4 6 7 8
ProCNP38 200nmol/kg: - + + + + +
B:
cGMPpmol/ml/mg
1.5
1.0
0.5
0.0
Single treatment on days: 1 1 1,2 1,3
ProCNP38 200nmol/kg: - + + +

FIGURE 76

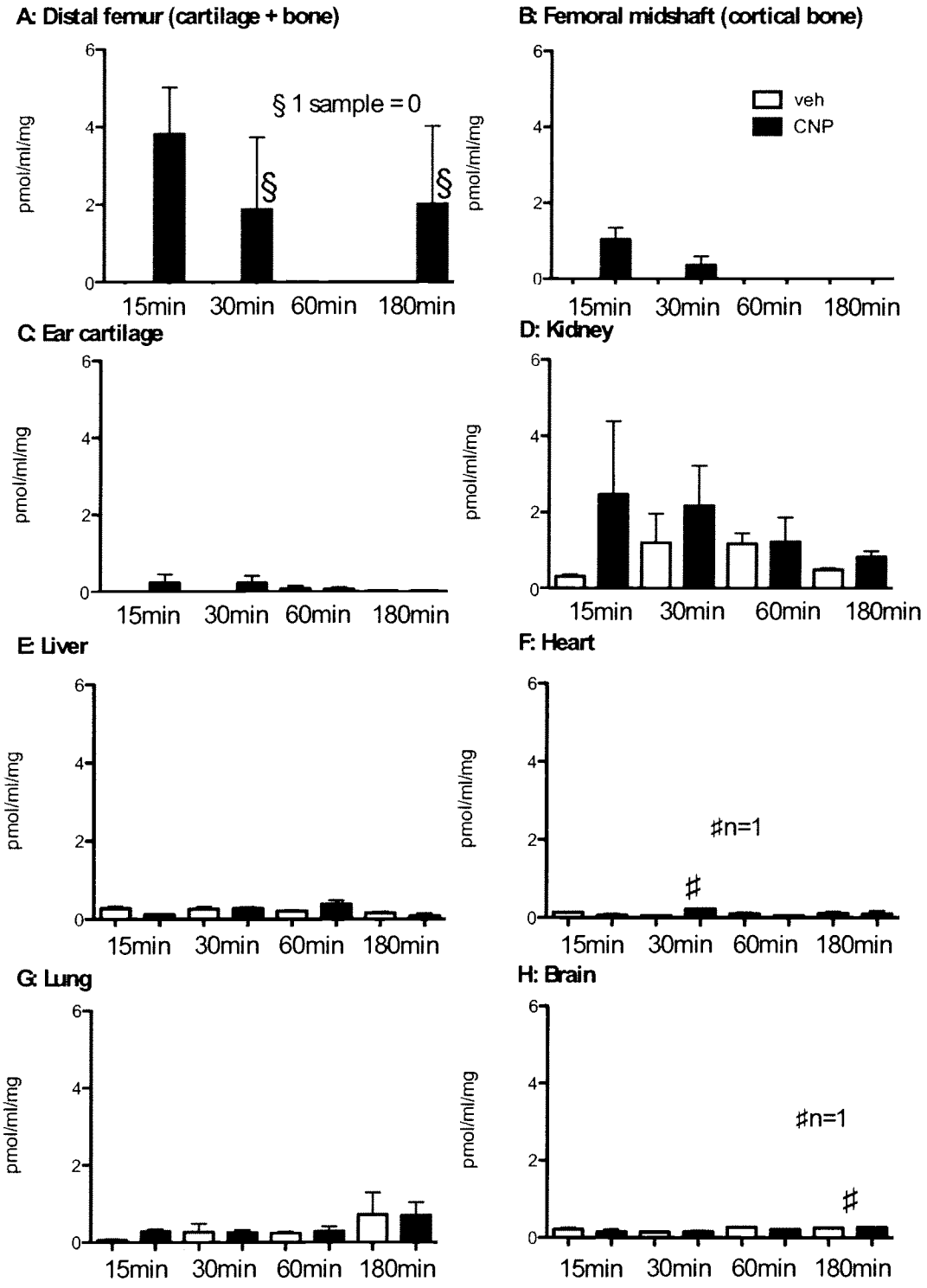

A: Distal femur (cartilage + bone)
§ 1 sample = 0
pmol/ml/mg
15min 30min 60min 180min
B: Femoral midshaft (cortical bone)
veh
CNP
C: Ear cartilage
D: Kidney
E: Liver
F: Heart
#n=1
G: Lung
H: Brain
#n=1

FIGURE 77

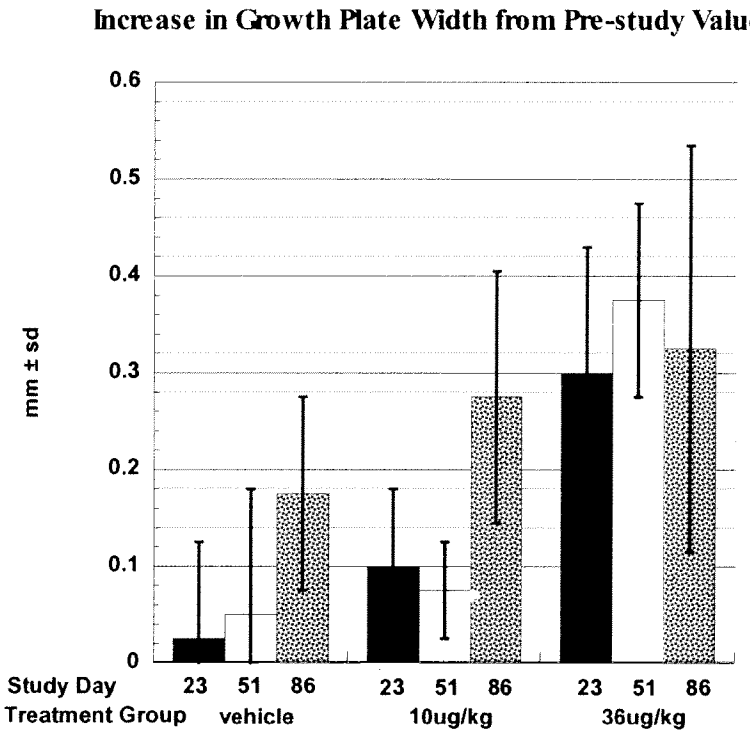


FIGURE 79

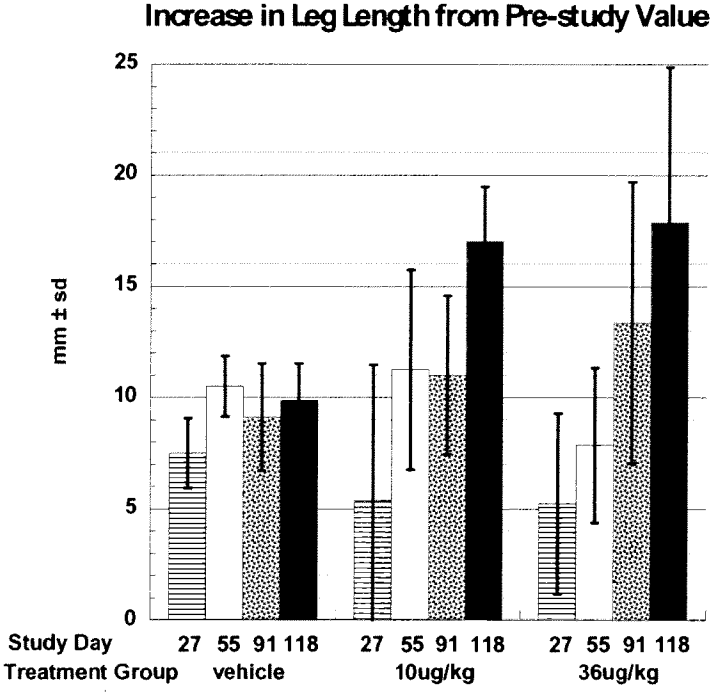
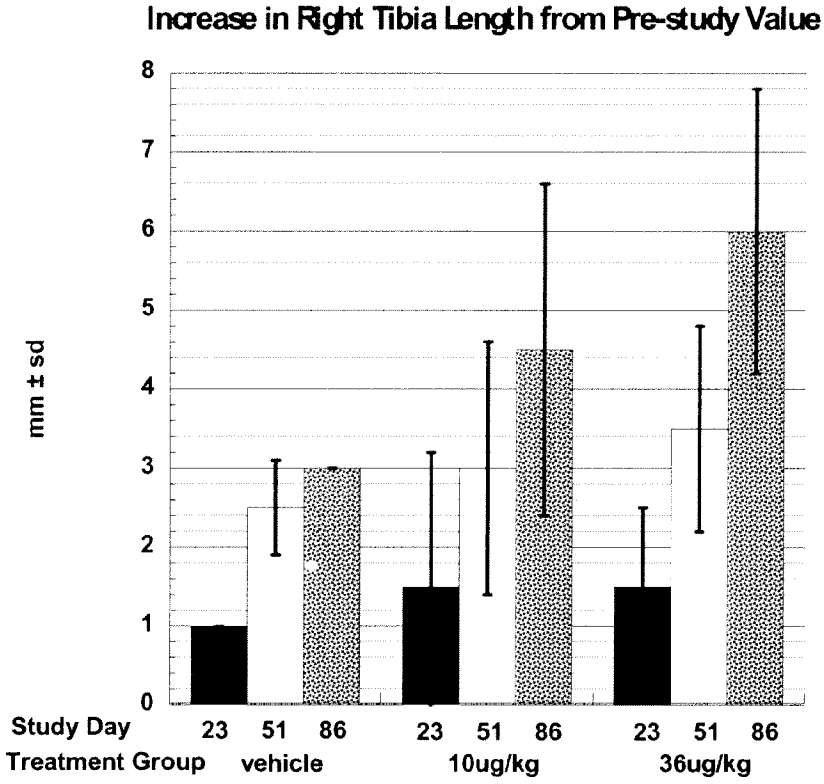


FIGURE 78

A.


Increase in Right Tibia Length from Pre-study Value
mm ± sd
0
1
2
3
4
5
6
7
8
Study Day
23 51 86 23 51 86 23 51 86
Treatment Group
vehicle
10ug/kg
36ug/kg

B.

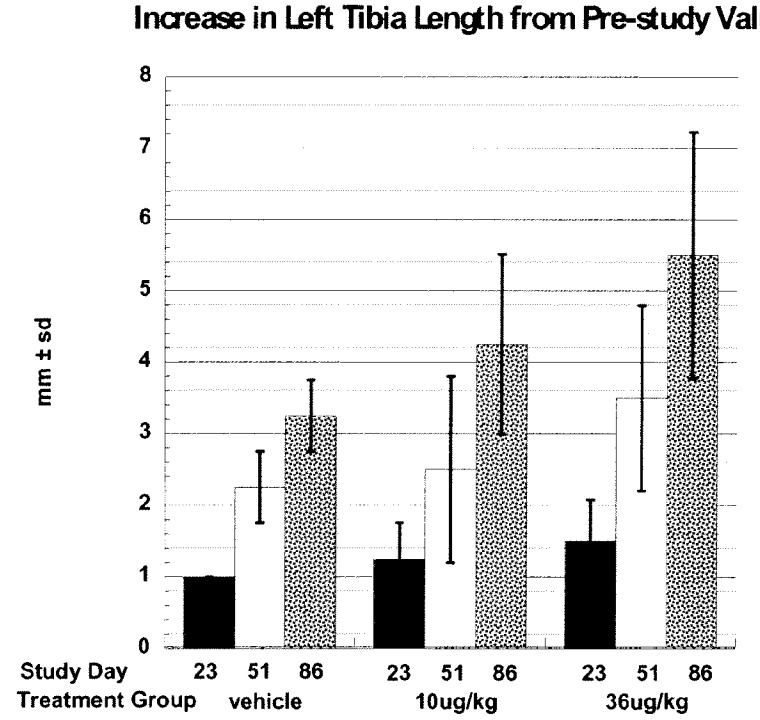

Increase in Left Tibia Length from Pre-study Value
mm ± sd
0
1
2
3
4
5
6
7
8
Study Day
23 51 86 23 51 86 23 51 86
Treatment Group
vehicle
10ug/kg
36ug/kg

FIGURE 80

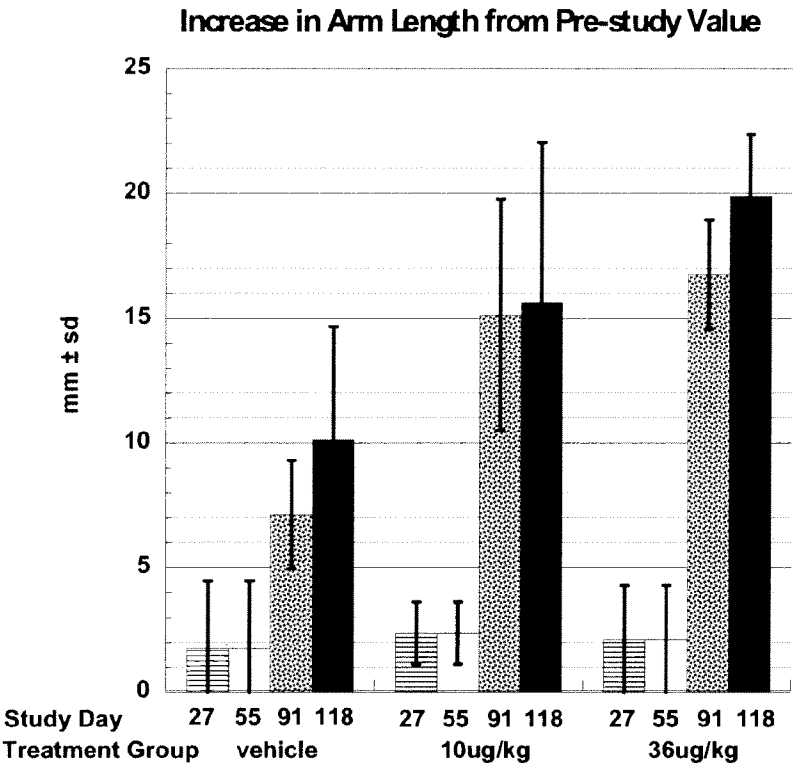


FIGURE 81

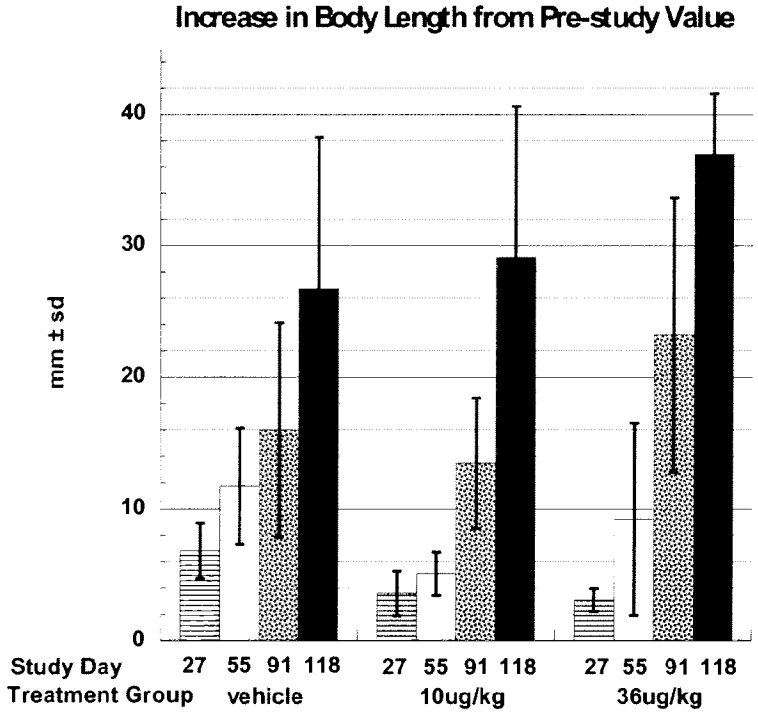


FIGURE 82

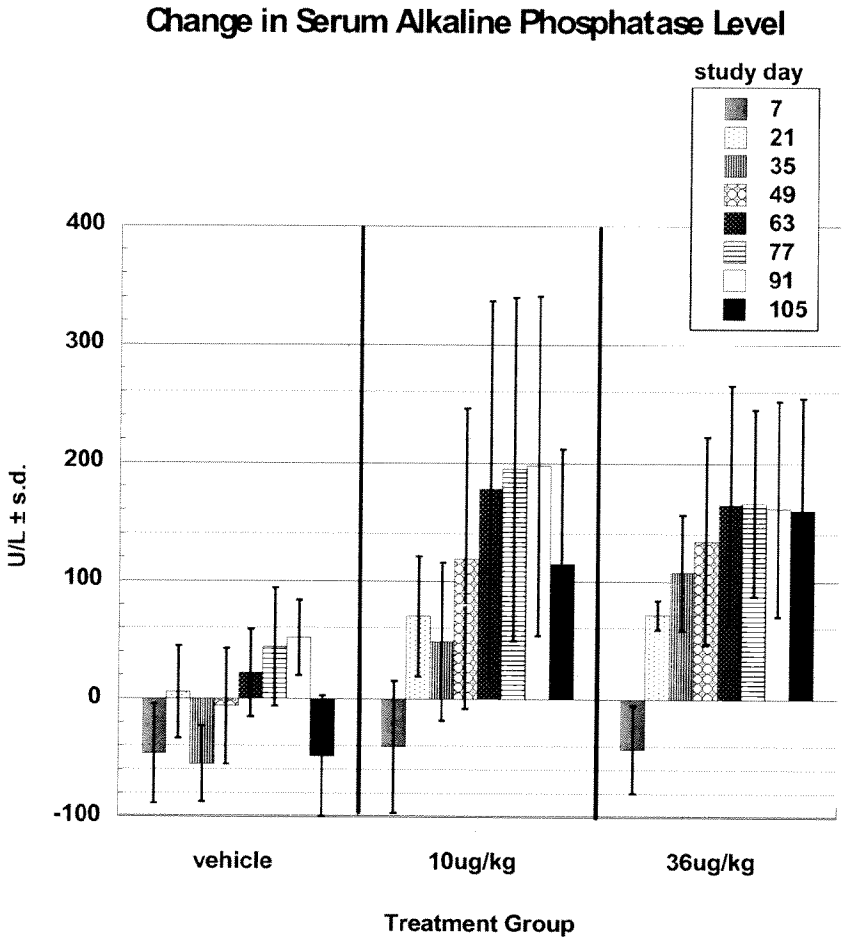
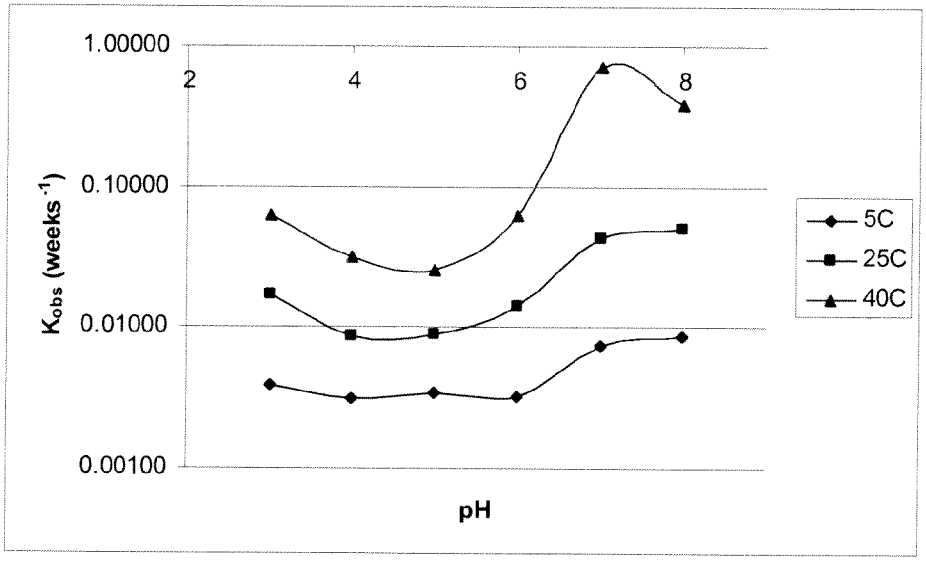


FIGURE 83



VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue; a claim printed with strikethrough indicates that the claim was canceled, disclaimed, or held invalid by a prior post-patent action or proceeding.

CROSS-REFERENCE TO RELATED APPLICATIONS

[The present application] *This application is a continuation reissue of Reissue application Ser. No. 14/604,262, which is an application for reissue of U.S. Pat. No. 8,598,121, issued Dec. 3, 2013, which is a continuation of U.S. patent application Ser. No. 12/784,117, now U.S. Pat. No. 8,198,242, issued Jun. 12, 2012, which* claims the priority and benefit of U.S. Provisional Application No. 61/254,563, filed on Oct. 23, 2009, and U.S. Provisional Application No. 61/180,112, filed on May 20, 2009, the disclosure of each of which is incorporated herein by reference in its entirety.

FIELD OF THE DISCLOSURE

The field of the disclosure relates, in general, to variants of C-type natriuretic peptide (CNP), compositions comprising CNP variants, methods of making CNP variants, and methods of using CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as skeletal dysplasias (e.g., achondroplasia) and vascular smooth muscle disorders.

BACKGROUND OF THE DISCLOSURE

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP) (Genbank Accession No. NP_006163, for the ANP precursor protein, NPPA), brain natriuretic peptide (BNP) (GenBank Accession No. NP_002512, for the BNP precursor protein, NPPB), and C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)). These small, single chain peptides (ANP, BNP, CNP) have a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. ANP and BNP bind to and activate the natriuretic peptide receptor A (NPR-A), also termed guanalyl cyclase A (GC-A), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Likewise, CNP interacts with NPR-B (GC-B) to stimulate the generation of cGMP (J. Hypertens., 10: 1111-1114 (1992)). A third type of receptor, NPR-C, binds each of the natriuretic peptides with high affinity and functions primarily to capture the peptides from the extracellular compartment and deposit the peptides into lysosomes, where they are degraded (Science, 238: 675-678 (1987)). ANP and BNP are produced primarily within the muscle cells of the heart, and are believed to have important roles in cardiovascular homeostasis (Science, 252: 120-123 (1991)). CNP is expressed more widely, including in the central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

In humans, CNP is initially produced from the natriuretic peptide precursor C(NPPC) gene as a single chain 126-amino acid pre-pro polypeptide (Biochem. Biophys. Res. Commun., 168: 863-870 (1990)). Removal of the signal peptide yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP-22) (Wu, J. Biol. Chem. 278: 25847-852 (2003)). CNP-53 and CNP-22 differ in their distribution, with CNP-53 predominating in tissues, while CNP-22 is mainly found in plasma and cerebrospinal fluid (J. Alfonzo, Recept. Signal. Transduct. Res., 26: 269-297 (2006)). The predominant CNP form in cartilage is unknown. Both CNP-53 and CNP-22 bind similarly to NPR-B. Furthermore, they both induce cGMP production in a dose-dependent and similar fashion (V T Yeung, Peptides, 17: 101-106 (1996)).

Natural CNP gene and polypeptide have been previously described. U.S. Pat. No. 5,352,770 discloses isolated and purified CNP-22 from porcine brain identical in sequence to human CNP and its use in treating cardiovascular indications. U.S. Pat. No. 6,034,231 discloses the human gene and polypeptide of proCNP (126 amino acids) and the human CNP-53 gene and polypeptide.

Clearance of CNP from the extracellular space occurs through the action of membrane-bound neutral endopeptidase (NEP), which rapidly degrades CNP (Biochem. J., 291 (Pt 1): 83-88 (1993)), and through NPR-C, which binds to and deposits CNP into lysosomes, where CNP is degraded. CNP has been shown to have an in vivo half-life of 2.6 min in the normal human (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). The low plasma concentration of CNP (J. Bone Moner. Res., 19 (Suppl. 1) S20 (2004)) and its co-expression with NPR-B in a number of tissues suggests that CNP functions primarily through an autocrine/paracrine mechanism.

As stated above, CNP binds to and activates natriuretic peptide receptor B (NPR-B), also termed guanylyl cyclase B (GC-B), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Downstream signaling mediated by cGMP generation influences a diverse array of biological processes that include endochondral ossification. Accordingly, elevated or depressed levels of any of the components in this pathway may lead to aberrant bone growth. For example, knockout of either CNP or NPR-B in mouse models results in animals having a dwarfed phenotype with shorter long bones and vertebrae. Mutations in human NPR-B that block proper CNP signaling have been identified and result in dwarfism (Olney, et al., J. Clin. Endocrinol. Metab. 91(4): 1229-1232 (2006); Bartels, et al., Am. J. Hum. Genet. 75: 27-34 (2004)). In contrast, mice engineered to produce elevated levels of CNP display elongated long bones and vertebrae.

Achondroplasia is a result of an autosomal dominant mutation in the gene for fibroblast growth factor receptor 3 (FGFR-3), which causes an abnormality of cartilage formation. FGFR-3 normally has a negative regulatory effect on chondrocyte growth, and hence bone growth. In achondroplasia, the mutated form of FGFR-3 is constitutively active, which leads to severely shortened bones. Both chondrocyte proliferation and differentiation appear to be disturbed, leading to remarkably short growth plate cartilage (P. Krejci et al., J. Cell Sci. 118: 5089-5100 (2005)). Endochondral ossification is the process that governs longitudinal long-bone growth. There are four zones of the growth plate—resting, proliferative, hypertrophic and zone of calcification. In the growth plate, NPR-B is expressed by proliferative

cells while NPR-C is expressed by hypertrophic cells (Yamashite et al., J. Biochem. 127: 177-179 (2000)). In normal endochondral bone growth, chondrocytes organize in columns and proliferate in the proliferative zone of the growth plate. These columns are disorganized in achondroplasia patients. Additionally, the hypertrophic zone is where the cells become large and eventually apoptose (lyse), leading to osteocyte invasion and mineralization. The hypertrophic chondrocytes and the overall size of the zone are much smaller in achondroplasia patients than in normal patients. CNP is an agonist for NPR-B, a positive regulator of chondrocyte and bone growth. Downstream signaling of CNP/NPR-B inhibits the FGFR-3 pathway at the level of mitogen-activated protein kinase (MAP K). Inhibition at MAP K promotes proliferation and differentiation of the chondrocytes in the proliferative and hypertrophic zones of the growth plate, resulting in bone growth.

In humans activating mutations of FGFR-3 are the primary cause of genetic dwarfism. Mice having activated FGFR-3 serve as a model of achondroplasia, the most common form of the skeletal dysplasias, and overexpression of CNP rescues these animals from dwarfism. Accordingly, CNP and functional variants of CNP are potential therapeutics for treatment of the various skeletal dysplasias.

Therapeutic use of CNP is currently limited by its short plasma half-life, which has been shown to be 2.6 minutes in vivo in humans (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). To increase CNP concentration above intrinsic levels (about 5 pM) typically found in human plasma, continuous infusion has been necessary in all human and animal studies using systemically administered CNP. A CNP variant having a longer in vivo serum half-life and exhibiting similar or improved activity to that of wild-type CNP is important for a sustainable therapeutic strategy. Two mechanisms by which the half-life of CNP is reduced in human plasma are degradation by neutral endopeptidase (NEP) and clearance by natriuretic peptide receptor C(NPR-C) (Growth Horm. & IGF Res., 16: S6-S14 (2006)). Modifications of peptides reportedly can improve resistance to endopeptidase and exopeptidase cleavage (Amino Acids, 30: 351-367 (2006); Curr. Opin. Biotech., 17: 638-642 (2006)).

The biological activities of various analogs and derivatives of CNP have been evaluated. By substituting S-methyl Cys in place of both Cys_6 and Cys_{22}, cyclization of the peptide via a Cys6-Cys22 disulfide linkage was reportedly shown to be important for the activity of CNP in stimulating cGMP formation (Biochem. Biophys. Res. Comm., 183: 964-969 (1992), also using alanine scanning to identify amino acids important for CNP functionality). A significant additional enhancement of activity reportedly results from the combined presence of the amino acids Leu_9, Lys_{10},and Leu_{11}. U.S. Pat. No. 5,434,133 describes CNP analogs comprising CNP-22 with substitutions at amino acid position 6, 7, 9, 11, or 22, wherein the amino acid is selected from Cys or Pmp (pentacyclomercaptopropionic acid) at position 6, Phe, 4-chloro-Phe, 4-fluoro-Phe, 4-nitro-Phe, or Cha (3-cyclohexyl-Ala) at position 7, Gly, Val, Aib, or tLeu at position 9, Leu or Ile at position 11, and Cys or Pmp at position 22.

U.S. Patent Publication No. 2004/0138134 (now U.S. Pat. No. 7,276,481) describes CNP variants comprising amino acids Cys_6 to Cys_{22} of CNP-22 ("CNP-17") which include at least one substitution for another natural amino acid at position 9, 10, 11, 16, 17, 19, or 20, CNP variants with insertions and deletions, such as addition of a His residue at the reported primary site of NEP cleavage, between Cys_6 and Phe_7, and methods of using such variants for increasing the size of a bone growth plate in abnormal bone and elongation of an abnormal bone. However, no significant gains in activity as measured by cGMP production were obtained for these variants, and activity was diminished for nearly all of the variants, as observed in an in vitro cell-based method (Example 7). Further no supportive data, such as for example in vitro stability or in vivo determination of improved pharmacokinetics (PK) were provided to substantiate the asserted NEP resistance and NPR-C resistance of the CNP analogs. U.S. Pat. No. 6,743,425 discloses substances for treating achondroplasia which activate NPR-B/GC-B and are peptides or low molecular weight compounds, including the C-type natriuretic peptides CNP-22 and CNP-53. PCT Publication No. WO 94/20534 discloses a chimera of CNP-22 and the 5-amino acid C-terminus ofANP designated as the vasonatrin peptide (VNP), a limited number of amino acid substitutions and cyclic chimeric peptides that result from formation of a disulfide or double bond.

Approaches for improving the half-life of other natriuretic peptide family members include decreasing the affinity of ANP for NPR-C (U.S. Pat. No. 5,846,932), utilizing pentapeptide antagonists of NPR-C (WO 00/61631), and co-administering NEP inhibitors such as thiorphan and candoxatril (Clin. Exp. Pharma. Physiol., 25: 986-991 (1997), Hyperten., 30: 184-190 (1997)). WO 2004/047871 describes conjugates of BNP and BNP variants to polyalkylene glycol moieties, sugar moieties, polysorbate moieties, polycationic moieties, and other hydrophilic polymer moieties that reportedly exhibit improved half-life in circulation and reportedly are useful for the treatment of acute congestive heart failure.

There have been no published reports, however, on a successful strategy for making CNP resistant to NEP while retaining its functionality.

SUMMARY OF THE DISCLOSURE

The present disclosure relates to variants of C-type natriuretic peptide (CNP) which are useful in the treatment of bone-related disorders (e.g., achondroplasia) and vascular smooth muscle disorders. The disclosure encompasses CNP variants having increased serum half-life, e.g. as a result of reduced ability to bind to neutral endopeptidase (NEP), greater resistance to proteolysis by NEP and/or reduced affinity to the clearance natriuretic peptide receptor C(NPR-C), while retaining the functionality of CNP.

The wild-type sequence of human CNP-22 (referred to herein as "hCNP22", "wtCNP22" or "CNP22") is set forth below:

(SEQ ID NO: 1)
(N-terminus) Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}.

Positions 6 to 22 of CNP22 form a cyclic domain by means of a disulfide bond between Cys6 and Cys22. The 17-amino acid cyclic structure has been shown to be important for binding of CNP to NPR-B (Schiller, Biochem. Biophys. Res. Commun., 138: 880-886 (1986)). The amino acid sequence of positions 6 to 22 of CNP22 is referred to herein as "CNP17" (SEQ ID NO: 2).

CNP is susceptible to NEP cleavage at a number of sites: Cys6-Phe7, Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20. In one embodiment, the disclosure encompasses a CNP variant that is (1) modified to increase its overall size or molecular weight, e.g., to a range from

about 2.6 kDa or 2.8 kDa to about 4 kDa, 4.2 kDa, 4.4 kDa, 4.6 kDa, 4.8 kDa, 5 kDa, 5.2 kDa, 5.4 kDa, 5.6 kDa, 5.8 kDa, 6 kDa, 6.2 kDa, 6.4 kDa, or to about 7 kDa, 7.2 kDa or about 8.2 kDa, and/or (2) modified at certain amino acid positions to reduce its susceptibility to NEP cleavage at 1, 2, 3, 4, 5 or all 6 of the sites listed above. The size or molecular weight of the CNP variant can be increased by various means, e.g., by conjugating additional amino acids and/or other kinds of chemical (e.g., natural or synthetic polymeric) groups to the peptide sequence at, e.g., the N-terminus, the C-terminus and/or side chain(s), and/or by using natural amino acids, unnatural amino acids, and/or peptidomimetics with bulkier side chains. The CNP variant is optionally further conjugated to other functional or structural moieties. Optionally in combination with any of the embodiments described herein, mutation(s) (e.g., substitution(s), addition(s), and/or deletion(s)) may be introduced to certain position(s) of CNP22 to reduce the CNP variants' affinity to NPR-C. Further modifications may be made without affecting NEP resistance or CNP activity, e.g., conservative substitutions, or other modifications known in the art.

In one embodiment, the CNP variant is represented by the general formula: (x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 5), wherein:

the CNP variant comprises one or more modified amino acids, which may result in modified peptide bonds (e.g., through use of peptide bond isosteres), at a position corresponding to one or more of the following CNP residues: Gly1, Lys4, Gly5, Cys6, Phe7, Gly8, Leu9, Lys10, Leu11, Ile14, Gly15, Ser16, Met17, Gly19, Leu20 and Gly21; and

(x) and (z) independently may be absent or may be an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, ANP, BNP) or a non-natriuretic polypeptide (e.g., human serum albumin (HSA), IgG, etc.).

In an embodiment, the CNP variant includes: (1) a modification at an amino acid position corresponding to one of positions 6, 7 or 8 (Cys6, Phe7 or Gly8) of CNP22, (2) optionally deletion, addition and/or substitution of any or all of the amino acids at positions 1-5 (Gly1, Leu2, Ser3, Lys4, and Gly5) and (3) optionally up to 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 further modifications (deletions, additions and/or substitutions) at positions corresponding to positions 6-22, of which 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 may be conservative substitutions or other substitutions described herein or known in the art.

It is understood that a reference to a particular amino acid position by number (e.g. position 7 of CNP22) refers to the corresponding amino acid position in any CNP variant, even if the number of the position in that CNP variant has changed due to preceding insertions or deletions. For example, a reference to "position 7" or "Phe7" would mean the corresponding position 2 for a CNP variant in which the first five amino acids had been deleted. Similarly, a reference to "position 7" would mean the corresponding position 8 for a CNP variant in which one amino acid had been added to the N-terminus.

In any of the embodiments described herein, the CNP variant may be cyclized through a covalent bond between positions corresponding to 6 and 22 of CNP22. It is contemplated that the covalent bond is formed using any methods known in the art. In another embodiment, the CNP variant may be cyclized through a covalent bond formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus (referred to as "terminal" amino acids for this purpose) of the peptide. In one embodiment, the covalent bond is formed between the side chains of the two terminal amino acids or the amino acids at positions corresponding to 6 and 22 of CNP22. In another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of each terminal amino acid. For example, head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail bonds are possible for the covalent bond formed between the terminal amino acids or between the amino acids at positions corresponding to 6 and 22 of CNP22.

In one embodiment, the disclosure provides a CNP variant having reduced affinity to NEP, and/or greater resistance to cleavage by NEP and/or increased in vivo serum half-life, while retaining functionality of CNP (e.g., stimulation of cGMP production). NEP preferably recognizes substrates smaller than about 3 kDa, due to the limited size of its active site cavity (Oefner, J. Mol. Biol., 296: 341-349 (2000)). In an embodiment, the CNP variants are modified to increase their overall molecular weight to a range from about 2.6 or 2.8 kDa to about 4, 4.6, 5, 5.2, 5.8, 6, 6.4 or 7 kDa, e.g., by adding about 0.6 to about 5 kDa of amino acids, hydrophilic or water-soluble polymers, hydrophobic acids (including fatty acids), and/or carbohydrates. In specific exemplary embodiments, the CNP variants have a molecular weight between about 2.6 kDa and about 7 kDa, or between about 2.8 kDa and 6 kDa, or between about 2.8 kDa and about 5.8 kDa. In certain embodiments, at least about 0.6, 0.8, 1, 1.2, 1.4, 1.6 or 1.8 kDa, or up to 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8 or 5 kDa are added, to increase the total molecular weight of the CNP variant, e.g., to a range from about 2.6 or 2.8 kDa up to about 4 kDa, 4.2 kDa, 4.4 kDa, 4.6 kDa, 4.8 kDa, 5 kDa, 5.2 kDa, 5.4 kDa, 5.6 kDa, 5.8 kDa, 6 kDa, 6.2 kDa, 7.2 kDa, 8.2 kDa or higher. In some embodiments, such CNP variants comprise an amino acid sequence at least about 70%, 75%. 80%, 85%, 90%, or 95% identical or homologous to amino acids 6-22 of CNP22. In other embodiments, such CNP variants comprise a substitution, insertion or deletion of 1, 2, 3, 4, 5, 6 or 7 amino acids with another natural or unnatural amino acid or peptidomimetic. While both conservative and non-conservative substitutions or insertions are envisioned at any position, introduction of modifications may commence, e.g., by conservative substitutions in regions that have been identified in the art as involved in CNP activity or NPR-B binding, while non-conservative substitutions may be made in those regions that have been previously shown to be tolerant of modification.

In another embodiment, the CNP variants comprise a CNP having an intact cyclized portion between Cys6 and Cys22, and N-terminal and/or C-terminal tails that contain about 1-40, 1-20, 5-40, 5-35, 10-35, 15-35, 5-31, 10-31, or 15-31 amino acids and are fragments derived from a CNP polypeptide and/or a non-CNP polypeptide. In an embodiment, such CNP variants have a molecular weight in a range from about 2.8 kDa to about 4, 4.6, 5, 5.2, 5.8, 6, 6.4 or 7 kDa. Non-limiting examples of such CNP variants include wild-type CNP22 or CNP22 with one or more amino acid substitutions (e.g., a K4R substitution), having an N-terminal and/or C-terminal extension derived from natriuretic peptide precursor sequences (e.g., ANP, BNP or CNP) from human or other species, a natriuretic peptide precursor C (NPPC) variant with amino acid substitutions, additions and/or deletions (e.g., the CNP variants may be truncations of CNP-53 which result in peptides with a molecular weight between about 2.8 kDa and 5.8 kDa), or other non-CNP

polypeptides such as, e.g., serum albumin or IgG protein (e.g., the CNP variants may be CNP chimeras containing fragments of serum albumin or IgG from human or other species).

In one embodiment, CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, are represented by the general formula:

(x)-Gly_1-Leu_2-Ser_3-(b)$_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-(h)$_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 6), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is polyethylene glycol (PEG, also called polyethylene oxide (PEO)), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or fibroblast growth factor 2 (FGF2);

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG); and

(b) and (h) independently may each be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser. In one embodiment, (b) is Arg. In another embodiment, for improved NEP resistance, (b) is not Gly. In yet another embodiment, (h) is not Arg.

Non-limiting examples of amino acid sequences derived from NPPC or variants thereof include:

Arg,

Glu-Arg,

(SEQ ID NO: 7)
Gly-Ala-Asn-Lys-Lys,

(SEQ ID NO: 8)
Gly-Ala-Asn-Arg-Arg,

(SEQ ID NO: 9)
Gly-Ala-Asn-Pro-Arg,

(SEQ ID NO: 10)
Gly-Ala-Asn-Gln-Gln,

(SEQ ID NO: 11)
Gly-Ala-Asn-Ser-Ser,

(SEQ ID NO: 12)
Gly-Ala-Asn-Arg-Gln,

(SEQ ID NO: 13)
Gly-Ala-Asn-Arg-Met,

(SEQ ID NO: 14)
Gly-Ala-Asn-Arg-Thr,

-continued

(SEQ ID NO: 15)
Gly-Ala-Asn-Arg-Ser,

(SEQ ID NO: 16)
Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala,

(SEQ ID NO: 17)
Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg,

(SEQ ID NO: 18)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg,

(SEQ ID NO: 19)
Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys,

(SEQ ID NO: 20)
Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg,

(SEQ ID NO: 21)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys,
and

(SEQ ID NO: 22)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg.

Non-limiting examples ofamino acid sequences derived from non-CNP polypeptides such as, e.g., ANP, BNP, serum albumin and IgG include:

(SEQ ID NO: 23)
Ser-Leu-Arg-Arg-Ser-Ser;

(SEQ ID NO: 24)
Asn-Ser-Phe-Arg-Tyr;

(SEQ ID NO: 25)
Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly;

(SEQ ID NO: 26)
Met-Val-Gln-Gly-Ser-Gly;

(SEQ ID NO: 27)
Lys-Val-Leu-Arg-Arg-Tyr;

(SEQ ID NO: 28)
Lys-Val-Leu-Arg-Arg-His;

(SEQ ID NO: 29)
Gly-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg;

(SEQ ID NO: 30)
Gly-Val-Pro-Gln-Val-Ser-Thr-Ser-Thr;

(SEQ ID NO: 31)
Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln;
and

(SEQ ID NO: 32)
Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser.

In an embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof independently may be conjugated to an amino acid extension containing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acids. In one embodiment, the amino acid extension is derived from NPPC, CNP53, ANP or BNP. In a specific embodiment, the amino acid extension is Gln-

Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 33). In a related embodiment, this 15-amino acid extension is added to the N-terminus to provide a CNP variant of the formula Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-$Gly1l_{15}$-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 34).

In one embodiment, the CNP variants comprise wtCNP22 or a variant thereof (e.g., one having addition(s), deletion(s), and/or substitution(s) such as, e.g., a K4R substitution) (SEQ ID NO: 35) conjugated at the N-terminus and/or C-terminus to a hydrophilic polymer (e.g., PEG) to increase their overall molecular size to a range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6 or 7 kDa. Such CNP variants are optionally further conjugated at the N-terminus and/or C-terminus to a polymeric group comprising, e.g., amino acids, carbohydrates, hydrophobic acids and/or phospholipids, a non-limiting example of which is an N-terminal amino acid extension containing 1 to 35, or 5 to 31, amino acids. In an embodiment, a hydrophilic polymeric (e.g., PEG) moiety of at least about 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6 or 1.8 kDa, or up to about 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8 or 5 kDa, is added to the N-terminus and/or C-terminus of wtCNP22 or a variant thereof.

As shown herein, conjugation of a hydrophilic or water-soluble PEG (or PEO) polymer of about 0.6 kDa or more to CNP22 or variants thereof generally increases resistance to NEP cleavage markedly. However, addition of PEG, even as small as 0.6 kDa, to wtCNP22 may reduce CNP functionality (e.g., stimulation of cGMP signaling), and addition of greater than about 2 or 3 kDa of PEG to CNP22 or variants thereof may reduce CNP functional activity in a size-dependent manner. But CNP functionality (at least comparable to that of wtCNP22) is retained when a PEG (or PEO) polymer of about 0.6 kDa to about 1.2 kDa, or potentially to about 2 kDa, is conjugated to a CNP variant having an N-terminal amino acid extension in which at least one relatively large amino acid that may potentially be positively charged under physiological conditions (e.g., arginine) immediately precedes the position corresponding to Gly1 of CNP22, such as, e.g., GANPR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), ER-CNP22 (SEQ ID NO: 38), ER-CNP22 (K4R) (SEQ ID NO: 39), R-CNP22 (SEQ ID NO: 40) and R-CNP22(K4R) (SEQ ID NO: 41).

Accordingly, in one embodiment, PEGylated CNP variants comprise at the N-terminus of CNP22 or a variant thereof (e.g., one having a K4R substitution) an amino acid extension containing at least 1, 2, 3, 4 or 5 amino acids, wherein the PEG polymer is conjugated to the N-terminus of the amino acid-extended CNP variant to result in a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP cleavage. In an embodiment, for enhanced CNP functionality, such pegylated, amino acid-extended CNP variants contain at least one relatively large natural or unnatural amino acid that may potentially be positively charged under physiological conditions, immediately preceding the position corresponding to Gly1 of CNP22. In a specific embodiment, the pegylated, amino acid-extended CNP variants contain at least one arginine residue immediately preceding the position corresponding to Gly1 of CNP22.

In addition to CNP variants conjugated at the N-terminus and/or C-terminus to a hydrophilic or water-soluble polymer such as, e.g., PEG (or PEO), the disclosure encompasses CNP variants conjugated to such a polymer at an internal site. For purposes of brevity here, PEG (or PEO) will be used as a representative example of a hydrophilic or water-soluble polymer. Various sites of PEGylation of a CNP variant are possible, including but not limited to: (1) PEGylation only at the N-terminus; (2) PEGylation only at the C-terminus; (3) PEGylation only at an internal site (e.g., Lys4); (4) PEGylation at both the N-terminus and the C-terminus; (5) PEGylation at the N-terminus and an internal site; and (6) PEGylation at the C-terminus and an internal site. For increased resistance to NEP degradation and retention of CNP functionality, in certain embodiments the total mass of PEGylatedCNP variants is characterized by the ranges described generally herein, e.g., in the range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6 or 7 kDa. In a particular embodiment, CNP17, CNP22, CNP37 (defined below) or variants thereof (including those having amino acid additions, substitutions and/or deletions) are PEGylated only at the N-terminus. In another embodiment, the CNP variants are PEGylated only at an internal site (e.g., Lys4). In yet another embodiment, the CNP variants are PEGylated at the N-terminus and an internal site (e.g., Lys4). In still another embodiment, for better functionality the CNP variants are not PEGylated at a site (e.g., Lys10) within the cyclic domain (corresponding to Cys6 to Cys22 of CNP22). To prevent PEGylation at an internal site, Lys4 and/or Lys10 can be substituted with a natural or unnatural amino acid or peptidomimetic that does not contain a reactive primary amino group on a side chain, such as, e.g., Gly, Ser, Arg, Asn, Gln, Asp, Glu or citrulline (Cit). In a particular embodiment, Lys4 and/or Lys10 are replaced with Arg. In another embodiment, Lys10 is not replaced with Arg.

The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG molecules) that may vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP peptide by means of N-hydroxy succinimide (NHS)-or aldehyde-based chemistry or other chemistry, as is known in the art. Such CNP variants can be generated using, e.g., wtCNP-22 (2.2 kDa), CNP-17 retaining only the cyclized region (residues 6-22) of wtCNP22, CNP variants having an amino acid extension at the N-terminus and/or C-terminus of CNP22, or variants with amino acid substitutions, additions and/or deletions, for example, GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22 (K4R) (SEQ ID NO: 37), R-CNP22 (SEQ ID NO: 40), R-CNP22(K4R) (SEQ ID NO: 41), ER-CNP22 (SEQ ID NO: 38) and ER-CNP22(K4R)

(SEQ ID NO: 39). In an embodiment, the PEG-CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, contain a monodispersed, linear PEG (or PEO) group conjugated at the N-terminus and/or C-terminus via NHS- or aldehyde-based chemistry, or a two-arm or three-arm branched PEG group conjugated at the N-terminus and/or C-terminus via NHS-based chemistry. The disclosure further contemplates negatively charged PEG-CNP variants designed for reduced renal clearance, including but not limited to carboxylated, sulfated and phosphorylated compounds.

In a related embodiment, the disclosure contemplates PEG-CNP conjugates comprising NHS- or aldehyde-based PEG of the formula $(CH_2CH_2O)_n$, wherein n is an integer from 12 to 50, and the PEG polymer is up to about 2.5 kDa in molecular weight. Ina specific embodiment, n is 12 or 24. In an embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the capping group is an alkyl group, e.g., a lower alkyl group such as methyl.

In an additional embodiment, the PEG polymers or derivatives thereof have a polymer number-average molecular weight in the range from about 0.4 kDa to about 2.5 kDa or from about 0.6 kDa to about 1.5 kDa.

In a further embodiment, the wtCNP or CNP variant peptide is conjugated to a moiety including, e.g., bisphosphonates, carbohydrates, hydrophobic acids (including fatty acids) or amino acid sequences. Such amino acid sequences include for example polyAsp or polyGlu useful in bone/cartilage targeting, or can be derived from bone proteins with elucidated bone-targeting domains or derivatives thereof, such as for example fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc. In embodiments described herein where CNP22 or a variant thereof is attached to a bone- or cartilage-targeting moiety, such a moiety is designed to promote getting the modified CNP peptide to chondrocytes of bone growth plates, where the peptide can bind and activate NPR-B on the chondrocytes.

In another embodiment, the disclosure provides CNP variants with a peptide bond that is less susceptible to cleavage by peptidases including NEP. The disclosure encompasses a CNP variant comprising at least one modified residue at a site of endopeptidase cleavage. In one embodiment, the Cys6-Phe7 peptide bond (—C(═O)—NH—) at an NEP cleavage site in CNP can be replaced with anyone of the following peptide-bond isosteres:

—CH_2—NH—,

—C(═O)—N(R)—, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

—C(═O)—NH—CH_2—,

—CH_2—S—,

—CH_2—$S(O)_n$—, where n is 1 or 2,

—CH═CH—,

—C(═O)—CH_2—,

—CH(CN)—NH—,

—CH(OH)—CH_2—,

—O—C(═O)—NH—, and

—NHC(═O)NH—.

In another embodiment, Phe7 is substituted with its enantiomer D-Phe. In yet another embodiment, D-enantiomers are introduced to one or more, up to all 22, positions within wtCNP-22. In a further embodiment, a beta amino acid such as 3-amino-2-phenylpropionic acid is substituted for Phe7, which increases the length of the backbone while reducing the length of the side chain. In yet another embodiment, the disclosure contemplates Cys analogs at the Cys6 position including but not limited to homocysteine, penicillamine, 2-mercaptopropionic acid, and 3-mercaptopropionic acid.

Even in the presence of a NEP-resistant bond between Cys6 and Phe7, other peptide bonds can be hydrolyzed by NEP, including Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20. Accordingly, the disclosure encompasses CNP analogs containing peptide bond isosteres at multiple locations in the backbone of the CNP analogs. In one embodiment, CNP analogs or variants comprise modifications at more than one peptidase cleavage site. In a further embodiment, such variant comprises a CNP with substitutions at amino acid residues important in binding to the NEP active site, thereby increasing resistance to NEP degradation. One or more NEP-binding residues, including but not limited to Gly8, Gly15, Ser18, Gly19 and/or Gly21, are replaced with larger-size natural or unnatural amino acid residues to reduce affinity to the NEP active site. In yet another embodiment, one or more hydrophobic residues essential in NEP recognition, including but not limited to Phe7, Leu9, Leu11, Ile14, Met17 and Leu20, are substituted with natural or unnatural amino acids and/or peptidomimetics that decrease NEP binding. In yet another embodiment, one to five of the first five amino acids of CNP can be deleted or substituted with any other natural amino acids or unnatural amino acids or peptidomimetics, or one or more natural or unnatural amino acids or peptidomimetics can be added to any one or to all of the first five positions of CNP.

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 46), wherein:

(x) may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone proteins with elucidated bone-targeting domains, such as, e.g., fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; polymeric or non-polymeric molecules that reduce renal clearance such as, e.g., charged PEG molecules; and extensions comprising, e.g., polymers (e.g., PEGs), carbohydrates, hydrophobic acids (including fatty acids), and/or amino acids, and wherein such amino acid extensions can contain, e.g., from 1 to 31, or 1 to 35, or 5 to 35, or 10 to 35, or 15 to 35 amino acid residues, and can be derived from NPPC, ANP, BNP, other non-CNP (poly) peptides such as, e.g., serum albumin or IgG, or variants of the aforementioned polypeptides having substitutions, additions and/or deletions, or combinations thereof;

(z) may be absent or may be selected from the group consisting of amino acid sequences useful in bone or cartilage targeting such as for example polyAsp and polyGlu, amino acid sequences from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin and sialoprotein, and amino acid sequences derived from non-CNP (poly)peptides such as, e.g., ANP or BNP;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide-bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog

are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, heterocyclyl, C_{6-14} aryl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3 -chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly, and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In one embodiment, the CNP variants comprise a modification at one or more of positions 6, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and/or 20, and may optionally have modifications at any of the other positions disclosed herein.

In embodiments described herein where CNP22 or variants thereof can be attached to a hydrophobic acid, the CNP peptides can be attached to one or hydrophobic acids. Nonlimiting examples of hydrophobic acids include straightchain or branched, saturated or unsaturated C_5-C_{12} carboxylic acids (e.g., pentanoic acid, heptanoic acid, etc.) and natural fatty acids. The hydrophobic acids can be attached to the N-terminus, the C-terminus, and/or the side chain of one or more amino acid residues. In one embodiment, the hydrophobic acids are conjugated to the N-terminus. In an embodiment, conjugation of CNP22 or a variant thereof to a hydrophobic acid is designed, inter alia, to promote non-specific interaction between the modified CNP peptide and serum albumin, thereby increasing the size of the CNP peptide and protecting it from cleavage by proteases such as, e.g., NEP. The interaction between the hydrophobic acid-conjugated CNP peptide and albumin is designed to be not too strong, so that the modified CNP peptide can diffuse through cartilage, get to chondrocytes of bone growth plates, and bind and activate NPR-B.

In a further embodiment, the disclosure provides CNP variants that in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), comprise at least one modified amino acid at position $(b)_6$, $(c)_7$ and/or $(d)_8$, and are represented by the general formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 47), wherein:

(x) may be absent or may be a peptide sequence containing one to five amino acids which is derived from a natriuretic polypeptide (e.g. NPPC, CNP, ANP or BNP) or a non-natriuretic polypeptide as described herein (e.g., HSA, IgG, a bone-targeting protein, etc.);

(z) may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone/cartilage targeting such as, e.g., polyAsp and polyGlu; bone proteins with bone-targeting domains and derivatives thereof, such as fusion proteins or peptides sequences of osteopontin, osteocalcin, and sialoprotein; molecules that reduce renal clearance, such as, e.g., charged PEGs; and molecules that increase resistance of CNP to NEP-mediated degradation, as described herein;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) may be Cys or descarboxy cysteine, or the $(b)_6$-$(c)_7$ peptide bond (—C(=O)—NH—) may be replaced with any one of the following peptide bond isosteres:

—CH_2—NH—,

—C(=O)—N(R)—, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

—C(=O)—NH—CH_2—,

—CH_2—S—,

—CH_2—$S(O)_n$—, where n is 1 or 2,

—CH_2—CH_2—,

—CH=CH—,

—C(=O)—CH_2—,

—CH(CN)—NH—,

—CH(OH)—CH_2—,

—O—C(=O)—NH—, or

—NHC(=O)NH—;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; peptide bond isosteres of Phe such as N-alkylated derivatives of Phe wherein the N-alkyl group is selected from the group consisting of methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl and tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-}6 alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5 -fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) is any natural or unnatural amino acid or peptidomiuretic that does not have a reactive primary amino group on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the disclosure encompasses CNP variants that in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP degradation, and are represented by the general formula:

(x)-(y)-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 48), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is polyethylene glycol (PEG), and a nonlimiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly) peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or FGF2;

(y) may be absent or may be one or more amino acids from Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (corresponding to positions 1 to 5 of CNP22) (SEQ ID NO: 1) and/or substitutions at one or more of those positions using natural or unnatural amino acids (e.g., K4R substitution);

(h) may be the wild-type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (h) is not Arg; and

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG).

In an embodiment, (x), (y) and (z) together contain from about 10 to about 40, or from about 15 to about 35 amino acids. In another embodiment, (x) is an amino acid sequence comprising from 1 to 40 amino acids, or from 1 to 20 amino acids.

Further contemplated are CNP variants that in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), and comprise the sequence:

(y)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22} (SEQ ID NO: 138), wherein:

(y) comprises one or more amino acids selected from Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (SEQ ID NO: 1) and/or substitutions at one or more of those positions using natural or unnatural amino acids (e.g., K4R substitution), and further comprises a hydrophilic or water soluble polymer of molecular weight from about 0.6 kDa to about 5 kDa. In an embodiment, the hydrophilic or water-soluble polymer is conjugated to the N-terminus of such amino acid-extended CNP variant. In a particular embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO).

In yet another embodiment, the disclosure provides CNP variants that in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g. 1 uM), wherein the CNP variants comprise an N-terminal and/or C-terminal peptide extension containing from 1 to 15 amino acids, and are conjugated to a hydrophilic or water soluble polymer. In an embodiment, the peptide extension contains from 5 to 10 amino acids. In a specific embodiment, the peptide extension contains 5 amino acids. In another specific embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO).

In a still further embodiment, the CNP variants of the disclosure in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g. 1 uM), and comprise at least a 15 amino acid fragment derived from natriuretic peptide precursor C(NPPC), wherein the fragment is at least 70% homologous to a sequence from wild type NPPC containing the same number of amino acid residues.

In still another embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased NEP resistance, and are represented by the formula:

(x)-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 49), wherein:

(x) may be absent (i.e., the N-terminus ends with an —NH_2 group) or may be selected from the group consisting of a sequence of 1, 2, 3, 4 or 5 amino acids from the peptide Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (SEQ ID NO: 1); amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; bone-targeting domains from bone proteins such as for example osteopontin, osteocalcin or sialoprotein; molecules that reduce renal clearance such as hydrophilic or water-soluble polymers, including but not limited to charged PEG molecules; and moieties comprising PEG, carbohydrates, hydrophobic acids, amino acids, or combinations thereof, wherein such moieties can be amino acid extensions including but not limited to amino acid sequences derived from NPPC or non-CNP (poly)peptides such as, e.g., BNP, ANP, serum albumin or IgG;

(z) may be absent or may be selected from the group consisting of amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; amino acid sequences derived from bone-targeting proteins, such as for example osteopontin, osteocalcin or sialoprotein; and amino acid sequences derived from NPPC or non-CNP (poly) peptides, as described herein;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3 -chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased NEP resistance, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-(a)$_4$-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-(e)$_9$-(f)$_{10}$-(g)$_{11}$-Asp_{12}-Arg_{13}-(h)$_{14}$-(i)$_{15}$-Ser_{16}-(j)$_{17}$-Ser_{18}-Gly_{19}-(k)$_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 50), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins and derivatives thereof, such as for example fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; moieties that reduce renal clearance, including but not limited to hydrophilic or water-soluble polymers such as, e.g., charged PEG molecules; and moieties comprising, e.g., hydrophilic polymers (e.g., PEG), carbohydrates, hydrophobic acids, and/or amino acids;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3 -chloro-5 -fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres such as, e.g., N-M-Ile;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the CNP variants can have amino acid substitution(s) at one or more of any of positions 1 to 22 of CNP22. In one embodiment, Gly1is substituted with Arg or Glu. In another embodiment, Lys4 is replaced with Arg. In still another embodiment, Gly5 is substituted with Arg, Gln or Ser. In yet another embodiment, Gly15 is substituted with Ser, Asn, Arg or Cit. In a further embodiment, Gly19 is substituted with Ser, Arg or Asn. In yet another embodiment, Gly21 is substituted with Ser, Thr, or Arg.

In one embodiment, the CNP variant is selected from the group consisting of GLSKGC(CH_2NH)FGLKLDRIGSMSGLGC (formed using descarboxy-Cys) (SEQ ID NO: 56), GLSKGC-(N-Me-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO: 57), GLSKGC-(D-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO:136), GLSKGCF-(tBuG)-LKLDRIGSMSGLGC (SEQ ID NO: 58), GLSKGC-(3-Cl-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO:137), and GLSKGC-[NHCH_2CH(Ph)

CO]-GLKLDRIGSMSGLGC (formed using 3-amino-2-phenylpropionic acid) (SEQ ID NO: 59). In a further embodiment, a disulfide bond exists between Cys6, descarboxy-Cys or another sulfhydryl-containing cysteine analog at the Cys6 position, and Cys22 of any CNP variant described herein.

In another embodiment, the CNP variants contain an amino cid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, including but not limited to:

(SEQ ID NO: 4)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (CNP-53);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-37, Analog BL);

(SEQ ID NO: 61)
AAWARLLQEHPNAGLSKGCFGLKLDRIGSMSGLGC (Analog CA);

(SEQ ID NO: 62)
AAWARLLQEHPNARGLSKGCFGLKLDRIGSMSGLGC (Analog CB);

(SEQ ID NO: 63)
DLRVDTKSRAAWARGLSKGCFGLKLDRIGSMSGLGC (Analog CC);

(SEQ ID NO: 40)
RGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 38)
ERGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 64)
GANQQGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 65)
GANRRGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 66)
GANPRGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 67)
GANSSGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC
(sometimes designated "CNP27-HSA" or "HSA-CNP27"
in the Examples and FIGS.);

(SEQ ID NO: 68)
SPKMVQGSG-CNP17-KVLRRH (Analog CD) (CNP17 having
N-terminal and C-terminal tails derived from BNP).

In a further embodiment, the CNP variants have a K4R substitution at position 4 of CNP22. Non-limiting examples of CNP(K4R) variants include:

((SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC (Analog AY);

(SEQ ID NO: 37)
GANPRGLSRGCFGLKLDRIGSMSGLGC (Analog CI);

(SEQ ID NO: 41)
RGLSRGCFGLKLDRIGSMSGLGC (Analog AZ);

(SEQ ID NO: 39)
ERGLSRGCFGLKLDRIGSMSGLGC (Analog BA);

(SEQ ID NO: 69)
GANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CH);
and

(SEQ ID NO: 70)
GANSSGLSRGCFGLKLDRIGSMSGLGC (Analog CG).

In one embodiment, CNP variants having a PEG (or PEO) moiety and an amino acid extension at the N-terminus contain arginine at the position immediately preceding the position corresponding to Gly1 of CNP22. Such PEGylated CNP variants are designed for increased resistance to NEP degradation, reduced binding to serum albumin, and enhanced CNP functional activity (e.g., activation of cGMP signaling). Non-limiting examples of PEGylated CNP variants include PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO24-GANRR-CNP22(SEQ ID NO: 65), PEO12-GANRR-CNP22(SEQ ID NO: 65), PEO24-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO12-GANPR-CNP22 (K4R) (SEQ ID NO: 37), PEO24-GANPR-CNP22(SEQ ID NO: 37), PEO12-GANPR-CNP22 (SEQ ID NO: 66), PEO24-GANQQ-CNP22(SEQ ID NO: 64), PEO12-GANQQ-CNP22(SEQ ID NO: 64), PEO24-ER-CNP22 (K4R) (SEQ ID NO: 39), PEO12-ER-CNP22(K4R) (SEQ ID NO: 39), PEO24-ER-CNP22(SEQ ID NO: 38), PEO12-ER-CNP22(SEQ ID NO: 38), PEO24-R-CNP22 (K4R) (SEQ ID NO: 41), PEO12-R-CNP22(K4R) (SEQ ID NO: 41), PEO24-R-CNP22(SEQ ID NO: 40), and PEO12-R-CNP22(SEQ ID NO: 40), wherein PEO24 is a monodispersed 1.2 kDa PEG polymer and PEO12 is a monodispersed 0.6 kDa PEG polymer. In an embodiment, the PEG (or PEO) polymer is attached to the N-terminus of the CNP variants.

Additional CNP variants include, but are not limited to, derivatives of CNP37 having mutation(s) at the furin cleavage site (underlined), designed to improve in vivo resistance to the furin protease, and/or having glycine (underlined) preceding glutamine, designed to prevent pyroglutamine formation, including but not limited to:

(SEQ ID NO: 71)
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (An. CS);

(SEQ ID NO: 72)
GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (An. CT);

(SEQ ID NO: 73)
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (An. CU);

(SEQ ID NO: 74)
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (An. CW);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37, An. DB);

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37).

In another embodiment, the CNP variants are chimera comprising CNP22 and an N-terminal peptide fragment, including but not limited to:

(SEQ ID NO: 76)
GHHSHEQHPHGANQQGLSKGCFGLKLDRIGSMSGLGC (Analog CQ)
(histidine-rich glycoprotein (HRGP) fragment-
CNP22 chimera);

(SEQ ID NO: 77)
GAHHPHEHDTHGANQQGLSKGCFGLKLDRIGSMSGLGC (Analog CR)
(HRGP fragment-CNP22 chimera);

(SEQ ID NO: 78)
GHHSHEQHPHGANPRGLSKGCFGLKLDRIGSMSGLGC (Analog CX)
(HRGP fragment-CNP22 chimera);

-continued

(SEQ ID NO: 79)
GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC (Analog CF) ($IgG_1(F_c)$ fragment-CNP22 chimera);

(SEQ ID NO: 80)
GQHKDDNPNLPRGANPRGLSKGCFGLKLDRIGSMSGLGC (Analog CY) (human serum albumin (HSA) fragment-CNP22 chimera);

(SEQ ID NO: 81)
GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC (Analog CE) (HSA fragment-CNP22 chimera);

(SEQ ID NO: 82)
FGIPMDRIGRNPRGLSKGCFGLKLDRIGSMSGLGC (Analog CZ) (osteocrin "NPR C inhibitor" fragment-CNP22 chimera);
and

(SEQ ID NO: 83)
GKRTGQYKLGSKTGPGPKGLSKGCFGLKLDRIGSMSGLGC (Analog DA) (FGF2 "heparin-binding domain" fragment-CNP22 chimera).

In a further embodiment, the CNP variants are chimera comprising an N-terminal peptide fragment and CNP22 in which arginine is substituted for Lys4 of CNP22 ("CNP22 (K4R)"), including but not limited to:

(SEQ ID NO: 84)
GQPREPQVYTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CK) ($IgG_1(F_c)$ fragment-CNP22(K4R) chimera);

(SEQ ID NO: 85
GVPQVSTSTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CL) (HSA fragment-CNP22(K4R) chimera)

(SEQ ID NO: 86)
GQPSSSSQSTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CM) (fibronectin fragment-CNP22(K4R) chimera);

(SEQ ID NO: 87)
GQTHSSGTQSGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CN) (fibrinogen fragment-CNP22(K4R) chimera);

(SEQ ID NO: 88)
GSTGQWHSESGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CO) (fibrinogen fragment-CNP22(K4R) chimera);
and

(SEQ ID NO: 89)
GSSSSSSSSSGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CP) (zinc finger fragment-CNP22(K4R) chimera).

In yet another embodiment, the CNP variants are chimera, or fusion proteins, comprising a CNP peptide or variant, and a cleavable peptide or protein, or peptide tag. Exemplary cleavable proteins or peptides include, but are not limited to, histidine (e.g., hexa-His) tags; TAF12: human transcription factor TAF12; KSI: ketosteroid isomerase; MBP: maltose-binding protein; β-Gal: β-galactosidase; GST: glutathione-S-transferase; Trx: thioredoxin; CBD: chitin binding domain; BMPM: BMP-2 mutation, SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, c-Myc, poly(His), poly(Arg), poly(Asp), poly (Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and fragments thereof.

In yet another embodiment, the CNP variant may be a monomer or a dimer. In a related embodiment the monomers of dimeric CNP variants can be attached N-terminus to N-terminus via a linker or no linker, N-terminus to C-terminus via a linker or no linker, or C-terminus to C-terminus via a linker or no linker.

Chimera comprising an IgG fragment and CNP22 or a variant thereof are designed for, inter alia, increased resistance to NEP degradation and reduced binding to serum albumin. CNP chimera comprising a surface fragment of HSA are designed for, inter alia, reduced immunogenicity and reduced binding to serum albumin. HRGP-CNP22 and HRGP-CNP22(K4R) chimera containing a cationic, histidine-rich, non-lysine, non-arginine sequence at the N-terminus are designed for, inter alia, increased stability to proteases. Chimera containing an osteocrin fragment are designed to release, upon protease (e.g., furin) cleavage, the osteocrin fragment at bone growth plates, where the fragment would inhibit the clearance receptor NPR-C. With respect to chimera comprising an FGF2 heparin-binding fragment, heparin binding to the fragment is designed to protect the chimera from degradation, thereby providing a longer serum half-life. Chimera containing a fibronectin, fibrinogen, or zinc-finger fragment are designed for reduced binding to serum albumin, among other advantageous features.

Not intending to be bound by theory, a CNP variant of molecular weight from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa which has increased resistance to NEP degradation and has similar or improved functionality (e.g., binding to NPR-B and stimulation of cGMP signaling) as compared to wtCNP22, may be more effective if it does not bind tightly to plasma proteins such as serum albumin. A CNP variant that does not bind tightly to plasma proteins (e.g., serum albumin) may be more effective in diffusing through cartilage, getting to chondrocytes of bone growth plates, and binding to and activating NPR-B for cGMP signaling. In one embodiment, CNP variants designed for reduced binding to plasma proteins (e.g., serum albumin) are chimeras comprising CNP22 or a variant thereof and a peptide fragment from IgG. In another embodiment, CNP variants designed for reduced binding to plasma proteins are chimeras comprising CNP22 or CNP22(K4R) and a fragment from a polypeptide (e.g., IgG, HSA, fibronectin, fibrinogen, a zinc finger-containing polypeptide, etc.). In yet another embodiment, CNP variants designed for reduced binding to plasma proteins comprise CNP22 or a variant thereof conjugated to a hydrophilic or water-soluble polymer. In one embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO). In another embodiment, the hydrophilic or water-soluble polymer (e.g., PEG) is functionalized with one or more functional groups that impart a negative charge to the polymer under physiological conditions, such as, e.g, carboxyl, sulfate or phosphate groups, or a combination thereof.

In any of the embodiments disclosed herein, the CNP variants may have substantially the same or better biological activity than wild-type CNP22. For example, the CNP variants may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP22, or may have greater activity than CNP22, e.g., with respect to interaction with NPR-B (GC-B) to stimulate the generation of cGMP. Alternatively, or in addition, the CNP variants may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP22, or may have greater activity than CNP22, with respect to regulating endochondral bone growth and chondrocyte activity, including but not limited to

chondrocyte proliferation, chondrocyte differentiation, inhibition of the mitogen activated protein (MAP) kinase/MEK (Raf-1) kinase signaling pathway, and promoting endochondral ossification. In any of the embodiments described herein, the CNP variants may comprise an amino acid sequence that is at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or more identical or homologous to amino acids 6-22 or 1-22 of wild-type CNP22.

In a further embodiment, the disclosure provides variants of CNP22 having less affinity to the NPR-C clearance receptor while retaining the ability to bind and activate NPR-B. The present disclosure encompasses variants that were, or can be, generated from a homology-based structural model of the

NPR-B/CNP complex as described in the Detailed Description. In another embodiment, the CNP variants have substitution(s) at one or more Gly sites at positions 1, 5, 8, 15, 19 and 21, to reduce conformational flexibility, which may increase their specificity for binding to NPR-B over NPR-C. Variants of CNP having potentially reduced affinity to the NPR-C include but are not limited to those having one or more of the following substitutions: G1R, G1E, G5R, G5Q, G5S, F7Y, G8T, G6S, G8V, G8N, L9S, L9T, K10Cit, K10Q, K10S, I14N, G15R, G15S, G15N, G15Cit, S16Q, M17V,

M17N, G19S, G19R, G19N, L20V, L20R, L20T, L20S, G21S, G21T and G21R.

In yet another embodiment, the CNP variants have a modification and/or substitution at one or more of positions 5, 7, 8, 9, 10, 14, 15, 16, 17, 19, 20 and 21, and may optionally have modifications and/or substitutions at any of the other positions disclosed herein. In a further embodiment, the CNP variants can optionally have conjugation(s) or extension(s), e.g., at the N- and/or C-terminus to facilitate bone/cartilage targeting, reduce renal clearance, and/or increase resistance to NEP degradation. Such conjugation(s) or extension(s) can comprise molecules or sequences formed or derived from, e.g., polyAsp, polyGlu, bone- or cartilage-targeting peptides, osteopontin, osteocalcin, sialoprotein, PEGs, carbohydrates, hydrophobic acids, NPPC or non-CNP (poly)peptides, or combinations thereof.

In still another embodiment, the CNP variants are prepared by standard solid-phase peptide synthesis methods with natural or unnatural amino acid(s) or peptidomimetic(s) being substituted and/or added where appropriate. In another embodiment, the CNP variants are produced by recombinant synthesis processes, e.g., via fusion proteins containing a tag or carrier protein, wherein use of the tag or carrier protein facilitates, e.g., detection, isolation and/or purification of the fusion protein, and selective chemical or proteolytic cleavage of the tag or carrier protein from the fusion protein provides the target CNP variant. In a further embodiment, PEGylation of the CNP variants occurs following, or part of, chemical or biological synthesis with the conjugation reaction being performed by NHS- or aldehyde-based chemistry or other chemistry known in the art. In another embodiment, the CNP variants comprise a disulfide bond. In a related embodiment, the disulfide bond forms a cyclic peptide. In a particular embodiment, the disulfide bond is formed between cysteine residues at positions corresponding to positions 6 and 22 of CNP22.

It is further contemplated that the CNP variants can be conjugated to a hydrophobic polymeric or non-polymeric moiety, such as, e.g., heptanoic acid, pentanoic acid, or fatty acids. The hydrophobic moiety can be conjugated to the side chain of an amino acid residue, including but not limited to a lysine, a serine, a cysteine or a threonine, or can be attached to the N-terminus and/or C-terminus of the CNP variant.

In an embodiment, the CNP variants as described herein have a pI in the range from about 8 to about 10.5 or from about 8.5 to about 10.

In a further embodiment, the disclosure provides a pharmaceutical composition comprising a CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In some embodiments, the compositions are sterile pharmaceutical compositions suitable for parenteral injection. In some embodiments, the compositions comprise substantially pure CNP variant, e.g. at least about 90% or 95% pure. In some embodiments, the compositions contain less than about 5%, 4%, 3%, 2%, 1% or 0.5% contaminants, such as other human proteins, porcine proteins, or CNP53 or fragments thereof (other than the desired CNP variant). In certain embodiments, the sterile composition is administered to a subject for treating or preventing any of the CNP-responsive conditions or disorders disclosed herein.

CNP variants of the disclosure advantageously retain CNP activity and exhibit increased serum half-life. Retention of CNP activity can be shown, for example, as retention of desired in vivo biological effect, or retention of at least about 50%, 60%, 70%, 80%, 90%, 95% or at least about 100% of the cGMP stimulating activity of CNP22, under the same concentration (e.g., 1 uM of CNP peptide or greater than the ED80). In some embodiments, CNP variants exhibit at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold or 40-fold increase in serum half-life compared to CNP22.

In a related embodiment, the CNP variants described herein have increased NEP resistance and exhibit increased half-life compared to wild-type CNP22. In one embodiment, the half-life of the CNP variants is increased by about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or about 100% compared to wild-type CNP22.

In certain embodiments, the CNP variants described herein increase cGMP production in vitro, increase cGMP production in vivo, increase in vivo the level of one or more biomarkers associated with cartilage or bone formation or growth, increase resistance to NEP cleavage in vitro, increase plasma or serum half-life in vivo, increase bioavailability in vivo, or increase the length of particular bones in vivo, or effect combinations of such increases, by about 1.5-fold, about 2-fold, about 2.5-fold, about 3-fold, about 3.5-fold, about 4-fold, about 4.5-fold, or about 5-fold or more compared to wild-type CNP22.

In yet another embodiment, the disclosure provides methods of treating conditions or disorders responsive to CNP, comprising administering a therapeutically effective amount of a CNP variant or a composition comprising the same to a subject in need thereof. In one embodiment, disorders responsive to CNP are disorders of bone growth, including but not limited to skeletal dysplasias and inherited skeletal malformations such as disorders associated with fibroblast growth factor receptor 3 (FGFR-3) mutations. In a specific embodiment, the disorder associated with FGFR-3 mutation(s) is achondroplasia. In another embodiment, the disorders responsive to CNP are disorders associated with vascular smooth muscle cells and tissues. In a further embodiment, the CNP variants are useful for increasing the size of the growth plate of a bone (e.g., a limb bone). In another embodiment, the CNP variants are useful for elongating a bone or increasing long bone growth. In still another

embodiment, the CNP variants are useful for enhancing matrix production, proliferation and differentiation of chondrocytes.

In certain embodiments, the CNP variants described herein are administered at a dose in the range from about 5 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg. In some embodiments, the CNP variants are administered at a dose of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variants are administered at a dose of about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750 or 800 ug/kg, or about 1 mg/kg, 1.25 mg/kg, 1.5 mg/kg, 2 mg/kg or other dose deemed appropriate by the treating physician. The doses of CNP variants described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc.

In another embodiment, the CNP variants are administered in a single treatment or in multiple doses. The multiple doses may be administered daily, or in multiple doses over the course of treatment. In certain embodiments, it is contemplated that the CNP variant is administered, in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week, 3 times per week, weekly, bi-weekly, every 3 weeks, monthly, every 6 weeks, every 2 months, every 3 months or as deemed appropriate by a treating physician.

In certain embodiments, administration of the CNP variant is adjusted to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP variant may be administered subcutaneously, intravenously, or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP variant daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

In additional embodiments, the disclosure provides a method of treating a CNP-responsive condition or disorder, comprising administering a CNP peptide or variant to a subject, and monitoring the level of at least one bone- or cartilage-associated biomarker in the subject (e.g., in a biological sample from the subject), wherein an increase or decrease in the level of the bone- or cartilage-associated biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject. In some embodiments, when the level of a biomarker increases in association with bone or cartilage formation or growth, an increase in the level of that biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject. In other embodiments, when the level of a biomarker decreases in association with bone or cartilage formation or growth, a decrease in the level of that biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject.

In further embodiments, the therapeutic method further comprises adjusting the amount (or dose) or frequency of administration of the CNP peptide or variant, wherein:

(i) the amount (or dose) or frequency of administration of the CNP peptide or variant is increased if the level of the at least one bone- or cartilage-associated biomarker is below a target level, where the level of the biomarker increases in association with bone or cartilage formation or growth; or

(ii) the amount (or dose) or frequency of administration of the CNP peptide or variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is above a target level, where the level of the biomarker increases in association with bone or cartilage formation or growth; or

(iii) the amount (or dose) or frequency of administration of the CNP peptide or variant is increased if the level of the at least one bone- or cartilage-associated biomarker is above a target level, where the level of the biomarker decreases in association with bone or cartilage formation or growth; or

(iv) the amount (or dose) or frequency of administration of the CNP peptide or variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is below a target level, where the level of the biomarker decreases in association with bone or cartilage formation or growth.

It is contemplated that the target level of a biomarker refers to the level or range of levels of the biomarker that is associated with therapeutic effect in the subject and/or beneficial effect in alleviating or ameliorating symptoms of the disorder or condition. In certain embodiments, a level of a biomarker above or below a target level may be deleterious to the subject.

In other embodiments, the disclosure contemplates a method for assessing the effect of administration of a CNP peptide or variant on bone or cartilage formation or growth. In one embodiment, the method provides for assaying or measuring the level of at least one bone- or cartilage-associated biomarker in a subject that has been administered a CNP peptide or variant in order to assess the effect of the CNP peptide or variant on bone and cartilage formation and growth in vivo. In a related embodiment, an increase in the level of the at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP peptide or variant has a positive effect on bone or cartilage formation or growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (e.g, endogenous level of CNP-22 or CNP-53), cGMP, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In further embodiments, the disclosure contemplates a method for assessing the effect of a CNP peptide or variant on the level of at least one bone- or cartilage-associated biomarker in a subject, comprising assaying or measuring the level of the bone- or cartilage-associated biomarker in a biological sample from a subject that has been administered

a CNP peptide or variant. In some embodiments, the method further comprises administering the CNP peptide or variant to the subject before assaying or measuring the level of the bone- or cartilage-associated biomarker.

In certain embodiments, the at least one bone- or cartilage-associated biomarker is selected from the group consisting of CNP (e.g, endogenous level of CNP-22 or CNP-53), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In some embodiments of methods (e.g., therapeutic, diagnostic and assay methods) relating to bone- or cartilage-associated biomarkers, the CNP peptide or variant is CNP-22, CNP-53, or any of the CNP peptides and variants described herein. In certain embodiments of such methods, the CNP peptide or variant is not CNP-22 or CNP-53.

In other embodiments, the disclosure provides a method for recombinant production of a CNP variant, comprising culturing in a medium a host cell comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein, under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides. In a related embodiment, the host cell is transformed with an expression vector comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein.

In one embodiment, the vector is a plasmid. In still another embodiment, the plasmid is selected from the group consisting of pET-21a, pJexpress, pET-31b, pET-15b, pET-32a, pET-41a, pMAL, pQE-30, pET-SUMO, pET-22b, and pTYB11.

In certain embodiments, the cleavable peptide or protein comprises a polypeptide that is selected from the group consisting of a histidine tag, human transcription factor TAF12, ketosteroid isomerase, maltose-binding protein, β-galactosidase, glutathione-S-transferase, thioredoxin, chitin binding domain, and BMP-2 mutation, or fragments thereof

In a related embodiment, the cleavable peptide or protein is cleaved by a cleaving agent. In some embodiments, the cleaving agent is selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease. Additional exemplary cleaving agents include, but are not limited to, palladium, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase, enteropeptidase, Kex 2 protease, Omp T protease, subtilisin, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K, pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

In certain embodiments, the fusion polypeptide is expressed as a soluble protein or as an inclusion body. In a related embodiment, the disclosure contemplates isolating the expressed fusion polypeptide from the host cell or culture medium. In a further embodiment, the isolated fusion polypeptide is contacted with a cleaving agent as described herein.

In one embodiment, the disclosure provides a bacterial host cell comprising an expression vector, said vector comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein. In some embodiments, the cleavable peptide or protein is selected from the group consisting of a histidine tag, human transcription factor TAF12, ketosteroid isomerase, maltose-binding protein, β-galactosidase, glutathione-S-transferase, thioredoxin, chitin binding domain, and BMP-2 mutation, or fragments thereof.

In another embodiment, the host cell is a bacteria, such as E. coli. In a related embodiment, the E. coli cell is selected from the group consisting of BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41 [also called C41(DE3)], C43 [also called C43(DE3)], Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3). In still a further embodiment, the host cell comprises a vector as described above. In some embodiments, the host cell is transformed with the vector prior to cell culture.

In certain embodiments, it is contemplated that the host cell is cultured in a medium under conditions suitable for expression of a fusion polypeptide encoded by the polynucleotides. In one embodiment, the fusion polypeptide is expressed as a soluble protein or as inclusion body. In a related embodiment, the expressed fusion polypeptide is isolated from the host cell or culture medium. In still another embodiment, the isolated fusion polypeptide is contacted with a cleaving agent as described herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** shows expression of CNP fusion proteins in E. coli (FIG. **1**A: Coomassie blue stain, FIG. **1**B: Western blot). M: protein marker; T: total cell lysates; S: soluble supernatants; P: 2 ug CNP22; KSI: KSI-CNP(M/N) fusion protein expression (insoluble); N: Un-induced KSI-CNP fusion protein total lysates; KSI': KSI-P-CNP fusion protein expression (insoluble); Trx: Trx-P-CNP fusion protein expression (soluble); MBP: MBP-P-CNP fusion protein expression (soluble); TAF: TAF-P-CNP fusion protein expression (insoluble) (BL21); TAF': TAF-P-CNP fusion protein expression (insoluble) BL21(DE3), where CNP is Gly-CNP37.

FIG. **2** shows formic acid cleavage of TAF-CNP inclusion bodies. M: protein marker; 1: Gly-CNP37 positive control; 2: uncleaved TAF-CNP inclusion bodies; 3: 2% formic acid cleaved TAF-CNP inclusion bodies, where CNP is Gly-CNP37.

FIGS. **3**A-E depict expression of CNP fusion proteins in E. coli. M: protein marker; Tu: total un-induced cell lysates; Su: un-induced soluble supernatants; T: total induced cell lysates; S: soluble supernatants; Cl: CNP22; C: Gly-wtCNP37 ("CNP38"); P: insoluble pellets. A: KSI: KSI-CNP38(M/N) fusion protein expression (Insoluble); KSI': KSI-Pro-CNP38 (Pro-Gly-wtCNP37 is designated "Pro-CNP38") fusion protein expression (Insoluble); Trx: Trx-Pro-CNP38 fusion expression (Soluble); MBP: MBP-Pro-CNP38 fusion protein expression (Soluble); TAF: TAF-Pro-CNP38 fusion protein expression (Insoluble) from BL21 cell; TAF': TAF-Pro-CNP38 fusion protein expression (Insoluble) from BL21 (DE3) cell. B: TAF-Pro-CNP37 and BMP-Pro-CNP37 fusion protein expression. C: BMP-Pro-CNP38 fusion protein and BMP protein expression. D: TAF-Pro-HSA-CNP ("Pro-GH-KSEVAHRFK-wtCNP27 (SEQ ID NO: 188) is designated "Pro-HSA-CNP") fusion protein expression. E: TAF-Pro-CNP38 fusion protein and TAF protein expression.

FIGS. **4**A-C depict formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. A: 50% formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. M: protein marker; U: un-cleaved TAF-Pro-CNP38 inclusion bodies; 25° C., 37° C., 42° C., 55° C.: TAF-Pro-CNP38 inclusion bodies were cleaved in 50% formic acid at 25° C., 37° C., 42° C. or 55° C. for 24 hours. 37° C.-S and 55° C.-S: soluble supernatants from the cleavage reactions at 37° C. and 55° C. neutralized with 10N NaOH and centrifuged at 14,000 rpm for 15 minutes. B: 10% and 2% formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. M: protein marker; U: un-cleaved TAF-Pro-CNP38 inclusion bodies; C: formic acid cleaved TAF-Pro-CNP38; S: soluble supernatant after centrifuged at 14,000 rpm for 5 minutes without neutralization; P: insoluble pellet after centrifuged at 14,000 rpm for 5 minutes without neutralization. C: LC/MS analysis of 2% and 10% formic acid cleaved products from TAF-Pro-CNP38 inclusion bodies.

FIGS. **5**A-C depict formic acid cleavage of TAF-Pro-CNP38 inclusion bodies at different temperature and time of formic acid cleavage. M: protein marker; C: Gly-wtCNP37 ("CNP38") positive control; U: un-cleaved TAF-Pro-CNP38 inclusion bodies. A: 2% formic acid cleaved TAF-Pro-CNP38 at 42° C., 55° C. or 70° C. for 6, 24 or 48 hours. B: 2% formic acid cleaved TAF-Pro-CNP38 at 55° C., 60° C., 65° C. or 70° C. for 17 or 24 hours. C: LC/MS analysis of 2% formic acid cleaved products from TAF-Pro-CNP38 inclusion bodies.

FIG. **6** is an SDS-PAGE (Coomassie blue stain) for the expression of a TAF-CNP34 fusion protein. M: protein marker; C: control [Gly-CNP37 ("CNP38")]; T: total cell lysates; S: supernatant; TI: total cell lysates induced; SI: supernatant induced.

FIG. **7** is an SDS-PAGE (Coomassie blue stain) for the expression of fusion proteins TAF-NL-(C/A & 6D/6E)-Pro-CNP38, TAF(C/A & 10D/10E)-Pro-CNP38, and TAF-Pro-CNP53, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; C: control [Gly-CNP37 ("CNP38")]; T: total cell lysates; TI: total cell lysates induced; S: supernatant; SI: supernatant induced.

FIG. **8** is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF-CNP34 and TAF-Pro-CNP53. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

FIG. **9** is an LC/MS chromatogram showing the peak for CNP-34 after formic acid cleavage of TAF-CNP34.

FIG. **10** is an LC/MS chromatogram showing the peak for Pro-CNP53 after formic acid cleavage of TAF-Pro-CNP53.

FIG. **11** is an SDS-PAGE (Coomassie blue stain) for the expression of fusion proteins TAF(C/A & 4D/4E)-Pro-CNP38 and TAF(4D/4E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; C: control [Gly-CNP37 ("CNP38")]; T: total cell lysates; TI: total cell lysates induced; S: supernatant; SI: supernatant induced.

FIG. **12** is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF (4D/4E)-Pro-CNP38 and TAF(C/A & 4D/4E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

FIG. **13** is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

FIG. **14** is a Western blot, using an anti-CNP antibody, of a TAF-Pro-CNP38 fusion protein produced in a 10 L fermen-tation of BL21(DE3) cells, where the cells were induced at OD_{600}=64 and hour 17 and "Pro-CNP38" denotes Pro-Gly-CNP37.

FIG. **15** is an SDS-PAGE (Coomassie blue stain) of eluate fractions from SP-Sepharose cation-exchange column chromatography of a cruder Pro-Gly-CNP37 ("Pro-CNP38") product. A: TAF-Pro-CNP38 inclusion body (IB) in water; B: IB in formate; C: IB in formate, neutralized; D: neutralized pellet; E: neutralized supernatant; F: TMAE Hi-CAP load; G: TMAE Hi-CAP flow through/SP-Sepharose load; H: SP-Sepharose flow through; SP-Sepharose eluate fractions 1-47: 10 uL/lane.

FIG. **16** shows the degree of resistance of N-terminal PEGylated CNP22 conjugates to neutral endopeptidase (NEP) in vitro.

FIG. **17** depicts the degree of NEP resistance of CNP variants having an amino acid extension at the N-terminus ["CNP27" is GANRR-CNP22(K4R) (SEQ ID NO: 36)].

FIG. **18** illustrates the degree of NEP resistance of N-terminal PEGylated CNP17 and GANRR-CNP22(K4R) ("CNP27") (SEQ ID NO: 36).

FIG. **19** illustrates the degree of NEP resistance of wtCNP22 and CNP variants Gly-CNP37, GHKSEVAH-RFK-wtCNP27 ("CNP27-HSA" in the figures) (SEQ ID NO: 144) and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12" in the figures) (SEQ ID NO: 36).

FIG. **20** shows the ability of CNP variants having an N-terminal amino acid extension to stimulate cGMP production in NIH3T3 cells in vitro. The results are relative to the level of cGMP produced in the presence of 1 uM CNP22. "CNP27" is GANRR-CNP22(K4R) (SEQ ID NO: 36)

FIG. **21** displays the ability of N-terminal PEGylated CNP17 and GANRR-CNP22(K4R) ("CNP27") (SEQ ID NO: 36) to stimulate cGMP production in NIH3T3 cells.

FIG. **22** illustrates the effects of N-terminal PEGylation of CNP22 on cGMP production.

FIG. **23** illustrates the cGMP production induced by wtCNP22 and CNP variants Gly-CNP37, GHKSEVAHRF-KwtCNP27 ("CNP27-HSA") (SEQ ID NO: 144), wtCNP29 and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) in NIH3T3 cells.

FIGS. **24**A and B show that CNP-22 and Pro-Gly-CNP37 ("Pro-CNP38") stimulated cGMP production through NPR-B with similar dose-response curves, and to a much greater extent than through NPR-A, and exhibited a similar profile for NPR-B vs. NPR-C selectivity in in vitro signaling competition assays.

FIG. **25** demonstrates that exposure of rat chondrosarcoma cells to CNP22 1 hour once daily or 2 hours once daily has substantially similar effectiveness in reversing FGF2-induced arrest of chondrocyte growth as continuous exposure to CNP22.

FIGS. **26**A and B show results from a dose response study of CNP22 effects on FGF2-arrested rat chondrosarcoma (RCS) cells.

FIGS. **27**A-D show that addition of CNP22 to RCS cells arrested by FGF2 increases matrix synthesis and partly inhibits FGF2, as assessed by ^{35}S-sulfate and ^{3}H-Pro incorporation into or decrease from matrix. Panels A and C, synthesis; B and D, degradation; A and B, ^{35}S measurement; C and D, ^{3}H measurement. Statistically significant differences are highlighted (ANOVA; *$p<0.05$, **$p<0.01$).

FIGS. **28**A-C show the levels of aggrecan and fibronectin production (mRNA, panels A and C, and protein, panel B) in RCS cells cultured with FGF2 and CNP22.

FIG. **29** shows the efficacy of CNP37 and PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24" in the figures) (SEQ ID NO: 36) in stimulating longitudinal growth of wild-type femur in an ex vivo mouse organ model.

FIG. **30** shows longitudinal bone growth of 2-3 day-old wild-type mouse tibias treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means±SEM (n=8).

FIG. **31** shows longitudinal bone growth of 2-3 day old achondroplastic FGFR3^{ach} mouse tibias treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means±SEM (n=7-8).

FIG. **32** shows longitudinal bone growth of 2-3 day-old wild-type mouse femurs treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means±SEM (n=8).

FIG. **33** shows longitudinal bone growth of 2-3 day-old FGFR3^{ach} mouse femurs treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means±SEM (n=3-7).

FIGS. **34**A-I depict CNP37 biodistribution in FGFR3^{ach} mouse femurs treated ex vivo every two days. Panels A-C illustrate distribution in distal femurs, panels D-F illustrate distribution in articular chondrocytes and panels G-I illustrate distribution in hypertrophic chondrocytes.

FIGS. **35**A-C depict the cellularity of proliferating columns in growth plates after treatment of wild-type and FGFR3^{ach} mouse femurs with CNP22 or CNP37 every two days for 8 days. (A) no treatment, (B) cell numbers per column after treatment, (C) morphological studies after treatment. The panels in FIGS. **35**C(i) to C(vi). correspond to the sample order set out in FIG. **35**B. Data is represented as means±SEM (n=4-8).

FIGS. **36**A-C depict chondrocyte hypertrophy after ex vivo treatment of wild-type and FGFR3^{ach} mouse femurs with CNP22 or CNP37 every two days for 8 days. (A) no treatment, (B) cell size after treatment, (C) morphological studies after treatment. The panels in FIGS. **36**C(i) to C(vi) correspond to the sample order set out in FIG. **36**B. Data is represented as means±SEM (n=4-9).

FIGS. **37**A-I depict the biodistribution of CNP37 in FGFR3^{ach} mouse tibias treated in vivo. Panels A-C show distribution in distal femurs, panels D-F illustrate distribution in articular chondrocytes and panels G-I illustrate distribution in hypertrophic chondrocytes.

FIGS. **38**A-C illustrate the in vivo effects of CNP37 on FGFR3^{ach} mouse tibia growth plate: (A) total growth plate thickness, (B) proliferating zone thickness, and (C) hypertrophic zone thickness. Data is represented as means±SEM (n=7-15).

FIG. **39** shows cGMP levels in conditioned media from wild-type mouse femurs treated ex vivo with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) ($p<0.01$).

FIG. **40** shows cGMP levels in conditioned media from FGFR3^{ach} mouse femurs treated ex vivo with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p<0.01$).

FIG. **41** depicts cGMP levels in conditioned media from wild-type mouse tibias treated ex vivo with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p<0.01$).

FIG. **42** shows cGMP levels in conditioned media from FGFR3^{ach} mouse tibias treated ex vivo with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p<0.01$).

FIG. **43** demonstrates that ex vivo exposure of wild-type and FGFR3^{ach} mouse femurs to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) increased the levels of cleaved collagen type II in the conditioned media ($p<0.05$).

FIG. **44** depicts the hypertrophic region of femoral bones isolated from wild-type mice and FGFR3^{Y367C} mice (a mouse model of severe achondroplasia) and treated ex vivo with vehicle or 1 uM Pro-Gly-CNP37 ("ProCNP38") for 6 days, demonstrating that Pro-Gly-CNP37 treatment resulted in increase in bone growth and expansion in the growth plate.

FIG. **45** shows that CNP37 and PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) intravenously (i.v.) administered to rats have a much longer half-life and a much greater bioavailability in the plasma than CNP22.

FIG. **46** illustrates that PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) subcutaneously (s.c.) administered to rats also has a much longer half-life and a much greater bioavailability in the plasma than CNP22.

FIG. **47** demonstrates that i.v. administered CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) stimulate a much greater level of cGMP production in rats than CNP22.

FIG. **48** shows that s.c. administered PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and, to a lesser extent, CNP37 are substantially more effective in stimulating cGMP production in rats than CNP22.

FIG. **49** shows body weight measurements of wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22 (K4R) (SEQ ID NO: 36).

FIG. **50** shows tail length measurements of wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22 (K4R) (SEQ ID NO: 36).

FIG. **51** illustrates the effect of treatment of FGFR3^{ach} mice with CNP22, CNP37 or PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) on body length ($p=0.02$, 1-tailed t-test, unequal variance).

FIG. **52** shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on tail length in FGFR3^{ach} mice.

FIGS. **53**A and B show the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on the length of distal long bones (A, ulna; B, tibia) in FGFR3^{ach} mice ($p<0.01$, one-tailed t-test, unequal variance).

FIGS. **54**A and B show the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on the length of proximal bones (A, humerus; B, femur) in FGFR3^{ach} mice ($p<0.01$, one-tailed t-test, unequal variance).

FIG. **55** illustrates that CNP37 administration corrects rhizomelia (disproportion of the length of the proximal limbs) as assessed by the femur:tibia ratio observed in FGFR3^{ach} mice ($p<0.01$, one-tailed t-test, unequal variance).

FIG. **56** shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on head length in FGFR3^{ach} mice ($p<0.01$ one-tailed t-test, unequal variance).

FIG. **57** shows that treatment of FGFR3^{ach} mice with CNP37 increases the size of the external auditory meatus (EAM) ($P=0.03$, one-tailed t-test, unequal variance).

FIG. **58** shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on spinal length in achondroplasic mice, expressed as extension of vertebral bodies (e.g., lumbar vertebra 5).

FIG. **59** shows that treatment of $FGFR3^{ach}$ mice with CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) results in increased cGMP plasma levels 15-min post-dose.

FIG. **60** illustrates the serum levels of cleaved collagen type II in $FGFR3^{ach}$ mice treated 5 weeks with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

FIG. **61** shows the serum levels of osteocalcin in $FGFR3^{ach}$ mice treated 5 weeks with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

FIG. **62** shows cGMP plasma levels 15-min post-dose from wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p<0.05$).

FIG. **63** shows the serum levels of cleaved collagen type II in wild-type mice treated 5 weeks with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36).

FIGS. **64-66** display the levels of cGMP, cleaved collagen type II and alkaline phosphatase after administration of vehicle or 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37 ("Pro-CNP38") to wild-type mice.

FIGS. **67** and **68** depict cleaved collagen type II and total alkaline phosphatase levels after administration of vehicle or Pro-Gly-CNP37 ("Pro-CNP38") under different dosing regimens.

FIG. **69** illustrates the relative increase in body length of Pro-Gly-CNP37 ("Pro-CNP38") and vehicle treated animals in two separate studies (S1 and S2) at Day 37 vs Day 1.

FIGS. **70**A and B show changes in bone mineral density (A) and bone mineral content (B) after administration of Pro-Gly-CNP37 ("Pro-CNP38").

FIG. **71** shows plasma cGMP levels 15 min after the last subcutaneous dose of CNP variant, Day 36, where Gly-wtCNP37 is "CNP38", Pro-Gly-wtCNP37 is "Pro-CNP38", and GHKSEVAHRFK-wtCNP27 (SEQ ID NO: 144) is "HSA-CNP27" in FIGS. **71-73**.

FIG. **72** shows serum levels of cleaved collagen type II from mice treated with Gly-CNP37, Pro-Gly-CNP37 or GHKSEVAHRFK-CNP27 (SEQ ID NO: 144).

FIG. **73** shows serum levels of alkaline phosphatase from mice treated with Gly-CNP37, Pro-Gly-CNP37 or GHKSEVAHRFK-CNP27 (SEQ ID NO: 144).

FIGS. **74**A and B depict desensitization of the cGMP response after acute (A) or chronic (B) treatment with 1 uM Gly-CNP37.

FIG. **75**A demonstrates that daily treatment of wild-type mice with 200 nmol/kg of Pro-Gly-CNP37 ("Pro-CNP38") for 8 days did not desensitize the cGMP response. FIG. **75**B shows that treatment of the mice with 200 nmol/kg of Pro-Gly-CNP37 once a day for two consecutive days potentiated the cGMP response.

FIGS. **76**A-D show that treatment of wild-type mice with 200 nmol/kg of Gly-CNP37 stimulated cGMP secretion in distal femurs (cartilage and bone) (A), femoral cortices (bone) (B), ear pinna (cartilage) (C), and kidney (D). FIGS. **76**E-H show that liver (E), heart (F), lung (G) and brain (H) tissues did not exhibit appreciable cGMP secretion in response to Gly-CNP37 relative to vehicle control at the studied time points.

FIGS. **77-82** show results from an on-going study in normal juvenile cynomolgus monkeys subcutaneously injected daily with vehicle or 10 or 36 ug/kg of Pro-Gly-CNP37. Both doses of Pro-Gly-CNP37 have increased growth plate width (FIG. **77**), increased right and left tibia lengths (FIGS. **78**A and B), increased leg length (FIG. **79**), increased arm length (FIG. **80**), increased body length (FIG. **81**), and increased the serum level of alkaline phosphatase (FIG. **82**).

FIG. **83** depicts the observed plot of degradation rate constant (K_{obs}) vs. pH at pH 3-8 and 5° C., 25° C. and 40° C. for Gly-CNP37 formulations.

DETAILED DESCRIPTION OF THE DISCLOSURE

The present disclosure relates to novel variants of CNP having reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleavage by NEP and/or clearance by NPR-C, pharmaceutical compositions comprising such CNP variants, and methods of using such

CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as achondroplasia and disorders associated with vascular smooth muscle cells and tissues.

A. Definitions

Unless otherwise stated, the following terms used in this application, including the specification and claims, have the definitions given below.

As used in the specification and the appended claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents unless the context clearly dictates otherwise.

The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range. Whenever the term "about" or "approximately" precedes the first numerical value in a series of two or more numerical values, it is understood that the term "about" or "approximately" applies to each one of the numerical values in that series.

The terms "ambient temperature" and "room temperature" are used interchangeably herein and refer to the temperature of the surrounding environment (e.g., the room in which a reaction is conducted or a composition is stored). In certain embodiments, ambient temperature or room temperature is a range from about 15° C. to about 28° C., or from about 15° C. to about 25° C., or from about 20° C. to about 28° C., or from about 20° C. to about 25° C., or from about 22° C. to about 28° C., or from about 22° C. to about 25° C. In other embodiments, ambient temperature or room temperature is about 15° C., 16° C., 17° C., 18° C., 19° C., 20° C., 21° C., 22° C., 23° C., 24° C., 25° C., 26° C., 27° C. or 28° C.

Definition of standard chemistry terms may be found in reference works, including Carey and Sundberg, Advanced Organic Chemistry, 3rd Edition, Vols. A and B (Plenum Press, New York 1992). The practice of the present disclosure may employ, unless otherwise indicated, certain conventional methods of synthetic organic chemistry, mass spectrometry, preparative and analytical chromatography, protein chemistry, biochemistry, recombinant DNA technology and pharmacology, within the skill of the art. See, e.g., T. E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A. L. Lehninger,

Biochemistry (Worth Publishers, Inc., 4th Edition, 2004); Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pa.: Mack Publishing Company, 1990).

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

"Polypeptide" and "protein" refer to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof, linked via peptide bonds or peptide bond isosteres. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The terms "polypeptide" and "protein" are not limited to a minimum length of the product. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms "polypeptide" and "protein" also include postexpression modifications of the polypeptide or protein, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present disclosure, a "polypeptide" can include "modifications," such as deletions, additions, substitutions (which may be conservative in nature or may include substitutions with any of the 20 amino acids that are commonly present in human proteins, or any other naturally or non-naturally-occurring or atypical amino acids), and chemical modifications (e.g., addition of or substitution with peptidomimetics), to the native sequence. These modifications may be deliberate, as through site-directed mutagenesis, or through chemical modification of amino acids to remove or attach chemical moieties, or may be accidental, such as through mutations arising with hosts that produce the proteins or through errors due to PCR amplification.

Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

"Conservative substitution" refers to substitution of an amino acid in a polypeptide with a functionally, structurally or chemically similar natural or unnatural amino acid. In one embodiment, the following groups each contain natural amino acids that are conservative substitutions for one another:

(1), Alanine (A) Serine (S), Threonine (T);

(2) Aspartic acid (D), Glutamic acid (E);

(3) Asparagine (N), Glutamine (Q);

(4) Arginine (R), Lysine (K);

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

(6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). In another embodiment, the following groups each contain natural amino acids that are conservative substitutions for one another.

(1) Glycine (G), Alanine (A);

(2) Aspartic acid (D), Glutamic acid (E);

(3) Asparagine (N), Glutamine (Q);

(4) Arginine (R), Lysine (K);

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V), Alanine (A);

(6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); and

(7) Serine (S), Threonine (T), Cysteine (C).

In a further embodiment, amino acids may be grouped as set out below.

(1) hydrophobic: Met, Ala, Val, Leu, Ile, Phe, Trp;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence backbone orientation: Gly, Pro; and

(6) aromatic: Trp, Tyr, Phe, His.

In one embodiment, the peptides or polypeptides described herein are generated via recombinant means, using a polynucleotide encoding a CNP variant. The disclosure thus encompasses polynucleotides encoding any of the CNP variants described herein, host cells or vectors comprising such polynucleotides, optionally linked to expression control sequences, and methods of using such polynucleotides, vectors or host cells to produce CNP variants of the disclosure. CNP variants expressed by such polynucleotides may be produced by methods including growing host cells in culture medium under conditions suitable for expression of the polynucleotide encoding a CNP variant, and isolating the expression product from the host cells or culture medium. Actual expression products may vary slightly from the encoded protein product depending on any post-translational processing.

"Polynucleotide" refers to a polymer composed of nucleotide units. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") as well as nucleic acid analogs. The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), the nucleotide sequence also encompasses an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T." "cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

"Expression control sequence" refers to a nucleotide sequence that regulates the expression of a nucleotide sequence operatively linked thereto. "Operatively linked" refers to a functional relationship between two parts in which the activity of one part (e.g., the ability to regulate transcription) results in an action on the other part (e.g., transcription of the sequence). Expression control sequences can include, for example and without limitation, sequences of promoters (e.g., inducible or constitutive), enhancers, transcription terminators, a start codon (i.e., ATG), splicing signals for introns, and stop codons.

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to

transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, e.g., a "recombinant polypeptide." A recombinant polynucleotide may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well.

"Chimera" as used herein refers to a polynucleotide or polypeptide comprising at least two heterologous polynucleotide or polypeptide sequences (i.e. derived from different sources or not associated with each other as a naturally-occurring sequence) which are directly or indirectly attached or linked together using techniques commonly known in the art, e.g., recombinant expression or chemical crosslinking. In one embodiment, the heterologous sequence can comprise a protein or peptide directly or indirectly linked to a CNP peptide or variant, including proteins or peptides which are cleavable from the CNP peptide or variant. In a related embodiment, CNP variants are chimera as described herein.

In certain embodiments, chimeras include CNP fusion proteins comprising a cleavable carrier protein or peptide tag. The term "cleavable carrier protein" or "cleavable peptide tag" refers to a peptide or polypeptide sequence that may be fused, directly or indirectly via a linker, to a heterologous polypeptide sequence, and is removable from the heterologous sequence using an agent that cleaves or separates the cleavable peptide or polypeptide from the heterologous polypeptide or protein. In some embodiments, the cleavable carrier protein or peptide tag improves generation, purification and/or detection of the fusion protein or the heterologous polypeptide. Exemplary cleavable carrier proteins and peptide tags include, but are not limited to, human transcription factor TAF12 (TAF12), ketosteroid isomerase (KSI), maltose-binding protein (MBP), β-galactosidase ((β-Gal), glutathione-S-transferase (GST), thioredoxin (Trx), chitin-binding domain (CBD), BMP-2 mutation (BMPM), SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, c-Myc, poly(His), poly (Arg), poly(Asp), poly(Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and fragments thereof.

A "cleaving agent" is an agent that is useful to cleave or separate, e.g., a cleavable peptide or polypeptide from a heterologous polypeptide or protein. Exemplary cleaving agents include, but are not limited to, palladium, cyanogen bromide (CNBr), formic acid, hydroxylamine, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase, enterokinase (enteropeptidase), Kex 2 protease, Omp T protease, Factor Xa protease, subtilisin, pro-TEV, SUMO protease, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K, pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

The terms "identical" and percent "identity", in the context of two or more polynucleotide or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially homologous" or "substantially identical", in the context of two nucleic acids or polypeptides, generally refers to two or more sequences or subsequences that have at least 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 98% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. In certain embodiments, the substantial homology or identity exists over regions of the sequences that are at least about 25, 50, 100 or 150 residues in length. In another embodiment, the sequences are substantially homologous or identical over the entire length of either or both comparison biopolymers.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are inputted into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math., 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol., 48: 443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA, 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection. One example of a useful algorithm is PILEUP, which uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol., 35: 351-360 (1987) and is similar to the method described by Higgins & Sharp, CABIOS, 5: 151-153 (1989). Another algorithm useful for generating multiple alignments of sequences is Clustal W (Thompson et al., Nucleic Acids Research, 22: 4673-4680 (1994)). An example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm (Altschul et al., J. Mol. Biol., 215: 403-410 (1990); Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA, 89: 10915 (1989); Karlin & Altschul, Proc. Natl. Acad. Sci. USA, 90: 5873-5787 (1993)). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

A further indication that two nucleic acid sequences or polypeptides are substantially homologous or identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two polypeptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described herein.

"Substantially pure" or "isolated" means an object species is the predominant species present (i.e., on a molar basis, more abundant than any other individual macromolecular species in the composition), and a substantially purified

fraction is a composition wherein the object species comprises at least about 50% (on a molar basis) of all macromolecular species present. In one embodiment, a substantially pure composition means that the species of interest comprises at least about 70%, 75%, 80%, 85%, 90%, 95%, 98% or more of the macromolecular species present in the composition on a molar or weight basis. The object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) if the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), stabilizers (e.g., BSA), and elemental ion species are not considered macromolecular species for purposes of this definition. In an embodiment, the compounds of the disclosure are substantially pure or isolated. In another embodiment, the compounds of the disclosure are substantially pure or isolated with respect to the macromolecular starting materials used in their production. In yet another embodiment, the pharmaceutical compositions of the disclosure comprise a substantially pure or isolated CNP variant admixed with one or more pharmaceutically acceptable excipients, carriers or diluents, and optionally with another biologically active agent.

"Naturally occurring" as applied to an object refers to the fact that the object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) and which has not been intentionally modified by man in the laboratory is naturally occurring. In one embodiment, a "naturally occurring" substance is of human origin.

"Wild-type" (wt) is a term referring to the natural form, including sequence, of a polynucleotide, polypeptide or protein in a species. A wild-type form is distinguished from a mutant form of a polynucleotide, polypeptide or protein arising from genetic mutation(s).

In one embodiment, a first polypeptide that is an "analog" or "variant" or "derivative" of a second polypeptide is a polypeptide having at least about 50%, 60% or 70% sequence homology, but less than 100% sequence homology, with the second polypeptide. Such analogs, variants or derivatives may be comprised of non-naturally occurring amino acid residues, including without limitation, homoarginine, ornithine, penicillamine, and norvaline, as well as naturally occurring amino acid residues. Such analogs, variants or derivatives may also be composed of one or a plurality of D-amino acid residues, and may also contain peptidomimetics or peptide bond isosteres such as non-peptide linkages between two or more amino acid or peptidomimetic residues. In another embodiment, a first polypeptide is an "analog", "variant" or "derivative" of a second polypeptide if the first polypeptide is not a known cleavage product of the second polypeptide or is not a known precursor of the second polypeptide, even if the first polypeptide has 100% sequence homology to the second polypeptide or has a wild-type sequence.

In an embodiment, the term "derived from" as used herein refers to a polypeptide or peptide sequence that is based on a wild type or naturally occurring polypeptide or peptide sequence and can have one or more deletions, additions, and/or substitutions with natural amino acids, unnatural amino acids or peptidomimetics. In one embodiment, the derivative sequence shares at least about 40%, 50%, 60% or 70%, but less than 100%, sequence similarity to the wild-type or naturally occurring sequence. In another embodiment, the derivative may be a fragment of a polypeptide, wherein the fragment is substantially homologous (e.g., at least about 70%, 75%, 80%, 85%, 90%, or 95% homologous) to the wild-type polypeptide over a length of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 amino acids. In still another embodiment, a polypeptide is "derived from" a wild-type polypeptide if it has a moiety (e.g., a polymer such as, e.g., PEG) directly or indirectly attached to it which is not present on the wild-type polypeptide, even if both polypeptides share 100% homology in their amino acid sequence.

As used herein, an "NPPC-derived" polypeptide refers to a polypeptide derived from the natriuretic peptide precursor C(NPPC) polypeptide, which is a single chain 126-amino acid pre-pro polypeptide, and which upon cleavage ultimately results in wtCNP22. Removal of the signal peptide from NPPC yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP, or CNP-22). Therefore, CNP22 itself is an "NPPC-derived" polypeptide by virtue of being derived from NPPC. In one embodiment, an NPPC-derived polypeptide is at least about 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% homologous to the wild type NPPC over the same number of amino acid residues. It is further contemplated that an NPPC-derived peptide may comprise from about 1 to about 53, or 1 to 37, or 1 to 35, or 1 to 31, or 1 to 27, or 1 to 22, or to 35, or about 15 to about 37 residues of the NPPC polypeptide. In one embodiment, an NPPC-derived peptide may comprise a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, or 53 amino acids derived from the NPPC polypeptide.

The term "effective amount" means a dosage sufficient to produce a desired result on a health condition, pathology, or disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. "Therapeutically effective amount" refers to that amount of an agent effective to produce the intended beneficial effect on health. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation. It will be understood that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors, including the activity of the specific compound employed; the bioavailability, metabolic stability, rate of excretion and length of action of that compound; the mode and time of administration of the compound; the age, body weight, general health, sex, and diet of the patient; and the severity of the particular condition.

"Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment. In certain embodiments, "treatment" refers to administration of a compound or composition to a subject for therapeutic, prophylactic or diagnostic purposes.

A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology. The compounds or compositions of the disclosure may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional or physical, subjective or objective.

The compounds of the disclosure may also be given as a therapeutic treatment or for diagnosis.

"Diagnostic" means identifying the presence, extent and/or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

"Bone- or cartilage-associated biomarker" or "bone- or cartilage-associated marker" refers to a growth factor, enzyme, protein, or other detectable biological substance or moiety whose level is increased or decreased in association with, e.g., cartilage turnover, cartilage formation, cartilage growth, bone resorption, bone formation, bone growth, or combinations thereof. Such biomarkers may be measured before, during and/or after administration of a CNP variant as described herein. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, propeptides of collagen type I and fragments thereof, collagen type 1 and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), aggrecan chondroitin sulfate, and alkaline phosphatase. Cartilage- and bone-associated biomarkers can be measured in any appropriate biological sample, including but not limited to tissues, blood, serum, plasma, cerebrospinal fluid, synovial fluid and urine. In some embodiments, the biomarkers are measured in blood, plasma or serum from animals undergoing efficacy/pharmacodynamic in vivo studies and/or from the conditioned media of ex vivo studies.

In certain embodiments, the level of at least one bone- or cartilage-associated biomarker is measured and the amount or frequency of administration of CNP variant administered to a subject can be adjusted according to the level of the biomarker measured. In some embodiments, the level of biomarker is "below a target level" or "above a target level." A target level of a biomarker is a level or range of levels of the biomarker at which a therapeutic effect is observed in the subject receiving the CNP variant. In certain embodiments, the target level of a biomarker for a subject having a CNP-responsive disorder or condition is the level or range of levels of the biomarker observed in a normal, non-affected subject. In other embodiments, to indicate a therapeutic effect, the target level of a biomarker need not be equivalent to the level or range of levels of the biomarker observed in a normal subject, but can be within, e.g., 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% or 5% of the "normal" level or range of levels of the biomarker observed in a non-affected subject.

For example, if the level of a biomarker increases in association with bone or cartilage formation or growth, the target level of the biomarker indicating a therapeutic effect may be higher than the level of the biomarker in patients suffering from a CNP-responsive disorder who have not been administered a CNP variant, and may optionally be lower than the "normal" level(s), at about the "normal" level(s), or above the "normal" level(s) of the biomarker in subjects not suffering from that disorder. In one embodiment, if the level of a biomarker is below a target level, it indicates an inadequate therapeutic effect, which may require an increase in the amount or frequency of administration of CNP variant administered. In a related embodiment, if the biomarker is above a target level, it indicates that more CNP variant than necessary has been administered, which may require a decrease in the amount or frequency of administration of the CNP variant administered.

As another example, if the level of a biomarker decreases in association with bone or cartilage formation or growth, the target level of the biomarker indicating a therapeutic effect may be lower than the level of the biomarker in patients suffering from a CNP-responsive disorder who have not been administered a CNP variant, and may optionally be higher than the "normal" level(s), at about the "normal" level(s), or below the "normal" level(s) of the biomarker in subjects not suffering from that disorder. In such a case, the converse of the above adjustments in CNP variant amount and frequency of administration may apply.

"Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a therapeutically effective amount of a CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In an embodiment, a pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present disclosure encompass any composition made by admixing a compound of the disclosure and a pharmaceutically acceptable excipient, carrier or diluent.

"Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and the like, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions (e.g., an oil/water or water/oil emulsion). Non-limiting examples of excipients include adjuvants, binders, fillers, diluents, disintegrants, emulsifying agents, wetting agents, lubricants, glidants, sweetening agents, flavoring agents, and coloring agents. Suitable pharmaceutical carriers, excipients and diluents are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (e.g., oral) or parenteral (e.g., subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration).

A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use, including but not limited to metal salts (e.g., sodium, potassium, magnesium, calcium, etc.) and salts of ammonia or organic amines.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual without causing any undesirable biological effects or without interacting in a deleterious manner with any of the components of the composition in which it is contained or with any components present on or in the body of the individual.

The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of a compound of the disclosure calculated in an amount sufficient to produce the desired effect, optionally in association with a pharmaceutically acceptable excipient, diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present disclosure depend on the particular

compound employed and the effect to be achieved, and the pharmaco-dynamics associated with each compound in the host.

"Physiological conditions" refer to conditions in the body of an animal (e.g., a human). Physiological conditions include, but are not limited to, body temperature and an aqueous environment of physiologic ionic strength, pH and enzymes. Physiological conditions also encompass conditions in the body of a particular subject which differ from the "normal" conditions present in the majority of subjects, e.g., which differ from the normal human body temperature of approximately 37° C. or differ from the normal human blood pH of approximately 7.4.

By "physiological pH" or a "pH in a physiological range" is meant a pH in the range of approximately 7.0 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

As used herein, the term "subject" encompasses mammals and non-mammals. Examples of mammals include, but are not limited to, any member of the mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. The term does not denote a particular age or gender.

The terms "polyethylene glycol", "PEG", "polyethylene oxide" and "PEO" are used interchangeably herein unless indicated otherwise. A CNP peptide (CNP22 or a variant thereof) conjugated via an amino group to a "PEOn" polymer associated with the number n, in general has the formula: CH_3—[—O—CH_2CH_2—]$_n$—C(=O) NHR, where n is the number of ethylene oxide units and R denotes the rest of the peptide. The "PEOn" polymer can optionally have an alkylene group, $(CH_2)_m$, where m is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. Such a "PEOn" (e.g., PEO12 or PEO24) polymer is monodispersed, i.e., is a single discrete polymer of a particular molecular weight. Similarly, a CNP peptide conjugated via an amino group to a "PEGnK" polymer associated with the number nK, in general has the formula: CH_3—[—O—CH_2CH_2—]$_p$—C(=O)—NHR, where p is an integer greater than 1. The "PEGnK" polymer also can optionally have an alkylene group, $(CH_2)_m$, where m is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. However, such a "PEGnK" (e.g., PEG1K, PEG2K, PEG55K or PEG20K) polymer is polydispersed, i.e., contains a mixture of polymers having a distribution of molecular weights, where the number nK denotes the polymer numberaverage molecular weight (M_n) in kilo Daltons. For example, "PEG2K" conjugated to a CNP peptide denotes a polydispersed PEG polymer having a polymer number-average molecular weight of around 2 kDa.

When a range of the mass of a polymer (e.g., PEG) is given (e.g., in units of kDa), the range refers to a range of polymer number-average molecular weights, not to a range of molecular weights of multiple polymers in a polydispersed mixture, unless expressly indicated otherwise.

The term "halogen", "halide" or "halo" refers to fluorine, chlorine, bromine, and/or iodine.

The term "alkyl" refers to a linear or branched saturated monovalent hydrocarbon radical, wherein the alkyl may optionally be substituted with one or more substituents Q as described herein. In certain embodiments, the alkyl is a linear saturated monovalent hydrocarbon radical that has 1 to 20 (C_{1-20}), 1 to 15 (C_{1-15}), 1 to 12 (C_{1-12}), 1 to 10 (C_{1-10}), or 1 to 6 (C_{1-6}) carbon atoms, or a branched saturated monovalent hydrocarbon radical of 3 to 20 (C_{3-20}), 3 to 15 (C_{3-15}), 3 to 12 (C_{3-12}), 3 to 10 (C_{3-10}), or 3 to 6 (C_{3-6}) carbon atoms. As used herein, linear C_{1-6} and branched C_{3-6} alkyl groups are also referred as "lower alkyl." Examples of alkyl groups include, but are not limited to, methyl, ethyl, propyl (including all isomeric forms, including n-propyl and isopropyl), butyl (including all isomeric forms, including n-butyl, isobutyl, sec-butyl and tert-butyl), pentyl (including all isomeric forms), and hexyl (including all isomeric forms). For example, C_{1-6} alkyl refers to a linear saturated monovalent hydrocarbon radical of 1 to 6 carbon atoms or a branched saturated monovalent hydrocarbon radical of 3 to 6 carbon atoms.

The term "alkoxy" refers to an —O-alkyl group. In certain embodiments, an alkoxy group may optionally be substituted with one or more substituents Q as described herein.

The term "haloalkyl" refers to an anlky group that is substituted with one or more halide atoms. In certain embodiments, a haloalkyl group is substituted with one, two, three, four, five or six halide atoms. In certain embodiments, a haloalkyl group may optionally be substituted with one or more additional substituents Q as described herein.

The term "cycloalkyl" refers to a cyclic saturated bridged and/or non-bridged monovalent hydrocarbon radical, which may be optionally substituted with one or more substituents Q as described herein. In certain embodiments, the cycloalkyl has from 3 to 20 (C_{3-20}), from 3 to 15 (C_{3-15}), from 3 to 12 (C_{3-12}), from 3 to 10 (C_{3-10}), or from 3 to 7 (C_{3-7}) carbon atoms. Examples of cycloalkyl groups include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, decalinyl, and adamantyl.

The term "heterocyclyl" or "heterocyclic" refers to a monocyclic non-aromatic ring system or a multicyclic ring system that contains at least one non-aromatic ring, wherein one or more of the non-aromatic ring atoms are heteroatoms independently selected from O, S, or N, and the remaining non-aromatic ring atoms are carbon atoms. In certain embodiments, the heterocyclyl or heterocyclic group has from 3 to 20, from 3 to 15, from 3 to 10, from 3 to 8, from 4 to 7, or from 5 to 6 ring atoms. In certain embodiments, the heterocyclyl is a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which may include a fused or bridged ring system, and in which the nitrogen or sulfur atoms may be optionally oxidized, the nitrogen atoms may be optionally quaternized, and some rings may be partially or fully saturated, or aromatic. The heterocyclyl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. Examples of heterocyclic groups include, but are not limited to, acridinyl, azepinyl, benzimidazolyl, benzindolyl, benzoisoxazolyl, benzisoxazinyl, benzodioxanyl, benzodioxolyl, benzofuranonyl, benzofuranyl, benzonaphthofuranyl, benzopyranonyl, benzopyranyl, benzotetrahydrofuranyl, benzotetrahydrothienyl, benzothiadiazolyl, benzothiazolyl, benzothiophenyl, benzotriazolyl, benzothiopyranyl, benzoxazinyl, benzoxazolyl, benzothiazolyl, β-carbolinyl, carbazolyl, chromanyl, chromonyl, cinnolinyl, coumarinyl, decahydroisoquinolinyl, dibenzofuranyl, dihydrobenzisothiazinyl, dihydrobenzisoxazinyl, dihydrofuryl, dihydropyranyl, dioxolanyl, dihydropyrazinyl, dihydropyridinyl, dihydropyrazolyl, dihydropyrimidinyl, dihydropyrrolyl, dioxolanyl, 1,4-dithianyl, furanonyl, furanyl, imidazolidinyl, imidazolinyl, imidazolyl, imidazopyridinyl, imidazothiazolyl, indazolyl, indolinyl, indolizinyl, indolyl, isobenzotetrahydro-

furanyl, isobenzotetrahydrothienyl, isobenzothienyl, isochromanyl, isocoumarinyl, isoindolinyl, isoindolyl, isoquinolinyl, isothiazolidinyl, isothiazolyl, isoxazolidinyl, isoxazolyl, morpholinyl, naphthyridinyl, octahydroindolyl, octahydroisoindolyl, oxadiazolyl, oxazolidinonyl, oxazolidinyl, oxazolopyridinyl, oxazolyl, oxiranyl, perimidinyl, phenanthridinyl, phenathrolinyl, phenarsazinyl, phenazinyl, phenothiazinyl, phenoxazinyl, phthalazinyl, piperazinyl, piperidinyl, 4-piperidonyl, pteridinyl, purinyl, pyrazinyl, pyrazolidinyl, pyrazolyl, pyridazinyl, pyridinyl, pyridopyridinyl, pyrimidinyl, pyrrolidinyl, pyrrolinyl, pyrrolyl, quinazolinyl, quinolinyl, quinoxalinyl, quinuclidinyl, tetrahydrofuryl, tetrahydrofuranyl, tetrahydroisoquinolinyl, tetrahydropyranyl, tetrahydrothienyl, tetrazolyl, thiadiazolopyrimidinyl, thiadiazolyl, thiamorpholinyl, thiazolidinyl, thiazolyl, thienyl, triazinyl, triazolyl, and 1,3,5-trithianyl. In certain embodiments, a heterocyclic group may optionally be substituted with one or more substituents Q as described herein.

The term "aryl" refers to a monocyclic aromatic group or a multicyclic monovalent aromatic group that contain at least one aromatic hydrocarbon ring. In certain embodiments, the aryl has from 6 to 20 (C_{6-20}), from 6 to 15 (C_{6-15}), or from 6 to 10 (C_{6-10}) ring atoms. Examples of aryl groups include, but are not limited to, phenyl, naphthyl, fluorenyl, azulenyl, anthryl, phenanthryl, pyrenyl, biphenyl, and terphenyl. Aryl also refers to bicyclic or tricyclic carbon rings, where at least one of the rings is aromatic and the others may be saturated, partially unsaturated, or aromatic, for example, dihydronaphthyl, indenyl, indanyl, and tetrahydronaphthyl (tetralinyl). In certain embodiments, an aryl group may optionally be substituted with one or more substituents Q as described herein.

The term "heteroaryl" refers to a monocyclic aromatic group or a multicyclic aromatic group that contain at least one aromatic ring, wherein at least one aromatic ring contains one or more heteroatoms independently selected from O, S, and N. Each ring of a heteroaryl group can contain one or two O atoms, one or two S atoms, and/or one to four N atoms, provided that the total number of heteroatoms in each ring is four or less and each ring contains at least one carbon atom. The heteroaryl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. In certain embodiments, the heteroaryl has from 5 to 20, from 5 to 15, or from 5 to 10 ring atoms. Examples of monocyclic heteroaryl groups include, but are not limited to, pyrrolyl, pyrazolyl, pyrazolinyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, thiadiazolyl, isothiazolyl, furanyl, thienyl, oxadiazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, and triazinyl. Examples of bicyclic heteroaryl groups include, but are not limited to, indolyl, benzothiazolyl, benzoxazolyl, benzothienyl, quinolinyl, tetrahydroisoquinolinyl, isoquinolinyl, benzimidazolyl, benzopyranyl, indolizinyl, benzofuranyl, isobenzofuranyl, chromonyl, coumarinyl, cinnolinyl, quinoxalinyl, indazolyl, purinyl, pyrrolopyridinyl, furopyridinyl, thienopyridinyl, dihydroisoindolyl, and tetrahydroquinolinyl. Examples of tricyclic heteroaryl groups include, but are not limited to, carbazolyl, benzindolyl, phenanthrollinyl, acridinyl, phenanthridinyl, and xanthenyl. In certain embodiments, a heteroaryl group may optionally be substituted with one or more substituents Q as described herein.

The term "optionally substituted" is intended to mean that a group, including alkyl, alkoxy, haloalkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, may be substituted with one or more substituents Q (in one embodiment, one, two, three or four substituents Q), where each Q is independently selected from the group consisting of cyano, halo, oxo, nitro, C_{1-6} alkyl, C_{1-6} alkoxy, halo-C_{1-6} alkyl, C_{3-7} cycloalkyl, heterocyclyl, C_{6-14} aryl, heteroaryl, $—C(O)R^e$, $—C(O)OR^e$, $—C(O)NR^fR^g$, $—C(NR^e)NR^fR^g$, $—OR^e$, $—OC(O)R^e$, $—OC(O)OR^e$, $—OC(O)NR^fR^g$, $—OC(=NR^e)NR^fR^g$, $—OS(O)R^e$, $—OS(O)_2R^e$, $—OS(O)NR^fR^g$, $—OS(O)_2NR^fR^g$, $—NR^fR^g$, $—NR^eC(O)R^f$, $—NR^eC(O)OR^f$, $—NR^eC(O)NR^fR^g$, $—NR^eC(=NR^h)NR^fR^g$, $—NR^eS(O)R^f$, $—NR^eS(O)_2R^f$, $NR^eS(O)NR^fR^g$, $—NR^eS(O)_2NR^fR^g$, $—SR^e$, $S(O)R^e$, $—S(O)_2R^e$, and $—S(O)_2NR^fR^g$, wherein each R^e, R^f, R^g, and R^h is independently hydrogen, C_{1-6} alkyl, C_{3-7} cycloalkyl, heterocyclyl, C_{6-14} aryl, or heteroaryl; or R^f and R^g, together with the N atom to which they are attached, form heterocyclyl.

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B. CNP Variants

The use of CNP22 as a therapeutic is limited by its short half-life in plasma (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). In human plasma, the concentration of CNP22 typically is less than five picomolar. CNP22 is degraded and cleared from circulation by NEP and NPR-C in humans (Growth Hormone & IGF Res., 16: S6-S14). In all human and animal studies using systemically administered CNP22, continuous infusion has been used to increase the CNP22 concentration in the subjects. A CNP peptide having a longer half-life and at least a similar level of functionality would be beneficial to a CNP-based therapeutic strategy. CNP variants are also disclosed in related International Application No. PCT/US08/84270, specifically incorporated herein by reference.

The present disclosure provides CNP variants which have reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleavage by NEP and/or clearance by NPR-C, but which have substantially similar or better functionality than wild-type CNP22. Reduced susceptibility of CNP variants to cleavage by NEP and/or clearance by NPR-C would increase the plasma or serum half-life of the variants, thereby increasing the opportunity for the variants to distribute to the target tissues and sites and effectuate the desired pharmacological effects. In certain embodiments, the CNP variants described herein have reduced susceptibility to cleavage by NEP and/or clearance by NPR-C in vitro or in vivo by at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, or 5-fold compared to wtCNP22, and have increased plasma or serum half-life in vivo by at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, or 5-fold compared to wtCNP22, while retaining at least about 50%, 60%, 70%, 80%, 90% or 100% of the functionality of wtCNP22, or having at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5fold, 4-fold, 4.5-fold, or 5-fold greater functionality than wtCNP22. CNP functionality can be evaluated in terms of, e.g., the level of one or more biomarkers (e.g., cGMP) associated with cartilage or bone formation or growth in an in vitro or in vivo study, the length of particular bones in an ex vivo or in vivo study, etc.

Natural substrates of NEP are small and natriuretic peptides (about 2.2 to about 3.2 kDa) are the largest of the natural substrates. According to X-ray crystallographic analyses, the NEP active-site is buried deep inside a central cavity, effectively restricting the size of substrate molecules to no more than about 3 kDa (Oefner et al., J. Mol. Biol., 296: 341-349 (2000)). Based on NPR-B signaling studies, variants of CNP-22, such as CNP-17 (retaining only the cyclic domain, Cys6-Cys22, of CNP22) and CNP-53 (CNP-22 with a 31-amino acid extension at the N-terminus), can still bind and activate NPR-B similarly to the 2.2 kDa

wtCNP-22. Accordingly, the disclosure encompasses CNP variants conjugated to a natural (e.g., peptide) and/or synthetic (e.g., PEG) polymer at the N-terminus and/or C-terminus of CNP22 or variants thereof, which exhibit increased NEP resistance but retain the ability to bind and activate the NPR-B signaling receptor. 55

In one embodiment, the disclosure encompasses CNP variants represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:139), or

(x)-Gly_1Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_8-$Glyl_{19}$-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:140), wherein:

(x) and (z) each independently are a natural polymer (e.g., a peptide sequence containing at least one amino acid) and/or a synthetic polymer (e.g., PEG) as described herein, such that the total mass of the CNP variant is characterized by the ranges described generally herein, e.g., in the range from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa. In one embodiment, the residues from Cys6 to Cys22 form a cyclic portion. In an embodiment, (x) and/or (z) comprise an amino acid extension derived from NPPC or a non-CNP polypeptide (e.g., ANP, BNP, IgG, etc.), wherein the extension contains 1 to 40, 1 to 35, 1 to 31, 5 to 35, 5 to 31 or 5 to 15 amino acids. In another embodiment, the CNP variants comprise one or more modifications and/or substitutions with another natural amino acid, an unnatural amino acid, a peptidomimetic and/or a peptide bond isostere at one or more of the following positions of CNP22: Gly1, Lys4, Gly5, Cys6, Phe7, Gly8, Leu9, Lys10, Leu11, Ile14, Gly15, Ser16, Met17, Gly19, Leu20 and Gly21.

In another embodiment, CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, are represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-$Leu_{20}Gly_{21}$-Cys_{22}-(z) (SEQ ID NO: 141), or

(x)-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 6), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG (or PEO), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or fibroblast growth factor 2 (FGF2);

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG); and

(b) and (h) independently may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxynorleucine, citrulline (Cit), Gln, Glu or Ser. In one embodiment, (b) is Arg. In another embodiment, for improved NEP resistance, (b) is not Gly. In yet another embodiment, (h) is not Arg.

Non-limiting examples of amino acid sequences derived from NPPC or variants thereof include:

Arg,

Glu-Arg,

(SEQ ID NO: 7)
Gly-Ala-Asn-Lys-Lys,

(SEQ ID NO: 8)
Gly-Ala-Asn-Arg-Arg,

(SEQ ID NO: 9)
Gly-Ala-Asn-Pro-Arg,

(SEQ ID NO: 10)
Gly-Ala-Asn-Gln-Gln,

(SEQ ID NO: 11)
Gly-Ala-Asn-Ser-Ser,

(SEQ ID NO: 12)
Gly-Ala-Asn-Arg-Gln,

(SEQ ID NO: 13)
Gly-Ala-Asn-Arg-Met,

(SEQ ID NO: 14)
Gly-Ala-Asn-Arg-Thr,

(SEQ ID NO: 15)
Gly-Ala-Asn-Arg-Ser,

(SEQ ID NO: 16)
Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala,

(SEQ ID NO: 17)
Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg,

(SEQ ID NO: 18)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg,

(SEQ ID NO: 19)
Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys,

(SEQ ID NO: 20)
Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg,

(SEQ ID NO: 21)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys,
and

(SEQ ID NO: 22)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg.

Non-limiting examples of amino acid sequences derived from non-CNP polypeptides such as, e.g., ANP, BNP, serum albumin and IgG include:

(SEQ ID NO: 23)
Ser-Leu-Arg-Arg-Ser-Ser;

(SEQ ID NO: 24)
Asn-Ser-Phe-Arg-Tyr;

-continued

(SEQ ID NO: 25)
Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly;

(SEQ ID NO: 26)
Met-Val-Gln-Gly-Ser-Gly;

(SEQ ID NO: 27)
Lys-Val-Leu-Arg-Arg-Tyr;

(SEQ ID NO: 28)
Lys-Val-Leu-Arg-Arg-His;

(SEQ ID NO: 29)
Gly-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg;

(SEQ ID NO: 30)
Gly-Val-Pro-Gln-Val-Ser-Thr-Ser-Thr;

(SEQ ID NO: 31)
Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln;
and

(SEQ ID NO: 32)
Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser.

In an embodiment, the N-terminal (x) group and/or the C-terminal (z) group of any of the CNP variants having an (x) and/or (z) group, as described herein, independently comprise an amino acid sequence that contains a small number of, if any, acidic natural or unnatural amino acids (e.g., Asp or Glu). In another embodiment, (x) and/or (z) are enriched in basic natural or unnatural amino acids (e.g., Lys, Arg or His) to maintain an alkaline pI similar to the pI of CNP22 (pI 8.9). In one embodiment, the pI of the CNP variants is in the range from about 8 to about 10.5, designed so that the CNP variants can diffuse more readily through the extracellular matrix surrounding chondrocytes of bone growth plates. In narrower embodiments, the pI of the CNP variants is from about 8.5 to about 10.5, or from about 8.5 to about 10, or from about 9 to about 10.

In yet another embodiment, (x) and/or (z) are enriched in polar natural or unnatural amino acids, designed for increased aqueous solubility. In still another embodiment, (x) and/or (z) contain a small number of, if any, hydrophobic natural or unnatural amino acids (e.g., Ala, Val, Leu, Ile or Met).

In a further embodiment, the N-terminus of the CNP variants terminates in at least one glycine residue, designed for increased serum half-life. In a related embodiment, to prevent pyroglutamine formation, the N-terminus of CNP variants terminates in a glycine residue if it would otherwise terminate in glutamine. In one embodiment, the (x) group contains an amino acid extension whose N-terminus terminates in at least one glycine residue. In another embodiment, (x) and/or (z) do not contain two adjacent basic natural or unnatural amino acids (e.g., Lys-Lys or Arg-Arg), designed to reduce susceptibility to cleavage by the protease furin. In an embodiment, (x) does not contain two adjacent basic amino acids immediately preceding the position corresponding to Gly1 of CNP22.

In still another embodiment, the (x) group and/or the (z) group of the CNP variants comprise an amino acid sequence derived from NPPC (e.g., derived from CNP53). In an embodiment, (x) comprises an amino acid sequence derived from the N-terminal tail of ANP or BNP. In another embodiment, (z) comprises an amino acid sequence derived from the C-terminal tail of ANP or BNP. In a further embodiment, (x) and/or (z) comprise an amino acid sequence derived from a non-natriuretic polypeptide such as, e.g., IgG, human serum albumin (HSA), histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, FGF-2, and bone-targeting proteins (e.g., osteocrin, osteopontin, osteocalcin, and sialoprotein).

In any embodiment described herein in which CNP22 or a variant thereof can have an N-terminal (x) group and/or a C-terminal (z) group, (x) and/or (z) independently can contain an amino acid sequence derived from the functional domain of a bone morphogenetic protein (BMP). An N-terminal and/or C-terminal amino acid extension derived from the functional domain of a BMP can increase the NEP resistance, and hence the serum half-life of the CNP variant, by increasing the total mass of the CNP variant to characterized by the ranges described generally herein, e.g., a range from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa. In addition, since certain BMPs are growth factors and cytokines that induce the formation of bone and cartilage, a fragment derived from the functional domain of a BMP can promote chondrocyte, cartilage or bone growth by a mechanism distinct from activation of the guanylyl cyclase function of NPR-B by the cyclic domain of CNP22 or a variant thereof. Non-limiting examples of BMPs that promote bone formation and development, cartilage formation and development, and/or osteoblast differentiation include BMP1, BMP2, BMP3, BMP5, BMP7 and BMP8a. In an embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof independently are conjugated to an amino acid sequence derived from the last 140 amino acids in the C-terminal portion of BMP1, BMP2, BMP3, BMP5, BMP7 or BMP8a.

In one embodiment, the CNP variants contain an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, including but not limited to:

(SEQ ID NO: 4)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (CNP-53);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37, Analog BL);

(SEQ ID NO: 61)
AAWARLLQEHPNAGLSKGCFGLKLDRIGSMSGLGC (Analog CA);

(SEQ ID NO: 62)
AAWARLLQEHPNARGLSKGCFGLKLDRIGSMSGLGC (Analog CB);

(SEQ ID NO: 63)
DLRVDTKSRAAWARGLSKGCFGLKLDRIGSMSGLGC (Analog CC);

(SEQ ID NO: 40)
RGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 38)
ERGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 64)
GANQQGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 65)
GANRRGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 66)
GANPRGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 67)
GANSSGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC;

-continued

(SEQ ID NO: 68)
SPKMVQGSG-CNP17-KVLRRH (Analog CD) (CNP17 having N-terminal and C-terminal tails derived from BNP).

In another embodiment, the CNP variants have a K4R substitution at position 4 of CNP22. Non-limiting examples of CNP(K4R) variants include:

((SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC (Analog AY);

(SEQ ID NO: 37)
GANPRGLSRGCFGLKLDRIGSMSGLGC (Analog CI);

(SEQ ID NO: 41)
RGLSRGCFGLKLDRIGSMSGLGC (Analog AZ);

(SEQ ID NO: 39)
ERGLSRGCFGLKLDRIGSMSGLGC (Analog BA);

(SEQ ID NO: 69)
GANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CH);
and

(SEQ ID NO: 70)
GANSSGLSRGCFGLKLDRIGSMSGLGC (Analog CG).

In further embodiments, the CNP variants are chimeras comprising CNP22, or a variant thereof having amino acid addition(s), deletion(s) and/or substitution(s), and a peptide fragment derived from a polypeptide or protein other than CNP, or the whole non-CNP polypeptide or protein, to the N-terminus of the CNP peptide, wherein CNP22 or the variant thereof may optionally have an N-terminal amino acid extension of one or more amino acid residues. In certain embodiments, the CNP chimeras comprise CNP22 or a variant thereof that has an N-terminal amino acid extension of one or more amino acid residues. In certain embodiments, the CNP chimeras contain lysine-lysine (KK) residues or GANKK residues immediately preceding the first position of CNP22 (Gly in the case of CNP22) or a variant thereof. In other embodiments, the CNP chimeras contain one or two residues different from lysine-lysine immediately preceding the first position of CNP22 or a variant thereof. Non-limiting examples of residues that can immediately precede the first position of CNP22 or a variant thereof include KP, PK, PR, PQ, QK, QQ, RR, SS, GANKP(SEQ ID NO: 200), GANPK (SEQ ID NO: 201), GANPR (SEQ ID NO: 9), GANPQ (SEQ ID NO: 202), GANQK (SEQ ID NO: 203), GANQQ (SEQ ID NO: 10), GANRR (SEQ ID NO: 8), and GANSS (SEQ ID NO: 11).

In another embodiment, the CNP variants are chimera comprising CNP22 and an N-terminal peptide fragment, including but not limited to:

(SEQ ID NO: 76)
GHHSHEQHPHGANQQGLSKGCFGLKLDRIGSMSGLGC (Analog CQ) (histidine-rich glycoprotein (HRGP) fragment-CNP22 chimera);

(SEQ ID NO: 77)
GAHHPHEHDTHGANQQGLSKGCFGLKLDRIGSMSGLGC (Analog CR) (HRGP fragment-CNP22 chimera);

(SEQ ID NO: 78)
GHHSHEQHPHGANPRGLSKGCFGLKLDRIGSMSGLGC (Analog CX) (HRGP fragment-CNP22 chimera);

(SEQ ID NO: 79)
GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC (Analog CF) ($IgG_1(F_c)$ fragment-CNP22 chimera);

(SEQ ID NO: 80)
GQHKDDNPNLPRGANPRGLSKGCFGLKLDRIGSMSGLGC (Analog CY) (human serum albumin (HSA) fragment-CNP22 chimera);

(SEQ ID NO: 81)
GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC (Analog CE) (HSA fragment-CNP22 chimera);

(SEQ ID NO: 82)
FGIPMDRIGRNPRGLSKGCFGLKLDRIGSMSGLGC (Analog CZ) (osteocrin "NPR C inhibitor" fragment-CNP22 chimera);
and

(SEQ ID NO: 83)
GKRTGQYKLGSKTGPGPKGLSKGCFGLKLDRIGSMSGLGC (Analog DA) (FGF2 "heparin-binding domain" fragment-CNP22 chimera).

In yet another embodiment, the CNP variants are chimera comprising an N-terminal peptide fragment and CNP22 in which arginine is substituted for Lys4 of CNP22 ("CNP22 (K4R)"), including but not limited to:

(SEQ ID NO: 84)
GQPREPQVYTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CK) ($IgG_1(F_c)$ fragment-CNP22(K4R) chimera);

(SEQ ID NO: 85)
GVPQVSTSTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CL) (HSA fragment-CNP22(K4R) chimera)

(SEQ ID NO: 86)
GQPSSSSQSTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CM) (fibronectin fragment-CNP22(K4R) chimera);

(SEQ ID NO: 87)
GQTHSSGTQSGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CN) (fibrinogen fragment-CNP22(K4R) chimera);

(SEQ ID NO: 88)
GSTGQWHSESGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CO) (fibrinogen fragment-CNP22(K4R) chimera);
and

(SEQ ID NO: 89)
GSSSSSSSSSGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CP) (zinc finger fragment-CNP22(K4R) chimera).

Chimera comprising IgG and CNP22 or a variant thereof are designed for, inter alia, increased resistance to NEP degradation and reduced binding to serum albumin. CNP chimera comprising a surface fragment of HSA are designed for, inter alia, reduced immunogenicity and reduced binding to serum albumin. HRGP-CNP22 and HRGP-CNP22(K4R) chimera containing a cationic, histidine-rich, non-lysine, non-arginine sequence at the N-terminus are designed for, inter alia, increased stability to proteases. Chimera containing an osteocrin fragment are designed to release, upon protease (e.g., furin) cleavage, the osteocrin fragment at bone growth plates, where the fragment would inhibit the clearance receptor NPR-C. With respect to chimera comprising an FGF2 heparin-binding fragment, heparin binding to the fragment is designed to protect the chimera from degradation, thereby providing a longer serum half-life. Chimera containing a fibronectin, fibrinogen, or zinc-finger fragment are designed for reduced binding to serum albumin, among other features.

Not intending to be bound by theory, a CNP variant of molecular weight from about 2.6 or 2.8 kDa to about 6 or 7

kDa which has increased resistance to NEP degradation and has similar or improved functionality (e.g., binding to NPR-B and stimulation of cGMP signaling) as compared to wtCNP22, may be more effective if it does not bind tightly to plasma proteins such as serum albumin. A CNP variant that does not bind tightly to plasma proteins (e.g., serum albumin) may be more effective in diffusing through cartilage, getting to chondrocytes of bone growth plates, and binding to and activating NPR-B for cGMP signaling. In one embodiment, CNP variants designed for reduced binding to plasma proteins (e.g., serum albumin) are chimeras comprising CNP22 or a variant thereof and a peptide fragment from IgG. In another embodiment, CNP variants designed for reduced binding to plasma proteins are chimeras comprising CNP22 or CNP22(K4R) and a fragment from a polypeptide (e.g., IgG, HSA, fibronectin, fibrinogen, a zinc finger-containing polypeptide, etc.). In yet another embodiment, CNP variants designed for reduced binding to plasma proteins comprise CNP22 or a variant thereof conjugated to a hydrophilic or water-soluble polymer. In one embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO). In another embodiment, the hydrophilic or water-soluble polymer (e.g., PEG) is functionalized with one or more functional groups that impart a negative charge to the polymer under physiological conditions, such as, e.g, carboxyl, sulfate or phosphate groups, or a combination thereof.

In a further embodiment, CNP variants of the disclosure include truncated CNP peptides ranging from human CNP-17 (hCNP-17) to human CNP-53 (hCNP-53), and having wildtype amino acid sequences derived from hCNP-53. Such truncated CNP peptides include:

(SEQ ID NO: 4)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSG
LGC (CNP-53);

(SEQ ID NO: 146)
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGL
GC (CNP-52);

(SEQ ID NO: 147)
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGL
GC (CNP-51);

(SEQ ID NO: 148)
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-50);

(SEQ ID NO: 149)
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-49);

(SEQ ID NO: 150)
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-48);

(SEQ ID NO: 151)
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47);

(SEQ ID NO: 152)
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46);

(SEQ ID NO: 153)
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45);

-continued

(SEQ ID NO: 154)
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-44);

(SEQ ID NO: 155)
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-43);

(SEQ ID NO: 156)
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-42);

(SEQ ID NO: 157)
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-41);

(SEQ ID NO: 158)
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40);

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37);

(SEQ ID NO: 161)
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36);

(SEQ ID NO: 162)
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35);

(SEQ ID NO: 163)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

(SEQ ID NO: 164)
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33);

(SEQ ID NO: 165)
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32);

(SEQ ID NO: 166)
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31);

(SEQ ID NO: 167)
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30);

(SEQ ID NO: 168)
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29);

(SEQ ID NO: 169)
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28);

(SEQ ID NO: 170)
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27);

(SEQ ID NO: 171)
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26);

(SEQ ID NO: 172)
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25);

(SEQ ID NO: 173)
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24);

(SEQ ID NO: 174)
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23);

(SEQ ID NO: 1)
GLSKGCFGLKLDRIGSMSGLGC (CNP-22);

(SEQ ID NO: 175)
LSKGCFGLKLDRIGSMSGLGC (CNP-21);

(SEQ ID NO: 176)
SKGCFGLKLDRIGSMSGLGC (CNP-20);

(SEQ ID NO: 177)
KGCFGLKLDRIGSMSGLGC (CNP-19);

-continued

(SEQ ID NO: 178)
GCFGLKLDRIGSMSGLGC (CNP-18);
and

(SEQ ID NO: 2)
CFGLKLDRIGSMSGLGC (CNP-17).

In certain embodiments, CNP variants do not include CNP17, CNP-22 or CNP-53.

In another embodiment, the truncated CNP peptides ranging from hCNP-17 to hCNP-53 can contain amino acid addition(s), deletion(s) and/or substitution(s) with natural or unnatural amino acid(s) or peptidomimetic(s) (e.g., peptide bond isostere(s)), as described herein, at any one or more of the amino acid positions of the particular truncated CNP peptides. In yet another embodiment, the truncated CNP peptides having wild-type sequences or amino acid addition(s), deletion(s) and/or substitution(s), can be conjugated at the N-terminus, C-terminus and/or internal site(s) to any of the moieties described herein, including but not limited to bone- or cartilage-targeting moieties (e.g., bisphosphonates, bone- or cartilage-targeting peptide sequences (e.g., polyAsp, pol-yGlu), peptide sequences derived from bone-targeting domains of bone proteins (e.g., osteopontin, osteocalcin, sialoprotein)), peptide sequences derived from the functional domains of bone morphogenetic proteins (e.g., BMP2, BMP3, BMP5, BMP7, BMP8a), peptide sequences derived from natriuretic polypeptides (e.g., NPPC, ANP, BNP), peptide sequences derived from polypeptides of non-natriuretic origin (e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, FGF-2, osteocrin), moieties that reduce renal clearance (e.g., negatively charged PEG moieties), hydrophilic polymers (e.g., PEG), carbohydrates (e.g., carbohydrates recognized by receptors on the surface of cells at bone growth plates), hydrophobic acids (e.g., C_5-C_{12} carboxylic acids, natural fatty acids), phospholipids, and combinations thereof. In an embodiment, the truncated CNP peptides having wildtype sequences or amino acid addition(s), deletion(s) and/or substitution(s), and optionally conjugated to one or more moieties at the N-terminus, C-terminus and/or internal site(s), have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa.

In a further embodiment, the CNP variants are derivatives of CNP37, which is QEHPNARKYKGANKK-CNP22 (SEQ ID NO: 60). The CNP37 variants can contain amino acid addition(s), deletion(s), and/or substitution(s) with natural or unnatural amino acid(s) or peptidomimetic(s) (e.g., peptide bond isostere(s)) at any one or more of the 37 positions of CNP37. Non-limiting examples of substitutions that can be made in CNP37, based on the numbering of CNP22, include K4R, G5S, G5R, G8S, K10R, G15S, S16Q, M17N, G19R, and combinations thereof. In an embodiment, the CNP37 derivatives contain a substitution of Met17 to a natural (e.g., asparagine) or unnatural amino acid or peptidomimetic, designed in part to avoid oxidation of the sulfur atom of methionine. In another embodiment, the CNP37 variants contain substitution(s) of Lys8, Lys10, Lys14 and/or Lys15 (based on numbering from the N-terminus of CNP37) to non-basic natural or unnatural amino acid(s) or peptiomimetic(s), designed in part to reduce albumin binding.

In addition or alternatively to amino acid addition(s), deletion(s) and/or substitution(s), the CNP37 derivatives can be conjugated at the N-terminus, C-terminus, and/or an internal site to any of the moieties described herein, including but not limited to bone- or cartilage-targeting moieties (e.g., bone-targeting peptide domains), moieties that reduce renal clearance (e.g., negatively charged PEG moieties), hydrophilic polymers (e.g., PEG), amino acid sequences comprising one or more amino acids (e.g., osteocrin "NPR-C inhibitor" fragment), carbohydrates (e.g., carbohydrates recognized by receptors on the surface of cells at bone growth plates), hydrophobic acids (e.g., C_5-C_{12} carboxylic acids and natural fatty acids), and combinations thereof.

In one embodiment, the CNP variants are modified CNP37 peptides having mutation(s)/substitution(s) at the furin cleavage site (underlined), designed to improve in vivo resistance to the furin protease, and/or containing glycine (underlined) at the N-terminus, designed to improve plasma stability and prevent pyroglutamine formation. Such CNP37 variants include but are not limited to:

(SEQ ID NO: 71)
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (An. CS);

(SEQ ID NO: 72)
GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (An. CT);

(SEQ ID NO: 73)
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (An. CU);

(SEQ ID NO: 74)
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (An. CW);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37, An. DB);
and

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37)

In a further embodiment, the CNP variants of the disclosure include CNP peptides and variants thereof that can be produced by the fusion protein process described herein. Non-limiting examples of CNP variants that can be produced by the fusion protein process described herein, using chemical or proteolytic cleavage or protein self-cleavage, include:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Gly-wtCNP53);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-wtCNP37);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (wtCNP37);

(SEQ ID NO: 144)
GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC
(HSA fragment-wtCNP27);

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)];

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSG
LGC [CNP53(M48N)];

(SEQ ID NO: 181)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[Gly-CNP37(M32N)];

-continued

(SEQ ID NO: 182)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP37(M32N)];

(SEQ ID NO: 183)
GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSNSGLGC
[HSA-CNP27(M22N)];

(SEQ ID NO: 184)
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R,
M22N)];

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Pro-wtCNP53);

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-wtCNP37);

(SEQ ID NO: 186)
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-wtCNP37);

(SEQ ID NO: 187)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (wtCNP34);

(SEQ ID NO: 188)
P-GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-HSA-wtCNP27);

(SEQ ID NO: 189)
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)];

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Met-wtCNP53);

(SEQ ID NO: 191)
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-Gly-wtCNP37);

(SEQ ID NO: 192)
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-wtCNP37);

(SEQ ID NO: 193)
M-GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-HSA-wtCNP27);

(SEQ ID NO: 194)
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)].

Other CNP variants, including truncated CNP peptides ranging from hCNP-17 to hCNP-53 and having wild-type sequences or amino acid addition(s), deletion(s) and/or substitution(s), can also be produced by the fusion protein process described herein, so long as the intended site of chemical or proteolytic cleavage of the fusion protein is not present within the amino acid sequence of the target CNP variant itself. As a non-limiting example, the fusion protein process described herein can be employed to produce truncated wtCNP34 using formic acid cleavage.

In additional embodiments, for any of the CNP peptides and CNP variants described herein that have asparagine (Asn/N) residue(s) and/or glutamine (Gln/Q) residue(s), whether they have a wild-type sequence or a non-natural amino acid sequence, any Asn residue(s) and/or any Gln residue(s) can independently be substituted with any other natural or unnatural amino acids, including conservative substitutions such as Asn to Gln. Such substitution(s) are designed in part to minimize or avoid any potential deamidation of asparagine and/or glutamine. Non-limiting examples of CNP peptides and variants in which any Asn residue(s) and/or any Gln residue(s) can independently be substituted with any other natural or unnatural amino acids, including conservative substitutions such as Asn to Gln, include wtCNP34, wtCNP37, Gly-wtCNP37, Pro-wtCNP37, Pro-Gly-wtCNP37, GHKSEVAHRFK-wtCNP27 (SEQ ID NO: 144), Pro-GHKSE-VAHRFK-wtCNP27 (SEQ ID NO: 188), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36). In certain embodiments, an asparagine residue of the CNP peptides and CNP variants described herein is not substituted with glutamine, aspartic acid or glutamic acid. In certain embodiments, a glutamine residue of the CNP peptides and CNP variants described herein is not substituted with asparagine, aspartic acid or glutamic acid. As a non-limiting example, asparagine residues 7 and/or 15 of Pro-Gly-wtCNP37 (PGQEHP NARKYKGANKKGLSKGCF-GLKLDRIGSMSGLGC) SEQ ID NO: 145) can independently be substituted with any other natural or unnatural amino acids, including glutamine, to avoid any potential deamidation of the asparagine residue(s) to aspartic acid or isoaspartic acid. In certain embodiments, asparagine residues 7 and/or 15 of Pro-Gly-wtCNP37 are not substituted with glutamine, aspartic acid or glutamic acid.

It is understood, however, that the present disclosure encompasses CNP variants in which any one or more, up to all, residues susceptible to deamidation or a deamidation-like reaction (e.g., isomerization) may be converted to other residue(s) via deamidation or a deamidation-like reaction to any extent, up to 100% conversion per converted residue. In certain embodiments, the disclosure encompasses CNP variants in which:

(1) any one or more, up to all, asparagine (Asn/N) residues may be converted to aspartic acid or aspartate, and/or to isoaspartic acid or isoaspartate, via deamidation up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(2) any one or more, up to all, glutamine (Gln/Q) residues may be converted to glutamic acid or glutamate, and/or to isoglutamic acid or isoglutamate, via deamidation up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(3) any one or more, up to all, aspartic acid or aspartate (Asp/D) residues may be converted to isoaspartic acid or isoaspartate via a deamidation-like reaction (also called isomerization) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(4) any one or more, up to all, glutamic acid or glutamate (Glu/E) residues may be converted to isoglutamic acid or isoglutamate via a deamidation-like reaction (also called isomerization) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(5) a combination of the above.

As a non-limiting example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of Pro-Gly-wtCNP37 [PGQEHPNARKYKGA NKKGLSKGCFGLKLDRIGSMSGLGC] (SEQ ID NO: 145) may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue, as described above.

As a further example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine and/or aspartic acid residues of Pro-Gly-wtCNP37 [PGQEHP NARKYKGANKKGLSKGCFGLKL DRIGSMSGLGC] (SEQ ID NO: 145) may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, and/or (2) isoaspartic acid/isoaspartate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As another example, the present disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of Gly-wtCNP37 [GQEHPNARKYKGA NKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 75)] may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As yet another example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of wtCNP37 [QEHPNARKYKGANKKGLSKGCFGLKL DRIGSMSGLGSEQ ID NO: 60)] may be converted to (1) partic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As a further example, the present disclosure encompasses CNP variants in which any one or more, up to all, asparagine, aspartic acid, and/or glutamic acid residues of an HSA-wtCNP27 chimera, GHKSEVAHRFKGA NKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 144), may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) isoaspartic acid/isoaspartate, and/or (3) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As a still further example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, aspartic acid, and/or glutamic acid residues of a Pro-HSA-wtCNP27 chimera, PGHKSEVAHRFKGA NKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 188), may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) isoaspartic acid/isoaspartate, and/or (3) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

In addition, the present disclosure encompasses CNP variants in which any one or more, up to all, methionine (Met/M) residues may be oxidized to any chemically feasible oxidized form (e.g., sulfoxide and/or sulfone) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% transformation per oxidized residue.

In another embodiment, the CNP variants comprise CNP22 or variants thereof conjugated at the N-terminus and/or C-terminus to moiet(ies) that facilitate translocation of the variants across a cell membrane or cell barrier. In one embodiment, the CNP variants are conjugated at the N-terminus and/or C-terminus to peptide sequence(s) that facilitate transport of the variants across a cell membrane or cell barrier, including via active peptide transporters.

In a further embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof are conjugated to chemical moieties such as, e.g., natural and/or synthetic polymers, to increase the total mass of the modified CNP peptide to the ranges described generally herein, e.g., a range from about 2.6 or 2.8 kDa to about 6 or 7 kDa. In one embodiment, the chemical moieties are biocompatible hydrophilic or water-soluble natural (e.g., peptides, carbohydrates) or synthetic (e.g., PEG (or PEO)) polymers.

In a particular embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof are conjugated to PEG (or PEO) polymers to result in a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa. Pegylation of CNP22 or a variant thereof is designed, inter alia, to reduce immunogenicity and improve half-life by reducing renal clearance and increasing protease resistance. A PEG moiety can be attached to the N- and/or C-terminus of CNP22 or any variant described herein, including but not limited to CNP-17 (the Cys6-Cys22 cyclized portion of CNP22), CNP37, and variants of CNP17, CNP22 or CNP37 having N- and/or C-terminal amino acid extension(s), amino acid substitution(s) and/or amino acid deletion(s). In an embodiment, the Lys4 and/or Lys10 residues of CNP17, CNP22 or CNP37, or variants thereof, are substituted with a natural or unnatural amino acid (e.g., Arg, Gly, Ser, Gln, Glu or Cit) or peptidomimetic that does not contain a reactive primary amine on a side chain, to preclude any potential PEGylation of these lysine residues. In one embodiment, the Lys4 and/or Lys10 residues of the CNP peptides are substituted withArg. In another embodiment, the Lys10 residue is not substituted with Arg.

In a further embodiment, CNP variants (including CNP22 and variants thereof) having a PEG (or PEO) moiety and an amino acid extension at the N-terminus contain arginine at the position immediately preceding the position corresponding to Gly1 of CNP22. Such PEGylated CNP variants are designed for increased resistance to NEP degradation, reduced binding to serum albumin, and enhanced CNP functional activity (e.g., activation of cGMP signaling). Non-limiting examples of PEGylated CNP variants include PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO24-GANRR-CNP22(SEQ ID NO: 36), PEO12-GANRR-CNP22(SEQ ID NO: 36), PEO24-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO12-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO24-GANPR-CNP22(SEQ ID NO: 37), PEO12-GANPR-CNP22 (SEQ ID NO: 37), PEO24-GANQQ-CNP22(SEQ ID NO: 64), PEO12-GANQQ-CNP22(SEQ ID NO: 64), PEO24-ER-CNP22(K4R) (SEQ ID NO: 39), PEO12-ER-CNP22 (K4R) (SEQ ID NO: 39), PEO24-ER-CNP22(SEQ ID NO: 39), PEO12-ER-CNP22(SEQ ID NO: 39), PEO24-R-CNP22 (K4R) (SEQ ID NO: 41), PEO12-R-CNP22(K4R) (SEQ ID NO: 41), PEO24-R-CNP22(SEQ ID NO: 41), and PEO12-R-CNP22(SEQ ID NO: 41), wherein PEO24 is a monodispersed 1.2 kDa PEG polymer and PEO12 is a monodispersed 0.6 kDa PEG polymer. In one embodiment, the PEG (or PEO) polymer is conjugated to the N-terminus of the CNP variants.

The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG) that can vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as,

e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP peptide by means of N-hydroxy succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. Such CNP variants can be generated using, e.g., wtCNP22 (2.2 kDa), CNP17 retaining only the cyclized region (residues 6-22) of wtCNP22, CNP variants having an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, or variants having amino acid substitutions, additions and/or deletions such as, e.g., GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), R-CNP22(SEQ ID NO: 40), R-CNP22 (K4R) (SEQ ID NO: 41), ER-CNP22(SEQ ID NO: 38) and ER-CNP22(K4R) (SEQ ID NO: 39). In an embodiment, the PEG-CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, contain a monodispersed, linear PEG (or PEO) moiety conjugated at the N-terminus and/or C-terminus via NHS- or aldehyde-based chemistry, or a two-arm or three-arm branched PEG moiety conjugated at the N-terminus and/or C-terminus via NHS-based chemistry. The disclosure also encompasses negatively charged PEG-CNP variants designed for reduced renal clearance, including but not limited to carboxylated, sulfated and phosphorylated compounds (Caliceti, Adv. Drug Deliv. Rev., 55: 1261-77 (2003); Perlman, J. Clin. Endo. Metab., 88: 3227-35 (2003); Pitkin, Antimicrob. Ag. Chemo., 29: 440-444 (1986); Vehaskari, Kidney Int'l, 22: 127-135 (1982)). In one embodiment, the PEG (or PEO) moiety contains carboxyl group(s), sulfate group(s), and/or phosphate group(s).

In another embodiment, the PEG (or PEO) moieties conjugated to the N-terminus, C-terminus and/or internal site(s) of CNP variants described herein contain one or more functional groups that are positively charged under physiological conditions. Such PEG moieties are designed, inter alia, to improve distribution of such PEGylated CNP variants to cartilage tissues. In one embodiment, such PEG moieties contain one or more primary, secondary or tertiary amino groups, quaternary ammonium groups, and/or other amine-containing (e.g., urea) groups.

In an embodiment, the disclosure encompasses CNP22 or variants thereof conjugated via NHS- or aldehyde-based chemistry to PEG (or PEO) of the formula $(CH_2CH_2O)_n$, wherein n is an integer from about 6 to about 100, and the PEG polymer is from about 0.3 kDa to about 5 kDa. In another embodiment, n is an integer from about 12 to about 50, and the PEG polymer is from about 0.6 kDa to about 2.5 kDa. In yet another embodiment, n is from about 12 to about 24, and the PEG polymer is from about 0.6 kDa to about 1.2 kDa. In still another embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the end-capping group is an alkyl group, e.g., a lower alkyl group such as methyl.

In a further embodiment, the disclosure provides CNP variants having one or more peptide bonds or peptide bond isosteres that have reduced susceptibility to cleavage by peptidases including neutral endopeptidase (NEP). NEP is a membrane-bound zinc-dependent endopeptidase that cleaves a substrate peptide bond at the amino end of large hydrophobic residues. Thus, modification of a peptide bond at a cleavage site for NEP to an unnatural peptide or non-peptide bond may preclude or decrease the efficiency of NEP cleavage.

For ANP and CNP, NEP cleavage is reported to occur first at the Cys6-Phe7 bond within the cyclized region, then elsewhere throughout the remainder of the structures. For BNP, cleavage is reported to occur first at the peptide N-terminus, then within the cyclic structure. Although the primary NEP cleavage site on CNP is reported to be the Cys6-Phe7 bond, when wtCNP22 was exposed to NEP digestion for 2.5 minutes in vitro, all possible sites were unexpectedly hydrolyzed, with the Cys6-Phe7 and Gly8-Leu9 peptide bonds being slightly most labile, as described in Example 2.

Substrate specificity of NEP is primarily determined by two substrate-binding subsites, S1' and S2' (Oefner et al., J. Mol. Biol. 296:341-349 (2000)). The S1' site accepts a large hydrophobic P1' residue of which the N-terminal peptide bond is subjected to hydrolysis (e.g., Phe, Leu, Be and Met). The S2' site generally prefers a smaller residue, termed P2' (e.g., Gly or Ser). In the case of CNP, Phe7 is reported to be the preferred P1' residue for the NEP SP site, while Gly8 is the preferred P2' residue for the S2' site. Because these two subsites can together accommodate only a certain total side chain size, any increase in the total size of the P1'-P2' residues of CNP can potentially disrupt NEP binding. For example, addition of a chloride atom at the 3-position of the P1' Phe7 aromatic ring (i.e., 3-Cl-Phe7) can potentially modify (e.g., destabilize) interactions between CNP and the NEP cleavage sites, for example at the S1' subsite. Addition of a tertiary butyl group to the smaller P2' residue Gly8 (i.e., tBu-Gly8) can potentially disrupt the interaction between CNP and the S2' subsite.

Accordingly, on one embodiment, CNP variant of the disclosure include CNP having an increase in the size of the P1'-P2' residues, such as Phe7-Gly8, to interfere with substrate recognition at the active site, thereby reducing susceptibility to NEP cleavage. Natural amino acids, unnatural amino acids and/or peptidomimetic moieties are substituted for one or more large P1' hydrophobic residues, including but not limited to Phe7, Leu9, Leu11, Ile14, Met17 and Leu20, and/or for one or more smaller P2' residues, including but not limited to Cys6, Gly8, Gly15, Ser16 and Gly19.

The disclosure encompasses CNP variants comprising at least one modified amino acid and/or at least one modified peptide bond, at at least one residue involved in substrate recognition and/or cleavage by NEP, wherein the modified amino acids and modified peptide bonds can be natural amino acids, unnatural amino acids, peptidomimetics and/or peptide bond isosteres. In one embodiment, the NEP cleavage site on CNP between Cys6 and Phe7 is modified. In a related embodiment, the peptide bond (—C(═O)—NH—) between Cys6 and Phe7 is replaced with one of the following peptide bond isosteres:

—CH_2—NH—,

—C(═O)—N(R)—, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

—C(═O)—NH—CH_2,

—CH_2—S—,

—CH_2—$S(O)_n$—, where n is 1 or 2,

—CH_2—CH_2,

—CH═CH—,

—C(═O)—CH_2—,

—CH(CN)—NH—,

—CH(OH)—CH_2—,

—O—C(═O)—NH—, or
—NHC(═O)NH—.

In another embodiment, the CNP variants are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 90), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin, and sialoprotein (Wang et al., Adv. Drug Delivery Rev., 57: 1049-76 (2005)); polymeric and non-polymeric molecules that reduce renal clearance such as, e.g., negatively charged PEGs; and natural polymers (e.g., those containing amino acids, fatty acids and/or carbohydrates) and synthetic polymers (e.g., PEGs) that increase resistance of the CNP variant to NEP degradation by increasing the total mass of the CNP variant to the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa;

(b) and (c) may be the wild-type Cys6 and Phe7, another natural amino acid or an unnatural amino acid, or may contain a peptide bond isostere as described herein to increase resistance to NEP cleavage; and

(d) may be the wild-type Gly8, or may be a larger natural or unnatural (e.g., t-Bu-Gly) amino acid or peptidomimetic to reduce binding to NEP.

In one embodiment, such CNP variants contain at least one modified amino acid at (b), (c) and/or (d).

Other peptide bonds within CNP may be cleaved even if CNP22 or a variant thereof has an NEP-resistant peptide bond or peptide bond isostere at Cys6-Phe7, including the Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17, and Gly19-eu20 bonds. Therefore, the disclosure encompasses CNP variants having peptide bond isostere(s) at one or more other NEP cleavages sites in addition to the Cys6-Phe7 bond, wherein the peptide bond isosteres include those described herein.

In another embodiment, the disclosure encompasses CNP variants having a cysteine analog at Cys6 and/or Cys22, including but not limited to homocysteine, penicillamine, 2-mercaptopropionic acid, and 3-mercaptopropionic acid. In an embodiment, such CNP variants have a cyclic domain formed by a disulfide bond between the wild-type Cys6 or analog and Cys22 or analog.

In yet another embodiment, one or more residues of CNP22 or a variant thereof, up to all the residues, are substitoted with a D-amino acid. Substitution of an L-amino acid with a D-amino acid essentially moves the side chain about 120 degrees from its original position, thereby potentially disrupting the binding of the CNP peptide to NEP. In a specific embodiment, L-Phe at Phe7 is substituted with its D-enantiomer, D-Phe.

In still another embodiment, a beta amino acid such as, e.g., 3-amino-2-phenylpropionic acid (or 2-phenyl-beta-alanine), replaces the wild-type alpha-amino acid Phe7. Use of a betaamino acid effectively increases the peptide backbone length by one methylene unit. Protease resistance can result from the change in substrate conformation or the increased distance between amino acid side chains.

Non-limiting examples of variants of CNP22 having an unnatural alpha-amino acid, a beta-amino acid or a peptide bond isostere include:

(SEQ ID NO: 56)
GLSKGC(CH_2NH)FGLKLDRIGSMSGLGC (Analog A),

(SEQ ID NO: 57)
GLSKGC-(N-Me-Phe)-GLKLDRIGSMSGLGC (Analog B),

(SEQ ID NO: 136)
GLSKGC-(D-Phe)-GLKLDRIGSMSGLGC (Analog E),

(SEQ ID NO: 58)
GLSKGCF-(tBu-Gly)-LKLDRIGSMSGLGC (Analog F),

(SEQ ID NO: 137)
GLSKGC-(3-Cl-Phe)-GLKLDRIGSMSGLGC (Analog G), and

(SEQ ID NO: 59)
GLSKGC-[NHCH$_2$CH(Ph)CO]-GLKLDRIGSMSGLGC (Analog H, formed using 3-amino-2-phenylpropionic acid).

In a further embodiment, the CNP variants have a total so mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 46), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting com-pounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin, and sialoprotein; polymeric and non-polymeric moieties that reduce renal clearance such as, e.g., negatively charged PEGs; polymers containing, e.g., amino acids, hydrophobic acids, and/or carbohydrates; and synthetic hydrophilic polymers such as, e.g., PEGs;

(a) may be the wild-type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide-bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, heterocyclyl, C_{6-14} aryl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3 -chloro-5 -fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly, and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In another embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP cleavage, and are represented by the formula:

(x)-Gly_1-Leu_2-Se_3-(a)$_4$$Gly_5$-(b)$_6(c)_7$-(d)$_8$-(e)$_9$-(f)$_{10}$-(g)$_{11}$-$Asp_{12}$-$Arg_{13}$-(h)$_{14}$-(i)$_{15}$-$Ser_{16}$-(j)$_{17}$-$Ser_{18}$-$Gly_{19}$-(k)$_{20}$-$Gly_{21}$-$Cys_{22}$-(z) (SEQ ID NO:143), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins and derivatives thereof, such as, e.g., fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; moieties that reduce renal clearance, including but not limited to hydrophilic or water-soluble polymers such as, e.g., charged PEG molecules; and moieities comprising, e.g., PEGs, amino acids, carbohydrates, and/or hydrophobic acids;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chlorophenylalanine, 3 -chloro-5 -fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres such as, e.g., N-Me-Leu;

(f) is selected from the group consisting of Lys, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln and Ser;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres such as, e.g., N-Me-Leu.

To improve the delivery of the CNP variants to the target sites of bone-related disorders (e.g., skeletal dysplasias), the CNP variants can be attached (e.g., at the N-terminus and/or C-terminus) to bone- or cartilage-targeting moieties. Non-limiting examples of bone- or cartilage-targeting moieties include bisphosphonates; hydroxyapatite; glucosamine; collagen (e.g., collagen type X); polyAsp; polyGlu; and amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteocrin, osteopontin, osteocalcin, and sialoprotein.

In addition to being less susceptible to NEP cleavage, the CNP variants potentially have reduced affinity to the NPR-C clearance receptor, while retaining CNP functionality. Besides NEP-mediated degradation, the half-life of CNP22 is influenced by the clearance receptor, NPR-C, which shares 58% sequence homology with the extracellular peptide-binding domain of NPR-B. CNP22 binds tightly to not only NPR-B (7-30 pM affinity), but also NPR-C (11-140 pM) (Bennett, B. D. et al., J. Biol. Chem., 266: 23060-67 (1991); Koller K. J. & Goeddel, D. V., Circulation, 86: 1081-88 (1992); Suga, S. et al., Endocrinology, 130: 229-39 (1992)). Even though the NPR-B crystal structure has yet to be reported, sequence homology as well as similarities between the NPR-C and NPR-A crystal structures (He, X.-L. et al., Science, 293(5535): 1657-62 (2001); Ogawa, H. et al., J. Biol. Chem., 279(27): 28625-31 (2004); He, X.-L., J. Mol. Biol., 361(4): 698-714 (2006)) suggest that NPR-B likely assumes a similar overall structural fold.

Therefore, an NPR-B homology model was built based on structure-based sequence alignment and crystallographic structures of the following related systems: CNP bound to NPR-C, ANP bound to NPR-A, and ANP bound to NPR-C (He, X.-L. et al., Science, 293(5535): 1657-62 (2001); Ogawa, H. et al., J. Biol. Chem., 279(27): 28625-31 (2004); He, X.-L., J. Mol. Biol., 361(4): 698-714 (2006)). Based on observations that the receptor appears to determine the bound peptide conformation, and that NPR-B most closely resembles NPR-A with respect to primary structure and functional properties, the NPR-B/CNP homology model was built with the NPR-A/ANP crystal structure as a model. Published signaling data of CNP variants (U.S. Pat. No. 5,434,133 and US Patent Application Publication No. 2004/0138134 A1), and of functional ANP variants that no longer bind to NPR-C (Cunningham, EMBO 13(11) 2508-15, 1994) were used to refine and interpret the NPR-B/CNP model.

The present disclosure encompasses CNP variants designed for improved NPR-B selectivity based on a homology-based structural model of the NPR-B/CNP complex. Combining the experimental and computational structure data of natriuretic peptides bound to the various receptors with the published functional data, CNP variants were generated that continue to bind to NPR-B, but can potentially have reduced affinity to the NPR-C clearance receptor. For example, NPR-C has a unique insertion in a loop structure in the peptide-binding site, placing its loop residues closer to such peptide residues as CNP Gly8 (orANP Gly9), compared to respective loop residues in NPR-A and NPR-B. Earlier studies indicated that the G9T mutation in ANP contributes to reduce affinity to NPR-C, thereby improving NPR-A selectivity (Cunningham, EMBO J., 13(11): 2508-15 (1994)). Accordingly, CNP variants were generated to replace the corresponding Gly8 residue with a larger residue (Ser, Val, Thr or Asn) to disrupt the CNP binding to NPR-C without affecting its binding to NPR-B. Further, one or more mutations were introduced at the C-terminal end of CNP, encompassing Gly15 to Gly21, which is predicted to interact with receptor-specific residues, based on the detailed structural analyses of the receptor/peptide complexes. For example, a G19R mutation in CNP22 does not result in a significant loss of NPR-B signaling activity. This mutation, however, cannot be modeled into the available crystal structure of NPR-C/CNP without altering the conformations of neighboring residues. These observations suggest that the G19R mutation may selectively disrupt the binding of CNP to a particular receptor, such as NPR-C.

In an embodiment, the CNP variants have substitution(s) at one or more Gly sites at positions 1, 5, 8, 15, 19 and 21, to reduce conformational flexibility and thereby increase receptor specificity. Comparative analyses of crystal structures of ANP bound to NPR-C and NPR-A (Ogawa, H. et al., J. Biol. Chem., 279: 28625-31 (2004); He, X.-L., J. Mol. Biol., 361: 698-714 (2006)) indicate that the conformational flexibility of ANP may play an important role in determining the receptor selectivity.

In one embodiment, functional CNP variants with potentially reduced affinity to NPR-C have one or more of the following amino acid substitutions: G1R, G1E, G5R, G5Q, G5S, F7Y, G8T, G8S, G8V, G8N, L9S, L9T, K10Cit, K10Q, K10S, 114N, G15R, G15S, G15N, G15Cit, S16Q, M17V, M17N, G19S, G19R, G19N, L20V, L20R, L20T, L20S, G21S, G21T and G21R. In an embodiment, the CNP variants have multipoint substitutions at positions 1, 5, 7, 8, 9, 10, 14, 15, 16, 17, 19, 20 and/or 21, and may optionally have modifications at any of the other positions in the peptide sequence of the variant.

In a further embodiment, the CNP variants described herein may be conjugated to moieties, up to a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, at the N-terminus, the C-terminus and/or an internal site, to facilitate bone/cartilage targeting, reduce NPR-C and renal clearance, increase resistance to NEP degradation, and/or improve CNP functionality. In one embodiment, the CNP variants are not conjugated to a polymeric moiety at a site within the cyclic region (corresponding to Cys6 to Cys22 of CNP22). Non-limiting examples of polymeric or non-polymeric moieties that can be conjugated to the CNP variants include synthetic e-targeting compounds such as, e.g., bisphosphonates; bone/cartilage targeting peptide sequences such as, e.g., pol-yAsp and polyGlu; peptide sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin and sialoprotein; peptide sequences derived from the functional domains of bone morphogenetic proteins such as, e.g., BMP2, BMP3, BMPS, BMP7 and BMP8a; peptide sequences derived from polypeptides of natriuretic origin such as, e.g., NPPC, ANP and BNP; other natural polymeric or non-polymeric moieties such as, e.g., carbohydrates, fatty acids and phospholipids; biocompatible synthetic hydrophilic polymers such as, e.g., PEG (or PEO); hydrophobic polymeric or non-polymeric moieties such as, e.g., heptanoic acid and pentanoic acid; and combinations thereof.

The CNP variants described herein can have substantially similar or better functional activity than CNP22, e.g., with respect to stimulation of cGMP production and signaling. In one embodiment, the CNP variants in vitro or in vivo stimulate the production of at least about 50% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM). In certain embodiments, the CNP variants retain at least about 50%, 60%, 70%, 80%, 90%, 95% or 100% of the cGMP-stimulation activity of wild-type CNP22 in vitro or in vivo. In another embodiment, the CNP variants have improved cGMP- stimulation activity compared to CNP22. In certain embodiments, the CNP variants in vitro or in vivo stimulate the production of at least about 110%, 120%, 130%, 140%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500% or more of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM).

Optionally excluded from the present disclosure are any of the natriuretic (e.g., CNP) peptides, fragments and variants specifically disclosed, and any of the natriuretic (e.g., CNP) peptides, fragments and variants actually produced, in any of the prior publications referenced herein, including but not limited to, U.S. Pat. Nos. 5,434,133, 6,034,231, 6,020,168, 6,743,425, 7,276,481, WO 94/20534, WO 02/047871, WO 2005/098490, WO 2004/047871, EP 0497368, EP 0466174, and Furuya et al., Biochem. Biophys. Res. Comm. 183: 964-969 (1992)). All such documents are incorporated by reference herein in their entirety.

In one embodiment, the present disclosure optionally excludes all known wild-type CNP-53, wild-type CNP-22, wild-type CNP-17, wild-type BNP, and wild-type ANP of human origin and non-human origin. For example, in an embodiment the disclosure optionally excludes human CNP-17, human CNP-22, chicken CNP-22 (corresponding to hCNP-22(Leu9Val)), trout and eel CNP-22 (corresponding to hCNP-22(Leu2Trp, Ser3Asn, Lys4Arg)), frog CNP22-I (corresponding to hCNP-22(Leu2Tyr, Lys4Arg, Leu9Val, Serl6Ala, Met17Phe)), frog CNP22-II (corresponding to hCNP-22(Leu2Thr, Ser16Ala)), human CNP-53, and pig and rat CNP-53 (corresponding to hCNP-53 (Gln17His, Ala28Gly)). In another embodiment, the disclosure optionally excludes fragments of NPPC, proCNP and CNP-53 which are produced by proteolytic cleavage in vivo in humans and non-human animals. In yet another embodiment, optionally excluded from the disclosure are the following truncated fragments of wild-type human CNP-53: CNP-50, CNP-46, CNP-44, CNP-39, CNP-30, CNP-29, CNP-28, CNP-27 and CNP-26.

In a further embodiment, the present disclosure optionally excludes CNP peptides and fragments thereof isolated or sought from the shark species Triakis scyllia and Scyliorhinus canicula (see, e.g., M. Takano et al., Zool. Sci., 11: 451-454 (1994)), including:

(SEQ ID NO: 204)
RLLKDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC
(CNP-41);

(SEQ ID NO: 205)
LKDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-39);

(SEQ ID NO: 206)
KDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-38);
and

(SEQ ID NO: 207)
GPSRGCFGVKLDRIGAMSGLGC (CNP-22).

In another embodiment, optionally excluded from the disclosure are CNP peptides and fragments thereof isolated or sought from the shark species Lamna ditropis (see, e.g., M. Takano et al., Zool. Sci., 11: 451-454 (1994)), including:

(SEQ ID NO: 208)
RLLKDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC
(CNP-41);

(SEQ ID NO: 209)
LKDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-39);

(SEQ ID NO: 210)
KDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-38);

(SEQ ID NO: 211)
FKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-29);
and

(SEQ ID NO: 212)
GPSRGCFGVKLDRIGAMSGLGC (CNP-22).

In still another embodiment, optionally excluded from the disclosure are CNP peptides and fragments thereof isolated from the shark species Squalus acanthias (see, e.g., M. Takano et al., Zool. Sci., 11: 451-454 (1994)), including:

(SEQ ID NO: 213)
RLLQDLSNNPLRFKGRSKKGPSRSCFGLKLDRIGAMSGLGC
(CNP-41);
and

(SEQ ID NO: 214)
GPSRSCFGLKLDRIGAMSGLGC (CNP-22).

In a further embodiment, the present disclosure optionally excludes CNP peptides isolated from medaka and puffer fish, designated "CNP-1", "CNP-2", "CNP-3" and "CNP-4" in K. Inoue et al., Proc. Nat. Acad. Sci., 100(17): 10079-10084 2003):

(SEQ ID NO: 215)
GWNRGCFGLKLDRIGSMSGLGC (medaka and puffer fish
CNP-1);

(SEQ ID NO: 216)
PMVAGGGCFGMKMDRIGSISGLGC (medaka CNP-2);

(SEQ ID NO: 217)
GRSSMVGGRGCFGMKIDRIGSISGLGC (puffer fish CNP-2);

(SEQ ID NO: 218)
GGMRSCFGVRLERIGSFSGLGC (medaka CNP-3);

(SEQ ID NO: 219)
GGLRSCFGVRLARIGSFSGLGC (puffer fish CNP-3);

(SEQ ID NO: 220)
GGSTSRSGCFGHKMDRIGTISGMGC (medaka CNP-4);
and

-continued

(SEQ ID NO: 221)
GGSSRSGCFGHKMDRIGTISGMGC (puffer fish CNP-4).

In a still further embodiment, optionally excluded from the disclosure are CNP-39 isolated from platypus venom and the CNP-22 fragment thereof, designated "ovCNP-39" and "ovCNP-39(18-39)" in G. de Plater et al., Toxicon., 36(6): 847-857 (1998):

(SEQ ID NO: 222)
LLHDHPNPRKYKPANKKGLSKGCFGLKLDRIGSTSGLGC
(ovCNP-39);
and

(SEQ ID NO: 223)
GLSKGCFGLKLDRIGSTSGLGC (ovCNP-39(18-39).

In another embodiment, the present disclosure optionally excludes the following peptides as specifically disclosed in US 2007/0197434:

(SEQ ID NO: 224)
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-
Arg-Ile-Gly-Ala-Met-Ser-Gly-Leu-Gly-Cys;

(SEQ ID NO: 225)
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-
Arg-Ile-Gly-Ser-Gln-Ser-Gly-Leu-Gly-Cys;

(SEQ ID NO: 226)
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-
Arg-Ile-Gly-Ser-Ala-Ser-Gly-Leu-Gly-Cys;

(SEQ ID NO: 227)
Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Leu-Lys-Leu-
Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys;

(SEQ ID NO: 228)
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-
Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-Asn-Ser-
Phe-Arg-Tyr;
and

(SEQ ID NO: 229)
Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Gln-
Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr.

In yet another embodiment, the disclosure optionally excludes peptides of SEQ ID NO:10, disclosed generically in US 2007/0197434, wherein such peptides are CNP-17 variants having certain natural amino acid substitution(s) at position(s) 4, 5, 6, 11, 12, 14 and/or 15. In still another embodiment, optionally excluded from the disclosure are peptides corresponding to hCNP-53(Ser47Ala), hCNP-53 (Met48Gln), hCNP-53(Met48Ala), and hCNP-53(C-term.)-Asn-Ser-Phe-Arg-Tyr.

In an embodiment, the present disclosure optionally excludes the peptides of SEQ ID NOs 1-4 and 6-71 as specifically disclosed in U.S. Pat. No. 7,276,481. In another embodiment, the disclosure optionally excludes peptides of SEQ ID NO 5, disclosed generically in U.S. Pat. No. 7,276,481, wherein such peptides are variants of CNP17 having at least one natural amino acid substitution at Leu9, Lys10, Leu11, Ser16, Met17, Gly19, and/or Leu20. In still another embodiment, optionally excluded are CNP17 variants in which CNP17 or variants thereof contain N-Me-Phe7, or N-Me-Phe7 and N-Me-Leu11. In a further embodiment, the disclosure optionally excludes CNP17 variants of SEQ ID NO 5, as disclosed in U.S. Pat. No. 7,276,481, which are fused or conjugated to growth hormone (GH), insulin-like growth factor 1 (IGF-1), or thyroid hormone

(TH). In yet another embodiment, optionally excluded are CNP22 variants in which CNP22 is fused to GH, IGF-1 or TH, or attached to GH, IGF-1 or TH via a linker (e.g., a peptide linker). In still another embodiment, optionally excluded are CNP17 variants in which CNP17 or variants thereof are conjugated to biotin or fluorescein at the N-terminus or the C-terminus.

In a further embodiment, the present disclosure optionally excludes the peptides of Compound Nos. 1-27, and SEQ ID NOs 1-17, 22-24, 30, 31 and 40-42 as specifically disclosed in U.S. Pat. No. 5,434,133. In another embodiment, the disclosure optionally excludes peptides of SEQ ID NOs 18-21 and 25-29, disclosed generically in U.S. Pat. No. 5,434,133. In still another embodiment, the disclosure optionally excludes the peptides of SEQ ID NOs 1-4 and 9 as specifically disclosed in WO 94/20534.

In some embodiments, however, the disclosure still encompasses methods of use of the natriuretic (e.g., CNP) peptides, fragments and variants optionally excluded herein, as well as pharmaceutical compositions (including sterile pharmaceutical compositions) comprising such natriuretic (e.g., CNP) peptides, fragments and variants.

C. Synthesis and Purification of CNP Variants

In some embodiments, the CNP variants described herein are produced by recombinant expression, using certain techniques known in the art in certain embodiments. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y. (1989)); DNA Cloning: A Practical Approach, Volumes I and II, D. N. Glover, Ed. (1985); and Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

In certain embodiments, the CNP variants described herein are produced by a recombinant process that comprises culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant polypeptide linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant polypeptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker. In some embodiments, the host cell is transformed with an expression vector comprising the polynucleotide encoding the CNP variant polypeptide linked to the polynucleotide encoding the cleavable peptide or protein. In certain embodiments, the fusion polypeptide is expressed as a soluble protein or as an inclusion body. The expressed fusion polypeptide can be isolated from the host cell or culture medium, and the isolated fusion polypeptide can be contacted with a cleaving agent to release the CNP variant.

Host cells used to produce CNP variants can be bacterial, yeast, insect, non-mammalian vertebrate, or mammalian cells. Bacterial cells include without limitation E. coli cell lines and strains. Non-limiting examples of E. coli cell lines and strains include BL21, BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41 [also called C41(DE3)], C43 [also called C43(DE3)], Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3). In an embodiment, CNP variants and CNP fusion proteins are produced using BL21(DE3) cells. Mammalian cells include, but are not limited to, hamster, monkey, chimpanzee, dog, cat, bovine, porcine, mouse, rat, rabbit, sheep and human cells. The host cells can be immortalized cells (a cell line) or non-immortalized (primary or secondary) cells and can be any of a wide variety of cell types, such as, but not limited to, fibroblasts, keratinocytes, epithelial cells (e g , mammary epithelial cells, intestinal epithelial cells), ovary cells (e.g., Chinese hamster ovary or CHO cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), chondrocytes and other bone-derived cells, and precursors of these somatic cell types. Host cells containing the CNP variant DNA or RNA are cultured under conditions appropriate for growth of the cells, expression of the DNA or RNA and identification/selection of cells expressing the CNP variant.

In some embodiments, the host cells are grown or cultured for a time period at a temperature from about 10° C. to about 40° C., or from about 20° C. to about 40° C., or from about 30° C. to about 40° C. In certain embodiments, the host cells are grown or cultured for a time period at about 20° C., 22° C., 25° C., 28° C., 30° C., 35° C. or 37° C. In certain embodiments, the host cells are grown or cultured for a time period at about 35° C. or 37° C.

Recombinant polynucleotides encoding CNP variant polypeptides (including CNP fusion proteins) are expressed in an expression vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in vitro expression system. Expression vectors include all those known in the art, including without limitation cosmids, plasmids (e.g., naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide. The expression vector is inserted into an appropriate host cell, via transformation or transfection, for expression of the polynucleotide encoding the polypeptide (see, e.g., Sambrook et al. (supra)).

Non-limiting examples of expression vectors contemplated for production of CNP variants, including cleavable CNP fusion proteins, include pJexpress, pJexpress401, pJexpress404, pET-15b, pET-21a, pET-22b, pET-31b, pET-32a, pET-41a, pMAL, pMAL-c2X, pQE-30, pET-SUMO, and pTYB11. Expression of particular constructs can generate soluble CNP variants (including CNP fusion proteins) or insoluble CNP variants (including CNP fusion proteins) in the form of inclusion bodies.

In some embodiments, expression of the polynucleotide(s) encoding a CNP variant or CNP fusion protein is enhanced using an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible vector. In some embodiments, the host cells are grown or cultured for a time period at a temperature from about 10° C. to about 40° C., or from about 20° C. to about 40° C., or from about 30° C. to about 40° C., in the presence of IPTG. In certain embodiments, the host cells are grown or cultured for a time period at about 20° C., 22° C., 25° C., 28° C., 30° C., 35° C. or 37° C. in the presence of IPTG. In certain embodiments, the host cells are grown or cultured for a time period at about 35° C. or 37° C. in the presence of 1 mM IPTG.

In further embodiments, the host cells are cultured with IPTG at a concentration from about 0.4 mM to about 2 mM, or from about 0.4 mM to about 1.5 mM, or from about 0.4 mM to about 1 mM. In certain embodiments, the IPTG is at a concentration of about 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9 or 2 mM. In an embodiment, the concentration of IPTG is about 1 mM.

In certain embodiments, the CNP variants described herein are recombinantly expressed as fusion proteins comprising a CNP variant polypeptide and a cleavable carrier protein or cleavable tag (e.g., peptide tag), wherein the

fusion protein comprises the CNP variant polypeptide directly linked to the cleavable carrier protein or tag or indirectly linked thereto via a linker. Use of a carrier protein or tag facilitates, e.g., detection, isolation and/or purification of the fusion protein. Cleavable carrier proteins and tags include, but are not limited to, histidine (e.g., hexa-His) tags; human transcription factor TAF12 (TAF12), TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12 (10D/10E), TAF12(C/A & D/E), TAF12C/A & 4D/4E), TAF12(C/A & 6D/6E), TAF12 (C/A & 10D/10E); ketosteroid isomerase (KSI); maltose-binding protein (MBP); β-galactosidase (β-Gal); glutathione-S-transferase (GST); thioredoxin (Trx); chitin binding domain (CBD); BMP-2, BMP-2 mutants, BMP-2 (C/A); SUMO; and mutants and fragments thereof.

An expression construct can express a fusion protein comprising a CNP variant and a carrier protein or tag. The tag can be an amino acid sequence that confers a useful property to the fusion protein. In one embodiment, the tag is a ligand-binding domain that can be used to purify the fusion protein by applying the fusion protein to separation media containing the ligand. For example, a fusion protein comprising a glutathione-S-transferase (GST) domain can be applied to a chromatographic column containing glutathione-linked separation media. As another example, a fusion protein comprising maltose-binding protein (MBP) as a tag can be applied to separation media containing maltose. As a further example, a fusion protein comprising a polyhistidine tag may be applied to a nickel column, whereby chelation of the polyhistidine tag to the nickel column facilitates purification of the fusion protein. In another embodiment, the tag is a ligand. For example, a fusion protein can comprise glutathione as a tag and be applied to a chromatographic column containing glutathione-S-transferase-linked separation media. Non-limiting examples of carrier proteins and tags for use in fusion proteins include human transcription factor TAF12 (TAF12), ketosteroid isomerase (KSI), maltose-binding protein (MBP), β-galactosidase (β-Gal), glutathione-S-transferase (GST), thioredoxin (Trx), chitin-binding domain (CBD), BMP-2 mutation (BMPM), SUMO, CAT, TrpE, staphylococcal proteinA, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, poly(His), poly(Arg), poly(Asp), poly (Gln), poly (Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and mutants and fragments thereof.

To generate the target CNP variant, the carrier protein or tag can be cleaved from the fusion protein by means of chemical cleavage, protease cleavage, or protein self-cleavage. Exemplary chemical and proteolytic cleavage agents (cleavage sites in parenthesis) include, but are not limited to, formic acid (Asp-Pro), cyanogen bromide (CNBr) (Met-X), hydroxylamine (Asn-Gly), Factor Xa (IEGR-X) (SEQ ID NO: 230), Enterokinase (DDDDK-X) (SEQ ID NO: 231), ProTEV (EXXYXQ-G) (SEQ ID NO: 232), and SUMO protease. Due to the nature of the particular kinds of chemical cleavage, cleavage using formic acid may generate Pro-CNP, CNBr may generate CNP having Met-to-Asn substitution, and hydroxylamine may generate Gly-CNP. Alternatively, chemical or protease cleavage may be avoided by using particular constructs (e.g., pET-21a-CNP) that express CNP variants not as fusion proteins. Expression of pET-21a-CNP may produce Met-CNP. Or certain fusion proteins (e.g., those containing intein-CBD) can undergo self-cleavage to generate CNP.

In further embodiments, a fusion protein comprises a cleavable peptide linker between a CNP variant and a carrier protein or tag (e.g., peptide tag). In certain embodiments, the cleavable peptide linker is selected from the group consisting of Asp-Pro, Asn-Gly, Met-X, Val-Asp-Asp-Arg (SEQ ID NO: 233), Gly-Ser-Asp-Arg (SEQ ID NO: 234), Ile-Thr-Asp-Arg (SEQ ID NO: 235), Pro-Gly-Asp-Arg (SEQ ID NO: 236), Ile-Glu-Gly-Arg-X (SEQ ID NO: 230), Asp-Asp-Asp-Asp-Lys-X (SEQ ID NO: 231), Glu-X-X-Tyr-X-Gln-Gly (SEQ ID NO: 232), Ala-Phe-Leu-Gly-Pro-Gly-Asp-Arg (SEQ ID NO: 237), and MGSSHHHHHHSSGLVPRGSHT-GDDDDKHMD (pET-15b linker) (SEQ ID NO: 95), where X denotes an amino acid. In some embodiments, the cleavable peptide linker is cleaved by a cleaving agent selected from the group consisting of palladium, cyanogen bromide (CNBr), formic acid, hydroxylamine, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase, enterokinase (enteropeptidase), Kex 2 protease, Omp T protease, Factor Xa protease, subtilisin, proTEV, SUMO protease, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K, pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

In certain embodiments, the cleavable carrier protein, tag (e.g., peptide tag) or peptide linker is cleaved using formic acid to release the CNP variant from the fusion protein. In some embodiments, the formic acid is at a concentration from about 1% to about 20%, or from about 1% to about 15%, or from about 2% to about 15%, or from about 1% to about 10%, or from about 2% to about 10%, or from about 1% to about 5%, or from about 2% to about 5%. In certain embodiments, the formic acid is at a concentration of about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14% or 15%. In certain embodiments, the formic acid is at a concentration of about 2%, 5% or 10%.

In further embodiments, cleavage of the CNP fusion protein in the presence of formic acid is conducted at a temperature from about 20° C. to about 80° C., or from about 30° C. to about 75° C., or from about 40° C. to about 75° C., or from about 50° C. to about 75° C., or from about 50° C. to about 70° C., or from about 55° C. to about 70° C., or from about 50° C. to about 60° C. In some embodiments, cleavage in the presence of formic acid is conducted at about 20° C., 22° C., 25° C., 30° C., 35° C., 37° C., 40° C., 42° C., 45° C., 50° C., 55° C., 60° C., 65° C., 70° C., 75° C. or 80° C. In certain embodiments, cleavage in the presence of formic acid is conducted at about 50° C., 55° C., 60° C., 65° C. or 70° C. In certain embodiments, cleavage in the presence of formic acid is conducted at about 55° C. or 70° C.

In additional embodiments, cleavage of the CNP fusion protein in the presence of formic acid is performed for a time period from about 3 hr to about 48 hr, or from about 5 hr to about 48 hr, or from about 5 hr to about 36 hr, or from about 5 hr to about 24 hr, or from about 5 hr to about 18 hr, or from about 20 hr to about 24 hr, or for about 6 hr to about 10 hr. In certain embodiments, cleavage in the presence of formic acid is performed for about 5 hr, 6 hr, 12 hr, 15 hr, 18 hr, 20 hr or 24 hr.

In some embodiments, cleavage of the CNP fusion protein is conducted in the presence of about 2%, 5% or 10% formic acid at about 55° C. for about 20 hr to about 36 hr, or at about 60° C. for about 15 hr to about 24 hr, or at about 65° C. for about 10 hr to about 21 hr, or at about 70° C. for about 6 hr to about 18 hr. In certain embodiments, cleavage of the CNP fusion protein is conducted in the presence of about 2% formic acid at about 55° C. for about 20 hr to about 24 or 36 hr, or at about 60° C. for about 15 hr to about 24 hr, or at about 65° C. for about 10 hr to about 18 hr, or at about 70° C. for about 6 hr to about 10 hr.

The present disclosure provides mild conditions for cleavage of CNP fusion proteins using formic acid to afford high yields of CNP variants. The conditions described herein for fusion protein cleavage using formic acid are also suitable for cleavage of fusion proteins comprising polypeptides or proteins other than CNP, where the fusion proteins contain an Asp-Pro peptide bond.

In further embodiments, soluble CNP fusion proteins or CNP fusion protein inclusion bodies are treated with a buffer and/or a detergent prior to chemical cleavage (e.g., using formic acid) or proteolytic cleavage of the CNP fusion protein. Non-limiting examples of buffers include B-PER II; diluted B-PER II (e.g., 1/20 dilution); B-PER; B-PER phosphate buffer; buffers containing Tris (e.g., 25 mM Tris, pH 7.5); buffers containing Tris and NaCl (e.g., 25 mM Tris, 150 mM NaCl, pH 7.9); and PBS. In an embodiment, the buffer is B-PER II. Non-limiting examples of detergents include octylsucrose, Triton X-100, Tween-20, NP-40, and CA-630. The detergent can be in a buffer (e.g., 1% detergent in 25 mM Tris buffer, pH 7.5). In certain embodiments, the detergent is Triton X-100 or CA-630.

It is understood that any of the methods and conditions described above may be used in combination with any of the other methods and conditions described above to generate a CNP variant disclosed herein.

In other embodiments, the CNP variants described herein are synthesized using a peptide synthesizer and purified according to methods known in the art, e.g., according to the methods of Atherton and Sheppard, Solid Phase Peptide Synthesis: a Practical Approach, IRL Press (Oxford, England (1989)).

Peptides can be synthesized based on, e.g., the following peptide sequence of CNP: G^1LS(K or R)$GC^6F^7G^8L$(K or R or Nle or 6-OH-Nle)$LDRIGSMSGLGC^{22}$.

Exemplary CNP variants include but are not limited to:

Analog A (GLSKGC(CH2NH)FGLKLDRIGSMSGLGC) (SEQ ID NO: 56) was made by converting the backbone "—C═O" group of C^6 to a "—CH_2" group;

Analog B (GLSKGC(N—Me—Phe)GLKLDRIGSMSGLGC) (SEQ ID NO: 57) was made by converting the backbone "—NH" group of F^7 to an "—N—CH_3" group;

Analog E (GLSKGC(D-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO: 136) was made using D-Phe at F^7;

Analog F (GLSKGCF(tBu-Gly)LKLDRIGSMSGLGC) (SEQ ID NO: 58) was made using a tertiary-butyl-Gly at G^8;

Analog G (GLSKGC(3-Cl-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO: 137) was made by adding a chloride atom to a meta position of the phenyl ring of F^7 (similar variants can be generated by making ortho, meta and/or para substitutions of the phenyl ring of Phe7 with Cl, F, Br, OH and/or CH_3);

-continued

and

Analog H (GLSKGC[$NHCH_2CH$(Ph)CO]GLKLDRIGSMSGLGC) (SEQ ID NO: 59) was made using (±)-3-(amino)-2-phenylpropionic acid at F^7.

Examples of CNP variants having, e.g., amino acid extensions, substitutions with natural or unnatural amino acids or peptide bond isosteres, and/or conjugations to polymers or hydrophobic moieties, include without limitation:

Analog J (SEQ ID NO: 91)
C6—CH2—NH, N—Me-L9, N—Me-L20

Analog K (SEQ ID NO: 92)
N—Me-L9, N—Me-L20

Analog L (SEQ ID NO: 93)
N—Me-L9, N—Me-L11, N—Me-L20

Analog M (SEQ ID NO: 94)
N—Me-L9, N—Me-L11

Analog Z (SEQ ID NO: 95)
K4R, F7Y

Analog AA (SEQ ID NO: 96)
K4R, G8V

Analog AB (SEQ ID NO: 97)
K4R, G8S

Analog AC (SEQ ID NO: 98)
K4R, G8T

Analog AD (SEQ ID NO: 99)
K4R, L9T

Analog AE (SEQ ID NO: 100)
K4R, G15R

Analog AF (SEQ ID NO: 101)
K4R, G15Cit

Analog AG (SEQ ID NO: 102)
K4R, M17V

Analog AH (SEQ ID NO: 35)
K4R

Analog AJ (SEQ ID NO: 103)
K4R, L20V

Analog AK (SEQ ID NO: 104)
K4R, L20t-Bu-Ala

Analog AT (SEQ ID NO: 105)
G1E, K4E

-continued

Analog AV
(SEQ ID NO: 106)
G1E, K4E - pentanoic acid (attached at the N-terminus)

Analog AW
(SEQ ID NO: 107)
G1E, K4E - heptanoic acid (attached at the N-terminus)

Analog AX
(SEQ ID NO: 2)
CNP17 (delta N-term)

Analog AY
(SEQ ID NO: 36)
GANRR-CNP22(K4R)

Analog AZ
(SEQ ID NO: 41)
R-CNP22(K4R)

Analog BB
(SEQ ID NO: 108)
G1E - heptanoic acid (attached at the N-terminus)

Analog BC
(SEQ ID NO: 109)
G1E - pentanoic acid (attached at the N-terminus)

Analog BF
(SEQ ID NO: 110)
K4R, K10Cit

Analog BG
(SEQ ID NO: 111)
K4R, K10Q

Analog BH
(SEQ ID NO: 112)
K4R, K10R

Analog BJ
(SEQ ID NO: 113)
K4R, G15N

Analog BK
(SEQ ID NO: 114)
K4R, G15S

Analog BL
(SEQ ID NO: 60)
CNP-37
(SEQ ID NO: 4)
CNP-53

Analog CA
(SEQ ID NO: 61)
AAWARLLQEHPNA-CNP22

Analog CB
(SEQ ID NO: 62)
AAWARLLQEHPNAR-CNP22

Analog CC
(SEQ ID NO: 63)
DLRVDTKSRAAWAR-CNP22

Analog CD
(SEQ ID NO: 68)
SPKMVQGSG-CNP17-KVLRRH (N- and C-terminal BNP tails)

Analog CE
(SEQ ID NO: 81)
GERAFKAWAVARLSQ-CNP22 (HSA-CNP22)

-continued

Analog CF
(SEQ ID NO: 79)
GQPREPQVYTLPPS-CNP22
PEG(24K)-CNP22
PEG(20K)-CNP22
PEG(5K)-CNP22
PEG(2K)-CNP22
PEG(2K)-CNP17

(SEQ ID NO: 36)
PEG(1K)-GANRR-CNP22(K4R)
PEG(1K)-CNP22
PEO4-(PEO12)3(branched)-CNP22
PEO12-CNP22

(SEQ ID NO: 36)
PEO12-GANRR-CNP22(K4R)

(SEQ ID NO: 36)
PEO24-GANRR-CNP22(K4R);
and

SEQ ID NOs: 1 to 6 and 34 to 144, and variants thereof that comprise up to 1, 2, 3, 4, or 5 further modifications.

In one embodiment, the CNP variants are cyclized via formation of a disulfide bond between Cys^6 and Cys^{22}. Cys^6 can be a cysteine analog such as, e.g., homocysteine or penicillamine. In a further embodiment, the CNP variants can be cyclized by a covalent bond formed head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail. In an embodiment, the covalent bond is formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus of the peptide (referred to as "terminal" amino acids in this context). In another embodiment, the covalent bond is formed between the side chains of the two terminal amino acids. In yet another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of the two terminal amino acids.

Head-to-tail cyclization of the terminal amine to the terminal carboxyl group can be carried out using a number of methods, e.g., using p-nitrophenyl ester, 2,4,5-trichlorophenyl ester, pentafluorophenyl ester, the azide method, the mixed anhydride method, HATU, a carbodimide (e.g., DIC, EDC or DCC) with a catalyst such as HOBt, HONSu or HOAt, or on-resin cyclization.

In addition, the cyclic structure can be formed via a bridging group involving the side chains of amino acid residues of the CNP variant and/or the terminal amino acid residues. A bridging group is a chemical moiety that allows cyclization of two portions of the peptide. Non-limiting examples of bridging groups include amides, thioethers, thioesters, disulfides, ureas, carbamates, sulfonamides, and the like. A variety of methods are known in the art for incorporation of units having such bridging groups. For example, a lactam bridge (i.e., a cyclic amide) can be formed between the N-terminal amino group or an amino group on a side chain and the C-terminal carboxylic acid or a carboxyl group on a side chain, e.g., the side chain of lysine or ornithine and the side chain of glutamic acid or aspartic acid. A thioester can be formed between the C-terminal carboxyl group or a carboxyl group on a side chain and the thiol group on the side chain of cysteine or a cysteine analog.

Alternatively, a cross link can be formed by incorporating a lanthionine (thio-dialanine) residue to link alanine residues that are covalently bonded together by a thioether bond. In another method, a cross-linking agent, such as a dicarbox-

ylic acid (e.g., suberic acid (octanedioic acid)), can link the functional groups of amino acid side chains, such as free amino, hydroxyl, and thiol groups.

Enzyme-catalyzed cyclization can also be used. For example, it has been reported that the thioesterase domain of tyrocidine synthetase can be used to cyclize a thioester precursor, a subtilisin mutant can be utilized to cyclize peptide glycolate phenylalanylamide esters, and the antibody ligase 16G3 can be employed to cyclize a p-nitrophenylester. For a review of peptide cyclization, see Davies, J. Peptide Sci., 9: 471-501 (2003), incorporated herein by reference in its entirety.

In certain embodiments, the final cyclized product has a purity of at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least about 99%.

D. Chemically Modified CNP Variants

Chemical modification of CNP22 or variants thereof can potentially impart advantageous properties to the modified CNP peptides, such as increased stability and half-life and reduced immunogenicity (for a general discussion of chemical modification of therapeutic proteins, see Pharmazie, 57(1): 5-29 (2002)). For example, attaching natural or synthetic, polymeric or non-polymeric moieties (e.g., PEG) to CNP peptides, to increase the total mass of the CNP peptides to the ranges described generally herein, e.g., a range from about 2.6 or 2.8 kDa to about 6 or 7 kDa, can reduce the susceptibility of the modified peptides to in vivo cleavage by exopeptidases and/or endopeptidases (e.g., NEP). In addition to PEGylation, glycosylation and other chemical derivatization procedures, e.g., modification by phosphorylation, amidation, carboxylation, acetylation, methylation, and creation of acid-addition salts, amides, esters and N-acyl derivatives, may also mask potentially immunogenic regions and/or proteolytically sensitive regions (Science, 303: 480-482 (2004)).

Examples of chemical modifications include, without limitation, the polymer addition method of Bednarsaki and the cross-linking method of Altus Corporation for improving stability and protease resistance and reducing immunogenicity. Bednarsaki showed that polymer addition can improve protein temperature stability (J. Am. Chem. Soc., 114(1): 378-380 (1992)), and Altus Corporation found that glutaraldehyde cross-linking can improve enzyme stability.

Chemical modification of polypeptides can be performed in a non-specific fashion (leading to mixtures of derivatized species) or in a site-specific fashion (e.g., based on wild-type macromolecule reactivity-directed derivatization and/or site-selective modification using a combination of site-directed mutagenesis and chemical modification) or, alternatively, using expressed protein ligation methods (Curr. Opin. Biotechnol., 13(4): 297-303 (2002)).

Pegylated CNP Variants

In one embodiment, for increased stability (e.g., resistance to NEP degradation), CNP22 or variants thereof (including those having amino acid additions, substitutions and/or deletions) are conjugated to hydrophilic, natural or synthetic polymers, to increase the total mass of the modified CNP peptides to a range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6, 7 or higher kDa. In certain embodiments, the added hydrophilic polymers have a total mass of about 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8, or about 5 kDa.

In an embodiment, the hydrophilic polymers are water-soluble so that the CNP peptides conjugated thereto do not precipitate out in an aqueous (e.g., physiological) environment. Further, the hydrophilic polymers are biocompatible, i.e., do not cause injury, toxicity or an immunological reaction in vivo.

The hydrophilic polymers can be branched or unbranched.

In one embodiment, the hydrophilic polymers are not branched.

Various sites of conjugation of CNP22 or variants thereof to a hydrophilic polymer are possible, including but not limited to: (1) only at the N-terminus; (2) only at the C-terminus; (3) only at an internal site (e.g., Lys4); (4) at both the N-terminus and the C-terminus; (5) at the N-terminus and an internal site; and (6) at the C-terminus and an internal site. In one embodiment, CNP22 or variants thereof are conjugated to a hydrophilic polymer only at the N-terminus. In another embodiment, conjugation is only at an internal site (e.g., Lys4). In yet another embodiment, conjugation is at the N-terminus and an internal site (e.g., Lys4). In still another embodiment, for better functionality the CNP peptides are not conjugated to a hydrophilic polymer at a site (e.g., Lys10) within the cyclic domain (corresponding to Cys6 to Cys22 of CNP22). If conjugation to a hydrophilic polymer is based on bond formation with a reactive primary amino group on the CNP peptide, conjugation at an internal site (e.g., Lys4 and/or Lys10) can be prevented by substitution of Lys4 and/or Lys10 with a natural or unnatural amino acid or peptidomimetic that does not contain a reactive primary amino group on a side chain, such as, e.g., Gly, Ser, Arg, Asn, Gln, Asp, Glu or citrulline (Cit). In a particular embodiment, Lys4 and/or Lys10 are replaced with Arg. In another embodiment, Lys10 is not replaced with Arg.

Non-limiting examples of hydrophilic polymers include polymers formed from carboxylic acid-bearing monomers (e.g., methacrylic acid (MA) and acrylic acid (AA)), polyvinyl alcohols, polymers formed from hydroxyl-bearing monomers (e.g., hydroxyethyl methacrylate (HEMA), hydroxypropyl methacrylate (HPMA), hydroxypropyl methacrylamide, and 3-trimethylsilylpropyl methacrylate (TMSPMA)), polyalkylene oxides, polyoxyethylated polyols (e.g., glycerol), poly(ethylene glycol) (PEG), poly(propylene glycol), mono-C_1-C_{10} alkoxy-PEGs (e.g., monomethoxy-PEG), tresyl monomethoxy-PEG, aryloxy-PEGs, PEG acrylate (PEGA), PEG methacrylate, PEG propionaldehyde, bis-succinimidyl carbonate PEG, copolymers of 2-methacryloyloxyethyl-phosphorylcholine (MPC) and N-vinyl pyrrolidone (VP), hydroxy functional poly(N-vinyl pyrrolidone) (PVP), SIS-PEG (SIS is polystyrene-polyisobutylene-polystyrene block copolymer), polystyrene-PEG, polyisobutylene-PEG, PCL-PEG (PCL is polycaprolactone), PLA-PEG (PLA is polylactic acid), PMMA-PEG (PMMA is poly(methyl methacrylate)), PDMS-PEG (PDMS is polydimethyloxanone), PVDF-PEG (PVDF is polyvinylidene fluoride), PLURONIC™ surfactants (polypropylene oxideco-polyethylene glycol), poly(tetramethylene glycol), poly (L-lysine-g-ethylene glycol) (PLL-g-PEG), poly (L-lysine-g-hyaluronic acid) (PLL-g-HA), poly(L-lysine-g-phosphoryl choline) (PLL-g-PC), poly(L-lysine-g-vinyl pyrrolidone) (PLL-g-PVP), poly(ethylimine-g-ethylene glycol) (PEI-g-PEG), poly(ethylimine-g-hyaluronic acid) (PEI-g-HA), poly (ethylimine-g-phosphoryl choline) (PEI-g-PC), poly(ethylimine-g-vinyl pyrrolidone) (PEI-g-PVP), PLL-co-HA, PLL-co-PC, PLL-co-PVP, PEI-co-PEG, PEI-co-HA, PEI-co-PC, PEI-co-PVP, cellulose and derivatives thereof (e.g., hydroxyethyl cellulose), dextran, dextrins, hyaluronic acid and derivatives thereof (e.g., sodium hyaluronate), elastin, chitosan, acrylic sulfate, acrylic sulfonate, acrylic sulfamate, methacrylic sulfate, methacrylic

sulfonate, methacrylic sulfamate, polymers and copolymers thereof, and polymers and copolymers of combinations thereof.

In a particular embodiment, the hydrophilic polymer is poly(ethylene glycol) (PEG), also called poly(ethylene oxide) (PEO). As used herein, the term "PEG" or "PEO" encompasses all the forms of PEG, branched and unbranched, which can be used to derivatize polypeptides, including without limitation mono-(C_1-C_{10}) alkoxy-PEGs and aryloxy-PEGs.

In one embodiment, the PEG-CNP conjugates comprise a PEG (or PEO) polymer of the formula $(CH_2CH_2O)_n$, wherein n is an integer from about 6 to about 100, and the PEG polymer is from about 0.3 kDa to about 5 kDa. In another embodiment, n is an integer from about 12 to about 50, and the PEG polymer is from about 0.6 kDa to about 2.5 kDa. In yet another embodiment, n is from about 12 to about 24, and the PEG polymer is from about 0.6 kDa to about 1.2 kDa. In a further embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the end-capping group is an alkyl group, e.g., a lower alkyl group such as methyl, so that the PEG polymer terminates in an alkoxy group. In an embodiment, the PEG polymer is not branched. In another embodiment, CNP22 or variants thereof are conjugated to a PEG polymer only at the N-terminus.

PEGs and PEOs potentially include molecules with a distribution of molecular weights, i.e., they are potentially polydispersed, depending on the manner in which they are prepared. The size/mass distribution of a polymeric preparation can be characterized statistically by its weight average molecular weight (M_w) and its number average molecular weight (M_n), the ratio of which is called the polydispersity index (M_w/M_n). M_w and M_n can be measured by mass spectroscopy. PEG-CNP variants conjugated to a PEG moiety larger than 1.5 kDa may exhibit a range of molecular weights due to the polydispersed nature of the parent PEG molecule. For example, in the case of mPEG2K (Sunbright ME-020HS, NOF Co.), the molecular masses of the PEG molecules are distributed over a range from about 1.5 kDa to about 3 kDa, with a polydispersity index of 1.036. By contrast, the PEGs conjugated to CNP22 or variants thereof using MS $(PEG)_n$ reagents (n=4, 8, 12 or 24, denoted as, e.g., "PEO12" or "PEO24") from Pierce Biotechnology (Rockford, Ill.) are monodispersed, having discrete chain length and defined molecular weight.

Methods for generating polypeptides comprising a PEG moiety are known in the art (see, e.g., U.S. Pat. No. 5,824,784). Methods for preparing PEGylated CNP peptides generally comprise the steps of (a) reacting CNP22 or a variant thereof with a PEGylation reagent under conditions suitable for attaching PEG to the CNP peptide (e.g., at the N-terminus), and (b) obtaining the reaction product(s). Because PEGylating a CNP peptide might significantly alter its binding to NPR-B, depending on the size of the PEG moiety and the location of PEGylation, different kinds of PEG and PEGylation reaction conditions can be explored. The chemistry that can be used for PEGylation of a CNP peptide includes acylation of reactive primary amine(s) of the peptide using the NHS-ester of methoxy-PEG (O—[(N-Succinimidyloxycarbonyl)-methyl]-O'-methylpolyethylene glycol). Acylation with methoxy-PEG-NHS or methoxy-PEG-SPA results in an amide linkage that eliminates any charge of the original primary amine. PEG-CNP peptides designated with the symbol "PEO12" or "PEO24", as well as those designated with the symbol "PEG1K", "PEG2K", "PEG5K" or "PEG20K", are PEGylated via reaction of a primary amino group on the peptide with an NHS ester-activated, methoxyend capped PEG reagent. PEG-CNP variants can also be prepared by other methods, e.g., via reductive amination involving a primary amino group on the peptide and a PEG aldehyde, such as, e.g., PEG-propionaldehyde, or mono-C_1-C_{10} alkoxy or aryloxy derivatives thereof (see U.S. Pat. No. 5,252,714).

Unlike ribosome protein synthesis, synthetic peptide synthesis proceeds from the C-terminus to the N-terminus. Accordingly, Boc-PEG (containing tert-butyloxycarbonyl (Boc)) is one method to attach PEG to the C-terminus of a peptide (R. B. Merrifield, J. Am. Chem. Soc., 85(14): 2149-2154 (1963)). Alternatively, Fmoc (fluorenylmethoxycarbonyl) chemistry can be employed (E. Atherton and R. C. Sheppard, Solid Phase Peptide Synthesis: a Practical Approach, IRL Press (Oxford, England (1989)).

The present methods for preparing PEG-CNP variants provide a substantially homogenous mixture of polymer-protein conjugates. After purification, discrete PEG-CNP preparations are sufficiently pure for in vitro and in vivo testing of biological properties. As demonstrated herein, certain PEGCNP variants exhibit reduced susceptibility to NEP cleavage and substantially similar or better functionality (e.g., stimulation of cGMP production).

As described herein, PEGylation reactions of CNP22 or variants thereof, using appropriate PEGylation reagent/CNP peptide ratios and reaction conditions, provide PEG-CNP derivatives. The nature and extent of PEGylation can be determined using, e.g., PAGE and HPLC analysis. In certain embodiments, at least about 50%, 60%, 70%, 80%, 90%, 95% or 99% of CNP22 or variants thereof are mono-PEGylated at the N-terminus. To optimize the beneficial effects of PEGylation on the biological properties of a CNP peptide, the polymer length, conformation (e.g., branched or linear), and/or functionalization (e.g., adding a negatively charged group) of a PEG moiety can be varied. PEGylated CNP variants are tested for NEP resistance, pharmacokinetics and bioactivity (e.g., the ability to bind to NPR-B and stimulate the generation of cGMP). PEGylated CNP variants that show improved NEP resistance and at least about 50% of the cGMP-stimulating activity of CNP22 can be further tested, e.g., in vitro in a rat chondrosarcoma cell-based achondroplasia model and in vivo in a murine achondroplasia animal model.

E. Methods of Using CNP Variants, Pharmaceutical Compositions of CNP Variants, and Routes of Administration

Methods of Using CNP Variants

Bone-Related Disorders

Fibroblast growth factors (FGFs) play important roles in bone formation, and mutations in FGF receptor genes (FGFR 1, 2 and 3) give rise to a variety of inherited skeletal malformations (Curr. Biol., 5: 500-507 (1995)). In particular, activating mutations in FGFR-3 are responsible for disorders of the long bones, including achondroplasia, the most common form of human genetic dwarfism (Nature, 371: 252-254 (1994); Cell, 78: 335-342 (1994)), the milder disorder hypochondroplasia (Ann. N.Y. Acad. Sci., 785: 182-187 (1996)), and the more severe and neonatal lethal thanatophoric dysplasia (TD) types I and II (Hum. Mol. Genet., 5: 509-512 (1996); Nat. Genet., 9: 321-328 (1995)). Mouse models over-expressing FGF-2, and consequentially activating FGFR-3, show shortened long bones and macrocephaly (Mol. Biol. Cell, 6: 1861-73 (1995)). Consistent

with this model, mice deficient in FGFR-3 show remarkable skeletal overgrowth with wider growth plates (Nature Genet., 12: 390-397 (1996)).

Complementary experiments with CNP, NPR-B and NPR-C suggest a link between the peptide ligand, the corresponding receptors, and bone growth. Activation of NPR-B by elevated plasma concentrations of CNP in transgenic mice causes skeletal overgrowth (Nat. Med., 10: 80-86 (2004)) histologically similar to that of the growth plate cartilage of FGFR-3 knockout mice (Nat. Genet., 4: 390-397 (1996)). In NPR-C knockout mice, NPR-C-mediated clearance of CNP should be eliminated; consistent with this prediction, the knockout animals show elongated long bones and elongated vertebrae with kyphosis (Proc. Natl. Acad. Sci. USA 96: 7403-08 (1999)). Conversely, CNP knockout mice are dwarfed with shorter long bones and vertebrae, a phenotype histologically similar to that of achondroplasia, and have increased mortality as a result of malocclusion and pulmonary restriction from the small rib cage (Proc. Natl. Acad. Sci. USA, 98: 4016-4021 (2001)). Consistent with the proposed role of CNP as an activator of NPR-B, the NPR-B knockout mouse has the same dwarfed skeletal phenotype and increased mortality as the CNP knockout mouse (Proc. Natl. Acad. Sci. USA, 101: 17300-05 (2004)). Furthermore, in a mouse model of achondroplasia with activated FGFR-3 in the cartilage, targeted overexpression of CNP in chondrocytes counteracts dwarfism (Yasoda et al., Nat. Med., 10: 80-86 (2004)). Additionally, CNP has been show to play a role in regulating endochondral bone growth and chondrocyte activity, including but not limited to chondrocyte proliferation and differentiation, inhibition of the mitogen activated protein (MAP) kinase/MEK (Raf-1) kinase signaling pathway, and promotion of endochondral ossification (Yasoda et al., Nat. Med., 10: 80-86 (2004)). These results suggest that activation of the CNP/NPR-B system is a potential therapeutic strategy for treatment of human achondroplasia.

By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variants of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders and skeletal dysplasias include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansentype metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondy-loepimetaphyseal dysplasia. Further, the CNP variants are useful as an adjunct or alternative to growth hormone for treating idiopathic short stature and other skeletal dysplasias.

In addition, the CNP variants are useful for treating other bone-related conditions and disorders, such as rickets, hypophosphatemic rickets [including X-linked hypophosphatemic rickets (also called vitamin D-resistant rickets) and autosomal dominant hypophosphatemic rickets], and osteomalacia [including tumor-induced osteomalacia (also called oncogenic osteomalacia or oncogenic hypophosphatemic osteomalacia)].

The CNP variants of the disclosure can also be used to treat osteoarthritis. Osteoarthritis is a degenerative disease of the articular cartilage and occurs frequently in the elderly. Osteoarthritis involves destruction of the cartilage and proliferative change in the bone and cartilage resulting from degeneration of articular components, with the change resulting in a secondary arthritis (e.g., synovitis). The extracellular matrix proteins, which are the functional entity of the cartilage, are reduced, and the number of chondrocytes decreases in osteoarthritis (Arth. Rheum. 46(8): 1986-1996 (2002)). By promoting the matrix production, growth and differentiation of chondrocytes, the CNP variants are useful for countering the undesired effects of FGF-2 and increasing matrix synthesis in subjects suffering from arthritis, including osteoarthritis, thereby treating arthritis, including osteoarthritis.

Vascular Smooth Muscle Disorders

CNP and other vasoactive peptides (including ANP, BNP and urodilatin) have vasodilator and diuretic properties and play an important role in cardiovascular homeostasis (J. Cardiovasc. Pharmacol., 117: 1600-06 (1998); Kidney Int., 49: 1732-37 (1996); Am. J. Physiol., 275: H1826-1833 (1998)). CNP is widely distributed in the cardiovascular system, especially in high concentration in vascular endothelial cells (J. Cardiovasc. Pharmacol., 117: 1600-06 (1998)). CNP is a potent relaxant of vascular smooth muscle, particularly in the coronary circulation (Biochem. Biophys. Res. Commun., 205: 765-771 (1994)), and is an inhibitor of smooth muscle cell proliferation (Biochem. Biophys. Res. Commun., 177: 927-931 (1991)). Although the vasodilator effect of CNP is less potent than that of ANP (about 1:100) (Hypertens. Res., 21: 7-13 (1998); Am. J. Physiol., 275: L645-L652 (1998)), CNP mRNA is increased in response to shear stress (FEBS Lett., 373: 108-110 (1995)) and plasma levels of CNP are elevated in inflammatory cardiovascular pathologies (Biochem. Biophys. Res. Commun., 198: 1177-1182 (1994)). CNP has been shown to suppress inflammation through inhibition of macrophage infiltration in injured carotid arteries of rabbits (Circ. Res., 91: 1063-1069 (2002)) and to directly inhibit cardiac fibroblast proliferation through an NPR-B/cGMP-dependent pathway (Endocrinology, 144: 2279-2284 (2003)).

The cardiovascular actions of CNP are mediated via activation of the NPR subtypes, NPR-B and NPR-C (Endocrinology, 130: 229-239 (1992)), the latter accounting for 95% of NPRs expressed in vivo (Science, 293: 1657-1662 (2001)). The CNP/NPR-B pathway leads to elevation of cGMP, a well-established secondary messenger in the cardiovascular system. NPR-C's 37-amino acid portion from the C-terminus has a consensus sequence that interacts with the heterotrimeric G protein G_i (J. Biol. Chem., 274: 17587-17592 (1999)), which has been shown to regulate adenylate cyclase and phospholipase C activity (J. Biol. Chem., 276: 22064-70 (2001); Am. J. Physiol., 278: G974-980 (2000); J. Biol. Chem., 271: 19324-19329 (1996)). CNP mediates smooth muscle hyperpolarization and relaxation via activation of NPR-C and the opening of a G protein-regulated, inwardly rectifying K^+ channels (Proc. Natl. Acad. Sci. USA, 100: 1426-1431 (2003)). Likewise, CNP has important anti-proliferative effects in cardiac fibroblasts and, through interaction with NPR-C, regulates local blood flow

and systemic blood pressure by hyperpolarizing smooth muscle cells (R. Rose and W. Giles, J. Physiol. 586: 353-366 (2008)).

By binding to NPR-B on vascular smooth muscle cells, CNP22 stimulates the production of cGMP, which acts as an intracellular secondary messenger to cause ultimately the relaxation of blood vessels. Based on the hypotensive actions of CNP, the CNP variants of the disclosure are useful for treating hypertension, congestive heart failure, cardiac edema, nephredema, hepatic edema, acute and chronic renal insufficiency, and so on. In addition, activation of cGMP signaling suppresses the growth of vascular smooth muscle cells. Accordingly, the CNP variants of the disclosure can be used to treat conditions or diseases caused by the abnormal growth of vascular smooth muscle cells, including but not limited to restenosis and arteriosclerosis.

The studies described above suggest that CNP may be a potential therapeutic candidate for vascular smooth muscle relaxation and remodeling. Pharmacological effects of CNP concerning certain disorders have been attributed, in part, to vasoprotective effects rather than to vasodilator activity (Am. J. Respir. Crit. Care Med., 170: 1204-1211 (2004)). Therefore, the CNP variants of the present disclosure are useful for treating conditions, e.g., vascular smooth muscle disorders, in which CNP may have a vasoprotective effect, including without limitation inducing smooth muscle relaxation and inhibiting infiltration of macrophages into cardiac tissue. In one embodiment, the CNP variants are used to treat heart failure, including but not limited to acute decompensated heart failure and acute congestive heart failure. In another embodiment, the CNP variants are used to treat asthma, cardiomyopathy, and restenosis of coronary arteries (by increasing smooth muscle cell relaxation and decreasing proliferation of smooth muscle cells).

Pharmaceutical Compositions of CNP Variants

In additional embodiments, the disclosure provides pharmaceutical compositions comprising a CNP variant, and one or more pharmaceutically acceptable excipients, carriers and/or diluents. In certain embodiments, the compositions further comprise one or more other biologically active agents (e.g., inhibitors of proteases, receptor tyrosine kinases, and/or the clearance receptor NPR-C).

In some embodiments, the compositions comprise the desired CNP variant in at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% purity. In certain embodiments, the compositions contain less than about 10%, 5%, 4%, 3%, 2%, 1% or 0.5% of macromolecular contaminants, such as other mammalian (e.g., human) proteins and other CNP variants.

Non-limiting examples of excipients, carriers and diluents include vehicles, liquids, buffers, isotonicity agents, additives, stabilizers, preservatives, solubilizers, surfactants, emulsifiers, wetting agents, adjuvants, and so on. The compositions can contain liquids (e.g., water, ethanol); diluents of various buffer content (e.g., Tris-HCl, phosphate, acetate buffers, citrate buffers), pH and ionic strength; detergents and solubilizing agents (e.g., Polysorbate 20, Polysorbate 80); anti-oxidants (e.g., methionine, ascorbic acid, sodium metabisulfite); preservatives (e.g., Thimerosol, benzyl alcohol, m-cresol); and bulking substances (e.g., lactose, mannitol, sucrose). The use of excipients, diluents and carriers in the formulation of pharmaceutical compositions is known in the art; see, e.g., Remington's Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pa. (1990)), which is incorporated herein by reference in its entirety.

For example, carriers include without limitation diluents, vehicles and adjuvants, as well as implant carriers, and inert, non-toxic solid or liquid fillers and encapsulating materials that do not react with the active ingredient(s). Non-limiting examples of carriers include phosphate buffered saline, physiological saline, water, and emulsions (e.g., oil/water emulsions). A carrier can be a solvent or dispersing medium containing, e.g., ethanol, a polyol (e.g., glycerol, propylene glycol, liquid polyethylene glycol, and the like), a vegetable oil, and mixtures thereof.

In some embodiments, the compositions are liquid formulations. In certain embodiments, the formulations comprise a CNP variant in a concentration range from about 0.1 mg/ml to about 20 mg/ml, or from about 0.5 mg/ml to about 20 mg/ml, or from about 1 mg/ml to about 20 mg/ml, or from about 0.1 mg/ml to about 10 mg/ml, or from about 0.5 mg/ml to about 10 mg/ml, or from about 1 mg/ml to about 10 mg/ml.

In further embodiments, the compositions comprise a buffer solution or buffering agent to maintain the pH of a CNP-containing solution or suspension within a desired range. Non-limiting examples of buffer solutions include phosphate buffered saline, Tris buffered saline, and Hank's buffered saline. Buffering agents include without limitation sodium acetate, sodium phosphate, and sodium citrate. Mixtures of buffering agents can also be used. In certain embodiments, the buffering agent is acetic acid/acetate or citric acid/citrate. The amount of buffering agent suitable in a composition depends in part on the particular buffer used and the desired pH of the solution or suspension. For example, acetate is a more efficient pH buffer at pH 5 than pH 6, so less acetate may be used in a solution at pH 5 than at pH 6. In some embodiments, the buffering agent has a concentration of about 10 mM±5 mM. In certain embodiments, the pH of a composition is from about pH 3 to about pH 7.5, or from about pH 3.5 to about pH 7, or from about pH 3.5 to about pH 6.5, or from about pH 4 to about pH 6, or from about pH 4 to about pH 5, or is at about pH 5.0±1.0.

In other embodiments, the compositions contain an isotonicity-adjusting agent to render the solution or suspension isotonic and more compatible for injection. Non-limiting examples of isotonicity agents include NaCl, dextrose, glucose, glycerin, sorbitol, xylitol, and ethanol. In certain embodiments, the isotonicity agent is NaCl. In certain embodiments, NaCl is in a concentration of about 160±20 mM, or about 140 mM±20 mM, or about 120±20 mM, or about 100 mM±20 mM, or about 80 mM±20 mM, or about 60 mM±20 mM.

In yet other embodiments, the compositions comprise a preservative. Preservatives include, but are not limited to, m-cresol and benzyl alcohol. In certain embodiments, the preservative is in a concentration of about 0.4%±0.2%, or about 1%±0.5%, or about 1.5%±0.5%, or about 2.0%±0.5%.

In still other embodiments, the compositions contain an anti-adsorbent (e.g., to mitigate adsorption of a CNP variant to glass or plastic). Anti-adsorbents include without limitation benzyl alcohol, Polysorbate 20, and Polysorbate 80. In certain embodiments, the anti-adsorbent is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%.

In additional embodiments, the compositions comprise a stabilizer. Non-limiting examples of stabilizers include glycerin, glycerol, thioglycerol, methionine, and ascorbic acid and salts thereof. In some embodiments, when the stabilizer

is thioglycerol or ascorbic acid or a salt thereof, the stabilizer is in a concentration from about 0.1% to about 1%. In other embodiments, when the stabilizer is methionine, the stabilizer is in a concentration from about 0.01% to about 0.5%, or from about 0.01% to about 0.2%. In still other embodiments, when the stabilizer is glycerin, the stabilizer is in a concentration from about 5% to about 100% (neat).

In further embodiments, the compositions contain an antioxidant. Exemplary anti-oxidants include without limitation methionine and ascorbic acid. In certain embodiments, the molar ratio of antioxidant to CNP variant is from about 0.1:1 to about 15:1, or from about 1:1 to about 15:1, or from about 0.5:1 to about 10:1, or from about 1:1 to about 10:1 or from about 3:1 to about 10:1.

Pharmaceutically acceptable salts can be used in the compositions, including without limitation mineral acid salts (e.g., hydrochloride, hydrobromide, phosphate, sulfate), salts of organic acids (e.g., acetate, propionate, malonate, benzoate, mesylate, tosylate), and salts of amines (e.g., isopropylamine, trimethylamine, dicyclohexylamine, diethanolamine). A thorough discussion of pharmaceutically acceptable salts is found in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Company, (Easton, Pa. (1990)).

The pharmaceutical compositions can be administered in various forms, such as tablets, capsules, granules, powders, solutions, suspensions, emulsions, ointments, and transdermal patches. The dosage forms of the compositions can be tailored to the desired mode of administration of the compositions. For oral administration, the compositions can take the form of, e.g., a tablet or capsule (including softgel capsule), or can be, e.g., an aqueous or nonaqueous solution, suspension or syrup. Tablets and capsules for oral administration can include one or more commonly used excipieints, diluents and carriers, such as mannitol, lactose, glucose, sucrose, starch, corn starch, sodium saccharin, talc, cellulose, magnesium carbonate, and lubricating agents (e.g., magnesium stearate, sodium stearyl fumarate). If desired, flavoring, coloring and/or sweetening agents can be added to the solid and liquid formulations. Other optional ingredients for oral formulations include without limitation preservatives, suspending agents, and thickening agents. Oral formulations can also have an enteric coating to protect the CNP variant from the acidic environment of the stomach. Methods of preparing solid and liquid dosage forms are known, or will be apparent, to those skilled in this art (see, e.g., Remington's Pharmaceutical Sciences, referenced above).

Formulations for parenteral administration can be prepared, e.g., as liquid solutions or suspensions, as solid forms suitable for solubilization or suspension in a liquid medium prior to injection, or as emulsions. For example, sterile injectable solutions and suspensions can be formulated according to techniques known in the art using suitable diluents, carriers, solvents (e.g., buffered aqueous solution, Ringer's solution, isotonic sodium chloride solution), dispersing agents, wetting agents, emulsifying agents, suspending agents, and the like. In addition, sterile fixed oils, fatty esters, polyols and/or other inactive ingredients can be used. As further examples, formulations for parenteral administration include aqueous sterile injectable solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions, which can contain suspending agents and thickening agents.

Compositions comprising a CNP variant can also be lyophilized formulations. In certain embodiments, the lyophilized formulations comprise a buffer and bulking agent, and optionally an antioxidant. Exemplary buffers include without limitation acetate buffers and citrate buffers. Exemplary bulking agents include without limitation mannitol, sucrose, dexran, lactose, trehalose, and povidone (PVP K24). In certain embodiments, mannitol is in an amount from about 3% to about 10%, or from about 4% to about 8%, or from about 4% to about 6%. In certain embodiments, sucrose is in an amount from about 6% to about 20%, or from about 6% to about 15%, or from about 8% to about 12%. Exemplary anti-oxidants include, but are not limited to, methionine and ascorbic acid.

The disclosure also provides kits containing, e.g., bottles, vials, ampoules, tubes, cartridges and/or syringes that comprise a liquid (e.g., sterile injectable) formulation or a solid (e.g., lyophilized) formulation. The kits can also contain pharmaceutically acceptable vehicles or carriers (e.g., solvents, solutions and/or buffers) for reconstituting a solid (e.g., lyophilized) formulation into a solution or suspension for administration (e.g., by injection), including without limitation reconstituting a lyophilized formulation in a syringe for injection or for diluting concentrate to a lower concentration. Furthermore, extemporaneous injection solutions and suspensions can be prepared from, e.g., sterile powder, granules, or tablets comprising a CNP-containing composition. The kits can also include dispensing devices, such as aerosol or injection dispensing devices, pen injectors, autoinjectors, needleless injectors, syringes, and/or needles.

As a non-limiting example, a kit can include syringes having a single chamber or dual chambers. For single-chamber syringes, the single chamber can contain a liquid CNP formulation ready for injection, or a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) that can be reconstituted into a solution or suspension for injection. For dual-chamber syringes, one chamber can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection.

As a further example, a kit can include one or more pen injector or autoinj ector devices, and dual-chamber cartridges. One chamber of a cartridge can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection. A cartridge can comprise an amount of the CNP variant that is sufficient for dosing over a desired time period (e.g., 1 day, 2 days, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, etc.). The pen injector or autoinjector can be adjusted to administer a desired amount of the CNP formulation from a cartridge.

In addition, pharmaceutical compositions comprising a CNP variant can be formulated as a slow release, controlled release or sustained release system for maintaining a relatively constant level of dosage over a desired time period, such as 1 week, 2 weeks, 3 weeks, 1 month, 2 months, or 3

months. Slow release, controlled release and sustained release formulations can be prepared using, e.g., biodegradable polymeric systems {which can comprise, e.g., hydrophilic polymers [e.g., polylactide, polyglycolide, poly(lactide-glycolide)]}, and can take the form of, e.g., microparticles, microspheres or liposomes, as is known in the art.

Dosages and Frequency of Dosing

As used herein, the term "therapeutically effective amount" of an active agent (e.g., a CNP variant) refers to an amount that provides therapeutic benefit to a patient. The amount may vary from one individual to another and may depend upon a number of factors, including the overall physical condition of the patient. A therapeutically effective amount of a CNP variant can be readily ascertained by one skilled in the art, using publicly available materials and procedures. For example, the amount of a CNP variant used for therapy should give an acceptable rate of growth based on growth charts for children ages 0-17 years with achondroplasia (214 females and 189 males), which list height for age, head circumference, and segmental growth (Horton W A et al., Standard growth curves for achondroplasia, J. Pediatr., 93: 435-8 (1978)). CDC charts can be used to assess weight for age and weight for height or BMI for age. Secondary outcomes with courses that are more chronic in nature can also be measured.

Having a longer serum half-life than wild-type CNP22, the CNP variants can potentially be administered less frequently than CNP22. The dosing frequency for a particular individual may vary depending upon various factors, including the disorder being treated and the condition and response of the individual to the therapy. In certain embodiments, a pharmaceutical composition containing a CNP variant is administered to a subject about one time per day, one time per two days, one time per three days, or one time per week. In one embodiment, for treatment of bone-related disorders (e.g., skeletal dysplasias, including achondroplasia), a daily or weekly dose of a CNP variant is administered to patients until and/or through adulthood.

The CNP variants described herein can be administered to patients at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia) and conditions (e.g., vascular smooth muscle disorders) in which CNP can provide a vaso-protective effect. The safety and therapeutic efficacy of the CNP variants can be determined by standard pharmacological procedures in cell cultures or experimental animals, such as, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Active agents exhibiting a large therapeutic index are normally preferred.

Data obtained from cell culture assays and animal studies can be used to formulate a range of dosage for use in humans. The dosage normally lies within a range of circulating concentrations that include the ED_{50}, with little or minimal toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. The therapeutically effective dose can be determined from cell culture assays and animal studies.

In certain embodiments, the CNP variants described herein are administered at a dose in the range from about 5 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg. In some embodiments, the CNP variants are administered at a dose of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 125, 130, 140, 150, 160, 170, 175, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variants are administered at a dose of about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000 ug/kg, or about 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 mg/kg, or other dose deemed appropriate by the treating physician. The doses of CNP variants described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc.

The frequency of dosing/administration of a CNP variant for a particular subject may vary depending upon various factors, including the disorder being treated and the condition and response of the subject to the therapy. The CNP variant can be administered in a single dose or in multiple doses per dosing. In certain embodiments, the CNP variant is administered, in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week, 3 times per week, weekly, bi-weekly, every 3 weeks, monthly, every 6 weeks, every 2 months, every 3 months, or as deemed appropriate by the treating physician.

In some embodiments, a CNP variant is administered so as to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP variant may be administered intravenously, subcutaneously or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP variant daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

Modes of Administration

The CNP variants, or pharmaceutical compositions comprising them, can be administered to subjects in various ways such as, e.g., by injection subcutaneously, intravenously, intra-arterially, intraperitoneally, intramuscularly, intraderwally, or intrathecally. In an embodiment, the CNP variants are administered by a single subcutaneous, intravenous, intraarterial, intraperitoneal, intramuscular, intradermal or intrathecal injection once a day.

The CNP variants can also be administered by direct injection at or near the site of disease. Further, the CNP variants can be administered by implantation of a depot at the target site of action (e.g., an abnormal or dysplasic bone). Alternatively, the CNP variants can be administered sublingually under the tongue (e.g., sublingual tablet) or by inhalation into the lungs (e.g., inhaler or aerosol spray), by delivery into the nasal cavity (e.g., intranasal spray), by

delivery into the eye (e.g., eye drop), or by transdermal delivery (e.g., by means of a patch on the skin). The CNP variants may also be administered orally in the form of microspheres, microcapsules, liposomes (uncharged or charged (e.g., cationic)), polymeric microparticles (e.g., polyamides, polylactide, polyglycolide, poly(lactide-glycolide)), microemulsions, and the like.

A further method of administration is by osmotic pump (e.g., an Alzet pump) or mini-pump (e.g., an Alzet miniosmotic pump), which allows for controlled, continuous and/or slow-release delivery of the CNP variant or pharmaceutical composition over a pre-determined period. The osmotic pump or mini-pump can be implanted subcutaneously, or near the target site (e.g., the long bones of limbs, the epiphyses, etc).

As explained above, the CNP variants can be used to treat conditions or diseases caused by the abnormal growth of vascular smooth muscle cells, including but not limited to restenosis and arteriosclerosis. For local delivery of a CNP variant to the diseased bodily vessel (e.g., blood vessel), the CNP variant can be delivered by means of a medical device (e.g., a stent) implanted at the diseased site. In one embodiment, the CNP variant is impregnated in a polymeric matrix or polymeric coating disposed over a stent. In another embodiment, the CNP variant is contained in reservoirs or channels formed in the body of a stent and covered by a porous polymeric membrane or layer through which the CNP variant can diffuse. The polymeric matrix, coating, membrane or layer can comprise at least one biodegradable (e.g., hydrophilic) polymer, as is known in the art. In a further embodiment, the CNP variant can be contained in micropores in the body of a stent. The CNP variant can be delivered from a stent by burst release, pulse release, controlled release or sustained release, or a combination thereof. For example, the stent can locally deliver the CNP variant to the diseased site in a burst release followed by a sustained release. Sustained release can be over a period up to about 2 weeks, 1 month, 2 months, 3 months, 6 months or 1 year.

It will be apparent to one skilled in the art that the CNP variants or compositions thereof can also be administered by other modes. Determination of the most effective mode of administration of the CNP variants or compositions thereof is within the skill of the skilled artisan.

The CNP variants can be administered as pharmaceutical formulations suitable for, e.g., oral (including buccal and sub-lingual), rectal, nasal, topical, pulmonary, vaginal or parenteral (including intramuscular, intraarterial, intrathecal, subcutaneous and intravenous) administration, or in a form suitable for administration by inhalation or insufflation. Depending on the intended mode of administration, the pharmaceutical formulations can be in the form of solid, semisolid or liquid dosage forms, such as tablets, suppositories, pills, capsules, powders, liquids, suspensions, emulsions, creams, ointments, lotions, and the like. The formulations can be provided in unit dosage form suitable for single administration of a precise dosage. The formulations comprise an effective amount of a CNP variant, and one or more pharmaceutically acceptable excipients, carriers and/or diluents, and optionally one or more other biologically active agents.

Combination Therapy

In one embodiment, a CNP variant can be used in combination with one or more other active agents useful for treating, ameliorating or preventing CNP-responsive conditions or disorders such as, e.g., bone-related disorders (e.g., skeletal dysplasias) and vascular smooth muscle disorders. The other active agent(s) can enhance the effects of the CNP variant and/or exert other pharmacological effects in addition to those of the CNP variant. Non-limiting examples of active agents that can be used in combination with the CNP variants described herein are other natriuretic peptides (e.g., BNP) and inhibitors (e.g., antagonists) of peptidases and proteases (e.g., NEP and furin), NPR-C and tyrosine kinases (e.g., FGFR-3). By preventing NEP cleavage of the CNP variant, an NEP inhibitor can prolong the half-life of the variant. Examples of NEP inhibitors include, without limitation, thiorphan and candoxatril. Co-use of an NPR-C inhibitor can also prolong the half-life of the CNP variant via inhibition of the variant's clearance by NPR-C. A non-limiting example of an NPR-C inhibitor is the fragment FGIPMDRIGRNPR (SEQ ID NO: 82), which would be released at the target site (e.g., bone growth plate) upon proteolytic cleavage of the FGIPMDRIGRNPR-CNP22 chimera (Analog CZ) (SEQ ID NO: 82) or similar chimeras comprising variants of CNP22 (e.g., those containing amino acid substitution(s), addition(s), and/or deletion(s) relative to CNP22). Co-use of a tyrosine kinase inhibitor can accentuate the effects of a CNP therapy by inhibiting the tyrosine kinase receptor FGFR-3, a negative regulator of chondrocyte and bone growth. Non-limiting examples of tyrosine kinase inhibitors include those disclosed in U.S. Pat. Nos. 6,329,375 and 6,344,459.

To achieve the appropriate therapeutic outcome in the combination therapies, one would generally administer to the subject the CNP composition and other therapeutic(s) in a combined amount effective to produce the desired therapeutic outcome (e.g., restored bone growth). This process may involve administering the CNP composition and other therapeutic agent(s) at the same time. Simultaneous administration can be achieved by administering a single composition or pharmacological protein formulation that includes both the CNP variant and other therapeutic agent(s). Alternatively, the other therapeutic agent(s) can be taken separately at about the same time as a pharmacological formulation (e.g., tablet, injection or drink) of the CNP variant. The CNP variant can also be formulated into a foodstuff such as brownies, pancakes, or cake, suitable for ingestion.

In other alternatives, administration of the CNP variant can precede or follow administration of the other therapeutic agent(s) by intervals ranging from minutes to hours. In embodiments where the other therapeutic agent(s) and the CNP composition are administered separately, one would generally ensure that the CNP variant and the other therapeutic agent(s) are administered within an appropriate time of one another so that both the CNP variant and the other therapeutic agent(s) can exert, synergistically or additively, a beneficial effect on the patient. For example, one can administer the CNP composition within about 0.5-6 hours (before or after) of the other therapeutic agent(s). In one embodiment, the CNP composition is administered within about 1 hour (before or after) of the other therapeutic agent(s).

Identifying and Monitoring Patient Populations

Protocols can be established to identify subjects suitable for CNP therapy and to determine whether a given patient is responsive to CNP therapy. For example, for treatment of bone-related disorders, indicators of growth can be measured, such as long bone growth measurements in utero and neonatal and measurements of bone growth biomarkers such as CNP, cGMP, Collagen II, osteocalcin, and Proliferating Cell Nuclear Antigen (PCNA).

One CNP signaling marker is cGMP (guano sine 3',5' cyclic monophosphate). The level of this intracellular signaling molecule increases after CNP binds to and activates

its cognate receptor NPR-B. Elevated levels of cGMP can be measured from cell culture extracts (in vitro) after CNP exposure, conditioned media from bone ex-plant studies (ex vivo) after CNP exposure, and in the plasma (in vivo) within minutes of CNP administration subcutaneously, intravenously, or via other routes of administration known in the art.

Cartilage and bone-specific analytes (or cartilage- and bone-associated markers) can also be measured to assess CNP efficacy. For example, fragments of cleaved collagen type II are a cartilage-specific marker for cartilage turnover. Type II collagen is the major organic constituent of cartilage and fragments of type II collagen (cleaved collagen) are released into circulation, and subsequently secreted into the urine, following cartilage turnover. Cartilage turnover preceeds new bone formation.

A bone-specific biomarker for bone formation which can be measured is N-terminal propeptides of type I procollagen (PINP). The synthesis of type I collagen is an important step in bone formation, as type I collagen is the major organic component in bone matrix. During collagen synthesis, propeptides are released from the procollagen molecule and can be detected in serum. In addition, fragments of collagen type I can be measured as a marker for bone resorption.

Other potential biomarkers for cartilage and bone formation and growth include aggrecan chondroitin sulfate (cartilage-specific marker for cartilage turnover), propeptides of type TT collagen (cartilage-specific marker for cartilage formation), alkaline phosphatase (bone-specific) and osteocalcin (bone-specific marker for bone formation). Cartilage- and bone-associated biomarkers can be measured, e.g., in serum from efficacy/pharmacodynamic in vivo studies and from the conditioned media of ex vivo studies, using commercially available kits.

In one embodiment, the level of at least one bone- or cartilage-associated biomarker is assayed or measured in a subject that has been administered a CNP variant in order to monitor the effects of the CNP variant on bone and cartilage formation and growth in vivo. For example, an increase in the level of at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP variant has a positive effect on bone growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (e.g, endogenous levels of CNP), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In an embodiment, biomarkers are measured by obtaining a biological sample from a subject who will be administered, is being administered or has been administered a CNP variant. Biomarkers can be measured using techniques known in the art, including, but not limited to, Western Blot, enzyme linked immunosorbant assay (ELISA), and enzymatic activity assay. The biological sample can be blood, serum, urine, or other biological fluids.

Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

F. Examples

Example 1

Synthesis of CNP Variants

CNP variants were prepared using the methods described herein. Substitutions with natural or unnatural amino acids or peptidomimetics were made, as indicated in Tables 1-3 (shown in Example 3), at the respective amino acid residues in the wild-type sequence of CNP22. In certain variants, additional amino acids were added to the N-terminal and/or C-terminal ends of the whole or a portion of the wild-type CNP22 sequence (see Table 3).

Also prepared were CNP variants in which a PEG (or PEO) moiety was conjugated to the N-terminus of CNP22 or variants thereof (see Table 4, shown in Example 3). PEGylation reagents can be obtained from the commercial sources shown in Table 5.

TABLE 5

Vendor	Product Name	Name	MW (Da)	PEGylation Reagent
NOF	Sunbright ME-200CS	mPEG20K	20,000	CH3(CH2CH2O)450-(CH2)5COO—NHS
NOF	Sunbright ME-050CS	mPEG5K	5,000	CH3(CH2CH2O)110-(CH2)5COO—NHS
Pierce	(Methyl-PEG12)3-PEG4-NHS Ester	(mPEG12)3-PEG4	2,400	[CH3(CH2CH2O)12]3-(CH2CH2O)4-NHCO(CH2)3-COO—NHS
NOF	Sunbright ME-020HS	mPEG2K	2,000	CH3(CH2CH2O)45-(CH2)5COO—NHS
NOF	Sunbright ME-020CS	mPEG2K	2,000	CH3(CH2CH2O)45-(CH2)5COO—NHS
NOF	Sunbright ME-010HS	mPEG1K	1,000	CH3(CH2CH2O)23-CO(CH2)2COO—NHS
Pierce	Methyl PEG24-NHS Ester	MS(PEG)24	1,200	CH3(CH2CH2O)24-(CH2)2COO—NHS
Pierce	EZ-Link NHS-PEG12-Biotin	PEO12-Biotin	940	Biotin-(CH2CH2O)12-(CH2)2COO—NHS
Pierce	Methyl PEG12-NHS Ester	MS(PEG)12	690	CH3(CH2CH2O)12-(CH2)2COO—NHS
Pierce	EZ-Link NHS-PEG4-Biotin	PEO4-biotin	590	Biotin-(CH2CH2O)4-(CH2)2COO—NHS
Pierce	Mono(lactosylamido) mono(succinimidyl)suberate	LSS	590	

TABLE 5-continued

Vendor	Product Name	Name	MW (Da)	PEGylation Reagent
Pierce	EZ-link NHS-LC-LC-Biotin	LC-LC-Biotin	570	
Pierce	EZ-link NHS-LC-Biotin	LC-Biotin (LC = long chain)	450	
Pierce	EZ-link NHS-Biotin	Biotin	340	

The PEG (also called PEO) polymers purchased from Pierce Biotechnology (Rockford, Ill.) are monodispersed—i.e., they contain a single discrete polymer of a particular molecular weight. By contrast, the PEG polymers purchased from NOF (Nippon Oil and Fat) are polydispersed—i.e., they contain a mixture of polymers having a distribution of molecular weights.

To PEGylate CNP22 or variants thereof, reaction and purification conditions are optimized for each PEG-CNP conjugate. According to a general PEGylation procedure, reaction mixtures contain about 1 mM CNP22 or a variant thereof, and about 1 to 5 mM NHS-activated PEG in potassium phosphate buffer, pH between about 5.0 and 6.5. To mono-PEGylate selectively at the peptide N-terminus and minimize PEGylation at an internal site (e.g., Lys4 of CNP22), the PEGylation reaction can be conducted under more acidic conditions (e.g., at a pH between about 5.5 and 6.5) to protonate selectively and hence deactivate the more basic primary amino group on the lysine side chain. After about 1 to 3 hours of incubation at room temperature, the PEGylation reaction is quenched by addition of aqueous glycine buffer. Reaction products are then separated by reverse-phase HPLC, optimized for each PEG-CNP conjugate. Fractionation samples are speedvacced to dryness, and reconstituted/formulated in 1 mM HCl. Identification and purity of each PEG-CNP product are determined by liquid chromatography-mass spectrometry (LC/MS).

Example 2A

Recombinant Production of CNP Variants

CNP variants can be produced using recombinant technology. In certain embodiments, the CNP variants are produced as fusion proteins comprising a cleavable peptide, carrier protein or tag. Exemplary methods for recombinantly producing CNP fusion proteins are disclosed below.

Materials and Methods

Cloning of CNP Fusion Proteins into Expression Vectors

CNP DNA fragments were amplified using polymerase chain reaction (PCR) and the amplified PCR fragments were digested with Nde I and BamHI and cloned into pET21a vector (Novagen, Gibbstown, N.J.). CNP fusion protein DNA was synthesized by DNA2.0 and cloned into different expression vectors (Table 6).

TABLE 6

Construct	Vector	Product	Chemical cleavage	Final product	Expression *E. coli* strain
pJexpress-TAF-CNP	pJexpress401	TAF-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP	BL21; BL21(DE3)
pJexpress-KSI-CNP(M/N)	pJexpress404	KSI-CNP(M/N) inclusion bodies	CNBr (Met-X)	CNP(M/N)	BL21
pET-31b-KSI-CNP	pET-31b	KSI-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)
pET-32a-Trx-CNP	pET-32a	Trx-CNP fusion protein (soluble)	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)
pMAL-CNP	pMAL-c2X	MBP-CNP fusion protein (soluble)	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)

CNP: GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Gly-CNP37 (SEQ ID NO: 75);
TAF: human transcription factor TAF12;
KSI: ketosteroid isomerase;
MBP: maltose-binding protein;
Trx: thioredoxin

Expression of CNP Fusion Proteins in E. coli

CNP fusion protein expression plasmids were transformed into E. coli BL21 or BL21(DE3). Transformed cells were plated on LB plates containing 100 ug/ml carbeniciline or 50 ug/ml kanamycin and incubated overnight at 37° C. One single colony was picked and cultured in 4 ml LB medium containing 100 ug/ml of carbeniciline or 50 ug/ml kanamycin at 37° C. with shaking. When an OD_{600} of bacterial culture reached 0.6, 1 mM isopropyl β-D-1-thio-galactopyranoside (IPTG) was added to the cell media and the media was incubated at 37° C. for 3 hours with shaking. For cell harvest, bacterial cells were centrifuged at 4000 rpm for 10 minutes and the cell pellets were stored at −80° C. Cell pellets were lysed with B-PER II Bacterial Extraction Reagent (PIERCE, 0.4 ml per 4 ml of bacterial culture) and Benzonase Nuclease (Novagen, 0.025 U/ml) at room temperature for 10 minutes. Bacterial crude extract was reserved and centrifuged to obtain supernatant. Supernatant and crude extract were assayed for CNP fusion protein expression and solubility by SDS-PAGE and Western Blot.

Detection of CNP Fusion Protein Expression with SDS-PAGE and Western Blot

Ten uL of cell lysates or soluble supernatants was run on sodium dodecyl sulfate-polyacrylimide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, Calif., NuPAGE 4-12%

Bis-tris Gel, MES SDS buffer). The gel was stained using 20 ml Imperial Protein Stain (Thermo Fisher, Rockford, Ill.) at room temperature for 1 hour and de-stained with water. For Western blot, the protein was transferred to membrane with Gel blot (Invitrogen). The membrane was blocked in TBS buffer with 5% milk at room temperature for 1 hour. Rabbit anti-CNP22 antibody (1:2500 dilution) (Bachem, Torrance, Calif.) was added to the membrane, which was then incubated at room temperature with shaking for 2 hours, and then the membrane was washed 3 times with TBS buffer.

Alkaline phosphate (AP) conjugated anti-rabbit IgG (1:5000 dilution) was added to the membrane, which was then incubated at room temperature with shaking for 1 hour, and then the membrane was washed 3 times with TBS buffer. Ten ml WESTERN BLUE®Stabilized Substrate (Promega, Madison, Wis.) was added to the membrane, which was then incubated at room temperature with shaking for 1 to 5 min, and then the membrane was washed with TBS buffer to remove excess stain.

Expression of TAF-CNP Fusion Protein in E. coli BL21

Cells (E. coli strain BL21) expressing TAF-CNP fusion protein were obtained from glycerol stock stored at −80° C. and were grown in 4 ml LB medium containing 50 ug/ml of kanamycin at 37° C. overnight with shaking (250 rpm). Four ml of overnight grown cell culture was transferred to 200 ml LB medium containing 50 ug/ml of kanamycin and was grown at 37° C. with shaking (250 rpm). When the OD_{600} reached 0.6, IPTG was then added to a final concentration of 1 mM to induce protein expression at 37° C. with shaking (250 rpm) for 3 hours. Cells were then spun down at 3000 rpm for 10 minutes and the resulting cell pellet was frozen at −80° C.

Purification of TAF-CNP Inclusion Bodies and Formic Acid Cleavage

The cell pellet (from 200 ml culture) was resuspended in 25 ml of B-PER II buffer (PIERCE), the pellet was sonicated for 10 minutes (50%, 1 second, pause 2 seconds) on ice, centrifuged at 12000 rpm for 20 minutes at 4° C., and then the pellet was resuspended in 25 ml 20× diluted B-PER II buffer. This was repeated until the supernatant became clear (3-5 times). One mL of resuspended TAF-CNP inclusion bodies was transferred to a 1.5 ml tube and centrifuged at 14000 rpm for 15 min. The supernatant was discarded and the pellet was dissolved with 10 ul of 88% formic acid, and then 490 ul of Millipore filtered water was added immediately. The pellet was mixed well by vortex and incubated at 55° C. for 20 to 24 hours (70° C./6 hours are alternative conditions). The products of the formic acid cleavage were assayed by SDS-PAGE and LC/MS (C4RP).

LC/MS Sample Preparation

Inclusion bodies were isolated from about 8 mL of culture (about 1.5 OD) and the pellet was solubilized in 10 uL nt formate. Resolubilized pellet was immediately diluted to 2% or 10% final formate concentration (0.5 mL) and incubated at 55° C. for 21 hours (pH 2) (cloudiness was more evident in the 2% formate sample the next day). Both samples were centrifuged at 15000 rpm for 2 minutes. Twenty uL of supernatant was injected into an LC/MS (C4 RP) apparatus.

Results

CNP Fusion Proteins were Expressed in E. coli

All CNP fusion proteins were expressed in E. coli induced with 1 mM IPTG at 37° C. for 3 hours (FIG. **1**). The constructs pJexpress-TAF-CNP, pJexpress-KSI-CNP(M/N) and pET31b-KSI-CNP were expressed as inclusion bodies, while the constructs pET-32a-Trx-CNP and pMAL-CNP were expressed as soluble fusion proteins. Western blot using anti-CNP22 antibody confirmed the expression of the CNP fusion proteins (FIG. **1**).

CNP was Produced from TAF-CNP Inclusion Bodies by Formic Acid Cleavage

TAF-CNP inclusion bodies were partially purified and treated with 2% formic acid at 55° C. for 20 to 24 hours (70° C./6 hours are alternative conditions). The majority of TAF-CNP was cleaved and one extra band having similar size as Gly-CNP**37** peptide appeared on SDS-PAGE (FIG. **2**). The cleaved sample was further analyzed by LC/MS (C4 RP). The LC/MS results showed that CNP was released in soluble form from TAF-CNP inclusion bodies after formic acid cleavage. LC/MS analysis indicated that formic acid cleavage of the CNP fusion proteins resulted in formation of cyclized Pro-Gly-CNP37 (MW=4102). Calculation of protein amounts based on analysis suggested that about 60 ug of formic acid-generated CNP was produced from 8 mL of very low OD culture. From a small scale (e.g., about 8 mL) of low OD (e.g., 1.2 OD) cell culture, approximately 8 ug/ml CNP was produced, while fermentation of a larger scale (e.g., about 8 L) of higher OD (e.g., 380D) cell culture can produce approximately 1 mg/ml CNP.

Conclusion

Five expression constructs were generated to express CNP fusion proteins. Expression of all five constructs produced soluble (Trx and MBP) or insoluble (TAF and KSI) CNP fusion proteins. Approximately 1 mg/ml soluble CNP can be produced from TAF-CNP inclusion bodies by a simple formic acid cleavage procedure.

Example 2B

Production of Additional CNP Variants in E. coli

Recombinant production of CNP variants was carried out as described in Example 2A. In this example, additional CNP constructs were generated with a QuikChange II XL sitedirected mutagenesis kit (Stratagene) or were synthesized by DNA2.0. Additional CNP constructs and expression vectors are listed in Table 7.

TABLE 7

Construct	Vector	Product	Chemical cleavage	Final product	Expression *E. coli* strain
pJexpress-TAF(C/A)-Pro-CNP38	pJexpress401	TAF(C/A)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF(4D/4E)-Pro-CNP38	pJexpress401	TAF(4D/4E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)

TABLE 7-continued

Construct	Vector	Product	Chemical cleavage	Final product	Expression *E. coli* strain
pJexpress-TAF (C/A&4D/4E)-Pro-CNP38	pJexpress401	TAF(C/A&4D/4E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF (C/A&10D/10E)-Pro-CNP38	pJexpress401	TAF(C/A&10D/10E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF-NL-(C/A & 6D/6E)-Pro-CNP38	pJexpress401	TAF-NL-(C/A & 6D/6E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-BMP-Pro-CNP38	pJexpress401	BMP-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21; BL21(DE3)
pJexpress-TAF-Pro-CNP37	pJexpress401	TAF-Pro-CNP37 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP37	BL21; BL21(DE3)
pJexpress-BMP-Pro-CNP37	pJexpress401	BMP-Pro-CNP37 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP37	BL21; BL21(DE3)
pJexpress-TAF Pro-HSA-CNP	pJexpress401	TAF-Pro-HSA-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-HSA-CNP	BL21
pJexpress-TAF	pJexpress401	TAF inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-BMP	pJexpress401	BMP inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF(C/A)	pJexpress401	TAF(C/A) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF(4D/4E)	pJexpress401	TAF(4D/4E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF (C/A&4D/4E)	pJexpress401	TAF(C/A&4D/4E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF (C/A&10D/10E)	pJexpress401	TAF(C/A&10D/10E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF-NL-(C/A & 6D/6E)	pJexpress401	TAF-NL-(C/A&6D/6E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF-Pro-CNP53	pJexpress401	TAF-Pro-CNP53 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP53	BL21(DE3)
pJexpress-TAF-CNP34	pJexpress401	TAF-CNP34 inclusion bodies	Formic acid (Asp-Pro)	CNP34	BL21(DE3)

CNP38: GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Gly-CNP37 (SEQ ID NO: 75)];

Pro-CNP38: PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Pro-Gly-CNP37] (SEQ ID NO: 145);

CNP37: QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 60);

HSA-CNP: GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC [HSA-CNP27 (SEQ ID NO: 144)];

Pro-CNP53: PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIG-SMSGLGC (SEQ ID NO: 185);

CNP34: PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 163);

TAF-Pro-CNP38: MVLTKKKLQDLVREVCPNEQLDEDVEEMLLQIADDFIESVVTAA-CQLARHRKSSTLEVKDVQLHLERQWNM-WIMGSSHHHHHHSSGLVPRGSHT-GDDDDKHMDPGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 196);
TAF: human transcription factor TAF12 histone fold domain (HFD) and linker from pET-15b vector;

TAF-NL: TAF12 HFD without a linker;

TAF12 HFD: VLTKKKLQDLVREVCPNEQLDEDVEEMLLQIADDFIESVVTAACQLA-RHRKSSTLEVKDVQLHLERQWNMWI (SEQ ID NO: 197);
pET-15b linker: MGSSHHHHHHSSGLVPRGSHTGDDDDKHMD (SEQ ID NO: 195);

TAF(C/A): cysteine in TAF is changed to alanine;

TAF(D/E): aspartic acid in TAF is changed to glutamic acid (number indicates which amino acid residue is changed);
TAF(C/A & D/E): cysteine and aspartic acid in TAF are changed to alanine and glutamic acid, respectively;
BMP: bone morphogenetic protein 2 with seven C/A (cysteine to alanine) mutations;

KSI: ketosteroid isomerase;

MBP: maltose-binding protein;

TRX: thioredoxin

Results

All CNP fusion proteins were expressed in E. coli induced with 1 mM IPTG at 37° C. for 3 hours. Constructs pJexpress-BMP-Pro-CNP38, pJexpress-TAF-Pro-CNP37, pJexpress-BMP-Pro-CNP37, pJexpress-Pro-HSA-CNP, pJexpress-TAF and pJexpress-BMP were expressed as inclusion bodies. A Western Blot with anti-CNP antibody was used to confirm the expression (FIG. **3**).

Pro-Gly-CNP37 ("Pro-CNP38") was Produced from TAF-Pro-CNP38 Inclusion Bodies by Formic Acid Cleavage

Formic acid is used as a denaturant for inclusion body proteins and can specifically cleave the peptide bond between Asp and Pro under optimized conditions. TAF-Pro-CNP inclusion bodies were partially purified as described above and treated with 50% formic acid at 25° C., 37° C., 42° C. and 55° C. for 24 hours. Most of the TAF-Pro-CNP38 was cleaved and one extra band with a similar size as the Gly-CNP37 ("CNP38") peptide showed on SDS-PAGE from the 37° C., 42° C. and 55° C. cleavages (FIG. **4**A). The cleavage reactions at 37° C. and 55° C. were neutralized with 10 M NaOH and centrifuged at 14,000 rpm for 15 minutes. The un-cleaved TAF-Pro-CNP38, TAF and other inclusion bodies precipitated in the pellets. The supernatants contained soluble Pro-CNP38 and were further analyzed by LC/MS. The LC/MS result showed the supernatants contained a mixture of non-specific cleaved peptides generated by excess acidic hydrolysis.

When TAF-Pro-CNP inclusion bodies were treated with 2% and 10% formic acid at 55° C. for 20 hours, the majority of TAF-Pro-CNP38 was cleaved and one extra band having a similar size as Gly-CNP37 was observed on SDS-PAGE (FIG. **4**B). The cleaved sample was further analyzed by LC/MS. The LC/MS analysis showed that correct Pro-CNP38 was released in soluble form from TAF-Pro-CNP38 inclusion bodies after formic acid cleavage. The yields of Pro-CNP38 from 2% and 10% formic acid cleavages were similar (FIG. **4**C).

Formic Acid Cleavage and Neutralization for Pro-Gly-CNP37 ("Pro-CNP38") Production and Purification

Formic acid can dissolve and cleave TAF-Pro-CNP38, BMP-Pro-CNP38 and other inclusion bodies. The pH neutralization results in precipitation of insoluble contaminating proteins/peptides (un-cleaved TAF-Pro-CNP38 and BMP-Pro-CNP38, TAF, BMP and others). The soluble Pro-CNP38 stays in the supernatant after centrifugation. TAF-Pro-CNP38 and BMP-Pro-CNP38 were cleaved in 2% formic acid at 55° C. or 70° C. for 24 hours. The cleavage reactions were neutralized with 1:1 ratio of 0.5 M Tris buffer and centrifuged at 14,000 rpm for 15 minutes. Results showed that the supernatants contained almost pure peptides and there was no observed recovery loss of Pro-CNP38 upon neutralization. This is a simple and efficient step for Pro-CNP38 purification.

Analysis of Varying Conditions for Formic Acid Cleavage of TAF-Pro-CNP38 Inclusion Bodies for Pro-Gly-CNP37 ("Pro-CNP38") Production

Formic acid can specifically cleave the peptide bond between Asp and Pro under optimized conditions. Non-specific cleavage of peptide bonds between Asp and any other amino acids or even non-specific cleavage of any peptide bond may occur if the formic acid cleavage conditions are not optimized. TAF-Pro-CNP38 inclusion bodies were cleaved with 2% formic acid at 42° C., 55° C. or 70° C. for 6, 24 or 48 hours. FIG. **5**A shows that TAF-Pro-CNP38 was cleaved completely at 70° C. for 24 hours or at 55° C. for 48 hours. The 70° C. cleavage could be completed within 17 hours (FIG. **5**B). Although the 70° C./24 h cleavage gave the highest yield, the non-specific cleavage products (for example, the peptide with molecular weight of 3142 generated from ProCNP38 by cleaving between peptide bond Asp and Arg) increased dramatically (FIG. **5**C).

The yield and purity of Pro-CNP38 production were improved when TAF-Pro-CNP38 inclusion bodies were purified or treated with B-PER II buffer before formic acid cleavage. Because B-PER II buffer contains detergent octylthioglucoside and is relatively expensive, other commonly used detergents or buffers were tested for large-scale Pro-CNP38 production. TAF-Pro-CNP38 inclusion bodies were re-suspended in different detergents (Octylsucrose; Triton x-100; Tween-20; NP-40; CA-630) or buffers (B-PER II; B-PER II 1/20 dilution; B-PER; B-PER phosphate buffer; 25 mM tris, 150 mM NaCl, pH 7.9; 25 mM tris, pH 7.5; filtered water; PBS) and incubated at room temperature (RT) for 24 hours. All detergents were 1% in 25 mM tris buffer, pH 7.5. After incubation in detergent or buffer, TAF-Pro-CNP38 inclusion bodies were cleaved by 2% formic acid at 55° C. for 22 hours. Results showed that BPER II continued to exhibit good yield, and positive results were also obtained with CA630 and Triton X-100.

Pro-Gly-CNP37 ("Pro-CNP38") Proteolytic Cleavage Products

One unidentified protease, possibly a membrane associated protease, cleaved the Pro-CNP38 peptide (produced from BL21 strain, MW 4102) into two peptides during Pro-CNP38 purification, resulting in peptides PGQEHPNAR (MW 1004) (SEQ ID NO: 198) and KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (MW 3115) (SEQ ID NO: 199). Not to be bound by theory, one possible reason why detergents may improve the yield and purity of Pro-CNP38 is that detergents can remove most, but possibly not all, of the unidentified protease. High temperature, basic pH and EDTA were tested to identify if these agents could inhibit the protease cleavage. TAF-Pro-CNP38 inclusion bodies were incubated at RT or 120° C. for 2 hrs and centrifuged at 14000 rpm for 15 min. The pellets were re-suspended in 2% formic acid and incubated at 55° C. or 70° C. for 18 hours. A 1:1 ratio of 0.5 M Tris was added to neutralize the cleavage. The neutralized samples were centrifuged at 14000 rpm for 5 min and the supernatants were left at RT for 6 hrs or 22 hrs with or without 10 mM EDTA, pH 10. The proteolytic cleavage was assayed by LC/MS (Table 8).

TABLE 8

LC/MS result of Pro-CNP38 proteolytic cleavage

Sample		A210 Peak Area	Protein Conc. (mg/mL)		Percent MW4102	Percent MW3115
1	H 55 C.	942	0.04	*	91.8	8.2
2	H 70 0	2878	0.11	*	79.2	20.7
3	H 70 6H	2675	0.10	*	80.6	19.4
4	H 70 24	2741	0.11	*	79.2	20.7
5	H 70 EDTA	2385	0.09	*	80.8	19.2
6	H 70 pH 10	1917	0.07	*	81.2	18.8
7	55 C.	1291	0.05		61.1	38.9
8	70 C. 0	4533	0.18		96.8	3.2
9	70 C. 6H	4120	0.16		97.5	2.5
10	70 C. 24	4108	0.16		96.5	3.5
11	70 C. EDTA	4336	0.17		96.9	3.1
12	70 C. pH 10	3425	0.13		97.5	2.4

All samples (8-12) cleaved at 70° C. showed limited proteolytic cleavage (less than 4% cleavage of Pro-CNP38). Almost 40% of Pro-CNP38 was cleaved by protease when cleavage was carried out at 55° C. (sample 7). Basic pH and EDTA did not influence the non-specific proteolytic cleavage. High temperature (120° C. for 2 hrs) non-specifically cleaved Pro-CNP38.

It should be noted that the BL21(DE3) strain from Stratagene does not have the unidentified protease.

Production of Pro-Gly-CNP37 ("Pro-CNP38") and Other CNP Variants from Different Constructs:

Pro-CNP38 can be produced in large scale in E. coli by means of overexpression of a TAF-Pro-CNP38 fusion protein as inclusion bodies followed by formic acid cleavage of the fusion protein. Following the methods described herein, other TAF-CNP fusion proteins (TAF-CNP34 and TAF-Pro-CNP53) were expressed as inclusion bodies and then cleaved with formic acid to generate the CNP variants CNP34 and Pro-CNP53. FIG. **6** depicts the expression of TAF-CNP34, FIG. **7** the expression of TAF-Pro-CNP53, FIG. **8** the products of formic acid cleavage of TAF-CNP34 and TAF-Pro-CNP53, FIG. **9** the peak for CNP-34 in an LC/MS chromatogram, and FIG. **10** the peak for Pro-CNP53 in an LC/MS chromatogram.

Use of formic acid may result in unspecific cleavage(s) at peptide bond(s) other than the targeted Asp-Pro bond. To improve the purity and overall titer of the desired formic acid cleavage product, different residues of aspartic acid in TAF12 or fragments thereof were changed to glutamic acid. Moreover, one or more cysteine residues in TAF12 or fragments thereof were changed to alanine to prevent unspecific disulfide bond formation. All TAF-Pro-CNP38 fusion proteins having such mutations in TAF12 were expressed as inclusion bodies and cleaved with formic acid to produce Pro-CNP38. FIG. **7** shows the expression of TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38, FIG. **11** the expression of TAF(C/A & 4D/4E)-Pro-CNP38 and TAF(4D/4E)-Pro-CNP38, FIG. **12** the products of formic acid cleavage of TAF(4D/4E)-Pro-CNP38 and TAF(C/A & 4D/4E)-Pro-CNP38, and FIG. **13** the products of formic acid cleavage of TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38. Table 9 summarizes the purity (prior to purification) and titer of Pro-CNP38 obtained from the various TAF-Pro-CNP38 constructs.

TABLE 9

Construct	Purity	Titer (ug/mL)
pJexpress-TAF-Pro-CNP38	32%	44
pJexpress-TAF(C/A)-Pro-CNP38	41%	50
pJexpress-TAF(4D/4E)-Pro-CNP38	36%	52
pJexpress-TAF(C/A & 4D/4E)-Pro-CNP38	42%	58
pJexpress-TAF(C/A & 10D/10E)-Pro-CNP38*	32%	26
pJexpress-TAF-NL-(C/A & 6D/6E)-Pro-CNP38	50%	55

*Cells with pJexpress-TAF(C/A&10D/10E)-Pro-CNP38 growed more slowly and the final cell density (OD_{600}) was lower compared to other TAF-Pro-CNP38 constructs.

Large-scale Production of Pro-Gly-CNP37 ("Pro-CNP38") by Fermentation and Formic Acid Cleavage

BL21(DE3) cells comprising the pJexpress-TAF-CNP construct were grown in a 10 liter fermenter at 37° C. for about 16-17 hours until OD_{600} reached 64. The cells were then grown/cultured in the presence of 1 mM IPTG at about 35-37° C., to induce expression of the TAF-Pro-CNP38 fusion protein, for about 7-8 hours until OD_{600} reached 160. The fermentation produced a titer of 9 g/L TAF-Pro-CNP38. FIG. **14** displays a Western blot of the TAF-Pro-CNP38 fusion protein produced in the fermentation.

A cell pellet recovered from 750 mL cell culture from the 10 L fermentation was resuspended in phosphate-buffered saline, pH 7.4 (PBS) and lysed by three passes through a pressure homogenizer (10,000 bar). The resulting lysate was centrifuged at 6,500 g for 10 minutes and the supernatant was discarded. The pellet fraction containing insoluble TAF-Pro-CNP38 fusion protein inclusion bodies was resuspended in 500 mL PBS with a rotostator. The suspension was centrifuged at 6,500 g for 10 minutes and the supernatant was discarded. The resulting inclusion body pellet was resuspended in 500 mL water with a roto stator and incubated at 55° C. for 30 minutes. 250 mL 6% formic acid was added to the warmed inclusion body suspension for a final concentration of 2% formic acid and incubated at 55° C. for 20-24 hours. 50 mL of 400 mM Na_2HPO_4 was added after 20-24 hours to begin the neutralization of the formic acid cleavage reaction, and the resulting mixture was titrated with 50% w/v NaOH to pH 6.9-7.4 and left at room temperature for 30 minutes. Upon neutralization, a heavy precipitate formed and was removed by centrifugation at 6,500 g for 10 minutes. The supernatant was retained and contained on average 1.3 g/L culture of 80% pure Pro-CNP38. The majority of the E. coli and TAF-related proteins and peptides remained in the pellet.

The soluble Pro-CNP38 resulting from the formic acid cleavage and neutralized supernatant was 80% pure and contained a mixture of linear and cyclized Pro-CNP38 peptides along with other product-related impurities. The pH neutral supernatant containing Pro-CNP38 was sterile-filtered. Further purification by anion-exchange chromatography using a Fractogel TMAE Hi-CAP column (EMD Biosciences) to remove DNA, endotoxins, and peptide contaminants, which should bind to the column, was performed at pH 7-7.4. The flow-through fraction contained partially cyclized Pro-CNP38. Cupric sulfate was added to the flow-through fraction to a final concentration of 10 uM and incubated at room temperature for 1 hour. The addition of Cu^{++} at neutral pH catalyzed the oxidation of free cysteine sulfhydryl groups on the peptide to form an intramolecular disulfide bond, resulting in 100% cyclized Pro-CNP38 and no detectable linear peptide. The conductivity of the solution was adjusted to <15 mS/cm by the addition of water. Cation-exchange chromatography using an SP-Sepharose column (GE Healthcare) and a sodium phosphate buffer (pH 7) was then performed to remove any remaining DNA and endotoxins in the flowthrough and further purify Pro-CNP38 to about 95-96% purity with <0.5% non-product related impurities. FIG. **15** is an SDS-PAGE of eluate fractions from the SP-Sepharose column; the highest concentrations of Pro-CNP38 were found in fractions 22 to 30. Reverse-phase HPLC/MS analysis of non-pooled fraction 24 indicated the presence of 90% Pro-CNP38, 5% Pro-CNP38 with an oxidized methionine residue, 3% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17, and 1.6% Pro-CNP38 cleaved at the Asp-Arg bond in the cyclic domain. The yield of pure Pro-CNP38 peptide upon final purification was 0.9 g/L cell culture (36% total recovery).

Pro-CNP38 collected from five separate purifications was pooled for formulation. The pooled product contained 93.5% Pro-CNP38, 3.3% Pro-CNP38 with an oxidized methionine residue, 1.3% deamidated Pro-CNP38, and 1% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17. Samples were diluted with 50 mM sodium phosphate (pH 7) to a conductivity of 10 mS/cm and loaded onto a CM-Sepharose column (GE-Healthcare) for concentration and buffer exchange. The weak cation-exchange property of the CM-Sepharose resin allows peptides to dissociate from the column with weak acid solutions rather than typical salt gradients. Acid concentrations required for dissociation of Pro-CNP38 from the CM-Sepharose column depended upon column loading. At 50 mg Pro-CNP38 per mL of resin, 10 mM HCl was sufficient for Pro-CNP38 elution. At 9 mg Pro-CNP38 per mL of resin, 50 mM HCl was required for

elution. When the loading was 9 mg Pro-CNP38 per mL of resin, Pro-CNP38 eluted in less than one column volume, which significantly concentrated the peptiude. The eluate fraction contained 20.3 mg/mL of 95% pure Pro-CNP38, along with 3% Pro-CNP38 with an oxidized methionine residue, 1% deamidated Pro-CNP38, and <1% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17. The concentrated solution of Pro-CNP38 in weak acid is suitable for dilution into appropriate buffers for either liquid or lyophilized formulations. cl Example 3

Cleavage of CNP Variants by Neutral Endopeptidase In Vitro

To determine the effects of amino acid substitutions, amino acid extensions, backbone modifications, side chain modifications and PEGylation on the susceptibility of CNP variants to neutral endopeptidase (NEP) cleavage, peptide cleavage assays were carried out using an in vitro assay that monitored disappearance of the non-cleaved CNP variant.

Recombinant human NEP (1 ug/mL final concentration) was added to 100 uM CNP variant diluted in 0.1 M Tris, pH 7. The reaction mixture was incubated at 37° C. for various periods of time, and the reaction was quenched with EDTA (10 mM final) followed by heat denaturation. The reaction mixture was reduced and then the reaction products were analyzed using HPLC and mass spectroscopy. The half-life of the CNP variant was calculated based on the disappearance of intact CNP variant over time. The results for digested CNP variants were compared to a parallel wtCNP22 digestion and normalized to the results for 100 uM CNP22 digested by 1 mg/mL NEP ($t_{1/2}$=80 min).

Table 1 lists the half-lives, based on the in vitro NEP cleavage assay, of various CNP variants having backbone or side chain modifications. Removal of three of the six NEP cleavage sites in Analog L nevertheless resulted in a substantially shorter half-life. Of tested CNP variants, the greatest resistance to NEP cleavage was exhibited by Analog N, which contains the D-enantiomer of all 22 amino acids of CNP22, and by Analog M, which has an N-methylated amide bond at both Leu9 and Leu11. However, both Analogs N and M failed to stimulate production of cGMP (see below).

Comparing the half-lives of Analogs A, B, E, F, G and H to one another, half-lives were determined to be about 1.5- to about 2.5-fold longer for Analogs E and G compared to those for Analogs A, B, F and H. All of these six analogs showed resistance or improved resistance to cleavage at the Cys6-Phe7 bond relative to wtCNP22 (data not shown). The rank order of analog resistance to NEP at 1 ug/ml, based on half-life, is Analog G (3-Cl-Phe)≤Analog E (D-Phe)>Analog H ("beta-2 Phe"), Analog B (N-Me-Phe), and Analog F (t-Bu-Gly)=wtCNP22>Analog A (Cys-CH_2—NH). Analogs E and G have about 1.5 times longer half-life in comparison to wtCNP22. Besides resistance to cleavage of the Cys6-Phe7 bond, Analogs B, E, F, G and H also exhibited resistance to cleavage of the Gly8-Leu9 bond in the presence of 1 ug/mL NEP (data not shown). These results indicate that CNP variants having backbone or side chain modifications between Cys6 and Gly8 can be resistant to NEP cleavage of the Cys6-Phe7 bond and/or Gly8-Leu9 bond, but do not necessarily have improved overall resistance to NEP or a longer half-life than CNP22. The results seem to be contrary to reports in the literature that NEP first cleaves at the Cys6-Phe7 bond of CNP22 and then elsewhere.

TABLE 1

Analog	Backbone and Side Chain Modifications / Natriuretic Peptide	cGMP Response rel. to 1 uM CNP22[1] 10 nM	cGMP Response rel. to 1 uM CNP22[1] 1 uM	NEP Cleavage ($t_{1/2}$, min)
	CNP22 (SEQ ID NO: 1)	46 ± 10	100 ± 13	80[2]
N	D-CNP22 (all D-amino acids) (SEQ ID NO: 115)	2	1	>>160
A	CNP22, C6—CH2—NH (reduced carbonyl) (SEQ ID NO: 56)	6	66	55
B	CNP22, N-methyl-F7 (methylated amide) (SEQ ID NO: 57)	2	38	80
BD	CNP22, N-methyl-L9 (SEQ ID NO: 116)	2	8	ND
BN	CNP22, N-methyl-L11 (SEQ ID NO: 117)	10	51	ND
BE	CNP22, N-methyl-L20 (SEQ ID NO: 118)	2	5	ND
M	CNP22, N-methyl-L9, N-methyl-L11 (SEQ ID NO: 94)	1	11	>>160
K	CNP22, N-methyl-L9, N-methyl-L20 (SEQ ID NO: 92)	1	1	80
L	CNP22, N-methyl-L9, N-methyl-L11, N-methyl-L20 (SEQ ID NO: 93)	18	10	30
J	CNP22, C6—CH2—NH, N-methyl-L9, N-methyl-L20 (SEQ ID NO: 91)	ND	ND	50
E	CNP22, D-F7 (D-Phe) (SEQ ID NO: 136)	2	6	130
H	CNP22, Beta-2-F7 (3-amino-2-phenylpropionyl) (SEQ ID NO: 57)	2	2	80
G	CNP22, 3-chloro-F7 (SEQ ID NO: 137)	17	93	135
F	CNP22, t-butyl-G8 (SEQ ID NO: 58)	2	18	80
V	CNP22, K4G, 3,4-dichloro-F7 (SEQ ID NO: 119)	ND	ND	68
X	CNP22, K4G, 3-methyl-F7 (SEQ ID NO: 120)	ND	ND	68
	ANP	10	23	ND

[1]Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2]CNP22 NEP resistance $t_{1/2}$ averaged 80 min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80 min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 2 lists the half-lives, based on the in vitro NEP cleavage assay, of various CNP variants having substitutions with natural and/or unnatural amino acids. Of tested variants, the greatest resistance to NEP cleavage was shown by Analog BK, which has K4R and G15S substitutions, and Analog BJ, which has K4R and G15N substitutions.

TABLE 2

Analog	Specificity Mutations Natriuretic Peptide	cGMP Response relative to 1 uM CNP22[1] 10 nM	 1uM	NEP Cleavage ($t_{1/2}$, min)
	CNP22 (SEQ ID NO: 1)	46 ± 10	100 ± 13	80
AH	CNP22, K4R (SEQ ID NO: 35)	59	121	80
BP	CNP22, K4R, G5S (SEQ ID NO: 121)	45	ND	ND
BO	CNP22, K4R, G5R (SEQ ID NO: 122)	18	80	ND
P	CNP22, K4G (SEQ ID NO: 123)	ND	ND	68
Z	CNP22, K4R, F7Y (SEQ ID NO: 95)	2	18	ND
AB	CNP22, K4R, G8S (SEQ ID NO: 97)	26 ± 26	86 ± 17	ND
AA	CNP22, K4R, G8V (SEQ ID NO: 96)	3	25	ND
AC	CNP22, K4R, G8T (SEQ ID NO: 98)	11 ± 2	66 ± 16	80
AD	CNP22, K4R, L9T (SEQ ID NO: 99)	4	68	ND
BH	CNP22, K4R, K10R (SEQ ID NO: 112)	12	80	ND
BF	CNP22, K4R, K10Cit (SEQ ID NO: 110)	6	33	ND
BG	CNP22, K4R, K10Q (SEQ ID NO: 111)	9	45	ND
BY	CNP22, K4R, K10S (SEQ ID NO: 124)	16	53	ND
BK	CNP22, K4R, G15S (SEQ ID NO: 114)	13 ± 1	71 ± 11	≥160
BJ	CNP22, K4R, G15N (SEQ ID NO: 113)	4	41	150
AE	CNP22, K4R, G15R (SEQ ID NO: 100)	0.3	0.3	ND
AF	CNP22, K4R, G15Cit (SEQ ID NO: 101)	1.4	2	ND
BZ	CNP22, K4R, S16Q (SEQ ID NO: 125)	42	116	ND
BX	CNP22, K4R, M17N (SEQ ID NO: 126)	40 ± 2	103 ± 17	ND
AG	CNP22, K4R, M17V (SEQ ID NO: 102)	10	65	ND
BQ	CNP22, K4R, G19S (SEQ ID NO: 127)	21	63	ND
BR	CNP22, K4R, G19R (SEQ ID NO: 128)	22 ± 6	84 ± 10	ND
AJ	CNP22, K4R, L20V (SEQ ID NO: 103)	0.2	8	ND
AK	CNP22, K4R, L20t-butyl-Ala (SEQ ID NO: 104)	1	21	ND
AT	CNP22, G1E, K4E (SEQ ID NO: 105)	11	54	60
BS	CNP22, K4R, L20R (SEQ ID NO: 129)	11	8	ND
BT	CNP22, K4R, G21S (SEQ ID NO: 130)	7	39	ND
BU	CNP22, K4R, G21T (SEQ ID NO: 131)	6	21	ND
BW	CNP22, K4R, G21R (SEQ ID NO: 132)	20	21	ND
	ANP	10	23	ND

[1]Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2]CNP22 NEP resistance $t_{1/2}$ averaged 80 min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80 min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 3 lists the half-lives, based on the in vitro NEP cleavage assay, of CNP variants having N-terminal and/or C-terminal modifications, including amino acid extensions. Of the analogs tested, Analogs AZ, CC, CF, BL, CS, CK and CL, Pro-Gly-CNP37 and HSA-CNP27 were most resistant to NEP degradation.

TABLE 3

	N- and C-Terminal Modifications Natriuretic Peptide	cGMP Response rel. to 1 uM CNP22[1] 10 nM	 1 uM	NEP Cleavage ($t_{1/2}$, min)
	CNP22	46 ± 10	100 ± 13	80
BC	Pentanoic acid (N-term.)-CNP22, G1E (SEQ ID NO: 109)	ND	ND	ND
BB	Heptanoic acid (N-term.)-CNP22, G1E (SEQ ID NO: 108)	32 ± 4	84 ± 19	45-65
AV	Pentanoic acid (N-term.)-CNP22, G1E, K4E (SEQ ID NO: 106)	ND	ND	120
AW	Heptanoic acid (N-term.)-CNP22, G1E, K4E (SEQ ID NO: 107)	ND	ND	<20

TABLE 3-continued

	N- and C-Terminal Modifications	cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
	Natriuretic Peptide	10 nM	1 uM	($t_{1/2}$, min)
AX	CNP17 (delta N-term) (SEQ ID NO: 2)	18	69	ND
	R-CNP22 (SEQ ID NO: 40)	ND	ND	ND
AZ	R-CNP22, K4R (SEQ ID NO: 41)	54 ± 11	106 ± 15	≥160
	ER-CNP22 (SEQ ID NO: 38)	ND	ND	ND
BA	ER-CNP22, K4R (SEQ ID NO: 39)	38 ± 10	113 ± 10	90
	GANRR-CNP22 (SEQ ID NO: 65)	ND	ND	ND
AY	GANRR-CNP22, K4R (SEQ ID NO: 36)	59 ± 9	105 ± 20	65
	GANQQ-CNP22 (SEQ ID NO: 64)	ND	ND	ND
CH	GANQQ-CNP22, K4R (SEQ ID NO: 69)	44 ± 8	95 ± 11	ND
	GANPR-CNP22 (SEQ ID NO: 66)	ND	ND	ND
CI	GANPR-CNP22, K4R (SEQ ID NO: 37)	50 ± 1	105 ± 12	ND
	GANSS-CNP22 (SEQ ID NO: 67)	ND	ND	ND
CG	GANSS-CNP22, K4R (SEQ ID NO: 70)	27 ± 1	88 ± 1	95
CA	AAWARLLQEHPNA-CNP22 (SEQ ID NO: 61)	24	76	ND
CB	AAWARLLQEHPNAR-CNP22 (SEQ ID NO: 62)	36	84	ND
CC	DLRVDTKSRAAWAR-CNP22 (SEQ ID NO: 63)	34	101	>160
CF	GQPREPQVYTLPPS-CNP22 (IgG1(Fc) fragment) (SEQ ID NO: 79)	23 ± 9	72 ± 19	>160
	PNARKYKGANKK-CNP22 (CNP34)	ND	ND	ND
BL	QEHPNARKYKGANKK-CNP22 (CNP37) (SEQ ID NO: 60)	43 ± 15	97 ± 27	>>160
	PQEHPNARKYKGANKK-CNP22 (Pro-CNP37)	ND	ND	ND
CE	GERAFKAWAVARLSQ-CNP22 (HSA fragment) (SEQ ID NO: 81)	15	87	ND
CY	GQHKDDNPNLPRGANPR-CNP22 (HSA fragment) (SEQ ID NO: 80)	ND	ND	ND
CQ	GHHSHEQHPHGANQQ-CNP22 (HRGP fragment) (SEQ ID NO: 76)	16	95	ND
CX	GHHSHEQHPHGANPR-CNP22 (HRGP fragment) (SEQ ID NO: 78)	ND	ND	ND
CS	GQEHPNARKYKGANPK-CNP22 (modified CNP37) (SEQ ID NO: 129)	19	61	>>160
CT	GQEHPNARKYKGANQK-CNP22 (modified CNP37) (SEQ ID NO: 130)	60	121	ND
CU	GQEHPNARKYKGANQQ-CNP22 (modified CNP37) (SEQ ID NO: 131)	9	57	ND
DB	GQEHPNARKYKGANKK-CNP22 (Gly-CNP37) (SEQ ID NO: 75)	50 ± 14	98 ± 17	>>160
	PGQEHPNARKYKGANKK-CNP22 (Pro-Gly-CNP37)	49 ± 6	103 ± 17	>>160
CW	GQEHPNARKYKGANKP-CNP22 (modified CNP37) (SEQ ID NO: 74)	ND	ND	ND
CR	GAHHPHEHDTHGANQQ-CNP22 (HRGP fragment) (SEQ ID NO: 128)	14 ± 5	77 ± 12	ND
CZ	FGIPMDRIGRNPR-CNP22 (osteocrin "NPR-C inhibitor") (SEQ ID NO: 82)	ND	ND	ND

TABLE 3-continued

	N- and C-Terminal Modifications	cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
	Natriuretic Peptide	10 nM	1 uM	($t_{1/2}$, min)
DA	GKRTGQYKLGSKTGPGPK-CNP22 (FGF2 "heparin-binding domain" fragment) (SEQ ID NO: 83)	ND	ND	ND
CK	GQPREPQVYTGANQQ-CNP22, K4R (IgG1(Fc) fragment) (SEQ ID NO: 84)	2	32	≥160
CL	GVPQVSTSTGANQQ-CNP22, K4R (HSA fragment) (SEQ ID NO: 85)	3	35	>160
	GHKSEVAHRFKGANKK-CNP22 (HSA-CNP27) (SEQ ID NO: 144)	51 ± 9	109 ± 15	>>160
	PGHKSEVAHRFKGANKK-CNP22 (Pro-HSA-CNP27)	32	107	ND
CN	GQTHSSGTQSGANQQ-CNP22, K4R (fibrinogen) (SEQ ID NO: 87)	12	115	ND
CM	GQPSSSSQSTGANQQ-CNP22, K4R (fibronectin) (SEQ ID NO: 86)	ND	ND	ND
CO	GSTGQWHSESGANQQ-CNP22, K4R (fibrinogen) (SEQ ID NO: 88)	2	33	ND
CP	GSSSSSSSSSGANQQ-CNP22, K4R (zinc finger) (SEQ ID NO: 89)	ND	ND	ND
CD	SPKMVOGSG-CNP17-KVLRRH ("BNP tails") (SEQ ID NO: 68)	25	102	ND
CJ	RSSCFGGRIDRIGAC ("C-ANP4-23", ANP-derived) (SEQ ID NO: 133)	ND	ND	ND
	CNP22, K4R, K10R, N.-term.--N-term. dimer/disuccinimidyl glutarate (SEQ ID NO: 134)	19	44	ND
	CNP22, K4R, K10R, N-term.--N.-term. dimer/Bis-PEO5 (SEQ ID NO: 135)	19	41	ND
BM	CNP53 (SEQ ID NO: 4)	61	101	>>160
	ANP	10	23	ND

[1]Stimulation of cGMP production in N1H3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22
[2]CNP22 NEP resistance $t_{1/2}$ averaged 80 min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80 min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.
ND = Not Determined

Table 4 lists the half-lives, based on the in vitro NEP cleavage assay, of CNP variants conjugated to PEG (or PEO) polymers at the N-terminus. All the PEGylated CNP variants tested and shown in Table 4 displayed resistance or enhanced resistance to NEP cleavage except for PEO12-GANPR-CNP22(K4R), which had the same half-life as wtCNP22. N-terminal PEGylation of CNP22 having a K4G substitution does not seem to confer substantial improvement in NEP resistance. For example, PEG2K-CNP22 (K4G) was only slightly more resistant to NEP cleavage than CNP22 (data not shown), whereas PEG2K-CNP22 had a much longer half-life in vitro than CNP22.

TABLE 4

N-Terminal PEGylation		cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
Natriuretic Peptide	PEG	10 nM	1 uM	($t_{1/2}$, min)
CNP22		46 ± 10	100 ± 13	80
CNP22	PEG20K	0	15	>>160
CNP22	PEG5K	8 ± 1	20 ± 7	>>160
CNP22	PEG2K	6 ± 2	32 ± 4	>>160
CNP22	PEO4-$(PEO12)_3$ (branched)	17 ± 1	52 ± 6	>>160
CNP22	PEO24 (1.2 kDa)	8 ± 5	46 ± 10	>>160
CNP22	PEG1K	15 ± 3	68 ± 17	>160
CNP22	PEO12 (0.6 kDa)	12 ± 7	57 ± 18	160
CNP22	(PEO12)-Biotin	19	81	140

TABLE 4-continued

N-Terminal PEGylation		cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
Natriuretic Peptide	PEG	10 nM	1 uM	($t_{1/2}$, min)
CNP22, K4G	(PEO12)-Biotin	10	27	100
CNP22, K4R	PEO24	15	56	ND
CNP22, K4R	PEO12	13	44	ND
CNP-17	PEG2K	5	50	>
R-CNP22, K4R (SEQ ID NO: 41)	PEO24	15 ± 2	75 ± 12	ND
R-CNP22, K4R (SEQ ID NO: 41)	PEO12	23 ± 2	93 ± 19	≥160
ER-CNP22, K4R (SEQ ID NO: 39)	PEO24	6 ± 2	60 ± 10	ND
ER-CNP22, K4R (SEQ ID NO: 39)	PEO12	20 ± 1	92 ± 25	ND
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEG2K	15 ± 2	45 ± 18	ND
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEO24	28 ± 9	82 ± 18	>>160
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEG1K	15 ± 0.4	56 ± 23	>160
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEO12	40 ± 2	99 ± 13	>160
GANQQ-CNP22, K4R (SEQ ID NO: 69)	PEO24	16 ± 13	73 ± 30	ND
GANQQ-CNP22, K4R (SEQ ID NO: 69)	PEO12	30	78	ND
GANPR-CNP22, K4R (SEQ ID NO: 37)	PEO24	ND	ND	ND
GANPR-CNP22, K4R (SEQ ID NO: 37)	PEO12	ND	ND	80
GANSS-CNP22, K4R (SEQ ID NO: 70)	PEO24	8 ± 5	46 ± 21	ND
GANSS-CNP22, K4R (SEQ ID NO: 70)	PEO12	8 ± 0.3	52 ± 13	ND

[1]Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22
[2]CNP22 NEP resistance $t_{1/2}$ averaged 80 min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80 min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.
ND = Not Determined

FIG. **16** shows the NEP resistance profile of five N-terminal PEGylated conjugates of CNP22. The CNP22 peptides conjugated to PEG (or PEO) polymers of increasing mass exhibited increasing resistance to NEP degradation. In particular, PEO24-CNP22, PEG2K-CNP22 and PEG5K-CNP22 were resistant to NEP degradation over the assay period of 160 minutes.

FIG. **17** displays the NEP resistance profile of CNP variants CNP37 (Analog BL), CNP53 and GANRR-CNP22 (K4R) (SEQ ID NO: 36) having an N-terminal amino acid extension. As can be clearly seen, both CNP37 and CNP53 were resistant to NEP degradation in this in vitro assay, whereas GANRR-CNP22(K4R) (SEQ ID NO: 36) had the same lability to NEP hydrolysis as CNP22.

FIG. **18** depicts the NEP resistance profile of CNP17 and GANRR-CNP22(K4R) (SEQ ID NO: 36) conjugated to a PEG (or PEO) moiety at the N-terminus. PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) greatly improved the NEP resistance of this CNP variant, with PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) being completely resistant to NEP cleavage over the assay period of 160 minutes. Increasing the mass of the PEO moiety from about 0.6 kDa (PEO12) to about 1.2 kDa (PEO24) improved the NEP resistance of PEGylated GANRR-CNP22(K4R) (SEQ ID NO: 36). PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) to a monodispersed PEO24 moiety rather than a polydispersed PEG1K moiety also improved NEP resistance. Finally, although both PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) and PEG2K-CNP17 have a similar total mass (keeping in mind that PEG2K is polydispersed), the former displayed substantially better NEP resistance.

NEP resistance assays were also performed on wtCNP22 and CNP variants G-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA", SEQ ID NO: 144) and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36). FIG. **19** shows that G-CNP37 and CNP27-HSA were completely resistant to NEP cleavage, and CNP27-PEO12 exhibited much greater stability to NEP degradation compared to wtCNP22.

Example 4

CNP Variant Stimulation of cGMP Production in NIH3T3 Cells

To determine the functional activity of CNP variants, the production of cGMP was measured in NIH3T3 cells exposed to the CNP variants. Murine NIH3T3 cells express endogenously the CNP signaling receptor, NPR-B, which shares 98% protein sequence identity with human NPR-B. NIH3T3 cells were cultured in high glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and antibiotics at 37° C. with 5% CO). Twenty four to 48 hours prior to signaling, cells were passaged to 12-well plates with a density of 2-5×10^5 cells per well at the time of the assay. CNP variants were resuspended in 1 mM HCl to a stock concentration of 1 mg/mL (455 uM for wtCNP22) and subsequently diluted to a 30 uM working stock solution with phosphate-buffered saline (PBS). Ten-fold serial dilutions were prepared in phosphate-buffered saline. Culture medium was removed from the cells and replaced with 0.4 mL PBS/Dulbecco's modified Eagle medium (50/50, v/v) containing 0.75 mM isobutylmethylxanine. Plates were incubated at 37° C., 5% CO_2 for 15 minutes before addition of 0.2 mL CNP variant in PBS and continued incubation at 37° C. for 15 minutes. Reactions were stopped by the addition of 0.2 mL lysis buffer supplied with the CatchPoint cGMP assay kit (Molecular Devices), and cGMP production was determined with the CatchPoint cGMP Assay (Molecular Devices). All stimulation experiments were performed in duplicate.

Tables 1-4 summarize the ability of CNP variants having backbone or side chain modifications, amino acid substitutions, N-terminal amino acid extensions, and/or N-terminal PEGylation, respectively, to stimulate cGMP production in NIH3T3 cells. In all four tables, the values for cGMP production in NIH3T3 cells exposed to 10 nM or 1 uM CNP variant are normalized to cell number and cGMP production in the presence of 1 uM wtCNP22.

Regarding the results in Table 1, only Analog G having 3-Cl-Phe at position 7 displayed substantially the same NPR-B stimulation activity at 1 uM as wtCNP22. With respect to Table 2, various CNP variants with amino acid substitutions, including Analogs AH, BO, AB, BH, BZ, BX and BR, showed substantially similar NPR-B stimulation activity as wtCNP22.

Considering the results in Table 3, many CNP variants having N-terminal and/or C-terminal modifications, including amino acid extensions, exhibited comparable NPR-B stimulation activity as wtCNP22. The functional CNP variants include Analog BB, which is CNP22(G1E) attached to heptanoic acid at the N-terminus, and Analog CD, which is the cyclic domain of CNP22 ("CNP17" retaining the Cys6 to Cys22 sequence) conjugated to the N-terminal and C-terminal "tails" of BNP. FIG. **20** illustrates that GANRR-CNP22(K4R) (SEQ ID NO: 36), CNP37 (Analog BL) and CNP53 all had similar NPR-B stimulation activity as wtCNP22 in the in vitro assay.

Of note from Table 3 is that among the CNP variants assayed for both CNP functionality and NEP resistance, Analog AZ (R-CNP22(K4R)), Analog CC, Analog CF, Analog BL (CNP37), Analog DB (Gly-CNP37) and GHKSEVAHRFK-CNP27 (HSA-CNP27) (SEQ ID NO: 144) all had substantially similar NPR-B stimulation activity as CNP22 while being substantially more resistant to NEP cleavage than CNP22.

With regard to the results in Table 4, nine N-terminal PEGylated CNP variants at 1 uM stimulated cGMP production to at least about 70% of the level achieved by wtCNP22. Several noteworthy aspects appear in Table 4. First, N-terminal PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) with a monodispersed PEO polymer (PEO12 is about 0.6 kDa, PEO24 about 1.2 kDa) resulted in better NPR-B functionality than that with a polydispersed PEG polymer (PEG1K has a polymer number average molecular weight (M_n) of around 1 kDa, PEG2K around 2 kDa) (see also FIG. **21**). Second, N-terminal PEGylation of wtCNP22 with a polydispersed PEG polymer of increasing M_n (PEG1K, PEG2K, PEG5K and PEG20K) or with a monodispersed PEO polymer of greater mass (PEO12 and PEO24) correspondingly decreased the NPR-B activation ability of the CNP variants (see also FIG. **22**). Third, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), having the N-terminal GANRR (SEQ ID NO: 8) extension, stimulated greater cGMP production than PEO24-CNP22 and PEO24-CNP22 (K4R). Also of note is that among the N-terminal PEGylated CNP variants assayed for both CNP functionality and NEP resistance, PEO12-R-CNP22(K4R), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) all had substantially similar NPR-B stimulation activity as CNP22 while being much more resistant to NEP degradation than CNP22.

Among the CNP variants listed in Tables 1-4 and assayed for both CNP functionality and NEP resistance, Analogs G, BK, AZ, CC, CF, BL and DB, Pro-Gly-CNP37, HSA-CNP27 (GHKSEVAHRFK-CNP27) (SEQ ID NO: 144), PEG1K-CNP22, (PEO12)-biotin-CNP22, PEO12-R-CNP22 (K4R), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) all had substantially similar NPR-B stimulation activity as wtCNP22 while being substantially more resistant to NEP cleavage than wtCNP22.

cGMP production assays were also carried out on wtCNP22 and CNP variants G-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA", SEQ ID NO: 144), wtCNP29 and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36). FIG. **23** shows that CNP22 and all the CNP variants assayed induced production of similar cGMP levels at either low or high dose of CNP.

Example 5

Binding Specificity for NPR-A, NPR-B and NPR-C

Signaling Competition Assay

To determine the binding specificity of CNP variants for the clearance receptor NPR-C, a signaling competition assay is carried out. Expression plasmids for human NPR-B or NPR-C (each purchased from OriGene) are transiently transfected and receptors are expressed in HEK293T cells. Forty hours after transfection, NPR-B, NPR-C and native HEK293T cells are harvested, counted and plated at a ratio of 1:1 (NPR-B cells:competing cells (either NPR-C or native HEK293T cells)) in 12-well or 96-well plates. Twenty hours after plating, cells are processed for the NPR-B/cGMP stimulation assay described in Example 4. If present, the natriuretic clearance receptor, NPR-C, is expected to bind and internalize CNP, thereby reducing the overall CNP concentration available for signaling through NPR-B, resulting in decreased GMP production and a shift in the dose-response curve to the right. A rightward shift in the dose-response curve has been verified for wtCNP22. CNP variants having reduced affinity for NPR-C are not expected to induce a shift, or are expected to induce a smaller shift, in the dose-response curve to the right. This signaling competition assay is similar to that previously described by Cunningham (U.S. Pat. No. 5,846,932; B. Cunningham, EMBO J. 13(11): 2508-2515 (1994); H. Jin et al., J. Clin. Invest. 98(4): 969-976 (1996)).

The cGMP stimulation activity of wtCNP-22, Pro-Gly-wtCNP37 and ANP through NPR-B and NPR-A, and their selectivity for NPR-B vs. NPR-C and for NPR-A vs. NPR-C, were evaluated in signaling competition assays. NPR-A, NPR-B and NPR-C individually were transiently tranfected into HEK293T cells. Thirty hours after transfection, the cells were plated into 96-well plates: (A) 20,000 NPR-B cells+20,000 mock transfected cells; (B) 20,000 NPR-B cells+20,000 NPR-C cells; (C) 20,000 NPR-A cells+20,000 mock transfected cells; and (D) 20,000 NPR-A cells+20,000 NPR-C cells. Twenty hours after plating, the culture media was removed and replaced with serum-free media:PBS (1:1)+0.75 uM IBMX for 15 minutes. For cGMP signaling through NPR-B, dose series for ANP, CNP-22 and Pro-Gly-CNP37 were added and incubated at 37° C. for 12 minutes before the assay was stopped by cell lysis. For cGMP signaling through NPR-A, dose series for CNP-22 and Pro-Gly-CNP37 were incubated at 37° C. for 12 minutes, while dose series for ANP were incubated at 37° C. for 6 minutes (because NPR-A appeared to be a "faster" guanylyl cyclase than NPR-B, the incubation time for ANP was shortened in order not to max out (use up all cellular GTP) too soon when signaling with ANP). FIGS. **24**A and B show that CNP-22 and Pro-Gly-CNP37 ("Pro-CNP38") stimulated cGMP production through NPR-B with similar dose-response curves, and to a much greater extent than through NPR-A, and exhibited a similar profile for NPR-B vs. NPR-C selectivity in the signaling competition assays.

Determination of Binding Affinities (K_i) for NPR-A, NPR-B and NPR-C

The binding affinities (K_i) of CNP variants for NPR-A, NPR-B and NPR-C are determined in a heterologous competition binding assay (U.S. Pat. No. 5,846,932; B. Cunningham, EMBO J. 13(11): 2508-2515 (1994); H. Jin et al.,

J. Clin. Invest. 98(4): 969-976 (1996)). Membranes from HEK293 cells, or another suitably transfectable cell line (e.g., HeLa cells), expressing human NPR-A, NPR-B or NPR-C are prepared for radio-labeled ligand binding assays. Membrane preparations are diluted in an appropriate buffer and varying concentrations of wtCNP22 or CNP variant (competitor) are added with I^{125}-labeled wtCNP22 (Bachem). Samples are incubated at room temperature to allow for ligand/receptor equilibration and bound peptide is separated from free peptide by filtration through PVDF filter membranes. Filters are washed before the addition of scintillant and counting by a scintillation counter. Binding is measured in duplicate for each concentration of competitor peptide. CNP variant affinity (K_i, equilibrium dissociation constant) and B_{max}, (receptor number) are calculated by non-linear regression analysis and/or the Cheng-Prusoff equation.

CNP variants exhibiting reduced affinity to NPR-C are expected to have reduced susceptibility to clearance by NPR-C and thus a longer plasma or serum half-life. Increased half-life of the CNP variants in circulation would increase the availability of the variants for therapeutic activity.

Example 6

Effect of CNP Variants on the Growth of Rat Chondrosarcoma (RCS) Cells and cGMP Production in RCS Cells

To assess the ability of CNP variants to stimulate bone growth, skeletal dysplasia is simulated in cell culture by treating rat chondrosarcoma (RCS) cells with fibroblast growth factor 2 (FGF-2), which activates fibroblast growth factor receptor 3 (FGFR-3) and induces growth arrest (Krejci et al., J. Cell Sci., 118(21):5089-5100 (2005)).

Optimal CNP treatment parameters are determined by varying CNP concentration (0.05, 0.1, 0.2 and 0.5 uM), and treatment duration and interval (continuous; 2.5 min, 10 min, 30 min, 1 hr, 2 hr, 4 hr and 8 hr once a day; 2.5 min, 10 min, 30 min, 1 hr, 2 hr and 4 hr twice a day). After 72 hours, cells are counted using an automated cell counter, and the amount of extracellular matrix is estimated using alcian blue staining.

RCS cells are then treated with a CNP variant using the optimal conditions determined from the growth experiments with wtCNP22. The concentration of cGMP is measured by competitive ELISA for untreated RCS cells, RCS cells treated with CNP, and RCS cells treated with the CNP variant. Cell growth and matrix synthesis resulting from treatment with the CNP variant are also monitored and compared to those resulting from CNP treatment.

To assess the effect of CNP variants in a human cell culture system, primary re-differentiated human chondrocytes in alignate beads are treated with wtCNP22 and CNP variants, and cGMP concentration is determined by competitive ELISA as a measure of effective CNP signaling.

The methods described herein can be employed to assess the ability of CNP variants to stimulate cGMP production in and growth of rat chondrosarcoma cells in vitro.

Example 7

Dose Response Study in Rat Chondrosarcoma Cells

The tyrosine kinase receptor fibroblast growth factor receptor 3 (FGFR-3), a negative regulator of chondrocyte growth, is contitutively on in achondroplasia subjects. Stimulation of the FGFR-3 receptor with FGF-2 causes growth arrest by prolonged activation of Erk MAPK, and causes decreased matrix synthesis and loss of matrix, as well as a change in cell shape. Continuous exposure of rat chondrosarcoma (RCS) cells to fibroblast growth factor 2 (FGF-2) simulates achondroplasia in cell culture by activating FGFR-3 and inducing growth arrest (Krejci et al., J. Cell Sci., 118(21): 5089-5100 (2005)). To determine the dose of CNP variant and frequency of dosing that stimulate sufficient growth of bone cells, a dose response study was performed using the RCS cell assay as described in Example 6.

RCS cells were seeded at 10×10^3 cells per well in 24-well plates, grown for 24 hr, treated for 72 hr, and then counted. RCS cells were continuously exposed to FGF-2 (5 ng/mL) to simulate a constitutively active FGFR-3, which induced cell growth arrest (see bar #5 in FIG. **25**). Wild-type CNP22 (0.2 uM) was cultured continuously (72 hr), 1 hr daily or 2 hr daily. All stimulants were changed daily Continuous exposure of RCS cells to 0.2 uM CNP22 in the presence of 5.0 ng/mL FGF-2 partially reversed FGF2-induced growth arrest, leading to the growth of approximately 200×10^3 cells per well (bar #6 in FIG. **25**), compared to approximately 100×10^3 cells per well in the absence of CNP22 (bar #5 in FIG. **25**).

Both 1 hr exposure to CNP22 (0.2 uM) once a day and 2 hr exposure to CNP22 (0.2 uM) once a day achieved about 84% of the effect of continuous CNP22 (0.2 uM) exposure on chondrocyte growth (bars #7 and 8 in FIG. **25**). These results demonstrate that continuous exposure of growth-arrested chondrocytes to CNP22 is not required for reversal of cell growth arrest. Additionally, dose response studies demonstrate that lower doses of CNP22 are capable of reversing growth arrest (FIG. **26**A).

Furthermore, histological and cell morphological analysis of the extracellular matrix showed that CNP22 treatment antagonized FGF2-mediated loss of chondrosarcoma extracellular matrix and increased matrix synthesis. Exposure to FGF-2 decreased matrix synthesis and increased degradation, while addition of CNP22 to the FGF-2 cell culture increased matrix synthesis and partially inhibited FGF-2, as assessed by ^{35}S-sulfate and ^{3}H-Pro incorporation into, or decrease from, matrix (FIGS. **27**A-D). Analysis of aggrecan and fibronectin production (mRNA and protein) in RCS cells cultured with FGF-2 and CNP22 shows that FGF-2 decreased aggrecan level and increased fibronectin level, which was inhibited by addition of CNP22 (FIGS. **28**A-C). FGF-2 induces and activates matrix-processing molecules predominantly via Erk, and addition of CNP22 shows some effect on this activation.

Additional high-throughput assays for measuring growth arrest, such as crystal violet staining, are useful for measuring the effects of CNP22 and variants thereof on RCS cells.

Similar dose response studies can be conducted with the CNP variants described herein to determine their effective dose for reversing FGF2-induced growth arrest of RCS cells.

Example 8A

Ex Vivo Stimulation of Growth of Tibia and Femur from Mice with Mild Achondroplasia

Mouse tibial organ culture model has been used to demonstrate the efficacy of wild-type CNP22 in stimulating longitudinal bone growth. Treatment of wild-type tibiae with

CNP22 at 10^{-8}, 10^{-7} or 10^{-6} M for 6 days increased longitudinal growth by 31%, 40% and 42%, respectively. Histological evaluation also showed expansion of the hypertrophic zone, e.g., an increase in the number and size of hypertrophic chondrocytes in the growth plate (Agoston et al., BMC Dev. Biol. 7:18 (2007)). Similar findings were observed in tibiae isolated from $FGFR3^{Ach}$ mice (Yasoda et al., Nat. Med. 10: 80-86 (2004)).

To determine the efficacy of CNP variants in stimulating longitudinal bone growth, CNP variants were tested in a mouse organ culture model of endochondral bone growth in wild-type mice and transgenic mice having a G380R mutation in the human FGFR-3 gene ($FGFR3^{wt/Ach}$ heterozygote) which represent a mouse model of mild achondroplasia. In brief, the pharmacological activity of wild-type CNP22 and CNP variants was compared in an organ culture model of embryonic or neonatal mouse tibiae, isolated from wild-type and $FGFR3^{wt/Ach}$ littermates. Overall bone growth and histological changes within the growth plate were assessed. Conditioned culture medium is also assessed for biomarkers of intracellular signaling (cGMP), cartilage metabolism (type II collagen, other collagens, aggrecan chondroitin sulfate), bone metabolism (bone alkaline phosphatase, osteocalcin, type I collagen [C-telopeptide, N-telopeptide]), and inflammation (interleukin-1, interleukin 6, interleukin-11).

Effective CNP variants are identified by their ability, e.g., to stimulate production of cGMP, and bone growth as measured by increased longitudinal bone length and expansion of the cells in the hypertrophic zone of the growth plate.

Measurement of Bone Growth

The efficacy of wtCNP22, CNP37 and PEO24-GANRRCNP22(K4R) (SEQ ID NO: 36) in stimulating longitudinal femoral growth was evaluated in the mouse organ culture model. For these experiments femora were isolated from 2-3 day old wild-type mice and cultured in alphaMEM supplemented with 0.2% BSA, 0.5 mM L-glutamine, 40 units penicillin/mL and 40 ug streptomycin/mL, for 8 days in the presence of vehicle, CNP22 or CNP variants. The treatment commenced at day 0 and was repeated every two days thereafter, as the medium was changed. Bones were measured prior to treatment and every two days thereafter, using a dissection microscope fitted with a 1 cm eye-piece reticule. Conditioned medium was used for biomarker analysis. At day 8 bones were fixed in 4% paraformaldehyde for 24 hr, decalcified in 5% formic acid for 24 hrs, dehydrated and embedded in paraffin. Bones were sectioned at 5 um (microns), which were then deparaffinized, rehydrated, and stained with Alcian Blue for 30 min (pH 2.5; MasterTech). Alcian Blue stains cartilage blue. Stained sections were visualized and photographed by brightfield microscopy. The thickness of the hypertrophic region of the growth plate cartilage was determined by image analysis.

FIG. **29** illustrates the effect of wtCNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on longitudinal growth of 3-day old wild-type mouse femurs treated with the CNP peptides every two days. The results were normalized to measurements prior to treatment (day 0). The studies were performed in triplicate (vehicle) or quadruplicate (CNP peptides). As shown in FIG. **29**, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), as well as CNP22, were effective in stimulating longitudinal femoral growth, with the N-terminal PEGylated CNP variant being the most effective.

The growth of wild-type and $FGFR3^{ach}$ mouse femur and tibia in response to CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) was also assessed. Culture of either wild-type or achondroplastic ($FGFR3^{ach}$) mouse tibia showed that CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) both increased the longitudinal growth of the tibia compared to vehicle or CNP22 (FIGS. **30** and **31**). CNP22, CNP37 and PE024-GANRR-CNP22(K4R) (SEQ ID NO: 36) also stimulated the growth of wild-type mouse femur (FIG. **32**). Moreover, each of CNP22, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and CNP37 increased the longitudinal growth of $FGFR3^{ach}$ mouse femur compared to vehicle (FIG. **33**).

Furthermore, ex vivo distribution of CNP37 in the growth plate of $FGFR3^{ach}$ mouse tibia was evaluated. Bone samples were prepared as described above. Paraffin sections were cut and heat fixed for 1 hour at 60° C. Antigen retrieval with 1% hyaluronidase at 37° C. (30 minutes) was followed by a 1 hour serum block (10% Normal Goat Serum). CNP22 antibody (1:500 dilution; Peninsula Laboratories Inc., San Carlos, Calif.) was applied overnight at 4° C. For immunodetection, Vectastain ELITE ABC kit (Vector, Burlingame, Calif.) was used according to the manufacturer's recommendations. Specific bound peroxidase was visualized by incubation with DAB substrate kit (Vector) and the reaction was developed for 3 minutes. Slides were then dehydrated and mounted and photographed using a brightfield microscope. Staining for CNP in the growth plate of $FGFR3^{ach}$ mouse tibia showed that CNP immunoreactivity was increased in the regions of articular and hypertrophic chondrocytes (FIG. **34**), indicating that CNP37 was delivered to the chondrocytes.

In addition to the distribution of CNP variants in the bone growth plate, the ex vivo effects of CNP37, CNP22 and vehicle on cells in the $FGFR3^{ach}$ and wild-type growth plate, e.g., hypertrophic cell size and cellularity of proliferating zone, were also evaluated. Bone samples were prepared, including Alcian Blue staining, as described above. Images of the entire proximal growth plate were taken at 4× magnitude. The growth plate is divided into three zones, starting from the epiphyseal side of cartilage: the resting zone (individual small chondrocytes), proliferating zone (columns of stacked chondrocytes parallel to the long axis of the bone), and hypertrophic zone (large chondrocytes and thin septa between the chondrocytes). In these regions, measurements were made by ImageJ software, including the number of proliferating chondrocytes per column and the density of hypertrophic chondrocytes. A test square (4×4 mm^2) at five different regions of the hypertrophic zone was used to determine the density of hypertrophic chondrocytes. The cell size of hypertrophic chondrocytes was calculated by 1 over determined cell density. Cellularity of proliferating columns was increased by CNP37 and CNP22 in both wild-type and $FGFR3^{ach}$ mice (FIGS. **35**B and C). Chondrocyte hypertrophy in $FGFR3^{ach}$ mice was also increased as a result of culture with CNP22 or CNP37 (FIGS. **36**B and C).

Ex vivo studies of cultures of mouse bones indicated that CNP37 was delivered to the growth plate and was able to increase chondrocyte cellularity and hypertrophy, which are associated with growth plate expansion and longitudinal bone growth. To assess the biodistribution of CNP37 in the bone growth plate in vivo and the in vivo effects of CNP37 on the growth plate (including total growth plate thickness, hypertrophic zone thickness, and cellularity of proliferating zone), bone samples were obtained from $FGFR3^{ach}$ mice treated with vehicle or CNP37 as described above. For biodistribution and in vivo effects studies, tibias were fixed and stored in 70% ethanol. For immunohistochemistry, the samples were decalcified in 5% formic acid for 2 days,

dehydrated and embedded in paraffin. Bones were sectioned at 5 um (microns), which were then deparaffinized, rehydrated, and used for CNP immunohistochemistry as described above. For cellular image analysis, bones were sectioned at 5 (microns), and then deparaffinized, rehydrated, and stained with Alcian Blue for 30 minutes (pH 2.5; MasterTech) and Hematoxylin &

Eosin for 30 seconds. Stained sections were visualized and photographed by brightfield microscopy. Thicknesses of the growth plate and the proliferating and hypertrophic zones were measured using ImageJ software.

In vivo biodistribution studies demonstrated that, similar to the ex vivo studies, CNP immunoreactivity was increased in the regions of articular and hypertrophic chondrocytes in the tibia growth plate of FGFR3^{ach} mice treated with CNP37, indicating that CNP37 was delivered in vivo to the growth plate of FGFR3^{ach} mouse tibia (FIG. **37**). Furthermore, CNP37 treatment significantly increased the total growth plate thickness, proliferating zone thickness and hypertrophic zone thickness of FGFR3^{ach} mouse tibia in vivo (FIGS. **38**A-C).

These results demonstrate that CNP variants of the disclosure penetrate into the growth plate of wild-type and achondroplastic animals, increase the number and size of chondrocytes, increase the thickness of the proliferating zone and the hypertrophic zone of the growth plate, and increase longitudinal bone growth in treated wild-type and achondroplasic animals. Therefore, the CNP variants are useful for stimulating bone growth in achondroplastic subjects.

Measurement of Biomarkers

In addition to measurement of bone growth in response to CNP variants, assay of the levels of biomarkers for cartilage and bone formation and growth induced in response to CNP variants is useful for evaluating the effect of CNP variants on bone growth.

Femurs and tibias were isolated from wild-type and FGFR3^{ach} mice as described above. Bones were cultured with CNP22 or a variant thereof for eight days with the replacement of media every two days. On the eighth day, media was collected and analyzed for the biomarkers cGMP (cyclic guanosine 3', 5' cyclic monophosphate) and fragments of cleaved collagen type II, a cartilage-specific marker for cartilage turnover. Both markers were measured using commercially available enzyme-linked immunosorbent assays (ELISA) for cGMP (Cayman Chemical Co., Ann Arbor, Mich.) and cleaved collagen type II (Cartilaps) (Immunodiagnostic Systems, Fountain Hills, Ariz.), following the manufacturer's protocol.

Levels of cGMP and collagen type II fragments were measured from cell culture extracts after exposure to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36). FIGS. **39-42** show a large increase ($p<0.01$) in the levels of cGMP in the media after exposure of ex-planted wild-type and FGFR3^{ach} mouse femurs and tibias to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36). In addition, exposure of wild-type and FGFR3^{ach} mouse femurs to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) increased the levels of cleaved collagen type II, with the treated FGFR3^{ach} mouse femurs showing significantly increased ($p<0.05$) levels of collagen type II fragments (FIG. **43**). Elevated levels of collagen type II fragments indicate a turn-over of the cartilage matrix, and cartilage turnover typically precedes new bone formation in growing bones.

Example 8B

Ex Vivo Stimulation of Growth of Femur from Mice with Severe Achondroplasia

The effect of a CNP variant on the growth of bones from mice with severe achondroplasia was evaluated ex vivo. Used in the study were transgenic mice expressing a human FGFR-3gene having a Y367C mutation (FGFR3^{Y367C}) [S. Pannier et al., Biochim. Biophys. Acta, 1792(2): 140-147 (2009)], which represent a mouse model of severe achondroplasia. Femurs were isolated at embryonic day 16.5 and cultured for 6 days in the presence of 1 uM Pro-Gly-CNP37. Bone lengths were measured at Day 1 and Day 7. The bones were then paraffin-embedded, sectioned and stained with hematoxylin and eosin to assess histological changes and cellular morphology. Treatment of bone explants isolated from FGFR3^{Y367C} mice with Pro-Gly-CNP37 ("ProCNP38") resulted in increase in bone growth and expansion in the growth plate (FIG. **44**). Femurs from FGFR3^{Y367C} mice treated with vehicle for 6 days showed an 18% deficiency in growth length-wise compared to wild-type femurs treated with vehicle. Treatment of femurs from FGFR3^{Y367C} mice with 1 uM Pro-Gly-CNP37 for 6 days reduced the growth deficiency to just 11%, i.e., reduced the growth defect by around 40%.

Example 9

Serum/Plasma Stability of CNP Variants In Vitro

In preparation for pharmacokinetics (PK) studies, the stability of CNP variants in serum and/or plasma is evaluated.

Briefly, the analyte is isolated by the removal of serum or plasma proteins by either a 2% trichloroacetic acid precipitation or a 1:3 serum:acetonitrile precipitation. The precipitation mixture is vortexed at 14,000 rpm for five minutes, and a portion of the supernatant is removed and diluted with water prior to transfer to a silanized autosampler vial for analysis. Serum extracts are then analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) with electro-spray ionization mass spectrometry (ESI-MS). A single mass (m/z), shown to be specific for the CNP variant, is monitored for quantitation purposes.

Initially, analytical stability and recovery is determined. Analytical (RP-HPLC and ESI-MS) parameters are optimized through the analysis of matrix standards (serum extracts fortified with analyte post-precipitation). After optimization, analytical recovery is determined by spiking serum samples at known concentrations and comparison of the analyte response to that of matrix standards prepared at similar concentrations. Analyte stability in serum extracts is also determined to assure no significant losses occur after serum precipitation and prior to actual analysis. To test the effect of freezing on serum stability, a two-cycle freeze/thaw study is also performed. In this study a serum sample is spiked with CNP variant and analyzed prior to freezing overnight at −20° C. The sample is then thawed at room temperature and re-analyzed. The process is repeated for a second freeze/thaw cycle.

Serum stability of CNP variant is determined by spiking of serum/plasma samples with CNP variant at a concentration of 10 ug/mL. The sample is placed in a 37° C. water bath for a period of three hours. At 30 minute intervals duplicate aliquots of serum are removed and analyzed. If

rapid losses of analyte are evident (>50% in 30 minutes), the study may be repeated with 10 minute timepoints.

In an exemplary method for determining the stability of CNP variants in murine plasma, a mixture of CNP variant (10 uL of a stock solution of about 2.5-5.0 mg/mL), heparinized murine plasma (50 uL, Bioreclamation, CD-1 Lith Hep 62231), and 5 M NaCl (10 uL) is incubated at 37° C. and 5% CO_2 for 0-5 hr, and then quenched with 10× protease inhibitor cocktail (15 uL, Sigma P2714). For extraction, 150 uL of MeOH/0.1% FA is added to 85 uL of the reaction mixture, and the resulting mixture is vortexed for 1 min and then centrifuged at 15° C. for 15 min. 75 uL of the supernatant is added to 300 uL of aqueous 0.1% FA. A small portion of the resulting mixture is subjected to analysis by LC/MS.

Example 10

Pharmacokinetics and cGMP Production in Rats and Mice

Studies were conducted in normal rats to evaluate the pharmacokinetics (PK) profile of CNP22 and certain CNP variants and the time courses of plasma cGMP concentration after single intravenous (i.v.) or subcutaneous (s.c.) administration of the CNP peptides. Plasma CNP immunoreactivity was determined by using a competitive radioimmunoassay (RIA) with an anti-CNP rabbit polyclonal antibody. Plasma cGMP concentration was determined by RIA using a commercially available kit (YAMASA cyclic GMP Assay kit, YAMASA Corporation).

Normal male rats, 7-8 weeks of age, were used. Recombinant wild-type CNP22, CNP37 and PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) were evaluated. A dosage of 20 nmol/kg of each CNP peptide as a solution in 5% mannitol was intravenously injected once into the tail, or a dosage of 50 nmol/kg of each CNP peptide as a solution in 0.03 mol/L acetic acid buffer solution, pH 4.0, containing 1% (w/v) benzyl alcohol and 10% (w/v) sucrose, was subcutaneously injected once into the back.

Plasma CNP immunoreactivity was determined by the competitive RIA using anti-CNP rabbit polyclonal antibody. Standard and QC samples were prepared. Fifty uL of the standard, QC and assay samples were added, respectively, to test tubes containing 50 uL of RIA buffer. Diluted anti-CNP rabbit polyclonal antibody (100 uL) was added to the tubes. All tubes were kept at 4° C. overnight. ^{125}I-[Tyr^{0}]-CNP22 solution (100 uL) and rabbit IgG solution (100 uL) were added and left at approximately 4° C. overnight. One milliliter of anti-rabbit IgG goat serum containing 10% polyethylene glycol was added, vortexed and left at approximately 4° C. for at least 1 hour, and then the insoluble fraction was precipitated by centrifugation. After aspiration of the supernatant, the amount of radiation (gamma line) in the sediment was measured by a gamma-counter. Each sample was measured in duplicate, and the mean was adopted as the value determined.

Plasma cGMP concentrations in the sample at 5, 30, 60 and 90 minutes after i.v. dosing, or at 5, 30, 60, 120 and 180 minutes after s.c. dosing, were determined by the competitive RIA using anti-cGMP monoclonal antibody. Standard samples were prepared. 100 uL of the assay samples (standard solutions for the calibration curve or the diluted plasma samples for cGMP determination) were transferred to test tubes. Then 100 uL of anti-cGMP monoclonal antibody solution and 100 uL of ^{125}I-labeled succinyl cGMP tyrosine methyl ester solution were added to the tubes, respectively. All tubes were kept at 4° C. overnight. After the addition of 500 uL dextran charcoal solution, the tubes were vortexed and then placed on ice for 10 minutes. The reaction mixture was centrifuged and 500 uL of the supernatant was transferred from each sample to a new test tube. The amount of radiation (gamma line) in the supernatant was measured by a gamma-counter. Each sample was measured in duplicate, and the mean was adopted as the value determined.

Plasma CNP immunoreactivity was employed for pharmacokinetics (PK) analysis. PK analysis was performed using WINNONLIN® Professional (Pharsight Corporation). The PK profiles of CNP22, CNP37 and PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) after i.v. administration were calculated using PK parameters such as concentration at 0 hour (C_0: extrapolation, pmol/mL), total body clearance (CL_{tot}: mL/min/kg), distribution volume at steady state (V_{dss}: mL/kg), area under the plasma concentration-time curve (AUC: pmol·min/mL), mean residence time (MRT: min), and half-life ($T_{1/2}$: min). The PK profiles of the CNP peptides after s.c. administration were calculated using PK parameters such as maximum plasma concentration (C_{max}: pmol/mL), time to reach C_{max} (T_{max}: min), area under the plasma concentration-time curve (AUC: pmol·min/mL), mean residence time (MRT: min), and half-life ($T_{1/2}$: min).

In plasma spike recovery experiments, the RIA detected CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) similarly (data not shown).

Procedures similar to those described above are employed to study in mice the PK profiles of CNP22 and variants thereof and their ability to stimulate cGMP production.

The PK profiles of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) after i.v. administration in three rats are illustrated in FIG. **45**. As shown by FIG. **45**, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a much longer half-life and a much greater bioavailability than CNP22. The half-life, $T_{1/2}$ (min), was 1.42 (±0.45) for CNP22, 22.3 (±1.5) for PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36), and 49.5 (±28.0) for CNP37. The area under the curve, AUC (pmol·min/mL), was 320 (±54) for CNP22, 1559 (±568) for CNP37, and 2084 (±424) for PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

The PK profiles of the three CNP peptides after s.c. administration in three rats are depicted in FIG. **46**. Compared to CNP22, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a much longer half-life (78.1 min (±16.4) vs. 10.0 (±5.0)) and a much greater bioavailability (60% (±6%) vs. 19% (±9%)).

The time courses of plasma cGMP concentrations after i.v. administration of the three CNP peptides in three rats are displayed in FIG. **47**. FIG. **47** clearly demonstrates that i.v. administration of CNP37 and PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) resulted in much higher plasma levels of cGMP at 30, 60 and 90 minutes than i.v. administration of CNP22.

The time profiles of plasma cGMP concentrations after s.c. administration of the three CNP peptides in three rats are shown in FIG. **48**. Subcutaneous administration of PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and CNP37 also resulted in substantially higher plasma concentrations of cGMP than s.c. administration of CNP22, with the difference relative to CNP22 increasing over time for PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), but decreasing over time for CNP37.

The rat studies indicate that compared to wtCNP22, the CNP variants CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a substantially longer half-life in vivo, had a substantially greater bioavailability in vivo, and stimu-

lated substantially higher levels of cGMP production in vivo for an extended period of time. The resistance of CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) to NEP degradation correlates to a longer plasma half-life in vivo, which in turn correlates to prolonged NPR-B/cGMP signaling in vivo. These results show that compared to CNP22, the CNP variants of the disclosure, administered by i.v. or s.c. injection (e.g., once daily), can be more effective in treating CNP-responsive conditions or disorders, such as bone-related disorders and vascular smooth muscle disorders.

Example 11

Pharmacokinetics Study in Mice

To determine CNP variants having increased NEP resistance, for efficacy study in FGFR3^{ach} mice (see Example 13), a pharmacokinetics (PK) study is carried out that compares the pharmacokinetics properties of CNP variants to wild-type CNP22. The FGFR3^{ach} mouse is a mutant mouse model of mild achondroplasia, containing a single transgene on a background of FVB mice.

Wild-type CNP22 or variant thereof is administered as a single intravenous (i.v.) dose in 6-week old wild-type FVB mice. Exemplary PK studies were conducted using wtCNP22. Six-week old FVB/N mice were intravenously administered wtCNP22 in a single dose at 100 nmol/kg. Mean plasma levels of CNP22 were calculated, and the estimated half-life of CNP22 was determined to be from 0.76 min to 1.03 min.

CNP variants displaying greater resistance to NEP degradation are expected to exhibit increased serum concentrations over time and a longer half-life in vivo.

Example 12

Efficacy of CNP Variants in Wild-Type Mice

The in vivo effects of CNP variants on bone growth were assessed in wild-type mice. Three week old FVB wild-type male mice received daily subcutaneous (s.c.) injection of either vehicle, G-CNP37 (200 nmol/kg) or PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) (200 nmol/kg) for 5 weeks. Body weight was measured at least once weekly. Tail length was measured at least once weekly using digital caliper readings, and body length (naso-anal length), bone length (tibia, femur, ulna and humerus), skull length (anterior to posterior cranial segment) and lumbar vertebrae 5 (LV5) length were measured after 5 weeks of treatment using a caliper. X-rays were taken at baseline and after 5 weeks of treatment.

Five weeks of treatment of wild-type mice with G-CNP37 resulted in significant body weight gain, with increased body weight being observed beginning at Day 9 ($p<0.05$) (FIG. 49). Treatment with G-CNP37 also resulted in significantly increased tail length, beginning at the second week after treatment ($p<0.01$) (FIG. **50**).

Table 10 shows the percentage change in tail length, body (naso-anal) length, skull (anterior to posterior cranial segment) length, bone (femur, tibia, humerus and ulna) lengths, and lumbar vertebrae 5 (LV5) length in wild-type mice injected s.c. once daily with 200 nmol/kg of either G-CNP37 or PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) for 5 weeks, relative to a value of 100% for wild-type mice treated with vehicle only.

TABLE 10

	Tail	Naso-Anal	Anterior to Posterior (Skull)	Femur	Tibia	Humerus	Ulna	LV5
G-CNP37	119%**	114%**	104%*	109%**	105%**	104%**	101%	108%*
CNP27-PEO12	101%	104%**	101%	102%*	100%	102%*	96%*	103%

**p < 0.01,
*p < 0.05

Treatment with G-CNP37 resulted in significantly increased tail length, body (naso-anal) length, skull length, proximal bone (femur and humerus) length, distal bone (tibia) length, and vertebral (lumbar vertebrae 5) length compared to treatment with vehicle.

Lower doses of Pro-Gly-CNP37 dosed daily s.c. at 5 nmol/kg, 20 nmol/kg or 70 nmol/kg for five weeks resulted in a dose-dependent increase in tail length, body (naso-anal) length, and bone lengths compared to vehicle. Table 11 shows the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in wild-type mice injected s.c. once daily with 5 nmol/kg, 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37 for 5 weeks, relative to a value of 100% for wild-type mice treated with vehicle only.

TABLE 11

	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
5 nmol/kg	107.6%**	102.7%**	103.3%**	101.7%**	101.1%	100.9%*
20 nmol/kg	107.8%**	107.6%**	107.1%**	103.4%**	102.3%**	102.5%**
70 nmol/kg	113.6%**	112.5%**	109.3%**	105.9%**	103.6%**	104.1%**

**p < 0.01,
*p < 0.05

In another study, various dosing regimens of Pro-Gly-CNP37 were administered for nine weeks followed by one week of recovery. Wild-type FVB mice were dosed s.c. with:

(1) vehicle daily for nine weeks, followed by one week of recovery;

(2) 20 nmol/kg Pro -Gly-CNP37 daily for one week, followed by three doses per week for eight weeks and one week of recovery;

(3) 20 nmol/kg Pro-Gly-CNP37 on alternating weeks; or

(4) 5 nmol/kg Pro-Gly-CNP37 daily for nine weeks followed by one week of recovery.

Increased growth in tail length, body length, and bone lengths was observed in all treatment groups (Groups 2, 3 and 4) at the end of the study. Table 12 shows the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in wild-type mice administered Pro-Gly-CNP37 ("Pro-CNP38") under the dose regimens described above, relative to a value of 100% for wt mice treated with vehicle only.

TABLE 12

	Total Pro-CNP38 received (nmol/kg)	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
Group 2	620	104.8%**	106.0%**	105.3%**	100.8%	102.5%	101.7%
Group 3	700	106.7%**	106.5%**	104.1%**	102.5%**	102.6%*	102.8%*
Group 4	350	104.9%**	105.1%**	107.3%**	102.7%**	102.3%**	103.6%**

**p < 0.01,
*p < 0.05

While all dose regimens of Pro-Gly-CNP37 increased axial and appendicular growth parameters measured, daily dosing of Pro-Gly-CNP37 promoted appendicular growth (femur, tibia, humerus, and ulna) at a lower total dose level (Group 4) compared to regimens with less frequent dosing (Groups 2 and 3).

Example 13

Efficacy in Mouse Model of Mild Achondroplasia

The efficacy of CNP variants in enhancing growth and correcting achondroplasia was tested in a mouse model of mild achondroplasia, using a strain of transgenic mice expressing a human FGFR-3 gene having a G380R mutation ($FGFR3^{ach}$) (Wang et al., Proc. Natl. Acad. Sci. USA, 96(8): 4455-4460 (1999); Naski et al., Development USA, 125: 4977-4988 (1998); U.S. Pat. Nos. 6,265,632 and 6,136,040).

At 3 weeks of age, $FGFR3^{ach}$ mice and their wild-type littermates were anesthetized to have lateral whole-body X-ray images taken by Faxitron, and randomized by body weight into the following treatment groups (n=8/group): (1) wild-type/vehicle, (2) $FGFR3^{ach}$/vehicle, (3) $FGFR3^{ach}$/CNP37, and (4) $FGFR3^{ach}$/PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36). The mice received once daily subcutaneous (s.c.) administration of designated test article (vehicle or 200 nmol/kg CNP variant) for 5 weeks. Satellite groups (n=3) were used to confirm exposure of each test article following a single subcutaneous administration on Day 1. Wild-type male FVB mice that received daily s.c. injection of vehicle for 5 weeks were used as a control for normal growth.

X-ray measurements at baseline and at the end of the study were performed to determine change in the head length, area of the skull, and the external auditory meatus (EAM, the ear canal running from the outer ear to the middle ear). Body weight and tail length were measured at least once weekly, using a digital caliper to measure tail length, and body (nasoanal) length was measured after 5 weeks of treatment. Bone (tibia, femur, ulna and humerus) lengths were measured using a digital caliper at necropsy.

On Day 37, all mice were sacrificed by terminal anesthesia and whole-animal photographs and X-ray images by Faxitron were taken. Left and right tibia, femur, humerus, and ulna were collected and measured using a digital caliper. The left portions of each bone were processed for histology, and the right portions were snap-frozen for archival. Samples obtained from the bones were used to evaluate the effects of NP variants on endochondral bone growth.

Treatment of $FGFR3^{ach}$ mice with CNP37 by once daily s.c. injection for 5 weeks resulted in significantly increased body length (FIG. **51**), tail length (FIG. **52**), distal bone (ulna and tibia) lengths (FIGS. **53**A and B), and proximal bone (humerus and femur) lengths (FIGS. **54**A and B). Moreover, treatment with CNP37 increased the head length (FIG. **56**), area of the external auditory meatus (FIG. **57**), and spine length (FIG. **58**) via extension of the vertebral bodies. Additionally, treatment with CNP37 corrected rhizomelia (disproportion of the length of the proximal limbs) of $FGFR3^{ach}$ mice, i.e., restored proportional growth of proximal bones, as assessed by the femur:tibia ratio (FIG. **55**). Table 13 summarizes the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in $FGFR3^{ach}$ mice injected s.c. once daily with 200 nmol/kg of either CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) for 5 weeks, relative to a value of 100% for $FGFR3^{ach}$ mice treated with vehicle only.

TABLE 13

	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
CNP37	115%**	109%**	112%**	107%**	105%*	106%**
CNP27-PEO24	100%	99%	100%	102%	100%	101%

**p < 0.01,
*p < 0.05

The results of these studies show that CNP37 can stimulate spinal and long bone growth, help to correct rhizomelia by preferentially increasing the length of the femur over the tibia, and help to restore craniofacial proportions in $FGFR3^{ach}$ mice. These results indicate that CNP37 and potentially other CNP variants may be effective in correcting the symptoms of achondroplasia and treating subjects having defects in bone growth or in need of increased bone growth.

Example 14

Measurement of Biomarkers and Evaluation of Immunogenicity in Wild-Type and Achondroplastic Mice

Measurement of Biomarkers After CNP Administration

The levels of bone growth biomarkers were measured in wild-type and achondroplastic ($FGFR3^{ach}$) mice.

Transgenic FVB $FGFR3^{ach}$ mice (a mouse model of mild achondroplasia) were treated daily for 5 weeks by subcutaneous injection with either vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0), CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) at 200 nmol/kg for each CNP compound, as described above. Plasma and serum were collected during the study. Harvested plasma was stored with 10× protease inhibitor at −80° C. until analysis. cGMP levels were measured 15 minutes post-injection on Day 36 from K2-ETDA plasma collection tubes. Fragments of cleaved collagen type II (cartilage-associated biomarkers), osteocalcin (bone-associated biomarker), and IgG (relating to immunogenicity) were measured from terminal bleed serum at the end of the study (Day 37). cGMP was measured using a commercially available ELISA kit (Cayman Chemical Co., Cat. No. 581021.1), cleaved collagen type II (Cartilaps) was measured using a commercially available kit from Immunodiagnostic Systems (Cat. No. 3CAL4000), and osteocalcin was measured using a commercially available kit from Biomedical Technologies Inc. (Stoughton, Mass.).

FIG. **59** shows an increase in cGMP plasma levels in $FGFR3^{ach}$ mice treated with CNP37 or PEO24-GANRRCNP22(K4R) (SEQ ID NO: 36) compared to vehicle. FIGS. **60** and **61** show that administration of CNP37 resulted in the greatest elevation of serum levels of cleaved collagen type II and osteocalcin. These results indicate that administration of the CNP peptides, in particular CNP37, to $FGFR3^{ach}$ mice led to increased levels of bone growth markers, suggesting increased bone formation and growth in CNP peptide-treated $FGFR3^{ach}$ mice.

For evaluation of biomarkers in wild-type mice, wild-type FVB mice were treated daily for 5 weeks by subcutaneous injection with either vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0), 200 nmol/kg G-CNP37, 200 nmol/kg PEO12-GANRR-CNP22 (K4R) ("CNP27-PEO12") (SEQ ID NO: 36), or 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37. Plasma and serum were collected during the study. Harvested plasma was stored with 10× protease inhibitor at −80° C. until analysis. cGMP levels were measured 15 minutes post-injection on Day 36 from K2-ETDA plasma collection tubes. The levels of cleaved collagen type II, bone-specific alkaline phosphatase, and IgG (relating to immunogenicity) were measured from terminal bleed serum at the end of the study (Day 37). cGMP and cleaved collagen type II (Cartilaps) were measured as described above. Bone-specific alkaline phosphatase was measured using a commercially available kit (Cusabio, Cat. No. CSB-E11914m).

Administration of G-CNP37 significantly increased ($p<0.05$) the level of cGMP (FIG. **62**) and particularly the level of fragments of cleaved collagen type II (FIG. **63**) in wild-type mice compared to vehicle. The significantly higher level of collagen type II fragments resulting from administration of G-CNP37 indicates turnover of the cartilage matrix, suggesting that G-CNP37 stimulated new bone formation in growing bones in wild-type mice.

Both doses of Pro-Gly-CNP37 ("Pro-CNP38"), 20 and 70 nmol/kg, significantly increased ($p<0.05$) plasma cGMP level 15 minutes after administration compared to wild-type mice treated with vehicle (FIG. **64**). Administration of the higher dose (70 nmol/kg) of Pro-Gly-CNP37 also significantly increased ($p<0.05$) the level of cleaved collagen type II compared to vehicle-treated mice (FIG. **65**), suggesting that the higher dose of Pro-Gly-CNP37 stimulated cartilage matrix turnover prior to new bone formation in wild-type mice. Furthermore, administration of the higher dose (70 nmol/kg) of Pro-Gly-CNP37 increased ($p<0.05$) the level of bone-specific alkaline phosphatase compared to vehicle-treated mice (FIG. **66**), suggesting that the higher dose of Pro-Gly-CNP37 increased bone remodeling in wild-type mice.

Evaluation of Immunogenicity of CNP Variants

Because the CNP variants are peptide derivatives, it is possible that administration of the peptides may lead to an immunogenic response in vivo. To assess whether an immune response occurred after successive administrations of CNP variant, measurement of serum antibody levels was performed.

An IgG assay was performed to assess whether an IgG immune response was triggered by 5 week exposure of achondroplastic $FGFR3^{ach}$ mice to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36). IgG is the most predominant immunoglobulin in mouse and human serum and is produced as part of the secondary immune response to an antigen. The IgG response to administration of the CNP peptides was determined as follows. 96-well plates were coated with 100 ng/mL CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) in BupH PBS buffer (Pierce/Thermo, Cat. No. 28372, Rockford, Ill.). After overnight incubation, plates were blocked with Casein-PBS blocking buffer (Pierce/Thermo, Cat. No. 37528) for two hours at room temperature with shaking at 300 rpm. After washing with wash buffer (1×PBS with 0.05% Tween), diluted serum samples from a terminal bleed (diluted 1:25) were added to the plate. Positive and negative controls were also loaded onto the plate. Positive control was 1:25 diluted serum (pooled from 6 individual FVB mice) with anti-CNP22 antibody added (Bachem rabbit anti-CNP22 IgG, Cat. No. T-4222) at 1:1000 dilution. Negative control was the diluted pooled serum. After two hours of incubation, the plates were washed and secondary antibody diluted in blocking buffer was added to the wells. For the mouse serum samples, anti-mouse IgG Fcγ (peroxidase-conjugated affinipure goat anti-mouse IgG, Fcγ fragment, Cat. No. 115-035-071, Jackson Immunoresearch, West Grove, Pa.) was added at a 1:10,000 dilution. For the positive and negative controls, anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Cat. No. SC-2004, Santa Cruz, Calif.) was added to blocking buffer. After two hours of incubation at room temperature with shaking at 300 rpm, plates were washed with wash buffer. 100 uL of TMB (One-step TMB, Pierce/Thermo, Cat. No. 34022) was added to all wells. Plates were incubated at room temperature for 15 minutes with shaking at 300 rpm. Colorimetric reactions were stopped by the addition of 100 uL of 2 N H_2SO_4. Plates were read at 450 nm (Spectramax, Molecular Devices, Sunnyvale, Calif.) and data was analyzed using SoftMax Pro software (Molecular Devices).

Serum samples from $FGFR3^{ach}$ mice treated with CNP22 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) indicated no positive IgG immune response in any of the mice. Only one out of nine CNP37-treated $FGFR3^{ach}$ mice showed a slightly positive IgG response.

The immunogenic response of wild-type mice administered G-CNP37, PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) or Pro-Gly-CNP37, as measured by an increase in serum IgG level, was also assessed by examining terminal bleed serum samples as described above. None of the wild-type mice treated with PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) or Pro-Gly-CNP37 (20 or 70 nmol/kg) showed a positive IgG immune response, and only one out of six wild-type mice administered G-CNP37 showed a slightly positive IgG response.

Measurement of Biomarkers Under Different CNP Dosing Regimens

Wild-type FVB mice were treated daily for 9 weeks by subcutaneous (s.c.) injection of vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0) or Pro-Gly-CNP37 ("Pro-CNP38") under different dosing regimens. Group 1 included vehicle-treated mice injected s.c. daily for 9 weeks followed by 1 week of no treatment. Group 2 included mice treated with Pro-Gly-CNP37 (20 nmol/kg) once daily for 1 week, followed by three doses per week for 8 weeks, followed by 1 week of no treatment. Group 3 contained mice treated with Pro-Gly-CNP37 (20 nmol/kg) once daily in alternate weeks (weeks 1, 3, 5, 7 and 9), with 1 week of no treatment following each week of treatment. Group 4 contained mice treated with Pro-Gly-CNP37 (5 nmol/kg) once daily for 9 weeks followed by 1 week of no treatment. Finally, Group 5 included mice treated with Pro-Gly-CNP37 (5 nmol/kg) once daily for 5 weeks without any week of no treatment. Terminal bleed serum was collected from the mice, and cleaved collagen type II, total alkaline phosphatase and total antibody levels were measured therefrom. Cleaved collagen type II levels were measured as described above. Total alkaline phosphatase levels, predominantly from the liver and bones, were measured with the assistance of a veterinary diagnostic laboratory testing facility (Antech).

A total antibody assay was developed to evaluate potential immune response. The platform used for the total antibody assay was an electrochemiluminescent assay (ECLA). The ECLA platform utilizes a biotin-labeled drug (here, biotin-Pro-Gly-CNP37) and a ruthenium-labeled drug (here, Ru Pro-Gly-CNP37). The biotin-labeled drug binds to a streptavidin-coated plate that contains electrodes, and the ruthenium-labeled drug functions as the detection component of the assay, as ruthenium can be electrochemically stimulated. A drug-specific antibody (here, a CNP-specific antibody) binds to the biotin-labeled drug and the ruthenium-labeled drug and "bridges" the two labeled drugs. Among the advantages of the ECLA platform, any isotype of antibody (IgG, IgM, etc.) can be detected, and the ECLA assay is species-independent.

The total antibody assay was performed as follows. Pro-Gly-CNP37 was labeled with biotin in a 4:1 challenge ratio, and Pro-Gly-CNP37 was also labeled separately with ruthenium in a 10:1 challenge ratio. Both separate labeling reactions were quenched by the addition of glycine, and the samples from both reactions were buffer-exchanged to PBS. Low and high QCs were prepared by using commercially available anti-CNP22 antibody (Bachem rabbit anti-CNP22 IgG, Cat. No. T-4222) added at low and high concentrations to 5% diluted FVB mouse serum. Mouse serum samples were diluted to 5% using assay diluent (5% BSA in PBS, MSD Cat. No. R93AA-1). A working solution was prepared by adding biotin-labeled Pro-Gly-CNP37 to assay diluent, then adding ruthenium-labeled Pro -Gly-CNP37 to assay diluent, and then combining the two solutions together. Twenty-five uL of low and high QCs were loaded onto the plate as controls. Then 25 uL of sample was added to a non-binding 96-well plate, followed by the addition of 50 uL of working solution to all wells. Samples and QCs were incubated with the working solution at room temperature for 2 hours with shaking at 350 rpm. In the meantime, an MSD streptavidin plate (MSD Cat. No. L13SA-1) was blocked with blocking buffer (MSD Cat. No. R93AA-1) at room temperature for 2 hours with shaking at 350 rpm. At the end of the two hour incubation, the MSD streptavidin plate was washed, and then 50 uL of sample or QC was transferred to the MSD plate. The plate was then incubated at room temperature for 1 hour with shaking at 350 rpm. At the end of the 1 hour incubation, the MSD plate was washed and 150 uL of 2× read buffer (MSD Cat. No. R92TC-2) was added to the plate. The plate was read using an MSD PR400 machine.

All five groups of mice exhibited substantially comparable levels of cleaved collagen type II (FIG. **67**). Treatment of the mice in Group 5 with 5 nmol/kg Pro-Gly-CNP37 once daily for 5 weeks significantly increased ($p<0.001$) total alkaline phosphatase level (FIG. **68**), which is indicative of bone remodeling. Serum samples from each of the four groups of mice treated with Pro-Gly-CNP37 did not show a positive antibody response in the total antibody assay.

Not to be bound by any theory, there are possible explanations as to why the four groups of CNP-treated mice did not exhibit statistically significant difference in cleaved collagen type II level compared to vehicle-treated mice, and why only the CNP-treated mice in Group 5 exhibited a statistically significant increase in total alkaline phosphatase level compared to vehicle-treated mice. It is possible that because the vehicle-treated mice in the study were also growing, cleaved collagen type II and alkaline phosphatase, which are biomarkers of growth, were also produced in the vehicle-treated mice. In addition, there was a one week period of no treatment for the mice in each of Groups 1 to 4, which possibly diluted out any changes in the cleaved collagen type II and total alkaline phosphatase levels between the CNP-treated mice in Groups 1-3 and the vehicle-treated mice. There was no period of no treatment for the CNP-treated mice in Group 5, and those mice exhibited significantly ($p<0.001$) greater total alkaline phosphatase level compared to vehicle-treated mice.

Example 15

Dose Responses of CNP Variants in Mice

The effects of varying doses of CNP variants were assessed in wild type FVB mice.

For the dose study, in two separate studies (S1 and S2), groups of 10 mice were administered Pro-Gly-wtCNP37 ("Pro-CNP38") at 20 and 70 nmol/kg subcutaneously once daily for 36 injections. Tail length and body weight were measured over the course of treatment. Animals were sacrificed at the end of the experiment and bone length assessed.

Tail length of vehicle treated animals was approximately 8 cm at day 36, whereas animals treated with 20 nmol/kg Pro-CNP38 exhibited tail length of approximately 8.75 cm and 70 nmol/kg Pro-CNP38 increased tail length to approximately 9.5 cm. Administration of Pro-CNP38 in either dose induces a significant ($p<0.05$) relative increase in total body length compared to control treated animals, demonstrating approximately a 130% increase in growth velocity at 20 nmol/kg and approximately a 160% increase in growth velocity at 70 nmol/kg Pro-CNP38 (FIG. **69**).

Treatment with Pro-CNP 38 also significantly increased bone length in most of the long bones assessed as well as total naso-anal (body length) of the animal. Table 14 is representative of the relative % increase in bone length in treated animals in comparison to vehicle treated animals.

TABLE 14

Pro-CNP38	Tail	Naso-Anal	LV4-6	Femur	Tibia	Humerus	Ulna
20 nmol/kg	8%*	7%*	10%*	7%*	4%*	3%*	3%
70 nmol/kg	16%*	13%*	13%*	10%*	7%*	4%*	5%*

Relative % increase,
*p < 0.05 ANNOVA (Dunnett's) v vehicle

Bone mineral density (BMD) and bone mineral content (BMC) were also evaluated after administration of Pro-CNP38 at different doses. Results (FIG. **70**) showed that administration of Pro-CNP38 at 70 nmol/kg significantly decreased bone mineral density (FIG. **70**A) and increased bone mineral content (FIG. **70**B), suggesting there is a delay in bone mineralization in treated animals, but the mineralization process itself is not adversely affected by treatment with CNP.

There were no significant changes in organ weight between Pro-CNP38 or vehicle treated animals.

Bioanalytical Studies of Dose Response in Mice

Bioanalytical studies were carried out to measure markers of CNP activity after in vivo administration of varying doses of Pro-CNP38. Plasma cGMP levels, serum levels of collagen type II and serum levels of alkaline phosphatase from in vivo samples were analyzed. Wild type mice were administered 20 and 70 nmol/kg Gly-wtCNP37 ("CNP38"), 20 and 70 nmol/kg Pro-Gly-wtCNP37 ("Pro-CNP38") and 70 and 200 nmol/kg of GHKSEVAHRFK-wtCNP27 ("HSA-CNP27") (SEQ ID NO: 144) subcutaneously once daily for 36 days. Plasma was collected 15 minutes after the last injection on day 36 and mice were sacrified 24 hr later. At sacrifice, terminal bleed serum was collected and used for biomarker analysis as described previously.

FIG. **71** shows that 20 nmol/kg CNP38 and 70 nmol/kg HSA-CNP27 signficantly increased plasma cGMP ($p<0.01$), raising plasma cGMP levels to approximately 300 pmol and 400 pmol, respectively. Adminsitration of 70 nmol/kg CNP38 increased cGMP to approximately 500 pmol ($p<0.01$), while administration of 70 nmol/kg Pro-CNP38 increased cGMP to approximately 575 pmol ($p<0.001$). Administration of 200 nmol/kg HSA-CNP27 increased cGMP to approximately 675 pmol ($p<0.001$).

CNP variants also significantly increased serum levels of cleaved collagen type II (FIG. **72**). CNP38 at 20 nmol/kg increased collagen to approximately 9 pg/ml ($p<0.05$), CNP38 at 70 nmol/kg increased collagen to approximately 8 pg/ml ($p<0.05$), Pro-CNP38 at 20 nmol/kg increased collagen to approximately 12 pg/ml ($p<0.05$), Pro-CNP38 at 70 nmol/kg increased collagen to approximately 16 pg/ml ($p<0.05$), HSA-CNP27 at 70 nmol/kg increased collagen to approximately 10 pg/ml, and HSA-CNP27 at 200 nmol/kg increased collagen to approximately 10 pg/ml ($p<0.5$).

Serum alkaline phosphatase (AP) levels also increased after administration of CNP variants (FIG. **73**). CNP38 at 20 nmol/kg increased AP to approximately 130 IU/L, CNP38 at 70 nmol/kg increased AP to approximately 160 IU/L ($p<0.001$), Pro-CNP38 at 20 nmol/kg increased AP to approximately 155 IU/L ($p<0.001$), Pro-CNP38 at 70 nmol/kg increased AP to approximately 180 IU/L ($p<0.001$), HSA-CNP27 at 70 nmol/kg increased AP to approximately 120 IU/L, and HSA-CNP27 at 200 nmol/kg increased AP to approximately 140 IU/L ($p<0.01$). Table 15 illustrates the percent of total AP that is bone specific.

TABLE 15

	Total AP IU/L	Bone-specific AP IU/L	% of Total Alk Phos
Vehicle	109.8	24.7	22.5
CNP38 (20 nmol/kg)	135.9	47.9	35.2
CNP38 (70 nmol/kg)	166.3	63.5	38.2
Pro-CNP38 (20 nmol/kg)	159.9	65.9	41.2
Pro-CNP38 (70 nmol/kg)	183.6	78.4	42.7
HSA-CNP27 (20 nmol/kg)	121.4	51.7	42.6
HSA-CNP27 (70 nmol/kg)	136.1	76.5	56.2

Analysis of anti-CNP antibodies showed that only HSA--CNP27 elicited and IgG antibody response in mice.

The results above illustrate that administration of CNP variants increases the concentration of collagen type II and alkaline phosphatase in serum, indicating that CNP increases factors relevant for increased bone growth, and suggest that administration of CNP variants at doses as low as 20 nmol/kg are effective in increasing bone growth in vivo.

cGMP Response After Different Dose Regimens

Bioanalytical analysis was also assessed at different times after administration of Pro-Gly-wtCNP37 ("Pro-CNP38") to wild type CD-1 mice, 8-10 weeks old, (n=3 per treatment group). Pro-CNP38 was given in a single subcutaneous dose of 200 nmol/kg and levels of cGMP measured at 15 minutes, 3 hours, 1 day, 2 days and 3 days post injection in the plasma, epiphysis, cortical bone (marrow removed), lung and brain. Blood was collected on K2EDTA. Tibial and femoral epiphysis and cortical bone, ear pinna, brains, kidneys and lungs were harvested, placed in boiling water for 5 minutes, then frozen to −70° C. Both plasma and tissue were assayed for cGMP (Cayman Chemical cyclic GMP ELISA kit).

Results showed that cGMP levels increased at 15 minutes post injection in plasma (approximately 1300 pmol/ml) and epiphysis (approximately 2.5 pmol/ml/mg). By 3 hours, plasma levels had decreased approximately to control levels, while levels in the epiphysis were approximately 3 fold lower than cGMP levels at 15 minutes post injection, but higher than control levels. Levels of cortical bone increased to approximately 0.5 pmol/ml/mg at 15 minutes and remained at this level at 3 hours. Levels of cGMP at all timepoints were back to control levels by 1 day after injection. Little to no cGMP was detected in lung or brain at any timepoint.

cGMP levels were also measured in mice administered multiple injections of Pro-CNP38. Groups of mice (n=3) were given Pro-CNP38 as follows: 20 nmol/kg single dose, subcutaneously; 200 nmol/kg single dose, subcutaneously; 20 nmol/kg subcutaneously on days 0 and 1; 200 nmol/kg subcutaneously on days 0 and 1; 20 nmol/kg subcutaneously on days 0 and 3; 200 nmol/kg subcutaneously on days 0 and 3. Mice were sacrificed 15 minutes after the final dose of Pro-CNP38 and plasma levels of cGMP analyzed. There does not appear to be a modulation of the plasma cGMP signal due to the different dose regimens. cGMP responses in the cartilage are also investigated.

Evaluation of Potential Desensitization of NPR-B Receptor

Histological analysis shows that CNP, when administered daily at 200 nmol/kg, accumulates in the growth plate of animals, based on increased CNP immunoreactivity. It is

possible that this accumulation, or daily stimulation of the CNP receptor, in the growth plate could desensitize the CNP receptor.

To determine if multiple dosing desensitizes the NPR-B receptor in vitro, normal human articular chondrocytes were cultured with Pro-Gly-wtCNP37 ("Pro-CNP38") for varying times and cGMP secretion measured.

Primary normal human chondrocytes isolated from articular cartilage were cultured as recommended by the supplier (Lonza). At 60-80% confluence the chondrocytes were treated with 1 uM Pro-CNP38 twice, with increasing amounts of time between treatments (first treatment at time 0, then at 15 min, 30 min, 60 min, 2 hrs, 3 hrs, 4 hrs, 6 hrs after the initial treatment). In the following experiment the treatments were either applied twice, with increasing amounts of time between treatment (first treatment at time 0, then at 6, 16, 24, 48 hrs after initial treatment) or only once, in parallel to the second treatment (only at 6, 16, 24 and 48 hrs-naive responses). Treatments were either applied for 15 min only (acute treatment), or for the duration of the experiment (chronic treatment when CNP was left in the medium). Cell lysates and conditioned medium were collected and analyzed for total cGMP secretion (Molecular Devices ELISA).

In a short term experiment, cells were stimulated with Gly-wtCNP37 ("CNP38") 1 uM two times for 15 minutes (acute treatment), or two times with CNP38 throughout culture (chronic treatment). The periods between treatments were 15 min, 30 min, 60 min, 2 hrs, 3 hrs, 4 hrs, and 6 hrs. Peak cGMP stimulation was obtained by treating cells once only, at the last time point of the experiment, (6 hours; >0.1 pmol/well in the acute experiment and 0.2 pmol/well in the chronic experiment). In the acute experiment, when the cells are treated twice, the response decreases to 0.1 pmol/well if the cells are treated at time 0 and then again at 15 minutes. In the acute experiment, when the cells are treated twice, the response decreases to approximately 0.5 pmol/well if the cells are treated at time 0 and again at 30 min, 60 min, 2, 3, and 4 hours. In the chronic experiment, when the cells are treated twice, the response decreases to approximately 0.16 pmol/well if the cells are treated at time 0 and at 15 minutes. In the chronic experiment, when the cells are treated twice, the response decreases to approximately 0.6 pmol/well if the cells are treated at time 0 and again at 30 min, 60 min, or 2 hours. In the chronic experiment, when the cells are treated twice, the response decreases to <0.5 pmol/well if the cells are treated at time 0 and again at 2, 4 or 6 hours.

Long term studies were also carried out. For acute treatment, 1 uM CNP38 was added to cell culture as described above. For chronic treatment, 1 uM CNP38 was added to cell culture throughout the experiment duration as described above. Results indicate that the NPR-B receptor can desensitize after repeated, daily CNP administration in vitro, and 48 hours between doses is sufficient to recover to 60% of the maximial NPR-B response to CNP38, if the CNP is removed after the first dose, and to <40% if the CNP is incubated throughout the experiment (FIG. **74**).

To evaluate whether treatment with a CNP variant desensitizes the NPR-B receptor in vivo, experiments were conducted with wild-type mice. CD-1 male mice 8-10 weeks of age were injected subcutaneously with vehicle control or Pro-Gly-wtCNP37 ("Pro-CNP38") at 200 nmol/kg on the appropriate days. The mice were either injected with Pro-CNP38 daily for up to 8 days, or were injected with Pro-CNP38 on the first day, the first and second days, or the first and third days of the study. Fifteen minutes following the final injection, the mice (n=3 per treatment group) were deeply anesthetized and ex-sanguinated via thoracotomy and aortic cannulation. Circulating blood was flushed from the body with PBS via an aortic cannula. The kidneys and right tibias, right femurs and left femurs were collected, boiled in water for 5 minutes and/or snap frozen in liquid nitrogen and stored on dry ice or in a −70° C. freezer until cGMP assay. For estimation of cGMP production in cartilage, distal femurs and/or proximal tibias were dissected, weighed and pulverized using Covaris Cryoprep CP02. Powdered samples were homogenized using a Covaris E210 sonicator in 5% perchloric acid, and neutralized in 60% KOH. Samples were then centrifuged at 10,000 rpm at 4° C. for 5 minutes, and the supernatant was used for the cGMP assay (Cyclic GMP Enzyme Immunoassay Kit, Cayman, Mich.). Additionally, left tibias were collected, fixed in 10% normal buffered formalin, and saved for further immunohistochemical analysis.

FIG. **75**A shows that repeated daily treatment of wild-type mice with 200 nmol/kg of Pro-CNP38 for 1, 4, 6, 7 and 8 days did not result in desensitization of the cGMP response. To the contrary, potentiation of the cGMP response was observed after 4 days of daily treatment. Further daily treatment resulted in a plateau of the cGMP reponse, up to 8 days. The results indicate that daily treatment of wild-type mice with 200 nmol/kg of Pro -CNP38 for up to 8 days do not desensitize the cGMP response.

The kinetics of the cGMP response in distal femoral cartilage after treatment of the wild-type mice with Pro-CNP38 was also investigated. Treatment of the mice once a day for two days potentiated the cGMP response to Pro-CNP38, compared with the cGMP response to a single treatment (FIG. **75**B). When the mice were treated on the first and third days, but not on the first and second days, the cGMP response after the second treatment (on the third day) was substantially similar to the cGMP response observed after a single treatment on day one (FIG. **75**B). The results suggest that dosing on consecutive days is beneficial for potentiating the cGMP response to Pro-CNP38 in this mouse study.

Activation of NPR-B in Different Tissues

To evaluate potential activation of NPR-B in different tissues by a CNP variant, wild-type male CD-1 mice were injected with 200 nmol/kg of Gly-CNP37, and the cGMP response was measured in certain tissues at different time points. Two mice were used for each treatment group. Each mouse received one subcutaneous injection of Gly-CNP37 or vehicle control. Fifteen, 30 or 60 minutes or 3 hours following the injection, the mice were deeply anesthetized and ex-sanguinated via thoracotomy and aortic cannulation. Circulating blood was removed by a PBS flush with an aortic cannula. Heart, liver, lung, kidney, ear pinna, aorta and brain were collected. All tissues were boiled in water for 5 minutes, finely dissected (including flushing of marrow from femoral cortices with PBS), weighed, cooled in liquid nitrogen, and pulverized in a BioPulverizer. The resulting powdered samples were homogenized with a Polytron in 6% pre-chilled perchloric acid and neutralized with 60% KOH. Samples were then centrifuged at 10,000 rpm and 4° C. for 5 minutes, and the supernatant was used for the assay of cGMP (Cyclic GMP Enzyme Immunoassay Kit, Cayman Chemical Company, Ann Arbor, Mich.). The results were normalized for tissue weight.

Secretion of cGMP in response to treatment with Gly-CNP37 ("CNP" in FIG. **76**) was detectable in distal femurs (cartilage and bone), femoral cortices (bone), ear pinna (cartilage), and kidney (FIGS. **76**A-D). Maximal cGMP responses in those tissues were observed 15 min after

treatment. Liver, heart, lung and brain tissues did not exhibit appreciable cGMP secretion in response to Gly-CNP37 relative to vehicle control at the studied time points (FIGS. **76**E-H). The results indicate that treatment with 200 nmol/kg of Gly-CNP37 stimulated cGMP secretion in cartilage, bone and renal tissues.

Example 16

Dose Responses of CNP Variants in Monkeys

The effects of the CNP variant Pro-Gly-CNP37 on bone growth and the levels of bone growth-related biomarkers are evaluated in cynomolgus monkeys. Eight normal juvenile cynomolgus monkeys (about 2.5 years of age at the start of the on-going study) are subcutaneously injected daily with 10 or 36 μg/kg/day of Pro-Gly-CNP37 (n=4 per dose group). Four such monkeys are administered vehicle as control. The total length of treatment is 6 months. Various measures of growth plate expansion and bone growth are made by digital X-ray and magnetic resonance imaging, and by measurement of limb and body lengths externally. Blood and urine samples are collected periodically for clinical pathology and measurement of levels of Pro-Gly-CNP37 and biomarkers. Following termination of the study, gross pathology is performed and tissue samples are evaluated histologically for assessment of efficacy and safety.

Data obtained thus far in the on-going study show that both doses of Pro-Gly-CNP37 have increased growth plate width by digital X-ray (FIG. **77**), increased right and left tibia lengths by digital X-ray (FIGS. **78**A and B), increased leg length by external measurement (FIG. **79**), increased arm length by external measurement (FIG. **80**), increased body length by external measurement (FIG. **81**), and increased the serum level of alkaline phosphatase, a biomarker for bone formation (FIG. **82**). The data demonstrate that Pro-Gly-CNP37 can stimulate bone growth in normal juvenile cynomolgus monkeys at hemodynamically acceptable doses.

Example 17

Effects of CNP Variants on Cardiovascular System in Mice

Natriuretic peptides such as CNP have been reported to affect the cardiovascular system. Wang et al. (Eur J Heart Fail. 9:548-57. 2007) describe that CNP has been shown to have a cardioprotective effect in preventing myocardial ischaemia/reperfusion injury and improving cardiac remodelling after myocardial infarction in rats. Wang demonstrated that mice overexpressing CNP have reduced incidence of cardiac hypertrophy caused by myocardial infarction. Additionally, CNP has been shown to cause endothelium-independent vasodilation (M. Honing et al., Hypertension, 37:1179-1183 (2001)) and therefore may transiently decrease blood pressure in vivo.

To assess the effects of CNP variants on the cardiovascular system, the blood pressure and heart rate in anesthetized wild-type FVB mice is studied following subcutaneous injection of the variants.

After a pilot study to define a broad dose range of cardiovascular activity, a dose-response study is conducted to examine the effects of three different dose levels of each CNP variant. Three male FVB mice aged 8 weeks comprise each treatment group. Doses are administered subcutaneously to anesthetized mice, and systolic, diastolic and mean arterial pressure (MAP), as well as heart rate, are monitored via implanted intraarterial pressure transducers.

Example 18

Formulation of CNP Variants

CNP preformulation studies were carried out to assess the stability of CNP variant Gly-wtCNP37 ("CNP38") at different pH's (pH 3, 4, 5, 6, 7 and 8) and temperatures (5° C., 25° C., and 40° C.) overtime. CNP38 exhibited greater stability at pH 4-6 than at the other pH's in the studies. CNP38 was stable at 5° C. at pH 4-6, with about 95% of CNP38 remaining after 15 weeks. When the temperature was raised to 25° C., at pH 4 about 85% of CNP38 remained after 15 weeks, at pH 5 about 85% remained after 15 weeks, and at pH 6 about 80% remained after 15 weeks. When the temperature was raised to 40° C., at pH 4 about 55-60% of CNP38 remained after 15 weeks, at pH 5 about 65% remained after 15 weeks, and at pH 6 about 40% remained after 15 weeks. FIG. **83** illustrates the observed plot of pseudo-first order degradation rate constant (K_{obs}) vs. pH from pH 3 to 8 and at 5° C., 25° C. and 40° C. The stability data for CNP38 in the preformulation studies suggests CNP formulations having a pH in the range from about 4 to about 6. An acidic pH (e.g., pH≤about 6) can promote the stability of a CNP variant by, e g , minimizing or avoiding deamidation of asparagine and/or glutamine residue(s), isomerization of aspartic acid residue(s), or degradation of the CNP variant by other pathways.

CNP variants can be formulated in pharmaceutical carriers for administration to subjects affected by, e.g., bone growth conditions. In some embodiments, liquid formulations of CNP variants are formulated according to any combinations of the ingredients and their amounts or concentrations in Table 16.

TABLE 16

Ingredient Class	Ingredient	Concentration Range
Active ingredient	CNP variant	10 mg/mL ± 9.9 mg/mL
Buffering agent	Acetic acid/acetate	10 mM ± 5 mM, or pH 5 ± 1
Buffering agent	Citric acid/citrate	10 mM ± 5 mM, or pH 5 ± 1
Isotonicity-adjusting agent	NaCl	140 mM ± 20 mM
Isotonicity-adjusting agent	Sucrose	10% ± 5%
Preservative	m-Cresol	0.4% ± 0.1% or 0.2%
Preservative/anti-adsorbent	Benzyl alcohol	1.5% ± 0.5%
Stabilizer	Glycerin (glycerol)	5%-100% (neat)[1]
Stabilizer	Methionine	0.01%-0.2%
Stabilizer	Ascorbic acid/ ascorbate salt	0.1%-1%
Stabilizer	Thioglycerol	0.1%-1%
Anti-adsorbent	Polysorbate 20	0.001%-0.5%
	Polysorbate 80	0.001%-0.5%
	Benzyl alcohol	0.5%-1.5%

[1]Glycerin is used to minimize or prevent water-driven hydrolysis, deamidation, isomerization or cleavage of CNP variants. For lyophilized formulations, 4-6% or 6-20% mannitol or sucrose can be substituted for NaCl.

In certain embodiments, lyophilized formulations of CNP variants are prepared from formulations formulated according to any combinations of the ingredients and their amounts or concentrations in Table 17.

TABLE 17

Ingredient Class	Ingredient	Concentration Range
Active ingredient	CNP variant	10 mg/mL ± 9.9 mg/mL
Buffering agent	Acetic acid/acetate	10 mM ± 5 mM, or pH 5 ± 1
Buffering agent	Citric acid/citrate	10 mM ± 5 mM, or pH 5 ± 1
Isotonicity-adjusting agent/bulking agent	Sorbitol	5% ± 3%
Isotonicity-adjusting agent/bulking agent	Mannitol	5% ± 3%
Isotonicity-adjusting agent/bulking agent	Sucrose	10% ± 5%
Preservative	m-Cresol	0.4% ± 0.2%
Preservative/anti-adsorbent	Benzyl alcohol	1.5% ± 0.5%
Stabilizer	Glycerin (glycerol)	5%-100% (neat)[1]
Stabilizer	Methionine	0.01%-0.2%
Stabilizer	Ascorbic acid/ ascorbate salt	0.1%-1%
Stabilizer	Thioglycerol	0.1%-1%
Anti-adsorbent	Polysorbate 20	0.001%-0.5%
	Polysorbate 80	0.001%-0.5%
	Benzyl alcohol	0.5%-1.5%

[1]Glycerin is used to minimize or prevent water-driven hydrolysis, deamidation, isomerization or cleavage of CNP variants.

In certain embodiments, a formulation comprising a CNP variant has a pH of about 3-7, or about 3-6, or about 3.5-6.5, or about 4-6, or about 4-5, or about 4.5-5.5. In some embodiments, for pH 4-5.5 a suitable buffering agent is acetic acid/acetate (e.g., sodium acetate), and for pH 5.5-6 a suitable buffering agent is citric acid/citrate. Citric acid/citrate (e.g., sodium citrate) is also a suitable buffering agent in the range of pH 3-6 or pH 4-6. In certain embodiments, the buffering agent has a concentration in the formulation of about 2-50 mM, or about 2-40 mM, or about 2-30 mM, or about 5-30 mM, or about 2-20 mM, or about 5-20 mM, or about 5-15 mM.

To minimize or avoid deamidation of a CNP variant, the variant can be formulated in pharmaceutically acceptable organic cosolvents, such as glycerin, ethanol, and propylene glycol. Because deamidation occurs by hydrolysis, substitution of an organic cosolvent for water minimizes contact of the CNP variant with water. The concentration of one or more organic solvents in an organic-aqueous solvent system can be, e.g., from about 10% to about 99%, or about 100% if water is not used.

Also to minimize or avoid deamidation of a CNP variant, water can be removed from the formulation by lyophilization. In some embodiments, lyophilized formulations contain any combinations of the following components:

- buffer: sodium acetate and acetic acid, or sodium citrate and citric acid;
- isotonicity/bulking agent: mannitol (e.g., 3-10%, 2-8% or 4-6%);
  sucrose (e.g., 6-20%, 5-15% or 8-12%);
- antioxidants: methionine and/or ascorbic acid with molal ratio of each antioxidant to CNP variant from about 0.1:1 to about 1:1, or from about 0.5:1 to about 5:1, or from about 1:1 to about 15:1, or from about 1:1 to about 10:1, or from about 3:1 to about 10:1.

Deamidation can also be minimized or avoided by storing a CNP composition (e.g., a liquid formulation or a lyophilized formulation) at lower temperature, such as at about 5° C., 0° C., −10° C., −20° C., −30° C., −40° C., −50° C., −60° C., −70° C., −80° C., −90° C., or −100° C.

To minimize or avoid oxidation of oxidizable residues (e.g., methionine) in a CNP variant, the variant can be formulated with one or more antioxidants. Exemplary antioxidants include, but are not limited to, methionine, ascorbic acid, and thioglycerol. Oxidation of, e.g., methionine residues can also be minimized or prevented by purging oxygen from a liquid medium (if a liquid formulation) with nitrogen or argon, and/or by purging oxygen from a container or packaging with nitrogen or argon.

In some embodiments, to minimize or prevent adsorption (e.g., adsorption of a CNP variant to plastic or glass), Polysorbate 20, Polysorbate 80 or benzyl alcohol, or a combination thereof, is added to a CNP formulation. In certain embodiments, each of the anti-adsorbent(s) is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%. Exemplary range(s) of anti-adsorbent(s) in the formulation include without limitation from about 0.001% to about 0.5% of Polysorbate 20, from about 0.001% to about 0.5% of Polysorbate 80, and/or from about 0.5% to about 1.5% of benzyl alcohol.

In certain embodiments, a liquid CNP formulation comprises, or a lyophilized CNP formulation is prepared from a formulation that comprises, (1) an acetic acid/acetate (e.g., sodium acetate) buffer having a concentration of about 30 mM±5 or 10 mM buffering agent and a pH of about 4±0.5 or 1, and (2) benzyl alcohol (e.g., as a preservative and/or antiadsorbent) at a concentration of about 1%±0.5%, and optionally (3) sucrose at a concentration of about 10%±5%.

Example 19

Clinical Evaluation of CNP Variants

The following example provides guidance on the parameters to be used for the clinical evaluation of compositions comprising CNP22 or variants thereof in the therapeutic methods of the present disclosure. As discussed herein, CNP22 or variants thereof will be used in the treatment of disorders responsive to CNP, including disorders of the bone and vascular smooth muscle. Clinical trials will be conducted which will provide an assessment of doses of CNP22 or variants thereof for safety, pharmacokinetics, and initial response of both surrogate and defined clinical endpoints. The trial will be conducted for a minimum, but not necessarily limited to, 24 weeks to collect sufficient safety information on about 100 evaluable patients. The initial dose for the trials will vary from about 0.001 to about 1.0 mg/kg/week, or any of the doses described herein. In the event that the initial dose in this range does not produce a significant direct clinical benefit, the dose should be increased within this range or beyond this range as necessary, and maintained for an additional minimal period of, but not necessarily limited to, 24 weeks to establish safety and to evaluate efficacy further.

Measurements of safety will include adverse events, allergic reactions, complete clinical chemistry panel (including kidney and liver functions), urinalysis, and CBC with differential. In addition, other parameters relevant to clinical benefit will be monitored. The present example also includes the determination of pharmacokinetic parameters of CNP22 or variants thereof, including absorption, distribution, metabolism, excretion, and half-life and bioavailability in the blood. It is anticipated that such analyses will help relate dose to clinical response.

Methods

Patients will undergo a baseline medical history and physical exam, and a standard set of clinical laboratory tests

(including CBC, Panel 20, CH50, and UA). The patients will be followed closely with weekly visits to the clinic. The patients will return to the clinic for a complete evaluation one week after completing the treatment period. Should dose escalation be required, the patients will follow the same schedule outlined above. Safety will be monitored throughout the trial.

Diagnosis and Inclusion Criteria

The patients may be male or female, with a documented diagnosis of a potentially CNP-responsive disorder. A specific example of a potentially CNP-responsive, bone-related disorder is achondroplasia, which may be confirmed by genetic testing and other evidence of an FGFR-3 mutation or dysfunction. The ideal age range of achondroplasia patients will be from infant (<1 year of age) to pre-adolescent (<13 years of age). A patient will be excluded from this study if the patient is pregnant or lactating; has received an investigational drug within 30 days prior to study enrollment; or has a medical condition, serious intercurrent illness, or other extenuating circumstance that may significantly decrease study compliance.

Safety

Therapy with CNP22 or variants thereof will be determined to be safe if no significant acute or chronic drug reactions occur during the course of the study. The longer-term administration of the drug will be determined to be safe if no significant abnormalities are observed in the clinical examinations, clinical labs, or other appropriate studies.

It has been shown that compared to wild-type CNP22, certain CNP variants of the disclosure are much more resistant to NEP degradation in vitro, have a much longer plasma half-life and bioavailability in rats, stimulate a much higher level of cGMP production in rats, and/or induce a significantly greater increase in long bone length and body length in achondroplastic mice. Furthermore, it has been shown that short duration dose regimen treatments with CNP22 are nearly as effective as continuous CNP22 treatment in reversing FGF2-induced arrest of chondrocyte growth in vitro. These results, among others described herein, demonstrate the utility of CNP variants of the disclosure in treating CNP-responsive conditions or disorders such as, e.g., bone-related disorders and vascular smooth muscle disorders.

It is understood that every embodiment of the disclosure described herein may optionally be combined with any one or more of the other embodiments described herein. Every patent literature and every non-patent literature cited herein are incorporated herein by reference in their entirety.

Numerous modifications and variations to the disclosure, as set forth in the embodiments and illustrative examples described herein, are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the disclosure.

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35 40 45

Ser Gly Leu Gly Cys
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Met Ser Gly Leu Gly Cys
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Ser Gly Leu Gly Cys
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Met Ser Gly Leu Gly Cys
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<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANRR-CNP22(K4R) - CNP27(Arg4)

<400> SEQUENCE: 36

Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 37
<211> LENGTH: 27

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANPR-CNP22(K4R) - CNP27(Pro4) - Analog CI

<400> SEQUENCE: 37

Gly Ala Asn Pro Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 38
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: ER-CNP22

<400> SEQUENCE: 38

Glu Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
1 5 10 15

Gly Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 39
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BA - ER-CNP22(K4R)

<400> SEQUENCE: 39

Glu Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
1 5 10 15

Gly Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 40
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: R-CNP22

<400> SEQUENCE: 40

Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 41
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AZ - R-CNP22(K4R)

<400> SEQUENCE: 41

Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 42
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP22 (K4R)(K10R)

<400> SEQUENCE: 42

Gly Leu Ser Arg Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 43
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP-22 (Cys6-Phe7 variable bond)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced
with -CH2-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced
with -C(=O)-N(R)- where R is methyl, ethyl, n-propyl, isopropyl,
cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced
with -C(=O)-NH-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced
with -CH2-S-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced
with -CH2-S(O)n where n is 1 or 2
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced
with -CH2-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced
with -CH=CH-
<220> FEATURE:

-continued

<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -C(=O)-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH(CN)-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH(OH)-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -O-C(=O)-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with NHC(=O)NH-

<400> SEQUENCE: 43

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 44
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is 3-amino-2-phenylpropionic acid or D-Phe

<400> SEQUENCE: 44

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 45
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP22 - modified Cys6
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys, homocysteine, penicillamine, 2-mercaptopropionic acid, or 3-mercaptopropionic acid.

<400> SEQUENCE: 45

Gly Leu Ser Lys Gly Xaa Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 46

-continued

<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with a bone or cartilage targeting compound, a biphosphonate, polyAsp, polyGlu, osteopontin, osteocalcin, or sialoprotein peptides, polymers, PEG, carbohydrates, hydrophobic acids, or amino acids
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser, or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Xaa is Cys with peptide bond isosteres between positions 6 and 7
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, 3-amino-2-phenylpropionic acid, or N-alkylated derivatives of Phe
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: Xaa is Leu or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa is Ile, tBu-Gly and peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa is Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) or 2-amino-isobutyric acid (Aib)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with a bone or cartilage targeting compound, a bisphosphonate, polyAsp, polyGlu, osteopontin, osteocalcin or sialoprotein peptides, or amino acid sequences derived from non-CNP polypeptides

<400> SEQUENCE: 46

Gly Leu Ser Xaa Gly Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Gly Ser
1 5 10 15

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Xaa Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 47
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with a peptide sequence containing one to five amino acids from a natriuretic or non-natriuretic polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with a peptide sequence containing one to five amino acids from a natriuretic or non-natriuretic polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid or peptidomimetic, Arg, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser, or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys or decarboxy Cys
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -CH2-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -C(=O)-N(R)- where R is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -C(=O)-NH-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -CH2-S-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -CH2-S(O)n where n is 1 or 2
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -CH2-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -CH=CH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -C(=O)-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -CH(CN)-NH-

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<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -CH(OH)-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -O-C(=O)-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be replaced with -NHC(=O)NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, 3-amino-2-phenylpropionic acid, or N-alkylated derivatives of Phe
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: Xaa is Leu or peptide isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa is Ile, tBu-Gly or peptide isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa is Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) or 2-amino-isobutyric acid (Aib)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with a synthetic bone targeting compound, a bisphosphonate, polyAsp, polyGlu, osteopontin, osteocalcin or sialoprotein peptides, or charged PEG molecules

<400> SEQUENCE: 47

Gly Leu Ser Xaa Gly Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Gly Ser
1 5 10 15

Xaa Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 48
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:

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<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus is optionally modified with a synthetic or natural polymeric group of combination thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa is Gly or is absent
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa is Leu or is absent
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: Xaa is Ser or is absent
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or is absent
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa is Gly or is absent
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Lys, a conservative amino acid substitution, any natural or unnatural amino acid or peptidomimetic that does have a reactive primary amine on a side chain
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with a synthetic or natural polymeric group or a combination thereof

<400> SEQUENCE: 48

Xaa Xaa Xaa Xaa Xaa Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 49
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus is optionally modified with a peptide fragment or water soluble polymer
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa is Cys or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, 3-amino-2-phenylpropionic acid or N-alkylated derivatives of Phe
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or N-Me-Leu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid

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substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Leu and peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Ile, tBu-Gly or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (12)..(12)
<223> OTHER INFORMATION: Xaa is Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) or 2-amino-isobutyric acid (Aib)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Cys is optionally modified with amino acid sequences useful in bone/cartilage targeting, polyAsp or polyGlu, osteopontin, osteocalcin, sialoprotein or sequences derived from NPPC or non-CNP polypeptides

<400> SEQUENCE: 49

Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Gly Ser Xaa Ser Gly Xaa Gly
1 5 10 15

Cys

<210> SEQ ID NO 50
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with bone targeting compound, moieties to reduce renal clearance, charged PEG molecules or hydrophilic polymers
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys or peptide isosteres between positions 6 and 7
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, 3-amino-2-phenylpropionic acid or N-alkylated derivatives of Phe
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly, Thr, Ser, Val or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)

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<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: Xaa is Leu, Asn or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa is Ile, tert-butyl-Gly (tBu-Gly), Asn or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Gly, Arg, Ser or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa is Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) or 2-amino-isobutyric acid (Aib)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser or peptide bond isoteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with bone targeting compound, moieties to reduce renal clearance, charged PEG molecules or hydrophilic polymers

<400> SEQUENCE: 50

Gly Leu Ser Xaa Gly Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Xaa Ser
1 5 10 15

Xaa Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 51
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa is Arg or Glu

<400> SEQUENCE: 51

Xaa Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 52
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa is Arg, Gln or Ser

<400> SEQUENCE: 52

Gly Leu Ser Lys Xaa Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

-continued

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 53
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Ser, Asn, Arg, or Cit

<400> SEQUENCE: 53

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Xaa Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 54
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: Xaa is Ser, Arg, or Asn

<400> SEQUENCE: 54

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Xaa Leu Gly Cys
20

<210> SEQ ID NO 55
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (21)..(21)
<223> OTHER INFORMATION: Xaa is Ser, Thr, or Arg

<400> SEQUENCE: 55

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Xaa Cys
20

<210> SEQ ID NO 56
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog A
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: CH2NH

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<400> SEQUENCE: 56

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 57
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analogs B
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa replaced with N-Me-Phe

<400> SEQUENCE: 57

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 58
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog F
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: tBu

<400> SEQUENCE: 58

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 59
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog H
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is replaced with NHCH2-CH(Phe)CO

<400> SEQUENCE: 59

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 60

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<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BL - CNP37

<400> SEQUENCE: 60

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 61
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CA

<400> SEQUENCE: 61

Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Gly Leu Ser
1 5 10 15

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
20 25 30

Leu Gly Cys
35

<210> SEQ ID NO 62
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CB

<400> SEQUENCE: 62

Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Gly Leu
1 5 10 15

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
20 25 30

Gly Leu Gly Cys
35

<210> SEQ ID NO 63
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CC

<400> SEQUENCE: 63

Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Gly Leu
1 5 10 15

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Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
20 25 30

Gly Leu Gly Cys
35

<210> SEQ ID NO 64
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANQQ-CNP22

<400> SEQUENCE: 64

Gly Ala Asn Gln Gln Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 65
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANRR-CNP22

<400> SEQUENCE: 65

Gly Ala Asn Arg Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 66
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANPR-CNP22

<400> SEQUENCE: 66

Gly Ala Asn Pro Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 67
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANSS-CNP22

<400> SEQUENCE: 67

Gly Ala Asn Ser Ser Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 68
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequene
<220> FEATURE:
<223> OTHER INFORMATION: Sythetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CD
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus BNP tail
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (32)..(32)
<223> OTHER INFORMATION: N-terminus BNP tail

<400> SEQUENCE: 68

Ser Pro Lys Met Val Gln Gly Ser Gly Cys Phe Gly Leu Lys Leu Asp
1 5 10 15

Arg Ile Gly Ser Met Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His
20 25 30

<210> SEQ ID NO 69
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANQQ-CNP22(K4R) - Analog CH

<400> SEQUENCE: 69

Gly Ala Asn Gln Gln Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 70
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANSS-CNP22(K4R) - Analog CG

<400> SEQUENCE: 70

Gly Ala Asn Ser Ser Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 71
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CS

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<400> SEQUENCE: 71

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Pro Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 72
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CT

<400> SEQUENCE: 72

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Gln Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 73
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CU

<400> SEQUENCE: 73

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Gln Gln
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 74
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CW

<400> SEQUENCE: 74

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Pro
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

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<210> SEQ ID NO 75
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 75

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 76
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CQ

<400> SEQUENCE: 76

Gly His His Ser His Glu Gln His Pro His Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 77
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CR

<400> SEQUENCE: 77

Gly Ala His His Pro His Glu His Asp Thr His Gly Ala Asn Gln Gln
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 78
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequencce
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CX

<400> SEQUENCE: 78

Gly His His Ser His Glu Gln His Pro His Gly Ala Asn Pro Arg Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met

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```
            20                  25                  30

Ser Gly Leu Gly Cys
        35


<210> SEQ ID NO 79
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CF

<400> SEQUENCE: 79

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gly Leu
1               5                   10                  15

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
            20                  25                  30

Gly Leu Gly Cys
        35


<210> SEQ ID NO 80
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CY

<400> SEQUENCE: 80

Gly Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg Gly Ala Asn Pro
1               5                   10                  15

Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
            20                  25                  30

Ser Met Ser Gly Leu Gly Cys
        35


<210> SEQ ID NO 81
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CE

<400> SEQUENCE: 81

Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Gly
1               5                   10                  15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
            20                  25                  30

Ser Gly Leu Gly Cys
        35


<210> SEQ ID NO 82
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
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<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CZ

<400> SEQUENCE: 82

Phe Gly Ile Pro Met Asp Arg Ile Gly Arg Asn Pro Arg Gly Leu Ser
1 5 10 15

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
20 25 30

Leu Gly Cys
35

<210> SEQ ID NO 83
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog DA

<400> SEQUENCE: 83

Gly Lys Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly
1 5 10 15

Pro Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
20 25 30

Gly Ser Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 84
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CK

<400> SEQUENCE: 84

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 85
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CL

<400> SEQUENCE: 85

Gly Val Pro Gln Val Ser Thr Ser Thr Gly Ala Asn Gln Gln Gly Leu
1 5 10 15

Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
20 25 30

Gly Leu Gly Cys
35

<210> SEQ ID NO 86
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CM

<400> SEQUENCE: 86

Gly Gln Pro Ser Ser Ser Ser Gln Ser Thr Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 87
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CN

<400> SEQUENCE: 87

Gly Gln Thr His Ser Ser Gly Thr Gln Ser Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 88
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CO

<400> SEQUENCE: 88

Gly Ser Thr Gly Gln Trp His Ser Glu Ser Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 89
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CP

<400> SEQUENCE: 89

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Gly Ser Ser Ser Ser Ser Ser Ser Ser Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 90
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with bone targeting compound, a bisphosphonates, polyAsp, polyGlu, osteopontin, osteocalcin, or sialoprotein peptides, negatively charged PEG molecules or natural polymers
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys or another natural or unnatural amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is Phe or another natural or unnatural amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, a larger natural or unnatural amino acid or peptidomimetic
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with bone targeting compound, a bisphosphonates, polyAsp, polyGlu, osteopontin, osteocalcin, or sialoprotein peptides, negatively charged PEG molecules or natural polymers

<400> SEQUENCE: 90

Gly Leu Ser Lys Gly Xaa Xaa Xaa Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 91
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog J
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is CH2-NH
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 91

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Gly Leu Ser Lys Gly Xaa Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 92
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog K
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 92

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 93
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog L
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 93

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 94
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog M
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me

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<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 94

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 95
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog Z

<400> SEQUENCE: 95

Gly Leu Ser Arg Gly Cys Tyr Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 96
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AA

<400> SEQUENCE: 96

Gly Leu Ser Arg Gly Cys Phe Val Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 97
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AB

<400> SEQUENCE: 97

Gly Leu Ser Arg Gly Cys Phe Ser Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 98
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE

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<223> OTHER INFORMATION: Analog AC

<400> SEQUENCE: 98

Gly Leu Ser Arg Gly Cys Phe Thr Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 99
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AD

<400> SEQUENCE: 99

Gly Leu Ser Arg Gly Cys Phe Gly Thr Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 100
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AE

<400> SEQUENCE: 100

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Arg Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 101
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AF
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Cit

<400> SEQUENCE: 101

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Xaa Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 102
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:

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<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AG

<400> SEQUENCE: 102

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Val Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 103
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AJ

<400> SEQUENCE: 103

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Val Gly Cys
20

<210> SEQ ID NO 104
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AK
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is tBu-Ala

<400> SEQUENCE: 104

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 105
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AT

<400> SEQUENCE: 105

Glu Leu Ser Glu Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 106
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

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<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AV
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus modified with pentanoic acid

<400> SEQUENCE: 106

Glu Leu Ser Glu Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 107
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AW
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Heptanoic acid modifying N-terminus

<400> SEQUENCE: 107

Glu Leu Ser Glu Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 108
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BB
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus modified with heptanoic acid

<400> SEQUENCE: 108

Glu Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 109
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BC
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus modified with pentanoic acid

<400> SEQUENCE: 109

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Glu Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 110
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BF
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Cit

<400> SEQUENCE: 110

Gly Leu Ser Arg Gly Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 111
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BG

<400> SEQUENCE: 111

Gly Leu Ser Arg Gly Cys Phe Gly Leu Gln Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 112
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BH

<400> SEQUENCE: 112

Gly Leu Ser Arg Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 113
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BJ

<400> SEQUENCE: 113

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Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Asn Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 114
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BK

<400> SEQUENCE: 114

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Ser Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 115
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog N
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)..(22)
<223> OTHER INFORMATION: All D-amino acids

<400> SEQUENCE: 115

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 116
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BD
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 116

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 117
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

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<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BN
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 117

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 118
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BE
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 118

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 119
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog V
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: 3,4-dichloro

<400> SEQUENCE: 119

Gly Leu Ser Gly Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 120
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog X
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: 3-methyl

<400> SEQUENCE: 120

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Gly Leu Ser Gly Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 121
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BP

<400> SEQUENCE: 121

Gly Leu Ser Arg Ser Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 122
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BO

<400> SEQUENCE: 122

Gly Leu Ser Arg Arg Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 123
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog P

<400> SEQUENCE: 123

Gly Leu Ser Gly Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 124
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BY

<400> SEQUENCE: 124

Gly Leu Ser Arg Gly Cys Phe Gly Leu Ser Leu Asp Arg Ile Gly Ser
1 5 10 15

-continued

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 125
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BZ

<400> SEQUENCE: 125

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Gln
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 126
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BX

<400> SEQUENCE: 126

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Asn Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 127
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BQ

<400> SEQUENCE: 127

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Ser Leu Gly Cys
20

<210> SEQ ID NO 128
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BR

<400> SEQUENCE: 128

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Arg Leu Gly Cys
20

<210> SEQ ID NO 129
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BS

<400> SEQUENCE: 129

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Arg Gly Cys
20

<210> SEQ ID NO 130
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BT

<400> SEQUENCE: 130

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Ser Cys
20

<210> SEQ ID NO 131
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BU

<400> SEQUENCE: 131

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Thr Cys
20

<210> SEQ ID NO 132
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BW

<400> SEQUENCE: 132

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Arg Cys
20

<210> SEQ ID NO 133
<211> LENGTH: 15

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CJ

<400> SEQUENCE: 133

Arg Ser Ser Cys Phe Gly Gly Arg Ile Asp Arg Ile Gly Ala Cys
1 5 10 15

<210> SEQ ID NO 134
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminal linked to CNP22 by disuccinimidyl glutarate

<400> SEQUENCE: 134

Gly Leu Ser Arg Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 135
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminal linked to CNP22 by Bis-PEO5

<400> SEQUENCE: 135

Gly Leu Ser Arg Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 136
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog E
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is D-Phe

<400> SEQUENCE: 136

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 137

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<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog G
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is 3-Cl-Phe

<400> SEQUENCE: 137

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 138
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa comprises one or more amino acids selected from Gly-Leu-Ser-Lys-Gly or substitutions at one or more of these amino acids and further comprises a hydrophilic or water soluable polymer

<400> SEQUENCE: 138

Xaa Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
1 5 10 15

Gly Cys

<210> SEQ ID NO 139
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Cys is modified with a natural and/or synthetic polymer
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Cys is modified with a natural and/or synthetic polymer

<400> SEQUENCE: 139

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
1 5 10 15

Cys

<210> SEQ ID NO 140
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is modified with a natural and/or synthetic

polymer
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is modified with a natural and/or synthetic polymer

<400> SEQUENCE: 140

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 141
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Cys is modified with a natural and/or synthetic polymeric group or combination thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Cys is optionally modified with a natural and/or synthetic polymeric group or combination thereof

<400> SEQUENCE: 141

Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
1 5 10 15

Cys

<210> SEQ ID NO 142
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa is optionally modified with bone- or cartilage-targeting moieties, moieties that reduce renal clearance, hydrophilic polymers, carbohydrates, hydrophobic acids and combinations thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa is absent or may comprise 1 to 5 amino acids from Gly-Leu-Ser-Lys-Gly, optionally substituted with a natural or unnatural amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is Lys, any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: Xaa is optionally modified with bone- or cartilage-targeting moieties, moieties that reduce renal clearance, hydrophilic polymers, carbohydrates, hydrophobic acids and combinations thereof

<400> SEQUENCE: 142

Xaa Xaa Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser Met Ser Gly
1 5 10 15

Leu Gly Cys

<210> SEQ ID NO 143
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with cartilage or bone targeting compounds, a bisphosphonate, polyAsp, polyGlu, osteopontin peptides, osteocalcin peptides, sialoprotein peptides, charged PEG molecules or hydrophilic polymers
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys or peptide isosteres between positions 6 and 7
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, N-alkylated Phe, or Phe analog
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly, Thr, Ser, Val or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Gly, Arg, Ser or Asn
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser or peptide bond isoteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)

-continued

<223> OTHER INFORMATION: Cys is optionally modified with cartilage or bone targeting compounds, a bisphosphonate, polyAsp, polyGlu, osteopontin peptides, osteocalcin peptides, sialoprotein peptides, charged PEG molecules or hydrophilic polymers

<400> SEQUENCE: 143

Gly Leu Ser Xaa Gly Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Xaa Ser
1 5 10 15

Xaa Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 144
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 144

Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 145
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 145

Pro Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 146
<211> LENGTH: 52
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 146

Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln
1 5 10 15

Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu
20 25 30

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
35 40 45

Gly Leu Gly Cys
50

<210> SEQ ID NO 147
<211> LENGTH: 51
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 147

Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu
1 5 10 15

His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser
20 25 30

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
35 40 45

Leu Gly Cys
50

<210> SEQ ID NO 148
<211> LENGTH: 50
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 148

Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His
1 5 10 15

Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys
20 25 30

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
35 40 45

Gly Cys
50

<210> SEQ ID NO 149
<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 149

Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro
1 5 10 15

Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly
20 25 30

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
35 40 45

Cys

<210> SEQ ID NO 150
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 150

Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn
1 5 10 15

Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys
20 25 30

Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40 45

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<210> SEQ ID NO 151
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 151

Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala
1 5 10 15

Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe
20 25 30

Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40 45

<210> SEQ ID NO 152
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 152

Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg
1 5 10 15

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
20 25 30

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40 45

<210> SEQ ID NO 153
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 153

Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys
1 5 10 15

Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu
20 25 30

Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40 45

<210> SEQ ID NO 154
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 154

Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr
1 5 10 15

Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys
20 25 30

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 155
<211> LENGTH: 43
<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 155

Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys
1 5 10 15

Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
20 25 30

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 156
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 156

Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly
1 5 10 15

Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp
20 25 30

Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 157
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 157

Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala
1 5 10 15

Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg
20 25 30

Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 158
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 158

Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn
1 5 10 15

Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
20 25 30

Gly Ser Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 159
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

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<400> SEQUENCE: 159

Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 160
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 160

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 161
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 161

Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu
1 5 10 15

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
20 25 30

Gly Leu Gly Cys
35

<210> SEQ ID NO 162
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 162

His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser
1 5 10 15

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
20 25 30

Leu Gly Cys
35

<210> SEQ ID NO 163
<211> LENGTH: 34
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 163

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Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys
1 5 10 15

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys

<210> SEQ ID NO 164
<211> LENGTH: 33
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 164

Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly
1 5 10 15

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
20 25 30

Cys

<210> SEQ ID NO 165
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 165

Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys
1 5 10 15

Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 166
<211> LENGTH: 31
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 166

Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe
1 5 10 15

Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 167
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 167

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
1 5 10 15

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 168
<211> LENGTH: 29
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 168

Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu
1 5 10 15

Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 169
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 169

Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys
1 5 10 15

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 170
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 170

Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 171
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 171

Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp
1 5 10 15

Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 172
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 172

Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg
1 5 10 15

Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 173
<211> LENGTH: 24
<212> TYPE: PRT

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 173

Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
1 5 10 15

Gly Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 174
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 174

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 175
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 175

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
1 5 10 15

Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 176
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 176

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
1 5 10 15

Gly Leu Gly Cys
20

<210> SEQ ID NO 177
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 177

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
1 5 10 15

Leu Gly Cys

<210> SEQ ID NO 178
<211> LENGTH: 18
<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 178

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
1 5 10 15

Gly Cys

<210> SEQ ID NO 179
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 179

Gly Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 180
<211> LENGTH: 53
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 180

Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu
1 5 10 15

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
20 25 30

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Asn
35 40 45

Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 181
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 181

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Asn Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 182
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence

-continued

<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 182

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Asn
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 183
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetic Polypeptide

<400> SEQUENCE: 183

Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Asn Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 184
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 184

Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Asn Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 185
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 185

Pro Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 186
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

-continued

<400> SEQUENCE: 186

Pro Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 187
<211> LENGTH: 34
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 187

Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys
1 5 10 15

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys

<210> SEQ ID NO 188
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 188

Pro Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 189
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 189

Pro Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys
1 5 10 15

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 190
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 190

Met Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

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Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 191
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 191

Met Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 192
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 192

Met Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 193
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 193

Met Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 194
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 194

Met Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys
1 5 10 15

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 195
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 195

Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val Pro
1 5 10 15

Arg Gly Ser His Thr Gly Asp Asp Asp Asp Lys His Met Asp
20 25 30

<210> SEQ ID NO 196
<211> LENGTH: 142
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 196

Met Val Leu Thr Lys Lys Lys Leu Gln Asp Leu Val Arg Glu Val Cys
1 5 10 15

Pro Asn Glu Gln Leu Asp Glu Asp Val Glu Glu Met Leu Leu Gln Ile
20 25 30

Ala Asp Asp Phe Ile Glu Ser Val Val Thr Ala Ala Cys Gln Leu Ala
35 40 45

Arg His Arg Lys Ser Ser Thr Leu Glu Val Lys Asp Val Gln Leu His
50 55 60

Leu Glu Arg Gln Trp Asn Met Trp Ile Met Gly Ser Ser His His His
65 70 75 80

His His His Ser Ser Gly Leu Val Pro Arg Gly Ser His Thr Gly Asp
85 90 95

Asp Asp Asp Lys His Met Asp Pro Gly Gln Glu His Pro Asn Ala Arg
100 105 110

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
115 120 125

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
130 135 140

<210> SEQ ID NO 197
<211> LENGTH: 72
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 197

Val Leu Thr Lys Lys Lys Leu Gln Asp Leu Val Arg Glu Val Cys Pro
1 5 10 15

Asn Glu Gln Leu Asp Glu Asp Val Glu Glu Met Leu Leu Gln Ile Ala
20 25 30

Asp Asp Phe Ile Glu Ser Val Val Thr Ala Ala Cys Gln Leu Ala Arg
35 40 45

His Arg Lys Ser Ser Thr Leu Glu Val Lys Asp Val Gln Leu His Leu
50 55 60

Glu Arg Gln Trp Asn Met Trp Ile
65 70

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<210> SEQ ID NO 198
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 198

Pro Gly Gln Glu His Pro Asn Ala Arg
1 5

<210> SEQ ID NO 199
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 199

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
1 5 10 15

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 200
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 200

Gly Ala Asn Lys Pro
1 5

<210> SEQ ID NO 201
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 201

Gly Ala Asn Pro Lys
1 5

<210> SEQ ID NO 202
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 202

Gly Ala Asn Pro Gln
1 5

<210> SEQ ID NO 203
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 203

Gly Ala Asn Gln Lys

-continued

1 5

<210> SEQ ID NO 204
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 204

Arg Leu Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg
1 5 10 15

Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg
20 25 30

Ile Gly Ala Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 205
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 205

Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg Ser Lys
1 5 10 15

Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly
20 25 30

Ala Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 206
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 206

Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg Ser Lys Lys
1 5 10 15

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 207
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 207

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 208
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 208

Arg Leu Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg
1 5 10 15

Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg

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            20                  25                  30

Ile Gly Ala Met Ser Gly Leu Gly Cys
        35                  40


<210> SEQ ID NO 209
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 209

Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg Ser Lys
1               5                   10                  15

Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly
            20                  25                  30

Ala Met Ser Gly Leu Gly Cys
        35


<210> SEQ ID NO 210
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 210

Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg Ser Lys Lys
1               5                   10                  15

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
            20                  25                  30

Met Ser Gly Leu Gly Cys
        35


<210> SEQ ID NO 211
<211> LENGTH: 29
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 211

Phe Lys Gly Arg Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val
1               5                   10                  15

Lys Leu Asp Arg Ile Gly Ala Met Ser Gly Leu Gly Cys
            20                  25


<210> SEQ ID NO 212
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 212

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
1               5                   10                  15

Met Ser Gly Leu Gly Cys
            20


<210> SEQ ID NO 213
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Squalus acanthias

<400> SEQUENCE: 213

Arg Leu Leu Gln Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg
1               5                   10                  15

Ser Lys Lys Gly Pro Ser Arg Ser Cys Phe Gly Leu Lys Leu Asp Arg
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            20                  25                  30

Ile Gly Ala Met Ser Gly Leu Gly Cys
        35                  40


<210> SEQ ID NO 214
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Squalus acanthias

<400> SEQUENCE: 214

Gly Pro Ser Arg Ser Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ala
1               5                   10                  15

Met Ser Gly Leu Gly Cys
            20


<210> SEQ ID NO 215
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 215

Gly Trp Asn Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1               5                   10                  15

Met Ser Gly Leu Gly Cys
            20


<210> SEQ ID NO 216
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 216

Pro Met Val Ala Gly Gly Gly Cys Phe Gly Met Lys Met Asp Arg Ile
1               5                   10                  15

Gly Ser Ile Ser Gly Leu Gly Cys
            20


<210> SEQ ID NO 217
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 217

Gly Arg Ser Ser Met Val Gly Gly Arg Gly Cys Phe Gly Met Lys Ile
1               5                   10                  15

Asp Arg Ile Gly Ser Ile Ser Gly Leu Gly Cys
            20                  25


<210> SEQ ID NO 218
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 218

Gly Gly Met Arg Ser Cys Phe Gly Val Arg Leu Glu Arg Ile Gly Ser
1               5                   10                  15

Phe Ser Gly Leu Gly Cys
            20


<210> SEQ ID NO 219
<211> LENGTH: 22
<212> TYPE: PRT
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<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 219

Gly Gly Leu Arg Ser Cys Phe Gly Val Arg Leu Ala Arg Ile Gly Ser
1 5 10 15

Phe Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 220
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 220

Gly Gly Ser Thr Ser Arg Ser Gly Cys Phe Gly His Lys Met Asp Arg
1 5 10 15

Ile Gly Thr Ile Ser Gly Met Gly Cys
20 25

<210> SEQ ID NO 221
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 221

Gly Gly Ser Ser Arg Ser Gly Cys Phe Gly His Lys Met Asp Arg Ile
1 5 10 15

Gly Thr Ile Ser Gly Met Gly Cys
20

<210> SEQ ID NO 222
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Ornithorhynchus anatinus

<400> SEQUENCE: 222

Leu Leu His Asp His Pro Asn Pro Arg Lys Tyr Lys Pro Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Thr Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 223
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Ornithorhynchus anatinus

<400> SEQUENCE: 223

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Thr Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 224
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 224

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Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 225
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 225

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Gln Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 226
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 226

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Ala Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 227
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 227

Ser Leu Arg Arg Ser Ser Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 228
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 228

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr
20 25

<210> SEQ ID NO 229
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 229

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Gln Ser Gly Leu Gly
1 5 10 15

Cys Asn Ser Phe Arg Tyr
20

<210> SEQ ID NO 230
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetnic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 230

Ile Glu Gly Arg Xaa
1 5

<210> SEQ ID NO 231
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 231

Asp Asp Asp Asp Lys Xaa
1 5

<210> SEQ ID NO 232
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(3)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 232

Glu Xaa Xaa Tyr Xaa Gln Gly
1 5

<210> SEQ ID NO 233
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 233

Val Asp Asp Arg
1

<210> SEQ ID NO 234
<211> LENGTH: 4
<212> TYPE: PRT

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 234

Gly Ser Asp Arg
1

<210> SEQ ID NO 235
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 235

Ile Thr Asp Arg
1

<210> SEQ ID NO 236
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 236

Pro Gly Asp Arg
1

<210> SEQ ID NO 237
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 237

Ala Phe Leu Gly Pro Gly Asp Arg
1 5

What is claimed is:

[1. A variant of C-type natriuretic peptide (CNP) selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Gly-CNP53);

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Pro-CNP53);

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Met-CNP53)

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNS
GLGC [CNP-53(M48N)].]

[2. A pharmaceutical composition comprising a CNP variant of claim 1, and a pharmaceutically acceptable excipient, carrier or diluent.]

[3. The composition of claim 2, which is a lyophilized formulation prepared from a formulation that comprises a citric acid/citrate buffer or an acetic acid/acetate buffer having a pH from about 4 to about 6.]

[4. The composition of claim 3, wherein the lyophilized formulation is prepared from a formulation that further comprises (a) an isotonicity-adjusting agent or a bulking agent or (b) an antioxidant.]

[5. A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a CNP variant to a subject in need thereof, wherein the CNP variant is a CNP variant according to claim 1, and wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.]

[6. A method for recombinant production of a CNP variant, comprising culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant polypeptide linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant polypeptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker, herein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Gly-CNP53);

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Pro-CNP53);

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Met-CNP53);

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNS
GLGC [CNP-53(M48N)].]

[7. The method of claim 6, wherein the cleavable peptide or protein is selected from the group consisting of histidine tags, human transcription factor TAF12, TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12 (6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12 (C/A & 4D/4E), TAF12(C/A & 6D/6E), TAF12(C/A & 10D/10E), ketosteroid isomerase, maltose-binding protein, β-galactosidase, glutathione-S-transferase, thioredoxin, chitin-binding domain, BMP-2, BMP-2 mutants, BMP-2(C/A), and mutants and fragments thereof.]

[8. The method of claim 6, wherein the host cell is bacterial.]

[9. The method of claim 6, wherein the fusion polypeptide is expressed as a soluble protein or as an inclusion body.]

[10. The method of claim 6, further comprising isolating the expressed fusion polypeptide from the host cell or culture medium.]

[11. The method of claim 10, further comprising contacting the isolated fusion polypeptide with a cleaving agent selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease.]

[12. A CNP variant produced according to the method of claim 6, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Gly-CNP53));

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Pro-CNP53));

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Met-CNP53));

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSG
LGC [CNP-53(M48N)]).]

[13. A method of increasing long bone growth, comprising administering a CNP variant according to claim 1 to a subject in need thereof, where the administration increases long bone growth.]

[14. A CNP variant according to claim 1 useful for increasing long bone growth or treating achondroplasia, hypochondroplasia, short stature, dwarfism, or homozygous achondroplasia in a subject in need thereof.]

15. A macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 158)
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40);

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37);

(SEQ ID NO: 161)
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36);

(SEQ ID NO: 162)
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35);

(SEQ ID NO: 163)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

(SEQ ID NO: 164)
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33);

(SEQ ID NO: 165)
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32);

(SEQ ID NO: 166)
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31);

(SEQ ID NO: 167)
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30);

(SEQ ID NO: 182)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP37(M32N)];

(SEQ ID NO: 186)
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37);

(SEQ ID NO: 192)
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37);

(SEQ ID NO: 181)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[Gly-CNP37(M32N)];

(SEQ ID NO: 191)
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-Gly-CNP37);

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27);

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEG1K-CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO12-CNP27(K4,5,9R)];
and

(SEQ ID NO: 36)
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO24-CNP27(K4,5,9R)],

and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

16. The macromolecule of claim 15, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

17. The macromolecule of claim 16, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

18. A sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 158)
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40);

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37);

(SEQ ID NO: 161)
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36);

(SEQ ID NO: 162)
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35);

(SEQ ID NO: 163)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

(SEQ ID NO: 164)
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33);

(SEQ ID NO: 165)
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32);

(SEQ ID NO: 166)
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31);

(SEQ ID NO: 167)
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30);

(SEQ ID NO: 182)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP37(M32N)];

(SEQ ID NO: 186)
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37);

(SEQ ID NO: 192)
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37);

(SEQ ID NO: 181)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[Gly-CNP37(M32N)];

(SEQ ID NO: 191)
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-Gly-CNP37);

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27);

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEG1K-CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO12-CNP27(K4,5,9R)];
and

-continued

(SEQ ID NO: 36)
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO24-CNP27(K4,5,9R)].

19. The sustained release formulation of claim 18, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

20. The sustained release formulation of claim 19, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

21. A method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 158)
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40);

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37);

(SEQ ID NO: 161)
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36);

(SEQ ID NO: 162)
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35);

(SEQ ID NO: 163)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

(SEQ ID NO: 164)
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33);

(SEQ ID NO: 165)
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32);

(SEQ ID NO: 166)
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31);

(SEQ ID NO: 167)
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30);

(SEQ ID NO: 182)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP37(M32N)];

(SEQ ID NO: 186)
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37);

(SEQ ID NO: 192)
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37);

(SEQ ID NO: 181)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[Gly-CNP37(M32N)];

(SEQ ID NO: 191)
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-Gly-CNP37);

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27);

-continued

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEG1K-CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO12-CNP27(K4,5,9R)];
and

(SEQ ID NO: 36)
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO24-CNP27(K4,5,9R)].

22. A method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 158)
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40);

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37);

(SEQ ID NO: 161)
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36);

(SEQ ID NO: 162)
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35);

(SEQ ID NO: 163)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

(SEQ ID NO: 164)
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33);

(SEQ ID NO: 165)
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32);

(SEQ ID NO: 166)
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31);

(SEQ ID NO: 167)
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30);

(SEQ ID NO: 182)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP37(M32N)];

(SEQ ID NO: 186)
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37);

(SEQ ID NO: 192)
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37);

(SEQ ID NO: 181)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[Gly-CNP37(M32N)];

(SEQ ID NO: 191)
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-Gly-CNP37);

-continued

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27);

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEG1K-CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO12-CNP27(K4,5,9R)];
and

(SEQ ID NO: 36)
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO24-CNP27(K4,5,9R)].

23. A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 158)
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40);

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37);

(SEQ ID NO: 161)
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36);

(SEQ ID NO: 162)
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35);

(SEQ ID NO: 163)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

(SEQ ID NO: 164)
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33);

(SEQ ID NO: 165)
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32);

(SEQ ID NO: 166)
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31);

(SEQ ID NO: 167)
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30);

(SEQ ID NO: 182)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP37(M32N)];

(SEQ ID NO: 186)
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37);

(SEQ ID NO: 192)
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37);

-continued

(SEQ ID NO: 181)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[Gly-CNP37(M32N)];

(SEQ ID NO: 191)
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-Gly-CNP37);

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27);

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEG1K-CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO12-CNP27(K4,5,9R)];
and

(SEQ ID NO: 36)
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO24-CNP27(K4,5,9R)],

and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

24. The method of claim 23, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

25. The method of claim 24, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

26. The method of claim 25, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

27. A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 158)
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-40);

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-39);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-38);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-37);

(SEQ ID NO: 161)
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36);

(SEQ ID NO: 162)
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35);

(SEQ ID NO: 163)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

(SEQ ID NO: 164)
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33);

(SEQ ID NO: 165)
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32);

(SEQ ID NO: 166)
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31);

(SEQ ID NO: 167)
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30);

(SEQ ID NO: 182)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP37(M32N)];

(SEQ ID NO: 186)
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-CNP37);

(SEQ ID NO: 192)
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-CNP37);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37);

(SEQ ID NO: 181)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[Gly-CNP37(M32N)];

(SEQ ID NO: 191)
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-Gly-CNP37);

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC
(HSA-CNP27);

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEG1K-CNP27(K4,5,9R)];

(SEQ ID NO: 36)
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO12-CNP27(K4,5,9R)];
and

(SEQ ID NO.36)
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC
[PEO24-CNP27(K4,5,9R)].

28. The method of claim 27, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

29. The method of claim 28, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

30. The method of claim 29, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

31. The macromolecule of claim 15, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37)

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);
and

-continued

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

32. The macromolecule of claim 15, wherein the CNP variant is

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

33. The macromolecule of claim 32, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

34. The macromolecule of claim 33, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

35. The macromolecule of claim 15, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37)

and

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

36. The macromolecule of claim 35, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

37. The macromolecule of claim 36, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

38. The macromolecule of claim 15, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37).

39. The macromolecule of claim 15, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

40. The sustained release formulation of claim 18, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37).

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);
and

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

41. The sustained release formulation of claim 18, wherein the CNP variant is

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

42. The sustained release formulation of claim 41, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

43. The sustained release formulation of claim 42, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

44. The sustained release formulation of claim 18, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37)
and
(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-38).

45. The sustained release formulation of claim 44, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

46. The sustained release formulation of claim 45, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

47. The sustained release formulation of claim 18, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37).

48. The sustained release formulation of claim 18, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

49. The method of claim 21, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37)

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);
and

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

50. The method of claim 21, wherein the CNP variant is

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

51. The method of claim 21, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37)
and

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

52. The method of claim 21, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37).

53. The method of claim 21, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

54. The method of claim 22, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37)

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39); and

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

55. The method of claim 22, wherein the CNP variant is

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

56. The method of claim 22, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) and
(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

57. The method of claim 22, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37).

58. The method of claim 22, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)

59. The method of claim 23, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37)

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39); and

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

60. The method of claim 23, wherein the CNP variant is

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

61. The method of claim 60, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

62. The method of claim 61, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

63. The method of claim 23, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) and
(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

64. The method of claim 63, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

65. The method of claim 64, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

66. The method of claim 23, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37).

67. The method of claim 23, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

68. The method of claim 27, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37)

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39); and

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

69. The method of claim 27, wherein the CNP variant is

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

70. The method of claim 69, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

71. The method of claim 70, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

72. The method of claim 27, wherein the CNP variant is selected from the group consisting of

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) and

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38).

73. The method of claim 72, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

74. The method of claim 73, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

75. The method of claim 27, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37).

76. The method of claim 27, wherein the CNP variant comprises an amino acid sequence at least 95% identical to

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37).

* * * * *

Exhibit 2

Curriculum Vita – Jonathan L. Sessler

Education and Employment Summary: Prof. Jonathan L. Sessler (sessler@cm.utexas.edu; PH: 512 471-5009; cell PH: 512 203-2121) was born in Urbana, Illinois, USA on May 20, 1956. He received a B.S. degree (with Highest Honors) in chemistry in 1977 from the University of California, Berkeley. He obtained a Ph.D. in organic chemistry from Stanford University in 1982 (supervisor: Prof. J. P. Collman). He was a NSF-CNRS and NSF-NATO Postdoctoral Fellow with Prof. Jean-Marie Lehn at L'Université Louis Pasteur de Strasbourg, France. He was then a JSPS Visiting Scientist in Prof. Tabushi's group in Kyoto, Japan. In September 1984 he accepted a position as Assistant Professor of Chemistry at the University of Texas at Austin, where he is currently the Doherty-Welch Chair. He served as a WCU Professor at Yonsei University from 2007-2012 and as a Visiting Distinguished Professor and Director of the Center for Supramolecular Chemistry and Catalysis at Shanghai University from Sept. 2015 until August of 2020.

Output: Dr. Sessler has authored >960 research publications, of which over 200 are in the *J. Am. Chem. Soc.* (plus >45 in *Angew. Chem.*, 10 in *Chem (Cell Press)*, 7 in *Nature Chem.*, 3 in *Nature* and 1 in *Science*). Dr. Sessler has co-authored two books (with Dr. Steve Weghorn and Drs. Philip Gale and Won-Seob Cho, respectively), edited two others (with Drs. Susan Doctrow, Tom McMurry, and Stephen J. Lippard and Drs. Mei-Xiang Wang and Placido Neri), and been an inventor on over 80 U.S. Patents. Dr. Sessler's work has been featured on more than 60 journal or book covers. His current Web of Science h-index is 125. His work has been cited >50,000 times not counting self-citations. He has given >700 invited/named/plenary lecturers. He currently has 5 grants (NIH, DOE(2x), and NSF(2x)) and has raised many millions of dollars over the course of his career.

Entrepreneurial Activities: Dr. Sessler was a co-founder (with Dr. Richard A. Miller) of Pharmacyclics, Inc., a publicly traded company (PCYC; NASDQ) dedicated to developing new cancer therapies that was acquired by AbbVie for $21B in 2015. A drug developed by Pharmacyclics, Inc. (Imbruvica) is being used to treat ca. 40% of all leukemia patients. In September of 2019, technology from his laboratory designed to overcome platinum-resistant solid tumors was licensed to the IQ Global Group. That technology is now the basis for a new company, InnovoTEX, of which Dr. Sessler is a co-founder.

Synergistic and Outreach Activities: Dr. Sessler served two full terms on NIH Study Sections and part of a 3rd full term (June 2019-July 2020). He was the co-organizer of several international conferences in porphyrin, supramolecular, and macrocyclic chemistry and numerous ACS symposia. Dr. Sessler was an Associate Editor for *ChemComm* for 11 years and has been or is a member of the editorial advisory boards of *Acc. Chem. Res.*, *Inorg. Chem.*, *Chem.*, and *J. Organic Chemistry*. Dr. Sessler is the US National Representative for the *Society of Porphyrins and Phthalocyanines* and a member of the Organizing Committee for the International Symposium on Macrocyclic and Supramolecular Chemistry, the Calixarenes, Molecular Sensors and Logic Devices, and Chemistry Applied to Sensing and the Environment meetings. In addition to English, Dr. Sessler speaks French, Hebrew, Spanish, some German and Japanese and a tiny bit of Korean.

Contributions to Education: Dr. Sessler is an award-winning teacher who has mentored more than 250 PhD and postdoctorals, and dozens of undergraduate students. Many are pursuing academic careers, including Toronto (Hargrove), Trinity (Urbach), Oregon (Johnson), Arizona (Tomat), Tulane (Jayawickramarajah), Texas Tech (Gorden), Tokyo

(Shionoya), Kyushu (Hisaeda, Furuta), Kansai (Ohya), Tokyo Metropolitan (Kubo), Zhejiang (Li), Beijing Normal (Gong, Ke), Sydney TU (Gale), ENS Lyon (Bucher), Université Lyon (Andrioletti), Kings College London (Sedgwick).

Honors and Fellowships: 1984 Camille and Henry Dreyfus Foundation Award for Newly Appointed Faculty; 1985 Univ. of Texas at Austin (UT Austin), College of Natural Science (CNS) Teaching Excellence Award; 1986 National Science Foundation Presidential Young Investigator; 1988 UT Austin, President's Associates Teaching Excellence Award; 1988 Camille and Henry Dreyfus Foundation Teacher-Scholar Award; 1989 Sloan Fellow, 1989-1991; 1990 UT Austin, CNS Teaching Excellence Award; 1991 Arthur C. Cope Scholar Award; 1992 Outstanding Professor, CNS, UT Austin; 1992 Japanese Society for the Promotion of Science, Visiting Senior Scientist; 1992 Alexander von Humboldt Stifung, Senior Visiting Scientist; 1994 Watkins Distinguished Visiting Professorship, Wichita State University; 1999 Fellow of the American Association for the Advancement of Science; 2000 UT Austin, CNS Teaching Excellence Award; 2001 Visiting Professor, Université de Paris VI; 2001 Izatt-Christensen Award in Macrocyclic Chemistry; 2002 Fishel Memorial Lecturer, Vanderbilt University; 2002 Visiting Professor, University of Southampton, 2002-2008; 2003 Leermakers Symposium, Distinguished Lecturer; 2003 Sidney Pollack Lecturer, Technion University; 2003 Spanish National Visiting Professorship, Universidad Autonoma de Madrid; 2004 Japanese Society for the Promotion of Science, Visiting Senior Scientist; 2005 Visiting Professor, Université Louis Pasteur de Strasbourg; 2005 40th Anniversary *Chem. Commun.* Lecturer; 2007 Grollman Lecturer, Univ. of Maryland School of Pharmacy; 2009 Evans Fellow, University of Otago, New Zealand; 2009 Prof. Invité, Université de Grenoble; 2009 Fulbright Specialist; 2009 Distinguished Visiting Professor, University of Auckland, New Zealand; 2009 WCU Professor, Yonsei University; 2010 Landsdowne Lecturer, University of Victoria, BC Canada; 2010 Fellow, Royal Society of Chemistry; 2011 Centenary Award, Royal Society of Chemistry; 2011 Rhoads/Raulins Lecture, Univ. of Wyoming; 2012 Honorary Professor, Jiangsu University, China; 2013 Honorary Member, Israel Chemical Society; 2013 Southwest Regional ACS Award; 2014 Molecular Sensors and Molecular Logic Gates Award (with Prof. Shinkai); 2015 Ken Raymond Lecturer, UC Berkeley; 2015 Fellow National Academy of Inventors; 2016 Hans A. Fischer Award, Society of Porphyrins and Phthalocyanines; 2016 Emanuel Vogel Memorial Lecture, Unversität zu Köln; 2016 Inventor of the Year, UT Austin; 2017 Klemmer Lecture, Univ. of Oregon; 2017 Xinda Lecturer Peking University; 2017 CASE-2017 Award; 2017 Roegnik Lecture, Ohio University; 2018 Thomas Dougherty Award in Photodynamic Therapy; 2018 Howard Lecturer Universities of Sydney and New South Wales; 2018 Elected, European Academy of Sciences; 2018 McGavock Lecture, Trinity University; 2018-2023 Clarivate Highly Cited Researcher; 2019 C. David Gutsche Award in Calixarene Chemistry; 2019 Foreign Associate Award, Asian Society for Porphyrins and Phthalocyanines; 2019 UT Austin Co-op Career Research Award (highest campus-wide research honor); 2019 Fellow of the ACS; 2019 Asian Conference on Chemosensors and Imaging Probes Distinguished Lectureship Award; 2019 Who's Who Albert N. Marquis Lifetime Achiever; 2020, American Institute of Chemists, Pioneer Award; 2021 ACS Breslow Award in Biomimetic Chemistry; 2021 Elected U.S. National Academy of Sciences; 2022 Elected American Academy of Arts and Sciences; 2022 RSC Mond-Nyholm Prize; 2022 RSC-MASC Supramolecular Chemistry Award; 2023 Elected Foreign Member, Chinese Academy of Sciences; 2024 Stoddart Science Fund Chemist Award; 2025 Fellow, Chinese Chemical Society.

Expert Witness Work and Testimony (Jonathan L. Sessler):

Completed >5 Years Ago

Nutrasep v. Thumb Oil Cooperative (hired by attorneys for Nutrasep); expert report; deposition and trial in Texas. This case related to alleged intellectual theft.

United States v. Procter & Gamble (hired by attorneys for P & G); expert report; deposition and hearing. This case involved phthalocyanine PDT agents

Sanofi-Aventis v. Teva (hired by attorneys for Teva); expert report; trial in Israel. This case centered around issues of drug patent invalidity.

Sanofi-Aventis v. Dr. Reddy's (hired by attorneys for Dr. Reddy's); invalidity and infringement; deposition; PI and trial in New Jersey. This case centered around issues of drug patent invalidity and infringement.

Sanofi-Aventis v. Impax, Mylan, and Amino D'Pharma (joint action; hired by attorneys for Mylan); deposition. This case centered around issues of drug patent invalidity and infringement.

United States v. Bodapadi (hired by US Attorney's Office, Utah); sentencing trial. This case related to alleged intellectual theft.

Genentech-Roche v. Sandoz (hired by attorneys for Sandoz); deposition. This case centered around issues of drug patent invalidity and infringement.

Novartis v. Watson (hired by attorneys for Watson); deposition and trial in Delaware. This case centered around issues of drug patent invalidity and infringement.

Johnson-Matthey v. Shire (hired by attorneys for Johnson-Matthey); invalidity; expert report

Mylan Pharmaceuticals v. Novartis (hired by attorneys for Mylan); expert report. This case centered around issues of drug patent invalidity and infringement.

Teva v. Gilead (hired by attorneys for Teva); two separate patentability evaluations

Kowa v. Apotex, *et al.* (hired by attorneys for Apotex, *et al.*); deposition and trial in New York. This case centered around issues of drug patent invalidity and infringement.

Sandoz A/S v. F. Hoffmann-La Roche AG Swedish proceedings (hired by attorneys for Sandoz); expert report and trial in Sweden. This case centered around issues of drug patent invalidity and infringement.

Mylan and Apicore v. Aurobindo (hired by attorneys for Mylan and Apicore); deposition and preliminary injunction bench hearing in Texas; IPR report. This case centered around issues of drug patent invalidity and infringement.

Teva v. Sankyo (hired by attorneys for Teva); two separate patentability evaluations

Sandoz A/S v. F. Hoffmann-La Roche AG Polish proceedings (hired by attorneys for Sandoz); two expert reports. This case centered around issues of drug patent invalidity and infringement.

Luitpold Pharma vs. Apicore US, LLC (hired by attorneys for Apicore); IPR proceedings

Provence v. Wista (hired by attorneys for Wista); IPR proceedings. This case involved issues of alleged patent invalidity.

Beloteca, Inc, v. Apicore US, LLC and Mylan Institutional, LLC (hired by attorneys for Apicore); expert report; Preliminary Injunction hearing. This case centered around issues of drug patent invalidity and infringement.

Euticals v. Novel (hired by attorneys for Novel); expert analysis. This case involved alleged theft of intellectual property.

Epizyme Inc. v. GlaxoSmithKline LLC; IPR proceedings (hired by attorneys for Epizyme

Completed ≤5 Years Ago

RetroVirox Inc. v. The Scripps Research Institute, *et al.* (hired by attorneys for RetroVirox); deposition and trial testimony. This case involved alleged theft of intellectual property.

AstraZeneca AB, et al. v. MSN Pharma, Inc., et al. (hired by attorneys for MSN); patent validity analysis.

Illumina, Inc. v. BGI (hired by attorneys for Illumina); deposition testimony. This case centered around issues of drug patent invalidity and infringement.

Mylan Institutional LLC, et al. v. Hong Kong King-Friend Industrial (hired by attorneys for Mylan); two expert reports; patent validity analysis.

Teva's opposition to Israeli Patent Application No. 241440 (hired by attorneys for Teva); invalidity analysis.

Teva Branded Pharma, et al. v. Aurobindo Pharma Ltd., et al. (hired by attorneys for Teva). This case involved issues of alleged patent invalidity.

Alzheon Inc. v Risen (Suzhou) Pharma Tech; IPR proceeding (hired by attorneys for Risen); deposition testimony. This case involved issues of alleged patent invalidity.

Guerbet v. Bracco (hired by attorneys for Guerbet); patent analysis

Anacor Pharmaceuticals, Inc. et al. v. Alkem Laboratories Ltd. (hired by attorneys for Alkem); patent analysis

Avion Pharmaceuticals, LLC et al. v. Lupin Limited et al (1-22-cv-00729, DDE) (assisted Perkins Cole, attorneys for Avion Pharmaceuticals, LLC; Albion Laboratories, Inc.); infringement analysis

Valinject GmbH (hired by the law firm of Procopio, Cory, Hargreaves, and Savitch LLP); infringement analysis regarding US patent 7,420,051.

Inter Partes Review of U.S. Patent Nos. 9,486,432 and RE47,045 (hired by law firm representing patent owner). Note: Dr. Sessler is subject to a confidentiality agreement and not at liberty to disclose the parties at suit or their attorneys. **Please keep this entry confidential and use this information only to make sure there are no conflicts.**

Vertex Pharmaceuticals v. Royalty Pharma (hired by the law firm Gibson-Dunn representing Royalty Pharma); technical support for contract dispute.

Current

Amide Beverage Co. v. Amazon, et al. (hired by the law firm Devlin representing Amide Beverage Co.); infringement and validity analysis of US patent 8,350,077.

Thermolife International. v. Catalyst Nutraceuticals, LLC and Pacific Northwest Naturals, LLC (hired by the law firm Devlin representing Thermolife); consulting advice for arbitration hearing involving US patent 8,455,53.

Corium, LLC (presently being considered for retention by the law firm Choate, Hall & Stewart LLP); consulting advice in connection with litigation concerning Azstarys®.

Publications (Jonathan L. Sessler):

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9. Collman, J. P.; Brauman, J. I.; Iverson, B. L.; Sessler, J. L.; Morris, R.; Gibson, Q. H. "Comparison of O_2 and CO Binding to Iron(II) 'Picket Fence' and 'Pocket' Porphyrins,"

Biomimetic Chemistry; Yoshida, Z.-I.; Ise, N., Eds.; Kodansha: Tokyo, 1983; pp 37-49.

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15. Hamilton, A.; Lehn, J.-M.; Sessler, J. L. "Coreceptor Molecules. Synthesis of Metalloreceptors Containing Porphyrin Subunits and Formation of Mixed Substrate Supermolecules by Binding of Organic Substrates and of Metal Ions," *J. Am. Chem. Soc.* **1986**, *108*, 5158-5167. **DOI:** 10.1021/ja00277a021. This work was subsequently featured on the cover of Prof. Lehn's Nobel Award lecture writeup in *Angew. Chem.*

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72. "Method and compositions for treating atheroma, tumors and other neoplastic tissue," Sessler, J. L.; Magda, D.; Mody, T. D.; Hemmi, G. University of Texas and Pharmacyclics, Inc. U.S. Patent Application filed August 16, 2006; issued as U.S. Patent 7,579,338 on August 25, 2009.

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73. "Water-soluble zinc ionophores, zinc chelators, and/or zinc complexes and use for treating cancer," Magda, D. J.; Sessler, J. L., Jonathan L. Sessler, Assignee. US patent application filed March 20, 2007; issued as U.S. Patent 7,838,536, November 23, 2010.

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74. "Water-soluble zinc ionophores, zinc chelators, and/or zinc complexes and use for treating cancer," Magda, D. J.; Sessler, J. L., Jonathan L. Sessler, Assignee. US

patent application filed October 15, 2010. Issued as US patent 8,507,531 on August 15, 2013.

75. "Functionalized Expanded Porphyrins," Sessler, J. L.; Pantos, P. J. University of Texas. U.S. Patent Application filed June 23, 2008. Issued as U.S. Patent 8,580,845 on November 12, 2013.

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76. "Polymers functionalized with specific recognition elements," Sessler, J. L.; Bielawski, C. W.; Aydogan, A.; Coady, D., U.S. Provisional Patent Application filed November 23, 2009. Issued as U.S. Patent 8,802,074, August 12, 2014.

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77. "Texaphyrin-Pt(IV) Conjugates and Compositions for use in Overcoming Platinum Resistance," Sessler, J. L.; Arambula, J.; Siddik, Z.; Thiabaud, G. University of Texas at Austin and MD Anderson. Patent application filed as PCT/US2015/035229. Issued as United States Patent No. 10,406,167 on September 10, 2019.

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78. "Melanocortin 1 Receptor Ligands and Methods of Use," Gillies, R. J.; Morse, D. L.; Barkey, N. B.; Sill, K. N.; Vagner, J.; Tafreshi. N. K.; Sessler, J. L.; Hruby, V. J.; Moffit Cancer Center and University of Texas at Austin. Issued as U.S. Patent No. 9,441,013, September 13, 2016.

79. "Texaphyrin and Antitumor Antibiotic Conjugates," Sessler, J. L.; Lee, M. H. The University of Texas at Austin, U.S. Patent Application no. 20190231888. PCT/US2017/054919.

2017

80. "Polymers Functionalized with Specific Recognition Elements," Sessler, J. L.; Bielawski, C. W.; Aydogan, A.; Coady, D., U.S. Provisional Patent Application filed November 23, 2009. Issued as U.S. Patent 9,585,910, March 7, 2017.

2018

81. "Naphthoquinone Containing Gold Carbene Complexes and Methods of Uses Thereof" by Arambula, J.; Sessler, J. L.; Aramugan, K.; Bielawski, C. The University of Texas System and Wright State PCT/US2018/040331 filed based on U.S. Serial No. 62/527,606. Issued as U.S. Patent 11,332,483, May 17, 2022.

82. "Compounds for the Selective Solid-Liquid Extraction and Liquid-Liquid Extraction of Lithium Chloride," by Sessler, J.; He, Q.; Kim, S. K. The University of Texas System, U.S. Provisional Patent Application and PCT Application PCT/US2019/033690 filed.

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83. "Functionalized Gold Carbene Napthaquinone Complexes for Use in the Treatment of Cancer," by Sessler, J. L.; Arambula, J.; Sen, S.; Cui, Z. The University of Texas System, U.S. Provisional and PCT Patent Applications filed. Published as US20220340601.

84. "Melanocortin 1 Receptor Ligands and Methods of Use," Gillies, R. J.; Morse, D. L.; Barkey, N. B.; Sill, K. N.; Vagner, J.; Tafreshi. N. K.; Sessler, J. L.; Hruby, V. J.; Moffit Cancer Center and University of Texas at Austin. Issued as U.S. Patent No. 10,329,326, June 25, 2019.

85. "Anticancer Prodrug for Overcoming Drug Resistance," Kim, J. S.; Sharma, A.; Lee, M. G.; Won, M.; Lee, J. Y.; Chi, S.-G.; Sessler, J. L. Korea University, August 22, 2019. Issued as U.S. Patent 11,241,501, February 8, 2022.

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86. "Manganese Texaphyrin Derivatives for Chemotherapy, Photoacoustic Imaging, and Photothermal Therapy," by Sessler, J. L.; Sedgwick, A. C.; Thiabaud, G.; Arambula, J. F. The University of Texas System, U.S. Provisional Patent and PCT applications filed.

87. "Cell Activatable Iron Chelators," by Sessler, J. L.; Steinbrueck, A.; Sedgwick, A. C. The University of Texas System, U.S. Provisional Patent application filed.

2021

88. "Lithium Selective Organogels," by Sessler, J. L.; Wang, H.; Page, Z. The University of Texas System, U.S. Provisional Patent and PCT applications filed.

89. "Melanocortin 1 Receptor Ligands and Methods of Use," Gillies, R. J.; Morse, D. L.; Barkey, N. B.; Sill, K. N.; Vagner, J.; Tafreshi. N. K.; Sessler, J. L.; Preihs, C.; Hruby, V. J.; Moffit Cancer Center, Intezyne, Inc., the University of Arizona, and The University of Texas System. Issued as U.S. Patent No. 11,230,568, January 25, 2022.

90. "Functionalized Resin for $LiPF_6$ Recovery," Sessler, J. L.; Page, Z. A.; Huang, S.-Y.; Wang, H. The University of Texas System, U.S. Provisional Patent filed.

2022

91. "Texaphyrin-Pt(IV) Conjugates and Compositions for use in Overcoming Platinum Resistance," Sessler, J. L.; Arambula, J.; Siddik, Z.; Thiabaud, G. University of Texas at Austin and MD Anderson. Patent application filed as PCT/US2015/035229. Issued as United States Patent No. 11,389,459, July 19, 2022.

92. “3D Printed Porous Supramolecular Sorbents,” Page, Z.; Sessler, J. L.; Mason, K.; Huang, S.-Y. The University of Texas at Austin. PCT patent application filed July 29, 2024.

93. “Compounds for the Selective Solid-liquid and Liquid-liquid extraction of Lithium Chloride,” Kim, S. K.; Sessler, J. L.; He, Q. The University of Texas at Austin, Gyeongsang National University. Preliminary patent application Serial No. 63/680,349, filed August 7, 2024.

95. “Melanocortin 1 Receptor Ligands and Methods of Use,” Gillies, R. J.; Morse, D. L.; Barkey, N. B.; Sill, K. N.; Vagner, J.; Tafreshi. N. K.; Sessler, J. L.; Preihs, C.; Hruby, V. J.; Moffit Cancer Center, Intezyne, Inc., the University of Arizona, and The University of Texas System. Issued as U.S. Patent No. 12,173,087, December 24, 2024.

Invited Lectures, 1985 - Present

1. "Organized Organic Chemistry: A 'Twin Template' Approach to Chemical Catalysis," Procter and Gamble Co., Cincinnati, Ohio; March, 1985.

2. "Recent Advances in Macrocyclic Chemistry," General Electric Co., Schenectady, New York; November, 1986.

3. "The Synthesis of Novel Porphyrin and Porphyrin-like Macrocycles," University of Houston, Houston, Texas; January, 1987.

4. "The Synthesis of Novel Porphyrin and Porphyrin-like Macrocycles," University of Texas, San Antonio, Texas; February, 1987.

5. "Bioorganic Chemistry in The Sessler Group," Celanese Co., Corpus Christi, Texas; February, 1987.

6. "The Synthesis of Novel Porphyrin and Porphyrin-like Macrocycles," University of Texas, Dallas, Texas; February, 1987.

7. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," Max Planck Institut für Medizinische Forschung, Heidelberg, West Germany; March, 1987.

8. "The Synthesis of Novel Porphyrin and Porphyrin-like Macrocycles," Université Louis Pasteur de Strasbourg, Strasbourg, France; March, 1987. This lecture was delivered in French.

9. "Bioorganic Chemistry in The Sessler Group," Allied Signal Co., Des Plaines, IL; April, 1987.

10. "The Synthesis of Novel Porphyrin and Porphyrin-like Macrocycles," Northwestern University, Evanston, Illinois; April, 1987.

11. "The Synthesis of Novel Porphyrin and Porphyrin-like Macrocycles," Southern Methodist University, Dallas, Texas; April, 1987.

12. "Synthetic Models for The Reaction Center of *Rhodopseudomonas viridis*," Tabushi Memorial Symposium on Bioorganic and Bioinorganic Chemistry, Kyoto, Japan; July, 1987. (This was an invited main session lecture.)

13. "The Synthesis of Novel Porphyrin and Porphyrin-like Macrocycles," 12th Kansai Symposium on Bioinorganic Chemistry, Kanazawa, Japan; July, 1987.

14. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," Osaka University, Osaka, Japan; July, 1987.

15. "Molecular Engineering: The Design of Specifically Tailored Molecular Systems," Southern Methodist University, Dallas, Texas, September, 1987.

16. "Approaches to Molecular Recognition: Selective Ligand Design and 'Artificial Oligonucleotides'," U.S.-Japan Joint Seminar on Molecular Recognition of Neutral and Cationic Substrates, Miami, FL; November 1987. (This was an invited main session lecture.)

17. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," 53rd Israeli Chemical Society Meeting, Ber Sheva, Israel; February 1988. (This was an invited Main Lecture.)

18. "The Synthesis of New Porphyrin and Porphyrin-like Macrocycles," Hebrew University of Jerusalem, Israel; February 1988.

19. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," Weizmann Institute, Rehovot, Israel; February 1988.

20. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," Georgia State University, Atlanta, Georgia; February 1988.

21. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," Emory University, Atlanta, Georgia; February 1988.

22. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," South Carolina University, Columbia, South Carolina; February 1988.

23. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," University of North Carolina, Chapel Hill, North Carolina; February 1988.

24. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," Virginia Commonwealth University, Richmond, Virginia; March 1988.

25. "Synthesis of New Porphyrin and Porphyrin-like Systems," Brigham Young University, Provo, Utah; March 1988.

26. "An 'Expanded Porphyrin': The Synthesis of a Novel Pentadentate Porphyrin-like Ligand," Los Alamos National Laboratory, Los Alamos, New Mexico; April 1988.

27. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," University of Kansas, Lawrence, Kansas; August, 1988.

28. "Synthèses des Nouvelles Porphyrines et Nouvelles Composées Porphyriniques," Institut Curie, Orsay, France; September, 1988. This lecture was delivered in French.

29. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Max Planck Institute für Medizinische Forschung, Heidelberg, W. Germany; September, 1988.

30. "Artificial Oligonucleotides: Molecular Recognition by Base Pairing Interactions," Fifth Internaltional Symposium on Inclusion Phenomena and Molecular Recognition, Orange Beach, Alabama; September, 1988. (This was a plenary lecture.)

31. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," Carnegie Mellon University, Pittsburgh, Pennsylvania, October, 1988.

32. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," University of California at Berkeley, Berkeley, California, October, 1988.

33. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Stanford University, Stanford, California, October, 1988.

34. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," 10th International Meeting on Photobiology, Jerusalem, Israel, November, 1988. (This was an invited Main Lecture.)

35. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Universidad Autonoma de Madrid, Madrid, Spain, November 1988.

36. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," State University of New York at Stony Brook, Stony Brook, New York, November, 1988.

37. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems, University of Cincinnati, Cincinnati, Ohio, November, 1988.

38. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Princeton University, Princeton, New Jersey, December, 1988.

39. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Squibb Medical Institute, New Jersey, December, 1988.

40. "Quinone Substituted Porphyrin Dimers: New Photosynthetic Model Systems," Exxon Research Laboratory, New Jersey, December, 1988.

41. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Texas Tech University, Lubbock, Texas, February, 1989.

42. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Oklahoma University, Norman, Oklahoma, March, 1989.

43. "Quinone Substituted Porphyrin Dimers: Evidence for 'Superexchange' Mediated Electron Transfer Reactions in New Photosynthetic Model Systems," University of California at San Diego, La Jolla, California, April, 1989.

44. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," University of California at Los Angeles, Los Angeles, California, April, 1989.

45. "Quinone Substituted Porphyrin Dimers: Evidence for Superexchange in New, Multichromophore Photosynthetic Model Systems," California Insitute of Technology, Pasadena, CA, April, 1989.

46. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," University of California at Irvine, Irvine, California, April, 1989.

47. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Texas A & M University, College Station, Texas, April, 1989.

48. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," University of Texas at Arlington, Fort Worth, Texas, May, 1989.

49. "Quinone Substituted Porphyrin Dimers: Evidence for Superexchange in New, Multichromophore Photosynthetic Model Systems," University of Auckland, Auckland, New Zealand, June, 1989.

50. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," XIV International Symposium on Macrocyclic Chemistry, Townsville, Australia, June, 1989.

51. "Evidence for Superexchange in New, Multichromophore Photosynthetic Model Systems," 4th International Conference on Bioinorganic Chemistry, Boston, Massachusetts, July, 1989.

52. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Mitsubishi Petrochemical Co., Tsukuba, Japan, August, 1989.

53. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Mitsubishi Kasei Co., Yokahama, Japan, August, 1989.

54. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Tokyo Institute of Technology, Tokyo, Japan, August, 1989.

55. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Institute for Molecular Science, Okazaki, Japan, August, 1989.

56. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Osaka University, Osaka, Japan, August, 1989.

57. "Quinone Substituted Porphyrin Dimers: Evidence for Superexchange in New, Multichromophore Photosynthetic Model Systems," Kyoto University, Kyoto, Japan, August, 1989.

58. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Gifu University, Gifu, Japan, August, 1989.

59. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," Kyoto University, Kyoto, Japan, August, 1989.

60. "Quinone Substituted Porphyrin Dimers: Evidence for Superexchange in New, Multichromophore Photosynthetic Model Systems," Hiroshima University, Hiroshima, Japan, August, 1989.

61. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," XIV Post-ISNA Symposium on Macrocyclic Chemistry, Fukuoka, Japan, August, 1989. (This was a plenary lecture.)

62. "'Texaphyrin': A Novel 22 π-Electron Pentadentate Porphyrin-like Ligand," 10th Annual UERP Bioorganic Symposium, Procter & Gamble Co., Cincinnati, Ohio, November, 1989. (This was a plenary lecture.)

63. "Quinone Substituted Porphyrin Dimers: Evidence for Superexchange in New, Multichromophore Photosynthetic Model Systems," Texas Christian University, December, 1989.

64. "Synthesis and Biomedical Properties of 'Expanded Porphyrins'," 2nd International Meeting of the Pacific Basin Chemical Societies, Honolulu, Hawaii, December, 1989.

65. "Photodynamic Inactivation of Enveloped Viruses with 'Expanded Porphyrins'," 1990 Meeting of the Society for Optical Engineering, Los Angeles, California, January, 1990.

66. "Photodynamic Inactivation of Enveloped Viruses with 'Expanded Porphyrins'," Department of Medicinal Chemistry, University of Texas at Austin, February, 1990.

67. "Artificial Oligonucleotides: Molecular Recognition *via* Base-Pairing," 5th Annual Symposium on Biofunctional Chemistry, Hiroshima, Japan, June 1990. (This was an invited plenary lecture.)

68. "Synthesis and Biomedical Properties of 'Expanded Porphyrins'," Shionogi Research Center, Osaka, Japan, June 1990.

69. "Artificial Oligonucleotides: Molecular Recognition *via* Base-Pairing," Kyoto University, Kyoto, Japan, June, 1990.

70. "Artificial Oligonucleotides: Molecular Recognition *via* Base-Pairing," Teijin Research Center, Tokyo, Japan, June 1990.

71. "Texaphyrins and Sapphyrins: Novel 'Expanded Porphyrins'," University of Tokyo, Tokyo, Japan, June 1990.

72. "Photodynamic Inactivation of Enveloped Viruses with 'Sapphyrins'," American Society for Photobiology, Annual Meeting, Vancouver, Canada, June 1990. (This was an invited Main Lecture.)

73. "Artificial Oligonucleotides: Molecular Recognition *via* Base-Pairing," Annual NSF Synthesis Workshop, Boulder, Colorado, July 1990.

74. "Texaphyrins and Sapphyrins: Novel 'Expanded Porphyrins'," Gordon Conference on Pyrroles, Brewster Academy, New Hampshire, July 1990. (This was an invited main-session lecture.)

75. "Synthesis and Spectroscopic Properties of 'Expanded Porphyrins'" Hebrew University, Jerusalem, September 1990.

76. "Texaphyrins et Sapphyrins: Des Nouvelles Porphyrines Expandées au Style Texan," Institute Curie, Orsay, France, October 1990. This lecture was delivered in French.

77. "Texaphyrins et Sapphyrins: Des Nouvelles Porphyrines Expandées au Style Texan," Université de Paris VII, Paris, France, October 1990. This lecture was delivered in French.

78. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," Cologne University, Cologne, Germany, November, 1990.

79. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," University of Paderborn, Paderborn, Germany, November 1990.

80. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," Bayer AG, Leverkeusen, Germany, November 1990.

81. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," Bonn University, Bonn, Germany, November 1990.

82. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," Ruhr University, Bochum, Germany, November 1990.

81. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," Max Planck Institut für Medizinische Forschung, Heidelberg, Germany, November 1990.

82. "Texaphyrins et Sapphyrins: Des Nouvelles Porphyrines Expandées au Style Texan," Institute Louis Pasteur de Strasbourg, Strasbourg, France, November 1990. This lecture was delivered in French.

83. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," University of Twente, Hengelo, Holland, November 1990.

84. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," University of Basel, Basel, Switzerland, November 1990.

85. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," Weizmann Institute of Science, Rehovot, Israel, December 1990.

86. "Texaphyrins and Sapphyrins: Novel Texas-sized 'Expanded Porphyrins'," Ben Gurion University of the Negev, Ber Sheva, Israel, December 1990. This lecture was delivered in Hebrew.

87. "Porphyrin-mediated Superexchange in Quinone Substituted Porphyrin Dimers and Trimers," Southwest Regional Meeting on Photosynthesis, Tempe, Arizona, January 1991.

88. "'Expanded Porphyrins': Potential New Photosensitizers for Use in Photodynamic Therapy," 1991 Meeting of the Society for Optical Engineering, Los Angeles, California, January, 1991.

89. "Preparation and Properties of 'Expanded Porphyrins'," Purdue University, West Lafayette, IN, February, 1991.

90. "Expanded Porphyrins: Macrocyclic Ligands for the Lanthanides and Actinides," Symposium on Metal Selective Ligands, 201st Meeting of the American Chemical Society, Atlanta, GA, April, 1991.

91. "Preparation and Properties of 'Expanded Porphyrins'," Northwestern, Evanston, IL, April, 1991.

92. "Non-covalent Approaches to Photosynthetic Model Systems," Annual Meeting, American Society for Photobiology, San Antonio, TX, July, 1991.

93. "Synthesis and Properties of Expanded Porphyrins," 13th International Symposium on Heterocyclic Chemistry, Corvalis Oregon, August, 1991.

94. "Synthesis and Properties of Expanded Porphyrins," Cope Award Symposium, 202nd Meeting of the American Chemical Society, New York, NY, August, 1991. (This was a plenary lecture.)

95. "Synthesis and Properties of Expanded Porphyrins," 13th International Symposium on Macrocyclic Chemistry, Sheffield, UK, September, 1991. (This was a plenary lecture.)

96. "New Approaches to Photosynthetic Model Systems," NATO ASI Summer School, September, 1991, Assois, France. (This was a plenary lecture.)

97. "Synthesis and Properties of Expanded Porphyrins," Johnson-Matthey Co., Westchester, PA, November, 1991.

98. "Molecular Recognition via Base-pairing," Department of Chemistry, Texas A & M University, College Station, TX, March, 1992.

99. "Synthesis and Properties of Expanded Porphyrins," 49th Industrial Affiliates Symposium, Department of Chemistry, Stanford University, April, 1992. (This was a plenary lecture.)

100. "Synthesis and Properties of Expanded Porphyrins," University of Basel, Switzerland, April, 1992.

101. "The Development of New Photosensitizers for Photodynamic Therapy," Ciba Geigy Inc., Basel, Switzerland, April, 1992.

102. "Synthesis and Properties of Expanded Porphyrins," University of Heidelberg, Germany, April, 1992.

103. "Synthesis and Properties of Expanded Porphyrins," Rohm & Haas Co., Spring House, PA, April, 1992.

104. "Molecular Recognition via Base-pairing," Department of Chemistry, University of Victoria, Victoria, Canada, May, 1992.

105. "Synthesis and Properties of Expanded Porphyrins," Department of Chemistry, University of British Columbia, Vancouver, Canada, May, 1992.

106. "Molecular Recognition via Base-pairing," Department of Chemistry, University of Washington, Seattle, WA, May, 1992.

107. "Photo-inactivation of Enveloped Viruses using Expanded Porphyrin Photosensitizers," 43rd Midwestern Regional ACS Meeting, Cincinnati, OH, May, 1992. (This was a plenary lecture.)

108. "Molecular Recognition via Base-pairing," Kochi University, Kochi, Japan, July, 1992.

109. "Synthesis and Properties of Expanded Porphyrins," Gifu University, Gifu, Japan, July, 1992.

110. "Synthesis and Properties of Expanded Porphyrins," Doshisha University, Kyoto, Japan, July, 1992.

111. "Synthesis and Properties of Expanded Porphyrins," Industrial Products Research Institute, Tskuba, Japan, July, 1992.

112. "Molecular Recognition via Base-pairing," Mitsubishi Kasei Ltd., Tokyo, Japan, July, 1992.

113. "Synthesis and Properties of Expanded Porphyrins," Kyoritsu College of Pharmacy, Tokyo, Japan, July, 1992.

114. "Synthesis and Properties of Expanded Porphyrins," Nagaoka Technical University, Nagaoka, Japan, July, 1992.

115. "Non-covalent Approaches to Photosynthetic Model Systems," Kyoto University, Kyoto, Japan, July, 1992.

116. "Synthesis and Properties of Expanded Porphyrins," Hiroshima University of Medicine, Hiroshima, Japan, July, 1992.

117. "Molecular Recognition via Base-pairing," Oita University, Oita, Japan, July, 1992.

118. "Molecular Recognition via Base-pairing," 7th International Symposium on Molecular Recognition and Inclusion, Kyoto, Japan, July, 1992. (This was a plenary lecture.)

119. "Anion Binding: A New Chapter in Porphyrin-related Research" 14th International Symposium on Macrocyclic Chemistry, Provo, UT, August, 1992. (This was a plenary lecture.)

120. "Molecular Recognition via Base-pairing," University of Michigan, Ann Arbor, MI, October, 1992.

121. "Synthesis and Properties of Expanded Porphyrins," Michigan State University, East Lansing, MI, October, 1992.

122. "Synthesis and Properties of Expanded Porphyrins," New York Academy of Sciences, New York, NY, October, 1992.

123. "Molecular Recognition via Base-pairing," New York University, New York, October, 1992.

124. "Synthesis and Properties of Expanded Porphyrins," Univ. of Texas at Dallas, Dallas, TX, November, 1992.

125. "Molecular Recognition via Base-pairing," Univ. of Houston, Houston, TX, November, 1992.

126. "Diamagnetic Texaphyrins as Potential New Photosensitizers for Photodynamic Therapy," Univ. of Texas Medical Branch at Galveston, Galveston, TX, November, 1992.

127. "Biomedical Applications of Expanded Porphyrins," Tetrapyrrole Study Symposium, Cambridge, UK, January, 1993. This was an invited, plenary lecture.

128. "Synthesis and Properties of Expanded Porphyrins," RSC Macrocycles Meeting, Oxford, UK, January, 1993. This was an invited, plenary lecture.

129. "Molecular Recognition via Base-pairing," University of Birmingham, Birminham, UK, January, 1993.

130. "Non-covalent Approaches to Photosynthetic Modeling," CFKR Conference on Spectroscopy, University of Texas at Austin, Austin, TX, January 1993.

131. "Synthesis and Properties of Expanded Porphyrins," Asahi Chemical Co., Ltd., Okayama, Japan, February 1993.

132. "Synthesis and Properties of Expanded Porphyrins," Special Honorary Symposium on Bioinorganic and Bioorganic Chemistry, Hiroshima University, Hiroshima, Japan, February 1993. This was an invited, plenary lecture.

133. "Synthesis and Properties of Expanded Porphyrins," University of Illinois, Urbana, IL, February 1993.

134. "Non-covalent Approaches to Photosynthetic Modeling," BSF Conference on New Developments in Primary Photosynthesis, Jerusalem, Israel, March, 1993. This was an invited, plenary lecture.

135. "Synthesis and Properties of Expanded Porphyrins," University of Pennsylvania, Philadelphia, PA, March, 1993.

136. "Synthesis and Properties of Expanded Porphyrins," Arco Chemical Company, Philadelphia, PA, March, 1993.

137. "Molecular Recognition via Base-pairing," Symposium on Molecular Recognition, 204th ACS Meeting, Denver, CO, March, 1993. This was an invited lecture.

138. "Synthesis and Applications of Expanded Porphyrins," Universität Köln, Cologne, Germany, April, 1993.

139. "Anion Binding by Expanded Porphyrins. A New Direction in Porphyrin-related Research," Universität Köln, Cologne, Germany, April, 1993.

140. "Non-covalent Approaches to Photosynthetic Modeling," Frei Universität, Berlin, Germany, April, 1993.

141. "Synthesis and Applications of Expanded Porphyrins," Universität Bremen, Bremen, Germany, April, 1993.

142. "Non-covalent Approaches to Photosynthetic Modeling," Universität Köln, Cologne, Germany, May, 1993.

143. "Synthesis and Applications of Expanded Porphyrins," Ruhr Universität, Bochum, Germany, May, 1993.

144. "Synthesis and Applications of Expanded Porphyrins," Universität Münster, Münster, Germany, May, 1993.

145. "Anion Binding by Expanded Porphyrins. A New Direction in Porphyrin-related Research," Université Luois Pasteur de Strasbourg, Strasbourg, France, May, 1993. This lecture was delivered in French.

146. "Anion Binding by Expanded Porphyrins. A New Direction in Porphyrin-related Research," Universität Heidelberg, Heidelberg, Germany, May, 1993.

147. "Molecular Recognition via Base-pairing: A New Approach to Photosynthetic Modeling," Forum for Young Chemists in Biofunctional Chemistry: Frontiers and Futures in Bioorganic Chemistry, Fukuoka, Japan, June 1993. This was a Plenary Lecture.

148. "Interactions of Expanded Porphyrins with DNA," 2nd International Symposium on Bioorganic Chemistry, Fukuoka, Japan, June 1993. This was an Invited Lecture.

149. "Synthesis and Properties of Expanded Porphyrins," Okayama University, Okayama, Japan, June, 1993.

150. "Synthesis and Properties of Expanded Porphyrins," Shizuoka University, Shizuoka, Japan, June, 1993.

151. "Synthesis and Properties of Expanded Porphyrins," Idemitsu Petrochemical Co., Chiba, Japan, June, 1993.

152. "Non-covalent Approaches to Photosynthetic Model Systems," Industrial Products Research Institute, Tsukuba, Japan, June, 1993.

153. "Synthesis and Applications of Expanded Porphyrins," Yale University, New Haven, CT, October, 1993.

154. "Synthesis and Applications of Expanded Porphyrins," University of Nevada, Reno, November, 1993.

155. "Synthesis and Applications of Expanded Porphyrins," University of Texas, San Antonio, TX, November, 1993.

156. "Synthesis and Applications of Expanded Porphyrins," George Washington University, Washington, D.C., February, 1994.

157. "Synthesis and Applications of Expanded Porphyrins," Southwest Texas University, San Marcos, TX, March, 1994.

158. "Molecular Recognition of Nucleotides and Oligonucleotides *via* Expanded Porphyrins," NATO Advanced Workshop on Transition Metal Complexes, Genoa, Italy, April, 1994. The was an Invited Lecture.

159. "Expanded Porphyrins. Big Ligands for Big Metals," Seaborg Institute, Lawrence Livermore Laboratory, Livermore, CA, May, 1994.

160. "Phosphate Chelation by Sapphyrin: A New Mode of Small Molecule-DNA Interaction," XIX International Symposium on Macrocyclic Chemistry, Lawrence, KS, June 1994.

161. "Synthesis and Applications of Expanded Porphyrins," Universität Bonn, Germany, July, 1994.

162. "Synthesis and Applications of Expanded Porphyrins," Technische Universität, Darmstadt, Germany, July, 1994.

163. "Biomedical Applications of Expanded Porphyrins," Bracco s. p. a., Milan, Italy, July, 1994.

164. "Expanded Porphyrins: New Reagents for the Recognition and Manipulation of Oligonucleotides," Musèum d'Histoire Naturelle, Paris, France, July, 1994. This lecture was delivered in French.

165. "Molecular Recognition *via* Base-pairing," RSC Perkin Division Symposium on Recognition Processes Marking the Centenary of Emil Fischer's Lock-and-Key Metaphor, Birmingham, England, July, 1994. The was an invited Plenary Lecture.

166. "Molecular Recognition *via* Base-pairing," Universität Bonn, Germany, August, 1994.

167. "Synthesis and Applications of Expanded Porphyrins," Max-Planck-Institut für Strahlenchemie, Germany, August, 1994.

168. "Non-covalent Approaches to Photosynthetic Model Systems," Université Louis Pasteur, Strasbourg, France, August, 1994. This lecture was delivered in French.

169. "Synthesis and Applications of Expanded Porphyrins," European Symposium on Molecular Recognition, Mainz, Germany, August, 1994. The was an Invited Lecture.

170. "Biomedical Applications of Expanded Porphyrins," Bayer Ag, Leverkuesen, Germany, August, 1994.

171. "Non-covalent Approaches to Photosynthetic Model Systems," Gordon Conference on Electron Donor-Acceptor Interactions, Newport, RI, August, 1994. The was an Invited Lecture.

172. "Texaphyrins: Large Texas-sized Molecules," "Synthesis and Applications of Expanded Porphyrins," and "Non-covalent Approaches to Photosynthetic Model Systems," Watkins Memorial Lectures, Wichita State University, October, 1994.

173. "Expanded Porphyrins as Molecular Recognition Units," International Conference on Bioorganic Chemistry, Fukuoka, Japan, October, 1994. The was an invited Plenary Lecture.

174. "Molecular Recognition via Base-Pairing," Université de Genève, November, 1994.

175. "Biomedical Applications of Expanded Porphyrins," Seventh Inter-American Photochemical Society Winter Conference, Clearwater Beach. Florida, January, 1995.

176. "Non-covalent Electron Transfer Model Systems," 14th Enzyme Mechanism Conference, Scottsdale, Arizona, January, 1995. The was an Invited Lecture.

177. "Receptors and Carriers for Antiviral Agents, National Institutes of Health (NIAID), Bethesda, Maryland, June, 1995.

178. "New Applications of Expanded Porphyrins," XXth Symposium on Macrocyclic Chemistry, Jerusalem, Israel, July, 1995. The was an invited Plenary Lecture.

179. "Synthesis and Applications of Expanded Porphyrins," 210th American Chemical Society National Meeting, Inorganic Division, Chicago, Illinois, August, 1995.

180. "Biomedical Applications of Expanded Porphyrins," 7th International Bioinorganic Conference (IBIC 7), Lübbeck, Germany, September, 1995. The was an Invited Lecture.

181. "Non-covalent Electron Transfer Model Systems," Max Planck Institute für Strahlenchemie, Mülheim, Germany, September, 1995.

182. "Synthesis and Properties of New Expanded Porphyrins," Universität zu Köln, Cologne, Germany, October, 1995.

183. "Biomedical Applications of Texaphyrins," Max Planck Institute für Strahlenchemie, Mülheim, Germany, October, 1995.

184. "Texaphyrins: A New Class of Radiation Sensitizer?," Institute Gustave Roussy, Paris, France, October, 1995. This lecture was delivered in French.

185. "Synthesis and Properties of Expanded Porphyrins," Kyoto University, Kyoto, Japan, November, 1995.

186. "New Approaches to Antisense Reagent Development," Osaka University, Osaka, Japan, November, 1995.

187. "Recent Developments in Texaphyrin Chemistry," Gifu University, Gifu, Japan, November, 1995.

188. "Synthesis and Properties of Expanded Porphyrins," Okazaki National Research Institute, Okazaki, Japan, November, 1995.

189. "Biomedical Applications of Texaphyrins," Mitsubishi Chemical Company, Yokohama, Japan, November, 1995.

190. "Synthesis and Applications of Expanded Porphyrins," Tokyo University, Tokyo, Japan, November, 1995.

191. "Synthesis and Applications of Expanded Porphyrins," National Institutes for Materials and Research, Tsukuba, Japan, November, 1995.

192. "Synthesis and Applications of Expanded Porphyrins," National Electronics Corporation, Tsukuba, Japan, November, 1995.

193. "Biomedical Applications of Texaphyrins," Stanford University, Stanford, California, April, 1996

194. "Synthesis and Properties of Expanded Porphyrins," University of California, Davis, California, April, 1996.

195. "Synthesis and Applications of Expanded Porphyrins," Andy Derome Memorial Lecture, Oxford University, UK, June, 1996.

196. "Non-covalent Electron Transfer Model Systems, Andy Derome Memorial Lecture, Oxford University, UK, June, 1996.

197. "Biomedical Applications of Expanded Porphyrins," Cambridge University, UK, June, 1996.

198. "Anion Binding with Expanded Porphyrins: From Simple Phosphates to DNA," Ciba Geigy Inc., Basel, Switzerland, June, 1996.

199. "New Developments in the Chemistry of Expanded Porphyrins," Polish Academy of Sciences, Warsaw, Poland, June, 1996.

200. "Synthesis and Applications of Expanded Porphyrins," Wroclaw University, Poland, June, 1996.

201. "Biomedical Applications of Texaphyrins," Technical University of Munich, Germany, June, 1996.

202. "Biomedical Applications of Lanthanide(III) Texaphyrins," 21st Rare Earth Research Conference, Duluth, Minnesota, July, 1996. This was an Invited Lecture.

203. "Biomedical Applications of Lanthanide(III) Texaphyrins," 2nd European COST D1 Workshop on MRI, Utricht, Holland, October, 1996. This was an invited Plenary Lecture.

204. "Anion Binding with Expanded Porphyrins: From Simple Phosphates to DNA," Italian Chemical Society Meeting (Lombardi Section), Milan, Italy, November, 1996. This was an invited Plenary Lecture.

205. "Texaphyrin-based Anticancer Agents," Washington University School of Medicine, St. Louis, March, 1997.

206. "Advances in Expanded Porphyrin Chemistry," University of Missouri, Columbia, Missouri, March, 1997.

207. "Expanded Porphyrin and Calixpyrrole Based Anion Separations," 213th American Chemical Society Meeting, San Francisco, California, April, 1997. This was an Invited Lecture.

208. "A Coordination Chemist's View of Anion Binding," 1997 Canadian Chemistry Society Meeting, Windsor, Canada, June, 1997. This was an Invited Lecture.

209. "Calixpyrroles: An Old But New Class of Anion Binding Agents," XXII International Symposium on Macrocyclic Chemistry, Seoul, Korea, August, 1997. This was an invited Plenary Lecture.

210. "Advances in Expanded Porphyrin Chemistry," 16th International Congress of Heterocyclic Chemistry, Bozeman, Montana, August, 1997.

211. "Calixpyrroles: An Old But New Class of Anion Binding Agents," 4th International Meeting on Calixarenes, Parma, Italy, September, 1997. This was an invited Plenary Lecture.

212. "Texaphyrin. A New Approach to Anticancer Drug Development," Parents Day, University of Texas, Austin, Texas, October, 1997.

213. "Polypyrrole-based Anion Binding Agents," Workshop on Supramolecular Chemistry, Tel Aviv, Israel, January, 1998. This was an invited Plenary Lecture.

214. "Texaphyrin. A New Approach to Anticancer Drug Development," Bar Ilan University, Ramat Aviv, Israel, January, 1988.

215. "Texaphyrin. A New Approach to Anticancer Drug Development," 150th Annual Meeting of the American Academy of Arts and Sciences (AAAS), Philadelphia, February, 1998. This was an Invited Lecture.

216. "Texaphyrin. A New Approach to Anticancer Drug Development," University of California at Berkeley, February, 1998.

217. "Non-covalent Electron Transfer Model Systems," American Chemical Society Meeting, Dallas, Texas, April, 1998. This was an Invited Lecture.

218. "Calixpyrroles. Old but New Anion Binding Agents," University of New South Wales, July, 1998.

219. "Molecular Recognition via Base-pairing," University of Adelaide, Australia, July, 1998.

220. "Polypyrrole-based Anion Binding Agents," University of Perth, Australia, July, 1998.

221. Biannual Australian National Organic Chemistry Meeting, New South Wales, Australia, July, 1998. This was an invited Plenary Lecture.

222. "Polypyrrole-based Anion Binding Agents," Australian National University, Canberra, Australia, July, 1998.

223. "Expanded Porphyrins" 8th International Symposium on Novel Aromatics, Hong Kong, China, August, 1998. This was an Invited Lecture.

224. "Calixpyrroles. Old but New Anion Binding Agents," Hong Kong University of Science and Technology, August, 1998.

225. "Polypyrrole-based Anion Binding Agents," International Meeting of the Global Supramolecular Chemistry Network, Zapkone, Poland, October, 1998. This was an Invited Lecture.

226. "Polypyrrole-based Anion Binding Agents," Oregon State University, October, 1998.

227. "Texaphyrin. A New Approach to Anticancer Drug Development," University of Oregon, October, 1998.

228. "Texaphyrin. A New Approach to Anticancer Drug Development," University of Texas, San Angelo, November, 1998.

229. "Texaphyrin. A New Approach to Anticancer Drug Development," University of California, San Diego, November, 1998.

230. "Polypyrrole-based Anion Binding Agents," University of California, Riverside, November 1998.

231. "Texaphyrins. A New Approach to Anticancer Drug Development," Caltech, Pasadena, CA, November, 1998.

232. "Polypyrrole-based Anion Binding Agents, University of California, Los Angeles, November, 1998.

233. "Texaphyrins. A New Approach to Anticancer Drug Development," Special Meeting on Photodynamic Therapy, University of Frankfurt, Frankfurt, Germany, December, 1998. This was an invited Plenary Lecture.

233. "Texaphyrins. New Cancer Specific MRI Contrast Agents," COST D1 Meeting on MRI, Torino, Frankfurt, Italy, December, 1998. This was an Invited Lecture.

234. "Texaphyrins. A New Approach to Anticancer Drug Development," IC'99 Wellington, New Zealand, February, 1999. This was an invited Plenary Lecture.

235. "Biomedical Applications of Expanded Porphyrins," American Chemical Society Meeting, Inorganic Division, Porphyrin Symposium, Anaheim, California, March, 1999. This was an Invited Lecture.

236. "Calix[4]pyrrole-Functionalized Silica Gels. Novel Supports for the HPLC-Based Separation of Anions," Industrial Chemistry & Engineering Division, Calixarene-Based Separations Symposium, Anaheim, California, March, 1999. This was an Invited Lecture.

237. "Biomedical Applications of Expanded Porphyrins," Notre Dame University, April, 1999.

238. "Texaphyrins. A New Approach to Anticancer Drug Development," University of New Orleans, May, 1998.

239. "Biomedical Applications of Expanded Porphyrins," International Symposium on Functional Dyes, Osaka, Japan, June, 1999. This was an invited plenary lecture.

240. "Polypyrrole-based Anion Binding Agents," Kansai University, Osaka, Japan, June, 1999.

241. "Polypyrrole-based Anion Binding Agents," University of Kyoto, Kyoto, Japan, June, 1999.

242. "Polypyrrole-based Anion Binding Agents," University of Tokyo, Tokyo, Japan, June, 1999.

243. "Polypyrrole-based Anion Binding Agents," University of Texas at Dallas, Dallas, Texas, July, 1999.

244. "Expanded Porphyrins in Three Dimensions," XXIV International Symposium on Macrocyclic Chemistry, Barcelona, Spain, July, 1999. This was an Invited Lecture.

245. "Biomedical Applications of Expanded Porphyrins," IUPAC '99, Berlin, Germany, August, 1999. This was an Invited Lecture.

246. "Lanthanide Texaphyrins. A New Approach to Drug Development," Schering, AG, Berlin, Germany, August, 1999.

247. "Functionalized Calixpyrroles," Calix[99], Perth, Western Australia, September, 1999. This was an Invited Lecture.

248. "Texaphyrins. A New Approach to Anticancer Drug Development," Western Regional American Chemical Society Meeting, Ontario, California, October, 1999. This was an Invited Lecture.

249. "Biomedical Applications of Expanded Porphyrins," University of Texas Southwestern Medical Center, Dallas, Texas, October, 1999.

250. "Texaphyrins. A New Approach to Anticancer Drug Development," Princeton University, Princeton, New Jersey, December, 1999.

251. "Biomedical Applications of Expanded Porphyrins," Abbott Laboratories, Chicago, Illinois, January, 2000.

252. "Expanded Porphyrins: Synthesis and Applications," Wake Forest University, Winston-Salem, North Carolina, February, 2000.

253. "Non-covalent Electron Transfer Model Systems," National Institute of Materials Research, Tsukuba, Japan, February, 2000.

254. Polypyrrole-based Anion Binding Agents," Saitama University, Saitama, Japan, March, 2000.

255. "Calixpyrrole-based Separations," National Meeting of the American Chemical Society, San Francisco, March, 2000. This was an Invited Lecture.

256. "Lanthanide Texaphyrins. A New Approach to Drug Development," Argonne National Laboratories, Argonne, Illinois, April, 2000.

257. "Polypyrrole-based Anion Binding Agents," Kraft Foods, Glenview, Illinois, April, 2000.

258. "Lanthanide Texaphyrins. A New Approach to Drug Development," Université de Dijon, Dijon, France, April, 2000. This lecture was delivered in French.

259. "Template-based Syntheses of Polypyrrole Macrocycles," Central Regional ACS Meeting, Cincinnati, Ohio, May, 2000. This was an Invited Lecture.

260. "Texaphyrins. A New Approach to Anticancer Drug Development," University of Leiden, Leiden, Holland, June, 2000.

261. "Porphyrin Analogue Chemistry," International Congress on Porphyrins and Phthalocyanines, Dijon, France, June, 2000.

262. "Base-Pairing Motifs in Porphyrin-based Supramolecular Systems," International Congress on Porphyrins and Phthalocyanines, Dijon, France, June, 2000. This was an Invited Lecture.

263. "New Developments in Expanded Porphyrin Chemistry," International Congress on Coordination Chemistry, St Andrews, Scotland, July 2000. This was an invited Golden Jubilee Lecture.

264. "Polypyrrole Receptors. Versatile Cation and Anion Chelating Agents," Pall, Inc., Port Washington, NY, October, 2000.

265. "Polypyrrole-based Anion Binding Agents," Regional Am. Chem. Soc. Meeting, St. Louis, MO, October, 2000. This was an invited lecture.

266. "Polypyrrole-based Anion Binding Agents," Regional Am. Chem. Soc. Meeting, New Orleans, LA, December, 2000.

267. "Polypyrrole Receptors. Versatile Cation and Anion Chelating Agents," Meeting of the Pacific Basins Chemical Societies, December, 2000.

268. "Synthesis and Applications of Polypyrrole Macrocycles," UK Macrocycles Meeting, Southampton, UK, January 2001.

269. "Polypyrrole-based Anion Binding Agents," Stanford University, Stanford, California, January, 2001.

270. "Heavy Element Coordination Using Expanded Porphyrins," Oak Ridge National Laboratory, February, 2001.

271. "Polypyrrole Based Macrocycles," Unversity of Tennessee, Knoxville, Febuary, 2001.

272. "Polypyrrole-based Anion Binding Agents," University of Michigan, April, 2001.

273. "Synthesis and Applications of Polypyrrole Macrocycles," University of Bern, Bern, Switzerland, April, 2001.

274. "Non-covalent Electron Transfer Model Systems," Université de Neuchâtel, Neuchâtel, Swizerland, April, 2001.

275. "Texaphyrins. A New Approach to Drug Development," Ecole Polytechnique de Lausanne, Lausanne, Swizerland, April, 2001. This lecture was delivered in French.

276. "New Expanded Porphyrins," Université de Fribourg, Fibourg, Swizerland, April, 2001. This lecture was delivered in French.

277. "Polypyrrole-based Anion Binding Agents," Basel Universität, Basel, Swizerland, April, 2001.

278. "Lanthanide Texaphyrins: A New Approach to Drug Development," Lonzo AG, Visp, Switzerland, May, 2001. This lecture was delivered in German.

279. "Biomedical Applications of Expanded Porphyrins," University of Toronto, ON, Canada, May, 2001.

280. "Polypyrrole-based Anion Binding Agents," University of York, ON, Canada, May, 2001.

281. "Synthesis and Applications of Expanded Porphyrins," Massey University, Palmerston North, New Zealand, June, 2001.

282. "Expanded Porphyrins: Novel Ligands for Large Cations," University of Auckland, Auckland, New Zealand, June, 2001.

283. "Synthesis and Applications of Expanded Porphyrins," James Cook University, Townsville, Australia, June, 2001.

284. "Polypyrrole-based Anion Binding Agents," 38th World Congress of the IUPAC, Brisbane, Australia, July, 2001. This was an Invited Keynote Lecture.

285. "Non-covalent Photosynthetic Model Systems," Symposium on Supramolecular Chemistry for the New Millinium, Hiroshima, Japan, July, 2001. This was an Invited Plenary Lecture.

286. "Biomedical Applications of Expanded Porphyrins," XXV International Symposium on Macrocyclic Chemistry, Fukuoka, Japan, July 2001. This was the Izatt-Christensen Award Lecture.

287. "Synthesis and Applications of Expanded Porphyrins," Wright Patterson Air Force Base, Dayton, OH, August, 2001.

288. "Expanded Porphryins: Novel Ligands for the Complexation of Large Cations," American Chemical Society Meeting, Inorganic Session, Chicago, IL, August, 2001. This was an invited lecture.

289. "Polypyrrole-based Anion Binding Agents," American Chemical Society Meeting, Industrial and Chemical Engineering Section, Chicago, IL, August, 2001. This was an invited one-hour Tutorial Lecture.

290. "Polypyrrole-based Anion Binding Agents," US-Czech Conference on Chemical Sensors, Prague, Czech Republic, October, 2001. This was an invited Plenary Lecture.

291. "Synthesis and Applications of Expanded Porphyrins," University of Seattle, Seattle, WA, October, 2001.

292. "Synthesis and Applications of Expanded Porphyrins," University of Texas, Arlington, February, 2002.

293. "Synthesis and Applications of Expanded Porphyrins," Universidad Autonoma de Madrid, February, 2002.

294. "Synthesis and Applications of Expanded Porphyrins," Louisiana State University, February, 2002.

295. "Lanthanide and Actinide Complexes of Expanded Porphyrins," Los Alamos National Laboratory, New Mexico, February, 2002.

296. "Molecular Recognition via Base Pairing," Boston Univeristy, Boston, MA, February, 2002.

297. "Polypyrrole-based Anion Binding Agents," Boston College, Boston, MA, March, 2002.

298. "Synthesis and Applications of Expanded Porphyrins," MIT, Cambridge, MA, February, 2002.

299. "Biomedical Applications of Expanded Porphyrins," Johns Hopkins University, Baltimore, MD, March, 2002.

300. "Pyrrole-based Self-assembly Systems," American Chemical Society meeting, Orlando, FL, March, 2002. This was an Invited Lecture.

301. "Synthesis and Applications of Expanded Porphyrins," Carruthers Memorial Symposium, Exeter, UK, April, 2002. This was an Invited Lecture.

302. "Polypyrrole-based Anion Binding Agents," Royal Society Workshop, Southampton University, Southampton, UK, April, 2002. This was an Invited Lecture.

303. "Polypyrrole-based Anion Binding Agents," Université Paris Sud, Orsay, France, May, 2002. This lecture was delivered in French.

304. "Synthesis and Applications of Expanded Porphyrins," Université Marie Curie, Paris VI, Paris, France, May, 2002. This lecture was delivered in French.

305. "Lanthanide Texaphyrins: A New Approach to Drug Development," 2002 GECOM Conference, Paris, France, May, 2002. This was a Plenary Lecture. This lecture was delivered in French.

306. "Cyclo[8]pyrrole," International Conference on Function Pi Systems, Ulm, Germany, June, 2002. This was an Invited Lecture.

307. "Polypyrrole-based Macrocycles: Novel Cation and Anion Receptors," Gordon Conference on Bioorganic Chemistry, New Hampshire, June, 2002. This was an invited Plenary Lecture.

308. "Pyrrole-based Self-assembly Systems," International Symposium on Macrocyclic Chemistry, Park City, UT, June, 2002. This was a Plenary Lecture.

309. "Calixphyrins: Hybrid Macrocycles at the Crossroads of Porphyrins and Calixpyrroles," 2nd International Conference on Porphyrins and Phthalocyanines, Kyoto, Japan, July, 2002. This was an invited Keynote Lecture.

310. "Synthesis and Applications of Expanded Porphyrins," Kangwon National University, Chun-Chon, South Korea, July, 2002.

311. "Synthesis and Applications of Expanded Porphyrins," Gordon Conference on Tetrapyrroles, Rhode Island, July, 2002. This was an invited Keynote Lecture.

312. "Biomedical Applications of Expanded Porphyrins," Metals in Medicine Gordon Conference, New Hampshire, July, 2002. This was an invited Plenary Lecture.

313. "Synthesis and Applications of Expanded Porphyrins," International Conference on Coordination Chemistry, Heidelberg, Germany, July, 2002. This was an invited Plenary Lecture.

314. "Cyclo[8]pyrrole," International Conference on Function Pi Systems, BASF, Ludwigshafen, Germany, July, 2002.

315. "Synthesis and Applications of Expanded Porphyrins," The University of Texas, Austin, TX, August, 2002.

316. "Polypyrrole-based Macrocycles: Novel Cation and Anion Receptors," XII International Symposium on Supramolecular Chemistry, Eilat, Israel, October, 2002. This was an invited Plenary Lecture.

317. "Synthesis and Applications of Expanded Porphyrins," University of Missouri, St. Louis, MO, November, 2002.

318. "Polypyrrole-based Anion Receptors," Texas Christian University, Ft. Worth, TX, November, 2002.

319. "Synthesis and Applications of Expanded Porphyrins," University of Chicago, Chicago, IL, January 24, 2003. This was the Closs Memorial Lecture.

320. "Expanded Porphyrins. From Cation Coordination to Anion Recognition." Vanderbilt University, Nashville, TN, February, 2003. This was the Fistel Memorial Lecture.

321. "Synthesis and Applications of Expanded Porphyrins," University of California, Irvine, Irvine, CA, February, 2003.

322. "Texaphyrins: A New Approach to Drug Development," Institute for Cellular and Molecular Biology, The University of Texas at Austin, March, 2003.

323. "Texaphyrins: A New Approach to Drug Development," School of Pharmacy, The Hebrew University of Jerusalem, Israel, April, 2003.

324. "Synthetic ensembles based on cytosine, guanine, pyrrole, and biimidazole," Technion, Israel, April, 2003.

325. "Synthesis and Applications of Expanded Porphyrins," Bar Ilan University, Israel, April, 2003.

326. "Texaphyrins: A New Approach to Drug Development," Technion, Israel, April, 2003. This was the Sidney Pollock Award Lectureship.

327. "Synthesis and Applications of Expanded Porphyrins," University of Akron, OH, April, 2003.

328. "Synthetic Receptors for Actinides and Pertechnetate," DOE Contractor's Meeting, Sante Fe, NM, April, 2003.

329. "Lanthanide Texaphyrins: A New Approach to Drug Development," Leermakers Symposium, Wesleyan University, Wesleyan, CN, May, 2003. This was a plenary lecture.

330. "Oligopyrroles: New Materials with Potential Practical Utility," Spanish Materials Science Summer School, Cartegena, Spain, June, 2003. This was a plenary lecture.

331. "Lanthanide Texaphyrins: A New Approach to Drug Development," Santa Maria Workshop on Bioinorganic Chemistry, Santa Maria del Mar, Cuba, July, 2003. This was a plenary lecture.

332. "Gadolinium(III) Texaphyrins: From Synthesis to Medical Applications," International Conference on Bioinorganic Chemistry, Cairns, Australia, July 2003. This was an invited, main session lecture.

333. "Calixpyrroles," International Conference on Calixarenes, Vancouver, BC, Canada, August, 2003. This was a plenary lecture.

334. "Synthetic Ensembles Based on Cytosine, Guanine, Pyrrole, and Biimidazole," Fall 2003 American Chemical Society Meeting, New York, NY, September, 2003. This was an invited symposium lecture.

335. "Lanthanide Texaphyrins: A New Approach to Drug Development," Swathmore College, Swathmore, PA, September, 2003.

336. "Pyrrole-based Anion Binding Agents," Lull University, Barcelona, Spain, September, 2003.

337. "Synthetic Ensembles Based on Purine, Pyrimidine, and Pyrrole," Midwestern Regional ACS Meeting, Columbia, MO, November, 2003. This was an invited symposium lecture.

338. "Expanded Porphyrins. A New Approach to Drug Development," Universidad Autonoma de Madrid, Madrid, Spain, December, 2003.

339. "Synthesis of Porphyrins and Porphyrin Precursors," Universidad Autonoma de Madrid, Madrid, Spain, December, 2003.

340. "Synthesis of Expanded Porphyrins," Universidad Autonoma de Madrid, December, 2004.

341. "Synthesis and Applications of Expanded Porphyrins," Universidad de Zaragosa, Zaragosa, Spain, February, 2004.

342. "Synthesis and Applications of Expanded Porphyrins," Pennsylvania State University, February, 2004.

343. "Pyrrole-based Anion Binding Agents," Spring 2004 American Chemical Society Meeting, Anaheim, CA, March, 2004. This was an invited symposium lecture.

344. "Synthesis and Applications of Expanded Porphyrins," University of Cologne, Cologne, Germany, May, 2004.

345. "Biomedical Applications of Expanded Porphyrins," University of Karlsruhe, Karlsruhe, Germany, May, 2004.

346. "Expanded Porphyrins. From Cation Coordination and Anion Recognition to Novel Materials Development," University of Strasbourg, Materials Development Center, Strasbourg, France, May, 2004. This lecture was delivered in French.

347. "Synthesis and Applications of Expanded Porphyrins," University of Grenoble, Grenoble, France, May, 2004. This lecture was delivered in French.

348. "Synthesis and Applications of Expanded Porphyrins," University of Seville, Seville, Spain, June, 2004. This lecture was delivered in Spanish.

349. "Synthesis and Applications of Oligopyrrole Macrocycles," University of Oviedo, Oveido, Spain, June, 2004. This lecture was delivered in Spanish.

350. "Expanded Porphyrins. From Cation Coordination and Anion Recognition to Novel Materials Development," University of Santiago, Santiago de Compuestela, Spain, June, 2004.

351. "Biomedical Applications of Expanded Porphyrins," University of Aveila, Aveila, Portugal, June, 2004.

352. "Synthesis and Applications of Oligopyrrole Macrocycles," Gordon Research Conference on Heterocyclic Chemistry, Salve Regina College, Newport, RI, July, 2004. This was an after-banquet humorous lecture.

353. "Synthetic Ensembles Based on Purine, Pyrimidine, and Pyrrole," 3rd International Conference on Porphyrins and Phthalocyanines, New Orleans, LA, July 2004. This was an invited symposium lecture.

354. "Pyrrole-based Receptors and Assemblies," 13th International Symposium on Supramolecular Chemistry, South Bend, IN, July, 2004. This was an invited main-session lecture.

355. "Calixpyrroles and Related Anion Receptors," 4th International Symposium on Supramolecular Science and Technology, Prague, Czech Republic, September, 2004. This was a plenary lecture.

356. "Calixpyrroles," 2004 Southwest Regional ACS Meeting, Fort Worth, TX, September, 2004. This was an invited symposium lecture.

357. "Expanded Porphyrins," MichlFest Symposium, University of Utah, Salt Lake City, UT, October, 2004.

358. "Synthesis and Applications of Expanded Porphyrins," University of California, Santa Barbara, Santa Barbara, CA, October, 2004.

359. "Synthesis and Applications of Expanded Porphyrins," University of Texas at El Paso, El Paso, TX, October, 2004.

360. "Biomedical Applications of Expanded Porphyrins," 2004 Kyushu University Organic Chemistry Symposium, Fukuoka, Japan, November, 2004. This was a plenary lecture.

361. "Texaphyrins. A New Approach to Drug Development," 1st International Conference on Biomimetic Chemistry, Kyoto, Japan, November, 2004. This was a plenary lecture.

362. "Synthesis and Applications of Expanded Porphyrins," Osuka University, Osaka, Japan, November, 2004.

363. "Synthesis and Applications of Expanded Porphyrins," Kansai University, Osaka, Japan, November, 2004.

364. "Synthetic Advances in Expanded Porphyrin Chemistry," Kyoto University, Kyoto, Japan, November, 2004.

365. "Noncovalent Electron Transfer Model Systems," Erlangen-Nürnberg University, Erlangen, Germany, February, 2005.

366. "Expanded Porphyrins: From Cation Coordination and Anion Binding to Designed Drug Development," Erlangen-Nürnberg University, Erlangen, Germany, February, 2005.

367. "Recent Synthetic Advances in the Area of Expanded Porphyrin Chemistry," The Technion, Haifa, Israel, March, 2005.

368. "Synthesis and Applications of Expanded Porphyrins," The Hebrew University of Jerusalem, Jerusalem, Israel, March, 2005.

339. "Synthesis and Applications of Expanded Porphyrins," Tel Aviv University, Tel Aviv, Israel, March, 2005.

340. "Synthesis and Applications of Expanded Porphyrins," The University of Florida, Gainsville, Florida, April, 2005.

341. "Synthesis and Applications of Expanded Porphyrins," Texas A & M University, College Station, Texas, April, 2005.

342. "Synthetic Actinide Receptors," DOE Contractors' Meeting, Washington, D.C., April, 2005.

343. "Calixpyrroles and Related Anion Binding Agents," First INEST Meeting, Richmond, Virginia, May, 2005.

344. "Synthesis and Applications of Expanded Porphyrins," Université Louis Pasteur, Strasbourg, France, June, 2005.

345. "Molecular Recognition via Base-Pairing," Université Louis Pasteur, Strasbourg, France, June, 2005.

346. "Expanded Porphyrins: Novel Ligands for the Lanthanides and Actinides," Rare Earths Research Conference, Keystone, Colorado, June, 2005. This was an invited lecture.

347. "Synthesis and Applications of Expanded Porphyrins," Kekulé Institute, Universität zu Bonn, Germany, July 2005.

348. "Polypyrrole-based Anion Binding Agents," Second International Southampton Symposium on Supramolecular Chemistry, Southampton, UK, July 2005. This was an invited Plenary Lecture.

349. "New Developments in the Area of Oligopyrrole Macrocycles," International Symposium Symposium on Macrocyclic Chemistry, Dresden, Germany, July 2005. This was an invited Plenary Lecture.

350. "Calixpyrroles and Related Macrocycles," Calix-2005 International Calixarene Symposium, Prague, Czech Republic, July 2005. This was a Chem. Commun. 40 Award Lecture.

351. "Synthesis and Applications of Expanded Porphyrins," University of California, Berkeley, Berkeley, California, September, 2005.

352. "Non-covalent Electron Transfer Models," Universidad Autonoma de Madrid, Madrid, Spain, September, 2005.

353. "Synthesis and Applications of Expanded Porphyrins," The Weizmann Institute, Rehovot, Israel, September, 2005.

354. "Calixpyrroles and Related Macrocycles," Hebrew University, Jerusalem, Israel, September, 2005.

355. "Lanthanide Texaphyrins. A New Approach to Anticancer Drug Development," Pacific Basin Chemical Societies Meeting, Honolulu, Hawaii, December, 2005. This was an invited talk.

356. "Molecular Recognition via Base-pairing," Pacific Basin Chemical Societies Meeting, Honolulu, Hawaii, December, 2005. This was an invited talk.

357. "Lanthanide Texaphyrins. A New Approach to Anticancer Drug Development,"Metals in Biology Gordon Research Conference, Ventura, California, January 2006. This was an invited talk.

358. "Synthesis and Applications of Expanded Porphyrins," The University of Auburn, Auburn, Alabama, February 2006.

359. "Synthesis and Applications of Expanded Porphyrins," Yonsei University, Seoul South Korea, April 2006.

360. "Synthesis and Applications of Expanded Porphyrins," Kangwon National University, Chun Chun, South Korea, April 2006.

361. "Synthesis and Applications of Expanded Porphyrins," Seoul National University, Seoul, South Korea, April, 2006.

362. "Calixpyrroles and Related Macrocycles," Korean Chemical Society National Meeting, Seoul, South Korea, April 2006. This was an invited keynote lecture.

363. "Synthesis and Applications of Expanded Porphyrins," Electrochemical Society Annual Meeting, Denver, Colorado, May 2006.

364. "Sort Course on Supramolecular Chemistry (4 lectures)," The University of Auckland, Auckland, New Zealand, May 2006.

365. "New Developments in the Chemistry of Expanded Porphyrins," The University of Auckland, Auckland, New Zealand, May 2006.

366. "Oligopyrroles. Novel Prodigiosin Analogues," Canadian Chemical Society Annual Meeting, Nova Scotia, New Foundland, Canada, May 2006.

367. "Calixpyrroles and Related Macrocycles," The University of Oviedo, Oviedo, Spain, June 2006.

368. "New Developments in the Chemistry of Expanded Porphyrins," 4th International Conference on Porphyrins and Phthalocyanines, Rome, Italy, July 2006. This was an invited, plenary lecture.

369. "Synthesis and Applications of Expanded Porphyrins," Clemson University, Clemson, South Carolina, July 2006.

370. "Synthesis and Applications of Expanded Porphyrins," Industrial Affiliates Meeting, Stanford University, Stanford, California, September 2006.

371. "Lanthanide Texaphyrins. A New Approach to Drug Development," Universidad Cuenervaca, Mexico, September 2006.

372. "Oligopyrrole-based Anion Binding Agents," Mexican Chemical Society, National Meeting, Mexico City, Mexico, September 2006. This was an invited symposium lecture.

373. "Synthesis and Applications of Expanded Porphyrins," Universidad Autonoma de Mexico, Mexico City, Mexico, September 2006.

374. "Oligopyrroles. Smart Receptors for Anionic and Neutral Substrates," Kyushu University International Symposium on Supramolecular Chemistry, Kyushu, Japan, November 2006. This was an invited plenary lecture.

375. "Lanthanide Texaphyrins. A New Approach to Drug Development," University of Texas Alumni Lecture, Fredricksburg, Texas, November 2006.

376. "Synthesis and Applications of Expanded Porphyrins," Université de Bordeaux, Bordeaux, France, February 2007.

377. "Synthesis and Applications of Expanded Porphyrins," University of South Florida, Tampa, Florida, March 2007.

378. "Calixpyrroles. Recent Advances and Applications," American Chemical Society Meeting, Chicago, Illinois, March 2007. This was an invited symposium lecture.

379. "Calixpyrroles. Recent Advances and Applications," Second International Romanian Workshop on Supramolecular Chemistry, Cluj, Romania, April 2007. This was an invited plenary lecture.

380. "New Texaphyrin Derivatives. Towards Second Generation Systems," Israeli COST Workshop on Medicinal Inorganic Chemistry, Jerusalem, Israel, April 2007.

381. "New Texaphyrin Derivatives. Towards Second Generation Systems," Canadian Chemical Society Annual Meeting, Winnipeg, Canada, May 2007. This was an invited symposium lecture.

382. "Oligopyrroles as 'Smart' Receptors," International Symposium on Macrocyclic Chemistry, Salice Therme, Italy, June 2007. This was an invited symposium lecture.

383. "Oligopyrrole-based Anion Binding Agents," Chinese Academy of Sciences, Institute of Chemistry, Laboratory of Molecular Recognition and Selective Synthesis, Beijing, China, July 2007. This was a Molecular Science Forum Lecture.

384. "Synthesis and Applications of Expanded Porphyrins," Beijing University, Beijing, China, July, 2007.

385. "Oligopyrroles as 'Smart' Receptors," Tsinghua University, Beijing, China, July 2007.

386. "Texaphyrins. A New Approach to Drug Development," Medipharm, Ltd, Shanghai, China, July 2007.

387. "Synthesis and Applications of Expanded Porphyrins," Shanghai Institute of Organic Chemistry, Shanghai, China, July 2007.

388. "Texaphyrins. A New Approach to Drug Development," Simcere, Inc., Nanging, China, July 2007.

389. "Recent Advances in the Chemistry of Expanded Porphyrins," Kyoto University, Kyoto, Japan, July 2007.

390. "Synthesis and Applications of Expanded Porphyrins," University of Tokyo, Tokyo, Japan, July 2007.

391. "Supramolecular Chemistry I," Osaka University, Osaka, Japan, July 2007. This was a Center of Excellence Invited Short Course Lecture.

392. "Supramolecular Chemistry II," Osaka University, Osaka, Japan, July 2007. This was a Center of Excellence Invited Short Course Lecture.

393. "Calixpyrroles" Calixarenes 2007 – International Symposium on Calixarene Chemistry, College Park Maryland, August 2007. This was an invited lecture.

394. "Oligopyrrole-based Anion Binding Agents," American Chemical Society National Meeting, Boston, Massachusettes, August 2007. This was an invited symposium lecture. It was also featured in C & E News in the "News of the Week" section: C & E News, **2007**, *85(35)*, 11.

395. "Expanded Porphyrins. Synthesis and Applications," Wichita State University, September, 2007.

396. "Oligopyrrole Macrocycles. Synthesis and Potential Applications, Karolina Institute, Stockholm, Sweden, October, 2007.

397. "Biomedical Applications of Expanded Porphyrins," Uppsula University, Sweden, October 2007.

398. "Oligopyrrole-based Anion Binding Agents," Southwest Regional ACS meeting, Lubbock, Texas, November, 2007. This was an invited symposium lecture.

399. "Oligopyrrole-based Anion Binding Agents," Midwest Regional ACS meeting, Kansas City, Missouri, November, 2007. This was an invited lecture for the Awards Symposium honoring George Gokel.

400. "Oligopyrrole Receptors and Sensors for Potentially Hazardous Materials," Univ. of Illinois, Urbana-Champaign, November, 2007.

401. "Oligopyrroles: Novel Sensors for Neutral and Anionic Analytes," Univ. of Oregon, NSF-IGERT retreat, Salisan Lodge, Oregon, December, 2007.

402. "Oligopyrrole Macrocycles. Receptors for Cationic, Neutral, and Anionic Substrates," BYU University, Logan, Utah, February, 2008.

403. "Oligopyrrole Macrocycles. Receptors for Cationic, Neutral, and Anionic Substrates," The Univ. of Utah, Salt Lake City, Utah, February, 2008.

404. "Hydrogen Bond Receptors for Neutral and Anionic Substrates," University of Puerto Rico, San Juan, Puerto Rico, March, 2008.

405. "Oligopyrrole-based Anion Binding Agents," ACS National Meeting, New Orleans, LA, April, 2008. This was an invited Inorganic Division lecture.

406. "Synthesis and Applications of Expanded Porphyrins," Bowling Green State University, Bowling Green, Ohio, April, 2008.

407. "Pyrrole-based Receptors. Synthesis and Applications," University of Arkansas, Fayetteville, AK, April, 2008.

408. "New Approaches to Anticancer Drug Development," University of Texas Health Sciences Center, Houston, TX, April, 2008.

409. "Medical and Nonmedical Applications of Oligopyrrole Macrocycles," University of Maryland School of Pharmacy, Baltimore, MD, May, 2008. This was the annual Ellis Grollman lecture.

410. "Oligopyrrole Macrocycles. Receptors for Cationic, Neutral, and Anionic Substrates," Johns Hopkins University, Baltimore, MD, May, 2008.

411. "Oligopyrrole-based Anion Receptors," Trinity University, Dublin, Ireland, May, 2008.

412. "Oligopyrrole Macrocycles. Receptors for Potentially Hazardous Substances," Université d'Angers, May, 2008.

413. "Oligopyrrole Macrocycles. Receptors for Potentially Hazardous Substances," Intelligent Optical Systems, Los Angeles, CA, May, 2008.

414. "Oligopyrrole Macrocycles. Receptors for Potentially Hazardous Substances," Los Alamos National Laboratory, New Mexico, May, 2008.

415. "Oligopyrrole Macrocycles. Receptors for Potentially Hazardous Substances," Universidad Autonoma de Madrid, Madrid, Spain, June, 2008.

416. "Oligopyrrole-based Anion Receptors," University of Mallorca, Palma, Spain, June, 2008.

417. "Oligopyrrole Macrocycles. Receptors for Potentially Hazardous Substances," Biannual Meeting of the Spanish Organic Chemistry Group, Taragona, Spain, June, 2008. This was a plenary lecture.

418. "New Sapphyrin Derivatives," 5th International Congress on Porphyrins and Phthalocyanines, Moscow, Russia, July, 2008. This was a keynote lecture.

419. "Oligopyrrole Anion Receptors," 5th International Congress on Porphyrins and Phthalocyanines, Moscow, Russia, July, 2008.

420. "Oligopyrrole-based Sensors for Actinides and Pertechnetate Anion," Am. Chem. Soc. Annual Fall Meeting, Philadelphia, PA, August, 2008. This was an invited lecture in a special symposium on pyrrolic ligands.

421. "Texaphyrin and Related Approaches to Anticancer Drug Development," Schulich Symposium on Drug Development, Technion, Israel, November, 2008.

422. "Oligopyrroles: Novel Anion Receptors," Yonsei University, Korea, November, 2008.

423. "Oligopyrroles: Novel Anion Receptors," KIST, Korea, November, 2008.

424. "Oligopyrroles: Novel Anion Receptors," POSTECH, Korea, November, 2008.

425. "TTF-Calixpyrroles: Receptors for Electron Deficient Substrates," Marquette University, Milwaukee, WI, February, 2009.

426. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Univ. of Massachusettes, Amherst, MA, February, 2009.

427. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Univ. of Colorado, Boulder, CO, February, 2009.

428. "Texaphyrin and Related Approaches to Anticancer Drug Development," University of Otago, Dunedin, New Zealand, April, 2009.

429. "Oligopyrroles: Receptors for Potentially Hazardous Materials," University of Indiana, Bloomington, IA, April, 2009.

430. "Oligopyrroles: Receptors for Potentially Hazardous Materials," PLU Lecture, Kansas State University, Manhattan, KS, April, 2009.

431. "Oligopyrroles: Receptors for Potentially Hazardous Materials," University of Kansas, Lawrence, KS, April, 2009.

432. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Universität Basel, Basel, Switzerland, May, 2009.

433. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Universität Zurich, Zurich, Switzerland, May, 2009.

434. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Université Genève, Geneva, Switzerland, May, 2009.

435. "Oligopyrroles: Receptors for Potentially Hazardous Materials," ETH, Zurich, Switzerland, May, 2009.

436. "Oligopyrroles: Receptors for Potentially Hazardous Materials," University of Grenoble, Grenoble, France, May, 2009. This lecture was delivered in French.

437. "Oligopyrroles: Receptors for Potentially Hazardous Materials," University of Dijon, Dijon, France, May, 2009. This lecture was delivered in French.

438. "Oligopyrroles: Receptors for Potentially Hazardous Materials," International Symposium on Macrocyclic Chemistry, Maastricht, Holland, June, 2009. This was an invited lecture.

439. "Oligopyrroles-based Materials," Gordon Research Conference on Supramolecular Assemblies, Colby College, Maine, June, 2009. This was an invited lecture.

440. "New Advances in the Chemistry of Calixpyrroles," International Symposium on Calixarenes, Seoul, Korea Chemistry, July, 2009. This was a plenary lecture.

441. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Kyushu University, July, 2009.

442. "Chelation-based Approaches to Anticancer Drug Development," 14th International Meeting on Bioinorganic Chemistry, Nagoya, Japan, July, 2009. This was an invited lecture.

443. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Gifu University, Gifu, Japan, July, 2009.

444. "Oligopyrroles: Receptors for Potentially Hazardous Materials," University of Auckland, Auckland, New Zealand, August, 2009.

445. Oligopyrroles: Receptors for Potentially Hazardous Materials," Waikato University, Hamilton, New Zealand, August, 2009.

446. Oligopyrroles: Receptors for Potentially Hazardous Materials," Victoria University, Wellington, New Zealand, August, 2009.

447. Oligopyrroles: Receptors for Potentially Hazardous Materials," Massey University, Palmerston North, New Zealand, August, 2009.

448. "Chelation-based Approaches to Anticancer Drug Development," University of Auckland, Auckland, New Zealand, August, 2009.

449. "Pyrrole-based Anion Receptors," Yonsei University, Korea, September, 2009.

450. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Suwon University, Korea, September, 2009.

451. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Puson University, Korea, October, 2009.

452. "Oligopyrroles: Receptors for Potentially Hazardous Materials," Ewha University, Korea, November, 2009.

453. University of Singapore, Singapore, December 2009

454. "Receptors for Potentially Hazardous Materials", Singapore International Chemistry Conference, December, 2009. This was a plenary lecture.

455. "Receptors for Potentially Hazardous Materials," Rutgers University, New Jersey, February, 2010.

456. "Receptors for Potentially Hazardous Materials," University of Pittsburg, Pittsburg, PA, February, 2010.

457. "Synthesis and Applications of Expanded Porphyrins," University of Victoria, Victoria, British Columbia, Canada, March, 2010. This was one of two Landsowne Lectures presented at U. Vic.

458. "Receptors for Potentially Hazardous Materials," University of Victoria, Victoria, British Columbia, Canada, March, 2010. This was one of two Landsowne Lectures presented at U. Vic.

459. "Pyrrole-based Receptors for Lanthanide and Actinide Cations," Am. Chem. Soc. National Meeting, San Francisco, March, 2010. This was an invited lecture.

460. "Donor-acceptor Assemblies," International Symposium on Macrocyclic and Supramolecular Chemistry, Nara, Japan, June, 2010. This was an invited lecture.

461. "Functionalized Oligopyrroles: Receptors for Potentially Hazardous Materials," Kansai University, Osaka, Japan, June, 2010.

462. "Chelation-based Approaches to Anticancer Drug Development," Kyoto University, Kyoto, Japan, June, 2010.

463. "Fun With Calixpyrroles: New Tricks for Old Molecules," Osaka University, Osaka, Japan, June, 2010.

464. "Functionalized Oligopyrroles: Receptors for Potentially Hazardous Materials," Global COE Conference, Kyushu University, Kyushu, Japan, June, 2010. This was an invited plenary lecture.

465. "Chelation-based Approaches to Anticancer Drug Development," WCU International Conference on Biofunctional Materials, Yonsei University, Seoul, Korea, June, 2010.

466. "Functionalized Oligopyrroles: Receptors for Potentially Hazardous Materials," Heterocycles Gordon Conference, Salve Regina, RI, June, 2010.

467. "Self-assembly and Electron Transfer Properties of Oligopyrroles," 6th International Conference on Porphyrins and Phthalocyanines, Tamaya, New Mexico, July, 2010. This was an invited lecture.

468. "Globalization and Human Rights: What the American Chemical Society Could Do," Am. Chem. Soc. National Meeting, Boston, MA, August, 2010. This was an invited presidential-level presentation.

469. "Anion-based Self-Assembly: A Texas-sized Approach," Am. Chem. Soc. National Meeting, Boston, MA, August, 2010. This was an invited lecture.

470. "Donor-Acceptor Receptors: From Sensors to Self-Assembly," Kangwon National University, Chun-Cheon, Korea, September, 2010.

471. "Non-covalent Electron Transfer Model Systems," 60th Japanese Coordination Chemical Society Meeting, Osaka, Japan, October, 2010. This was an invited lecture.

472. "Expanded Porphyrin-based Drug Development," ChemComm Symposium, Ewha University, Seoul, Korea, October, 2010. This was an invited lecture.

473. "Anion-based Self-Assembly: A Texas-sized Approach," Korean Chemical Society Meeting, Daegu, Korea, October, 2010.

474. "Donor-Acceptor Receptors: From Sensors to Self-Assembly," Symposium on Functional Supramolecular Materials, Yonsei University, Seoul, Korea, October 2010. This was an invited lecture.

475. "Functionalized Calixpyrroles," Inha University, Inchon, Korea, November, 2010.

476. "Donor-Acceptor Receptors: From Sensors to Self-Assembly," Symposium on pi-Functional Materials, Kyoto University, Kyoto, Japan, November, 2010. This was a plenary lecture.

477. "Polypyrrole-based Schiff Base Macrocycles," Pacifichem, Honolulu, HI, December, 2010. This was an invited lecture.

478. "Poplypyrroles: Sensors for Potentially Hazardous Materials," Pacifichem, Honolulu, HI, December, 2010. This was an invited lecture.

479. "Polypyrrole-based Anion Binding Agents," Pacifichem, Honolulu, HI, December, 2010. This was an invited lecture.

480. "Functionalized Calixpyrroles," University of Toronto, Main Campus, Toronto, Canada, January, 2011.

481. "Functionalized Calixpyrroles," University of Toronto, Mississuaga Campus, Toronto, Canada, January, 2011.

482. "Pyrrole-based Receptors and Carriers," CASH 2011 Symposium, University of Bath, UK, February, 2011. This was an invited plenary lecture.

483. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," Univ. of Houston, Houston, TX. February, 2011.

484. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," National University of Singapore, March, 2011.

485. "Receptors for Early Actinides and Ion Pairs," DOE Contractors Meeting, Baltimore, MD, April, 2011. This was an invited lecture.

486. "Texaphyrins: Death and Possible Resurection," Univ. of Texas, Southwestern Medical School, May, 2011.

487. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," Northwestern University, Evanston, IL May, 2011.

488. "Pyrrole-based Receptors and Carriers," National Chiao Tung University, Taiwan, May, 2011.

489. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," Academica Sinica, Taiwan, May, 2011.

490. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," National Taiwan University, May 2011.

491. "Pyrrole-based Self-Assembled Materials," Canadian Society of Chemistry Annual Meeting, Montreal, Canada, June, 2011. This was an invited lecture.

492. "Pyrrole-based Self-Assembled Materials," Gordon Conference on Polymers, Mount Holyoke College, MA, June, 2011. This was an invited lecture.

493. "Pyrrole-based Receptors and Carriers: A New Approach to Drug Development?" 6th International Symposium on Macrocyclic and Supramolecular Chemistry, Brighton, UK., July, 2011. This was an invited lecture.

494. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," Saitama University, Saitama, Japan, September 9, 2011.

495. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," National Institute for Material Science, Tsukuba, Japan, September 12, 2011.

496. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," Tokyo Metropolitan University, Saitama, Japan, September 13, 2011.

497. "Pyrrolic Receptors: From Sensors and Recognition to Self-Assembly," DOES Distinguished Lecture Series SKKU, Korea, September 23, 2011.

498. "Pyrrole-based Anion Receptors," Joint Korean-Japanese Inorganic Symposium, Gyeoungju, South Korea, October 1, 2011. This was an invited lecture.

499. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," NanoUT 2011, Salt Lake City, Utah, October 13, 2011. This was an invited plenary lecture.

500. "Functionalized Calixpyrroles: From Simple Anion Receptors to Molecular Switches," POSTECH, Korea, October 26, 2012 .

501. "Functionalized Calixpyrroles: From Simple Anion Receptors to Molecular Switches," GNU, Korea November 4, 2012.

502. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," Southwest Regional ACS Meeting, Austin, Texas, November 10, 2011. This was an invited lecture.

503. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," University of Durham, United Kingdom, November 14, 2011. This was a Centenary Award Lecture.

504. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," University of Sheffield, United Kingdom, November 15, 2011. This was a Centenary Award Lecture.

505. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," University of York, United Kingdom, November 16, 2011. This was a Centenary Award Lecture.

506. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," University of Wyoming, Laramie, Wyoming, November 18, 2011. This was the Rhoads/Raulins Distinguished Lecture.

507. "Oligo Pyrroles and Related Macrocyles: From Receptors and Sensors to Self-assembly," Modern Trends in Inorganic Chemistry-14, Hyderbad, India, December 13, 2011. This was a plenary lecture.

508. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," IIT-Bombay, December 14, 2011.

509. "Pyrrole-based Anion Recognition," The 11th Global COE International Symposium: Bio-Environmental Chemistry Osaka University, December 21, 2011.

510. "Supramolecular Chemistry as Applied to Anion Recognition," 22nd Special Global COE Lecture Kyushu University, Fukuoka, Japan, January 11, 2012.

511. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," 16th International Workshop, Kyushu University, Fukuoka, Japan, January 12, 2012.

512. "Calixpyrroles as Chemical Switches," International Symposium on Macrocyclic and Supramolecular Chemistry, Dunedin, New Zealand, February 2, 2012.

513. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," University of Auckland, Auckland, New Zealand, February 3, 2012.

514. "Pyrrole-based Anion Binding Agents: From Receptors and Carriers to Self-assembled Materials," University of Texas, El Paso, El Paso, Texas, February 9, 2012.

515. "Calixpyrroles: From Sensors to Switches and Responsive Materials," University of Reno, Reno, Nevada, March 9th, 2012.

516. "Calixpyrroles: From Sensors to Switches and Responsive Materials," American Chemical Society Meeting, San Diego, March 26, 2012. This was an invited lecture in a session honoring Julius Rebek.

517. "Texaphyrins. Death and Attempts at Resurrection," American Chemical Society Meeting, San Diego, March 27, 2012. This was an invited lecture in a symposium on bioinorganic chemistry.

518. "Calixpyrroles: From Sensors to Switches and Responsive Materials," Wageningen Symposium, Wagningen, Holland, April 12, 2012. This was a plenary lecture.

519. "Texaphyrins. Death and Attempts at Resurrection," Gordon Stone Memorial Symposium, Baylor University, Waco, Texas, May 17, 2012. This was an invited lecture.

520. "Metal Complex-based Approaches to Cancer Drug Development," 3rd International Symposium on Synthesis and Drug Development (ISOSDD), Changzhou, May 21, 2012. This was an invited lecture.

521. "Calixpyrroles: From Sensors to Switches and Responsive Materials," Jiangsu University, Zhenjiang, China, May 24, 2012. This lecture was associated with the awarding of an honorary professorship.

522. "Calixpyrroles: From Sensors to Switches and Responsive Materials," Nanjing University, Nanjing, China, May 25, 2012.

523. "Calixpyrroles: From Sensors to Switches and Responsive Materials," East China Normal University, Shanghai, China, May 30, 2012.

534. "Calixpyrroles: From Sensors to Switches and Responsive Materials," Fudan University, Shanghai, China, May 31, 2012.

535. "Calixpyrroles: From Sensors to Switches and Responsive Materials," Shanghai University, Shanghai, China, June 1, 2012.

536. "Calixpyrroles: From Sensors to Switches and Responsive Materials," East China Institute of Science and Technology, Shanghai, China, June 4, 2012.

537. "Calixpyrroles: From Sensors to Switches and Responsive Materials," Osaka University, Osaka, Japan, June 27, 2012.

538. "New Studies of Texaphyrins and Other Expanded Porphyrins," ICPP Satellite Meeting, Kyoto University, Kyoto, Japan, June 29, 2012. This was an invited lecture.

539. "Calixpyrroles: Anion Switchable Building Blocks for Self-Assembly," 7th International Conference on Porphyrins and Phthalocyanines, Jeju Island, Korea, July 4, 2012. This was an invited lecture.

540. "Calixpyrroles: From Sensors to Analyte-responsive Materials," 3rd Molecular Sensor and Molecular Logic Gate symposium, Korea University, Seoul, Korea, July 9, 2012. This was an invited keynote lecture.

541. "Texaphyrins as Carriers for Platinum-based Anticancer Drugs," Seoul National University, Seoul, Korea, July 12, 2012.

542. "Texaphyrins as Carriers for Platinum-based Anticancer Drugs," Kangwon National University, ChunCheon, South Korea, July 13, 2012.

543. "Calixpyrroles: From Sensors to Switches and Responsive Materials," Universidad Autonoma de Madrid, Madrid, Spain, July 19, 2012.

544. "Calixpyrroles: From Sensors to Supramolecular Assembly," PacifiKen II, Timberline Lodge, Oregon, August 2, 2012. This was an invited lecture.

545. "Calixpyrroles: From Sensors to Supramolecular Assembly," 1st International Conference on Supramolecular Technologies, Tsinghua University, Beijing, China, December, 2012. This was an invited plenary lecture.

546. "Calixpyrroles: From Sensors to Supramolecular Assembly," Israel Chemical Society Annual Meeting, Tel Aviv, Israel, February, 2013. This was an invited plenary lecture.

547. "Calixpyrroles: From Sensors to Supramolecular Assembly," Scripps Research Institute, La Jolla, California, May, 2013.

548. "Calixpyrroles: From Sensors to Supramolecular Assembly," The Hebrew University of Jerusalem, Jerusalem, Israel, June 2013.

549. "Calixpyrroles as Functional Ion Pair Receptors," Calix-2013, St. Johns, Canada, July 2013. This was an invited plenary lecture.

550. "Texaphyrins: Towards the Development of Redox Active Drugs," 1st International Meeting on Redox-based Drug Development, Erlangen, Germany, July, 2013. This was an invited plenary lecture.

551. "Calixpyrroles: From Sensors to Supramolecular Assembly," The University of Sao Carlos, Sao Carlos, Brazil, August, 2013.

552. "Oligopyrrole-based Anion Receptors," ACS Central Texas Section Meeting, Austin, TX, November, 2013.

553. "Coordination Chemistry-based Drug Development," ACS Regional Meeting, Waco, TX, November, 2013 (this was an invited award lecture).

554. "Calixpyrroles: From Sensors to Supramolecular Assembly," Polish Academy of Sciences, Warsaw, Poland, December, 2013.

556. "Calixpyrroles: From Sensors to Supramolecular Assembly," Centre Nationale de Recherches, Lyon, France, February, 2014.

557. "New Advances in the Chemistry of Expanded Porphyrins," University de Lyon I, Lyon, France, February, 2014.

558. "Calixpyrroles: From Sensors to Supramolecular Assembly," Université Louis Pasteur de Strasbourg, Strasbourg, France, February, 2014.

559. "Calixpyrroles: From Sensors to Supramolecular Assembly," Karlsruhe Insitute of Technology, Karlsruhe, Germany, February, 2014.

560. "Non-covalent Electron Transfer Model Systems," University of North Texas, Denton, TX, March, 2014.

561. "New Advances in the Chemistry of Expanded Porphyrins," Korean Chemical Society Meeting, Seoul, Korea, April, 2014 (this was an invited keynote lecture).

562. "Calixpyrroles: From Sensors to Supramolecular Assembly," Yonsei University Satellite Symposium on Biofunctional Materials, Seoul, Korea, April, 2014 (this was an invited plenary lecture).

563. "Pyrrole-based Anion Recognition and Transport," Canadian Chemical Society Meeting, Vancouver, Canada, June, 2014.

564. "Expanded Porphyrins—An Update," International Symposium on Macrocyclics and Supramolecular Chemistry, Shanghai, China, June, 2014 (this was an invited lecture).

565. "Calixpyrroles: From Sensors to Supramolecular Assembly," 1st Purple Mountain Research Conference, Nanjing, China, June 2014 (this was an invited plenary lecture).

566. "Non-covalent Electron Transfer Model Systems," International Conference on Porphyrins and Phthalocyanines, Istanbul, Turkey, June, 2014 (this was an invited lecture).

567. "Texaphyrin-based Drug Development," Technion Shulich Summer School, Haifa, Israel, July, 2014 (this was an invited lecture).

568. "Texaphyrin-based Drug Development," International Conference on Coordination Chemistry, Singapore, July, 2014 (this was an invited lecture).

569. "Sensors for Biological Thiols," National University of Singapore, Singapore, July, 2014.

570. "Calixpyrroles: From Sensors to Supramolecular Assembly," University of Tokyo, Tokyo, Japan, July, 2014.

571. "Calixpyrroles: Easy-to-Prepare Anion Binding Agents," Kanazawa University, Kanazawa, Japan, July, 2014.

572. "Expanded Porphyrins: Macrocycles Based on Pyrrole, Pyridine, and Furan," Michinoku International Symposium on Porphyrins, Phthalocyanines, and Functional π Molecules, Sendai, Japan, October, 2014. This was an invited lecture.

573. "Calixpyrroles: From Sensors to Supramolecular Assembly," International Symposium on Molecular Sensor and Molecular Logic Gates 2014, East China University of Science and Technology, Shanghai, China, November, 2014. This was an award lecture.

574. "Texaphyrins: Adventures in Academic Entrepreneurship," Shanghai University, Shanghai, China, November, 2014.

575. "Calixpyrroles: From Sensors to Supramolecular Assembly," Shanghai University, Shanghai, China, November, 2014.

576. "Expanded Porphyrins: Macrocycles Based on Pyrrole, Pyridine, and Furan," Henan University, Kaifeng, China, November, 2014.

577. "Platinum Texaphyrin Conjugates," University of Arizona, Tucson, AZ, November, 2014.

578. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Technion, Haifa, Israel, December, 2014.

579. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," University of Alabama, Tusculoosa, Alabama, February, 2015.

580. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Texas State, San Marcos, Texas, March, 2015.

581. "Expanded Porphyrins: A Personal Journey," University of California, Berkeley, California, March, 2015. This was the inaugural Ken Raymond Lecture.

582. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Oxford University, Oxford, UK, March, 2015.

583. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Texas A&M, Kingsville, April, 2015. Note: This was the Garland Lecture.

584. "Calixpyrrole-based Ion Pair Receptors," DOE Contractors' Meeting, Gaithesberg, VA, April, 2015.

585. "Platinum Texaphyrin Conjugates," International Conference on Cutting Edge Molecules for Biology and Material Application, Hong Kong, April, 2015. This was a Plenary Lecture.

586. "Expanded Porphyrins: A Personal Journey," Prof. Shunichi Fukuzumi Retirement Symposium, Osaka, Japan, April, 2015. This was a Plenary Lecture.

587. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Kyushu University, May, 2015.

588. "Advances in Expanded Porphyrin Chemistry," Electrochemical Society Annual Meeting, Chicago, IL, May, 2025. This was an invited lecture.

589. "Ion Pair Receptors—A Tutorial," National Science Foundation Workshop on Water, Garden City, MD, June, 2015.

590. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Zhejiang University, Hanzhou, China, June, 2015

591. "Platinum Texaphyrin Conjugates," 7th Chinese-American Chemists Symposium, Suzchou, China, June 2015.

592. "Pyrrole Macrocycle-based Self-Assembly," Internation Symposium on Macrocyclic and Supramolecular Chemistry, Strasbourg, France, June, 2015. This was an invited lecture.

593. "Pyrrole Macrocycle-based Self-Assembly," Wais Hosseini 60th Birthday Symposium, Strasbourg, France, 2015. This was an invited lecture.

594. "Calixpyrrole-based Ion Pair Receptors," International Conference on Calixarenes 2015, Sicily, Italy, July, 2015. This was an invited lecture.

595. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Hiroshima University, July 2015.

596. "Platinum Texaphyrin Conjugates," Kyoto University, Kyoto, Japan, July, 2015.

597. "Platinum Texaphyrin Conjugates," Medicinal Inorganic Chemistry Symposium, Hebrew University of Jerusalem, September, 2015.

597. "Platinum Texaphyrin Conjugates," Universidad Autonoma de Madrid, Madrid, Spain, July, 2015

598. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Institute Catalonia di Qimica, Tarragona, Spain, July, 2015.

599. "New Advances in the Chemistry of Expanded Porphyrins," Institute Qimica de Serona, Universidad Ramon Llul, Barcelona, Spain, July, 2015.

600. "Platinum Texaphyrin Conjugates," Medicinal Inorganic Chemistry Symposium, Hebrew University of Jerusalem, September, 2015.

601. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Brigham Young University, September, 2015.

602. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Schulich Winter School in Honor Harry Gray's 80th Birthday, Technion, Israel, December, 2015.

603. "Calixpyrrole-based Anion Receptors," Pacifichem, Honolulu, HI, December, 2015.

604. "Pyrrole-based Self-Assembled Materials," Pacifichem, Honolulu, HI, December, 2015.

605. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," Gyeongsang National University, Jinju, South Korea, January, 2016.

606. "Expanded Porphyrins: A Personal Journey," Symposium on Phthalocyanines and Close Friends Honoring Prof. Tomas Torres' 65th Birthday, Elche, Spain, January, 2016.

607. "Expanded Porphyrins: A Personal Journey," Am. Chem. Society, Spring 2016 Meeting, San Diego, CA. This was an invited symposium lecture.

608. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," University of Minnesota, MN, April, 2016

609. "Texaphyrins: Life, Death, and Attempts at Resurrection," MIT, Cambridge, MA, May, 2016.

610. "Expanded Porphyrins: A Personal Journey," Dombay Organic Chemistry Conference, Dombay, Russia, May, 2016. This was a plenary lecture.

611. "Expanded Porphyrins: A Personal Journey," Shinshu University, Ueda, Japan, June, 2016.

612. "Expanded Porphyrins: A Personal Journey," Hunan Normal University, Changsha, China, June, 2016.

613. "Non-Porphyrin Macrocycles Based on Pyridine, Furan, and Pyrrole," International Conference on Porphyrins and Phthalocyanines, Nanjing, China, July, 2016. This was an award lecture.

614. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," East China University of Science and Technology, Shanghai, China, July 2016.

615. "Medicinal Macrocyclic Chemistry," International Symposium on Supramolecular and Macrocyclic Chemistry, Seoul, Korea, July, 2016.

614. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials," East China Normal University, Shanghai, China, July, 2016.

615. "Adventures in Self-Assembly: Is There Logic Here?" Molecular Sensors and Molecular Logic Gates symposium, Bath, UK, July, 2016. This was a plenary lecture.

616. "Calixpyrroles: From Anion Receptors to Self-assembled Materials," University of Texas at Dallas, September 2016.
617. "Calixpyrroles and Self-Assembly," ICREA Conference on Functional Nanocontainers, Taragona, Spain, October, 2016. This was an invited plenary lecture.

618. "Expanded Porphyrins: A Personsal Journey," 2nd Kanazawa University Symposium on Supramolecular Chemistry and Materials, October, 2016. This was an invited plenary lecture.

619. "Expanded Porphyrins: A Personal Journey," Shinju University, Japan, October, 2016.

620. "Expanded Porphyrins: A Personal Journey," Emanuel Vogel Memorial Lecture, Universität zu Köln, December, 2016.

621. "Calixpyrroles: From Anion Receptors to Self-Assembly and Logic Devices," Universität Bonn, December, 2016.

622. "Calixpyrroles: From Anion Receptors to Self-Assembly and Logic Devices," 1st Shanghai University Symposium on Supramolecular Chemistry, Shanghai, December, 2016. This was an invited plenary lecture.

623. "Texaphyrin-based Photodynamic Therapy and Drug Development" 2017 Conference on the Physics of Quantum Electronics, Snowbird, UT, January 2017. This was an invited lecture.

624. "Expanded Porphyrins: A Personal Journey," Klemmer Lecture, University of Oregon, Eugene, OR, February, 2017.

625. "Calixpyrroles: From Anion Receptors to Self-Assembly and Logic Devices," University of Oregon Skiposium, Sun River, OR, February, 2017.

626. "Expanded Porphyrins: A Personal Journey," Kangwon National University, Chuncheon, S. Korea, February, 2017.

627. "Expanded Porphyrins: A Personal Journey," Gyeongsang National University, Jinju, S. Korea, February, 2017.

628. "Expanded Porphyrins: A Personal Journey," Postech, Pohang, Korea, February, 2017.

629. "Cible, Inc." Livestrong Cancer Institute, Austin, TX, March, 2017.

630. "Calixpyrroles: From Anion Receptors to Self-Assembled Materials" Dartmouth College, Hannover, Vermont, April, 2017.

631. "Calixpyrrole-based Logic Devices," Nanoscience 2017, Snowbird, UT, April, 2017.

632. "Expanded Porphyrins: A Personal Journey," Auburn University, Auburn, AL, April, 2017

633. "Calixpyrroles: From Anion Receptors to Self-Asembly and Materials," Auburn University, Auburn, AL, April, 2017.

634. "Expanded Porphyrins: A Personal Journey," Yale University, New Haven, CT, May, 2017.

635. "Calixpyrrole-based Receptors: Is There Logic Here?" King Abdullah Univesity of Science and Technology, Saudia Arabia, May 2017.

636. "Texaphyrins: An Overview" Chemistry Applied to Sensing and the Environment (CASE 2017), Shanghai, China. Note: This was the CASE 2017 Award Lecture.

637. "Expanded Porphyrins: A Personal Journey," Peking University, Beijing, China, May, 2017.

638. "Expanded Porphyrins: A Personal Journey," Beijing Polytechnical University, Beijing, China, May, 2017.

639. "Texas-sized Chemistry," Beijing Normal University, Beijing, China, May, 2017.

640. "Short Course on Supramolecular Chemistry," Beijing Normal University, Beijing, China, May, 2017.

641. "Calixpyrroles: From Anion Receptors to Self-assembled Materials and Logic Devices," Institute of Chemistry, Chinese Academy of Sciences," Beijing, China, May, 2017.

642. "Calixpyrroles-based Molecular Logic Devices," Gordon Conference on Molecular Switches, Holderness, NH, June, 2017. Note: This was an evening invited lecture.

643. "Texaphyrin-based Drug Development," Rare Earth Research Conference, Ames, Iowa, June 2017. Note: This was an invited lecture in the Spedding Award session honoring Prof. Ken Raymond (UC Berkeley).

644. "Towards 3D Aromaticity," International Symposium on Macrocyclic and Supramolecular Chemistry, Cambridge, UK, July 2017. Note: This was an invited oral talk.

645. "Self-assembled Gels and 3D Patterning," Symposium on Beyond the Non-covalent Bond, Jesus College, Cambridge, July 2017.

646. "Self-assembled Gels and 3D Patterning," Symposium on Supramolecular Chemistry, Tsinghua University, Beijing, China, August, 2017

647. "Calixpyrrole-based Logic Devices," International Conference on Calixarene Chemistry, Tianjin, China, August, 2017. This was an invited plenary lecture.

648. "Short Course on Photodynamic Therapy," Universidad de Sonoro, Hermosillo, Mexico, September, 2017.

649. "Receptors for Lithium, Hydroxide, and Uranyl Ions," Encuentro de Quimica Inorganica de Mexico, Universidad de Sonoro, Hermosillo, Mexico, September, 2017.

650. "Adventures in Self-Assembly: Is there Logic Here?" Roegnik Lecture, Ohio University, Athens, Ohio, October, 2017. This was a named lecture.

651. "Texaphyrins: Life, Death, and Attempts and Resurrection," Live Strong Cancer Institute annual retreat, Austin, Texas, November 2017. This was an invited lecture.

652. "Texaphyrin Conjugates: A Case Study in the Value of CPRIT Support," American Cancer Society 2017 Public Forum, Austin, Texas, November 2017.

653. "Expanded Porphyrins: A Personal Journey," Université Laval, Quèbec, Canada, December, 2017.

654. "Expanded Porphyrins: A Personal Journey," South University of Science and Technology of China, Shenzhen, China, December, 2017.

655. "Expanded Porphyrins: A Personal Journey," Paul Beer 60th Birthday Fest, Oxford University, Oxford, UK, January, 2018. This was an invited lecture.

656. "Texaphyrin-based Theranostics: An Update," Metals in Biology Gordon Research Conference. Ventura, CA, January, 2018. This was an invited lecture.

657. "Expanded Porphyrins: A Personal Journey," Burns Night Lecture, University of New Orleans, New Orleans, LA, January, 2018.

658. "Expanded Porphyrins as Drug Candidates," Texas 4000 Public Lecture, The University of Texas at Austin, Austin, TX, February, 2018.

659. "Expanded Porphyrins: A Personal Journey," Symposium on Expanded, Contracted, and Isomeric Porphyrins, Kyushu University, Kyushu, Japan, February, 2018.

660. "Adventures in Self-Assembly: Is there Logic Here?" Symposium on Molecular Capsules, ACS National Meeting, New Orleans, LA, March, 2018. This was an invited lecture.

661. "Adventures in Self-Assembly: Is there Logic Here?" Wayne State University, Detroit, MI, April, 2018.

662. "Texaphyrins: Life, Death, and Attempts at Ressurection," Howard Lecture I, University of Sydney, Sydney, Australia, May, 2018.

663. "Adventures in Self-Assembly: Is there Logic Here?" Howard Lecture II, University of New South Wales, Sydney, Australia, May, 2018.

664. "Adventures in Self-Assembly: Is there Logic Here?" Molecular Sensors and Molecular Logic Gates 2018, Dalian, China, June, 2018. This was an invited plenary lecture.

665. "Expanded Porphyrins as Ligands and Probes for (Anti)aromaticity," Peking University, Beijing, China, June 2018.

666. "Expanded Porphyrins as Ligands and Probes for (Anti)aromaticity," Inorganic Gordon Research Conference, Maine, June, 2018. This was an invited lecture.

667. "Metal-based Drug Discovery: From Gadolinium Texaphyrins to Platinum Conjugates and Gold Carbenes," Metals in Medicine Gordon Research Conference, New Hampshire, June, 2018. This was an invited lecture.

668. "Oligopyrrole-based Advances in Photodynamic and Photothermal Therapy," International Conference on Porphyrins and Phthalocyanines, Munich, German, July, 2018. This was the Thomas Dougherty Award Lecture.

669. "Oligopyrrole-based Advances in Drug Discovery," Kyoto University, Kyoto, Japan, July, 2018.

670. "Adventures in Self-Assembly: Is there Logic Here?" Onsen Symposium, Ehime University, Matsuyama, Japan, July, 2018. This was an invited plenary lecture.

672. "Oligopyrrole-based Advances in Drug Discovery," Ningbo University, Ningbo, China, August, 2018.

673. "Oligopyrrole Research in the Sessler Group," Sichuan University, Chengdu, China, August, 2018.

674. "Oligopyrrole Macrocycles as Theranostics," Student-organized Symposium on Advances in Supramolecular Chemistry. Division of Analytical Chemistry, American Chemical Society National Fall Meeting, Boston, MA, August, 2018.

675. "Gadolinium Texaphyrin-based Drug Discovery Efforts," Imaging 2020, Jackson Hole, WY, September, 2018. This was an invited talk.

676. "Macrocycle-based Adventures in Self-Assembly," New York University Abu Dhabi, UAE, September, 2018.

677. "Expanded Porphyrin-based Efforts at Drug Discovery," Istanbul Technical University, Istanbul, Turkey, October, 2018.

678. "Texaphyrins: Life, Death, and Attempts at Resurrection," Symposium in Honor of Dan Meyerstein, Ariel University, Ariel, Israel, October, 2018. This was an invited lecture.

679. "Adventures in Self-Assembly: Is there Logic Here?" Weizmann Institute of Science, Rehovot, Israel, October, 2018.

680. "Expanded Porphyrin-based Efforts at Drug Discovery," McGavock Lecture, Trinity University, San Antonio, Texas, October, 2018

681. "Texaphyrins: Life, Death, and Attempts at Resurrection," University of Tennessee, TN, November, 2018

682. "Calixpyrroles: From Ion Recognition to Self-Assembly," Oak Ridge National Laboratory, TN, November, 2018.

683. "Expanded Porphyrins as Testbeds for Aromaticity," Aromaticity2018, Cancun, Mexico, November, 2018

684. "Texaphyrins as Drug Discovery Candidates," AsBic9, Singapore, December, 2018. This was an invited Keynote lecture.

685. "Texaphyrins as Drug Discovery Candidates," Huazhong University of Science and Technology (HUST), December, 2018.

686. "Calixpyrroles: From Ion Recognition to Self-Assembly," Wuhan University, December, 2018.

687. "Texaphyrins as Drug Discovery Candidates," South-Central University of Nationalities, December, 2018

688. "Calixpyrroles: From Ion Recognition to Self-Assembly," Zhejiang University, December, 2018.

689. "Calixpyrroles: From Ion Recognition to Self-Assembly," Burns Night Symposium, University of Chicago, January, 2019.

690. "Texas-inspired Approaches to Drug Discovery," Seattle Genetics, Seattle, WA, March 2019.

691. "Calixpyrroles: From Ion Recognition to Self-Assembly," University of Washington, Seattle, WA, March 2019.

692. "Calixpyrroles: From Anion Recognition to Ion Pair Extraction," American Chemical Society Meeting, Orlando, FL, March, 2019. Note this was an invited lecture in the Separations Award symposium honoring Sheng Dai.

693. "Texas-inspired Approaches to Drug Discovery," Xinda Lecture no 562, Peking University, Beijing, China, March 2019.

694. "On Adventures in Expanded Porphyrin Chemistry," Xuetang Lectureship, Tsinghua University, Beijing, China, March 2019.

695. "Texas-inspired Approaches to Drug Discovery," *ChemComm* Symposium, University of Auckland, April, 2019

696. "Calixpyrrole-based Anion Transporters," SupraBio2019, Barcelona, Spain, May, 2019. This was a plenary lecture.

697. "Texas-inspired Approaches to Drug Discovery," CanBic19, Parry Sound, Ontario, Canada, May, 2019. This was an invited lecture.

698. "Texas-inspired Approaches to Drug Discovery," Unversität zu Regensburg, Germany, May, 2019.

699. "Calixpyrrole-based Anion Transporters," Unversität zu Erlangen, Germany, May, 2019.

700. "Macrocycle-based Adventures in Self-Assembly," International François Diederich Farewell Symposium, ETH, Zurich, Switzerland, June, 2019.

701. "Calixpyrroles: From Anion Recognition to Self-Assembly," Calix19, Casis, France, June, 2019. This was an award lecture.

702. "Calixpyrroles: From Anion Recognition to Self-Assembly," Changhee Lee Retirement Symposium, Seoul, Korea, June, 2019. This was an invited lecture.

703. "Texas-inspired Approaches to Drug Discovery," Shanghai Jiaotong University, July, 2019.

704. "New Developments in Pyrrolic Macrocyclic Chemistry," Kyoto University, August, 2019.

705. "Texas-inspired Approaches to Drug Discovery," ICBIC-2019, Interlaken, Switzerland, August, 2019. This was an invited lecture.

706. "Texas-inspired Approaches to Drug Discovery," Shanghai Science Forum, August, 2019. This was a general audience outreach lecture.

707. "Receptors for Ion Pairs and Early Actinides," DOE Contractors' Meeting, Gaithersburg, MD, September, 2019.

708. "Texas-inspired Approaches to Drug Discovery," Asian Conference on Porphyrins and Phthalocyanines, Yanqi Lake, China, August, 2019. This was an award lecture.

709. "Calixpyrroles: From Anion Recognition to Self-Assembly and Logic Devices," University of Calgery, October, 2019.

710. "Texas-inspired Approaches to Drug Discovery," Symposium Honoring Jean-Pierre Sauvage's 75th Birthday, University of Strasbourg, October, 2019.

711. "Texas-sized Molecules as Ligands for the Lanthanide and Actinides," Baker Symposium, Cornell University, October, 2019.

712. "Calixpyrroles as Sensors and Logic Devices," 3rd Asian Conference on Chemosensors and Imaging Probes, Amritsar, India, November, 2019. This was an invited Award Lecture.

713. "Texas-inspired Approaches to Drug Discovery," Zhejiang University, Hangzhou, China, December, 2019.

714. "Calixpyrroles: From Anion Recognition to Self-Assembly and Logic Devices," Sustech, Shenzhen, China, December, 2019. This was a University Lecture.

715. "Texas-inspired Approaches to Drug Discovery," University of Oregon, January, 2019.

716. "Texas-inspired Approaches to Drug Discovery," King Abdullah University of Science and Technology, January, 2019.

717. "Texas Style Metal-based Drug Discovery," LSU, October 2020 (virtual).

718. "Expanded Porphyrins as Ligands for the Lanthanides and Early Actinides," University of Manitoba, February, 2021 (virtual).

719. "Macrocycle-based Adventures in Self-Assembly," United Arab Emirates Universities Innovation Week. February, 2021. This was a plenary lecture.

720. "Anion Pair Receptors," Am. Chem. Soc. spring meeting (virtual), April, 2021. This was an invited talk for the Inorganic Chemistry Award symposium honoring Kristin Bowman-James.

721. "Texas Style Metal-based Drug Discovery," University of British Columbia, April, 2021 (virtual).

722. "Texaphyrin Drug Conjugates," American Institute of Chemists. May 2021 (virtual). This was a Pioneer Award lecture.

723. "Aromatic, Antiaromatic, and Bicyclic Porphyrinoids," International Conference on Porphyrins and Phthalocyanines (virtual), June, 2021.

724. "Texas Style Metal-based Drug Discovery," RSC's Macrocyclic and Supramolecular Chemistry, June, 2021. This was a plenary lecture.

735. "Anion Pair Receptors," University of Oregon (in person), August, 2021.

736. "Texaphyrin-based Drug Discovery Efforts," Croucher Advanced Study Institute 2021 Metals in Biology and Medicine: From Molecular Imaging to Drug Resistance. This was a plenary lecture (virtual for meeting in Hong Kong). September, 2021.

737. "Texas-Inspired Drug Discovery Efforts," Prof. Lechosław Latos-Grażyński's 70th Birthday Symposium. This was an invited virtual lecture for a meeting held in Poland. September, 2021.

738. "Expanded Porphyrins: Studies of Systems with Biomedical Potential and Unusual Electronic Features," 31st Physical Organic Chemistry Symposium. This was a virtual plenary lecture for a meeting held online from Tsukuba, Japan. September, 2021.

739. "Texas-Inspired Drug Discovery Efforts," Royal Society of Chemistry Lecture, Australian National University (virtual). October, 2021.

740. "Texas-Inspired Drug Discovery Efforts," Nanjing University (virtual). November 2021.

741. "Biomedical Applications of Expanded Porphyrins," Pacifichem Virtual Meeting, December, 2021.

742. "Macrocycle-based Ion Pair Receptors: From Recognition to Self-Assembly and Materials Chemistry," Pacifichem Virtual Meeting, December, 2021.

743. "Texas-Inspired Drug Discovery Efforts," Université de Paris Descartes, February, 2022.

744. "Pyrrolic Macrocycles: Adventures in Self-Assembly and Functionalized Materials," Université Aix-Marseille, St. Joseph, February, 2022

745. "Texas-Inspired Drug Discovery Efforts," Université Aix-Marseille, Luminy, February, 2022.

746. "Pyrrolic Macrocycles: Adventures in Self-Assembly and Functionalized Materials," Université de Lyon, February, 2022.

747. "Texas-Inspired Drug Discovery Efforts," Université de Dijon, March, 2022

748. "Pyrrolic Macrocycles: Adventures in Self-Assembly and Functionalized Materials," École Normale Supérieure de Lyon, March, 2022.

749. "Pyrrolic Macrocycles: Adventures in Self-Assembly and Functionalized Materials," Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), March, 2022.

750. "Pyrrolic Macrocycles: Adventures in Self-Assembly and Functionalized Materials," Université de Strasbourg, March, 2022.

751. "Texas-Inspired Drug Discovery Efforts," Université de Strasbourg, March, 2022.

752. "Texas-Inspired Drug Discovery Efforts," École Normale Supérieure de Lyon, March, 2022.

753. "Macrocycle-based Ion Pair Receptors: From Recognition to Self-Assembly and Materials Chemistry," Breslow Award Lecture, ACS Spring Meeting, San Diego, CA, March, 2022.

754. "Macrocycle-based Ion Pair Receptors: From Recognition to Self-Assembly and Materials Chemistry," Washington University, St. Louis, MO, April, 2022.

755. "Texas-Inspired Drug Discovery Efforts," RISE Lecture University of Puerto Rico, Rio Peidras, San Juan, Puerto Rico, April, 2022.

756. "Macrocycle-based Ion Pair Receptors: From Recognition to Self-Assembly and Materials Chemistry," University of South Florida, Tampa, FL, April, 2022.

757. "Pyrrolic Macrocycles: Adventures in Self-Assembly and Functionalized Materials," Zhejiang University Materials Symposium. April, 2022. This was a virtual plenary lecture.

758. "Pyrrolic Macrocycles: Adventures in Self-Assembly and Functionalized Materials," University of Houston, Houston, TX, May, 2022. This was a student-invited lecture.

759. "Texas-Inspired Drug Discovery Efforts," Israeli Science Foundation Workshop on Medicinal Inorganic Chemistry, Jerusalem, Israel, June, 2022. This was a plenary lecture.

760. "Texas-Inspired Drug Discovery Efforts," International Symposium on Macrocyclic and Supramolecular Chemistry, Eugene, OR, June, 2022. Note: All lectures at this meeting were plenary, including this one.

761. "Lanthanide Texaphyrins: An Update on Drug Development Efforts," Rare Earths Research Conference, Philadelphia, PA, June, 2022. This was an invited lecture.

762. "*Meso*-free Expanded Porphyrins" International Symposium on Novel Aromaticy, Warsaw, Poland, July, 2022 (delivered by Zoom). This was an invited plenary lecture.

763. "Calixpyrroles," Calix-2022, New Orlean, LA, July, 2022 (delivered by Zoom). This was an invited plenary lecture.

764. "*Meso*-free Aromatic, Antiaromatic, and Non-topographically Planar Porphyrinoids," 12th International Conference on Porphyrins and Phthalocyanines, Madrid, Spain, July, 2022. This was an invited lecture.

765. "Texas-inspired Theranostics," Molecular Sensors and Molecular Logic Gates 2022, Dublin, Ireland, July, 2022. This was an invited plenary lecture honoring Prof. A. P. de Silva.

766. "Texas-inspired Drug Discovery Efforts," Xi'an Shiyou University, August, 2022 (delivered by Zoom).

767. "An Update on Texas-inspired Drug Discovery," Fall 2022 American Chemical Society Meeting, Chicago, IL, August, 2022. This was an invited lecture in a symposium on medicinal inorganic chemistry organized by Eszter Boros and Eric Gale.

768. "Texas-inspired Theranostics," The 2nd International Forum on Medicinal Chemistry of Natural Active Ligand Metal-Based Drugs from Chemotherapy to Chemoimmunotherapy organized by Guangxi Normal University, August, 2022 (delivered by Zoom). This was an invited plenary lecture.

769. "Texas-inspired Approaches to Drug Discovery," Pauling Symposium Honoring Cynthia Burrows, Portland, OR, October, 2022. This was an invited plenary lecture.

770. "Texas-inspired Approaches to Drug Discovery," Andrew Hamilton 70th Birthday Celebration, New York University, New York, NY, November, 2022. This was an invited plenary lecture.

771. "Studies of Meso-free Expanded Porphyrins," Dongho Kim 65th Birthday Symposium, Yonsei University, Seoul, South Korea, November, 2022. This was an invited plenary lecture.

772. "Pyrrole-based Porous Materials (and Friends)," Texas Symposium on Porous Materials, Texas A&M University, College Station, TX, December, 2022. This was an invited plenary lecture.

773. "Pyrrole-based Porous Materials (and Friends)," Macrocycle and Supramolecular Chemistry meeting of the Royal Society of Chemistry, Nottingham, UK, December, 2022. This was a plenary award lecture.

774. "Texas-inspired Approaches to Drug Discovery," Austin Community College, Austin, TX, March 2023. This was a public outreach lecture.

775. "Texas-inspired Approaches to Drug Discovery," University of Birmingham, Birmingham, UK, April, 2023.

776. "Texas-inspired Approaches to Drug Discovery," Dalton Division meeting of the Royal Society of Chemistry, Warwick, UK, April 2023. This was a plenary award lecture.

777. "Pyrrole-based Porous Materials (and Friends)," KAUST, Jeddah, Saudi Arabia, May, 2023.

778. "Texas-inspired Approaches to Drug Discovery and Sustainability," International Symposium on Sustainability, Technion, Israel, May 2023. This was a plenary lecture.

779. "Texas-inspired Approaches to Drug Discovery," University of Durham, Durham, UK, May 2023.

780. "Texas-inspired Approaches to Drug Discovery," University of Aberdeen, Aberdeen, UK, May 2023.

781. "Texas-inspired Approaches to Drug Discovery," Queens University, Belfast, UK, May 2023.

782. "Pyrrolic Macrocycles: Adventures in Self-assembly and Functionalized Materials," International Symposium on Supramolecular and Macrocyclic Chemistry, Reykjavik, Iceland, June 2023. This was a plenary lecture.

783. "Calix[4]pyrroles and Friends," Zhejiang University of Technology, July 2023. This lecture was delivered virtually.

784. "Biomedical Applications of Expanded Porphyrins," Northwestern University, July 2023. This was the first of three Ibers Memorial Lectures.

785. "Calix[4]pyrroles and Friends," Northwestern University, July 2023. This was the second of three Ibers Memorial Lectures.

786. "Adventures in Self-Assembly," Northwestern University, July 2023. This was the third of three Ibers Memorial Lectures.

787. "Pyrrole-based Porous Materials (and Friends)," Calix2023. Tel Aviv, Israel, July 2023. This was a plenary lecture.

788. "Critical Materials and Sustainability," Huazhong University of Science and Technology, Wuhan, China. August 2023. This was a six hour mini-course on the topic.

789. "Texas-inspired Drug Discovery Efforts," Zhejiang University, Hangzhou, China, August 2023

790. "Calix[4]pyrroles and Friends," International Symposium on "Smart Molecules". Henan, China. August 2023. This was a plenary lecture delivered virtually.

791. "Calix[4]pyrroles and Friends," Rathore Memorial Lecture, Marquette University, Milwaukee, Wisconsin, October 2023.

792. "Texas-inspired Drug Discovery Efforts," Texas A&M Freshman Exploration Lecture, College Station, TX, October 2023.

793. "Texas-inspired Drug Discovery Efforts," Karcher-Barton Lecture, Oklahoma University, Norman, OK, November 2023.

794. "Texas-inspired Drug Discovery Efforts," Kansas University, Lawrence, KS, November 2023.

795. "Calix[4]pyrroles and Friends," University of Rhode Island, South Kingstown, RI, November, 2023.

796. "Pyrrole-based Porous Materials," Material Research Society, Annual Meeting, Boston, 2023, November, 2023

797. "Calix[4]pyrroles and Friends," North American Supramolecular Conference, Tulane, LA, December 2023. This was a plenary lecture.

798. "Texas-inspired Drug Discovery Efforts," Asian Coordination Chemistry Conference, Bangkok, Thailand, February, 2024. This was a plenary lecture.

799. "Texas-inspired Drug Discovery Efforts," Texas Tech University, Lubbock, TX, March 2024.

800. "Expanded Porphyrin Adventures," International Symposium on Macrocyclic and Supramolecular Chemistry, Hangzhou, China, May 2024. This was the Stoddart Family Award Lecture.

801. "Calix[4]pyrrole-based Materials," Shanghai University, Shanghai, China, May 2024.

802. "Texas-inspired Drug Discovery Efforts," Tongji University, Shanghai, China, May 2024. This was the Tongji University Lecture, 2024.

803. "Calix[4]pyrrole-based Materials," Tsinghua University, Beijing, China, May 2024.

804. "Expanded Porphyrin Adventures," Peking University, Beijing, China, May 2024.

805. "Texas-inspired Drug Discovery Efforts," Shaanxi University of Science & Technology, Xi'an, China. May 2024.

806. "Texas-inspired Drug Discovery Efforts," International Conference on Porphyrins and Phthalocyanines, Niagara Falls, NY, June 2024.

807. "Receptors for Critical Materials," DOE Contractors Meeting, Gaithersburg, MD September, 2024.

808. "Texas-inspired Drug Discovery Efforts," Asian Bioinorganic Chemistry meeting, Guilin, China, December 2024. This was a plenary lecture delivered virtually.

809. "Receptors for PFAS," American Chemical Society meeting, San Diego, CA, March 2025. This was an invited lecture.

810. "Advances in Calixpyrrole Chemistry," Joint Conference on Calixarenes and Cucurbiturils, June 2025. This was a plenary lecture delivered virtually.

811. "Playing Ball with Electrons," Gordon Research Conference on Molecular Switches and Machines, July 2025. This was an invited plenary lecture.

Exhibit 3

Advanced Organic Chemistry

THIRD EDITION

Part A: Structure and Mechanisms

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4

Study and Description of Organic Reaction Mechanisms

Introduction

The chapters that follow this one will be devoted largely to the description of specific reactions. The development of a working understanding of organic chemistry requires the mastery of certain fundamental reaction types that occur in a wide variety of individual reactions. Most organic reactions occur in several steps; these steps constitute the *reaction mechanism.* Knowledge of the detailed mechanism of reactions often reveals close relationships between reactions that otherwise might appear to be unrelated. Consideration of reaction mechanism is also usually the basis for development of new transformations and improvement of existing procedures. In this chapter, the ways in which organic reactions can be studied in order to determine aspects of the reaction mechanism will be discussed. The types of experimental studies that provide data and the methods by which it is possible to develop information about reaction mechanism from the data will be considered.[1]

1. Extensive discussions of techniques for studying reaction mechanisms are presented in E. S. Lewis (ed.), *Investigation of Rates and Mechanisms of Reactions, Techniques of Organic Chemistry,* Third Edition, Vol. VI, Part I, Wiley-Interscience , New York, 1974; C. F. Bernasconi (ed.), *Investigation of Rates and Mechanisms of Reactions, Techniques of Organic Chemistry,* Fourth Edition, Vol. VI, Part I, Wiley-Interscience, New York, 1986.

4.1. Thermodynamic Data

Any reaction will have associated with it a change in enthalpy (ΔH), entropy (ΔS), and free energy (ΔG). The principles of thermodynamics assure us that ΔH, ΔS, and ΔG are independent of the reaction path. They are interrelated by the fundamental equation:

$$\Delta G = \Delta H - T\,\Delta S \tag{4.1}$$

Furthermore, the value of ΔG is related to the equilibrium constant K for the reaction:

$$\Delta G = -RT \ln K \tag{4.2}$$

Since these various quantities are characteristics of the reactants and products but are *independent* of the reaction path, they cannot provide insight into mechanisms. Information about ΔG, ΔH, and ΔS does, however, indicate the feasibility of any specific reaction. The enthalpy of a given reaction can be estimated from tabulated thermochemical data or from bond energy data such as those in Table 1.3 (p. 12). The example below indicates the use of bond energy data for estimating the enthalpy of a reaction.

Example 4.1. Calculate the enthalpy change associated with hydrogenation of butene.

$$CH_3CH{=}CHCH_3 + H_2 \longrightarrow CH_3CH_2CH_2CH_3$$

$$-\Delta H = \Sigma \text{ bond energies}_{(\text{formed})} - \Sigma \text{ bond energies}_{(\text{broken})}$$

Bonds formed:			Bonds broken:	
2	C—H	196.4	H—H	103.2
	C—C	80.5	C=C	145
		276.9		248.2

$$\Delta H = -276.9 - (-248.2) = -28.7 \text{ kcal/mol}$$

The hydrogenation is therefore calculated to be exothermic by about 29 kcal/mol.

Calculations of this type can provide only an approximate indication of the enthalpy change that is associated with a given reaction. The generalized bond energies given in Table 1.3 assume that a bond energy is independent of the structure of the rest of the molecule. This is only approximately correct, as can be judged by observing the variations in C—H and C—C bond energies as a function of structure in Part B of Table 1.3.

More accurate calculation of the thermodynamic parameters of a reaction can be done on the basis of tabulated thermochemical data. There are extensive compilations of ΔH_f° and ΔG_f° for many compounds. The subscript f designates these as, respectively, the enthalpies and free energies of formation of the compound from its constituent elements. The superscript ° is used to designate data that refer to the substance in its standard state, i.e., the pure substance at 25°C and 1 atm. The

compiled data can be used to calculate the enthalpy or free energy of a given reaction if the data are available for each reactant and product:

$$\Delta H^\circ = \Sigma \Delta H^\circ_{f_{\text{products}}} - \Sigma \Delta H^\circ_{f_{\text{reactants}}}$$

or

$$\Delta G^\circ = \Sigma \Delta G^\circ_{f_{\text{products}}} - \Sigma \Delta G^\circ_{f_{\text{reactants}}}$$

In the case of hydrogenation of 2-butane, ΔH°_f for butane (gas) is -30.15 kcal/mol, ΔH°_f for *trans*-2-butene (gas) is -2.67 kcal/mol, and ΔH°_f for H_2 is 0. Thus, ΔH° of the hydrogenation reaction at standard conditions is -27.5 kcal/mol.

If the thermodynamic data for a compound of interest have not been determined and tabulated, it may be possible to estimate ΔH_f or ΔG_f from tabulated data pertaining to individual structural fragments. Procedures have been developed for estimating thermodynamic characteristics of hydrocarbons and some derivatives by summing the contributions of the constituent groups.[2]

Estimation of the free-energy change associated with a reaction permits the calculation of the equilibrium position for a reaction and indicates the feasibility of a given chemical process. A positive ΔG° imposes a limit on the extent to which a reaction can occur. For example, as can be calculated using Eq. (4.2), a ΔG° of 1.0 kcal/mol limits conversion to product at equilibrium to 18%. An appreciably negative ΔG° indicates that the reaction is thermodynamically favorable.

Molecular orbital calculations provide another approach to obtaining estimates of thermodynamic data. The accuracy with which the various computational methods reproduce molecular energies differs. The total stabilization energies calculated even for small hydrocarbons, relative to the separate nuclei and electrons, are very large numbers (typically 50,000 and 100,000 kcal/mol for C_2 and C_4 species, respectively). This is the total energy that comes directly out of an MO calculation. The energy differences that are of principal chemical interest, such as ΔH for a reaction, are likely to be in the range of 0–50 kcal/mol. A very small error, relative to the total energy, in a MO calculation becomes a very large error in a calculated ΔH value. Fortunately, the absolute errors for compounds of similar type are likely to be comparable so that errors will cancel in calculation of the *energy differences* between two related molecules. Table 4.1 gives some calculated ΔH values for some simple reactions and the corresponding experimental values. It is clear from the variation in results among the calculations and the deviation from experimental data that the ability to produce reliable estimates depends upon the computational method that is chosen.

Calculations are frequently done on the basis of *isodesmic reactions* in order to provide for maximum cancellation of errors in the total energies. An isodesmic reaction is defined as a process in which the number of formal bonds of each type is kept constant; that is, the number of C—H, C=C, C=O, etc., bonds on each

2. G. J. Janz, *Thermodynamic Properties of Organic Compounds*, Academic Press, New York, 1967; D. R. Stull, E. F. Westrum, Jr., and G. C. Sinke, *The Chemical Thermodynamics of Organic Compounds*, John Wiley, New York, 1969.

Table 4.1. ΔH for Some Reactions Calculated by MO Methods[a]

Reaction	Calculation method					
	CNDO/2	MNDO	STO-3G	4-31G	6-31G	Exp
$CH_3{-}CH_3 + H_2 \rightarrow 2CH_4$					−22.0	−15
$CH_3CH{=}CH_2 \rightarrow$ 					+7.8	+7
$C_2H_2 + H_2 \rightarrow CH_2{=}CH_2$	−157	−42.7	−64.4	−57.3		−41
$C_2H_2 + C_2H_4 \rightarrow CH_2{=}CHCH{=}CH_2$	−203	−3.7	−57.9	−41.3		−41

a. Enthalpy given in kcal/mol. Data for CNDO/2, STO-3G, and 4-31G from T. A. Halgren, D. A. Kleier, J. H. Jr., L. D. Brown, and W. N. Lipscomb, *J. Am. Chem. Soc.* **100**, 6595 (1978); data for MNDO from M. J. S. D and G. P. Ford, *J. Am. Chem. Soc.* **101**, 5558 (1979); data for 6-31G from J. A. Pople, *J. Am. Chem. Soc* **97**, 5306 (1975).

Table 4.2. Calculated and Experimental ΔH Values for Some Isodesmic Reactions

Reaction	Calculated ΔH (4-31G)[a] (kcal/mol)	Calculated ΔH (6-31G)[b] (kcal/mol)	Experimental ΔH (kcal/mol)
$CH_3CH_2CH_3 + CH_4 \rightarrow 2CH_3CH_3$	1.0	0.8	2.6
 $+ 3CH_4 \rightarrow 2CH_3CH_3 + CH_2{=}CH_2$	−58	−50.4	−43.9
$H_2C{=}C{=}O + CH_4 \rightarrow CH_2{=}CH_2 + H_2C{=}O$	12.8	13.3	15.0
$CH_3CN + CH_4 \rightarrow CH_3CH_3 + HCN$	12.0	11.7	14.4

a. Data from W. J. Hehre, R. Ditchfield, L. Radom, and J. A. Pople, *J. Am. Chem. Soc.* **92**, 4796
b. From W. J. Hehre, L. Radom, P. v. R. Schleyer, and J. Pople. *Ab Initio Molecular Orbital Theory*, Wiley, New York, 1986, pp 299-305.

side of the equation is identical.[3] Although the reaction may not correspond to any real chemical process, the calculation can provide a test of the reliability of the computational method because of the additivity of enthalpies of formation. The "experimental" ΔH of the process can be obtained by summation of the tabulated ΔH_f° values of the participating molecules. Table 4.2 illustrates some isodesmic reactions and shows ΔH values calculated at the 4-31G and 6-31G levels.

Of the semiempirical methods, only MINDO,[4] MNDO,[5] and AM-1[6] are claimed to reliably estimate energies, and the range of reliability is open to some discussion.[7] With the *ab initio* method, the 4-31G and 6-31G basis sets achieve a level of accuracy that permits comparison of energy data. Users of MO data, however, must critically assess the reliability of the method being applied in the *particular case* under study.

Whether ΔH for a projected reaction is based on bond energy data, tabulated thermochemical data, or MO computations, there remain some fundamental prob-

3. W. J. Hehre, R. Ditchfield, L. Radom, and J. A. Pople, *J. Am. Chem. Soc.* **92**, 4796 (1970).
4. R. C. Bingham, M. J. S. Dewar, and D. H. Lo, *J. Am. Chem. Soc.* **97**, 1294 (1975).
5. M. J. S. Dewar and G. P. Ford, *J. Am. Chem. Soc.* **101**, 5558 (1979).
6. M. J. S. Dewar, E. G. Zoebisch, E. F. Healy, and J. J. P. Stewart, *J. Am. Chem. Soc.* **107**, 3902 (1985).
7. J. A. Pople, *J. Am. Chem. Soc.* **97**, 5307 (1975); T. A. Halgren, D. A. Kleier, J. H. Hall, Jr., L. D. Brown, and W. L. Lipscomb, *J. Am. Chem. Soc.* **100**, 6595 (1978); M. J. S. Dewar and D. M. Storch, *J. Am. Chem. Soc.* **107**, 3898 (1985).

lems which prevent a final conclusion about a reaction's feasibility. In the first place, most reactions of interest occur in solution, and the enthalpy, entropy, and free energy associated with any reaction depend strongly on the solvent medium. There is only a limited amount of tabulated thermochemical data that is directly suitable for treatment of reactions in organic solvents. Molecular orbital calculations usually refer to the isolated (gas phase) molecule. Estimates of solvation effects must be made in order to apply either experimental or theoretical data to reactions occurring in solution.

There is an even more basic limitation to the usefulness of thermodynamic data for making predictions about reactions: Thermodynamics provides no information about the energy requirements of the pathways that a potential reaction can follow; that is, thermodynamics provides no information about the *rates of chemical reactions*. In the absence of a relatively low-energy pathway, two molecules that can potentially undergo a highly favorable reaction will coexist without reacting. Thus, even if a reaction is thermodynamically feasible, it may not occur at a significant rate. It is therefore extremely important to develop an understanding of reaction mechanisms and the energy requirements and rates of the various steps by which organic reactions proceed.

4.2. Kinetic Data

Kinetic data are capable of providing much detailed insight into reaction mechanisms. The rate of a given reaction can be determined by following the disappearance of a reactant or the appearance of product. The extent of reaction is often measured spectroscopically, since spectroscopic techniques provide a rapid, continuous means of monitoring changes in concentration. Numerous other methods are available, however, and may be preferable in certain cases. For example, continuous pH measurement or acid–base titration can be used to follow the course of reactions that consume or generate acid or base. Conductance measurements provide a means for determining the rate or reactions that generate ionic species; polarimetry is a convenient way of following reactions involving optically active materials. In general, any property that can be measured and related to the concentration of a reactant or product can be used to determine a reaction rate.

The goal of a kinetic study is to establish the quantitative relationship between the concentration of reactants and catalysts and the rate of the reaction. Typically, such a study involves rate measurements at enough different concentrations of each reactant so that the *kinetic order* with respect to each reactant can be assessed. A complete investigation allows the reaction to be described by a rate law, which is an algebraic expression containing one or more *rate constants* as well as the concentrations of all reactant species that are involved in the rate-determining step and steps prior to the rate-determining step. In the rate law, each concentration has an exponent that is the order of the reaction with respect to that component. The overall kinetic order of the reaction is equal to the sum of all the exponents in the

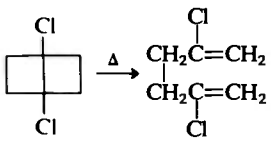
rate expression. Several examples of rate laws which illustrate the variety observed are presented in Scheme 4.1. Some are simple; others are more complex.

The relationship between a kinetic expression and a reaction mechanism can be appreciated by considering the several individual steps that constitute the overall reaction mechanism. The expression for the rate of any *single step* in a reaction mechanism will contain a term for the concentration of each reacting species. Thus, for the reaction sequence

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \xrightarrow{k_2} D \xrightarrow{k_3} E + F$$

Scheme 4.1. Some Representative Rate Laws

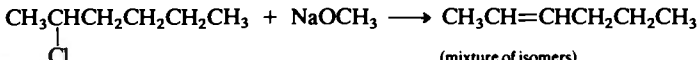
1[a]



A

rate = k[**A**]

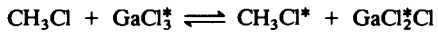
2[b]



B

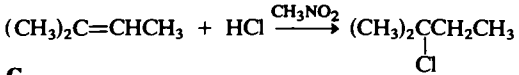
rate = k[**B**][$NaOCH_3$]

3[c]



rate = $k[CH_3Cl][GaCl_3^*]^2$

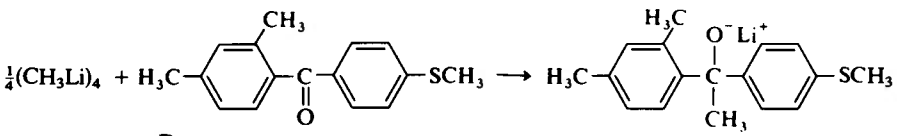
4[d]



C

rate = k[**C**][HCl]^2

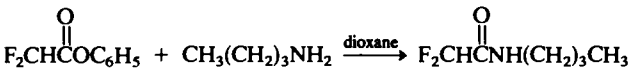
5[e]



D

rate = $k[(CH_3Li)_4]^{1/4}$[**D**]

6[f]



E **F**

rate = k_1[**E**][**F**] + k_2[**E**][**F**]^2

a. E. N. Cain and R. K. Solly, *J. Am. Chem. Soc.* **95**, 7884 (1973).
b. R. A. Bartsch and J. F. Bunnett, *J. Am. Chem. Soc.* **90**, 408 (1968).
c. F. P. DeHaan, H. C. Brown, D. C. Conway, and M. G. Gibby, *J. Am. Chem. Soc.* **91**, 4854•(1969).
d. Y. Pocker, K. D. Stevens, and J. J. Champoux, *J. Am. Chem. Soc.* **91**, 4199 (1969).
e. S. G. Smith, L. F. Charbonneau, D. P. Novak, and T. L. Brown, *J. Am. Chem. Soc.* **94**, 7059 (1972).
f. A. S. A. S. Shawali and S. S. Biechler, *J. Am. Chem. Soc.* **89**, 3020 (1967).

the rates for the successive steps are

$$\text{step 1:} \quad \frac{d[C]}{dt} = k_1[A][B] - k_{-1}[C]$$

$$\text{step 2:} \quad \frac{d[D]}{dt} = k_2[C]$$

$$\text{step 3:} \quad \frac{d[E]}{dt} = \frac{d[F]}{dt} = k_3[D]$$

Let us further specify that the first step is a very rapid but unfavorable equilibrium, and that $k_2 \ll k_3$, i.e., that the second step is slow relative to the third step. Under these circumstances, the overall rate of the reaction will depend on the rate of the second step, and the second step is called the *rate-determining step.*

Kinetic data provide information only about the rate-determining step and steps preceding it. In the hypothetical reaction under consideration, the final step follows the rate-determining step, and since its rate will not affect the rate of the overall reaction, k_3 will not appear in the overall rate expression. The rate of the overall reaction is governed by the second step, which is the bottleneck in the process. The rate of this step is equal to k_2 multiplied by the molar concentration of intermediate C, which may not be directly measurable. It is therefore necessary to express the rate in terms of the concentration of reactants. In the case under consideration, this can be done by recognizing that [C] is related to [A] and [B] by an equilibrium constant

$$K = \frac{[C]}{[A][B]}$$

Furthermore, K is related to k_1 and k_{-1} by the requirement that no net change in composition occur at equilibrium.

$$k_{-1}[C] = k_1[A][B]$$

$$[C] = \frac{k_1}{k_{-1}}[A][B]$$

The rate of step 2 can therefore be written in terms of [A] and [B]:

$$\frac{d[D]}{dt} = k_2[C] = k_2\frac{k_1}{k_{-1}}[A][B] = k_{obs}[A][B]$$

Experimentally, it would be observed that the reaction rate would be proportional to both [A] and [B]. Kinetic data are normally handled using the integrated forms of the differential equations. The integrated rate equations for simple first-order and second-order reactions are

$$\text{first-order reaction:} \quad k = \frac{1}{t}\ln\left(\frac{c_0}{c}\right)$$

$$\text{second-order reaction:} \quad k = \frac{1}{t(a_0 - b_0)}\ln\frac{b_0(a)}{a_0(b)}$$

where a, b, and c refer to concentrations of reactants at time t, and a_0, b_0, and c_0 to initial concentrations. As reaction mechanisms become more complex, the mathematical form of their rate expressions and their solutions become increasingly complex. For the simple rate expressions, graphical or numerical analysis of the data directly provides a value for the appropriate rate constant. More complex rate expressions may be analyzed by a variety of graphical or analytical techniques, in conjunction with sufficient changes in the reactant concentrations and other variables to determine the kinetic expression and rate constants.

Most organic reactions involve more than one step. It is therefore necessary to consider the kinetic expressions that arise from some of the more important cases of multistep reactions. There may be a rapid equilibrium preceding the rate-determining step. Such a mechanism may operate, for example, in the reaction of an alcohol with hydrobromic acid to give an alkyl bromide:

$$\mathrm{ROH} + \mathrm{H^+} \underset{k_{-1}}{\overset{\text{fast } k_1}{\rightleftharpoons}} \mathrm{R\dot{O}H_2}$$

$$\mathrm{R\dot{O}H_2} + \mathrm{Br^-} \xrightarrow[k_2]{\text{slow}} \mathrm{RBr} + \mathrm{H_2O}$$

The overall rate being measured is that of step 2, but there may be no means of directly measuring $[\mathrm{ROH_2^+}]$. The concentration of the protonated intermediate $\mathrm{ROH_2^+}$ can be expressed in terms of the concentration of the starting material by taking into consideration the equilibrium constant, which relates [ROH], $[\mathrm{Br^-}]$, and $[\mathrm{H^+}]$:

$$K = \frac{[\mathrm{ROH_2^+}]}{[\mathrm{ROH}][\mathrm{H^+}]}$$

$$[\mathrm{ROH_2^+}] = K[\mathrm{ROH}][\mathrm{H^+}]$$

$$\text{rate} = k_2K[\mathrm{ROH}][\mathrm{H^+}][\mathrm{Br^-}] = k_{\text{obs}}[\mathrm{ROH}][\mathrm{H^+}][\mathrm{Br^-}]$$

A useful approach that is often used in analysis and simplification of kinetic expressions is the *steady-state approximation.* It can be illustrated with a hypothetical reaction scheme:

$$\mathrm{A} + \mathrm{B} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{C}$$

$$\mathrm{C} + \mathrm{D} \xrightarrow{k_2} \mathrm{E} + \mathrm{F}$$

$$\overline{\mathrm{A} + \mathrm{B} + \mathrm{D} \longrightarrow \mathrm{E} + \mathrm{F}}$$

If C is a reactive, unstable species, its concentration will never be very large. It must then be consumed at a rate that closely approximates the rate at which it is formed. Under these conditions, it is a valid approximation to set the rate of formation of C equal to its rate of destruction:

$$k_1[\mathrm{A}][\mathrm{B}] = k_2[\mathrm{C}][\mathrm{D}] + k_{-1}[\mathrm{C}]$$

Rearrangement of this equation provides an expression for [C]:

$$\frac{k_1[A][B]}{k_2[D] + k_{-1}} = [C]$$

By substituting into the rate for the second step, the following expression is obtained:

$$\text{rate} = k_2[C][D] = k_2 \frac{k_1[A][B]}{k_2[D] + k_{-1}}[D]$$

If $k_2[D]$ is much greater than k_{-1}, the rate expression simplifies to

$$\text{rate} = \frac{k_2 k_1[A][B][D]}{k_2[D]} = k_1[A][B]$$

On the other hand, if $k_2[D]$ is much less than k_{-1}, the observed rate expression becomes

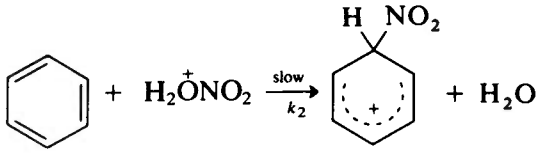
$$\text{rate} = \frac{k_1 k_2[A][B][D]}{k_{-1}}$$

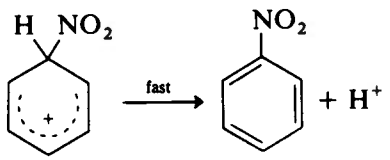
The first situation corresponds to the first step being rate-determining. In the second case, it is the second step that is rate-determining, with the first step being a preequilibrium.

The normal course of a kinetic investigation involves the postulation of likely mechanisms and comparison of the observed rate law with those expected for the various mechanisms. Those mechanisms that are incompatible with the observed kinetics can be eliminated as possibilities. Let us consider aromatic nitration by nitric acid in an inert solvent as a typical example and restrict the mechanisms being considered to the three shown below. In an actual case, such arbitrary restriction would not be imposed, but instead all mechanisms compatible with existing information would be considered.

(A)

$$2\,HONO_2 \underset{k_{-1}}{\overset{k_1}{\underset{\text{fast}}{\rightleftharpoons}}} H_2\overset{+}{O}NO_2 + NO_3^-$$





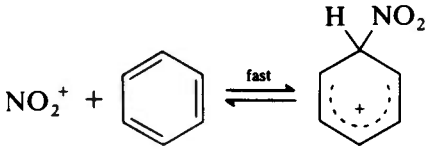
$$\text{rate} = k_2[H_2\overset{+}{O}NO_2][\text{benzene}] = \frac{k_2 k_1}{k_{-1}} \frac{[HONO_2]^2}{[NO_3^-]}[\text{benzene}]$$

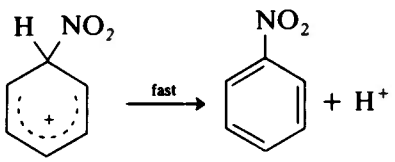
$$= k_{\text{obs}} \frac{[HONO_2]^2}{[NO_3^-]}[\text{benzene}]$$

(B)

$$2\,HONO_2 \underset{k_{-1}}{\overset{\text{fast } k_1}{\rightleftharpoons}} H_2\overset{+}{O}NO_2 + NO_3^-$$

$$H_2\overset{+}{O}NO_2 \xrightarrow[k_2]{\text{slow}} H_2O + NO_2^+$$

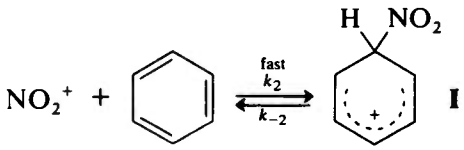


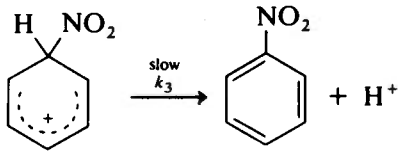


$$\text{rate} = \frac{k_1k_2}{k_{-1}}\frac{[HONO_2]^2}{[NO_3^-]} = k_{obs}\frac{[HONO_2]^2}{[NO_3^-]}$$

(C)

$$2\,HONO_2 \underset{k_{-1}}{\overset{\text{fast } k_1}{\rightleftharpoons}} NO_2^+ + NO_3^- + H_2O$$





The third step is rate-controlling, so

$$\text{rate} = k_3[\mathbf{I}]$$

[I] can be expressed in terms of the rapid equilibria involved in its formation:

$$k_{-2}[\mathbf{I}] = k_2[NO_2^+][\text{benzene}]$$

$$[NO_2^+] = \frac{k_1[HNO_3]^2}{k_{-1}[NO_3^-][H_2O]}$$

$$\text{rate} = k_3\frac{k_2[\text{benzene}]k_1[HNO_3]^2}{k_{-2}k_{-1}[NO_3^-][H_2O]}$$

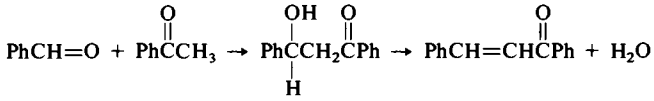
$$\text{rate} = \frac{k_{obs}[HNO_3]^2[\text{benzene}]}{[NO_3^-][H_2O]}$$

$$= k_{obs}\frac{[HNO_3]^2[\text{benzene}]}{[NO_3^-]} \qquad \text{if } [H_2O] \gg [\text{benzene}]$$

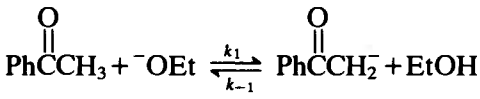
Mechanism B has the distinctive feature that it is zero-order in the reactant benzene, since the rate-determining step occurs prior to the involvement of benzene. Mechanism B has, in fact, been established for nitration of benzene in several organic solvents, and the absence of a benzene concentration term in the rate law is an important part of the evidence for this mechanism.[8]

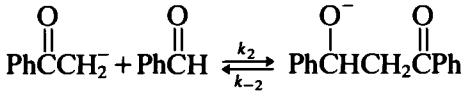
Mechanisms A and C, on the other hand, provide kinetic expressions that are rather similar in form, differing only in the inclusion of water in the expression for mechanism C. This might not be a detectable difference. If the concentration of water is several times larger than that of benzene, its overall concentration will change little during the course of the reaction. In this circumstance, the term for the concentration of water would disappear (by becoming a component of the observed rate constant k) so that the form of the kinetic expression alone would not distinguish between mechanisms A and C.

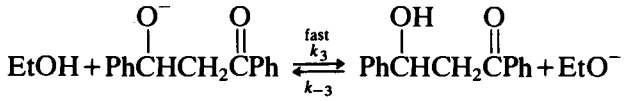
To illustrate the development of a kinetic expression from a postulated reaction mechanism, let us consider the base-catalyzed reaction of benzaldehyde and acetophenone.

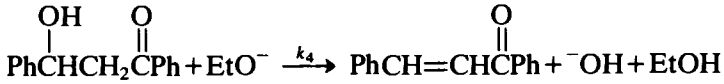


Based on general knowledge of base-catalyzed reactions of carbonyl compounds, a reasonable sequence of steps can be written, but the relative rates of the steps are an open question. Furthermore, it is known that reactions of this type are generally reversible so that the potential reversibility of each step must be taken into account. A completely reversible mechanism is as follows:

(1) 

(2) 

(3) 

(4) 

8. J. H. Ridd, *Acc. Chem. Res.* **4**, 248 (1971); J. H. Ridd, in *Studies on Chemical Structure and Reactivity*, J. H. Ridd (ed.), John Wiley and Sons, New York, 1966, Chapter 7; J. G. Hoggett, R. B. Moodie, J. R. Penton, and K. Schofield, *Nitrogen and Aromatic Reactivity*, Cambridge University Press, Cambridge, 1971; K. Schofield, *Aromatic Nitration*, Cambridge University Press, Cambridge, 1980.

Since proton transfer reactions between oxygen atoms are usually very fast, step 3 can be assumed to be a rapid equilibrium. With the above mechanism assumed, let us examine the rate expressions that would result, depending upon which of the steps is rate-determining.

If step 1 is rate-controlling, the rate expression would be

$$\text{rate} = k_1[\text{PhCOCH}_3][^-\text{OEt}]$$

Under these conditions, the concentration of the second reactant, benzaldehyde, would not enter into the rate expression.

If step 1 is an equilibrium and step 2 is rate-controlling, we obtain the rate expression

$$\text{rate} = k_2[\text{PhCOCH}_2^-][\text{PhCHO}]$$

which on substituting in terms of the rapid prior equilibrium gives

$$\text{rate} = k_2K_1[\text{PhCOCH}_3][^-\text{OEt}][\text{PhCHO}]$$

since

$$[\text{PhCOCH}_2^-] = K_1[\text{PhCOCH}_3][^-\text{OEt}]$$

where K_1 is the equilibrium constant for the deprotonation in the first step.

If the final step is rate-controlling, the rate is

$$\text{rate} = k_4[^-\text{OEt}][\text{PhCH(OH)CH}_2\text{COPh}]$$

The concentration of the intermediate

$$\text{PhCH(OH)CH}_2\text{COPh}$$

can be expressed in terms of the three prior equilibria. Using **I** for the intermediate and $\mathbf{I}^-$ for its conjugate base and neglecting [EtOH], since EtOH is the solvent and its concentration will remain constant, gives the relationships:

$$K_3 = \frac{[\mathbf{I}][^-\text{OEt}]}{[\mathbf{I}^-]} \qquad \text{and} \qquad [\mathbf{I}] = K_3\frac{[\mathbf{I}^-]}{[^-\text{OEt}]}$$

and, since $[\mathbf{I}^-] = K_2[\text{PhCOCH}_2^-][\text{PhCHO}]$, substituting for $[\mathbf{I}^-]$ gives

$$[\mathbf{I}] = K_3\frac{K_2[\text{PhCOCH}_2^-][\text{PhCHO}]}{[^-\text{OEt}]}$$

Substituting for $[\text{PhCOCH}_2^-]$ from the equilibrium expression for step 1 gives

$$[\mathbf{I}] = \frac{K_3K_2[\text{PhCHO}]}{[^-\text{OEt}]}K_1[\text{PhCOCH}_3][^-\text{OEt}] = K'[\text{PhCHO}][\text{PhCOCH}_3]$$

and this provides the final rate expression

$$\text{rate} = k_{\text{obs}}[^-\text{OEt}][\text{PhCHO}][\text{PhCOCH}_3]$$

The form of this third-order kinetic expression is identical to that in the case where the second step is rate-determining.

Experimental studies of this base-catalyzed condensation have revealed that it is third-order, indicating that either the second or fourth step must be rate-determining. Studies on the intermediate **I** obtained by an alternative synthesis have shown that k_4 is about four times as large as k_{-3} so that about 80% of the intermediate goes on to product. These reactions are faster than the overall reaction under the same conditions, so the second step must be rate-controlling.[9]

These examples illustrate the relationship between kinetic results and the determination of reaction mechanism. Kinetic results can exclude from consideration all mechanisms that require a rate law different from the observed one. It is often true, however, that related mechanisms give rise to identical predicted rate expressions. In this case, the mechanisms are "kinetically equivalent," and a choice between them is not possible on the basis of kinetic data. A further limitation on the information that kinetic studies provide should also be recognized. Although the data can give the *composition* of the activated complex for the rate-determining step and preceding steps, they provide no information about the *structure* of the intermediate. Sometimes the structure can be inferred from related chemical experience, but it is never established by kinetic data alone.

The nature of the rate constants k_r can be discussed in terms of *transition state theory*. This is a general theory for analyzing the energetic and entropic components of a reaction process. In transition state theory, a reaction is assumed to involve the attainment of an activated complex that goes on to product at an extremely rapid rate. The rate of decomposition of the activated complex has been calculated from the assumptions of the theory to be $6 \times 10^{12}\ s^{-1}$ at room temperature and is given by the expression[10]

$$\text{rate of activated complex decomposition} = \frac{\kappa kT}{h} \tag{4.3}$$

in which κ is the transmission coefficient, which is usually taken to be 1, k is Boltzmann's constant, h is Planck's constant, and T is absolute temperature.

$$\text{rate of reaction} = \frac{\kappa kT}{h}[\text{activated complex}]$$

If the activated complex is considered to be in equilibrium with its component molecules, the attainment of the transition state (T.S.) can be treated as being

9. E. Coombs and D. P. Evans, *J. Chem. Soc.*, 1295 (1940); D. S. Noyce, W. A. Pryor, and A. H. Bottini, *J. Am. Chem. Soc.* 77, 1402 (1955).
10. For a complete development of these relationships, see M. Boudart, *Kinetics of Chemical Processes*, Prentice-Hall, Englewood Cliffs, New Jersey, 1968, pp. 35-46; I. Amdur and G. G. Hammes, *Chemical Kinetics, Principles and Selected Topics*, McGraw-Hill, New York, 1966, pp. 43-58; J. W. Moore and R. G. Pearson, *Kinetics and Mechanism*, Wiley, New York, 1981, pp. 159-169; M. M. Kreevoy and D. G. Truhlar, in *Investigation of Rates and Mechanisms of Reactions*, C. F. Bernasconi (ed.), *Techniques of Chemistry*, Fourth Edition, Vol. VI, Part 1, Wiley-Interscience, New York, 1986.

analogous to a bimolecular reaction:

$$A + B \rightarrow C$$

$$A + B \rightleftharpoons T.S. \rightarrow C$$

$$K^{+} = \frac{[T.S.]}{[A][B]}$$

The position of this equilibrium is related to the free energy required for attainment of the transition state. The superscript sign ($^{+}$) is used to specify that it is a process involving a transition state or "activated complex" that is under discussion:

$$\Delta G^{+} = -RT \ln K^{+}$$

This free energy is referred to as the *free energy of activation.* The rate of the reaction is then given by

$$\text{rate} = \frac{\kappa kT}{h}[T.S.]$$

$$[T.S.] = K^{+}[A][B]$$

Since

$$K^{+} = e^{-\Delta G^{+}/RT}$$

$$\text{rate} = \frac{\kappa kT}{h} e^{-\Delta G^{+}/RT}[A][B] \tag{4.4}$$

Comparison with the form of the expression for the rate of any single reaction step:

$$\text{rate} = k_r[A][B]$$

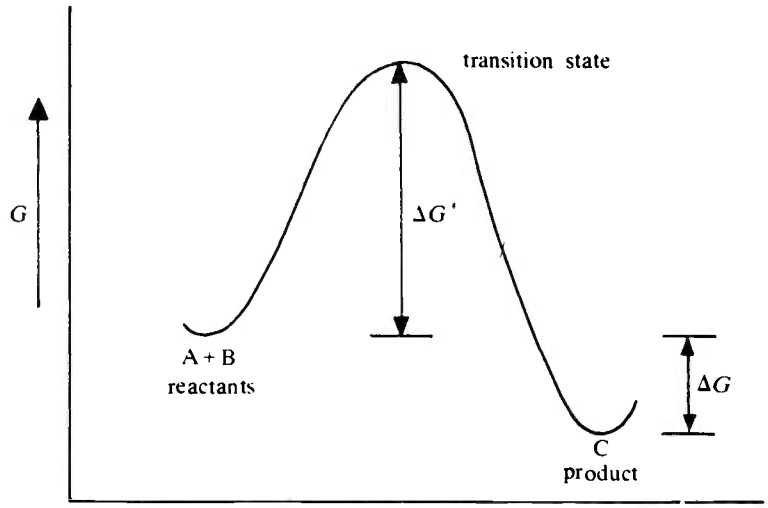
reveals that the magnitude of ΔG^{+} will be the factor that determines the magnitude of k_r at any given temperature.

Qualitative features of reaction mechanisms are often described in the context of transition state theory and illustrated with potential energy diagrams. The potential energy diagrams for a hypothetical one-step bimolecular reaction and for a two-step reaction are shown in Fig. 4.1. The lower diagram depicts a two-step reaction in which an intermediate having a finite lifetime is involved. Two transition states are then involved. The higher activation energy of the first transition state implies that the first step would be slower and therefore rate-determining. These two-dimensional diagrams are useful devices for qualitative discussion of reaction mechanisms. The line plots the free energy of the reaction complex as it progresses along the reaction coordinate from reactants to products.

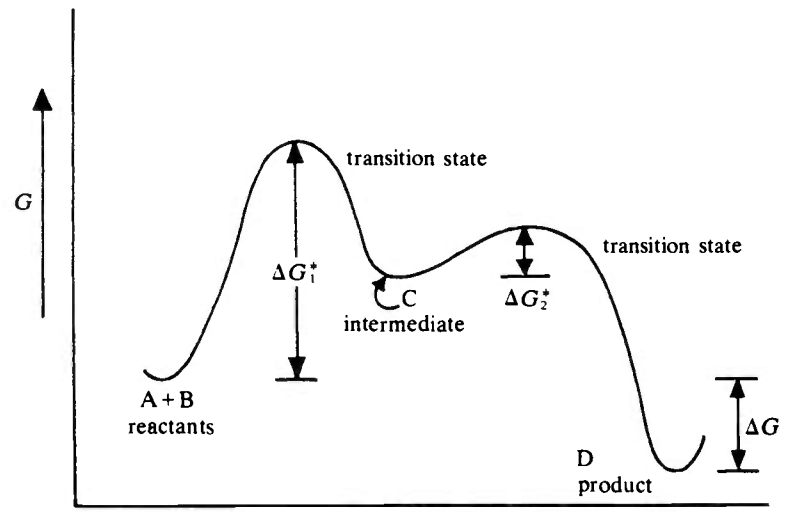
Such diagrams make clear the difference between an intermediate and a transition state. An intermediate lies in a depression on the potential energy curve. Thus, it will have a finite lifetime. The actual lifetime will depend on the depth of the depression. A shallow depression implies a low activation energy for the subsequent step, and therefore a short lifetime. The deeper the depression, the longer will be the lifetime of the intermediate. The situation at a transition state is quite different.

It has only fleeting existence and represents an energy maximum on the reaction path.

There is one path between reactants and products that has a lower energy maximum than any other; this is the pathway that the reaction will follow. The line in a two-dimensional potential energy plot represents this lowest-energy pathway. It represents a path across an energy surface describing energy as a function of the spatial arrangement of the atoms involved in the reaction. The *principle of microscopic reversibility* arises directly from transition state theory. *The same pathway that is traveled in the forward direction of a reaction will be traveled in the reverse direction,*



Reaction coordinate for a single-step reaction



Reaction coordinate for a two-step reaction

Fig. 4.1. Potential energy diagrams for single-step and two-step reactions.

since it affords the lowest energy barrier for either process. Thus, information about the nature of a transition state or intermediate deduced by a study of a forward reaction is applicable to the discussion of the reverse process occurring under the same conditions.

Since transition states cannot be observed, there is no experimental method for establishing their structures. In recent years, theoretical descriptions of molecules have been applied to this problem. By applying one of the MO methods, structures can be calculated for successive geometries which gradually transform the reactants into products. Exploration of a range of potential geometries and calculation of the energy of the resulting ensembles can, in principle, locate and describe the minimum-energy pathway. To the extent that the calculations accurately reflect the molecular reality, this provides a structural description of the reaction path and transition state.

The temperature dependence of reaction rates permits evaluation of the enthalpy and entropy components of the free energy of activation. The terms in Eq. (4.4) corresponding to k_r can be expressed as

$$k_r = \frac{\kappa kT}{h}(e^{-\Delta H^{\ddagger}/RT})(e^{\Delta S^{\ddagger}/R}) \tag{4.5}$$

The term $(\kappa kT/h)\, e^{\Delta S^{\ddagger}/R}$ varies only slightly with T compared to $e^{-\Delta H^{\ddagger}/RT}$ because of the exponential nature of the latter. To a good approximation, then

$$\frac{k_r}{T} = C\, e^{-\Delta H^{\ddagger}/RT} \tag{4.6}$$

$$\ln \frac{k_r}{T} = \frac{-\Delta H^{\ddagger}}{RT} + C' \tag{4.7}$$

A plot of $\ln (k_r/T)$ versus $(1/T)$ is then a straight line, and its slope is $-\Delta H^{\ddagger}/R$. Once $\Delta H^{\ddagger}$ is determined in this manner, $\Delta S^{\ddagger}$ is available from the relationship

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger}}{T} + R \ln \frac{hk_r}{\kappa kT} \tag{4.8}$$

which can be obtained by rearranging Eq. (4.5).

The temperature dependence of reactions can also be expressed in terms of the Arrhenius equation

$$k_r = A\, e^{-E_a/RT} \tag{4.9}$$

$$\ln k_r = -E_a/RT + \ln A \tag{4.10}$$

Comparison of the form of Eq. (4.9) with Eq. (4.5) indicates that A in the Arrhenius equation corresponds to $(\kappa kT/h)e^{\Delta S^{\ddagger}/R}$. The Arrhenius equation shows that a plot of $\ln k_r$ versus $1/T$ will have the slope $-E_a/R$.[11] For reactions in solution at constant pressure, $\Delta H^{\ddagger}$ and E_a are related by

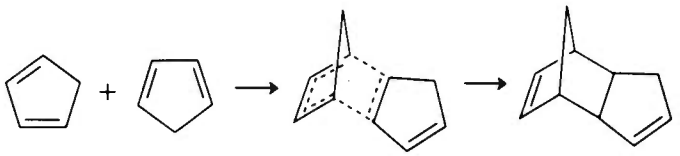
$$E_a = \Delta H^{\ddagger} + RT \tag{4.11}$$

11. For full consideration of the relationship between Eqs. (4.5) and (4.9), see I. Amdur and G. G. Hammes, *Chemical Kinetics, Principles and Selected Topics*, McGraw-Hill, New York, 1966, pp. 53–58.

The magnitudes of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ reflect transition state structure. Atomic positions in the transition state do not correspond to those in the ground state. In particular, the reacting bonds will be partially formed and partially broken. This is reflected in the higher energy content of the activated complex and corresponds to the enthalpy of activation, $\Delta H^{\ddagger}$. The entropy of activation is a measure of the degree of order produced in the formation of the activated complex. If translational, vibrational, or rotational degrees of freedom are lost in going to the transition state, there will be a decrease in the total entropy of the system. Conversely, an increase of translational, vibrational, or rotational degrees of freedom will result in a positive entropy of activation.

Wide variation in enthalpy and entropy of activation for different reaction systems is possible, as illustrated by the following two reactions.

Dimerization of cyclopentadiene[12]:



gas phase $\Delta H^{\ddagger} = 15.5$ kcal/mol

$\Delta S^{\ddagger} = -34$ eu

Decomposition of 1,1′-azobutane[13]:

$$\text{Bu—N=N—Bu} \rightarrow 2\text{Bu}\cdot + \text{N}_2$$

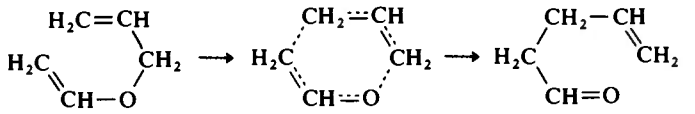
gas phase $\Delta H^{\ddagger} = 52$ kcal/mol

$\Delta S^{\ddagger} = +19$ eu

The relatively low $\Delta H^{\ddagger}$ term for the dimerization of cyclopentadiene is characteristic of concerted reactions (see Chapter 11), in which bond making accompanies bond breaking. It differs markedly from $\Delta H^{\ddagger}$ for the thermal decomposition of 1,1′-azobutane, in which the rate-determining step is a homolytic cleavage of a C—N bond, with little new bond making to compensate for the energy cost of the bond breaking. The entropy of activation, on the other hand, is more favorable in the 1,1′-azobutane decomposition, since a translational degree of freedom is being gained in the transition state as the molecular fragments separate. The dimerization of cyclopentadiene is accompanied by a very negative entropy of activation because of the loss of translational and rotational degrees of freedom. The two reacting molecules must attain a specific geometry to permit the bonding interactions that occur as the transition state is approached.

12. A. Wassermann, *Monatsh. Chem.* **83**, 543 (1952).
13. A. U. Blackham and N. L. Eatough, *J. Am. Chem. Soc.* **84**, 2922 (1962).

Unimolecular reactions that take place by way of cyclic transition states typically have negative entropies of activation because of the loss of rotational degrees of freedom associated with the highly ordered transition state. For example, thermal isomerization of allyl vinyl ether to 4-pentenal has $\Delta S^{\ddagger} = -8$ eu.[14]



It is important to remember that the enthalpy and entropy of activation reflect the response of the reacting system *as a whole* to formation of the activated complex. As a result, the interpretation of these parameters is more complicated for reactions taking place in solution than for gas phase reactions. This complexity is particularly true for processes involving formation or destruction of charged species. The solvolysis of *t*-butyl chloride in 80% aqueous ethanol, for example, has as its rate-determining step unimolecular ionization of the carbon-chlorine bond to form chloride ion and the *t*-butyl cation. One might guess that this ionization should lead to a positive entropy of activation, since two particles are being generated. In fact, the entropy of activation is −6.6 eu. Because of its polar character, the transition state requires a greater ordering of solvent molecules than the nonpolar reactant.[15] It turns out to be generally true that reactions which generate charged species exhibit negative entropies of activation in solution. The reverse is true for reactions in which charged reactants lead to a neutral transition state.

4.3. Substituent Effects and Linear Free-Energy Relationships

In Chapter 1, Section 1.2 (p. 16), the effect of substituent groups on the acid strength of acetic acid derivatives was discussed qualitatively. It was noted in particular that the presence of groups more electronegative than hydrogen increased the acid strength relative to acetic acid. A number of important relationships between substituent groups and chemical properties have been developed. In many cases, such relationships can be expressed quantitatively and are useful both for interpretation of reaction mechanisms and for prediction of reaction rates and equilibria.

The most widely applied of these relationships is the *Hammett equation* which relates rates and equilibria for many reactions of compounds containing substituted phenyl groups. It was noted in the 1930s that there is a relationship between the acid strengths of substituted benzoic acids and the rates of many other chemical reactions, for instance, the rates of hydrolysis of substituted ethyl benzoates. The correlation is illustrated graphically in Fig. 4.2, which shows $\log k/k_0$, where k_0 is the rate constant for hydrolysis of ethyl benzoate and k is the rate constant for the

14. F. W. Schuler and G. W. Murphy, *J. Am. Chem. Soc.* **72**, 3155 (1950).
15. E. Grunwald and S. Winstein, *J. Am. Chem. Soc.* **70**, 846 (1948).

substituted esters, plotted against $\log K/K_0$, where K and K_0 are the corresponding acid dissociation constants. Analogous plots for many other reactions of aromatic compounds show a similar linear correlation with the acid dissociation constants of the corresponding benzoic acids. Neither the principles of thermodynamics nor theories of reaction rates predict or require that there should be such linear relationships. There are, in fact, numerous reaction series that fail to show such correlations. Some insight into the origin of the correlation can be gained by considering the relationship between the correlation equation and the free-energy changes involved in the two processes. The line in Fig. 4.2 defines the following equation, in which m is the slope of the line:

$$m \log \frac{K}{K_0} = \log \frac{k}{k_0} \tag{4.12}$$

Substituting for K and k with the appropriate free energy or free energy of activation:

$$m(\log K - \log K_0) = \log k - \log k_0$$

$$m(-\Delta G/2.3RT + \Delta G_0/2.3RT) = -\Delta G^{\ddagger}/2.3RT + \Delta G_0^{\ddagger}/2.3RT$$

$$m(-\Delta G + \Delta G_0) = -\Delta G^{\ddagger} + \Delta G_0^{\ddagger}$$

$$m\Delta\Delta G = \Delta\Delta G^{\ddagger} \tag{4.13}$$

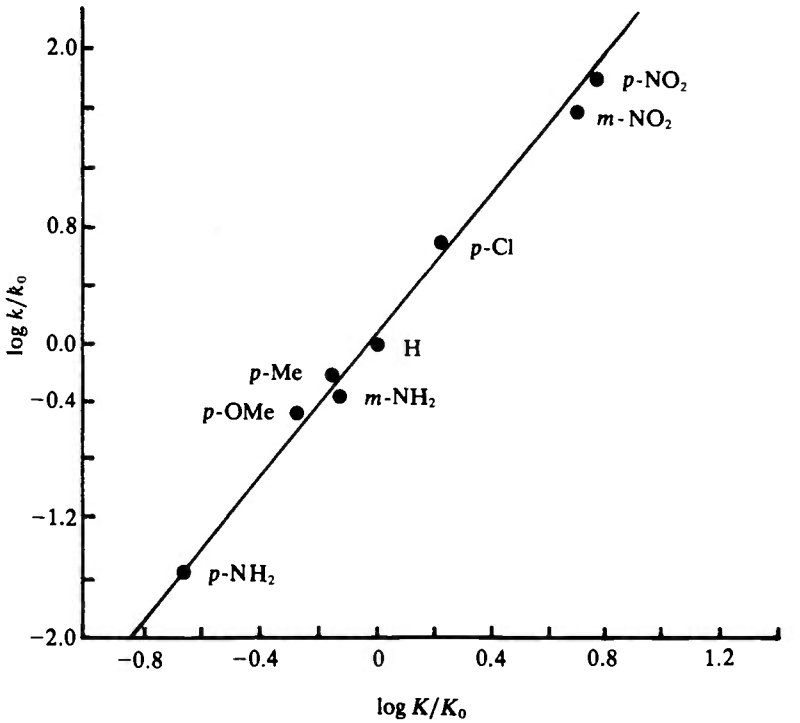


Fig. 4.2. Correlation of acid dissociation constants of benzoic acids with rates of alkaline hydrolysis of ethyl benzoates. [From L. P. Hammett, *J. Am. Chem. Soc.* **59**, 96 (1937).]

The linear correlation therefore indicates that the change in the free energy of activation for hydrolysis of ethyl benzoate on introduction of a series of substituent groups is *directly proportional* to the change in the free energy of ionization that is caused by the same series of substituents on benzoic acid. The various correlations arising from such directly proportional changes in free energies are called *linear free-energy relationships*.

Since ΔG and $\Delta G^{\ddagger}$ are combinations of enthalpy and entropy terms, a linear free-energy relationship between two reaction series can result from one of three circumstances: (1) ΔH is constant and the ΔS terms are proportional for the two series, (2) ΔS is constant and the ΔH terms are proportional, or (3) ΔH and ΔS are linearly related. Dissection of the free-energy changes into enthalpy and entropy components has often shown the third case to be true.[16]

The Hammett free-energy relationship is expressed in the following equations for equilibria and for rate data, respectively:

$$\log \frac{K}{K_0} = \sigma\rho \tag{4.14}$$

$$\log \frac{k}{k_0} = \sigma\rho \tag{4.15}$$

The numerical values of the terms σ and ρ are defined by selection of the reference reaction, the ionization of benzoic acids. This reaction is arbitrarily assigned a value of the *reaction constant*, ρ, of 1. The *substituent constant*, σ can then be determined for a series of substituent groups by measurement of the acid dissociation constant of the substituted benzoic acids. The σ values so defined are used in the correlation of other reaction series, and the ρ values of the reactions are thus determined.

The relationship between Eqs. (4.12) and (4.14) is evident when the Hammett equation is expressed in terms of free energy. For the standard reaction, $\log [K/K_0] = \sigma\rho$:

$$-\Delta G/2.3RT + \Delta G_0/2.3RT = \sigma\rho = \sigma$$

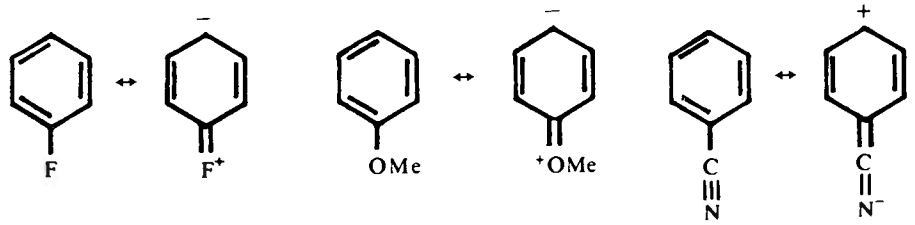
since $\rho = 1$ for the standard reaction. Substituting into Eq. (4.12):

$$\begin{aligned} m\sigma &= -\Delta G^{\ddagger}/2.3RT + \Delta G_0^{\ddagger}/2.3RT \\ m\sigma &= \log k - \log k_0 \\ m\sigma &= \log \frac{k}{k_0} \\ m &= \rho \end{aligned} \tag{4.16}$$

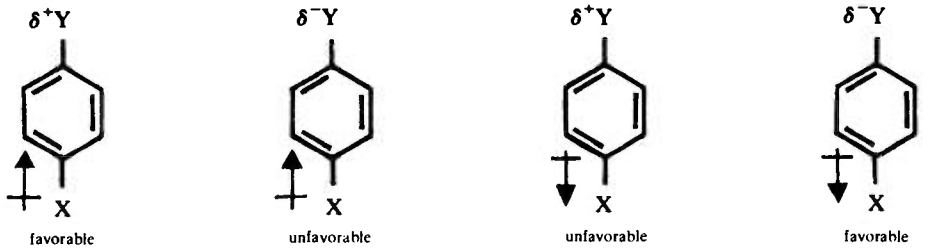
The value of σ reflects the effect the substituent group has on the free energy of ionization of the substituted benzoic acid. The effect of the substituent is believed to represent a combination of factors. A substituent group can cause a polarization

16. P. D. Bolton, K. A. Fleming, and F. M. Hall, *J. Am. Chem. Soc.* **94**, 1033 (1972); J. E. Leffler, *J. Org. Chem.* **20**, 1202 (1955).

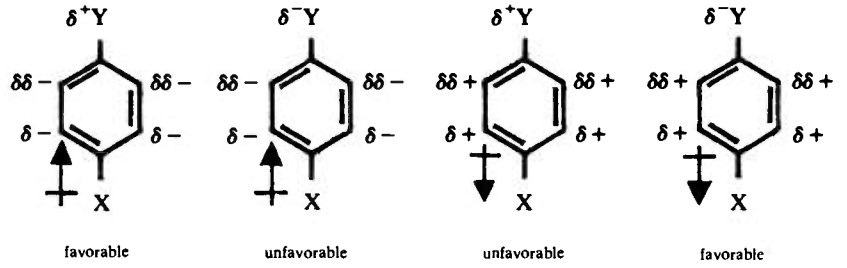
of charge density around the ring through the π system in both the reactant and the product. This will affect the position of the equilibrium. In the case of a reaction rate, the relative effects on the reactant and the transition state will determine the effect on the energy of activation. One mechanism for polarization and charge redistribution is the *resonance effect*, which is illustrated in Fig. 4.3a for several substituents. There is also an effect that originates with the bond dipoles between groups of differing electronegativity. Substituents with electronegativity greater than an aromatic carbon will place a net positive charge on the substituted carbon atom, whereas atoms less electronegative than an aromatic carbon will have the opposite effect. The resulting dipoles can perturb the electronic situation at the reaction site in two ways. The presence of the charge separation will influence the energy associated with development of charge elsewhere in the molecule. This is the result of through-space electrostatic interaction and is called a *field effect*. Depending on



a. Resonance effects



b. Field effects



c. Inductive effects

Fig. 4.3. Resonance, field, and inductive components of substituent effects in substituted benzenes.

the orientation of the dipole and of the charge developing at the reaction site, a substituent can either favor or disfavor the reaction, as illustrated in Fig. 4.3b. Another possible means of interaction of a substituent and the reaction site is called the *inductive effect.* This is transmission of bond dipoles through the intervening bonds by successive polarization of each bond. The experimental and theoretical results presently available indicate that the field effect outweighs the inductive effect as the primary means of transmission of the effect of bond dipoles.[17]

The Hammett equation in the form Eq. (4.14) or Eq. (4.15) is free of complications due to steric effects, since it is applied only to *meta* and *para* substituents. The geometry of the benzene ring ensures that groups in these positions cannot interact sterically with the site of reaction. Tables of σ values for many substituents have been collected; some values are shown in Table 4.3. The σ value for any substituent reflects the interaction of the substituent with the reacting site by a combination of resonance and field interactions. Table 4.4 shows a number of ρ values. The ρ value reflects the sensitivity of the particular reaction to substituent effects. The examples that follow illustrate some of the ways in which the Hammett equation can be used.

Example 4.2. The pK_a of *p*-chlorobenzoic acid is 3.98; that of benzoic acid is 4.19. Calculate σ for *p*-Cl.

$$\sigma = \log \frac{K_{p\text{-Cl}}}{K_{\text{H}}} = \log K_{p\text{-Cl}} - \log K_{\text{H}}$$

$$= -\log K_{\text{H}} - (-\log K_{p\text{-Cl}})$$

$$= pK_{a_{\text{H}}} - pK_{a_{p\text{-Cl}}}$$

$$= 4.19 - 3.98 = 0.21$$

Example 4.3. If the ρ value for alkaline saponification of methyl esters of substituted benzoic acids is 2.38, and the rate constant for saponification of methyl benzoate under the conditions of interest is $2 \times 10^{-4}\ M^{-1}\ s^{-1}$, calculate the rate constant for hydrolysis of methyl *m*-nitrobenzoate.

$$\log \frac{k_{m\text{-NO}_2}}{k_{\text{H}}} = \sigma_{m\text{-NO}_2}(\rho) = (0.70)(2.38) = 1.69$$

$$\frac{k_{m\text{-NO}_2}}{k_{\text{H}}} = 49$$

$$k_{m\text{-NO}_2} = 98 \times 10^{-4}\ M^{-1}\ s^{-1}$$

17. M. J. S. Dewar and P. J. Grisdale, *J. Am. Chem. Soc.* **84**, 3548 (1962); M. J. S. Dewar and A. P. Marchand, *J. Am. Chem. Soc.* **88**, 354 (1966); H. D. Holtz amd L. M. Stock, *J. Am. Chem. Soc.* **86**, 5188 (1964); C. L. Liotta, W. F. Fisher, G. H. Greene, Jr., and B. L. Joyner, *J. Am. Chem. Soc.* **94**, 4891 (1972); C. F. Wilcox and C. Leung, *J. Am. Chem. Soc.* **90**, 336 (1968); W. F. Reynolds, *Prog. Phys. Org. Chem.* **14**, 165 (1983).

Table 4.3. Substituent Constants[a]

Substituent group		σ_m	σ_p	σ^+	σ^-	$\mathcal{F}$	$\mathcal{R}$	σ_I	σ_R^0
Acetamido	CH_3CONH	**0.14**	**0.0**	−0.6	0.47	−0.27			
Acetoxy	CH_3CO_2	0.39	0.31	0.18		0.68	−0.07		
Acetyl	CH_3CO	**0.36**	**0.47**		0.82	0.53	0.20	0.20	0.16
Amino	NH_2	**−0.09**	**−0.30**	−1.3		0.04	−0.68	0.12	−0.50
Bromo	Br	0.37	**0.26**	0.15		0.72	−0.18	0.44	−0.16
t-Butyl	$(CH_3)_3C$	**−0.09**	**−0.15**	−0.26		−0.10	−0.14		
Carbomethoxy	CH_3O_2C	**0.35**	**0.44**		0.74			0.20	0.16
Carboxy	HO_2C	**0.35**	**0.44**		0.73	0.55	0.14		
Chloro	Cl	**0.37**	**0.24**	0.11		0.69	−0.16	0.46	−0.18
Cyano	CN	**0.62**	**0.70**		0.99	0.85	0.18	0.56	0.08
Ethoxy	C_2H_5O	0.1	−0.14	−0.82		0.36	−0.44		
Ethyl	C_2H_5	**−0.08**	**−0.13**	−0.30		−0.07	−0.11		
Fluoro	F	**0.34**	**0.15**	−0.07		0.71	−0.34	0.50	−0.31
Hydrogen	H	**0**	**0**	0	0	0	0	0	0
Hydroxy	OH	0.13	−0.38	−0.92		0.49	−0.64		
Methanesulfonyl	CH_3SO_2	**0.64**	**0.73**		1.05	0.90	0.21	0.60	0.12
Methoxy	CH_3O	**0.10**	−0.12	−0.78		0.41	−0.50	0.27	−0.42
Methyl	CH_3	**−0.06**	**−0.14**	−0.31		−0.05	−0.14	−0.04	−0.13
Nitro	NO_2	**0.71**	**0.81**		1.23	1.11	0.16	0.65	0.15
Phenyl	C_6H_5	**0.05**	**0.05**	−0.18	0.08	0.14	0.09		
Trifluoromethyl	CF_3	**0.46**	**0.53**		0.74	0.63	0.19	0.42	0.08
Trimethylammonio	$(CH_3)_3N^+$	0.99	0.96			1.46	0.0		
Trimethylsilyl	$(CH_3)_3Si$	−0.04	−0.07			−0.05	−0.04		

a. Values of σ_m, σ_p, σ^+, and σ^- from O. Exner in *Correlation Analysis in Chemistry*, N. B. Chapman and J. Shorter (eds.), Plenum Press, New York, 1978, Chap. 10. $\mathcal{F}$ and $\mathcal{R}$ from C. G. Swain and E. C. Lupton, *J. Am. Chem. Soc.* **90**, 4328 (1968). Values of σ_I and $\sigma^0{}_R$ from J. Bromilow, R. T. C. Brownlee, V. O. Lopez, and R. W. Taft, *J. Org. Chem.* **44**, 4766 (1979). Values of σ_m and σ_p shown in boldface type are regarded as particularly reliable.

Example 4.4. Using data in Tables 4.3 and 4.4, calculate how much faster *p*-bromobenzyl chloride will solvolyze in water than *p*-nitrobenzyl chloride.

$$\log \frac{k_{p\text{-Br}}}{k_H} = (-1.31)(0.26), \qquad \log \frac{k_{p\text{-NO}_2}}{k_H} = (-1.31)(0.81)$$

$$\log k_{Br} - \log k_H = -0.34, \qquad \log k_{NO_2} - \log k_H = -1.06$$

$$\log k_{Br} + 0.34 = \log k_H, \qquad \log k_{NO_2} + 1.06 = \log k_H$$

$$\log k_{Br} + 0.34 = \log k_{NO_2} + 1.06$$

$$\log k_{Br} - \log k_{NO_2} = 0.72$$

$$\log \frac{k_{Br}}{k_{NO_2}} = 0.72$$

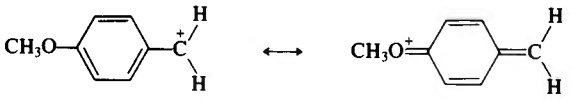
$$\frac{k_{Br}}{k_{NO_2}} = 5.25$$

Given in Table 4.3 in addition to the σ_m and σ_p values used with the classical Hammett equation are σ^+ and σ^-. These are substituent constant sets which reflect a recognition that the extent of resonance participation can vary for different reactions. The σ^+ values are used for reactions in which there is direct resonance interaction between an electron donor substituent and a cationic reaction center, whereas the σ^- set pertains to reactions in which there is a direct resonance interaction between the substituent and an electron-rich reaction site. These are cases where the resonance component of the substituent effect will be particularly important.

Table 4.4. Reaction Constants[a]

Reaction	ρ
$ArCO_2H \rightleftharpoons ArCO_2^- + H^+$, water	1.00
$ArCO_2H \rightleftharpoons ArCO_2^- + H^+$, EtOH	1.57
$ArCH_2CO_2H \rightleftharpoons ArCH_2CO_2^- + H^+$, water	0.56
$ArCH_2CH_2CO_2H \rightleftharpoons ArCH_2CH_2CO_2^- + H^+$, water	0.24
$ArOH \rightleftharpoons ArO^- + H^+$, water	2.26
$ArNH_3^+ \rightleftharpoons ArNH_2 + H^+$, water	3.19
$ArCH_2NH_3^+ \rightleftharpoons ArCH_2NH_2 + H^+$, water	1.05
$ArCO_2Et + {}^-OH \longrightarrow ArCO_2^- + EtOH$	2.61
$ArCH_2CO_2Et + {}^-OH \longrightarrow ArCH_2CO_2^- + EtOH$	1.00
$ArCH_2Cl + H_2O \longrightarrow ArCH_2OH + HCl$	−1.31
$ArC(Me)_2Cl + H_2O \longrightarrow ArC(Me)_2OH + HCl$	−4.48
$ArNH_2 + PhCOCl \longrightarrow ArNHCOPh + HCl$	−3.21

a. From P. R. Wells, *Linear Free Energy Relationships*, Academic Press, New York, 1968, pp. 12, 13.



Direct resonance interaction with a cationic center



Direct resonance interaction with an anionic center

The underlying physical basis for the failure of Hammett σ_m and σ_p values to correlate certain reaction series is that all substituent interactions are some mixture of resonance and field effects. When direct resonance interaction is possible, the extent of the resonance increases and the substituent constants appropriate to a more "normal" mix of resonance and field effects then fail. There have been many attempts to develop sets of σ values that take into account extra resonance interactions.

One approach is to correct for the added resonance interaction. This is done in a modification of the Hammett equation known as the Yukawa-Tsuno equation[18]:

$$\log \frac{K}{K_0} = \rho\sigma + \rho(r)(\sigma^+ - \sigma) \tag{4.17}$$

The additional parameter r is adjusted from reaction to reaction; it reflects the extent of the additional resonance contribution. A large r corresponds to a reaction with a large resonance component, whereas when r goes to zero, the equation is identical to the original Hammett equation. When there is direct conjugation with an electron-rich reaction center, an equation analogous to Eq. (4.17) can be employed, but σ^- is used instead of σ^+.

The Yukawa-Tsuno relationship expanded to include both the σ^+ and σ^- constants is called the LArSR equation[19]:

$$\log \frac{k}{k_0} = \rho(\sigma^0 + r^+\Delta\sigma_R{}^+ + r^-\Delta\sigma_R{}^-)$$

In this equation, the substituent parameters $\Delta\sigma_R{}^+$ and $\Delta\sigma_R{}^-$ reflect the incremental resonance interaction with electron-demanding and electron-releasing reaction centers, respectively. The variables r^+ and r^- are established for a reaction series by regression analysis and are measures of the extent of the extra resonance contribution: the larger their value, the greater the extra resonance contribution. Since both donor and acceptor capacity will not contribute in a single reaction process, either r^+ or r^- would be expected to be zero.

18. Y. Yukawa and Y. Tsuno, *Bull. Chem. Soc. Jpn.* **32**, 971 (1959); J. Hine, *J. Am. Chem. Soc.* **82**, 4877 (1960); B. M. Wepster, *J. Am. Chem. Soc.* **95**, 102 (1973).
19. Y. Yukawa, Y. Tsuno, and M. Sawada, *Bull. Chem. Soc. Jpn.* **39**, 2274 (1966); Y. Yukawa, Y. Tsuno, and M. Sawada, *Bull. Chem. Soc. Jpn.* **45**, 1210 (1972).

Another approach to the treatment of the variability of resonance and field effects was devised by Swain and Lupton.[20] Their approach is to partition substituent effects into pure resonance and field contributions. The substituent constant would then be expressed as a sum of the field and resonance contributions.

$$\sigma = f\mathcal{F} + r\mathcal{R}$$

This treatment requires that *meta*- and *para*-substituted compounds be treated as separate reaction series, since they will have different relative resonance and field components, with resonance being stronger in the *para* series. Since the Swain–Lupton treatment uses four parameters, f, r, $\mathcal{F}$, and $\mathcal{R}$, in place of the single σ of the Hammett equation, the mathematical manipulations are somewhat more complex, but they can be easily handled with an appropriate calculator program. The result of the calculation is a "best-fit" correlation of the rate or equilibrium data with the terms f, r, $\mathcal{F}$, and $\mathcal{R}$. The computation also gives a "percent resonance" by comparing the magnitudes of f and r. The numerical reliability of both the substituent constants, $\mathcal{F}$ and $\mathcal{R}$, and the question of whether complete separation of resonance and field effects is achieved is a matter of some dispute.[21] The derived substituent constants are nevertheless qualitatively useful in comparing the properties of individual substituent groups and making at least a rough comparison of the interplay of resonance and field effects in different reactions. $\mathcal{F}$ and $\mathcal{R}$ values are included in Table 4.3 (p. 201). Certain substituents exhibit opposing resonance and field effects. Fluoro and methoxy groups are two examples (others can be located by examination of Table 4.3). This arises because these substituents act as electron donors by the resonance mechanism but the dipole associated with the substituent operates in the opposite direction.

The most elaborate and accurate treatment of substituent effects has been the development of a series of substituent constants, σ_R, chosen to reflect the resonance contribution of the substituent under various structural circumstances. This substituent constant is then used in conjunction with a second one σ_I, which reflects the inductive (and field) component of the overall substituent effect. The modified equation, called a *dual-substituent-parameter equation*, takes the form

$$\log \frac{K}{K_0} \quad \text{or} \quad \log \frac{k}{k_0} = \sigma_I \rho_I + \sigma_R \rho_R$$

where ρ_I and ρ_R are the reaction constants, which reflect the sensitivity of the system to inductive (and field) and resonance effects.[22] The σ_I values have been defined from studies in aliphatic systems where no resonance component should be present.

20. C. G. Swain and E. C. Lupton, *J. Am. Chem. Soc.* **90**, 4328 (1968); C. G. Swain, S. H. Unger, N. R. Rosenquist, and M. S. Swain, *J. Am. Chem. Soc.* **105**, 492 (1983); C. Hansch, A. Leo, S. H. Unger, K. H. Kim, D. Nikaitani, and E. J. Lien, *J. Med. Chem.* **16**, 1207 (1973).
21. W. F. Reynolds and R. D. Topsom, *J. Org. Chem.* **49**, 1989 (1984); A. J. Hoefnagel, W. Osterbeek, and B. M. Wepster, *J. Org. Chem.* **49**, 1993 (1984); M. Charton, *J. Org. Chem.* **49**, 1997 (1984); S. Marriott, W. F. Reynolds, and R. D. Topsom, *J. Org. Chem.* **50**, 741 (1985); C. G. Swain, *J. Org. Chem.* **49**, 2005 (1984).
22. S. Ehrenson, R. T. C. Brownlee, and R. W. Taft, *Prog. Phys. Org. Chem.* **10**, 1 (1973).

By properly scaling the σ_I values with σ values from aromatic systems, it is possible to assign values such that

$$\sigma = \sigma_I + \sigma_R$$

Statistical analysis of data from many reaction series has shown that no single σ_R is applicable to the entire range of reactions. This again reflects the fact that the resonance component is variable and responds to the nature of the particular reaction. Therefore, a series of four σ_R values was established, each of which applies to various reaction types, ranging from direct conjugation with electron-deficient reaction centers to the other extreme. We will discuss only one of these, σ_R^0, which applies in cases of minimal perturbation of the aromatic ring by charge development at the reaction site. The σ_R^0 values given in Table 4.3 are based on the use of ^{13}C chemical shifts as a measure of the sum of resonance and inductive effects. The chemical shift data of substituted benzenes were analyzed to provide the best correlation with the dual-substituent-parameter equation. In nonpolar solvents, which presumably best reflect the inherent molecular properties, $\rho_I = 3.74$ for cyclohexane and 3.38 for carbon tetrachloride. The corresponding values of ρ_R are 20.59 and 20.73. The relative magnitudes of ρ_I and ρ_R indicate that the ^{13}C chemical shift is more responsive to the resonance character of the substituent than to the inductive character.[23]

In general, the dissection of substituent effects need not be limited to resonance and field components, which are of special prominence in reactions of aromatic compounds. Any type of substituent interaction with a reaction center could be characterized by a substituent constant characteristic of the particular type of interaction and a reaction parameter indicating the sensitivity of the reaction series to that particular type of interaction. For example, it has been suggested that electronegativity and polarizability can be treated as substituent effects separate from field and resonance effects. This gives rise to the equation

$$\log \frac{k}{k_0} = \sigma_F \rho_F + \sigma_R \rho_R + \sigma_\chi \rho_\chi + \sigma_\alpha \rho_\alpha$$

where σ_F is the field, σ_R is the resonance, σ_χ is the electronegativity, and σ_α is the polarizability substituent constant.[24] We will, in general, emphasize the resonance and field components in our discussion of substituent effects.

The various multiparameter equations can usually improve the correlation of data that give poor correlations with the single-parameter Hammett equation. It must be recognized that this in part may be a direct consequence of the introduction of additional variable parameters. To derive solid mechanistic insight into the basis of improved correlation requires a critical appraisal of the results, and statistical analysis may be necessary. The details of critical statistical evaluation of free-energy

23. J. Bromilow, R. T. C. Brownlee, V. O. Lopez, and R. W. Taft, *J. Org. Chem.* **44**, 4766 (1979).
24. R. W. Taft and R. D. Topsom, *Prog. Phys. Org. Chem.* **16**, 1 (1987).

correlations have been extensively discussed, but this topic is beyond the scope of our coverage.[25]

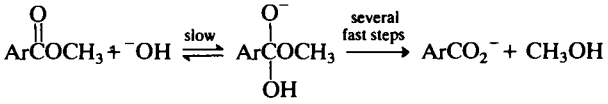
It should be recognized that all of the approaches to linear free-energy relationships have been developed on an empirical basis. The reaction constants, substituent constants, and other parameters involved are specified by the definitions of the correlation equation. The data are then treated by the equation, and the variable parameters arise from the best fit to the correlation equation. In the case of the Hammett equation, each reaction series gives rise to its characteristic ρ value. For the Swain–Lupton equation, the *meta* series and the *para* series would each give rise to f and r.

We might ask if insight into substituent effects can be obtained by analyzing the structural perturbations produced by substituents by molecular orbital calculations. This is a fundamentally challenging task because of the uncertainty of the structure at the transition state and also a large task because of the large number of calculations that would be involved. In one approach to the problem, the stabilizing (or destabilizing) effect of substituents as a positive charge (representing an electrophile) or a negative charge (representing a nucleophile) approaches a benzene ring was calculated.[26] The effect of the substituents, as reflected in the calculated stabilization or destabilization, was parallel to that indicated by linear free-energy correlations. The amino, hydroxy, and fluoro groups, for example, were found to provide extra stabilization for the approach of an electrophile in comparison with other substituents where a strong resonance interaction would not be expected. For the approach of a negative charge, these substituents were destabilizing, but extra stabilization was found for groups such as nitro, cyano, and sulfonyl. While detailed calculations have been undertaken for only a limited number of substituents, it appears that the MO calculations give rise to the same patterns as found on the basis of empirical correlation. The MO calculations support the idea that substituent effects in aromatic compounds are a combination of field effects and resonance effects.[27]

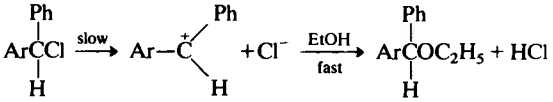
Let us now consider how linear free-energy relationships can provide insight into reaction mechanisms. The choice of benzoic acid ionization as the reference reaction for the Hammett equation leads to $\sigma > 0$ for electron-withdrawing groups and $\sigma < 0$ for electron-releasing groups, since electron-withdrawing groups favor the ionization of the acid and electron-releasing groups have the opposite effect. Further inspection of the Hammett equation shows that ρ will be positive for all reactions that are favored by electron-withdrawing groups and negative for all

25. For detailed discussion of analysis of substituent effects, see J. Shorter, in *Correlation Analysis in Chemistry*, Plenum Press, New York, 1978; M. Charton, *Prog. Phys. Org. Chem.* **13**, 119 (1981); W. F. Reynolds, *Prog. Phys. Org. Chem.* **13**, 119 (1981); W. F. Reynolds, *Prog. Phys. Org. Chem.* **14**, 165 (1983).
26. E. R. Vorpagel, A. Streitwieser, Jr., and S. D. Alexandratos, *J. Am. Chem. Soc.* **103**, 3777 (1981).
27. R. D. Topsom, *Prog. Phys. Org. Chem.* **16**, 125 (1987); H. Agren and P. S. Bagus, *J. Am. Chem. Soc.* **107**, 134 (1985); R. D. Topsom, *Acc. Chem. Res.* **16**, 292 (1983); W. J. Reynolds, P. Dais, D. W. MacIntyre, R. D. Topsom, S. Marriott, E. v. Nagy-Felsobuki, and R. W. Taft, *J. Am. Chem. Soc.* **105**, 378 (1983); A. Pross and L. Radom, *Prog. Phys. Org. Chem.* **13**, 1 (1980).

reactions that are favored by electron-releasing groups. If the rates of a reaction series show a satisfactory correlation, both the sign and the magnitude of ρ provide information about the transition state for the reaction. In Example 4.3 (p. 200), the ρ value for saponification of substituted methyl benzoates is +2.38. This indicates that electron-withdrawing groups facilitate the reaction and that the reaction is somewhat more sensitive to substituent effects than the ionization of benzoic acids. The conclusion that the reaction is favored by electron-withdrawing substituents is in agreement with the accepted mechanism for ester saponification. The tetrahedral intermediate is negatively charged. Its formation should therefore be favored by substituents that can stabilize the developing charge.

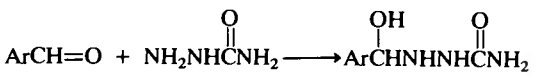


The solvolysis of diarylmethyl chlorides in ethanol shows a ρ value of -5.0, indicating that electron-releasing groups strongly facilitate the reaction. This ρ value provides support for a mechanism involving ionization of the halides in the rate-determining step. Electron-releasing groups can facilitate the ionization by a stabilizing interaction with the electron-deficient carbon that develops as ionization proceeds.

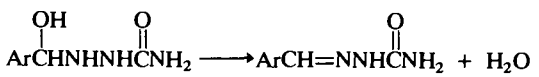


The relatively large ρ shows that the reaction is very sensitive to substituent effects and implies that there is a relatively large redistribution of charge in the transition state.

Not all reactions can be fitted by the Hammett equation or the multiparameter variants. There can be several reasons for this. The most common is that there is a change in mechanism as substituents vary. In a multistep reaction, for example, one step may be rate-determining in the region of electron-withdrawing substituents, but a different step may become rate-limiting as the substituents become electron-releasing. The rate of the semicarbazone formation reaction of benzaldehydes, for example, shows a nonlinear Hammett plot with ρ of about 3.5 for electron-releasing groups, but with ρ near -0.25 for electron-withdrawing groups.[28] The change in ρ is believed to be the result of a change in the rate-limiting step.



(rate-controlling for electron-releasing substituents)



(rate-controlling for electron-attracting substituents)

28. D. S. Noyce, A. T. Bottini, and S. G. Smith, *J. Org. Chem.* **23**, 752 (1958).

Any reaction which shows a major shift in transition state structure over the substituent series would be expected to give a curved Hammett plot, since a variation in the extent of resonance participation would then be expected.

The substituent constants recorded in Table 4.3 (p. 201), provide valuable insight into the electronic nature of the various functional groups. By comparing σ with σ^+, one gains a qualitative impression of the ability of a given substituent to act as an electron-donating group by resonance. The $\mathcal{F}$ and σ_I values are indicators of the field and inductive characteristics of the various substituents. It should be noted that some groups, such as OMe and NH_2, that have strong electron-releasing capacity by resonance are electron-withdrawing when only field and inductive effects are considered. If $\mathcal{F}$ and $\mathcal{R}$ are taken as the best indicators of field versus resonance interactions, the substituents can be classified as in Table 4.5. By comparing σ_m values with σ_p values (Table 4.3), one can see that field effects usually dominate with *meta* substituents, while resonance effects are more important for *para* substituents. Thus σ_m constants of substituents such as hydroxy and methoxy have the positive signs while the σ_p constants are negative. This relationship is reasonable in terms of structure, sine the *para* substituents are more favorably situated for resonance interaction.

There is a system for designating the electron-releasing or -attracting properties of substituents. This system is illustrated in Table 4.5. The symbols $+M$ and $-M$ have been used to designate resonance interactions (the M comes from "mesomerism," a synonym for resonance) and the symbols $+I$ and $-I$ for combined field and inductive effects (I for inductive because the importance of field effects was recognized only after the system was established).

The development of linear free-energy relationships in purely aliphatic molecules is complicated because steric and conformation factors come into play along with electronic effects. A number of successful treatment of aliphatic systems have been developed by separating polar effects from steric effects. We will not

Table 4.5. Classification of Substituent Groups

Resonance: Electron-releasing ($-M$)	Electron-releasing ($-M$)			Electron-withdrawing ($+M$)
Field: Electron-releasing ($-I$)	Electron-withdrawing ($+I$)			Electron-withdrawing ($+I$)
Me	AcNH	Br	OH	Ac
Et	AcO	Cl	MeO	CN
$(Me)_3C$	NH_2	F	EtO	NO_2
			Ph	CF_3
				$(Me)_3N^+$

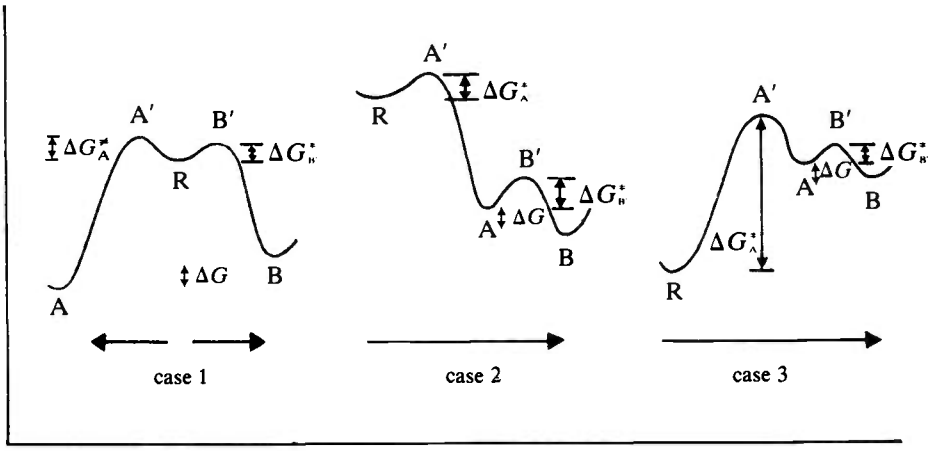


Fig. 4.4. Kinetic versus thermodynamic control.

discuss these methods but there are reviews available which can be consulted for information about this area.[29]

4.4. Basic Mechanistic Concepts: Kinetic versus Thermodynamic Control, Hammond's Postulate, and the Curtin–Hammett Principle

Use of two-dimensional potential energy diagrams can provide insight into the important general concepts listed in the heading of this section. There are many organic reactions in which the energy requirements for competing reaction paths are rather similar. It is important to be able to analyze the factors that may permit a particular reaction path to dominate.

4.4.1. Kinetic versus Thermodynamic Control

Product composition may be governed by the equilibrium thermodynamics of the system. When this is true, the product composition is governed by *thermodynamic control.* Alternatively, product composition may be governed by competing rates of formation of products. This is called *kinetic control.*

Let us consider cases 1-3 in Fig. 4.4. In case 1, $\Delta G^{\ddagger}$'s for formation of transition states A′ and B′ from the reactant R are much less than $\Delta G^{\ddagger}$'s for formation of A′ and B′ from A and B, respectively. If the latter two $\Delta G^{\ddagger}$'s are sufficiently large that the competitively formed products B and A do not return to R, the ratio of the products A and B at the end of the reaction will not depend on their relative

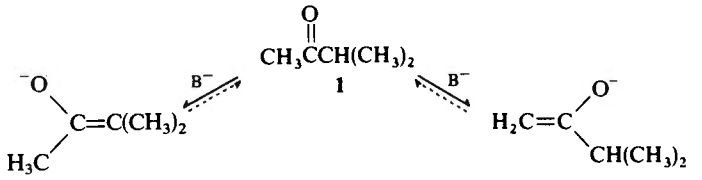
29. J. Hine, *Physical Organic Chemistry*, McGraw-Hill, New York, 1962, pp. 95-98; P. R. Wells, *Linear Free Energy Relationships*, Academic Press, New York, 1968, pp. 35-44; M. Charton, *Prog. Phys. Org. Chem.* **10**, 81 (1973).

stabilities, but only on their relative rates of formation. The formation of A and B is effectively irreversible in these circumstances. The energy plot in case 1 corresponds to this situation. This is a case of kinetic control. The relative amounts of products A and B will depend on the heights of the activation barriers $\Delta G_A^{\ddagger}$ and $\Delta G_b^{\ddagger}$.

In case 2, the lowest $\Delta G^{\ddagger}$ is that for formation of A′ from R. However, the $\Delta G^{\ddagger}$ for formation of B′ from A is not much larger. System 2 might be governed by either kinetic or thermodynamic factors. Conversion of R to A will be only slightly more favorable than conversion of A to B. If the reaction conditions are carefully adjusted, it will be possible for A to accumulate and not proceed to B. Under such conditions, A will be the dominant product and the reaction will be under kinetic control. Under somewhat more energetic conditions, for example, at a higher temperature, A will be transformed to B, and under these conditions the reaction will be under thermodynamic control. A and B will equilibrate, and the product ratio will depend on the equilibrium constant determined by ΔG.

In case 3, the barrier separating A and B is very small relative to that for formation of A′ from R. In this case, A and B will equilibrate more rapidly than R is converted to A. Adjustment of temperature or other reaction conditions would not change the A:B ratio at the end of the reaction very much.

The idea of kinetic versus thermodynamic control can be illustrated by discussing briefly the case of formation of enolate anions from unsymmetrical ketones. This is a very important case for synthesis and will be discussed more fully in Chapter 1 of Part B. Any ketone with more than one type of α-hydrogen can give rise to more than one enolate. Many studies have shown that the ratio of the possible enolates depends on the reaction conditions.[30] This can be illustrated for the case of methyl isopropyl ketone (3-methyl-2-butanone). If the base chosen is a strong, sterically hindered one and the solvent is aprotic, the major enolate formed is **3**. If a protic solvent is used or if a weaker base (one comparable in basicity to the ketone enolate) is used, the dominant enolate is **2**. Enolate **3** is the "kinetic enolate" while **2** is the thermodynamically favored enolate.



2 **3**

The structural and mechanistic basis for the relationships between kinetic versus thermodynamic control and the reaction conditions is as follows. The α-hydrogens of the methyl group are less hindered sterically than the α-hydrogen of the isopropyl group. As a result, abstraction of one of these hydrogens as a proton is faster than

30. J. d'Angelo, *Tetrahedron* **32**, 2979 (1976); H. O. House, *Modern Synthetic Reactions*, Second Edition, W. A. Benjamin, Menlo Park, California, 1972.

for the isopropyl hydrogen. This effect is magnified when the base is sterically hindered so that it is particularly sensitive to the difference in the steric situation of the competing hydrogens. If the base is very strong, the enolate will not be reconverted to the ketone because the enolate will be too weak a base to regain the proton. These conditions correspond to case 1 in Fig. 4.4 and represent a case of kinetic control. If a weaker base is used or if the solvent is protic, protons can be transferred reversibly between the isomeric enolates and the base (because the base strengths of the enolate and the base are comparable). Under these conditions, the more stable enolate will be the predominant one because the enolates are equilibrated. The more substituted enolate **2** is the more stable of the pair, just as more substituted alkenes are more stable than terminal alkenes.

4.4.2. Hammond's Postulate

Because of the crucial role played by the energy of the transition state in determining the rates of chemical reactions, information about the structure of transition states is crucial to understanding reaction mechanisms. However, because transition states have only transitory existence, it has not been possible to make experimental measurements that would provide direct information about the structure of transition states. Hammond has discussed the circumstances under which it is valid to relate transition state structure to the structure of reactants, intermediates, and products.[31] His statements concerning transition state structure are known as *Hammond's postulates.* Discussing individual steps in a reaction mechanism, Hammond's postulate states "if two states, as for example, a transition state and an unstable intermediate, occur consecutively during a reaction process and have nearly the same energy content, their interconversion will involve only a small reorganization of molecular structure."

This statement can best be discussed with reference to potential energy diagrams. Case 1 in Fig. 4.5 represents a highly exothermic step with a low activation energy. It follows from Hammond's postulate that in this step the transition state will structurally resemble the reactant since they are close in energy and therefore interconverted by a small structural change. This is depicted in the potential energy diagram by a small displacement toward product along the reaction coordinate. Case 2 describes a step in which the transition state is a good deal higher in energy than either the reactant or the product. In this case, neither the reactant nor the product will be a good model of the transition state. Case 3 illustrates an endothermic step such as would occur in the formation of an unstable intermediate. In this case, the energy of the transition state is similar to that of the intermediate and the transition state should be similar in structure to the intermediate.

The significance of the concept incorporated in Hammond's postulate then is that, in appropriate cases, it permits discussion of transition state structure in terms

31. G. S. Hammond, *J. Am. Chem. Soc.* 77, 334 (1955).

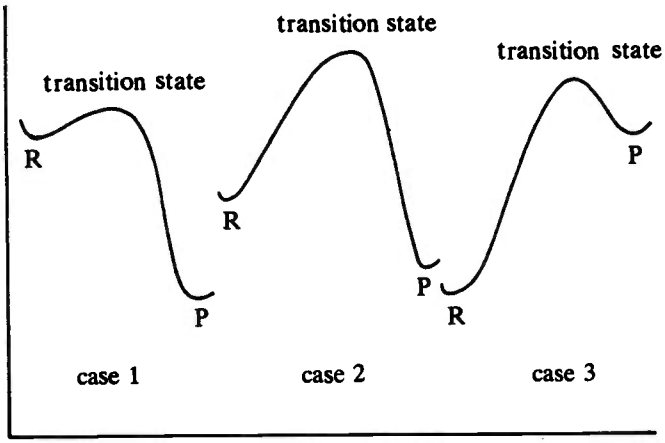
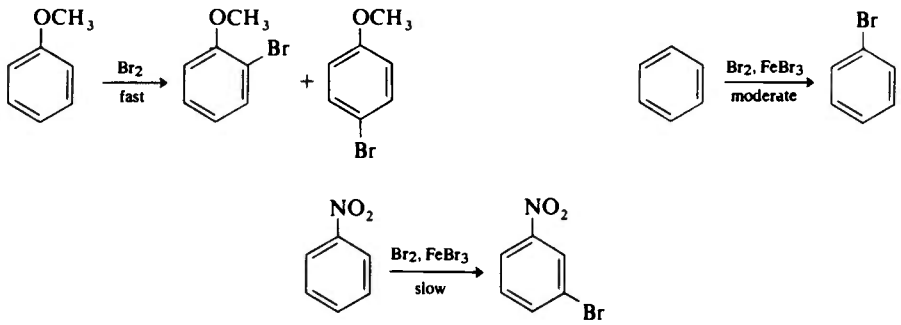


Fig. 4.5. Some typical potential energy diagrams that illustrate the application of Hammond's postulate.

of the reactants, intermediates, or products in a multistep reaction sequence. The postulate indicates that the cases where such comparison is appropriate are those in which the transition state is close in energy to the reactant, intermediate, or product.

The case of electrophilic aromatic substitution can illustrate a situation in which it is useful to discuss transition state structure in terms of a reaction intermediate. The *ortho–para*- and *meta*-directing effects of aromatic substituents were among the first structure–reactivity relationships to be developed in organic chemistry. Certain functional groups were found to activate aromatic rings toward substitution and to direct the entering electrophile to the *ortho* and *para* positions whereas others were deactivating and led to substitution in the *meta* position. The bromination of anisole (methoxybenzene), benzene, and nitrobenzene can serve as cases for discussion.

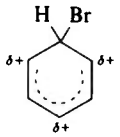


It can be demonstrated that the reactions are kinetically controlled. It is therefore the $\Delta G^{\ddagger}$ value that holds the key to the connection between the rate effects and the substituent's directing effects. But to discuss $\Delta G^{\ddagger}$ satisfactorily, we must know something about the reaction mechanism and the nature of the competing transition states. Electrophilic aromatic substitution will be discussed in detail in Chapter 10.

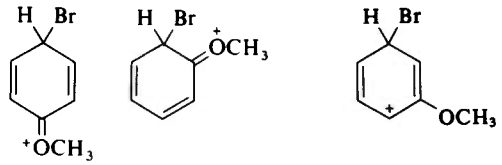
Evidence presented there will indicate that electrophilic aromatic substitution involves a distinct intermediate and two less well-defined states. The potential energy diagram in Fig. 4.6 is believed to be a good representation of the energy changes that occur during bromination. By application of the Hammon postulate, we can conclude that the rate-determining step involves formation of a transition state that should closely resemble the intermediate σ complex. It is therefore legitimate to discuss effects of substituents on the transition state in terms of the structure of this intermediate.

Since the product composition is kinetically controlled, the isomer ratio will be governed by the relative magnitudes of ΔG_o^+, ΔG_m^+, and ΔG_p^+, the energies of activation for the *ortho*, *meta*, and *para* transition states, respectively. In Fig. 4.7 a qualitative comparison of these ΔG^+ values is made.

At the transition state, a considerable positive charge is present on the benzene ring, primarily at positions 2, 4, and 6 in relation to the entering bromine.



The electron-releasing methoxy group can interact directly to delocalize the charge and stabilize the intermediates leading to *o*- and *p*-bromoanisole. It cannot stabilize the intermediate leading to *m*-bromoanisole.



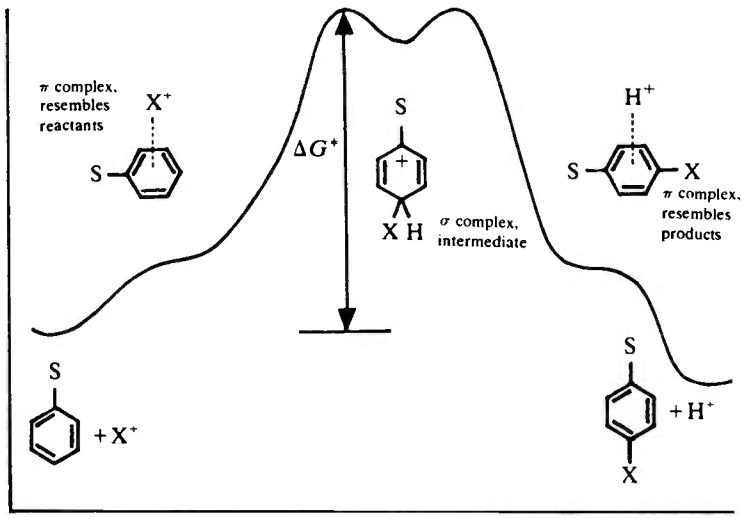
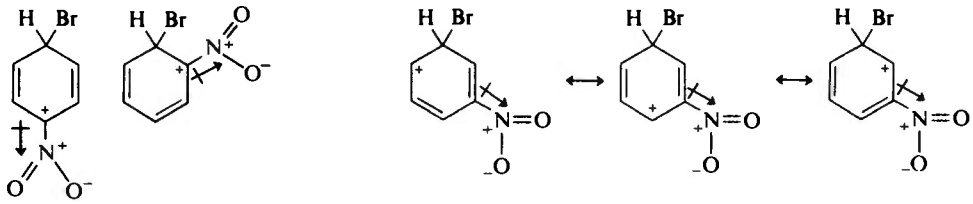


Fig. 4.6. Potential energy diagram for electrophilic aromatic substitution.

The *ortho* and *para* intermediates are therefore stabilized relative to benzene but the *meta* intermediate is not, as is illustrated in Fig. 4.7. As a result, anisole reacts faster than benzene, and the products are mainly the *ortho* and *para* isomers.

In the case of nitrobenzene, the electron-withdrawing nitro group is not able to stabilize the positive charge in the σ-complex intermediate. In fact, it strongly destabilizes the intermediate. This destabilization is greatest in the *ortho* and *para* intermediates, which place positive charge on the nitro-substituted carbon. The *meta* transition state is also destablized relative to benzene, but not as much as the *ortho* and *para* transition states. As a result, nitrobenzene is less reactive than benzene and the product is mainly the *meta* isomer



The substituent effects in aromatic electrophilic substitution can be analyzed in terms of resonance effects. In other systems, stereoelectronic effects or steric

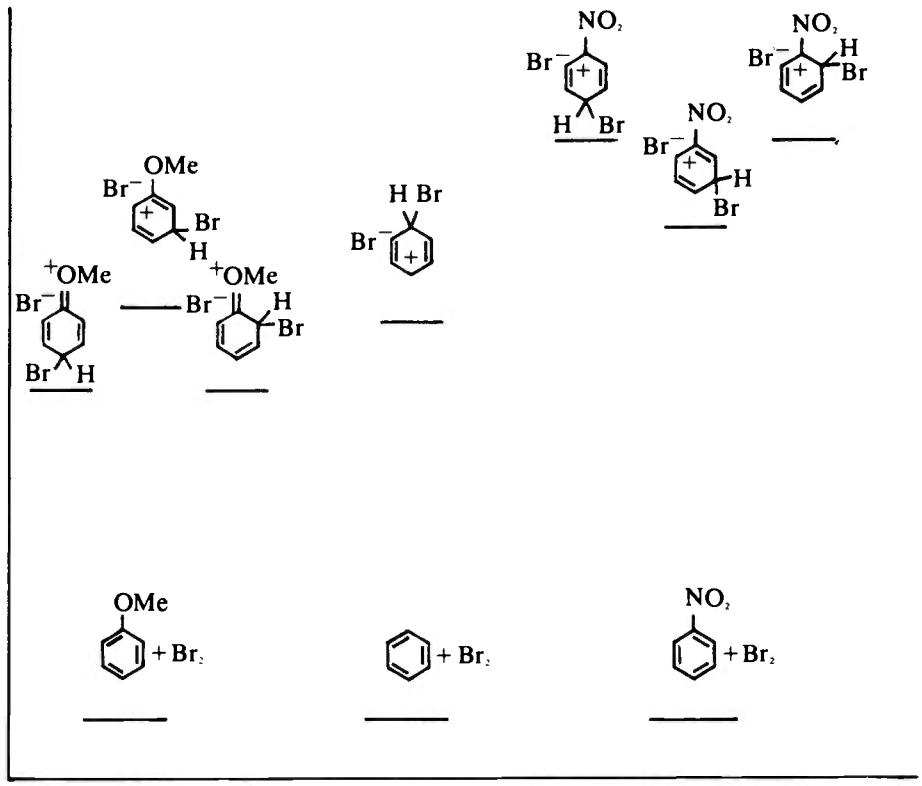


Fig. 4.7. Transition state energies in bromination.

effects might be more important. Whatever the nature of the substituent effects, the Hammond postulate insists that structural discussion of transition states in terms of reactants, intermediates, or products is valid only when their energies are similar.

4.4.3. The Curtin–Hammett Principle

In Chapter 3, equilibria among conformers of organic molecules were discussed. At this point, let us consider in a general way the effect that conformational equilibria can have on a chemical reaction. Under what circumstances can the position of the conformational equilibrium for a reactant determine which of two competing reaction paths will be followed? A potential energy diagram is shown in Fig. 4.8. In most cases, the energy of activation for a chemical reaction will be greater than that for a conformational equilibrium as is illustrated in the figure. If this is the case, $\Delta G_a^{\ddagger}$ and $\Delta G_b^{\ddagger} \gg \Delta G_c$. The conformers of the reactant are in equilibrium and are interconverted at a rate much faster than that at which the competing reactions occur.

$$\mathrm{B} \rightleftharpoons \mathrm{A} \qquad K_c = \frac{[\mathrm{A}]}{[\mathrm{B}]}$$

$$\text{rate of formation of product } P_\mathrm{A} = \frac{dP_\mathrm{A}}{dt} = k_a[\mathrm{A}] = k_a K_c[\mathrm{B}]$$

$$\text{rate of formation of product } P_\mathrm{B} = \frac{dP_\mathrm{B}}{dt} = k_b[\mathrm{B}]$$

$$\text{product ratio} = \frac{dP_\mathrm{A}/dt}{dP_\mathrm{B}/dt} = \frac{k_a K_c[\mathrm{B}]}{k_b[\mathrm{B}]} = \frac{k_a K_c}{k_b}$$

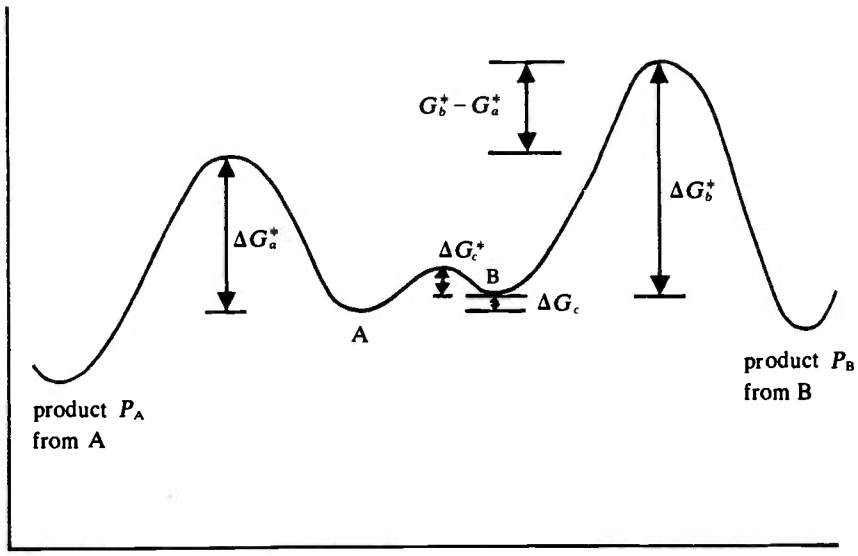


Fig. 4.8. Effect of conformation on product distribution.

According to transition state theory,

$$k_r = \frac{\kappa kT}{h} e^{-\Delta G^{\ddagger}/RT} \qquad \text{and} \qquad K_c = e^{-(-\Delta G_c)/RT}$$

$$\text{product ratio} = \frac{(\kappa kT/h)\, e^{-\Delta G_a^{\ddagger}/RT}\, e^{+\Delta G_c/RT}}{(\kappa kT/h)\, e^{-\Delta G_b^{\ddagger}/RT}}$$

$$= e^{(-\Delta G_a^{\ddagger}+\Delta G_b^{\ddagger}+\Delta G_c)/RT}$$

But from Fig. 4.8,

$$\Delta G_b^{\ddagger} - \Delta G_a^{\ddagger} + \Delta G_c = G_b^{\ddagger} - G_a^{\ddagger}$$

The product ratio is therefore not determined by ΔG_c but instead primarily on the relative energy of the two transition states leading to A and B.

The conclusion that the ratio of products formed from conformational isomers is not determined by the conformation population ratio is known as the *Curtin–Hammett principle*.[32] While the rate of the formation of the products is dependent upon the relative concentration of the two conformers, since $\Delta G_b^{\ddagger}$ is decreased relative to $\Delta G_a^{\ddagger}$ to the extent of the difference in the two conformational energies, the conformational preequilibrium is established rapidly, relative to the two competing product-forming steps.[33] The position of the conformational equilibrium cannot control the product ratio. The reaction may proceed through a minor conformation if it is the one which provides access to the lowest-energy transition state.

The same arguments can be applied to other energetically facile interconversions of two potential reactants. For example, many organic molecules undergo rapid proton shifts (tautomerism) and the chemical reactivity of the two isomers may be quite different. It is not valid, however, to deduce the ratio of two tautomers on the basis of subsequent reactions which have activation energies greater than that of the tautomerism. Just as in the case of conformational isomerism, the ratio of products formed in subsequent reactions will not primarily be controlled by the position of the facile equilibrium.

4.5. Isotope Effects

A special type of substituent effect that has proved very valuable in the study of reaction mechanisms is the replacement of an atom by one of its isotopes. Isotopic substitution has most often involved replacing protium by deuterium (or tritium),

32. D. Y. Curtin, *Rec. Chem. Prog.* **15**, 111 (1954); E. L. Eliel, *Stereochemistry of Carbon Compounds*, Mc-Graw-Hill, New York, 1962, pp. 151–152, 237–238.
33. For a more complete discussion of the relationship between conformational equilibria and reactivity, see J. I. Seeman, *Chem. Rev.* **83**, 83 (1983).

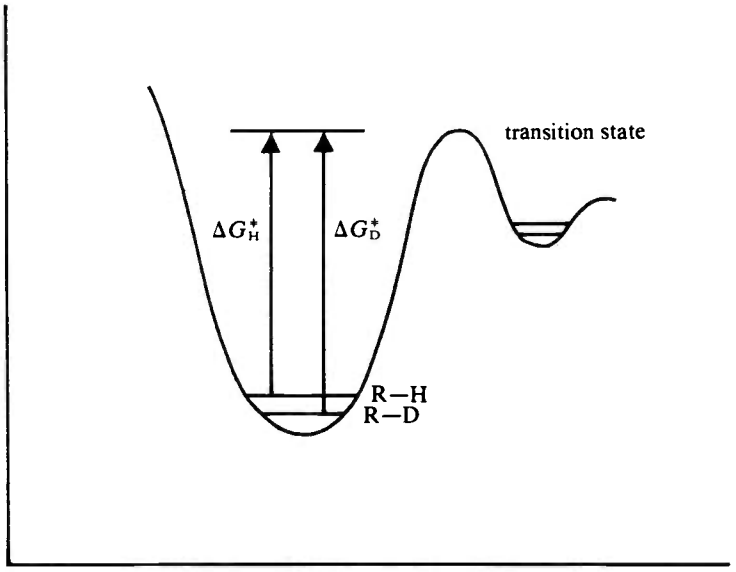


Fig. 4.9. Differing zero-point energies of protium- and deuterium-substituted molecules as the cause of primary kinetic isotope effects.

but the principle is applicable to nuclei other than hydrogen. The quantitative differences are largest, however, for hydrogen. Isotopic substitution has no effect on the qualitative chemical reactivity of the substrate, but it often has an easily measured effect on the rate at which reaction occurs. Let us consider how this modification of the rate arises. Initially, the discussion will concern *primary kinetic isotope effects*, those in which a bond to the isotopically substituted atom is broken in the rate-determining step. We will use C—H bonds as the specific topic of discussion, but the same concepts apply for other elements.

Any C—H bond has characteristic vibrations which impart some energy to the molecule in its normal state. This energy is called the *zero-point* energy. The energy associated with these vibrations is related to the mass of the vibrating atoms. Because of the greater mass of deuterium, the vibrations associated with a C—D bond contribute less to the zero-point energy than do those of the corresponding C—H bond. For this reason, substitution of protium by deuterium lowers the zero-point energy of a molecule. For a reaction involving cleavage of a bond to hydrogen (or deuterium), a vibrational degree of freedom in the normal molecule is converted to a translational degree of freedom on passing through the transition state. The energy difference due to this vibration disappears at the transition state. The transition state has the same energy for the protonated and deuterated species. Since the deuterated molecule had the lower zero-point energy, it necessarily has a higher activation energy to reach the transition state. This is illustrated in Fig. 4.9.

Just how large the rate difference is depends on the nature of the transition state. The maximum effect occurs when the hydrogen being transferred is bound about equally to two other atoms at the transition state. The calculated maximum

for the isotope effect k_H/k_D involving C—H bonds is about 7 at room temperature.[34] When bond breaking is more or less than half complete at the transition state, the value is less and can be close to 1 if the transition state is very reactant-like or very product-like. Primary isotope effects can provide two very useful pieces of information about a reaction mechanism. First, the existence of a substantial isotope effect—that is, if k_H/k_D is 2 or more—is strong evidence that the bond to the isotopically substituted hydrogen atom is being broken in the rate-determining step. Second, the magnitude of the isotope effect provides a qualitative indication of where the transition state lies with respect to product and reactant. A relatively low primary isotope effect implies that the bond to hydrogen is either only slightly or nearly completely broken at the transition state. That is, the transition state must occur quite close to reactant or to product. An isotope effect near the theoretical maximum is good evidence that the transition state involves strong bonding of the hydrogen to both its new and old bonding partner.

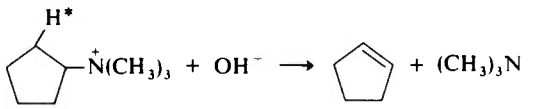
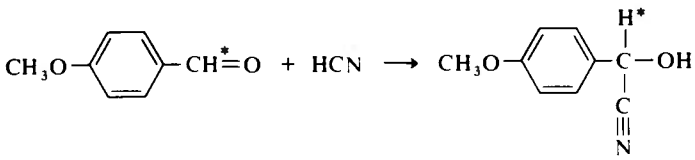
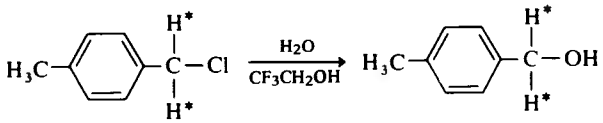
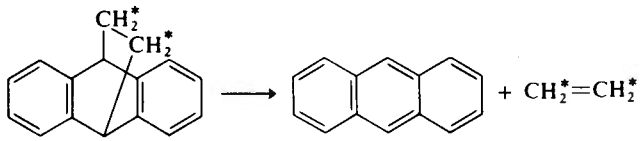
Isotope effects may be observed even when the substituted hydrogen atom is not directly involved in the reaction. Such effects are called *secondary kinetic isotope effects.* Secondary isotope effects are smaller than primary ones and are usually in the range of $k_H/k_D = 0.7$–1.5. Secondary isotope effects may be normal ($K_H/K_D > 1$) or inverse ($k_H/k_D < 1$). They are also classified as α, β, etc., depending on whether the isotopic substitution is on the reacting carbon or farther away. Secondary isotope effects result from a tightening or loosening of the C—H bond at the transition state. The strength of the bond may change because of a hybridization change or a change in the extent of hyperconjugation, for example. If sp^3-hybridized carbon is converted to sp^2 as reaction occurs, a hydrogen bound to the carbon will experience decreased resistance to C—H bending. The freeing of the vibration for a C—H bond is greater than that for a C—D bond because the C—H bond is slightly longer and the vibration therefore has a larger amplitude. This will result in a normal isotope effect. Entry 5 in Scheme 4.2 is an example of such a reaction since it proceeds through a carbocation intermediate.

An inverse isotope effect will occur if coordination at the reaction center increases in the transition state. The bending will become more restricted. Entry 4 in Scheme 4.2 exemplifies such a case involving conversion of a tricoordinate carbonyl group to a tetravelent cyanohydrin. In this case the secondary isotope effect is 0.73.

Secondary isotope effects at the β position have been especially thoroughly studied in nucleophilic substitution reactions. When carbocations are involved as intermediates, substantial isotope effects are observed. This is because the hyperconjugative stabilization by the β-hydrogens weakens the C—H bond.[35] The observed

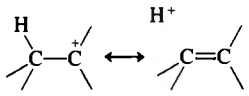
34. K. B. Wiberg, *Chem. Rev.* **55**, 713 (1955); F. H. Westheimer, *Chem. Rev.* **61**, 265 (1961).
35. V. J. Shiner, W. E. Buddenbaum, B. L. Murr, and G. Lamaty, *J. Am. Chem. Soc.* **90**, 809 (1968); A. J. Kresge and R. J. Preto, *J. Am. Chem. Soc.* **89**, 5510 (1967); G. J. Karabatsos, G. C. Sonnichsen, C. G. Papaioannou, S. E. Scheppele, and R. L. Shone, *J. Am. Chem. Soc.* **89**, 463 (1967); D. D. Sunko and W. J. Hehre, *Prog. Phys. Org. Chem.* **14**, 205 (1983).

Scheme 4.2. Some Representative Kinetic Isotope Effects

	Reaction	k_H/k_D(°C)[a]
A. Primary kinetic isotope effects		
1[b]	$PhCH_2{-}H^* + Br\cdot \longrightarrow Ph{-}CH_2\cdot + H^*{-}Br$	4.6 (77)
2[c]	$(CH_3)_2C(H^*){-}C(=O){-}C(H^*)(CH_3)_2 + OH^- \longrightarrow (CH_3)_2C(H^*){-}C(O^-)=C(CH_3)_2$	6.1 (25)
3[d]		4.0 (191)
B. Secondary kinetic isotope effects		
4[e]		0.73 (25)
5[f]		1.30 (25)
6[g]		1.37 (50)

a. Temperature of measurement is indicated in parentheses.
b. K. B. Wiberg and L. H. Slaugh, *J. Am. Chem. Soc.* **80**, 3033 (1958).
c. R. A. Lynch, S. P. Vincenti, Y. T. Lin, L. D. Smucker, and S. C. Subba Rao, *J. Am. Chem. Soc.* **94**, 8351 (1972).
d. W. H. Saunders, Jr., and T. A. Ashe, *J. Am. Chem. Soc.* **91**, 4473 (1969).
e. L. do Amaral, H. G. Bull, and E. H. Cordes, *J. Am. Chem. Soc.* **94**, 7579 (1972).
f. V. J. Shiner, Jr., M. W. Rapp, and H. R. Pinnick, Jr., *J. Am. Chem. Soc.* **92**, 232 (1970).
g. M. Taagepera and E. R. Thornton, *J. Am. Chem. Soc.* **94**, 1168 (1972).

secondary isotope effects are normal as would be predicted since the bond is weakened.



Detailed analysis of isotope effects reveals that there are many other factors which can contribute to the overall effect in addition to the dominant change in bond vibrations. For that reason, it is not possible to quantitatively predict the magnitude of either primary or secondary isotope effects for a given reaction.

Furthermore, there is not a sharp numerical division between primary and secondary effects, especially in the range between 1 and 2. For these reasons, isotope effects are usually used in conjugation with other criteria in the description of reaction mechanisms.[36]

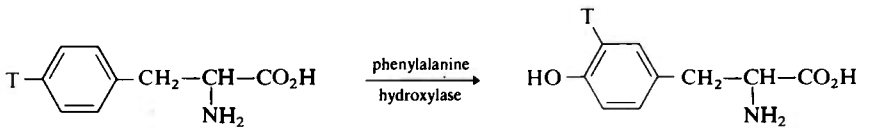
4.6. Isotopes in Labeling Experiments

A quite different use of isotopes in mechanistic studies involves their use as labels for ascertaining the location of a given atom involved in a reaction. As in kinetic experiments, the isotopic substitution will not qualitatively affect the course of the reaction. The nuclei most commonly used for isotopic tracer experiments in organic chemistry are deuterium, tritium, and the ^{13}C and ^{14}C isotopes of carbon. There are several means of locating isotopic labels. Deuterium can frequently be located by analysis of NMR spectra. In contrast to the normal ^{1}H isotope, deuterium does not show an NMR signal under the normal operating circumstances. The absence of a specific signal can therefore be used to locate deuterium. Both mass spectrometry and infrared spectroscopy also can be used to locate deuterium. Tritium and ^{14}C and other radioactive isotopes are usually located on the basis of the radioactivity. This is a very sensitive method. It should be borne in mind that, in most experiments in which radioactive labels are used, only a small fraction of the atoms at the site of substitution are the radioactive nuclide. The high sensitivity of radioactive atoms to detection makes the method practical. Usually, the location of ^{14}C requires a degradation process of some sort to separate the atoms that might conceivably be labeled. Carbon-13 has become an important isotope for tracer experiments relatively recently. Unlike normal ^{12}C, ^{13}C has a nuclear magnetic moment and can be detected in NMR spectrometers of the proper frequency. This avoids the necessity of developing a degradation scheme to separate specific carbon atoms.

There are many excellent examples of isotopic labeling experiments in both organic chemistry and biochemistry.[37] An interesting example is the case of hydroxylation of the amino acid phenylalanine which is carried out by the enzyme phenyl-

36. For more complete discussion of isotope effects, see W. H. Saunders, in *Investigation of Rates and Mechanisms of Reactions*, E. S. Lewis (ed.), *Techniques of Chemistry*, Third Edition, Vol. VI, Part 1, Wiley-Interscience, New York, 1974, pp. 211-255; L. Melander and W. H. Saunders, Jr., *Reaction Rates of Isotopic Molecules*, Wiley, New York, 1980; W. H. Saunders, in *Investigation of Rates and Mechanisms of Reactions*, C. F. Bernasconi (ed.), *Techniques of Chemistry*, Fourth Edition, Vol. VI, Part 1, Wiley-Interscience, New York, 1986, Chapter VIII.

37. For examples of use of isotopic labels in mechanistic studies, see V. F. Raaen, in *Investigation of Rates and Mechanisms of Reactions*, E. S. Lewis (ed.), *Techniques of Chemistry*, Vol VI, Part 1, Wiley-Interscience, New York, 1974, pp. 257-284, and *Isotopes in Organic Chemistry*, Vols. 1-4, E. Buncel and C. C. Lee, (eds.), Elsevier, New York, 1975-1978; C. Wentrup, in *Investigation of Rates and Mechanisms of Reactions*, C. F. Bernasconi (ed.), *Techniques of Chemistry*, Fourth Edition, Vol. VI, Part 1, Wiley-Interscience, New York, 1986, Chapter IX.



When this reaction was studied by use of tritium, the phenylaline was labeled with tritium at the 4-position of the phenyl ring. When the product, tyrosine, was isolated, it retained much of the original radioactivity, even though the 4-position was now substituted by a hydroxyl group. When this was studied in detail, it was found that the ^3H originally at the 4-position had rearranged to the 3-position in the course of oxidation. This hydrogen shift, called the *NIH shift*,[38] has subsequently been found to occur in many biological oxidations of aromatic compounds.

4.7. Characterization of Reaction Intermediates

Identification of the intermediates in a multistep reaction is a major objective of studies of reaction mechanisms. When the nature of each intermediate is fairly well understood, a great deal is known about the reaction mechanism. The amount of an intermediate present in a reacting system at any instant of time will depend on the rates of the steps by which it is formed and the rate of its subsequent reaction. A qualitative indication of the relationship between intermediate concentration and the kinetics of the reaction can be gained by considering a simple two-step reaction mechanism:

$$\text{reactants} \xrightarrow{k_1} \text{intermediate} \xrightarrow{k_2} \text{products}$$

In some reactions, the situation $k_1 > k_2$ exists. Under these conditions, the concentration of the intermediate will build up as it goes on more slowly to product. The possibility of isolating, or at least observing, the intermediate then exists. If both k_1 and k_2 are large, the reaction may proceed too rapidly to permit isolation of the intermediate, but spectroscopic studies, for example, should reveal the existence of two distinct stages for the overall reaction. It should be possible to analyze such a system and determine the two rate constants.

If the two steps are of about equal rates, only a small concentration of the intermediate will exist at any time. It is sometimes possible to interrupt such a reaction by lowering the temperature rapidly or adding a reagent that stops the reaction and isolate the intermediate. Intermediates can also be "trapped." In this approach, a compound that is expected to react specifically with the intermediate is added to the reaction system. If trapping occurs, the intermediate is diverted from

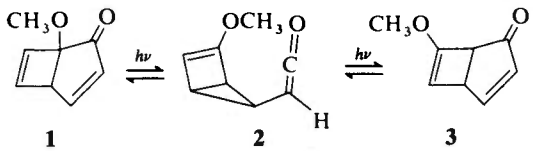
38. From its discovery at the National Institutes of Health (NIH); for an account of this discovery, see G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, B. Witkop, and S. Udenfriend, *Science* **157**, 1524 (1967).

its normal course, and evidence for the existence of the intermediate is obtained if the structure of the trapped product is consistent with expectation.

Often it is more practical to study intermediates present in low concentration by spectroscopic methods. The theory and practice of instrumental methods of detection of intermediates will be discussed here only very briefly. Instrumental techniques have become very important in the study of reaction mechanisms. The most common methods in organic chemistry include ultraviolet-visible (UV-VIS), infrared (IR), nuclear magnetic resonance (NMR), and electron paramagnetic resonance (EPR) spectroscopy.

UV-VIS spectrometers can rapidly scan the electronic region of the spectrum and provide evidence of the development of characteristic *chromophores*. The major limitation imposed is that the compound to be detected must have a characteristic absorbance in the range 220–700 nm. Absorption of energy in this region is associated with promotion of electrons to higher energy states. In organic molecules, absorption of energy in this region usually requires the presence of multiple bonds, especially two or more such bonds in conjugation. Saturated molecules normally do not absorb significantly in this region. The amount of an intermediate that can be detected depends on how strongly it absorbs relative to other components of the reaction systems. In favorable cases, concentrations as low as 10^{-6} M can be detected.

Infrared (IR) spectrometers measure absorption of energy by excitation of molecular vibrations, including stretching, bending, and twisting of various parts of the molecule. Most organic molecules have a large number of bands in the IR spectrum. While it is usually not possible to assign every band to a specific vibration, individual bands can be highly characteristic of a specific molecule. Nearly all of the organic functional groups also have one or more regions of characteristic absorption. If it is suspected that a particular functional group is present in an intermediate, examination of the changes in the spectrum in the characteristic region of the functional group may permit detection. An example of IR detection of intermediates can be drawn from a study of the photochemical conversion of **1** to **3**. It was suspected that the ketene **2** might be an intermediate.[39]



1 **2** **3**

Ketenes absorb near 2100–2130 cm^{-1}. When the photolysis was carried out and the IR spectrum of the solution monitored, it was found that a band appeared at 2118 cm^{-1}, grew, and then decreased as photolysis proceeded. The observation of this characteristic absorption constitutes good evidence for a ketone intermediate. As with UV-VIS spectroscopy, the amount of intermediate that can be detected depends both on the intensity of the absorption band and the presence of interfering bands. In general, IR requires somewhat higher concentration for detection than does UV-VIS.

71042 39. O. L. Chapman and J. D. Lassila, *J. Am. Chem. Soc.* **90**, 2449 (1968).

Either UV-VIS or IR spectroscopy can be combined with the technique of *matrix isolation* to detect and identify highly unstable intermediates. In this method, the intermediate is trapped in a solid inert matrix, usually one of the inert gases, at very low temperatures. Since each molecule is surrounded by inert gas atoms, there is no possibility for intermolecular reactions and the rates of intramolecular reactions are slowed by the low temperature. Matrix isolation is a very useful method for looking for intermediates in photochemical reactions. The method can also be used for gas phase reactions that can be conducted in such a way that the intermediates can be rapidly condensed into the matrix.

Nuclear magnetic resonance (NMR) is very widely used for detection of intermediates in organic reactions. Proton magnetic resonance is most useful because ^1H provides the greatest sensitivity of detection among the nuclei of interest in organic chemistry. Fluorine-19 and phosphorus-31 are other elements that provide high sensitivity. Carbon-13, oxygen-17, and nitrogen-15 provide relatively lower sensitivity, but isotopic enrichment is possible. Initially, NMR suffered from very low sensitivity relative to other spectroscopic methods. However, the development of high-field instruments and the use of Fourier transform methods have greatly increased sensitivity so that NMR can now be used to detect many reaction intermediates.

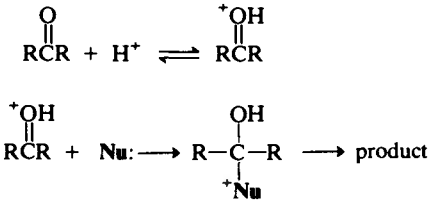
Free radicals and other intermediates with unpaired electrons can be detected in extremely low concentration by electron paramagnetic resonance (EPR). This technique measures the energy absorbed to reorient an electron's spin in a magnetic field. It provides structural information on the basis of splitting of the signal by adjacent nuclei, much as in NMR interpretation. EPR is not only extremely sensitive, but also very specific. Diamagnetic molecules present in solution give no signals, and the possibility for interference is therefore greatly decreased. The method can only be applied to reactions involving paramagnetic intermediates.

All other spectroscopic methods are equally applicable, in principle, to the detection of reaction intermediates so long as the method provides sufficient structural information to assist in the identification of the transient species. In the use of all methods, including those discussed above, it must be remembered that simple detection does not prove the species is an intermediate. It also must be shown that the species is converted to product. In favorable cases, this may be done by isolation or trapping experiments. More often, it may be necessary to determine the kinetic behavior of the appearance and disappearance of the intermediate and demonstrate that it is consistent with the species being an intermediate.

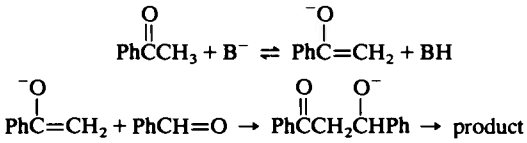
4.8. Catalysis by Acids and Bases

A detailed understanding of a reaction mechanism requires knowledge of the role catalysts play in the reaction. Catalysts cannot affect the position of equilibrium of a reaction. They function by increasing the rate of one or more steps in a reaction mechanism by providing a reaction path having a lower activation energy. The most

general family of catalytic processes are those that involve transfer of a proton. Many reactions involving neutral reactants are strongly catalyzed by proton donors (Brønsted acids) or proton acceptors (Brønsted bases). Catalysis occurs when the conjugate base or conjugate acid of the substrate is a more reactive molecule than the neutral species. For example, reactions involving nucleophilic attack at carbonyl groups are often accelerated by acids. This type of catalysis occurs because the conjugate acid of the carbonyl compound is much more electrophilic than the neutral molecule.



Many important organic reactions involve nucleophilic carbon species (carbanions). The properties of carbanions will be discussed in detail in Chapter 7 and in Part B, Chapters 1 and 2. Most C—H bonds are very weakly acidic and have no tendency to ionize spontaneously to form carbanions. Reactions that involve carbanion intermediates are therefore usually carried out by reaction of the neutral organic molecule and the electrophile in the presence of a base that can generate the more reactive carbanion intermediate. Base-catalyzed condensation reactions of carbonyl compounds provide many examples of this type of reaction. The reaction between acetophenone and benzaldehyde which was considered in Section 4.2, for example, requires a basic catalyst to proceed, and the kinetics of the reaction show that the rate is proportional to the catalyst concentration. This is because the neutral acetophenone molecule is not nucleophilic and does not react with benzaldehyde. The enolate (carbanion) formed by deprotonation is much more nucleophilic.



The role that acid and base catalysts play can be quantitatively studied by kinetic techniques. It is possible to recognize several distinct types of catalysis by acids and bases. The term *specific acid catalysis* is used when the reaction rate is dependent on the equilibrium for protonation of the reactant. This type of catalysis is independent of the concentration and specific structure of the various proton donors present in solution. Specific acid catalysis is governed by the *hydrogen ion concentration* (pH) of the solution. For example, for a series of reactions in an aqueous buffer system, the rate of the reaction would be a function of the pH, but not of the concentration or identity of the acidic and basic components of the buffer. The kinetic expression for any such reaction will include a term for hydrogen ion concentration, $[H^+]$. When the nature and concentration of proton donors present

in solution affect the reaction rate, the term used is *general acid catalysis*. The kinetic expression for such a reaction will include terms for each of the potential proton donors that act as catalysts. The terms *specific base catalysis* and *general base catalysis* apply in the same way to base-catalyzed reactions.

Specific acid catalysis:

$$\text{rate} = k[H^+][X][Y], \qquad \text{where } [X][Y] = \text{concentration of the reactants}$$

General acid catalysis:

$$\text{rate} = k_1[H^+][X][Y] + k_2[HA^1][X][Y] + k_3[HA^2][X][Y],$$

where HA^1, HA^2 ... are all kinetically significant proton donors

The experimental detection of general acid catalysis is done by rate measurements at constant pH but differing buffer concentration. Since under these circumstances $[H^+]$ is constant but the weak acid component(s) of the buffer (HA^1, HA^2, etc.) changes, the observation of a change in rate is evidence of general acid catalysis. If the rate remains constant, the reaction exhibits specific acid catalysis. Similarly, general base-catalyzed reactions will show a dependence of the rate on the concentration and identity of the basic constituents of the buffer system.

Specific acid catalysis is observed when a reaction proceeds only through a protonated intermediate which is in equilibrium with its conjugate base. Since the position of this equilibrium is a function of the concentration of solvated protons, only a single acid-dependent term appears in the kinetic expression. For example, in a two-step reaction involving rate-determining reaction of one reagent with the conjugate acid of a second, the kinetic expression will be as follows:

$$B + H^+ \overset{\text{fast}}{\rightleftharpoons} BH^+ \qquad BH^+ + C \overset{\text{slow}}{\longrightarrow} B{-}C + H^+$$

$$\text{rate} = k_2[BH^+][C] = k_2[C]K[B][H^+]$$

$$= k_{obs}[H^+][B][C]$$

Several situations can lead to the observation of general acid catalysis. General acid catalysis can occur as a result of hydrogen bonding between the reactant R and a proton donor D—H to form a reactive complex {D-H ... R} which then reacts with a substance Z:

$$D{-}H + R \rightleftharpoons D{-}H\cdots R$$

$$D{-}H\cdots R + Z \overset{\text{slow}}{\longrightarrow} D{-}H + R{-}Z$$

Under these circumstances, a distinct contribution to the overall rate will be seen for each potential hydrogen bond donor D—H. General acid catalysis is also observed when a rate-determining proton transfer occurs from acids other than the solvated proton:

$$R + HA \overset{\text{slow}}{\longrightarrow} {}^+RH + A^-$$

$${}^+RH \rightarrow \text{product}$$

Each acid HA^1, HA^2, etc., will then make a contribution to the overall rate of the reaction.

A kinetic expression which is equivalent to that for general acid catalysis also occurs if a prior equilibrium between reactant and the acids is followed by rate-controlling proton transfer. Each individual conjugate base will appear in the overall rate expression:

$$R + HA \rightleftharpoons {}^{+}RH + A^{-}$$

$${}^{+}RH + A^{-} \xrightarrow{\text{slow}} \text{product} + HA$$

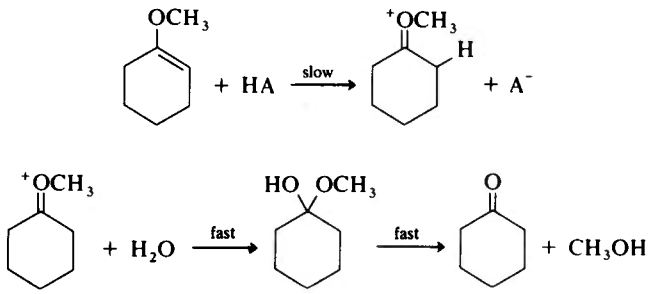
Notice that specific acid catalysis describes a situation where the reactant is in equilibrium with regard to proton transfer, and proton transfer is not rate-determining. On the other hand, each case that leads to general acid catalysis involves proton transfer in the rate-determining step. Because of these differences, the study of rates as a function of pH and buffer concentrations can permit conclusions about the nature of proton transfer processes and their relationship to the rate-determining step in a reaction.

As might be expected intuitively, there is a relationship between the effectiveness of general acid catalysts and the acidity of a proton donor as measured by its acid dissociation constant. This relationship is expressed by the following equation, which is known as the *Brønsted catalysis law*:

$$\log k_{\text{cat}} = \alpha \log K_a + b \tag{4.19}$$

An analogous equation holds for catalysis by bases. This equation requires that the free energies of activation for the catalytic step for a series of acids be directly proportional to the free energies of dissociation for the same series of acids. The proportionality constant σ is an indication of the sensitivity of the catalytic step to structural changes relative to the effect of the same structural changes on acid dissociation. It is often found that a single proportionality constant α is restricted to only structurally related types of acids and that α values of different magnitude are revealed by each type.

Figure 4.10 is plot of the Brønsted relationship for hydrolysis of an enol ether. The plot shows that the effectiveness of the various carboxylic acids as catalysts is related to their dissociation constants. In this particular case, the constant α is 0.79[40]:



40. A. J. Kresge, H. L. Chen, Y. Chiang, E. Murrill, M. A. Payne, and D. S. Sagatys, *J. Am. Chem. Soc.* **93**, 413 (1971).

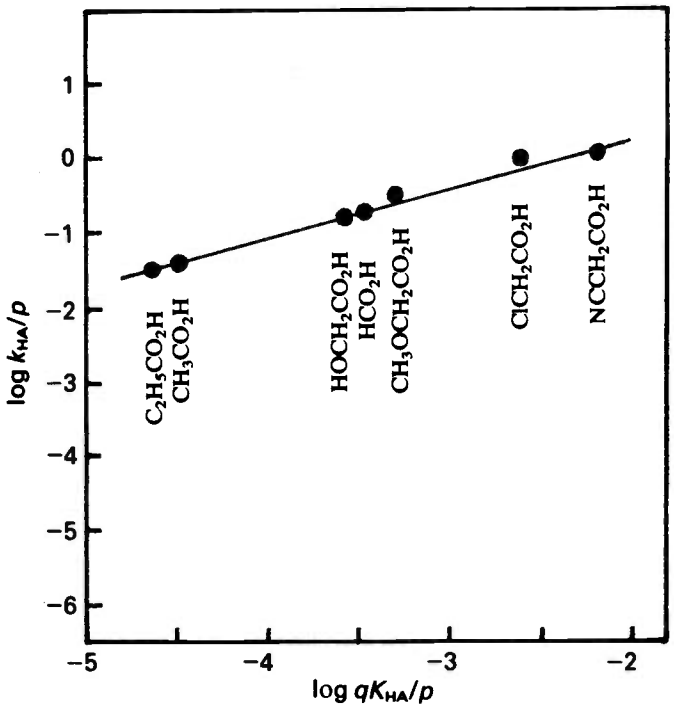


Fig. 4.10. Brønsted relation for the hydrolysis of methyl cyclohexenyl ether. (Adapted from Ref. 40 by permission of the American Chemical Society.)

Since α relates the sensitivity of the proton transfer to that of dissociation of the acid, it is frequently assumed that the value of α can be used as an indicator of transition state structure. The closer α approaches unity, the greater would be the degree of proton transfer in the transition state. There are limits to the generality of this interpretation, however.[41]

The details of proton transfer processes can also be probed by examination of *solvent isotope effects*, for example, by comparing the rates of a reaction in H_2O versus D_2O. The solvent isotope effect can be either normal or inverse, depending on the nature of the proton transfer process in the reaction mechanism. D_3O^+ is a stronger acid than H_3O^+. As a result, reactants in D_2O solution are somewhat more extensively protonated than in H_2O at identical acid concentration. A reaction that involves a rapid equilibrium protonation will proceed faster in D_2O than in H_2O, because of the higher concentration of the protonated reactant. On the other hand, if proton transfer is part of the rate-determining step, the reaction will be faster in H_2O than in D_2O because of the normal primary kinetic isotope effect of the type considered in Section 4.5.

41. A. J. Kresge, *J. Am. Chem. Soc.* **92**, 3210 (1970); R. A. Marcus, *J. Am. Chem. Soc.* **91**, 7224 (1969); F. G. Bordwell and W. J. Boyle, Jr., *J. Am. Chem. Soc.* **94**, 3907 (1972); D. A. Jencks and W. P. Jencks, *J. Am. Chem. Soc.* **99**, 7948 (1977); A. Pross, *J. Org. Chem.* **49**, 1811 (1984).

The interpretation of solvent isotope effects can be complicated by the large number of secondary isotope effects that can conceivably operate when it is the solvent molecule that is the site of isotopic substitution. The quantitative evaluation of solvent isotope effects is a very difficult problem. The relationship between the magnitude of the solvent isotope effect and the occurrence of equilibrium protonation as opposed to rate-limiting proton transfer is sufficiently general to be of value in mechanistic studies. As with nearly all mechanistic criteria, however, there are circumstances that permit exceptions, so corroborating evidence from other studies is always desirable.

Many organic reactions involve acid concentrations considerably higher than can be accurately measured on the pH scale, which applies to relatively dilute aqueous solutions. It is not difficult to prepare solutions in which the formal proton concentration is 10 *M* or more, but these formal concentrations are not a suitable measure of the *activity* of protons in such solutions. For this reason, it has been necessary to develop *acidity functions* to measure the proton-donating strength of concentrated acidic solutions. The activity of the hydrogen ion (solvated proton) can be related to the extent of protonation of a series of bases by the equilibrium expression for the protonation reaction:

$$B + H^+ \rightleftharpoons {}^+BH$$

$$K = \frac{(a^+_{BH})}{(a_{H^+})(a_B)} = \frac{[{}^+BH]\gamma^+_{BH}}{a_{H^+} + [B]\gamma_B}$$

where γ is the activity coefficient for the base and its conjugate acid. A common measure of acidity is referred to as h_0 and is defined by measuring the extent of protonation of a series of bases for which K has been measured. The relative concentration of the base and its conjugate acid then defines h_0 for any particular acidic solution.

$$h_0 = \frac{[{}^+BH]\gamma^+_{BH}}{K[B]\gamma_B}$$

The quantity H_0, defined as $-\log h_0$, is commonly tabulated, and it corresponds to the "pH" of very concentrated acidic solutions.

The problem of determining K independently of measurement of H_0 is the principal issue to be faced in establishing the H_0 scale for a series of acidic solutions. What is done is to measure K for some base in aqueous solution where $H_0 \approx$ pH. This base can then be used to find the H_0 of a somewhat more acidic solution. The K of a second, somewhat weaker base is then determined in the more acidic solution. This second base can then be used to extend H_0 into a still more acidic solution. The process is continued by using a series of bases to establish H_0 for successively more acidic solution. The H_0 is thereby referenced to the original aqueous measurement.[42] The assumption involved in this procedure is that the ratio of the activity

42. For reviews and discussion of acidity functions, see E. M. Arnett, *Prog. Phys. Org. Chem.* **1**, 223 (1963); C. H. Rochester, *Acidity Functions*, Academic Press, New York, 1970; R. A. Cox and K. Yates, *Can. J. Chem.* **61**, 225 (1983); C. D. Johnson and B. Stratton, *J. Org. Chem.* **51**, 4100 (1986).

coefficients for the series of bases and the series of cations does not change from solvent to solvent, that is,

$$\frac{\gamma^{+}_{B_1H}}{\gamma_{B_1}} = \frac{\gamma^{+}_{B_2H}}{\gamma_{B_2}} = \frac{\gamma^{+}_{B_3H}}{\gamma_{B_3}} \ldots \text{etc.}$$

Not unexpectedly, this procedure reveals some dependence on the particular type of base used, so no absolute H_0 scale can be established. Nevertheless, this technique provides a very useful measure of the relative hydrogen ion activity of concentrated acid solutions that can be used in the study of reactions that proceed only at high acid concentration. Table 4.6 gives H_0 values for some water–sulfuric acid mixtures.

4.9. Lewis Acid Catalysis

Lewis acids are defined as molecules which act as electron pair acceptors. The proton is an important special case, but many other species can play an important role in the catalysis of organic reactions. The most important in organic reactions are metal cations and covalent compounds of metals. Metal cations which play prominent roles as catalysts include the alkali metal monocations Li^+, Na^+, K^+, Cs^+, and Rb^+, the divalent ions Mg^{2+}, Ca^{2+}, and many of the transition metal cations. The most commonly employed of the covalent compounds include boron trifluoride, aluminum trichloride, titanium tetrachloride, and stannic tetrachloride. Various other derivatives of boron, aluminum, and titanium also are employed as Lewis acid catalysts.

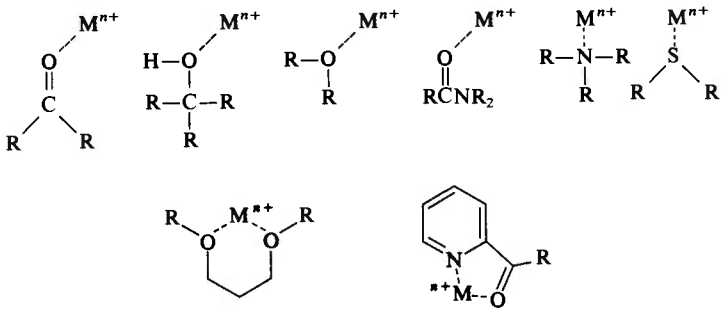
The catalytic activity of metal ions originates in the formation of a donor–acceptor complex between the cation and the reactant, which must act as a Lewis base. The result of the complexation is that the donor atom becomes effectively more electronegative. All functional groups that have unshared electron pairs are

Table 4.6. H_0 as a Function of Composition of Aqueous Sulfuric Acid[a]

$\%H_2SO_4$	H_0	$\%H_2SO_4$	H_0
5	0.24	55	−3.91
10	−0.31	60	−4.46
15	−0.66	65	−5.04
20	−1.01	70	−5.80
25	−1.37	75	−6.56
30	−1.72	80	−7.34
35	−2.06	85	−8.14
40	−2.41	90	−8.92
45	−2.85	95	−9.85
50	−3.38	98	−10.41

a. From M. J. Jorgenson and D. R. Hartter, *J. Am. Chem. Soc.* **85**, 878 (1963).

potential electron donors, but especially prominent in reaction chemistry are carbonyl (sp^2) oxygens, hydroxyl and ether (sp^3) oxygens, and nitrogen- and sulfur-containing functional groups. Halogen substituents can act as donors to very strong Lewis acids. The presence of two potential donor atoms in a favorable geometric relationship permits formation of bidentate 'chelate" structures and may lead to particularly strong complexes.



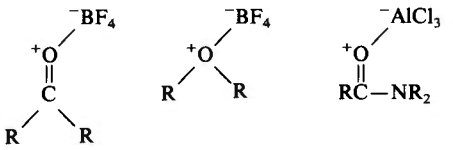
If the complexation process is regarded as resulting in a full covalent bond between the donor and the Lewis base, there is a net transfer of one unit of formal charge to the metal ion from the donor atom. This enhances the electronegativity of the donor atom. The complexes of carbonyl groups, for example, are more reactive to nucleophilic attack. Hydroxyl groups complexed to metal cations are stronger acids than the uncomplexed hydroxyl. Ether or sulfide groups complexed with metal ions are better leaving groups.

The strength of the complexation is a function both of the donor atom and the metal ion. The solvent medium is also an important factor because solvent molecules that have potential electron donor atoms can compete for the Lewis acid. Qualitative predictions about the strength of donor-acceptor complexation can be made on the basis of the hard-soft-acid-base concept. The better matched the donor and acceptor, the stronger is the complexation. Scheme 4.3 gives an ordering or hardness and softness for some neutral and ionic Lewis acids and bases.

Scheme 4.3. Relative Ordering of Hardness and Softness

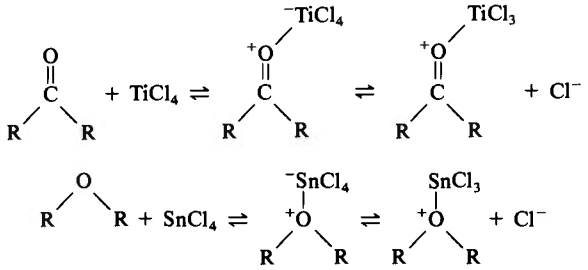
	Lewis acids		Lewis bases	
	Cationic	Neutral	Neutral	Anionic
Hard	H^+	BF_3, $AlCl_3$	H_2O	F^-, SO_4^-
	Li^+, Na^+, Ca^{2+}	R_3B	Alcohols, ketones, ethers	Cl^-
				Br^-
	Zn^{2+}, Cu^{2+}		Amines (aliphatic)	N_3^-
			Amines (aromatic)	
	Pd^{2+}, Hg^{2+}, Ag^+			CN^-
	RS^+, RSe^+			I^-
Soft	I^+		Sulfides	S^{2-}

Compounds such as boron trifluoride and aluminum chloride form complexes by accepting an electron from the donor molecule. The same functional groups that act as lone pair donors to metal cations form complexes with boron trifluoride, aluminum trichloride, and related compounds.

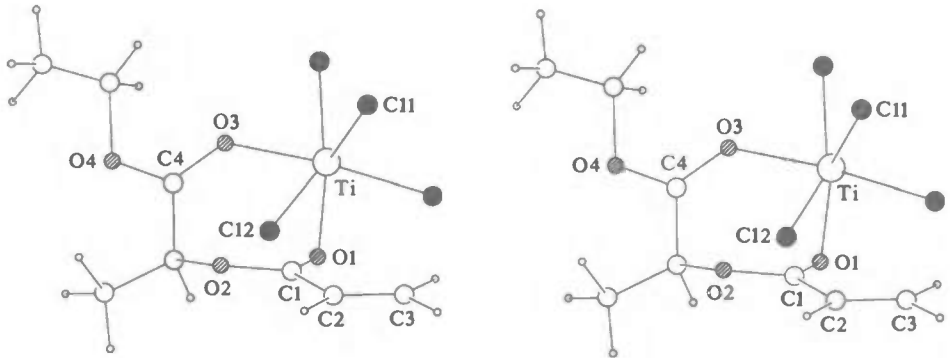


Since in this case the complex is formed between two neutral species, it, too, is neutral, but a formal positive charge develops on the donor atom, and a formal negative charge on the acceptor atom. The result is to increase the effective electronegativity of the donor atom and increase the electrophilicity of the complexed functional group.

Titanium tetrachloride and stannic chloride can form complexes that are related in character to both those formed by metal ions and those formed by neutral Lewis acids. Complexation can occur with displacement of a chloride from the metal coordination sphere or by an increase in the coordination number at the Lewis acid.

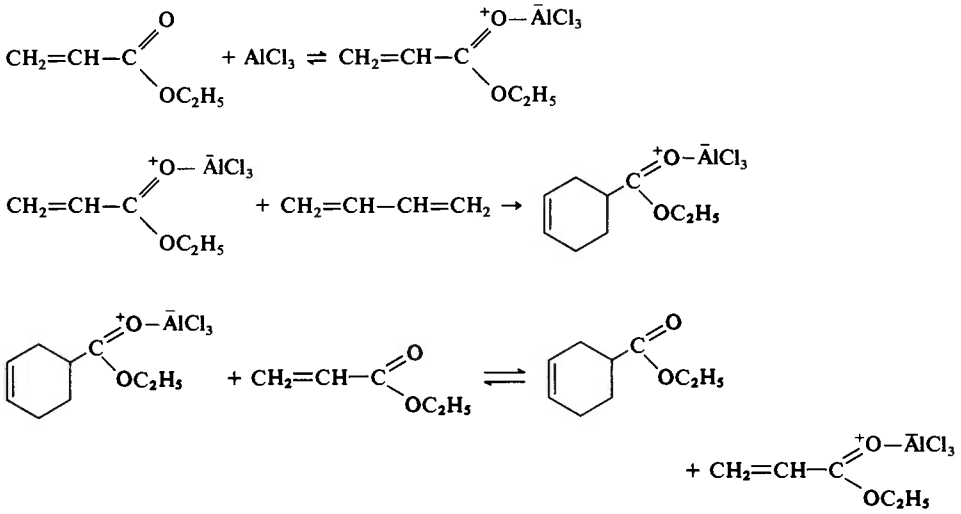


The crystal structure of the adduct of titanium tetrachloride and the ester formed from ethyl 2-hydroxypropanoate (ethyl lactate) and acrylic acid has been solved.[43] It is a chelated structure with the oxygen donor atoms being incorporated into the titanium coordination sphere along with the four chloride anions.



43. T. Poll, J. O. Melter, and G. Helmchen, *Angew. Chem. Int. Ed. Engl.* **24**, 112 (1985).

Diels–Alder reactions represent a type of reaction in which a Lewis acid is often used in catalytic quantities. The complexed ester (ethyl acrylate in the example given below) is substantially more reactive than the uncomplexed molecule, and the reaction proceeds through the complex. The reactive complex is regenerated by exchange of the Lewis acid from the adduct.



4.10. Solvent Effects

Most organic reactions are done in solution, and it is therefore important to recognize some of the general ways in which solvent can affect the course and rates of reactions. Some of the more common solvents can be roughly classified as in Table 4.7 on the basis of their structure and dielectric constants. There are important differences between *protic* solvents—solvents that contain relatively mobile protons such as those bonded to oxygen, nitrogen, or sulfur—and *aprotic* solvents, in which all hydrogen is bonded to carbon. Similarly, *polar* solvents, those that have high dielectric constants, have effects on reaction rates that are different from those of *nonpolar* solvent media.

When discussing solvent effects, it is important to distinguish between the macroscopic effects of the solvent and effects that depend upon details of structure. Macroscopic properties refer to properties of the bulk solvent. An important example is the dielectric constant, which is a measure of the ability of the bulk material to increase the capacitance of a condenser. In terms of structure, the dielectric constant is a function of both the permanent dipole of the molecule and its *polarizability.* Polarizability refers to the ease of distortion of the molecule's electron density. Dielectric constants increase with dipole moment and with polarizability. An important property of solvent molecules with regard to reactions is the response of the solvent to changes in charge distribution as the reaction occurs. The dielectric

Table 4.7. Dielectric Constants of Some Common Solvents[a]

Aprotic solvents				Protic solvents	
Nonpolar		Polar			
Hexane	1.9	Pyridine	12	Acetic acid	6.1
Carbon tetrachloride	2.2	Acetone	21	Trifluoroacetic acid	8.6
Dioxane	2.2	Hexamethyl phosphoramide	30	*tert*-Butyl alcohol	12.5
Benzene	2.3	Nitromethane	36	Ammonia	(22)
Diethyl ether	4.3	Dimethylformamide	37	Ethanol	24.5
Chloroform	4.8	Acetonitrile	38	Methanol	32.7
Tetrahydrofuran	7.6	Dimethyl sulfoxide	47	Water	78

a. Dielectric constant data are abstracted from the compilation of solvent properties in J. A. Riddick and W. B. Bunger (eds.), *Organic Solvents*, Vol. II of *Techniques of Organic Chemistry*, Third Edition, Wiley-Interscience, New York, 1970.

constant of a solvent is a good indicator of the ability of the solvent to accommodate separation of charge. It is not the only factor, however, since, being a macroscopic property, it conveys little information about the ability of the solvent molecules to interact with the solute molecules at close range. These direct solute-solvent interactions will depend on the specific structures of the molecules.

Let us consider how solvent might affect the solvolysis of *t*-butyl chloride. Much evidence, which will be discussed in detail in Chapter 5, indicates that the rate-determining step of the reaction is ionization of the carbon-chlorine bond to give a carbocation and the chloride ion. The transition state must reflect some of the charge separation that occurs in the ionization. Figure 4.11 gives a schematic interpretation of the solvation changes which would take place during the ionization of *t*-butyl chloride, with S representing surrounding solvent molecules.

The bulk dielectric constant may be a poor indicator of the ability of solvent molecules to facilitate the charge separation in the transition state. The fact that the carbon and chlorine remain partially bonded at the transition state prevents the solvent molecules from actually intervening between the developing centers of charge. Instead, the solvent molecules must stabilize the charge development by acting around the periphery of the activated complex. This interaction will depend

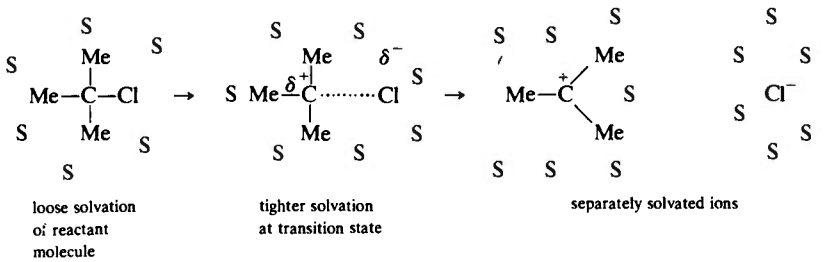


Fig. 4.11. Solvation changes during ionization of *t*-butyl chloride.

Table 4.8. *Y* Values for Some Solvent Systems[a]

Ethanol–Water		Methanol–Water			
(% ethanol)	*Y*	(% methanol)	*Y*	Other solvents	*Y*
100	−2.03	100	−1.09	Acetic acid	−1.64
80	0.0	80	0.38	Formic acid	2.05
50	1.65	50	1.97	*t*-Butyl alcohol	−3.2
20	3.05	10	3.28	90% Acetone–Water	−1.85
0	3.49			90% Dioxane–Water	−2.03

a. From A. H. Fainberg and S. Winstein, *J. Am. Chem. Soc.* **78**, 2770 (1956).

upon the detailed structure of the activated complex and the solvent. The ability of a number of solvents to stabilize the transition state of *t*-butyl chloride ionization has been measured by comparing the rate of the reaction in the various solvents. The reference solvent was taken as 80:20 ethanol–water. The *Y* value of other solvents is defined by the equation

$$\log \frac{k_{\text{solvent}}}{k_{80\%\text{ ethanol}}} = Y$$

Table 4.8 lists the *Y* values for some alcohol–water mixtures and for some other solvents. Notice that among the solvents listed there is a spread of more than 10^6 in the measured rate of reaction between *t*-butyl alcohol and water. This large range of reaction rates demonstrates how important solvent effects can be. The *Y* value reflects primarily the ionization power of the solvent. It is largest for polar solvents such as water and formic acid and becomes progressively smaller and eventually negative as the solvent becomes less polar and contains more (or larger) nonpolar alkyl groups.

Solvents that fall in the nonpolar aprotic class are not effective at stabilizing the development of charge separation. These molecules have small dipole moments and do not have hydrogens capable of forming hydrogen bonds. Reactions that involve charge separation in the transition state therefore usually proceed much more slowly in this class of solvents than in protic or polar aprotic solvents. The reverse is true for reactions in which species having opposite charges come together in the transition state. Since in this case the transition state is less highly charged than the individual reactants, it is favored by the poor solvation, which leaves the oppositely charge reactants in a very reactive state. Arguing along these lines, the broad relationships between reactivity and solvent type shown in Scheme 4.4 can be deduced.

Many other measures of solvent polarity have been developed.[44] One of the most useful is based on shifts in the absorption spectrum of a reference dye. The positions of absorption bands in general are sensitive to solvent polarity because the electronic distribution, and therefore the polarity, of the excited state is different

44. C. Reichardt, *Angew. Chem. Int. Ed. Engl.* **18**, 98 (1979); C. Reichardt, *Solvent Effects in Organic Chemistry*, Verlag Chemie, Weinheim, 1979.

Table 4.9. $E_T(30)$, an Empirical Measure of Polarity, Compared with Dielectric Constant

	$E_T(30)$	ε		$E_T(30)$	ε
Water	63.1	78	Dimethylformamide	43.8	37
CF_3CH_2OH	59.5		Acetone	42.2	21
Methanol	55.5	32.7	CH_2Cl_2	41.1	8.9
80:20 ethanol-water	53.7		$CHCl_3$	39.1	4.8
Ethanol	51.9	24.5	$CH_3CO_2C_2H_5$	38.1	6.0
Acetic acid	51.2	6.1	Tetrahydrofuran	37.4	7.6
Isopropanol	48.6	19.9	Diethyl ether	34.6	4.3
Acetonitrile	46.7	38	Benzene	34.5	2.3
Nitromethane	46.3	36	Hexane	30.9	1.0
Dimethyl sulfoxide	45.0	47			

a. Data from C. Reichardt, *Angew. Chem. Int. Ed. Engl.* **18**, 98 (1979).

Scheme 4.4. Effect of Solvent Polarity on Reactions of Various Charge Types

$A^- + B^+ \longrightarrow \overset{\delta-}{A}\cdots\overset{\delta+}{B} \longrightarrow A\text{–}B$	Favored by nonpolar solvent
$A\text{–}B \longrightarrow \overset{\delta-}{A}\cdots\overset{\delta+}{B} \longrightarrow A^- + B^+$	Favored by polar solvent
$A + B \longrightarrow A\cdots B \longrightarrow A\text{–}B$	Relatively insensitive to solvent polarity
$A\text{–}B^+ \longrightarrow \overset{\delta+}{A}\cdots\overset{\delta+}{B} \longrightarrow A + B^+$	Slightly favored by polar solvent
$A^+ + B \longrightarrow \overset{\delta+}{A}\cdots\overset{\delta+}{B} \longrightarrow A\text{–}B^+$	Slightly favored by nonpolar solvent

from that of the ground state. The shifts in the absorption maxima reflect the effect of solvent on the energy gap between the ground state and excited state molecules. An empirical solvent polarity measure called $E_T(30)$ is based on this concept.[45] Some values for common solvents are given in Table 4.9 along with the dielectric constants for the solvents. It can be seen that there is a rather different order of polarity given by these two quantities.

The electrostatic solvent effects discussed in the preceding paragraphs are not the only possible modes of interaction of solvent with reactants and transition states. Specific structural effects may cause either the reactants or transition state to be particularly strongly solvated. Figure 4.12 shows how such solvation can affect the relative energies of the ground state and transition state and cause rate variations from solvent to solvent. Unfortunately, no general theory for quantitatively predicting such specific effects has been developed to date.

Since a solvent may affect the rates of two competing reactions to different extents, a change in solvent may strongly modify the composition of a product

45. C. Reichardt and K. Dimroth, *Fortshr. Chem. Forsch.* **11**, 1 (1968); C. Reichardt, *Justus Liebigs Ann. Chem.* **752**, 64 (1971).

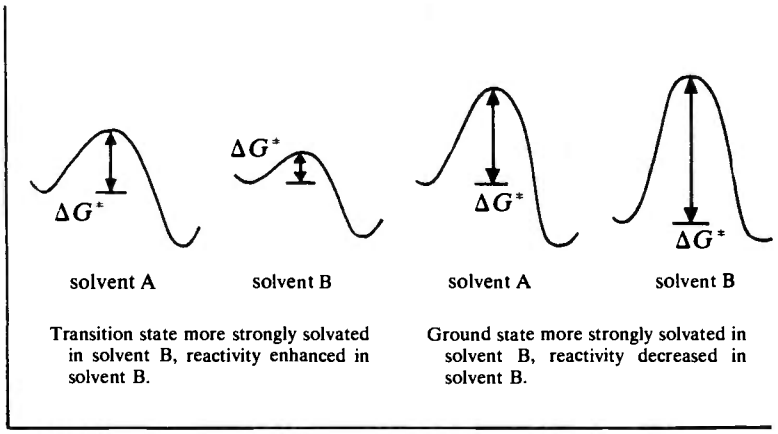
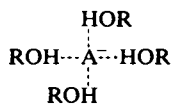
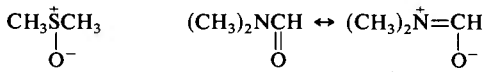


Fig. 4.12. Potential energy diagrams showing effect of preferential solvation of (a) transition state and (b) ground state on the activation energy.

mixture arising from competing reaction paths. Many such instances have been encountered in synthetic chemistry. An important example of solvent effects is the enhanced nucleophilicity of many anions in polar aprotic solvents as compared with protic solvents.[46] In protic solvents, anions are strongly solvated by hydrogen bonding. This is particularly true for anions that have a high concentration of charge on oxygen or nitrogen. This hydrogen bonding decreases the availability of the electrons of the anion to participate in reactions as a nucleophile.



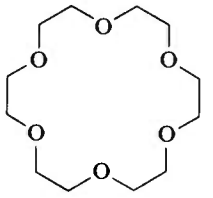
In aprotic solvents no hydrogens suitable for hydrogen bonding are present. As a result, the electrons of the anion are more easily available for reaction. Stated another way, the anion is at a higher energy level because of the absence of solvent stabilization. The polarity of the aprotic solvents is important because ionic compounds have very low solubility in nonpolar solvents. Dissolved ionic compounds are likely to be present as ion pairs or larger aggregates in which the reactivity of the anion is diminished by the electrostatic interaction with the cation. Energy must be expended against this electrostatic attraction to permit the anion to react as a nucleophile. Metal cations such as K^+ and Na^+ are strongly solvated by polar aprotic solvents such as dimethyl sulfoxide and dimethylformamide. The oxygen atoms in these molecules act as electron donors toward the cations. The dissolved salts are dissociated and as a result the anions are highly reactive because they are poorly solvated and not associated with cations.



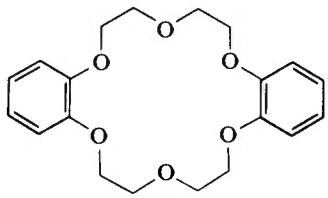
46. A. J. Parker, *Q. Rev. Chem. Soc.* **16**, 163 (1962); C. D. Ritchie, in *Solute-Solvent Interactions*, J. F. Coetzee and C. D. Ritchie (eds.), Marcel Dekker, New York, 1969, Chapter 4; E. Buncel and H. Wilson, *Adv. Phys. Org. Chem.* **14**, 133 (1977).

The realization that the nucleophilicity of anions is strongly enhanced in polar aprotic solvents has led to important improvements of several types of synthetic processes that involve nucleophilic substitutions or additions.

Particularly striking examples of the effect of specific solvation can be cited from among the *crown ethers.* These are macrocyclic polyethers that have the property of specifically solvating cations such as Na^+ and K^+.



18-crown-6



dibenzo-18-crown-6

When added to nonpolar solvents, the crown ethers increase the solubility of ionic materials. For example, in the presence of 18-crown-6, potassium fluoride is soluble in benzene and acts as a reactive nucleophile:

$$CH_3(CH_2)_7Br + KF \xrightarrow[\text{benzene}]{\text{18-crown-6}} CH_3(CH_2)_7F \qquad \text{Ref. 47}$$

In the absence of the polyether, potassium fluoride is insoluble in benzene and unreactive toward alkyl halides. Similar enhancement of solubility and reactivity of other salts is observed in the presence of crown ethers. The solubility and reactivity enhancement results because the ionic compound is dissociated to a tightly complexed cation and a "naked" anion. Figure 4.13 shows the tight coordination that can be achieved with a typical crown ether. The complexed cation, since it is surrounded by the nonpolar crown ether, has high solubility in the nonpolar media. To maintain electroneutrality, the anion is also transported into the solvent. The cation is shielded from interaction with the anion by the surrounding crown ether molecule. As a result, the anion is unsolvated and at a relatively high energy and therefore highly reactive.

A closely related solvation phenomenon is the basis for *phase transfer catalysis.* Phase transfer catalysts are salts in which one of the ions (usually the cation) has large nonpolar substituent groups which confer good solubility in organic solvents. The most common examples are tetraalkylammonium and tetraalkylphosphonium ions. In two-phase systems consisting of water and a nonpolar organic solvent, these cations are extracted into the organic phase and, as a result, anions are also present in the organic phase. The anions are weakly solvated and again display high reactivity. Reactions are carried out between a salt containing the desired nucleophilic anion and an organic reactant, typically, an alkyl halide. The addition of the phase transfer catalysts causes migration of the anion into the organic phase, and, because of the high nucleophilicity of the anion, reaction occurs under excep-

47. C. Liotta and H. P. Harris, *J. Am. Chem. Soc.* **96**, 2250 (1974).

tionally mild conditions. Section 3.2 of Part B gives some specific examples of the use of phase transfer catalysis in nucleophilic displacement reactions.

It should always be borne in mind that solvent effects can modify the energy of both the reactants and the transition state. It is the *difference* in the two solvation effects that is the basis for changes in activation energies and reaction rates. Thus, although it is common to see solvent effects discussed solely in terms of reactant solvation or transition state solvation, this is usually an oversimplification. One case that illustrates this point is the hydrolysis of esters by hydroxide ion.

$$^{-}OH + CH_3\overset{\overset{O}{\|}}{C}OC_2H_5 \rightleftharpoons H_3C-\overset{\overset{O^{\delta-}}{\vdots}}{\underset{\underset{HO^{\delta-}}{\vdots}}{C}}-OC_2H_5 \longrightarrow \text{product}$$

The reaction is found to be much more rapid in dimethyl sulfoxide–water than in ethanol–water. Reactant solvation can be separated from transition state solvation by calorimetric measurement of the heat of solution of the reactants in each solvent system. The data in Fig. 4.14 compare the energies of the reactants and the transition state for ethyl acetate and hydroxide ion reacting in aqueous ethanol versus aqueous dimethyl sulfoxide. It can be seen that both the reactants and the transition state

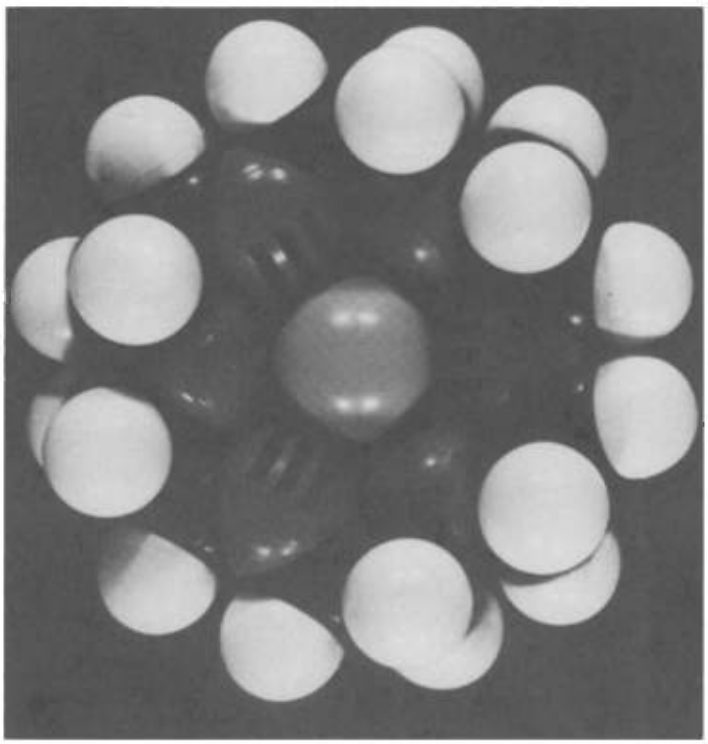


Fig. 4.13. Space-filling molecular model depicting a metal cation complexed by 18-crown-6.

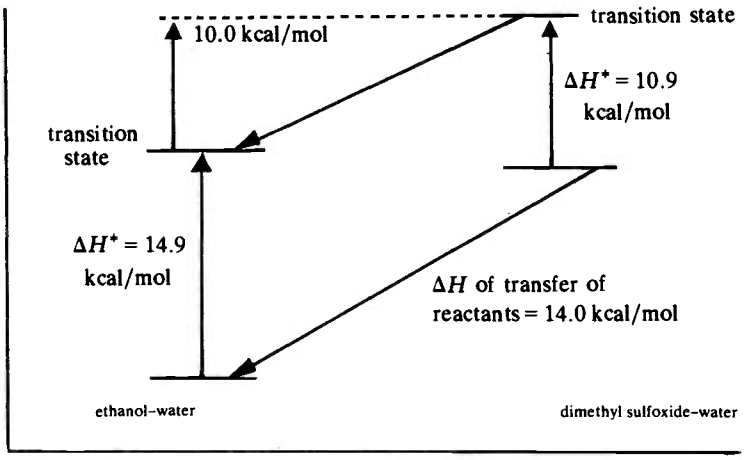


Fig. 4.14. Reactant and transition state solvation in the reaction of ethyl acetate with hydroxide ion. [From P. Haberfield, J. Friedman, and M. F. Pinkston, *J. Am. Chem. Soc.* **94**, 71 (1972).]

are more strongly solvated in the ethanol-water medium. The enhancement in reaction rate comes from the fact that the difference is greater for the small hydroxide ion than for the larger anionic species present at the transition state. It is generally true that solvation forces are strongest for the small, hard anions and decrease with size and softness.

4.11. Structural Effects in the Gas Phase

Having considered how solvents can strongly affect the properties of molecules in solution, let us consider some of the special features that arise in the gas phase, where solvation effects are totally eliminated. Although the majority of organic preparative reactions and mechanistic studies have been conducted in solution, some important reactions are carried out in the gas phase. Also, since current theoretical calculations generally do not treat solvent effects, experimental data from the gas phase are the most appropriate basis for comparison with theoretical results. Frequently, quite different trends in substituent effects are seen when systems in the gas phase are compared to similar systems in solution.

It is possible to measure equilibrium constants and heats of reaction in the gas phase by using mass spectrometers of special configuration.[48] With proton transfer reactions, for example, the equilibrium constant can be determined by measuring the ratio of two reactant species competing for protons. Table 4.10 compares ΔH_{gas}

48. Discussion of the techniques for gas phase equilibrium measurements can be found in T. A. Lehman and M. M. Bursey, *Ion Cyclotron Resonance Spectrometry*, Wiley-Interscience, New York, 1976, and in M. T. Bowers (ed.), *Gas Phase Ion Chemistry*, Vols. 1 and 2, Academic Press, New York, 1979.

Table 4.10. Comparison of Substituent Contributions to Phenol Ionization in the Gas Phase and Solution[a]

X	Substituent increment in kcal/mol[b]			
	ΔG_{gas}	ΔH_{gas}	ΔG_{H_2O}	ΔH_{H_2O}
m-CH_3	+0.4	+0.4	+0.18	+0.02
p-CH_3	+1.3	+1.3	+0.42	+0.02
m-Cl	−7.9	−7.9	−1.2	−0.3
p-Cl	−6.6	−6.6	−0.7	−0.2
p-NO_2	−25.8	−25.8	−3.8	−0.8

a. Data are from T. B. McMahon and P. Kebarle, *J. Am. Chem. Soc.* **99**, 2222 (1977).
b. The tabulated increments give the change in ΔG and ΔH resulting from replacement of hydrogen by the substituent specified.

with ΔH_{H_2O} for a series of phenol ionizations.



A key point to recognize is that the relative magnitude of the substituent effects is much larger in the gas phase. In general terms, this can be explained on the basis that all sovents effects have been removed. Whereas a phenolate anion in aqueous solution is stabilized by hydrogen bonding, there is no such stabilization in the gas phase. Since the solvent stabilization will be rather similar, on an absolute scale, for all the phenolate anions, the magnitudes of the substituent effects are "leveled" to some extent. In contrast, in the gas phase the importance of the internal substituent effect on stability of the anion is undiminished. The importance of the solvation can also be judged by noting that entropy makes a larger contribution to ΔG than enthalpy for the reaction series in solution. This reflects the extensive solvent organization that accompanies solvation.[49]

A comparison of phenol acidity in dimethyl sulfoxide versus the gas phase also shows an attenuation of substituent effects, but not nearly as much as in water. While the effect of substituents on ΔG for deprotonation in aqueous solution is about one-sixth that in the gas phase, the ratio for dimethyl sulfoxide is about one-third. This result points to hydrogen bonding of the phenolate anion by water as the major difference in the solvating properties of water and dimethyl sulfoxide.[50]

Another example of enhanced sensitivity to substituent effects in the gas phase can be seen in a comparison of the gas phase basicity for a series of substituted acetophenones and methyl benzoates. It was found that sensitivity of the free energy

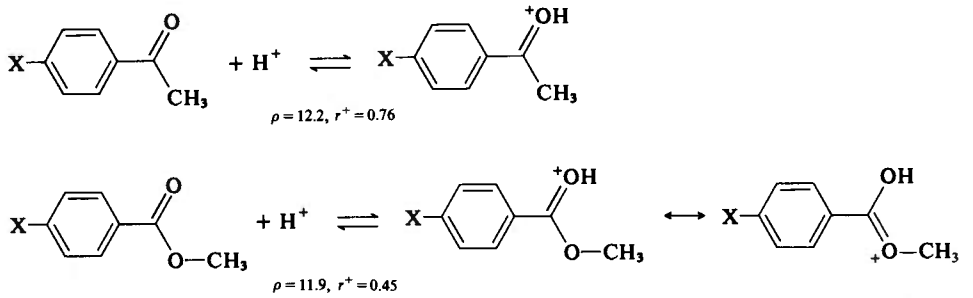
49. L. P. Fernandez and L. G. Hepler, *J. Am. Chem. Soc.* **81**, 1783 (1959); C. L. Liotta, H. P. Hopkins, Jr., and P. T. Kasudia, *J. Am. Chem. Soc.* **96**, 7153 (1974).
50. M. Mashima, R. T. McIver, Jr., R. W. Taft, F. G. Bordwell and W. N. Olmstead, *J. Am. Chem. Soc.* **106**, 2717 (1984); M. Fujio, R. T. McIver, and R. W. Taft, *J. Am. Chem. Soc.* **103**, 4017 (1981).

Table 4.11. Acidities of Simple Alcohols in Solution[a]

	pK_a	
	H_2O	DMSO
H_2O	15.7	31.4
CH_3OH	15.5	29.0
C_2H_5OH	15.9	29.8
$(CH_3)_2CHOH$		30.2
$(CH_3)_3COH$		32.2

a. Data are from W. N. Olmstead, Z. Margolin, and F. G. Bordwell, *J. Org. Chem.* **45**, 3295 (1980).

to substituent changes was about four times that in solution, as measured by the comparison of ΔG for each substituent.[51] The gas phase data for both series were correlated by the Yukawa-Tsuno equation. For both series, the ρ value was about 12. However, the parameter r^+, which reflects the contribution of extra resonance effects, was greater in the acetophenone series than in the methyl benzoate series. This can be attributed to the substantial resonance stabilization provided by the methoxy groups in the esters. This diminishes the extent of conjugation with the substituents.



Another area of gas phase substituent effects which has attracted interest is the acidity of simple alcohols. In the gas phase, the order is *t*-BuOH > EtOH > MeOH ≫ H_2O.[52] This is different from the order in solution as revealed by the pK data in water and DMSO shown in Table 4.11.

These changes in relative acidity can again be traced to solvation effects. In the gas phase, any substituent effect can be analyzed directly in terms of its stabilizing or destabilizing effect on the anion. Two factors are believed to be of primary importance. The inductive effect arising from the H—C bond dipoles should be destabilizing since they tend to increase electron density at the carbon bonded to oxygen. This dipolar interaction is removed by replacing hydrogens with methyl

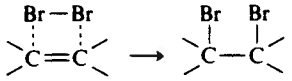
51. M. Mishima, M. Fujo, and Y. Tsuno, *Tetrahedron Lett.* **27**, 939, 951 (1986).
52. J. I. Brauman and L. K. Blair, *J. Am. Chem. Soc.* **92**, 5986 (1970); J. E. Bartmess and R. T. McIver, Jr., *Gas Phase Ion Chemistry*, Vol. 2, M. T. Bowers (ed.), Academic Press, New York, 1979.

substituents seen in many other contexts. This effect cannot be the dominant one, however, since it would lead to a gas phase acidity opposite to that observed. The dominant effect is believed to be polarizability. The methyl substituents, each consisting of four atoms, are better able to undergo local electronic distortion (induced polarizability) to accommodate the negative charge than is a single hydrogen atom. Thus, each substitution of a methyl for a hydrogen increases gas phase acidity.[53] An additional factor which determines the greater acidity of alcohols compared with water is the fact that the water O—H bond is somewhat stronger. In solution, these factors are outweighed by solvation effects, and the observed order of acidity is MeOH > H_2O > EtOH > *i*-PrOH > *t*-BuOH. This order results from the fact that the small anion can be better solvated than the more substituted ones.[54] The fact that water's position in the relative acidity order is most drastically changed in going from solution to the gas phase reflects the high sensitivity of the small hydroxide ion to solvation effects.

Within an isomeric series of alcohols, the gas phase acidity order is *sec* > *tert* > *pri* for C_4 and C_5 alcohols, although the differences between the isomeric *sec* and *tert* alcohols are small.[55] Analysis of a large number of alcohols showed the effect of adding an additional methyl substituent at the α, β, and γ positions. The effect at the α carbon was the greatest, but methyl groups at the β and γ positions were also stabilizing. This is again indicative of the importance of the entire molecule in stabilizing the charge in the gas phase.[56]

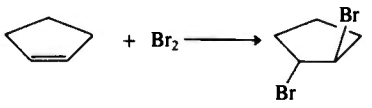
4.12. Stereochemistry

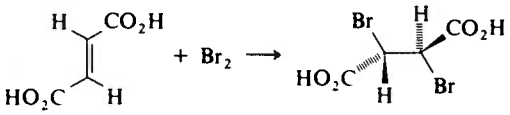
The study of the stereochemical course of organic reactions often leads to detailed insight into reaction mechanisms. Instrumental techniques including IR and NMR spectroscopy, optical rotatory dispersion, and circular dichroism make it possible to determine the stereochemical course of many reactions. Mechanistic postulates frequently make distinctive predictions about the stereochemical outcome of the reaction. Throughout the chapters dealing with specific types of reactions, consideration will be given to the stereochemistry of the reaction and its relationship to the reaction mechanism. As an example, the bromination of alkenes can be cited. A very simple mechanism for bromination is given below:



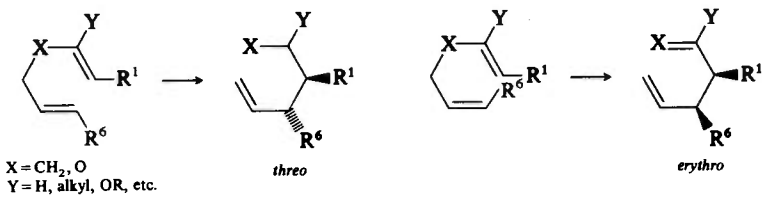
53. R. W. Taft, M. Taagepera, J. L. M. Abboud, J. F. Wolf, D. J. DeFrees, W. J. Hehre, J. E. Bartmess, and R. T. McIver, *J. Am. Chem. Soc.* **100**, 7765 (1978).
54. W. N. Olmstead, Z. Margolin, and F. G. Bordwell, *J. Org. Chem.* **45**, 3295 (1980).
55. G. Boaud, R. Houriet, and T. Gäuman, *J. Am. Chem. Soc.* **105**, 2203 (1983).
56. For a broad review of substituent and solvent effects on acidity and basicity, see R. W. Taft, *Prog. Phys. Org. Chem.* **14**, 247 (1983).

According to this mechanism, a molecule of bromine becomes complexed to the double bond of the alkene, and reorganization of the bonding electrons gives the product. This mechanism can be shown to be incorrect for most alkenes on the basis of stereochemistry. Most alkenes give bromination products in which the two added bromines are on opposite sides of the former carbon-carbon double bond. The above mechanism does not predict this.

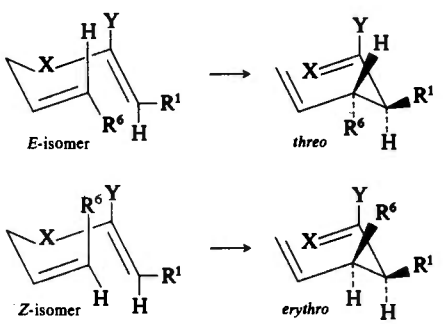




Another example of a reaction in which the stereochemistry of the process provides some valuable information about the mechanism is the thermal rearrangement of 1,5-dienes and substituted analogs.



These reactions will be discussed in more detail under the topic of 3,3-sigmatropic rearrangements in Chapter 11. For the present, we simply want to focus on the fact that the reaction is *stereospecific*; the *E*-isomer will give one diastereomeric product while the related *Z*-isomer will give another one. The stereochemical relationship between reactants and products can be explained if the reaction occurs through a cyclic transition state in which the configurational relationships in the original double bonds are maintained.



A large number of stereochemical results on the 3,3-sigmatropic family of reactions have been correlated by this type of analysis.[57] The basic assumption of the analysis is that the transition state is similar to a cyclohexane ring in its response to steric effects. The success of the model in interpretation of the stereochemical results supports its correctness. It can be concluded that the transition state must be a rather tightly organized arrangement of the atoms with strong bonding between both atoms 3 and 4 and atoms 1 and 6.

4.13. Conclusion

To conclude this chapter, it is important to emphasize a logical point about the determination of reaction mechanisms. *A proposed mechanism can never really be proven; rather, it is a case of alternative mechanisms being eliminated.* Having in mind a mechanism that explains all the facts does not constitute proof that the mechanism is correct. That conclusion is possible only when all alternatives have been excluded. A key stage in a mechanistic investigation then is the enumeration of the various possible mechanisms and the design of experiments that distinguish between them. The principal basis for enumerating mechanistic possibilities is accumulated chemical experience with related systems and the inherent structural features of the system. A chemist approaching a mechanistic study must cast as broad as possible vision on the problem so as not to exclude possibilities.

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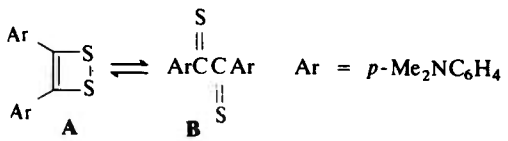
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Problems

(*References for these problems will be found on page* 776.)

1. Measurement of the equilibrium constant for the interconversion of the dithiete **A** and the dithione **B** yielded the data given below. Calculate $\Delta G°$, $\Delta H°$, and $\Delta S°$.

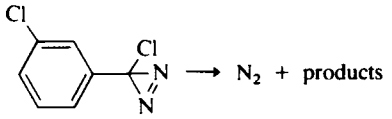


Temp. (°C)	K
−2.9	16.9
11.8	11.0
18.1	8.4
21.9	7.9
29.3	6.5
32.0	6.1
34.9	5.7
37.8	5.3
42.5	4.6

2. (a) Calculate the enthalpy and entropy of activation ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) at 40°C for the acetolysis of *m*-chlorobenzyl *p*-toluenesulfonate from the data given:

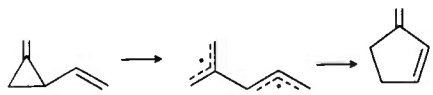
Temp. (°C)	$k \times 10^5$ (s^{-1})
25.0	0.0136
40.0	0.085
50.1	0.272
58.8	0.726

(b) Calculate the activation parameters ΔE_a, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ at 100°C from the data given for the reaction shown below:

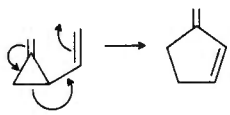


Temp. (°C)	$k \times 10^4$ (s^{-1})
60.0	0.30
70.0	0.97
75.0	1.79
80.0	3.09
90.0	8.92
95.0	15.90

3. 2-Vinylmethylenecyclopropane rearranges in the gas phase to 3-methylenecyclopentene. Two possible reaction mechanisms are:



or



(a) Sketch a reaction energy diagram for each process.
(b) How might an isotopic labeling experiment distinguish between these mechanisms?

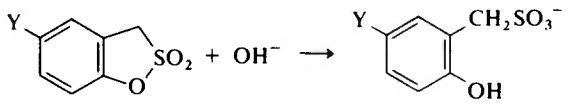
4. In Table 4.3 the phenyl group is assigned both a σ^+ and a σ^- value. Furthermore, the signs of σ^+ and σ^- are different. Discuss the reasons that the phenyl group has both a σ^+ and a σ^- value and explain why they are of a different sign.

5. Match the ρ values with the appropriate reactions. Explain your reasoning.
Reaction constants: +2.45, +0.75, −2.39, −7.29
Reactions:

(a) nitration of substituted benzenes

(b) ionization of substituted benzenthiols
(c) ionization of substituted benzenephosphonic acids
(d) reaction of substituted *N,N*-dimethylanilines with methyl iodide.

6. (a) Determine the value of ρ for the reaction shown from the data given:

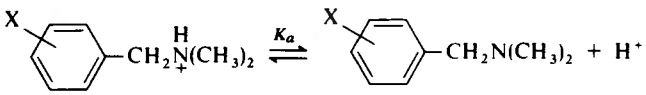


Y	$k(M^{-1}\,s^{-1})$
H	37.4
CH_3O	21.3
CH_3	24.0
Br	95.1
NO_2	1430

(b) The pseudo-first-order rate constants for the acid-catalyzed hydration of substituted styrenes in 3.5 *M* $HClO_4$ at 25° are given. Plot the data against σ and σ^+ and determine ρ and ρ^+. Interpret the significance of the results.

Substituent	$k \times 10^8$ (sec^{-1})
p-CH_3O	488,00
p-CH_3	16,400
H	811
p-Cl	318
p-NO_2	1.44

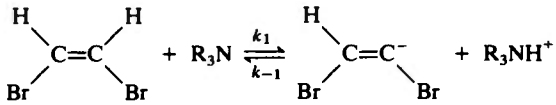
(c) The basicity of a series of substituted benzyldimethylamines has been measured. Determine whether these basicity data are correlated by the Hammett equation. What is the value of ρ? What interpretation do you put on its sign?

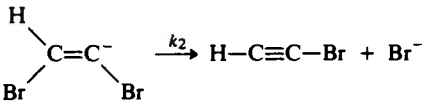


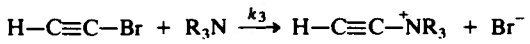
X	pK_a
p-CH_3O	9.32
p-CH_3	9.22
p-F	8.94
H	9.03
m-NO_2	8.19
p-NO_2	8.14
p-Cl	8.83
m-Cl	8.67

7. Write the rate law that would describe the rate of product formation for each of the following systems:

(a)

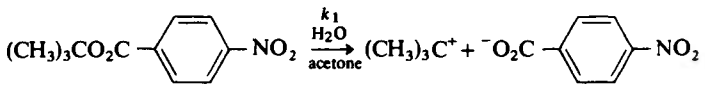


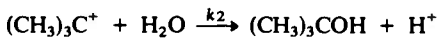




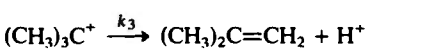
if the second step is rate-controlling and the first step is a preequilibrium.

(b)



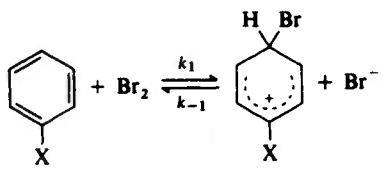


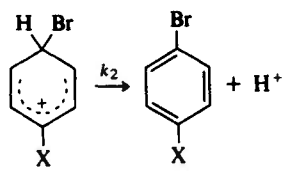
and

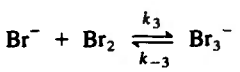


if the competing product-forming steps are faster than the first step.

(c)







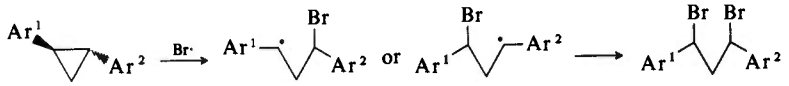
assuming that the σ complex is a steady-state intermediate. The final step is a rapid equilibrium that converts some of the initial Br_2 to unreactive Br_3^-. What is the rate expression if the intermediate goes to product much faster than it reverts to starting material and if the equilibrium constant for tribromide ion formation is large?

(d) $PhC(=O)CH(CH_3)_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PhC(OH)=C(CH_3)_2$

$PhC(OH)=C(CH_3)_2 + Cr(VI) \xrightarrow{k_2}$ products

where no assumption is made as to the relative magnitude of k_1, k_{-1}, and k_2.

8. The rates of brominolysis of a series of 1,2-diarylcyclopropanes under conditions where the rate is determined by Br· attack and leads to 1,3-dibromo-1,3-diarylpropanes are given below.



Set up an equation which you would expect to correlate the observed rate of reaction with both the Ar^1 and the Ar^2 substituent. Check the performance of your equation by comparing the correlation with the data given below. Discuss the results of the correlation.

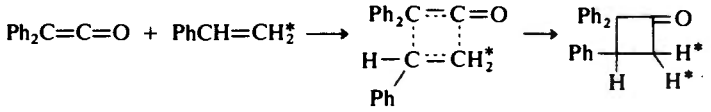
Ar^1	Ar^2	Relative rate	Ar^1	Ar^2	Relative rate
p-MeO	*p*-MeO	1.6×10^4	*p*-Cl	*p*-Ph	48
p-Ph	*p*-MeO	4.4×10^3	*p*-CN	*p*-Ph	45
H	*p*-MeO	2.5×10^3	*p*-Br	*p*-Cl	18
p-Cl	*p*-MeO	2.3×10^3	*p*-Br	*p*-Br	15
m-Br	*p*-MeO	2.1×10^3	*m*-Br	H	10
p-CN	*p*-MeO	1.6×10^3	*m*-Br	*p*-Cl	4.8
p-NO_2	*p*-MeO	1.3×10^3	*p*-CN	H	1.8
H	*p*-Ph	6.8×10^2	*p*-NO_2	H	0.88
p-Cl	*p*-Ph	4.3×10^2	*p*-Cl	*p*-CN	0.55
m-Br	*p*-Ph	1.8×10^2	*p*-Cl	*p*-NO_2	0.37
H	H	1.4×10^2	*p*-CN	*p*-CN	0.046

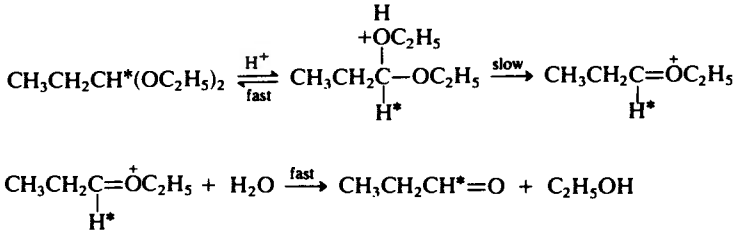
9. Predict whether normal or inverse isotope effects will be observed for each reaction below. Explain. Indicate any reactions in which you would expect $k_H/k_D > 2$. The isotopically substituted hydrogens are marked with asterisks.

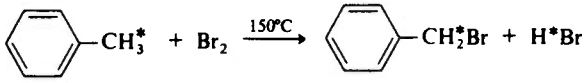
(a) $CH_3CH_2^*CH(OSO_2Ar)CH_2^*CH_3 \xrightarrow[H_2O]{EtOH} CH_3CH_2^*\overset{+}{C}HCH_2^*CH_3$

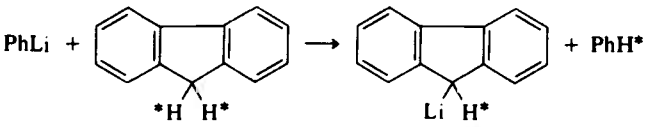
$\longrightarrow CH_3CH_2^*CH(OH)CH_2^*CH_3 + CH_3CH_2^*CH(OCH_2CH_3)CH_2^*CH_3$

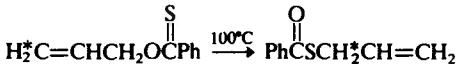
(b) $CH_3^*CH(NO_2)CH_3^* + {}^-OH \longrightarrow CH_3^*C(=NO_2^-)CH_3^* + H_2O$

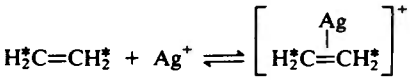
(c) 

(d) 

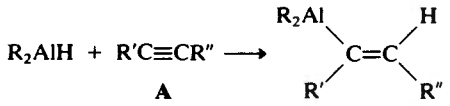
(e) 

(f) 

(g) 

(h) 

10. Reactions of dialkylaluminum hydrides with acetylenes give addition products:

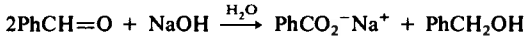


The rate expression for the reaction is

$$-\frac{d[\mathrm{A}]}{dt} = k[\mathrm{A}][(\mathrm{R_2AlH})_3]^{1/3}$$

Propose a mechanism that could account for the overall four-thirds order kinetics and the appearance of the dialkylaluminum hydride concentration to the one-third power.

11. The Cannizzaro reaction is a disproportionation which takes place in strongly basic solution and converts benzaldehyde to benzyl alcohol and sodium benzoate.



Several mechanisms have been postulated, all of which propose a *hydride ion* transfer as a key step. On the basis of the following results, postulate one or

more mechanisms which are consistent with all the data provided. Indicate the significance of each observation with respect to the mechanism(s) you postulate.

(1) When the reaction is carried out in D_2O, the benzyl alcohol contains no deuterium in the methylene group.
(2) When the reaction is carried out in $H_2{}^{18}O$, both the benzyl alcohol and sodium benzoate contain ^{18}O.
(3) The reaction rate is given by the expression

$$\text{rate} = k_{obs}[PhCH{=}O]^2[^-OH]$$

(4) The rates of substituted benzaldehydes are correlated by the Hammett equation with $\rho = +3.76$.
(5) The solvent isotope effect k_{D_2O}/k_{H_2O} is 1.90.

12. A mechanism for olefin arylation by palladium(II) is given below. The isotope effect k_H/k_D was found to be 5 when benzene-d_6 was used. When styrene-β,β-d_2 was used, no isotope effect was observed. Which step is rate-determining?



$$+ Pd(O_2CCH_3)_2 \xrightarrow{k_1} Ph{-}PdO_2CCH_3 + CH_3CO_2H$$

$$Ph{-}PdO_2CCH_3 + PhCH{=}CH_2 \xrightarrow{k_2} Ph{-}Pd{-}CH{=}CHPh + CH_3CO_2H$$

$$Ph{-}Pd{-}CH{=}CHPh \xrightarrow{k_3} PhCH{=}CHPh + Pd$$

13. A scale for solvent ionizing power, Y^+, applicable in solvolysis reactions of cationic substrates, has been developed. For example,

$$S + (C_2H_5)_3O^+PF_6^- \rightarrow (C_2H_5)_2O + C_2H_5S^+ + PF_6^-$$

S = solvent

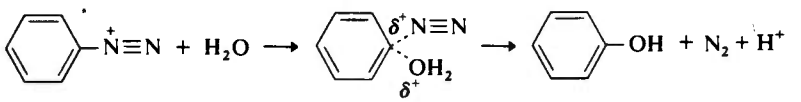
The numerical values of Y^+ are found to be related to Y, the measure of solvent ionizing power for neutral substrates, by the equation

$$Y^+ = -0.09\ Y$$

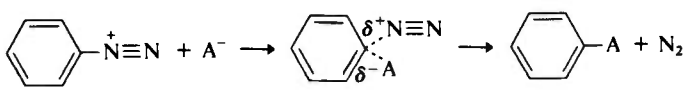
Explain, in qualitative terms, (a) why Y^+ is negative with respect to Y and (b) why Y^+ is smaller in magnitude than Y (as is indicated by the coefficient of 0.09).

14. Two mechanisms are among those that have been postulated for decomposition of aryl diazonium salts in aqueous solution containing nucleophilic anions, A^-:

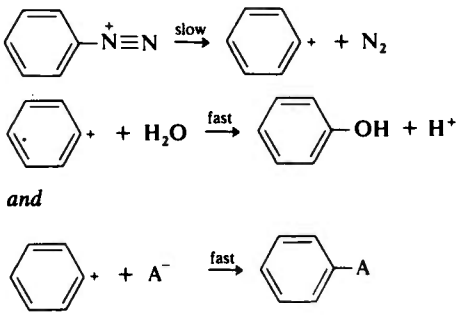
Mechanism A



and



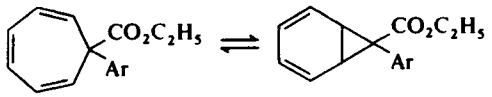
Mechanism B



Indicate how each of the following techniques might be applied to distinguishing between these mechanisms:

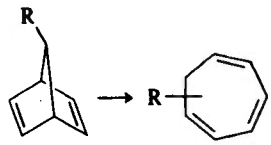
(a) kinetic studies
(b) rate and product composition as a function of $[A^-]$
(c) solvent isotope effect studies
(d) isotope effect resulting from substitution of D for H at *ortho* positions
(e) substituent effect studies

15. Cycloheptatrienes are in many cases in rapid equilibrium with an isomeric bicyclo[4.1.0] heptadiene. The thermodynamics of the valence isomerism has been studied in a number of instances and some of the data are given below. Calculate the equilibrium constant for each case at 25°C. Calculate the temperature at which $K = 1$ for each system. Are the signs of the enthalpy and entropy as you would expect them to be? Can you discern any pattern of substituent effects from the data?



Ar	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)
Phenyl	−5.4	−16.8
p-Nitrophenyl	−3.5	−11.0
p-Methoxyphenyl	−2.3	−7.4

16. Bicyclo[2.2.1]heptadiene rearranges at elevated temperatures to cycloheptatriene and toluene. The reaction is facilitated by substituents at C-7 such as phenyl and alkoxy, in which case cycloheptatrienes are the dominant products.



For R = *t*-butoxy, the rate data are given for several temperatures in decane. The reaction is about 50% faster in ethoxyethanol than in decane. Calculate the

Temp. (°C)	k (s^{-1})
139.8	7.28×10^{-6}
154.8	3.37×10^{-5}
170.3	1.43×10^{-4}

activation parameters at 150°C. Although precisely comparable data are not available, E_a for the gas phase isomerization of norbornadiene is ~50 kcal/mol. Draw a sketch showing the degree of transition state stabilization or destabilization caused by the alkoxy substituent. Is there any basis for regarding the bond cleavage in the rate-determining step to be heterolytic or homolytic? How do you propose that the effect of the substuent group R operates? Can you propose an experiment that might support your proposal?

17. A study of the aromatic nitration reaction in aqueous nitric acid revealed that when no aromatic substrate was present, an incorporation of ^{18}O from labeled water into nitric acid occurred. The rate of this exchange process was identical with the rate of nitration of several reactive aromatic hydrocarbons. Discuss how this result is consistent with mechanism B on page 188, but not with mechanisms A or C.

$$HNO_3 + H_2{}^{18}O \longrightarrow HN^{18}O_3 + H_2O$$

18. Comparison of several series of solvolysis reactions which proceed via carbocation intermediates reveals that an α-cyano group retards the reactions by a factor of about 10^3. A β-cyano group is even more strongly rate-retarding, with the factor being as high as 10^7. Why are both α- and β-cyano groups rate-retarding? What might cause the β-cyano group to be more rate-retarding than the α-cyano group?

19. Comparison of the gas phase acidity of benzoic acids with pK_a values of the same compounds in aqueous solution provides some interesting relationships.

    (1) The trend in acidity as a function of substituent is the same but the magnitude of the substituent effects is much larger in the gas phase. (The $\Delta\Delta G°$ for any given substituent is about ten times larger in the gas phase.)
    (2) Whereas acetic acid and benzoic acid are of comparable acidity in water, benzoic acid is much more acidic in the gas phase.
    (3) While the substituent effect in the gas phase is assumed to be nearly entirely an enthalpy effect, it can be shown that in solution the substituent effect is largely the result of changes in ΔS.

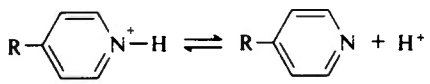
    Discuss how the change from gas phase to water solution can cause each of these effects.

20. It has been suggested that the chemical shift of aromatic ring carbons might provide a good indication of the intrinsic electron-releasing or electron-attracting capacity of substituents in circumstances where there is no perturbation by an approaching reagent. Such a perturbation is always present in substituent effects determined on the basis of reactivity. The measured chemical shifts from benzene for the carbon *para* to the substituent are given below. Plot these against σ, σ^+, and what conclusions do you draw from these plots? If you have access to an appropriate program and computer, determine the "percent resonance" associated with the chemical shift, as determined by the Swain-Lupton equation.

R	$\Delta\delta^a$	R	$\Delta\delta^a$
NH_2	−9.86	CN	3.80
OCH_3	−7.75	CCH_3	4.18
F	−4.49	CO_2CH_3	4.12
Cl	−2.05	SO_2CH_3	4.64
Br	−1.62	NO_2	5.53
CH_3	−2.89	CH=O	5.51
CF_3	3.19		

a. $\Delta\delta$ is the change in chemical shift in CCl_4 from benzene in ppm; a negative sign indicates increased shielding.

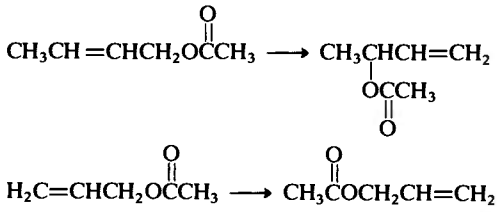
21. The ionization of a series of 4-substituted pyridines has been studied, and both equilibrium acidities (pK_a) and enthalpies of ionization have been recorded at 25°C:



R	pK_a	$\Delta H°$ (kcal/mol)
H	5.21	4.8
NH_2	9.12	11.3
OCH_3	6.58	6.8
CH_3	6.03	6.1
Cl	3.83	3.6
Br	3.75	3.5
CN	1.86	1.3

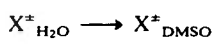
Calculate $\Delta S°$ for ionization of each compound. Comment on the contribution of $\Delta H°$ and $\Delta S°$ terms to the free energy of ionization. Test the data for linear free-energy correlations. Are the linear free-energy correlations dominated by entropy or enthalpy terms?

22. Allyl esters undergo rearrangement reactions at 300°C and above. Two examples are shown, one of which is "degenerate," since the product and reactant are identical:



At least three distinct mechanisms can be written for these reactions. Write down some possible mechanisms, and suggest isotopic labeling studies that could distinguish among the possibilities you have proposed.

23. Estimates of the heat of solvation of various species in DMSO as compared to water have been made, and can be expressed as enthalpies of transfer. Some data are given below. Discuss their significance.



X	$\Delta H_{transfer}$ (kcal/mol)
K^+	−8.8
Na^+	−7.1
Cl^-	+4.9

Exhibit 4

Advanced Organic Chemistry

THIRD EDITION

Part A: Structure and Mechanisms

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and RICHARD J. SUNDBERG

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Charlottesville, Virginia

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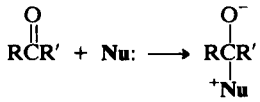
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8

Reactions of Carbonyl Compounds

The carbonyl group is one of the most prevalent of the functional groups. The carbonyl group is involved in many synthetically important reactions. Reactions involving carbonyl groups are also exceptionally important in biological processes. Most of the reactions of aldehydes, ketones, esters, amides, and the other carboxylic acid derivatives directly involve the carbonyl group. In Chapter 7, the role of the carbonyl group in stabilizing carbanions was discussed. In this chapter, the primary topic for discussion will be the characteristic mechanistic patterns of reactions at carbonyl centers. The first two chapters of Part B deal mainly with the use of carbonyl compounds in synthesis to form carbon-carbon bonds.

In many reactions at carbonyl groups, a key step is addition of a nucleophile, generating a tetracoordinate carbon atom. The overall course of the reaction is then determined by the fate of this *tetrahedral intermediate.*



The reactions of the specific classes of carbonyl compounds are related by the decisive importance of tetrahedral intermediates, and differences in reactivity can often be traced to structural features present in the intermediates.

8.1. Hydration and Addition of Alcohols to Aldehydes and Ketones

For most simple carbonyl compounds, the equilibrium constant for addition of water to the carbonyl group is unfavorable.

Table 8.1. Hydration of Carbonyl Compounds

$$K = \frac{[\text{hydrate}]^{a}}{[\text{carbonyl}]}$$

Carbonyl compound	K (in water, 25°C)
CH_2O	2.28×10^{3}[b]
CH_3CHO	1.06[b]
CH_3CH_2CHO	0.85[b]
$(CH_3)_2CHCHO$	0.61[b]
$(CH_3)_3CCHO$	0.23[b]
CF_3CHO	2.9×10^{4}[b]
C_6H_5CHO	8×10^{-3}[c]
CH_3COCH_3	1.4×10^{-3}[b]
FCH_2COCH_3	0.11[c]
$ClCH_2COCH_3$	0.11[b]
CF_3COCH_3	35[b]
CF_3COCF_3	1.2×10^{6}[b]
$C_6H_5COCH_3$	9.3×10^{-6}[c]
$C_6H_5COCF_3$	78[b]
$CH_3COCOCH_3$	0.6[d]
$CH_3COCO_2CH_3$	0.8[d]

a. $K = K_{eq}[H_2O] = 55.5\,K_{eq}$
b. J. P. Guthrie, *Can. J. Chem.* **53**, 898 (1975).
c. J. P. Guthrie, *Can. J. Chem.* **56**, 962 (1978).
d. T. J. Burkey and R. C. Fahey, *J. Am. Chem. Soc.* **105**, 868 (1983).

$$\underset{\displaystyle\underset{\displaystyle O}{\|}}{RCR'} + H_2O \rightleftharpoons \underset{\displaystyle OH}{\overset{\displaystyle OH}{|}}{RCR'} \qquad K < 1$$

Exceptions are formaldehyde, which is nearly completely hydrated in aqueous solution, and aldehydes and ketones with highly electronegative groups such as trichloroacetaldehyde and hexafluoroacetone. The data given in Table 8.1 illustrate that the equilibrium constant for hydration decreases with increasing alkyl substitution.

Although the equilibrium constant for hydration is unfavorable, the equilibrium between an aldehyde or ketone and its hydrate is established rapidly and can be detected by isotopic exchange, using water labeled with ^{18}O, for example.

$$R_2C{=}O + H_2O^{*} \rightleftharpoons \underset{}{\overset{*OH}{|}}{R_2COH} \rightleftharpoons R_2C{=}O^{*} + H_2O$$

The hydration reaction has been extensively studied because it is the mechanistic prototype for many reactions at carbonyl centers that involve more complex molecules.[1] For acetaldehyde, the half-life of the exchange reaction is on the order of one minute under neutral conditions, but the reaction if considerably faster in

1. R. P. Bell, *Adv. Phys. Org. Chem.* **4**, 1 (1966); W. P. Jencks, *Chem. Rev.* **72**, 705 (1972).

acidic or basic media. The second-order rate constant for acid-catalyzed hydration of acetaldehyde is on the order of $500\ M^{-1}\ s^{-1}$.[2] Acid catalysis involves either protonation or hydrogen bonding at the carbonyl oxygen. Both specific acid catalysis and general acid catalysis can be observed.[3] (Review Section 4.8 for the discussion of specific and general acid catalysis.)

Specific acid-catalyzed hydration

$$RCH{=}O + H^+ \rightleftharpoons RCH{=}\overset{+}{O}H$$

$$RCH{=}\overset{+}{O}H + H_2O \overset{\text{slow}}{\rightleftharpoons} \underset{\displaystyle {}^{+}OH_2}{RCHOH} \rightleftharpoons RCH(OH)_2 + H^+$$

General acid-catalyzed hydration

$$RCH{=}O + HA \rightleftharpoons RCH{=}O\cdots HA$$

$$RCH{=}O\cdots HA + H_2O \overset{\text{slow}}{\rightleftharpoons} \underset{\displaystyle {}^{+}OH_2}{RCHOH} + A^- \rightleftharpoons RCH(OH)_2 + HA$$

Basic catalysts function by deprotonating water to give the more nucleophilic hydroxide ion.

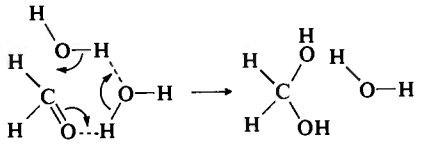
Base-catalyzed hydration

$$B^- + H_2O + RCH{=}O \rightleftharpoons \underset{\displaystyle OH}{RCHO^-} + BH$$

$$\underset{\displaystyle OH}{RCHO^-} + H_2O \rightleftharpoons RCH(OH)_2 + {}^-OH$$

$$BH + {}^-OH \rightleftharpoons B^- + H_2O$$

Molecular orbital (STO-3G) calculations on the gas phase hydrogen reaction of formaldehyde identify a concerted process involving two water molecules as a low-energy mechanism for hydration.



The calculated activation energy for this process in water is 16 kcal/mol, which is in good agreement with the experimentally observed value.[4]

Aldehydes and ketones undergo reversible addition reactions with alcohols. The product of addition of one mole of alcohol to an aldehyde or ketone is referred

2. P. Greenzaid, Z. Luz, and D. Samuel, *J. Am. Chem. Soc.* **89**, 756 (1967).
3. L. H. Funderburk, L. Aldwin, and W. P. Jencks, *J. Am. Chem. Soc.* **100**, 5444 (1978); R. A. McClelland and M. Coe, *J. Am. Chem. Soc.* **105**, 2718 (1983).
4. I. H. Williams, D. Spangler, D. A. Femec, G. M. Maggiora, and R. L. Schowen, *J. Am. Chem. Soc.* **105**, 31 (1983).

to as a *hemiacetal* or *hemiketal*, respectively. Dehydration followed by addition of a second molecule of alcohol gives an *acetal* or *ketal*. This second phase of the process can be catalyzed only by acids, since a necessary step is elimination of hydroxide (as water) from the tetrahedral intermediate. There is no low-energy mechanism for base assistance of this elimination step. Since there is no mechanism for basic catalysis, acetals and ketals are stable toward hydrolysis in alkaline aqueous solution but are hydrolyzed rapidly in acidic solution.

$$R_2C{=}O + R'OH \rightleftharpoons R_2C(OH)OR' \quad \textit{hemiketal}$$

$$R_2C(OH)OR' + H^+ \rightleftharpoons R_2C(^+OH_2)OR'$$

$$R_2C(^+OH_2)OR' \rightleftharpoons R_2C{=}\overset{+}{O}R' + H_2O$$

$$R_2C{=}\overset{+}{O}R' + R'OH \rightleftharpoons R_2C(\overset{+}{O}HR')OR' \rightleftharpoons R_2C(OR')_2 + H^+ \quad \textit{ketal}$$

The equilibrium constants for addition of alcohols to carbonyl compounds to give hemiacetals or hemiketals show the same response to structural features as for the hydration reaction. Equilibrium constants for addition of methanol to acetaldehyde in both water and chloroform solution are near 0.8 M^{-1}. The comparable value for addition of water is about 0.02 M^{-1}.[5] The overall equilibrium constant for formation of the dimethyl acetal of acetaldehyde is 1.58 M^{-1}. Because the position of the equilibrium does not strongly favor product, the formation of acetals and ketals must be carried out in such a way as to drive the reaction to completion. One approach is to use a dehydrating reagent or azeotropic distillation so that the water that is released is irreversibly removed from the system.

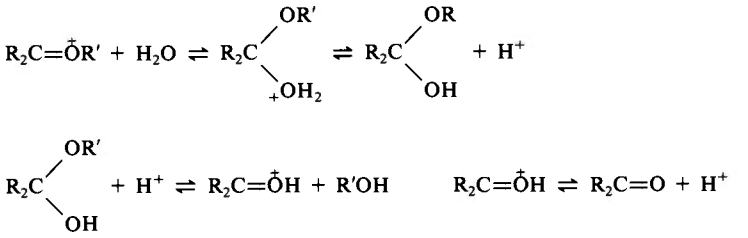
Because of the unfavorable equilibrium constant in aqueous solution and the relative facility of the hydrolysis, acetals and ketals are rapidly converted to aldehydes and ketones in acidic aqueous solution.

$$R_2C(OR')_2 + H_2O \xrightarrow{H^+} R_2C{=}O + 2R'OH$$

The mechanism of this hydrolysis reaction has been studied in great detail.[6] The mechanism is the reverse of that for acetal or ketal formation.

$$R_2C(OR')_2 + H^+ \rightleftharpoons R_2C(OR')(^+HOR') \rightleftharpoons R_2C{=}\overset{+}{O}R' + R'OH$$

5. R. Bone, P. Cullis, and R. Wolfenden, *J. Am. Chem. Soc.* **105**, 1339 (1983).
6. E. H. Cordes and H. G. Bull, *Chem. Rev.* **74**, 581 (1974).



Some of the evidence that has helped to establish the general mechanism is as follows:

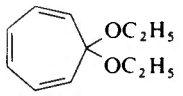
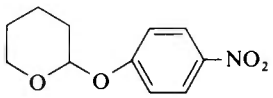
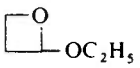
1. Isotopic labeling experiments have established that C—O bond rupture occurs between the carbonyl carbon and oxygen; therefore, substitution at the alcohol C—O bond is not involved.
2. For most acetals and ketals, the reaction is *specific-acid-catalyzed.* This is consistent with the existence of a preequilibrium in which the ketal is protonated. The role of the proton is to assist the departure of the alkoxy group by converting it to a better leaving group. In essence, this cleavage step is an S_N1 reaction with the remaining alkoxy group stabilizing the carbocation formed by ionization.
3. Hammett treatments show good correlations with large negative ρ values for the hydrolysis of acetals of aromatic aldehydes. This is consistent with the development of a positive charge at the carbonyl center in the rate-determining step.
4. Solvent isotope effects are usually in the range $k_{D_3O^+}/k_{H_3O^+} = 2\text{–}3$. These values reflect the greater equilibrium acidity of deuterated acids (Section 4.8) and indicate that the initial protonation is a fast preequilibrium.

Acetal and ketal hydrolyses usually exhibit specific acid catalysis, in agreement with a mechanism involving rate-determining cleavage of the conjugate acid of the reactant. General acid catalysis is observed, however, in certain acetals and ketals in which special structural features reduce the energy required for C—O bond cleavage.[7] Thus, hydrolysis of each of the acetals shown in Scheme 8.1 exhibits general acid catalysis, and each acetal has a special structural feature that facilitates C—O bond heterolysis. Easing the energy requirement for C—O bond cleavage permits the proton transfer step to become partially rate-determining, which results in the observation of general acid catalysis.

Three-dimensional potential energy diagrams of the type introduced in connection with the variable E2 transition state theory for elimination reactions can be used to consider structural effects on the reactivity of carbonyl compounds and the tetrahedral intermediates involved in carbonyl group reactions. Many of these reactions involve the formation or breaking of two separate bonds. This is the case in the first stage of acetal hydrolysis, which involves both a proton transfer and breaking of a C—O bond. The overall reaction might take place in several ways.

7. T. H. Fife, *Acc. Chem. Res.* **5**, 264 (1972).

Scheme 8.1. Acetals and Ketals That Exhibit General Acid Catalysis in Hydrolysis

	Acetal or ketal	Special structural feature
1[a]		Very stable carbocation (stabilized by both alkoxy function and aromaticity)
2[b]		Good leaving group
3[c]		Ring strain relieved in cleavage step
4[d]	$(Ar)_2C(OC_2H_5)_2$	Aryl substituents stabilize carbocation ion
5[e]	$PhCH[OC(CH_3)_3]_2$	Aryl stabilization and relief of steric strain

a. E. Anderson and T. H. Fife, *J. Am. Chem. Soc.* **91**, 7163 (1969).
b. T. H. Fife and L. H. Brod, *J. Am. Chem. Soc.* **92**, 1681 (1970).
c. R. F. Atkinson and T. C. Bruice, *J. Am. Chem. Soc.* **96**, 819 (1974).
d. R. H. DeWolfe, K. M. Ivanetich, and N. F. Perry, *J. Org. Chem.* **34**, 848 (1969).
e. E. Anderson and T. H. Fife, *J. Am. Chem. Soc.* **93**, 1701 (1971).

There are two mechanistic extremes:

1. The proton could be completely transferred and then the departing alcohol molecule would leave to form a carbocation in a distinct second step. This is the usual specific-acid-catalyzed mechanism.
2. The acetal might undergo ionization with formation of an alkoxide ion and a carbocation. In a second step the alkoxide would be protonated. This mechanism is extremely rare if not impossible, because an alkoxide is a poor leaving group.

There could be an intermediate mechanism between these extremes. This is a general acid-catalyzed mechanism in which the proton transfer and the C—O bond rupture occur as a *concerted* process. The concerted process need not be perfectly synchronous; that is, proton transfer might be more complete at the transition state than C—O rupture, or vice versa. These ideas are represented in the three-dimensional energy diagram in Fig. 8.1.

The two paths around the edge of the diagram represent the stepwise processes described as the mechanistic extremes 1 and 2. We know that process 2 represented by path (a) is a high-energy process so that the upper-left corner of the diagram would have a high energy. The lines designated (b) and (c) indicate concerted but nonsynchronous mechanisms in which there is both partial proton transfer and partial C—O bond rupture at the transition state. In path (b), C—O cleavage is more complete than proton transfer at the transition state, while the reverse is true for path (c). Both these paths represent concerted, general-acid-catalyzed processes.

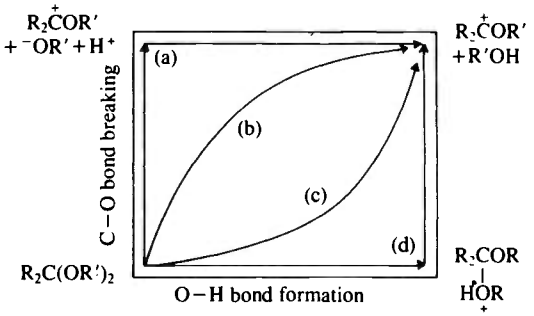


Fig. 8.1. Representation of transition states for the first stage of acetal hydrolysis. (a) Initial C—O bond breaking; (b) concerted mechanism with C—O bond breaking leading O—H bond formation; (c) concerted mechanism with proton transfer leading C—O bond breaking; (d) initial proton transfer.

Path (d) represents the specific-acid-catalyzed process in which proton transfer precedes C—O cleavage.

If it is possible to estimate or calculate the energy of the reacting system at various stages, the energy dimension can be added as in Fig. 8.2. The actual mechanism will then be the process which proceeds over the lowest-energy barrier. The energy dimension can be shown as contours. The diagram in Fig. 8.2 shows the initial ionization to an alkoxide and carbocation as very high in energy. The stepwise path of protonation followed by ionization is shown with small barriers with the protonated ketal as an intermediate. The lowest-energy path is shown as a concerted process represented by the dashed line. The transition state, which lies at the highest-energy point on this line, would exhibit more complete proton transfer than C—O cleavage.

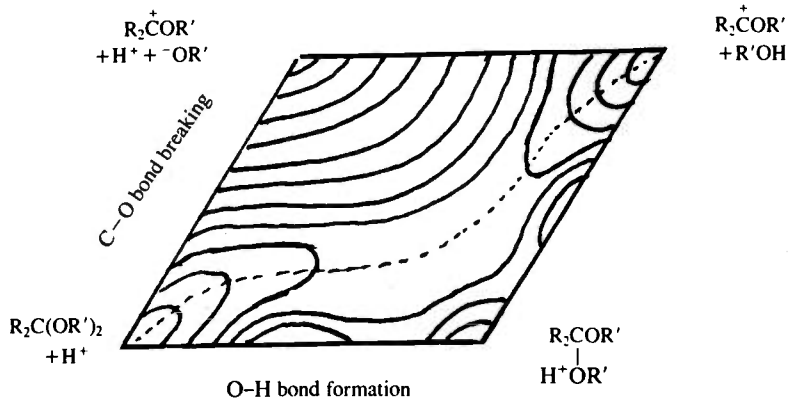
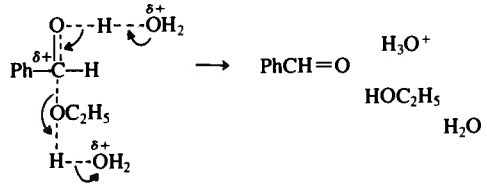


Fig. 8.2. Contour plot showing a favored concerted mechanism for the first step in acetal hydrolysis, in which proton transfer is more complete in the transition state than C—O bond breaking.

Structural effects can now be discussed by asking how they will affect the position of the transition state on the potential energy surface. The stepwise path via the protonated acetal should be followed in the case of alcohols that are poor leaving groups. As the alcohol becomes more acidic and its conjugate base becomes a better leaving group, the transition state will be shifted to a point where C—O bond breaking has begun before proton transfer is complete. This would mean that the mechanism has become concerted, although the transition state would still have much of the character of a carbocation. Three-dimensional reaction energy diagrams can be used to describe how structural changes can affect the nature of the transition state. Just as the two-dimensional diagrams can give meaning to such phrases as an "early" or a "late" transition state, the three-dimensional diagrams are illustrative of statements such as "C—O cleavage is more advanced than proton transfer."

Consideration of the types of acetals shown in Scheme 8.1, which exhibit general acid catalysis, indicates why the concerted mechanism operates in these molecules. The developing aromatic character of the cation formed in the case of entry 1 will lower the energy requirement for C—O bond rupture. The bond can begin to break before protonation is complete. Entry 2 represents a case where a good leaving group (a stable phenolate anion) reduces the energy requirement for C—O bond cleavage. In entry 3 the four-membered ring is broken in the reaction. Cleavage in this case is facilitated by release of strain energy. Entries 4 and 5 are similar to entry 1 because the aryl groups provide stabilization for developing cationic character.

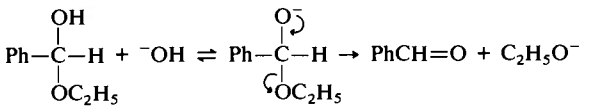
The second step in acetal and ketal hydrolysis is conversion of the hemiacetal or hemiketal to the carbonyl compound. The mechanism of this step is similar to that of the first step. Usually, the second step is faster than the initial one.[8] Hammett σ–ρ plots and solvent isotope effects both indicate that the transition state has less cationic character than is the case for the first step. These features of the mechanism suggest that a concerted removal of the proton at the hydroxyl group occurs as the alcohol is eliminated.



This would disperse the positive charge over several atoms and diminish the sensitivity of the reaction to substituent effects. The ρ values that are observed are consistent with this interpretation. While ρ is -3.25 for acetal hydrolysis, it is only -1.9 for hemiacetal hydrolysis.[9]

In contrast to acetals, which are base stable, hemiacetals undergo base-catalyzed hydrolysis. In the alkaline pH range, the mechanism shifts toward a base-catalyzed

8. Y. Chiang and A. J. Kresge, *J. Org. Chem.* **50**, 5038 (1985).
9. T. J. Przystas and T. H. Fife, *J. Am. Chem. Soc.* **103**, 4884 (1981).



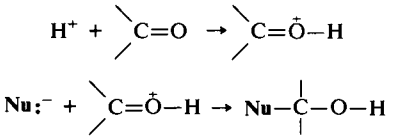
There are two competing substituent effects on this reaction. Electron-attracting groups favor the deprotonation but disfavor the elimination step. The observed substituent effects are small, and under some conditions the Hammett plot is nonlinear.[10]

8.2. Addition–Elimination Reactions of Aldehydes and Ketones

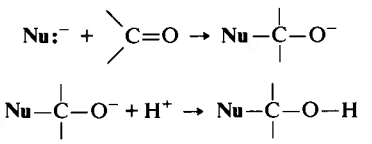
The mechanistic pattern established by study of hydration and alcohol addition reactions of ketones and aldehydes is followed in a number of other reactions of carbonyl compounds. Reactions at carbonyl centers usually involve a series of addition and elimination steps proceeding through tetrahedral intermediates. These steps can be either acid-catalyzed or base-catalyzed. The overall result of the reaction is determined by the reactivity of these tetrahedral intermediates.

In general terms, there are three possible mechanisms for addition of a nucleophile and a proton to give a tetrahedral intermediate in a carbonyl addition reaction.

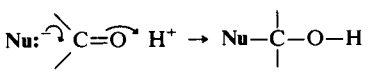
(a) Protonation followed by nucleophilic attack on the protonated carbonyl group:



(b) Nucleophilic addition at the carbonyl group followed by protonation:



(c) Concerted proton transfer and nucleophilic attack:



10. R. L. Finley, D. G. Kubler, and R. A. McClelland, *J. Org. Chem.* **45**, 644 (1980).

There are examples of each of these mechanisms, and a three-dimensional potential energy diagram can provide a useful general framework within which to consider the specific addition reactions that we will encounter. The breakdown of a tetrahedral intermediate involves the same processes but operates in the opposite direction, so the principles that are developed will apply equally well to the reactions of the tetrahedral intermediates. Let us examine the three general mechanistic cases in relation to the energy diagram in Fig. 8.3.

Case (a) should be favored for weak nucleophiles. The protonated carbonyl compound will be much more highly reactive toward such nucleophiles. Case (b) should be favored for strong nucleophiles. Such nucleophiles will generally be more basic than carbonyl groups and would be protonated in preference to the carbonyl group. In such systems, proton donors would diminish the overall reaction rate by decreasing the amount of nucleophile that is available for reaction. The reactions of primary amines are generally not catalyzed by acids because the protonated amines are not nucleophilic toward the carbonyl group and the carbonyl group does not compete with the amine as a site for protonation. The concerted mechanism (c) is observed for less basic nucleophiles. The simultaneous transfer of the proton at the carbonyl oxygen assists in achieving addition by species that are not sufficiently nucleophilic to react by mechanism (b). The general trend then is that the weaker and less basic the nucleophile, the more important is partial or complete protonation of the carbonyl group. If we consider the reverse process, the same general relationships will hold. The good leaving groups can be expected to follow path (a), poor leaving groups will follow path (b), and intermediate cases are likely to react by the concerted mechanism.

Certain nucleophilic species add to carbonyl groups to give tetrahedral intermediates that are unstable and break down to form a new double bond. An important group of such reactions are those with compounds containing primary amino groups. Scheme 8.2 lists some of the more familiar classes of such reactions. In general,

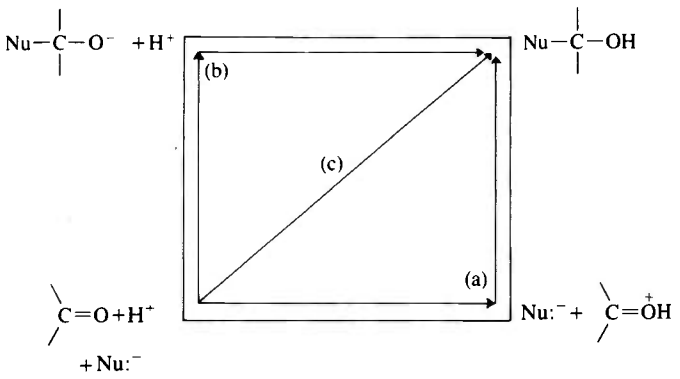
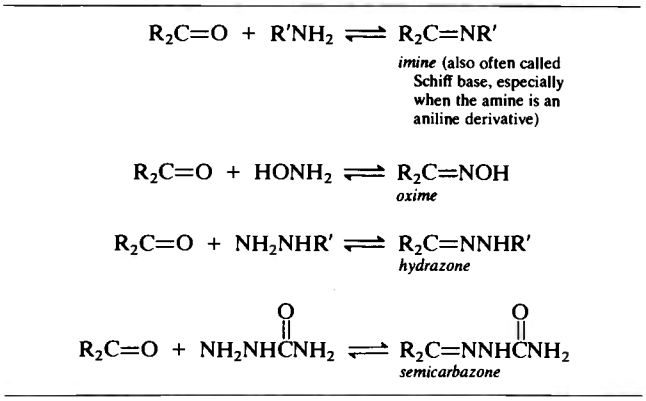


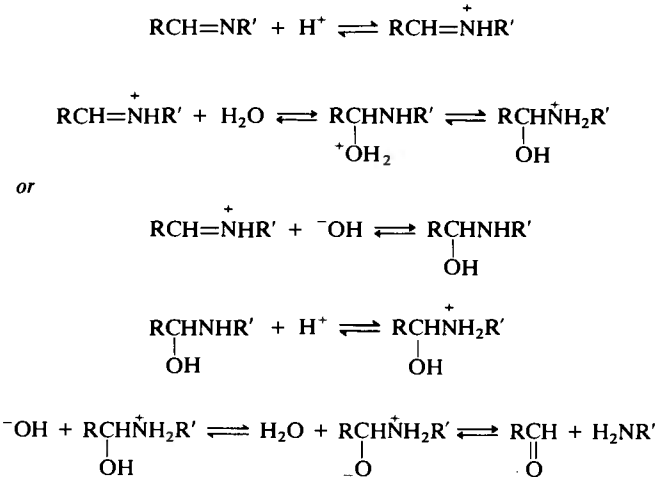
Fig. 8.3. Three-dimensional potential energy diagram for addition of a proton and nucleophile to a carbonyl group. (a) Proton transfer complete before nucleophilic addition begins; (b) nucleophilic addition complete before proton transfer begins; (c) concerted proton transfer and nucleophilic addition.

Scheme 8.2. Some Addition–Elimination Reactions of Aldehydes and Ketones



these reactions are reversible, and mechanistic information can be obtained by study of either the forward or the reverse process.

The hydrolysis of simple imines occurs readily in aqueous acid and has been studied in great detail by kinetic methods. The precise mechanism is a function of the reactant structure and the pH of the solution. The overall mechanism consists of an addition of water to the C=N bond, followed by expulsion of the amine from a tetrahedral intermediate.[11]



The relative rates of the various steps are a function of the pH of the solution and the basicity of the imine. In the alkaline range, the rate-determining step is usually nucleophilic attack by hydroxide ion on the protonated C=N bond. At intermediate pH values, water replaces hydroxide as the dominant nucleophile. In acidic solution, the rate-determining step becomes the breakdown of the tetrahedral

11. J. Hine, J. C. Craig, Jr., J. G. Underwood II, and F. A. Via, *J. Am. Chem. Soc.* **92**, 5194 (1970); E. H. Cordes and W. P. Jencks, *J. Am. Chem. Soc.* **85**, 2843 (1963).

intermediate. A mechanism of this sort, in which the overall rate is sensitive to pH, can be usefully studied by constructing a pH-rate profile, which is a plot of the observed rate constants versus pH. Figure 8.4 shows the pH-rate profiles for hydrolysis of a series of imines derived from substituted aromatic aldehydes and *t*-butylamine. The form of pH-rate profiles can be predicted on the basis of the detailed mechanism of the reaction. The value of the observed rate can be calculated quantitatively as a function of pH if a sufficient number of the individual rate constants and the acid dissociation constants of the participating species are known. Agreement between the calculated and observed pH-rate profiles can serve as a sensitive test of the adequacy of the postulated mechanism. Alternatively, one may begin with the experimental pH-rate profile and deduce details of the mechanism from it.

Complete understanding of the shape of the curves in Fig. 8.4 requires a kinetic expression somewhat more complicated than we wish to deal with here. The nature of the extremeties of the curves can be understood, however, on the basis of qualitative arguments. The rate decreases with decreasing pH in the acidic region because formation of the zwitterionic tetrahedral intermediate is required for

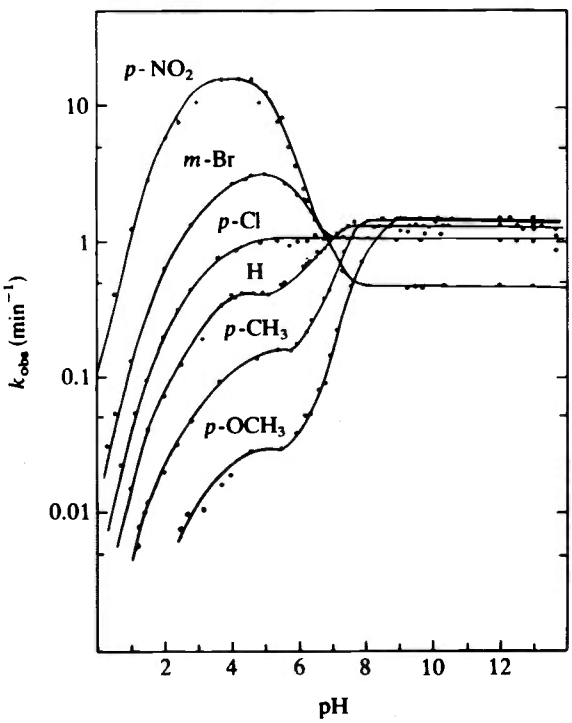


Fig. 8.4. Logarithm of the first-order rate constants for the hydrolysis of substituted benzylidene-1,1-dimethyl-ethylamines as a function of pH. [Reproduced from *J. Am. Chem. Soc.* **85**, 2843 (1963) by permission of the American Chemical Society.]

explusion of the amine. The concentration of the zwitterionic species decreases with increasing acidity, since its concentration is governed by an acid-base equilibrium:

$$K = \frac{[H^+][RCH(O^-)\overset{+}{N}H_2R]}{[RCH(OH)\overset{+}{N}H_2R]}$$

As the hydrogen ion concentration increases, the concentration of the reactive form of the intermediate decreases.

In the alkaline region, the rate is pH independent. In this region, the rate-controlling step is attack of the hydroxide ion on the protonated imine. The concentration of both of these species is pH dependent, but in opposite, compensating, ways. The overall rate is therefore pH independent in the alkaline range. (Work Problem 6 to establish that this is so.)

The formation of imines takes place by a mechanism that is the reverse of the hydrolysis. Preparative procedures often ensure completion of the reaction by removing water as it is formed by azeotropic distillation or by the use of a dehydrating agent.

The other C=N systems included in Scheme 8.2 are more stable to aqueous hydrolysis than are the imines. For many cases, the equilibrium constants for formation are high, even in aqueous solution. The additional stability can be attributed to the participation of the atom adjacent to the nitrogen in delocalized bonding.

$$R_2C{=}N{-}\ddot{O}H \longleftrightarrow R_2\bar{C}{-}N{=}\overset{+}{O}H$$

$$R_2C{=}N{-}\ddot{N}H_2 \longleftrightarrow R_2\bar{C}{-}N{=}\overset{+}{N}H_2$$

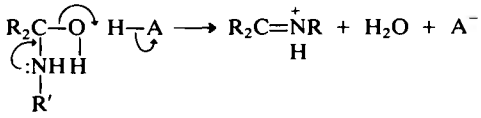
The formation of oximes, hydrazones, and related imine derivatives is usually catalyzed by both general acids and general bases. General base catalysis of dehydration of the tetrahedral intermediate involves nitrogen deprotonation concerted with elimination of hydroxide ion.[12]

$$R_2C{=}O + NH_2R' \overset{fast}{\rightleftharpoons} R_2C(OH)NHR'$$

$$B: + H{-}N{-}C(R)(R'){-}OH \xrightarrow{slow} \overset{+}{B}H + R'N{=}CR_2 + \bar{O}H$$

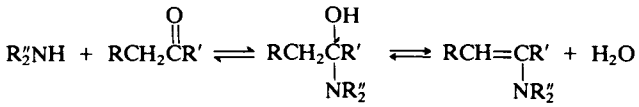
12. W. P. Jencks, *Prog. Phys. Org. Chem.* **2**, 63 (1964); J. M. Sayer, M. Peskin, and W. P. Jencks, *J. Am. Chem. Soc.* **95**, 4277 (1973).

General acid catalysis of the breakdown of the carbinolamine intermediate occurs by assistance of the expulsion of water.

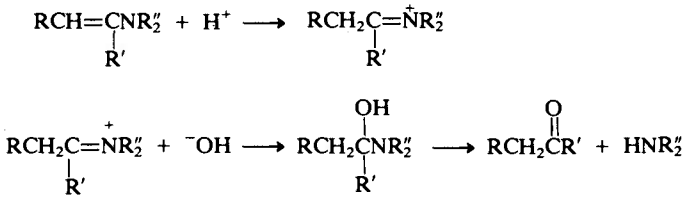


As with simple imines, the identity of the rate-limiting step changes with solution pH. As the pH decreases, the rate of the addition decreases because protonation of the amino compound reduces the concentration of the nucleophilic unprotonated form. Thus, while the dehydration step is normally rate-determining in neutral and basic solution, addition becomes rate-determining in acidic solutions.

Secondary amines cannot form imines, and dehydration proceeds to give carbon-carbon double bonds bearing amino substituents (enamines). Enamines were mentioned in Chapter 7 as examples of nucleophilic carbon species, and their synthetic utility is illustrated in Part B, Chapter 1. The equilibrium for the reaction between secondary amines and carbonyl compounds ordinarily lies far to the left in aqueous solution, but the reaction can be driven forward by dehydration methods.



The mechanism of hydrolysis of enamines has been studied kinetically over a range of pH. In alkaline solution, rate-determining C-protonation is followed by attack of hydroxide ion on the resulting iminium ion. The carbinolamine intermediate then breaks down as in imine hydrolysis. In the neutral and weakly acidic pH range, water attack on the C-protonated enamine becomes rate-limiting. As in imine hydrolysis, decomposition of the tetrahedral intermediate becomes rate-limiting in strongly acidic solution.[13]



Certain reactions between carbonyl compounds and nucleophiles are catalyzed by amines. Some of these reactions are of importance for forming carbon-carbon bonds, and these are discussed in Section 2.2 of Part B. The mechanistic principle can be illustrated by considering the catalysis of the reaction between ketones and

13. P. Y. Sollenberger and R. B. Martin, *J. Am. Chem. Soc.* **92**, 4261 (1970); W. Maas, M. J. Janssen, E. J. Stamhuis, and H. Wynberg, *J. Org. Chem.* **32**, 1111 (1967); E. J. Stamhuis and W. Maas, *J. Org. Chem.* **30**, 2156 (1965).

hydroxylamine by aniline derivatives.

$$ArCH{=}O + NH_2OH \xrightarrow{Ar'NH_2} ArCH{=}NOH + H_2O$$

Analysis of the kinetics of this catalysis points to the protonated imine as a key intermediate.

$$ArCH{=}O + Ar'NH_2 + H^+ \rightarrow ArCH{=}\overset{+}{N}HAr' + H_2O$$
$$ArCH{=}\overset{+}{N}HAr' + NH_2OH \xrightarrow{fast} ArCH{=}NOH + Ar\overset{+}{N}H_3$$

Because the imine is much more basic than the original carbonyl compound, it is more extensively protonated at any given pH than is the aldehyde. The protonated imine is also more reactive as an electrophile than the neutral aldehyde. The four distinct electrophiles present in the system are

$$ArCH{=}\overset{+}{O}H > ArCH{=}\overset{+}{N}HAr' > ArCH{=}O > ArCH{=}NAr'$$
$$\xrightarrow[\text{decreasing reactivity}]{}$$

The protonated imine is the dominant reactive form. Although the protonated aldehyde is more reactive, its concentration is very low since it is much less basic than the imine or the reactant hydroxylamine. On the other hand, even though the aldehyde may be present in a greater concentration than the protonated imine, its reactivity is sufficiently less so that the iminium ion is the major reactant.[14]

8.3. Addition of Carbon Nucleophiles to Carbonyl Groups

The addition of carbon nucleophiles, including organometallic compounds, enolates, and enols, to carbonyl groups is one of the main methods of formation of carbon–carbon bonds. Such reactions are extremely important in synthesis and will be discussed extensively in Part B. Here, we will examine some of the fundamental mechanistic aspects of addition of carbon nucleophiles to carbonyl groups.

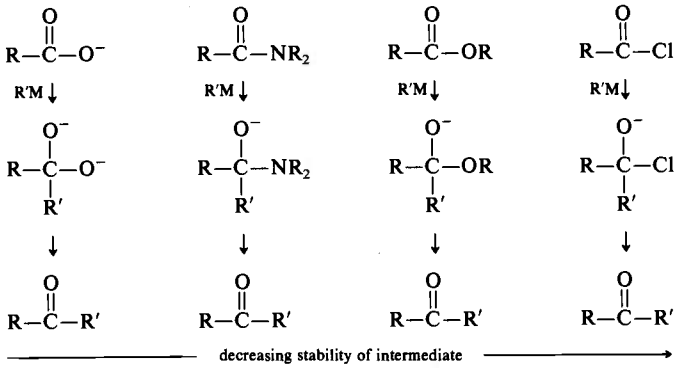
Organolithium and organomagnesium reagents are highly reactive toward most carbonyl compounds. With aldehydes and ketones, the tetrahedral adduct is stable, and alcohols are isolated after hydrolysis of the product alkoxide salt.

$$R'M + R_2C{=}O \longrightarrow R'{-}\overset{R}{\underset{R}{C}}{-}O^-M^+ \xrightarrow{H^+} R'{-}\overset{R}{\underset{R}{C}}{-}OH$$

In the case of esters, carboxylate anions, amides, and acid chlorides, the tetrahedral adduct may undergo elimination. The elimination forms a ketone, permitting a subsequent addition to occur. The rate at which breakdown of the tetrahedral adduct

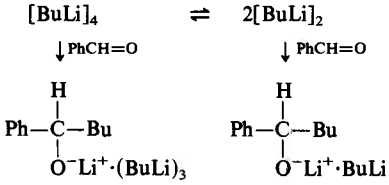
14. E. H. Cordes and W. P. Jencks, *J. Am. Chem. Soc.* **84**, 826 (1962); J. Hine, R. C. Dempsey, R. A. Evangelista, E. T. Jarvi, and J. M. Wilson, *J. Org. Chem.* **42**, 1593 (1977).

occurs is a function of the reactivity of the heteroatom substituent as a leaving group. The order of stability of the tetrahedral intermediates is shown below.



Im most cases, the product ratio can be controlled by choice of reaction conditions. Ketones are isolated under conditions where the tetrahedral intermediate is stable until hydrolyzed, whereas alcohols are formed when the tetrahedral intermediate decomposes in the presence of unreacted organometallic reagent. Examples of synthetic application of this class of reactions will be discussed in Section 7.2 of Part B.

The reaction of organolithium reagents with simple carbonyl compounds is very fast, and there is relatively little direct kinetic evidence concerning the details of the reaction. It would be expected that one important factor in determining reactivity would be the degree of aggregation of the organolithium reagent. It has been possible to follow the reaction of benzaldehyde with *n*-butyllithium at −85°C, using NMR techniques that are capable of monitoring rapid reactions. The reaction occurs over a period of 50–300 ms. It has been concluded that the dimer of *n*-butyllithium is more reactive than the tetramer by a factor of about 10. As the reaction proceeds, the product alkoxide ion is incorporated into butyllithium aggregates. This gives rise to an additional species with a different reactivity.[15]



The rate laws for the reaction of several aromatic ketones with alkyllithium reagents has been examined. The reaction of 2,4-dimethyl-4′-methylthiobenzophenone with methyllithium in ether exhibits the rate law

$$\text{rate} = k[\text{MeLi}]^{1/4}[\text{ketone}]$$

15. J. F. McGarrity, C. A. Ogle, Z. Brich, and H.-R. Loosli, *J. Am. Chem. Soc.* **107**, 1810 (1985).

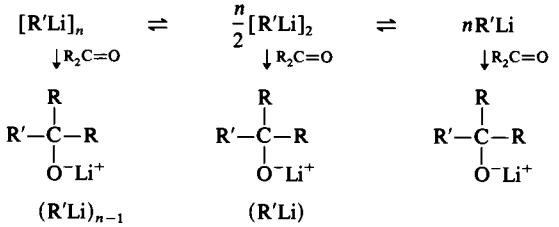
This is consistent with a mechanism in which monomeric methyllithium in equilibrium with the tetramer is the reactive nucleophile.[16]

$$[\mathrm{MeLi}]_4 \rightleftharpoons 4\,\mathrm{MeLi}$$

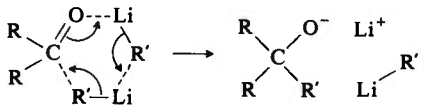
$$\mathrm{MeLi} + \text{ketone} \xrightarrow{\text{slow}} \text{product}$$

Most other studies have led to considerably more complex behavior. The rate data for reaction of 3-methyl-1-phenylbutanone with *s*-butyllithium and *n*-butyllithium in cyclohexane can be fit to a mechanism involving product formation both through a complex of the ketone with alkyllithium aggregate and through reaction with dissociated alkyllithium.[17] Evidence for the initial formation of a complex can be observed in the form of a shift in the carbonyl absorption band in the infrared spectrum. Complex formation presumably involves a Lewis acid–base interaction between the carbonyl oxygen and lithium ions in the alkyllithium cluster.

In general terms, it appears likely that alkyllithium reagents have the possibility of reacting through any of several aggregated and dissociated forms. The precise distribution will depend on the specific conditions of reaction.

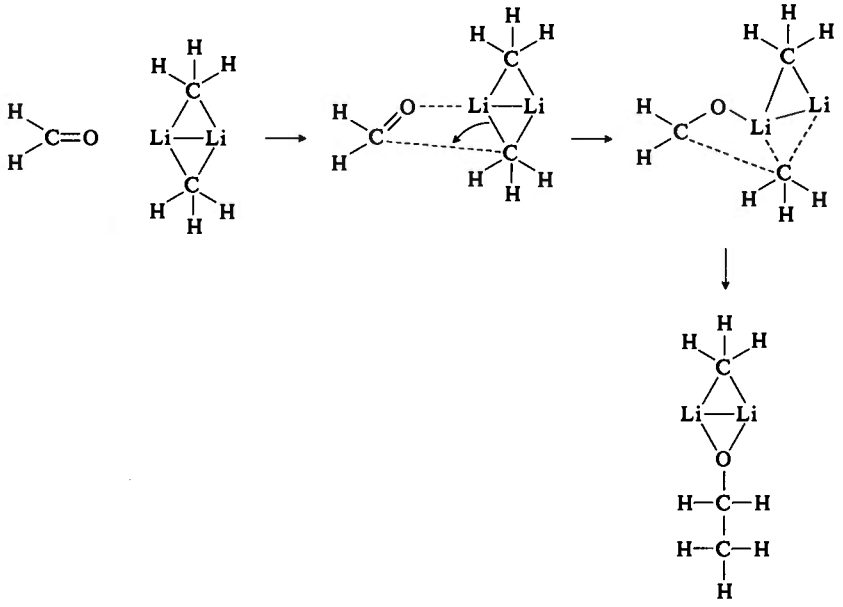


A cyclic mechanism can be written for reaction with a dimeric species.



Molecular orbital modeling of the reaction of organolithium compounds with carbonyl groups has examined the interaction of formaldehyde with the dimer of methyllithium. The reaction is predicted to proceed by initial complexation of the carbonyl group at lithium, followed by a rate-determining step involving formation of the new carbon–carbon bond. The cluster then reorganizes to incorporate the newly formed alkoxide ion.[18]

16. S. G. Smith, L. F. Charbonneau, D. P. Novak, and T. L. Brown, *J. Am. Chem. Soc.* **94**, 7059 (1972).
17. M. A. Al-Aseer and S. G. Smith, *J. Org. Chem.* **49**, 2608 (1984).
18. E. Kaufman, P. v. R. Schleyer, K. N. Houk, and Y.-D. Wu, *J. Am. Chem. Soc.* **107**, 5560 (1985).



The transition state is reached very early in the second step with only slight formation of the C—C bond.

The kinetics of addition of alkyllithium reagents has been studied using a series of ethyl benzoates. The rates show a rather complex dependence on both alkyllithium concentration and the nature of aryl substituents in the ester. The rapid formation of an initial ester-alkyllithium complex can be demonstrated. It is believed that product can be formed by reaction of both aggregated and monomeric alkyllithium reagent. *N,N,N,N*-Tetramethylethylenediamine greatly accelerates the reaction, presumably by dissociating the organometallic aggregate (see Section 7.1).

$$(RLi)_n + n\text{TMEDA} \rightleftharpoons \frac{n}{2}[(RLi)_2 \cdot 2\text{TMEDA}]$$

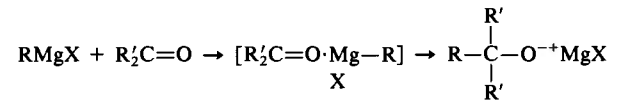
$$[(RLi)_2 \cdot 2\text{TMEDA}] + R'O_2CPh \rightarrow \left[R-\underset{\displaystyle Ph}{\overset{\displaystyle O-R'}{\underset{|}{\overset{|}{C}}}}-O^-Li^+ \cdot RLi \cdot 2\text{TMEDA} \right]$$

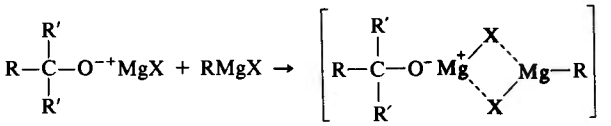
The kinetics of reaction of Grignard reagents with ketones are also subject to a number of complications. The purity of the magnesium metal used in the preparation of the Grignard reagent is crucial since trace transition metal impurities have a major effect on the observed reaction rates. One of the most thorough studies available involves the reaction of methylmagnesium bromide with 2-methylbenzophenone in diethyl ether.[19] The results suggest the reaction mechanism is similar to that discussed for alkyllithium reactions. There is initial complexation between the

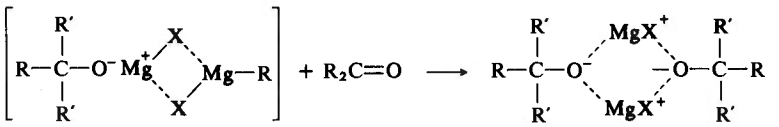
19. E. C. Ashby, J. Laemmle, and H. M. Neumann, *J. Am. Chem. Soc.* **94**, 5421 (1972).

ketone and Grignard reagent. The main Grignard species CH_3MgBr is in equilibrium with $(CH_3)_2Mg$, and this species can contribute to the overall rate. Finally, the product alkoxide complexes with the Grignard reagent to give another reactive species. The general mechanistic scheme is outlined below.

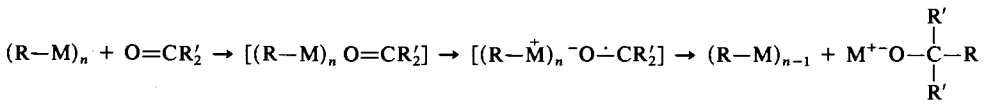
$$2RMgX \rightleftharpoons MgX_2 + R_2Mg$$







There is another mechanism for addition of organometallic reagents to carbonyl compounds. This involves a discrete electron transfer step.[20]

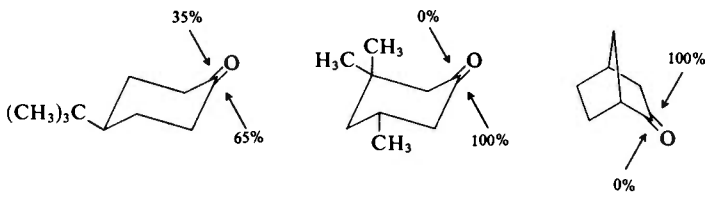


The distinguishing feature of this mechanism is the second step, in which an electron is transferred from the organometallic reagent to the carbonyl compound to give the radical anion of the carbonyl compound. Subsequent collapse of the ion pair gives the same product as is formed in the normal mechanism. The electron transfer mechanism would be expected to be favored by structural features that stabilize the radical anion. Aryl ketones and diones fulfill this requirement, and much evidence for the electron transfer mechanism has been accumulated for such ketones. In several cases, it is possible to observe the intermediate radical anion by EPR spectroscopy.[21]

The stereochemistry of organometallic additions with carbonyl compounds fits into the general pattern for nucleophilic attack discussed in Chapter 3. With 4-*t*-butylcyclohexanone, there is a preference for equatorial attack but the selectivity

20. E. C. Ashby, *Pure Appl. Chem.* **52**, 545 (1980); E. C. Ashby, J. Laemmle, and H. M. Neumann, *Acc. Chem. Res.* **7**, 272 (1974).
21. K. Maruyama and T. Katagiri, *J. Am. Chem. Soc.* **108**, 6263 (1986); E. C. Ashby and A. B. Goel, *J. Am. Chem. Soc.* **103**, 4983 (1981).

is low. Enhanced steric factors promote exclusive equatorial addition.

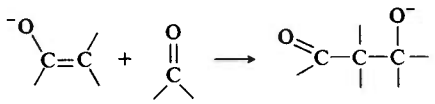


Ref. 22

Stereoselectivity in approach of methyllithium to cyclic ketones.

Addition of Grignard reagents to ketones and aldehydes was one of the reactions that led to the formulation of Cram's rule.[23] (See Section 3.10.) Many ketones and aldehydes have subsequently been subjected to studies to determine the degree of stereoselectivity. Cram's rule is obeyed when no special complexing functional groups are present near the reaction site. One such series of studies is summarized in Table 8.2.

The addition reaction of enolates with carbonyl compounds is of very broad scope and is of great synthetic importance. Essentially all of the enolates introduced in Chapter 7 are capable of adding to carbonyl groups. The reaction is known as the *generalized aldol condensation.*



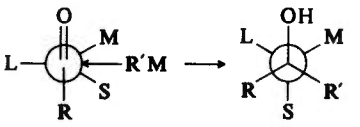
Enolates of aldehydes, ketones, and esters and the carbanions of nitriles and nitro compounds as well as phosphorus- and sulfur-stabilized carbanions undergo the reaction. The synthetic application of this group of reactions will be discussed in detail in Chapter 2 of Part B. In this section, we will discuss the fundamental mechanistic issues using the reaction of ketone enolates with aldehydes and ketones.

The aldol condensation can be carried out under either of two broad sets of conditions which lead to the product being determined by either kinetic or thermodynamic factors. To achieve *kinetic control,* the enolate which is to serve as the nucleophile is generated stoichiometrically, usually with lithium as the counterion in an aprotic solvent. Under these conditions, enolates are both structurally and stereochemically stable. The electrophilic carbonyl compound is then added. Under these conditions, the structure of the reaction product is determined primarily by two factors: (1) the structure of the initial enolate and (2) the stereoselectivity of the addition to the electrophilic carbonyl group.

The other broad category of reaction conditions results in *thermodynamic control.* This can result from several factors. Aldol condensations can be effected for many compounds using less than a stoichiometric amount of base. Under these conditions,

22. E. C. Ashby and S. A. Noding, *J. Org. Chem.* **44**, 4371 (1979).
23. D. J. Cram and F. A. Abd Elhafez, *J. Am. Chem. Soc.* **74**, 5828 (1952); D. J. Cram and J. D. Knight, *J. Am. Chem. Soc.* **74**, 5835 (1952); F. A. Abd Elhafez and D. J. Cram, *J. Am. Chem. Soc.* **74**, 5846 (1952).

Table 8.2. Stereoselectivity in Addition of Organometallic Reagents to Some Chiral Aldehydes and Ketones[a]

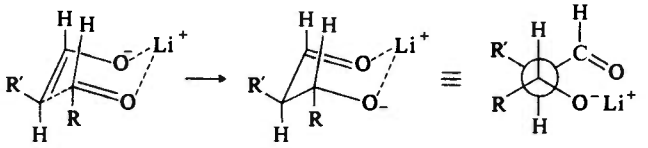


R	L	M	S	R'M	Percent of favored product
H	Ph	CH_3	H	CH_3MgBr	71
H	Ph	CH_3	H	PhMgBr	78
H	t-C_4H_9	CH_3	H	PhMgBr	98
CH_3	Ph	CH_3	H	C_2H_5Li	93
CH_3	Ph	CH_3	H	C_2H_5MgBr	88
CH_3	Ph	CH_3	H	t-C_4H_9MgBr	96
C_2H_5	Ph	CH_3	H	CH_3MgBr	86
C_2H_5	Ph	CH_3	H	CH_3Li	94
C_2H_5	Ph	CH_3	H	PhLi	85
i-C_3H_7	Ph	CH_3	H	CH_3MgBr	90
i-C_3H_7	Ph	CH_3	H	CH_3Li	96
i-C_3H_7	Ph	CH_3	H	PhLi	96
t-C_4H_9	Ph	CH_3	H	CH_3MgBr	96
t-C_4H_9	Ph	CH_3	H	CH_3Li	97
t-C_4H_9	Ph	CH_3	H	PhLi	98
Ph	Ph	CH_3	H	CH_3MgBr	87
Ph	Ph	CH_3	H	CH_3Li	97
Ph	Ph	CH_3	H	t-C_4H_9MgBr	96

a. Data from O. Arjona, R. Perez-Ossorio, A. Perez-Rubalcaba, and M. L. Quiroga, *J. Chem. Soc., Perkin Trans. 2,* 597 (1981); C. Alvarez-Ibarra, P. Perez-Ossorio, A. Perez-Rubalcaba, M. L. Quiroga, and M. J. Santesmases, *J. Chem. Soc., Perkin Trans. 2,* 1645 (1983).

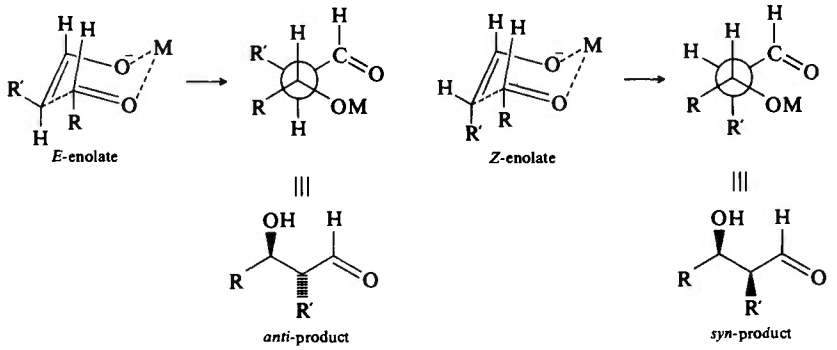
the aldol reaction is reversible and the product ratio will be determined by the relative stability of the various possible products. Thermodynamic conditions also permit equilibration between all the enolates of the nucleophile. The conditions which permit equilibration include higher reaction temperatures, the presence of protic solvents, and the use of less tightly coordinating cations.

The fundamental mechanistic concept around which the course of aldol reactions under conditions of kinetic control has been interpreted involves the postulation of a cyclic transition state in which both the carbonyl and the enolate oxygen are coordinated to a Lewis acid. We will use the Li^+ cation in our discussion, but another metal cation or electron-deficient atom may play the same role.



According to this concept, the aldol condensation normally occurs through a chairlike transition state. It is further assumed that the structure of this transition state is sufficiently similar to that of chair cyclohexane that the conformational concepts developed for cyclohexane derivatives can be applied. Thus, in the example above, the reacting aldehyde is shown with R rather than H in the equatorial-like position. The differences in stability of the various transition states, and therefore the product ratios, are governed by the steric interactions between substituents.

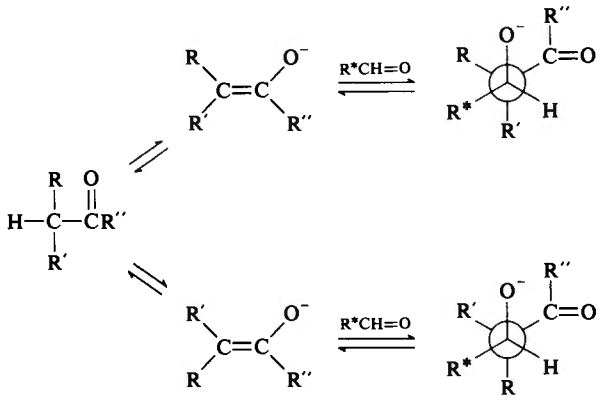
A consequence of this mechanism is that the reaction if *stereospecific* with respect to the *E*- or *Z*-configuration of the enolate. The *E*-enolate will give the *anti* aldol product while the *Z*-enolate will give the *syn* aldol.



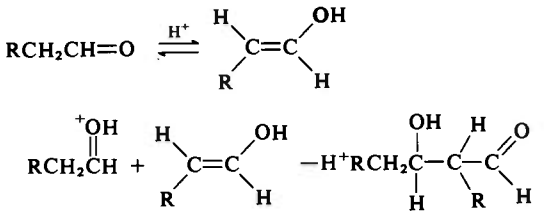
Numerous observations on the reactions of enolates of both ketones and esters are consistent with this general concept.[24] However, the prediction or interpretation of the *specific ratio* of *syn* to *anti* product from any given reaction process requires assessment of several variables. These include: (1) What is the stereochemistry of the enolate that is reacting? (2) How strong is the selectivity between the two faces of the carbonyl group? This is a function of the steric interactions in the diastereomeric transition states. (3) Does the Lewis acid promote a tight coordination with both the carbonyl and enolate oxygen atoms? (4) Are there any special structural features, such as additional Lewis base coordination sites, which could override steric effects? (5) Are the reaction conditions such as to promote kinetic control? Chapter 2 of Part B will give a more complete discussion of the ways in which these factors can be controlled to provide specific reaction products.

When the aldol reaction is carried out under thermodynamic conditions, the product selectivity is often not as high as under kinetic conditions. All the regioisomeric and stereoisomeric enolates may participate as nucleophiles. The adducts can return to reactants, and so only the difference in stability of the stereoisomeric *anti* and *syn* products will influence the product composition.

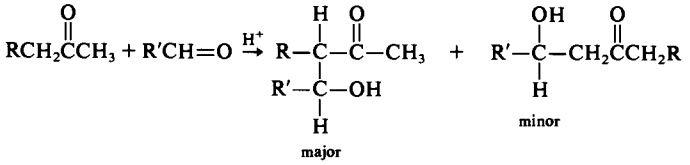
24. C. H. Heathcock, in *Asymmetric Syntheses*, Vol. 3, J. D. Morrison (ed.), Academic Press, New York, 1984, Chapter 2; C. H. Heathcock, in *Comprehensive Carbanion Chemistry*, Part B, E. Buncel and T. Durst (eds.), Elsevier, Amsterdam, 1984, Chapter 4; T. Mukaiyama, *Org. React.* **28**, 203 (1982); D. A. Evand, J. V. Nelson, and T. R. Taber, *Top Stereochem.* **13**, 1 (1982); A. T. Nielsen and W. J. Houlihan, *Org. React.* **16**, 1 (1968).



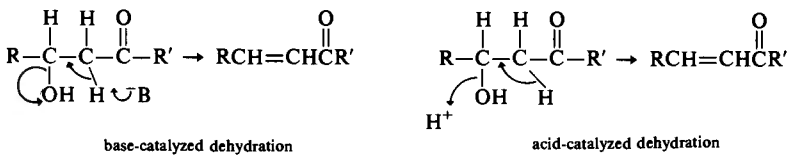
It is also possible to carry out the aldol condensation under acidic conditions. The reactive nucleophile is then the enol. The mechanism, as established in detail for acetaldehyde,[25] involves nucleophilic attack of the enol on the protonated carbonyl tautomer.



There has been little study of the stereochemistry of the reaction under acidic conditions. When regioisomeric enols are possible, acid-catalyzed reactions tend to go through the more substituted of the enols.

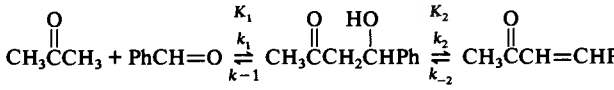


Under both basic and acidic conditions, the aldol condensation can proceed to dehydrated product.



25. L. M. Baigrie, R. A. Cox, H. Slebocka-Tilk, M. Tencer, and T. T. Tidwell, *J. Am. Chem. Soc.* **107**, 3640 (1985).

The dehydration reactions require somewhat higher activation energies than the addition step and are not usually observed under strictly kinetic conditions. Detailed rate studies have provided rate and equilibrium constants for the individual steps in some cases. The results for the acetone-benzaldehyde system in the presence of hydroxide ion are given below.



Ref. 26

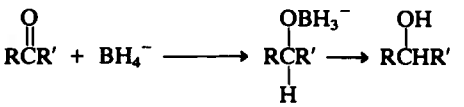
$K_1 = 0.83M^{-1}$ $\quad$ $K_2 = 24$

$k_1 = 0.17M^{-2}\,s^{-1}$ $\quad$ $k_2 = 7.5 \times 10^{-3}M^{-1}\,s^{-1}$

$k_{-1} = 1.4 \times 10^{-2}M^{-1}\,s^{-1}$ $\quad$ $k_{-2} = 3.2 \times 10^{-4}M^{-1}\,s^{-1}$

8.4. Reactivity of Carbonyl Compounds toward Addition

We would like at this point to consider some general relationships concerning the reactivity of carbonyl compounds. The discussion of the nucleophilic addition reactions in Sections 8.2 and 8.3 indicates that many factors influence the overall rate of a reaction under typical conditions. Among the crucial factors are (1) the role of protons or other Lewis acids in activating the carbonyl group toward nucleophilic attack, (2) the reactivity of the nucleophilic species and its influence on the reaction mechanism and (3) the stability of the tetrahedral intermediate and the extent to which it proceeds to product rather than reverting to starting material. Since consideration of all of these factors complicates the interpretation of the inherent reactivity of the carbonyl compound itself, an irreversible process where the addition product is stable affords the most direct means of comparing the reactivity of different carbonyl compounds. Under these conditions, the relative rates of reaction of different carbonyl compounds can be directly compared. One such reaction is hydride reduction. In particular, reduction by sodium borohydride in alcohol solvents is a fast, irreversible reaction which has provided a convenient basis for comparing the reactivity of different carbonyl compounds.[27]



The reaction is second-order overall, with the rate given by $k[R_2C{=}O][NaBH_4]$. The interpretation of the rates is complicated slightly by the fact that the alkoxyborohydrides produced by the first addition can also function as reducing agents, but this has little apparent effect on the relative reactivity of the carbonyl compounds. Table 8.3 presents some of the rate data obtained from these studies.

26. J. P. Guthrie, J. Cossar, and K. F. Taylor, *Can. J. Chem.* **62**, 1958 (1984).
27. H. C. Brown, O. H. Wheeler, and K. Ichikawa, *Tetrahedron* **1**, 214 (1957); H. C. Brown and K. Ichikawa, *Tetrahedron* **1**, 221 (1957).

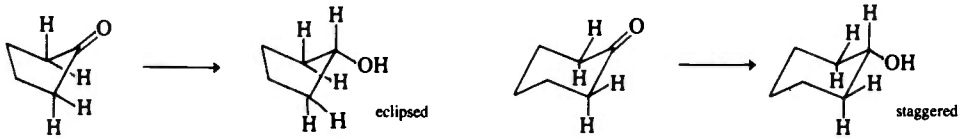
Table 8.3. Rates of Reduction of Aldehydes and Ketones by Sodium Borohydride

Carbonyl compound	$k_2 \times 10^4$ $(M^{-1} s^{-1})^a$
Benzaldehyde	12,400[b]
Benzophenone	1.9
Acetophenone	2.0
Acetone	15.1
Cyclobutanone	264
Cyclopentanone	7
Cyclohexanone	161

a. In isopropyl alcohol at 0°C.
b. Extrapolated from data at lower temperatures.

Reductions by $NaBH_4$ are characterized by low enthalpies of activation (8–13 kcal/mol) and large negative entropies of activation (−28 to −40 eu). Aldehydes are substantially more reactive than ketones as can be seen by comparison of the rates for benzaldehyde and acetophenone. This relative reactivity is characteristic for nearly all carbonyl addition reactions. The lesser reactivity of ketones can be attributed primarily to steric effects. Not only does the additional substituent increase the steric restrictions to approach of the nucleophile, but also it gives rise to larger steric interaction in the tetrahedral product as the hybridization changes from trigonal to tetrahedral.

Among the cyclic ketones, the reactivity of cyclobutanone is enhanced because of the strain of the four-membered ring, which is decreased in going from sp^2 to sp^3 hybridization. The greater reactivity of cyclohexanone as compared to cyclopentanone is also quite general for carbonyl addition reactions. The major factor responsible for the large difference in this case is the change in torsional strain as addition occurs. As the hybridization goes from sp^2 to sp^3, the torsional strain is increased in cyclopentanone. The opposite is true for cyclohexanone. The equatorial hydrogens are nearly eclipsed with the carbonyl oxygen in cyclohexanone, but the chair structure of cyclohexanol allows all bonds to be in staggered arrangements.



The borohydride reduction rate data are paralleled by many other carbonyl addition reactions. In fact, for a series of ketones, most of which are cyclic, a linear free-energy correlation of the form

$$\log k = A \log k_0 + B$$

Table 8.4. Relative Reactivity of Some Ketones toward Addition of Nucleophiles

Ketone	B = log relative reactivity[a]
Cyclobutanone	0.09
Cyclohexanone	0.00[a]
4-*t*-butylcyclohexanone	−0.008
Adamantanone	−0.46
Cycloheptanone	−0.95
Cyclopentanone	−1.18
Acetone	−1.19
Bicyclo[2.2.1]heptan-2-one	−1.48
3,3,5,5-Tetramethylcyclohexanone	−1.92

a. A. Finiels and P. Geneste, *J. Org. Chem.* **44**, 1577 (1979); reactivity relative to cyclohexanone as a standard.

exists for nucleophiles such as NH_2OH, CN^-, $HOCH_2CH_2S^-$, and SO_3^-.[28] These nucleophiles span a wide range of reactivity and represent nitrogen, carbon, and sulfur atoms acting as the nucleophile. The free-energy relationship implies that in this series of ketones the same structural features govern reactivity toward each of the nucleophiles. To a good approximation, the parameter $A = 1$, which reduces the correlation to

$$\log(k/k_0) = B$$

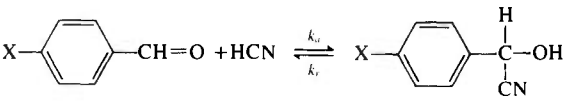
This equation implies that the relative reactivity is independent of the specific nucleophile and that relative reactivity is insensitivie to changes in position of the transition state. Table 8.4 lists some of the B values for some representative ketones. The parameter B indicates relative reactivity on a log scale. Cyclohexanone is seen to be a particularly reactive ketone, being almost as reactive as cyclobutanone and more than 10 times as reactive as acetone.

The same structural factors come into play in determining the position of equilibria in reversible additions to carbonyl compounds. The best studied of such equilibrium processes is probably addition of cyanide to give cyanohydrins.

$$R_2C{=}O + HCN \rightleftharpoons N{\equiv}C{-}\overset{\displaystyle R}{\underset{\displaystyle R}{\overset{|}{\underset{|}{C}}}}{-}OH$$

For cyclopentanone, cyclohexanone, and cycloheptanone the K values for addition are 48, 1000, and 8 M^{-1}, respectively.[29] For aromatic aldehydes, the equilibria are affected by the electronic nature of the aryl substituent. Electron donors disfavor addition by stabilizing the aldehyde whereas electron-accepting substituents have

28. A. Finiels and P. Geneste, *J. Org. Chem.* **44**, 1577 (1979).
29. V. Prelog and M. Kobelt, *Helv. Chim. Acta* **32**, 1187 (1949).

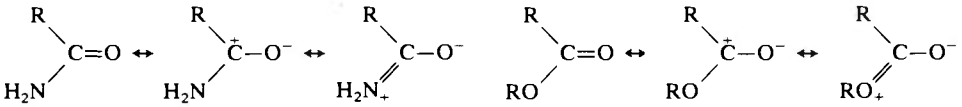


Ref. 30

K is correlated by Hammett equation with σ^+, $\rho = 1.01$

k_a is correlated by Hammett equation with σ^+, $\rho = 1.18$

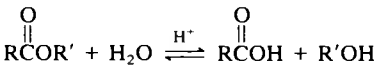
There are large differences in the reactivity of the various carboxylic acid derivatives, such as amides, esters, and acyl chlorides. One important factor is the resonance stabilization provided by the heteroatom. This is in the order N > O > Cl. Electron donation reduces the electrophilicity of the carbonyl group, and the corresponding stabilization is lost in the tetrahedral intermediate.



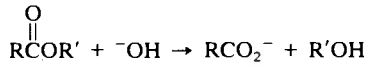
The high reactivity of the acyl chlorides also reflects the electron-withdrawing inductive effect of the chlorine, which more than outweighs the small π-donor effect.

8.5. Ester Hydrolysis

Esters can be hydrolyzed in either basic or acidic solution. In acidic solution, the reaction is reversible.



The position of the equilibrium depends on the relative concentration of water and the alcohol. In aqueous solution, hydrolysis occurs. In alcohlic solution, the equilibrium is shifted in favor of the ester. In alkaline aqueous solution, ester hydrolysis is essentially irreversible:

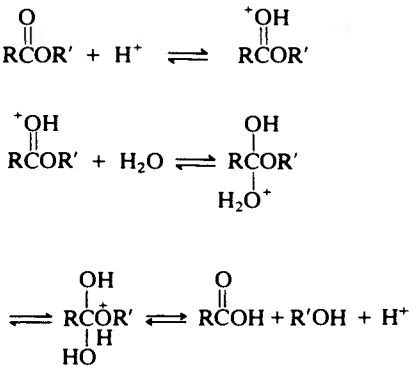


The carboxylic acid is converted to its anion under these conditions, and the position of the equilibrium lies far to the right. The mechanistic designations $A_{AC}2$ and $B_{AC}2$ are given to the acid- and base-catalyzed hydrolysis mechanisms, respectively. The A denotes acid catalysis, while B indicates base catalysis. The AC designation indicates acyl-oxygen bond cleavage occurs. The digit 2 has its usual significance,

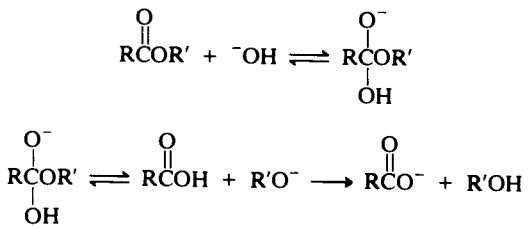
30. W.-M. Ching, and R. G. Kallen, *J. Am. Chem. Soc.* **100**, 6119 (1978).

indicating the bimolecular nature of the rate-determining step.

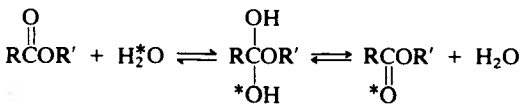
$A_{AC}2$ mechanism



$B_{AC}2$ mechanism



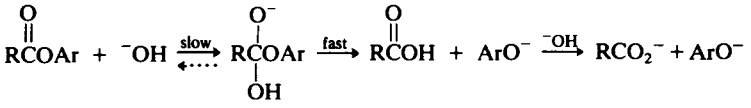
Esters without special structural features hydrolyze by these mechanisms. Among the evidence supporting these mechanisms are kinetic studies that show the expected dependence on hydrogen ion or hydroxide ion concentration and isotopic labeling studies that prove that it is the acyl-oxygen, not the alkyl-oxygen, bond that is cleaved during hydrolysis.[31] Acid-catalyzed hydrolysis of esters is accompanied by some exchange of oxygen from water into the ester. This exchange occurs by way of the tetrahedral intermediate when expulsion of water is competitive with expulsion of the alcohol.



Substituent effects come into play at several points in the ester hydrolysis mechanism. In the base-catalyzed reaction, electron-withdrawing substituents in either the acyl or the alkoxy group facilitate hydrolysis. Since the tetrahedral intermediate formed in the rate-determining step is negatively charged, it and the transition state leading to it are stabilized by electron withdrawal. If the carbonyl group is conjugated with an electron-releasing group, reactivity is decreased by

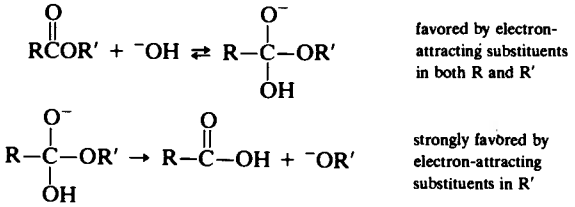
31. M. L. Bender, *Chem. Rev.* **60**, 53 (1960).

ground state stabilization. The partitioning of the tetrahedral intermediate between reversion to starting material by loss of hydroxide ion and formation of product by expulsion of the alkoxide is strongly affected by substituents in the alkoxy group. Electron-withdrawing groups shift the partitioning in favor of loss of the alkoxide and favor hydrolysis. For this reason, exchange of carbonyl oxygen with solvent does not occur in basic hydrolyses when the alkoxy group is a good leaving group. This has been demonstrated, for example, for esters of phenols. Because phenols are much stronger acids than alcohols, their conjugate bases are better leaving groups than simple alkoxide ions. Aryl esters are hydrolyzed faster than alkyl esters and without observable exchange of carbonyl oxygen with solvent.

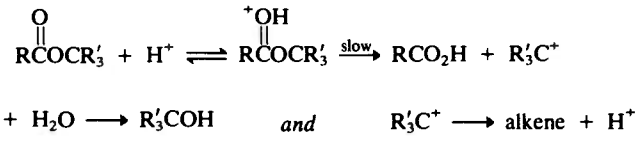


Even simple alkyl benzoate esters give only a small amount of exchange under basic hydrolysis conditions. This means that reversal of the hydroxide addition must be slow relative to the forward breakdown of the tetrahedral intermediate.[32]

These substituent effects can be summarized in a general way for the B_{AC} mechanism by noting the effect of substituents on each step of the mechanism:



It is possible to shift ester hydrolysis away from the normal $A_{AC}2$ or $B_{AC}2$ mechanisms by structural changes in the reactant. When the ester is derived from a tertiary alcohol, acid-catalyzed hydrolysis often occurs by a mechanism involving alkyl-oxygen fission. The change in mechanism is due to the stability of the tertiary carbocation that can be formed by alkyl-oxygen cleavage.[33] When this mechanism occurs, alkenes as well as alcohols may be produced, since the carbocation can react by substitution or elimination. This mechanism is referred to as $A_{AL}1$, reflecting the fact that the alkyl-oxygen bond is broken.

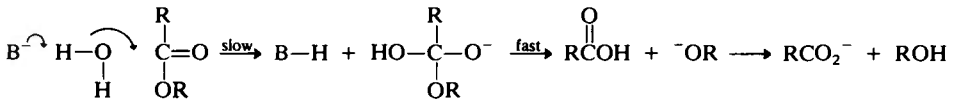


The change of mechanism of tertiary alkyl esters is valuable in synthetic methodology since it permits certain esters to be hydrolyzed very selectively. The usual situation

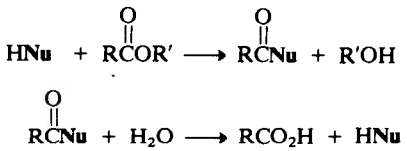
32. R. A. McClelland, *J. Am. Chem. Soc.* **106**, 7579 (1984).
33. A. G. Davies and J. Kenyon, *Q. Rev. Chem. Soc.* **9**, 203 (1955).

involves the use of *t*-butyl esters, which can be cleaved to carboxylic acids by action of acids such as *p*-toluenesulfonic acid or trifluoroacetic acid under anhydrous conditions where other esters are stable.

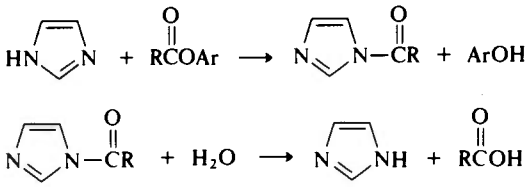
In the preceding paragraphs, the ester hydrolysis mechanisms discussed pertained to aqueous solutions of strong acids and strong bases. These are conditions under which specific acid catalysis or specific base catalysis is expected to be dominant. In media in which other acids or bases are present, the possible occurrence of general-acid-catalyzed and general-base-catalyzed hydrolysis must be considered. General base catalysis has been observed in the case of esters in which the acyl group carries electron-attracting substituents.[34] The transition state for esters undergoing hydrolysis by a general-base-catalyzed mechanism involves partial proton transfer from the attacking water molecule to the general base during formation of the tetrahedral intermediate.



Ester hydrolysis can also be promoted by *nucleophilic catalysis.* If a component of the reaction system is a more effective nucleophile toward the carbonyl group than hydroxide ion or water under a given set of conditions, an acyl transfer reaction can take place to form an intermediate.

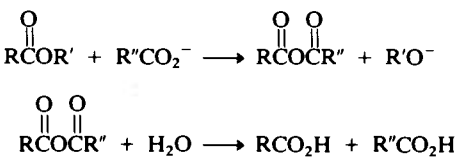


If this intermediate, in turn, is more rapidly attacked by water or hydroxide ion than the original ester, the overall reaction will be faster in the presence of the nucleophile than in its absence. These are the requisite conditions for nucleophilic catalysis. Esters of relatively acidic alcohols (in particular, phenols) are hydrolyzed by the nucleophilic catalysis mechanism in the presence of imidazole.[35]

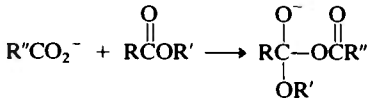


34. W. P. Jencks and J. Carriuolo, *J. Am. Chem. Soc.* **83**, 1743 (1961).
35. T. C. Bruice and G. L. Schmir, *J. Am. Chem. Chem. Soc.* **79**, 1663 (1957); M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.* **79**, 1652, 1656 (1957).

Carboxylate anions can also serve as nucleophilic catalysts.[36] In this case, an anhydride is the reactive intermediate.



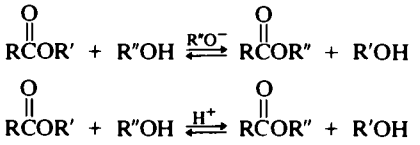
The nucleophilic catalysis mechanism only operates when the alkoxy group being hydrolyzed is not much more basic than the nucleophilic catalyst. This relationship can be understood by considering the tetrahedral intermediate generated by attack of the potential catalyst on the ester:



The relative leaving group abilities of $R'O^-$ and $R''CO_2^-$ are strongly correlated with the basicity of the two anions. If $R''CO_2^-$ is a much better leaving group than $R'O^-$, it will be eliminated preferentially form the tetrahedral intermediate and no hydrolysis will occur.

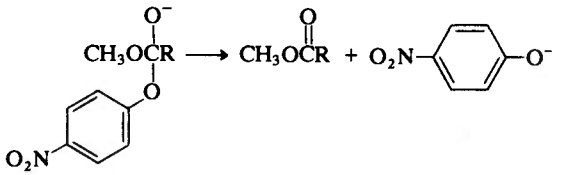
The preceding discussion has touched on the most fundamental aspects of ester hydrolysis mechanisms. Much effort has been devoted to establishing some of the finer details, particularly concerning proton transfers during the formation and breakdown of the tetrahedral intermediates. These studies have been undertaken in part because of the fundamental importance of hydrolytic reactions in biological systems. These biological hydrolyses are catalyzed by enzymes. The detailed mechanistic studies of ester hydrolysis lay the groundwork for understanding the catalytic mechanisms of the hydrolytic enzymes. Discussion of the biological mechanisms and their relationship to the fundamental mechanistic studies is available in several books that discuss enzyme catalysis in terms of molecular mechanisms.[37]

Esters react with alcohols in either acidic or basic solution to exchange alkoxy groups (ester interchange) by mechanisms which parallel those described for hydrolysis. The alcohol or alkoxide takes the role of the nucleophilic species:



36. V. Gold, D. G. Oakenfull, and T. Riley, *J. Chem. Soc., B*, 515 (1968).
37. T. C. Bruice and S. J. Benkovic, *Bioorganic Mechanisms*, Vol. 1, W. A. Benjamin, New York, 1966, pp. 1-258; W. P. Jencks, *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York, 1969; M. L. Bender, *Mechanisms of Homogeneous Catalysis from Protons to Proteins*, Wiley-Interscience, New York, 1971; C. Walsh, *Enzymatic Reaction Mechanisms*, W. H. Freeman, San Francisco, 1979; A. Fersht, *Enzyme Structure and Mechanism*, Second Edition, W. H. Freeman, New York, 1985.

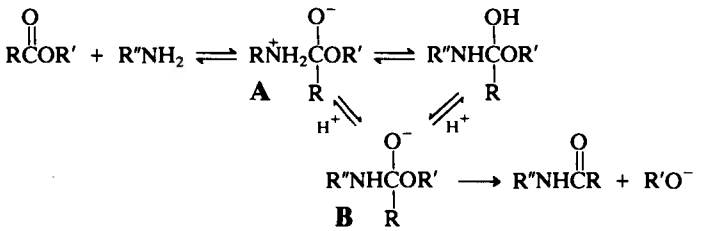
As in the case of hydrolysis, there has been a good deal of study of substituent effects, solvent effects, isotopic exchange, kinetics, and the catalysis of these processes.[38] The alcoholysis reaction is reversible in both acidic and basic solution, in contrast to hydrolysis. The key intermediate is the tetrahedral addition product. Its fate is determined largely by the relative basicity of the two alkoxy groups. A tetrahedral intermediate generated by addition of methoxide ion to a *p*-nitrophenyl ester, for example, breaks down exclusively by elimination of much less basic *p*-nitrophenoxide ion.



In general, the equilibrium in a base-catalyzed alcohol exchange reaction lies in the direction of incorporation of the less acidic alcohol into the ester.

8.6. Aminolysis of Esters

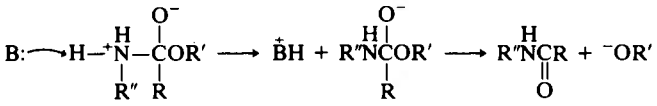
Esters react with ammonia and amines to give amides. The mechanism is similar to that of hydroxide-ion-catalyzed ester hydrolysis and involves nucleophilic attack of the amine at the carbonyl group, followed by expulsion of alkoxide ion from the tetrahedral intermediate. The identity of the rate-determining step depends primarily on the leaving group ability of the alkoxy group.[39] With relatively good leaving groups such as phenolate or acidic alcohols such as trifluoroethanol, the slow step is expulsion of the oxygen leaving group from a zwitterionic tetrahedral intermediate **A**. With poorer leaving groups, breakdown of the tetrahedral intermediate occurs only after formation of the anionic species **B**. For such systems, the deprotonation of **A** is rate-determining.



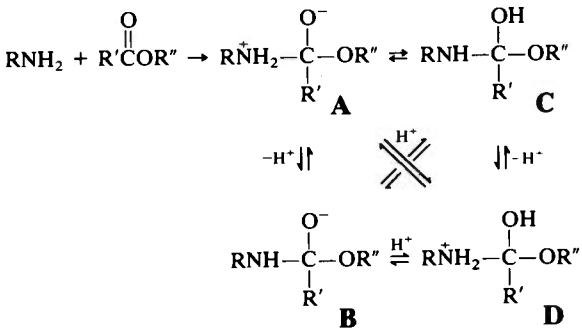
Aminolysis of esters often reveals general base catalysis and in particular a contribution to the reaction rate from terms that are second-order in the amine. The

38. C. G. Mitton, R. L. Schowen, M. Gresser, and J. Shapely, *J. Am. Chem. Soc.* **91**, 2036 (1969); C. G. Mitton, M. Gresser, and R. L. Schowen, *J. Am. Chem. Soc.* **91**, 2045 (1969).
39. F. M. Menger and J. H. Smith, *J. Am. Chem. Soc.* **94**, 3824 (1972); A. C. Satterthwait and W. P. Jencks, *J. Am. Chem. Soc.* **96**, 7018 (1974).

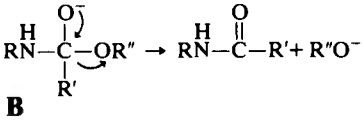
general base is believed to function by deprotonating the zwitterionic tetrahedral intermediate.[40] Deprotonation of the nitrogen facilitates breakdown of the tetrahedral intermediate, since the increased electron density at nitrogen favors expulsion of an anion.



Detailed mechanistic studies have been carried out on aminolysis of substituted aryl acetates and aryl carbonates.[41] Aryl esters are considerably more reactive than alkyl esters because the less basic phenoxide ions are better leaving groups than alkoxide ions. The tetrahedral intermediate formed in aminolysis can exist in several different forms differing in extent and site of protonation.

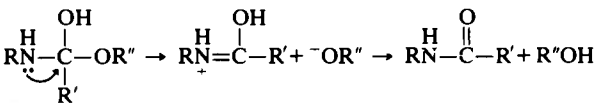


In **A** and **D** the best leaving group is the neutral amine, whereas in **B** and **C** the group $R''O^-$ would be expected to be a better leaving group than RNH^-. Furthermore, in **B** and **C** the lone pair on nitrogen can assist in elimination. In **A** the negatively charged oxygen also has the capacity to assist by "pushing" with re-formation of the carbonyl group. Precisely how the intermediate proceeds to product depends upon pH and the identity of the groups RNH_2 and $R''O^-$. When $R''O^-$ is a relatively poor leaving group, as would be the case for alkyl esters, reaction usually occurs through **B** or **C**.



B

or

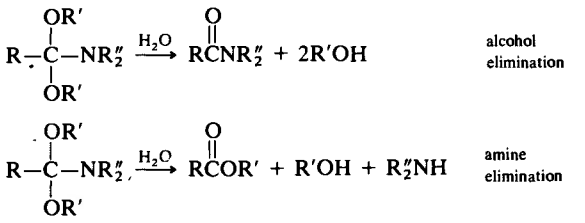


C

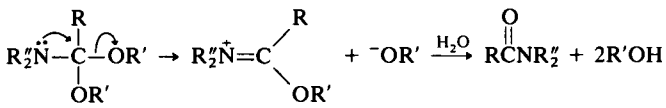
40. W. P. Jencks and M. Gilchrist, *J. Am. Chem. Soc.* **88**, 104 (1966); J. F. Kirsch and A. Kline, *J. Am. Chem. Soc.* **91**, 1841 (1969).
41. W. P. Jencks and M. Gilchrist, *J. Am. Chem.. Soc.* **90**, 2622 (1968); A. Satterthwait and W. P. Jencks, *J. Am. Chem. Soc.* **96**, 7018 (1974); A. Satterthwait and W. P. Jencks, *J. Am. Chem. Soc.* **96**, 7031 (1974); M. J. Gresser and W. P. Jencks, *J. Am. Chem. Soc.* **99**, 6970 (1977).

When the leaving group is better, breakdown can occur directly from **A**. This is the case when $R''O^-$ is a phenolate anion. The mechanism will also depend upon the pH and the presence of general acids and bases since the position of the equilibria among the tetrahedral intermediates and rates of breakdown will be determined by these factors.

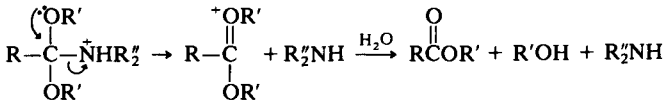
Insight into the factors that govern breakdown of tetrahedral intermediates has also been gained by studying the hydrolysis of amide acetals. If the amine is expelled, an ester is formed, whereas elimination of an alcohol gives an amide.



The pH of the solution is of overwhelming importance in determining the course of these hydrolysis.[42] In basic solution, oxygen elimination is dominant. This is because the unprotonated nitrogen substituent is a poor leaving group and is also more effective at stabilizing the intermediate resulting from alkoxide elimination.



In acidic solution, nitrogen is protonated and becomes a better leaving group and also loses its ability to assist in the elimination of the alkoxide. Under these circumstances, nitrogen elimination is favored.

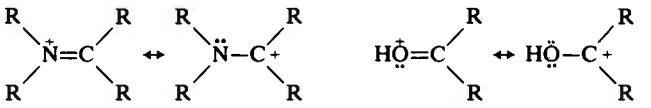


In analyzing the behavior of these types of tetrahedral intermediates, it must always be kept in mind that proton transfer reactions are usually fast relative to other steps. This permits the possibility that a minor species in equilibrium with the major species may be the primary intermediate. Detailed studies of kinetics, solvent isotope effects, and the nature of catalysis are the best tools for investigating the various possibilities.

It is useful to recognize that the dissociation of tetrahedral intermediates in carbonyl chemistry is related to the generation of carbocations by ionization pro-

42. R. A. McClelland, *J. Am. Chem. Soc.* **100**, 1844 (1978).

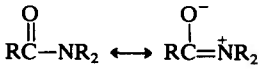
cesses. Thus, the question of which substituent on a tetrahedral intermediate is the best leaving group is similar to the issues raised in comparing the reactivity of S_N1 reactants on the basis of leaving group ability. Poorer leaving groups can react in the case of tetrahedral intermediates because of the assistance provided by the oxygen or nitrogen substituents that are present. For example, the iminium ions and protonated carbonyl compounds that are generated by breakdown of tetrahedral intermediates can be recognized as being examples of stabilized carbocations.



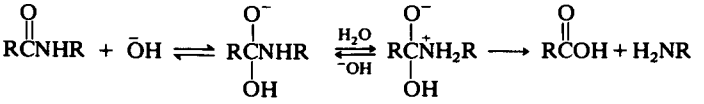
Keeping these relationships in mind should be helpful in understanding the reactivity of tetrahedral intermediates.

8.7. Amide Hydrolysis

The hydrolysis of amides to carboxylic acids and amines requires considerably more vigorous conditions than ester hydrolysis.[43] The reason is that the electron-releasing nitrogen substituent imparts a very significant ground state stabilization to the carbonyl group, and this stabilization is lost in the transition state leading to the tetrahedral intermediate.



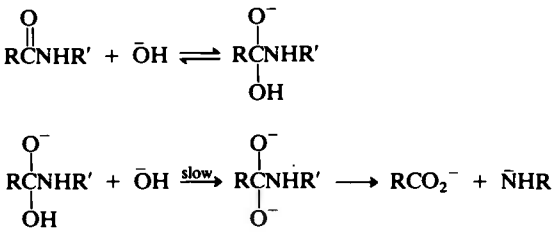
In basic solution, a mechanism similar to the $B_{AC}2$ mechanism for ester hydrolysis is believed to operate[44]:



The principal difference lies in the poorer ability of amide ions to act as leaving groups, compared to alkoxides. As a result, protonation at nitrogen is required for breakdown of the tetrahedral intermediate. Also, exchange between the carbonyl oxygen and water is extensive because reversion of the tetrahedral intermediate to reactants is faster than decomposition to products.

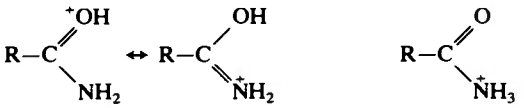
43. C. O'Connor, *Q. Rev. Chem. Soc.* **24**, 553 (1970).
44. M. L. Bender and R. J. Thomas, *J. Am. Chem. Soc.* **83**, 4183 (1961).

In some amide hydrolyses, the breakdown of the tetrahedral intermediate in the forward direction may proceed through formation of a dianion.[45]

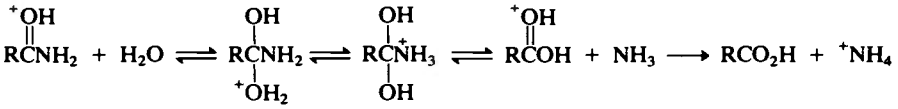


This variation from the ester hydrolysis mechanism also reflects the poorer leaving ability of amide ions as compared to alkoxide ions. The evidence for the involvement of the dianion comes from kinetic studies and from solvent isotope effects, which suggest that a rate-limiting proton transfer is involved.[46] The reaction is also higher than first-order in hydroxide ion under these circumstances, which is consistent with the dianion mechanism.

The mechanism for acid-catalyzed hydrolysis of amides involves attack by water on the protonated amide. An important feature of the chemistry of amides is that the most basic site in an amide is the carbonyl oxygen. Very little of the N-protonated form is present.[47] The major factor that contributes to the stability of the O-protonated form is the π-electron delocalization over the O—C—N system. No such delocalization is possible in the N-protonated form.



The usual hydrolysis mechanism in strongly acidic solution involves addition of water to the O-protonated amide followed by breakdown of the tetrahedral intermediate.



By using *N*-acyltrialkylammonium ions as models of the N-protonated amide, it has been possible to show that it would be kinetically impossible for acid-catalyzed

45. R. M. Pollack and M. L. Bender, *J. Am. Chem. Soc.* **92**, 7190 (1970).
46. R. L. Schowen, H. Jayaraman, L. Kershner, and G. W. Zuorick, *J. Am. Chem. Soc.* **88**, 4008 (1966).
47. R. J. Gillespie and T. Birchall, *Can. J. Chem.* **41**, 148, 2642 (1963); A. R. Fersht, *J. Am. Chem. Soc.* **93**, 3504 (1971); R. B. Martin, *J. Chem. Soc., Chem. Commun.*, 793 (1972); A. J. Kresge, P. H. Fitzgerald, and Y. Chiang, *J. Am. Chem. Soc.* **96**, 4698 (1974).

hydrolysis to proceed via the N-protonated form.[48] There is almost no exchange of oxygen with water during acid-catalyzed hydrolysis of amides.[49] Since a tetrahedral intermediate is involved, the lack of exchange requires that the intermediate must dissociate exclusively by elimination of the nitrogen substituent. This requirement is reasonable, since the amino group is the most basic site and is the preferred site of protonation in the tetrahedral intermediate. The protonated amine will be a better leaving group than hydroxide ion.

8.8. Acylation of Nucleophilic Oxygen and Nitrogen Groups

The conversion of alcohols to esters by O-acylation and of amines to amides by N-acylation are fundamental organic reactions. These reactions are the reverse of the hydrolytic procedures discussed in the preceding sections. In Section 3.4 of Part B, these reactions are discussed from the point of view of synthetic applications and methods.

Although the previous two sections of this chapter emphasized hydrolytic processes, two mechanisms that led to O- or N-acylation were considered. In the discussion of acid-catalyzed ester hydrolysis, it was pointed out that this reaction is reversible. Thus, it is possible to acylate alcohols by reaction with a carboxylic acid. To drive the reaction forward, the alcohol is usually used in large excess, and it may also be necessary to remove water as it is formed. This can be done by azeotropic distillation in some cases.

$$\mathrm{RCOH} + \mathrm{R'OH} \overset{\mathrm{H^+}}{\rightleftharpoons} \mathrm{RCOR'} + \mathrm{H_2O}$$

The second reaction that should be recalled is the aminolysis of esters. This reaction leads to the formation of amides by N-acylation.

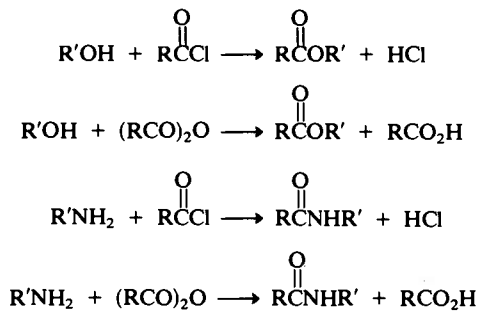
$$\mathrm{RCOR'} + \mathrm{R''NH_2} \longrightarrow \mathrm{RCNHR''} + \mathrm{R'OH}$$

The equilibrium constant for this reaction is ordinarily favorable, but the reaction is rather slow.

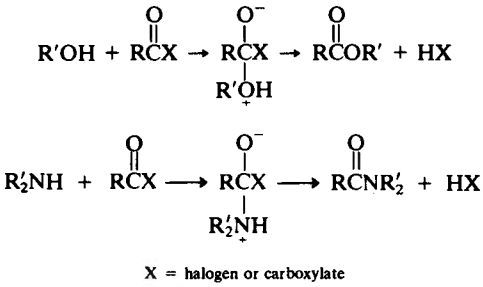
The most common O- and N-acylation procedures use acylating agents that are more reactive than carboxylic acids or their esters. Carboxylic acid chlorides and anhydrides react rapidly with most unhindered hydroxy and amino groups to

48. A. Williams, *J. Am. Chem. Soc.* **97**, 5281 (1975).
49. R. A. McClelland, *J. Am. Chem. Soc.* **97**, 5281 (1975); for cases in which some exchange does occur, see H. Slebocka-Tilk, R. S. Brown, and J. Olekszyk, *J. Am. Chem. Soc.* **109**, 4620 (1987).

give esters and amides, respectively.

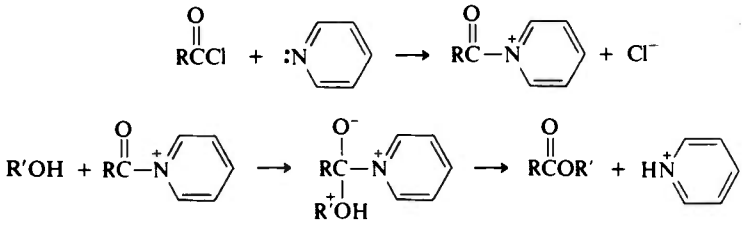


The general mechanisms are well known.[50] The nucleophilic species undergoes addition at the carbonyl group, followed by elimination of the halide or carboxylate group. Acyl halides and anhydrides are reactive acylating reagents because of a combination of the inductive effect of the halogen or oxygen substituent on the reactivity of the carbonyl group and the ease with which the tetrahedral intermediate can expel such relatively good leaving groups.



X = halogen or carboxylate

Acylation of alcohols is usually performed in the presence of an organic base such as pyridine. The base serves two purposes. It neutralizes the protons generated in the reaction and prevents the development of high acid concentrations. Pyridine also becomes directly involved in the reaction as a *nucleophilic catalyst.*

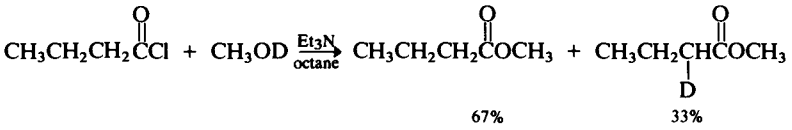


Pyridine is more nucleophilic than an alcohol toward the carbonyl center of an acid chloride. The product that results, an acylpyridinium ion, is, in turn, more reactive toward an alcohol than the original acid chloride. The conditions required for

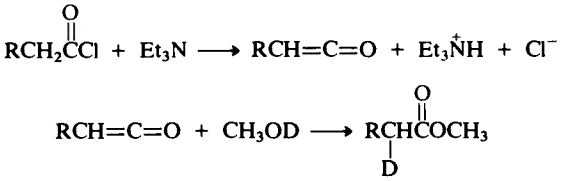
50. D. P. N. Satchell, *Q. Rev. Chem. Soc.* **17**, 160 (1963).

nucleophilic catalysis therefore exist, and acylation of the alcohol by acid chloride is faster in the presence of pyridine than in its absence. Among the evidence that supports this mechanism is spectroscopic observation of the acetylpyridinium ion.[51]

With more strongly basic tertiary amines such as triethylamine, another mechanism can come into play. It has been found that when methanol deuterated on oxygen reacts with acid chlorides in the presence of triethylamine, some deuterium is found α to the carbonyl group in the ester.

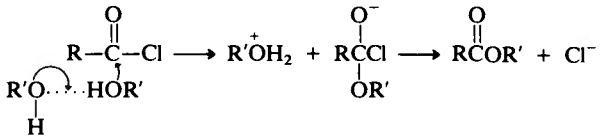


This finding suggests that some of the ester is formed via a ketene intermediate.[52]



Ketenes undergo rapid addition by nucleophilic attack at the *sp*-carbon atom. The reaction of tertiary amines and acid halides, in the absence of nucleophiles, is a general preparative route to ketenes.[53]

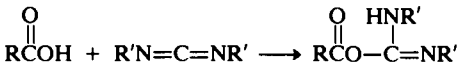
Kinetic studies of the reaction of alcohols with acid chlorides in polar solvents in the absence of basic catalysts generally reveal terms both first-order and second-order in alcohol.[54] Transition states in which the second alcohol molecule acts as a proton acceptor have been proposed.

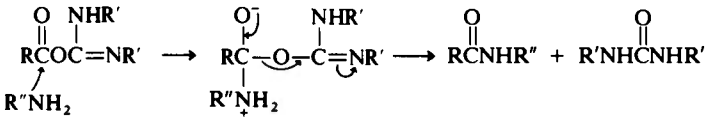


In addition to acid chlorides and acid anhydrides, there are a number of other types of compounds that are reactive acylating agents. Many have been developed to facilitate the synthesis of polypeptides, in which mild conditions and high selectivity are required. An important group of reagents for converting carboxylic acids to active acylating agents are the carbodiimides, such as dicyclohexyl-

51. A. R. Fersht and W. P. Jencks, *J. Am. Chem. Soc.* **92**, 5432, 5442 (1970).
52. W. E. Truce and P. S. Bailey, *J. Org. Chem.* **34**, 1341 (1969).
53. R. N. Lacey, in *The Chemistry of Alkenes*, S. Patai (ed.), Wiley-Interscience Publishers, New York, 1964, pp. 1168-1170; W. E. Hanford and J. C. Sauer, *Org. React.* **3**, 108 (1947).
54. D. N. Kevill and F. D. Foss, *J. Am. Chem. Soc.* **91**, 5054 (1969); S. D. Ross, *J. Am. Chem. Soc.* **92**, 5998 (1970).

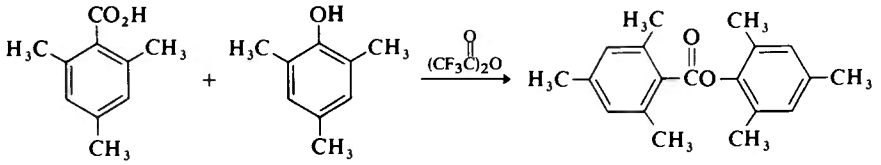
carbodiimide. The mechanism for carbodiimide-promoted amide bond formation is shown below.





The first step is addition of the carboxylic acid to the C=N bond of the carbodiimide, which generates an O-acylated urea derivative. This is a reactive acylating agent because there is a strong driving force for elimination of the urea unit, with formation of the very stable amide carbonyl group.[55] The amine reacts with the active acylating agent. In the absence of an amine, the acid would be converted to the anhydride with a second molecule of the carboxylic acid serving as the nucleophile.

Carboxylic acids react with trifluoroacetic anhydride to give mixed anhydrides that are especially useful for the acylation of hindered alcohols and phenols.



The active acylating agent may be the protonated mixed anhydride,[56] or, alternatively, the anhydride may dissociate to the acylium and trifluoroacetate ions[57]:

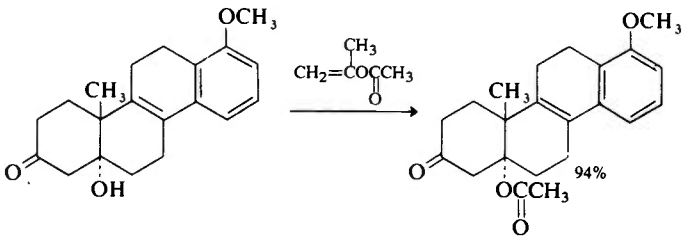


Either mechanism explains why trifluoroacetylation of the nucleophile does not occur. Protonation of the anhydride would occur selectively at the more electron-rich carbonyl oxygen, rather than at the carbonyl flanked by the very electron-withdrawing trifluoromethyl group. Similarly, cleavage of the unsymmetrical anhydride would occur to give the more stable acylium ion. The trifluoroacetylium ion would be highly unstable.

Another useful family of acylating agents are the enol esters. The acetate of the enol form of acetone, isopropenyl acetate, is the most commonly used member of this group of compounds. These compounds act as acylating agents in the presence of a trace amount of an acid catalyst and are reactive toward weak nucleophiles

55. D. F. DeTar and R. Silverstein, *J. Am. Chem. Soc.* **88**, 1013, 1020 (1966).
56. R. C. Parish and L. M. Stock, *J. Org. Chem.* **30**, 927 (1965).
57. J. M. Tedder, *Chem. Rev.* **55**, 787 (1955).

such as hindered hydroxyl groups.



Ref. 58

The active acylating agent is presumably the C-protonated enol ester. This species would be highly reactive owing to the presence of a positively charged oxygen. An alternative possibility is that the protonated enol ester decomposes to acetone and an acylium ion, which then acts as the acylating agent.

$$(CH_3)_2C{=}\overset{+}{O}{-}\overset{O}{\overset{\|}{C}}CH_3 \rightleftharpoons CH_3\overset{O}{\overset{\|}{C}}CH_3 + \overset{+}{O}{\equiv}CCH_3$$

Section 3.4 of Part B gives additional examples of synthetically useful acylating reagents.

8.9. Intramolecular Catalysis

The reactions of carbonyl compounds have provided the testing ground for developing the facts and interpretation of intramolecular catalysis. Studies in intramolecular catalysis have been designed to determine how much more efficiently a given functional group acts as a catalyst when it is part of the reacting molecule and located in such a position that encounter between the catalytic group and the reaction center is facilitated. These studies are important to understanding biological mechanisms because enzymes are believed to act as exceedingly efficient catalysts by bringing together at the "active site" various basic, acidic, or nucleophilic groups in such a geometry that the particular reaction is highly favorable. This section will illustrate some of the facts that have emerged from these studies and the mechanistic conclusions that have been drawn.

It was pointed out in the mechanistic discussion concerning acetal and ketal hydrolysis that general acid catalysis occurs only for acetals and ketal having special structural features. Usually, specific acid catalysis operates. The question of whether general acid catalysis could be observed in intramolecular reactions has been of interest because intramolecular general acid catalysis is postulated to play a part in the mechanism of action of the enzyme lysozyme, which hydrolyzes the acetal linkage present in certain polysacharides. One group of molecules that has been examined as a model system are acetals derived from *o*-hydroxybenzoic acid (sali-

58. W. S. Johnson, J. Ackerman, J. F. Eastham, and H. A. DeWalt, Jr., *J. Am. Chem. Soc.* **78**, 6302 (1956).

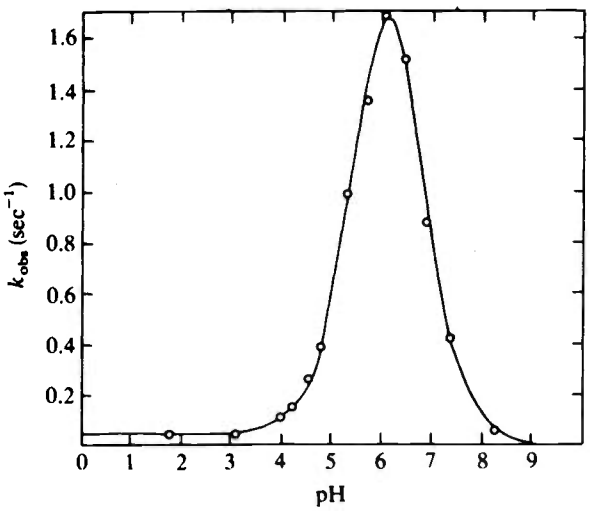
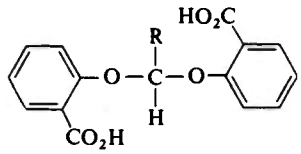
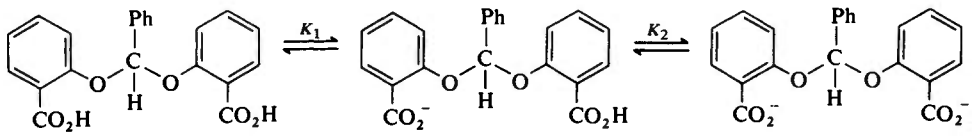


Fig. 8.5. pH-Rate profile for release of salicyclic acid from benzaldehyde disalicyl acetal. [From E. Anderson and T. H. Fife, *J. Am. Chem. Soc.* **95**, 6437 (1973). Reproduced by permission of the American Chemical Society.]

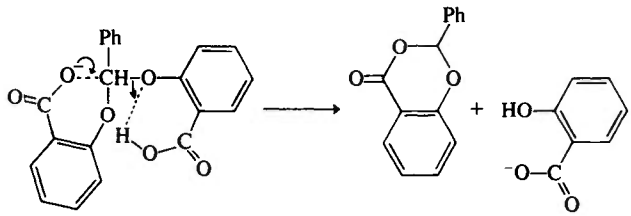
cyclic acid):



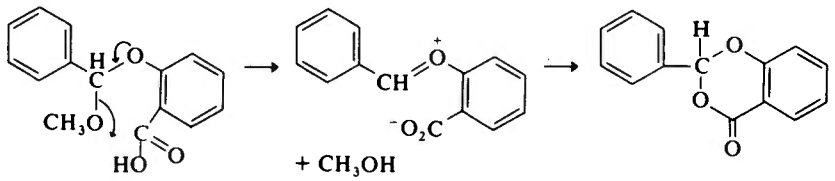
The pH-rate profile (see Fig. 8.5) indicates that of the species that are available, the monoanion of the acetal is the most reactive. The concentration of this species is at a maximum in the intermediate pH range. The neutral molecule decreases in concentration with increasing pH; the converse is true of the dianion.



The transition state for the rapid hydrolysis of the monoanion has been depicted as involving an intramolecular general acid catalysis by the carboxylic acid group, with participation by the anionic carboxylate group which becomes bound at the developing electrophilic center.

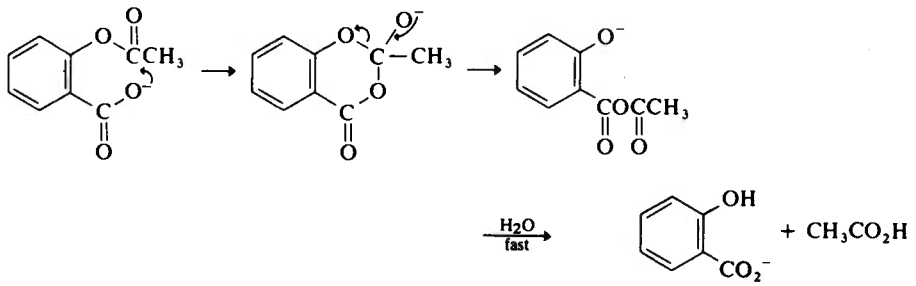


A mixed acetal of benzaldehyde, methanol, and salicyclic acid has also been studied.[59] It, too, shows a marked rate enhancement attributable to intramolecular general acid catalysis.

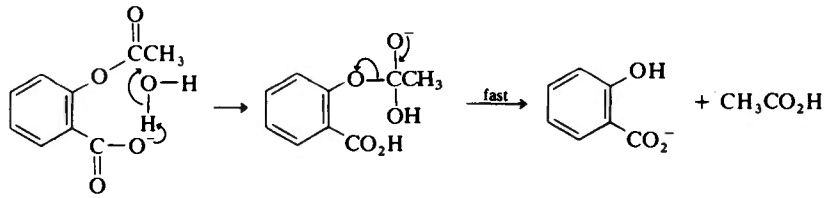


The case of intramolecular participation in ester hydrolysis has been extensively studied using acetylsalicyclic acid (aspirin) and its derivatives. The kinetic data show that the anion is hydrolyzed more rapidly than the neutral species, indicating that the carboxylate group becomes involved in the reaction in some way. Three mechanisms can be considered:

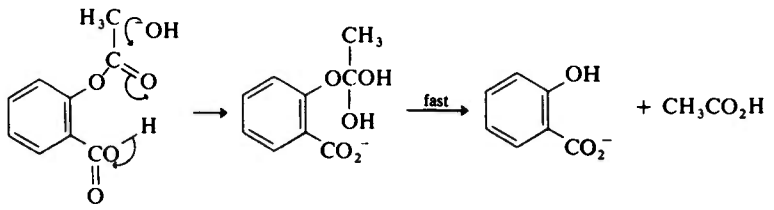
I. Nucleophilic catalysis



II. General base catalysis

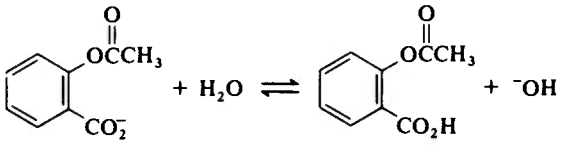


III. General acid catalysis of hydroxide ion attack

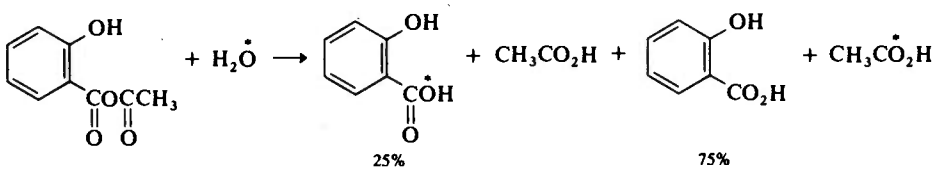


59. T. H. Fife and E. Anderson, *J. Am. Chem. Soc.* **93**, 6610 (1971).

Mechanism III cannot be distinguished from the first two on the basis of kinetics alone, because the reactive species shown is in rapid equilibrium with the anion and therefore equivalent to it in terms of reaction kinetics.

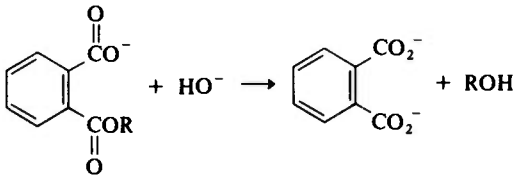


Mechanism I has been ruled out by an isotopic labeling experiment. The mixed anhydride of salicyclic acid and acetic acid is an intermediate if nucleophilic catalysis occurs by mechanism I. This molecule is known to hydrolyze in water with about 25% incorporation of solvent water into the salicyclic acid.



Hydrolysis of aspirin in $H_2{}^{18}O$ leads to no incorporation of ^{18}O into the product salicyclic acid, ruling out the anhydride as an intermediate and thereby excluding mechanism I.[60] The general acid catalysis of mechanism III can be ruled out on the basis of failure of other nucleophiles to show evidence for general acid catalysis by the neighboring carboxylic acid group. Since there is no reason to believe hydroxide should be special in this way, mechanism III is eliminated. Thus, mechanism II, general base catalysis of hydroxide ion attack, is believed to be the correct description of the hydrolysis of aspirin.

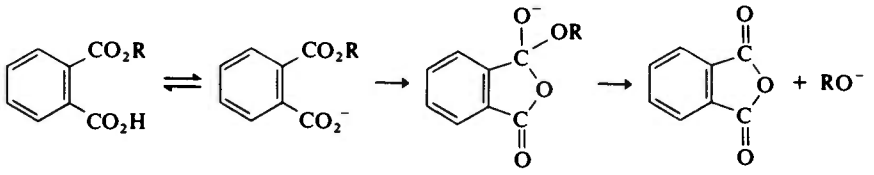
The extent to which intramolecular nucleophilic catalysis of the type depicted in mechanism I is important is a function of the leaving ability of the alkoxy group. This has been demonstrated by the study of the hydrolysis of a series of monoesters of phthalic acid.



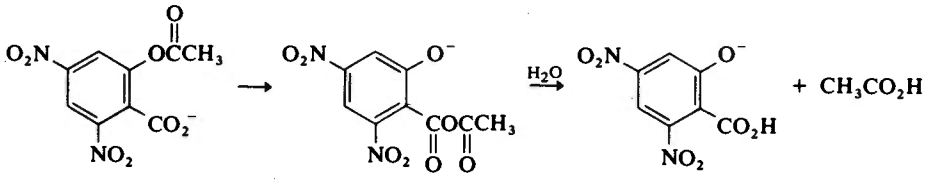
Nucleophilic participation is important only for esters of alcohols that have $pK_a \leq 13$. Specifically, phenyl and trifluoroethyl esters show nucleophilic catalysis, but methyl and 2-chloroethyl esters do not.[61] This result reflects the fate of the tetrahedral intermediate that results from nucleophilic participation. For relatively acidic

60. A. R. Fersht and A. J. Kirby, *J. Am. Chem. Soc.* **89**, 4857 (1967).
61. J. W. Thanassi and T. C. Bruice, *J. Am. Chem. Soc.* **88**, 747 (1966).

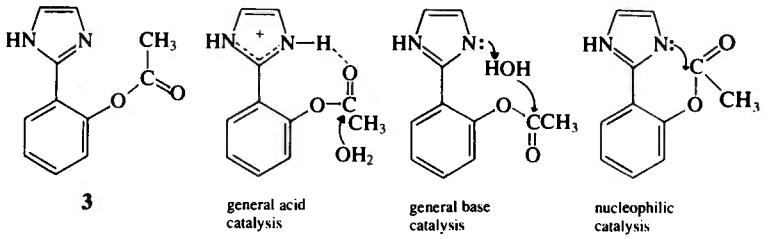
alcohols, the alkoxide group can be eliminated, leading to hydrolysis via nucleophilic catalysis.



For less acidic alcohols, nucleophilic participation is ineffective because of the low tendency for such alcohols to function as leaving groups. The tetrahedral intermediate formed by intramolecular addition simply returns to starting material because the carboxylate is a much better leaving group than the alkoxide. In contrast to aspirin itself, acetyl salicylates with electron-withdrawing groups (*o*- and *p*-nitro analogs) hydrolyze via the nucleophilic catalysis mechanism.[62]



Intramolecular catalysis of ester hydrolysis by nitrogen nucleophiles is also important. The role of imidazole rings in intramolecular catalysis has received particularly close scrutiny. There are two major reasons for this. One is that the imidazole ring of the histidine residue in proteins is believed to frequently be involved in enzyme-catalyzed hydrolyses. Secondly, the imidazole ring has several possible catalytic functions including action as a general acid in the protonated form, action as a general base in the neutral form, and action as a nucleophile in the neutral form. A study of a number of derivatives of structure **3** was undertaken to distinguish between the importance of these various possible mechanisms as a function of pH.[63]



The relative importance of the potential catalytic mechanisms depends on pH, which also determines the concentration of the other participating species such as water,

62. A. R. Fersht and A. J. Kirby, *J. Am. Chem. Soc.* **89**, 5960 (1967); **90**, 5818 (1968).
63. G. A. Rogers and T. C. Bruice, *J. Am. Chem. Soc.* **96**, 2463 (1974).

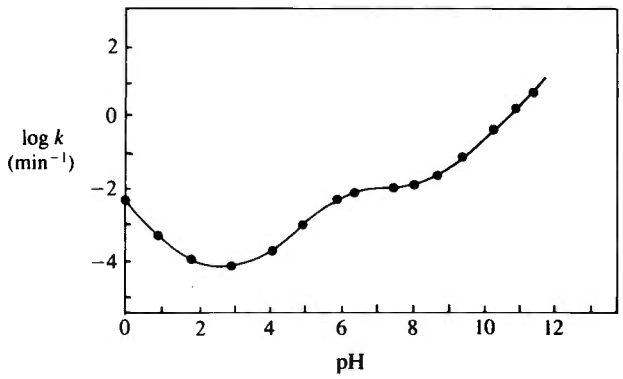


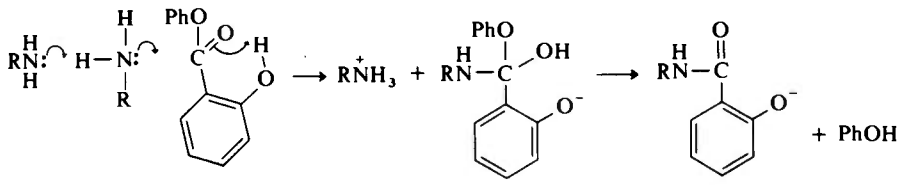
Fig. 8.6. pH-Rate profile for compound **3**. Reproduced with permission from Ref. 63.

hydronium ion, and hydroxide ion. At low pH, the general acid catalysis mechanism dominates, and comparison with analogous systems where the intramolecular proton transfer is not available suggests that the intramolecular catalysis results in a 25–100-fold rate enhancement. At neutral pH, the intramolecular general base mechanism begins to operate. It is estimated that the catalytic effect for this mechanism is a factor of about 10^4. Although the nucleophilic catalysis mechanism was not observed in the parent compound, it occurred in certain substituted derivatives. The change in mechanism with pH for compound **3** gives rise to the pH–rate profile shown in Fig. 8.6.

The rates at the extremeties pH < 2 and pH > 9 are proportional to $[H^+]$ and $[^-OH]$, respectively, and represent the specific proton-catalyzed and hydroxide-catalyzed mechanisms. The region at pH 2–4 is the area where intramolecular general acid catalysis operates. At pH 6–8, the intramolecular general base catalysis mechanism is dominant. In the absence of the intramolecular catalytic mechanisms, the rates of the H^+- and ^-OH-catalyzed reactions would decrease in proportion to the concentration of the catalytic species to a minimum value representing the "uncatalyzed water hydrolysis." An estimate of the effectiveness of the intramolecular mechanisms can be made by extrapolating the lines which are proportional to $[H^+]$ and $[^-OH]$. The extent to which the actual rate lies above these extrapolated lines in the pH range 2–7 represents the contribution from the intramolecular catalysis.

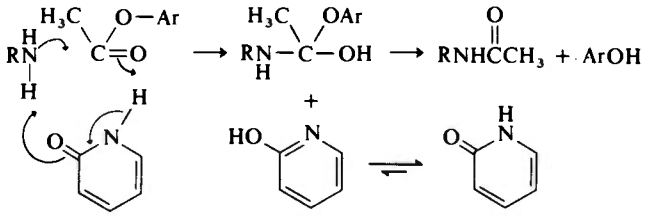
Intramolecular participation of the *o*-hydroxy group in aminolysis of phenyl salicylate has been established by showing that such compounds are more reactive than analogs lacking the hydroxyl substituent. This reaction exhibits overall third-order kinetics, second-order in the reacting amine. Similar kinetics are observed in the aminolysis of simple esters. Both intermolecular general base catalysis (by the second amine molecule) and intramolecular general acid catalysis (by the hydroxyl group) apparently occur.[64]

64. F. M. Menger and J. H. Smith, *J. Am. Chem. Soc.* **91**, 5346 (1969).

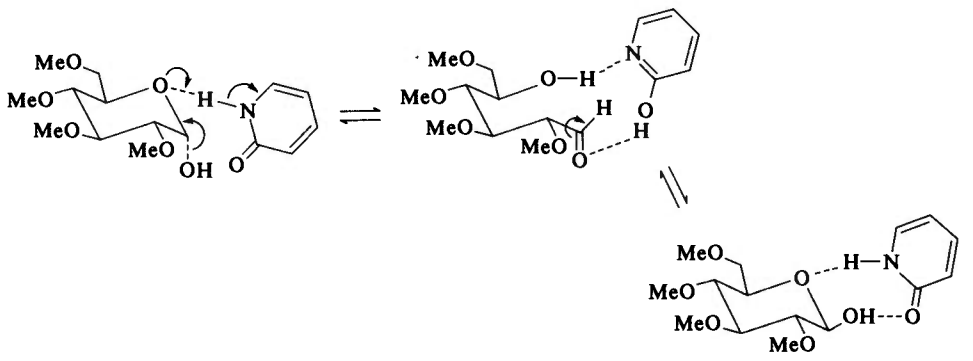


This mechanism can reduce the activation energy of the reaction in at least two ways. The partial transfer of a proton to the carbonyl oxygen increases the electrophilicity of the carbonyl. Likewise, partial deprotonation of the amino group increases its nucleophilicity.

Certain molecules that can permit concerted proton transfers are efficient catalysts for reaction at carbonyl centers. An example that can be cited is the catalytic effect that 2-pyridone has on the aminolysis of esters. Although neither a strong base ($pK_{a_H}{}^{+} = 0.75$) nor a strong acid ($pK_a = 11.6$), 2-pyridone is an effective catalyst of the reaction of *n*-butylamine with 4-nitrophenyl acetate.[65] The overall rate is more than 500 times greater when 2-pyridone acts as the catalyst than when a second molecule of butylamine (acting as a general base) is present in the transition state. 2-Pyridone has been called a *tautomeric catalyst* to emphasize its role in proton transfer. Such molecules are also called *bifunctional catalysts*, since two atoms in the molecule are involved in the proton transfer process.

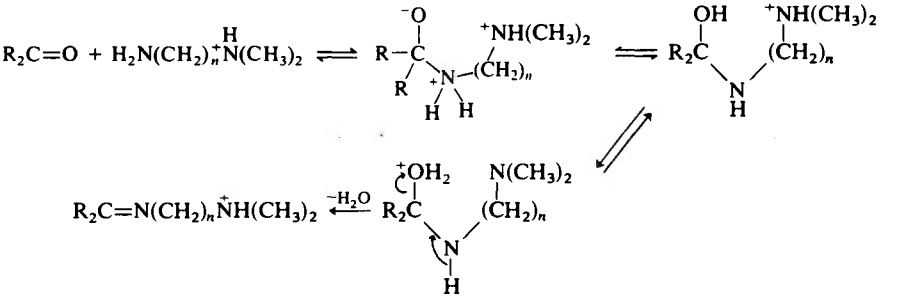


2-Pyridone also catalyzes epimerization of the anomeric position of the tetramethyl ether of glucose. The mechanism involves two double proton transfers. The first leads to a ring-opened intermediate, and the second results in ring closure to the isomerized product.

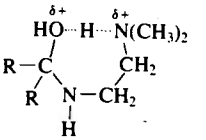


65. P. R. Rony, *J. Am. Chem. Soc.* **91**, 6090 (1969).

Another type of bifunctional catalysis has been noted with α, ω-diamines in which one of the amino groups is primary and the other tertiary. These substituted diamines are from several times to as much as 100 times more reactive toward imine formation that similar monofunctional amines.[66] This is attributed to a catalytic intramolecular proton transfer.



The rate enhancement is greatest for $n = 2$ (1000) but still significant for $n = 3$ (a factor of 10). As the chain is lengthened to $n = 4$ and $n = 5$, the rate enhancement, if any, is minor. This relationship reflects the fact that when $n = 4$ or 5, the transition state for the intramolecular proton transfer would have to involve rings of nine and ten atoms, respectively, and would not be geometrically advantageous. The particularly rapid reaction when $n = 2$ corresponds to the possibility for a proton transfer via a seven-membered cyclic transition state. Assuming that the proton is transferred in a colinear fashion through a hydrogen bond, this represents a favorable transition state geometry.



These examples serve to introduce the idea of intramolecular catalysis and the fact that favorable juxtaposition of acidic, nucleophilic, or basic sites can markedly accelerate some of the common reactions of carbonyl compounds. It is believed that nature has evolved a similar strategy of optimal placement of functional groups to achieve the catalytic activity of enzymes. The functional groups employed to accomplish this are those present on the amino acid residues found in proteins. The acidic sites available include phenolic or carboxylic acid groups from tyrosine, glutamic acid, and aspartic acid. Basic sites include the imidazole ring in histidine and the ε-amino group of lysine. This latter group and the amidine group in arginine are normally protonated at physiological pH and can serve as cationic centers or general acids, as well. Thiol (cysteine) and hydroxyl (threonine and serine) groups and the deprotonated carboxyl groups of glutamic acid and aspartic acid are potential

66. J. Hine, R. C. Dempsey, R. A. Evangelista, E. T. Jarvi, and J. M. Wilson, *J. Org. Chem.* **42**, 1593 (1977); J. Hine and Y. Chou, *J. Org. Chem.* **46**, 649 (1981).

nucleophilic sites. The student interested in examining further the mechanism of enzymatic reactions will find these topics covered in detail in several of the texts listed as general references.

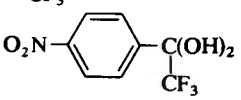
General References

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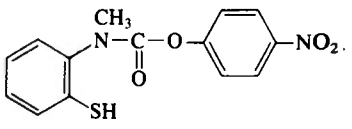
Problems

(*References for these problems will be found on page* 780.)

1. The hydrates of aldehydes and ketones are considerably more acidic than normal alcohols (pK ≈ 16-19). How would you account for this fact? Some reported values are shown below. Explain the order of relative acidity.

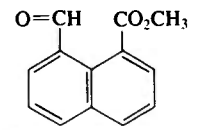
Hydrate	pK
$H_2C(OH)_2$	13.4
$Cl_3CCH(OH)_2$	10.0
$PhC(OH)_2$ CF_3	10.0
	9.2

2. Suggest explanations for each of the following observations.
   (a) The equilibrium constant for cyanohydrin formation from 3,3-dimethyl-2-butanone is 40 times larger than that for acetophenone.
   (b) The rate of release of *p*-nitrophenoxide from compound **A** is independent of pH in aqueous solution of pH > 10.

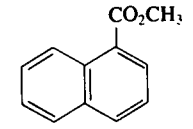


A

(c) Ester **B** undergoes alkaline hydrolysis 8300 times faster than ester **C** in aqueous dioxane.

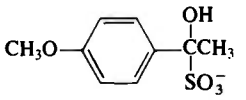


B

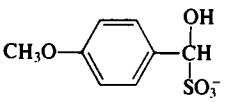


C

(d) Under comparable conditions, the general-base-catalyzed elimination of bisulfite ion from **D** is about ten times greater than from **E**.

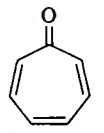


D

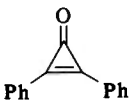


E

(e) The rate of isotopic exchange of the carbonyl group in tropone (**F**) and 2,3-diphenylcyclopropenone (**G**) is much less than for acetophenone.



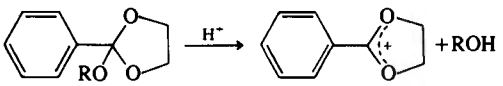
F



G

3. Arrange the carbonyl compounds in each group in order of decreasing rate of hydrolysis of their respective diethyl acetals or ketals. Explain your reasoning.
   (a) acetaldehyde, chloroacetaldehyde, crotonaldehyde
   (b) acetaldehyde, formaldehyde, acetone
   (c) cyclopentanone, cyclohexanone, camphor
   (d) acetone, methyl *t*-butyl ketone, methyl neopentyl ketone
   (e) benzaldehyde, *p*-methoxybenzaldehyde, butyraldehyde.

4. The acid-catalyzed hydrolysis of 2-alkoxy-2-phenyl-1,3-dioxolanes has been studied. The initial step is rate-determining under certain conditions and is described by the rate law given below, which reveals general acid catalysis.



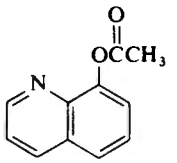
$$k_{obs} = k_{H^+}[H^+] + k_{H_2O}[H_2O] + k_{HA}[HA]$$

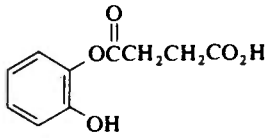
By determining k_{HA} for several different buffer catalysts and each of several alkoxy leaving groups, it was determined that there was a relationship between the Brønsted coefficient α and the structure of the alkoxy leaving group. The data are given and show that α decreases as the alkoxy group becomes less basic.

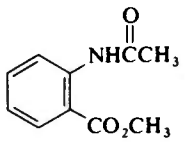
RO—	α
Cl_2CHCH_2O—	0.69
$ClCH_2CH_2O$—	0.80
$CH_3OCH_2CH_2O$—	0.85
CH_3O—	0.90

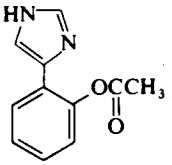
What information is provided by the fact that the Brønsted α decreases as the acidity of the alcohol increases? Discuss these results in terms of a three-dimensional potential energy diagram with the extent of O—H bond formation and the extent of C—O bond breaking taken as the reaction progress coordinates.

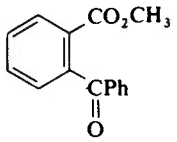
5. Each of the following molecules has been considered to be capable of some form of intramolecular catalysis of ester hydrolysis. For each substrate indicate one or more mechanisms by which intramolecular catalysis might occur. Depict a transition state arrangement that shows this catalysis.

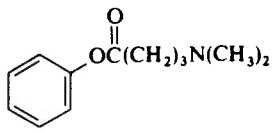
(a) 

(b) 

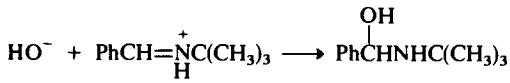
(c) 

(d) 

(e) 

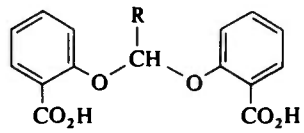
(f) 

6. Consider the alkaline pH region of the pH-rate profile in Fig. 8.4 (p. 450), which indicates a rate independent of pH. The rate-controlling reaction in this region is



Show that the rate of this reaction is pH independent, despite the involvement of two species, the concentrations of which are pH dependent.

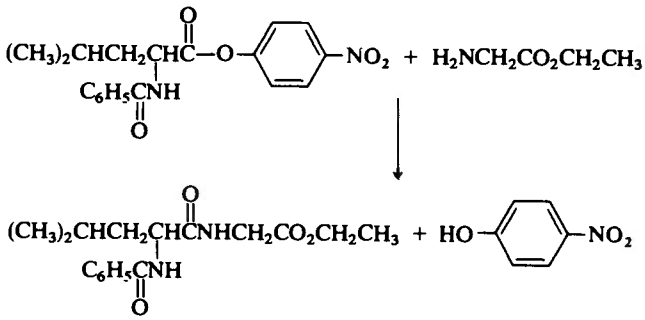
7. Derive the general expression for the observed rate constant for hydrolysis of **A** as a function of pH. Assume, as is the case experimentally, that intramolecular



A

general acid catalysis completely outweighs intermolecular catalysis by hydronium ion in the pH range of interest. Does the form of your expression agree with the pH-rate profile given for this reaction in Fig. 8.5 (p. 480)?

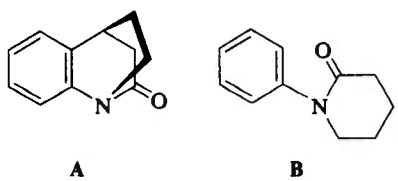
8. Optically pure dipeptide is obtained when the *p*-nitrophenyl ester of *N*-benzolyl-L-leucine is coupled with glycine ethyl ester in ethyl acetate:



If, however, the *p*-nitrophenyl ester of *N*-benzoyl-L-leucine is treated with l-methylpiperidine in chloroform for 30 min, then coupled with glycine ethyl ester, the dipeptide isolated is almost completely racemic. Furthermore, treatment of the *p*-nitrophenyl ester of *N*-benzoyl-L-leucine with 1-methylpiperidine alone leads to the formation of a crystalline material, $C_{13}H_{15}NO_2$, having strong IR bands at 1832 and 1664 cm^{-1}. Explain these observations, and suggest a reasonable structure for the crystalline product.

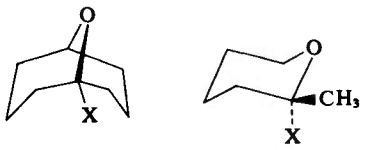
9. Offer as complete as possible explanations, based on structural and mechanistic concepts, of the following observations.

   (a) The bicyclic lactam **A** hydrolyzes 10^7 times faster than the related monocyclic compound **B**.



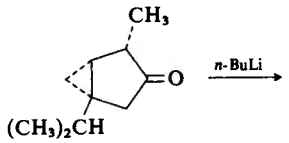
A **B**

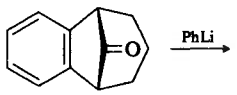
(b) Leaving groups, X, solvolyze from structure **C** at a rate which is 10^{-13} less than that for monocyclic model **D**.

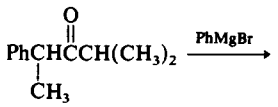


C **D**

10. Analyze the factors which would determine stereoselectivity in the addition of organometallic compounds to the following carbonyl compounds. Predict the major product.

(a) 

(b) 

(c) 

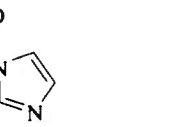
11. Indicate which compound in each of the following pairs will have the more negative standard free-energy change for hydrolysis at pH 7:

(a) $CH_3CO_2CH_3$ *or* $CH_3\overset{\overset{O}{\|}}{C}SCH_3$

(b) $CH_3CO_2CH_3$ *or* $CH_3CO_2PO_3H_2$

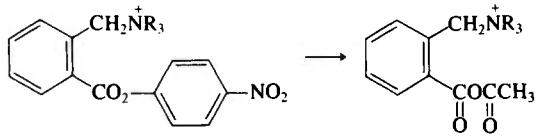
(c) $CH_3CO_2CH_3$ *or* $H_2NCH_2CO_2CH_3$

(d) $CH_3\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}SCH_3$ *or* $CH_3\overset{\overset{O}{\|}}{C}SCH_3$

(e) $CH_3CO_2CH_3$ *or* 

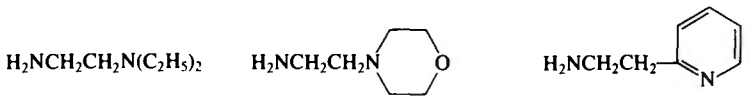
(f) $CH_3CO_2CH_3$ *or* $CH_3\overset{\overset{O}{\|}}{C}N(CH_3)_2$

12. Sodium acetate reacts with *p*-nitrophenyl benzoates to give mixed anhydrides if the reaction is conducted in a polar aprotic solvent in the presence of a crown ether. The reaction is strongly accelerated by quaternary nitrogen groups substituted at the *ortho* position. Explain the basis for the enhanced reactivity of these compounds.



Introduction of $^{+}NR_3$ for H accelerates the reaction by a factor of $> 10^3$

13. The kinetics of the hydrolysis of some imines derived from benzophenone and primary amines revealed the normal dependence of mechanism on pH with rate-determining nucleophilic attack at high pH and rate-determining decomposition of the tetrahedral intermediate at low pH. The simple primary amines show a linear correlation between the rate of nucleophilic addition and the basicity of the amine. Several diamines which were included in the study, in particular **A**, **B**, and **C**, all showed a positive (more reactive) deviation from the correlation line for the simple amines. Why might these amines be more reactive than predicted on the basis of their basicity?

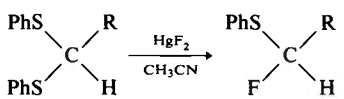


A **B** **C**

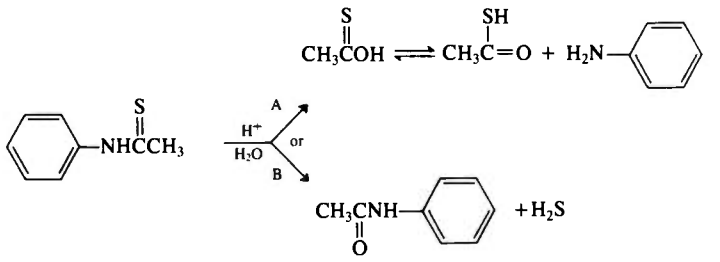
14. The following data give the dissociation constants for several acids that catalyze hydration of acetaldehyde. Also given are the rate constants for the hydration reaction catalyzed by each acid. Treat the data according to the Brønsted equation, and comment on the mechanistic significance of the result.

Acid	K	k_{hydr}
Formic	1.77×10^{-4}	1.74
Phenylacetic	4.9×10^{-5}	0.91
Acetic	1.75×10^{-5}	0.47
Pivalic	9.4×10^{-6}	0.33

15. 1,1-Diphenylthioalkanes react with mercuric fluoride to give 1-fluoro-1-phenylthioalkanes. Provide a detailed description of a likely mechanism for this reaction. Consider such questions as: (1) Is an S_N1 or an S_N2 process most likely to be involved? (2) Would NaF cause the same reaction to occur? (3) Why is only one of the phenylthio groups replaced?



16. The acid-catalyzed hydrolysis of thioacetanilide can follow two courses.

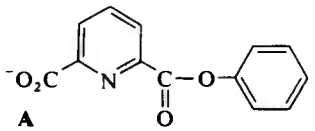


The product analysis permits determination of the amount of product formed by each path, as a function of the acidity of the solution. The results are as shown:

H_2SO_4 (% by weight)	3.2	6.1	12	18	36	48
% following path A	50	55	65	75	96	100

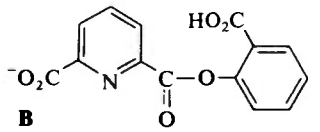
Provide a mechanism in sufficient detail to account for the change in product ratio with acid strength.

17. The rates of hydrolysis of the ester group in compounds **A** and **B** have been compared. The effect of an added metal ion (Ni^{2+}) on the rate of hydrolysis has been studied, and the observed rate constants for attack by ^-OH are tabulated. Suggest the most favorable transition state structure for the addition step of the hydrolysis reaction with each substrate under each set of conditions. Discuss the relationship between the structures of these transition states and the relative rates of attack by hydroxide ion.



A

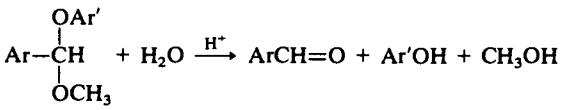
k_{HO^-}: $3.0 \times 10^2\ M^{-1}\ s^{-1}$
k_{HO^-} in presence of excess Ni^{2+}: $2.8 \times 10^6\ M^{-1}\ s^{-1}$



B

k_{HO^-}: $7.1 \times 10^1\ M^{-1}\ s^{-1}$
k_{HO^-} at pH values where salicylic acid group is not ionized: $2.7 \times 10^5\ M^{-1}\ s^{-1}$
k_{HO^-} when salicylic acid group is not ionized and excess Ni^{2+} is present: $2.7 \times 10^7\ M^{-1}\ s^{-1}$

18. Data pertaining to substituent effects on the acid-catalyzed hydrolysis of mixed methyl aryl acetals of benzaldehyde are given below. The reactions exhibited general acid catalysis, and the Brønsted α values are tabulated. Discuss the information provided by these data about the transition state for the first hydrolysis step, making reference to a diagram showing the location of the transition state as a function of O—H bond formation and C—O bond breaking.



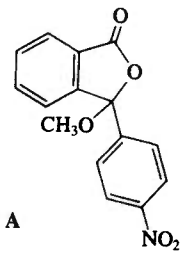
Series I, substituent in Ar			Series II, substituent in Ar'		
X	k_{cat}[a]	α	X	k_{cat}[a]	α
m-NO_2	2.7×10^{-4}	1.05	m-NO_2	8.85×10^{-2}	0.49
m-F	2.2×10^{-3}	0.92	*m*-Br	4.7×10^{-2}	0.65
m-CH_3O	9.6×10^{-3}	0.78	*m*-F	2.45×10^{-2}	0.67
H	1.3×10^{-2}	0.77	*m*-CH_3O	2.55×10^{-2}	0.71
p-CH_3	1.1×10^{-1}	0.72	H	1.3×10^{-2}	0.77
p-CH_3O	2.8×10^{-1}	0.68	*p*-CH_3	1.3×10^{-2}	0.88
			p-CH_3O	1.65×10^{-2}	0.96

a. Rate constant for catalysis by acetic acid.

19. The hydrolysis of the lactone **A** shows a significant catalysis by acetate ion in acetate buffer, with the rate expression being

$$k_{obs} = 1.6 \times 10^{-6} + 6.4 \times 10^{-4}[H^+] + 2.08 \times 10^{-5}[OAc^-] + 49[^-OH]$$

This results in a pH–rate profile as shown in Fig. 8.P19, with the acetate catalysis being significant in the pH range 3–6. Discuss how this catalysis by acetate ion might occur. What are the most likely mechanisms for hydrolysis at pH $\lesssim$ 2 and pH > 7, where the rates are linear in $[H^+]$ and $[^-OH]$, respectively?



A

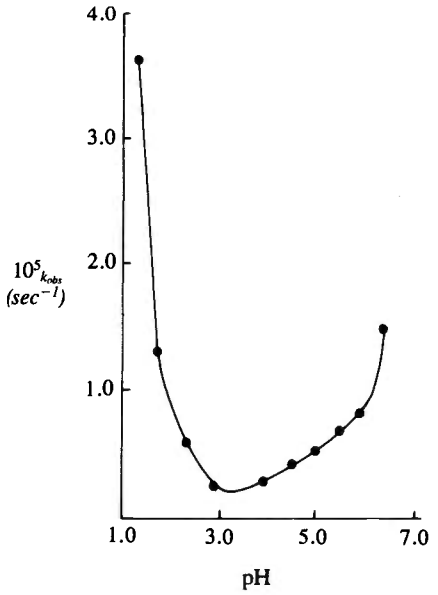
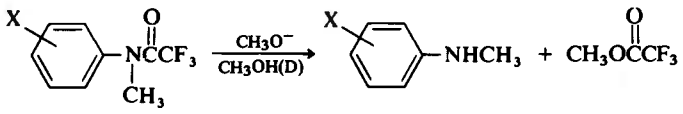


Fig. 8.P19. pH-Rate profile for hydrolysis of A in buffered aqueous solution at 70°C.

20. Some data on substituent effects for the reaction of trifluoroacetanilides with methoxide ion in methanol and methanol-O-*d* are given below. Calculate the isotope effect for each system. Plot the rate data against appropriate Hammett substituent constants. What facets of the data are in agreement with a normal addition-elimination sequence passing through a tetrahedral intermediate? What facets of the data indicate additional complications? Can you propose a mechanism that is consistent with all the data given?

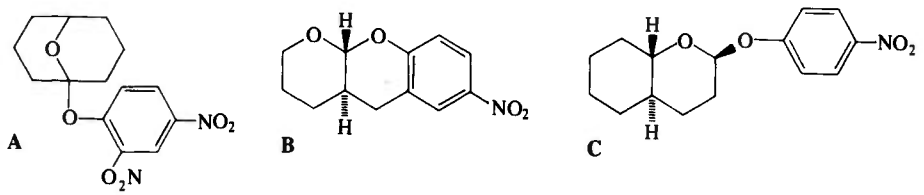


X	k_{CH_3OH} [a]	k_{CH_3OD} [a]
m-NO_2	5.75	8.13
m-Br	0.524	0.464
p-Cl	0.265	0.274
p-Br	0.349	0.346
m-Cl	0.513	0.430
m-OCH_3	0.110	0.101
H	0.104	0.0899
m-CH_3	0.0833	0.0595
p-CH_3	0.0729	0.0451
p-OCH_3	0.0564	0.0321

a. Second-order rate constants in $M^{-1}\,sec^{-1}$

21. The halogenation of simple ketones such as acetone can proceed through the enol or enolate. By applying the steady-state condition to the enolate, derive a kinetic expression for reaction of acetone with any halogenating agent X-Y in a buffered solution where both C-protonation and O-protonation of the enolate can compete with halogenation. Show that this rate expression predicts that halogenation will be zero-order in halogenating agent under some conditions but first-order in halogenating agent under other conditions.

22. The order of reactivity toward hydrolysis of the cyclic acetals shown below is **A** ≪ **B** ≪ **C**. Offer an explanation for this difference in reactivity.



23. Examine the structure of the reactants given and the pH–rate profiles (Figs. 8.P23a-d) of the reactions in question. Offer explanations for the response of the observed reaction rate to the pH for each case.

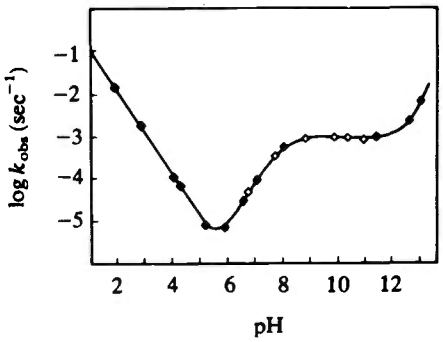


Fig. 8.P23a. (Reproduced from problem reference 23a by permission of the American Chemical Society.)

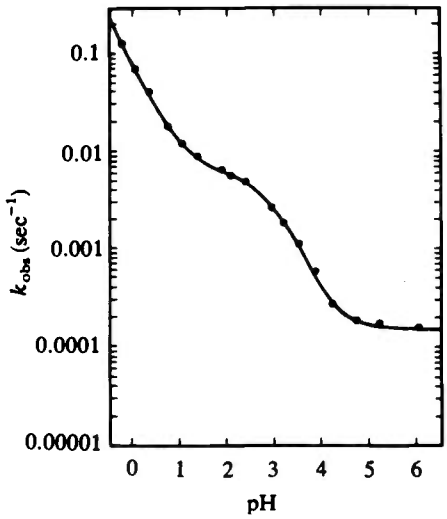


Fig. 8.P23b. (Reproduced from problem reference 23b by permission of the American Chemical Society.)

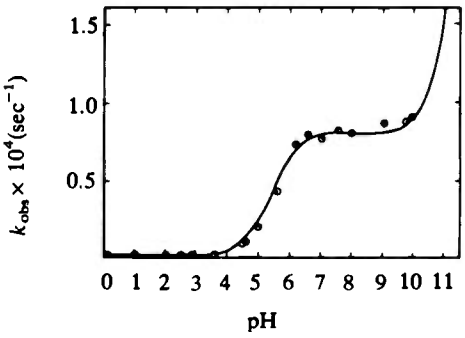


Fig. 8.P23c. (Reproduced from problem reference 23c by permission of the American Chemical Society.)

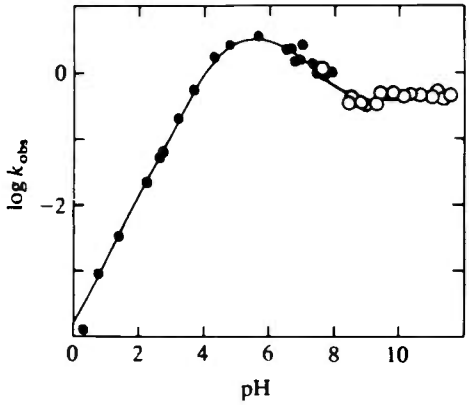
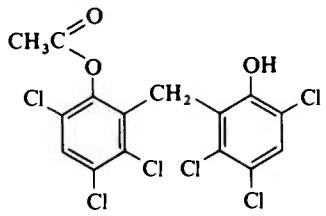


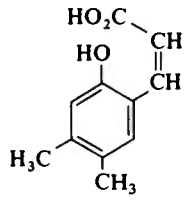
Fig. 8.P23d. (Reproduced from problem reference 23d by permission of the American Chemical Society.)

(a)



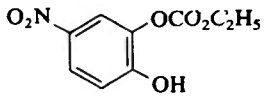
ester hydrolysis

(b)



lactonization

(c)



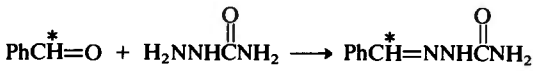
ester hydrolysis

(d) $(CH_3)_2CHCH{=}NCH_3$

hydrolysis

24. The rates of both formation and hydrolysis of dimethyl acetals of *p*-substituted benzaldehydes are substituent dependent. Do you expect k_{form} to increase or decrease with increasing electron-attracting capacity of the *para* substituent? Do you expect the k_{hydrol} to increase or decrease with the electron-attracting power of the substituent? How do you expect K, the equilibrium constant for acetal formation, to vary with the nature of the substituent?

25. Consider the kinetic effect that would be observed in the reaction of semicarbazide with benzaldehyde:



Would you expect to find k_H/k_D to be normal or inverse? Would you expect k_H/k_D to be constant, or would it vary with pH?

26. Figure 8.P26 gives the pH-rate profile for conversion of the acid **A** to the anhydride **B** in aqueous solution. The reaction shows no sensitivity to buffer concentration. Notice that the reaction rate increases with the size of the alkyl substituent, and in fact the derivative with $R^1 = R^2 = CH_3$ is still more reactive. Propose a mechanism which is consistent with the pH-rate profile and the structure of the initially formed product (which is subsequently hydrolyzed to the diacid). How do you account for the effect of the alkyl substituents on the rate?

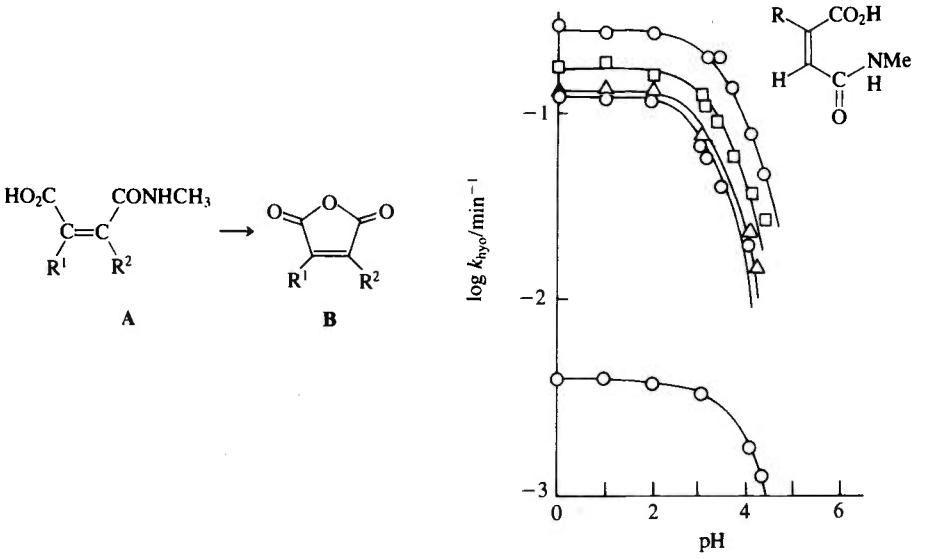


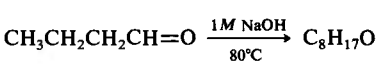
Fig. 8.P26. pH-Rate profiles for the hydrolysis of alkyl-*N*-methylmaleamic acids at 39°C and ionic strength 1.0. In increasing order of reactivity, R = H, Me, Et, *i*-Pr, and *t*-Bu.

27. Assume that the usual mechanism for hydrolysis of an imine, Im, is operative, i.e., that the hydrolysis occurs through a tetrahedral intermediate, TI:

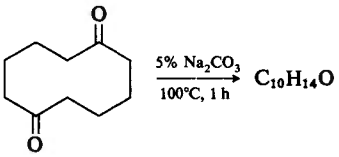
Assume that the steady-state approximation can be applied to the intermediate TI. Derive the kinetic expression for hydrolysis of the imine. How many variables must be determined to construct the pH–rate profile? What simplifying assumptions are justified at very high and very low pH values? What are the kinetic expressions that result from these assumptions?

28. Give a specific structure, including all stereochemical features, for the product expected for each of the following reactions.

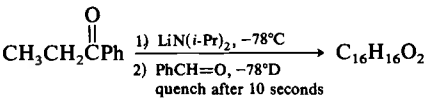
(a)



(b)



(c)



(d)

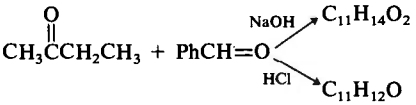


Exhibit 5

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The Impact of PEGylation on Biological Therapies

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Abstract

The term PEGylation describes the modification of biological molecules by covalent conjugation with polyethylene glycol (PEG), a non-toxic, non-immunogenic polymer, and is used as a strategy to overcome disadvantages associated with some biopharmaceuticals. PEGylation changes the physical and chemical properties of the biomedical molecule, such as its conformation, electrostatic binding, and hydrophobicity, and results in an improvement in the pharmacokinetic behavior of the drug. In general, PEGylation improves drug solubility and decreases immunogenicity. PEGylation also increases drug stability and the retention time of the conjugates in blood, and reduces proteolysis and renal excretion, thereby allowing a reduced dosing frequency. In order to

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benefit from these favorable pharmacokinetic consequences, a variety of therapeutic proteins, peptides, and antibody fragments, as well as small molecule drugs, have been PEGylated.

This paper reviews the chemical procedures and the conditions that have been used thus far to achieve PEGylation of biomedical molecules. It also discusses the importance of structure and size of PEGs, as well as the behavior of linear and branched PEGs. A number of properties of the PEG polymer – e.g. mass, number of linking chains, the molecular site of PEG attachment – have been shown to affect the biological activity and bioavailability of the PEGylated product. Releasable PEGs have been designed to slowly release the native protein from the conjugates into the blood, aiming at avoiding any loss of efficacy that may occur with stable covalent PEGylation.

Since the first PEGylated drug was developed in the 1970s, PEGylation of therapeutic proteins has significantly improved the treatment of several chronic diseases, including hepatitis C, leukemia, severe combined immunodeficiency disease, rheumatoid arthritis, and Crohn disease. The most important PEGylated drugs, including pegademase bovine, pegaspargase, pegfilgrastim, interferons, pegvisomant, pegaptanib, certolizumab pegol, and some of the PEGylated products presently in an advanced stage of development, such as PEG-uricase and PEGylated hemoglobin, are reviewed. The adaptations and applications of PEGylation will undoubtedly prove useful for the treatment of many previously difficult-to-treat conditions.

1. Peptides as Therapeutic Agents

The precise structure of peptides allows them to play important roles as activators or inhibitors of biological mechanisms, or as biocatalysts in metabolic processes. As such, they are an obvious choice as new therapeutic agents. The development of genetic engineering has allowed the production of peptides and proteins in amounts that could only be dreamed of previously, thus making these new drugs affordable for therapeutic use. The intrinsic properties of these macromolecules, however, present drawbacks that limit their administration, such as instability, degradation by proteolytic enzymes, rapid excretion, and potential immunogenicity.

Over the years, several strategies have been devised to overcome these limitations. Among these were the entrapment of peptides into particles, gels or liposomes, genetic substitution of amino acids to stabilize the structure or reduce immunogenicity, and modification of the surface by covalent conjugation with low- or high-molecular-weight compounds such as simple acylating agents, sugars, or polymers. The latter approach proved the most successful, as initially demonstrated by the safer use of neocarcinostatin as an antitumor agent[1] and of streptokinase for blood clot dissolution[2] following conjugation to a synthetic acrylic polymer or to dextran, respectively.

2. Development of PEGylation

The most successful and general strategy in polymer conjugation came from the pioneering studies of Abuchowski et al.[3] in the 1970s, which were soon followed by a number of studies and improvements by other groups, as summarized in table I. The researchers took advantage of the unique properties of polyethylene glycol (PEG), a non-toxic, non-immunogenic polymer approved by the US FDA for internal use. The advantage of PEG resides in the fact that it possesses, in addition to its favorable biological properties, very good solubility in both aqueous and organic milieu, great flexibility, high hydration that increases its hydrodynamic volume, and a range of molecular weight species with low polydispersity.[4,5] All of these properties are acquired by compounds to which PEG is covalently bound. As a consequence of the water cloud surrounding the polymer, proteins conjugated with PEG exhibit increased solubility, become resistant to antibodies, proteolytic enzymes and cells, and, because of their increased size (figure 1), are ultra-filtered by the kidneys more slowly.

Proteins are delicate molecules, easy to denature and inactivate. The first obstacle to achieving a successful coupling of PEG to proteins, a process called 'PEGylation', was to find a chemistry of binding that was mild enough not to damage the protein. Several chemical strategies for converting the PEG terminal hydroxyl group to one reactive with the protein amino acid residues under gentle conditions have been described.[12] The protein amino groups have been primarily exploited for conjugation, since they are always present and generally at the protein surface and exposed to the solvent. Cysteine thiol residues, when present, are also very convenient because, being seldom present in proteins, they allow site-specific conjugation; furthermore, they can be

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Table I. History of PEGylation

Decade	PEGs	General observation	Applications
1970–80	PEG-chloro triazine[3] PEG-succinimidyl succinate PEG-tresyl	Immunogenic or toxic starting material, highly polydispersed PEG, lack of selectivity	Research studies, enzyme modification for biocatalysis
1980–90	PEG-aldehyde[6] PEG-succinimidyl carbonate PEG-pNO_2 phenyl carbonate PEG-AA-NHS[7] PEG-carbonylimidazole, etc.	Site-specific conjugation, less polydispersed PEG, absence of diols	Enzyme replacement therapy
1990–2000[8]	Branched PEG PEG-NHS PEG-maleimide PEG-OPSS	Improved selectivity, marketing of PEGylated drug	Cytokines, hormones, anticancer drugs targeting
2000 onwards	Enzymatic coupling[9] Disulfide coupling[10] Releasable PEGs[11] Forked PEGs Star PEGs Monodisperse PEGs	Detailed chemical and biological characterization of conjugates, combination of genetic engineering and PEGylation in the design and discovery of new drugs, more stringent regulatory requirements	Non-protein drugs PEGylation, oligonucleotide PEGylation, cell PEGylation

AA = amino acids; **NHS** = N-hydroxysuccinimide; **OPSS** = ortho-pyridyldisulfide; **PEG** = polyethylene glycol.

conveniently introduced by genetic engineering at desired sites in the sequence.

Improvement of the PEG properties was another very important consideration, namely the synthesis of a less polydisperse product or species of specific structure (the branched form being the most successful). In this respect, one can see that the improvement of chemistry of PEGylation parallels the application and the success of biological therapies (table I). PEGylation has led to the success of many therapeutic proteins and oligonucleotides, including several products that have become blockbusters (table II).

In addition, since PEG is an amphiphilic polymer (both hydrophilic and hydrophobic), the conjugated proteins become soluble in some organic solvents, allowing the use of conjugated enzymes as new biocatalysts. What is interesting in this respect is the unexpected applications of lipase or chymotrypsin in catalyzing synthetic processes.[21]

A number of different parameters must be taken into consideration when producing a PEG-protein conjugate; these are reviewed in the following sections and typical examples of polymer-modified proteins are described. However, for more complete documentation, the reader is referred to some recent reviews,[22-24] and a discussion of small molecule conjugation can be found in the reviews by Greenwald[25] and by Pasut et al.[26]

3. Chemistry of PEGylation

3.1 Amino Group Modification

The amino groups of proteins are the most exploited residues for PEG-polymer conjugation by alkylation or acylation. The process used to achieve PEGylation may negatively influence the surface character of the protein; alkylation maintains a positively charged amine, whereas acylation yields a neutral amide. In some

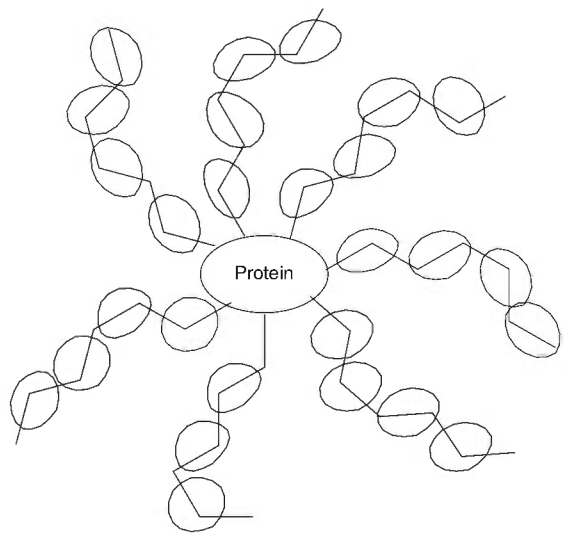


Fig. 1. Schematic representation of a protein with bound polyethylene glycol. The circles surrounding the polymer mimic the cloud of water coordinated to the ether-oxygen groups of the polymer.

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Table II. PEGylated proteins or oligonucleotides approved by the US FDA

Trade name	Generic name	Parent drug	Size of PEG moiety (kDa)	Indication	Year of approval	Reference
Adagen®	Pegademase	Adenosine deaminase	5	SCID	1990	13
Oncaspar®	Pegaspargase	Asparaginase	5	Leukemia	1994	14
Neulasta®	Pegfilgrastim	G-CSF	20	Neutropenia	2002	15
PegIntron®	Peginterferon-α-2b	Interferon-α-2b	12	Hepatitis C	2000	16
Pegasys®	Peginterferon-α-2a	Interferon-α-2a	40	Hepatitis C	2001	17
Somavert®	Pegvisomant	hGH	5	Acromegaly	2003	18
Macugen®	Pegaptanib	Anti-VEGF aptamer	40	AMD	2004	19
Mircera®	PEG-EPO	Erythropoietin	40	Anemia	2007	
Cimzia®	Certolizumab pegol	Anti-TNFα Fab′	40	Rheumatoid arthritis and Crohn disease	2008	20

AMD = age-related macular degeneration; **EPO** = erythropoietin; **G-CSF** = granulocyte colony-stimulating factor; **hGH** = human growth hormone; **SCID** = severe combined immunodeficiency disease; **TNF** = tumor necrosis factor; **VEGF** = vascular endothelial growth factor.

cases, such modification was demonstrated to be critical for the maintenance of the biological activity of the protein.[27,28]

A PEG activator that maintains the charge of the amino groups is trichlorotriazine (figure 2a), which was used by Abuchowski et al. in their pioneering work[3] but later abandoned because of toxicity.

The aldehyde (figure 2b) and the epoxide PEGs represent valid alternatives. In particular, the aldehyde reacts with amines to yield an unstable Schiff base, which is in turn reduced to a stable secondary amine.[29]

While the role of charge is generally important for the clearance of peptides or non-peptide drugs,[30] this is not so critical for proteins, especially if they are large, because several lysine residues and strongly charged arginines are also present. PEGs terminating with a carboxylic group activated with N-hydroxysuccinimide (NHS) [figure 3a] are particularly important acylating agents. In some cases, between the polymer and the activated moiety, spacers may be present to introduce specific properties to the conjugate.[7,31]

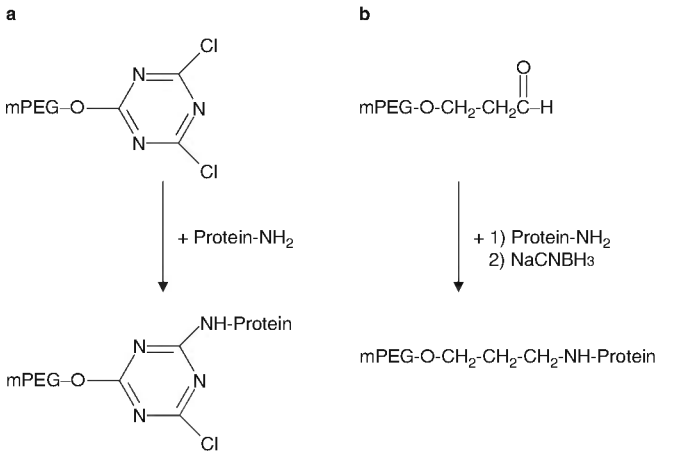


Fig. 2. Amino group modification that maintains the positive charge of the protein residue: (**a**) trichlorotriazine; (**b**) aldehyde. **PEG** = polyethylene glycol.

PEGs activated as carbonate (e.g. p-nitrophenylchloroformate, benzotriazole, succinimidyl carbonate) [figures 3b–d] are also frequently used,[32,33] yielding a less stable urethane linkage with the protein.

It is interesting to note that the ε-amino groups of lysine possess a higher acid dissociation constant (pKa) than the protein α-amino terminal; this allows a preferential site of conjugation and produces more defined and more biologically active products if the reaction is carried out at low pH, where only the α-amino group is reactive. This was exploited with success in the case of pegfilgrastim (see section 6.1.3).[6]

3.2 Conjugation to Imidazole Group of Histidine

The secondary amine group of histidine has a lower pKa than the primary amine. At a pH close to neutrality, a properly activated PEG can be conjugated at this level. The acyl-histidine bond, however, is not stable, and the conjugate behaves as a prodrug, slowly releasing the free protein, as in the case of Peginterferon-α-2b.[34]

3.3 Cysteine Thiol Residue Conjugation

PEG-cysteine reagents can conjugate proteins by forming either stable thio-ethers (e.g. maleimide [figure 4a] and vinylsulfone [figure 4b]) or disulfides (e.g. PEG-pyridyldisulfide [figure 4c]). The latter yields a protein-PEG conjugate that can be

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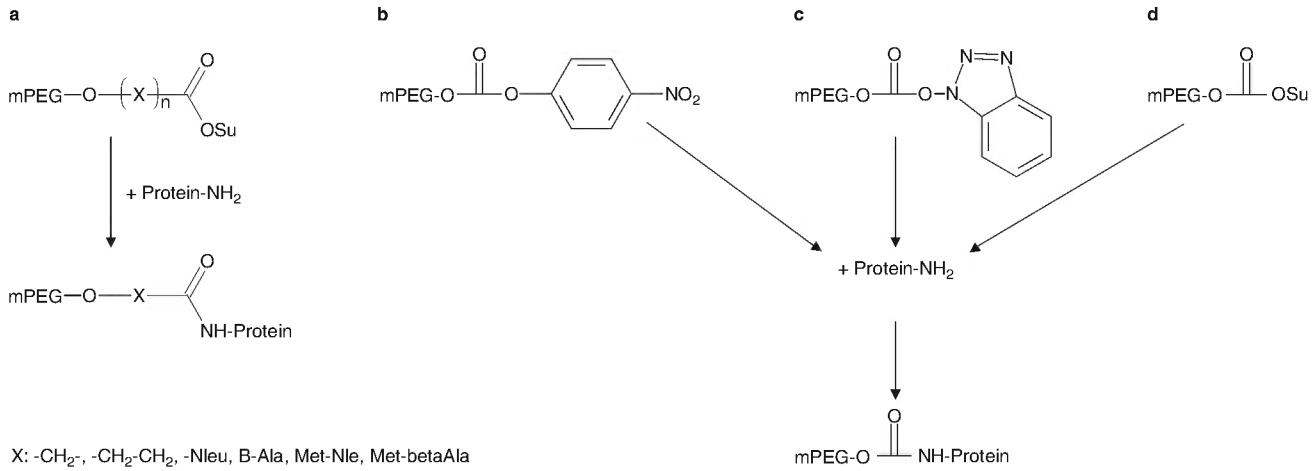


Fig. 3. Amino group modification that leads to loss of the positive charge of the protein residue: (**a**) N-hydroxysuccininimide; (**b**) p-nitrophenyl-chloroformate; (**c**) benzotriazole; (**d**) succinimydil carbonate. **PEG** = polyethylene glycol.

released under reducing conditions that may also be found in the body.[35,36]

There are only rare examples of available free cysteine residues in proteins being used for conjugation, because this amino acid, when present, is generally involved in catalysis or in disulfide bridges. One example is granulocyte colony-stimulating factor (G-CSF), where a free cysteine is partially exposed and the conjugation takes place only after partial reversible denaturation of the native protein.[37] On the other hand, there are several examples of cysteine being introduced by genetic engineering in a convenient position of the protein sequence.[38]

Very recently, a clever strategy was devised[10] to take advantage of the cysteines when present as disulfide bridges. The cysteine is first freed by a reduction reaction, and the free cysteine is reacted with a bifunctional thiol-reactive PEG to give a new stable bridge with the polymer attached.

3.4 Conjugation to Carboxylic Group, Arginine and Hydroxyl Residues, or Sugars

Direct coupling of PEG-NH_2 to activated protein carboxylic groups is not possible, because these would react with the amines of the protein itself or of another nearby protein molecule to yield intra- or inter-molecular linkages. An original solution came from the studies of Zalipsky and Meno-Rudolph[39] by the use of PEG hydrazide, which is reactive at low pH as an amino donor. However, to date, no useful application has been found.

There are few examples of arginine-reactive PEGs (reported in proprietary literature only); these are mainly based on PEG with

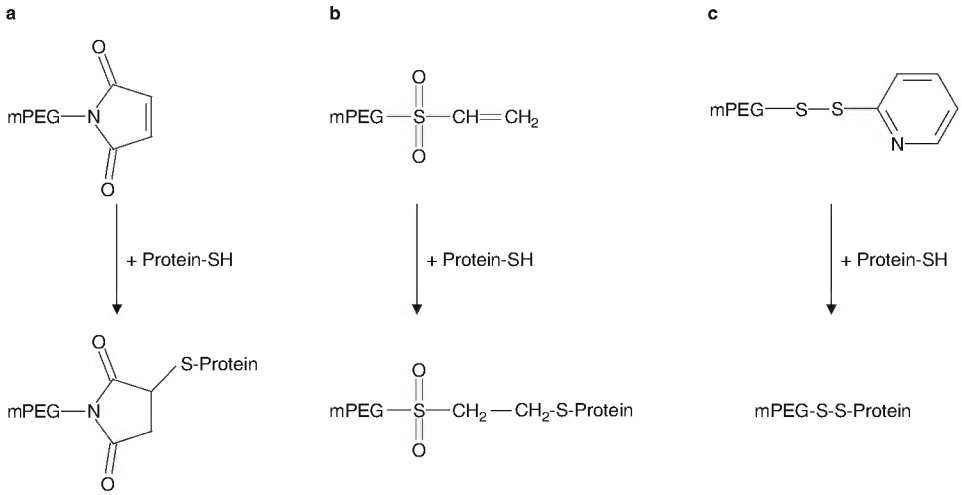


Fig. 4. Polyethylene glycols (PEGs) reactive towards thiol groups: (**a**) maleimide; (**b**) vinylsulfone; (**c**) pyridyldisulfide.

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vicinal dicarbonylic groups, although the method has not achieved useful application because of a lack of specificity.

The alcohol groups of serine and threonine and the phenolic group of tyrosine are potential sites for modification by acylating PEGs,[40,41] but for serine and threonine modifications the proteins generally cannot withstand the necessary harsh conditions of the chemical reaction. Specific conjugation at these residues, however, has been achieved recently by taking advantage of an enzymatic approach, namely the sequential use of glutamine transferase and sialyl transferase to link a properly modified sialyl PEG.[42] The method is still awaiting large-scale application.

In the case of tyrosine, the phenol ester obtained by acylation is unstable in water. For this reason, many researchers remove any unstable ester links at the end of the amino PEGylation using a mild treatment with hydroxylamine.[43]

For PEG linkage to the sugar residues of glycoproteins, only rather unspecific methods have been described.[39] These are based on oxidative opening of the sugar ring to yield an aldehyde that is reactive with PEG hydrazide. The coupling reaction is carried out at low pH in order to avoid reaction with the protein amino residues that are protonated under these circumstances.

3.5 Glutamine Residue Conjugation

While there is no chemical method for specific reaction of the glutamine amide, conjugation can be achieved enzymatically by the use of specific or nonspecific transglutaminase (an enzyme present in all organisms and extracted from microbial sources). This involves a transamidase reaction between the glutamine of the protein and a PEG-NH_2 as the nucleophyle donor.[9] This PEGylation is very specific, since it requires not only a particular sequence around the glutamine residue but, as we recently discovered, a correlation between the site of specific conjugation and the flexibility of the protein chain.[44] Alternatively, the same enzyme may catalyze the conjugation of a PEG-glutamine to the ε-group of lysine.[45]

3.6 Releasable PEGs

A method for the slow release of the native protein from a PEG-conjugate into the blood (as in the classical low-molecular-weight prodrug strategy) was devised to avoid the loss of activity that accompanies stable covalent PEGylation. Releasable PEGs have been known for a long time, e.g. by succinimidyl succinate conjugation, but only recently has the strategy been seriously considered and intelligent chemistries designed to allow protein release at a specified rate. These methods are generally based on a triggering moiety that, by enzymatic reaction or water hydrolysis, releases a group that catalyzes the detachment of the polymer, leaving the native protein.[11] By carefully choosing the chemical groups in the trigger moiety, long retention in the blood of the inactive conjugate and a slow, controlled release of the native protein can be achieved.

4. Critical Parameters to Consider for Protein PEGylation

4.1 PEG Structure and Size

Although it became clear that the size of the polymer plays a significant role in determining the biological behavior of PEG conjugates, the only polymer used for several years was 5 kDa in size, and was end-capped at one side with a methoxyl group and terminated with a hydroxyl group. This choice was based on the fact that this polymer is easy to activate, and conjugation can be achieved in biological laboratories and not just by skilled polymer chemists. It was also recognized that the mass of bound PEG was important for determining the time it remains in the blood. The required mass can be reached by adding either several small PEG chains to the protein, or alternatively one PEG chain of high mass. For a long time, conjugation was complicated by the presence of a significant amount of diol impurities in the samples and high polydispersity (the presence in the polymer of species of different length). These two factors led to heterogeneity in the final product: covalent protein aggregates in the first case and conjugates with different mass in the second. This problem was later overcome at the end of the 1990s by the marketing of a pure, low polydisperse, activated, very-high-mass polymer (30–40 kDa) [Shearwater Polymers, Inc.] that was more suitable for medical applications. Pharmaceutical grade PEG should have a polydispersity spanning from 1.01 for a product of 5 kDa, up to 1.1 for a product of 50 kDa. Note that the value for a monodisperse product is 1.00.

A new form of polymer, called branched PEG (PEG_2), characterized by two chains of polymer linked to one activated moiety, was also discovered (figure 5). The importance of this product stems from the fact that this form allows for higher masking and protection of the protein surface based on the so-called 'umbrella-like effect'.[46,47]

In addition, PEG_2 allows for linking a double mass of polymer by the modification of only one amino acid residue in the protein, which leaves it more biologically active. This form of PEG was very successful, giving rise to three important products, peg-interferon-α-2a, pegaptanib, and certolizumab pegol (table II),

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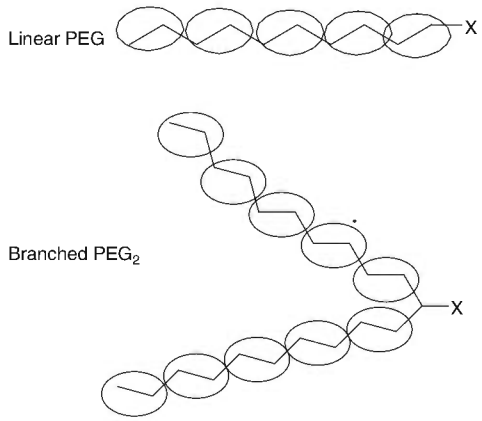


Fig. 5. Schematic representation of linear polyethylene glycol (PEG) and the branched form (PEG_2). The circles around the PEG chain represent the bound water and X represents the protein reactive groups.

while several others are now in advanced stages of clinical investigation.

Development of this idea resulted in the proposal of a new hyper-branched form of PEG, called polyPEG® (Warwick Effect Polymers Ltd, Coventry, UK),[1] and biological results for this product are awaited.[48]

4.2 Targeted Conjugation in the Protein Sequence

Since there are usually several PEG chains linked to the protein surface, there is a high chance of masking the catalytic sites (in the case of enzymes) or recognition sites (in the case of receptors). Therefore, for each new product, it is necessary to find the mass and number of linking chains that maintain the biological activity and bioavailability as much as possible.[49,50] For this reason, many researchers are trying to produce genetic variants with amino acids not present in the natural protein (generally a cysteine residue), inserted at sites far from those involved in biological activity and using specific reagents to direct the PEG conjugation to that site only.[38]

Unfortunately, there are few chemical strategies for site-specific conjugation that can be utilized for all proteins. One such strategy is conjugation to the amino terminal group of the protein at low pH (see section 3.1).[6]

Alternatively, the number of potential NH_2 isomers can be reduced using genetic engineering to replace lysine with a non-reactive residue. When these methods do not give satisfactory results, it may be possible to carry out the conjugation reaction in the presence of soluble or insoluble inhibitors to reversibly mask the biologically active sites.[51]

However, in some cases, the activity is maintained despite the presence of the PEG chain at, or close to, the protein catalytic or recognition site, because of the flexibility of the chain.

All of these parameters must be taken into consideration in approaching a new protein PEGylation, and guidelines suitable for young researchers can be found in more detailed papers.[19,52]

5. Biological Consequences of PEGylation

In general, after PEGylation, the retention time of protein conjugates in blood is increased and immunogenicity is reduced. There are, however, interesting examples where unexpected behaviors were observed after modification. For instance, increased activity is observed with PEGylated trypsin for low-molecular-weight substrates and for PEGylated amino oxidase.[46,53] These results were explained as an increased affinity for the substrates as a result of the new microenvironment created by the amphiphilic PEG or by favorable modification of the three-dimensional structure.

Another unexpected behavior was observed for PEGylation of a peptide-based chelating agent; addition of PEG yielded stronger aggregation and micelle formation.[54] There have also been instances in which PEGylation of a protein gave rise to new biological properties. For instance, PEGylation of cholesterol oxidase changed its substrate specificity from total cholesterol to high-density lipoprotein (HDL)-cholesterol, and PEGylation of a growth hormone, pegvisomant, changed the activity from agonist to antagonist.[55]

PEGylation changes the physical and chemical properties of the proteins so that they may become soluble in organic solvents. In the case of enzymes, this opens up the possibility of conducting highly selective reactions in non-aqueous solvents, which is very useful in organic syntheses.[28]

Approved by the FDA for many years as a safe product for external and internal use, PEG is used as an excipient in many preparations for medical use, e.g. formulations to relieve constipation, and products for colonoscopy (at very high doses).[56,57] Parenterally administered PEG is excreted by the kidney at a rate dependent on the molecular weight and the site of injection.[58] PEGs over 40 kDa in size have a greatly reduced elimination rate, but excretion via the kidney is still possible due to the flexibility of the chain or by removal of the PEG chains by the reticuloendothelial system. PEG is also slowly oxidized by alcohol dehydrogenase[59] or by other oxidases such as cytochrome P-450.[60] It can also be excreted in bile.

1 The use of trade names is for product identification purposes only and does not imply endorsement.

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The protein-PEG conjugates are degraded in the liver, as suggested by the fact that small peptide fragments have been found in the liver following administration. A thorough discussion on acute, sub-chronic or other toxicities, as well as metabolism and disposition of protein-PEG conjugates, has already been presented.[61] From their review of the literature, the authors concluded that PEG is safe for parenteral administration either in imaging agents or as a component of long-term circulating liposomes.[62] However, as with any new chemical entity, the safety depends on the chemical nature (the PEG-activating moiety, PEG conjugates, PEG chain length, etc.), dose, and mode of administration. Therefore, any new derivative requires independent studies. Administration of PEGylated proteins at high doses in animals has been associated with the occurrence of renal tubular vacuolization, although there was no alteration of clinical pathology or functional markers.[63] A more recent study demonstrated that PEG associated with a protein or another biological molecule does not pose an additional risk to humans.[64] Furthermore, we must remember that, relative to the high potency of protein drugs, the amount of PEG administered in the conjugates is usually very small and should therefore be of minimum concern for therapeutic use.

6. Successfully PEGylated Drugs

6.1 Approved Products

Among the many protein drugs being improved by PEGylation, those that have already reached the market are briefly described here.

6.1.1 Pegademase Bovine

PEGylated bovine adenosine deaminase (pegademase bovine; Adagen®, Enzon Inc.) was the first PEGylated protein to be marketed successfully. It was approved by the FDA in 1990 as a replacement therapy to treat severe combined immunodeficiency disease (SCID). Bovine adenosine deaminase, the enzyme that removes toxic adenosine metabolites from blood, was randomly modified with PEG 5 kDa to extend the time it remains in plasma by several-fold and to reduce its immunogenicity. The favorable pharmacokinetic and pharmacodynamic behavior of the conjugate support once-weekly subcutaneous administration in patients with SCID, replacing the need for multiple blood transfusions and consequently avoiding the associated risk of viral infections. This was an important landmark in PEG technology because it demonstrated for the first time the feasibility of a technology that was previously an appealing but unproven idea, and encouraged further applications.[13]

6.1.2 Pegaspargase

The second biologically active protein to be conjugated with PEG was L-asparaginase (pegaspargase; Oncaspar®, Enzon Inc.), also an enzyme. Pegaspargase was approved by the FDA in 1994 for patients in whom the unmodified protein elicited an allergic reaction. Again, conjugation with multiple strands of PEG 5 kDa increases the elimination half-life by about three times compared with the native protein. The PEGylated product was as effective as the native drug in treating patients with acute lymphoblastic leukemia, and showed a lower degree of immunogenicity.[14,15]

Unfortunately, the methods utilized for the PEGylation of bovine adenosine deaminase and L-asparaginase are based on nonselective attachment of the polymer. This results in heterogeneous populations with differing degrees of PEGylation, the presence of isomers, and inter-batch variability, which may be reflected in variability of pharmacokinetic behavior. From these initial products, more homogenous and better characterized products were prepared to satisfy the stringent requirements of the regulatory agencies.

6.1.3 Pegfilgrastim

G-CSF is the major regulator of granulopoiesis *in vivo*. It has become pharmaceutically important for the stimulation of proliferation, differentiation, and survival of cells of the granulocyte lineage.[65,66] Recombinant G-CSF (filgrastim) is a protein of 18.7 kDa comprising 174 amino acids, which lacks glycosylation.[67] It has a relatively short half-life (3.5–3.8 hours), and so multiple daily doses are required.

The PEG-conjugated form, pegfilgrastim (Neulasta®, Amgen Inc.), was produced by site-specific attachment of a PEG 20 kDa molecule to the α-amino groups of the N-terminal methionine residue of filgrastim by reductive alkylation of the protein with PEG aldehyde under acidic conditions. Pegfilgrastim remains in plasma long enough to allow a single subcutaneous injection per chemotherapy treatment, rather than the multiple injections needed of the unconjugated protein, and has the added advantage of preventing the high clearance of G-CSF via internalization of the receptor-ligand complex in neutrophil cells, which is in some way related to the number of circulating neutrophils. Pegfilgrastim, which remains in the bloodstream for a prolonged time, stimulates the proliferation of neutrophils and consequently causes its own clearance when the therapeutic target is achieved.[68,69] Pegfilgrastim also has a decreased rate of aggregation under physiological conditions (i.e. neutral pH and 37°C) and no detectable insoluble precipitation with loss of biological activity compared with native G-CSF.[70]

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6.1.4 Interferons

Interferons combined with oral ribavirin are the treatments most used for viral infections and cell-proliferative disorders.[71] However, standard interferons have a short half-life (4–5 hours) and are undetectable in the blood after 24 hours. Therefore, administration three times weekly by subcutaneous injection is required to achieve negligible fluctuations in plasma concentrations and to minimize the duration of intervals during which the protein concentration is low enough to allow the virus to replicate and develop resistance mutations. Plasma levels of the PEGylated interferon, however, are sustained at constant levels for several days, providing constant antiviral protection. Currently, two different PEG-interferons are commercially available and in widespread clinical use. Both conjugates are a mixture of monoconjugate isomers obtained by random linkage of reactive PEGs at the nucleophilic sites of the protein; they differ in the size and structure of PEG and in the conditions of reaction.[72]

PEG-Interferon-α-2b

PEG-interferon-α-2b (PEG-IFNα-2b; PegIntron®, Schering-Plough Corporation) was obtained by covalently linking IFNα-2b with PEG-SC 12 kDa via a urethane bond,[16] with the PEG chain attached mainly to His^{34}. This linkage is hydrolytically unstable in plasma and, once injected, the free protein is slowly released, thus avoiding the frequent problems of PEGylation in terms of binding to the receptor, drug metabolism, and renal excretion. However, as a consequence of the instability of the linkage, the conjugates must be stored as a powder and reconstituted just before use.

PEG-Interferon-α-2a

PEG-IFNα-2a (Pegasys®, Hoffmann La Roche Inc.) was obtained by covalently linking branched PEG_2-NHS 40 kDa by amide bonds to the IFNα-2a lysine residues. This produced a mixture of four major mono-PEGylated isomers at Lys^{31}, Lys^{121}, Lys^{131}, and Lys^{134}.[17] The amide bond has greater stability, so the injected conjugate circulates intact, and it is therefore the conjugate itself that interacts with the receptor rather than the free native protein.

Recently, the isomers of PEG-IFNα-2a were separated chromatographically and the activity was evaluated; the isomers, Lys^{31}, and Lys^{134}, were found to be the most active. Based on the observations of these studies, it is hoped that it will be possible to design new PEG-interferon conjugates that retain more of the biological activity of the native protein.

The pharmacokinetics of PEG-IFNα-2b and PEG-IFNα-2a are summarized in table III. PEG-IFNα-2b has a prolonged serum half-life relative to the unconjugated protein, due to the greater polymer mass and the stability of the linkage. The different chemistries through which PEGylation is achieved, and in particular the unstable PEG-histidine urethane linkage, lead to different *in vitro* activities of the conjugated proteins. For example, the activity relative to the native protein is 28% for PEG-IFNα-2b and only 7% for PEG-IFNα-2a.[72]

Table III. Biochemical and chemical properties of the two PEGylated interferons

PK parameters	IFNα	PEG-IFNα-2a	PEG-IFNα-2b
Elimination half-life (h)	6–9	72–96	32–40
Clearance (mL/h)	6000	60–100	725
Absorption half-life (h)	2.4	50	4.6

IFN = interferon; **PEG** = polyethylene glycol; **PK** = pharmacokinetic.

However, the extended pharmacokinetics of PEG-IFNα-2a overcome its reduced *in vitro* potency, giving rise to more prolonged *in vivo* activity. Once-weekly administration with either PEGylated form was found to produce significantly higher rates of viral eradication than the parent IFNα administered three times weekly, without compromising tolerability.

Detailed investigations of the PEG isomers have revealed the structural requirements for maintaining IFNα activity. This clearly demonstrated the problem of designing successful protein drugs that act through interaction with receptors. Here, the recognition is based on an extended surface interaction between protein and cell receptors that may be prevented or reduced when the bulky molecule of PEG is present. Other examples – including insulin PEGylation[73] and, more recently, the elegant work carried out on PEGylated growth hormone antagonists[18] – stress the need to use site-specific PEGylation methods (as previously described for pegfilgrastim [section 6.1.3]) or engineered mutants (G-CSF, growth hormone, erythropoietin),[74] where PEGylation takes place only at a specific site in the protein.

6.1.5 Pegvisomant

Pegvisomant (Somavert®, Pfizer Inc.), the first specific growth hormone receptor antagonist, was developed for the treatment of acromegaly and now represents a first-in-class therapy for the treatment of this disorder. Acromegaly is a syndrome arising from excess production by the pituitary gland of human growth hormone (hGH), generally as the result of a pituitary adenoma, and it is characterized by abnormal enlargement of the hands, feet, skull, and jaw.

Growth hormone contains two distinct, high- and low-affinity binding sites for the receptor.[75] The first growth hormone mutant tested as an antagonist was G120K, in which a lysine was substi-

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tuted for a glycine. G120K binds the growth hormone receptor with high affinity but prevents signal transduction; unfortunately, like growth hormone itself, it has a very short half-life. In order to prolong its plasma circulating time, a random conjugation with PEG 5 kDa was carried out. This achieved a protein with a half-life of about 100 hours but with a low affinity for the receptor. To address this, further mutations were introduced to reduce the number of potential PEG binding sites. From these, B2036 was chosen for further development. B2036 is an antagonist with increased affinity at one binding site and lowered affinity at another, but does not allow the necessary conformational changes required for signal transduction. In order to delay plasma clearance, 4–6 PEG 5kDa chains were conjugated, increasing the hydrodynamic volume of the protein.[55] PEGylation decreased the antagonistic activity of B2036 by about 28-fold, but the effect was balanced by the much improved plasma half-life. It is worth noting that the binding of pegvisomant to the soluble extracellular portion of the receptor was reduced by 4.5-fold compared with B2036, strongly supporting the idea that PEG causes a steric hindrance at the level of the membrane receptor.

A multicenter study[76] assessed pegvisomant in 112 patients with acromegaly who had previously been treated with surgery or radiation therapy. Compared with placebo, 12 weeks of treatment with pegvisomant caused dose-dependent reductions in serum levels of total insulin-like growth factor-I (IGF-I), IGF binding protein-III (IGFBP-III), and its acid-labile subunit, whose mitogenic properties are responsible for the growth and metabolic clinical manifestations of acromegaly. In 2003, the FDA approved pegvisomant as a second-line therapy for the treatment of acromegaly.

6.1.6 Pegaptanib

Oligonucleotide-protein interactions, ubiquitous in nature, are now of great interest for the development of a new class of therapeutic agents. Aptamers are DNA- or RNA-modified oligonucleotides with high affinity and specificity towards proteins that are selected and amplified by a procedure called systemic evolution of ligands by exponential enrichment (SELEX).[19]

In general, aptamers have the drawback of possessing too short an *in vivo* half-life because of nuclease attack and rapid clearance. In contrast, they may present advantages that derive from their versatility, high sensitivity, and selectivity to protein, as well as ease of screening and production. Aptamers show very high specificity (being able to distinguish between closely related proteins, and between peptide enantiomers) and they are non-immunogenic despite being administered at high doses. Furthermore, their large-scale synthesis is fairly straightforward and low-cost; the process is free from cell-based expression systems.

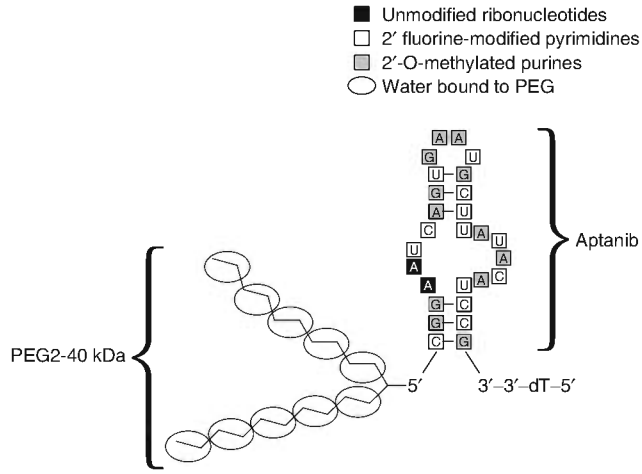


Fig. 6. Pegaptanib structure and the site of attachment of 40 kDa polyethylene glycol (PEG) chain are shown.

Pegaptanib (Macugen®, Eyetech Inc. and Pfizer Inc.) [figure 6] is an aptamer directed against vascular endothelial growth factor (VEGF), a protein responsible for pathological ocular neovascularization and vascular permeability leading to a form of macular degeneration. The initial anti-VEGF aptamer chosen for binding the protein showed the classic aptamer drawback: a low half-life resulting from nuclease degradation and rapid clearance by the kidney. The incorporation of modified unnatural nucleotides reduced the nuclease attack, increased the half-life from 1.4 to 131 hours, and increased binding affinity for VEGF.[77] Conjugation to a 40 kDa branched PEG decreased the binding affinity by 4-fold but prolonged the time it remained in tissue and the plasma half-life. For full efficacy, the drug requires intravitreal injection to reach the local VEGF that causes neovascularization. PEGylation allows slow diffusion out of the vitreous humor, thus maximizing efficacy and minimizing systemic exposure.

Pegaptanib was approved by the FDA in 2004 for the treatment of all types of neovascular age-related macular degeneration (AMD). Treatment is required every 6 weeks. This represents the first successful application of PEGylation to a new class of biotechnology drugs, the oligonucleotides, demonstrating once again the versatility of the approach.

6.1.7 Certolizumab Pegol

Currently, antibody therapies are used mainly for chronic diseases that require doses of antibody to be administered repeatedly over several months or years, with consequently high costs. One approach used to increase stability and reduce costs has been to express fragments of antibodies, such as Fab′ and scFv, in a

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microbial expression system such as *Escherichia coli*. This strategy obviates the potential rate-reducing steps in clinical studies: the construction of intact immunoglobulin G and its difficult expression in mammalian cells. However, antibody fragments, although cheaper to produce on a large scale (because of the larger capacity of microbial, compared with mammalian, cell fermentation), are more rapidly excreted.

Antibody fragments have been PEGylated in order to reduce their antigenicity and glomerular filtration.[78] Although the addition of PEG did improve the circulating half-lives of the fragments, their ability to bind antigen was reduced as more PEG was attached. For example, in a typical case,[79] the binding was reduced to 62% when three PEG 5 kDa chains were attached, and further reduced to 17% when six PEG 5 kDa chains were attached. This was overcome by site-specific attachment of PEG of larger molecular mass at the Fab′ hinge region so that the *in vivo* half-life was still increased but without loss of antigen-binding affinity. Moreover, the linking of PEG to one or two sites only in the hinge region also facilitates the production of well defined conjugates that are identical from batch to batch and hence suitable for large-scale manufacturing.[78]

Certolizumab pegol (Cimzia®, UCB S.A.) was developed based on this rationale (figure 7). It is a PEGylated Fab′ fragment of a humanized anti-tumor necrosis factor (TNF)-α monoclonal antibody that binds and neutralizes membrane-bound or soluble TNFα after binding.

In this case, the conjugation with a branched PEG (PEG_2) enables retention of recognition capacity, high circulating time in blood, and a possible reduction of immunogenicity. Moreover, since site-specific PEGylation of the Fab′ fragment is directed to a site well away from the antigen-binding region, the conjugate exhibits the same affinity to TNFα as the native antibody without loss of biologic activity.[20] The conjugation takes place at a lone, free thiol cysteine residue, using PEG_2 40 kDa maleimide as the reagent.

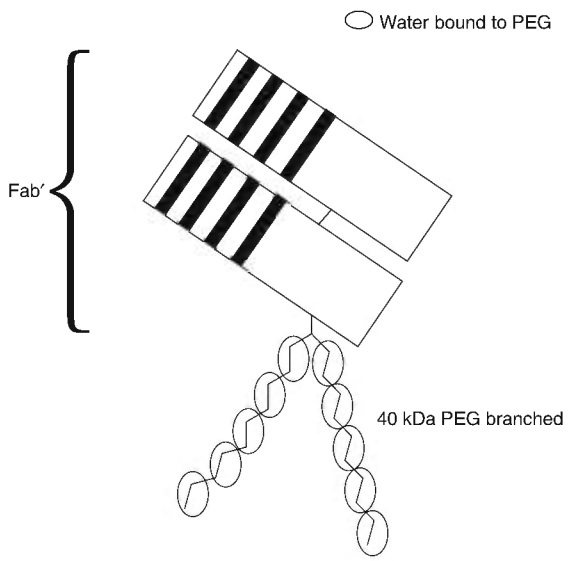


Fig. 7. Structure of polyethylene glycol (PEG) conjugated to anti-tumor necrosis factor (TNF) antigen binding fragment (Fab′).

Certolizumab pegol was approved by the FDA in April 2008 for patients with moderate-to-severe Crohn disease who have not responded to conventional therapies, and now it is in clinical development for rheumatoid arthritis and psoriasis. All of these diseases are chronic, inflammatory disorders characterized by elevated TNFα levels. Detailed evaluation of Crohn disease safety and efficacy demonstrated positive results for certolizumab pegol compared with placebo in moderate-to-severe active disease.[80-82] In addition, data from trials in all indications suggest that certolizumab pegol is well tolerated.[80,83-85]

Certolizumab pegol has the advantage of monthly subcutaneous administration while maintaining high binding affinity for both soluble and membrane-bound TNFα. Certolizumab pegol does not demonstrate apoptosis of lymphocytes and monocytes. Furthermore, antibodies to infliximab do not cross-react with certolizumab pegol. A further advantage observed with certolizumab pegol is its preferential accumulation in inflamed tissue relative to uninflamed tissue, a behavior probably related to the enhanced permeability and retention (EPR) effect,[1] which was not observed with the non-PEGylated Fab′.

An alternative strategy applied to TNF antibody conjugation, which has not reached marketing success so far, was developed by Amgen Inc. (pegsunercept). Pegsunercept was obtained by linking a 30 kDa methoxy PEG aldehyde to the N-terminal group of the recombinant human soluble TNF receptor type I.[86]

A PEGylated anti-VEGF antibody (CDP791) has been developed using the same rationale and is in advanced clinical trials for the treatment of solid tumors. Again, PEG conjugation at a hinge region cysteine residue reduces the immunogenicity relative to the parent IgG and increases the half-life in plasma by reducing kidney glomerular filtration.[87]

These results demonstrate the potential of proper PEGylation to reduce many limitations of the manufacture, cost, and toxicity of the full antibodies, further widening the potential for therapies in this important class of drugs.

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6.2 Products in an Advanced Stage of Development

There are many other products in advanced stages of investigation. Here we discuss a representative selection to demonstrate the suitability of PEGylation in different therapeutic areas: (i) PEG-uricase, a PEGylated enzyme that has a potentially huge application due to the prevalence of gout in Western populations; and (ii) PEG-hemoglobin, which extends the use of this oxygen carrier outside of red blood cells.

6.2.1 PEG-Uricase

Gout is characterized by persistent hyperuricemia, which results in deposition of uric acid crystals in the joint and periarticular structures and development of uric acid calculi in the kidney, causing subsequent pain. Further diseases may be associated with hyperuricemia, e.g. tumor lysis syndrome and compromised renal function, which prevents uric acid excretion.

Allopurinol, an inhibitor of the enzyme xanthine oxidase, which catalyzes the last steps of the metabolism to more soluble purines, was introduced for the treatment of gout in 1960. Some patients, however, had an exfoliative reaction to this drug. It was suggested that administration of uricase (the enzyme that hydrolyses uric acid to the more soluble allantoin that can be easily excreted by the kidney) could be a possible alternative. Uricase is not naturally present in humans and was obtained from heterologous sources such as bacteria or animals, and consequently gave rise to allergic responses.[88-91] In 2002, a recombinant version, rasburicase (Ellitek®, Sanofi-Aventis), was produced in *Saccharomyces cerevisiae* and became available in the US for the treatment of hyperuricemia caused by tumor lysis syndrome. Being a heterologous protein, however, it was associated with immunogenicity.[92]

Several researchers suggested PEGylation as a means of diminishing the immunogenicity of proteins and of improving the half-life and patient compliance.[88-91] PEG-uricase (Puricase®, Mountain View Pharmaceuticals) was finally produced by the random PEGylation of recombinant mammalian uricase (an enzyme with a primary sequence similar to that of porcine uricase) with nine polymer chains of PEG 10 kDa per subunit.[93,94] The number and size of PEG chains were chosen to preserve enzyme activity and to minimize anti-uricase antibody formation. In 2003, PEG-uricase clinical studies commenced; data from phase I and II trials demonstrated that PEG-uricase led to substantial and sustained lowering of plasma uric acid levels.[93] The most effective dose was found to be 8 mg every 2 weeks. Surprisingly, low levels of anti-PEG antibodies were detected in a few patients. This may be explained by the finding that six or seven PEG ethylene oxide units are antigenic determinants in the polymer, and/or alternatively that the terminating methoxy-groups are involved in the formation of low-affinity antibodies.[95-97]

6.2.2 Hemoglobin

Modified hemoglobin solutions are being considered for use as acellular oxygen carriers to sustain oxygen-carrying capacity and restore intravascular volume during blood loss or hemodilution. A major limitation in the use of hemoglobin is its extravasation into the interstitial spaces and subsequent scavenging of nitric oxide.[98]

The random chemical modification of hemoglobin initially studied was not a success;[99] again, it became apparent that site-specific modification was important in order to obtain reproducible and homogenous products. This was achieved by linking PEG to the free thiol cysteine (Cys^{93})[100] using PEG maleimide of 5, 10, and 20 kDa to obtain PEGylated hemoglobins with two PEG chains per tetramer. The conjugate showed a slight increase in oxygen binding without any difference between the various species.

A further site-specific modification was achieved using a PEG-aldehyde to obtain a derivative with six PEG chains. Tryptic-peptide mapping showed that the reaction was selective for the four amino termini of the protein; the remaining two chains were distributed among four lysine residues – Lys^{11} (α chain), Lys^{40} (α), Lys^{56} (α), and Lys^{8} (β chain) – which were shown by molecular modeling studies to be those most exposed to solvent.[101] This derivative showed very high oxygen binding activity compared with the Cys^{93} PEGylated protein, suggesting a role of this cysteine amino acid in oxygen binding. It is anticipated that this new class of PEGylated hemoglobin will soon be available as a potential blood substitute carrier offering a treatment option for individuals who are opposed to blood transfusions for cultural reasons.

7. Conclusions

PEGylation is a versatile technique that has proved to be important for increasing the utility of proteins as therapeutic agents. With the development of techniques to achieve site-specific PEGylation and to reduce the loss of activity that had previously limited a general application of the method, there is a huge potential for the development of PEGylated biopharmaceuticals. The ideal PEG-conjugated drug is one where the improvement of residence time and organ or cell specificity is reached without any decrease in biological activity. So far, the best balance between the pharmacokinetics and pharmacodynamics has been reached in the most recent drugs by using mono-PEGylated proteins and high-

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molecular-weight branched PEGs, such as in PEG-IFNα-2, certolizumab pegol, and pegaptanib.

Based on the excellent results obtained with PEGylation of proteins and oligonucleotides and the unique favourable properties of this polymer, one can expect that PEG modification of small peptides and organic drugs will also soon yield marketed products. Problems in this area are related to the PEG drug loading limitation, the easy loss of activity following conjugation and the difficulty in targeting. Very promising results, however, have already been obtained by Enzon Inc. in the development of camptothecin conjugation, where the addition of PEG permitted an increase in solubility and in tumor targeting by EPR.[26,102,103]

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Exhibit 6

The Facts On File

DICTIONARY of ORGANIC CHEMISTRY

Edited by
John Daintith



Facts On File, Inc.

The Facts On File Dictionary of Organic Chemistry

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forming zigzag chains and rings. This is caused by hydrogen bonds that form between the hydrogen and the highly electronegative fluoride ions. Hydrogen fluoride is used extensively as a catalyst in the petroleum industry.

hydrogen ion A positively charged hydrogen atom, H^+, i.e. a proton. Hydrogen ions are produced by all acids in water, in which they are hydrated to hydroxonium (hydronium) ions, H_3O^+. *See* acid; pH.

hydrogen peroxide (H_2O_2) A colorless syrupy liquid, usually used in solution in water. Although it is stable when pure, on contact with bases such as manganese(IV) oxide it gives off oxygen, the manganese(IV) oxide acting as a catalyst:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Hydrogen peroxide can act as an oxidizing agent, converting iron(II) ions to iron(III) ions, or as a reducing agent with potassium manganate(VII). It is used as a bleach and in rocket fuel. The strength of solutions is usually given as *volume strength* – the volume of oxygen (dm^3) at STP given by decomposition of 1 dm^3 of the solution.

hydrogensulfate (bisulfate, HSO_4^-) An acidic salt or ester of sulfuric acid (H_2SO_4), in which only one of the acid's hydrogen atoms has been replaced by a metal or organic radical. An example is sodium hydrogensulfate, $NaHSO_4$.

hydrogen sulfide (sulfuretted hydrogen, H_2S) A colorless very poisonous gas with an odor of bad eggs. Hydrogen sulfide is prepared by reacting hydrochloric acid with iron(II) sulfide. It is tested for by mixing with lead nitrate, with which it gives a black precipitate. Its aqueous solution is weakly acidic. Hydrogen sulfide reduces iron(III) chloride to iron(II) chloride, forming hydrochloric acid and a yellow precipitate of sulfur. Hydrogen sulfide precipitates insoluble sulfides, and is used in qualitative analysis. It burns with a blue flame in oxygen to form sulfur(IV) oxide and water. Natural gas contains some hydrogen sulfide, which is removed before supply to the consumer.

hydrogensulfite (bisulfite, HSO_3^-) An acidic salt or ester of sulfurous acid (H_2SO_3), in which only one of the acid's hydrogen atoms has been replaced by a metal or organic radical. An example is sodium hydrogensulfite, $NaHSO_3$.

hydrolysis A reaction between a compound and water. An example is the hydrolysis of an ESTER to give a carboxylic acid and an alcohol:

$$CH_3COOC_2H_5 + H_2O \rightleftharpoons CH_3COOH + C_2H_5OH$$

hydron The positive ion H^+. The name is used when the isotope is not relevant, i.e. a hydron could be a proton, deuteron, or triton.

hydrophilic Water attracting. *See* lyophilic.

hydrophobic Water repelling. *See* lyophobic.

hydroquinone *See* benzene-1,4-diol.

hydrosol A colloid in aqueous solution.

hydroxide A compound containing the ion OH^- or the group –OH.

hydroxonium ion *See* hydrogen ion.

hydroxybenzene *See* phenol.

hydroxybenzoate *See* salicylate.

hydroxybenzoic acid *See* salicylic acid.

2-hydroxypropanoic acid *See* lactic acid.

hydroxyl group A group (–OH) containing hydrogen and oxygen, characteristic of alcohols and phenols, and some hydroxides. It should not be confused with the hydroxide ion (OH^-).

Exhibit 7

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ORGANIC CHEMISTRY DIVISION
COMMISSION ON PHYSICAL ORGANIC CHEMISTRY*

GLOSSARY OF TERMS USED IN PHYSICAL ORGANIC CHEMISTRY

(IUPAC Recommendations 1994)

Prepared for publication by

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List of General Symbols

A	pre-exponential factor in Arrhenius equation (see *energy of activation*).
α	order of reaction with respect to reactant A, or Brønsted coefficient (see *order of reaction*, *Brønsted relation*).
β	order of reaction with respect to reactant B or Brønsted coefficient (see *order of reaction*, *Brønsted relation*).
C_p	heat capacity (at constant pressure).
e	base of natural logarithms (e = 2.718).
E_a	energy of activation (see *energy of activation*).
G	Gibbs energy.
H	enthalpy.
h	Planck constant.
K	Kelvin (unit of thermodynamic temperature).
K^o	equilibrium constant.
k	rate constant (see *order of reaction*).
k_B	Boltzmann constant.
ln	natural logarithm.
lg	decadic logarithm.
λ	wavelength.
ν	frequency.
^{o}	(superscript) relating to thermodynamic standard state.
p	pressure.
R	gas constant.
S	entropy.
s	second (unit of time).
Σ	summation.
T	thermodynamic temperature.
t	time.
v	rate of reaction (see *rate of reaction*).
‡	(superscript) relating to transition state.
$\leftrightarrow$	See *delocalization*.
[X]	amount concentration of X.

See also IUPAC QUANTITIES (1988).

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GLOSSARY OF TERMS USED IN PHYSICAL ORGANIC CHEMISTRY

2nd Edition, 1994

Synopsis. This glossary contains definitions and explanatory notes for terms used in the context of research and publications in physical organic chemistry. Its aim is to provide guidance on physical organic chemical terminology with a view to achieve a far-reaching consensus on the definitions of useful terms and on the abandonment of unsatisfactory ones. As a consequence of the development of physical organic chemistry, and of the increasing use of physical organic terminology in other fields of chemistry, the 1994 revision of the Glossary is much expanded in comparison to the previous edition, and it also includes terms from cognate fields. A few definitions have been refined, some others totally revised in the light of comments received from the scientific community.

INTRODUCTION TO THE 1994 REVISION

General Remarks

The "Glossary of Terms Used In Physical Organic Chemistry" was published in provisional form in 1979 (IUPAC PHYSICAL ORGANIC GLOSSARY (1979)) and in revised form in 1983. A historical account of procedure and progress up to that stage was outlined in the provisional publication. The 1983 revision incorporated modifications agreed by the IUPAC Commission III.2 (Physical Organic Chemistry), partly in response to comments received since the provisional publication. The terms defined in the 1983 Glossary are incorporated in the IUPAC "COMPENDIUM OF CHEMICAL TERMINOLOGY" (1987) (Victor Gold Book).

The present revision was undertaken with the long-term objective of compiling a compendium of organic chemical terminology. For this reason the criteria for inclusion of terms were much expanded. The criteria for the 1983 edition were that the meaning of a term was either not given in non-scientific dictionaries or standard textbooks or differed significantly from the definition in such reference works, or that the term was associated with some ambiguity or uncertainty with respect to its meaning. The present compendium tries to be comprehensive, but without including trivial terms and without redefining IUPAC approved terms. In particular, terms from cognate fields which are used by physical organic chemists, are included. The terminology for the various spectroscopic methods of interest to organic chemists is not treated in the present document, except for some terms from NMR spectroscopy, but may be the subject of a separate study. As a matter of policy, named reactions (such as Diels-Alder) and symbolic representation of reaction mechanisms (S_N1, etc.) have been excluded with a few exceptions. These topics have been treated in separate reports of this Commission (IUPAC REACTION MECHANISMS (1989), IUPAC TERMINOLOGY FOR TRANSFORMATIONS (1989)).

The work has been coordinated with that of other Commissions within the Division of Organic Chemistry, which deal with terminology, (e.g., IUPAC PHOTOCHEMICAL GLOSSARY (1988), and its revision (1992), Commission III.3; IUPAC TERMINOLOGY OF STEREOCHEMISTRY (1993), joint project of Commissions III.1 and III.2, and IUPAC CLASS NAMES (1993), joint project of Commissions III.1 and III.2), and with that of Commission I.5. Discrepancies in the views between the Commissions have been eliminated. Whenever there was overlap or disagreement between the 1983 glossary and one of the more recent ones, the best available definitions were incorporated in the present text with a reference to their origin. Otherwise this glossary was not expanded into areas where other Commissions or working parties are already active. It is hoped that a merged compendium will be published after approval of the various glossaries.

The aim of the Glossary is to provide guidance on physical-organic chemical terminology with a view to achieving a far-reaching consensus on the definitions of useful terms and the abandonment of unsatisfactory ones. The Commission is anxious to emphasize that it cannot (and would not wish to) impose rules or restrictions which might hinder rather than help the precise formulation of new ideas.

Generally speaking, operational definitions were preferred to definitions couched in terms of theoretical models. We have tried to avoid taking sides on issues of scientific, as distinct from semantic, disagreement.

The Commission is pleased to acknowledge the generous contributions of many scientists who helped by proposing or defining new terms, or by criticizing existing ones. The following have contributed to this revision:

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The Commission thanks Mrs. M. Wyss and Dr. S. Motallebi for their help in the preparation of the manuscript of this Glossary.

Arrangement, Abbreviations and Symbols

The arrangement is simply alphabetical, terms beginning with Greek letters following those beginning with Latin ones. Italicized words in the body of a definition, as well as those cited at the end, point to relevant cross-references. No distinction is made between singular and plural in cross-referencing. Literature references should direct the reader either to the original literature where the term was originally defined, or to pertinent references where it is used. Underlining of words means that the words should be emphasized in the particular context of the entry. The appearance of a term in quotation marks in the body of a definition indicates that no further information will be found under that heading. Capitalized names indicate references.

A cross (+) against the title of an entry implies that the Commission recommends discontinuation of the term.

In accordance with general practice Kekulé structures are normally used in this Glossary to represent the formulae of aromatic compounds. A single hexagon with a full circle inside is used in cases where delocalization is emphasized. A full circle is also used in the case of monocyclic aromatic ions, but fused aromatics are represented by Kekulé structures, because in this case each circle would imply the presence of 6 π electrons in each hexagon. Thus naphthalene with two circles would appear to have 12 π electrons, while it has only 10 in reality. Dashed lines indicate delocalization of positive or negative charge or of the spin of an unpaired electron. Curved singly headed arrows represent formal movement of electron pairs, while doubly headed arrows between formulae symbolize resonance.

In accordance with previous IUPAC recommendations (IUPAC QUANTITIES (1988)) the symbol ‡ to indicate transition states ("double dagger") is used as a prefix to the appropriate quantities, e.g. $\Delta^{\ddagger}G$ rather than the more often used $\Delta G^{\ddagger}$.

Relative positions are indicated by post-slashed Arabic numerals. For example, 1/4/dibromo-addition may refer to addition at positions 9 and 10 of anthracene, or at positions 2 and 5 of hexa-2,4-diene (IUPAC TERMINOLOGY FOR TRANSFORMATIONS (1989)).

Alphabetical Entries

abstraction

A *chemical reaction* or *transformation*, the main feature of which is the bimolecular removal of an atom (neutral or charged) from a molecular entity. For example:

$$CH_3COCH_3 + (i\text{-}C_3H_7)_2N^- \longrightarrow (CH_3COCH_2)^- + (i\text{-}C_3H_7)_2NH$$

(proton abstraction from acetone)

$$CH_4 + Cl^{\bullet} \longrightarrow H_3C^{\bullet} + HCl$$

(hydrogen abstraction from methane)

See *detachment*.

acceptor number (AN)

A quantitative measure, devised by GUTMANN (1976), of *Lewis acidity*.

acid

A *molecular entity* or *chemical species* capable of donating a hydron (proton) (see *Brønsted acid*) or capable of forming a covalent bond with an electron pair (see *Lewis acid*).

See also *hard acid*.

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acidity

(1) Of a compound:

For *Brønsted acids* it means the tendency of a compound to act as a *hydron* donor. It can be quantitatively expressed by the acid dissociation constant of the compound in water or some other specified medium. For *Lewis acids* it relates to the association constants of *Lewis adducts* and π *adducts*.

(2) Of a medium:

The use of the term is mainly restricted to a medium containing *Brønsted acids*, where it means the tendency of the medium to hydronate a specific reference base. It is quantitatively expressed by the appropriate *acidity function*.

acidity function

Any function that measures the thermodynamic *hydron*-donating or -accepting ability of a solvent system, or a closely related thermodynamic property, such as the tendency of the *lyate ion* of the solvent system to form *Lewis adducts*. (The term "basicity function" is not in common use in connection with basic solutions.) Acidity functions are not unique properties of the solvent system alone, but depend on the solute (or family of closely related solutes) with respect to which the thermodynamic tendency is measured.

Commonly used acidity functions refer to concentrated acidic or basic solutions. Acidity functions are usually established over a range of composition of such a system by UV/VIS spectrophotometric or NMR measurements of the degree of hydronation (protonation or Lewis adduct formation) for the members of a series of structurally similar indicator bases (or acids) of different strength: the best known of these functions is the Hammett acidity function H_0 (for uncharged indicator bases that are primary aromatic amines). For detailed information on other acidity functions, on the evaluation of acidity functions, and on the limitations of the concept, see ROCHESTER (1970), COX and YATES (1983).

HAMMETT (1940, 1970).

activated complex

An activated complex, often characterized by the superscript ‡, is defined as that assembly of atoms which corresponds to an arbitrary infinitesimally small region at or near the col (saddle point) of a potential energy surface.

See also *transition state*.

activation energy

See *energy of activation*.

addend

See *addition* reaction.

addition

(1) Refers to *addition reaction* or addition *transformation*.

(2) Loosely, the formation of an *adduct*. (For an example, see *Lewis acid*.)

(3) Loosely, any *association* or *attachment*.

addition reaction

A *chemical reaction* of two or more reacting *molecular entities*, resulting in a single reaction product containing all atoms of all components, with formation of two chemical bonds and a net reduction in bond multiplicity in at least one of the reactants. The reverse process is called an *elimination* reaction. The addition may occur at only one site (α-*addition*, 1/1/addition), at two adjacent sites (1/2/addition) or at two non-adjacent sites (1/3/- or 1/4/addition, etc.). For example

$$\text{(a) } H^+ + Br^- + (CH_3)_2C{=}CH_2 \longrightarrow (CH_3)_2CBr\text{-}CH_3 \quad \text{(1/2/addition)}$$

$$\text{(b) } Br_2 + CH_2{=}CH\text{-}CH{=}CH_2 \longrightarrow BrCH_2\text{-}CH{=}CH\text{-}CH_2Br \quad \text{(1/4/addition)}$$

$$\text{and } BrCH_2\text{-}CH(Br)\text{-}CH{=}CH_2 \quad \text{(1/2/addition)}$$

If the reagent or the source of the addends of an addition are not specified, then it is called an addition *transformation*.

See also *addition*, α-*addition*, *cheletropic reaction*, *cycloaddition*.

additivity principle

The hypothesis that each of several structural features of a *molecular entity* makes a separate and additive contribution to a property of the substance concerned. More specifically, it is the hypothesis that each of the several *substituent* groups in a parent molecule makes a separate and additive contribution to the standard Gibbs energy change (or *Gibbs energy of activa-*

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tion) corresponding to a particular equilibrium (or *rate of reaction*). For further information and examples see BENSON (1976).

See also *transferability*.

adduct

A new *chemical species* AB, each *molecular entity* of which is formed by direct combination of two separate molecular entities A and B in such a way that there is change in *connectivity*, but no loss, of atoms within the *moieties* A and B. Stoichiometries other than 1:1 are also possible, e.g. a bis-adduct (2:1). An "*intramolecular* adduct" can be formed when A and B are *groups* contained within the same molecular entity.

This is a general term which, whenever appropriate, should be used in preference to the less explicit term *complex*. It is also used specifically for products of an *addition reaction*. For examples see *Lewis adduct*, *Meisenheimer adduct*, *π-adduct*.

***A*-factor**

See *energy of activation*.

agostic

The term designates structures in which a hydrogen atom is bonded to both a carbon atom and a metal atom. The term is also used to characterize the interaction between a CH bond and an unsaturated metal centre, and to describe similar bonding of a transition metal with Si-H compounds. The expression "μ-hydrido-bridged" is also used to describe the bridging hydrogen.

BROOKHART and GREEN (1983); LUO and CRABTREE (1989).

alcoholysis

See *solvolysis*.

allylic substitution reaction

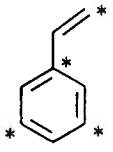
A *substitution reaction* occurring at position 1/ of an allylic system, the double bond being between positions 2/ and 3/. The incoming group may be attached to the same atom 1/ as the *leaving group*, or the incoming group becomes attached at the relative position 3/, with movement of the double bond from 2/3 to 1/2. For example:

(written as a *transformation*).

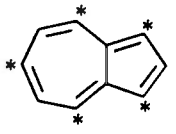
alternant

A *conjugated system* of π-electrons is termed alternant if its atoms can be divided into two sets so that no atom of one set is directly linked to any other atom of the same set.

Example of alternant π system:



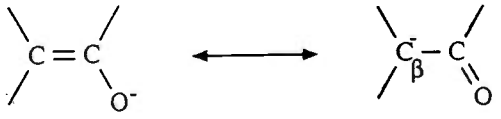
Example of non-alternant π system:



(two atoms of unstarred set are directly linked)

ambident

A description applied to a *chemical species* whose *molecular entities* each possess two alternative and strongly interacting distinguishable reactive centres, to either of which a *bond* may be made in a reaction: the centres must be connected in such a way that reaction at either site stops or greatly retards subsequent attack at the second site. The term is most commonly applied to *conjugated nucleophiles*, for example the enolate ion



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(which may react with *electrophiles* either at the β-carbon atom or at oxygen) or γ-pyridones, and also to the vicinally ambident cyanide ion, cyanate ion, thiocyanate ion, sulfinate ion, nitrite ion, and unsymmetrical hydrazines. Ambident electrophiles are exemplified by carboxylic esters $RC(O)OCR_3$ which react with nucleophiles either at the carbonyl carbon or the alkoxy carbon.

Molecular entities, such as dianions of dicarboxylic acids, containing two non-interacting (or feebly interacting) reactive centres, are not generally considered to be ambident and are better described as "bifunctional".

The Latin root of the word implies two reactive centres, but the term has in the past also incorrectly been applied to chemical species with more than two reactive centres. For such species the existing term "polydent" (or, better, "multident") is more appropriate.

GOMPPER (1964); SMITH and DeWALL (1977).

See also *chelation*.

amphiphilic

A compound containing a large organic cation or anion which possesses a long unbranched hydrocarbon chain, e.g.

$$H_3C(CH_2)_nCO_2^-M^+ \quad H_3C(CH_2)_nSO_3^-M^+ \quad H_3C(CH_2)_nN(CH_3)_3^+X^- \qquad (n > 7).$$

The existence of distinct polar (hydrophilic) and non polar (hydrophobic) regions in the molecule promotes the formation of *micelles* in dilute aqueous solution.

amphiprotic (solvent)

Self-ionizing solvent possessing both characteristics of *Brønsted acids* and *bases*, for example H_2O and CH_3OH, in contrast to *aprotic solvent*.

amphoteric

A *chemical species* that behaves both as an acid and as a base is called amphoteric. This property depends upon the medium in which the species is investigated: H_2SO_4 is an acid when studied in water, but becomes amphoteric in *superacids*.

anchimeric assistance

See *neighbouring group participation*.

anionotropic rearrangement (or anionotropy)

A rearrangement in which the migrating group moves with its electron pair from one atom to another.

anion radical

See *radical ion*.

annelation

Alternative, but less desirable term for *annulation*. The term is widely used in German and French language.

annulation

A *transformation* involving fusion of a new ring to a molecule via two new bonds.

Some authors use the term "annelation" for the fusion of an additional ring to an already existing one, and "annulation" for the formation of a ring from one or several acyclic precursors, but this distinction is not made generally.

DANHEISER, GERE and SARD (1982), JUNG (1976).

See also *cyclization*.

annulene

Mancude (i.e. having formally the **ma**ximum number of **non**cu**m**ulative **d**ouble bonds) monocyclic hydrocarbon without side chains of the general formula C_nH_n (n is an even number) or C_nH_{n+1} (n is an odd number). Note that in systematic nomenclature an annulene with seven or more carbon atoms may be named [n]annulene, where n is the number of carbon atoms, e.g. [9]annulene for cyclonona-1,3,5,7-tetraene.

IUPAC CLASS NAMES (1993).

See *aromatic, Hückel (4n + 2) rule*.

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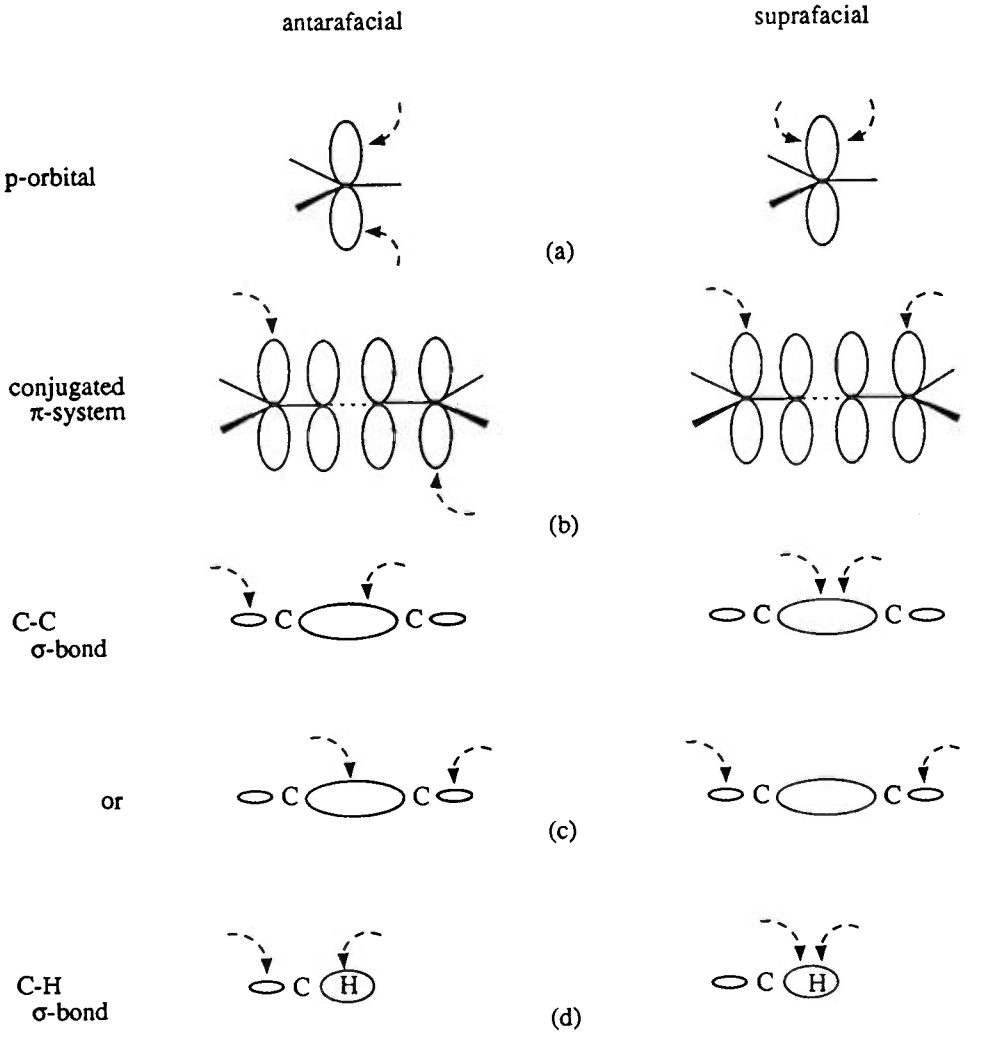
antarafacial, suprafacial

When a part of a molecule ("molecular fragment") undergoes two changes in bonding (*bond*-making or bond-breaking), either to a common centre or to two related centres, external to itself, these bonding changes may be related in one of two spatially different ways. These are designated as "antarafacial" if opposite faces of the molecular fragment are involved, and "suprafacial" if both changes occur at the same face. The concept of "face" is clear from the diagrams in the cases of planar (or approximately planar) frameworks with isolated or interacting p-orbitals (Figs. a and b below).

The terms antarafacial and suprafacial are, however, also employed in cases in which the essential part of the molecular fragment undergoing changes in bonding comprises two atoms linked only by a σ-bond. In these cases it is customary to refer to the phases of the local σ-bonding orbital: occurrence of the two bonding changes at sites of like orbital phase is regarded as suprafacial, whereas that at two sites of opposite phase is antarafacial. The possibilities are shown for C-C and C-H σ-bonds in Figs. c and d. There may be two distinct and alternative stereochemical outcomes of a suprafacial process involving a σ-bond between saturated carbon atoms, i.e. either retention or inversion at both centres. The antarafacial process results in inversion at one centre and retention at the second.

For examples of the use of these terms see *cycloaddition*, *sigmatropic rearrangement*.

See also *anti*, *sigma*, *pi*.



anti

In the representation of *stereochemical* relationships "anti" means "on opposite sides" of a reference plane, in contrast to "syn" which means "on the same side", as in the following examples.

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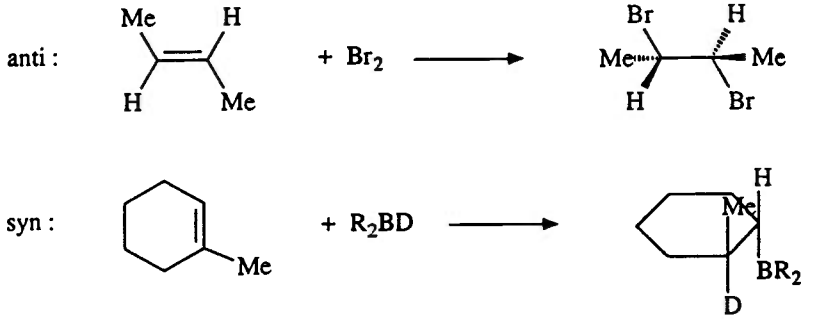
(A) Two substituents attached to atoms joined by a single *bond* are anti if the torsion angle (dihedral angle) between the bonds to the substituents is greater than 90°, or syn if it is less than 90°. (A further distinction is made between antiperiplanar, synperiplanar, anticlinal and synclinal.)

IUPAC ORGANIC RULES (1979); IUPAC STEREOCHEMICAL TERMINOLOGY (1993); KLYNE and PRELOG (1960).

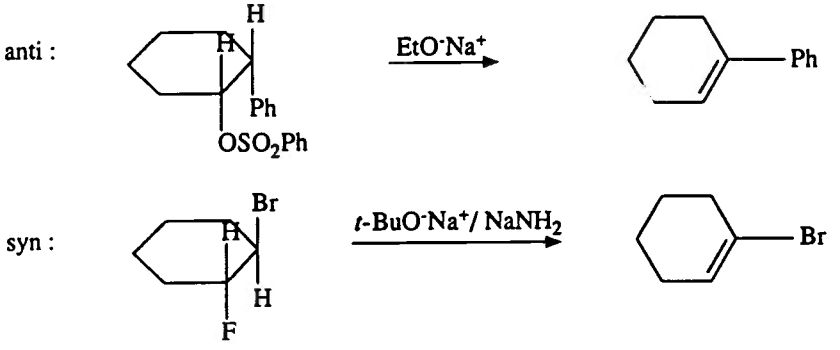
(B) In the older literature the terms anti and syn were used to designate stereoisomers of oximes and related compounds. That usage was superseded by the terms "trans" and "cis" or *E* and *Z*, respectively.

(C) When the terms are used in the context of *chemical reactions* or *transformations*, they designate the relative orientation of substituents in the substrate or product:

(1) Addition to a carbon-carbon double bond:



(2) Alkene-forming elimination:



IUPAC ORGANIC RULES (1979).

In the examples described under (1) and (2) anti processes are always *antarafacial*, and syn processes are *suprafacial*. See also IUPAC STEREOCHEMICAL TERMINOLOGY (1993) for further meaning of the term.

antiaromatic

See *aromatic*.

anti-Hammond effect

See *More O'Ferrall-Jencks diagram*.

anti-Markownikoff addition

See *Markownikoff rule*.

aprotic (solvent)

Non-*protogenic* (in a given situation). (With extremely strong *Brønsted acids* or *bases*, solvents that are normally aprotic may accept or lose a proton. For example, acetonitrile is in most instances an aprotic solvent, but it is *protophilic* in the presence of concentrated sulfuric acid and *protogenic* in the presence of potassium *tert*-butoxide. Similar considerations apply to benzene, trichloromethane, etc.)

See also *dipolar aprotic solvent*.

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aquation

The incorporation of one or more integral molecules of water into another species with or without displacement of one or more other atoms or groups. For example the incorporation of water into the inner *ligand* sphere of an inorganic *complex* is an aquation reaction.

See also *hydration*.

aromatic, aromaticity

(1) In the traditional sense, "having a chemistry typified by benzene".

(2) A cyclically *conjugated molecular entity* with a stability (due to *delocalization*) significantly greater than that of a hypothetical localized structure (e.g. *Kekulé structure*) is said to possess aromatic character. If the structure is of higher energy (less stable) than such a hypothetical classical structure, the molecular entity is "antiaromatic".

The most widely used method for determining aromaticity is the observation of diatropicity in the ^1H NMR spectrum.

ATKINS (1974); GARRATT (1986).

See also *Hückel (4n + 2) rule, Möbius aromaticity*.

(3) The terms aromatic and antiaromatic have been extended to describe the stabilization or destabilization of *transition states* of *pericyclic reactions*. The hypothetical reference structure is here less clearly defined, and use of the term is based on application of the *Hückel (4n + 2) rule* and on consideration of the topology of orbital overlap in the transition state. Reactions of molecules in the *ground state* involving antiaromatic transition states proceed, if at all, much less easily than those involving aromatic transition states.

DEWAR (1971); ZIMMERMANN (1971).

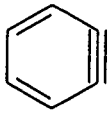
Arrhenius equation

See *energy of activation*.

aryne

A hydrocarbon derived from an arene by abstraction of two hydrogen atoms from adjacent carbon atoms; thus 1,2-didehydroarene. Arynes are commonly represented with a formal triple bond. The analogous heterocyclic compounds are called heteroarynes or hetarynes.

E.g., benzyne



Arynes are usually transient species.

IUPAC CLASS NAMES (1993).

association

The assembling of separate *molecular entities* into any aggregate, especially of oppositely charged free ions into *ion pairs* or larger and not necessarily well-defined clusters of ions held together by electrostatic attraction. The term signifies the reverse of *dissociation*, but is not commonly used for the formation of definite *adducts* by *colligation* or *coordination*.

asymmetric induction

The traditional term describing the preferential formation in a *chemical reaction* of one *enantiomer* or *diastereoisomer* over the other as a result of the influence of a chiral feature in the substrate, reagent, *catalyst* or environment. The term also refers to the formation of a new chiral feature preferentially in one *configuration* under such influence.

IUPAC STEREOCHEMICAL TERMINOLOGY (1993).

atomic orbital

A one-electron wavefunction describing an electron in the effective field provided by a nucleus and the other electrons present.

See also *molecular orbital*.

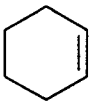
attachment

A *transformation* by which one *molecular entity* (the *substrate*) is converted into another by the formation of one (and only one) two-centre *bond* between the substrate and another molecular entity and which involves no other changes in *connectivity* in the substrate. For example, the formation of an acyl cation by attachment of carbon monoxide to a *carbenium ion* (R^+):

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$$R^+ + CO \longrightarrow (RCO)^+$$

The product of an attachment may also be the *adduct* of the two reactants, but not all adducts can be represented as the products of an attachment. (For example, the Diels-Alder *cycloaddition*

$$CH_2{=}CH\text{-}CH{=}CH_2 + H_2C{=}CH_2 \longrightarrow$$ 

results in an adduct of buta-1,3-diene and ethene, but the reaction cannot be described as an attachment since bonds are formed between more than two centres.)

See also *colligation*, *electron attachment*.

autocatalytic reaction

A *chemical reaction* in which a product (or a reaction *intermediate*) also functions as *catalyst*. In such a reaction the observed *rate of reaction* is often found to increase with time from its initial value.

See *order of reaction*.

automerization

Synonymous with *degenerate rearrangement*.

autoprotolysis

A *proton* (hydron) *transfer* reaction between two identical molecules (usually a solvent), one acting as a *Brønsted acid* and the other as a *Brønsted base*. For example:

$$2H_2O \longrightarrow H_3O^+ + OH^-$$

autoprotolysis constant

The product of the activities (or, more approximately, concentrations) of the species produced as the result of *autoprotolysis*. For solvents in which no other ionization processes are significant the term is synonymous with "ionic product". The autoprotolysis constant for water, K_w, is equal to the product of activities

$$a(H_3O^+)a(OH^-) = 1.0 \times 10^{-14} \text{ at } 25\ ^\circ C.$$

"A" value

The conformational preference of an equatorial compared to an axial substituent in a monosubstituted cyclohexane. This steric substituent parameter equals $\Delta_r G^\circ$ in kcal/mol for the equatorial to axial equilibration on cyclohexane. The values are also known as "Winstein-Holness" A values.

HIRSCH (1967); CAREY and SUNDBERG (1990).

+ azacarbene

See *nitrene*.

+ azene

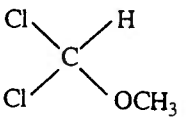
See *nitrene*.

+ azylene

See *nitrene*.

α-addition (alpha-addition)

A *chemical reaction* resulting in a single reaction product from two or three reacting chemical species, with formation of two new chemical *bonds* to the same atom in one of the reactant *molecular entities*. The synonymous term 1/1/addition is also used. For example:

$$Cl_2C{:} + CH_3OH \longrightarrow$$ 

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(This particular example can also be viewed as an *insertion reaction*.) In inorganic chemistry such α-addition reactions, generally to a metallic central atom, are known as "oxidative additions".

α-Addition is the reverse of *α-elimination* or 1/1/elimination.

See also *addition, elimination*.

α-effect

A positive deviation of an α-nucleophile (a nucleophile bearing an unshared pair of electrons on an atom adjacent to the nucleophilic site) from a Brønsted-type plot of log k_{nuc} *vs.* pK_a constructed for a series of related normal nucleophiles. More generally, it is the influence of the atom bearing a lone pair of electrons on the reactivity at the adjacent site.

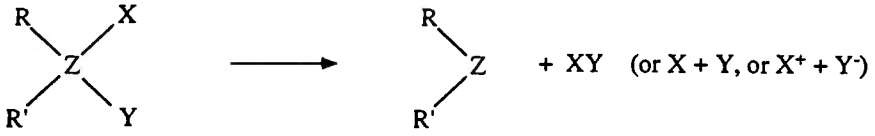
HOZ and BUNCEL (1985).

See also *Brønsted relation*.

The use of the term has been extended to include the effect of any substituent on an adjacent reactive centre, for example in the case of the "α-silicon effect".

α-elimination

A *transformation* of the general type



where the central atom Z is commonly carbon. The reverse reaction is called *α-addition*.

Baldwin's rules

A set of empirical rules for certain formations of 3- to 7-membered rings. The predicted pathways are those in which the length and nature of the linking chain enables the terminal atoms to achieve the proper geometries for reaction. The disfavoured cases are subject to severe distortions of bond angles and bond distances.

BALDWIN (1976).

base

A *chemical species* or *molecular entity* having an available pair of electrons capable of forming a *covalent bond* with a *hydron* (proton) (see *Brønsted base*) or with the vacant orbital of some other species (see *Lewis base*).

See also *hard base, superbase*.

basicity

For *Brønsted bases* it means the tendency of a compound to act as *hydron* (proton) acceptor. The basicity of a *chemical species* is normally expressed by the *acidity* of the conjugate acid (see *conjugate acid-base pair*). For *Lewis bases* it relates to the association constants of *Lewis adducts* and *π-adducts*.

basicity function

See *acidity function*.

bathochromic shift (effect)

Shift of a spectral band to lower frequencies (longer wavelengths) owing to the influence of substitution or a change in environment. It is informally referred to as a red shift and is opposite to *hypsochromic shift* (blue shift).

IUPAC PHOTOCHEMICAL GLOSSARY (1992).

Bell-Evans-Polanyi principle

The linear relation between *energy of activation* (E_a) and enthalpy of reaction (ΔH_r) sometimes observed within a series of closely related reactions.

$$E_a = A + B\Delta H_r$$

DEWAR (1969), JENCKS (1985).

benzyne

1,2-Didehydrobenzene (the *aryne* derived from benzene) and its derivatives formed by substitution. The terms *m*- and *p*-benzyne are occasionally used for 1,3- and 1,4-didehydrobenzene, respectively.

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bifunctional catalysis

Catalysis (usually for *hydron* transfer) by a bifunctional *chemical species* involving a mechanism in which both *functional groups* are implicated in the *rate-controlling step*, so that the corresponding *catalytic coefficient* is larger than that expected for catalysis by chemical species containing only one of these functional groups.

The term should not be used to describe the *concerted* action of two different catalysts ("concerted catalysis").

bimolecular

See *molecularity*.

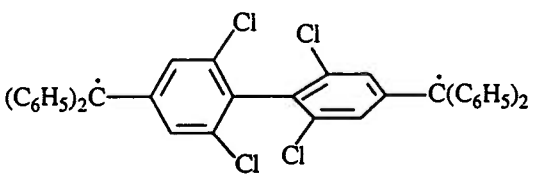
binding site

A specific region (or atom) in a *molecular entity* that is capable of entering into a stabilizing interaction with another molecular entity. An example of such an interaction is that of an active site in an enzyme with its *substrate*. Typical forms of interaction are by *hydrogen bonding*, *coordination*, and *ion pair* formation.

Two binding sites in different molecular entities are said to be complementary if their interaction is stabilizing.

biradical

An even-electron *molecular entity* with two (possibly delocalized) radical centres which act nearly independently of each other, e.g.



Species in which the two radical centres interact significantly are often referred to as "biradicaloids". If the two radical centres are located on the same atom, the species are more properly referred to by their generic names: carbenes, nitrenes, etc.

The lowest-energy triplet state of a biradical lies below or at most only a little above its lowest singlet state (usually judged relative to k_BT., the product of the Boltzmann constant k_B and the absolute temperature T). The states of those biradicals whose radical centres interact particularly weakly are most easily understood in terms of a pair of local doublets.

Theoretical descriptions of low-energy states of biradicals display the presence of two unsaturated valences (biradicals contain one fewer bond than permitted by the rules of valence): the dominant valence bond structures have two dots, the low energy molecular orbital *configurations* have only two electrons in two approximately nonbonding molecular orbitals, two of the natural orbitals have occupancies close to one, etc.

The term is synonymous with "diradical".

IUPAC PHOTOCHEMICAL GLOSSARY (1993).

See also *carbene*, *nitrene*.

Bodenstein approximation

See *steady state*.

bond

There is a chemical bond between two atoms or groups of atoms in case that the forces acting between them are such as to lead to the formation of an aggregate with sufficient stability to make it convenient for the chemist to consider it as an independent "molecular species". In the context of this Glossary, the term refers usually to the *covalent bond*.

PAULING (1967).

See also *agostic*, *coordination*, *hydrogen bond*, *multi-centre bond*.

bond dissociation

See *heterolysis*, *homolysis*.

(In ordinary usage the term refers to homolysis only.)

bond-dissociation energy, D (SI unit: kJ mol^{-1}, or J (per molecule))

The enthalpy (per mole) required to break a given *bond* of some specific *molecular entity* by *homolysis*, e.g. for $CH_4 \rightarrow H_3C^{\cdot} + H^{\cdot}$, symbolized as $D(CH_3\text{-}H)$ (cf. *heterolytic bond dissociation energy*).

See also *bond energy*.

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bond energy (mean bond energy)

The average value of the gas-phase *bond dissociation energies* (usually at a temperature of 298 K) for all *bonds* of the same type within the same *chemical species*. The mean bond energy for methane, for example, is one-fourth the enthalpy of reaction for

$$CH_4(g) \longrightarrow C(g) + 4H(g)$$

Tabulated bond energies are generally values of bond energies averaged over a number of selected typical chemical species containing that type of bond.

bond migration

See *migration*.

bond number

The number of electron-pair *bonds* between two nuclei in any given *Lewis formula*. For example in ethene the bond number between the carbon atoms is two, and between the carbon and hydrogen atoms is one.

bond order

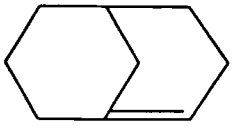
A theoretical index of the degree of bonding between two atoms relative to that of a normal single bond, i.e. the bond provided by one localized electron pair. In the valence-bond theory it is a weighted average of the bond numbers between the respective atoms in the *contributing structures*. In molecular-orbital theory it is calculated from the weights of the atomic orbitals in each of the occupied molecular orbitals. For example, in valence-bond theory (neglecting other than Kekulé structures) the bond order between adjacent carbon atoms in benzene is 1.5; in Hückel molecular orbital theory it is 1.67. Other variants of molecular orbital theory provide other values for bond orders.

borderline mechanism

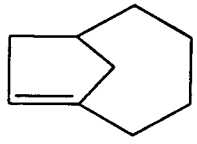
A mechanism intermediate between two extremes, for example a nucleophilic substitution intermediate between S_N1 and S_N2, or intermediate between electron transfer and S_N2.

Bredt's rule

A double bond cannot be placed with one terminus at the bridgehead of a bridged ring system unless the rings are large enough to accommodate the double bond without excessive *strain*. For example, while bicyclo[2.2.1]hept-1-ene is only capable of existence as a *transient*, its higher homologues having a double bond at the bridgehead position have been isolated: e.g.



Bicyclo[3.3.1]non-1-ene



Bicyclo[4.2.1]non-1(8)-ene

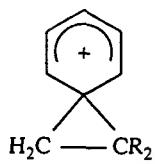
BREDT (1924); see also FAWCETT (1950); WISEMAN (1967); KEESE (1972).
For an alternative formulation see WISEMAN and CHONG (1969).

bridged carbocation

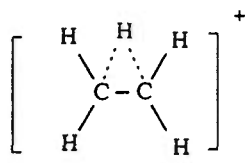
A *carbocation* (real or hypothetical) in which there are two (or more) carbon atoms that could in alternative *Lewis formulae* be designated as *carbenium centres* but which is instead represented by a structure in which a *group* (a hydrogen atom or a hydrocarbon residue, possibly with substituents in non-involved positions) bridges these potential carbenium centres. One may distinguish "electron-sufficient bridged carbocations" and "electron-deficient bridged carbocations". Examples of the former are phenyl-bridged ions (for which the trivial name "phenonium ion" has been used), such as (A). These ions are straightforwardly classified as *carbenium ions*. The latter type of ion necessarily involves three-centre bonding. Structures (C) and (D) contain five-coordinate carbon atoms. The "hydrogen-bridged carbocation" (B) contains a two-co-ordinate hydrogen atom. Hypercoordination, which includes two-coordination for hydrogen and five- but also higher coordination for carbon is generally observed in bridged carbocations.

OLAH, SURYA PRAKASH, WILLIAMSON, FIELD, and WADE (1987).
See also *carbonium ion, multi-centre bond, neighbouring group participation*.

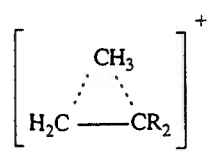
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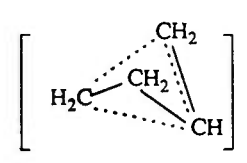
(A)



(B)



(C)



(D)

bridging ligand

A *ligand* attached to two or more, usually metallic, central atoms.
IUPAC INORGANIC NOMENCLATURE (1990).

Brønsted acid (Brönsted acid)

A *molecular entity* capable of donating a *hydron* (proton) to a base, (i.e. a "hydron donor") or the corresponding *chemical species*. For example: H_2O, H_3O^+, CH_3CO_2H, H_2SO_4, HSO_4^-, HCl, CH_3OH, NH_3.
See also *conjugate acid-base pair.*

Brønsted base (Brönsted base)

A *molecular entity* capable of accepting a *hydron* (proton) from an acid (i.e. a "hydron acceptor") or the corresponding *chemical species*. For example: OH^-, H_2O, $CH_3CO_2^-$, HSO_4^-, SO_4^{2-}, Cl^-.
See also *conjugate acid-base pair.*

Brønsted relation (Brönsted relation)

The term applies to either of the equations

$$k_{HA}/p = G(K_{HA}q/p)^{\alpha}$$

$$k_A/q = G(K_{HA}q/p)^{-\beta}$$

(or their logarithmic forms) where α, β and G are constants for a given reaction series (α and β are called "Brønsted exponents"), k_{HA} and k_A are *catalytic coefficients* (or rate coefficients) of reactions whose rates depend on the concentrations of HA and/or of A^-. K_{HA} is the acid dissociation constant of the acid HA, p is the number of equivalent acidic protons in the acid HA, and q is the number of equivalent basic sites in its conjugate base A^-. The chosen values of p and q should always be specified. (The charge designations of HA and A^- are only illustrative.)

The Brønsted relation is often termed the "Brønsted *catalysis* law" (or the "Catalysis Law"). Although justifiable on historical grounds, this name is not recommended, since Brønsted relations are known to apply to many uncatalysed and pseudo-catalysed reactions (such as simple proton (*hydron*) *transfer reactions*). The term "pseudo-Brønsted relation" is sometimes used for reactions which involve *nucleophilic catalysis* instead of acid-base catalysis. Various types of Brønsted parameters have been proposed such as β_{lg}, β_{nuc}, β_{eq} for leaving group, nucleophile and equilibrium constants, respectively.
See also *linear free-energy relation.*

Bunnett-Olsen equations

The equations for the relation between $\lg([SH^+]/[S]) + H_0$ and $H_0 + \lg [H^+]$ for base S in aqueous mineral acid solution, where H_0 is Hammett's *acidity function* and $H_0 + \lg[H^+]$ represents the activity function $\lg(\gamma_S\gamma_{H^+})/\gamma_{SH^+}$ for the nitroaniline reference bases to build H_0.

$$\lg([SH^+]/[S]) - \lg[H^+] = (\Phi - 1)(H_0 + \lg[H^+]) + pK_{SH^+}$$

$$\lg([SH^+]/[S]) + H_0 = \Phi(H_0 + \lg[H^+]) + pK_{SH^+}$$

BUNNETT and OLSEN (1966); HAMMETT (1970).
See also *Cox-Yates equation.*

cage

An aggregate of molecules, generally in the condensed phase, that surrounds the fragments formed, for example, by thermal or photochemical dissociation. Because the cage hinders the separation of the fragments by diffusion, they may preferentially react with one another ("cage effect") but not necessarily to re-form the precursor species. For example,

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$$R\text{-}N{=}N\text{-}R \xrightarrow{\Delta} [R^{\cdot} + N \equiv N + R^{\cdot}]_{cage} \longrightarrow R\text{-}R + N_2$$

See also *geminate recombination.*

cage compound

A polycyclic compound having the shape of a cage (see OLAH, 1990). The term is also used for *inclusion compounds.*

canonical form

See *contributing structure.*

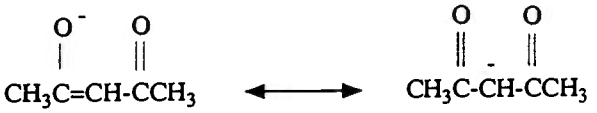
captodative effect

Effect on the stability of a carbon centred *radical* determined by the combined action of a captor (electron withdrawing) and a dative (electron releasing) substituent, both attached to the radical centre. The term is also used for certain unsaturated compounds.

BORDWELL and LYNCH (1989); SUSTMANN and KORTH (1990); VIEHE, JANOUSEK, MERENY, and STELLA (1985).

carbanion

Generic name for anions containing an even number of electrons and having an unshared pair of electrons on a tervalent carbon atom (e.g. Cl_3C^- or $HC{\equiv}C^-$) or - if the ion is mesomeric (see *mesomerism*) - having at least one significant *contributing structure* with an unshared pair of electrons on a tervalent carbon atom, for example,



See also *radical ion.*

carbene

Generic name for the species H_2C: and substitution derivatives thereof, containing an electrically neutral bivalent carbon atom with two nonbonding electrons. The nonbonding electrons may have antiparallel spins (singlet state) or parallel spins (triplet state). Use of the alternative name "methylene" as a generic term is not recommended.

See also *biradical.*

carbenium centre

The three-coordinate carbon atom in a *carbenium ion* to which the excess positive charge of the ion (other than that located on heteroatoms) may be formally considered to be largely attributed, i.e., which has one vacant p-orbital. (N.B. It is not always possible to uniquely identify such an atom.) This formal attribution of charge often does not express the real charge distribution.

carbenium ion

A generic name for *carbocations*, real or hypothetical, that have at least one important *contributing structure* containing a tervalent carbon atom with a vacant p-orbital. (The name implies a protonated carbene or a substitution derivative thereof.)

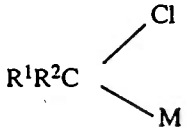
The term was proposed (and rejected) as a replacement for the traditional usage of the name *carbonium ion.*

To avoid ambiguity, the name should not be used as the root for the systematic nomenclature of carbocations. The corresponding difficulty confused carbonium ion nomenclature for many years. For example, the term "ethylcarbonium ion" has at times been used to refer either to $CH_3CH_2^+$ (ethyl cation) or (correctly) to $CH_3CH_2CH_2^+$ (propyl cation).

For the nomenclature of carbenium ions see IUPAC NOMENCLATURE GUIDE (1993).

carbenoid

A *carbene* like *chemical species* but with properties and *reactivity* differing from the free carbene itself, e.g.



M = metal

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carbocation

A cation containing an even number of electrons with a significant portion of the excess positive charge located on one or more carbon atoms. This is a general term embracing *carbenium ions*, all types of *carbonium ions*, vinyl cations, etc. Carbocations may be named by adding the word "cation" to the name of the corresponding *radical* [IUPAC ORGANIC RULES (1979), IUPAC NOMENCLATURE GUIDE (1993).] Such names do not imply structure (e.g. whether three-coordinated or five-coordinated carbon atoms are present).

OLAH and SCHLEYER (1972).

See also *bridged carbocation*, *radical ion*.

carbonium ion

The term should be used with great care since several incompatible meanings are currently in use. It is not acceptable as the root for systematic nomenclature for *carbocations*.

(1) In most of the existing literature the term is used in its traditional sense for what is here defined as *carbenium ion*.

(2) A carbocation, real or hypothetical, that contains at least one five-coordinate carbon atom.

(3) A carbocation, real or hypothetical, whose structure cannot adequately be described by two-electron two-centre *bonds* only. (The structure may involve carbon atoms with a *coordination number* greater than five.)

OLAH and SCHLEYER (1972).

carbyne

Generic name for the species

$$HC\dot{:}$$

and substitution derivatives thereof, such as

$$EtO_2C\text{-}C\dot{:}$$

containing an electrically neutral univalent carbon atom with three non-bonding electrons. Use of the alternative name "methylidyne" as a generic term is not recommended.

catalysis

The action of a *catalyst*.

catalysis law

See *Brønsted relation*.

catalyst

A substance that participates in a particular *chemical reaction* and thereby increases its rate but without a net change in the amount of that substance in the system. At the molecular level, the catalyst is used and regenerated during each set of *microscopic chemical events* leading from a *molecular entity* of reactant to a molecular entity of product.

See also *autocatalytic reaction*, *bifunctional catalysis*, *catalytic coefficient*, *electron-transfer catalysis*, *general acid catalysis*, *general base catalysis*, *intramolecular catalysis*, *micellar catalysis*, *Michaelis-Menten kinetics*, *phase-transfer catalysis*, *pseudo-catalysis*, *rate of reaction*, *specific catalysis*.

catalytic coefficient

If the *rate of reaction* (v) is expressible in the form

$$v = (k_0 + \Sigma k_i[C_j]^{n_i})\,[A]^{\alpha}\,[B]^{\beta}\ldots$$

where A, B, ... are reactants and C_i represents one of a set of catalysts, then the proportionality factor k_i is the catalytic coefficient of the particular *catalyst* C_i. Normally the *partial order* of reaction (n_i) with respect to a catalyst will be unity, so that k_i is an $(\alpha+\beta+\ldots+1)$th order rate coefficient. The proportionality factor k_0 is the $(\alpha+\beta+\ldots)$th order rate coefficient of the uncatalysed component of the total reaction.

cation radical

See *radical ion*.

cationotropic rearrangement (cationotropy)

See *tautomerism*.

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chain reaction

A reaction in which one or more reactive reaction *intermediates* (frequently radicals) are continuously regenerated, usually through a repetitive cycle of elementary steps (the "propagation step"). For example, in the chlorination of methane by a radical *mechanism*, Cl· is continuously regenerated in the chain propagation steps:

$$Cl^{\cdot} + CH_4 \longrightarrow HCl + H_3C^{\cdot} \qquad \text{Propagation steps}$$

$$H_3C^{\cdot} + Cl_2 \longrightarrow CH_3Cl + Cl^{\cdot}$$

In chain polymerization reactions, reactive intermediates of the same types, generated in successive steps or cycles of steps, differ in relative molecular mass, as in

$$RCH_2\dot{C}HPh + H_2C{=}CHPh \longrightarrow RCH_2CHPhCH_2\dot{C}HPh$$

See also *chain transfer*, *initiation*, *termination*.

chain transfer

The abstraction, by the *radical* end of a growing chain-polymer, of an atom from another molecule. The growth of the polymer chain is thereby terminated but a new radical, capable of chain propagation and polymerization, is simultaneously created. For the example of alkene polymerization cited for a *chain reaction*, the reaction

$$RCH_2\dot{C}HPh + CCl_4 \longrightarrow RCH_2CHClPh + Cl_3C^{\cdot}$$

represents a chain transfer, the radical $Cl_3C^{\cdot}$ inducing further polymerization:

$$CH_2{=}CHPh + Cl_3C^{\cdot} \longrightarrow Cl_3CCH_2\dot{C}HPh$$

$$Cl_3CCH_2\dot{C}HPh + CH_2{=}CHPh \longrightarrow Cl_3CCH_2CHPhCH_2\dot{C}HPh \quad \text{etc.}$$

The phenomenon occurs also in other chain reactions such as cationic polymerization.
See also *telomerization*.

charge density

See *electron density*.

charge population

The net electric charge on a specified atom in a *molecular entity*, as determined by some prescribed definition, e.g. that of MULLIKEN (1955).
See also *electron density*.

charge-transfer complex

A ground state *adduct* which exhibits an observable charge transfer absorption band.
IUPAC PHOTOCHEMICAL GLOSSARY (1992).

chelation

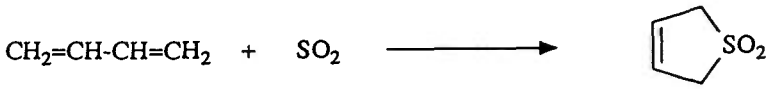
The formation or presence of *bonds* (or other attractive interactions) between two or more separate *binding sites* within the same ligand and a single central atom. A *molecular entity* in which there is chelation (and the corresponding *chemical species*) is called a "chelate". The terms bidentate (or didentate), tridentate, tetradentate... multidentate are used to indicate the number of potential binding sites of the ligand, at least two of which must be used by the ligand in forming a "chelate". For example, the bidentate ethylenediamine forms a chelate with CuI in which both nitrogen atoms of ethylenediamine are bonded to copper. (The use of the term is often restricted to metallic central atoms.)

The phrase "separate binding sites" is intended to exclude cases such as $[PtCl_3(CH_2{=}CH_2)]^-$, ferrocene, and (benzene)tricarbonylchromium in which ethene, the cyclopentadienyl group, and benzene, respectively, are considered to present single binding sites to the respective metal atom, and which are not normally thought of as chelates (see *hapto*).
See also *cryptand*.

cheletropic reaction

A form of *cycloaddition* across the terminal atoms of a fully *conjugated system* with formation of two new σ-bonds to a single atom of the ("monocentric") reagent. There is formal loss of one π-bond in the substrate and an increase in *coordination number* of the relevant atom of the reagent. An example is the *addition* of sulfur dioxide to butadiene:

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The reverse of this type of reaction is designated "cheletropic elimination". WOODWARD and HOFFMANN (1969).

chelotropic reaction

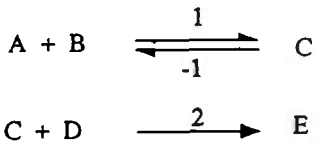
Alternative (and etymologically more correct) name for *cheletropic reaction*. DEWAR (1971).

chemical flux

A concept related to *rate of reaction*, particularly applicable to the progress in one direction only of component reaction steps in a complex system or to the progress in one direction of reactions in a system at dynamic equilibrium (in which there are no observable concentration changes with time). Chemical flux (φ) is a derivative with respect to time, and has the dimensions of amount of substance per unit volume transformed per unit time.

The sum of all the chemical fluxes leading to destruction of B is designated the "total chemical flux out of B" (symbol $\Sigma\varphi_{-B}$); the corresponding formation of B by concurrent elementary reactions is the "total chemical flux into B or A" (symbol $\Sigma\varphi_B$).

For the mechanism



the total chemical flux into C is caused by the single reaction (1):

$$\Sigma\varphi_C = \varphi_1$$

whereas the chemical flux out of C is a sum over all reactions that remove C:

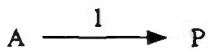
$$\Sigma\varphi_{-C} = \varphi_{-1} + \varphi_2$$

where φ_{-1} is the "chemical flux out of C into B (and/or A)" and φ_2 is the "chemical flux out of C into E". The rate of appearance of C is then given by

$$d[C]/dt = \Sigma\varphi_C - \Sigma\varphi_{-C}$$

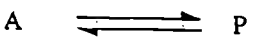
In this system φ_1 (or $\Sigma\varphi_{-A}$) can be regarded as the hypothetical rate of decrease in the concentration of A due to the single (unidirectional) reaction (1) proceeding in the assumed absence of all other reactions.

For a non-reversible *reaction*



$$-d[A]/dt = \varphi_1$$

If two substances A and P are in *chemical equilibrium*



then $\Sigma\varphi_A = \Sigma\varphi_{-A} = \Sigma\varphi_P = \Sigma\varphi_{-P}$

and $-d[A]/dt = d[P]/dt = 0$

GOLD (1979).

See also *order of reaction*, *rate-limiting step*, *steady state*.

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chemical reaction

A process that results in the interconversion of *chemical species*. Chemical reactions may be *elementary reactions* or *stepwise reactions*. (It should be noted that this definition includes experimentally observable interconversions of conformers.)

Detectable chemical reactions normally involve sets of *molecular entities*, as indicated by this definition, but it is often conceptually convenient to use the term also for changes involving single molecular entities (i.e. "microscopic chemical events").

See also *identity reaction*.

chemical relaxation

If the equilibrium mixture of a *chemical reaction* is disturbed by a sudden change, especially of some external parameter (such as temperature, pressure, or electrical field strength), the system will readjust itself to a new position of the chemical equilibrium or return to the original position, if the perturbation is temporary. The readjustment is known as chemical relaxation.

In many cases, and in particular when the displacement from equilibrium is slight, the progress of the system towards equilibrium can be expressed as a first-order law

$$[C_t - (C_{eq})_2] = [(C_{eq})_1 - (C_{eq})_2]\, e^{-t/\tau}$$

where $(c_{eq})_1$ and $(c_{eq})_2$ are the equilibrium concentrations of one of the chemical species involved in the reaction before and after the change in the external parameter, and c_t is its concentration at time t. The time parameter τ, named relaxation time, is related to the *rate constants* of the chemical reaction involved.

Measurements of the relaxation times by relaxation methods [involving a temperature jump (T-jump), pressure jump, electric field jump or a periodic disturbance of an external parameter, as in ultrasonic techniques] are commonly used to follow the kinetics of very fast reactions.

See BERNASCONI (1976); LEFFLER and GRUNWALD (1963); see also *relaxation*.

chemical shift (NMR), δ (SI unit: 1)

The variation of the resonance frequency of a nucleus in nuclear magnetic resonance (NMR) spectroscopy in consequence of its magnetic environment. The chemical shift of a nucleus, δ, is expressed in ppm by its frequency, ν_{cpd}, relative to a standard, ν_{ref}, and defined as

$$\delta = \frac{\nu_{cpd} - \nu_{ref}}{\nu_o} \times 10^6$$

where ν_o is the operating frequency of the spectrometer. For ^1H and ^{13}C NMR the reference signal is usually that of tetramethylsilane ($SiMe_4$). Other references are used in the older literature and in other solvents, such as D_2O.

If a resonance signal occurs at lower frequency or higher applied field than an arbitrarily selected reference signal, it is said to be upfield, and if resonance occurs at higher frequency or lower applied field, the signal is downfield. Resonance lines upfield from $SiMe_4$ have positive, and resonance lines downfield from $SiMe_4$ have negative δ-values.

chemical species

An ensemble of chemically identical *molecular entities* that can explore the same set of molecular energy levels on the time scale of the experiment. The term is applied equally to a set of chemically identical atomic or molecular structural units in a solid array.

For example, two conformational *isomers* may be interconverted sufficiently slowly to be detectable by separate NMR spectra and hence to be considered to be separate chemical species on a time scale governed by the radiofrequency of the spectrometer used. On the other hand, in a slow chemical reaction the same mixture of conformers may behave as a single chemical species, i.e. there is virtually complete equilibrium population of the total set of molecular energy levels belonging to the two conformers.

Except where the context requires otherwise, the term is taken to refer to a set of molecular entities containing isotopes in their natural abundance.

The wording of the definition given in the first paragraph is intended to embrace both cases such as graphite, sodium chloride, or a surface oxide, where the basic structural units may not be capable of isolated existence, as well as those cases where they are.

In common chemical usage, and in this Glossary, generic and specific chemical names (such as *radical* or hydroxide ion) or chemical formulae refer either to a chemical species or to a *molecular entity*.

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chemically induced dynamic nuclear polarization (CIDNP)

See *CIDNP.*

chemoselective, chemoselectivity

Chemoselectivity is the preferential reaction of a chemical reagent with one of two or more different functional groups. A reagent has a high chemoselectivity if reaction occurs with only a limited number of different functional groups. For example, sodium tetrahydroborate is a more chemoselective reducing agent than is lithium tetrahydroaluminate. The concept has not been defined in more quantitative terms. The term is also applied to reacting molecules or intermediates which exhibit selectivity towards chemically different reagents.

Some authors use the term chemospecificity for 100 % chemoselectivity. However, this usage is discouraged.

See TROST (1980).

See also *regioselectivity, stereoselectivity, stereospecificity.*

+ chemospecificity

See *chemoselectivity.*

chromophore

The part (atom or group of atoms) of a *molecular entity* in which the electronic transition responsible for a given spectral band is approximately localized. The term arose in the dyestuff industry, referring originally to the groupings in the molecule that are responsible for the dye's colour.

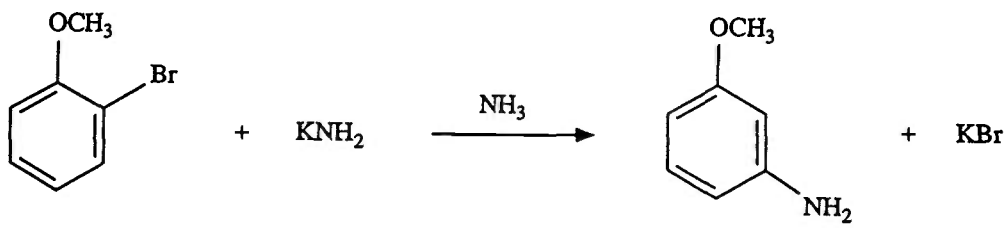
WITT (1876).

CIDNP (Chemically Induced Dynamic Nuclear Polarization)

Non-Boltzmann nuclear spin state distribution produced in thermal or photochemical reactions, usually from *colligation* and diffusion, or *disproportionation* of *radical pairs*, and detected by NMR spectroscopy by enhanced absorption or emission signals.

cine-substitution

A *substitution reaction* (generally *aromatic*) in which the entering group takes up a position adjacent to that occupied by the *leaving* group. For example,



See also *tele-substitution.*

class (a) metal ion

A metal ion that combines preferentially with *ligands* containing ligating atoms that are the lightest of their Periodic Group.

See also *class (b) metal ion, hard acid.*

class (b) metal ion

A metal ion that combines preferentially with *ligands* containing ligating atoms other than the lightest of their Periodic Group.

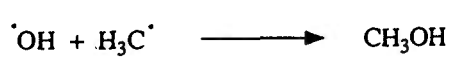
See also *class (a) metal ion, hard acid.*

clathrate

See *host, inclusion compound.*

colligation

The formation of a covalent bond by the combination or recombination of two *radicals* (the reverse of *unimolecular homolysis*). For example:



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common-ion effect (on rates)

A reduction in the *rate* of certain reactions of a *substrate* RX in solution [by a path that involves a *pre-equilibrium* with formation of R^+ (or R^-) ions as reaction intermediates] caused by the addition to the reaction mixture of an electrolyte solute containing the "common ion" X^- (or X^+). For example, the rate of solvolysis of diphenylmethyl chloride in acetone-water is reduced by the addition of salts of the common ion Cl^- which causes a decrease in the quasi-equilibrium concentration of the diphenylmethyl cation in the scheme:

$$Ph_2CHCl \underset{-1}{\overset{1}{\rightleftharpoons}} Ph_2CH^+ + Cl^- \qquad \text{(free ions, not ion pairs)}$$

$$Ph_2CH^+ + OH_2 \xrightarrow{2} Ph_2CHOH + H^+ \qquad \text{(solvated)}$$

This phenomenon is a direct consequence of the *mass-law effect* on ionization equilibria in electrolytic solution.

More generally, the common-ion effect is the influence of the "common ion" on the reactivity due to the shift of the dissociation equilibrium. It may also lead to an enhancement of the *rate of reaction*.

compensation effect

In a considerable number of cases plots of $T\Delta^{\ddagger}S$ *vs.* $\Delta^{\ddagger}H$, for a series of reactions, e.g. for a reaction in a range of different solvents, are straight lines of approximately unit slope. Therefore, the terms $\Delta^{\ddagger}H$ and $T\Delta^{\ddagger}S$ in the expression partially compensate, and $\Delta^{\ddagger}G = \Delta^{\ddagger}H - T\Delta^{\ddagger}S$ often is a much simpler function of solvent (or other) variation than $\Delta^{\ddagger}H$ or $T\Delta^{\ddagger}S$ separately.

CONNOR (1991).

See also *isokinetic relationship*.

complementary binding sites

See *binding site*.

complex

A *molecular entity* formed by loose *association* involving two or more component molecular entities (ionic or uncharged), or the corresponding *chemical species*. The bonding between the components is normally weaker than in a covalent *bond*.

The term has also been used with a variety of shades of meaning in different contexts: it is therefore best avoided when a more explicit alternative is applicable. In inorganic chemistry the term "coordination entity" is recommended instead of "complex" (IUPAC INORGANIC NOMENCLATURE (1990)). For the different usage of "complex" in inorganic chemistry, see IUPAC INORGANIC RULES (1970); Rule 2.24.

See also *activated complex, adduct, charge transfer complex, electron-donor-acceptor complex, encounter complex, inclusion complex, σ-adduct, π-adduct, transition state*.

composite reaction

A *chemical reaction* for which the expression for the rate of disappearance of a reactant (or rate of appearance of a product) involves rate constants of more than a single *elementary reaction*. Examples are "opposing reactions" (where rate constants of two opposed *chemical reactions* are involved), "parallel reactions" (for which the rate of disappearance of any reactant is governed by the rate constants relating to several simultaneous reactions to form different respective products from a single set of reactants), and *stepwise reactions*.

comproportionation

The reverse of *disproportionation*. The term "symproportionation" is also used.

HARTMANNS, KLENKE, and METZGER (1986).

concerted process

Two or more *primitive changes* are said to be concerted (or to constitute a concerted process) if they occur within the same *elementary reaction*. Such changes will normally (though perhaps not inevitably) be "energetically coupled". (In the present context the term "energetically coupled" means that the simultaneous progress of the primitive changes involves a *transition state* of lower energy than that for their successive occurrence.) In a concerted process the primitive changes may be *synchronous* or asynchronous.

See also *bifunctional catalysis, potential energy (reaction) surface*.

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condensation reaction

A (usually stepwise) reaction in which two or more reactants (or remote reactive sites within the same *molecular entity*) yield a single main product with accompanying formation of water or of some other small molecule, e.g. ammonia, ethanol, acetic acid, hydrogen sulfide.

The *mechanism* of many condensation reactions has been shown to comprise consecutive *addition* and *elimination reactions*, as in the base-catalysed formation of (*E*)-but-2-enal (crotonaldehyde) from acetaldehyde, via 3-hydroxybutanal (aldol). The overall reaction in this example is known as the aldol condensation.

The term is sometimes also applied to cases where the formation of water or another simple molecule does not occur, as in "benzoin condensation".

configuration (electronic)

A distribution of the electrons of an atom or a *molecular entity* over a set of one-electron wavefunctions called *orbitals*, according to the Pauli principle. From one configuration several states with different multiplicities may result. For example, the ground electronic configuration of the oxygen molecule (O_2) is

$$1\sigma_g^2,\ 1\sigma_u^2,\ 2\sigma_g^2,\ 2\sigma_u^2,\ 1\pi_u^4,\ 3\sigma_g^2,\ 1\pi_g^2$$

resulting in the

$$^3\Sigma_g^-,\ ^1\Delta_g,\ \text{and}\ ^1\Sigma_g^+\ \text{multiplets}.$$

IUPAC PHOTOCHEMICAL GLOSSARY (1992).

configuration (molecular)

In the context of stereochemistry, the term is restricted to the arrangements of atoms of a *molecular entity* in space that distinguishes stereoisomers, the isomerism of which is not due to conformational differences.

IUPAC STEREOCHEMICAL TERMINOLOGY (1993).

conformation

The spatial arrangements of atoms affording distinction between stereoisomers which can be interconverted by rotation about formally single bonds. Some authorities extend the term to include inversion at trigonal bipyramidal centres and other "polytopal rearrangements".

IUPAC STEREOCHEMICAL TERMINOLOGY (1993).

conjugate acid-base pair

The *Brønsted acid* BH^+ formed on protonation of a base B is called the conjugate acid of B, and B is the conjugate base of BH^+. (The conjugate acid always carries one unit of positive charge more than the base, but the absolute charges of the species are immaterial to the definition.) For example: the Brønsted acid HCl and its conjugate base Cl^- constitute a conjugate acid-base pair.

conjugated system, conjugation

In the original meaning a conjugated system is a molecular entity whose structure may be represented as a system of alternating single and multiple bonds: e.g.

$$CH_2{=}CH{-}CH{=}CH_2 \qquad\qquad CH_2{=}CH{-}C{\equiv}N$$

In such systems, conjugation is the interaction of one p-orbital with another across an intervening σ-bond in such structures. (In appropriate molecular entities d-orbitals may be involved.) The term is also extended to the analogous interaction involving a p-orbital containing an unshared electron pair, e.g.

$$:Cl{-}CH{=}CH_2$$

See also *delocalization*, *homoconjugation*, *resonance*.

conjugative mechanism

See *electronic effect*.

connectivity

In a chemical context, the information content of a *line formula*, but omitting any indication of *bond* multiplicity.

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constitution

The description of the identity and connectivity (and corresponding bond multiplicities) of the atoms in a *molecular entity* (omitting any distinction from their spatial arrangement).

conrotatory

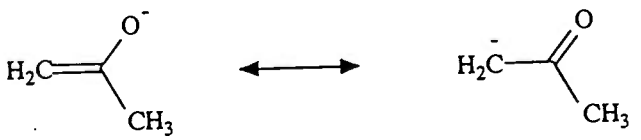
See *electrocyclic reaction.*

contact ion pair

See *ion pair.*

contributing structure

The definition is based on the valence-bond formulation of the quantum mechanical idea of the wavefunction of a molecule as composed of a linear combination of wavefunctions, each representative of a formula containing bonds that are only single, double or triple with a particular pairing of electron spins. Each such formula represents a contributing structure, also called "resonance structure" to the total wavefunction, and the degree to which each contributes is indicated by the square of its coefficient in the linear combination. The contributing structures, also called "canonical forms", themselves thus have a purely formal significance: they are the components from which wavefunctions can be built. Structures may be covalent (or non-polar) or ionic (or polar). The representation is frequently kept qualitative so that we speak of important or major contributing structures and minor contributing structures. For example, two major non-equivalent contributing structures for the conjugate base of acetone are



See also *delocalization, Kekulé structure, resonance.*

coordinate covalence (coordinate link)

See *coordination.*

coordination

The formation of a covalent *bond*, the two shared electrons of which have come from only one of the two parts of the *molecular entity* linked by it, as in the reaction of a *Lewis acid* and a *Lewis base* to form a *Lewis adduct*; alternatively, the bonding formed in this way. In the former sense, it is the reverse of *unimolecular heterolysis.* "Coordinate covalence" and "coordinate link" are synonymous (obsolescent) terms. The synonym "dative bond" is obsolete.

(The origin of the bonding electrons has by itself no bearing on the character of the bond formed. Thus, the formation of methyl chloride from a methyl cation and a chloride ion involves coordination; the resultant bond obviously differs in no way from the C-Cl bond in methyl chloride formed by any other path, e.g. by colligation of a methyl radical and a chlorine atom.)

The term is also used to describe the number of ligands around a central atom without necessarily implying two-electron bonds.

See also *dipolar bond, π-adduct.*

coordination number

The coordination number of a specified atom in a *chemical species* is the number of other atoms directly linked to that specified atom [cf. IUPAC INORGANIC NOMENCLATURE (1990); Rule I-10.2.5]. For example, the coordination number of carbon in methane is four, and it is five in protonated methane, CH_5^+. (The term is used in a different sense in the crystallographic description of ionic crystals.)

coordinatively saturated

A transition metal complex that has formally 18 outer shell electrons at the central metal atom.

coordinatively unsaturated

A transition metal complex that possesses fewer ligands than exist in the coordinatively saturated complex. These complexes usually have fewer than 18 outer shell electrons at the central metal atom.

coronate

See *crown.*

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correlation analysis

The use of empirical correlations relating one body of experimental data to another, with the objective of finding quantitative estimates of the factors underlying the phenomena involved. Correlation analysis in organic chemistry often uses *linear free-energy relations* for rates or equilibria of reactions, but the term also embraces similar analysis of physical (most commonly spectroscopic) properties and of biological activity.

CHAPMAN and SHORTER (1972, 1978).

See also *Quantitative Structure-Activity Relationships (QSAR).*

cosphere

See *cybotactic region.*

coupling constant (spin-spin coupling constant), J (SI unit: Hz (NMR))

A quantitative measure for nuclear spin-spin, nuclear-electron (hyperfine coupling) and electron-electron (fine coupling in EPR) coupling in magnetic resonance spectroscopy. The "indirect" or scalar NMR coupling constants are in a first approximation independent of the external magnetic field and are expressed in Hz.

covalent bond

A region of relatively high electron density between nuclei which arises at least partly from sharing of electrons and gives rise to an attractive force and characteristic internuclear distance.

See also *agostic, coordination , hydrogen bond, multi-centre bond.*

Cox-Yates equation

A modification of the *Bunnett-Olsen equation* of the form

$$\lg([SH^+])/[S]) - \lg[H^+] = m^*X + pK_{SH^+}$$

where X is the activity function $\lg(\gamma_S\gamma_{H^+})/\gamma_{SH^+})$ for an arbitrary reference base. The function X is called the excess acidity because it gives a measure of the difference between the acidity of a solution and that of an ideal solution of the same concentration. In practice $X = -(H_o + \lg[H^+])$ and $m^* = 1 - \Phi$.

COX and YATES (1978, 1981); LUCCHINI, MODENA, SCORRANO, COX and YATES (1982).

See also *Bunnett-Olsen equations.*

critical micelle concentration (cmc)

There is a relatively small range of concentrations separating the limit below which virtually no *micelles* are detected and the limit above which virtually all additional surfactant molecules form micelles. Many properties of surfactant solutions, if plotted against the concentration, appear to change at a different rate above and below this range. By extrapolating the loci of such a property above and below this range until they intersect, a value may be obtained known as the critical micellization concentration (critical micelle concentration), symbol c_M, abbreviation cmc (or c.m.c.). As values obtained using different properties are not quite identical, the method by which the cmc is determined should be clearly stated.

See IUPAC MANUAL APPENDIX II (1972).

See also *inverted micelle.*

cross-conjugation

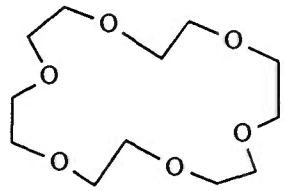
In a system XC_6H_4GY this is conjugation involving the substituent X, the benzene ring, and the side-chain connective-plus-reaction site GY, i.e. either X is a $+R$ group and GY is a $-R$ group, or X is a $-R$ group and GY is a $+R$ group. In Hammett correlations this situation can lead to the need to apply exalted substituent constants σ^+ or σ^-, respectively, as in electrophilic or nucleophilic aromatic substitution, respectively. The term "through resonance" is synonymous. Cross conjugation has also been used to describe the interactions occurring in 2-phenylallyl and and similar systems (DEWAR (1969)).

crown

A *molecular entity* comprising a monocyclic *ligand* assembly that contains three or more *binding sites* held together by covalent bonds and capable of binding a *guest* in a central (or nearly central) position. The *adducts* formed are sometimes known as "coronates". The best known members of this group are macrocyclic polyethers, such as "18-crown-6", containing several repeating units $-CR_2-CR_2O-$ (where R is most commonly H), and known as crown ethers.

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PEDERSEN (1967); CRAM et al. (1986).
See also *host*.

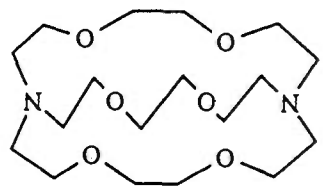


"18-crown-6"

cryptand

A *molecular entity* comprising a cyclic or polycyclic assembly of *binding sites* that contains three or more *binding sites* held together by *covalent bonds*, and which defines a molecular cavity in such a way as to bind (and thus "hide" in the cavity) another molecular entity, the *guest* (a cation, an anion or a neutral species), more strongly than do the separate parts of the assembly (at the same total concentration of binding sites). The *adduct* thus formed is called a "cryptate". The term is usually restricted to bicyclic or oligocyclic molecular entities.

Example



Corresponding monocyclic ligand assemblies (*crowns*) are sometimes included in this group, if they can be considered to define a cavity in which a guest can hide. The terms "podand" and "spherand" are used for certain specific ligand assemblies. Coplanar cyclic polydentate ligands, such as porphyrins, are not normally regarded as cryptands.

DIETRICH, LEHN and SAUVAGE (1969).
See also *host*.
For a contribution to the evolving terminology, see also VÖGTLE (1980).

Curtin-Hammett principle

In a *chemical reaction* that yields one product (X) from one conformational isomer (A') and a different product (Y) from another conformational isomer (A") (and provided these two isomers are rapidly interconvertible relative to the rate of product formation, whereas the products do not undergo interconversion) the product composition is not in direct proportion to the relative concentrations of the conformational isomers in the *substrate*; it is controlled only by the difference in standard free energies ($\delta\Delta^{\ddagger}G$) of the respective *transition states*.

It is also true that the product composition is formally related to the relative concentrations of the conformational isomers A' and A" (i.e. the conformational equilibrium constant) and the respective rate constants of their reactions; these parameters are generally - though not invariably - unknown.

The diagram below represents the energetic situation for transformation of interconverting isomers A and A' into products X and Y.

$$X \xleftarrow{k_X} A' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A'' \xrightarrow{k_Y} Y$$

$$k_1 >> k_X \qquad k_{-1} >> k_Y$$

ELIEL (1962); see also SEEMAN et al. (1980), SEEMAN (1983).

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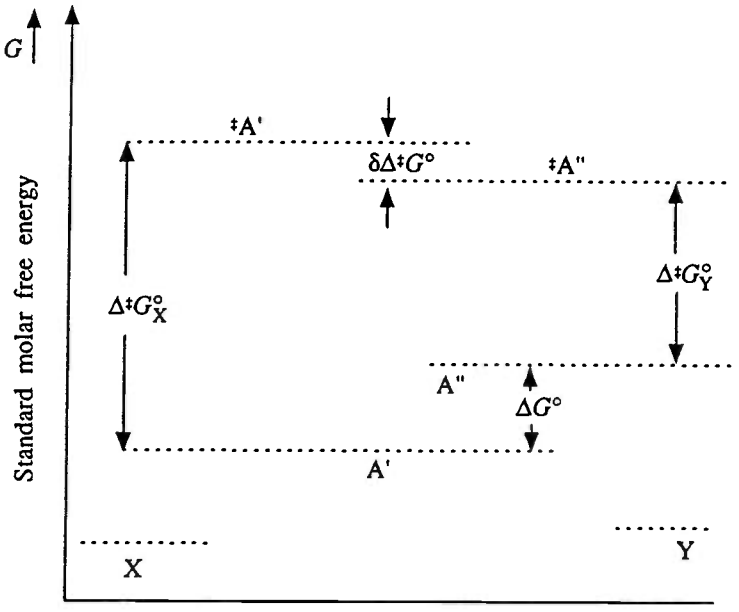


Figure. Curtin-Hammett principle. Transformation of interconverting isomers A' and A" into products X and Y.

cybotactic region

That part of a solution in the vicinity of a solute molecule in which the ordering of the solvent molecules is modified by the presence of the solute molecule. The term solvent "cosphere" of the solute has also been used.

KOSOWER (1968); STEWART and MORROW (1927).

See also *solvation.*

cyclization

Formation of a ring compound from a chain by formation of a new bond.

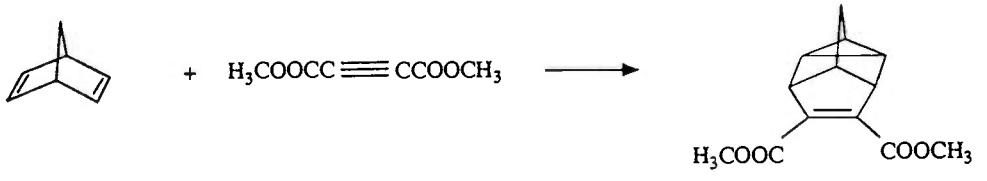
See also *annulation.*

cycloaddition

A reaction in which two or more unsaturated molecules (or parts of the same molecule) combine with the formation of a cyclic *adduct* in which there is a net reduction of the bond multiplicity.

The following two systems of notations have been used for the more detailed specification of cycloadditions, of which the second, more recent system (described under (2)) is preferred:

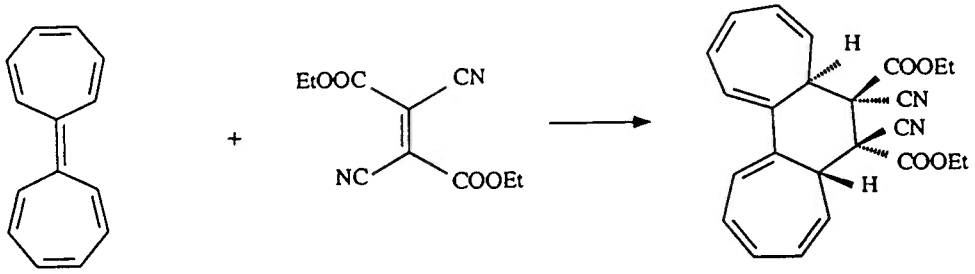
(1) A (*i*+*j*+...) cycloaddition is a reaction in which two or more molecules (or parts of the same molecule), respectively, provide units of *i*, *j*,... linearly connected atoms: these units become joined at their respective termini by new σ-bonds so as to form a cycle containing (*i*+*j*+...) atoms. In this notation, (a) a Diels-Alder reaction is a (4+2) cycloaddition, (b) the initial reaction of ozone with an alkene is a (3+2) cycloaddition, and (c) the reaction shown below is a (2+2+2) cycloaddition. (N.B.: parentheses (...) are used in the description based on numbers of atoms.)



(2) The symbolism [*i*+*j*+...] for a cycloaddition identifies the numbers *i*, *j*,... of electrons in the interacting units that participate in the transformation of reactants to products. In this notation the reaction (a) and (b) of the preceding paragraph would both be described as [2+4] cycloadditions, and (c) as a [2+2+2] cycloaddition. The symbol a or s (a = *antarafacial*, s = suprafacial) is often added (usually as a subscript after the number to designate the stereochemistry of addition to each fragment. A subscript specifying the orbitals, viz., σ, π (sigma, pi) with their usual significance) or n (for an orbital associated with a single atom only), may be

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added as a subscript before the number. Thus the normal Diels-Alder reaction is a $[4_s+2_s]$ or $[_\pi 4_s + _\pi 2_s]$ cycloaddition, whilst the reaction



would be a $[14_a+2_s]$ or $[_\pi 14_a + _\pi 2_s]$ cycloaddition. (N.B. Square brackets [...] are used in the descriptions based on numbers of electrons.)

Cycloadditions may be *pericyclic reactions* or (non-*concerted*) *stepwise reactions*. The term "dipolar cycloaddition" is used for cycloadditions of 1,3-dipolar compounds.

HUISGEN (1968); HUISGEN, GRASHEY and SAUER (1964); WOODWARD and HOFFMANN (1969).

See also *cheletropic reactions*.

cycloelimination

The reverse of *cycloaddition*. The term is preferred to the synonyms "cycloreversion", "retro-addition", and "retrocycloaddition".

+ cycloreversion

See *cycloelimination*.

+ dative bond

See *coordination*.

decomposition, chemical

The breakdown of a single entity (normal molecule, reaction *intermediate*, etc.) into two or more fragments.

+ de-electronation

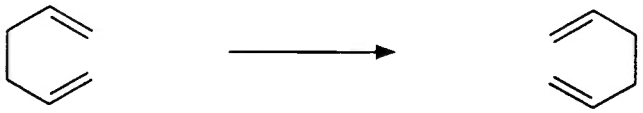
See *oxidation* (1).

degenerate chemical reaction

See *identity reaction*.

degenerate rearrangement

A *molecular rearrangement* in which the principal product is indistinguishable (in the absence of isotopic labelling) from the principal reactant. The term includes both "degenerate *intramolecular* rearrangements" and reactions that involve *intermolecular* transfer of atoms or groups ("degenerate intermolecular rearrangements"): both are degenerate *isomerizations*. The occurrence of degenerate rearrangements may be detectable by isotopic labelling or by dynamic NMR techniques. For example: the [3,3]*sigmatropic* rearrangement of hexa-1,5-diene (Cope rearrangement),



Synonymous but less preferable terms are "automerization", "permutational isomerism", "isodynamic transformation", "topomerization".

BINSCH, ELIEL, and KESSLER (1971).

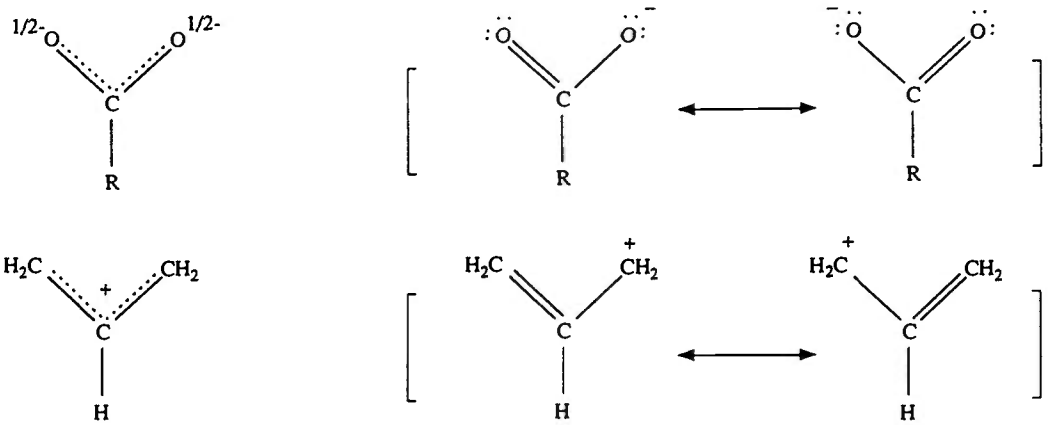
See also *fluxional*, *molecular rearrangement*, *valence isomer*.

delocalization

A quantum mechanical concept most usually applied in organic chemistry to describe the π-bonding in a *conjugated system*. This bonding is not localized between two atoms: instead, each link has a "fractional double bond character" or *bond order*. There is a corresponding "delocalization energy", identifiable with the stabilization of the system compared with a

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hypothetical alternative in which formal (localized) single and double *bonds* are present. Some degree of delocalization is always present and can be estimated by quantum mechanical calculations. The effects are particularly evident in *aromatic* systems and in symmetrical *molecular entities* in which a lone pair of electrons or a vacant p-orbital is conjugated with a double bond (e.g. carboxylate ions, nitro compounds, enamines, the allyl cation). Delocalization in such species may be represented by partial bonds or as *resonance* (here symbolized by a two-headed arrow) between *contributing structures*.



These examples also illustrate the concomitant delocalization of charge in ionic conjugated systems. Analogously, delocalization of the spin of an unpaired electron occurs in conjugated *radicals*.

See also *mesomerism*.

deshielding

See *shielding*.

detachment

The reverse of an *attachment*.

See also *electron attachment*.

detailed balancing, principle of

When equilibrium is reached in a reaction system (containing an arbitrary number of components and reaction paths), as many atoms, in their respective *molecular entities*, will pass forward, as well as backwards, along each individual path in a given finite time interval. Accordingly, the reaction path in the reverse direction must in every detail be the reverse of the reaction path in the forward direction (provided always that the system is at equilibrium).

The principle of detailed balancing is a consequence for macroscopic systems of the principle of *microscopic reversibility*.

diamagnetic

Substances having a negative magnetic susceptibility are diamagnetic. They are repelled out of a magnetic field.

See also *paramagnetic*.

diastereoisomerism

Stereoisomerism other than *enantiomerism*. Diastereoisomers (or diastereomers) are stereoisomers not related as mirror images. Diastereoisomers are characterized by differences in physical properties, and by some differences in chemical behaviour toward achiral as well as chiral reagents.

IUPAC STEREOCHEMICAL TERMINOLOGY (1993).

dielectric constant

A measure for the effect of a medium on the potential energy of interaction between two charges. It is measured by comparing the capacity of a capacitor with and without the sample present.

dienophile

The olefin component of a Diels-Alder reaction.

See *cycloaddition*.

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diffusion-controlled rate

See *encounter-controlled rate, microscopic diffusion control.* See also *mixing control.*

dimerization

The *transformation* of a *molecular entity* A to give a molecular entity A_2. For example:

$$H_3C^{\bullet} + {}^{\bullet}CH_3 \longrightarrow CH_3CH_3$$

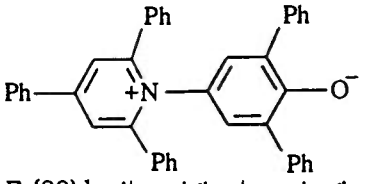
$$2\ CH_3COCH_3 \longrightarrow (CH_3)_2C(OH)CH_2COCH_3$$

$$2\ RCOOH \longrightarrow (RCOOH)_2$$

See also *association.*

Dimroth-Reichardt E_T parameter

A measure of the *ionizing power* (loosely *polarity*) of a solvent, based on the maximum wavenumber of the longest wavelength electronic absorption band of



in a given solvent. E_T, called $E_T(30)$ by its originators, is given by

$$E_T = 2.859 \times 10^{-3}\nu$$

$$= 2.859 \times 10^{4}\lambda^{-1}$$

where E_T is in kcal/mol, ν is in cm^{-1} and λ is in nm.

The so-called normalized E_T^N scale is defined as

$$E_T^N = \frac{E_T(\text{solvent}) - E_T(SiMe_4)}{E_T(\text{water}) - E_T(SiMe_4)} = [E_T(\text{solvent}) - 30.7] / 32.4$$

DIMROTH, REICHARDT, SIEPMANN and BOHLMANN (1963); REICHARDT (1988).

See also *Grunwald-Winstein equation, Z-value.*

dipolar aprotic solvent

A solvent with a comparatively high relative permittivity (or dielectric constant), greater than ca. 15, and a sizable permanent dipole moment, that cannot donate suitably labile hydrogen atoms to form strong hydrogen bonds, e.g. dimethyl sulfoxide. The term (and its alternative "polar aprotic solvent") is a misnomer and is therefore discouraged. Such solvents are usually not *aprotic*, but *protophilic* (and at most weakly *protogenic*). In describing a solvent it is better to be explicit about its essential properties, e.g. dipolar and non-protogenic.

dipolar bond

A *bond* formed (actually or conceptually) by *coordination* of two neutral moieties, the combination of which results in charge-separated structures, e.g.,

$$R_3N: + \ddot{O}: \longrightarrow R_3N^{+}\text{—}\ddot{O}^{-}$$

The term is preferred to the obsolescent synonyms "coordinate link", "co-ordinate co-valence", "dative bond", "semipolar bond".

dipolar cycloaddition

See *cycloaddition.*

dipole-dipole interaction

Intermolecular or intramolecular interaction between molecules or groups having a permanent electric dipole moment. The strength of the interaction depends on the distance and relative orientation of the dipoles. The term applies also to intramolecular interactions between bonds having permanent dipole moments.

See also *van der Waals forces.*

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dipole-induced dipole forces

See *van der Waals forces.*

diradical

See *biradical.*

direct effect

See *field effect.*

dismutation

See *disproportionation.*

dispersion forces

See *London forces.*

disproportionation

Any *chemical reaction* of the type A + A → A' + A'' where A, A' and A'' are different *chemical species.* For example:

$$2\ ArH^+ \longrightarrow ArH + ArH^{2+}$$

The reverse of disproportionation is called *comproportionation.*

A special case of disproportionation (or "dismutation") is "radical disproportionation", exemplified by

$$^{\cdot}CH_2CH_3 + {}^{\cdot}CH_2CH_3 \longrightarrow CH_2{=}CH_2 + CH_3CH_3$$

Reactions of the more general type

$$R\dot{C}HCH_3 + R'\dot{C}HCH_3 \longrightarrow RCH{=}CH_2 + R'CH_2CH_3$$

are also loosely described as radical disproportionations.

(A somewhat more restricted usage of the term prevails in inorganic chemistry.)

disrotatory

See *electrocyclic reaction.*

dissociation

(1) The separation of a *molecular entity* into two or more molecular entities (or any similar separation within a polyatomic molecular entity). Examples include *unimolecular heterolysis* and *homolysis*, and the separation of the constituents of an *ion pair* into free ions.

(2) The separation of the constituents of any aggregate of molecular entities.

In both senses dissociation is the reverse of *association.*

distonic radical cation

A radical cation in which charge and radical sites are separated.

YATES, BOUMA and RADOM (1986).

donor number (DN)

A quantitative measure of *Lewis basicity* devised by GUTMANN (1976).

driving force (also called the affinity) of a reaction, A (SI unit: kJ mol^{-1})

The decrease in Gibbs energy on going from the reactants to the products of a *chemical reaction* ($-\Delta G$).

downfield

See *chemical shift.*

dual substituent-parameter equation

In a general sense, this is any equation which expresses substituent effects in terms of two parameters. However, in practice the term is used more specifically for an equation for summarizing the effects of *meta-* or *para*-substituents (i = *m* or *p*) X on chemical reactivity, spectroscopic properties, etc. of a probe site Y in benzene or other aromatic system.

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$$P^i = \rho_I^i \sigma_I + \rho_R^i \sigma_R$$

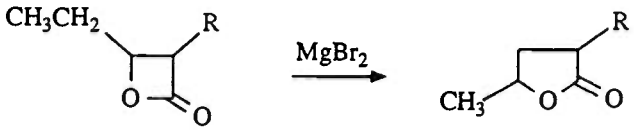
P is the magnitude of the property Y for substituent X, expressed relative to the property for X=H; σ_I and σ_R are inductive or polar and resonance substituent constants, respectively, there being various scales for σ_R; ρ_I and ρ_R are the corresponding regression coefficients.

EHRENSON, BROWNLEE, and TAFT (1973); TAFT and TOPSOM (1987).

See also *extended Hammett equation.*

dyotropic rearrangement

An uncatalyzed process in which two σ-bonds simultaneously migrate intramolecularly, e.g.



REETZ (1972).

E_T-value

See *Dimroth-Reichardt E_T parameter, Z-value.*

+ educt

Term used mainly in the German literature for starting material (reactant). It should be avoided in English, because there it means "something that comes out" and not "something that goes in". The german use of the term is in fact also incorrect.

effective charge

Change in effective charge is a quantity obtained by comparison of the *polar effect* of substituents on the free energies of rate or equilibrium processes with that on a standard ionization equilibrium. Provided the effective charge on the states in the standard equilibrium are defined, then it is possible to measure effective charges for states in the reaction or equilibrium under consideration.

WILLIAMS (1984, 1992).

effective molarity (or effective concentration)

The ratio of the first-order rate constant of an *intramolecular* reaction involving two functional groups within the same *molecular entity* to the second-order *rate constant* of an analogous *intermolecular elementary reaction.* This ratio has the dimension of concentration. The term can also apply to an equilibrium constant.

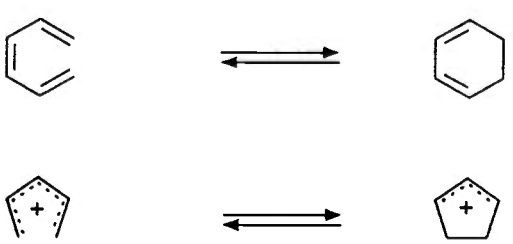
See also *intramolecular catalysis.*

eighteen-electron rule

An electron-counting rule to which an overwhelming majority of stable diamagnetic transition metal complexes adhere. The number of nonbonded electrons at the metal plus the number of electrons in the metal-ligand bonds should be 18. The 18 electron rule in transition metal chemistry is a full analogue of the "Lewis octet rule".

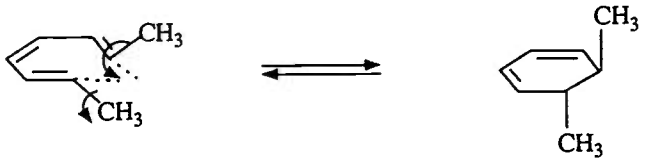
electrocyclic reaction

A *molecular rearrangement* that involves the formation of a σ-bond between the termini of a fully *conjugated* linear π-electron system (or a linear fragment of a π-electron system) and a decrease by one in the number of π-bonds, or the reverse of that process. For example:

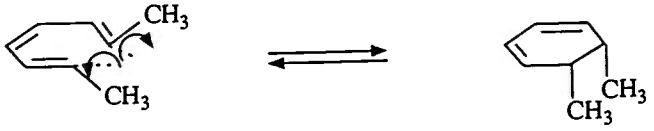


The stereochemistry of such a process is termed "conrotatory" or *antarafacial* if the substituents at the interacting termini of the conjugated system both rotate in the same sense, e.g.

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or "disrotatory" (or *suprafacial*) if one terminus rotates in a clockwise and the other in a counter-clockwise sense, e.g.



See also *pericyclic reaction*.

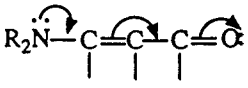
electrofuge

A *leaving group* that does not carry away the bonding electron pair. For example, in the nitration of benzene by NO_2^+, H^+ is the electrofuge. The adjective of electrofuge is electrofugal.

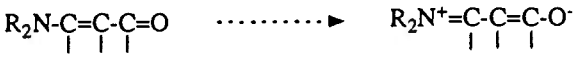
See also *electrophile*, *nucleofuge*.

electromeric effect

A molecular polarizability effect occurring by an *intramolecular* electron displacement (sometimes called the "conjugative mechanism" and, previously, the "tautomeric mechanism") characterized by the substitution of one electron pair for another within the same atomic octet of electrons. It can be indicated by curved arrows symbolizing the displacement of electron pairs, as in



which represents the hypothetical electron shift



INGOLD (1953).

The term has been deemed obsolescent or even obsolete (see *mesomeric effect*, *resonance effect*). It has long been custom to use phrases such as "enhanced substituent resonance effect" which imply the operation of the electromeric effect, without using the term, and various modern theoretical treatments parametrize the response of substituents to "electronic demand", which amounts to considering the electromeric effect together with the *inductomeric effect*.

EHRENSON, BROWNLEE and TAFT (1973); TAFT and TOPSOM (1987); CHARTON (1987).

electron acceptor

(1) A substance to which an electron may be transferred; for example 1,4-dinitrobenzene or the dication 1,1'-dimethyl-4,4'-bipyridyldiium .

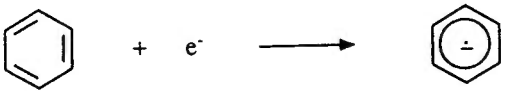
+ (2) A Lewis acid. This usage is discouraged.

electron affinity

The energy released when an additional electron (without excess energy) attaches itself to a *molecular entity* (usually an electrically neutral molecular entity). (The direct measurement of this quantity involves molecular entities in the gas phase.)

electron attachment

The transfer of an electron to a *molecular entity*, resulting in a molecular entity of (algebraically) increased negative charge. (It is not an *attachment*, as defined in this Glossary.)



See also *oxidation* (1), *reduction*.

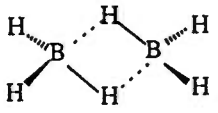
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electron configuration

See *configuration (electronic).*

electron-deficient bond

A single bond between adjacent atoms that is formed by less than two electrons, as in B_2H_6:



The B-H-B bonds are called a "two-electron three-centre bonds".

electron density

If $P(x,y,z)\,dx\,dy\,dz$ is the probability of finding an electron in the volume element $dx\,dy\,dz$ at the point of a molecular entity with coordinates x,y,z, then $P(x,y,z)$ is the electron density at this point. For many purposes (e.g., X-ray scattering, forces on atoms) the system behaves exactly as if the electrons were spread out into a continuously distributed charge. The term has frequently been wrongly applied to negative *charge population.*

See also *charge density.*

electron detachment

The reverse of an *electron attachment.*

electron donor

(1) A *molecular entity* that can transfer an electron to another molecular entity, or to the corresponding *chemical species.*

+ (2) A Lewis base. This use is discouraged.

+ electron-donor-acceptor complex

A term sometimes employed instead of *charge-transfer complex* or *Lewis adduct.*

See also *adduct, coordination.*

electron-pair acceptor

A synonym for *Lewis acid.*

electron-pair donor

A synonym for *Lewis base.*

electron transfer

The transfer of an electron from one *molecular entity* to another, or between two localized sites in the same *molecular entity.*

See also *inner sphere (electron transfer), outer sphere (electron transfer), Marcus equation.*

electron-transfer catalysis

The term indicates a sequence of reactions such as shown in equations (1)-(3), leading from A to B:

$$A + e^- \longrightarrow A^{\cdot -} \qquad (1)$$

$$A^{\cdot -} \longrightarrow B^{\cdot -} \qquad (2)$$

$$B^{\cdot -} + A \longrightarrow B + A^{\cdot -} \qquad (3)$$

An analogous sequence involving radical cations ($A^{\cdot +}$, $B^{\cdot +}$) is also observed.

The most notable example of electron-transfer catalysis is the $S_{RN}1$ (or $T+D_N+A_N$) reaction of aromatic halides.

The term has its origin in a suggested analogy to acid-base catalysis, with the electron instead of the proton. However, there is a difference between the two catalytic mechanisms, since the electron is not a true catalyst, but rather behaves as the initiator of a *chain reaction.* "Electron-transfer induced chain reaction" is a more appropriate term for the situation described by equations (1)-(3).

EBERSON (1987).

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electronation

See *electron attachment, reduction.*

electronegativity

A measure of the power of an atom or a group of atoms to attract electrons from other parts of the same *molecular entity*. The concept has been quantified by a number of authors, including especially Pauling and Mulliken.

See ATKINS (1974).

electroneutrality principle

The principle expresses the fact that all pure substances carry a net charge of zero.

electronic effect of substituents: symbols and signs

The *inductive effect* has universally been represented by the symbol I. This is now commonly taken to include both through-bonds and through-space transmission, but I is also used specifically for through-bonds transmission; through-space transmission is then symbolized as F (for *field effect*). The symbols for the influence of substituents exerted through electron delocalization have variously been M (*mesomeric*), E (*electromeric*), T (tautomeric), C (conjugative), K (konjugativ), and R (*resonance*). Since the present fashion is to use the term *resonance effect*, R is the most commonly used symbol, although M is still seen quite often.

Both the possible sign conventions are in use. The Ingold sign convention associates *electronegativity* (relative to hydrogen atom) with a negative sign, electropositivity with a positive sign. Thus the nitro group is described as electron-withdrawing by virtue of its -I and -M effects; chloro is described as a -I, +M substituent, etc. For *correlation analysis* and *linear free-energy relationships* this convention has been found inconvenient, for it is in contradiction to the sign convention for polar substituent constants (σ-*constants*). Authors concerned with these fields often avoid this contradiction by adopting the opposite sign convention originally associated with Robinson, for electronic effects. This practice is almost always associated with the use of R for the electron delocalization effect: thus the nitro group is a +I, +R substituent; chloro a +I, -R substituent, etc.

electrophile, electrophilic

An electrophile (or electrophilic reagent) is a reagent that forms a *bond* to its reaction partner (the *nucleophile*) by accepting both bonding electrons from that reaction partner.

An "electrophilic *substitution reaction*" is a *heterolytic* reaction in which the reagent supplying the entering group acts as an electrophile. For example

$$\underset{\text{electrophile}}{C_6H_6 + NO_2^+} \longrightarrow \underset{\text{electrofuge}}{C_6H_5NO_2 + H^+}$$

Electrophilic reagents are *Lewis acids*. "Electrophilic catalysis" is catalysis by Lewis acids.

The term "electrophilic" is also used to designate the apparent polar character of certain *radicals*, as inferred from their higher relative reactivities with reaction sites of higher electron density.

See also *electrophilicity*.

electrophilicity

(1) The property of being *electrophilic* (see *electrophile*).

(2) The relative reactivity of an electrophilic reagent. (It is also sometimes referred to as "electrophilic power".) Qualitatively, the concept is related to Lewis acidity. However, whereas Lewis acidity is measured by relative equilibrium constants, electrophilicity is measured by relative *rate constants* for reactions of different electrophilic reagents towards a common *substrate* (usually involving attack at a carbon atom).

See also *nucleophilicity*.

element effect

The ratio of the rate constants of two reactions that differ only in the identity of the element of the atom in the *leaving group*, e.g., k_{Br}/k_{Cl}. As for *isotope effects*, a ratio of unity is regarded as a "null effect".

elementary reaction

A reaction for which no *reaction intermediates* have been detected or need to be postulated in order to describe the chemical reaction on a molecular scale. An elementary reaction is assumed to occur in a single step and to pass through a single *transition state*.

IUPAC CHEMICAL KINETICS (1981).

See also *stepwise reaction*.

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elimination

The reverse of an *addition reaction* or *transformation*.

In an elimination two groups (called eliminands) are lost most often from two different centres (1/2/elimination or 1/3/elimination, etc.) with concomitant formation of an unsaturation in the molecule (double bond, triple bond) or formation of a new ring.

If the groups are lost from a single centre (α-elimination, 1/1/*elimination*) the resulting product is a *carbene* or a "carbene analogue".

See also *α-elimination*.

enantiomer

One of a pair of *molecular entities* which are mirror images of each other and non-superimposable.

enantioselectivity

See *stereoselectivity*.

encounter complex

A *complex* of *molecular entities* produced at an *encounter-controlled rate*, and which occurs as an intermediate in a reaction mechanism. When the complex is formed from two molecular entities it is called an "encounter pair". A distinction between encounter pairs and (larger) encounter complexes may be relevant in some cases, e.g. for mechanisms involving *pre-association*.

encounter-controlled rate

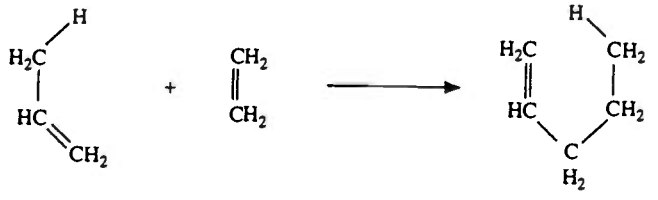
A *rate of reaction* corresponding to the rate of encounter of the reacting *molecular entities*. This is also known as "diffusion-controlled rate" since rates of encounter are themselves controlled by diffusion rates (which in turn depend on the viscosity of the *medium* and the dimensions of the reactant molecular entities).

For a *bimolecular* reaction between solutes in water at 25 °C an encounter-controlled rate is calculated to have a second-order *rate constant* of about 10^{10} dm^3 mol^{-1} s^{-1}.

See also *microscopic diffusion control*.

ene reaction

The addition of a compound with a double bond having an allylic hydrogen (the "ene") to a compound with a multiple bond (the "enophile") with transfer of the allylic hydrogen and a concomitant reorganization of the bonding, as illustrated below for propene (the "ene") and ethene (the "enophile"). The reverse is a "retro-ene" reaction.



energy of activation (Arrhenius energy of activation; activation energy), E_a (SI unit: kJ mol^{-1})

An operationally defined quantity expressing the dependence of a rate constant on temperature according to

$$E_a = RT^2(\partial \ln k / \partial T)_p$$

as derived from the "Arrhenius equation", $k = A \exp(-E_a/RT)$, where A (SI unit: as for the corresponding rate constant) is termed the "pre-exponential factor".

See also *enthalpy of activation*.

energy profile

See *Gibbs energy diagram, potential-energy profile*.

enforced concerted mechanism

Variation of reaction parameters in a series of reactions proceeding in non-concerted steps may lead to a situation, where the putative intermediate will possess a lifetime shorter than a bond vibration, so that the steps become concerted. The *transition state* structure will lie on the coordinate of the *More O'Ferrall-Jencks diagram* leading to that of the putative intermediate.

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enophile

See *ene reaction*.

entering group

An atom or *group* that forms a *bond* to what is considered to be the main part of the *substrate* during a reaction. For example: the attacking *nucleophile* in a bimolecular nucleophilic *substitution reaction*.

enthalpy of activation (standard entalpy of activation), $\Delta^{\ddagger}H^{o}$ (SI unit: kJ mol^{-1})

The standard enthalpy difference between the *transition state* and the ground state of the reactants at the same temperature and pressure. It is related to the temperature coefficient of the rate constant according to the equation:

$$\Delta^{\ddagger}H = RT^2(\partial \ln k/\partial T)_p - RT = E_a - RT$$

$$= -R(\partial \ln(k/T)/\partial(1/T))_p$$

where E_a is the *energy of activation*, providing that the rate constants for reactions other than first-order reactions are expressed in temperature-independent concentration units (e.g., mol dm^{-3}, measured at a fixed temperature and pressure). If ln$k$ is expressed as

$$\ln k = (a/T) + b + c \ln T + dT,$$

then

$$\Delta^{\ddagger}H = -aR + (c - 1)RT + dRT^2.$$

If enthalpy of activation and *entropy of activation* are assumed to be temperature independent, then

$$\Delta^{\ddagger}H = -aR$$

If the concentration units are mol dm^{-3}, the true and apparent enthalpies of activation differ by $(n - 1)/(\alpha RT^2)$, where n is the *order of reaction* and α the thermal expansivity.

See also *entropy of activation*, *Gibbs energy of activation*.

entropy of activation, (standard entropy of activation), $\Delta^{\ddagger}S^{o}$ (SI unit: J mol^{-1} K^{-1})

The standard entropy difference between the *transition state* and the ground state of the reactants, at the same temperature and pressure.

It is related to the *Gibbs energy of activation* and *enthalpy of activation* by the equations

$$\Delta^{\ddagger}S = (\Delta^{\ddagger}H - \Delta^{\ddagger}G)/T$$

$$= \Delta^{\ddagger}H/T - R \ln (k_B/h) + R \ln (k/T)$$

or, if ln k is expressed as $\ln k = a/T + b + c \ln T + dT$,

$$\Delta^{\ddagger}S = R [b - \ln (k_B/h) + (c - 1)(1 + \ln T) + 2\, dT]$$

provided that rate constants for reactions other than first-order reactions are expressed in temperature-independent concentration units (e.g., mol dm^{-3}, measured at a fixed temperature and pressure). The numerical value of $\Delta^{\ddagger}S$ depends on the standard state (and therefore on the concentration units selected). If entropy of activation and *enthalpy of activation* are assumed to be temperature-independent,

$$\Delta^{\ddagger}S = R[b - \ln(k_B/h)]$$

Strictly speaking, the quantity defined is the entropy of activation at constant pressure from which the entropy of activation at constant volume can be deduced.

The information represented by the entropy of activation may alternatively be conveyed by the pre-exponential factor A (see *energy of activation*).

epimer

A *diastereoisomer* that has the opposite *configuration* at only one of two or more tetrahedral "stereogenic" centres present in the respective *molecular entity*.

IUPAC STEREOCHEMICAL GLOSSARY (1993).

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epimerization

Interconversion of epimers by reversal of the configuration at one of the "stereogenic" centres.

equilibrium, chemical

Reversible processes (processes which may be made to proceed in the forward or reverse direction by the (infinitesimal) change of one variable, ultimately reach a point where the rates in both directions are identical, so that the system gives the appearance of having a static composition at which the Gibbs energy, G, is a minimum. At equilibrium the sum of the chemical potentials of the reactants equals that of the products, so that

$$\Delta G_r = \Delta G_r^\circ + RT \ln K = 0$$

$$\Delta G_r^\circ = -RT \ln K$$

The equilibrium constant, K, is given by the *mass-law effect.*

equilibrium control

See *thermodynamic control.*

equilibrium isotope effect

See *isotope effect.*

excess acidity

See *Bunnett-Olsen equations, Cox-Yates equation.*

excimer

An excited dimer, "non-bonding" in the ground state. For example, a complex formed by the interaction of an excited *molecular entity* with a ground state counterpart of this entity.

IUPAC PHOTOCHEMICAL GLOSSARY (1992).

See also *exciplex.*

exciplex

An electronically excited complex of definite stoichiometry, "non-bonding" in the ground state. For example, a complex formed by the interaction of an excited *molecular entity* with a ground state counterpart of a different structure.

IUPAC PHOTOCHEMICAL GLOSSARY (1992).

See also *excimer.*

excited state

State of a system with energy higher than that of the ground state. This term is most commonly used to characterize a molecule in one of its electronically excited states, but can also refer to vibrational and/or rotational excitation in the electronic ground state.

IUPAC ANALYTICAL CHEMISTRY (1982).

extended Hammett equation

This term applies in a general way to any multiparametric extension of the *Hammett equation.* It is sometimes used specifically for a form of *dual substituent-parameter equation* in which the actual value of the correlated property P under the influence of the substituent X is used, rather than the value relative to that for X = H. An intercept term h corresponding to the value of P for X = H is introduced, e.g.

$$P = \alpha\sigma_I + \beta\sigma_R + h$$

The equation may be applied to systems for which the inclusion of further terms to represent other effects, e.g. steric, is appropriate

CHAPMAN and SHORTER (1972, 1978); CHARTON (1987).

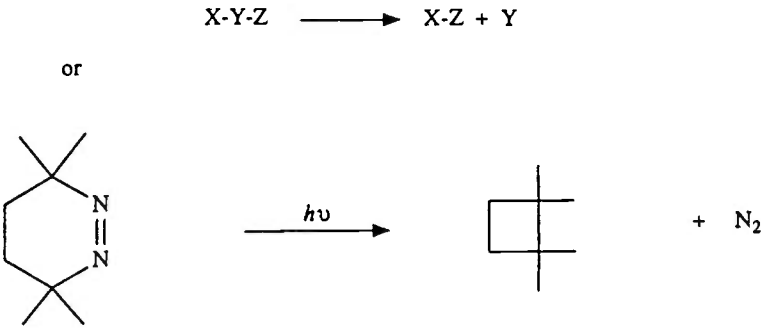
external return

See ion-pair return.

extrusion transformation

A *transformation* in which an atom or *group* Y connected to two other atoms or groups X and Z is lost from a molecule, leading to a product in which X is bonded to Z, e.g.,

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The reverse of an extrusion is called *insertion*.
See *cheletropic reaction*.

η-symbol ("eta")

See *hapto*.

fast-atom bombardment (FAB) mass spectroscopy

A method in which ions are produced in a mass spectrometer from nonvolatile or thermally fragile organic molecules by bombarding the compound in the condensed phase with energy-rich neutral particles.
IUPAC MASS SPECTROSCOPY (1991).

field effect

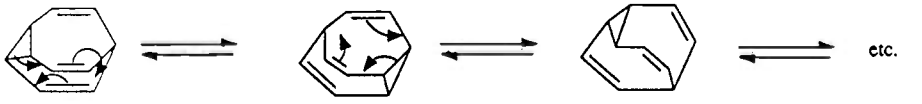
An experimentally observable effect symbolized by F (on reaction rates, etc.) of *intramolecular* coulombic interaction between the centre of interest and a remote unipole or dipole, by direct action through space rather than through bonds. The magnitude of the field effect (or "direct effect") depends on the unipolar charge/dipole moment, orientation of dipole, shortest distance between the centre of interest and the remote unipole or dipole, and on the effective dielectric constant. An approach to its calculation was described by KIRKWOOD and WESTHEIMER (1938) and has been elaborated in more recent years.
See also *electronic effect*, *inductive effect*, *polar effect*.

flash vacuum pyrolysis (FVP)

Thermal reaction of a molecule by exposing it to a short thermal shock at high temperature, usually in the gas phase.

fluxional

A *chemical species* is said to be fluxional if it undergoes rapid *degenerate rearrangements* (generally detectable by methods which allow the observation of the behaviour of individual nuclei in a rearranged chemical species, e.g. NMR, X-ray).
Example: Bullvalene (1 209 600 interconvertible arrangements of the ten CH groups).



The term is also used to designate positional change among ligands of complex compounds and organometallics. In these cases, the change is not necessarily degenerate.
See also *valence tautomerization*.

force-field calculations

See *molecular mechanics calculation*.

fractionation factor, isotopic

The ratio $(x_1/x_2)_A/(x_1/x_2)_B$, where x is the abundance, expressed as the atom fraction of the isotope distinguished by the subscript numeral, when the two isotopes are equilibrated between two different *chemical species* A and B (or between specific sites A and B in the same or different chemical species). The term is most commonly met in connection with deuterium solvent *isotope effects*, when the fractionation factor Φ expresses the ratio

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$$\Phi = (x_D/x_H)_{solute}/(x_D/x_H)_{solvent}$$

for the exchangeable hydrogen atoms in the chemical species (or sites) concerned. The concept is also applicable to *transition states.*

GOLD (1969).

fragmentation

(1) The *heterolytic* cleavage of a molecule according to the general reaction

$$a\text{-}b\text{-}c\text{-}d\text{-}X \longrightarrow (a\text{-}b)^+ + c{=}d + X^-$$

where a-b is an *electrofuge* and X is a *nucleofuge* (which may emerge from the reaction in combined form), and the middle group affords the unsaturated fragment c=d. For example,

$$Ph_3C\text{-}CO_2H + H^+ \longrightarrow Ph_3C^+ + C{=}O + H_2O$$

GROB (1969).

(2) The breakdown of a *radical* into a diamagnetic molecule or ion and a smaller radical, e.g.,

$$(CH_3)_3C\text{-}O\cdot \longrightarrow (CH_3)_2C{=}O + H_3C\cdot$$

$$[ArBr]^{\cdot -} \longrightarrow Ar\cdot + Br^- \quad \text{(solution)}$$

(3) The breakdown of a *radical ion* in a mass spectrometer or in solution, forming an ion of lower molar mass and a radical, e.g.,

$$[(CH_3)_3C\text{-}OH]^{\cdot +} \longrightarrow (CH_3)_2C{=}O^+H + H_3C\cdot \quad \text{(mass spectrometer)}$$

free radical

See *radical.*

frontier orbitals

The *H*ighest-energy *O*ccupied *M*olecular *O*rbital (HOMO) (filled or partly filled) and *L*owest-energy *U*noccupied *M*olecular *O*rbital (LUMO) (completely or partly vacant) of a *molecular entity.* Examination of the mixing of frontier molecular orbitals of reacting molecular entities affords an approach to the interpretation of reaction behaviour; this constitutes a simplified perturbation *molecular orbital* theory of chemical behaviour.

FUKUI, YONEZAWA, and SHINGU (1952); FLEMING (1976).

See also *SOMO, subjacent orbital.*

functional group

Organic compounds are thought of as consisting of a relatively unreactive backbone, for example a chain of sp^3 hybridized carbon atoms, and one or several functional groups. The functional group is an atom, or a group of atoms that has similar chemical properties whenever it occurs in different compounds. It defines the characteristic physical and chemical properties of families of organic compounds.

gas-phase acidity

The negative of the Gibbs energy (ΔG_r°) change for the reaction

$$A\text{-}H \longrightarrow A^- + H^+$$

in the gas phase.

BARTMESS and MC IVER (1979).

gas-phase basicity

The negative of the Gibbs energy (ΔG_r°) change associated with the reaction

$$B + H^+ \longrightarrow BH^+$$

in the gas phase. Also called absolute or intrinsic basicity.

See also *proton affinity.*

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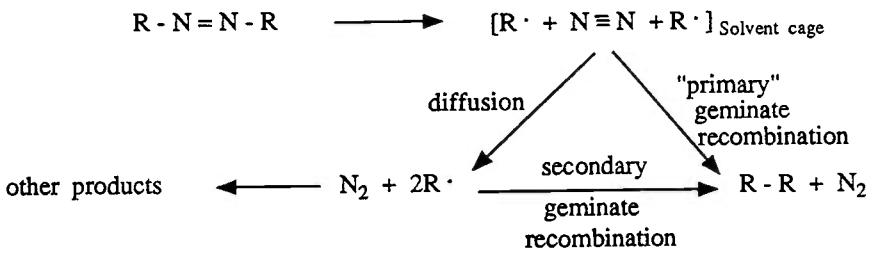
geminate pair

Pair of *molecular entities* in close proximity in solution within a solvent cage and resulting from reaction (e.g. bond scission, electron transfer, group transfer) of a precursor that constitutes a single kinetic entity.

See also *ion pair, radical pair.*

geminate recombination

The reaction with each other of two *transient molecular entities* produced from a common precursor in solution. If reaction occurs before any separation by diffusion has occurred, this is termed "primary geminate recombination". If the mutually reactive entities have been separated, and come together by diffusion, this is termed "secondary geminate recombination".



general acid catalysis

The *catalysis* of a chemical reaction by a series of *Brønsted acids* (which may include the solvated hydrogen ion) so that the rate of the catalysed part of the reaction is given by $\Sigma k_{HA}[HA]$ multiplied by some function of *substrate* concentrations. (The acids HA are unchanged by the overall reaction.) General catalysis by acids can be experimentally distinguished from *specific catalysis* by hydrogen cations (*hydrons*) by observation of the *rate of reaction* as a function of buffer concentration.

See also *catalysis, catalytic coefficient, intramolecular catalysis, pseudo-catalysis, specific catalysis.*

general base catalysis

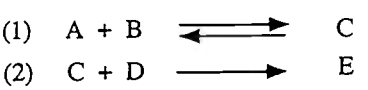
The catalysis of a *chemical reaction* by a series of *Brønsted bases* (which may include the *lyate* ion) so that the rate of the catalysed part of the reaction is given by $\Sigma k_B[B]$ multiplied by some function of *substrate* concentration.

See also *general acid catalysis.*

Gibbs energy diagram

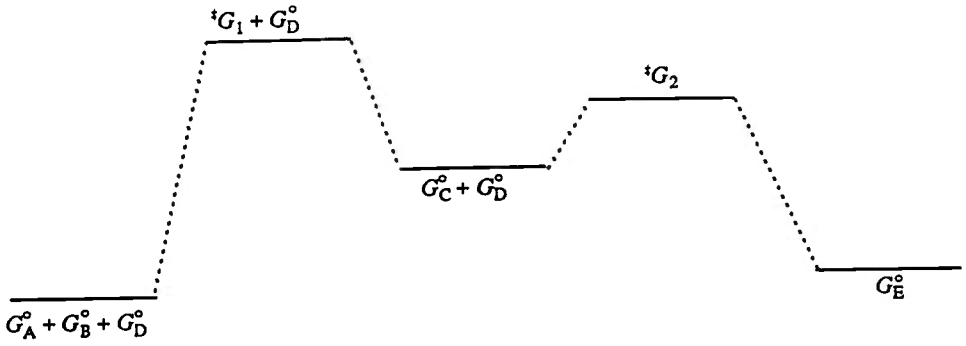
A diagram showing the relative standard Gibbs energies of reactants, *transition states*, reaction *intermediates*, and products, in the same sequence as they occur in a chemical reaction. These points are often connected by a smooth curve (a "Gibbs energy profile", commonly still referred to as a "free energy profile") but experimental observation can provide information on relative standard Gibbs energies only at the maxima and minima and not at the configurations between them. The abscissa expresses the sequence of reactants, products, reaction intermediates and transition states and is usually undefined or only vaguely defined by the *reaction coordinate* (extent of bond breaking or bond making). In some adaptations the abscissas are however explicitly defined as *bond orders*, Brønsted exponents, etc.

Contrary to statements in many text books, the highest point on a Gibbs energy diagram does not necessarily correspond to the transition state of the *rate-limiting step*. For example, in a *stepwise reaction* consisting of two reaction steps



one of the transition states of the two reaction steps must (in general) have a higher standard Gibbs energy than the other, whatever the concentration of D in the system. However, the value of that concentration will determine which of the reaction steps is rate-limiting. If the particular concentrations of interest, which may vary, are chosen as the standard state, then the rate-limiting step is the one of highest Gibbs energy.

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See also *potential energy profile, potential energy (reaction) surface, reaction coordinate.*

Gibbs energy of activation (standard free energy of activation), $\Delta^{\ddagger}G$ (SI unit: kJ mol^{-1})

The standard Gibbs energy difference between the *transition state* of a reaction (either an *elementary reaction* or a *stepwise reaction*) and the ground state of the reactants. It is calculated from the experimental rate constant k via the conventional form of the absolute rate equation:

$$\Delta^{\ddagger}G = RT\,[\ln(k_B/h) - \ln(k/T)]$$

where k_B is the Boltzmann constant and h the Planck constant ($k_B/h = 2.08358 \times 10^{10}$ K^{-1} s^{-1}). The values of the rate constants, and hence Gibbs energies of activation, depend upon the choice of concentration units (or of the thermodynamic standard state).

See also *enthalpy of activation, entropy of activation.*

ground state

The state of lowest Gibbs energy of a system.

IUPAC ANALYTICAL CHEMISTRY (1982).

See also *excited state.*

group

A defined linked collection of atoms or a single atom within a *molecular entity.* This use of the term in physical organic and general chemistry is less restrictive than the definition adopted for the purpose of nomenclature of organic compounds. [See IUPAC ORGANIC RULES (1979), Section C]

See also *substituent.*

Grunwald-Winstein equation

The *linear free energy relation*

$$\log(k_s/k_0) = mY$$

expressing the dependence of the rate of solvolysis of a substrate on ionizing power of the solvent. The rate constant k_0 applies to the reference solvent (ethanol-water, 80:20, v/v) and k_s to the solvent s, both at 25 °C. The parameter m is characteristic of the substrate and is assigned the value unity for *tert*-butyl chloride. The value Y is intended to be a quantitative measure of the *ionizing power* of the solvent s. The equation was later extended by WINSTEIN, GRUNWALD and JONES (1951) to the form

$$\log(k_s/k_0) = mY + lN$$

where N is the *nucleophilicity* of the solvent and l its susceptibility parameter. The equation has also been applied to reactions other than solvolysis. [For the definition of other Y-scales, see BENTLEY and SCHLEYER (1977), BENTLEY and LLEWELLYN (1990).]

GRUNWALD and WINSTEIN (1948); FAINBERG and WINSTEIN (1956).

See also *Dimroth-Reichardt E_T parameter, polarity, Z-value.*

guest

An organic or inorganic ion or molecule that occupies a cavity, cleft or pocket within the molecular structure of a *host molecular entity* and forms a *complex* with it or that is trapped in a cavity within the crystal structure of a host.

See also *crown, cryptand, inclusion compound.*

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half-life, $t_{1/2}$ (SI unit: s)

In a kinetic experiment, the time required for the concentration of a particular reacting *species* to fall to one-half of its initial value. (Its dependence on initial concentration depends upon the *order of reaction*. It is independent of initial concentration only for a first-order process.)

See also *lifetime*.

halochromism

Halochromism means the colour change which occurs on addition of *acid* (or *base*, or a salt) to a solution of a compound. A chemical reaction (e.g. ion formation) transforms a colourless compound into a coloured one.

FALBE and REGITZ (1990); REICHARDT, ASHARIN-FORD, and SCHAEFER. (1993).

Hammett acidity function

See *acidity function*.

Hammett equation (or Hammett relation)

The equation in the form

$$\lg(k/k_o) = \rho\sigma$$

or

$$\lg(K/K_o) = \rho\sigma$$

applied to the influence of *meta*- or *para*-substituents X on the reactivity of the functional group Y in the benzene derivative *m*- or *p*-XC_6H_4Y. k or K is the rate or equilibrium constant, respectively, for the given reaction of *m*- or *p*-XC_6H_4Y; k_o or K_o refers to the reaction of C_6H_5Y, i.e. X = H; σ is the *substituent constant* characteristic of *m*- or *p*-X; ρ is the *reaction* constant characteristic of the given reaction of Y. The equation is often encountered in a form with $\lg k_o$ or $\lg K_o$ written as a separate term on the right hand side, e.g.

$$\lg k = \rho\sigma + \lg k_o$$

or

$$\lg K = \rho\sigma + \lg K_o$$

It then signifies the intercept corresponding to X = H in a regression of $\lg k$ or $\lg K$ on σ.

HAMMETT (1940, 1970).

See also ρ-*value*, σ-*constant*, *Taft equation*, *Yukawa-Tsuno equation*.

Hammond principle (or Hammond postulate)

The hypothesis that, when a *transition state* leading to an unstable *reaction intermediate* (or product) has nearly the same energy as that intermediate, the two are interconverted with only a small reorganization of molecular structure. Essentially the same idea is sometimes referred to as "Leffler's assumption", namely, that the transition state bears the greater resemblance to the less stable species (reactant or reaction intermediate/product). Many text books and physical organic chemists, however, express the idea in Leffler's form, but attribute it to Hammond.

As a corollary, it follows that a factor stabilising a reaction *intermediate* will also stabilize the *transition state* leading to that *intermediate*.

The acronym "Bemahapothle" (Bell, Marcus, Hammond, Polanyi, Thornton, Leffler) is sometimes used in recognition of the principal contributors towards expansion of the original idea of the Hammond postulate.

HAMMOND (1955); LEFFLER (1953); WILLIAMS (1984). See also FARCASIU (1975).

See also *More O'Ferrall-Jencks diagram*.

Hansch constant

A measure of the capability of a solute for *hydrophobic* (*lipophilic*) interaction based on the partition coefficient P for distribution of the solute between octan-1-ol and water. The most general way of applying P in *correlation analysis*, QSAR, etc. is as $\log P$, but the behaviour of substituted benzene derivatives may be quantified by a substituent constant scale, π, which is defined in a way analogous to the Hammett σ scale. There are various π scales, depending on the substrate series used as reference.

HANSCH and LEO (1979).

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hapto

The hapto symbol, η with numerical superscript, provides a topological description for the bonding of hydrocarbons and other π-electron systems to metals, by indicating the connectivity between the ligand and the central atom. For example, η^3 indicates that three atoms of the *ligand* are bonded to the central atom.

IUPAC INORGANIC NOMENCLATURE (1990).

hard acid

A *Lewis acid* with an acceptor centre of low *polarizability*. Other things being approximately equal, complexes of *hard acids* and *bases* or *soft acids* and *bases* have an added stabilization (sometimes called "HSAB" rule). For example the hard O- (or N-) bases are preferred to their S- (or P-) analogues by hard acids. Conversely a "soft acid" possesses an acceptor centre of high polarizability and exhibits the reverse preference for coordination of a soft base. These preferences are not defined in a quantitative sense.

See PEARSON (1963, 1973); HO (1977).

See also *class (a) metal ion, hard base.*

hard base

A *Lewis base* with a donor centre (e.g. an oxygen atom) of low *polarizability*; the converse applies to "soft bases".

See also *hard acid.*

heat capacity of activation, $\Delta^{\ddagger}C_p^{\circ}$ (SI unit: J mol^{-1} K^{-1})

A quantity related to the temperature coefficient of $\Delta^{\ddagger}H$ (*enthalpy of activation*) and $\Delta^{\ddagger}S$ (*entropy of activation*) according to the equations:

$$\Delta^{\ddagger}C_p = (\partial\Delta^{\ddagger}H/\partial T)_p = T\,(\partial\Delta^{\ddagger}S/\partial T)_p$$

If the rate constant is expressible in the form $\ln k = a/T + b + c \ln T + dT$,

then $$\Delta^{\ddagger}C_p = (c - 1)\,R + 2dRT$$

See KOHNSTAM (1967).

+ hemileptic

See *homoleptic.*

Henderson-Hasselbach equation

An equation of the form

$$\mathrm{pH} = \mathrm{p}K_a - \lg([\mathrm{HA}]/[\mathrm{A}^-])$$

for the calculation of the pH of solutions where the ratio $[HA]/[A^-]$ is known.

heterobimetallic complex

A metal complex having two different metal atoms.

heteroconjugation

+(1) Association between a base and the conjugate acid of a different base through a *hydrogen bond* ($B'\cdots HB^+$ or $A'H\cdots A^-$). The term has its origin in the *conjugate acid-base pair* and is in no way related to *conjugation* of *orbitals*. Heteroassociation is a more appropriate term.

+(2) Some authors refer to *conjugated systems* containing a heteroatom, e.g. pyridine, as "heteroconjugated systems". This usage is discouraged since it inappropriately suggests an analogy to *homoconjugation* (2), and conflicts with the currently accepted definition of that term.

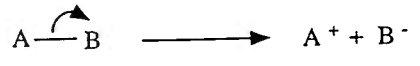
heteroleptic

Transition metal od Main Group compounds having more than one type of *ligand.*

See also *homoleptic.*

heterolysis, heterolytic

The cleavage of a *covalent bond* so that both bonding electrons remain with one of the two fragments between which the bond is broken, e.g.



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Heterolytic bond fission is a feature of many *bimolecular reactions* in solution (e.g., *electrophilic* substitution, *nucleophilic* substitution).

See also *homolysis*, *heterolytic bond-dissociation energy*.

heterolytic bond-dissociation energy

The energy required to break a given *bond* of some specific compound by *heterolysis*. For the *dissociation* of a neutral molecule AB in the gas phase into A^+ and B^- the heterolytic *bond-dissociation energy* $D(A^+B^-)$ is the sum of the bond dissociation energy, $D(A\text{-}B)$, and the adiabatic ionization energy of the radical $A^\cdot$ minus the electron affinity of the radical $B^\cdot$.

heterovalent hyperconjugation

See *hyperconjugation*.

hidden return

See *ion-pair return*.

Hildebrand parameter

A parameter measuring the cohesion of a solvent (energy required to create a cavity in the solvent).

CHASTRETTE, RAJZMANN, CHANON, and PURCELL (1985).

Hofmann rule

"The principal alkene formed in the decomposition of quaternary ammonium hydroxides that contain different primary alkyl groups is always ethylene, if an ethyl group is present." Originally given in this limited form by A.W. Hofmann, the rule has since been extended and modified as follows: "When two or more alkenes can be produced in a β-*elimination* reaction, the alkene having the smallest number of alkyl groups attached to the double bond carbon atoms will be the predominant product." This orientation described by the Hofmann rule is observed in elimination reactions of quaternary ammonium salts and tertiary sulfonium salts, and in certain other cases.

HOFMANN (1851).

See also *Zaitsev rule*.

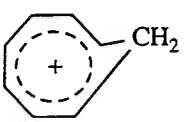
homo

(1) An acronym for *H*ighest *O*ccupied *M*olecular *O*rbital. See *frontier orbitals*.

(2) A prefix (consisting of lower case letters, homo,) used to indicate a higher homologue of a compound.

homoaromatic

Whereas in an *aromatic* molecule there is continuous overlap of p-orbitals over a cyclic array of atoms, in a homoaromatic molecule there is a formal discontinuity in this overlap resulting from the presence of a single sp^3 hybridized atom at one or several positions within the ring; p-orbital overlap apparently bridges these sp^3 centres, and features associated with aromaticity are manifest in the properties of the compound. Pronounced homoaromaticity is not normally associated with neutral molecules, but mainly with species bearing an electrical charge, e.g., the "homotropylium" cation, $C_8H_9^+$,



In bis, tris, (etc.) homoaromatic species, two, three, (etc.) single sp^3 centres separately interrupt the π-electron system.

See also *homoconjugation* (2).

homoconjugation

+ (1) Association between a base and its *conjugate acid* through a *hydrogen bond* ($B\cdots HB^+$ or $AH\cdots A^-$). Homoassociation is a more appropriate term for this phenomenon.

(2) The orbital overlap of two π-systems separated by a non-conjugating group, such as CH_2.

See also *conjugate acid-base pair*, *conjugated system*, *homoaromatic*.

homoleptic

Transition metal or Main Group compounds having only one type of *ligand* are said to be homoleptic, e.g. $TaMe_5$.

See also *heteroleptic*.

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homolysis, homolytic

The cleavage of a *bond* ("homolytic cleavage" or "homolytic fission") so that each of the molecular fragments between which the bond is broken retains one of the bonding electrons. A *unimolecular* reaction involving homolysis of a bond (not forming part of a cyclic structure) in a *molecular entity* containing an even number of (paired) electrons results in the formation of two radicals:



It is the reverse of *colligation*. Homolysis is also commonly a feature of *bimolecular substitution reactions* (and of other reactions) involving radicals and molecules.

See also *bond dissociation energy, heterolysis*.

host

A *molecular entity* that forms *complexes* with organic or inorganic guests, or a chemical species that can accommodate guests within cavities of its crystal structure. Examples include *cryptands* and *crowns* (where there are ion-dipole attractions between heteroatoms and positive ions), *hydrogen-bonded* molecules that form "clathrates" (e.g. hydroquinone and water), and host molecules of *inclusion compounds* (e.g. urea or thiourea). *van der Waals forces* and *hydrophobic interactions* bind the guest to the host molecule in clathrates and inclusion compounds.

Hückel (4*n* + 2) rule

Monocyclic planar (or almost planar) systems of trigonally (or sometimes digonally) hybridized atoms that contain (4*n* + 2) π-electrons (where *n* is a non-negative integer) will exhibit *aromatic* character. The rule is generally limited to $n = 0$-5.

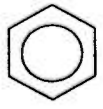
This rule is derived from the Hückel MO calculation on planar monocyclic conjugated hydrocarbons $(CH)_m$ where *m* is an integer equal to or greater than 3 according to which (4*n* + 2) π-electrons are contained in a closed-shell system. Examples of systems that obey the Hückel rule include:



Cyclopropenyl cation
($m = 3, n = 0$)



Cyclopentadienyl anion
($m = 5, n = 1$)



Benzene
($m = 6, n = 1$)

Systems containing 4*n* π-electrons (such as cyclobutadiene and the cyclopentadienyl cation) are "antiaromatic".

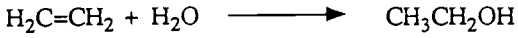
See also *conjugation, Möbius aromaticity*.

hybridization

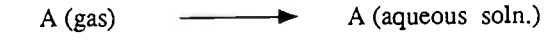
Linear combination of *atomic orbitals* on an atom. Hybrid orbitals are often used in organic chemistry to describe the bonding molecules containing tetrahedral (sp^3), trigonal (sp^2), and digonal (sp) atoms.

hydration

Addition of water or of the elements of water (i.e. H and OH) to a *molecular entity*. For example, hydration of ethene:



The term is also used in a more restricted sense for the process:



cf. the use of the term in inorganic/physical chemistry to describe the state of the ions of an electrolyte in aqueous solution.

AHRLAND (1979).

See also *aquation, solvation*.

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hydrogen bond

The hydrogen bond is a form of *association* between an electronegative atom and a hydrogen atom attached to a second, relatively electronegative atom. It is best considered as an electrostatic interaction, heightened by the small size of hydrogen, which permits proximity of the interacting dipoles or charges. Both electronegative atoms are usually (but not necessarily) from the first row of the Periodic Table, i.e., N, O, or F. Hydrogen bonds may be *intermolecular* or *intramolecular*. With a few exceptions, usually involving fluorine, the associated energies are less than 20-25 kJ mol^{-1} (5-6 kcal mol^{-1}).

hydrolysis

Solvolysis by water.

hydron

General name for the ion H^+ either in natural abundance, or where it is not desired to distinguish between the isotopes, as opposed to proton for $^1H^+$, deuteron for $^2H^+$ and triton for $^3H^+$.

IUPAC NAMES FOR HYDROGEN ATOMS (1988).

hydrophilic

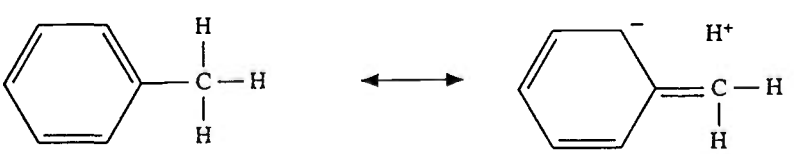
"Water loving". The capacity of a *molecular entity* or of a *substituent* to interact with polar solvents, in particular with water, or with other polar groups.

hydrophobic interaction

The tendency of hydrocarbons (or of *lipophilic* hydrocarbon-like groups in solutes) to form *intermolecular* aggregates in an aqueous *medium*, and analogous intramolecular interactions. The name arises from the attribution of the phenomenon to the apparent repulsion between water and hydrocarbons. However, the phenomenon ought to be attributed to the effect of the hydrocarbon-like groups on the water-water interaction. The misleading alternative term "hydrophobic *bond*" is discouraged.

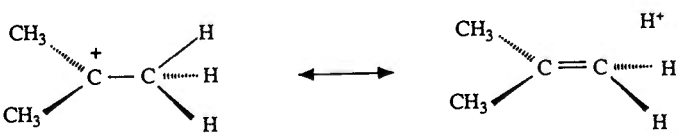
hyperconjugation

In the formalism that separates bonds into σ and π types, hyperconjugation is the interaction of σ-bonds (e.g. C-H, C-C, etc.) with a π network. This interaction is customarily illustrated by *contributing structures*, e.g. for toluene (below), sometimes said to be an example of "heterovalent" or "sacrificial hyperconjugation", so named because the contributing structure contains one two-electron bond less than the normal *Lewis formula* for toluene



At present, there is no evidence for sacrificial hyperconjugation in neutral hydrocarbons.

The concept of hyperconjugation is also applied to *carbenium ions* and *radicals*, where the interaction is now between σ-bonds and an unfilled or partially filled π- or p-orbital. A contributing structure illustrating this for the *tert*-butyl cation is:

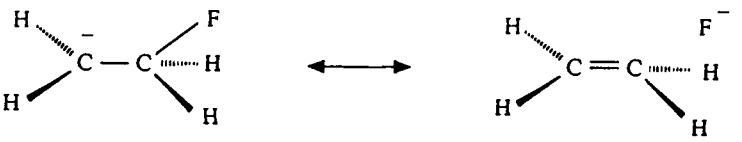


This latter example is sometimes called an example of "isovalent hyperconjugation" (the contributing structure containing the same number of two-electron bonds as the normal Lewis formula).

Both structures shown on the right hand side are also examples of "double bond- no-bond resonance".

The interaction between filled π or p orbitals and adjacent antibonding σ^* orbitals is referred to as "negative hyperconjugation", as for example in the fluoroethyl anion:

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RADOM (1982).
See also *sigma, pi,* n-σ* *delocalization.*

hypsochromic shift

Shift of a spectral band to higher frequency or shorter wavelength upon substitution or change in medium. It is informally referred to as blue shift.
IUPAC PHOTOCHEMICAL GLOSSARY (1988).
See also *bathochromic shift.*

identity reaction

A *chemical reaction* whose products are chemically identical with the reactants, for example the bimolecular self exchange reaction of CH_3I with I^-.
See also *degenerate rearrangement.*

imbalance

The situation in which *reaction* parameters that characterize different bond forming or bond breaking processes in the same reaction have developed to different extents as the *transition state* is approached along some arbitrarily defined reaction coordinate. For example, in the nitroalkane anomaly, the Brønsted β exponent for proton removal is smaller than the Brønsted α for the nitroalkane, because of imbalance between the amount of bond breaking and resonance delocalization in the transition state. Imbalance is common in reactions such as elimination, addition and other complex reactions that involve proton (hydron) transfer.
BERNASCONI (1992).
See also *synchronous, synchronization (principle of imperfect synchronization).*

+ imene

See *nitrene.*

+ imidogen

See *nitrene.*

+ imidonium ion

See *nitrenium ion.*

+ imin

See *nitrene.*

+ imine radical

See *nitrene.*

inclusion compound (or inclusion complex)

A *complex* in which one component (the *host*) forms a cavity or, in the case of a crystal, a crystal lattice containing spaces in the shape of long tunnels or channels in which molecular entities of a second *chemical species* (the *guest*) are located. There is no covalent bonding between guest and host, the attraction being generally due to *van der Waals forces.* If the spaces in the host lattice are enclosed on all sides so that the guest species is "trapped" as in a cage, such compounds are known as "clathrates" or "cage" compounds".

induction period

The initial slow phase of a *chemical reaction* which later accelerates. Induction periods are often observed with radical reactions, but they may also occur in other systems (for example before steady-state concentration of the reactants is reached).

inductive effect

In strict definition, an experimentally observable effect (on rates of reaction, etc.) of the transmission of charge through a chain of atoms by electrostatic induction. A theoretical distinction may be made between the *field effect,* and the inductive effect as models for the Coulomb interaction between a given site within a *molecular entity* and a remote unipole or

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dipole within the same entity. The experimental distinction between the two effects has proved difficult, except for molecules of peculiar geometry, which may exhibit "reversed field effects". Ordinarily the inductive effect and the field effect are influenced in the same direction by structural changes in the molecule and the distinction between them is not clear. This situation has led many authors to include the field effect in the term "inductive effect". Thus the separation of σ values into inductive and *resonance* components does not imply the exclusive operation of a through-bonds route for the transmission of the non-conjugative part of the substituent effect. To indicate the all-inclusive use of the term inductive, the phrase "so-called inductive effect" is sometimes used. Certain modern theoretical approaches suggest that the "so-called inductive effect" reflects a field effect rather than through-bonds transmission.

EHRENSON, BROWNLEE and TAFT (1973); TAFT and TOPSOM (1987).

See also *field effect, mesomeric effect, polar effect.*

inductomeric effect

A molecular polarizability effect occurring by the inductive mechanism of electron displacement. The consideration of such an effect and the descriptive term have been regarded as obsolescent or even obsolete, but in recent years theoretical approaches have reintroduced substituent polarizability as a factor governing reactivity, etc. and its parametrization has been proposed.

See TAFT and TOPSOM (1987); INGOLD (1953).

inert

Stable and *unreactive* under specified conditions.

inhibition

The decrease in *rate of reaction* brought about by the addition of a substance (inhibitor), by virtue of its effect on the concentration of a reactant, *catalyst* or *reaction intermediate*. For example, molecular oxygen and *p*-benzoquinone can react as "inhibitors" in many reactions involving *radicals* as *intermediates* by virtue of their ability to act as *scavengers* toward these radicals.

If the rate of a reaction in the absence of inhibitor is v_0 and that in the presence of a certain amount of inhibitor is v, the degree of inhibition (i) is given by

$$i = (v_0 - v)/v_0$$

See also *mechanism based inhibition.*

initiation

A reaction or process generating free *radicals* (or some other *reactive reaction intermediates*) which then induce a *chain reaction*. For example, in the chlorination of alkanes by a radical mechanism the initiation step is the *dissociation* of molecular chlorine.

inner-sphere (electron transfer)

Historically an electron transfer between two metal centres sharing a ligand or atom in their respective coordination shells. The definition has more recently been extended to any situation in which the interaction between the donor and acceptor centres in the *transition state* is significant (>20 kJ mol^{-1}).

IUPAC PHOTOCHEMICAL GLOSSARY (1992).

See also *outer-sphere electron transfer.*

insertion

A *chemical reaction* or *transformation* of the general type

$$X\text{-}Z + Y \longrightarrow X\text{-}Y\text{-}Z$$

in which the connecting atom or *group* Y replaces the bond joining the parts X and Z of the reactant XZ. An example is the carbene insertion reaction

$$R_3C\text{-}H + H_2C: \longrightarrow R_3C\text{-}CH_3$$

The reverse of an insertion is called an *extrusion.*

See also *α-addition.*

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intermediate

A *molecular entity* with a *lifetime* appreciably longer than a molecular vibration (corresponding to a local potential energy minimum of depth greater than RT) that is formed (directly or indirectly) from the reactants and reacts further to give (either directly or indirectly) the products of a chemical reaction; also the corresponding *chemical species*.

See *reaction step, elementary reaction, stepwise reaction.*

intermolecular

(1) Descriptive of any process that involves a transfer (of atoms, *groups*, electrons, etc.) or interactions between two or more *molecular entities*.

(2) Relating to a comparison between different molecular entities.

See also *intramolecular.*

internal return

See *ion-pair return.*

intimate ion pair

See *ion pair.*

intramolecular

(1) Descriptive of any process that involves a transfer (of atoms, *groups*, electrons, etc.) or interactions between different parts of the same *molecular entity*.

(2) Relating to a comparison between atoms or groups within the same molecular entity.

See also *intermolecular.*

intramolecular catalysis

The acceleration of a chemical transformation at one site of a *molecular entity* through the involvement of another *functional* ("catalytic") *group* in the same molecular entity, without that group appearing to have undergone change in the reaction product. The use of the term should be restricted to cases for which analogous *intermolecular catalysis* by *chemical species* bearing that catalytic group is observable. Intramolecular catalysis can be detected and expressed in quantitative form by a comparison of the reaction rate with that of a comparable model compound in which the catalytic group is absent, or by measurement of the *effective molarity* of the catalytic group.

See also *effective molarity, neighbouring group participation.*

intrinsic barrier

The Gibbs energy of activation ($\Delta^{\ddagger}G$) in the limiting case where $\Delta G^{o} = 0$, i.e. when the effect of thermodynamic driving force is eliminated. According to the *Marcus equation*, the intrinsic barrier is related to the *reorganization energy*, λ, of the reaction by the equation

$$\Delta^{\ddagger}G = \lambda/4$$

CANNON (1980); SCHLESENER, AMATORE and KOCHI (1986).

inverse kinetic isotope effect

See *isotope effect.*

inverted micelle

The reversible formation of association colloids from surfactants in non-polar solvents leads to aggregates termed inverted (or inverse, reverse or reversed) *micelles*. Such association is often of the type

$$\text{Monomer} \rightleftharpoons \text{Dimer} \rightleftharpoons \text{Trimer} \rightleftharpoons \ldots\ldots\ n\text{-mer}$$

and the phenomenon of *critical micelle concentration* (or an analogous effect) is consequently not observed.

In an inverted micelle the polar *groups* of the surfactants are concentrated in the interior and the *lipophilic* groups extend towards and into the non-polar solvent.

ion pair

A pair of oppositely charged ions held together by Coulomb attraction without formation of a *covalent bond*. Experimentally, an ion pair behaves as one unit in determining conductivity, kinetic behaviour, osmotic properties, etc.

Following Bjerrum, oppositely charged ions with their centres closer together than a distance

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$$q = 8.36 \times 10^6 \, z^+ z^- / (\varepsilon_r T) \text{ pm}$$

are considered to constitute an ion pair ("Bjerrum ion pair"). [z^+ and z^- are the charge numbers of the ions, and ε_r is the relative permittivity (or dielectric constant) of the medium.]

An ion pair, the constituent ions of which are in direct contact (and not separated by an intervening solvent or other neutral molecule) is designated as a "tight ion pair" (or "intimate" or "contact ion pair"). A tight ion pair of X^+ and Y^- is symbolically represented as X^+Y^-.

By contrast, an ion pair whose constituent ions are separated by one or several solvent or other neutral molecules is described as a "loose ion pair", symbolically represented as $X^+ \| Y^-$. The members of a loose ion pair can readily interchange with other free or loosely paired ions in the solution. This interchange may be detectable (e.g., by isotopic labelling) and thus afford an experimental distinction between tight and loose ion pairs.

A further conceptual distinction has sometimes been made between two types of loose ion pairs. In "solvent-shared ion pairs" the ionic constituents of the pair are separated by only a single solvent molecule, whereas in "solvent-separated ion pairs" more than one solvent molecule intervenes. However, the term "solvent-separated ion pair" must be used and interpreted with care since it has also widely been used as a less specific term for "loose" ion pair.

See also *common-ion effect, dissociation, ion-pair return, special salt effect.*

ion pair return

The recombination of a pair of ions R^+ and Z^- formed from ionization of RZ.

If the ions are paired as a tight *ion pair* and recombine without prior separation into a loose ion pair this is called "internal ion-pair return":

$$R^+Z^- \rightleftharpoons RZ$$

tight ion pair — covalent molecule

It is a special case of "primary *geminate recombination*".

If the ions are paired as a loose ion pair and form the covalent chemical species via a tight ion pair, this is called "external ion-pair return":

$$R^+ \| Z^- \rightleftharpoons R^+Z^- \rightleftharpoons RZ$$

loose ion pair — tight ion pair — covalent molecule

It is a special case of "secondary *geminate recombination*".

When the covalent molecule RZ is reformed without direct evidence of prior partial racemization or without other direct evidence of prior formation of a tight ion pair, (e.g., without partial racemization if the group R is suitably chiral) the internal ion-pair return is sometimes called a "hidden return".

External (unimolecular) ion-pair return is to be distinguished from "external (bimolecular) ion return", the (reversible) process whereby dissociated ions are converted into loose ion pairs:

$$R^+ + Z^- \rightleftharpoons R^+ \| Z^-$$

ionic strength, I (SI unit: mol dm^{-3})

In a solution of fully dissociated electrolytes the ionic strength is defined as $I = 0.5\Sigma_i c_i z_i^2$, in which c_i is the concentration and z_i the charge number of ionic species i. μ is also defined as $I_m = 0.5\Sigma_i m_i z_i^2$, where m_i is the molality.

ionization

The generation of one or more ions. It may occur, e.g. by loss of an electron from a neutral *molecular entity*, by the *unimolecular heterolysis* of such an entity into two or more ions, or by a heterolytic *substitution reaction* involving neutral molecules, such as

$$CH_3CO_2H + H_2O \longrightarrow H_3O^+ + CH_3CO_2^-$$

$$Ph_3CCl + AlCl_3 \longrightarrow Ph_3C^+ + AlCl_4^- \quad \text{(electrophile assisted)}$$

$$Ph_3CCl \longrightarrow Ph_3C^+Cl^- \quad \text{(ion pair, in benzene)}$$

The loss of an electron from a singly, doubly, etc. charged cation is called second, third, etc. ionization. This terminology is used especially in mass spectroscopy.

See also *dissociation, ionization energy.*

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ionization energy, E_i (SI unit kJ mol^{-1} or J per molecule)

The minimum energy required to remove an electron from an isolated *molecular entity* (in its vibrational ground state) in the gaseous phase. If the resulting molecular entity is considered to be in its vibrational ground state, one refers to the energy as the "adiabatic ionization energy". If the molecular entity produced possesses the vibrational energy determined by the Franck-Condon principle (according to which the electron ejection takes place without an accompanying change in molecular geometry), the energy is called the "vertical ionization energy". The name ionization energy is preferred to the somewhat misleading earlier name "ionization potential".

See also *ionization*.

ionizing power

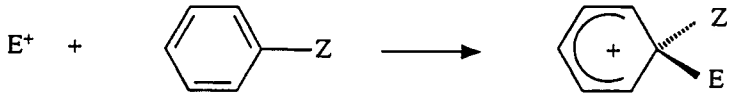
A term to denote the tendency of a particular solvent to promote *ionization* of an uncharged or, less often, charged solute. The term has been used both in a kinetic and in a thermodynamic context.

See also *Dimroth-Reichardt E_T parameter*, *Grunwald-Winstein equation*, *Z-value*.

ipso-attack

The attachment of an entering group to a position in an aromatic compound already carrying a *substituent group* (other than hydrogen). The entering group may displace that substituent group but may also itself be expelled or migrate to a different position in a subsequent step. The term "ipso-substitution" is not used, since it is synonymous with substitution.

For example:



where E$^+$ is an *electrophile* and Z is a substituent (other than hydrogen).

See also *cine-substitution*, *tele-substitution*.

isodesmic reaction

A reaction (actual or hypothetical) in which the types of bonds that are made in forming the products are the same as those which are broken in the reactants, e.g.

(a) $PhCOOH + p\text{-}ClC_6H_4COO^- \longrightarrow PhCOO^- + p\text{-}ClC_6H_4COOH$

(b) $ClCH{=}CH_2 + ClCH_2CH_2Cl \longrightarrow CH_2{=}CH_2 + Cl_2CHCH_2Cl$

Such processes have advantages for theoretical treatment. The *Hammett equation* as applied to equilibria (cf. (a)) essentially deals with isodesmic processes.

For the use of isodesmic processes in quantum chemistry, see HEHRE et al. (1970).

isoelectronic

Two or more *molecular entities* are described as isoelectronic if they have the same number of valence electrons and the same structure, i.e. number and *connectivity* of atoms, but differ in some of the elements involved. thus

CO, N_2 and NO^+ are isoelectronic

$CH_2{=}C{=}O$ and $CH_2{=}N{=}N$ are isoelectronic

CH_3COCH_3 and $CH_3N{=}NCH_3$ have the same number of electrons, but have different structures, hence they are not described as isoelectronic.

isoentropic

A reaction series is said to be isoentropic if the individual reactions of the series have the same standard *entropy of activation*.

isoequilibrium relationship

A relationship analogous to the *isokinetic relationship* but applied to equilibrium data. The equation defining the isoequilibrium temperature β is

$$\Delta_r H - \beta\Delta_r S = \text{constant}$$

where ΔH and ΔS are enthalpy and entropy of reaction, respectively.

See also *isokinetic relationship*.

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isokinetic relationship

When a series of structurally related substrates undergo the same general reaction or when the reaction conditions for a single substrate are changed in a systematic way, the *enthalpies* and *entropies of activation* sometimes satisfy the relation

$$\Delta^{\ddagger}H - \beta\Delta^{\ddagger}S = \text{constant}$$

where the parameter β is independent of temperature. This equation (or some equivalent form) is said to represent an "isokinetic relationship". The temperature $T = \beta$ (at which all members of a series obeying the isokinetic relationship react at the same rate) is termed the "isokinetic temperature".

Supposed isokinetic relationships as established by direct correlation of $\Delta^{\ddagger}H$ with $\Delta^{\ddagger}S$ are often spurious and the calculated value of β is meaningless, because errors in $\Delta^{\ddagger}H$ lead to compensating errors in $\Delta^{\ddagger}S$. Satisfactory methods of establishing such relationships have been devised.

EXNER (1973); LEFFLER (1955).

See also *compensation effect, isoequilibrium relationship, isoselective relationship.*

isolobal

The term is used to compare molecular fragments with each other and with familiar species from organic chemistry. Two fragments are isolobal if the number, symmetry properties, approximate energy, and shape of the *frontier orbitals* and the number of electrons in them are similar.

See *isoelectronic.*

isomer

One of several species (or *molecular entities*) that have the same atomic composition (molecular formula) but different line formulae or different stereochemical formulae and hence different physical and/or chemical properties.

isomerization

A *chemical reaction*, the principal product of which is isomeric with the principal reactant. An *intramolecular* isomerization that involves the breaking or making of bonds is a special case of a *molecular rearrangement.*

Isomerization does not necessarily imply *molecular rearrangement* (e.g. in the case of the interconversion of conformational isomers).

isosbestic point

This term is usually employed with reference to a set of absorption spectra, plotted on the same chart for a set of solutions in which the sum of the concentrations of two principal absorbing components, A and B, is constant. The curves of absorbance against wavelength (or frequency) for such a set of mixtures often all intersect at one or more points, called isosbestic points.

Isosbestic points are commonly met when electronic spectra are taken (a) on a solution in which a *chemical reaction* is in progress (in which case the two absorbing components concerned are a reactant and a product, A + B), or (b) on a solution in which the two absorbing components are in equilibrium and their relative proportions are controlled by the concentration of some other component, typically the concentration of hydrogen ions, e.g., an acid-base indicator equilibrium.

$$A \rightleftharpoons B + H^{+}_{aq}$$

The effect may also appear (c) in the spectra of a set of solutions of two unrelated non-interacting components having the same total concentration. In all these examples, A (and/or B) may be either a single *chemical species* or a mixture of chemical species present in invariant proportion.

If A and B are single chemical species, isosbestic points will appear at all wavelengths at which their molar absorption coefficients (formerly called extinction coefficients) are the same. (A more involved identity applies when A and B are mixtures of constant proportion.)

If absorption spectra of the types considered above intersect not at one or more isosbestic points but over progressively changing wavelength, this is prima facie evidence in case (a) for the formation of a *reaction intermediate* in substantial concentration ($A \rightarrow C \rightarrow B$), in case (b) for the involvement of a third absorbing species in the equilibrium, e.g.

$$A \rightleftharpoons B + H^{+}_{aq} \rightleftharpoons C + 2H^{+}_{aq},$$

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or in case (c) for some interaction of A and B, e.g.,

$$A + B \rightleftharpoons C$$

isoselective relationship

A relationship analogous to the *isokinetic relationship*, but applied to *selectivity* data of reactions. At the isoselective temperature, the selectivities of the series of reactions following the relationship are identical.

GIESE (1984).

See also *isoequilibrium relationship, isokinetic relationship.*

isotope effect

The effect on the rate or equilibrium constant of two reactions that differ only in the isotopic composition of one or more of their otherwise chemically identical components is referred to as a kinetic isotope effect (see *isotope effect, kinetic*) or a thermodynamic (or equilibrium) isotope effect (see *isotope effect, thermodynamic*), respectively.

isotope effect, equilibrium

see *isotope effect, thermodynamic.*

isotope effect, heavy atom

An *isotope effect* due to isotopes other than those of hydrogen.

isotope effect, intramolecular

A kinetic *isotope effect* observed when a single substrate, in which the isotopic atoms occupy equivalent reactive positions, reacts to produce a non-statistical distribution of *isotopomeric* products. In such a case the isotope effect will favor the pathway with lower force constants for displacement of the isotopic nuclei in the *transition state.*

isotope effect, inverse

A kinetic *isotope effect* which $k^l/k^h < 1$, i.e. the heavier substrate reacts more rapidly than the lighter one, as opposed to the more usual "normal" isotope effect, in which $k^l/k^h > 1$. The isotope effect will normally be "normal" when the frequency differences between the isotopic *transition states* are smaller than in the reactants. Conversely, in inverse isotope effect can be taken as evidence for an increase in the corresponding force constants on passing from the reactant to the transition state.

isotope effect, kinetic

The effect of isotopic substitution on a rate constant is referred to as a kinetic isotope effect.

For example in the reaction

$$A + B \longrightarrow C$$

the effect of isotopic substitution in reactant A is expressed as the ratio of rate constants k^l/k^h, where the superscripts l and h represent reactions in which the molecules A contain the light and heavy isotopes, respectively.

Within the framework of *transition state* theory in which the reaction is rewritten as

$$A + B \rightleftharpoons [TS]^{\ddagger} \longrightarrow C$$

and with neglect of isotopic mass on *tunnelling* and the transmission coefficient, k^l/k^h can be regarded as if it were the equilibrium constant for an isotope exchange reaction between the transition state [TS‡] and the isotopically substituted reactant A, and calculated from their vibrational frequencies as in the case of a thermodynamic isotope effect (see *isotope effect, thermodynamic*).

Isotope effects like the above, involving a direct or indirect comparison of the rates of reaction of *isotopologues*, are called "intermolecular", in contrast to intramolecular isotope effects (see *isotope effect, intramolecular*), in which a single substrate reacts to produce a non-statistical distribution of *isotopomeric* product molecules.

See WOLFSBERG (1972).

isotope effect, primary

A kinetic isotope effect attributable to isotopic substitution of an atom to which a bond is made or broken in the *rate-controlling step* or in a *pre-equilibrium* step of a specified reaction is

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termed a primary isotope effect. The corresponding isotope effect on the equilibrium constant of a reaction in which one or more bonds to isotopic atoms are broken, is called a "primary equilibrium isotope effect".

See also *isotope effect, secondary.*

isotope effect, secondary

A kinetic isotope effect that is attributable to isotopic substitution of an atom to which bonds are neither made nor broken in the *rate-controlling step* or in a *pre-equilibrium* step of a specified reaction, and is therefore not a primary isotope effect, is termed a secondary isotope effect. One speaks of α, β (etc.) secondary isotope effects, where α, β (etc.) denote the position of isotopic substitution relative to the reaction centre. The corresponding isotope effect on the equilibrium constant of such a reaction is called a "secondary equilibrium isotope effect".

Secondary isotope effects have been discussed in terms of the conventional electronic effects of physical organic chemistry, e.g. induction, *hyperconjugation, hybridization,* etc., since these properties are determined by the electron distribution, that depends on vibrationally averaged bond lengths and angles which vary slightly with isotopic substitution. While this usage is legitimate, the term "electronic isotope effect" should be avoided, because of the misleading implication that such an effect is electronic rather than vibrational in origin.

See also *isotope effect, steric.*

isotope effect, solvent

A kinetic or equilibrium isotope effect resulting from change in the isotopic composition of the solvent.

isotope effect, steric

A secondary isotope effect attributed to the different vibrational amplitudes of *isotopologues.* For example, both the mean and mean-square amplitudes of vibrations associated with C-H bonds are greater than those of C-D bonds. The greater effective bulk of molecules containing the former may be manifested by a *steric effect* on a rate or equilibrium constant.

isotope effect, thermodynamic

The effect of isotopic substitution on an equilibrium constant is referred to as a thermodynamic (or equilibrium) isotope effect.

For example, the effect of isotopic substitution in reactant A that participates in the equilibrium:

$$A + B \rightleftharpoons C$$

is the ratio K^l/K^h of the equilibrium constant for the reaction in which A contains the light isotope to that in which it contains the heavy isotope. The ratio can be expressed as the equilibrium constant for the isotopic exchange reaction:

$$A^l + C^h \rightleftharpoons A^h + C^l$$

in which reactants such as B that are not isotopically substituted do not appear.

The potential energy surfaces of isotopic molecules are identical to a high degree of approximation, so thermodynamic isotope effects can only arise from the effect of isotopic mass on the nuclear motions of the reactants and products, and can be expressed quantitatively in terms of partition function ratios for nuclear motion:

$$\frac{K^l}{K^h} = \frac{(Q^l_{nuc} / Q^h_{nuc})_C}{(Q^l_{nuc} / Q^h_{nuc})_A}$$

Although the nuclear partition function is a product of the translational, rotational and vibrational partition functions, the isotope effect is determined almost entirely by the last named, specifically by vibrational modes involving motion of isotopically different atoms. In the case of light atoms (i.e. protium *vs.* deuterium or tritium) at moderate temperatures, the isotope effect is dominated by zero-point energy differences.

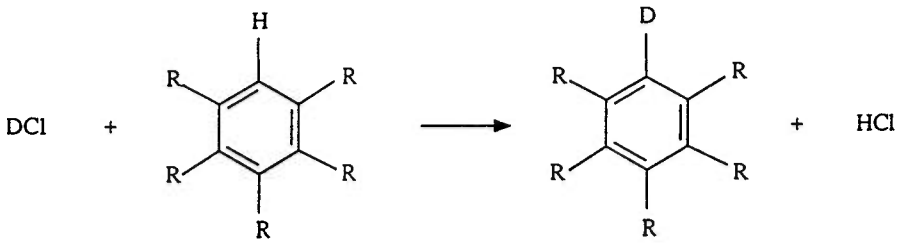
WOLFSBERG (1972).

See also *fractionation factor.*

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isotope exchange

A *chemical reaction* in which the reactant and product *chemical species* are chemically identical but have different isotopic composition. In such a reaction the isotope distribution tends towards equilibrium (as expressed by fractionation factors) as a result of transfers of isotopically different atoms or groups. For example,



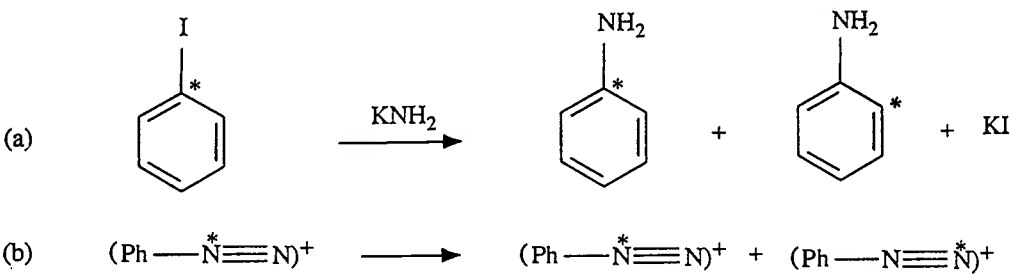
isotopic perturbation, method of

NMR shift difference measurement of the isotope effect on a fast (degenerate) equilibrium between two species which are equivalent except for isotopic substitution. This can be used to distinguish a rapidly equilibrating mixture with time-averaged symmetry from a single structure with higher symmetry.

SIEHL (1987).

isotopic scrambling

The achievement, or the process of achieving, an equilibrium distribution of isotopes within a specified set of atoms in a *chemical species* or group of chemical species. For example,



(* denotes position of an isotopically different atom.)

See also *fractionation factor.*

isotopologue

A *molecular entity* that differs only in isotopic composition (number of isotopic substitutions), e.g. CH_4, CH_3D, CH_2D_2... .

isotopomer

Isomers having the same number of each isotopic atom but differing in their positions. The term is a contraction of "isotopic isomer".

Isotopomers can be either constitutional isomers (e.g. $CH_2DCH{=}O$ and $CH_3CD{=}O$) or isotopic stereoisomers (e.g. (*R*)- and (*S*)-CH_3CHDOH or (*Z*)- and (*E*)-$CH_3CH{=}CHD$).

IUPAC STEREOCHEMICAL TERMINOLOGY (1993).

isovalent hyperconjugation

See *hyperconjugation.*

Kamlet-Taft solvent parameters

Parameters of the Kamlet-Taft solvatochromic relationship which measure separately the hydrogen bond donor (α), hydrogen bond acceptor (β), and dipolarity/*polarizability* (π^*) properties of solvents as contributing to overall solvent *polarity.*

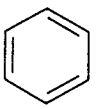
KAMLET, ABBOUD and TAFT (1981).

Kekulé structure (for aromatic compounds)

A representation of an *aromatic molecular entity* (such as benzene), with fixed alternating single and double bonds, in which interactions between multiple bonds are assumed to be absent.

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For benzene,

 and 

are the Kekulé structures.

kinetic control (of product composition)

The term characterizes conditions (including reaction times) that lead to reaction products in a proportion governed by the relative rates of the parallel (forward) reactions in which the products are formed, rather than by the respective overall equilibrium constants.

See also *thermodynamic control.*

kinetic electrolyte effect (kinetic ionic-strength effect)

The general effect of an added electrolyte (i.e. an effect other than, or in addition to, that due to its possible involvement as a reactant or catalyst) on the observed rate constant of a reaction in solution. At low concentrations (when only long-range coulombic forces need to be considered) the effect on a given reaction is determined only by the *ionic strength* of the solution and not by the chemical identity of the ions. For practical purposes, this concentration range is roughly the same as the region of validity of the Debye-Hückel limiting law for activity coefficients. At higher concentrations, the effect of an added electrolyte depends also on the chemical identity of the ions. Such specific action can usually be interpreted as the incursion of a *reaction path* involving an ion of the electrolyte as reactant or catalyst, in which case the action is not properly to be regarded just as a kinetic electrolyte effect.

Kinetic electrolyte effects are usually (too restrictively and therefore incorrectly) referred to as "kinetic salt effects".

A kinetic electrolyte effect ascribable solely to the influence of the ionic strength on activity coefficients of ionic reactants and transition states is called a "primary kinetic electrolyte effect".

A kinetic electrolyte effect arising from the influence of the ionic strength of the solution upon the *pre-equilibrium* concentration of an ionic species that is involved in a subsequent *rate-limiting step* of a reaction is called a "secondary kinetic electrolyte effect". A common case encountered in practice is the effect on the concentration of hydrogen ion (acting as catalyst) produced from the *ionization* of a weak acid in a buffer solution.

See also *common-ion effect, order of reaction.*

kinetic ambiguity

Synonymous with *kinetic equivalence.*

kinetic equivalence

Two reaction schemes are kinetically equivalent if they imply the same *rate law.*

For example, consider the two schemes (i) and (ii) for the formation of C from A:

$$\text{(i)} \qquad A \underset{k_{-1},\ OH^-}{\overset{k_1,\ OH^-}{\rightleftharpoons}} B \xrightarrow{k_2} C$$

Providing that B does not accumulate as a *reaction intermediate.*

$$\frac{d[C]}{dt} = \frac{k_1 k_2 [A]\,[OH^-]}{k_2 + k_{-1}[OH^-]} \qquad (1)$$

$$\text{(ii)} \qquad A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow[OH^-]{k_2} C$$

Providing that B does not accumulate as a reaction intermediate

$$\frac{d[C]}{dt} = \frac{k_1 k_2 [A]\,[OH^-]}{k_{-1} + k_2 [OH^-]} \qquad (2)$$

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Both equations for $d[C]/dt$ are of the form

$$\frac{d[C]}{dt} = \frac{r\,[A]\,[OH^-]}{1 + s\,[OH^-]} \qquad (3)$$

where r and s are constants (sometimes called "coefficients in the rate equation"). The equations are identical in their dependence on concentrations and do not distinguish whether OH^- catalyses the formation of B, and necessarily also its reversion to A, or is involved in its further transformation to C. The two schemes are therefore kinetically equivalent under conditions to which the stated provisos apply.

kinetic isotope effect

See *isotope effect.*

Koppel-Palm solvent parameters

Parameters to measure separately the ability of a solvent to enter into non-specific solvent-solute interactions (permittivity ε and refractive index n_D) and specific solvent-solute interaction (solvent basicity or nucleophilicity B and solvent acidity or electrophilicity E) as contributing to overall solvent polarity.

KOPPEL and PALM (1972).

Kosower *Z*-value

See *Z-value.*

labile

The term has loosely been used to describe a relatively *unstable* and *transient chemical species* or (less commonly) a relatively *stable* but reactive species. It must therefore not be used without explanation of the intended meaning.

See also *inert, persistent, reactive, unreactive.*

least nuclear motion, principle of

The hypothesis that, for given reactants, the reactions involving the smallest change in nuclear positions will have the lowest *energy of activation.* (It is also often simply referred to as principle of least motion.)

HINE (1977).

leaving group

An atom or *group* (charged or uncharged) that becomes detached from an atom in what is considered to be the residual or main part of the *substrate* in a specified reaction.

For example, in the heterolytic *solvolysis* of benzyl bromide in acetic acid

$$PhCH_2Br + AcOH \longrightarrow PhCH_2OAc + HBr$$

the leaving group is Br^- ; in the reaction

$$MeS^- + PhCH_2N^+Me_3 \longrightarrow MeSCH_2Ph + NMe_3$$

the leaving group is NMe_3; in the *electrophilic* nitration of benzene, it is H^+. The term has meaning only in relation to a specified reaction. The leaving group is not, in general, the same as the *substituent group* present in the substrate (e.g. bromo and trimethylammonio in the substrates of the first two examples above.)

A slightly different usage of the term prevails in the (non-mechanistic) naming of transformations, where the actual substituent group present in the substrate (and also in the product) is referred to as the leaving group.

See also *electrofuge, entering group, nucleofuge.*

Leffler's assumption

See *Hammond principle.*

left-to-right convention

Arrangement of the structural formulae of the reactants so that the bonds to be made or broken form a linear array in which the electrons move from left to right.

IUPAC REACTION MECHANISMS (1989).

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levelling effect

The tendency of a solvent to make all *Brønsted acids* whose *acidity* exceeds a certain value appear equally acidic. It is due to the complete transfer to a *protophilic* solvent of a hydron from a dissolved acid stronger than the *conjugate acid* of the solvent. The only acid present to any significant extent in all such solutions is the *lyonium ion*. For example, the solvent water has a levelling effect on the acidities of $HClO_4$, HCl, and HI: aqueous solutions of these acids at the same (moderately low) concentrations have the same acidities. A corresponding levelling effect applies to strong bases in *protogenic* solvents.

Lewis acid

A *molecular entity* (and the corresponding *chemical species*) that is an electron-pair acceptor and therefore able to react with a *Lewis base* to form a *Lewis adduct*, by sharing the electron pair furnished by the Lewis base. For example:

$$\underset{\text{Lewis acid}}{Me_3B} + \underset{\text{Lewis base}}{:NH_3} \longrightarrow \underset{\text{Lewis adduct}}{Me_3B\text{-}\overset{-}{N}H_3\overset{+}{}}$$

See also *coordination*, *dipolar bond*.

Lewis acidity

The thermodynamic tendency of a substrate to act as a *Lewis acid*. Comparative measures of this property are provided by the equilibrium constants for Lewis adduct formation of a series of Lewis acids with a common reference *Lewis base*.

See also *acceptor number (AN)*, *electrophilicity*.

Lewis adduct

The *adduct* formed between a *Lewis acid* and a *Lewis base*.

Lewis base

A *molecular entity* (and the corresponding *chemical species*) able to provide a pair of electrons and thus capable of *coordination* to a *Lewis acid*, thereby producing a *Lewis adduct*.

Lewis basicity

The thermodynamic tendency of a substance to act as a *Lewis base*. Comparative measures of this property are provided by the equilibrium constants for *Lewis adduct* formation for a series of Lewis bases with a common reference *Lewis acid*.

See also *donor number (DN)*, *nucleophilicity*.

Lewis formula (electron dot or Lewis structure)

Molecular structure in which the valency electrons are shown as dots so placed between the bonded atoms that one pair of dots represents two electrons or one *covalent* (single) *bond*, e.g.

$$H : \underset{\cdot\cdot}{\overset{\cdot\cdot}{Cl}} :$$

A double bond is represented by two pairs of dots, etc. Dots representing non-bonded outer-shell electrons are placed adjacent to the atoms with which they are associated, but not between the atoms. Formal charges (e.g. +, -, 2+, etc.) are attached to atoms to indicate the difference between the positive nuclear charge (atomic number) and the total number of electrons (including those in the inner shells), on the formal basis that bonding electrons are shared equally between atoms they join. (Bonding pairs of electrons are usually denoted by lines, representing *covalent bonds*, as in *line formulae*.)

lifetime (mean lifetime), τ

The lifetime of a *chemical species* which decays in a first-order process is the time needed for a concentration of this species to decrease to 1/e of its original value. Statistically, it represents the mean life expectancy of an excited species. In a reacting system in which the decrease in concentration of a particular chemical species is governed by a first-order *rate law*, it is equal to the reciprocal of the sum of the (pseudo)unimolecular rate constants of all processes which cause the decay. When the term is used for processes which are not first order, the lifetime depends on the initial concentration of the species, or of a quencher, and should be called apparent lifetime instead.

See also *chemical relaxation*, *half-life*, *rate of reaction*.

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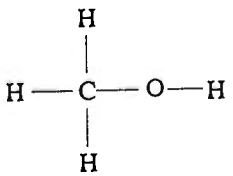
ligand

If it is possible to indicate a "central atom" in a polyatomic *molecular entity*, the atoms or *groups* bound to that atom are called ligands. (Cf. IUPAC INORGANIC NOMENCLATURE (1990); Rule I-10.2.3.) The term is generally used in connection with metallic "central atoms".

In biochemistry a part of a polyatomic molecular entity may be considered central, and atoms, groups or molecules bound to that part are considered ligands (Cf. BIOCHEMICAL NOMENCLATURE (1992)).

line formula

A two-dimensional representation of *molecular entities* in which atoms are shown joined by lines representing single or multiple bonds, without any indication or implication concerning the spatial direction of bonds. For example, methanol is represented as



(The term should not be confused with the representation of chemical formulae by the "Wiswesser line notation", a method of string notation. Formulae in this notation are also known as "Wiswesser line formulae".)

linear free-energy relation

A linear correlation between the logarithm of a rate constant or equilibrium constant for one series of reactions and the logarithm of the rate constant or equilibrium constant for a related series of reactions. Typical examples of such relations (also known as linear Gibbs energy relations) are the *Brønsted relation*, and the *Hammett equation* (see also *σ-value*).

The name arises because the logarithm of an equilibrium constant (at constant temperature and pressure) is proportional to a standard free energy (Gibbs energy) change, and the logarithm of a rate constant is a linear function of the free energy (Gibbs energy) of activation.

It has been suggested (IUPAC PHYSICAL ORGANIC GLOSSARY (1983)) that this name should be replaced by *Linear Gibbs Energy Relation*, but at present there is little sign of acceptance of this change.

The area of physical organic chemistry which deals with such relations is commonly referred to as "Linear Free-Energy Relationships".

linear Gibbs energy relation

See *linear free-energy relation*.

linear solvation energy relationships

Equations involving the application of *solvent parameters* in linear or multiple (linear) regression expressing the solvent effect on the rate or equilibrium constant of a reaction.

See *Dimroth-Reichardt E_T parameter*, *Kamlet-Taft solvent parameter*, *Koppel-Palm solvent parameter*, *Z-value*.

line-shape analysis

Determination of rate constants for a chemical exchange from the shapes of spectroscopic lines of dynamic processes. The method is most often used in nuclear magnetic resonance spectroscopy.

Lineweaver-Burk plot

See *Michaelis-Menten kinetics*.

lipophilic

Literally "fat-loving". Applied to *molecular entities* (or parts of molecular entities) having a tendency to dissolve in fat-like (e.g. hydrocarbon) solvents.

See also *hydrophilic*, *hydrophobic interaction*.

London forces

Attractive forces between apolar molecules, due to their mutual polarizability. They are also components of the forces between polar molecules. Also called "dispersion forces".

See also *van der Waals forces*.

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lone (electron) pair

Two paired electrons localized in the valence shell on a single atom. Lone pairs should be designated with two dots.

The term "nonbonding electron pair" is more appropriate, and is found in many modern text books.

loose ion pair

See *ion pair.*

LUMO

See *frontier orbitals.*

lyate ion

The anion produced by *hydron* removal from a solvent molecule. For example, the hydroxide ion is the lyate ion of water.

lyonium ion

The cation produced by hydronation of a solvent molecule. For example, $CH_3OH_2^+$ is the lyonium ion of methanol.

See also *onium ion.*

macroscopic diffusion control

See *mixing control.*

magic acid

See *superacid.*

magnetic equivalence

Nuclei having the same resonance frequency in nuclear magnetic resonance spectroscopy and also identical spin-spin interactions with the nuclei of a neighbouring group are magnetically equivalent. The spin-spin interaction between magnetically equivalent nuclei does not appear, and thus has no effect on the multiplicity of the respective NMR signals. Magnetically equivalent nuclei are necessarily also chemically equivalent, but the reverse is not necessarily true.

magnetization transfer

NMR method for determining kinetics of chemical exchange by perturbing the magnetization of nuclei in a particular site or sites and following the rate at which magnetic equilibrium is restored. The most common perturbations are saturation and inversion, and the corresponding techniques are often called "saturation transfer" and "selective inversion-recovery".

See also *saturation transfer.*

Marcus equation

A general expression which correlates the Gibbs energy of activation ($\Delta^{\ddagger}G$) with the driving force ($\Delta_r G^{o\prime}$) of the reaction:

$$\Delta^{\ddagger}G = (\lambda/4)(1 + \Delta_r G^{o\prime}/\lambda)^2$$

where λ is the *reorganization energy* and $\Delta_r G^{o\prime}$ is the standard free energy of the reaction corrected for the electrostatic work required to bring the reactants together. $\lambda/4$ is the *intrinsic barrier of the reaction.* Originally developed for *outer-sphere electron transfer* reactions, the Marcus equation has later been applied also to atom and group transfer reactions.

MARCUS (1964); ALBERY (1980).

Markownikoff rule

"In the addition of hydrogen halides to unsymmetrically constituted [unsaturated] hydrocarbons, the halogen atom becomes attached to the carbon bearing the lesser number of hydrogen atoms." Originally formulated by Markownikoff (Markovnikov) to generalize the orientation in additions of hydrogen halides to simple alkenes, this rule has been extended to polar *addition reactions* as follows. " In the *heterolytic* addition of a polar molecule to an alkene or alkyne, the more electronegative (nucleophilic) atom (or part) of the polar molecule becomes attached to the carbon atom bearing the smaller number of hydrogen atoms."

This is an indirect statement of the common mechanistic observation, that the more electropositive (electrophilic) atom (or part) of the polar molecule becomes attached to the end of the multiple bond that would result in the more stable *carbenium ion* (whether or not a carbe-

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nium ion is actually formed as a *reaction intermediate* in the addition reaction). Addition in the opposite sense is commonly called "anti-Markovnikov addition".

MARKOWNIKOFF (1870).

mass-law effect

At equilibrium, the product of the activities (or concentrations) of the reacting species is constant. Thus for the equilibrium

$$\alpha A + \beta B \rightleftharpoons \gamma C + \delta D$$

$$K = [C]^{\gamma} [D]^{\delta} / [A]^{\alpha} [B]^{\beta}$$

GULDBERG and WAAGE (1879).

See also *common-ion effect, equilibrium.*

matrix isolation

A term which refers to the isolation of a reactive or unstable species by dilution in an inert matrix (argon, nitrogen, etc.), usually condensed on a window or in an optical cell at low temperature, to preserve its structure for identification by spectroscopic or other means.

IUPAC ATMOSPHERIC GLOSSARY (1990).

mean lifetime

See *lifetime.*

mechanism

A detailed description of the process leading from the reactants to the products of a reaction, including a characterization as complete as possible of the composition, structure, energy and other properties of *reaction intermediates*, products, and *transition states*. An acceptable mechanism of a specified reaction (and there may be a number of such alternative mechanisms not excluded by the evidence) must be consistent with the reaction stoichiometry, the *rate law*, and with all other available experimental data, such as the stereochemical course of the reaction. Inferences concerning the electronic motions which dynamically interconvert successive species along the *reaction path* (as represented by curved arrows, for example) are often included in the description of a mechanism.

It should be noted that for many reactions all this information is not available and the suggested mechanism is based on incomplete experimental data. It is not appropriate to use the term mechanism to describe a statement of the probable sequence in a set of stepwise reactions. That should be referred to as a reaction sequence, and not a mechanism.

See also *Gibbs energy diagram.*

mechanism-based inhibition

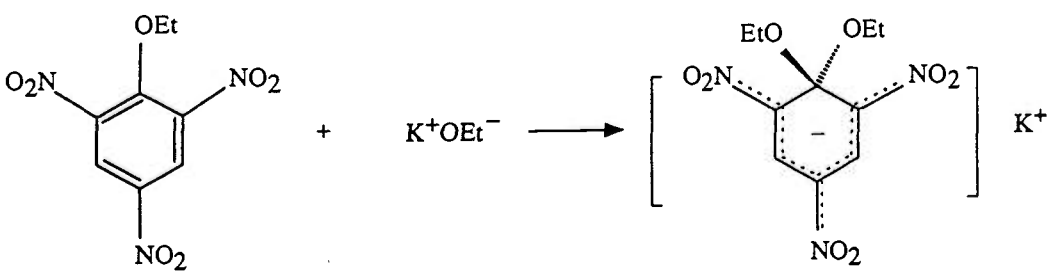
Irreversible *inhibition* of an enzyme due to its catalysis of the reaction of an artificial substrate. Also called "suicide inhibition".

medium

The phase (and composition of the phase) in which *chemical species* and their reactions are studied in a particular investigation.

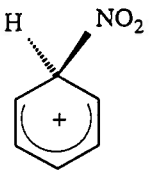
Meisenheimer adduct

A cyclohexadienyl derivative formed as *Lewis adduct* from a *nucleophile* (*Lewis base*) and an *aromatic* or heteroaromatic compound, also called Jackson-Meisenheimer adduct. In earlier usage the term "Meisenheimer complex" was restricted to the typical Meisenheimer alkoxide *adducts* of nitro-substituted aromatic ethers, e.g.,



Analogous cationic adducts, such as

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considered to be *reaction intermediates* in *electrophilic* aromatic *substitution reactions*, are called "Wheland intermediates", and sometimes, inappropriately, σ-complexes.

JACKSON and GAZZOLO (1900); BUNCEL, CRAMPTON, STRAUSS and TERRIER. (1984).

See also σ-*adduct*.

melting point (corrected/uncorrected)

The term originally signified that a correction was made (not made) for the emergent stem of the thermometer. In current usage it often means that the accuracy of the thermometer was (was not) verified. This current usage is inappropriate and should be abandoned.

mesolytic cleavage

Cleavage of a bond in a *radical ion* whereby a *radical* and an ion are formed. The term reflects the mechanistic duality of the process, which can be viewed as homolytic or heterolytic depending on how the electrons are attributed to the fragments.

See MASLAK and NARVAEZ (1990).

mesomeric effect

The effect (on reaction rates, ionization equilibria, etc.) attributed to a substituent due to overlap of its p- or π-orbitals with the p- or π-orbitals of the rest of the *molecular entity*. *Delocalization* is thereby introduced or extended, and electronic charge may flow to or from the substituent. The effect is symbolized by M.

Strictly understood, the mesomeric effect operates in the ground electronic state of the molecule. When the molecule undergoes electronic excitation or its energy is increased on the way to the *transition state* of a *chemical reaction*, the mesomeric effect may be enhanced by the *electromeric effect*, but this term is not much used, and the mesomeric and electromeric effects tend to be subsumed in the term *resonance effect* of a *substituent*.

See also *electronic effect*, *field effect*, *inductive effect*.

mesomerism

Essentially synonymous with *resonance*. The term is particularly associated with the picture of π-electrons as less localized in an actual molecule than in a *Lewis formula*.

The term is intended to imply that the correct representation of a structure is intermediate between two or more Lewis formulae.

See also *aromatic* (2), *delocalization*.

mesophase

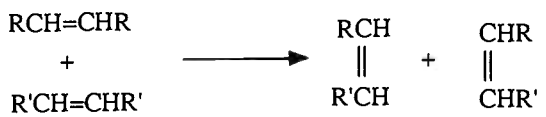
The phase of a liquid crystalline compound between the crystalline and the isotropic liquid phase.

metastable (chemical species)

See *transient (chemical species)*.

metathesis

A *bimolecular* process formally involving the exchange of a bond (or bonds) between similar interacting *chemical species* so that the bonding affiliations in the products are identical (or closely similar) to those in the reactants. For example:



(The term has its origin in inorganic chemistry with a different meaning, but this older usage is not applicable in physical organic chemistry.)

+ methylene

See *carbene*.

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+ methylidyne

See *carbyne*.

micellar catalysis

The acceleration of a *chemical reaction* in solution by the addition of a surfactant at a concentration higher than its *critical micelle concentration* so that the reaction can proceed in the environment of surfactant aggregates (*micelles*). (Rate enhancements may be due, for example, to higher concentration of the reactants in that environment, more favourable orientation and solvation of the species, or enhanced rate constants in the micellar pseudophase of the surfactant aggregate.) Micelle formation can also lead to a decreased reaction rate.

See also *catalyst*.

micelle

Surfactants in solution are often association colloids, that is, they tend to form aggregates of colloidal dimensions, which exist in equilibrium with the molecules or ions from which they are formed. Such aggregates are termed micelles.

See also *inverted micelle*.

IUPAC MANUAL APPENDIX II (1972).

Michaelis-Menten kinetics

The dependence of an initial *rate of reaction* upon the concentration of a *substrate* S that is present in large excess over the concentration of an enzyme or other *catalyst* (or reagent) E with the appearance of saturation behaviour following the Michaelis-Menten equation,

$$v = V[S]/(K_m + [S]),$$

where v is the observed initial rate, V is its limiting value at substrate saturation (i.e., $[S] >> K_m$), and K_m the substrate concentration when $v = V/2$. The definition is experimental, i.e., it applies to any reaction that follows an equation of this general form. The symbols V_{max} or v_{max} are sometimes used for V.

The parameters V and K_m (the "Michaelis constant") of the equation can be evaluated from the slope and intercept of a linear plot of v^{-1} against $[S]^{-1}$ (a "Lineweaver-Burk plot") or from slope and intercept of a linear plot of v against $v/[S]$ ("Eadie-Hofstee plot").

A Michaelis-Menten equation is also applicable to the condition where E is present in large excess, in which case the concentration [E] appears in the equation instead of [S].

The term has sometimes been used to describe reactions that proceed according to the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} \text{Products}$$

in which case $K_m = (k_{-1} + k_{cat})/k_1$ (Briggs-Haldane conditions). It has more usually been applied only to the special case in which $k_{-1} >> k_{cat}$ and $K_m = k_{-1}/k_1 = K_s$; in this case K_m is a true dissociation constant (Michaelis-Menten conditions).

See also *rate-determining step*.

microscopic chemical event

See *chemical reaction, molecularity*.

microscopic diffusion control (encounter control)

The observable consequence of the limitation that the rate of a bimolecular *chemical reaction* in a homogeneous medium cannot exceed the rate of encounter of the reacting *molecular entities*.

If (hypothetically) a *bimolecular* reaction in a homogeneous medium occurred instantaneously when two reactant molecular entities made an encounter, the *rate of reaction* would be an *encounter-controlled rate*, determined solely by rates of diffusion of reactants. Such a hypothetical "fully diffusion controlled rate" is also said to correspond to "total microscopic diffusion control", and represents the asymptotic limit of the rate of reaction as the rate constant for the chemical conversion of the encounter pair into product (or products) becomes large relative to the rate constant for separation (or dissociation) of the encounter pair.

"Partial microscopic diffusion control" is said to operate in a homogeneous reaction when the rates of chemical conversion and of separation are comparable. (The degree of microscopic diffusion control cannot usually be determined with any precision.)

See also *mixing control*.

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microscopic reversibility, principle of

In a reversible reaction, the mechanism in one direction is exactly the reverse of the mechanism in the other direction. This does not apply to reactions that begin with a photochemical excitation.

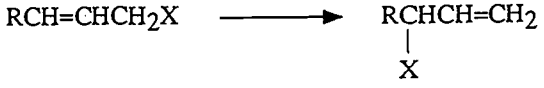
See also *chemical reaction, detailed balancing.*

migration

(1) The (usually *intramolecular*) transfer of an atom or *group* during the course of a *molecular rearrangement.*

(2) The movement of a *bond* to a new position, within the same *molecular entity*, is known as "bond migration".

Allylic rearrangements, e.g.,



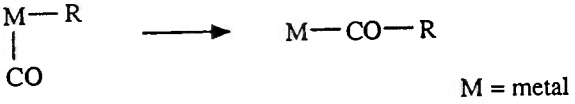
exemplify both types of migration.

migratory aptitude

The term is applied to characterize the relative tendency of a group to participate in a rearrangement. In nucleophilic rearrangements (*migration* to an electron-deficient centre), the migratory aptitude of a group is loosely related to its capacity to stabilize a partial positive charge, but exceptions are known, and the position of hydrogen in the series is often unpredictable.

migratory insertion

A combination of *migration* and *insertion.* The term is mainly used in organometallic chemistry.



minimum structural change, principle of

See *molecular rearrangement.*

mixing control

The experimental limitation of the *rate of reaction* in solution by the rate of mixing of solutions of the two reactants. It can occur even when the reaction rate constant is several powers of 10 less than that for an *encounter-controlled* rate. Analogous (and even more important) effects of the limitation of reaction rates by the speed of mixing are encountered in heterogeneous (solid/liquid, solid/gas, liquid/gas) systems.

See also *microscopic diffusion control, stopped flow.*

Möbius aromaticity

A monocyclic array of orbitals in which there is a single out-of-phase overlap (or, more generally, an odd number of out-of-phase overlaps) reveals the opposite pattern of *aromatic* character to Hückel systems; with $4n$ electrons it is stabilized (aromatic), whereas with $4n + 2$ it is destabilized (antiaromatic). In the excited state $4n + 2$ Möbius π-electron systems are stabilized, and $4n$ systems are destabilized. No examples of ground-state Möbius π-systems are known, but the concept has been applied to *transition states* of *pericyclic reactions* [see *aromatic* (3)].

The name is derived from the topological analogy of such an arrangement of orbitals to a Möbius strip.

HEILBRONNER (1964); ZIMMERMAN (1971).

See also *Hückel (4n + 2) rule.*

moiety

In physical organic chemistry moiety is generally used to signify part of a molecule, e.g. in an ester R^1COOR^2 the alcohol moiety is R^2O. The term should not be used for a small fragment of a molecule.

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molecular entity

Any constitutionally or isotopically distinct atom, *molecule*, ion, *ion pair*, *radical*, *radical ion*, *complex*, conformer etc., identifiable as a separately distinguishable entity.

Molecular entity is used in this glossary as a general term for singular entities, irrespective of their nature, while *chemical species* stands for sets or ensembles of molecular entities. Note that the name of a compound may refer to the respective molecular entity or to the chemical species, e.g. methane, may mean a single molecule of CH_4 (molecular entity) or a molar amount, specified or not (chemical species), participating in a reaction.

The degree of precision necessary to describe a molecular entity depends on the context. For example "hydrogen molecule" is an adequate definition of a certain molecular entity for some purposes, whereas for others it is necessary to distinguish the electronic state and/or vibrational state and/or nuclear spin, etc. of the hydrogen molecule.

molecular mechanics calculation

An empirical calculational method intended to give estimates of structures and energies for conformations of molecules. The method is based on the assumption of "natural" bond lengths and angles, deviation from which leads to strain, and the existence of torsional interactions and attractive and/or repulsive *van der Waals* and dipolar forces between non-bonded atoms. The method is also called "(empirical) force-field calculations".

BURKERT and ALLINGER (1982).

molecular metal

A non-metallic material whose properties resemble those of metals, usually following oxidative doping; e.g. polyacetylene following oxidative doping with iodine.

molecular orbital

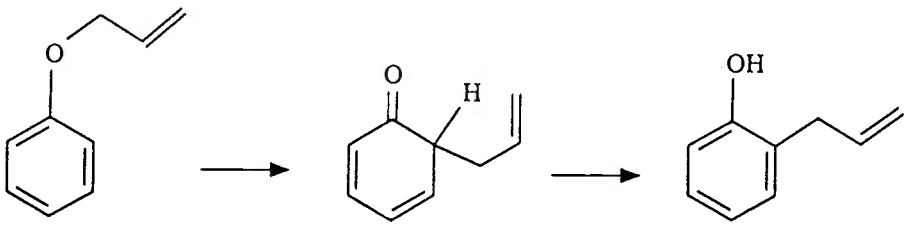
A one-electron wavefunction describing an electron moving in the effective field provided by the nuclei and all other electrons of a *molecular entity* of more than one atom. Such molecular orbitals can be transformed in prescribed ways into component functions to give "localized molecular orbitals". Molecular orbitals can also be described, in terms of the number of nuclei (or "centres") encompassed, as two-centre, multi-centre, etc. molecular orbitals, and are often expressed as a linear combination of *atomic orbitals*.

An orbital is usually depicted by sketching contours on which the wavefunction has a constant value (contour map) or by indicating schematically the envelope of the region of space in which there is an arbitrarily fixed high (say 96%) probability of finding the electron occupying the orbital, giving also the algebraic sign (+ or -) of the wavefunction in each part of that region.

molecular rearrangement

The term is traditionally applied to any reaction that involves a change of connectivity (sometimes including hydrogen), and violates the so-called "principle of minimum structural change". According to this oversimplified principle, *chemical species* do not isomerize in the course of a *transformation*, e.g. *substitution*, or the change of a functional *group* of a chemical species into a different functional group is not expected to involve the making or breaking of more than the minimum number of bonds required to effect that transformation. For example, any new substituents are expected to enter the precise positions previously occupied by displaced groups.

The simplest type of rearrangement is an *intramolecular* reaction in which the product is isomeric with the reactant (one type of "intramolecular isomerization"). An example is the first step of the Claisen rearrangement:



The definition of molecular rearrangement includes changes in which there is a *migration* of an atom or bond (unexpected on the basis of the principle of minimum structural change), as in the reaction

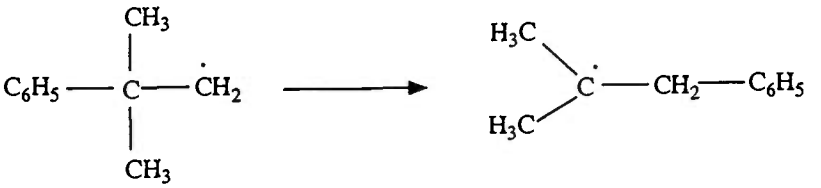
$$CH_3CH_2CH_2Br + AgOAc \longrightarrow (CH_3)_2CHOAc + AgBr$$

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where the *rearrangement stage* can formally be represented as the "1,2-shift" of hydride between adjacent carbon atoms in the carbocation

$$CH_3CH_2CH_2^+ \longrightarrow CH_3CH^+CH_3$$

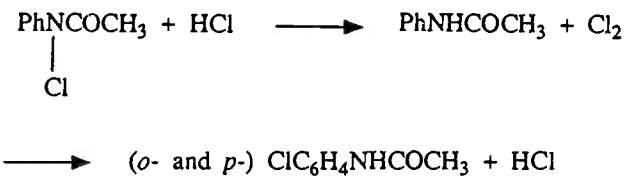
Such migrations occur also in radicals, e.g.:



The definition also includes reactions in which an *entering group* takes up a different position from the *leaving group*, with accompanying bond migration. An example of the latter type is the "allylic rearrangement":

$$(CH_3)_2C{=}CHCH_2Br + OH^- \longrightarrow (CH_3)_2C(OH)CH{=}CH_2 + Br^-$$

A distinction is made between "intramolecular rearrangements" (or "true molecular rearrangements") and "*intermolecular* rearrangements" (or "apparent rearrangements"). In the former case the atoms and groups that are common to a reactant and a product never separate into independent fragments during the rearrangement stage (i.e. the change is intramolecular), whereas in an "intermolecular rearrangement" a migrating group is completely free from the parent molecule and is re-attached to a different position in a subsequent step, as in the Orton reaction:



MAYO (1980).

molecularity

The number of reactant *molecular entities* that are involved in the "microscopic chemical event" constituting an *elementary reaction*. (For reactions in solution this number is always taken to exclude molecular entities that form part of the *medium* and which are involved solely by virtue of their solvation of solutes.) A reaction with a molecularity of one is called "unimolecular", one with a molecularity of two "bimolecular" and of three "termolecular".

See also *chemical reaction, order of reaction.*

molecule

An electrically neutral entity consisting of more than one atom ($n > 1$). Rigorously, a molecule, in which $n > 1$ must correspond to a depression on the potential energy surface that is deep enough to confine at least one vibrational state.

See also *molecular entity.*

More O'Ferrall-Jencks diagram

Visualization of the potential energy surfaces for a reacting system, as a function of two chosen coordinates. It is particularly useful to discuss structural effects on the *transition state* geometry for processes occurring either by stepwise or concerted routes. The use of such diagrams, first suggested for elimination reactions (MORE O'FERRALL (1970)), was later extended to acid-base catalysis and to certain other reactions (JENCKS (1972, 1980)).

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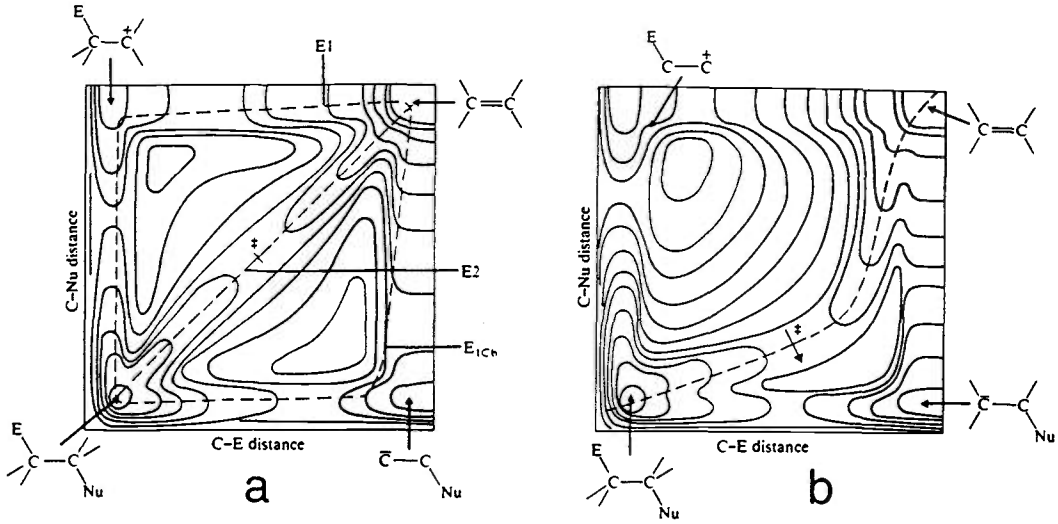


Figure. More O'Ferrall-Jencks diagrams of energy contours for β-elimination reactions as a function of lengths of the two bonds broken. Reaction coordinates follow the dotted lines. **a**: E_2 mechanism with "central" character, simultaneous fission of C_a-Nu and C_b-H bonds. **b**: The result of stabilization of the carbanion, Nu-C-C⁻; a continuation of this trend would result in a switch to the $E1_{cb}$ mechanism (Reproduced from N. S. ISAACS, "Physical Organic Chemistry", Longman Scientific, Essex, UK, (1987), with permission of Longman Scientific).

Structural changes influencing vibrational modes of the transition states cause changes in transition state geometry. Changes in the direction of the reaction coordinate (reactant or product stabilizing- or destabilizing factors) cause changes according to the *Hammond principle*. Structural changes perpendicular to the reaction coordinate (anti-Hammond effects, perpendicular effects) cause changes opposite to the Hammond behaviour, i.e., the easier the process related to the structural change, the more advanced it will be at the transition state.

See WINEY and THORNTON (1975).

multi-centre bond

Representation of some *molecular entities* solely by localized two-electron two-centre *bonds* appears to be unsatisfactory. Instead, multi-centre bonds have to be considered in which electron pairs occupy orbitals encompassing three or more atomic centres. Examples include the three-centre bonds in diborane, the delocalized π-bonding of benzene, and *bridged carbocations*.

+ multi-centre reaction

A synonym for *pericyclic reaction*. The number of "centres" is the number of atoms not bonded initially, between which single bonds are breaking or new bonds are formed in the *transition state*. This number does not necessarily correspond to the ring size of the transition state for the pericyclic reaction. Thus, a Diels-Alder reaction is a "four-centre reaction". This terminology has largely been superseded by the more detailed one developed for the various pericyclic reactions.

See *cycloaddition*, *sigmatropic rearrangement*.

multident

See *ambident*.

μ (mu) and **μ**

Notation for a ligand (prefix) that bridges two or more metal centres. The symbol **μ** is used for dipole moments.

+ narcissistic reaction

A *chemical reaction* that can be described as the conversion of a reactant into its mirror image, without rotation or translation of the product, so that the product enantiomer actually coincides with the mirror image of the reactant molecule. Examples of such reactions are cited under the entries *fluxional* and *degenerate rearrangement*.

SALEM (1971).

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neighbouring group participation

The direct interaction of the reaction centre (usually, but not necessarily, an incipient *carbenium* centre) with a lone pair of electrons of an atom or with the electrons of a σ- or π-bond contained within the parent molecule but not conjugated with the reaction centre. A distinction is sometimes made between n-, σ- and π-participation.

A rate increase due to neighbouring group participation is known as "anchimeric assistance". "Synartetic acceleration" is the special case of anchimeric assistance ascribed to participation by electrons binding a substituent to a carbon atom in a β-position relative to the leaving group attached to the α-carbon atom. According to the underlying model, these electrons then provide a three-centre bond (or "bridge") "fastening together" (as the word "synartetic" is intended to suggest) the α- and β-carbon atoms between which the charge is divided in the intermediate *bridged ion* formed (and in the *transition state* preceding its formation). The term synartetic acceleration is not widely used.

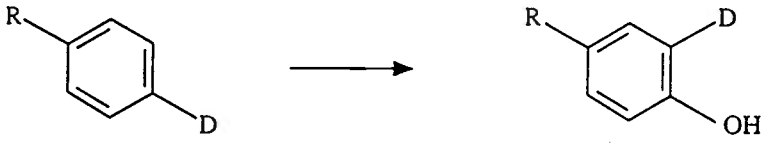
See also *intramolecular catalysis, multi-centre bond.*

NHOMO

See *subadjacent orbital.*

NIH shift

The *intramolecular* hydrogen *migration* which can be observed in enzymatic and chemical hydroxylations of aromatic rings. It is evidenced by appropriate deuterium labelling, i.e.



In enzymatic reactions the NIH shift is generally thought to derive from the rearrangement of arene oxide intermediates, but other pathways have been suggested.

(NIH stands for National Institutes of Health where the shift was discovered.)

HANZLIK, HOGBERG and JUDSON (1984).

nitrene

Generic name for HN: and substitution derivatives thereof, containing an electrically neutral univalent nitrogen atom with four non-bonding electrons. Two of these are paired; the other two may have parallel spins (triplet state) or antiparallel spins (singlet state). The name is the strict analogue of *carbene* and, as a generic name, it is preferred to a number of alternatives proposed ("imene", "imine radical", "azene", "azylene", "azacarbene", "imin", "imidogen").

See LWOWSKI (1970).

nitrenium ion

The cation H_2N^+ and its *N*-hydrocarbyl derivatives R_2N^+, in which the nitrogen has a positive charge, and two unshared electrons. A synonymous term is aminylium ion.

IUPAC CLASS NAMES (1993).

no-bond resonance

See *hyperconjugation.*

nonclassical carbocation

A *carbocation* the ground state of which has delocalized (bridged) bonding π- or σ-electrons. (N.B.: Allylic and benzylic carbocations are not considered nonclassical.)

BARTLETT (1965).

normal kinetic isotope effect

See *isotope effect.*

nucleofuge

A *leaving group* that carries away the bonding electron pair. For example, in the *hydrolysis* of an alkyl chloride, Cl^- is the nucleofuge. The tendency of atoms or groups to depart with the bonding electron pair is called nucleofugality.

The adjective is nucleofugal.

See also *electrofuge, nucleophile.*

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nucleophile, nucleophilic

A nucleophile (or nucleophilic reagent) is a reagent that forms a bond to its reaction partner (the *electrophile*) by donating both bonding electrons.

A "nucleophilic substitution reaction" is a *heterolytic* reaction in which the reagent supplying the entering group acts as a nucleophile. For example

$$\underset{\text{nucleophile}}{MeO^- + Et\text{-}Cl} \longrightarrow \underset{\text{nucleofuge}}{MeO\text{-}Et + Cl^-}$$

The term "nucleophilic" is also used to designate the apparent polar character of certain *radicals*, as inferred from their higher relative reactivity with reaction sites of lower electron density.

Nucleophilic reagents are *Lewis bases*.

nucleophilic catalysis

Catalysis by a *Lewis base*, involving formation of a *Lewis adduct* as a *reaction intermediate*. For example, the hydrolysis of acetic anhydride in aqueous solution catalysed by pyridine:

$$C_5H_5N + (CH_3CO)_2O \longrightarrow [C_5H_5NCOCH_3]^+ + CH_3CO_2^-$$

$$[C_5H_5NCOCH_3]^+ + H_2O \longrightarrow C_5H_5N + CH_3CO_2H + H^+_{aq}$$

See also *electrophilic*, *nucleophilicity*.

nucleophilicity

(1) The property of being *nucleophilic*.

(2) The relative reactivity of a *nucleophilic* reagent. (It is also sometimes referred to as "nucleophilic power".) Qualitatively, the concept is related to Lewis basicity. However, whereas Lewis basicity is measured by relative equilibrium constants,

$$B: + A \underset{}{\overset{K}{\rightleftharpoons}} B^+\text{-}A^-$$

nucleophilicity of a Lewis base is measured by relative *rate constants* of different nucleophilic reagents towards a common *substrate*, most commonly involving formation of a bond to carbon,

$$B: + A\text{-}Z \longrightarrow B^+\text{-}A^- + Z:^-$$

See also *electrophilicity*, *Ritchie equation*, *Swain-Scott equation*.

n-σ* delocalization (or n-σ* no bond resonance)

Delocalization of a free electron pair (n) into an antibonding σ-orbital (σ*).

See *hyperconjugation*, *resonance*.

onium ion

(1) A cation (with its counterion) derived by addition of a hydron to a mononuclear parent hydride of the nitrogen, chalcogen and halogen family, e.g. H_4N^+ ammonium ion.

(2) Derivatives formed by substitution of the above parent ions by univalent groups, e.g. $(CH_3)_2S^+H$ dimethylsulfonium, $(CH_3CH_2)_4N^+$ tetraethylammonium.

(3) Derivatives formed by substitution of the above parent ions by groups having two or three free valencies on the same atom. Such derivatives are, whenever possible, designated by a specific class name. E.g. $R_2C{=}NH_2^+$ iminium ion.

IUPAC CLASS NAMES (1993).

See also *carbenium ion*, *carbonium ion*.

opposing reactions

See *composite reactions*.

optical yield

In a *chemical reaction* involving chiral reactants and products, the ratio of the optical purity of the product to that of the precursor, reactant or catalyst. This should not be confused with "enantiomeric excess". The optical yield is in no way related to the chemical yield of the reaction.

IUPAC STEREOCHEMICAL TERMINOLOGY (1993).

See *stereoselectivity*.

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orbital

See *atomic orbital, molecular orbital.*

orbital steering

A concept expressing that the stereochemistry of approach of two reacting species is governed by the most favourable overlap of their appropriate *orbitals.*

orbital symmetry

The behaviour of an atomic or localized *molecular orbital* under molecular symmetry operations characterizes its orbital symmetry. For example, under a reflection in an appropriate symmetry plane, the phase of the orbital may be unchanged (symmetric), or it may change sign (antisymmetric), i.e. the positive and negative lobes are interchanged.

A principal context for the use of orbital symmetry is the discussion of chemical changes that involve "conservation of orbital symmetry". If a certain symmetry element (e.g. the reflection plane) is retained along a reaction pathway, that pathway is "allowed" by orbital symmetry conservation if each of the occupied orbitals of the reactant(s) is of the same symmetry type as a similarly (e.g. singly or doubly) occupied orbital of the product(s). This principle permits the qualitative construction of correlation diagrams to show how molecular orbitals transform (and how their energies change) during idealized chemical changes (e.g. *cycloadditions*).

An idealized single bond is a σ-bond, i.e., it has cylindrical symmetry, whereas a p-orbital or π-bond orbital has π-symmetry, i.e. it is antisymmetric with respect to reflection in a plane passing through the atomic centres with which it is associated. In ethene, the π-bonding orbital is symmetric with respect to reflection in a plane perpendicular to and bisecting the C-C bond, whereas the π^*-antibonding orbital is antisymmetric with respect to this operation.

Considerations of orbital symmetry are frequently grossly simplified in that, for example, the p-orbitals of a carbonyl group would be treated as having the same symmetry as those of ethene, and the fact that the carbonyl group in, for example, camphor, unlike that in formaldehyde, has no mirror planes would be ignored. These simplified considerations nevertheless afford the basis of one approach to the understanding of the rules which indicate whether *pericyclic reactions* are likely to occur under thermal or photochemical conditions.

WOODWARD and HOFFMANN (1969); HALEVI (1992).

See also *sigma, pi.*

order of reaction, n (SI unit: 1)

If the macroscopic (observed, empirical or phenomenological) *rate of reaction* (v) for any reaction can be expressed by an empirical differential rate equation (or *rate law*) which contains a factor of the form $k\,[A]^{\alpha}[B]^{\beta}\ldots$ (expressing in full the dependence of the rate of reaction on the concentrations [A], [B]...) where α, β are constant exponents (independent of concentration and time) and k is independent of [A] and [B] etc. (rate constant, rate coefficient), then the reaction is said to be of order α with respect to A, of order β with respect to B,..., and of (total or overall) order $n = \alpha+\beta+\ldots$. The exponents α, β,... can be positive or negative integral or rational nonintegral numbers. They are the reaction orders with respect to A, B,... and are sometimes called "partial orders of reaction". Orders of reaction deduced from the dependence of initial rates of reaction on concentration are called "orders of reaction with respect to concentration"; orders of reaction deduced from the dependence of the rate of reaction on time of reaction are called "orders of reaction with respect to time".

The concept of order of reaction is also applicable to chemical rate processes occurring in systems for which concentration changes (and hence the rate of reaction) are not themselves measurable, provided it is possible to measure a *chemical flux.* For example, if there is a dynamic equilibrium according to the equation

$$a\mathrm{A} \rightleftharpoons p\mathrm{P}$$

and if a chemical flux is experimentally found, (e.g. by NMR *line shape analysis*) to be related to concentrations by the equation

$$\varphi_{-\mathrm{A}}/a = k[\mathrm{A}]^{\alpha}[\mathrm{L}]^{\lambda}.$$

then the corresponding reaction is of order α with respect to A... and of total (or overall) order n $(= \alpha + \lambda + \ldots)$.

The proportionality factor k above is called the (nth order) "rate coefficient".

Rate coefficients referring to (or believed to refer to) *elementary reactions* are called "rate constants" or, more appropriately "microscopic" (hypothetical, mechanistic) rate constants.

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The (overall) order of a reaction cannot be deduced from measurements of a "rate of appearance" or "rate of disappearance" at a single value of the concentration of a species whose concentration is constant (or effectively constant) during the course of the reaction. If the overall rate of reaction is, for example, given by

$$v = k[A]^{\alpha}[B]^{\beta}$$

but [B] stays constant, then the order of the reaction (with respect to time), as observed from the concentration change of A with time, will be α, and the rate of disappearance of A can be expressed in the form

$$v_A = k_{obs}[A]^{\alpha}$$

The proportionality factor k_{obs} deduced from such an experiment is called the "observed rate coefficient" and it is related to the $(\alpha + \beta)$th order rate coefficient k by the equation

$$k_{obs} = k[B]^{\beta}$$

For the common case when $\alpha = 1$, k_{obs} is often referred to as a "pseudo-first order rate coefficient" (k_{ψ}).

For a simple (*elementary*) reactions a partial order of reaction is the same as the stoichiometric number of the reactant concerned and must therefore be a positive integer (see *rate of reaction*). The overall order is then the same as the *molecularity*. For *stepwise reactions* there is no general connection between stoichiometric numbers and partial orders. Such reactions may have more complex rate laws, so that an apparent order of reaction may vary with the concentrations of the *chemical species* involved and with the progress of the reaction: in such cases it is not useful to speak of orders of reaction, although apparent orders of reaction may be deducible from initial rates.

In a *stepwise reaction*, orders of reaction may in principle always be assigned to the elementary steps.

See also *kinetic equivalence*.

outer-sphere (electron transfer)

An outer-sphere electron transfer is a reaction in which the electron transfer takes place with no or very weak (4 -16 kJ mol^{-1}) electronic interaction between the reactants in the transition state. If instead the donor and the acceptor exhibit a strong electronic coupling, the reaction is described as inner-sphere electron transfer. The two terms derive from studies concerning metal complexes and it has been suggested that for organic reactions the term "nonbonded" and "bonded" electron transfer should be used.

See also *inner-sphere electron transfer*.

EBERSON (1987); LITTLER (1970).

oxidation

(1) The complete, net removal of one or more electrons from a *molecular entity* (also called "de-electronation").

(2) an increase in the *oxidation number* of any atom within any *substrate* (see HENDRICKSON, CRAM and HAMMOND (1970)).

(3) Gain of oxygen and/or loss of hydrogen of an organic *substrate*.

All oxidations meet criteria (1) and (2), and many meet criterion (3), but this is not always easy to demonstrate.

Alternatively, an oxidation can be described as a *transformation* of an organic substrate that can be rationally dissected into steps or *primitive changes*. The latter consist in removal of one or several electrons from the substrate followed or preceded by gain or loss of water and/or *hydrons* or hydroxide ions, or by *nucleophilic* substitution by water or its reverse and/or by an *intramolecular molecular rearrangement*.

This formal definition allows the original idea of oxidation (combination with oxygen), together with its extension to removal of hydrogen, as well as processes closely akin to this type of transformation (and generally regarded in current usage of the term in organic chemistry to be oxidations and to be effected by "oxidizing agents") to be descriptively related to definition (1). For example the oxidation of methane to chloromethane may be considered as follows:

$$CH_4 - 2e^- - H^+ + OH^- = CH_3OH \xrightarrow{\text{reversal of hydrolysis}} CH_3Cl$$

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oxidation number

See IUPAC INORGANIC RULES (1970), Rule 0.1; IUPAC INORGANIC NOMENCLATURE (1990), Rules I-5.5.2 and I-10.2.7.

See also *oxidation*.

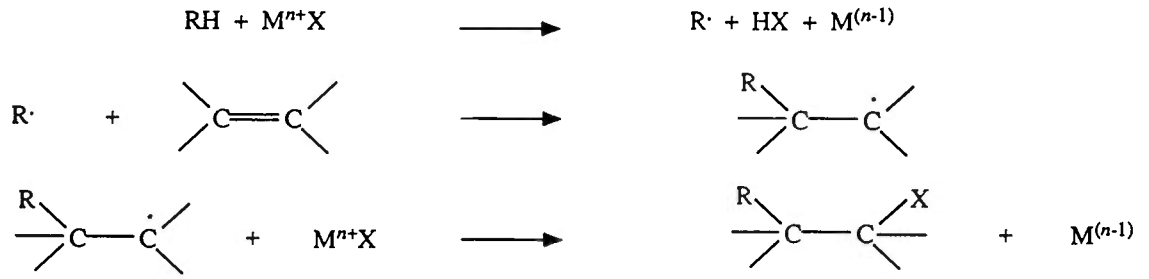
oxidative addition

The *insertion* of a metal of a metal complex into a covalent bond involving formally an overall two-electron loss on one metal or a one-electron loss on each of two metals, i.e.,

$$L_nM^m + XY \longrightarrow L_nM^{m+2}(X)(Y), \quad \text{or}$$

$$2\,L_nM^m + XY \longrightarrow L_nM^{m+1}(X) + L_nM^{m+1}(Y)$$

In free-*radical* chemistry, the term is used to indicate a free radical addition to a carbon-carbon double bond, under oxidative conditions. For example:



oxidative coupling

The coupling of two *molecular entities* through an oxidative process, usually catalysed by a transition metal compound and involving dioxygen as the oxidant; e.g.,

$$2\,CO + 2\,MeOH + 1/2\,O_2 \xrightarrow{\text{catalyst}} MeOOCCOOMe + H_2O$$

parallel reaction

See *composite reaction*.

paramagnetic

Substances having a magnetic susceptibility greater than 0 are paramagnetic. They are drawn into a magnetic field.

See also *diamagnetic*.

partial rate factor

The rate of substitution at one specific site in an *aromatic* compound relative to the rate of substitution at one position in benzene. For example, the partial rate factor f_p^Z for *para*-substitution in a monosubstituted benzene C_6H_5Z is related to the rate constants $k(C_6H_5Z)$ and $k(C_6H_6)$ for the total reaction (i.e. at all positions) of C_6H_5Z and benzene, respectively, and % *para* (the percentage *para*-substitution in the total product formed from C_6H_5Z) by the relation

$$f_p^Z = \frac{6k(C_6H_5Z)}{k(C_6H_6)} \frac{\%\ para}{100}$$

Similarly for *meta*-substitution:

$$f_m^Z = \frac{6k(C_6H_5Z)}{2k(C_6H_6)} \frac{\%\ meta}{100}$$

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(The symbols p_f^Z, m_f^Z, o_f^Z are also in use.) The term applies equally to the *ipso* position, and it can be extended to other substituted *substrates* undergoing parallel reactions at different sites with the same reagent according to the same *rate law*.

INGOLD (1953); STOCK and BROWN (1963).

See also *selectivity*.

pericyclic reaction

A *chemical reaction* in which *concerted* reorganization of bonding takes place throughout a cyclic array of continuously bonded atoms. It may be viewed as a reaction proceeding through a fully *conjugated* cyclic transition state. The number of atoms in the cyclic array is usually six, but other numbers are also possible. The term embraces a variety of processes, including *cycloadditions*, *cheletropic reactions*, *electrocyclic reactions* and *sigmatropic rearrangements*, etc. (provided they are *concerted*).

See also *multi-centre reaction*.

periselectivity

The differentiation between two symmetry-allowed processes, for example the [2+4] *vs.* [4+6] *cycloaddition* of cyclopentadiene to tropone.

perpendicular effect

See *More O'Ferrall-Jencks diagram*.

persistent

The term persistent is used to characterize *radicals* which have lifetimes of several minutes or greater in dilute solution in inert solvents. Persistence is a kinetic or reactivity property. In contrast, radical *stability*, which is a thermodynamic property, is expressed in terms of the C-H bond strength of the appropriate hydrocarbon. The lifetime of a radical is profoundly influenced by steric shielding of the radical centre by bulky substituents.

GRILLER and INGOLD (1976).

See also *transient*.

pH-rate profile

A plot of observed rate coefficient, or more usually its decadic logarithm, against pH of solution, other variables being kept constant.

phase-transfer catalysis

The phenomenon of rate enhancement of a reaction between *chemical species* located in different phases (immiscible liquids or solid and liquid) by addition of a small quantity of an agent (called the "phase-transfer *catalyst*") that extracts one of the reactants, most commonly an anion, across the interface into the other phase so that reaction can proceed. These catalysts are salts of "*onium ions*" (e.g. tetraalkylammonium salts) or agents that complex inorganic cations (e.g. *crown* ethers). The catalyst cation is not consumed in the reaction although an anion exchange does occur.

phenonium ion

See *bridged carbocation*.

photolysis

The cleavage of one or more covalent *bonds* in a *molecular entity* resulting from absorption of light, or a photochemical process in which such cleavage is an essential part. For example:

$$Cl_2 \longrightarrow 2\,Cl^{\bullet}$$

The term is used incorrectly to describe irradiation of a sample, although in the combination flash photolysis this usage is accepted.

IUPAC PHOTOCHEMICAL GLOSSARY (1992).

+ polar aprotic solvent

See *dipolar aprotic*.

polar effect

For a reactant molecule RY, the polar effect of the group R comprises all the processes whereby a substituent may modify the electrostatic forces operating at the reaction centre Y, relative to the standard R^oY. These forces may be governed by charge separations arising from differences in the *electronegativity* of atoms (leading to the presence of dipoles), the presence of

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unipoles, or electron *delocalization*. It is synonymous with *electronic effect* or "electrical effect" of a substituent as distinguished from other substituent effects, e.g. *steric effects*.

Sometimes, however, the term "polar effect" is taken to refer to the influence, other than steric, that non-conjugated substituents exert on reaction rates, i.e. effects connected with electron delocalization between a substituent and the molecular framework to which it is attached are excluded. Polar effect is then not synonymous with electronic effect

See also *field effect, inductive effect, mesomeric effect*.

polar solvent

See *polarity*.

polarity

When applied to solvents, this rather ill-defined term covers their overall solvation capability (solvation power) for solutes (i.e. in chemical equilibria: reactants and products; in reaction rates: reactants and *activated complex*; in light absorptions: ions or molecules in the ground and excited state), which in turn depends on the action of all possible, nonspecific and specific, intermolecular interactions between solute ions or molecules and solvent molecules, excluding such interactions leading to definite chemical alterations of the ions or molecules of the solute. Occasionally, the term solvent polarity is restricted to nonspecific solute/solvent interactions only (i.e. to *van der Waals forces*).

See also *Dimroth-Reichardt E_T parameter, Grunwald-Winstein equation, ionizing power, Kamlet-Taft solvent parameters, van der Waals forces, Z-value*.

REICHARDT (1965, 1988).

polarizability

The ease of distortion of the electron cloud of a *molecular entity* by an electric field (such as that due to the proximity of a charged reagent). It is experimentally measured as the ratio of induced dipole moment (μ_{ind}) to the field E which induces it:

$$\alpha = \mu_{ind}/E$$

The units of α are $\mathbf{C}^2\ m^2\ \mathbf{V}^{-1}$. In ordinary usage the term refers to the "mean polarizability", i.e., the average over three rectilinear axes of the molecule. Polarizabilities in different directions (e.g. along the bond in Cl_2, called "longitudinal polarizability", and in the direction perpendicular to the bond, called "transverse polarizability") can be distinguished, at least in principle. Polarizability along the bond joining a substituent to the rest of the molecule is seen in certain modern theoretical approaches as a factor influencing chemical *reactivity*, etc., and parametrization thereof has been proposed.

See INGOLD (1953); TAFT and TOPSOM (1987).

polydent

See *ambident*.

potential-energy profile

A curve describing the variation of the potential energy of the system of atoms that make up the reactants and products of a reaction as a function of one geometric coordinate, and corresponding to the "energetically easiest passage" from reactants to products (i.e. along the line produced by joining the paths of steepest descent from the *transition state* to the reactants and to the products). For an *elementary reaction* the relevant geometric coordinate is the *reaction coordinate*; for a *stepwise reaction* it is the succession of reaction coordinates for the successive individual reaction steps. (The reaction coordinate is sometimes approximated by a quasi-chemical index of reaction progress, such as "degree of atom transfer" or *bond order* of some specified bond.)

See also *potential-energy (reaction) surface, Gibbs energy diagram*.

potential-energy (reaction) surface

A geometric hypersurface on which the potential energy of a set of reactants is plotted as a function of the coordinates representing the molecular geometries of the system.

For simple systems two such coordinates (characterizing two variables that change during the progress from reactants to products) can be selected, and the potential energy plotted as a contour map.

For simple *elementary reactions*, e.g. A-B + C → A + B-C, the surface can show the potential energy for all values of the A, B, C geometry, providing that the ABC angle is fixed.

For more complicated reactions a different choice of two coordinates is sometimes preferred, e.g. the *bond orders* of two different *bonds*. Such a diagram is often arranged so that reactants are located at the bottom left corner and products at the top right. If the trace of the

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representative point characterizing the route from reactants to products follows two adjacent edges of the diagram, the changes represented by the two coordinates take place in distinct succession; if the trace leaves the edges and crosses the interior of the diagram, the two changes are *concerted*. In many qualitative applications it is convenient (although not strictly equivalent) for the third coordinate to represent the standard Gibbs energy rather than potential energy.

Using bond orders is, however, an oversimplification, since these are not well-defined, even for the transition state. (Some reservations concerning the diagrammatic use of Gibbs energies are noted under *Gibbs energy diagram*.)

The energetically easiest route from reactants to products on the potential-energy contour map defines the *potential-energy profile*.

ALBERY (1967); MORE O'FERRALL (1970); JENCKS (1972, 1985).

See also *reaction coordinate*.

pre-association

A step on the *reaction path* of some *stepwise reactions* in which the *molecular entity* C is already present in an *encounter pair* or *encounter complex* with A during the formation of B from A, e.g.

$$A + C \underset{}{\overset{\text{Pre - association}}{\rightleftharpoons}} \underbrace{(A \cdots\cdots C)}_{\text{encounter complex}} \xrightarrow{\text{rapid}} B$$

In this mechanism the *chemical species* C may but does not necessarily assist the formation of B from A, which may itself be a *bimolecular* reaction with some other reagent.

Pre-association is important when B is too short-lived to permit B and C to come together by diffusion.

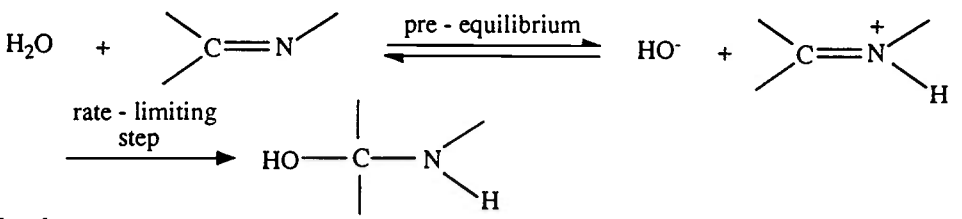
See also *microscopic diffusion control, spectator mechanism*.

precursor complex

See *encounter complex*.

pre-equilibrium (or prior equilibrium)

A rapidly reversible step preceding the *rate-limiting step* in a *stepwise reaction*. For example



See also *kinetic equivalence*.

pre-exponential factor

See *energy of activation, entropy of activation*.

primary kinetic electrolyte effect

See *kinetic electrolyte effect*.

primary kinetic isotope effect

See *isotope effect*.

primitive change

One of the conceptually simpler molecular changes into which an *elementary reaction* can be notionally dissected. Such changes include *bond* rupture, bond formation, internal rotation, change of bond length or bond angle, bond *migration*, redistribution of charge, etc.

The concept of primitive changes is helpful in the detailed verbal description of elementary reactions, but a primitive change does not represent a process that is by itself necessarily observable as a component of an elementary reaction.

IUPAC REACTION MECHANISMS (1989).

prior equilibrium

See *pre-equilibrium*.

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product-determining step

The step of a *stepwise reaction*, in which the product distribution is determined. The product-determining step may be identical to, or occur later than, the *rate-controlling step* on the reaction coordinate.

product development control

The term is used for reactions under *kinetic control* where the *selectivity* parallels the relative (thermodynamic) stabilities of the products. Product development control is usually associated with a *transition state* occurring late on the *reaction coordinate*.

See also *steric-approach control*, *thermodynamic control*.

promotion

See *pseudo-catalysis*.

propagation

See *chain reaction*.

protic

See *protogenic*.

protogenic (solvent)

Capable of acting as a proton (hydron) donor strongly or weakly acidic (as a *Brønsted acid*). The term is preferred to the synonym "protic" or the more ambiguous expression "acidic" by itself. Also called HBD (hydrogen bond donor) solvent.

See *protophilic solvent*.

+ protolysis

This term has been used synonymously with proton (hydron)-transfer reaction. Because of its misleading similarity to *hydrolysis*, *photolysis*, etc., its use is discouraged.

See also *autoprotolysis*.

proton affinity

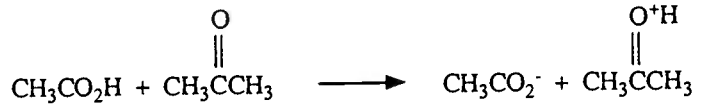
The negative of the enthalpy change in the gas phase reaction (real or hypothetical) between a proton (more appropriately *hydron*) and the *chemical species* concerned, usually an electrically neutral species to give the *conjugate acid* of that species. Proton affinity is often, but unofficially, abbreviated as PA.

LIAS, LIEBMAN, and LEVIN (1984).

See also *gas phase basicity*.

proton transfer reaction

A *chemical reaction*, the main feature of which is the *intermolecular* or *intramolecular* transfer of a proton (*hydron*) from one *binding site* to another. For example,



In the detailed description of proton transfer reactions, especially of rapid proton transfers between electronegative atoms, it should always be specified whether the term is used to refer to the overall process (including the more-or-less *encounter-controlled* formation of a hydrogen bonded complex and the separation of the products; see *microscopic diffusion control*) or just to the proton transfer event (including solvent rearrangement) by itself.

See also *autoprotolysis*, *tautomerism*.

protophilic (solvent)

Capable of acting as proton acceptor, strongly or weakly basic (as a *Brønsted base*). Also called HBA (hydrogen bond acceptor) solvent.

See also *protogenic solvent*.

prototropic rearrangement (or prototropy)

See *tautomerism*.

pseudo-catalysis

If an acid or base is present in nearly constant concentration throughout a reaction in solution (owing to buffering or the use of a large excess), it may be found to increase the rate of

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that reaction and also to be consumed during the process. The acid or base is then not a *catalyst* and the phenomenon cannot be called *catalysis* according to the well-established meaning of these terms in chemical kinetics, although the *mechanism* of such a process is often intimately related to that of a catalysed reaction. It is recommended that the term pseudo-catalysis be used in these and analogous cases (not necessarily involving acids or bases). For example, if a *Brønsted acid* accelerates the hydrolysis of an ester to a carboxylic acid and an alcohol, this is properly called acid catalysis, whereas the acceleration, by the same acid, of hydrolysis of an amide should be described as pseudo-catalysis by the acid: the "acid pseudo-catalyst" is consumed during the reaction through formation of an ammonium ion. The terms "general acid pseudo-catalysis" and "general base pseudo-catalysis" may be used as the analogues of *general acid catalysis* and *general base catalysis*.

The term "base-promoted", "base-accelerated, or "base-induced" is sometimes used for reactions that are pseudo-catalysed by bases. However, the term "promotion" also has a different meaning in other chemical contexts.

pseudo-first order rate coefficient

See *order of reaction*.

+ pseudomolecular rearrangement

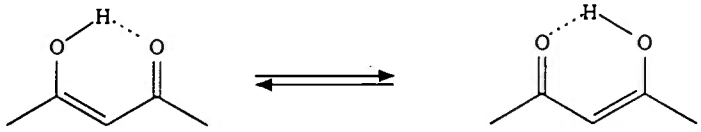
The use of this awkwardly formed term is discouraged. It is synonymous with "intermolecular rearrangement".

See *molecular rearrangement*.

pseudopericyclic

A *concerted transformation* is pseudopericyclic if the primary changes in bonding occur within a cyclic array of atoms at one (or more) of which nonbonding and bonding atomic orbitals interchange roles.

A formal example is the enol → enol prototropy of pentane-2,4-dione (acetylacetone).



Because the π- and σ-atomic orbitals that interchange roles are orthogonal, such a reaction does not proceed through a fully *conjugated transition state* and is thus not a pericyclic reaction and therefore not governed by the rules that express *orbital symmetry* restrictions applicable to pericyclic reactions.

ROSS, SEIDERS and LEMAL (1976).

+ pseudo-unimolecular

A term sometimes used as synonymous with pseudo-first order, but is inherently meaningless.

See *molecularity*, *order of reaction*.

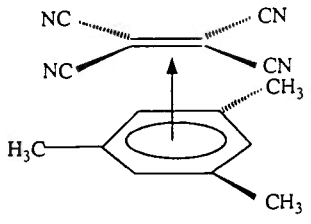
pyrolysis

Thermolysis, usually associated with exposure to a high temperature.

See also *flash vacuum pyrolysis*.

π-adduct (pi-adduct)

An *adduct* formed by electron-pair donation from a π-orbital into a σ-orbital, or from a σ-orbital into a π-orbital, or from a π-orbital into a π-orbital. For example:



Such an adduct has commonly been known as a "π-complex", but, as the bonding is not necessarily weak, it is better to avoid the term *complex*, in accordance with the recommendations in this Glossary.

See also *coordination*.

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π-bond (pi bond)

See *sigma, pi.*

+ π-complex

See *π-adduct.*

π-electron acceptor, π-electron donor group

A *substituent* capable of a +R (e.g. NO_2) or -R (e.g. OCH_3) effect, respectively.
See *electronic effect, polar effect, σ-constant.*

π-orbital

See sigma, pi.

quantitative structure-activity relationships (QSAR)

The building of structure-biological activity models by using regression analysis with physicochemical constants, indicator variables or theoretical calculations. The term has been extended by some authors to include chemical reactivity, i.e. activity is regarded as synonymous with reactivity. This extension is, however, discouraged.
See CHARTON (1989).
See also *correlation analysis.*

quantum yield

The number of defined events which occur per photon absorbed by the system. The integral quantum yield is

$$\phi = \frac{\text{number of events}}{\text{number of photons absorbed}}$$

For a photochemical reaction,

$$\phi = \frac{\text{moles of reactant consumed or product formed}}{\text{moles of photons absorbed}}$$

The differential quantum yield is

$$\phi = \frac{d[x]/dt}{n}$$

where $d[x]/dt$ is the rate of change of the amount of (substance) concentration of a measurable quantity, and n the amount of photons (mol or its equivalent *einsteins*) absorbed per unit time. Φ can be used for photophysical processes or photochemical reactions.
IUPAC PHOTOCHEMICAL GLOSSARY (1992).

radical (or free radical)

A *molecular entity* such as $\cdot CH_3$, $\cdot SnH_3$, $Cl\cdot$ possessing an unpaired electron. (In these formulae the dot, symbolizing the unpaired electron, should be placed so as to indicate the atom of highest spin density, if this is possible.) Paramagnetic metal ions are not normally regarded as radicals. However, in the "isolobal analogy" (see HOFFMANN (1982)), the similarity between certain paramagnetic metal ions and radicals becomes apparent.
At least in the context of physical organic chemistry, it seems desirable to cease using the adjective "free" in the general name of this type of *chemical species* and *molecular entity*, so that the term "free radical" may in future be restricted to those radicals which do not form parts of radical pairs.
Depending upon the core atom that possesses the unpaired electron, the radicals can be described as carbon-, oxygen-, nitrogen-, metal-centred radicals. If the unpaired electron occupies an orbital having considerable s or more or less pure p character, the respective radicals are termed σ- or π-radicals.
In the past, the term "radical" was used to designate a *substituent group* bound to a *molecular entity*, as opposed to "free radical", which nowadays is simply called radical. The bound entities may be called *groups* or *substituents*, but should no longer be called radicals.
IUPAC NOMENCLATURE GUIDE (1993).
See also *biradical.*

radical anion

See *radical ion.*

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radical centre(s)

The atom (or group of atoms) in a polyatomic radical on which an unpaired electron is largely localized. Attachment of a monovalent atom to a radical centre gives a molecule for which it is possible to write a *Lewis formula* in which the normal stable valencies are assigned to all atoms.

radical combination

See *colligation.*

radical ion

A radical that carries an electric charge. A positively charged radical is called a "radical cation" (e.g., the benzene radical cation $C_6H_6^{\cdot+}$); a negatively charged radical is called a "radical anion" (e.g., the benzene radical anion $C_6H_6^{\cdot-}$ or the benzophenone radical anion $Ph_2C\text{-}O^{\cdot-}$). Commonly, but not necessarily, the odd electron and the charge are associated with the same atom.

Unless the positions of unpaired spin and charge can be associated with specific atoms, superscript dot and charge designations should be placed in the order .+ or .- suggested by the name "radical ion", (e.g $C_3H_6^{\cdot+}$).

Note: In the previous edition of the Glossary, it was recommended to place the charge designation directly above the centrally placed dot. However, this format is now discouraged because of the difficulty of extending it to ions bearing more than one charge, and/or more than one unpaired electron.

In mass spectroscopic usage the symbol for the charge precedes the dot representing the unpaired electron.

IUPAC MASS SPECTROMETRY (1991).

radical pair (or geminate pair)

The term is used to identify two *radicals* in close proximity in solution, within a solvent *cage.* They may be formed simultaneously by some *unimolecular* process, e.g., peroxide decomposition, or they may have come together by diffusion. While the radicals are together, correlation of the unpaired electron spins of the two species cannot be ignored: this correlation is responsible for the *CIDNP* phenomenon.

See also *geminate recombination.*

radiolysis

The cleavage of one or several bonds resulting from exposure to high-energy radiation. The term is also often used loosely to specify the method of irradiation ("pulse radiolysis") used in any radiochemical reaction, not necessarily one involving bond cleavage.

rate coefficient

See *order of reaction, kinetic equivalence.*

rate-controlling step

A rate-controlling (rate-determining or rate-limiting) step in a reaction occurring by a composite reaction sequence is an *elementary reaction* the rate constant for which exerts a strong effect - stronger than that of any other rate constant - on the overall rate. It is recommended that the expressions rate-controlling, *rate-determining* and *rate-limiting* be regarded as synonymous, but some special meanings sometimes given to the last two expressions are considered under a separate heading.

A rate-controlling step can be formally defined on the basis of a control function (or control factor) CF, identified for an elementary reaction having a rate constant k_i by

$$CF = (\partial \ln \upsilon / \partial \ln k_i)_{K_j,\, k_j}$$

where υ is the overall rate of reaction. In performing the partial differentiation all equilibrium constants K_j and all rate constants except k_i are held constant. The elementary reaction having the largest control factor exerts the strongest influence on the rate υ, and a step having a CF much larger than any other step may be said to be rate-controlling.

A rate-controlling step defined in the way recommended here has the advantage that it is directly related to the interpretation of *kinetic isotope effects.*

IUPAC CHEMICAL KINETICS (1991)

As formulated this implies that all rate constants are of the same dimensionality. Consider however the reaction of A and B to give an intermediate C, which then reacts further with D to give products:

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$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \qquad (1)$$

$$C + D \xrightarrow{k_2} \text{Products} \qquad (2)$$

Assuming that C reaches a *steady state*, then the observed rate is given by

$$v = \frac{k_1 k_2 [A] [B] [D]}{k_{-1} + k_2 [D]}$$

Considering $k_2[D]$ a pseudo-first order rate constant, then $k_2[D] >> k_{-1}$, and the observed rate $v = k_1[A][B]$ and $k_{obs} = k_1$

Step (1) is said to be the rate-controlling step.

If $k_2[D] << k_{-1}$, then the observed rate

$$v = \frac{k_1 k_2}{k_{-1}} [A] [B] [D]$$

$$= K k_2 [A] [B] [D]$$

where K is the equilibrium constant for the pre-equilibrium (1) and is equal to k_1/k_{-1}, and $k_{obs} = Kk_2$.

Step (2) is said to be the rate-controlling step.

See also *Gibbs energy diagram, microscopic diffusion control, mixing control, rate-determining step, rate-limiting step.*

rate constant, k (SI unit: s^{-1} $(dm^3\ mol^{-1})^{n-1}$)

See *order of reaction.*

rate-determining step, rate-limiting step

These terms are best regarded as synonymous with *rate-controlling step.* However, other meanings that have been given to them should be mentioned, as it is necessary to be aware of them in order to avoid confusion:

Sometimes the term rate-determining is used as a special case of rate-controlling, being assigned only to an initial slow step which is followed by rapid steps. Such a step imposes an upper limit on the rate, and has also been called rate-limiting.

In view of the considerable danger of confusion when special meanings are applied to rate-determining and rate-limiting, it is recommended that they be regarded as synonymous, with the meaning explained under the entry *rate-controlling step.* See *Michaelis-Menten kinetics.*

IUPAC CHEMICAL KINETICS (1991).

rate law (or empirical differential rate equation)

An expression for the *rate of reaction* of a particular reaction in terms of concentrations of *chemical species* and constant parameters (normally *rate coefficients* and partial *orders of reaction*) only. For examples of rate laws see equations (1) to (3) under *kinetic equivalence*, and (1) under *steady state.*

rate of appearance

See *rate of reaction.*

rate of reaction

For the general *chemical reaction*

$$aA + bB \rightarrow pP + qQ...$$

occurring under constant-volume conditions, without an appreciable build-up of reaction *intermediates*, the rate of reaction v is defined as

$$v = -\frac{1}{a}\frac{d[A]}{dt} = -\frac{1}{b}\frac{d[B]}{dt} = +\frac{1}{p}\frac{d[P]}{dt} = +\frac{1}{q}\frac{d[Q]}{dt}$$

where symbols placed inside square brackets denote amount (or amount of substance) concentrations (conventionally expressed in units of mol dm^{-3}). The symbols R and r are also commonly used in place of v. It is recommended that the unit of time should always be the second.

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In such a case the rate of reaction differs from the rate of increase of concentration of a product P by a constant factor (the reciprocal of its coefficient in the stoichiometric equation, p) and from the rate of decrease of concentration of the reactant A by $1/a$. [It should be noted that all coefficients in the stoichiometric equation are positive; those for products (p, q...) will therefore differ in sign from the stoichiometric numbers defined in IUPAC MANUAL (1979).]

The quantity

$$\dot{\xi} = \frac{d\xi}{dt}$$

defined by the equation

$$\dot{\xi} = -\frac{1}{a}\frac{dn_A}{dt} = -\frac{1}{b}\frac{dn_B}{dt} = +\frac{1}{p}\frac{dn_P}{dt} = +\frac{1}{q}\frac{dn_Q}{dt}$$

(where n_A designates the amount of substance A, conventionally expressed in units of mole) may be called the "rate of conversion" and is appropriate when the use of concentrations is inconvenient, e.g. under conditions of varying volume. In a system of constant volume, the rate of reaction is equal to the rate of conversion per unit volume throughout the reaction.

For a *stepwise reaction* this definition of "rate of reaction" (and "extent of reaction", ξ) will apply only if there is no accumulation of intermediate or formation of side products. It is therefore recommended that the term "rate of reaction" be used only in cases where it is experimentally established that these conditions apply. More generally, it is recommended that, instead, the terms "rate of disappearance" or "rate of consumption" of A (i.e. $-d[A]/dt$, the rate of decrease of concentration of A) or "rate of appearance" of P (i.e. $d[P]/dt$, the rate of increase of concentration of product P) be used, depending on the concentration change of the particular *chemical species* that is actually observed. In some cases reference to the *chemical flux* observed may be more appropriate.

The symbol v (without lettered subscript) should be used only for rate of reaction; v with a lettered subscript (e.g. v_A) refers to a rate of appearance or rate of disappearance (e.g. of the chemical species A).

N.B. This definition is consistent with CODATA (1974) recommendations and with IUPAC MANUAL APPENDIX V (1981), but differs from the unconventional terminology in the earlier IUPAC MANUAL (1979).

See also *chemical relaxation, lifetime, order of reaction.*

reacting bond rules

(1) For an internal motion of a *molecular entity* corresponding to progress over a *transition state* (energy maximum), any change that makes the motion more difficult will lead to a new molecular geometry at the energy maximum, in which the motion has proceeded further. Changes that make the motion less difficult will have the opposite effect. (This rule corresponds to the *Hammond principle.*

(2) For an internal motion of a *molecular entity* that corresponds to a vibration, any change that tends to modify the equilibrium point of the vibration in a particular direction will actually shift the equilibrium in that direction.

(3) Effects on reacting bonds (bonds made or broken in the reaction) are the most significant. The bonds nearest the site of structural change are those most strongly affected.

THORNTON (1967).

See also *More O'Ferrall-Jencks diagram.*

reaction

See *chemical reaction.*

reaction coordinate

A geometric parameter that changes during the conversion of one (or more) reactant *molecular entities* into one (or more) product molecular entities and whose value can be taken for a measure of the progress of an *elementary reaction* (for example, a bond length or bond angle or a combination of bond lengths and/or bond angles; it is sometimes approximated by a non-geometric parameter, such as the *bond order* of some specified bond).

In the formalism of "*transition-state* theory", the reaction coordinate is that coordinate in a set of curvilinear coordinates obtained from the conventional ones for the reactants which, for each reaction step, leads smoothly from the configuration of the reactants through that of the transition state to the configuration of the products. The reaction coordinate is typically chosen to follow the path along the gradient (path of shallowest ascent/deepest descent) of potential energy from reactants to products.

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The term has also been used interchangeably with the term transition coordinate, applicable to the coordinate in the immediate vicinity of the potential energy maximum. Being more specific, the name transition coordinate is to be preferred in that context.

MARCUS (1966).

See also *potential-energy profile, potential-energy reaction surface.*

reaction mechanism

See *mechanism.*

reaction path

(1) A synonym for *mechanism.*
(2) A trajectory on the *potential-energy surface.*
(3) A sequence of synthetic steps.

reaction stage

A set of one or more (possibly experimentally inseparable) *reaction steps* leading to and/or from a detectable or presumed reaction *intermediate.*

reaction step

An *elementary reaction,* constituting one of the stages of a *stepwise reaction* in which a reaction *intermediate* (or, for the first step, the reactants) is converted into the next reaction intermediate (or, for the last step, the products) in the sequence of intermediates between reactants and products.

See also *rate-limiting step, reaction stage.*

reactive, reactivity

As applied to a *chemical species,* the term expresses a kinetic property. A species is said to be more reactive or to have a higher reactivity in some given context than some other (reference) species if it has a larger rate constant for a specified *elementary reaction.* The term has meaning only by reference to some explicitly stated or implicitly assumed set of conditions. It is not to be used for reactions or reaction patterns of compounds in general.

The term is also more loosely used as a phenomenological description not restricted to elementary reactions. When applied in this sense the property under consideration may reflect not only rate, but also equilibrium, constants.

See also *stable, unreactive, unstable.*

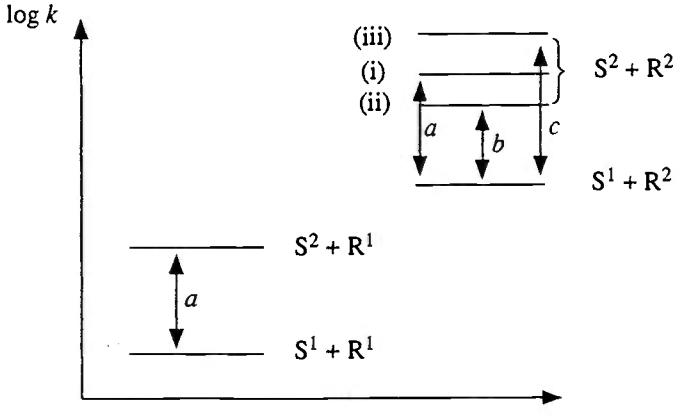
reactivity index

Any numerical index derived from quantum mechanical model calculations that permits the prediction of relative reactivities of different molecular sites. Many indices are in use, based on a variety of theories and relating to various types of reaction. The more successful applications have been to the *substitution reactions* of *conjugated systems* where relative reactivities are determined largely by changes of π-electron energy.

reactivity-selectivity principle (RSP)

This idea may be expressed loosely as: the more *reactive* a reagent is, the less selective it is.

Consider two substrates S^1 and S^2 undergoing the same type of reaction with two reagents R^1 and R^2, S^2 being more reactive than S^1, and R^2 more reactive than R^1 in the given type of reaction. The relative reactivities (in log units, see *selectivity*) for the four possible reactions may notionally be represented as follows:



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With the positions of (S^1 + R^1), (S^2 + R^1), and (S^1 + R^2) fixed, there are three types of positions for (S^2 + R^2):

In position (i) the selectivity of R^2 for the two substrates, measured by a is the same as the selectivity of R^1 for the two substrates, also a.

In position (ii) the selectivity of R^2 for the two substrates, measured by b, is less than the selectivity of R^1 for the two substrates, i.e. $b < a$. It is this situation which is in accord with the RSP.

In position (iii) the selectivity of R^2 for the two substrates, measured by c, is greater than the selectivity of R^1 for the two substrates, i.e. $c > a$. This situation may be described as anti-RSP.

There are many examples in which the RSP is followed, but there are also many examples corresponding to situations (i) and (iii). The RSP is in accord with intuitive feeling and certainly holds in the limiting case when reactivity is controlled by diffusion. However, the validity of the RSP is a matter of great controversy "...and diverse opinions have been expressed, from declaring the reactivity-selectivity principle as a universal law up to 'virtually useless in practice as a general rule'." [EXNER (1988)].

ARGILE, CAREY, FUKATA, HARCOURT, MORE O'FERRAL AND MURPHY (1985); BUNCEL and WILSON (1987); JOHNSON (1975); STOCK and BROWN (1963).

rearrangement

See *degenerate rearrangement, molecular rearrangement, sigmatropic rearrangement.*

rearrangement stage

The *elementary reaction* or *reaction stage* (of a *molecular rearrangement*) in which there is both making and breaking of bonds between atoms common to a reactant and a reaction product or a reaction *intermediate*. If the rearrangement stage consists of a single elementary reaction, this is a "rearrangement step".

reduction

The complete transfer of one or more electrons to a *molecular entity* (also called "electronation"), and, more generally, the reverse of the processes described under *oxidation* (2), (3).

reductive elimination

The reverse of *oxidative addition.*

regioselectivity, regioselective

A regioselective reaction is one in which one direction of bond making or breaking occurs preferentially over all other possible directions. Reactions are termed completely (100 %) regioselective if the discrimination is complete, or partially (x %), if the product of reaction at one site predominates over the product of reaction at other sites. The discrimination may also semi-quantitatively be referred to as high or low regioselectivity.

(Originally the term was restricted to *addition* reactions of unsymmetrical reagents to unsymmetrical alkenes.)

In the past, the term "regiospecificity" was proposed for 100 % regioselectivity. This terminology is not recommended owing to inconsistency with the terms *stereoselectivity* and *stereospecificity* .

See ADAMS (1992); HASSNER (1968).

See also *chemoselectivity.*

relaxation

Passage of an excited or otherwise perturbed system towards or into thermal equilibrium with its environment.

IUPAC PHOTOCHEMICAL GLOSSARY (1988).

See also *chemical relaxation.*

reorganization energy

In a one-electron transfer reaction

$$A + D \rightleftharpoons A^{\cdot -} + D^{\cdot +}$$

the reorganization energy λ is the energy required for all structural adjustments (in the reactants and in the surrounding solvent molecules) which are needed in order that A and D assume the configuration required for the transfer of the electron.

See *intrinsic barrier, Marcus equation.*

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resonance

In the context of chemistry, the term refers to the representation of the electronic structure of a *molecular entity* in terms of *contributing structures*. Resonance among contributing structures means that the wavefunction is represented by "mixing" the wavefunctions of the contributing structures. The concept is the basis of the quantum mechanical valence bond methods. The resulting stabilization is linked to the quantum mechanical concept of "resonance energy". The term resonance is also used to refer to the delocalization phenomenon itself.

See ATKINS (1974).

See also *mesomerism*.

resonance effect

This is the term most commonly used to describe the influence (on reactivity, spectra, etc.) of a *substituent* through electron *delocalization* into or from the substituent. The use of the term obviates the need to attempt to distinguish between the operation of the *mesomeric effect* and the *electromeric effect*. (An alternative term with essentially the same meaning is "conjugative effect". At one time "tautomeric effect" was also used, but was abandoned because *tautomerism* implies reorganization of the atomic nuclei.) The effect is symbolized by R.

resonance energy

The difference in potential energy between the actual *molecular entity* and the *contributing structure* of lowest potential energy. The resonance energy cannot be measured, but only estimated, since contributing structures are not observable *molecular entities*.

See *resonance*.

+ retroaddition

See *cycloelimination*.

+ retrocycloaddition

See *cycloelimination*.

retro-ene reaction

See *ene* reaction.

reverse micelle (or reversed micelle)

See *inverted micelle*.

Ritchie equation

The *linear free energy relation*

$$\log k_N = \log k_0 + N_+$$

applied to the reactions between nucleophiles and certain large and relatively stable organic cations, e.g. arenediazonium, triarylmethyl, and aryltropylium cations in various solvents. k_N is the rate constant for reaction of a given cation with a given nucleophilic system (i.e. given nucleophile in a given solvent). k_0 is the rate constant for the same cation with water in water, and N_+ is a parameter which is characteristic of the nucleophilic system and independent of the cation. A surprising feature of the equation is the absence of a coefficient of N_+, characteristic of the substrate (cf. the s in the *Swain-Scott equation*), even though values of N_+ vary over 13 log units. The equation thus involves a gigantic breakdown of the *reactivity-selectivity principle*. The equation has been extended both in form and in range of application.

RITCHIE (1972, 1978, 1986).

See also CHAPMAN and SHORTER (1978).

ρ-value (rho-value)

A measure of the susceptibility to the influence of *substituent* groups on the rate constant or equilibrium constant of a particular organic reaction involving a family of related *substrates*. Defined by Hammett for the effect of ring substituents in *meta*- and *para*-positions of aromatic side-chain reactions by the empirical "ρσ-equation" of the general form

$$\log(k_X/k_H) = \rho\sigma_X$$

in which σ_X is a constant characteristic of the substituent X and of its position in the reactant molecule.

More generally (and not only for aromatic series), ρ-values (modified with appropriate subscripts and superscripts) are used to designate the susceptibility of reaction series for

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families of various organic compounds to any substituent effects, as given by the modified set of σ-constants in an empirical ρσ-correlation.

Reactions with a positive ρ-value are accelerated (or the equilibrium constants of analogous equilibria are increased) by substituents with positive σ-constants. Since the sign of σ was defined so that substituents with a positive σ increase the acidity of benzoic acid, such substituents are generally described as attracting electrons away from the aromatic ring. It follows that reactions with a positive ρ-value are considered to involve a transition state (or reaction product) so that the difference in energy between this state and the reactants is decreased by a reduction in electron density at the reactive site of the substrate.

See also *Hammett equation*, *σ-constant*, *Taft equation*.

ρσ-equation (rho-sigma equation)

See *Hammett equation*, *ρ-value*, *σ-constant*, *Taft equation*.

sacrificial hyperconjugation

See *hyperconjugation*.

+ salt effect

See *kinetic electrolyte effect*.

saturation transfer

A term used in nuclear magnetic resonance. When a nucleus is strongly irradiated, its spin population may partly be transferred to another nucleus by an exchange process.

See *magnetization transfer*.

Saytzeff rule

Dehydrohalogenation of secondary- and tertiary-alkyl halides proceeds by the preferential removal of the β-hydrogen from the carbon that has the smallest number of hydrogens. Originally formulated by A. Saytzeff (Zaitsev) to generalize the orientation in *β-elimination reactions* of alkyl halides, this rule has been extended and modified, as follows: When two or more olefins can be produced in an elimination reaction, the thermodynamically most *stable* alkene will predominate. Exceptions to the Saytzeff rule are exemplified by the *Hofmann rule*.

SAYTZEFF (1875).

See also *Markownikoff rule*.

scavenger

A substance that reacts with (or otherwise removes) a trace component (as in the scavenging of trace metal ions) or traps a reactive reaction *intermediate*.

IUPAC COMPENDIUM (1987).

See also *inhibition*.

scrambling

See *isotopic scrambling*.

secondary kinetic electrolyte effect

See *kinetic electrolyte effect*.

secondary kinetic isotope effect

See *isotope effect*.

selectivity

The discrimination shown by a reagent in competitive attack on two or more *substrates* or on two or more positions in the same substrate. It is quantitatively expressed by ratios of *rate constants* of the competing reactions, or by the decadic logarithms of such ratios.

See also *isoselective relationship*, *partial rate factor*, *regioselectivity*, *selectivity factor*, *stereoselectivity*.

selectivity factor

A quantitative representation of *selectivity* in *aromatic substitution reactions* (usually *electrophilic*, for monosubstituted benzene derivatives). If the *partial rate factor*, *f*, expresses the reactivity of a specified position in the aromatic compound PhX relative to that of a single position in benzene, then the selectivity factor S_f (expressing discrimination between *p*- and *m*-positions in PhX) is defined as

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$$S_f = \log (f_p^X / f_m^X).$$

STOCK and BROWN (1963).

shielding

In the context of NMR spectroscopy shielding is the effect of the electron shells of the observed and the neighbouring nuclei on the external magnetic field. The external field induces circulations in the electron cloud. The resulting magnetic moment is oriented in the opposite direction to the external field, so that the local field at the central nucleus is weakened, although it may be strengthened at other nuclei (deshielding).

The phenomenon is the origin of the structural dependence of the resonance frequencies of the nuclei.

See also *chemical shift*.

sigma, pi (σ, π)

The terms are symmetry designations, π molecular orbitals being antisymmetric with respect to a defining plane containing at least one atom (e.g. the molecular plane of ethene), and σ molecular orbitals symmetric with respect to the same plane. In practice the terms are used both in this rigorous sense (for orbitals encompassing the entire molecule) and also for localized two-centre orbitals or bonds, and it is necessary to make a clear distinction between the two usages.

In the case of two-centre bonds, a π-bond has a nodal plane that includes the internuclear bond axis, whereas a σ-bond has no such nodal plane. (A δ-bond in organometallic or inorganic molecular species has two nodes.) *Radicals* are classified by analogy into σ- and π-radicals.

Such two-centre orbitals may take part in molecular orbitals of σ- or π-symmetry. For example, the methyl group in propene contains three C-H bonds, each of which is of local σ-symmetry (i.e. without a nodal plane including the internuclear axis), but these three "σ-bonds" can in turn be combined to form a set of group orbitals one of which has π-symmetry with respect to the principal molecular plane and can accordingly interact with the two-centre orbital of π-symmetry (π-bond) of the double-bonded carbon atoms, to form a molecular orbital of π-symmetry.

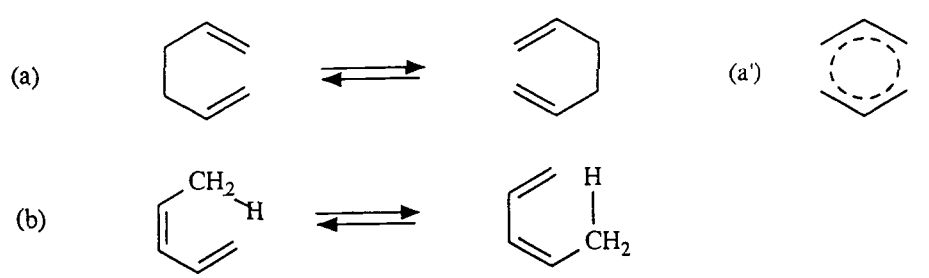
Such an interaction between the CH_3 group and the double bond is an example of what is called *hyperconjugation*. This cannot rigorously be described as "σ-π conjugation" since σ and π here refer to different defining planes, and interaction between orbitals of different symmetries (with respect to the same defining plane) is forbidden.

See also JORGENSEN and SALEM (1973).

sigmatropic rearrangement

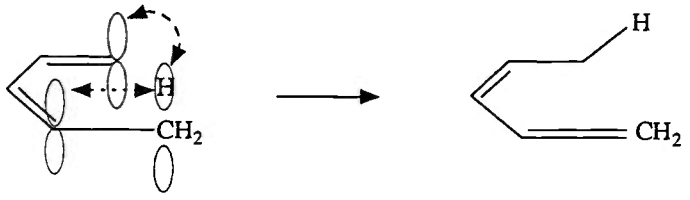
A *molecular rearrangement* that involves both the creation of a new σ-bond between atoms previously not directly linked and the breaking of an existing σ-bond. There is normally a concurrent relocation of π-bonds in the molecule concerned, but the total number of π- and σ-bonds does not change. The term was originally restricted to *intramolecular pericyclic reactions*, and many authors use it with this connotation. It is, however, also applied in a more general, purely structural, sense.

If such reactions are *intramolecular*, their *transition state* may be visualized as an *association* of two fragments connected at their termini by two partial σ-bonds, one being broken and the other being formed as, for example, the two allyl fragments in (a'). Considering only atoms within the (real or hypothetical) cyclic array undergoing reorganization, if the numbers of these in the two fragments are designated i and j, then the rearrangement is said to be a sigmatropic change of order $[i,j]$ (conventionally $[i] \leq [j]$). Thus the rearrangement (a) is of order [3,3], whilst reaction (b) is a [1,5]sigmatropic shift of hydrogen. (N.B.: By convention square brackets [...] here refer to numbers of atoms, in contrast with current usage in the context of cycloaddition.)



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The descriptors a and s (*antarafacial* and *suprafacial*) may also be annexed to the numbers *i* and *j*; (b) is then described as a [1s,5s] sigmatropic rearrangement, since it is suprafacial with respect both to the hydrogen atom and to the pentadienyl system:



The prefix "homo" (meaning one extra atom, interrupting *conjugation* - cf. "*homoaromaticity*") has frequently been applied to sigmatropic rearrangements, but is misleading.

See also *cycloaddition*, *tautomerism*.

silylene

(1) Generic name for H_2Si: and substitution derivatives thereof, containing an electrically neutral bivalent silicon atom with two non-bonding electrons. (The definition is analogous to that given for *carbene*.)

(2) The silanediyl group (H_2Si), analogous to the methylene group (H_2C).

single-electron transfer mechanism (SET)

A reaction *mechanism* characterized by the transfer of a single electron between the species occurring on the *reaction coordinate* of one of the *elementary steps*.

single-step reaction

A reaction that proceeds through a single *transition state*.

Slater-type orbital

An approximate atomic orbital that attempts to allow for electron-electron repulsion by scaling the nuclear charge for each orbital.

soft acid

See *hard acid*.

soft base

See *hard base*.

solvation

Any stabilizing interaction of a solute (or solute *moiety*) and the solvent or a similar interaction of solvent with groups of an insoluble material (i.e., the ionic groups of an ion-exchange resin). Such interactions generally involve electrostatic forces and *van der Waals forces*, as well as chemically more specific effects such as *hydrogen bond* formation.

See also *cybotactic region*.

solvatochromic relationship

A *linear free-energy relationship* based on *solvatochromism*.

See also *Kamlet-Taft solvent parameters*.

solvatochromism

The (pronounced) change in position and sometimes intensity of an electronic absorption or emission band, accompanying a change in the polarity of the medium. Negative (positive) solvatochromism corresponds to a *hypsochromic* (*bathochromic*) shift with increasing solvent *polarity*.

BUNCEL and RAJAGOPAL (1990); REICHARDT (1988).

See also *Dimroth-Reichardt E_T parameter*, *Z-value*.

solvent isotope effect

See *isotope effect*.

solvent parameter

Quantitative measures of the capability of solvents for interaction with solutes. Such parameters have been based on numerous different physico-chemical quantities, e.g. *rate constants*, solvatochromic shifts in ultraviolet/visible spectra, solvent-induced shifts in infrared

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frequencies, etc. Some solvent parameters are purely empirical in nature, i.e. they are based directly on some experimental measurement. It may be possible to interpret such a parameter as measuring some particular aspect of solvent-solute interaction or it may be regarded simply as a measure of solvent *polarity*. Other solvent parameters are based on analysing experimental results. Such a parameter is considered to quantify some particular aspect of solvent capability for interaction with solutes.

See REICHARDT (1965).

See also *Dimroth-Reichardt E_T parameter, Grunwald-Winstein equation, Kamlet-Taft solvent parameters, Koppel-Palm solvent parameters, solvophobicity parameter, Z-value.*

solvent polarity

See *polarity.*

solvent-separated ion pair

See *ion pair.*

solvolysis

Generally, reaction with a solvent, or with a *lyonium ion* or *lyate ion*, involving the rupture of one or more bonds in the reacting solute. More specifically the term is used for *substitution, elimination* and *fragmentation reactions* in which a solvent species is the *nucleophile* ("alcoholysis" if the solvent is an alcohol, etc.).

solvophobicity parameter

A *solvent parameter* defined by

$$Sp = 1 - M/M(\text{hexadecane})$$

derived from the Gibbs energy of transfer ($\Delta_t G^o$) of a series of solutes from water to numerous aqueous-organic mixtures and to pure solvents:

$$\Delta_t G^o(\text{to solvent}) = MR_T + D$$

where R_T is a solute parameter, and M and D characterize the solvent. The M values are used to define a solvent solvophobic effect so that Sp values are scaled from unity (water) to zero (hexadecane).

ABRAHAM, GRELLIER, and MCGILL (1988).

SOMO

A *S*ingly *O*ccupied *M*olecular *O*rbital (such as the half-filled *HOMO* of a radical).

See also *frontier orbitals.*

sonication

Irradiation with (often ultra)sound waves, e.g. to increase the rate of a reaction or to prepare vesicles in mixtures of surfactants and water.

special salt effect

The initial steep rate increase observed in the *kinetic electrolyte effect* on certain solvolysis reactions, upon addition of some non-common ion salts, especially $LiClO_4$.

species

See *chemical species.*

specific catalysis

The acceleration of a reaction by a unique *catalyst*, rather than by a family of related substances. The term is most commonly used in connection with specific hydrogen-ion or hydroxide-ion (*lyonium ion* or *lyate ion*) catalysis.

See also *general acid catalysis, general base catalysis, pseudo-catalysis.*

spectator mechanism

A *pre-association* mechanism in which one of the *molecular entities*, C, is already present in an *encounter pair* with A during formation of B from A, but does not assist the formation of B, e.g.

$$A + C \underset{}{\overset{\text{Pre association}}{\rightleftharpoons}} \underbrace{(A \cdots C)}_{\text{encounter complex}} \longrightarrow \underbrace{(B \cdots C)}_{\text{encounter complex}} \xrightarrow{\text{rapid}} \text{Product}$$

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The formation of B from A may itself be a bimolecular reaction with some other reagent. Since C does not assist the formation of A, it is described as being present as a spectator, and hence such a mechanism is sometimes referred to as a spectator mechanism.

See also *microscopic diffusion control*.

spin adduct

See *spin trapping*.

spin counting

See *spin trapping*.

spin density

The unpaired *electron density* at a position of interest, usually at carbon, in a *radical*. It is often measured experimentally by electron paramagnetic resonance (EPR, ESR (electron spin resonance)) spectroscopy through hyperfine coupling constants of the atom or an attached hydrogen.

See also *radical centre*.

spin label

A *stable* paramagnetic *group* (typically a nitryl radical) that is attached to a part of a *molecular entity* whose microscopic environment is of interest and may be revealed by the electron spin resonance (ESR) spectrum of the spin label.

When a simple paramagnetic molecular entity is used in this way without covalent attachment to the molecular entity of interest it is frequently referred to as a "spin probe".

spin trapping

In certain reactions in solution a *transient radical* will interact with a diamagnetic reagent to form a more *persistent* radical. The product radical accumulates to a concentration where detection and, frequently, identification are possible by EPR/ESR spectroscopy. The key reaction is usually one of *attachment*; the diamagnetic reagent is said to be a "spin trap", and the persistent product radical is then the "spin *adduct*". The procedure is referred to as spin trapping, and is used for monitoring reactions involving the intermediacy of *reactive* radicals at concentrations too low for direct observation. Typical spin traps are *C*-nitroso compounds and nitrones, to which *reactive* radicals will rapidly add to form nitryl radicals. A quantitative development, in which essentially all reactive radicals generated in a particular system are intercepted, has been referred to as "spin counting". Spin trapping has also been adapted to the interception of radicals generated in both gaseous and solid phases. In these cases the spin adduct is in practice transferred to a liquid solution for observation in order to facilitate interpretation of the EPR/ESR spectra of the radicals obtained.

stable

As applied to *chemical species*, the term expresses a thermodynamic property, which is quantitatively measured by relative molar standard Gibbs energies. A chemical species A is more stable than its isomer B if $\Delta_r G^\circ > 0$ for the (real or hypothetical) reaction $A \rightarrow B$, under standard conditions. If for the two reactions

$$\begin{array}{ll} P \rightarrow X + Y & (\Delta_r G_1^\circ) \\ Q \rightarrow X + Z & (\Delta_r G_2^\circ) \end{array}$$

$\Delta_r G_1^\circ > \Delta_r G_2^\circ$, P is more stable relative to the product Y than is Q relative to Z. Both in qualitative and quantitative usage the term stable is therefore always used in reference to some explicitly stated or implicitly assumed standard.

The term should not be used as a synonym for *unreactive* or "less reactive" since this confuses thermodynamics and kinetics. A relatively more stable chemical species may be more *reactive* than some reference species towards a given reaction partner.

See also *inert*, *unstable*.

stationary state

(1) In quantum mechanics: A state that does not evolve with time.

(2) In kinetics: See *steady state*.

steady state (or stationary state)

(1) In a kinetic analysis of a complex reaction involving *unstable* intermediates in low concentration, the rate of change of each such *intermediate* is set equal to zero, so that the *rate equation* can be expressed as a function of the concentrations of *chemical species* present in

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macroscopic amounts. For example, assume that X is an unstable intermediate in the reaction sequence:

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X$$

$$X + C \xrightarrow{k_2} D$$

Conservation of mass requires that:

$$[A] + [X] + [D] = [A]_0$$

which, since $[A]_0$ is constant, implies:

$$-d[X]/dt = d[A]/dt + d[D]/dt.$$

Since [X] is negligibly small, the rate of formation of D is essentially equal to the rate of disappearance of A, and the rate of change of [X] can be set equal to zero. Applying the steady state approximation ($d[X]/dt = 0$) allows the elimination of [X] from the kinetic equations, whereupon the rate of reaction is expressed:

$$d[D]/dt = -d[A]/dt = \frac{k_1 k_2 [A][C]}{k_{-1} + k_2 [C]}$$

Note: The steady-state approximation does not imply that [X] is even approximately constant, only that its absolute rate of change is very much smaller than that of [A] and [D]. Since according to the reaction scheme $d[D]/dt = k_2[X][C]$, the assumption that [X] is constant would lead, for the case in which C is in large excess, to the absurd conclusion that formation of the product D will continue at a constant rate even after the reactant A has been consumed.

(2) In a stirred flow reactor a steady state implies a regime so that all concentrations are independent of time.

stepwise reaction

A *chemical reaction* with at least one reaction *intermediate* and involving at least two consecutive *elementary reactions*.

See also *composite reaction*.

stereoelectronic

Pertaining to the dependence of the properties (especially the energy) of a *molecular entity* in a particular electronic state (or of a *transition state*) on relative nuclear geometry. The electronic ground state is usually considered, but the term can apply to excited states as well. Stereoelectronic effects are ascribed to the different alignment of electronic orbitals in different arrangements of nuclear geometry.

stereoelectronic control

Control of the nature of the products of a *chemical reaction* (or of its rate) by *stereoelectronic* factors. The term is usually applied in the framework of an orbital approximation. The variations of *molecular orbital* energies with relative nuclear geometry (along a *reaction coordinate*) are then seen as consequences of variations in basis-orbital overlaps.

stereoselectivity, stereoselective

Stereoselectivity is the preferential formation in a chemical reaction of one stereoisomer over another. When the stereoisomers are enantiomers, the phenomenon is called enantioselectivity and is quantitatively expressed by the enantiomer excess; when they are diastereoisomers, it is called diastereoselectivity and is quantitatively expressed by the diastereomer excess. Reactions are termed (100%) stereoselective if the discrimination is complete or partially (*x*%) stereoselective if one product predominates. The discrimination may also be referred to semiquantitatively as high or low stereoselectivity.

ELIEL (1962); IUPAC STEREOCHEMICAL TERMINOLOGY (1993).

stereospecificity, stereospecific

(1) A reaction is termed stereospecific if starting materials differing only in their *configuration* are converted into stereoisomeric products. According to this definition, a stereospecific process is necessarily *stereoselective* but not all *stereoselective* processes are stereospecific. Stereospecificity may be total (100%) or partial. The term is also applied to

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situations where reaction can be performed with only one stereoisomer. For example the exclusive formation of *trans*-1,2-dibromocyclohexane upon bromination of cyclohexene is a stereospecific process, although the analogous reaction with (*E*)-cyclohexene has not been performed.

(2) The term has also been applied to describe a reaction of very high stereoselectivity, but this usage is unnecessary and is discouraged.

ELIEL (1962).

[For the use of the term "stereospecific polymerization" see IUPAC POLYMERS (1981)]

steric-approach control

Control of *stereoselectivity* of a reaction by steric hindrance towards attack of the reagent, which is directed to the less hindered face of the molecule. Partial bond making is strong enough at the *transition state* for steric control to take place. This suggests that the transition state should not be close to products.

See also *product development control.*

steric effect

The effect on a chemical or physical property (structure, rate or equilibrium constant) upon introduction of substituents having different steric requirements. The steric effect in a reaction is ascribed to the difference in steric energy between, on the one hand, reactants and, on the other hand, a *transition state*, (or products). A steric effect on a rate process may result in a rate increase ("steric acceleration") or a decrease ("steric retardation"). (The adjective "steric" is not to be confused with *stereochemical.*)

Steric effects arise from contributions ascribed to *strain* as the sum of (1) non-bonded repulsions, (2) bond angle strain, and (3) bond stretches or compressions.

For the purpose of *correlation analysis* or *linear free-energy relations* various scales of steric parameters have been proposed, notably *A values*, Taft's E_s and Charton's υ scales.

In a reactant molecule RY and an appropriate reference molecule R°Y, the "primary steric effect" of R is the direct result of differences in compressions which occur because R differs from R° in the vicinity of the reaction centre Y. A "secondary steric effect" involves the differential moderation of electron delocalization by non-bonded compressions.

Some authors make a distinction between "steric" effects attributed to van der Waals repulsions alone, and "strain" effects, attributed to deviations of bond angles from "ideal" values.

CHARTON (1987).

See *Taft equation, van der Waals forces.*

steric isotope effect

See *isotope effect, steric.*

steric hindrance

The original term for a *steric effect* arising from crowding of substituents.

stoichiometric number

See *rate of reaction.*

stopped flow

A technique for following the kinetics of reactions in solution (usually in the millisecond time range) in which two reactant solutions are rapidly mixed by being forced through a mixing chamber. The flow of the mixed solution along a uniform tube is then suddenly arrested. At a fixed position along the tube the solution is monitored (as a function of time following the stoppage of the flow) by some method with a rapid response (e.g. photometry).

See *mixing control.*

strain

Strain is present in a *molecular entity* or *transition structure* if the energy is enhanced because of unfavourable bond lengths, bond angles, or dihedral angles ("torsional strain") relative to a standard.

It is quantitatively defined as the standard enthalpy of a structure relative to a strainless structure (real or hypothetical) made up from the same atoms with the same types of bonding. (The enthalpy of formation of cyclopropane is 53.6 kJ mol^{-1}, whereas the enthalpy of formation based on three "normal" methylene groups, from acyclic models, is -62 kJ mol^{-1}. On this basis cyclopropane is destabilized by *ca.* 115 kJ mol^{-1} of strain energy.)

See *molecular mechanics calculation.*

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subjacent orbital

The *N*ext-to-*H*ighest *O*ccupied *M*olecular *O*rbital ("*NHOMO*", also called "HOMO^{-}") and the *S*econd *L*owest *U*noccupied *M*olecular *O*rbital (SLUMO). Subjacent orbitals are sometimes found to play an important role in the interpretation of molecular interactions according to the *frontier orbital* approach.

BERSON (1972).

substituent electronegativity

See *electronegativity*.

substituent

An atom or *group* of bonded atoms that can be considered to have replaced a hydrogen atom (or two hydrogen atoms in the special case of bivalent groups) in a parent *molecular entity* (real or hypothetical).

substitution reaction

A *reaction*, *elementary* or *stepwise*, in which one atom or *group* in a *molecular entity* is replaced by another atom or group. For example,

$$CH_3Cl + OH^- \longrightarrow CH_3OH + Cl^-$$

substrate

A *chemical species*, the reaction of which with some other chemical reagent is under observation (e.g., a compound that is transformed under the influence of a *catalyst*). The term should be used with care. Either the context or a specific statement should always make it clear which chemical species in a reaction is regarded as the substrate.

See also *transformation*.

successor complex

The radical ion pair which forms by the transfer of an electron from the donor D to the acceptor A after these species have diffused together to form the *precursor* or *encounter complex*:

$$A + D \longrightarrow (A\ D) \longrightarrow (A^{\cdot -}\ D^{\cdot +})$$

suicide inhibition

See *mechanism-based inhibition*.

superacid

A medium having a high *acidity*, generally greater than that of 100 wt.-% sulfuric acid. The common superacids are made by dissolving a powerful Lewis acid (e.g. SbF_5) in a suitable *Brønsted acid*, such as HF or HSO_3F. (An equimolar mixture of HSO_3F and SbF_5 is known by the trade name "magic acid".)

In a biochemical context "superacid catalysis" is sometimes used to denote catalysis by metal ions analogous to catalysis by hydrogen ions.

By analogy, a compound having a very high *basicity*, such as lithium diisopropylamide, is called a "superbase".

GILLESPIE (1968); OLAH (1985); OLAH and OLAH (1970).

superbase

See *superacid*.

suprafacial

See *antarafacial*.

supramolecule

A system of two or more *molecular entities* held together and organized by means of intermolecular (noncovalent) binding interactions.

LEHN (1993).

Swain-Scott equation

The *linear free-energy relation* of the form

$$\lg(k/k_0) = sn$$

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applied to the variation of *reactivity* of a given *electrophilic substrate* towards a series of nucleophilic reagents. n is characteristic of the reagent (i.e. a measure of its *nucleophilicity*) and s is characteristic of the substrate (i.e. a measure of its sensitivity to the nucleophilicity of the reagent). A scale of n values is based on the rate coefficients k for the reaction of methyl bromide with nucleophiles in water at 25 °C, s being defined as 1.00 for these reactions and n being defined as 0.00 for the hydrolysis of methyl bromide. (Other scales have been devised.)

SWAIN and SCOTT (1953).

Swain-Lupton equation

A dual parameter approach to the correlation analysis of substituent effects, which involves a field constant ($\mathcal{F}$) and a resonance constant ($\mathcal{R}$). The original treatment was modified later.

SWAIN and LUPTON (1968); SWAIN, UNGER, ROSENQUIST and SWAIN (1983).

The procedure has been considerably applied, but also much criticized (see REYNOLDS and TOPSOM (1984); HOEFNAGEL, OSTERBEEK and WEPSTER (1984); CHARTON (1984); SWAIN (1984); HANSCH, LEO, and TAFT (1991).

symbiosis

The term was originally applied to describe the maximum flocking of either hard or soft *ligands* in the same complexes. For hydrocarbon molecules, symbiosis implies that those containing a maximum number of C-H bonds (e.g. CH_4) or C-C bonds (e.g. Me_4C) are the most stable.

HO (1977).

symproportionation

Synonymous with *comproportionation*.

syn

See *anti*.

synartetic acceleration

See *neighbouring group participation*.

synchronization (principle of nonperfect synchronization)

This principle applies to reactions in which there is a lack of synchronization between bond formation or bond rupture and other *primitive changes* that affect the stability of products and reactants, such as *resonance*, *solvation*, electrostatic, *hydrogen bonding* and *polarizability* effects. The principle states that a product-stabilizing factor whose development lags behind bond changes at the *transition state*, or a reactant-stabilizing factor whose loss is ahead of bond changes at the *transition state*, increases the *intrinsic barrier* and decreases the "intrinsic rate constant" of a reaction. For a product-stabilizing factor whose development is ahead of bond changes, or reactant factors whose loss lags behind bond changes, the opposite relations hold. The reverse effects are observable for factors that destabilize a reactant or product.

BERNASCONI (1992).

See also *imbalance*, *synchronous*.

synchronous

A *concerted* process in which the *primitive changes* concerned (generally bond rupture and bond formation) have progressed to the same extent at the *transition state* is said to be synchronous. The term figuratively implies a more or less synchronized progress of the changes. However, the progress of the bonding change (or other primitive change) has not been defined quantitatively in terms of a single parameter applicable to different bonds or different bonding changes. The concept is therefore in general only qualitatively descriptive and does not admit an exact definition except in the case of concerted processes involving changes in two identical bonds.

See also *imbalance*.

σ-adduct (sigma adduct)

The product formed by the *attachment of* an *electrophilic* or *nucleophilic entering group* or of a *radical* to a ring carbon of an aromatic species so that a new σ-bond is formed and the original *conjugation* is disrupted. (This has generally been called a "σ-complex", but adduct is more appropriate than complex according to the definitions given.) The term may also be used for analogous adducts to unsaturated (and conjugated) systems in general.

See also *Meisenheimer adduct*.

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σ-bond (sigma bond)

See *sigma, pi.*

σ-constant (sigma constant)

Specifically the substituent constant for *meta-* and for *para*-substituents in benzene derivatives as defined by Hammett on the basis of the ionization constant of a substituted benzoic acid in water at 25°C, i.e. $\log(K_a/K_a^o)$, where K_a is the ionization constant of a *m-* or *p*-substituted benzoic acid and K_a^o that of benzoic acid itself.

The term is also used as a collective description for related electronic substituent constants based on other standard reaction series, of which, σ^+, σ^- and σ^o are typical; also constants which represent dissected electronic effects such as σ_I and σ_R. For this purpose it might be better always to spell out the term in full, i.e. as "Hammett sigma constant", and restrict σ-constants to the scale of substituent constants which is based on benzoic acid.

A large positive σ-value implies high electron-withdrawing power by inductive and/or resonance effect, relative to H; a large negative σ-value implies high electron-releasing power relative to H.

CHAPMAN and SHORTER (1972, 1978); JOHNSON (1973); SHORTER (1973).

See also *Hammett equation, ρ-value, Taft equation.*

σ-orbital (sigma orbital)

See *sigma, pi.*

T-jump

See *chemical relaxation.*

Taft equation

Various equations are associated with R.W. Taft, but the term is most often used to designate the family of equations that emerged from Taft's analysis of the reactivities of aliphatic esters, and which involved the polar substituent constant σ^* and the steric substituent constant E_s

$$\log k = \log k_o + \rho^*\sigma^* + \delta E_s$$

or the one-parameter forms applicable when the role of either the polar term or the steric term may be neglected. Nowadays σ^* is usually replaced by the related constant σ_I.

TAFT (1952, 1953).

See also *Hammett equation, ρ-value, σ-constant.*

+ tautomeric effect

See *electromeric effect.*

tautomerism

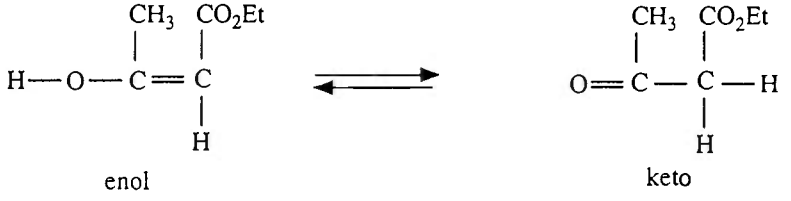
Isomerism of the general form

$$\text{G-X-Y=Z} \rightleftharpoons \text{X=Y-Z-G}$$

where the isomers (called tautomers) are readily interconvertible; the atoms connecting the groups X,Y,Z are typically any of C, H, O, or S, and G is a group which becomes an *electrofuge* or *nucleofuge* during isomerization. The commonest case, when the electrofuge is H^+, is also known as "prototropy".

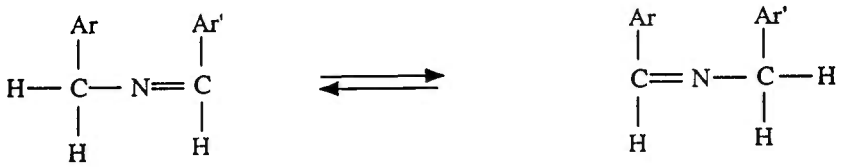
Examples, written so as to illustrate the general pattern given above, include:

Keto-enol tautomerism, such as



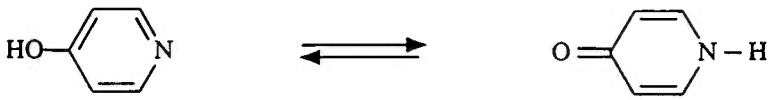
(G = H, X = O, Y = CCH_3, Z = $CHCO_2Et$)

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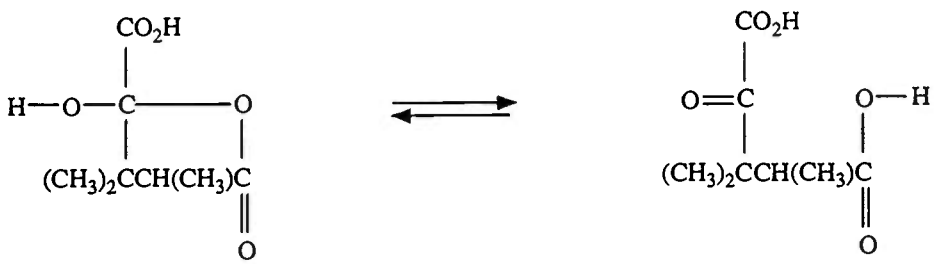
(G = H, X = CHAr, Y = N, Z = CHAr')

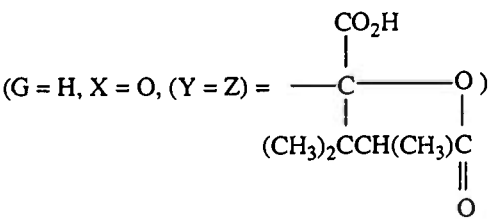
The grouping Y may itself be a three-atom (or five-atom) chain extending the conjugation, as in



(G = H, X = O, Y = C-CH=CH, Z = N)

The double bond between Y and Z may be replaced by a ring, when the phenomenon is called ring-chain tautomerism, as in





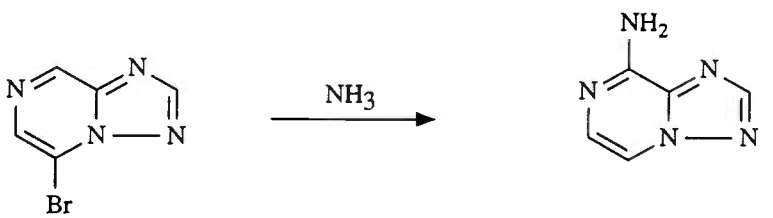
INGOLD (1953).
See also *ambident, sigmatropic rearrangement, tautomerization, valence tautomerization.*

tautomerization

The *isomerization* by which tautomers are interconverted. It is a *heterolytic molecular rearrangement* and is frequently very rapid.
See *tautomerism.*

tele-substitution

A *substitution reaction* in which the *entering group* takes up a position more than one atom away from the atom to which the *leaving group* was attached.

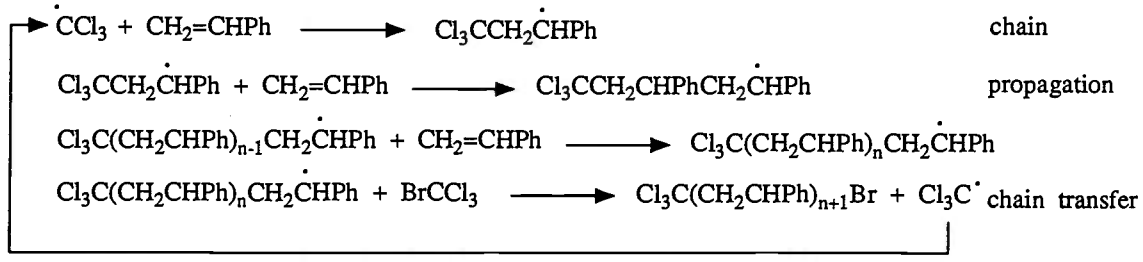


See also *cine-substitution.*

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telomerization

The formation of an *addition* oligomer, having uniform end groups X'...X", by a *chain reaction* in which a *chain transfer* limits the length of the polymer ("telomer") produced. An example is the polymerization of styrene in bromotrichloromethane solution (X' = CCl_3, X" = Br), where $Cl_3C\cdot$ radicals are formed in the initiation step to produce $Cl_3C[CH_2CHPh]_nBr$, with n greater than 1 and often less than ca. 10:



termination

The steps in a *chain reaction* in which reactive *intermediates* are destroyed or rendered inactive, thus ending the chain.

tetrahedral intermediate

A reaction *intermediate* in which the bond arrangement around an initially double-bonded carbon atom (typically a carbonyl carbon atom) has been transformed from trigonal to tetrahedral. For example, aldol in the *condensation reaction* of acetaldehyde (but most tetrahedral intermediates have a more fleeting existence).

thermodynamic control (of product composition)

The term characterizes conditions that lead to reaction products in a proportion governed by the equilibrium constant for their interconversion and/or for the interconversion of reaction *intermediates* formed in or after the *rate-limiting step*. (Some workers prefer to describe this phenomenon as "equilibrium control".)

See also *kinetic control*.

thermolysis

The uncatalysed cleavage of one or more covalent *bonds* resulting from exposure of a compound to a raised temperature, or a process in which such cleavage is an essential part.

See also *pyrolysis*.

tight-ion pair

See *ion pair*.

torquoselectivity

The term refers to the preference for "inward" or "outward" rotation of substituents in conrotatory or disrotatory electrocyclic ring opening reactions.

JEFFORD, BERNARDINELLI, WANG, SPELLMEYER, BUDA, and HOUK (1992).

transferability

Transferability assumes invariance of properties, associated conceptually with an atom or a fragment present in a variety of molecules. The property, such as *electronegativity*, nucleophilicity, NMR *chemical shift*, etc. is held as retaining a similar value in all these occurrences.

transformation

The conversion of a *substrate* into a particular product, irrespective of reagents or *mechanisms* involved. For example, the transformation of aniline ($C_6H_5NH_2$) into *N*-phenylacetamide ($C_6H_5NHCOCH_3$) may be effected by use of acetyl chloride or acetic anhydride or ketene. A transformation is distinct from a reaction, the full description of which would state or imply all the reactants and all the products.

For nomenclature of transformations see IUPAC TERMINOLOGY FOR TRANSFORMATIONS (1989).

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transient (chemical) species

Relating to a short-lived reaction *intermediate*. It can be defined only in relation to a time scale fixed by the experimental conditions and the limitations of the technique employed in the detection of the intermediate. The term is a relative one.

Transient species are sometimes also said to be "metastable". However, this latter term should be avoided, because it relates a thermodynamic term to a kinetic property, although most transients are also thermodynamically *unstable* with respect to reactants and products.

See also *persistent*.

transition coordinate

The *reaction coordinate* at the *transition state* corresponding to a vibration with an imaginary frequency. Motion along it in the two opposite senses leads towards the reactants or towards the products.

See also *reaction coordinate*, *transition state*.

transition state

In theories describing *elementary reactions* it is usually assumed that there is a transition state of more positive molar Gibbs energy between the reactants and the products through which an assembly of atoms (initially composing the *molecular entities* of the reactants) must pass on going from reactants to products in either direction. In the formalism of "transition state theory" the transition state of an elementary reaction is that set of states (each characterized by its own geometry and energy) which an assembly of atoms, when randomly placed there, would have an equal probability of forming the reactants or of forming the products of that elementary reaction. The transition state is characterized by one and only one imaginary frequency. The assembly of atoms at the transition state has been called an *activated complex*. (It is not a *complex* according to the definition in this Glossary.)

It may be noted that the calculations of reaction rates by the transition state method and based on calculated *potential-energy surfaces* refer to the potential energy maximum at the saddle point, as this is the only point for which the requisite separability of transition state coordinates may be assumed. The ratio of the number of assemblies of atoms that pass through to the products to the number of those that reach the saddle point from the reactants can be less than unity, and this fraction is the "transmission coefficient" κ. (There are also reactions, such as the gas-phase *colligation* of simple *radicals*, that do not require "activation" and which therefore do not involve a transition state.)

See also *Gibbs energy of activation*, *Hammond principle*, *potential energy profile*, *transition structure*.

transition state analogue

A substrate designed to mimic the properties or the geometry of the *transition state* of reaction.

transition structure

A saddle point on a *potential-energy surface*. It has one negative force constant in the harmonic force constant matrix.

See also *activated complex*, *transition state*.

transmission coefficient

See *transition state*.

transport control

See *microscopic diffusion control*.

trapping

The interception of a *reactive* molecule or reaction *intermediate* so that it is removed from the system or converted into a more *stable* form for study or identification.

See also *scavenger*.

tunnelling

The process by which a particle or a set of particles crosses a barrier on its potential energy surface without having the energy required to surmount this barrier. Since the rate of tunnelling decreases with increasing reduced mass, it is significant in the context of *isotope effects* of hydrogen isotopes.

umpolung

Any process by which the normal alternating donor and acceptor reactivity pattern of a chain, which is due to the presence of O or N heteroatoms, is interchanged. Reactivity

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umpolung is most often achieved by temporary exchange of heteroatoms (N, O) by others, such as P, S, and Se.

The original meaning of the term has been extended to the reversal of any commonly accepted reactivity pattern. For example, reaction of R-C≡CX (X = halide) as a synthon for "R-C≡C$^+$ (i.e. *electrophilic* acetylene) is an umpolung of the normal more common acetylide, R-C≡C$^-$ (i.e. *nucleophilic*) reactivity.

SEEBACH (1979).

unimolecular

See *molecularity*.

unreactive

Failing to react with a specified *chemical species* under specified conditions. The term should not be used in place of *stable*, since a relatively more stable species may nevertheless be more *reactive* than some reference species towards a given reaction partner.

unstable

The opposite of *stable*, i.e. the *chemical species* concerned has a higher molar Gibbs energy than some assumed standard. The term should not be used in place of *reactive* or *transient*, although more reactive or transient species are frequently also more unstable.

(Very unstable chemical species tend to undergo exothermic *unimolecular* decompositions. Variations in the structure of the related chemical species of this kind generally affect the energy of the *transition states* for these decompositions less than they affect the stability of the decomposing chemical species. Low stability may therefore parallel a relatively high rate of unimolecular decomposition.)

upfield

See *chemical shift*.

valence

The maximum number of univalent atoms (originally hydrogen or chlorine atoms) that may combine with an atom of the element under consideration, or with a fragment, or for which an atom of this element can be substituted.

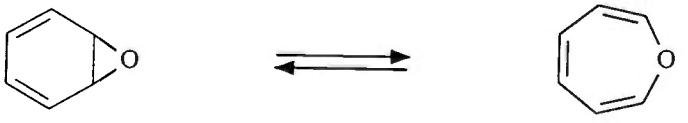
valence isomer

A constitutional isomer interrelated with another by *pericyclic reactions*. For example, Dewar benzene, prismane and benzvalene are valence isomers of benzene.

See *tautomerism*.

valence tautomerization

The term describes simple reversible and generally rapid *isomerizations* or *degenerate rearrangements* involving the formation and rupture of single and/or double *bonds*, without *migration* of atoms or *groups*; e.g.



TISLER (1973)

See also *fluxional*, *tautomerism*.

van der Waals forces

The attractive or repulsive forces between *molecular entities* (or between groups within the same molecular entity) other than those due to *bond* formation or to the electrostatic interaction of ions or of ionic *groups* with one another or with neutral molecules. The term includes: *dipole-dipole*, *dipole-induced dipole* and *London* (instantaneous induced dipole-induced dipole) *forces*.

The term is sometimes used loosely for the totality of nonspecific attractive or repulsive intermolecular forces.

volume of activation, $\Delta^{\ddagger}V$

A quantity derived from the pressure dependence of the *rate constant* of a reaction (mainly used for reactions in solution), defined by the equation

$$\Delta^{\ddagger}V = -RT(\partial \ln k/\partial p)_T$$

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providing that the rate constants of all reactions (except first-order reactions) are expressed in pressure-independent concentration units, such as mol dm^{-3} at a fixed temperature and pressure.

The volume of activation is interpreted, according to *transition state* theory as the difference between the partial molar volumes of the transition state ($\ddagger V$) and the sums of the partial volumes of the reactants at the same temperature and pressure, i.e.,

$$\Delta\ddagger V = \ddagger V - \Sigma(rV_R)$$

where r is the order in the reactant R and V_R its partial molar volume.

Wheland intermediate

See *Meisenheimer adduct, σ-adduct.*

Woodward-Hoffmann rules

See *orbital symmetry.*

Ylide

A *chemical species* produced (actually or notationally) by loss of a *hydron* from an atom directly attached to the central atom of an "*onium*" *ion*, e.g.

$$Ph_3P^+\text{–}CHRR' \longrightarrow \left(Ph_3P^+C^-RR' \longleftrightarrow Ph_3P{=}CRR' \right)$$

ylide

Yukawa-Tsuno equation

A multiparameter extension of the *Hammett equation* to quantify the role of *enhanced resonance* effects on the reactivity of *meta-* and *para*-substituted benzene derivatives, e.g.

$$\lg k = \lg k_o + \rho[\sigma + r(\sigma^+ - \sigma)]$$

The parameter r gives the enhanced resonance effect on the scale $(\sigma^+-\sigma)$, or $(\sigma^--\sigma)$, respectively.

See also *ρ-value, σ-constant.*

See YUKAWA and TSUNO (1959); SHORTER (1973).

Z-value

An index of the *ionizing power* of a solvent based on the frequency of the longest wavelength electronic absorption maximum of 1-ethyl-4-methoxycarbonylpyridinium iodide in the solvent. The Z-value is defined by

$$Z = 2.859 \times 10^4/\lambda$$

where Z is in kcal mol^{-1} and λ is in nm.

KOSOWER (1958).

See also *Dimroth-Reichardt E_T parameter, Grunwald-Winstein equation.*

Zaitsev rule

See *Saytzeff rule.*

zwitterionic compound

A neutral compound having electrical charges of opposite sign, delocalized or not on adjacent or nonadjacent atoms. Zwitterionic compounds have no uncharged canonical representations. Sometimes referred to as inner salts, ampholytes, dipolar ions (a misnomer).

For example: $H_3N^+CH_2C(=O)O^-$, glycine.

See also *ylide.*

Zucker-Hammett hypothesis

This hypothesis states that, if in an acid catalyzed reaction, lg k_1 (first-order rate constant of the reaction) is linear in H_o (Hammett *acidity function*), water is not involved in the *transition state* of the *rate-controlling step.* However, if log k_1 is linear in log[H^+], then water is involved. This has been shown to be incorrect by Hammett himself (see HAMMETT (1970)).

LONG and PAUL (1957).

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Grade: USP (3%), technical (3, 6, 27.5, 30, 35, 50, and 90%), FCC. Most common commercial strengths are 27.5, 35, 50, and 70%.
Hazard: Dangerous fire and explosion risk, strong oxidizing agent. Concentrated solutions are highly toxic and strongly irritating. TLV: TWA 1 ppm; animal carcinogen.
Use: Bleaching and deodorizing of textiles, wood pulp, hair, fur, etc.; source of organic and inorganic peroxides; pulp and paper industry; plasticizers; rocket fuel; foam rubber; manufacture of glycerol; antichlor; dyeing; electroplating; antiseptic; laboratory reagent; epoxidation, hydroxylation, oxidation, and reduction; viscosity control for starch and cellulose derivatives; refining and cleaning metals; bleaching and oxidizing agent in foods; neutralizing agent in wine distillation; seed disinfectant; substitute for chlorine in water and sewage treatment.
See "Kastone" *[Du Pont]*.

hydrogen phosphide. See phosphine.

hydrogen, phosphoretted. See phosphine.

hydrogen selenide.
CAS: 7783-07-5. H_2Se.
Properties: Colorless gas. Bp –42C; fp –64C; d 2.00 (air = 1). Soluble in water, carbon disulfide, phosgene.
Grade: 98% pure.
Hazard: Dangerous fire and explosion risk; reacts violently with oxidizing materials. Toxic by inhalation, strong irritant to skin, damaging to lungs and liver. TLV: 0.05 ppm (as selenium).
Use: Preparation of metallic selenides and organoselenium compounds; in doping as mix for preparation of semiconductor materials containing controlled amounts of significant impurities.

hydrogen slush. A mixture of solid and liquid hydrogen at the hydrogen triple point 13.8K and 1.02 psia. It is denser and less hazardous than liquid hydrogen.

hydrogen sodium selenite. See selenious acid, monosodium salt.

hydrogen sulfide. (sulfuretted hydrogen).
CAS: 7783-06-4. H_2S.
Properties: Colorless gas; offensive odor. D 1.189 (air = 1.00), fp –83.8C, bp –60.2C, sp vol 11.23 cu ft/lb (21.1C, 1 atm), autoign temp 500F (260C). Soluble in water and alcohol.
Derivation: (1) By the action of dilute sulfuric acid on a sulfide, usually iron sulfide; (2) by direct union of hydrogen and sulfur vapor at a definite temperature and pressure; (3) as a by-product of petroleum refining.
Grade: Technical 98.5%, purified 99.5% min, CP.
Hazard: Highly flammable, dangerous fire risk, explosive limits in air 4.3–46%. Toxic by inhalation, strong irritant to eyes and mucous membranes. TLV: TWA 10 ppm; STEL 15 ppm.
Use: Purification of hydrochloric acid and sulfuric acid, precipitating sulfides of metals, analytical reagent, source of sulfur and hydrogen.

hydrogen tellurate. See telluric acid.

hydrogen telluride. H_2Te.
Properties: Colorless gas. D (liquid): 2.57 (–20C), fp –49C, bp –2C. Soluble in water but unstable; soluble in alcohol and alkalies.
Hazard: See hydrogen selenide.

"Hydroholac" ***[Rohm & Haas].*** TM for plasticized nitrocellulose lacquer emulsions, including clear finishes, binders and colors. Produce flexible, lacquer-type, cleanable leather finishes from aqueous systems.
Use: Finishes on glove, garment, handbag and shoe leather.

α-hydro-ω-hydroxypoly(oxy(methyl-1,2-ethanediyl)) ether with bis((2-(hydroxyethyl)amino)methyl)phenol (3:1).
CAS: 68909-26-2.
Hazard: A moderate eye irritant.

hydrol. See tetramethyldiaminobenzhydrol.

hydrolase. (hydrase). An enzyme which catalyzes the removal of water from the substrate.
See enzyme.

"Hydrolin" ***[Olin].*** TM for ammonium nitrate preparations.
Hazard: See ammonium nitrate.
Use: Bleaching agent for pulp, clay, and mineral processing.

hydroliquefaction. Production of liquid hydrocarbon fuels by hydrogenation of coal.
See gasification; Oil/Gas Process.

hydrolube. A water-glycol base noncombustible hydraulic fluid.

hydrolysis. A chemical reaction in which water reacts with another substance to form two or more new substances. This involves ionization of the water molecule as well as splitting of the compound hydrolyzed, e.g., $CH_3COOC_2H_5 + H_2O \rightarrow CH_3COOH + C_2H_5OH$. Examples are conversion of starch to glucose by water in the presence of suitable catalysts; conversion of sucrose (cane sugar) to glucose and fructose by reaction with water in the presence of an enzyme or acid catalyst; conversion of

natural fats into fatty acids and glycerol by reaction with water in one process of soap manufacture; and reaction of the ions of a dissolved salt to form various products, such as acids, complex ions, etc.

hydrolyzed vegetable proteins. See "Hydrotriticum" *[Croda].*

hydrometer. Device for measuring the density of liquids.
See Baumé.

hydronium ion. An ion (H_3O^+) formed by the transfer of a proton (hydrogen nucleus) from one molecule of water to another; a companion ion (OH^-) is also formed, the reaction is $2H_2O \rightarrow H_3O^+ + OH^-$. Formation of such ions is statistically rare, resulting from the interaction of water molecules in a ratio of 1:556 million.

hydropathy index. A scale that expresses the relative hydrophobic and hydrophilic tendencies of amino acid R groups. Used to predict membrane-spanning regions.

hydroperoxide. An organic peroxide having the generalized formula ROOH. An example is ethyl hydroperoxide (C_2H_5OOH). Methyl and ethyl hydroperoxides are unstable and thus are strong oxidizing agents and explosion hazards; those of higher molecular weight are more stable. Hydroperoxides can be derived by oxidation of saturated hydrocarbons, or by alkylating hydrogen peroxide in a strongly acidic environment. They are used as polymerization initiators.

hydrophilic. Having a strong tendency to bind or absorb water, which results in swelling and formation of reversible gels. This property is characteristic of carbohydrates, such as algin, vegetable gums, pectins, and starches, and of complex proteins such as gelatin and collagen.

hydrophobic. (1) Antagonistic to water; incapable of dissolving in water. This property is characteristic of all oils, fats, waxes, and many resins, as well as of finely divided powders like carbon black and magnesium carbonate.
(2) The association of nonpolar groups or compounds with each other in aqueous systems due to their insolubility in water.

hydroponics. See nutrient solution.

hydroquinol. See hydroquinone.

hydroquinone. (quinol; hydroquinol; *p*-dihydroxybenzene).
CAS: 123-31-9. $C_6H_4(OH)_2$.
Properties: White crystals. D 1.330, mp 170C, bp 285C, flash p 329F (165C), autoign temp 960F (515.5C). Soluble in water, alcohol, and ether. Combustible.
Derivation: Aniline is oxidized to quinone by manganese dioxide and is then reduced to hydroquinone.
Grade: Technical, photographic.
Hazard: Toxic by ingestion and inhalation, irritant. TLV: TWA 2 mg/m³; animal carcinogen.
Use: Photographic developer (except color film); dye intermediate; inhibitor; stabilizer in paints and varnishes, motor fuels, and oils; antioxidant for fats and oils; inhibitor of polymerization; skin hyperpigmentation.

hydroquinone benzyl ether. See *p*-benzyloxyphenol.

hydroquinone dibenzyl ether.
CAS: 103-16-2. $C_6H_5CH_2OC_6H_4OCH_2C_6H_5$.
Properties: Tan powder. Mp 119C (min). Purity 90% (min). Insoluble in water; soluble in acetone, benzene, and chlorobenzene. Combustible.
Use: Solvent; perfumes, soap, plastics, and pharmaceuticals.

hydroquinone di-*n*-butyl ether. (1,4-dibutoxybenzene). $C_6H_4[O(CH_2)_3CH_3]_2$.
Properties: White flakes; no appreciable odor. Mp 45–46C; bp 124C (1.3 mm Hg), 158C (15.0 mm Hg). Insoluble in water; soluble in benzene, acetone, ethyl acetate, and alcohol. Combustible.

hydroquinone diethyl ether. (1,4-diethoxybenzene). $C_6H_4(OC_2H_5)_2$.
Properties: White granular solid with anise-like odor. Mp 71–72C, bp 246C. Neither boiling caustic nor acid solution causes any hydrolysis. Absorbs UV light. Insoluble in water; soluble in benzene, acetone, ethyl acetate, and alcohol. Combustible.

hydroquinone di(β-hydroxyethyl) ether.
(*p*-di-[2-hydroxyethoxy]benzene).
$C_6H_4(OC_2H_4OH)_2$.
Properties: White solid. Mp 99C, bp 185–200C (0.3 mm Hg). Slightly soluble in water and most organic solvents; miscible with water at 80C. Combustible.
Use: Preparation of polyester, polyolefins, polyurethanes, and hard waxy resins, organic synthesis.

hydroquinone dimethyl ether. (1,4-dimethoxybenzene; DMB; dimethyl hydroquinone).
CAS: 654-42-2. $C_6H_4(OCH_3)_2$.
Properties: White flakes with sweet clover odor. Bp 213C, mp 56C, d 1.0293 (65C), viscosity 1.04 cP (65C), dielectric constant 2.8, absorbs UV light in range 2800–3000Å. Soluble in benzene and alcohol; insoluble in water. Combustible.
Use: Weathering agent in paints and plastics, fixative in perfumes, dyes, resin intermediate, cosmetics, especially suntan preparations, flavoring.

hydroquinine mono-*n*-butyl ether.
$CH_3(CH_2)_3OC_6H_4OH$.

Exhibit 9

ARTICLES
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Maleimide–thiol adducts stabilized through stretching

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Maleimide–thiol reactions are widely used to produce protein–polymer conjugates for therapeutics. However, maleimide–thiol adducts are unstable in vivo or in the presence of thiol-containing compounds because of the elimination of the thiosuccinimide linkage through a retro-Michael reaction or thiol exchange. Here, using single-molecule force spectroscopy, we show that applying an appropriate stretching force to the thiosuccinimide linkage can considerably stabilize the maleimide–thiol adducts, in effect using conventional mechanochemistry of force-accelerated bond dissociation to unconventionally stabilize an adjacent bond. Single-molecule kinetic analysis and bulk structural characterizations suggest that hydrolysis of the succinimide ring is dominant over the retro-Michael reaction through a force-dependent kinetic control mechanism, and this leads to a product that is resistant to elimination. This unconventional mechanochemical approach enabled us to produce stable polymer–protein conjugates by simply applying a mechanical force to the maleimide–thiol adducts through mild ultrasonication. Our results demonstrate the great potential of mechanical force for stimulating important productive chemical transformations.

Maleimides are broadly applicable for coupling with cysteines or reactive thiol moieties in proteins, peptides and drugs via Michael-type addition reactions[1,2]. Owing to its high selectivity, fast reaction kinetics and mild reaction conditions, this specific covalent conjugation has been commonly employed in the field of bio-labelling[3,4], surface science[5,6], materials science[7–9] and drug delivery[10–14]. In particular, the use of maleimide–thiol adducts in antibody–drug conjugates (ADCs), which combine the high selectivity of therapeutic antibodies with the high potency of drugs, has proven to be an efficient strategy in cancer therapy[15–17]. Notably, several FDA-approved ADCs incorporate a maleimide–thiol-formed thiosuccinimide linkage as the means of conjugation, such as ado-trastuzumab emtansine (Kadcyla) for Her2-positive breast cancer[18] and brentuximab vedotin (Adcentris) for relapsed Hodgkin lymphoma and anaplastic large-cell lymphoma[19–21], along with the approved antibody PEGylated conjugate (Cimzia)[22].

Even so, the thiosuccinimide linkage is well recognized as being unstable under physiological conditions or in the presence of free thiols, as it is eliminated through a retro-Michael reaction or thiol exchange[23–25]. Nearly all maleimide–thiol adducts in ADCs suffer from measurable drug loss, limiting their in vivo stability and efficacy[17,21]. Yet the stability of maleimide–thiol adducts can also be dramatically increased through the hydrolysis of the thiosuccinimide five-membered ring, resulting in a stable hydrolysate that is resistant to elimination of the maleimide–thiol bond[23,25–27]. These two competing reaction pathways lead to products with disparate chemical stabilities (Fig. 1a).

Considerable efforts have been devoted to accelerating the spontaneous ring-opening hydrolysis of maleimide–thiol adducts after conjugation to stabilize the bonds. The addition of special catalysts[28] or treatment with base[23,25,29] can efficiently trigger ring-opening hydrolysis, but the harsh reaction conditions are not biocompatible. Introducing an electron-withdrawing group to the thiosuccinimide ring[26,30] or selectively engineering cysteines in the positively charged environment of proteins[27] can also promote the self-hydrolysis of the thiosuccinimide ring, successfully preventing elimination of the maleimide–thiol bond; these methods are biocompatible but require complex molecular designs and syntheses. Thus, there is currently no general and practical way to stabilize maleimide–thiol conjugates. A versatile and broadly applicable method for tuning the chemistry of maleimide–thiol adducts remains in high demand.

We considered whether we could stabilize the maleimide–thiol adducts by simply applying force to the bonds. Tensile force is usually considered a destructive factor, as it can cause bond rupture[31], but recently this force has been demonstrated to be productive if the compounds are properly designed[32–37]. For example, some force-responsive compounds (or mechanophores) with specific structural elements, such as strained rings[38–43], weak bonds[44–47] or isomerizable bonds[48,49], are force-activable to change colour[46], emit light[44], release small molecules[47] or trigger new reactions[39] if an appropriate force is applied. Moreover, force can bias reaction pathways and generate products that are otherwise inaccessible through thermal- or light-activated processes[39,50]. A recent study further showed that the effect of force on the stability of a bond depends on the direction of the force relative to the reaction coordinate[51]. Inspired by these findings, we reasoned that the ring-open hydrolysis and retro-Michael reaction may have different force dependencies because the force directions with respect to the two reaction coordinates are altered by the five-membered succinimide ring. Although the force-accelerated rupture of maleimide–thiol adducts through the retro-Michael reaction is well recognized, we reasoned that if the ring-opening reaction can be kinetically accelerated over the retro-Michael reaction by force, we should unconventionally be able to obtain stable hydrolysates that are resistant to elimination.

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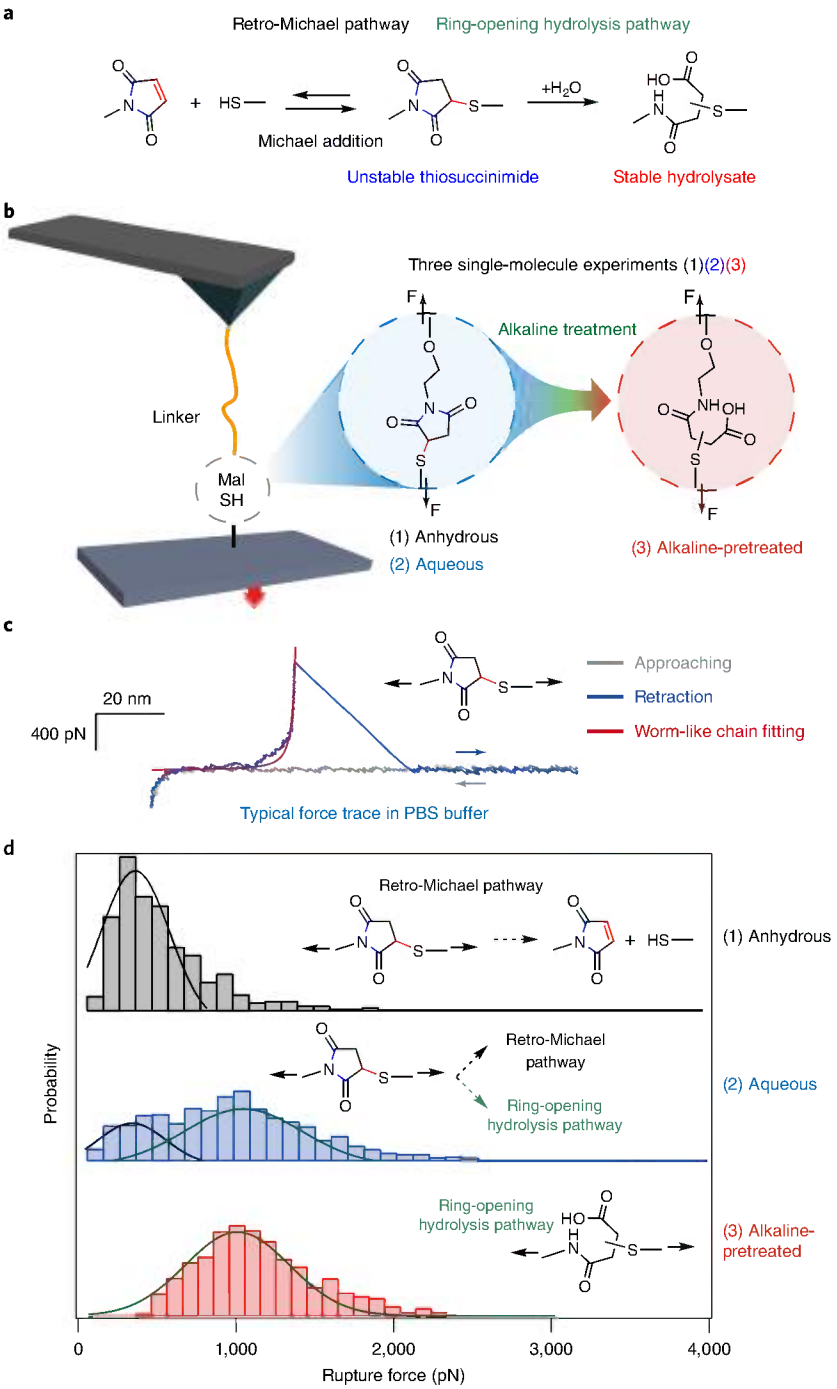


Fig. 1 | Single-molecule force spectroscopy of maleimide–thiol adducts. a, Michael addition of the thiol to the maleimide results in the maleimide–thiol conjugate, the thiosuccinimide. The reaction is fast, but the conjugate is relatively unstable and can undergo further reaction via one of two pathways: (i) it can undergo irreversible ring-opening hydrolysis to yield a stable hydrolysate (succinamic acid thioether), which prevents elimination of the maleimide–thiol conjugate; (ii) it can undergo a retro-Michael conversion back to the starting thiol and maleimide. **b**, Schematic of the single-molecule force spectroscopy experiments conducted under three different conditions (anhydrous, aqueous and alkaline-pretreated). In the first two experiments, the newly formed thiosuccinimide was immediately stretched in anhydrous acetonitrile (1) and in neutral aqueous PBS (2). In the third experiment, the thiosuccinimide was completely ring-opened (hydrolysed) by alkaline treatment before being stretched in PBS (3). **c**, A typical force trace of the newly formed thiosuccinimide in PBS (pH 7.4) obtained in single-molecule force spectroscopy experiments. A worm-like chain model (red curve) was used to fit the single rupture peak of the retraction force-extension curve (blue curve). **d**, Rupture force histograms for newly formed thiosuccinimide in anhydrous acetonitrile (top) and in neutral aqueous PBS (middle) and the rupture force of the hydrolysate (bottom) analysed based on the force peak values. Numbers of independent single-molecule events: $n = 477$, 1,002 and 1,031, respectively. The average rupture forces are 389 ± 207, 900 ± 516 and 1,072 ± 359 pN (means ± s.d.), respectively. Moreover, the force distribution of thiosuccinimide cleavage obtained in aqueous PBS can be deconvoluted into two peaks corresponding to the retro-Michael (black) and ring-opening hydrolysis pathways (green). Unless otherwise stated, the bin size of the histograms is 100 pN.

Using atomic force microscopy (AFM)-based single-molecule force spectroscopy, we show here that force can largely stabilize maleimide–thiol adducts through ring-opening hydrolysis. This observation was verified at the bulk level by applying solvodynamic forces to the polymers containing maleimide–thiol conjugates, through ultrasonication. We further demonstrated the applicability of this method to stabilize maleimide–thiol linked antibody–polyethylene glycol (PEG) conjugates using ultrasonication. This method demanded neither additional chemical synthesis steps nor harsh reaction conditions. Therefore, we anticipate this method will be widely applicable in drug delivery, bio-labelling, surface modification and soft-materials manufacture. The discovery of these counter-intuitive force-stabilized maleimide–thiol adducts may also broaden the scope of using force as a productive means of accelerating chemical transformations.

Results

Distinct mechanical stability of unhydrolysed and hydrolysed maleimide–thiol adducts. AFM-based single-molecule force spectroscopy has been widely used to measure the mechanical strength of both weak biomolecular interactions (for example, receptor–ligand interactions and intercellular adhesion molecules) and strong chemical-bond cleavage[52–55]. We used this technique to directly measure the bond strength of maleimide–thiol adducts under different conditions to reveal the effect of a stretching force on the mechanical stability of the bonds. We show here that, in aqueous solution, the application of force to the unhydrolysed maleimide–thiol adducts can substantially accelerate ring-opening hydrolysis to the point where it is favoured over the retro-Michael pathway.

Three experiments were designed to apply a stretching force to the maleimide–thiol conjugate under various conditions (Fig. 1b): (1) anhydrous, where the maleimide–thiol conjugates are stretched in anhydrous acetonitrile, in which maleimide–thiol adducts cannot be hydrolysed and rupture can only occur via the retro-Michael pathway; (2) aqueous, where the maleimide–thiol adducts are stretched in neutral aqueous PBS, in which some newly formed adducts can be hydrolysed before the final detachment; (3) alkaline-pretreated, where the maleimide–thiol adducts are first completely hydrolysed by treatment with basic PBS and then stretched in neutral aqueous PBS. The detailed experimental conditions and surface modification schemes are provided in the Methods and Supplementary Fig. 1. Typical force traces from the three experiments are shown in Fig. 1c and Supplementary Fig. 2a–c. We have employed practical controls to ensure that these traces indeed correspond to single molecule events (Supplementary Note 1). Worm-like chain (WLC) fitting of the force curves (red curves) provided the contour length L_c (~35 nm) and the persistence length p (~0.38 nm, Supplementary Fig. 2d,e) of the PEG linker used to attach the maleimide and thiol groups to the cantilever tip and the substrate, which were consistent with the values reported in the literature for a single PEG chain[56]. The sharp peak on each retraction trace was assigned as the rupture peak for the maleimide–thiol adduct, which was further confirmed by a few control experiments using a quenching molecules-treated cantilever or different surface modifications (Supplementary Note 2 and Supplementary Figs. 3 and 4). The rupture-force histograms are presented in Fig. 1d. The contact time, pressure or attaching geometry of the maleimide–thiol conjugate between the cantilever tip and the substrate did not affect the rupture forces (Supplementary Figs. 5 and 6). We have also carried out single-molecule experiments using four repeats of protein GB1 as the fingerprint to unambiguously identify single molecule events[57]. The rupture forces measured in this way agreed well with those obtained using PEG linkers (Supplementary Note 3 and Supplementary Fig. 7). The rupture forces of newly formed thiosuccinimides in aqueous solution (900 ± 516 pN; hereafter written as mean ± s.d. unless otherwise indicated) were higher than those observed in anhydrous solution (389 ± 207 pN) at the same pulling speed (4 μm s^{-1}), suggesting that some maleimide–thiol adducts were hydrolysed before being ruptured. The rupture forces (1,072 ± 359 pN) for the completely hydrolysed samples were the highest under these conditions and were independent of the solvent condition (Supplementary Fig. 8). Moreover, the force distribution obtained in an aqueous solution can be deconvoluted into two peaks corresponding to the retro-Michael pathway (black) and the rupture of the ring-opened hydrolysates of the maleimide–thiol adducts (green) (Fig. 1d). Note that, in aqueous solution, the maleimide–thiol adducts were stretched immediately after they were formed, yet a significant portion of the ring-opened hydrolysates were detected, indicating that a stretching force can reduce the reaction's half-life from >100 h (ref. [23]) to a number of seconds.

To further confirm that the hydrolysates gave rise to higher rupture forces, we performed the single-molecule experiments in aqueous solutions at different pH levels (pH 6.4, 7.4 and 8.1). As expected, more high rupture-force events were observed at higher pH levels due to the elevated rate of hydrolysis (Supplementary Fig. 9).

Because of the distinct reversibility of the two reaction pathways, the single-molecule pick-up ratios were dramatically affected by buffer conditions. In a continuous, long-term single-molecule force spectroscopy measurement conducted in anhydrous acetonitrile, cleavage of the thiosuccinimide underwent a reversible retro-Michael reaction, leading to low rupture forces with steady single-molecule pick-up ratios throughout the single-molecule experiment (Supplementary Fig. 10). However, the single-molecule pick-up ratio dropped gradually when the buffer was changed to aqueous PBS, indicating that the maleimide–thiol conjugates were irreversibly hydrolysed and the broken bonds were not able to reform (Supplementary Fig. 10 and Supplementary Note 4). It is worth mentioning that the gradual decrease in the single-molecule pick-up ratio in a timescale of a few hours was due to the force-activated hydrolysis of maleimide–thiol adduct instead of the spontaneous degradation of the maleimide in water, as the spontaneous degradation has a half-life of ~48 h (ref. [30]). The single-molecule pick-up ratios of all experiments are provided in Supplementary Table 1.

Force-dependent rupture kinetics of the unhydrolysed and hydrolysed maleimide–thiol adducts. Dynamic force spectroscopy was used to map the free-energy landscape of the force-induced rupture of the maleimide–thiol conjugates[54]. The rupture-force distributions of the newly formed (unhydrolysed) maleimide–thiol conjugates in acetonitrile and the hydrolysates in PBS at different loading rates are shown in Supplementary Fig. 11. The former data set corresponds to the retro-Michael pathway and the latter corresponds to the rupture of the ring-opened hydrolysates. The average rupture forces (f^*) under both conditions increased logarithmically with increasing loading rates (Supplementary Fig. 12), and these data could be fitted by an equation derived from the Bell–Evans model[58,59]

$$f^* = f_\beta \ln \frac{r_F}{k f_\beta} = f_\beta \ln r_F - f_\beta \ln k f_\beta$$

where $f_\beta = k_B T/\Delta x^\ddagger$ (k_B is the Boltzmann constant, T is the temperature and $\Delta x^\ddagger$ is the extension of the bond along the force axis in the transition state), k is the rate constant at zero force, and r_F is the loading rate. The fitting parameters are summarized in Supplementary Table 2. The value of $\Delta x^\ddagger$ for the retro-Michael cleavage is 0.559 Å, which is close to that of the rupture of the ring-opened hydrolysate (0.505 Å). The values of $\Delta x^\ddagger$ for both pathways are shorter than those of other mechanically activated reactions[50,60,61], and they are close to the transition-state distance for the rupture of covalent bonds[62]. The

rate constant of the hydrolysate obtained by the Bell–Evans model is $3.5 \times 10^{-4}\,s^{-1}$, which is far lower than the retro-Michael reaction rate constant ($2.1\,s^{-1}$), indicating that the mechanical stability is enhanced by hydrolysis of the thiosuccinimide.

The parameters from dynamic force spectroscopy were further confirmed by the model-free analysis method introduced by Craig and Oesterhelt[63,64] (Supplementary Fig. 13 and Supplementary Table 2). Moreover, the obtained kinetic parameters can be used to adequately reproduce the rupture force distributions at different force loading rates by Monte Carlo simulation (Supplementary Fig. 14).

The rupture-force distributions for the newly formed maleimide–thiol adducts at different loading rates can also be roughly fitted by two Gaussian distributions, corresponding to the retro-Michael reaction and the rupture of the hydrolysates (Supplementary Fig. 15). This further indicated that a substantial amount of thiosuccinimide was hydrolysed in PBS by force on an experimental timescale of <1 s.

Force-dependent ring-opening hydrolysis of maleimide–thiol conjugates by pre-stretching. To elucidate how force accelerates the ring-opening hydrolysis of thiosuccinimide, we designed a pre-stretching experiment in aqueous solution (Fig. 2a). We found that applying a pre-stretching force did accelerate the ring-opening hydrolysis, leading to more stable hydrolysed maleimide–thiol conjugates. The final rupture-force distributions of the maleimide–thiol conjugates after different pre-stretching forces (from ~60 pN to ~600 pN for 100 ms) are summarized in Fig. 2b. The histograms can be deconvoluted by two Gaussian distributions corresponding to events from the retro-Michael reaction and the cleavage of hydrolysate, respectively. With the increase of pre-stretching force, the relative ratio between the two peaks decreased gradually, leading to an increase in the overall rupture forces. This indicates that the application of a pre-stretching force could accelerate the ring-opening hydrolysis. When a pre-stretching force of more than 300 pN was applied for 100 ms, the average rupture forces reached a plateau at ~1,200 pN, which was close to the rupture force of the hydrolysate (Fig. 2c). This indicated complete conversion of the thiosuccinimide to the hydrolysate by force. However, if the solvent was changed to anhydrous acetonitrile, the final average rupture force was close to the retro-Michael pathway and became independent of the pre-stretching force (Supplementary Fig. 16). Moreover, the final rupture force also increased with increasing pre-stretching time at different pre-stretching forces in aqueous solution (Fig. 2d).

Assuming that the hydrolysis of thiosuccinimide is a first-order reaction, the lifetimes of the substrate at different pre-stretching forces can be obtained by exponentially fitting the time-dependent pre-stretching data or bimodal fitting the pre-stretching distributions, as summarized in Fig. 2e. The lifetimes of the retro-Michael dissociation of the thiosuccinimide and the rupture of the hydrolysates at different forces obtained from dynamic force spectroscopy experiments are also plotted in the same figure for comparison. All lines were generated based on the Bell–Evans model. At forces lower than 270 pN, the retro-Michael reaction is the major pathway (Fig. 2e, inset), and force destabilizes the maleimide–thiol adducts. However, if the stretching forces are higher than 270 pN, the hydrolysis pathway becomes dominant. Once the thiosuccinimide is hydrolysed, the lifetime of the adduct is greatly increased (Fig. 2e), leading to force-stabilized maleimide–thiol conjugates.

Force-induced ring-opening hydrolysis of maleimide–thiol adducts by ultrasound. To further confirm that force can induce hydrolysis of maleimide–thiol adducts and lead to stable conjugates at the bulk level, we directly characterized the force-induced hydrolysates at the bulk level using ultrasound-based mechanochemistry. The solvodynamic shear force along the polymer chain in solution was generated by solvent cavitation, or the rapid nucleation, growth and collapse of microbubbles under ultrasound, which has been used to explore a number of mechanically responsive polymers[65].

We coupled a maleimide-terminated PEG (M_w of 5 kDa) and a thiol-terminated PEG (M_w of 5 kDa) to yield **P1**, which contained a maleimide–thiol adduct in the middle (see Supplementary Methods for details) (Fig. 3a). The small-molecule maleimide–thiol adduct, **S1**, was also synthesized as a control by coupling *N*-ethylmaleimide and 2-mercaptoethanol. **P1** and **S1** were treated with basic PBS buffer (pH 8.1) at 37 °C for 5 days until the ring-opening process was complete to obtain completed hydrolysed products **AP1** and **AS1**, respectively, for comparison. Ultrasound-treated **P1**, **UP1**, was obtained by applying pulsed ultrasound for just 30 min (Fig. 3a).

These small-molecule compounds and polymers were then characterized by Fourier-transform infrared (FT-IR) and ^{1}H NMR spectroscopy. FT-IR spectroscopy showed the appearance of new transmittance peaks at 1,695, 1,650 and 1,550 cm^{-1} (two asymmetric carbonyls after ring-opening and the NH of the CONH moiety, respectively) after alkaline treatment (**AS1**), whereas the spectrum of the as-synthesized model compounds (**S1**) showed only one peak at 1,685 cm^{-1} in this region (two symmetric carbonyls on the maleimide five-membered ring) (Supplementary Fig. 17). The spectra of the polymers containing a central thiosuccinimide after alkaline treatment (**AP1**) or ultrasonication (**UP1**) also showed a peak at 1,650 cm^{-1}, whereas the peak at 1,550 cm^{-1} was covered by the peak from the PEG (Fig. 3b). ^{1}H NMR spectroscopy could not be used to identify the hydrolysis product, as the signal from the PEG chain dominated the 3.0–4.0 ppm region, masking the signals from the hydrolysed thiosuccinimide (Supplementary Fig. 18).

To separate the signals from the thiosuccinimide and PEG in the ^{1}H NMR spectra, another model compound was synthesized, **P2**, containing two symmetric thiosuccinimides with adjacent benzene rings, along with its small-molecule analogue (**S2**) (see Supplementary Methods for details). Formation of the Michael-type adduct (**S2** and **P2**) and its ring-opened hydrolysate (**AS2** and **AP2**) can easily be identified in the ^{1}H NMR spectra, providing a facile means of following the addition and hydrolysis reactions (Fig. 3c). Following the addition of 2-mercaptoethanol and thiol-PEG, the aromatic protons shifted downfield and upfield, respectively (two peaks centred at 7.370 and 7.190 ppm), while the 7.165 ppm peak vanished, indicating production of the thiosuccinimide. After alkaline treatment, the ring-opened product (**AS2** and **AP2**) was evidenced by the shifts in aromatic protons from 7.370 and 7.190 ppm to 7.475 and 7.146 ppm, respectively (Fig. 3d and Supplementary Fig. 19). Trace amounts of ring-opened hydrolysates were observed before any treatment due to the hydrolysis of **P2** by moisture in the air. The two thiosuccinimide rings can be hydrolysed either symmetrically or asymmetrically, leading to the splitting of each resonance peak to four separate peaks. Following ultrasonication, similar chemical shifts were observed for ultrasound-treated **P2**, **UP2** (Fig. 3d). Approximately 75% of the thiosuccinimide in the polymers was mechanically hydrolysed by ultrasonication (based on the integrals of the b and d peak areas, Supplementary Fig. 20), while a portion of the thiosuccinimide underwent retro-Michael cleavage as indicated by the appearance of small peaks at ~7.310 ppm. This finding was further confirmed by UV–vis (Supplementary Fig. 21) and FT-IR spectra, which showed the appearance of new transmittance peaks between 1,500 and 1,700 cm^{-1} (Supplementary Fig. 22). Furthermore, the hydrolysis reaction was confirmed by the pH change of the solution after ultrasonication (Supplementary Fig. 23). The hydrolysis generated an additional hydroxyl group, which is expected to decrease the pH of the solution.

NMR spectra also show that the force-induced ring-opening might give two regio-isomers (four peaks splitting of the aromatic protons), while alkaline treatment favours breaking the distal, instead of proximal, N–C bonds (two peaks splitting of the aromatic protons) (Fig. 3c and Supplementary Fig. 19). In the alkaline

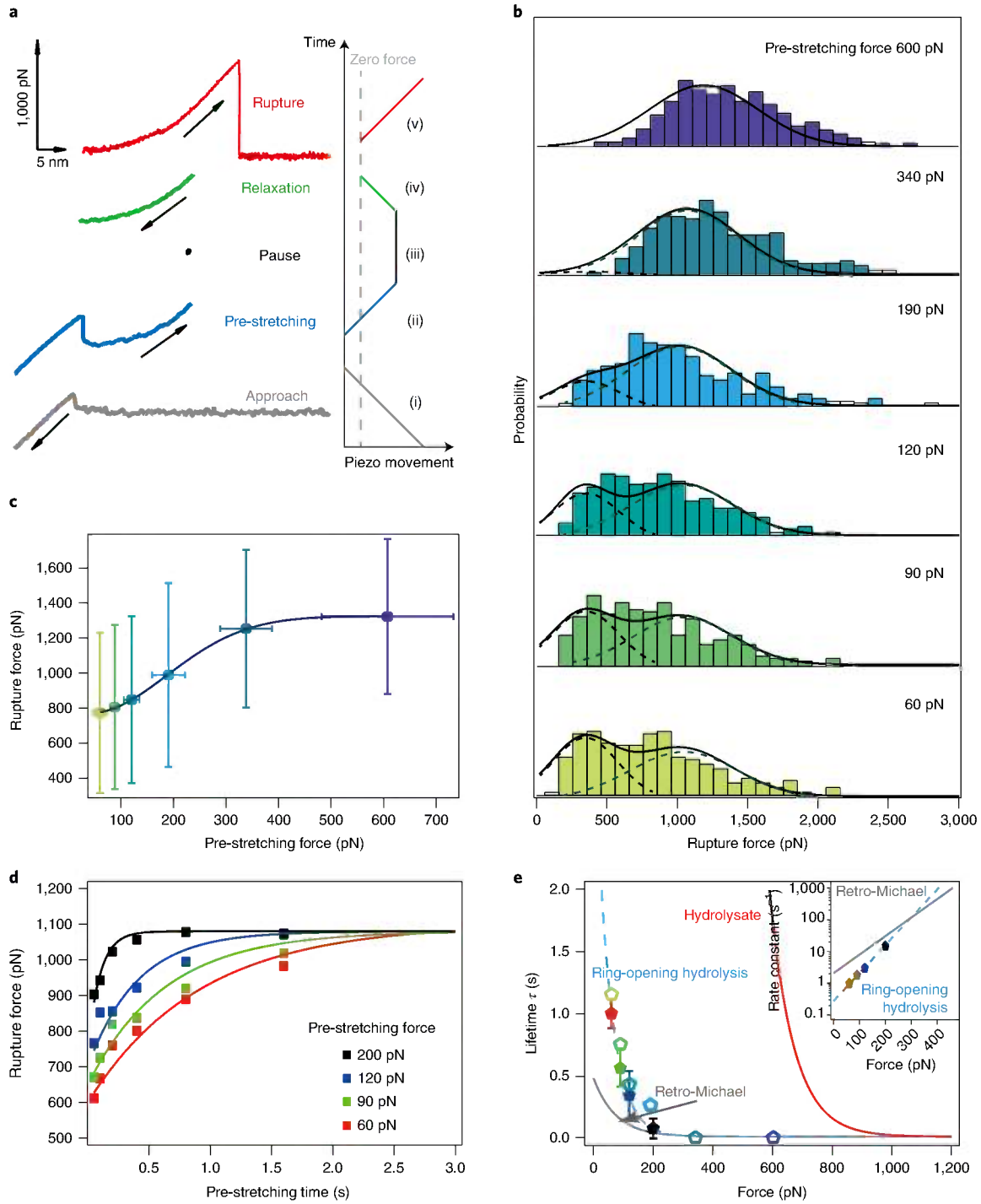


Fig. 2 | Pre-stretching experiment of maleimide–thiol conjugates. a, Illustration of the pre-stretching-force traces and corresponding piezo movements, where the first trace is at the bottom and the last trace at the top. The maleimide-functionalized cantilever was brought into contact with the thiol-modified glass substrate (i). The cantilever was then retracted to a pre-stretching force (ii) and held for 100 ms to allow hydrolysis of the thiosuccinimide under the pre-stretching force (iii). The cantilever was relaxed to zero force (iv) and retracted again to break the maleimide–thiol adduct and determine the rupture force after pre-stretching (v). **b**, Rupture-force histograms for maleimide–thiol adducts after different pre-stretching forces ($n = 299$, 300, 600, 300, 300 and 346, respectively, from ~60 pN to ~600 pN). The histograms were fitted by bimodal distributions (black solid line) of two Gaussian functions corresponding to the retro-Michael reaction (black dashed line) and the cleavage of hydrolysate (green dashed line), respectively. **c**, Dependence of rupture force on the pre-stretching force. Error bars, mean ± s.d. **d**, Time-dependent pre-stretching experiments at different pre-stretching forces. The data were fitted by a two-state sigmoid model to calculate the lifetime, τ, and the rate constant of the ring-opening hydrolysis after different pre-stretching forces. **e**, Force-dependent lifetimes of the maleimide–thiol adducts for the retro-Michael reaction (grey line), ring-opening hydrolysis from time-dependent pre-stretching (filled coloured markers) and bimodal fitting of the pre-stretching distribution (open coloured markers), and cleavage of the hydrolysate (red line). Inset, variation of the rate constants of the retro-Michael reaction and ring-opening hydrolysis with force. The solid lines of the retro-Michael reaction and cleavage of the hydrolysate were simulated from dynamic force spectroscopy results using the Bell–Evans model. The dashed lines are from exponential fitting of the lifetime of ring-opening hydrolysis. Error bars, mean ± s.d.

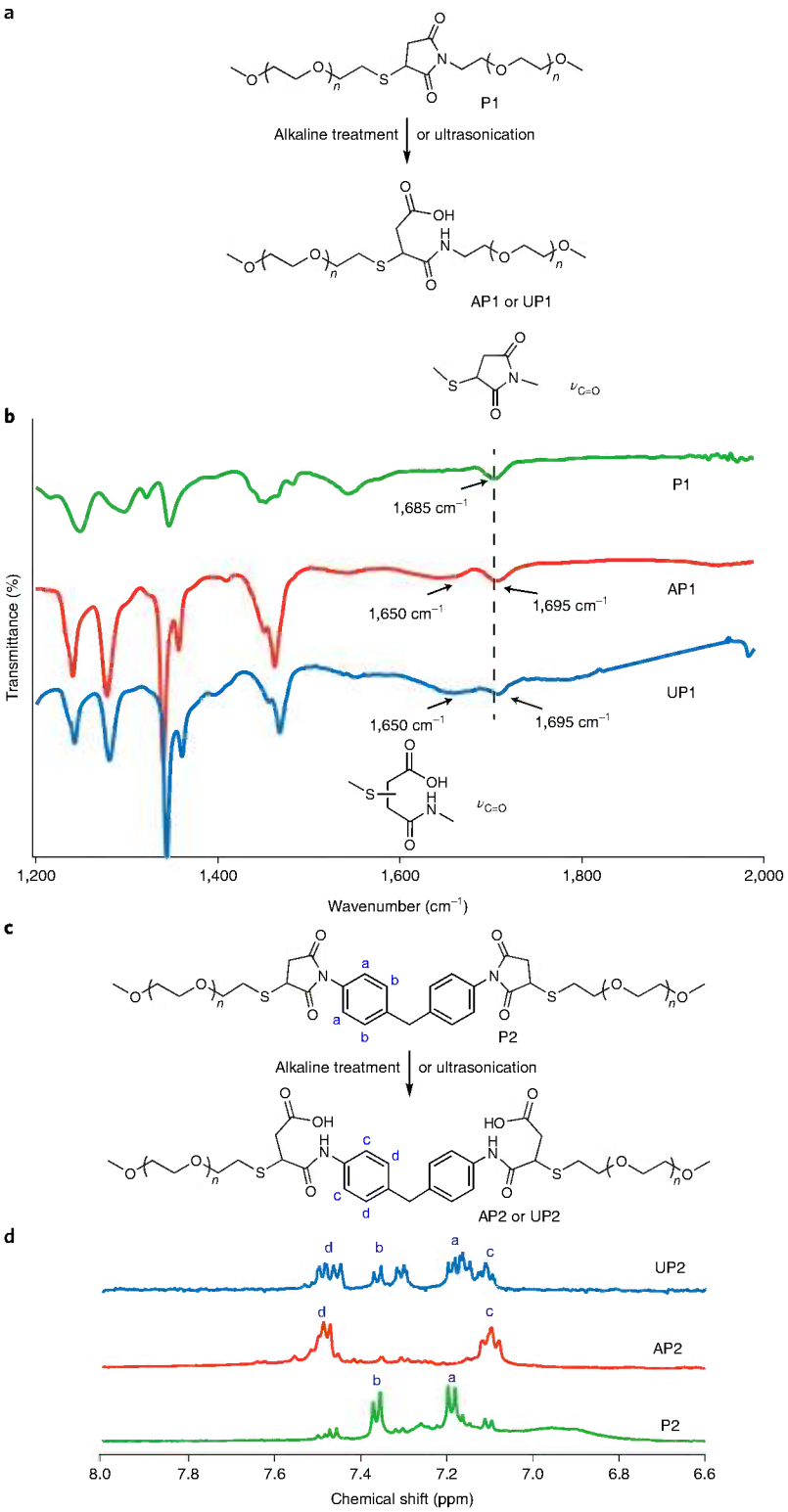


Fig. 3 | Chemical characterization of the force-induced hydrolysis of the maleimide–thiol conjugates. a, Model polymer, **P1**, with a maleimide–thiol adduct at the centre, was synthesized by coupling a maleimide-terminated PEG (M_w, 5 kDa) with a thiol-terminated PEG (M_w, 5 kDa). The ring-opening hydrolysis of **P1** was achieved either through alkaline treatment in basic PBS buffer (pH 8.1) at 37 °C for 5 days or ultrasonication under pulsed ultrasound (11.81 W cm^{-2} for 30 min; switched on and off every second) to yield **AP1** or **UP1**. **b**, FT-IR transmittance spectra of **P1** containing the core thiosuccinimide before (green) and after (**AP1**, red) alkaline treatment or after ultrasonication (**UP1**, blue). The peak at 1,650 cm^{-1} corresponds to the ring-opened thiosuccinimide. **c**, **P2**, containing two symmetric thiosuccinimide moieties with adjacent benzene rings, was synthesized by treating a thiol-terminated PEG (M_w, 5 kDa) with 4,4′-bis-maleimidodiphenylmethane. The ring-opening hydrolysis of **P2** was achieved either through alkaline treatment (**AP2**) or ultrasonication (**UP2**). **d**, ^{1}H NMR spectra in dimethyl sulfoxide-d_6 (DMSO-d_6) of **P2** (green), **P2** after alkaline treatment (**AP2**, red) and **P2** after ultrasonication for 30 min (**UP2**, blue). The chemical shifts of the aromatic protons, labelled a, b, c and d, are consistent with the chemical shifts of the model compounds (Supplementary Fig. 19), confirming that force can accelerate the hydrolysis of thiosuccinimide.

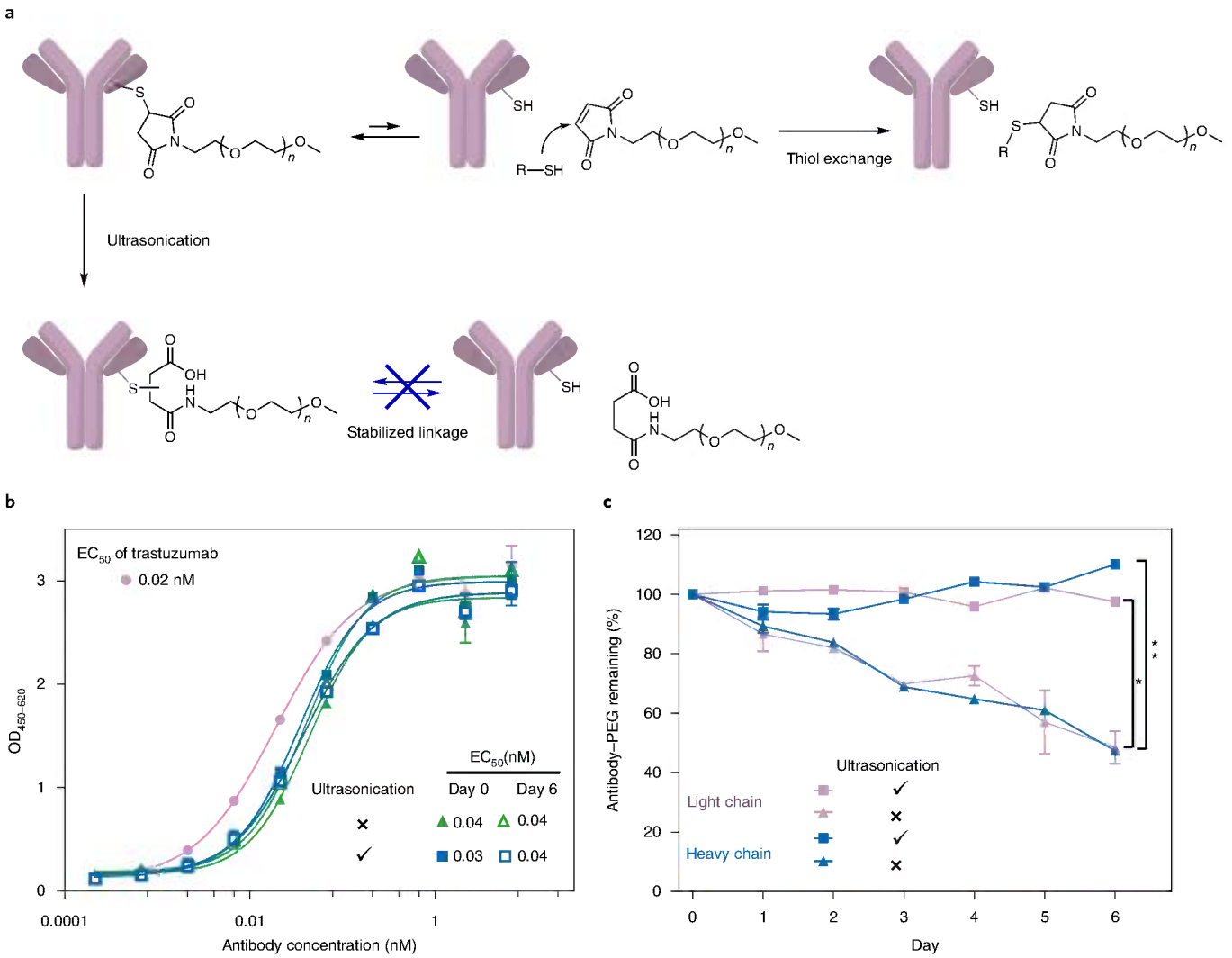


Fig. 4 | Ultrasonication increases the stability of maleimide–thiol-based antibody–PEG conjugates. a, Proposed reaction scheme for the improved chemical stability of ultrasound-treated antibody–PEG (Ab-PEG) conjugates. **b**, Estimation of the binding activity of ultrasound-treated (blue) and untreated (green) Ab-PEG to its antigen Her2 protein in comparison with the unmodified trastuzumab antibody (pink) by ELISA assay. Open and filled markers correspond to the data for freshly prepared Ab-PEG and Ab-PEG incubated in PBS buffer at 37 °C for 6 days, respectively. The representative data shown are mean ± s.d. of $n = 2$ biological replicate samples and EC_{50} values were calculated from four-parameter curve fitting to the data. The ELISA experiments were repeated independently four times with similar results. OD, optical density. **c**, Quantitation of the remaining Ab-PEG conjugates (light chain, pink; heavy chain, blue) by western blot analysis. The ratios of Ab-PEG remaining were calculated based on the western blot analysis shown in Supplementary Fig. 24 and normalized to the signal at day 0. Each data point represents the mean of $n = 3$ biologically independent experiments. Error bars, mean ± s.d. $^{**}P = 0.0012$; $^{*}P = 0.0124$ (95% confidence intervals). *P* values were determined by unpaired two-sided Student's *t*-test.

treatment, the distal N–C bond has less steric hindrance than the proximal one for attack by OH^-. Therefore, the major product is the distal N–C bond hydrolysed product. However, force may greatly change the reactivity of the two N–C bonds, leading to a hydrolysis rate of the proximal bond comparable to the distal one in ultrasound experiments.

Force-induced hydrolysis improves the stability of maleimide–thiol-based antibody–PEG conjugates. Under physiological conditions, the relatively high concentration of exogenous thiol nucleophiles (R-SH) (for example, cysteine, glutathione and serum albumin) slowly reduces the loading efficiency, which becomes the key therapeutic limitation of maleimide–thiol-based antibody conjugates[13,23,25–27,29,30]. Encouraged by the finding that solvodynamic shear forces counter-intuitively strengthen and stabilize maleimide–thiol conjugates in polymers, we endeavoured to engineer stable maleimide–thiol-based antibody–PEG (Ab-PEG) conjugates via ultrasonication (Fig. 4a).

Compared with the precise stretching forces provided by AFM at the single-molecule level, the solvodynamic shear forces generated by ultrasound are not homogeneous[65] and may lead to bond scission through the retro-Michael pathway, as seen for **P2** (Fig. 3d). Fortunately, because the Michael addition and retro-Michael reaction are reversible, the dissociated maleimide–thiol adducts can re-form in the absence of force (Fig. 1a). Thus, we used excess maleimide–PEG and multiple ultrasonication cycles to generate Ab–PEG conjugates with a majority of the maleimide–thiol linkers hydrolysed.

We used trastuzumab, a major antibody targeting Her2-positive breast cancer, as the model antibody. Trastuzumab was reduced to expose free cysteine residues. Then, the reduced antibody was treated with maleimide–PEG to yield the Ab–PEG conjugates.

A dilute solution of the purified Ab–PEG conjugates (~2 mg ml^{-1}) was subjected to pulsed ultrasound (switched on and off every second) in the presence of a 10-fold excess of maleimide–PEG (M_w, 5 kDa) for 30 min in an ice-water bath to generate the ultrasound-treated sample. The sample was then purified and condensed to the same concentration as that of the untreated sample. The stabilities of the ultrasound-treated and untreated Ab–PEG samples were evaluated in vitro in a PBS solution (pH 7.4) containing 1 mM of reduced glutathione at 37 °C. The binding curve of ultrasound-treated Ab–PEG was similar to untreated conjugates as well as the unmodified trastuzumab. The half maximal effective concentrations (EC_{50}) of ultrasound-treated and untreated Ab–PEG were in a range of 0.03–0.04 nM, on the same scale as that of the unmodified trastuzumab (0.02 nM) (Fig. 4b). Enzyme-linked immunosorbent assay (ELISA) results indicate that ultrasonication did not cause the unfolding and loss of function of the antibody conjugates. The loss of PEG chains from the antibody can be evaluated by the decrease in molecular weight based on western blot analysis (Supplementary Fig. 24). The Ab–PEG samples not subjected to ultrasonication lost ~50% of their payload in one week, whereas the Ab–PEG samples treated by ultrasound retained over 90% of their payload over the same period for both light and heavy chains (Fig. 4c). The stability of ultrasound-treated antibody conjugates was comparable to that of the alkaline-treated samples[29] (Supplementary Fig. 25). These results clearly show that force-induced ring-opening hydrolysis by ultrasonication can be used as an efficient means of improving the stability of maleimide–thiol-based antibody conjugates.

Discussion

In this work, we report the force-induced hydrolysis of maleimide–thiol adducts, leading to products with improved mechanical and chemical stabilities under physiological conditions. Due to the presence of a competing retro-Michael reaction, this reaction pathway is not accessible in the absence of force and only becomes dominant when the applied force reaches a certain threshold.

Although there are many previous theoretical and experimental examples in which force is used to alter reaction pathways, the mechanism of the force-induced hydrolysis of maleimide–thiol adducts presented here is fundamentally different. In this reaction, force does not change the reaction mechanism of either the retro-Michael reaction or the hydrolysis of the thiosuccinimide, but it differentially alters their reaction kinetics. The selectivity of the final product is kinetically controlled instead of thermodynamically controlled due to the force-dependent kinetics of the two competing reaction pathways. In other words, our results show that the application of force can selectively favour the pathway that shows a stronger force-dependency. If the effects observed in this work can be shown to be general, then such a mechanism of force-dependent kinetic control expands our current understanding of using force to control mechanochemistry and may inspire the implementation of different mechanophores in the same polymer to achieve a complex mechanical response. It is expected that the activation of multiple mechanophores can be finely controlled by the amplitude and direction of force.

The discovery of the stabilization of maleimide–thiol adducts by applying a stretching force provides a potentially convenient solution to the instability of maleimide–thiol conjugates by simple ultrasonication. Notably, the apparent solvodynamic shear forces introduced by ultrasonication depend largely on the size of the polymers and proteins, as well as the position of the maleimide–thiol linkage[39,65]. On ultrasonication, although the forces exerted on the thiosuccinimide ring can be as high as a few hundred piconewtons[35], the forces on the antibody protein are much lower because they are not at the centre of the adducts, and they are in a compact folded conformation. We did not observe any measurable loss in the functionality of the protein–polymer conjugates following the ultrasonication procedure used in our experiments. Moreover, the undesired retro-Michael reaction products can be avoided by using excess maleimide-containing polymers. Despite these advantages, practically controlling the force applied to the maleimide–thiol adducts for different systems remains challenging. Rigorous optimization of this method is currently underway for practical translation.

Conclusion

We discovered that the application of force to maleimide–thiol adducts can counter-intuitively increase their mechanical and chemical stability. This was achieved through the force-accelerated ring-opening hydrolysis of the thiosuccinimide ring, which generates a product that is resistant to retro-Michael reactions and thiol exchange. Using single-molecule force spectroscopy, we have quantitatively illustrated how and to what extent force can favour one of the two competing pathways and afford products that are not observed in thermally activated pathways. Our proof-of-principle experiments also demonstrated that this force-controlled reaction may be used to produce stable antibody–polymer conjugates using ultrasound-induced solvodynamic force. We envision that other types of force are also applicable to stabilize maleimide–thiol conjugates, including hydrodynamic force from flow, compressive or stretching forces from materials deformation, and the friction forces at diverse interfaces. The concept of using force-dependent kinetic control to obtain products inaccessible by conventional chemical reaction pathways may also greatly extend the current scope of mechanochemistry.

Methods

Single-molecule AFM measurements. Single-molecule force spectroscopy experiments were carried out on a commercial AFM instrument (JPK Force Robot 300) at room temperature (~22 °C). Standard silicon nitride (Si_3N_4) cantilevers were purchased from Bruker (type: MLCT). D cantilevers (spring constant of ~0.05 N m^{-1}) were used in all experiments, and the spring constant was calibrated using an equipartition theorem for each experiment. Three experiments were undertaken in different solvents to measure the rupture force of the thiosuccinimide and its hydrolysate. In the first two experiments, a maleimide-functionalized cantilever was brought into contact with a thiol-functionalized glass substrate at a set-point force of 1.0 nN and left there for 1.0 s to trigger the formation of a maleimide–thiol conjugate between the cantilever tip and the substrate. In the third experiment, the maleimide–thiol conjugate-functionalized substrate was incubated in basic PBS buffer (pH 8.1, 5 days, 37 °C) until the five-membered ring was completely hydrolysed. An NHS (*N*-hydroxysuccinimide)–PEG functionalized cantilever was used to pick up the amino group at the very end of the hydrolysate-containing linker using a constant contact force of 1.0 nN for 1.0 s. The cantilever was then retracted from the substrate at a constant speed of 4.0 μm s^{-1} to obtain the force–extension curves. All force curves were collected by commercial software (JPK) and analysed using a custom-written protocol in Igor 6.35 (Wavemetrics). Details on the AFM measurements and analysis procedures are provided in the Supplementary Information.

Pre-stretching single-molecule force spectroscopy experiments. The pre-stretching single-molecule force spectroscopy experiments were carried out using a maleimide-functionalized cantilever and a thiol-coated substrate in anhydrous acetonitrile and PBS (pH 7.4) solution. The maleimide-functionalized cantilever was first brought into contact with the thiol-coated glass surface at a constant force of 1.0 nN for 1.0 s. The cantilever was then retracted to a preset force (pre-stretching force) and held there for a certain duration (pre-stretching time). Next, the cantilever was relaxed to zero force (close but not touching the substrate). Finally, the cantilever was fully retracted to rupture the maleimide–thiol conjugate. The pre-stretching force was set between 60 and 600 pN, and the pre-stretching time was varied from 50 ms to 2.0 s. The relaxation and stretching speeds were 4.0 μm s^{-1}. Events that did not survive the pre-stretching procedure were excluded from data analysis. Because the molecule was always relaxed to zero force before the final stretch, the pre-stretching protocol does not artificially shift the force population to a higher force if no chemical reaction occurs during the pre-stretching. (Supplementary Fig. 26)

Synthesis and characterization of maleimide–thiol-containing model compounds and polymers. Model compound small-molecule adducts **S1** and **S2**, polymers **P1** and **P2** and their alkaline-treated hydrolysates (**AS1**, **AS2**, **AP1**, **AP2**) were synthesized following previous reports[1,23,29] (for details see Supplementary

Information). Compounds and polymers were dissolved at high concentrations (~20 mg ml^{-1} for small-molecule compounds and ~100 mg ml^{-1} for polymers) before chemical characterization. ^{1}H NMR spectra were recorded on a Bruker 500 MHz NMR spectrometer in dimethyl sulfoxide-d_6 (DMSO-d_6). ^{1}H NMR spectra of the polymers were merged from more than 128 scans to improve the signal-to-noise ratio. FT-IR spectra were recorded on a Nicolet iS50 FT-IR (Thermo Scientific) with ATR (attenuated total reflection). Samples for FT-IR were dissolved in $CDCl_3$, and ~20 µl of solution was added to the detector after baseline correction. The spectra were recorded from 400 to 4,000 cm^{-1} with a bandwidth of 0.4 cm^{-1}.

Pulsed ultrasonication. Ultrasonication was performed using an ultrasonic apparatus XQ-1000D operating at 20 kHz with a 6 mm tip probe. The homemade Suslick-like cells were oven-dried before use. The ultrasonication experiments were carried out in ~10 ml PBS (pH 7.4) containing 2.0 mg ml^{-1} of **P1** or **P2** in an ice/water bath to maintain a temperature of ~6–9 °C during ultrasonication. Nitrogen was introduced into the cell using a syringe needle, and the solution was degassed with bubbling N_2 for 30 min prior to and during each experiment. The ultrasound was pulsed (1.0 s on and 1.0 s off, 11.81 W cm^{-2}) in each experiment to prevent the temperature from increasing. After 30 min of ultrasonication (total ultrasound time of 15 min), the resulting solution was withdrawn from the Suslick-like cell and filtered through a 0.22 µm syringe filter. The crude product was purified using a desalination column. Purified fractions were collected and freeze-dried before characterization.

Stability of the Ab–PEG conjugate. The monoclonal antibody anti-Her2 trastuzumab was diluted to 200 µM in PBS (pH 7.4, containing 2 mM EDTA). The resulting antibody was treated with ~4–5 equiv. of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 10 mM in distilled water) and incubated at 37 °C for 2 h. The reduced antibodies with a mixture of heavy chains (containing three free cysteine groups) and light chains (containing one free cysteine group) were treated with 20 equiv. of maleimide–PEG (M_w, 5 kDa). The reaction was stirred at 4 °C for 12 h to generate the Ab–PEG conjugates. Due to the steric hindrance, we obtained the Ab–PEG with different conjugation ratios. For the heavy chain, the conjugation ratios were in the range of 0–3. For the light chain, the conjugation ratios were in the range of 0–1.

The conjugates were diluted to ~2.0 mg ml^{-1} and combined with a 10-fold excess of maleimide–PEG (M_w, 5 kDa). The solution used for pulsed ultrasonication was kept in an ice/water bath and degassed with bubbling N_2 for 30 min before as well as during the experiment. The ultrasound treatment lasted for 30 min (11.81 W cm^{-2}; switched on and off every second). Both the ultrasound-treated and untreated conjugates were purified using Millipore ultrafiltration centrifugal tubes and then concentrated to ~25 µM. The constructions of the Ab–PEG conjugates were reproducible in phosphate-free buffer (Tris buffer saline, pH 7.4) (Supplementary Fig. 27).

The stability of the ultrasound-treated and untreated Ab–PEG samples was evaluated in vitro at a concentration of 20 µM in a PBS solution (pH 7.4) containing 1 mM reduced glutathione. GB1 protein (M_w, ~8 kDa) as an internal reference was added into the solutions to monitor the protein degradation. The solutions were filtered using a 0.22 µm filter and incubated at 37 °C. At each time point, a 100 µl aliquot was removed and frozen at −80 °C. After the final time point, the binding activity of samples from day 0 and day 6 were evaluated by ELISA assay. All samples were fully reduced before the western blot analysis. The loss of PEG chains from the antibody could be evaluated by the decrease in molecular weights based on western blot analysis using Gelpro. The average drug:antibody ratios (DAR) of the light chains and heavy chains were obtained and the Ab–PEG remaining (%) each day was calculated and normalized to the average DAR at day 0. Details on the ELISA and western blot analysis are provided in the Supplementary Information.

Statistics and reproducibility. The numbers of biological replicates are indicated in the figure legends and Methods. Data are presented as mean ± s.d., as indicated in the legends of the figures and Supplementary figures. Student's *t*-test was performed with GraphPad Prism 5.0. The reproducibility of repeated independent experiments is indicated in the legends of the figures and Supplementary figures.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability

Igor procedures for single-molecule experiment analyses of this study are available from the corresponding author upon request.

Data availability

All data generated and analysed during this study are included in this article and its Supplementary Information, and are also available from the authors upon reasonable request.

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Author contributions

Yi.C., W.H. and X.G. conceived the project and designed the experiments. W.H., X.G. and H.L. performed the single-molecule experiments and analysed the data. W.H., Y.Y., Z.Z., Y.L.C. and Y.S. performed the ultrasound experiments and analysed the data. W.H., X.W. and Y.Y. performed the antibody stability experiments. Yi.C., W.W. and M.Q. supervised the project. W.H. and Yi.C. wrote the paper with contributions from all authors.

Competing interests

The authors declare no competing interests.

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Data exclusions	N/A
Replication	N/A
Randomization	N/A
Blinding	N/A

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☒	☐ Unique biological materials
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

1. Anti-Her2 positive trastuzumab was purchased from efe-bio (shanghai), CAS 180288-69-1, Lot E122375
2. Goat anti-Human IgG antibody-HRP was purchased from Beyotime, China, Cat: A0201, diluted as 1:1000 ratio for ELISA
3. Mouse anti-Human IgG1 Fc secondary antibody-Horse Radish Peroxidase (HRP) (Cat: A-10648, ThermoFisher, USA), anti-Human (κ-chain specific), goat F(ab')2 fragment-HRP antibody (Cat: SAB3701414, Sigma-Aldich, Germany) and his-tag (27E8) mouse monoclonal antibody-HRP conjugate (Cat: 9991, Cell Signaling Technology, USA) were diluted by 1:1000 ratio for western blot.

Validation

1. Trastuzumab is a humanized monoclonal antibody for patients with invasive breast cancers that overexpress HER2. Trastuzumab has been clinically used to treat Her2 positive metastatic breast cancer and HER2 positive gastric cancer.
2. Goat anti-Human IgG antibody-HRP is the secondary antibody targeting human IgG (trastuzumab in this paper), and can be detected by catalyzing TMB (3,3',5,5'-Tetramethylbenzidine Liquid Substrate, Cat: T4444, Sigma-Aldrich, Germany) to blue product in ELISA.
3. Mouse anti-Human IgG1 Fc secondary antibody-HRP is the secondary antibody targeting human IgG1 Fc domain (heavy chain of trastuzumab in this paper), anti-Human (κ-chain specific), goat F(ab')2 fragment-HRP antibody is the secondary antibody targeting human κ-chain (light chain of trastuzumab in this paper), and his-tag (27E8) mouse monoclonal antibody-HRP conjugate detects recombinant proteins containing the 6xHis epitope tag (internal reference 6xhis-GB1 protein in this paper). Three of them can be detected using ECL™ Prime Western Blotting System.

Exhibit 10

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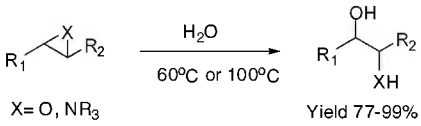
Hot Water-Promoted Ring-Opening of Epoxides and Aziridines by Water and Other Nucleopliles

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Received November 10, 2007



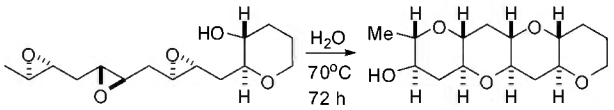
Effective hydrolysis of epoxides and aziridines was conducted by heating them in water at 60 or 100 °C. Other types of nucleophile such as amines, sodium azide, and thiophenol could also efficiently open epoxides and aziridines in hot water. It was proposed that hot water acted as a modest acid catalyst, reactant, and solvent in the hydrolysis reactions.

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Introduction

Although water is cheap, safe, and clean compared with organic solvents, water is not a regular choice for the medium of organic reactions because of the insolubility of reactants and incompatibility of reaction intermediates. In certain situations, the special physical and chemical properties of water, such as high dielectric constant, high heat capacity, hydrophobic interactions between reactants, hydrogen bond formation between water and reactant, and acid or base characters may be utilized to promote organic reactions. In the early 1980s, Breslow's group reported that Diels−Alder reaction was remarkably accelerated in water owing to the hydrophobic effect.[1] This important finding dramatically changed chemists' attitudes toward the use of water as solvent for organic reactions. After that, a number of organic reactions were found to proceed very well in water.[2] A very recent work from Jamison's research group revealed that the cascade epoxide-opening yielding ladder polyether could be promoted by water. This finding presented a highly efficient synthesis of natural trans-fused polycyclic ethers as well as provided new evidence for the biosynthesis of this kind of natural products (Scheme 1).[3] In this paper, we report that the ring-opening reactions of epoxides and aziridines can be promoted by hot water. A general acid-catalyzed mechanism was proposed based on the observed selectivity of the reactions in hot water.

SCHEME 1. Epoxide-Opening Cascades Promoted by Water



Results and Discussion

Hydrolysis of epoxides is a pivotal protocol for making synthetically useful vicinal diols. Compared with the use of protic acid,[4] the hydrolysis of epoxides now can be conducted under milder condition by using solid or solid-supported Lewis acids,[5] one-electron-transfer reagents,[6] and other newly discovered reagents.[7] In the course of our research to finding catalysts for enantioselective ring-opening of epoxides, we accidentally found that styrene oxide could be hydrolyzed in hot water (60 °C) within 3.5 h and the corresponding vicinal diol was obtained in 98% yield (Scheme 2). A literature search showed that in 1993, Kotsuki's group reported a catalyst-free hydrolysis

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SCHEME 2. Hydrolysis of Styrene Oxide in Hot Water

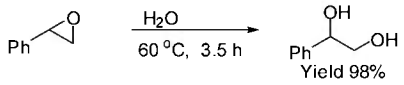


TABLE 1. Hydrolysis of Styrene Oxide and Cyclohexene Oxide in Water or a 1:1 Mixture of Water and Other Solvents[a]

epoxide $\xrightarrow{H_2O:\text{organic solvent} = 1:1}$ vicinal diol

entry	epoxides	other solvents	yield (%)[b]
1	styrene oxide	pure water	98
2	styrene oxide	MeOH	70
3	styrene oxide	EtOH	60
4	styrene oxide	*i*-PrOH	40
5	styrene oxide	*t*-BuOH	50
6	styrene oxide	ethylene glycol	96
7	styrene oxide	CH_3CN	9
8	styrene oxide	THF	1
9	styrene oxide	acetone	20
10	styrene oxide	DMF	6
11	styrene oxide	DMSO	38
12	styrene oxide	PEG400	40
13	styrene oxide	1,4-dioxane	95
14[c]	cyclohexene oxide	pure water	89
15[c]	cyclohexene oxide	1,4-dioxane	47
16[c]	cyclohexene oxide	ethylene glycol	85

Reaction conditions: 2 mmol of epoxides in water or a 1:1 mixture of water and organic solvent was heated at 60 °C for 3.5 h. [b] GC yield. [c] The reaction was carried out at 80 °C for 12 h, isolated yield.

of epoxides in the mixed solvent of acetone and water at 60 °C under 10 kbar, but the substrate scope was limited and the yield was unsatisfied.[8]

Solvent screening was carried out by employing an equal volume of water and water miscible organic solvent (Table 1). For the hydrolysis of styrene oxide, water gave the highest yield while the introduction of CH_3CN, THF, DMF, acetone, or DMSO gave little conversion of styrene oxide. The addition of different alcohols gave moderate conversion. It seemed that a 1:1 mixture of water and 1,4-dioxane or ethylene glycol was also a good solvent system in which a similar yield was obtained compared with that of water. However, when a relatively inert substrate cyclohexene oxide was hydrolyzed, an 89% yield of 1,2-dihydroxycyclohexane was obtained in water compared with an 47% and 85% yield in the mixture of water and 1,4-dioxane or ethylene glycol, respectively. Thereby, water is the choice of solvent.

We also found that the amount of water had a significant influence on reaction rate. As shown in Figure 1a, for the hydrolysis of styrene oxide, the product yield climbed higher following the increase of water amount and the curve went to a plateau after the ratio of water to substrate was raised to 6 mL of water per millimole of substrate. This critical amount of water requirement was also observed in recently reported *N*-*tert*-butyloxycarbonylation of amines in which water was also used as solvent.[9] Temperature effect was also examined with cyclohexene oxide being a test substrate; superior yield was obtained at higher temperature and the reaction rate reached a maximum in boiling water (Figure 1b).

A variety of epoxides could be hydrolyzed by heating them in water (Table 2). All aromatic epoxides (entries 1 to 6) showed high reactivity and nearly quantitative yields of diols were obtained. Acyclic aliphatic terminal epoxides (entries 7 to 9) could be hydrolyzed efficiently at 100 °C. A large-scale reaction was carried out by applying 6 mmol of 6,7-epoxycitronellol in 12 mL of water, and the epoxide could be transformed to the corresponding triol in 97% yield. The acetyl-protected 6,7-epoxycitronellol (entry 11) could also be hydrolyzed without removal of the acetyl group. α-Hydroxyl epoxides (entries 12 to 14) also reacted smoothly with excellent yields. Cyclopentene oxide and cyclohexene oxide were hydrolyzed with excellent stereoselectivity since only trans vicinal diols were detected (entries 15 and 16). For trisubstituted 1,2-epoxylimonene (entry 17), 36 h of reaction in boiling water gave the hydrolyzed product in 90% yield. Cyclic α,β epoxy ketone (entry 18) was hydrolyzed to trans-diol in 77% yield (compared to 47% in Kotsuki's method).[8]

Further studies showed that this method is suitable for the hydrolysis of aziridines as well.[10] As shown in Table 3, various aziridines, including aromatic, aliphatic, and cyclic aliphatic aziridines, could be hydrolyzed successfully and the yields were excellent. For *N*-tosyl-2-phenylaziridine (entry 1), exclusive product was obtained from water attacking at the benzylic position. The alkyl terminal aziridine (entry 2) was preferentially attacked at the less substituted position. In the case of cycloalkyl aziridines (entries 3, 4, and 5), the stereochemistry of the products was determined to be trans by comparing with the reported data. Nonactivated aziridines such as *N*-benzylcyclohexanoaziridine were also tested, but the reactions were very slow.

Recently the ring-opening reactions of epoxides or aziridines with various types of nucleophiles have been extensively studied.[11] Several methods with different catalysts employed water as solvent and it was believed that water was essential for the success of the reactions.[12] In 2005, Saidi's group reported an effective aminolysis of epoxides in water around room temperature without additional catalyst.[13] To extend the generality of our simple method, three representative substrates were treated with different nucleophiles in hot water without additional catalyst (Table 4). In Saidi's report, the additions of aromatic amines to alkyl epoxides were sluggish at room temperature. We found that at 60 °C, both benzylamine and aniline could equally and effectively add to styrene oxide and cyclohexene oxide.[14] Moreover, the addition of amines to *N*-tosylcyclohexanoaziridine proceeded very well and gave quantitative yield of products.[15] Instead of using expensive $TMSN_3$, cheap and water-soluble NaN_3 could be added to both

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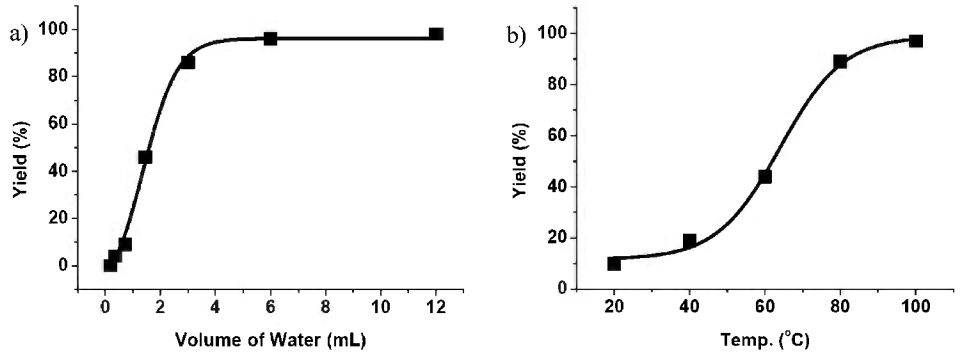


FIGURE 1. (a) A 2 mmol sample of styrene oxide was hydrolyzed in the indicated volume of water at 60 °C for 3.5 h and the yields were analyzed by GC. (b) A 2 mmol sample of cyclohexene oxide was hydrolyzed in 12 mL of water at the indicated temperature for 12 h and the yields were analyzed by GC.

epoxides and aziridines, giving azidolysis products at neutral condition.[16] It has been reported that thiophenol could be efficiently added to epoxides in water around room temperature.[17] We found that the ring-opening of aziridine by thiophenol could proceed in neutral hot water without catalyst and gave 73% of sulfur attacking product accompanied by the diol byproduct.[15a−c,18]

Efforts were also made to understand the role of hot water in the above reactions. One possibility is that the hydrogen-bonding formation between epoxide oxygen and water may activate epoxides based on the reports, showing that the hydrogen bond between alcohol (or phenol) and epoxide can accelerate their ring-opening reactions.[14b,19] In several reported catalytic ring-opening reactions of epoxides or aziridines operating in ambient water, including the newly published cascade epoxide-opening in water at 70 °C, the hydrogen bond between water and epoxide or aziridine was also used to explain the rate enhancement.[3,12b,13] But in the present studies, the rates of hydrolysis reaction reached the maximum in boiling water, while actually the hydrogen bond donating ability of water drops quickly from 25 to 100 °C because the hydrogen-bonding formation is exothermic.[20] An alternative and more likely explanation of the rate acceleration in hot water may be that the self-ionization of water enhances as temperature rises, the −log Kw (Kw is the self-ionization constant of water) value of water at 100 °C is 12,[21] where both H^+ and OH^- are 10 times more abundant than that in ambient water (−log Kw = 14 at 25 °C), therefore water itself can act as a modest acid or base catalyst.[22] It was reported that some traditionally acid- or base-catalyzed reactions, such as hydrolysis of esters and ethers[23] and dehydration of alcohols,[24] could take place in neutral high-temperature water (>200 °C, −log Kw = 11 at 200 °C) without additional acid or base. As the ring-opening reactions of epoxides or aziridines are conventional acid or base catalyzed, it seems reasonable to assume that it can also be facilitated where the self-ionization of water is progressively enhanced along with the increase of temperature. Acid catalysis dominates the reported organic reactions in high-temperature water.[23a,c] The observed cis/trans ratio of diol products of entries 4 and 5 in Table 2 was found to be similar with the product distribution in the protic acid-catalyzed hydrolysis (cis-diols are predominant in the hydrolyzed products).[25] If hot water acts as a modest acid, we predicted that an intramolecular hydroxyl group should open epoxide in hot water, with higher reaction rate since these reactions are also traditionally acid catalyzed.

To test our hypothesis, two simple epoxides with a hydroxyl group at the terminal site were prepared. Both *trans*-4,5-epoxyhexanols **1** and *cis*-4,5-epoxyhexanols **3** were cyclized in water at 60 °C in 0.5 h with almost quantitative yield. The 5-exo-opening of epoxide was preferred and the observed ratio of oxalane to oxane product produced from trans and cis substrates was exactly the same compared with the reported product distribution from the BF_3·Et_2O-catalyzed cyclization of the same substrates (Scheme 3).[26] The ratio of oxalane to oxane product was not changed in the reactions conducted in water at 0 °C

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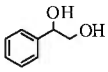
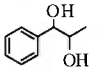
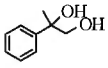
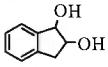
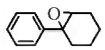
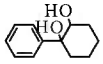
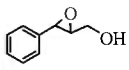
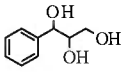
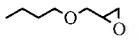
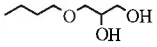
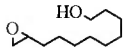
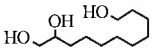
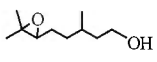
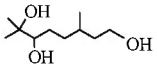
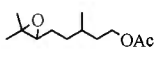
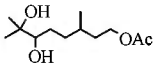
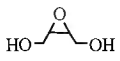
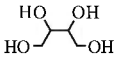
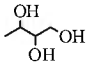
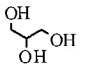
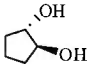
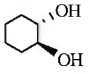
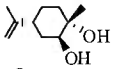
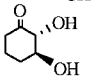
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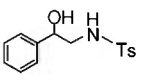
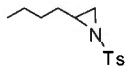
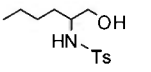
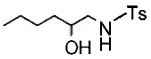
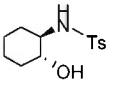
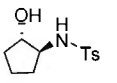
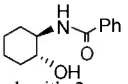
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TABLE 2. Hydrolysis of Epoxides in Hot Water[a]

Entry	Epoxide	Product	Temp (°C)	Time (h)	Yield (%)[b]
1			60	3.5	98
2[c]			60	1	98
3			60	1	97
4[d]			60	3	98
5[e]			60	1	97
6[f]			60	2	99
7			100	9	96
8			100	12	97
9			100	4	99
10[g]			60	1	97
11			60	1.5	96
12			100	9	99
13			60	4	98
14			60	4	98
15			100	18	96
16			100	12	98
17[h]			100	36	90
18			100	10	77

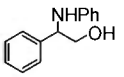
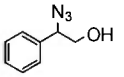
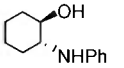
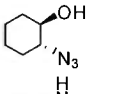
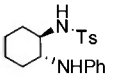
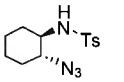
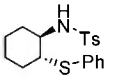
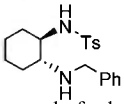
[a] The reaction was conducted with 2 mmol of epoxide in 12 mL of water at the indicated temperature. [b] Isolated yield; the ratio of the isomers was determined by integrations of the signals in the ^{1}H NMR spectrum. [c] Threo/erythro: 60/40. [d] Trans/cis: 25/75. [e] Trans/cis: 25/75. [f] Threo/erythro: 75/25. [g] 6 mmol of epoxide in 12 mL of water. [h] 1 mmol of a 1:1 mixture of *trans*-(*R*)-(+)-limonene oxide and *cis*-(*R*)-(+)-limonene oxide in 12 mL of water.

TABLE 3. Hydrolysis of Aziridines in Hot Water[a]

Entry	Aziridine	Product	Time (h)	Yield (%)[b]
1			7	99
2			48	62
				31
3			7	99
4			5	99
5			4	99

[a] The reaction was conducted with 2 mmol of aziridine in 12 mL of water at 100 °C. [b] Isolated yield; the ratio of the isomers was determined by integrations of the signals in the ^{1}H NMR

TABLE 4. Ring-Opening Reactions of Epoxides and Aziridines in Hot Water[a]

Entry	Substrate	Nucleophile	Product	Time (h)	Yield (%)[b]
1				3	92
2		NaN_3		3	93
3				3	93
4		NaN_3		3	97
5				5	98
6		NaN_3		5	99
7				72	73
8				5	99

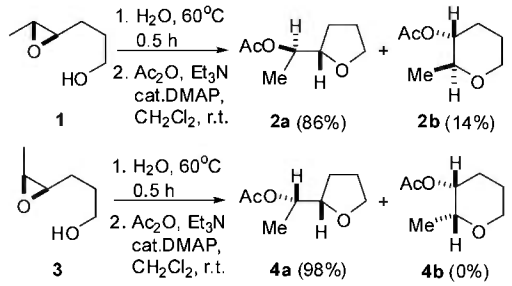
[a] The reaction was conducted with 1 mmol of substrate and 2 mmol of nucleophile in 6 mL of water at 60 °C. [b] Isolated yield.

but more than 15 h was required for completion of the same reactions. Another reported Lewis acid-catalyzed cyclization of diepoxide was also tested in hot water.[27] A diastereomeric mixture of 1,5-diepoxides **5**[28] was completely consumed in water at 60 °C in 0.5 h (Scheme 4). Acylation of the resulting crude mixture gave 44% of seven-member-ring product **6a** and 24% of five-member-ring product **6b**, which was parallel with the result from BF_3-etherate-promoted cyclization. The same reaction could also complete in water at 100 °C within 10 min or more than 24 h at 0 °C with the same product distribution. This

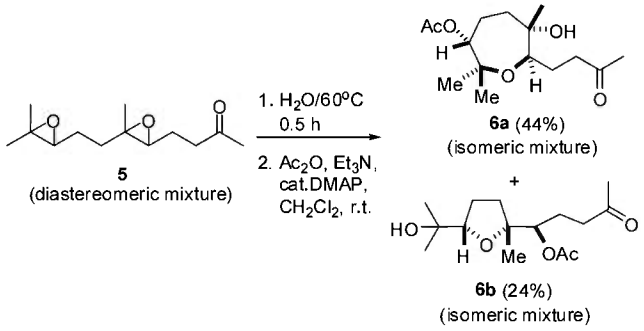
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SCHEME 3. Hydroxy-Epoxide Cyclization in Water at 60 °C



SCHEME 4. Oxacyclization of 1,5-Diepoxide



evidence implies that the ring-opening reactions in hot water was acid catalyzed.

The dielectric constant of ambient water is 80 and this number quickly reduces to 55 at 100 °C, which implies that water is more like a polar organic solvent at high temperature. Therefore, some substrates that were originally water insoluble dissolve and react at elevated temperatures.[29]

In conclusion, efficient hydrolysis of epoxides and aziridines has been achieved by just heating them in water. Other nucleophiles such as amines, sodium azide, and thiophenol could also effectively open epoxides and aziridines in hot water. Hydroxyl epoxides could also undergo cyclization to give cyclic ether in hot water. Simple workup (without the wash step) or direct evaporation of water led to pure ring-opening products and the yields are generally over 90% for a variety of substrates. Moreover, the present method avoided the use and subsequent disposal of additional catalyst, which will satisfy both laboratory and industrial operations. Furthermore, the present finding revealed another type of organic reaction that may benefit from the enhanced self-ionization of water. The utilization of manageable hot water (60−100 °C) rather than high-temperature water (>200 °C) makes the present reactions safe and practical.

(28) Compound **5** is an inseparable 3.7:1 mixture of two diastereomers. It was prepared from geranylacetone by Shi enantioselective double epoxidation. Wang, Z.-X.; Tu, Y.; Frohn, M.; Zhang, J.-R.; Shi, Y. *J. Am. Chem. Soc.* **1997**, *119*, 11224.

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Experiment Section

A Typical Procedure of Hydrolysis of Epoxide in Hot Water (Table 2, entry 1). The suspension of styrene oxide (1 mmol, 120 mg) in distilled water (6 mL) in a 10 mL flask with a condenser was stirred at 60 °C and monitored by TLC. After completion, the mixture was extracted with EtOAc, washed with brine, dried over $MgSO_4$, and then concentrated to give the crude product. Purification by silica gel flash column chromatography provided diol product (135 mg, 98%).

1-Phenyl-1, 2-ethanediol: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31−7.37 (m, 5H), 4.83 (dd, J = 3.2, 8.0 Hz, 1H), 3.76 (dd, J = 3.0, 11.4 Hz, 1H), 3.66 (dd, J = 8.2, 11.1 Hz, 1H), 2.69 (br s, 1H), 2.27 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.34, 128.36, 127.77, 126.00, 74.63, 67.88.

A Typical Procedure of Hydrolysis of 2-Phenyl-1-tosylaziridine in Hot Water (Table 3, entry 1). The suspension of 2-phenyl-1-tosylaziridine (2 mmol, 546 mg) in distilled water (12 mL) in a 25 mL flask with a condenser was stirred at 100 °C and monitored by TLC. After completion, the mixture was extracted with EtOAc, washed with brine, dried over $MgSO_4$, and then concentrated to give the crude product. Purification by silica gel flash column chromatography provided product (576 mg, 99%).

2-(*N*-Tosylamino)-1-phenyl-1-ethanol: ^{1}HNMR (400 MHz, $CDCl_3$) δ 7.69 (d, J = 8.0 Hz, 2H), 7.23−7.25 (m, 7H), 5.68 (s, 1H), 4.76 (dd, J = 2.4, 8.4 Hz, 1H), 3.48 (br s, 1H), 3.14−3.17 (m, 1H), 2.94−3.00 (m, 1H), 2.37 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.40, 140.76, 136.48, 129.65, 128.39, 127.89, 126.94, 125.78, 72.58, 50.18, 21.39.

A Typical Procedure of the Ring-Opening Reactions of Epoxides or Aziridines by Other Nucleophiles (Table 4, entry 4). To a stirred suspension of cyclohexene oxide (1 mmol, 98 mg) in distilled water (6 mL) was added the sodium azide (2 mmol, 130 mg) in a 10 mL flask with a condenser; the resulting mixture was stirred at 60 °C and was monitored by TLC. After completion, the mixture was extracted with EtOAc, washed with brine, dried over $MgSO_4$, and then concentrated to give the crude product. Purification by silica gel flash column chromatography provided product (137 mg, 97%).

2-Azidocyclohexanol: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.42−3.35 (m, 1H), 3.22−3.15 (m, 1H), 2.52 (s, 1H), 2.07−2.00 (m, 2H), 1.78−1.72 (m, 2H), 1.36−1.19 (m, 4H).

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Supporting Information Available: General experimental procedures and ^{1}H NMR and ^{13}C NMR spectra of compounds in Tables 2−4 and Schemes 3 and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Anti-Markovnikov hydration of alkenes over platinum-loaded titanium oxide photocatalyst†

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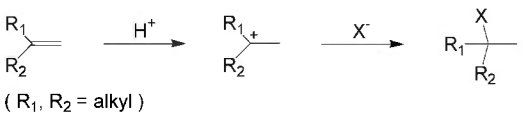
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Hydration of various alkenes proceeded over a platinum-loaded titanium oxide photocatalyst. It is notable that, unlike conventional acid-catalysts, the photocatalyst preferentially promoted the hydration according to the anti-Markovnikov rule. The results of some reaction experiments and semiempirical molecular orbital calculations suggested the reaction path of this hydration as follows: an electrophilic surface oxygen radical species formed on the photoexcited titanium oxide attacks a carbon double bond of the alkene to produce an intermediate, followed by the addition of a hydrogen radical formed on the platinum sites to produce an alcohol. The anti-Markovnikov selectivity for each investigated alkene would be determined by the stability of the radical intermediate.

1. Introduction

Hydration of alkenes (water addition to carbon double bond) is an important industrial process for the direct synthesis of alcohols, which are widely used as the chemical intermediates for resins, agricultural chemicals, surfactants, and so on. Although the selective hydration to produce branched alcohols is possible by using homogeneous and heterogeneous acid catalysts, *e.g.*, sulfonic acid,[1a] heteropolyacid,[1b] zeolite,[1c] ion-exchanged resin,[1d] *etc.*, the acid catalysts are unable to produce primary alcohols (except for ethylene to ethanol) or less substituted alcohols because of the strict regioselectivity known as the Markovnikov rule. According to this rule, as shown in eqn (1),[2] the proton (H^+) is firstly added to the carbon bonding to less alkyl substituents in the carbon double bond, followed by the addition of a functional group (X^-, anion or neutral molecule) to the carbon with more alkyl substituents. In the case of the hydration, after the addition of the proton, a water molecule adds to the carbocation intermediate, followed by the deprotonation. This selectivity is determined by the stability of the carbocation intermediate.



(R_1, R_2 = alkyl) (1)

On the other hand, various processes to produce anti-Markovnikov type alcohols from alkenes have been studied for a long time. Hydroboration by borane reagents followed by oxidation,[3] and hydroformylation by transition metal catalysts[4] followed by hydrogenation are the very famous routes to give the excellent anti-Markovnikov selectivity. However, both are multistep processes and especially the former consumes the boranes. Recently, one-pot syntheses of primary alcohols have been attained, *e.g.*, successive hydroformylation/hydrogenation with the Rh/xantphos and Shvo's catalyst,[5] and Wacker-type oxidation/hydrolysis/hydrogenation with the $PdCl_2$ and Shvo's catalyst.[6] In these syntheses, however, strictly defined reaction conditions were required for each elementary process. So far, direct hydration processes have been realised only for the alkenes having an electron withdrawing group, such as photohydration of nitro-substituted styrenes in the presence/absence of a photosensitiser,[7a] photohydration of 1,1-diphenylpropene in the presence of a photosensitising cyclodextrin host,[7b] and hydration of such alkenes with phosphine catalysts.[8] Thus, the development of new simple processes that follow the anti-Markovnikov rule are still required.

Photocatalysis has received much attention because of its unique potential to enable various organic syntheses in mild conditions by using photoformed hole (h^+) and electron (e^-),[9] and could directly produce alcohols from alkanes (cyclohexane, *etc.*) on titanium oxide,[10a] V_2O_5/Al_2O_3,[10b] Cr–Si binary oxide,[10c]

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$CuCl_2$,[10d] and so on. However, primary alcohols were obtained as minor products.[10b,d] To our knowledge, photocatalytic hydration of alkenes has not been reported yet.

In our previous study, we succeeded in the selective aromatic ring hydroxylation by using water with a platinum-loaded titanium oxide photocatalyst and clarified that the oxygen radical species produced from water were electrophilic.[11] Thus, in the present study, we applied this photocatalytic system to the hydration of alkenes, and found that the photocatalytic hydration of alkenes proceeded and the regioselectivity preferentially followed the anti-Markovnikov rule.

2. Experimental section

2.1. Reagents

Titanium oxide sample, JRC-TIO-8 (anatase, 338 $m^2 g^{-1}$) and JRC-TIO-6 (rutile, 100 $m^2 g^{-1}$) were supplied by Catalysis Society of Japan and the former was used unless stated. Precursors of the cocatalyst were chloroplatinic acid hexahydrate ($H_2PtCl_6 \cdot 6H_2O$, Kishida, 99.9%), palladium chloride ($PdCl_2$, Kishida, 99%), and gold chloride acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$, Kishida, 99%). These reagents were used without further purification. Other reagents were 1-butene (TCI, 98%), 1,3-butadiene (TCI, 95%), 1-hexene (Aldrich, 99%), cyclohexene (Wako, 97%), 1-methyl-1-cyclohexene (TCI, 98%), 2-methyl-1,3-butadiene (TCI, 99%), 1,5-hexadiene (TCI, 97%), thiophene (Aldrich, 99%), D_2O (Acros Organics, 99.95%), and $H_2{}^{18}O$ (Euriso-top, 95.4%). These reagents were used after purification by distillation, except for the isotopic-labeled water.

2.2. Preparation of metal-loaded titanium oxide photocatalysts

Metal-loaded titanium oxide was prepared by a photodeposition method. The titanium oxide powder (4 g) was dispersed into an aqueous methanol solution (400 ml, methanol concentration 25%) containing the metal precursor in a 500 ml beaker with vigorous stirring, followed by irradiation from the top with a 300 W xenon lamp, which emitted both UV and visible light, at room temperature for 3 h. Then, the suspension was filtered off with suction, washed with water, and dried at 323 K overnight. In this article, x wt% of metal (M)-loaded titanium oxide is described as M(x)/TiO_2. Measurements of ICP-AES with an Optima 3300 DV (Perkin-Elmer Japan) confirmed that the objective amount of metal was correctly loaded on the titanium oxide. The average platinum particle size on the Pt/TiO_2 samples was estimated by using a CO-pulse method in a flow system after the reductive pretreatment with hydrogen at 423 K for 20 min.

2.3. Photocatalytic reaction tests

Photocatalytic reaction tests were carried out by using two kinds of reactors; one was a closed reactor connected with a vacuum line and the other was a closed reactor connected with an argon gas flow line. In the former system, the photocatalytic reactions of gaseous substrates were investigated. The prepared M(x)/TiO_2 sample in a quartz cell was irradiated with the 300 W xenon lamp from beneath in an air atmosphere for 1 h followed by evacuation. Then, a gaseous substrate (1-butene or 1,3-butadiene, 1.8 mmol) and liquid water (mainly 10 ml, corresponding to 560 mmol) were introduced to the cell, where the substrate should be partly dissolved in the water and the photocatalyst also existed in the liquid phase, and the cell was irradiated with the 300 W xenon lamp with magnetic stirring of the liquid phase at 314 K. After the irradiation, the gas phase products were collected by a gas tight syringe and analysed by a GC-TCD (Shimadzu, GC-8A). Then, the products in the liquid phase were analysed by a GC-MS (Shimadzu, QP-5050A) and a GC-FID (Shimadzu, GC-14B). In the latter reactor, liquid substrates were investigated. The prepared Pt(0.1)/TiO_2 sample in the quartz cell was irradiated with the 300 W xenon lamp for 1 h in the presence of air. After the air in the reactor was purged by argon, the substrate (1.0 ml, corresponding to 8.0–10 mmol) except for thiophene (2.0 ml, corresponding to 24 mmol) and water (1.0 ml or 10 ml) were introduced to the cell, and the cell was irradiated with the 300 W xenon lamp in an argon atmosphere with magnetic stirring at 314 K for 60 or 180 min. After the irradiation, the gas phase products were analysed by the GC-TCD (Shimadzu, GC-8A). Then, methanol was added to the liquid phase and analysed by the GC-MS (Shimadzu, QP-5050A) and the GC-FID (Shimadzu, GC-14B). In both systems, when the irradiation light wavelength was limited, an optical band pass filter permitting the light transmission of 360 ± 5 nm in wavelength or a cut-off filter permitting that of longer than 370 nm in wavelength was used and the reaction temperature was 305 K. As the control experiments, reaction tests without the photocatalyst or the photoirradiation were also carried out in the absence of the photocatalyst or in the dark. These experiments confirmed that the detected products in this article were produced by the photocatalysis on the M(x)/TiO_2 samples.

3. Results and discussion

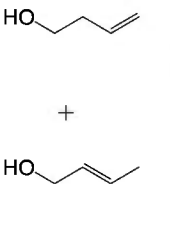
3.1. Outline of the photocatalytic reaction

Table 1 shows the result of the photocatalytic reaction of 1,3-butadiene over the M(0.1)/TiO_2 samples. We found that some kinds of metal-loaded TiO_2 photocatalysts were able to promote hydration of 1,3-butadiene. When the Pt(0.1)/TiO_2 sample was used under irradiation without the limitation of the wavelength (Table 1, entry 1), the products were 5.6 μmol of primary alcohols (2-butne-1-ol and 3-butene-1-ol), and 1.4 μmol of 2-butenal, 3.2 μmol of coupling products and an excess amount (110 μmol) of hydrogen, and thus the alcohol selectivity was low (42%). On the other hand, when the irradiation wavelength was limited to 360 ± 5 nm, the coupling products and the excess amount of hydrogen was suppressed and the higher alcohol selectivity (76%) was obtained (Table 1, entry 2). This would be mainly because the direct photoexcitation of 1,3-butadiene adsorbed on the titanium oxide resulting in the formation of byproducts was suppressed.[11b] It is notable that, although the hydration reaction with various acid catalysts usually follows the Markovnikov rule,[1] the current photocatalytic hydration of 1,3-butadiene proceeded according to the anti-Markovnikov

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Table 1 Results of the reaction tests for the photocatalytic hydration of 1,3-butadiene over the M(0.1)/TiO_2 samples

		Products/μmol								
		Anti-Markovnikov type		Markovnikov type						
										
Entry	Loading metal					CP[a]	H_2	Alcohol yield[b] (%)	Alcohol selec.[c] (%)	Anti-Markovnikov selec.[d] (%)
1[e]	Pt	5.6 (2.1 + 3.5)[f]	1.4 (1.4 + n.d.)[g]	n.d.	n.d.	3.2	110	0.31	42	100
2[h]	Pt	5.0 (1.5 + 3.5)[f]	0.8 (0.7 + 0.1)[g]	n.d.	n.d.	0.4	5.8	0.28	76	100
3[h]	Pd	5.2 (2.0 + 3.2)[f]	0.9 (0.8 + 0.1)[g]	0.1	0.1	2.1	6.1	0.29	50	97
4[h]	Au	1.0 (0.5 + 0.5)[f]	0.4 (0.4 + n.d.)[g]	n.d.	n.d.	0.8	5.0	0.056	33	100

[a] Coupling products, mainly C_8 hydrocarbons. The yields of other coupling products were very small. [b] Alcohol yield (%) = 100 × $Y_{Alcohols}$/(introduced 1,3-butadiene), where Y stands for the yielded amount of product. [c] Alcohol selectivity (%) = 100 × ($Y_{Primary\ alcohols}$ + $Y_{2\text{-}Butanol}$)/($Y_{Primary\ alcohols}$ + $Y_{2\text{-}Butanol}$ + $Y_{Alehydes}$ + $Y_{2\text{-}Butanone}$ + 2 × $Y_{Coupling\ products}$). [d] Anti-Markovnikov selectivity (%) = 100 × ($Y_{Primary\ alcohols}$ + $Y_{Aldehydes}$)/($Y_{Primary\ alcohols}$ + $Y_{2\text{-}Butanol}$ + $Y_{Aldehydes}$ + $Y_{2\text{-}Butanone}$). [e] Catalyst, 0.2 g; 1,3-butadiene, 1.8 mmol; water, 10 ml (560 mmol); reaction time, 180 min; reaction temperature, 314 K; irradiated light wavelength was not limited. Irradiated light intensities were 13, 54, and 148 mW cm^{-2} when measured at 254 ± 10, 365 ± 15, and 405 ± 30 nm, respectively. The detection limits of the organic compounds, hydrogen and carbon dioxide were 0.01, 0.1 and 1.0 μmol, respectively. [f] The left and right values in the parenthesis correspond to the yield of 3-buten-1-ol and 2-butene-1-ol, respectively. [g] The left and right values in the parenthesis correspond to the yield of 2-butenal and butanal, respectively. n.d. = not detected. [h] Reaction temperature, 305 K; irradiated light wavelength, 360 ± 5 nm; irradiated light intensity was 8.4 mW cm^{-2} when measured at 365 ± 15 nm. The other conditions were the same as the footnote e.

rule very selectively (almost 100%) regardless of the irradiated light wavelength (Table 1, entries 1 and 2), *i.e.*, only primary alcohols were produced while no secondary alcohols were observed. In addition, such high anti-Markovnikov selectivity was also observed on the samples with the other loading metals, such as palladium and gold (97 and 100%, Table 1, entries 3 and 4), although they promoted the reaction with less alcohol selectivity even under the light of limited wavelength.

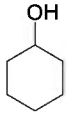
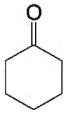
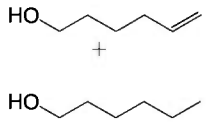
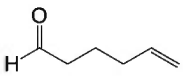
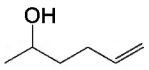
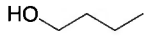
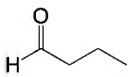
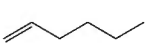
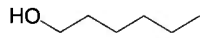
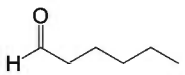
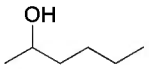
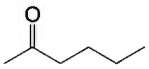
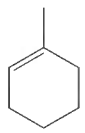
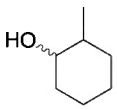
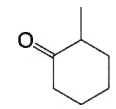
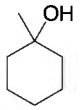
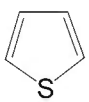
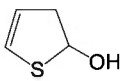
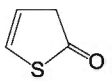
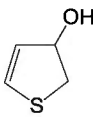
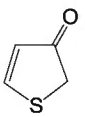
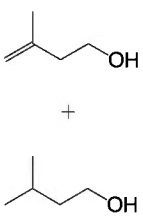
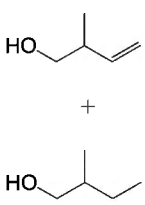
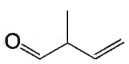
Table 2 shows the results of the photocatalytic reaction tests of various alkenes in the optimised conditions for each substrate. From all of the investigated alkenes, the corresponding alcohols were produced, indicating that this photocatalytic system is available for various alkenes in both gas and liquid states. The alcohols were observed in the liquid phase in each case. Further, the anti-Markovnikov type products were preferentially (65–100%) produced from all of the substrates except for the cyclohexene having a symmetric double bond, although the anti-Markovnikov selectivity was different between the listed substrates. This will be discussed later. In the case of 1-methyl-1-cyclohexene, the alcohol according to the anti-Markovnikov rule (1.2 μmol) consisted of *cis*- and *trans*-isomers of similar amounts, 0.7 and 0.5 μmol, respectively (Table 2, entry 5), which will be also discussed later. For the cyclohexene, the alcohol selectivity was especially low (17%) because it is difficult to inhibit the coupling reaction in this condition. For the other substrates, the alcohol selectivity widely varied (38–83%) because of the yield of the carbonyl compounds.

Fig. 1 shows the effect of the platinum loading amount on the photocatalytic reaction of 1,3-butadiene. As shown in Fig. 1a, the highest amount of alcohol was obtained on the Pt(0.1)/TiO_2 sample. In principle, the smaller platinum nanoparticles having a larger work function would be effective for the separation of the photoformed hole–electron pairs, and the larger amount of such effective small nanoparticles would provide the best performance.[12] The size and number of the platinum nanoparticles determined by the CO adsorption method were shown in Fig. 1c. The Pt(0.1)/TiO_2 sample had the largest number of platinum nanoparticles with the almost smallest size among them, which agrees with the above discussion. On the other hand, the high anti-Markovnikov selectivity did not vary with increasing platinum loading amount although the average diameter of the platinum also increased (Fig. 1b and c). Thus, the loading amount, *i.e.*, the diameter and the number of the platinum nanoparticles, would not be the controlling factor for the anti-Markovnikov selectivity, as discussed later.

Fig. S1 and S2, (ESI†) show the influence of the crystal phase and the specific surface area of titanium oxide on the photocatalytic activity for the hydration of 1-hexene (Fig. S1, ESI†) and thiophene (Fig. S2, ESI†). As for the specific surface area of the Pt(0.1)/TiO_2(anatase) sample, the total yield increased with increasing the specific surface area while the anti-Markovnikov selectivity was almost constant for both substrates (Fig. S1b and S2b, ESI†). This would be because catalytically active sites on the titanium oxide would increase with increasing the specific surface area as reported in the literature.[12,13] As for the crystal phase of titanium oxide, the anatase exhibited higher photocatalytic activity than the rutile having almost the same specific surface area (Fig. S1b and S2b, ESI†). Since the potential of the photoformed hole in the rutile (3.04 V *vs.* NHE)[14a] is lower than that in the anatase (3.12 V *vs.* NHE),[14b] the rutile would less promote the oxidation of water (*e.g.*, $H_2O + h^+ \rightarrow {}^{\bullet}OH + H^+$, 2.8 V *vs.* NHE)[14c] than the anatase. Thus, anatase titanium

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Table 2 Results of the reaction tests for the photocatalytic hydration of various alkenes over the Pt(0.1)/TiO_2 sample

Entry	Substrate	Products/μmol				CP[b]	H_2	Alcohol yield[c] (10^{-2}%)	Alcohol selec.[d] (%)	Anti-Markovnikov selec.[e] (%)
1[a]										
		3.7	2.6			7.7[f]	35	3.7	17	—
		Anti-Markovnikov type		Markovnikov type						
2[g,h]										
		1.4 (1.3 + 0.1)[i]	0.1	0.8		0.9[f]	23	2.6	38	65
3[j]										
		10	3.4	3.5	1.7	n.d.[k]	11	75	73	72
4[a]										
		0.3	0.3	0.1	0.1	0.1[l]	6.6	0.50	40	75
5[a]										
		1.2[m] [*cis*-0.7 *trans*-0.5]	1.3	0.6		n.d.[k]	2.6	2.1	58	81
6[n]										
		25.2	0.3	n.d.[k]	n.d.[k]	0.1[o]	62	11	83	100
		Anti-Markovnikov type								
7[g]										
		1.3 (1.1 + 0.2)[i]	0.9 (0.8 + 0.1)[i]	0.1		0.2[p]	7.4	2.2	81	100

[a] Catalyst, 0.2 g; substrate, 1.0 ml (8.0–9.9 mmol); water, 1.0 ml (56 mmol); reaction time, 60 min; reaction temperature, 305 K; irradiated light wavelength, > 370 nm; irradiated light intensities were 3, 10 and 84 mW cm^{-2} when measured at 254 ± 10, 365 ± 15 and 405 ± 30 nm, respectively. The detection limit of the organic compounds was 0.01 μmol, that of hydrogen was 0.1 μmol, and that of carbon dioxide was 1.0 μmol. [b] CP = coupling products. [c] Alcohol yield (%) = 100 × $Y_{Alcohols}$/(introduced substrate), where Y stands for the yielded amount of product. [d] Alcohol selectivity (%) = 100 × $Y_{Alcohols}/(Y_{Alcohols} + Y_{Carbonyl\ products} + 2 \times Y_{CP})$. [e] Anti-Markovnikov selectivity (%) = 100 × $(Y_{Anti\text{-}Markovnikov\ type\ alcohol} + Y_{Anti\text{-}Markovnikov\ type\ carbonyl\ product})/(Y_{Alcohols} + Y_{Carbonyl\ products})$. [f] Dimer ($C_{12}$) hydrocarbons were produced. The detailed structure was not analysed. [g] Catalyst, 0.2 g; substrate, 1.0 ml (8.4–10 mmol); water, 10 ml (560 mmol); reaction time, 180 min; reaction temperature, 305 K; irradiated light wavelength, 360 ± 5 nm; irradiated light intensity was 8.4 mW cm^{-2} when measured at 365 ± 15 nm. [h] Also detected was 10 μmol of carbon dioxide. [i] The left and right values in the parenthesis corresponds to the yield of unsaturated and saturated alcohol in the two structural formulas. [j] 1-Butene, 1.8 mmol; water, 10 ml (560 mmol); the other conditions were the same as the footnote g. [k] n.d. = not detected. [l] 5-Methyl undecane. The yields of other coupling products were very small. [m] *Cis*, *trans* isomers were produced. The yield of each product is shown in the parenthesis. [n] Thiophene, 2.0 ml (24 mmol); water, 8.0 ml (440 mmol); reaction time, 60 min; reaction temperature, 314 K; irradiated light intensities were 23, 80, and 176 mW cm^{-2} when measured at 254 ± 10, 365 ± 15 and 405 ± 30 nm, respectively. Also detected was 18 μmol of carbon dioxide. [o] Bithiophenes. [p] Dimer (C_{10}) hydrocarbons were produced. The detailed structure was not analysed.

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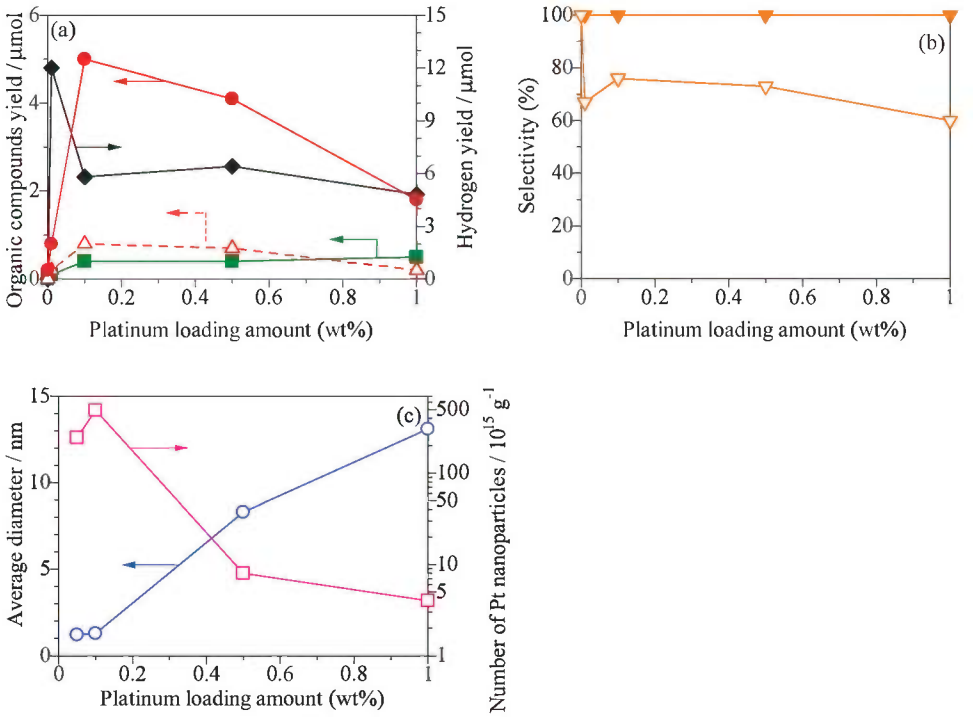


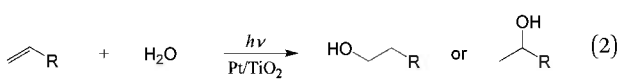
Fig. 1 The effect of the platinum loading amount on the products yield (a) and selectivity (b) in the photocatalytic hydration of 1,3-butadiene over the Pt(*x*)/TiO_2 samples. The red filled circles, red blank triangles, green filled squares and black filled diamonds in panel a correspond to the total yield of 2-butene-1-ol and 3-butene-1-ol, that of 2-butenal and butanal, that of the coupling products, and the yield of hydrogen, respectively. The orange filled inverse triangles and orange blank inverse triangles in panel b correspond to the anti-Markovnikov selectivity and the alcohol selectivity, respectively. The blue blank circles and pink blank squares in panel c correspond to the average diameter and the number of the platinum nanoparticles, respectively. The average diameter of the platinum nanoparticles was determined by the CO pulse method. As for the reaction conditions, see the footnote a of Table 1.

oxide with a large surface area would be most effective for the hydration.

The magnitude of the influence by the specific surface area was different between the substrates, which would be derived from the properties of the substrates although the details were not clarified in the present study.

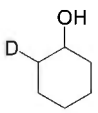
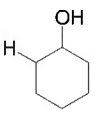
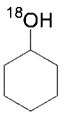
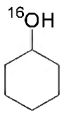
3.2. Active species

Table 3 shows the results of the isotopic experiments in the photocatalytic hydration of cyclohexene. When the reaction was carried out by using D_2O, the mass number (*m*/*z*) of the molecular ion peak for most of the produced cyclohexanol (>99%) increased from 100 to 101 (Table 3, entry 1). In the present analysis method, the D atom of the hydroxyl group in the produced alcohol can be easily exchanged with the H atom of methanol that was added for homogenisation of the liquid phase after the reaction before the analysis. Thus, the increase of the mass number would originate from the D atom of D_2O, which was directly added to the carbon double bond. This result suggests that the alcohol was dominantly produced through photocatalytic hydration (water addition) of the carbon double bond as shown in eqn (2).



(2)

Table 3 Results of the isotopic experiments in the photocatalytic hydration of cyclohexene over the Pt(0.1)/TiO_2 sample[a]

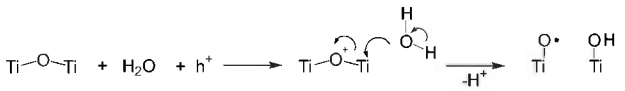
Entry	Water	Distribution of produced cyclohexanol (%)			
1[b]	D_2O		>99		n.d.[c]
2[d]	$H_2{}^{18}O$		87		13

[a] Catalyst, 0.2 g; cyclohexene, 1.0 ml (9.9 mmol); water, 1.0 ml (56 mmol); reaction time, 60 min; reaction temperature, 305 K; irradiated light wavelength, >370 nm; irradiated light intensities were 3, 10 and 84 mW cm^{-2} when measured at 254 ± 10, 365 ± 15 and 405 ± 30 nm, respectively. The detection limit of the organic compounds was 0.01 μmol. [b] In the present analysis method, the D atom of the hydroxyl group in the produced alcohol can be exchanged easily with H of the methanol that was introduced for analysis after the reaction. [c] n.d. = not detected. [d] Before the reaction, the photocatalyst was evacuated at 473 K for 2 h to remove the physisorbed water on the Pt(0.1)/TiO_2 sample.

When the reaction was carried out by using $H_2{}^{18}O$, the produced cyclohexanol contained 87% of ^{18}O atom and 13% of ^{16}O in the hydroxyl group (Table 3, entry 2). This indicates that the introduced water dominantly reacts with the alkene, while the lattice oxygen on the titanium oxide surface also

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reacts with the alkene. In the literature,[15] it was reported that upon photoirradiation the reaction between water molecules and the titanium oxide surface could produce an active surface oxygen species and the mechanism in the aqueous solution of acidic or neutral pH was proposed as follows: a bridging Ti–O–Ti site on the surface would trap the photoformed hole, and receive the nucleophilic attack of water to produce the surface oxygen radical (eqn (3)). The surface oxygen radical species would have an electrophilic property and preferably attack the carbon double bond having high electron density.[11b]



(3)

On the other hand, the photoformed electron would reduce the proton in the solution to produce a hydrogen radical at the platinum sites (eqn (4)).

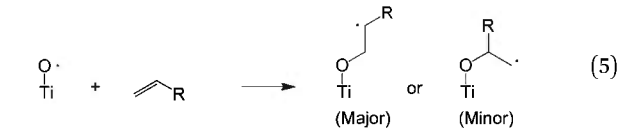


(4)

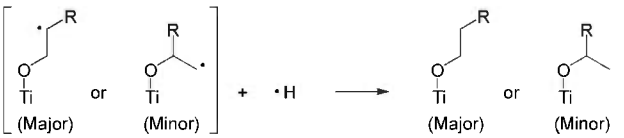
Considering eqn (3) and (4), and the results of the isotopic experiments (Table 3), the hydration of the carbon double bond would proceed through the addition of the electrophilic surface oxygen radical and the hydrogen radical. This implies that the reaction would preferably progress on the titanium oxide surface around the deposited platinum nanoparticles.

3.3. Radical intermediates

To discuss the order of the addition of these two radical species to the carbon double bond, we calculated the final heat of formation of the possible intermediate species, *i.e.*, the active oxygen adduct and the hydrogen radical adduct (Table 4), by using PM7 method[16] in MOPAC 2012.[17] In this calculation, the hydroxyl radical (•OH) was employed instead of the surface active oxygen species (eqn (3)) in order to simplify the system because they have similar thermodynamic properties to each other.[11b] In the case of the hydroxyl radical adduct, the radical intermediate in the anti-Markovnikov hydration (Table 4, A) was more stable than that in the Markovnikov hydration (Table 4, C) for all the investigated substrates. On the other hand, in the case of the hydrogen radical adduct, the radical intermediate in the Markovnikov hydration (Table 4, D) was more stable than that in the anti-Markovnikov hydration (Table 4, B). These results clearly indicate that if the hydrogen radical attacks first to the carbon double bond before the surface oxygen radical does, the product would not follow the anti-Markovnikov rule, which was inconsistent with the fact. Thus, it is suggested that the surface oxygen radical would be first added to the carbon double bond (eqn (5)), followed by the addition of the hydrogen radical (eqn (6)). In this way, the order of the radicals addition to the carbon double bond, *i.e.*, the surface oxygen radical and then hydrogen radical, would lead to the anti-Markovnikov products. Although one may consider another possibility instead of eqn (6), that the intermediate species abstracts a hydrogen atom from water, this could not occur because the bond energy of O–H in water (497.10 ± 0.29 kJ mol^{-1} at 298 K) is larger than that of C–H in hydrocarbons (*e.g.*, butane 421.3 and 411.1 ± 2.2 kJ mol^{-1}, cyclohexane 416.3 kJ mol^{-1} at 298 K).[18]



(5)

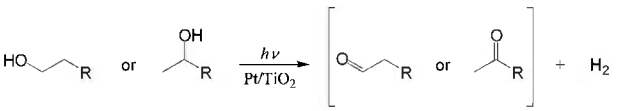


(6)

The differences of the final heat of formation for the two possible isomers of the hydroxyl radical adduct were also listed (Table 4, A–C). It is notable that the value decreased with increasing anti-Markovnikov selectivity, meaning that a larger difference between the two possible radical intermediates resulted in a higher anti-Markovnikov selectivity. Thus, the difference of the stability between the intermediates (eqn (5)) would determine the regioselectivity of the hydroxyl group for each substrate.

3.4. Successive reactions

Table 5 shows the results of the photocatalytic reaction tests for the successive reactions of 1-butene conversion by using the alcohols and the carbonyl compounds as the reactants. Butanal and 2-butanone were converted not to alcohols but to hydrogen and carbon dioxide (Table 5, entries 1 and 2), where only the complete oxidation with water occurred. On the other hand, 1-butanol and 2-butanol were oxidised into the carbonyl compounds (Table 5, entries 3 and 4), that is, the successive oxidation could occur after the hydration. Thus, the alcohol can be oxidised to carbonyl compounds as shown in eqn (7), and further oxidised to the final products, *i.e.*, hydrogen and carbon dioxide.



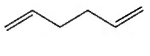
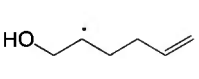
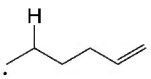
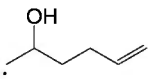
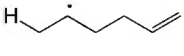
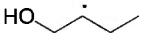
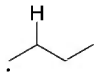
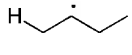
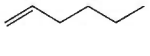
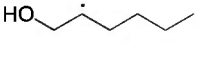
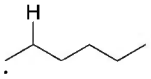
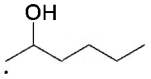
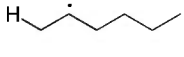
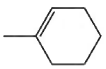
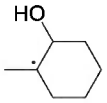
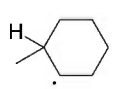
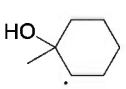
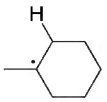
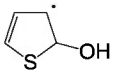
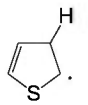
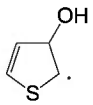
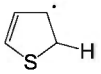
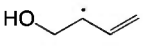
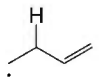
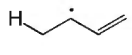
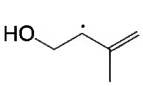
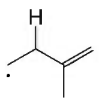
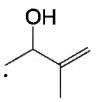
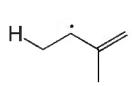
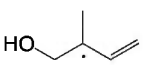
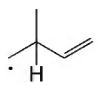
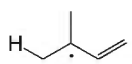
(7)

Fig. 2 shows the time course of the products yield in the photocatalytic hydration of 1-butene in the closed reactor. Although the total production rate of the alcohols and the carbonyl products decreased once after 1 h, it became constant at least until 24 h (Fig. 2b, purple filled squares). It is notable that the carbonyl compounds were produced at a constant rate even in the initial stage of the photocatalytic reaction. If the carbonyl compounds were produced only through the successive oxidation of the alcohols (eqn (7)), the production of the carbonyl compounds should be delayed compared to that of the alcohols, which is inconsistent with the results. Thus, the carbonyl compounds are also produced directly and competitively. In the previous study for the aromatic ring hydroxylation,[11b]

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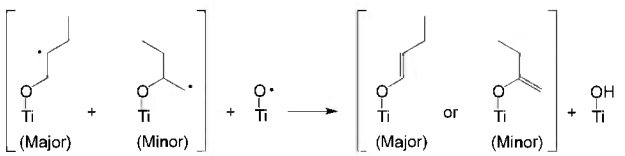
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Table 4 Relationship between anti-Markovnikov selectivity in the photocatalytic hydration and the final heat of formation of the possible radical intermediates[a]

		Final heat of formation/kJ mol^{-1}						
		Anti-Markovnikov type		Markovnikov type		Difference		
Entry	Substrate	•OH adduct (A)	•H adduct (B)	•OH adduct (C)	•H adduct (D)	(A–C)	(B–D)	Anti-Markovnikov selec.[b] (%)
1		 −89.5	 99.0	 −77.1	 71.6	−12.4	27.4	65
2		 −163.0	 25.1	 −143.4	 −1.74	−19.6	26.8	72
3		 −205.9	 −17.2	 −185.8	 −44.4	−20.1	27.2	75
4		 −218.8	 −19.9	 −197.7	 −42.1	−21.1	12.2	81
5		 −38.4	 151.9	 −14.4	 130.2	−24.0	21.7	100
6		 −74.5	 142.4	 −27.7	 86.0	−46.8	56.4	100
7[c]		 −111.2	 102.3	 −60.5	 51.0	−50.7	51.3	100
8		−113.5	 121.8	 −56.4	 45.0	−57.1	76.8	100

[a] Final heat of formation was calculated by PM7 method[16] in MOPAC2012.[17] As for the reaction conditions, see the footnotes in Tables 1 and 2. [b] See the foot note e in Table 2.

it was suggested that the hydrogen atom could be abstracted from the intermediate by a radical species. In a similar way, the carbonyl compounds would be produced through the abstraction of hydrogen from the intermediate species as shown in eqn (8).



(8)

Considering these reaction routes to produce carbonyl compounds (eqn (7) and (8)), the regioselectivity of the carbonyl group would be the same as that of the hydroxyl group in the produced alcohol because of the difference of the stability between the intermediates as mentioned above.

3.5. Effect of water quantity

Fig. 3 shows the influence of water quantity on the photocatalytic hydration of 1-butene as a gaseous reactant, where it was a three phase reaction system (gas–liquid–solid). With increasing the quantity of the liquid water to 10 ml in volume,

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Table 5 Results of photocatalytic reaction tests of hydration products over the Pt(0.1)/TiO_2 sample[a]

		Products/μmol					
Entry	Substrate	HO	O H	OH	O	H_2	CO_2
1	O H	n.d.	4.8	n.d.	n.d.	54	11
2	O	n.d.	n.d.	n.d.	6.8	43	8.2
3	HO	13.0	7.6	n.d.	n.d.	54	4.9
4	OH	n.d.	n.d.	14.2	4.7	51	5.6

[a] Catalyst, 0.2 g; substrate, 25 μmol in the aqueous solution (10 ml); reaction time, 180 min; reaction temperature, 314 K; light intensities were 13, 54, and 148 mW cm^{-2} when measured at 254 ± 10, 365 ± 15, and 405 ± 30 nm, respectively. n.d. = not detected.

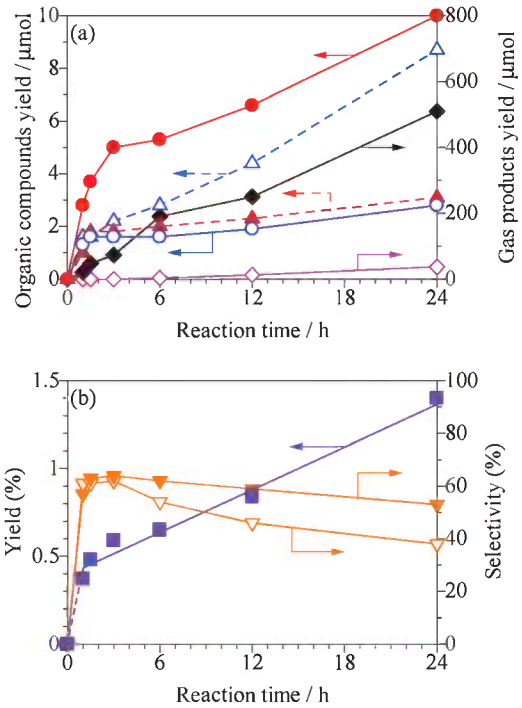


Fig. 2 The time course of the products yield (a), and the total yield and selectivity (b) in the photocatalytic hydration of 1-butene over the Pt(0.1)/TiO_2 sample. The red filled circles, red filled triangles, blue blank circles, blue blank triangles, black filled diamonds and pink blank diamonds in panel a correspond to the yields of 1-butanol, butanal, 2-butanol, 2-butanone, hydrogen and carbon dioxide, respectively. The purple filled squares, orange filled inverse triangles and orange blank inverse triangles in panel b correspond to the total yield of the alcohols and the carbonyl products, the anti-Markovnikov selectivity and the alcohol selectivity, respectively. Catalyst, 0.2 g; 1-butene, 1.8 mmol; water, 10 ml (560 mmol); reaction temperature, 314 K; irradiated light intensities were 13, 54, and 148 mW cm^{-2} when measured at 254 ± 10, 365 ± 15 and 405 ± 30 nm, respectively.

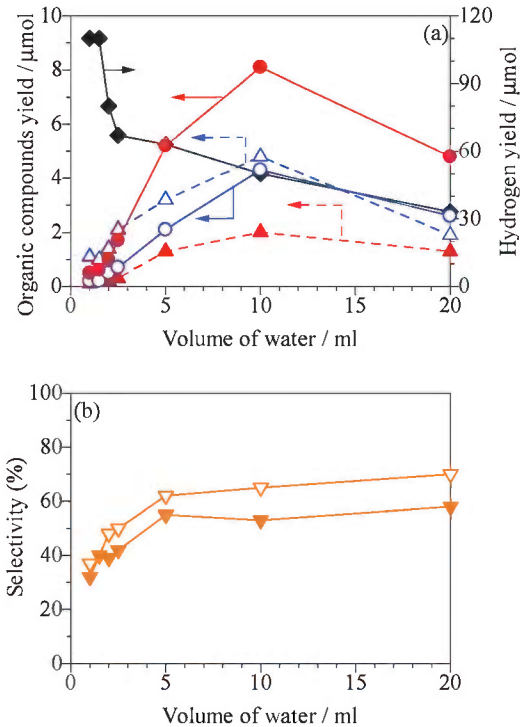


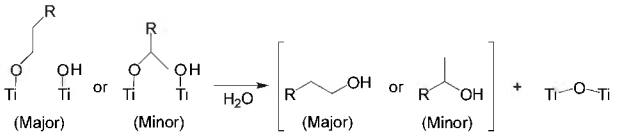
Fig. 3 The influence of water quantity on the photocatalytic hydration of 1-butene over the Pt(0.1)/TiO_2 sample. The red filled circles, red filled triangles, blue blank circles, blue blank triangles and black filled diamonds in panel a correspond to the yields of 1-butanol, butanal, 2-butanol, 2-butanone and hydrogen, respectively. The orange filled inverse triangles and orange blank inverse triangles in panel b correspond to the anti-Markovnikov selectivity and the alcohol selectivity, respectively. Catalyst, 0.2 g; 1-butene, 1.8 mmol; reaction time, 180 min; reaction temperature, 314 K; light intensities were 13, 54, and 148 mW cm^{-2} when measured at 254 ± 10, 365 ± 15 and 405 ± 30 nm, respectively.

the yield of the alcohols and carbonyl products increased and that of excess hydrogen decreased (Fig. 3a). In addition, the alcohol selectivity and the anti-Markovnikov selectivity also increased. When the introduced amount of water was very small (1.0 ml), the color of the catalyst changed, implying that undetectable byproducts (*e.g.*, coupling products) would be produced from the 1-butene and the hydration products. This means that the presence of water would contribute to not only the formation of alcohol and carbonyl products (eqn (9) and (10))

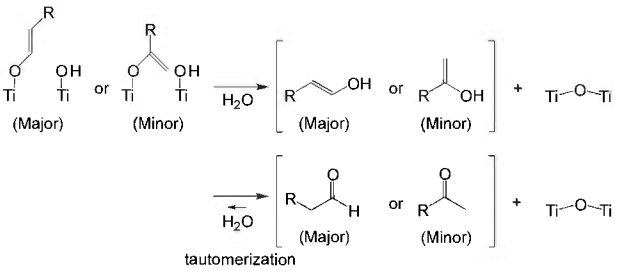
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but also the desorption of the products from the surface as a solvent to prohibit the excess adsorption of the products and side reactions such as successive oxidation and coupling reactions.



(9)



(10)

On the other hand, when the volume of water was larger than 10 ml, the yield of the hydration products decreased although the selectivity was not changed. With an increased volume of water in the closed system, the decreased concentration of 1-butene in the aqueous solution might reduce the reaction rate.

3.6. Proposed reaction mechanism

From the above investigations and our previous study, the proposed reaction mechanism of the photocatalytic hydration of the carbon double bond is summarised in Fig. 4. In the current three phase reaction systems (gas–water–solid or oil–water–solid), the catalyst obviously exists in the aqueous phase. Thus, water molecules are always adsorbed on the titanium oxide surface (Fig. 4, i). The titanium oxide is photoexcited to produce an electron and a hole, and then the electron migrates to the platinum sites and the hole is trapped by the surface bridge oxygen sites of titanium oxide as usually proposed (Fig. 4, ii).[15] The trapped hole induces the nucleophilic attack of the adsorbed water to produce a surface oxygen radical and a surface hydroxyl group. An alkene molecule would interact with the hydrogen of the adsorbed water or the surface hydroxyl group (Fig. 4, iii). The electrophilic surface oxygen radical attacks the double bond to produce a radical intermediate (Fig. 4, iv or iv′). It should be noted that the position selectivity of the surface oxygen radical to the two carbon atoms at the carbon double bond almost follows the anti-Markovnikov rule according to the stability of the radical intermediates (Fig. 4, iv). Finally, the electron in the platinum sites reduces a proton to produce a hydrogen radical, which adds to the intermediate species to produce a surface alkoxide species, and it desorbs as an alcohol (Fig. 4, v and v′), which is promoted by water. In this mechanism, the lattice oxygen atom of the titanium oxide surface is introduced to the product while the oxygen atom in

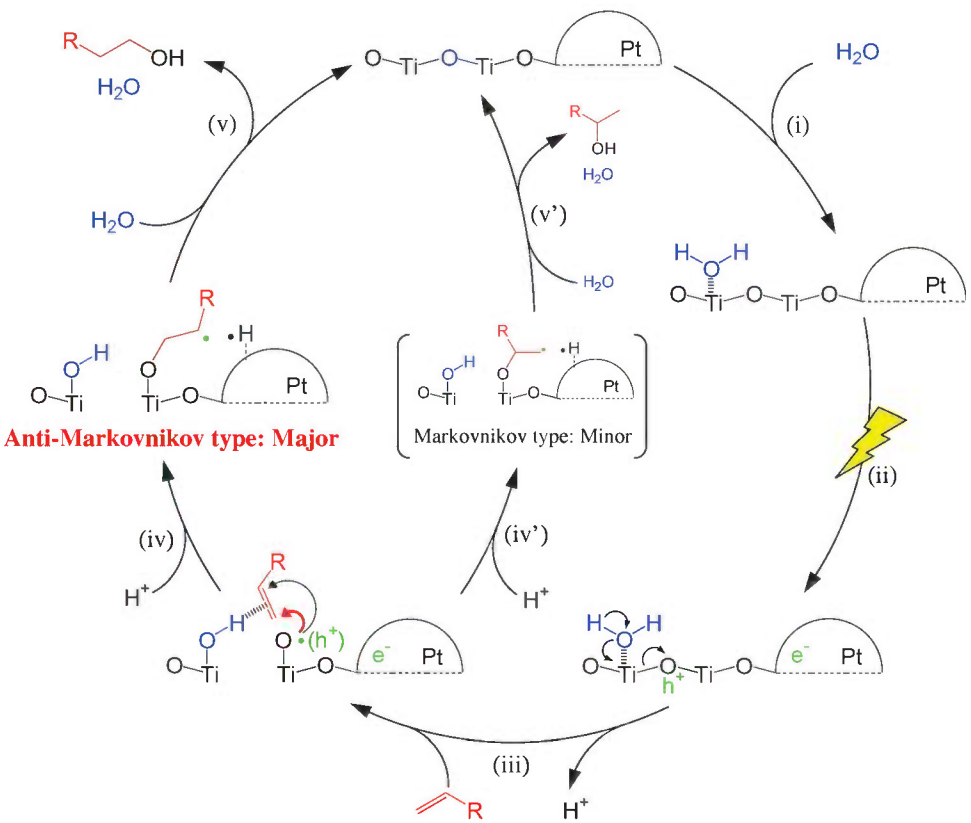


Fig. 4 The proposed reaction mechanism of the photocatalytic hydration of alkene to produce alcohol.

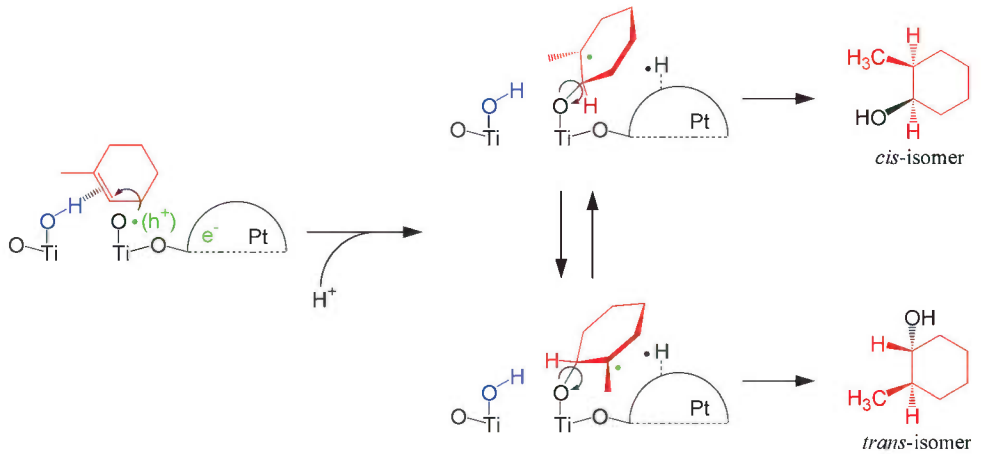


Fig. 5 The proposed mechanism of the photocatalytic hydration of 1-methyl-1-cyclohexene to produce the *cis–trans* isomers of 2-methylcyclohexanol. The above steps correspond to the steps iv and v in Fig. 4.

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the water molecule is introduced to the surface in the first cycle, and then the surface oxygen atoms that originated from the water molecule are introduced into the product in the successive cycles. This is supported by the isotopic experiment with $H_2{}^{18}O$ (Table 3, entry 2) and the previous study.[11b]

In this hydration reaction, anti-Markovnikov selectivity was not influenced by the platinum loading amount (the average size and the number of the platinum nanoparticle, Fig. 1). Since the key step of determining the anti-Markovnikov selectivity (the addition of the surface oxygen radical) would take place on the titanium oxide surface, this step would not be significantly influenced by the platinum loading amount.

From 1-methyl-1-cyclohexene, *cis–trans*-isomers of 2-methylcyclohexanol were produced in the present photocatalytic anti-Markovnikov hydration. The above hydration mechanism and the almost equal yields of *cis–trans* isomers suggest that the intermediate species can freely rotate in the C–O bond and be attacked by the hydrogen radical from both sides as shown in Fig. 5.

A part of the produced alcohol is further oxidised to produce carbonyl compounds probably with the formation of hydrogen (eqn (7)). On the other hand, the direct production of carbonyl compounds would competitively occur. The mechanism is proposed in Fig. S3, (ESI†), in which the steps (iv) and (v) are different from the above. After the formation of the intermediate (Fig. S3, iii, ESI†), titanium oxide is further photoexcited to form a hole and an electron (Fig. S3, iv and iv′, ESI†). The hole abstracts the hydrogen atom from the intermediate to produce an adsorbed enoxy species (Fig. S3, v and v′, ESI†). Finally, the enol is desorbed from the surface, and the tautomerisation occurs to produce a carbonyl compound (Fig. S3, vi and vi′, ESI†) while the electrons in the platinum sites reduce protons to form a hydrogen molecule (Fig. S3, vii, ESI†).

4. Conclusion

We investigated the hydration of various alkenes (from C4 to C7, and thiophene) over the 0.1 wt% platinum-loaded titanium oxide photocatalyst. In all of the investigated alkenes, the hydration products were obtained. Interestingly, the obtained regioselectivity of the hydroxyl group preferentially followed the anti-Markovnikov rule, *i.e.*, the primary alcohols or the less substituted alcohols were predominantly produced.

The surface oxygen participates in the present hydration to be involved in the products. For the anti-Markovnikov hydration, the order of the radical addition is important, *i.e.*, the electrophilic surface oxygen radical, which is produced through the reaction between water and the lattice oxygen of the titanium oxide surface trapping the photoformed hole, should attack the double bond of the alkene to form the radical intermediate before the addition of the hydrogen radical, which is produced from the proton and the photoformed electron on the platinum sites. At that time, the anti-Markovnikov selectivity would be determined by the stability of the radical intermediate, which depends on the nature of substrate.

The carbonyl compounds as byproducts were formed by successive oxidation or by direct photocatalytic oxidation, which also followed the anti-Markovnikov rule, since the reaction mechanism would be similar to the hydration.

The water would also function as a solvent to assist the desorption of the products, resulting in the decrease of the formation of undesired byproducts.

Acknowledgements

We thank Dr Kazunari Takahashi and Dr Hidenobu Kajitani (Mitsubishi Chemical Corporation) for the valuable discussion. This research was financially supported by Mitsubishi Chemical Corporation, Grant-in-Aid for JSPS fellows (22–7799) and the fund for doctoral students in Nagoya University.

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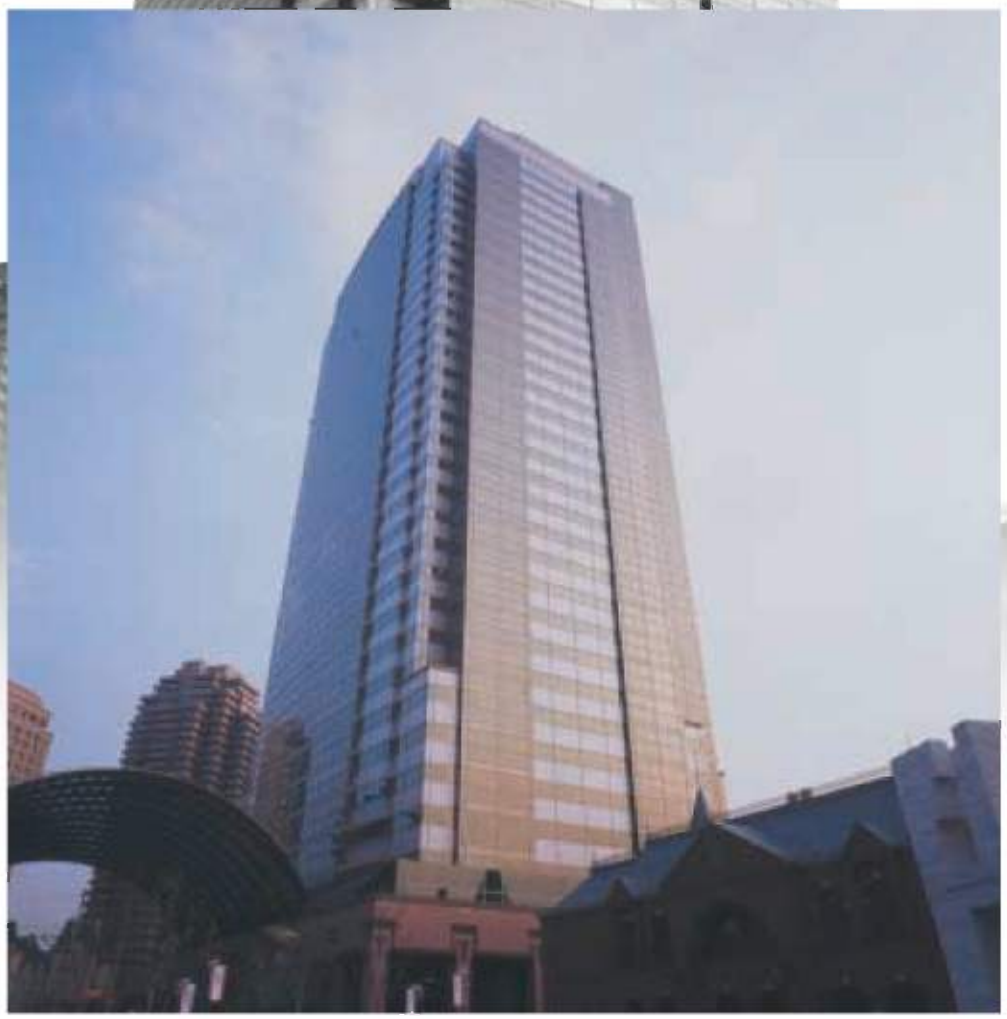
Exhibit 12

CATALOGUE Ver.8

WORLD-WIDE LEADER in DDS

Drug Delivery Systems

PEG DERIVATIVES, PHOSPHOLIPIDS AND DRUG DELIVERY MATERIALS
FOR PHARMACEUTICAL PRODUCTS AND FORMULATIONS



NOF CORPORATION

ASCENDIS EX1074
U.S. Patent No. RE48,267

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NOF CORPORATION: Our introduction

Innovating Drug Delivery Systems

As an integrated chemical manufacturer featuring diverse activities from "Biosphere to Outer Space", NOF CORP. has now focused on the development of innovative drug modifiers, carriers and solubilizers such as PEG, phospholipids, highly purified oleic acid nonionic surfactants, cholesterol pullulan, MPC polymers and chitosan. As a worldwide leader in drug delivery systems (DDS), NOF provides excellent product quality for the pharmaceutical formulations field.

革新的DDSへの挑戦
"バイオから宇宙まで"のキャッチフレーズの基に幅広い分野で社会に貢献する当社が、世界に誇るPEG、リン脂質、高純度オレイン酸系非イオン活性剤、コレステロールプルラン、及びMPCポリマーを駆使し、世界の医薬品関連分野に向けてDDS事業開発部を発足させました。DDSにおける世界のリーダーとして、当社はGMPグレードの優れた商品を提供しています。

PRODUCT OVERVIEW

I. Activated PEGs and PEGylation technology

We produce excellent purity methoxypolyethylene glycols (mPEGs) ranging from 2,000 to 30,000 molecular weight and also activated PEG derivatives including methoxy-PEG amines, maleimides and carboxylic acids. By offering various PEG terminal groups, many customers' drugs can be modified. In addition, drug circulating half-life and biodistribution in the blood stream can be prolonged when our PEG-modified phospholipids are used and injected as liposomal formulations.

分子量2,000から30,000までの極めて高純度のメトキシポリエチレングリコールを製造できるだけでなく、これらの原料を用いたメトキシPEGアミン、マレイミド、カルボン酸などの活性化PEG誘導体も高純度、高品質で製造しています。PEGの末端基を変化させることにより、お客様の医薬品を様々に修飾することができます。また、当社の高純度リン脂質と高純度PEGを融合させたPEG化リン脂質を用いますと、医薬品の血中半減期を延長することができます。

II. Phospholipids and Liposome formulation technology

We have successfully supplied our quality GMP-grade phospholipids worldwide for drug products for decades with solid reliability and outstanding reputation. We not only develop new types of phospholipids, but also formulate lyophilized liposomes, so-called EMPTY LIPOSOMES, with which customers readily encapsulate their drugs with their methods.
Our LIPONIZER liposome production systems allow reliable batch processing of customer formulations into liposomes. (LIPONIZER is registered mark of Nomura Micro Science Co., Ltd.)

1990年代初期からGMPグレードのリン脂質を世界に向けて供給し、市販のリポソーム医薬品に用いられ高い評価を得ています。また、顧客サイドで、その医薬品を容易にリポソーム化できる中空リポソームを開発しました。リポナイザー®を使用すればバッチ毎のバラツキの無いリポソームが作製出来ます。

III. High-purity Oleic Acid Derivatives

Hiht-purity Oleic Acid Derivatives (NOFABLE® Series) derived from highly purified oleic acid have been developed so that customers can dissolve and formulate their sensitive drugs while suppressing degradation due to oxidation.

独自の高純度に精製したオレイン酸を用いて、誘導体の非イオン活性剤（NOFABLEシリーズ）を開発しました。酸化に由来する不純物を除去していますので、医薬品の分解や変性などが防げます。さらに、NOFABLEシリーズを用いてワクチン用のオイルアジュバントも開発しています。

IV. Low molecular weight MACROGOL (PEG200, 300, and 400)

NOF has developed high quality polyethylene glycols specific for pharmaceutical excipient use. Our SUNBRIGHT® DKH series is specifically synthesized and purified to reduce ethylene glycol and diethylene glycol contaminants found in competitors' products.

当社は、EG(エチレングリコール)およびDEG(ジエチレングリコール)の極めて少ない医薬品添加剤用ポリエチレングリコールを開発しました。

V. Cholesterol pullulan

Pullulan partially modified with cholesterol have been developed to enable fabrication of highly purified nanoparticles by dispersing into water. Antigens, peptides and proteins can be encapsulated into nanoparticles, and these conjugates can activate the immune system as adjuvant-like excipients in vaccine or immuno-stimulating products.

多糖のプルランをコレステロールで修飾し部分的に疎水化させた後、水に均一に分散させることにより高純度のナノ粒子を作製する技術を開発しました。抗原やタンパク質をナノ粒子中に包埋でき、得られた複合体は生体の免疫細胞を活性化させます。

VI. MPC polymers and solubilizers for poorly water-soluble drugs

Unique water-soluble phosphorylcholine-containing MPC polymers in which lipid-like phosphorylcholine groups copolymerized with alkyl hydrophobic units have been developed. Using our MPC polymers, poorly water-soluble drugs can be dissolved in aqueous media.

親水性のホスホリルコリン基を疎水系のメタクリル酸ポリマーに導入することにより、水溶性のMPCポリマーを開発しました。このポリマーを使用して、難水溶性薬物を可溶化することができます。

VII. Hydrophobic drug solubilization kits

We have developed new solubilization kits for poorly water-soluble drugs using our proprietary sophisticated drug delivery technologies. Drug solubilizing procedures are easy so that the kit is suitable for NCE screening or toxicological testing.

種々のDDS技術を結集し、難水溶性薬物の可溶化キットを開発しました。キットの使用方法は極めて簡単で、安全性試験、新規開発薬剤の初期テストのスクリーニングを容易に行うことができます。

VIII. Chitosan with low endotoxin contamination

Highly purified chitosan is now introduced for gene delivery or hydrogel use. Using sophisticated purification methods, endotoxin-free chitosan (less than 100EU/g) is available for the DDS field.

高純度のキトサンを遺伝子導入用およびハイドロジェル用途にご紹介いたします。独自の精製技術によって開発されたエンドトキシンフリーのキトサンはDDS分野に安心して御使用いただけます。

IX. Biocompatible anchors for membrane incorporation (BAM)

We have developed our BAM concept for modifying cell membranes. Using BAM, cell or liposomal membranes can be modified with proteins or drugs without causing damage. Our new BAM technology allows live cell immobilization on surfaces of various kinds of materials.

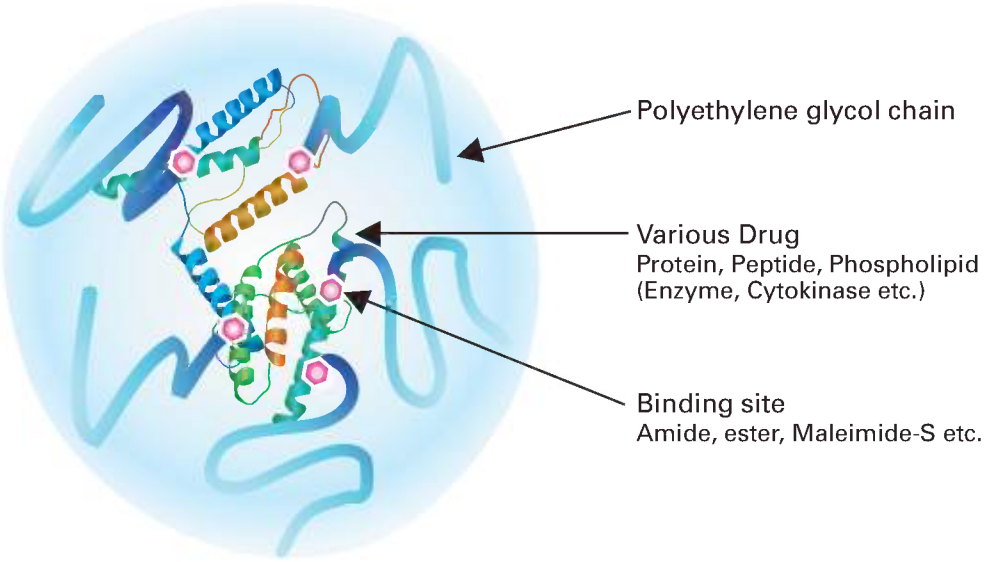
細胞にダメージを与えない細胞修飾剤を開発しました。BAM を用いることにより細胞や組織にダメージを与えることなく細胞や組織の表面を蛋白質や薬剤などの生理活性物質で修飾することや細胞や組織を生きたまま各種材料表面に固定化することができます。

Using these high-quality products, NOF CORPORATION provides custom formulation expertise and assistance on request.

I. High Quality PEGs and Activated PEGs <SUNBRIGHT® Series>

NOF has developed the following superior quality activated PEG (SUNBRIGHT Series) well-suited for preparing pharmaceuticals and biomedical products. Our SUNBRIGHT Series comprises extraordinarily purified terminally functional polyalkylene glycols, the most appropriate and versatile DDS formulation materials for PEG-modified drugs.

医薬品および蛋白質などの生理活性物質の調製に最適な高品質のポリエチレングリコール(PEG)修飾剤ーSUNBRIGHT Seriesーを開発しました。SUNBRIGHTシリーズは、極めて高純度で多様な官能基をもつPEGであり、薬剤のPEG修飾に最も適したDDS基剤です。

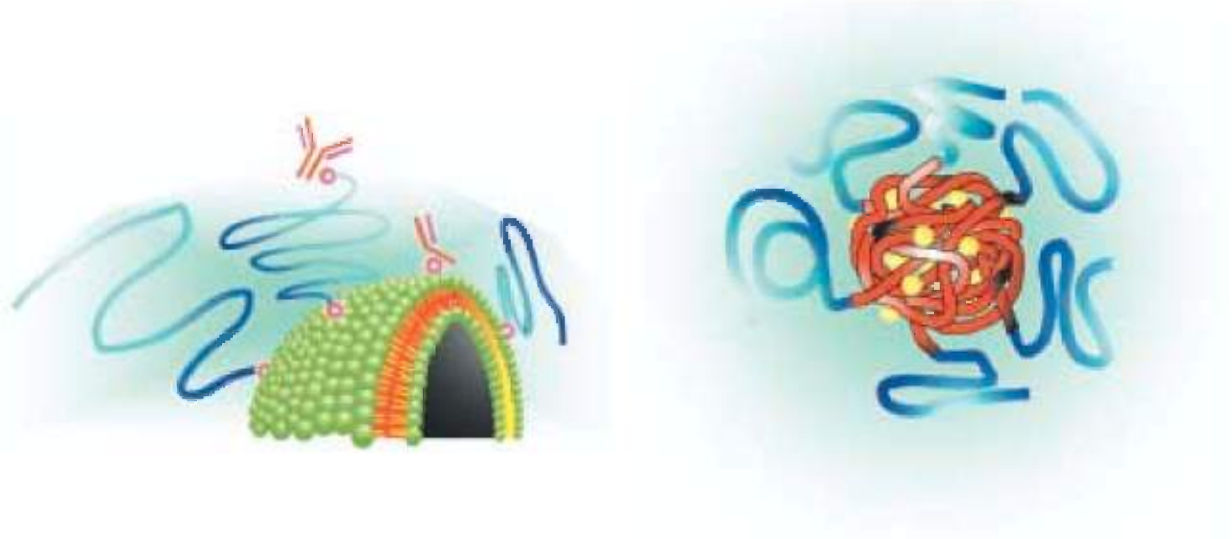


Drugs, enzymes, phospholipids and other biomaterials can be covalently conjugated with our activated PEGs by various terminal coupling groups as shown above, facilitating versatile drug formulation.

この図に示しますように、末端に種々の官能基をもつPEG修飾剤を用いることにより、薬剤、酵素、リン脂質、その他生体物質にPEG鎖を導入することが可能です。また疎水性の高分子等とPEG修飾剤を結合させることにより、高分子ミセル体を形成し、疎水性薬剤等を水溶液中に安定に分散させることができます。さらに、リン脂質と結合させたリン脂質ーPEGをリポソームに用いることにより、リポソーム表面に水和層を付加することができ、薬剤等を内包したリポソーム製剤を安定に分散させることができます。

Our activated PEGs in the SUNBRIGHT series can be used for tethering to or masking colloidal or vesicle surfaces for targeting (left) or in polymeric self-assembled micellar vesicles as a hydrophilic corona right).

以上のように、PEG修飾をすることにより、薬剤等の安定化、抗原性の低下、投与量の低減等を図ることができ、さらに抗体等を表面に結合させることによりターゲッティング性を高めることができます。



Summary of NOF PEG Products

Product Classification	Description
1. High-purity single-arm PEGs	High-purity Methoxy-PEG in the SUNBRIGHT® MEH Series contains the lowest levels of diol PEG, with the narrowest polydispersity in the world. 不純物がなくかつ分子量分布もシャープなＳＵＮＢＲＩＧＨＴ　ＭＥＨシリーズの高純度MethoxyPEGはジオールPEG含量が極めて少なく、分子量分布もシャープです。
2. High-purity multi-arm PEGs	We manufacture a variety of multi-arm PEG derivatives (2-arm, 3-arm, 4-arm and 8-arm PEGs), which are used as raw materials of multi-arm activated PEGs. 当社は、2-アーム、3-アーム、4-アーム、8-アームなど種々のマルチアーム PEG誘導体製品を製造致しております。
3. High-purity activated PEGs	
3-1 High-purity Monofunctional activated PEGs	Mono-functional SUNBRIGHT Series contains very low levels of bi-functional PEG impurities, so that crosslinking of drugs during PEG modification is completely suppressed. SUNBRIGHTシリーズの一官能活性化タイプは、高純度のメトキシ PEGを原料として用いていますので不純物であるジオール由来の2官能PEGをほとんど含んでいません。その為PEG修飾時に問題となる薬物の架橋反応を防止できます。
3-2 High-purity multi-arm activated PEGs	We provide next-generation multi-arm (2,3,4 and 8 arm) PEG derivatives with terminals activated by various functional groups. 当社は、上記に示しました2-アーム、3-アーム、4-アーム、8-アームなど種々のマルチアームPEGの末端に種々の官能性基を付与した誘導体製品を供給致しております。multi-arm PEGは、ハイドロゲルの原料だけでなく、難溶性薬剤に応用することにより溶解性の向上が計れます。
4. Branched activated PEGs	Our BRANCHED PEG is a cutting-edge reagent for your new sustained-release PEGylated drugs. In addition, NOF has many patents and know-how for utilizing our new BRANCHED PEGs for your applications. 新しい2鎖型及び3鎖型PEG誘導体の開発をおこないました。末端官能基がマレイミド基、アルデヒド基、アミノ基、NHS活性化のものにつきまして供給をいたします。
5. Heterofunctional activated PEGs	Our new HETERO-FUNCTIONAL PEG DERIVATIVES have different functional groups introduced to each PEG terminal. These include reactive esters(NHS), aldehydes, hydroxy, carboxylic acids. 当社は、両末端に異なる反応性基を付与したヘテロ活性化PEG誘導体を供給いたします。
6. Comb-shaped PEG polymers (carboxylic anhydride type)	These alternating copolymers introduce many reactive anhydride groups and PEG chains into a single polymeric molecule (Comb-shaped polymers). このシリーズは、酸無水物基とポリアルキレングリコール鎖をもつ櫛形構造の新しいタイプのポリマーです。

1. High-purity single-arm PEGs

NOF manufactures various molecular weights of high-purity mono-functionalized methoxy-PEG (mPEG) with narrow polydispersity. These high-purity mPEGs have already been commercialized into known PEG drugs such as PEG-INTERFERON and PEG-GCSF, and other PEG-DRUGs.

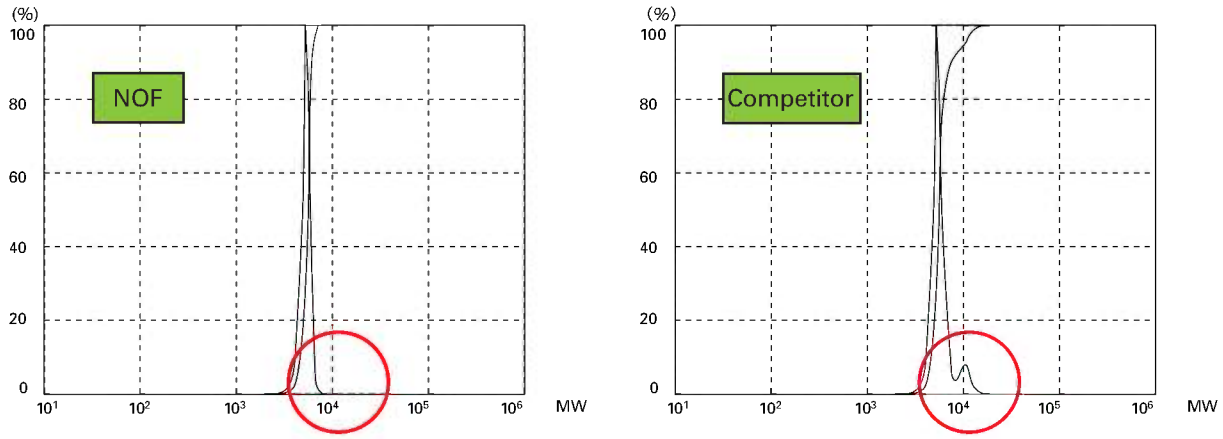
不純物がなくかつ分子量分布もシャープな高純度メトキシPEGを製造しています。これらの高純度メトキシPEGは、これまでにPEG-インターフェロンPEG-GCSFなどの種々のPEG修飾医薬品に使われています。

Methoxy-polyethyleneglycol with low-diol content & narrow polydispersity

$$CH_3O(CH_2CH_2O)_n\text{-}H$$

NOF's mPEGs have the lowest levels of contaminating diol content in the world. HPLC data in Figure 1 clearly show our superiority in comparison to other mPEG-5000 products. Importantly, the polydispersity of our mPEGs is also the narrowest in the world. Our other activated PEG derivatives produced using our SUNBRIGHT MEH Series achieve excellent drug modification with high-purity and high-activity. NOF manufactures these exclusive high-purity mPEGs with molecular weights from 2,000 to 30,000.

メトキシPEG、SUNBRIGHT® MEHシリーズは、他社品に比べてジオール含量が極めて少ないことが特徴です。下図に示しますように、当社のメトキシPEG-5000は、他社品に比べて不純物であるジオール含量が極めて少なく、分子量分布がシャープであることが特徴です。当社のすべてのPEG誘導体は、このMEHシリーズを原料PEGとして用いていますので、修飾剤として高純度で高活性を示します。当社は、分子量2000から30000までの高純度メトキシPEGを提供しています。



Comparisons of GPC chart of mPEG (MW5,000)
between NOF product and competitor's.

NOF CORPORATION is the sole worldwide patent holder of these high-purity mPEGs all their derivatives.

高純度メトキシポリエチエレングリコール及びその誘導体につきましては、各国での特許を取得しております。
※Patent No.：JP3050228, U.S.Patent 6455639, KR0358276

2. High-purity Multi-Arm PEGs (2-arm, 3-arm, 4-arm and 8-arm type PEGs)

We manufacture a variety of multi-arm PEG derivatives (2-arm, 3-arm, 4-arm and 8-arm PEGs). Per our tradition, these derivatives are supplied with low impurities and narrow polydispersity.

当社は、2-アーム、3-アーム、4-アーム、8-アームタイプの種々のマルチアームPEG誘導体製品を製造致しております。これらのマルチアームPEGは、マルチアーム活性化PEG誘導体の原料として使用されます。

2-arm type

DKH Series
(Polyethylene glycol)

$H\text{-}(OCH_2CH_2)_n\text{-}OH$

4-arm type

PTE Series
(Pentaerythritol, tetra-polyethylene glycol ether)

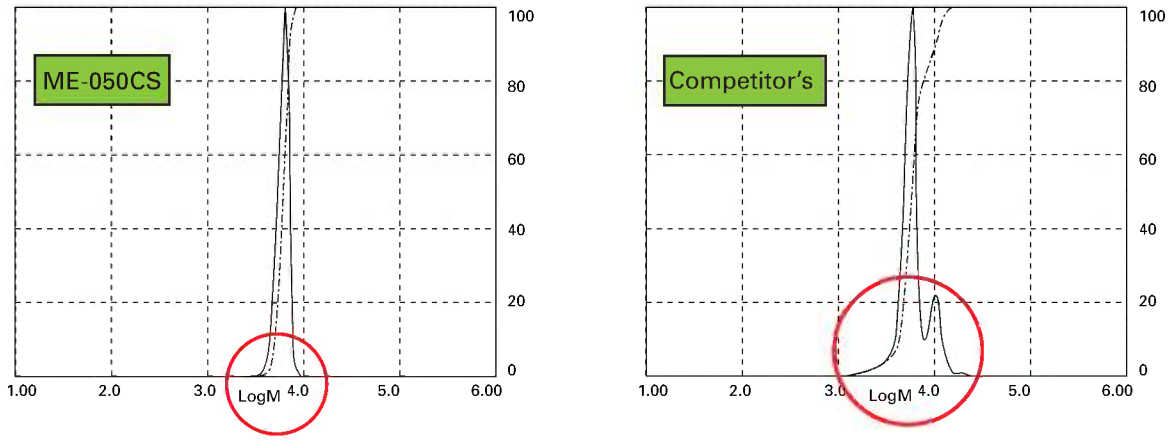
8-arm type

HGEO Series
(Hexa-glycerine, octa-polyethylene glycol ether)

3. High-purity functional Acivated PEGs

Our mono-functional SUNBRIGHT® Series contains very low levels of bi-functional PEG impurities, so that undesirable crosslinking of drugs during PEG modification is completely suppressed. Also, NOF can adjust the molecular weight and supply multi-functional PEG derivatives (bi-functional, 3-functional, 4-functional, 8-functional and hetero-functional) according to customer needs. NOF plans and produces these derivatives from carefully selected raw materials. Moreover, we can make other functional or custom polyalkylene glycol derivatives such as PPG, PBG, PPG-PEG block or random polymers for your new drug modifications.

また当社はPEG誘導体をベースから合成設計していますので、ご要望に応じて、分子量の変更や多官能性タイプ、ヘテロタイプ等の合成など種々の対応をすることができます。さらにその他PPG(PolyPropylene Glycol)、PBG(PolyButylene Glycol)、PPG-PEGブロック／ランダムポリマー等の他のポリアルキレングリコール誘導体を用いた活性化ポリアルキレングリコールも製造することが可能です。



GPC chromatograms comparing functional PEG derivative purity, showing NOF superior quality (NHS-activated carboxyl-PEGs: M-PEG-X-NHS)

Application Chart for PEG Derivatives

当社は種々の官能基を持つPEG誘導体を取り揃えております。

Product name	Reactive group	Target groups
CS series	-CO-CH_2CH_2-COO-**NHS**	-NH_2,-OH,-SH
GS series	-CO-$CH_2CH_2CH_2$-COO-**NHS**	-NH_2,-OH,-SH
AS series	-CH_2-COO-**NHS**	-NH_2,-OH,-SH
HS series	-$CH_2CH_2CH_2CH_2CH_2$-COO-**NHS**	-NH_2,-OH,-SH
NP series	-CO_2-phenyl-NO_2	-NH_2
AL series	-CH_2CH_2-CHO	-NH_2
PA series	-$CH_2CH_2CH_2NH_2$	-COOH
DE series	-$CH_2CH_2CH(OC_2H_5)_2$	-NH_2
SH series	-CH_2CH_2SH	-SH,-Maleimide,-COOH
MA series	-$CH_2CH_2CH_2NHCOCH_2CH_2$-**Maleimide**	-SH

Coupling of PEG derivatives to Proteins: example conditions

上記の種々の官能基を持つPEG誘導体を用いた蛋白修飾の代表例を以下に示します。

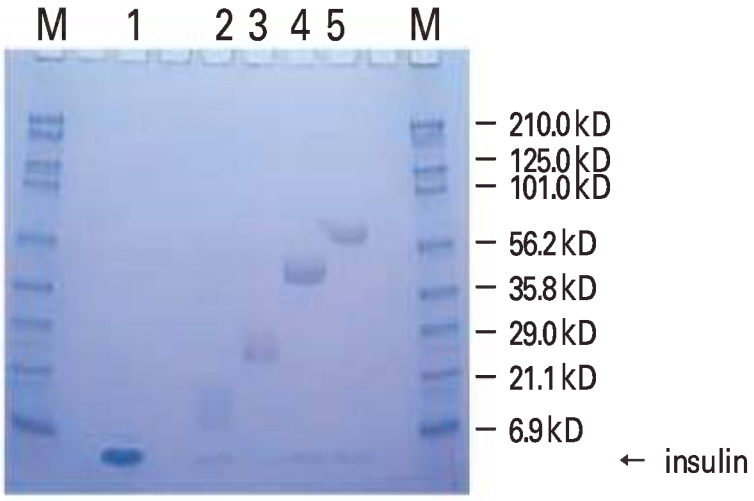
1.Coupling of PEG-NHS derivatives to protein amines (PEG-NHS+ Protein-NH2)
Condition 1: 50mM Phosphate buffer (pH7.2), 4C, 6hrs[*1] Condition 2: Borate-phosphate buffer (pH8.0), 25C, 2hrs[*2]
2. Coupling of PEG-Aldehyde derivatives to protein amines (PEG-Ald+ Protein-NH2)
Condition : Sodium cyanoborohydride (10eq.), 4C, 20hrs[*3]
3.Coupling of PEG-Maleimide derivatives to protein cysteine (PEG-Maleimide+ Protein-SH)
Condition : 100mM Phosphate buffer (pH6.5), 4C, 4hrs
4. Coupling of PEG-NH2 derivatives to protein carboxylates (PEG-NH2+ Protein-COOH)
Condition : 50mM Phosphate buffer (pH7.2),WSC(2eq), 4C, 10hrs
5. Coupling of PEG-p-Nitrophenylcarbonate derivatives to protein amines (PEG-NP+ Protein-NH2)
Condition : Borate-phosphate buffer (pH8.0-8.3), r.t, overnight[*2]

References:

[*1] Abuchowski, A., et al. (1984) *Cancer Biochem. Biophys.* **7**,175
[*2] Sartore, L., et al. (1991) *Appl.Biochem.Biotechnol.* **27**,45
[*3] U.S.Patent 5,824,784

PEG Conjugation Capabilities

PEG conjugation is known to help enhance drug performance by optimizing pharmacokinetics, increasing bioavailability, decreasing immunogenicity and decreasing administration frequency with stable PEG linkages. In addition, NOF can provide various linkage chemistries in our SUNBRIGHT Series to reliably link with your chosen candidate drug molecule site-specifically. Figure 2 shows reliable clean PEG-aldehyde conjugation with human insulin across several PEG molecular weights.



Analysis of PEG-ALD-insulin by SDS-PAGE with Coomassie brilliant blue.

Insulin was reacted with NOF PEG-ALD at 4℃. The PEGylated insulin was purified on Q Sepharose Fast Flow column.

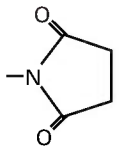
lane M	molecular weight markers	lane 3	PEG(ME-100AL)-insulin eluate
lane 1	insulin	lane 4	PEG(ME-200AL)-insulin eluate
lane 2	PEG(ME-050AL)-insulin eluate	lane 5	PEG(ME-300AL)-insulin eluate

3-1. High-purity Monofunctional Activated PEGs

1) SUNBRIGHT® CS, GC, AS, and HS Series (Carboxylated-mPEGs; NHS active esters)

NOF provides four types of carboxyl group linkages in PEG-(X)-NHS. Customers can choose appropriate products according to their needs and the desired stability of the PEGylated drug.

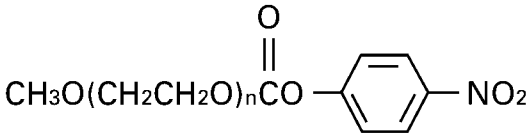
カルボキシル基の結合形式(x)が異なる4タイプのNHS活性化エステル-PEGをそろえています。従って目的に応じて4タイプの中から適切なものをお選び頂けます。

$CH_3O(CH_2CH_2O)_n$-X-*NHS*　　NHS: 

Product name	X		MW
SUNBRIGHT ME-020CS	succinate	-CO-CH_2CH_2-COO-	2,000
SUNBRIGHT ME-050CS			5,000
SUNBRIGHT ME-120CS			12,000
SUNBRIGHT ME-200CS			20,000
SUNBRIGHT MEGC-20HS	glutarate	-CO-$CH_2CH_2CH_2$-COO-	2,000
SUNBRIGHT MEGC-50HS			5,000
SUNBRIGHT MEGC-10TS			10,000
SUNBRIGHT MEGC-20TS			20,000
SUNBRIGHT MEGC-30TS			30,000
SUNBRIGHT ME-020AS	carboxymethyl	-CH_2-COO-	2,000
SUNBRIGHT ME-050AS			5,000
SUNBRIGHT ME-050HS	carboxypentyl	-$(CH_2)_5$-COO-	5,000
SUNBRIGHT ME-100HS			10,000
SUNBRIGHT ME-200HS			20,000

SUNBRIGHT®

2) SUNBRIGHT® NP Series (p-Nitrophenyl-PEGs)



Product name	MW
SUNBRIGHT MENP-20H	2,000
SUNBRIGHT MENP-50H	5,000
SUNBRIGHT MENP-10T	10,000
SUNBRIGHT MENP-20T	20,000
SUNBRIGHT MENP-30T	30,000

3) SUNBRIGHT® AL Series (Aldehyde-PEGs)

NOF owns rights to state-of-the-art synthetic methods for making amine-reactive mPEG-aldehydes that avoid the formation of common bifunctional impurities.

当社は最新の技術を駆使し不純物である2官能性-PEGを含まないM-PEG-アルデヒドを製造しています。

$$CH_3O(CH_2CH_2O)_n\text{-}CH_2CH_2CHO$$

Product name	MW
SUNBRIGHT ME-050AL	5,000
SUNBRIGHT ME-100AL	10,000
SUNBRIGHT ME-200AL	20,000
SUNBRIGHT ME-300AL	30,000

4) SUNBRIGHT® PA Series (Amino-PEGs)

NOF offers propylamine-functionalized amino-terminal PEGs.

当社はプロピルアミンタイプのアミノ-PEGを提供しています。

$$CH_3O(CH_2CH_2O)_n\text{-}CH_2CH_2CH_2NH_2$$

Product name	MW
SUNBRIGHT MEPA-20H	2,000
SUNBRIGHT MEPA-50H	5,000
SUNBRIGHT MEPA-12T	12,000
SUNBRIGHT MEPA-20T	20,000
SUNBRIGHT MEPA-30T	30,000

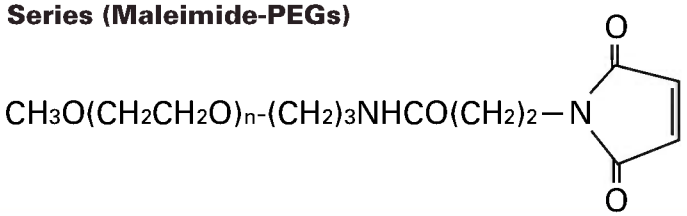
SUNBRIGHT®

5) SUNBRIGHT® SH Series (Thiol-PEGs)

$$CH_3O(CH_2CH_2O)_n\text{-}CH_2CH_2SH$$

Product name	MW
SUNBRIGHT ME-020SH	2,000
SUNBRIGHT ME-050SH	5,000
SUNBRIGHT ME-100SH	10,000
SUNBRIGHT ME-200SH	20,000
SUNBRIGHT ME-300SH	30,000

6) SUNBRIGHT® MA Series (Maleimide-PEGs)



Product name	MW
SUNBRIGHT ME-020MA	2,000
SUNBRIGHT ME-050MA	5,000
SUNBRIGHT ME-120MA	12,000
SUNBRIGHT ME-200MA	20,000
SUNBRIGHT ME-300MA	30,000

3-2. High-purity Multi-Arm Activated PEGs

2-arm type **Bi-Functional PEG Series**

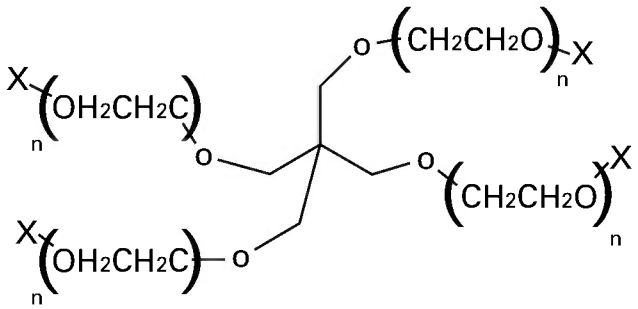
$$X\text{-}(OCH_2CH_2)_n\text{-}X$$

Product name	X	Arms	MW
SUNBRIGHT DE-034AS*	Succinimidylcarboxymethyl -CH_2-COO-**NHS**	2	3,400
SUNBRIGHT DE-030CS	Succinimidylsuccinate -CO-CH_2CH_2-COO-**NHS**		3,000
SUNBRIGHT DE-050CS			5,000
SUNBRIGHT DE-034GS*	Succinimidylglutarate -CO-$CH_2CH_2CH_2$COO-**NHS**		3,400
SUNBRIGHT DE-050GS			5,000
SUNBRIGHT DE-100GS			10,000
SUNBRIGHT DE-200GS			20,000
SUNBRIGHT DE-034HS*	Succinimidylcarboxypentyl -CH_2CH_2-$CH_2CH_2CH_2$-COO-**NHS**		3,400
SUNBRIGHT DE-050HS			5,000
SUNBRIGHT DE-100HS			10,000
SUNBRIGHT DE-200HS			20,000
SUNBRIGHT DE-100MA	Maleimide -NHCO-CH_2CH_2-**Maleimide**		10,000
SUNBRIGHT DE-200MA			20,000
SUNBRIGHT DE-010PA	Propylamine -$CH_2CH_2CH_2$**NH_2**		1,000
SUNBRIGHT DE-020PA			2,000
SUNBRIGHT DE-034PA			3,400
SUNBRIGHT DE-050PA			5,000
SUNBRIGHT DE-100PA			10,000
SUNBRIGHT DE-200PA			20,000
SUNBRIGHT DE-300PA			30,000
SUNBRIGHT DE-050SH*	Ethanthiol -CH_2CH_2**SH**		5,000
SUNBRIGHT DE-100SH*			10,000
SUNBRIGHT DE-200SH*			20,000

SUNBRIGHT® * : custom synthesis

4-arm type **4-arm-Functional PEG Series**

PTE Series (Pentaerythritol, tetra-polyethylene glycol ether)



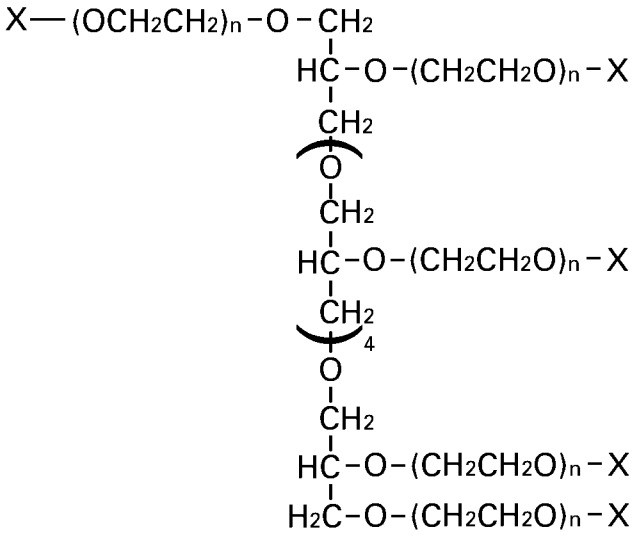
Product name	X	Arms	MW
SUNBRIGHT PTE-100CS	Succinimidylsuccinate -CO-CH_2CH_2-COO-**NHS**	4	10,000
SUNBRIGHT PTE-100GS	Succinimidylglutarate -CO-$CH_2CH_2CH_2$-COO-**NHS**		10,000
SUNBRIGHT PTE-200GS			20,000
SUNBRIGHT PTE-100HS	Succinimidylcarboxypentyl -$CH_2CH_2CH_2CH_2CH_2$-COO-**NHS**		10,000
SUNBRIGHT PTE-200HS			20,000
SUNBRIGHT PTE-100NP	p-Nitophenylcarbonate -CO_2-phenyl-**NO_2**		10,000
SUNBRIGHT PTE-200NP			20,000
SUNBRIGHT PTE-200MA*	Maleimide -NHCO-CH_2CH_2-**Maleimide**		20,000
SUNBRIGHT PTE-100PA	Propylamine -$CH_2CH_2CH_2$**NH_2**		10,000
SUNBRIGHT PTE-200PA			20,000
SUNBRIGHT PTE-100SH	Ethanethiol -CH_2CH_2**SH**		10,000
SUNBRIGHT PTE-200SH			20,000

SUNBRIGHT® * : custom synthesis

8-arm type

8-arm-Functional PEG Series

HGEO Series (Hexa-glycerine, octa-polyethylene glycol ether)



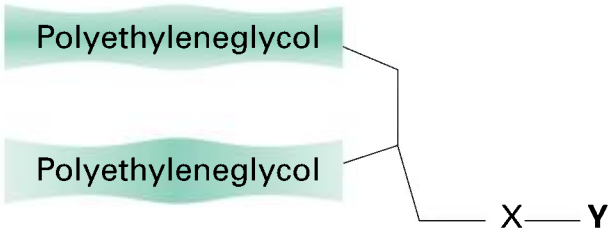
Product name	X	Arms	MW
SUNBRIGHT HGEO-200GS*	Succinimidylglutarate -CO-$CH_2CH_2CH_2$-COO-**NHS**	8	20,000
SUNBRIGHT HGEO-200NP*	p-Nitophenylcarbonate -CO_2-phenyl-**NO_2**		20,000

* : custom synthesis

4. Branched Activated PEGs

NOF has developed novel branched PEG products for sustained-release PEGylated drugs.
NOF offers various types of functional group, such as aldehyde, maleimide, carbonate, or carboxylate activated esters (Y), for activated branched PEGs. A range og spacers (X) for each functional group is also available. NOF's branched PEGs are our original and patented products.

当社は、下記に示しましたように新しい2鎖型PEG誘導体の開発をおこないました。それぞれのPEG鎖の分子量または官能基までのリンカー部分を調節することが出来ます。末端官能基としては、アミノ基、アルデヒド基、マレイミド基、カーボネート基、活性エステル化カルボキシル基を揃えております



X: Spacer
Y: Functional group

Product name	Functional end Y	MW
SUNBRIGHT GL2-200MA	Maleimide	20,000
SUNBRIGHT GL2-400MA		40,000
SUNBRIGHT GL2-200PA	Amine	20,000
SUNBRIGHT GL2-400PA		40,000
SUNBRIGHT GL2-200GS2	NHS-glutaryl	20,000
SUNBRIGHT GL2-400GS2		40,000
SUNBRIGHT GL2-200HS	NHS-carboxymethyl	20,000
SUNBRIGHT GL2-400HS		40,000
SUNBRIGHT GL2-200NP*	Carbonate	20,000
SUNBRIGHT GL2-400NP*		40,000
SUNBRIGHT GL3-400AL2	Aldehyde	40,000
SUNBRIGHT GL3-400MA*	Maleimide	40,000
SUNBRIGHT GL3-400PA*	Amine	40,000

SUNBRIGHT® * : custom synthesis

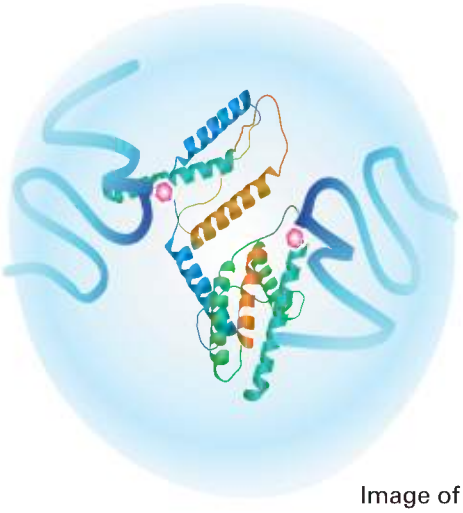


Image of Branched PEG-drug

Branched PEG modification offers ability to custom modify bioactive agents and surfaces site-selectively with dual PEG chains per site as depicted above.

5. Heterofunctional PEGs

1) Hydroxy-PEG-Amine

$$HO(CH_2CH_2O)_n\text{-}CH_2CH_2CH_2NH_2$$

Product name	MW
SUNBRIGHT HO-020PA	2,000
SUNBRIGHT HO-034PA	3,400
SUNBRIGHT HO-050PA	5,000

2) Amino-PEG-Carboxylic acid

$$HCl \cdot H_2N\text{-}CH_2CH_2CH_2O(CH_2CH_2O)_n\text{-}(CH_2)_5COOH$$

Product name	MW
SUNBRIGHT PA-020HC	2,000
SUNBRIGHT PA-034HC	3,400
SUNBRIGHT PA-050HC	5,000

3) Boc-protected-Amino-PEG-Carboxyl-NHS

$$Boc\text{-}NHCH_2CH_2CH_2O(CH_2CH_2O)n\text{-}(CH_2)_5COO\text{-}NHS$$

Boc = tert-butoxycarbonyl

Product name	MW
SUNBRIGHT BO-100HS*	10,000

* : custom synthesis

4) Maleimide-PEG-Carboxyl-NHS

$$Maleimide\text{-}(CH_2)_3CONHCH_2CH_2CH_2O(CH_2CH_2O)n\text{-}(CH_2)_5COO\text{-}NHS$$

Product name	MW
SUNBRIGHT MA-050HS*	5,000

* : custom synthesis

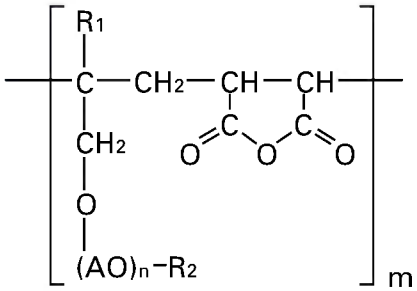
5) Hydroxy-PEG-Aldehyde

$$HO(CH_2CH_2O)_n\text{-}CH_2CH_2CHO$$

Product name	MW
SUNBRIGHT HO-050AL	5,000
SUNBRIGHT HO-100AL	10,000
SUNBRIGHT HO-200AL	20,000
SUNBRIGHT HO-300AL	30,000

6. Comb-shaped Co-polymers (carboxylic anhydride type)

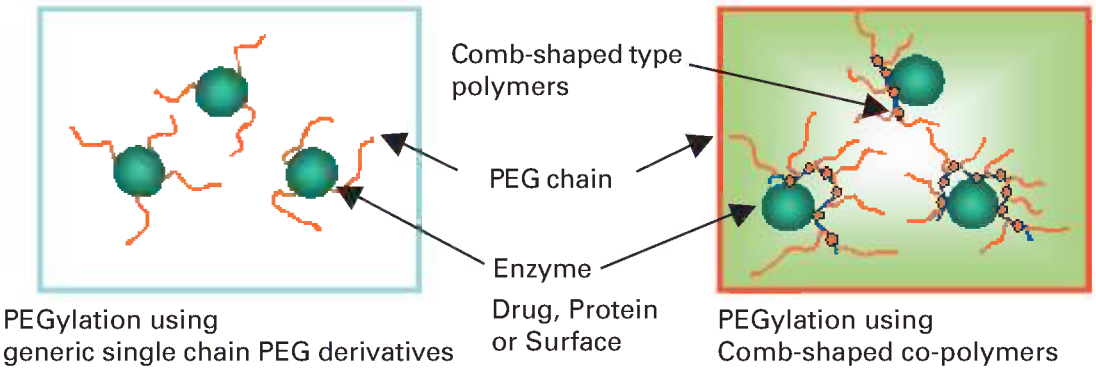
These alternating copolymers are developed for introducing many reactive anhydride groups and PEG chains into a single polymeric molecule (comb-shaped polymers) as shown below.



R_1 : H or CH_3
R_2 : CH_3 or other alkyl group
AO : alkylene oxide group

When this polymer reacts with proteins and enzymes, many PEG chains can be introduced to the biomolecule or drug as shown below.

このポリマーを酵素の修飾に用いますと、一本鎖のPEG修飾剤を用いた場合に比べて、少ない結合で酵素表面により多くのPEG鎖を導入することができます。その結果、修飾酵素の残存活性が高くなります。



Moreover, polymer hydrophilic/lipophilic balance (HLB) can be controlled by balancing propylene oxide and ethylene oxide ratios. Selecting appropriate polymers, customer modified proteins, enzymes or drugs can be dissolved and stabilized in organic solvents.

さらにこの化合物は、骨格内のポリアルキレングリコールのエチレンオキサイドとプロピレンオキサイドの組成比を変えることにより、親水性ー親油性のバランスを調整することができます。使用する酵素に応じて最適なポリマーを選択することにより、修飾酵素を有機溶剤に溶解することも可能です。

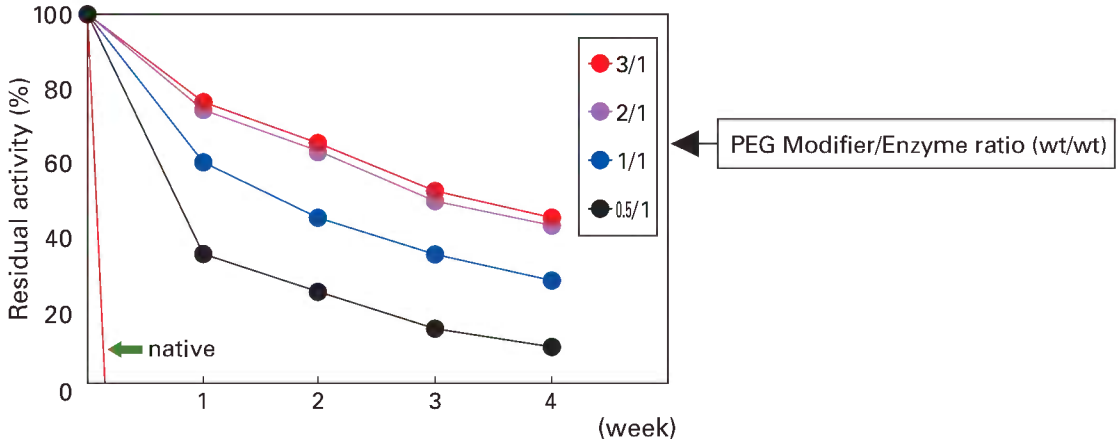
General Characteristics for PEG SUNBRIGHT AM series

Method of Test	AM-0530K	AM-1510K	AM-2090P
Mw of AO (PEG)	about 500	about 1,500	about 2,000
DP(1)	about 30 ~ 40	about 10 ~ 15	about 10 ~ 20
Total Mw	About 15,000 ~ 20,000	About 15,000 ~ 20,000	about 20,000 ~ 40,000

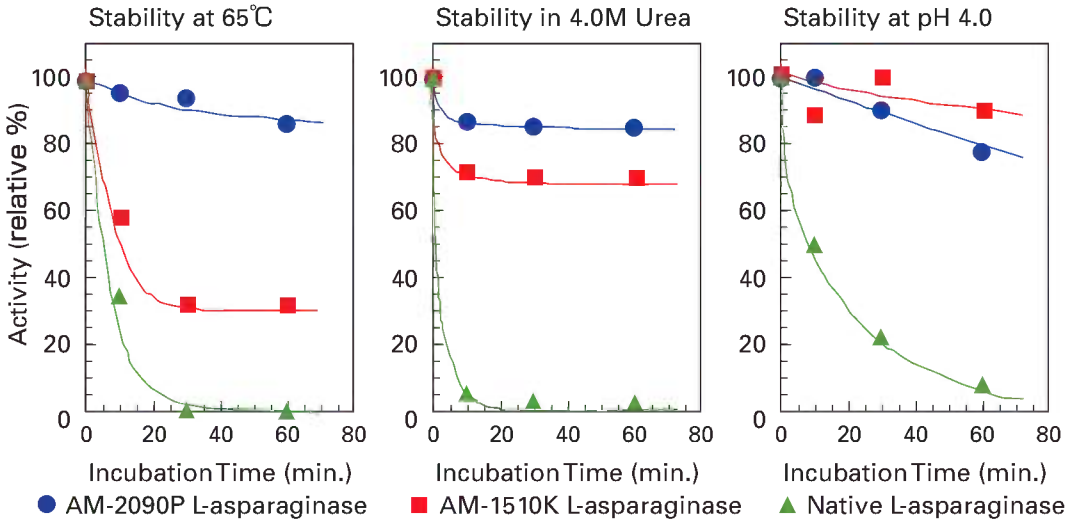
(1) Degree of Polymerization

Efficacy of Comb-type PEG Enzyme Conjugation

Stability of Modified Protease in water (at 40˚C)



Stability of modified L-Asparaginase



Residual activity and Antigenicity of Modified L-Asparaginase

Modifier Type	*Modification ratio	Residual activity %	Antigenicity
Native	0(0)	100	100
PEG1	79(73)	0.9	0
PEG2	57(52)	11.0	0
AM-1510K	50(46)	45.5	0
AM-2090P	34(31)	85.3	0

*Number of amino groups conjugated to PEG modifier.
(Total number of amino groups in ASPase= 92).

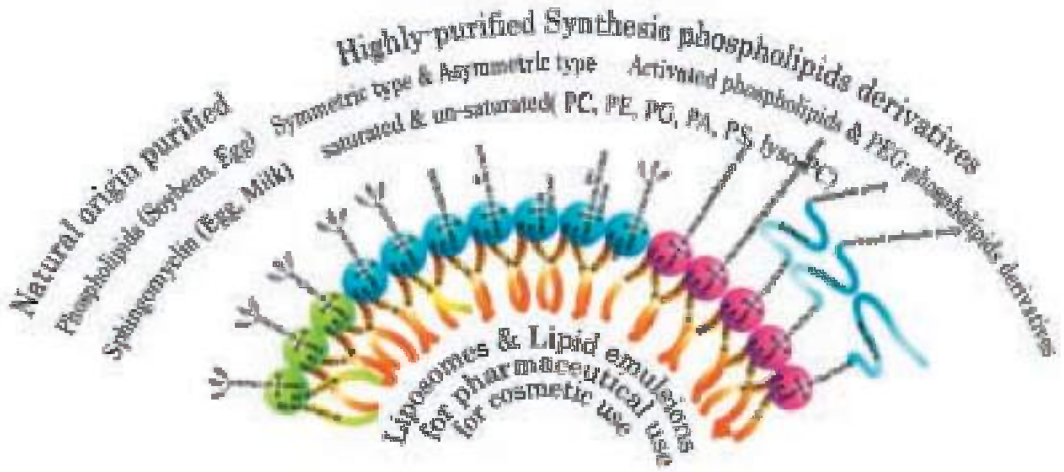
References: for modified enzymes using SUNBRIGHT AM-series:
(1) Kajiuchi, J., Park, J. W. (1992) *J. Chem. Eng.* Japan. **25**, 202
(2) Masunaga, T., et al. (1993) *Journal of SCCJ.* **27**, No.3 276
(3) Sasaki, H., et al. (1993) *Biochem. Biophys. Res. Commun.* **197**, 287
(4) Kodera, Y., et al. (1994) *Bioconjugate Chem.* **5**. 283
(5) Matsuo, K., et al. (1994) *Adv. Bioseparation Eng.* 1993, **3**, 56

II. Phospholipid derivatives and Liposome Formulations

<COATSOME® series>

NOF supplies the high-purity phospholipid derivatives (COATSOME Series) suitable for lipid emulsion and liposome formulations used for pharmaceuticals and cosmetics. Using state-of-the-art synthesis and purification technology, NOF has developed various kinds and grades of phospholipids for pharmaceuticals and cosmetics. Also, NOF offers custom-made synthesis of phospholipids. Furthermore, NOF provides unique research kits for liposomal drug delivery systems (DDS) with which customers can easily make and study liposomal formulations. Both liposome suspensions and equipment for liposome fabrication are also available from NOF (see LIPONIZER®, section X).

当社は、医薬品や化粧品などの脂肪乳剤処方やリポソーム処方に適した高純度リン脂質誘導体（COATSOME シリーズ）を、世界トップレベルの最新合成技術・精製技術を用いて提供しています。医薬・化粧品用リン脂質については豊富な品種とグレードを取り揃えています。また、お客様の要望にあわせた構造のリン脂質についてもカスタム合成いたします。さらに、簡単に薬剤のリポソーム化ができる中空リポソームキットやリポソーム処方液、リポソーム製造機も販売しています。



NOF Phospholipid Product Summary

1.Phospholipidsfromnaturalorigin 天然系リン脂質 1-1. SoybeanPhospholipids 1-2. EggyolkPhospholipids	NOFpurifiesphospholipidsfromnaturaloriginandcarefullyselectedstartingmaterials. 天然系リン脂質は厳選された原料を用いて、高度に精製して純度を上げたものです。 NOFusesnon-GMOproductsfromplantorigin,andvirus-freeproductsfromeggorigin. 植物由来のものは非遺伝子組み替え、卵黄由来はウィルスフリーの原料を使用しています。
1-3.Sphingomyelin	NOF provides pure products from both milk and egg origin. 原料としてミルク由来と卵黄由来の2種類を揃えています。
2.Syntheticphospholipids 合成系リン脂質	NOF's synthetic phospholipids are all plant origin and not animal origin. Saturated or unsaturated acyl chains, both symmetric and asymmetric types, reactive group-attached phospholipids and PEG-phospholipids are available. 合成リン脂質は、動物性原料を使わずに合成したものです。 飽和系、不飽和系、対称型、非対称型、反応性リン脂質、PEGーリン脂質など多くの品種を取り揃えています。
3.PEGylatedandFunctionalized Phospholipids	PEGylated and functionalized phospholipids are available for long-circulation and targeting use. 血中滞留性向上リポソームやターゲティング用に、PEGリン脂質及び末端活性化基結合リン脂質も揃えています。
4.LipidMixtureforTumorTargeting	Lipid Mixture containing transferrin attached phospholipid is supplied for tumor targeting liposome preparation. 癌細胞へのターゲティング用にトランスフェリンを結合したリン脂質を含む脂質混合物を供給します。
5.EmptyLiposomekit	Customer's water soluble drugs are easily formulated as liposomes with this kit. 簡単手軽にお手持ちの薬剤をリポソーム化することができます。
6. LIPONIZER (seeSectionX)	For liposome sizing, NOF provides the Liponizer™ of which small scale to commercial scale production is available. 小スケールから工業生産まで対応できる製造機器です。

NOF offers liposome formulations suitable for cosmetics or topical use of pharmaceuticals with colorless and odorless suspensions. There include both liquid and powder forms.

当社は、高純度なリン脂質を用いた色や臭いがない化粧品や外用剤に適したリポソーム処方を提供しています。液体・粉体の両方のタイプがあります。

1. Purified phospholipids from natural origins <COATSOME® NC series>

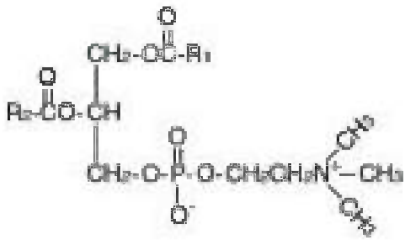
1-1. Soybean Phospholipids

植物（大豆）由来リン脂質誘導体

NOF supplies high-purity phospholipids of high phosphatidylcholine content with less color and less odor using carefully selected NON-GMO soybeans. Hydrogenated forms are more stable with saturation fatty acids and easier to handle in both powder.

遺伝子組換え原料を使用していない厳選された天然大豆を原料に用いて、ホスファチジルコリン含量の高い高純度なレシチンを提供しています。高純度品であるため、色や臭いに優れていて、水素添加によりアシル鎖の不飽和基を少なくしていますので、非常に安定性に優れ、粉末形態であるため使いやすくなっています。

Basic Structure : di-acyl-phosphatidylcholine



R_1COO, R_2COO : C12~C20 fatty acids

Product		Reference data		
Name	Type	PC purity	I.V.*	Appearance
COATSOME NC-21E	Hydrogenated	98% up	Max 1	White powder
COATSOME NC-21	Hydrogenated	90% up	Max 1	White powder

*I.V.= Iodine Value

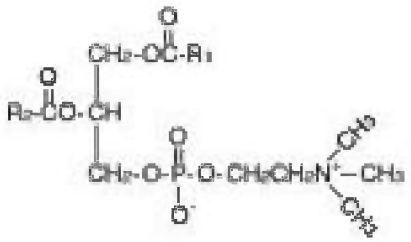
1-2. Egg yolk Phospholipids

卵黄由来リン脂質誘導体

Phospholipids from egg yolk, purified of impurities and supplied as virus-free, are characterized by saturated acyl chain at the α-position and unsaturated acyl chains at the β–position are basic structure. Therefore, these lipids can be easily dispersed in the water to perform excellent surface-active effects, so that they are also formulated in lipid microspheres.

ウィルスフリーの卵黄を原料として、高度精製して不純物を除去しています。卵黄リン脂質の特徴は、リン脂質中の二つのアシル基がそれぞれ飽和と不飽和と異なっている構造のリン脂質が主成分であることです。そのため、水分散性が良く界面活性能に優れています。また、リピドマイクロスフェアーの主要構成成分の一つとして、親油性薬物のキャリアーに用いられています。

Basic Structure : di-acyl-phosphatidylcholine



R_1COO : C12~C22 saturated fatty acids
R_2COO : C12~C20 unsaturated fatty acids

Product		Reference data	
Name	**Type**	**PC purity**	**Appearance**
COATSOME NC-50	Non-hydrogenated	95% up	White to off-white solid / powder
COATSOME NC-11	Hydrogenated	95% up	White powder

1-3. Sphingomyelin from natural sources <COATSOME® NM series>

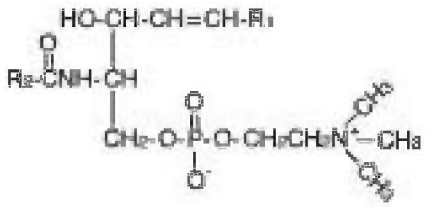
高純度天然スフィンゴミエリン

Phospholipids are the primary constituents of biological membranes. Recently, phospholipids have been directly implicated in the important biological functions as well as biomembrane structure. Sphingomyelin in particular was found to be vital for formation and maintenance of lipid rafts, where immune responses, various kinds of signaling, and certain material transport take place. Sphingomyelin is thus involved in expression of specific cellular functions, such as intracellular information transmission and maintenance of membrane structure. Sphingomyelin, with long fatty acid chains and low phase transition temperatures (Tc), is easy to prepare into liposomes.

Highly pure sphingomyelin of either the milk or yolk origin is available from NOF.
Sphingomyelin from yolk has fatty acid chains consisting of about 80% palmitic acid joined by amide bonds, while that from milk contains many kinds of fatty acids whose chains are longer than palmitic acid. These two sphingomyelin products slightly differ in physical properties, and can be selected according to needs.

リン脂質は、生体膜を構成する主要な物質ですが、近年、これらの脂質が構成脂質の役割にとどまらず、膜機能に直接関与する重要な役割を担っていることがわかってきました。中でも、スフィンゴミエリンは、ラフトの形成と維持に非常に重要であることが明らかにされています。ラフトは、免疫応答を始めさまざまなシグナリングの場として、また特定の物質輸送の場として機能していて、スフィンゴミエリンが、生体膜の構造維持とともに細胞内情報伝達系への関与など細胞の機能発現にも大きく関わる物質として大変注目されています。またスフィンゴミエリンは脂肪酸鎖長が長い割に、相転移温度（Tc）が低いという特徴があり、リポソーム化しやすい基剤です。
当社は注目度の高いスフィンゴミエリンを牛乳由来のものと卵黄由来のものを高度精製して提供します。
卵黄由来では、アミド結合した脂肪酸鎖の約80%がパルミチン酸であるのに対し、牛乳由来では、パルミチン酸以上の長鎖の脂肪酸が幅広く含まれていて、物理的な性質が若干異なるので、用途に応じて選択できます。

Sphingomyelin Structure



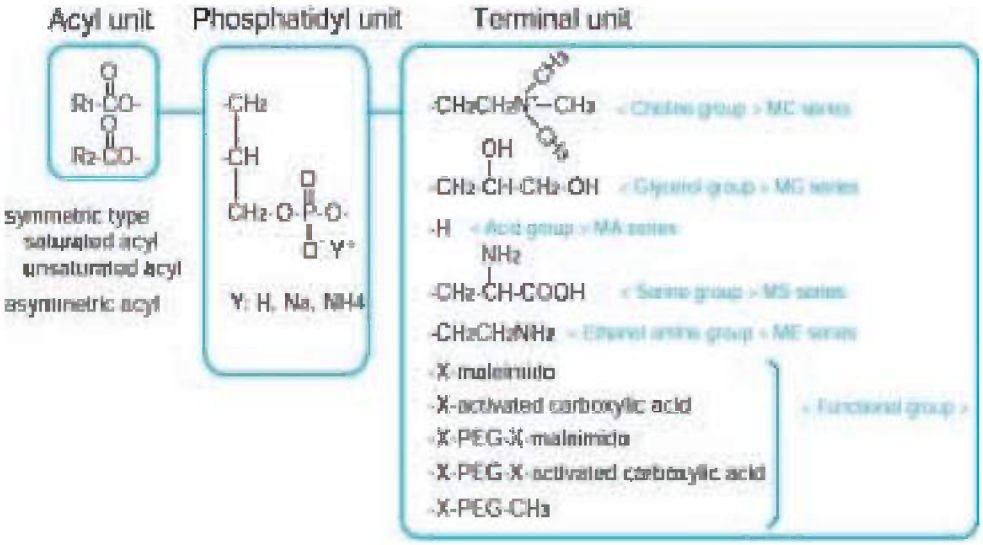
Product		Reference data	
Name	**Origin**	**purity**	**Alkyl composition**
COATSOME NM-10	Egg	98% up	R1: C13 R2: C15:0, C17:0
COATSOME NM-70	Milk	98% up	R1: C13-C24 R2: C15:0, C17:0, C21:0, C22:0, C23:0

2. Highly-purified synthetic phospholipid derivatives

NOF produces high-purity fatty acids including unsaturated fatty acids using their own technology. Diverse synthetic phospholipid derivatives of various fatty acid compositions are available from NOF. These phospholipids contain no fatty acid materials of animal origin, are produced in FDA-inspected facilities under cGMP, and are safe for pharmaceutical use.
NOF also synthesizes new phospholipid derivatives at customer request. NOF is also highly experienced in DMF registration of new derivatives and supplies lipids in both small and commercial scale quantities for approved pharmaceutical products.

原料の脂肪酸を自社で高純度に精製して、各種リン脂質誘導体を製造していますので、様々な脂肪酸組成の誘導体を合成することができます。当社の合成リン脂質は、動物由来原料を使用していません。また、FDAの認可を受けたGMP管理下の工場で製造していますので、安心して医薬品に使うことが出来ます。
新しいリン脂質誘導体についても受託合成致し、新規誘導体のDMF登録も数多く行っていますので、試薬ステージから臨床ステージ、さらには承認医薬品用途まで供給できます。

NOF Synthetic phospholipid derivatives



The figure above divides the traditional phospholipids into three characteristic parts. Each of these can be modified and produced by NOF to yield various derivatives. Custom properties can be obtained with the combination of these parts for different end-goals.

合成リン脂質は大きく三つに分かれていて、これらを組み合わせることにより、いろいろな物性の誘導体を合成することが出来ます。



Liposomes were imaged by FEI company Ltd.,
with using Cryo-TEM ,TECNAI F20TWIN Cryo.

•Synthetic phospholipid derivatives: symmetric acyl chains

Symmetric-type synthetic phospholipids have two identical acyl groups (R1, R2). Natural phospholipids show distributions of fatty acids that constitute acyl groups, usually different fatty acids, resulting in fluctuation of lipid purity and quality depending on the materials source. By contrast, synthetic phospholipids do not show such fluctuations. They are highly uniform and optimal for producing drugs. Two kinds of symmetric-type synthetic phospholipids are available from NOF: saturated and unsaturated phospholipids.

対称型はリン脂質の構造中の二つのアシル基が同一の脂肪酸から構成される合成リン脂質です。天然のリン脂質の場合、アシル基の構成脂肪酸が分布を持っていて、二つのアシル基が不均一である場合が多いため、原料の産地等によって品質がばらつく場合がありますが、合成リン脂質はそのようなばらつきはありません。均一性が厳しく要求される医薬品に最適です。対称型の合成リン脂質としては飽和脂肪酸系と不飽和脂肪酸系の2種類がございます。

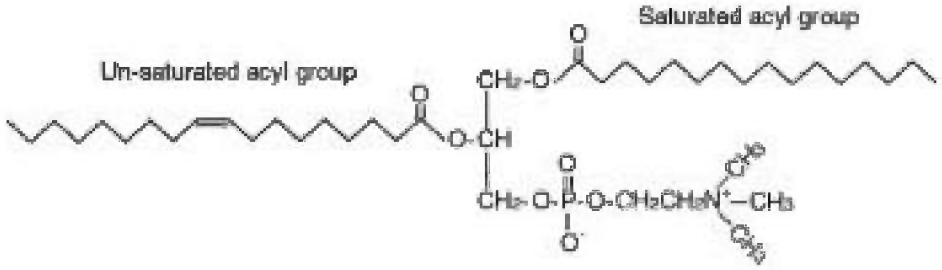
•Synthetic phospholipids derivatives: asymmetric acyl chains

Asymmetric-type synthetic phospholipids have two different fatty acids introduced into the individual R1 and R2 acyl positions. Introducing pure fatty acids into the a and b positions results in consistent characteristics such as phase transition temperatures. Using the highly pure fatty acids from NOF and strictly controlling the introduction of fatty acids, where the a position is saturated and the b position is unsaturated, NOFs lipid quality and purity are unique. This yields two characteristics: (1) stability as in saturated phospholipid derivatives, and (2) ease-of-handle, like unsaturated phospholipids.
Another asymmetric type also available from NOF is a high-quality lyso phospholipid with only the a acyl chain position substituted (see below).

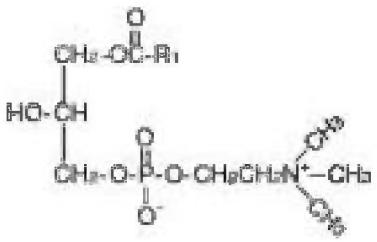
非対称型の合成リン脂質は、リン脂質の持つ二つのアシル基に異なる種類の脂肪酸を導入したものです。1位と2位に純粋な脂肪酸を導入することにより、相転移温度が一定なリン脂質となります。特に、当社の持つ高純度脂肪酸を原料として、1位の位置に飽和脂肪酸、2位の位置に不飽和脂肪酸を厳しくコントロールして導入したタイプは、飽和系リン脂質誘導体の安定性と不飽和系リン脂質の取り扱い易さを併せ持つ第2世代リン脂質と言えます。
また、別の非対称型として1位のアシル基のみを結合させたリゾリン脂質も提供していります。

Typical chemical structure for asymmetric phospholipids

Asymmetric (saturated/unsaturated) acyl group type (POPC):



Asymmetric saturated acyl group single chain type (Lyso phospholipid type)



•Saturated Unsaturated acyl group types

飽和脂肪酸系リン脂質誘導体と不飽和脂肪酸系リン脂質誘導体

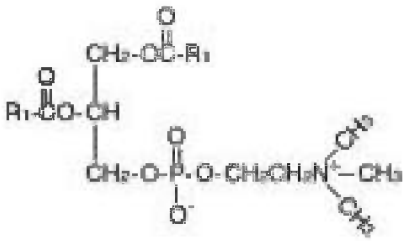
NOF uses no animal materials to produce phospholipid derivatives of saturated fatty acids.

当社のリン脂質誘導体に使用している飽和脂肪酸は、動物原料を一切使用しておりません。

In general, highly pure unsaturated fatty acids are difficult to produce. Thus, lipid products usually contain impurities and are unstable against oxidization. However, NOF produces highly pure mono-unsaturated fatty acids such as oleic or erucic acids, very stable against oxidation. Phospholipid derivatives produced using these highly pure mono-unsaturated fatty acids are also highly pure and very stable.
All unsaturated fatty acid products from NOF are produced from plant origins.

一般的に不飽和脂肪酸は精製が困難で高純度な原料は得られにくく様々な不純物が混在するといわれています。そのため不飽和脂肪酸は酸化安定性が悪いと言われています。最新技術を駆使して安定性の極めて高い高純度モノ不飽和脂肪酸を製造していて、それを用いたリン脂質誘導体は、高純度かつ酸化安定性に非常に優れています。
また、原料に使用している不飽和脂肪酸は、すべて植物を原料としたものです。

2-1. Phosphatidylcholine



R_1 = identical fatty acid

Saturated acyl groups: Symmetric type (DDPC, DLPC, DMPC, DPPC, DSPC)

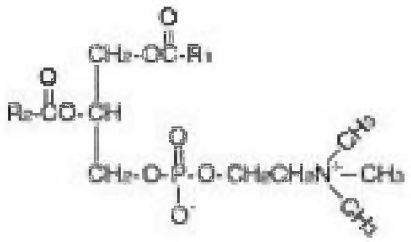
Product		Reference data				
Abbreviation	Name	PC purity	Mw	Character of acyl group (R1)(purity)		Tc
DDPC	COATSOME MC-1010 *	99% up	565.7	C10:0	99%	-6
DLPC	COATSOME MC-2020	99% up	621.8	C12:0	99%	0
DMPC	COATSOME MC-4040	99% up	677.9	C14:0	99%	23
DPPC	COATSOME MC-6060	99% up	734.0	C16:0	99%	42
DSPC	COATSOME MC-8080	99% up	790.2	C18:0	99%	55

* : custom synthesis

Unsaturated acyl groups: Symmetric type (DOPC, DEPC)

Product		Reference data				
Abbreviation	Name	PC purity	Mw	Character of acyl group (R1)(purity)		Tc
DOPC	COATSOME MC-8181	99% up	786.1	C18:1	99%	-22
DEPC	COATSOME MC-2121AL	99% up	898.3	C22:1	98%	-17

Saturated acyl groups: Asymmetric type (MPPC, MSPC, PMPC, PSPC, SMPC, SPPC)



R_1 and R_2 are different fatty acid residues

Product		Reference data			
Abbreviation	Name	R_1COO	R_2COO	PC purity	Mw
MPPC	COATSOME MC-4060*	C14:0	C16:0	99% up	706.0
MSPC	COATSOME MC-4080*	C14:0	C18:0	99% up	734.0
PMPC	COATSOME MC-6040*	C16:0	C14:0	99% up	706.0
PSPC	COATSOME MC-6080*	C16:0	C18:0	99% up	762.1
SMPC	COATSOME MC-8040*	C18:0	C14:0	99% up	734.0
SPPC	COATSOME MC-8060*	C18:0	C16:0	99% up	762.1

* : custom synthesis

Lyso lipids (M-LysoPC, P-LysoPC, S-LysoPC, HS-LysoPC)

Product		Reference data		
Abbreviation	Name	R_1COO	PC purity	Mw
M-LysoPC	COATSOME MC-40H*	C14:0	99% up	467.6
P-LysoPC	COATSOME MC-60H*	C16:0	99% up	495.6
S-LysoPC	COATSOME MC-80H*	C18:0	99% up	523.7
HS-LysoPC	COATSOME MC-70H*	HS**	99% up	518.1

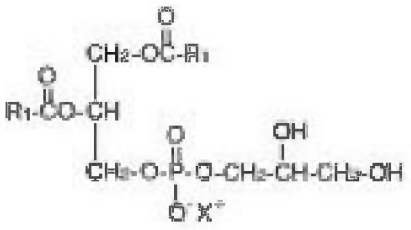
* : custom synthesis HS** : Hydrogenated Soybean, Palmitoyl/Stearoyl = 1/4

Mixed acyl groups: Asymmetric type (MOPC, POPC, SOPC, HSOPC)

Product		Reference data			
Abbreviation	Name	R_1COO	R_2COO	PC purity	Mw
MOPC	COATSOME MC-4081*	C14:0	C 18:1	99% up	732.0
POPC	COATSOME MC-6081	C16:0	C 18:1	99% up	760.1
SOPC	COATSOME MC-8081*	C18:0	C 18:1	99% up	788.1
HSOPC	COATSOME MC-7081*	HS**	C 18:1	99% up	784.3

* : custom synthesis HS** : Hydrogenated Soybean, Palmitoyl/Stearoyl = 1/4

2-2. Phosphatidylglycerol



R_1 = identical fatty acid

X : Na or NH_4

Saturated acyl groups: Symmetric type (DLPG, DMPG, DPPG, DSPG)

Product		Reference data			
Abbreviation	Name	PG purity	Mw	Character of acyl group (purity)	
DLPG-Na	COATSOME MG-2020LS	99% up	632.7	C12:0	99% up
DLPG-NH_4	COATSOME MG-2020LA	99% up	627.8	C12:0	99% up
DMPG-Na	COATSOME MG-4040LS	99% up	688.9	C14:0	99% up
DMPG-NH_4	COATSOME MG-4040LA	99% up	683.9	C14:0	99% up
DMPG-NH_4/Na	COATSOME MGLM-4040	99% up	684.6	C14:0	99% up
DPPG-Na	COATSOME MG-6060LS	99% up	745.0	C16:0	99% up
DPPG-NH_4	COATSOME MG-6060LA	99% up	740.0	C16:0	99% up
DSPG-Na	COATSOME MG-8080LS	99% up	801.1	C18:0	98% up
DSPG-NH_4	COATSOME MG-8080LA	99% up	796.1	C18:0	98% up

* : custom synthesis

Unsaturated acyl groups: Symmetric type (DOPG, DEPG)

Product		Reference data			
Abbreviation	Name	PG purity	Mw	Character of acyl group (purity)	
DOPG-Na	COATSOME MG-8181LS*	98% up	797.0	C 18:1	98% up
DEPG-Na	COATSOME MG-2121LS*	98% up	909.2	C 22:1	98% up

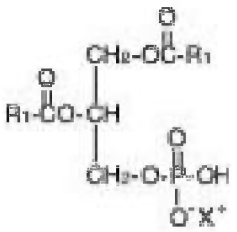
* : custom synthesis

Mixed acyl groups: Asymmetric type (POPG)

Product		Reference data			
Abbreviation	Name	R_1COO	R_2COO	PG purity	Mw
POPG-Na	COATSOME MG-6081LS*	C16:0	C 18:1	98% up	771.0

* : custom synthesis

2-3. Phosphatidic acid



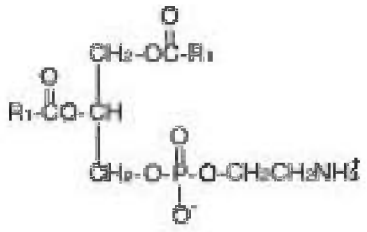
R_1 = identical fatty acid

X : Na

Saturated acyl groups: Symmetric type (DMPA, DPPA, DSPA)

Product		Reference data			
Abbreviation	**Name**	**PA purity**	**Mw**	**Character of acyl group (purity)**	
DMPA-Na	COATSOME MA-4040LS	99% up	614.8	C14:0	99% up
DPPA-Na	COATSOME MA-6060LS	99% up	670.9	C16:0	99% up
DSPA-Na	COATSOME MA-8080LS	99% up	727.0	C18:0	99% up

2-4. Phosphatidylethanolamine



R_1 and R_2 are different fatty acid residues

Saturated acyl groups: Symmetric type (DMPE, DPPE, DSPE)

Product		Reference data			
Abbreviation	**Name**	**PE purity**	**Mw**	**Character of acyl group (purity)**	
DMPE	COATSOME ME-4040	99% up	635.9	C14:0	99% up
DPPE	COATSOME ME-6060	99% up	692.0	C16:0	99% up
DSPE	COATSOME ME-8080	99% up	748.1	C18:0	99% up

* : custom synthesis

Unsaturated acyl groups: Symmetric type (DOPE, DEPE)

Product		Reference data			
Abbreviation	**Name**	**PE purity**	**Mw**	**Character of acyl group (purity)**	
DOPE	COATSOME ME-8181	99% up	744.0	C18:1	99% up
DEPE	COATSOME ME-2121AL*	99% up	856.3	C22:1	98% up

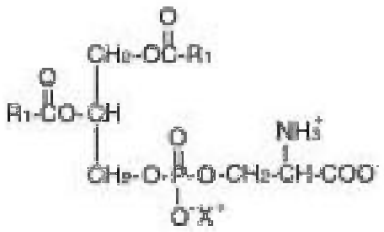
* : custom synthesis

Mixed acyl groups: Asymmetric type (POPE)

Product		Reference data			
Abbreviation	**Name**	**R_1COO**	**R_2COO**	**PC purity**	**Mw**
POPE	COATSOME ME-6081*	C16:0	C 18:1	99% up	718.0

* : custom synthesis

2.5. Phosphatidylserine



R_1 = identical fatty acid

X : Na

Saturated acyl groups: Symmetric type (DMPS, DPPS, DSPS)

Product		Reference data			
Abbreviation	**Name**	**PS purity**	**Mw**	**Character of acyl group (purity)**	
DMPS-Na	COATSOME MS-4040LS*	80% up	701.8	C14:0	99% up
DPPS-Na	COATSOME MS-6060LS*	80% up	758.0	C16:0	99% up
DSPS-Na	COATSOME MS-8080LS*	80% up	814.1	C18:0	99% up

* : custom synthesis

Unsaturated acyl groups: Symmetric type (DOPS)

Product		Reference data			
Abbreviation	**Name**	**PS purity**	**Mw**	**Character of acyl group (purity)**	
DOPS-Na	COATSOME MS-8181LS*	97% up	810.0	C 18:1	99% up

* : custom synthesis

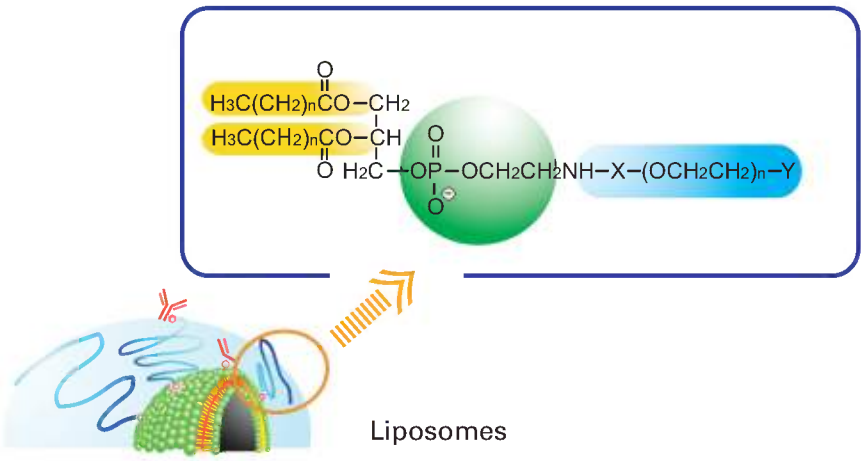
3. PEGylated and Functionalized Phospholipids

3-1. PEGylated lipid derivatives

NOF's unique PEGYLATED LIPIDS can be applied for formulating liposomal drugs for prolonged circulation in vivo. Coating the liposome surface with polyethylene glycol extends the circulation time of liposomal drugs in blood by preventing capture by the body's reticuloendothelial system.

These derivatives are used not only for liposomes, but also for emulsifiers and stabilizers of microspheres in solution. High-purity PEG-phospholipids are produced with our high-purity methoxy-PEGs (SUNBRIGHT Series)(low-diol content) and high-purity phospholipids (COATSOME Series).
Our PEG-phospholipids are obtained with very low impurity content and narrow polydispersity important to pharmaceutical use.

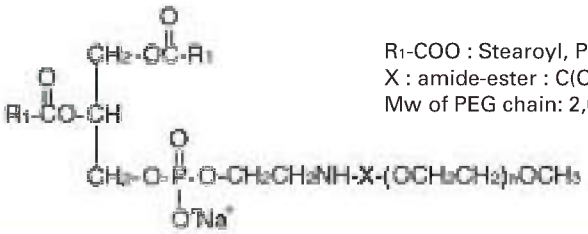
PEG修飾脂質は、体内投与において血中滞留性を高める目的でリポソーム薬剤に用いることができます。ポリエチレングリコールでリポソーム表面を覆うことにより、細網内皮系への取り込みを抑え、血中での滞留時間を延長することができます。
また、乳化剤および水溶液中での微粒子の安定剤としても使用することができます。



3-1-1. PEG-phospholipids

NOF PEG-PHOSPHOLIPIDs have three types of chemistry linking PEG with the lipid polar phosphorylethanolamine group: two are amide esters in which the ester bond may be cleaved at a certain pH, the third carbamate bond is stable enough to be used for commercial PEG-modified liposomes (see below).

PEGーリン脂質には、PEG鎖とリン脂質の結合様式の違いにより2タイプがあります。一つはアミド結合とエステル結合からなるタイプであり、pHに依存して加水分解されますが、間の炭素数によってその分解速度が異なります。もう一つのカーバメート結合タイプは加水分解に対してさらに安定であり、現在市販されているPEGーリポソームに用いられています。



R_1-COO : Stearoyl, Palmitoyl, Oleoyl groups
X : amide-ester : $C(O)(CH_2)_2C(O)$, $C(O)(CH_2)_3C(O)$ or Carbamate linkage: C(O)
Mw of PEG chain: 2,000 to 12,000

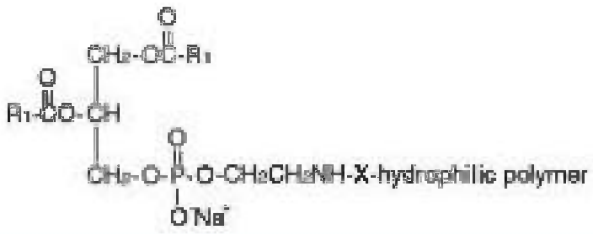
Product		Reference data		
Abbreviation	**Name**	**R_1COO**	**PEG chain Mw**	**X : Linker**
DSPE-PEG	SUNBRIGHT DSPE-020C	C18:0	2000	$CO(CH_2)_2CO$
	SUNBRIGHT DSPE-050C		5000	
	SUNBRIGHT DSPE-020G		2000	$CO(CH_2)_3CO$
	SUNBRIGHT DSPE-050G		5000	
	SUNBRIGHT DSPE-020CN		2000	CO
	SUNBRIGHT DSPE-050CN		5000	

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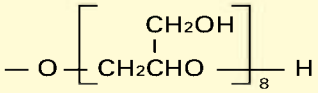
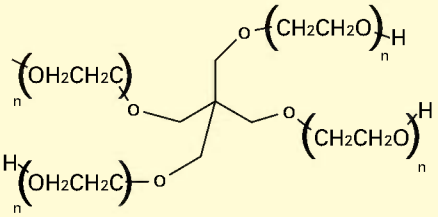
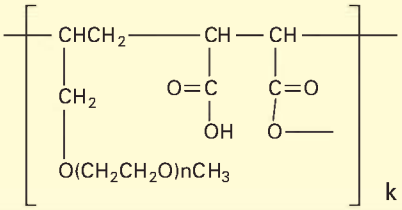
3-1-2. New hydrophilic phospholipid derivatives

As shown below, we can also provide new phospholipid derivatives attached to hydrophilic polymers such as polyglycerine and multi-arm PEGs. These have been shown to provide liposome performance advantages in vivo (see below).

次に示したように、水溶性ポリマーとしてポリグリセリンおよびマルチアームPEGを付与した新しいタイプのリン脂質誘導体を供給します。



X : Linker

Product		Reference data		
Abbreviation	Name	R_1COO	Hydrophilic polymer	
			MW	Structure
DSPE-Polyglycerine	SUNBRIGHT DSPE-PG8G	C18:0	700	
DSPE-Multi-arm PEG	SUNBRIGHT DSPE-PTE020		2,000	
DSPE-Comb-shaped PEG	SUNBRIGHT DSPE-AM0530K		12,000	

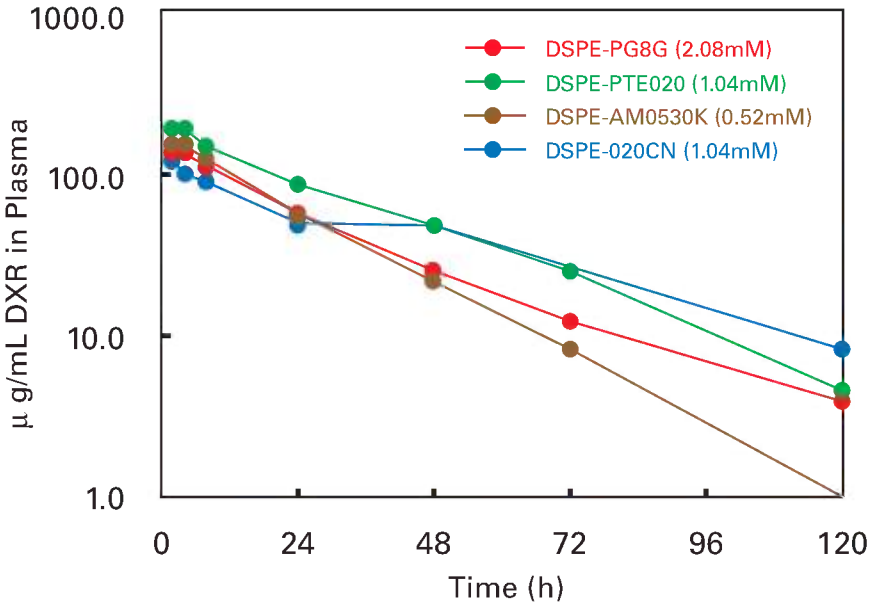
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Physicochemical Properties of New Type Liposomes

New type hydrophilic phospholipid derivatives	Particle Size (nm)	Encapsulation Efficiency of DXR† (%)
DSPE-PTE020	108	100.0
DSPE-PG8G	78	96.6
DSPE-AM0530K	92	93.4

Lipid Composition: DSPE-PTE020 /HSPC/Chol= 1.04mM/11.28mM/7.68mM
DSPE-PG8G/HSPC/Chol =2.08mM/11.28mM/7.68mM
DSPE-AM0530K/HSPC/Chol=0.520mM/11.28mM/7.68mM

†DXR: doxorubicin (anti cancer drug)



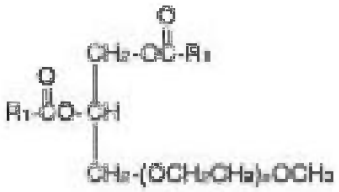
Plasma Clearance Profile for PEG-based Liposomes and Polyglyceline

3-1-3. Un-charged PEG-lipid derivatives

a) Diacylglycerol-PEG

These products lack phosphorylethanolamine groups and the extra linkage is relatively more stable against hydrolysis. Furthermore, because of the nonionic characteristics of the backbone, these products are suitable to use for drugs which are sensitive to ionic charges.

このシリーズは、ジアシルグリセロールにPEG鎖を直接付与しているので、フォスフォリツエタノールアミン基およびその他の結合部位を含まないため、加水分解に対して安定であり、電荷が問題となる薬剤への使用に適しています。



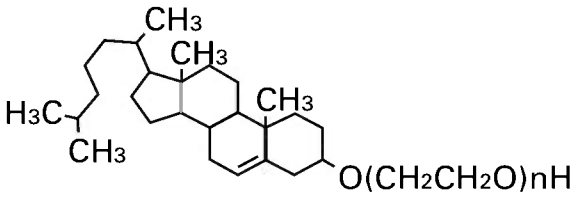
Product	Reference data	
name	R_1-COO	PEG Mw
SUNBRIGHT GS-020	stearoyl $CH_3(CH_2)_{16}COO$	2,000
SUNBRIGHT GS-050		5,000

SUNBRIGHT®

b) Cholesterol-PEG derivatives

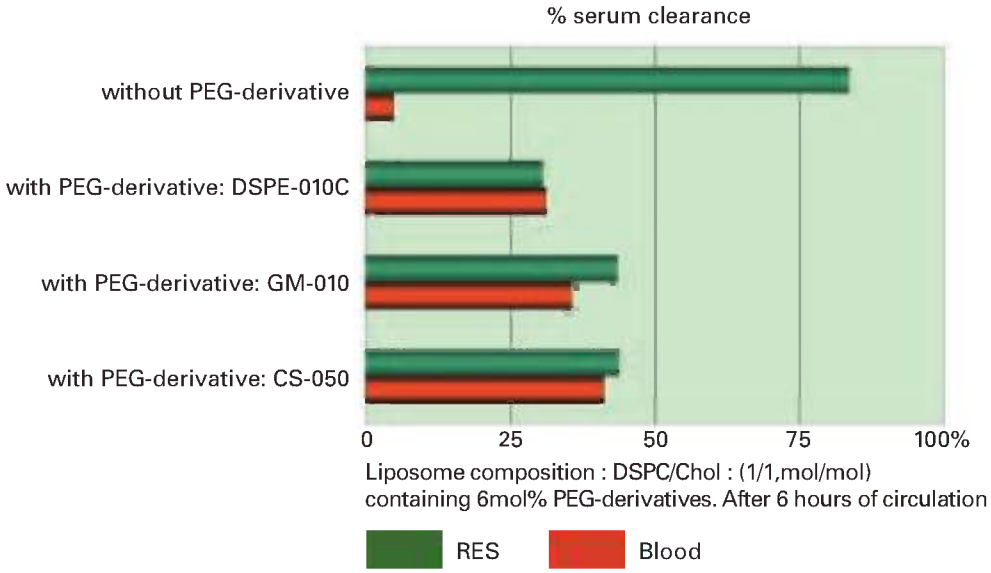
NOF has several high-purity mono-alkylated polyethylene glycols other than our quality mPEG. Our cholesterol-modified PEGs provide a versatile alternative to mPEG. Using more than 8-carbon chain alkylated PEGs, several unique surface-active effects are observed. These products or materials can be used not only for surface modification of LIPOSOMES, but also for peptide PEGylation by attaching peptide functional groups to the PEG chain ends.

メトキシポリエチレングリコール以外にも、さまざまな種類の高純度モノアルキル置換ポリエチレングリコールを開発しています。アルキル基として炭素数が8以上の炭化水素基を用いると、種々の界面活性効果が出てきます。これらの誘導体はそのままリポソームなどの表面修飾に用いることができるのみではなく、末端に種々の官能基を導入してたん白質などのPEG化にも使うことができます。



Typical product name	MW
SUNBRIGHT CS-010	1,000
SUNBRIGHT CS-020	2,000
SUNBRIGHT CS-050	5,000

Serum clearance efficiency of new PEG-Lipid derivatives in Liposomes

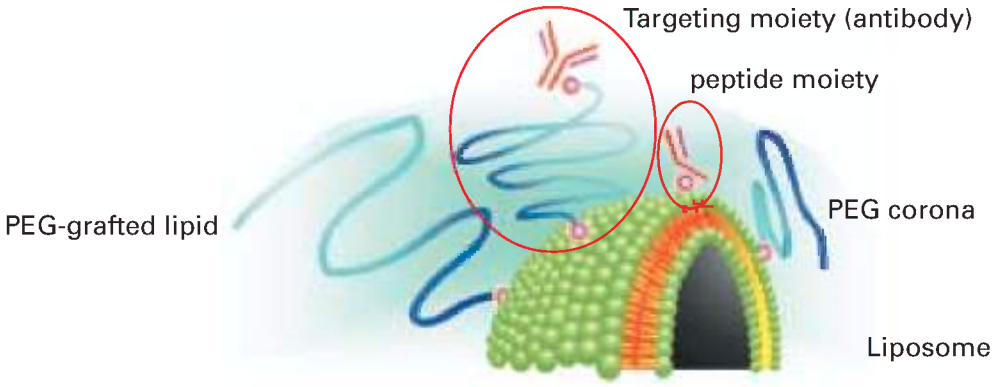


Reference : K. Maruyama., et al.(1996) *Bio. Pharm. Bull.* **19**, 10, 1347

3-2. Functionalized phospholipid derivatives

Functionalized phospholipid derivatives with activated groups attached at either the lipid polar end or attached PEG's terminal end are now available from NOF. Antibodies, peptides, etc may be covalently attached to the surface of liposomes or lipid emulsions using these reactive chemistries. These result in particles for targeting or extending circulation in blood. NOF supplies various kinds of these high purity derivatives utilizing our own high purity PEGs, state-of-the-art synthetic technologies, and pure reagents.

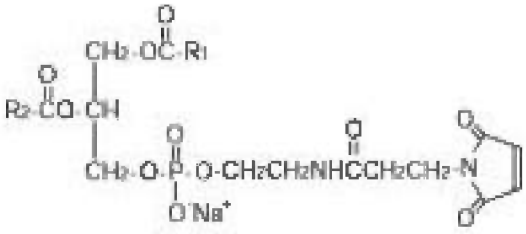
機能性リン脂質を用いると、脂肪乳剤やリポソームの表面に種々のペプチドや抗体などを結合させtarget性を付与したり、PEG鎖を導入して処方の血中滞留性をあげることができます。当社は原料PEGから合成しておりますので、高純度な種々の誘導体を提供いたします。



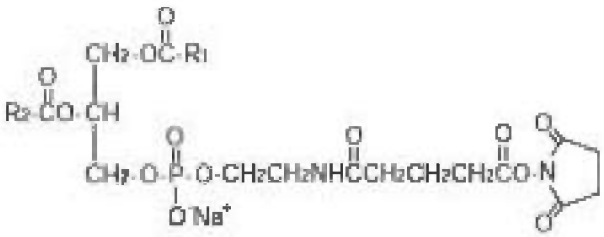
3-2-1. Activated phospholipids

NOF supplies both reactive maleimido and carboxylic acid group activated phospholipids suitable for reactions with sulfhydryl groups on antibodies or amino groups of peptides, respectively.

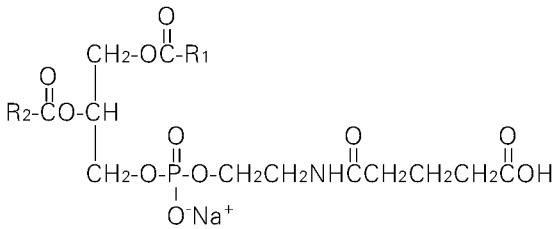
活性化リン脂質には、抗体などのSH基と反応するマレイミド基を持つタイプと、たんぱく質などのNH_2基やSH基と反応する活性化カルボキシル基を持つタイプがあります。



Maleimido (MAL) derivative for SH group reaction



Activated carboxylic acid (NHS) for NH_2 group reaction

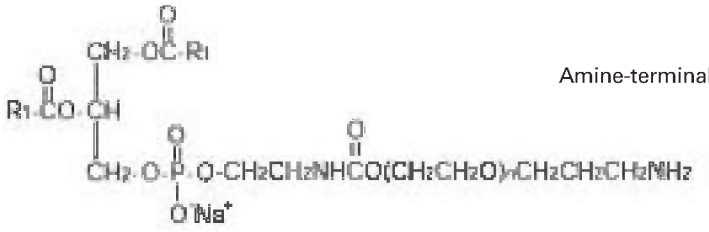


Carboxylic acid derivative for NH_2 group reaction

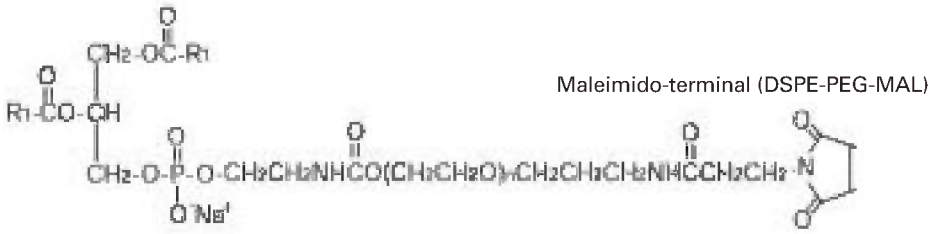
Product		Reference data		
Abbreviation	Name	Target group	R_1COO	R_2COO
DPPE-MAL	COATSOME FE-6060MA3*	SH group	C16:0	C16:0
DSPE-MAL	COATSOME FE-8080MA3*		C18:0	C18:0
POPE-MAL	COATSOME FE-6081MA3*		C16:0	C18:1
DMPE-MAL	COATSOME FE-4040MA3*		C14:0	C14:0
DPPE-MAL	COATSOME FE-6060MA3*		C16:0	C16:0
DOPE-MAL	COATSOME FE-81812MA3*		C18:1	C18:1
DSPE-NHS	COATSOME FE-8080SU5*	NH_2 group	C18:0	C18:0
POPE-NHS	COATSOME FE-6081SU5*		C16:0	C18:1
DMPE-NHS	COATSOME FE-4040SU5*		C14:0	C14:0
DPPE-NHS	COATSOME FE-6060SU5*		C16:0	C16:0
DOPE-NHS	COATSOME FE-8181SU5*		C18:1	C18:1
DPPE-Glu	COATSOME FE-6060GL*		C16:0	C16:0
DSPE-Glu	COATSOME FE-8080GL*		C18:0	C18:0
DOPE-Glu	COATSOME FE-8181GL*		C18:1	C18:1

COATSOME® * : custom synthesis

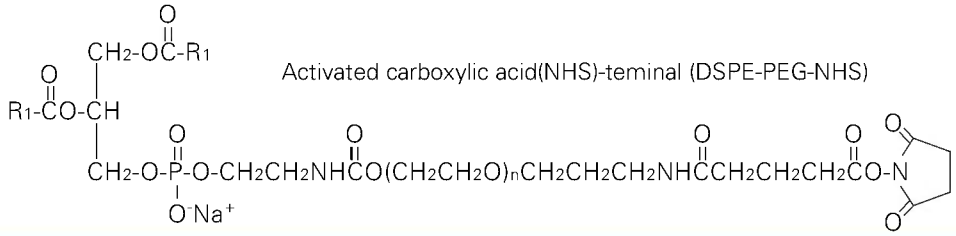
3-2-2. Activated PEG phospholipids



Amine-terminal (DSPE-PEG-NH_2)



Maleimido-terminal (DSPE-PEG-MAL)

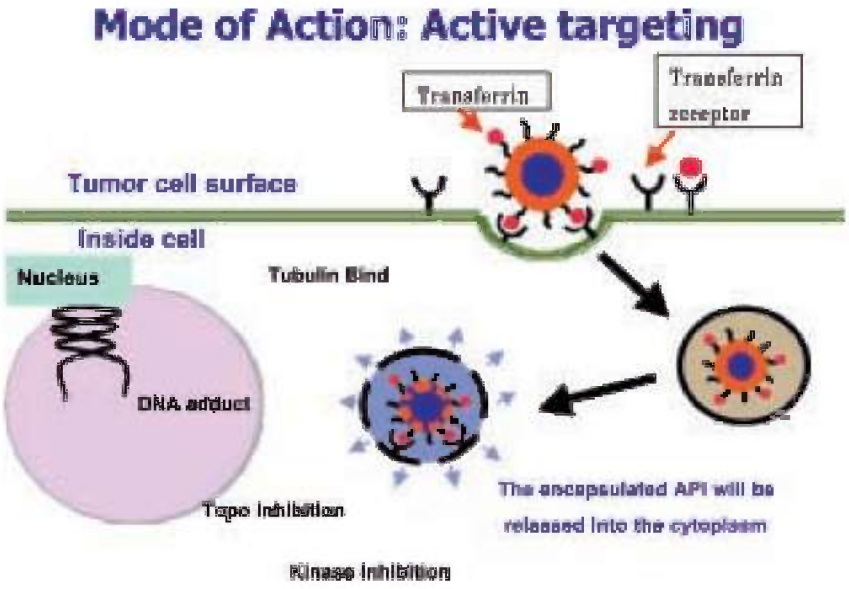


Activated carboxylic acid(NHS)-teminal (DSPE-PEG-NHS)

Product		Reference data		
Abbreviation	Name	Target group	R_1COO	PEG chain Mw
DSPE-PEG-NH_2	SUNBRIGHT DSPE-020PA	COOH group	C18:0	2000
	SUNBRIGHT DSPE-050PA			5000
DSPE-PEG-MAL	SUNBRIGHT DSPE-020MA	SH group		2000
	SUNBRIGHT DSPE-050MA			5000
DSPE-PEG-NHS	SUNBRIGHT DSPE-020GS	NH_2 group		2000

SUNBRIGHT®

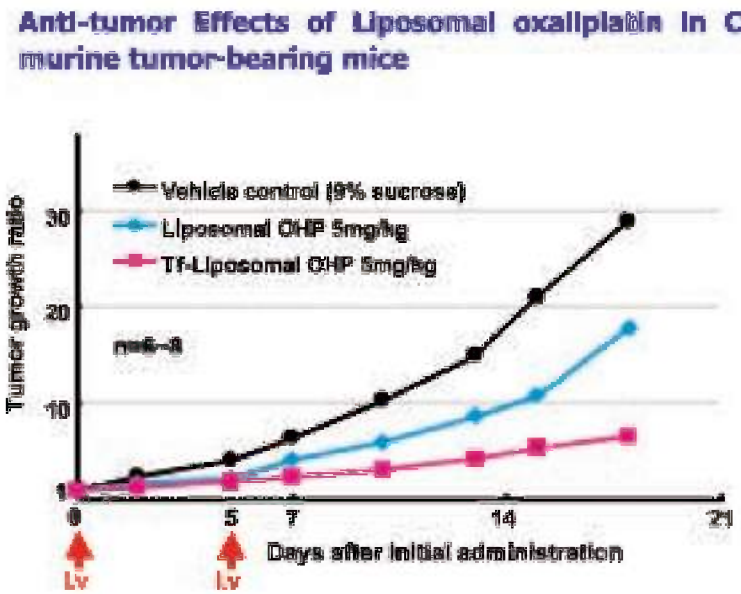
4. Lipid Mixture for Tumor Targeting



Ordinary tumor cells are abundant with transferrin receptors. This means that liposomes with transferrin attached on the outside surface can be a potential carrier of anti-cancer drugs to tumor cells. NOF has developed a tumor targeting Lipid Mixture called COATSOME PL-001T, which can be prepared as a liposomal suspension in the customer's laboratory.
COATSOME PL-001T consists of PC and other suitable lipids whereby transferrin is attached with activated phospholipid through chemical bonding.
The attached document describes the liposomal preparation of COATSOME PL-001T for tumor targeting.

癌細胞表面には一般的にトランスフェリンレセプターが多く存在することから、外側表面にトランスフェリンを化学結合させたリポソームを用いることにより、癌細胞へのターゲテイングが可能になります。
日本油脂の提供する脂質混合物を使用すれば、癌細胞標的能を有するリポソームをラボにて調製できます。
COATSOME　PL-001T　は、ホスファチジルコリンやトランスフェリンの結合したリン脂質などを含む特殊な脂質混合体であります。
ご購入の際に添付される方法に従って調製頂ければ、癌細胞標的能を有するリポソームが作製できます。

Product Name	Characteristics
COATSOME PL-001T	Dried powder consists of PC and other suitable lipids containing transferrin attached phospholipid



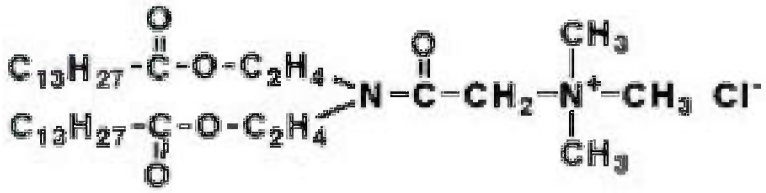
5. Empty Liposomes <COATSOME® EL Series>

Liposomes for Basic Research



NOF has developed a unique research kit for liposomal drug delivery system (DDS) development. Generally, researchers using liposome systems intended for DDS have difficulties consistently encapsulating liposomal drugs principally because development of a liposomal drug requires conditions that carefully control the formulation of phospholipids, particle size, and encapsulation efficiency.
NOF has formulated and manufactured ready-to-use freeze-dried liposomes, called Empty Liposomes, in vial form. Empty Liposomes can encapsulate drug by adding a drug solution to our Empty Liposomes and then gentle shaking. This allows the researcher to prepare liposomal drugs without complicated methods (e.g., extrusion). Encapsulation efficiency for Empty Liposomes has been found to be especially high with anthracycline and aminoglycosides.
In addition, we have developed new cationic Empty Liposomes <COATSOME EL-01-D> for gene delivery. This novel cationic liposome contains *O,O'*-ditetradecanoyl-*N*-(α-trimethylammonioacetyl) diethanolamine chloride (DC-6-14, see below), DOPE and cholesterol, and has demonstrated efficient transfection activity both *in vitro* in serum-containing medium and *in vivo*.

一般に、リポソーム化医薬品を作製するためには、リン脂質組成、粒径調整、内包化率などの調整が必要であり、研究者にとってリポソーム化医薬品が容易に処方できない原因となっていました。当社はこれらの問題を解決すべくリポソーム型DDS検討用にユニークな研究用キットを開発しました。
中空リポソームと称するバイアルに入った凍結乾燥リポソーム粉末は、数種類のリン脂質と電荷物質で構成されていて、医薬品を溶解させた溶液をバイアルに注入し緩やかに混和することにより、簡単に医薬品をリポソーム中に内包させることができます。中空リポソームを用いることによりエクスツルージョン等の特殊な技術を用いることなく、特にアントラサイクリンやアミノクルコシドなどの医薬品を高収率で容易に内包できます。
また、遺伝子デリバリー用として新しいカチオニックリポソーム<COATSOME EL-01-D>を開発しました。COATSOME EL-01-Dは、カチオン性脂質であるO,O' -ditetradecanoyl-N-(α-trimethylammonioacetyl)diethanolamine chloride (DC-6-14)に加え、DOPEとコレステロールを含有し、in vitroの血清添加培地中やin vivoアッセー系においても高い遺伝子導入・発現活性を有している新規なカチオン性リポソームです。

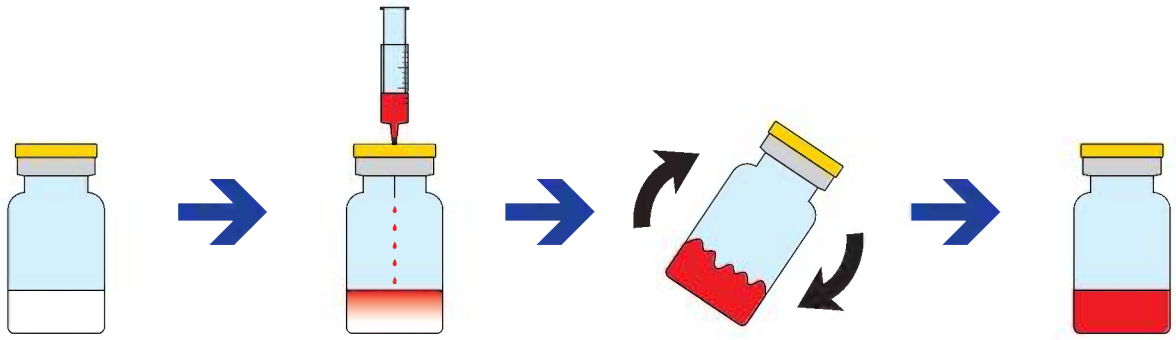


O,O'-ditetradecanoyl-*N*-(α-trimethylammonioacetyl)diethanolamine chloride (DC-6-14)

Product characteristics are shown below. Cationic charged liposomes are prepared using stearyl amine as a charged lipid. Nonionic and anionic charged liposomes are prepared using DPPG as charged lipids.
When drug solutions (using distilled water for injection) are prepared, the molarity of charged lipid required determines the drug concentration. Please refer to Suggestions for Use in section 3-2 below.

製品の組成と特性を以下に示します。カチオン性リポソームは電荷脂質であるステアリルアミンを処方に入れて作製しています。ノニオン性リポソームとアニオン性リポソームは、電荷脂質であるDPPGのモル数を変化させて作製しています。
薬物を水（注射用蒸留水）に溶解させて薬物水溶液を作製する際には、電荷脂質のモル数に応じて濃度を決める必要があります。詳細は2項の作製法をご覧ください。

Liposome Preparation is simple



(1) (2) (3) (4)

(1)Remove COATSOME EL from a cool storage temperature and allow it to come to room temperature.
(2)Add a drug aqueous solution in the temperature range of 16˚C to 40˚C.
(3)Shake the vial gently three to five times by hand.
(4)The liposomal drug is now ready for use.

(1)COATSOME ELを冷蔵庫から取り出し、室温に達するまで放置します。
(2)温度16℃から40℃の範囲内で、薬物水溶液を注入します。
(3)バイアルを手に持ち、3回から5回振ります。
(4)リポソーム化医薬品の出来上がり。

5-1. EL Series Product Characteristics

a) COATSOME EL-01-C, EL-01-N, EL-01-A

Product Name	EL-01-C	EL-01-N	EL-01-A
Type of Electrical charge	Cationic	Nonionic (Slightly anionic)	Anionic
Lipid components (μmol/vial)	DPPC:Cholesterol:Stearyl amine=52:40:8	DPPC:Cholesterol:DPPG =54:40:6	DPPC:Cholesterol:DPPG =30:40:30
Total amount of lipids	57 mg	61 mg	61 mg
Charged lipid (μmol/vial)	Stearyl amine (8μmol/vial)	DPPG (6μmol/vial)	DPPG (30μmol/vial)
Particle size (nm)	100-300	100-300	100-300
Osmotic pressure ratio*	0.8-1.1	0.8-1.1	0.8-1.1

*) liposome after addition of 2mL of distilled water

b) COATSOME EL-11-C, EL-11-N, EL-11-A

Product Name	EL-11-C	EL-11-N	EL-11-A
Type of Electrical charge	Cationic	Nonionic (Slightly anionic)	Anionic
Lipid components (μmol/vial)	POPC:Cholesterol:Stearyl amine=52:40:8	POPC:Cholesterol:POPG =54:40:6	POPC:Cholesterol:POPG =30:40:30
Total amount of lipids	57 mg	61 mg	61 mg
Charged lipid (μmol/vial)	Stearyl amine (8μmol/vial)	POPG (6μmol/vial)	POPG (30μmol/vial)
Particle size (nm)	50-250	50-250	50-250
Osmotic pressure ratio*	0.8-1.1	0.8-1.1	0.8-1.1

*) liposome after addition of 2mL of distilled water

New COATSOME EL-11-C, EL-11-N and EL-11-A contain POPC (COATSOME MC-6081, see p24) which is similar to EPC (egg yolk phospholipid). Our POPC synthesized from high quality plant origin materials is virus-free.

COATSOME EL-11-C、EL-11-N、EL-11-Aは、卵黄リン脂質に似た性質を持つPOPC (COATSOME MC-6081、p24参照)を配合した新しい製品です。このPOPCは、高い品質の植物系原料を用いて合成されたウィルスフリーのリン脂質です。

c) COATSOME EL-01-PN, EL-01-PA (Long-circulating Liposomes)

Product Name	EL-01-PN	EL-01-PA
Type of Electrical charge	Slightly anionic	Anionic
Lipid components (μmol/vial)	DSPE-PG8G:DPPC:Cholesterol:DPPG =4.2 : 20.5 : 15.2 : 2.3	DSPE-PG8G:DPPC:Cholesterol:DPPG =4.2 : 11.4 : 15.2 : 11.4
Total amount of lipids	29 mg	29 mg
Charged lipid (μmol/vial)	DPPG, (DSPE-PG8G) (2.3μmol/vial), (4.2μmol/vial)	DPPG, (DSPE-PG8G) (11.4μmol/vial), (4.2μmol/vial)
Particle size (nm)	50-250	50-250
Osmotic pressure ratio*	0.8-1.1	0.8-1.1

*) liposome after addition of 2mL of distilled water

New COATSOME EL-01-PN and EL-01-PA contain DSPE-Polyglycerine (SUNBRIGHT DSPE-PG8G, see p29) which has similar effect with DSPE-PEG regarding long circulation in blood.

COATSOME EL-01-PN、EL-01-PAは、ポリグリセリン-DSPE (SUNBRIGHT DSPE-PG8G、p29参照)を配合した新しい製品です。このポリグリセリン-DSPEは、PEG-DSPEのように血中滞留性を高める効果がありますので、血中滞留型リポソームを簡単にご使用いただけます。

d) COATSOME EL-01-D (For the delivery of DNA)

Cationic Liposomes for the delivery of DNA with a particle size of 100-200nm and osmotic pressure ratio of 0.8-1.1
DOPE:Cholesterol:DC-6-14=0.75:0.75:1.00(μmol/vial) Total amount of lipids:1.51mg.
Charged lipid is DC-6-14 (1.00μmol/vial) in this formulation.
Instructions for encapsulation are attached to the products in this case.

COATSOME EL-01-Dについては、別途調整方法が添付されます。

5-2. Suggestions for use

(1) For COATSOME EL-01-C, EL-01-N, EL-01-A, EL-11-C, EL-11-N, EL-11-A, EL-01-PN and EL-01-PA

a) After 2ml of distilled water for injection are added to the vial, the osmotic pressure ratio of the product will be 0.8 to 1.1 Adjust the osmotic pressure ratio according to the intended experiment (e.g., influence of osmotic pressure in in vivo testing).
b) To enhance the encapsulation efficiency of the drug by electrostatic interaction, drug solution should be added according to amounts of the charged lipids representing stearyl amine, DPPG respectively. The charged lipid/drug molar ratio should be no less than 2 and preferably no less than 3.
c) The aqueous solution of drug must be kept in the temperature range of 16˚C to 40˚C.
d) The prepared drug encapsulated in the liposomes should be used immediately after preparation. (Not for human use)

使用方法
(a) 2mlの蒸留水を添加することにより浸透圧が0.8-1.1となるように調整してあります。目的の実験によっては浸透圧の調整が必要です。
(b) 電気的相互作用により内包率を向上させるためには、電荷脂質(ステアリルアミン、DPPGのいずれかが該当)の医薬品溶液に対するモル比が重要であり、そのモル比は2以上で好ましくは3以上です。
(C) 医薬品の水溶液は16℃から40℃の範囲に調整しておきます。
(d) 調整したリポソーム化医薬品は直ちにご使用ください。(人には使用できません)

5-3. Analysis of encapsulation efficiency

1) Method to remove unencapsulated drugs

a) Dialysis
Prepared suspension of drug liposomes is placed into a dialyzing tube and dialyzed against an isotonic solution. Drug not encapsulated by liposomes is discharged into the isotonic solution outside the tube.

b) Gel Filtration
Prepared suspension of drug in liposomes is passed through a column filled with a gel filtration carrier under isotonic saline (150mM NaCl). Liposomes will pass through first and unencapsulated drugs will elute later. Select gel filtration (e.g. Sephadex G-50 for low molecular weight, Sepharoce 4B for high molecular weight) media according to the molecular weight of the drug to be removed.

c) Centrifugation
Centrifugation is carried out by high rotation (100,000 rpm) with isotonic saline solution (150 mM NaCl). Liposomal drug will settle to the bottom. Unencapsulated drug remains in the supernatant. Repeat the procedure two or three times at 100,000 rpm.

In order to obtain good results it is extremely important to equalize the osmotic pressure both inside and outside the liposomes. Temperature must be kept below liposome phase transition temperature during the entire procedure (below 40˚C).

内包化率の測定

（1）未内包化医薬品の除去による方法

a）透　析
調整したリポソーム液はを透析チューブに入れて、等張液により透析します。
未内包医薬品は等張液中に漏出します。

b）ゲルろ過
調整したリポソーム液は、ゲルろ過用充填剤を入れたカラム中を通過させます。
リポソームが先に、未内包医薬品が後からカラムを通過して出てきます。
医薬品の分子量によって、充填剤の種類（例:Sephadex G-50:低分子用、Sepharoce 4B:高分子用）を選択します。

c）遠心分離
生理食塩水（150mM NaCl）を加えて高速度下で遠心分離を行うと、リポソーム化医薬品は沈んで得られます。上澄みを除去した後再度生理食塩水を加え、通常は100,000回転で2回から3回繰り返します。
これらのどの方法においても、リポソームの内層と外層を等張に維持することが必須であり、調整工程を通じて温度はリン脂質の相転移温度以下に保たなければなりません。この場合は40℃以下で行います。

2) Quantification of encapsulated drug

After separating and removing nonencapsulated drug in liposomes according to a method mentioned above in (1), drug encapsulated in liposomes can be quantified by destroying a sample of liposomes by adding an appropriate quantity of surface active agent (Triton X-100) or by adding solvent (chloroform). When 2 ml of chloroform is used, 2 ml of methanol and 1.2 ml of water are also added into the tube. Then vortex, followed by centrifugation for 5 minutes under 3000 rpm. After destroying the liposomes by one of these methods, released drugs are quantified by ordinary methods such as HPLC.

（2）内包医薬品の定量

上述したいずれかの方法で未内包医薬品を除去した後、リポソームを破壊する方法によってリポソーム中に内包された医薬品の定量を行います。リポソームを破壊するには、界面活性剤を添加する方法と溶剤（クロロホルム）を入れて水相と溶剤相とに分離する方法があります。いずれにしても、リポソームを破壊した後は、通常の方法にて医薬品の定量を行います。

5-4. Application Data for the COATSOME® EL Series

(1) Cationic Liposomes, COATSOME EL-01-C

Drug	Particle Size (nm)	Encapsulation Efficiency (%)
Bucladesine sodium (0.5mg/ml)	173	20-25
Sodium salicylate (0.16mg/ml)	162	40-45
DNA (Salmon Testes, 100ug/ml)	942	100

(2) Nonionic Liposomes, COATSOME EL-01-N

If higher encapsulation efficiency is desired, the concentration of cationic drug must be below 1 mM. The DPPG (3mM)/drug molar ratio must be higher than 3 in order to obtain high encapsulation efficiency.

内包効率を向上させるためには、カチオニック医薬品の濃度は1mM以下とする必要があります。高い内包効率を得るためには、DPPG（3mM）と医薬品とのモル比は3以上とする必要があります。

(3) Anionic Liposomes, COATSOME EL-01-A

Drug	Particle Size (nm)	Encapsulation Efficiency (%)
Doxorubicin hydrochloride (1mg/ml)	150	97-100
Amikacin sulfate (1mg/ml)	140	98-100
Streptomycin sulfate (1mg/ml)	145	95-100
Procainamide hydrochloride (1mg/ml)	152	80- 85
Epirubicin hydrochloride (1mg/ml)	146	97-100
Pirarubicin hydrochloride (1mg/ml)	129	97-100

If higher encapsulation efficiency is desired, the concentration of cationic drug must be below 5 mM. The DPPG (15mM)/drug molar ratio must be higher than 3 in order to obtain high encapsulation efficiency.

内包効率を向上させるためには、カチオニック医薬品の濃度は5mM以下とする必要があります。高い内包効率を得るためは、DPPG（15mM）と医薬品とのモル比は3以上とする必要があります。

Comparison of activity in vivo*

Drug Maker	Liposome	AUC (%dose/ml-min)	MRT (min)	CL tot (ml/min/Kg)
Mannitol	EL Series	237	448	0.174
	Bangham Method	2204	398	0.179
Inulin	EL Series	2137	408	0.175
	Bangham Method	2315	373	0.174

*K.Yachi et al, *Biopharm. & Drug Dispos.*, **17**, 589-605(1996):
Composition:DPPC:DPPG:Chol=27:53:20 (Anionic Liposomes)

(4) Cationic Liposomes for gene delivery (COATSOME EL-01-D)

Transfection Activities *in vitro*[*1] by Luciferase Assay[*5] (Light units/mg protein·sec)

	HRA	MEIIL	ES-2
Serum (-)	6,048	6,291	1,325
Serum (+)	11,378	2,913	689

Transfection Activities *in vivo*[*2] in intraperitoneal disseminated tumors[*5]

	Percentage of LacZ-positive cells	n
COATSOME EL-01-D	1.00 ± 0.11	5
Commercially available A	0.38 ± 0.26	3
Commercially available B	0.62 ± 0.21	3
Commercially available C	0.23 ± 0.23	3

Relationship Between Transfection Efficiencies and Cell Mitotic Activities[*3,*5]

Cell lines	Percentage of LacZ-positive cells		Labeling index (%)
	COATSOME EL-01-D	Commercially available C	
Cancer cell lines			
HRA	42.9 ± 3.8	4.7 ± 1.0	68.2 ± 1.6
mEIIL	4.5 ± 1.0	0.6 ± 0.1	41.0 ± 1.2
ES-2	23.7 ± 1.9	3.3 ± 0.9	41.9 ± 4.5
OVHS-1	0.8 ± 0.4	0.9 ± 0.3	6.1 ± 1.4
MCAS	1.3 ± 0.1	< 0.1	51.5 ± 6.1
SKOV3	11.6 ± 1.8	0.2 ± 0.2	35.2 ± 6.7
OVCAR3	12.3 ± 1.1	0.4 ± 0.4	34.4 ± 3.7
KK	2.7 ± 0.7	0.1 ± 0.1	26.2 ± 4.3
KOC-3S	5.9 ± 2.1	2.6 ± 1.6	ND[*4]
Nakajima	4.9 ± 1.7	0.1 ± 0.1	ND[*4]
KF	15.5 ± 2.3	0.1 ± 0.1	ND[*4]
SW626	26.8 ± 4.6	0.2 ± 0.1	ND[*4]
Colo320DM	32.3 ± 3.2	5.2 ± 1.1	ND[*4]
HRA	42.9 ± 3.8	4.7 ± 1.0	ND[*4]
mEIIL	4.5 ± 1.0	0.6 ± 0.1	ND[*4]
Normal cell lines			
Fibroblast (TIG)	9.6 ± 2.2	0.9 ± 0.2	42.4 ± 10.7
Fibroblast (IMR)	2.5 ± 0.4	1.6 ± 0.5	14.2 ± 3.2
HUVEC0103	< 0.1	< 0.1	22.2 ± 1.6
HUVEC1204	< 0.1	0.2 ± 0.1	11.3 ± 0.2
HUVEC0923	< 0.1	< 0.1	18.2 ± 4.1

*1) Transfection performed in the absence or presence of 10% fetal bovine serum (FBS). Data shown represent mean of three experiments.

*2) mEIIL cells growing in peritoneal cavities of nude mice were transfected with liposome/CAG-lacZ (20μg) and the percentage of lacZ-positive cells was determined. Data shown represent mean: ± SD.

*3) Percentages of LacZ-positive cells and labeling indexes were determined. Transfection was performed in the presence of 10% FBS. Data shown represent mean ± SD. n=3

*4) ND = Not done

*5) Reference: A. Kikuchi et al., *HUMAN GENE THERAPY*, **10**: 947-955 (1999).

III. High-purity Polysorbate 80

NOFABLE ESO-9920 of NOF Corporation is an extra highly purified Polysorbate 80 and an extremely low impurity profile based on both of a purification technology of Oleic Acid (99% up) and a noble ethoxylation technology.
NOFABLE ESO-9920 provides a low allergic reaction among Polysorbate 80. New study indicates that NOFABLE ESO-9920 has a low degranulation characteristics compare with competitor's injectable Poylsorbate 80 due to its low impurity profile.

日本油脂の高純度オレイン酸誘導体ポリソルベート80（NOFABLE ESO-9920）は純度が99%以上という、非常に純度の高いオレイン酸を使用しているため、脂肪酸に由来する不純物が極端に少ない製品となっています。
この特徴は安全性にもつながっており、最新のデータではラット肥満細胞を用いた試験において一般のポリソルベート80に比較してヒスタミン遊離が少ないという結果が得られていて、アレルギーを起こしにくいポリソルベートとして注目を集めています。

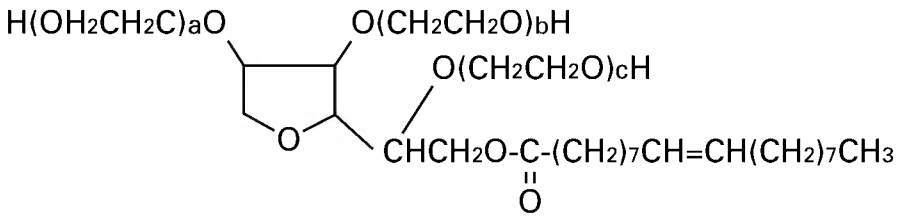


Appearance comparison of High-purity Polysorbate 80

Characteristics of High-purity Polysorbate 80

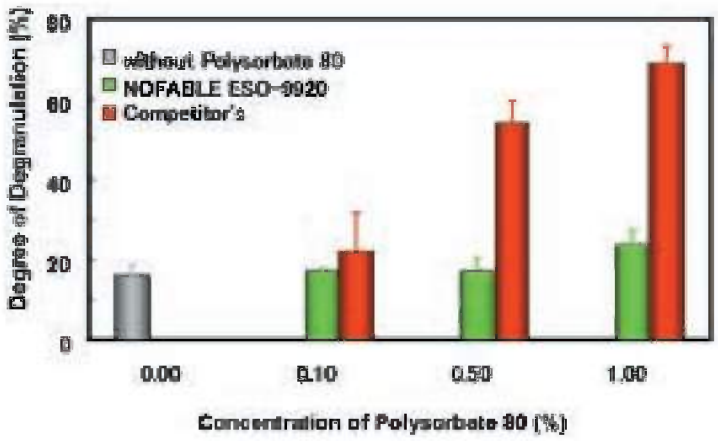
	NOFABLE ESO-9920	Conventional Polysorbate 80
Purity of Oleic acid (%)	99	65
Odor	-	+++

Polyoxyethylene Sorbitan Oleate (Polysorbate 80)



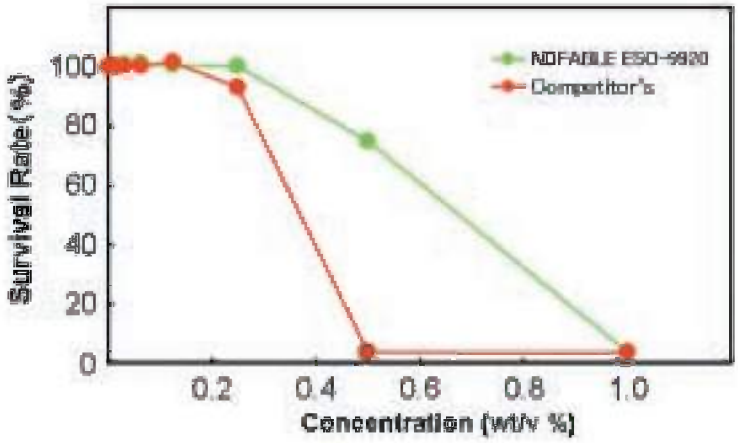
Product Name	Trade Name	Oleic acid Content	CAS NO.
Polyoxyethylene Sorbitan monooleate	NOFABLE ESO-9920	>99%	9005-65-6

Degranulation Test (An allergic study model)



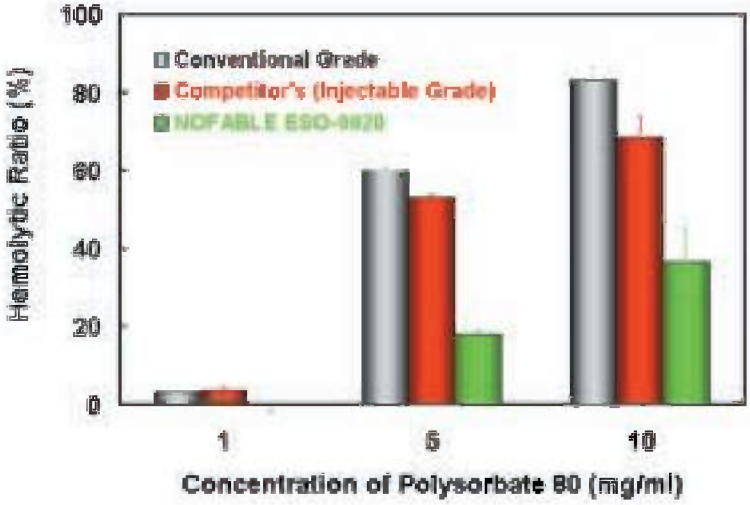
Effect of Polysorbate 80 on degranulation in RBL-2H3 mast cell. The cells were treated with different concentration of polysorbate 80 for 60 mins. The degree of Degranulation is determined by measurement of released β -hexosaminidase into supernatants.

Cell Toxicity Test



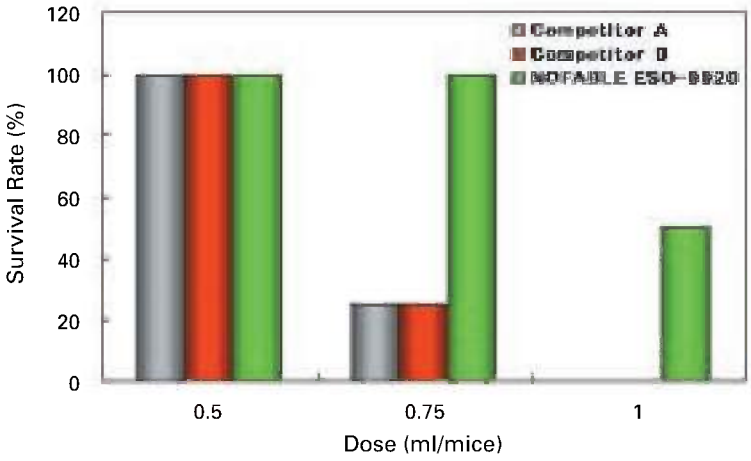
Effect of concentration of Polysorbate 80 on cell toxicity using SIRC Cell. The cells were treated with each Polysorbate 80 for 24 hrs. The viable cells are determined by Neutral Red Uptake method.

Hemolysis Test



Effect of Concentration of Polysorbate 80 on Hemolytic Ratio. Red Blood cells from guinea pig are treated with Polysorbate 80 for 60 mins. Each hemolytic ratio is determined by absorbance of 576nm in the solution.

Survival Rate



Effect of concentration of Polysorbate 80 on Survival Rate in BALB/c mice. Polysorbate 80 is diluted by PBS and injected into mice with intravinous administration.

Status of European Pharmacopoeia (EP)

NOFABLE ESO-9920 which consists of high purity Oleic Acid (99% up), has not met EP 5th Edition compendium due to a cap of purity (85% max.) of Oleic Acid of Polysorbate 80 in the compendium. The cap of the purity of Oleic Acid has been eliminated in EP 5.4th Edition through negotiations between European Medicals Agency (EMEA) and NOF CORPORATION.
Now, NOFABLE ESO-9920 (Polysorbate 80) meets Multi-compendial-NF, EP and JP

European Pharmacopoeia (EP) 5th EDITIONに収載されているポリソルベート80にはオレイン酸純度の上限が規定(58～85%)されており、ESO-9920はその高純度(99%以上)が故にEPの規格から外れるという矛盾が生じておりました。
日本油脂がEP当局に働きかけた結果、EP追補5.4 にてオレイン酸純度の上限が撤廃されました。
これにより、日本油脂のポリソルベート80(ESO-9920)は3極(JP、EP、NF)に対応した製品となります。

IV. High-purity Oleic Acid Derivatives <NOFABLE® Series>

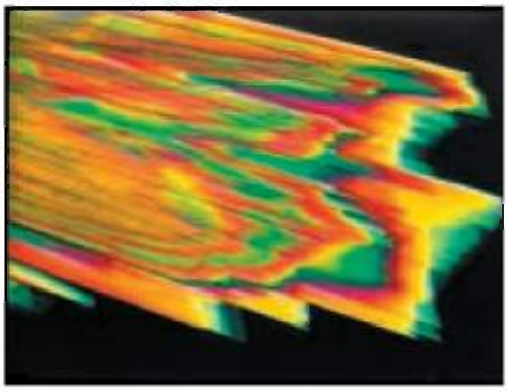
NOF has succeeded to produce 99% purity of oleic acid commercially. NOF also produces highly purified oleic acid derivatives (glycerol-oleate, ethyl-oleate, polyoxyethylene-sorbitan-monooleate, etc.) for pharmaceutical use. These products contain reduced amounts of impurities such as peroxides or aldehydes typically seen in oleic acid product.
NOFABLE Series is obtained colorless and odorless products. Our NOFABLE ESO-9920 (polysorbate 80) is highly recommended as a nonionic emulsifier (injection grade) for drug products because degradation of drugs by oxidation is suppressed.

99%のオレイン酸を工業的なプロセスで生産する方法を開発しました。
酸化され易い不飽和脂肪酸を除去しているので、酸化に対する安定性も向上し、過酸化物やアルデヒド含量の少ない誘導体が得られ、医薬品用途には最適です。
NOFABLEシリーズは写真にも示したようにほとんど無色透明かつ無臭に近い製品です。特に、NOFABLE ESO-9920（ポリソルベート80）は、酸化物の少ない点から医薬品の分解を極力防止できる製品として、注射医薬品の非イオン系乳化剤として推奨します。

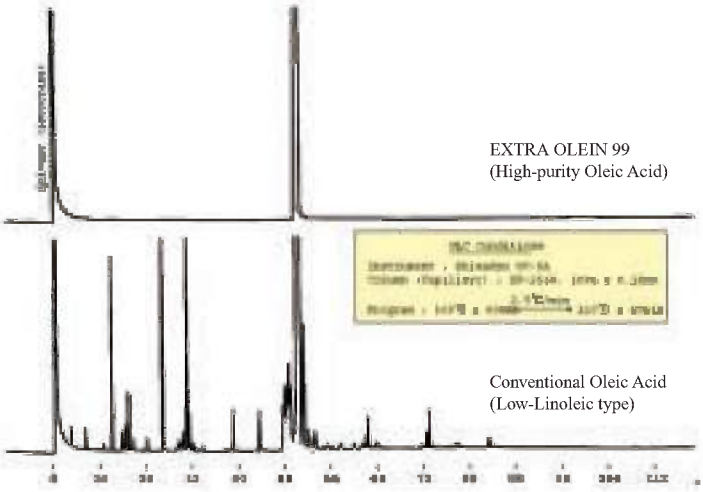
1. High-purity Oleic Acid <EXTRA OLEIN® 99>

$$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$$

Product Name	Trade Name	Oleic acid Content	CAS NO.
Oleic Acid	EXTRA OLEIN 99	>99%	112-80-1

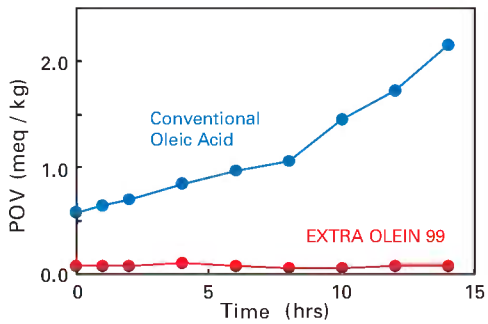


Crystal of Oleic Acid



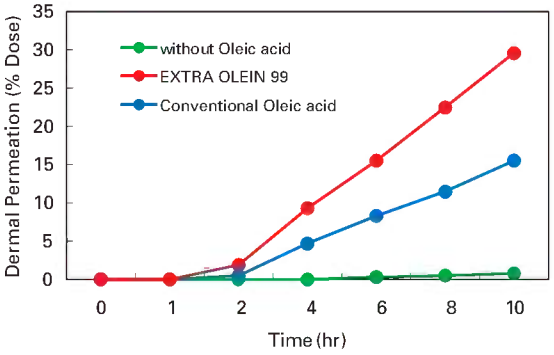
Gas chromatogram for NOF Oleic acid compared to conventional product

Oxidation Stability of Oleic Acid



Formation of peroxides in oleic acid during incubation at 50℃.

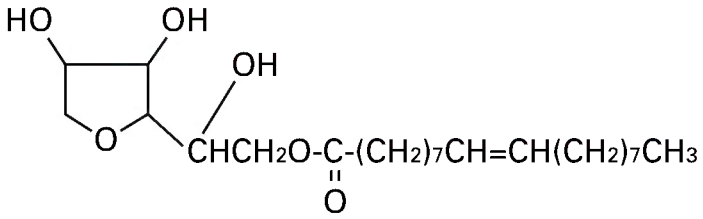
Dermal Absorption Enhancement with Oleic Acid



Dermal Absorption Enhancement of Indomethacin with Highly Purified Oleic Acid (EXTRA Series).

2. High-purity Oleic Acid Derivatives

2-1. Sorbitan Oleate



Product Name	Trade Name	Oleic acid Content	CAS NO.
Sorbitan monooleate	NOFABLE SO-991	>99%	1338-43-8

2-2. Glycerol Oleate

$$HO\text{-}CH_2\text{-}CH(OH)\text{-}CH_2\text{-}OCO\text{-}(CH_2)_7CH{=}CH(CH_2)_7CH_3$$

Product Name	Trade Name	Oleic acid Content	CAS NO.
Glycerol monooleate	NOFABLE GO-991	>99%	25496-72-4

2-3. Ethyl Oleate

$$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOCH_2CH_3$$

Product Name	Trade Name	Oleic acid Content	CAS NO.
Ethyl oleate	NOFABLE EO-99	>99%	111-62-6

Product Name	Regulatory status*1
Oleic acid	JPE
Sorbitan monooleate	JPE
Polyoxyethylene sorbitan monooleate	JP EP NF
Glycerol monooleate	JPE
Ethyl oleate	JPE

*1 JP : Japanese Pharmacopeia
JPE: Japanese Pharmaceutical Excipients
EP : European Pharmacopeia
NF : Natural Formura

V. High-purity low molecular weight MACROGOL

(PEG-200,300,400)
SUNBRIGHT® DKH-02HB,DKH-03HB,DKH-04HB

NOF has developed high quality low molecular weight Polyethylene Glycols specific for pharmaceutical excipients.
European Pharmacopoeia (EP) and U.S. Pharmacopoeia (USP) both have regulations for EG (Ethylene Glycol) and DEG (Diethylene Glycol) content, due to their toxicity (EP not more than 0.4%, USP not more than 0.25%). However, it is very difficalt to achieve low levels of EG and DEG contaminants in low molecular weight PEGs.
Our SUNBRIGHT DKH series has a much reduced EG, DEG content than competitors, so NOF can guarantee more strict specifications than EP, USP regulations. Moreover, we have eliminated other common impurities, such as ethylene oxide, dioxane, peroxide and aldehyde derivatives.
Our specifications are shown below. NOF can also supply PEGs that meet JP, EP and USP specifications.

EG(エチレングリコール)およびDEG(ジエチレングリコール)の極めて少ない医薬品添加剤用ポリエチレングリコールを開発しました。毒性の点からEGとDEGの含有量をEPは4000ppm以下、USPは2500ppm以下に規制しています。当社、EP、USP規格を大きく下回るEG、DEGの含有量を保証します。さらにエチレンオキサイド、ジオキサン、過酸化物、アルデヒド誘導体などの不純物を取り除いています。EP、USP、JP、に適合したPEG200、300、400も供給できます。

1) EG, DEG content in PEG 200

		DKH-02HB (NOF)	company A	company B
EG	(ppm)	**61**	217	11,494
DEG	(ppm)	**181**	29,095	43,548

EG, DEG content of PEG 400

		DKH-04HB (NOF)	company A	company B
EG	(ppm)	**41**	740	630
DEG	(ppm)	**31**	836	753

2) Specifications

Parameter	Method	Specification		
		DKH-02HB	DKH-03HB	DKH-04HB
Completeness and color solution	USP	Pass		
Viscosity		3.9-4.8	5.4-6.4	6.8-8.0
Average molecular weight		190-210	285-315	380-420
pH		4.5-7.5		
Residue on ignition (%)		NMT 0.1		
Heavy metals (ppm)		NMT 5		
Free ethylene oxide and 1,4-dioxane (ppm)		NMT 10 (Ethylene oxide : NMT 1, 1,4-Dioxane : NMT 10)		
Limit of ethylene glycol and diethylene glycol (%)		NMT 0.05		
Organic volatile impurities		Pass		

3) Other specifications

Peroxide value (meq/Kg)	NOF	NMT 3
Formaldehyde (ppm)	NOF	NMT 5

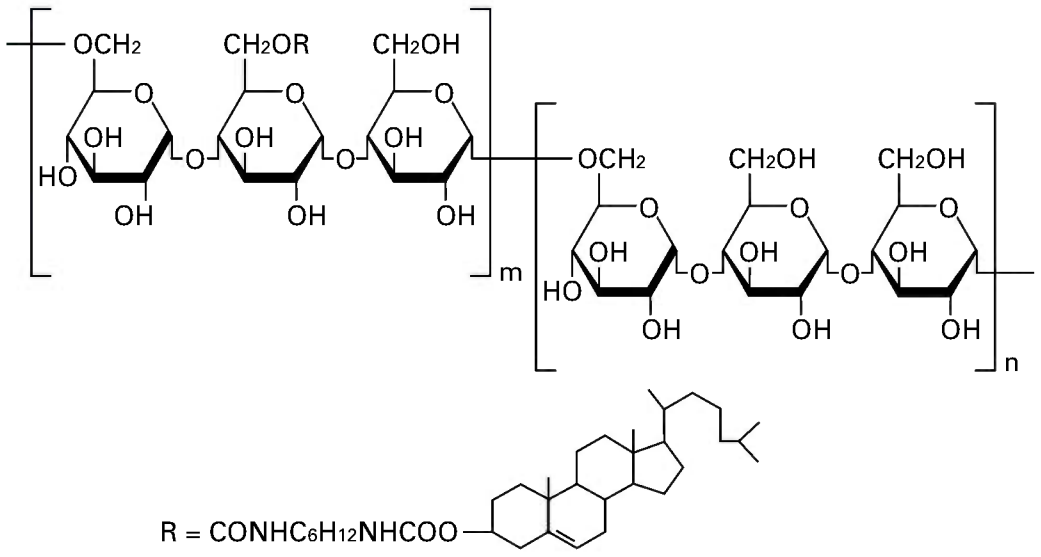
NOF can also supply products without anti-oxidant agent, BHT.

VI. Cholesterol Pullulan

(PUREBRIGHT® CP-100T)

NOF has developed unique hydrogel-nanoparticles based upon hydrophobic polysaccharides (PUREBRIGHT CP) for DDS in which specific drugs and/or peptides can be encapsulated. Pullulan starch partly modified with cholesterol (CP) shows unique characteristics described below.
In water, attached cholesterol molecules form hydrophobic cores, then these cores cause polymer self-aggregation localizing attached pullulan outside. Resulting CP-nanoparticles can stabilize proteins and/or peptides by forming hybrid-complexes[1] shown below. These particles also stimulate the immune system through interactions with dendritic cells, so that vaccine therapy for cancer may be demonstrated by encapsulating cancer-specific antigens, such as Her2[2].
CP structure and characteristics are shown below.

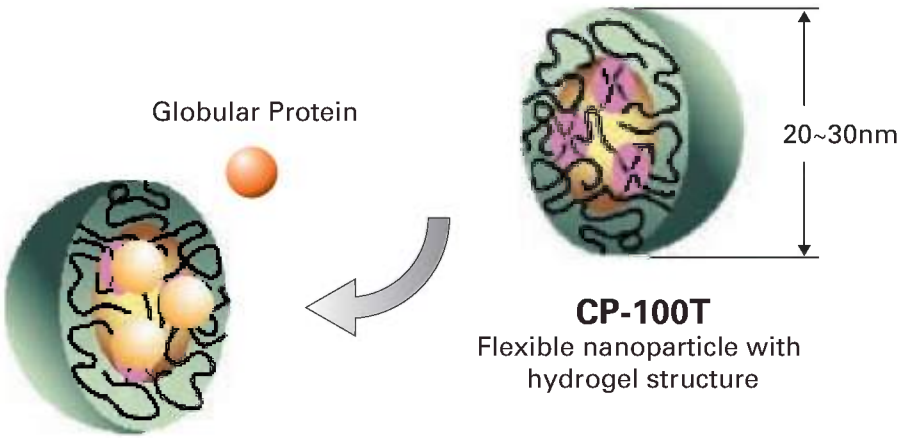
薬物やタンパク質等を内包可能な、疎水化多糖(PUREBRIGHT CP)からなるユニークなハイドロゲルナノ微粒子を開発いたしました。コレステリル基を導入したプルラン(CP)は、水中でCP中のコレステリル基を非共有結合の架橋点として数分子間で自己会合することにより、単分散なナノ微粒子を形成します。さらに、このCPナノ微粒子は、下図に示すような様々な球状タンパク質と複合体を形成することから、酵素の安定化や薬物のキャリアとしての有用性が明らかとなっています[1]。また、癌抗原タンパク(Her2)を複合化することで、生体の免疫システムを活性化させる癌ワクチン療法の開発にも成功しています[2]。CPの構造と特長を以下に示します。



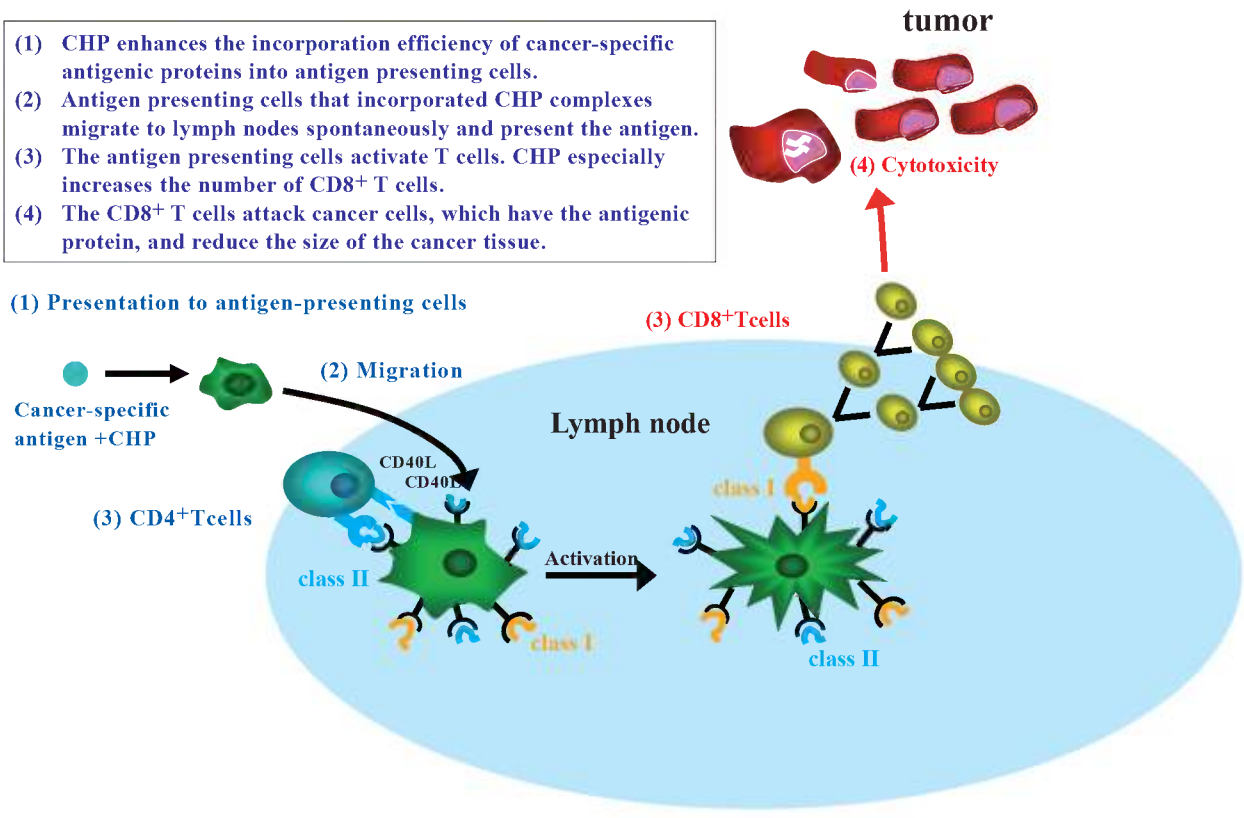
Structure of PUREBRIGHT CP-100T

Molecular weight of pullulan : ca. 100,000
1.0~2.0 cholesteryl groups per 100 glucose units is substituted

References
1) K.Akiyoshi and J.Sunamoto, *Supramolecular Science,* **3**, 157-163 (1996)
2) H.Shiku, et al, *Cancer Chemotherapy and Pharmacology,* **46**, S77-S82, JUN(2000)



$$\text{CP-n (Protein) Complex} \underset{}{\overset{K}{\rightleftharpoons}} \text{CP agg + n (Protein)}$$

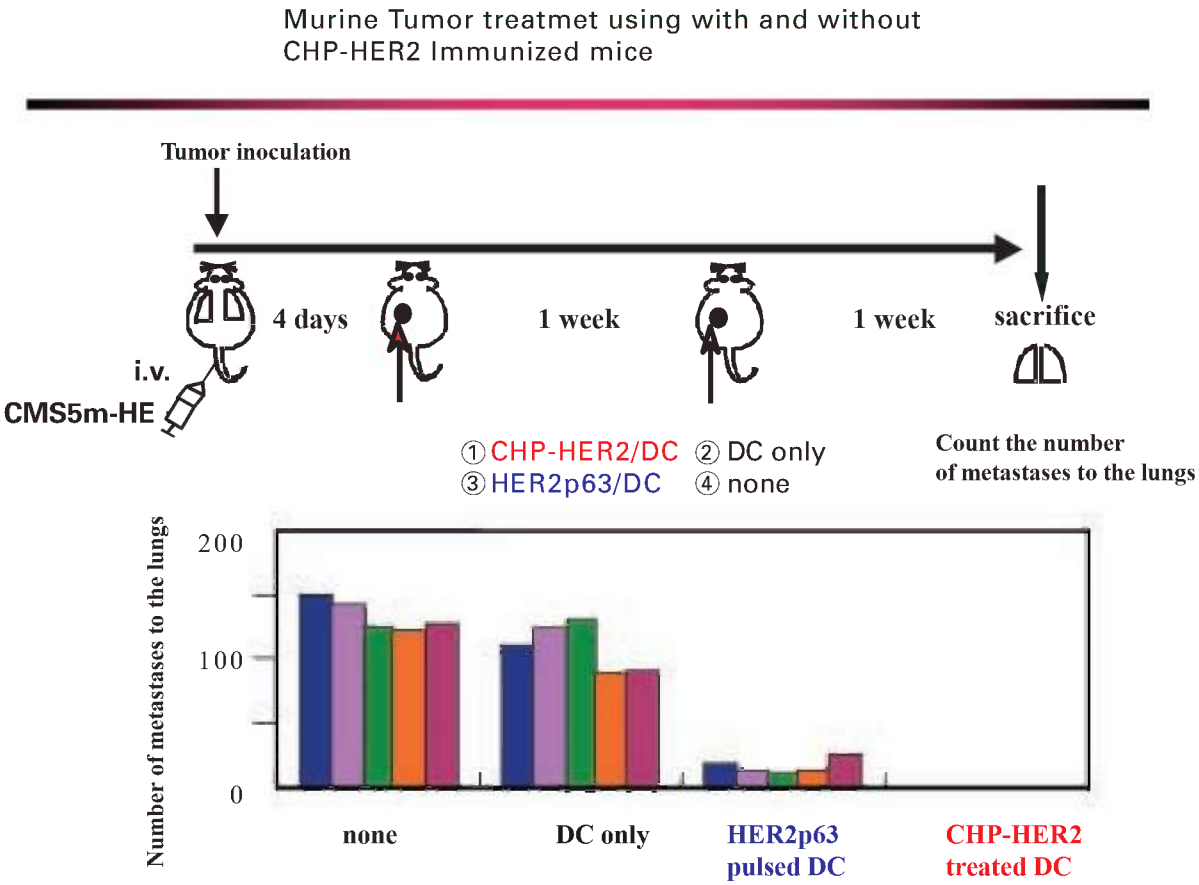


Proposed adjuvant activity of CP-100T particles loaded with antigene

Clinical Studies is using CHP-HER2 (Prof. Shiku , Department of Medicine, Mie University, Japan)

*Pre-clinical studies

CHP-(146Her2) complexes were used in a solid tumor mouse model in vivo.
CHP-HER2 complexes effectively suppressed metastasis of cancer cells to the lungs.



Phase I clinical study led by physicians in humans.

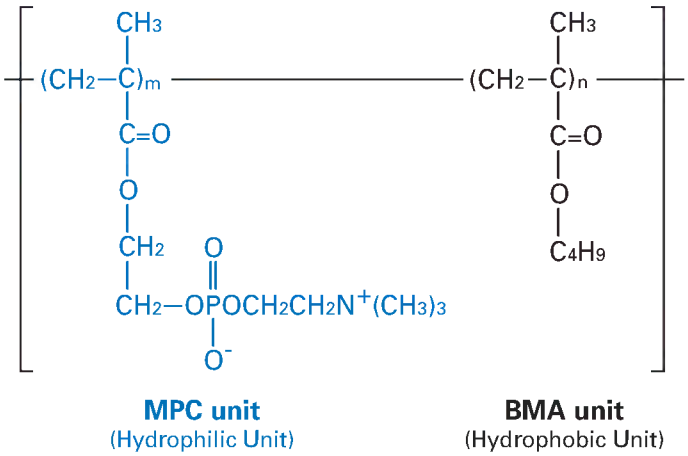
CHP-(146Her2) complexes were used in a safety and efficacy study.
Safety:
Test conditions: 300 μg of CHP-Her2 complexes was parenterally injected at intervals of two weeks.
Results: Safety of CHR-Her2 complexes was confirmed.
Antibody Titer:
Antibody titers against 146Her2 in blood were measured using the ELISA method, and increases in antibody titers was confirmed.
Antibody expression:
Presence of the antibody against 146Her2 in the blood was confirmed using the Western blotting method.
Activation of $CD8^+$ T cells:
Number of $CD8^+$ T cells were confirmed to increase as well as $CD4^+$ T cells in vaccinated subjects by flow cytometry.

VII. MPC Polymers

(PUREBRIGHT® MB Series)

NOF is developing water-soluble polymers (PUREBRIGHT MB) to dissolve drugs of low aqueous solubility. Polymers comprise 2-methacryloyloxyethyl phosphorylcholine (MPC) and n-butyl methacrylate (BMA).

水に難溶性の医薬品を可溶化させることのできるユニークな水溶性ポリマー(PUREBRIGHT MB)を開発しました。2-メタクロイルオキシホスホリルコリン(MPC)とn-ブチルメタクリレート(BMA)とのコポリマーで構成されていますが、疎水性のBMAユニットを導入することにより疎水結合を介して可溶化されます。



MPC ; 2-Methacryloyloxyethyl phosphorylcholine　BMA ; n-Butyl methacrylate

Structure of NOF's MPC polymer

Product name	
PUREBRIGHT MB-37-50T	For parenteral use
PUREBRIGHT MB-37-100T	For oral use

How to use

STEP 1 Prepare 30mg/ml of PUREBRIGHT MB aqueous solution(Concentration can be varied according to the solubility of drugs). Warm aqueous solution to 60˚C in case not dissolve completely.
STEP 2 Add suitable amounts of drugs into PUREBRIGHT MB solution with stirring.
STEP 3 Warm the solution at 60˚C for 5 minutes(Temperature and period of time may be varied according to the solubility and stability of drugs)

使用方法

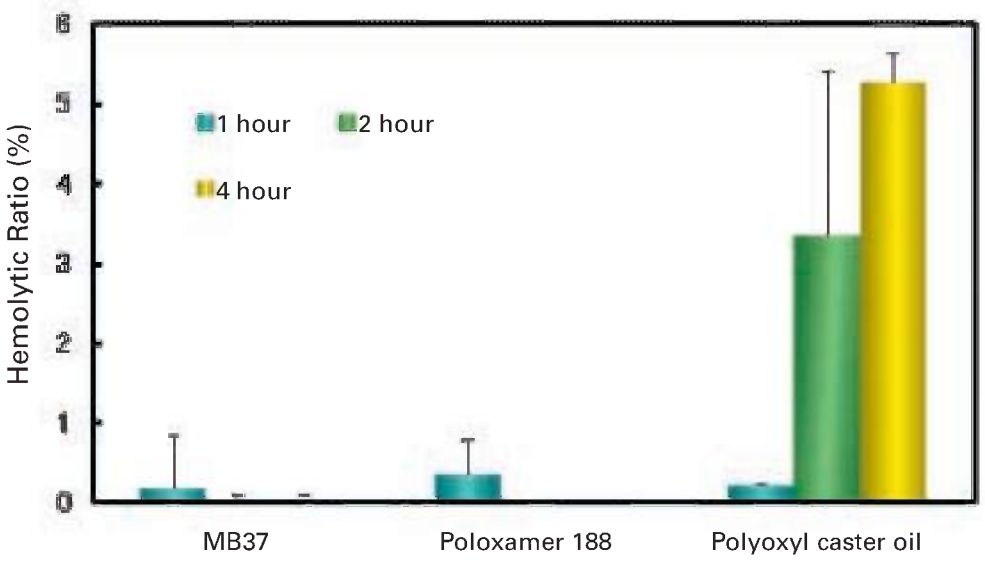
Step1 30mg/mlの濃度になるようにPUREBRIGHT MBの水溶液を作成する(濃度は薬剤の溶解性に応じて増減する)。溶けにくい場合は60℃に加熱し、完全に溶解させる。
Step2 Step-1で作成したPUREBRIGHT MBの水溶液を攪拌しながら、希望濃度になるように薬剤を添加する。
Step3 60℃で5分間、加熱処理をする(温度と時間は薬剤の熱安定性と溶解性に応じて増減する)。

We would like to describe toxicological data as follows.

1. Toxicological Data
   PUREBRIGHT® MB-37 series show only slight hemolization in comparison with other solubilizers such as poloxamer and polyoxyethylene castor oil. LD_{50} are obtained as more than 25,000mg/kg (mice, orally) and more than 1000mg/kg, 8rats, iv).
2. Solubilizing ability
   PUREBRIGHT MB-37 series can dissolve poorly water-soluble drugs in aqueous medium. (Refer to Section VII, PUREBRIGHT SL-110)

Toxicological Information of PUREBRIGHT MB-37 Series

Acute toxicity (100T**)	Oral LD_{50} (Mice) > 25,000 mg/Kg
Acute toxicity (50T*)	Intravenous injection LD_{50} (Rats) > 1,000 mg/Kg
Antigenecity (50T*)	Negative
Mutagenecity (50T*)	Negative
Skin sensitization (100T**)	Non skin sensitization (Guinea pig)
Skin toxicity (100T**)	Non-primary irritation (Rabbit)
Eye stimulative (100T**)	Practically non-stimulative to eye mucosa (Rabbit)
Hemolysis (50T*)	Refer to next figure



Effect of Solubilizing Agent on Hemolytic potential

Red blood cells(guinea pig) are exposed to test compounds (5%) for each hours, and the hemolytic ratio is determined by absorbance of 576nm. References:

1) K.Ishihara, et al., *J.Biomed.Mater.Res.* ,**65A**, 2. 210-215 (2003)
2) T.Konno, et al., *Proceed.Int'l. Symp. Control.Rel.Bioact.Mater.*, **29**, 464 (2002)

VIII. Hydrophobic Drug Solubilization Kit

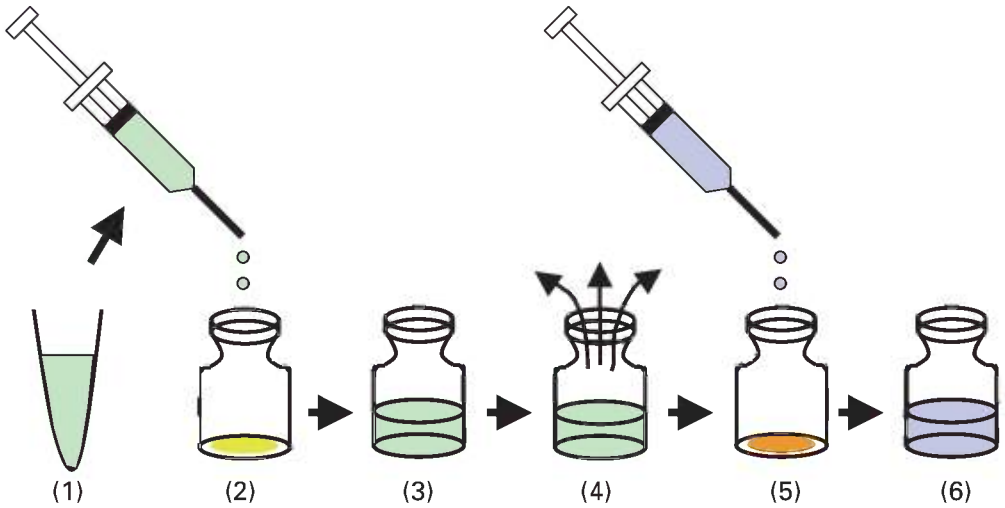
(PUREBRIGHT® SL Series)

NOF CORPORATION has developed a new Solubilization Kit for poorly water-soluble drugs using our various sophisticated drug delivery technology. Drug solubilizing procedure is simple, facilitating NCE screening or toxicological testing.

当社はさまざまなDDS技術を結集し、難水溶性薬剤の可溶化キットを開発しました。
キットの使用方法は極めて簡単ですので、安全性試験など、新規開発薬剤の初期テストのスクリーニングを容易に行うことができます。



PUREBRIGHT SL-000-S
(2 vials of each: total 10 vials)
Content:
PUREBRIGHT SL-110: MPC polymer based
PUREBRIGHT SL-220: PEG-phospholipid based
PUREBRIGHT SL-310: PEG-cholesterol based1
PUREBRIGHT SL-350: PEG-cholesterol based2
PUREBRIGHT SL-411: Phospholipid complex based
Bulk supply also available.



< Preparation >
The following instructions describe preparation of 5 vials of test solution at once.

(1) Weigh drugs into test tube or cuvette according to desired concentration in the finished solution. If you wish to make 1mg/mL finished solution, weigh 12mg of drug, add 3mL of ethanol (select other solvent when drug is not soluble in ethanol) into test tube, and then dissolve completely by gentle shaking.

希望の最終濃度の4倍濃度の薬剤/溶剤溶液を調製する。
例) 希望最終濃度　1mg/mLの場合、4mg/mLのエタノール溶液を調製する。

(2) Take 500 µL of drug solution with pipette, and pour the solution into each vial of PUREBRIGHT SL Kit respectively.

PUREBRIGHT SLバイアルに(1)で調製した溶液を500µL加える。

(3) Dissolve them completely with shaking. Warm the vial to 50˚C* to achieve a clear solution.

十分に攪拌し、完全に溶解させる。溶解しにくい場合は50℃程度まで加温して溶解させる。

(4) Evaporate the solvent from the vial according to one of the following methods.
a) Place the vial into vacuum chamber previously warmed to 50°C*, then evaporate the solvent under vacuum.
b) Evaporate the solvent with N_2 gas at 50°C where vials are placed in a warm bath. Please be careful with handling to avoid the fire or accident with volatile solvent.

エバポレーションにより溶媒を除去する。
例1）50℃程度まで加温しながら真空乾燥機でエバポレートする（引火性のある溶媒を用いるときには窒素ガスを流す等して充分に注意してください）。
例2）50℃程度まで加温しながら窒素ガス等によりエバポレートする。

(5) Add 2 mL of deionized water into the vial, and the dissolve completely with shaking. Add 1.8 mL of deionized water in case of using PUREBRIGHT SL-411.

イオン交換水を2mL（PUREBRIGHT SL-411は1.8mL）添加後、十分攪拌を行い、可溶化させる。

Drug in aqueous solution (e.g. 1mg/mL) is now ready.

1mg/mLの濃度の薬物溶液の完成。

Remarks: *Temperature could be changed depending upon drug stability.

注）温度は薬剤の熱安定性により調節してください。

Drug Solubilization with PUREBRIGHT® (薬剤の可溶化例)

Drug	Pep	IND	MPS	CLF	NFD	PRB	CLM	PTX
Concentration (mg/mL)	6.0	2.5	0.75	7.5	2.5	5.0	0.5	1.0
PUREBRIGHT SL-110	I	I	S	I	I	A	I	S
PUREBRIGHT SL-220	S	S	N	I	I	I	I	S
PUREBRIGHT SL-310	S	S	S	S	S	I	S	S
PUREBRIGHT SL-350	I	I	N	N	I	I	I	S
PUREBRIGHT SL-411	S	N	N	N	N	S	N	N
Polyoxyl 35 Castor oil	I	I	I	A	I	I	I	I

PUREBRIGHT & Polyoxyl 35 Castor oil : 3% (w/w)

Pep: Peptide type Drug
IND: Indomethacin
MPS: Medroxyprogesterone acetate
CLF: Clofibrate
NFD: Nifedipine
PRB: Probucol
CLM: Chlormadinone acetate
PTX: Paclitaxel

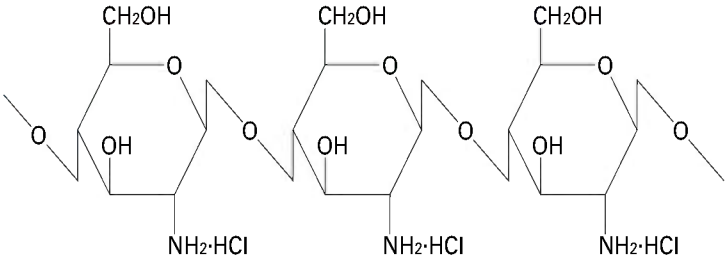
S: Soluble
A: Almost soluble
I : Insoluble
N: No data

IX. High purity CHITOSANs for DDS

(CHITOSAN GH Series)

Chitosan is a cationic polysaccharide obtained from arthropod exoskeleton, consisting of linear poly(1,4) N-acetyl-D-glucosamine corresponding to the deacetylated form of chitin. It is applied in diverse industrial areas such as food, cosmetics and agricultural additives. Other chitosan applications such as hydrogels or gene carrier are published recently. Chitosan has rarely been considered for pharmaceutical use due to large amounts of endotoxin contaminants. Using sophisticated purification technology, NOF and Yaizu Suisankagaku Industry CO., LTD. (YSK) have succeeded in producing chitosan hydrochloride CHITOSAN GH-400EF in which endotoxin levels are remarkably low.

キトサンは、キチンの脱アセチル化体で、グルコサミン残基がβ1-4結合により直鎖状に連なった構造をもつカチオン性の高分子多糖類です。その物理的・生理的機能性を利用して食品原料、化粧品原料、工業原料、農業資材等様々な分野で応用されています。また医薬品分野においても、キトサンの持つユニークな物性が近年注目を集めており、ハイドロゲルや遺伝子導入用キャリヤーなどの各種の応用例が発表されてきています。一方キトサンはエンドトキシンを吸着しやすい性質から、これまで医薬品分野での応用においては支障がありました。NOFと焼津水産化学工業株式会社(YSK)は独自の製法により、エンドトキシン量を大幅に低減化した医薬品グレードのキトサン塩酸塩「CHITOSAN GH-400EF」を開発しました。





CHITOSAN GH-400EF is obtained by further purification to remove endotoxin from food additive quality chitosan, produced in a sanitized factory starting from shells of *pandalus borealis* captured from the North Atlantic Ocean near Norway.

CHITOSAN GH-400EFは、ノルウェイ沖北大西洋上で捕獲される北極エビPandalus borealis の殻を出発原料として、衛生管理の行き届いた工場で製造された食品用キトサンを原料として、独自の製法により精製し、エンドトキシンを低減しています。

Typical Residual Endotoxin

CHITOSAN GH-400EF	Generic Chitosan (Industrial Grade)
50EU/g	2,300EU/g

Specifications

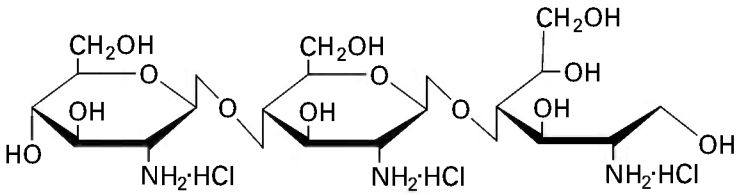
ITEM	SPECIFICATION	TEST
Matter insoluble in water	NMT 0.5%	EP
pH	4.0~6.0	EP
Viscosity	NMT 100 cps	NOF
Degree of deacetylation	NLT 80%	EP
Chlorides	10.0~20.0%	EP
Heavy metals	NMT 40ppm	EP
Loss on drying	NMT 10%	EP
Sulphated ash	NMT 1.0%	EP
Endotoxin	NMT 100 EU/g	NOF

CHITOSAN GH-003

CHITOSAN GH-003 is a short oligo-saccharide whose structure contains ß-1,4 bonds linking 3 D-glucosamine molecules shown below. Our high purity (>99% determined by HPLC) CHITOSAN GH-003 is more stable against degradation than ordinary chitosan oligosaccharides due to its open ring structure, and furthermore, our NOF product is suitable for pharmaceuticals, cosmetics and laboratory reagent use.

CHITOSAN GH-003は、D-グルコサミン残基3分子がβ-1、4結合したキトサンオリゴ糖です。本製品は糖アルコール体である為、通常のキトサンオリゴ糖よりも物質安定性が向上しており、更に多段階に渡る精製により高純度化（HPLC純度99%以上）しているため、研究用試薬、医薬品、または化粧品原料等の分野でご利用頂けます。

Structure of CHITOSAN GH-003



Molecular Weight: 612.89 Appearance: White Powder
Purity: NLT 99%(HPLC)



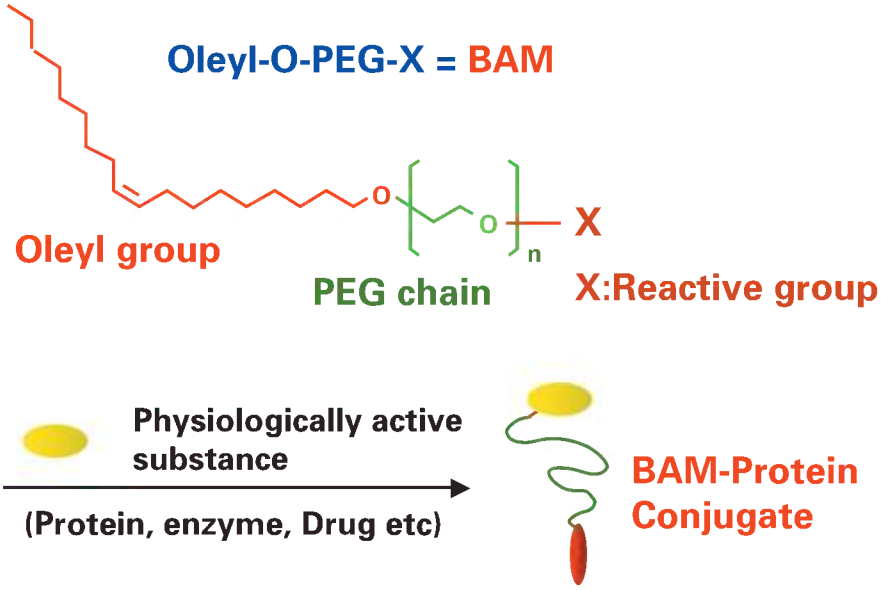
Reference: Dynamic Contrast-Enhanced MRI of Implanted VX2 tumor in rabbit muscle; Comparison of Gd-DTPA and NMS60, *Magnetic Resonance Imaging*, **17**, pp. 1297-1305, (1999)

X. Biocompatible PEG Anchors for cell membrane <BAM>

(SUNBRIGHT® OE Series)

NOF has developed Biocompatible Anchor for cell Membrane (BAM) mimics consisting of oleyl groups as a hydrophobic cell membrane anchor and activated polyethylene glycol (PEG) for increasing water solubility and reacting with either physiologically active substances (PAS) or material surfaces at the PEG terminal. Using BAM, membranes of cells or tissues can be modified with PAS such as proteins, enzymes or drugs without causing damage. Utilizing our new technologies, cells or tissues can be also immobilized alive on surfaces of various kinds of materials. Possessing excellent functions beyond conventional concepts, our new technologies should provide wide application in pharmaceuticals and cosmetics.

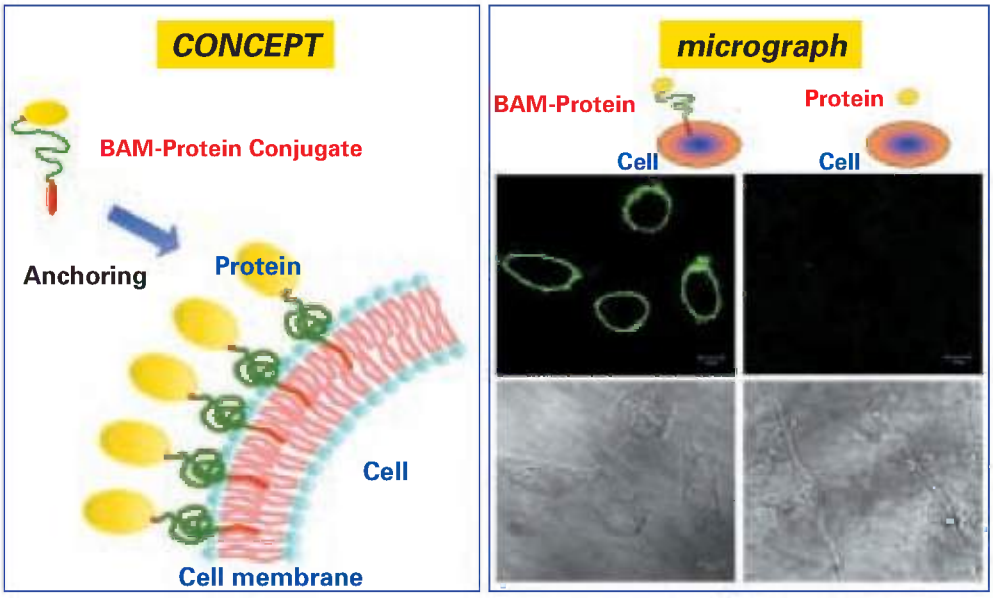
細胞にダメージを与えない細胞膜修飾剤（BAM：Biocompatible Anchor for cell Membrane）を開発しました。BAMは細胞膜へのアンカーとして脂質オレイル基と、水溶性を高めるためのポリエチレングリコール（PEG）からなり、生理活性物質や材料表面に結合させるためPEG鎖末端に各種の反応性基を導入した構造を有します。BAMを用いることにより、細胞や組織にダメージを与えることなく、細胞や組織の表面を蛋白質や薬剤などの生理活性物質で修飾することや、細胞や組織を生きたまま各種材料表面に固定化することが可能となりました。従来のコンセプトを超えた機能を持っており、医薬品、化粧品分野で広い用途が期待されます。



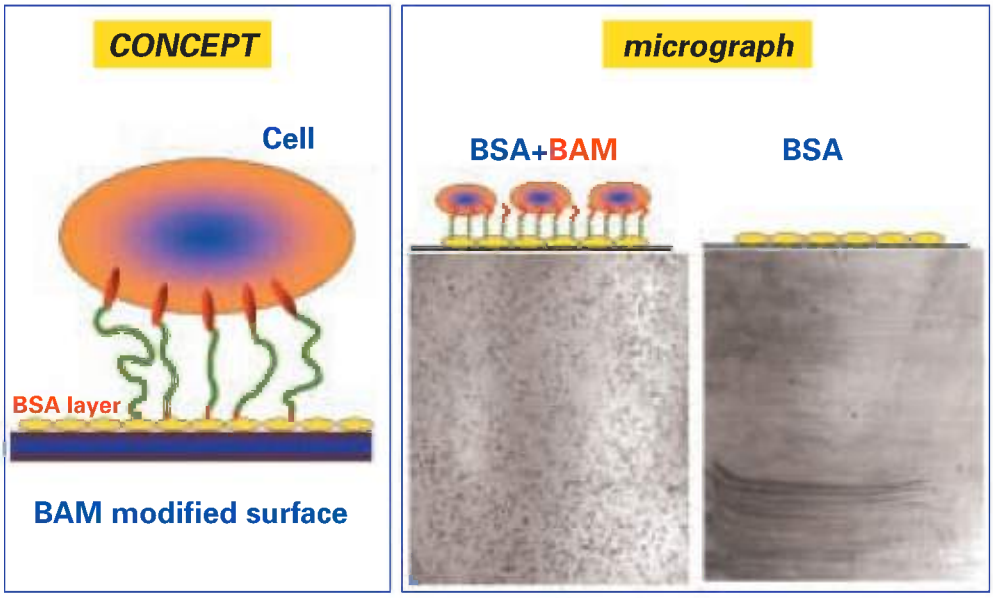
Biocompatible Anchor for cell Membrane (BAM)

Product name	Reactive Group (X)	Mw
SUNBRIGHT OE-020CS	-CO-CH_2CH_2-COO-NHS	2,000
SUNBRIGHT OE-040CS		4,000
SUNBRIGHT OE-080CS		8,000

Application I : Anchoring of BAM-Protein conjugate to the cell membrane



Application II : Anchoring of cell on the BAM modified surface



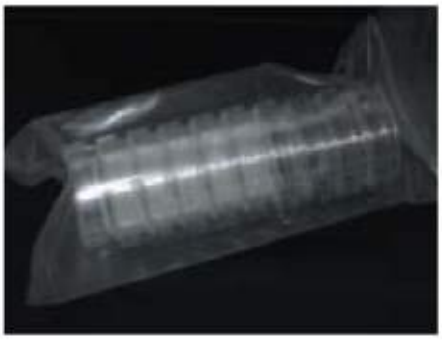
New Non-adherent Cell immobilizing Culture Dishes

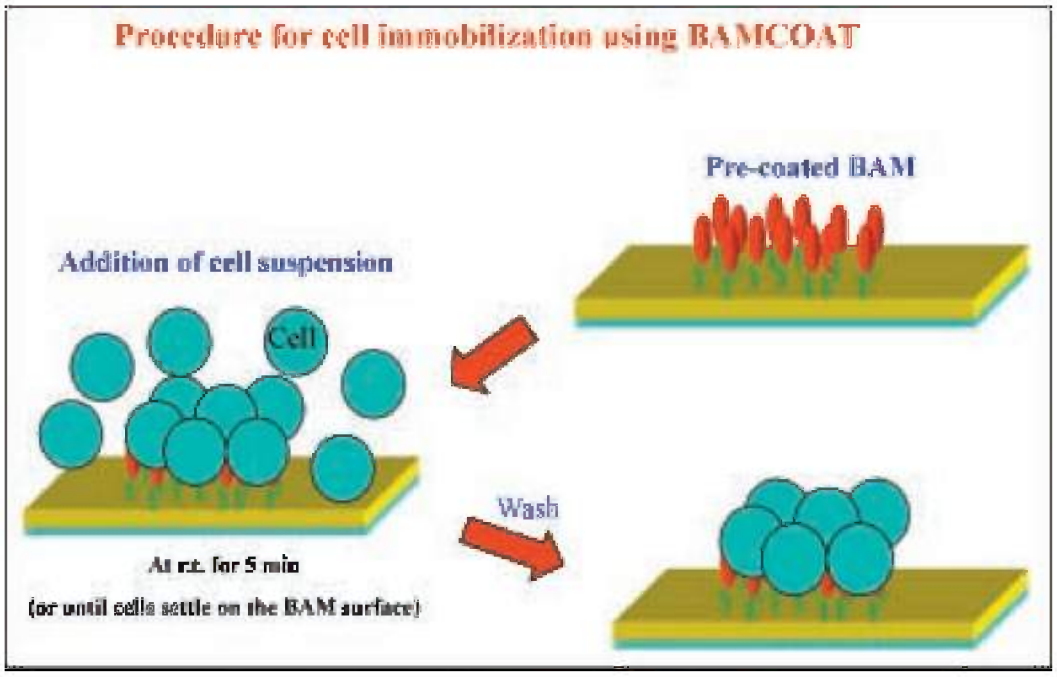
BAMCOAT DI-035G

1) New technology for cell immobilizing systems without cell damage.
2) Easy-to-use pre-coated glass bottom dishes.
3) Available for numerous applications for observation of non-adherent cells in culture.

ex. Observation of intracellular calcium
Observation of Green Fluorescence Protein(GFP) localization







参考文献
1) K. Kato, et. al, *Biotechnol. Prog.*, **20**, 897-904 (2004)
2) K. Kato, et. al, *BioTechniques* **35**,1014-1021 (2003)

XI. LIPONIZER® for Liposome production

In order to reliably produce liposomes with certain size distributions, NOF would like to recommend our LIPONIZER. By altering filter sizes, desirable sizes of liposomes (200nm) can be resproducibly obtained. NOF has currently two sizes of LIPONIZERs. LP-90 for small scale (90mm diameter) and LP-293 for larger scale (293 mm). Specification and pictures are shown below.

LIPONIZER LP-90-500
(For Small Scale)



Manufacturing System



Technical Specifications:

Max. Pressure	30kgf/cm^2
Approx. flow rate	300ml/min
Max liquid concentration	200mg/ml
Temperature range	5-80˚C

LIPONIZER LP-293



Product Specifications:

Weight	230kg
Max. Pressure	30kgf/cm^2
Approx. Flow Rate	100ml/min
Max. Liquid Concentration	300mg/ml
Temp. Range	5-80˚C

LIPONIZER® is registered mark of Nomura Micro Science Co., Ltd.

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DDS Development Dept.

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Exhibit 13

Alexandre Mebazaa
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Joseph E. Parrillo *Editors*

Acute Heart Failure



Springer

ASCENDIS EX1030
U.S. Patent No. RE48,267

Alexandre Mebazaa, Mihai Gheorghiade,
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Acute Heart Failure

Springer

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Foreword

Acute heart failure is a leading cause of morbidity and mortality worldwide, and its incidence is increasing as the population ages. This represents a huge clinical and economic burden for all health care systems. With its high prevalence and associated morbidity and mortality, acute heart failure requires better therapies. Greater awareness of the disease process, better understanding of the underlying mechanisms, and improved management of patients with heart failure are urgently needed to ensure that the outcomes of patients with acute heart failure are optimized.

In recent years, the concept of acute heart failure as a single disease entity has changed, and the definition and classification of acute heart failure have gradually been modified with the increased realization that the term *acute heart failure* incorporates several acute heart failure syndromes, which differ in presentation, etiology, pathophysiology, and outcome. Clinicians and investigators around the world are now collaborating to establish more uniform definitions, collect relevant epidemiologic data, and develop more standardized guidelines and recommendations for the management of patients with acute heart failure. Recent epidemiologic studies have helped delineate key characteristics of patients with acute heart failure, highlight important risk factors, and identify national and international approaches to management.

This new book on acute heart failure is thus very timely, drawing together the most recent data and concepts in this field, and providing a fresh look at the complexities of acute heart failure. The editors, who are indeed authorities in the field, have succeeded in producing a truly complete review of this vast and important subject. The book is divided into two main parts, the first concentrating largely on epidemiology and pathophysiology, and the second focusing on diagnosis and management. In addition to the chapters covering the more traditional features of heart failure, other chapters address the nutritional, psychological, and ethical aspects. The text is supported throughout by ample and appropriate imaging and relevant references.

The 85 chapters are written by a broad cross-section of recognized experts from the different clinical arenas involved in the investigation and treatment of patients with acute heart failure, including cardiologists, cardiac and thoracic surgeons, intensivists, anesthesiologists, pharmacologists, nephrologists, and pulmonologists. The wide range of specialties represented by the

authors highlights the multidisciplinary nature of this disease process, which can affect almost all organ systems, creating a heterogeneous and complex entity. In addition, the international nature of the authors, including contributors from some 15 countries, helps make this text relevant for clinicians worldwide.

This highly comprehensive book is an important contribution to the literature in this field and will serve as a valuable text for all those involved in the care of patients with acute heart failure.

Jean-Louis Vincent
Head
Department of Intensive Care
Erasme Hospital
Free University of Brussels Belgium
Belgium

Preface

One challenge in editing a textbook is that by the time the the book is published, it is outdated. We believe that the present textbook on AHFS stands up to this challenge. AHFS is a new and rapidly evolving field. At present, we are just starting to learn about AHFS and to develop paradigms similar to those created in the early 1980s for chronic heart failure, one of the fastest changing areas in cardiovascular medicine during the last 20 years. Researchers have only started to measure how important a burden AHFS is on health care.

We are beginning to investigate some reasonable pathophysiological hypotheses. Only recently, the ESC and ACC/AHA issued the first set of guidelines. None of these guidelines is truly evidence based, as only a few clinical trials have investigated new treatments for AHFS and even fewer have yielded positive results. Therefore, the editors felt the need to assemble in a single compendium a baseline document that establishes the current knowledge of this syndrome. In so doing, we have also tried to highlight unmet needs and to delineate areas for future research. Finally, the editors addressed the large heterogeneity of this syndrome by inviting a variety of contributors with different backgrounds and from different continents to participate.

Alexandre Mebazaa
Mihai Gheorghiade
Faiez M. Zannad
Joseph E. Parrillo

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Natriuretic Peptides

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Peptide hormones belonging to the natriuretic peptide (NP) family play an important role in maintaining cardiac and renal function by regulating fluid volume, pressure, and sodium concentration. The principal members of this family are atrial natriuretic peptide (ANP); urodilatin (URO); brain natriuretic peptide (BNP); and C-type natriuretic peptide (CNP). The physiologic effects of these molecules generally lead to vasodilation, natriuresis, and diuresis, and regulate sodium and water homeostasis, rendering them promising candidates for potential therapeutic use in cardiovascular and renal dysfunction. This chapter reviews the biochemistry, physiology, and pharmacologic potential of the major natriuretic peptides, with particular emphasis on their clinical importance in the treatment of acute heart failure syndrome (AHFS).

Biochemistry

Structure and Synthesis

The natriuretic peptides are characterized by a highly conserved 17-amino-acid ring structure that varies little even among different species. Whereas the amino- and carboxy-terminal amino acid sequences vary markedly, the ring structure differs in only five positions between ANP/URO, BNP, and CNP (Fig. 56.1). The biologically active forms of these molecules are processed from precursor proteins encoded by two genes on chromosome 1 (ANP/URO and BNP) and a third gene on chromosome 2 (CNP). Atrial natriuretic peptide and URO share the same amino acid sequence from position 99 to 126 of the ANP-prohormone; however, URO has an additional N-terminal extension of 4 amino acids.

Atrial Natriuretic Peptide

Human ANP is a 28-amino-acid peptide synthesized in the myoendocrine cells of the heart atria, derived from a 151-amino-acid preprohormone through a series of proteolytic processing steps. An N-terminal, 126-amino-acid precursor, pro-ANP ($ANP_{1–126}$), is first derived from the preprohormone and stored in atrial myocyte granules. Pro-ANP is then cleaved into a 98-amino-acid N-terminal fragment and the biologically active, 28-amino-acid C-terminal atrial natriuretic peptide ($ANP_{99–126}$); both are released by exocytosis into the circulation.[1,2] Under normal conditions, ANP gene expression in the ventricles is seen only at very low levels (about 7% that in the atria), but increased ANP messenger RNA (mRNA) production may occur in pathologic conditions such as heart failure and left ventricular (LV) hypertrophy.[3] Atrial natriuretic peptide release is induced by elevations in cardiac-wall tension,[4] which may be due to physical exercise, neurohumoral stimuli, and other physiologic factors.[1]

Urodilatin

Urodilatin (URO/$ANP_{95–126}$) is a 32-amino-acid peptide that is produced by differential processing of the pro-ANP precursor peptide in distal renal tubule cells; therefore, it is identical to ANP except for the presence of four additional N-terminal amino acids. Urodilatin is found in urine

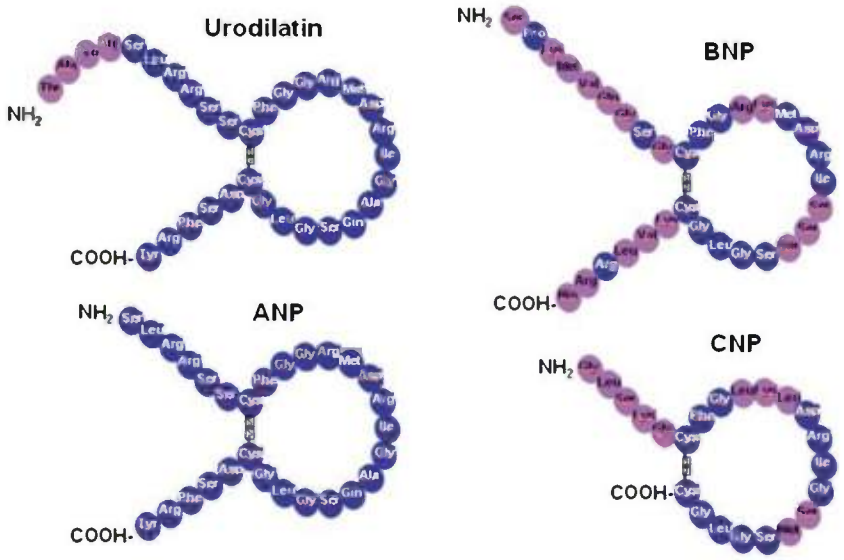


FIGURE 56.1. Structure of the natriuretic peptides ANP_{99-126}, urodilatin/ANP_{95-126}, BNP_{77-108}, and $CNP_{105-126}$ showing the conserved 17-amino-acid ring configuration. Nonconserved residues are indicated in pink.

and has not been detected and isolated from plasma. It is secreted into the tubular lumen and acts as a paracrine hormone, binding further downstream to receptors in the inner medullary collecting duct and inhibiting sodium reabsorption.[5,6] Urodilatin release occurs in response to acute volume load and selective increases in sodium concentration and dietary sodium intake,[7,8] and is closely correlated with the circadian rhythm of urinary sodium excretion.[9] In congestive heart failure (CHF), urinary urodilatin excretion was found to be increased, apparently to counteract antinatriuresis and fluid retention.[10]

Brain Natriuretic Peptide

Brain natriuretic peptide was originally isolated from porcine brain extracts and later identified from human cardiac tissue. It consists of 32 amino acids (BNP_{77-108}) derived from a 108-amino-acid pro-BNP precursor that is found, in humans, predominantly in the ventricles of the heart and in much smaller amounts in the atria, brain, and amniotic tissue.[11,12] N-terminal pro-BNP and mature BNP molecules are released from ventricular myocytes at a 1:1 molar ratio and circulate in plasma. Unlike ANP, which is stored in the form of the 126-amino-acid precursor pro-ANP, BNP is stored mainly as the mature peptide.[4,13] Whereas there is a threefold greater concentration of BNP mRNA in the ventricles than in the atria, the ventricles have only 1% of the amount of mature BNP that is in the atria.[14] Brain natriuretic peptide mRNA is transcribed in response to stretching of ventricle and atrial walls from increased blood pressure or elevated venous volume.[12,15–17]

C-Type Natriuretic Peptide

Processing of CNP starts with the removal of a 23-amino-acid signal peptide from the 126-amino acid preprohormone (prepro-CNP), converting it into pro-CNP (CNP_{24-126}). Pro-CNP is then cleaved to produce a 53-amino-acid intermediate peptide, CNP-53 (CNP_{74-126}); further cleavage of CNP-53 produces biologically active CNP-22 ($CNP_{105-126}$).[18] CNP-22 is produced in brain and endothelial cells and acts primarily as an autocrine or paracrine hormone that regulates vascular tone and blood pressure.[4,19] It is found in much lower concentrations than either ANP or BNP in the heart or in plasma. Like ANP, CNP is stored mainly in the 104-amino-acid propeptide (pro-CNP) form.[18]

Atrial natriuretic peptide and BNP secretion is regulated mainly by the degree of atrial and ventricular wall stress that results from volume or pressure overload and by several endogenous

hormones, neurotransmitters, proinflammatory cytokines, and vasoactive factors. Both CNP production and secretion in vitro are stimulated by ANP, 20-fold more potently by BNP, and by several cytokines and growth factors, including transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and lipopolysaccharide (LPS), although no relation has been established between CNP and any pathologic effects caused by these cytokines.[2]

Natriuretic Peptide Receptors and Metabolism

The three types of natriuretic peptide receptors (NPRs) are NPR-A, NPR-B, and NPR-C. NPR-A and NPR-B are expressed at high levels in the atria, ventricles, aorta, peripheral blood vessels, platelets, lungs, skin, tubules, smooth muscles of the kidney, and presynaptic sympathetic nerve fibers. Both are highly homologous transmembrane proteins composed of an extracellular binding domain (~440 amino acids), a transmembrane domain (37 amino acids), and intracellular domains with kinase (~280 amino acids) and guanylyl cyclase (~250 amino acids) activity. The guanylyl cyclase domain catalyzes the generation of cyclic guanosine monophosphate (cGMP), which mediates most of the physiologic effects of natriuretic peptides.[4] Atrial natriuretic peptide, BNP, and urodilatin all bind predominantly to NPR-A; ANP is the natural ligand for NPR-A and binds to it with a 10-fold higher affinity than BNP. C-type natriuretic peptide binds most strongly to NPR-B.[2,14]

Natriuretic peptide receptor C is expressed in cells of the kidney, adrenal gland, brain, lungs, and blood vessel walls. It is more abundantly expressed than any other NP receptor type, constituting 90% of all NP receptors in target tissues.[4] It has an extracellular binding domain that is approximately 30% homologous to its NPR-A and NPR-B counterparts and a short, noncatalytic 37-amino-acid intracellular domain that shares no homology with the corresponding domains of the other two receptor types. Natriuretic peptide receptor C is considered a "clearance" receptor whose action is geared toward internalization of receptor-ligand complexes and subsequent lysosomal degradation of the ligand.[20,21] One function of NPR-C may be the removal of excess natriuretic peptides in the circulation, although it may achieve other effects through signaling pathways involving other second messengers such as cyclic adenosine monophosphate (cAMP) or by coupling with G proteins. All natriuretic peptides bind avidly to NPR-C, although ANP and CNP bind with a somewhat higher affinity than BNP.[4,22,23]

Natriuretic peptide binding to NP receptors stimulates cGMP production and elevation of intracellular cGMP levels. Cellular responses depend on downstream cGMP signal transduction, mediated by cGMP-dependent protein kinases (PKGs) that catalyze phosphorylation of target proteins (reviewed by D'Souza et al.[4]). Two protein kinases, PKG-I and PKG-II, appear to be involved in this signaling pathway. PKG-I is a soluble cytoplasmic enzyme that modulates intracellular calcium levels and is present in cardiac myocytes, vasculature, lung, cerebellum, kidney, adrenal glands, and uterus. PKG-II regulates fluid homeostasis at the cell membrane and is found in brain, intestine, lung, kidney, and bone but is absent from the heart and vascular tissue. Cyclic GMP may also activate cAMP-dependent protein kinase A (PKA) or cGMP-regulated phosphodiesterases (PDEs) in certain tissues such as the heart, lung, liver, adrenals, brain, platelets, and smooth muscle.

Natriuretic peptides are cleared from the circulation by NPR-C uptake and the enzymatic action of neutral endopeptidase (NEP), a zinc metallopeptidase enzyme present in high concentrations in the brush border of renal proximal tubular cells and also in the vascular endothelium. Neutral endopeptidase digests other vasodilators such as bradykinin, substance P, and adrenomedullin, and vasoconstrictors such as angiotensin II and endothelin 1 (ET-1) (reviewed in Schmitt et al.[14]).

Physiology and Pharmacology

Atrial Natriuretic Peptide and Brain Natriuretic Peptide

Atrial natriuretic peptide and lower levels of BNP are produced in the atria and ventricles in response to changes in transmural atrial pressure or stretch

as a consequence of pressure or volume expansion. Neurohumoral stimuli such as vasopressin, angiotensin II, endothelin (ET), enkephalin, morphine, and TNF-α also induce ANP release.[24–28]

The effects of ANP and BNP on the cardiovascular and renal systems are well documented.[17,29–36] Under physiologic conditions, ANP and BNP have similar physiologic effects, including vasodilation; suppression of the renin-angiotensin-aldosterone system (RAAS), endothelin, and sympathicoadrenergic systems; reduced blood pressure and peripheral vascular resistance; and enhanced diuresis, natriuresis, and kaliuresis.[29,30,36] In canine models and isolated arterial strips, ANP relaxes coronary arteries[37] and appears to be more selective for the renal arteries when compared to others (vertebral, femoral, and common carotid).[38] In humans, however, results of studies on the effects of BNP on coronary arteries and coronary flow rate have been equivocal.[39,40]

As natural antagonists of the RAAS, ANP and BNP inhibit Na^+ reabsorption in the inner medullary collecting duct and also modify the renal hemodynamics by increasing preglomerular vasodilation and postglomerular vasoconstriction, thereby increasing the glomerular filtration rate (GFR). All these actions have the end effect of enhancing natriuresis and diuresis. The pro-ANP fragment $ANP_{31–67}$ also stimulates the release of prostaglandins, which induce natriuresis indirectly by blocking adenosine triphosphatase (ATPase)-dependent sodium exchange in the inner medullary collecting duct of the kidney.

Atrial natriuretic peptide or BNP infusions in healthy subjects increase urinary flow rate and Na^+ excretion in a dose-dependent manner and inhibit plasma renin secretion.[34,41] Brain natriuretic peptide also has predominantly central hemodynamic effects, such as decreased stroke volume and mean arterial pressure and increased heart rate, but it does not seem to affect the peripheral microcirculation in the skin and conjunctiva, capillary density, or skin oxygen capacity.[36]

Atrial natriuretic peptide also appears to play an important role in organogenesis and embryogenesis by regulating the differentiation and regulation of multiple organ systems, including the skeletal system, immune response, natural killer cell activation, histamine release, reproductive system, and central nervous system (reviewed in Schmitt et al.[14]). Additionally, other studies emphasize the potential role of ANP and BNP as important factors in cardiovascular remodeling as a result of their antiproliferative activity.[4] The mechanism by which these NPs modulate proliferation, cell growth, and apoptosis in cardiac myocytes, smooth muscle cells, and fibroblasts, as well as extracellular matrix production, is currently the subject of intensive research.

The biological half-life of ANP is approximately 3.5 minutes, whereas that of BNP is nearly seven times longer at 22.6 minutes as a result of its slower degradation by NEP and lower affinity for the NPR-C.[2]

Urodilatin

Because they are highly homologous to each other and are derived from the same precursor, the biologic actions of urodilatin and ANP are similar; however, the effects of urodilatin on renal sodium excretion are more critical than those of ANP. Urodilatin infusion in animal models results in a much stronger natriuretic and diuretic effect than that produced by an equimolar ANP infusion.[42,43] This is most likely due to the lesser susceptibility of urodilatin to degradation by the neutral endopeptidase as it passes the proximal tubules in the kidney.[44]

Urodilatin causes dilation of preglomerular and constriction of postglomerular vessels[45] and also increases GFR in animal models of acute renal failure.[46] In healthy human volunteers, urinary excretion of urodilatin is directly correlated to increased sodium intake and renal sodium excretion.[7] After prolonged diets with varying sodium doses, infused urodilatin at 20 ng/kg/min for 60 minutes increased diuresis and natriuresis in proportion to the amount of sodium in the diet, further suggesting an important role for endogenous urodilatin in sodium homeostasis.[35] The renal response to urodilatin appears to be exerted at the collecting duct where sodium reabsorption is inhibited. Even lower doses (5 and 10 ng/kg/min) of intravenously administered urodilatin to healthy subjects with normal LV function have significant systemic effects on the cardiovascular, endocrine, and renal systems. In contrast to the response in heart failure patients, in healthy volunteers these low doses led to a reduction in the

cardiac output by 7% and 16%; in stroke volume by 10% and 20%, respectively, most likely due to venodilation; and in renal blood flow (hippurate clearance) by 13% to 37%, with no changes in arterial pressure or GFR.[47]

In addition to its effects on the kidney, in healthy volunteers, urodilatin at ≤20 ng/kg/min inhibits the neurohumoral axis, resulting in decreased secretion of renin, angiotensin II, aldosterone, vasopressin, and BNP into the plasma. At higher dosages, plasma renin, angiotensin II, aldosterone, and vasopressin levels actually increase in a compensatory fashion because of the decrease in blood pressure.[5,47,48]

In a randomized, placebo-controlled phase II study of subjects with bronchial asthma, urodilatin at various doses, alone or in combination with albuterol, improved bronchodilation parameters, including forced expiratory volume (FEV), maximum expiratory flow (MEF), and peak expiratory flow (PEF), and showed potential as an adjunct to β_2-adrenergic agonists for asthma management.[49]

Unlike ANP, urodilatin is more resistant to NEP degradation, probably because of the four additional amino acids at its N-terminal tail, and it seems to be cleared from circulation also by binding to NPR-C.[50] Infused urodilatin has a plasma half-life of approximately 5 to 24 minutes; with bolus injections, however, the half-life is shortened to 2 to 3 minutes.[48]

C-Type Natriuretic Peptide

C-type natriuretic peptide is produced in endothelial cells, acts in a paracrine/autocrine manner, and is rapidly inactivated so that plasma measurements do not always reflect changes in CNP levels. C-type natriuretic peptide has been shown to regulate vascular tone and induce arterial vasodilation and relaxation. Its potency varies with the size of the blood vessel and the type of NP receptor expressed on the vessel. It is more potent than ANP in relaxing smooth muscle and likely plays a more important role in dilating blood vessel walls than in inducing diuresis or natriuresis.[19]

Infused CNP has been shown to lower blood pressure in animal models as well as in healthy human volunteers.[18] C-type natriuretic peptide knockout mice, however, appear to compensate with increased ANP production so that they are not hypertensive as expected; rather, they are mildly hypotensive. C-type natriuretic peptide also suppresses adrenocorticotropic hormone (ACTH), vasopressin, and aldosterone synthesis.

C-type natriuretic peptide plays an important role in modulating vascular inflammation and in other vascular protective mechanisms (reviewed in Scotland et al.[18]). C-type natriuretic peptide production is stimulated by inflammatory cytokines such as IL-1 and TNF and by LPS; septic shock patients actually have significantly higher levels of circulating CNP. The antiinflammatory effect of CNP includes suppression of proinflammatory prostaglandin E_2 (PGE_2) production in isolated cells, reduced expression of vascular adhesion molecules, and inhibition of leukocyte migration, or "rolling." Vascular protection by CNP may also involve its beneficial effects on the vascular endothelium and smooth muscle and inhibition of coagulation and thrombus formation.

Clinical Significance

Studies of NPs have significantly expanded our understanding of homeostatic mechanisms underlying fluid volume and pressure regulation. Alterations in circulating levels of the cardiac NPs have been used as diagnostic and prognostic markers in various cardiovascular conditions, such as CHF, hypertension, LV dysfunction, acute coronary syndromes, and aortic valve diseases[2,51–53] (reviewed in D'Souza et al.[4]).

Natriuretic Peptides as Disease Indicators

Congestive Heart Failure

Increased mechanical strain in the atria and ventricles is a major stimulus for the synthesis and release of both ANP and BNP, resulting in a marked increase in ANP and BNP levels in circulation during the development of heart failure. Plasma ANP and BNP are negatively correlated with left ventricular ejection fraction (LVEF). Several studies have shown that plasma BNP, as well as the N-terminal pro-BNP fragment (NT-pro-BNP), may be a more powerful prognostic

indicator of development of LV systolic or diastolic dysfunction and LV hypertrophy than is ANP.[2,17,51,52,54]

Rapid (15-minute) BNP immunofluorescence assays (Biosite Diagnostics, San Diego, CA, and Bayer Diagnostics, Tarrytown, NY) are currently approved as a tool for the diagnosis and prognosis of patients with acute coronary syndromes, including acute decompensated heart failure. There is also an 18-minute electrochemiluminescence assay for NT-pro-BNP (Roche Diagnostics, GmbH, Basel, Switzerland). Although BNP testing does not replace echocardiography and a full cardiologic assessment and should be used only within the clinical context, heart failure appears more likely with plasma BNP concentrations above 100 pg/mL.[51,52] At or above this level (100 pg/mL), BNP was more accurate than the National Health and Nutrition Examination Survey (NHANES) and Framingham criteria in diagnosing heart failure.[54] The ratio of plasma ANP to plasma BNP is significantly higher in patients with obstructive airway disease than in those who have heart failure, perhaps because of increased right atrial pressures secondary to severe primary lung disease.[4,55]

Serum BNP levels are useful guides for risk stratification for predicting mortality and for designing heart failure treatment regimens by titrating doses of standard medications to a desired BNP level.[56] The BNP levels rise in direct proportion to the severity of symptoms and cardiac damage, and BNP is an independent predictor of mortality in CHF patients. A pilot study of patients with decompensated heart failure has suggested that BNP concentration measured before discharge may help determine the risk of readmission within the month following discharge.[57]

Because BNP concentration is a good indicator of elevated end-diastolic pressure and LV wall tension, which in turn closely correlates with dyspnea, it also correlates well with the New York Heart Association (NYHA) classification scheme. However, tests are currently in progress to evaluate the prognostic and diagnostic value of NT-pro-BNP, which may be a better indicator of abnormal cardiac function than BNP itself in NYHA class I and II patients. In NYHA class III and IV patients, sensitivity and specificity are comparable. The NT-pro-BNP levels may also be more tightly linked to renal function, and negative predictive values for NT-pro-BNP are >95%.[54] The diagnostic value of NT-pro-BNP, however, depends on the patient's age, because NT-pro-BNP levels change with age and declining GFR (the cutoff for detecting CHF rises sharply from 125 pg/mL to 450 pg/mL after age 75), and there is a gray period coinciding with the peak incidence of CHF between 65 and 85 years of age when NT-pro-BNP is of little use to the clinician. Clearly, further research is required to determine how NT-pro-BNP measurements can be best applied in cardiovascular and cardiorenal management.

Acute Myocardial Infarction

Acute myocardial infarction is associated with compensatory activation of the RAAS and sympathetic nervous system, a low to moderate rise in ANP, and a 100-fold increase in plasma BNP via rapid synthesis in infarcted and periinfarcted areas. The ANP and BNP levels correlate with pulmonary capillary wedge pressure (PCWP), but BNP levels at 72 hours correlate inversely with the cardiac index (CI) and LVEF at day 5 and month 3 post-MI.[4] Although BNP gene expression and de novo synthesis occur rapidly in both ventricles, ANP is secreted from storage in the atrium, further indicating distinct pathologic roles for ANP and BNP in AMI.

Myocardial Ischemia and Coronary Artery Disease

In vivo models of experimental ischemia have shown that even brief periods of hypoxia generated by coronary artery occlusion trigger the rapid release of both ANP and BNP without any correlation to myocardial wall stress. In patients undergoing percutaneous transluminal coronary angioplasty (PTCA), inflation of the PTCA balloon induces transient myocardial ischemia, followed immediately by a rapid and transient increase in plasma BNP levels. However, no correlation was found between the magnitude of increase in BNP levels and the duration of myocardial ischemia from this procedure. The ANP levels also increased slightly after balloon inflation. Plasma BNP and pro-BNP levels are also elevated in patients with

chronic coronary artery disease CAD without LV dysfunction.[58]

Natriuretic Peptides as Therapeutic Agents in Acute Heart Failure Syndrome

Acute heart failure syndrome (AHFS) comprises a set of severe, life-threatening symptoms resulting from congestion or fluid retention in the lungs and inadequate perfusion of tissues and organs.[59] Specific signs and symptoms of AHFS include dyspnea or orthopnea, fatigue, pulmonary congestion, rales, coughing, cachexia, peripheral edema, hypoperfusion, and jugular vein distention. Patients with AHFS may not have any previously known cardiac dysfunction but may suffer from symptoms following an acute coronary event such as a myocardial infarction (new-onset AHFS), or they may experience acute decompensation of chronic heart failure.[60]

Current Therapy of Acute Heart Failure Syndrome

The goals of AHFS therapy are (1) to improve clinical symptoms and hemodynamics without increasing the risk of short- and long-term mortality, (2) to preserve or improve renal function, and (3) to minimize myocardial damage. Current treatment strategies focus on rapidly improving hemodynamics and relieving congestion by reducing fluid volume with diuretics; increasing contractility and cardiac output with digoxin and inodilators such as dobutamine, dopamine, and milrinone; and enhancing pulmonary and peripheral circulation with vasodilators such as nitroglycerin and angiotensin-converting enzyme (ACE) inhibitors such as lisinopril and enalapril.

Current therapies have limitations, however. Controlled clinical trials have shown that hemodynamic improvements observed with AHFS interventions do not translate into long-term patient benefit, with most resulting in minimal or no benefit, some deleterious effects, or high mortality rates. For instance, vasodilators generally used when hypoperfusion is accompanied by congestion, normal or high blood pressure, and insufficient response to diuretics alone can also cause hypotension and induce tolerance with prolonged use. High-dose diuretics are associated with increased risk of renal deterioration and neurohormonal activation of the angiotensin-aldosterone and sympathetic nervous systems, perhaps affecting long-term outcomes such as mortality.[61] Some inodilators such as dobutamine and milrinone may increase the risk of ventricular arrhythmias, postdischarge morbidity, and mortality.[61,62] Digoxin increases cardiac contractility and improves symptoms with possible a reduction in the number of hospitalizations, but its long-term clinical outcomes are neutral at best.[63] Therefore, new therapeutic approaches that improve short-term outcomes without negatively affecting renal and cardiac function and long-term survival are desired.

Newer therapies include levosimendan, an inotropic, Ca^{2+}-sensitizing, K^+ channel opener with antiischemic and anti-stunning effects that increases myocardial contraction without increasing myocardial oxygen consumption, and tolvaptan, a vasopressin V2-receptor antagonist that increases urine volume and serum sodium with little or no sodium loss (reviewed in Gheorghiade et al.[64]). Levosimendan is well tolerated. Most common adverse effects include headache, hypotension, nausea, and dizziness believed to be secondary to its vasodilatory effects and slight reductions in red blood cell count, hemoglobin, and serum potassium, but no proarrhythmic effects.[65,66] However, a multicenter randomized trial (Survival of Patients with Acute Heart Failure in Need of Intravenous Inotropic Support [SURVIVE]) evaluating its efficacy in improving survival in AHFS patients showed no significant reduction in mortality when compared with dobutamine.[67]

Natriuretic Peptide Therapy for Acute Heart Failure Syndrome

Three natriuretic peptide agents are currently approved or undergoing development for the management of acute heart failure (Table 56.1). Carperitide and ularitide are synthetic analogues of ANP and urodilatin, respectively, and nesiritide is a recombinant form of human BNP. All three

TABLE 56.1. Characteristics of therapeutic natriuretic peptides

	Carperitide	Nesiritide	Ularitide
Peptide analogue	ANP_{99-126}	BNP_{77-108}	Urodilatin (ANP_{95-126})
Source	Synthetic	Recombinant	Synthetic
Development phase	Approved in Japan for acute heart failure	Approved in the U.S. for ADHF	Phase II–III
Secreted by	Atrial and ventricular myocytes	Ventricular myocytes	Renal tubular cells
Stimulus for secretion	↑ Blood pressure, venous volume; atrial wall stretching	↑ Blood pressure, venous volume; ventricular wall stretching	↑ Plasma Na^+; ↑ blood pressure
Receptor	NPR-A, NPR-C	NPR-A, NPR-C	NPR-A, NPR-C
Plasma half-life	2–5 min	18–23 min	2–24 min
Sensitivity to NEP	Yes	Yes	Yes, but less than ANP
Physiologic targets	Vascular wall endothelium and smooth muscle	Vascular-wall endothelium and smooth muscle	Collecting ducts
Pharmacologic effects: mechanism of action	↓ Na^+ reabsorption, ↓ renal perfusion rate; ↑ GFR; ↓ RAAS; predominantly venous vasodilation; bronchodilation	↓ Na^+ reabsorption, ↓ renal perfusion rate; ↑ GFR; ↓ RAAS; vasodilation; bronchodilation	↓ Na^+ reabsorption, ↓ renal perfusion rate; ↑ GFR; ↓ RAAS; vasodilation; bronchodilation
Clinical effects in ADHF patients	↓ PCWP, LVFP; ↑ CI	↓ PCWP and systemic arterial pressure; relief from dyspnea	↓ PCWP, PAP, and SVR; ↑ CI; ↑ relief from dyspnea
Adverse effects	Hypotension; renal dysfunction	Hypotension; renal dysfunction (azotemia, ↑ serum creatinine); potential ↑ mortality	Sweating; hypotension; nausea; dizziness; bradycardia

ADHF, acute decompensated heart failure; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; LVFP, left ventricular filling pressure; NPR-A and NPR-C, natriuretic peptide receptor-A and -C; CI, cardiac index; RAAS, renin-angiotensin-aldosterone system; GFR, glomerular filtration rate; NEP, neutral endopeptidase; PAP, pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; SVR, systemic vascular resistance.

restore hemodynamic balance and improve cardiac or renal function in patients experiencing AHFS.

Carperitide (Synthetic Atrial Natriuretic Peptide)

Carperitide is a synthetic form of human ANP that was approved for use in Japan in 1995 for the management of acute heart failure (AHF). In a 6-year open-label study of 3777 patients with AHF treated with 0.085 μg/kg/min carperitide for 65 hours, 82% of patients experienced clinical improvement in terms of severity of Killip classification and renal dysfunction.[68] A much smaller, earlier study in patients who had severe heart failure with AMI also demonstrated a decrease in PCWP by almost 50% (21 to 11 mm Hg) and plasma aldosterone from 148 to 56 pg/mL after carperitide infusion (0.05 to 1.0 μg/kg/min) without altering heart rate and systemic blood pressure.[69]

Studies on the effect of ANP infusion in CHF patients vary widely in design, patient population, and type of ANP analogue used, making interpretation difficult.[14] They all show an invariable reduction in cardiac filling pressure, however, that occurs too early to be due to a fluid shift or changes in diuresis and pulmonary or peripheral vascular resistance and has been attributed to ANP-induced venous vasodilation. This reinforces the concept of ANP as a vasodilator on veins rather than arteries in patients with high PCWP and plasma noradrenaline.[70]

Nesiritide (Recombinant Brain Natriuretic Peptide)

Nesiritide (Natrecor®) is a recombinant human B-type natriuretic peptide (hBNP) that was approved in the United States in 2001 for the treatment of AHFS patients with dyspnea at rest or minimal activity. The recommended dose is an intravenous bolus of 2 μg/kg followed by a continuous infusion of 0.01 μg/kg/min. Nesiritide improves short-term dyspnea and produces dose-dependent reductions in PCWP and systemic arterial pressure in patients with heart failure.[71] In clinical trials, the decrease in PCWP was observed within 15 minutes of the start of administration, and most of the effect was achieved at 60 minutes of the infusion.

Unlike dobutamine, nesiritide did not cause ventricular or supraventricular arrhythmias in patients with decompensated heart failure, and it actually reduced ventricular ectopy or had a neutral effect (Prospective Randomized Evaluation of Cardiac Ectopy with Dobutamine or Natrecor Therapy [PRECEDENT] study).[72] Urine output (mean change in volume status) during the first 24 hours of nesiritide infusion did not differ from that obtained with nitroglycerin treatment. Nesiritide may also cause hypotension (Vasodilation in the Management of Acute Congestive Heart Failure [VMAC] study).[73] The VMAC study also showed that relief from dyspnea was indeed reduced by nesiritide treatment but at levels not significantly different from the reduction caused by the relatively low dose of nitroglycerin used for comparison.

Controversy remains, however, as to whether nesiritide is associated with a significantly increased risk of worsening renal function and death after treatment.[74,75] This finding was based on meta-analyses of all available randomized clinical trials evaluating nesiritide in acute decompensated heart failure. Its use has not translated into long-term improvement in clinical outcome.[73]

Ularitide

Ularitide is a synthetic version of urodilatin that is currently under clinical development for the treatment of AHFS. Ularitide has the same ANP_{95-126} amino acid sequence as urodilatin. Like urodilatin, the synthetic version ularitide also binds to NPR-A receptors. Maximum concentration is reached 1.5 to 2 hours after the start of infusion. Various studies have reported a half-life range of 2 to 24 minutes.[48,76,77]

The primary pharmacodynamic effects of ularitide are preload and afterload reduction due to vasodilation of peripheral veins and arteries and increased diuresis and natriuresis at higher doses, lowering blood pressure. These effects result from its direct action on vascular smooth-muscle cells, causing dilation of renal, pulmonary, and coronary arterial vessels, and from inhibition of sodium reabsorption in the renal tubules. Ularitide activity is largely mediated by binding to NPR-A receptors, leading to increased intracellular cGMP levels. Consistent with this mode of action, dose-dependent ularitide-induced increases in plasma cGMP levels have been observed in both healthy subjects and subjects with AHFS.[78]

Ularitide restores fluid hemostasis by inducing diuresis and natriuresis, and vasodilation. In healthy volunteers, ularitide increased the renal filtration fraction by increasing GFR and reducing the effective renal plasma flow, and it increased the fractional excretion of sodium and the rate of urinary sodium excretion.[48] Additionally, administration of ularitide in healthy subjects at dosages up to 20 ng/kg/min leads to a decrease in plasma concentrations of renin, aldosterone, and angiotensin II, suggesting that ularitide may also have a downregulating effect on the RAAS that inhibits aldosterone secretion from the adrenal cortex.[47,48] Ularitide infusion into healthy volunteers also led to reduced cardiac preload, pulmonary arterial pressure (PAP) and PCWP, increased CI, and reduced systemic vascular resistance (SVR).[79] These mechanisms suggest that ularitide may provide a potential benefit to patients with AHF characterized by elevated left and right ventricular filling pressures and neuroendocrine activation.

The clinical efficacy of ularitide in patients with ADHF was evaluated in SIRIUS II, a parallel-dose, placebo-controlled, double-blinded study in 221 patients randomized to placebo or one of three ularitide dosing arms. Ularitide was administered by 24-hour intravenous infusions at 7.5, 15, or 30 ng/kg/min. Compared with placebo for change from baseline to +6 hours, all three ularitide doses were associated with significantly greater reductions in the co-primary efficacy end point of mean PCWP. Dyspnea at 6 hours (the second of two co-primary end points) was rated moderately or markedly improved by a greater percentage of subjects in each ularitide dose group than in the placebo group.[80]

Adverse effects of ularitide include increased sweating, nausea, dizziness, and hypotension (decreased blood pressure) with infusions of 5 ng/kg/min or greater. In the studies where these were observed, all side effects resolved without vasoconstrictor treatment. No apparent worsening of renal function was observed through 72 hours in the SIRIUS II study.

Conclusion

Natriuretic peptides are a family of peptide hormones with distinct biomolecular properties and clinical effects that have been well studied as a treatment modality for AHFS. Structurally, each has a 17-amino-acid loop differing in up to five positions among the different natriuretic peptides.

The vasodilators nesiritide and carperitide have been approved in the United States and Japan, respectively, for acute heart failure. The natural analogues of carperitide and nesiritide (ANP and BNP) are both produced by the atria and ventricles of the heart in response to increased blood pressure and stretching of the cardiac wall and generally cause vasodilation, RAAS inhibition, and reduction in blood pressure without affecting fluid volume. Plasma BNP has also been used as a diagnostic or prognostic marker for acute and chronic cardiac syndromes.

Ularitide, a synthetic form of the renal NP urodilatin, is currently undergoing clinical development. In contrast to the other peptides, urodilatin (ularitide), is produced in the kidney and acts in a paracrine fashion on collecting ducts, resulting in reduced Na^+ reabsorption and thereby increased natriuresis and diuresis. Intravenously applied ularitide also causes vasodilation, reduction in blood pressure, and bronchodilation.

Because of the capacity of the natriuretic peptides to restore hemodynamic balance and fluid homeostasis, they hold promise in the management of cardiopulmonary and renal symptoms of AHFS, although further studies are required to understand more completely their impacts on short- and long-term morbidity and mortality in AHFS patients.

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Exhibit 14

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Review

Developmental actions of natriuretic peptides in the brain and skeleton

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Abstract. The concept that atrial natriuretic peptide (ANP) and the closely related peptides BNP and CNP might be involved in the ontogeny of several organ systems emerged in the late 1980s. While many of the reported in vitro actions have not been examined in the context of organ development in vivo, recent studies demonstrate that mice which lack or overexpress natriuretic peptides or receptors exhibit pronounced skeletal growth defects. This article discusses how natriuretic peptides and other factors appear to regulate bone growth as an example of how natriuretic peptides might participate in the ontogeny of other organ systems. Evidence indicating that natriuretic peptides regulate neural development is then reviewed. Natriuretic peptides and receptors exhibit complex expression patterns in the developing nervous system, where they have been shown to act on neural cells as early as at the embryonic neural tube stage. Interestingly, both bone and brain growth appear to utilize primarily CNP and the CNP-specific type B receptor, and perhaps the type C receptor. In vitro data indicate that CNP may act on developing neurons, astrocytes and Schwann cells like a classical growth factor, regulating proliferation, patterning, phenotypic specification, survival and axonal pathfinding. Natriuretic peptides might also have roles in the vascularization of the embryonic brain, establishment of the blood-brain and blood-nerve barriers, and perhaps in nerve regeneration.

Key words. Atrial natriuretic peptide (ANP); BNP; CNP; receptor; embryo.

Introduction

Natriuretic peptides constitute a family of the three structurally related molecules: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and the type-C natriuretic peptide (CNP) [1–4]. The founding member of this peptide family, ANP, was first known as a hormone produced by the heart that regulated vascular tone, sodium and water balance, and other cardiovascular functions through action on the kidney and vascular smooth muscle cells (VSMCs). However, all three peptides are expressed in the adult brain, where one function appears to be central control of the cardiovascular system. There are three known mammalian receptors for peptides in the ANP family [3, 4]. Two of these, type A and B (NPR-A and NPR-B, respectively), are single transmembrane spanning proteins that contain guanylyl cyclase (GC) activity in their intracellular domain. The third, type C (NPR-C), is also a single transmembrane spanning protein, but contains only a short 37-amino acid intracellular domain, and lacks GC activity. Because NPR-C has a short intracellular domain devoid of GC activity, and is internalized after binding ANP, it has been referred to as the 'clearance' receptor. However, more recent data obtained with specific analogs for this receptor indicate NPR-C can transmit signals by inhibiting cyclic AMP (cAMP) formation, stimulating intracellular calcium levels, and/or by inhibiting a MAPK signaling pathway [5,

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reviewed in 4]. Natriuretic peptide receptors differ in their relative affinity for ANP analogs. NPR-A binds to ANP and BNP with high affinity, and to CNP with very low affinity. NPR-B, on the other hand, is relatively selective for CNP. NPR-C binds all natriuretic peptides with high affinity. The molecular pathways purportedly utilized by natriuretic peptides to regulate growth have recently been reviewed [6]

This paper focuses on natriuretic peptide control of bone and brain development. Evidence that natriuretic peptides regulate cardiac development and fetal cardiovascular homeostasis have been reviewed elsewhere [7, 8].

Early studies on the proliferative actions of natriuretic peptides on cultured cells

Early data indicating that natriuretic peptides might perform regulatory actions in the development of several organ systems were reviewed in 1990 [9]. The first report suggesting that natriuretic peptides might have growth factor-like actions appeared in 1985, and showed that ANP potently stimulated DNA synthesis in cultures of bovine adrenal zona glomerulosa cells with a significant increase at 10^{-12} M [10]. This interesting finding seems to have received little subsequent attention. Three years later, two groups showed that ANP, at concentrations as low as 10^{-11} M, inhibited the proliferation of rat kidney mesangial cells [11, 12]. Among other early studies, it was demonstrated that natriuretic peptides exerted antiproliferative effects on cultured vascular smooth muscle [13, 14] and endothelial cells [15]. Whereas the significance of the effects on mesangial and vascular cells during development remains to be determined, in vivo studies in mature animals suggest that the antiproliferative effects observed in vitro may be important after renal and vascular injury, respectively. For example, transgenic mice overexpressing BNP in the liver were found to exhibit greatly reduced mesangial expansion and cell proliferation compared to wild-type mice in two different models of glomerulonephritis [16, 17]. In the vascular system, CNP expression was found to be induced in carotid arteries after balloon injury [18, 19], and adenoviral delivery of CNP (but not control vector expressing β-galactosidase) to injured carotid arteries reduced neointimal formation by 90% [20]. Moreover, ANP and NPR-A knockout mice exhibited increased muscularization of pulmonary arterioles under normal and hypoxic conditions [21, 22],

Natriuretic peptide regulation of skeletal growth

The regulation of longitudinal bone growth has become the most well documented developmental action of natriuretic peptides. This comes as a result of data obtained from classical approaches and from transgenic and knockout mouse models of natriuretic peptides, receptors and their downstream signaling components. These studies are discussed in some detail here because the deduced models may serve in some ways as an example for how natriuretic peptides might pattern the brain and other organ systems. To help explain the mechanisms by which natriuretic peptides regulate bone growth, some background on bone development in the embryo is presented here [23, 24]. Bone formation in vertebrates proceeds by two distinct mechanisms. One of these, termed intramembraneous ossification is utilized primarily to form the flat bones of the skull. In this process, mesenchymal cells differentiate directly into osteoblasts, which deposit bone matrix to form ossification centers. There is currently no evidence that natriuretic peptides are involved in this process, so it will not be discussed further. The other mechanism, called endochondrial ossification, is the primary mechanism used to form the long bones and vertebrae. In this case, mesenchymal cells initially condense to form primordial bone. Cells in the core differentiate into chondrocytes, which then, as discussed below, undergo a program of sequential proliferation, hypertrophy, calcification, apoptosis and finally replacement with bone marrow. Surrounding the core, mesenchymal cells differentiate to become the cells of the perichondrium, which later differentiate into osteoblasts. These eventually replace most of the cartilage with bone. The chondrocyte lineage is a key cellular component driving the longitudinal growth of bones. Chondrocytes at the center of the primordial bone first differentiate into prehypertrophic and then hypertrophic chondrocytes. In contrast, chondroctyes outside of the central zone continue to proliferate along future longitudinal bone axis. As bone development proceeds, chondrocytes near the central zone begin to differentiate into hypertrophic chondrocytes, whereas older hypertrophic chondrocytes lying closer to the center induce mineralization of the surrounding matrix and undergo cell death. Thus, fronts of proliferating and differentiating chondrocytes (together called the epiphyseal growth plates) advance in opposing directions as the bone develops.

Many aspects of long bone development are dictated by autocrine or paracrine signals in these growth plates. Among these signals are Indian hedgehog (IHH), parathyroid hormone-related peptide (PTHrP), and members of the bone morphogenetic protein (BMP) family [25–28]. IHH is produced in chondrocytes as they progress from proliferative to hypertrophic states (fig. 1). PTHrP is expressed in articular perichondrium and signals to receptors on proliferating and prehypertrophic chondrocytes. IHH and PTHrP signaling is necessary to maintain a normal proliferative zone of chondrocytes in the advancing epiphyseal plate, and to prevent premature

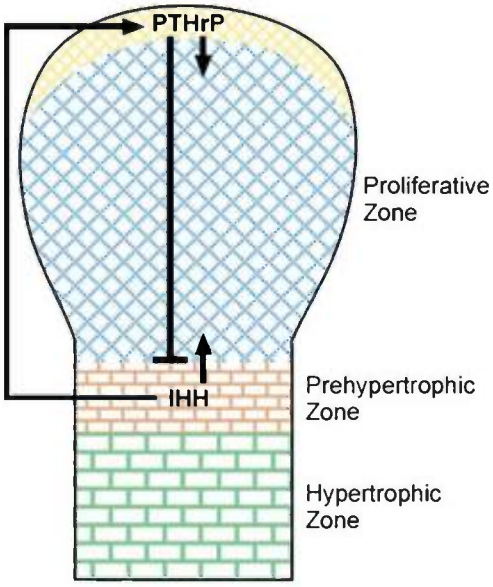


Figure 1. Paracrine regulation of long bone growth by Indian hedgehog (IHH) and parathyroid hormone related peptide (PTHrP). Only the end of the bone where growth is taking place is shown, i.e. the area of ossification underneath the hypertrophic zone is not shown. IHH is expressed in prehypertrophic chondrocytes (orange) as they progress from a proliferative (blue) to a hypertrophic (green) state. IHH acts directly on chondrocytes to positively regulate their proliferation, and indirectly by stimulating production of PTHrP in the articular perichondrium (yellow). PTHrP inhibits the differentiation of chondrocytes, thereby preventing hypertrophy from occurring in the growth zone near the articular surface. Thus, the range of PTHrP signaling determines the distance to the articular surface from which hypertrophy begins. Bone morphogenetic proteins (see text) as well as other secreted proteins also appear to have roles in long bone growth. As discussed in detail in this review, transgenic and knockout mouse experiments have confirmed that natriuretic peptides are important regulators of bone growth. CNP and its specific receptor (NPR-B) are expressed in both proliferating and prehypertrophic chondrocytes, but the way in which they interact with IHH/PTHrP signaling to regulate long bone growth is currently unknown.

differentiation of proliferating chondrocytes into hypertrophic chondrocytes. Both IHH [29] and PTHrP [30, 31] knockout mouse strains show a smaller zone of proliferating cells, resulting in hypertrophy occurring relatively close to the articular surface. In IHH knockout mice, PTHrP gene expression is absent in the articular perichondrium, suggesting that at least part of IHH action is mediated by PTHrP. Transgenic expression of a constitutively active form of the PTHrP receptor in chondrocytes prevented the premature chondrocyte hypertrophy in IHH knockout mice, but not the defects on chondrocyte proliferation and overall limb size [31]. Based on these and other functional studies and on gene expression patterns, a model has emerged (fig. 1) in which IHH expression in prehypertrophic chodrocytes positively regulates the proliferation of chondrocytes independent of PTHrP. At the same time, IHH signals to the articular perichondrium to induce expression of PTHrP, which in turn prevents hypertrophy from occurring in the growth zone near the articular surface. In this way, PTHrP maintains chondrocytes near the articular surface in a proliferative state. Moreover, its range of signaling determines the distance to the articular surface where hypertrophy begins. As chondrocytes proliferate, cells at the trailing edge of the growth plate fall out of range of the PTHrP signals, and thus are allowed to differentiate. BMPs appear to positively regulate IHH expression, but also appear to act in parallel with IHH and IHH/PTHrP to regulate chondrocyte proliferation and differentiation, respectively [28].

The first indication that natriuretic peptides might regulate bone growth was a report in 1988 demonstrating that ANP inhibited both basal and parathyroid hormone-stimulated DNA synthesis in cultures derived from avian tibia epiphyseal growth plates (presumably containing primarily cells of the chondrocyte lineage) [32]. Later, Hagiwara and co-workers showed that cultured rat chondrocytes expressed NPR-B, and that the preferred ligand for this receptor, CNP, was produced in these cultures and potently inhibited DNA synthesis [33]. Consistent with the antiproliferative effect being mediated by NPR-B, ANP was much less potent. Seemingly in conflict with these in vitro studies suggesting that natriuretic peptides might inhibit bone growth, transgenic mice which overexpressed BNP in the liver were found to exhibit skeletal overgrowth [34]. Investigators in the latter study felt that BNP acted promiscuously on NPR-B, because CNP was more potent than BNP in inducing growth of mouse tibial explants. They also argued against the involvement of NPR-C, because the actions in explant cultures were blocked by HS-142-1, an agent which appears to selectively block NPR-A and NPR-B. Consistent with a model in which CNP regulates bone growth via NPR-B, the CNP and NPR-B genes were found by Northern analysis to be expressed at high levels in embryonic mouse tibias, whereas no ANP, BNP, NPR-C and very little NPR-A gene expression were detected [35]. Moreover, in situ hybridization revealed that both CNP and NPR-B were localized in proliferative and prehypertrophic chondrocytes [36].

Further evidence that natriuretic peptides positively regulate bone growth in vivo came from reports indicating that three different strains of mice with spontaneous or chemical induced mutations in the NPR-C gene [37], and mice with a targeted deletion in the NPR-C gene [38] exhibited skeletal overgrowth. Because NPR-C functions to clear natriuretic peptides, the investigators proposed that the skeletal defects were due to elevated local levels of natriuretic peptides. Finally, CNP knockout mice showed a dwarf phenotype with a marked reduction in the heights of proliferative and hypertrophic chondrocyte zones [36]. The bone growth defect in CNP knockout mice was rescued by transgenic expression of CNP in chondrocytes, providing evidence that CNP acts locally on bone growth. Mice with targeted deletions of ANP, BNP and NPR-A

show no obvious skeletal defects, strengthening the notion that CNP, acting via the NPR-B receptor, is the primary natriuretic peptide regulator of longitudinal bone growth.

CNP appears to regulate bone growth in vivo primarily via cyclic GMP (cGMP)-dependent protein kinase II (cGK-II). Like CNP knockout animals, mice with a targeted mutation in the cGK-II gene had a dwarf phenotype which, in this case, could not be rescued by targeted expression of CNP in growth plate chondrocytes [39]. In summary, an abundance of data strongly support a model in which chondrocyte-derived CNP acts via NPR-B, cGMP and cGK-II within the epiphyseal growth plate to promote proliferation and/or delay differentiation of chondrocytes. However, despite knowledge gained from the pronounced skeletal phenotypes of natriuretic peptide ligand and receptor knockout and overexpressing mice, the relationships of this ligand receptor signaling system with other factors regulating bone growth such as IHH, PTHrP or BMPs are uncertain. Clearly these ligand receptors systems are non-redundant, because targeted deletion of each of the respective genes produces a strong phenotype. The interaction of these factors is likely to be addressed in the near future, with new epistasis information obtained by, for example, interbreeding of respective mice and/or analyses in other vertebrate models.

Natriuretic peptide action in the developing brain

The first indication that natriuretic peptides might have actions in brain development was a study published in 1990 in which Tong and Pelletier [40] used receptor autoradiography to show that high-affinity binding sites for ^{125}I-ANP were present in the embryonic day (E) 13 rat telencephalon ventricular zone (VZ). This zone is the specific area in the embryonic telencephalon where neuroblasts actively proliferate. Moreover, E13 is a time of peak neurogenesis in the rat. Subsequently, three other groups provided receptor autoradiographic evidence that high-affinity natriuretic peptide receptors are present in the developing rat nervous system, and associated blood vessels [41–43]. Two groups focused specifically within the rat brain and again reported their presence of ^{125}I-ANP binding sites in the telencephalon VZ [42, 43]. Radioligand displacement studies with different ANP analogs indicated that these latter sites exhibited a 'NPR-C-like' receptor peptide binding profile [42, 43], but were coupled to cGMP production [43].

In another more detailed study performed in embryonic day E14 mice, ^{125}I-ANP binding sites were observed over the developing blood vessels and the vascular plexus around the developing brain and in the meningeal layer [41]. In the area of the spinal cord, bilateral accumulations of silver grains were observed adjacent to (or over) the floor plate and in the roof plate. Radioligand binding sites were also observed over the boundary caps, DRG and peripheral nerves. The investigators reported that these latter binding sites were in Schwann cell precursors, satellite cells and Schwann cells respectively.

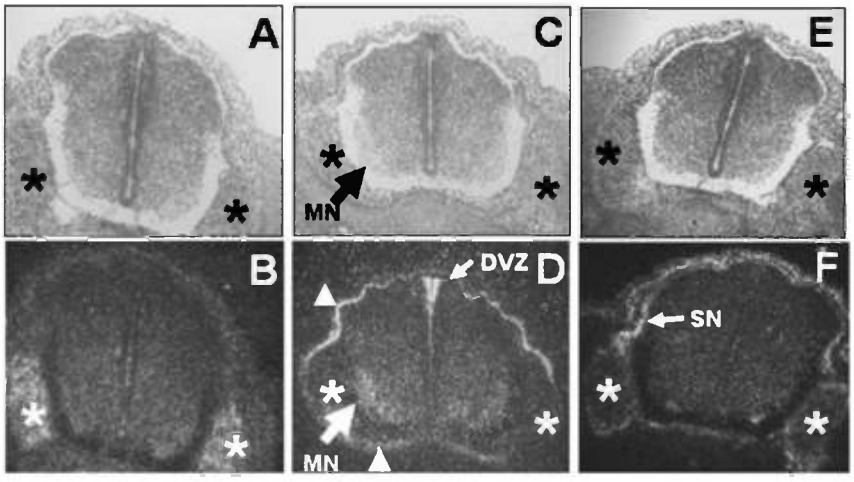


Figure 2. NPR-B (*A*, *B*), CNP (*C*, *D*) and NPR-C (*E*, *F*) gene expression in the area of the spinal cord in E14.5 mice. *A*, *C* and *E* are bright field micrographs; *B*, *D* and *F* are the same sections show in dark field. DRG are indicated by asterisks (*) in all panels, and are strongly labeled by the NPR-B riboprobe in *A* and *B*. A population of NPR-B-mRNA-hybridizing motor neurons is also indicated by large arrows in *C* and *D*. Arrowheads in *C* and *D* point to a band of CNP mRNA-positive cells that surrounds the spinal cord and DRG and may correspond to dura and/or chondrocytes in the growth plate of primordial bone. The small arrow in *D* points to an area of intense CNP gene expression in the dorsal VZ (DVZ) of the spinal cord. Among the sites of NPR-C gene expression were the DRG and spinal nerves (SN) (*E* and *F*) (panels *A*–*D* are taken from [44].

Localization of specific receptor and ligand subtypes in the developing nervous system by in situ hybridization

Using in situ hybridization, we recently examined the regional gene expression patterns for each of the specific natriuretic peptide receptors and ligands in the early embryonic mouse brain (E10.5 to E14.5) [44]. In the peripheral nervous system, NPR-B messenger RNA (mRNA) was abundant in sensory ganglia from E10.5 to E14.5 [fig. 2A, B show expression in E14.5 dorsal root ganglia (DRG)]. Tissue culture studies discussed below indicate that these receptors might mediate sensory neuron survival and/or neurite outgrowth. Within the brain, NPR-B mRNA was detected at E10.5 in cells just outside the VZ of the hindbrain [44], a region where post-mitotic neurons are undergoing differentiation. Receptors in this location may be involved in cell cycle exit, specification of cell lineage and/or patterning events (see below). NPR-C mRNA was found to be abundantly expressed at several sites within or near the nervous system. Among the prominent sites of expression were the trigeminal and DRG. Gene transcripts were localized within these ganglia, but also in the associated nerves, most likely in Schwann cells (fig. 2E, F, figs 3 and 4A). In vitro data discussed below suggest that natriuretic peptides might be a proliferative signal for these cells. Intense signals for NPR-C mRNA were also observed in the notochord (figs 3 and 4B), hinting that natriuretic peptides might play a role in spinal cord dorsal/ventral patterning. Moreover, signals were also observed in the roof plate of the spinal cord and in bilateral stripes in the VZ just lateral to the floor plate (fig. 3). The latter site of expression was reminiscent of Nkx and other homeobox transcription factor-expressing domains that give rise to oligodendrocytes or specific subclasses of ventral neurons [45, 46]. Given this complex pattern of NPR- C expression, it will be important to determine if this receptor serves in vivo just to clear or limit the diffusion of natriuretic peptides, or to signal in a direct way to regulate some aspect of development. In addition to expression in neuronal and glial cells, small discrete clusters of NPR-C gene transcripts were also observed within the CNS parenchyma, most likely over invading capillaries [44]. High levels of NPR-C expression were also observed in the perineural vascular plexus surrounding the brain [44]. NPR-A mRNA was not detected within the brain or any ganglia, although transcripts were observed in many large blood vessels.

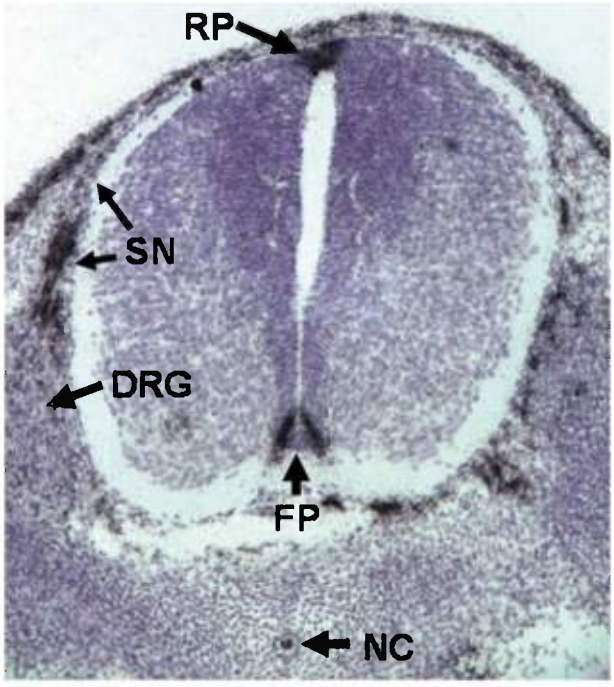


Figure 3. NPR-C receptor gene expression in the spinal cord in E12.5 mouse embryos. All panels are transverse sections. Intense gene expression is localized to the roof plate (RP), in stripes surrounding the floorplate (FP), in the spinal nerve (SN) and notochord (NC). A lower-level signal is present in the dorsal root ganglia (DRG). See figure 4 for higher magnification views (data adapted from [44].

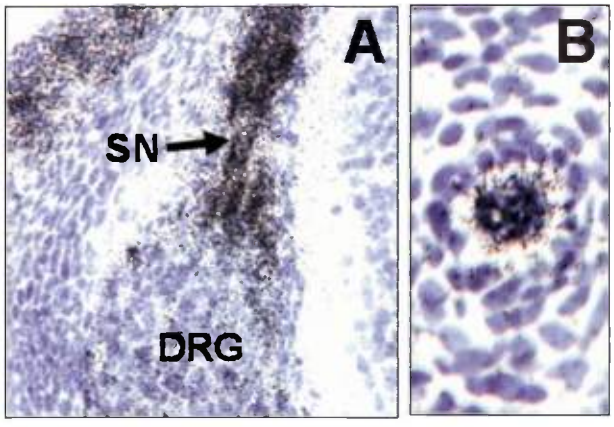


Figure 4. High magnification photos of selected areas of figure 3 showing NPR-C receptor gene expression in the area of spinal cord in E12.5 mouse embryos.

It may be instructive to compare the in situ hybridization results with the previously described receptor localizations by receptor autoradiography. Because ANP is a poor ligand for NPR-B, ^{125}I-ANP would not be expected to recognize, and did not localize to the NPR-B gene expression sites in the hindbrain that we observed by in situ hybridization. On the other hand, NPR-C gene transcripts showed an almost perfect one-to one spatial relationship with the ^{125}I-ANP binding sites reported by Scott and Jared [41, see above]. It is intriguing, however, that we were unable to detect gene expression for any of the receptors in the telencephalon VZ, whereas autoradiographic approaches by three different groups detected highly abundant ^{125}I-ANP binding sites at this location

[40, 42, 43]. One potential explanation for this apparent discrepancy is that these binding sites correspond to an unknown receptor. Binding and displacement profiles of natriuretic peptide analogs on both embryonic brain extracts and autoradiographic sections do suggest the existence of a novel high-affinity natriuretic peptide receptor [42, 43]. Moreover, an NPR-C specific analog potently inhibited the proliferation of a mouse neuroblastoma cell line in which no NPR-C gene transcripts could be detected by reverse transcription – polymerase chain reaction using multiple primer sets [47]. Because the human genome does not seem to contain other sequences with extensive homology to known natriuretic receptors, such an atypical receptor, if it exists, is likely to have a primary structure considerably different than NPR-A, NPR-B and NPR-C.

The above receptor localization data suggest that natriuretic peptides might regulate neural development via NPR-B, NPR-C and/or novel receptors, but what could be the source of the peptide? We detected high levels of CNP gene expression at all levels of the CNS caudal to the mesencephalon beginning at E10.5 [44], in agreement with a previous observation of Cameron [48]. Specifically, CNP transcripts were detected at E10.5 in the dorsal and intermediate VZ in these regions. Later, at E14.5, CNP expression was still observed at high levels in the dorsal VZ, although new signals were seen laterally in cells in the dorsal hindbrain, reflecting either new cellular expression or radial cell migration. Specific hybridization signals were also observed in cells surrounding the neural tube, apparently in the dura layer of the meninges (fig. 2C, D). CNP gene transcripts were also observed in the cartilage of the neural arch at E14.5, which is juxtaposed to Schwann cells in developing spinal nerves, and in a layer on the outside or just lateral to the DRG. We did not examine expression earlier than E10.5, but another group detected CNP mRNA in RNA isolated from embryonic mouse heads at E.9.5 [49].

In contrast to CNP, we did not detect ANP and BNP gene transcripts in the nervous system at E10.5 to E14.5, but they were expressed at high levels in the embryonic heart. Although we did not extend our experiments beyond E14.5, another group reported that ANP mRNA was present in the mouse DRG at E15.5 [48]. CNP is also reported to be expressed in vascular endothelial cells in adults [50], although we could not detect CNP gene transcripts in capillaries that invaded the developing brain. In any case, CNP may be available locally from several sources to regulate neural development in the embryo: in the dorsal CNS VZ and parenchyma, dura and cartilage adjacent to peripheral spinal nerves, and possibly in endothelial cells of capillaries invading the brain, peripheral nerves and ganglia. ANP produced in DRG cells and ANP and BNP from the heart might also be sources of natriuretic peptides that regulate neural development. The latter endocrine source might be especially important at early stages, before the development of blood-brain and blood-nerve barriers.

Postnatal time courses of ANP, BNP and CNP gene expression in the rat brain have also been reported [51]. Interestingly, ANP and CNP gene expression in numerous brain regions was transient or significantly higher between P4 and P13 than at P60, suggesting that natriuretic peptides may play one or more general roles in postnatal development, such as regulation of synaptogenesis or myelination.

Analysis of natriuretic peptide action using tissue culture models

Embryonic CNS cultures

On the basis of the above in situ hybridization data showing NPR-B gene expression in the E10.5 hindbrain, we established cultures of neural precursors isolated from this region and treated cells with different natriuretic peptides [44]. CNP potently inhibited DNA synthesis in these cultures, with an EC_{50} of approximately 10^{-10} M. ANP was much less potent than CNP, whereas the NPR-C specific agonist des-[Gln(18),Ser(19),Gly(20),Leu(21),Gly(22)]-ANP(4-23)-NH(2) ($desANP_{4-23}$) exhibited a level of potency between CNP and ANP. The potent inhibitory action of CNP was consistent with in situ hybridization studies showing the presence of NPR-B gene expression in this embryonic brain region. The fact that the NPR-C-specific analog $desANP_{4-23}$ also inhibited DNA synthesis indicated that at least some of the antiproliferative actions may have been mediated through a NPR-C or NPR-C-like receptor. Although we could not localize NPR-C mRNA in the embryonic hindbrain by in situ hybridization, we previously detected its expression in this region by RT-PCR [47].

Using a chick neural plate explant model, it was recently reported that 'chick' natriuretic peptide was able to enhance the ability of the patterning factor sonic hedgehog (Shh) to induce ventral phenotypes in the dorsal explants [52]. To determine if a similar interaction between Shh and natriuretic peptides might occur in the mouse embryonic hindbrain, we studied the ability of Shh and CNP to regulate expression of the sonic Shh target gene gli-1 in our embryonic mouse hindbrain cultures. We found that both CNP and Shh induced the expression of gli-1, and that the combined induction was additive [44]. These data, plus the fact that NPR-B mRNA was detected in the early embryonic hindbrain and that NPR-C gene expression was localized to the notochord, bilateral stripes just above the anterior spinal cord floor plate and in the roof plate (see above), suggest that natriuretic peptides might have a role in dorsal/ventral patterning.

E14.5 rat DRG

The receptor localization studies showing that NPR-B and NPR-C genes were expressed in the embryonic DRG suggested that developing sensory neurons might also be a target of natriuretic peptide action during development. Thus, the actions of these peptides were examined using cells isolated from E14.5 rat DRG [44]. In cultures containing both neurons and Schwann cells, CNP elicited a twofold increase in DNA synthesis, with a peak activity at 10^{-8} M. Significantly, $desANP_{4-23}$, exhibited far greater potency, with an EC50 of approximately 10^{-10} M. In contrast, while ANP also increased DNA synthesis, effects were not observed until a dose of 10^{-7} M. The high potency of both CNP and $desANP_{4-23}$ in the mitogenic stimulation of DRG cells suggested that effects were mediated by both NPR-B and NPR-C receptors. Labeling with BrdU and cell-specific markers indicated that the cells undergoing proliferation in these cultures were Schwann cells. We also determined the ability of 10^{-7} M CNP to regulate neuron survival and neurite outgrowth in DRG cultures. CNP had modest positive effects on neuron survival and significantly inhibited neurite outgrowth. Thus natriuretic peptides appear to have the ability to regulate the proliferation of Schwann cells and sensory neuron survival, and might have a function in sensory neuron process elaboration. The latter might have significance in the regulation of sensory neuron axon pathfinding, an idea supported by the fact that cGMP exhibits potent action in growth cone tuning assays [53, 54]. Because CNP inhibits process elongation in vitro, CNP might function in vivo as a repulsive signal. The expression data discussed above suggest one possibility: as axons leave the DRG they encounter CNP in either the dura or cartilage surrounding the spinal cord (fig. 2C, D). Because of this they avoid entering into this tissue and instead traverse up towards and eventually into the cord. Sensory axons then terminate in the dorsal cord, a consequence potentially reinforced by inhibitory CNP signals emanating from the VZ and ventral cord (fig. 2C, D).

Cultured astrocytes

Although natriuretic peptide receptors (mRNA or protein) have not been demonstrated on astrocytes in vivo, several papers indicate that astrocyte proliferation can be regulated by natriuretic peptides via NPR-C. Levin and Frank first showed in 1991 that natriuretic peptides inhibit DNA synthesis in cultured astrocytes [55]. The antiproliferative action was produced with very low concentrations of an analog specific for NPR-C, and was associated with an inhibition of MAP kinase activity [5]. An NPR-C analog was also found to inhibit the proliferation of a mouse neuroblastoma cell line [47]. This antiproliferative effect was lost when MAP kinase activity was previously inhibited with the MEK1/2 antagonist PD98059. The data suggest that a MAP kinase growth factor pathway constitutively operated in these cells. In the presence of PD98059, this growth factor pathway may have been blocked, preventing any further inhibitory action of an NPR-C agonist. That a MAP kinase growth pathway was constitutively active in these cells was evidenced by the fact the PD98059 alone inhibited basal and serum-stimulated proliferation in these cells.

Cultured olfactory sensory neurons

Olfactory sensory neurons in rodents undergo continuous proliferation and turnover throughout adult life. Thus, the expression patterns and growth-related actions of natriuretic peptides and receptors were examined in the rat olfactory epithelium by Ronnett and co-workers [56]. NPR-B was localized to olfactory neurons in the upper one-half of the epithelium of the adult rat, where immature and maturing neurons reside. NPR-B was also expressed at and just above the interface of the olfactory epithelium and the lamina propria. The ligand CNP was associated with sustentacular cells, most prominently in the cell foot processes. CNP was also associated with olfactory sensory neuron axon bundles in the lamina propria leading into the bulb. In contrast to NPR-B, CNP was also expressed in the olfactory bulb, in periglomerular cells that surround the glomeruli and, in some cases, within the mitral cells deeper within the bulb.

The expression of CNP in sustentacular cells and in cells surrounding glomeruli and of NPR-B in olfactory neurons located in the different layers of the epithelium, suggested that CNP acts as a paracrine factor that could regulate olfactory sensory neuronal proliferation, differentiation or survival. To study the effect of natriuretic peptides on developing olfactory neurons, primary neonatal rat olfactory neuron cultures were utilized [56]. CNP by itself had no effect on BrdU incorporation into olfactory precursors, but strongly inhibited the proliferative effect of BDNF. CNP also induced differentiation in BDNF-treated cultures, as monitored by the percentage of cells containing processes and the expression of a marker of differentiated olfactory neurons. CNP also appeared to promote the survival of mature neurons. Taken with data obtained from other culture models, these results indicate that natriuretic peptides exhibit diverse action of neural cells that depend on cell type and developmental stage.

Vascularization of the brain

Although no published studies appear to have addressed the role of natriuretic peptides on angiogenesis specifically in the brain, considerable evidence indicates that natriuretic peptides regulate several general aspects of angiogenesis during development and/or after vascular in-

jury. Blood vessel formation in a particular organ or tissue can begin by either vasculogenesis (formation of blood vessels from mesodermal tissue de novo) or angiogenesis (generation of new vessels by extension or modification of existing vessels). The former process occurs by the differentiation of mesodermal cells into endothelial cells, expansion and the condensation of these cells into a primitive capillary tubelike network. Subsequent steps include recruitment of smooth muscle cells and pericytes, secretion of extracellular matrix proteins and vascular remodeling. The vascular system in the brain appears to occur exclusively by angiogenesis.

The molecular steps involved in the formation of blood vessels are beginning to be elucidated [57–60]. Vascular endothelial growth factor (VEGF) and its receptors lie at a center of a regulatory network controlling blood vessel growth and differentiation. In the process of vasculogenesis, VEGF is secreted by tissue in response to hypoxia, and acts on local mesoderm-derived angioblasts to induct their differentiation and proliferation. VEGF appears to also be important at later steps in vasculogenesis and angiogenesis (see below). Another set of important factors include the angiopoietins and their receptors (Tie1/2). Activation of Tie 2 receptors expressed on endothelial cells appears to result in release of factors that recruit primordial VSMCs. Gene knockout and transgenic overexpression studies indicate that the VEGF and angiopoietin ligand receptor systems are critical for blood vessel development, but the precise actions for these ligands and receptors have not yet been elucidated (reviewed in [59]). Other transgenic studies implicate PDGF-α and perhaps TGF-β in the recruitment/proliferation/differentiation of VSMCs. *Eph* receptors and their ligands, classically believed to direct migration of neurons and axons, may also direct vascular network assembly, affecting endothelial migration, capillary morphogenesis and angiogenesis [61].

Although certain fundamental processes in angiogenesis are probably used in the vascularization of all organs, each tissue must carefully regulate this process to suit its specific needs. One aspect of angiogenesis that is unique in the brain is that blood vessels must eventually develop and maintain a blood-brain barrier (BBB) [62]. Nerves leaving or entering the brain must also develop a barrier, in this case, called the blood-nerve barrier (BNB). These barriers include endothelial cells specialized to contain high-resistance tight junctions, specific transporters, metabolizing enzymes and other proteins [63]. In contact with these endothelial cells are pericytes and astrocyte foot pads, which appear to provide factors which contribute to the endothelial cell specialization [62–66]. The BBB and BNB develop gradually during development, and are not fully mature until the postnatal period [62, 67, 69].

Initial vascularization of the brain begins about the time of neural tube closure with the formation of a primitive endothelial capillary network over the surface of the brain (called the perineural vascular plexus) [66, 68–70]. The perineural vascular plexus arises in part from angioblasts that migrate from the splanchnopleuric mesoderm to the surface of the neural tube. New blood vessels invade the brain by a process called sprouting angiogenesis. Vascularization of the brain thus requires the proliferation and recruitment of both endothelial and VSMCs/pericytes derived from the perineural vascular plexus, and mechanisms which direct sprouting vessels into proper locations in the developing brain.

In embryonic mice, it has been shown that VEGF is transiently expressed in the ventricular zone (VZ) [71], while a VEGF receptor (VEGFR2/*flk-1*) is expressed in the perineural vascular plexus and in the endothelial cells that have invaded the developing brain tissue towards the VZ [66]. This has led to the suggestion that VEGF in the VZ stimulates the ingrowth of capillaries from the perineural vascular plexus.

Vascularization of the retina has recently been modeled in the rat, cat and human [72–75]. These studies suggest that VEGF, produced in retinal glial cells, guides capillary growth in neural tissue. A primary stimulus for angiogenesis during the development of the retina (like many other tissues) is believed to be hypoxia, and is mediated by VEGF and other angiogenic factors. In the retina, transient VEGF expression is observed specifically in glial cells just ahead, and at the leading edge, of advancing blood vessels. One of its receptors (VEGF2/flk-1), on the other hand, is expressed in the endothelial cells at the leading edge and just behind the advancing blood vessel. A short period of time after the primitive vessel is formed, pericytes from the established part of the vessel proliferate and migrate along the vessel sprout. The latter also appears to be a VEGF-mediated action, but may also involve PDGF-α Blood vessel formation and glial VEGF expression have been shown to be coordinately regulated in response to changes in atmospheric oxygen in rats and mice. Cultured glial cells exposed to hypoxic conditions respond with an increase in VEGF gene expression. Thus, glial cell appear to be a critical mediator of angiogenesis in the retina.

Natriuretic peptides and blood vessel formation

Although the role of natriuretic peptides in brain angiogenesis or BBB formation has not been specifically addressed, several potential roles of natriuretic peptide and their receptors in blood vessel formation have been proposed based on in vitro studies. As previously discussed, ANP and BNP are expressed early in the developing heart (E11 in the rat [76]), whereas CNP appears to be produced by vascular endothelial cells. Natriuretic peptides have been shown to inhibit the spontaneous as well as hypoxia- and endothelin-stimulated proliferation of puri-

fied VSMCs [9, 77–81], to inhibit the proliferation of endothelial cells [82] and to inhibit the recruitment of VSMCs [83, 84]. Natriuretic peptides were shown to inhibit the production and release of the VEGF from cultured VSMCs [77], suggesting one potential mechanism for natriuretic peptide regulation of angiogenesis. Functional receptors also appear to be present on pericytes [85].

As previously discussed, an early described action of ANP was inhibition of vascular endothelial cell proliferation. A recent study indicates that ANP can inhibit VEGF signaling in these cells [86]. Interestingly, an extended dose-response analysis showed that low concentrations of natriuretic peptides can increase, rather than decrease endothelial cell proliferation and migration in vitro [87]. The effects were significant at ANP concentrations as low as 10^{-13} M, and were blocked by inhibition of PKG and a MAP kinase pathway. This underscores the importance of considering a full concentration range of peptides in such studies.

Summary

Soon after the initial characterization of ANP about 20 years ago, it was proposed that natriuretic peptides play important roles in the development of certain organ systems. Recent reports indicating that natriuretic transgenic and knockout mice exhibited severe skeletal defects and showed impaired recovery after vascular and renal injury have added strong evidence that natriuretic peptides can act in growth-related capacities. With respect to brain development, there is abundant data indicating that high-affinity natriuretic binding sites and receptor gene transcripts are expressed in the early developing brain and associated blood vessels. Further, natriuretic peptides have been shown to potently regulate the proliferation, survival and neurite outgrowth of cultured neuronal and/or glial cells, and to regulate the proliferation and recruitment of blood vessel cell components. In vivo models should clarify the significance of in vitro studies in the next few years. In addition, given that natriuretic peptides appear to exert antiproliferative actions after renal and vascular injury, it will be of interest to determine if these peptides have roles in nerve injury and regeneration.

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Exhibit 15

Natriuretic Peptides in Health and Disease

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7 Cellular and Molecular Aspects of C-Type Natriuretic Peptide (CNP)

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EXPRESSION AND DISTRIBUTION OF CNP

Structure of CNP

C-type natriuretic peptide (CNP) was originally isolated from porcine brain extracts *(1)*. CNP has a primary structure similar to α-ANP and BNP consisting of 22 amino acids,

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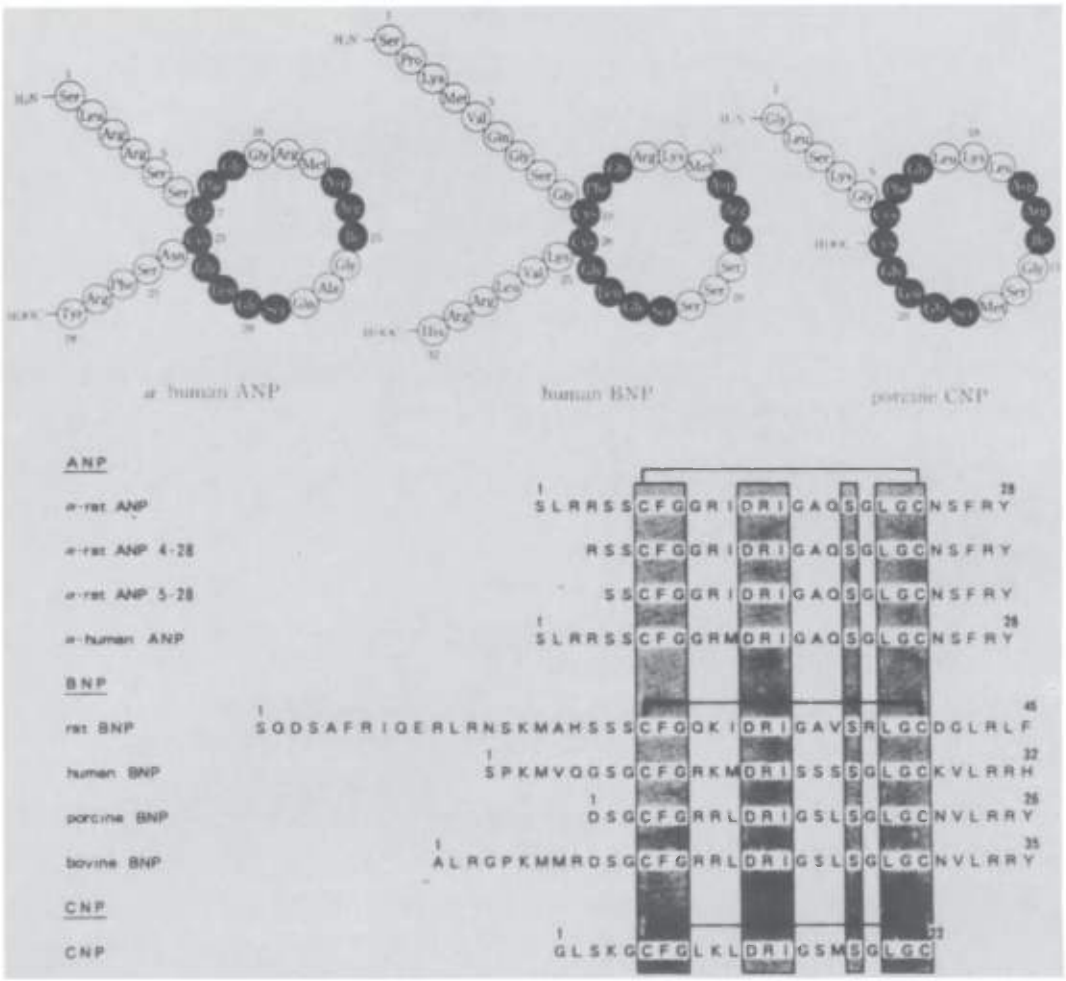


Fig. 1. Amino acid sequences and primary structures of the natriuretic peptide family. Shadowed area shows homology among mammalian ANP, BNP, and CNP.

and the ring portion consisting of 17 amino acids is highly homologous to α-ANP and BNP (Fig. 1). Different from ANP and BNP, CNP lacks the C-terminal tail, and has a Cys residue at the C-terminus. Another species of CNP is CNP-53, which has the N-terminal extension of 31 amino acids compared with CNP-22 *(2)*. Molecular cloning of the CNP precursor in the pig, rat, human, and mouse has revealed that the primary structure of CNP-22 is identical in these species *(1,3–5)*. However, two amino acid substitutions are noted in CNP-53 between human and porcine/rat/mouse precursors. ProCNP consists of 103 amino acids and a single arginine residue precedes CNP-53 and CNP-22. Using reverse-phase high-performance liquid chromatography (HPLC), we demonstrated that the major molecular forms in the human brain are CNP-22 and CNP-53 *(4)*.

Distribution of CNP

Using the specific radioimmunoassay (RIA) for CNP, it was elucidated that immunoreactive CNP was detected in the porcine, rat, and human brains, but not in the periph-

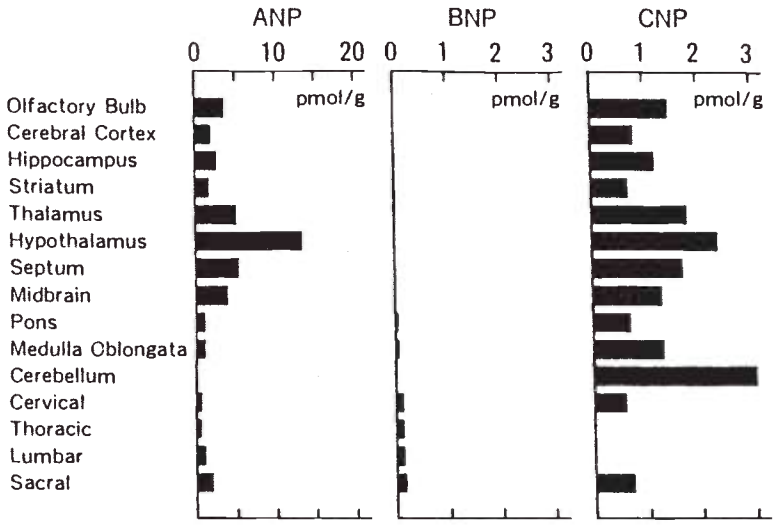


Fig. 2. Regional distribution of ANP-, BNP-, and CNP-like immunoreactivities in rat brain areas.

Table 1
ANP-, BNP-, and CNP-Like Immunoreactivities (LI)
in Discrete Regions of the Human Brain[a]

	Natriuretic peptide		
Brain regions	*ANP-LI*	*BNP-LI*	*CNP-LI*
Cerebral cortex	<0.07	<0.06	0.61
Thalamus	<0.07	<0.06	2.90
Hypothalamus	0.13	<0.06	4.02
Midbrain	<0.07	<0.06	3.28
Pons	0.15	<0.06	<0.4
Medulla oblongata	0.08	<0.06	2.78
Cerebellar cortex	<0.07	<0.06	0.85

[a]All values are pmol/g of wet tissue.

eral organs, including the heart *(4–6)*. As shown in Fig. 2, the CNP concentration in the rat brain is highest in the cerebellum, followed by the hypothalamus, thalamus, septum, and olfactory bulb. In the human brain, the highest concentration was found in the hypothalamus, followed by the midbrain and thalamus (Table 1). As shown in Table 1, the concentration of CNP in the human brain is higher than that of ANP or BNP by at least one order of magnitude. Reflecting the high level of CNP in the brain, the CNP concentration in the human cerebrospinal fluid is about 10 times higher than the ANP or BNP concentration *(7)*. CNP is, therefore, considered to be the major natriuretic neuropeptide. By using Northern blotting, CNP mRNA could be detected in the porcine and rat brains, but not in the heart *(1,3)*. In the rat brain, CNP mRNA is abundantly expressed in the anteroventral preoptic area and arcuate nucleus *(8)*. CNP is also highly expressed in the human, rat, and porcine pituitary glands *(1,3,4,6)*.

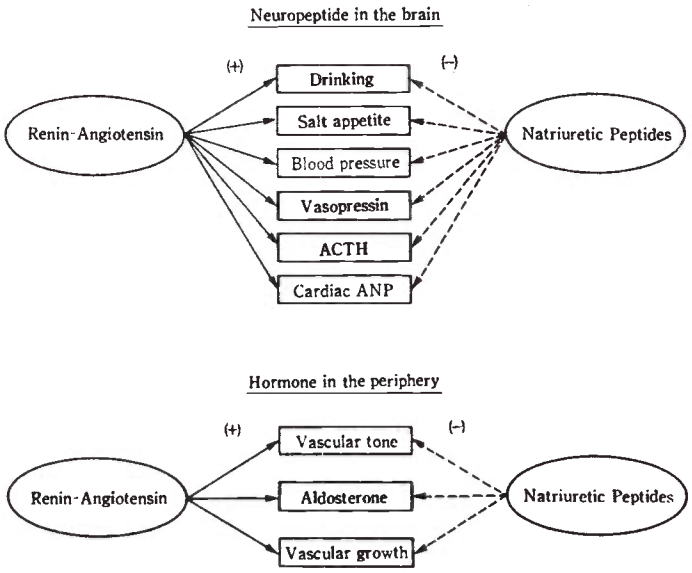


Fig. 3. Antagonistic relationship between the natriuretic peptide system and the renin–angiotensin system in the central nervous system and the periphery.

BIOLOGICAL ACTIONS OF CNP

Central Actions of CNP

By the intracerebroventricular (icv) injection of ANP and BNP into unrestrained conscious rats, we and others have demonstrated that natriuretic peptides can exert inhibitory actions on water drinking, induction of salt appetite, centrally mediated blood pressure increase, and vasopressin and ACTH secretions *(9–12)*. These actions of natriuretic peptides are antagonistic to those of angiotensin II (AII). In the periphery, AII causes vasoconstriction and enhances aldosterone secretion, thus acting to increase body fluid and raise blood pressure. ANP induces vasodilation, diuresis, and natriuresis and inhibits aldosterone secretion, hence acting to decrease body fluid and lower blood pressure. Thus, the renin–angiotensin system and the natriuretic peptide system have similar actions in both the central nervous system (CNS) and the periphery and regulate body fluid and cardiovascular homeostasis in an antagonistic way (Fig. 3) *(13,14)*.

Among the natriuretic peptide receptors, it was elucidated that guanylyl cyclase-B (GC-B) is selectively activated by CNP and considered to be the specific receptor for CNP, whereas guanylyl cyclase-A (GC-A) shows high affinity to ANP and BNP *(15,16)*. Figure 4 shows the mRNA expressions of three subtypes of the natriuretic peptide receptor in the rat brain by the *in situ* hybridization technique. Reflecting the abundant expression of GC-B in the CNS, the icv injection of CNP is demonstrated to exert similar inhibitory action on AII-induced vasopressin secretion *(17)*. However, the potency of centrally administered CNP was about one-tenth that of ANP. Figure 5 shows the effect of the icv injection of the antisense oligonucleotide directed against rat GC-B mRNA in conscious rats on AII-induced vasopressin secretion, to block the endogenous expression of GC-B. As clearly shown in Fig. 5, the increase of plasma vasopressin level induced by AII was significantly potentiated by the administration of the GC-B antisense oligonucleotide compared to the control oligonucleotide. This result indicates the physiological role



Fig. 4. Regional expression of three subtypes of the natriuretic peptide receptor (GC-A, GC-B, and C-receptor) mRNAs in the rat brain by *in situ* hybridization.

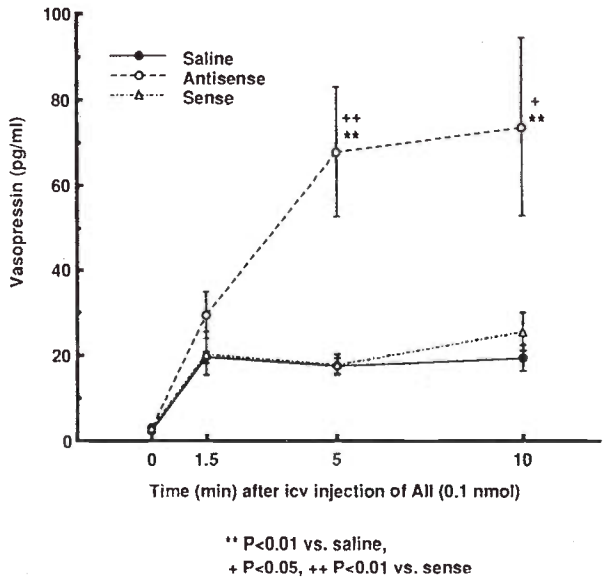


Fig. 5. Effect of the icv injection of the antisense oligonucleotide against GC-B mRNA on angiotensin II-induced vasopressin secretion in conscious, unrestrained rats.

of CNP/GC-B in modulating vasopressin secretion in the brain. As shown in Fig. 4, the distribution of GC-B is not the same of GC-A. Furthermore, differential expression of GC-B and GC-A in neuronal and glial cells is also demonstrated *(18)*. Therefore, the different roles of CNP in the CNS is well expected.

Peripheral Actions of CNP

To examine whether CNP can elicit biological actions in the periphery, we investigated the effect of synthetic CNP administered intravenously in humans and rats and compared it with that of ANP *(19,20)*. The iv injection of CNP (2 nmol/rat) into the conscious rat increased the plasma level of cyclic GMP (cGMP) from 8.8 ± 1.6 pmol/mL to 39 ± 2.2 pmol/mL 5 min after CNP injection. In contrast, the iv administration of ANP at the same dose increased the plasma cGMP level up to 258 ± 15 pmol/mL 5 min after the injection. In humans, the administration of CNP (0.43 nmol/kg) elicited significant

Table 2
Chromosomal Localization of Natriuretic Peptide System

Ligand	*Mouse/human*	*Receptor*	*Human*
ANP	4/1	ANP-A receptor	1
BNP	4/1	ANP-B receptor	9
CNP	1/2	Clearance receptor	5

increase of plasma cGMP level (from 4.77 ± 1.25 to 8.33 ± 1.59 pmol/mL 15 min after administration) and of urinary cGMP excretion (from 30.7 ± 4.3 to 74.9 ± 13.4 nmol/30 min). Intravenously administered CNP exerted significant diuretic, natriuretic, kalliuretic, and chloriuretic actions with the increase of endogenous creatinine clearance. The CNP also elicited significant hypotensive actions (ΔBPs/ΔBPd: –4.3 ± 1.3/–4.1 ± 1.0 mmHg) with concomitant increase of heart rate (+7.6 ± 2.6 bpm). Plasma aldosterone concentration significantly decreased from 45.4 ± 2.3 to 35.4 ± 4.9 pg/mL 30 min after administration. The biological activities of CNP administered intravenously in humans and rats were, therefore, weaker than that of ANP *(21–24)*, suggesting the reduced abundancy of GC-B, to which intravenously injected CNP can access.

The CNP was also demonstrated to exert potent antigrowth effect on cultured vascular smooth muscle cells (VSMC) *(14,25–27)* or migrating and proliferating VSMC in the neointima after balloon-injury in vivo that possess synthetic phenotype and highly express GC-B as described below *(28)*.

STRUCTURE AND CHROMOSOMAL ASSIGNMENT OF CNP GENE (TABLE 2)

We cloned mouse and human CNP genes *(4,5)*. The mouse CNP gene is composed of at least two exons and one intron. The 5'-flanking region contains an array of *cis*-acting regulatory elements, including Y box, GC box, and CRE-like sequence as well as a dinucleotide CA repeat (microsatellite). On the basis of the polymerase chain reaction (PCR)-analyzed microsatellite-length polymorphisms among recombinant inbred strains of mice, the CNP gene *(Nppc)* was assigned to mouse chromosome 1 (cen-*Acrg*-0.79 ± 0.82c *M-Nppcl(Sag)*-3.33 ± 2.67c *M-ln*). In contrast, using somatic hybrid cell methodology, the human CNP gene *(Nppc)* was assigned to chromosome 2 (2q24-qter). Thus, chromosomal assignment of the CNP locus in mice and humans has added another locus to the conserved syntenic group in mice and humans. It has been reported that both the ANP and the BNP genes, which are expressed predominantly in the heart, are localized on human chromosome 1 *(29,30)*. Furthermore, it is also demonstrated that these genes are tightly linked on mouse chromosome 4 *(29–31)*. In contrast, CNP does not act as a cardiac hormone but as a local regulator or a neuropeptide. The CNP gene is shown to be localized on mouse chromosome 1/human chromosome 2, which is physically separated from the ANP and BNP genes. Therefore, CNP is functionally and evolutionally distinct from ANP and BNP. Given the remarkable structural conservation of CNP among species, and even among nonmammals, it is tempting to speculate that CNP is a prototype of the natriuretic peptide family, from which ANP and BNP originated through gene duplication.

DEMONSTRATION OF VASCULAR NATRIURETIC PEPTIDE SYSTEM

Secretion of CNP from Vascular Endothelial Cells

Using specific RIA for CNP, we detected CNP-like immunoreactivity (-LI) in the culture medium conditioned with endothelial cells derived from the bovine carotid artery *(32)*. The CNP-LI in the conditioned medium was accumulated in a time-dependent manner and reached 17.8 ± 1.6 fmol/10^6 cells/24 h. The endothelial cellular content of CNP-LI was 0.15 ± 0.05 fmol/10^6 cells. High-performance gel-permeation chromatographic analysis revealed that CNP-LI in the condition medium from the endothelial cells consisted of two components corresponding to synthetic CNP and CNP-53. Northern blot analysis detected CNPmRNA with a size of 1.2 kb in the cultured endothelial cells. These results demonstrate the synthesis and secretion of CNP by the cultured endothelial cells.

Occurrence of CNP in the In Vivo Blood Vessels

We examined whether CNP gene expression occurs in the in vivo blood vessel wall by reverse transcription and polymerase chain reaction (RT-PCR) *(19,33)*. Our RT-PCR revealed that CNP gene transcripts were detected in blood vessels in vivo of a wide range of mammals, including not only the bovine but also human, rabbit, rat, and hamster aortae. Furthermore, the gene transcript for GC-B was simultaneously detected with that for CNP in human arteries at various sites *(33,34)*. More recently, by the immunohistochemical method using our monoclonal antibody (MAb) against CNP (KY-CNP-I) *(35)*, we demonstrated the presence of CNP in the endothelial cells in human coronary arteries *(36)*. Detection of the expressions of CNP and GC-B in in vivo vascular walls strongly indicates the presence of the "vascular natriuretic peptide system," in which CNP serves as a local regulator of vascular tone and growth (Fig. 6), like "the vascular renin–angiotensin system" *(37)*. The CNP can, therefore, be considered to be novel endothelium-derived relaxing factor (EDRF) *(19,38)*.

Regulation of Endothelial CNP Secretion in the Interaction of Endothelial Cells/Vascular Smooth Muscle Cells/Macrophages (Fig. 7)

The endothelial secretion of CNP was regulated by various vasoactive substances, including growth factors and cytokines. Among them, transforming growth factor-β (TGF-β), one of the crucial factors involved in vascular remodeling, induced more than two orders of magnitude increase of endothelial CNP secretion *(32)*. In addition, the endothelial secretion of CNP is potently stimulated by tumor necrosis factor-α (TNF-α), one of the central cytokines for inflammation and also by lipopolysaccharide (LPS), the endotoxin itself *(39)*. We also demonstrated that shear stress at the physiological range augmented CNP expression in human, bovine, and mouse endothelial cells *(40)*. Actually, within the mouse CNP gene we cloned *(5)* are NF-κ β (which is crucial for TNF-α induced gene expression), TGF-β, and shear-stress responsive elements. In contrast, endothelial CNP secretion was potently inhibited by oxidized LDL *(41)*, insulin *(42)*, and vascular-endothelial growth factor (VEGF), a novel endothelium-specific angiogenic factor *(43)*.

We investigated the interaction of endothelial cells (EC), VSMC, and macrophages (M ϕ) for the regulation of endothelial CNP production, its molecular mechanisms, and CNP action on vascular growth, using the coculture system *(44)*. The concentration of CNP in the medium of EC/VSMC coculture was increased 60-fold, compared with that

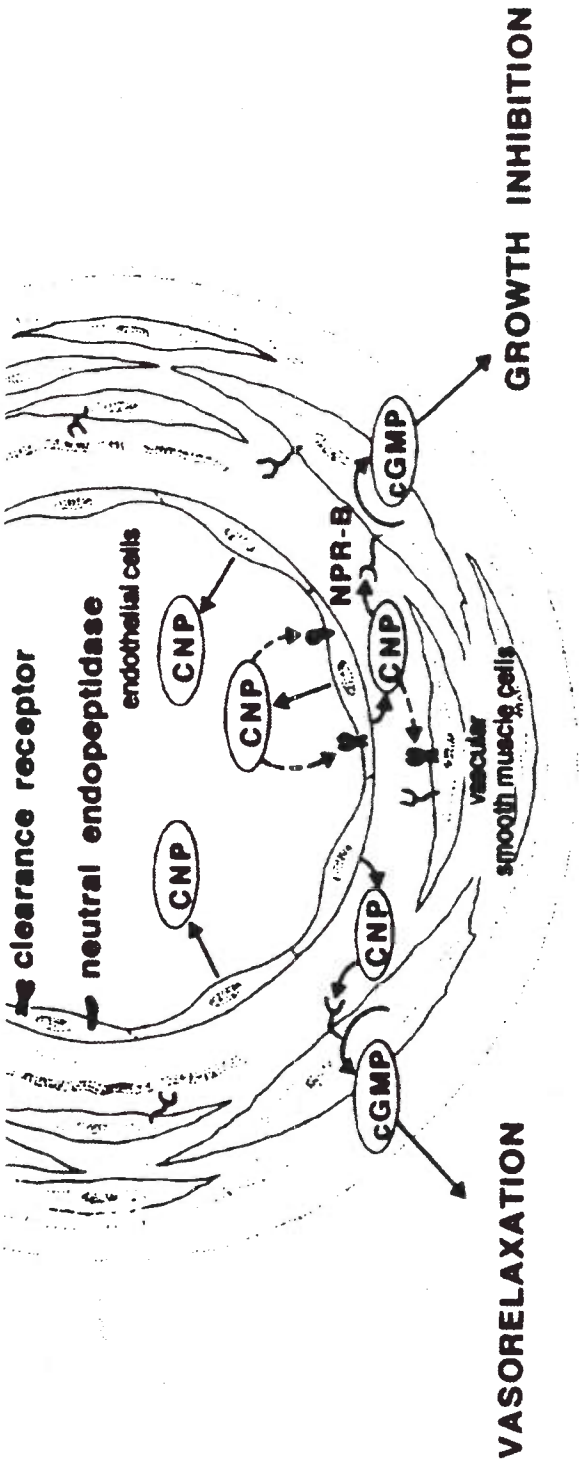


Fig. 6. Existence of the vascular natriuretic peptide system.

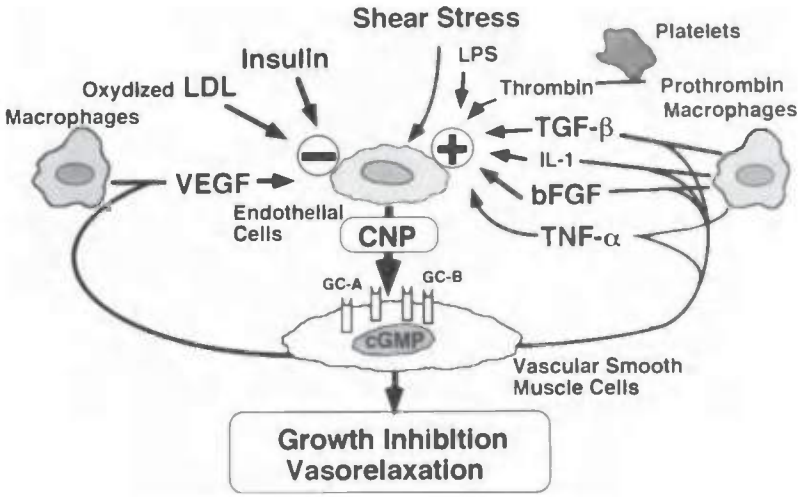


Fig. 7. Regulation of endothelial secretion of CNP.

of EC alone. The endothelial production of CNP was also augmented 10-fold in EC/M ϕ (THP-1, a human monocytic leukemia cell line, treated with phorbol ester) coculture (Fig. 8A). As shown in Fig. 8B, Northern blot analysis revealed augmented expression of CNPmRNA in EC/M ϕ cocultures. The accumulation of intracellular cGMP in the medium was increased in EC/VSMC coculture. This response was blocked by the anti-CNP MAb, and HS-142-1, the nonpeptide natriuretic-peptide receptor antagonist *(45)*. The concentration of biologically active TGF-β in the medium of EC/VSMC coculture was elevated to the level to stimulate the endothelial production of CNP. Actually, the neutralizing antibody against TGF-β abrogated the cGMP accumulation in the coculture. In contrast, the augmentation of the endothelial production of CNP in EC/M ϕ coculture was suppressed by the neutralizing antibody against TNF-α. These results show that the endothelial production of CNP in EC/VSMC and EC/M ϕ cocultures is at least in part regulated by TGF-β and TNF-α, respectively. Furthermore, the conditioned medium from EC was demonstrated to have the growth inhibitory effect on VSMC, which was abolished by the anti-CNP MAb and HS-142-1. The treatment of the anti-CNP MAb and HS-142-1 also significantly increased the cell number of the EC/VSMC coculture. These results reveal the pathophysiological significance of endothelial CNP as a novel paracrine/autocrine vascular local regulator for vascular growth in the interaction of EC, VSMC, and M ϕ.

DETECTION OF PLASMA CNP AND ITS REGULATION

We examined the presence of CNP in circulation in humans and rats *(19,46)*. The dilution curves of the plasma extracts from both humans and rats were parallel to the standard curve of CNP in the RIA. The plasma CNP-LI level of CNP in healthy persons was 1.4 ± 0.6 fmol/mL and that of male Wistar rats (200–300 g) was 1.2 ± 0.2 fmol/mL. Whereas there was no alteration in the plasma CNP-LI level in patients with congestive heart failure or hypertension, the plasma level of CNP was increased in septic patients and further elevated in septic shock (Fig. 9). TNF-α is recognized to be the central mediator

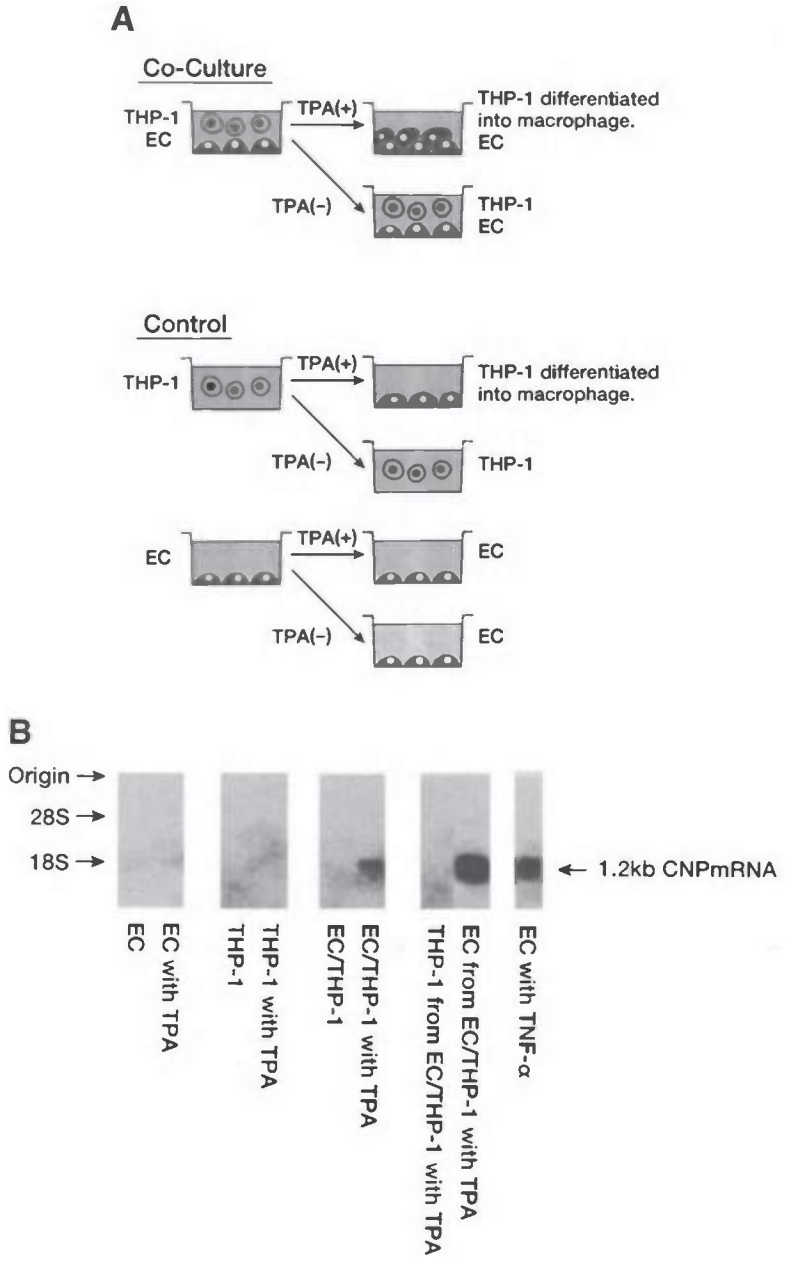


Fig. 8. Endothelial production of CNP in the interaction of endothelial cells and macrophages. **(A)** Experimental protocol of the coculture of endothelial cells and THP-1 treated with TPA (which differentiated into macrophages). **(B)** Expression of CNPmRNA in endothelial cells/THP-1 with and without the treatment of TPA.

of the septic shock and its production is enhanced in the syndrome. The increased plasma level of CNP in sepsis is, therefore, at least in part the consequence of augmented CNP secretion from vascular endothelial cells, since TNF-α is one of the potent stimulators of

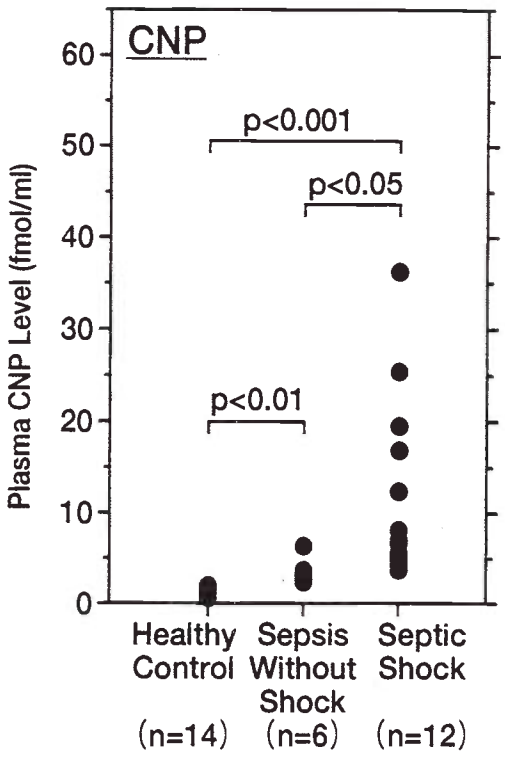


Fig. 9. Plasma CNP level in septic patients.

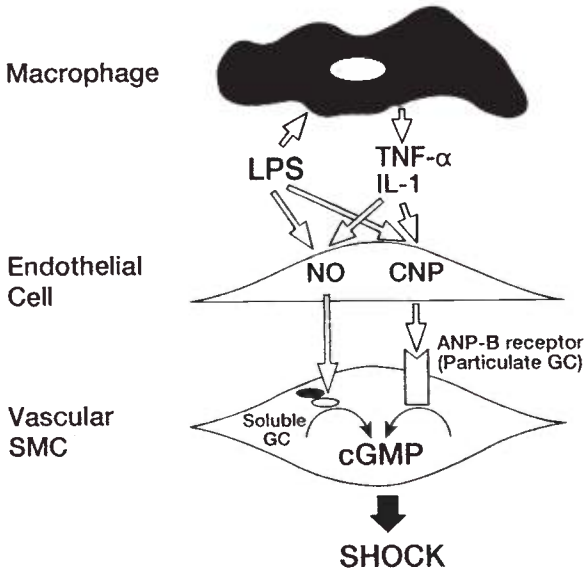


Fig. 10. Possible pathophysiological implication of endothelial CNP in endotoxin shock.

endothelial CNP secretion *(39)*. So far, nitric oxide (NO), EDRF itself, is recognized to be the central mediator of septic shock. Our results suggest that CNP can also be involved in the pathophysiology of shock in concert with NO (Fig. 10).

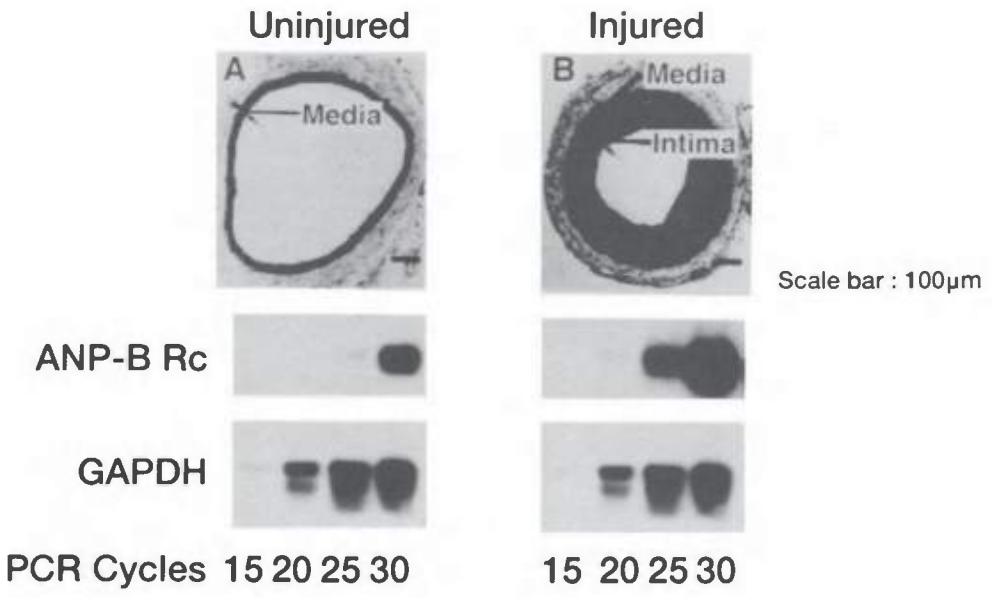


Fig. 11. GC-B (ANP-BRc) gene expression in rat balloon-injured carotid arteries evaluated by RT-PCR/Southern blotting (2 wk after the balloon injury).

SIGNIFICANCE OF VASCULAR NATRIURETIC SYSTEM IN VASCULAR REMODELING

To elucidate the role of CNP in human proliferative vascular lesions, we examined vascular expression of CNP by immunohistochemical analysis *(36)*, using the anti-CNP MAb (KY-CNP-I), which we developed *(35)*. In normal coronary arteries, intense CNP staining was observed in EC. In contrast, atherosclerotic lesions showed a marked decrease in positivity for staining of EC. However, M ϕ and VSMC, which were recruited in the lesions, showed positive staining for CNP, indicating CNP production in these cells. Actually, besides EC, these are some reports that show the production of CNP in cultured human and murine macrophages *(47,48)*. In restenotic coronary lesions after PTCA, regenerating EC also exhibited attenuated CNP expression, whereas infiltrating M ϕ and VSMC with synthetic phenotype showed CNP positivity.

The gene expression of natriuretic peptide receptor subtypes was also investigated. In VSMC obtained from the media of the intact rat aorta that possess the contractile phenotype, GC-A is highly expressed. In contrast, in cultured VSMC that show the synthetic phenotype, GC-A expression is downregulated and, in turn, GC-B and C-receptor expressions are much augmented *(49)*. The subtype switching of the natriuretic-peptide receptor in the phenotype change of VSMC was assessed in intimal-thickening lesions after carotid injury in rats. A Fogarty catheter was introduced into the left carotid artery, inflated, and withdrawn to produce endothelial denudation on male SD rats. The mRNA expression of GC-B was significantly augmented in the injured carotid arteries (Fig. 11) *(14,28)*. There is also a report that shows the upregulation of C-receptor expression in balloon-injured lesions *(50)*.

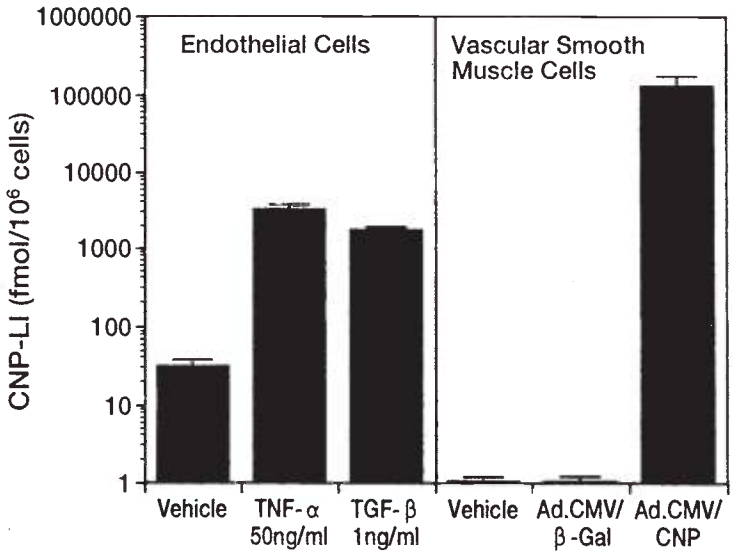


Fig. 12. CNP secretion from vascular smooth muscle cells transacted with adenovirus overexpressing CNP gene in comparison with CNP secretion from endothelial cells. Ad.CMV/CNP: adenovirus expressing CNP gene. Ad.CMV/β-Gl: control adenovirus expressing β-galactosidase gene.

IN VIVO GENE TRANSFER OF CNP INTO BLOOD VESSELS— APPLICATION OF VASCULAR NATRIURETIC PEPTIDE SYSTEM TO GENE THERAPY FOR PROLIFERATIVE VASCULAR LESIONS

As described above, we revealed that in human atheromatous arteries, VSMC with synthetic phenotype overexpress GC-B. In contrast, CNP expression in EC was reduced in these lesions. Therefore, to selectively suppress the growth of VSMC with synthetic phenotype in vascular lesions, we aimed at overexpression of CNP gene in VSMC by adenovirus-mediated gene transfer, expecting the autocrine/paracrine action of CNP. Rat aortic VSMC (5×10^3 cells/well) were infected with a replication-deficient recombinant adenovirus containing cytomegalovirus promoter-driving rat CNP cDNA, (advCMV/CNP) (40 MOI). The advCMV/CNP-transacted VSMC produced 122 ± 44 pmol/10^4 cells of CNP, which was 4000 times higher than EC (32 ± 5 fmol/10^4 cells) (Fig. 12).

The augmentation of cGMP production and almost complete growth arrest was observed (84% reduction) in AdCMV/CNP-treated VSMC. Furthermore, CNP overexpression at the site of balloon injury of the rabbit femoral artery actually significantly suppressed the formation of the neointima in vivo. These results indicate the profound autocrine/paracrine growth-inhibitory property of CNP chronically secreted from adenoviral-transfected VSMC and suggest the usefulness of CNP as an autocrine/paracrine growth inhibitor for gene therapy of proliferative vascular lesions.

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Exhibit 16

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STRUCTURAL REQUIREMENTS OF C-TYPE NATRIURETIC PEPTIDE FOR ELEVATION OF CYCLIC GMP IN CULTURED VASCULAR SMOOTH MUSCLE CELLS

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SUMMARY : C-type natriuretic peptide (CNP), which was recently found to be a selective ligand for one of the two known natriuretic peptide receptor guanylyl cyclases (NPR-B), potently stimulates cGMP production in cultured rat vascular smooth muscle cells (VSMC) and exerts potent antiproliferative effects on the cells. To investigate the structural requirements of CNP for stimulation of cGMP accumulation *via* NPR-B, we prepared CNP analogs and tested them on cultured rat VSMC. Our results indicate that only the ring portion of CNP with a disulfide bond (CNP(6-22)) participates in stimulation of cGMP accumulation, especially the sequence Leu^{9}-Lys^{10}-Leu^{11} in the ring portion executes essential roles for both elevation of cGMP and selectivity of the ligand for NPR-B. We also found a good correlation between the activities of the CNP analogs for stimulation of cGMP accumulation and inhibition of DNA synthesis. © 1992 Academic Press, Inc.

The natriuretic peptides are a family of homologous peptide hormones that exert natriuretic/diuretic and hypotensive effects *in vivo* through the activation of the cell surface receptor guanylyl cyclases (1). Molecular cloning studies have so far identified two types of natriuretic peptide receptor guanylyl cyclases, which were designated as NPR-A (GC-A) and NPR-B (GC-B) (2-5), and very recently it has been demonstrated that both atrial natriuretic peptide (A-type natriuretic peptide, ANP) (6) and brain natriuretic peptide (B-type natriuretic peptide, BNP) (7) are selective ligands for NPR-A, whereas C-type natriuretic peptide (CNP), which has recently been identified as a third member of the natriuretic peptide family (8), is a selective ligand for NPR-B (9). The structure-activity relationships of ANP have already been investigated well (10-12), however, little is known for those of CNP.

Recently we have found that CNP exhibits more potent activities for stimulation of cGMP accumulation and inhibition of DNA synthesis than α-human ANP (α-hANP) in cultured rat

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Abbreviations: CNP, C-type natriuretic peptide, pro-CNP[105-126]; ANP, atrial natriuretic peptide (A-type natriuretic peptide); α-hANP, human pro-ANP[99-126]; BNP, brain natriuretic peptide (B-type natriuretic peptide); VSMC, vascular smooth muscle cells.

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vascular smooth muscle cells (VSMC), whereas α-hANP exerts more potent activities for elevation of cGMP content and vasorelaxation than CNP in intact rat aorta (13, 14). These observations suggest that functional NPR-B and NPR-A receptors are dominantly expressed in cultured VSMC and intact aorta, respectively. In the present investigation, we focused on the effects of CNP on cultured rat VSMC, and intended to define the structural requirements of CNP for stimulation of cGMP accumulation *via* NPR-B. Structurally CNP has a 17-residue ring structure formed by an intramolecular disulfide linkage which is a common structural motif of the natriuretic peptides. Only 5 of the 17-residues in the ring portion differ between CNP and α-hANP, whereas the N- and C-terminals vary in both amino acid composition and length. Especially noteworthy is the C-terminal structures, CNP ends at the second cysteine residue, while α-hANP has 5 amino acids extension from the ring structure. With these structural differences in mind, we prepared CNP analogs with an emphasis on modification of the peptide at residues characteristic to CNP, and examined the activity of the analogs for stimulation of cGMP accumulation in cultured rat VSMC. The relationship between the activities of the analogs for cGMP accumulation and inhibition of DNA synthesis in VSMC will also be presented.

MATERIALS AND METHODS

Peptides : CNP, α-hANP and all the analogs were synthesized by the solid phase method as described previously (6, 8, 12). Gene and mRNA analyses across species have shown that the amino acid sequences of human, porcine and rat CNP with 22 amino acids (CNP-22) are identical (15-17). All peptides were characterized by amino acid analysis. The amino acid sequences of the analogs are listed in Table 1.

Table 1. Amino acid sequences of CNP analogs

Compound No.	Amino acid sequences
	1 6 9 10 11 16 17 22
CNP	G L S K G C F G L K L D R I G S M S G L G C
	1 7 10 11 12 17 18 23 28
α-hANP	S L R R S S C F G G R M D R I G A Q S G L G C N S F R Y
1	G L S K G C F G G R M D R I G A Q S G L G C N S F R Y
2	G L S K G C F G L K L D R I G S M S G L G C N S F R Y
3	S L R R S S C F G L K L D R I G S M S G L G C
4	C F G L K L D R I G S M S G L G C
5	G L S K G *MeC* F G L K L D R I G S M S G L G *MeC*
6	G L S K G C F G G K L D R I G S M S G L G C
7	G L S K G C F G L R L D R I G S M S G L G C
8	G L S K G C F G L K M D R I G S M S G L G C
9	G L S K G C F G L K L D R I G A M S G L G C
10	G L S K G C F G L K L D R I G S Q S G L G C
11	G L S K G C F G G K M D R I G S M S G L G C
12	G L S K G C F G L R M D R I G S M S G L G C
13	G L S K G C F G G R L D R I G S M S G L G C
14	G L S K G C F G G R M D R I G S M S G L G C
15	S L R R S S C F G L K L D R I G A Q S G L G C N S F R Y

One-letter symbols for amino acids are used. Amino acid residues in α-hANP are underlined.
MeC ; S-methyl cysteine.
All peptides except analog 5 have a 17-amino acid residue ring structure formed by an intramolecular disulfide linkage between two cysteine residues.

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cGMP determinations in rat VSMC : Rat VSMC were derived from explants of male Sprague-Dawley rats thoracic aorta (18), and were cultured in 24-well plates. The cells were incubated with the peptides in the presence of 0.5 mM 1-methyl-3-isobutylxanthine for 15 min at 37°C. The incubation was terminated by aspirating the medium and adding 6% perchloric acid. Intracellular cGMP was measured by using a cGMP radioimmunoassay kit (Yamasa Shoyu) as described previously (12).

DNA synthesis of rat VSMC : DNA synthesis in rat VSMC was assayed by measuring the incorporation of [methyl-^{3}H]-thymidine into acid-insoluble materials of the cells (19). VSMC were subcultured in 24-well plates and quiescent cells were incubated with the peptides for 20 hr followed by the addition of 37 kBq of [methyl-^{3}H]-thymidine (ca. 740 MBq/mmole, Amersham) and further 4 hr incubation. The reaction was stopped by adding 10% trichloroacetic acid, and the radioactivity in the acid-insoluble materials was determined with a liquid scintillation spectrometer.

Vasorelaxation in rat aorta : Aortic strips were prepared from thoracic aorta of Sprague-Dawley rats. The strips was contracted with 2×10^{-7} M norepinephrine and vasorelaxant activity of the peptide was determined (12).

RESULTS AND DISCUSSION

Cyclic GMP content in cultured rat VSMC was potently increased by CNP as reported previously (13), and the maximum elevation induced by 10^{-6} M of CNP was 6.27-fold higher than that induced by 10^{-6} M of α-hANP (Table 2). We first intended to clarify whether the exocyclic N-terminal portion or the ring portion of CNP was responsible for cGMP elevation in

Table 2. Biological activities of CNP analogs

Peptide or Compound No.	cGMP accumulation in VSMC (pmole/4x10^5 cells)[a]	[%][b]	Inhibition of DNA synthesis in VSMC (%)[c]	Vasorelaxation in aorta EC_{50}(x10^{-9}M)[d]	[%][e]
CNP	10.43	[100]	59	83.7	[100]
α-hANP	1.68	[16]	39	3.8	[2202]
1	2.47	[24]	28	11.9	[705]
2	11.20	[107]	58	31.7	[264]
3	11.13	[107]	61	27.1	[308]
4	10.34	[99]	60	63.3	[132]
5	0.64	[6]	15	N.D.	
6	5.78	[55]	51	128.4	[65]
7	6.93	[67]	61	106.7	[78]
8	8.34	[80]	59	84.3	[99]
9	11.87	[114]	59	N.D.	
10	11.06	[106]	63	N.D.	
11	1.19	[11]	33	N.D.	
12	4.58	[44]	56	N.D.	
13	0.97	[9]	25	105.3	[80]
14	0.39	[4]	18	90.5	[92]
15	9.39	[90]	68	4.0	[2110]

Each value represents the mean of three to five experiments. a ; cGMP concentration in VSMC induced by 10^{-6} M of the peptide. b ; relative potency to CNP. c ; Inhibition of [^{3}H]-thymidine incorporation into VSMC by 10^{-7} M of the peptides. Thymidine incorporation without the peptides are defined as 100%, and the results are expressed as percent inhibition. d ; The concentration of the peptide required for half-maximum relaxation of rat aorta contracted by norepinephrine (2×10^{-7} M). e ; relative potency to CNP. N.D. ; not determined. The numbers of the analogs correspond to those in Table 1.

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cultured rat VSMC. For this purpose, the N-terminal portions of α-hANP and CNP were exchanged, the C-terminal portion of α-hANP was added to CNP, and the N-terminal portion of CNP was deleted (Table 1, analogs **1-4**). As shown in Table 2, the analog in which the N-terminal portion of α-hANP was substituted with that of CNP (analog **1**) elicited cGMP elevation comparable to α-hANP. The analogs which contain the ring portion of CNP (analogs **2-4**) all caused potent elevation of cGMP content similar to CNP. The addition of the C-terminal residues of α-hANP to CNP (analog **2**) did not affect the activity for stimulation of cGMP accumulation, and substitution of the N-terminal portion of CNP with that of α-hANP or deletion of the N-terminal residues of CNP (analogs **3, 4**) did not alter the activity either. In addition, we found that both human and porcine CNP-53, which are N-terminal extended forms of CNP (15, 20), stimulated cGMP accumulation in VSMC as potently as CNP (data not shown). On the other hand, cleavage of the disulfide bond of CNP (analog **5**) resulted in a marked reduction in potency, suggesting that the ring structure of CNP with a disulfide bond was essential for the activity. These results indicate that only the ring portion of CNP with a disulfide bond (CNP(6-22), analog **4**) participates in stimulation of cGMP accumulation and that neither the exocyclic N-terminal portion nor the C-terminally truncated structure of CNP are responsible for elevation of cGMP.

In the ring portion of CNP, there are 5 different amino acids in the sequence compared to α-hANP (Table 1). To determine the amino acids in CNP crucial for stimulation of cGMP accumulation, we next performed single amino acid substitutions in the ring portion of CNP with the corresponding amino acid of α-hANP (analogs **6-10**). As shown in Table 2, the analog in which Ser or Met at position 16 or 17 was replaced by Ala or Gln (analogs **9, 10**) exhibited the potent activity for elevation of cGMP in VSMC similar to CNP. Substitution of the amino acid at position 9, 10 or 11 (analogs **6-8**) resulted in a reduction in potency. These results indicate that the amino acids Leu, Lys and Leu at positions 9 to 11 in CNP but not the amino acids at positions 16 and 17 execute an important role for stimulation of cGMP accumulation in VSMC. But none of the single amino acid substituted analogs (analogs **6-8**) achieved as much reduction in activity as α-hANP does. This suggests that at least two amino acids at positions 9 to 11 in CNP participates in stimulation of cGMP accumulation through activating NPR-B. Therefore, the role of the 3 amino acid residues at position 9 through 11 in CNP was examined further. Simultaneous substitutions of 2 amino acids in CNP by the corresponding ones of α-hANP (analogs **11-13**) resulted in a marked decrease in potency (Table 2). When all the 3 amino acids at positions 9 to 11 were substituted (analog **14**), the ability for cGMP elevation was mostly diminished (less than 1/20 of CNP). Furthermore, analog **15** in which the sequence Gly-Arg-Met at positions 10 to 12 of α-hANP were replaced by a sequence Leu-Lys-Leu elevated cGMP levels as potently as CNP. From these results, we concluded that the sequence Leu^{9}-Lys^{10}-Leu^{11} in the ring portion of CNP executes essential roles for both stimulation of cGMP accumulation and selectivity of the ligand for NPR-B.

Table 2 also compares the activity of the CNP analogs for vasorelaxation in isolated rat aorta, in which NPR-A is dominantly expressed. As was expected, the amino acid(s) substituted analogs (analogs **6-14**) exhibited weak vasorelaxant effects when compared to α-hANP. Surprisingly, the vasorelaxant activity of [Leu^{10}, Lys^{11}, Leu^{12}]-α-hANP (analog **15**)

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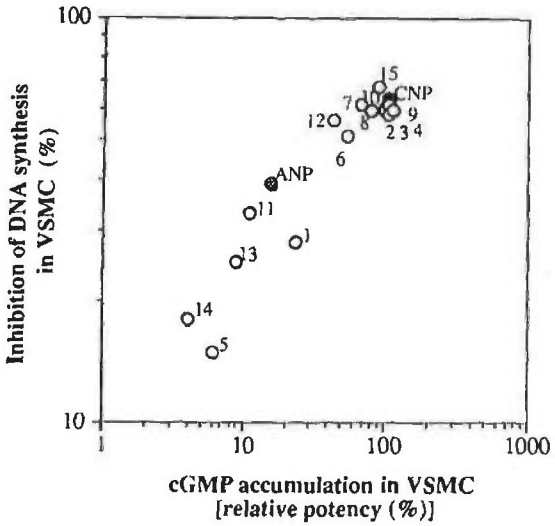


Figure 1. Comparison between the activities of the CNP analogs for cGMP accumulation and inhibition of DNA synthesis in cultured VSMC. The numbers of the analogs correspond to those described in Table 1. Relative potencies of the analogs for cGMP accumulation in VSMC are expressed as the ratio to those of CNP and are shown in Table 2. The potencies of the peptides for inhibition of DNA synthesis are also shown in Table 2.

was equivalent to that of α-hANP, whereas the activity of [Gly^{9}, Arg^{10}, Met^{11}]-CNP (analog **14**) was as low as CNP (Table 2). These observations indicate that the sequence Gly^{10}-Arg^{11}-Met^{12} in α-hANP alone is not sufficient for the NPR-A selectivity of the ligand and additional residues are involved. It should be noted that [Leu^{10}, Lys^{11}, Leu^{12}]-α-hANP (analog **15**) is the first ligand that fully activates both the two known natriuretic peptide receptor guanylyl cyclases, NPR-A and NPR-B.

Recently we have demonstrated that CNP inhibits mitogenesis and proliferation of VSMC through elevation of cGMP (14). We examined the activity of the CNP analogs for inhibition of DNA synthesis in cultured VSMC, and as shown in Table 2 and Figure 1, the inhibitory action of the analogs correlated well with that for cGMP accumulation (r^2=0.800, P<0.05), supporting our interpretation that antimitogenic effect on VSMC is mediated by cGMP.

In conclusion, the ring portion of CNP with a disulfide bond (CNP(6-22)) is the minimum structure required for stimulation of cGMP accumulation in cultured VSMC, especially the sequence Leu^{9}-Lys^{10}-Leu^{11} in the ring portion of CNP executes important roles for both stimulation of cGMP accumulation and selectivity of the ligand for NPR-B. To confirm this conclusion further, we are currently planning to investigate the ability of the CNP analogs for binding to NPR-A or NPR-B using mammalian cells transfected with each of the receptor cDNAs. The structure-activity relationships deduced here may be useful for elucidation of the molecular mechanism of the ligand (CNP)-receptor (NPR-B) interaction. Furthermore, the data described here should be helpful for the development of a more potent agonist to NPR-B which is expected to be effective for the prevention of proliferation of vascular smooth muscle cells after vascular injury or during the progression of atherosclerosis.

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Natriuretic Peptides, Their Receptors, and Cyclic Guanosine Monophosphate-Dependent Signaling Functions

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Natriuretic peptides are a family of structurally related but genetically distinct hormones/paracrine factors that regulate blood volume, blood pressure, ventricular hypertrophy, pulmonary hypertension, fat metabolism, and long bone growth. The mammalian members are atrial natriuretic peptide, B-type natriuretic peptide, C-type natriuretic peptide, and possibly osteocrin/musclin. Three single membrane-spanning natriuretic peptide receptors (NPRs) have been identified. Two, NPR-A/GC-A/NPR1 and NPR-B/GC-B/NPR2, are transmembrane guanylyl cyclases, enzymes that catalyze the synthesis of cGMP. One, NPR-C/NPR3, lacks intrinsic enzymatic activity and controls the local concentrations of natriuretic peptides through constitutive receptor-mediated internalization and degradation. Single allele-inactivating mutations in the promoter of human NPR-A are associated with hypertension and heart failure, whereas homozygous inactivating mutations in human NPR-B cause a form of short-limbed dwarfism known as acromesomelic dysplasia type Maroteaux. The physiological effects of natriuretic peptides are elicited through three classes of cGMP binding proteins: cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channels. In this comprehensive review, the structure, function, regulation, and biological consequences of natriuretic peptides and their associated signaling proteins are described. (*Endocrine Reviews* 27: 47–72, 2006)

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Abbreviations: ANP, Atrial natriuretic peptide; AVP, arginine-vasopressin; BNP, B-type natriuretic peptide; CNG, cyclic nucleotide-gated; CNP, C-type natriuretic peptide; FGF3, fibroblast growth factor-3; GC-A, guanylyl cyclase A; HSP, heat shock protein; IP_3, inositol 1,4,5-trisphosphate; MMP, matrix metalloproteinase; NPR, natriuretic peptide receptor; NPR-C, natriuretic peptide clearance receptor; PDE, phosphodiesterase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate.

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I. Introduction and Historical Background

ALTHOUGH PHYSIOLOGICAL EXPERIMENTS had long predicted a humoral link between the heart and kidneys (1), De Bold *et al.* (2) reported the first direct evidence for such a substance in 1981. They found that the iv injection of atrial, but not ventricular, homogenates into rats elicited a rapid decrease in blood pressure that was accompanied by increased renal sodium and water excretion. After this seminal observation, several groups purified peptides of varying sizes from atrial tissue that possess both natriuretic and smooth muscle-relaxing activity (3–6). These peptides were given a number of different names such as atrial natriuretic factor, cardionatrin, cardiodilatin, atriopeptin, and atrial natriuretic peptide (ANP); the latter description is most often used today. B-type natriuretic peptide (BNP), which was

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originally called brain natriuretic peptide (7), and C-type natriuretic peptide (CNP) (8) were subsequently purified from porcine brain extracts based on their ability to relax smooth muscle. All three members contain the conserved sequence CFGXXXDRIXXXXGLGC (Fig. 1) where X is any amino acid. The flanking cysteines form a 17-amino-acid disulfide-linked ring that is required for biological activity (3).

In 1984, ANP was shown to elevate cGMP concentrations in rat tissues, primary cell cultures, and urine (9). During the same year, ANP was reported to activate particulate, but not soluble, guanylyl cyclase activity in various rat tissue homogenates (10, 11) in a manner that correlated with vascular smooth muscle relaxation (11).

Initial photoaffinity labeling and/or chemical cross-linking of ^{125}I-labeled ANP to whole cells or membranes revealed proteins of 60 and 120–140 kDa as estimated by reducing SDS-PAGE (12–15). Purification of the smaller protein (16) and subsequent cloning of its cDNA (17) predicted a disulfide-linked homodimeric receptor with a large extracellular binding domain, a single membrane-spanning region, and only 37 intracellular amino acids. This receptor is generally referred to as the natriuretic peptide clearance receptor, NPR-C or NPR3. Purification of the higher molecular weight protein revealed that the ANP binding activity cofractionated with guanylyl cyclase activity (18–21). Cloning of the cDNA for this receptor, known as natriuretic peptide receptor-A (NPR-A), guanylyl cyclase A (GC-A) or natriuretic

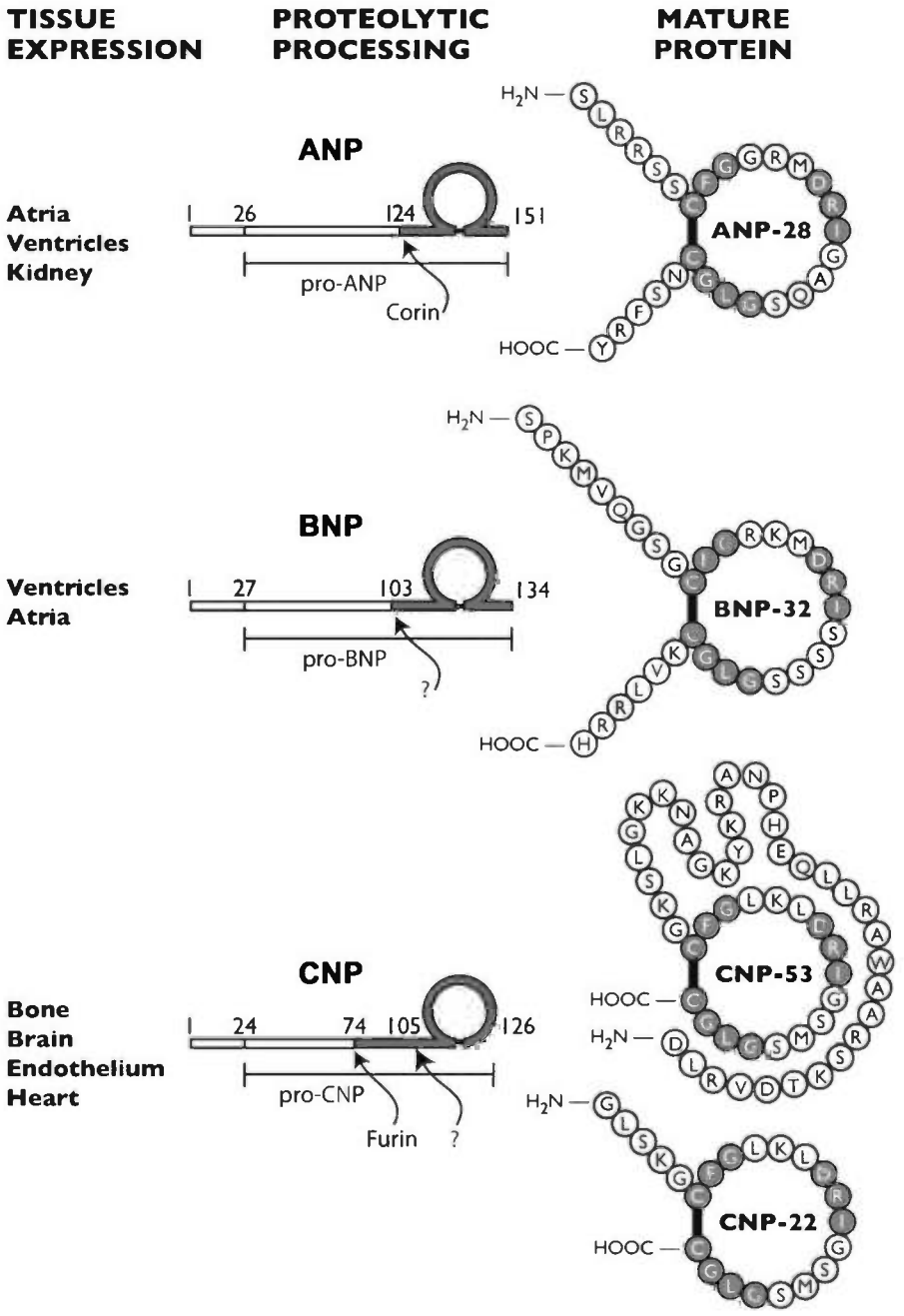


FIG. 1. Natriuretic peptide expression, processing, and structure. ANP, BNP, and CNP are expressed in the indicated tissues as pre-pro-hormones. The signal sequences are cleaved to form pro-ANP, -BNP, and -CNP. The peptides are further proteolytically processed to form mature peptides. ANP is cleaved by corin. The enzyme responsible for BNP cleavage has not been definitively identified. Cleavage of pro-CNP by furin *in vitro* results in a 53-amino-acid peptide. An unknown enzyme further processes CNP to a 22-amino-acid form as well. All three mature peptides contain a conserved 17-residue disulfide-linked ring structure that is required for activity. The disulfide bond is shown in *black*, and invariant residues within the ring are *shaded*.

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peptide receptor 1 (NPR1) was obtained by probing a rat brain cDNA library with a sea urchin receptor guanylyl cyclase homolog (22, 23). Surprisingly, sequence analysis suggested that the hormone binding and guanylyl cyclase domains resided within the same polypeptide. This was confirmed when cells transfected with the NPR-A cDNA, but not with empty vector, displayed marked ^{125}I-ANP binding and ANP-dependent cGMP elevations. As a result of the same library screen, a second guanylyl cyclase-linked natriuretic peptide receptor was identified and was called guanylyl cyclase B (GC-B), natriuretic peptide receptor-B (NPR-B), or natriuretic peptide receptor 2 (NPR 2) (24, 25). The specificity of the ligand-guanylyl cyclase receptor interaction was determined in transfected cells. ANP and BNP stimulate NPR-A, whereas CNP stimulates NPR-B (26, 27) (Fig. 2).

II. Natriuretic Peptides

In mammals, there are generally three natriuretic peptides: ANP, BNP, and CNP, although CNP does not stimulate "natriuresis" at physiological concentrations. Teleosts have a novel family member called ventricular natriuretic peptide instead of BNP, whereas only CNP is expressed in sharks (28). Evolutionary analysis indicates that ANP and BNP evolved from CNP gene duplication events (29). Hence, CNP is the most ancient family member.

A. Atrial natriuretic peptide

All natriuretic peptides are synthesized as preprohormones (Fig. 1). Human preproANP is 151 amino acids in length. Cleavage of the amino terminal signal sequence results in the 126-amino-acid proANP, which is the predominant form stored in atrial granules. ProANP is rapidly cleaved upon secretion by the transmembrane cardiac serine protease called corin to form the biologically active carboxyl-terminal 28-amino-acid peptide (30). Mice lacking corin have undetectable levels of the mature form of ANP in heart tissue and are hypertensive (31). Alternative processing of proANP by an unknown protease in the kidney generates a 32-residue peptide called urodilatin, which may be important in regulating renal sodium and water excretion (32).

ANP is primarily expressed and stored in granules in the atria, although it is present at lower concentrations in other tissues such as the ventricles and kidney (Fig. 1). The primary stimulant for ANP release is atrial wall stretch resulting from increased intravascular volume (33, 34). Once secreted, ANP perfuses into the coronary sinus, which facilitates distribution to its various target organs in a true endocrine manner. In addition, hormones such as endothelin (35), angiotensin (36), and arginine-vasopressin (AVP) (37) stimulate ANP release (38), as do water immersion (39) and head down tilt (40). Plasma levels of ANP in normal patients are approximately 10 fmol/ml and are elevated 10- to 30-fold in patients with congestive heart failure (41, 42) (Table 1).

The human ANP gene is found on chromosome 1p36.2 (Table 1). The mouse gene is located on chromosome 4 (43). The ANP gene, like the BNP and CNP genes, contains three exons. Disruption of the murine ANP gene (Nppa) results in marked hypertension, which was initially suggested to be salt-sensitive (Table 2) (44). However, a subsequent report from the same laboratory found that blood pressures in ANP-deficient animals are not influenced by dietary salt intake (45).

B. B-type natriuretic peptide

BNP was initially purified from porcine brain extracts and given the name brain natriuretic peptide (7). However, it was subsequently found in much higher concentrations in cardiac ventricles from patients or animals undergoing cardiac stress such as congestive heart failure or myocardial infarction (42).

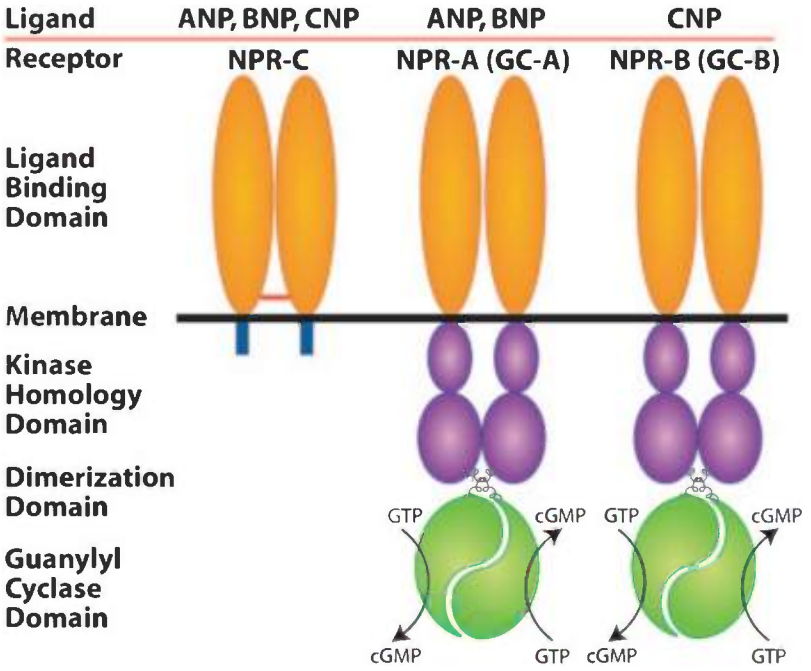


FIG. 2. Natriuretic peptide receptor topology and ligand preferences. Natriuretic peptides bind three proteins, NPR-A, NPR-B, and NPR-C. NPR-A and NPR-B are membrane-bound guanylyl cyclases consisting of an extracellular ligand binding domain, a single hydrophobic transmembrane region, and intracellular kinase homology, dimerization, and carboxyl-terminal guanylyl cyclase domains. The catalytic domain is hypothesized to form a dimer in a head-to-tail arrangement that contains two active sites. NPR-C is approximately 30% identical to NPR-A and NPR-B in the extracellular ligand-binding domain but contains only 37 intracellular amino acids. *Red horizontal line* indicates an intermolecular disulfide bond.

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TABLE 1. Human natriuretic peptide gene locus, plasma concentration, and half-life

	ANP	BNP	CNP
Gene name	NPPA	NPPB	NPPC
Gene locus	1p36.2	1p36.2	2q24-qter
Plasma half life (min)	~2 (1.7 –3.1) [328, 329]	~20 (19.5 –22.6) [330]	2.6 [331]
Plasma concentration (pmol/liter)			
Normal	6.4 ± 0.9 [42] (1.1 –13.7) [42, 332–334]	0.9 ± 0.007 [42] (0.9 –6) [42, 332–335]	1.4 ± 0.6 [332] (1.4 –1.9) [332, 336, 337]
In congestive heart failure	87 ± 12 [321] (26 –164) [42, 321, 332]	87 ± 11 [321] (3.9 –267) [42, 321, 332, 335]	1.4 ± 0.2 [332] (1.4 –1.85) [332, 338]
In myocardial infarction	33.4 ± 6.1 [321] (33.4 –55.3) [321, 337, 339]	60 ± 9.4 [287] (26.6 –62.2) [321, 337, 339]	N.D.
In pulmonary arterial hypertension	14.0 [260] (8.8 –20.5) [260]	15.3 [260] (9.2 –49.4) [260]	N.D.
In chronic renal failure	43 ± 11 [332] (43 –48) [321, 332]	130 ± 37.4 [332] (28 –130) [321, 332, 335, 340]	3.0 ± 0.4 [332]
In subarachnoid hemorrhage	5.9 ± 1.0 [341] (5.46 –10.5) [341, 342]	15.1 ± 3.8 [341] (0.64 –23.2) [341, 342]	2.0 –2.6 [343] (0.91 –9.1) [342, 343]
In cirrhosis	27.98 ± 3.71 [333]	16.0 ± 1.91 [333] (1.2 –43.1) [333, 335]	1.36 ± 0.18 [336]

The gene loci for each human natriuretic peptide are shown. The approximate plasma half-life and plasma concentration of each natriuretic peptide in normal and various disease states also are shown. Values represent the mean patient value from a representative study. Values in *parentheses* represent the range of mean values from several studies. Reference numbers are in *brackets*. N.D., Not determined.

For this reason, it is currently referred to as BNP or "B-type natriuretic peptide," but not "brain natriuretic peptide." Human BNP is synthesized as a preprohormone of 134 residues containing a signal sequence that is cleaved to yield a 108-amino-acid prohormone (Fig. 1). Additional cleavage by an unknown protease results in an inactive 76-residue amino-terminal (nt) fragment and a 32-residue carboxyl-terminal biologically active peptide. Fully processed BNP length varies between species. Human, pig, and dog BNP is 32 amino acids (46, 47), whereas rat and mouse BNP is 45 amino acids (48, 49).

Although BNP is stored with ANP in atrial granules, BNP is not stored in granules in the ventricles. Instead, ventricular BNP production is transcriptionally regulated by cardiac wall stretch resulting from volume overload. The nuclear transcription factor, GATA 4, plays a dominant role in regulating this process (50, 51). Healthy individuals have plasma BNP concentrations of approximately 1 fmol/ml (3.5 pg/ml) or about one tenth that of ANP (Table 1). In contrast, plasma BNP concentrations of patients with congestive heart failure are elevated between 200- and 300-fold. The enormous range of plasma BNP concentrations between normal and sick individuals makes it an ideal indicator of cardiac stress (42). Several studies indicate that elevated BNP levels correlate with poor prognoses (see *Section XI*).

The human BNP gene is only 8 kb upstream of the ANP gene on chromosome 1p36.2 (Table 1). The mouse gene is located on chromosome 4. Disruption of both alleles of the murine BNP gene (Nppb) yields normotensive animals that develop pressure-sensitive ventricular fibrosis (Table 2) (52). Hence, at least in mice, BNP is not an endocrine regulator of blood pressure but rather a paracrine regulator of the heart.

TABLE 2. Phenotypes of mice and humans with inactivation mutations in genes that code for natriuretic peptides and their receptors

Gene	Mouse knockout phenotype	Human diseases
ANP	Hypertension Cardiac hypertrophy	
BNP	Ventricular fibrosis	
CNP	Dwarfism	
NPR-A	Hypertension Ventricular fibrosis Cardiac hypertrophy	Hypertension Ventricular fibrosis
NPR-B	Dwarfism, seizures Female sterility Decreased adiposity	Dwarfism (AMDM)
NPR-C	Giantism Hypotension	

Knockout mice for each of the natriuretic peptides and receptors have been generated. The characteristic phenotype of each knockout animal is listed. Human genetic mutations have been identified for two of the receptors as shown. AMDM, Acromesomelic dysplasia, type Maroteaux.

C. C-type natriuretic peptide

CNP is the most highly expressed natriuretic peptide in the brain and is found in high concentrations in chondrocytes (53, 54) and cytokine-exposed endothelial cells (55). It is not stored in granules. In cultured endothelial cells, its secretion is up-regulated by TNF-α (56), TGF-β (55), IL-I (56), and sheer stress (57) and suppressed by insulin (58). CNP is the most conserved natriuretic peptide. For instance, both 22- and 53-amino-acid versions of CNP are identical in humans, pigs, and rats. Human proCNP contains 103 residues, and the intracellular endoprotease furin has been shown to process proCNP to the mature 53-amino-acid peptide *in vitro* (Fig. 1) (59). In some tissues, CNP-53 is cleaved to CNP-22 by an unknown extracellular enzyme. Although CNP-22 and CNP-53 elicit similar if not identical functions (60, 61), their tissue expression differs. CNP-53 is the major form in the brain (62), endothelial cells (63), and heart (64), whereas CNP-22 predominates in human plasma (63) and cerebral spinal fluid (65). Normal plasma CNP concentrations (both forms) are in the low femtomole per milliliter range (63) and are minimally (66), if at all (67), elevated in patients with congestive heart failure.

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The human CNP gene is located between 2q24 and the 2q terminus (Table 1) (68, 69). The mouse gene is located on chromosome 1 (69). Disruption of the murine CNP gene (Nppc) results in normotensive mice that display severe dwarfism and undergo early death as a result of impaired endochondral ossification (Table 2) (see Fig. 9) (70).

D. Osteocrin/musclin

Using signal-trap strategies, two different groups identified a peptide with limited similarity to natriuretic peptides. Interestingly, one group found it primarily in bone and named it osteocrin (71), whereas another group found it primarily in muscle and named it musclin (72). In a paper presented at the American Society of Bone and Mineral Research Conference in 2004 (73), the osteocrin group reported that osteocrin binds with high affinity to NPR-C, but not to NPR-A or NPR-B, in a manner that is competitive with ANP. When NPR-A and NPR-C were expressed in the same cells, osteocrin increased ANP-dependent cGMP elevations, presumably by blocking NPR-C mediated ANP degradation. Transgenic expression of osteocrin under the bone specific collagen type I promoter resulted in mice with elongated bones and marked kyphosis, which is similar to the phenotype of mice transgenically overexpressing BNP (74) or CNP (75) or lacking NPR-C (76, 77). These data suggest that osteocrin increases local CNP levels in the growth plate by blocking binding to NPR-C (see *Section X.L*).

III. Natriuretic Peptide Receptors

There are three known natriuretic peptide binding proteins in mammals: NPR-A, NPR-B, and NPR-C (Fig. 2). They are also known as GC-A, GC-B, and the clearance receptor, or as NPR1, NPR2, and NPR3, respectively. NPR-A and NPR-B represent two of the five transmembrane guanylyl cyclases found in humans (78). The other members of the family are GC-C, the receptor for the intestinal peptides guanylin and uroguanylin, and Ret-GC-1 and Ret-GC-2, retinal enzymes that regulate the photoreceptor dark cycle. The third natriuretic peptide receptor, NPR-C, does not possess any known intrinsic enzymatic activity.

A. Natriuretic peptide receptor A

Human and rat NPR-A mRNA are highly expressed in kidney, adrenal, terminal ileum, adipose, aortic, and lung tissues (Table 3) (23, 25, 79). *In situ* hybridization analysis of rhesus monkey tissues indicated that NPR-A mRNA is prevalent in the kidney, adrenal glomerulosa, adrenal medulla, pituitary, cerebellum, and endocardial endothelial cells (80). In the brain, NPR-A mRNA was observed in the mitral cell layer of the olfactory bulb, medial habenula, subfornical organ, and area postrema (81, 82). It was also observed in forebrain white matter tracts, suggesting synthesis in glial cells. Western blot analysis detected high NPR-A protein levels in rodent lung, kidney, adrenal, testis, and liver tissue (Table 3) (83, 84). NPR-A was purified to apparent homogeneity from rat lung (18) and bovine adrenal cortex (19, 85). In cultured cells, NPR-A is readily found in primary vascular smooth muscle and kidney mesangial cells. Its expression decreases dramatically with continued propagation (86). In fact, we are not aware of any immortalized cell line that expresses high levels of this receptor, although low expression is observed in some human embryonic kidney 293 (87) and rat PC-12 pheochromocytoma (88) cell lines (Table 3).

TABLE 3. Natriuretic peptide receptor tissue and cell line expression

Receptor	Tissue expression	Cell line expression
NPR-A	Adrenal, brain, VSM, lung, kidney, adipose, heart	Primary VSMC Primary renal mesangial PC-12 Some 293 cell lines
NPR-B	Chondrocytes, brain, lung, VSM, uterus	Primary VSMC Smooth muscle (A10, A7R5) Fibroblast (NIH3T3, Balb3T3) Chondrocyte (ATDC5)
NPR-C	Most tissues	A10 VSMC 3T3 fibroblast

The tissue expression pattern of each natriuretic peptide receptor is shown. Natriuretic peptide receptor expression in commonly studied cell lines also is listed. VSM, Vascular smooth muscle; VSMC, VSM cell.

The guanylyl cyclase-linked natriuretic peptide receptors have a growth factor receptor-like topology consisting of an extracellular ligand-binding domain of approximately 450 amino acids, a 20- to 25-residue single hydrophobic membrane-spanning region, and an intracellular domain of approximately 570 amino acids (Fig. 2). The latter is made of a 250-amino-acid kinase homology domain, a roughly 40-residue coiled-coil dimerization domain, and approximately 250-amino-acid carboxyl-terminal guanylyl cyclase catalytic domain (78). The rank order of NPR-A activation by natriuretic peptides is ANP $\geq$ BNP $\gg$ CNP (26, 27).

The extracellular domain of rat NPR-A contains three intramolecular disulfide bonds between Cys-60/Cys-86, Cys-164/Cys-215, and Cys-423/Cys-432 (see Ref. 89 for graphic depiction of disulfide bonds), but no intermolecular disulfide bonds (90). When fractionated by SDS-PAGE, NPR-A exhibits considerable size heterogeneity, which is primarily due to differential N-linked glycosylation. Sequencing of the amino termini of human natriuretic peptide receptor-IgG fusion proteins purified from Chinese hamster ovary cells indicated that Asn-2 and Asn-13 of NPR-A are glycosylated (91). A soluble extracellular domain of rat NPR-A purified from Cos cells is glycosylated on Asn-13, Asn-180, Asn-306, Asn-347, and Asn-395 (92). The role of glycosylation in the regulation of NPR-A is controversial. Some investigators found that terminal glycosylation affects ligand binding (93, 94), whereas others did not (92, 95, 96).

Under basal conditions, NPR-A is phosphorylated on four serines (Ser-497, Ser-502, Ser-506, and Ser-510) and two threonines (Thr-500 and Thr-513) within a stretch of 17 amino acids at the amino-terminal portion of its kinase homology domain. Conversion of any phosphorylated residue to alanine decreases receptor-associated phosphate, changes tryptic phosphopeptide mapping patterns, and reduces hormone-dependent guanylyl cyclase activity. The mutation of four or more phosphorylation sites to alanine yields a hormonally unresponsive receptor, indicating that phosphorylation of

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NPR-A is absolutely required for hormonal activation (87). Whether NPR-A contains additional phosphorylation sites is currently unknown but remains a possibility because sea urchin homologs have stoichiometries of 15–17 moles of phosphate per mole of receptor (97, 98).

The crystal structure of the glycosylated, unliganded, dimerized extracellular domain of rat NPR-A was solved at 2.0 Å resolution (99). The monomer contains a type I periplasmic binding protein fold and consists of two interconnected subdomains with each containing a central β-sheet flanked by α-helices. An apparent chloride ion is buried within the amino portion of each monomer. Chloride was reported to be absolutely required for ANP binding to NPR-A (100); however, this observation has not been confirmed. Although originally proposed to adopt a tail-to-tail V-shaped dimer with the apex being closest to the membrane, the crystal structure of the extracellular domain bound to a truncated form of ANP revealed that the receptor forms a head-to-head, A-like dimer with a stoichiometry of one molecule of ANP to two molecules of receptor (101). Data from studies where the proposed dimerization interfaces were mutated are consistent with an A-shaped, not V-shaped, model (102, 103). Because ANP has no internal symmetry, binding of ANP to NPR-A is asymmetric.

NPR-A has been shown to associate with a limited number of partners. NPR-A expressed in 293 cells interacts with heat shock proteins 70 and 90 (HSP70 and HSP90), molecular chaperones required for proper protein folding and/or trafficking (104). HSP90 is hypothesized to bind within the NPR-A kinase homology domain because deletion of the intracellular or the kinase homology domains disrupts the interaction. Inhibition of HSP90 activity decreases ANP-stimulated cGMP production, presumably due to decreased processing and/or folding of NPR-A (104). Additionally, the kinase homology domain of NPR-A was shown to associate with protein phosphatase 5 (105) and cGMP-dependent protein kinase Iα (106) in two-hybrid screens. To date, neither interaction has been reported in mammalian cells. cGMP-dependent protein kinase Iα was suggested to phosphorylate and activate NPR-A in a feed forward mechanism (106). However, we find that neither overexpression nor lack of expression (null animals) of cGMP-dependent protein kinase Iα has any effect on the phosphorylation status or guanylyl cyclase activity of NPR-A (344).

The human NPR-A gene is approximately 16 kb, contains 22 exons and 21 introns, and is located on chromosome 1q21–22 (23, 107). The rat NPR-A gene (Npr1) spans about 17.5 kb and also contains 22 exons and 21 introns (108). It lacks a definitive TATA box but contains three putative Sp1 binding sites. The murine NPR-A gene has been disrupted by two separate laboratories (109, 110). The null animals have high blood pressure, cardiac hypertrophy, and ventricular fibrosis (Table 2) (109, 110). One group also found that male mice lacking NPR-A died at 6 months of age due to a catastrophic cardiovascular event (110), but this was later attributed to the genetic background of the mice (111). In humans, a single allele mutation was identified in the promoter of the NPR-A gene that decreases receptor expression by about 70% (112). Interestingly, of the eight Japanese patients identified with this mutation, seven had hypertension and one had congestive heart failure. Hence, every time a loss of function mutation was identified in the NPR-A gene, it was associated with disease. In contrast, a separate study involving 498 New Zealand patients failed to observe this mutation, suggesting that it may be rare outside of Japan (113).

B. Natriuretic peptide receptor B

NPR-B mRNA was found in lung, brain, adrenal, kidney, uterus, and ovary tissue (Table 3) (25, 79, 114). *In situ* hybridization studies found detectable NPR-B mRNA in the adrenal medulla, pituitary, cerebellum, and skin (80). NPR-B is the predominant natriuretic peptide receptor in the brain. NPR-B mRNA was detected throughout the neuroaxis, being abundantly expressed in the limbic cortex, neocortex, olfactory bulb, hippocampus, and amygdala (81). Intense staining was found in preoptic-hypothalamic neuroendocrine circuits and in motor nuclei of cranial nerves. In a separate study, high levels of NPR-B mRNA were found throughout the hypothalamus and the neural lobe of the pituitary (82). NPR-B protein has been found at relatively high concentrations in fibroblasts (Table 3) (115–117).

NPR-B has the same overall topology as NPR-A (Fig. 2). The disulfide-bonding pattern of NPR-B has not been chemically determined, but mutagenesis-based studies are consistent with intramolecular disulfide bonds between Cys-53 and Cys-79, Cys-205 and Cys-314, as well as Cys-417 and Cys-426 (118). Similarly, the glycosylation sites of NPR-B have not been chemically determined, but mutagenesis studies suggest that five of the seven extracellular asparagines are glycosylated (119, 120). The mutation of Asn-24 to Asp resulted in a 90% loss in CNP binding, which is probably due to improper folding or cellular targeting of the receptor (120). NPR-B is phosphorylated on Thr-513, Thr-516, Ser-518, Ser-523, and Ser-526 (121). Similarly to NPR-A, mutating any of these residues to alanine reduces hormone-dependent guanylyl cyclase activity. Whether additional unidentified phosphorylation sites exist is unknown. No crystal structure has been reported for any domain of NPR-B. Multiple splice variants of NPR-B have been identified, including a species lacking enzymatic activity that can function in a dominant-negative manner (122, 123). Whether these truncated variants participate in CNP signaling is unknown. The rank order of activation of NPR-B by natriuretic peptides is CNP $\gg$ ANP $\geq$ BNP. To date, studies on purified NPR-B have not been reported.

Two loss of function mouse models exist for NPR-B (Table 2). Targeted deletion of exons 3 through 7, which encode the carboxyl-terminal half of the extracellular domain and transmembrane segment of NPR-B, by homologous recombination results in dwarfism and female sterility (124). The heterozygous animals were significantly shorter than the wild-type animals as well. A spontaneous mutation resulting from a T to G transversion, which causes the substitution of a highly conserved leucine with an arginine in the guanylyl cyclase domain of NPR-B, also results in dwarfism in mice containing two defective alleles (*cn/cn*) (125). Female infertility was not noted in this mouse model. Interestingly, the targeted deletion of CNP or NPR-B resulted in mice that had

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significantly higher mortality rates than the single mutation-containing *cn/cn* mice. One possible explanation for these seemingly disparate results is that CNP binding to NPR-B signals through another mechanism in addition to cGMP synthesis.

The human NPR-B gene spans about 16.5 kb, contains 22 exons, and is located on chromosome 9p21–12 (126). Similar to NPR-A, the NPR-B promoter lacks a defined TATA box but contains multiple putative Sp1 binding sites. The mouse gene, Npr2, is found on chromosome 4. Homozygous loss of function mutations in human NPR-B have been identified in patients with a rare form of short-limbed dwarfism called acromesomelic dysplasia, type Maroteaux (Table 2) (127). Sterility was not noted in these patients. Similarly to the "knockout" mice, patients with a single mutated NPR-B allele were statistically shorter than the average person from their respective populations (127).

C. Natriuretic peptide clearance receptor

NPR-C mRNA is found in atrial, mesentery, placenta, lung, kidney, and venous tissue (79, 128) and in aortic smooth muscle and aortic endothelial cells (Table 3) (17). *In situ* hybridization studies found detectable NPR-C mRNA in kidney, adrenal, heart, cerebral cortex, and cerebellum tissue (80). NPR-C protein was purified to apparent homogeneity from bovine lung (129) and vascular smooth muscle cells (16).

The extracellular domain of NPR-C is about 30% identical to NPR-A and NPR-B (130). However, unlike the cyclase-linked receptors, it contains only 37 intracellular amino acids and no guanylyl cyclase activity (17) (Fig. 2). The extracellular domain of human NPR-C is glycosylated on Asn-41, Asn-248, and Asn-349 and contains two sets of intramolecular disulfide bonds between Cys63-Cys91 and Cys168-Cys216 that are conserved in NPR-A and NPR-B (131). One intermolecular bond was identified in bovine NPR-C at Cys469 (132), whereas in human NPR-C two intermolecular disulfide bonds were found at Cys-428 and Cys-431 (131). Hence, unlike NPR-A and NPR-B, NPR-C is a disulfide-linked homodimer (Fig. 2). NPR-C is phosphorylated on serine residues when overexpressed in hamster cells (133).

The crystal structures of the unbound and CNP-bound versions of the NPR-C extracellular domain indicate a ligand to receptor stoichiometry of 1:2 with a membrane distal dimerization interface or A-shaped dimer (134). Hormone binding was found to induce a 20-Å closure of the membrane proximal domains of the dimer.

The affinity of NPR-C for natriuretic peptides is ANP $\geq$ CNP $>$ BNP in both humans and rats (27, 91). Dissociation constants range from 10 to 140 pM (27). The differential affinity of NPR-C for the cardiac family members may contribute to the longer serum half-life of BNP compared with ANP (Table 1). NPR-C, but not NPR-A or NPR-B, also binds the synthetic ANP analog, c-ANF (ANP 4–23), which is missing the carboxyl-terminal tail and a portion of the disulfide ring structure (135). Hence, functions that are stimulated by c-ANF, but not ANP, have been suggested to result from NPR-C-dependent signaling. However, caution is advised when interpreting these experiments because c-ANF indirectly increases NPR-A-dependent responses by blocking NPR-C-dependent ANP degradation (135).

Loss of function mutations in mice indicate that the major function of NPR-C is to clear natriuretic peptides from the circulation or extracellular milieu through receptor-mediated internalization and degradation (76, 77). Like many nutrient type transmembrane receptors, such as the transferrin or low-density lipoprotein receptors, the internalization of NPR-C is constitutive. In other words, it is independent of ligand binding (136). NPR-C internalization is abolished by hypertonic sucrose treatment, which causes clathrin disassembly, suggesting that the endocytosis is mediated by clathrin-coated pits (137). ^{125}I-ANP hydrolysis also is disrupted by cellular treatment with NH_4Cl or chloroquine, suggesting that NPR-C-bound ligand undergoes lysosomal hydrolysis followed by receptor recycling to the cell surface (136, 138).

In contrast, a number of laboratories have reported signaling functions for NPR-C (139). The NPR-C selective agonist c-ANF (ANP 4–23) reduces adenylyl cyclase activity in membranes or cAMP concentrations in whole cells (140). This effect is inhibited by pertussis toxin treatment, which is consistent with a requirement for Gi- or Go-protein activation (141). The inhibition was blocked with an antibody specific for the intracellular domain of NPR-C (142), whereas small peptide fragments of the NPR-C intracellular domain mimic the inhibition (143). Similarly, the ability of CNP to inhibit catecholamine efflux from pheochromocytoma cells is dependent on NPR-C protein levels and is inhibited by an antibody against the intracellular portion of NPR-C (144, 145). NPR-C has been shown to stimulate phospholipase C in a G protein-dependent manner as well (146–148).

The human NPR-C gene is located on chromosome 5p14-p13, spans more than 65 kb, and contains eight exons and seven introns (149). The mouse NPR-C gene, Npr3, is located on chromosome 15. Multiple loss of function mouse models exist for NPR-C (Table 2). Targeted inactivation of both alleles of the NPR-C gene by homologous recombination results in animals that have a reduced ability to clear ^{125}I-ANP from their circulation (two thirds longer half-life), reduced ability to concentrate urine, and long bone overgrowth (77). However, circulating levels of ANP and BNP in the knockout animals are similar to those in wild-type animals, suggesting the existence of a feedback mechanism for ANP and BNP synthesis. In addition to the targeted deletion model, three different strains have been identified that contain recessive loss of function mutations in the gene for NPR-C (76). Longjohn mice contain a 36-bp in-frame deletion between positions 195 and 232 that results in a 12-amino acid deletion. Longjohn2 animals contain a C to T transition at position 283 that results in a premature stop codon. The *strigosus* strain, which is Latin for long and emaciated, has a C to A transversion at position 502 that results in an Asp to His substitution. All mutations are found in the extracellular ligand-binding domain and presumably disrupt ligand binding, although this has not been formally demonstrated. Like the animals with the targeted deletion, these animals exhibited marked skeletal overgrowth. A lack of body fat deposits also was noted upon necropsy. Interestingly, none of the animals with homozygous loss of function NPR-C mutations were

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impaired in any known natriuretic peptide response. On the contrary, these animals display phenotypes associated with exaggerated NPR-A and NPR-B actions, for example, hypotension and gigantism, respectively. These data suggest that NPR-A or NPR-B mediates the known effects of natriuretic peptides that have been identified to date, at least in mice. However, it is possible that NPR-C mediates some yet to be discovered natriuretic peptide function. In our opinion, the demonstration of natriuretic peptide functions that are intact in NPR-A- and NPR-B-expressing animals, but absent in animals lacking functional NPR-C, is essential to support a signaling role for NPR-C in mice.

IV. Activation of NPR-A

In the basal state, NPR-A is a higher-ordered oligomer, and its guanylyl cyclase activity is tightly repressed (Fig. 3). Evidence for dimers, trimers, and quatramers exists (150–152) (Fig. 3). Unlike growth factor receptors, ligand binding does not lead to further oligomerization (150, 152). Analysis of the crystal structure of NPR-A indicates that one molecule of ANP binds per two molecules of NPR-A and causes a Ferris wheel-like translocation of the two juxtamembrane domains with little change in interdomain distance (101). However, these data are not consistent with a report showing that a version of NPR-A containing a mutant unpaired juxtamembrane cysteine forms a disulfide dimer upon hormone binding, suggesting that ANP binding decreases the distance between the juxtamembrane regions of the monomers (153). Through an unknown mechanism, this activation signal is transmitted across the plasma membrane, which initiates a series of subsequent events. First, the normal repression exerted by the kinase homology domain is relieved. The kinase homology domain is thought to repress NPR-A because receptors lacking this domain are constitutively active (154, 155). At this point, it is hypothesized that the guanylyl cyclase domains come together in a head to tail arrangement to form two active sites per dimer. This idea is based on the crystal structure of adenylyl cyclase, not guanylyl cyclase, because the latter does not exist. However, a similar structure is likely between the two cyclases because a surprisingly few number of amino acid changes were shown to convert a guanylyl cyclase to an adenylyl cyclase (156) and vice versa (157). Second, the affinity of the hormone-binding domain for ligand decreases, which increases the dissociation rate (158, 159). Finally, the regulatory phosphorylation sites on the kinase homology domain are dephosphorylated, which desensitizes the receptor (Fig. 3) (160). Quantitation of thiophosphate incorporation into active and desensitized forms of NPR-A suggests that the dephosphorylation is primarily the result of reduced receptor phosphorylation, with only slight increases in receptor dephosphorylation (161). Currently, the identities of these regulatory enzymes are unknown. However, NPR-A is dephosphorylated by two separate phosphatase activities in crude membranes. One is inhibited by microcystin and does not require a metal cofactor for activity. The other requires magnesium or manganese for activity but is not inhibited by microcystin (162).

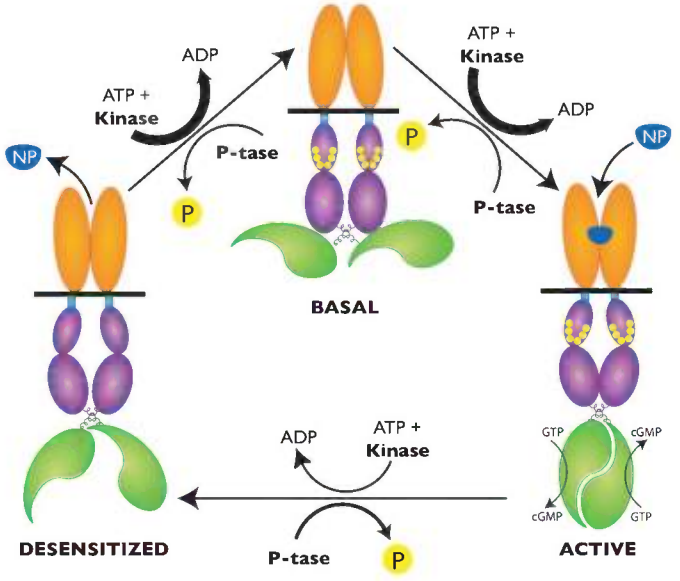


FIG. 3. Hypothetical model for NPR-A and NPR-B activation and desensitization. Three states of receptor activation are labeled "basal," "active," and "desensitized." In the basal state, NPR-A and NPR-B are higher ordered oligomers (shown here as dimers for simplicity). In the basal state, they are phosphorylated on five (NPR-B) or six (NPR-A) known sites within the kinase homology domain (*purple*). Phosphates are indicated by the *small yellow spheres*. It is hypothesized that phosphorylation "licenses" the receptor for hormonal activation. The rate of phosphorylation or dephosphorylation is indicated by the thickness of the respective *arrows*. Natriuretic peptide (NP; *blue*) binding to the highly phosphorylated, inactive basal receptor induces a conformational change that brings the juxtamembranes regions of the extracellular domain together. This activation signal is transduced across the membrane, which is hypothesized to relieve the repression of the kinase homology domain on the guanylyl cyclase domain (*green*). This allows the cyclase domains to dimerize. Each dimer is envisioned to contain two active sites. Prolonged ligand exposure stimulates receptor dephosphorylation, which results in reduced activity via a process called desensitization. The dephosphorylation primarily results from inhibition of the phosphorylation process. Release of ligand and rephosphorylation returns the enzyme to its basal state.

ANP was originally shown to increase guanylyl cyclase activity in crude membranes in the absence of ATP (10). However, a few years later, several groups observed that including ATP in the reaction mixture dramatically increased ANP-dependent cyclase activity (163–165). Because AMP-PNP, a nonhydrolyzable ATP analog that presumably cannot substitute for ATP in protein kinase reactions, also increased ANP-dependent activity, it was suggested that ATP directly binds and activates NPR-A (164). Subsequent reports found that ATP was absolutely required for NPR-A activation (166–168). Similar data were reported for NPR-B (169). This led to a two-stage activation model for natriuretic peptide receptors where natriuretic peptide binding to the extracellular domain facilitates ATP binding to the kinase homology domain, which ultimately brings the catalytic domains together to form an active site.

More recent studies suggest that the ATP-dependent regulation of the kinase homology domains of NPR-A and NPR-B also involves changes in their phosphorylation state, a process that is required for natriuretic peptide receptor activation (87, 121). For instance, mutations that disrupt the putative ATP regulator module in NPR-B reduce the phos-

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phate content of the receptor (121). Additionally, ATPγS was found to sensitize NPR-A to subsequent activation by ANP and AMPPNP, indicating that in broken cell assays ATP is serving as a substrate for the protein kinase that phosphorylates NPR-A (170). Recently, we reported that NPR-A or NPR-B in membranes prepared in the presence of phosphatase inhibitors is activated up to 200-fold in the absence of ATP (171). Importantly, the addition of ATP did not increase initial enzymatic rates, but did increase activities measured at longer time periods. These data indicate that ATP stabilizes, but does not activate, natriuretic peptide receptors. Whether ATP binds directly to the receptors or to other regulatory proteins is not known.

V. Desensitization of NPR-A and NPR-B

Hormone-dependent guanylyl cyclase activities of both NPR-A and NPR-B are reduced due to chronic exposure to ligand, a process known as homologous desensitization. Incubation of HEK293 cells stably expressing NPR-A or NPR-B in the presence of ANP or CNP, respectively, leads to a time-dependent reduction in ligand-dependent guanylyl cyclase activity (desensitization) that correlates with receptor dephosphorylation (95, 160, 161, 172). *In vitro* treatment of membranes with a purified protein phosphatase also results in NPR-A dephosphorylation and inhibition (160). Tryptic phosphopeptide analyses of NPR-A or NPR-B isolated from cells treated in the presence or absence of hormones are qualitatively similar (172, 173). Thus, the dephosphorylation cannot be attributed to the loss of a specific phosphopeptide(s) despite a clear decrease in receptor-associated phosphate. One explanation for this apparent contradiction is that ligand exposure results in complete dephosphorylation of a receptor population subset, whereas the rest of the receptor population is not dephosphorylated. Another possibility is that a specific site(s) is dephosphorylated in response to ligand binding, but the phosphopeptide that contains this site is lost during the purification process. Hence, this phosphopeptide does not appear on the tryptic phosphopeptide maps of NPR-A or NPR-B from either control or desensitized cells.

To test the absolute requirement of dephosphorylation in hormone-dependent NPR-A desensitization, a mutant version of NPR-A was constructed where the known NPR-A phosphorylated residues were replaced with glutamate to mimic the negative charge of phosphate. This mutant was approximately one fifth as hormonally responsive as the wild type but was resistant to the effects of microcystin and ANP-dependent desensitization (174). These data indicate that dephosphorylation is a mechanism of NPR-A and NPR-B desensitization.

Classic heterologous desensitization, *i.e.*, the ability of other cGMP-elevating enzymes to desensitize NPR-A, does not seem to occur (175). These data suggest that cGMP elevations are not sufficient for homologous desensitization, which are consistent with studies showing that whole cell exposure to cGMP analogs does not affect NPR-A activity (173).

VI. Inhibition of NPR-A and NPR-B (Receptor Cross-Talk)

In general, hormones or growth factors that stimulate vasoconstriction or promote cellular growth or proliferation antagonize the actions of natriuretic peptides. Examples of factors that inhibit NPR-A and/or NPR-B are: angiotensin II, AVP, lysophosphatidic acid, sphingosine-1-phosphate, platelet-derived growth factor, basic fibroblast growth factor, and endothelin (115–117, 176–180). These agents bind either a tyrosine kinase or serpentine receptor that activates phospholipase C, converting phosphotidylinositol 1,4-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Diacylglycerol is a direct activator of the classical and novel protein kinase C (PKC) isoforms, whereas IP_3 binds receptors on the sarcoplasmic or endoplasmic reticulum to increase intracellular calcium concentrations.

Initially, PKC was implicated in the mechanism of heterologous desensitization because treatment of cells expressing NPR-A or NPR-B with phorbol 12-myristate 13-acetate (PMA), a pharmacological PKC activator, markedly decreased whole cell hormone-dependent cGMP elevations and membrane-associated guanylyl cyclase activity but did not alter receptor numbers (173, 181). Subsequently, the PMA-dependent decrease in NPR-A activity was correlated with receptor dephosphorylation, and a relatively specific PKC inhibitor was shown to block both the PMA-dependent desensitization and dephosphorylation (173). The specific PKC isozyme involved in natriuretic peptide receptor inhibition has not been reported. In contrast to tryptic phosphopeptide maps associated with natriuretic peptide-dependent (homologous) desensitization, PKC activation results in the dephosphorylation of a single or small subset of the total phosphorylation sites (173, 181). Tryptic phosphopeptide mapping analysis of NPR-B isolated from HEK293 cells treated with or without PMA indicated that Ser-523 is dephosphorylated and that the phosphorylation of Ser-518 is increased in response to PKC activation (181). The mutation of Ser-523 to glutamate prevented the inhibition, indicating that dephosphorylation was required for PKC-dependent desensitization of NPR-B (181).

Evidence for a PKC-independent NPR-B desensitization pathway also exists. Incubation of A10 vascular smooth muscle cells that endogenously express NPR-B with AVP causes a decrease in intracellular cGMP synthesis and a reduction of guanylyl cyclase activity (115). These effects are independent of PKC because neither the PKC inhibitor, GF-109203X, nor the chronic down-regulation of PKC was able to block the desensitization. These observations suggest that, in addition to the diacylglycerol-PKC arm of the phospholipase C pathway, the inositol triphosphate-calcium arm also plays a role in the desensitization of NPR-B. In fact, AVP exposure elevates intracellular calcium concentrations in these cells (115). Furthermore, ionomycin, a calcium-ionophore, mimics the effects of AVP; and a cell-permeable calcium-chelator blocks the AVP-dependent desensitization. Together, these data suggest that calcium elevations, not PKC activation, are required for the AVP-dependent inhibition of NPR-B. Interestingly, calcium-dependent NPR-B desensitization has also been observed for the serum components lysophosphatidic

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acid (182) and sphingosine-1-phosphate (176) as well as in response to hyperosmotic stimuli (182). In the latter scenario, calcium elevations were shown to stimulate NPR-B dephosphorylation (182).

The mechanisms for PKC- and calcium-dependent desensitization of NPR-B are unique. The former results from reduced phosphorylation of a known site and primarily affects the affinity of NPR-B for CNP and GTP (183). The latter is associated with reductions in maximal velocities by a mechanism that does not involve inhibition of NPR-B phosphorylation and requires a process in addition to the dephosphorylation of the known sites (183). Growth factor-dependent inhibition of NPR-B is also correlated with receptor dephosphorylation, but the involvement of individual phosphorylation sites in this process has not been reported (117).

VII. Internalization of NPR-A and NPR-B

Ligand-mediated internalization and degradation are also mechanisms for terminating surface receptor-mediated signaling. For natriuretic peptide signaling, there is some controversy as to whether internalization and degradation of NPR-A and NPR-B occur, whereas it is widely accepted that NPR-C internalizes and recycles back to the plasma membrane (see *Section III.C*). Early studies conducted on PC-12 pheochromocytoma cells suggested that both NPR-A and NPR-C internalize ANP and that both receptors are recycled back to the cell surface (184). Pandey and colleagues (185–188), using Leydig, Cos, and 293 cell lines, reported that ANP binding to NPR-A stimulates its internalization, which results in the majority of the receptors being degraded with a smaller portion being recycled to the plasma membrane. In contrast, Maack and co-workers (159, 189) reported that NPR-A in primary kidney or stably expressing Chinese hamster ovary cells is a constitutively membrane resident protein that neither undergoes endocytosis nor mediates lysosomal hydrolysis of ANP. Similarly, Jewett *et al.* (158) found that 293 cells expressing NPR-A bound less ANP over time but concluded that the reduced binding was due to a diminished affinity of NPR-A for ANP and not to decreased amounts of NPR-A at the cell surface. Finally, Fan *et al.* (138) failed to observe internalization of NPR-A, recycling of NPR-A, or significantly degraded ANP products in the media bathing NPR-A-expressing 293 cells. Only one study has addressed the receptor trafficking properties of NPR-B; it found no evidence for receptor internalization or recycling (138).

VIII. Degradation of Natriuretic Peptides

All three natriuretic peptides are degraded through two accepted processes: 1) NPR-C-mediated internalization followed by lysosomal degradation as discussed above; and 2) enzymatic degradation by neutral endopeptidase 24.11 (neprilysin), a zinc-dependent enzyme expressed on the plasma membrane that has broad substrate specificity and tissue distribution. In sheep, the enzymatic and receptor-mediated processes contribute equally to the degradation of ANP and BNP (190). Human BNP is more resistant to hydrolysis by neprilysin than ANP (191). Phosphoramidon, a potent inhibitor of this neutral endopeptidase, blocked the degradation of ANP in 293 cells expressing NPR-A but not NPR-C, indicating that NPR-C and neutral endopeptidase employ different degradation mechanisms (138). Addition of phosphoramidon to murine kidney slices increased the EC_{50} for ANP-dependent, but not BNP-dependent, activation of NPR-A, suggesting that ANP is a better neutral endopeptidase substrate than BNP (192). Targeted deletion of neutral endopeptidase 24.11 (193) does not lead to skeletal overgrowth like the targeted deletion of NPR-C (77), which suggests that CNP concentrations in the growth plate are primarily controlled by NPR-C in mice.

IX. Receptor-Specific Agonists and Antagonists

ANP, BNP, and CNP bind NPR-C and NPR-A or NPR-B. In an effort to identify a specific ligand for the guanylyl cyclase receptors, a phage library was screened for ANP variants that preferentially bind human NPR-A over human NPR-C (194). A variant was identified that has 1,000- to 10,000-fold greater affinity for NPR-A than for NPR-C. This analog was used to demonstrate that NPR-A, not NPR-C, is required for ANP-dependent inhibition of aldosterone synthesis in a human glomerulosa cell line (195). A similar approach was used to identify an ANP variant that had a 200-fold binding preference for rat NPR-A over rat NPR-C. Infusion of this analog into rats resulted in greater renal effects than the same concentration of natural ANP, presumably due to its reduced ability to be degraded through the NPR-C internalization pathway (196).

The best-studied antagonist of natriuretic peptides is a microbial polysaccharide known as HS-142-1 (197, 198). It inhibits ligand binding and activation of both NPR-A and NPR-B through a novel allotopic (allosteric), not simple competition, mechanism (199). It has no effect on natriuretic peptide binding to NPR-C (198). Current availability of HS-142-1 is unknown. Two other peptide-based antogonists to NPR-A, A71915 (200) and A74186 (201), also have been reported. The effect of these peptides on NPR-B activity is not known. To our knowledge, there is no specific antagonist that completely blocks the guanylyl cyclase activity of either NPR-A or NPR-B.

X. Physiological Effects of Natriuretic Peptides

Natriuretic peptides are often described simply as peptides involved in the regulation of blood pressure and volume. However, the effects of these peptides are widespread, and their levels change in response to a variety of pathological conditions (Table 1). The changes shown in Table 1 may be due to changes in intravascular volume or cardiac adrenergic tone as a result of the disease state or may be due to compensatory mechanisms causing increased production of the peptides. The levels of each peptide—ANP, BNP, or CNP—are also regulated by receptor activity, which can be altered by genetic mutation. Therefore, the downstream effects of each natriuretic peptide and the regulation of their

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circulating levels is likely to be much more complex than originally anticipated.

ANP and BNP have overlapping functions when administered iv to mammals, including humans. However, studies in murine knockout models clearly demonstrate separate functions for ANP and BNP. Nevertheless, their roles in human physiology remain to be definitively defined. This section will describe in more detail where and how the natriuretic peptides are acting in the body as well as describe some of the implications of these actions (Fig. 4).

A. Cyclic GMP binding effectors

Natriuretic peptides elicit their physiological responses through the synthesis of cGMP, a classic intracellular second messenger that was originally identified in rat urine in 1963 (202). There are three known cGMP binding proteins: cGMP-dependent protein kinases (PKG), cGMP binding phosphodiesterases (PDEs), and cyclic nucleotide-gated ion channels (Fig. 5). The best-studied cGMP signaling effects occur through PKGs, serine and threonine kinases that are activated by cGMP binding (203, 204). There are two PKG genes. The PKGI gene is alternatively spliced to produce α and β isoforms that differ in their amino termini. Both PKGI isozymes are mostly cytosolic and are highly expressed in platelets, smooth muscle, cardiomyocytes, and brain. Deletion of functional PKGI by homologous recombination in mice results in loss of cGMP-dependent vascular smooth muscle relaxation and juvenile (205), but not adult (204), hypertension. PKGII is myristoylated at glycine-2, and therefore, mostly membrane bound. It is found in high concentrations in the intestine, kidney, brain, chondrocytes, and bone (206). Deletion of functional PKGII in mice (207) or rats (208) results in normotensive animals with dwarfism and resistance to infection by heat-stable enterotoxin from *Escherichia coli*.

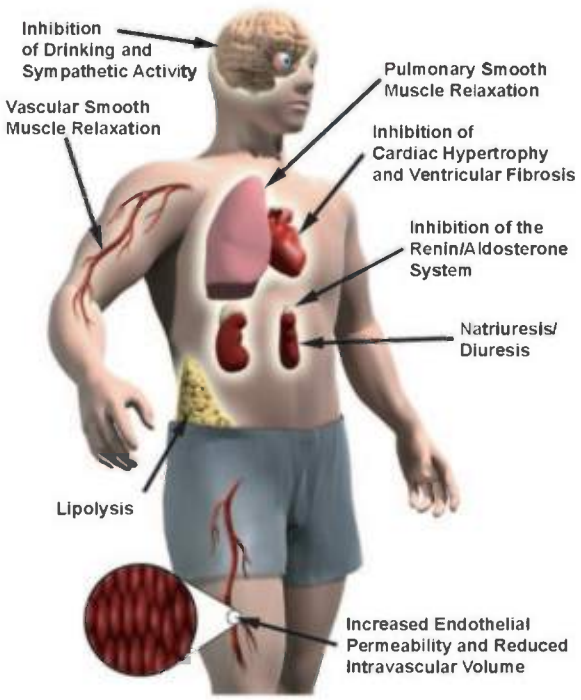


FIG. 4. Physiological consequences associated with NPR-A activation. See *Section X* for detailed description of physiological processes that are regulated by NPR-A.

Cyclic nucleotide PDEs are crucial regulators of cyclic nucleotide signaling because they degrade cyclic nucleotides into inactive 5′-nucleotide monophosphates. Hence, PDEs regulate concentrations of intracellular second messengers. There are 11 different families of PDEs, containing at least 25 different mammalian proteins (209, 210). The families are organized according to their substrate specificity (whether they degrade cAMP, cGMP, or both) and how they are activated or inhibited. For example, PDE1, -2, -3, -10, and -11 degrade both cGMP and cAMP; PDE4, -7, and -8 specifically hydrolyze cAMP; whereas PDE5, -6, and -9 only degrade cGMP (211). Some PDEs are regulated allosterically by cGMP. For instance, cGMP binding to PDE5 (the target of Viagra, Levitra, and Cialis) increases its activity and accelerates cGMP degradation in a feed-forward mechanism (Fig. 5). Alternatively, allosteric activation can facilitate cross-talk between the cGMP and cAMP pathways. For example, cGMP binding activates PDE2, which results in decreased intracellular cAMP concentrations, whereas cGMP binding inhibits PDE3 activity, resulting in increased cAMP levels.

cGMP mediates cellular responses through the regulation of cyclic nucleotide-gated (CNG) ion channels, a family of nonselective cation channels containing a carboxyl-terminal cyclic nucleotide-binding domain that binds cAMP or cGMP (212). They are most noted for their ability to control the dark cycle in photoreceptor cells, but they are also found in chemosensory cells, brain, airway epithelial cells, and the kidney. There are six known human genes encoding CNG channels, which can be broken into two groups designated A and B. The former group is comprised of those subunits that can form functional channels on their own, whereas the latter group cannot. To our knowledge, data linking CNG channels to specific natriuretic peptide functions have not been reported.

B. Effects of the ANP/NPR-A system on blood pressure

Mice completely lacking ANP (44) or NPR-A (109, 110) have blood pressures 20 to 40 mm Hg higher than normal, whereas animals transgenically expressing higher than normal amounts of ANP (213) or BNP (48) have blood pressures 20–30 mm Hg lower than normal. These data clearly indicate that the ANP/NPR-A system regulates basal blood pressures in mice. Some of the most striking data on this issue come from members of Smithies' group (214) who demonstrated that ANP-dependent guanylyl cyclase activities and blood pressures are directly proportional to NPR-A gene dosage over a range of 0 to 4 alleles. Although ANP was initially suggested to regulate blood pressure in a salt-sensitive manner, more recent data suggest that this is not the case (45, 109). Its combined effects on intravascular volume, vasorelaxation, natriuresis, and diuresis mediate the hypotensive nature of ANP as discussed below.

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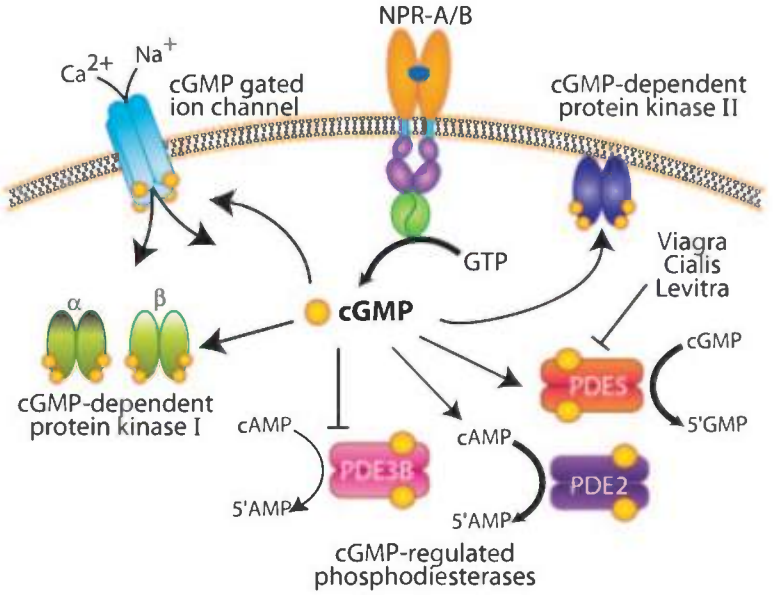


FIG. 5. Cyclic GMP effectors. cGMP mediates its effects by binding three known classes of proteins: cGMP-gated ion channels, cGMP-dependent protein kinases (type Iα, Iβ or type II) and phosphodiesterases (PDEs). Binding of cGMP to the different families of PDEs can induce degradation of cGMP (PDE5, the target of Viagra, Cialis, and Levitra), activate degradation of cAMP (PDE2), or inhibit degradation of cAMP (PDE3B), thereby regulating both cGMP and cAMP levels in cells.

C. Effects of ANP/NPR-A on endothelium permeability and intravascular volume

In the original article of De Bold *et al.* (2) describing the discovery of ANP, vascular atrial extract injections were shown to increase hematocrit levels (2). Subsequent studies indicated that the ANP-dependent vascular volume contraction does not require the natriuretic or diuretic effects of ANP because it precedes urination and occurs in nephrectomized animals (215–217). Additional experiments indicated that ANP increased capillary hydraulic conductivity (218) and permeability of the endothelium to macromolecules like albumin (219) (Fig. 4). However, data from cell culture-derived models are conflicted on this latter issue, with some reports suggesting that cGMP increases (220) and other reports suggesting that it decreases permeability (221). Consistent with ANP increasing cell permeability, mice specifically lacking NPR-A in their vascular endothelium are slightly hypertensive (10–15 mm Hg) and are volume expanded by 11–13% (222). This suggests that NPR-A in the endothelium accounts for about one third of the total hypotensive effects of ANP because animals completely lacking NPR-A are severely hypertensive (30–40 mm Hg) and volume expanded by 30%. Wild-type mice cleared radioiodinated serum albumin from their circulation in an ANP-dependent manner, whereas animals deficient in endothelial NPR-A did not (222). Strikingly, ANP increased the hematocrit levels in wild-type but not mutant animals, indicating that the ability of ANP to increase hematocrit levels absolutely requires endothelial NPR-A. Together, these data suggest that ANP regulates chronic transvascular fluid balance by increasing microvascular permeability. The mechanism for this phenomenon is currently unknown.

D. Effects of ANP and BNP on cardiac hypertrophy and fibrosis

ANP and BNP have direct effects on the heart. Mice lacking ANP (44) or NPR-A (110, 223) have enlarged hearts, whereas animals overexpressing ANP (213, 224) have smaller hearts. Initially, it was unclear whether the cardiac hypertrophy observed in the knockout animals resulted from prolonged exposure to systemic hypertension or from the loss of a local inhibitory effect on heart growth; it is likely that both processes lead to cardiac hypertrophy. The first evidence supporting a local effect involved NPR-A knockout mice that were treated with antihypertensive drugs from birth (111). These animals were normotensive but still had cardiac hypertrophy. In a separate study, the selective transgenic replacement of NPR-A in the heart of NPR-A knockout animals reduced cardiomyocyte size without affecting hypertension (225). Conversely, in a third elegant study, the selective deletion of NPR-A from the heart using Cre/lox technology resulted in mice with decreased blood pressure but mild cardiac hypertrophy (226). The reason for the reduced blood pressures in these animals likely results from elevated cardiac and plasma levels of ANP and BNP, which provides evidence for a local NPR-A-dependent feedback regulatory system for cardiac natriuretic peptide synthesis and/or secretion.

Early studies indicated that BNP inhibits the proliferation of cardiac fibroblasts in culture (227). This observation was validated *in vivo* when mice lacking BNP were shown to display pressure-sensitive ventricular fibrosis (52). The mechanism involved in the BNP-dependent regulation of fibroblasts is controversial. One group using a BNP transgene model system suggested that BNP attenuates angiotensin II-dependent fibrosis by inhibiting MAPK activity (228) whereas another group found that BNP inhibits transforming growth factor β-dependent fibrotic processes by activating MAPKs (229). Recent evidence suggests that the cardiac fibrosis involves matrix metalloproteinases (MMPs) because both ANP and BNP regulate MMP levels (229–231). Mice lacking NPR-A (*Npr1*$^{-/-}$) have increased expression and activity of MMP-2 and MMP-9. Furthermore, increased activity correlates with increased expression of nuclear factor-κB (NF-κB) (232). Several reports indicate that the ANP/

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BNP/NPR-A system inhibits pressure-induced cardiac remodeling as well (111, 226, 233). Hence, drugs that activate this pathway or block the inactivation of this pathway may be of significant clinical benefit to patients with failing hearts.

E. Effects of ANP on natriuresis and diuresis

In the kidney, ANP increases glomerular filtration rate, inhibits sodium and water reabsorption, and reduces renin secretion (Fig. 6). ANP-dependent diuresis and natriuresis are mediated exclusively by NPR-A in mice because these effects are completely lost in NPR-A knockout animals (234). ANP increases the glomerular filtration rate by elevating the pressure in the glomerular capillaries through coordinated afferent arteriolar dilation and efferent arteriolar constriction (235). In addition to these hydraulic effects, ANP inhibits sodium and water reabsorption throughout the nephron. In the proximal tubules, ANP inhibits angiotensin II-stimulated sodium and water transport (236). In collecting ducts, it reduces sodium adsorption by inhibiting an amiloride-sensitive cation channel (237). The effect of ANP on both transport processes is cGMP-dependent.

F. Effects of ANP and CNP on vascular relaxation and remodeling

The ability of the cardiac natriuretic peptides to relax precontracted aortic rings requires NPR-A because preparations from animals lacking this receptor are unresponsive to ANP and BNP (238). CNP relaxes aortic rings by a process that does not require NPR-A, presumably by activating NPR-B (238, 239). Unlike wild-type animals, mice selectively lacking NPR-A in vascular smooth muscle cells as a result of Cre/lox technology do not undergo an acute reduction in blood pressure in response to a bolus injection of ANP (240). However, resting blood pressures in these Cre/loxed mice do not differ from their wild-type littermates, indicating that NPR-A-stimulated vasorelaxation is important for acute, but not chronic, blood pressure regulation.

The mechanism of ANP-dependent vasorelaxation has been well studied (Fig. 7). Consistent with the requirement of PKGI in this pathway, PKGI knockout mice do not vasodilate in response to cGMP-elevating agents like ANP or nitric oxide generators (205). Interestingly, the adult animals are normotensive (204), which suggests that the hypertensive phenotype of the ANP or NPR-A knockout animals must result from a PKGI-independent effect. PKGI stimulates vascular smooth muscle cell relaxation by decreasing intracellular calcium levels and by decreasing the calcium sensitivity of the contractile system. To lower calcium concentrations, PKGI acts on several calcium channels. PKGIα directly phosphorylates and activates (opens) calcium-activated potassium channels (241, 242), which increases potassium efflux and causes membrane hyperpolarization. The hyperpolarization then inhibits calcium influx through nearby voltage-gated calcium channels. PKGI is also thought to directly inhibit the voltage-gated calcium channels through phosphorylation of the channel or an associated regulatory protein. At the endoplasmic reticulum, PKGI directly phosphorylates the inositol (1, 4, 5) trisphosphate receptor (243) and the inositol (1, 4, 5) trisphosphate receptor-associated PKGI substrate to inhibit calcium release from this storage vesicle (244). PKGI also activates the calcium/ATPase membrane-associated pump via an unknown mechanism to pump calcium out of the cell, thus reducing intracellular calcium lev-

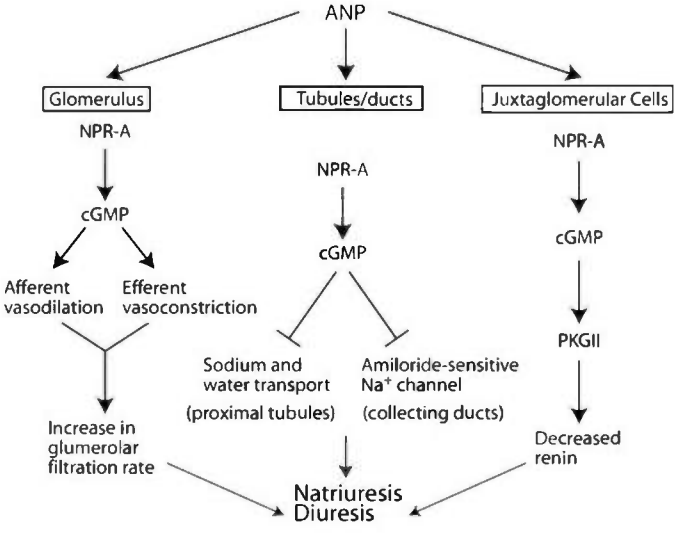


FIG. 6. ANP regulation of the kidney. Renal function is modulated by ANP in at least three ways. First, ANP increases the glomerular filtration rate by differentially regulating the tone of glomerular afferent and efferent blood vessels. Second, it decreases sodium reabsorption in the proximal tubules and collecting duct through cGMP-dependent modulation of sodium channels and transporters. Third, it decreases renin secretion from the juxtaglomerular cells via a PKGII-dependent process. Together, these processes reduce natriuresis, diuresis, and renin secretion.

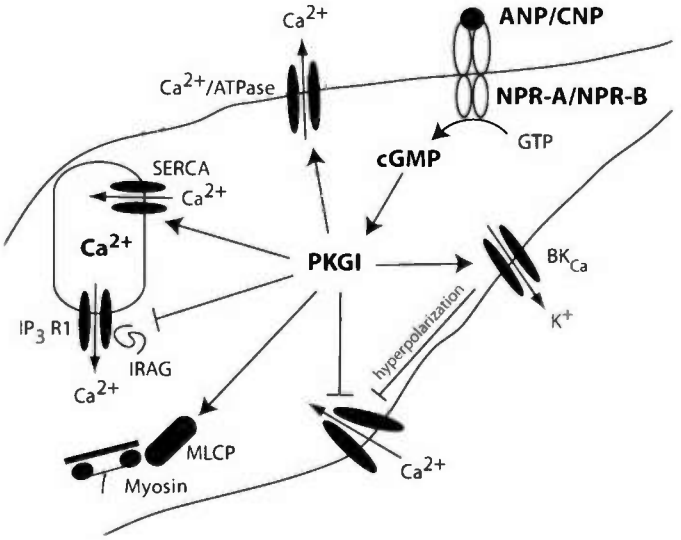


FIG. 7. Natriuretic peptide-dependent smooth muscle relaxation. ANP and CNP stimulation of their cognate receptors, NPR-A and NPR-B, respectively, increases intracellular cGMP concentrations. cGMP activates protein kinase GI (PKGI), which phosphorylates target proteins. PKGI inhibits the IP_3 receptor and stimulates the plasma membrane calcium/ATPase, the sarcoplasmic reticulum calcium/ATPase (SERCA), and the potassium/calcium channel (BK_{Ca}) to decrease intracellular calcium concentrations. PKGI phosphorylation and activation of myosin light chain phosphatase (MLCP) increases the calcium levels necessary for contraction, which lowers calcium sensitivity.

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els. PKGI phosphorylates phospholamban, which activates the calcium/ATPase (SERCA), resulting in calcium sequestration into the sarcoplasmic reticulum (245). However, mice lacking phospholamban vasodilate normally in response to cGMP-elevating agents (246). Finally, PKGIα decreases the calcium sensitivity of the contractile system by phosphorylating and activating myosin light chain phosphatase (247), which decreases myosin light chain phosphorylation. Together, these effects stimulate vascular smooth muscle relaxation (Fig. 5) (reviewed in Refs. 248 and 249).

CNP also is a vasodilator and is released in response to vascular injury (56). NPR-B is present in aortic vascular smooth muscle and mediates CNP relaxation of precontracted rat aorta (239). Furthermore, CNP inhibits vascular smooth muscle proliferation (250) and oxidized low-density lipoprotein-induced migration of cultured human coronary artery smooth muscle cells (251) in a cGMP-dependent manner. In recent years, the therapeutic potential of CNP-dependent regulation of vascular tone has been explored. CNP is expressed in rat carotid neointimal but not medial vascular smooth muscle cells (252), suggesting that it may act in a paracrine fashion to regulate neointimal formation. Consistent with this idea, CNP suppresses intimal growth caused by several types of arterial injury (253–255). Finally, recent reports suggest that CNP is the long sought after "endothelium-derived hyperpolarizing factor" (256) and that it inhibits myocardial ischemia/reperfusion injury (257) as well as platelet-leukocyte interactions (258). Interestingly, these processes were suggested to require signaling through NPR-C, not NPR-B.

G. Effects of natriuretic peptides in the lung

All three natriuretic peptide receptors are highly expressed in the lung. ANP stimulates the dilation of pulmonary airways and blood vessels. Infusion or inhalation of ANP stimulates bronchodilation in normal and asthmatic patients (reviewed in Ref. 259). ANP and BNP are elevated in patients with pulmonary hypertension and are indicative of increased right ventricular strain (260–262). Mice overexpressing ANP are resistant to hypoxia-induced hypertension (263), whereas ANP-deficient mice exhibited increased pulmonary hypertension in response to chronic hypoxia (264). CNP also reduces pulmonary hypertension (265) and fibrosis (266).

H. ANP-dependent antagonism of the renin-aldosterone system

ANP regulates blood pressure, in part, through the inhibition of the renin-angiotensin II-aldosterone system (Fig. 6). Renin is a protease secreted from renal juxtaglomerular cells. It cleaves angiotensinogen to angiotensin I, which is subsequently converted to angiotensin II by angiotensin-converting enzyme in the pulmonary vascular endothelium. Angiotensin II then stimulates vasoconstriction and the release of aldosterone, the major hormone responsible for regulating sodium reabsorption in the renal cortical collecting ducts. High doses of ANP do not reduce renin levels in humans, presumably because of compensatory responses associated with the dramatic decreases in arterial blood pressure. However, physiological doses of ANP suppress both renin and aldosterone levels (267). In dogs, intrarenal ANP infusion markedly inhibits the renin secretion rate (268). Inhibition of cAMP-stimulated renin secretion requires PKGII (269). Mice lacking PKGII, but not PKGI, have higher renin expression than their wild-type littermates and are resistant to 8-bromo-cGMP-dependent inhibition of basal and forskolin-induced renin secretion (270). At birth, NPR-A null mice have elevated kidney renin and angiotensin II levels, which is consistent with the known antagonizing effects of ANP and NPR-A on the renin-angiotensin-aldosterone system (271). However, in the adult male NPR-A$^{-/-}$ mice, the renal and systemic levels of renin are decreased, whereas adrenal renin activity and aldosterone levels remain elevated, suggesting that the reduced renal and systemic renin levels result as a compensatory mechanism to increased blood pressure.

In addition to inhibiting renin secretion, ANP directly inhibits aldosterone production in the adrenal gland (Fig. 8). In the adrenal glomerulosa, ANP reduces ACTH-stimulated, angiotensin II-stimulated, and basal aldosterone levels (272–276). The involvement of cGMP in ANP-dependent inhibition of aldosterone production has been controversial because in some studies the ANP effect was mimicked by a cell-permeable cGMP analog (277), but in other studies, cGMP analogs were ineffective (278). Additional experiments indicated that the ability of ANP to reduce aldosterone levels could be mimicked with a NPR-C-specific ligand and blocked by the Gi/Go inhibitor, pertussis toxin. However, recent reports employing a natriuretic peptide variant that

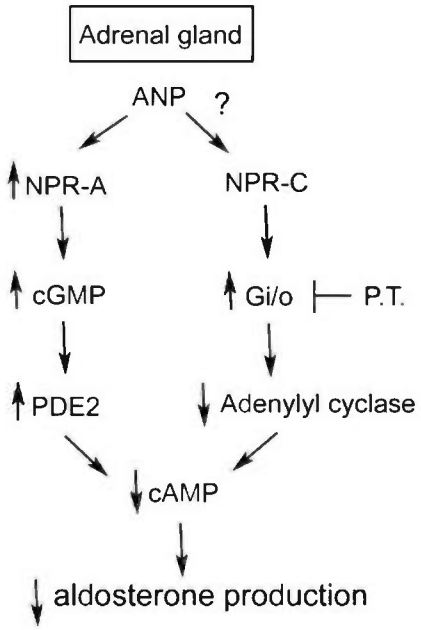


FIG. 8. ANP regulation of the adrenal gland. ANP-dependent decreases in aldosterone secretion from the adrenal gland require reductions in cAMP concentrations. There are two proposed mechanisms for this effect. One involves ANP-dependent activation of NPR-A, which produces cGMP and stimulates cAMP-hydrolyzing PDE2, whereas another involves NPR-C-dependent inhibition of adenylyl cyclase via a pertussis toxin-sensitive G protein-dependent pathway. P.T., Pertussis toxin.

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has a 1000-fold higher binding constant for NPR-A compared with NPR-C (195) or catalytically active or inactive guanylyl cyclase C receptors (279) suggests that NPR-A is responsible for the ANP-dependent reductions in aldosterone levels. Consistent with this assessment, mice lacking NPR-A have plasma aldosterone levels about 2-fold higher than wild-type littermates (271). The mechanism for the ANP-dependent reductions in aldosterone may involve PDE2, a cGMP-activated PDE that is highly expressed in the glomerulosa layer of the adrenal gland (Fig. 8) (280). In this scenario, ANP binds to NPR-A causing intracellular cGMP elevations and PDE2 activation. Activated PDE2 then degrades cAMP, which is the major intracellular determinant for aldosterone synthesis. An alternative possibility involves the steroidogenic acute regulatory protein because ANP inhibits the synthesis (281) and phosphorylation (282) of the steroidogenic acute regulatory protein in adrenal glomerulosa cells as well.

I. Effects of ANP on fat metabolism

Many studies have shown an association between obesity and hypertension; however, the effect of natriuretic peptides on fat tissue is only now beginning to emerge. In the late 1980s, ANP-dependent cGMP elevations were measured in rat mammary gland fat cells (283) and rat brown adipose tissue (284). However, in both studies, scientists were unable to demonstrate ANP-stimulated lipolysis. In a later study, ANP-stimulated lipolysis was demonstrated both in isolated human fat cells and *in vivo* by peptide infusion (285). Subsequently, it was determined that ANP-stimulated lipolysis is specific to primates (286) presumably because primates contain a higher NPR-A to NPR-C ratio.

The mechanism and the pathophysiological relevance of these observations are beginning to be elucidated. ANP stimulation of lipolysis is mimicked by 8-bromo-cGMP and is independent of PDE3B, the main enzyme involved in the degradation of cAMP in the adipocyte (285). This suggests that ANP-stimulated lipolysis involves cGMP, but not cAMP elevations as is required for epinephrine-induced lipolysis. A recent study suggested that PKGI is the cGMP effector in the ANP-dependent lipolytic response because pharmacological inhibition of PKGI decreases ANP-dependent lipolysis in primary human preadipocytes (287). Similar to cAMP-dependent lipolysis, the ANP/NPR-A/cGMP-dependent pathway stimulated the phosphorylation of hormone-sensitive lipase, the major regulated enzyme in fat responsible for the hydrolysis of triglycerides into free fatty acids. Increased phosphorylation of lipid droplet-binding protein, perilipin, in response to ANP was also observed. Whether the same sites are phosphorylated on these enzymes in response to cGMP as are phosphorylated in response to cAMP is not known.

Recent papers have examined the metabolic role of ANP-dependent lipolysis to establish a possible link between obesity and hypertension. Unlike catecholamine-dependent dysregulation of lipolysis, which is associated with obesity, the lipid-mobilizing effects of ANP are not related to obesity in young men (288). However, obese women have increased ANP- and isoproterenol-dependent lipolysis when fed a low-caloric diet (289).

J. Neurological effects of natriuretic peptides

All natriuretic peptides and natriuretic peptide receptors have been found in the brain, although CNP and NPR-B appear to be particularly abundant. Consistent with the systemic volume-depleting effects, injection of ANP into the third ventricle of the hypothalamus inhibits water intake induced by overnight dehydration or angiotensin II exposure (Fig. 4) (290). Intracerebroventricular infusion of ANP suppresses salt appetite (291) as well as AVP release from the hypothalamus (292). ANP-dependent suppression of sympathetic activity in the brain stem also has been observed (293, 294). Specifically, ANP was shown to sensitize vagal afferents and dampen the arterial baro receptor response (293, 295, 296). Finally, CNP and cell permeable cGMP analogs have been reported to stimulate GH release in rat anterior pituitary cells (297) and pituitary-derived GH_3 cells (298).

K. Immunological effects of natriuretic peptides

Natriuretic peptides and their receptors are found in many immune cells; however, the significance of these peptides in the immune system is only now emerging. Current evidence suggests a role for ANP in the allergen response of asthma and in immune-related postischemic damage.

The most-studied role of natriuretic peptides in the immune response has been observed in macrophages and dendritic cells. ANP elicits its antiinflammatory effect by reducing production of proinflammatory cytokines (TNF-α and IL-12) while enhancing production of IL-10 (299, 300). ANP increases neutrophil migration *in vitro* (301), and NPR-A knockout mice exhibit decreased neutrophil infiltration to cardiac tissue after injury compared with wild-type mice by decreasing activation of the transcription factor NF-κB (302). Excessive neutrophil infiltration after ischemia can lead to further tissue damage, thus lending a cardioprotective function to blocking ANP signaling after ischemia. NPR-A knockout mice also exhibit decreased eosinophil accumulation in the lungs after allergic challenge with ovalbumin (303), suggesting that ANP signaling may play a role in asthma.

L. The CNP/NPR-B/cGMP/PKGII system and long bone growth

The most obvious physiological effect of CNP is to stimulate long bone growth (Fig. 9). It regulates many types of bone cells, but its major target appears to be the chondrocyte as described below. In a mouse osteoclast model, 1,25-dihydroxyvitamin D3 stimulated CNP expression, cGMP elevations, and osteoclast bone resorptive activity (304). In osteoblasts, CNP elevated differentiation markers like alkaline phosphatase and increased the mineralization of nodules (305). In chondrocytes, CNP elevated cGMP concentrations (53), and in fetal mouse tibia cultures, CNP induced endochondrial ossification (306).

The genetic data supporting the CNP/NPR-B/cGMP bone growth system in mice are striking. Inactivating mutations in the genes coding for CNP (70) or NPR-B (124, 125) cause dwarfism, whereas superphysiological levels of natriuretic peptides resulting from transgenic overexpression (74, 75,

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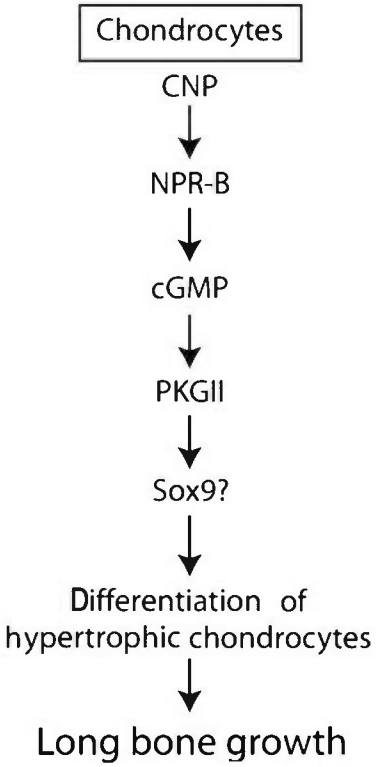


FIG. 9. CNP-dependent long bone growth. CNP-dependent long bone growth requires CNP binding and activation of NPR-B, cGMP binding, and activation of PKGII and PKGII-dependent increases in the proliferation of hypertrophic chondrocytes. The substrate(s) for PKGII in this process has not been identified. One possible substrate is the chondrocyte differentiation factor, Sox9.

307) or reduced clearance (76, 77) cause skeletal overgrowth. No growth abnormalities are observed in any of these mutant animals at birth, suggesting that the CNP/NPR-B/cGMP system only stimulates postpartum bone growth. Growth plates from animals lacking functional CNP or NPR-B (70, 124) are thinner due to reductions in the proliferative and hypertrophic zones, whereas growth plates in the transgenic mice are expanded (74, 307).

Targeted deletion of PKGII by homologous recombination in mice (207) or spontaneous loss of function mutations of PKGII in rats (208) also causes dwarfism. However, unlike the CNP and NPR-B knockout animals, the growth plates of these animals are expanded. One explanation for the growth plate differences is that other cGMP effectors besides PKGII also are required for normal CNP-dependent long bone growth, but this remains to be determined. The substrate(s) of PKGII that mediate its bone growth-promoting properties is not known. However, a recent report suggests that the "master" inhibitor of chondrocyte differentiation, SOX9, is a reasonable candidate because PKGII expression inactivates SOX9 by causing its translocation from the nucleus to the cytoplasm (208). Neither the sites of phosphorylation nor the identity of the kinase (PKGII or other) that phosphorylates SOX9 has been determined.

Multiple putative loss of function mutations in the gene encoding NPR-B were recently identified in human patients with the autosomal recessive disease, acromesomelic dysplasia, type Maroteaux (127). The frequency of this disease is rare (1/2000,000); but because carriers are shorter than matched controls, the effect of these mutations on the stature of the general population is significant. The most common form of human dwarfism, achondroplasia, results from autosomal dominant mutations in the gene coding for fibroblast growth factor-3 (FGF3) receptor, which causes constitutive activation of the signal transducer and activator of transcription 1 and MAPK pathways in chondrocytes (308). Mice expressing constitutively active FGF3 receptors are dwarfed, and their growth plates resemble those of mice lacking CNP or NPR-B (309). In contrast, mice lacking a functional version of the FGF3 receptor exhibit skeletal overgrowth, similarly to mice overexpressing CNP (309). Transgenic overexpression of CNP in growth plates partially reverses the dwarfism phenotype of mice expressing a constitutive active FGF3 receptor (75). The ability of CNP to stimulate bone growth in mice expressing the constitutively active FGF3 receptor mutant may result from its ability to inhibit MAPK signaling because mice expressing the CNP transgene had reduced MAPK kinase, but not signal transducer and activator of transcription 1, phosphorylation (75). The mechanism involved in the CNP-dependent MAPK inhibition is unknown.

XI. Therapeutic Applications of Natriuretic Peptides

As hormone/paracrine factors that regulate intravascular volume, blood pressure, natriuresis, diuresis, and long bone growth, the potential use of the natriuretic peptides for therapeutic benefit is promising. A search of the literature yields a list of over 100 reviews discussing the therapeutic use and potential of these peptides. Initial studies tested the use of ANP and BNP as potential therapeutic agents for the treatment of congestive heart failure, hypertension, and renal failure. The infusion of synthetic ANP, clinically known as anaritide and by the trade name Carperitide, into patients with hypertension (310) or chronic heart failure (41, 311) resulted in elevated sodium and water excretion and decreased blood pressure. Long term (48 h) anaritide infusions of patients with acute heart failure resulted in beneficial hemodynamic responses without tolerance, suggesting that ANP injections may be a clinically useful treatment for heart failure (312).

Anaritide was also used to treat patients with acute renal failure. However, data from these trials has proven contradictory. An early report found that ANP treatment did not improve the dialysis-free survival rate in critically ill patients with acute tubular necrosis (313). In fact, it was associated with decreased survival. However, a subsequent report found that administration of ANP to patients with acute ischemic renal failure resulting from complicated cardiac surgery significantly increased renal function and decreased the need for dialysis (314).

Human recombinant BNP, clinically known as nesiritide and by the trade name Natrecor, mimics the actions of endogenous BNP and has been shown to cause potent vasorelaxation accompanied with increases in natriuresis and diuresis, as well as decreases in plasma aldosterone and endothelin levels in patients with acute heart failure (re-

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viewed in Ref. 315). Thus, BNP has emerged as a new tool to manage heart failure (261, 316, 317). The U.S. Food and Drug Administration approved the use of BNP (nesiritide) for the treatment of acutely decompensated heart failure in 2001. Because the half-life of nesiritide (BNP) is significantly longer than that of anaritide (ANP), it is thought to be the better of the two drugs (Table 1). Unfortunately, the widespread use of nesiritide has recently come under scrutiny due to the increased risk of renal dysfunction and mortality in patients undergoing BNP treatment (318, 319). Additional clinical trials are necessary to evaluate this situation and to more narrowly define the benefits, risks, and parameters required for optimal BNP treatment in humans.

The other clinical benefit of natriuretic peptides comes from their diagnostic use. ANP and BNP levels are increased in patients with heart failure and in many patients with hypertension and chronic renal failure (320–322). Because BNP plasma levels correlate more closely than ANP levels with left ventricular function, a common indicator of heart disease, BNP is considered a better diagnostic marker of heart failure. Immunoassays that measure the level of BNP or pro-BNP are commonly used clinically (322). The measurement of BNP levels in both emergency and primary care settings has been used to rule out or confirm a heart failure diagnosis in patients. In emergency care, patients presenting shortness of breath were evaluated for BNP level, and its correlation with heart failure was used to rule out heart failure *vs.* other pulmonary causes of dyspnea (323). Elevated BNP levels correlate with poor prognoses from other diseases as well. For example, BNP levels have been successfully used to predict poststroke mortality (324), postcardiac surgery atrial fibrillation (325), as well as the risk of death in patients with heart failure (326). Finally, based on its ability to effectively measure the benefit of various treatments in patients with right ventricular overload and pulmonary hypertension, BNP has been suggested to be a better guide for optimal treatment of heart failure than classical clinical measurements (261, 322).

Although many of the clinically therapeutic roles for natriuretic peptides have been centered on the current and potential uses of ANP and BNP, the therapeutic uses of CNP have yet to be explored. One potential use for CNP therapy is in the treatment of dwarfism, especially acromesomelic dysplasia type Maroteaux, which is the result of loss of function mutations in NPR-B. Activation of the CNP/NPR-B pathway downstream of the mutation could stimulate the expansion of the growth plates as was seen in the transgenic mice models. Another potential use of the CNP pathway may be to speed the healing of bone fractures, because the bones of rats lacking functional PKGII heal much slower than those from wild-type animals (208). Finally, CNP may hold promise as a cardiovascular drug because recent evidence indicates that it can prevent cardiac remodeling after myocardial infarction in mice (327).

XII. Concluding Comments and Future Directions

Over the past 25 yr, natriuretic peptides and their cognate receptors were discovered and purified, and the genes encoding these proteins were cloned and "knocked out." Additional studies investigated the structure and regulation of the individual participants in these signal transduction pathways; ultimately yielding a tremendous body of literature on natriuretic peptide-dependent regulation of physiological and pathophysiological processes as well as at least two drugs, anaritide and nesiritide.

During the next decade, we anticipate that further discoveries will be made regarding the molecular nature of these pathways as well as their clinical applications. Regarding the former, structural information on the guanylyl cyclase and kinase homology domains of NPR-A and NPR-B would be informative, as would be the identity of molecules such as kinases and phosphatases that regulate these receptors. Identifying downstream participants in ANP-dependent lipolysis and CNP-dependent bone growth pathways also will be of extraordinary importance, as would be the identification of physiological events that are specifically regulated by NPR-C. On the clinical side, we anticipate the discovery of small molecule activators or inhibitors (antagonist) of these pathways that may be used to treat diseases like systemic hypertension, obesity, pulmonary hypertension, heart failure, and skeletal growth disorders as well as diseases yet to be associated with these pleiotropic signaling systems.

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Exhibit 18

REMARKS

Claims 15-30 are pending and under examination. In the Action, the Examiner rejected claims 15-30 as assertedly being based on a defective reissue Oath or Declaration. The claims were also rejected on grounds of non-statutory double patenting. Reconsideration is requested in light of the following remarks.

I. Summary of Interview Pursuant to MPEP §713.04 and 37 CFR §1.133

Applicant would like to thank Examiners Railey and Witz for the courtesy of the telephone discussion on June 25, 2019.

During the interview, the defective Oath and Declaration rejection was discussed. As noted in the Interview Summary of July 3, 2019, the Examiners indicated that the subject matter of claims 15-30 was previously not claimed nor subject to a restriction requirement in a previous application. Applicant thanks the Examiners for the helpful discussion.

II. The Rejection of the Claims in View of a Defective Oath or Declaration Should Be Withdrawn

In the Action, the Examiner maintained the rejection of claims 15-30 on the basis that the Oath or Declaration filed in the reissue application was defective under 35 USC §251. The Examiner asserted that the declaration does not describe a sufficient error in the patent claims as prescribed under 37 CFR 1.175, and asserted that the restriction requirement in parent application 13/366,672 ("the '672 application") required election of CNP species, having no generic or linking claim, and that the CNP variant could be conjugated to a hydrophobic acid and an amino acid sequence from 1-20 amino acids, which is substantially identical to the reissue claims. Applicant respectfully disagrees.

35 USC §251 states that the error upon which a reissue is based must be one which causes the patent to be "deemed wholly or partly inoperative or invalid, by reason of a defective specification or drawing, or by reason of the patentee claiming more or less than he had a right to claim in the patent." See also, MPEP §1402.

The Oath filed with the present reissue application indicated that the patent was defective by virtue of patentee claiming less than he had a right to claim. Pending claims 15 and 18 are directed to a macromolecule or sustained release composition, respectively, comprising a CNP variant and a synthetic polymeric group coupled to the CNP

variant through a hydrolysable linkage. As noted in the Interview Summary, the feature of a synthetic polymeric group is a feature that was previously not claimed in the original patent and was not subject to restriction by the examiner in the '672 application. Currently pending claims 21 and 22 were not presented in the '672 application (i.e., were previously unclaimed as acknowledged by the Examiner in the Interview Summary) and were not subject to a restriction requirement. Further currently pending method claims 23 and 27 require the presence of the synthetic polymeric group coupled to a hydrolysable linkage, which was also not the subject of a restriction requirement. As such, the claims are directed to subject matter that was not previously claimed in or subject to a restriction requirement in a parent application, and Applicant is entitled to pursue the overlooked aspects of the invention claimed herein. (MPEP §1412.01(II)).

Therefore, the Oath accurately reflects that patentee is claiming less than he had a right to claim, and the rejection of the Oath or Declaration as defective under 35 USC §251 should be withdrawn and the application examined accordingly.

III. The Rejection of the Claims on the Grounds of Non-statutory Double Patenting

Applicant acknowledges the rejections of the claims on the grounds of non-statutory double patenting and maintains the request that the rejections be held in abeyance until notification of allowable subject matter.

IV. Conclusion

Applicant submits that the pending application is in condition for allowance and respectfully requests notification of the same.

Dated: July 10, 2019

Respectfully submitted,

By /Katherine L. Neville/
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Exhibit 19

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/646,822	07/11/2017	Daniel J. Wendt	30610/44556C1-R2	7727

90849 7590 07/03/2019

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233 South Wacker Drive
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Chicago, IL 60606

EXAMINER	
RAILEY, JOHNNY F	

ART UNIT	PAPER NUMBER
3991	

NOTIFICATION DATE	DELIVERY MODE
07/03/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

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Applicant-Initiated Interview Summary	Application No. 15/646,822	Applicant(s) Wendt et al.	
	Examiner JOHNNY F RAILEY II	Art Unit 3991	AIA (FITF) Status No

All participants (applicant, applicants representative, PTO personnel):

(1) JOHNNY F. RAILEY II.

(2) JEAN WITZ.

(3) KATE NEVILLE.

(4) AJAY SINGH.

Date of Interview: 25 June 2019.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☑Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 15-30.

Identification of prior art discussed: NONE.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Discussed the rejection of claims 15-30 based upon a defective declaration under 35 USC 251. Applicant noted that claim 15 requires that the CNP variant comprise a synthetic polymeric group coupled to a hydrolysable linkage. This is a feature that was previously not claimed in the original patent and was not subject to restriction by the examiner in application 13/466672. Previous unclaimed methods, i.e. claims 21 and 22, do not require the presence of a synthetic polymeric group coupled to a hydrolysable linkage. These methods were not subject to the restriction requirement. Other method claims, i.e. claims 23 and 27 require the presence of the synthetic polymeric group coupled to a hydrolysable linkage. Applicant's response after final will address these issues and will be considered. The rejection of claims 15-30 based upon a defective declaration should be withdrawn. Whether or not a restriction/election by original presentation of claims 15-30 will be addressed upon the response. The non-statutory double patenting rejections of claims 15-30 remain of record and should be addressed in applicant's response..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/JOHNNY F RAILEY II/ Patent Reexamination Specialist, Art Unit 3991	/Jean C. Witz/ Supervisory Patent Reexamination Specialist CRU 3991

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

Exhibit 20

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/646,822	07/11/2017	Daniel J. Wendt	30610/44556C1-R2	7727

90849 7590 11/26/2019

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
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Chicago, IL 60606

EXAMINER	
CAMPELL, BRUCE R	

ART UNIT	PAPER NUMBER
3991	

NOTIFICATION DATE	DELIVERY MODE
11/26/2019	ELECTRONIC

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Office Action Summary	Application No. 15/646,822	Applicant(s) Wendt et al.	
	Examiner BRUCE R CAMPELL	Art Unit 3991	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 10 October 2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 15-30 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-30 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 10/10/2019.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

Reissue Applications

For reissue applications filed before September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the law and rules in effect on September 15, 2012. Where specifically designated, these are "pre-AIA" provisions.

For reissue applications filed on or after September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the current provisions.

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on October 10, 2019 has been entered.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claim(s) 15-30 are rejected under pre-AIA 35 U.S.C. 102(e) as being anticipated by Wendt, U.S. Patent 8,377,884.

The applied reference has a common assignee and inventors with the instant application. Based upon the earlier effective U.S. filing date of the reference, it constitutes prior art under pre-AIA 35 U.S.C. 102(e). This rejection under pre-AIA 35 U.S.C. 102(e) might be overcome either by a showing under 37 CFR 1.132 that any invention disclosed but not claimed in the reference was derived from the inventor or joint inventors (i.e., the inventive entity) of this application and is thus not the invention "by another," or if the same invention is not being claimed, by an appropriate showing under 37 CFR 1.131(a).

Wendt discloses variants of C type natriuretic peptide (CNP) useful for treating achondroplasia. CNP22 can be extended at the N-terminus by 1-40 amino acids derived from NPPC or CNP53 (col. 8, lines 59-65). These variants can be conjugated to PEG at the N-terminus (col. 9, lines 8-14). Thus Wendt anticipates the pegylated forms of SEQ ID Nos. 60 and 146-174 (claims 15-20). With regard to claim 21, Wendt teaches that CNP stimulates cGMP production (col. 2, lines 23-26), and directly demonstrates this with CNP22, CNP27, CNP37 and CNP53 (Figs. 4-6, 9-12). With regard to claim 22, CNP reverses growth arrest induced by activated FGFR3 (Fig. 7). With regard to claims 23-30, Wendt teaches that the CNP variants are useful for treating skeletal dysplasias, including achondroplasia (col. 61, lines 37-59).

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to

consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claims 15-30 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over U.S. patent 6,743,425 ("Nakao '425") taken with U.S. 2007/0197434 ("Nakao '434"), Golembo, James, Potter, Yeung, Totsune, Eun, Szoka and Huang.

State of the Art Regarding CNP

Potter teaches the structure of C type natriuretic peptide (CNP). CNP is synthesized as a 126 amino acid pre-pro-hormone, which is cleaved by furin to release a 53 amino acid polypeptide (CNP53) having an N-terminal aspartate residue. CNP53 can be further cleaved by an unidentified peptidase to yield CNP22. See Fig. 1. CNP binds to two receptors, NPR-A and NPR-B, which are transmembrane guanylyl cyclases (p. 51). Loss of NPR-B function is associated with dwarfism in both humans and mice (pp. 52-53). CNP stimulates bone growth by increasing proliferation of hypertrophic chondrocytes, and dwarfism in mice caused by constitutively active FGF3 receptor can be partially reversed by transgenic expression of CNP (p. 62). Potter suggests using CNP to treat dwarfism (p. 63).

Yeung compared the effects of CNP22 and CNP53 on cGMP production in cultured mouse astrocytes. The two forms of CNP were found to have very similar binding characteristics and effects on cGMP production (see Figs. 1, 3, 4), which did not appear to be caused by conversion of CNP53 to CNP22. It was concluded that CNP53 is biologically active itself, i.e. it is not simply a precursor to CNP22 (p. 105).

Totsune teaches that the methionine residue found in both CNP53 and CNP22 is susceptible to oxidation (p. 39, Fig. 3; also see Potter Fig. 1).

State of the Art Regarding Recombinant Protein Production

Eun reviews protein synthesis and turnover. Eun teaches that a protein's stability and susceptibility to degradation are affected by the amino acid residues at the N-terminal end. Methionine and glycine have a stabilizing effect, while aspartate is destabilizing. See pp. 13-14.

Szoka teaches that small peptides can be produced as fusion proteins in bacteria (p. 11) and discloses a method for cleaving fusion proteins to release the peptide of interest. An expression vector is designed so that the N-terminal of the peptide of interest is joined to the C-terminal of a bacterial protein, linked by an aspartate-proline dipeptide (Fig. 3). Acid treatment cleaves the dipeptide, releasing the peptide of interest with a proline residue added at the N-terminus (pp. 16-17). When bovine growth hormone was produced by this method, presence of the N-terminal protein did not prevent the hormone from binding its receptor (Fig. 7).

Huang teaches, "It is well known that hSA, the most abundant protein in plasma, is a good carrier protein with a substantial circulatory half-life (up to 19 days in the human blood) and is widely distributed in vivo. A number of proteins have been fused to albumin to improve their circulating half-lives and stability characteristics" (p. 2, citations omitted).

Suggestion to Make and Use the Claimed Inventions

Nakao '425 discloses a method for treating achondroplasia caused by constitutive activation of FGFR3 by administering a compound that activates guanylyl cyclase B, such as CNP22 or CNP53 (col. 2, line 48 – col. 3, line 20; col. 8, lines 1-48; claim 5). Nakao also discloses transgenic mice overexpressing FGFR3 which can be used to test potential therapeutics (col. 14, lines 44-46). Nakao '425 does not teach a method in which the administered compound is a CNP analog (i.e. not CNP22 or CNP53) conjugated to a synthetic polymer.

Nakao '434 discloses another use for CNP peptides, treatment of rheumatoid arthritis. Nakao '434 teaches that CNP22 or CNP53 could be modified by addition, deletion or substitution of amino acid residues to yield biologically active analogs (¶¶ 29, 35, 47, 102-115).

Golembo discloses a method for treating skeletal dysplasias by administering natriuretic peptides. The administered peptide can be CNP or a CNP variant (p. 5, lines 26-28; p. 7, lines 14-17). Golembo teaches that deletion of the 5 N-terminal residues from CNP22 (to yield what the instant application calls CNP17) yields a peptide as active as CNP22, and further teaches substitution of the methionine residue, which is a

neutral endopeptidase cleavage site (p. 7, line 29 – p. 8, line 15; pp. 21-24). Golembo teaches that CNP has a short half life in vivo (p. 3, line 31) but can be conjugated to an agent to prolong its half life in circulation (p. 6, line 20).

James discloses natriuretic peptides and analogs conjugated to PEG (¶¶ 4, 49, 50, claim 1). Natriuretic peptide analogs can include an N-terminal "tail" comprising all or part of the native natriuretic peptide sequence (¶ 70). James teaches that the use of pegylated proteins as therapeutics is well established and provides advantages such as increased half life in the bloodstream (¶¶ 13, 16).

It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the method of Nakao '425 by administering a CNP analog.

Many of the claimed peptides are truncated forms of CNP53 (SEQ ID Nos. 60, 145—178). Yeung established that CNP22 and CNP53 are equally effective, and Golembo found that CNP17 is also as effective as CNP22, leading to the conclusion that the CNP17 ring structure is all that is required to activate the NPR-B receptor. Accordingly, Nakao '434 and Golembo both teach that CNP53 or CNP22 analogs with a longer or shorter "tail" attached to the CNP17 ring can be used. There was a reasonable expectation that truncated forms of CNP53 (CNP52, CNP51, etc.) would be active because the longest tail (CNP53) does not interfere with receptor binding, as taught by Yeung. Furthermore, a truncated form of CNP53 should still be cleaved to CNP22 by an endopeptidase, as taught by Potter. It would also have been obvious to include an N-terminal "tail" as an attachment site for HSA or PEG, as taught by James, to avoid steric interference with the CNP17 ring which is involved with receptor binding.

Some of the claimed peptides are truncated forms of CNP53 which have been further modified.

The substitution of the methionine with a different amino acid (SEQ ID Nos. 180-184) is suggested by Totsune, which teaches that the methionine residue is susceptible to oxidation, and Golembo, which teaches that this residue is susceptible to cleavage by endopeptidase.

With regard to SEQ ID Nos. 75, 179, 181, 190, 191, 193 and 194, it would have been obvious to append a glycine or methionine to the N-terminus of any peptide because these residues have a stabilizing effect, as taught by Eun. It was especially obvious to do so for CNP53 because the N-terminal aspartate of CNP53 would have a destabilizing effect.

If the CNP analog were produced by the conventional method of expression as part of a fusion protein (taught by Szoka), an N-terminal proline would remain after cleavage of the fusion protein, as in SEQ ID Nos. 145, 185, 186, 188 and 189.

With regard to SEQ ID Nos. 144, 183, 188 and 193, it was obvious to provide a CNP analog as a fusion with HSA since this was known to increase the half life of circulating proteins, as taught by Huang.

Regarding all the claimed peptides, pegylation is an art recognized method for prolonging the half life of proteins in the bloodstream, and James discloses pegylation of "virtually any natriuretic peptide." James also teaches that undesired conjugation of PEG can be prevented by substituting lysine residues with arginine (¶ 65), a modification found in SEQ ID Nos. 36, 184, 189 and 194.

With regard to the peptides' properties which are recited in the claim preambles, James Fig. 2 discloses pegylated natriuretic peptides in which the PEG can be fully hydrolyzed to release the peptide, as recited in claims 15 and 18. Stimulation of cGMP production (claim 21) and overcoming constitutive activation of FGFR3 (claim 22) were known properties of CNP, and treating skeletal dysplasia including achondroplasia (claim 23) had already been suggested (Potter, Yeung, Golembo, Nakao '425).

Thus the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made.

Claims 15-30 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over U.S. patent 6,743,425 ("Nakao '425") taken with U.S. 2007/0197434 ("Nakao '434"), Golembo, James, Potter, Yeung, Totsune, Eun, Szoka and Huang, and further in view of Wendt.

Nakao '425, Nakao '434, Golembo, James, Potter, Yeung, Totsune, Eun, Szoka and Huang are relied upon as described in the rejection above.

Wendt is relied upon as cited above. Wendt is cited here because it specifically suggests replacing the methionine residue (residue 48 of CNP53 or residue 17 of CNP22) with asparagine (e.g. col. 43, lines 23-25). This is relevant to SEQ ID Nos. 180-184.

Wendt is also cited because the test results showing increased half life of CNP37 and pegylated CNP27 compared to CNP22 (e.g. Figs. 9-12) provide further expectation of success regarding extension of CNP22 by including all or part of the remaining CNP53 sequence, and pegylation of these peptides. This is relevant to SEQ ID Nos. 60 and 145-178.

Thus the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made.

Conclusion

No claim is allowed.

Correspondence

Any inquiry concerning this communication or earlier communications from the examiner should be directed to BRUCE CAMPELL whose telephone number is 571-272-0974. The examiner can normally be reached on Monday - Thursday from 9:00 to 5:00, Central Time. The examiner can also be reached on alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Timothy Speer, can be reached on 313-446-4825.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR.

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Exhibit 21

nature medicine

Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway

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Achondroplasia is the most common genetic form of human dwarfism, for which there is presently no effective therapy. C-type natriuretic peptide (CNP) is a newly identified molecule that regulates endochondral bone growth through GC-B, a subtype of particulate guanylyl cyclase. Here we show that targeted overexpression of CNP in chondrocytes counteracts dwarfism in a mouse model of achondroplasia with activated fibroblast growth factor receptor 3 (FGFR-3) in the cartilage. CNP prevented the shortening of achondroplastic bones by correcting the decreased extracellular matrix synthesis in the growth plate through inhibition of the MAPK pathway of FGF signaling. CNP had no effect on the STAT-1 pathway of FGF signaling that mediates the decreased proliferation and the delayed differentiation of achondroplastic chondrocytes. These results demonstrate that activation of the CNP–GC-B system in endochondral bone formation constitutes a new therapeutic strategy for human achondroplasia.

Achondroplasia is the most common genetic form of human dwarfism, with a prevalence at birth of about 1/26,000 (ref. 1). Effective therapy for this condition has not as yet been established. Recent advances in molecular genetics have shown that constitutively active mutations in the gene encoding FGFR-3 are responsible for human achondroplasia[2–4]. Constitutive activation of FGFR-3 stimulates various intracellular signaling pathways, including the MAPK and STAT-1 pathways[5]. It has been reported that the STAT-1 pathway of FGFR-3 signaling inhibits endochondral bone growth[6,7]; the contribution of the MAPK pathway, another major component of FGFR-3 signaling, remains unclear.

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and CNP[8]. They exert biological actions by accumulation of intracellular cGMP mediated by two subtypes of particulate guanylyl cyclase: guanylyl cyclase-A (GC-A) for ANP and BNP and guanylyl cyclase-B (GC-B) for CNP[9,10]. Although the natriuretic peptide system has mainly been implicated in regulating the cardiovascular system, we have shown that the CNP–GC-B system is an important regulator of endochondral bone growth[11–14]. Activation of the CNP–GC-B system in transgenic mice with elevated plasma concentrations of BNP[11] or in mice depleted of the clearance receptor for natriuretic peptides[15,16] results in very similar skeletal overgrowth. By contrast, inactivation of the CNP–GC-B pathway in mice depleted of CNP[13] or cGMP-dependent protein kinase II[14], a downstream mediator of the CNP–GC-B system, results in dwarfism caused by defects in endochondral ossification.

Growth plates stimulated by the CNP–GC-B system have widened proliferative and hypertrophic chondrocyte layers, much like those in FGFR-3-depleted bones[12,17,18]. CNP depletion, by contrast, leads to impaired bone growth resembling that of achondroplastic bones, with a similar histological picture of decreased width in both the proliferative and hypertrophic chondrocyte layers of the growth plate[13,19]. These observations raise the possibility that the CNP–GC-B system in endochondral ossification reverses the inhibitory effect of the FGFs–FGFR-3 system in skeletogenesis. As for the interaction of these systems, marked elevation of cGMP induced by CNP has been reported to block activation of the MAPK cascade induced by FGFs in fibroblasts[20] and mesangeal cells[21].

Here, we developed transgenic mice (*Nppc* mice) with targeted overexpression of CNP in growth-plate cartilage using a transgene containing *Col2a1*, a cartilage-specific promoter[22], and *Nppc*, the gene encoding CNP. Using a mouse model of achondroplasia with activated FGFR-3 in cartilage (*Fgfr3*^{ach} mice)[19], we generated and analyzed doubly transgenic *Nppc Fgfr3*^{ach} mice that overexpress CNP in achondroplastic growth-plate chondrocytes to assess the growth-promoting effect of the CNP–GC-B system on achondroplastic bones and the molecular mechanisms by which this occurs.

RESULTS

Generation of *Nppc* mice

We obtained two strains of *Nppc*-transgenic founders of similar phenotype, and one of them, carrying ten copies of the transgene (*Col2a1-*

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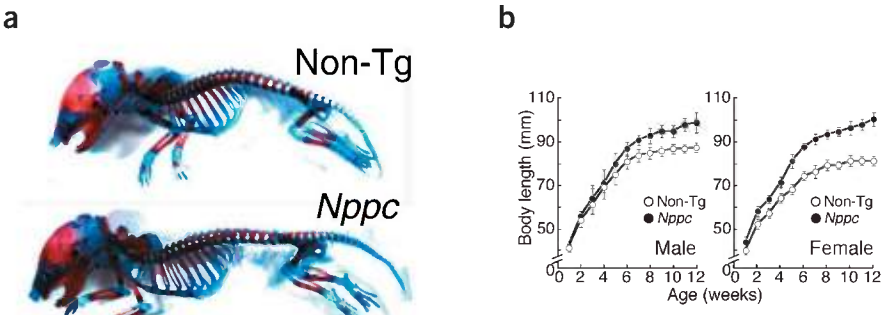
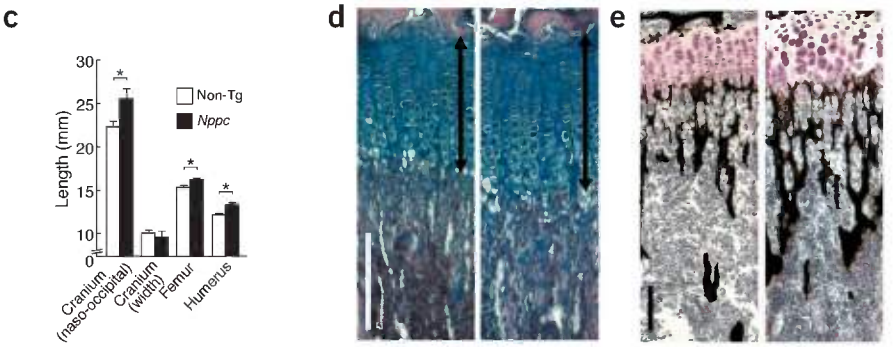


Figure 1 Generation of *Nppc* mice. (**a**) Whole skeletons of nontransgenic (Non-Tg) and *Nppc* mice at birth. (**b**) Growth curves of male (left) and female (right) nontransgenic and *Nppc* mice. (**c**) Bone lengths of 3-month-old female mice. *, $P < 0.05$, $n = 4–5$. (**d**,**e**) Histological analysis of the tibial growth plate of 2-week-old mice. Nontransgenic (left) and *Nppc* (right) in each panel. Alcian blue and H&E staining (**d**) and von Kossa staining (**e**). Arrows indicate growth-plate cartilage in **d**. Scale bar, 200 μm (**d**) or 100 μm (**c**).



Nppc), was used for further analysis. Targeted overexpression of CNP in the cartilage of these *Nppc* mice was confirmed by RT-PCR (see **Supplementary Fig. 1** online). The production of cGMP, the second messenger of CNP, was about nine times higher in the cartilage of *Nppc* mice than in that of their nontransgenic littermates (18.5 ± 1.5 versus 2.1 ± 0.7 fmol/mg protein, $P < 0.01$).

At birth, the whole skeleton of *Nppc* mice showed a substantial longitudinal overgrowth of bones, including long bones of limbs, vertebrae and skull (**Fig. 1a**). No delay in ossification was observed in the periphery of limbs at this stage. Longitudinal overgrowth of *Nppc* mice became prominent in adulthood. The growth curve shows that the naso-anal lengths of 10-week-old male and female *Nppc* mice were 10% and 19% larger, respectively, than those of their nontransgenic littermates (**Fig. 1b**). The growth-promoting effect in *Nppc* mice was more prominant in females than in males, so we used female mice in later experiments. The length of bones formed through endochondral ossification (cranium, femur and humerus) were significantly larger in *Nppc* mice than in nontransgenic mice, whereas the width of the cranium, which is determined by membranous ossification, did not differ between the two genotypes (**Fig. 1c**).

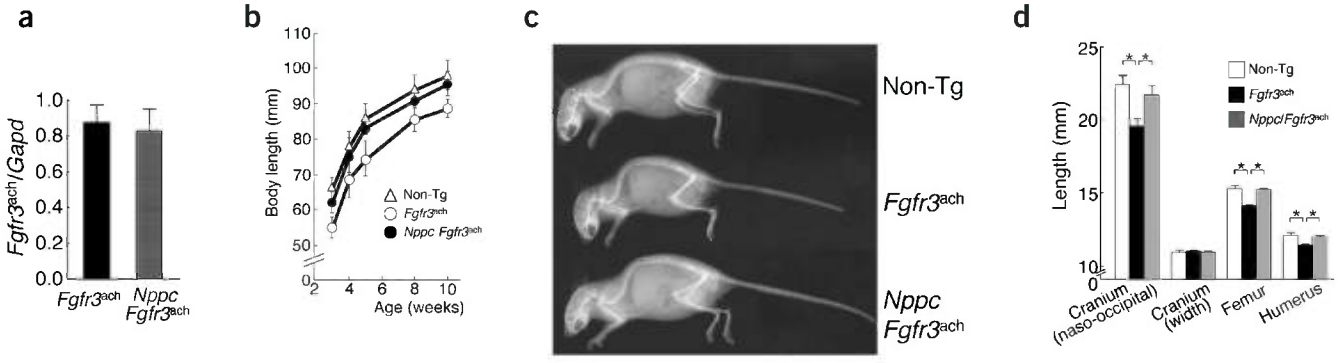
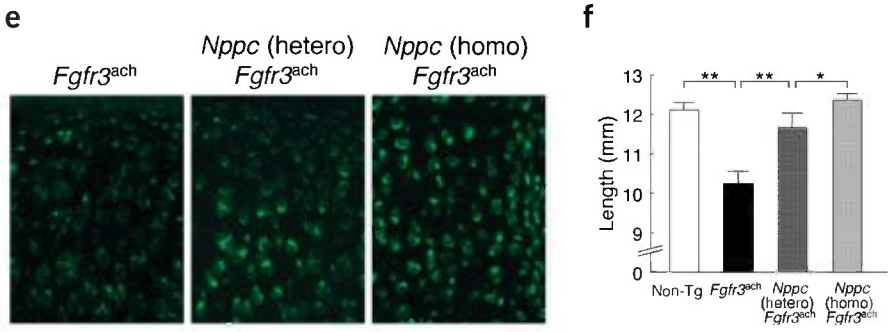


Figure 2 Skeletal rescue of *Fgfr3*^{ach} mice by overexpression of CNP in cartilage. (**a**) Expression of *Fgfr3*^{ach} transgene estimated by quantitative RT-PCR in cartilage of *Fgfr3*^{ach} and *Nppc Fgfr3*^{ach} mice. (**b**) Growth curve of female nontransgenic, *Fgfr3*^{ach} and *Nppc Fgfr3*^{ach} mice. (**c**) Whole skeletons. (**d**) Bone lengths. *, $P < 0.05$, $n = 4–5$. (**e**,**f**) Dose-response effect of CNP on achondroplastic bones. Immunostaining of CNP in neonatal rib cartilage (**e**) and ulnar lengths in 2-month-old female nontransgenic, *Fgfr3*^{ach}, *Nppc*-heterozygous (hetero) *Fgfr3*^{ach} and *Nppc*-homozygous (homo) *Fgfr3*^{ach} mice (**f**). * and ** in **f**, $P < 0.05$ and $P < 0.01$, respectively, $n = 3–5$.



Histological examination showed that *Nppc* mice had wider growth plates than their nontransgenic littermates (**Fig. 1d**). In the tibiae of 2-week-old mice, the proliferative and hypertrophic chondrocyte layers express type II and X collagens, respectively, as shown by *in situ* hybridization (see **Supplementary Fig. 2** online). These layers were significantly wider in *Nppc* mice than in nontransgenic mice (proliferative: 234 ± 12 versus 207 ± 14 μm, $P < 0.05$; hypertrophic: 215 ± 3 versus 193 ± 16 μm, $P < 0.05$). The intensities of *Col2a1* and *Col10a1* mRNA expression did not differ between the two genotypes. Each hypertrophic chondrocyte was enlarged in *Nppc* mice as compared with their nontransgenic littermates (**Fig. 1d** and **Supplementary Fig. 2** online). Von Kossa staining of the proximal tibiae showed that subchondral trabeculae were more abundant and longer in *Nppc* mice than in nontransgenic mice (**Fig. 1e**). In addition, assessment by peripheral quantitative computed tomography (pQCT) showed that the bone mineral density of trabecular bones in the proximal tibiae of 12-week-old *Nppc* mice is significantly larger than that of their nontransgenic littermates (202.2 ± 6.4 versus 188.3 ± 5.3 g/mm^3, $P < 0.05$).

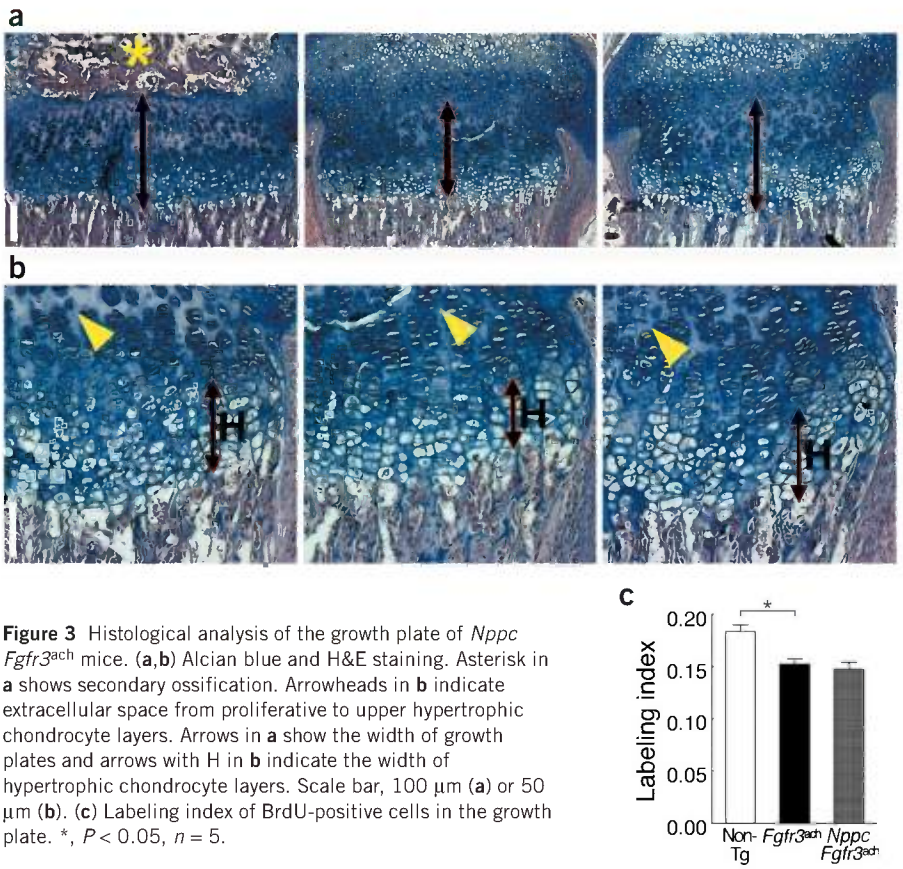


Figure 3 Histological analysis of the growth plate of *Nppc Fgfr3*^{ach} mice. (**a**,**b**) Alcian blue and H&E staining. Asterisk in **a** shows secondary ossification. Arrowheads in **b** indicate extracellular space from proliferative to upper hypertrophic chondrocyte layers. Arrows in **a** show the width of growth plates and arrows with H in **b** indicate the width of hypertrophic chondrocyte layers. Scale bar, 100 μm (**a**) or 50 μm (**b**). (**c**) Labeling index of BrdU-positive cells in the growth plate. *, $P < 0.05$, $n = 5$.

Gross phenotypes of *Nppc Fgfr3*^{ach} mice

To study the effects of CNP on dwarfism in *Fgfr3*^{ach} mice, we created *Fgfr3*^{ach} mice with targeted overexpression of CNP in cartilage by cross-mating *Fgfr3*^{ach} mice with *Nppc* mice. We confirmed the expression of the *Col2a1-Nppc* transgene in the cartilage of the resultant double transgenic (*Nppc Fgfr3*^{ach}) mice (data not shown). We also confirmed, by quantitative RT-PCR analysis, that the amount of *Fgfr3*^{ach} expression in the cartilage of *Nppc Fgfr3*^{ach} mice is no different from that in the cartilage of *Fgfr3*^{ach} mice, indicating that overexpression of CNP does not alter the expression of the *Fgfr3*^{ach} transgene (**Fig. 2a**).

The growth curve and soft x-ray analysis of *Nppc Fgfr3*^{ach} mice showed that the shortening of *Fgfr3*^{ach} mice is rescued by overexpression of CNP in the growth-plate cartilage (**Fig. 2b**,**c**). At the age of 10 weeks, the naso-anal length of *Nppc Fgfr3*^{ach} mice was 94.7 ± 4.0 mm, 8% larger than that of *Fgfr3*^{ach} mice (87.7 ± 2.6 mm) and comparable to that of their nontransgenic littermates (97.0 ± 4.2 mm) (**Fig. 2b**). The shortening of bones formed through endochondral ossification in 3-month-old female *Fgfr3*^{ach} mice, measured on the soft x-ray film, was rescued in *Nppc Fgfr3*^{ach} mice (**Fig. 2d**). The width of the cranium did not differ among the three genotypes (**Fig. 2d**).

To investigate the dose-response effect of CNP on the growth of achondroplastic bones, we generated *Fgfr3*^{ach} mice homozygous for the *Col2a1-Nppc* transgene. The relative abundance of CNP protein in the cartilage of neonatal mice, as estimated by immunohistochemical staining, was *Nppc*-homozygous *Fgfr3*^{ach} > *Nppc*-heterozygous *Fgfr3*^{ach} > *Fgfr3*^{ach} (**Fig. 2e**). The immunostaining intensities measured using NIH Image software[23] were 145% and 127% as high in the cartilage of *Nppc*-homozygous *Fgfr3*^{ach} and *Nppc*-heterozygous *Fgfr3*^{ach} mice, respectively, as in that of *Fgfr3*^{ach} mice. The dwarfing bones of *Fgfr3*^{ach} mice were elongated dose-dependently in *Nppc*-homozygous *Fgfr3*^{ach} and *Nppc*-heterozygous *Fgfr3*^{ach} mice (**Fig. 2f**).

Histological analysis of *Nppc Fgfr3*^{ach} mice

Microscopic analysis of tibial growth plates from 2-week-old mice showed that the decreased width of the growth plate of *Fgfr3*^{ach} mice (169 ± 15 μm) is rescued in *Nppc Fgfr3*^{ach} mice (229 ± 21 μm) and is comparable to that of their nontransgenic littermates (220 ± 15 μm) (**Fig. 3a**). Higher magnification showed that the decreased extracellular space between the proliferative and the upper hypertrophic chondrocyte layers of *Fgfr3*^{ach} mice was restored in *Nppc Fgfr3*^{ach} mice and could be as large as in nontransgenic littermates (**Fig. 3b**). *In situ* hybridization analysis showed that the width of the hypertrophic chondrocyte layer expressing *Col10a1* mRNA was also restored in *Nppc Fgfr3*^{ach} mice, although there was no appreciable difference in expression intensity between genotypes (see **Supplementary Fig. 3** online). There was also no difference in the intensity of *Col2a1* mRNA expression (data not shown). In the proximal tibia of 2-week-old mice, the secondary ossification center was well organized in nontransgenic mice, whereas it was not yet formed in either their *Fgfr3*^{ach} or *Nppc Fgfr3*^{ach} littermates, indicating that overexpression of CNP does not affect the delayed differentiation of chondrocytes in achondroplastic growth plates (**Fig. 3a**). The secondary ossification centers in the tibiae of both *Fgfr3*^{ach} and *Nppc Fgfr3*^{ach} mice were formed about 3 d later than in nontransgenic tibia. The number of bromodeoxyuridine (BrdU)-positive chondrocytes was significantly lower in the growth plates of *Fgfr3*^{ach} mice, as previously reported[19], whereas there was no difference in the number of BrdU-positive chondrocytes in the growth plates of *Fgfr3*^{ach} and *Nppc Fgfr3*^{ach} mice (**Fig. 3c** and **Supplementary Fig. 4** online). This indicates that overexpression of CNP does not change the decreased proliferation of chondrocytes in *Fgfr3*^{ach} mice.

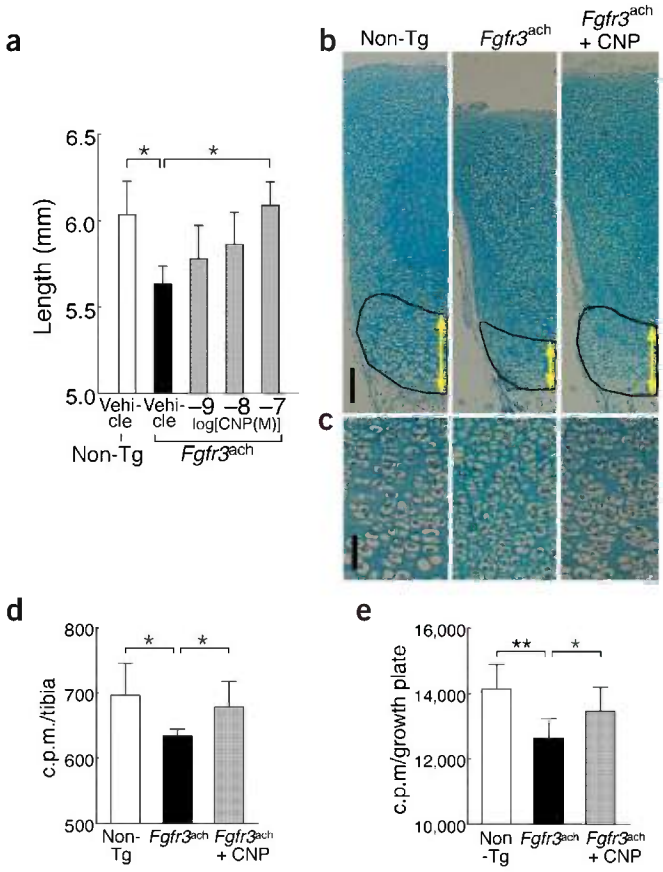


Figure 4 Effects of CNP on cultured tibiae from *Fgfr3*^{ach} mice. (**a**) Tibial lengths at the end of culture period. *, $P < 0.05$, $n = 5–6$. (**b**) Histological pictures of tibiae of nontransgenic mice, tibiae of *Fgfr3*^{ach} mice and CNP-treated tibiae of *Fgfr3*^{ach} mice. Arrows indicate the width of hypertrophic chondrocyte layers demarcated in the figures. (**c**) Highlight of the prehypertrophic chondrocyte layers from **b**. Scale bar, 200 μm (**b**) or 50 μm (**c**).(**d**,**e**) [^{35}S]sulfate (**d**) and [^{3}H]proline (**e**) incorporation into cartilage into tibiae of nontransgenic mice, *Fgfr3*^{ach} mice and *Fgfr3*^{ach} mice with CNP treatment. * and **, $P < 0.05$ and $P < 0.01$, respectively, $n = 5–6$ (**d**) and 9 (**e**).

Effects of CNP on cultured tibiae from *Fgfr3*^{ach} mice

To confirm the direct effect of CNP on bone growth in *Fgfr3*^{ach} mice, we carried out organ culture experiments using tibiae from fetal *Fgfr3*^{ach} mice. The fetal tibiae of *Fgfr3*^{ach} mice at 16.5 days post coitus (dpc) were shorter than those of their nontransgenic littermates (data not shown). CNP (10^{-9}–10^{-7} M) caused elongation of explanted tibiae from fetal *Fgfr3*^{ach} mice in a dose-dependent manner; those treated with 10^{-7} M CNP reached lengths as great as those of vehicle-treated tibiae from nontransgenic mice by the end of the culture period (**Fig. 4a**). Microscopic analysis showed that the width of the hypertrophic chondrocyte layer in cultured tibiae from *Fgfr3*^{ach} mice, which is lower than that in tibiae of nontransgenic mice, was restored by treatment with 10^{-7} M CNP (**Fig. 4b**). With higher magnification, we observed that the decreased extracellular space between the proliferative and the prehypertrophic chondrocyte layers of the tibiae of *Fgfr3*^{ach} mice was normalized by 10^{-7} M CNP (**Fig. 4c**). In fact, although the basal synthesis of glycosaminoglycan, measured by [^{35}S]sulfate incorporation, was much lower in tibiae from *Fgfr3*^{ach} mice than in those from nontransgenic mice, 10^{-7} M CNP increased glycosaminoglycan synthesis in tibiae from *Fgfr3*^{ach} mice to a level as high as in vehicle-treated tibiae from nontransgenic mice (**Fig. 4d**). The same dose of CNP also increased the synthesis of cartilage collagen, as estimated from the uptake of [^{3}H]proline into growth plates from *Fgfr3*^{ach} mice, to a level comparable to that in the growth plates from nontransgenic mice (**Fig. 4e**).

Effects of CNP on signaling pathways of FGF

We next investigated the effects of CNP on FGF signaling pathways in growth-plate chondrocytes using cultured tibiae from fetal mice. The treatment of tibial explants with CNP (10^{-6} and 10^{-7} M) or its second messenger, cGMP (10^{-5} M), before addition of FGF-2 (2 ng/ml) markedly decreased the FGF-2-induced phosphorylation of ERK1/2 in a dose-dependent fashion without decreasing the amount of ERK protein (**Fig. 5a**). In contrast, the same doses of either CNP or cGMP did not alter the FGF-2-induced phosphorylation of STAT-1 (**Fig. 5b**). Essentially identical results were obtained using the chondrogenic cell line ATDC5 (ref. 24) (data not shown). To further elucidate the effect of CNP on phosphorylation of ERK1/2 *in vivo*, we examined the amounts of phosphorylated ERK1/2 in the cartilage of nontransgenic, *Fgfr3*^{ach}, *Nppc* and *Nppc Fgfr3*^{ach} mice. The abundance of phosphorylated ERK1/2 was greater in the cartilage of *Fgfr3*^{ach} mice, and smaller in the cartilage of *Nppc* mice, than in the cartilage of nontransgenic mice. *Nppc Fgfr3*^{ach} mice, in which the shortening of achondroplastic bones was rescued, showed substantially less elevation of ERK1/2 phosphorylation than was seen in the cartilage of *Fgfr3*^{ach} mice (**Fig. 5c**). PD98059, an inhibitor of the MAPK cascade[25], increased the total length and width of the growth-plate cartilage of tibiae from both fetal *Fgfr3*^{ach} and nontransgenic mice in organ culture (**Fig. 5d**). PD98059 increased the length of tibiae from *Fgfr3*^{ach} mice more than that of tibiae from nontransgenic mice, in parallel with the level of cartilage matrix synthesis estimated from [^{35}S]sulfate and [^{3}H]proline incorporation (**Fig. 5e–g**). There was no difference in the proliferation of chondrocytes in tibiae, measured by BrdU uptake, between PD98059- and vehicle-treated groups of either genotype (labeling indexes: nontransgenic, 0.252 ± 0.029 versus 0.248 ± 0.025, respectively; *Fgfr3*^{ach}, 0.221 ± 0.025 versus 0.225 ± 0.027, respectively). We obtained the same results using U0126, another MEK inhibitor (data not shown).

DISCUSSION

This study demonstrates that targeted overexpression of CNP in cartilage counteracts dwarfism in *Fgfr3*^{ach} mice, a model of human achondroplasia. We previously showed, using transgenic and knockout mice, that the CNP–GC-B system is important in endochondral ossification[11–13]. The *Nppc* mice developed in this study showed skeletal overgrowth with widened growth plates, a phenotype resembling that of FGFR-3-depleted mice[17,18] and opposite to those of *Fgfr3*^{ach} mice[19] and *Nppc* knockout mice[13]. This indicates that the CNP–GC-B system may counteract the FGFs–FGFR-3 system in endochondral ossification. Here, we show that activation of the CNP–GC-B system reverses the FGFR-3-mediated inhibition of the endochondral ossification in achondroplasia. Activation of the CNP–GC-B system in chondrocytes of the growth plate therefore represents a potential new therapeutic strategy for human achondroplasia caused by a constitutively active mutation in *FGFR3*.

Constitutive activation of FGFR-3 has been reported to inhibit the growth of long bones made through endochondral ossification by decreasing the proliferation and delaying the differentiation of

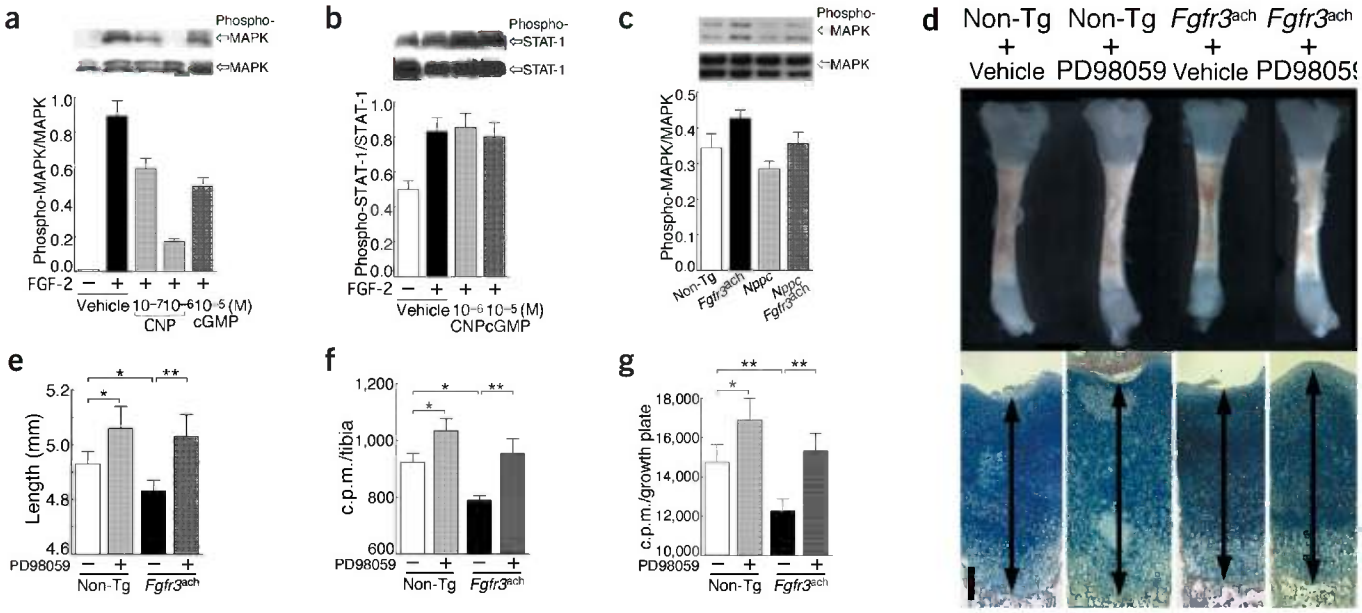


Figure 5 Intracellular signaling analysis. (**a–c**) Western blotting for phosphorylated MAPK (**a**) and STAT-1 (**b**) in explanted tibiae, and phosphorylated MAPK in growth-plate cartilage from neonatal mice (**c**). (**d–g**) Effects of PD98059 on cultured *Fgfr3*^{ach} tibiae. Gross appearance (upper) and histological picture of the growth plate (**d**), tibial length (**e**), [^{35}S]sulfate incorporation (**f**) and [^{3}H]proline uptake (**g**) in each group. Arrows in **d** indicate the width of the growth-plate cartilage. * and ** in **e–g**, $P < 0.05$ and $P < 0.01$, respectively, $n = 4–5$. Scale bar, 200 μm (**d**).

growth-plate chondrocytes via the STAT-1 pathway[6,19]. In the present study, however, we determined that CNP does not affect the amount of phosphorylated STAT-1 induced by FGF-2 either in explanted tibiae or in the chondrogenic cell line ATDC5. In addition, we showed that overexpressed CNP does not restore either the decreased BrdU uptake or the delayed formation of secondary ossification centers in the growth-plate cartilage of *Fgfr3*^{ach} mice (**Fig. 3**). Thus, CNP does not correct either the decreased proliferation or the delayed differentiation of achondroplastic growth-plate chondrocytes *in vivo*. These results show that CNP does not correct the STAT-1-mediated bone growth inhibition of activated FGFR-3. On the other hand, we show that constitutive activation of FGFR-3 inhibits longitudinal bone growth by decreasing the extracellular matrix synthesis through the MAPK pathway, indicating that MEK inhibitors (i) increase the extracellular matrix synthesis of explanted tibiae, as estimated by incorporation of [^{35}S]sulfate and [^{3}H]proline, more strongly in tibiae of *Fgfr3*^{ach} mice than in those of nontransgenic mice; (ii) do not alter the proliferation of chondrocytes, as estimated by BrdU uptake, in the tibiae of both *Fgfr3*^{ach} and nontransgenic mice; and (iii) elongate cultured tibiae from *Fgfr3*^{ach} mice more strongly than those from nontransgenic mice (**Fig. 5e–g**). In addition, we show that CNP markedly decreases the FGF-2-induced phosphorylation of ERK1/2 both in explanted tibiae and in ATDC5 cells, confirming that CNP interferes with activation of the MAPK pathway downstream of FGFR-3 signaling in the growth-plate cartilage, as we and other groups have previously reported in mesangeal cells[21] and fibroblasts[20]. We have further confirmed this finding using *in vivo* animal models, including *Nppc Fgfr3*^{ach} mice. Accordingly, we conclude that the mechanism by which CNP counteracts the dwarfism of achondroplastic bones in *Fgfr3*^{ach} mice is by restoring the MAPK-mediated inhibition of extracellular matrix production in the growth-plate cartilage of achondroplastic bones (**Fig. 6**). The role of the MAPK pathway in FGF signaling in chondrocytes has not been extensively studied so far, and the results of previous studies using cell culture were complicated and contradictory[26,27]. In the present study, we used both *ex vivo* organ culture and cell culture to elucidate the role of the MAPK pathway in endochondral bone elongation directly and in detail, and the two sets of results corresponded well. De Crombrugghe *et al.* have recently reported that constitutive activation of MEK1 in chondrocytes induces dwarfism that resembles achondroplasia (S. Murakami, S. McKinney, D. Givol, and B. de Crombrugghe, personal communication). These results support our hypothesis that the CNP–GC-B system rescues achondroplastic dwarfism by inhibiting the MAPK pathway of FGFR-3 signaling in the regulation of bone growth.

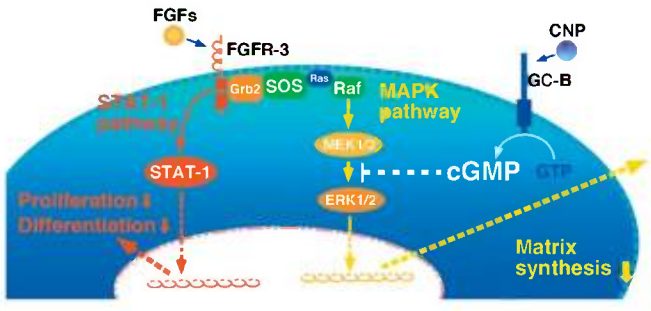


Figure 6 Schematic representation of the mechanism by which CNP compensates for FGFR-3-mediated shortening of bones. FGFR-3 inhibits endochondral bone growth by inhibiting proliferation and differentiation of growth-plate chondrocytes through the STAT-1 pathway and by decreasing extracellular matrix synthesis through the MAPK pathway. cGMP, the second messenger of CNP–GC-B, inhibits the MAPK pathway of FGFR-3 signaling, restores the decreased synthesis of the extracellular matrix and partially counteracts the dwarfism of achondroplastic bones.

Bone morphogenetic protein-2 (BMP-2) and BMP-4 have recently been reported to counteract the growth-inhibitory effect of FGFR-3 on explanted bones from the same achondroplastic model mice by restoring the proliferation of achondroplastic growth-plate chondrocytes[28]. Although the nature of intracellular interactions between FGF and BMP signaling in chondrocytes remains unclear, CNP and BMP might complementarily reverse the inhibitory effect of FGF on bone growth suggesting the possibility of a new combined therapy for achondroplasia.

Current therapies for achondroplasia include distraction osteogenesis[29,30], an orthopedic procedure, and administration of growth hormone[31]. Although distraction osteogenesis gives better results, it limits the quality of a patient's life. Growth hormone has only minimal effect on achondroplasia, has been reported to aggravate body disproportion in some case[32] and is very costly. According to our unpublished results, the maximal growth-promoting effect of CNP (10^{-7} M) on cultured fetal bones is much stronger than that of growth hormone (100 ng/ml), IGF-I (100 ng/ml)[33] or other skeletal growth regulators, including BMP-2 (100 ng/ml), BMP-4 (100 ng/ml), TGF-β_2 (100 ng/ml)[34] and PTH (100 ng/ml)[35], under identical experimental conditions. Activation of the CNP–GC-B system in chondrocytes may thus be a prospective means to treat achondroplasia. Activation of GC-B resulting from elevated plasma concentrations of BNP[11] or CNP (unpublished data) in transgenic mice using a promoter of serum amyloid P component causes skeletal overgrowth. This indicates that specific activators of the CNP–GC-B system could be delivered via the general circulation as therapy for achondroplasia. Considering the minimal hypotensive effect of CNP compared with the other natriuretic peptides ANP and BNP, systemic administration of CNP could be of practical use as a means to activate the CNP–GC-B system in the growth-plate chondrocytes. In addition, overexpression of CNP in cartilage increased bone mineral density, as estimated by pQCT, in the trabecular bone adjacent to the growth-plate cartilage, suggesting that the activation of the CNP–GC-B system in endochondral cartilage template formation positively affects the properties of bone produced from this template. This effect could be beneficial to patients with achondroplasia treated with CNP.

We observed a gender difference in the promoting effect of CNP on bone growth in the present study, but the reasons for this observation are unclear at present. Further studies are ongoing in our laboratory.

In conclusion, the present study demonstrates a potential new therapeutic strategy for human achondroplasia involving activation of the CNP–GC-B system in the chondrocytes of the growth plate.

METHODS

Generation and identification of *Nppc* mice. A 489-base-pair mouse *Nppc* cDNA[36] was inserted into a 6.5-kilobase segment of the *Col2a1* promoter region[22], at a site within the first intron. The assembled transgene (*Col2a1-Nppc*) was microinjected into fertilized C57BL/6 oocytes and the resultant transgenic mice were identified by Southern blotting. *Sac* I digestion of mouse genomic DNA containing *Col2a1-Nppc* yielded additional hybridizing DNA species of ~2.1 kilobases, whereas the intrinsic *Nppc* gene was recognized as DNA species of ~3.0 kilobases[37]. We assessed the transgene copy number on the basis of the density of the band compared with that of the intrinsic *Nppc* band as determined using densitometry.

Generation of the doubly transgenic mice of *Col2a1-Nppc* and *Fgfr3*^{ach}. Generation of transgenic mice that express an activated FGFR-3 in the growth plate (*Fgfr3*^{ach} mice) using *Col2a1* promoter and enhancer sequences was reported previously[19]. In all experiments using this achondroplastic model, we used transgenic mice carrying the heterozygous *Fgfr3*^{ach} transgene. To generate doubly transgenic mice for *Col2a1-Nppc* and *Fgfr3*^{ach}, *Nppc* mice were mated with *Fgfr3*^{ach} mice. The resultant *Nppc Fgfr3*^{ach} mice and *Nppc* mice were then mated to generate *Fgfr3*^{ach} mice homozygous for *Col2a1-Nppc*. All of the experimental procedures were approved by the Kyoto University Graduate School of Medicine committee on animal research.

Detection of mRNA expression of transgenes. To detect expression of *Col2a1-Nppc*, we carried out RT-PCR using as the forward primer a sequence located in the exon I of the *Col 2a1* gene and as the reverse primer a sequence from *Nppc* cDNA. The resultant PCR product of 450 base pairs was then recognizable as a transcript of the transgene from which the ~3-kilobase intron I of *Col2a1* had been spliced out. Expression of the *Fgfr3*^{ach} transgene was estimated by a quantitative RT-PCR method based on kinetic analysis[38,39], using primers for *Fgfr3*^{ach} and *Gapd*, as previously reported[19,39].

Skeletal morphology and pQCT analysis. For visualization of the whole skeleton, cleared skeletons of newborn mice were stained with Alizarin red S and Alcian blue[11]. To observe skeletons in adulthood, 3-month-old female mice were subjected to soft x-ray analysis[11], and the lengths of bones were measured on the soft x-ray film. To evaluate the bone mineral density of trabecular bones in the proximal tibiae, tibiae were subjected to pQCT analysis, using an XCT Research SA instrument (Stratec Medizintechnik), as previously reported[40].

Histological analysis. For light microscopy, sections were cut from paraffin-embedded specimens and stained with Alcian blue and hematoxylin-eosin (H&E). For BrdU staining, 2-week-old mice were injected intraperitoneally with BrdU (100 μg per gram body weight) and were killed 1 h later[19]. The labeling index in the proliferative chondrocyte layer was analyzed using National Institutes of Health (NIH) freeware. To evaluate the mineralized stage, von Kossa staining was done on undecalcified sections[13]. Immunohistochemical staining for CNP was carried out on frozen sections of neonatal mouse ribs using the ImmunoStaining Detection Kit (Peninsula). Intensity of immunostaining was analyzed using NIH freeware as previously described[23]. *In situ* hybridization analysis was carried out on 2-week-old mice tibiae using digoxigenin-labeled antisense and sense riboprobes obtained from rat *Col2a1* and *Col10a1* cDNA fragments[41].

Organ culture. Tibiae from *Fgfr3*^{ach} fetuses or their nontransgenic littermates were dissected out at 16.5 dpc and cultured for 4 d by the suspension culture technique[12] with the indicated doses of CNP or with biochemical inhibitors of MEK, PD98059 (50 μM, Cell Signaling) or U0126 (10 μM, Promega). Glycosaminoglycan synthesis in the cultured tibiae was assessed by measuring [^{35}S]sulfate incorporation as previously described[42], using 5 μCi/ml [^{35}S]sulfate in the medium. The synthesis of cartilage collagen was assessed by measuring uptake of [^{3}H]proline (20 μCi/ml in the medium) into cartilage collagen isolated from papain digests of the proximal tibial growth-plate cartilage[43]. The proliferation of chondrocytes in explanted tibiae was assessed by measuring BrdU incorporation as described before[12]. cGMP production was measured by radioimmunoassay[44] using cultured tibiae from *Nppc* or nontransgenic fetuses of 16.5 dpc at the end of the 4-d culture period.

Intracellular signaling analysis. Tibial explants from fetal mice of 16.5 dpc were incubated with 10^{-7}–10^{-6} M CNP or 10^{-5} M cGMP for 1 h, and then FGF-2 (2 ng/ml) was added for 5 min. ATDC5 cells on day 11 after confluency were incubated with the same doses of CNP or cGMP for 1 h, and then FGF-2 (1 ng/ml) was added for 3 min. Explanted tibiae, ATDC5 cells and tibial growth plates freshly dissected out from neonatal nontransgenic, *Fgfr3*^{ach}, *Nppc* and *Nppc Fgfr3*^{ach} mice were homogenized and lysed, and then western blotting was carried out using antibodies to phosphorylated ERK1/2 (Promega), ERK1/2 (BD Bioscience), phosphorylated STAT-1 (Cell Signaling) or STAT-1 (Santa Cruz Biotechnology).

Statistics. Data were expressed as mean ± s.d. and the statistical significance of differences in mean values was assessed by Student's *t*-test or analysis of variance (ANOVA) with Fisher's protected least-significant-difference test, as appropriate. Differences among means were considered significant at values of $P < 0.01$ or $P < 0.05$.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Exhibit 22

Docket No.: 30610/44556C1-R2
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Reissue Application of:
Daniel J. Wendt et al.

Application No.: Not Yet Assigned	Confirmation No.: Not Yet Assigned
Filed: Concurrently Herewith	Art Unit: Not Yet Assigned
For: Variants of C-Type Natriuretic Peptide	Examiner: Not Yet Assigned

PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115

MS Reissue
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page **2** of this paper.

Amendments to the Claims begin on page **3** of this paper.

Remarks/Arguments begin on page **28** of this paper.

Exhibit 23

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/646,822	07/11/2017	Daniel J. Wendt	30610/44556C1-R2	7727

90849 7590 05/10/2019

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

EXAMINER	
RAILEY, JOHNNY F	

ART UNIT	PAPER NUMBER
3991	

NOTIFICATION DATE	DELIVERY MODE
05/10/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mflores@bmrn.com
mgbdocket@marshallip.com

Office Action Summary	Application No. 15/646,822	Applicant(s) Wendt et al.	
	Examiner JOHNNY F RAILEY II	Art Unit 3991	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 09 October 2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 15-30 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-30 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

For reissue applications filed before September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the law and rules in effect on September 15, 2012. Where specifically designated, these are "pre-AIA" provisions.

For reissue applications filed on or after September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the current provisions.

Priority

This application is a continuation reissue of reissue application 14/604,262, now U.S. Patent RE46,707 E, which is an reissue application for U.S. Patent 8,598,121, filed as application no. 13/466,672 and issued on 3 December 2013, which is a continuation of U.S. Patent Application 12/784,117, now U.S. Patent 8,198,242, issued on 12 June 2012, which claims priority and benefit of U.S. Provisional Application 61/254,563, filed on 23 October 2009, and U.S. Provisional Application 61/180,112, filed on 20 May 2009.

Status

The amendments to the specification and claims filed on 9 October 2018 are not in compliance with 37 CFR 1.173. The amendment to the specification as presented at page 2 of the response contains crossed-through words. The claims 15, 18, 21-23, and 27 contain crossed-through and double-bracketed material as follows: [[145]] 191. New claims 15-30 should remain underlined. All amendments in reissue applications are made relative to the patent. See MPEP 1453.

Claims

Pending claims are 15-30. Original patent claims 1-14 have been canceled. The independent claims are 15, 18, 21-23, and 27 are amended and summarized as follows:

Claim 15 is drawn to a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

Claim 18 is drawn to a sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

Claim 21 is drawn to a method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

Claim 22 is drawn to a method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

Claim 23 is drawn to a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP is variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

Claim 27 is drawn to a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

Defective Declaration/Oath

The reissue oath/declaration filed with this application is defective because the error which is relied upon to support the reissue application is not an error upon which a reissue can be based. See 37 CFR 1.175 and MPEP § 1414. During the prosecution of application 13/466,672, a restriction requirement was held in which claims drawn to the elected species of SEQ ID NO: 185, a C-type natriuretic peptide of 53 amino acids (CNP53) were elected without traverse. The examiner also indicated that the variants of CNP-53 claimed as SEQ ID NOs: 179, 190, and 180 would be rejoined with the elected invention. See the Notice of Allowability mailed on 30 July 2013 in that application. The examiner also rejoined method claims for the elected composition claims and withdrew from further consideration claims drawn to other non-elected subject matter. The addition of new claims 15-30 is not an error which is relied upon to support the reissue application. See MPEP 1412.01 I:

> Where a restriction (or an election of species) requirement was made in an application and applicant permitted the elected invention to issue as a patent without filing a divisional application on the non-elected invention(s), the non-elected invention(s) cannot be recovered by filing a reissue application. A reissue applicant's failure to timely file a divisional application covering the non-elected invention(s) in response to a restriction (or an election of species) requirement is not considered to be error causing a patent granted on the elected claims to be partially inoperative by reason of claiming less than the applicant had a right to claim. Accordingly, this is not correctable by reissue of the original patent under 35 U.S.C. 251. In re Watkinson, 900 F.2d 230, 14 USPQ2d 1407 (Fed. Cir. 1990); In re Orita, 550 F.2d 1277, 1280, 193 USPQ 145, 148 (CCPA 1977); See also In re Mead, 581 F.2d 251, 198 USPQ 412 (CCPA 1978). In this situation, the reissue claims should be rejected under 35 U.S.C. 251 for lack of defect in the original patent and lack of error in obtaining the original patent.

Claim Rejections - 35 USC § 251

Claims 15-30 are rejected as being based upon a defective reissue declaration under 35 U.S.C. 251 as set forth above. See 37 CFR 1.175.

The nature of the defect(s) in the declaration is set forth in the discussion above in this Office action. This rejection is maintained from the previous office action.

Applicant's Response

Beginning at page 29 of the response, applicants argue that the reissue oath/declaration properly identifies an error upon which a reissue can be based, wherein that reason is that patentee is claiming less than it had a right to claim in the patent. The pending claims are broadened and are directed to embodiments disclosed but not claimed in the 13/466,672 application that issued as U.S. Patent No. 8,598,121. Applicants cite MPEP §1412.03 (I) and (IV) to support the broadening aspect of a reissue.

Applicant notes that claims in the 13/466,672 application (which issued as U.S. Patent 8,598,121) were drawn to a variant of C-type natriuretic peptide (CNP) with the specific SEQ ID NOs: 179; 185; 190; and 180. Pharmaceutical compositions and methods of treating a bone-related disorder or skeletal dysplasia were claimed. A method for recombinant production of a CNP variants and a method of increasing long bone growth were also claimed. Compositions and methods were limited to the species having SEQ ID NOs: 179; 185; 190; and 180. Applicants argue that the independent claims of the current continuation reissue application are directed to a macromolecule capable of releasing a CNP variant, in particular comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage. Sustained release CNP variants having the hydrolysable linkage are also claimed, as well as various methods of using these CNP variants to treat cells, conditions or disorders. See pages 30-31 in the response.

It is argued that pending claims 15-30 represent previously unclaimed subject matter in the form of invention embodiments substantially different from those claimed in U.S. Patent No. 8,598,121. Further, applicant states that subject matter included in this continuation reissue includes subject matter not covered by patent claims, therefore the claims at reissue can be said to enlarge the scope of the patent claims. The reissue claims are held to be not substantially identical to those of the nonelected groups identified in the restriction requirement and represent overlooked aspects of the invention. Applicant distinguishes the instant case from the decision *In re Orita*, 550 F.2d 1277, 193 USPQ145 (CCPA 1977) by arguing that the claims at reissue in *Orita* were substantially identical to the originally non-elected claims. Applicant states: "By contrast, as noted above, the pending continuation reissue claims 15-30 cover subject matter that was disclosed but had never been presented in the original application and that is substantially different from the non-elected claims." Applicant argues that the independent claims of the instant continuation reissue comprise CNP variants with a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage. This is previously unclaimed subject

matter that is substantially different from those originally claimed in the '672 application. Citing *In re Youman*, 679 F.3d 1335, 102 USPQ2d 1862 (Fed. Cir. 2012), applicant argues that the recapture rule applies when surrendered subject matter is being reclaimed, overlooked aspects by definition were never claimed and thus never surrendered. See pages 32-33 in the response.

Applicant submits that the current reissue claims should be evaluated in view of the findings of *In re Doyle* 293 F.3d 1355, 63 USPQ2d 1161 (Fed. Cir. 2002). With respect to *Doyle*, the MPEP states that "the court permitted the patentee to file a reissue application to present a so-called linking claim, a claim broad enough to read on or link the invention elected (and patented) together with the invention not elected." Further, MPEP §1412.01(II) states that "[c]laims to separate inventions/embodiments/species that were disclosed but never covered by the claims in the original application prosecution are claims to overlooked aspects." Applicant argues:

> As in *Doyle*, the instant continuation reissue claims are neither identical to nor substantially similar to the non-elected claims, most notably with respect to the composition claims directed to CNP variants, are species that fall within the new genus claims, directed to, for example, macromolecules capable of releasing a CNP variants, sustained release CNP variant formulations, methods of stimulating cGMP production in a cell, and methods of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3). As such, the continuation reissue claims are substantially broader than the claims of the nonelected groups with species elections. In contrast to *Orita*, and similar to *Doyle*, Applicants never asserted the continuation reissue claims or anything similar to them in the original application, and also never agreed to prosecute the reissue claims in a divisional application.

See pages 34-35 in the response. Finally, applicant notes on page 36: "*Orita* extends only to claims that are identical to or of substantially similar scope to those of the non-elected group."

Examiner's Comments

A review of the content of the claims and the restriction requirement as presented in application 13/366,672 is in order:

Claims 1-4, 7, 9, 10, 16, 18, 22-24, 30, 31, 35, 39, 41, and 44-46 were subject to a restriction requirement in the paper mailed on 13 February 2013. The restriction requirement set forth the following groups of inventions as independent and distinct:

> Restriction to one of the following inventions is required under 35 U.S.C. 121:
>
> I. Claims 1-4, 30, 45 and 46, drawn to a variant of C-type natriuretic peptide (CNP), classified in class 530, subclass 300.
>
> II. Claim 7, drawn to a method of treating a bone-related disorder or skeletal dysplasia, classified in class 514, subclass 12.4.
>
> III. Claim 9, drawn to a method of treating a vascular smooth muscle disorder, classified in class 514, subclass 12.4.

IV. Claims 10, 16, 18, 22-24, 31,35, drawn to a method for recombinant production of a CNP variant and a host cell used in said method, classified in class 435, subclass 69.1/325.

V. Claims 39 and 41, drawn to a method of treating a CNP-responsive condition or disorder, classified in class 514, subclass 12.4.

VI. Claim 44, drawn to a method of increasing long bone growth, classified in class 514, subclass 12.4.

The subject matter of claims 1-4, 30, 45, and 46, drawn to a variant of C-type natriuretic peptide (CNP), were regarded and the invention of Group I.

Claim 1 requires that the CNP variant be conjugated to a moiety selected from a hydrophobic acid and an amino acid sequence from 1 to 20 amino acids. The CNP variants are identified specifically by the following SEQ ID NOs: 60, 75, 144, 146-180, 183-185, 188-190, 193 and 194.

Claim 30 requires that the CNP variant is produced from a polynucleotide sequence (encoding the CNP variant polypeptide) linked to a second polynucleotide sequence (encoding a cleavable peptide) whereby a fusion protein is produced. The CNP variants are identified specifically by the following SEQ ID NOs: 34, 36, 37, 39, 41, 60-63, 68, 69, 71, 75-77, 79, 81, 87, 144, 179, 180, 183-185, 188-190, 193, 194, and 146-178.

Claim 46 requires that the CNP variant be conjugated to a moiety selected from a hydrophobic acid and an amino acid sequence from 1 to 20 amino acids. The CNP variants are identified specifically by the following SEQ ID NOs: 145, 181, 182, 186, 191, and 192.

In the restriction requirement on page 4, it was noted:

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention.

On page 5, applicant was also required to select a patentably distinct species:

This application contains claims directed to the following patentably distinct species: sequences as disclosed in claims 1, 9, 10, 30, 31, 39, and 46. The species are independent or distinct because each sequence is a distinct separate polypeptide. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species, or a single grouping of patentably indistinct species, for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, no claim is generic.

There is a search and/or examination burden for the patentably distinct species as set forth above because at least the following reason(s) apply:

The species are independent or distinct because each sequence is a distinct separate polypeptide; a search for the recited sequences would not be coextensive.

In the response to the restriction requirement that was filed on 12 April 2013, applicant elected Group I and the species Pro-CNP53 (SEQ ID NO: 185) for examination. Applicant advised that claim 45 should be in Group VI, and therefore the claims that read upon the elected subject matter are claims 1-4, 30, and 46. "Applicants request rejoinder of the non-elected method claims (Groups II-VI) upon notification of allowance of the elected composition claims." There was no traversal of the restriction and election of species requirements.

At allowance mailed on 30 July 2013 held:

Applicants' election, of Group I, claims 1-4, 30, 45 and 46, drawn to a variant of C-type natriuretic peptide (CNP), in the reply filed on 12 April 2013, is acknowledged. In response to a requirement for species election, Applicants elect the species Pro-CNP53 (SEQ ID NO: 185) for continued examination. Because Applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Additionally, Applicants request rejoinder of the non-elected method claims (Groups ll-VI) upon notification of allowance of the elected composition claims. Said Groups will be rejoined and examined upon the identification of allowable subject matter.

Claims 1-4, 7, 9, 10, 16, 18, 22-24, 30, 35, 39, 41 and 44-46 are pending in the instant application. Claims 7, 9,10,16,18, 22-24, 31,35, 39, 41,45 and 46 have been withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Claims 1-4, 30 and 45 are under consideration to the extent they read on the elected invention, SEQ ID NO: 185.

Based upon this review of the file record in the '672 application, applicant was given an opportunity to traverse the restriction and election of species requirements. This was not done. Applicant was free to file any divisional applications on the non-elected subject matter drawn to independent and distinct inventions. This also was not done. The claims in the current reissue are drawn now to that non-elected subject matter, including all of the species of CNP variants listed by SEQ ID NO as was found in the original claims of the '672 application. The methods of treatment in the current reissue claims also are not substantially different from the methods of the non-elected inventions. The same CNP variants as originally presented in the '672 application are used to the same effects as the current claims: all involve using these CNP variants to treat a subject. There is no structural difference in the variants found in the original claims of the '672 application and the effects of administering these variants to a subject involve the same inherent properties. Note, for example, original claim 10 in the '672 application which broadly requires administering the CNP peptide or variant to a subject to produce a "therapeutic effect" in the subject by increasing the level of at least one bone- or cartilage-associated biomarker. Applicant's

attempts to distinguish the instant CNP variants from those of the '672 application are unpersuasive. See page 30, last paragraph in the response. The presence of a "hydrolysable linkage" is not distinct from the claims of the '672 application. See claims 10, 23, 24, 30, and 46. The CNP variants contain a hydrophobic acid or an amino acid sequence (cleavable peptide) conjugated to the CNP protein. That "cleavable peptide" can be removed by a hydrolysis reaction. Long-chain peptides contain amino acids linked via peptide bonds, a type of amide bond, that can be broken by amide hydrolysis, wherein the bonds are cleaved with the addition of a water molecule. Living organisms have enzymes capable of both forming and breaking peptide bonds. Therefore any claims to a "hydrolysable linkage" does not distinguish the reissue claims from the "cleavable peptides" of the '672 claims.

The reissue claims are substantially identical to those of the nonelected groups identified in the restriction requirement of the '672 application. In this regard, the findings of *Orita* are relevant. *Orita* precludes a reissue applicant from obtaining substantially identical claims to those of nonelected groups identified in a restriction requirement when such claims could not have been prosecuted in the application from which they were restricted. The claims in *Doyle* were different from those of the applicant's in *Orita* because *Doyle*'s new claims were neither identical nor substantially similar to the nonelected claims. The current reissue claims do not embrace any "overlooked aspects of the invention," while they are substantially similar if not identical to the nonelected canceled claims of the '672 application. The reissue claims are drawn to one or more of the non-elected groups and species of the original restriction requirement. The claims are not genus claims reading on elected and non-elected claims, but are claims comprised of a Markush groupings of specific SEQ ID NOs, which were present in the non-elected inventions in the '672 application. The current claims are not broader than the original non-elected claims of the '672 application, but rather represent species of the non-elected inventions. Unlike in *Doyle*, the claims presented in the current reissue application are not linking claims, but fall within the scope of the non-elected claims in the '672 application.

Claim Rejections – Nonstatutory Double Patenting

The previous office action held the following nonstatutory double patenting rejections:

1. Claims 15-20 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 2 of U.S. Patent No. RE46,707 E in view of Tsubery.

2. Claim 21 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Komatsu.

3. Claim 22 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Krejci.

4. Claims 23-30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Tsubery.

Applicant's Response

Applicant requests that these rejections be held in abeyance until allowable subject matter has been identified.

Examiner's Comments

The nonstatutory double patenting rejections remain of record, as no allowable subject matter has been indicated.

CONCLUSION

Claims 15-30 are rejected. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

ONGOING DUTY TO DISCLOSE

Applicant is reminded of the continuing obligation under 37 CFR 1.178(b), to timely apprise the Office of any prior or concurrent proceeding in which Patent No. RE46,707 E is or was involved. These proceedings would include interferences, reissues, reexaminations, and litigation.

Applicant is further reminded of the continuing obligation under 37 CFR 1.56, to timely apprise the Office of any information which is material to patentability of the claims under consideration in this reissue application.

These obligations rest with each individual associated with the filing and prosecution of this application for reissue. See also MPEP §§ 1404, 1442.01 and 1442.04.

INTERVIEWS

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice. The fax phone number for the organization where this application or proceeding is assigned is 571-273-9900.

FUTURE CORRESPONDENCE

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Johnny F. Railey II** whose telephone number is (571)272-0985. The examiner can

normally be reached on Monday through Thursday from 9:00 a.m. to 6:30 p.m. The examiner can also be reached on alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Jean Witz**, can be reached on (571) 272-0927.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://www.uspto.gov/patents/process/status/index.jsp . Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

All correspondence relating to this proceeding should be directed to:

By EFS: Registered users may submit via the electronic filing system EFS-Web at:

https://efs.uspto.gov/efile/myportal/efs-registered

By mail to: Mail Stop Reexam/Reissue
Central Reexamination Unit
Commissioner for Patents
United States Patent & Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

By hand: Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

/Johnny F. Railey II/
Patent Reexamination Specialist
CRU - Art Unit 3991

/Padmashri Ponnaluri/
Patent Reexamination Specialist
CRU - Art Unit 3991

/Jean C. Witz/
Supervisory Patent Reexamination Specialist
CRU - Art Unit 3991

Exhibit 24

Unified Patent Court
Einheitliches Patentgericht
Juridiction unifiée du brevet

2025-01644

Court of First Instance
Local Division Munich

Munich, 28th January 2025

Justitsministeriet
Slotholmsgade 10
1216 København K
Dänemark



FORMULAR A

ANMODNING OM FORKYNDELSE AF DOKUMENTER
(artikel 8, stk. 2, i Europa-Parlamentets og Rådets forordning (EU) 2020/1784 af 25. november 2020 om forkyndelse i medlemsstaterne af retslige og udenretslige dokumenter i civil- og handelsretlige sager (forkyndelse af dokumenter) ([1]))

Den fremsendende instans" journalnummer:

ACT_1613/2025 UPC_CFI_18/2025

1. FREMSENDENDE INSTANS

1.1. Navn:

Unified Patent Court - Local Division Munich

1.2. Adresse:

1.2.1. Gade + nummer/postboks:

Denisstrasse 3

1.2.2. Postnummer + bynavn:

80335 München

1.2.3. Land:

Tyskland

1.3. Tlf. :

+49 89 6142100 14

1.4. Fax : (*)

1.5. E-mail:

contact_munich.loc@unifiedpatentcourt.org

2. MODTAGENDE INSTANS

2.1. Navn:

Justitsministeriet

2.2. Adresse:

2.2.1. Gade + nummer/postboks:

Slotholmsgade 10

2.2.2. Postnummer + bynavn:

1216 København K

2.2.3. Land:

Danmark

2.3. Tlf.:

+45 72 26 84 00

2.4. Fax: (*)

2.5. E-mail:

jm@jm.dk

3. REKVIRENT(ER) ([2])

3.1. Navn:

Lawyers Jones Day

3.2. Adresse:

3.2.1. Gade + nummer/postboks:

Prinzregentenstrasse 11

3.2.2. Postnummer + bynavn:

80538 München

3.2.3. Land:

Generated by the European e-Justice Portal

Tyskland

3.3. Tlf.: (*)

+49 89 20 60 42 200

3.4. Fax : (*)

+49 89 20 60 42 293

3.5. E-mail: (*)

munichreception@jonesday.com

4. ADRESSAT

4.1. Navn:

Ascendis Pharma A/S

4.1.1. Fødselsdato (hvis denne oplysning foreligger):

4.2. Adresse:

4.2.1. Gade + nummer/postboks:

Tuborg Boulevard 12

4.2.2. Postnummer + bynavn:

2900 Hellerup

4.2.3. Land:

Danmark

4.3. Tlf. : (*)

4.4. Fax : (*)

4.5. E-mail: (*)

4.6. Identifikationsnummer/socialsikringsnummer/virksomhedsnummer/eller tilsvarende (*):

4.7. Eventuelle andre oplysninger vedrørende adressaten (*):

represented by the managing director Jan Møller Mikkelsen

5. FORKYNDELSESMÅDE

☑ 5.1. I overensstemmelse med modtagerstatens nationale ret

☐ 5.2. På følgende særlige måde

5.2.1. Hvis denne måde er uforenelig med modtagerstatens nationale ret, bør dokumentet eller dokumenterne forkyndes i overensstemmelse med den pågældende medlemsstats nationale ret:

☐ 5.2.1.1. Ja

☐ 5.2.1.2. Nej

6. DOKUMENT, DER SKAL FORKYNDES

6.1. Dokumentets art

☑ 6.1.1. retsligt

☐ 6.1.1.1. stævning

☐ 6.1.1.2. afgørelse/dom

☐ 6.1.1.3. appel

☑ 6.1.1.4. Andet

1. Letter dated 28th January 2025, 2. Infringement Action dated 10th January 2025

☐ 6.1.2. udenretsligt

6.2. Dato eller frist, hvorefter forkyndelse ikke længere er nødvendig (*):

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6.3. Sprog, som dokumentet er affattet på

6.3.1. original

- [ ] Bulgarsk(bg)
- [ ] Tjekkisk(cs)
- [ ] Dansk(da)
- [ ] Tysk(de)
- [ ] Græsk(el)
- [x] Engelsk(en)
- [ ] Spansk(es)
- [ ] EstiskPolsk(et)
- [ ] Finsk(fi)
- [ ] Fransk(fr)
- [ ] Kroatisk(hr)
- [ ] Ungarsk(hu)
- [ ] Italiensk(it)
- [ ] Litauisk(lt)
- [ ] Lettisk(lv)
- [ ] Maltesisk(mt)
- [ ] Nederlandsk(nl)
- [ ] Polsk(pl)
- [ ] Portugisisk(pt)
- [ ] Rumænsk(ro)
- [ ] Slovakisk(sk)
- [ ] Slovensk(si)
- [ ] Svensk(sv)
- [ ] Andet

6.3.2. oversættelse

- [ ] Bulgarsk(bg)
- [ ] Tjekkisk(cs)
- [ ] Dansk(da)
- [ ] Tysk(de)
- [ ] Græsk(el)
- [ ] Engelsk(en)
- [ ] Spansk(es)
- [ ] EstiskPolsk(et)
- [ ] Finsk(fi)
- [ ] Fransk(fr)
- [ ] Kroatisk(hr)
- [ ] Ungarsk(hu)
- [ ] Italiensk(it)
- [ ] Litauisk(lt)
- [ ] Lettisk(lv)
- [ ] Maltesisk(mt)
- [ ] Nederlandsk(nl)
- [ ] Polsk(pl)
- [ ] Portugisisk(pt)
- [ ] Rumænsk(ro)
- [ ] Slovakisk(sk)

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- [ ] Slovensk(si)
- [ ] Svensk(sv)
- [ ] Andet

6.4. Antal bilag:

2

7. SPROG TIL UNDERRETTELSE AF ADRESSATEN OM RETTEN TIL AT NÆGTE AT MODTAGE DOKUMENTET

Med henblik på artikel 12, stk. 2, i forordning (EU) 2020/1784 angiv venligst på hvilket af følgende sprog, ud over sproget i afsenderstaten, oplysningerne skal anføres:

7.1. Det officielle sprog eller et af de officielle sprog i afsenderstaten

- [ ] Bulgarsk(bg)
- [ ] Tjekkisk(cs)
- [ ] Dansk(da)
- [x] Tysk(de)
- [ ] Græsk(el)
- [ ] Engelsk(en)
- [ ] Spansk(es)
- [ ] EstiskPolsk(et)
- [ ] Finsk(fi)
- [ ] Fransk(fr)
- [ ] Kroatisk(hr)
- [ ] Ungarsk(hu)
- [ ] Italiensk(it)
- [ ] Litauisk(lt)
- [ ] Lettisk(lv)
- [ ] Maltesisk(mt)
- [ ] Nederlandsk(nl)
- [ ] Polsk(pl)
- [ ] Portugisisk(pt)
- [ ] Rumænsk(ro)
- [ ] Slovakisk(sk)
- [ ] Slovensk(si)
- [ ] Svensk(sv)

7.2. Det officielle sprog i en anden medlemsstat, som adressaten muligvis forstår

- [ ] Bulgarsk(bg)
- [ ] Tjekkisk(cs)
- [ ] Dansk(da)
- [ ] Tysk(de)
- [ ] Græsk(el)
- [ ] Engelsk(en)
- [ ] Spansk(es)
- [ ] EstiskPolsk(et)
- [ ] Finsk(fi)
- [ ] Fransk(fr)
- [ ] Kroatisk(hr)
- [ ] Ungarsk(hu)
- [ ] Italiensk(it)
- [ ] Litauisk(lt)
- [ ] Lettisk(lv)
- [ ] Maltesisk(mt)

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☐ Nederlandsk(nl)
☐ Polsk(pl)
☐ Portugisisk(pt)
☐ Rumænsk(ro)
☐ Slovakisk(sk)
☐ Slovensk(si)
☐ Svensk(sv)

8. EN KOPI AF DOKUMENTET SKAL RETURNERES SAMMEN MED ATTESTEN OM FORKYNDELSE (artikel 5, stk. 4, i forordning (EU) 2020/1784)

☐ 8.1. Ja (i så fald skal det dokument, der skal forkyndes, fremsendes i to eksemplarer)

☑ 8.2. Nej

9. GRUNDE TIL IKKE AT FREMSENDE GENNEM DET DECENTRALE IT-SYSTEM (artikel 5, stk. 4, i forordning (EU) 2020/1784) (4) (*)

Elektronisk fremsendelse var ikke mulig på grund af:

☐ forstyrrelse af IT-systemet

☐ ekstraordinære omstændigheder

1. I henhold til artikel 11, stk. 2, i forordning (EU) 2020/1784 skal De træffe alle nødvendige foranstaltninger, for at dokumentet kan forkyndes hurtigst muligt og under alle omstændigheder senest en måned efter modtagelsen. Har det ikke har været muligt for Dem at forkynde dokumentet senest en måned efter modtagelsen, skal De underrette nærværende instans om dette forhold ved angivelse i punkt 2 i attesten vedrørende forkyndelse eller manglende forkyndelse af dokumenter.

2. Kan De ikke imødekomme denne anmodning om forkyndelse på grundlag af de fremsendte oplysninger eller dokumenter, skal De i henhold til artikel 10, stk. 2, i forordning (EU) 2020/1784 + tage kontakt til nærværende instans for at indhente de manglende oplysninger eller dokumenter ved brug af formular E i bilag I til forordning (EU) 2020/1784.

Udfærdiget i:

München

Den:

28.01.2025

Underskrift og/eller stempel eller elektronisk signatur og/eller elektronisk segl:

Unified Patent Court
Einheitliches Patentgericht
Juridiction unifiée du brevet

(1)EUT L 405 af 2.12.2020, s. 1

(*)Valgfrit.

(2)Hvor der er mere end én sagsøger, anføre venligst de oplysninger, der er angivet i punkt 4.1-4.5.

(3) Denne formular gælder kun for de medlemsstater, der yder bistand i overensstemmelse med artikel 7, stk. 1, litra a), i forordning (EU) 2020/1784.

(4)Denne formular gælder kun for de medlemsstater, der yder bistand i overensstemmelse med artikel 7, stk. 1, litra a), i forordning (EU) 2020/1784.

Generated by the European e-Justice Portal

Unified Patent Court
Einheitliches Patentgericht
Juridiction unifiée du brevet

Court of First Instance
Local Division Munich

Munich, 28th January 2025

Ascendis Pharma A/S
Tuborg Boulevard 12
2900 Hellerup
Denmark

Dear Sir or Madam,

Ascendis Pharma A/S has been named as a defendant in the action

ACT_1613/2025 UPC_CFI_18/2025

before the Unified Patent Court (UPC; Local Division Munich) related to the patent EP3175863. The claimant is BioMarin Pharmaceutical Inc. The statement of claim and other documents you will find enclosed.

If you want to defend yourself against the action, you have to submit your statement of defence **in electronic form** within

three months

after service of the statement of claim (Rules 4 and 23 of the Rules of Procedure of the Unified Patent Court – RoP). The statement of defence shall contain the informations stated in Rule 24 RoP. According to Article 48 of the Agreement on a Unified Patent Court (UPCA) you have to be represented before the UPC by lawyers (Art. 48 Paragraph 1 UPCA) or an European Patent Attorney (Art. 48 Paragraph 2 UPCA).

Please provide your authorised representative with the following access code:

29d180af-1a92-4420-ace5-31f03970b778

This access code enables your authorised representative, to submit your statement of defence electronically.

Please instruct your representative to log in to the UPC Case management system at https://cms.unified-patent-court.org, to go to the "Representation" section in the main menu and click on the "Access CMS Case" menu item. If the Representation has been confirmed by the UPC, the case files will be fully visible for your representative.

Please note that a decision by default may be given against company Ascendis Pharma A/S, if you fail to take the necessary steps within the time limit mentioned above.

Sincerely yours,

Unified Patent Court

JONES DAY

RECHTSANWÄLTE • ATTORNEYS-AT-LAW • PATENTANWÄLTE
PRINZREGENTENSTRASSE 11 • D-80538 MÜNCHEN
TELEFON: (49) 89-20 60 42-200 • TELEFAX: (49) 89-20 60 42-293

January 10, 2025

Via CMS
Unified Patent Court
Munich Local Division
Denisstr. 3
80335 Munich

DR. CHRISTIAN PAUL
Munich Office
Ass: Petra Glawleschkoff
Phone +49 89 206042 205
Our reference:
120024.000065

INFRINGEMENT ACTION

In the matter of

BioMarin Pharmaceutical Inc., 105 Digital Drive, Novato, CA 94949, United States of America, represented by the managing director Alexander Hardy, ibid.

- Claimant -

Attorneys of record:	JONES DAY, Rechtsanwälte, Prinzregentenstr. 11, 80538 München, Dr. Christian Paul, Dr. Tobias Mandler, Pascal Grandé
contributing:	JONES DAY, Patent Attorneys, Prinzregentenstr. 11, 80538 Munich, Dr. Olga Bezzubova, Dr. Ping Li, Dr. Sven Rihm
supported by:	PAUL HASTINGS (EUROPE) LLP, 100 Bishopsgate, London, United Kingdom, EC2N 4AG, Jason Raeburn, Alex Morgan

against

1. **Ascendis Pharma A/S**, Tuborg Boulevard 12, 2900 Hellerup, Denmark, represented by the managing director Jan Møller Mikkelsen, ibid.

- Defendant 1) -

2. **Ascendis Pharma Growth Disorders A/S**, Tuborg Boulevard 12, 2900 Hellerup, Denmark, represented by the managing director Jan Møller Mikkelsen, ibid.

- Defendant 2) -

regarding patent infringement (EP 3 175 863 B1)

Value in dispute (preliminary estimate): EUR 5,000,000

We hereby declare that we represent the Claimant, with the assistance of the patent attorneys of our firm as named above.

For the content of this statement of claim, please refer to the table of contents at the end of this pleading.

To facilitate the "*formal check*", an appropriately completed table with the summarized information is provided as

Exhibit JD 1.

We provide a list of exhibits as

Exhibit JD 2.

The court fee was transferred to the court's bank account in accordance with Art. 70 UPCA, R. 15.2, R. 370 f. RoP. We enclose the proof of payment as

Exhibit JD 3.

Service of process can be effected in English, R. 270 RoP, Art. 8 ff. of Regulation (EU) 2020/1784. As part of an international cooperation that is a publicly traded company on Nasdaq, the Defendants are engaging with their customers and partners regularly in English on the global market. The Defendants, including their managing directors, are therefore capable of understanding English, as further evidenced by their English website (Exhibit JD 6) as well as their annual report (Exhibit JD 5) and investor communication (e.g. Exhibits JD 4, JD 8), all published in English.

We request, in the name of the Claimant, the Court to:

I. order the Defendants to cease, desist and be prohibited, with immediate effect, from making, offering, placing on the market or using or storing for those purposes a variant of C-type natriuretic peptide (CNP) within the territory of Germany, France, Italy, Netherlands, Austria, Belgium, Denmark, Estonia, Finland, Luxembourg, Portugal and Sweden, wherein

the variant CNP is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160), further comprising a hydrophilic polymer, wherein the hydrophilic polymer is polyethylene glycol (PEG), and a hydrolysable linkage conjugating the hydrophilic polymer to the CNP variant,

(claims 2, 3, 4 and 7 as granted, claim 1 as upheld)

especially if this is done in the form of "TransCon CNP" (navepegritide),

especially if

the hydrophilic polymer is conjugated to the CNP variant at a location selected from the group consisting of (1) only at the N-terminus; (2) only at the C-terminus; (3) only at an internal site; (4) at both the N-terminus and the C-terminus; (5) at the N-terminus and an internal site; and (6) at the C-terminus and an internal site

(claim 5 as granted, claim 2 as upheld)

especially if

the pharmaceutical composition also comprises a pharmaceutically acceptable excipient, carrier, or diluent

(claim 8 as granted, claim 3 as upheld)

especially if

the CNP variant is used in a method for the treatment of achondroplasia

(claim 10 as granted, claim 4 as upheld)

with the exception of the aforementioned variant that is offered to, placed on the market for, used by or stored for such purposes for end consumers that have already received treatment with the aforementioned variant in a clinical trial before the service of the decision and who require continuous treatment with the

aforementioned variant to treat their achondroplasia, in connection with and pursuant to an existing clinical trial protocol.

II. order the Defendants under a daily penalty of up to EUR 50,000 for each day of delay within a period of 30 days from the date of service of the notification within the meaning of R. 118 (8) sentence 1 RoP and, if applicable, the certified translation, to provide the Claimant with information on the extent to which it has committed the acts of infringement under section I., since 1. December 2021, for each of the countries listed in section I., in the form of a list – in electronic form in a native file format (e.g. Excel) which can be analyzed with the aid of a computer –, of the following information structured for each month of a calendar year and according to the products referred to in section I:

1. the origin and distribution channels, including sales offices, for the products under section I., especially for "TransCon CNP";

2. the quantity of products and parts thereof manufactured or having been manufactured, in particular by Wacker Biotech GmbH and Vetter Pharma-Fertigung GmbH & Co. KG, delivered, received or ordered, as well as the respective prices paid;

3. the individual offers, broken down by offered quantities, times, prices and type designations, as well as the names and addresses of the offerees;

4. the individual deliveries, broken down by delivery quantities, times and prices and the respective destinations;

5. the identity of all third parties involved in the manufacture or distribution of the infringing products;

6. the number and dates of the products offered;

7. the advertising carried out, broken down by advertising medium, its distribution, the distribution period and the distribution area; including evidence of these advertising activities and the domains used; in the case of internet advertising, the domain, search engines and other marketing tools used to register the websites concerned individually or collectively; the access figures and the placement periods of each campaign;

8. the costs, broken down by individual cost factors, explanations of the calculation of these costs and the profits realized for each act pursuant to section I.;

whereby copies of the corresponding purchase and sales documents, namely invoices, must be submitted in electronic form which can be analyzed with the aid of a computer as proof of the information, whereby details requiring confidentiality outside the data subject to disclosure and notification may be redacted, insofar as invoices are not available or do not indicate the required information, delivery notes of the individual deliveries;

III. order the Defendants under penalty of a periodic fine of EUR 2,000 for each day of delay for each item below respectively within a period of one week from the date of service of the decision and at the Defendants' expense:

1. to recall the products under section I. delivered to commercial customers since 1. December 2021 from the distribution channels within two weeks from the date of service of this decision and to this end to send a registered letter or email (with cc to the Claimant's attorneys) to its commercial customers containing the following content, or a translation into the language of the commercial customer:

> "*Dear customer,*
>
> *By decision of [insert date], the Court of First Instance of the Unified Patent Court, Local Division Munich, ruled that Ascendis Pharma A/S / Ascendis Pharma Growth Disorders A/S (hereinafter "Ascendis") has infringed the patent EP 3 175 863 B1 of BioMarin Pharmaceutical Inc. by making, offering, placing on the market or using or importing or storing for those purposes the product "TransCon CNP" as a variant of C-type natriuretic peptide (CNP) within the territory of Germany, France, Italy, Netherlands, Austria, Belgium, Denmark, Estonia, Finland, Luxembourg, Portugal and Sweden. Ascendis will therefore no longer market TransCon CNP in these countries and hereby requests that you no longer offer these (whether online or offline) and return any of these products in your possession to Ascendis within 7 days from the date of this letter. Any products that are offered to, placed on the market for, used by or stored for such purposes for end consumers that have already received treatment with the aforementioned product in a clinical trial before the service of the decision and who require continuous treatment with the aforementioned product to treat their achondroplasia, in connection with and pursuant to an existing clinical trial protocol, are exempt. Ascendis will refund the purchase price and all costs associated with the return of these products, including costs for transport and customs.*"

and to provide copies of any communications to the Claimant's attorneys for verification purposes;

2. to permanently remove the products under section I. from the distribution channels;

IV. order the Defendants to pay the Claimant an amount of EUR 150,000 as interim damages.

V. declare that Defendants are obliged on the merits to compensate the Claimant for any damage that it has suffered or will suffer in future for all past and future acts pursuant to section I. since 1. December 2021.

VI. order the respective Defendant for each individual case of non-compliance with the order under section I. to pay a recurring penalty payment of up to EUR 250,000 to the Court (repeatedly if necessary) and to be determined by the Local Division in Munich upon Claimant's request.

VII. order the Defendants to pay the Claimant's costs of the proceedings.

VIII. order the immediate enforcement of the decision after the Claimant has notified the Court which part of the Orders it intends to enforce and has submitted a certified translation of the Orders into the official language of the Contracting Member State in which enforcement is to take place and after the Defendants have been served with the notification and the (respective) certified translation.

In the event that one or both Defendants fails to file written submissions in defense or fails to appear at the oral hearing after having been served with a document instituting proceedings or with an equivalent document, we request the Court to issue a decision by default pursuant to Article 37(1) UPCA respectively.

REASONS

1 The Claimant takes action against the Defendants for direct infringement of claims 1-4 of the patent *in suit* as upheld in opposition proceedings before the EPO with respect to the Defendants' product "*TransCon CNP*" in the territory of Germany, France, Italy, Netherlands, Austria, Belgium, Denmark, Estonia, Finland, Luxembourg, Portugal and Sweden. In detail:

A. FACTS

I. The parties to the legal dispute

2 The parties are biopharmaceutical companies. They are competitors on the market for *inter alia* treatments for the rare disease achondroplasia, a genetic condition that causes severely short stature and disproportionate growth.

1. The Claimant

3 The Claimant is an innovative biopharmaceutical company headquartered in California, USA. It is engaged in the research, development and distribution of biotechnological therapies, in particular for the treatment of rare genetic diseases.

4 Among other things, the Claimant has developed and manufactures the drug Voxzogo® (Vosoritide). The Claimant's Voxzogo® product is the first drug to receive regulatory approval for treatment of achondroplasia (also referred to as "ACH") in the USA and in the EU.

2. The Defendants

5 The Defendants are part of the Ascendis Pharma Group, headquartered in Denmark, and are originators (i.e., not generic drug companies) that have developed the originator drug TransCon CNP that stands in competition with the Claimant's drug Voxzogo®.

6 Defendant 1) is the parent company of the Ascendis group and is publicly traded on the Nasdaq stock exchange. Defendant 1) maintains offices in, *inter alia,* Denmark, Germany (Heidelberg and Berlin) and the USA. Defendant 1) describes itself as "*a leading, fully integrated biopharma company*" (https://investors.ascendispharma.com/news-releases/news-release-details/ascendis-pharma-receives-orphan-designation-transcontm-cnp):

About Ascendis Pharma A/S

Ascendis Pharma is applying its innovative platform technology to build a leading, fully integrated biopharma company focused on making a meaningful difference in patients' lives. Guided by its core values of patients, science and passion, the company utilizes its TransCon™ technologies to create new and potentially best in class therapies.

Ascendis Pharma currently has a pipeline of three independent endocrinology rare disease product candidates in clinical development and is advancing oncology as its second therapeutic area of focus. The company continues to expand into additional therapeutic areas to address unmet patient needs.

Ascendis is headquartered in Copenhagen, Denmark, with additional offices in Heidelberg and Berlin, Germany, and in Palo Alto and Redwood City, California.

For more information, please visit www.ascendispharma.com

Exhibit JD 4.

7 Defendant 2) is a wholly owned subsidiary, with the primary focus of developing products relating to growth disorders such as achondroplasia. The 2023 Annual Report of Ascendis Pharma A/S confirms the corporate relationship on page 8 as follows,

Exhibit JD 5:

Our Organization

Certain of our operations are conducted through our following wholly-owned subsidiaries:

Wholly-owned subsidiaries	Domicile
Ascendis Pharma GmbH	Germany
Ascendis Pharma Endocrinology GmbH	Germany
Ascendis Pharma, Inc.	USA
Ascendis Pharma Endocrinology, Inc.	USA
Ascendis Pharma Ophthalmology Division A/S	Denmark
Ascendis Pharma Endocrinology Division A/S	Denmark
Ascendis Pharma Bone Diseases A/S	Denmark
Ascendis Pharma Growth Disorders A/S	Denmark
Ascendis Pharma Oncology Division A/S	Denmark
Ascendis Pharma Nordics A/S	Denmark
Ascendis Pharma Europe A/S	Denmark
Ascendis Pharma UK Limited	United Kingdom
Ascendis Pharma Iberia S.L.	Spain

8 The Defendants have developed the drug "*TransCon CNP*" (navepegritide), which is administered as a once-weekly injection used to promote bone growth in patients with achondroplasia. According to the press release **Exhibit JD 4**, Defendant 1) received "*Orphan Designation for TransCon CNP for the Treatment of Achondroplasia in Europe*".

a. International marketing

9 The Defendants regularly update the market on new developments relating to the details, features and status of TransCon CNP and provide their website in English, Danish, Japanese, German and French (https://ascendispharma.com/pipeline/endocrinology/transcon-cnp/):

TRANSCON CNP

TransCon CNP is an investigational prodrug of CNP in development for the treatment of ACH in children. Designed to provide continuous CNP exposure, the goal of TransCon CNP is to optimize efficacy with a safe and convenient once-weekly dose. TransCon CNP has orphan designation for the treatment of ACH in both the U.S and Europe.

Program Status

We are conducting the ACcomplisH Trial, a global phase 2 clinical trial designed to evaluate the safety and efficacy of TransCon CNP in children with ACH from ages two to ten. In addition to height, we are evaluating various endpoints, including body proportionality and patient-reported outcome (PRO) measures using a tool developed with extensive input from those affected by ACH. These measures provide patient related perspectives on the impact of a disease or treatment.



The New Frontier of Growth

We are committed to leveraging the CNP pathway not only for achondroplasia but for other growth disorders. With the possibility of a sustained release profile and continuous exposure enabled by our TransCon technology, we are opening up new frontiers to investigate CNP in our future research.

10 As indicated above, the Defendants emphasize that TransCon CNP "*has orphan designation for the treatment of ACH in both the U.S and Europe*" and that the "*ACcomplisH Trial*"—a "*global phase 2 clinical trial*"—is ongoing. We provide excerpts of the website, including an English translation (from page 29), as

Exhibit JD 6.

b. Manufacturing in Germany

11 The Defendants have entered into contracts with Wacker Biotech GmbH ("Wacker") in Jena, Germany, to manufacture TransCon CNP in Germany. Wacker is producing the drug according to the Defendants' orders and under the oversight of the Defendants. Wacker has announced the contractual relationship in a press release, submitted as

Exhibit JD 7.

12 There, Wacker has admitted that it was contracted by the Defendants to manufacture TransCon CNP:

> "*TransCon™ CNP drug substance, a transiently conjugated peptide using TransCon™ technology, is* ***being made by Wacker Biotech GmbH in Jena, Germany***."
>
> "*Ascendis Pharma is being supported by Wacker Biotech GmbH,* ***which Ascendis Pharma has contracted to manufacture TransCon™ CNP***."
>
> "*Wacker Biotech* ***has been working with Ascendis Pharma since 2017****. The process for making the candidate based on Ascendis Pharma's proprietary TransCon technology was transferred successfully to Wacker Biotech GmbH in*

> *Jena at the start of this partnership.* ***TransCon™ CNP is manufactured there*** *in compliance with pharmaceutical GMP (Good Manufacturing Practice) requirements to ensure the high quality necessary for the approval of clinical trials or for market approval* ***by the FDA or EMA****. Wacker Biotech made the material both for the successful phase 1 trial and for the phase 2 trial that has now been applied for...*" (emphasis added)

13 The fill and finish for the drug, *i.e.*, filling the drug in vials, is done by Vetter Pharma-Fertigung GmbH & Co. KG ("Vetter") under contract with the Defendants, also in Germany.

14 These are the only TransCon CNP production sites of the Defendants. As the Defendants state in a stock exchange prospectus of 18 September 2024, submitted as

Exhibit JD 8,

> "*...CNP drug substance is supplied by* ***Wacker Biotech****, Germany. Our TransCon CNP drug product in vials is manufactured* ***by Vetter*** *pursuant to our agreement with Vetter.* ***We do not currently have any other suppliers for the drug substance, drug product or other components of our*** *TransCon hGH, TransCon PTH and* ***TransCon CNP****, although we believe that there are alternate sources of supply that could satisfy our clinical and commercial requirements,...*" (**Exhibit JD 8**, page S-40). (emphasis added)

c. Clinical trials

15 The Defendants jointly conduct far-progressed clinical trials, with sites and consequently usage of TransCon CNP also, inter alia, in Germany.

16 As achondroplasia is a rare ("orphan") disease, there are limited numbers of achondroplasia patients per country. The prevalence rate for the disease is approximately 0.0035%. Consequently, the patients enrolled in the current trials conducted by the Defendants already amount to approximately 10% of the total available patient population.

17 In more detail:

aa) ACcomplisH trial

18 The AComplisH trial is a Phase 2 multicenter trial of subcutaneous doses of TransCon CNP. During the trial, TransCon CNP is administered once weekly for 52 weeks in prepubertal children with achondroplasia, followed by a so-called Open-Label Extension

Period. The interim results for this trial have been published in the clinical trials register, submitted as

Exhibit JD 9.

19 The publication specifies Defendant 2) as the sponsor and Defendant 1) as the public and scientific contact:

Sponsors

Sponsor organisation name	Ascendis Pharma Growth Disorders A/S
Sponsor organisation address	Tuborg Blvd 12, Hellerup, Denmark, DK 2900
Public contact	Clinical Trial Information Desk, Ascendis Pharma A/S, 0045 70222244, clinhelpdesk@ascendispharma.com
Scientific contact	Clinical Trial Information Desk, Ascendis Pharma A/S, 0045 70222244, clinhelpdesk@ascendispharma.com

20 As can be seen from the interim results, the trial has sites in Australia, New Zealand, USA, Portugal, Austria, Denmark, Germany and Ireland (**Exhibit JD 9**, page 2).

bb) AttaCH trial

21 AttaCH is an ongoing "*Phase 2, Multicenter, Long-Term, Open Label Extension Trial Evaluating Safety, Tolerability, and Efficacy of Subcutaneous Doses of TransCon CNP Administered Once Weekly in Children and Adolescents with Achondroplasia*", according to the information in the EU clinical trials database, submitted as

Exhibit JD 10.

22 The AttaCH trial has trial sites in Denmark, Austria, Germany, Ireland, Portugal and Spain. Defendant 2) is listed as the sponsor of this trial (**Exhibit JD 10**, page 1 and 6). The email address "clinhelpdesk@ascendispharma.com" that is listed both as the scientific and public contact point for the trial (**Exhibit JD 10**, page 10) is an email address under the domain of the website operated by Defendant 1).

cc) reACHin trial

23 The Defendants are also conducting a so-called reACHin trial as "*A Phase 2, Multicenter, Double-Blind, Randomized, Placebo-controlled Trial, evaluating Safety, Tolerability, and Efficacy of Subcutaneous Doses of TransCon CNP Administered Once Weekly for 52 Weeks in Infants (0 to <2 years of age) with Achondroplasia followed by an Open Label Extension (OLE) period*", according to the information in the EU clinical trials database, submitted as

Exhibit JD 11.

24 The trial has trial sites in Denmark, Norway, Spain, Italy, Sweden, Ireland, France, Germany, Austria, USA, United Kingdom, Australia and New Zealand. Again, Defendant 2) is listed as the sponsor of this trial (**Exhibit JD 11**, page 1 and 11), and the email address "clinhelpdesk@ascendispharma.com" that is listed both as the scientific and public contact point for the trial (**Exhibit JD 11**, page 11) is an email address under the domain of the website operated by Defendant 1).

25 For the reACHin trial, the planned number of patients to be enrolled comprises a total of 17% of the age cohort of 0 to <2 years in the countries where the trial is conducted. In some countries, the planned patients for this trial cover even higher proportions of the total patient population, being approximately 40% (Austria, Denmark) and up to approximately 80% (Sweden) of the relevant age cohort of the total patient population.

dd) teACH trial

26 The Defendants are further conducting the so-called teACH trial. Currently the trial is recruiting primarily in Denmark. The protocol for this trial has been published and is submitted as

Exhibit JD 12.

27 The published protocol states on page 11 that after the trial, participants will be offered long-term open label or compassionate use treatment with navepegritide (TransCon CNP):

> **Brief Summary:**
>
> The purpose of this clinical trial is to evaluate efficacy and safety of once weekly subcutaneous (SC) doses of navepegritide 100 µg/kg compared to placebo after a 52-week double-blind, placebo-controlled treatment period in adolescents aged 12 to <18 years with genetically confirmed ACH. Eligible participants who complete the 52-week double-blind treatment period and have not reached epiphyses closure will be offered long-term open label or compassionate use treatment with navepegritide 100 µg/kg/week.
>
> Prior to enrollment, participants must have a minimum of 6 months historical height data for the calculation of baseline AGV.
>
> Clinical trial details include:
>
> - The clinical trial duration will be up to 62 weeks (screening and follow-up period included)
> - The treatment duration will be up to 52 weeks.
> - The visit frequency according to the Schedule of Activities (SoA) (Section 1.3)

28 It is further stated on page 32 that a participant will be offered continued navepegritide treatment after clinical trial completion, either *via* a compassionate use program or in a long-term extension trial:

> **6.7. CONTINUED ACCESS TO TRIAL TREATMENT AFTER THE END OF THE CLINICAL TRIAL**
>
> A participant will be offered continued navepegritide treatment after clinical trial completion, either via a compassionate use programme or in a long-term extension trial, if the following conditions are met:
>
> - Have completed the trial without permanently discontinuing IMP treatment during the 52-week treatment period.
> - Open epiphyses at the Week 52 visit.
>
> The compassionate use program or the long-term extension trial will be carried out in accordance with national laws and regulations.

29 Defendant 2) is listed as the sponsor of this trial (page 1). The link in the protocol for reporting any adverse events (page 2) directs to "*safety.ascendispharma.com*", the website of Defendant 1).

II. The Patent *in suit*

30 The Claimant is the proprietor of European patent EP 3 175 863 B1 (in short, the "Patent") entitled "Variants of C-Type Natriuretic Peptide". The Patent was filed on 20 May 2010, claiming priorities from 20 May 2009 and 23 October 2009. The Patent was granted with effect from 1 December 2021 and is currently in force, *inter alia*, in Germany, France, Italy, Netherlands, Austria, Belgium, Denmark, Estonia, Finland, Luxembourg, Portugal and Sweden.

31 We enclose the Patent document as

Exhibit JD 13

and current extracts from the respective national registers for the Patent as

Exhibit JD 14.

32 The Patent is currently subject to opposition proceedings before the EPO. The opposition had been filed by Defendant 2). Pursuant to the decision of 23 September 2024, the EPO opposition division upheld the Patent in an amended form. Claim 1 as upheld is a combination of granted claims 2, 3, 4 and 7. We submit the decision of the opposition division, including Annex B with the auxiliary request 1 as upheld which specifies the claims 1-4 raised herein, as

Exhibit JD 15.

33 Both parties have appealed the opposition decision. As at the date of this Complaint, grounds for the appeals have not yet been filed.

34 The Patent had initially been opted out of the jurisdiction of the UPC. The opt-out has been withdrawn prior to filing of this complaint.

III. Technical background

35 The invention relates to the technical field of using peptides, and prodrugs of such peptides, in medicine. As an introduction into this technical field, a short guidance document on the basics and the technical background ("tech primer") is provided as

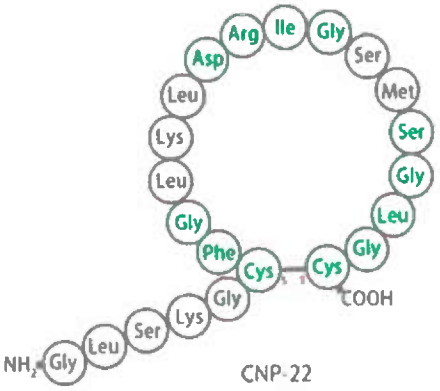
Exhibit JD 16.

36 As explained in para. [0001], the Patent relates to variants of "*C-type natriuretic peptide*" (CNP), including compositions, methods of making and using CNP variants to treat bone-related disorders, including dwarfism.

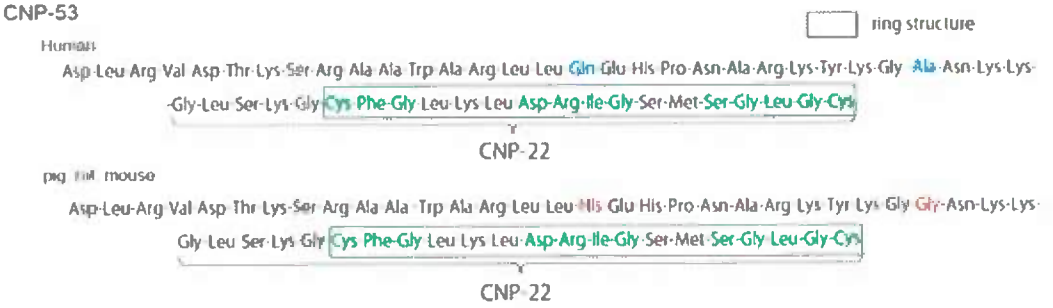
37 Achondroplasia is the most common form of dwarfism, which is characterized by a large head, short limbs, and a normal-sized torso. This genetic disorder is caused by activating mutations of the fibroblast growth factor receptor (FGFR-3) (para. [0007]). CNP modulates the activity of FGFR3 through inhibition of the FDFR3 signaling pathway. Overexpression of CNP was shown to counter the effects of FGFR-3 mutations and rescue animals from dwarfism. Therefore, CNP and functional analogues of CNP are potential therapeutics for these rare diseases (para. [0008]).

38 CNP is a peptide from the natriuretic peptide family of small, single-chain peptides which play important roles in multiple biological processes. CNP interacts with the natriuretic peptide receptor B ("NPR-B") to stimulate the generation of cyclic guanosidine monophosphate ("cGMP") (para. [0002]), an important messenger molecule in multiple cellular signaling pathways. CNP exerts a number of physiological effects in the cardiovascular system, neuronal development and reproduction, but its primary role appears to be the regulation of bone growth.

39 In humans, CNP is initially produced from the natriuretic peptide precursor C ("NPPC") gene as a single chain pre-pro polypeptide of 126-amino acids ("CNP-126"; the CNP nomenclature adds the length of the peptide to its name). Removal of the signal peptide, which is only required for polypeptide transport within the cell, produces proCNP, which consists of 103 amino acids. proCNP is then further broken down by an enzyme called furin into two components, an inactive NT-proCNP and an active CNP-53 peptide. CNP-53 is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide CNP-22 (para. [0003]). Both CNP-53 and CNP-22 retain the 17-amino-acid ring (cyclized portion) stabilized by disulfide bonds, which is crucial for its biological activity:

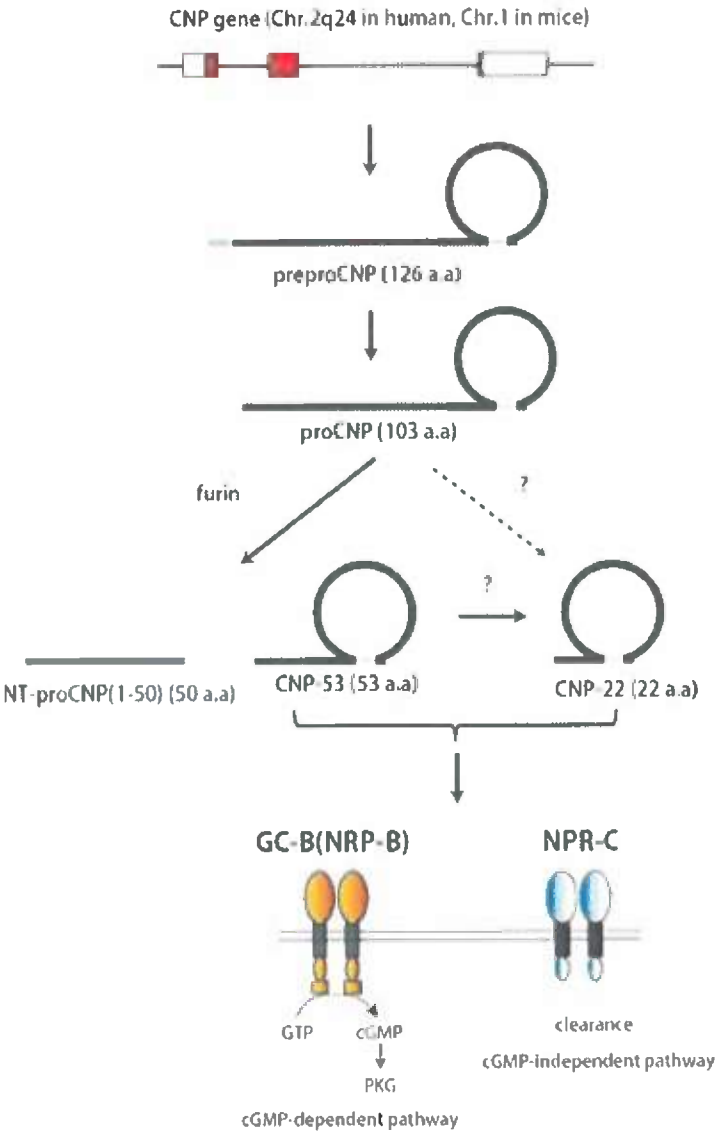


CNP-22



40 CNP-53 and CNP-22 bind similarly to NPR-B and induce cGMP production in a dose-dependent fashion. The two peptides differ in their distribution, with CNP-53 predominantly found in tissues and CNP-22 in plasma. Clearance of CNP from the extracellular space occurs by two mechanisms, enzymatic degradation by membrane-bound neutral endopeptidase ("NEP"), and binding by natriuretic peptide receptor C (NPR-C), which deposits it in lysosomes, where CNP is degraded (para. [0005], [0009]).

41 A schematic representation of CNP production and its receptors is reproduced below from a review by Nakagawa and Nishikimi (Biology 2022, 11, 986; https://doi.org/10.3390/biology11070986):



42 However, the therapeutic utility of the native CNP-22 peptide is limited by its short plasma half-life, which has been shown to be just 2.6 minutes *in vivo* in humans. Therefore, continuous systemic infusions have been necessary in human studies to maintain CNP concentration above intrinsic levels. Various attempts have been made in the prior art for improving the half-life of natriuretic peptides, but there have been no published reports on a successful strategy for making CNP resistant to NEP while retaining its functionality (para. [0013]).

IV. The teaching of the Patent

1. The problem of the invention

43 The Patent identifies the need for active CNP analogues of the naturally occurring CNP-22 peptide, the use of which as a therapeutic is limited by its short half-life in plasma (para. [0157]).

2. The solution according to the Patent

44 The Claimant's inventors were able to solve this problem. The Patent provides recombinant (i.e., non-native) CNP peptides and their modifications, collectively referred to in the Patent as CNP variants, that have an extended half-life compared to the naturally occurring CNP-22 and are useful in the treatment of bone-related disorders (*e.g.*, achondroplasia). The increased persistence of CNP variants in the circulation is, *inter alia,* due to their reduced ability to bind NEP, greater resistance to proteolysis by NEP and/or reduced affinity to NPR-C (para. [0021]).

45 The CNP variants taught by the Patent are (1) modified to increase the overall size or molecular weight of CNP-22 by attaching further moieties to it, and/or (2) modified at certain amino acid positions within the CNP peptide by way of mutation (para. [0023]). More specifically, CNP-22 may be extended at the N-terminus by CNP-derived amino acid sequences (para. [0024], [0030]). Examples of CNP variants ("extended" CNP-22 peptides) are provided in para. [0058], [0170] and [0177]. They include CNP-38, which is the active agent of the molecule of claim 1 as upheld. Extended CNP-22 peptides are also referred to in the Patent as "truncated CNP peptides" because they are shorter than their precursors, CNP-126 and CNP-53.

46 The extended CNP-22 peptide variants, such as CNP-38, may be further optimized for drug delivery by attaching/conjugating them to a hydrophilic (water attracting) polymer such as polyethylene glycol (PEG) (para. [0023], [0038], [0176], [0179], [0267]). The resultant molecule is often referred to as "*conjugate*".

47 PEG is known to increase the solubility and stability of conjugated peptides. Depending on the linkage between the PEG and the peptide, PEG may be attached to the peptide either permanently or transiently. In the latter case, the PEG moiety is split off from the peptide in the body, and the active peptide is released from the conjugate (para. [0038], [0198]). Such PEGylated conjugates act as so-called "prodrugs". Prodrugs are inactive or less active forms of drugs that are converted into their active state after being metabolized or otherwise modified in the body. A PEGylated prodrug of CNP-38 is the subject of the invention of claim 1 as upheld.

48 A feature analysis of claims 1-4 as upheld by the Opposition Division is provided as

Exhibit JD 17.

49 As noted above, claim 1 as upheld in opposition proceedings is a combination of granted claims 2, 3, 4 and 7. According to claim 1 as upheld, the problem of a therapeutically useful CNP analogue has been solved by a recombinant CNP-38 peptide which is attached/ conjugated to a hydrophilic polymer PEG. The resultant conjugate acts as a CNP prodrug: the active CNP-38 peptide is released from the conjugate by splitting off the PEG moiety.

50 Claim 1 as upheld defines the structure of the CNP conjugate by three features reflected in the following feature analysis:

1. A variant of C-type natriuretic peptide (CNP), wherein the variant CNP is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160),
   further comprising
2. a hydrophilic polymer wherein the hydrophilic polymer is polyethylene glycol (PEG), and
3. a hydrolysable linkage conjugating the hydrophilic polymer to the CNP variant.

(claims 2, 3, 4 and 7 as granted)

51 Claim 2 as upheld further specifies the variant CNP of claim 1 by the location at the peptide where the hydrophilic polymer conjugates:

1. The variant CNP of claim 1, wherein the hydrophilic polymer is conjugated to the CNP variant at a location selected from the group consisting of
   (1) only at the N-terminus;
   (2) only at the C-terminus;
   (3) only at an internal site;
   (4) at both the N-terminus and the C-terminus;
   (5) at the N-terminus and an internal site; and
   (6) at the C-terminus and an internal site.

(claim 5 as granted)

52 Claim 3 as upheld is directed to a pharmaceutical composition comprising the CNP variant:

1. A pharmaceutical composition comprising
2. a CNP variant of any of the preceding claims, and
3. a pharmaceutically acceptable excipient, carrier, or diluent.

(claim 8 as granted)

53 Claim 4 as upheld is directed to the use of the CNP variants as a drug for the treatment of, inter alia, achondroplasia:

> 1. A CNP variant according to any one of claim 1 or claim 2 or a pharmaceutical composition according to claim 3 for use in a method for the treatment of a bone-related disorder or skeletal dysplasia
>
> 2. selected from the group consisting of … achondroplasia….

(claim 10 as granted)

In claim 4 as upheld, two medical conditions recited in claim 10 as granted were deleted during the opposition proceedings.

3. The skilled person

54 The teaching of the Patent is directed to a person skilled in the relevant field. In this case, the skilled person may be defined as a group of scientists in the field of peptide drug development with several years of experience in the development of peptide drugs and prodrugs of peptides.

4. Interpretation of claim 1 as upheld

55 The Patent specification explains the function and meaning of the individual features of claim 1 as upheld:

a. Feature 1

> "A variant of C-type natriuretic peptide (CNP), wherein the variant CNP is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160)"

56 The Patent teaches several truncated CNP peptides ranging in length from CNP-17 to CNP-53 (para. [0177]). Amino acid sequences for these peptides are provided in para. [0177]. The truncated CNP peptide recited in feature 1 of claim 1 as upheld is CNP-38, which consists of the 38 C-terminal amino acids of the native CNP-126 polypeptide. The amino acid sequence of CNP-38 is defined by SEQ ID NO: 160, which is also recited in this feature 1.

b. Feature 2

> "further comprising a hydrophilic polymer wherein the hydrophilic polymer is polyethylene glycol (PEG)"

57 According to feature 2 of claim 1 as upheld, the CNP-38 peptide is further modified to form a conjugate with a hydrophilic polymer, namely PEG. As the Patent explains, the term "PEG" encompasses all forms of PEG which can be used to derivatize polypeptides (para. [0272]). PEG is a common tool for designing prodrugs of active peptide substances. A PEG moiety, when conjugated to the peptide, will alter the properties of the peptide, *e.g.*, reduce its binding to plasma proteins or shield the drug from its environment entirely. Because PEG is hydrophilic, it retains the ability of the peptide to be water-soluble. According to para. [0196], "PEGylation of CNP-22 or a variant thereof is designed, *inter alia*, to reduce immunogenicity and improve half-life by reducing renal clearance and increasing protease resistance".

c. Feature 3

> "further comprising a hydrolysable linkage conjugating the hydrophilic polymer to the CNP variant."

58 According to feature 3 of claim 1 as upheld, the PEG is conjugated to the CNP-38 peptide by a hydrolysable linkage. The hydrolysable linkage thus connects ("*conjugates*") the CNP-38 peptide with the PEG moiety.

59 The term "*hydrolysable linkage*" is disclosed in the context of conjugation of CNP variants to hydrophilic or water-soluble polymers discussed in paragraph [0037]. Examples of linkages for connecting the PEG to CNP variants are provided in paragraph [0038] and, identically, in paragraph [0198] of the Patent, as follows:

> "[0038] *The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG molecules) that may vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa).* (...)" (emphasis added)

60 The Patent lists several specific bonds as examples of bonds that can provide a hydrolysable or stable linkage. While alkylene bonds are stable, amide bonds, imine bonds, aminal bonds and ester bonds are hydrolysable. This is well known to the skilled person.

61 The inherent feature of these hydrolysable bonds (unlike stable bonds) is that they are susceptible to breakage under physiological conditions in the human body. In prodrug design, such bonds are included as predetermined breaking points that allow for a controlled separation of a conjugated moiety, also called "carrier", such as PEG from the active drug to be released (the so-called "parent drug"). Hydrolysable bonds also allow a separation of the carrier from the parent drug that leaves no residual trace on the parent drug, *i.e.*, the separation yields the parent drug without any chemical modifications ("tags"), as is desired and preferred in effective prodrug design.

5. Interpretation of claim 2 as upheld

62 Claim 2 as upheld is directed to the location at which the peptide is conjugated to the hydrophilic polymer:

> 1. The variant CNP of claim 1, wherein the hydrophilic polymer is conjugated to the CNP variant at a location selected from the group consisting of
>
> (1) only at the N-terminus;
> (2) only at the C-terminus;
> (3) only at an internal site;
> (4) at both the N-terminus and the C-terminus;
> (5) at the N-terminus and an internal site; and
> (6) at the C-terminus and an internal site.

63 According to claim 2, the hydrophilic polymer, which is a PEG according to feature 2 of claim 1, can be attached (conjugated) to the CNP variant, which is the CNP-38 peptide according to feature 1 of claim 1, at any location within the peptide. In other words, the PEG can be attached to any amino acid of the CNP-38 peptide with a reactive chemical group, regardless of its position in the peptide, whether internally or at its ends. Moreover, the claim allows for more than one PEG moiety to be conjugated to CNP-38, again at any location within the peptide (para. [0023], [0034], [0037], [0179]). In paragraph [0037], the Patent provides a specific example of CNP-37 or a longer version thereof, PEGylated only at an internal site, e.g., at amino acid lysine (Lys/K) at position 4, using the amino acid numbering of CNP-22.

6. Interpretation of claim 3 as upheld

64 Claim 3 as upheld is directed to a pharmaceutical composition as follows:

1. A pharmaceutical composition comprising
2. a CNP variant of any of the preceding claims, and
3. a pharmaceutically acceptable excipient, carrier, or diluent.

65 According to claim 3, the PEGylated CNP-38 peptide is provided in the form of a pharmaceutical composition (para. [0075]). Pharmaceutical compositions comprise substances (*e.g.*, excipients, carriers, diluents) that allow the active ingredient, presently the conjugate of claim 1, to be safely delivered to a human or animal body. A definition of "pharmaceutical composition" is provided in paragraph [0138], whereas examples of "pharmaceutically acceptable carries" are provided in paragraph [0139].

7. Interpretation of claim 4 as upheld

66 Claim 4 as upheld is directed to the use of the CNP variant or the pharmaceutical composition as follows:

1. A CNP variant according to any one of claim 1 or claim 2 or a pharmaceutical composition according to claim 3 for use in a method for the treatment of a bone-related disorder or skeletal dysplasia
2. selected from the group consisting of osteoarthritis, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondroplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelttype mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia.

67 The list of bone-related disorders and skeletal dysplasias can be found in the specification in para. [0281]. Achondroplasia, which is the most common form of the skeletal dysplasia and includes dwarfism, is in the focus of the Patent (para. [0001], [0021], [0079], [0099], [0279], [0280]).

V. Infringement of the Patent

68 The Defendants infringe the Patent by manufacturing and placing on the market the contested embodiment "TransCon CNP" which realizes all the features of claims 1-4 as upheld.

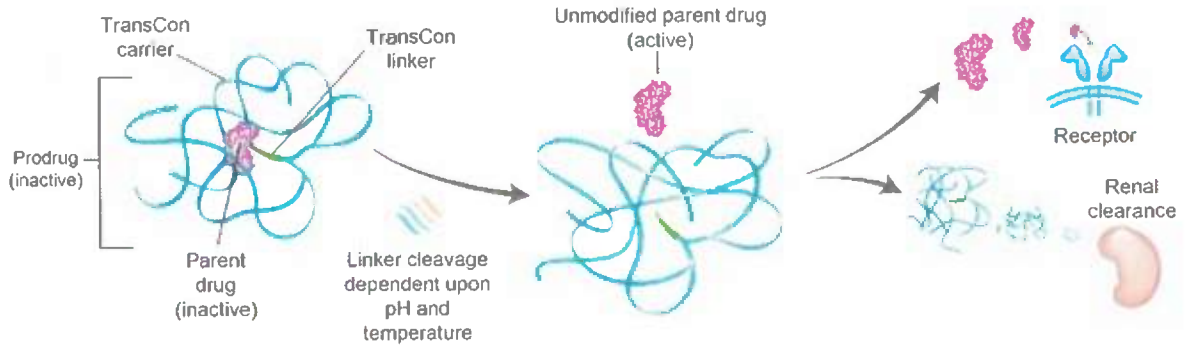
1. The contested embodiment

69 The Defendants' drug "*TransCon CNP*", also known as "*navepegritide*", infringes claims 1-4 as upheld by the opposition division.

70 The following information on TransCon CNP is taken from the Defendants' own publications. In the annual report already provided as **Exhibit JD 5**, Defendant 1) states the following with respect to TransCon CNP (*see*, **Exhibit JD 5**, page 25):

> "*Our Solution: TransCon CNP TransCon CNP (navepegritide) is an investigational* ***prodrug of CNP*** *administered once weekly and designed to provide sustained release of active CNP supporting continuous exposure for the treatment of achondroplasia. TransCon CNP is* ***designed to provide*** *effective* ***shielding of CNP from neutral endopeptidase degradation*** *in subcutaneous tissue and the blood compartment,* ***minimize binding of CNP to the NPR-C receptor*** *to decrease clearance, reduce binding of CNP to the NPR-B receptor in the cardiovascular system to avoid hypotension, and* ***release unmodified CNP****, which is small enough in size to allow effective penetration into growth plates...*" (emphasis added)

71 In the same annual report, Defendant 1) has published a conceptual illustration of TransCon CNP (*see*, **Exhibit JD 5**, page 14):

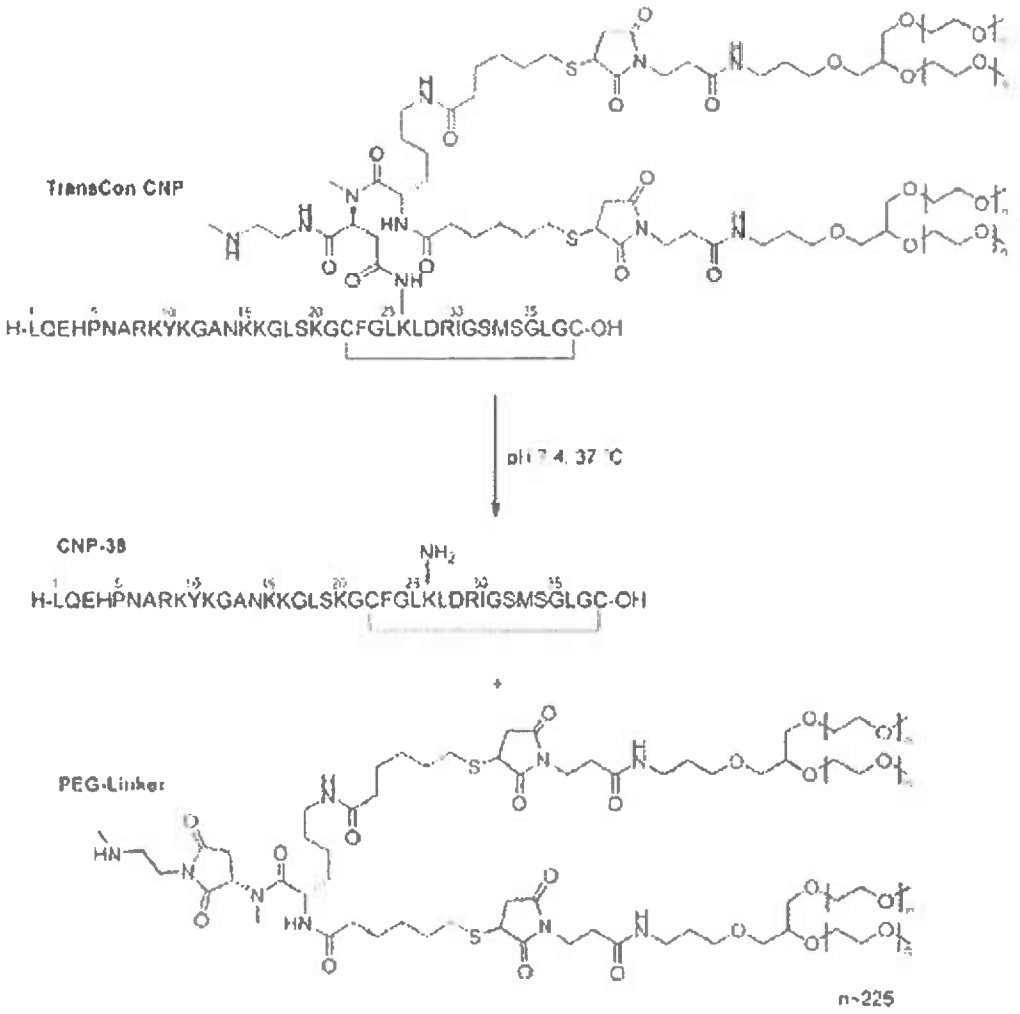


72 The illustration shows that TransCon CNP comprises the "parent drug" (the CNP peptide), to which the "TransCon carrier" is conjugated *via* a "TransCon linker". The "parent drug" is transiently bound to the moiety consisting of the "TransCon linker" and the "TransCon carrier", which shields the parent drug from clearance. The linker cleavage is dependent upon pH and temperature: following injection, the TransCon linker is designed to cleave at a specific rate to predictably release the unmodified parent drug.

73 The exact structure of TransCon CNP was disclosed by Defendant 2) in the course of the opposition proceedings in a declaration (D3) by one of its scientists, submitted as

Exhibit JD 18.

74 On page 2, **Exhibit JD 18** discloses the following schematic conversion of TransCon CNP into CNP-38:



75 As the Defendants' declaration shows, TransCon CNP comprises CNP-38 and a PEG-Linker. The connecting bond between the lysine (K) at position 26 of CNP-38 and the PEG-Linker is shown to be an amide bond.

76 In the regulatory documentation for one of their trials (the so-called "ACcomplisH" trial), submitted as

Exhibit JD 19,

the Defendants characterize TransCon CNP as

> "TransCon CNP 3.9 mg CNP-38/vial" (**Exhibit JD 19**, para. D.3.1 "product name") (emphasis added)

and as

> "C-Type natriuretic peptide conjugated to multi-arm polyethylene glycol carrier through a cleavable linker" (**Exhibit JD 19**, para. D.3.8 and D.3.9.3, "INN – Proposed INN" and "other descriptive name") (emphasis added):

D.3 Description of the IMP		
D.3.1	Product name	TransCon CNP 3.9 mg CNP-38/vial
D.3.2	Product code	TransCon CNP
D.3.4	Pharmaceutical form	Powder and solvent for solution for injection
D.3.4.1	Specific paediatric formulation	No
D.3.7	Routes of administration for this IMP	Subcutaneous use
D.3.8 to D.3.10 IMP Identification Details (Active Substances)		
D.3.8	INN - Proposed INN	C-TYPE NATRIURETIC PEPTIDE CONJUGATED TO MULTI-ARM POLYETHYLENE GLYCOL CARRIER THROUGH A CLEAVABLE LINKER
D.3.9.1	CAS number	2413551-27-4
D.3.9.2	Current sponsor code	TransCon CNP
D.3.9.3	Other descriptive name	C-TYPE NATRIURETIC PEPTIDE CONJUGATED TO MULTI-ARM POLYETHYLENE GLYCOL CARRIER THROUGH A CLEAVABLE LINKER

2. Realization of the claim features of claim 1 as upheld

77 According to the Defendants' own publications **Exhibit JD 18** and **Exhibit JD 19**, TransCon CNP infringes claim 1 as upheld, which recites the CNP-38 variant sequence and was upheld by the opposition division in the opposition proceedings:

a. Feature 1

> "A variant of C-type natriuretic peptide (CNP), wherein the variant CNP is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160)"

78 The declaration D3 from the opposition proceedings (**Exhibit JD 18**) and the statements in the regulatory documentation to the ACcomplisH trial (**Exhibit JD 19**) show that TransCon CNP comprises the "CNP-38" variant that is recited in claim 1 as upheld.

b. Feature 2

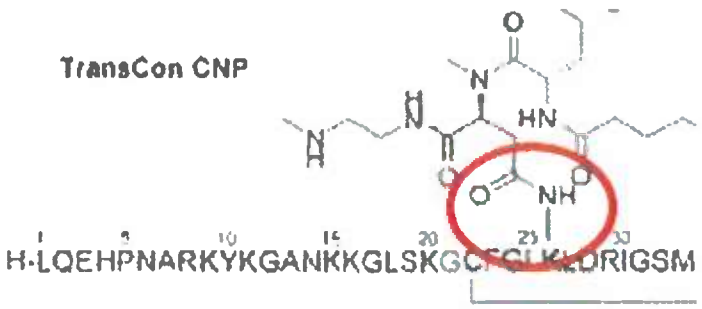
"further comprising a hydrophilic polymer wherein the hydrophilic polymer is polyethylene glycol (PEG)"

79 The regulatory documentation to the ACcomplisH trial (**Exhibit JD 19**) confirms that the CNP variant (*i.e.*, "C-Type natriuretic peptide") is "conjugated to multi-arm polyethylene glycol carrier". The polyethylene glycol (PEG) structure is also depicted as part of the detailed TransCon CNP structure in the declaration D3 (**Exhibit JD 18**).

c. Feature 3

"further comprising a hydrolysable linkage conjugating the hydrophilic polymer to the CNP variant."

80 The declaration D3 (**Exhibit JD 18**) also shows that the PEG carrier is connected to the CNP-38 peptide with an amide bond (O=C-NH-, circled below):



81 The amide bond is explicitly listed as an example of a bond that provides a hydrolysable linkage in para. [0038] of the Patent specification ("*hydrolysable ... linkage such as, e.g., amide ... bond*"). The PEG in TransCon CNP is therefore conjugated to the CNP peptide variant by means of a hydrolysable linkage.

3. Realization of the claim features of claim 2 as upheld

1. The variant CNP of claim 1, wherein the hydrophilic polymer is conjugated to the CNP variant at a location selected from the group consisting of
(1) only at the N-terminus;
(2) only at the C-terminus;
(3) only at an internal site;
(4) at both the N-terminus and the C-terminus;
(5) at the N-terminus and an internal site; and
(6) at the C-terminus and an internal site.

82 As shown in Defendants' declaration D3 in the opposition proceedings (**Exhibit JD 18**), the hydrophilic polymer is conjugated to CNP-38 at the lysine (K) at position 26 of CNP-38. This is an internal site according to option (3) of claim 2.

4. Realization of the claim features of claim 3 as upheld

1. A pharmaceutical composition comprising
2. a CNP variant of any of the preceding claims, and
3. a pharmaceutically acceptable excipient, carrier, or diluent.

83 As the specification of the drug administered in the ACcomplisH trial shows (**Exhibit JD 19**), the TransCon CNP vials contain "TransCon CNP 3.9 mg CNP-38/vial". The vials contain a powder for injection (**Exhibit JD 12**, page 28). This is a pharmaceutical composition as it will be administered to patients (feature 1 of claim 3). The pharmaceutical composition contains the CNP-38 prodrug according to claim 1 in a concentration of 3.9 mg (feature 2 of claim 3). The powder into which the 3.9 mg CNP-38 is mixed is a pharmaceutically acceptable excipient or carrier (feature 3 of claim 3).

5. Realization of the claim features of claim 4 as upheld

1. A CNP variant according to any one of claim 1 or claim 2 or a pharmaceutical composition according to claim 3 for use in a method for the treatment of a bone-related disorder or skeletal dysplasia
2. selected from the group consisting of … achondroplasia…,

84 In the Defendants' product, the CNP variant according to claim 1 and 2 as upheld, in the form of the pharmaceutical composition according to claim 3 as upheld, is specifically provided for the treatment of achondroplasia.

B. LEGAL ASSESSMENT

85 The Defendants' drug "*TransCon CNP*" makes literal use of all features of claims 1-4 as upheld (cf. above) and therefore infringes the Patent. The requests are justified as a consequence. In detail:

I. Regarding the requests of the Claimant

86 The claim for injunctive relief pursuant to section I. follows from Art. 63 (1) UPCA. TransCon CNP is manufactured by Wacker and Vetter in Germany on behalf of—and therefore attributable to—the Defendants (cf. above). The manufactured products are then supplied by the Defendants (or their agents) to the respective member states and thus made, offered, placed on the market as well as used and stored for those purposes in connection

with the clinical trials. This is done against the background of TransCon CNP's EU-wide orphan drug designation which acts as an indicator that there is significant potential risk of further infringing acts being committed within the member states. In the context of the eligible patient population in the countries where the trials are conducted, the Defendants' trials already cover up to ten percent of the relevant patients and/or market for these products.

87 The claim to information and accounting according to section II. follows from Art. 25 lit. (a), 67 UPCA. The requested information is necessary to investigate the full scope of the infringement and calculate the respective damages. Pursuant to R 354.3 RoP the Court's decisions and orders may provide for periodic penalty payments to be made to the Court in the event that a party fails to comply with the terms of the order or an earlier order. The penalty payment can be imposed for subsequent periods (day, week) in which the failure to comply continues, *i.e.*, daily penalty payments for the duration of the failure to comply (cf. LD Munich, Order of August 27, 2024, UPC_CFI_201/2024).

88 The request for recall and removal from the channels of distribution and periodic penalties in case of non-compliance according to section III. follows from Art. 64 (2) lit. (e) UPCA and R 354.3 RoP.

89 The Claimant is entitled to interim damages in accordance with the request under section IV pursuant to Art. 68 UPCA in combination with R. 119 RoP. The requested amount reflects a minimum amount to cover the expected costs of the procedure for determining the award of damages and compensation on the part of the successful party and is thus appropriate.

90 The Claimant is also entitled to damages according to the request in section V., cf. Art. 68 (1) UPCA in combination with R. 118.1 RoP. The Defendants are clearly aware of the Patent from their own opposition filed against the Patent. As the Claimant is not yet able to quantify the damage incurred, a legitimate interest in a declaration on the Defendants' liability exists.

91 The order in case of non-compliance with the request under section I. in the request in section VI. follows from Art. 63(2) UPCA and R 354.3 RoP.

92 The Defendant should bear the costs of the proceedings in accordance with the request in section VII. pursuant to. Art. 69 (1) UPCA.

93 A decision on enforceability of the decision follows from R. 354.1 RoP. The Claimant's interest in an immediate enforcement clearly outweighs the Defendants' interest.

II. Miscellaneous

94 The Patent is in force. Therefore, the UPCA is applicable (Art. 3 lit. c UPCA). The international jurisdiction arises from Art. 31 UPCA in conjunction with Art. 71a f. Brussels Ia Regulation.

95 Local and material jurisdiction of the Unified Patent Court - Local Division Munich - follows from Art. 33 (1) subparagraph 1 lit. a) in conjunction with Art. 32 (1) lit. a) UPCA and the respective acts of infringement of the Defendants in Germany.

Dr. Christian Paul
Rechtsanwalt

CHRISTIAN JOHANNES REINHARD PAUL
Digitally signed by CHRISTIAN JOHANNES REINHARD PAUL
Date: 2025.01.13 11:56:30 +01'00'

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Exhibit 25

US008598121B2

(12) **United States Patent**
Wendt et al.

(10) **Patent No.: US 8,598,121 B2**
(45) **Date of Patent: Dec. 3, 2013**

(54) **VARIANTS OF C-TYPE NATRIURETIC PEPTIDE**

(75) Inventors: **Daniel J. Wendt**, Walnut Creek, CA (US); **Shinong Long**, Milpitas, CA (US); **Sianna Castillo**, San Francisco, CA (US); **Christopher P. Price**, Kentfield, CA (US); **Mika Aoyagi-Scharber**, Mill Valley, CA (US); **Michel Claude Vellard**, San Rafael, CA (US); **Augustus O. Okhamafe**, Concord, CA (US)

(73) Assignee: **Biomarin Pharmaceutical Inc.**, Novato, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/466,672**

(22) Filed: **May 8, 2012**

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(51) **Int. Cl.**

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A61K 38/00	(2006.01)
A61K 38/16	(2006.01)
C12P 21/04	(2006.01)

(52) **U.S. Cl.**

USPC **514/12.4**; 514/16.7; 514/21.3; 435/69.4; 435/69.7; 435/70.1; 530/324

(58) **Field of Classification Search**

None

See application file for complete search history.

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(Continued)

Primary Examiner — Shulamith H Shafer

(74) *Attorney, Agent, or Firm* — Marshall, Gerstein & Borun LLP

(57) **ABSTRACT**

The present disclosure provides variants of C-type natriuretic peptide (CNP), pharmaceutical compositions comprising CNP variants, and methods of making CNP variants. The CNP variants are useful as therapeutic agents for the treatment of diseases responsive to CNP, including but not limited to bone-related disorders, such as skeletal dysplasias (e.g., achondroplasia), and vascular smooth muscle disorders (e.g., restenosis and arteriosclerosis).

14 Claims, 61 Drawing Sheets

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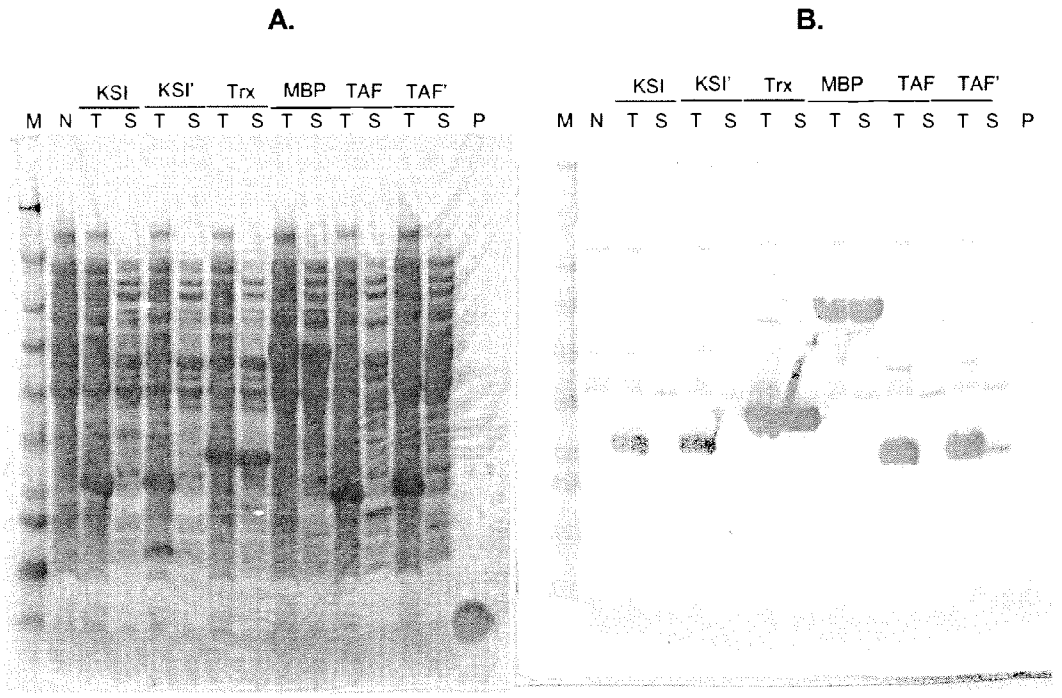
Yamashita et al., Concentration of mRNA for the natriuretic peptide receptor-C in hypertrophic chondrocytes of the fetal mouse tibia, *J. Biochem.*, 127:177-9 (2000).

Yasoda et al., Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway, *Nat. Med.*, 10:80-6 (2004).

Yeung et al., Binding of CNP-22 and CNP-53 to cultured mouse astrocytes and effects on cyclic GMP, *Peptides*, 17:101-6 (1996).

* cited by examiner

FIGURE 1



Coomassie blue stain

Western blot with anti-CNP antibody

FIGURE 2

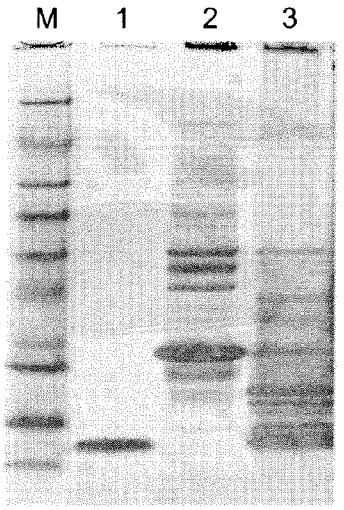


FIGURE 3

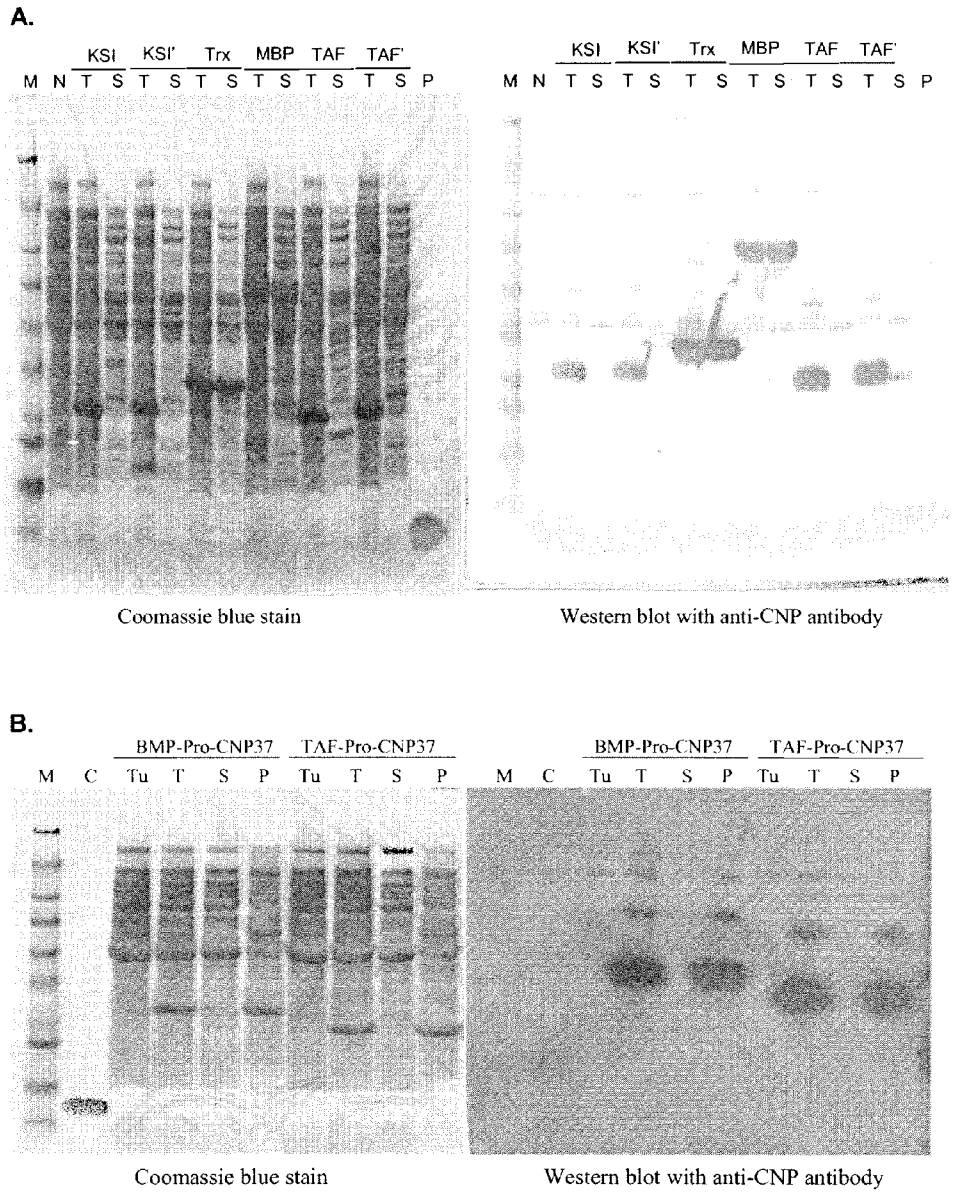

A.
KSI KSI' Trx MBP TAF TAF'
M N T S T S T S T S T S T S P
KSI KSI' Trx MBP TAF TAF'
M N T S T S T S T S T S T S P
Coomassie blue stain
Western blot with anti-CNP antibody
B.
BMP-Pro-CNP37 TAF-Pro-CNP37
M C Tu T S P Tu T S P
BMP-Pro-CNP37 TAF-Pro-CNP37
M C Tu T S P Tu T S P
Coomassie blue stain
Western blot with anti-CNP antibody

FIGURE 3 continued

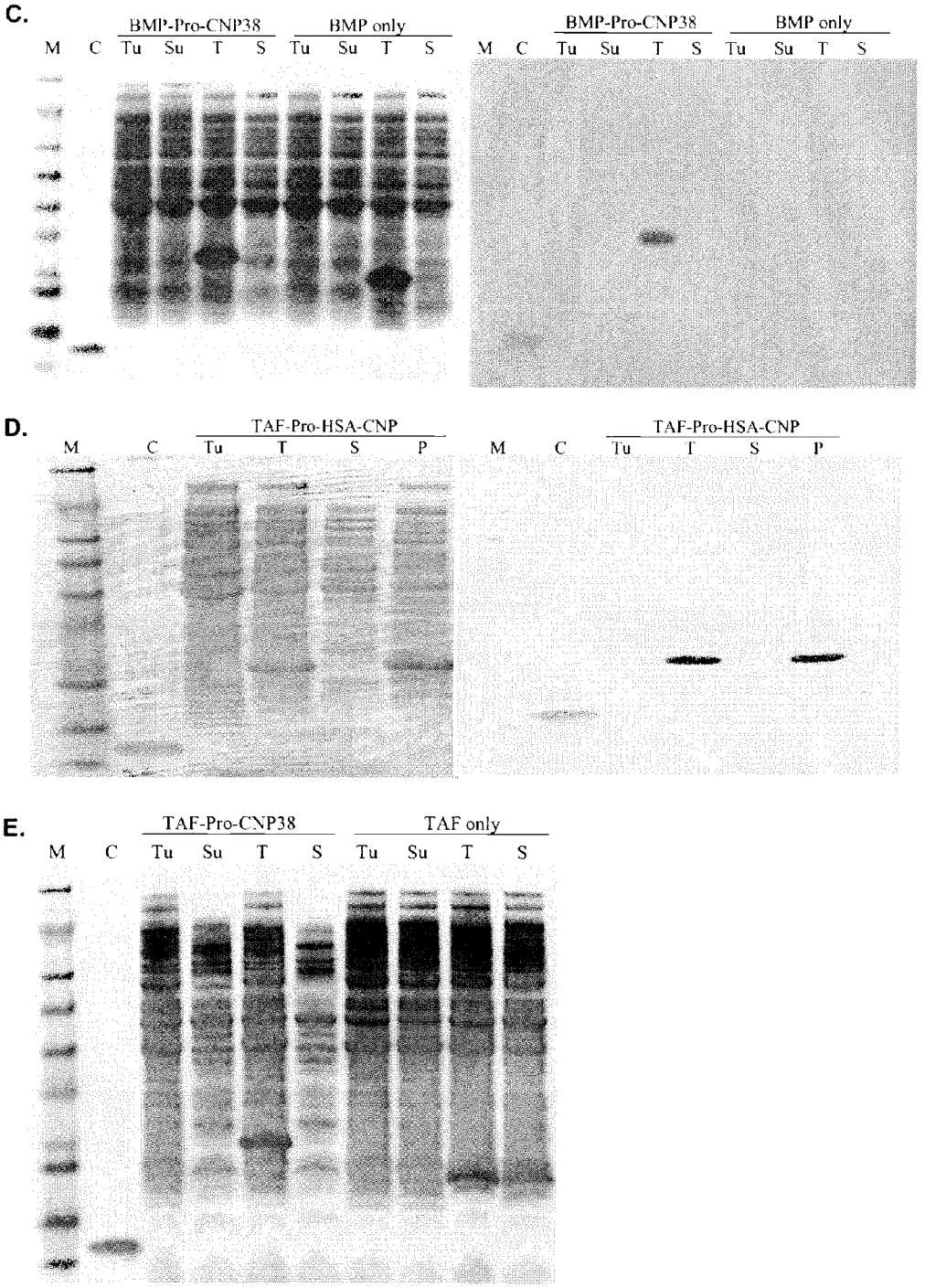


FIGURE 4

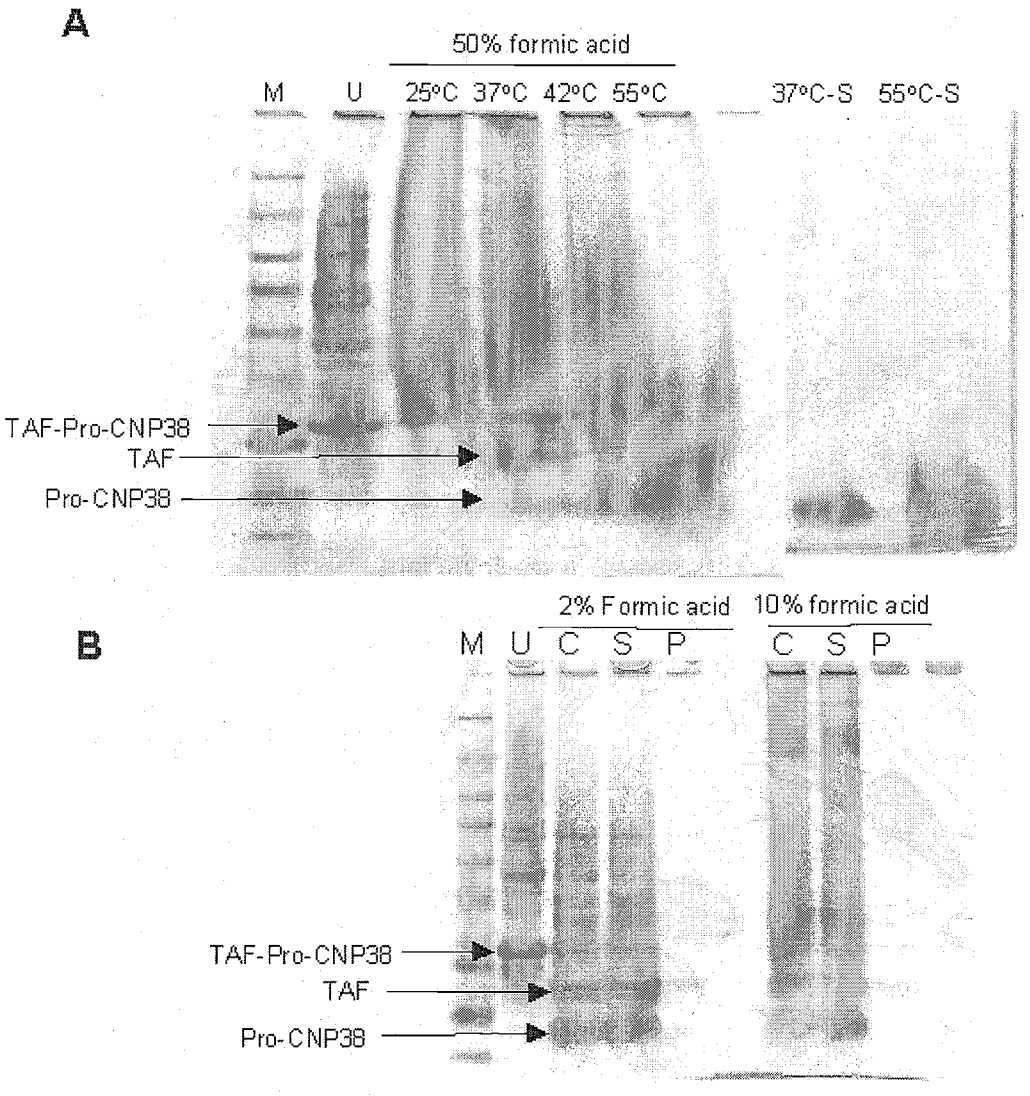

A
50% formic acid
M
U
25°C
37°C
42°C
55°C
37°C-S
55°C-S
TAF-Pro-CNP38
TAF
Pro-CNP38
B
2% Formic acid
10% formic acid
M
U
C
S
P
C
S
P
TAF-Pro-CNP38
TAF
Pro-CNP38

FIGURE 4C

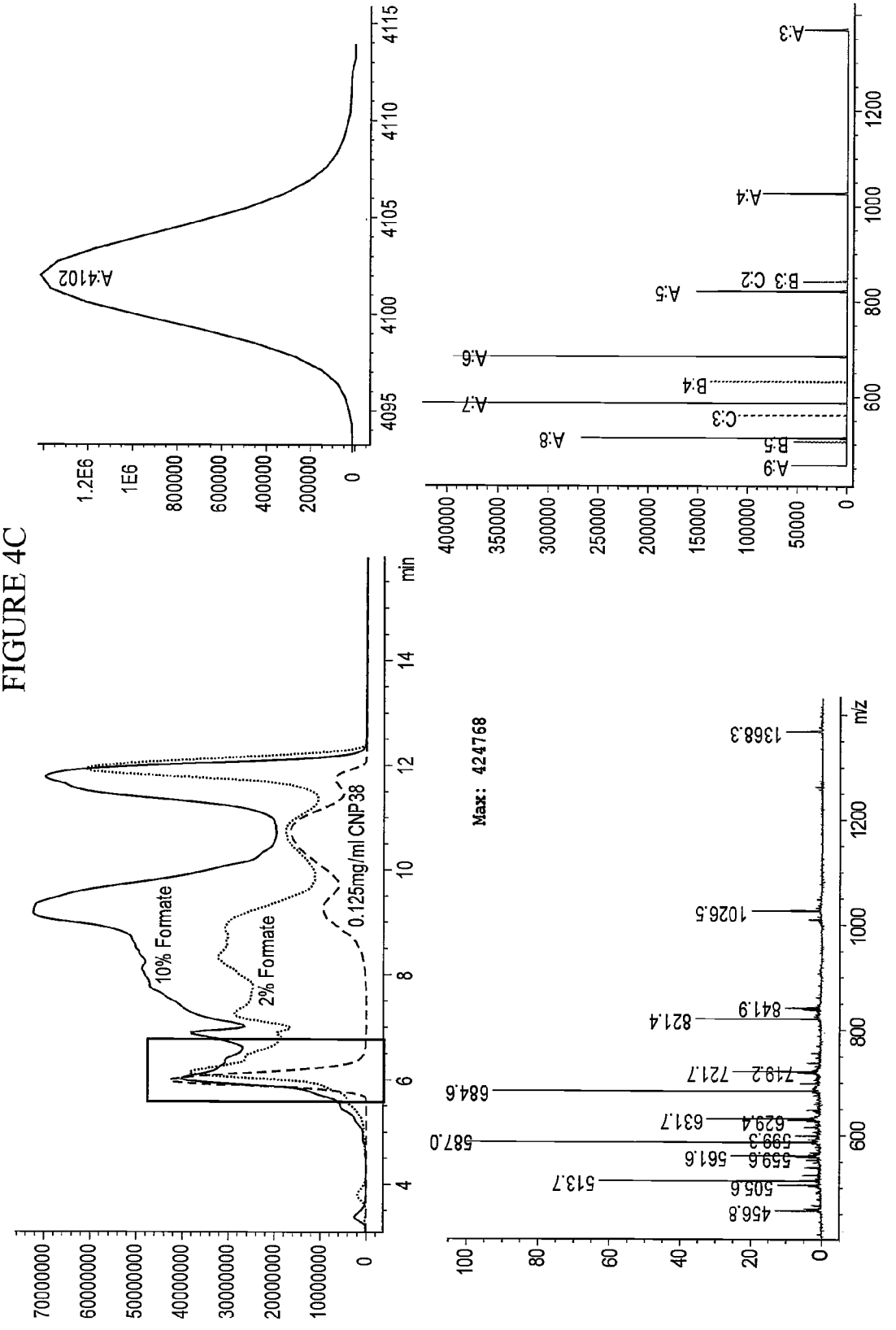

10% Formate
2% Formate
0.125mg/ml CNP38
min
A:4102
Max: 424768
m/z
A:3
A:4
B:3 C:2
A:5
A:6
B:4
A:7
C:3
A:8
B:5
A:9
1368.3
1026.5
841.9
821.4
749.2
721.7
684.6
687.0
599.3
559.6
561.6
629.4
631.7
513.7
505.6
456.8

FIGURE 5

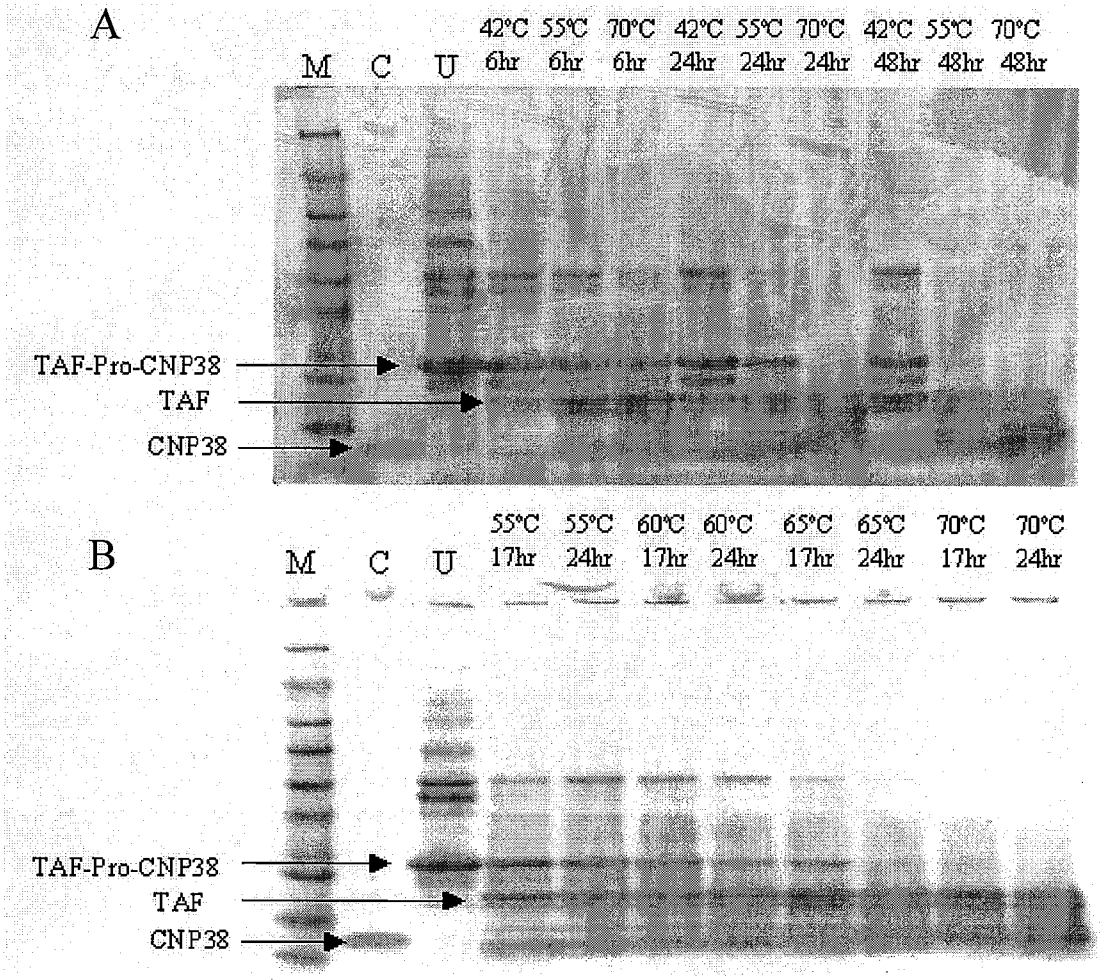

A
M
C
U
42°C 6hr
55°C 6hr
70°C 6hr
42°C 24hr
55°C 24hr
70°C 24hr
42°C 48hr
55°C 48hr
70°C 48hr
TAF-Pro-CNP38
TAF
CNP38
B
M
C
U
55°C 17hr
55°C 24hr
60°C 17hr
60°C 24hr
65°C 17hr
65°C 24hr
70°C 17hr
70°C 24hr
TAF-Pro-CNP38
TAF
CNP38

FIGURE 5C

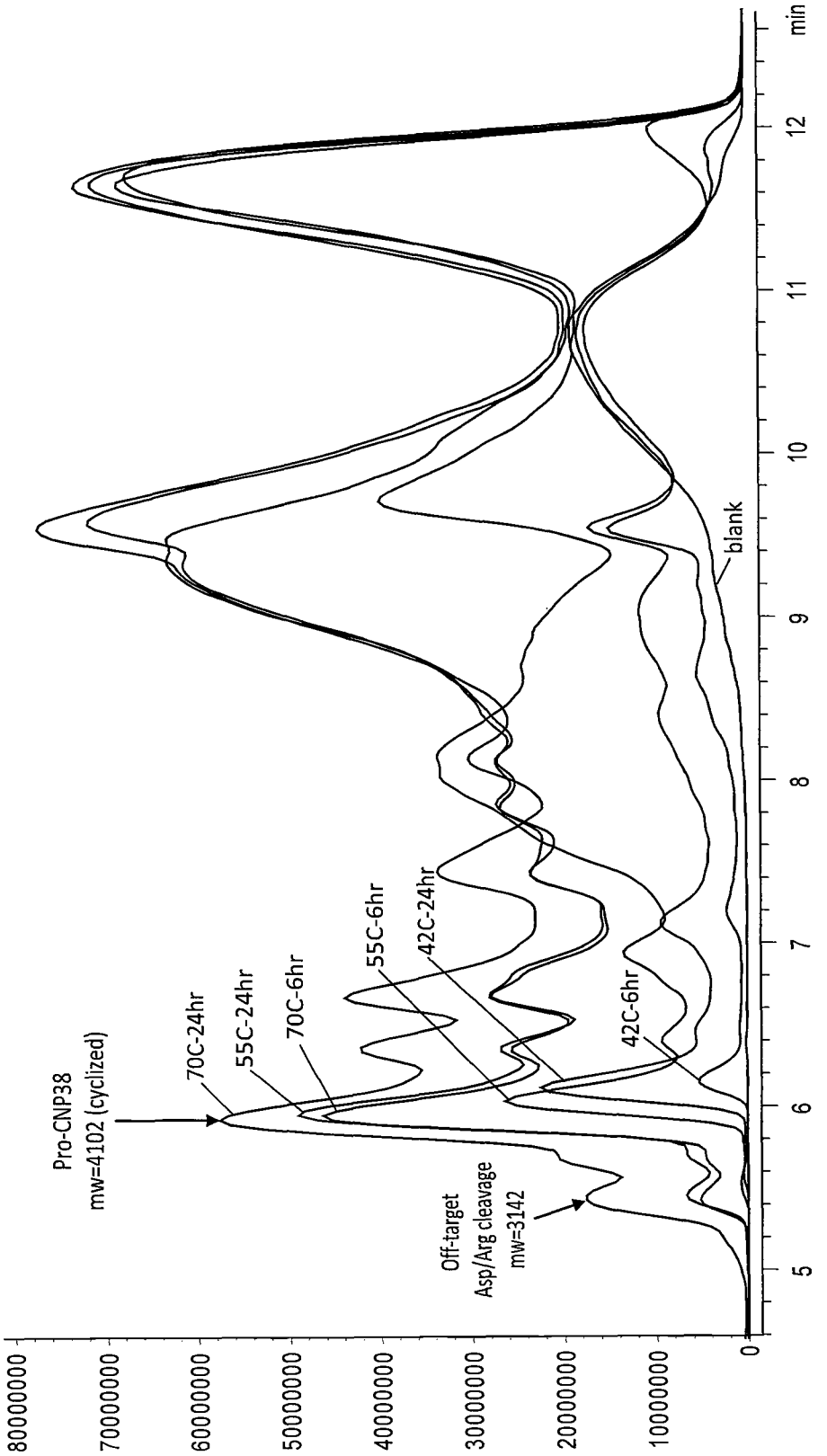

Pro-CNP38
mw=4102 (cyclized)
70C-24hr
55C-24hr
70C-6hr
55C-6hr
42C-24hr
42C-6hr
blank
Off-target
Asp/Arg cleavage
mw=3142
80000000
70000000
60000000
50000000
40000000
30000000
20000000
10000000
0
5
6
7
8
9
10
11
12
min

FIGURE 6

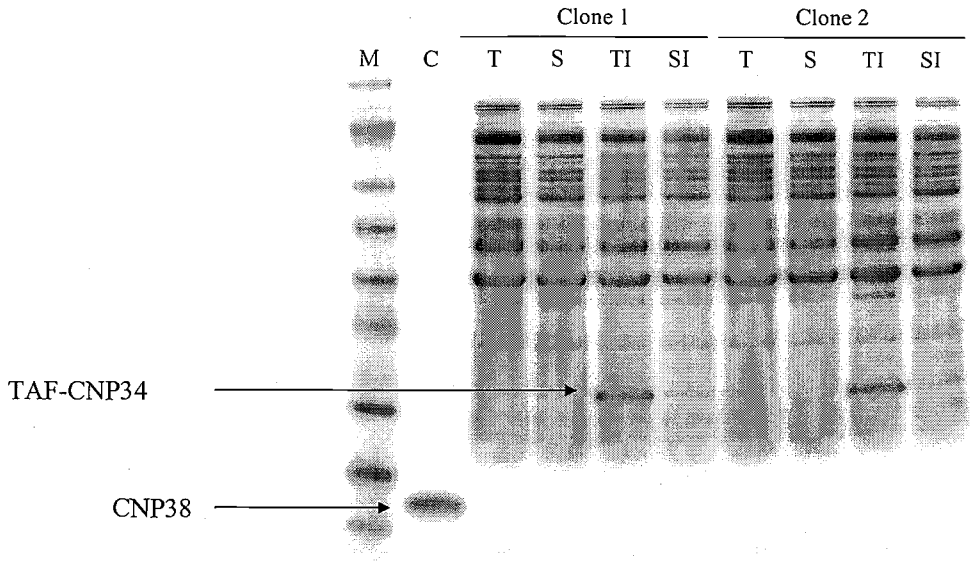


FIGURE 7

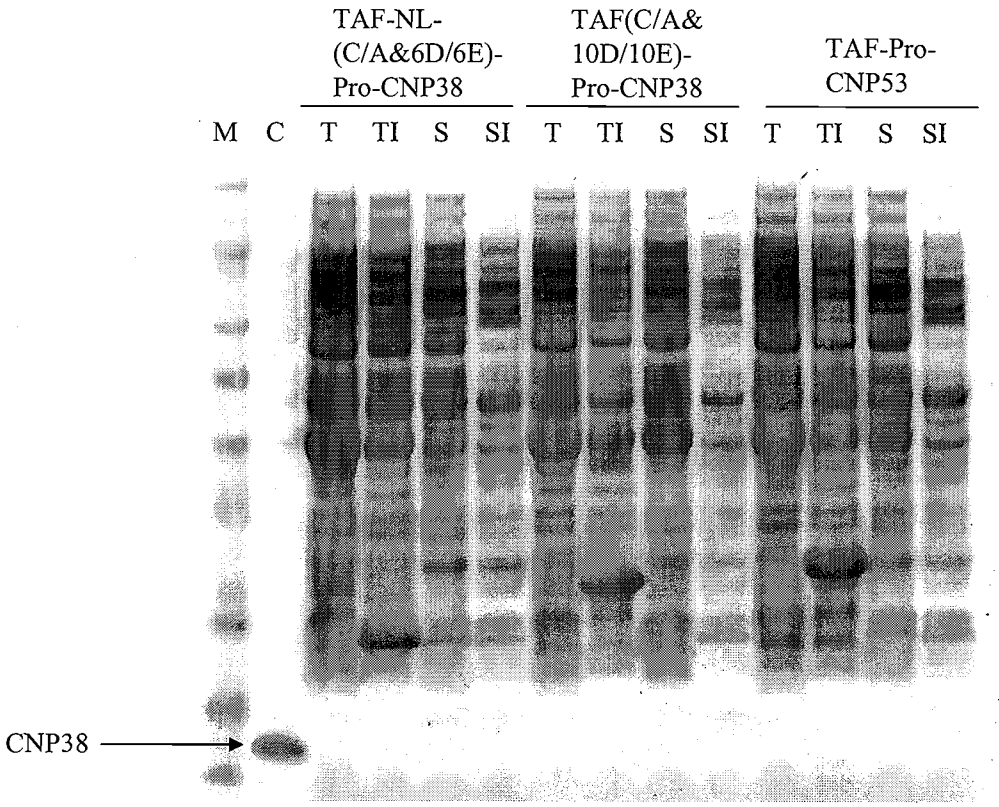


FIGURE 8

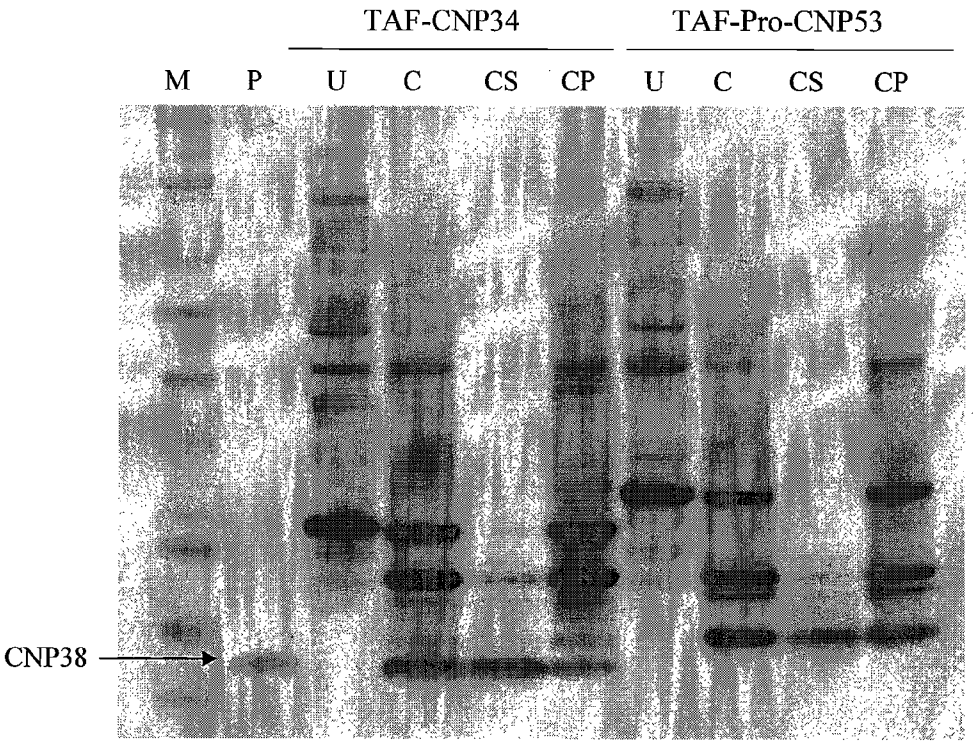


FIGURE 9

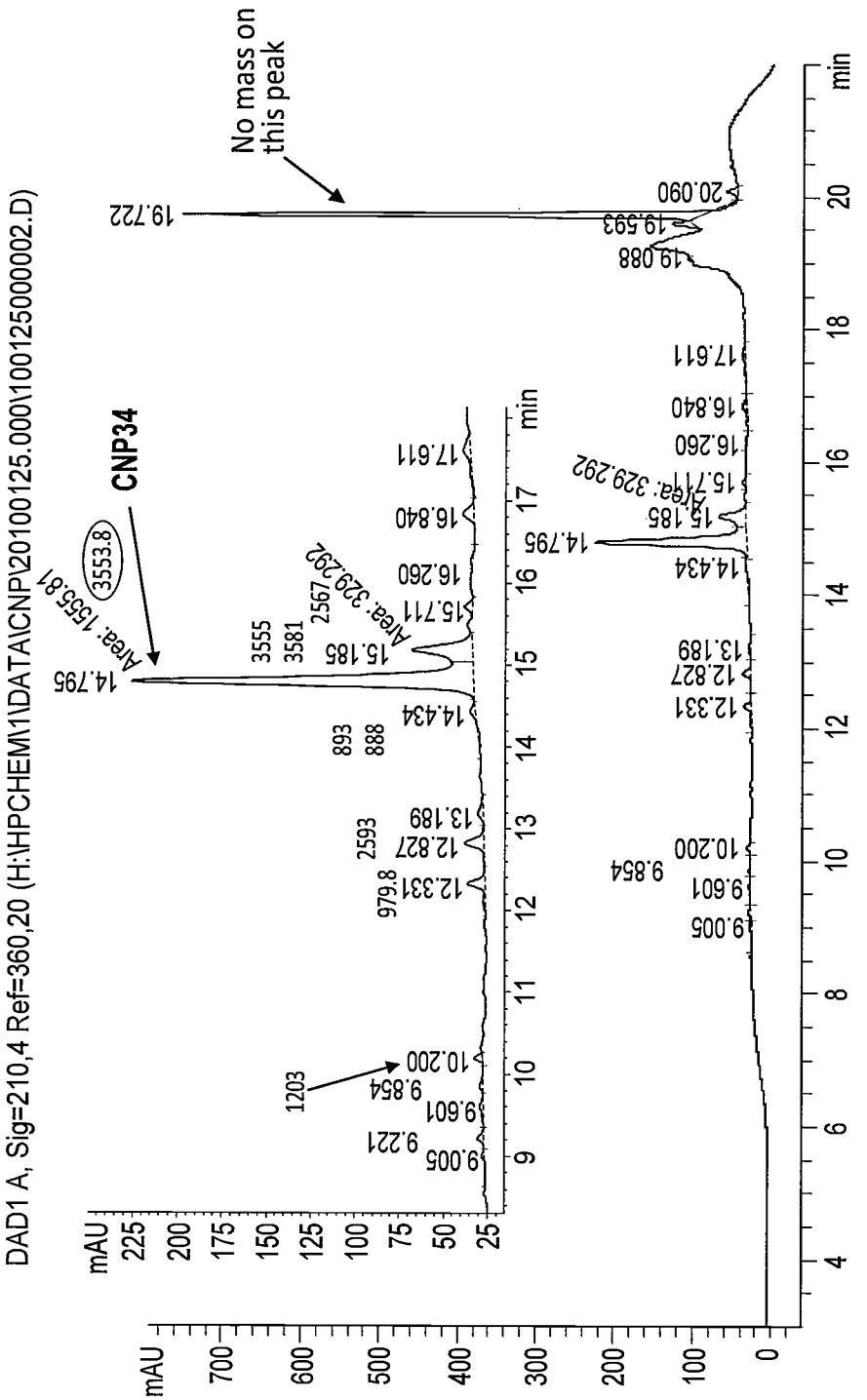

Calc. CNP34 monoisotopic mass = 3553.88
DAD1 A, Sig=210,4 Ref=360,20 (H:\HPCHEM\1\DATA\CNP\20100125.000\100125000002.D)
CNP34
3553.8
Area: 1555.81
No mass on this peak
Area: 329.292
mAU
min

FIGURE 10

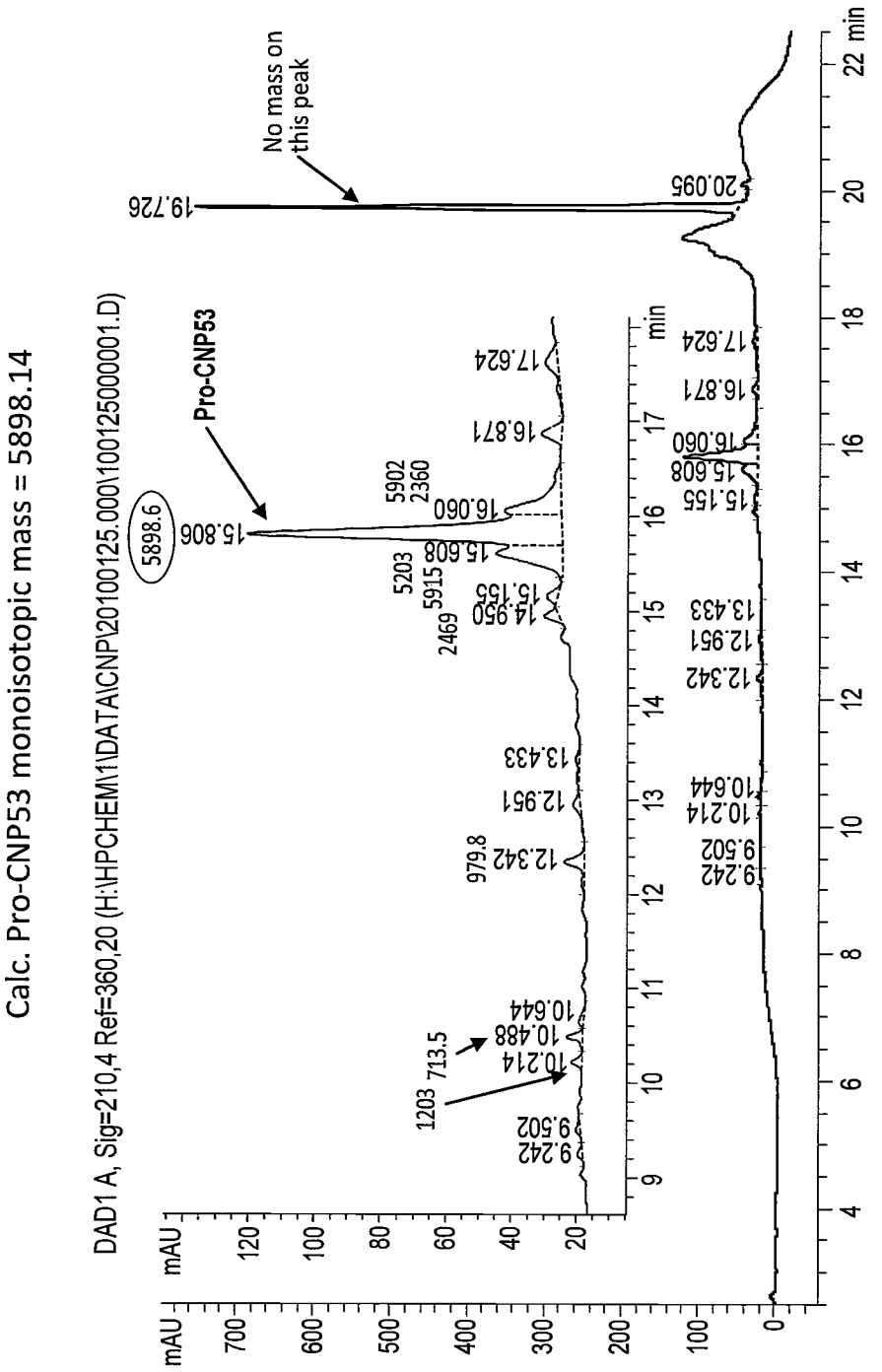

Calc. Pro-CNP53 monoisotopic mass = 5898.14
DAD1 A, Sig=210,4 Ref=360,20 (H:\HPCHEM\1\DATA\CNP\20100125.000\100125000001.D)
Pro-CNP53
No mass on this peak
5898.6
19.726
15.806
16.060
15.608
15.155
14.950
13.433
12.951
12.342
10.644
10.488
10.214
9.502
9.242
17.624
16.871
20.095
713.5
979.8
mAU
min
22 min

FIGURE 11

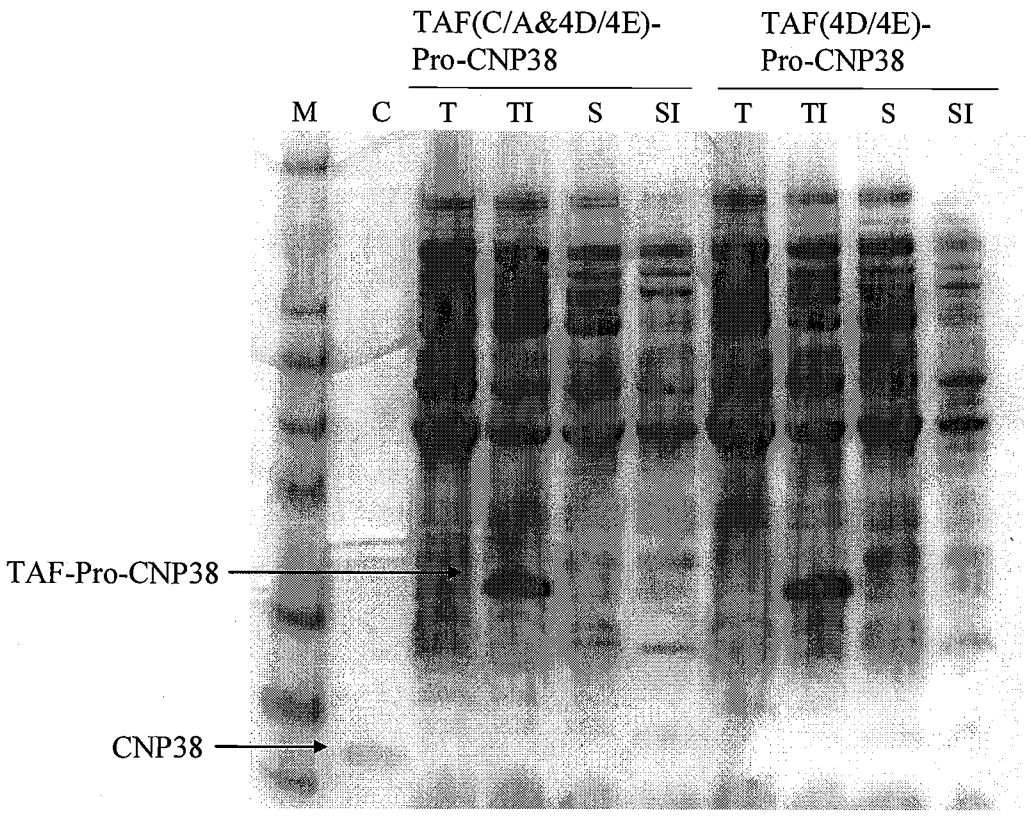

TAF(C/A&4D/4E)-
Pro-CNP38
TAF(4D/4E)-
Pro-CNP38
M
C
T
TI
S
SI
T
TI
S
SI
TAF-Pro-CNP38
CNP38

FIGURE 12

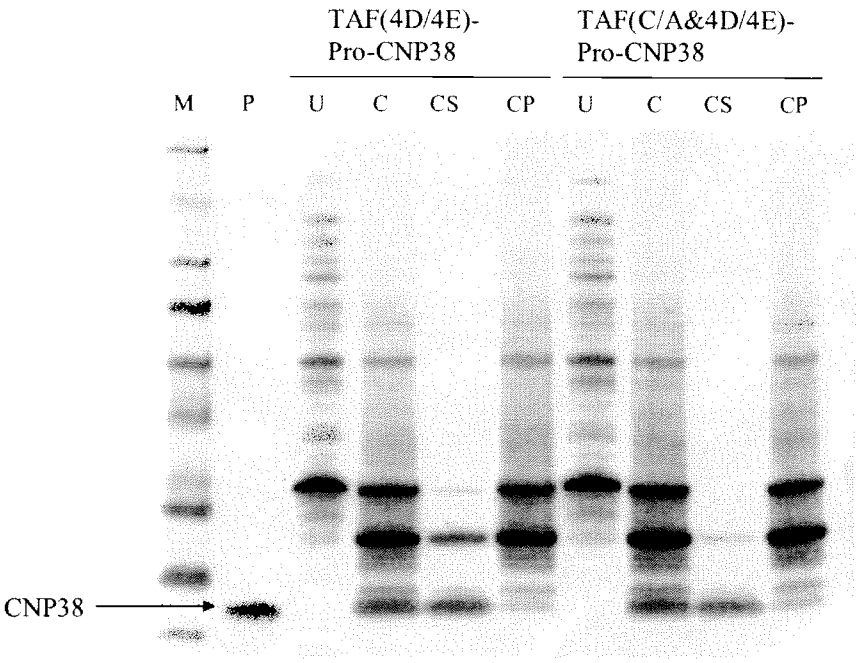


FIGURE 13

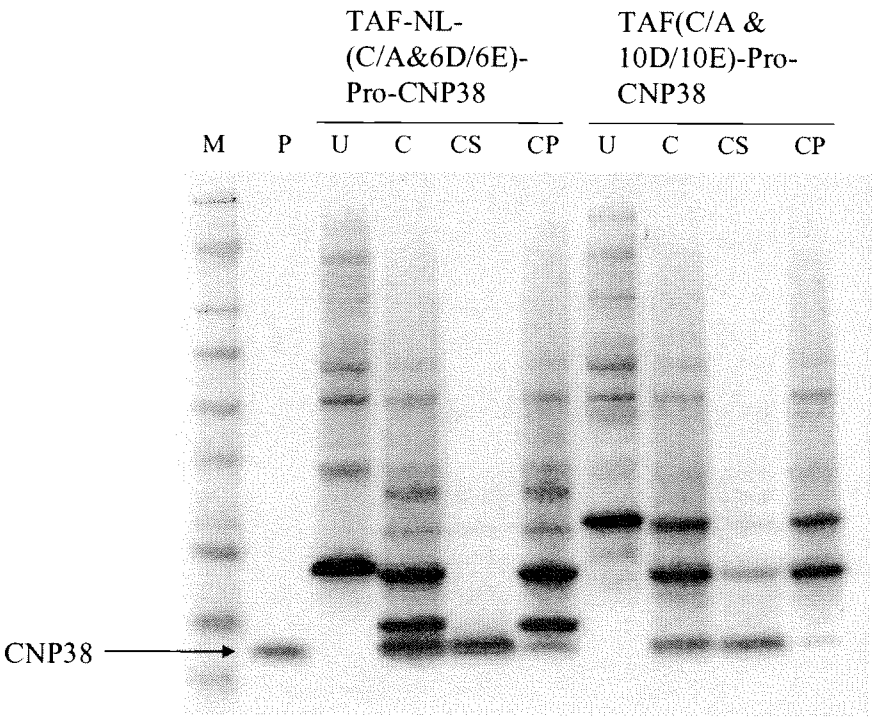


FIGURE 14

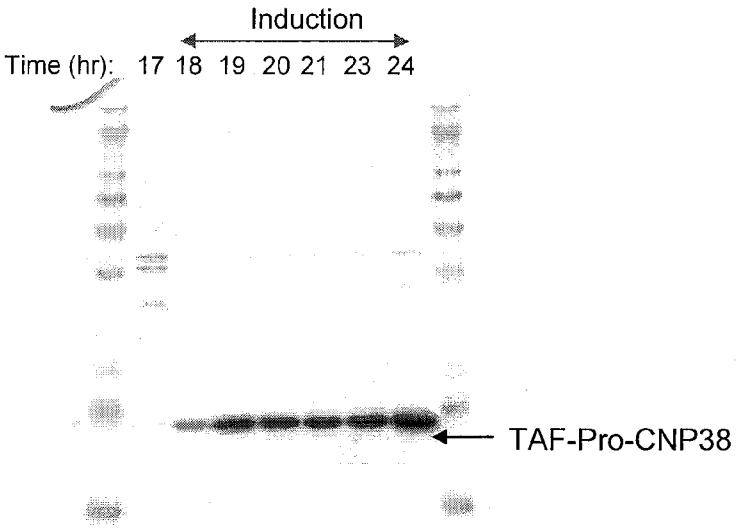


FIGURE 15

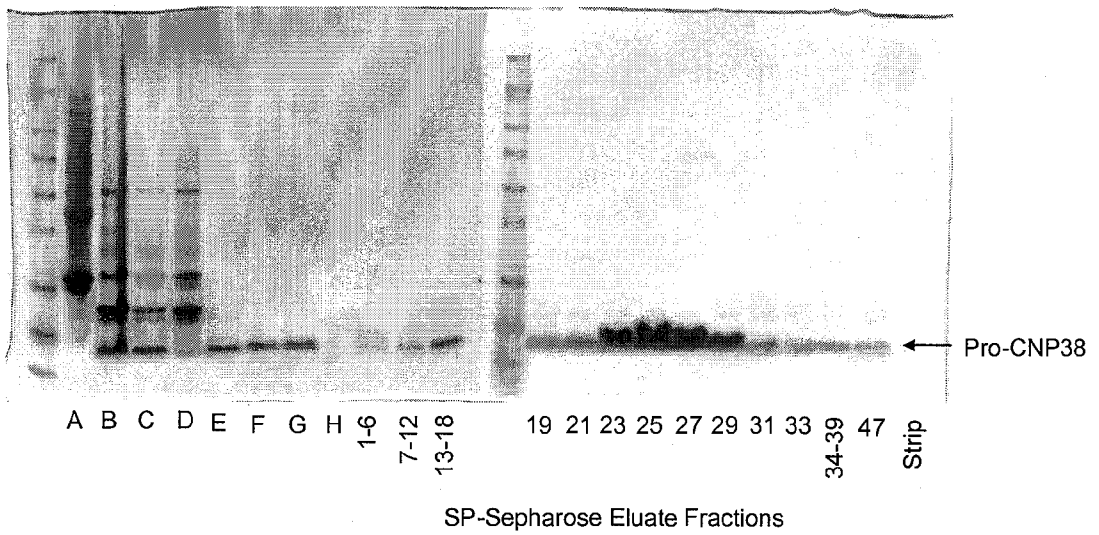


FIGURE 16

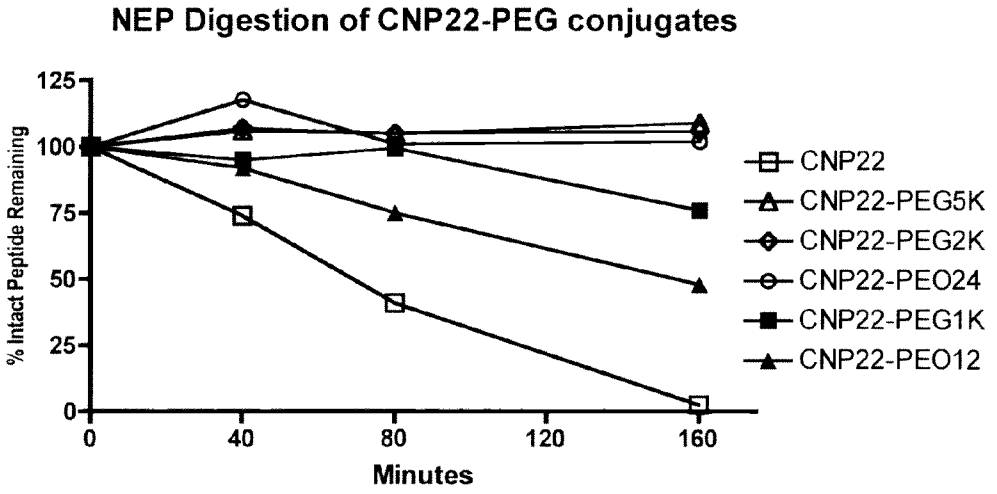


FIGURE 17

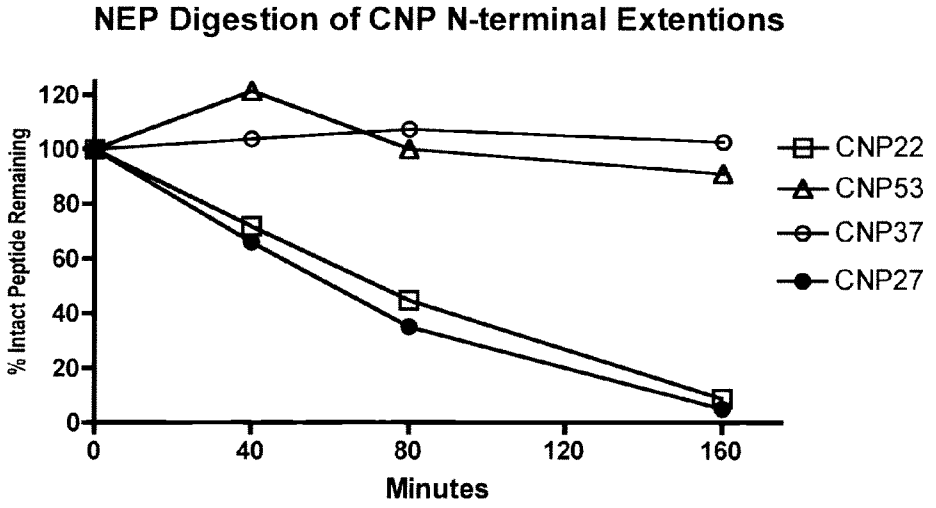


FIGURE 18

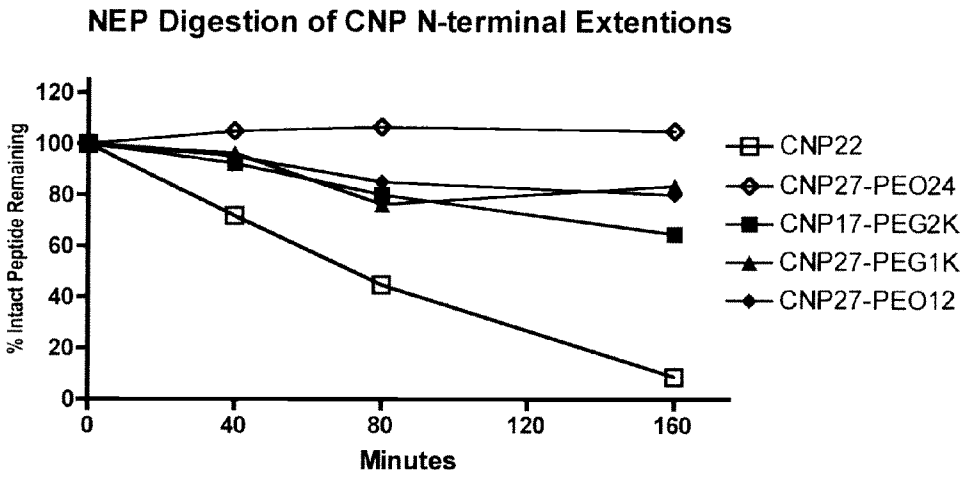


FIGURE 19

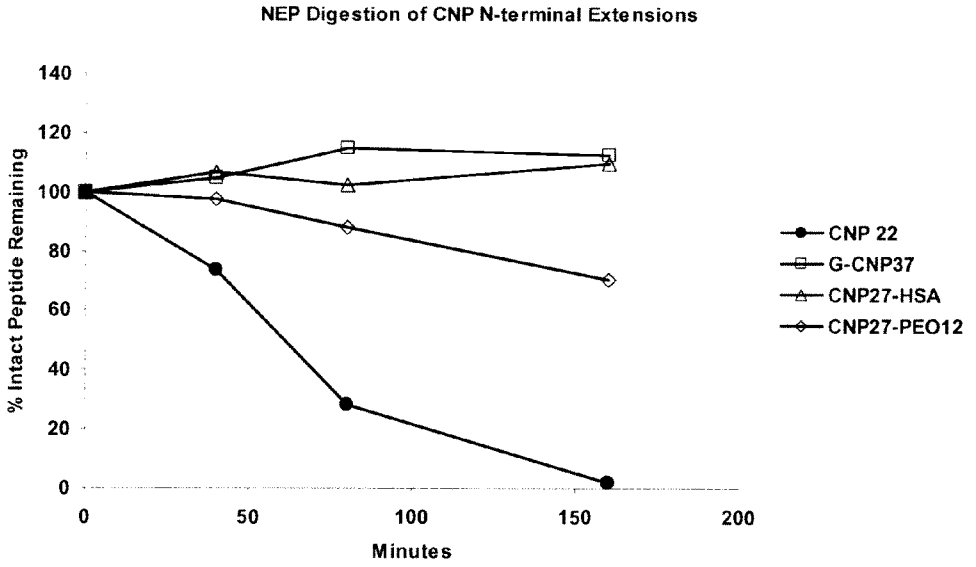


FIGURE 20

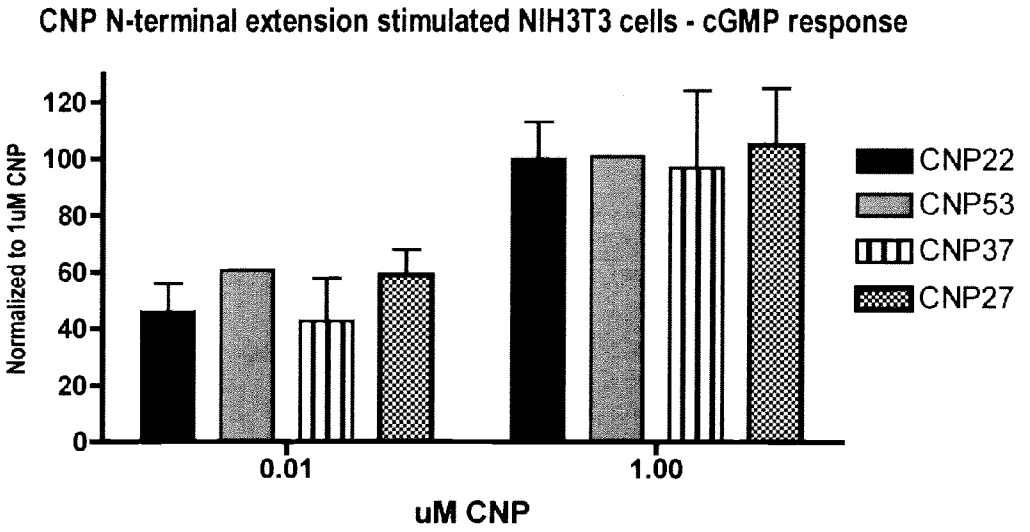


FIGURE 21

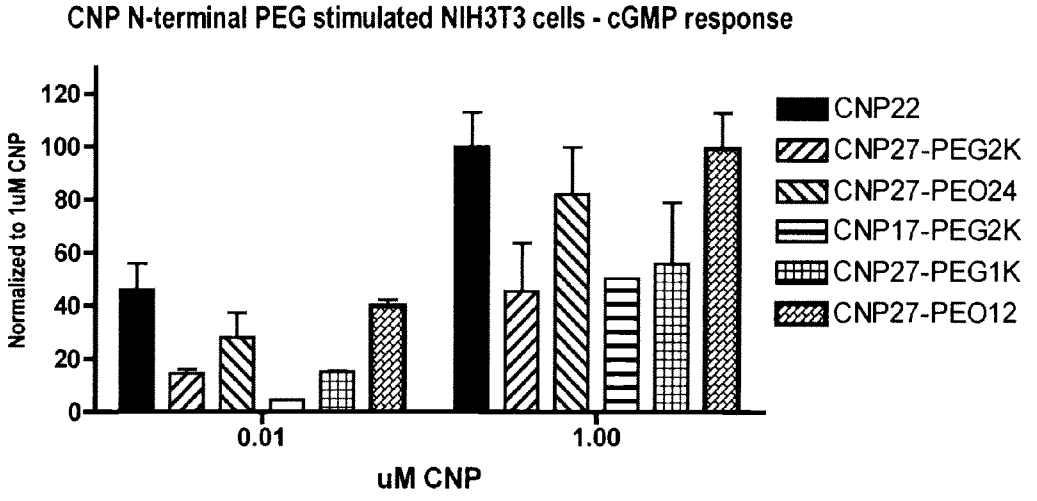


FIGURE 22

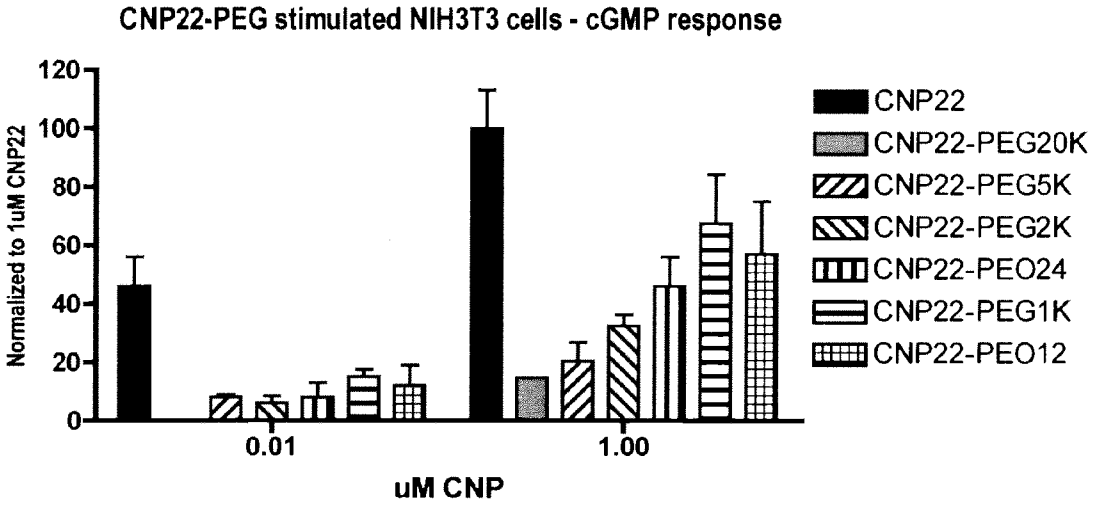


FIGURE 23

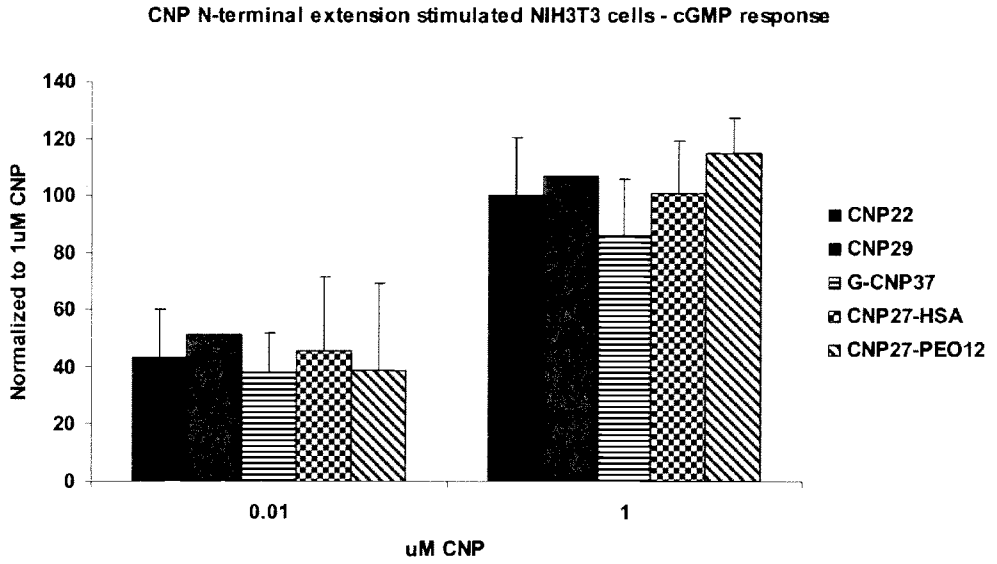
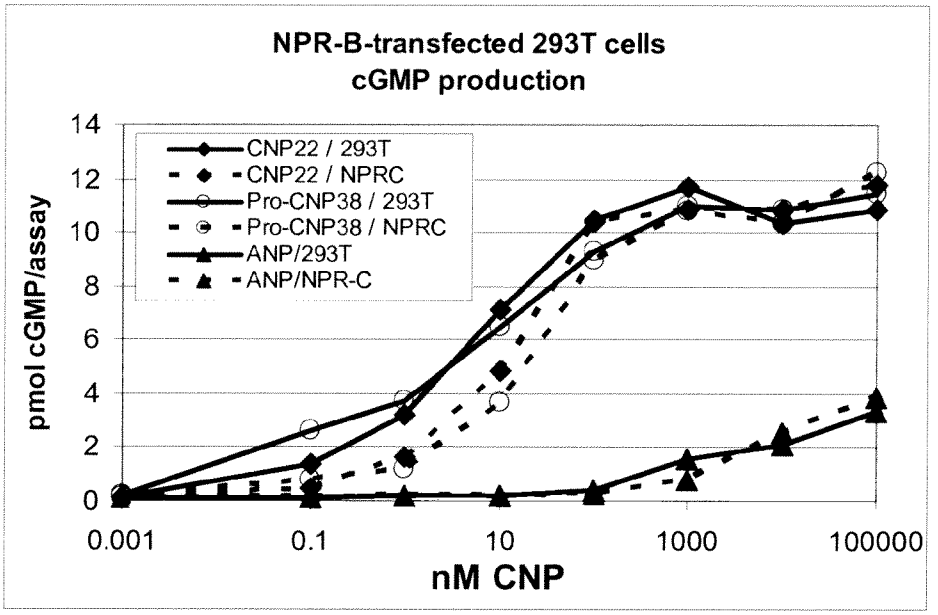


FIGURE 24

A.


NPR-B-transfected 293T cells
cGMP production
pmol cGMP/assay
nM CNP
CNP22 / 293T
CNP22 / NPRC
Pro-CNP38 / 293T
Pro-CNP38 / NPRC
ANP/293T
ANP/NPR-C
0
2
4
6
8
10
12
14
0.001
0.1
10
1000
100000

B.

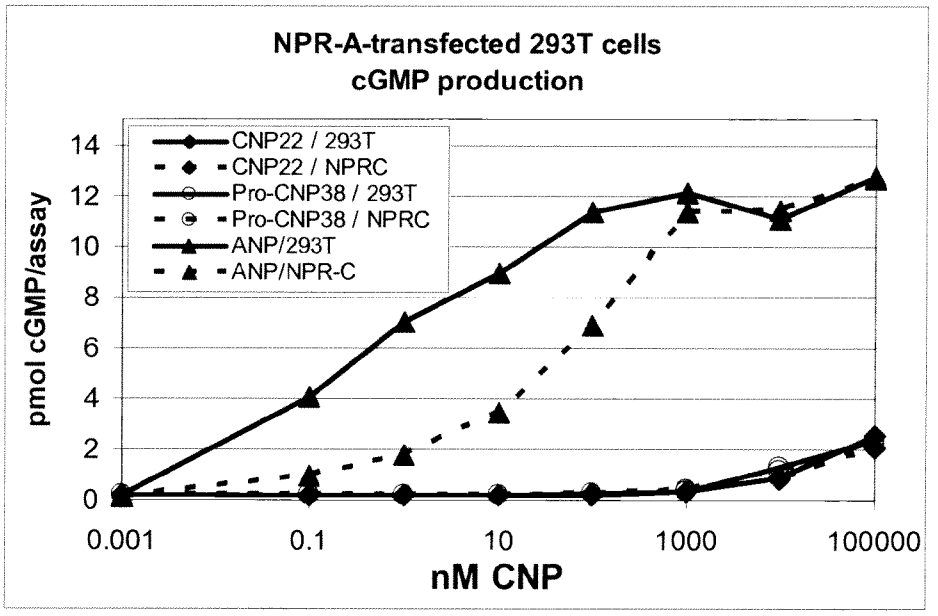
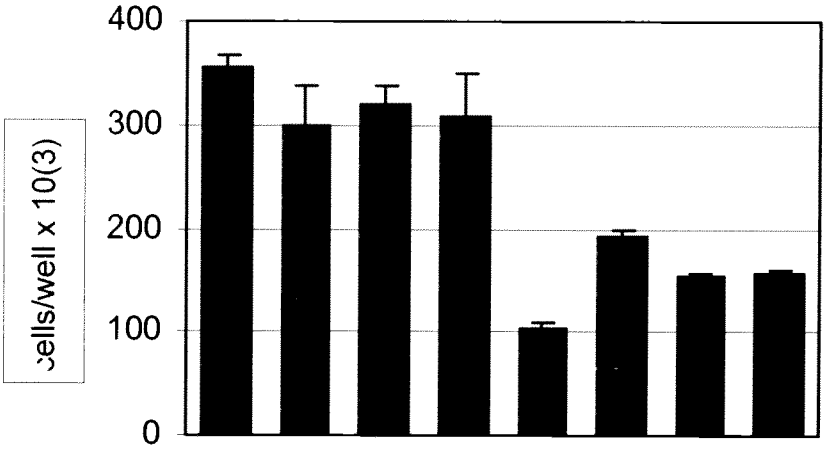

NPR-A-transfected 293T cells
cGMP production
pmol cGMP/assay
nM CNP
CNP22 / 293T
CNP22 / NPRC
Pro-CNP38 / 293T
Pro-CNP38 / NPRC
ANP/293T
ANP/NPR-C
0
2
4
6
8
10
12
14
0.001
0.1
10
1000
100000

FIGURE 25



Bars from left to right:

1 control (no CNP22, no FGF2)
2 CNP22 (0.2 uM) continuous
3 CNP22 (0.2 uM) 1 hr once daily
4 CNP22 (0.2 uM) 2 hr once daily
5 FGF2 (5 ng/mL) continuous
6 FGF2 + CNP22 (0.2 uM) continuous
7 FGF2 + CNP22 (0.2 uM) 1 hr daily
8 FGF2 + CNP22 (0.2 uM) 2 hr daily

FIGURE 26

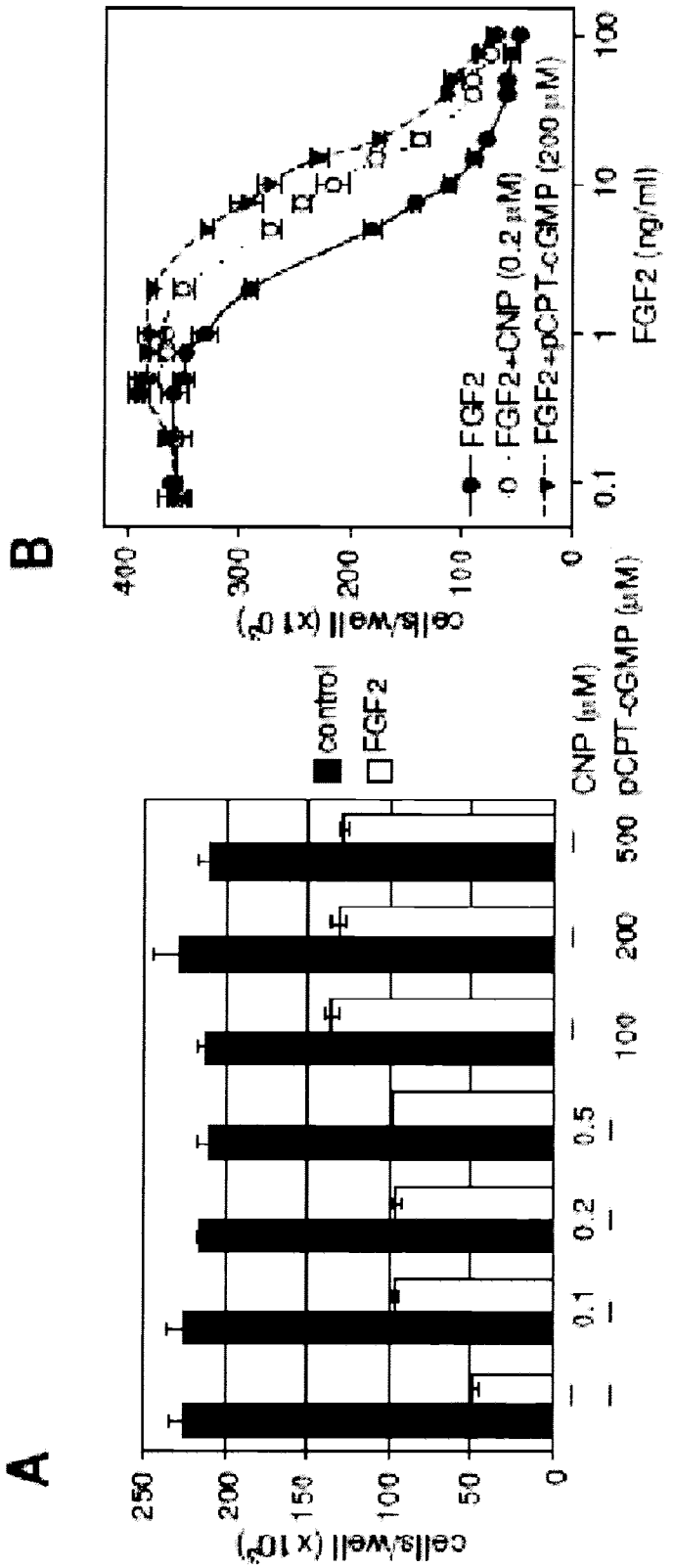

A
cells/well (x 10⁴)
250
200
150
100
50
0
control
FGF2
—
0.1
0.2
0.5
100
200
500
CNP (μM)
pCPT-cGMP (μM)
B
cells/well (x 10⁴)
400
300
200
100
0
FGF2
FGF2+CNP (0.2 μM)
FGF2+pCPT-cGMP (200 μM)
0.1
1
10
100
FGF2 (ng/ml)

FIGURE 27

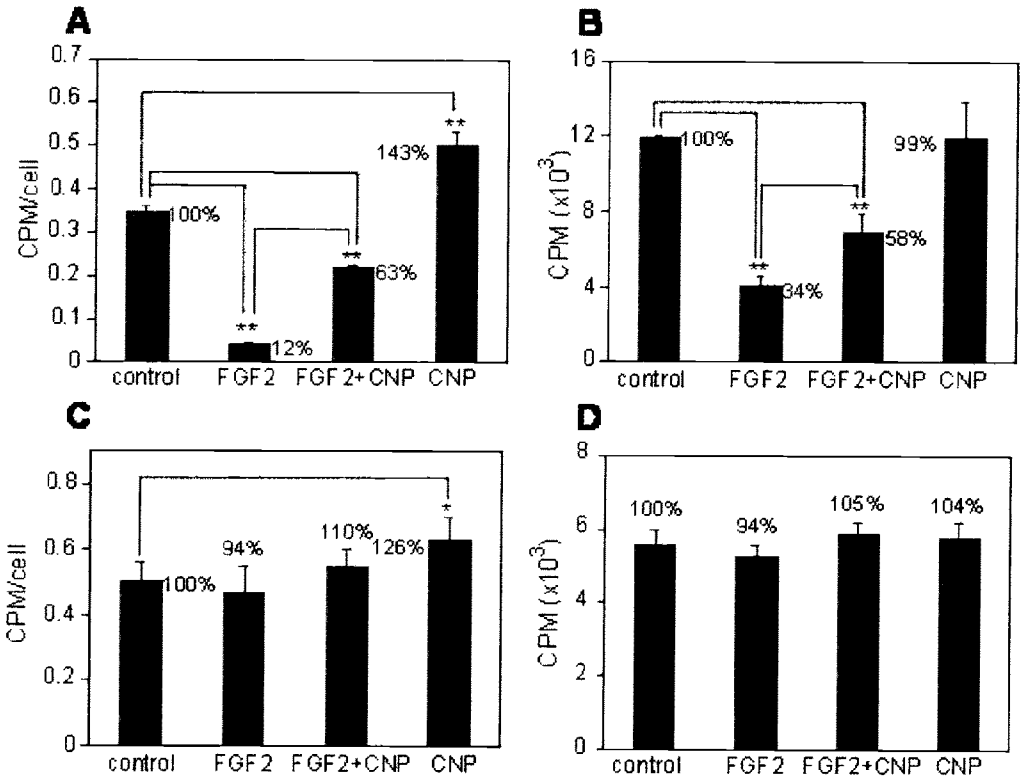

A
CPM/cell
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100%
12%
63%
143%
**
control
FGF2
FGF2+CNP
CNP
B
CPM (x10^3)
16
12
8
4
0
100%
34%
58%
99%
control
FGF2
FGF2+CNP
CNP
C
CPM/cell
0.8
0.6
0.4
0.2
0
100%
94%
110%
126%
control
FGF2
FGF2+CNP
CNP
D
CPM (x10^3)
8
6
4
2
0
100%
94%
105%
104%
control
FGF2
FGF2+CNP
CNP

FIGURE 28

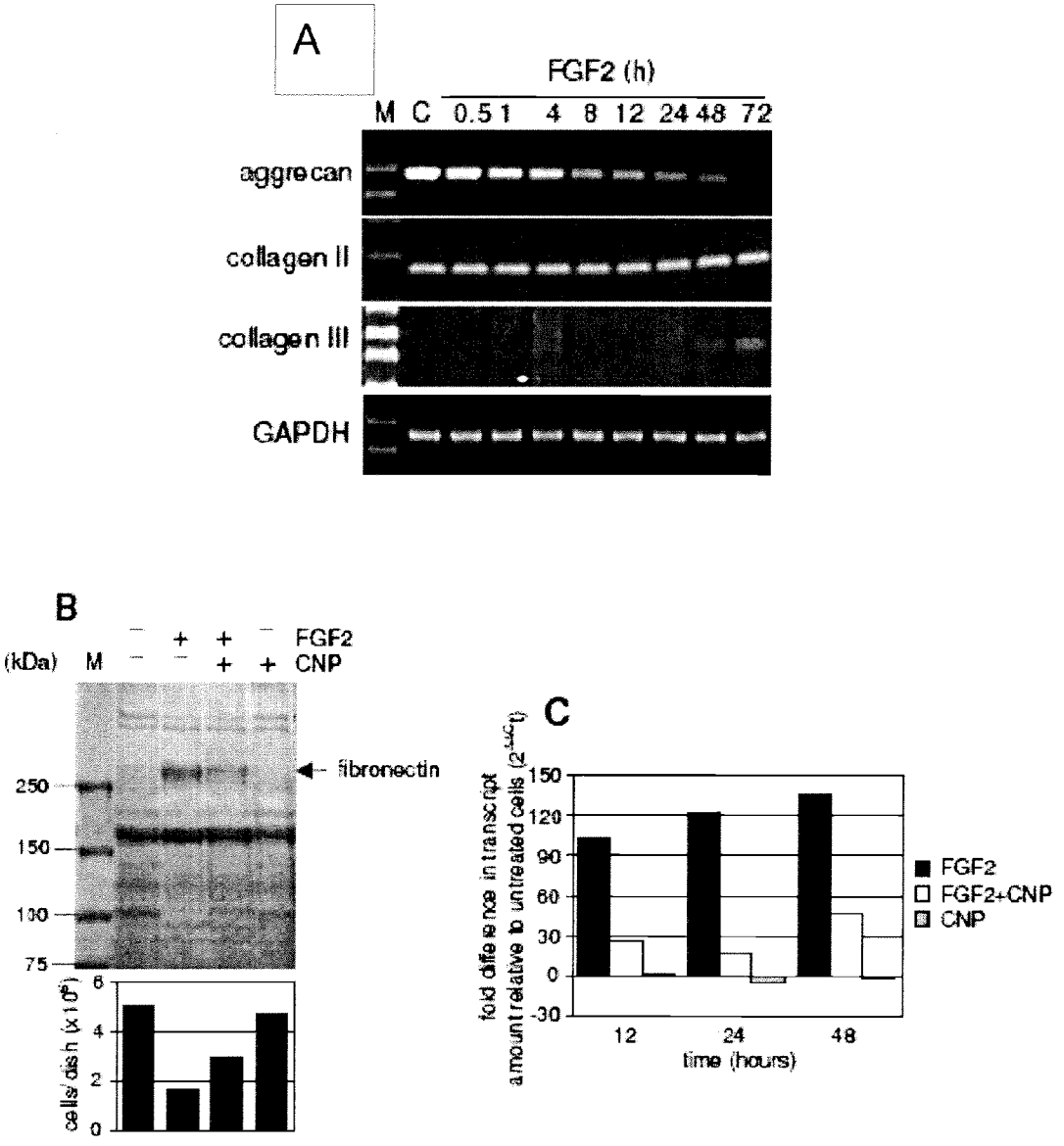

A
FGF2 (h)
M C 0.5 1 4 8 12 24 48 72
aggrecan
collagen II
collagen III
GAPDH
B
(kDa) M
FGF2
CNP
fibronectin
250
150
100
75
cells/dish (x10⁵)
C
fold difference in transcript amount relative to untreated cells
time (hours)
FGF2
FGF2+CNP
CNP

FIGURE 29

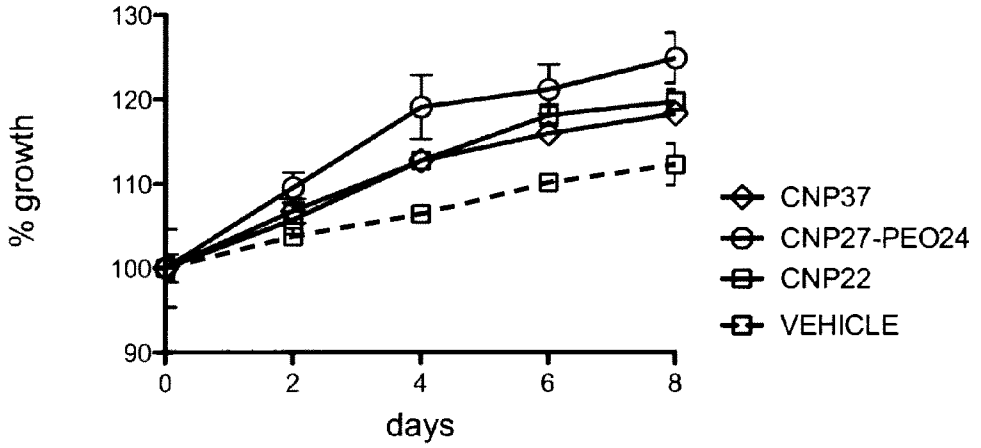


FIGURE 30

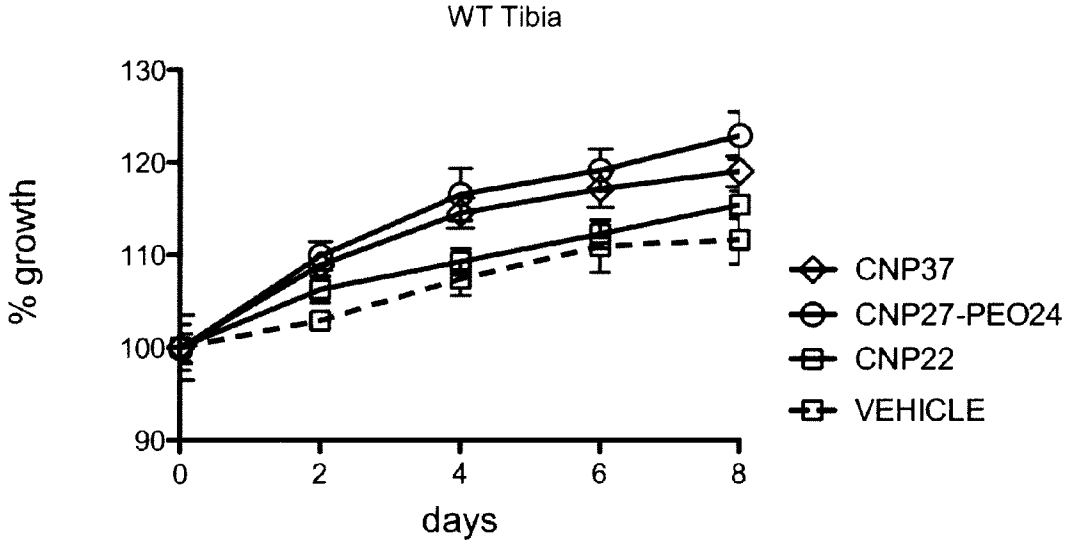


FIGURE 31

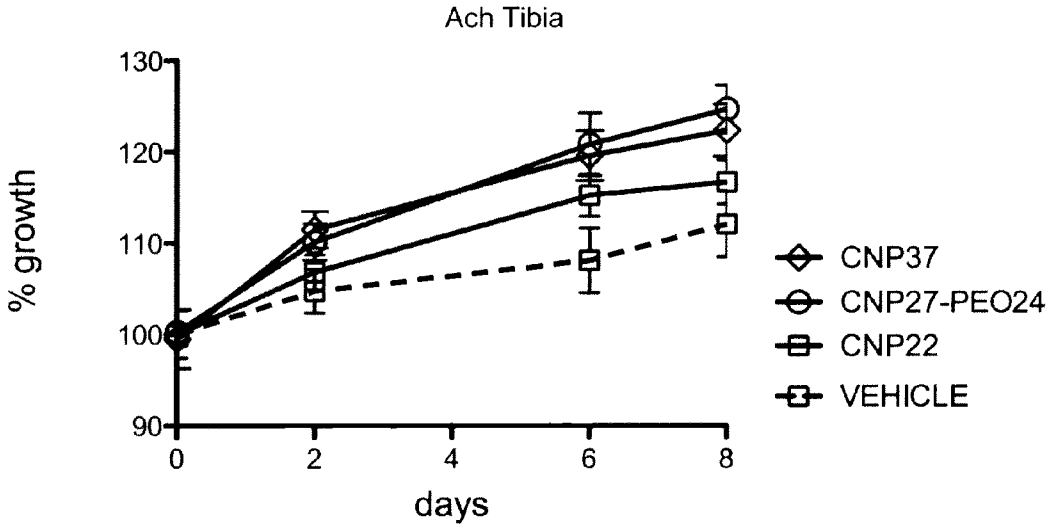


FIGURE 32

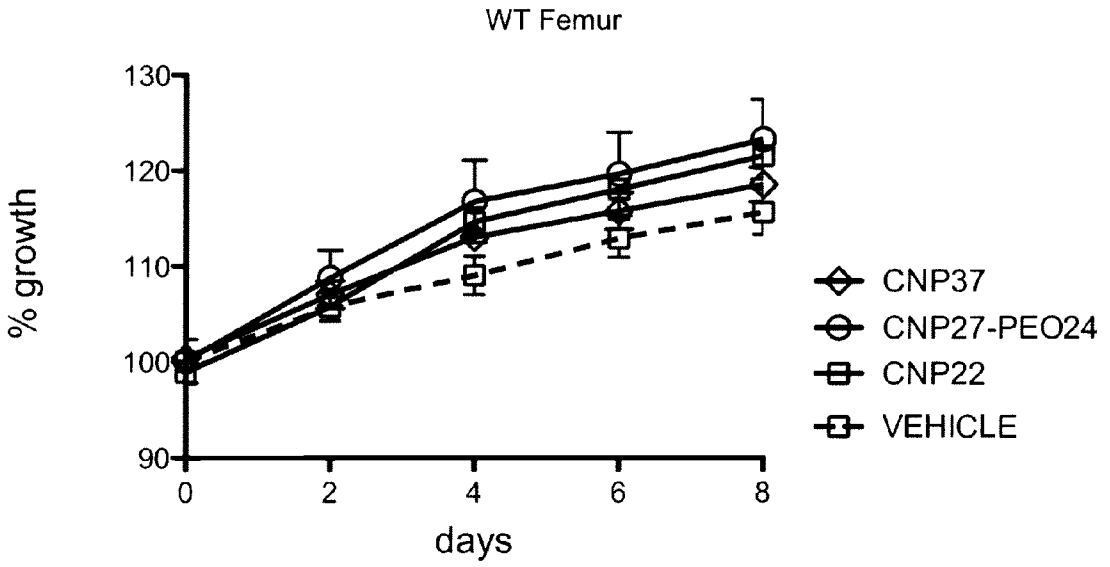


FIGURE 33

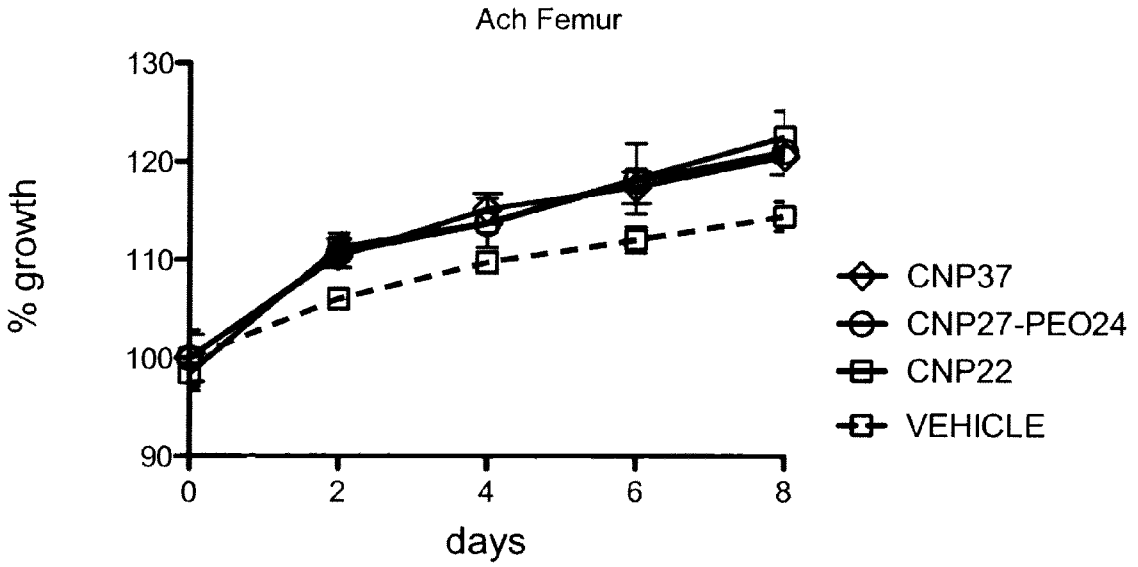

Ach Femur
% growth
130
120
110
100
90
0
2
4
6
8
days
CNP37
CNP27-PEO24
CNP22
VEHICLE

FIGURE 34

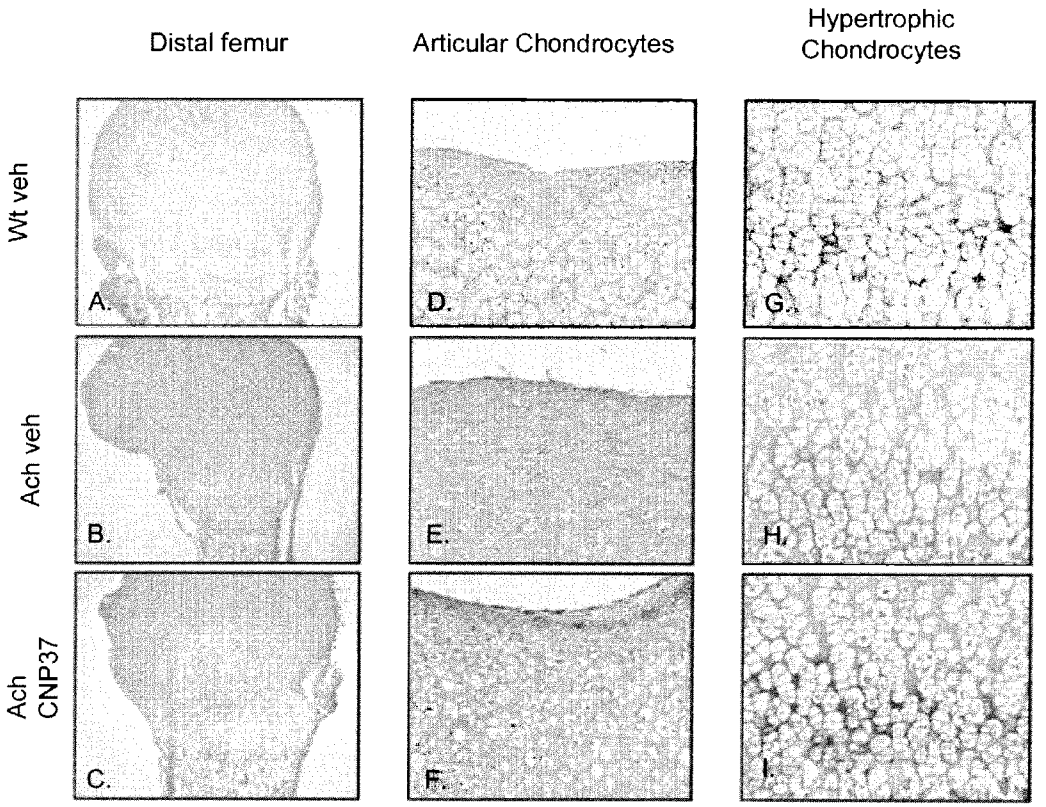

Distal femur
Articular Chondrocytes
Hypertrophic Chondrocytes
Wt veh
Ach veh
Ach CNP37
A.
B.
C.
D.
E.
F.
G.
H.
I.

FIGURE 35

Cellularity of Proliferating Columns

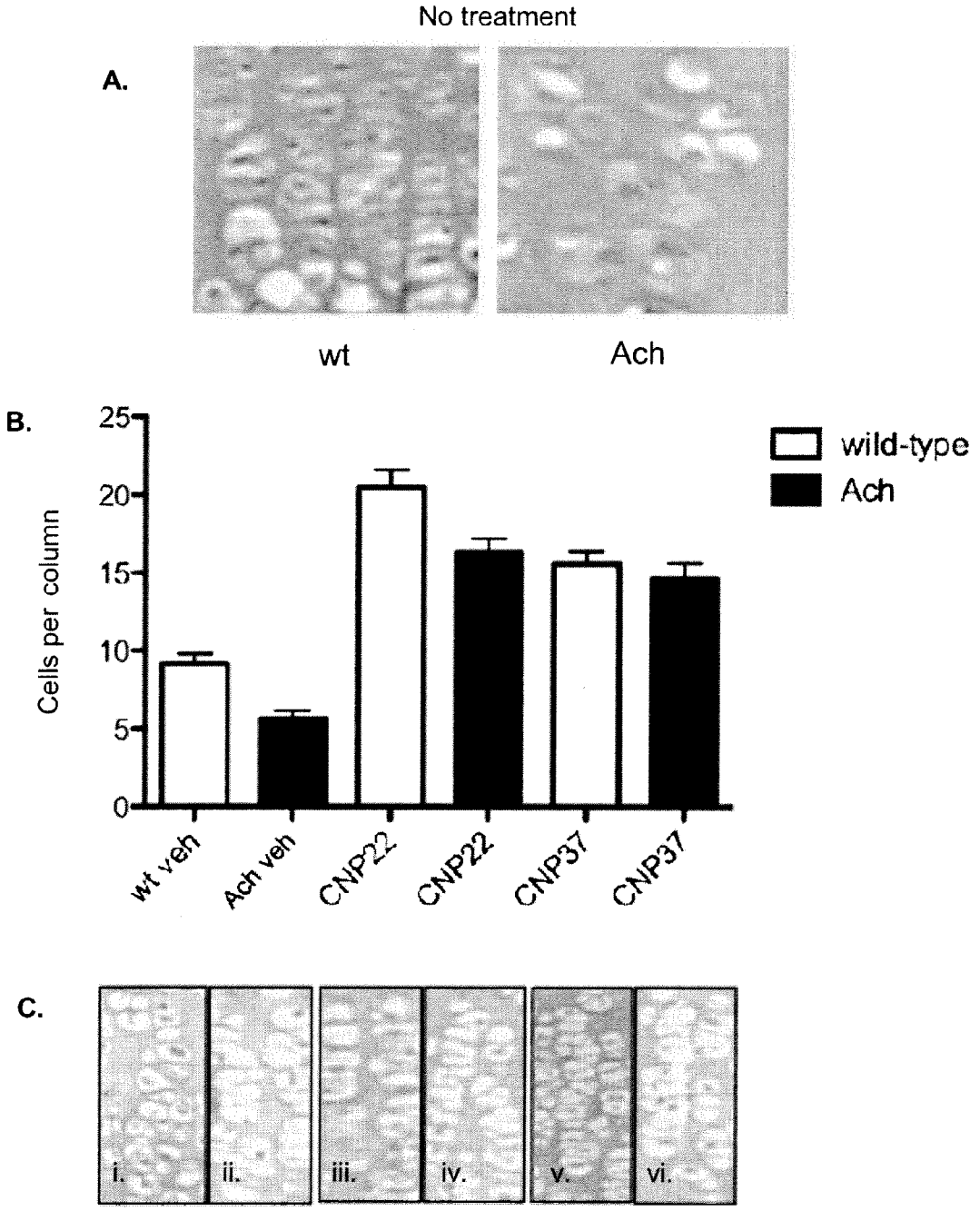


FIGURE 36

Chondrocyte Hypertrophy

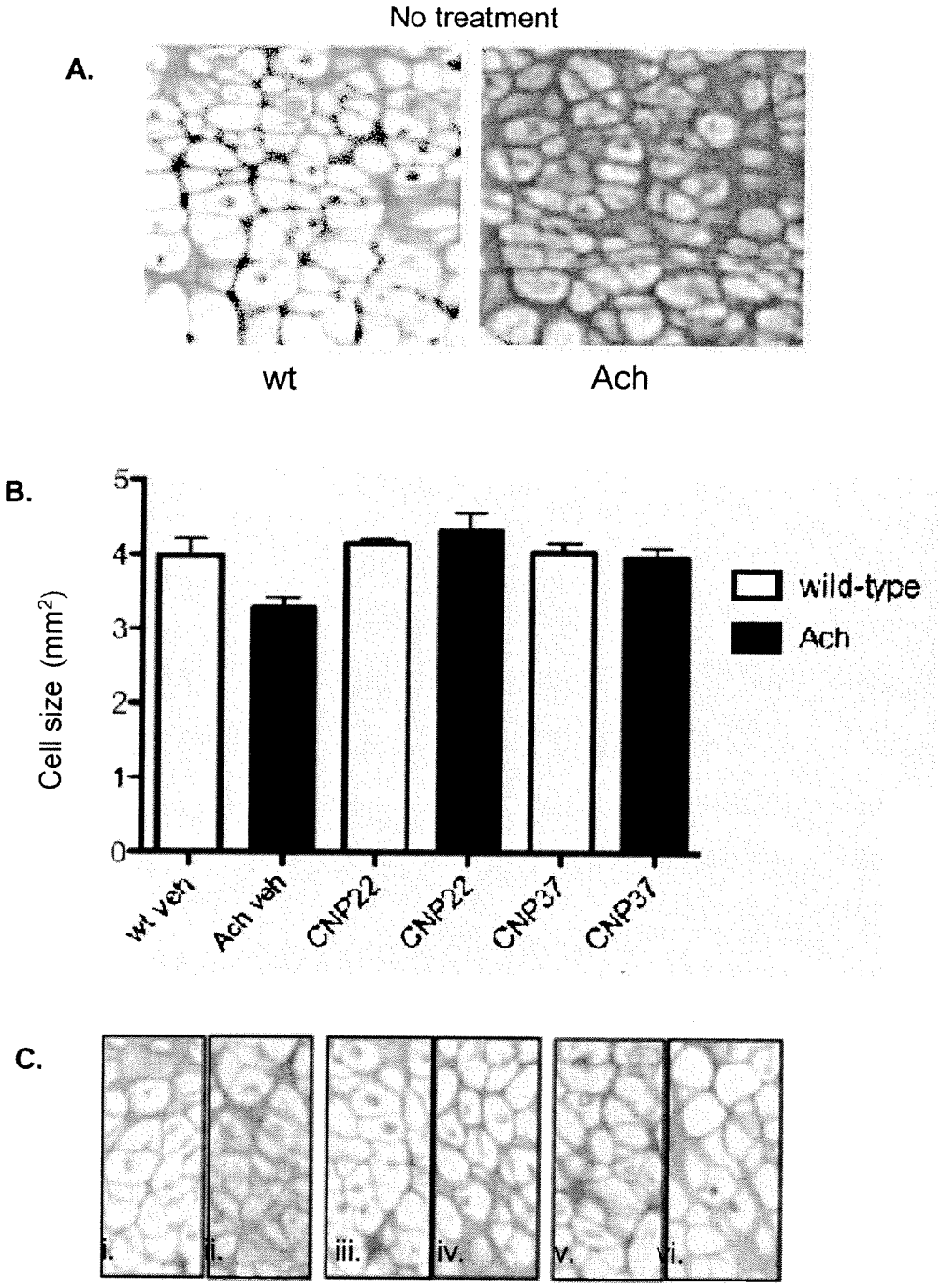


FIGURE 37

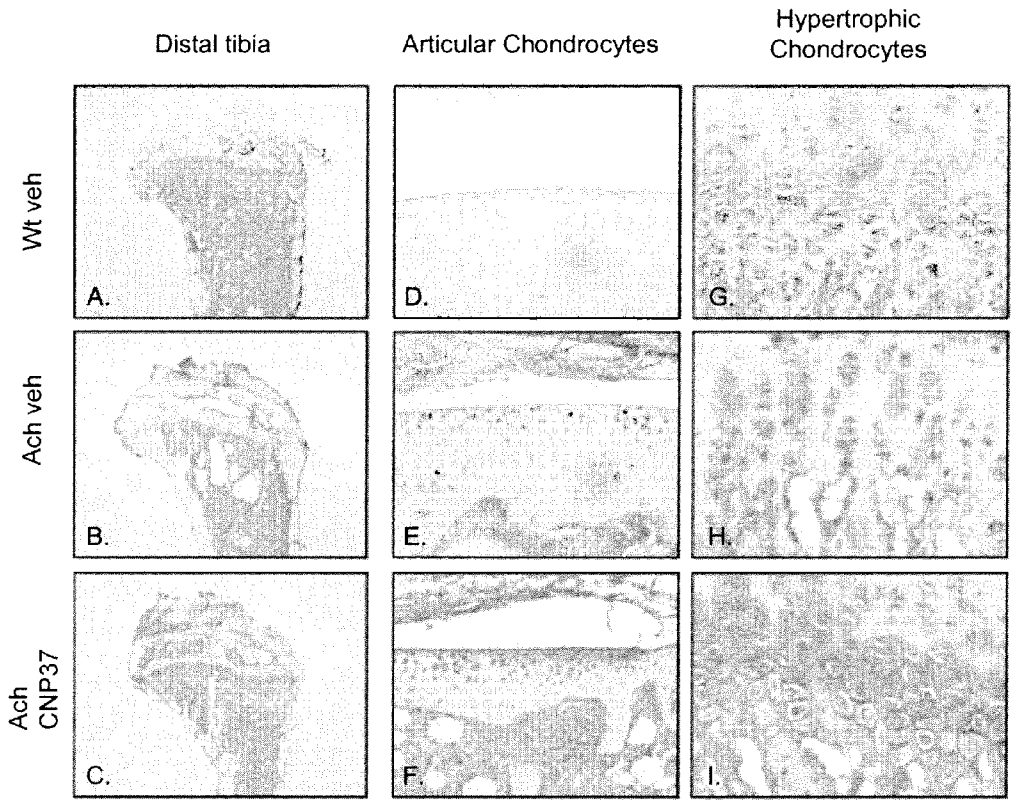
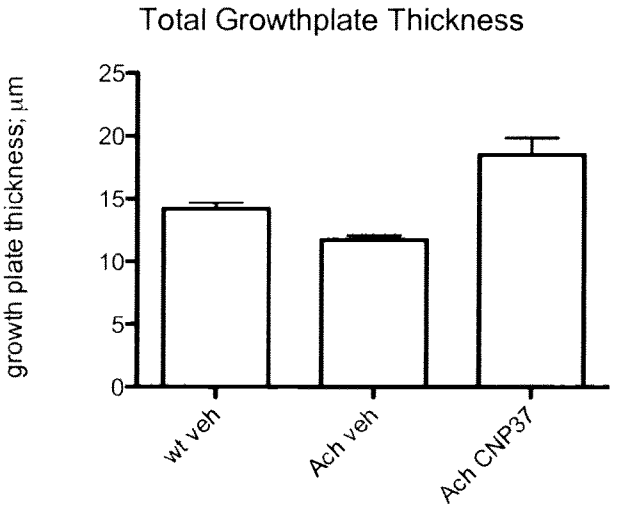
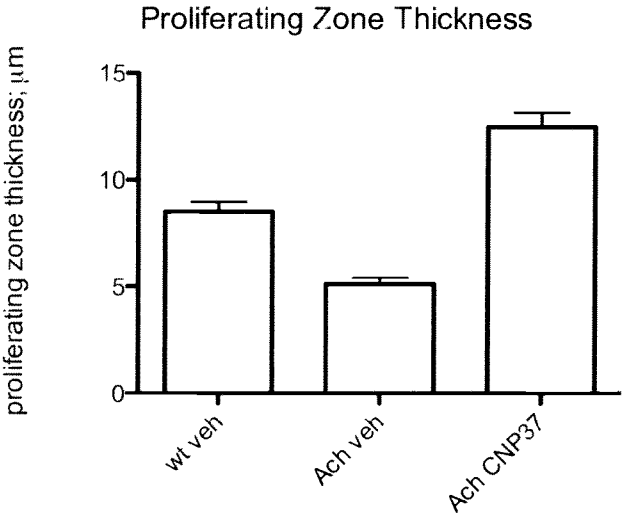

Distal tibia
Articular Chondrocytes
Hypertrophic Chondrocytes
Wt veh
Ach veh
Ach CNP37
A.
B.
C.
D.
E.
F.
G.
H.
I.

FIGURE 38

A.



B.



C.

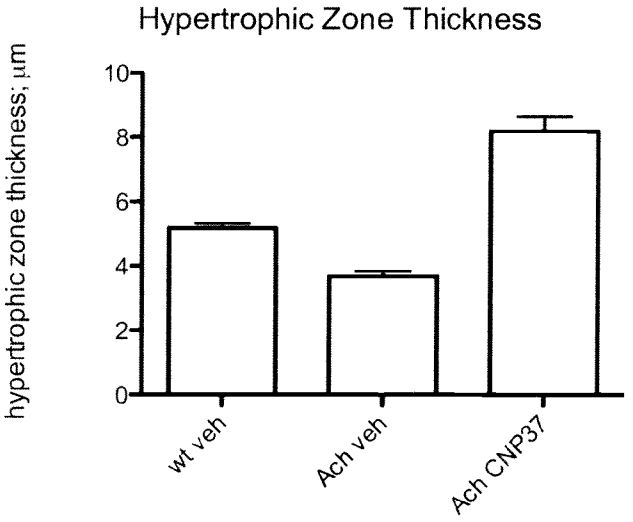


FIGURE 39

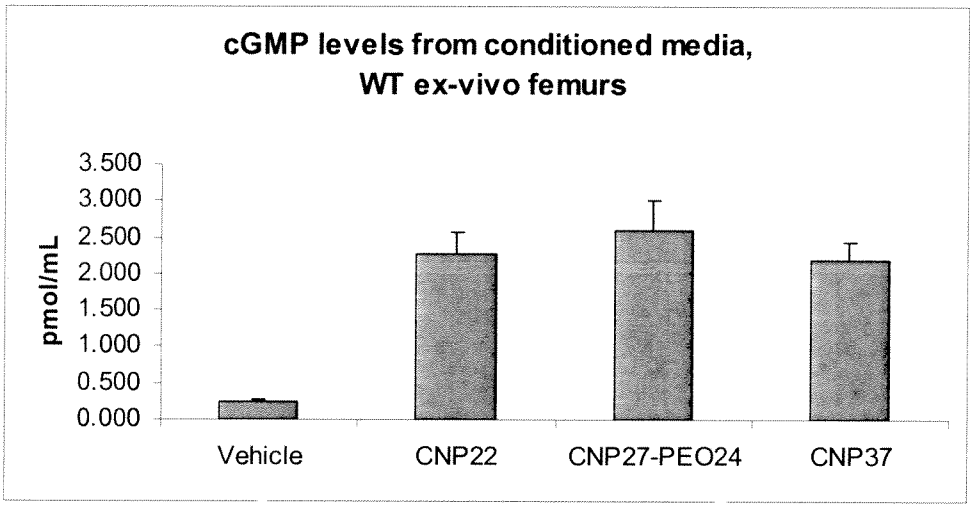


FIGURE 40

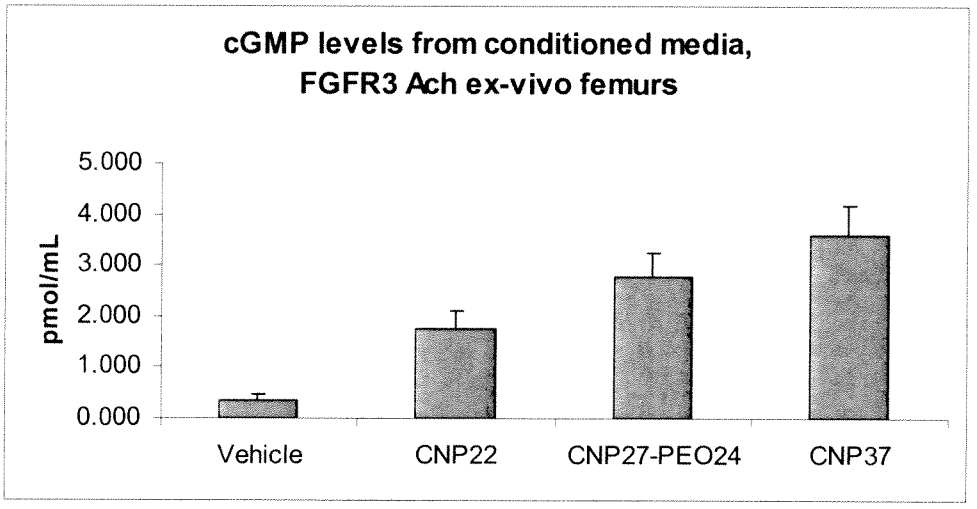


FIGURE 41

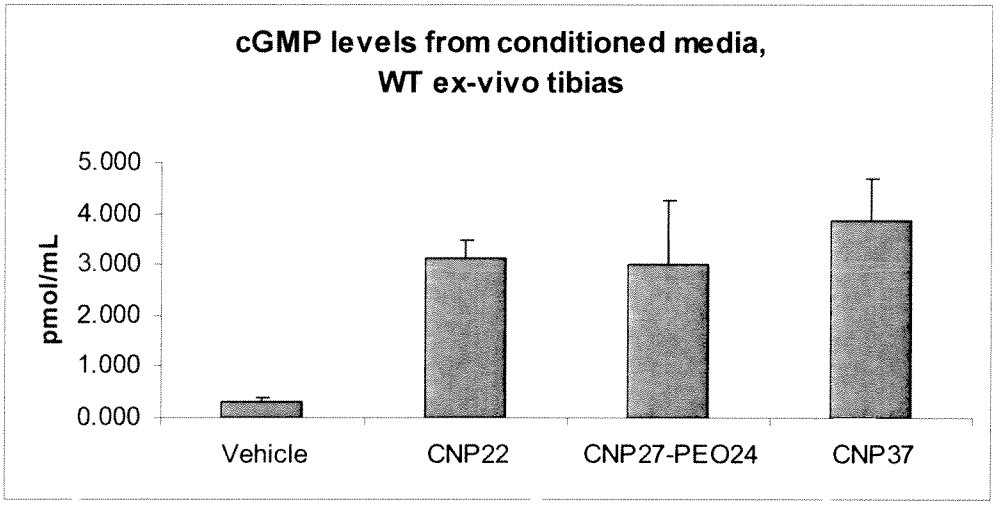


FIGURE 42

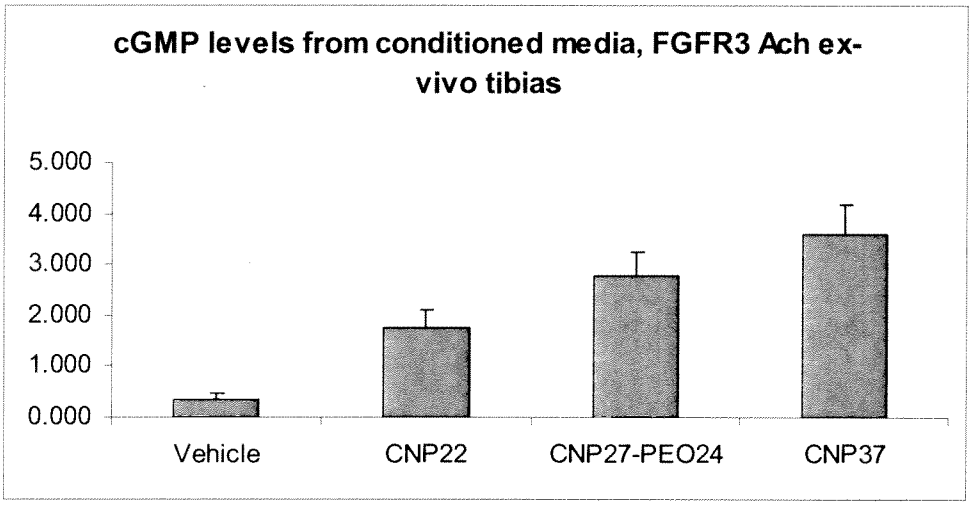


FIGURE 43

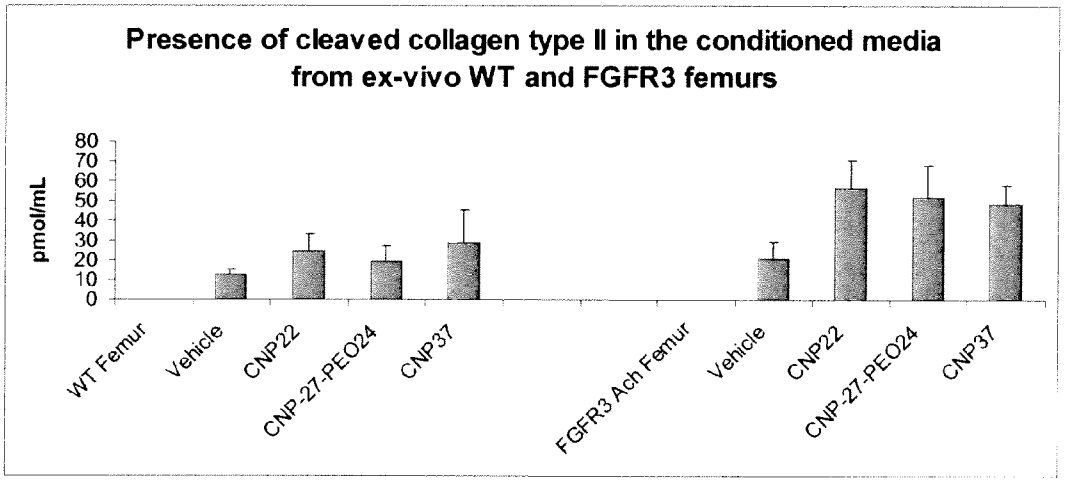


FIGURE 44

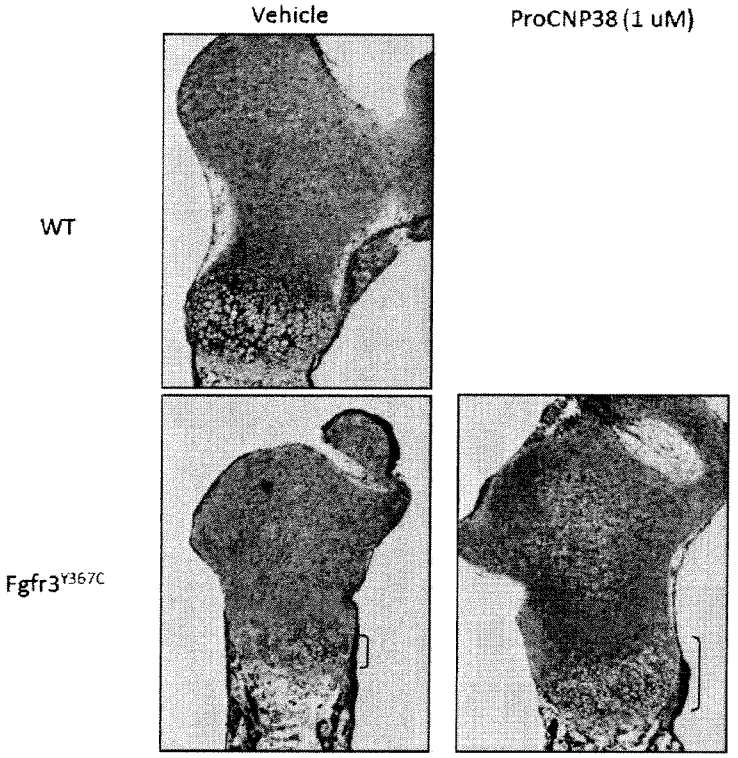


FIGURE 45

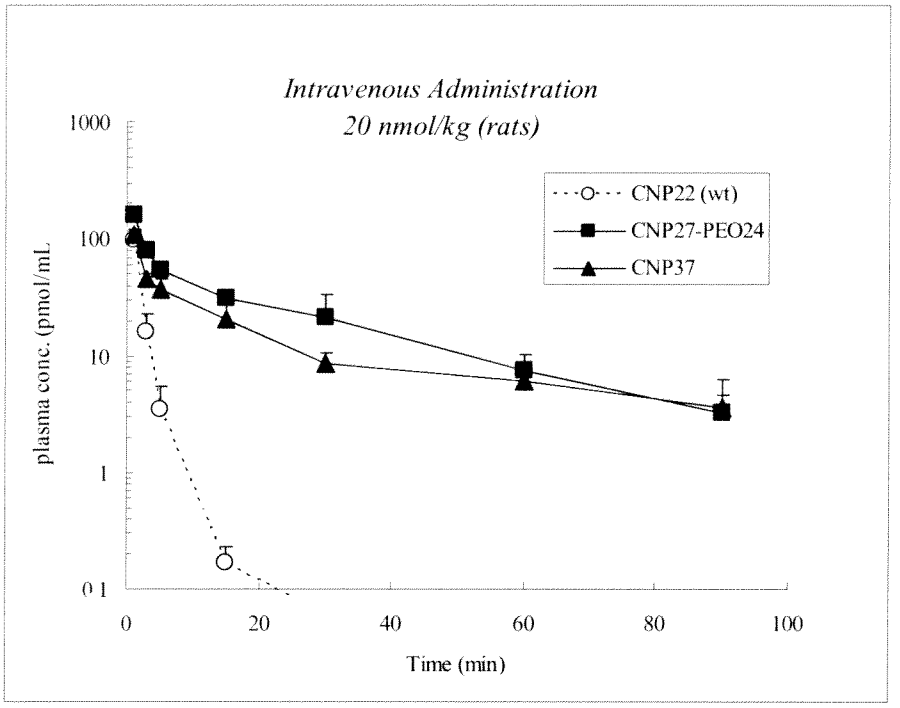


FIGURE 46

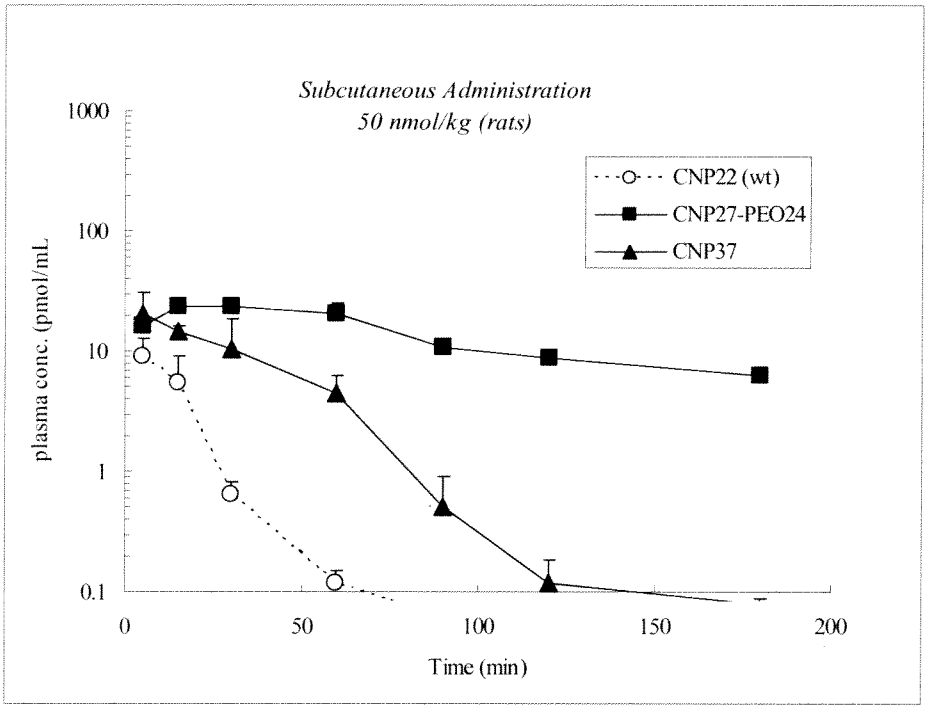


FIGURE 47

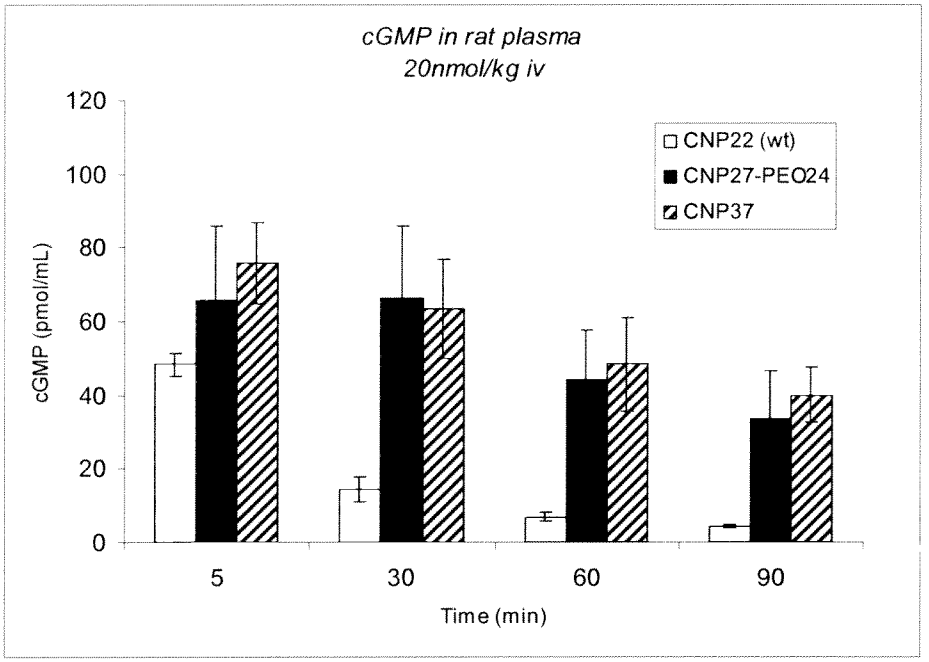


FIGURE 48

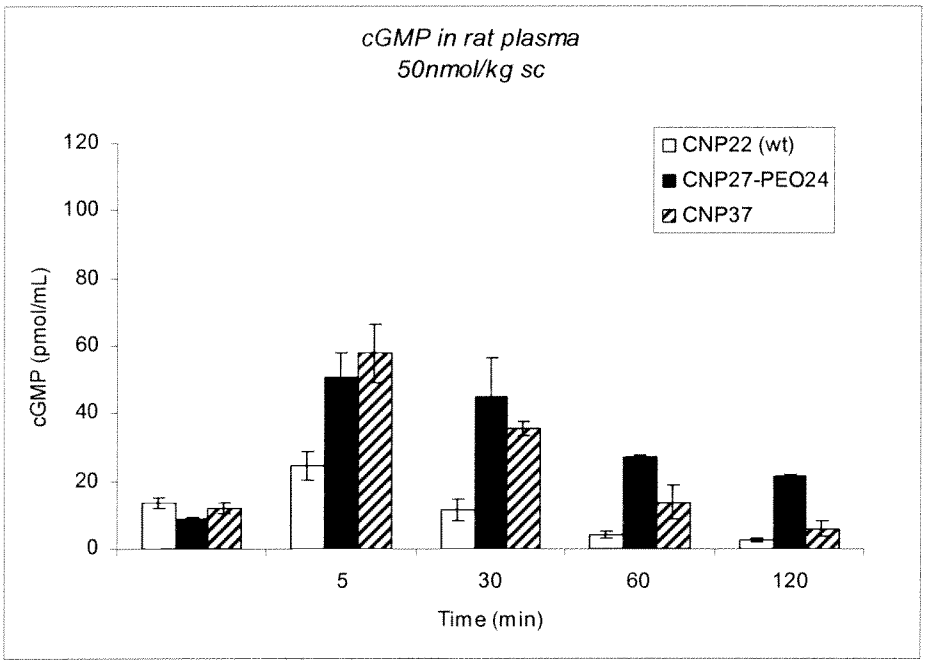


FIGURE 49

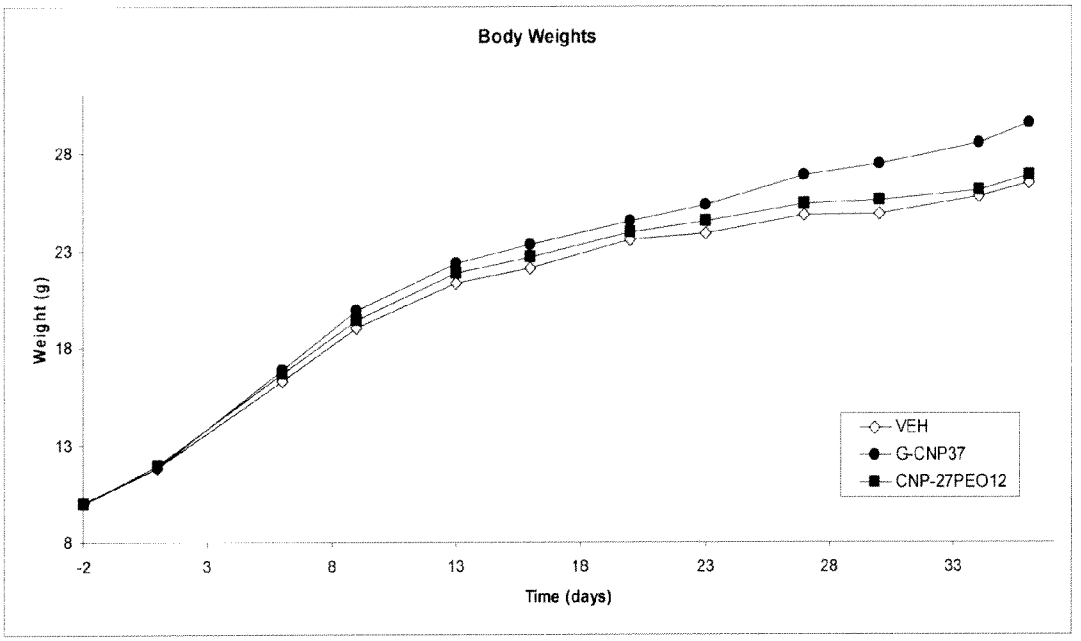


FIGURE 50

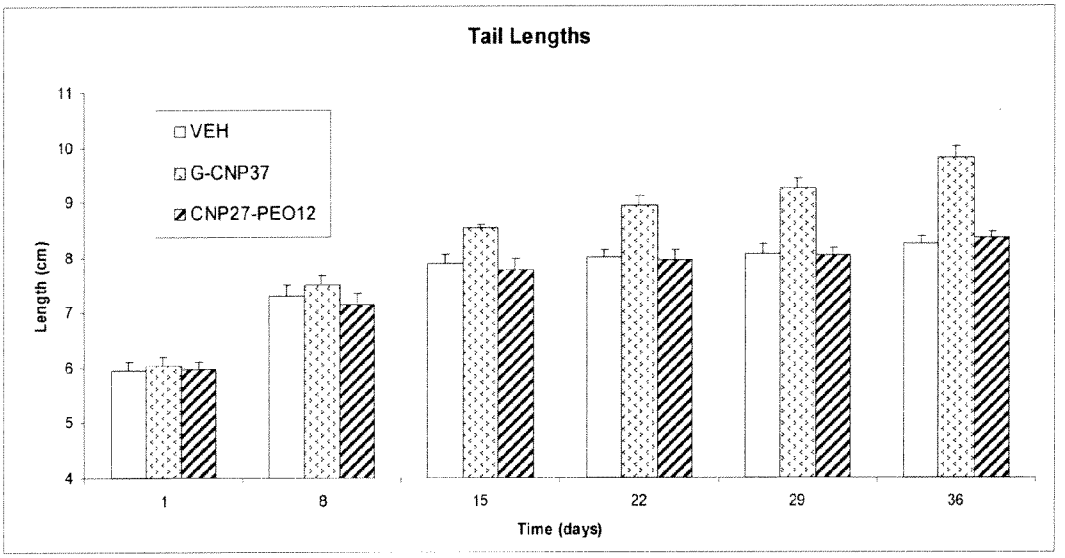


FIGURE 51

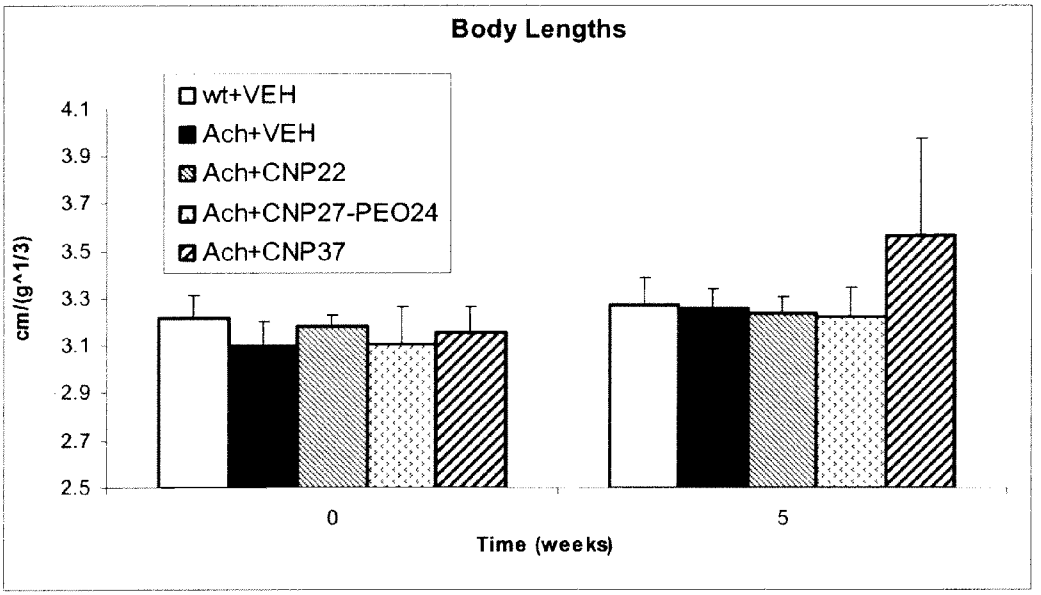


FIGURE 52

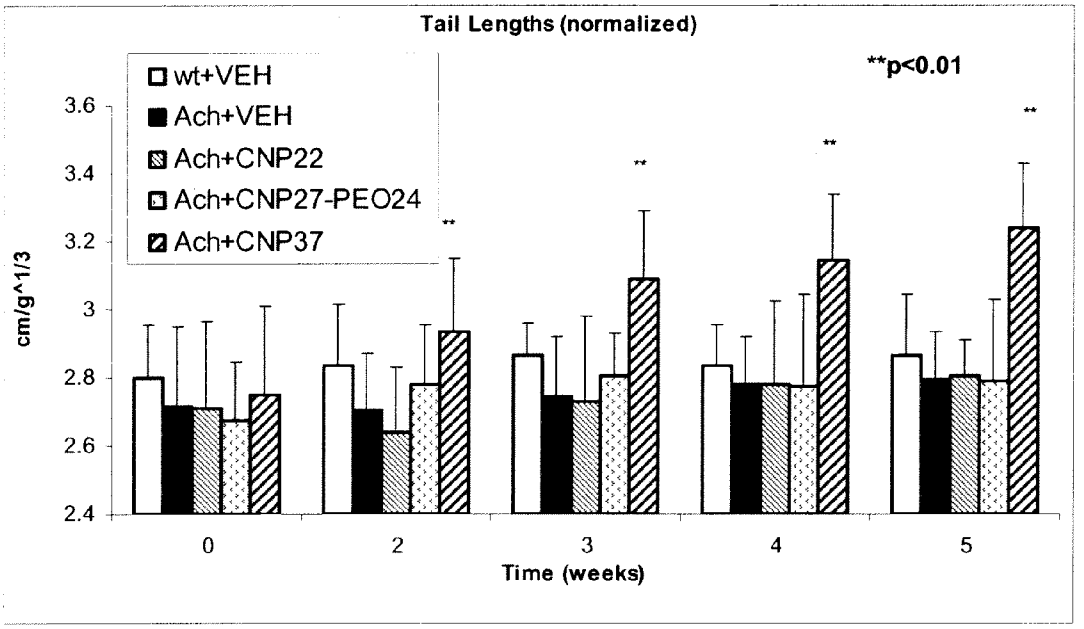
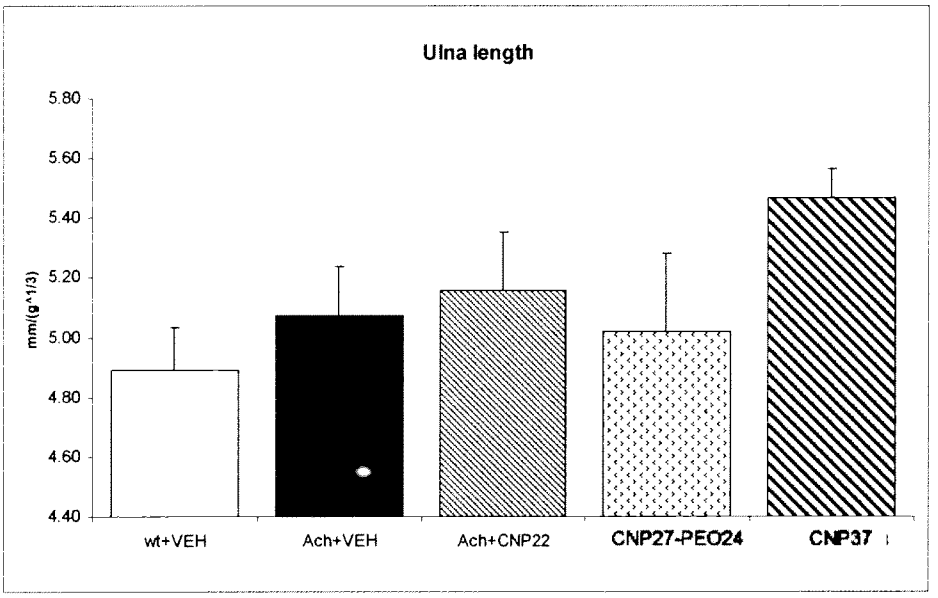


FIGURE 53

A.


Ulna length
5.80
5.60
5.40
5.20
5.00
4.80
4.60
4.40
mm/(g^1/3)
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

B.

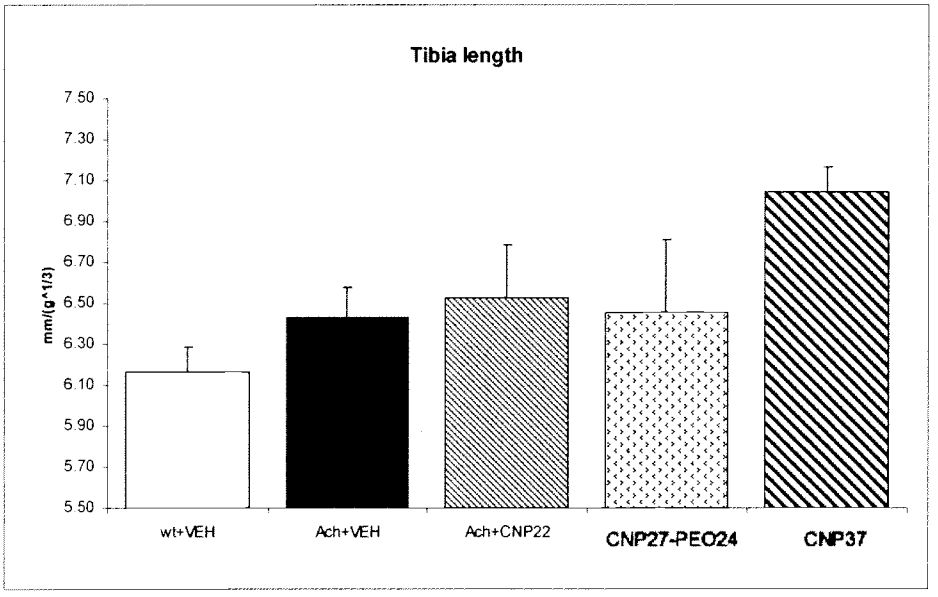
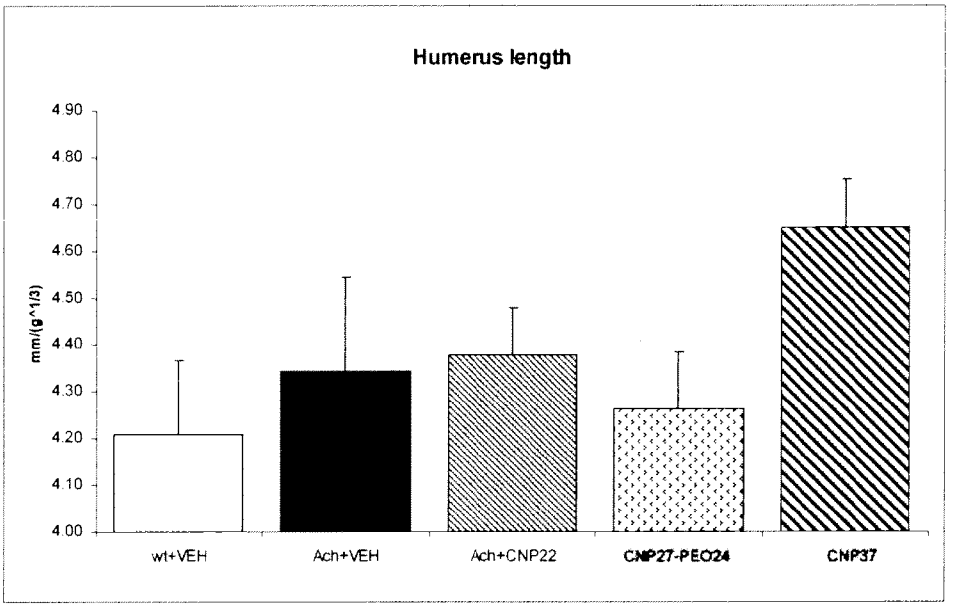

Tibia length
7.50
7.30
7.10
6.90
6.70
6.50
6.30
6.10
5.90
5.70
5.50
mm/(g^1/3)
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

FIGURE 54

A.


Humerus length
mm/(g^1/3)
4.90
4.80
4.70
4.60
4.50
4.40
4.30
4.20
4.10
4.00
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

B.

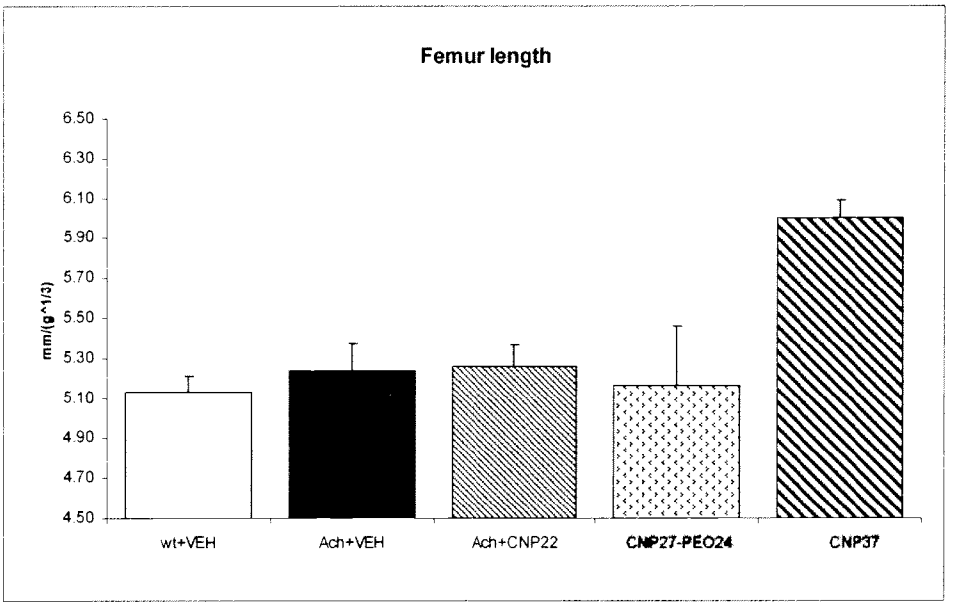

Femur length
mm/(g^1/3)
6.50
6.30
6.10
5.90
5.70
5.50
5.30
5.10
4.90
4.70
4.50
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

FIGURE 55

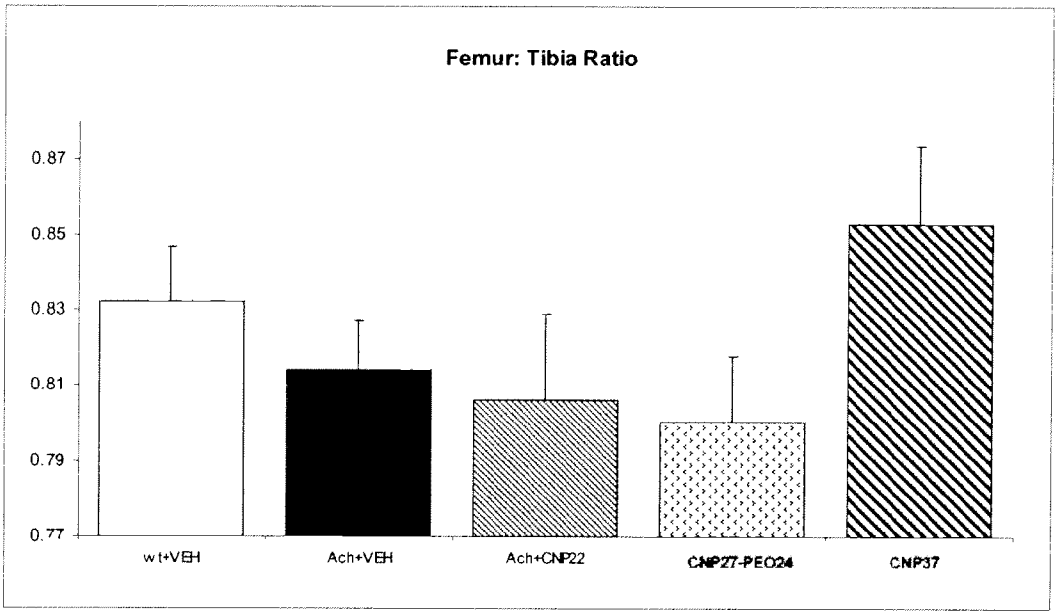


FIGURE 56

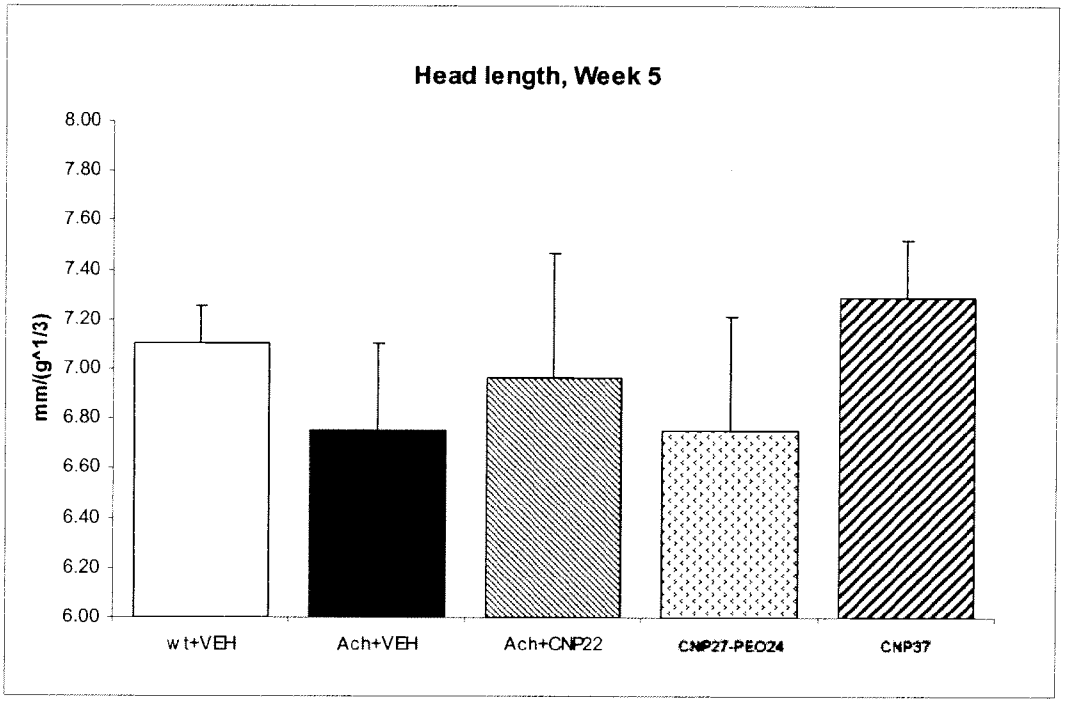


FIGURE 57

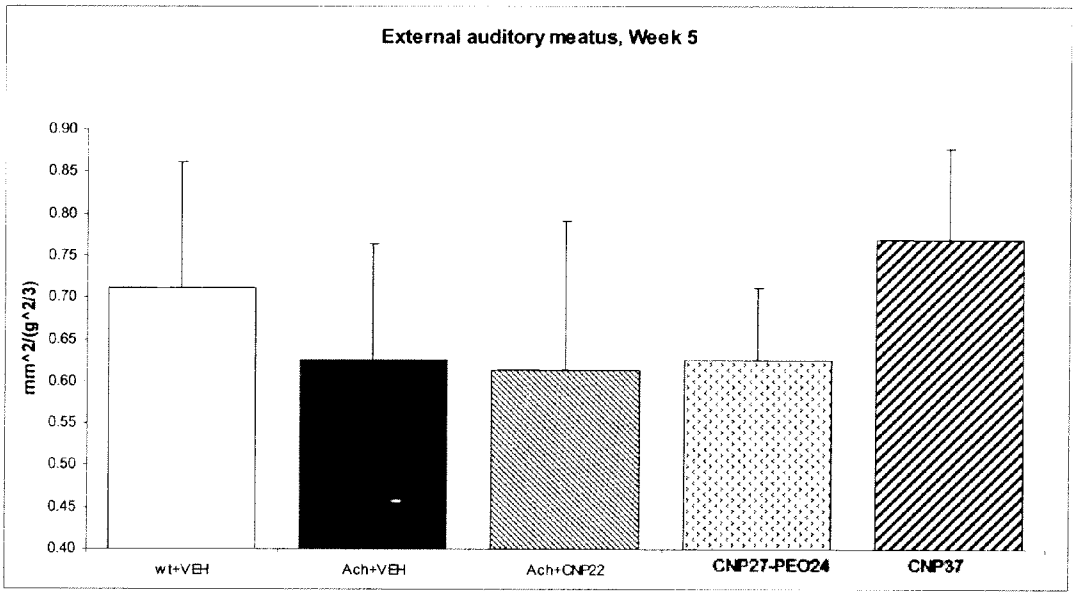


FIGURE 58

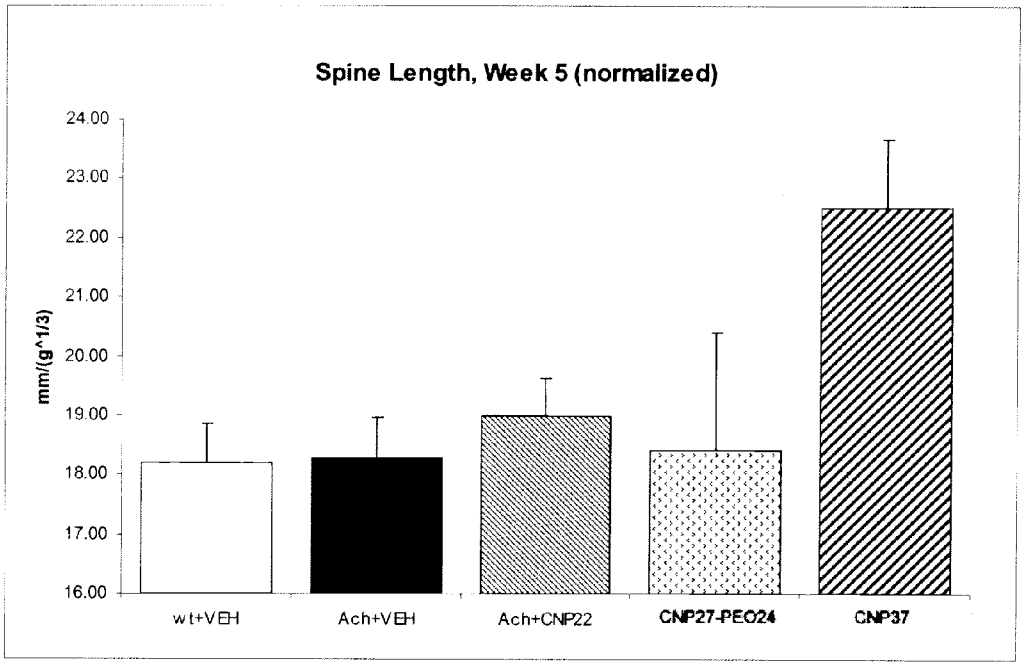


FIGURE 59

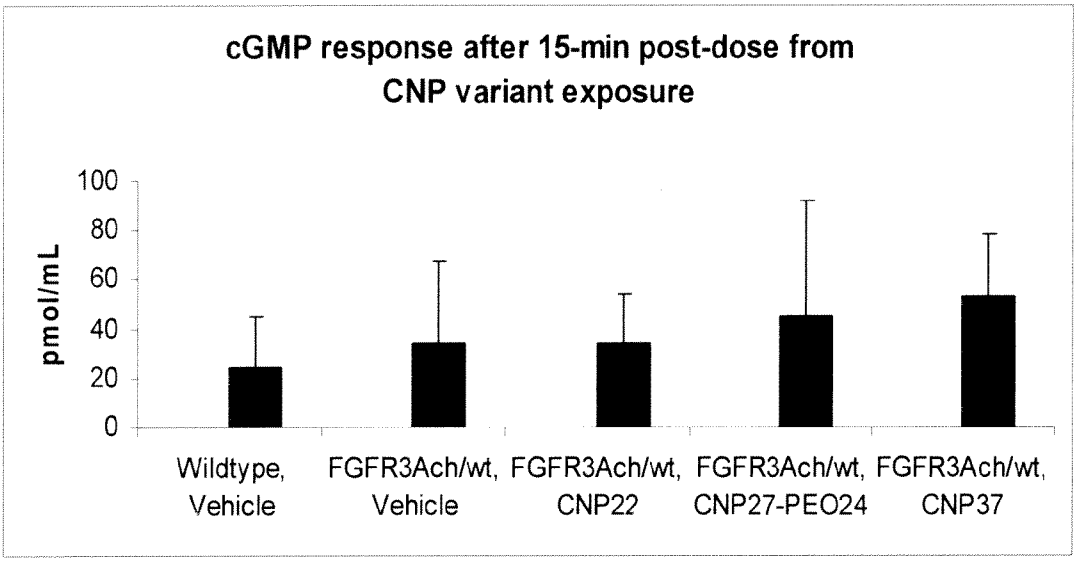


FIGURE 60

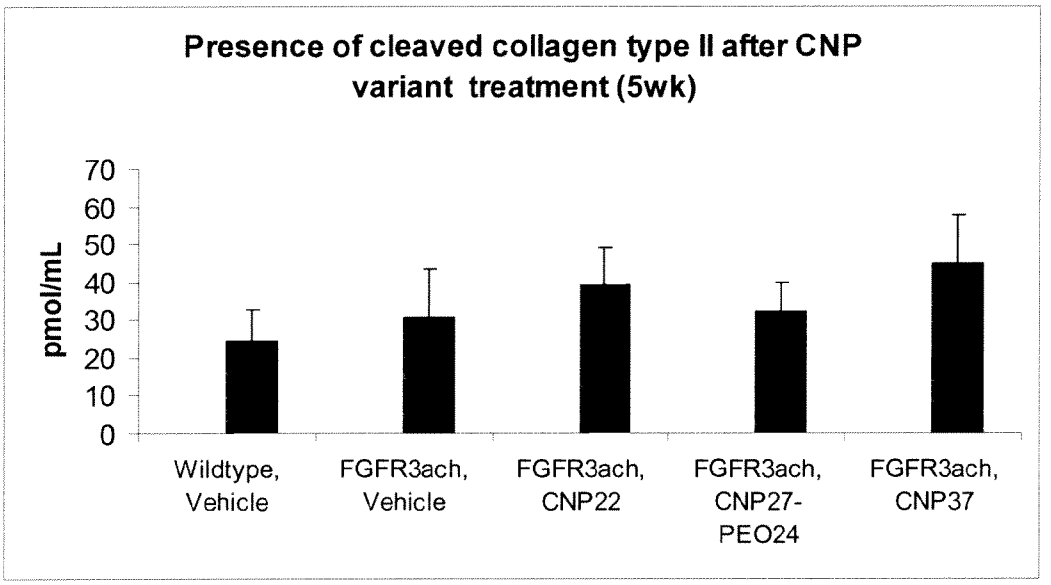


FIGURE 61

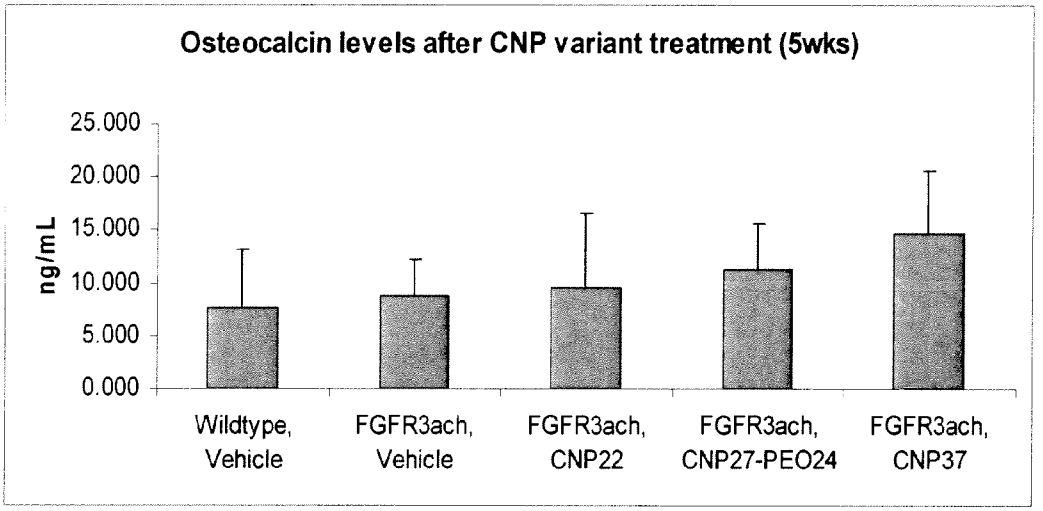


FIGURE 62

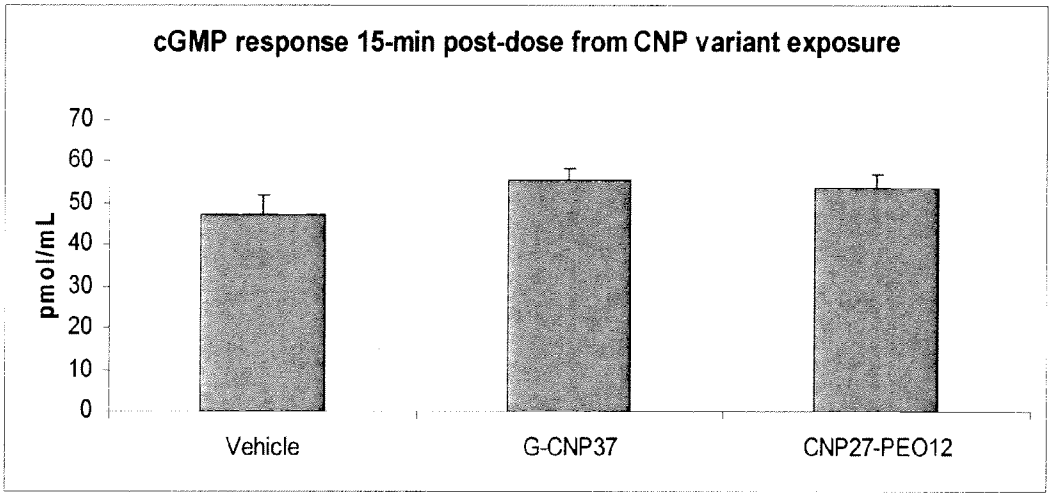


FIGURE 63

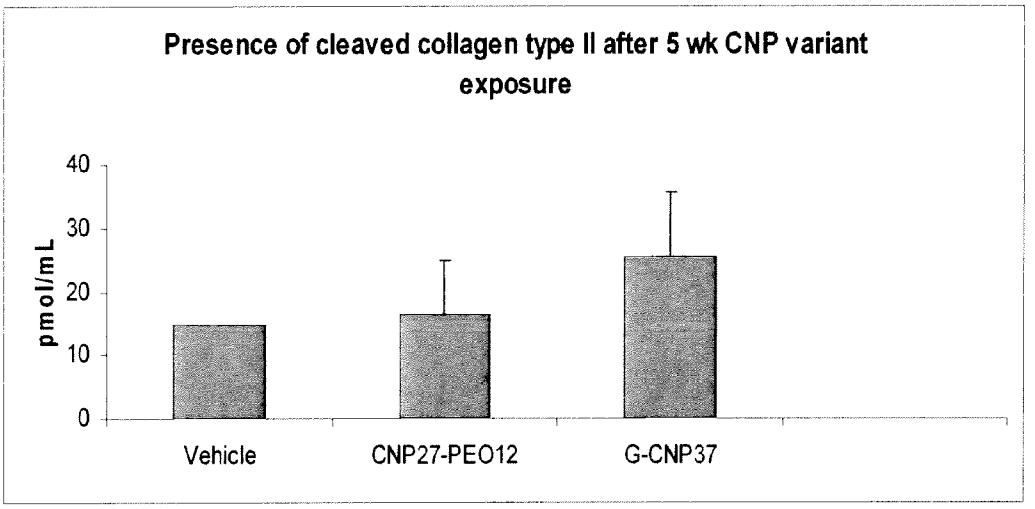
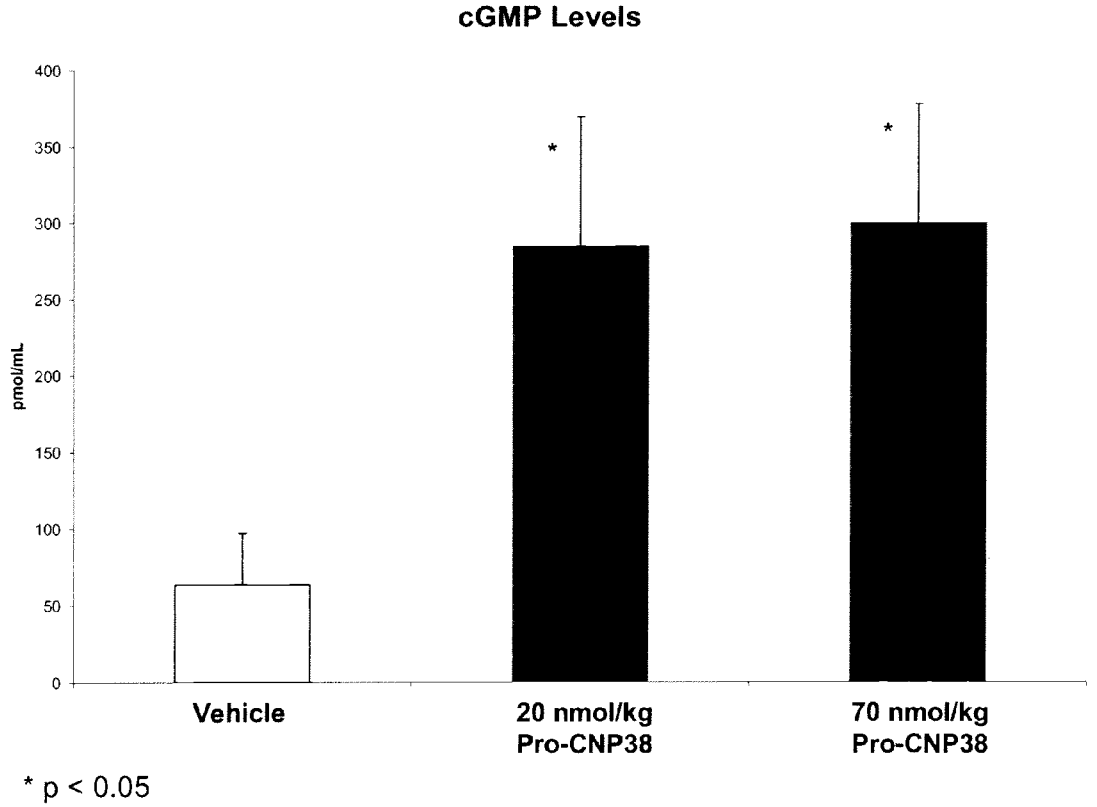


FIGURE 64



* $p < 0.05$

FIGURE 65

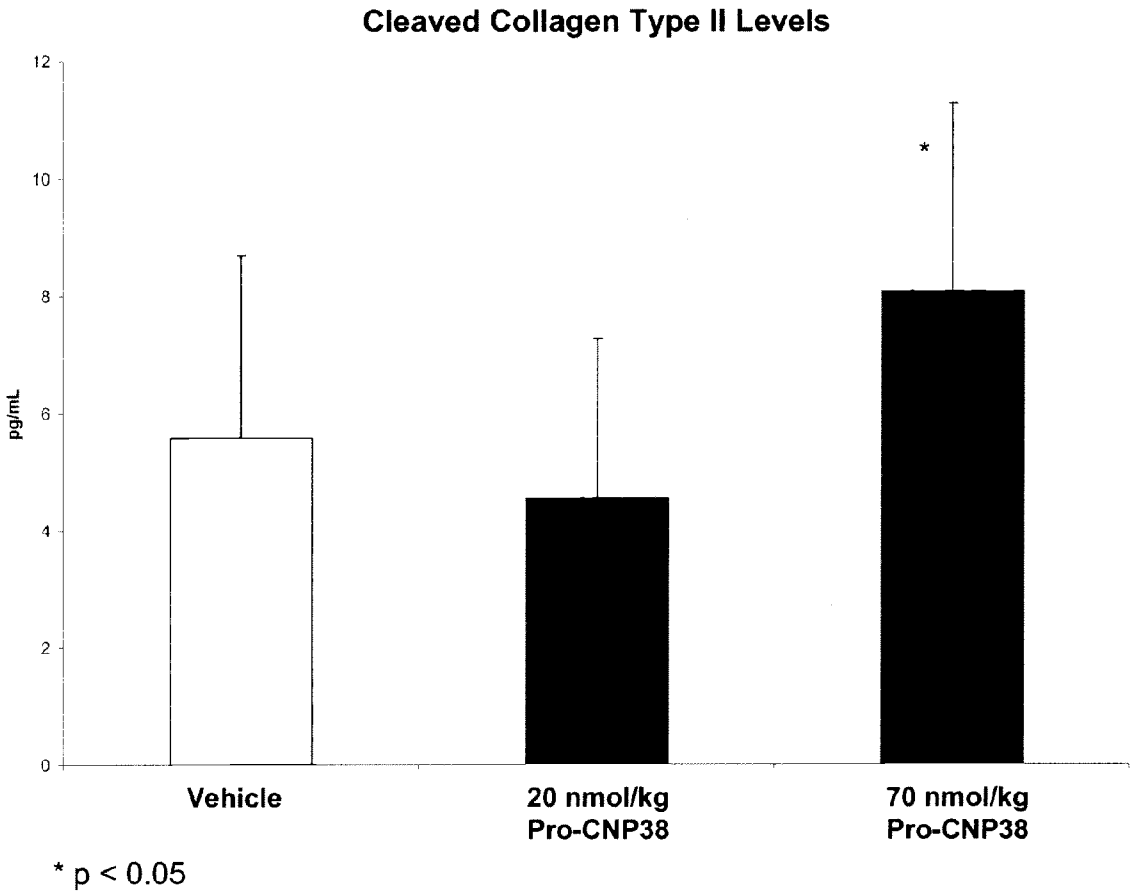

Cleaved Collagen Type II Levels
pg/mL
12
10
8
6
4
2
0
*
Vehicle
20 nmol/kg Pro-CNP38
70 nmol/kg Pro-CNP38
* p < 0.05

FIGURE 66

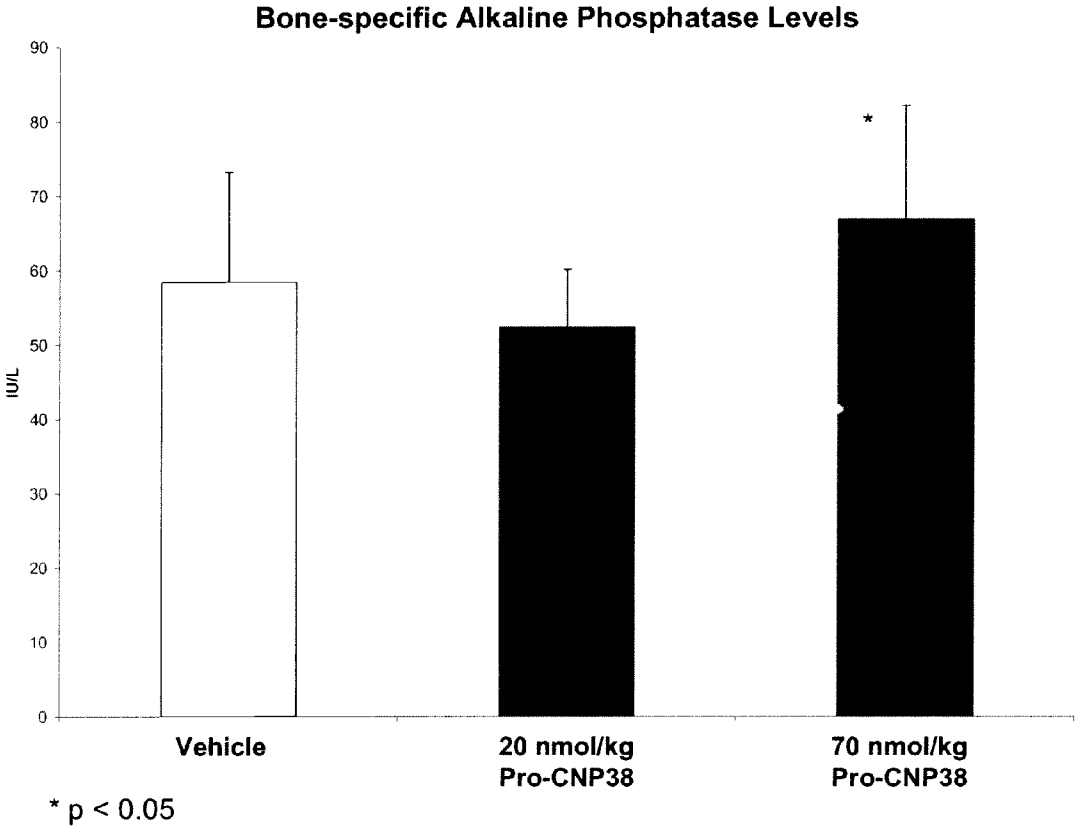

Bone-specific Alkaline Phosphatase Levels
IU/L
90
80
70
60
50
40
30
20
10
0
*
Vehicle
20 nmol/kg Pro-CNP38
70 nmol/kg Pro-CNP38
* p < 0.05

FIGURE 67

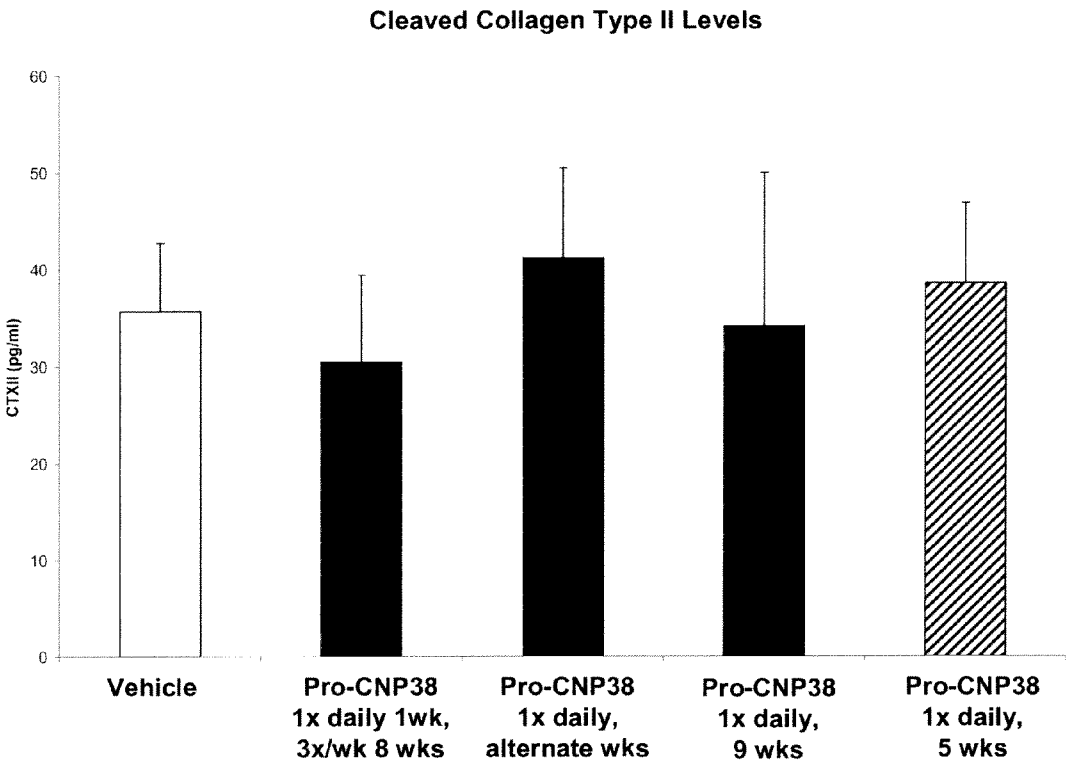

Cleaved Collagen Type II Levels
CTXII (pg/ml)
60
50
40
30
20
10
0
Vehicle
Pro-CNP38 1x daily 1wk, 3x/wk 8 wks
Pro-CNP38 1x daily, alternate wks
Pro-CNP38 1x daily, 9 wks
Pro-CNP38 1x daily, 5 wks

FIGURE 68

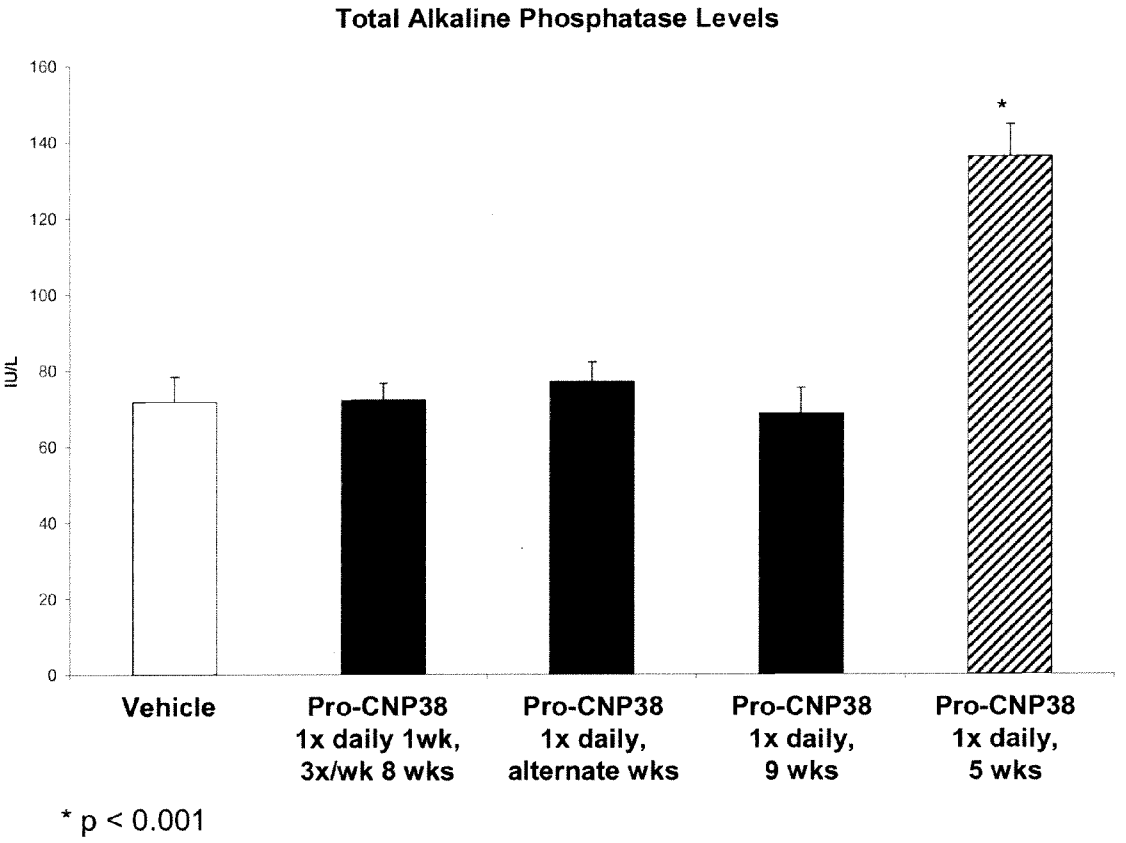

Total Alkaline Phosphatase Levels
160
140
120
100
80
60
40
20
0
IU/L
*
Vehicle
Pro-CNP38 1x daily 1wk, 3x/wk 8 wks
Pro-CNP38 1x daily, alternate wks
Pro-CNP38 1x daily, 9 wks
Pro-CNP38 1x daily, 5 wks
* p < 0.001

FIGURE 69

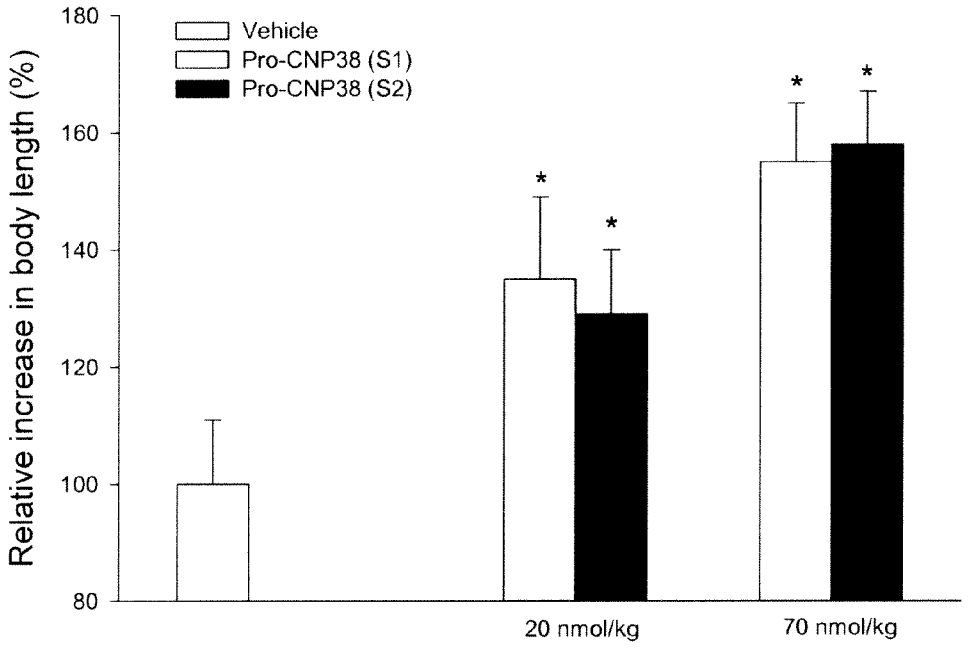
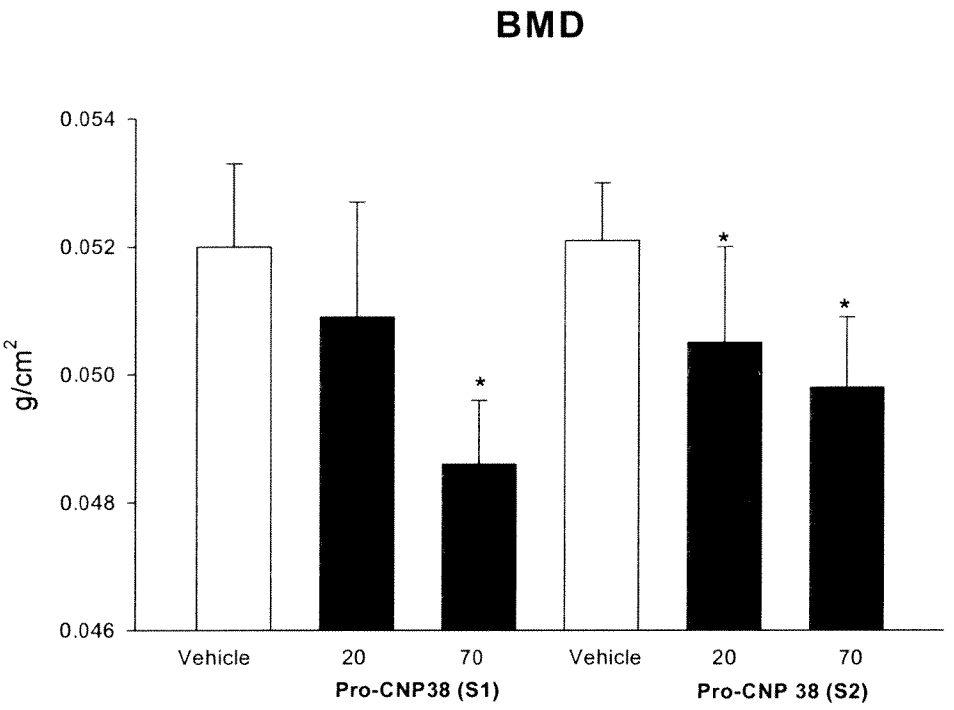

Vehicle
Pro-CNP38 (S1)
Pro-CNP38 (S2)
Relative increase in body length (%)
180
160
140
120
100
80
*
*
*
*
20 nmol/kg
70 nmol/kg

FIGURE 70

A.


BMD
0.054
0.052
0.050
0.048
0.046
g/cm2
*
*
*
Vehicle
20
70
Vehicle
20
70
Pro-CNP38 (S1)
Pro-CNP 38 (S2)

B.

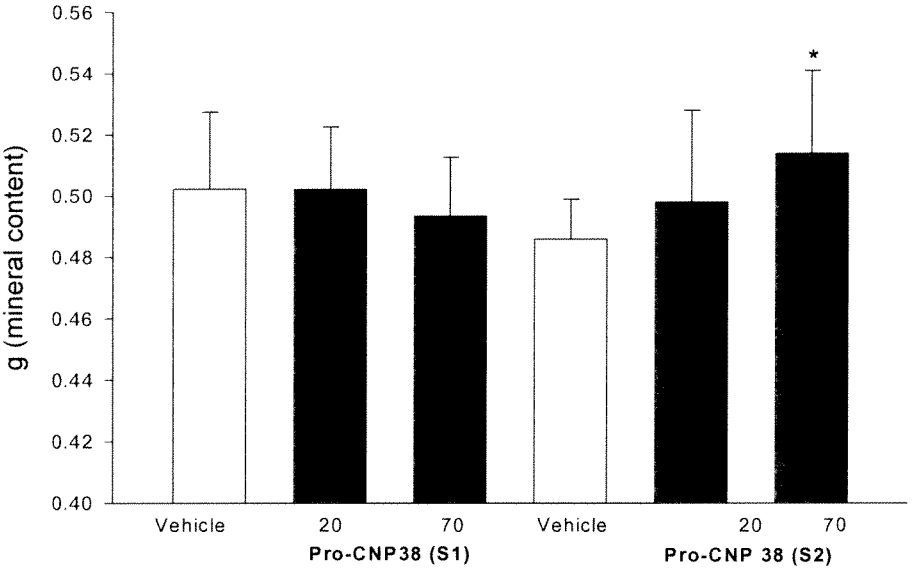

BMC
0.56
0.54
0.52
0.50
0.48
0.46
0.44
0.42
0.40
g (mineral content)
*
Vehicle
20
70
Vehicle
20
70
Pro-CNP38 (S1)
Pro-CNP 38 (S2)

FIGURE 71

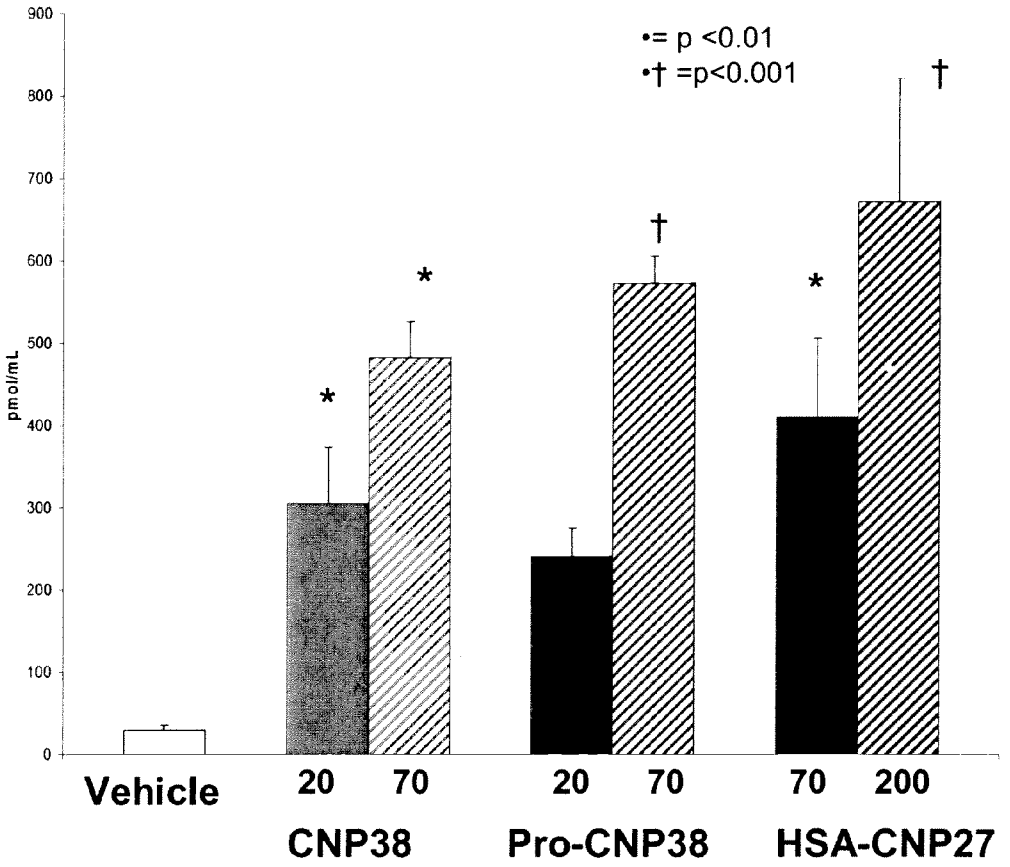

•= p <0.01
•† =p<0.001
pmol/mL
900
800
700
600
500
400
300
200
100
0
*
*
*
†
†
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 72

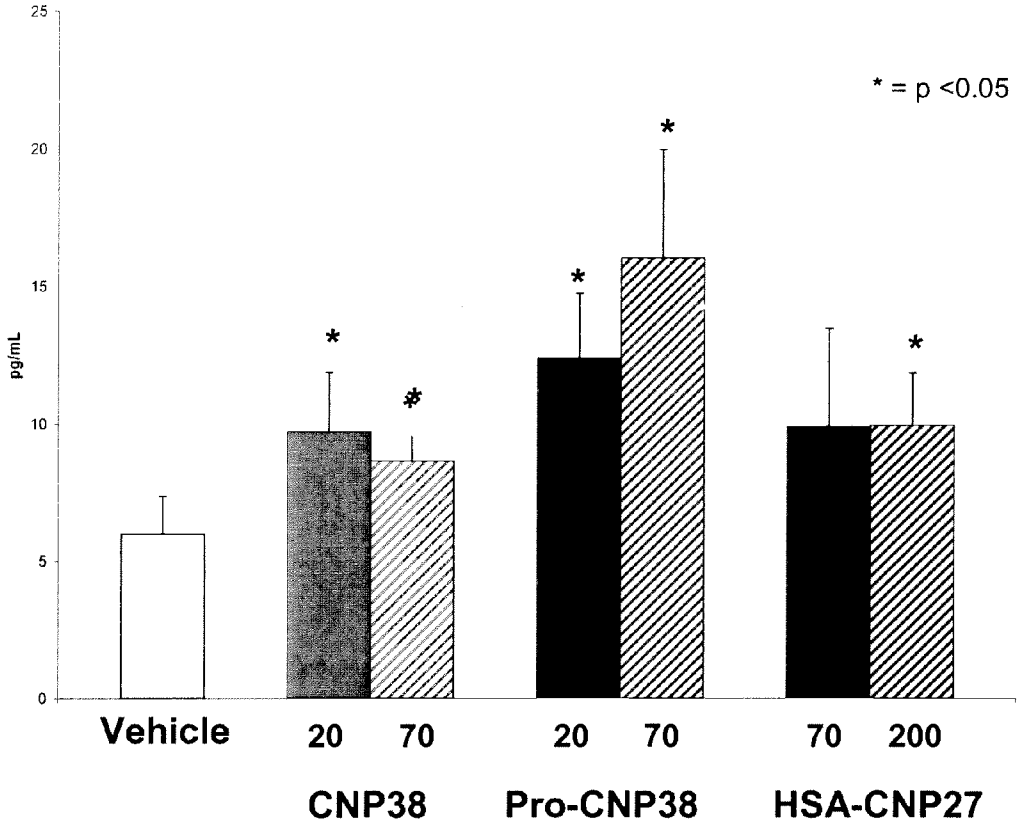

* = p <0.05
pg/mL
25
20
15
10
5
0
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 73

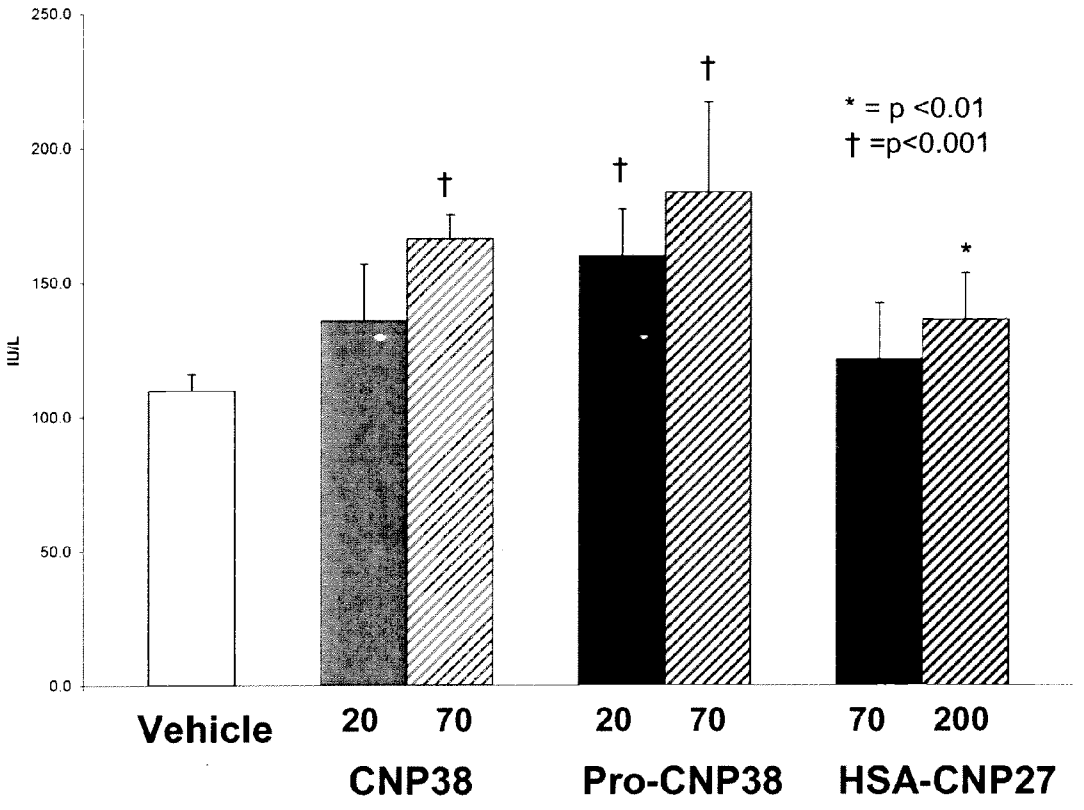
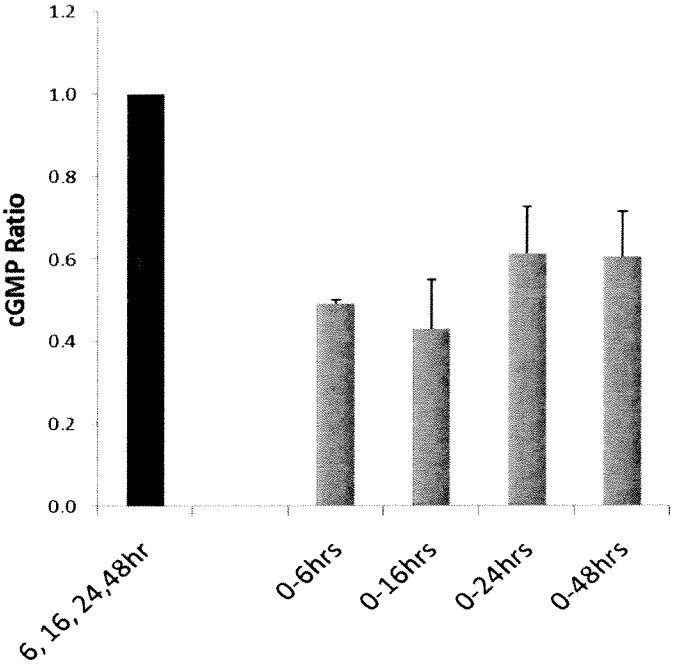

250.0
200.0
150.0
100.0
50.0
0.0
IU/L
* = p <0.01
† =p<0.001
†
†
†
*
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 74

A.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
cGMP Ratio
6, 16, 24,48hr
0-6hrs
0-16hrs
0-24hrs
0-48hrs

B.

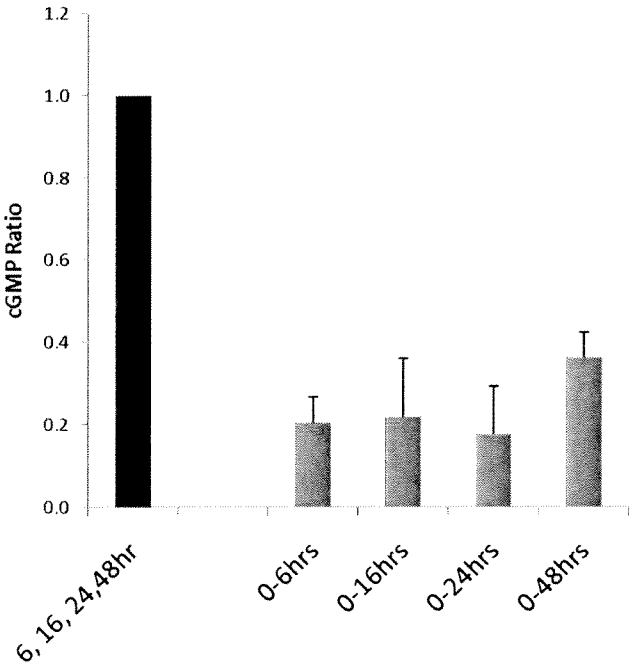

1.2
1.0
0.8
0.6
0.4
0.2
0.0
cGMP Ratio
6, 16, 24,48hr
0-6hrs
0-16hrs
0-24hrs
0-48hrs

FIGURE 75

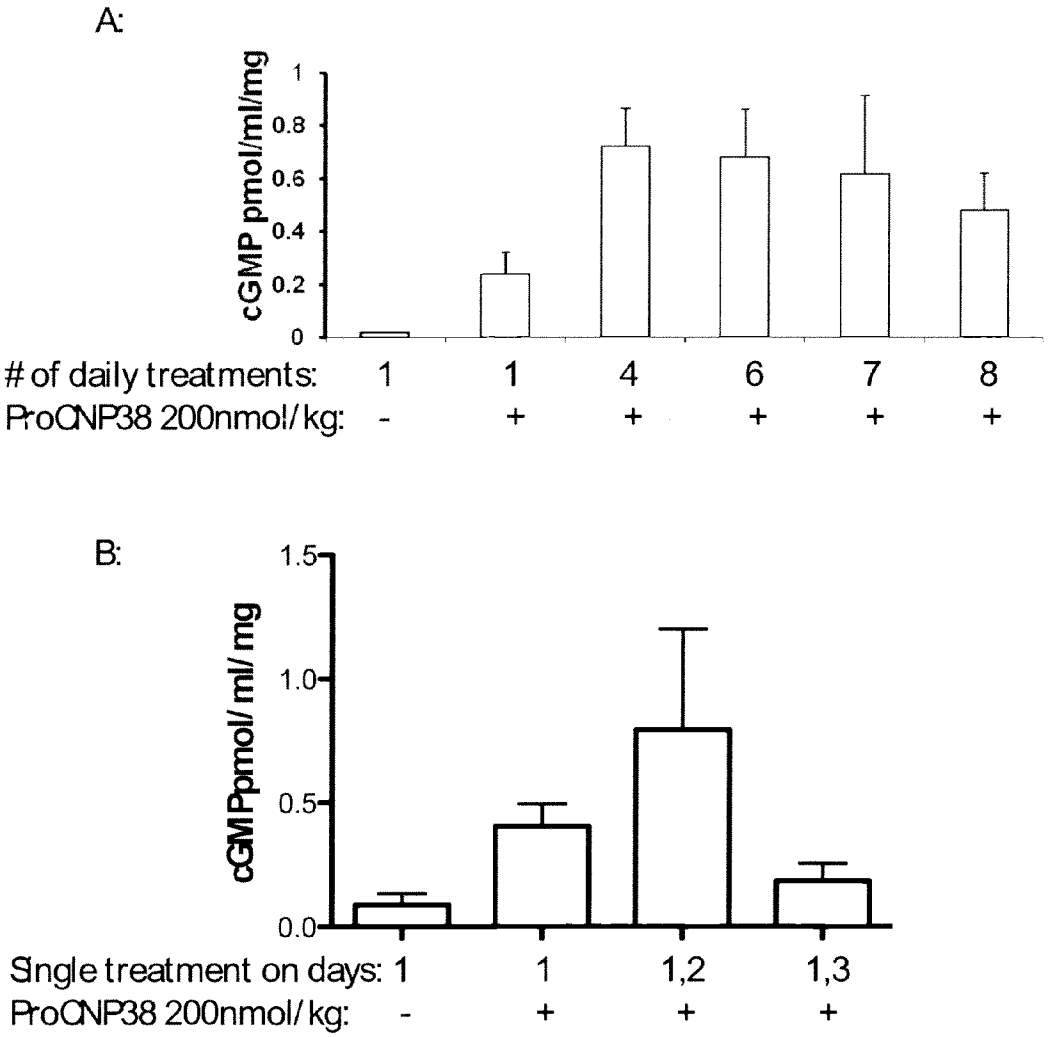

A:
cGMP pmol/ml/mg
1
0.8
0.6
0.4
0.2
0
of daily treatments: 1 1 4 6 7 8
ProCNP38 200nmol/kg: - + + + + +
B:
cGMPpmol/ml/mg
1.5
1.0
0.5
0.0
Single treatment on days: 1 1 1,2 1,3
ProCNP38 200nmol/kg: - + + +

FIGURE 76

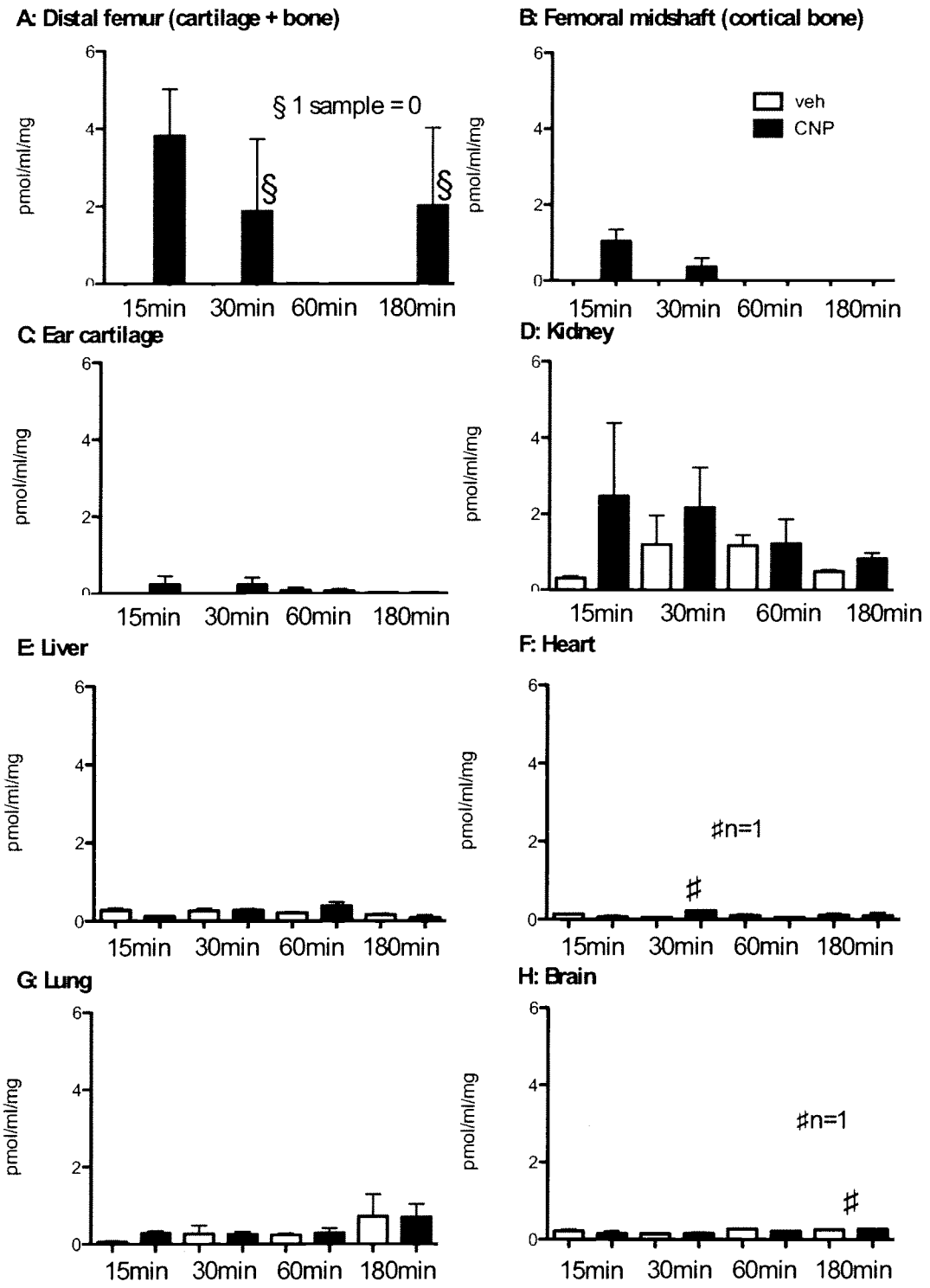

A: Distal femur (cartilage + bone)
§ 1 sample = 0
§
§
pmol/ml/mg
15min 30min 60min 180min
B: Femoral midshaft (cortical bone)
veh
CNP
C: Ear cartilage
D: Kidney
E: Liver
F: Heart
#n=1
#
G: Lung
H: Brain
#n=1
#

FIGURE 77

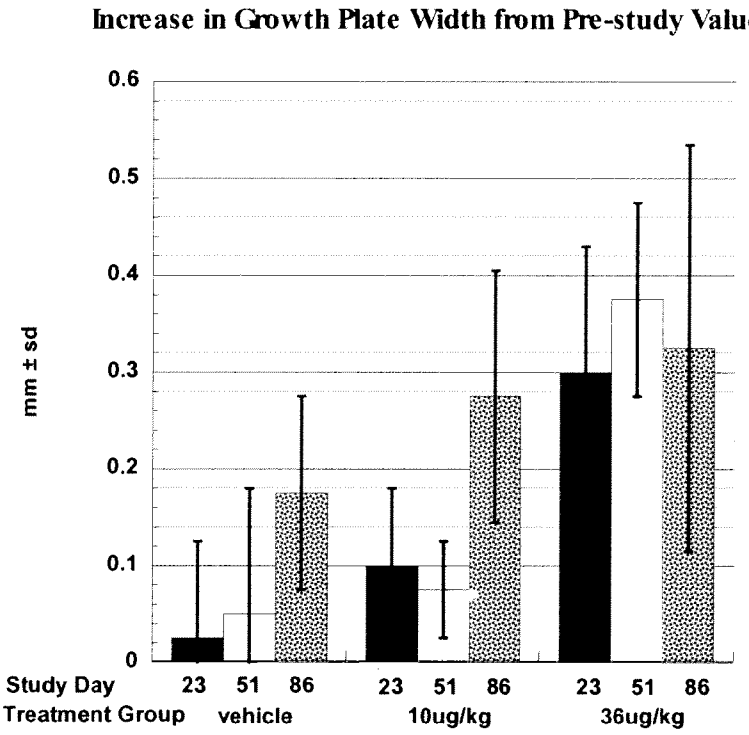


FIGURE 79

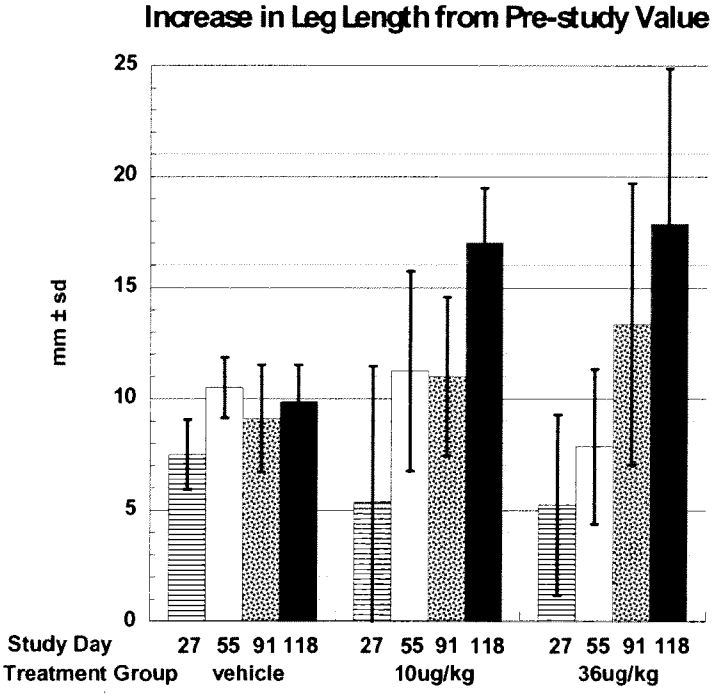
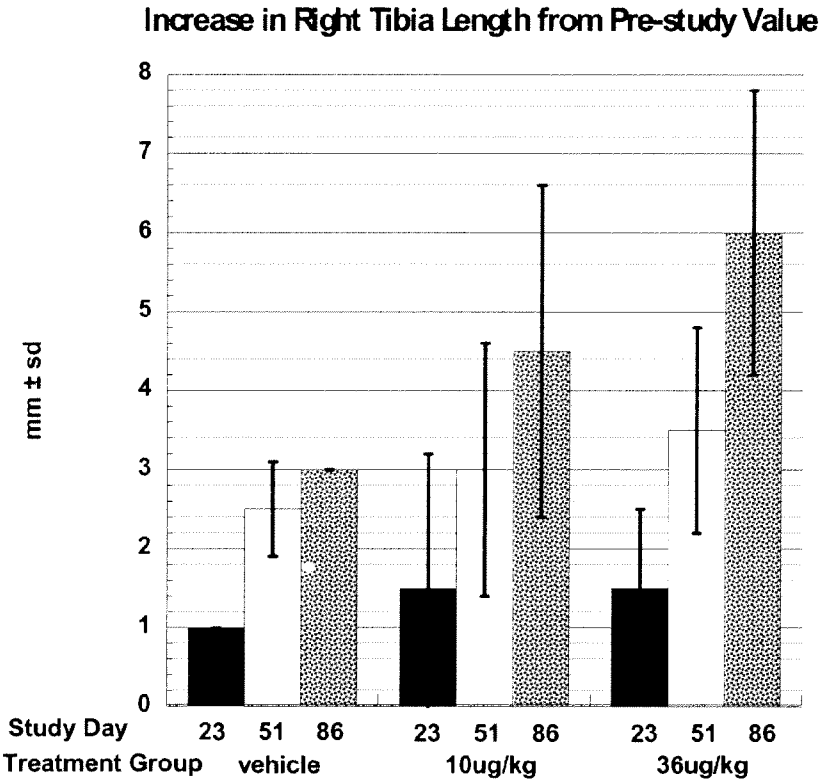


FIGURE 78

A.


Increase in Right Tibia Length from Pre-study Value
mm ± sd
0
1
2
3
4
5
6
7
8
Study Day
23 51 86 23 51 86 23 51 86
Treatment Group
vehicle
10ug/kg
36ug/kg

B.

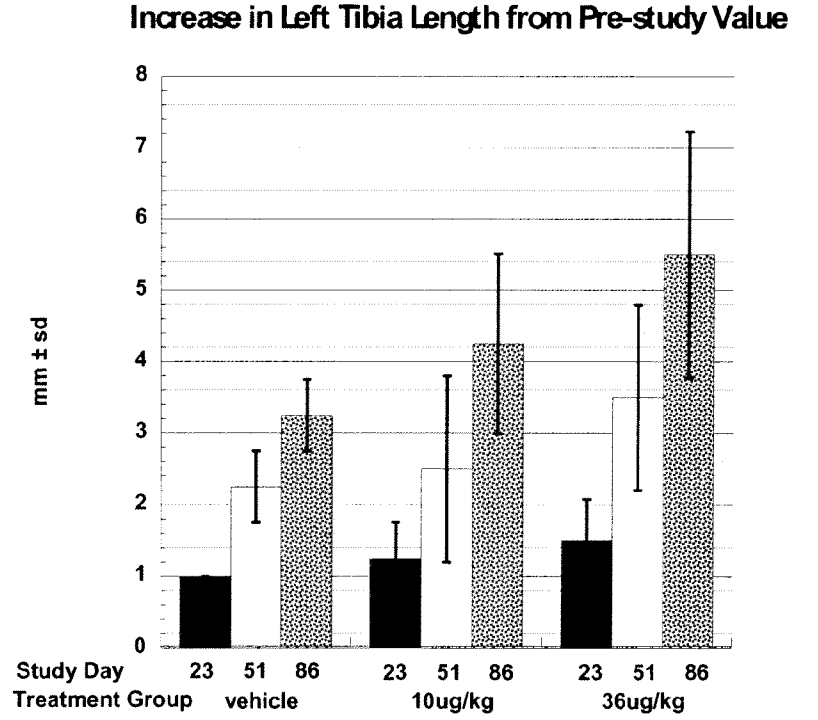

Increase in Left Tibia Length from Pre-study Value
mm ± sd
0
1
2
3
4
5
6
7
8
Study Day
23 51 86 23 51 86 23 51 86
Treatment Group
vehicle
10ug/kg
36ug/kg

FIGURE 80

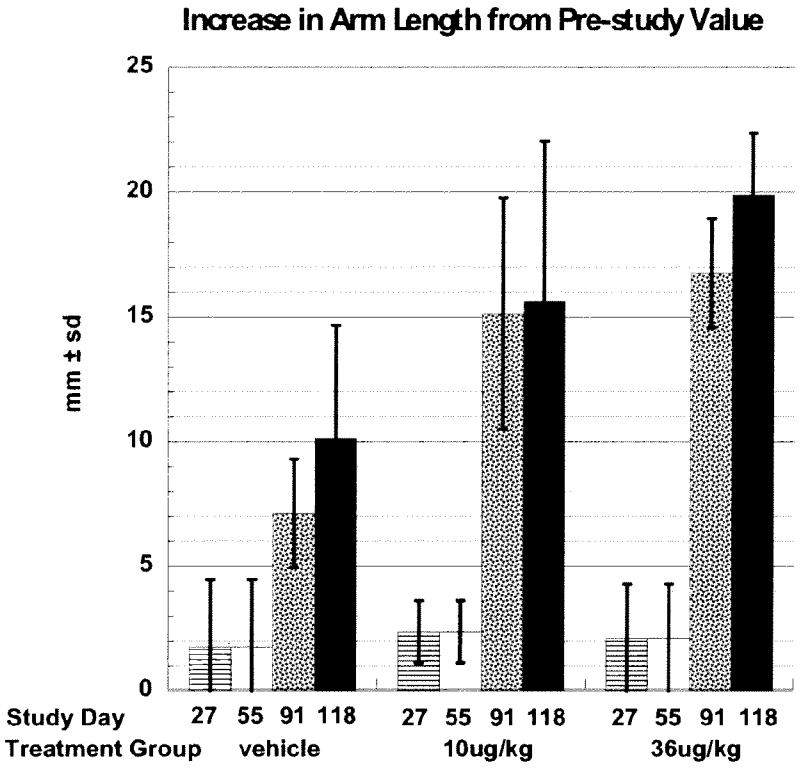


FIGURE 81

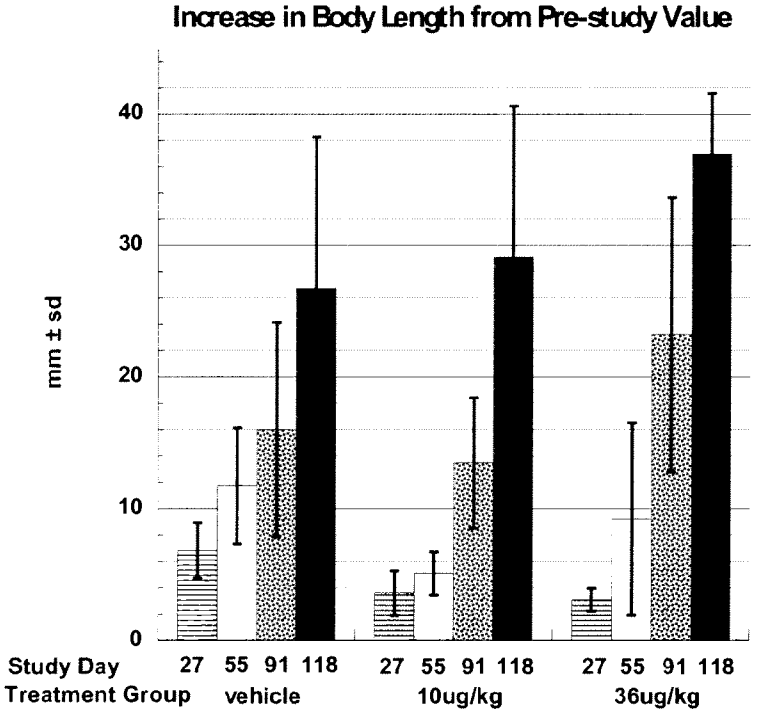


FIGURE 82

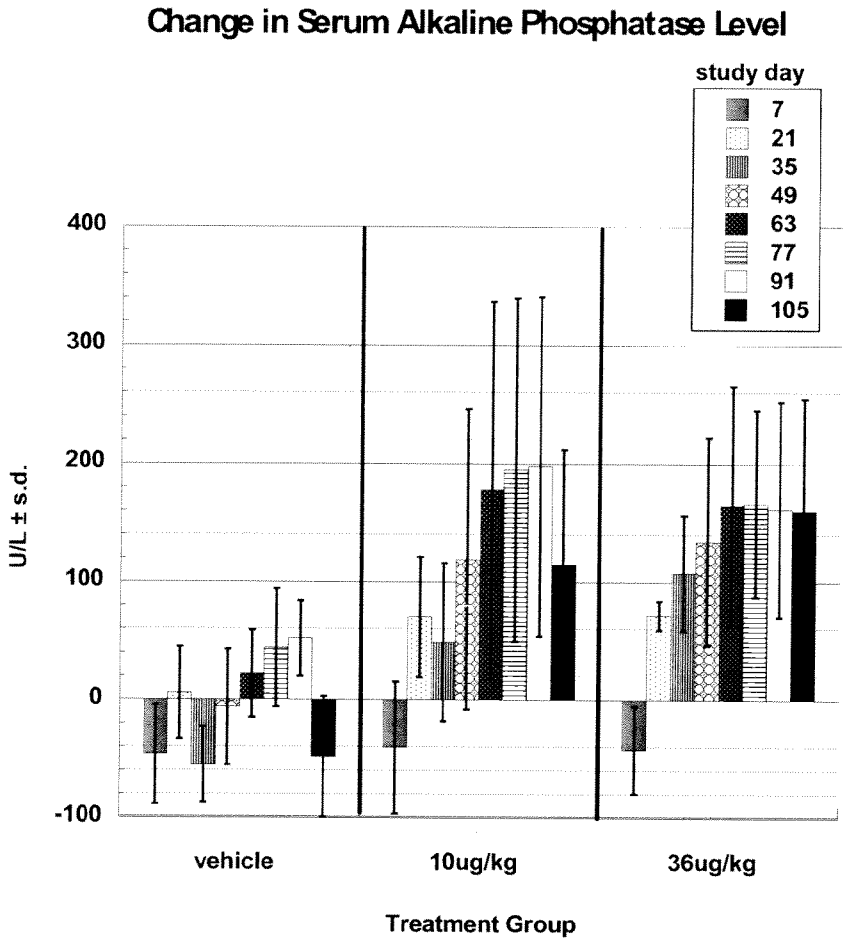
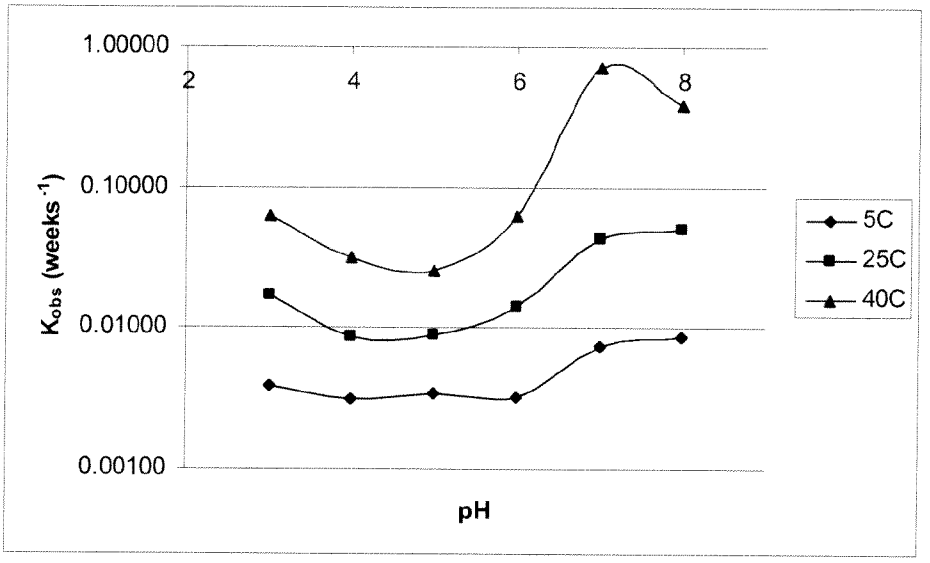


FIGURE 83



VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the priority and benefit of U.S. Provisional Application No. 61/254,563, filed on Oct. 23, 2009, and U.S. Provisional Application No. 61/180,112, filed on May 20, 2009, the disclosure of each of which is incorporated herein by reference in its entirety.

FIELD OF THE DISCLOSURE

The field of the disclosure relates, in general, to variants of C-type natriuretic peptide (CNP), compositions comprising CNP variants, methods of making CNP variants, and methods of using CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as skeletal dysplasias (e.g., achondroplasia) and vascular smooth muscle disorders.

BACKGROUND OF THE DISCLOSURE

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP) (Genbank Accession No. NP_006163, for the ANP precursor protein, NPPA), brain natriuretic peptide (BNP) (GenBank Accession No. NP_002512, for the BNP precursor protein, NPPB), and C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)). These small, single chain peptides (ANP, BNP, CNP) have a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. ANP and BNP bind to and activate the natriuretic peptide receptor A (NPR-A), also termed guanalyl cyclase A (GC-A), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Likewise, CNP interacts with NPR-B (GC-B) to stimulate the generation of cGMP (J. Hypertens., 10: 1111-1114 (1992)). A third type of receptor, NPR-C, binds each of the natriuretic peptides with high affinity and functions primarily to capture the peptides from the extracellular compartment and deposit the peptides into lysosomes, where they are degraded (Science, 238: 675-678 (1987)). ANP and BNP are produced primarily within the muscle cells of the heart, and are believed to have important roles in cardiovascular homeostasis (Science, 252: 120-123 (1991)). CNP is expressed more widely, including in the central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

In humans, CNP is initially produced from the natriuretic peptide precursor C(NPPC) gene as a single chain 126-amino acid pre-pro polypeptide (Biochem. Biophys. Res. Commun., 168: 863-870 (1990)). Removal of the signal peptide yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP-22) (Wu, J. Biol. Chem. 278: 25847-852 (2003)). CNP-53 and CNP-22 differ in their distribution, with CNP-53 predominating in tissues, while CNP-22 is mainly found in plasma and cerebrospinal fluid (J. Alfonzo, Recept. Signal. Transduct. Res., 26: 269-297 (2006)). The predominant CNP form in cartilage is unknown. Both CNP-53 and CNP-22 bind similarly to NPR-B. Furthermore, they both induce cGMP production in a dose-dependent and similar fashion (V T Yeung, Peptides, 17: 101-106 (1996)).

Natural CNP gene and polypeptide have been previously described. U.S. Pat. No. 5,352,770 discloses isolated and purified CNP-22 from porcine brain identical in sequence to human CNP and its use in treating cardiovascular indications. U.S. Pat. No. 6,034,231 discloses the human gene and polypeptide of proCNP (126 amino acids) and the human CNP-53 gene and polypeptide.

Clearance of CNP from the extracellular space occurs through the action of membrane-bound neutral endopeptidase (NEP), which rapidly degrades CNP (Biochem. J., 291 (Pt 1): 83-88 (1993)), and through NPR-C, which binds to and deposits CNP into lysosomes, where CNP is degraded. CNP has been shown to have an in vivo half-life of 2.6 min in the normal human (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). The low plasma concentration of CNP (J. Bone Moner. Res., 19 (Suppl. 1) S20 (2004)) and its co-expression with NPR-B in a number of tissues suggests that CNP functions primarily through an autocrine/paracrine mechanism.

As stated above, CNP binds to and activates natriuretic peptide receptor B (NPR-B), also termed guanylyl cyclase B (GC-B), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Downstream signaling mediated by cGMP generation influences a diverse array of biological processes that include endochondral ossification. Accordingly, elevated or depressed levels of any of the components in this pathway may lead to aberrant bone growth. For example, knockout of either CNP or NPR-B in mouse models results in animals having a dwarfed phenotype with shorter long bones and vertebrae. Mutations in human NPR-B that block proper CNP signaling have been identified and result in dwarfism (Olney, et al., J. Clin. Endocrinol. Metab. 91(4): 1229-1232 (2006); Bartels, et al., Am. J. Hum. Genet. 75: 27-34 (2004)). In contrast, mice engineered to produce elevated levels of CNP display elongated long bones and vertebrae.

Achondroplasia is a result of an autosomal dominant mutation in the gene for fibroblast growth factor receptor 3 (FGFR-3), which causes an abnormality of cartilage formation. FGFR-3 normally has a negative regulatory effect on chondrocyte growth, and hence bone growth. In achondroplasia, the mutated form of FGFR-3 is constitutively active, which leads to severely shortened bones. Both chondrocyte proliferation and differentiation appear to be disturbed, leading to remarkably short growth plate cartilage (P. Krejci et al., J. Cell Sci. 118: 5089-5100 (2005)). Endochondral ossification is the process that governs longitudinal long-bone growth. There are four zones of the growth plate—resting, proliferative, hypertrophic and zone of calcification. In the growth plate, NPR-B is expressed by proliferative cells while NPR-C is expressed by hypertrophic cells (Yamashite et al., J. Biochem. 127: 177-179 (2000)). In normal endochondral bone growth, chondrocytes organize in columns and proliferate in the proliferative zone of the growth plate. These columns are disorganized in achondroplasia patients. Additionally, the hypertrophic zone is where the cells become large and eventually apoptose (lyse), leading to osteocyte invasion and mineralization. The hypertrophic chondrocytes and the overall size of the zone are much smaller in achondroplasia patients than in normal patients. CNP is an agonist for NPR-B, a positive regulator of chondrocyte and bone growth. Downstream signaling of CNP/NPR-B inhibits the FGFR-3 pathway at the level of mitogen-activated protein kinase (MAP K). Inhibition at MAP K promotes proliferation and differ-

entiation of the chondrocytes in the proliferative and hypertrophic zones of the growth plate, resulting in bone growth.

In humans activating mutations of FGFR-3 are the primary cause of genetic dwarfism. Mice having activated FGFR-3 serve as a model of achondroplasia, the most common form of the skeletal dysplasias, and overexpression of CNP rescues these animals from dwarfism. Accordingly, CNP and functional variants of CNP are potential therapeutics for treatment of the various skeletal dysplasias.

Therapeutic use of CNP is currently limited by its short plasma half-life, which has been shown to be 2.6 minutes in vivo in humans (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). To increase CNP concentration above intrinsic levels (about 5 pM) typically found in human plasma, continuous infusion has been necessary in all human and animal studies using systemically administered CNP. A CNP variant having a longer in vivo serum half-life and exhibiting similar or improved activity to that of wild-type CNP is important for a sustainable therapeutic strategy. Two mechanisms by which the half-life of CNP is reduced in human plasma are degradation by neutral endopeptidase (NEP) and clearance by natriuretic peptide receptor C(NPR-C) (Growth Horm. & IGF Res., 16: S6-S14 (2006)). Modifications of peptides reportedly can improve resistance to endopeptidase and exopeptidase cleavage (Amino Acids, 30: 351-367 (2006); Curr. Opin. Biotech., 17: 638-642 (2006)).

The biological activities of various analogs and derivatives of CNP have been evaluated. By substituting S-methyl Cys in place of both Cys_6 and Cys_{22}, cyclization of the peptide via a Cys6-Cys22 disulfide linkage was reportedly shown to be important for the activity of CNP in stimulating cGMP formation (Biochem. Biophys. Res. Comm., 183: 964-969 (1992), also using alanine scanning to identify amino acids important for CNP functionality). A significant additional enhancement of activity reportedly results from the combined presence of the amino acids Leu_9, Lys_{10}, and Leu_{11}. U.S. Pat. No. 5,434,133 describes CNP analogs comprising CNP-22 with substitutions at amino acid position 6, 7, 9, 11, or 22, wherein the amino acid is selected from Cys or Pmp (pentacyclomercaptopropionic acid) at position 6, Phe, 4-chloro-Phe, 4-fluoro-Phe, 4-nitro-Phe, or Cha (3-cyclohexyl-Ala) at position 7, Gly, Val, Aib, or tLeu at position 9, Leu or Ile at position 11, and Cys or Pmp at position 22.

U.S. Patent Publication No. 2004/0138134 (now U.S. Pat. No. 7,276,481) describes CNP variants comprising amino acids Cys_6 to Cys_{22} of CNP-22 ("CNP-17") which include at least one substitution for another natural amino acid at position 9, 10, 11, 16, 17, 19, or 20, CNP variants with insertions and deletions, such as addition of a His residue at the reported primary site of NEP cleavage, between Cys_6 and Phe_7, and methods of using such variants for increasing the size of a bone growth plate in abnormal bone and elongation of an abnormal bone. However, no significant gains in activity as measured by cGMP production were obtained for these variants, and activity was diminished for nearly all of the variants, as observed in an in vitro cell-based method (Example 7). Further no supportive data, such as for example in vitro stability or in vivo determination of improved pharmacokinetics (PK) were provided to substantiate the asserted NEP resistance and NPR-C resistance of the CNP analogs. U.S. Pat. No. 6,743,425 discloses substances for treating achondroplasia which activate NPR-B/GC-B and are peptides or low molecular weight compounds, including the C-type natriuretic peptides CNP-22 and CNP-53. PCT Publication No. WO 94/20534 discloses a chimera of CNP-22 and the 5-amino acid C-terminus of ANP designated as the vasonatrin peptide (VNP), a limited number of amino acid substitutions and cyclic chimeric peptides that result from formation of a disulfide or double bond.

Approaches for improving the half-life of other natriuretic peptide family members include decreasing the affinity of ANP for NPR-C (U.S. Pat. No. 5,846,932), utilizing pentapeptide antagonists of NPR-C (WO 00/61631), and co-administering NEP inhibitors such as thiorphan and candoxatril (Clin. Exp. Pharma. Physiol., 25: 986-991 (1997), Hyperten., 30: 184-190 (1997)). WO 2004/047871 describes conjugates of BNP and BNP variants to polyalkylene glycol moieties, sugar moieties, polysorbate moieties, polycationic moieties, and other hydrophilic polymer moieties that reportedly exhibit improved half-life in circulation and reportedly are useful for the treatment of acute congestive heart failure.

There have been no published reports, however, on a successful strategy for making CNP resistant to NEP while retaining its functionality.

SUMMARY OF THE DISCLOSURE

The present disclosure relates to variants of C-type natriuretic peptide (CNP) which are useful in the treatment of bone-related disorders (e.g., achondroplasia) and vascular smooth muscle disorders. The disclosure encompasses CNP variants having increased serum half-life, e.g. as a result of reduced ability to bind to neutral endopeptidase (NEP), greater resistance to proteolysis by NEP and/or reduced affinity to the clearance natriuretic peptide receptor C(NPR-C), while retaining the functionality of CNP.

The wild-type sequence of human CNP-22 (referred to herein as "hCNP22", "wtCNP22" or "CNP22") is set forth below:

(SEQ ID NO: 1)
(N-terminus) Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}.

Positions 6 to 22 of CNP22 form a cyclic domain by means of a disulfide bond between Cys6 and Cys22. The 17-amino acid cyclic structure has been shown to be important for binding of CNP to NPR-B (Schiller, Biochem. Biophys. Res. Commun., 138: 880-886 (1986)). The amino acid sequence of positions 6 to 22 of CNP22 is referred to herein as "CNP17" (SEQ ID NO: 2).

CNP is susceptible to NEP cleavage at a number of sites: Cys6-Phe7, Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20. In one embodiment, the disclosure encompasses a CNP variant that is (1) modified to increase its overall size or molecular weight, e.g., to a range from about 2.6 kDa or 2.8 kDa to about 4 kDa, 4.2 kDa, 4.4 kDa, 4.6 kDa, 4.8 kDa, 5 kDa, 5.2 kDa, 5.4 kDa, 5.6 kDa, 5.8 kDa, 6 kDa, 6.2 kDa, 6.4 kDa, or to about 7 kDa, 7.2 kDa or about 8.2 kDa, and/or (2) modified at certain amino acid positions to reduce its susceptibility to NEP cleavage at 1, 2, 3, 4, 5 or all 6 of the sites listed above. The size or molecular weight of the CNP variant can be increased by various means, e.g., by conjugating additional amino acids and/or other kinds of chemical (e.g., natural or synthetic polymeric) groups to the peptide sequence at, e.g., the N-terminus, the C-terminus and/or side chain(s), and/or by using natural amino acids, unnatural amino acids, and/or peptidomimetics with bulkier side chains. The CNP variant is optionally further conjugated to other functional or structural moieties. Optionally in combination with any of the embodiments described herein, mutation(s) (e.g., substitution(s), addition(s), and/or deletion(s))

may be introduced to certain position(s) of CNP22 to reduce the CNP variants' affinity to NPR-C. Further modifications may be made without affecting NEP resistance or CNP activity, e.g., conservative substitutions, or other modifications known in the art.

In one embodiment, the CNP variant is represented by the general formula: (x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 5), wherein:

the CNP variant comprises one or more modified amino acids, which may result in modified peptide bonds (e.g., through use of peptide bond isosteres), at a position corresponding to one or more of the following CNP residues: Gly1, Lys4, Gly5, Cys6, Phe7, Gly8, Leu9, Lys10, Leu11, Ile14, Gly15, Ser16, Met17, Gly19, Leu20 and Gly21; and

(x) and (z) independently may be absent or may be an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, ANP, BNP) or a non-natriuretic polypeptide (e.g., human serum albumin (HSA), IgG, etc.).

In an embodiment, the CNP variant includes: (1) a modification at an amino acid position corresponding to one of positions 6, 7 or 8 (Cys6, Phe7 or Gly8) of CNP22, (2) optionally deletion, addition and/or substitution of any or all of the amino acids at positions 1-5 (Gly1, Leu2, Ser3, Lys4, and Gly5) and (3) optionally up to 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 further modifications (deletions, additions and/or substitutions) at positions corresponding to positions 6-22, of which 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 may be conservative substitutions or other substitutions described herein or known in the art.

It is understood that a reference to a particular amino acid position by number (e.g. position 7 of CNP22) refers to the corresponding amino acid position in any CNP variant, even if the number of the position in that CNP variant has changed due to preceding insertions or deletions. For example, a reference to "position 7" or "Phe7" would mean the corresponding position 2 for a CNP variant in which the first five amino acids had been deleted. Similarly, a reference to "position 7" would mean the corresponding position 8 for a CNP variant in which one amino acid had been added to the N-terminus.

In any of the embodiments described herein, the CNP variant may be cyclized through a covalent bond between positions corresponding to 6 and 22 of CNP22. It is contemplated that the covalent bond is formed using any methods known in the art. In another embodiment, the CNP variant may be cyclized through a covalent bond formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus (referred to as "terminal" amino acids for this purpose) of the peptide. In one embodiment, the covalent bond is formed between the side chains of the two terminal amino acids or the amino acids at positions corresponding to 6 and 22 of CNP22. In another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of each terminal amino acid. For example, head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail bonds are possible for the covalent bond formed between the terminal amino acids or between the amino acids at positions corresponding to 6 and 22 of CNP22.

In one embodiment, the disclosure provides a CNP variant having reduced affinity to NEP, and/or greater resistance to cleavage by NEP and/or increased in vivo serum half-life, while retaining functionality of CNP (e.g., stimulation of cGMP production). NEP preferably recognizes substrates smaller than about 3 kDa, due to the limited size of its active site cavity (Oefner, J. Mol. Biol., 296: 341-349 (2000)). In an embodiment, the CNP variants are modified to increase their overall molecular weight to a range from about 2.6 or 2.8 kDa to about 4, 4.6, 5, 5.2, 5.8, 6, 6.4 or 7 kDa, e.g., by adding about 0.6 to about 5 kDa of amino acids, hydrophilic or water-soluble polymers, hydrophobic acids (including fatty acids), and/or carbohydrates. In specific exemplary embodiments, the CNP variants have a molecular weight between about 2.6 kDa and about 7 kDa, or between about 2.8 kDa and 6 kDa, or between about 2.8 kDa and about 5.8 kDa. In certain embodiments, at least about 0.6, 0.8, 1, 1.2, 1.4, 1.6 or 1.8 kDa, or up to 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8 or 5 kDa are added, to increase the total molecular weight of the CNP variant, e.g., to a range from about 2.6 or 2.8 kDa up to about 4 kDa, 4.2 kDa, 4.4 kDa, 4.6 kDa, 4.8 kDa, 5 kDa, 5.2 kDa, 5.4 kDa, 5.6 kDa, 5.8 kDa, 6 kDa, 6.2 kDa, 7.2 kDa, 8.2 kDa or higher. In some embodiments, such CNP variants comprise an amino acid sequence at least about 70%, 75%. 80%, 85%, 90%, or 95% identical or homologous to amino acids 6-22 of CNP22. In other embodiments, such CNP variants comprise a substitution, insertion or deletion of 1, 2, 3, 4, 5, 6 or 7 amino acids with another natural or unnatural amino acid or peptidomimetic. While both conservative and non-conservative substitutions or insertions are envisioned at any position, introduction of modifications may commence, e.g., by conservative substitutions in regions that have been identified in the art as involved in CNP activity or NPR-B binding, while non-conservative substitutions may be made in those regions that have been previously shown to be tolerant of modification.

In another embodiment, the CNP variants comprise a CNP having an intact cyclized portion between Cys6 and Cys22, and N-terminal and/or C-terminal tails that contain about 1-40, 1-20, 5-40, 5-35, 10-35, 15-35, 5-31, 10-31, or 15-31 amino acids and are fragments derived from a CNP polypeptide and/or a non-CNP polypeptide. In an embodiment, such CNP variants have a molecular weight in a range from about 2.8 kDa to about 4, 4.6, 5, 5.2, 5.8, 6, 6.4 or 7 kDa. Non-limiting examples of such CNP variants include wild-type CNP22 or CNP22 with one or more amino acid substitutions (e.g., a K4R substitution), having an N-terminal and/or C-terminal extension derived from natriuretic peptide precursor sequences (e.g., ANP, BNP or CNP) from human or other species, a natriuretic peptide precursor C (NPPC) variant with amino acid substitutions, additions and/or deletions (e.g., the CNP variants may be truncations of CNP-53 which result in peptides with a molecular weight between about 2.8 kDa and 5.8 kDa), or other non-CNP polypeptides such as, e.g., serum albumin or IgG protein (e.g., the CNP variants may be CNP chimeras containing fragments of serum albumin or IgG from human or other species).

In one embodiment, CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, are represented by the general formula:

(x)-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 6), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is polyethylene glycol (PEG, also called polyethylene oxide (PEO)), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin,

fibrinogen, zinc finger-containing polypeptides, osteocrin or fibroblast growth factor 2 (FGF2);

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG); and

(b) and (h) independently may each be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser. In one embodiment, (b) is Arg. In another embodiment, for improved NEP resistance, (b) is not Gly. In yet another embodiment, (h) is not Arg.

Non-limiting examples of amino acid sequences derived from NPPC or variants thereof include:

Arg,

Glu-Arg,

(SEQ ID NO: 7)
Gly-Ala-Asn-Lys-Lys,

(SEQ ID NO: 8)
Gly-Ala-Asn-Arg-Arg,

(SEQ ID NO: 9)
Gly-Ala-Asn-Pro-Arg,

(SEQ ID NO: 10)
Gly-Ala-Asn-Gln-Gln,

(SEQ ID NO: 11)
Gly-Ala-Asn-Ser-Ser,

(SEQ ID NO: 12)
Gly-Ala-Asn-Arg-Gln,

(SEQ ID NO: 13)
Gly-Ala-Asn-Arg-Met,

(SEQ ID NO: 14)
Gly-Ala-Asn-Arg-Thr,

(SEQ ID NO: 15)
Gly-Ala-Asn-Arg-Ser,

(SEQ ID NO: 16)
Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala,

(SEQ ID NO: 17)
Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg,

(SEQ ID NO: 18)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg,

(SEQ ID NO: 19)
Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys,

(SEQ ID NO: 20)
Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg,

(SEQ ID NO: 21)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys,

-continued

and

(SEQ ID NO: 22)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg.

Non-limiting examples of amino acid sequences derived from non-CNP polypeptides such as, e.g., ANP, BNP, serum albumin and IgG include:

(SEQ ID NO: 23)
Ser-Leu-Arg-Arg-Ser-Ser;

(SEQ ID NO: 24)
Asn-Ser-Phe-Arg-Tyr;

(SEQ ID NO: 25)
Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly;

(SEQ ID NO: 26)
Met-Val-Gln-Gly-Ser-Gly;

(SEQ ID NO: 27)
Lys-Val-Leu-Arg-Arg-Tyr;

(SEQ ID NO: 28)
Lys-Val-Leu-Arg-Arg-His;

(SEQ ID NO: 29)
Gly-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg;

(SEQ ID NO: 30)
Gly-Val-Pro-Gln-Val-Ser-Thr-Ser-Thr;

(SEQ ID NO: 31)
Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln;

and

(SEQ ID NO: 32)
Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser.

In an embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof independently may be conjugated to an amino acid extension containing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acids. In one embodiment, the amino acid extension is derived from NPPC, CNP53, ANP or BNP. In a specific embodiment, the amino acid extension is Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 33). In a related embodiment, this 15-amino acid extension is added to the N-terminus to provide a CNP variant of the formula Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 34).

In one embodiment, the CNP variants comprise wtCNP22 or a variant thereof (e.g., one having addition(s), deletion(s), and/or substitution(s) such as, e.g., a K4R substitution) (SEQ ID NO: 35) conjugated at the N-terminus and/or C-terminus to a hydrophilic polymer (e.g., PEG) to increase their overall molecular size to a range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6 or 7 kDa. Such CNP variants are optionally further conjugated at the N-terminus and/or C-terminus to a polymeric group comprising, e.g., amino acids, carbohydrates, hydrophobic acids and/or phospholipids, a non-limiting example of which is an N-terminal amino acid extension containing 1 to 35, or 5 to 31, amino acids. In an embodiment, a hydrophilic polymeric (e.g., PEG) moiety of at least about

0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6 or 1.8 kDa, or up to about 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8 or 5 kDa, is added to the N-terminus and/or C-terminus of wtCNP22 or a variant thereof.

As shown herein, conjugation of a hydrophilic or water-soluble PEG (or PEO) polymer of about 0.6 kDa or more to CNP22 or variants thereof generally increases resistance to NEP cleavage markedly. However, addition of PEG, even as small as 0.6 kDa, to wtCNP22 may reduce CNP functionality (e.g., stimulation of cGMP signaling), and addition of greater than about 2 or 3 kDa of PEG to CNP22 or variants thereof may reduce CNP functional activity in a size-dependent manner. But CNP functionality (at least comparable to that of wtCNP22) is retained when a PEG (or PEO) polymer of about 0.6 kDa to about 1.2 kDa, or potentially to about 2 kDa, is conjugated to a CNP variant having an N-terminal amino acid extension in which at least one relatively large amino acid that may potentially be positively charged under physiological conditions (e.g., arginine) immediately precedes the position corresponding to Gly1 of CNP22, such as, e.g., GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), ER-CNP22 (SEQ ID NO: 38), ER-CNP22 (K4R) (SEQ ID NO: 39), R-CNP22 (SEQ ID NO: 40) and R-CNP22(K4R) (SEQ ID NO: 41).

Accordingly, in one embodiment, PEGylated CNP variants comprise at the N-terminus of CNP22 or a variant thereof (e.g., one having a K4R substitution) an amino acid extension containing at least 1, 2, 3, 4 or 5 amino acids, wherein the PEG polymer is conjugated to the N-terminus of the amino acid-extended CNP variant to result in a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP cleavage. In an embodiment, for enhanced CNP functionality, such pegylated, amino acid-extended CNP variants contain at least one relatively large natural or unnatural amino acid that may potentially be positively charged under physiological conditions, immediately preceding the position corresponding to Gly1 of CNP22. In a specific embodiment, the pegylated, amino acid-extended CNP variants contain at least one arginine residue immediately preceding the position corresponding to Gly1 of CNP22.

In addition to CNP variants conjugated at the N-terminus and/or C-terminus to a hydrophilic or water-soluble polymer such as, e.g., PEG (or PEO), the disclosure encompasses CNP variants conjugated to such a polymer at an internal site. For purposes of brevity here, PEG (or PEO) will be used as a representative example of a hydrophilic or water-soluble polymer. Various sites of PEGylation of a CNP variant are possible, including but not limited to: (1) PEGylation only at the N-terminus; (2) PEGylation only at the C-terminus; (3) PEGylation only at an internal site (e.g., Lys4); (4) PEGylation at both the N-terminus and the C-terminus; (5) PEGylation at the N-terminus and an internal site; and (6) PEGylation at the C-terminus and an internal site. For increased resistance to NEP degradation and retention of CNP functionality, in certain embodiments the total mass of PEGylatedCNP variants is characterized by the ranges described generally herein, e.g., in the range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6 or 7 kDa. In a particular embodiment, CNP17, CNP22, CNP37 (defined below) or variants thereof (including those having amino acid additions, substitutions and/or deletions) are PEGylated only at the N-terminus. In another embodiment, the CNP variants are PEGylated only at an internal site (e.g., Lys4). In yet another embodiment, the CNP variants are PEGylated at the N-terminus and an internal site (e.g., Lys4). In still another embodiment, for better functionality the CNP variants are not PEGylated at a site (e.g., Lys10) within the cyclic domain (corresponding to Cys6 to Cys22 of CNP22). To prevent PEGylation at an internal site, Lys4 and/or Lys10 can be substituted with a natural or unnatural amino acid or peptidomimetic that does not contain a reactive primary amino group on a side chain, such as, e.g., Gly, Ser, Arg, Asn, Gln, Asp, Glu or citrulline (Cit). In a particular embodiment, Lys4 and/or Lys10 are replaced with Arg. In another embodiment, Lys10 is not replaced with Arg.

The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG molecules) that may vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP peptide by means of N-hydroxy succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. Such CNP variants can be generated using, e.g., wtCNP-22 (2.2 kDa), CNP-17 retaining only the cyclized region (residues 6-22) of wtCNP22, CNP variants having an amino acid extension at the N-terminus and/or C-terminus of CNP22, or variants with amino acid substitutions, additions and/or deletions, for example, GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), R-CNP22 (SEQ ID NO: 40), R-CNP22(K4R) (SEQ ID NO: 41), ER-CNP22 (SEQ ID NO: 38) and ER-CNP22(K4R) (SEQ ID NO: 39). In an embodiment, the PEG-CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, contain a monodispersed, linear PEG (or PEO) group conjugated at the N-terminus and/or C-terminus via NHS- or aldehyde-based chemistry, or a two-arm or three-arm branched PEG group conjugated at the N-terminus and/or C-terminus via NHS-based chemistry. The disclosure further contemplates negatively charged PEG-CNP variants designed for reduced renal clearance, including but not limited to carboxylated, sulfated and phosphorylated compounds.

In a related embodiment, the disclosure contemplates PEG-CNP conjugates comprising NHS- or aldehyde-based PEG of the formula $(CH_2CH_2O)_n$, wherein n is an integer from 12 to 50, and the PEG polymer is up to about 2.5 kDa in molecular weight. In a specific embodiment, n is 12 or 24. In an embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the capping group is an alkyl group, e.g., a lower alkyl group such as methyl.

In an additional embodiment, the PEG polymers or derivatives thereof have a polymer number-average molecular weight in the range from about 0.4 kDa to about 2.5 kDa or from about 0.6 kDa to about 1.5 kDa.

In a further embodiment, the wtCNP or CNP variant peptide is conjugated to a moiety including, e.g., bisphosphonates, carbohydrates, hydrophobic acids (including fatty acids) or amino acid sequences. Such amino acid sequences include for example polyAsp or polyGlu useful in bone/cartilage targeting, or can be derived from bone proteins with elucidated bone-targeting domains or derivatives thereof, such as for example fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc. In embodiments described herein where CNP22 or a variant thereof is attached to a bone- or cartilage-targeting moiety, such a moiety is designed to promote getting the modified CNP peptide to

chondrocytes of bone growth plates, where the peptide can bind and activate NPR-B on the chondrocytes.

In another embodiment, the disclosure provides CNP variants with a peptide bond that is less susceptible to cleavage by peptidases including NEP. The disclosure encompasses a CNP variant comprising at least one modified residue at a site of endopeptidase cleavage. In one embodiment, the Cys6-Phe7 peptide bond (—C(═O)—NH—) at an NEP cleavage site in CNP can be replaced with anyone of the following peptide-bond isosteres:

—CH_2—NH—,

—C(═O)—N(R)—, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

—C(═O)—NH—CH_2—,

—CH_2—S—,

—CH_2—S(O)$_n$—, where n is 1 or 2,

—CH═CH—,

—C(═O)—CH_2—,

—CH(CN)—NH—,

—CH(OH)—CH_2—,

—O—C(═O)—NH—, and

—NHC(═O)NH—.

In another embodiment, Phe7 is substituted with its enantiomer D-Phe. In yet another embodiment, D-enantiomers are introduced to one or more, up to all 22, positions within wtCNP-22. In a further embodiment, a beta amino acid such as 3-amino-2-phenylpropionic acid is substituted for Phe7, which increases the length of the backbone while reducing the length of the side chain. In yet another embodiment, the disclosure contemplates Cys analogs at the Cys6 position including but not limited to homocysteine, penicillamine, 2-mercaptopropionic acid, and 3-mercaptopropionic acid.

Even in the presence of a NEP-resistant bond between Cys6 and Phe7, other peptide bonds can be hydrolyzed by NEP, including Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20. Accordingly, the disclosure encompasses CNP analogs containing peptide bond isosteres at multiple locations in the backbone of the CNP analogs. In one embodiment, CNP analogs or variants comprise modifications at more than one peptidase cleavage site. In a further embodiment, such variant comprises a CNP with substitutions at amino acid residues important in binding to the NEP active site, thereby increasing resistance to NEP degradation. One or more NEP-binding residues, including but not limited to Gly8, Gly15, Ser18, Gly19 and/or Gly21, are replaced with larger-size natural or unnatural amino acid residues to reduce affinity to the NEP active site. In yet another embodiment, one or more hydrophobic residues essential in NEP recognition, including but not limited to Phe7, Leu9, Leu11, Ile14, Met17 and Leu20, are substituted with natural or unnatural amino acids and/or peptidomimetics that decrease NEP binding. In yet another embodiment, one to five of the first five amino acids of CNP can be deleted or substituted with any other natural amino acids or unnatural amino acids or peptidomimetics, or one or more natural or unnatural amino acids or peptidomimetics can be added to any one or to all of the first five positions of CNP.

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 46), wherein:

(x) may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone proteins with elucidated bone-targeting domains, such as, e.g., fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; polymeric or non-polymeric molecules that reduce renal clearance such as, e.g., charged PEG molecules; and extensions comprising, e.g., polymers (e.g., PEGs), carbohydrates, hydrophobic acids (including fatty acids), and/or amino acids, and wherein such amino acid extensions can contain, e.g., from 1 to 31, or 1 to 35, or 5 to 35, or 10 to 35, or 15 to 35 amino acid residues, and can be derived from NPPC, ANP, BNP, other non-CNP (poly)peptides such as, e.g., serum albumin or IgG, or variants of the aforementioned polypeptides having substitutions, additions and/or deletions, or combinations thereof;

(z) may be absent or may be selected from the group consisting of amino acid sequences useful in bone or cartilage targeting such as for example polyAsp and polyGlu, amino acid sequences from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin and sialoprotein, and amino acid sequences derived from non-CNP (poly)peptides such as, e.g., ANP or BNP;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide-bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, heterocyclyl, C_{6-14} aryl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly, and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In one embodiment, the CNP variants comprise a modification at one or more of positions 6, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and/or 20, and may optionally have modifications at any of the other positions disclosed herein.

In embodiments described herein where CNP22 or variants thereof can be attached to a hydrophobic acid, the CNP peptides can be attached to one or hydrophobic acids. Non-limiting examples of hydrophobic acids include straight-chain or branched, saturated or unsaturated C_5-C_{12} carboxylic acids (e.g., pentanoic acid, heptanoic acid, etc.) and natural fatty acids. The hydrophobic acids can be attached to the N-terminus, the C-terminus, and/or the side chain of one or more amino acid residues. In one embodiment, the hydrophobic acids are conjugated to the N-terminus. In an embodiment, conjugation of CNP22 or a variant thereof to a hydrophobic acid is designed, inter alia, to promote non-specific interaction between the modified CNP peptide and serum albumin, thereby increasing the size of the CNP peptide and protecting it from cleavage by proteases such as, e.g., NEP. The interaction between the hydrophobic acid-conjugated CNP peptide and albumin is designed to be not too strong, so that the modified CNP peptide can diffuse through cartilage, get to chondrocytes of bone growth plates, and bind and activate NPR-B.

In a further embodiment, the disclosure provides CNP variants that in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), comprise at least one modified amino acid at position $(b)_6$, $(c)_7$ and/or $(d)_8$, and are represented by the general formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 47), wherein:

(x) may be absent or may be a peptide sequence containing one to five amino acids which is derived from a natriuretic polypeptide (e.g. NPPC, CNP, ANP or BNP) or a non-natriuretic polypeptide as described herein (e.g., HSA, IgG, a bone-targeting protein, etc.);

(z) may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone/cartilage targeting such as, e.g., polyAsp and polyGlu; bone proteins with bone-targeting domains and derivatives thereof, such as fusion proteins or peptides sequences of osteopontin, osteocalcin, and sialoprotein; molecules that reduce renal clearance, such as, e.g., charged PEGs; and molecules that increase resistance of CNP to NEP-mediated degradation, as described herein;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) may be Cys or descarboxy cysteine, or the $(b)_6$-$(c)_7$ peptide bond (—C(═O)—NH—) may be replaced with any one of the following peptide bond isosteres:

—CH_2—NH—,
—C(═O)—N(R)—, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,
—C(═O)—NH—CH_2—,
—CH_2—S—,
—CH_2—$S(O)_n$—, where n is 1 or 2,
—CH_2—CH_2—,
—CH═CH—,
—C(═O)—CH_2—,
—CH(CN)—NH—,
—CH(OH)—CH_2—,
—O—C(═O)—NH—, or
—NHC(═O)NH—;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; peptide bond isosteres of Phe such as N-alkylated derivatives of Phe wherein the N-alkyl group is selected from the group consisting of methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl and tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-$C_{1-}6$ alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) is any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amino group on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the disclosure encompasses CNP variants that in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP degradation, and are represented by the general formula:

(x)-(y)-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 48), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic

polymeric group is polyethylene glycol (PEG), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or FGF2;

(y) may be absent or may be one or more amino acids from Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (corresponding to positions 1 to 5 of CNP22) (SEQ ID NO: 1) and/or substitutions at one or more of those positions using natural or unnatural amino acids (e.g., K4R substitution);

(h) may be the wild-type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (h) is not Arg; and

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG).

In an embodiment, (x), (y) and (z) together contain from about 10 to about 40, or from about 15 to about 35 amino acids. In another embodiment, (x) is an amino acid sequence comprising from 1 to 40 amino acids, or from 1 to 20 amino acids.

Further contemplated are CNP variants that in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), and comprise the sequence:

(y)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22} (SEQ ID NO: 138), wherein:

(y) comprises one or more amino acids selected from Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (SEQ ID NO: 1) and/or substitutions at one or more of those positions using natural or unnatural amino acids (e.g., K4R substitution), and further comprises a hydrophilic or water soluble polymer of molecular weight from about 0.6 kDa to about 5 kDa. In an embodiment, the hydrophilic or water-soluble polymer is conjugated to the N-terminus of such amino acid-extended CNP variant. In a particular embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO).

In yet another embodiment, the disclosure provides CNP variants that in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g. 1 uM), wherein the CNP variants comprise an N-terminal and/or C-terminal peptide extension containing from 1 to 15 amino acids, and are conjugated to a hydrophilic or water soluble polymer. In an embodiment, the peptide extension contains from 5 to 10 amino acids. In a specific embodiment, the peptide extension contains 5 amino acids. In another specific embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO).

In a still further embodiment, the CNP variants of the disclosure in vitro or in vivo stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g. 1 uM), and comprise at least a 15 amino acid fragment derived from natriuretic peptide precursor C(NPPC), wherein the fragment is at least 70% homologous to a sequence from wild type NPPC containing the same number of amino acid residues.

In still another embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased NEP resistance, and are represented by the formula: (x)-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 49), wherein:

(x) may be absent (i.e., the N-terminus ends with an —NH_2 group) or may be selected from the group consisting of a sequence of 1, 2, 3, 4 or 5 amino acids from the peptide Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (SEQ ID NO: 1); amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; bone-targeting domains from bone proteins such as for example osteopontin, osteocalcin or sialoprotein; molecules that reduce renal clearance such as hydrophilic or water-soluble polymers, including but not limited to charged PEG molecules; and moieties comprising PEG, carbohydrates, hydrophobic acids, amino acids, or combinations thereof, wherein such moieties can be amino acid extensions including but not limited to amino acid sequences derived from NPPC or non-CNP (poly)peptides such as, e.g., BNP, ANP, serum albumin or IgG;

(z) may be absent or may be selected from the group consisting of amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; amino acid sequences derived from bone-targeting proteins, such as for example osteopontin, osteocalcin or sialoprotein; and amino acid sequences derived from NPPC or non-CNP (poly) peptides, as described herein;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased NEP resistance, and are represented by the formula: (x)-Gly_1-Leu_2-Ser_3-(a)$_4$-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-(e)$_9$-(f)$_{10}$-(g)$_{11}$-Asp_{12}-Arg_{13}-(h)$_{14}$-(i)$_{15}$-Ser_{16}-(j)$_{17}$-Ser_{18}-Gly_{19}-(k)$_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 50), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins and derivatives thereof, such as for example fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; moieties that reduce renal clearance, including but not limited to hydrophilic or water-soluble polymers such as, e.g., charged PEG molecules; and moieties comprising, e.g., hydrophilic polymers (e.g., PEG), carbohydrates, hydrophobic acids, and/or amino acids;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the CNP variants can have amino acid substitution(s) at one or more of any of positions 1 to 22 of CNP22. In one embodiment, Gly1 is substituted with Arg or Glu. In another embodiment, Lys4 is replaced with Arg. In still another embodiment, Gly5 is substituted with Arg, Gln or Ser. In yet another embodiment, Gly15 is substituted with Ser, Asn, Arg or Cit. In a further embodiment, Gly19 is substituted with Ser, Arg or Asn. In yet another embodiment, Gly21 is substituted with Ser, Thr, or Arg.

In one embodiment, the CNP variant is selected from the group consisting of GLSKGC(CH_2NH)FGLKLDRIGSMSGLGC (formed using descarboxy-Cys) (SEQ ID NO: 56), GLSKGC-(N-Me-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO: 57), GLSKGC-(D-Phe)-GLKLDRIGSMSGLGC(SEQ ID NO:136), GLSKGCF-(tBuG)-LKLDRIGSMSGLGC (SEQ ID NO: 58), GLSKGC-(3-Cl-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO:137), and GLSKGC-[NHCH_2CH(Ph)CO]-GLKLDRIGSMSGLGC (formed using 3-amino-2-phenylpropionic acid) (SEQ ID NO: 59). In a further embodiment, a disulfide bond exists between Cys6, descarboxy-Cys or another sulfhydryl-containing cysteine analog at the Cys6 position, and Cys22 of any CNP variant described herein.

In another embodiment, the CNP variants contain an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, including but not limited to:

(SEQ ID NO: 4)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-53);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-37, Analog BL);

(SEQ ID NO: 61)
AAWARLLQEHPNAGLSKGCFGLKLDRIGSMSGLGC (Analog CA);

(SEQ ID NO: 62)
AAWARLLQEHPNARGLSKGCFGLKLDRIGSMSGLGC (Analog CB);

(SEQ ID NO: 63)
DLRVDTKSRAAWARGLSKGCFGLKLDRIGSMSGLGC (Analog CC);

(SEQ ID NO: 40)
RGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 38)
ERGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 64)
GANQQGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 65)
GANRRGLSKGCFGLKLDRIGSMSGLGC;

-continued

(SEQ ID NO: 66)
GANPRGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 67)
GANSSGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC
(sometimes designated "CNP27-HSA" or "HSA-CNP27"
in the Examples and figures);

(SEQ ID NO: 68)
SPKMVQGSG-CNP17-KVLRRH (Analog CD) (CNP17 having
N-terminal and C-terminal tails derived from BNP).

In a further embodiment, the CNP variants have a K4R substitution at position 4 of CNP22. Non-limiting examples of CNP(K4R) variants include:

((SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC (Analog AY);

(SEQ ID NO: 37)
GANPRGLSRGCFGLKLDRIGSMSGLGC (Analog CI);

(SEQ ID NO: 41)
RGLSRGCFGLKLDRIGSMSGLGC (Analog AZ);

(SEQ ID NO: 39)
ERGLSRGCFGLKLDRIGSMSGLGC (Analog BA);

(SEQ ID NO: 69)
GANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CH);
and

(SEQ ID NO: 70)
GANSSGLSRGCFGLKLDRIGSMSGLGC (Analog CG).

In one embodiment, CNP variants having a PEG (or PEO) moiety and an amino acid extension at the N-terminus contain arginine at the position immediately preceding the position corresponding to Gly1 of CNP22. Such PEGylated CNP variants are designed for increased resistance to NEP degradation, reduced binding to serum albumin, and enhanced CNP functional activity (e.g., activation of cGMP signaling). Non-limiting examples of PEGylated CNP variants include PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO24-GANRR-CNP22(SEQ ID NO: 65), PEO12-GANRR-CNP22(SEQ ID NO: 65), PEO24-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO12-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO24-GANPR-CNP22(SEQ ID NO: 37), PEO12-GANPR-CNP22 (SEQ ID NO: 66), PEO24-GANQQ-CNP22(SEQ ID NO: 64), PEO12-GANQQ-CNP22(SEQ ID NO: 64), PEO24-ER-CNP22(K4R) (SEQ ID NO: 39), PEO12-ER-CNP22(K4R) (SEQ ID NO: 39), PEO24-ER-CNP22(SEQ ID NO: 38), PEO12-ER-CNP22(SEQ ID NO: 38), PEO24-R-CNP22 (K4R) (SEQ ID NO: 41), PEO12-R-CNP22(K4R) (SEQ ID NO: 41), PEO24-R-CNP22(SEQ ID NO: 40), and PEO12-R-CNP22(SEQ ID NO: 40), wherein PEO24 is a monodispersed 1.2 kDa PEG polymer and PEO12 is a monodispersed 0.6 kDa PEG polymer. In an embodiment, the PEG (or PEO) polymer is attached to the N-terminus of the CNP variants.

Additional CNP variants include, but are not limited to, derivatives of CNP37 having mutation(s) at the furin cleavage site (underlined), designed to improve in vivo resistance to the furin protease, and/or having glycine (underlined) preceding glutamine, designed to prevent pyroglutamine formation, including but not limited to:

(SEQ ID NO: 71)
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (An. CS);

(SEQ ID NO: 72)
GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (An. CT);

(SEQ ID NO: 73)
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (An. CU);

(SEQ ID NO: 74)
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (An. CW);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37, An. DB);

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37).

In another embodiment, the CNP variants are chimera comprising CNP22 and an N-terminal peptide fragment, including but not limited to:

(SEQ ID NO: 76)
GHHSHEQHPHGANQQGLSKGCFGLKLDRIGSMSGLGC (Analog CQ)
(histidine-rich glycoprotein (HRGP) fragment-
CNP22 chimera);

(SEQ ID NO: 77)
GAHHPHEHDTHGANQQGLSKGCFGLKLDRIGSMSGLGC (Analog CR)
(HRGP fragment-CNP22 chimera);

(SEQ ID NO: 78)
GHHSHEQHPHGANPRGLSKGCFGLKLDRIGSMSGLGC (Analog CX)
(HRGP fragment-CNP22 chimera);

(SEQ ID NO: 79)
GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC (Analog CF)
($IgG_1(F_c)$ fragment-CNP22 chimera);

(SEQ ID NO: 80)
GQHKDDNPNLPRGANPRGLSKGCFGLKLDRIGSMSGLGC (Analog
CY) (human serum albumin (HSA) fragment-CNP22
chimera);

(SEQ ID NO: 81)
GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC (Analog CE)
(HSA fragment-CNP22 chimera);

(SEQ ID NO: 82)
FGIPMDRIGRNPRGLSKGCFGLKLDRIGSMSGLGC (Analog CZ)
(osteocrin "NPR C inhibitor" fragment-CNP22
chimera);
and

(SEQ ID NO: 83)
GKRTGQYKLGSKTGPGPKGLSKGCFGLKLDRIGSMSGLGC
(Analog DA) (FGF2 "heparin-binding domain"
fragment-CNP22 chimera).

In a further embodiment, the CNP variants are chimera comprising an N-terminal peptide fragment and CNP22 in which arginine is substituted for Lys4 of CNP22 ("CNP22 (K4R)"), including but not limited to:

(SEQ ID NO: 84)
GQPREPQVYTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CK)
($IgG_1(F_c)$ fragment-CNP22(K4R) chimera);

(SEQ ID NO: 85
GVPQVSTSTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CL)
(HSA fragment-CNP22(K4R) chimera)

-continued

(SEQ ID NO: 86)
GQPSSSSQSTGANQQGLSRGCFGLKLDRIGSMSGLGC
(Analog CM) (fibronectin fragment-CNP22(K4R)
chimera);

(SEQ ID NO: 87)
GQTHSSGTQSGANQQGLSRGCFGLKLDRIGSMSGLGC
(Analog CN) (fibrinogen fragment-CNP22(K4R)
chimera);

(SEQ ID NO: 88)
GSTGQWHSESGANQQGLSRGCFGLKLDRIGSMSGLGC
(Analog CO) (fibrinogen fragment-CNP22(K4R)
chimera);
and

(SEQ ID NO: 89)
GSSSSSSSSSGANQQGLSRGCFGLKLDRIGSMSGLGC
(Analog CP) (zinc finger fragment-CNP22(K4R)
chimera).

In yet another embodiment, the CNP variants are chimera, or fusion proteins, comprising a CNP peptide or variant, and a cleavable peptide or protein, or peptide tag. Exemplary cleavable proteins or peptides include, but are not limited to, histidine (e.g., hexa-His) tags; TAF12: human transcription factor TAF12; KSI: ketosteroid isomerase; MBP: maltose-binding protein; β-Gal: β-galactosidase; GST: glutathione-S-transferase; Trx: thioredoxin; CBD: chitin binding domain; BMPM: BMP-2 mutation, SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, c-Myc, poly(His), poly(Arg), poly(Asp), poly (Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and fragments thereof.

In yet another embodiment, the CNP variant may be a monomer or a dimer. In a related embodiment the monomers of dimeric CNP variants can be attached N-terminus to N-terminus via a linker or no linker, N-terminus to C-terminus via a linker or no linker, or C-terminus to C-terminus via a linker or no linker.

Chimera comprising an IgG fragment and CNP22 or a variant thereof are designed for, inter alia, increased resistance to NEP degradation and reduced binding to serum albumin. CNP chimera comprising a surface fragment of HSA are designed for, inter alia, reduced immunogenicity and reduced binding to serum albumin. HRGP-CNP22 and HRGP-CNP22(K4R) chimera containing a cationic, histidine-rich, non-lysine, non-arginine sequence at the N-terminus are designed for, inter alia, increased stability to proteases. Chimera containing an osteocrin fragment are designed to release, upon protease (e.g., furin) cleavage, the osteocrin fragment at bone growth plates, where the fragment would inhibit the clearance receptor NPR-C. With respect to chimera comprising an FGF2 heparin-binding fragment, heparin binding to the fragment is designed to protect the chimera from degradation, thereby providing a longer serum half-life. Chimera containing a fibronectin, fibrinogen, or zinc-finger fragment are designed for reduced binding to serum albumin, among other advantageous features.

Not intending to be bound by theory, a CNP variant of molecular weight from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa which has increased resistance to NEP degradation and has similar or improved functionality (e.g., binding to NPR-B and stimulation of cGMP signaling) as compared to wtCNP22, may be more effective if it does not bind tightly to plasma proteins such as serum albumin. A CNP variant that does not bind tightly to plasma proteins (e.g., serum albumin) may be more effective in diffusing through cartilage, getting to chondrocytes of bone growth plates, and binding to and activating NPR-B for cGMP signaling. In one embodiment, CNP variants designed for reduced binding to plasma proteins (e.g., serum albumin) are chimeras comprising CNP22 or a variant thereof and a peptide fragment from IgG. In another embodiment, CNP variants designed for reduced binding to plasma proteins are chimeras comprising CNP22 or CNP22(K4R) and a fragment from a polypeptide (e.g., IgG, HSA, fibronectin, fibrinogen, a zinc finger-containing polypeptide, etc.). In yet another embodiment, CNP variants designed for reduced binding to plasma proteins comprise CNP22 or a variant thereof conjugated to a hydrophilic or water-soluble polymer. In one embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO). In another embodiment, the hydrophilic or water-soluble polymer (e.g., PEG) is functionalized with one or more functional groups that impart a negative charge to the polymer under physiological conditions, such as, e.g, carboxyl, sulfate or phosphate groups, or a combination thereof.

In any of the embodiments disclosed herein, the CNP variants may have substantially the same or better biological activity than wild-type CNP22. For example, the CNP variants may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP22, or may have greater activity than CNP22, e.g., with respect to interaction with NPR-B (GC-B) to stimulate the generation of cGMP. Alternatively, or in addition, the CNP variants may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP22, or may have greater activity than CNP22, with respect to regulating endochondral bone growth and chondrocyte activity, including but not limited to chondrocyte proliferation, chondrocyte differentiation, inhibition of the mitogen activated protein (MAP) kinase/MEK (Raf-1) kinase signaling pathway, and promoting endochondral ossification. In any of the embodiments described herein, the CNP variants may comprise an amino acid sequence that is at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or more identical or homologous to amino acids 6-22 or 1-22 of wild-type CNP22.

In a further embodiment, the disclosure provides variants of CNP22 having less affinity to the NPR-C clearance receptor while retaining the ability to bind and activate NPR-B. The present disclosure encompasses variants that were, or can be, generated from a homology-based structural model of the NPR-B/CNP complex as described in the Detailed Description. In another embodiment, the CNP variants have substitution(s) at one or more Gly sites at positions 1, 5, 8, 15, 19 and 21, to reduce conformational flexibility, which may increase their specificity for binding to NPR-B over NPR-C. Variants of CNP having potentially reduced affinity to the NPR-C include but are not limited to those having one or more of the following substitutions: G1R, G1E, G5R, G5Q, G5S, F7Y, G8T, G8S, G8V, G8N, L9S, L9T, K10Cit, K10Q, K10S, I14N, G15R, G15S, G15N, G15Cit, S16Q, M17V, M17N, G19S, G19R, G19N, L20V, L20R, L20T, L20S, G21S, G21T and G21R.

In yet another embodiment, the CNP variants have a modification and/or substitution at one or more of positions 5, 7, 8, 9, 10, 14, 15, 16, 17, 19, 20 and 21, and may optionally have modifications and/or substitutions at any of the other positions disclosed herein. In a further embodiment, the CNP variants can optionally have conjugation(s) or extension(s), e.g., at the N- and/or C-terminus to facilitate bone/cartilage targeting, reduce renal clearance, and/or increase resistance to NEP degradation. Such conjugation(s) or extension(s) can comprise molecules or sequences formed or derived from,

e.g., polyAsp, polyGlu, bone- or cartilage-targeting peptides, osteopontin, osteocalcin, sialoprotein, PEGs, carbohydrates, hydrophobic acids, NPPC or non-CNP (poly)peptides, or combinations thereof.

In still another embodiment, the CNP variants are prepared by standard solid-phase peptide synthesis methods with natural or unnatural amino acid(s) or peptidomimetic(s) being substituted and/or added where appropriate. In another embodiment, the CNP variants are produced by recombinant synthesis processes, e.g., via fusion proteins containing a tag or carrier protein, wherein use of the tag or carrier protein facilitates, e.g., detection, isolation and/or purification of the fusion protein, and selective chemical or proteolytic cleavage of the tag or carrier protein from the fusion protein provides the target CNP variant. In a further embodiment, PEGylation of the CNP variants occurs following, or part of, chemical or biological synthesis with the conjugation reaction being performed by NHS- or aldehyde-based chemistry or other chemistry known in the art. In another embodiment, the CNP variants comprise a disulfide bond. In a related embodiment, the disulfide bond forms a cyclic peptide. In a particular embodiment, the disulfide bond is formed between cysteine residues at positions corresponding to positions 6 and 22 of CNP22.

It is further contemplated that the CNP variants can be conjugated to a hydrophobic polymeric or non-polymeric moiety, such as, e.g., heptanoic acid, pentanoic acid, or fatty acids. The hydrophobic moiety can be conjugated to the side chain of an amino acid residue, including but not limited to a lysine, a serine, a cysteine or a threonine, or can be attached to the N-terminus and/or C-terminus of the CNP variant.

In an embodiment, the CNP variants as described herein have a pI in the range from about 8 to about 10.5 or from about 8.5 to about 10.

In a further embodiment, the disclosure provides a pharmaceutical composition comprising a CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In some embodiments, the compositions are sterile pharmaceutical compositions suitable for parenteral injection. In some embodiments, the compositions comprise substantially pure CNP variant, e.g. at least about 90% or 95% pure. In some embodiments, the compositions contain less than about 5%, 4%, 3%, 2%, 1% or 0.5% contaminants, such as other human proteins, porcine proteins, or CNP53 or fragments thereof (other than the desired CNP variant). In certain embodiments, the sterile composition is administered to a subject for treating or preventing any of the CNP-responsive conditions or disorders disclosed herein.

CNP variants of the disclosure advantageously retain CNP activity and exhibit increased serum half-life. Retention of CNP activity can be shown, for example, as retention of desired in vivo biological effect, or retention of at least about 50%, 60%, 70%, 80%, 90%, 95% or at least about 100% of the cGMP stimulating activity of CNP22, under the same concentration (e.g., 1 uM of CNP peptide or greater than the ED80). In some embodiments, CNP variants exhibit at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold or 40-fold increase in serum half-life compared to CNP22.

In a related embodiment, the CNP variants described herein have increased NEP resistance and exhibit increased half-life compared to wild-type CNP22. In one embodiment, the half-life of the CNP variants is increased by about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or about 100% compared to wild-type CNP22.

In certain embodiments, the CNP variants described herein increase cGMP production in vitro, increase cGMP production in vivo, increase in vivo the level of one or more biomarkers associated with cartilage or bone formation or growth, increase resistance to NEP cleavage in vitro, increase plasma or serum half-life in vivo, increase bioavailability in vivo, or increase the length of particular bones in vivo, or effect combinations of such increases, by about 1.5-fold, about 2-fold, about 2.5-fold, about 3-fold, about 3.5-fold, about 4-fold, about 4.5-fold, or about 5-fold or more compared to wild-type CNP22.

In yet another embodiment, the disclosure provides methods of treating conditions or disorders responsive to CNP, comprising administering a therapeutically effective amount of a CNP variant or a composition comprising the same to a subject in need thereof. In one embodiment, disorders responsive to CNP are disorders of bone growth, including but not limited to skeletal dysplasias and inherited skeletal malformations such as disorders associated with fibroblast growth factor receptor 3 (FGFR-3) mutations. In a specific embodiment, the disorder associated with FGFR-3 mutation(s) is achondroplasia. In another embodiment, the disorders responsive to CNP are disorders associated with vascular smooth muscle cells and tissues. In a further embodiment, the CNP variants are useful for increasing the size of the growth plate of a bone (e.g., a limb bone). In another embodiment, the CNP variants are useful for elongating a bone or increasing long bone growth. In still another embodiment, the CNP variants are useful for enhancing matrix production, proliferation and differentiation of chondrocytes.

In certain embodiments, the CNP variants described herein are administered at a dose in the range from about 5 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg. In some embodiments, the CNP variants are administered at a dose of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variants are administered at a dose of about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750 or 800 ug/kg, or about 1 mg/kg, 1.25 mg/kg, 1.5 mg/kg, 2 mg/kg or other dose deemed appropriate by the treating physician. The doses of CNP variants described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc.

In another embodiment, the CNP variants are administered in a single treatment or in multiple doses. The multiple doses may be administered daily, or in multiple doses over the course of treatment. In certain embodiments, it is contemplated that the CNP variant is administered, in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week, 3 times per week, weekly, bi-weekly, every 3 weeks, monthly, every 6 weeks, every 2 months, every 3 months or as deemed appropriate by a treating physician.

In certain embodiments, administration of the CNP variant is adjusted to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP variant may be administered subcutaneously, intravenously, or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP

variant daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

In additional embodiments, the disclosure provides a method of treating a CNP-responsive condition or disorder, comprising administering a CNP peptide or variant to a subject, and monitoring the level of at least one bone- or cartilage-associated biomarker in the subject (e.g., in a biological sample from the subject), wherein an increase or decrease in the level of the bone- or cartilage-associated biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject. In some embodiments, when the level of a biomarker increases in association with bone or cartilage formation or growth, an increase in the level of that biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject. In other embodiments, when the level of a biomarker decreases in association with bone or cartilage formation or growth, a decrease in the level of that biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject.

In further embodiments, the therapeutic method further comprises adjusting the amount (or dose) or frequency of administration of the CNP peptide or variant, wherein:

(i) the amount (or dose) or frequency of administration of the CNP peptide or variant is increased if the level of the at least one bone- or cartilage-associated biomarker is below a target level, where the level of the biomarker increases in association with bone or cartilage formation or growth; or

(ii) the amount (or dose) or frequency of administration of the CNP peptide or variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is above a target level, where the level of the biomarker increases in association with bone or cartilage formation or growth; or

(iii) the amount (or dose) or frequency of administration of the CNP peptide or variant is increased if the level of the at least one bone- or cartilage-associated biomarker is above a target level, where the level of the biomarker decreases in association with bone or cartilage formation or growth; or

(iv) the amount (or dose) or frequency of administration of the CNP peptide or variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is below a target level, where the level of the biomarker decreases in association with bone or cartilage formation or growth.

It is contemplated that the target level of a biomarker refers to the level or range of levels of the biomarker that is associated with therapeutic effect in the subject and/or beneficial effect in alleviating or ameliorating symptoms of the disorder or condition. In certain embodiments, a level of a biomarker above or below a target level may be deleterious to the subject.

In other embodiments, the disclosure contemplates a method for assessing the effect of administration of a CNP peptide or variant on bone or cartilage formation or growth. In one embodiment, the method provides for assaying or measuring the level of at least one bone- or cartilage-associated biomarker in a subject that has been administered a CNP peptide or variant in order to assess the effect of the CNP peptide or variant on bone and cartilage formation and growth in vivo. In a related embodiment, an increase in the level of the at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP peptide or variant has a positive effect on bone or cartilage formation or growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (e.g, endogenous level of CNP-22 or CNP-53), cGMP, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In further embodiments, the disclosure contemplates a method for assessing the effect of a CNP peptide or variant on the level of at least one bone- or cartilage-associated biomarker in a subject, comprising assaying or measuring the level of the bone- or cartilage-associated biomarker in a biological sample from a subject that has been administered a CNP peptide or variant. In some embodiments, the method further comprises administering the CNP peptide or variant to the subject before assaying or measuring the level of the bone- or cartilage-associated biomarker.

In certain embodiments, the at least one bone- or cartilage-associated biomarker is selected from the group consisting of CNP (e.g, endogenous level of CNP-22 or CNP-53), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In some embodiments of methods (e.g., therapeutic, diagnostic and assay methods) relating to bone- or cartilage-associated biomarkers, the CNP peptide or variant is CNP-22, CNP-53, or any of the CNP peptides and variants described herein. In certain embodiments of such methods, the CNP peptide or variant is not CNP-22 or CNP-53.

In other embodiments, the disclosure provides a method for recombinant production of a CNP variant, comprising culturing in a medium a host cell comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein, under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides. In a related embodiment, the host cell is transformed with an expression vector comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein.

In one embodiment, the vector is a plasmid. In still another embodiment, the plasmid is selected from the group consisting of pET-21a, pJexpress, pET-31b, pET-15b, pET-32a, pET-41a, pMAL, pQE-30, pET-SUMO, pET-22b, and pTYB11.

In certain embodiments, the cleavable peptide or protein comprises a polypeptide that is selected from the group consisting of a histidine tag, human transcription factor TAF12, ketosteroid isomerase, maltose-binding protein, β-galactosidase, glutathione-S-transferase, thioredoxin, chitin binding domain, and BMP-2 mutation, or fragments thereof.

In a related embodiment, the cleavable peptide or protein is cleaved by a cleaving agent. In some embodiments, the cleaving agent is selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease. Additional exemplary cleaving agents include, but are not limited to, palladium, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase,

enteropeptidase, Kex 2 protease, Omp T protease, subtilisin, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K, pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

In certain embodiments, the fusion polypeptide is expressed as a soluble protein or as an inclusion body. In a related embodiment, the disclosure contemplates isolating the expressed fusion polypeptide from the host cell or culture medium. In a further embodiment, the isolated fusion polypeptide is contacted with a cleaving agent as described herein.

In one embodiment, the disclosure provides a bacterial host cell comprising an expression vector, said vector comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein. In some embodiments, the cleavable peptide or protein is selected from the group consisting of a histidine tag, human transcription factor TAF12, ketosteroid isomerase, maltose-binding protein, β-galactosidase, glutathione-S-transferase, thioredoxin, chitin binding domain, and BMP-2 mutation, or fragments thereof.

In another embodiment, the host cell is a bacteria, such as *E. coli*. In a related embodiment, the *E. coli* cell is selected from the group consisting of BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41 [also called C41(DE3)], C43 [also called C43(DE3)], Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3). In still a further embodiment, the host cell comprises a vector as described above. In some embodiments, the host cell is transformed with the vector prior to cell culture.

In certain embodiments, it is contemplated that the host cell is cultured in a medium under conditions suitable for expression of a fusion polypeptide encoded by the polynucleotides. In one embodiment, the fusion polypeptide is expressed as a soluble protein or as inclusion body. In a related embodiment, the expressed fusion polypeptide is isolated from the host cell or culture medium. In still another embodiment, the isolated fusion polypeptide is contacted with a cleaving agent as described herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** shows expression of CNP fusion proteins in *E. coli* (FIG. **1**A: Coomassie blue stain, FIG. **1**B: Western blot). M: protein marker; T: total cell lysates; S: soluble supernatants; P: 2 ug CNP22; KSI: KSI-CNP(M/N) fusion protein expression (insoluble); N: Un-induced KSI-CNP fusion protein total lysates; KSI': KSI-P-CNP fusion protein expression (insoluble); Trx: Trx-P-CNP fusion protein expression (soluble); MBP: MBP-P-CNP fusion protein expression (soluble); TAF: TAF-P-CNP fusion protein expression (insoluble) (BL21); TAF': TAF-P-CNP fusion protein expression (insoluble) BL21(DE3), where CNP is Gly-CNP37.

FIG. **2** shows formic acid cleavage of TAF-CNP inclusion bodies. M: protein marker; 1: Gly-CNP37 positive control; 2: uncleaved TAF-CNP inclusion bodies; 3: 2% formic acid cleaved TAF-CNP inclusion bodies, where CNP is Gly-CNP37.

FIGS. **3**A-E depict expression of CNP fusion proteins in *E. coli*. M: protein marker; Tu: total un-induced cell lysates; Su: un-induced soluble supernatants; T: total induced cell lysates; S: soluble supernatants; C1: CNP22; C: Gly-wtCNP37 ("CNP38"); P: insoluble pellets. A: KSI: KSI-CNP38(M/N) fusion protein expression (Insoluble); KSI': KSI-Pro-CNP38 (Pro-Gly-wtCNP37 is designated "Pro-CNP38") fusion protein expression (Insoluble); Trx: Trx-Pro-CNP38 fusion expression (Soluble); MBP: MBP-Pro-CNP38 fusion protein expression (Soluble); TAF: TAF-Pro-CNP38 fusion protein expression (Insoluble) from BL21 cell; TAF': TAF-Pro-CNP38 fusion protein expression (Insoluble) from BL21 (DE3) cell. B: TAF-Pro-CNP37 and BMP-Pro-CNP37 fusion protein expression. C: BMP-Pro-CNP38 fusion protein and BMP protein expression. D: TAF-Pro-HSA-CNP ("Pro-GH-KSEVAHRFK-wtCNP27 (SEQ ID NO: 188) is designated "Pro-HSA-CNP") fusion protein expression. E: TAF-Pro-CNP38 fusion protein and TAF protein expression.

FIGS. **4**A-C depict formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. A: 50% formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. M: protein marker; U: un-cleaved TAF-Pro-CNP38 inclusion bodies; 25° C., 37° C., 42° C., 55° C.: TAF-Pro-CNP38 inclusion bodies were cleaved in 50% formic acid at 25° C., 37° C., 42° C. or 55° C. for 24 hours. 37° C.-S and 55° C.-S: soluble supernatants from the cleavage reactions at 37° C. and 55° C. neutralized with 10N NaOH and centrifuged at 14,000 rpm for 15 minutes. B: 10% and 2% formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. M: protein marker; U: un-cleaved TAF-Pro-CNP38 inclusion bodies; C: formic acid cleaved TAF-Pro-CNP38; S: soluble supernatant after centrifuged at 14,000 rpm for 5 minutes without neutralization; P: insoluble pellet after centrifuged at 14,000 rpm for 5 minutes without neutralization. C: LC/MS analysis of 2% and 10% formic acid cleaved products from TAF-Pro-CNP38 inclusion bodies.

FIGS. **5**A-C depict formic acid cleavage of TAF-Pro-CNP38 inclusion bodies at different temperature and time of formic acid cleavage. M: protein marker; C: Gly-wtCNP37 ("CNP38") positive control; U: un-cleaved TAF-Pro-CNP38 inclusion bodies. A: 2% formic acid cleaved TAF-Pro-CNP38 at 42° C., 55° C. or 70° C. for 6, 24 or 48 hours. B: 2% formic acid cleaved TAF-Pro-CNP38 at 55° C., 60° C., 65° C. or 70° C. for 17 or 24 hours. C: LC/MS analysis of 2% formic acid cleaved products from TAF-Pro-CNP38 inclusion bodies.

FIG. **6** is an SDS-PAGE (Coomassie blue stain) for the expression of a TAF-CNP34 fusion protein. M: protein marker; C: control [Gly-CNP37 ("CNP38")]; T: total cell lysates; S: supernatant; TI: total cell lysates induced; SI: supernatant induced.

FIG. **7** is an SDS-PAGE (Coomassie blue stain) for the expression of fusion proteins TAF-NL-(C/A & 6D/6E)-Pro-CNP38, TAF(C/A & 10D/10E)-Pro-CNP38, and TAF-Pro-CNP53, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; C: control [Gly-CNP37 ("CNP38")]; T: total cell lysates; TI: total cell lysates induced; S: supernatant; SI: supernatant induced.

FIG. **8** is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF-CNP34 and TAF-Pro-CNP53. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

FIG. **9** is an LC/MS chromatogram showing the peak for CNP-34 after formic acid cleavage of TAF-CNP34.

FIG. **10** is an LC/MS chromatogram showing the peak for Pro-CNP53 after formic acid cleavage of TAF-Pro-CNP53.

FIG. **11** is an SDS-PAGE (Coomassie blue stain) for the expression of fusion proteins TAF(C/A & 4D/4E)-Pro-CNP38 and TAF(4D/4E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; C: control [Gly-

CNP37 ("CNP38")]; T: total cell lysates; TI: total cell lysates induced; S: supernatant; SI: supernatant induced.

FIG. **12** is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF (4D/4E)-Pro-CNP38 and TAF(C/A & 4D/4E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

FIG. **13** is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

FIG. **14** is a Western blot, using an anti-CNP antibody, of a TAF-Pro-CNP38 fusion protein produced in a 10 L fermentation of BL21(DE3) cells, where the cells were induced at OD_{600}=64 and hour 17 and "Pro-CNP38" denotes Pro-Gly-CNP37.

FIG. **15** is an SDS-PAGE (Coomassie blue stain) of eluate fractions from SP-Sepharose cation-exchange column chromatography of a cruder Pro-Gly-CNP37 ("Pro-CNP38") product. A: TAF-Pro-CNP38 inclusion body (IB) in water; B: IB in formate; C: IB in formate, neutralized; D: neutralized pellet; E: neutralized supernatant; F: TMAE Hi-CAP load; G: TMAE Hi-CAP flow through/SP-Sepharose load; H: SP-Sepharose flow through; SP-Sepharose eluate fractions 1-47: 10 uL/lane.

FIG. **16** shows the degree of resistance of N-terminal PEGylated CNP22 conjugates to neutral endopeptidase (NEP) in vitro.

FIG. **17** depicts the degree of NEP resistance of CNP variants having an amino acid extension at the N-terminus ["CNP27" is GANRR-CNP22(K4R) (SEQ ID NO: 36)].

FIG. **18** illustrates the degree of NEP resistance of N-terminal PEGylated CNP17 and GANRR-CNP22(K4R) ("CNP27") (SEQ ID NO: 36).

FIG. **19** illustrates the degree of NEP resistance of wtCNP22 and CNP variants Gly-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA" in the figures) (SEQ ID NO: 144) and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12" in the figures) (SEQ ID NO: 36).

FIG. **20** shows the ability of CNP variants having an N-terminal amino acid extension to stimulate cGMP production in NIH3T3 cells in vitro. The results are relative to the level of cGMP produced in the presence of 1 uM CNP22. "CNP27" is GANRR-CNP22(K4R) (SEQ ID NO: 36)

FIG. **21** displays the ability of N-terminal PEGylated CNP17 and GANRR-CNP22(K4R) ("CNP27") (SEQ ID NO: 36) to stimulate cGMP production in NIH3T3 cells.

FIG. **22** illustrates the effects of N-terminal PEGylation of CNP22 on cGMP production.

FIG. **23** illustrates the cGMP production induced by wtCNP22 and CNP variants Gly-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA") (SEQ ID NO: 144), wtCNP29 and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) in NIH3T3 cells.

FIGS. **24**A and B show that CNP-22 and Pro-Gly-CNP37 ("Pro-CNP38") stimulated cGMP production through NPR-B with similar dose-response curves, and to a much greater extent than through NPR-A, and exhibited a similar profile for NPR-B vs. NPR-C selectivity in in vitro signaling competition assays.

FIG. **25** demonstrates that exposure of rat chondrosarcoma cells to CNP22 1 hour once daily or 2 hours once daily has substantially similar effectiveness in reversing FGF2-induced arrest of chondrocyte growth as continuous exposure to CNP22.

FIGS. **26**A and B show results from a dose response study of CNP22 effects on FGF2-arrested rat chondrosarcoma (RCS) cells.

FIGS. **27**A-D show that addition of CNP22 to RCS cells arrested by FGF2 increases matrix synthesis and partly inhibits FGF2, as assessed by ^{35}S-sulfate and ^{3}H-Pro incorporation into or decrease from matrix. Panels A and C, synthesis; B and D, degradation; A and B, ^{35}S measurement; C and D, ^{3}H measurement. Statistically significant differences are highlighted (ANOVA; *p<0.05, **p<0.01).

FIGS. **28**A-C show the levels of aggrecan and fibronectin production (mRNA, panels A and C, and protein, panel B) in RCS cells cultured with FGF2 and CNP22.

FIG. **29** shows the efficacy of CNP37 and PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24" in the figures) (SEQ ID NO: 36) in stimulating longitudinal growth of wild-type femur in an ex vivo mouse organ model.

FIG. **30** shows longitudinal bone growth of 2-3 day-old wild-type mouse tibias treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means±SEM (n=8).

FIG. **31** shows longitudinal bone growth of 2-3 day old achondroplastic FGFR3^{ach} mouse tibias treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means±SEM (n=7-8).

FIG. **32** shows longitudinal bone growth of 2-3 day-old wild-type mouse femurs treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means±SEM (n=8).

FIG. **33** shows longitudinal bone growth of 2-3 day-old FGFR3^{ach} mouse femurs treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means±SEM (n=3-7).

FIGS. **34**A-I depict CNP37 biodistribution in FGFR3^{ach} mouse femurs treated ex vivo every two days. Panels A-C illustrate distribution in distal femurs, panels D-F illustrate distribution in articular chondrocytes and panels G-I illustrate distribution in hypertrophic chondrocytes.

FIGS. **35**A-C depict the cellularity of proliferating columns in growth plates after treatment of wild-type and FGFR3^{ach} mouse femurs with CNP22 or CNP37 every two days for 8 days. (A) no treatment, (B) cell numbers per column after treatment, (C) morphological studies after treatment. The panels in FIGS. **35**C(i) to C(vi). correspond to the sample order set out in FIG. **35**B. Data is represented as means±SEM (n=4-8).

FIGS. **36**A-C depict chondrocyte hypertrophy after ex vivo treatment of wild-type and FGFR3^{ach} mouse femurs with CNP22 or CNP37 every two days for 8 days. (A) no treatment, (B) cell size after treatment, (C) morphological studies after treatment. The panels in FIGS. **36**C(i) to C(vi) correspond to the sample order set out in FIG. **36**B. Data is represented as means±SEM (n=4-9).

FIGS. **37**A-I depict the biodistribution of CNP37 in FGFR3^{ach} mouse tibias treated in vivo. Panels A-C show distribution in distal femurs, panels D-F illustrate distribution in articular chondrocytes and panels G-I illustrate distribution in hypertrophic chondrocytes.

FIGS. **38**A-C illustrate the in vivo effects of CNP37 on FGFR3^{ach} mouse tibia growth plate: (A) total growth plate thickness, (B) proliferating zone thickness, and (C) hypertrophic zone thickness. Data is represented as means±SEM (n=7-15).

FIG. **39** shows cGMP levels in conditioned media from wild-type mouse femurs treated ex vivo with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) ($p<0.01$).

FIG. **40** shows cGMP levels in conditioned media from FGFR3^{ach} mouse femurs treated ex vivo with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p<0.01$).

FIG. **41** depicts cGMP levels in conditioned media from wild-type mouse tibias treated ex vivo with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p<0.01$).

FIG. **42** shows cGMP levels in conditioned media from FGFR3^{ach} mouse tibias treated ex vivo with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p<0.01$).

FIG. **43** demonstrates that ex vivo exposure of wild-type and FGFR3^{ach} mouse femurs to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) increased the levels of cleaved collagen type II in the conditioned media ($p<0.05$).

FIG. **44** depicts the hypertrophic region of femoral bones isolated from wild-type mice and FGFR3^{Y367C} mice (a mouse model of severe achondroplasia) and treated ex vivo with vehicle or 1 uM Pro-Gly-CNP37 ("ProCNP38") for 6 days, demonstrating that Pro-Gly-CNP37 treatment resulted in increase in bone growth and expansion in the growth plate.

FIG. **45** shows that CNP37 and PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) intravenously (i.v.) administered to rats have a much longer half-life and a much greater bioavailability in the plasma than CNP22.

FIG. **46** illustrates that PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) subcutaneously (s.c.) administered to rats also has a much longer half-life and a much greater bioavailability in the plasma than CNP22.

FIG. **47** demonstrates that i.v. administered CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) stimulate a much greater level of cGMP production in rats than CNP22.

FIG. **48** shows that s.c. administered PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and, to a lesser extent, CNP37 are substantially more effective in stimulating cGMP production in rats than CNP22.

FIG. **49** shows body weight measurements of wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22 (K4R) (SEQ ID NO: 36).

FIG. **50** shows tail length measurements of wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36).

FIG. **51** illustrates the effect of treatment of FGFR3^{ach} mice with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on body length ($p=0.02$, 1-tailed t-test, unequal variance).

FIG. **52** shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on tail length in FGFR3^{ach} mice.

FIGS. **53**A and B show the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on the length of distal long bones (A, ulna; B, tibia) in FGFR3^{ach} mice ($p<0.01$, one-tailed t-test, unequal variance).

FIGS. **54**A and B show the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on the length of proximal bones (A, humerus; B, femur) in FGFR3^{ach} mice ($p<0.01$, one-tailed t-test, unequal variance).

FIG. **55** illustrates that CNP37 administration corrects rhizomelia (disproportion of the length of the proximal limbs) as assessed by the femur:tibia ratio observed in FGFR3^{ach} mice ($p<0.01$, one-tailed t-test, unequal variance).

FIG. **56** shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on head length in FGFR3^{ach} mice ($p<0.01$ one-tailed t-test, unequal variance).

FIG. **57** shows that treatment of FGFR3^{ach} mice with CNP37 increases the size of the external auditory meatus (EAM) ($P=0.03$, one-tailed t-test, unequal variance).

FIG. **58** shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on spinal length in achondroplasic mice, expressed as extension of vertebral bodies (e.g., lumbar vertebra 5).

FIG. **59** shows that treatment of FGFR3^{ach} mice with CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) results in increased cGMP plasma levels 15-min post-dose.

FIG. **60** illustrates the serum levels of cleaved collagen type II in FGFR3^{ach} mice treated 5 weeks with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

FIG. **61** shows the serum levels of osteocalcin in FGFR3^{ach} mice treated 5 weeks with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

FIG. **62** shows cGMP plasma levels 15-min post-dose from wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p<0.05$).

FIG. **63** shows the serum levels of cleaved collagen type II in wild-type mice treated 5 weeks with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36).

FIGS. **64**-**66** display the levels of cGMP, cleaved collagen type II and alkaline phosphatase after administration of vehicle or 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37 ("Pro-CNP38") to wild-type mice.

FIGS. **67** and **68** depict cleaved collagen type II and total alkaline phosphatase levels after administration of vehicle or Pro-Gly-CNP37 ("Pro-CNP38") under different dosing regimens.

FIG. **69** illustrates the relative increase in body length of Pro-Gly-CNP37 ("Pro-CNP38") and vehicle treated animals in two separate studies (S1 and S2) at Day 37 vs Day 1.

FIGS. **70**A and B show changes in bone mineral density (A) and bone mineral content (B) after administration of Pro-Gly-CNP37 ("Pro-CNP38").

FIG. **71** shows plasma cGMP levels 15 min after the last subcutaneous dose of CNP variant, Day 36, where Gly-wtCNP37 is "CNP38", Pro-Gly-wtCNP37 is "Pro-CNP38", and GHKSEVAHRFK-wtCNP27 (SEQ ID NO: 144) is "HSA-CNP27" in FIGS. **71**-**73**.

FIG. **72** shows serum levels of cleaved collagen type II from mice treated with Gly-CNP37, Pro-Gly-CNP37 or GHKSEVAHRFK-CNP27 (SEQ ID NO: 144).

FIG. **73** shows serum levels of alkaline phosphatase from mice treated with Gly-CNP37, Pro-Gly-CNP37 or GHKSEVAHRFK-CNP27 (SEQ ID NO: 144).

FIGS. **74**A and B depict desensitization of the cGMP response after acute (A) or chronic (B) treatment with 1 uM Gly-CNP37.

FIG. **75**A demonstrates that daily treatment of wild-type mice with 200 nmol/kg of Pro-Gly-CNP37 ("Pro-CNP38") for 8 days did not desensitize the cGMP response. FIG. **75**B shows that treatment of the mice with 200 nmol/kg of Pro-Gly-CNP37 once a day for two consecutive days potentiated the cGMP response.

FIGS. **76**A-D show that treatment of wild-type mice with 200 nmol/kg of Gly-CNP37 stimulated cGMP secretion in distal femurs (cartilage and bone) (A), femoral cortices (bone) (B), ear pinna (cartilage) (C), and kidney (D). FIGS.

76E-H show that liver (E), heart (F), lung (G) and brain (H) tissues did not exhibit appreciable cGMP secretion in response to Gly-CNP37 relative to vehicle control at the studied time points.

FIGS. **77-82** show results from an on-going study in normal juvenile cynomolgus monkeys subcutaneously injected daily with vehicle or 10 or 36 ug/kg of Pro-Gly-CNP37. Both doses of Pro-Gly-CNP37 have increased growth plate width (FIG. **77**), increased right and left tibia lengths (FIGS. **78**A and B), increased leg length (FIG. **79**), increased arm length (FIG. **80**), increased body length (FIG. **81**), and increased the serum level of alkaline phosphatase (FIG. **82**).

FIG. **83** depicts the observed plot of degradation rate constant (K_{obs}) vs. pH at pH 3-8 and 5° C., 25° C. and 40° C. for Gly-CNP37 formulations.

DETAILED DESCRIPTION OF THE DISCLOSURE

The present disclosure relates to novel variants of CNP having reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleavage by NEP and/or clearance by NPR-C, pharmaceutical compositions comprising such CNP variants, and methods of using such

CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as achondroplasia and disorders associated with vascular smooth muscle cells and tissues.

A. Definitions

Unless otherwise stated, the following terms used in this application, including the specification and claims, have the definitions given below.

As used in the specification and the appended claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents unless the context clearly dictates otherwise.

The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range. Whenever the term "about" or "approximately" precedes the first numerical value in a series of two or more numerical values, it is understood that the term "about" or "approximately" applies to each one of the numerical values in that series.

The terms "ambient temperature" and "room temperature" are used interchangeably herein and refer to the temperature of the surrounding environment (e.g., the room in which a reaction is conducted or a composition is stored). In certain embodiments, ambient temperature or room temperature is a range from about 15° C. to about 28° C., or from about 15° C. to about 25° C., or from about 20° C. to about 28° C., or from about 20° C. to about 25° C., or from about 22° C. to about 28° C., or from about 22° C. to about 25° C. In other embodiments, ambient temperature or room temperature is about 15° C., 16° C., 17° C., 18° C., 19° C., 20° C., 21° C., 22° C., 23° C., 24° C., 25° C., 26° C., 27° C. or 28° C.

Definition of standard chemistry terms may be found in reference works, including Carey and Sundberg, Advanced Organic Chemistry, 3rd Edition, Vols. A and B (Plenum Press, New York 1992). The practice of the present disclosure may employ, unless otherwise indicated, certain conventional methods of synthetic organic chemistry, mass spectrometry, preparative and analytical chromatography, protein chemistry, biochemistry, recombinant DNA technology and pharmacology, within the skill of the art. See, e.g., T. E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A. L. Lehninger, Biochemistry (Worth Publishers, Inc., 4th Edition, 2004); Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pa.: Mack Publishing Company, 1990).

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

"Polypeptide" and "protein" refer to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof, linked via peptide bonds or peptide bond isosteres. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The terms "polypeptide" and "protein" are not limited to a minimum length of the product. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms "polypeptide" and "protein" also include postexpression modifications of the polypeptide or protein, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present disclosure, a "polypeptide" can include "modifications," such as deletions, additions, substitutions (which may be conservative in nature or may include substitutions with any of the 20 amino acids that are commonly present in human proteins, or any other naturally or non-naturally-occurring or atypical amino acids), and chemical modifications (e.g., addition of or substitution with peptidomimetics), to the native sequence. These modifications may be deliberate, as through site-directed mutagenesis, or through chemical modification of amino acids to remove or attach chemical moieties, or may be accidental, such as through mutations arising with hosts that produce the proteins or through errors due to PCR amplification.

Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

"Conservative substitution" refers to substitution of an amino acid in a polypeptide with a functionally, structurally or chemically similar natural or unnatural amino acid. In one

embodiment, the following groups each contain natural amino acids that are conservative substitutions for one another:

(1), Alanine (A) Serine (S), Threonine (T);

(2) Aspartic acid (D), Glutamic acid (E);

(3) Asparagine (N), Glutamine (Q);

(4) Arginine (R), Lysine (K);

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

(6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

In another embodiment, the following groups each contain natural amino acids that are conservative substitutions for one another:

(1) Glycine (G), Alanine (A);

(2) Aspartic acid (D), Glutamic acid (E);

(3) Asparagine (N), Glutamine (Q);

(4) Arginine (R), Lysine (K);

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V), Alanine (A);

(6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); and

(7) Serine (S), Threonine (T), Cysteine (C).

In a further embodiment, amino acids may be grouped as set out below.

(1) hydrophobic: Met, Ala, Val, Leu, Ile, Phe, Trp;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence backbone orientation: Gly, Pro; and

(6) aromatic: Trp, Tyr, Phe, His.

In one embodiment, the peptides or polypeptides described herein are generated via recombinant means, using a polynucleotide encoding a CNP variant. The disclosure thus encompasses polynucleotides encoding any of the CNP variants described herein, host cells or vectors comprising such polynucleotides, optionally linked to expression control sequences, and methods of using such polynucleotides, vectors or host cells to produce CNP variants of the disclosure. CNP variants expressed by such polynucleotides may be produced by methods including growing host cells in culture medium under conditions suitable for expression of the polynucleotide encoding a CNP variant, and isolating the expression product from the host cells or culture medium. Actual expression products may vary slightly from the encoded protein product depending on any post-translational processing.

"Polynucleotide" refers to a polymer composed of nucleotide units. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") as well as nucleic acid analogs. The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), the nucleotide sequence also encompasses an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T." "cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

"Expression control sequence" refers to a nucleotide sequence that regulates the expression of a nucleotide sequence operatively linked thereto. "Operatively linked" refers to a functional relationship between two parts in which the activity of one part (e.g., the ability to regulate transcription) results in an action on the other part (e.g., transcription of the sequence). Expression control sequences can include, for example and without limitation, sequences of promoters (e.g., inducible or constitutive), enhancers, transcription terminators, a start codon (i.e., ATG), splicing signals for introns, and stop codons.

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, e.g., a "recombinant polypeptide." A recombinant polynucleotide may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well.

"Chimera" as used herein refers to a polynucleotide or polypeptide comprising at least two heterologous polynucleotide or polypeptide sequences (i.e. derived from different sources or not associated with each other as a naturally-occurring sequence) which are directly or indirectly attached or linked together using techniques commonly known in the art, e.g., recombinant expression or chemical crosslinking. In one embodiment, the heterologous sequence can comprise a protein or peptide directly or indirectly linked to a CNP peptide or variant, including proteins or peptides which are cleavable from the CNP peptide or variant. In a related embodiment, CNP variants are chimera as described herein.

In certain embodiments, chimeras include CNP fusion proteins comprising a cleavable carrier protein or peptide tag. The term "cleavable carrier protein" or "cleavable peptide tag" refers to a peptide or polypeptide sequence that may be fused, directly or indirectly via a linker, to a heterologous polypeptide sequence, and is removable from the heterologous sequence using an agent that cleaves or separates the cleavable peptide or polypeptide from the heterologous polypeptide or protein. In some embodiments, the cleavable carrier protein or peptide tag improves generation, purification and/or detection of the fusion protein or the heterologous polypeptide. Exemplary cleavable carrier proteins and peptide tags include, but are not limited to, human transcription factor TAF12 (TAF12), ketosteroid isomerase (KSI), maltose-binding protein (MBP), β-galactosidase (β-Gal), glutathione-S-transferase (GST), thioredoxin (Trx), chitin-binding domain (CBD), BMP-2 mutation (BMPM), SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, c-Myc, poly(His), poly (Arg), poly(Asp), poly(Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and fragments thereof.

A "cleaving agent" is an agent that is useful to cleave or separate, e.g., a cleavable peptide or polypeptide from a heterologous polypeptide or protein. Exemplary cleaving agents include, but are not limited to, palladium, cyanogen bromide (CNBr), formic acid, hydroxylamine, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase, enterokinase (enteropeptidase), Kex 2 protease, Omp T protease, Factor Xa protease, subtilisin, proTEV, SUMO protease, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K, pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

The terms "identical" and percent "identity", in the context of two or more polynucleotide or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially homologous" or "substantially identical", in the context of two nucleic acids or polypeptides, generally refers to two or more sequences or subsequences that have at least 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 98% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. In certain embodiments, the substantial homology or identity exists over regions of the sequences that are at least about 25, 50, 100 or 150 residues in length. In another embodiment, the sequences are substantially homologous or identical over the entire length of either or both comparison biopolymers.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are inputted into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math., 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol., 48: 443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA, 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection. One example of a useful algorithm is PILEUP, which uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol., 35: 351-360 (1987) and is similar to the method described by Higgins & Sharp, CABIOS, 5: 151-153 (1989). Another algorithm useful for generating multiple alignments of sequences is Clustal W (Thompson et al., Nucleic Acids Research, 22: 4673-4680 (1994)). An example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm (Altschul et al., J. Mol. Biol., 215: 403-410 (1990); Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA, 89: 10915 (1989); Karlin & Altschul, Proc. Natl. Acad. Sci. USA, 90: 5873-5787 (1993)). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

A further indication that two nucleic acid sequences or polypeptides are substantially homologous or identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two polypeptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described herein.

"Substantially pure" or "isolated" means an object species is the predominant species present (i.e., on a molar basis, more abundant than any other individual macromolecular species in the composition), and a substantially purified fraction is a composition wherein the object species comprises at least about 50% (on a molar basis) of all macromolecular species present. In one embodiment, a substantially pure composition means that the species of interest comprises at least about 70%, 75%, 80%, 85%, 90%, 95%, 98% or more of the macromolecular species present in the composition on a molar or weight basis. The object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) if the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), stabilizers (e.g., BSA), and elemental ion species are not considered macromolecular species for purposes of this definition. In an embodiment, the compounds of the disclosure are substantially pure or isolated. In another embodiment, the compounds of the disclosure are substantially pure or isolated with respect to the macromolecular starting materials used in their production. In yet another embodiment, the pharmaceutical compositions of the disclosure comprise a substantially pure or isolated CNP variant admixed with one or more pharmaceutically acceptable excipients, carriers or diluents, and optionally with another biologically active agent.

"Naturally occurring" as applied to an object refers to the fact that the object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) and which has not been intentionally modified by man in the laboratory is naturally occurring. In one embodiment, a "naturally occurring" substance is of human origin.

"Wild-type" (wt) is a term referring to the natural form, including sequence, of a polynucleotide, polypeptide or protein in a species. A wild-type form is distinguished from a mutant form of a polynucleotide, polypeptide or protein arising from genetic mutation(s).

In one embodiment, a first polypeptide that is an "analog" or "variant" or "derivative" of a second polypeptide is a polypeptide having at least about 50%, 60% or 70% sequence homology, but less than 100% sequence homology, with the second polypeptide. Such analogs, variants or derivatives may be comprised of non-naturally occurring amino acid residues, including without limitation, homoarginine, ornithine, penicillamine, and norvaline, as well as naturally occurring amino acid residues. Such analogs, variants or derivatives may also be composed of one or a plurality of D-amino acid residues, and may also contain peptidomimetics or peptide bond isosteres such as non-peptide linkages between two or more amino acid or peptidomimetic residues. In another embodiment, a first polypeptide is an "analog", "variant" or "derivative" of a second polypeptide if the first polypeptide is not a known cleavage product of the second polypeptide or is not a known precursor of the second polypeptide, even if the first polypeptide has 100% sequence homology to the second polypeptide or has a wild-type sequence.

In an embodiment, the term "derived from" as used herein refers to a polypeptide or peptide sequence that is based on a wild type or naturally occurring polypeptide or peptide sequence and can have one or more deletions, additions, and/or substitutions with natural amino acids, unnatural amino acids or peptidomimetics. In one embodiment, the derivative sequence shares at least about 40%, 50%, 60% or 70%, but less than 100%, sequence similarity to the wild-type or naturally occurring sequence. In another embodiment, the

derivative may be a fragment of a polypeptide, wherein the fragment is substantially homologous (e.g., at least about 70%, 75%, 80%, 85%, 90%, or 95% homologous) to the wild-type polypeptide over a length of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 amino acids. In still another embodiment, a polypeptide is "derived from" a wild-type polypeptide if it has a moiety (e.g., a polymer such as, e.g., PEG) directly or indirectly attached to it which is not present on the wild-type polypeptide, even if both polypeptides share 100% homology in their amino acid sequence.

As used herein, an "NPPC-derived" polypeptide refers to a polypeptide derived from the natriuretic peptide precursor C(NPPC) polypeptide, which is a single chain 126-amino acid pre-pro polypeptide, and which upon cleavage ultimately results in wtCNP22. Removal of the signal peptide from NPPC yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP, or CNP-22). Therefore, CNP22 itself is an "NPPC-derived" polypeptide by virtue of being derived from NPPC. In one embodiment, an NPPC-derived polypeptide is at least about 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% homologous to the wild type NPPC over the same number of amino acid residues. It is further contemplated that an NPPC-derived peptide may comprise from about 1 to about 53, or 1 to 37, or 1 to 35, or 1 to 31, or 1 to 27, or 1 to 22, or to 35, or about 15 to about 37 residues of the NPPC polypeptide. In one embodiment, an NPPC-derived peptide may comprise a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, or 53 amino acids derived from the NPPC polypeptide.

The term "effective amount" means a dosage sufficient to produce a desired result on a health condition, pathology, or disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. "Therapeutically effective amount" refers to that amount of an agent effective to produce the intended beneficial effect on health. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation. It will be understood that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors, including the activity of the specific compound employed; the bioavailability, metabolic stability, rate of excretion and length of action of that compound; the mode and time of administration of the compound; the age, body weight, general health, sex, and diet of the patient; and the severity of the particular condition.

"Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment. In certain embodiments, "treatment" refers to administration of a compound or composition to a subject for therapeutic, prophylactic or diagnostic purposes.

A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology. The compounds or compositions of the disclosure may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional or physical, subjective or objective. The compounds of the disclosure may also be given as a therapeutic treatment or for diagnosis.

"Diagnostic" means identifying the presence, extent and/or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

"Bone- or cartilage-associated biomarker" or "bone- or cartilage-associated marker" refers to a growth factor, enzyme, protein, or other detectable biological substance or moiety whose level is increased or decreased in association with, e.g., cartilage turnover, cartilage formation, cartilage growth, bone resorption, bone formation, bone growth, or combinations thereof. Such biomarkers may be measured before, during and/or after administration of a CNP variant as described herein. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, propeptides of collagen type I and fragments thereof, collagen type 1 and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), aggrecan chondroitin sulfate, and alkaline phosphatase. Cartilage- and bone-associated biomarkers can be measured in any appropriate biological sample, including but not limited to tissues, blood, serum, plasma, cerebrospinal fluid, synovial fluid and urine. In some embodiments, the biomarkers are measured in blood, plasma or serum from animals undergoing efficacy/pharmacodynamic in vivo studies and/or from the conditioned media of ex vivo studies.

In certain embodiments, the level of at least one bone- or cartilage-associated biomarker is measured and the amount or frequency of administration of CNP variant administered to a subject can be adjusted according to the level of the biomarker measured. In some embodiments, the level of biomarker is "below a target level" or "above a target level." A target level of a biomarker is a level or range of levels of the biomarker at which a therapeutic effect is observed in the subject receiving the CNP variant. In certain embodiments, the target level of a biomarker for a subject having a CNP-responsive disorder or condition is the level or range of levels of the biomarker observed in a normal, non-affected subject. In other embodiments, to indicate a therapeutic effect, the target level of a biomarker need not be equivalent to the level or range of levels of the biomarker observed in a normal subject, but can be within, e.g., 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% or 5% of the "normal" level or range of levels of the biomarker observed in a non-affected subject.

For example, if the level of a biomarker increases in association with bone or cartilage formation or growth, the target level of the biomarker indicating a therapeutic effect may be higher than the level of the biomarker in patients suffering from a CNP-responsive disorder who have not been administered a CNP variant, and may optionally be lower than the "normal" level(s), at about the "normal" level(s), or above the "normal" level(s) of the biomarker in subjects not suffering from that disorder. In one embodiment, if the level of a biomarker is below a target level, it indicates an inadequate therapeutic effect, which may require an increase in the amount or frequency of administration of CNP variant administered. In a related embodiment, if the biomarker is above a target level, it indicates that more CNP variant than necessary has been administered, which may require a decrease in the amount or frequency of administration of the CNP variant administered.

As another example, if the level of a biomarker decreases in association with bone or cartilage formation or growth, the

target level of the biomarker indicating a therapeutic effect may be lower than the level of the biomarker in patients suffering from a CNP-responsive disorder who have not been administered a CNP variant, and may optionally be higher than the "normal" level(s), at about the "normal" level(s), or below the "normal" level(s) of the biomarker in subjects not suffering from that disorder. In such a case, the converse of the above adjustments in CNP variant amount and frequency of administration may apply.

"Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a therapeutically effective amount of a CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In an embodiment, a pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present disclosure encompass any composition made by admixing a compound of the disclosure and a pharmaceutically acceptable excipient, carrier or diluent.

"Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and the like, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions (e.g., an oil/water or water/oil emulsion). Non-limiting examples of excipients include adjuvants, binders, fillers, diluents, disintegrants, emulsifying agents, wetting agents, lubricants, glidants, sweetening agents, flavoring agents, and coloring agents. Suitable pharmaceutical carriers, excipients and diluents are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (e.g., oral) or parenteral (e.g., subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration).

A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use, including but not limited to metal salts (e.g., sodium, potassium, magnesium, calcium, etc.) and salts of ammonia or organic amines.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual without causing any undesirable biological effects or without interacting in a deleterious manner with any of the components of the composition in which it is contained or with any components present on or in the body of the individual.

The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of a compound of the disclosure calculated in an amount sufficient to produce the desired effect, optionally in association with a pharmaceutically acceptable excipient, diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present disclosure depend on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

"Physiological conditions" refer to conditions in the body of an animal (e.g., a human). Physiological conditions include, but are not limited to, body temperature and an aqueous environment of physiologic ionic strength, pH and enzymes. Physiological conditions also encompass conditions in the body of a particular subject which differ from the "normal" conditions present in the majority of subjects, e.g., which differ from the normal human body temperature of approximately 37° C. or differ from the normal human blood pH of approximately 7.4.

By "physiological pH" or a "pH in a physiological range" is meant a pH in the range of approximately 7.0 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

As used herein, the term "subject" encompasses mammals and non-mammals. Examples of mammals include, but are not limited to, any member of the mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. The term does not denote a particular age or gender.

The terms "polyethylene glycol", "PEG", "polyethylene oxide" and "PEO" are used interchangeably herein unless indicated otherwise. A CNP peptide (CNP22 or a variant thereof) conjugated via an amino group to a "PEOn" polymer associated with the number n, in general has the formula: CH_3—[—O—CH_2CH_2—]$_n$—C(=O)—NHR, where n is the number of ethylene oxide units and R denotes the rest of the peptide. The "PEOn" polymer can optionally have an alkylene group, $(CH_2)_m$, where m is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. Such a "PEOn" (e.g., PEO12 or PEO24) polymer is monodispersed, i.e., is a single discrete polymer of a particular molecular weight. Similarly, a CNP peptide conjugated via an amino group to a "PEGnK" polymer associated with the number nK, in general has the formula: CH_3—[—O—CH_2CH_2—]$_p$—C(=O)—NHR, where p is an integer greater than 1. The "PEGnK" polymer also can optionally have an alkylene group, $(CH_2)_m$, where m is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. However, such a "PEGnK" (e.g., PEG1K, PEG2K, PEG5K or PEG20K) polymer is polydispersed, i.e., contains a mixture of polymers having a distribution of molecular weights, where the number nK denotes the polymer number-average molecular weight (M_n) in kilo Daltons. For example, "PEG2K" conjugated to a CNP peptide denotes a polydispersed PEG polymer having a polymer number-average molecular weight of around 2 kDa.

When a range of the mass of a polymer (e.g., PEG) is given (e.g., in units of kDa), the range refers to a range of polymer number-average molecular weights, not to a range of molecular weights of multiple polymers in a polydispersed mixture, unless expressly indicated otherwise.

The term "halogen", "halide" or "halo" refers to fluorine, chlorine, bromine, and/or iodine.

The term "alkyl" refers to a linear or branched saturated monovalent hydrocarbon radical, wherein the alkyl may optionally be substituted with one or more substituents Q as described herein. In certain embodiments, the alkyl is a linear saturated monovalent hydrocarbon radical that has 1 to 20 (C_{1-20}), 1 to 15 (C_{1-15}), 1 to 12 (C_{1-12}), 1 to 10 (C_{1-10}), or 1 to 6 (C_{1-6}) carbon atoms, or a branched saturated monovalent hydrocarbon radical of 3 to 20 (C_{3-20}), 3 to 15 (C_{3-15}), 3 to 12 (C_{3-12}), 3 to 10 (C_{3-10}), or 3 to 6 (C_{3-6}) carbon atoms. As used herein, linear C_{1-6} and branched C_{3-6} alkyl groups are also referred as "lower alkyl." Examples of alkyl groups include,

but are not limited to, methyl, ethyl, propyl (including all isomeric forms, including n-propyl and isopropyl), butyl (including all isomeric forms, including n-butyl, isobutyl, sec-butyl and tert-butyl), pentyl (including all isomeric forms), and hexyl (including all isomeric forms). For example, C_{1-6} alkyl refers to a linear saturated monovalent hydrocarbon radical of 1 to 6 carbon atoms or a branched saturated monovalent hydrocarbon radical of 3 to 6 carbon atoms.

The term "alkoxy" refers to an —O-alkyl group. In certain embodiments, an alkoxy group may optionally be substituted with one or more substituents Q as described herein.

The term "haloalkyl" refers to an alkyl group that is substituted with one or more halide atoms. In certain embodiments, a haloalkyl group is substituted with one, two, three, four, five or six halide atoms. In certain embodiments, a haloalkyl group may optionally be substituted with one or more additional substituents Q as described herein.

The term "cycloalkyl" refers to a cyclic saturated bridged and/or non-bridged monovalent hydrocarbon radical, which may be optionally substituted with one or more substituents Q as described herein. In certain embodiments, the cycloalkyl has from 3 to 20 (C_{3-20}), from 3 to 15 (C_{3-15}), from 3 to 12 (C_{3-12}), from 3 to 10 (C_{3-10}), or from 3 to 7 (C_{3-7}) carbon atoms. Examples of cycloalkyl groups include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, decalinyl, and adamantyl.

The term "heterocyclyl" or "heterocyclic" refers to a monocyclic non-aromatic ring system or a multicyclic ring system that contains at least one non-aromatic ring, wherein one or more of the non-aromatic ring atoms are heteroatoms independently selected from O, S, or N, and the remaining non-aromatic ring atoms are carbon atoms. In certain embodiments, the heterocyclyl or heterocyclic group has from 3 to 20, from 3 to 15, from 3 to 10, from 3 to 8, from 4 to 7, or from 5 to 6 ring atoms. In certain embodiments, the heterocyclyl is a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which may include a fused or bridged ring system, and in which the nitrogen or sulfur atoms may be optionally oxidized, the nitrogen atoms may be optionally quaternized, and some rings may be partially or fully saturated, or aromatic. The heterocyclyl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. Examples of heterocyclic groups include, but are not limited to, acridinyl, azepinyl, benzimidazolyl, benzindolyl, benzoisoxazolyl, benzisoxazinyl, benzodioxanyl, benzodioxolyl, benzofuranonyl, benzofuranyl, benzonaphthofuranyl, benzopyranonyl, benzopyranyl, benzotetrahydrofuranyl, benzotetrahydrothienyl, benzothiadiazolyl, benzothiazolyl, benzothiophenyl, benzotriazolyl, benzothiopyranyl, benzoxazinyl, benzoxazolyl, benzothiazolyl, β-carbolinyl, carbazolyl, chromanyl, chromonyl, cinnolinyl, coumarinyl, decahydroisoquinolinyl, dibenzofuranyl, dihydrobenzisothiazinyl, dihydrobenzisoxazinyl, dihydrofuryl, dihydropyranyl, dioxolanyl, dihydropyrazinyl, dihydropyridinyl, dihydropyrazolyl, dihydropyrimidinyl, dihydropyrrolyl, dioxolanyl, 1,4-dithianyl, furanonyl, furanyl, imidazolidinyl, imidazolinyl, imidazolyl, imidazopyridinyl, imidazothiazolyl, indazolyl, indolinyl, indolizinyl, indolyl, isobenzotetrahydrofuranyl, isobenzotetrahydrothienyl, isobenzothienyl, isochromanyl, isocoumarinyl, isoindolinyl, isoindolyl, isoquinolinyl, isothiazolidinyl, isothiazolyl, isoxazolidinyl, isoxazolyl, morpholinyl, naphthyridinyl, octahydroindolyl, octahydroisoindolyl, oxadiazolyl, oxazolidinonyl, oxazolidinyl, oxazolopyridinyl, oxazolyl, oxiranyl, perimidinyl, phenanthridinyl, phenathrolinyl, phenarsazinyl, phenazinyl, phenothiazinyl, phenoxazinyl, phthalazinyl, piperazinyl, piperidinyl, 4-piperidonyl, pteridinyl, purinyl, pyrazinyl, pyrazolidinyl, pyrazolyl, pyridazinyl, pyridinyl, pyridopyridinyl, pyrimidinyl, pyrrolidinyl, pyrrolinyl, pyrrolyl, quinazolinyl, quinolinyl, quinoxalinyl, quinuclidinyl, tetrahydrofuryl, tetrahydrofuranyl, tetrahydroisoquinolinyl, tetrahydropyranyl, tetrahydrothienyl, tetrazolyl, thiadiazolopyrimidinyl, thiadiazolyl, thiamorpholinyl, thiazolidinyl, thiazolyl, thienyl, triazinyl, triazolyl, and 1,3,5-trithianyl. In certain embodiments, a heterocyclic group may optionally be substituted with one or more substituents Q as described herein.

The term "aryl" refers to a monocyclic aromatic group or a multicyclic monovalent aromatic group that contain at least one aromatic hydrocarbon ring. In certain embodiments, the aryl has from 6 to 20 (C_{6-20}), from 6 to 15 (C_{6-15}), or from 6 to 10 (C_{6-10}) ring atoms. Examples of aryl groups include, but are not limited to, phenyl, naphthyl, fluorenyl, azulenyl, anthryl, phenanthryl, pyrenyl, biphenyl, and terphenyl. Aryl also refers to bicyclic or tricyclic carbon rings, where at least one of the rings is aromatic and the others may be saturated, partially unsaturated, or aromatic, for example, dihydronaphthyl, indenyl, indanyl, and tetrahydronaphthyl (tetralinyl). In certain embodiments, an aryl group may optionally be substituted with one or more substituents Q as described herein.

The term "heteroaryl" refers to a monocyclic aromatic group or a multicyclic aromatic group that contain at least one aromatic ring, wherein at least one aromatic ring contains one or more heteroatoms independently selected from O, S, and N. Each ring of a heteroaryl group can contain one or two O atoms, one or two S atoms, and/or one to four N atoms, provided that the total number of heteroatoms in each ring is four or less and each ring contains at least one carbon atom. The heteroaryl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. In certain embodiments, the heteroaryl has from 5 to 20, from 5 to 15, or from 5 to 10 ring atoms. Examples of monocyclic heteroaryl groups include, but are not limited to, pyrrolyl, pyrazolyl, pyrazolinyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, thiadiazolyl, isothiazolyl, furanyl, thienyl, oxadiazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, and triazinyl. Examples of bicyclic heteroaryl groups include, but are not limited to, indolyl, benzothiazolyl, benzoxazolyl, benzothienyl, quinolinyl, tetrahydroisoquinolinyl, isoquinolinyl, benzimidazolyl, benzopyranyl, indolizinyl, benzofuranyl, isobenzofuranyl, chromonyl, coumarinyl, cinnolinyl, quinoxalinyl, indazolyl, purinyl, pyrrolopyridinyl, furopyridinyl, thienopyridinyl, dihydroisoindolyl, and tetrahydroquinolinyl. Examples of tricyclic heteroaryl groups include, but are not limited to, carbazolyl, benzindolyl, phenanthrollinyl, acridinyl, phenanthridinyl, and xanthenyl. In certain embodiments, a heteroaryl group may optionally be substituted with one or more substituents Q as described herein.

The term "optionally substituted" is intended to mean that a group, including alkyl, alkoxy, haloalkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, may be substituted with one or more substituents Q (in one embodiment, one, two, three or four substituents Q), where each Q is independently selected from the group consisting of cyano, halo, oxo, nitro, C_{1-6} alkyl, C_{1-6} alkoxy, halo-C_{1-6} alkyl, C_{3-7} cycloalkyl, heterocyclyl, C_{6-14} aryl, heteroaryl, $—C(O)R^e$, $—C(O)OR^e$, $—C(O)NR^fR^g$, $—C(NR^e)NR^fR^g$, $—OR^e$, $—OC(O)R^e$, $—OC(O)OR^e$, $—OC(O)NR^fR^g$, $—OC(=NR^e)NR^fR^g$, $—OS(O)R^e$, $—OS(O)_2R^e$, $—OS(O)NR^fR^g$, $—OS(O)_2NR^fR^g$, $—NR^fR^g$, $—NR^eC(O)R^f$, $—NR^eC(O)OR^f$, $—NR^eC(O)NR^fR^g$, $—NR^eC(=NR^h)NR^fR^g$, $—NR^eS(O)R^f$, $—NR^eS(O)_2R^f$, $—NR^eS(O)NR^fR^g$, $—NR^eS(O)_2NR^fR^g$, $—SR^e$, $—S(O)R^e$, $—S(O)_2R^e$, and $—S(O)_2NR^fR^g$, wherein each R^e, R^f, R^g, and R^h is independently hydrogen, C_{1-6} alkyl, C_{3-7} cycloalkyl, heterocyclyl, C_{6-14} aryl, or heteroaryl; or R^f and R^g, together with the N atom to which they are attached, form heterocyclyl.

B. CNP Variants

The use of CNP22 as a therapeutic is limited by its short half-life in plasma (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). In human plasma, the concentration of CNP22 typically is less than five picomolar. CNP22 is degraded and cleared from circulation by NEP and NPR-C in humans (Growth Hormone & IGF Res., 16: S6-S14). In all human and animal studies using systemically administered CNP22, continuous infusion has been used to increase the CNP22 concentration in the subjects. A CNP peptide having a longer half-life and at least a similar level of functionality would be beneficial to a CNP-based therapeutic strategy. CNP variants are also disclosed in related International Application No. PCT/US08/84270, specifically incorporated herein by reference.

The present disclosure provides CNP variants which have reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleavage by NEP and/or clearance by NPR-C, but which have substantially similar or better functionality than wild-type CNP22. Reduced susceptibility of CNP variants to cleavage by NEP and/or clearance by NPR-C would increase the plasma or serum half-life of the variants, thereby increasing the opportunity for the variants to distribute to the target tissues and sites and effectuate the desired pharmacological effects. In certain embodiments, the CNP variants described herein have reduced susceptibility to cleavage by NEP and/or clearance by NPR-C in vitro or in vivo by at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, or 5-fold compared to wtCNP22, and have increased plasma or serum half-life in vivo by at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, or 5-fold compared to wtCNP22, while retaining at least about 50%, 60%, 70%, 80%, 90% or 100% of the functionality of wtCNP22, or having at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, or 5-fold greater functionality than wtCNP22. CNP functionality can be evaluated in terms of, e.g., the level of one or more biomarkers (e.g., cGMP) associated with cartilage or bone formation or growth in an in vitro or in vivo study, the length of particular bones in an ex vivo or in vivo study, etc.

Natural substrates of NEP are small and natriuretic peptides (about 2.2 to about 3.2 kDa) are the largest of the natural substrates. According to X-ray crystallographic analyses, the NEP active-site is buried deep inside a central cavity, effectively restricting the size of substrate molecules to no more than about 3 kDa (Oefner et al., J. Mol. Biol., 296: 341-349 (2000)). Based on NPR-B signaling studies, variants of CNP-22, such as CNP-17 (retaining only the cyclic domain, Cys6-Cys22, of CNP22) and CNP-53 (CNP-22 with a 31-amino acid extension at the N-terminus), can still bind and activate NPR-B similarly to the 2.2 kDa wtCNP-22. Accordingly, the disclosure encompasses CNP variants conjugated to a natural (e.g., peptide) and/or synthetic (e.g., PEG) polymer at the N-terminus and/or C-terminus of CNP22 or variants thereof, which exhibit increased NEP resistance but retain the ability to bind and activate the NPR-B signaling receptor.

In one embodiment, the disclosure encompasses CNP variants represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:139), or

(x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:140), wherein:

(x) and (z) each independently are a natural polymer (e.g., a peptide sequence containing at least one amino acid) and/or a synthetic polymer (e.g., PEG) as described herein, such that the total mass of the CNP variant is characterized by the ranges described generally herein, e.g., in the range from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa. In one embodiment, the residues from Cys6 to Cys22 form a cyclic portion. In an embodiment, (x) and/or (z) comprise an amino acid extension derived from NPPC or a non-CNP polypeptide (e.g., ANP, BNP, IgG, etc.), wherein the extension contains 1 to 40, 1 to 35, 1 to 31, 5 to 35, 5 to 31 or 5 to 15 amino acids. In another embodiment, the CNP variants comprise one or more modifications and/or substitutions with another natural amino acid, an unnatural amino acid, a peptidomimetic and/or a peptide bond isostere at one or more of the following positions of CNP22: Gly1, Lys4, Gly5, Cys6, Phe7, Gly8, Leu9, Lys10, Leu11, Ile14, Gly15, Ser16, Met17, Gly19, Leu20 and Gly21.

In another embodiment, CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, are represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 141), or

(x)-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 6), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG (or PEO), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or fibroblast growth factor 2 (FGF2);

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG); and

(b) and (h) independently may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxynorleucine, citrulline (Cit), Gln, Glu or Ser. In one embodiment, (b) is Arg. In another embodiment, for improved NEP resistance, (b) is not Gly. In yet another embodiment, (h) is not Arg.

Non-limiting examples of amino acid sequences derived from NPPC or variants thereof include:

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Arg,

Glu-Arg,

                                        (SEQ ID NO: 7)
Gly-Ala-Asn-Lys-Lys,

                                        (SEQ ID NO: 8)
Gly-Ala-Asn-Arg-Arg,

                                        (SEQ ID NO: 9)
Gly-Ala-Asn-Pro-Arg,
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-continued

(SEQ ID NO: 10)
Gly-Ala-Asn-Gln-Gln,

(SEQ ID NO: 11)
Gly-Ala-Asn-Ser-Ser,

(SEQ ID NO: 12)
Gly-Ala-Asn-Arg-Gln,

(SEQ ID NO: 13)
Gly-Ala-Asn-Arg-Met,

(SEQ ID NO: 14)
Gly-Ala-Asn-Arg-Thr,

(SEQ ID NO: 15)
Gly-Ala-Asn-Arg-Ser,

(SEQ ID NO: 16)
Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala,

(SEQ ID NO: 17)
Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg,

(SEQ ID NO: 18)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg,

(SEQ ID NO: 19)
Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys,

(SEQ ID NO: 20)
Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg,

(SEQ ID NO: 21)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys,
and

(SEQ ID NO: 22)
Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg.

Non-limiting examples of amino acid sequences derived from non-CNP polypeptides such as, e.g., ANP, BNP, serum albumin and IgG include:

(SEQ ID NO: 23)
Ser-Leu-Arg-Arg-Ser-Ser;

(SEQ ID NO: 24)
Asn-Ser-Phe-Arg-Tyr;

(SEQ ID NO: 25)
Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly;

(SEQ ID NO: 26)
Met-Val-Gln-Gly-Ser-Gly;

(SEQ ID NO: 27)
Lys-Val-Leu-Arg-Arg-Tyr;

(SEQ ID NO: 28)
Lys-Val-Leu-Arg-Arg-His;

(SEQ ID NO: 29)
Gly-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg;

(SEQ ID NO: 30)
Gly-Val-Pro-Gln-Val-Ser-Thr-Ser-Thr;

-continued

(SEQ ID NO: 31)
Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln;

(SEQ ID NO: 32)
Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser.

In an embodiment, the N-terminal (x) group and/or the C-terminal (z) group of any of the CNP variants having an (x) and/or (z) group, as described herein, independently comprise an amino acid sequence that contains a small number of, if any, acidic natural or unnatural amino acids (e.g., Asp or Glu). In another embodiment, (x) and/or (z) are enriched in basic natural or unnatural amino acids (e.g., Lys, Arg or His) to maintain an alkaline pI similar to the pI of CNP22 (pI 8.9). In one embodiment, the pI of the CNP variants is in the range from about 8 to about 10.5, designed so that the CNP variants can diffuse more readily through the extracellular matrix surrounding chondrocytes of bone growth plates. In narrower embodiments, the pI of the CNP variants is from about 8.5 to about 10.5, or from about 8.5 to about 10, or from about 9 to about 10.

In yet another embodiment, (x) and/or (z) are enriched in polar natural or unnatural amino acids, designed for increased aqueous solubility. In still another embodiment, (x) and/or (z) contain a small number of, if any, hydrophobic natural or unnatural amino acids (e.g., Ala, Val, Leu, Ile or Met).

In a further embodiment, the N-terminus of the CNP variants terminates in at least one glycine residue, designed for increased serum half-life. In a related embodiment, to prevent pyroglutamine formation, the N-terminus of CNP variants terminates in a glycine residue if it would otherwise terminate in glutamine. In one embodiment, the (x) group contains an amino acid extension whose N-terminus terminates in at least one glycine residue. In another embodiment, (x) and/or (z) do not contain two adjacent basic natural or unnatural amino acids (e.g., Lys-Lys or Arg-Arg), designed to reduce susceptibility to cleavage by the protease furin. In an embodiment, (x) does not contain two adjacent basic amino acids immediately preceding the position corresponding to Gly1 of CNP22.

In still another embodiment, the (x) group and/or the (z) group of the CNP variants comprise an amino acid sequence derived from NPPC (e.g., derived from CNP53). In an embodiment, (x) comprises an amino acid sequence derived from the N-terminal tail of ANP or BNP. In another embodiment, (z) comprises an amino acid sequence derived from the C-terminal tail of ANP or BNP. In a further embodiment, (x) and/or (z) comprise an amino acid sequence derived from a non-natriuretic polypeptide such as, e.g., IgG, human serum albumin (HSA), histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, FGF-2, and bone-targeting proteins (e.g., osteocrin, osteopontin, osteocalcin, and sialoprotein).

In any embodiment described herein in which CNP22 or a variant thereof can have an N-terminal (x) group and/or a C-terminal (z) group, (x) and/or (z) independently can contain an amino acid sequence derived from the functional domain of a bone morphogenetic protein (BMP). An N-terminal and/or C-terminal amino acid extension derived from the functional domain of a BMP can increase the NEP resistance, and hence the serum half-life of the CNP variant, by increasing the total mass of the CNP variant to characterized by the ranges described generally herein, e.g., a range from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa. In addition, since certain BMPs are growth factors and cytokines that

induce the formation of bone and cartilage, a fragment derived from the functional domain of a BMP can promote chondrocyte, cartilage or bone growth by a mechanism distinct from activation of the guanylyl cyclase function of NPR-B by the cyclic domain of CNP22 or a variant thereof. Non-limiting examples of BMPs that promote bone formation and development, cartilage formation and development, and/or osteoblast differentiation include BMP1, BMP2, BMP3, BMP5, BMP7 and BMP8a. In an embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof independently are conjugated to an amino acid sequence derived from the last 140 amino acids in the C-terminal portion of BMP1, BMP2, BMP3, BMP5, BMP7 or BMP8a.

In one embodiment, the CNP variants contain an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, including but not limited to:

(SEQ ID NO: 4)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (CNP-53);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-37, Analog BL);

(SEQ ID NO: 61)
AAWARLLQEHPNAGLSKGCFGLKLDRIGSMSGLGC (Analog CA);

(SEQ ID NO: 62)
AAWARLLQEHPNARGLSKGCFGLKLDRIGSMSGLGC (Analog CB);

(SEQ ID NO: 63)
DLRVDTKSRAAWARGLSKGCFGLKLDRIGSMSGLGC (Analog CC);

(SEQ ID NO: 40)
RGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 38)
ERGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 64)
GANQQGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 65)
GANRRGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 66)
GANPRGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 67)
GANSSGLSKGCFGLKLDRIGSMSGLGC;

(SEQ ID NO: 144)
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC;
and

(SEQ ID NO: 68)
SPKMVQGSG-CNP17-KVLRRH (Analog CD) (CNP17 having
N-terminal and C-terminal tails derived from BNP).

In another embodiment, the CNP variants have a K4R substitution at position 4 of CNP22. Non-limiting examples of CNP(K4R) variants include:

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC (Analog AY);

(SEQ ID NO: 37)
GANPRGLSRGCFGLKLDRIGSMSGLGC (Analog CI);

(SEQ ID NO: 41)
RGLSRGCFGLKLDRIGSMSGLGC (Analog AZ);

(SEQ ID NO: 39)
ERGLSRGCFGLKLDRIGSMSGLGC (Analog BA);

-continued

(SEQ ID NO: 69)
GANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CH);
and

(SEQ ID NO: 70)
GANSSGLSRGCFGLKLDRIGSMSGLGC (Analog CG).

In further embodiments, the CNP variants are chimeras comprising CNP22, or a variant thereof having amino acid addition(s), deletion(s) and/or substitution(s), and a peptide fragment derived from a polypeptide or protein other than CNP, or the whole non-CNP polypeptide or protein, to the N-terminus of the CNP peptide, wherein CNP22 or the variant thereof may optionally have an N-terminal amino acid extension of one or more amino acid residues. In certain embodiments, the CNP chimeras comprise CNP22 or a variant thereof that has an N-terminal amino acid extension of one or more amino acid residues. In certain embodiments, the CNP chimeras contain lysine-lysine (KK) residues or GANKK residues immediately preceding the first position of CNP22 (Gly in the case of CNP22) or a variant thereof. In other embodiments, the CNP chimeras contain one or two residues different from lysine-lysine immediately preceding the first position of CNP22 or a variant thereof. Non-limiting examples of residues that can immediately precede the first position of CNP22 or a variant thereof include KP, PK, PR, PQ, QK, QQ, RR, SS, GANKP(SEQ ID NO: 200), GANPK (SEQ ID NO: 201), GANPR (SEQ ID NO: 9), GANPQ (SEQ ID NO: 202), GANQK (SEQ ID NO: 203), GANQQ (SEQ ID NO: 10), GANRR (SEQ ID NO: 8), and GANSS (SEQ ID NO: 11).

In another embodiment, the CNP variants are chimera comprising CNP22 and an N-terminal peptide fragment, including but not limited to:

(SEQ ID NO: 76)
GHHSHEQHPHGANQQGLSKGCFGLKLDRIGSMSGLGC (Analog CQ)
(histidine-rich glycoprotein (HRGP) fragment-CNP22
chimera);

(SEQ ID NO: 77)
GAHHPHEHDTHGANQQGLSKGCFGLKLDRIGSMSGLGC (Analog CR)
(HRGP fragment-CNP22 chimera);

(SEQ ID NO: 78)
GHHSHEQHPHGANPRGLSKGCFGLKLDRIGSMSGLGC (Analog CX)
(HRGP fragment-CNP22 chimera);

(SEQ ID NO: 79)
GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC (Analog CF)
(IgG$_1$(F$_c$) fragment-CNP22 chimera);

(SEQ ID NO: 80)
GQHKDDNPNLPRGANPRGLSKGCFGLKLDRIGSMSGLGC
(Analog CY) (human serum albumin (HSA) fragment-
CNP22 chimera);

(SEQ ID NO: 81)
GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC
(Analog CE) (HSA fragment-CNP22 chimera);

(SEQ ID NO: 82)
FGIPMDRIGRNPRGLSKGCFGLKLDRIGSMSGLGC (Analog CZ)
(osteocrin "NPR C inhibitor" fragment-CNP22
chimera);
and

(SEQ ID NO: 83)
GKRTGQYKLGSKTGPGPKGLSKGCFGLKLDRIGSMSGLGC
(Analog DA) (FGF2 "heparin-binding domain"
fragment-CNP22 chimera).

In yet another embodiment, the CNP variants are chimera comprising an N-terminal peptide fragment and CNP22 in which arginine is substituted for Lys4 of CNP22 ("CNP22 (K4R)"), including but not limited to:

(SEQ ID NO: 84)
GQPREPQVYTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CK) ($IgG_1(F_c)$ fragment-CNP22(K4R) chimera);

(SEQ ID NO: 85)
GVPQVSTSTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CL) (HSA fragment-CNP22(K4R) chimera);

(SEQ ID NO: 86)
GQPSSSSQSTGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CM) (fibronectin fragment-CNP22(K4R) chimera);

(SEQ ID NO: 87)
GQTHSSGTQSGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CN) (fibrinogen fragment-CNP22(K4R) chimera);

(SEQ ID NO: 88)
GSTGQWHSESGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CO) (fibrinogen fragment-CNP22(K4R) chimera); and

(SEQ ID NO: 89)
GSSSSSSSSSGANQQGLSRGCFGLKLDRIGSMSGLGC (Analog CP) (zinc finger fragment-CNP22(K4R) chimera).

Chimera comprising IgG and CNP22 or a variant thereof are designed for, inter alia, increased resistance to NEP degradation and reduced binding to serum albumin. CNP chimera comprising a surface fragment of HSA are designed for, inter alia, reduced immunogenicity and reduced binding to serum albumin. HRGP-CNP22 and HRGP-CNP22(K4R) chimera containing a cationic, histidine-rich, non-lysine, non-arginine sequence at the N-terminus are designed for, inter alia, increased stability to proteases. Chimera containing an osteocrin fragment are designed to release, upon protease (e.g., furin) cleavage, the osteocrin fragment at bone growth plates, where the fragment would inhibit the clearance receptor NPR-C. With respect to chimera comprising an FGF2 heparin-binding fragment, heparin binding to the fragment is designed to protect the chimera from degradation, thereby providing a longer serum half-life. Chimera containing a fibronectin, fibrinogen, or zinc-finger fragment are designed for reduced binding to serum albumin, among other features.

Not intending to be bound by theory, a CNP variant of molecular weight from about 2.6 or 2.8 kDa to about 6 or 7 kDa which has increased resistance to NEP degradation and has similar or improved functionality (e.g., binding to NPR-B and stimulation of cGMP signaling) as compared to wtCNP22, may be more effective if it does not bind tightly to plasma proteins such as serum albumin. A CNP variant that does not bind tightly to plasma proteins (e.g., serum albumin) may be more effective in diffusing through cartilage, getting to chondrocytes of bone growth plates, and binding to and activating NPR-B for cGMP signaling. In one embodiment, CNP variants designed for reduced binding to plasma proteins (e.g., serum albumin) are chimeras comprising CNP22 or a variant thereof and a peptide fragment from IgG. In another embodiment, CNP variants designed for reduced binding to plasma proteins are chimeras comprising CNP22 or CNP22(K4R) and a fragment from a polypeptide (e.g., IgG, HSA, fibronectin, fibrinogen, a zinc finger-containing polypeptide, etc.). In yet another embodiment, CNP variants designed for reduced binding to plasma proteins comprise CNP22 or a variant thereof conjugated to a hydrophilic or water-soluble polymer. In one embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO). In another embodiment, the hydrophilic or water-soluble polymer (e.g., PEG) is functionalized with one or more functional groups that impart a negative charge to the polymer under physiological conditions, such as, e.g, carboxyl, sulfate or phosphate groups, or a combination thereof.

In a further embodiment, CNP variants of the disclosure include truncated CNP peptides ranging from human CNP-17 (hCNP-17) to human CNP-53 (hCNP-53), and having wild-type amino acid sequences derived from hCNP-53. Such truncated CNP peptides include:

(SEQ ID NO: 4)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-53);

(SEQ ID NO: 146)
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52);

(SEQ ID NO: 147)
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51);

(SEQ ID NO: 148)
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50);

(SEQ ID NO: 149)
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49);

(SEQ ID NO: 150)
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48);

(SEQ ID NO: 151)
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47);

(SEQ ID NO: 152)
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46);

(SEQ ID NO: 153)
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45);

(SEQ ID NO: 154)
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44);

(SEQ ID NO: 155)
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43);

(SEQ ID NO: 156)
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42);

(SEQ ID NO: 157)
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41);

(SEQ ID NO: 158)
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40);

(SEQ ID NO: 159)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39);

(SEQ ID NO: 160)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37);

(SEQ ID NO: 161)
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36);

-continued

(SEQ ID NO: 162)
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35);

(SEQ ID NO: 163)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

(SEQ ID NO: 164)
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33);

(SEQ ID NO: 165)
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32);

(SEQ ID NO: 166)
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31);

(SEQ ID NO: 167)
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30);

(SEQ ID NO: 168)
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29);

(SEQ ID NO: 169)
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28);

(SEQ ID NO: 170)
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27);

(SEQ ID NO: 171)
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26);

(SEQ ID NO: 172)
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25);

(SEQ ID NO: 173)
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24);

(SEQ ID NO: 174)
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23);

(SEQ ID NO: 1)
GLSKGCFGLKLDRIGSMSGLGC (CNP-22);

(SEQ ID NO: 175)
LSKGCFGLKLDRIGSMSGLGC (CNP-21);

(SEQ ID NO: 176)
SKGCFGLKLDRIGSMSGLGC (CNP-20);

(SEQ ID NO: 177)
KGCFGLKLDRIGSMSGLGC (CNP-19);

(SEQ ID NO: 178)
GCFGLKLDRIGSMSGLGC (CNP-18);
and

(SEQ ID NO: 2)
CFGLKLDRIGSMSGLGC (CNP-17).

In certain embodiments, CNP variants do not include CNP-17, CNP-22 or CNP-53.

In another embodiment, the truncated CNP peptides ranging from hCNP-17 to hCNP-53 can contain amino acid addition(s), deletion(s) and/or substitution(s) with natural or unnatural amino acid(s) or peptidomimetic(s) (e.g., peptide bond isostere(s)), as described herein, at any one or more of the amino acid positions of the particular truncated CNP peptides. In yet another embodiment, the truncated CNP peptides having wild-type sequences or amino acid addition(s), deletion(s) and/or substitution(s), can be conjugated at the N-terminus, C-terminus and/or internal site(s) to any of the moieties described herein, including but not limited to bone- or cartilage-targeting moieties (e.g., bisphosphonates, bone- or cartilage-targeting peptide sequences (e.g., polyAsp, polyGlu), peptide sequences derived from bone-targeting domains of bone proteins (e.g., osteopontin, osteocalcin, sialoprotein)), peptide sequences derived from the functional domains of bone morphogenetic proteins (e.g., BMP2, BMP3, BMP5, BMP7, BMP8a), peptide sequences derived from natriuretic polypeptides (e.g., NPPC, ANP, BNP), peptide sequences derived from polypeptides of non-natriuretic origin (e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, FGF-2, osteocrin), moieties that reduce renal clearance (e.g., negatively charged PEG moieties), hydrophilic polymers (e.g., PEG), carbohydrates (e.g., carbohydrates recognized by receptors on the surface of cells at bone growth plates), hydrophobic acids (e.g., C_5-C_{12} carboxylic acids, natural fatty acids), phospholipids, and combinations thereof. In an embodiment, the truncated CNP peptides having wild-type sequences or amino acid addition(s), deletion(s) and/or substitution(s), and optionally conjugated to one or more moieties at the N-terminus, C-terminus and/or internal site(s), have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa.

In a further embodiment, the CNP variants are derivatives of CNP37, which is QEHPNARKYKGANKK-CNP22 (SEQ ID NO: 60). The CNP37 variants can contain amino acid addition(s), deletion(s), and/or substitution(s) with natural or unnatural amino acid(s) or peptidomimetic(s) (e.g., peptide bond isostere(s)) at any one or more of the 37 positions of CNP37. Non-limiting examples of substitutions that can be made in CNP37, based on the numbering of CNP22, include K4R, G5S, G5R, G8S, K10R, G15S, S16Q, M17N, G19R, and combinations thereof. In an embodiment, the CNP37 derivatives contain a substitution of Met17 to a natural (e.g., asparagine) or unnatural amino acid or peptidomimetic, designed in part to avoid oxidation of the sulfur atom of methionine. In another embodiment, the CNP37 variants contain substitution(s) of Lys8, Lys10, Lys14 and/or Lys15 (based on numbering from the N-terminus of CNP37) to non-basic natural or unnatural amino acid(s) or peptiomimetic(s), designed in part to reduce albumin binding.

In addition or alternatively to amino acid addition(s), deletion(s) and/or substitution(s), the CNP37 derivatives can be conjugated at the N-terminus, C-terminus, and/or an internal site to any of the moieties described herein, including but not limited to bone- or cartilage-targeting moieties (e.g., bone-targeting peptide domains), moieties that reduce renal clearance (e.g., negatively charged PEG moieties), hydrophilic polymers (e.g., PEG), amino acid sequences comprising one or more amino acids (e.g., osteocrin "NPR-C inhibitor" fragment), carbohydrates (e.g., carbohydrates recognized by receptors on the surface of cells at bone growth plates), hydrophobic acids (e.g., C_5-C_{12} carboxylic acids and natural fatty acids), and combinations thereof.

In one embodiment, the CNP variants are modified CNP37 peptides having mutation(s)/substitution(s) at the furin cleavage site (underlined), designed to improve in vivo resistance to the furin protease, and/or containing glycine (underlined) at the N-terminus, designed to improve plasma stability and prevent pyroglutamine formation. Such CNP37 variants include but are not limited to:

(SEQ ID NO: 71)
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (An. CS);

(SEQ ID NO: 72)
GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (An. CT);

(SEQ ID NO: 73)
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (An. CU);

-continued

(SEQ ID NO: 74)
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (An. CW);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-CNP37, An. DB);
and

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-CNP37)

In a further embodiment, the CNP variants of the disclosure include CNP peptides and variants thereof that can be produced by the fusion protein process described herein. Non-limiting examples of CNP variants that can be produced by the fusion protein process described herein, using chemical or proteolytic cleavage or protein self-cleavage, include:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Gly-wtCNP53);

(SEQ ID NO: 75)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Gly-wtCNP37);

(SEQ ID NO: 60)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (wtCNP37);

(SEQ ID NO: 144)
GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC
(HSA fragment-wtCNP27);

(SEQ ID NO: 36)
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)];

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSG
LGC [CNP53(M48N)];

(SEQ ID NO: 181)
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[Gly-CNP37(M32N)];

(SEQ ID NO: 182)
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP37(M32N)];

(SEQ ID NO: 183)
GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSNSGLGC
[HSA-CNP27(M22N)];

(SEQ ID NO: 184)
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R,
M22N)];

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Pro-wtCNP53);

(SEQ ID NO: 145)
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-Gly-wtCNP37);

(SEQ ID NO: 186)
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-wtCNP37);

(SEQ ID NO: 187)
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (wtCNP34);

(SEQ ID NO: 188)
P-GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC
(Pro-HSA-wtCNP27);

(SEQ ID NO: 189)
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)];

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Met-wtCNP53);

(SEQ ID NO: 191)
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-Gly-wtCNP37);

(SEQ ID NO: 192)
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-wtCNP37);

(SEQ ID NO: 193)
M-GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-HSA-wtCNP27);

(SEQ ID NO: 194)
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)].

Other CNP variants, including truncated CNP peptides ranging from hCNP-17 to hCNP-53 and having wild-type sequences or amino acid addition(s), deletion(s) and/or substitution(s), can also be produced by the fusion protein process described herein, so long as the intended site of chemical or proteolytic cleavage of the fusion protein is not present within the amino acid sequence of the target CNP variant itself. As a non-limiting example, the fusion protein process described herein can be employed to produce truncated wtCNP34 using formic acid cleavage.

In additional embodiments, for any of the CNP peptides and CNP variants described herein that have asparagine (Asn/N) residue(s) and/or glutamine (Gln/Q) residue(s), whether they have a wild-type sequence or a non-natural amino acid sequence, any Asn residue(s) and/or any Gln residue(s) can independently be substituted with any other natural or unnatural amino acids, including conservative substitutions such as Asn to Gln. Such substitution(s) are designed in part to minimize or avoid any potential deamidation of asparagine and/or glutamine. Non-limiting examples of CNP peptides and variants in which any Asn residue(s) and/or any Gln residue(s) can independently be substituted with any other natural or unnatural amino acids, including conservative substitutions such as Asn to Gln, include wtCNP34, wtCNP37, Gly-wtCNP37, Pro-wtCNP37, Pro-Gly-wtCNP37, GHKSEVAHRFK-wtCNP27 (SEQ ID NO: 144), Pro-GHKSEVAHRFK-wtCNP27 (SEQ ID NO: 188), PEO12-GANRRCNP22(K4R) (SEQ ID NO: 36), and PEO24-GANRRCNP22(K4R) (SEQ ID NO: 36). In certain embodiments, an asparagine residue of the CNP peptides and CNP variants described herein is not substituted with glutamine, aspartic acid or glutamic acid. In certain embodiments, a glutamine residue of the CNP peptides and CNP variants described herein is not substituted with asparagine, aspartic acid or glutamic acid. As a non-limiting example, asparagine residues 7 and/or 15 of Pro-Gly-wtCNP37 (PGQEHP NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC) (SEQ ID NO: 145) can independently be substituted with any other natural or unnatural amino acids, including glutamine, to avoid any potential deamidation of the asparagine residue(s) to aspartic acid or isoaspartic acid. In certain embodiments, asparagine residues 7 and/or 15 of Pro-Gly-wtCNP37 are not substituted with glutamine, aspartic acid or glutamic acid.

It is understood, however, that the present disclosure encompasses CNP variants in which any one or more, up to all, residues susceptible to deamidation or a deamidation-like reaction (e.g., isomerization) may be converted to other residue(s) via deamidation or a deamidation-like reaction to any

extent, up to 100% conversion per converted residue. In certain embodiments, the disclosure encompasses CNP variants in which:

(1) any one or more, up to all, asparagine (Asn/N) residues may be converted to aspartic acid or aspartate, and/or to isoaspartic acid or isoaspartate, via deamidation up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(2) any one or more, up to all, glutamine (Gln/Q) residues may be converted to glutamic acid or glutamate, and/or to isoglutamic acid or isoglutamate, via deamidation up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(3) any one or more, up to all, aspartic acid or aspartate (Asp/D) residues may be converted to isoaspartic acid or isoaspartate via a deamidation-like reaction (also called isomerization) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(4) any one or more, up to all, glutamic acid or glutamate (Glu/E) residues may be converted to isoglutamic acid or isoglutamate via a deamidation-like reaction (also called isomerization) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(5) a combination of the above.

As a non-limiting example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of Pro-Gly-wtCNP37 [PGQEHPNARKYKGA NKKGLSKGCFGLKLDRIGSMSGLGC] (SEQ ID NO: 145) may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue, as described above.

As a further example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine and/or aspartic acid residues of Pro-Gly-wtCNP37 [PGQEHP NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC] (SEQ ID NO: 145) may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, and/or (2) isoaspartic acid/isoaspartate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As another example, the present disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of Gly-wtCNP37 [GQEHPNARKYKGA NKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 75)] may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As yet another example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of wtCNP37 [QEHPNARKYKGANKKGLSKGCFGLKL DRIGSMSGLGC (SEQ ID NO: 60)] may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As a further example, the present disclosure encompasses CNP variants in which any one or more, up to all, asparagine, aspartic acid, and/or glutamic acid residues of an HSA-wtCNP27 chimera, GHKSEVAHRFKGA NKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 144), may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) isoaspartic acid/isoaspartate, and/or (3) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As a still further example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, aspartic acid, and/or glutamic acid residues of a Pro-HSA-wtCNP27 chimera, PGHKSEVAHRFKGA NKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 188), may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) isoaspartic acid/isoaspartate, and/or (3) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

In addition, the present disclosure encompasses CNP variants in which any one or more, up to all, methionine (Met/M) residues may be oxidized to any chemically feasible oxidized form (e.g., sulfoxide and/or sulfone) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% transformation per oxidized residue.

In another embodiment, the CNP variants comprise CNP22 or variants thereof conjugated at the N-terminus and/or C-terminus to moiet(ies) that facilitate translocation of the variants across a cell membrane or cell barrier. In one embodiment, the CNP variants are conjugated at the N-terminus and/or C-terminus to peptide sequence(s) that facilitate transport of the variants across a cell membrane or cell barrier, including via active peptide transporters.

In a further embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof are conjugated to chemical moieties such as, e.g., natural and/or synthetic polymers, to increase the total mass of the modified CNP peptide to the ranges described generally herein, e.g., a range from about 2.6 or 2.8 kDa to about 6 or 7 kDa. In one embodiment, the chemical moieties are biocompatible hydrophilic or water-soluble natural (e.g., peptides, carbohydrates) or synthetic (e.g., PEG (or PEO)) polymers.

In a particular embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof are conjugated to PEG (or PEO) polymers to result in a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa. Pegylation of CNP22 or a variant thereof is designed, inter alia, to reduce immunogenicity and improve half-life by reducing renal clearance and increasing protease resistance. A PEG moiety can be attached to the N- and/or C-terminus of CNP22 or any variant described herein, including but not limited to CNP-17 (the Cys6-Cys22 cyclized portion of CNP22), CNP37, and variants of CNP17, CNP22 or CNP37 having N- and/or C-terminal amino acid extension(s), amino acid substitution(s) and/or amino acid deletion(s). In an embodiment, the Lys4 and/or Lys10 residues of CNP17, CNP22 or CNP37, or variants thereof, are substituted with a natural or unnatural amino acid (e.g., Arg,

Gly, Ser, Gln, Glu or Cit) or peptidomimetic that does not contain a reactive primary amine on a side chain, to preclude any potential PEGylation of these lysine residues. In one embodiment, the Lys4 and/or Lys10 residues of the CNP peptides are substituted with Arg. In another embodiment, the Lys10 residue is not substituted with Arg.

In a further embodiment, CNP variants (including CNP22 and variants thereof) having a PEG (or PEO) moiety and an amino acid extension at the N-terminus contain arginine at the position immediately preceding the position corresponding to Gly1 of CNP22. Such PEGylated CNP variants are designed for increased resistance to NEP degradation, reduced binding to serum albumin, and enhanced CNP functional activity (e.g., activation of cGMP signaling). Non-limiting examples of PEGylated CNP variants include PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO24-GANRR-CNP22(SEQ ID NO: 36), PEO12-GANRR-CNP22(SEQ ID NO: 36), PEO24-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO12-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO24-GANPR-CNP22(SEQ ID NO: 37), PEO12-GANPR-CNP22 (SEQ ID NO: 37), PEO24-GANQQ-CNP22(SEQ ID NO: 64), PEO12-GANQQ-CNP22(SEQ ID NO: 64), PEO24-ER-CNP22(K4R) (SEQ ID NO: 39), PEO12-ER-CNP22(K4R) (SEQ ID NO: 39), PEO24-ER-CNP22(SEQ ID NO: 39), PEO12-ER-CNP22(SEQ ID NO: 39), PEO24-R-CNP22 (K4R) (SEQ ID NO: 41), PEO12-R-CNP22(K4R) (SEQ ID NO: 41), PEO24-R-CNP22(SEQ ID NO: 41), and PEO12-R-CNP22(SEQ ID NO: 41), wherein PEO24 is a monodispersed 1.2 kDa PEG polymer and PEO12 is a monodispersed 0.6 kDa PEG polymer. In one embodiment, the PEG (or PEO) polymer is conjugated to the N-terminus of the CNP variants.

The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG) that can vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP peptide by means of N-hydroxy succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. Such CNP variants can be generated using, e.g., wtCNP22 (2.2 kDa), CNP17 retaining only the cyclized region (residues 6-22) of wtCNP22, CNP variants having an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, or variants having amino acid substitutions, additions and/or deletions such as, e.g., GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), R-CNP22(SEQ ID NO: 40), R-CNP22 (K4R) (SEQ ID NO: 41), ER-CNP22(SEQ ID NO: 38) and ER-CNP22(K4R) (SEQ ID NO: 39). In an embodiment, the PEG-CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, contain a monodispersed, linear PEG (or PEO) moiety conjugated at the N-terminus and/or C-terminus via NHS- or aldehyde-based chemistry, or a two-arm or three-arm branched PEG moiety conjugated at the N-terminus and/or C-terminus via NHS-based chemistry. The disclosure also encompasses negatively charged PEG-CNP variants designed for reduced renal clearance, including but not limited to carboxylated, sulfated and phosphorylated compounds (Caliceti, Adv. Drug Deliv. Rev., 55: 1261-77 (2003); Perlman, J. Clin. Endo. Metab., 88: 3227-35 (2003); Pitkin, Antimicrob. Ag. Chemo., 29: 440-444 (1986); Vehaskari, Kidney Int'l, 22: 127-135 (1982)). In one embodiment, the PEG (or PEO) moiety contains carboxyl group(s), sulfate group(s), and/or phosphate group(s).

In another embodiment, the PEG (or PEO) moieties conjugated to the N-terminus, C-terminus and/or internal site(s) of CNP variants described herein contain one or more functional groups that are positively charged under physiological conditions. Such PEG moieties are designed, inter alia, to improve distribution of such PEGylated CNP variants to cartilage tissues. In one embodiment, such PEG moieties contain one or more primary, secondary or tertiary amino groups, quaternary ammonium groups, and/or other amine-containing (e.g., urea) groups.

In an embodiment, the disclosure encompasses CNP22 or variants thereof conjugated via NHS- or aldehyde-based chemistry to PEG (or PEO) of the formula $(CH_2CH_2O)_n$, wherein n is an integer from about 6 to about 100, and the PEG polymer is from about 0.3 kDa to about 5 kDa. In another embodiment, n is an integer from about 12 to about 50, and the PEG polymer is from about 0.6 kDa to about 2.5 kDa. In yet another embodiment, n is from about 12 to about 24, and the PEG polymer is from about 0.6 kDa to about 1.2 kDa. In still another embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the end-capping group is an alkyl group, e.g., a lower alkyl group such as methyl.

In a further embodiment, the disclosure provides CNP variants having one or more peptide bonds or peptide bond isosteres that have reduced susceptibility to cleavage by peptidases including neutral endopeptidase (NEP). NEP is a membrane-bound zinc-dependent endopeptidase that cleaves a substrate peptide bond at the amino end of large hydrophobic residues. Thus, modification of a peptide bond at a cleavage site for NEP to an unnatural peptide or non-peptide bond may preclude or decrease the efficiency of NEP cleavage.

For ANP and CNP, NEP cleavage is reported to occur first at the Cys6-Phe7 bond within the cyclized region, then elsewhere throughout the remainder of the structures. For BNP, cleavage is reported to occur first at the peptide N-terminus, then within the cyclic structure. Although the primary NEP cleavage site on CNP is reported to be the Cys6-Phe7 bond, when wtCNP22 was exposed to NEP digestion for 2.5 minutes in vitro, all possible sites were unexpectedly hydrolyzed, with the Cys6-Phe7 and Gly8-Leu9 peptide bonds being slightly most labile, as described in Example 2.

Substrate specificity of NEP is primarily determined by two substrate-binding subsites, S1' and S2' (Oefner et al., J. Mol. Biol. 296:341-349 (2000)). The S1' site accepts a large hydrophobic P1' residue of which the N-terminal peptide bond is subjected to hydrolysis (e.g., Phe, Leu, Be and Met). The S2' site generally prefers a smaller residue, termed P2' (e.g., Gly or Ser). In the case of CNP, Phe7 is reported to be the preferred P1' residue for the NEP S1' site, while Gly8 is the preferred P2' residue for the S2' site. Because these two subsites can together accommodate only a certain total side chain size, any increase in the total size of the P1'-P2' residues of CNP can potentially disrupt NEP binding. For example, addition of a chloride atom at the 3-position of the P1' Phe7 aromatic ring (i.e., 3-Cl-Phe7) can potentially modify (e.g., destabilize) interactions between CNP and the NEP cleavage sites, for example at the S1' subsite. Addition of a tertiary butyl group to the smaller P2' residue Gly8 (i.e., tBu-Gly8) can potentially disrupt the interaction between CNP and the S2' subsite.

Accordingly, in one embodiment, CNP variants of the disclosure include CNP having an increase in the size of the

P1'-P2' residues, such as Phe7-Gly8, to interfere with substrate recognition at the active site, thereby reducing susceptibility to NEP cleavage. Natural amino acids, unnatural amino acids and/or peptidomimetic moieties are substituted for one or more large P1' hydrophobic residues, including but not limited to Phe7, Leu9, Leu11, Ile14, Met17 and Leu20, and/or for one or more smaller P2' residues, including but not limited to Cys6, Gly8, Gly15, Ser16 and Gly19.

The disclosure encompasses CNP variants comprising at least one modified amino acid and/or at least one modified peptide bond, at at least one residue involved in substrate recognition and/or cleavage by NEP, wherein the modified amino acids and modified peptide bonds can be natural amino acids, unnatural amino acids, peptidomimetics and/or peptide bond isosteres. In one embodiment, the NEP cleavage site on CNP between Cys6 and Phe7 is modified. In a related embodiment, the peptide bond (—C(=O)—NH—) between Cys6 and Phe7 is replaced with one of the following peptide bond isosteres:

—CH_2—NH—,

—C(=O)—N(R)—, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

—C(=O)—NH—CH_2—,

—CH_2—S—,

—CH_2—S(O)$_n$—, where n is 1 or 2,

—CH_2—CH_2—,

—CH=CH—,

—C(=O)—CH_2—,

—CH(CN)—NH—,

—CH(OH)—CH_2—,

—O—C(=O)—NH—, or

—NHC(=O)NH—.

In another embodiment, the CNP variants are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 90), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin, and sialoprotein (Wang et al., Adv. Drug Delivery Rev., 57: 1049-76 (2005)); polymeric and non-polymeric molecules that reduce renal clearance such as, e.g., negatively charged PEGs; and natural polymers (e.g., those containing amino acids, fatty acids and/or carbohydrates) and synthetic polymers (e.g., PEGs) that increase resistance of the CNP variant to NEP degradation by increasing the total mass of the CNP variant to the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa;

(b) and (c) may be the wild-type Cys6 and Phe7, another natural amino acid or an unnatural amino acid, or may contain a peptide bond isostere as described herein to increase resistance to NEP cleavage; and

(d) may be the wild-type Gly8, or may be a larger natural or unnatural (e.g., t-Bu-Gly) amino acid or peptidomimetic to reduce binding to NEP.

In one embodiment, such CNP variants contain at least one modified amino acid at (b), (c) and/or (d).

Other peptide bonds within CNP may be cleaved even if CNP22 or a variant thereof has an NEP-resistant peptide bond or peptide bond isostere at Cys6-Phe7, including the Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17, and Gly19-Leu20 bonds. Therefore, the disclosure encompasses CNP variants having peptide bond isostere(s) at one or more other NEP cleavages sites in addition to the Cys6-Phe7 bond, wherein the peptide bond isosteres include those described herein.

In another embodiment, the disclosure encompasses CNP variants having a cysteine analog at Cys6 and/or Cys22, including but not limited to homocysteine, penicillamine, 2-mercaptopropionic acid, and 3-mercaptopropionic acid. In an embodiment, such CNP variants have a cyclic domain formed by a disulfide bond between the wild-type Cys6 or analog and Cys22 or analog.

In yet another embodiment, one or more residues of CNP22 or a variant thereof, up to all the residues, are substituted with a D-amino acid. Substitution of an L-amino acid with a D-amino acid essentially moves the side chain about 120 degrees from its original position, thereby potentially disrupting the binding of the CNP peptide to NEP. In a specific embodiment, L-Phe at Phe7 is substituted with its D-enantiomer, D-Phe.

In still another embodiment, a beta amino acid such as, e.g., 3-amino-2-phenylpropionic acid (or 2-phenyl-beta-alanine), replaces the wild-type alpha-amino acid Phe7. Use of a beta-amino acid effectively increases the peptide backbone length by one methylene unit. Protease resistance can result from the change in substrate conformation or the increased distance between amino acid side chains.

Non-limiting examples of variants of CNP22 having an unnatural alpha-amino acid, a beta-amino acid or a peptide bond isostere include:

(SEQ ID NO: 56)
GLSKGC(CH_2NH)FGLKLDRIGSMSGLGC (Analog A),

(SEQ ID NO: 57)
GLSKGC-(N-Me-Phe)-GLKLDRIGSMSGLGC (Analog B),

(SEQ ID NO: 136)
GLSKGC-(D-Phe)-GLKLDRIGSMSGLGC (Analog E),

(SEQ ID NO: 58)
GLSKGCF-(tBu-Gly)-LKLDRIGSMSGLGC (Analog F),

(SEQ ID NO: 137)
GLSKGC-(3-Cl-Phe)-GLKLDRIGSMSGLGC (Analog G), and

(SEQ ID NO: 59)
GLSKGC-[NHCH_2CH(Ph)CO]-GLKLDRIGSMSGLGC (Analog H, formed using 3-amino-2-phenylpropionic acid).

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 46), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin, and sialoprotein; polymeric and non-polymeric moieties that reduce renal clearance such as, e.g., negatively charged PEGs; polymers containing, e.g., amino acids, hydrophobic acids, and/or carbohydrates; and synthetic hydrophilic polymers such as, e.g., PEGs;

(a) may be the wild-type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide-bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, heterocyclyl, C_{6-14} aryl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly, and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In another embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP cleavage, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-$(i)_{15}$-Ser_{16}-$(j)_{17}$-Ser_{18}-Gly_{19}-$(k)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:143), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins and derivatives thereof, such as, e.g., fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; moieties that reduce renal clearance, including but not limited to hydrophilic or water-soluble polymers such as, e.g., charged PEG molecules; and moieities comprising, e.g., PEGs, amino acids, carbohydrates, and/or hydrophobic acids;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2—NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para-positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres such as, e.g., N-Me-Leu;

(f) is selected from the group consisting of Lys, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln and Ser;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres such as, e.g., N-Me-Leu.

To improve the delivery of the CNP variants to the target sites of bone-related disorders (e.g., skeletal dysplasias), the CNP variants can be attached (e.g., at the N-terminus and/or C-terminus) to bone- or cartilage-targeting moieties. Non-limiting examples of bone- or cartilage-targeting moieties include bisphosphonates; hydroxyapatite; glucosamine; collagen (e.g., collagen type X); polyAsp; polyGlu; and amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteocrin, osteopontin, osteocalcin, and sialoprotein.

In addition to being less susceptible to NEP cleavage, the CNP variants potentially have reduced affinity to the NPR-C clearance receptor, while retaining CNP functionality. Besides NEP-mediated degradation, the half-life of CNP22 is influenced by the clearance receptor, NPR-C, which shares 58% sequence homology with the extracellular peptide-binding domain of NPR-B. CNP22 binds tightly to not only NPR-B (7-30 pM affinity), but also NPR-C (11-140 pM)

(Bennett, B. D. et al., J. Biol. Chem., 266: 23060-67 (1991); Koller K. J. & Goeddel, D. V., Circulation, 86: 1081-88 (1992); Suga, S. et al., Endocrinology, 130: 229-39 (1992)). Even though the NPR-B crystal structure has yet to be reported, sequence homology as well as similarities between the NPR-C and NPR-A crystal structures (He, X.-L. et al., Science, 293(5535): 1657-62 (2001); Ogawa, H. et al., J. Biol. Chem., 279(27): 28625-31 (2004); He, X.-L., J. Mol. Biol., 361(4): 698-714 (2006)) suggest that NPR-B likely assumes a similar overall structural fold.

Therefore, an NPR-B homology model was built based on structure-based sequence alignment and crystallographic structures of the following related systems: CNP bound to NPR-C, ANP bound to NPR-A, and ANP bound to NPR-C (He, X.-L. et al., Science, 293(5535): 1657-62 (2001); Ogawa, H. et al., J. Biol. Chem., 279(27): 28625-31 (2004); He, X.-L., J. Mol. Biol., 361(4): 698-714 (2006)). Based on observations that the receptor appears to determine the bound peptide conformation, and that NPR-B most closely resembles NPR-A with respect to primary structure and functional properties, the NPR-B/CNP homology model was built with the NPR-A/ANP crystal structure as a model. Published signaling data of CNP variants (U.S. Pat. No. 5,434,133 and US Patent Application Publication No. 2004/0138134 A1), and of functional ANP variants that no longer bind to NPR-C (Cunningham, EMBO 13(11) 2508-15, 1994) were used to refine and interpret the NPR-B/CNP model.

The present disclosure encompasses CNP variants designed for improved NPR-B selectivity based on a homology-based structural model of the NPR-B/CNP complex. Combining the experimental and computational structure data of natriuretic peptides bound to the various receptors with the published functional data, CNP variants were generated that continue to bind to NPR-B, but can potentially have reduced affinity to the NPR-C clearance receptor. For example, NPR-C has a unique insertion in a loop structure in the peptide-binding site, placing its loop residues closer to such peptide residues as CNP Gly8 (or ANP Gly9), compared to respective loop residues in NPR-A and NPR-B. Earlier studies indicated that the G9T mutation in ANP contributes to reduce affinity to NPR-C, thereby improving NPR-A selectivity (Cunningham, EMBO J., 13(11): 2508-15 (1994)). Accordingly, CNP variants were generated to replace the corresponding Gly8 residue with a larger residue (Ser, Val, Thr or Asn) to disrupt the CNP binding to NPR-C without affecting its binding to NPR-B. Further, one or more mutations were introduced at the C-terminal end of CNP, encompassing Gly15 to Gly21, which is predicted to interact with receptor-specific residues, based on the detailed structural analyses of the receptor/peptide complexes. For example, a G19R mutation in CNP22 does not result in a significant loss of NPR-B signaling activity. This mutation, however, cannot be modeled into the available crystal structure of NPR-C/CNP without altering the conformations of neighboring residues. These observations suggest that the G19R mutation may selectively disrupt the binding of CNP to a particular receptor, such as NPR-C.

In an embodiment, the CNP variants have substitution(s) at one or more Gly sites at positions 1, 5, 8, 15, 19 and 21, to reduce conformational flexibility and thereby increase receptor specificity. Comparative analyses of crystal structures of ANP bound to NPR-C and NPR-A (Ogawa, H. et al., J. Biol. Chem., 279: 28625-31 (2004); He, X.-L., J. Mol. Biol., 361: 698-714 (2006)) indicate that the conformational flexibility of ANP may play an important role in determining the receptor selectivity.

In one embodiment, functional CNP variants with potentially reduced affinity to NPR-C have one or more of the following amino acid substitutions: G1R, G1E, G5R, G5Q, G5S, F7Y, G8T, G8S, G8V, G8N, L9S, L9T, K10Cit, K10Q, K10S, 114N, G15R, G15S, G15N, G15Cit, S16Q, M17V, M17N, G19S, G19R, G19N, L20V, L20R, L20T, L20S, G21S, G21T and G21R. In an embodiment, the CNP variants have multipoint substitutions at positions 1, 5, 7, 8, 9, 10, 14, 15, 16, 17, 19, 20 and/or 21, and may optionally have modifications at any of the other positions in the peptide sequence of the variant.

In a further embodiment, the CNP variants described herein may be conjugated to moieties, up to a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, at the N-terminus, the C-terminus and/or an internal site, to facilitate bone/cartilage targeting, reduce NPR-C and renal clearance, increase resistance to NEP degradation, and/or improve CNP functionality. In one embodiment, the CNP variants are not conjugated to a polymeric moiety at a site within the cyclic region (corresponding to Cys6 to Cys22 of CNP22). Non-limiting examples of polymeric or non-polymeric moieties that can be conjugated to the CNP variants include synthetic bone-targeting compounds such as, e.g., bisphosphonates; bone/cartilage targeting peptide sequences such as, e.g., polyAsp and polyGlu; peptide sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin and sialoprotein; peptide sequences derived from the functional domains of bone morphogenetic proteins such as, e.g., BMP2, BMP3, BMP5, BMP7 and BMP8a; peptide sequences derived from polypeptides of natriuretic origin such as, e.g., NPPC, ANP and BNP; other natural polymeric or non-polymeric moieties such as, e.g., carbohydrates, fatty acids and phospholipids; biocompatible synthetic hydrophilic polymers such as, e.g., PEG (or PEO); hydrophobic polymeric or non-polymeric moieties such as, e.g., heptanoic acid and pentanoic acid; and combinations thereof.

The CNP variants described herein can have substantially similar or better functional activity than CNP22, e.g., with respect to stimulation of cGMP production and signaling. In one embodiment, the CNP variants in vitro or in vivo stimulate the production of at least about 50% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM). In certain embodiments, the CNP variants retain at least about 50%, 60%, 70%, 80%, 90%, 95% or 100% of the cGMP-stimulation activity of wild-type CNP22 in vitro or in vivo. In another embodiment, the CNP variants have improved cGMP-stimulation activity compared to CNP22. In certain embodiments, the CNP variants in vitro or in vivo stimulate the production of at least about 110%, 120%, 130%, 140%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500% or more of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM).

Optionally excluded from the present disclosure are any of the natriuretic (e.g., CNP) peptides, fragments and variants specifically disclosed, and any of the natriuretic (e.g., CNP) peptides, fragments and variants actually produced, in any of the prior publications referenced herein, including but not limited to, U.S. Pat. Nos. 5,434,133, 6,034,231, 6,020,168, 6,743,425, 7,276,481, WO 94/20534, WO 02/047871, WO 2005/098490, WO 2004/047871, EP 0497368, EP 0466174, and Furuya et al., Biochem. Biophys. Res. Comm. 183: 964-969 (1992)). All such documents are incorporated by reference herein in their entirety.

In one embodiment, the present disclosure optionally excludes all known wild-type CNP-53, wild-type CNP-22, wild-type CNP-17, wild-type BNP, and wild-type ANP of

human origin and non-human origin. For example, in an embodiment the disclosure optionally excludes human CNP-17, human CNP-22, chicken CNP-22 (corresponding to hCNP-22(Leu9Val)), trout and eel CNP-22 (corresponding to hCNP-22(Leu2Trp, Ser3Asn, Lys4Arg)), frog CNP22-I (corresponding to hCNP-22(Leu2Tyr, Lys4Arg, Leu9Val, Ser16Ala, Met17Phe)), frog CNP22-II (corresponding to hCNP-22(Leu2Thr, Ser16Ala)), human CNP-53, and pig and rat CNP-53 (corresponding to hCNP-53(Gln17His, Ala28Gly)). In another embodiment, the disclosure optionally excludes fragments of NPPC, proCNP and CNP-53 which are produced by proteolytic cleavage in vivo in humans and non-human animals. In yet another embodiment, optionally excluded from the disclosure are the following truncated fragments of wild-type human CNP-53: CNP-50, CNP-46, CNP-44, CNP-39, CNP-30, CNP-29, CNP-28, CNP-27 and CNP-26.

In a further embodiment, the present disclosure optionally excludes CNP peptides and fragments thereof isolated or sought from the shark species *Triakis scyllia* and *Scyliorhinus canicula* (see, e.g., M. Takano et al., Zool. Sci., 11: 451-454 (1994)), including:

(SEQ ID NO: 204)
RLLKDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-41);

(SEQ ID NO: 205)
LKDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-39);

(SEQ ID NO: 206)
KDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-38);
and

(SEQ ID NO: 207)
GPSRGCFGVKLDRIGAMSGLGC (CNP-22).

In another embodiment, optionally excluded from the disclosure are CNP peptides and fragments thereof isolated or sought from the shark species *Lamna ditropis* (see, e.g., M. Takano et al., Zool. Sci., 11: 451-454 (1994)), including:

(SEQ ID NO: 208)
RLLKDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-41);

(SEQ ID NO: 209)
LKDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-39);

(SEQ ID NO: 210)
KDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-38);

(SEQ ID NO: 211)
FKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-29);
and

(SEQ ID NO: 212)
GPSRGCFGVKLDRIGAMSGLGC (CNP-22).

In still another embodiment, optionally excluded from the disclosure are CNP peptides and fragments thereof isolated from the shark species *Squalus acanthias* (see, e.g., M. Takano et al., Zool. Sci., 11: 451-454 (1994)), including:

(SEQ ID NO: 213)
RLLQDLSNNPLRFKGRSKKGPSRSCFGLKLDRIGAMSGLGC (CNP-41);
and

(SEQ ID NO: 214)
GPSRSCFGLKLDRIGAMSGLGC (CNP-22).

In a further embodiment, the present disclosure optionally excludes CNP peptides isolated from medaka and puffer fish, designated "CNP-1", "CNP-2", "CNP-3" and "CNP-4" in K. Inoue et al., Proc. Nat. Acad. Sci., 100(17): 10079-10084 (2003):

(SEQ ID NO: 215)
GWNRGCFGLKLDRIGSMSGLGC (medaka and puffer fish CNP-1);

(SEQ ID NO: 216)
PMVAGGGCFGMKMDRIGSISGLGC (medaka CNP-2);

(SEQ ID NO: 217)
GRSSMVGGRGCFGMKIDRIGSISGLGC (puffer fish CNP-2);

(SEQ ID NO: 218)
GGMRSCFGVRLERIGSFSGLGC (medaka CNP-3);

(SEQ ID NO: 219)
GGLRSCFGVRLARIGSFSGLGC (puffer fish CNP-3);

(SEQ ID NO: 220)
GGSTSRSGCFGHKMDRIGTISGMGC (medaka CNP-4);
and

(SEQ ID NO: 221)
GGSSRSGCFGHKMDRIGTISGMGC (puffer fish CNP-4).

In a still further embodiment, optionally excluded from the disclosure are CNP-39 isolated from platypus venom and the CNP-22 fragment thereof, designated "ovCNP-39" and "ovCNP-39(18-39)" in G. de Plater et al., Toxicon., 36(6): 847-857 (1998):

(SEQ ID NO: 222)
LLHDHPNPRKYKPANKKGLSKGCFGLKLDRIGSTSGLGC (ovCNP-39);
and

(SEQ ID NO: 223)
GLSKGCFGLKLDRIGSTSGLGC (ovCNP-39(18-39).

In another embodiment, the present disclosure optionally excludes the following peptides as specifically disclosed in US 2007/0197434:

(SEQ ID NO: 224)
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ala-Met-Ser-Gly-Leu-Gly-Cys;

(SEQ ID NO: 225)
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Gln-Ser-Gly-Leu-Gly-Cys;

(SEQ ID NO: 226)
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Ala-Ser-Gly-Leu-Gly-Cys;

(SEQ ID NO: 227)
Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys;

(SEQ ID NO: 228)
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr;
and

(SEQ ID NO: 229)
Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr.

In yet another embodiment, the disclosure optionally excludes peptides of SEQ ID NO:10, disclosed generically in US 2007/0197434, wherein such peptides are CNP-17 vari-

ants having certain natural amino acid substitution(s) at position(s) 4, 5, 6, 11, 12, 14 and/or 15. In still another embodiment, optionally excluded from the disclosure are peptides corresponding to hCNP-53(Ser47Ala), hCNP-53 (Met48Gln), hCNP-53(Met48Ala), and hCNP-53(C-term.)-Asn-Ser-Phe-Arg-Tyr.

In an embodiment, the present disclosure optionally excludes the peptides of SEQ ID NOs 1-4 and 6-71 as specifically disclosed in U.S. Pat. No. 7,276,481. In another embodiment, the disclosure optionally excludes peptides of SEQ ID NO 5, disclosed generically in U.S. Pat. No. 7,276,481, wherein such peptides are variants of CNP17 having at least one natural amino acid substitution at Leu9, Lys10, Leu11, Ser16, Met17, Gly19, and/or Leu20. In still another embodiment, optionally excluded are CNP17 variants in which CNP17 or variants thereof contain N-Me-Phe7, or N-Me-Phe7 and N-Me-Leu11. In a further embodiment, the disclosure optionally excludes CNP17 variants of SEQ ID NO 5, as disclosed in U.S. Pat. No. 7,276,481, which are fused or conjugated to growth hormone (GH), insulin-like growth factor 1 (IGF-1), or thyroid hormone (TH). In yet another embodiment, optionally excluded are CNP22 variants in which CNP22 is fused to GH, IGF-1 or TH, or attached to GH, IGF-1 or TH via a linker (e.g., a peptide linker). In still another embodiment, optionally excluded are CNP17 variants in which CNP17 or variants thereof are conjugated to biotin or fluorescein at the N-terminus or the C-terminus.

In a further embodiment, the present disclosure optionally excludes the peptides of Compound Nos. 1-27, and SEQ ID NOs 1-17, 22-24, 30, 31 and 40-42 as specifically disclosed in U.S. Pat. No. 5,434,133. In another embodiment, the disclosure optionally excludes peptides of SEQ ID NOs 18-21 and 25-29, disclosed generically in U.S. Pat. No. 5,434,133. In still another embodiment, the disclosure optionally excludes the peptides of SEQ ID NOs 1-4 and 9 as specifically disclosed in WO 94/20534.

In some embodiments, however, the disclosure still encompasses methods of use of the natriuretic (e.g., CNP) peptides, fragments and variants optionally excluded herein, as well as pharmaceutical compositions (including sterile pharmaceutical compositions) comprising such natriuretic (e.g., CNP) peptides, fragments and variants.

C. Synthesis and Purification of CNP Variants

In some embodiments, the CNP variants described herein are produced by recombinant expression, using certain techniques known in the art in certain embodiments. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y. (1989)); DNA Cloning: A Practical Approach, Volumes I and II, D. N. Glover, Ed. (1985); and Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

In certain embodiments, the CNP variants described herein are produced by a recombinant process that comprises culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant polypeptide linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant polypeptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker. In some embodiments, the host cell is transformed with an expression vector comprising the polynucleotide encoding the CNP variant polypeptide linked to the polynucleotide encoding the cleavable peptide or protein. In certain embodiments, the fusion polypeptide is expressed as a soluble protein or as an inclusion body. The expressed fusion polypeptide can be isolated from the host cell or culture medium, and the isolated fusion polypeptide can be contacted with a cleaving agent to release the CNP variant.

Host cells used to produce CNP variants can be bacterial, yeast, insect, non-mammalian vertebrate, or mammalian cells. Bacterial cells include without limitation *E. coli* cell lines and strains. Non-limiting examples of *E. coli* cell lines and strains include BL21, BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41 [also called C41(DE3)], C43 [also called C43(DE3)], Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3). In an embodiment, CNP variants and CNP fusion proteins are produced using BL21(DE3) cells. Mammalian cells include, but are not limited to, hamster, monkey, chimpanzee, dog, cat, bovine, porcine, mouse, rat, rabbit, sheep and human cells. The host cells can be immortalized cells (a cell line) or non-immortalized (primary or secondary) cells and can be any of a wide variety of cell types, such as, but not limited to, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), ovary cells (e.g., Chinese hamster ovary or CHO cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), chondrocytes and other bone-derived cells, and precursors of these somatic cell types. Host cells containing the CNP variant DNA or RNA are cultured under conditions appropriate for growth of the cells, expression of the DNA or RNA and identification/selection of cells expressing the CNP variant.

In some embodiments, the host cells are grown or cultured for a time period at a temperature from about 10° C. to about 40° C., or from about 20° C. to about 40° C., or from about 30° C. to about 40° C. In certain embodiments, the host cells are grown or cultured for a time period at about 20° C., 22° C., 25° C., 28° C., 30° C., 35° C. or 37° C. In certain embodiments, the host cells are grown or cultured for a time period at about 35° C. or 37° C.

Recombinant polynucleotides encoding CNP variant polypeptides (including CNP fusion proteins) are expressed in an expression vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in vitro expression system. Expression vectors include all those known in the art, including without limitation cosmids, plasmids (e.g., naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide. The expression vector is inserted into an appropriate host cell, via transformation or transfection, for expression of the polynucleotide encoding the polypeptide (see, e.g., Sambrook et al. (supra)).

Non-limiting examples of expression vectors contemplated for production of CNP variants, including cleavable CNP fusion proteins, include pJexpress, pJexpress401, pJexpress404, pET-15b, pET-21a, pET-22b, pET-31b, pET-32a, pET-41a, pMAL, pMAL-c2X, pQE-30, pET-SUMO, and pTYB11. Expression of particular constructs can generate soluble CNP variants (including CNP fusion proteins) or insoluble CNP variants (including CNP fusion proteins) in the form of inclusion bodies.

In some embodiments, expression of the polynucleotide(s) encoding a CNP variant or CNP fusion protein is enhanced using an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible vector. In some embodiments, the host cells are grown or cultured for a time period at a temperature from

about 10° C. to about 40° C., or from about 20° C. to about 40° C., or from about 30° C. to about 40° C., in the presence of IPTG. In certain embodiments, the host cells are grown or cultured for a time period at about 20° C., 22° C., 25° C., 28° C., 30° C., 35° C. or 37° C. in the presence of IPTG. In certain embodiments, the host cells are grown or cultured for a time period at about 35° C. or 37° C. in the presence of 1 mM IPTG.

In further embodiments, the host cells are cultured with IPTG at a concentration from about 0.4 mM to about 2 mM, or from about 0.4 mM to about 1.5 mM, or from about 0.4 mM to about 1 mM. In certain embodiments, the IPTG is at a concentration of about 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9 or 2 mM. In an embodiment, the concentration of IPTG is about 1 mM.

In certain embodiments, the CNP variants described herein are recombinantly expressed as fusion proteins comprising a CNP variant polypeptide and a cleavable carrier protein or cleavable tag (e.g., peptide tag), wherein the fusion protein comprises the CNP variant polypeptide directly linked to the cleavable carrier protein or tag or indirectly linked thereto via a linker. Use of a carrier protein or tag facilitates, e.g., detection, isolation and/or purification of the fusion protein. Cleavable carrier proteins and tags include, but are not limited to, histidine (e.g., hexa-His) tags; human transcription factor TAF12 (TAF12), TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12(C/A & 4D/4E), TAF12(C/A & 6D/6E), TAF12(C/A & 10D/10E); ketosteroid isomerase (KSI); maltose-binding protein (MBP); β-galactosidase (β-Gal); glutathione-S-transferase (GST); thioredoxin (Trx); chitin binding domain (CBD); BMP-2, BMP-2 mutants, BMP-2(C/A); SUMO; and mutants and fragments thereof.

An expression construct can express a fusion protein comprising a CNP variant and a carrier protein or tag. The tag can be an amino acid sequence that confers a useful property to the fusion protein. In one embodiment, the tag is a ligand-binding domain that can be used to purify the fusion protein by applying the fusion protein to separation media containing the ligand. For example, a fusion protein comprising a glutathione-S-transferase (GST) domain can be applied to a chromatographic column containing glutathione-linked separation media. As another example, a fusion protein comprising maltose-binding protein (MBP) as a tag can be applied to separation media containing maltose. As a further example, a fusion protein comprising a polyhistidine tag may be applied to a nickel column, whereby chelation of the polyhistidine tag to the nickel column facilitates purification of the fusion protein. In another embodiment, the tag is a ligand. For example, a fusion protein can comprise glutathione as a tag and be applied to a chromatographic column containing glutathione-S-transferase-linked separation media. Non-limiting examples of carrier proteins and tags for use in fusion proteins include human transcription factor TAF12 (TAF12), ketosteroid isomerase (KSI), maltose-binding protein (MBP), β-galactosidase (β-Gal), glutathione-S-transferase (GST), thioredoxin (Trx), chitin-binding domain (CBD), BMP-2 mutation (BMPM), SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, poly(His), poly(Arg), poly(Asp), poly (Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and mutants and fragments thereof.

To generate the target CNP variant, the carrier protein or tag can be cleaved from the fusion protein by means of chemical cleavage, protease cleavage, or protein self-cleavage. Exemplary chemical and proteolytic cleavage agents (cleavage sites in parenthesis) include, but are not limited to, formic acid (Asp-Pro), cyanogen bromide (CNBr) (Met-X), hydroxylamine (Asn-Gly), Factor Xa (IEGR-X) (SEQ ID NO: 230), Enterokinase (DDDDK-X) (SEQ ID NO: 231), ProTEV (EXXYXQ-G) (SEQ ID NO: 232), and SUMO protease. Due to the nature of the particular kinds of chemical cleavage, cleavage using formic acid may generate Pro-CNP, CNBr may generate CNP having Met-to-Asn substitution, and hydroxylamine may generate Gly-CNP. Alternatively, chemical or protease cleavage may be avoided by using particular constructs (e.g., pET-21a-CNP) that express CNP variants not as fusion proteins. Expression of pET-21a-CNP may produce Met-CNP. Or certain fusion proteins (e.g., those containing intein-CBD) can undergo self-cleavage to generate CNP.

In further embodiments, a fusion protein comprises a cleavable peptide linker between a CNP variant and a carrier protein or tag (e.g., peptide tag). In certain embodiments, the cleavable peptide linker is selected from the group consisting of Asp-Pro, Asn-Gly, Met-X, Val-Asp-Asp-Arg (SEQ ID NO: 233), Gly-Ser-Asp-Arg (SEQ ID NO: 234), Ile-Thr-Asp-Arg (SEQ ID NO: 235), Pro-Gly-Asp-Arg (SEQ ID NO: 236), Ile-Glu-Gly-Arg-X (SEQ ID NO: 230), Asp-Asp-Asp-Asp-Lys-X (SEQ ID NO: 231), Glu-X-X-Tyr-X-Gln-Gly (SEQ ID NO: 232), Ala-Phe-Leu-Gly-Pro-Gly-Asp-Arg (SEQ ID NO: 237), and MGSSHHHHHHSSGLVPRGSHTGDDDDKHMD (pET-15b linker) (SEQ ID NO: 95), where X denotes an amino acid. In some embodiments, the cleavable peptide linker is cleaved by a cleaving agent selected from the group consisting of palladium, cyanogen bromide (CNBr), formic acid, hydroxylamine, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase, enterokinase (enteropeptidase), Kex 2 protease, Omp T protease, Factor Xa protease, subtilisin, proTEV, SUMO protease, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K, pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

In certain embodiments, the cleavable carrier protein, tag (e.g., peptide tag) or peptide linker is cleaved using formic acid to release the CNP variant from the fusion protein. In some embodiments, the formic acid is at a concentration from about 1% to about 20%, or from about 1% to about 15%, or from about 2% to about 15%, or from about 1% to about 10%, or from about 2% to about 10%, or from about 1% to about 5%, or from about 2% to about 5%. In certain embodiments, the formic acid is at a concentration of about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14% or 15%. In certain embodiments, the formic acid is at a concentration of about 2%, 5% or 10%.

In further embodiments, cleavage of the CNP fusion protein in the presence of formic acid is conducted at a temperature from about 20° C. to about 80° C., or from about 30° C. to about 75° C., or from about 40° C. to about 75° C., or from about 50° C. to about 75° C., or from about 50° C. to about 70° C., or from about 55° C. to about 70° C., or from about 50° C.

to about 60° C. In some embodiments, cleavage in the presence of formic acid is conducted at about 20° C., 22° C., 25° C., 30° C., 35° C., 37° C., 40° C., 42° C., 45° C., 50° C., 55° C., 60° C., 65° C., 70° C., 75° C. or 80° C. In certain embodiments, cleavage in the presence of formic acid is conducted at about 50° C., 55° C., 60° C., 65° C. or 70° C. In certain embodiments, cleavage in the presence of formic acid is conducted at about 55° C. or 70° C.

In additional embodiments, cleavage of the CNP fusion protein in the presence of formic acid is performed for a time period from about 3 hr to about 48 hr, or from about 5 hr to about 48 hr, or from about 5 hr to about 36 hr, or from about 5 hr to about 24 hr, or from about 5 hr to about 18 hr, or from about 20 hr to about 24 hr, or for about 6 hr to about 10 hr. In certain embodiments, cleavage in the presence of formic acid is performed for about 5 hr, 6 hr, 12 hr, 15 hr, 18 hr, 20 hr or 24 hr.

In some embodiments, cleavage of the CNP fusion protein is conducted in the presence of about 2%, 5% or 10% formic acid at about 55° C. for about 20 hr to about 36 hr, or at about 60° C. for about 15 hr to about 24 hr, or at about 65° C. for about 10 hr to about 21 hr, or at about 70° C. for about 6 hr to about 18 hr. In certain embodiments, cleavage of the CNP fusion protein is conducted in the presence of about 2% formic acid at about 55° C. for about 20 hr to about 24 or 36 hr, or at about 60° C. for about 15 hr to about 24 hr, or at about 65° C. for about 10 hr to about 18 hr, or at about 70° C. for about 6 hr to about 10 hr.

The present disclosure provides mild conditions for cleavage of CNP fusion proteins using formic acid to afford high yields of CNP variants. The conditions described herein for fusion protein cleavage using formic acid are also suitable for cleavage of fusion proteins comprising polypeptides or proteins other than CNP, where the fusion proteins contain an Asp-Pro peptide bond.

In further embodiments, soluble CNP fusion proteins or CNP fusion protein inclusion bodies are treated with a buffer and/or a detergent prior to chemical cleavage (e.g., using formic acid) or proteolytic cleavage of the CNP fusion protein. Non-limiting examples of buffers include B-PER II; diluted B-PER II (e.g., 1/20 dilution); B-PER; B-PER phosphate buffer; buffers containing Tris (e.g., 25 mM Tris, pH 7.5); buffers containing Tris and NaCl (e.g., 25 mM Tris, 150 mM NaCl, pH 7.9); and PBS. In an embodiment, the buffer is B-PER II. Non-limiting examples of detergents include octyl-sucrose, Triton X-100, Tween-20, NP-40, and CA-630. The detergent can be in a buffer (e.g., 1% detergent in 25 mM Tris buffer, pH 7.5). In certain embodiments, the detergent is Triton X-100 or CA-630.

It is understood that any of the methods and conditions described above may be used in combination with any of the other methods and conditions described above to generate a CNP variant disclosed herein.

In other embodiments, the CNP variants described herein are synthesized using a peptide synthesizer and purified according to methods known in the art, e.g., according to the methods of Atherton and Sheppard, *Solid Phase Peptide Synthesis: a Practical Approach*, IRL Press (Oxford, England (1989)).

Peptides can be synthesized based on, e.g., the following peptide sequence of CNP: G^1LS(K or R)$GC^6F^7G^8L$(K or R or Nle or 6-OH-Nle)$LDRIGSMSGLGC^{22}$.

Exemplary CNP variants include but are not limited to:

Analog A (GLSKGC(CH2NH)FGLKLDRIGSMSGLGC) (SEQ ID NO: 56) was made by converting the backbone "—C═O" group of C^6 to a "—CH_2" group;

Analog B (GLSKGC(N—Me—Phe)GLKLDRIGSMSGLGC) (SEQ ID NO: 57) was made by converting the backbone "—NH" group of F^7 to an "—N—CH_3" group;

Analog E (GLSKGC(D-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO: 136) was made using D-Phe at F^7;

Analog F (GLSKGCF(tBu-Gly)LKLDRIGSMSGLGC) (SEQ ID NO: 58) was made using a tertiary-butyl-Gly at G^8;

Analog G (GLSKGC(3-Cl-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO: 137) was made by adding a chloride atom to a meta position of the phenyl ring of F^7 (similar variants can be generated by making ortho, meta and/or para substitutions of the phenyl ring of Phe7 with Cl, F, Br, OH and/or CH_3);
and

Analog H (GLSKGC[NHCH$_2$CH(Ph)CO]GLKLDRIGSMSGLGC) (SEQ ID NO: 59) was made using (±)-3-(amino)-2-phenylpropionic acid at F^7.

Examples of CNP variants having, e.g., amino acid extensions, substitutions with natural or unnatural amino acids or peptide bond isosteres, and/or conjugations to polymers or hydrophobic moieties, include without limitation:

Analog J (SEQ ID NO: 91)
C6—CH2—NH, N—Me-L9, N—Me-L20

Analog K (SEQ ID NO: 92)
N—Me-L9, N—Me-L20

Analog L (SEQ ID NO: 93)
N—Me-L9, N—Me-L11, N—Me-L20

Analog M (SEQ ID NO: 94)
N—Me-L9, N—Me-L11

Analog Z (SEQ ID NO: 95)
K4R, F7Y

Analog AA (SEQ ID NO: 96)
K4R, G8V

Analog AB (SEQ ID NO: 97)
K4R, G8S

Analog AC (SEQ ID NO: 98)
K4R, G8T

Analog AD (SEQ ID NO: 99)
K4R, L9T

Analog AE (SEQ ID NO: 100)
K4R, G15R

Analog AF (SEQ ID NO: 101)
K4R, G15Cit

-continued

Analog AG	(SEQ ID NO: 102)
K4R, M17V	
Analog AH	(SEQ ID NO: 35)
K4R	
Analog AJ	(SEQ ID NO: 103)
K4R, L20V	
Analog AK	(SEQ ID NO: 104)
K4R, L20t-Bu-Ala	
Analog AT	(SEQ ID NO: 105)
G1E, K4E	
Analog AV	(SEQ ID NO: 106)
G1E, K4E - pentanoic acid (attached at the N-terminus)	
Analog AW	(SEQ ID NO: 107)
G1E, K4E - heptanoic acid (attached at the N-terminus)	
Analog AX	(SEQ ID NO: 2)
CNP17 (delta N-term)	
Analog AY	(SEQ ID NO: 36)
GANRR-CNP22(K4R)	
Analog AZ	(SEQ ID NO: 41)
R-CNP22(K4R)	
Analog BB	(SEQ ID NO: 108)
G1E - heptanoic acid (attached at the N-terminus)	
Analog BC	(SEQ ID NO: 109)
G1E - pentanoic acid (attached at the N-terminus)	
Analog BF	(SEQ ID NO: 110)
K4R, K10Cit	
Analog BG	(SEQ ID NO: 111)
K4R, K10Q	
Analog BH	(SEQ ID NO: 112)
K4R, K10R	
Analog BJ	(SEQ ID NO: 113)
K4R, G15N	
Analog BK	(SEQ ID NO: 114)
K4R, G15S	
Analog BL	(SEQ ID NO: 60)
CNP-37	(SEQ ID NO: 4)
CNP-53	

-continued

Analog CA	(SEQ ID NO: 61)
AAWARLLQEHPNA-CNP22	
Analog CB	(SEQ ID NO: 62)
AAWARLLQEHPNAR-CNP22	
Analog CC	(SEQ ID NO: 63)
DLRVDTKSRAAWAR-CNP22	
Analog CD	(SEQ ID NO: 68)
SPKMVQGSG-CNP17-KVLRRH (N- and C-terminal BNP tails)	
Analog CE	(SEQ ID NO: 81)
GERAFKAWAVARLSQ-CNP22 (HSA-CNP22)	
Analog CF	(SEQ ID NO: 79)
GQPREPQVYTLPPS-CNP22	
PEG(24K)-CNP22	
PEG(20K)-CNP22	
PEG(5K)-CNP22	
PEG(2K)-CNP22	
PEG(2K)-CNP17	
	(SEQ ID NO: 36)
PEG(1K)-GANRR-CNP22(K4R)	
PEG(1K)-CNP22	
PEO4-(PEO12)3(branched)-CNP22	
PEO12-CNP22	
	(SEQ ID NO: 36)
PEO12-GANRR-CNP22(K4R)	
	(SEQ ID NO: 36)
PEO24-GANRR-CNP22(K4R); and	
SEQ ID NOs: 1 to 6 and 34 to 144, and variants thereof that comprise up to 1, 2, 3, 4, or 5 further modifications.	

In one embodiment, the CNP variants are cyclized via formation of a disulfide bond between Cys^6 and Cys^{22}. Cys^6 can be a cysteine analog such as, e.g., homocysteine or penicillamine. In a further embodiment, the CNP variants can be cyclized by a covalent bond formed head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail. In an embodiment, the covalent bond is formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus of the peptide (referred to as "terminal" amino acids in this context). In another embodiment, the covalent bond is formed between the side chains of the two terminal amino acids. In yet another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of the two terminal amino acids.

Head-to-tail cyclization of the terminal amine to the terminal carboxyl group can be carried out using a number of methods, e.g., using p-nitrophenyl ester, 2,4,5-trichlorophenyl ester, pentafluorophenyl ester, the azide method, the mixed anhydride method, HATU, a carbodimide (e.g., DIC, EDC or DCC) with a catalyst such as HOBt, HONSu or HOAt, or on-resin cyclization.

In addition, the cyclic structure can be formed via a bridging group involving the side chains of amino acid residues of the CNP variant and/or the terminal amino acid residues. A bridging group is a chemical moiety that allows cyclization of two portions of the peptide. Non-limiting examples of bridg-

ing groups include amides, thioethers, thioesters, disulfides, ureas, carbamates, sulfonamides, and the like. A variety of methods are known in the art for incorporation of units having such bridging groups. For example, a lactam bridge (i.e., a cyclic amide) can be formed between the N-terminal amino group or an amino group on a side chain and the C-terminal carboxylic acid or a carboxyl group on a side chain, e.g., the side chain of lysine or ornithine and the side chain of glutamic acid or aspartic acid. A thioester can be formed between the C-terminal carboxyl group or a carboxyl group on a side chain and the thiol group on the side chain of cysteine or a cysteine analog.

Alternatively, a cross link can be formed by incorporating a lanthionine (thio-dialanine) residue to link alanine residues that are covalently bonded together by a thioether bond. In another method, a cross-linking agent, such as a dicarboxylic acid (e.g., suberic acid (octanedioic acid)), can link the functional groups of amino acid side chains, such as free amino, hydroxyl, and thiol groups.

Enzyme-catalyzed cyclization can also be used. For example, it has been reported that the thioesterase domain of tyrocidine synthetase can be used to cyclize a thioester precursor, a subtilisin mutant can be utilized to cyclize peptide glycolate phenylalanylamide esters, and the antibody ligase 16G3 can be employed to cyclize a p-nitrophenylester. For a review of peptide cyclization, see Davies, J. Peptide Sci., 9: 471-501 (2003), incorporated herein by reference in its entirety.

In certain embodiments, the final cyclized product has a purity of at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least about 99%.

D. Chemically Modified CNP Variants

Chemical modification of CNP22 or variants thereof can potentially impart advantageous properties to the modified CNP peptides, such as increased stability and half-life and reduced immunogenicity (for a general discussion of chemical modification of therapeutic proteins, see Pharmazie, 57(1): 5-29 (2002)). For example, attaching natural or synthetic, polymeric or non-polymeric moieties (e.g., PEG) to CNP peptides, to increase the total mass of the CNP peptides to the ranges described generally herein, e.g., a range from about 2.6 or 2.8 kDa to about 6 or 7 kDa, can reduce the susceptibility of the modified peptides to in vivo cleavage by exopeptidases and/or endopeptidases (e.g., NEP). In addition to PEGylation, glycosylation and other chemical derivatization procedures, e.g., modification by phosphorylation, amidation, carboxylation, acetylation, methylation, and creation of acid-addition salts, amides, esters and N-acyl derivatives, may also mask potentially immunogenic regions and/or proteolytically sensitive regions (Science, 303: 480-482 (2004)).

Examples of chemical modifications include, without limitation, the polymer addition method of Bednarsaki and the cross-linking method of Altus Corporation for improving stability and protease resistance and reducing immunogenicity. Bednarsaki showed that polymer addition can improve protein temperature stability (J. Am. Chem. Soc., 114(1): 378-380 (1992)), and Altus Corporation found that glutaraldehyde cross-linking can improve enzyme stability.

Chemical modification of polypeptides can be performed in a non-specific fashion (leading to mixtures of derivatized species) or in a site-specific fashion (e.g., based on wild-type macromolecule reactivity-directed derivatization and/or site-selective modification using a combination of site-directed mutagenesis and chemical modification) or, alternatively, using expressed protein ligation methods (Curr. Opin. Biotechnol., 13(4): 297-303 (2002)).

Pegylated CNP Variants

In one embodiment, for increased stability (e.g., resistance to NEP degradation), CNP22 or variants thereof (including those having amino acid additions, substitutions and/or deletions) are conjugated to hydrophilic, natural or synthetic polymers, to increase the total mass of the modified CNP peptides to a range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6, 7 or higher kDa. In certain embodiments, the added hydrophilic polymers have a total mass of about 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8, or about 5 kDa.

In an embodiment, the hydrophilic polymers are water-soluble so that the CNP peptides conjugated thereto do not precipitate out in an aqueous (e.g., physiological) environment. Further, the hydrophilic polymers are biocompatible, i.e., do not cause injury, toxicity or an immunological reaction in vivo.

The hydrophilic polymers can be branched or unbranched. In one embodiment, the hydrophilic polymers are not branched.

Various sites of conjugation of CNP22 or variants thereof to a hydrophilic polymer are possible, including but not limited to: (1) only at the N-terminus; (2) only at the C-terminus; (3) only at an internal site (e.g., Lys4); (4) at both the N-terminus and the C-terminus; (5) at the N-terminus and an internal site; and (6) at the C-terminus and an internal site. In one embodiment, CNP22 or variants thereof are conjugated to a hydrophilic polymer only at the N-terminus. In another embodiment, conjugation is only at an internal site (e.g., Lys4). In yet another embodiment, conjugation is at the N-terminus and an internal site (e.g., Lys4). In still another embodiment, for better functionality the CNP peptides are not conjugated to a hydrophilic polymer at a site (e.g., Lys10) within the cyclic domain (corresponding to Cys6 to Cys22 of CNP22). If conjugation to a hydrophilic polymer is based on bond formation with a reactive primary amino group on the CNP peptide, conjugation at an internal site (e.g., Lys4 and/or Lys10) can be prevented by substitution of Lys4 and/or Lys10 with a natural or unnatural amino acid or peptidomimetic that does not contain a reactive primary amino group on a side chain, such as, e.g., Gly, Ser, Arg, Asn, Gln, Asp, Glu or citrulline (Cit). In a particular embodiment, Lys4 and/or Lys10 are replaced with Arg. In another embodiment, Lys10 is not replaced with Arg.

Non-limiting examples of hydrophilic polymers include polymers formed from carboxylic acid-bearing monomers (e.g., methacrylic acid (MA) and acrylic acid (AA)), polyvinyl alcohols, polymers formed from hydroxyl-bearing monomers (e.g., hydroxyethyl methacrylate (HEMA), hydroxypropyl methacrylate (HPMA), hydroxypropyl methacrylamide, and 3-trimethylsilylpropyl methacrylate (TMSPMA)), polyalkylene oxides, polyoxyethylated polyols (e.g., glycerol), poly(ethylene glycol) (PEG), poly(propylene glycol), mono-C_1-C_{10} alkoxy-PEGs (e.g., monomethoxy-PEG), tresyl monomethoxy-PEG, aryloxy-PEGs, PEG acrylate (PEGA), PEG methacrylate, PEG propionaldehyde, bis-succinimidyl carbonate PEG, copolymers of 2-methacryloyloxyethyl-phosphorylcholine (MPC) and N-vinyl pyrrolidone (VP), hydroxy functional poly(N-vinyl pyrrolidone) (PVP), SIS-PEG (SIS is polystyrene-polyisobutylene-polystyrene block copolymer), polystyrene-PEG, polyisobutylene-PEG, PCL-PEG (PCL is polycaprolactone), PLA-PEG (PLA is polylactic acid), PMMA-PEG (PMMA is poly(methyl methacrylate)), PDMS-PEG (PDMS is polydimethyloxanone), PVDF-PEG (PVDF is polyvinylidene

fluoride), PLURONIC™ surfactants (polypropylene oxide-co-polyethylene glycol), poly(tetramethylene glycol), poly (L-lysine-g-ethylene glycol) (PLL-g-PEG), poly(L-lysine-g-hyaluronic acid) (PLL-g-HA), poly(L-lysine-g-phosphoryl choline) (PLL-g-PC), poly(L-lysine-g-vinyl pyrrolidone) (PLL-g-PVP), poly(ethylimine-g-ethylene glycol) (PEI-g-PEG), poly(ethylimine-g-hyaluronic acid) (PEI-g-HA), poly (ethylimine-g-phosphoryl choline) (PEI-g-PC), poly(ethylimine-g-vinyl pyrrolidone) (PEI-g-PVP), PLL-co-HA, PLL-co-PC, PLL-co-PVP, PEI-co-PEG, PEI-co-HA, PEI-co-PC, PEI-co-PVP, cellulose and derivatives thereof (e.g., hydroxyethyl cellulose), dextran, dextrins, hyaluronic acid and derivatives thereof (e.g., sodium hyaluronate), elastin, chitosan, acrylic sulfate, acrylic sulfonate, acrylic sulfamate, methacrylic sulfate, methacrylic sulfonate, methacrylic sulfamate, polymers and copolymers thereof, and polymers and copolymers of combinations thereof.

In a particular embodiment, the hydrophilic polymer is poly(ethylene glycol) (PEG), also called poly(ethylene oxide) (PEO). As used herein, the term "PEG" or "PEO" encompasses all the forms of PEG, branched and unbranched, which can be used to derivatize polypeptides, including without limitation mono-(C_1-C_{10}) alkoxy-PEGs and aryloxy-PEGs.

In one embodiment, the PEG-CNP conjugates comprise a PEG (or PEO) polymer of the formula $(CH_2CH_2O)_n$, wherein n is an integer from about 6 to about 100, and the PEG polymer is from about 0.3 kDa to about 5 kDa. In another embodiment, n is an integer from about 12 to about 50, and the PEG polymer is from about 0.6 kDa to about 2.5 kDa. In yet another embodiment, n is from about 12 to about 24, and the PEG polymer is from about 0.6 kDa to about 1.2 kDa. In a further embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the end-capping group is an alkyl group, e.g., a lower alkyl group such as methyl, so that the PEG polymer terminates in an alkoxy group. In an embodiment, the PEG polymer is not branched. In another embodiment, CNP22 or variants thereof are conjugated to a PEG polymer only at the N-terminus.

PEGs and PEOs potentially include molecules with a distribution of molecular weights, i.e., they are potentially polydispersed, depending on the manner in which they are prepared. The size/mass distribution of a polymeric preparation can be characterized statistically by its weight average molecular weight (M_w) and its number average molecular weight (M_n), the ratio of which is called the polydispersity index (M_w/M_n). M_w and M_n can be measured by mass spectroscopy. PEG-CNP variants conjugated to a PEG moiety larger than 1.5 kDa may exhibit a range of molecular weights due to the polydispersed nature of the parent PEG molecule. For example, in the case of mPEG2K (Sunbright ME-020HS, NOF Co.), the molecular masses of the PEG molecules are distributed over a range from about 1.5 kDa to about 3 kDa, with a polydispersity index of 1.036. By contrast, the PEGs conjugated to CNP22 or variants thereof using MS $(PEG)_n$ reagents (n=4, 8, 12 or 24, denoted as, e.g., "PEO12" or "PEO24") from Pierce Biotechnology (Rockford, Ill.) are monodispersed, having discrete chain length and defined molecular weight.

Methods for generating polypeptides comprising a PEG moiety are known in the art (see, e.g., U.S. Pat. No. 5,824,784). Methods for preparing PEGylated CNP peptides generally comprise the steps of (a) reacting CNP22 or a variant thereof with a PEGylation reagent under conditions suitable for attaching PEG to the CNP peptide (e.g., at the N-terminus), and (b) obtaining the reaction product(s). Because PEGylating a CNP peptide might significantly alter its binding to NPR-B, depending on the size of the PEG moiety and the location of PEGylation, different kinds of PEG and PEGylation reaction conditions can be explored. The chemistry that can be used for PEGylation of a CNP peptide includes acylation of reactive primary amine(s) of the peptide using the NHS-ester of methoxy-PEG (O—[(N-Succinimidyloxycarbonyl)-methyl]-O'-methylpolyethylene glycol). Acylation with methoxy-PEG-NHS or methoxy-PEG-SPA results in an amide linkage that eliminates any charge of the original primary amine. PEG-CNP peptides designated with the symbol "PEO12" or "PEO24", as well as those designated with the symbol "PEG1K", "PEG2K", "PEG5K" or "PEG20K", are PEGylated via reaction of a primary amino group on the peptide with an NHS ester-activated, methoxy-end capped PEG reagent. PEG-CNP variants can also be prepared by other methods, e.g., via reductive amination involving a primary amino group on the peptide and a PEG aldehyde, such as, e.g., PEG-propionaldehyde, or mono-C_1-C_{10} alkoxy or aryloxy derivatives thereof (see U.S. Pat. No. 5,252,714).

Unlike ribosome protein synthesis, synthetic peptide synthesis proceeds from the C-terminus to the N-terminus. Accordingly, Boc-PEG (containing tert-butyloxycarbonyl (Boc)) is one method to attach PEG to the C-terminus of a peptide (R. B. Merrifield, J. Am. Chem. Soc., 85(14): 2149-2154 (1963)). Alternatively, Fmoc (fluorenylmethoxycarbonyl) chemistry can be employed (E. Atherton and R. C. Sheppard, *Solid Phase Peptide Synthesis: a Practical Approach*, IRL Press (Oxford, England (1989)).

The present methods for preparing PEG-CNP variants provide a substantially homogenous mixture of polymer-protein conjugates. After purification, discrete PEG-CNP preparations are sufficiently pure for in vitro and in vivo testing of biological properties. As demonstrated herein, certain PEG-CNP variants exhibit reduced susceptibility to NEP cleavage and substantially similar or better functionality (e.g., stimulation of cGMP production).

As described herein, PEGylation reactions of CNP22 or variants thereof, using appropriate PEGylation reagent/CNP peptide ratios and reaction conditions, provide PEG-CNP derivatives. The nature and extent of PEGylation can be determined using, e.g., PAGE and HPLC analysis. In certain embodiments, at least about 50%, 60%, 70%, 80%, 90%, 95% or 99% of CNP22 or variants thereof are mono-PEGylated at the N-terminus. To optimize the beneficial effects of PEGylation on the biological properties of a CNP peptide, the polymer length, conformation (e.g., branched or linear), and/or functionalization (e.g., adding a negatively charged group) of a PEG moiety can be varied. PEGylated CNP variants are tested for NEP resistance, pharmacokinetics and bioactivity (e.g., the ability to bind to NPR-B and stimulate the generation of cGMP). PEGylated CNP variants that show improved NEP resistance and at least about 50% of the cGMP-stimulating activity of CNP22 can be further tested, e.g., in vitro in a rat chondrosarcoma cell-based achondroplasia model and in vivo in a murine achondroplasia animal model.

E. Methods of Using CNP Variants, Pharmaceutical Compositions of CNP Variants, and Routes of Administration

Methods of Using CNP Variants

Bone-Related Disorders

Fibroblast growth factors (FGFs) play important roles in bone formation, and mutations in FGF receptor genes (FGFR 1, 2 and 3) give rise to a variety of inherited skeletal malfor-

mations (Curr. Biol., 5: 500-507 (1995)). In particular, activating mutations in FGFR-3 are responsible for disorders of the long bones, including achondroplasia, the most common form of human genetic dwarfism (Nature, 371: 252-254 (1994); Cell, 78: 335-342 (1994)), the milder disorder hypochondroplasia (Ann. N.Y. Acad. Sci., 785: 182-187 (1996)), and the more severe and neonatal lethal thanatophoric dysplasia (TD) types I and II (Hum. Mol. Genet., 5: 509-512 (1996); Nat. Genet., 9: 321-328 (1995)). Mouse models overexpressing FGF-2, and consequentially activating FGFR-3, show shortened long bones and macrocephaly (Mol. Biol. Cell, 6: 1861-73 (1995)). Consistent with this model, mice deficient in FGFR-3 show remarkable skeletal overgrowth with wider growth plates (Nature Genet., 12: 390-397 (1996)).

Complementary experiments with CNP, NPR-B and NPR-C suggest a link between the peptide ligand, the corresponding receptors, and bone growth. Activation of NPR-B by elevated plasma concentrations of CNP in transgenic mice causes skeletal overgrowth (Nat. Med., 10: 80-86 (2004)) histologically similar to that of the growth plate cartilage of FGFR-3 knockout mice (Nat. Genet., 4: 390-397 (1996)). In NPR-C knockout mice, NPR-C-mediated clearance of CNP should be eliminated; consistent with this prediction, the knockout animals show elongated long bones and elongated vertebrae with kyphosis (Proc. Natl. Acad. Sci. USA 96: 7403-08 (1999)). Conversely, CNP knockout mice are dwarfed with shorter long bones and vertebrae, a phenotype histologically similar to that of achondroplasia, and have increased mortality as a result of malocclusion and pulmonary restriction from the small rib cage (Proc. Natl. Acad. Sci. USA, 98: 4016-4021 (2001)). Consistent with the proposed role of CNP as an activator of NPR-B, the NPR-B knockout mouse has the same dwarfed skeletal phenotype and increased mortality as the CNP knockout mouse (Proc. Natl. Acad. Sci. USA, 101: 17300-05 (2004)). Furthermore, in a mouse model of achondroplasia with activated FGFR-3 in the cartilage, targeted overexpression of CNP in chondrocytes counteracts dwarfism (Yasoda et al., Nat. Med., 10: 80-86 (2004)). Additionally, CNP has been show to play a role in regulating endochondral bone growth and chondrocyte activity, including but not limited to chondrocyte proliferation and differentiation, inhibition of the mitogen activated protein (MAP) kinase/MEK (Raf-1) kinase signaling pathway, and promotion of endochondral ossification (Yasoda et al., Nat. Med., 10: 80-86 (2004)). These results suggest that activation of the CNP/NPR-B system is a potential therapeutic strategy for treatment of human achondroplasia.

By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variants of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders and skeletal dysplasias include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia. Further, the CNP variants are useful as an adjunct or alternative to growth hormone for treating idiopathic short stature and other skeletal dysplasias.

In addition, the CNP variants are useful for treating other bone-related conditions and disorders, such as rickets, hypophosphatemic rickets [including X-linked hypophosphatemic rickets (also called vitamin D-resistant rickets) and autosomal dominant hypophosphatemic rickets], and osteomalacia [including tumor-induced osteomalacia (also called oncogenic osteomalacia or oncogenic hypophosphatemic osteomalacia)].

The CNP variants of the disclosure can also be used to treat osteoarthritis. Osteoarthritis is a degenerative disease of the articular cartilage and occurs frequently in the elderly. Osteoarthritis involves destruction of the cartilage and proliferative change in the bone and cartilage resulting from degeneration of articular components, with the change resulting in a secondary arthritis (e.g., synovitis). The extracellular matrix proteins, which are the functional entity of the cartilage, are reduced, and the number of chondrocytes decreases in osteoarthritis (Arth. Rheum. 46(8): 1986-1996 (2002)). By promoting the matrix production, growth and differentiation of chondrocytes, the CNP variants are useful for countering the undesired effects of FGF-2 and increasing matrix synthesis in subjects suffering from arthritis, including osteoarthritis, thereby treating arthritis, including osteoarthritis.

Vascular Smooth Muscle Disorders

CNP and other vasoactive peptides (including ANP, BNP and urodilatin) have vasodilator and diuretic properties and play an important role in cardiovascular homeostasis (J. Cardiovasc. Pharmacol., 117: 1600-06 (1998); Kidney Int., 49: 1732-37 (1996); Am. J. Physiol., 275: H1826-1833 (1998)). CNP is widely distributed in the cardiovascular system, especially in high concentration in vascular endothelial cells (J. Cardiovasc. Pharmacol., 117: 1600-06 (1998)). CNP is a potent relaxant of vascular smooth muscle, particularly in the coronary circulation (Biochem. Biophys. Res. Commun., 205: 765-771 (1994)), and is an inhibitor of smooth muscle cell proliferation (Biochem. Biophys. Res. Commun., 177: 927-931 (1991)). Although the vasodilator effect of CNP is less potent than that of ANP (about 1:100) (Hypertens. Res., 21: 7-13 (1998); Am. J. Physiol., 275: L645-L652 (1998)), CNP mRNA is increased in response to shear stress (FEBS Lett., 373: 108-110 (1995)) and plasma levels of CNP are elevated in inflammatory cardiovascular pathologies (Biochem. Biophys. Res. Commun., 198: 1177-1182 (1994)). CNP has been shown to suppress inflammation through inhibition of macrophage infiltration in injured carotid arteries of rabbits (Circ. Res., 91: 1063-1069 (2002)) and to directly inhibit cardiac fibroblast proliferation through an NPR-B/cGMP-dependent pathway (Endocrinology, 144: 2279-2284 (2003)).

The cardiovascular actions of CNP are mediated via activation of the NPR subtypes, NPR-B and NPR-C (Endocrinology, 130: 229-239 (1992)), the latter accounting for 95% of NPRs expressed in vivo (Science, 293: 1657-1662 (2001)). The CNP/NPR-B pathway leads to elevation of cGMP, a well-established secondary messenger in the cardiovascular system. NPR-C's 37-amino acid portion from the C-terminus has a consensus sequence that interacts with the heterotrimeric G protein G_i (J. Biol. Chem., 274: 17587-17592 (1999)), which has been shown to regulate adenylate cyclase and phospholipase C activity (J. Biol. Chem., 276: 22064-70 (2001); Am. J. Physiol., 278: G974-980 (2000); J. Biol.

Chem., 271: 19324-19329 (1996)). CNP mediates smooth muscle hyperpolarization and relaxation via activation of NPR-C and the opening of a G protein-regulated, inwardly rectifying K^+ channels (Proc. Natl. Acad. Sci. USA, 100: 1426-1431 (2003)). Likewise, CNP has important anti-proliferative effects in cardiac fibroblasts and, through interaction with NPR-C, regulates local blood flow and systemic blood pressure by hyperpolarizing smooth muscle cells (R. Rose and W. Giles, J. Physiol. 586: 353-366 (2008)).

By binding to NPR-B on vascular smooth muscle cells, CNP22 stimulates the production of cGMP, which acts as an intracellular secondary messenger to cause ultimately the relaxation of blood vessels. Based on the hypotensive actions of CNP, the CNP variants of the disclosure are useful for treating hypertension, congestive heart failure, cardiac edema, nephredema, hepatic edema, acute and chronic renal insufficiency, and so on. In addition, activation of cGMP signaling suppresses the growth of vascular smooth muscle cells. Accordingly, the CNP variants of the disclosure can be used to treat conditions or diseases caused by the abnormal growth of vascular smooth muscle cells, including but not limited to restenosis and arteriosclerosis.

The studies described above suggest that CNP may be a potential therapeutic candidate for vascular smooth muscle relaxation and remodeling. Pharmacological effects of CNP concerning certain disorders have been attributed, in part, to vasoprotective effects rather than to vasodilator activity (Am. J. Respir. Crit. Care Med., 170: 1204-1211 (2004)). Therefore, the CNP variants of the present disclosure are useful for treating conditions, e.g., vascular smooth muscle disorders, in which CNP may have a vasoprotective effect, including without limitation inducing smooth muscle relaxation and inhibiting infiltration of macrophages into cardiac tissue. In one embodiment, the CNP variants are used to treat heart failure, including but not limited to acute decompensated heart failure and acute congestive heart failure. In another embodiment, the CNP variants are used to treat asthma, cardiomyopathy, and restenosis of coronary arteries (by increasing smooth muscle cell relaxation and decreasing proliferation of smooth muscle cells).

Pharmaceutical Compositions of CNP Variants

In additional embodiments, the disclosure provides pharmaceutical compositions comprising a CNP variant, and one or more pharmaceutically acceptable excipients, carriers and/or diluents. In certain embodiments, the compositions further comprise one or more other biologically active agents (e.g., inhibitors of proteases, receptor tyrosine kinases, and/or the clearance receptor NPR-C).

In some embodiments, the compositions comprise the desired CNP variant in at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% purity. In certain embodiments, the compositions contain less than about 10%, 5%, 4%, 3%, 2%, 1% or 0.5% of macromolecular contaminants, such as other mammalian (e.g., human) proteins and other CNP variants.

Non-limiting examples of excipients, carriers and diluents include vehicles, liquids, buffers, isotonicity agents, additives, stabilizers, preservatives, solubilizers, surfactants, emulsifiers, wetting agents, adjuvants, and so on. The compositions can contain liquids (e.g., water, ethanol); diluents of various buffer content (e.g., Tris-HCl, phosphate, acetate buffers, citrate buffers), pH and ionic strength; detergents and solubilizing agents (e.g., Polysorbate 20, Polysorbate 80); anti-oxidants (e.g., methionine, ascorbic acid, sodium metabisulfite); preservatives (e.g., Thimerosol, benzyl alcohol, m-cresol); and bulking substances (e.g., lactose, mannitol, sucrose). The use of excipients, diluents and carriers in the formulation of pharmaceutical compositions is known in the art; see, e.g., Remington's Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pa. (1990)), which is incorporated herein by reference in its entirety.

For example, carriers include without limitation diluents, vehicles and adjuvants, as well as implant carriers, and inert, non-toxic solid or liquid fillers and encapsulating materials that do not react with the active ingredient(s). Non-limiting examples of carriers include phosphate buffered saline, physiological saline, water, and emulsions (e.g., oil/water emulsions). A carrier can be a solvent or dispersing medium containing, e.g., ethanol, a polyol (e.g., glycerol, propylene glycol, liquid polyethylene glycol, and the like), a vegetable oil, and mixtures thereof.

In some embodiments, the compositions are liquid formulations. In certain embodiments, the formulations comprise a CNP variant in a concentration range from about 0.1 mg/ml to about 20 mg/ml, or from about 0.5 mg/ml to about 20 mg/ml, or from about 1 mg/ml to about 20 mg/ml, or from about 0.1 mg/ml to about 10 mg/ml, or from about 0.5 mg/ml to about 10 mg/ml, or from about 1 mg/ml to about 10 mg/ml.

In further embodiments, the compositions comprise a buffer solution or buffering agent to maintain the pH of a CNP-containing solution or suspension within a desired range. Non-limiting examples of buffer solutions include phosphate buffered saline, Tris buffered saline, and Hank's buffered saline. Buffering agents include without limitation sodium acetate, sodium phosphate, and sodium citrate. Mixtures of buffering agents can also be used. In certain embodiments, the buffering agent is acetic acid/acetate or citric acid/citrate. The amount of buffering agent suitable in a composition depends in part on the particular buffer used and the desired pH of the solution or suspension. For example, acetate is a more efficient pH buffer at pH 5 than pH 6, so less acetate may be used in a solution at pH 5 than at pH 6. In some embodiments, the buffering agent has a concentration of about 10 mM±5 mM. In certain embodiments, the pH of a composition is from about pH 3 to about pH 7.5, or from about pH 3.5 to about pH 7, or from about pH 3.5 to about pH 6.5, or from about pH 4 to about pH 6, or from about pH 4 to about pH 5, or is at about pH 5.0±1.0.

In other embodiments, the compositions contain an isotonicity-adjusting agent to render the solution or suspension isotonic and more compatible for injection. Non-limiting examples of isotonicity agents include NaCl, dextrose, glucose, glycerin, sorbitol, xylitol, and ethanol. In certain embodiments, the isotonicity agent is NaCl. In certain embodiments, NaCl is in a concentration of about 160±20 mM, or about 140 mM±20 mM, or about 120±20 mM, or about 100 mM±20 mM, or about 80 mM±20 mM, or about 60 mM±20 mM.

In yet other embodiments, the compositions comprise a preservative. Preservatives include, but are not limited to, m-cresol and benzyl alcohol. In certain embodiments, the preservative is in a concentration of about 0.4%±0.2%, or about 1%±0.5%, or about 1.5%±0.5%, or about 2.0%±0.5%.

In still other embodiments, the compositions contain an anti-adsorbent (e.g., to mitigate adsorption of a CNP variant to glass or plastic). Anti-adsorbents include without limitation benzyl alcohol, Polysorbate 20, and Polysorbate 80. In certain embodiments, the anti-adsorbent is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%.

In additional embodiments, the compositions comprise a stabilizer. Non-limiting examples of stabilizers include glycerin, glycerol, thioglycerol, methionine, and ascorbic acid and salts thereof. In some embodiments, when the stabilizer is thioglycerol or ascorbic acid or a salt thereof, the stabilizer is in a concentration from about 0.1% to about 1%. In other embodiments, when the stabilizer is methionine, the stabilizer is in a concentration from about 0.01% to about 0.5%, or from about 0.01% to about 0.2%. In still other embodiments, when the stabilizer is glycerin, the stabilizer is in a concentration from about 5% to about 100% (neat).

In further embodiments, the compositions contain an anti-oxidant. Exemplary anti-oxidants include without limitation methionine and ascorbic acid. In certain embodiments, the molar ratio of antioxidant to CNP variant is from about 0.1:1 to about 15:1, or from about 1:1 to about 15:1, or from about 0.5:1 to about 10:1, or from about 1:1 to about 10:1 or from about 3:1 to about 10:1.

Pharmaceutically acceptable salts can be used in the compositions, including without limitation mineral acid salts (e.g., hydrochloride, hydrobromide, phosphate, sulfate), salts of organic acids (e.g., acetate, propionate, malonate, benzoate, mesylate, tosylate), and salts of amines (e.g., isopropylamine, trimethylamine, dicyclohexylamine, diethanolamine). A thorough discussion of pharmaceutically acceptable salts is found in *Remington's Pharmaceutical Sciences,* 18th Edition, Mack Publishing Company, (Easton, Pa. (1990)).

The pharmaceutical compositions can be administered in various forms, such as tablets, capsules, granules, powders, solutions, suspensions, emulsions, ointments, and transdermal patches. The dosage forms of the compositions can be tailored to the desired mode of administration of the compositions. For oral administration, the compositions can take the form of, e.g., a tablet or capsule (including softgel capsule), or can be, e.g., an aqueous or nonaqueous solution, suspension or syrup. Tablets and capsules for oral administration can include one or more commonly used excipieints, diluents and carriers, such as mannitol, lactose, glucose, sucrose, starch, corn starch, sodium saccharin, talc, cellulose, magnesium carbonate, and lubricating agents (e.g., magnesium stearate, sodium stearyl fumarate). If desired, flavoring, coloring and/or sweetening agents can be added to the solid and liquid formulations. Other optional ingredients for oral formulations include without limitation preservatives, suspending agents, and thickening agents. Oral formulations can also have an enteric coating to protect the CNP variant from the acidic environment of the stomach. Methods of preparing solid and liquid dosage forms are known, or will be apparent, to those skilled in this art (see, e.g., Remington's Pharmaceutical Sciences, referenced above).

Formulations for parenteral administration can be prepared, e.g., as liquid solutions or suspensions, as solid forms suitable for solubilization or suspension in a liquid medium prior to injection, or as emulsions. For example, sterile injectable solutions and suspensions can be formulated according to techniques known in the art using suitable diluents, carriers, solvents (e.g., buffered aqueous solution, Ringer's solution, isotonic sodium chloride solution), dispersing agents, wetting agents, emulsifying agents, suspending agents, and the like. In addition, sterile fixed oils, fatty esters, polyols and/or other inactive ingredients can be used. As further examples, formulations for parenteral administration include aqueous sterile injectable solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions, which can contain suspending agents and thickening agents.

Compositions comprising a CNP variant can also be lyophilized formulations. In certain embodiments, the lyophilized formulations comprise a buffer and bulking agent, and optionally an antioxidant. Exemplary buffers include without limitation acetate buffers and citrate buffers. Exemplary bulking agents include without limitation mannitol, sucrose, dexran, lactose, trehalose, and povidone (PVP K24). In certain embodiments, mannitol is in an amount from about 3% to about 10%, or from about 4% to about 8%, or from about 4% to about 6%. In certain embodiments, sucrose is in an amount from about 6% to about 20%, or from about 6% to about 15%, or from about 8% to about 12%. Exemplary anti-oxidants include, but are not limited to, methionine and ascorbic acid.

The disclosure also provides kits containing, e.g., bottles, vials, ampoules, tubes, cartridges and/or syringes that comprise a liquid (e.g., sterile injectable) formulation or a solid (e.g., lyophilized) formulation. The kits can also contain pharmaceutically acceptable vehicles or carriers (e.g., solvents, solutions and/or buffers) for reconstituting a solid (e.g., lyophilized) formulation into a solution or suspension for administration (e.g., by injection), including without limitation reconstituting a lyophilized formulation in a syringe for injection or for diluting concentrate to a lower concentration. Furthermore, extemporaneous injection solutions and suspensions can be prepared from, e.g., sterile powder, granules, or tablets comprising a CNP-containing composition. The kits can also include dispensing devices, such as aerosol or injection dispensing devices, pen injectors, autoinjectors, needleless injectors, syringes, and/or needles.

As a non-limiting example, a kit can include syringes having a single chamber or dual chambers. For single-chamber syringes, the single chamber can contain a liquid CNP formulation ready for injection, or a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) that can be reconstituted into a solution or suspension for injection. For dual-chamber syringes, one chamber can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection.

As a further example, a kit can include one or more pen injector or autoinjector devices, and dual-chamber cartridges. One chamber of a cartridge can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection. A cartridge can comprise an amount of the CNP variant that is sufficient for dosing over a desired time period (e.g., 1 day, 2 days, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, etc.). The pen injector or autoinjector can be adjusted to administer a desired amount of the CNP formulation from a cartridge.

In addition, pharmaceutical compositions comprising a CNP variant can be formulated as a slow release, controlled release or sustained release system for maintaining a relatively constant level of dosage over a desired time period, such as 1 week, 2 weeks, 3 weeks, 1 month, 2 months, or 3 months. Slow release, controlled release and sustained release formulations can be prepared using, e.g., biodegrad-

able polymeric systems {which can comprise, e.g., hydrophilic polymers [e.g., polylactide, polyglycolide, poly(lactide-glycolide)]}, and can take the form of, e.g., microparticles, microspheres or liposomes, as is known in the art.

Dosages and Frequency of Dosing

As used herein, the term "therapeutically effective amount" of an active agent (e.g., a CNP variant) refers to an amount that provides therapeutic benefit to a patient. The amount may vary from one individual to another and may depend upon a number of factors, including the overall physical condition of the patient. A therapeutically effective amount of a CNP variant can be readily ascertained by one skilled in the art, using publicly available materials and procedures. For example, the amount of a CNP variant used for therapy should give an acceptable rate of growth based on growth charts for children ages 0-17 years with achondroplasia (214 females and 189 males), which list height for age, head circumference, and segmental growth (Horton W A et al., Standard growth curves for achondroplasia, J. Pediatr., 93: 435-8 (1978)). CDC charts can be used to assess weight for age and weight for height or BMI for age. Secondary outcomes with courses that are more chronic in nature can also be measured.

Having a longer serum half-life than wild-type CNP22, the CNP variants can potentially be administered less frequently than CNP22. The dosing frequency for a particular individual may vary depending upon various factors, including the disorder being treated and the condition and response of the individual to the therapy. In certain embodiments, a pharmaceutical composition containing a CNP variant is administered to a subject about one time per day, one time per two days, one time per three days, or one time per week. In one embodiment, for treatment of bone-related disorders (e.g., skeletal dysplasias, including achondroplasia), a daily or weekly dose of a CNP variant is administered to patients until and/or through adulthood.

The CNP variants described herein can be administered to patients at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia) and conditions (e.g., vascular smooth muscle disorders) in which CNP can provide a vasoprotective effect. The safety and therapeutic efficacy of the CNP variants can be determined by standard pharmacological procedures in cell cultures or experimental animals, such as, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Active agents exhibiting a large therapeutic index are normally preferred.

Data obtained from cell culture assays and animal studies can be used to formulate a range of dosage for use in humans. The dosage normally lies within a range of circulating concentrations that include the ED_{50}, with little or minimal toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. The therapeutically effective dose can be determined from cell culture assays and animal studies.

In certain embodiments, the CNP variants described herein are administered at a dose in the range from about 5 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg. In some embodiments, the CNP variants are administered at a dose of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 125, 130, 140, 150, 160, 170, 175, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variants are administered at a dose of about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000 ug/kg, or about 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 mg/kg, or other dose deemed appropriate by the treating physician. The doses of CNP variants described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc.

The frequency of dosing/administration of a CNP variant for a particular subject may vary depending upon various factors, including the disorder being treated and the condition and response of the subject to the therapy. The CNP variant can be administered in a single dose or in multiple doses per dosing. In certain embodiments, the CNP variant is administered, in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week, 3 times per week, weekly, bi-weekly, every 3 weeks, monthly, every 6 weeks, every 2 months, every 3 months, or as deemed appropriate by the treating physician.

In some embodiments, a CNP variant is administered so as to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP variant may be administered intravenously, subcutaneously or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP variant daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

Modes of Administration

The CNP variants, or pharmaceutical compositions comprising them, can be administered to subjects in various ways such as, e.g., by injection subcutaneously, intravenously, intra-arterially, intraperitoneally, intramuscularly, intradermally, or intrathecally. In an embodiment, the CNP variants are administered by a single subcutaneous, intravenous, intra-arterial, intraperitoneal, intramuscular, intradermal or intrathecal injection once a day.

The CNP variants can also be administered by direct injection at or near the site of disease. Further, the CNP variants can be administered by implantation of a depot at the target site of action (e.g., an abnormal or dysplasic bone). Alternatively, the CNP variants can be administered sublingually under the tongue (e.g., sublingual tablet) or by inhalation into the lungs (e.g., inhaler or aerosol spray), by delivery into the nasal cavity (e.g., intranasal spray), by delivery into the eye (e.g., eye drop), or by transdermal delivery (e.g., by means of a patch on the skin). The CNP variants may also be administered orally in the form of microspheres, microcapsules, liposomes (uncharged or charged (e.g., cationic)), polymeric microparticles (e.g., polyamides, polylactide, polyglycolide, poly(lactide-glycolide)), microemulsions, and the like.

A further method of administration is by osmotic pump (e.g., an Alzet pump) or mini-pump (e.g., an Alzet mini-osmotic pump), which allows for controlled, continuous and/or slow-release delivery of the CNP variant or pharmaceutical composition over a pre-determined period. The osmotic pump or mini-pump can be implanted subcutaneously, or near the target site (e.g., the long bones of limbs, the epiphyses, etc.).

As explained above, the CNP variants can be used to treat conditions or diseases caused by the abnormal growth of vascular smooth muscle cells, including but not limited to restenosis and arteriosclerosis. For local delivery of a CNP variant to the diseased bodily vessel (e.g., blood vessel), the CNP variant can be delivered by means of a medical device (e.g., a stent) implanted at the diseased site. In one embodiment, the CNP variant is impregnated in a polymeric matrix or polymeric coating disposed over a stent. In another embodiment, the CNP variant is contained in reservoirs or channels formed in the body of a stent and covered by a porous polymeric membrane or layer through which the CNP variant can diffuse. The polymeric matrix, coating, membrane or layer can comprise at least one biodegradable (e.g., hydrophilic) polymer, as is known in the art. In a further embodiment, the CNP variant can be contained in micropores in the body of a stent. The CNP variant can be delivered from a stent by burst release, pulse release, controlled release or sustained release, or a combination thereof. For example, the stent can locally deliver the CNP variant to the diseased site in a burst release followed by a sustained release. Sustained release can be over a period up to about 2 weeks, 1 month, 2 months, 3 months, 6 months or 1 year.

It will be apparent to one skilled in the art that the CNP variants or compositions thereof can also be administered by other modes. Determination of the most effective mode of administration of the CNP variants or compositions thereof is within the skill of the skilled artisan.

The CNP variants can be administered as pharmaceutical formulations suitable for, e.g., oral (including buccal and sub-lingual), rectal, nasal, topical, pulmonary, vaginal or parenteral (including intramuscular, intraarterial, intrathecal, subcutaneous and intravenous) administration, or in a form suitable for administration by inhalation or insufflation. Depending on the intended mode of administration, the pharmaceutical formulations can be in the form of solid, semi-solid or liquid dosage forms, such as tablets, suppositories, pills, capsules, powders, liquids, suspensions, emulsions, creams, ointments, lotions, and the like. The formulations can be provided in unit dosage form suitable for single administration of a precise dosage. The formulations comprise an effective amount of a CNP variant, and one or more pharmaceutically acceptable excipients, carriers and/or diluents, and optionally one or more other biologically active agents.

Combination Therapy

In one embodiment, a CNP variant can be used in combination with one or more other active agents useful for treating, ameliorating or preventing CNP-responsive conditions or disorders such as, e.g., bone-related disorders (e.g., skeletal dysplasias) and vascular smooth muscle disorders. The other active agent(s) can enhance the effects of the CNP variant and/or exert other pharmacological effects in addition to those of the CNP variant. Non-limiting examples of active agents that can be used in combination with the CNP variants described herein are other natriuretic peptides (e.g., BNP) and inhibitors (e.g., antagonists) of peptidases and proteases (e.g., NEP and furin), NPR-C and tyrosine kinases (e.g., FGFR-3). By preventing NEP cleavage of the CNP variant, an NEP inhibitor can prolong the half-life of the variant. Examples of NEP inhibitors include, without limitation, thiorphan and candoxatril. Co-use of an NPR-C inhibitor can also prolong the half-life of the CNP variant via inhibition of the variant's clearance by NPR-C. A non-limiting example of an NPR-C inhibitor is the fragment FGIPMDRIGRNPR (SEQ ID NO: 82), which would be released at the target site (e.g., bone growth plate) upon proteolytic cleavage of the FGIPMDRIGRNPR-CNP22 chimera (Analog CZ) (SEQ ID NO: 82) or similar chimeras comprising variants of CNP22 (e.g., those containing amino acid substitution(s), addition(s), and/or deletion(s) relative to CNP22). Co-use of a tyrosine kinase inhibitor can accentuate the effects of a CNP therapy by inhibiting the tyrosine kinase receptor FGFR-3, a negative regulator of chondrocyte and bone growth. Non-limiting examples of tyrosine kinase inhibitors include those disclosed in U.S. Pat. Nos. 6,329,375 and 6,344,459.

To achieve the appropriate therapeutic outcome in the combination therapies, one would generally administer to the subject the CNP composition and other therapeutic(s) in a combined amount effective to produce the desired therapeutic outcome (e.g., restored bone growth). This process may involve administering the CNP composition and other therapeutic agent(s) at the same time. Simultaneous administration can be achieved by administering a single composition or pharmacological protein formulation that includes both the CNP variant and other therapeutic agent(s). Alternatively, the other therapeutic agent(s) can be taken separately at about the same time as a pharmacological formulation (e.g., tablet, injection or drink) of the CNP variant. The CNP variant can also be formulated into a foodstuff such as brownies, pancakes, or cake, suitable for ingestion.

In other alternatives, administration of the CNP variant can precede or follow administration of the other therapeutic agent(s) by intervals ranging from minutes to hours. In embodiments where the other therapeutic agent(s) and the CNP composition are administered separately, one would generally ensure that the CNP variant and the other therapeutic agent(s) are administered within an appropriate time of one another so that both the CNP variant and the other therapeutic agent(s) can exert, synergistically or additively, a beneficial effect on the patient. For example, one can administer the CNP composition within about 0.5-6 hours (before or after) of the other therapeutic agent(s). In one embodiment, the CNP composition is administered within about 1 hour (before or after) of the other therapeutic agent(s).

Identifying and Monitoring Patient Populations

Protocols can be established to identify subjects suitable for CNP therapy and to determine whether a given patient is responsive to CNP therapy. For example, for treatment of bone-related disorders, indicators of growth can be measured, such as long bone growth measurements in utero and neonatal and measurements of bone growth biomarkers such as CNP, cGMP, Collagen II, osteocalcin, and Proliferating Cell Nuclear Antigen (PCNA).

One CNP signaling marker is cGMP (guanosine 3',5' cyclic monophosphate). The level of this intracellular signaling molecule increases after CNP binds to and activates its cognate receptor NPR-B. Elevated levels of cGMP can be measured from cell culture extracts (in vitro) after CNP exposure, conditioned media from bone ex-plant studies (ex vivo) after CNP exposure, and in the plasma (in vivo) within minutes of CNP administration subcutaneously, intravenously, or via other routes of administration known in the art.

Cartilage and bone-specific analytes (or cartilage- and bone-associated markers) can also be measured to assess CNP efficacy. For example, fragments of cleaved collagen type II are a cartilage-specific marker for cartilage turnover.

Type II collagen is the major organic constituent of cartilage and fragments of type II collagen (cleaved collagen) are released into circulation, and subsequently secreted into the urine, following cartilage turnover. Cartilage turnover preceeds new bone formation.

A bone-specific biomarker for bone formation which can be measured is N-terminal propeptides of type I procollagen (PINP). The synthesis of type I collagen is an important step in bone formation, as type I collagen is the major organic component in bone matrix. During collagen synthesis, propeptides are released from the procollagen molecule and can be detected in serum. In addition, fragments of collagen type I can be measured as a marker for bone resorption.

Other potential biomarkers for cartilage and bone formation and growth include aggrecan chondroitin sulfate (cartilage-specific marker for cartilage turnover), propeptides of type TT collagen (cartilage-specific marker for cartilage formation), alkaline phosphatase (bone-specific) and osteocalcin (bone-specific marker for bone formation). Cartilage- and bone-associated biomarkers can be measured, e.g., in serum from efficacy/pharmacodynamic in vivo studies and from the conditioned media of ex vivo studies, using commercially available kits.

In one embodiment, the level of at least one bone- or cartilage-associated biomarker is assayed or measured in a subject that has been administered a CNP variant in order to monitor the effects of the CNP variant on bone and cartilage formation and growth in vivo. For example, an increase in the level of at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP variant has a positive effect on bone growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (e.g, endogenous levels of CNP), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In an embodiment, biomarkers are measured by obtaining a biological sample from a subject who will be administered, is being administered or has been administered a CNP variant. Biomarkers can be measured using techniques known in the art, including, but not limited to, Western Blot, enzyme linked immunosorbant assay (ELISA), and enzymatic activity assay. The biological sample can be blood, serum, urine, or other biological fluids.

Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

F. Examples

Example 1

Synthesis of CNP Variants

CNP variants were prepared using the methods described herein. Substitutions with natural or unnatural amino acids or peptidomimetics were made, as indicated in Tables 1-3 (shown in Example 3), at the respective amino acid residues in the wild-type sequence of CNP22. In certain variants, additional amino acids were added to the N-terminal and/or C-terminal ends of the whole or a portion of the wild-type CNP22 sequence (see Table 3).

Also prepared were CNP variants in which a PEG (or PEO) moiety was conjugated to the N-terminus of CNP22 or variants thereof (see Table 4, shown in Example 3). PEGylation reagents can be obtained from the commercial sources shown in Table 5.

TABLE 5

Vendor	Product Name	Name	MW (Da)	PEGylation Reagent
NOF	Sunbright ME-200CS	mPEG20K	20,000	CH3(CH2CH2O)450-(CH2)5COO—NHS
NOF	Sunbright ME-050CS	mPEG5K	5,000	CH3(CH2CH2O)110-(CH2)5COO—NHS
Pierce	(Methyl-PEG12)3-PEG4-NHS Ester	(mPEG12)3-PEG4	2,400	[CH3(CH2CH2O)12]3-(CH2CH2O)4-NHCO(CH2)3-COO—NHS
NOF	Sunbright ME-020HS	mPEG2K	2,000	CH3(CH2CH2O)45-(CH2)5COO—NHS
NOF	Sunbright ME-020CS	mPEG2K	2,000	CH3(CH2CH2O)45-(CH2)5COO—NHS
NOF	Sunbright ME-010HS	mPEG1K	1,000	CH3(CH2CH2O)23-CO(CH2)2COO—NHS
Pierce	Methyl PEG24-NHS Ester	MS(PEG)24	1,200	CH3(CH2CH2O)24-(CH2)2COO—NHS
Pierce	EZ-Link NHS-PEG12-Biotin	PEO12-Biotin	940	Biotin-(CH2CH2O)12-(CH2)2COO—NHS
Pierce	Methyl PEG12-NHS Ester	MS(PEG)12	690	CH3(CH2CH2O)12-(CH2)2COO—NHS
Pierce	EZ-Link NHS-PEG4-Biotin	PEO4-biotin	590	Biotin-(CH2CH2O)4-(CH2)2COO—NHS
Pierce	Mono(lactosylamido) mono(succinimidyl)suberate	LSS	590	
Pierce	EZ-link NHS-LC-LC-Biotin	LC-LC-Biotin	570	
Pierce	EZ-link NHS-LC-Biotin	LC-Biotin (LC = long chain)	450	
Pierce	EZ-link NHS-Biotin	Biotin	340	

The PEG (also called PEO) polymers purchased from Pierce Biotechnology (Rockford, Ill.) are monodispersed—i.e., they contain a single discrete polymer of a particular molecular weight. By contrast, the PEG polymers purchased from NOF (Nippon Oil and Fat) are polydispersed—i.e., they

contain a mixture of polymers having a distribution of molecular weights.

To PEGylate CNP22 or variants thereof, reaction and purification conditions are optimized for each PEG-CNP conjugate. According to a general PEGylation procedure, reaction mixtures contain about 1 mM CNP22 or a variant thereof, and about 1 to 5 mM NHS-activated PEG in potassium phosphate buffer, pH between about 5.0 and 6.5. To mono-PEGylate selectively at the peptide N-terminus and minimize PEGylation at an internal site (e.g., Lys4 of CNP22), the PEGylation reaction can be conducted under more acidic conditions (e.g., at a pH between about 5.5 and 6.5) to protonate selectively and hence deactivate the more basic primary amino group on the lysine side chain. After about 1 to 3 hours of incubation at room temperature, the PEGylation reaction is quenched by addition of aqueous glycine buffer. Reaction products are then separated by reverse-phase HPLC, optimized for each PEG-CNP conjugate. Fractionation samples are speedvacced to dryness, and reconstituted/formulated in 1 mM HCl. Identification and purity of each PEG-CNP product are determined by liquid chromatography-mass spectrometry (LC/MS).

Example 2A

Recombinant Production of CNP Variants

CNP variants can be produced using recombinant technology. In certain embodiments, the CNP variants are produced as fusion proteins comprising a cleavable peptide, carrier protein or tag. Exemplary methods for recombinantly producing CNP fusion proteins are disclosed below.

Materials and Methods

Cloning of CNP Fusion Proteins into Expression Vectors

CNP DNA fragments were amplified using polymerase chain reaction (PCR) and the amplified PCR fragments were digested with Nde I and BamHI and cloned into pET21a vector (Novagen, Gibbstown, N.J.). CNP fusion protein DNA was synthesized by DNA2.0 and cloned into different expression vectors (Table 6).

TABLE 6

Construct	Vector	Product	Chemical cleavage	Final product	Expression *E. coli* strain
pJexpress-TAF-CNP	pJexpress401	TAF-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP	BL21; BL21(DE3)
pJexpress-KSI-CNP(M/N)	pJexpress404	KSI-CNP(M/N) inclusion bodies	CNBr (Met-X)	CNP(M/N)	BL21
pET-31b-KSI-CNP	pET-31b	KSI-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)
pET-32a-Trx-CNP	pET-32a	Trx-CNP fusion protein (soluble)	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)
pMAL-CNP	pMAL-c2X	MBP-CNP fusion protein (soluble)	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)

CNP: GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Gly-CNP37 (SEQ ID NO: 75);
TAF: human transcription factor TAF12;
KSI: ketosteroid isomerase;
MBP: maltose-binding protein;
Trx: thioredoxin

Expression of CNP Fusion Proteins in *E. coli*

CNP fusion protein expression plasmids were transformed into *E. coli* BL21 or BL21(DE3). Transformed cells were plated on LB plates containing 100 ug/ml carbeniciline or 50 ug/ml kanamycin and incubated overnight at 37° C. One single colony was picked and cultured in 4 ml LB medium containing 100 ug/ml of carbeniciline or 50 ug/ml kanamycin at 37° C. with shaking. When an OD_{600} of bacterial culture reached 0.6, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cell media and the media was incubated at 37° C. for 3 hours with shaking. For cell harvest, bacterial cells were centrifuged at 4000 rpm for 10 minutes and the cell pellets were stored at −80° C. Cell pellets were lysed with B-PER II Bacterial Extraction Reagent (PIERCE, 0.4 ml per 4 ml of bacterial culture) and Benzonase Nuclease (Novagen, 0.025 U/ml) at room temperature for 10 minutes. Bacterial crude extract was reserved and centrifuged to obtain supernatant. Supernatant and crude extract were assayed for CNP fusion protein expression and solubility by SDS-PAGE and Western Blot.

Detection of CNP Fusion Protein Expression with SDS-PAGE and Western Blot

Ten uL of cell lysates or soluble supernatants was run on sodium dodecyl sulfate-polyacrylimide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, Calif., NuPAGE 4-12% Bis-tris Gel, MES SDS buffer). The gel was stained using 20 ml Imperial Protein Stain (Thermo Fisher, Rockford, Ill.) at room temperature for 1 hour and de-stained with water. For Western blot, the protein was transferred to membrane with Gel blot (Invitrogen). The membrane was blocked in TBS buffer with 5% milk at room temperature for 1 hour. Rabbit anti-CNP22 antibody (1:2500 dilution) (Bachem, Torrance, Calif.) was added to the membrane, which was then incubated at room temperature with shaking for 2 hours, and then the membrane was washed 3 times with TBS buffer.

Alkaline phosphate (AP) conjugated anti-rabbit IgG (1:5000 dilution) was added to the membrane, which was then incubated at room temperature with shaking for 1 hour, and then the membrane was washed 3 times with TBS buffer. Ten ml WESTERN BLUE®Stabilized Substrate (Promega, Madison, Wis.) was added to the membrane, which was then incubated at room temperature with shaking for 1 to 5 min, and then the membrane was washed with TBS buffer to remove excess stain.

Expression of TAF-CNP Fusion Protein in *E. coli* BL21

Cells (*E. coli* strain BL21) expressing TAF-CNP fusion protein were obtained from glycerol stock stored at −80° C. and were grown in 4 ml LB medium containing 50 ug/ml of kanamycin at 37° C. overnight with shaking (250 rpm). Four ml of overnight grown cell culture was transferred to 200 ml

LB medium containing 50 ug/ml of kanamycin and was grown at 37° C. with shaking (250 rpm). When the OD_{600} reached 0.6, IPTG was then added to a final concentration of 1 mM to induce protein expression at 37° C. with shaking (250 rpm) for 3 hours. Cells were then spun down at 3000 rpm for 10 minutes and the resulting cell pellet was frozen at −80° C.

Purification of TAF-CNP Inclusion Bodies and Formic Acid Cleavage

The cell pellet (from 200 ml culture) was resuspended in 25 ml of B-PER II buffer (PIERCE), the pellet was sonicated for 10 minutes (50%, 1 second, pause 2 seconds) on ice, centrifuged at 12000 rpm for 20 minutes at 4° C., and then the pellet was resuspended in 25 ml 20× diluted B-PER II buffer. This was repeated until the supernatant became clear (3-5 times). One mL of resuspended TAF-CNP inclusion bodies was transferred to a 1.5 ml tube and centrifuged at 14000 rpm for 15 min. The supernatant was discarded and the pellet was dissolved with 10 ul of 88% formic acid, and then 490 ul of Millipore filtered water was added immediately. The pellet was mixed well by vortex and incubated at 55° C. for 20 to 24 hours (70° C./6 hours are alternative conditions). The products of the formic acid cleavage were assayed by SDS-PAGE and LC/MS (C4RP).

LC/MS Sample Preparation

Inclusion bodies were isolated from about 8 mL of culture (about 1.5 OD) and the pellet was solubilized in 10 uL nt formate. Resolubilized pellet was immediately diluted to 2% or 10% final formate concentration (0.5 mL) and incubated at 55° C. for 21 hours (pH 2) (cloudiness was more evident in the 2% formate sample the next day). Both samples were centrifuged at 15000 rpm for 2 minutes. Twenty uL of supernatant was injected into an LC/MS (C4 RP) apparatus.

Results

CNP Fusion Proteins were Expressed in *E. coli*

All CNP fusion proteins were expressed in *E. coli* induced with 1 mM IPTG at 37° C. for 3 hours (FIG. **1**). The constructs pJexpress-TAF-CNP, pJexpress-KSI-CNP(M/N) and pET-31b-KSI-CNP were expressed as inclusion bodies, while the constructs pET-32a-Trx-CNP and pMAL-CNP were expressed as soluble fusion proteins. Western blot using anti-CNP22 antibody confirmed the expression of the CNP fusion proteins (FIG. **1**).

CNP was Produced from TAF-CNP Inclusion Bodies by Formic Acid Cleavage

TAF-CNP inclusion bodies were partially purified and treated with 2% formic acid at 55° C. for 20 to 24 hours (70° C./6 hours are alternative conditions). The majority of TAF-CNP was cleaved and one extra band having similar size as Gly-CNP37 peptide appeared on SDS-PAGE (FIG. **2**). The cleaved sample was further analyzed by LC/MS (C4 RP). The LC/MS results showed that CNP was released in soluble form from TAF-CNP inclusion bodies after formic acid cleavage. LC/MS analysis indicated that formic acid cleavage of the CNP fusion proteins resulted in formation of cyclized Pro-Gly-CNP37 (MW=4102). Calculation of protein amounts based on analysis suggested that about 60 ug of formic acid-generated CNP was produced from 8 mL of very low OD culture. From a small scale (e.g., about 8 mL) of low OD (e.g., 1.2 OD) cell culture, approximately 8 ug/ml CNP was produced, while fermentation of a larger scale (e.g., about 8 L) of higher OD (e.g., 380D) cell culture can produce approximately 1 mg/ml CNP.

Conclusion

Five expression constructs were generated to express CNP fusion proteins. Expression of all five constructs produced soluble (Trx and MBP) or insoluble (TAF and KSI) CNP fusion proteins. Approximately 1 mg/ml soluble CNP can be produced from TAF-CNP inclusion bodies by a simple formic acid cleavage procedure.

Example 2B

Production of Additional CNP Variants in *E. coli*

Recombinant production of CNP variants was carried out as described in Example 2A. In this example, additional CNP constructs were generated with a QuikChange II XL site-directed mutagenesis kit (Stratagene) or were synthesized by DNA2.0. Additional CNP constructs and expression vectors are listed in Table 7.

TABLE 7

Construct	Vector	Product	Chemical cleavage	Final product	Expression *E. coli* strain
pJexpress-TAF(C/A)-Pro-CNP38	pJexpress401	TAF(C/A)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF(4D/4E)-Pro-CNP38	pJexpress401	TAF(4D/4E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF(C/A&4D/4E)-Pro-CNP38	pJexpress401	TAF(C/A&4D/4E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF(C/A&10D/10E)-Pro-CNP38	pJexpress401	TAF(C/A&10D/10E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF-NL-(C/A & 6D/6E)-Pro-CNP38	pJexpress401	TAF-NL-(C/A & 6D/6E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-BMP-Pro-CNP38	pJexpress401	BMP-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21; BL21(DE3)

TABLE 7-continued

Construct	Vector	Product	Chemical cleavage	Final product	Expression *E. coli* strain
pJexpress-TAF-Pro-CNP37	pJexpress401	TAF-Pro-CNP37 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP37	BL21; BL21(DE3)
pJexpress-BMP-Pro-CNP37	pJexpress401	BMP-Pro-CNP37 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP37	BL21; BL21(DE3)
pJexpress-TAF Pro-HSA-CNP	pJexpress401	TAF-Pro-HSA-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-HSA-CNP	BL21
pJexpress-TAF	pJexpress401	TAF inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-BMP	pJexpress401	BMP inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF(C/A)	pJexpress401	TAF(C/A) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF(4D/4E)	pJexpress401	TAF(4D/4E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF (C/A&4D/4E)	pJexpress401	TAF(C/A&4D/4E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF (C/A&10D/10E)	pJexpress401	TAF(C/A&10D/10E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF-NL-(C/A & 6D/6E)	pJexpress401	TAF-NL-(C/A&6D/6E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF-Pro-CNP53	pJexpress401	TAF-Pro-CNP53 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP53	BL21(DE3)
pJexpress-TAF-CNP34	pJexpress401	TAF-CNP34 inclusion bodies	Formic acid (Asp-Pro)	CNP34	BL21(DE3)

CNP38: GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Gly-CNP37 (SEQ ID NO: 75)];

Pro-CNP38: PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Pro-Gly-CNP37] (SEQ ID NO: 145);

CNP37: QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 60);

HSA-CNP: GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC [HSA-CNP27 (SEQ ID NO: 144)];

Pro-CNP53: PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIG-SMSGLGC (SEQ ID NO: 185);

CNP34: PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 163);

TAF-Pro-CNP38: MVLTKKKLQDLVREVCPNEQLDEDVEEMLLQIADDFIESVVTAA-CQLARHRKSSTLEVKDVQLHLERQWNM-WIMGSSHHHHHHSSGLVPRGSHT-GDDDDKHMDPGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 196);
TAF: human transcription factor TAF12 histone fold domain (HFD) and linker from pET-15b vector;

TAF-NL: TAF12 HFD without a linker;

TAF12 HFD: VLTKKKLQDLVREVCPNEQLDEDVEEMLLQIADDFIESVVTAACQLA-RHRKSSTLEVKDVQLHLERQWNMWI (SEQ ID NO: 197);
pET-15b linker: MGSSHHHHHHSSGLVPRGSHTGDDDDKHMD (SEQ ID NO: 195);

TAF(C/A): cysteine in TAF is changed to alanine;

TAF(D/E): aspartic acid in TAF is changed to glutamic acid (number indicates which amino acid residue is changed);
TAF(C/A & D/E): cysteine and aspartic acid in TAF are changed to alanine and glutamic acid, respectively;
BMP: bone morphogenetic protein 2 with seven C/A (cysteine to alanine) mutations;

KSI: ketosteroid isomerase;

MBP: maltose-binding protein;

TRX: thioredoxin

Results

All CNP fusion proteins were expressed in *E. coli* induced with 1 mM IPTG at 37° C. for 3 hours. Constructs pJexpress-BMP-Pro-CNP38, pJexpress-TAF-Pro-CNP37, pJexpress-BMP-Pro-CNP37, pJexpress-Pro-HSA-CNP, pJexpress-TAF and pJexpress-BMP were expressed as inclusion bodies. A Western Blot with anti-CNP antibody was used to confirm the expression (FIG. **3**).

Pro-Gly-CNP37 ("Pro-CNP38") was Produced from TAF-Pro-CNP38 Inclusion Bodies by Formic Acid Cleavage

Formic acid is used as a denaturant for inclusion body proteins and can specifically cleave the peptide bond between Asp and Pro under optimized conditions. TAF-Pro-CNP inclusion bodies were partially purified as described above and treated with 50% formic acid at 25° C., 37° C., 42° C. and 55° C. for 24 hours. Most of the TAF-Pro-CNP38 was cleaved and one extra band with a similar size as the Gly-CNP37 ("CNP38") peptide showed on SDS-PAGE from the 37° C., 42° C. and 55° C. cleavages (FIG. **4**A). The cleavage reactions at 37° C. and 55° C. were neutralized with 10 M NaOH and centrifuged at 14,000 rpm for 15 minutes. The un-cleaved TAF-Pro-CNP38, TAF and other inclusion bodies precipitated in the pellets. The supernatants contained soluble Pro-CNP38 and were further analyzed by LC/MS. The LC/MS

result showed the supernatants contained a mixture of non-specific cleaved peptides generated by excess acidic hydrolysis.

When TAF-Pro-CNP inclusion bodies were treated with 2% and 10% formic acid at 55° C. for 20 hours, the majority of TAF-Pro-CNP38 was cleaved and one extra band having a similar size as Gly-CNP37 was observed on SDS-PAGE (FIG. **4**B). The cleaved sample was further analyzed by LC/MS. The LC/MS analysis showed that correct Pro-CNP38 was released in soluble form from TAF-Pro-CNP38 inclusion bodies after formic acid cleavage. The yields of Pro-CNP38 from 2% and 10% formic acid cleavages were similar (FIG. **4**C).

Formic Acid Cleavage and Neutralization for Pro-Gly-CNP37 ("Pro-CNP38") Production and Purification

Formic acid can dissolve and cleave TAF-Pro-CNP38, BMP-Pro-CNP38 and other inclusion bodies. The pH neutralization results in precipitation of insoluble contaminating proteins/peptides (un-cleaved TAF-Pro-CNP38 and BMP-Pro-CNP38, TAF, BMP and others). The soluble Pro-CNP38 stays in the supernatant after centrifugation. TAF-Pro-CNP38 and BMP-Pro-CNP38 were cleaved in 2% formic acid at 55° C. or 70° C. for 24 hours. The cleavage reactions were neutralized with 1:1 ratio of 0.5 M Tris buffer and centrifuged at 14,000 rpm for 15 minutes. Results showed that the supernatants contained almost pure peptides and there was no observed recovery loss of Pro-CNP38 upon neutralization. This is a simple and efficient step for Pro-CNP38 purification.

Analysis of Varying Conditions for Formic Acid Cleavage of TAF-Pro-CNP38 Inclusion Bodies for Pro-Gly-CNP37 ("Pro-CNP38") Production

Formic acid can specifically cleave the peptide bond between Asp and Pro under optimized conditions. Non-specific cleavage of peptide bonds between Asp and any other amino acids or even non-specific cleavage of any peptide bond may occur if the formic acid cleavage conditions are not optimized. TAF-Pro-CNP38 inclusion bodies were cleaved with 2% formic acid at 42° C., 55° C. or 70° C. for 6, 24 or 48 hours. FIG. **5**A shows that TAF-Pro-CNP38 was cleaved completely at 70° C. for 24 hours or at 55° C. for 48 hours. The 70° C. cleavage could be completed within 17 hours (FIG. **5**B). Although the 70° C./24 h cleavage gave the highest yield, the non-specific cleavage products (for example, the peptide with molecular weight of 3142 generated from Pro-CNP38 by cleaving between peptide bond Asp and Arg) increased dramatically (FIG. **5**C).

The yield and purity of Pro-CNP38 production were improved when TAF-Pro-CNP38 inclusion bodies were purified or treated with B-PER II buffer before formic acid cleavage. Because B-PER II buffer contains detergent octylthioglucoside and is relatively expensive, other commonly used detergents or buffers were tested for large-scale Pro-CNP38 production. TAF-Pro-CNP38 inclusion bodies were re-suspended in different detergents (Octylsucrose; Triton x-100; Tween-20; NP-40; CA-630) or buffers (B-PER II; B-PER II 1/20 dilution; B-PER; B-PER phosphate buffer; 25 mM tris, 150 mM NaCl, pH 7.9; 25 mM tris, pH 7.5; filtered water; PBS) and incubated at room temperature (RT) for 24 hours. All detergents were 1% in 25 mM tris buffer, pH 7.5. After incubation in detergent or buffer, TAF-Pro-CNP38 inclusion bodies were cleaved by 2% formic acid at 55° C. for 22 hours. Results showed that BPER II continued to exhibit good yield, and positive results were also obtained with CA630 and Triton X-100.

Pro-Gly-CNP37 ("Pro-CNP38") Proteolytic Cleavage Products

One unidentified protease, possibly a membrane associated protease, cleaved the Pro-CNP38 peptide (produced from BL21 strain, MW 4102) into two peptides during Pro-CNP38 purification, resulting in peptides PGQEHPNAR (MW 1004) (SEQ ID NO: 198) and KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (MW 3115) (SEQ ID NO: 199). Not to be bound by theory, one possible reason why detergents may improve the yield and purity of Pro-CNP38 is that detergents can remove most, but possibly not all, of the unidentified protease. High temperature, basic pH and EDTA were tested to identify if these agents could inhibit the protease cleavage. TAF-Pro-CNP38 inclusion bodies were incubated at RT or 120° C. for 2 hrs and centrifuged at 14000 rpm for 15 min. The pellets were re-suspended in 2% formic acid and incubated at 55° C. or 70° C. for 18 hours. A 1:1 ratio of 0.5 M Tris was added to neutralize the cleavage. The neutralized samples were centrifuged at 14000 rpm for 5 min and the supernatants were left at RT for 6 hrs or 22 hrs with or without 10 mM EDTA, pH 10. The proteolytic cleavage was assayed by LC/MS (Table 8).

TABLE 8

LC/MS result of Pro-CNP38 proteolytic cleavage

Sample		A210 Peak Area	Protein Conc. (mg/mL)		Percent MW4102	Percent MW3115
1	H 55 C.	942	0.04	*	91.8	8.2
2	H 70 0	2878	0.11	*	79.2	20.7
3	H 70 6H	2675	0.10	*	80.6	19.4
4	H 70 24	2741	0.11	*	79.2	20.7
5	H 70 EDTA	2385	0.09	*	80.8	19.2
6	H 70 pH 10	1917	0.07	*	81.2	18.8
7	55 C.	1291	0.05		61.1	38.9
8	70 C. 0	4533	0.18		96.8	3.2
9	70 C. 6H	4120	0.16		97.5	2.5
10	70 C. 24	4108	0.16		96.5	3.5
11	70 C. EDTA	4336	0.17		96.9	3.1
12	70 C. pH 10	3425	0.13		97.5	2.4

All samples (8-12) cleaved at 70° C. showed limited proteolytic cleavage (less than 4% cleavage of Pro-CNP38). Almost 40% of Pro-CNP38 was cleaved by protease when cleavage was carried out at 55° C. (sample 7). Basic pH and EDTA did not influence the non-specific proteolytic cleavage. High temperature (120° C. for 2 hrs) non-specifically cleaved Pro-CNP38.

It should be noted that the BL21(DE3) strain from Stratagene does not have the unidentified protease.

Production of Pro-Gly-CNP37 ("Pro-CNP38") and Other CNP Variants from Different Constructs:

Pro-CNP38 can be produced in large scale in *E. coli* by means of overexpression of a TAF-Pro-CNP38 fusion protein as inclusion bodies followed by formic acid cleavage of the fusion protein. Following the methods described herein, other TAF-CNP fusion proteins (TAF-CNP34 and TAF-Pro-CNP53) were expressed as inclusion bodies and then cleaved with formic acid to generate the CNP variants CNP34 and Pro-CNP53. FIG. **6** depicts the expression of TAF-CNP34, FIG. **7** the expression of TAF-Pro-CNP53, FIG. **8** the products of formic acid cleavage of TAF-CNP34 and TAF-Pro-CNP53, FIG. **9** the peak for CNP-34 in an LC/MS chromatogram, and FIG. **10** the peak for Pro-CNP53 in an LC/MS chromatogram.

Use of formic acid may result in unspecific cleavage(s) at peptide bond(s) other than the targeted Asp-Pro bond. To

improve the purity and overall titer of the desired formic acid cleavage product, different residues of aspartic acid in TAF12 or fragments thereof were changed to glutamic acid. Moreover, one or more cysteine residues in TAF12 or fragments thereof were changed to alanine to prevent unspecific disulfide bond formation. All TAF-Pro-CNP38 fusion proteins having such mutations in TAF12 were expressed as inclusion bodies and cleaved with formic acid to produce Pro-CNP38. FIG. **7** shows the expression of TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38, FIG. **11** the expression of TAF(C/A & 4D/4E)-Pro-CNP38 and TAF(4D/4E)-Pro-CNP38, FIG. **12** the products of formic acid cleavage of TAF(4D/4E)-Pro-CNP38 and TAF(C/A & 4D/4E)-Pro-CNP38, and FIG. **13** the products of formic acid cleavage of TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38. Table 9 summarizes the purity (prior to purification) and titer of Pro-CNP38 obtained from the various TAF-Pro-CNP38 constructs.

TABLE 9

Construct	Purity	Titer (ug/mL)
pJexpress-TAF-Pro-CNP38	32%	44
pJexpress-TAF(C/A)-Pro-CNP38	41%	50
pJexpress-TAF(4D/4E)-Pro-CNP38	36%	52
pJexpress-TAF(C/A & 4D/4E)-Pro-CNP38	42%	58
pJexpress-TAF(C/A & 10D/10E)-Pro-CNP38*	32%	26
pJexpress-TAF-NL-(C/A & 6D/6E)-Pro-CNP38	50%	55

*Cells with pJexpress-TAF(C/A&10D/10E)-Pro-CNP38 growed more slowly and the final cell density (OD_{600}) was lower compared to other TAF-Pro-CNP38 constructs.

Large-scale Production of Pro-Gly-CNP37 ("Pro-CNP38") by Fermentation and Formic Acid Cleavage

BL21(DE3) cells comprising the pJexpress-TAF-CNP construct were grown in a 10 liter fermenter at 37° C. for about 16-17 hours until OD_{600} reached 64. The cells were then grown/cultured in the presence of 1 mM IPTG at about 35-37° C., to induce expression of the TAF-Pro-CNP38 fusion protein, for about 7-8 hours until OD_{600} reached 160. The fermentation produced a titer of 9 g/L TAF-Pro-CNP38. FIG. **14** displays a Western blot of the TAF-Pro-CNP38 fusion protein produced in the fermentation.

A cell pellet recovered from 750 mL cell culture from the 10 L fermentation was resuspended in phosphate-buffered saline, pH 7.4 (PBS) and lysed by three passes through a pressure homogenizer (10,000 bar). The resulting lysate was centrifuged at 6,500 g for 10 minutes and the supernatant was discarded. The pellet fraction containing insoluble TAF-Pro-CNP38 fusion protein inclusion bodies was resuspended in 500 mL PBS with a rotostator. The suspension was centrifuged at 6,500 g for 10 minutes and the supernatant was discarded. The resulting inclusion body pellet was resuspended in 500 mL water with a rotostator and incubated at 55° C. for 30 minutes. 250 mL 6% formic acid was added to the warmed inclusion body suspension for a final concentration of 2% formic acid and incubated at 55° C. for 20-24 hours. 50 mL of 400 mM Na_2HPO_4 was added after 20-24 hours to begin the neutralization of the formic acid cleavage reaction, and the resulting mixture was titrated with 50% w/v NaOH to pH 6.9-7.4 and left at room temperature for 30 minutes. Upon neutralization, a heavy precipitate formed and was removed by centrifugation at 6,500 g for 10 minutes. The supernatant was retained and contained on average 1.3 g/L culture of 80% pure Pro-CNP38. The majority of the *E. coli* and TAF-related proteins and peptides remained in the pellet.

The soluble Pro-CNP38 resulting from the formic acid cleavage and neutralized supernatant was 80% pure and contained a mixture of linear and cyclized Pro-CNP38 peptides along with other product-related impurities. The pH neutral supernatant containing Pro-CNP38 was sterile-filtered. Further purification by anion-exchange chromatography using a Fractogel TMAE Hi-CAP column (EMD Biosciences) to remove DNA, endotoxins, and peptide contaminants, which should bind to the column, was performed at pH 7-7.4. The flow-through fraction contained partially cyclized Pro-CNP38. Cupric sulfate was added to the flow-through fraction to a final concentration of 10 uM and incubated at room temperature for 1 hour. The addition of Cu^{++} at neutral pH catalyzed the oxidation of free cysteine sulfhydryl groups on the peptide to form an intramolecular disulfide bond, resulting in 100% cyclized Pro-CNP38 and no detectable linear peptide. The conductivity of the solution was adjusted to <15 mS/cm by the addition of water. Cation-exchange chromatography using an SP-Sepharose column (GE Healthcare) and a sodium phosphate buffer (pH 7) was then performed to remove any remaining DNA and endotoxins in the flow-through and further purify Pro-CNP38 to about 95-96% purity with <0.5% non-product related impurities. FIG. **15** is an SDS-PAGE of eluate fractions from the SP-Sepharose column; the highest concentrations of Pro-CNP38 were found in fractions 22 to 30. Reverse-phase HPLC/MS analysis of non-pooled fraction 24 indicated the presence of 90% Pro-CNP38, 5% Pro-CNP38 with an oxidized methionine residue, 3% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17, and 1.6% Pro-CNP38 cleaved at the Asp-Arg bond in the cyclic domain. The yield of pure Pro-CNP38 peptide upon final purification was 0.9 g/L cell culture (36% total recovery).

Pro-CNP38 collected from five separate purifications was pooled for formulation. The pooled product contained 93.5% Pro-CNP38, 3.3% Pro-CNP38 with an oxidized methionine residue, 1.3% deamidated Pro-CNP38, and 1% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17. Samples were diluted with 50 mM sodium phosphate (pH 7) to a conductivity of 10 mS/cm and loaded onto a CM-Sepharose column (GE-Healthcare) for concentration and buffer exchange. The weak cation-exchange property of the CM-Sepharose resin allows peptides to dissociate from the column with weak acid solutions rather than typical salt gradients. Acid concentrations required for dissociation of Pro-CNP38 from the CM-Sepharose column depended upon column loading. At 50 mg Pro-CNP38 per mL of resin, 10 mM HCl was sufficient for Pro-CNP38 elution. At 9 mg Pro-CNP38 per mL of resin, 50 mM HCl was required for elution. When the loading was 9 mg Pro-CNP38 per mL of resin, Pro-CNP38 eluted in less than one column volume, which significantly concentrated the peptide. The eluate fraction contained 20.3 mg/mL of 95% pure Pro-CNP38, along with 3% Pro-CNP38 with an oxidized methionine residue, 1% deamidated Pro-CNP38, and <1% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17. The concentrated solution of Pro-CNP38 in weak acid is suitable for dilution into appropriate buffers for either liquid or lyophilized formulations.

Example 3

Cleavage of CNP Variants by Neutral Endopeptidase In Vitro

To determine the effects of amino acid substitutions, amino acid extensions, backbone modifications, side chain modifications and PEGylation on the susceptibility of CNP variants to neutral endopeptidase (NEP) cleavage, peptide cleavage assays were carried out using an in vitro assay that monitored disappearance of the non-cleaved CNP variant.

Recombinant human NEP (1 ug/mL final concentration) was added to 100 uM CNP variant diluted in 0.1 M Tris, pH 7. The reaction mixture was incubated at 37° C. for various periods of time, and the reaction was quenched with EDTA (10 mM final) followed by heat denaturation. The reaction mixture was reduced and then the reaction products were analyzed using HPLC and mass spectroscopy. The half-life of the CNP variant was calculated based on the disappearance of intact CNP variant over time. The results for digested CNP variants were compared to a parallel wtCNP22 digestion and normalized to the results for 100 uM CNP22 digested by 1 mg/mL NEP ($t_{1/2}$=80 min).

Table 1 lists the half-lives, based on the in vitro NEP cleavage assay, of various CNP variants having backbone or side chain modifications. Removal of three of the six NEP cleavage sites in Analog L nevertheless resulted in a substantially shorter half-life. Of tested CNP variants, the greatest resistance to NEP cleavage was exhibited by Analog N, which contains the D-enantiomer of all 22 amino acids of CNP22, and by Analog M, which has an N-methylated amide bond at both Leu9 and Leu11. However, both Analogs N and M failed to stimulate production of cGMP (see below).

Comparing the half-lives of Analogs A, B, E, F, G and H to one another, half-lives were determined to be about 1.5- to about 2.5-fold longer for Analogs E and G compared to those for Analogs A, B, F and H. All of these six analogs showed resistance or improved resistance to cleavage at the Cys6-Phe7 bond relative to wtCNP22 (data not shown). The rank order of analog resistance to NEP at 1 ug/ml, based on half-life, is Analog G (3-Cl-Phe)≥Analog E (D-Phe)>Analog H ("beta-2 Phe"), Analog B (N-Me-Phe), and Analog F (t-Bu-Gly)=wtCNP22>Analog A (Cys-CH_2—NH). Analogs E and G have about 1.5 times longer half-life in comparison to wtCNP22. Besides resistance to cleavage of the Cys6-Phe7 bond, Analogs B, E, F, G and H also exhibited resistance to cleavage of the Gly8-Leu9 bond in the presence of 1 ug/mL NEP (data not shown). These results indicate that CNP variants having backbone or side chain modifications between Cys6 and Gly8 can be resistant to NEP cleavage of the Cys6-Phe7 bond and/or Gly8-Leu9 bond, but do not necessarily have improved overall resistance to NEP or a longer half-life than CNP22. The results seem to be contrary to reports in the literature that NEP first cleaves at the Cys6-Phe7 bond of CNP22 and then elsewhere.

TABLE 1

Analog	Backbone and Side Chain Modifications Natriuretic Peptide	cGMP Response rel. to 1 uM CNP22[1] 10 nM	1 uM	NEP Cleavage ($t_{1/2}$, min)
	CNP22 (SEQ ID NO: 1)	46 ± 10	100 ± 13	80[2]
N	D-CNP22 (all D-amino acids) (SEQ ID NO: 115)	2	1	>>160
A	CNP22, C6—CH2—NH (reduced carbonyl) (SEQ ID NO: 56)	6	66	55
B	CNP22, N-methyl-F7 (methylated amide) (SEQ ID NO: 57)	2	38	80
BD	CNP22, N-methyl-L9 (SEQ ID NO: 116)	2	8	ND
BN	CNP22, N-methyl-L11 (SEQ ID NO: 117)	10	51	ND
BE	CNP22, N-methyl-L20 (SEQ ID NO: 118)	2	5	ND
M	CNP22, N-methyl-L9, N-methyl-L11 (SEQ ID NO: 94)	1	11	>>160
K	CNP22, N-methyl-L9, N-methyl-L20 (SEQ ID NO: 92)	1	1	80
L	CNP22, N-methyl-L9, N-methyl-L11, N-methyl-L20 (SEQ ID NO: 93)	18	10	30
J	CNP22, C6—CH2—NH, N-methyl-L9, N-methyl-L20 (SEQ ID NO: 91)	ND	ND	50
E	CNP22, D-F7 (D-Phe) (SEQ ID NO: 136)	2	6	130
H	CNP22, Beta-2-F7 (3-amino-2-phenylpropionyl) (SEQ ID NO: 57)	2	2	80
G	CNP22, 3-chloro-F7 (SEQ ID NO: 137)	17	93	135
F	CNP22, t-butyl-G8 (SEQ ID NO: 58)	2	18	80
V	CNP22, K4G, 3,4-dichloro-F7 (SEQ ID NO: 119)	ND	ND	68
X	CNP22, K4G, 3-methyl-F7 (SEQ ID NO: 120)	ND	ND	68
	ANP	10	23	ND

[1]Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2]CNP22 NEP resistance $t_{1/2}$ averaged 80 min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80 min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 2 lists the half-lives, based on the in vitro NEP cleavage assay, of various CNP variants having substitutions with natural and/or unnatural amino acids. Of tested variants, the greatest resistance to NEP cleavage was shown by Analog BK, which has K4R and G15S substitutions, and Analog BJ, which has K4R and G15N substitutions.

TABLE 2

Analog	Specificity Mutations Natriuretic Peptide	cGMP Response relative to 1 uM CNP22[1] 10 nM	1uM	NEP Cleavage ($t_{1/2}$, min)
	CNP22 (SEQ ID NO: 1)	46 ± 10	100 ± 13	80
AH	CNP22, K4R (SEQ ID NO: 35)	59	121	80
BP	CNP22, K4R, G5S (SEQ ID NO: 121)	45	ND	ND
BO	CNP22, K4R, G5R (SEQ ID NO: 122)	18	80	ND
P	CNP22, K4G (SEQ ID NO: 123)	ND	ND	68
Z	CNP22, K4R, F7Y (SEQ ID NO: 95)	2	18	ND
AB	CNP22, K4R, G8S (SEQ ID NO: 97)	26 ± 26	86 ± 17	ND
AA	CNP22, K4R, G8V (SEQ ID NO: 96)	3	25	ND
AC	CNP22, K4R, G8T (SEQ ID NO: 98)	11 ± 2	66 ± 16	80
AD	CNP22, K4R, L9T (SEQ ID NO: 99)	4	68	ND
BH	CNP22, K4R, K10R (SEQ ID NO: 112)	12	80	ND
BF	CNP22, K4R, K10Cit (SEQ ID NO: 110)	6	33	ND
BG	CNP22, K4R, K10Q (SEQ ID NO: 111)	9	45	ND
BY	CNP22, K4R, K10S (SEQ ID NO: 124)	16	53	ND
BK	CNP22, K4R, G15S (SEQ ID NO: 114)	13 ± 1	71 ± 11	≥160
BJ	CNP22, K4R, G15N (SEQ ID NO: 113)	4	41	150
AE	CNP22, K4R, G15R (SEQ ID NO: 100)	0.3	0.3	ND
AF	CNP22, K4R, G15Cit (SEQ ID NO: 101)	1.4	2	ND
BZ	CNP22, K4R, S16Q (SEQ ID NO: 125)	42	116	ND
BX	CNP22, K4R, M17N (SEQ ID NO: 126)	40 ± 2	103 ± 17	ND
AG	CNP22, K4R, M17V (SEQ ID NO: 102)	10	65	ND
BQ	CNP22, K4R, G19S (SEQ ID NO: 127)	21	63	ND
BR	CNP22, K4R, G19R (SEQ ID NO: 128)	22 ± 6	84 ± 10	ND
AJ	CNP22, K4R, L20V (SEQ ID NO: 103)	0.2	8	ND
AK	CNP22, K4R, L20t-butyl-Ala (SEQ ID NO: 104)	1	21	ND
AT	CNP22, G1E, K4E (SEQ ID NO: 105)	11	54	60
BS	CNP22, K4R, L20R (SEQ ID NO: 129)	11	8	ND
BT	CNP22, K4R, G21S (SEQ ID NO: 130)	7	39	ND
BU	CNP22, K4R, G21T (SEQ ID NO: 131)	6	21	ND
BW	CNP22, K4R, G21R (SEQ ID NO: 132)	20	21	ND
	ANP	10	23	ND

[1]Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22
[2]CNP22 NEP resistance $t_{1/2}$ averaged 80 min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80 min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.
ND = Not Determined

Table 3 lists the half-lives, based on the in vitro NEP cleavage assay, of CNP variants having N-terminal and/or C-terminal modifications, including amino acid extensions. Of the analogs tested, Analogs AZ, CC, CF, BL, CS, CK and CL, Pro-Gly-CNP37 and HSA-CNP27 were most resistant to NEP degradation.

TABLE 3

	N- and C-Terminal Modifications Natriuretic Peptide	cGMP Response rel. to 1 uM CNP22[1] 10 nM	1 uM	NEP Cleavage ($t_{1/2}$, min)
	CNP22	46 ± 10	100 ± 13	80
BC	Pentanoic acid (N-term.)-CNP22, G1E (SEQ ID NO: 109)	ND	ND	ND
BB	Heptanoic acid (N-term.)-CNP22, G1E (SEQ ID NO: 108)	32 ± 4	84 ± 19	45-65
AV	Pentanoic acid (N-term.)-CNP22, G1E, K4E (SEQ ID NO: 106)	ND	ND	120
AW	Heptanoic acid (N-term.)-CNP22, G1E, K4E (SEQ ID NO: 107)	ND	ND	<20
AX	CNP17 (delta N-term) (SEQ ID NO: 2)	18	69	ND
	R-CNP22 (SEQ ID NO: 40)	ND	ND	ND
AZ	R-CNP22, K4R (SEQ ID NO: 41)	54 ± 11	106 ± 15	≥160
	ER-CNP22 (SEQ ID NO: 38)	ND	ND	ND

TABLE 3-continued

	N- and C-Terminal Modifications Natriuretic Peptide	cGMP Response rel. to 1 uM CNP22[1] 10 nM	 1 uM	NEP Cleavage ($t_{1/2}$, min)
BA	ER-CNP22, K4R (SEQ ID NO: 39)	38 ± 10	113 ± 10	90
	GANRR-CNP22 (SEQ ID NO: 65)	ND	ND	ND
AY	GANRR-CNP22, K4R (SEQ ID NO: 36)	59 ± 9	105 ± 20	65
	GANQQ-CNP22 (SEQ ID NO: 64)	ND	ND	ND
CH	GANQQ-CNP22, K4R (SEQ ID NO: 69)	44 ± 8	95 ± 11	ND
	GANPR-CNP22 (SEQ ID NO: 66)	ND	ND	ND
CI	GANPR-CNP22, K4R (SEQ ID NO: 37)	50 ± 1	105 ± 12	ND
	GANSS-CNP22 (SEQ ID NO: 67)	ND	ND	ND
CG	GANSS-CNP22, K4R (SEQ ID NO: 70)	27 ± 1	88 ± 1	95
CA	AAWARLLQEHPNA-CNP22 (SEQ ID NO: 61)	24	76	ND
CB	AAWARLLQEHPNAR-CNP22 (SEQ ID NO: 62)	36	84	ND
CC	DLRVDTKSRAAWAR-CNP22 (SEQ ID NO: 63)	34	101	>160
CF	GQPREPQVYTLPPS-CNP22 (IgG1(Fc) fragment) (SEQ ID NO: 79)	23 ± 9	72 ± 19	>160
	PNARKYKGANKK-CNP22 (CNP34)	ND	ND	ND
BL	QEHPNARKYKGANKK-CNP22 (CNP37) (SEQ ID NO: 60)	43 ± 15	97 ± 27	>>160
	PQEHPNARKYKGANKK-CNP22 (Pro-CNP37)	ND	ND	ND
CE	GERAFKAWAVARLSQ-CNP22 (HSA fragment) (SEQ ID NO: 81)	15	87	ND
CY	GQHKDDNPNLPRGANPR-CNP22 (HSA fragment) (SEQ ID NO: 80)	ND	ND	ND
CQ	GHHSHEQHPHGANQQ-CNP22 (HRGP fragment) (SEQ ID NO: 76)	16	95	ND
CX	GHHSHEQHPHGANPR-CNP22 (HRGP fragment) (SEQ ID NO: 78)	ND	ND	ND
CS	GQEHPNARKYKGANPK-CNP22 (modified CNP37) (SEQ ID NO: 129)	19	61	>>160
CT	GQEHPNARKYKGANQK-CNP22 (modified CNP37) (SEQ ID NO: 130)	60	121	ND
CU	GQEHPNARKYKGANQQ-CNP22 (modified CNP37) (SEQ ID NO: 131)	9	57	ND
DB	GQEHPNARKYKGANKK-CNP22 (Gly-CNP37) (SEQ ID NO: 75)	50 ± 14	98 ± 17	>>160
	PGQEHPNARKYKGANKK-CNP22 (Pro-Gly-CNP37)	49 ± 6	103 ± 17	>>160
CW	GQEHPNARKYKGANKP-CNP22 (modified CNP37) (SEQ ID NO: 74)	ND	ND	ND
CR	GAHHPHEHDTHGANQQ-CNP22 (HRGP fragment) (SEQ ID NO: 128)	14 ± 5	77 ± 12	ND
CZ	FGIPMDRIGRNPR-CNP22 (osteocrin "NPR-C inhibitor") (SEQ ID NO: 82)	ND	ND	ND
DA	GKRTGQYKLGSKTGPGPK-CNP22 (FGF2 "heparin-binding domain" fragment) (SEQ ID NO: 83)	ND	ND	ND
CK	GQPREPQVYTGANQQ-CNP22, K4R (IgG1(Fc) fragment) (SEQ ID NO: 84)	2	32	≥160
CL	GVPQVSTSTGANQQ-CNP22, K4R (HSA fragment) (SEQ ID NO: 85)	3	35	>160

TABLE 3-continued

	N- and C-Terminal Modifications Natriuretic Peptide	cGMP Response rel. to 1 uM CNP22[1] 10 nM	 1 uM	NEP Cleavage ($t_{1/2}$, min)
	GHKSEVAHRFKGANKK-CNP22 (HSA-CNP27) (SEQ ID NO: 144)	51 ± 9	109 ± 15	>>160
	PGHKSEVAHRFKGANKK-CNP22 (Pro-HSA-CNP27)	32	107	ND
CN	GQTHSSGTQSGANQQ-CNP22, K4R (fibrinogen) (SEQ ID NO: 87)	12	115	ND
CM	GQPSSSSQSTGANQQ-CNP22, K4R (fibronectin) (SEQ ID NO: 86)	ND	ND	ND
CO	GSTGQWHSESGANQQ-CNP22, K4R (fibrinogen) (SEQ ID NO: 88)	2	33	ND
CP	GSSSSSSSSSGANQQ-CNP22, K4R (zinc finger) (SEQ ID NO: 89)	ND	ND	ND
CD	SPKMVQGSG-CNP17-KVLRRH ("BNP tails") (SEQ ID NO: 68)	25	102	ND
CJ	RSSCFGGRIDRIGAC ("C-ANP4-23", ANP-derived) (SEQ ID NO: 133)	ND	ND	ND
	CNP22, K4R, K10R, N.-term.--N-term. dimer/ disuccinimidyl glutarate (SEQ ID NO: 134)	19	44	ND
	CNP22, K4R, K10R, N-term.--N.-term. dimer/Bis-PEO5 (SEQ ID NO: 135)	19	41	ND
BM	CNP53 (SEQ ID NO: 4)	61	101	>>160
	ANP	10	23	ND

[1]Stimulation of cGMP production in N1H3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22
[2]CNP22 NEP resistance $t_{1/2}$ averaged 80 min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80 min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.
ND = Not Determined

Table 4 lists the half-lives, based on the in vitro NEP cleavage assay, of CNP variants conjugated to PEG (or PEO) polymers at the N-terminus. All the PEGylated CNP variants tested and shown in Table 4 displayed resistance or enhanced resistance to NEP cleavage except for PEO12-GANPR-CNP22(K4R), which had the same half-life as wtCNP22. N-terminal PEGylation of CNP22 having a K4G substitution does not seem to confer substantial improvement in NEP resistance. For example, PEG2K-CNP22(K4G) was only slightly more resistant to NEP cleavage than CNP22 (data not shown), whereas PEG2K-CNP22 had a much longer half-life in vitro than CNP22.

TABLE 4

N-Terminal PEGylation		cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
Natriuretic Peptide	PEG	10 nM	1uM	($t_{1/2}$, min)
CNP22		46 ± 10	100 ± 13	80
CNP22	PEG20K	0	15	>>160
CNP22	PEG5K	8 ± 1	20 ± 7	>>160
CNP22	PEG2K	6 ± 2	32 ± 4	>>160
CNP22	PEO4-$(PEO12)_3$ (branched)	17 ± 1	52 ± 6	>>160
CNP22	PEO24 (1.2 kDa)	8 ± 5	46 ± 10	>>160
CNP22	PEG1K	15 ± 3	68 ± 17	>160
CNP22	PEO12 (0.6 kDa)	12 ± 7	57 ± 18	160
CNP22	(PEO12)-Biotin	19	81	140
CNP22, K4G	(PEO12)-Biotin	10	27	100
CNP22, K4R	PEO24	15	56	ND
CNP22, K4R	PEO12	13	44	ND
CNP-17	PEG2K	5	50	>160
R-CNP22, K4R (SEQ ID NO: 41)	PEO24	15 ± 2	75 ± 12	ND
R-CNP22, K4R (SEQ ID NO: 41)	PEO12	23 ± 2	93 ± 19	≥160
ER-CNP22, K4R (SEQ ID NO: 39)	PEO24	6 ± 2	60 ± 10	ND
ER-CNP22, K4R (SEQ ID NO: 39)	PEO12	20 ± 1	92 ± 25	ND

TABLE 4-continued

N-Terminal PEGylation		cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
Natriuretic Peptide	PEG	10 nM	1uM	($t_{1/2}$, min)
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEG2K	15 ± 2	45 ± 18	ND
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEO24	28 ± 9	82 ± 18	>>160
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEG1K	15 ± 0.4	56 ± 23	>160
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEO12	40 ± 2	99 ± 13	>160
GANQQ-CNP22, K4R (SEQ ID NO: 69)	PEO24	16 ± 13	73 ± 30	ND
GANQQ-CNP22, K4R (SEQ ID NO: 69)	PEO12	30	78	ND
GANPR-CNP22, K4R (SEQ ID NO: 37)	PEO24	ND	ND	ND
GANPR-CNP22, K4R (SEQ ID NO: 37)	PEO12	ND	ND	80
GANSS-CNP22, K4R (SEQ ID NO: 70)	PEO24	8 ± 5	46 ± 21	ND
GANSS-CNP22, K4R (SEQ ID NO: 70)	PEO12	8 ± 0.3	52 ± 13	ND

[1]Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22
[2]CNP22 NEP resistance $t_{1/2}$ averaged 80 min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80 min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.
ND = Not Determined

FIG. **16** shows the NEP resistance profile of five N-terminal PEGylated conjugates of CNP22. The CNP22 peptides conjugated to PEG (or PEO) polymers of increasing mass exhibited increasing resistance to NEP degradation. In particular, PEO24-CNP22, PEG2K-CNP22 and PEG5K-CNP22 were resistant to NEP degradation over the assay period of 160 minutes.

FIG. **17** displays the NEP resistance profile of CNP variants CNP37 (Analog BL), CNP53 and GANRR-CNP22 (K4R) (SEQ ID NO: 36) having an N-terminal amino acid extension. As can be clearly seen, both CNP37 and CNP53 were resistant to NEP degradation in this in vitro assay, whereas GANRR-CNP22(K4R) (SEQ ID NO: 36) had the same lability to NEP hydrolysis as CNP22.

FIG. **18** depicts the NEP resistance profile of CNP17 and GANRR-CNP22(K4R) (SEQ ID NO: 36) conjugated to a PEG (or PEO) moiety at the N-terminus. PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) greatly improved the NEP resistance of this CNP variant, with PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) being completely resistant to NEP cleavage over the assay period of 160 minutes. Increasing the mass of the PEO moiety from about 0.6 kDa (PEO12) to about 1.2 kDa (PEO24) improved the NEP resistance of PEGylated GANRR-CNP22(K4R) (SEQ ID NO: 36). PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) to a monodispersed PEO24 moiety rather than a polydispersed PEG1K moiety also improved NEP resistance. Finally, although both PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and PEG2K-CNP17 have a similar total mass (keeping in mind that PEG2K is polydispersed), the former displayed substantially better NEP resistance.

NEP resistance assays were also performed on wtCNP22 and CNP variants G-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA", SEQ ID NO: 144) and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36). FIG. **19** shows that G-CNP37 and CNP27-HSA were completely resistant to NEP cleavage, and CNP27-PEO12 exhibited much greater stability to NEP degradation compared to wtCNP22.

Example 4

CNP Variant Stimulation of cGMP Production in NIH3T3 Cells

To determine the functional activity of CNP variants, the production of cGMP was measured in NIH3T3 cells exposed to the CNP variants. Murine NIH3T3 cells express endogenously the CNP signaling receptor, NPR-B, which shares 98% protein sequence identity with human NPR-B. NIH3T3 cells were cultured in high glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and antibiotics at 37° C. with 5% CO). Twenty four to 48 hours prior to signaling, cells were passaged to 12-well plates with a density of $2–5\times10^5$ cells per well at the time of the assay. CNP variants were resuspended in 1 mM HCl to a stock concentration of 1 mg/mL (455 uM for wtCNP22) and subsequently diluted to a 30 uM working stock solution with phosphate-buffered saline (PBS). Ten-fold serial dilutions were prepared in phosphate-buffered saline. Culture medium was removed from the cells and replaced with 0.4 mL PBS/Dulbecco's modified Eagle medium (50/50, v/v) containing 0.75 mM isobutylmethylxanine. Plates were incubated at 37° C., 5% CO_2 for 15 minutes before addition of 0.2 mL CNP variant in PBS and continued incubation at 37° C. for 15 minutes. Reactions were stopped by the addition of 0.2 mL lysis buffer supplied with the CatchPoint cGMP assay kit (Molecular Devices), and cGMP production was determined with the CatchPoint cGMP Assay (Molecular Devices). All stimulation experiments were performed in duplicate.

Tables 1-4 summarize the ability of CNP variants having backbone or side chain modifications, amino acid substitutions, N-terminal amino acid extensions, and/or N-terminal PEGylation, respectively, to stimulate cGMP production in NIH3T3 cells. In all four tables, the values for cGMP production in NIH3T3 cells exposed to 10 nM or 1 uM CNP variant are normalized to cell number and cGMP production in the presence of 1 uM wtCNP22.

Regarding the results in Table 1, only Analog G having 3-Cl-Phe at position 7 displayed substantially the same NPR-B stimulation activity at 1 uM as wtCNP22. With respect to Table 2, various CNP variants with amino acid substitutions, including Analogs AH, BO, AB, BH, BZ, BX and BR, showed substantially similar NPR-B stimulation activity as wtCNP22.

Considering the results in Table 3, many CNP variants having N-terminal and/or C-terminal modifications, including amino acid extensions, exhibited comparable NPR-B stimulation activity as wtCNP22. The functional CNP variants include Analog BB, which is CNP22(G1E) attached to heptanoic acid at the N-terminus, and Analog CD, which is the cyclic domain of CNP22 ("CNP17" retaining the Cys6 to Cys22 sequence) conjugated to the N-terminal and C-terminal "tails" of BNP. FIG. **20** illustrates that GANRR-CNP22

(K4R) (SEQ ID NO: 36), CNP37 (Analog BL) and CNP53 all had similar NPR-B stimulation activity as wtCNP22 in the in vitro assay.

Of note from Table 3 is that among the CNP variants assayed for both CNP functionality and NEP resistance, Analog AZ (R-CNP22(K4R)), Analog CC, Analog CF, Analog BL (CNP37), Analog DB (Gly-CNP37) and GHKSEVAHRFK-CNP27 (HSA-CNP27) (SEQ ID NO: 144) all had substantially similar NPR-B stimulation activity as CNP22 while being substantially more resistant to NEP cleavage than CNP22.

With regard to the results in Table 4, nine N-terminal PEGylated CNP variants at 1 uM stimulated cGMP production to at least about 70% of the level achieved by wtCNP22. Several noteworthy aspects appear in Table 4. First, N-terminal PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) with a monodispersed PEO polymer (PEO12 is about 0.6 kDa, PEO24 about 1.2 kDa) resulted in better NPR-B functionality than that with a polydispersed PEG polymer (PEG1K has a polymer number average molecular weight (M_n) of around 1 kDa, PEG2K around 2 kDa) (see also FIG. **21**). Second, N-terminal PEGylation of wtCNP22 with a polydispersed PEG polymer of increasing M_n (PEG1K, PEG2K, PEG5K and PEG20K) or with a monodispersed PEO polymer of greater mass (PEO12 and PEO24) correspondingly decreased the NPR-B activation ability of the CNP variants (see also FIG. **22**). Third, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), having the N-terminal GANRR (SEQ ID NO: 8) extension, stimulated greater cGMP production than PEO24-CNP22 and PEO24-CNP22 (K4R). Also of note is that among the N-terminal PEGylated CNP variants assayed for both CNP functionality and NEP resistance, PEO12-R-CNP22(K4R), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) all had substantially similar NPR-B stimulation activity as CNP22 while being much more resistant to NEP degradation than CNP22.

Among the CNP variants listed in Tables 1-4 and assayed for both CNP functionality and NEP resistance, Analogs G, BK, AZ, CC, CF, BL and DB, Pro-Gly-CNP37, HSA-CNP27 (GHKSEVAHRFK-CNP27) (SEQ ID NO: 144), PEG1K-CNP22, (PEO12)-biotin-CNP22, PEO12-R-CNP22(K4R), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) all had substantially similar NPR-B stimulation activity as wtCNP22 while being substantially more resistant to NEP cleavage than wtCNP22.

cGMP production assays were also carried out on wtCNP22 and CNP variants G-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA", SEQ ID NO: 144), wtCNP29 and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36). FIG. **23** shows that CNP22 and all the CNP variants assayed induced production of similar cGMP levels at either low or high dose of CNP.

Example 5

Binding Specificity for NPR-A, NPR-B and NPR-C

Signaling Competition Assay

To determine the binding specificity of CNP variants for the clearance receptor NPR-C, a signaling competition assay is carried out. Expression plasmids for human NPR-B or NPR-C (each purchased from OriGene) are transiently transfected and receptors are expressed in HEK293T cells. Forty hours after transfection, NPR-B, NPR-C and native HEK293T cells are harvested, counted and plated at a ratio of 1:1 (NPR-B cells:competing cells (either NPR-C or native HEK293T cells)) in 12-well or 96-well plates. Twenty hours after plating, cells are processed for the NPR-B/cGMP stimulation assay described in Example 4. If present, the natriuretic clearance receptor, NPR-C, is expected to bind and internalize CNP, thereby reducing the overall CNP concentration available for signaling through NPR-B, resulting in decreased cGMP production and a shift in the dose-response curve to the right. A rightward shift in the dose-response curve has been verified for wtCNP22. CNP variants having reduced affinity for NPR-C are not expected to induce a shift, or are expected to induce a smaller shift, in the dose-response curve to the right. This signaling competition assay is similar to that previously described by Cunningham (U.S. Pat. No. 5,846,932; B. Cunningham, EMBO J. 13(11): 2508-2515 (1994); H. Jin et al., J. Clin. Invest. 98(4): 969-976 (1996)).

The cGMP stimulation activity of wtCNP-22, Pro-Gly-wtCNP37 and ANP through NPR-B and NPR-A, and their selectivity for NPR-B vs. NPR-C and for NPR-A vs. NPR-C, were evaluated in signaling competition assays. NPR-A, NPR-B and NPR-C individually were transiently tranfected into HEK293T cells. Thirty hours after transfection, the cells were plated into 96-well plates: (A) 20,000 NPR-B cells+20,000 mock transfected cells; (B) 20,000 NPR-B cells+20,000 NPR-C cells; (C) 20,000 NPR-A cells+20,000 mock transfected cells; and (D) 20,000 NPR-A cells+20,000 NPR-C cells. Twenty hours after plating, the culture media was removed and replaced with serum-free media:PBS (1:1)+ 0.75 uM IBMX for 15 minutes. For cGMP signaling through NPR-B, dose series for ANP, CNP-22 and Pro-Gly-CNP37 were added and incubated at 37° C. for 12 minutes before the assay was stopped by cell lysis. For cGMP signaling through NPR-A, dose series for CNP-22 and Pro-Gly-CNP37 were incubated at 37° C. for 12 minutes, while dose series for ANP were incubated at 37° C. for 6 minutes (because NPR-A appeared to be a "faster" guanylyl cyclase than NPR-B, the incubation time for ANP was shortened in order not to max out (use up all cellular GTP) too soon when signaling with ANP). FIGS. **24**A and B show that CNP-22 and Pro-Gly-CNP37 ("Pro-CNP38") stimulated cGMP production through NPR-B with similar dose-response curves, and to a much greater extent than through NPR-A, and exhibited a similar profile for NPR-B vs. NPR-C selectivity in the signaling competition assays.

Determination of Binding Affinities (K_i) for NPR-A, NPR-B and NPR-C

The binding affinities (K_i) of CNP variants for NPR-A, NPR-B and NPR-C are determined in a heterologous competition binding assay (U.S. Pat. No. 5,846,932; B. Cunningham, EMBO J. 13(11): 2508-2515 (1994); H. Jin et al., J. Clin. Invest. 98(4): 969-976 (1996)). Membranes from HEK293 cells, or another suitably transfectable cell line (e.g., HeLa cells), expressing human NPR-A, NPR-B or NPR-C are prepared for radio-labeled ligand binding assays. Membrane preparations are diluted in an appropriate buffer and varying concentrations of wtCNP22 or CNP variant (competitor) are added with I^{125}-labeled wtCNP22 (Bachem). Samples are incubated at room temperature to allow for ligand/receptor equilibration and bound peptide is separated from free peptide by filtration through PVDF filter membranes. Filters are washed before the addition of scintillant and counting by a scintillation counter. Binding is measured in duplicate for each concentration of competitor peptide. CNP variant affinity (K_i, equilibrium dissociation constant) and B_{max}, (receptor number) are calculated by non-linear regression analysis and/or the Cheng-Prusoff equation.

CNP variants exhibiting reduced affinity to NPR-C are expected to have reduced susceptibility to clearance by NPR-C and thus a longer plasma or serum half-life. Increased half-life of the CNP variants in circulation would increase the availability of the variants for therapeutic activity.

Example 6

Effect of CNP Variants on the Growth of Rat Chondrosarcoma (RCS) Cells and cGMP Production in RCS Cells

To assess the ability of CNP variants to stimulate bone growth, skeletal dysplasia is simulated in cell culture by treating rat chondrosarcoma (RCS) cells with fibroblast growth factor 2 (FGF-2), which activates fibroblast growth factor receptor 3 (FGFR-3) and induces growth arrest (Krejci et al., J. Cell Sci., 118(21):5089-5100 (2005)).

Optimal CNP treatment parameters are determined by varying CNP concentration (0.05, 0.1, 0.2 and 0.5 uM), and treatment duration and interval (continuous; 2.5 min, 10 min, 30 min, 1 hr, 2 hr, 4 hr and 8 hr once a day; 2.5 min, 10 min, 30 min, 1 hr, 2 hr and 4 hr twice a day). After 72 hours, cells are counted using an automated cell counter, and the amount of extracellular matrix is estimated using alcian blue staining.

RCS cells are then treated with a CNP variant using the optimal conditions determined from the growth experiments with wtCNP22. The concentration of cGMP is measured by competitive ELISA for untreated RCS cells, RCS cells treated with CNP, and RCS cells treated with the CNP variant. Cell growth and matrix synthesis resulting from treatment with the CNP variant are also monitored and compared to those resulting from CNP treatment.

To assess the effect of CNP variants in a human cell culture system, primary re-differentiated human chondrocytes in alignate beads are treated with wtCNP22 and CNP variants, and cGMP concentration is determined by competitive ELISA as a measure of effective CNP signaling.

The methods described herein can be employed to assess the ability of CNP variants to stimulate cGMP production in and growth of rat chondrosarcoma cells in vitro.

Example 7

Dose Response Study in Rat Chondrosarcoma Cells

The tyrosine kinase receptor fibroblast growth factor receptor 3 (FGFR-3), a negative regulator of chondrocyte growth, is contitutively on in achondroplasia subjects. Stimulation of the FGFR-3 receptor with FGF-2 causes growth arrest by prolonged activation of Erk MAPK, and causes decreased matrix synthesis and loss of matrix, as well as a change in cell shape. Continuous exposure of rat chondrosarcoma (RCS) cells to fibroblast growth factor 2 (FGF-2) simulates achondroplasia in cell culture by activating FGFR-3 and inducing growth arrest (Krejci et al., J. Cell Sci., 118(21): 5089-5100 (2005)). To determine the dose of CNP variant and frequency of dosing that stimulate sufficient growth of bone cells, a dose response study was performed using the RCS cell assay as described in Example 6.

RCS cells were seeded at 10×10^3 cells per well in 24-well plates, grown for 24 hr, treated for 72 hr, and then counted. RCS cells were continuously exposed to FGF-2 (5 ng/mL) to simulate a constitutively active FGFR-3, which induced cell growth arrest (see bar #5 in FIG. **25**). Wild-type CNP22 (0.2 uM) was cultured continuously (72 hr), 1 hr daily or 2 hr daily. All stimulants were changed daily Continuous exposure of RCS cells to 0.2 uM CNP22 in the presence of 5.0 ng/mL FGF-2 partially reversed FGF2-induced growth arrest, leading to the growth of approximately 200×10^3 cells per well (bar #6 in FIG. **25**), compared to approximately 100×10^3 cells per well in the absence of CNP22 (bar #5 in FIG. **25**).

Both 1 hr exposure to CNP22 (0.2 uM) once a day and 2 hr exposure to CNP22 (0.2 uM) once a day achieved about 84% of the effect of continuous CNP22 (0.2 uM) exposure on chondrocyte growth (bars #7 and 8 in FIG. **25**). These results demonstrate that continuous exposure of growth-arrested chondrocytes to CNP22 is not required for reversal of cell growth arrest. Additionally, dose response studies demonstrate that lower doses of CNP22 are capable of reversing growth arrest (FIG. **26**A).

Furthermore, histological and cell morphological analysis of the extracellular matrix showed that CNP22 treatment antagonized FGF2-mediated loss of chondrosarcoma extracellular matrix and increased matrix synthesis. Exposure to FGF-2 decreased matrix synthesis and increased degradation, while addition of CNP22 to the FGF-2 cell culture increased matrix synthesis and partially inhibited FGF-2, as assessed by ^{35}S-sulfate and ^{3}H-Pro incorporation into, or decrease from, matrix (FIGS. **27**A-D). Analysis of aggrecan and fibronectin production (mRNA and protein) in RCS cells cultured with FGF-2 and CNP22 shows that FGF-2 decreased aggrecan level and increased fibronectin level, which was inhibited by addition of CNP22 (FIGS. **28**A-C). FGF-2 induces and activates matrix-processing molecules predominantly via Erk, and addition of CNP22 shows some effect on this activation.

Additional high-throughput assays for measuring growth arrest, such as crystal violet staining, are useful for measuring the effects of CNP22 and variants thereof on RCS cells.

Similar dose response studies can be conducted with the CNP variants described herein to determine their effective dose for reversing FGF2-induced growth arrest of RCS cells.

Example 8A

Ex Vivo Stimulation of Growth of Tibia and Femur from Mice with Mild Achondroplasia

Mouse tibial organ culture model has been used to demonstrate the efficacy of wild-type CNP22 in stimulating longitudinal bone growth. Treatment of wild-type tibiae with CNP22 at 10^{-8}, 10^{-7} or 10^{-6} M for 6 days increased longitudinal growth by 31%, 40% and 42%, respectively. Histological evaluation also showed expansion of the hypertrophic zone, e.g., an increase in the number and size of hypertrophic chondrocytes in the growth plate (Agoston et al., BMC Dev. Biol. 7:18 (2007)). Similar findings were observed in tibiae isolated from FGFR3^{Ach} mice (Yasoda et al., Nat. Med. 10: 80-86 (2004)).

To determine the efficacy of CNP variants in stimulating longitudinal bone growth, CNP variants were tested in a mouse organ culture model of endochondral bone growth in wild-type mice and transgenic mice having a G380R mutation in the human FGFR-3 gene (FGFR3$^{wt/Ach}$ heterozygote) which represent a mouse model of mild achondroplasia. In brief, the pharmacological activity of wild-type CNP22 and CNP variants was compared in an organ culture model of embryonic or neonatal mouse tibiae, isolated from wild-type and FGFR3$^{wt/Ach}$ littermates. Overall bone growth and histological changes within the growth plate were assessed. Conditioned culture medium is also assessed for biomarkers of intracellular signaling (cGMP), cartilage metabolism (type II collagen, other collagens, aggrecan chondroitin sulfate), bone metabolism (bone alkaline phosphatase, osteocalcin, type I collagen [C-telopeptide, N-telopeptide]), and inflammation (interleukin-1, interleukin 6, interleukin-11).

Effective CNP variants are identified by their ability, e.g., to stimulate production of cGMP, and bone growth as measured by increased longitudinal bone length and expansion of the cells in the hypertrophic zone of the growth plate.

Measurement of Bone Growth

The efficacy of wtCNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) in stimulating longitudinal femoral growth was evaluated in the mouse organ culture model. For these experiments femora were isolated from 2-3 day old wild-type mice and cultured in alphaMEM supple-

mented with 0.2% BSA, 0.5 mM L-glutamine, 40 units penicillin/mL and 40 ug streptomycin/mL, for 8 days in the presence of vehicle, CNP22 or CNP variants. The treatment commenced at day 0 and was repeated every two days thereafter, as the medium was changed. Bones were measured prior to treatment and every two days thereafter, using a dissection microscope fitted with a 1 cm eye-piece reticule. Conditioned medium was used for biomarker analysis. At day 8 bones were fixed in 4% paraformaldehyde for 24 hr, decalcified in 5% formic acid for 24 hrs, dehydrated and embedded in paraffin. Bones were sectioned at 5 um (microns), which were then deparaffinized, rehydrated, and stained with Alcian Blue for 30 min (pH 2.5; MasterTech). Alcian Blue stains cartilage blue. Stained sections were visualized and photographed by brightfield microscopy. The thickness of the hypertrophic region of the growth plate cartilage was determined by image analysis.

FIG. **29** illustrates the effect of wtCNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on longitudinal growth of 3-day old wild-type mouse femurs treated with the CNP peptides every two days. The results were normalized to measurements prior to treatment (day 0). The studies were performed in triplicate (vehicle) or quadruplicate (CNP peptides). As shown in FIG. **29**, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), as well as CNP22, were effective in stimulating longitudinal femoral growth, with the N-terminal PEGylated CNP variant being the most effective.

The growth of wild-type and FGFR3^{ach} mouse femur and tibia in response to CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) was also assessed. Culture of either wild-type or achondroplastic (FGFR3^{ach}) mouse tibia showed that CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) both increased the longitudinal growth of the tibia compared to vehicle or CNP22 (FIGS. **30** and **31**). CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) also stimulated the growth of wild-type mouse femur (FIG. **32**). Moreover, each of CNP22, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and CNP37 increased the longitudinal growth of FGFR3^{ach} mouse femur compared to vehicle (FIG. **33**).

Furthermore, ex vivo distribution of CNP37 in the growth plate of FGFR3^{ach} mouse tibia was evaluated. Bone samples were prepared as described above. Paraffin sections were cut and heat fixed for 1 hour at 60° C. Antigen retrieval with 1% hyaluronidase at 37° C. (30 minutes) was followed by a 1 hour serum block (10% Normal Goat Serum). CNP22 antibody (1:500 dilution; Peninsula Laboratories Inc., San Carlos, Calif.) was applied overnight at 4° C. For immunodetection, Vectastain ELITE ABC kit (Vector, Burlingame, Calif.) was used according to the manufacturer's recommendations. Specific bound peroxidase was visualized by incubation with DAB substrate kit (Vector) and the reaction was developed for 3 minutes. Slides were then dehydrated and mounted and photographed using a brightfield microscope. Staining for CNP in the growth plate of FGFR3^{ach} mouse tibia showed that CNP immunoreactivity was increased in the regions of articular and hypertrophic chondrocytes (FIG. **34**), indicating that CNP37 was delivered to the chondrocytes.

In addition to the distribution of CNP variants in the bone growth plate, the ex vivo effects of CNP37, CNP22 and vehicle on cells in the FGFR3^{ach} and wild-type growth plate, e.g., hypertrophic cell size and cellularity of proliferating zone, were also evaluated. Bone samples were prepared, including Alcian Blue staining, as described above. Images of the entire proximal growth plate were taken at 4× magnitude. The growth plate is divided into three zones, starting from the epiphyseal side of cartilage: the resting zone (individual small chondrocytes), proliferating zone (columns of stacked chondrocytes parallel to the long axis of the bone), and hypertrophic zone (large chondrocytes and thin septa between the chondrocytes). In these regions, measurements were made by ImageJ software, including the number of proliferating chondrocytes per column and the density of hypertrophic chondrocytes. A test square (4×4 mm^2) at five different regions of the hypertrophic zone was used to determine the density of hypertrophic chondrocytes. The cell size of hypertrophic chondrocytes was calculated by 1 over determined cell density. Cellularity of proliferating columns was increased by CNP37 and CNP22 in both wild-type and FGFR3^{ach} mice (FIGS. **35**B and C). Chondrocyte hypertrophy in FGFR3^{ach} mice was also increased as a result of culture with CNP22 or CNP37 (FIGS. **36**B and C).

Ex vivo studies of cultures of mouse bones indicated that CNP37 was delivered to the growth plate and was able to increase chondrocyte cellularity and hypertrophy, which are associated with growth plate expansion and longitudinal bone growth. To assess the biodistribution of CNP37 in the bone growth plate in vivo and the in vivo effects of CNP37 on the growth plate (including total growth plate thickness, hypertrophic zone thickness, and cellularity of proliferating zone), bone samples were obtained from FGFR3^{ach} mice treated with vehicle or CNP37 as described above. For biodistribution and in vivo effects studies, tibias were fixed and stored in 70% ethanol. For immunohistochemistry, the samples were decalcified in 5% formic acid for 2 days, dehydrated and embedded in paraffin. Bones were sectioned at 5 um (microns), which were then deparaffinized, rehydrated, and used for CNP immunohistochemistry as described above. For cellular image analysis, bones were sectioned at 5 (microns), and then deparaffinized, rehydrated, and stained with Alcian Blue for 30 minutes (pH 2.5; MasterTech) and Hematoxylin & Eosin for 30 seconds. Stained sections were visualized and photographed by brightfield microscopy. Thicknesses of the growth plate and the proliferating and hypertrophic zones were measured using ImageJ software.

In vivo biodistribution studies demonstrated that, similar to the ex vivo studies, CNP immunoreactivity was increased in the regions of articular and hypertrophic chondrocytes in the tibia growth plate of FGFR3^{ach} mice treated with CNP37, indicating that CNP37 was delivered in vivo to the growth plate of FGFR3^{ach} mouse tibia (FIG. **37**). Furthermore, CNP37 treatment significantly increased the total growth plate thickness, proliferating zone thickness and hypertrophic zone thickness of FGFR3^{ach} mouse tibia in vivo (FIGS. **38**A-C).

These results demonstrate that CNP variants of the disclosure penetrate into the growth plate of wild-type and achondroplastic animals, increase the number and size of chondrocytes, increase the thickness of the proliferating zone and the hypertrophic zone of the growth plate, and increase longitudinal bone growth in treated wild-type and achondroplasic animals. Therefore, the CNP variants are useful for stimulating bone growth in achondroplastic subjects.

Measurement of Biomarkers

In addition to measurement of bone growth in response to CNP variants, assay of the levels of biomarkers for cartilage and bone formation and growth induced in response to CNP variants is useful for evaluating the effect of CNP variants on bone growth.

Femurs and tibias were isolated from wild-type and FGFR3^{ach} mice as described above. Bones were cultured with CNP22 or a variant thereof for eight days with the replacement of media every two days. On the eighth day, media was

collected and analyzed for the biomarkers cGMP (cyclic guanosine 3', 5' cyclic monophosphate) and fragments of cleaved collagen type II, a cartilage-specific marker for cartilage turnover. Both markers were measured using commercially available enzyme-linked immunosorbent assays (ELISA) for cGMP (Cayman Chemical Co., Ann Arbor, Mich.) and cleaved collagen type II (Cartilaps) (Immunodiagnostic Systems, Fountain Hills, Ariz.), following the manufacturer's protocol.

Levels of cGMP and collagen type II fragments were measured from cell culture extracts after exposure to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36). FIGS. **39-42** show a large increase ($p<0.01$) in the levels of cGMP in the media after exposure of ex-planted wild-type and $FGFR3^{ach}$ mouse femurs and tibias to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36). In addition, exposure of wild-type and $FGFR3^{ach}$ mouse femurs to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) increased the levels of cleaved collagen type II, with the treated $FGFR3^{ach}$ mouse femurs showing significantly increased ($p<0.05$) levels of collagen type II fragments (FIG. **43**). Elevated levels of collagen type II fragments indicate a turnover of the cartilage matrix, and cartilage turnover typically precedes new bone formation in growing bones.

Example 8B

Ex Vivo Stimulation of Growth of Femur from Mice with Severe Achondroplasia

The effect of a CNP variant on the growth of bones from mice with severe achondroplasia was evaluated ex vivo. Used in the study were transgenic mice expressing a human FGFR-3gene having a Y367C mutation ($FGFR3^{Y367C}$) [S. Pannier et al., Biochim. Biophys. Acta, 1792(2): 140-147 (2009)], which represent a mouse model of severe achondroplasia. Femurs were isolated at embryonic day 16.5 and cultured for 6 days in the presence of 1 uM Pro-Gly-CNP37. Bone lengths were measured at Day 1 and Day 7. The bones were then paraffin-embedded, sectioned and stained with hematoxylin and eosin to assess histological changes and cellular morphology. Treatment of bone explants isolated from $FGFR3^{Y367C}$ mice with Pro-Gly-CNP37 ("ProCNP38") resulted in increase in bone growth and expansion in the growth plate (FIG. **44**). Femurs from $FGFR3^{Y367C}$ mice treated with vehicle for 6 days showed an 18% deficiency in growth length-wise compared to wild-type femurs treated with vehicle. Treatment of femurs from $FGFR3^{Y367c}$ mice with 1 uM Pro-Gly-CNP37 for 6 days reduced the growth deficiency to just 11%, i.e., reduced the growth defect by around 40%.

Example 9

Serum/Plasma Stability of CNP Variants In Vitro

In preparation for pharmacokinetics (PK) studies, the stability of CNP variants in serum and/or plasma is evaluated.

Briefly, the analyte is isolated by the removal of serum or plasma proteins by either a 2% trichloroacetic acid precipitation or a 1:3 serum:acetonitrile precipitation. The precipitation mixture is vortexed at 14,000 rpm for five minutes, and a portion of the supernatant is removed and diluted with water prior to transfer to a silanized autosampler vial for analysis. Serum extracts are then analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) with electrospray ionization mass spectrometry (ESI-MS). A single mass (m/z), shown to be specific for the CNP variant, is monitored for quantitation purposes.

Initially, analytical stability and recovery is determined. Analytical (RP-HPLC and ESI-MS) parameters are optimized through the analysis of matrix standards (serum extracts fortified with analyte post-precipitation). After optimization, analytical recovery is determined by spiking serum samples at known concentrations and comparison of the analyte response to that of matrix standards prepared at similar concentrations. Analyte stability in serum extracts is also determined to assure no significant losses occur after serum precipitation and prior to actual analysis. To test the effect of freezing on serum stability, a two-cycle freeze/thaw study is also performed. In this study a serum sample is spiked with CNP variant and analyzed prior to freezing overnight at −20° C. The sample is then thawed at room temperature and re-analyzed. The process is repeated for a second freeze/thaw cycle.

Serum stability of CNP variant is determined by spiking of serum/plasma samples with CNP variant at a concentration of 10 ug/mL. The sample is placed in a 37° C. water bath for a period of three hours. At 30 minute intervals duplicate aliquots of serum are removed and analyzed. If rapid losses of analyte are evident (>50% in 30 minutes), the study may be repeated with 10 minute timepoints.

In an exemplary method for determining the stability of CNP variants in murine plasma, a mixture of CNP variant (10 uL of a stock solution of about 2.5-5.0 mg/mL), heparinized murine plasma (50 uL, Bioreclamation, CD-1 Lith Hep 62231), and 5 M NaCl (10 uL) is incubated at 37° C. and 5% CO_2 for 0-5 hr, and then quenched with 10× protease inhibitor cocktail (15 uL, Sigma P2714). For extraction, 150 uL of MeOH/0.1% FA is added to 85 uL of the reaction mixture, and the resulting mixture is vortexed for 1 min and then centrifuged at 15° C. for 15 min. 75 uL of the supernatant is added to 300 uL of aqueous 0.1% FA. A small portion of the resulting mixture is subjected to analysis by LC/MS.

Example 10

Pharmacokinetics and cGMP Production in Rats and Mice

Studies were conducted in normal rats to evaluate the pharmacokinetics (PK) profile of CNP22 and certain CNP variants and the time courses of plasma cGMP concentration after single intravenous (i.v.) or subcutaneous (s.c.) administration of the CNP peptides. Plasma CNP immunoreactivity was determined by using a competitive radioimmunoassay (RIA) with an anti-CNP rabbit polyclonal antibody. Plasma cGMP concentration was determined by RIA using a commercially available kit (YAMASA cyclic GMP Assay kit, YAMASA Corporation).

Normal male rats, 7-8 weeks of age, were used. Recombinant wild-type CNP22, CNP37 and PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) were evaluated. A dosage of 20 nmol/kg of each CNP peptide as a solution in 5% mannitol was intravenously injected once into the tail, or a dosage of 50 nmol/kg of each CNP peptide as a solution in 0.03 mol/L acetic acid buffer solution, pH 4.0, containing 1% (w/v) benzyl alcohol and 10% (w/v) sucrose, was subcutaneously injected once into the back.

Plasma CNP immunoreactivity was determined by the competitive RIA using anti-CNP rabbit polyclonal antibody. Standard and QC samples were prepared. Fifty uL of the standard, QC and assay samples were added, respectively, to

test tubes containing 50 uL of RIA buffer. Diluted anti-CNP rabbit polyclonal antibody (100 uL) was added to the tubes. All tubes were kept at 4° C. overnight. ^{125}I-[Tyr^{0}]-CNP22 solution (100 uL) and rabbit IgG solution (100 uL) were added and left at approximately 4° C. overnight. One milliliter of anti-rabbit IgG goat serum containing 10% polyethylene glycol was added, vortexed and left at approximately 4° C. for at least 1 hour, and then the insoluble fraction was precipitated by centrifugation. After aspiration of the supernatant, the amount of radiation (gamma line) in the sediment was measured by a gamma-counter. Each sample was measured in duplicate, and the mean was adopted as the value determined.

Plasma cGMP concentrations in the sample at 5, 30, 60 and 90 minutes after i.v. dosing, or at 5, 30, 60, 120 and 180 minutes after s.c. dosing, were determined by the competitive RIA using anti-cGMP monoclonal antibody. Standard samples were prepared. 100 uL of the assay samples (standard solutions for the calibration curve or the diluted plasma samples for cGMP determination) were transferred to test tubes. Then 100 uL of anti-cGMP monoclonal antibody solution and 100 uL of ^{125}I-labeled succinyl cGMP tyrosine methyl ester solution were added to the tubes, respectively. All tubes were kept at 4° C. overnight. After the addition of 500 uL dextran charcoal solution, the tubes were vortexed and then placed on ice for 10 minutes. The reaction mixture was centrifuged and 500 uL of the supernatant was transferred from each sample to a new test tube. The amount of radiation (gamma line) in the supernatant was measured by a gamma-counter. Each sample was measured in duplicate, and the mean was adopted as the value determined.

Plasma CNP immunoreactivity was employed for pharmacokinetics (PK) analysis. PK analysis was performed using WINNONLIN® Professional (Pharsight Corporation). The PK profiles of CNP22, CNP37 and PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) after i.v. administration were calculated using PK parameters such as concentration at 0 hour (C_0: extrapolation, pmol/mL), total body clearance (CL_{tot}: mL/min/kg), distribution volume at steady state (V_{dss}: mL/kg), area under the plasma concentration-time curve (AUC: pmol·min/mL), mean residence time (MRT: min), and half-life ($T_{1/2}$: min). The PK profiles of the CNP peptides after s.c. administration were calculated using PK parameters such as maximum plasma concentration (C_{max}: pmol/mL), time to reach C_{max} (T_{max}: min), area under the plasma concentration-time curve (AUC: pmol·min/mL), mean residence time (MRT: min), and half-life ($T_{1/2}$: min).

In plasma spike recovery experiments, the RIA detected CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) similarly (data not shown).

Procedures similar to those described above are employed to study in mice the PK profiles of CNP22 and variants thereof and their ability to stimulate cGMP production.

The PK profiles of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) after i.v. administration in three rats are illustrated in FIG. **45**. As shown by FIG. **45**, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a much longer half-life and a much greater bioavailability than CNP22. The half-life, $T_{1/2}$ (min), was 1.42 (±0.45) for CNP22, 22.3 (±1.5) for PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36), and 49.5 (±28.0) for CNP37. The area under the curve, AUC (pmol·min/mL), was 320 (±54) for CNP22, 1559 (±568) for CNP37, and 2084 (±424) for PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

The PK profiles of the three CNP peptides after s.c. administration in three rats are depicted in FIG. **46**. Compared to CNP22, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a much longer half-life (78.1 min (±16.4) vs. 10.0 (±5.0)) and a much greater bioavailability (60% (±6%) vs. 19% (±9%)).

The time courses of plasma cGMP concentrations after i.v. administration of the three CNP peptides in three rats are displayed in FIG. **47**. FIG. **47** clearly demonstrates that i.v. administration of CNP37 and PEO24-GANRR-CNP22 (K4R) (SEQ ID NO: 36) resulted in much higher plasma levels of cGMP at 30, 60 and 90 minutes than i.v. administration of CNP22.

The time profiles of plasma cGMP concentrations after s.c. administration of the three CNP peptides in three rats are shown in FIG. **48**. Subcutaneous administration of PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and CNP37 also resulted in substantially higher plasma concentrations of cGMP than s.c. administration of CNP22, with the difference relative to CNP22 increasing over time for PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), but decreasing over time for CNP37.

The rat studies indicate that compared to wtCNP22, the CNP variants CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a substantially longer half-life in vivo, had a substantially greater bioavailability in vivo, and stimulated substantially higher levels of cGMP production in vivo for an extended period of time. The resistance of CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) to NEP degradation correlates to a longer plasma half-life in vivo, which in turn correlates to prolonged NPR-B/cGMP signaling in vivo. These results show that compared to CNP22, the CNP variants of the disclosure, administered by i.v. or s.c. injection (e.g., once daily), can be more effective in treating CNP-responsive conditions or disorders, such as bone-related disorders and vascular smooth muscle disorders.

Example 11

Pharmacokinetics Study in Mice

To determine CNP variants having increased NEP resistance, for efficacy study in FGFR3^{ach} mice (see Example 13), a pharmacokinetics (PK) study is carried out that compares the pharmacokinetics properties of CNP variants to wild-type CNP22. The FGFR3^{ach} mouse is a mutant mouse model of mild achondroplasia, containing a single transgene on a background of FVB mice.

Wild-type CNP22 or variant thereof is administered as a single intravenous (i.v.) dose in 6-week old wild-type FVB mice. Exemplary PK studies were conducted using wtCNP22. Six-week old FVB/N mice were intravenously administered wtCNP22 in a single dose at 100 nmol/kg. Mean plasma levels of CNP22 were calculated, and the estimated half-life of CNP22 was determined to be from 0.76 min to 1.03 min.

CNP variants displaying greater resistance to NEP degradation are expected to exhibit increased serum concentrations over time and a longer half-life in vivo.

Example 12

Efficacy of CNP Variants in Wild-Type Mice

The in vivo effects of CNP variants on bone growth were assessed in wild-type mice. Three week old FVB wild-type male mice received daily subcutaneous (s.c.) injection of either vehicle, G-CNP37 (200 nmol/kg) or PEO12-GANRR-

CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) (200 nmol/kg) for 5 weeks. Body weight was measured at least once weekly. Tail length was measured at least once weekly using digital caliper readings, and body length (naso-anal length), bone length (tibia, femur, ulna and humerus), skull length (anterior to posterior cranial segment) and lumbar vertebrae 5 (LV5) length were measured after 5 weeks of treatment using a caliper. X-rays were taken at baseline and after 5 weeks of treatment.

Five weeks of treatment of wild-type mice with G-CNP37 resulted in significant body weight gain, with increased body weight being observed beginning at Day 9 ($p<0.05$) (FIG. **49**). Treatment with G-CNP37 also resulted in significantly increased tail length, beginning at the second week after treatment ($p<0.01$) (FIG. **50**).

Table 10 shows the percentage change in tail length, body (naso-anal) length, skull (anterior to posterior cranial segment) length, bone (femur, tibia, humerus and ulna) lengths, and lumbar vertebrae 5 (LV5) length in wild-type mice injected s.c. once daily with 200 nmol/kg of either G-CNP37 or PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) for 5 weeks, relative to a value of 100% for wild-type mice treated with vehicle only.

TABLE 10

	Tail	Naso-Anal	Anterior to Posterior (Skull)	Femur	Tibia	Humerus	Ulna	LV5
G-CNP37	119%**	114%**	104%*	109%**	105%**	104%**	101%	108%*
CNP27-PEO12	101%	104%**	101%	102%*	100%	102%*	96%*	103%

**$p < 0.01$,

*$p < 0.05$

Treatment with G-CNP37 resulted in significantly increased tail length, body (naso-anal) length, skull length, proximal bone (femur and humerus) length, distal bone (tibia) length, and vertebral (lumbar vertebrae 5) length compared to treatment with vehicle.

Lower doses of Pro-Gly-CNP37 dosed daily s.c. at 5 nmol/kg, 20 nmol/kg or 70 nmol/kg for five weeks resulted in a dose-dependent increase in tail length, body (naso-anal) length, and bone lengths compared to vehicle. Table 11 shows the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in wild-type mice injected s.c. once daily with 5 nmol/kg, 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37 for 5 weeks, relative to a value of 100% for wild-type mice treated with vehicle only.

TABLE 11

	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
5 nmol/kg	107.6%**	102.7%**	103.3%**	101.7%**	101.1%	100.9%*
20 nmol/kg	107.8%**	107.6%**	107.1%**	103.4%**	102.3%**	102.5%**
70 nmol/kg	113.6%**	112.5%**	109.3%**	105.9%**	103.6%**	104.1%**

**$p < 0.01$,

*$p < 0.05$

In another study, various dosing regimens of Pro-Gly-CNP37 were administered for nine weeks followed by one week of recovery. Wild-type FVB mice were dosed s.c. with:

(1) vehicle daily for nine weeks, followed by one week of recovery;

(2) 20 nmol/kg Pro-Gly-CNP37 daily for one week, followed by three doses per week for eight weeks and one week of recovery;

(3) 20 nmol/kg Pro-Gly-CNP37 on alternating weeks; or

(4) 5 nmol/kg Pro-Gly-CNP37 daily for nine weeks followed by one week of recovery.

Increased growth in tail length, body length, and bone lengths was observed in all treatment groups (Groups 2, 3 and 4) at the end of the study. Table 12 shows the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in wild-type mice administered Pro-Gly-CNP37 ("Pro-CNP38") under the dose regimens described above, relative to a value of 100% for wt mice treated with vehicle only.

TABLE 12

	Total Pro-CNP38 received (nmol/kg)	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
Group 2	620	104.8%**	106.0%**	105.3%**	100.8%	102.5%	101.7%
Group 3	700	106.7%**	106.5%**	104.1%**	102.5%**	102.6%*	102.8%*
Group 4	350	104.9%**	105.1%**	107.3%**	102.7%**	102.3%**	103.6%**

**p < 0.01,
*p < 0.05

While all dose regimens of Pro-Gly-CNP37 increased axial and appendicular growth parameters measured, daily dosing of Pro-Gly-CNP37 promoted appendicular growth (femur, tibia, humerus, and ulna) at a lower total dose level (Group 4) compared to regimens with less frequent dosing (Groups 2 and 3).

Example 13

Efficacy in Mouse Model of Mild Achondroplasia

The efficacy of CNP variants in enhancing growth and correcting achondroplasia was tested in a mouse model of mild achondroplasia, using a strain of transgenic mice expressing a human FGFR-3 gene having a G380R mutation ($FGFR3^{ach}$) (Wang et al., Proc. Natl. Acad. Sci. USA, 96(8): 4455-4460 (1999); Naski et al., Development USA, 125: 4977-4988 (1998); U.S. Pat. Nos. 6,265,632 and 6,136,040).

At 3 weeks of age, $FGFR3^{ach}$ mice and their wild-type littermates were anesthetized to have lateral whole-body X-ray images taken by Faxitron, and randomized by body weight into the following treatment groups (n=8/group): (1) wild-type/vehicle, (2) $FGFR3^{ach}$/vehicle, (3) $FGFR3^{ach}$/CNP37, and (4) $FGFR3^{ach}$/PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36). The mice received once daily subcutaneous (s.c.) administration of designated test article (vehicle or 200 nmol/kg CNP variant) for 5 weeks. Satellite groups (n=3) were used to confirm exposure of each test article following a single subcutaneous administration on Day 1. Wild-type male FVB mice that received daily s.c. injection of vehicle for 5 weeks were used as a control for normal growth.

X-ray measurements at baseline and at the end of the study were performed to determine change in the head length, area of the skull, and the external auditory meatus (EAM, the ear canal running from the outer ear to the middle ear). Body weight and tail length were measured at least once weekly, using a digital caliper to measure tail length, and body (naso-anal) length was measured after 5 weeks of treatment. Bone (tibia, femur, ulna and humerus) lengths were measured using a digital caliper at necropsy.

On Day 37, all mice were sacrificed by terminal anesthesia and whole-animal photographs and X-ray images by Faxitron were taken. Left and right tibia, femur, humerus, and ulna were collected and measured using a digital caliper. The left portions of each bone were processed for histology, and the right portions were snap-frozen for archival. Samples obtained from the bones were used to evaluate the effects of CNP variants on endochondral bone growth.

Treatment of $FGFR3^{ach}$ mice with CNP37 by once daily s.c. injection for 5 weeks resulted in significantly increased body length (FIG. **51**), tail length (FIG. **52**), distal bone (ulna and tibia) lengths (FIGS. **53**A and B), and proximal bone (humerus and femur) lengths (FIGS. **54**A and B). Moreover, treatment with CNP37 increased the head length (FIG. **56**), area of the external auditory meatus (FIG. **57**), and spine length (FIG. **58**) via extension of the vertebral bodies. Additionally, treatment with CNP37 corrected rhizomelia (disproportion of the length of the proximal limbs) of $FGFR3^{ach}$ mice, i.e., restored proportional growth of proximal bones, as assessed by the femur:tibia ratio (FIG. **55**). Table 13 summarizes the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in $FGFR3^{ach}$ mice injected s.c. once daily with 200 nmol/kg of either CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) for 5 weeks, relative to a value of 100% for $FGFR3^{ach}$ mice treated with vehicle only.

TABLE 13

	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
CNP37	115%**	109%**	112%**	107%**	105%*	106%**
CNP27-PEO24	100%	99%	100%	102%	100%	101%

**p < 0.01,
*p < 0.05

The results of these studies show that CNP37 can stimulate spinal and long bone growth, help to correct rhizomelia by preferentially increasing the length of the femur over the tibia, and help to restore craniofacial proportions in $FGFR3^{ach}$ mice. These results indicate that CNP37 and potentially other CNP variants may be effective in correcting the symptoms of achondroplasia and treating subjects having defects in bone growth or in need of increased bone growth.

Example 14

Measurement of Biomarkers and Evaluation of Immunogenicity in Wild-Type and Achondroplastic Mice

Measurement of Biomarkers After CNP Administration

The levels of bone growth biomarkers were measured in wild-type and achondroplastic ($FGFR3^{ach}$) mice.

Transgenic FVB $FGFR3^{ach}$ mice (a mouse model of mild achondroplasia) were treated daily for 5 weeks by subcutaneous injection with either vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0), CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) at 200 nmol/kg for each CNP compound, as described above. Plasma and serum were collected during the study. Harvested plasma was stored with 10× protease inhibitor at −80° C. until analysis. cGMP levels were measured 15 minutes post-injection on Day 36 from K2-ETDA plasma collection tubes. Fragments of cleaved collagen type II (cartilage-associated biomarkers), osteocalcin (bone-associated biomarker), and IgG (relating to immunogenicity) were measured from terminal bleed serum at the

end of the study (Day 37). cGMP was measured using a commercially available ELISA kit (Cayman Chemical Co., Cat. No. 581021.1), cleaved collagen type II (Cartilaps) was measured using a commercially available kit from Immunodiagnostic Systems (Cat. No. 3CAL4000), and osteocalcin was measured using a commercially available kit from Biomedical Technologies Inc. (Stoughton, Mass.).

FIG. **59** shows an increase in cGMP plasma levels in FGFR3^{ach} mice treated with CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) compared to vehicle. FIGS. **60** and **61** show that administration of CNP37 resulted in the greatest elevation of serum levels of cleaved collagen type II and osteocalcin. These results indicate that administration of the CNP peptides, in particular CNP37, to FGFR3^{ach} mice led to increased levels of bone growth markers, suggesting increased bone formation and growth in CNP peptide-treated FGFR3^{ach} mice.

For evaluation of biomarkers in wild-type mice, wild-type FVB mice were treated daily for 5 weeks by subcutaneous injection with either vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0), 200 nmol/kg G-CNP37, 200 nmol/kg PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36), or 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37. Plasma and serum were collected during the study. Harvested plasma was stored with 10× protease inhibitor at −80° C. until analysis. cGMP levels were measured 15 minutes post-injection on Day 36 from K2-ETDA plasma collection tubes. The levels of cleaved collagen type II, bone-specific alkaline phosphatase, and IgG (relating to immunogenicity) were measured from terminal bleed serum at the end of the study (Day 37). cGMP and cleaved collagen type II (Cartilaps) were measured as described above. Bone-specific alkaline phosphatase was measured using a commercially available kit (Cusabio, Cat. No. CSB-E11914m).

Administration of G-CNP37 significantly increased ($p<0.05$) the level of cGMP (FIG. **62**) and particularly the level of fragments of cleaved collagen type II (FIG. **63**) in wild-type mice compared to vehicle. The significantly higher level of collagen type II fragments resulting from administration of G-CNP37 indicates turnover of the cartilage matrix, suggesting that G-CNP37 stimulated new bone formation in growing bones in wild-type mice.

Both doses of Pro-Gly-CNP37 ("Pro-CNP38"), 20 and 70 nmol/kg, significantly increased ($p<0.05$) plasma cGMP level 15 minutes after administration compared to wild-type mice treated with vehicle (FIG. **64**). Administration of the higher dose (70 nmol/kg) of Pro-Gly-CNP37 also significantly increased ($p<0.05$) the level of cleaved collagen type II compared to vehicle-treated mice (FIG. **65**), suggesting that the higher dose of Pro-Gly-CNP37 stimulated cartilage matrix turnover prior to new bone formation in wild-type mice. Furthermore, administration of the higher dose (70 nmol/kg) of Pro-Gly-CNP37 increased ($p<0.05$) the level of bone-specific alkaline phosphatase compared to vehicle-treated mice (FIG. **66**), suggesting that the higher dose of Pro-Gly-CNP37 increased bone remodeling in wild-type mice.

Evaluation of Immunogenicity of CNP Variants

Because the CNP variants are peptide derivatives, it is possible that administration of the peptides may lead to an immunogenic response in vivo. To assess whether an immune response occurred after successive administrations of CNP variant, measurement of serum antibody levels was performed.

An IgG assay was performed to assess whether an IgG immune response was triggered by 5 week exposure of achondroplastic FGFR3^{ach} mice to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36). IgG is the most predominant immunoglobulin in mouse and human serum and is produced as part of the secondary immune response to an antigen. The IgG response to administration of the CNP peptides was determined as follows. 96-well plates were coated with 100 ng/mL CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) in BupH PBS buffer (Pierce/Thermo, Cat. No. 28372, Rockford, Ill.). After overnight incubation, plates were blocked with Casein-PBS blocking buffer (Pierce/Thermo, Cat. No. 37528) for two hours at room temperature with shaking at 300 rpm. After washing with wash buffer (1×PBS with 0.05% Tween), diluted serum samples from a terminal bleed (diluted 1:25) were added to the plate. Positive and negative controls were also loaded onto the plate. Positive control was 1:25 diluted serum (pooled from 6 individual FVB mice) with anti-CNP22 antibody added (Bachem rabbit anti-CNP22 IgG, Cat. No. T-4222) at 1:1000 dilution. Negative control was the diluted pooled serum. After two hours of incubation, the plates were washed and secondary antibody diluted in blocking buffer was added to the wells. For the mouse serum samples, anti-mouse IgG Fcγ (peroxidase-conjugated affinipure goat anti-mouse IgG, Fcγ fragment, Cat. No. 115-035-071, Jackson Immunoresearch, West Grove, Pa.) was added at a 1:10,000 dilution. For the positive and negative controls, anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Cat. No. SC-2004, Santa Cruz, Calif.) was added to blocking buffer. After two hours of incubation at room temperature with shaking at 300 rpm, plates were washed with wash buffer. 100 uL of TMB (One-step TMB, Pierce/Thermo, Cat. No. 34022) was added to all wells. Plates were incubated at room temperature for 15 minutes with shaking at 300 rpm. Colorimetric reactions were stopped by the addition of 100 uL of 2 N H_2SO_4. Plates were read at 450 nm (Spectramax, Molecular Devices, Sunnyvale, Calif.) and data was analyzed using SoftMax Pro software (Molecular Devices).

Serum samples from FGFR3^{ach} mice treated with CNP22 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) indicated no positive IgG immune response in any of the mice. Only one out of nine CNP37-treated FGFR3^{ach} mice showed a slightly positive IgG response.

The immunogenic response of wild-type mice administered G-CNP37, PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) or Pro-Gly-CNP37, as measured by an increase in serum IgG level, was also assessed by examining terminal bleed serum samples as described above. None of the wild-type mice treated with PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) or Pro-Gly-CNP37 (20 or 70 nmol/kg) showed a positive IgG immune response, and only one out of six wild-type mice administered G-CNP37 showed a slightly positive IgG response.

Measurement of Biomarkers Under Different CNP Dosing Regimens

Wild-type FVB mice were treated daily for 9 weeks by subcutaneous (s.c.) injection of vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0) or Pro-Gly-CNP37 ("Pro-CNP38") under different dosing regimens. Group 1 included vehicle-treated mice injected s.c. daily for 9 weeks followed by 1 week of no treatment. Group 2 included mice treated with Pro-Gly-CNP37 (20 nmol/kg) once daily for 1 week, followed by three doses per week for 8 weeks, followed by 1 week of no treatment. Group 3 contained mice treated with Pro-Gly-CNP37 (20 nmol/kg) once daily in alternate weeks (weeks 1, 3, 5, 7 and 9), with 1 week of no treatment following each week of treatment. Group 4 contained mice treated with Pro-Gly-CNP37 (5 nmol/kg)

once daily for 9 weeks followed by 1 week of no treatment. Finally, Group 5 included mice treated with Pro-Gly-CNP37 (5 nmol/kg) once daily for 5 weeks without any week of no treatment. Terminal bleed serum was collected from the mice, and cleaved collagen type II, total alkaline phosphatase and total antibody levels were measured therefrom. Cleaved collagen type II levels were measured as described above. Total alkaline phosphatase levels, predominantly from the liver and bones, were measured with the assistance of a veterinary diagnostic laboratory testing facility (Antech).

A total antibody assay was developed to evaluate potential immune response. The platform used for the total antibody assay was an electrochemiluminescent assay (ECLA). The ECLA platform utilizes a biotin-labeled drug (here, biotin-Pro-Gly-CNP37) and a ruthenium-labeled drug (here, Ru—Pro-Gly-CNP37). The biotin-labeled drug binds to a streptavidin-coated plate that contains electrodes, and the ruthenium-labeled drug functions as the detection component of the assay, as ruthenium can be electrochemically stimulated. A drug-specific antibody (here, a CNP-specific antibody) binds to the biotin-labeled drug and the ruthenium-labeled drug and "bridges" the two labeled drugs. Among the advantages of the ECLA platform, any isotype of antibody (IgG, IgM, etc.) can be detected, and the ECLA assay is species-independent.

The total antibody assay was performed as follows. Pro-Gly-CNP37 was labeled with biotin in a 4:1 challenge ratio, and Pro-Gly-CNP37 was also labeled separately with ruthenium in a 10:1 challenge ratio. Both separate labeling reactions were quenched by the addition of glycine, and the samples from both reactions were buffer-exchanged to PBS. Low and high QCs were prepared by using commercially available anti-CNP22 antibody (Bachem rabbit anti-CNP22 IgG, Cat. No. T-4222) added at low and high concentrations to 5% diluted FVB mouse serum. Mouse serum samples were diluted to 5% using assay diluent (5% BSA in PBS, MSD Cat. No. R93AA-1). A working solution was prepared by adding biotin-labeled Pro-Gly-CNP37 to assay diluent, then adding ruthenium-labeled Pro-Gly-CNP37 to assay diluent, and then combining the two solutions together. Twenty-five uL of low and high QCs were loaded onto the plate as controls. Then 25 uL of sample was added to a non-binding 96-well plate, followed by the addition of 50 uL of working solution to all wells. Samples and QCs were incubated with the working solution at room temperature for 2 hours with shaking at 350 rpm. In the meantime, an MSD streptavidin plate (MSD Cat. No. L13SA-1) was blocked with blocking buffer (MSD Cat. No. R93AA-1) at room temperature for 2 hours with shaking at 350 rpm. At the end of the two hour incubation, the MSD streptavidin plate was washed, and then 50 uL of sample or QC was transferred to the MSD plate. The plate was then incubated at room temperature for 1 hour with shaking at 350 rpm. At the end of the 1 hour incubation, the MSD plate was washed and 150 uL of 2× read buffer (MSD Cat. No. R92TC-2) was added to the plate. The plate was read using an MSD PR400 machine.

All five groups of mice exhibited substantially comparable levels of cleaved collagen type II (FIG. **67**). Treatment of the mice in Group 5 with 5 nmol/kg Pro-Gly-CNP37 once daily for 5 weeks significantly increased ($p<0.001$) total alkaline phosphatase level (FIG. **68**), which is indicative of bone remodeling. Serum samples from each of the four groups of mice treated with Pro-Gly-CNP37 did not show a positive antibody response in the total antibody assay.

Not to be bound by any theory, there are possible explanations as to why the four groups of CNP-treated mice did not exhibit statistically significant difference in cleaved collagen type II level compared to vehicle-treated mice, and why only the CNP-treated mice in Group 5 exhibited a statistically significant increase in total alkaline phosphatase level compared to vehicle-treated mice. It is possible that because the vehicle-treated mice in the study were also growing, cleaved collagen type II and alkaline phosphatase, which are biomarkers of growth, were also produced in the vehicle-treated mice. In addition, there was a one week period of no treatment for the mice in each of Groups 1 to 4, which possibly diluted out any changes in the cleaved collagen type II and total alkaline phosphatase levels between the CNP-treated mice in Groups 1-3 and the vehicle-treated mice. There was no period of no treatment for the CNP-treated mice in Group 5, and those mice exhibited significantly ($p<0.001$) greater total alkaline phosphatase level compared to vehicle-treated mice.

Example 15

Dose Responses of CNP Variants in Mice

The effects of varying doses of CNP variants were assessed in wild type FVB mice.

For the dose study, in two separate studies (S1 and S2), groups of 10 mice were administered Pro-Gly-wtCNP37 ("Pro-CNP38") at 20 and 70 nmol/kg subcutaneously once daily for 36 injections. Tail length and body weight were measured over the course of treatment. Animals were sacrificed at the end of the experiment and bone length assessed.

Tail length of vehicle treated animals was approximately 8 cm at day 36, whereas animals treated with 20 nmol/kg Pro-CNP38 exhibited tail length of approximately 8.75 cm and 70 nmol/kg Pro-CNP38 increased tail length to approximately 9.5 cm. Administration of Pro-CNP38 in either dose induces a significant ($p<0.05$) relative increase in total body length compared to control treated animals, demonstrating approximately a 130% increase in growth velocity at 20 nmol/kg and approximately a 160% increase in growth velocity at 70 nmol/kg Pro-CNP38 (FIG. **69**).

Treatment with Pro-CNP 38 also significantly increased bone length in most of the long bones assessed as well as total naso-anal (body length) of the animal. Table 14 is representative of the relative % increase in bone length in treated animals in comparison to vehicle treated animals.

TABLE 14

Pro-CNP38	Tail	Naso-Anal	LV4-6	Femur	Tibia	Humerus	Ulna
20 nmol/kg	8%*	7%*	10%*	7%*	4%*	3%*	3%
70 nmol/kg	16%*	13%*	13%*	10%*	7%*	4%*	5%*

Relative % increase,
*$p < 0.05$ ANNOVA (Dunnett's) v vehicle

Bone mineral density (BMD) and bone mineral content (BMC) were also evaluated after administration of Pro-CNP38 at different doses. Results (FIG. **70**) showed that administration of Pro-CNP38 at 70 nmol/kg significantly decreased bone mineral density (FIG. **70**A) and increased bone mineral content (FIG. **70**B), suggesting there is a delay in bone mineralization in treated animals, but the mineralization process itself is not adversely affected by treatment with CNP.

There were no significant changes in organ weight between Pro-CNP38 or vehicle treated animals.

Bioanalytical Studies of Dose Response in Mice

Bioanalytical studies were carried out to measure markers of CNP activity after in vivo administration of varying doses

of Pro-CNP38. Plasma cGMP levels, serum levels of collagen type II and serum levels of alkaline phosphatase from in vivo samples were analyzed. Wild type mice were administered 20 and 70 nmol/kg Gly-wtCNP37 ("CNP38"), 20 and 70 nmol/kg Pro-Gly-wtCNP37 ("Pro-CNP38") and 70 and 200 nmol/kg of GHKSEVAHRFK-wtCNP27 ("HSA-CNP27") (SEQ ID NO: 144) subcutaneously once daily for 36 days. Plasma was collected 15 minutes after the last injection on day 36 and mice were sacrified 24 hr later. At sacrifice, terminal bleed serum was collected and used for biomarker analysis as described previously.

FIG. **71** shows that 20 nmol/kg CNP38 and 70 nmol/kg HSA-CNP27 signficantly increased plasma cGMP ($p<0.01$), raising plasma cGMP levels to approximately 300 pmol and 400 pmol, respectively. Adminsitration of 70 nmol/kg CNP38 increased cGMP to approximately 500 pmol ($p<0.01$), while administration of 70 nmol/kg Pro-CNP38 increased cGMP to approximately 575 pmol ($p<0.001$). Administration of 200 nmol/kg HSA-CNP27 increased cGMP to approximately 675 pmol ($p<0.001$).

CNP variants also significantly increased serum levels of cleaved collagen type II (FIG. **72**). CNP38 at 20 nmol/kg increased collagen to approximately 9 pg/ml ($p<0.05$), CNP38 at 70 nmol/kg increased collagen to approximately 8 pg/ml ($p<0.05$), Pro-CNP38 at 20 nmol/kg increased collagen to approximately 12 pg/ml ($p<0.05$), Pro-CNP38 at 70 nmol/kg increased collagen to approximately 16 pg/ml ($p<0.05$), HSA-CNP27 at 70 nmol/kg increased collagen to approximately 10 pg/ml, and HSA-CNP27 at 200 nmol/kg increased collagen to approximately 10 pg/ml ($p<0.05$).

Serum alkaline phosphatase (AP) levels also increased after administration of CNP variants (FIG. **73**). CNP38 at 20 nmol/kg increased AP to approximately 130 IU/L, CNP38 at 70 nmol/kg increased AP to approximately 160 IU/L ($p<0.001$), Pro-CNP38 at 20 nmol/kg increased AP to approximately 155 IU/L ($p<0.001$), Pro-CNP38 at 70 nmol/kg increased AP to approximately 180 IU/L ($p<0.001$), HSA-CNP27 at 70 nmol/kg increased AP to approximately 120 IU/L, and HSA-CNP27 at 200 nmol/kg increased AP to approximately 140 IU/L ($p<0.01$). Table 15 illustrates the percent of total AP that is bone specific.

TABLE 15

	Total AP IU/L	Bone-specific AP IU/L	% of Total Alk Phos
Vehicle	109.8	24.7	22.5
CNP38 (20 nmol/kg)	135.9	47.9	35.2
CNP38 (70 nmol/kg)	166.3	63.5	38.2
Pro-CNP38 (20 nmol/kg)	159.9	65.9	41.2
Pro-CNP38 (70 nmol/kg)	183.6	78.4	42.7
HSA-CNP27 (20 nmol/kg)	121.4	51.7	42.6
HSA-CNP27 (70 nmol/kg)	136.1	76.5	56.2

Analysis of anti-CNP antibodies showed that only HSA-CNP27 elicited an IgG antibody response in mice.

The results above illustrate that administration of CNP variants increases the concentration of collagen type II and alkaline phosphatase in serum, indicating that CNP increases factors relevant for increased bone growth, and suggest that administration of CNP variants at doses as low as 20 nmol/kg are effective in increasing bone growth in vivo.

cGMP Response After Different Dose Regimens

Bioanalytical analysis was also assessed at different times after administration of Pro-Gly-wtCNP37 ("Pro-CNP38") to wild type CD-1 mice, 8-10 weeks old, (n=3 per treatment group). Pro-CNP38 was given in a single subcutaneous dose of 200 nmol/kg and levels of cGMP measured at 15 minutes, 3 hours, 1 day, 2 days and 3 days post injection in the plasma, epiphysis, cortical bone (marrow removed), lung and brain. Blood was collected on K2EDTA. Tibial and femoral epiphysis and cortical bone, ear pinna, brains, kidneys and lungs were harvested, placed in boiling water for 5 minutes, then frozen to −70° C. Both plasma and tissue were assayed for cGMP (Cayman Chemical cyclic GMP ELISA kit).

Results showed that cGMP levels increased at 15 minutes post injection in plasma (approximately 1300 pmol/ml) and epiphysis (approximately 2.5 pmol/ml/mg). By 3 hours, plasma levels had decreased approximately to control levels, while levels in the epiphysis were approximately 3 fold lower than cGMP levels at 15 minutes post injection, but higher than control levels. Levels of cortical bone increased to approximately 0.5 pmol/ml/mg at 15 minutes and remained at this level at 3 hours. Levels of cGMP at all timepoints were back to control levels by 1 day after injection. Little to no cGMP was detected in lung or brain at any timepoint.

cGMP levels were also measured in mice administered multiple injections of Pro-CNP38. Groups of mice (n=3) were given Pro-CNP38 as follows: 20 nmol/kg single dose, subcutaneously; 200 nmol/kg single dose, subcutaneously; 20 nmol/kg subcutaneously on days 0 and 1; 200 nmol/kg subcutaneously on days 0 and 1; 20 nmol/kg subcutaneously on days 0 and 3; 200 nmol/kg subcutaneously on days 0 and 3. Mice were sacrificed 15 minutes after the final dose of Pro-CNP38 and plasma levels of cGMP analyzed. There does not appear to be a modulation of the plasma cGMP signal due to the different dose regimens. cGMP responses in the cartilage are also investigated.

Evaluation of Potential Desensitization of NPR-B Receptor

Histological analysis shows that CNP, when administered daily at 200 nmol/kg, accumulates in the growth plate of animals, based on increased CNP immunoreactivity. It is possible that this accumulation, or daily stimulation of the CNP receptor, in the growth plate could desensitize the CNP receptor.

To determine if multiple dosing desensitizes the NPR-B receptor in vitro, normal human articular chondrocytes were cultured with Pro-Gly-wtCNP37 ("Pro-CNP38") for varying times and cGMP secretion measured.

Primary normal human chondrocytes isolated from articular cartilage were cultured as recommended by the supplier (Lonza). At 60-80% confluence the chondrocytes were treated with 1 uM Pro-CNP38 twice, with increasing amounts of time between treatments (first treatment at time 0, then at 15 min, 30 min, 60 min, 2 hrs, 3 hrs, 4 hrs, 6 hrs after the initial treatment). In the following experiment the treatments were either applied twice, with increasing amounts of time between treatment (first treatment at time 0, then at 6, 16, 24, 48 hrs after initial treatment) or only once, in parallel to the second treatment (only at 6, 16, 24 and 48 hrs-naïve responses). Treatments were either applied for 15 min only (acute treatment), or for the duration of the experiment (chronic treatment when CNP was left in the medium). Cell lysates and conditioned medium were collected and analyzed for total cGMP secretion (Molecular Devices ELISA).

In a short term experiment, cells were stimulated with Gly-wtCNP37 ("CNP38") 1 uM two times for 15 minutes (acute treatment), or two times with CNP38 throughout culture (chronic treatment). The periods between treatments were 15 min, 30 min, 60 min, 2 hrs, 3 hrs, 4 hrs, and 6 hrs. Peak cGMP stimulation was obtained by treating cells once only, at the last time point of the experiment, (6 hours; >0.1 pmol/well in the acute experiment and 0.2 pmol/well in the chronic experiment). In the acute experiment, when the cells

are treated twice, the response decreases to 0.1 pmol/well if the cells are treated at time 0 and then again at 15 minutes. In the acute experiment, when the cells are treated twice, the response decreases to approximately 0.5 pmol/well if the cells are treated at time 0 and again at 30 min, 60 min, 2, 3, and 4 hours. In the chronic experiment, when the cells are treated twice, the response decreases to approximately 0.16 pmol/well if the cells are treated at time 0 and at 15 minutes. In the chronic experiment, when the cells are treated twice, the response decreases to approximately 0.6 pmol/well if the cells are treated at time 0 and again at 30 min, 60 min, or 2 hours. In the chronic experiment, when the cells are treated twice, the response decreases to <0.5 pmol/well if the cells are treated at time 0 and again at 2, 4 or 6 hours.

Long term studies were also carried out. For acute treatment, 1 uM CNP38 was added to cell culture as described above. For chronic treatment, 1 uM CNP38 was added to cell culture throughout the experiment duration as described above. Results indicate that the NPR-B receptor can desensitize after repeated, daily CNP administration in vitro, and 48 hours between doses is sufficient to recover to 60% of the maximial NPR-B response to CNP38, if the CNP is removed after the first dose, and to <40% if the CNP is incubated throughout the experiment (FIG. **74**).

To evaluate whether treatment with a CNP variant desensitizes the NPR-B receptor in vivo, experiments were conducted with wild-type mice. CD-1 male mice 8-10 weeks of age were injected subcutaneously with vehicle control or Pro-Gly-wtCNP37 ("Pro-CNP38") at 200 nmol/kg on the appropriate days. The mice were either injected with Pro-CNP38 daily for up to 8 days, or were injected with Pro-CNP38 on the first day, the first and second days, or the first and third days of the study. Fifteen minutes following the final injection, the mice (n=3 per treatment group) were deeply anesthetized and ex-sanguinated via thoracotomy and aortic cannulation. Circulating blood was flushed from the body with PBS via an aortic cannula. The kidneys and right tibias, right femurs and left femurs were collected, boiled in water for 5 minutes and/or snap frozen in liquid nitrogen and stored on dry ice or in a −70° C. freezer until cGMP assay. For estimation of cGMP production in cartilage, distal femurs and/or proximal tibias were dissected, weighed and pulverized using Covaris Cryoprep CP02. Powdered samples were homogenized using a Covaris E210 sonicator in 5% perchloric acid, and neutralized in 60% KOH. Samples were then centrifuged at 10,000 rpm at 4° C. for 5 minutes, and the supernatant was used for the cGMP assay (Cyclic GMP Enzyme Immunoassay Kit, Cayman, Mich.). Additionally, left tibias were collected, fixed in 10% normal buffered formalin, and saved for further immunohistochemical analysis.

FIG. **75**A shows that repeated daily treatment of wild-type mice with 200 nmol/kg of Pro-CNP38 for 1, 4, 6, 7 and 8 days did not result in desensitization of the cGMP response. To the contrary, potentiation of the cGMP response was observed after 4 days of daily treatment. Further daily treatment resulted in a plateau of the cGMP reponse, up to 8 days. The results indicate that daily treatment of wild-type mice with 200 nmol/kg of Pro-CNP38 for up to 8 days do not desensitize the cGMP response.

The kinetics of the cGMP response in distal femoral cartilage after treatment of the wild-type mice with Pro-CNP38 was also investigated. Treatment of the mice once a day for two days potentiated the cGMP response to Pro-CNP38, compared with the cGMP response to a single treatment (FIG. **75**B). When the mice were treated on the first and third days, but not on the first and second days, the cGMP response after the second treatment (on the third day) was substantially similar to the cGMP response observed after a single treatment on day one (FIG. **75**B). The results suggest that dosing on consecutive days is beneficial for potentiating the cGMP response to Pro-CNP38 in this mouse study.

Activation of NPR-B in Different Tissues

To evaluate potential activation of NPR-B in different tissues by a CNP variant, wild-type male CD-1 mice were injected with 200 nmol/kg of Gly-CNP37, and the cGMP response was measured in certain tissues at different time points. Two mice were used for each treatment group. Each mouse received one subcutaneous injection of Gly-CNP37 or vehicle control. Fifteen, 30 or 60 minutes or 3 hours following the injection, the mice were deeply anesthetized and ex-sanguinated via thoracotomy and aortic cannulation. Circulating blood was removed by a PBS flush with an aortic cannula. Heart, liver, lung, kidney, ear pinna, aorta and brain were collected. All tissues were boiled in water for 5 minutes, finely dissected (including flushing of marrow from femoral cortices with PBS), weighed, cooled in liquid nitrogen, and pulverized in a BioPulverizer. The resulting powdered samples were homogenized with a Polytron in 6% pre-chilled perchloric acid and neutralized with 60% KOH. Samples were then centrifuged at 10,000 rpm and 4° C. for 5 minutes, and the supernatant was used for the assay of cGMP (Cyclic GMP Enzyme Immunoassay Kit, Cayman Chemical Company, Ann Arbor, Mich.). The results were normalized for tissue weight.

Secretion of cGMP in response to treatment with Gly-CNP37 ("CNP" in FIG. **76**) was detectable in distal femurs (cartilage and bone), femoral cortices (bone), ear pinna (cartilage), and kidney (FIGS. **76**A-D). Maximal cGMP responses in those tissues were observed 15 min after treatment. Liver, heart, lung and brain tissues did not exhibit appreciable cGMP secretion in response to Gly-CNP37 relative to vehicle control at the studied time points (FIGS. **76**E-H). The results indicate that treatment with 200 nmol/kg of Gly-CNP37 stimulated cGMP secretion in cartilage, bone and renal tissues.

Example 16

Dose Responses of CNP Variants in Monkeys

The effects of the CNP variant Pro-Gly-CNP37 on bone growth and the levels of bone growth-related biomarkers are evaluated in cynomolgus monkeys. Eight normal juvenile cynomolgus monkeys (about 2.5 years of age at the start of the on-going study) are subcutaneously injected daily with 10 or 36 μg/kg/day of Pro-Gly-CNP37 (n=4 per dose group). Four such monkeys are administered vehicle as control. The total length of treatment is 6 months. Various measures of growth plate expansion and bone growth are made by digital X-ray and magnetic resonance imaging, and by measurement of limb and body lengths externally. Blood and urine samples are collected periodically for clinical pathology and measurement of levels of Pro-Gly-CNP37 and biomarkers. Following termination of the study, gross pathology is performed and tissue samples are evaluated histologically for assessment of efficacy and safety.

Data obtained thus far in the on-going study show that both doses of Pro-Gly-CNP37 have increased growth plate width by digital X-ray (FIG. **77**), increased right and left tibia lengths by digital X-ray (FIGS. **78**A and B), increased leg length by external measurement (FIG. **79**), increased arm length by external measurement (FIG. **80**), increased body length by external measurement (FIG. **81**), and increased the serum level of alkaline phosphatase, a biomarker for bone

formation (FIG. **82**). The data demonstrate that Pro-Gly-CNP37 can stimulate bone growth in normal juvenile cynomolgus monkeys at hemodynamically acceptable doses.

Example 17

Effects of CNP Variants on Cardiovascular System in Mice

Natriuretic peptides such as CNP have been reported to affect the cardiovascular system. Wang et al. (Eur J Heart Fail. 9:548-57. 2007) describe that CNP has been shown to have a cardioprotective effect in preventing myocardial ischaemia/reperfusion injury and improving cardiac remodelling after myocardial infarction in rats. Wang demonstrated that mice overexpressing CNP have reduced incidence of cardiac hypertrophy caused by myocardial infarction. Additionally, CNP has been shown to cause endothelium-independent vasodilation (M. Honing et al., Hypertension, 37:1179-1183 (2001)) and therefore may transiently decrease blood pressure in vivo.

To assess the effects of CNP variants on the cardiovascular system, the blood pressure and heart rate in anesthetized wild-type FVB mice is studied following subcutaneous injection of the variants.

After a pilot study to define a broad dose range of cardiovascular activity, a dose-response study is conducted to examine the effects of three different dose levels of each CNP variant. Three male FVB mice aged 8 weeks comprise each treatment group. Doses are administered subcutaneously to anesthetized mice, and systolic, diastolic and mean arterial pressure (MAP), as well as heart rate, are monitored via implanted intraarterial pressure transducers.

Example 18

Formulation of CNP Variants

CNP preformulation studies were carried out to assess the stability of CNP variant Gly-wtCNP37 ("CNP38") at different pH's (pH 3, 4, 5, 6, 7 and 8) and temperatures (5° C., 25° C., and 40° C.) over time. CNP38 exhibited greater stability at pH 4-6 than at the other pH's in the studies. CNP38 was stable at 5° C. at pH 4-6, with ≥about 95% of CNP38 remaining after 15 weeks. When the temperature was raised to 25° C., at pH 4 about 85% of CNP38 remained after 15 weeks, at pH 5 about 85% remained after 15 weeks, and at pH 6 about 80% remained after 15 weeks. When the temperature was raised to 40° C., at pH 4 about 55-60% of CNP38 remained after 15 weeks, at pH 5 about 65% remained after 15 weeks, and at pH 6 about 40% remained after 15 weeks. FIG. **83** illustrates the observed plot of pseudo-first order degradation rate constant (K_{obs}) vs. pH from pH 3 to 8 and at 5° C., 25° C. and 40° C. The stability data for CNP38 in the preformulation studies suggests CNP formulations having a pH in the range from about 4 to about 6. An acidic pH (e.g., pH≤about 6) can promote the stability of a CNP variant by, e.g., minimizing or avoiding deamidation of asparagine and/or glutamine residue(s), isomerization of aspartic acid residue(s), or degradation of the CNP variant by other pathways.

CNP variants can be formulated in pharmaceutical carriers for administration to subjects affected by, e.g., bone growth conditions. In some embodiments, liquid formulations of CNP variants are formulated according to any combinations of the ingredients and their amounts or concentrations in Table 16.

TABLE 16

Ingredient Class	Ingredient	Concentration Range
Active ingredient	CNP variant	10 mg/mL ± 9.9 mg/mL
Buffering agent	Acetic acid/acetate	10 mM ± 5 mM, or pH 5 ± 1
Buffering agent	Citric acid/citrate	10 mM ± 5 mM, or pH 5 ± 1
Isotonicity-adjusting agent	NaCl	140 mM ± 20 mM
Isotonicity-adjusting agent	Sucrose	10% ± 5%
Preservative	m-Cresol	0.4% ± 0.1% or 0.2%
Preservative/anti-adsorbent	Benzyl alcohol	1.5% ± 0.5%
Stabilizer	Glycerin (glycerol)	5%-100% (neat)[1]
Stabilizer	Methionine	0.01%-0.2%
Stabilizer	Ascorbic acid/ ascorbate salt	0.1%-1%
Stabilizer	Thioglycerol	0.1%-1%
Anti-adsorbent	Polysorbate 20	0.001%-0.5%
	Polysorbate 80	0.001%-0.5%
	Benzyl alcohol	0.5%-1.5%

[1]Glycerin is used to minimize or prevent water-driven hydrolysis, deamidation, isomerization or cleavage of CNP variants. For lyophilized formulations, 4-6% or 6-20% mannitol or sucrose can be substituted for NaCl.

In certain embodiments, lyophilized formulations of CNP variants are prepared from formulations formulated according to any combinations of the ingredients and their amounts or concentrations in Table 17.

TABLE 17

Ingredient Class	Ingredient	Concentration Range
Active ingredient	CNP variant	10 mg/mL ± 9.9 mg/mL
Buffering agent	Acetic acid/acetate	10 mM ± 5 mM, or pH 5 ± 1
Buffering agent	Citric acid/citrate	10 mM ± 5 mM, or pH 5 ± 1
Isotonicity-adjusting agent/bulking agent	Sorbitol	5% ± 3%
Isotonicity-adjusting agent/bulking agent	Mannitol	5% ± 3%
Isotonicity-adjusting agent/bulking agent	Sucrose	10% ± 5%
Preservative	m-Cresol	0.4% ± 0.2%
Preservative/anti-adsorbent	Benzyl alcohol	1.5% ± 0.5%
Stabilizer	Glycerin (glycerol)	5%-100% (neat)[1]
Stabilizer	Methionine	0.01%-0.2%
Stabilizer	Ascorbic acid/ ascorbate salt	0.1%-1%
Stabilizer	Thioglycerol	0.1%-1%
Anti-adsorbent	Polysorbate 20	0.001%-0.5%
	Polysorbate 80	0.001%-0.5%
	Benzyl alcohol	0.5%-1.5%

[1]Glycerin is used to minimize or prevent water-driven hydrolysis, deamidation, isomerization or cleavage of CNP variants.

In certain embodiments, a formulation comprising a CNP variant has a pH of about 3-7, or about 3-6, or about 3.5-6.5, or about 4-6, or about 4-5, or about 4.5-5.5. In some embodiments, for pH 4-5.5 a suitable buffering agent is acetic acid/acetate (e.g., sodium acetate), and for pH 5.5-6 a suitable buffering agent is citric acid/citrate. Citric acid/citrate (e.g., sodium citrate) is also a suitable buffering agent in the range of pH 3-6 or pH 4-6. In certain embodiments, the buffering agent has a concentration in the formulation of about 2-50 mM, or about 2-40 mM, or about 2-30 mM, or about 5-30 mM, or about 2-20 mM, or about 5-20 mM, or about 5-15 mM.

To minimize or avoid deamidation of a CNP variant, the variant can be formulated in pharmaceutically acceptable organic cosolvents, such as glycerin, ethanol, and propylene

glycol. Because deamidation occurs by hydrolysis, substitution of an organic cosolvent for water minimizes contact of the CNP variant with water. The concentration of one or more organic solvents in an organic-aqueous solvent system can be, e.g., from about 10% to about 99%, or about 100% if water is not used.

Also to minimize or avoid deamidation of a CNP variant, water can be removed from the formulation by lyophilization. In some embodiments, lyophilized formulations contain any combinations of the following components:

- buffer: sodium acetate and acetic acid, or sodium citrate and citric acid;
- isotonicity/bulking agent: mannitol (e.g., 3-10%, 2-8% or 4-6%);
  sucrose (e.g., 6-20%, 5-15% or 8-12%);
- antioxidants: methionine and/or ascorbic acid with molal ratio of each antioxidant to CNP variant from about 0.1:1 to about 1:1, or from about 0.5:1 to about 5:1, or from about 1:1 to about 15:1, or from about 1:1 to about 10:1, or from about 3:1 to about 10:1.

Deamidation can also be minimized or avoided by storing a CNP composition (e.g., a liquid formulation or a lyophilized formulation) at lower temperature, such as at about 5° C., 0° C., −10° C., −20° C., −30° C., −40° C., −50° C., −60° C., −70° C., −80° C., −90° C., or −100° C.

To minimize or avoid oxidation of oxidizable residues (e.g., methionine) in a CNP variant, the variant can be formulated with one or more antioxidants. Exemplary antioxidants include, but are not limited to, methionine, ascorbic acid, and thioglycerol. Oxidation of, e.g., methionine residues can also be minimized or prevented by purging oxygen from a liquid medium (if a liquid formulation) with nitrogen or argon, and/or by purging oxygen from a container or packaging with nitrogen or argon.

In some embodiments, to minimize or prevent adsorption (e.g., adsorption of a CNP variant to plastic or glass), Polysorbate 20, Polysorbate 80 or benzyl alcohol, or a combination thereof, is added to a CNP formulation. In certain embodiments, each of the anti-adsorbent(s) is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%. Exemplary range(s) of anti-adsorbent(s) in the formulation include without limitation from about 0.001% to about 0.5% of Polysorbate 20, from about 0.001% to about 0.5% of Polysorbate 80, and/or from about 0.5% to about 1.5% of benzyl alcohol.

In certain embodiments, a liquid CNP formulation comprises, or a lyophilized CNP formulation is prepared from a formulation that comprises, (1) an acetic acid/acetate (e.g., sodium acetate) buffer having a concentration of about 30 mM±5 or 10 mM buffering agent and a pH of about 4±0.5 or 1, and (2) benzyl alcohol (e.g., as a preservative and/or anti-adsorbent) at a concentration of about 1%±0.5%, and optionally (3) sucrose at a concentration of about 10%±5%.

Example 19

Clinical Evaluation of CNP Variants

The following example provides guidance on the parameters to be used for the clinical evaluation of compositions comprising CNP22 or variants thereof in the therapeutic methods of the present disclosure. As discussed herein, CNP22 or variants thereof will be used in the treatment of disorders responsive to CNP, including disorders of the bone and vascular smooth muscle. Clinical trials will be conducted which will provide an assessment of doses of CNP22 or variants thereof for safety, pharmacokinetics, and initial response of both surrogate and defined clinical endpoints. The trial will be conducted for a minimum, but not necessarily limited to, 24 weeks to collect sufficient safety information on about 100 evaluable patients. The initial dose for the trials will vary from about 0.001 to about 1.0 mg/kg/week, or any of the doses described herein. In the event that the initial dose in this range does not produce a significant direct clinical benefit, the dose should be increased within this range or beyond this range as necessary, and maintained for an additional minimal period of, but not necessarily limited to, 24 weeks to establish safety and to evaluate efficacy further.

Measurements of safety will include adverse events, allergic reactions, complete clinical chemistry panel (including kidney and liver functions), urinalysis, and CBC with differential. In addition, other parameters relevant to clinical benefit will be monitored. The present example also includes the determination of pharmacokinetic parameters of CNP22 or variants thereof, including absorption, distribution, metabolism, excretion, and half-life and bioavailability in the blood. It is anticipated that such analyses will help relate dose to clinical response.

Methods

Patients will undergo a baseline medical history and physical exam, and a standard set of clinical laboratory tests (including CBC, Panel 20, CH50, and UA). The patients will be followed closely with weekly visits to the clinic. The patients will return to the clinic for a complete evaluation one week after completing the treatment period. Should dose escalation be required, the patients will follow the same schedule outlined above. Safety will be monitored throughout the trial.

Diagnosis and Inclusion Criteria

The patients may be male or female, with a documented diagnosis of a potentially CNP-responsive disorder. A specific example of a potentially CNP-responsive, bone-related disorder is achondroplasia, which may be confirmed by genetic testing and other evidence of an FGFR-3 mutation or dysfunction. The ideal age range of achondroplasia patients will be from infant (<1 year of age) to pre-adolescent (<13 years of age). A patient will be excluded from this study if the patient is pregnant or lactating; has received an investigational drug within 30 days prior to study enrollment; or has a medical condition, serious intercurrent illness, or other extenuating circumstance that may significantly decrease study compliance.

Safety

Therapy with CNP22 or variants thereof will be determined to be safe if no significant acute or chronic drug reactions occur during the course of the study. The longer-term administration of the drug will be determined to be safe if no significant abnormalities are observed in the clinical examinations, clinical labs, or other appropriate studies.

It has been shown that compared to wild-type CNP22, certain CNP variants of the disclosure are much more resistant to NEP degradation in vitro, have a much longer plasma half-life and bioavailability in rats, stimulate a much higher level of cGMP production in rats, and/or induce a significantly greater increase in long bone length and body length in achondroplastic mice. Furthermore, it has been shown that short duration dose regimen treatments with CNP22 are nearly as effective as continuous CNP22 treatment in reversing FGF2-induced arrest of chondrocyte growth in vitro. These results, among others described herein, demonstrate the utility of CNP variants of the disclosure in treating CNP-responsive conditions or disorders such as, e.g., bone-related disorders and vascular smooth muscle disorders.

It is understood that every embodiment of the disclosure described herein may optionally be combined with any one or more of the other embodiments described herein. Every patent literature and every non-patent literature cited herein are incorporated herein by reference in their entirety.

Numerous modifications and variations to the disclosure, as set forth in the embodiments and illustrative examples described herein, are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the disclosure.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 237

<210> SEQ ID NO 1
<211> LENGTH: 22
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<220> FEATURE:
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<223> OTHER INFORMATION: wtCNP-22
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (6)..(22)
<223> OTHER INFORMATION: Cysteines in positions 6 and 22 form cyclic domain via disulfide bond

<400> SEQUENCE: 1

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 2
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP-17

<400> SEQUENCE: 2

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
1 5 10 15

Cys

<210> SEQ ID NO 3
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP-27

<400> SEQUENCE: 3

Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 4
<211> LENGTH: 53
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:

<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP-53

<400> SEQUENCE: 4

Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu
1 5 10 15

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
20 25 30

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
35 40 45

Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 5
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with an amino acid sequence derived from a natriuretic polypeptide, a non-natriuretic polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with an amino acid sequence derived from a natriuretic polypeptide, a non-natriuretic polypeptide

<400> SEQUENCE: 5

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 6
<211> LENGTH: 22
<212> TYPE: PRT
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is modified with a synthetic or natural polymeric group or combination thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser
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<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with a synthetic or natural polymeric group or combination thereof

<400> SEQUENCE: 6

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Gly Leu Ser Xaa Gly Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 7
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 7

Gly Ala Asn Lys Lys
1 5

<210> SEQ ID NO 8
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 8

Gly Ala Asn Arg Arg
1 5

<210> SEQ ID NO 9
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 9

Gly Ala Asn Pro Arg
1 5

<210> SEQ ID NO 10
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 10

Gly Ala Asn Gln Gln
1 5

<210> SEQ ID NO 11
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 11

Gly Ala Asn Ser Ser
1 5

<210> SEQ ID NO 12
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

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<400> SEQUENCE: 12

Gly Ala Asn Arg Gln
1 5

<210> SEQ ID NO 13
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 13

Gly Ala Asn Arg Met
1 5

<210> SEQ ID NO 14
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 14

Gly Ala Asn Arg Thr
1 5

<210> SEQ ID NO 15
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 15

Gly Ala Asn Arg Ser
1 5

<210> SEQ ID NO 16
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 16

Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala
1 5 10

<210> SEQ ID NO 17
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 17

Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg
1 5 10

<210> SEQ ID NO 18
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 18

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Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg
1 5 10

<210> SEQ ID NO 19
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 19

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

<210> SEQ ID NO 20
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 20

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Arg Arg
1 5 10 15

<210> SEQ ID NO 21
<211> LENGTH: 31
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 21

Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu
1 5 10 15

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

<210> SEQ ID NO 22
<211> LENGTH: 31
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 22

Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu
1 5 10 15

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Arg Arg
20 25 30

<210> SEQ ID NO 23
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 23

Ser Leu Arg Arg Ser Ser
1 5

<210> SEQ ID NO 24
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 24

Asn Ser Phe Arg Tyr
1 5

<210> SEQ ID NO 25
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 25

Ser Pro Lys Met Val Gln Gly Ser Gly
1 5

<210> SEQ ID NO 26
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 26

Met Val Gln Gly Ser Gly
1 5

<210> SEQ ID NO 27
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 27

Lys Val Leu Arg Arg Tyr
1 5

<210> SEQ ID NO 28
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 28

Lys Val Leu Arg Arg His
1 5

<210> SEQ ID NO 29
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 29

Gly Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg
1 5 10

<210> SEQ ID NO 30
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

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<400> SEQUENCE: 30

Gly Val Pro Gln Val Ser Thr Ser Thr
1 5

<210> SEQ ID NO 31
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 31

Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln
1 5 10 15

<210> SEQ ID NO 32
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 32

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
1 5 10

<210> SEQ ID NO 33
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Amino acid extention

<400> SEQUENCE: 33

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

<210> SEQ ID NO 34
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP-22 with amino acid extention
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid
substitution thereof, any natural or unnatural amino acid, Arg,
Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (25)..(25)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid
substitution thereof, any natural or unnatural amino acid, Arg,
Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (37)..(37)
<223> OTHER INFORMATION: Cys is optionally modified with a synthetic,
natural or combination polymeric group

<400> SEQUENCE: 34

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
1 5 10 15

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Leu Ser Xaa Gly Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 35
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP22 (K4R)

<400> SEQUENCE: 35

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 36
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANRR-CNP22(K4R) - CNP27(Arg4)

<400> SEQUENCE: 36

Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 37
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANPR-CNP22(K4R) - CNP27(Pro4) - Analog CI

<400> SEQUENCE: 37

Gly Ala Asn Pro Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 38
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: ER-CNP22

<400> SEQUENCE: 38

Glu Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
1 5 10 15

Gly Ser Met Ser Gly Leu Gly Cys

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20

<210> SEQ ID NO 39
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BA - ER-CNP22(K4R)

<400> SEQUENCE: 39

Glu Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
1 5 10 15

Gly Ser Met Ser Gly Leu Gly Cys
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<210> SEQ ID NO 40
<211> LENGTH: 23
<212> TYPE: PRT
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<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: R-CNP22

<400> SEQUENCE: 40

Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 41
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AZ - R-CNP22(K4R)

<400> SEQUENCE: 41

Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 42
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP22 (K4R)(K10R)

<400> SEQUENCE: 42

Gly Leu Ser Arg Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 43

<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP-22 (Cys6-Phe7 variable bond)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH2-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -C(=O)-N(R)- where R is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -C(=O)-NH-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH2-S-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH2-S(O)n where n is 1 or 2
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH2-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH=CH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -C(=O)-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH(CN)-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -CH(OH)-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with -O-C(=O)-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Cys6-Phe7 peptide bond (-C(=O)-NH-) replaced with NHC(=O)NH-

<400> SEQUENCE: 43

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 44

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<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is 3-amino-2-phenylpropionic acid or D-Phe

<400> SEQUENCE: 44

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 45
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: CNP22 - modified Cys6
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys, homocysteine, penicillamine, 2-mercaptopropionic acid, or 3-mercaptopropionic acid.

<400> SEQUENCE: 45

Gly Leu Ser Lys Gly Xaa Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 46
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with a bone or cartilage targeting compound, a biphosphonate, polyAsp, polyGlu, osteopontin, osteocalcin, or sialoprotein peptides, polymers, PEG, carbohydrates, hydrophobic acids, or amino acids
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser, or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Xaa is Cys with peptide bond isosteres between positions 6 and 7
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, 3-amino-2-phenylpropionic acid, or N-alkylated derivatives of Phe
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val or Asn
<220> FEATURE:

<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: Xaa is Leu or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa is Ile, tBu-Gly and peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa is Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) or 2-amino-isobutyric acid (Aib)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with a bone or cartilage targeting compound, a bisphosphonate, polyAsp, polyGlu, osteopontin, osteocalcin or sialoprotein peptides, or amino acid sequences derived from non-CNP polypeptides

<400> SEQUENCE: 46

Gly Leu Ser Xaa Gly Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Gly Ser
1 5 10 15

Xaa Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 47
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with a peptide sequence containing one to five amino acids from a natriuretic or non-natriuretic polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with a peptide sequence containing one to five amino acids from a natriuretic or non-natriuretic polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid or peptidomimetic, Arg, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser, or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys or decarboxy Cys
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be

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      replaced with -CH2-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -C(=O)-N(R)- where R is methyl, ethyl, n-propyl,
      isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -C(=O)-NH-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -CH2-S-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -CH2-S(O)n where n is 1 or 2
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -CH2-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -CH=CH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -C(=O)-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -CH(CN)-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -CH(OH)-CH2-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -O-C(=O)-NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: Peptide bond between positions 6 and 7 may be
      replaced with -NHC(=O)NH-
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, 3-amino-2-phenylpropionic
      acid, or N-alkylated derivatives of Phe
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly (tBu-Gly), Val, Ser,
      Thr or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or peptide bond isosteres
      thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Arg, Gly, 6-hydroxy-norleucine,
      citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (11)..(11)
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<223> OTHER INFORMATION: Xaa is Leu or peptide isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa is Ile, tBu-Gly or peptide isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa is Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) or 2-amino-isobutyric acid (Aib)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with a synthetic bone targeting compound, a bisphosphonate, polyAsp, polyGlu, osteopontin, osteocalcin or sialoprotein peptides, or charged PEG molecules

<400> SEQUENCE: 47

Gly Leu Ser Xaa Gly Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Gly Ser
1 5 10 15

Xaa Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa is a synthetic or natural polymeric group or combination thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa is absent or may be one or more amino acids from Gly-Leu-Ser-Lys-Gly and/or substitutions at one or more of these positions
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is Lys, a conservative amino acid substitution, any natural or unnatural amino acid or peptidomimetic that does have a reactive primary amine on a side chain
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: Cys is optionally modified with a synthetic or natural polymeric group or a combination thereof

<400> SEQUENCE: 48

Xaa Xaa Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser Met Ser Gly
1 5 10 15

Leu Gly Cys

<210> SEQ ID NO 49
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES

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<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa is absent or may be a peptide fragment or water soluble polymer
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa is Cys or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, 3-amino-2-phenylpropionic acid or N-alkylated derivatives of Phe
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or N-Me-Leu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is Leu and peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Ile, tBu-Gly or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (13)..(13)
<223> OTHER INFORMATION: Xaa is Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) or 2-amino-isobutyric acid (Aib)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (16)..(16)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (18)..(18)
<223> OTHER INFORMATION: Cys is optionally modified with amino acid sequences useful in bone/cartilage targeting, polyAsp or polyGlu, osteopontin, osteocalcin, sialoprotein or sequences derived from NPPC or non-CNP polypeptides

<400> SEQUENCE: 49

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Gly Ser Xaa Ser Gly Xaa
1 5 10 15

Gly Cys

<210> SEQ ID NO 50
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with bone targeting compound, moieties to reduce renal clearance, charged PEG molecules or hydrophilic polymers
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg,

Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys or peptide isosteres between positions 6 and 7
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, 3-amino-2-phenylpropionic acid or N-alkylated derivatives of Phe
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly, Thr, Ser, Val or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: Xaa is Leu, Asn or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa is Ile, tert-butyl-Gly (tBu-Gly), Asn or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Gly, Arg, Ser or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa is Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) or 2-amino-isobutyric acid (Aib)
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser or peptide bond isoteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with bone targeting compound, moieties to reduce renal clearance, charged PEG molecules or hydrophilic polymers

<400> SEQUENCE: 50

Gly Leu Ser Xaa Gly Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Xaa Ser
1 5 10 15

Xaa Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 51
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa is Arg or Glu

<400> SEQUENCE: 51

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Xaa Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 52
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa is Arg, Gln or Ser

<400> SEQUENCE: 52

Gly Leu Ser Lys Xaa Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 53
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Ser, Asn, Arg, or Cit

<400> SEQUENCE: 53

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Xaa Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 54
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: Xaa is Ser, Arg, or Asn

<400> SEQUENCE: 54

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Xaa Leu Gly Cys
20

<210> SEQ ID NO 55
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (21)..(21)
<223> OTHER INFORMATION: Xaa is Ser, Thr, or Arg

<400> SEQUENCE: 55

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Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Xaa Cys
20

<210> SEQ ID NO 56
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog A
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: CH2NH

<400> SEQUENCE: 56

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 57
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analogs B
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa replaced with N-Me-Phe

<400> SEQUENCE: 57

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 58
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog F
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: tBu

<400> SEQUENCE: 58

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 59
<211> LENGTH: 22
<212> TYPE: PRT

-continued

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog H
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is replaced with NHCH2-CH(Phe)CO

<400> SEQUENCE: 59

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 60
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BL - CNP37

<400> SEQUENCE: 60

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 61
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CA

<400> SEQUENCE: 61

Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Gly Leu Ser
1 5 10 15

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
20 25 30

Leu Gly Cys
35

<210> SEQ ID NO 62
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CB

<400> SEQUENCE: 62

Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Gly Leu
1 5 10 15

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser

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            20                  25                  30

Gly Leu Gly Cys
        35


<210> SEQ ID NO 63
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CC

<400> SEQUENCE: 63

Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Gly Leu
1               5                   10                  15

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
            20                  25                  30

Gly Leu Gly Cys
        35


<210> SEQ ID NO 64
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANQQ-CNP22

<400> SEQUENCE: 64

Gly Ala Asn Gln Gln Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1               5                   10                  15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
            20                  25


<210> SEQ ID NO 65
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANRR-CNP22

<400> SEQUENCE: 65

Gly Ala Asn Arg Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1               5                   10                  15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
            20                  25


<210> SEQ ID NO 66
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANPR-CNP22

<400> SEQUENCE: 66

Gly Ala Asn Pro Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1               5                   10                  15
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Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
            20                  25


<210> SEQ ID NO 67
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANSS-CNP22

<400> SEQUENCE: 67

Gly Ala Asn Ser Ser Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1               5                   10                  15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
            20                  25


<210> SEQ ID NO 68
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequene
<220> FEATURE:
<223> OTHER INFORMATION: Sythetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CD
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus BNP tail
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (32)..(32)
<223> OTHER INFORMATION: N-terminus BNP tail

<400> SEQUENCE: 68

Ser Pro Lys Met Val Gln Gly Ser Gly Cys Phe Gly Leu Lys Leu Asp
1               5                   10                  15

Arg Ile Gly Ser Met Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His
            20                  25                  30


<210> SEQ ID NO 69
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANQQ-CNP22(K4R) - Analog CH

<400> SEQUENCE: 69

Gly Ala Asn Gln Gln Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1               5                   10                  15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
            20                  25


<210> SEQ ID NO 70
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: GANSS-CNP22(K4R) - Analog CG
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<400> SEQUENCE: 70

Gly Ala Asn Ser Ser Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 71
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CS

<400> SEQUENCE: 71

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Pro Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 72
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CT

<400> SEQUENCE: 72

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Gln Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 73
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CU

<400> SEQUENCE: 73

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Gln Gln
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 74
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CW

<400> SEQUENCE: 74

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Pro
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 75
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 75

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 76
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CQ

<400> SEQUENCE: 76

Gly His His Ser His Glu Gln His Pro His Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 77
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CR

<400> SEQUENCE: 77

Gly Ala His His Pro His Glu His Asp Thr His Gly Ala Asn Gln Gln
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

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<210> SEQ ID NO 78
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequencce
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CX

<400> SEQUENCE: 78

Gly His His Ser His Glu Gln His Pro His Gly Ala Asn Pro Arg Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 79
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CF

<400> SEQUENCE: 79

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gly Leu
1 5 10 15

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
20 25 30

Gly Leu Gly Cys
35

<210> SEQ ID NO 80
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CY

<400> SEQUENCE: 80

Gly Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg Gly Ala Asn Pro
1 5 10 15

Arg Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 81
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CE

<400> SEQUENCE: 81

Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Gly
1 5 10 15

-continued

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 82
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CZ

<400> SEQUENCE: 82

Phe Gly Ile Pro Met Asp Arg Ile Gly Arg Asn Pro Arg Gly Leu Ser
1 5 10 15

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
20 25 30

Leu Gly Cys
35

<210> SEQ ID NO 83
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog DA

<400> SEQUENCE: 83

Gly Lys Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly
1 5 10 15

Pro Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
20 25 30

Gly Ser Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 84
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CK

<400> SEQUENCE: 84

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 85
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

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<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CL

<400> SEQUENCE: 85

Gly Val Pro Gln Val Ser Thr Ser Thr Gly Ala Asn Gln Gln Gly Leu
1 5 10 15

Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
20 25 30

Gly Leu Gly Cys
35

<210> SEQ ID NO 86
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CM

<400> SEQUENCE: 86

Gly Gln Pro Ser Ser Ser Ser Gln Ser Thr Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 87
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CN

<400> SEQUENCE: 87

Gly Gln Thr His Ser Ser Gly Thr Gln Ser Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 88
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CO

<400> SEQUENCE: 88

Gly Ser Thr Gly Gln Trp His Ser Glu Ser Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 89
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CP

<400> SEQUENCE: 89

Gly Ser Ser Ser Ser Ser Ser Ser Ser Ser Gly Ala Asn Gln Gln Gly
1 5 10 15

Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
20 25 30

Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 90
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with bone targeting compound, a bisphosphonates, polyAsp, polyGlu, osteopontin, osteocalcin, or sialoprotein peptides, negatively charged PEG molecules or natural polymers
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys or another natural or unnatural amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is Phe or another natural or unnatural amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, a larger natural or unnatural amino acid or peptidomimetic
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with bone targeting compound, a bisphosphonates, polyAsp, polyGlu, osteopontin, osteocalcin, or sialoprotein peptides, negatively charged PEG molecules or natural polymers

<400> SEQUENCE: 90

Gly Leu Ser Lys Gly Xaa Xaa Xaa Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 91
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog J
<220> FEATURE:
<221> NAME/KEY: MOD_RES

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<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is CH2-NH
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 91

Gly Leu Ser Lys Gly Xaa Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 92
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog K
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 92

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 93
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog L
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 93

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 94
<211> LENGTH: 22

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog M
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 94

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 95
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog Z

<400> SEQUENCE: 95

Gly Leu Ser Arg Gly Cys Tyr Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 96
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AA

<400> SEQUENCE: 96

Gly Leu Ser Arg Gly Cys Phe Val Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 97
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AB

<400> SEQUENCE: 97

Gly Leu Ser Arg Gly Cys Phe Ser Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 98
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AC

<400> SEQUENCE: 98

Gly Leu Ser Arg Gly Cys Phe Thr Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 99
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AD

<400> SEQUENCE: 99

Gly Leu Ser Arg Gly Cys Phe Gly Thr Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 100
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AE

<400> SEQUENCE: 100

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Arg Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 101
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AF
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Cit

<400> SEQUENCE: 101

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Xaa Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 102
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AG

<400> SEQUENCE: 102

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Val Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 103
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AJ

<400> SEQUENCE: 103

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Val Gly Cys
20

<210> SEQ ID NO 104
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AK
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is tBu-Ala

<400> SEQUENCE: 104

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 105
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AT

<400> SEQUENCE: 105

Glu Leu Ser Glu Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 106
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AV
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus modified with pentanoic acid

<400> SEQUENCE: 106

Glu Leu Ser Glu Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 107
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog AW
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Heptanoic acid modifying N-terminus

<400> SEQUENCE: 107

Glu Leu Ser Glu Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 108
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BB
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus modified with heptanoic acid

<400> SEQUENCE: 108

Glu Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 109
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BC

<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminus modified with pentanoic acid

<400> SEQUENCE: 109

Glu Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 110
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BF
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Cit

<400> SEQUENCE: 110

Gly Leu Ser Arg Gly Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 111
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BG

<400> SEQUENCE: 111

Gly Leu Ser Arg Gly Cys Phe Gly Leu Gln Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 112
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BH

<400> SEQUENCE: 112

Gly Leu Ser Arg Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 113
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BJ

<400> SEQUENCE: 113

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Asn Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 114
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BK

<400> SEQUENCE: 114

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Ser Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 115
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog N
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)..(22)
<223> OTHER INFORMATION: All D-amino acids

<400> SEQUENCE: 115

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 116
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BD
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 116

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 117

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<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BN
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 117

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 118
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BE
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: N-Me

<400> SEQUENCE: 118

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 119
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog V
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: 3,4-dichloro

<400> SEQUENCE: 119

Gly Leu Ser Gly Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 120
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog X
<220> FEATURE:
<221> NAME/KEY: BINDING
<222> LOCATION: (7)..(7)

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<223> OTHER INFORMATION: 3-methyl

<400> SEQUENCE: 120

Gly Leu Ser Gly Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 121
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BP

<400> SEQUENCE: 121

Gly Leu Ser Arg Ser Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 122
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BO

<400> SEQUENCE: 122

Gly Leu Ser Arg Arg Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 123
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog P

<400> SEQUENCE: 123

Gly Leu Ser Gly Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 124
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BY

<400> SEQUENCE: 124

-continued

Gly Leu Ser Arg Gly Cys Phe Gly Leu Ser Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 125
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BZ

<400> SEQUENCE: 125

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Gln
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 126
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BX

<400> SEQUENCE: 126

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Asn Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 127
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BQ

<400> SEQUENCE: 127

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Ser Leu Gly Cys
20

<210> SEQ ID NO 128
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BR

<400> SEQUENCE: 128

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Arg Leu Gly Cys

20

<210> SEQ ID NO 129
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BS

<400> SEQUENCE: 129

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Arg Gly Cys
20

<210> SEQ ID NO 130
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BT

<400> SEQUENCE: 130

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Ser Cys
20

<210> SEQ ID NO 131
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BU

<400> SEQUENCE: 131

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Thr Cys
20

<210> SEQ ID NO 132
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog BW

<400> SEQUENCE: 132

Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Arg Cys
20

<210> SEQ ID NO 133

<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog CJ

<400> SEQUENCE: 133

Arg Ser Ser Cys Phe Gly Gly Arg Ile Asp Arg Ile Gly Ala Cys
1 5 10 15

<210> SEQ ID NO 134
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminal linked to CNP22 by disuccinimidyl glutarate

<400> SEQUENCE: 134

Gly Leu Ser Arg Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 135
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: N-terminal linked to CNP22 by Bis-PEO5

<400> SEQUENCE: 135

Gly Leu Ser Arg Gly Cys Phe Gly Leu Arg Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 136
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog E
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is D-Phe

<400> SEQUENCE: 136

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 137

<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: Analog G
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is 3-Cl-Phe

<400> SEQUENCE: 137

Gly Leu Ser Lys Gly Cys Xaa Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 138
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa comprises one or more amino acids selected from Gly-Leu-Ser-Lys-Gly or substitutions at one or more of these amino acids and further comprises a hydrophilic or water soluable polymer

<400> SEQUENCE: 138

Xaa Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
1 5 10 15

Gly Cys

<210> SEQ ID NO 139
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Cys is modified with a natural and/or synthetic polymer
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Cys is modified with a natural and/or synthetic polymer

<400> SEQUENCE: 139

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
1 5 10 15

Cys

<210> SEQ ID NO 140
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is modified with a natural and/or synthetic polymer

<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is modified with a natural and/or synthetic polymer

<400> SEQUENCE: 140

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 141
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Cys is modified with a natural and/or synthetic polymeric group or combination thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Cys is optionally modified with a natural and/or synthetic polymeric group or combination thereof

<400> SEQUENCE: 141

Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
1 5 10 15

Cys

<210> SEQ ID NO 142
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Xaa is optionally modified with bone- or cartilage-targeting moieties, moieties that reduce renal clearance, hydrophilic polymers, carbohydrates, hydrophobic acids and combinations thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa is absent or may comprise 1 to 5 amino acids from Gly-Leu-Ser-Lys-Gly, optionally substituted with a natural or unnatural amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is Lys, any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: Xaa is optionally modified with bone- or cartilage-targeting moieties, moieties that reduce renal clearance, hydrophilic polymers, carbohydrates, hydrophobic acids and combinations thereof

<400> SEQUENCE: 142

Xaa Xaa Cys Phe Gly Leu Xaa Leu Asp Arg Ile Gly Ser Met Ser Gly
1 5 10 15

Leu Gly Cys

<210> SEQ ID NO 143
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: Gly is optionally modified with cartilage or bone targeting compounds, a bisphosphonate, polyAsp, polyGlu, osteopontin peptides, osteocalcin peptides, sialoprotein peptides, charged PEG molecules or hydrophilic polymers
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa is Cys or peptide isosteres between positions 6 and 7
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)..(7)
<223> OTHER INFORMATION: Xaa is L-Phe, D-Phe, N-alkylated Phe, or Phe analog
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa is Gly, tert-butyl-Gly, Thr, Ser, Val or Asn
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)..(9)
<223> OTHER INFORMATION: Xaa is Leu, Ser, Thr or peptide bond isosteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (10)..(10)
<223> OTHER INFORMATION: Xaa is Lys or a conservative amino acid substitution thereof, any natural or unnatural amino acid, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa is Gly, Arg, Ser or Asn
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa is Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser or peptide bond isoteres thereof
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Cys is optionally modified with cartilage or bone targeting compounds, a bisphosphonate, polyAsp, polyGlu, osteopontin peptides, osteocalcin peptides, sialoprotein peptides,

charged PEG molecules or hydrophilic polymers

<400> SEQUENCE: 143

Gly Leu Ser Xaa Gly Xaa Xaa Xaa Xaa Xaa Xaa Asp Arg Xaa Xaa Ser
1 5 10 15

Xaa Ser Gly Xaa Gly Cys
20

<210> SEQ ID NO 144
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 144

Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 145
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 145

Pro Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 146
<211> LENGTH: 52
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 146

Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln
1 5 10 15

Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu
20 25 30

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
35 40 45

Gly Leu Gly Cys
50

<210> SEQ ID NO 147
<211> LENGTH: 51
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 147

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Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu
1               5                   10                  15

His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser
            20                  25                  30

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
        35                  40                  45

Leu Gly Cys
    50


<210> SEQ ID NO 148
<211> LENGTH: 50
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 148

Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His
1               5                   10                  15

Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys
            20                  25                  30

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
        35                  40                  45

Gly Cys
    50


<210> SEQ ID NO 149
<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 149

Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro
1               5                   10                  15

Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly
            20                  25                  30

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
        35                  40                  45

Cys


<210> SEQ ID NO 150
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 150

Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn
1               5                   10                  15

Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys
            20                  25                  30

Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
        35                  40                  45


<210> SEQ ID NO 151
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 151

Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala
1 5 10 15

Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe
20 25 30

Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40 45

<210> SEQ ID NO 152
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 152

Ser Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg
1 5 10 15

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
20 25 30

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40 45

<210> SEQ ID NO 153
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 153

Arg Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys
1 5 10 15

Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu
20 25 30

Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40 45

<210> SEQ ID NO 154
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 154

Ala Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr
1 5 10 15

Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys
20 25 30

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 155
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 155

-continued

```
Ala Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys
1               5                   10                  15

Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
            20                  25                  30

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
        35                  40


<210> SEQ ID NO 156
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 156

Trp Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly
1               5                   10                  15

Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp
            20                  25                  30

Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
        35                  40


<210> SEQ ID NO 157
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 157

Ala Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala
1               5                   10                  15

Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg
            20                  25                  30

Ile Gly Ser Met Ser Gly Leu Gly Cys
        35                  40


<210> SEQ ID NO 158
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 158

Arg Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn
1               5                   10                  15

Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
            20                  25                  30

Gly Ser Met Ser Gly Leu Gly Cys
        35                  40


<210> SEQ ID NO 159
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 159

Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys
1               5                   10                  15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
```

20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 160
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 160

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 161
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 161

Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu
1 5 10 15

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
20 25 30

Gly Leu Gly Cys
35

<210> SEQ ID NO 162
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 162

His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser
1 5 10 15

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
20 25 30

Leu Gly Cys
35

<210> SEQ ID NO 163
<211> LENGTH: 34
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 163

Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys
1 5 10 15

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys

<210> SEQ ID NO 164
<211> LENGTH: 33
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 164

Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly
1 5 10 15

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
20 25 30

Cys

<210> SEQ ID NO 165
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 165

Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys
1 5 10 15

Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 166
<211> LENGTH: 31
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 166

Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe
1 5 10 15

Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 167
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 167

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
1 5 10 15

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 168
<211> LENGTH: 29
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 168

Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu
1 5 10 15

Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys

20 25

<210> SEQ ID NO 169
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 169

Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys
1 5 10 15

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 170
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 170

Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 171
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 171

Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp
1 5 10 15

Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 172
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 172

Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg
1 5 10 15

Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 173
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 173

Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile
1 5 10 15

Gly Ser Met Ser Gly Leu Gly Cys

20

<210> SEQ ID NO 174
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 174

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 175
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 175

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
1 5 10 15

Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 176
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 176

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
1 5 10 15

Gly Leu Gly Cys
20

<210> SEQ ID NO 177
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 177

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
1 5 10 15

Leu Gly Cys

<210> SEQ ID NO 178
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 178

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
1 5 10 15

Gly Cys

<210> SEQ ID NO 179
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 179

Gly Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 180
<211> LENGTH: 53
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 180

Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu
1 5 10 15

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
20 25 30

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Asn
35 40 45

Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 181
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 181

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Asn Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 182
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 182

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Asn
20 25 30

Ser Gly Leu Gly Cys

35

<210> SEQ ID NO 183
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetic Polypeptide

<400> SEQUENCE: 183

Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Asn Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 184
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 184

Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Asn Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 185
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 185

Pro Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 186
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 186

Pro Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 187

<211> LENGTH: 34
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 187

Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys
1 5 10 15

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys

<210> SEQ ID NO 188
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 188

Pro Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 189
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 189

Pro Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys
1 5 10 15

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 190
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 190

Met Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 191
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 191

Met Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 192
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 192

Met Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 193
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 193

Met Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 194
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 194

Met Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys
1 5 10 15

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 195
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 195

Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val Pro
1 5 10 15

Arg Gly Ser His Thr Gly Asp Asp Asp Asp Lys His Met Asp

20 25 30

<210> SEQ ID NO 196
<211> LENGTH: 142
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 196

Met Val Leu Thr Lys Lys Lys Leu Gln Asp Leu Val Arg Glu Val Cys
1 5 10 15

Pro Asn Glu Gln Leu Asp Glu Asp Val Glu Glu Met Leu Leu Gln Ile
20 25 30

Ala Asp Asp Phe Ile Glu Ser Val Val Thr Ala Ala Cys Gln Leu Ala
35 40 45

Arg His Arg Lys Ser Ser Thr Leu Glu Val Lys Asp Val Gln Leu His
50 55 60

Leu Glu Arg Gln Trp Asn Met Trp Ile Met Gly Ser Ser His His His
65 70 75 80

His His His Ser Ser Gly Leu Val Pro Arg Gly Ser His Thr Gly Asp
85 90 95

Asp Asp Asp Lys His Met Asp Pro Gly Gln Glu His Pro Asn Ala Arg
100 105 110

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
115 120 125

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
130 135 140

<210> SEQ ID NO 197
<211> LENGTH: 72
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 197

Val Leu Thr Lys Lys Lys Leu Gln Asp Leu Val Arg Glu Val Cys Pro
1 5 10 15

Asn Glu Gln Leu Asp Glu Asp Val Glu Glu Met Leu Leu Gln Ile Ala
20 25 30

Asp Asp Phe Ile Glu Ser Val Val Thr Ala Ala Cys Gln Leu Ala Arg
35 40 45

His Arg Lys Ser Ser Thr Leu Glu Val Lys Asp Val Gln Leu His Leu
50 55 60

Glu Arg Gln Trp Asn Met Trp Ile
65 70

<210> SEQ ID NO 198
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 198

Pro Gly Gln Glu His Pro Asn Ala Arg
1 5

<210> SEQ ID NO 199
<211> LENGTH: 30

<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 199

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
1 5 10 15

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 200
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 200

Gly Ala Asn Lys Pro
1 5

<210> SEQ ID NO 201
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 201

Gly Ala Asn Pro Lys
1 5

<210> SEQ ID NO 202
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 202

Gly Ala Asn Pro Gln
1 5

<210> SEQ ID NO 203
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 203

Gly Ala Asn Gln Lys
1 5

<210> SEQ ID NO 204
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 204

Arg Leu Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg
1 5 10 15

Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg
20 25 30

Ile Gly Ala Met Ser Gly Leu Gly Cys

35 40

<210> SEQ ID NO 205
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 205

Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg Ser Lys
1 5 10 15

Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly
20 25 30

Ala Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 206
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 206

Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg Ser Lys Lys
1 5 10 15

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 207
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 207

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 208
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 208

Arg Leu Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg
1 5 10 15

Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg
20 25 30

Ile Gly Ala Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 209
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 209

Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg Ser Lys
1 5 10 15

Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly
20 25 30

Ala Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 210
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 210

Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg Ser Lys Lys
1 5 10 15

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 211
<211> LENGTH: 29
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 211

Phe Lys Gly Arg Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val
1 5 10 15

Lys Leu Asp Arg Ile Gly Ala Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 212
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 212

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 213
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Squalus acanthias

<400> SEQUENCE: 213

Arg Leu Leu Gln Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg
1 5 10 15

Ser Lys Lys Gly Pro Ser Arg Ser Cys Phe Gly Leu Lys Leu Asp Arg
20 25 30

Ile Gly Ala Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 214
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Squalus acanthias

<400> SEQUENCE: 214

Gly Pro Ser Arg Ser Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 215
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 215

Gly Trp Asn Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 216
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 216

Pro Met Val Ala Gly Gly Gly Cys Phe Gly Met Lys Met Asp Arg Ile
1 5 10 15

Gly Ser Ile Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 217
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 217

Gly Arg Ser Ser Met Val Gly Gly Arg Gly Cys Phe Gly Met Lys Ile
1 5 10 15

Asp Arg Ile Gly Ser Ile Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 218
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 218

Gly Gly Met Arg Ser Cys Phe Gly Val Arg Leu Glu Arg Ile Gly Ser
1 5 10 15

Phe Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 219
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 219

Gly Gly Leu Arg Ser Cys Phe Gly Val Arg Leu Ala Arg Ile Gly Ser
1 5 10 15

Phe Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 220
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 220

Gly Gly Ser Thr Ser Arg Ser Gly Cys Phe Gly His Lys Met Asp Arg
1 5 10 15

Ile Gly Thr Ile Ser Gly Met Gly Cys
20 25

<210> SEQ ID NO 221
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 221

Gly Gly Ser Ser Arg Ser Gly Cys Phe Gly His Lys Met Asp Arg Ile
1 5 10 15

Gly Thr Ile Ser Gly Met Gly Cys
20

<210> SEQ ID NO 222
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Ornithorhynchus anatinus

<400> SEQUENCE: 222

Leu Leu His Asp His Pro Asn Pro Arg Lys Tyr Lys Pro Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Thr Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 223
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Ornithorhynchus anatinus

<400> SEQUENCE: 223

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Thr Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 224
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 224

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
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<210> SEQ ID NO 225
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 225

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Gln Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 226
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 226

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1 5 10 15

Ala Ser Gly Leu Gly Cys
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<210> SEQ ID NO 227
<211> LENGTH: 23
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 227

Ser Leu Arg Arg Ser Ser Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
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Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 228
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 228

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr
20 25

<210> SEQ ID NO 229
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 229

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Gln Ser Gly Leu Gly
1 5 10 15

Cys Asn Ser Phe Arg Tyr
20

<210> SEQ ID NO 230
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetnic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 230

Ile Glu Gly Arg Xaa

-continued

1 5

<210> SEQ ID NO 231
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 231

Asp Asp Asp Asp Lys Xaa
1 5

<210> SEQ ID NO 232
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(3)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 232

Glu Xaa Xaa Tyr Xaa Gln Gly
1 5

<210> SEQ ID NO 233
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 233

Val Asp Asp Arg
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<210> SEQ ID NO 234
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 234

Gly Ser Asp Arg
1

<210> SEQ ID NO 235
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 235

Ile Thr Asp Arg
1

-continued

<210> SEQ ID NO 236
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 236

Pro Gly Asp Arg
1

<210> SEQ ID NO 237
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 237

Ala Phe Leu Gly Pro Gly Asp Arg
1 5

What is claimed is:

1. A variant of C-type natriuretic peptide (CNP) selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Gly-CNP53);

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Pro-CNP53);

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Met-CNP53)

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNS GLGC [CNP-53(M48N)].

2. A pharmaceutical composition comprising a CNP variant of claim **1**, and a pharmaceutically acceptable excipient, carrier or diluent.

3. The composition of claim **2**, which is a lyophilized formulation prepared from a formulation that comprises a citric acid/citrate buffer or an acetic acid/acetate buffer having a pH from about 4 to about 6.

4. The composition of claim **3**, wherein the lyophilized formulation is prepared from a formulation that further comprises (a) an isotonicity-adjusting agent or a bulking agent or (b) an antioxidant.

5. A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a CNP variant to a subject in need thereof, wherein the CNP variant is a CNP variant according to claim **1**, and wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

6. A method for recombinant production of a CNP variant, comprising culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant polypeptide linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant polypeptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Gly-CNP53);

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Pro-CNP53);

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Met-CNP53);

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNS GLGC [CNP-53(M48N)].

7. The method of claim **6**, wherein the cleavable peptide or protein is selected from the group consisting of histidine tags, human transcription factor TAF12, TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12(C/A & 4D/4E), TAF12(C/A & 6D/6E), TAF12(C/A & 10D/10E), ketosteroid isomerase, maltose-binding protein, β-galactosidase, glutathione-S-transferase, thioredoxin, chitin-binding domain, BMP-2, BMP-2 mutants, BMP-2(C/A), and mutants and fragments thereof.

8. The method of claim **6**, wherein the host cell is bacterial.

9. The method of claim **6**, wherein the fusion polypeptide is expressed as a soluble protein or as an inclusion body.

10. The method of claim **6**, further comprising isolating the expressed fusion polypeptide from the host cell or culture medium.

11. The method of claim **10**, further comprising contacting the isolated fusion polypeptide with a cleaving agent selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease.

12. A CNP variant produced according to the method of claim **6**, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Gly-CNP53));

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Pro-CNP53));

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Met-CNP53));

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSG
LGC [CNP-53(M48N)].

13. A method of increasing long bone growth, comprising administering a CNP variant according to claim **1** to a subject in need thereof, where the administration increases long bone growth.

14. A CNP variant according to claim **1** useful for increasing long bone growth or treating achondroplasia, hypochondroplasia, short stature, dwarfism, or homozygous achondroplasia in a subject in need thereof.

* * * * *

Exhibit 26

CAMBRIDGE
Dictionary of American English

SECOND EDITION



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strong and is having a great effect on their life: *Consumed with guilt, Charles virtually begs to be punished.*

★**consumer** /kən'su:·mər/ *n* [C] a person who buys goods or services for their own use ○ *consumer goods/spending* ○ *American consumers are becoming informed about the safety of products made for children.*

consummate <COMPLETE> /'kɑn·sə·mət, kən'sʌm·ət/ *adj* perfect; complete in every way ○ *consummate skill*

consummate /'kɑn·sə,meɪt/ *v* [T] ○ *The deal was consummated with a handshake.*

<HAVE SEX> /'kɑn·sə,meɪt/ *v* [T] *literary* to make a marriage or romantic relationship complete by having sex

consumption /kən'sʌm·ʃən/ *n* [U] **1** an amount of something that is used, or the process of using something, esp. so that there is less of it ○ *Consumption of electricity is always higher during the summer months because of air-conditioning.* **2** Consumption is also the using of goods and services in an economy, or the amount of goods and services used.

★**contact** <COMMUNICATION> /'kɑn·tækt/ *n* [U] communication with someone, or with a group or organization ○ *Have you kept in contact with your friends from college?* ○ *The pilot was always in contact with an air traffic controller.*

★**contact** /'kɑn·tækt/ *v* [T] ○ *I tried to contact him at his office but he was out to lunch.*

USAGE

contact

Remember to use the preposition **with** when you use the phrases **make/be in/keep in/lose contact with** *someone.*

When you live abroad it's important not to lose contact with friends and family at home.

~~*When you live abroad it's important not to lose contact to friends and family at home.*~~

★<TOUCH> /'kɑn·tækt/ *n* [U] the touching of two objects or surfaces ○ *Don't let that glue come into contact with your skin.*

<HELPFUL PERSON> /'kɑn·tækt/ *n* [C] **1** a person whom you know and who may be able to help you in a useful way, esp. because of their influence with other people or their knowledge ○ *He tried to use his contacts to get a better job in advertising.* **2** A contact is also a person you meet: *My face-to-face contacts outside of the office had been mostly hotel clerks, policemen, and waitresses.*

contact lens *n* [C] a small, round, curved piece of transparent material, esp. plastic, that fits on the surface of an eye to improve sight ○ *I need new contact lenses.*

contagious /kən'teɪ·dʒəs/ *adj* **1** (of a disease) able to be caught by touching someone with the disease or something they have touched or worn, or (of a person) having this type of disease ○ *a highly contagious strain of flu* **2** *fig.* Contagious also means moving easily from one person to another: *The mood was contagious, and soon everyone was laughing.*

contain <HAVE INSIDE> /kən'teɪn/ *v* [T] (of an object or area) to have an amount of something inside or within it ○ *How much liquid does this bottle contain?* ○ *Each large crate contains 12 boxes.*

container /kən'teɪ·nər/ *n* [C] a hollow object, such as a box or a bottle, which can be used for holding something esp. for the purposes of carrying or storing ○ *plastic milk containers*

USAGE

contain or include?

Use **contain** to talk about objects that have something else inside them.

This folder contains important letters.

This soup contains garlic and onions.

Use **include** to say that something or someone is a part of something else but that there are other parts as well.

The team includes two new players.

The price of the ticket includes insurance and tax.

<INCLUDE> /kən'teɪn/ *v* [T] to have as a part, or be equal to; include ○ *The information contained on forms would be kept strictly confidential.* ○ *Each food serving contains 95 calories.* ○ *You could retrieve all files that contained certain key words.*

<CONTROL> /kən'teɪn/ *v* [T] to keep within limits; not to allow to spread ○ *Medical teams were scrambling to contain the illness that has already killed thousands in Latin America.*

containment /kən'teɪn·mənt/ *n* [U] ○ *I think there are some solid things that can be done in terms of cost containment* (= limiting the cost).

contaminant /kən'tæm·ə·nənt/ *n* [C] a substance that pollutes, spoils, or poisons something ○ *High levels of contaminants have been found in the groundwater, causing concerns about possible health risks to nearby neighborhoods.*

contaminate /kən'tæm·ə,neɪt/ *v* [T] to make something less pure or make it poisonous ○ *The disease can be caused by a variety of viruses, bacteria, and other small organisms that contaminate food or water.*

contaminated /kən'tæm·ə,neɪţ·əd/ *adj* ○ *contaminated water*

contamination /kən,tæm·ə'neɪ·ʃən/ *n* [U]

contemplate /'kɑnt·əm,pleɪt/ *v* [I/T] to spend time considering a possible future action, or to consider one particular thing for a long time in a serious way ○ [T] *The owner of the team contemplated moving his football club to another city.* ○ [T] *Sharon is contemplating going to graduate school.*

contemplation /,kɑnt·əm'pleɪ·ʃən/ *n* [U]

Exhibit 27

Concise Oxford English Dictionary

ELEVENTH EDITION, REVISED

Edited by
Catherine Soanes
Angus Stevenson

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consultancy ■ n. (pl. **consultancies**) a professional practice giving expert advice in a particular field.

consultant ■ n. **1** a person who provides expert advice professionally. **2** Brit. a hospital doctor of senior rank.

consultation /kɒnsəl'teɪʃ(ə)n/ ■ n. the action or process of formally consulting or discussing. ➤ a meeting with an expert or professional in order to seek advice.

consumable ■ adj. intended to be used up and then replaced. ■ n. a consumable commodity.

consume ■ v. **1** eat, drink, or ingest. ➤ use up. ➤ (especially of a fire) completely destroy. **2** buy (goods or services) for personal use. **3** (of a feeling) absorb all of the attention and energy of.
– DERIVATIVES **consuming** adj. **consumingly** adv.
– ORIGIN ME: from L. *consumere*, from *con-* 'altogether' + *sumere* 'take up'.

consumer ■ n. **1** a person or thing that eats or uses something. **2** a person who buys goods and services for personal use: [as modifier] *consumer demand.*

consumer durables ■ pl. n. products expected to have a long useful life.

consumerism ■ n. **1** the protection or promotion of the interests of consumers. **2** the preoccupation of society with the acquisition of goods.
– DERIVATIVES **consumerist** adj. & n. **consumeristic** adj.

consummate ■ v. /'kɒnsjʊmeɪt, -sə-/ **1** make (a marriage or relationship) complete by having sexual intercourse. **2** complete (a transaction). ■ adj. /kən'sʌmət, 'kɒnsʌmət/ showing great skill and flair.
– DERIVATIVES **consummately** adv. **consummation** n. **consummator** n.
– ORIGIN ME: from L. *consummat-*, *consummare* 'bring to completion', from *con-* 'altogether' + *summa* 'sum total', fem. of *summus* 'highest, supreme'.

consumption ■ n. **1** the action or process of consuming. ➤ an amount consumed. **2** dated a wasting disease, especially tuberculosis.
– DERIVATIVES **consumptive** adj. & n. (dated). **consumptively** adv. (dated).
– ORIGIN ME: from L. *consumptio(n-)*, from *consumere* (see **CONSUME**).

cont. ■ abbrev. **1** contents. **2** continued.

contact ■ n. /'kɒntakt/ **1** the state or condition of physical touching. ➤ [as modifier] caused by or operating through physical touch: *contact dermatitis.* **2** the action of communicating or meeting. ➤ a communication or relationship. ➤ a person whom one may ask for information or assistance. ➤ a person who has been associated with a patient with a contagious disease. **3** a connection for the passage of an electric current from one thing to another. **4** (**contacts**) contact lenses. ■ v. /'kɒntakt, kən'takt/ **1** get in touch or communication with. **2** touch.
– DERIVATIVES **contactable** adj.
– ORIGIN C17: from L. *contactus*, from *contact-*, *contingere* 'touch, grasp, border on', from *con-* 'together with' + *tangere* 'to touch'.

contactee ■ n. a person who claims to have been contacted by alien beings.

contact flight (also **contact flying**) ■ n. navigation of an aircraft by the observation of landmarks.

contact lens ■ n. a thin plastic lens placed directly on the surface of the eye to correct visual defects.

contact print ■ n. a photographic print made by placing a negative directly on to sensitized paper, glass, or film and illuminating it.

contact sheet ■ n. a sheet of contact prints.

contact sport ■ n. a sport in which the participants necessarily come into bodily contact with one another.

contagion /kən'teɪdʒ(ə)n/ ■ n. the communication of disease from one person or organism to another by close contact. ➤ dated a disease spread by close contact.
– ORIGIN ME: from L. *contagio(n-)*, from *con-* 'together with' + the base of *tangere* 'to touch'.

contagious ■ adj. **1** (of a disease) spread by direct or indirect contact of people or organisms. ➤ having a contagious disease. **2** (of an emotion, attitude, etc.) likely to spread to and affect others: *her enthusiasm is contagious.*
– DERIVATIVES **contagiously** adv. **contagiousness** n.

contagious abortion ■ n. a type of brucellosis which causes spontaneous abortion in cattle.

contain ■ v. **1** have or hold within. **2** control or restrain. ➤ prevent (a problem) from becoming worse. **3** (of a number) be divisible by (a factor) without a remainder.
– DERIVATIVES **containable** adj.
– ORIGIN ME: from OFr. *contenir*, from L. *continere*, from *con-* 'altogether' + *tenere* 'to hold'.

container ■ n. an object for holding or transporting something. ➤ a large standard-sized metal box for the transport of goods by road, rail, sea, or air.

containerize or **containerise** ■ v. [usu. as adj. **containerized**] pack into or transport by container.
– DERIVATIVES **containerization** n.

containment ■ n. the action of keeping something harmful under control. ➤ the action of preventing the expansion of a hostile country or influence.

contaminate ■ v. make (something) impure by exposure to or addition of a poisonous or polluting substance.
– DERIVATIVES **contaminant** n. **contamination** n. **contaminator** n.
– ORIGIN ME: from L. *contaminat-*, *contaminare* 'make impure', from *contamen* 'contact, pollution', from *con-* 'together with' + the base of *tangere* 'to touch'.

contango /kən'taŋgəʊ/ ■ n. Brit. Stock Exchange the normal situation in which the spot or cash price of a commodity is lower than the forward price. ➤ historical a percentage paid by a buyer of stock to postpone transfer to a future settling day.
– ORIGIN C19: prob. an arbitrary formation on the pattern of Latin verbs ending in *-o* in the first person sing., perh. with the idea 'I make contingent' (see **CONTINGENT**).

Conté /'kɒnteɪ/ ■ n. a kind of hard grease-free crayon used for artwork.
– ORIGIN C19: named after the French inventor Nicolas J. *Conté.*

conte /kɒnt/ ■ n. a short story as a form of literary composition. ➤ a medieval narrative tale.
– ORIGIN Fr., based on L. *computare* 'reckon, sum up'.

contemn /kən'tɛm/ ■ v. archaic treat or regard with contempt.
– DERIVATIVES **contemner** /-'tɛmə, -'tɛmnə/ n.
– ORIGIN ME: from L. *contemnere*, from *con-* (expressing intensive force) + *temnere* 'despise'.

contemplate /'kɒntɛmpleɪt, -təm-/ ■ v. look at thoughtfully. ➤ think about. ➤ think profoundly and at length. ➤ have as a probable intention.
– DERIVATIVES **contemplator** n.
– ORIGIN C16: from L. *contemplat-*, *contemplari* 'survey, observe, contemplate', based on *templum* 'place for observation'.

contemplation ■ n. **1** the action of contemplating. **2** religious meditation. ➤ (in Christian spirituality) a form of prayer in which a person seeks a direct experience of the divine.

contemplative /kən'tɛmplətɪv/ ■ adj. expressing or involving contemplation. ■ n. a person whose life is devoted to prayer, especially in a monastery or convent.
– DERIVATIVES **contemplatively** adv.

contemporaneous /kən,tɛmpə'reɪnɪəs, kɒn-/ ■ adj. existing at or occurring in the same period of time.
– DERIVATIVES **contemporaneity** /-'ni:ɪti, -'neɪɪti/ n. **contemporaneously** adv. **contemporaneousness** n.
– ORIGIN C17: from L., from *con-* 'together with' + *temporaneus* (from *tempus, tempor-* 'time') + **-ous**.

contemporary /kən'tɛmp(ə)r(ə)ri/ ■ adj. **1** living, occurring, or originating at the same time. **2** belonging to or occurring in the present. ➤ modern in style or design. ■ n. (pl. **contemporaries**) a person or thing existing at the same time as another. ➤ a person of roughly the same age as another.
– DERIVATIVES **contemporarily** adv. **contemporariness** n.
– ORIGIN C17: from med. L. *contemporarius*, from *con-* 'together with' + *tempus, tempor-* 'time' (on the pattern of L. *contemporaneus* and late L. *contemporalis*).

CERTIFICATE OF SERVICE

I, hereby certify that on this day, February 5, 2026 a true and correct copy of the foregoing **PUBLIC VERSION OF RESPONDENTS' OPENING CLAIM CONSTRUCTION BRIEF** was filed and served upon the following as indicated:

The Honorable Lisa R. Barton **Secretary to the Commission** U.S. International Trade Commission 500 E Street, S.W., Room 112 Washington, DC 20436	☒ Via EDIS ☐ Via Hand Delivery ☐ Via First Class Mail ☐ Via Electronic Mail ☐ Via Overnight Courier
The Honorable Clark S. Cheney **Chief Administrative Law Judge** U.S. International Trade Commission 500 E Street, S.W., Room 112 Washington, DC 20436 Cheney1447@usitc.gov	☐ Via Hand Delivery ☐ Via First Class Mail ☒ Via Electronic Mail ☒ Via Box
Jennifer Dienes, Esq. **Office of Unfair Import Investigations** U.S. International Trade Commission 500 E Street, S.W., Room 112 Washington, DC 20436 jennifer.dienes@usitc.gov anne.goalwin@usitc.gov	☐ Via Hand Delivery ☐ Via First Class Mail ☒ Via Electronic Mail ☒ Via Box
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/s/ Russell W. Warnick
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