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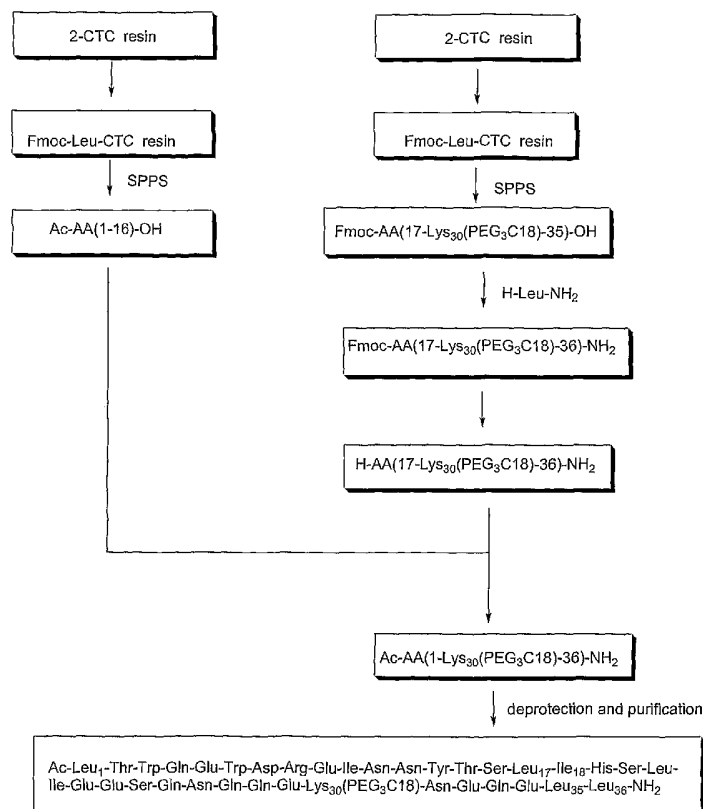
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(54) Title: COMPOSITIONS AND METHODS FOR SYNTHESIS OF PEPTIDE AND RELATED CONJUGATE

(57) Abstract: Provided are peptide
fragments which may be used in a method
of producing a synthetic peptide, or a
conjugate comprised of the synthetic
peptide operatively linked to fatty acid,
wherein the method comprises chemically
coupling 2 or more peptide fragments
together in a process of assembly to
produce the synthetic peptide or conjugate.



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COMPOSITIONS AND METHODS FOR SYNTHESIS OF PEPTIDE AND RELATED CONJUGATE

FIELD OF INVENTION

5 The present invention relates to novel methods of synthesis of a synthetic peptide having the amino acid sequence of SEQ ID NO:4, and of a conjugate comprised of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid. More particularly, the present invention relates to a process by which smaller peptides, each comprising a separate portion of the synthetic peptide desired to
10 be chemically synthesized (and therefore the smaller peptides are referred to as "peptide fragments" with respect to the synthetic peptide or conjugate which is produced by combining a set of peptide fragments), are covalently coupled in assembling the desired synthetic peptide having the amino acid sequence of SEQ ID NO:4, or a conjugate comprised thereof.

BACKGROUND OF THE INVENTION

It was discovered that peptides derived from the native sequence of either the HR1 region (having an amino acid sequence of SEQ ID NO:1) ("HR1 peptides") or HR2 region (having an amino acid sequence of SEQ ID NO:2) ("HR2 peptides") of HIV gp41
20 inhibit transmission of HIV to host cells both in *in vitro* assays and in *in vivo* clinical studies. For example, some HR2 peptides, as exemplified by DP178 (also known as T20, enfuvirtide, and Fuzeon®; SEQ ID NO:3) blocked infection of target cells with a potency of 0.5 ng/ml (EC₅₀ against HIV-1_{LAI}). Efforts have been made to improve the biological activity of HIV gp41-derived peptides, such as by trying to stabilize the helical structure of
25 the HR2 peptide. Additionally, various efforts have also been made to improve the pharmacological properties of HIV gp41-derived peptides.

Developed in conjunction with this invention are conjugates, each conjugate comprising a fatty acid operatively linked to synthetic peptide, wherein the synthetic peptide is derived from the HR2 region of HIV gp41 (as disclosed in a co-pending
30 application by Applicant). As compared to synthetic peptide alone (e.g., not part of a conjugate), such conjugates demonstrate one or more improved pharmaceutical activities (e.g., one or more of increased antiviral activity against HIV-1, increased antiviral potency against HIV strains resistant to current HIV fusion inhibitor peptides, a reduction in clearance, and a marked prolongation of terminal half-life).

35 A preferred conjugate comprises fatty acid operatively linked to a synthetic peptide having the amino acid sequence of SEQ ID NO:4 (see Table 3 herein). As this preferred conjugate comes to be used more extensively, for example as a therapeutic

and/or as a medicament, the need arises for an ability to synthesize the conjugate, and its component parts such as the synthetic peptide having the amino acid sequence of SEQ ID NO:4 (containing 36 amino acids), in large scale quantities. Current standard conditions for linear, solid phase synthesis of a peptide having about 30 amino acids or more can present some challenges in efficiency of synthesis, and in purity of the desired peptide (i.e., the amount of peptide produced having the desired amino acid sequence versus peptide having an amino acid sequence other than the desired amino acid sequence as a result of misincorporation of amino acids during long chain synthesis). Thus, there is a need for a process by which a synthetic peptide having the amino acid sequence of SEQ ID NO:4, or a conjugate comprised thereof, can be produced in large scale quantities.

SUMMARY OF THE INVENTION

There is provided, in accordance with the present invention, methods for synthesis of a synthetic peptide having the amino acid sequence of SEQ ID NO:4. Such methods utilize either solid phase and/or liquid phase synthesis procedures to synthesize specific peptide fragments, that may comprise a set of peptide fragments, and then combine the specific peptide fragments in a set of peptide fragments to yield the synthetic peptide having an amino acid sequence of SEQ ID NO:4. The methods of the present invention involve synthesizing specific (with respect to amino acid sequence), side chain protected (using chemical protecting groups), peptide fragments which serve as intermediates, which are then chemically coupled in a process of assembly in a manner to result in the desired synthetic peptide having an amino acid sequence of SEQ ID NO:4 having side chain protection. The method may further comprise deprotection of the side chains to yield a synthetic peptide having an amino acid sequence of SEQ ID NO:4, which may then be used to produce a conjugate comprised of the synthetic peptide operatively linked to fatty acid.

Also provided are at least 2 peptide fragments, each peptide fragment serving as an intermediate that may be covalently coupled with the one or more other peptide fragments of the at least 2 peptide fragments, to yield the synthetic peptide having an amino acid sequence of SEQ ID NO:4. In a preferred embodiment, the peptide fragments within a set of peptide fragments according to the present invention, are coupled in a solution phase process in a manner to result in the desired synthetic peptide having an amino acid sequence of SEQ ID NO:4. In another embodiment, at least one peptide fragment, used in the assembly of the synthetic peptide having an amino acid sequence of SEQ ID NO:4, may be operatively linked to fatty acid directly or through a linker, so that when the peptide fragments are combined, assembled is a conjugate

comprised of fatty acid operatively linked to a synthetic peptide having an amino acid sequence of SEQ ID NO:4.

Also provided is a novel modified amino acid comprising an amino acid, and more preferably a lysine, having covalently coupled thereto a linker-fatty acid combination, and more preferably a linker-fatty acid combination comprising (PEG-3)-C18. The modified amino acid is incorporated into one of the peptide fragments (preferably during solid phase synthesis) resulting in a peptide fragment comprising a peptide fragment-linker-fatty acid combination. The peptide fragment-linker-fatty acid combination is then combined with one or more other peptide fragments to yield a conjugate comprising the synthetic peptide having an amino acid sequence of SEQ ID NO:4, operatively linked to fatty acid through a linker. In a preferred embodiment, the peptide fragments (including a peptide fragment operatively linked to fatty acid), within a set of peptide fragments according to the present invention, are covalently coupled in a solution phase process in a manner to result in the desired conjugate comprised of a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to a fatty acid (directly or through a linker).

The present invention also provides the use of a set of peptide fragments according to the present invention, in the manufacture of a medicament for use within therapy of HIV infection (e.g., used in a method of inhibiting transmission of HIV, a method of inhibiting HIV fusion, or a method of treating HIV infection), as described herein. The medicament is preferably in the form of a conjugate produced according to the compositions and methods of the present invention together with a pharmaceutically acceptable carrier.

The present invention further extends to methods of making a conjugate comprising the steps of: (a) synthesizing at least two peptide fragments, wherein at least one of the peptide fragments comprises a peptide fragment operatively linked to fatty acid (directly or through a linker), and wherein when assembled together the at least two peptide fragments form a synthetic peptide having the amino acid sequence of SEQ ID NO:4 which is operatively linked to fatty acid; (b) chemically coupling the 2 or more peptide fragments in a process of assembly to produce the conjugate comprised of a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to a fatty acid.

The above and other objects, features, and advantages of the present invention will be apparent in the following Detailed Description of the Invention when read in conjunction with accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a comparison of the sequences contained within the HR2 region of HIV-1 gp41 for purposes of illustration, and not limitation, as determined from various laboratory strains and clinical isolates, wherein illustrated are some of the variations in amino acid sequence (e.g., polymorphisms), as indicated by the single letter amino acid code.

FIG. 2 is a schematic showing synthesis of a synthetic peptide, SEQ ID NO:4, using 3 peptide fragments (SEQ ID NOs:5, 6, & 7) assembled by chemically coupling in a fragment condensation approach.

FIG. 3 is a schematic showing synthesis of a synthetic peptide, SEQ ID NO:4, using 2 peptide fragments (SEQ ID NOs:9 & 10 or SEQ ID NOs:9 & 11) assembled by chemically coupling in a fragment condensation approach.

FIG. 4 is a schematic showing synthesis of a conjugate comprised of a synthetic peptide (SEQ ID NO:4), a linker, and a fatty acid, using 2 peptide fragments (e.g., SEQ ID NOs:13 & 20, or SEQ ID NOs:13 & 21) assembled by chemically coupling in a fragment condensation approach.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "amino acid" is meant, for purposes of the specification and claims and in reference to the synthetic peptide or peptide fragment used in the present invention, to refer to a molecule that has at least one free amine group and at least one free carboxyl group. The amino acid may have more than one free amine group, or more than one free carboxyl group, or may further comprise one or more free chemical reactive groups other than an amine or a carboxyl group (e.g., a hydroxyl, a sulfhydryl, etc.). The amino acid may be a naturally occurring amino acid (e.g., L-amino acid), a non-naturally occurring amino acid (e.g., D-amino acid), a synthetic amino acid, a modified amino acid, an amino acid derivative, an amino acid precursor, and a conservative substitution. One skilled in the art would know that the choice of amino acids incorporated into a peptide will depend, in part, on the specific physical, chemical or biological characteristics required of the antiviral peptide. Such characteristics are determined, in part, by determination of structure and function (e.g., antiviral activity; as described herein in more detail). For example, the skilled artisan would know from the descriptions herein that amino acids in a synthetic peptide may be comprised of one or more of naturally occurring (L)-amino acid and non-naturally occurring (D)-amino acid. A preferred amino acid may be used to the exclusion of amino acids other than the preferred amino acid. The term "internal", when used to refer to an amino acid (e.g., lysine at amino acid position 30 of SEQ ID NO:4)

means that the amino acid is other than the N-terminal amino acid and the C-terminal amino acid in an amino acid sequence (e.g., of a peptide fragment or synthetic peptide or conjugate). In other words, an internal amino acid is located in the amino acid sequence in a position in-between the N-terminal amino acid and the C-terminal amino acid.

5 A "conservative substitution", in relation to amino acid sequence of a synthetic peptide used in the present invention, is a term used hereinafter for the purposes of the specification and claims to mean one or more amino acids substitution in the sequence of the synthetic peptide such that its biological activity is substantially unchanged (e.g., if the peptide inhibits HIV gp41-mediated fusion at a concentration in the nanomolar range
10 before the substitution, after the substitution inhibition of HIV gp41-mediated fusion is still observed in the nanomolar range). As known in the art "conservative substitution" is defined by aforementioned function, and includes substitutions of amino acids having substantially the same charge, size, hydrophilicity, and/or aromaticity as the amino acid replaced. Such substitutions are known to those of ordinary skill in the art to include, but
15 are not limited to, glycine-alanine-valine; isoleucine-leucine; methionine-leucine; tryptophan-tyrosine; aspartic acid-glutamic acid; arginine-lysine; asparagine-glutamine; and serine-threonine. With particular relevance to the present invention, a conserved substitution is known in the art to also include substituting lysine with ornithine, in providing a free amine group (e.g., epsilon amine). For HIV gp41-derived peptides, such
20 substitutions may also comprise polymorphisms at the various amino acid positions along the relevant HR region (HR2) of gp41 found in any one or more of various clades, laboratory strains, or clinical isolates of HIV, which are readily available from public databases and are well known in the art (see also, FIG. 1 as illustrative example).

The term "fatty acid" when used herein for purposes of the specification and
25 claims, means a fatty acid comprising no less than 5, and no more than 30, carbon atoms; and is also used to mean a fatty acid analog produced by operatively linking fatty acid to either a linker or an amino acid. For example, as described herein in more detail, operatively linking a fatty acid to a C-terminal amino acid results in an alkylamide (also referred to herein as "fatty acid"). "A fatty acid" or "fatty acid", when used in reference to
30 operatively linking to a synthetic peptide or peptide fragment, means one or more fatty acids, unless otherwise specifically indicated herein. The more preferred fatty acids useful in this invention are the fatty acids of 12 to 20 carbon atoms. The fatty acid may comprise a monoacid or a diacid. The fatty acid may be saturated (Table 1, "S") or unsaturated (Table 1, "U"). Examples of fatty acids that may be useful in producing a
35 conjugate according to the present invention include, but are not limited to the following illustrated in Table 1.

Table 1

Monoacid	Abbreviation	Chemical name(s)
S	C6	Hexanoic acid
S	C8	(+/-)-2-ethylhexanoic acid; octanoic acid
S	C10	Decanoic acid
S	C12	dodecanoic acid; lauric acid
S	C14	tetradecanoic acid; myristic acid
S	C16	hexadecanoic acid; palmitic acid
S	C18	octadecanedioic acid; stearic acid
S	C20	Eicosanoic acid; arachadic acid
S	C22	docosanoic acid
S	C24	tetracosanoic acid
S	C28	octacosanoic acid
S	C30	triacontanoic acid
U	C10:1	3,7-dimethyl-6-octenoic acid; citronellic acid
U	C12:1	cis-5-dodecenoic acid
U	C14:1	Delta 9 cis-tetradecenoic acid; myristoleic acid
U	C16:1	Delta 9 cis-hexadecenoic acid; palmitoleic acid
U	C18:1 (cis-9)	Delta 11 cis-octadecenoic acid; cis-vaccenic acid
U	C18:1(trans-9)	Delta 9 trans-octadecenoic acid; elaidic acid
U	C18:1(cis-11)	Delta 11 cis-octadecenoic acid; cis-vaccenic acid
U	C18:2	delta 9 cis 12 cis octadecadienoic acid; linoleic acid
U	C18:3	delta 9 cis 12 cis 15 cis octa-decatrienoic acid; linolenic acid
U	C20:1	delta 11 cis-eicosenoic acid; cis-11-eicosenoic acid
U	C22:1	Delta 13 cis-docosenoic acid; erucic acid
U	C24:1	Delta 15 cis-tetracosenoic acid; nervonic acid
Diacid	C8 diacid	Octanedioic acid; suberic acid
Diacid	C9 diacid	nonanedioic acid; azelaic acid
Diacid	C10 diacid	decanedioic acid; sebacic acid
Diacid	C11 diacid	undecanedioic acid
Diacid	C12 diacid	dodecanedioic acid
Diacid	C14 diacid	tetradecanedioic acid; 1,12-dodecanedicarboxylic acid
Diacid	C16 diacid	hexadecanedioic acid
Diacid	C20 diacid	eicosanedioic acid; 1,18-octadecanedicarboxylic acid
Diacid	C22 diacid	docosanedioic acid
Diacid	C26 diacid	hexacosanedioic acid

A preferred fatty acid may be applied in the present invention to the exclusion of a fatty acid other than the preferred fatty acid.

5 The term "synthetic peptide" is used herein, and for the purposes of the specification and claims, to mean a peptide comprising an amino acid sequence, wherein the amino acid sequence has the amino acid sequence of SEQ ID NO:4. The term "isolated" when used in reference to a synthetic peptide means that it is substantially free (e.g., at least 90% pure, and more preferably at least 95% pure) of components which

have not become part of the integral structure of the peptide itself; e.g., such as substantially free of chemical precursors or other chemicals when chemically synthesized, produced, or modified using biological, biochemical, or chemical processes.

The terms "peptide fragment" and "intermediate" are used synonymously herein, in relation to a synthetic peptide having an amino acid sequence of SEQ ID NO:4, and for the purposes of the specification and claims, to mean a peptide comprising an amino acid sequence of no less than about 4 amino acids and no more than about 32 amino acid residues in length (and more preferably, no less than 5 amino acids, and no more than 30 amino acids), and comprises at least a portion (as contiguous amino acids) of the amino acid sequence of SEQ ID NO:4 (see Tables 2 and 4 for illustrative examples). As described herein (see, also, Tables 2 & 4), the term "peptide fragment" is also used herein to refer to a peptide fragment having operatively linked thereto fatty acid (either directly or through a linker). The term "isolated" when used in reference to a peptide fragment means that it is substantially free of components which have not become part of the integral structure of the peptide fragment itself; e.g., such as substantially free of chemical precursors or other chemicals when chemically synthesized, produced, or modified using biological, biochemical, or chemical processes. The peptide fragment may comprise, in its amino acid sequence, one or more conservative substitutions and/or one or more polymorphisms found in the corresponding amino acid sequence of HIV gp41. As apparent to one skilled in the art and from the teachings herein, a lysine in the amino acid sequence of a peptide may be substituted with another amino acid (naturally occurring or not naturally occurring) having a side chain with a free amino group (e.g., epsilon amine). Ornithine is an illustrative example of such amino acid that may be used to substitute a lysine. Further, while in a preferred embodiment peptide fragments (singly or when combined as a set to form a synthetic peptide) are synthesized such that peptidic bonds are formed between the amino acid residues, it is apparent to one skilled in the art that non-peptidic bonds may be formed using reactions known to those skilled in the art (e.g., imino, ester, hydrazide, azo, semicarbazide, and the like).

The present invention also encompasses particular sets of peptide fragments which act as intermediates in a method of synthesis of either (a) a synthetic peptide having the amino acid sequence of SEQ ID NO:4, or (b) a conjugate comprised of a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid. The peptide fragments, in a set of peptide fragments, are covalently coupled ("combined" or "assembled") in a method of synthesizing a synthetic peptide having an amino acid sequence of SEQ ID NO:4. Similarly, and wherein at least one peptide fragment, of a set of peptide fragments, comprises a peptide fragment operatively linked to fatty acid, the peptide fragments in a set of peptide fragments are combined in a

method of synthesizing a conjugate comprising a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid. The peptide fragments of the invention may include, but are not limited to, those having the amino acid sequences depicted in the following Table 2. Preferably, no less than 2 peptide fragments, and no more than 5 peptide fragments, are combined to form a synthetic having an amino acid sequence of SEQ ID NO:4, or a conjugate comprised of the synthetic peptide. Preferred peptide fragment(s) and sets(s) of peptide fragments may be used in the present invention to the exclusion of peptide fragment(s) and sets(s) of peptide fragments other than the preferred peptide fragment(s) and sets(s) of peptide fragments. The corresponding amino acids in SEQ ID NO:4 of each peptide fragment are also indicated.

Thus, as shown in Tables 2 & 4 and according to the method of the present invention of assembling at least 2 peptide fragments to form a synthetic peptide having the amino acid sequence of SEQ ID NO:4, each peptide fragment of the at least 2 peptide fragments is selected from a group consisting of a peptide fragment having an amino acid sequence of any one of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:22. As shown in Tables 2 & 4, each peptide fragment is made up of a number of contiguous amino acids of the amino acid sequence of SEQ ID NO:4. The sets of peptide fragments according to the invention include Sets 1-30, as designated in Table 2 (the numbering of a set is for ease of description only). As depicted in Tables 2 & 4, some peptide fragments (see e.g., Sets 9-16; SEQ ID NOs:16-21) comprise a peptide fragment operatively linked to fatty acid through a linker (a "peptide fragment-linker-fatty acid combination"). Such peptide fragment-linker-fatty acid combinations are illustrated (for purposes of ease of description only) by a lysine residue ("K") with a vertical line ("|") and an "X" appearing underneath the lysine residue; i.e., representing a peptide fragment to which is covalently coupled a linker ("|") via the epsilon amine of a lysine, and also covalently coupled to the linker is a fatty acid ("X"). As depicted in Tables 2 & 4, some peptide fragments (see e.g., Sets 17-30; SEQ ID NOs:24-31) comprise a peptide fragment operatively linked to fatty acid directly or through a linker at a terminal (C-terminal or N-terminal) amino acid of the peptide fragment (a "peptide fragment-fatty acid combination"). Such peptide fragment-fatty acid combinations are illustrated (for purposes of ease of description only) by an "X*", wherein X* is a fatty acid operatively linked either directly or through a linker to a terminal (C-, N-) amino acid of a peptide fragment.

Table 2

Set Number	Peptide fragments	Amino acid positions In SEQ ID NO:4
1	LTWQEWDREINN (SEQ ID NO:5) YTSLIHSLIEESQNQQ (SEQ ID NO: 6) EKNEQEL (SEQ ID NO:7)	1-12 13-28 29-35
2	LTWQEWDREINN (SEQ ID NO:5) YTSLIHSLIEESQNQQ (SEQ ID NO: 6) EKNEQELL (SEQ ID NO:8)	1-12 13-28 29-36
3	LTWQEWDREINNYTSLIHSL (SEQ ID NO:9) IEESQNQQEKNEQEL (SEQ ID NO:10)	1-20 21-35
4	LTWQEWDREINNYTSLIHSL (SEQ ID NO:9) IEESQNQQEKNEQELL (SEQ ID NO: 11)	1-20 21-36
5	LTWQEWDREINNYTSLIHSLIEESQNQQ (SEQ ID NO:12) EKNEQEL (SEQ ID NO:7)	1-28 29-35
6	LTWQEWDREINNYTSLIHSLIEESQNQQ (SEQ ID NO:12) EKNEQELL (SEQ ID NO:8)	1-28 29-36
7	LTWQEWDREINNYTSL (SEQ ID NO:13) IHSLIEESQNQQEKNEQEL (SEQ ID NO:14)	1-16 17-35
8	LTWQEWDREINNYTSL (SEQ ID NO:13) IHSLIEESQNQQEKNEQELL (SEQ ID NO:15)	1-16 17-36
9	LTWQEWDREINN (SEQ ID NO:5) YTSLIHSLIEESQNQQ (SEQ ID NO: 6) EKNEQEL (SEQ ID NO:16) I X	1-12 13-28 29-35
10	LTWQEWDREINN (SEQ ID NO:5) YTSLIHSLIEESQNQQ (SEQ ID NO: 6) EKNEQELL (SEQ ID NO:17) I X	1-12 13-28 29-36
11	LTWQEWDREINNYTSLIHSL (SEQ ID NO:9) IEESQNQQEKNEQEL (SEQ ID NO:18) I X	1-20 21-35
12	LTWQEWDREINNYTSLIHSL (SEQ ID NO:9) IEESQNQQEKNEQELL (SEQ ID NO:19) I X	1-20 21-36
13	LTWQEWDREINNYTSL (SEQ ID NO:13) IHSLIEESQNQQEKNEQEL (SEQ ID NO:20) I X	1-16 17-35
14	LTWQEWDREINNYTSL (SEQ ID NO:13) IHSLIEESQNQQEKNEQELL (SEQ ID NO:21) I X	1-16 17-36
15	LTWQEWDREINN (SEQ ID NO:5) YTSLIHSLIEESQNQQEKNEQELL (SEQ ID NO:22)	1-12 13-36

16	LTWQEWDREINN (SEQ ID NO:5) YTSLIHSLIEESQNQQEKNEQELL (SEQ ID NO:23) I X	1-12 13-36
17	X*LTWQEWDREINN (SEQ ID NO:24) YTSLIHSLIEESQNQQ (SEQ ID NO: 6) EKNEQEL (SEQ ID NO:7)	1-12 13-28 29-35
18	X*LTWQEWDREINN (SEQ ID NO:24) YTSLIHSLIEESQNQQ (SEQ ID NO: 6) EKNEQELL (SEQ ID NO:8)	1-12 13-28 29-36
19	LTWQEWDREINN (SEQ ID NO:5) YTSLIHSLIEESQNQQ (SEQ ID NO: 6) EKNEQELLX* (SEQ ID NO:28)	1-12 13-28 29-36
20	X*LTWQEWDREINNYTSLIHSL (SEQ ID NO:25) IEESQNQQEKNEQEL (SEQ ID NO:10)	1-20 21-35
21	X*LTWQEWDREINNYTSLIHSL (SEQ ID NO:25) IEESQNQQEKNEQELL (SEQ ID NO:11)	1-20 21-36
22	LTWQEWDREINNYTSLIHSL (SEQ ID NO:9) IEESQNQQEKNEQELLX* (SEQ ID NO:29)	1-20 21-36
23	X*LTWQEWDREINNYTSLIHSLIEESQNQQ (SEQ ID NO:26) EKNEQEL (SEQ ID NO:7)	1-28 29-35
24	X*LTWQEWDREINNYTSLIHSLIEESQNQQ (SEQ ID NO:26) EKNEQELL (SEQ ID NO:8)	1-28 29-36
25	LTWQEWDREINNYTSLIHSLIEESQNQQ (SEQ ID NO:12) EKNEQELLX* (SEQ ID NO:28)	1-28 29-36
26	X*LTWQEWDREINNYTSL (SEQ ID NO:27) IHSLIEESQNQQEKNEQEL (SEQ ID NO:14)	1-16 17-35
27	X*LTWQEWDREINNYTSL (SEQ ID NO:27) IHSLIEESQNQQEKNEQELL (SEQ ID NO:15)	1-16 17-36
28	LTWQEWDREINNYTSL (SEQ ID NO:13) IHSLIEESQNQQEKNEQELLX* (SEQ ID NO:30)	1-16 17-36
29	X*LTWQEWDREINN (SEQ ID NO:24) YTSLIHSLIEESQNQQEKNEQELL (SEQ ID NO:22)	1-12 13-36
30	LTWQEWDREINN (SEQ ID NO:5) YTSLIHSLIEESQNQQEKNEQELLX* (SEQ ID NO:31)	1-12 13-36

The term "reactive functionality", when used herein for purposes of the specification and claims, means a chemical group or chemical moiety that is capable of forming a bond with another chemical group or chemical moiety. For example, a reactive functionality of a molecule is reacted with a reactive functionality of another molecule in chemically coupling (or otherwise, operatively linking) the two molecules. Thus, with respect to an example of the present invention, a first reactive functionality of a linker is reacted with a reactive functionality of an amino acid being incorporated into the amino acid sequence of the peptide fragment, in chemically coupling the linker to the amino acid to be incorporated into the amino acid sequence of the peptide fragment. A second

reactive functionality of a linker is, or has been, reacted with a reactive functionality of a fatty acid in chemically coupling the linker to the fatty acid. With respect to chemical groups, a reactive functionality is known to those skilled in the art to comprise a group that includes, but is not limited to, maleimide, thiol, carboxylic acid, hydrogen, phosphoryl, acyl, hydroxyl, acetyl, hydrophobic, amine, amido, dansyl, sulfo, a succinimide, a thiol-
5 reactive, an amine-reactive, a carboxyl-reactive, and the like. A preferred reactive functionality may be used, in application to the present invention, to the exclusion of a reactive functionality other than the preferred reactive functionality.

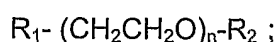
Each term "operatively linked" or "operatively linking", when used herein for purposes of the specification and claims, means that the two or more molecules are physically associated by a linking means that does not interfere with the ability of either of the linked molecules to function as described herein. For example, a synthetic peptide or peptide fragment may be operatively linked to fatty acid. "Operatively linked to fatty acid" means that the synthetic peptide or peptide fragment is either operatively linked directly to fatty acid, or is operatively linked to fatty acid through a linker. In the case of being
10 operatively linked directly to fatty acid, the synthetic peptide or peptide fragment, via its reactive functionality, may be operatively linked to a reactive functionality of a fatty acid using standard chemical techniques. In the case that the synthetic peptide or peptide fragment is operatively linked to fatty acid through a linker, an amino acid, as an amino acid already part of the amino acid sequence of a synthetic peptide or peptide fragment
15 or as an amino acid to be incorporated into the amino acid sequence of a synthetic peptide or peptide fragment, via its reactive functionality may be operatively linked to a linker via a reactive functionality of the linker. The linker may already be operatively linked to a fatty acid (a linker-fatty acid combination), or is subsequently operatively linked to a fatty acid. As known to those skilled in the art, and as will be more apparent by the following embodiments, there are several methods and compositions in which two or more molecules may be operatively linked utilizing reactive functionalities, including chemical means, and by genetic fusion (recombinant expression). Additionally, it is also known in the art that a bond formed by operatively linking two molecules may comprise,
20 but is not limited to, one or more of: covalent, ionic, hydrogen, van der Waals and the like.

Each term "coupling", or "covalently coupling", or "chemically coupling" or use of any of these terms in the past tense (e.g., "coupled"), when used herein for purposes of the specification and claims and in relation to assembling two or more peptide fragments, means that there is a chemical bonding, and more preferably a covalent chemical
25 bonding, between a peptide fragment with another peptide fragment in the process of assembling the peptide fragments to form a synthetic peptide having an amino acid sequence of SEQ ID NO:4, or a conjugate comprised of the synthetic peptide.

The term "linker", when used herein for purposes of the specification and claims, means a compound or moiety that acts as a molecular bridge to operatively link two different molecules. For example, with respect to the present invention, a first reactive functionality of a linker is covalently coupled to a reactive functionality of a fatty acid, and a second reactive functionality of the linker is covalently coupled to a reactive functionality of an amino acid, wherein the amino acid is then incorporated into the amino acid sequence of a peptide fragment during its synthesis, or the amino acid is already part of the amino acid sequence of the peptide fragment (in forming a "peptide fragment-linker-fatty acid combination"). The peptide fragment may then be assembled with other peptide fragments to form the synthetic peptide, in forming the conjugate according to the methods of the present invention. In general, there is no particular size or content limitations for the linker so long as it can fulfill its purpose as a molecular bridge. Linkers are known to those skilled in the art to include, but are not limited to, chemical chains, chemical compounds (e.g., reagents), amino acids, and the like. The linkers may include, but are not limited to, homobifunctional linkers, heterobifunctional linkers, biostable linkers, and biodegradable linkers, as well known in the art. Preferably, when a linker is used, it is a non-planar (e.g., so that the synthetic peptide in the conjugate is not rigidly fixed to the fatty acid in the conjugate but remains flexible in the linkage).

Heterobifunctional linkers, well known to those skilled in the art, contain one end having a first reactive functionality to specifically link a first molecule, and an opposite end having a second reactive functionality to specifically link to a second molecule. It will be evident to those skilled in the art that a variety of monofunctional, difunctional, and polyfunctional reagents (such as those described in the catalog of the Pierce Chemical Co., Rockford, Ill.) may be employed as a linker with respect to the present invention. Depending on such factors as the molecules to be linked, and the conditions in which the linking is performed, the linker may vary in length and composition for optimizing such properties as preservation of biological function stability, resistance to certain chemical and/or temperature parameters, and of sufficient stereo-selectivity or size. For example, the linker should not significantly interfere with the ability of the synthetic peptide (to which it is linked) to function as an inhibitor of either or both of HIV fusion and HIV transmission to a target cell.

Preferred linkers, as described herein in more detail, comprise from no less than 2 and no more than 100 units of ethylene glycol; as may be represented by the formula:



wherein R is a reactive functionality, and n is from 2 to 100, and more preferably, n is from 2 to 30. Preferred linkers include PEG3; discrete units comprised of PEG3 such as (PEG 3)₂, (PEG 3)₄, (PEG 3)₅, (PEG 3)₉; PEG13; (PEG 13)₂; PEG25; PEG29; a

combination of preferred linkers (e.g., a linker made by combining two or more PEG linkers together; for example, a linker comprised of a PEG13 operatively linked to a PEG3); and amino acid linkers (e.g., comprising combinations of Gly, Ser, and like amino acids; ranging from no less than 3 amino acids to no more than 15 amino acids). A preferred linker may be used, in application to the present invention, to the exclusion of a linker other than the preferred linker.

The term "chemical protecting group", when used herein for purposes of the specification and claims, means a chemical moiety that is used to block a reactive functionality from chemically reacting with another reactive functionality. Chemical protecting groups are well known by those in the art of peptide synthesis to include, but are not limited to, tBu (t-butyl), trt (triphenylmethyl(trityl)), OtBu (tert-butoxy), Boc or t-Boc (tert-butyloxycarbonyl), Fmoc (9-fluorenylmethoxycarbonyl), Aoc (t-amyoxy-carbonyl), TEOC (β -trimethylethyloxycarbonyl), CLIMOC (2-chloro-1-indanyl methoxyl carbonyl), BIMOC (benz-[f]-indene-3-methoxylcarbonyl), PBF (2,2,4,6,7-pentamethyldihydro-benzofuan-5-sulfonyl), 2-Cl-Z (chlorobenzyl-oxycarbonyl), Alloc (allyloxycarbonyl), Cbz (benzyloxycarbonyl), Adoc (adamantyloxy-carbonyl), Mcb (1-methylcyclobutyloxy-carbonyl), Bpoc (2-(p-biphenyl) propyl-2-oxycarbonyl), Azoc (2-(p-phenylazophenyl) propyl-2-oxycarbonyl), Ddz (2,2 dimethyl-3,5-dimethyloxybenzyl-oxycarbonyl), MTf (4-methoxy-2,3,6-trimethoylbenzenesulfonyl), PMC (2,2,5,7,8-pentamethylchroman-6-sulfonyl), Tos (tosyl), Hmb (2-hydroxyl-4-methoxybenzyl), Poc (2-phenylpropyl-2-oxycarbonyl), Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl), ivDde (1-(4,4-dimethyl-2,6-dioxo-cyclohex-1-ylidene)-3-methylbutyl), benzyl, dansyl, para-nitrobenzyl ester, and the like. A preferred chemical protecting group may be used, in application to the present invention, to the exclusion of a chemical protecting group other than the preferred chemical protecting group.

The term "deprotection", when used herein for purposes of the specification and claims, is known in the art to mean a process by which chemical protecting group(s) is removed from a molecule containing one or more chemical protecting groups, wherein the molecule comprises an amino acid, peptide fragment, synthetic peptide, or conjugate comprised of a synthetic peptide. Generally, the deprotection process involves reacting the molecule protected by one or more chemical protecting groups with a chemical agent that removes the chemical protecting group. For example, an N-terminal alpha amino group, which is protected by a chemical protecting group, may be reacted with a base to remove base labile chemical protecting groups (e.g., Fmoc, and the like). Chemical protecting groups (e.g., Boc, TEOC, Aoc, Adoc, Bopc, Ddz, Cbz, and the like) are removed by acid. Other chemical protecting groups, particularly those derived from carboxylic acids, may be removed by acid or a base.

The terms "first", "second", "third" and the like, may be used herein to: (a) indicate an order; or (b) to distinguish between molecules or chemical groups of a molecule (e.g., for ease of description); or (c) a combination of (a) and (b). However, the terms "first", "second", "third" and the like, are not otherwise to be construed as limiting the invention.

5 [End of formal Definition Section]

The present invention relates to methods and compositions (e.g., peptide fragments, Sets of peptide fragments, and a modified amino acid) for synthesizing a synthetic peptide, or conjugate comprising a synthetic peptide, having the amino acid sequence of SEQ ID NO:4. A conjugate comprised of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid may be represented by the following Formula 1, with B being operatively linked to the N-terminus, Z being operatively linked to the C-terminus, and J being operatively linked to an internal amino acid, lysine:

15 Formula 1

B-LTWQEWDRINNYTSLIHSLIEESQNQQEKNEQELL-Z

I
J

wherein B, Z, and J may each be a member selected from the group consisting of a fatty acid, a linker-fatty acid combination, and a chemical group. Preferably, no more than 2 of B, J, or Z comprise a fatty acid or linker-fatty acid combination. A fatty acid may comprise one or more fatty acid molecules. A linker-fatty acid combination may comprise one or more linkers and one or more fatty acid molecules. A chemical group may comprise, but is not limited to, one or more of: a reactive functionality, or a chemical protecting group. Techniques useful for introducing a chemical group at the N-terminus of a peptide fragment, or the C-terminus of a peptide fragment, or a combination of the N-terminus and C-terminus of a peptide fragment, are well known in the art.

In a preferred conjugate, B and Z each be comprise a chemical group, and J is a linker-fatty acid combination comprising PEG3-C18 which is operatively linked (via a reactive functionality of the PEG3 linker) to a synthetic peptide having the amino acid sequence of SEQ ID NO:4 via the lysine at amino acid position 30 of SEQ ID NO:4 (See also, Table 3, Ref# 4-2). When the antiviral activity of this preferred conjugate was compared with the antiviral activity of synthetic peptide alone (SEQ ID NO:4) against a panel of several clinical isolates of HIV-1, this preferred conjugate consistently demonstrated an increased antiviral potency, typically ranging from 2 fold more potent (e.g., 2 fold decrease in IC50 value) to 50 fold more potent. Additionally, this preferred conjugate was compared to synthetic peptide alone (SEQ ID NO:4; not part of a

conjugate) for activity against a representative HIV-2 strain (HIV2, _{NIHZ}). This preferred conjugate showed significant antiviral activity against HIV-2 (Category C) as compared to the synthetic peptide alone (Category D), and further demonstrated about a 10 fold increase in antiviral potency against HIV-2.

5 In another preferred conjugate, B is a fatty acid or linker-fatty acid combination operatively linked to the N-terminal amino acid of SEQ ID NO:4 (See, Table 3, Ref# 4-7 and Ref# 4-8). In another preferred conjugate, Z is a fatty acid or linker-fatty acid combination operatively linked to the C-terminal amino acid of SEQ ID NO:4 (See, Table 3, Ref# 4-9 and Ref# 4-10). In another preferred conjugate, more than one of B, J, and Z
10 is a fatty acid or linker-fatty acid combination operatively linked to the amino acid sequence of SEQ ID NO:4 (See, Table 3, Ref# 4-11). Table 3 shows a comparison of various conjugates and synthetic peptide alone, and that the conjugates demonstrate one or more improved pharmaceutical activities as compared to the synthetic peptide alone. Compared is antiviral activity in the absence of HSA (IC₅₀), antiviral activity in the
15 presence of HSA with fatty acids (IC₅₀ + HSA FrV), and pharmacokinetic properties of total body clearance (CL), and terminal elimination half-life (t_{1/2}). "Category A" is used herein to designate compositions with a calculated IC₅₀ value less than or equal to 0.005 µg/ml. "Category C" is used herein to designate compositions with a calculated IC₅₀ value less than or equal to 0.10 µg/ml, but greater than 0.005 µg/ml. "Category D" is
20 used herein to designate compositions with a calculated IC₅₀ value less than or equal to 1.0 µg/ml, but greater than 0.10 µg/ml.

Table 3

Ref#	FA	Linker	Linkage	IC ₅₀ III B	IC ₅₀ + HSA FrV	CL L/K/h	t _{1/2} (hr)
SEQ ID NO:4	None	None	None	C	C	0.30	0.6
4-1	C18	PEG25	K30	A	A	0.01	6.3
4-2	C18	PEG3	K30	A	A	0.007	8.9
4-3	C18	(PEG13) ₂	K30	A	A	0.009	7.28
4-4	C12	PEG3	K30	A	C	0.02	5.4
4-5	C16	PEG3	K30	A	C	0.004	15.4
4-6	C20	PEG3	K30	A	A		
4-7	C18	None	N-terminus	C	C	0.001	31.0
4-8	C18	PEG3	N-terminus	C	C	0.001	27.1
4-9	C18	None	C-terminus	A	A		
4-10	C18	PEG3	C-terminus	A	A	0.004	10.6
4-11	C18	PEG3	on each of K30, & N- terminus	C	C		

This invention is based, in part, on the unexpected discovery that certain combinations of peptide fragments may be utilized in a synthetic process, such as a combining (through chemically coupling) a set of peptide fragments (a synthetic process that may also be referred to as "combinatorial synthesis"), to allow for the manufacture of a synthetic peptide having the amino acid sequence of SEQ ID NO:4, or a conjugate comprised of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid directly or through a linker, on a large scale with high throughput and high yield (as compared to conventional linear solid phase synthesis only). Such peptide fragments and methods allow for manufacture of the synthetic peptide or a conjugate to be synthesized on a scale of kilogram amounts. Further, using peptide fragments and methods according to the present invention, the efficiency of synthesis of the synthetic peptide or conjugate may be increased, as compared to a standard automated solid phase synthesis on a peptide synthesizer (e.g., using approximately 3 to 5 fold less raw material and reagents). Further, the peptide fragments and methods according to the present invention reduce impurities, and therefore allow for synthetic peptides and conjugates to be synthesized in high purity, as compared to conventional techniques for producing such synthetic peptide or conjugate (e.g., solely conventional solid phase synthesis).

EXAMPLE 1

With respect to peptide synthesis, the individual peptide fragments themselves, and a synthetic peptide having the amino acid sequence of SEQ ID NO:4 produced from combining peptide fragments of a set of peptide fragments according to the present invention (or a conjugate comprised of the synthetic peptide), may each be made using techniques known to those skilled in the art for synthesizing peptide sequences. For example, in a novel approach of using peptide synthesis techniques, a conjugate comprised of synthetic peptide operatively linked to fatty acid may be produced solely using solid phase synthesis. In another example, in a preferred approach, the peptide fragments may be synthesized in solid phase, and then combined in solution phase in a process of assembly to produce the synthetic peptide or conjugate. In another approach, solution phase synthesis may be used to produce the peptide fragments, which then are combined in solid phase in a process of assembly to produce the synthetic peptide or conjugate. In still another approach, each peptide fragment may be synthesized using solid phase synthesis, and then combined in solid phase in a process of assembly to produce the synthetic peptide or conjugate. In a preferred embodiment, each peptide

fragment is produced using solid phase synthesis known to those skilled in the art. In a preferred embodiment, a synthetic peptide having the amino acid sequence of SEQ ID NO:4 produced using an assembly process that combines solid phase and solution phase techniques using a set of peptide fragments according to the present invention. In

- 5 another preferred embodiment, a conjugate comprised of a synthetic peptide having the amino acid sequence of SEQ ID NO:4, a linker, and a fatty acid, is produced using an assembly process that combines solid phase and solution phase techniques using a set of peptide fragments according to the present invention. Protected peptide fragments according to the present invention include, but are not limited to, the peptide fragments
- 10 listed in Table 4.

Table 4

SEQ ID NO:	Amino acid sequence	Amino acid positions in SEQ ID NO:4
5	Ac-LTWQEWDREINN	1-12
6	CPG-YTSLIHSLIEESQNQQ	13-28
7	CPG-EKNEQEL CPG	29-35
8	EKNEQELL-NH ₂	29-36
8	CPG-EKNEQELL-NH ₂	29-36
9	Ac-LTWQEWDREINNYTSLIHSL	1-20
10	CPG-IEESQNQQEKNEQEL	21-35
11	CPG-IEESQNQQEKNEQELL-NH ₂	21-36
11	IEESQNQQEKNEQELL-NH ₂	21-36
12	Ac-LTWQEWDREINNYTSLIHSLIEESQNQQ	1-28
13	Ac-LTWQEWDREINNYTSL	1-16
14	CPG-IHSLIEESQNQQEKNEQEL	17-35
15	IHSLIEESQNQQEKNEQELL-NH ₂	17-36
16	CPG-EKNEQEL X	29-35
17	CPG-EKNEQELL-NH ₂ X	29-36
17	EKNEQELL-NH ₂ X	29-36
18	CPG-IEESQNQQEKNEQEL X	21-35
19	IEESQNQQEKNEQELL-NH ₂ X	21-36
19	CPG-IEESQNQQEKNEQELL-NH ₂ X	21-36
20	CPG-IHSLIEESQNQQEKNEQEL	17-35

	I X	
21	IHSLIEESQNQQEKNEQELL-NH ₂	17-36
	I X	
21	CPG-IHSLIEESQNQQEKNEQELL-NH ₂	17-36
	I X	
22	CPG-YTSLIHSLIEESQNQQEKNEQELL-NH ₂	13-36
22	YTSLIHSLIEESQNQQEKNEQELL-NH ₂	13-36
23	CPG-YTSLIHSLIEESQNQQEKNEQELL-NH ₂	13-26
	I X	
28	CPG-EKNEQELLX*	29-36
29	CPG-IEESQNQQEKNEQELLX*	21-36
30	CPG-IHSLIEESQNQQEKNEQELLX*	17-36
31	CPG-YTSLIHSLIEESQNQQEKNEQELLX*	13-36

K – represents the epsilon amine of lysine protected by CPG
CPG

Ac- acetyl group, NH₂-amide group (but can be another chemical group as described in more detail in the "Definitions" section herein; see also, Formula 1 herein); CPG is chemical protecting group (e.g., Fmoc or other N-terminal chemical protecting group, as described in more detail in the "Definitions" section herein).

In the synthesis of an amino acid sequence (e.g., peptide fragment, synthetic peptide, conjugate) according to the present invention, any one or more of the side-chains of the amino acids in the amino acid sequence may be protected with a chemical protecting group. In a preferred embodiment, t-Bu comprises the chemical protecting group for side chains of amino acid residues tyrosine (Y), threonine (T), and serine (S). In a preferred embodiment, OtBu comprises the chemical protecting group for side chains of amino acid residues glutamic acid (E) and aspartic acid (D). In another preferred embodiment, trt comprises the chemical protecting group for side chains of amino acid residues glutamine (Q), histidine (H), and asparagine (N). In another preferred embodiment, Boc comprises the chemical protecting group for side chains of amino acid residues lysine (K), tryptophan (W), histidine (H), or arginine (R; using (Boc)₂). In a preferred embodiment, PBF comprises the chemical protecting group for side chains of amino acid residue arginine (R). In a preferred embodiment, it is not necessary to protect the side chain of leucine (L) or isoleucine (I).

A process of assembly, in which peptide fragments of a set of peptide fragments are combined to produce a synthetic peptide having the amino acid sequence of SEQ ID NO:4, or a conjugate comprised of such synthetic peptide operatively linked to a fatty

acid, is also referred to as a fragment condensation approach, or a fragment assembly approach. Briefly, each peptide fragment, of a set of peptide fragments, is preferably synthesized using solid phase peptide synthesis techniques. However, some peptide fragments may be preferred to be produced using solution phase synthesis, as will be described in more detail herein. Generally speaking, the fragment condensation approaches specifically described herein are preferred embodiments, wherein one approach involves assembly of 3 fragments (e.g., as illustrated in Example 2 herein, and FIG. 2), and another approach involves assembly of 2 fragments (e.g., as illustrated in Examples 3, 4, & 5 herein, and FIGs. 3 & 4). However, depending on length of the peptide fragments, and the amino acid sequence of each peptide fragment, it is preferable that no less than 2 peptide fragments and no more than 5 peptide fragments be synthesized, and then assembled to complete the synthesis of the synthetic peptide having the amino acid sequence of SEQ ID NO:4, or a conjugate comprised of such synthetic peptide operatively linked to a fatty acid.

EXAMPLE 2

In referring to Table 3 (Set 1 and Set 2) and FIG. 2, illustrated is a method for synthesis of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 using 3 specific peptide fragments (e.g., SEQ ID NOs:5-7 + Leu; or SEQ ID NOs:5, 6, and 8), and using a fragment condensation approach involving combining the 3 peptide fragments to produce the synthetic peptide. Surprisingly, each of these peptide fragments demonstrated physical properties and solubility characteristics that make them preferred peptide fragments to be used in a method for synthesis of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 in high yield and high purity. A peptide fragment having the amino acid sequence of SEQ ID NO:5, and comprising the first 12 amino acids of SEQ ID NO:4 was synthesized by standard solid phase synthesis (using a super acid sensitive resin; e.g., 4-hydroxy-methyl-3-methoxyphenoxy-butyric acid resin, or 2-chlorotrityl chloride resin- "CTC", FIG.2), with acetylation of ("Ac", as a chemical group) the N-terminus, while having a hydroxyl group (-OH) at the C-terminus (see, FIG. 2, "Ac-AA(1-12)-OH"). A peptide fragment having the amino acid sequence of SEQ ID NO:6, and comprising amino acids 13-28 of SEQ ID NO:4 was synthesized by standard solid phase synthesis with Fmoc at the N-terminus (as a chemical protecting group), and -OH at the C-terminus (see, FIG. 2, "Fmoc-AA(13-28)-OH"). A peptide fragment having the amino acid sequence of SEQ ID NO:7, and comprising amino acids 29-35 of SEQ ID NO:4 was synthesized by standard solid phase synthesis with Fmoc at the N-terminus (as a chemical protecting group), and -OH at the C-terminus (see, FIG. 2, "Fmoc-AA(29-35)-OH"). Each peptide fragment was cleaved from the resin used for its solid phase

synthesis by cleavage reagents, solvents, and techniques well known to those skilled in the art. Each peptide fragment was then isolated by removing the majority of above mentioned solvents by distillation and precipitating the peptide fragment by the addition of water with or without an alcohol containing-cosolvent. The resulting solid was isolated by
5 filtration, washed, reslurried in water or alcohol/water, refiltered, and dried in a vacuum oven

As shown in FIG. 2, a peptide fragment was produced by solution phase synthesis, when the peptide fragment having the amino acid sequence of SEQ ID NO:7 (see, FIG. 2, "Fmoc-AA(29-35)-OH") was chemically coupled to Leu, amino acid 36 of
10 SEQ ID NO:4 which has been amidated, in solution phase to result in a peptide fragment having the amino acid sequence of SEQ ID NO:8 (comprising amino acids 29-36 of SEQ ID NO:4) with amidation of the C-terminus (as a chemical group) (see, FIG. 2, "Fmoc-AA(29-36)-NH₂"). In summary of this solution phase reaction, the carboxy terminus of isolated peptide fragment Fmoc-AA(29-35)-OH is converted to an active ester of HOBt
15 (1-hydroxybenzotriazole hydrate), 6 Cl HOBt, or HOAT (1-Hydroxy-7-azabenzotriazole) using HBTU (O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate) or TBTU (O-benzotriazol-1-yl-N,N,N',N'-tetramethyltetrafluoro-borate) and HOBt, 6-Cl HOBt, or HOAT, respectively, in the presence of DIEA (diisopropylethyl amine) (or similar base such as NMM, collidine, and the like) and leucine amide (e.g., a combination
20 of coupling reagents and racemization suppressants). The reaction is run in a polar, aprotic solvent such as DMF (dimethyl formamide) or NMP (N-methyl pyrrolidinone) at 0°C to 30°C. At the completion of the reaction, alcohol or a water miscible solvent and/or water are added to precipitate the peptide fragment having the amino acid sequence of SEQ ID NO:8 with amidation of the C-terminus (Fmoc-AA(29-36)-NH₂).

As schematically illustrated in FIG. 2, to produce peptide fragment Fmoc-AA(29-36)-NH₂ using a peptide fragment Fmoc-AA(29-35)-OH combined with leucine amide (see, FIG. 2, "H-Leu-NH₂") in a solution phase process, the peptide fragment Fmoc-AA(29-35)-NH₂ (12.51 g, 6.4 mmol, 1 eq), H-Leu-NH₂*HCl (1.059 g, 6.4 mmol, 1 eq), and HOAT (1.30 g, 9.5 mmol, 1.5 eq) were dissolved in DMF (50 ml, 4 vol) treated with DIEA (5.39
30 ml, 31.8 mmol, 5 eq), and stirred at room temperature until dissolved (about 5 minutes). The solution was then cooled using an ice bath, and TBTU (2.45 g, 7.6 mmol, 1.2 eq) was added. The reaction was stirred for approximately 5 minutes at 0°C, then at room temperature for 2.5 hours. Analysis by HPLC (high performance liquid chromatography) showed no more starting fragment. The resulting preparation is Fmoc-AA(29-36)-NH₂ (see, e.g., Table 4, SEQ ID NO:8).

The Fmoc chemical protecting group of the peptide fragment Fmoc-AA(29-36)-NH₂ was removed using a base such as piperidine or potassium carbonate in organic

solvents such as DMF, NMP, or mixtures thereof. In this example, piperidine (1.5 ml, 15.2 mmol, 2.4 eq) was added to remove the Fmoc protecting group. The reaction was stirred overnight. The reaction mixture was then poured into 400 ml of water, and the solids formed were collected via filtration and dried. The dry solid was suspended in 400 ml 3:1 MTBE:hexanes and stirred for 4 hours, then collected and dried. The solids were then suspended in 400 mL 1:1 ethanol:water and stirred for 1 hour. The solids were collected and dried. As shown in FIG. 2 (see, e.g., Table 4, SEQ ID NO:8), the result was a substantially pure preparation of peptide fragment H-AA(29-36)-NH₂.

As illustrated in FIG. 2, a solution phase reaction was then performed in which peptide fragment H-AA(29-36)-NH₂ (SEQ ID NO:8) is combined with peptide fragment Fmoc-AA(13-28)-OH (SEQ ID NO:6) to yield a peptide fragment Fmoc-AA(13-36)-NH₂ (Table 4, SEQ ID NO:22 with chemical groups at each of the N-terminus and C-terminus). Peptide fragment H-AA(29-36)-NH₂ (8.74 g, 4.7 mmol, 1 eq), peptide fragment Fmoc-AA(13-28)-OH (16.32 g, 4.7 mmol, 1 eq), and HOAT (0.96 g, 7.1 mmol, 1.5 eq) were dissolved in DMF (100 ml, 20 vol), cooled with an ice bath, and treated with DIEA (4.0 ml, 23.5 mmol, 5 eq). Added to the reaction was TBTU (1.81 g, 5.6 mmol, 1.2 eq), and the reaction was stirred for about 5 minutes at 0°C, then 2 hours at room temperature. Analysis by HPLC showed no more starting material. Piperidine (2 ml, 20 mmol, 4 eq) was then added to remove the Fmoc, and the reaction stirred for 3 hours. The reaction was poured into 450 ml water and the solids filtered off and dried. The solids were then suspended in 450 ml 3:1 MTBE:hexanes and stirred for about 3 hours. The solids were collected and dried, then suspended in 450 ml 1:1 ethanol:water and stirred for about 1 hour. The solids were filtered off and dried affording H-AA(13-36)-NH₂ (SEQ ID NO:22; Table 4) as a substantially pure white solid as determined by HPLC analysis for purity.

As illustrated in FIG. 2, peptide fragment H-AA(13-36)-NH₂ (SEQ ID NO:22) was then assembled in a solution phase reaction with peptide fragment Ac-(1-12)-OH (SEQ ID NO:5) to yield a synthetic peptide having the amino acid sequence of SEQ ID NO:4 (see, e.g., FIG. 2, Ac-(1-36)-NH₂). Peptide fragment H-AA(13-36)-NH₂ (11.18 g, 2.20 mmol, 1 eq), peptide fragment Ac-AA(1-12)-OH (6.69 g, 2.20 mmol, 1 eq), and HOAT (0.450 g, 3.31 mmol, 1.5 eq) were dissolved in DMF (120 ml, 10 vol), cooled with an ice bath, and treated with DIEA (2.0 ml, 11.5 mmol, 5 eq). Added to the reaction was TBTU (0.847 g, 2.64 mmol, 1.2 eq). After stirring 5 minutes at 0°C, the reaction was stirred at room temperature for 2 hours, after which HPLC showed the reaction was complete. Hydrazine (10 mL, 321 mmol, 146 eq) was then added and the reaction stirred for about 20 minutes. Analysis by HPLC showed no more protected fragment, so the reaction was poured into 500 ml ice-water. The solids formed were collected and dried, and then purified by HLPC, affording a substantially pure preparation of synthetic peptide Ac-AA(1-

36)-NH₂ (SEQ ID NO:4).

As shown in FIG. 2, the side chain chemical protecting groups of synthetic peptide Ac-AA(1-36)-NH₂ may be removed by acidolysis or any other method known to those skilled in the art for deprotecting a peptide by removing side chain chemical protecting groups. In this example, synthetic peptide Ac-AA(1-36)-NH₂ (16.98 g, 2.09 mmol) was treated with TFA (trifluoroacetic acid):DTT(dithiothreitol):water (95:5:5; 200 ml) and stirred at room temperature for 4 hours. MTBE (approximately 500 ml) was added, and a precipitate was collected by filtration. The dried powder was then dissolved in 400 ml of 3:1% water/acetonitrile containing 1% HOAc (acetic acid), and was reacted for 20 hours to allow for substantially pure preparation (deprotected and decarboxylated) of synthetic peptide having an amino acid sequence of SEQ ID NO:4, as determined by HPLC analysis for purity.

EXAMPLE 3

In referring to Table 2 (Set 3 and Set 4) and FIG. 3, illustrated is a method for synthesis of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 using 2 specific peptide fragments (e.g., SEQ ID NOs:9-10 + Leu; or SEQ ID NOs:9 and 11), and using a fragment assembly approach involving combining the 2 peptide fragments by chemically coupling them to produce the synthetic peptide. Surprisingly, each of these peptide fragments demonstrated physical properties and solubility characteristics that make them preferred peptide fragments to be used in a method for synthesis of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 in high yield and greater purity. Additionally, because of such high yield and degree of purity obtained with this two fragment assembly approach, such an approach may be preferable over a three fragment assembly approach (when the two approaches are compared with each other) due to fewer isolations needed, and fewer impurities generated during the two fragment approach. Also, in selecting peptide fragments to be used in a two fragment assembly approach, it was discovered that having leucine and/or isoleucine residues at the point of juncture between the two fragments being assembled together (e.g., the C-terminal amino acid of a peptide fragment having the amino acid sequence of SEQ ID NO:9, and the N-terminal amino acid of a peptide fragment having the amino acid sequence of SEQ ID NO:10 or SEQ ID NO:11) favored assembly in the high yield and the degree of purity obtained.

A peptide fragment having the amino acid sequence of SEQ ID NO:9, and comprising the first 20 amino acids of SEQ ID NO:4 was synthesized by standard solid phase synthesis, with acetylation of ("Ac", as a chemical group) the N-terminus, while having a hydroxyl group (-OH) at the C-terminus (see, FIG. 3; also referred to herein as

"Ac-AA(1-20)-OH"). A peptide fragment having the amino acid sequence of SEQ ID NO:10, and comprising amino acids 21-35 of SEQ ID NO:4 was synthesized by standard solid phase synthesis with Fmoc at the N-terminus (as a chemical protecting group), and -OH at the C-terminus (see, FIG. 3; also referred to herein as "Fmoc-AA(21-35)-OH").

5 As shown in FIG. 3, a peptide fragment was produced by solution phase synthesis, when the peptide fragment Fmoc-AA(21-35)-OH was chemically coupled to Leu, amino acid 36 of SEQ ID NO:4 which has been amidated, in solution phase to result in a peptide fragment having the amino acid sequence of SEQ ID NO:11 (comprising amino acids 21-36 of SEQ ID NO:4) with amidation of the C-terminus (as a chemical group) ("Fmoc-AA(21-36)-NH₂").

10 To produce peptide fragment Fmoc-AA(21-36)-NH₂ using a peptide fragment Fmoc-AA(21-35)-OH combined with leucine ("H-Leu NH₂") in a solution phase process, the peptide fragment Fmoc-AA(21-35)-OH (200 g, 50.5 mmol, 1.0 eq), H-Leu-NH₂ (7.89 g, 60.6 mmol, 1.2 eq), and 6-Cl HOBT (10.3 g, 60.6 mmol, 1.2 eq) were dissolved
15 in DMF (2000 ml, 10 vol) treated with DIEA (13.2 ml 75.75 mmol, 1.5 eq) and stirred at room temperature until dissolved (about 30 minutes). The solution was cooled to 0 ± 5°C, and TBTU (19.5 g, 60.6 mmol, 1.2 eq) was added, stirred for 10 minutes at 0 ± 5°C, and then allowed to react at 25 ± 5°C for 30 minutes or until the reaction was shown complete by HPLC. The Fmoc chemical protecting group of the peptide fragment Fmoc-AA(21-36)-NH₂ (Table 4, SEQ ID NO:11) was then removed prior to isolation of the fragment H-AA(21-36)-NH₂ by adding piperidine (27.5 mL, 278 mmol, 5.5 eq), and the solution was
20 stirred for 1 hour at 25 ± 5°C or until analysis by HPLC showed that substantially all the Fmoc was removed from the peptide fragment. The reactor was cooled, and water (2000 ml, 10 vol) was added, and the free-flowing slurry was stirred 30 minutes at <10°C, and
25 then isolated by filtration. The collected solid was washed with 1:1 EtOH/water and dried in a vacuum oven at 35 ± 5°C. The peptide fragment was then reslurried in MTBE (2000 mL, 10 vol) at 30 ± 5°C for 4 hours and then isolated by filtration and redried. The MTBE reslurry may be repeated if necessary to remove additional piperidine. The result is a substantially pure preparation of peptide fragment H-AA(21-36)-NH₂ (see FIG.3; and
30 Table 4, SEQ ID NO:11).

As illustrated in FIG.3, a solution phase reaction was then performed in which peptide fragment H-AA(21-36)-NH₂ (SEQ ID NO:11) was combined with peptide fragment Ac-AA(1-20)-OH (SEQ ID NO:9; Table 4) to yield a synthetic peptide having the amino acid sequence of SEQ ID NO:4 (see, e.g., FIG. 3, Ac-(1-36)-NH₂). Peptide fragment H-AA(21-36)-NH₂ (195 g, 50.7 mmol, 1.07 eq), peptide fragment Ac-AA(1-20)-OH (210 g, 47.4 mmol, 1.0 eq), 6-Cl HOBT (16.1 g, 94.8 mmol, 2.0 eq) and DIEA (24.8 ml, 142.2 mmol, 3 eq) were dissolved in DMF (4200 ml, 20 vol), and cooled to 0 ± 5°C. Added to
35

the reaction was TBTU (18.3 g, 56.88 mmol, 1.2 eq). The reaction was stirred for 15 minutes at $0 \pm 5^\circ\text{C}$, and at $25 \pm 5^\circ\text{C}$ for 30 minutes or until the reaction was shown to be complete by HPLC. The reactor was cooled, and water (4200 ml, 20 vol) was slowly added. A slurry was formed and stirred at $<10^\circ\text{C}$ for at least 30 minutes. The solid was isolated by filtration and washed with additional water. The collected solid dried in a vacuum oven at $35 \pm 5^\circ\text{C}$. The result was a substantially pure preparation of fully protected synthetic peptide Ac-AA(1-36)-NH₂ (SEQ ID NO:4), as determined by HPLC analysis for purity. As illustrated in FIG.3, the synthetic peptide Ac-AA(1-36)-NH₂ was then deprotected (by removing the side chain chemical protecting groups) and decarboxylated (at the tryptophan residues) by using the methods described herein in Example 2, or any other method known to those skilled in the art, for deprotection and decarboxylation. The result was a substantially pure preparation (deprotected and decarboxylated) of synthetic peptide having an amino acid sequence of SEQ ID NO:4, as determined by HPLC analysis for purity.

EXAMPLE 4

Using similar techniques and conditions as described in Examples 2 and 3 herein, additional fragment assembly approaches have been used to produce the synthetic peptide having an amino acid sequence of SEQ ID NO:4 (See, e.g., Tables 2 & 4). For example, with reference to Table 2, peptide fragments in Set 5 (SEQ ID NO:12, "AA-1-28"; and SEQ ID NO:7, "AA-29-35", with addition of leucine ("AA-36-NH₂") in forming "AA-29-36-NH₂" (SEQ ID NO:8), see also Table 4) and Set 6 (SEQ ID NO:12, "AA-1-28"; and SEQ ID NO:8, "AA-29-36-NH₂", see also Table 4), have been used in a fragment condensation approach to produce the synthetic peptide having an amino acid sequence of SEQ ID NO:4. Synthesis of a peptide fragment Ac-AA(1-28)-OH was performed by standard solid phase synthesis ("SPPS"). Fmoc-AA(29-35)-OH was built on 25 g H-Leu-CTC resin (0.59 mmol/g) using standard SPPS conditions; however, chemical protecting group ivdde was used on the epsilon amine of lysine at amino acid position 30 ("Lys₃₀ivDde"). After cleavage with 1% TFA/DCM by standard conditions, and drying, recovered is a white powder of substantially pure Fmoc-AA(29-Lys₃₀ivDde-35)-OH (see, Table 4, SEQ ID NO:7).

Fmoc-AA(29-Lys₃₀ivDde-35)-OH (1.6 g, 1 eq) was dissolved in DMF (11 mL). H-Leu-NH₂ (127 mg, 1.2 eq) and 6 Cl-HOBt (152 mg, 1.1 eq) were added, DMF (2 mL) was added as a wash, and the solution was cooled to $0-5^\circ\text{C}$. DIEA (212 μL , 1.5 eq) was added followed by TBTU (287 mg, 1.1 eq). The reaction was stirred at $0-5^\circ\text{C}$ for 10 minutes. The solution was heated to $25 \pm 5^\circ\text{C}$ and stirred for 1 hour. A sample was analyzed by HPLC, indicating that the reaction was complete. Piperidine (402 μL , 4.0 eq)

was added, and the reaction was stirred at $25\pm 5^{\circ}\text{C}$ and stirred for 30 minutes. The deprotection was monitored by HPLC and deemed complete after 2 hours. Water (16 mL) was added to precipitate the product. The product was isolated by filtration, washed with water, and reslurried in methanol/water. The product was again isolated by filtration and washed with water/ethanol (1:1). The damp solid was reslurried in 50% EtOH/H₂O (50 mL) then filtered, washed with 50% EtOH/H₂O, and dried. The solids were reslurried twice in MTBE (8 vol). The solids were collected by filtration, washed with MTBE, and dried to yield H-AA(29-Lys₃₀ivDde-36)-NH₂.

Ac-AA(1-28)-OH (3.0 g, 1 eq) and H-AA(29-Lys₃₀ivDde-36)-NH₂ (888 mg, 1 eq) were dissolved in DMF (45 mL). 6 Cl-HOBt (97 mg, 1.2 eq) was added, DMF (5 mL) was added as a wash, and the solution was cooled to $0-5^{\circ}\text{C}$. DIEA (125 μL , 1.5 eq) was added followed by TBTU (184 mg, 1.2 eq). The reaction was stirred at $0-5^{\circ}\text{C}$ for 15 minutes. The solution was heated to $25\pm 5^{\circ}\text{C}$ and stirred for 1 hour. A sample was removed and analyzed by HPLC, indicating that the reaction was complete. Hydrazine hydrate (2.6 mL) was added to remove the ivDde, and the reaction was stirred at $25\pm 5^{\circ}\text{C}$ for 2 hours. Water (50 mL) was added to precipitate the product. The product was isolated by filtration, washed with water, and reslurried in ethanol/water (1:1). The product was again isolated by filtration, washed with water/ethanol (1:1), and dried to yield a substantially pure preparation of protected synthetic peptide having the amino acid sequence of SEQ ID NO:4 (Ac-AA(1-36)-NH₂; no ivDde). The peptide was then treated with TFA/water/DTT to remove the acid labile side chain protecting groups, and then purified by HPLC to yield a substantially pure preparation of synthetic peptide having the amino acid sequence of SEQ ID NO:4 (Ac-AA(1-36)-NH₂).

EXAMPLE 5

For purposes of illustration, presented are examples of operatively linking fatty acid to a terminal (C- or N-) amino acid of a peptide fragment, and use of that peptide fragment in a fragment condensation approach to produce a conjugate comprised of synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid. The first example illustrates operatively linking fatty acid to the C-terminus of the synthetic peptide by operatively linking the fatty acid to a peptide fragment used in the synthesis of synthetic peptide. In this example, the fatty acid was covalently coupled to the amino acid to be incorporated into the peptide fragment as the terminal amino acid. In this first example, produced is CPG-Leu-C18 to be incorporated as the C-terminal amino acid of a peptide fragment comprising amino acids 21-36 of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 ("AA(21-36)"). CPG-Leu-OH (1.05 g,

3.96 mmol, 1.0 eq) was dissolved in DMF (10 mL, 10 vol) and DCM (20 mL, 20 vol), and HOAT (1.076 g, 7.91 mmol, 2 eq) and DIEA (2.75 mL, 15.8 mmol, 4 eq) were added. The resulting yellow solution was cooled to $0 \pm 5^\circ\text{C}$, and then TBTU (1.525 g, 4.75 mmol, 1.2 eq) and octadecylamine (C18 fatty acid; 1.274 g, 4.73 mmol, 1.2 eq) were added, and the reaction was stirred at $0 \pm 5^\circ\text{C}$ for 5 minutes. The reaction had thickened, so it was allowed to heat to $25 \pm 5^\circ\text{C}$ and additional DCM (10 mL, 10 vol) was added. The reaction was stirred at $25 \pm 5^\circ\text{C}$ for 2 hours, after which HPLC showed no more starting material present. The dichloromethane was taken off under reduced pressure, and the resulting oily solid was suspended in 100 mL water. The oily solid was collected by filtration and dried over night. The result is a CPG-Leu-NH(CH₂)₁₇CH₃ ("CPG-Leu-C18"). A deprotection reaction was then performed to remove the chemical protecting group (CPG). CPG-Leu-C18 (1.002 g, 1.93 mmol, 1 eq) was dissolved in DCM (10 mL, 10 vol), and methanol (MeOH, 10 mL, 10 vol) and Pd/C (palladium on carbon; 0.257 g) was added. The reaction was placed under a hydrogen (H₂) atmosphere and stirred over night. HPLC showed no more starting material, so the reaction was filtered through celite, and the solvent taken off in vacuo, affording H-Leu-C18 as a solid.

To produce peptide fragment Fmoc-AA(21-36)-NHC18) (see Tables 2 & 4, SEQ ID NO:29) using a peptide fragment Fmoc-AA(21-35)-OH combined with H-Leu-C18 in a solution phase process, the peptide fragment Fmoc-AA(21-37)-OH (2.01 g, 0.508 mmol, 1.0 eq), H-Leu-C18 (0.234 g, 0.611 mmol, 1.2 eq), HOAT (1.04 g, 0.764 mmol, 1.5 eq) and DIEA (0.354 mL, 2.03 mmol, 4 eq) were dissolved in DMF (20 mL, 10 vol) and DCM (5 mL, 2.5 vol), and the reaction was cooled to $0 \pm 5^\circ\text{C}$. TBTU (0.197 g, 0.613 mmol, 1.2 eq) was added, stirred for 5 minutes at $0 \pm 5^\circ\text{C}$, and then allowed to react at $25 \pm 5^\circ\text{C}$ for 2 hours or until the reaction was shown complete by HPLC. The Fmoc chemical protecting group of the peptide fragment Fmoc-AA(21-36)-NHC18 was then removed prior to isolation of the fragment H-AA(21-36)-NHC18. In this deprotection reaction, piperidine (0.301 mL, 3.04 mmol, 6 eq) was added, and the solution was stirred for 1 hour at $25 \pm 5^\circ\text{C}$ or until analysis by HPLC showed that substantially all the Fmoc was removed from the peptide fragment. DCM was taken off in vacuo, and the remaining aqueous solution poured into 50 mL ice water. The gummy solid was isolated by filtration and dried over night. The peptide fragment is then reslurried in 1:1 EtOH/ water (20 mL, 10 vol) for 2 hours. The solids were collected and dried. The peptide fragment was then slurried in 3:1 hexanes:MTBE (20 mL, 10 vol) over night and then isolated by filtration and redried. The result is a substantially pure preparation of peptide fragment having fatty acid operatively linked thereto ("H-AA(21-36)-NHC18"; SEQ ID NO:29).

To produce a conjugate comprised of a synthetic peptide, having an amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid ("Ac-AA(1-36)-NHC18"), peptide

fragments of Table 2, Set 22 were combined in a fragment condensation approach. Peptide fragment H-AA(21-36)-NHC18 (SEQ ID NO:29; 1.501 g, 0.366 mmol, 1 eq), peptide fragment comprising amino acids 1-20 of SEQ ID NO:33 ("Ac-AA(1-20)-OH"; SEQ ID NO:9; 1.628 g, 0.368 mmol, 1.0 eq), and HOAT (0.075 g, 0.551 mmol, 1.5 eq) and DIEA (0.255 ml, 0.146 mmol, 4 eq) were dissolved in DMF (15 ml, 10 vol), and the reaction was cooled to $0 \pm 5^\circ\text{C}$. Added to the reaction was TBTU (0.141 g, 0.439 mmol, 1.2 eq). The reaction was stirred for 5 minutes at $0 \pm 5^\circ\text{C}$, and then at $25 \pm 5^\circ\text{C}$ for 3 hours or until the reaction was shown to be complete by HPLC. The reactor was cooled, and water (50 mL, 33 vol) was slowly added. The gummy solid was isolated by filtration and washed with additional water. The collected solid dried in a vacuum oven at $35 \pm 5^\circ\text{C}$. The conjugate Ac-AA(1-36)-NHC18 was then deprotected (by removing the side chain chemical protecting groups) and decarboxylated (at the tryptophan residues) by using the methods described herein, or any other method known to those skilled in the art, for deprotection and decarboxylation, with subsequent purification. The result was a substantially pure preparation (deprotected and decarboxylated) of a conjugate comprising a synthetic peptide operatively linked to a fatty acid, wherein the fatty acid is operatively linked to the C-terminus of the synthetic peptide.

Using peptide fragments in Set 20 (SEQ ID NO:25 & SEQ ID NO:10 + Leu36) or Set 21 (SEQ ID NO:25 & SEQ ID NO:11), produced by a fragment condensation approach is a conjugate comprising a synthetic peptide operatively linked to a fatty acid, wherein the fatty acid is operatively linked to the N-terminus of the synthetic peptide. The fragment approach is essentially the same as that described in this example for the conjugate comprising a synthetic peptide operatively linked to a fatty acid, wherein the fatty acid is operatively linked to the C-terminus of the synthetic peptide. The difference is that instead of using SEQ ID NO:29 for a peptide fragment comprising amino acids 21-36 of SEQ ID NO:4, used is SEQ ID NO:11. Instead of using SEQ ID NO:9 for amino acids 1-20, used is SEQ ID NO:25 having a fatty acid operatively linked to the N-terminal amino acid of the peptide fragment. Fatty acid can be operatively linked to the N-terminal amino acid of the peptide fragment by standard coupling methods known in the art. For example, H-AA(1-20)-CPG (SEQ ID NO:9) and fatty acid (C18-OPFP), and DIEA are dissolved in DMF, and stirred for 2 hours at room temperature. The reaction mixture is cooled by ice bath and pre-cooled water is added in slowly. The precipitated peptide fragment ("C18-AA(1-20)-CPG") is vacuum filtered, washed by 10% EtOH-water, and dried in the oven overnight. The preparation of conjugate is then deprotected, and purified using methods described herein.

In this embodiment, illustrated is a fragment condensation approach to synthesizing a conjugate, wherein the conjugate comprises synthetic peptide having the

amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid at two different locations of the synthetic peptide (see, e.g., Table 3, Ref# 4-11). Thus, more than one of the peptide fragments used in the fragment condensation approach is operatively linked to fatty acid. This example also illustrates use of peptide fragments from multiple sets as listed in Table 2. For example, illustrated is a peptide fragment having the amino acid sequence of SEQ ID NO:25 (Table 2, Set 20) combined with a peptide fragment having the amino acid sequence of SEQ ID NO:19 (Table 2, Set 12) to form the conjugate. To produce a conjugate comprising more than one fatty acid, a synthetic peptide having an amino acid sequence of SEQ ID NO:4 was synthesized by a fragment condensation approach using a peptide fragment having an amino acid sequence of SEQ ID NO:25 having operatively linked at the N-terminus a linker-fatty acid combination ("C18-PEG3"), and a peptide fragment having an amino acid sequence of SEQ ID NO:19 having a linker-fatty acid combination operatively linked to an internal lysine ("K30") ("AA(21-30(PEG3-C18)-36)").

One gram of Fmoc-AA(1-20)-O-2-CTC resin was put into peptide reactor, which was temperature-controlled at 30°C with circulating bath. 10vol of DCM was charged into the reactor, and the reaction was agitated with nitrogen for 15 minutes, and then drained. Fmoc protection group was removed by the treatment of 20% piperidine in NMP (N-methyl pyrrolidinone; 10vol) for 20 minutes, twice. NMP washed the resin until a negative chloranil test was obtained. The reactor was charged with pre-activated C18-PEG3-OH/6-Cl-HOBt/TBTU/DIEA (2/2/2/2.3eq) in DMF/DCM(1:1, 8 vol) and the mixture was agitated by nitrogen until a negative ninhydrine test was obtained. The resin was washed by NMP (10 vol x 4) and DCM (10 vol x 4). The reactor was charged with 1%TFA in DCM (10 vol) and the suspension was agitated by nitrogen for 2 minutes at 0°C, then DCM solution was drained into a flask with pyridine (1.26 vol relative to TFA). After this treatment was repeated ten times, the resin was washed by DCM twice and IPA twice. The solvent in the pooled solution was removed under reduced pressure. The residue was dissolved in EtOH, and precipitated with water. The solid was collected by vacuum filtration, washed by 10% EtOH-water and dried in the vacuum oven overnight. The resulting white solid is a preparation of C18-PEG3-AA(1-20)-OH (SEQ ID NO:25).

C18-PEG3-AA(1-20)-OH (SEQ ID NO:25; 113mg, 1eq), AA(21-30(PEG3-C18)-36)-NH₂ (SEQ ID NO:19; 100mg, 1eq), 6-Cl-HOBt (8.2mg, 2eq), DIEA (12.5ul, 3eq) and DMF (2ml, 20vol) were charged into a 25-ml flask. The resulted solution was cooled to 0°C by ice bath. TBTU (10mg, 1.3eq) was added, and solution was stirred for 1 hour with ice bath, and 3 hours at room temperature. The reaction mixture was cooled again by ice bath to 0°C and pre-cooled water was added in slowly. The precipitated peptide was vacuum filtered, washed by 10% EtOH-water, and then dried in the oven overnight. The

preparation of conjugate ("C18-PEG3-AA(1-30-(PEG3-C18)-36)-NH₂ ") was then deprotected, and purified. C18-PEG3-AA(1-30-(PEG3-C18)-36)-NH₂ (180mg) was dissolved in 15vol of TFA/DTT/water (90/5/5) and stirred at room temperature for 4.5 hours. Pre-cooled MTBE was added into the reactor slowly to maintain the temperature at less than 10 °C. The resulting slurry was stirred at room temperature for 30 minutes before vacuum filtration. After being washed with MTBE, the solid was reslurried in acetonitrile with pH adjust to 4 by AcOH and DIEA overnight. The suspension was diluted by 50mM NH₄OAc and the solution was filtered and directly loaded onto HPLC for purification. The result is a substantially homogeneous conjugate comprised of fatty acid operatively linked (through a linker) to the N-terminus, and fatty acid operatively linked (through a linker) to an internal amino acid (lysine in amino acid position 30), of a synthetic peptide having an amino acid sequence of SEQ ID NO:4.

EXAMPLE 6

In this embodiment, described are methods for producing linkers of discrete size, and for producing linker-fatty acid combinations. For example, a linker used in the present invention comprises (PEG3)_n, wherein n = 1-10. To illustrate the synthesis of the linker comprising (PEG3)_n (where n > 1), commercially obtained was starting material known as 8-amino-3,6 dioxaoctanoic acid, and what is referenced herein as "PEG3", generally represented by the structure:



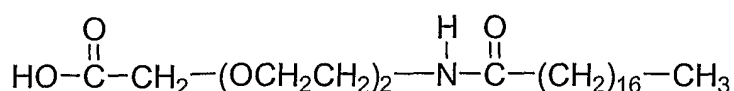
wherein R₁ and R₂ are each a reactive functionality, and more preferably a reactive functionality selected from the group consisting of a hydrogen, oxygen, a hydroxyl, an amine-reactive group, a carboxyl-reactive group, and a chemical protecting group ("CPG") used in peptide synthesis to protect a reactive functionality from further chemical reactivity. Various discrete length linkers, having PEG3 as a subunit, were produced by covalently coupling two or more PEG3 molecules together to get the desired discrete length of the linker. Thus, for example, to produce (PEG3)₂, two PEG3 units were covalently coupled; to produce (PEG3)₃, a PEG3 unit was covalently coupled to a (PEG3)₂; and successive PEG3 units may be covalently coupled to achieve a (PEG3)_n where n = 4-10. More preferably, the specific number of ethylene glycol units (i.e., -OCH₂CH₂) in the chemical backbone of the linker ranges from 2 to 30 ethylene glycol units, depending on the value of n, one or two of which ethylene glycol units may be broken or substituted for in forming a reactive functionality or for linking a molecule of PEG3 to a another molecule of PEG3 in forming (PEG3)_n. Methods by which a reactive

functionality of a PEG linker (e.g., PEG13, PEG25, PEG29, etc.) may be operatively linked to a reactive functionality of a molecule are essentially the same as those described herein for the (PEG3)₂ linker.

In an illustrative example, a (PEG3)₂ linker is produced by solid phase synthesis.

2-CTC resin (15g, 1.61 mmol/g, 24.15 mmol) was swelled in 150 ml of CH₂Cl₂ twice for 5 minutes each time. PEG3 (Fmoc-PEG3-OH; 3.32g, 9 mmol) was dissolved in 150 ml of CH₂Cl₂ and DIEA (N,N-diisopropylethylamine; 2.90 mL, 18 mmol). The solution containing PEG3 was added to the swelled resin, and the resin was agitated for 2 hours. The resin was then washed three times with CH₂Cl₂. After the last wash, added to the resin was 10% DIEA in methanol (50 ml), and the resin was agitated for another 45 minutes. The resin was then washed four times with methanol (100 ml) and twice with CH₂Cl₂ (100 ml). The resin was dried (e.g., overnight). Three grams of the dried resin was then swelled with CH₂Cl₂ (30 ml) for 5 minutes. The Fmoc was cleaved by adding 20% piperidine in NMP (N-methylpyrrolidinone; 30 ml) twice for 30 minutes each time. The resin was then washed six times with NMP (30 ml) until a negative chloranil test was achieved. To the resin was added PEG3 (Fmoc-PEG3-OH; 0.73 g, 1.9 mmol) dissolved in 30 ml of DMF (dimethylformamide) and TCTU (tetramethyluronium tetrafluoroborate; 0.37g, 1.9 mmol) and DIEA (0.64 mL, 3.8 mmol), and the resin was agitated until a negative Kaiser test (approximately 2 hours). The resin was then washed three times with NMP. The product (PEG3)₂ was cleaved from the resin using 2% TFA in CH₂Cl₂ (15 ml) for 15 minutes. The solvent was taken off under reduced pressure to afford a preparation of (PEG3)₂, which then was purified for high purity using LC-MS. Using the same methodologies outlined above, a linker may be produced which comprises a combination of any of PEG13, PEG3, and PEG25.

Also illustrated in this embodiment is a method of making a linker-fatty acid combination. An illustrative and preferred fatty acid combination comprises C18-PEG3-R, where R is a reactive functionality. C18-PEG3-OH may be represented by the formula:



A method of making C18-PEG3-OH is illustrated in Example 7. However, other reactive functionalities have been used besides a hydroxyl. For example, a linker-fatty acid combination comprising C18-PEG3-OPFP was produced as follows. C18-PEG3-OH (25.08g, 1eq) and pentafluorophenol (PFP, 10.74g, 1eq) were mixed in DCM (500ml). The resulted suspension was cooled to 0°C, then 1,3-diisopropylcarbodiimide (DIC, 9.14ml, 1eq) was added into it. The reaction mixture was stirred at 0°C for 30 minutes,

and then for another 5 hours at room temperature, or until the reaction was shown to be completed. DCM was partially removed by evaporation under reduced pressure. The precipitate was removed by vacuum filtration and residual DCM was further removed by evaporation under reduced pressure. The solid was dried under high vacuum at room temperature overnight or until a constant weight. A purified preparation of C18-PEG3-OPFP was obtained as white solid.

EXAMPLE 7

The present invention also provides a novel composition comprising a modified amino acid comprising a lysine which is operatively linked to fatty acid through a linker (i.e., an amino acid-linker-fatty acid combination) which is incorporated into the amino acid sequence of a peptide fragment or synthetic peptide during synthesis. The modified amino acid may be represented by the formula:



wherein n is from 2 to 100, and more preferably, n is from 3 to 30; wherein FA is a fatty acid (see, e.g., Table 1; a preferred fatty acid is in the range of C-12 to C-20); wherein R_1 is a reactive functionality, and more preferably a reactive functionality selected from the group consisting of a hydrogen, oxygen, a hydroxyl, an amine-reactive group (including, but not limited to an activated ester), a carboxyl-reactive group; and wherein CPG comprises a chemical protecting group, preferably a chemical protecting group useful in peptide synthesis. Preferred chemical protecting groups include, but are not limited to, Fmoc, Boc (Fmoc and Boc are preferred amine protection groups), benzyl (Bz), trityl, and *t*-butyl. Preferably, CPG is at the amino terminus of the lysine, the PEG linker (with attached fatty acid) is covalently coupled to the lysine epsilon amine, and R_1 is at the carboxy terminus of the lysine.

In an illustrative example, Lys-PEG3-C18 (wherein the epsilon amine of lysine is covalently coupled to one reactive functionality of the linker PEG3, and the fatty acid C18 is covalently coupled to another reactive functionality of the linker PEG3) may be represented as CPG-Lys(PEG3-C18)- R_1 . This modified lysine may be incorporated into a peptide fragment, or a synthetic peptide, of the present invention during its synthesis, at a position in the amino acid sequence position corresponding to the lysine in an amino acid sequence of any one of SEQ ID NOs:16-21, and 23, as further illustrated in Tables 2 & 4. Assembly of this peptide fragment with one or more peptide fragments in a set of peptide fragments can result in production of a conjugate comprised of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid.

As an illustration of this embodiment, CPG-Lys(PEG3-C18)- R_1 , as exemplified by Fmoc-Lys(PEG3-C18)-OH, was synthesized by a process which included utilizing a solid

phase coupling. Fmoc-PEG3-OH was coupled to a solid phase resin by dissolving Fmoc-PEG₃-OH (30.3 g, 218.4 mmol, 0.36 eq) and DIEA (76 ml, 218.4 mmol, 2 eq) in DCM (700 ml, 5 vol) and adding the solution to 2-CTC resin (140 g, 218.4 mmol Cl, 1 eq) which had been pre-swelled in DCM. The coupling was allowed to proceed with agitation for 40 minutes at 20°C to 25°C. The resin was drained and unreacted sites were capped by adding a solution of 9:1 methanol: DIEA (840 ml, 6 vol). After 45 minutes at 25 ± 5°C, the resin was drained, and washed with methanol. The Fmoc group was removed from the resin-bound PEG3 by reswelling the resin in DCM and then treating it twice with 10% piperidine in NMP (700 ml, 5 vol) for 20 to 30 minutes. The resin was then washed with NMP to a negative chloranil test. Stearic acid (C18) was activated by dissolving stearic acid (20.5 g, 72.2 mmol, 1.1 eq) in 1:1 NMP/DCM (840 ml, 6 vol) by the addition of DIEA (6.3 ml, 85.3 mmol, 1.3 eq). TBTU (23.2 g, 72.2 mmol, 1.1 eq) was added and the solution was stirred. To the deprotected, resin bound PEG3 was added the activated C18 solution and the mixture was allowed to react for 1 to 3 hours or until the reaction was complete by the Kaiser test. The resin was washed twice with 1:1 DCM/NMP, 3 times with DCM, and then the resultant compound comprising a linker-fatty acid combination (C-18-PEG3) was cleaved from the resin by treatment with 2% TFA in DCM for 45 to 50 minutes. The resin was drained and washed repeatedly with DCM. The DCM was removed by distillation and replaced by MTBE which caused the product to precipitate. The product was isolated by filtration, reslurried in MTBE, re-isolated, and dried. The resultant product was a substantially pure preparation of C18-PEG3-OH.

Alternate methods may be used to produce CPG-Lys(PEG3-C18)-R₁. For example, to CPG-Lys-OH (50 mg, 0.134 mmol, 1.0 eq) was added ethyl acetate (1 mL). To the suspension was added DIEA (57 µL, 0.339 mmol, 2.5 eq) and C18-PEG3-OH (80.8 mg, 0.134 mmol, 1.0 eq) and the reaction was stirred for 18 hours. Hexanes were added and the solution was decanted away from the resulting solids. The product was purified by flash chromatography using DCM/MeOH as the mobile phase. The resultant product was a preparation of isolated CPG-Lys(PEG3-C18)OH. In another method to produce CPG-Lys(PEG3-C18)-R₁, Fmoc-Lys-OH (11.65 g, 1 eq) and DIEA (5 ml, 1 eq) were mixed in DMF (86 ml, 5 vol). C18-PEG3-OPFP was added into the solution, and the reaction mixture was stirred at room temperature for 3 hours or until analysis of a sample showed that the reaction was complete. The reaction mixture was cooled to 0°C to 5°C. HCl (0.01N, 342 ml, 20 vol) was added to the reaction mixture. The resulting suspension was stirred for 1 hour before undergoing vacuum filtration. After washing with water, the solid was reslurried in MTBE (10 vol) for 2 hours. The product was filtered, and washed by MTBE until HPLC analysis showed no PFO to PFP peak in the solvent front. The solid was dried in a vacuum oven, and a preparation of isolated Fmoc-Lys(PEG3-C18)-OH was

obtained.

EXAMPLE 8

In this example, illustrated is a fragment condensation approach for synthesizing a conjugate comprised of fatty acid operatively linked to a synthetic peptide having an amino acid sequence of SEQ ID NO:4. The modified amino acid according to the present invention (as described in Example 7) is used as an amino acid in the synthesis of a peptide fragment according to the present invention. Thus, a method of making a conjugate comprises the steps of: (a) synthesizing two or more peptide fragments, wherein one of the peptide fragments is synthesized so as to comprise a peptide fragment operatively linked to fatty acid through a linker; (b) combining the peptide fragments by chemically coupling to produce the conjugate comprising a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid through a linker. As an illustration of a method for producing such conjugate, refer to FIG. 4 and Tables 2 & 4 (SEQ ID NO:13, and either SEQ ID NO:20 or SEQ ID NO:21). A peptide fragment having an amino acid sequence of SEQ ID NO:20 ("Fmoc-AA(17-Lys₃₀(PEG3-C18)-35)-OH") was built on H-Leu-CTC resin (0.59 mmol/g) using standard SPPS conditions and recovered as white solid. A peptide fragment having an amino acid sequence of SEQ ID NO:21 was synthesized. Fmoc-AA(17-Lys₃₀(PEG3-C18)-35)-OH (1.50 g, 1 eq), H-Leu-NH₂ (46.8 mg, 1.2 eq) and 6 Cl-HOBt (61 mg, 1.2 eq) were dissolved in DMF (15 mL). DIEA (78.4 μ L, 1.5 eq) was added. The solution was cooled to 0°C to 5°C and TBTU (115.6 mg, 1.2 eq) was added in. The reaction was stirred at 0°C to 5°C for 15 minutes. The solution was heated to 25 $\pm$ 5°C and stirred for 1 hour. A sample was removed and analyzed by HPLC, indicating that the reaction was complete. Piperidine (130 μ L, 6eq) was added and the reaction was stirred at 25 $\pm$ 5°C and stirred for 1 hour. A sample was removed and analyzed by HPLC, indicating that the reaction was complete. Water (50 mL) was added to precipitate the product. The product was isolated by filtration, washed with water, and dried in the vacuum over overnight. The solid was reslurried in MTBE (30ml) for 4 hours. The product was again isolated by filtration, washed with MTBE, and dried to yield a peptide fragment having the amino acid sequence of SEQ ID NO:21 (H-AA(17-Lys₃₀(PEG3-C18)-36)-NH₂).

As illustrated in FIG. 4, a conjugate comprised of a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid was produced by a fragment condensation approach when a peptide fragment having the amino acid sequence of SEQ ID NO:21 (H-AA(17-Lys₃₀(PEG3-C18)-36)-NH₂) was operatively linked (covalently coupled) to a peptide fragment having the amino acid sequence of SEQ ID

NO:13 ("Ac-AA(1-16)-OH"). Ac-AA(1-16)-OH (552mg, 1 eq) and H-AA(17-Lys₃₀(PEG3-C18)-36)-NH₂ (773 mg, 1.05 eq) and 6-Cl-HOBt (51mg, 2eq) were dissolved in DMF (10 mL). DIEA (78.4 μ L, 3eq) was added in and the solution was cooled to 0°C to 5°C. TBTU (57.8mg, 1.2 eq) was added in and the reaction was stirred at 0°C to 5°C for 15 minutes.

- 5 The solution was heated to 25 $\pm$ 5°C, and stirred for 1 hour. A sample was removed and analyzed by HPLC, indicating that the reaction was complete. Water (25 mL) was added to precipitate the product. The product was isolated by filtration, washed with water and dried to yield the conjugate (Ac-AA(1-Lys₃₀(PEG3-C18)-36)-NH₂). As illustrated in FIG. 4, the conjugate was then subjected to global side chain deprotection and decarboxylation.
- 10 A 90/5/5 TFA/water/dithiothreitol solution (15ml, 15vol) was prepared and cooled to 0°C, and the conjugate (1g) was added into the above solution in portion. The ice bath was removed and the solid was dissolved shortly. The reaction mixture was stirred at room temperature for 4.5 hours, and then cooled to 0°C to 5°C. The pre-cooled MTBE (50ml) was added into the reactor slowly to maintain the temperature below 10°C. The resulted
- 15 slurry was stirred for 30 minutes with ice bath, and 4 hours at room temperature. The solid was collected by vacuum filtration, washed by MTBE, and then returned to the flask for decarboxylation. Acetonitrile (10 mL) was added into the flask, then 150 μ L of DIEA and 100 μ L of HOAc. The suspension with pH at 3.5 to 4 was stirred at room temperature overnight. The crude conjugate was collected as an off-white solid by vacuum filtration.
- 20 Purification was achieved by HPLC. The result is a substantially pure preparation (deprotected and decarboxylated) of a conjugate comprised of synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid through a linker.

EXAMPLE 9

- 25 In this example, illustrated is a method of producing a conjugate comprised of a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to a fatty acid through a linker, wherein the method is by linear synthesis (linear fashion by standard solid phase synthesis), and the modified amino acid according to the present invention (as described in Example 7) is incorporated into the amino acid sequence at
- 30 position 30 of SEQ ID NO:4. While this illustrative example describes the use of one type of resin ("sieber resin", Fmoc-Xanthidrylamine resin; N-alkylated 9-aminoxanthenyln resin), other resins useful in this method of linear synthesis include, but not limited to, Rink amide resin (4-[(2,4-Dimethoxyphenyl) Fmoc-aminomethyl]phenoxyacetamido methyl resin), MBHA resin (4-hydroxymethylamine resin), Rink MBHA resin, HMBA resin
- 35 (4-hydroxy-methylbenzoic resin), DMA resin (Sarcosine dimethylacrylamide resin), Oxime resin (*p*-Nitrobenzophenone oxime resin), and Ramage resin (2-([5-Fmoc-dibenzo[A,D]cycloheptane-2-yl]oxy)-acetamido methyl resin, or tricyclic amide linker

resin). These resins are examples of peptide synthesis resins often referred to as "amide" resins, because synthesis of a peptide on these resins, followed by cleavage of the synthesized peptide, can provide a peptide with a C-terminal amide group, and in some cases, a peptide with a preferred chemical group, other than an amide, at the C-terminal amino acid.

In a preferred embodiment of the method of the present invention for producing a conjugate comprised of a synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid, the C-terminal amino acid of the synthetic peptide has a chemical group comprising an amide. Thus, in a method of synthesizing a conjugate according to the present invention using an amide resin, and incorporating the modified amino acid (lysine operatively linked to fatty acid through a linker) during the solid phase peptide synthesis reaction, avoided are (a) the need to add to the synthesized peptide an amidated C-terminal amino acid in a solution phase reaction (e.g., AcAA1-K₃₀(PEG3-C18)-35-OH + H-AA36-NH₂); and (b) the need to operatively link fatty acid to the synthetic peptide in a separate reaction following the synthesis of the synthetic peptide. According to the present invention, provided is a method of synthesizing a conjugate comprises: linearly synthesizing a synthetic peptide having an amino acid sequence of SEQ ID NO:4 using an amide resin and solid phase peptide synthesis; incorporating a modified amino acid comprising a lysine operatively linked to fatty acid as an amino acid in the amino acid sequence of SEQ ID NO:4 (e.g., at position 30 of SEQ ID NO:4) during synthesis of the synthetic peptide to produce the conjugate; and cleaving the resultant conjugate from the resin. As a result of the method according to the present invention, the conjugate may be made with higher throughput (e.g., fewer isolation steps); and fewer impurities (such as those that may be generated in solution phase reactions or additional coupling reactions).

To illustrate this method by example, 4.5 gram of Sieber resin was put into peptide reactor, which was temperature-controlled at 30°C with circulating bath. DCM (10 vol) was charged into the reactor and agitated with nitrogen for 15 minutes, and then drained. 20% piperidine in NMP (10 vol) was charged into the reactor and agitated by nitrogen for 25min, and then drained. This treatment was repeated once. NMP (10 vol) was charged into the reactor and agitated with nitrogen for 1 minute, then drained. This NMP wash was repeated 8 times or until a negative chlorinil test was obtained. The pre-activated Fmoc-Leu-OH (2.0eq, as amino acid 36 of SEQ ID NO:4) with 6-Cl-HOBt/TBTU/DIEA (2.0eq/2.0/2.3eq) in DMF/DCM (6vol/2vol) was charged into the reactor and agitated with nitrogen for 1.5 hours or until a negative ninhydrin test was obtained. NMP (10 vol) was charged into the reactor and agitated with nitrogen for 1 minute, then drained. This NMP wash was repeated for 2 times. The addition of each successive amino acid in the

peptide chain, including the modified lysine (Lys(PEG3-C18) involves the same operation described above: Fmoc removal by 20% piperidine, coupling of amino acid and NMP wash. If a negative ninhydrin test couldn't be obtained within 3 hours of coupling, a re-couple or an acetylation would be performed. By the end of the assembly, the N-terminus was acetylated by Ac₂O (5eq) and pyridine (5eq) in NMP.

The conjugate, synthesized on the resin, was cleaved from the resin, deprotected, and then purified. For example, in one method the resin was treated with 2% TFA in DCM (10 vol) at 0°C for 2 minutes. This treatment was repeated for 10 times. The resin was then washed with DCM (10vol) and IPA (10vol X 2). The solvent was removed on the rotavap under reduced pressure. The residue was re-dissolved in IPA (10 vol) and precipitated by water. After being re-slurried in 10% EtOH (30 vol) for 2 hours, the solid was collected by vacuum filtration and dried in vacuum oven overnight. 1.44g of white solid was collected after drying. 800mg of this white solid was deprotected in 12 vol of TFA/DTT/water(90/5/5) for 5 hours at room temperature. The solid was precipitated by MTBE and collected by vacuum filtration. It was decarboxylated in acetonitrile with pH adjustment by AcOH and DIEA overnight. 400mg of this crude material was purified on HPLC and the result is a substantially pure preparation of a conjugate comprised of a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to a fatty acid through a linker (92.9% purity). In another method, 20 vol of TFA/DTT/water (90/5/5) was charged into the reactor containing the resin, and agitated at 20°C for 4.5 hours. The solution was collected in a flask with pre-cooled MTBE. The solid was collected by vacuum filtration. It was decarboxylated in acetonitrile with pH adjustment by AcOH and DIEA overnight. The resulting solid was collected as a crude preparation of conjugate. This conjugate was purified on HPLC or using other chromatographic methods or purification methods known in the art of peptide synthesis, and the resulting white fluffy solid is a substantially pure preparation of conjugate comprised of a synthetic peptide having an amino acid sequence of SEQ ID NO:4 operatively linked to a fatty acid through a linker (92.6% purity).

The foregoing description of the specific embodiments of the present invention have been described in detail for purposes of illustration. In view of the descriptions and illustrations, others skilled in the art can, by applying current knowledge, readily modify and/or adapt the present invention for various applications without departing from the basic concept; and thus, such modifications and/or adaptations are intended to be within the meaning and scope of the appended claims.

What is claimed is:

1. A method for synthesizing a synthetic peptide having the amino acid sequence of B-LTWQEWDREINNYTSLIHSLIEESQNQQEKNEQELL-Z (SEQ ID NO:4), the method comprising coupling at least 2 peptide fragments together in assembling a synthetic peptide having the amino acid of SEQ ID NO:4; wherein a peptide fragment comprises no less than about 4 contiguous amino acids and no more than about 32 contiguous amino acids of the amino acid sequence of SEQ ID NO:4; wherein when assembled together, the at least 2 peptide fragments form the amino acid sequence of SEQ ID NO:4; and wherein B and Z are each a chemical group.

2. The method according to claim 1, wherein each peptide fragment of the at least 2 peptide fragments is selected from a group consisting of a peptide fragment having an amino acid sequence of any one of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:22.

3. The method according to claim 1, wherein the at least 2 peptide fragments coupled together to form a synthetic peptide having the amino acid of SEQ ID NO:4 are: a peptide fragment having the amino acid sequence of SEQ ID NO:5; a peptide fragment having the amino acid sequence of SEQ ID NO:6; and a peptide fragment having the amino acid sequence of SEQ ID NO:8.

4. The method according to claim 1, wherein the at least 2 peptide fragments coupled together to form a synthetic peptide having the amino acid of SEQ ID NO:4 are: a peptide fragment having the amino acid sequence of SEQ ID NO:9; and a peptide fragment having the amino acid sequence of SEQ ID NO:11.

5. The method according to claim 1, wherein the at least 2 peptide fragments coupled together to form a synthetic peptide having the amino acid of SEQ ID NO:4 are: a peptide fragment having the amino acid sequence of SEQ ID NO:12; and a peptide fragment having the amino acid sequence of SEQ ID NO:8.

6. The method according to claim 1, wherein the at least 2 peptide fragments coupled together to form a synthetic peptide having the amino acid of SEQ ID NO:4 are: a peptide fragment having the amino acid sequence of SEQ ID NO:13; and a peptide fragment having the amino acid sequence of SEQ ID NO:15.

7. The method according to claim 1, wherein the at least 2 peptide fragments coupled

together to form a synthetic peptide having the amino acid of SEQ ID NO:4 are: a peptide fragment having the amino acid sequence of SEQ ID NO:5; and a peptide fragment having the amino acid sequence of SEQ ID NO:22.

- 5 8. A set of peptide fragments, the set comprising at least 2 peptide fragments that can be assembled to form a synthetic peptide having the amino acid of SEQ ID NO:4, comprising a set selected from the group consisting of:
- (a) a peptide fragment having the amino acid sequence of SEQ ID NO:5, a peptide fragment having the amino acid sequence of SEQ ID NO:6, and a peptide fragment
 - 10 having the amino acid sequence of SEQ ID NO:8;
 - (b) a peptide fragment having the amino acid sequence of SEQ ID NO:5, a peptide fragment having the amino acid sequence of SEQ ID NO:6, and a peptide fragment having the amino acid sequence of SEQ ID NO:7;
 - (c) a peptide fragment having the amino acid sequence of SEQ ID NO:9, and a peptide
 - 15 fragment having the amino acid sequence of SEQ ID NO:11;
 - (d) a peptide fragment having the amino acid sequence of SEQ ID NO:9, and a peptide fragment having the amino acid sequence of SEQ ID NO:10;
 - (e) a peptide fragment having the amino acid sequence of SEQ ID NO:12, and a peptide fragment having the amino acid sequence of SEQ ID NO:8;
 - 20 (f) a peptide fragment having the amino acid sequence of SEQ ID NO:12, and a peptide fragment having the amino acid sequence of SEQ ID NO:7;
 - (g) a peptide fragment having the amino acid sequence of SEQ ID NO:13, and a peptide fragment having the amino acid sequence of SEQ ID NO:15;
 - (h) a peptide fragment having the amino acid sequence of SEQ ID NO:13, and a peptide
 - 25 fragment having the amino acid sequence of SEQ ID NO:14; and
 - (e) a peptide fragment having the amino acid sequence of SEQ ID NO:5, and a peptide fragment having the amino acid sequence of SEQ ID NO:22.

9. The set of peptide fragments according to claim 8, wherein at least one peptide
- 30 fragment, of a set of peptide fragments, has a chemical protecting group.

10. A method for synthesizing a conjugate comprised of synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid, the method comprising coupling at least 2 peptide fragments together in assembling a conjugate
- 35 comprised of synthetic peptide having the amino acid of SEQ ID NO:4; wherein a peptide fragment comprises no less than about 4 contiguous amino acids and no more than about 32 contiguous amino acids of the amino acid sequence of SEQ ID NO:4; wherein at least

one of the peptide fragments is operatively linked to fatty acid; and wherein when assembled together, the at least 2 peptide fragments form a synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid.

5 11. The method according to claim 10, wherein fatty acid is operatively linked directly or through a linker to the synthetic peptide having the amino acid sequence of SEQ ID NO:4.

12. The method according to claim 10 wherein more than one fatty acid molecule is
10 operatively linked to the synthetic peptide having the amino acid sequence of SEQ ID NO:4.

13. The method according to claim 12, wherein fatty acid is operatively linked to the synthetic peptide having the amino acid sequence of SEQ ID NO:4 by one or more of:
15 directly; and through a linker.

14. The method according to claim 10 wherein the conjugate comprises a chemical group at a location on the synthetic peptide selected from the group of an N-terminal amino acid, a C-terminal amino acid, and a combination thereof.

20

15. The method according to claim 10, wherein each peptide fragment of the at least 2 peptide fragments is selected from a group consisting of a peptide fragment having an amino acid sequence of any one of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID
25 NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, and SEQ ID NO:31.

30 16. The method according to claim 10, wherein the at least 2 peptide fragments coupled together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:5; a peptide fragment having the amino acid sequence of SEQ ID NO:6; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:17.

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17. The method according to claim 10, wherein the at least 2 peptide fragments coupled together to form the conjugate are: a peptide fragment having the amino acid sequence of

SEQ ID NO:9; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:19.

18. The method according to claim 10, wherein the at least 2 peptide fragments coupled
5 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:13; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:21.

19. The method according to claim 10, wherein the at least 2 peptide fragments coupled
10 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:5; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:23.

20. The method according to claim 10, wherein the at least 2 peptide fragments coupled
15 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:6; a peptide fragment having the amino acid sequence of SEQ ID NO:8; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:24.

21. The method according to claim 10, wherein the at least 2 peptide fragments coupled
20 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:5; a peptide fragment having the amino acid sequence of SEQ ID NO:6; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:28.

22. The method according to claim 10, wherein the at least 2 peptide fragments coupled
25 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:11; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:25.

23. The method according to claim 10, wherein the at least 2 peptide fragments coupled
30 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:9; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:29.

24. The method according to claim 10, wherein the at least 2 peptide fragments coupled
35 together to form the conjugate are: a peptide fragment having the amino acid sequence of

SEQ ID NO:8; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:26.

25. The method according to claim 10, wherein the at least 2 peptide fragments coupled
5 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:12; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:28.

26. The method according to claim 10, wherein the at least 2 peptide fragments coupled
10 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:15; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:27.

27. The method according to claim 10, wherein the at least 2 peptide fragments coupled
15 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:13; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:30.

28. The method according to claim 10, wherein the at least 2 peptide fragments coupled
20 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:22; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:24.

29. The method according to claim 10, wherein the at least 2 peptide fragments coupled
25 together to form the conjugate are: a peptide fragment having the amino acid sequence of SEQ ID NO:5; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:31.

30. The method according to claim 10, wherein the at least 2 peptide fragments coupled
30 together to form the conjugate are: a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:19; and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:25.

35 31. A set of peptide fragments, the set comprising at least 2 peptide fragments that can be assembled to form a conjugate comprised of synthetic peptide having the amino acid of SEQ ID NO:4 operatively linked to fatty acid, comprising a set selected from the group

consisting of:

- (a) a peptide fragment having the amino acid sequence of SEQ ID NO:5, a peptide fragment having the amino acid sequence of SEQ ID NO:6, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:17;
- (b) a peptide fragment having the amino acid sequence of SEQ ID NO:5, a peptide fragment having the amino acid sequence of SEQ ID NO:6, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:16;
- (c) a peptide fragment having the amino acid sequence of SEQ ID NO:9, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:19;
- (d) a peptide fragment having the amino acid sequence of SEQ ID NO:9, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:18;
- (e) a peptide fragment having the amino acid sequence of SEQ ID NO:13, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:21;
- (f) a peptide fragment having the amino acid sequence of SEQ ID NO:13, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:20;
- (g) a peptide fragment having the amino acid sequence of SEQ ID NO:5, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:23;
- (h) a peptide fragment having the amino acid sequence of SEQ ID NO:6, a peptide fragment having the amino acid sequence of SEQ ID NO:8, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:24;
- (i) a peptide fragment having the amino acid sequence of SEQ ID NO:6, a peptide fragment having the amino acid sequence of SEQ ID NO:7, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:24;
- (j) a peptide fragment having the amino acid sequence of SEQ ID NO:5, a peptide fragment having the amino acid sequence of SEQ ID NO:6, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:28;
- (k) a peptide fragment having the amino acid sequence of SEQ ID NO:11, and a peptide

fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:25;

(l) a peptide fragment having the amino acid sequence of SEQ ID NO:10, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid

5 sequence of SEQ ID NO:25;

(m) a peptide fragment having the amino acid sequence of SEQ ID NO:9, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:29;

(n) a peptide fragment having the amino acid sequence of SEQ ID NO:8, and a peptide

10 fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:26;

(o) a peptide fragment having the amino acid sequence of SEQ ID NO:7, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:26;

15 (p) a peptide fragment having the amino acid sequence of SEQ ID NO:12, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:28;

(q) a peptide fragment having the amino acid sequence of SEQ ID NO:15, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid

20 sequence of SEQ ID NO:27;

(r) a peptide fragment having the amino acid sequence of SEQ ID NO:14, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:27;

(s) a peptide fragment having the amino acid sequence of SEQ ID NO:13, and a peptide

25 fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:30;

(t) a peptide fragment having the amino acid sequence of SEQ ID NO:22, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:24;

30 (u) a peptide fragment having the amino acid sequence of SEQ ID NO:5, and a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:31; and

(v) a peptide fragment operatively linked to fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:19; and a peptide fragment operatively linked to

35 fatty acid, wherein the peptide fragment has the amino acid sequence of SEQ ID NO:25.

32. The set of peptide fragments according to claim 31, wherein at least one peptide

fragment, of a set of peptide fragments, has a chemical protecting group.

33. Use of a modified amino acid, wherein the modified amino acid comprises a lysine having operatively linked to fatty acid through a linker, wherein the modified amino acid is incorporated into a peptide fragment during its solid phase peptide synthesis to produce a peptide fragment operatively linked to fatty acid, wherein the peptide fragment operatively linked to fatty acid and containing the modified amino acid is used in the method of synthesizing a conjugate according to any one of claims 1 through 30.

34. The use of a modified amino acid according to claim 33, wherein the fatty acid operatively linked to the lysine through a linker is a fatty acid having between 12 carbon atoms and 20 carbon atoms, and wherein the linker is a polyethylene glycol linker having between 2 to 30 ethylene glycol units.

35. Use of a modified amino acid, wherein the modified amino acid comprises a lysine having operatively linked to fatty acid through a linker, wherein the modified amino acid is incorporated into a peptide fragment during its solid phase peptide synthesis to produce a peptide fragment operatively linked to fatty acid, wherein the peptide fragment operatively linked to fatty acid and containing the modified amino acid is a peptide fragment operatively linked to fatty acid contained in any one of the sets of peptide fragments according to claim 31.

36. The use of a modified amino acid according to claim 35, wherein the fatty acid operatively linked to the lysine through a linker is a fatty acid having between 12 carbon atoms and 20 carbon atoms, and wherein the linker is a polyethylene glycol linker having between 2 to 30 ethylene glycol units.

37. A method for synthesizing a conjugate comprised of synthetic peptide having the amino acid sequence of SEQ ID NO:4 operatively linked to fatty acid, the method comprising: linearly synthesizing a synthetic peptide having an amino acid sequence of SEQ ID NO:4 using an amide resin and solid phase peptide synthesis; incorporating a modified amino acid, comprising lysine operatively linked to fatty acid, in amino acid position 30 of the amino acid sequence of SEQ ID NO:4 during synthesis of the synthetic peptide resulting in synthesis of the conjugate; and cleaving the resultant conjugate from the resin.

38. The method according to claim 37, further comprising the steps of deprotecting and

decarboxylating the cleaved conjugate, followed by chromatographic purification of the conjugate.

39. The method according to claim 37, wherein the fatty acid operatively linked to the
5 lysine is operatively linked to lysine through a linker, wherein the fatty acid comprises a
fatty acid having between 12 carbon atoms and 20 carbon atoms, and wherein the linker
is a polyethylene glycol linker having between 2 to 30 ethylene glycol units.

40. Use of a modified amino acid in the linear synthesis of a conjugate, wherein the
10 modified amino acid comprises a lysine having operatively linked to fatty acid through a
linker, wherein the modified amino acid is incorporated into a synthetic peptide having the
amino acid sequence of SEQ ID NO:4 during its synthesis to produce a conjugate
comprising a synthetic peptide operatively linked to fatty acid.

15

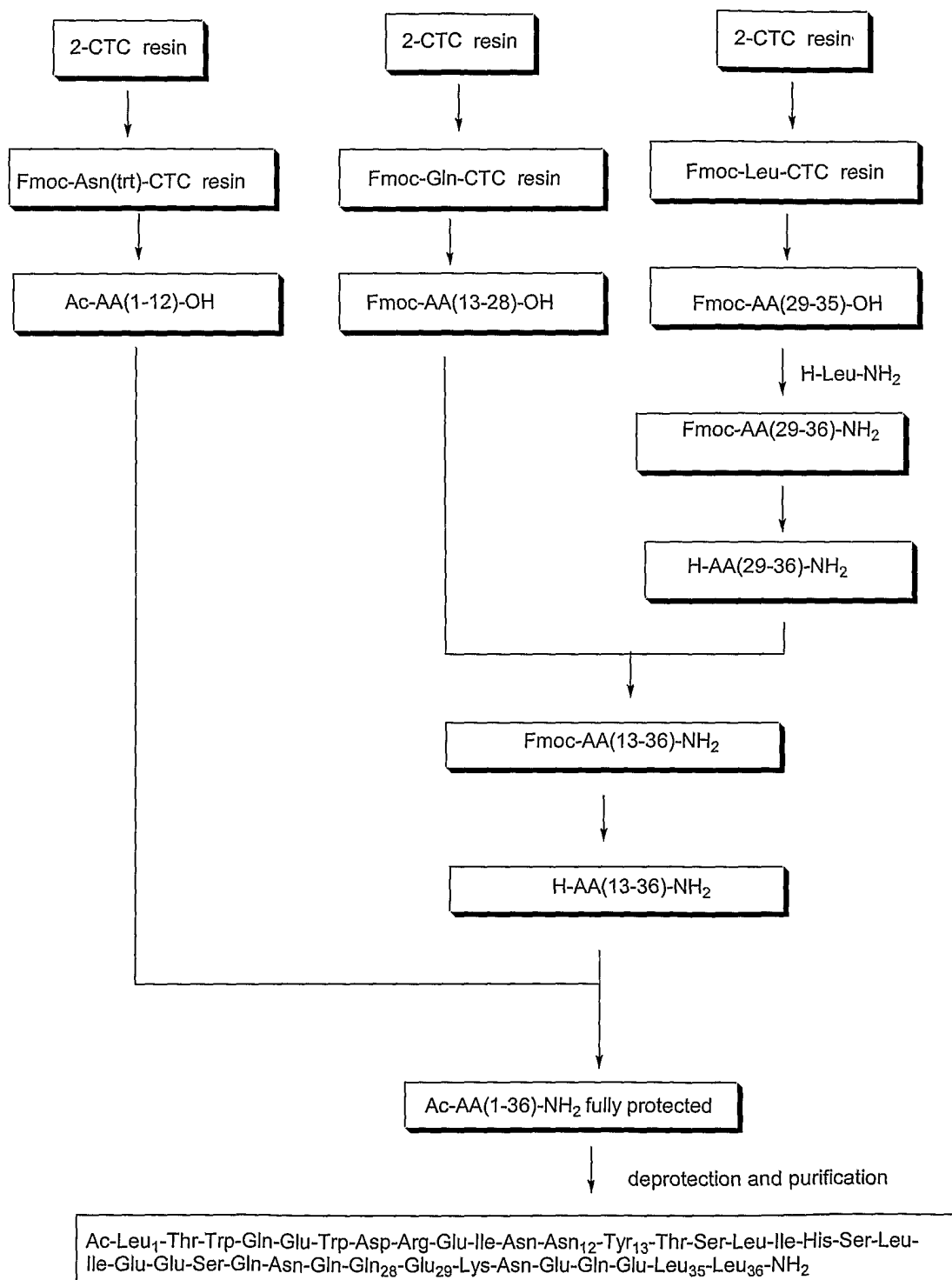
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FIG. 1

	MTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	
	-----+-----+-----+-----	
	10 20 30	
	-----+-----+-----+-----	
		<u>Isolate</u>
624	MTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	NL4-3
631	MTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	LAV1a
626	TTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	IIIB
626	TTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	HXB2
619	MTWMQWEKEINNYTGLIYNLIEESQNQQEKNEQELL	DH12
	MTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	BRU
	TTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	HXB2
	MTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	pNL4-3
625	MTWLQWDKEISNYTNIIYDLIEEAQNQQEKNEQDLL	Ug273-A
623	MTWMEWEREIDNYTNTIYTLLIEESQLQQEKNEQELL	Us2-B
619	MTWMQWDREISNYTGTIYRLLEDSONQQEKNEKDLL	Ug268-C
	MTWMEWEREIDNYTGLIYSLIEESQTQQEKNEQELL	Se365-D
620	MTWIEWEREISNYTNQIYEILTESQNQQDRNEKDLL	CM240-E
612	MTWMEWEKEISNYSYEIYRLIEQSONQQEKNEQELL	Bz126-F
613	MTWIQWDREISNYTQQIYSLIEESQNQQEKNEQDLL	HH8793-G

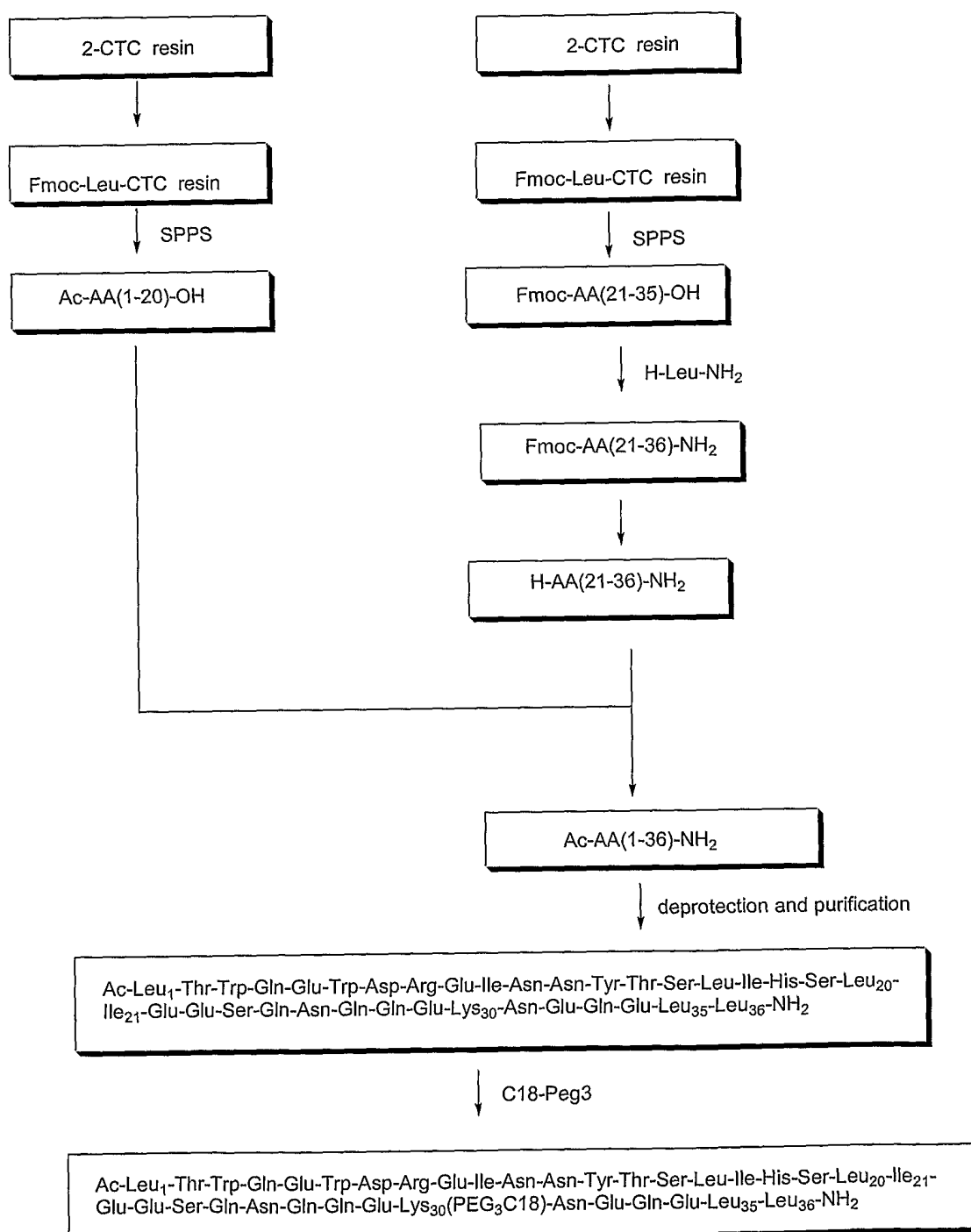
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FIG. 2



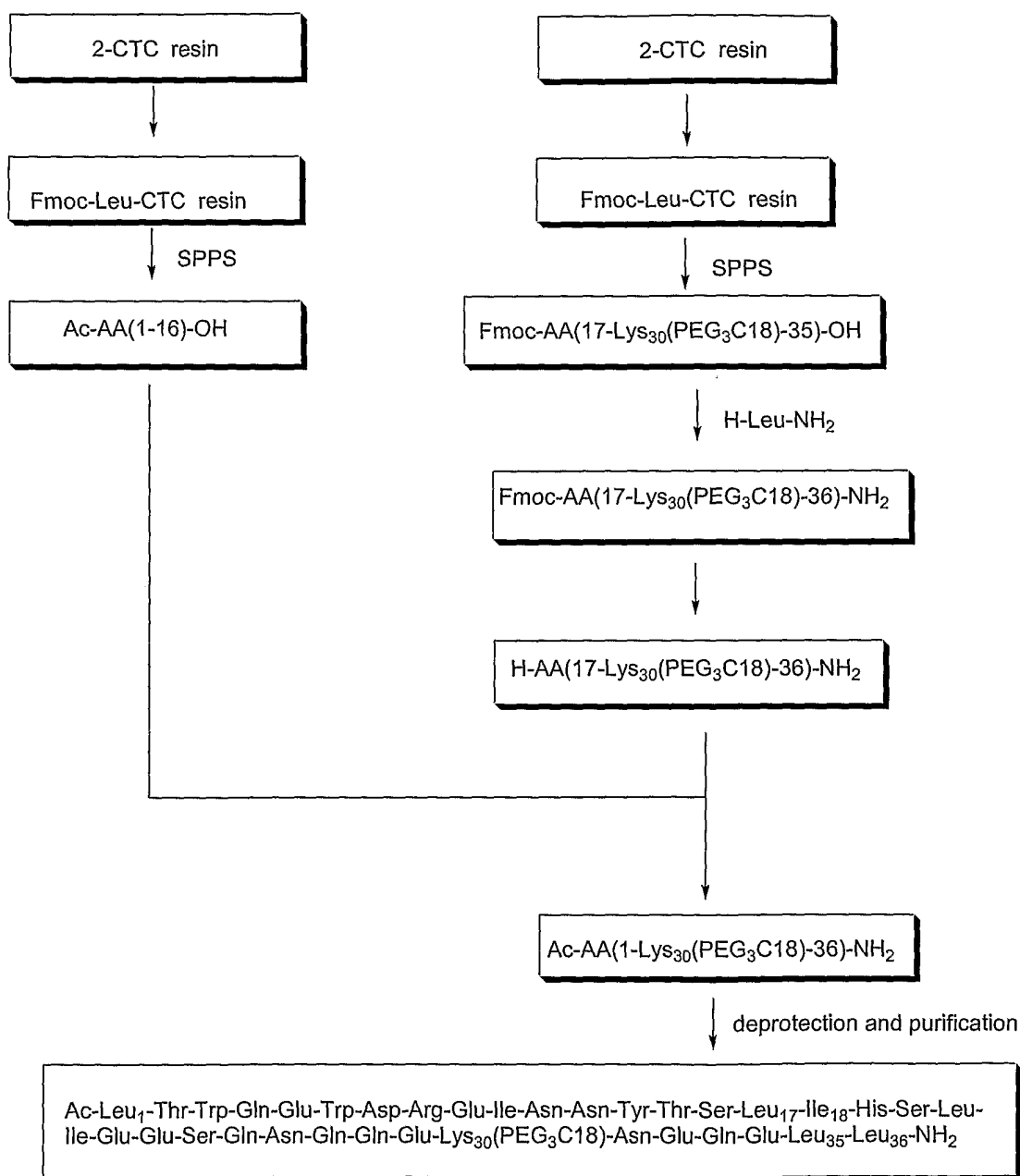
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FIG. 3



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FIG. 4



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<120> Compositions and methods for synthesis of peptide and related conjugate

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<213> Artificial

<220>

<223> synthesized

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Thr Leu Thr Val Gln Ala Arg Gln Leu Leu Ser Gly Ile Val Gln Gln
1 5 10 15

Gln Asn Asn Leu Leu Arg Ala Ile Glu Ala Gln Gln His Leu Leu Gln
20 25 30

Leu Thr Val Trp Gly Ile Lys Gln Leu Gln Ala Arg Ile Leu Ala Val
35 40 45

Glu Arg Tyr Leu Lys Asp Gln Gln Leu Leu Gly Ile
50 55 60

<210> 2

<211> 64

<212> PRT

<213> Artificial

<220>

<223> synthesized

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Trp Asn Ala Ser Trp Ser Asn Lys Ser Leu Glu Gln Ile Trp Asn Asn
1 5 10 15

Met Thr Trp Met Glu Trp Asp Arg Glu Ile Asn Asn Tyr Thr Ser Leu
20 25 30

Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu
35 40 45

Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn Trp Phe
50 55 60

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<220>
<223> synthesized

<400> 3

Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln
1 5 10 15

Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu
20 25 30

Trp Asn Trp Phe
35

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<220>
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chemical group

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Leu Thr Trp Gln Glu Trp Asp Arg Glu Ile Asn Asn Tyr Thr Ser Leu
1 5 10 15

Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu
20 25 30

Gln Glu Leu Leu
35

<210> 5
<211> 12
<212> PRT
<213> Artificial

<220>
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Leu Thr Trp Gln Glu Trp Asp Arg Glu Ile Asn Asn
1 5 10

<210> 6
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<213> Artificial

<220>
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Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln
1 5 10 15

<210> 7
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<212> PRT
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<220>
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<400> 7

Glu Lys Asn Glu Gln Glu Leu
1 5

<210> 8
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<212> PRT
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<220>
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<400> 8

Glu Lys Asn Glu Gln Glu Leu Leu
1 5

<210> 9
<211> 20
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<220>
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<400> 9

Leu Thr Trp Gln Glu Trp Asp Arg Glu Ile Asn Asn Tyr Thr Ser Leu
1 5 10 15

Ile His Ser Leu
20

<210> 10
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<213> Artificial

<220>
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<400> 10

Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Glu Leu
1 5 10 15

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chemical group

<400> 11

Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu
1 5 10 15

<210> 12
<211> 28
<212> PRT
<213> Artificial

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<400> 12

Leu Thr Trp Gln Glu Trp Asp Arg Glu Ile Asn Asn Tyr Thr Ser Leu
1 5 10 15

Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln
20 25

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<223> N-terminal Leu may be modified with a chemical group

<400> 13

Leu	Thr	Trp	Gln	Glu	Trp	Asp	Arg	Glu	Ile	Asn	Asn	Tyr	Thr	Ser	Leu
1				5					10					15	

<210> 14

<211> 19

<212> PRT

<213> Artificial

<220>

<223> synthesized

<220>

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<223> N-terminal Ile may be modified with a chemical group

<400> 14

Ile	His	Ser	Leu	Ile	Glu	Glu	Ser	Gln	Asn	Gln	Gln	Glu	Lys	Asn	Glu
1				5					10					15	

Gln Glu Leu

<210> 15

<211> 20

<212> PRT

<213> Artificial

<220>

<223> synthesized

<220>

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<223> C-terminal Leu may be modified with a chemical group

<400> 15

Ile	His	Ser	Leu	Ile	Glu	Glu	Ser	Gln	Asn	Gln	Gln	Glu	Lys	Asn	Glu
1				5					10					15	

Gln	Glu	Leu	Leu
			20

<210> 16

<211> 7
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may be modified with a chemical group

<400> 16

Glu Lys Asn Glu Gln Glu Leu
1 5

<210> 17
<211> 8
<212> PRT
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and/or C-terminal Leu may be modified with a chemical group

<400> 17

Glu Lys Asn Glu Gln Glu Leu Leu
1 5

<210> 18
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<212> PRT
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<220>
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<220>
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<223> Lys is coupled to a linker-fatty acid combination; N-terminal Ile
may be modified with a chemical group

<400> 18

Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Glu Leu
1 5 10 15

<210> 19
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<212> PRT
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<220>
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<223> Lys is coupled to a linker-fatty acid combination; N-terminal Ile
and/or C-terminal Leu may be modified with a chemical group

<400> 19

Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu
1 5 10 15

<210> 20
<211> 19
<212> PRT
<213> Artificial

<220>
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<223> Lys is coupled to a linker-fatty acid combination; N-terminal Ile
may be modified with a chemical group

<400> 20

Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu
1 5 10 15

Gln Glu Leu

<210> 21
<211> 20
<212> PRT
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<220>
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<223> Lys is coupled to a linker-fatty acid combination; N-terminal Ile and/or C-terminal Leu may be modified with a chemical group

<400> 21

Ile	His	Ser	Leu	Ile	Glu	Glu	Ser	Gln	Asn	Gln	Gln	Glu	Lys	Asn	Glu
1				5				10						15	

Gln	Glu	Leu	Leu
		20	

<210> 22
<211> 24
<212> PRT
<213> Artificial

<220>
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<220>
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<223> N-terminal Tyr and/or C-terminal Leu may be modified with a chemical group

<400> 22

Tyr	Thr	Ser	Leu	Ile	His	Ser	Leu	Ile	Glu	Glu	Ser	Gln	Asn	Gln	Gln
1				5				10						15	

Glu	Lys	Asn	Glu	Gln	Glu	Leu	Leu
			20				

<210> 23
<211> 36
<212> PRT
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<220>
<223> synthesized

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<223> Lys is coupled to a linker-fatty acid combination; N-terminal Leu and/or C-terminal Leu may be modified with a chemical group

<400> 23

Leu	Thr	Trp	Gln	Glu	Trp	Asp	Arg	Glu	Ile	Asn	Asn	Tyr	Thr	Ser	Leu
1				5				10						15	

Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu
20 25 30

Gln Glu Leu Leu
35

<210> 24
<211> 12
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or through a linker

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Leu Thr Trp Gln Glu Trp Asp Arg Glu Ile Asn Asn
1 5 10

<210> 25
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<223> N-terminal Leu may be operatively linked to fatty acid directly
or through a linker

<400> 25

Leu Thr Trp Gln Glu Trp Asp Arg Glu Ile Asn Asn Tyr Thr Ser Leu
1 5 10 15

Ile His Ser Leu
20

<210> 26
<211> 28
<212> PRT

<213> Artificial

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<223> synthesized

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<222> (1)..(1)

<223> N-terminal Leu may be operatively linked to fatty acid directly or through a linker

<400> 26

Leu	Thr	Trp	Gln	Glu	Trp	Asp	Arg	Glu	Ile	Asn	Asn	Tyr	Thr	Ser	Leu
1				5					10					15	

Ile	His	Ser	Leu	Ile	Glu	Glu	Ser	Gln	Asn	Gln	Gln
			20					25			

<210> 27

<211> 16

<212> PRT

<213> Artificial

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<223> synthesized

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<222> (1)..(1)

<223> N-terminal Leu may be operatively linked to fatty acid directly or through a linker

<400> 27

Leu	Thr	Trp	Gln	Glu	Trp	Asp	Arg	Glu	Ile	Asn	Asn	Tyr	Thr	Ser	Leu
1				5					10					15	

<210> 28

<211> 8

<212> PRT

<213> Artificial

<220>

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<223> C-terminal Leu may be operatively linked to fatty acid directly or through a linker; N-terminal Glu may be modified with a chemical group

<400> 28

Glu Lys Asn Glu Gln Glu Leu Leu
1 5

<210> 29
<211> 16
<212> PRT
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or through a linker; N-terminal Ile may be modified with a chemical
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<400> 29

Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu
1 5 10 15

<210> 30
<211> 20
<212> PRT
<213> Artificial

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or through a linker; N-terminal Ile may be modified with a chemical
group

<400> 30

Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu
1 5 10 15

Gln Glu Leu Leu
20

<210> 31
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<212> PRT
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<220>

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<220>

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<223> C-terminal Leu may be operatively linked to fatty acid directly
or through a linker; N-terminal Tyr may be modified with a chemical
group

<400> 31

Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln
1 5 10 15

Glu Lys Asn Glu Gln Glu Leu Leu
20