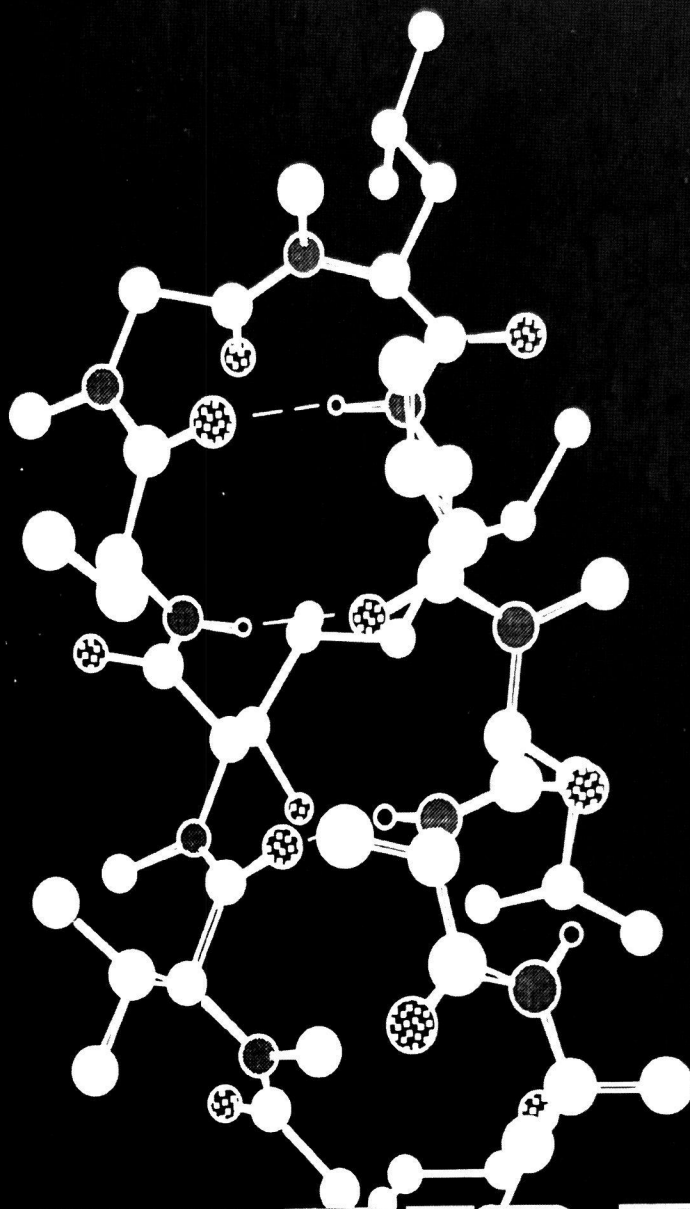




PEPTIDES

Synthesis, Structures,
and Applications

Edited by
BERND GUTTE

Peptides

Synthesis, Structures, and Applications

Edited by

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Cover art: Conformation of cyclosporin A observed in the crystal.
See Chapter 5 by Marshall *et al.*

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Contents

Contributors xi

Preface xiii

1

The History of Peptide Chemistry

Theodor Wieland

I. Introduction	1
II. Early Peptide Syntheses	3
III. A New Era in Peptide Chemistry	12
IV. Classic Peptide Syntheses	26
V. Conclusion	34
References	34

2

Amide Formation, Deprotection, and Disulfide Formation in Peptide Synthesis

Yoshiaki Kiso and Haruaki Yajima

I. Amide-Forming Reactions	40
II. Deprotection	53
III. Disulfide Bond Formation	61
Abbreviations	82
References	84

3

Solid-Phase Peptide Synthesis

Bruce Merrifield

I. Introduction	94
II. The Solid Support	94

III. Handles, Linkers, and Spacers	103
IV. Protecting Group Strategy	114
V. The Coupling Reaction	127
VI. Cause and Correction of Incomplete Coupling Reactions	137
VII. The Cleavage Reaction	143
VIII. Solid-Phase Synthesis of Peptides and Proteins	146
Abbreviations	156
References	159

4

α -Helix Formation by Peptides in Water

J. Martin Scholtz and Robert L. Baldwin

I. Introduction	171
II. Methods of Studying Helix Formation	172
III. Models for Helix-Coil Transition	175
IV. Helix Propensities	178
V. Side-Chain Interactions	183
VI. Capping Interactions	187
VII. Protein Helices	188
References	191

5

Peptide Conformation: Stability and Dynamics

Garland R. Marshall, Denise D. Beusen, and Gregory V. Nikiforovich

I. Introduction	193
II. Theoretical Studies	195
III. Experimental Analysis	209
IV. Goal-Directed Modifications of Conformational Flexibility	218
V. Examples of Experimental and Computational Techniques Applied to the Study of Peptide Conformational Stability and Dynamics	225
References	235

6

**Structure–Function Studies of Peptide Hormones:
An Overview***Victor J. Hruby and Dinesh Patel*

I. Introduction	247
II. Mammalian Peptide Hormones	248
III. Use of Classic Structure–Function Studies: General Considerations	250
IV. Use of Conformational Constraints: General Considerations	252
V. Selected Examples to Illustrate Approaches	257
VI. Summary and Conclusions	277
References	279

7

Neuropeptides: Peptide and Nonpeptide Analogs*Andrzej W. Lipkowski and Daniel B. Carr*

I. Biosynthesis of Neuropeptides	288
II. Neuropeptide Receptors	289
III. Opioids	290
IV. Tachykinins	297
V. Angiotensin II	301
VI. Oxytocin and Vasopressin	305
VII. Cholecystokinin	307
VIII. Conclusions	310
References	311

8

**Reversible Inhibitors of Serine Proteinases:
*Naturally Occurring Miniproteins, Semisynthetic Variants,
Recombinant Homologs, and Synthetic Peptides****Herbert R. Wenzel and Harald Tschesche*

I. Introduction	322
II. Serine Proteinase–Inhibitor Interactions	323
III. Naturally Occurring Proteinase Inhibitors	325

IV. Semisynthetic Proteinase Inhibitors	336
V. Proteinase Inhibitors by Genetic Engineering	343
VI. Chemically Synthesized Proteinase Inhibitors and Fragments	348
VII. Concluding Remarks and Outlook	354
References	355

9

Design of Polypeptides

Bernd Gutte and Stephan Klauser

I. Introduction	363
II. Recent Design Activities	366
III. Polypeptide Design: <i>Quo Vadis?</i>	389
References	390

10

Soluble Chemical Combinatorial Libraries: Current Capabilities and Future Possibilities

Richard A. Houghten

I. Introduction	396
II. Classes and Applications of Soluble Combinatorial Libraries of Peptides	401
III. Applications of Soluble Peptide Libraries	404
IV. Conclusion	414
References	415

11

Epitope Mapping with Peptides

Hans Rudolf Bosshard

I. Introduction	419
II. The Nature of Epitopes	420
III. Overview of Noncrystallographic Mapping Procedures	425
IV. Cross-Reactivity Studies between Proteins and Peptides Using Antiprotein Antibodies	427
V. Cross-Reactivity Studies between Proteins and Peptides Using Antipeptide Antibodies	444

Contents	ix
VI. Concluding Remarks	450
References	451
 12	
Synthesis and Applications of Branched Peptides in Immunological Methods and Vaccines	
<i>James P. Tam</i>	
I. Introduction	456
II. Types of Branched Peptides	456
III. Design and Physical Characteristics of Multiple Antigen Peptides	458
IV. Multiple Antigen Peptide Immunogenicity	460
V. Application of Multiple Antigen Peptides in Vaccines	469
VI. Other Applications	474
VII. Preparation of Multiple Antigen Peptides	477
References	493
 <i>Index</i>	 501

Amide Formation, Deprotection, and Disulfide Formation in Peptide Synthesis

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-
- I. Amide-Forming Reactions
 - A. Acid Chloride and Fluoride Methods
 - B. Active Ester Methods
 - 1. Phenyl Esters
 - 2. *N*-Hydroxylamine Esters
 - 3. Bifunctional Active Esters
 - C. Unsymmetrical Anhydride Methods
 - 1. Mixed Anhydride Method
 - 2. Phosphoric Mixed Anhydride Methods
 - D. Carbodiimide Methods
 - 1. DCC-Additive Methods
 - 2. Fragment Condensation at Glycine or Proline Residues
 - E. Azide Methods
 - 1. Protected Hydrazide Methods
 - 2. Azide Method Using Diphenylphosphorylazide
 - F. Phosphonium and Uronium Salt Type Coupling Reagents
 - G. Other Amide-Forming Reactions
 - II. Deprotection
 - A. Deprotection of α -Amino Protecting Groups
 - 1. Mild Acids
 - 2. Alkaline Conditions
 - B. Final Deprotection
 - 1. *tert*-Butoxycarbonyl/Benzyl
 - 2. 9-Fluorenylmethoxycarbonyl/*tert*-Butyl
 - 3. Other Strategies

III. Disulfide Bond Formation

A. Reduction-Reoxidation of Disulfide Bond-Containing Peptides and Proteins

1. Examination of Oxidation Conditions
2. Formation of Isomers

B. Disulfide Formation by Air Oxidation

1. Disulfide Formation by One-Step Deprotection
2. Disulfide Formation by Two-Step Deprotection

C. Disulfide Formation by Iodine Oxidation

D. Stepwise Disulfide Formation

1. Combination of 4-Methylbenzyl and Acetamidomethyl Groups
2. Combination of Trityl and Acetamidomethyl Groups
3. Combination of 3-Nitro-2-pyridosulphenyl and Acetamidomethyl Groups

E. Disulfide Formation by Silyl Chloride-Sulfoxide System

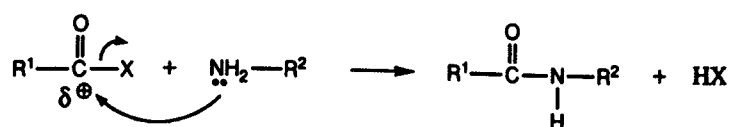
1. Disulfide Bond Formation Using Silyl Chloride-Sulfoxide System
2. Possible Mechanism
3. Synthesis of Human Brain Natriuretic Peptide (hBNP)
4. Directed Formation of Double Intrachain Disulfides
5. Directed Formation of Double Interchain Disulfides
6. Regioselective Formation of the Three Disulfide Bonds of Human Insulin

Abbreviations

References

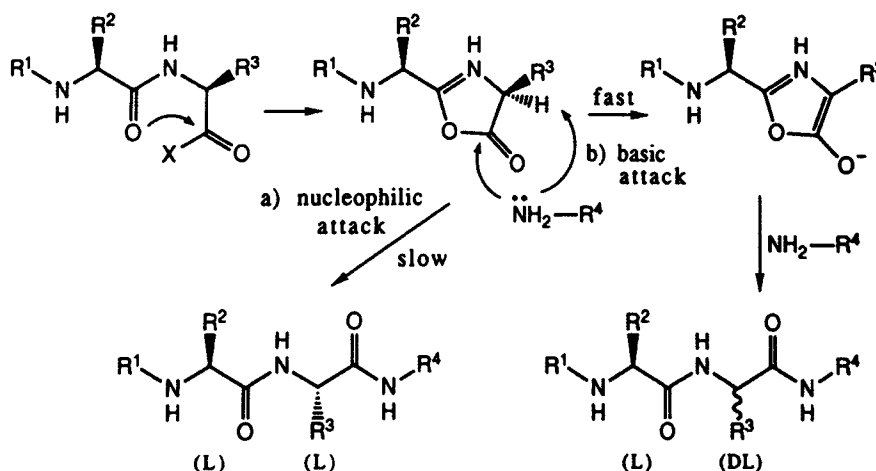
I. Amide-Forming Reactions

The amide-forming reaction, or coupling reaction, is one of the most important steps in peptide synthesis. Because harsh chemical conditions can damage complex peptides, appropriate **activation** is required to perform the coupling under mild conditions. Theoretically, two types of reactions are available, namely, amino activation and carboxyl activation. The former methods, however, have rarely been applied to practical peptide synthesis because of the drastic reaction conditions and racemization. Carboxyl activation has been used in almost all the more recent peptide couplings. The general mechanism of carboxyl activation is illustrated in Scheme 1, where X represents any kind of activating group or atom.



Scheme 1 Peptide bond formation by carboxyl activation.

There are two possible synthetic strategies: **stepwise elongation** and **fragment condensation**. A synthetic scheme which employs either or both methods is elaborated according to the size and nature of the peptide to be synthesized. In stepwise elongation, each amino acid bearing an N^α -amino protecting group is activated and coupled to the carboxyl-terminal amino acid one by one. This method is usually applied to the synthesis of relatively small peptides or peptide fragments of a larger peptide. Fragment condensation is used to construct a larger peptide from two peptide fragments. The method, however, is often accompanied by racemization unless appropriate care is taken. Antonovics and Young (1967) studied in detail the coupling reactions which proceeded via azlactone (oxazolone) formation after carboxyl activation (Scheme 2). From this result, it is clear that the carboxyl activation on an amino acid residue other than glycine or proline produces a racemized peptide unless means to inhibit racemization are provided.



Scheme 2 Racemization mechanism during fragment condensation.

Stepwise elongation and fragment condensation methods have been developed to circumvent the inevitable racemization. The latter methods, including the useful azide method, require distinctive practical considerations.

A. Acid Chloride and Fluoride Methods

The acid chloride method was first introduced by E. Fischer (1903). Most acid chlorides are prepared by treating amino acids with PCl_5 , PCl_3 , SOCl_2 (Pizey, 1974; Matsuda *et al.*, 1985), or $(\text{COCl})_2$. Any side-chain hydroxyl, thiol, as well as amino groups should be protected during coupling. The carboxyl

group, however, is activated so strongly that some side reactions limit the application of this method.

Acid chloride methods have been used efficiently in coupling of amino acids bearing N^α-protecting groups such as 9-fluorenylmethoxycarbonyl (Fmoc; Carpino *et al.*, 1986) and 2-(triphenylphosphino)ethyloxycarbonyl (Peoc; Kunz and Bechtolsheimer, 1982) which are stable under acidic conditions and cleavable under alkaline conditions. The stability of the protecting groups to acidic conditions made it possible to survive conversion to an acid chloride.

Amino acid fluorides used for solution and solid-phase syntheses are obtained by treatment with cyanuric fluoride (Bertho *et al.*, 1991; Carpino *et al.*, 1991) under mild conditions.

B. Active Ester Methods

The characteristics of the active ester method are as follows: (i) Two pathways of aminolysis are possible in the mixed anhydride method, whereas only one is found in the active ester method. (ii) The active ester can be stored. (iii) The ester can be coupled with an amine component that has free carboxyl groups. (iv) The method is especially useful for the synthesis of glutamine- and/or asparagine-containing peptides (Bodanszky and du Vigneaud, 1959). The side product generated on carboxyl activation, a nitrile derivative, can be eliminated by purification.

Active esters are usually prepared by the dicyclohexylcarbodiimide (DCC) or mixed anhydride method from N-protected amino acids and alcohol. Transesterification methods using trifluoroacetate (Sakakibara and Inukai, 1965; Rzeszotarska and Vlasov, 1967) and trichloroacetate (Fujino and Hatanaka, 1968) have also been reported.

1. Phenyl Esters

Generally, phenols which have electron-withdrawing substituent in the ortho or para position can be used as active esters (Wieland and Jaenicke, 1956; Wieland *et al.*, 1962). In practice, however, *p*-nitrophenol (Bodanszky, 1955; Bodanszky *et al.*, 1957), 2,4,5-trichlorophenol (Pless and Boissonnas, 1963a,b,c; Guttmann *et al.*, 1965; Strumer *et al.*, 1965; Huguenin, 1964), pentachlorophenol (Kovacs and Ceprini, 1965; Kovacs *et al.*, 1967a; Kupryszewski and Formela, 1961), and pentafluorophenol (Kovacs *et al.*, 1967b) are widely used because of their easy availability.

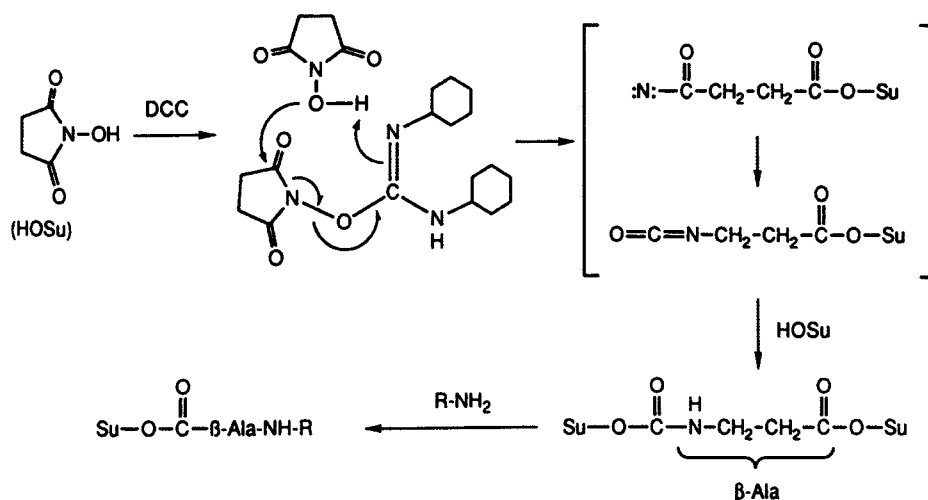
After the coupling reaction with a phenol-type active ester, the liberated acidic phenol must be removed by an appropriate method such as washing with aqueous sodium carbonate or recrystallization. If the desired peptide product is an oil, the phenol cannot be removed completely. Water-soluble active esters, such as 4-hydroxyphenyldimethylsulfonium methylsulfate (Kouge *et al.*, 1986; 1987), overcome these difficulties not only by allowing easy removal of the by-products, but also by undergoing the reaction in aqueous sol-

vents. It is noteworthy that an active ester acylates the phenolic hydroxyl group on tyrosine in the presence of a base (Agarwal *et al.*, 1969; Ramachandran and Li, 1963).

2. *N*-Hydroxylamine Esters

A series of *N*-hydroxylamine-type active esters represented by *N*-hydroxy-succinimide (HOSu) ester (Anderson *et al.*, 1963; 1964) have been described. These types of active esters are regarded as bifunctional active esters which rarely induce racemization of the α -carbon atom. In contrast to the above phenol esters, these esters eliminate *N*-hydroxylamine after coupling, which is easily soluble in water and thus removable by simple washing.

HOSu esters are prepared by coupling *N*-protected amino acids and HOSu by DCC. An undesired side reaction was reported to take place during the preparation (Gross and Bilk, 1968; Weygand *et al.*, 1968): the binding of DCC and HOSu produces β -alanine via Lossen rearrangement (Scheme 3). *N*-Hydroxy-5-norbornene-2,3-dicarboximide (HONB, 1 in Fig. 1) introduced by Fujino *et al.* (1974), rarely undergoes such side reactions because of its rigid structure.



Scheme 3 Side reaction on reacting HOSu with DCC.

N-Hydroxybenzotriazole (HOBt, 2 in Fig. 1; König and Geiger, 1970a,b) esters are widely used in peptide synthesis and are prepared using DCC. The racemization-suppressing effect and coupling accelerating effect of HOBt are excellent. Many groups have reported the reaction mechanism involving HOBt ester (Horiki, 1977; König and Geiger, 1973). The effects of HOBt as an additive are described in a later section (D.1.). 3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOObt, 3 in Fig. 1; König and Geiger, 1970c) is a reagent superior to HOBt with regard to racemization. However, HOObt esters have the disadvantage of undergoing Lossen rearrangement.

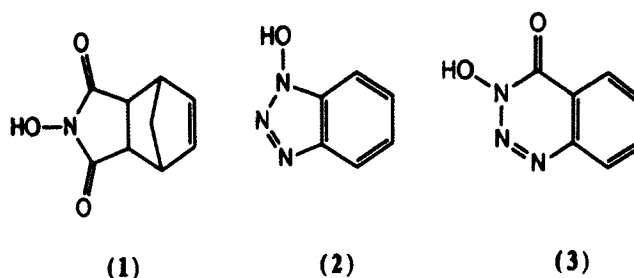


Figure 1 Structures of HONB (1), HOBt (2), and HOOBt (3).

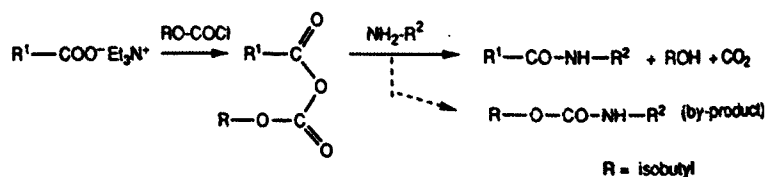
3. Bifunctional Active Esters

Various bifunctional active esters such as hydroxyquinoline (HOQ, 4 in Fig. 2; Jakubke, 1965; Jakubke and Voigt, 1966a,b), catechol (5 in Fig. 2, Lloyd and Young, 1968, 1971), and hydroxypyridine (6 in Fig. 2; Taschner *et al.*, 1965a,b), have been developed as racemization-free reagents for peptide fragment coupling. They can, of course, be applied to coupling by stepwise elongation and are readily removable after the reaction, taking advantage of their bifunctionality.

C. Unsymmetrical Anhydride Methods

1. Mixed Anhydride Method

The mixed anhydride method of peptide bond formation involves aminolysis of an anhydride consisting of an N-protected amino acid and another acid (Albertson, 1962; Meienhofer, 1979). The carboxylic-carboxylic mixed anhydride method and carbonic-carboxylic mixed anhydride methods were developed. Isobutylchloroformate (Vaughan and Osato, 1951) is commonly used for the preparation of carbonic-carboxylic mixed anhydrides (Scheme 4).



Scheme 4 Mixed anhydride method using isobutylchloroformate.

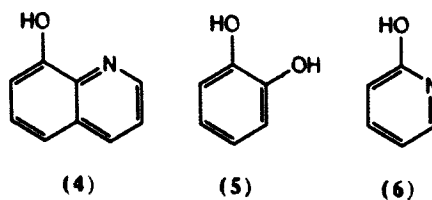
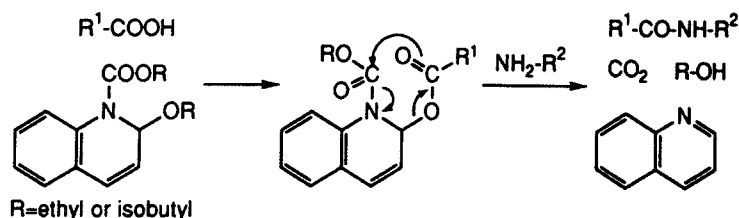


Figure 2 Structures of HOQ (4), catechol (5), and hydroxypyridine (6).

In mixed anhydride methods, the amide-forming reaction is complete within several minutes in an ice bath. The side-chain functional groups in the carboxyl component should be protected. The carboxyl groups in the amine component could be either in ester form or protected tentatively with a tertiary amine. In the latter case, the reaction can be performed in water-containing solvents, but the product should be carefully separated because of its acidity.

As a side reaction in mixed anhydride methods, urethane formation occurs in the amine component (Albertson, 1962; Wieland *et al.*, 1962; Wieland and Stimming, 1953), but it can be neglected under usual experimental conditions. Addition of HOBt to the reaction mixture before the amine component is added reduces urethane formation (Prasad *et al.*, 1985). Also, when Z-Gly (where Z represents the benzyloxycarbonyl group) is used as the carboxyl component, attention must be paid to the spontaneous formation of acylimide (Kopple and Renik, 1958; Schellenberg and Ullrich, 1959).

Mixed anhydrides are also obtained by the reaction of amino acids and dihydroquinoline derivatives. On reaction with a carboxyl component, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; Belleau and Malek, 1968) or 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline (IIDQ; Kiso and Yajima, 1972; Kiso *et al.*, 1973) is converted via a six-membered ring intermediate to quinoline and a carbonic-carboxylic mixed anhydride which immediately reacts with an amine component to give a peptide in good yield (Scheme 5). The by-products are carbon dioxide and quinoline as long as undesired urethane formation is negligible.



Scheme 5 Mixed anhydride method using EEDQ or IIDQ.

2. Phosphoric Mixed Anhydride Methods

The mixed anhydrides of amino acid and phosphoric acid are also useful for formation of the peptide bond. Many kinds of phosphoric reagents, for example, $(\text{PhO})_2\text{P}(\text{O})\text{Cl}$ (Albertson, 1962; Meienhofer, 1979) and $\text{Me}_2\text{P}(\text{S})\text{Cl}$ (Ueki *et al.*, 1979; 1988; Ueki and Inazu, 1982), have been developed. In this category, 3,3'-(chlorophosphoryl)bis(1,3-oxazolidin-2-one) (BOP-Cl, 7 in Fig. 3; Meseguer *et al.*, 1980; Cabre and Palomo, 1984; Omodei-Sale *et al.*, 1984) is particularly useful for coupling of imino acids (Van Der Auwera and Anteunis, 1987; Tung *et al.*, 1986) such as *N*-methyl amino acids (Tung and Rich, 1985) because of the strong reactivity and selectivity toward the amine component.

Norborn-5-ene-2,3-dicarboximido diphenyl phosphate (NDPP, 8 in Fig. 3) is a reagent for the so-called active ester-type mixed anhydride method (Kiso *et*

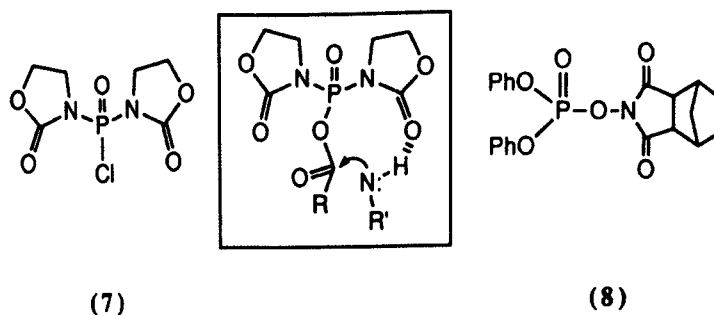
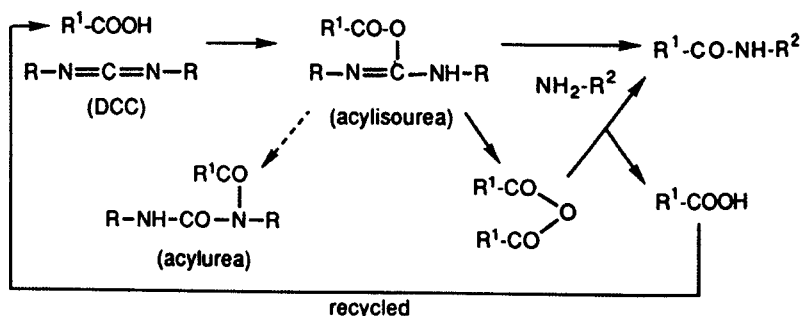


Figure 3 Structures of BOP-Cl (7) and NDPP (8).

al., 1980a). In the reaction of NDPP and an amino acid, the HONB ester is formed via a carboxylic-phosphoric mixed anhydride intermediate (Fig. 3).

D. Carbodiimide Methods

N,N'-Dicyclohexylcarbodiimide (DCC) has been widely used (Rich and Singh, 1979; Williams and Ibrahim, 1981) since Sheehan and Hess (1955) first applied it to peptide synthesis. The reaction mechanism involving DCC is illustrated in Scheme 6 (Schüssler and Zahn, 1962; Detar *et al.*, 1966). Briefly, an activated intermediate, *O*-acylisourea, which is produced by reaction of a protected amino acid and DCC, undergoes aminolysis to form a peptide and dicyclohexylurea (DCU). The reaction of an N-protected amino acid and DCC without an amine component gives another activated species, a symmetrical anhydride, which also reacts with amine components. The DCU produced in this reaction is insoluble in almost all common organic solvents and is thus easily removed from the reaction mixture by filtration.



Scheme 6 Reaction mechanism of DCC.

The noticeable side reaction is the formation of an acylurea by intramolecular rearrangement of the acylisourea. The extent of the side reaction is enhanced when the proton concentration of N-protected amino acid is decreased by excess triethylamine (which is usually used for neutralization of chloride ions bound to the amine component). The side reaction is profoundly influenced by

the choice of solvent as well as the base (Sheehan *et al.*, 1956). In the fragment condensation by DCC, this side reaction has to be considered seriously, but in the coupling of N-protected amino acids, it can be ignored because of its easy removal by recrystallization. Dehydration of the side-chain amide group of glutamine or asparagine also occurs during DCC activation.

To simplify the removal of DCU and unreacted carbodiimide, water-soluble carbodiimides such as 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide were developed; in such systems, both the reagent and the resulting urea derivative are soluble and easily removable by washing (Sheehan *et al.*, 1961). Diisopropylcarbodiimide (DIPCDI) is a useful reagent in solid-phase peptide synthesis (SPPS) because the diimide and the corresponding urea are soluble in organic solvents (Sheehan and Hess, 1955; Sheehan and Yang, 1958).

1. DCC-Additive Methods

Wünsch's group found that fragment condensation using DCC and HOSu under strictly controlled conditions preserves the chiral purity almost completely, with less than 1% racemization being reported (Weygand *et al.*, 1966; Wünsch and Drees, 1966). The method was applied to the synthesis of glucagon to obtain an active peptide (Wünsch, 1967; Wünsch and Wendlberger, 1968; Wünsch *et al.*, 1968). Strictly speaking, however, the synthesis was carried out with suppression of racemization, and an accurate racemization test is required to judge whether the product is absolutely optically pure.

The disadvantage of the DCC-HOSu method is the side reaction, Lossen rearrangement, which gives a β -alanine derivative by reaction with amine components (Gross and Bilk, 1968; Weygand *et al.*, 1968). To remedy this, König and Geiger (1970a,b,c) examined various analogous reagents and reported DCC-HOBT as the most suitable. This reagent suppresses not only racemization but also acylurea formation in fragment condensation. In these syntheses, use of water-soluble carbodiimide instead of DCC was recommended (Kimura *et al.*, 1981, 1988), as the urea or acylureas formed are more easily removed from the reaction products.

These methods are also useful for the coupling of amino acids with respect to increases in yield and suppression of side reactions. The suppression of racemization by this methodology has been reported by many researchers using various additives (Yajima *et al.*, 1973a,b; Fujino, 1976). However, the racemization test is carried out in a model system, and the results do not always agree with practical synthesis.

2. Fragment Condensation at Glycine or Proline Residues

Activation of glycine residues does not cause racemization because glycine has no chiral center, nor does activation of proline residues because proline has no imino hydrogen which would participate in azlactone formation. Therefore, whatever kind of coupling method is used, fragment condensation by activating the carboxyl group of glycine or proline never fails to preserve chiral

purity of the peptide, and these tactics are commonly used in synthetic schemes for larger peptides. When DCC is used as a coupling reagent in fragment condensation, however, acylurea formation becomes a problem. The removal of acylurea is fairly difficult. The addition of racemization-suppressing reagents such as *N*-hydroxysuccinimide also suppresses acylurea formation.

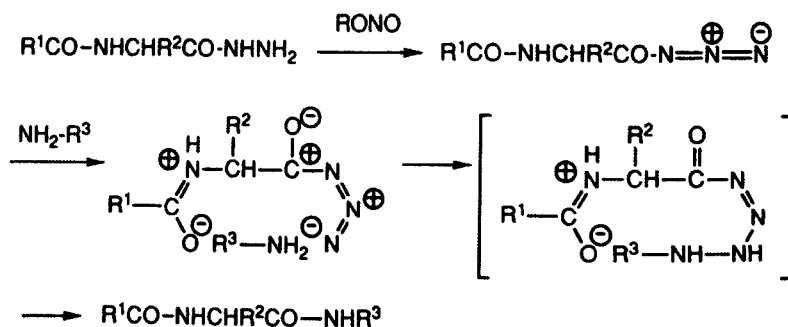
More recently, coupling reagents such as benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) have been employed instead of DCC (Castro *et al.*, 1975, 1977). The strong reactivity of the coupling reagents is advantageous for fragment condensation, since the reaction rate is slower than that of amino acid coupling. In addition, 4-(dimethylamino)pyridine can be used as a catalyst because of the lack of the problem of racemization at Gly or Pro residues.

E. Azide Methods

The azide method, first developed by Curtius (1902, 1904), is currently used not only for the stepwise coupling of troublesome amino acids such as serine or histidine, but also for fragment condensation, where it shows real merit. It is the most suitable method for fragment condensation because little racemization occurs, and side-chain protection can be kept at a minimum (only the ϵ -amino group of lysine and the thiol group of cysteine have to be protected) because azide is less reactive with side-chain functional groups such as hydroxyl groups.

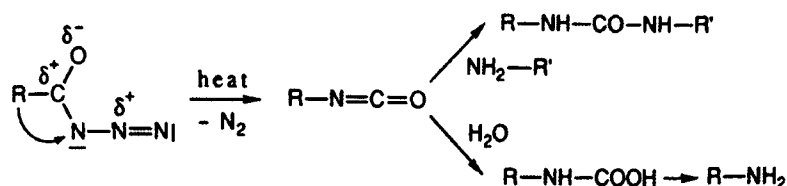
First, the hydrazide is prepared by treating an *N*-protected amino acid or peptide ester with excess hydrazine hydrate. The hydrazide is converted to azide by *tert*-butyl or isoamyl nitrite (Rudinger's variation; Honzl and Rudinger, 1961), and the azide reacts with the amine component without extraction with organic solvent. Because the azide is unstable at room temperature, the reaction with the amine component is carried out below 0°C for 40 hr after neutralization of hydrogen azide with a base such as triethylamine or *N*-methylmorpholine.

The resistance to racemization in this method is explained by the electron distribution of the N_3 group, which reduces the tendency of the $R-CO-NH$ group to participate in azlactone formation (Scheme 7). However, using an isotope dilution assay, Kemp *et al.* (1970) detected racemization even in the azide method.



Scheme 7 Suppression of racemization of azide.

The side reactions that generally occur in the azide procedure are as follows: (i) Curtius rearrangement at higher temperature, producing a urea derivative or an amine (Hofmann *et al.*, 1960) (Scheme 8), and (ii) hydrolysis of azide, giving an amide and nitric oxide (Honzl and Rudinger, 1961). In addition, side reactions which involve particular amino acids have also been reported (Gregory *et al.*, 1968). In conclusion, the azide procedure is an excellent coupling method; however, some side reactions have been reported, and the procedure is not simple.



Scheme 8 Curtius rearrangement of azide.

1. Protected Hydrazide Methods

Peptide esters containing Arg(NO₂), Asp(O-*t*-Bu), Asp(OBzl), or Glu(OBzl) (where *t*-Bu and Bzl represent *tert*-butyl and benzyl, respectively) cannot be converted to the corresponding hydrazide by hydrazinolysis. In such cases, a protected hydrazide is introduced at the C terminus of a peptide or carboxyl group of an amino acid. After elongation of the peptide chain, the substituent on the hydrazide is removed, and the resulting hydrazide is converted to an azide.

Various protected hydrazides have been reported. Naturally, the substituent chosen has to have a selectivity of removal different from that of the α -amino protecting group. The usual protecting groups for hydrazine are Z (Hofmann *et al.*, 1950, 1952), *tert*-butoxycarbonyl (Boc; Boissonnas *et al.*, 1960; Schwyzer *et al.*, 1960), 2,2,2-trichloroethoxycarbonyl (Troc; Yajima and Kiso, 1971; Watanabe *et al.*, 1974), and 2,2,2-trichloro-*tert*-butoxycarbonyl (Tcboc; Shimokura *et al.*, 1985).

2. Azide Method Using Diphenylphosphorylazide

Azides have long been obtained exclusively via hydrazides since their first preparation by Curtius. Shioiri *et al.* (1972), however, devised an interesting alternative to the hydrazide-mediated azide method, and reported that

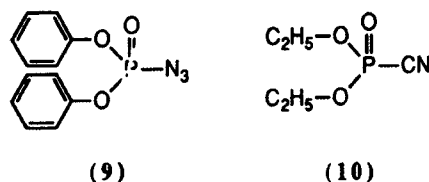


Figure 4 Structures of DPPA (9) and DEPC (10).

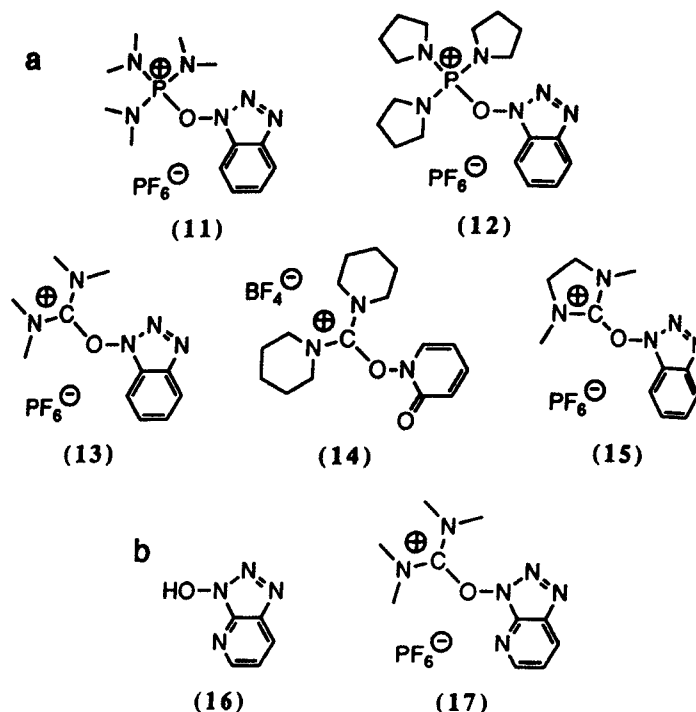


Figure 5 (a) Structures of BOP (11), PyBop (12), HBTU (13), TOPPipU (14), and BOI (15). (b) Structures of HOAt (16) and HATU (17).

diphenylphosphorylazide (DPPA, **9** in Fig. 4) directly converts a free carboxyl group to an azide (Shioiri and Yamada, 1974). The coupling reaction is considered to proceed via an azide rather than an acyloxyphosphoric mixed anhydride, which is also a likely intermediate. A similar reagent, diethylphosphorocyanidate (DEPC, **10** in Fig. 4) was also reported (Yamada *et al.*, 1973; Shioiri *et al.*, 1976a,b). The advantages of the reaction using DPPA or DEPC are no formation of insoluble by-products and less racemization in fragment condensation.

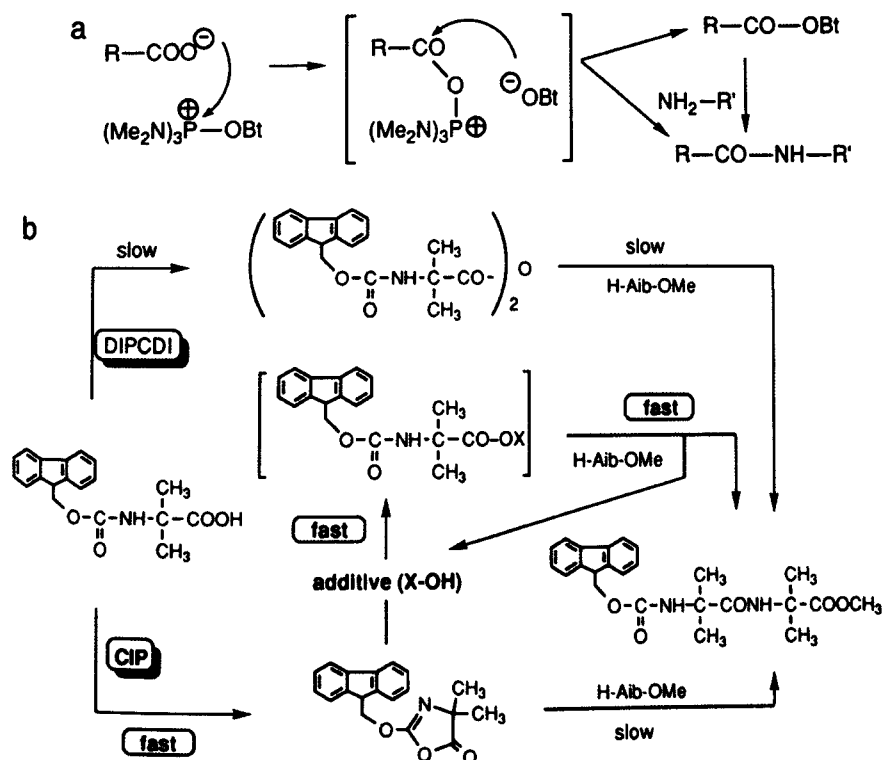
F. Phosphonium and Uronium Salt Type Coupling Reagents

Very high performance of coupling reagents has been found to be necessary, especially in the field of SPPS. Benzotriazole-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate (BOP, **11** in Fig. 5a), reported by Castro *et al.* (1975, 1977), is an excellent coupling reagent offering high reactivity and easy handling. The reaction between BOP and an N-protected amino acid proceeds via an acyloxyphosphonium as an intermediate to produce the corresponding benzotriazole ester and easily removable by-products such as hexamethylphosphoric triamide (HMPA) and salts (Scheme 9a). Under basic conditions, no side reaction accompanies the main reaction, in contrast to the use of DCC, and the reaction rate is generally very fast. For example, in the case of

coupling of Boc-Ile with Val-OBzl, the reaction is completed within a few minutes. BOP is used in both solution and solid-phase syntheses.

However, HMPA, the starting material in the synthesis and a by-product in the reaction of BOP, has been reported to have respiratory toxicity. It is therefore recommended to replace BOP by benzotriazole-1-yloxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP, **12** in Fig. 5a), which has similar reactivity to BOP and forms no carcinogenic by-products (Martinez *et al.*, 1988; Coste *et al.*, 1990a).

2-(1*H*-Benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, **13** in Fig. 5a; Knorr *et al.*, 1989), 2-[2-oxo-1(2*H*)-pyridyl]-1,1,3,3-bis(pentamethylenuronium) tetrafluoroborate (TOPPipU, **14** in Fig. 5a; Henklein *et al.*, 1991), and 2-(benzotriazole-1-yl)-oxy-1,3-dimethylimidazolidinium hexafluorophosphate (BOI, **15** in Fig. 5a; Kiso *et al.*, 1992b) have similar reactivities to BOP. Phosphonium is substituted by uronium in these reagents. Carpino (1993) reported that 1-hydroxy-7-azabenzotriazole (HOAt, **16** in Fig. 5b), an additive, and 2-(1*H*-7-azabenzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, **17** in Fig. 5b), a uronium-type coupling reagent containing the HOAt moiety, enhanced the coupling reaction rate and reduced the loss of chiral integrity. The nitrogen atom at the 7 position of HOAt has a neighboring group effect to accelerate the coupling reaction.



Scheme 9 (a) Reaction mechanism of BOP. (b) Reaction mechanism of CIP.

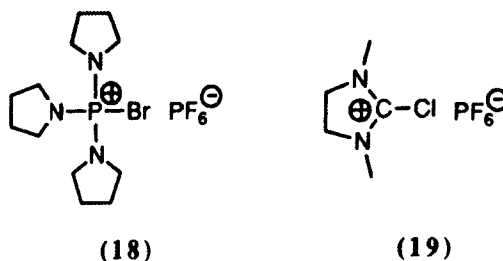


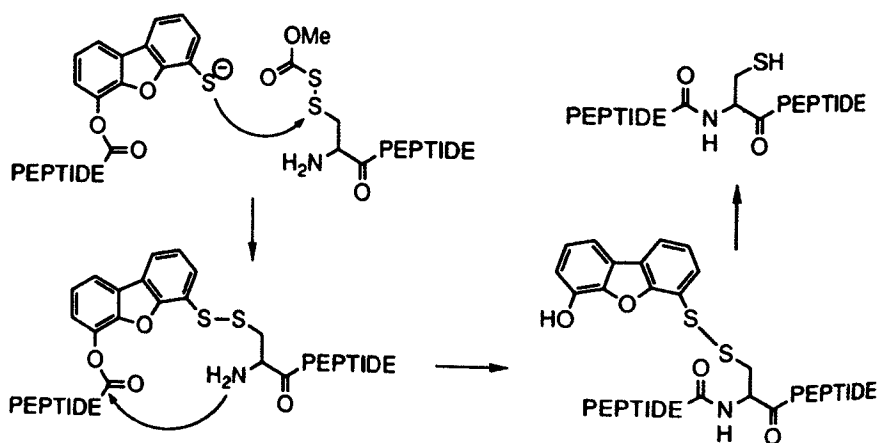
Figure 6 Structures of PyBroP (18) and CIP (19).

Bromotrispyrrolidinophosphonium hexafluorophosphate (PyBroP, 18 in Fig. 6; Coste *et al.*, 1990b) and 2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate (CIP, 19 in Fig. 6; Akaji *et al.*, 1992c, 1994) have been developed as coupling reagents for *N*-methylamino acids or α -dialkylamino acids and as esterification reagents (Scheme 9b).

G. Other Amide-Forming Reactions

Many reactions that form amide bonds are known besides those described above. In this section, those which are interesting from the viewpoint of organic chemistry or synthesis of large peptides are described.

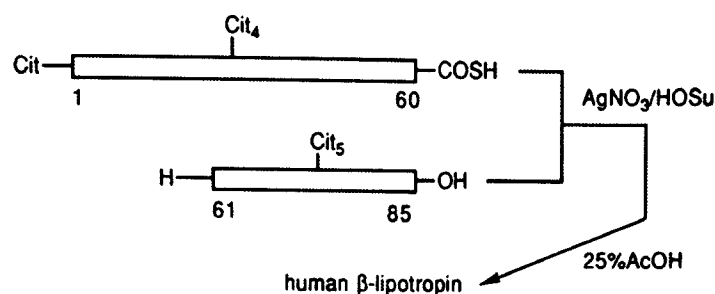
Kemp *et al.* reported a series of studies on peptide synthesis referred to as the "prior thiol capture method" (Kemp and Galakatos, 1986; Kemp *et al.*, 1986; McBride and Kemp, 1987; Kemp and Fotouhi, 1987). This method consists of intermolecular disulfide formation and subsequent intramolecular acyl rearrangement (Scheme 10).



Scheme 10 Prior thiol capture method.

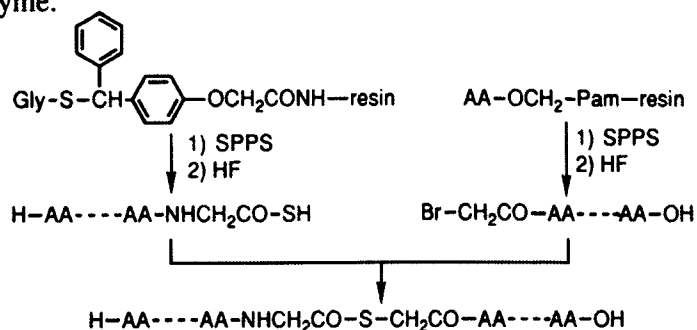
Blake (1981) proposed a new method for the assembly of peptide segments in aqueous medium by the use of the thiocarboxyl function. A partially protected C-terminal Gly thiocarboxyl segment is coupled with an amine component by the aid of silver ion or 2,2'-dipyridyl disulfide as an activator. In this reaction, the ϵ -amino group of lysine residues is protected by the citraconyl

group, but other functional side-chain groups are not protected. Thiocarboxyl peptides have been synthesized by the solid-phase method starting from Boc-Gly-S-resin. This method was successfully applied to the syntheses of human β -lipotropin (Scheme 11; Blake and Li, 1983), *S*-carbamoylmethyl bovine apocytocrome *c* (Blake, 1986) and α -inhibin-92 (Yamashiro and Li, 1986). In addition, Hojo and Aimoto (1991) employed an *S*-alkyl thioester of partially protected peptide segments for the synthesis of the DNA-binding domain of c-Myb protein(142–193)-amide.



Scheme 11 Thiocarboxylic acid method.

To obtain a small protein, Schnölzer and Kent (1992) reported an interesting method using substitution of a peptide bond and ligation at that site (Scheme 12). A thioester was employed instead of the peptide bond between Gly-51 and Gly-52 of human immunodeficiency virus type 1 (HIV-1) protease. This region has been reported to bear no relation on activity. The peptide carrying C-terminal thiocarboxyl glycine and the N-bromoacetylated peptide were prepared by a solid-phase technique. Ligation of the two segments in aqueous media gave an HIV-1 protease analog which had activity similar to that of the native enzyme.



Scheme 12 Chemical ligation.

II. Deprotection

Protection and deprotection steps are important in peptide synthesis. Deprotecting steps can be briefly categorized into two types: (1) deprotection of α -amino groups to give peptides for further extension and (2) final deprotection to give free peptides. The results of peptide syntheses are critically influenced by

the combination of these two types of deprotection along with the selection of protecting groups for α -amino and side-chain functional groups in a so-called strategy. Most frequently, strong acids such as HF or trifluoromethanesulfonic acid (TFMSA) have been used for the final deprotection in combination with selective deprotection of temporary α -amino protecting groups such as Boc using trifluoroacetic acid (TFA). Alkaline conditions have also been used for selective deprotection of temporary α -amino protecting Fmoc groups and acidic reagents such as TFA for the final deprotection. However, the synthesis of higher peptides with various functional groups requires new and different types of protection and deprotection. In this section, the deprotection methods are described in relation to their strategies.

A. Deprotection of α -Amino Protecting Groups

Selectivity is highly important in deprotection of α -amino protecting groups. Cleavage of the side-chain protecting groups is one of the most serious problems during peptide synthesis. Such side reactions are particularly troublesome in SPPS, which is carried out without purification of the reaction intermediates. Cleavage of the protected peptides from the resin is also a serious problem in solid-phase synthesis. In other words, the side-chain protecting groups should be kept intact up to the final deprotection stage, and selectivity of the α -amino deprotecting method is crucial for peptide chain elongation. When a peptide contains sensitive amino acids such as methionine or cysteine, it becomes even harder to cleave the α -amino protecting groups selectively.

1. Mild Acids

a. Trifluoroacetic Acid Trifluoroacetic acid is one of the most commonly used reagents for cleavage of α -protecting groups such as Boc and Z(OMe). Good solubility of peptides in TFA, fewer side reactions during deprotection, and mildness make it useful not only for removal of temporary protecting groups but also for the final deprotection step. The reagent in the form of 20–60% TFA in CH_2Cl_2 is often used for removal of α -Boc in solid-phase synthesis (Gutte and Merrifield, 1971). The side reactions encountered during the deprotection are (i) partial elimination of side-chain protecting groups, (ii) pyroglutamyl formation from peptides containing glutamine at the N terminus (Takashima *et al.*, 1968), and (iii) *tert*-butylation of the indole ring in tryptophan-containing peptides (Alakhov *et al.*, 1970).

b. Hydrochloric Acid/Dioxane The HCl/dioxane reagent is prepared by introducing hydrogen chloride gas into dioxane, and it is often used for removal of acid-sensitive temporary protecting groups. In the form of 4 N HCl/dioxane, it is used for cleavage of Boc groups (Guttmann, 1961; Stewart and Woolley, 1965). Pyroglutamyl formation from N-terminal glutamine-containing peptides is reduced greatly (Beyerman *et al.*, 1973).

c. Dilute Methanesulfonic Acid in Dioxane–Dichloromethane This diluted methanesulfonic acid (MSA) system, which uses 0.5 M MSA in 1:9 (v/v) dioxane–CH₂Cl₂ (Kiso *et al.*, 1992b), is primarily used in SPPS. The advantages are as follows: (i) elimination of side-chain protecting groups is reduced compared to the conventional 45% TFA/CH₂Cl₂ method, and (ii) pyroglutamyl formation from glutamine-containing peptides is similarly decreased relative to the use of 4 N HCl/dioxane. Using the MSA deprotection system, Kiso *et al.* (1990a) developed an efficient method for SPPS consisting of *in situ* neutralization and the rapid coupling reaction using BOP or BOI reagent activation (Kiso *et al.*, 1990a) (Fig. 7). Porcine brain natriuretic peptide (pBNP) was synthesized successfully using this method (Kiso *et al.*, 1992b).

2. Alkaline Conditions

a. Piperidine in Dimethylformamide The combination of 20–55% piperidine in dimethylformamide (DMF) is used mainly for removal of Fmoc groups in SPPS (Atherton *et al.*, 1981; Heimer *et al.*, 1981). Diketopiperazine formation is the most serious side reaction occurring during peptide chain elongation at the second residue (Roth and Mazanek, 1974; Bray *et al.*, 1990), and it is especially serious in peptides containing proline at the C terminus.

b. Tetra-*n*-butylammonium Fluoride Trihydrate in Dimethylformamide Dilute (0.02 M) tetra-*n*-butylammonium fluoride trihydrate (TBAF) in DMF is

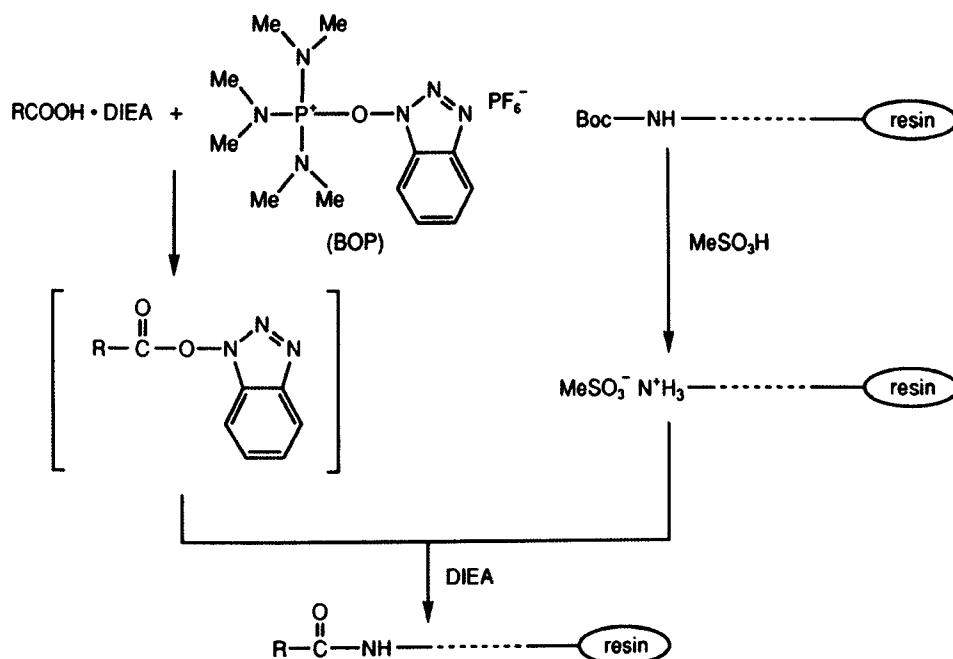


Figure 7 Coupling pathway for efficient SPPS employing *in situ* neutralization.

used for removal of Fmoc groups (Ueki and Amemiya, 1987) as exemplified in the synthesis of Leu-Ala-Gly-Val in continuous-flow system.

c. 1,8-Diazabicyclo[5.4.0]undec-7-ene in Dimethylformamide Another reagent employed for cleavage of Fmoc groups is 2% 1,8-diazabicyclo[5.4.0]undec-7-ene in DMF (Wade *et al.*, 1991). Peptides with about 20 residues have been synthesized in continuous-flow systems using this reagent.

B. Final Deprotection

The reagent for final deprotection should cleave all the protecting groups as completely as possible without side reactions. Particularly in the synthesis of higher peptides using various side-chain protecting groups, selection of appropriate protecting groups in combination with an unfailing final deprotecting reagent is extremely important.

1. *tert*-Butoxycarbonyl/Benzyl

a. Anhydrous Hydrogen Fluoride Anhydrous HF cleaves Boc or *t*-Bu groups within several minutes and Bzl-based, *N*^c-NO₂ and *N*^c-Tos groups in 30–60 min (Sakakibara *et al.*, 1967; Mazur, 1968). Owing to its ability to remove all the protecting groups, including that of cysteine, HF is widely utilized both in solid-phase and in solution synthesis. The reported side reactions are binding of anisole to glutamic acid (Sano and Kawanishi, 1975; Feinberg and Merrifield, 1975), formation of 3-benzyltyrosine (Sakakibara *et al.*, 1967; Erickson and Merrifield, 1973), and alkylation of tryptophan indole (Masui *et al.*, 1980). These side reactions can be greatly reduced, however, by adjusting the reaction conditions and by adding scavengers.

Tam *et al.* (1983a,b) introduced a two-step HF treatment referred to as the low–high HF method. 3-Benzilation of Tyr residues and succinimide formation from Asp(OBzl) residues are notably suppressed in comparison with the orthodox HF method, and reduction from Met(O) to Met and deformylation of Trp(CHO) are effectively accomplished by the low–high HF method.

b. Trifluoromethanesulfonic Acid in Trifluoroacetic Acid The 1 M TFMSA–thioanisole/TFA system completely cleaves almost all protecting groups currently used in peptide synthesis within 1 hr (Yajima *et al.*, 1974). Many biologically active peptides have so far been synthesized by this method, demonstrating its usefulness. The total synthesis of bovine ribonuclease A (Yajima and Fujii, 1980, 1981; Fujii and Yajima, 1981) can be regarded as a comprehensive study of this method. This facile method, which needs no special equipment, is widely applied in both solution and solid-phase syntheses.

c. Trimethylsilyl Trifluoromethanesulfonate in Trifluoroacetic Acid The 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)–thioanisole/TFA system almost completely cleaves various protecting groups currently used

in peptide synthesis (Fujii *et al.*, 1987b,c) in a short time. High recovery of bulky isoleucines from Boc-Ile-phenylacetamidomethyl (PAM) resin after 1 M TMSOTf–thioanisole/TFA treatment indicates that this is a promising final deprotecting method in solid-phase synthesis. This system has been employed in solution-phase syntheses of glucose-dependent insulinotropic polypeptide (GIP; Yajima *et al.*, 1985b, 1986), neuromedin U-25 (Fujii *et al.*, 1987a), sauvagine (Nomizu *et al.*, 1988), and human pancreatic polypeptide (Sugiyama *et al.*, 1987) as well as in the solid-phase synthesis of magainin (Matsuzaki *et al.*, 1989).

d. Tetrafluoroboric Acid in Trifluoroacetic Acid The system 1 M HBF_4 in TFA is a weaker acid than HBr in TFA. Kiso *et al.* (1989b) found the usefulness of this mild reagent in the presence of thioanisole (1 M HBF_4 –thioanisole in TFA at 4°C) as a final deprotecting reagent. Together with α -amino protecting groups such as Boc or 4-methoxybenzyloxycarbonyl [Z(OMe)], the Z or 2-chlorobenzyloxycarbonyl (Cl-Z) group at the side chain of Lys and the Bzl groups at Ser, Thr, Asp, and Glu were cleaved quantitatively within 60 min. Complete removal of the Cl_2 -Bzl group from Tyr, the benzyloxymethyl (Bom) group from His, and the diphenylphosphinothioyl (Ppt) group from Trp was also achieved within 60 min. However, a 60-min treatment at an elevated temperature (25°C) was necessary for complete removal of the cyclohexyl (cHex) group from Asp and the mesitylene-2-sulfonyl (Mts) group from Arg. HBF_4 in TFA also cleaves the amino acid amide from 4-methylbenzhydrylamine resin more effectively than TFMSA in TFA. Lamprey gonadotropin-releasing hormone (a 10-residue peptide amide) was synthesized using 1 M HBF_4 –thioanisole in TFA by both solution-phase and solid-phase methods (Kiso *et al.*, 1989b). Dynorphin A was also synthesized using this deprotecting reagent in the solid phase (Kiso *et al.*, 1991a).

2. 9-Fluorenylmethoxycarbonyl/*tert*-Butyl

a. Trifluoroacetic Acid As mentioned above, TFA is commonly used for cleavage of α -amino protecting groups such as Boc, whereas it is used as a final deprotecting reagent in the Fmoc/*t*-Bu strategy. The most notable advantage of TFA in Fmoc/*t*-Bu strategy is the reduced incidence of side reactions such as formation of 3-benzyltyrosine and succinimide formation on aspartate residues compared to strong acids such as HF and TFMSA. Addition of ethanedithiol can suppress the *tert*-butylation of indole rings to a considerable extent (Wünsch *et al.*, 1977).

With regard to protecting groups for arginine, it has been reported (Atherton *et al.*, 1983, 1985; Akaji *et al.*, 1990) that TFA cannot completely cleave the widely used 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group (Fujino *et al.*, 1980, 1981). 2,2,5,7,8-Pentamethylchroman-6-sulfonyl (Pmc; Ramage and Green, 1988) has been developed as an N^ϵ -protecting group efficiently cleavable by TFA. In addition, other reagents mentioned below can be used more efficiently for synthesis of longer Arg-containing peptides.

b. Trimethylsilyl Bromide in Trifluoroacetic Acid The 1 M trimethylsilyl bromide (TMSBr)-thioanisole/TFA system cleaves Bzl and Z groups nearly completely, and 2,6-dichlorobenzoyloxycarbonyl (Cl_2Bzl) and Mts groups up to 90% (Fujii *et al.*, 1987d). It also efficiently (80%) reduces Met(O) to Met and suppresses succinimide formation of Asp nearly perfectly.

c. Tetrafluoroboric Acid in Trifluoroacetic Acid In Fmoc-based peptide synthesis, 1 M HBF_4 -thioanisole in TFA was found to be useful (Akaji *et al.*, 1990). Complete removal of *t*-Bu groups from the side chains of Ser, Thr, Tyr, Asp, and Glu, removal of the Boc group from Lys, removal of the *tert*-butoxymethyl (Bm) group from His, the removal of 4,4-dimethoxybenzhydryl (Mbh) groups from Asn and Gln, and the cleavage of Mtr groups from Arg could be achieved within 30 min. In the presence of ethanedithiol, Trp was quantitatively regenerated from Boc-Trp after a 120-min treatment. Of the various S-protecting groups of Cys, 4-methylbenzyl (MeBzl) and *t*-Bu were cleaved quantitatively within 60 min, but 4-methoxybenzyl (MeOBzl) was cleaved incompletely. S-Linked acetamidomethyl (Acm) groups remained intact after 120 min of treatment even in the presence of thioanisole. Bulky amino acids including Ile could be cleaved from *p*-benzyloxybenzyl alcohol resin (Wang resin) by this reagent. Amino acid amides were cleaved from *p*-methylbenzhydrylamine (MBHA) resin within 60 min except for Val- NH_2 and Phe- NH_2 , for which longer treatment (120 min) was necessary for complete cleavage. However, Val- NH_2 was quantitatively cleaved from acid-labile 4-(2',4'-dimethoxyphenylaminomethyl)phenoxy-type (DMAMP) resin by treatment with this reagent for 30 min at 4°C. Human glucagon, oxytocin, human brain natriuretic peptide (BNP), and eel atrial natriuretic peptide (ANP) were synthesized in the solid phase using this deprotecting reagent (Kiso *et al.*, 1991a; Akaji *et al.*, 1992b).

3. Other Strategies

a. Fluoride Ion Deprotection Strategy In this method, the acid-sensitive Boc group is used for α -amino protection, and tetra-*n*-butylammonium fluoride trihydrate (TBAF)-sensitive protecting groups are employed for side chain functional groups. A TBAF-sensitive resin is also used in this orthogonal strategy (Kiso *et al.*, 1988a) (Fig. 8). Bradykinin potentiating peptide 5a (BPP5a) and α -neo endorphin have been synthesized in high yield (Kiso *et al.*, 1990a).

b. Reductive Acidolysis Strategy Kiso *et al.* (1979, 1980b,c) reported that scavengers such as thioanisole promote deprotection under acidic conditions, and that the deprotection reaction proceeds by the cooperative action of a hard electrophile and a soft nucleophile, the "push-pull" mechanism, well agreeing with Pearson's hard soft acids bases principle. In the course of exploring new deprotecting procedures based on this concept, Kiso *et al.* (1987a) found that chlorinated silanes-TFA scavengers had strong reductivity along with acidolytic activities.

A series of safety-catch type protecting groups based on the 4-methylsulfinylbenzyl group have been developed; 4-methylsulfinylbenzyloxycarbonyl

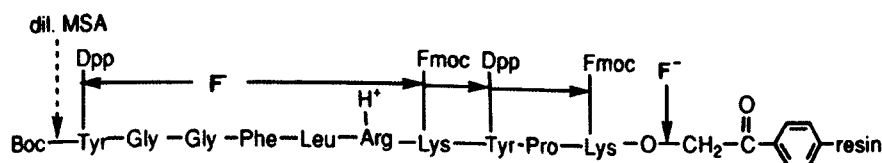
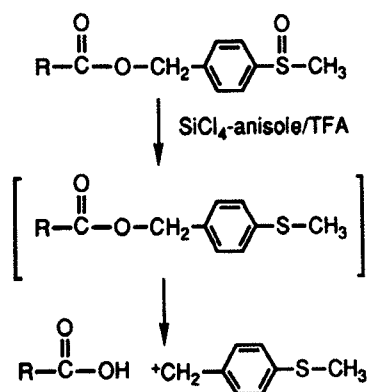


Figure 8 Two-dimensional orthogonal protection scheme for peptide synthesis using a fluoride ion final deprotection strategy.

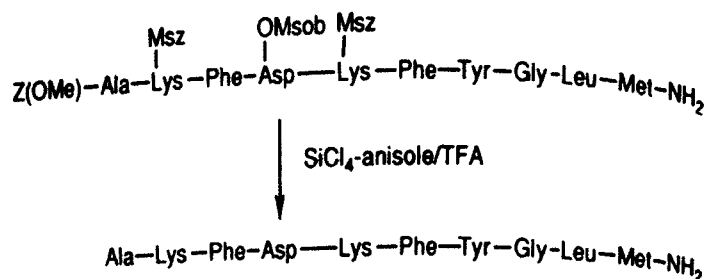
(Ms_z; Kiso *et al.*, 1989a) for amino groups, and 4-methylsulfinylbenzyl (Msob; Samanen and Brandeis, 1985, 1988; Kiso *et al.*, 1987b, 1988b, 1989a) for carboxyl and hydroxyl groups. These protecting groups are stable under acidic conditions but can be smoothly removed in a one-pot reaction involving reductive acidolysis using tetrachlorosilane (SiCl₄)-TFA scavengers (Kiso and Kimura, 1991). In the deprotecting reaction, the Ms_z group is first reduced to the TFA-labile 4-methylthiobenzyloxycarbonyl (Mtz) group and then cleaved by acidolysis; in other words, the Ms_z group is deprotected by the reduction of the sulfoxide moiety and subsequent acidolysis (reductive acidolysis). Deprotection of the Msob group proceeds in a similar manner (Scheme 13).



Scheme 13 Mechanism of reductive acidolysis.

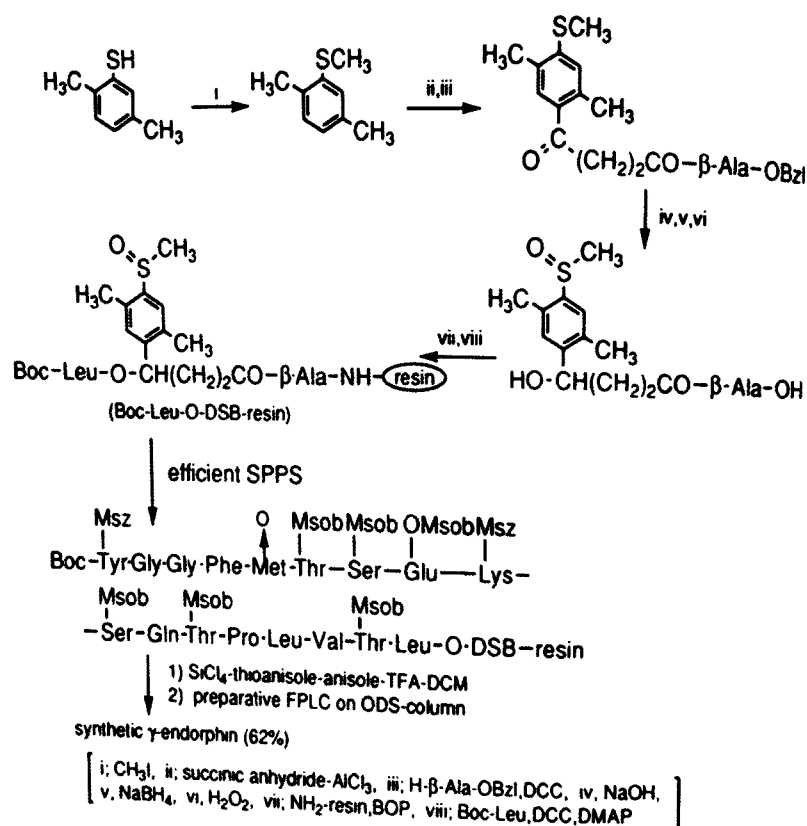
Using the acid-stable Ms_z group as the semipermanent side-chain protecting group in combination with an acid-labile temporary N α -protecting group such as Boc, a novel two-dimensional (2D) protection methodology in peptide synthesis has been developed by Kiso *et al.* (1989a). The method is based on a final deprotection strategy using reductive acidolysis. Scylliorhinin I was synthesized in solution using this system (Kiso *et al.*, 1989a) (Scheme 14).

Furthermore, Kiso *et al.* (1992c) have also developed a new 2D protection strategy in SPPS employing the safety-catch linkage to the resin, which is an orthogonal system based on an acid-labile temporary and reductive acidolysis-labile semipermanent protection scheme. Two types of resins carrying ester and substituted benzhydrylamide linkages are prepared and applied to practical peptide synthesis. To introduce a safety-catch ester linkage to aminomethylated-polystyrene resin, a new handle reagent 4-(2,5-dimethyl-4-methylsulfinylphenyl)-4-



Scheme 14 Synthesis of scyliorhinin I by reductive acidolysis.

hydroxybutanoic (DSB) acid, was developed. γ -Endorphin (17-amino acid peptide) was synthesized using the DSB-resin (Kiso *et al.*, 1992a,c, 1994) (Scheme 15).

Scheme 15 Preparation of DSB-resin and synthesis of γ -endorphin.

A safety-catch type of resin suitable for peptide amide synthesis by incorporation of an alkylsulfinyl group to the conventional benzhydrylamine type resin was also designed by Kiso *et al.* (1992c). A new handle reagent, *N*-Boc-(2 or 4)-(4'-methoxyphenylaminomethyl)-5-methoxyphenylsulfinylhexanoic acid, was prepared in seven steps starting from 3-methoxythiophenol. Coupling of the acid with $\beta\text{-Ala-NH-resin}$ gave *N*-Boc-dimethoxyalkylsulfinylbenzhydrylamine (DSA) resin. Using DSA-resin, buccalin ($\text{Gly-Met-Asp-Ser-Leu-Ala-Phe-Ser-Gly-Leu-NH}_2$) was successfully synthesized (Kiso *et al.*, 1992c). Another safety-catch acid labile (SCAL) handle was prepared for the synthesis of peptide

amides by the Boc/Fmoc strategy and was successfully applied to the synthesis of a portion of human calcitonin (Patek and Lebl, 1990, 1991, 1992; Patek, 1993).

In addition, using a TBAF-sensitive resin, γ -endorphin (Kiso *et al.*, 1991b) and thymic humoral factor γ_2 (THF γ_2) (Kiso *et al.*, 1993) were prepared in high yield by a stepwise deprotection method using reductive acidolysis followed by fluoride ion.

III. Disulfide Bond Formation

Disulfide bond formation is an important step that critically influences the synthesis of a cystine-containing peptide. Particularly in the syntheses of peptides and proteins containing two or more disulfides, the final disulfide forming reactions often occur in poor yields. This is attributed to the formation of side products such as polymers or isomers with disulfide locations different from those of the desired product. These side reactions are unavoidable whenever peptide chains are synthesized either by chemical means or by genetic engineering. For example, when a protein is synthesized by recombinant gene technology in *Escherichia coli*, the product forms granules in the bacteria, and production of the desired molecule with the correct spatial structure and the right disulfide combination is the essential step. In this section, aspects of the folding and disulfide formation of proteins are briefly introduced, and disulfide forming reactions in the chemical synthesis of cystine-containing peptides, including (1) air oxidation, (2) iodine oxidation, and (3) stepwise methods, are described.

The 20 kinds of amino acids contained in proteins determine the spatial structure and the minimum free energy by hydrogen-bonding and hydrophobic interactions between amino acid residues or between an amino acid residue and solvent. Among these amino acids, cysteines greatly contribute to the stabilization of the conformation by forming covalent disulfide bonds. Anfinsen (1973) showed using ribonuclease that the tertiary structure of proteins is predetermined basically by the primary structure. After reduction of the four disulfide bonds in ribonuclease in the presence of denaturant, removal of the denaturant and air oxidation regenerated predominantly the original protein with the correct disulfide bonds. Thus, they showed that refolding of the reduced and denatured protein produced mainly the natural form among the 105 possible isomers with different disulfide combinations.

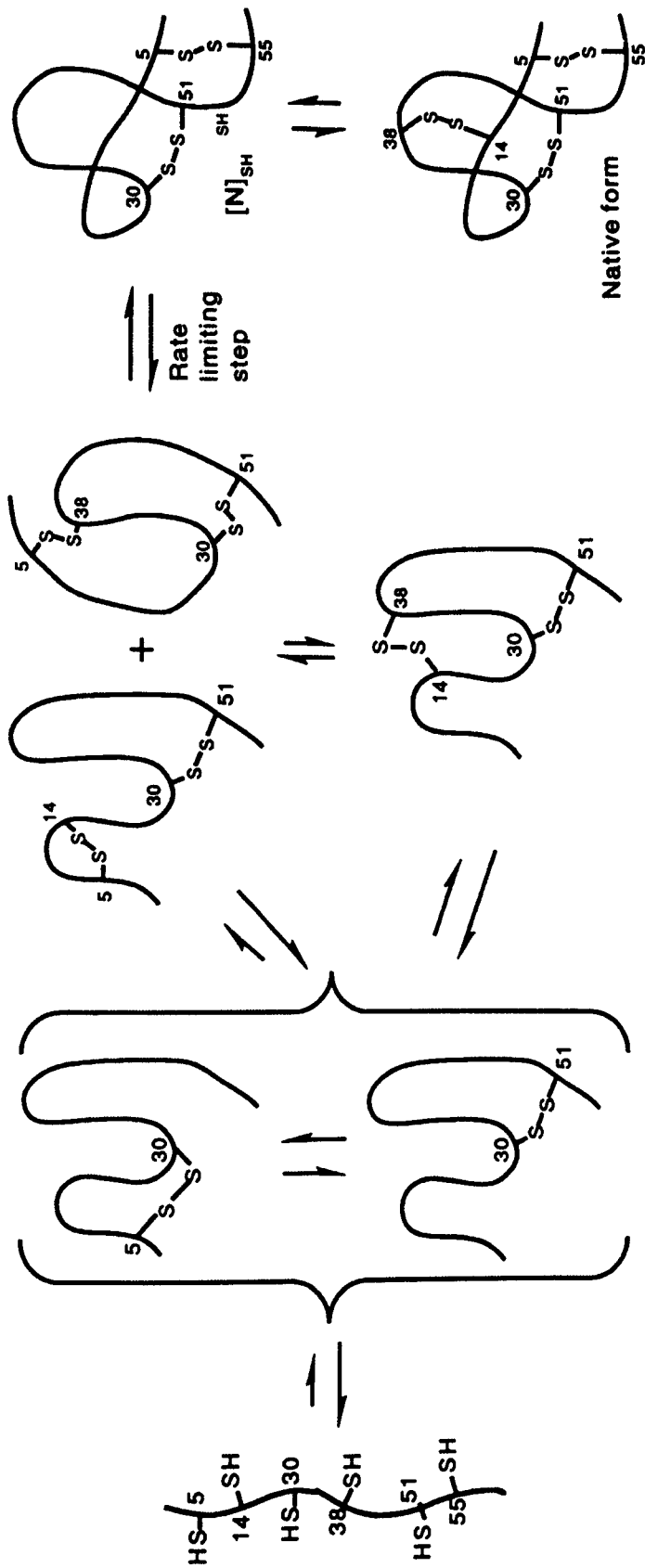
This result indicates that the appropriate disulfide bonds can be spontaneously formed from linear peptides with the correct amino acid sequence. In the laboratory, disulfide bonds are mostly formed by such natural refolding processes, although the reduction-reoxidation reaction requires a relatively long time. In biological systems, disulfide formation involves several accelerating factors, such as metal ions, disulfide exchanging enzymes, and low molecular weight SH or S-S compounds. These factors are applicable to chemical syntheses, and air oxidation in the presence of oxidized-reduced glutathione is often used in practical syntheses.

Disulfide formation processes have been intensively examined in some proteins, and the intermediates have been identified or inferred. For instance, Creighton (1980) conducted intensive research on bovine pancreatic trypsin inhibitor (BPTI) and described the formation process of three disulfides and the conformation of several intermediates (Creighton and Goldenberg, 1984). The natural type BPTI was obtained by air oxidation of reduced BPTI in the presence of oxidized-reduced glutathione. The intermediates produced during the reaction were trapped and identified by iodoacetic acid, and the disulfide bond formation process shown in Scheme 16 was deduced. Thus, among the six sulfhydryl groups in BPTI, formation of the first two disulfides occurs between residues 30 and 51 and between positions 5 and 30. The second group of bonds occurs between 14 and 38, between 5 and 14, and between 5 and 38. The natural disulfide between residues 5 and 55 has not yet been formed at this step and is not generated from the second three intermediates with two disulfides. The compound $[N]_{SH}^{SH}$ (Scheme 16) with the natively like conformation and two disulfide bonds (30-51 and 5-55) is formed only after the second intermediates undergo intramolecular rearrangement. The formation of $[N]_{SH}^{SH}$ is the rate-limiting step in the whole oxidation reaction. Thus, disulfide formation in BPTI follows a predetermined process; that is, intermediates with nonnative type disulfide orientations refold to form native type disulfide combinations. Furthermore, States *et al.* (1987) examined the intermediates by ^1H nuclear magnetic resonance (NMR) and discussed the relationship between disulfide formation and conformation.

The studies mentioned above concern the disulfide formation process *in vitro*, whereas recent experiments (Taniyama *et al.*, 1990) on expression and excretion of human lysozyme in yeast by genetic recombination imply that a similar process involving S-S exchange reactions also occurs *in vivo*. Human lysozyme consists of 130 amino acid residues and four disulfide bridges. Expression and excretion experiments involving a series of mutant proteins in which cysteines were converted to alanines showed the disulfide forming process of lysozyme via intermediates with nonnatural disulfide combinations.

A. Reduction-Reoxidation of Disulfide Bond-Containing Peptides and Proteins

As mentioned above, the amino acid sequence is supposed to dictate formation of the correct disulfide combination. However, not all proteins are oxidized as smoothly as ribonuclease. For example, oxidation of insulin or epidermal growth factor gives only a low yield, because these peptides are synthesized by proteolytic cleavage after the disulfide bonds are formed in the linear precursor protein. In the case of insulin, the disulfide forming reactions in proinsulin proceed more efficiently than in a mixture of the A and B chains after separate synthesis (Dixon and Wardlaw, 1960; Crea *et al.*, 1978; Santere *et al.*, 1981; Blohm *et al.*, 1988).



Scheme 16 Disulfide bond forming process in BPTI.

The formation of polymers and isomers to a certain extent during the oxidation reaction is inevitable and reduces the yield. In chemical synthesis, the yield is highly influenced by the purity of the protected peptide chain and the extent of side reactions during the final deprotection step. Therefore, for peptides or proteins with more than two disulfide bonds, it is very difficult to establish definite reaction conditions under which the air oxidation always proceeds smoothly, and it is necessary to find the best oxidation conditions for each compound. Generally, various factors concerning the reaction such as the extent of polymer and isomer formation, the solvent, pH, temperature, and the choice and concentration of additives such as glutathione are examined by reduction-reoxidation experiments.

1. Examination of Oxidation Conditions

Since Anfinsen (1973) performed the first reduction-reoxidation experiment, several peptides and proteins such as snake venom toxins (Menz *et al.*, 1980; Galat *et al.*, 1981; Gacond *et al.*, 1984), lysozyme (Acharya and Taniuchi, 1976; Perraudin *et al.*, 1983), and BPTI (Creighton and Goldenberg, 1984; Creighton, 1980) have been examined. This section describes the reduction-reoxidation experiments using scorpion toxin AaHII, in terms of improving the yield in chemical synthesis (Sabatier *et al.*, 1987).

AaHII, a peptide amide isolated from scorpion venom, consists of 64 amino acid residues and four disulfide bonds (Fig. 9). Normal air oxidation after reduction of AaHII gave mainly associated compounds along with very small amounts of the active peptide. To avoid the undesirable reactions, the reduced peptide was subjected to disulfide formation while it was dialyzed in buffer solution to solubilize the desired product, and the pH of the dialysis buffer and the influence of additives on the reaction were examined. The progress of the oxidation reaction was monitored by HPLC, and the product yield was calculated taking the toxicity of the soluble product as an indicator. As a result, the best yield of the active peptide was obtained when dialysis and air oxidation were carried out in the presence of dilute denaturant and oxidized-reduced glutathione at pH 8.0. The formation of associated compounds is often observed in the syntheses of other disulfide bond-containing peptides and proteins such as transforming growth factor (TGF) and epidermal growth factor (EGF), which are described later.

2. Formation of Isomers

Besides polymer formation, the formation of isomers which have the same amino acid sequence but have disulfide positions different from those of the native peptide is one of the most serious causes of reduced yield. In some cases, the isomers have been identified and the positions of disulfide bridges determined. Such identification of isomers is exemplified by the synthesis of insulin-like growth factor I (IGF-I), composed of 70 amino acid residues and three disulfide bridges, produced by recombinant methods in *E. coli*. Air oxidation of the reduced IGF-I synthesized in *E. coli* gives an isomer which elutes just before IGF-I, the main product, on HPLC (Saito *et al.*, 1987; Meng *et al.*, 1988). Comparison of peptide maps of the two products strongly implied that the isomer has

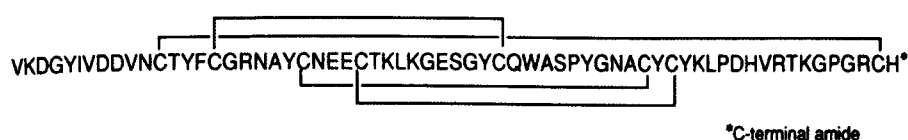


Figure 9 Structure of scorpion toxin AaIII.

two different disulfide positions relative to the native form. The disulfide positions were identified by chemical synthesis of the disulfide-containing peptide fragments with all possible disulfide combinations (Iwai *et al.*, 1989).

The synthesis of TGF- α , a peptide with 50 amino acids and three disulfide linkages, provides another example of the identification of isomers produced during chemical synthesis. Several groups synthesized TGF- α as described later. Among them, the group at Monsanto isolated and identified an isomer with two different disulfide bridges which was eluted after the main peak on HPLC (Tou *et al.*, 1990).

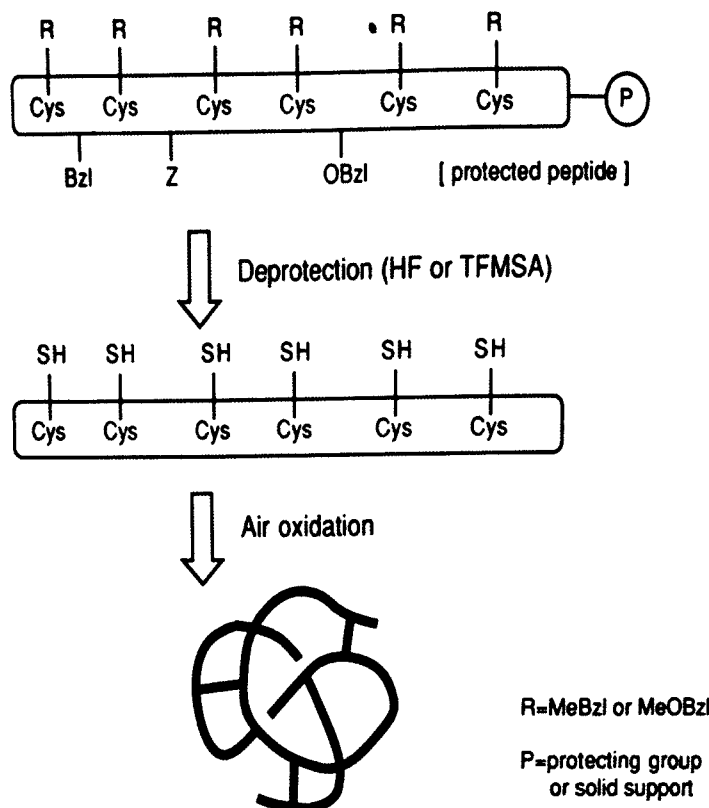
This section has briefly mentioned disulfide formation and the formation of polymers and isomers. In the next section, several important disulfide forming methods used in chemical synthesis of peptides and proteins are described along with some examples.

B. Disulfide Formation by Air Oxidation

Air oxidation is the most commonly used disulfide forming reaction. In particular, peptides and proteins with more than three disulfide bonds are synthesized by air oxidation in almost all cases. Depending on the thiol protecting groups used in peptide chain elongation, the reduced peptide for oxidation can be obtained either by one- or by two-step final deprotection.

1. Disulfide Formation by One-Step Deprotection

Scheme 17 shows the common synthetic scheme for disulfide-containing peptides using this method. At first, the protected peptide chain is constructed using solid-phase or solution methods. The MeBzl (Erickson and Merrifield, 1973) or MeOBzl groups (Akabori *et al.*, 1964) are employed as the thiol protecting group on cysteine, and benzyl alcohol-type protecting groups are used for other side-chain functional groups. After all the protecting groups, including those of cysteines, are removed by HF or TFMSA, reduction yields the peptide containing free SH groups. In almost all cases, the reduced peptide is subjected to air oxidation without purification to give the desired disulfide-containing peptide. An additive such as glutathione is included in the reaction mixture to accelerate the reaction and to improve the reaction yield. Generally, in this method the purity of the protected peptide critically influences the final yield, and only solution methods so far have given successful results in the synthesis of cysteine-containing proteins of approximately 100 residues.



Scheme 17 Disulfide bond formation by one-step deprotection.

One of the examples of disulfide formation by high-dilution air oxidation in the presence of glutathione is the total synthesis of ribonuclease A by Yajima and Fujii (1981). Oxidized-reduced glutathione is considered to accelerate the disulfide forming reaction by S-S exchange and is often used as an additive for disulfide formation involving higher peptides and proteins (Szajewski and Whitesides, 1980). In particular, Chavez and Scheraga (1980) and Ahmed *et al.* (1975) studied ribonuclease A intensively and found that the addition of glutathione increased the reaction yield by approximately 20% in comparison to the reaction without glutathione.

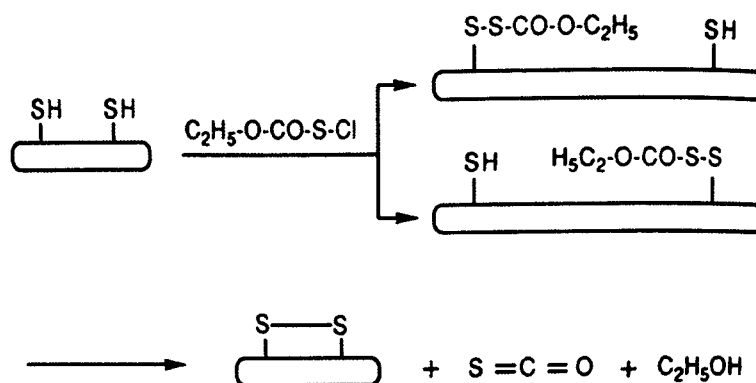
Although the above-mentioned high-dilution air oxidation in the presence of glutathione is a generally applicable method, glutathione interferes with proper disulfide formation in some cases. Yajima *et al.* (1984, 1985a) reported that the correct disulfide bonds are not formed by air oxidation in the presence of glutathione in the synthesis of mouse EGF, which has 53 amino acid residues and three disulfides. Reduced EGF has a high tendency to undergo association and polymer formation during air oxidation. Air oxidation of reduced EGF without glutathione does give the objective peptide, although the yield is low (4.1%). However, in the presence of glutathione, air oxidation gives exclusively glutathione-EGF adducts without any formation of the desired peptide. Heath and Merrifield (1986) also observed such glutathione adduct formation and association in solid-phase synthesis of mouse EGF. Addition of a denaturant to the air oxidation reaction mixture is also often used.

Human, rat, and bovine TGF- α have 50 amino acid residues and three intramolecular disulfide bonds, and they have been synthesized on a solid phase separately by the groups of Tam, Kent, and Tou (Monsanto). All three groups added a denaturant such as urea or guanidine during the air oxidation reaction. Kent and co-workers (Woo *et al.*, 1989) used 1 M guanidine hydrochloride in the synthesis of human TGF- α . They reported that air oxidation without guanidine gave a mixture which showed a complicated elution pattern on HPLC. In the syntheses of human and rat TGF- α by Tam's group (Tam *et al.*, 1986; Tam, 1987) and of bovine TGF- α by the Monsanto group (Tou *et al.*, 1990), the urea concentration of the reaction mixture in which the reduced TGF α was dissolved was gradually reduced by dialysis, and finally air oxidation was performed in the presence of 1–2 M urea.

In the air oxidation of TGF- α , oxidized–reduced glutathione is also added to the reaction mixture to facilitate disulfide formation by S–S exchange reaction. The Monsanto group reported that the overall yield without glutathione in the air oxidation step was 57% of that with glutathione. In these syntheses overall yields remained between 4.5 and 10%, whereas Tam *et al.* (1986) described a slightly higher yield. Further attempts included synthesis of a peptide corresponding to positions 26 to 81 of Shope fibromavirus growth factor (SFGE) using the same method (Lin *et al.*, 1988) as used for the synthesis of human and rat TGF- α . However, most of the reduced peptides were converted to the insoluble associated form. Although SFGE(26–80) is of similar size and has similar disulfide positions as does TGF- α , the reduced SFGE(26–80) peptide precipitates in 5 M urea. Tam *et al.* (1986) finally obtained the desired product by air oxidation in 1.5 M guanidine hydrochloride solution (peptide concentration of 0.1 mg/ml) but in only 3% overall yield.

As usual air oxidation reactions require a considerably long time, ranging from 1 to several days, oxidants such as $K_3Fe(CN)_6$ are often used to facilitate disulfide formation. This method is often employed in the synthesis of peptides with a single disulfide bond (Minamitake *et al.*, 1990; Spear *et al.*, 1989; Kazmierski *et al.*, 1988).

Le-Nguyen and Rivier (1986) used carboethoxy sulfonyl chloride (Sec-Cl) as an additive to accelerate the oxidation reaction, effectively utilizing the disulfide exchange reaction (Scheme 18). When this reagent was applied to the synthesis of turnip trypsin inhibitor containing 28 amino acid residues and three intramolecular disulfide bonds, the oxidation reaction was completed within 10 min, but the product yield was lowered by approximately 25% compared to usual air oxidation (50 hr) (Le-Nguyen *et al.*, 1989). Air oxidation has been employed in syntheses of many peptides with 20 to 50 residues and two or three disulfide bonds, such as conotoxins (Cruz *et al.*, 1989) and echistatin (Garsky *et al.*, 1989). The methods of syntheses of relatively small peptides with a single disulfide bond are extensively described in a review by Hiskey (1981).



Scheme 18 Disulfide bond formation using Scc-Cl.

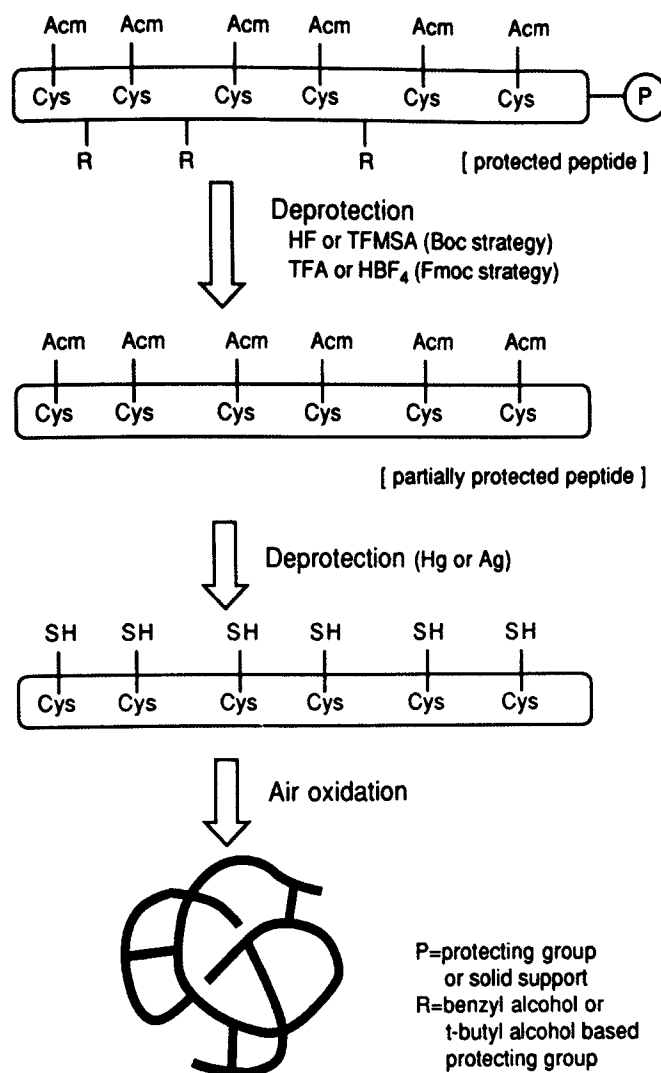
2. Disulfide Formation by Two-Step Deprotection

Scheme 19 shows a synthetic scheme for disulfide formation by two-step deprotection. A protected peptide chain is synthesized by the solid-phase or solution method based on either the Boc strategy (using benzylalcohol-type protecting groups) or the Fmoc strategy (*tert*-butylalcohol-type protecting groups). The Ac₂m group (Veber *et al.*, 1968) is used as the thiol protecting group of cysteine.

At the first deprotection step, protecting groups on side-chain functional groups are cleaved by acidolysis. This deprotection gives the thiol-protected peptide because the Ac₂m group is resistant to acid but is cleavable by Hg(OAc)₂ or I₂ (Ac represents acetyl). The trimethylacetamidomethyl (T_{ac}m) group (Kiso *et al.*, 1990b), which has chemical properties similar to those of Ac₂m, is also used for this type of synthesis.

Subsequently, after the T_{ac}m group is cleaved from the thiol-protected peptide, air oxidation forms the required disulfide bridges. This method has the advantage that the thiol-protected peptide can be purified before air oxidation. The syntheses of disulfide-containing higher peptides or proteins carry the possibility that the proper disulfide bridges will not be formed when purification is insufficient. In fact, Kenner's group (Galpin *et al.*, 1981) reported that the active peptide could not be obtained because of the insufficient removal of eight Ac₂m groups by Hg(OAc)₂ in the synthesis of a lysozyme analog consisting of 129 amino acids and containing four disulfides. It is presumed that the purification of protected peptides before the final deprotection was insufficient. The progress of purification methods using HPLC has made it possible to synthesize small proteins up to 123 residues long.

Removal of the Ac₂m or T_{ac}m groups used in this method is conducted by mercury salt such as Hg(OAc)₂ (Veber *et al.*, 1972) or Hg(OCOCF₃)₂ (Fujino and Nishimura, 1976; Nishimura *et al.*, 1978) or silver salt. Hg(OAc)₂ is most commonly used. The peptide mercuric salt obtained by deprotection is treated with a thiol compound such as mercaptoethanol to give a reduced peptide with free thiol groups, which is then subjected to air oxidation. Generally, mercuric compounds are highly toxic and require careful handling. Therefore, silver salts have been developed to remove the Ac₂m group. The counteranion of the silver cation greatly influences cleavage of the thiol protecting group, and silver trifluoromethanesulfonate (AgOTf; Fujii *et al.*, 1989) or silver tetrafluoroborate (AgBF₄; Yoshida *et al.*, 1990a) have been used in practical syntheses.

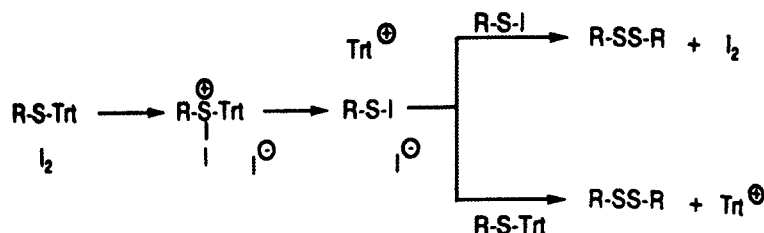


Scheme 19 Disulfide bond formation by two-step deprotection.

In contrast to mercuric compounds, AgOAc cannot cleave AcM. The AcM group is cleavable by iodine as mentioned below, although modification of tryptophan and oxidation of methionine also occur. The side reactions are largely avoided in the synthesis of Trp- or Met-containing peptides using two-step deprotection by AgBF₄ (Yoshida *et al.*, 1990c). AgOTf has been applied to the synthesis of chicken calcitonin (Fujii *et al.*, 1989), endothelin (Nomizu *et al.*, 1989), and tachyplesin (Akaji *et al.*, 1989), among others, and AgBF₄ has been used in the synthesis of somatostatin (Yoshida *et al.*, 1990c), porcine brain natriuretic peptide (pBNP-32; Yoshida *et al.*, 1990b), and others.

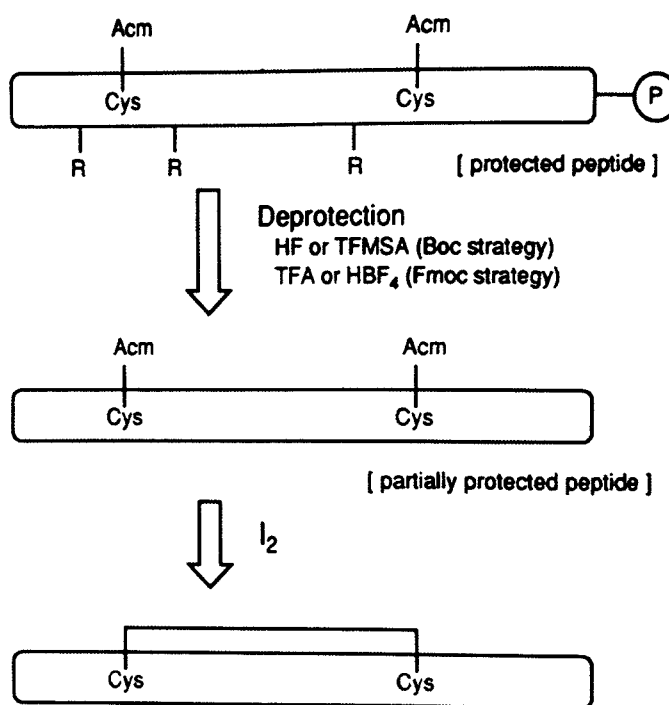
C. Disulfide Formation by Iodine Oxidation

The iodine oxidation method is based on the iodine treatment of Cys(AcM) or Cys(Trt) derivatives (where Trt is trityl) to give cystine via sulphenyl iodide (Scheme 20) (Kamber *et al.*, 1980). The general synthetic scheme using iodine



Scheme 20 Mechanism of the iodine oxidation method.

oxidation is illustrated in Scheme 21. As in the case of the two-step deprotection mentioned above, the peptide chain is constructed with AcM for thiol protection and then subjected to acidolysis to produce the thiol-protected peptide. Subsequent iodine treatment simultaneously facilitates cleavage of AcM groups and disulfide formation. The advantages of this method are the purification before SS formation and the simultaneous thiol deprotection and SS formation.



P=protecting group or solid support

R=benzyl alcohol or t-butyl alcohol based protecting group

Scheme 21 Disulfide bond formation by iodine oxidation.

However, disulfide formation by iodine is not a regioselective reaction, so it is not easy to build up more than two disulfides in one step. In fact, in the synthesis of human TGF- α with three disulfides by Scanlon *et al.* (1987), using iodine treatment of AcM removal and disulfide formation, gave a mixture of many products. The desired peptide was obtained by reoxidation after reduction, but

the overall yield remained as low as 0.1%. Because iodine treatment was reported (Yoshida *et al.*, 1990c) to be accompanied by oxidation of methionine and modification of tryptophan (mentioned later), the method shown in Scheme 21 has been mainly applied to the synthesis of peptides containing one cystine and lacking tryptophan and methionine. However, in the case of small proteins such as HIV protease (99 amino acids), it is difficult to apply the method, owing to very slow cleavage of Ac₁m by iodine (Nutt *et al.*, 1988).

Natriuretic peptides (Lyle *et al.*, 1987), BNP (Kiso *et al.*, 1990c), and some other peptides have been synthesized by the iodine method. More recently, iodine oxidation has been used instead in the stepwise disulfide formation mentioned in the following section, whereas mercuric salts and silver salts are exclusively employed for the deprotection of Ac₁m groups.

D. Stepwise Disulfide Formation

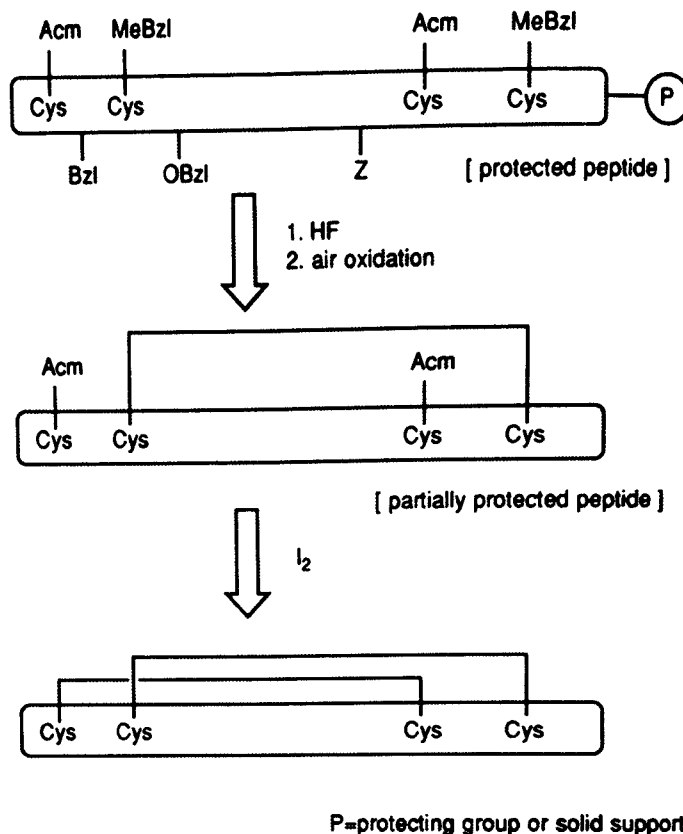
The methods described so far construct disulfide bonds in one step, whereas the stepwise method builds one disulfide bridge at each step. Theoretically, disulfide bonds can be formed at any intended positions by this method. Therefore, stepwise disulfide formation has been applied to the syntheses of newly isolated cystine-containing peptides and isomers having different disulfide positions, and thus has been used for the determination of disulfide positions in the native form (Iwai *et al.*, 1989).

In this method, each disulfide is bridged regioselectively. The second and subsequent disulfides must be formed under conditions which do not cleave the already connected disulfide bonds. Thus, alkaline conditions, use of thiol reagents, or long reaction times with the possibility of SS exchange should be avoided. Because of these limitations, established procedures to construct more than three disulfides have not yet been reported, although several papers have used the method to build two disulfide linkages using a combination of two types of protecting groups cleavable by different reagents. Three types of combinations of pairs of protecting groups are discussed in this section.

1. Combination of 4-Methylbenzyl and Acetamidomethyl Groups

The common MeBzl–Ac₁m combination is used in the stepwise disulfide forming method using air oxidation and iodine oxidation. The general synthetic scheme is shown in Scheme 22. The protected peptide chain is built up using MeBzl and Ac₁m groups for the protection of thiols corresponding to the two disulfide positions, and benzyl alcohol-type groups for the protection of other side-chain functional groups. HF treatment cleaves MeBzl and benzylalcohol type groups to give the partially protected peptide with two free SH groups, and subsequent air oxidation forms the first disulfide bond. $K_3Fe(CN)_6$ is added to accelerate the reaction at this stage. Tryptophan is often protected by the formyl group to avoid modification of the indole ring during iodine treatment to form

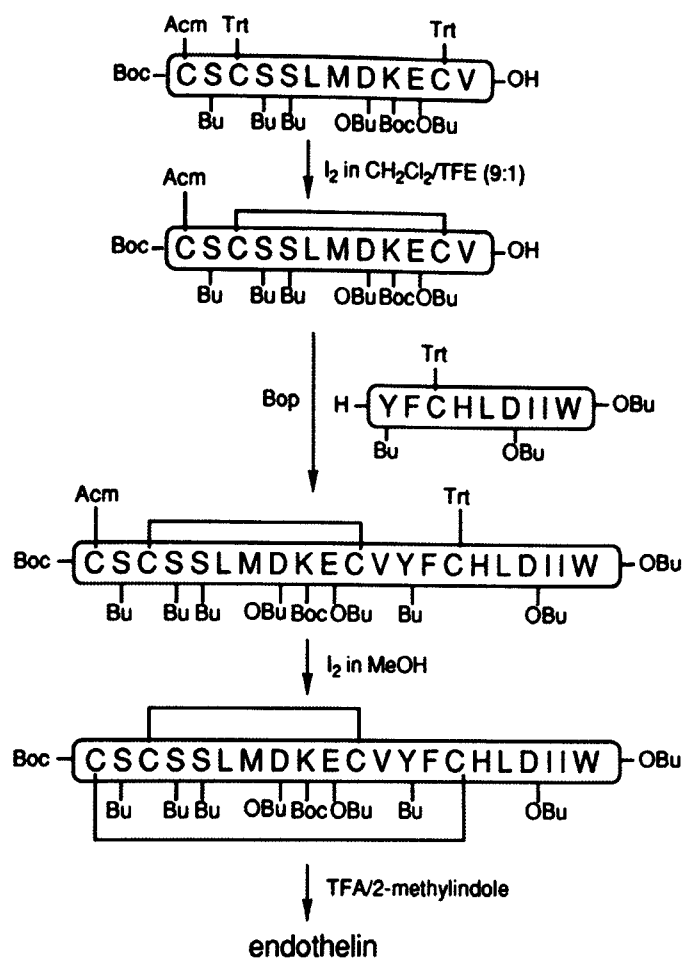
the second disulfide. The formyl group is cleavable by brief treatment with NaOH after formation of two disulfide bonds. This method was applied to the synthesis of endothelin (Kumagaye *et al.*, 1988), conotoxins and isomers with different disulfide positions (Nishiuchi and Sakakibara, 1982, 1983; Almquist *et al.*, 1989), mast cell degranulating peptide (Buku *et al.*, 1989), and others.



Scheme 22 Stepwise disulfide formation from MeBzl- and AcM-protected cysteines.

2. Combination of Trityl and Acetamidomethyl Groups

As mentioned in the section on iodine oxidation (Section III,C), the reaction rate of oxidative cleavage by iodine is greatly influenced by the reaction solvent (Scanlon *et al.*, 1987). Immer *et al.* (1988, 1989) utilized these properties to perform stepwise disulfide formation by selectively forming the first disulfide by cleavage of trityl group by iodine, leaving the AcM group intact. Endothelin and sarafotoxin S6b were synthesized by this method. The synthetic scheme for endothelin is illustrated in Scheme 23. The first disulfide is formed between two Cys(Trt) residues by treatment with iodine in methylene chloride-trifluoroethanol (TFE) for 5 min to cleave the Trt group, and then the second disulfide is formed between Cys(Trt) and Cys(AcM) by treating with iodine in methanol for 5 min. The yields of cyclizations are 70% for the first disulfide and 65% for the second.

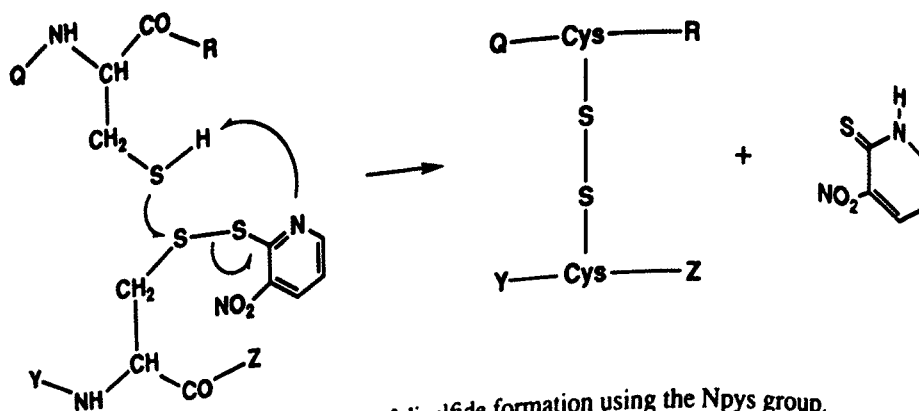


Scheme 23 Synthesis of endothelin using Trt- and AcM-protected cysteines.

3. Combination of 3-Nitro-2-pyridosulfenyl and Acetamidomethyl Groups

3-Nitro-2-pyridosulfenyl (Npys), a thiol protecting group developed by Matsueda and Walter (1980), is stable under acidic conditions and is cleavable by tri-*n*-butylphosphine in the presence of water (Matsueda *et al.*, 1981). It has another important characteristic in that it reacts readily with free sulfhydryl groups to form disulfide bonds (Matsueda *et al.*, 1982; Bernatowicz *et al.*, 1986) (Scheme 24). This property is applicable to regioselective intermolecular disulfide formation between Cys(Npys) and Cys with a free sulfhydryl group (Ruiz-Gayo *et al.*, 1988).

Sakakibara's group (Chino *et al.*, 1986) applied this stepwise method to the solution-phase synthesis of β -human atrial natriuretic peptide (β -hANP) with an antiparallel dimeric structure. They formed the first disulfide between Cys(Npys) and Cys, and the second by iodine oxidation. The free cysteine for the first step is generated by HF treatment of Cys(MeBzl). Albericio *et al.*

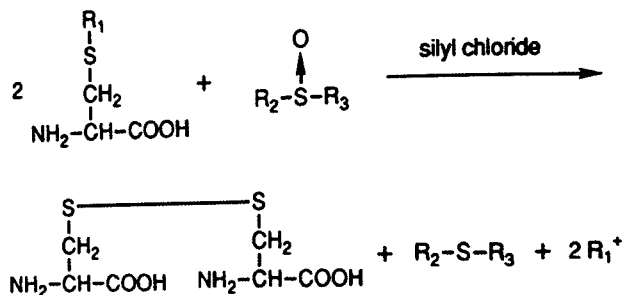


Scheme 24 Mechanism of disulfide formation using the Npys group.

(1989) intensively examined the stability of the Npys group under the conditions used in solid-phase synthesis.

E. Disulfide Formation by Silyl Chloride–Sulfoxide System

Akaji *et al.* (1991, 1992a,b, 1993a,b) demonstrated that methyltrichlorosilane in the presence of diphenylsulfoxide can cleave various thiol protecting groups of cysteine to form cystine directly (Scheme 25). This reaction is rapid and completed within 10 to 30 min in TFA. Most of the nucleophilic amino acids can be recovered unchanged after treatment with chlorosilane.



Scheme 25 Disulfide bond formation using silyl chloride–sulfoxide system.

1. Disulfide Bond Formation Using the Silyl Chloride–Sulfoxide System

As summarized in Table I, all three protecting groups of the amidomethyl type, namely, Ac_m (Veber *et al.*, 1972), Tac_m (Kiso *et al.*, 1990b), and benzamidomethyl (Bam; Chakravarty and Olsen, 1978), were cleaved to form cystine as a sole product within 30 min at 4°C. Among the benzyl type protecting groups, MeOBzl (Akabori *et al.*, 1964) was cleaved quantitatively to form cystine within 10 min at 4°C; however, MeBzl (Erickson and Merrifield, 1973) was cleaved incompletely, with 26% of the parent compound remaining after the 30 min treatment, and no cleavage of 4-nitrobenzyl (NO₂Bzl; Bachi and Ross-Petersen, 1972) was observed even after the 60 min treatment. Similarly, *t*-Bu

Table I Cleavage of Various Thiol Protecting Groups of Cysteine and Formation of Cystine by Methyltrichlorosilane–Diphenylsulfoxide at 4°C^a

Protected Cysteine	10 min		30 min		60 min	
	Starting compound	Cystine	Starting compound	Cystine	Starting compound	Cystine
	(%)	(%)	(%)	(%)	(%)	(%)
Cys(Acm)	15	79	0	93		
Cys(Tacm)	9	82	0	88		
Cys(Bam)	4	98	0	100		
Cys(<i>t</i> -Bu)	0	99				
Cys(<i>i</i> -Pr)	94	0	93	0	100	0
Cys(MeOBzl)	0	95				
Cys(MeBzl)	51	49	26	76	16	83
Cys(NO ₂ Bzl)	97	0	96	0	95	0

^aAcm, Acetamidomethyl; Tacm, trimethylacetamidomethyl; Bam, benzamidomethyl; MeOBzl, 4-methoxybenzyl; MeBzl, 4-methylbenzyl; NO₂Bzl, 4-nitrobenzyl.

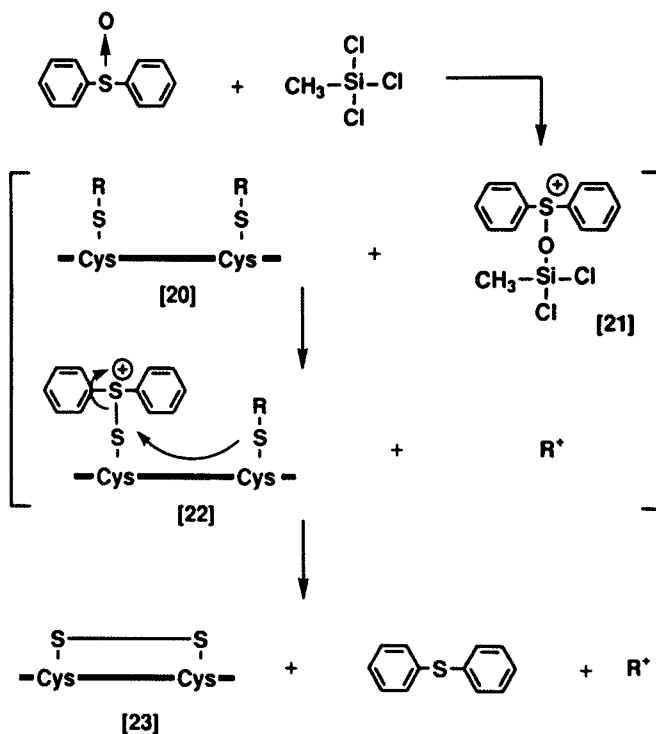
(Callahan *et al.*, 1963) was cleaved quantitatively within 10 min, whereas the more acid-stable isopropyl group (*i*-Pr; Gawron and Lieb, 1952) remained intact even after 60 min of treatment with silyl chloride–sulfoxide. Thus, the acid stability of the S-protecting group appears to influence the rate of cystine formation by silyl chloride–sulfoxide. Among the benzyl and alkylthioether type S-protecting groups, only those stable to HF were quantitatively recovered unchanged. Other less acid-stable S-protecting groups were cleaved to form cystine in more than 80% yield.

Met, His, and Tyr, as well as cystine, were recovered quantitatively even after 60 min of treatment with methyltrichlorosilane–diphenylsulfoxide at 25°C (Table II). No Met(O) was formed. Quantitative recovery of Trp(CHO) (Previero *et al.*, 1967) under the same reaction conditions was also confirmed.

2. Possible Mechanism

A proposed pathway for the synthesis of cystine peptides using the silyl chloride–sulfoxide system is shown in Scheme 26. The first interaction should be the formation of a sulfonium cation (**21**) derived from diphenylsulfoxide and an oxygenophilic silyl compound. The formation of sulfonium ions of this type has been shown and utilized for the reduction of sulfoxides using the trimethylsilylchloride/thiol system (Numata *et al.*, 1979). Subsequent electrophilic attack of **21** on the sulfur atom of S-protected cysteine (**20**) will lead to the formation of intermediate **22**. The nature of the silyl chloride employed should be

one of the main factors which influence the electrophilicity of **21**. The assumed intermediate sulfenyl compound (**22**) may then function as the electrophile and react with another S-protected cysteine to provide disulfide **23** and diphenylsulfide. This final step would be analogous to the reaction of a sulfenyl iodide with an S-protected cysteine in the iodine oxidation reaction studied by Kamber *et al.* (1980). In addition, the mechanism suggests that the ability of the S-protecting group to form a stabilized moiety on cleavage of the bond between the sulfur atom and itself, like the iminium ion from the S-Acm group (Kamber *et al.*, 1980), may influence the rate of disulfide bond formation in the silyl chloride-sulfoxide system. We consider the proposed mechanism to provide an explanation for our observation that the reaction rate of cystine formation largely depends on the nature of the silyl chloride and the S-protecting group.



Scheme 26 Possible mechanism of disulfide formation by silyl chloride-sulfoxide system.

3. Synthesis of Human Brain Natriuretic Peptide (hBNP)

Human BNP consists of 32 amino acid residues, including two Met and a His residue, and it contains one disulfide bond in the molecule (Sudoh *et al.*, 1989) (Fig. 10). The synthesis of hBNP appears to be a suitable test for the silyl chloride-sulfoxide method in practical synthesis of a cystine-containing peptide, as we have observed the formation of a large amount of the Met(O) derivative during synthesis of the structurally homologous porcine BNP using the iodine oxidation method (Yoshida *et al.*, 1990c).

The S-protected hBNP in TFA was treated with methyltrichlorosilane-diphenylsulfoxide at 25°C for 10 min to form the disulfide bond. The

Table II Recoveries of Various Nucleophilic Amino Acids After Treatment of Boc-Cys(Acm)-OH with Methyltri-chlorosilane-Diphenylsulfoxide

Amino acid	30 min	60 min
Cys(Acm)	0	0
Cystine	1.05	1.02
Met	1.02	1.02
His	1.00	1.01
Tyr	1.02	1.02
Trp(CHO)	0.92	0.95
Gly ^a	1.00	1.00

^a Internal standard.

crude product gave a single main peak on HPLC, showing that no significant modification occurred on the peptide chain. After purification, the homogeneous peptide had physicochemical properties identical to those of an authentic sample prepared by air oxidation (Yoshida *et al.*, 1990c).

4. Directed Formation of Double Intrachain Disulfides

Using silyl chloride, we have established a scheme for the directed formation of double intrachain disulfide bonds and synthesized conotoxin M1 (McIntosh *et al.*, 1982), a peptide neurotoxin consisting of 14 amino acid residues and containing two disulfide bonds per molecule (Scheme 27). The S-protected conotoxin M1 (**24**) was treated with iodine at 25°C for 15 min to construct the first disulfide bond between the cysteine residues protected by Acm groups, leaving the two *t*-Bu groups intact. The product (**25**) in TFA was then treated

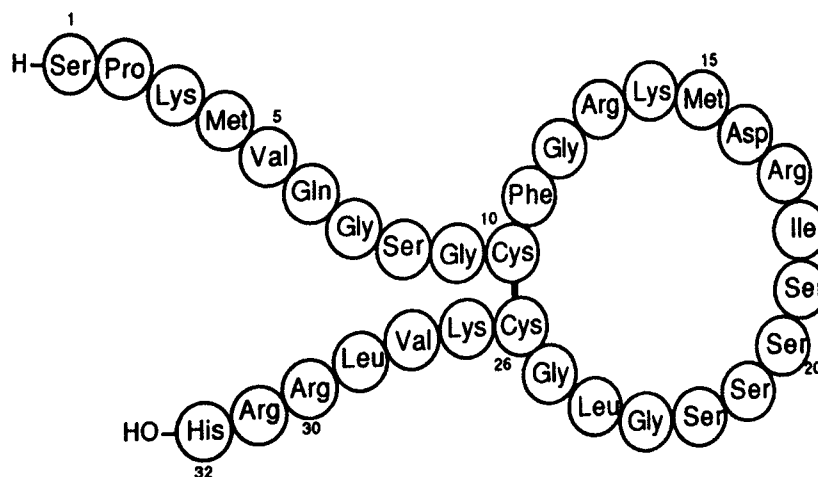
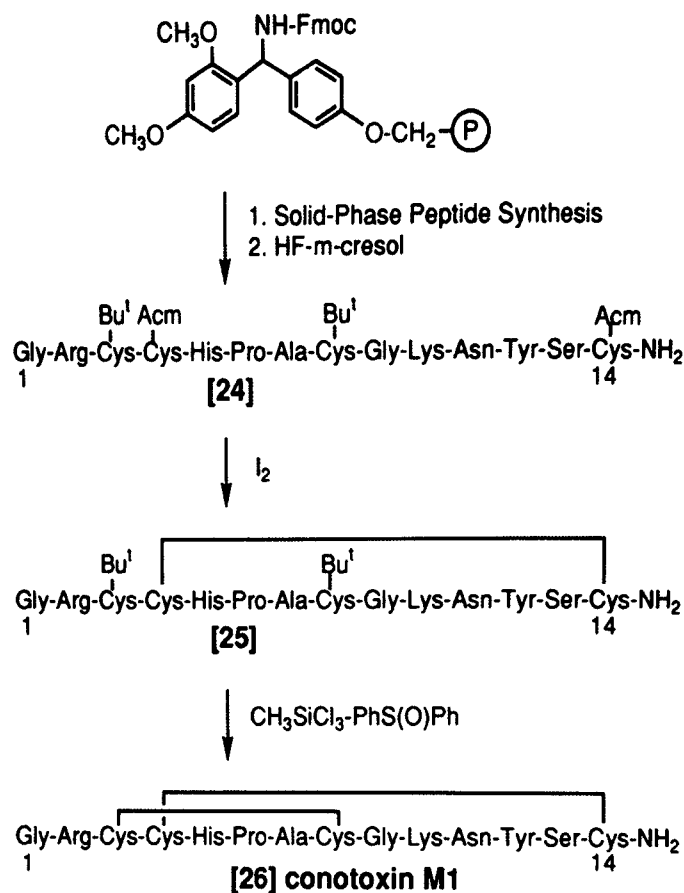


Figure 10 Amino acid sequence and disulfide pairing of hBNP.

with CH_3SiCl_3 in the presence of PhS(O)Ph at 25°C for 10 min to construct the second disulfide bond between the cysteine residues with the *t*-Bu groups. The crude product (26) gave a single main peak on HPLC, showing that no significant disulfide exchange occurred during the silyl chloride treatment.

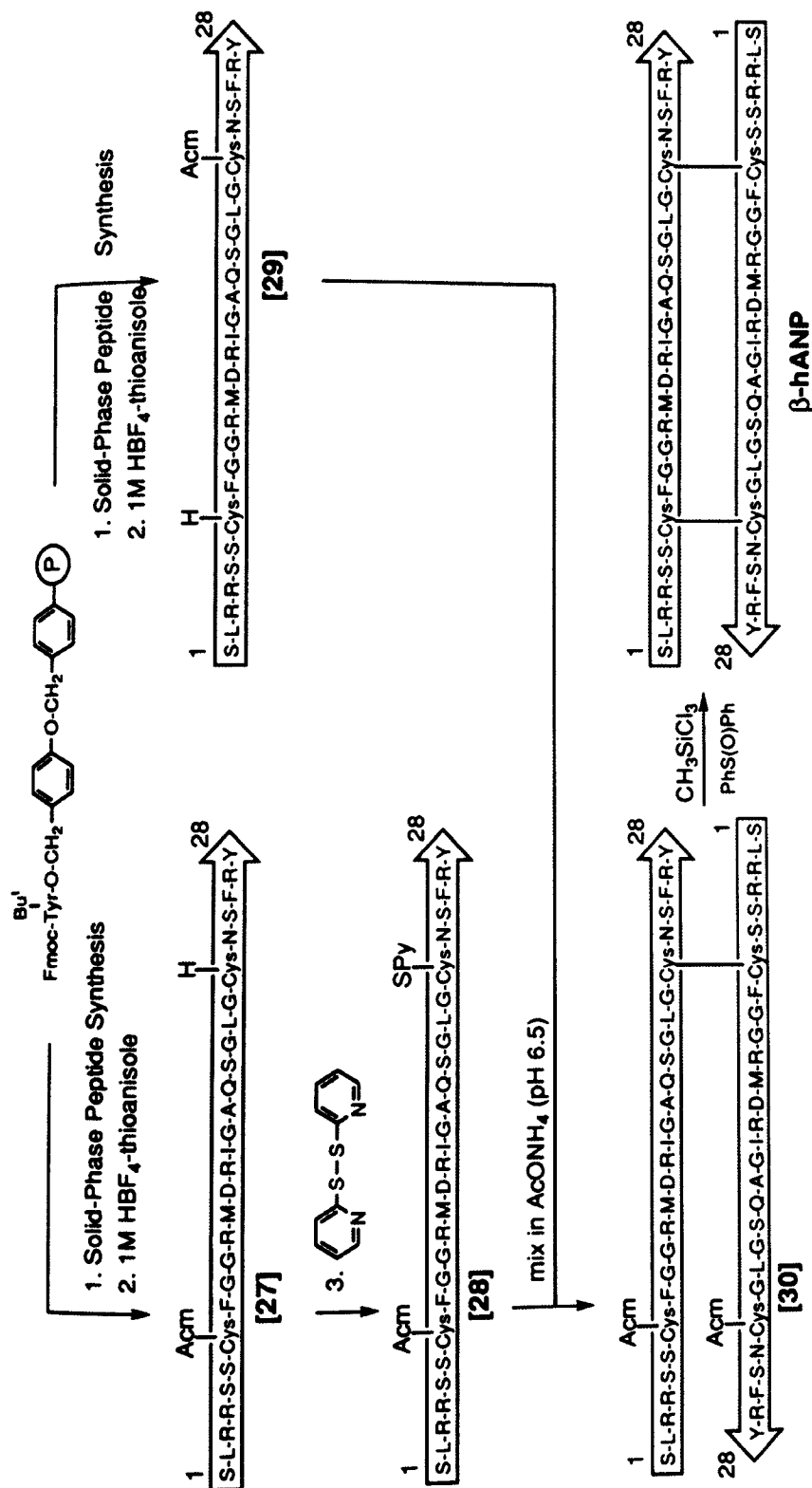


Scheme 27 Synthesis of conotoxin M1.

5. Directed Formation of Double Interchain Disulfides

Next, the silyl chloride method was applied to the stepwise formation of two interchain disulfide bonds. The Acm and Trt groups (Zervas and Photaki, 1962) were employed as the orthogonal thiol protecting groups of cysteine. The Trt group can be cleaved by a weak acid in the presence of scavengers (Pearson *et al.*, 1989; König and Kernebeck, 1979; Hiskey *et al.*, 1975; Photaki *et al.*, 1970), keeping the Acm group intact. By subsequent treatment with 2,2'-dithiodipyridine (Grassetti and Murray, 1967), the liberated thiol group can be converted to the corresponding *S*-2-pyridinesulphenyl cysteine [Cys(*S*-Py)] derivative. Thus, to achieve the directed formation of double interchain disulfides, the silyl chloride method was combined with disulfide formation by thiolysis of the *S*-2-pyridinesulphenyl cysteine. According to this scheme, we have synthesized β -hANP (Kangawa *et al.*, 1985), an antiparallel dimer of α -hANP (Scheme 28).

The [Cys(Acm)⁷, Cys²³]- α -hANP (27) was allowed to react with 2,2'-dithiodipyridine to give [Cys(Acm)⁷, Cys(*S*-Py)²³]- α -hANP (28), which was



Scheme 28 Synthesis of β-hANP by regioselective disulfide formation.

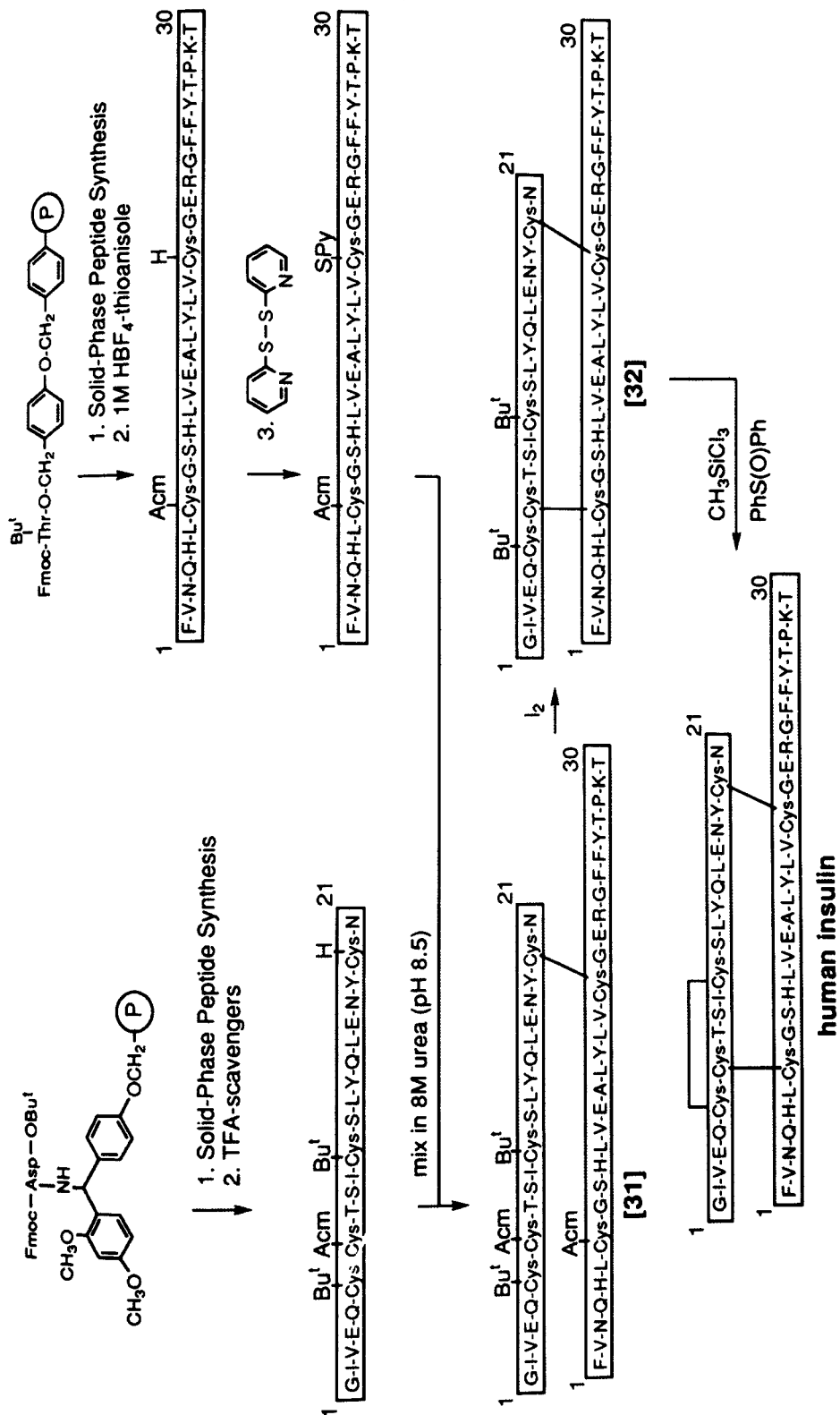
then mixed with [Cys⁷,Cys(Acm)²³]- α -hANP (29) at pH 6.5 for 30 min to construct the first disulfide bridge. After purification, the product (30) in TFA was treated with CH₃SiCl₃-PhS(O)Ph at 25°C for 10 min to form the second disulfide bond between the cysteine residues carrying the Acm groups. The crude product had a single main peak on HPLC, and no production of α -hANP was detected, indicating that no disulfide exchange had occurred during the silyl chloride treatment.

6. Regioselective Formation of the Three Disulfide Bonds of Human Insulin

By a combination of the above two schemes for directed double disulfide formation, we established a regioselective scheme to form the three disulfide bonds of human insulin, in which the disulfide bonds are constructed stepwise by successive reactions using thiolysis, iodine, and silyl chloride (Scheme 29). The three orthogonal thiol protecting groups of cysteine, Trt, Acm, and *t*-Bu groups are employed. As confirmed in both double disulfide formations, the Trt group is selectively cleavable with an acid in the presence of Acm and *t*-Bu groups, and the Acm group can be oxidatively cleaved with iodine to form cystine, leaving the *t*-Bu group.

According to this novel scheme, cleavage of the Trt group with a weak acid to liberate the cysteine side chain in position A20 and subsequent thiolysis of the *S*-2-pyridinesulfonyl cysteine in position B19 direct the formation of the first interchain disulfide bond (A20-B19). The second interchain disulfide bond (A7-B7) is formed between the cysteine residues carrying the Acm groups by treatment of the resulting [di-Acm, di-*t*-Bu, monodisulfide]human insulin (31) with iodine. The starting peptide (31) disappeared completely after the 60-min treatment to give a di-disulfide insulin (32). Finally, the product (32) was treated with silyl chloride to construct the intrachain disulfide bond (A6-A11) between the cysteine residues with the *t*-Bu groups. After the 15-min reaction, the starting peptide (32) disappeared completely to give a crude product which was easily purified by preparative HPLC. No significant formation of isomers or polymers was detected during the silyl chloride treatment. Synthetic human insulin gave a sharp single peak on an analytical HPLC column and had the same retention time as that of an authentic sample prepared by a semisynthetic method (Inouye *et al.*, 1979).

We consider that the scheme using silyl chloride makes it more feasible and efficient to construct three disulfide bonds regioselectively in peptides or proteins. The present regioselective method will be useful for the preparation of unnatural isomers with disulfide bonds at different positions and, hence, thermodynamical stability different from that of the corresponding natural peptide.



Scheme 29 Total synthesis of human insulin by regioselective disulfide formation.

Abbreviations

Acm	Acetamidomethyl
ANP	Atrial natriuretic peptide
Bam	Benzamidomethyl
Bm	<i>tert</i> -butoxymethyl
BNP	Brain natriuretic peptide
Boc	<i>tert</i> -butoxycarbonyl
BOI	2-(Benzotriazole-1-yl)-oxy-1,3-dimethylimidazolidinium hexafluorophosphate
Bom	Benzyloxymethyl
BOP	Benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
BOP-Cl	3,3'-(Chlorophosphoryl)bis(1,3-oxazolidin-2-one)
BPP5a	Bradykinin potentiating peptide 5a
BPTI	Bovine pancreatic trypsin inhibitor
<i>t</i> -Bu	<i>tert</i> -Butyl
Bzl	Benzyl
cHex	Cyclohexyl
CIP	2-Chloro-1,3-dimethylimidazolidinium hexafluorophosphate
Cl-Z	2-Chlorobenzyloxycarbonyl
Cl ₂ Bzl	2,6-Dichlorobenzyloxycarbonyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DCU	Dicyclohexylurea
DEPC	Diethylphosphorocyanidate
DIPCDI	Diisopropylcarbodiimide
DMAMP	4-(2',4'-dimethoxyphenylaminomethyl)phenoxy
DMF	Dimethylformamide
DPPA	Diphenylphosphorylazide
DSA	Dimethoxyalkylsulfinylbenzhydramine
DSB	4-(2,5-Dimethyl-4-methylsulfinylphenyl)-4-hydroxybutanoic acid
EEDQ	1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
EGF	Epidermal growth factor
Fmoc	9-Fluorenylmethoxycarbonyl
GIP	Glucose-dependent insulinotropic polypeptide
hANP	Human atrial natriuretic peptide
HATU	2-(1 <i>H</i> -7-azabenzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate
hBNP	Human brain natriuretic peptide
HBTU	2-(1 <i>H</i> -Benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate
HIV	Human immunodeficiency virus
HMPA	Hexamethylphosphoric triamide

HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	<i>N</i> -Hydroxybenzotriazole
HONB	<i>N</i> -Hydroxy-5-norbornene-2,3-dicarboximide
HOObt	3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriadine
HOSu	<i>N</i> -Hydroxysuccinimide
HOQ	Hydroxyquinoline
HPLC	High-performance liquid chromatography
IGF	Insulin-like growth factor
IIDQ	1-Isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline
Mbh	4,4-Dimethoxybenzhydryl
MBHA	<i>p</i> -Methylbenzhydrylamine
MeBzl	4-Methylbenzyl
MeOBzl	4-Methoxybenzyl
MSA	Methanesulfonic acid
Msob	4-Methylsulfinylbenzyl
MsZ	4-Methylsulfinylbenzyloxycarbonyl
Mtr	4-Methoxy-2,3,6-trimethylbenzenesulfonyl
Mts	Mesitylene-2-sulfonyl
Mtz	4-Methylthiobenzyloxycarbonyl
NDPP	Norborn-5-ene-2,3-dicarboximido diphenyl phosphate
NO ₂ Bzl	4-Nitrobenzyl
Npys	3-Nitro-2-pyridylsulfenyl
PAM	Phenylacetamidomethyl
pBNP	Porcine brain natriuretic peptide
Peoc	2-(Triphenylphosphino)ethyloxycarbonyl
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl
Ppt	Diphenylphosphinothioyl
<i>i</i> -Pr	Isopropyl
PyBOP	Benzotriazole-1-yloxytrispyrrolidinophosphonium hexafluoro-phosphate
PyBroP	Bromotrispyrrolidinophosphonium hexafluorophosphate
SCAL	Safety-catch acid labile
Sce-Cl	Carboethoxy sulfenyl chloride
SFGF	Shope fibromavirus growth factor
SPPS	Solid-phase peptide synthesis
Tacm	Trimethylacetamidomethyl
TBAF	Tetra- <i>n</i> -butylammonium fluoride trihydrate
Tcboc	2,2,2-Trichloro- <i>tert</i> -butoxycarbonyl
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid
TGF	Transforming growth factor
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TMSBr	Trimethylsilyl bromide
THF- γ_2	Thymic humoral factor γ_2

TOPPipU	2-[2-Oxo-1(2 <i>H</i>)-pyridyl]-1,1,3,3-bis(pentamethylenecarboxylate)tetrafluoroborate
Tos	<i>p</i> -Toluenesulfonyl
Troc	2,2,2-Trichloroethoxycarbonyl
Trt	Trityl
Z	Benzyloxycarbonyl
Z(OMe)	4-Methoxybenzyloxycarbonyl

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