Converting Peptides into Drug Leads by Lipidation

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Abstract: Lipidation is a posttranslational modification of proteins that has also found its use in designing peptide drugs. The presence of a lipid group in peptides modulates their hydrophobicity, secondary structures and self-assembling propensities while retaining their abilities to bind to target receptors. Lipidation improves peptides' metabolic stability, membrane permeability, bioavailability, and changes peptides' pharmacokinetic and pharmacodynamic properties. Herein, we review the applications of various lipidation strategies in peptide drug design, the effects of the chain length and anchor position of fatty acids in peptide lipidation, the physicochemical and biological properties of selected lipidated peptides and the synthesis strategies for peptide lipidation.

Keywords: ADME, lipidation, lipidated peptide, lipoamino acids, peptide, peptide SAR, peptide secondary structure, peptide self-assembling, peptide serum stability, peptide synthesis.

INTRODUCTION

Lipidation is a posttranslational modification of peptides and proteins. Some nonribosomally synthesized bacterial antibiotics [1] and ghrelin (octanoyl-Ser[3], Fig. **1a**) [2, 3] are naturally-occurring lipidated peptides. Protein lipidations, such as prenylation, *S*-acylation, *N*-myristoylation, *S*- and *N*-palmitoylation play important roles as posttranslational modifications of proteins to maintain and modulate their functions [4, 5]. For example, lipidation is necessary for RAS superfamily proteins to maintain their cellular functions [6]. Thus, due to the significant impacts on the properties of peptides following lipidation, this chemical modification has proven to be a useful tool in peptide drug design.

Peptide drugs offer many desirable drug properties such as potent bioactivity, high receptor binding affinity and specificity, and relatively low toxicity. However, most peptides are quickly degraded in serum and exhibit rapid clearance *in vivo*. Many strategies have been employed in an attempt to overcome these apparent disadvantages including lipidation, glycosylation, cyclization, backbone modification, unnatural or D-amino acid conjugation and PEGylation. Among these chemical modification methods, lipidation has proven to be one of the most robust strategies for the generation of new therapeutic peptide leads as discussed in this review paper. Lipidation can dramatically change peptides' physicochemical properties and pharmacological properties [7]. Additionally, lipidation increases peptides' plasma stability and decreases peptides' kidney clearance.

Four lipidized peptide drugs are currently approved for use in humans: two long-acting anti-diabetics human glucagon-like peptide-1 (GLP-1) analogue liraglutide (Victoza®, NN2211, Fig. **1b**) and insulin detemir (Levemir®, ($N^{\varepsilon,B29}$-tetradecanoyl)-des-(B30)-insulin, Fig. **1c**), and two antibiotics daptomycin and polymixin B. A number of lipidated peptides, lipoproteins and lipidated peptide vaccines such as Lymerix™ (against Lyme disease) are at different phases of clinical trials. As indicated in Table **1**, lipidation was extensively exploited in the drug developments of angiotensin II, desmopressin, enkephalin, galanin, neuropeptide Y (NPY), octreotide, thyrotropin releasing hormone (TRH), luteinizing hormone releasing hormone (LHRH), calcitonin, gastric inhibitory polypeptide (GIP), ghrelin, GLP-1, growth hormone-releasing hormone (GHRH), tetragastrin and insulin. In addition, lipidized peptides have also been used as protein function probes, gene delivery vectors and vaccine adjuvants [8, 9]; palmitoyl pentapeptide analogues were used in cosmetics [10].

Lipidation can be classified into three types based on lipid bond formation methods with peptide chain or side chains: amidation, esterification (*S*- or *O*-) and *S*-bond (ether or disulfide) formation. Amidation and *O*-esterification are strong and irreversible covalent bonds, whereas *S*-esterification and disulfide bond formation are weak and reversible covalent bonds. Non-natural lipoamino acids (LAA) (Fig. **2a**) were also extensively exploited in the development of peptide drugs and vaccines by the Toth group [59-62, 69-73]. Lipidation methods, alkyl chains, lipid anchor position and the spacer between lipids and peptides have significant impacts on the physicochemical properties, receptor binding affinities and bioactivities of lipidated peptides.

Several related review papers on protein lipidation [74-76], vaccine lipidation [77, 78], lipid core peptide delivery system [79], lipid-based delivery system [80, 81], anti-infective lipidated peptide drugs [82, 83], amphiphilic lipidated peptide self-assembling system [84], and lipopeptide molecular probes [85-87] were published. Herein, we review the peptide lipidation methods and discuss the structure-activity relationship (SAR) of peptide lipidation with particular focus on peptide fatty acid lipidation.

1. PHYSICOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF LIPIDATED PEPTIDES

1.1. Lipophilicity

Most naturally-occurring peptides are hydrophilic peptides. Lipidation provides a simple way to modulate peptide lipophilicity. Since lipophilicity affects the absorption, distribution, metabolism and excretion (ADME) and bioavailability of drugs, lipidation is an attractive strategy to convert peptides into drug leads. Partition between the aqueous phase and the membrane phase of drugs can be simulated through partition of drugs between two immiscible solvents [88]. The *n*-octanol-water partition coefficient (logD) is commonly used to evaluate the permeability of drugs across biological membranes. Many research groups have evaluated the hydrophobicity of lipidated peptides by determining their logD [8, 12, 14, 15, 18, 31, 55, 89, 90]. Lipidation of TRH with lauroyl (*N*-lauroyl-TRH, Fig. **2b**) increased the logD of TRH from 0.068 to 1.91 [38]. Lipidation of NPY-BBB1(K3) with palmitoyl (NPY-BBB1, Table **2**) changed the logD of NPY-BBB1(K3) from -1.38 to 2.82 [15]. All the palmitoyl lipidated galanin and neuropeptide W (NPW) analogous showed similar logD values, ~1.2 and ~1.6, respectively (Table **2**) [14, 31]. The logD was not affected by the anchor positions of the palmitoyl group on the KKKK-palmitoyl scaffold. More specifically, the logD was linearly related to the chain length of the fatty acids of the galanin lipidated Gal-B2 analogues from Gal-B2-C_8 to Gal-B2-C_{18} (Fig. **2c;** C_n, n is the carbon number of linear fatty acids) [18].

1.2. Secondary Structures

Lipidation can change the secondary structures of peptides through the hydrophobic interactions of the fatty acid chain with the

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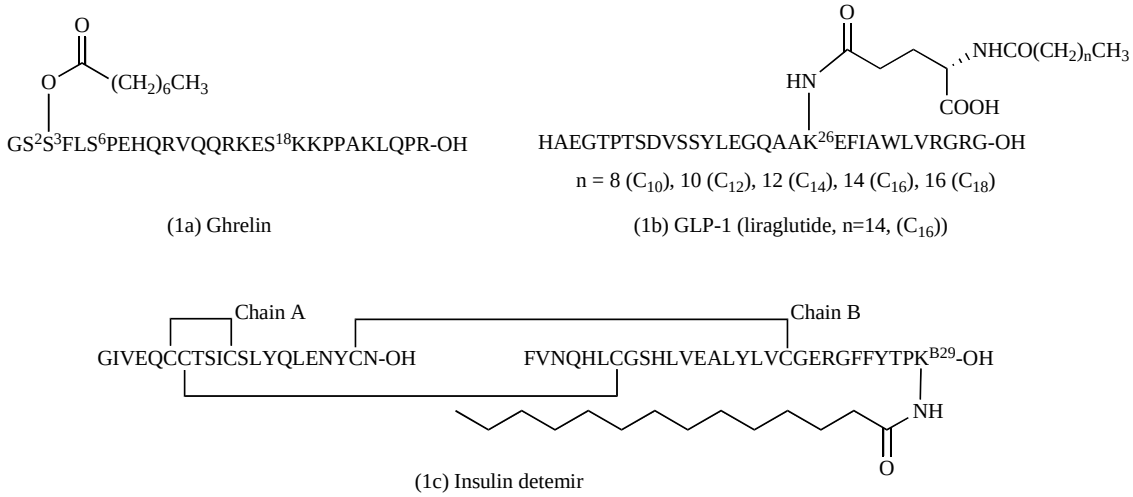


Fig. (1). Structures of ghrelin, GLP-1 (liraglutide) and insulin detemir.

Table 1. Lipidation Methods Applied in Peptide Modifications

Type			Peptides investigated
Fatty acid	linear	Amide	Enkephalin [11-13], Galanin [14-18], GIP [7, 19], GLP-1 (liraglutide) [20], hPP [21], IGF1R JM inhibitor [22], Insulin [23-29]; Leuteinizing hormone releasing hormone (LHRH) [30], NPW [31], NPY [15], NT [15], RC-160 [32, 33], STAT3 [34], Tetragastrin [35-37], Thyrotropin releasing hormone (TRH) [28, 30, 38, 39]
		Ester	Angiotensin II [40, 41], BBI [42-44], Desmopressin [45], Ghrelin [2, 46-48], Salmon calcitonin (sCT) [49-51], TRH [52], Octreotide [53]
	branched		Liraglutide [20]
Amino fatty acid			Conotoxin SIIIA [54], Ghrelin [46], Liraglutide [20], Met-Enkephalin [55],TAN-1511A [56], Tenecin-1 [57]
Acidic fatty acid (dicarboxylic acid)			Growth hormone-releasing hormone antagonists (GHRH) [58]
Halo-fatty acid			Ghrelin [46]
Lipoamino acid			Core peptide [59, 60], Thymopentin TP5 [61], Peptide A2 [62], Polycationic lipophilic core dendron excipient [63, 64]
Alkyl ether (*O*-, *S*-)			Angiotensin [41]
Alkyl amine			Compound 3 [65]
Adamantylation			Ghrelin [46]
Cholesterol			C34 [66], Insulin [67,68]

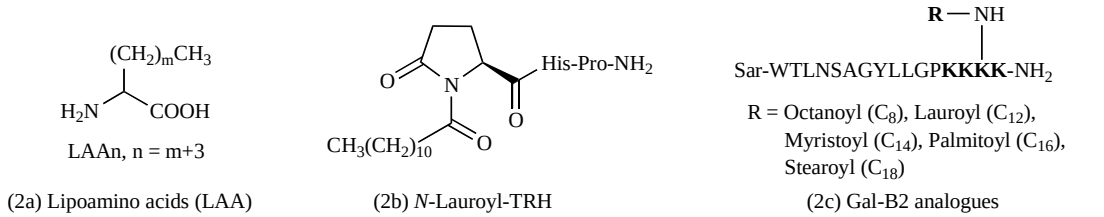


Fig. (2). Structures of lipoamino acids (LAA), *N*-lauroyl-TRH and Gal-B2 analogues.

peptide backbone or side chains [91, 92]. The lipidation analogues of random-coiled salmon calcitonin (sCT), REAL-sCT (Fig. **3a**) and Mal-sCT (Fig. **3b**) adopted α-helix secondary structures [49]. The octanoylation analogues on Lys^{16} or Lys^{28} of random-coiled human β-amyloid (1-40) (Fig. **3c**) adopted β-sheet structures [93]. Palmitoyl-kallidin (Fig. **3d**) adopted a non-ideal type I β-turn in the presence of dodecylphosphocholine (DPC) micelles while palmitoyl-Gly-kallidin adopted a γ-turn structure [94].

Long-chain fatty acid (>C_8) lipidations have strong effects on the secondary structures of peptides when compared to short-chain fatty acid lipidations, likely due to their strong hydrophobic interactions with peptides. For example, a random-coilded peptide **16r** acquired an α-helix structure after *N*-terminal lipidation with fatty acids (Fig. **3e**); the α-helical content of C_{16}**-16r** was higher than that of C_6**-16r** in water or 50% trifluoroethanol (TFE) [95]. The melting temperatures (T_m) of lipidated collagen derived peptides (Fig. **3e**) from triple helix state to random coil state were also correlated with the chain length of fatty acids [95]. The lipidated analogues of **16r**-Tyr-$SPARC_{119\text{-}122}$ (Fig. **3e**) showed similar increasing trends of α-helical contents and T_m with the elongation of fatty acid chain [96]. A de novo α-helix peptide **1α** (Fig. **3f**) acquired β-sheet conformations after *N*-terminal lipidation with long-chain fatty acids (from C_{12} to C_{16}), while the lipidated analogues of **1α** with short-chain fatty acids (from C_2 to C_{10}) resulted in random-coiled structures [97]. The analogues of a de novo α-helix dimer **2α** (Fig. **3f**) lipidated with long-chain fatty acids (from C_{12} to C_{16}) at its two *N*-termini maintained α-helix

Table 2. The SAR Study Analogues of Neuropeptide Galanin, NPW, NT and NPY (Lys-Pal, N^{ε}-Palmitoyl-Lysine; Ava, 5-Amino-Valeric Acid)

Analogue	Sequence
Gal-LAA [14]	(Sar)WTLNSAGYLLGP**HA(Lys-Pal)**-NH_2
Gal-(K)4	(Sar)WTLNSAGYLLGP**KKKK**-NH_2
Gal-B1	(Sar)WTLNSAGYLLGP**KKK(Lys-Pal)**-NH_2
Gal-B2	(Sar)WTLNSAGYLLGP**KK(Lys-Pal)K**-NH_2
Gal-B3	(Sar)WTLNSAGYLLGP**K(Lys-Pal)KK**-NH_2
Gal-B4	(Sar)WTLNSAGYLLGP**(Lys-Pal)KKK**-NH_2
Gal-B11	(Sar)WTLNSAGYLLG**KK(Lys-Pal)K**-NH_2
Gal-B12	(Sar)WTLNSAGYLL**KK(Lys-Pal)K**-NH_2
NPW-B2 [31]	Ac-WYK**(Lys-Pal)KKK**(Ava)GRAAGLLMGL-NH_2
NPW-B3	Ac-WYK**K(Lys-Pal)KK**(Ava)GRAAGLLMGL-NH_2
NPW-B4	Ac-WYK**KK(Lys-Pal)K**(Ava)GRAAGLLMGL-NH_2
NPW-B5	Ac-WYK**KKK(Lys-Pal)**(Ava)GRAAGLLMGL-NH_2
NT-BBB1(K4) [15]	Ac-**KKKK**PYIL-OH
NT-BBB1	Ac-**K(Lys-Pal)KK**PYIL-OH
NPY-BBB1(K3) [15]	Ac-Y**KKK(Ahx)**RHYINLITRQRY-NH_2
NPY-BBB1	Ac-Y**KK(Lys-Pal)(Ahx)**RHYINLITRQRY-NH_2
NPY-BBB2(K3)	Ac-Y**KKK(Ahx)A**RHYINLITRQRY-NH_2
NPY-BBB2	Ac-Y**KK(Lys-Pal)(Ahx)A**RHYINLITRQRY-NH_2

structures while the analogues of **2α** lipidated with short-chain fatty acids (from C_4 to C_{10}) induced β-sheet structures [97].

Lipid position was found to be important in stabilizing the conformations of lipidated peptides. The *N*-terminal lipidation analogue of α-conotoxin MII, LAA_{10}-MII (Fig. **3g**; $LAA_{10,}$ 2-amino-D,L-dodecanoic acid), exhibited consistent helical tertiary structure [69]. However, the analogue [Asn^5]LAA_{10}-MII (Fig. **3g**) lipidated in the middle of peptide chain did not adopt the bioactive conformation [69]. Lipidation of β-amyloid near one β-sheet region (Lys^{16}) or the bend region of the two β-sheets (Lys^{28}) yielded two analogues (C_8-Lys^{16})-β-amyloid(1-40) and (C_8-Lys^{28})-β-amyloid (1-40) (Fig. **3c**) with enhanced secondary conformations as compared to that of the native β-amyloid(1-40) [93]. Lipidation at the unstructured *N*-terminal region of β-amyloid, such as *N*-C_8-β-amyloid (1-40), did not affect the secondary conformations [93].

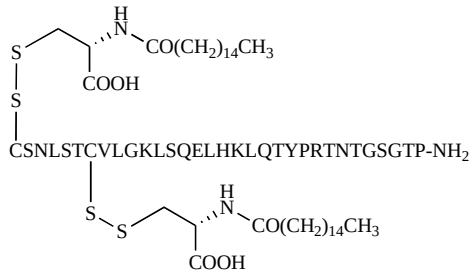
Unsaturated fatty acids and hydroxyl fatty acids exhibit less strong hydrophobic interactions with peptide chains when compared to the corresponding saturated fatty acids, resulting in less influences on the secondary structures of lipidated peptides. Oleoyl-**16r**-Tyr-$SPARC_{119-122}$ (Fig. **3e**) showed a lower helical content and T_m than that of C_{18}-**16r**-Tyr-$SPARC_{119-122}$ [96]. The helical content of ricinoleoyl-**16r**-Tyr-$SPARC_{119-122}$ is higher than that of oleoyl-**16r**-Tyr-$SPARC_{119-122}$ due to the compensation of the hydrogen bond of the hydroxyl group but is not strong than that of C_{18}-**16r**-Tyr-$SPARC_{119-122}$ [96].

The interactions of lipidated peptides with membranes significantly influence peptide secondary structures. SMOi2-9 (Ac-LTYAWHTSFKAL-NH_2) showed random conformations in aqueous solutions; lipidated peptide SMOi2-43 (Fig. **3h**) adopted a β-type conformation in aqueous solution and an α-helix conformation in DPC micelles [22]. *N*-Octanoyl-mastoparan-X (Fig. **3i**) and *N*-propanoyl-mastoparan-X adopted β-sheet conformations in HEPES solution. However, both analogues adopted α-helix conformations in 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) or 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) solutions with 50-60% α-helical contents [98]. The lipidated analogue of human lactoferrin C_{12}-LF11 (Fig. **3j**) adopted an α-helix conformation in DPC micelles but formed a loop in sodium dodecyl sulphate (SDS) micelles [99]. Lipidated peptides with specific conformation properties also appeared as useful molecular probes to study protein folding [100].

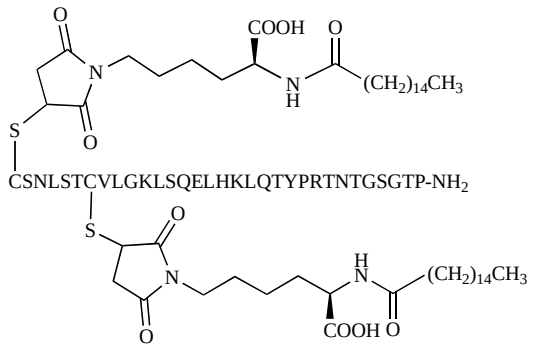
1.3. Oligomers and Self-Assembling Macromolecules

Lipidated peptides can form stable oligomers, self-assembling structures (such as micelles, tubules, vesicles, mono- or bilayers, nanofibers) and aggregates. *N*-Terminal lipidated analogues of dimer **2α** (from C_{10}- to C_{16}-, Fig. **3f**) formed stable oligomers [97]. Compound 3 (Fig. **4a**) formed hollow tubules or helical ribbons spontaneously; these self-assembled structures protected compound 3 from trypsin degradation [65]. Several reviews describing self-assembly mechanisms, compositions and applications of lipidated peptides were available [84, 101-104]. Herein, we briefly describe the effects of lipid anchor position and the chain length of fatty acids on the self-assembly and aggregation properties of lipidated peptide.

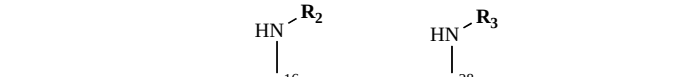
In lipidated peptides, long-chain fatty acid conjugations provide stronger hydrophobic driving force for aggregation formation as compared to short-chain fatty acid conjugations. *N*-Terminal lipidation of PA4 with hexanoic acid (C_6-) (Fig. **4b**) formed random size and shape precipitates at the concentrations as low as 1%, while *N*-terminal lipidation of PA4 with long-chain fatty acids (C_{10}-, C_{16}- or C_{22}-) formed different morphological nanofibers at the concentration of 0.25% [105]. In some cases long-chain fatty acids facilitated the formation of large particles regardless of the peptide sequences [95]. Lipidation strategies also have impacts on the particle formation of lipidated peptides. REAL-sCT (Fig. **3a**) and Mal-sCT (Fig. **3b**) formed particles with 30- to 40-fold differences in mean particle radii, 3.1 - 3.6 nm (11.2 - 1000 μM) and 105 - 134.3 nm (2.5 - 224 μM), respectively [49]. Lipidated analogues of the GBV-C/HGV NS3 protein fragments 505-514 (SAELSMQRRG) and 513-522 (RGRTGRGRSG) demonstrated different aggregation properties [106]. Palmitoyl-SAELSMQRRG formed fibrous aggregates in aqueous solution, whereas palmitoyl-RGRTGRGRSG failed to form any fibrous aggregates under the same conditions [106].



(3a) REAL-sCT



(3b) Mal-sCT



R_1 or R_2 or R_3 = C_8

(3c) beta-amyloid (1-40)

(3d) kallidin

RNH-KAEIEALKAEIEALKA-NH_2

16r

RNH-KAEIEALKAEIEALKAYKHGK-NH_2

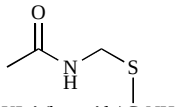
16r-Tyr-SPARC$_{119\text{-}122}$

RNH-(GP-Hyp)$_4$GVKGDKGDPGWPGAP(GP-Hyp)$_4$-OH

collagen derived peptides

R = Hexanoyl (C_6), C_8, C_{12}, C_{14}, C_{16}, C_{18}, Oleoyl, Ricinoleoyl; Hyp, Hydroxyproline

(3e) 16r and collagen derived peptides



RNH-(beta-Ala)ALEQKLAALEQKLA(beta-Ala)C-NH_2

1a

RNH-(beta-Ala)ALEQKLAALEQKLA(beta-Ala)C-NH_2

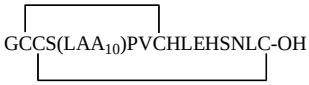
RNH-(beta-Ala)ALEQKLAALEQKLA(beta-Ala)C-NH_2

2a

R = Acetyl (C_2), Butanoyl (C_4), C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}

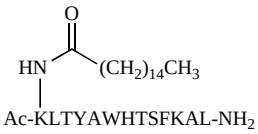
(3f) 1a and 2a

LAA$_{10}$-MII



[Asn^5]LAA$_{10}$-MII

(3g) LAA-MII



(3h) SMOi2-43

RNH-IDWKGIAAMAKKLL-NH_2

R = H, Propanoyl (C_3), C_8

(3i) Mastoparan-X

$CH_3(CH_2)_{10}CONH$-FQWQRNIRKVR-NH_2

(3j) C_{12}-LF11

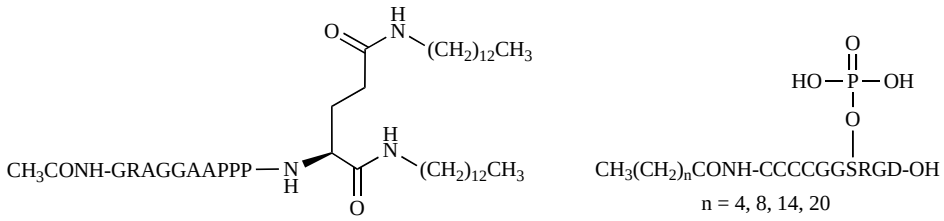
Fig. (3). Structures of the lipidated analogues of sCT (Real-sCT and Mal-sCT), β-amyloid, kallidin, 16r, collagen, 1α/2α, LAA-MII, SMOi2-43, mastoparan-X and C_{12}-LF11.

Lipid anchor positions have certain impacts on the critical micelle concentration (CMC) of lipidated peptides. For example, the CMCs of (N^{ε}-C_8-Lys^{16})-β-amyloid-(1-40) and (N^{ε}-C_8-Lys^{28})-β-amyloid-(1-40) (Fig. **3c**) were 10 μM and 8 μM, respectively; whereas the CMC of N-C_8-β-amyloid-(1-40) was equal to the CMC of the native β-amyloid (1-40), ~50 μM [93].

1.4. Membrane Binding

Lipidation affects peptide-membrane interactions [88]. The interactions may have tremendous effects on the physical properties of membranes, such as the fluidity, free volume, intrinsic curvature and morphologies of the membrane [106]. Lipidated analogues of the pentapeptide YIGSR-NH_2 (Fig. **5a**) showed weak disturbances in dipalmitoylphosphatidylcholine (DPPC) liposome bilayers due to the similarity of peptide lipid chains with DPPC lipid chains [107]. The interactions also affect the membrane trafficking, localization and signal transduction of lipidated peptides, as indicated in Ras proteins [108, 109].

The chain lengths of fatty acids have important impacts on peptide-membrane interactions. The fatty acid moieties of the lipidated analogues of tripeptide GHK (with C_6, C_{10} or C_{14}, Fig. **5b**) were inserted into the external bilayer of DPPC liposomes while the peptide chain protruded from the liposome surface [110]. The membrane binding energies of N-terminal acylated glycine analogues (N-acyl-Gly) were increased with the elongation of the



(4a) Compound 3 (4b) PA4

Fig. (4). Structure of compound 3 and PA4 analogues.

fatty acid chains [111]. The effective charges and the distance between membranes and peptide chains of lipidated peptides were decreased due to the strong hydrophobicity of lipidated peptides [98]. Lipidated analogues of mastoparan-X (Fig. **3i**) showed reduced free energy upon insertion in neutral POPC and negatively charged POPC/POPG (1:3) membranes as compared to that of the native mastoparan-X. Despite of the less hydrophobicity of propanoyl group compare to octanoyl group, *N*-propanoyl-mastoparan-X showed reduced free energy upon insertion of water into the membrane compared to *N*-octanoylation-mastoparan-X [98]. Lipidation may have negative effects on the membrane binding properties of lipidated peptides due to the increased aggregation propensity and the reduced effective monomer concentration [112].

1.5. Receptor Binding

The anchor positions and the chain lengths of fatty acids have great influence on the receptor binding ability of lipidated peptides. Lipidation on a noncritical terminus is an important strategy to maintain peptides' receptor binding ability. The *C*-terminus of endomorphin-1 is important to maintain its bioactivity; endomorphin-1-LAA_n (n = 8, 12; Fig. **6a**) showed decreased binding affinities to opioid receptors due to the blocking of the *C*-terminus whereas the affinities of LAA_8-endomorphin-1 were not affected [113]. DiC_{10}-Leu-enkephalin (Fig. **6b**) exhibited a remarkable decrease of the opioid receptor binding affinity due to the lipidation on the *N*-terminus which is critical for maintaining its bioactivity [13]. The strategy of lipidation on a noncritical terminus was applied in the modifications of galanin, NPY and neurotensin (NT) and yielded novel anticonvulsant neuropeptides without losing their receptor binding affinities [14, 15]. The lipidated galanin analogues Gal-B1 to Gal-B4 (Table **2**) showed a decreasing binding trend to the human GalR1 receptor (hGalR1) and GalR2 receptor (hGalR2) with the increasing distance between the palmitoyl moiety and the pharmacophore of galanin (pharmacophore: Sar-WTLNSAGYLLGP) [14].

Long-chain fatty acid lipidation can cause considerable decreases in peptide receptor binding affinity as compared to short-chain fatty acid lipidation. The hGalR1 receptor binding affinity of lipidated Gal-(K)4 analogues (from C_8 to C_{18}, Fig. **2c**) demonstrated a decreasing tendency with the elongation of the chain length of fatty acids; the hGalR1 receptor binding ability of C_{18}-Gal-B2 (*Ki* 4.0 ± 2 nM) was ten-fold less than that of Gal-(K)4 (*Ki* 0.4 ± 0.1 nM) [18]. Similarly, the somatostatin receptor affinities of C_4-RC-160 and C_{18}-RC-160 (Fig. **6c**) were decreased 10- and 30-fold, respectively, as compared to that of the native RC-160 [33].

Lipidation may have different receptor binding effects toward different receptor subtypes. [K^{13}(E-Pamitoyl), Q^{34}]hPP (Fig. **6d**), a lipidated analogue of hormone pancreatic polypeptide (hPP), showed a similar binding ability toward human neuropeptide Y2 and Y5 receptors and a modest decrease of binding ability toward human neuropeptide Y4 receptor as compared to that of the parent peptide [$K^{13,}$ Q^{34}]hPP [21]. The binding affinities of Gal-B2 toward hGalR1 and hGalR2 receptors were decreased modestly as compared to Gal-(K)4, 10- and 2-fold, respectively [14].

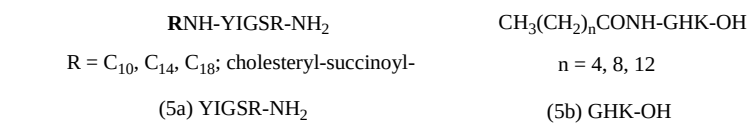
Despite the common negative effects, some positive effects of lipidation on peptides' receptor binding affinity were also reported in the literature. LAA_{10}-MII (Fig. **3g**) exhibited equal potency as the native MII in the inhibition of the neuronal nicotinic acetylcholine receptor subtype $\alpha3\beta2$ [69]. The lipidated analogues of RC-160 (with C_8-, C_{14}-, C_{16}-, Fig. **6c**) showed similar or modestly increased binding affinities toward somatostatin receptor as compared to that of the native RC-160 [32]. *N*-terminal lipidated GHRH antagonist analogues of JV-1-102 (from C_8- to C_{16}-, Fig. **6e**) exhibited 30- to 100-fold increases of binding abilities to the membrane receptors on rat anterior pituitary cells [58].

2. ADME PROPERTIES OF LIPIDATED PEPTIDES

2.1. Absorption

Lipidation has strong and diverse effects on the membrane permeabilities of lipidated peptides. The three lipidated tetragastrin (TG) analogues, C_2-TG, C_6-TG and C_{12}-TG (Fig. **7a**), have equal gastric acid output activities following *i.v.* administration [114]. However, the total gastric acid output amounts of C_2-TG and C_6-TG following large intestine loop administration were increased up to 133- and 74-fold, respectively, as compared to that of the native TG, whereas C_{12}-TG showed similar activity as the native TG [114]. The permeability across the upper small intestine of *N*-lauroyl-TRH (Fig. **2b**) was increased, but the permeabilities across the other portions of small intestine and large intestine were not affected [39].

Lipidation improves the *in vitro* and *in vivo* uptakes of lipidated peptides [115]. The transportations of tyrosine kinase inhibitors ABL-myristoyl (Fig. **7b**) and ABL-ss-myristoyl (Fig. **7c**) across B lymphocyte cell line BA/F3 were increased 12- and 22-fold, respectively, as compared to that of the native ABL [116]. The Caco-2 cellular uptake of [^{125}I]DiC_{10}-Leu-enkephalin (Fig. **6b**) was 131-fold higher than that of Leu-enkephalin [13]. C_2-TG and C_6-TG, exhibited increased penetration across the Caco-2 monolayers, the jejuna and colon tissues as compared to that of the native TG



(5a) YIGSR-NH_2 (5b) GHK-OH

Fig. (5). Structures of the lipidated analogues of YIGSR-NH_2 and GHK-OH.

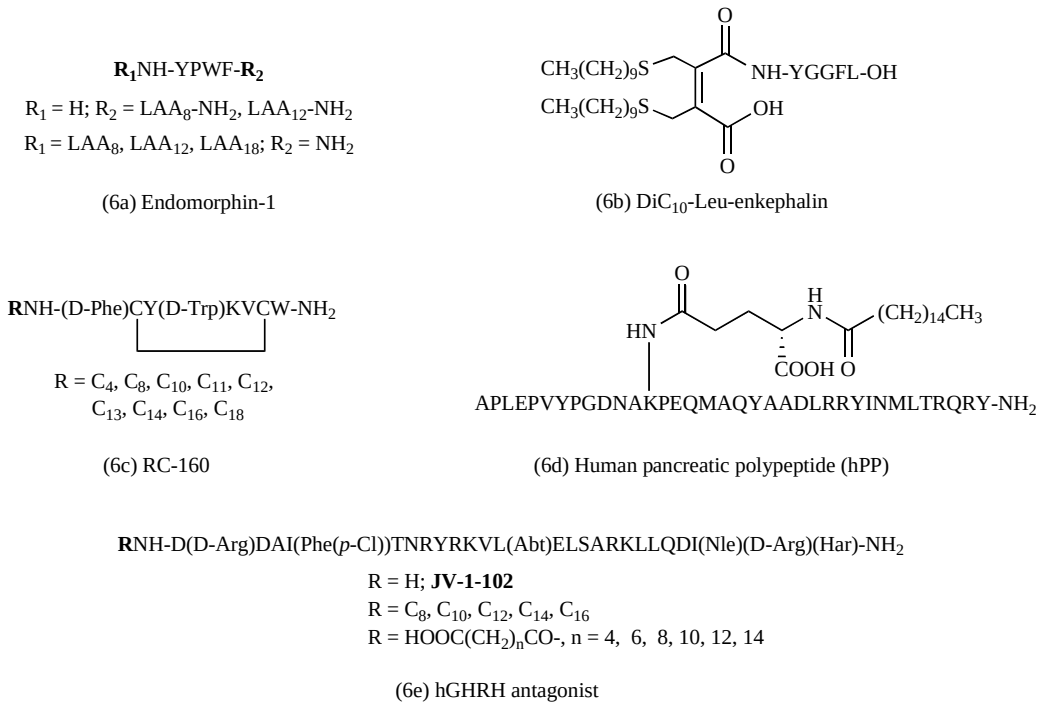


Fig. (6). Structures of the lipidated analogues of endomorphin-1, DiC_{10}-Leu-enkephalin, RC-160, hPP, hGHRH antagonists.

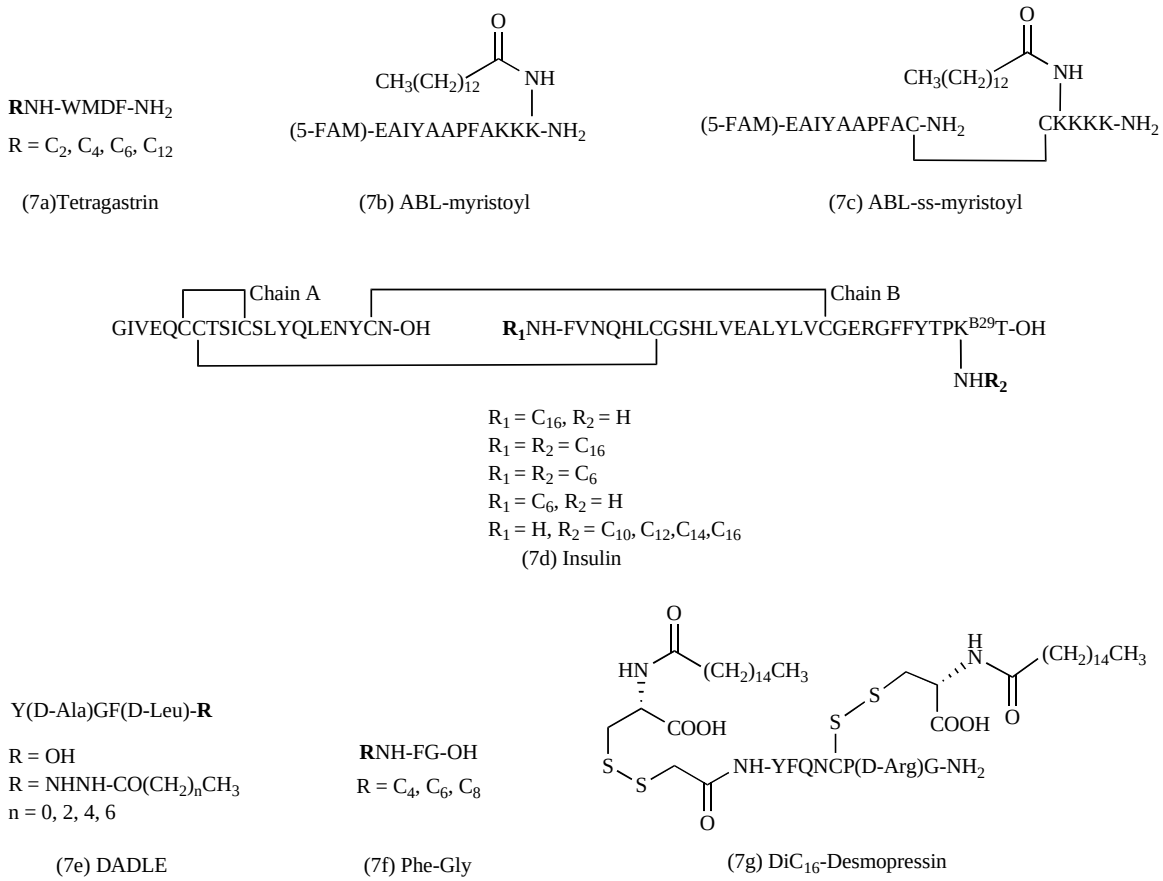


Fig. (7). Structures of the lipidated analogues of tetragastrin, ABL-myristoyl, ABL-ss-myristoyl, insulin, DADLE, Phe-Gly, and DiC16-desmopressin. (in figure 7b and 7c; 5-FAM, 5'-isomer of fluorescein).

[35-37]. (N^{B1}-palmitoyl)-insulin and (N^{B1}-palmitoyl)-(N^{B29}-palmitoyl)-insulin (Fig. **7d**) showed remarkably increased large intestinal absorptions and bioavailabilities when compared to that of the native insulin [25, 26, 28].

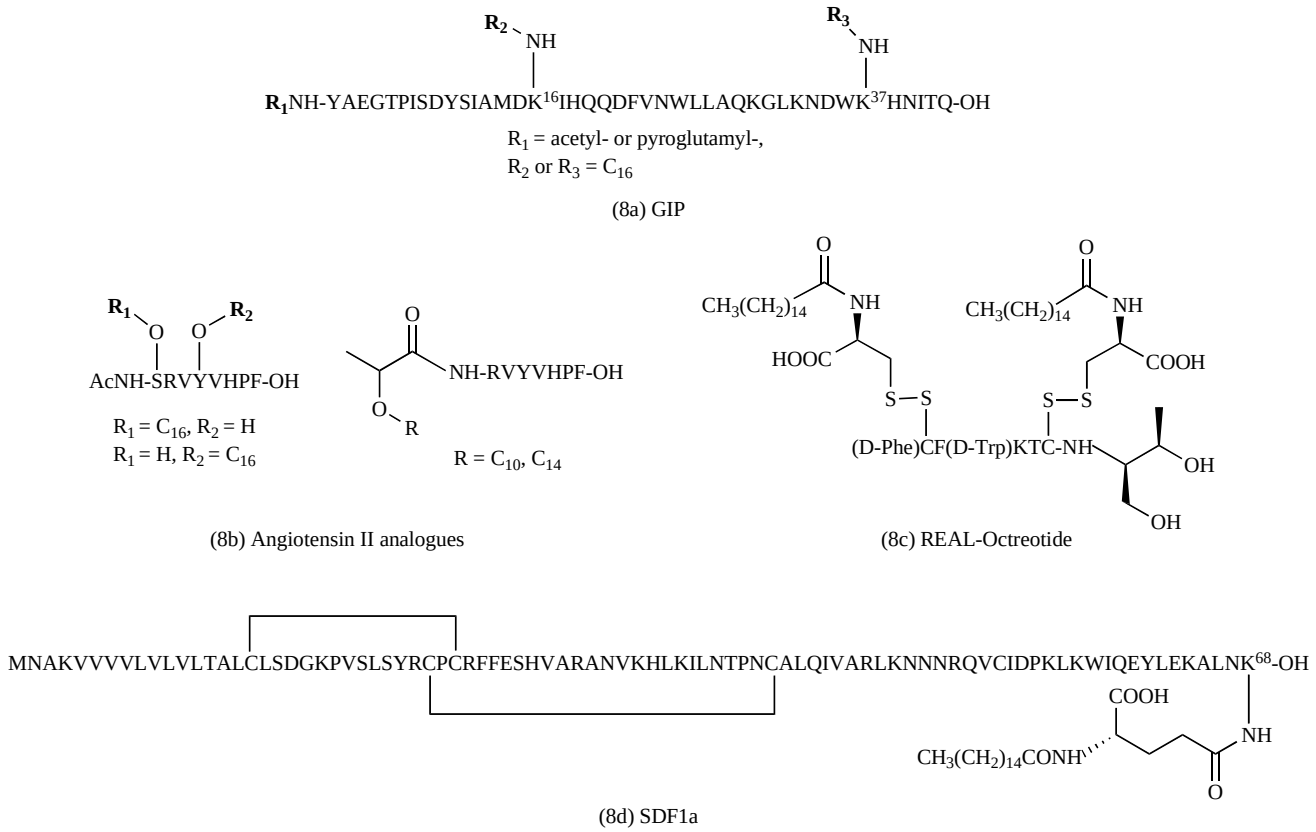


Fig. (8). Structures of the lipidated analogues of GIP, angiotensin II, REAL-octreotide and SDF1α.

Lipidation with long-chain fatty acids may limit the membrane transportation of lipidated peptides in some scenarios. The C_{12}-TG cannot pass through the jejunal and colonic mucosas, while C_2-TG and C_6-TG showed increased penetration across the jejunal and colonic mucosas [35-37]. "Bell-shape" profiles were found in the intestinal permeabilities of lipidated TG and DADLE analogues (C_2- to C_8-, Fig. **7e**) [12, 37]. Due to strong membrane binding, C_8-Phe-Gly (Fig. **7f**) was retained in the skin stratum corneum and its diffusion across intestinal epithelia was reduced as compared to that of C_4-Phe-Gly and C_6-Phe-Gly [89, 115]. Similarly, *N*-terminal lipidated TG analogues (Fig. **7a**) showed decreased transdermal permeabilities with the elongation of the fatty acid chain, C_2-TG > C_4-TG > C_6-TG [36]. Prolonged absorption phases were found in the pharmacokinetic study of DiC_{16}-desmopressin (Fig. **7g**, *i.v.* tail vein injection) [45], insulin detemir (subcutaneous injection, (*s.c.*)) [117], Mal-sCT and REAL-sCT (*s.c.*) [49].

2.2. Metabolic Stability

Lipidated peptides bind to serum albumin; this phenomenon affects serum metabolism of peptides. $N^{\varepsilon,B29}$-Palmitoyl-human insulin (Fig. **7d**) was co-eluted with albumin in size exclusion chromatography [29]. The insulin-secretion stimulating activity of the lipidated GIP analogue (Lys^{37}-Palmitoyl)GIP (Fig. **8a**) was correlated with the concentrations of albumin [19]. The uptake of lipidated Bowman-Birk protease inhibitor (BBI) analogue BBIssPal into the Caco-2 cell monolayers was increased up to 140-fold more than that of the native BBI in serum-free medium, but this uptake was reduced dramatically after adding fetal bovine serum [42]. The radioactivity of the supernatant of the mixture of lipidated hPP analogue ^{68}Ga-DOTA-[K^{13}(E-Pamitoyl),Q^{34}]hPP and plasma after precipitation was dramatically decreased compared to that of the supernatant of the mixture of non-lipidated hPP analogue ^{68}Ga-DOTA-[K^{13},Q^{34}]hPP and plasma (Fig. **6d**), indicating the strong binding of plasma protein with the lipopeptide [21].

The molecular biological mechanism of the interactions of lipidated peptides with serum albumin has not been fully studied. While the mechanism of the interactions of fatty acids with serum albumin shed a light on this unknown mechanism [118-121]. Seven common fatty acid binding sites among the identified eleven binding sites on human serum albumin were classified into three high-affinity binding sites (site V, IV and II) and four low-affinity binding sites (site I, III, VI and VII) [122-124]. Fatty acids have diverse binding abilities on those binding sites. Medium- and long-chain fatty acids prefer binding on sub-domains IIA and IIIA, respectively [122]. The binding of insulin detemir at IIA and IIIA domains can be displaced by long-chain fatty acids [27, 125].

Many lipidated peptides showed increased plasma stability, such as lipidated analogues of TG, RC-160, sCT, hPP, galanin, NT, NPY, NPW and insulin. The half-life of C_6-TG in rat plasma (10 mg protein per mL) was 300-fold higher than the half-life of the native TG, 32 h and 6.48 min, respectively [37]. The half-life of C_{12}-RC-160 in mouse serum (90%) was increased up to 12-fold compared to the half-life of the native RC-160, 24 h and 2 h, respectively [32]. The half-lives of lipidated analogues of galanin (17 mer, in 25% rat serum), salmon calcitonin (32 mer, mice, *s.c.*) and liraglutide (31 mer, pig, *s.c.*) were also increased but shorter than 12 h [18, 20, 51]. The half-life of ^{68}Ga-DOTA-[K^{13}(E-Palmitoyl), Q^{34}]hPP in rat after tail vein injection was 25 min, while the half-life of the non-lipidized analogue ^{68}Ga-DOTA-[K^{13}, Q^{34}]hPP was 3.6 min [21].

The chain length of fatty acids plays an important role in modulating the stability of lipidated peptides. Systemic study of the effects of the chain length of fatty acids on plasma stability of modified galanin analogues resulted in a bell-shape profile with the greatest stability achieved at myristoylation analogue (C_{14}-galanin), likely due to its albumin binding properties [18]. The stability of

Angiotensin II analogue [(*O*-palmitoyl) Lac^1-Val^5]Ang (Fig. **8b**) in lipase was 2-fold higher than that of (*O*-decanoyl) Lac^1-Val^5]Ang [40]. The half-life of highly hydrophobic diC_{10}-Leu-enkephalin (Fig. **6b**) in mouse plasma was increased up to ~193 h, while the half-life of the native Leu-enkephalin was only 6.7 min [13].

Lipidation also has significant impacts on the stability of lipidated peptides in tissues and organs. Lipidated peptides [Tyr^3]REAL-octreotide (Fig. **8c**) [53], Mal-sCT [50] and [K^{68}(E-Pam)]SDF1α (Fig. **8d**; SDF1α, stromal cell-derived factor-1α) [126] were more stable (2 to 12-fold) than their parent peptides in liver slices, liver fluid and liver homogenates, respectively. Lipidated analogues of Phe-Gly with C_4-, C_6- and C_8- (Fig. **7f**) showed similar stabilities in rat skin homogenates [89]. The stability trends of lipidated TG analogues (Fig. **7a**) were different in jejunum and colon homogenates (C_{12}-TG >> C_2-TG > C_6-TG >> TG) [37] and in Caco-2 homogenates (C_4-TG > C_2-TG > C_6-TG >> TG) [35]. The organ stabilities of C_6-TG were in rectum > liver > colon > jejunum [37]. The half-lives of diC_{10}-Leu-enkephalin in small intestinal mucosal homogenate, liver homogenate and plasma were ~12 min, ~80 min and ~193 h, respectively [13].

2.3. Distribution and Elimination

The distribution and elimination of lipidated peptides were protracted due to their serum albumin binding properties. DiC_{10}-Leu-enkephalin, DiC_{16}-desmopressin (Fig. **7g**), REAL-octreotide and ^{68}Ga-DOTA-[K^{13}(E-Palmitoyl),Q^{34}]hPP were accumulated in the liver [13, 21, 45, 53]. Hedgehog antagonist SMOi2–17 (Ac-AKFSTHWAYTL(N^{ε}-Pal-K), all-D) was accumulated in the liver and the lung after 30 min following *i.v.* administration [22]. The brain concentration of this lipidated peptide reached up to 10 nM; the blood concentration of this peptide was 400 nM [22]. DiC_{10}-Leu-enkephalin was eliminated into the small intestine with bile following *i.v.* administration, while the native Leu-enkephalin was quickly degraded in blood [13]. DiC_{16}-desmopressin showed a similar anti-diuretic linear dose-response relationship as the native desmopressin but at 250-fold less dosage [45].

3. BIOLOGICAL ACTIVITIES OF LIPIDATED PEPTIDES

3.1. *N*-Terminal Lipidation

N-Terminal myristoyl or palmitoyl conjugations are common strategies in peptide lipidation. *N*-Terminal palmitoyl lipidation analogue of the α-helix 2 fragment of STAT3 (a signal transducer and activator of transcription protein) showed an enhanced inhibition on the growth of breast cancer cells [34]. C_{14}-RC-160 (Fig. **6c**) showed similar anti-proliferation activities as RC-160 against three carcinoma cell lines DU145 (pancreatic), ECV304 (umbilical cord endothelioma) and MIA-PaCa2 (prostate) but at 1000-, 1000- and 100-fold lower concentrations, respectively. C_{14}-RC-160 also showed similar anti-angiogenic activity as RC-160 but at 10-fold lower concentration [32].

The chain length of fatty acids has strong impacts on the bioactivities of lipidated peptides. The long-chain fatty acid lipidation analogues of FDC-amide (Fig. **9a**) were more active than the corresponding short-chain fatty acid lipidation analogues in the inhibition of melittin-induced hemolysis [127]. Similarly, *N*-palmitoyl-yamamarin (Fig. **9b**) was 20-fold more effective than the native yamamarin on the growth inhibition of rat hepatoma cells while the short-chain fatty acid lipidation (i.e. C_2- and C_8-yamamarin) did not improve the bioactivities [128]. The growth hormone inhibitory activities of *N*-terminal lipidated RC-160 analogues (Fig. **6c**) were increased with the elongation of the fatty acid chains, but the bioactivities were saturated at C_{14}-RC-160 and C_{16}-RC-160 due to the strong binding effects of long-chain fatty acid moieties with albumin and/or adipose tissues [33].

3.2. Lysine Lipidation

Side chain lipidation on N^{ε}-lysine was extensively applied in many peptides. Lipidation of $N^{\varepsilon, B29}$-Lys of insulin (Fig. **7d**) with fatty acids (from C_{10}- to C_{16}-) increased the stability, the duration of action and the glucose-lowering effects of insulin [23-25, 27]. Insulin detemir, ($N^{\varepsilon,B29}$-Myristoyl)-des-30B-L-Thr-human insulin (Fig. **1c**), demonstrated a great serum albumin binding affinity and a blood glucose-lowering effect. This long-acting (up to 24 hours)

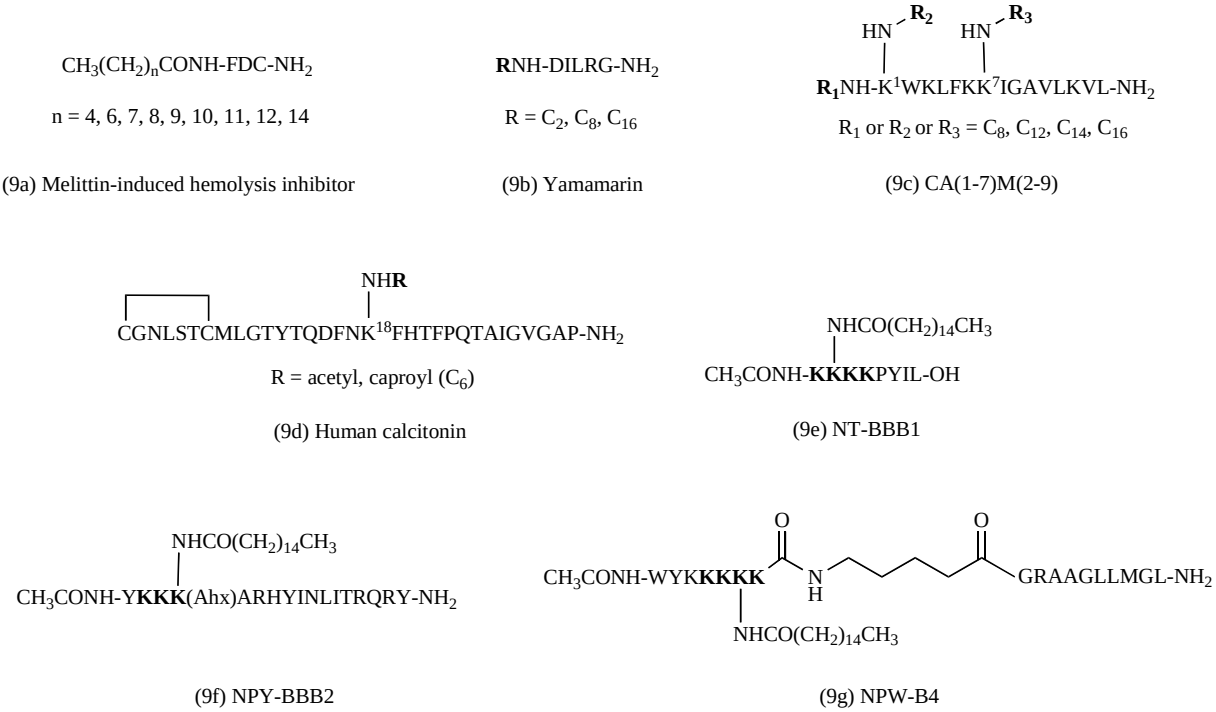


Fig. (9). Structures of the lipidated analogues of melittin-induced hemolysis inhibitor, yamamarin, CA(1-7)M(2-9), human calcitonin, NT, NPY and NPW.

anti-diabetic drug was launched in 2004 with a brand name Levemir®. (N^{B1}-Caproyl)-($N^{\varepsilon,B29}$-caproyl)-insulin (Fig. **7d**) showed significant absorptions in small intestine and large intestine *in vitro*; it also showed equal hypoglycemic activity when compared to (N^{B1}-caproyl)-insulin and the native insulin following *i.v.* injections in rats [23, 24]. The hypoglycemic activity was decreased slightly for the (N^{B1})- or (N^{B1}, $N^{\varepsilon,B29}$)-myristoyl and palmitoyl lipidated insulin analogues [24].

GIP is a promising glucose-dependent anti-diabetic drug candidate, but GIP is degraded rapidly by dipeptidyl peptidase IV (DPP-IV). Moreover, the metabolite GIP(3-42) is an antagonist to the GIP receptor. Palmitoylation on N^{ε}-Lys^{16} or N^{ε}-Lys^{37} of GIP and *N*-pGluGIP (Fig. **8a**) dramatically improved the metabolic stability and the antihyperglycemic and insulinotropic activities of GIP and *N*-pGluGIP following *i.p.* injections in mice [7, 19]. (N^{ε}-Palmitoyl-Lys^{16})*N*-pGluGIP and (N^{ε}-Palmitoyl-Lys^{37})*N*-pGluGIP were fully intact even after a 24 h incubation with DPP-IV, while the native GIP was completely degraded by DPP-IV in 8 h [7]. Similarly, the GIP receptor antagonist analogues, (Pro^{3})GIP and (Hyp^{3})GIP lipidated at N^{ε}-Lys^{16} with palmitoyl, showed significantly improved stabilities toward DPP-IV and enhanced inhibitions of GIP-stimulated cAMP production and cellular insulin secretions [129, 130].

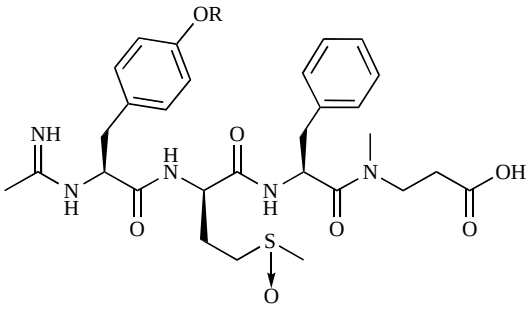
GLP-1 is a promising anti-diabetic drug candidate lacking hypoglycemic side effects, but GLP-1 is degraded rapidly by DPP-IV. The half-life of GLP-1 is only 2 min (*i.v.*) or 1 h (*s.c.*) [131-133]. Lipidations of GLP-1 led to the discovery of the recently launched once-daily (*s.c.*) anti-diabetic drug liraglutide (Arg^{34}-Lys^{26}-(N^{ε}-(γ-Glu(*N*-palmitoyl)))-GLP-1(7-37), Fig. **1b**). Liraglutide has a prolonged half-life (10~12 h in humans, *s.c.*) and equipotent receptor binding ability toward GLP-1 receptor as the native GLP-1 [134-136]. The lipidation analogues of GLP-1 (from C_{10}- to C_{18}-, Fig. **1b**) *via* the spacer *γ*-glutamic acid insertion have no discernible effects on GLP-1 receptor binding potency with the exception of the C_{18}-analogue [20]. The half-lives of lipidated GLP-1 analogues following *s.c.* administration in pigs were protracted with the elongation of the chain length of fatty acids. A phase I clinical trial for an oral variant of liraglutide, NN9924, was initiated recently. Inspired by the success of liraglutide, human pancreatic polypeptide (hPP) was modified with palmitoyl lipidation *via* the spacer γ-glutamic acid insertion at Lys^{13}; this lipidated hPP analogue (Fig. **6d**) demonstrated a prolonged duration of anorectic effect [21].

Lipidation analogue of cecropin A-melittin hybrid peptide CA(1-7)M(2-9) at N^{ε}-Lys^{7} with C_8-, C_{12}-, C_{14}- and C_{16}- (Fig. **9c**) showed impaired secondary structures and decreased bactericidal activities, whereas the analogues that were lipidated at terminal positions ($N^{1\alpha}$- or $N^{1\varepsilon}$-) exhibited 7-fold increases in the inhibition of promastigote or amastigote proliferation as compared to the native CA(1-7)M(2-9) [137].

The half-lives of lipidated human calcitonin analogues N^{ε}-acetyl-Lys^{18}-human calcitonin and N^{ε}-caproyl-Lys^{18}-human calcitonin (Fig. **9d**) were increased up to 3 to 10-folds compared to the native human calcitonin in small intestine and large intestine mucosal homogenates [138]. Both lipidated analogues showed improved hypocalcemic effects following administration into small intestinal loop, while the native human calcitonin was not active [138].

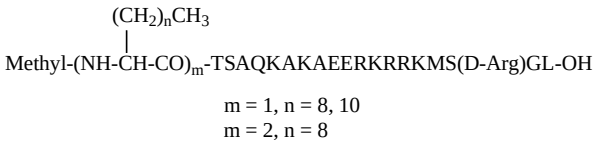
Our research group developed a peptide delivery method into the central nervous system (CNS) *via* the conjugation of a lipidated oligolysine motif (so called "cationization-lipidization motif") [14-18, 31, 139]. This strategy was successfully applied to four neuropeptides: galanin (Fig. **2c**) [14, 17, 18], NT (Fig. **9e**) [15], NPY (Fig. **9f**) [15] and NPW (Fig. **9g**) [31]. Neuropeptides modified with this strategy showed increased BBB penetration, improved serum stability, preserved peptide receptor binding properties and enhanced anticonvulsant activities.

Galanin is an endogenous neuropeptide exhibiting an anticonvulsant activity. Gal-LAA, lipidated at the C-termius of galanin (1-16) (Table **2**), did not demonstrate any anticonvulsant activity following systemic administration [14]. Surprisingly, a combination of lipidation and oligolysine "cationization" yielded



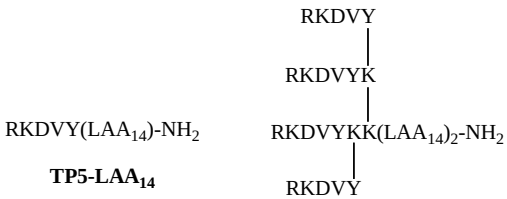
R = acetyl, methoxycarbonyl

(10a) Dermorphine



m = 1, n = 8, 10
m = 2, n = 8

(10b) Peptide A2



(10c) TP5 analogues

RNH-GLRILLLKV-OH

R = LAA_{10}, LAA_{12}, myristoyl, glucosesuccinate

(10d) Core peptide

Fig. (10). Structures of the lipidated analogues of dermorphine, peptide A2, TP5 and core peptide.

potent, systematically-active anticonvulsant compounds [14]. Interestingly, the anchor position of the palmitoyl moiety and the presence of cationic amino acids (Lys, Arg and/or His) significantly affected the bioactivities of the lipidated galanin analogues [14]. The SAR study of lipidated analogues of Gal-B2 (Fig. **2c**) revealed that the anticonvulsant activities were increased with the elongation of fatty acid chains from C_6 to C_{18} but were saturated at Gal-B2-C_{16} and Gal-B2-C_{18} in mice [18]. Several galanin analogues that exhibit promising activities in suppressing seizures in several animal models of epilepsy are under preclinical evaluations [17].

Anticonvulsant neuropeptides NPY and NT are not active *in vivo* after systemic delivery. The two neuropeptides conjugated with cationization-lipidation motif, NPY-BBB2 and NT-BBB1, exhibited potent anticonvulsant activities (less than 1 mg/kg, *i.p.*) in the 6 Hz epilepsy model [15]. Central insertion of the cationization-lipidation motif into NPW also yielded active anticonvulsant compounds [31]. Taken together, the cationization-lipidation conjugation provides an attractive modification method to improve peptides' CNS activity and to generate new drug leads.

3.3. Esterification with Fatty Acids

Esterification is often regarded as a reversible lipidation method due to its hydrolysis propensity *via* enzyme-mediated degradation resulting in the release of intact peptides. Reversible posttranslational *S*-acylation of proteins plays many important roles in protein membrane association, stability and cellular signaling [6]. The inactive analogue [Ac-(*O*-Palmitoyl)Ser^1-Val^5]Ang (Fig. **8b**) recovered its innate bioactivity after being hydrolyzed to [Ac-Ser^1-Val^5]Ang by mild saponification or lipolysis in an aorta strip contraction assay [41].

Ghrelin (Fig. **1a**) is the only known endogenous hormone which is naturally lipidized with octanoyl at Ser^3; decanoylation or decenoylation analogues were also exist [2, 47]. The hydrophobic interaction of the octanoyl group of ghrelin with human growth hormone secretagogue receptor type 1A (hGHSR1a) is essential for its anti-obesity bioactivity. Lipidations of [Ser^3]ghrelin with other fatty acids were also tolerable, such as octyl, 3-octenoyl, 2,4,6-octatrienoyl, 4-methylpentanoyl, 2-propylpentanoyl-, undecanoyl, 3-phenylpropionyl, adamantaneacetyl and other long-chain fatty acid lipidations [46, 48]. Site-specific activities were exhibited in the ghrelin analogues lipidated at different positions of serine [46]. [(*O*-Octanoyl)-Ser^2]ghrelin preserved equipotent activity as the native ghrelin in aequorin bioluminescence functional assay but [(*O*-octanoyl)-Ser^6]ghrelin and [(*O*-octanoyl)-Ser^{18}]ghrelin were not active [46].

Acylation on the hydroxy group of the tyrosine of dermorphin analogue with various fatty acids (Fig. **10a**) increased the oral bioavailabilities of the peptides; acetylation and methoxycarbonylation analogues showed similar antinociceptive activities as the parent peptide [140].

3.4. The REAL Technology and Cysteine Lipidation

The reversible aqueous lipidation technology (REAL) aims to lipidize peptides in aqueous solution by the disulfide bond formation of (*N*-palmitoylcysteinyl)-2-pyridyl-disulfide (Pal-CPD) with thiol groups of either Cys or a coupled thiopropionyl moiety on peptide chain [42]. The REAL technology increases peptides' plasma stability, blood circulation, gastrointestinal absorption and accumulation in the liver. The REAL technology was applied successfully in the modification of desmopressin (Fig. **7g**) [45], salmon calcitonin (Fig. **3a**)) [51, 53], octreotide (Fig. **8c**) [51, 53] and soy Bowman-Birk protease inhibitor (BBI) [42-44].

DiC_{16}-desmorpressin was synthesized by the conjugation of Pal-CPD with thiol groups of Cys^5 and *N*-thioacetyl of desmopressin [45]. DiC_{16}-desmopressin showed 4-fold duration of anti-diuretic activity without body weight loss in rats when compared to desmopressin (4 days vs. 1 day) [45]. REAL-sCT (Fig. **3a**) showed similar plasma calcium-lowering ability as the native sCT following *i.v.* administration [51]. Oral administration of REAL-sCT at a 10-fold higher dosage demonstrated similar effects as *s.c.* administration in stabilizing urinary deoxypyridinoline level. REAL-sCT was mainly retained in the blood and liver. REAL-octreotide was more stable in plasma and liver slices and more potent in the inhibition of growth hormone release following *s.c.* administration in rats as compared to that of the native octreotide [53].

BBIssPal was synthesized with REAL technology *via* the conjugation of Pal-CPD with 3-thiopropionyl-Lysine groups of BBI (8 KDa) [42, 43]. The blood concentration following *i.v.* tail injection in mice and the uptake across the Caco-2 cell monolayers of BBIssPal was increased up to 10-fold and140-fold, respectively, when compared to the native BBI. The potency of BBI in preventing the carcinogen-induced transformation of C3H10T1/2 cells was also maintained in BBIssPal. BBIssPal was accumulated predominantly in the liver. The other lipidated analogue of BBI modified with REAL technology on cysteines, BBI-Pal, exhibited a long retention time in the stomach following oral administration [44].

S-Alkylation of sCT with N^{ϵ}-maleimido-L-Lys(Palmitoyl)-OH in aqueous medium yielded Mal-sCT (Fig. **3b**) [49, 50]. Mal-sCT showed enhanced stabilities in the homogenates of intestine and liver, increased uptake across the Caco-2 cell monolayers, and extended hypocalcemic activity following oral administration as compared to that of the native sCT.

3.5. Lipoamino Acids (LAAs)

The application of unnatural lipoamino acids (LAAs, Fig. **2a**) were developed by the Toth group [73]. Lipidation of ryanodine receptor agonist [D-Arg^{18}]peptide A2 (Fig. **10b**) with LAA maintained peptides' α-helical structures, increased peptides' Ca^{2+} release activities and permeabilities across the Caco-2 cell monolayers as compared to that of the native peptide [62]. LAA conjugations enhanced the serum stability of thymopentin TP5 (RKDVY, Fig. **10c**) [61, 141]. The plasma half-lives of $[(TP5)_4$-$lys]_2$-lys-LAA_{14}-LAA_{14} and TP5-LAA_{14} were increased up to 7 and 18 min, respectively; while the half-life of the native TP5 was only ~1 min [61]. The *C*-terminal lipidated analogues of Glu-Trp-NH_2 with LAAs showed increased stabilities in Caco-2 cell homogenates and permeabilities across the Caco-2 monolayers [142]. Lipidated analogue of core peptide (CP, a transmembrane sequence of T-cell antigen receptor α-chain) LAA_{10}-CP (Fig. **10d**) showed increased permeability across Caco-2 cell monolayers and anti-arthritis activity in rats as compared to that of the native CP [59, 60]. The AUC and C_{max} of low molecular weight heparin were improved following co-administration of polylysine-$(LAA_{10})_2$ per oral [63]. Of note, the enantiomeric diversity of LAAs should be considered in LAA lipidation [30, 69, 70, 143].

3.6. Lipidation with Amino Fatty Acids

Lipidation with amino fatty acids produced mixed results on the modification of several peptides. The serum stability and the anti-bacterial activity of the pharmacophore of tenecin-1 were increased following *N*-terminal lipidation with 6-aminohexanoic acid (Ahx) (Fig. **11a**) but *N*-terminal acetylation or benzylation analogues showed decreased antibacterial activity due to lacking the *N*-terminal amino group [57]. Replacing the palmitoyl group of liraglutide (Fig. **1b**) with 11-aminoundecanoyl dramatically decreased its stability in pigs (half-lives 16 h *vs* 1.1 h, respectively) [20]. Replacing the octanoyl group of ghrelin (Fig. **1a**) with 7-aminoheptanoyl (Ahp) decreased its binding affinity and agonist activity on hGHSR1a [46].

Amino fatty acids were also exploited as backbone spacers to replace noncritical amino acids in peptide chain. Replacing Pyroglu[1]-Asn[2] and Gly[6]-Gly[7] of conotoxin SIIIA with Ahx yielded Ahx-SIIIA (Fig. **11b**). Ahx-SIIIA showed improved inhibitions of TTX-sensitive sodium current in dorsal root ganglia neurons and compound action potentials in the sciatic nerve and enhanced analgesic activity in the formalin pain model [54]. Replacing the non-essential Gly[2]-Gly[3] of Met-enkephalin with aminobutanoyl (Abt), aminopentanoyl (Apn), Ahx or Ahp (Fig. **11c**) yielded lipidated Met-enkephalin analogues with reduced affinities and agonist activities on opioid receptors [55]. However, these analogues showed analgesic and anticonvulsant activities comparable to the native Met-enkephalin. Replacing Gly-Gly-Gly moiety of TAN-1511A with 4-aminobenzoyl and 4-aminomethyl-benzoyl (Fig. **11d**) improved the peptide's proliferative activities in bone marrow cells, but this improvement was not found in the lipidated analogues substituted with Ahx and aminooctanoyl (Aot) [56]. Replacing non-critical amino acids of NPY with Ahx yielded lipidated NPY analogues (Table **2**) with similar receptor binding abilities [144].

3.7. Dicarboxylic Acid Modification

Dicarboxylic acid acylation transforms the positively charged cationic amine group into the negatively charged anionic acid group in the lipidated peptides. The most successful application of this strategy is the discovery of ultra-long-acting basal insulin Degludec ($N^{\varepsilon,29B}$-[*N*-(15-carboxypentadecanoyl)-L-γ-glutamyl] -des-30B-L-threonine-human insulin, NN1250, Fig. **11e**), which is currently being evaluated in a phase III clinical trial [145]. The *N*-terminal lipidation of growth hormone-releasing hormone antagonist GHRH(1-29)NH_2 (Fig. **6e**) with dicarboxylic acids (ranging from heptane diacid to hexadecane diacid) and fatty acids were systemically studied [58]. Decane diacid-GHRH(1-29)NH_2 showed similar binding affinity and inhibitory ability as compared to that of decanoyl-GHRH(1-29)NH_2. All analogues had significant anti-proliferative activities against MiaPaCa-2 human pancreatic cancer cell line [58].

3.8. Miscellaneous Lipidation Strategies

The *C*-terminus esterification analogues of DADLE *via* hydrazine spacer with C_2-, C_4-, C_6- or C_8- (Fig. **7e**) showed increased permeabilities across intestine and stabilities *in vitro* as compared with the native DADLE [12]. The *C*-terminal esterification of dermorphin (Fig. **10a**) with various aliphatic alcohols increased its oral bioavailability and maintained its antinociceptive activity [140]. Modification of core peptide (CP, Fig. **10d**) with *C*-terminal multi-palmitoyl groups *via* spacer Gly-Tris improved the binding ability to the lipid membrane; mono- and tri-palmitoyl-Tris-Gly-CP analogues inhibited IL-2 production more effectively than CP [59]. Lipidation on the imidazole group of histidine was reported in the chemical modification of thyrotropin-releasing hormone (TRH), but this strategy was not continued due to the unstable bond of acyl-imidazole [146, 147].

A novel isostere of palmitoyl cysteine, (S)-2-Amino-4-(2-pentadecyl-1,3-dioxolan-2-yl)-butanoic acid, was reported to substitute *S*-palmitoyl moiety of H-Ras protein [148]. Fmoc-Met-enkephalin exhibited increased BBB permeability, enhanced analgesic activity, and controlled release following spontaneous chemical hydrolysis *in vivo* [149]. Replacing the octanoyl group of human ghrelin with 1-adamantaneacetyl or 8-bromooctanoyl maintained its binding affinity to receptor hGHSR1a [46]. Conjugation on the C34 fragment of HIV fusion inhibitor gp41 with cholesterol moiety Gly-Ser-Gly-Cys(cholesterol) dramatically improved its antiviral potency [66]. Conjugation on N^{B29}-insulin with one or two deoxycholic acids maintained its binding to human hepatoma cell line HepG2; these analogues produced similar blood glucose nadir as the native insulin but with a longer duration following *i.v.* administration [67, 68].

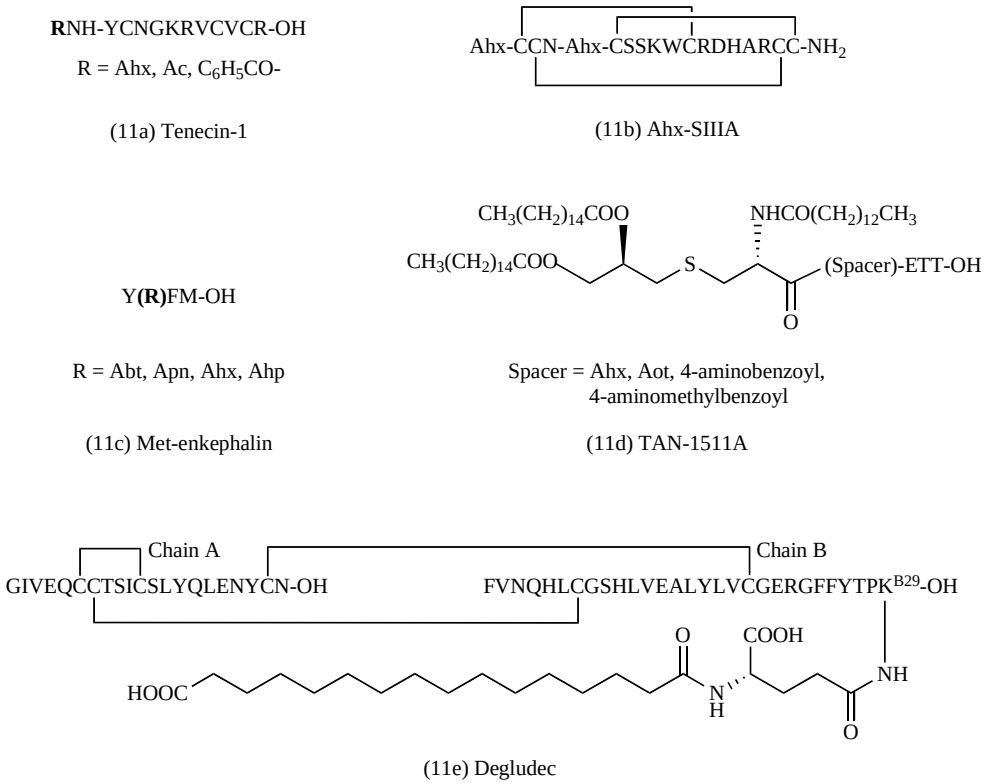


Fig. (11). Structures of the lipidated analogues of tenecin-1, Ahx-SIIIA, Met-enkephalin, TAN-1511A and Degludec.

Glycosylation compensates the low aqueous solubility of lipidated pepitdes and increases their membrane transportation through glucose transporters. Liposaccharide, the combination of LAA lipidation and glycosylation, is applied in the development of small-molecule drugs, peptides, vaccines and antibiotics [59, 72, 73, 80, 150-154]. Hemolytic potency of liposaccharides was correlated with the chain length of LAA [72]. Two naturally-occurring ghrelin-like peptides lipidated with octanoyl at Ser^3 and glycosylated with mucin-type sugar chain (*N*-acetyl-hexosamine)$_3$-(hexose)$_2$ at Thr^{11} or Ser^{10}-Thr^{11} were also found in red stingray *Dasyatis akajei* [155].

It is noteworthy that stapled peptides can also be considered as "lipidated" peptides with non-natural olefin amino acids inserted into the peptide backbone. The helical contents of the analogues of BH3 segment of BID stapled with alkenes 4-octene (i+4 span) or 4-tetradecene (i+7 span) were increased up to 5-fold [156]. Properties of the stapled peptides are reviewed in [157].

Insertion of glycine, glutamic acid, amino fatty acids, PEG or hydrazine between fatty acids and peptides is a useful strategy to minimize the disturbance of fatty acids on the bioactivities of lipidated peptides. For example, the spacer γ-Glu conjugated between Lys^{26} of Arg^{34}-GLP-1(7-37) and fatty acids was important in maintaining the receptor binding potency [20].

Reports on unfavorable results of lipidated peptides can provide useful information on designing peptide drug leads. The CNS activity of *N*-lauroyl-TRH were decreased in pentobarbital sleeping assay and the endocrine activity in thyroid-stimulating hormone releasing activity assay [38]. Replacing the palmitoyl moiety of liraglutide with 2-hexyldecanoyl, 10-cyclohexane-decanoyl, 10-benzyldecanoyl or 4-pentylbenzenesulfonyl abolished the peptides' receptor binding affinity [20, 41]. Alkyl ether [Sar^1,Tyr^4(*O*-$C_{18}H_{37}$)]Ang showed a very low binding affinity on bovine adrenocortical membranes as compared to human angiotension analogues [Sar^1]Ang and [Sar^1,Tyr^4(*O*-Pal)]Ang [41].

Hemolysis was found in long-chain fatty acid lipidated peptides due to their strong membrane binding affinity [98, 127]. Cytotoxicity and aggregation propensity were related to the position of the octanoyl group on *β*-amyloid [93]. The analogues of *β*-amyloid lipidated at Lys^{16} and Lys^{28} with octanoylation showed more toxic than the native *β*-amyloid and *N*-octanyl-*β*-amyloid toward Neuro2A cells [93]. Lipidation of 2α (Fig. **3f**) with 1-adamantanecarbonyl transformed the structure of 2α from *α*-helix into *β*-sheet and formed amyloid fibrils in aqueous solution [158].

4. CHEMICAL SYNTHESIS METHODS

Automated peptide synthesis on solid support is the most commonly used method for the syntheses of lipidated peptides. Building blocks such as N^{ε}-acyl-Lys and LAA can be chemically synthesized and directly coupled on resin by the Fmoc solid phase peptide synthesis (SPPS) method [18, 159-161]. *S*-Acylated cysteine is not recommended due to possible aminolysis by the α-amino group [162].

The on-resin lipidation is an applicable strategy for the syntheses of long-chain peptides *via* orthogonal protecting strategy. Fmoc-orthogonal protecting groups for the side chain of lysine can be selected from Dde-, Nosyl-, Alloc-, Mtt- and Mmt [163]. Dde group can be deprotected with hydrazine or $NH_2OH\cdot HCl$/imidazole in NMP/CH_2Cl_2 [161, 164, 165]. Nosyl group can be deprotected with *p*-methoxythiophenol/K_2CO_3 in CH_3CN/ DMSO [166, 167]. Alloc can be deprotected with Pd^0 catalysis [161]. Trityl protecting groups Mmt, Mtt and PhFI can be removed by 1%-3% TFA in CH_2Cl_2 with scavenger 2% TES [168, 169]. Mmt protecting strategy is also applicable in the side chain protection of Ser/Thr and Cys residues [170-172]. Meanwhile, Cys(Trt) needs 50% TFA to deprotect Trityl group [172]. 2-ClTrt protecting group on the side chain of Tyr is also sensitive to 2% TFA [173]. Deprotecting tert-Butylsulfanyl group of Cys(StBu) with PBu_3 may cause β-elimination [162].

Boc- group can be deprotected with trimethylsilyl trifluoromethanesulfonate (TMSOTf) with lutidine; this deprotecting method is orthogonal with Fmoc and unsaturated double bond [174]. Unsaturated fatty acid lipidations such as prenylation are sensitive to strong acids; special linkers such as trityl, oxime, hydrazino-benzoyl, (2-phenyl-2-trimethylsilyl)ethyl (PTMSEL) and ellman sulfonamide should be considered [6, 74, 169, 175].

Solution lipidation methods such as REAL technology are attractive strategies for peptides and proteins [13, 45, 49, 51]. Palmitoyl estertification of Ser or Cys can be accomplished by conjugating palmitoyl chloride to peptides in TFA solvent [176]. The TFA condition prevents acylation at amino groups. Rearrangement of *S*-acyl to *N*-acyl in terminal Cys lipidation can occur in the Fmoc deprotecting process with piperidine, so 1% DBU should be used to remove Fmoc [175]. On the other hand, the rearrangement can be exploited as a peptide lipidation method. Conjugating hydrazinoacetyl peptides with lipidated *α*-oxo-aldehyde is a convenient *in situ* lipidation method and can be applied in parallel synthesis (Fig. **12**) [177-180].

5. SUMMARY AND OUTLOOK

The modification of peptides and proteins *via* lipidation has been continuously growing since 1970's; the marketing of the lipidated peptide drugs is a considerable validation that lipidation is a successful strategy to develop more peptide drug leads in the future. As discussed herein, lipidation improved pharmacological and pharmaceutical properties of many peptides. It becomes apparent that even subtle changes in lipidation strategy, including the type of fatty acids, lipid anchor position or spacer insertion can produce profound impacts on the physicochemical properties and bioactivities of lipidated peptides.

Extensive SAR studies are necessary to take full advantage of lipidation to convert peptides into drugs, since mixed results on ADME properties of lipidated peptides emphasize the complexity of this modification strategy. With systematic and judicious approaches, lipidation will increase peptides' hydrophobicity, secondary structure, self-assembly, albumin binding, metabolic stability, bioavailabilty and ADME properties while maintaining peptides' superior bioactivity including potency and specificity for target receptors. With increased interest of exploring peptides as therapeutics, lipidation will continue to be one of the leading chemical modifications to produce clinical drug candidates.

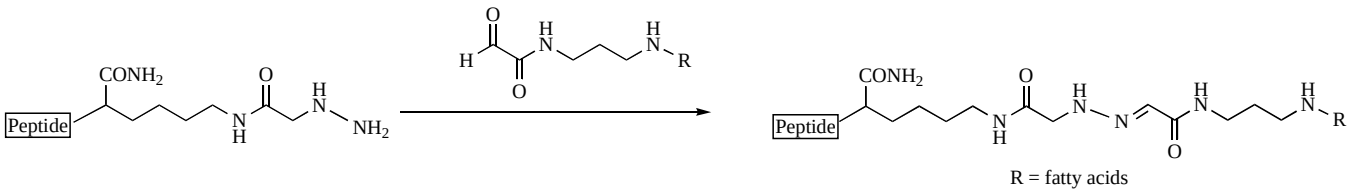


Fig. (12). In situ hydrazine lipidation strategy.

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ABBREVIATIONS

Abt = aminobutanoyl
ADME = absorption, distribution, metabolism and excretion
Ahp = aminoheptanoyl
Ahx = aminohexanoic acid
β-Alanine = 3-aminopropanoic acid
Alloc = allyloxycarbonyl
Aot = aminooctanoyl
Apn = aminopentanoyl
AUC = area under the curve
Ava = 5-amino-valeric acid
BBB = blood-brain barrier
BBI = Bowman-Birk protease inhibitor
C_2 = acetyl
C_3 = propanoyl
C_6 = hexanoyl or caproyl
C_8 = octanoyl
C_{10} = decanoyl
C_{12} = lauroyl
C_{14} = myristoyl
C_{16} = palmitoyl
C_{18} = stearoyl
CD = circular dichroism
2-ClTrt = 2-chlorotrityl
CMC = critical micelle concentration
CNS = central nervous system
CP = core peptide
Dde = 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl
DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene
DIC = *N,N'*-diisopropylcarbodiimide
DPC = dodecylphosphocholine
DPP-IV = dipeptidyl peptidase IV
DPPC = dipalmitoylphosphatidylcholine
5-FAM = 5'-isomer of fluorescein
Fmoc = *N*-(9-fluorenyl)methoxycarbonyl
GalR1 = galanin receptors subtype 1
GalR2 = galanin receptors subtype 2
GHRH = growth hormone-releasing hormone
GIP = gastric inhibitory polypeptide or glucose-dependent insulinotropic polypeptide
GLP-1 = glucogan-like peptide-1
GPCRs = G-protein coupled receptors
Har = homoarginine
hGHSR1a = human growth hormone secretagogue receptor type 1A
HOBt = 1-hydroxybenzotriazole
hPP = hormone pancreatic polypeptide
IGF1R JM = insulin-like growth factor 1 receptor juxtamembrane segment
i.p. = intraperitoneally
i.v. = intravenous
LAA = lipoamino acid
LAA_8 = 2-amino-D,L-octanic acid
LAA_{10} = 2-amino-D,L-dodecanoic acid
LAA_{12} = 2-amino-D,L-lauric acid
Mmt = 4-methoxytrityl
Mtt = 4-methyltrityl
Nle = norleucine
Nosyl = 2-nitro-benzenesulfonyl
NPY = neuropeptide Y
NT = neurotensin
Pal-CPD = (*N*-palmitoylcysteinyl)-2-pyridyl-disulfide
PBu_3 = tributylphosphine
Phe(*p*Cl) = *p*-chlorophenylalanine
pGlu = paroglutamyl
PhFI = 9-phenylfluoren- 9-yl
POPC = 1-palmitoyl-2-oleoyl-phosphatidylcholine
POPG = 1-palmitoyl-2-oleoyl-phosphatidylglycerol
PTMSEL = (2-phenyl-2-trimethylsilyl)ethyl
REAL = reversible aqueous lipidation
Sar = sarcosine
SAR = structure-activity relationship
s.c. = subcutaneous injection
sCT = salmon calcitonin
SDF1α = stromal cell-derived factor-1α
SDS = sodium dodecylsulphate
SPPS = solid phase peptide synthesis
STAT = signal transducer and activator of transcription protein
TES = triethylsilane
TFA = trifluoroacetic acid
TFE = 2,2,2-trifluoroethanol
TG = tetragastrin
T_m = melting temperature
TMSOTf = trimethylsilyl trifluoromethanesulfonate
TRH = thyrotropin-releasing hormone.

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