



(51) International Patent Classification:

A61K 38/26 (2006.01) C07K 14/605 (2006.01)

(21) International Application Number:

PCT/US2017/034961

(22) International Filing Date:

30 May 2017 (30.05.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/344,669 02 June 2016 (02.06.2016) US
 62/413,082 26 October 2016 (26.10.2016) US

(71) Applicant: INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION [US/US]; 518 Indiana Avenue, Indianapolis, Indiana 46202 (US).

(72) Inventors: DIMARCHI, Richard D.; 10890 Wilmington Drive, Carmel, Indiana 46033 (US). MAYER, John P.; 5839 Washington Blvd., Indianapolis, Indiana 46220 (US). MROZ, Piotr; 1207 Paper Birch Court, Bloomington, Indiana 47403 (US).

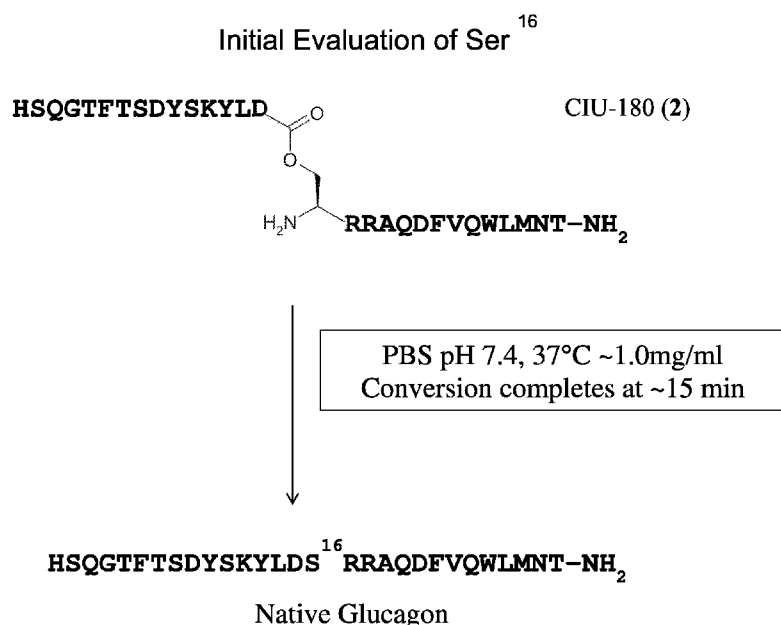
(74) Agent: BREEN, John P.; BARNES & THORNBURG LLP, 11 South Meridian Street, Indianapolis, Indiana 46204 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: AQUEOUSLY SOLUBLE AND CHEMICALLY STABLE DEPSI GLUCAGON AGONISTS

Fig. 3A



(57) Abstract: Modified glucagon peptides are disclosed having improved solubility. The glucagon peptides have been modified by the replacement of one or more of the native amide bonds with ester bonds. The modifications enhance the solubility of the glucagon peptide wherein the native amide bonds are reconstituted in vivo upon administration to a patient.



TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
- *of inventorship (Rule 4.17(iv))*

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

-1-

AQUEOUSLY SOLUBLE & CHEMICALLY STABLE DEPSI GLUCAGON AGONISTS

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to U.S. Provisional Patent Application Nos. 62/344,669 and 62/413,082, filed on June 2, 2016 and October 26, 2016, respectively, the disclosures of which are hereby expressly incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

10 Incorporated by reference in its entirety is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: 735 kilobytes acii (text) file named "265654seqlist_ST25.txt," created on May 26, 2017.

BACKGROUND

15 Pre-proglucagon is a 158 amino acid precursor polypeptide that is processed in different tissues to form a number of different proglucagon-derived peptides, including glucagon, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2) and oxyntomodulin (OXM), that are involved in a wide variety of physiological functions, including glucose homeostasis, insulin secretion, gastric emptying, and
20 intestinal growth, as well as the regulation of food intake. Glucagon is a 29-amino acid peptide that corresponds to amino acids 33 through 61 of pre-proglucagon, while GLP-1 is produced as a 37-amino acid peptide that corresponds to amino acids 72 through 108 of pre-proglucagon.

 Hypoglycemia occurs when blood glucose levels drops too low to provide
25 enough energy for the body's activities. In adults or children older than 10 years, hypoglycemia is uncommon except as a side effect of diabetes treatment, but it can result from other medications or diseases, hormone or enzyme deficiencies, or tumors. When blood glucose begins to fall, glucagon, a hormone produced by the pancreas, signals the liver to break down glycogen and release glucose, causing blood glucose
30 levels to rise toward a normal level. Thus, glucagon's most recognized role in glucose regulation is to counteract the action of insulin and maintain blood glucose

-2-

levels. However for diabetics, this glucagon response to hypoglycemia may be impaired, making it harder for glucose levels to return to the normal range.

Hypoglycemia is a life threatening event that requires immediate medical attention. The administration of glucagon is an established medication for treating acute hypoglycemia and it can restore normal levels of glucose within minutes of administration. When glucagon is used in the acute medical treatment of hypoglycemia, a crystalline form of glucagon is solubilized with a dilute acid buffer and the solution is injected intramuscularly. While this treatment is effective, the methodology is cumbersome and dangerous for someone that is semi-conscious.

Accordingly, there is a need for a glucagon analog that maintains the biological performance of the parent molecule but is sufficiently soluble and stable, under relevant physiological conditions, that it can be pre-formulated as a solution, ready for injection.

Additionally, diabetics are encouraged to maintain near normal blood glucose levels to delay or prevent microvascular complications. Achievement of this goal usually requires intensive insulin therapy. In striving to achieve this goal, physicians have encountered a substantial increase in the frequency and severity of hypoglycemia in their diabetic patients. Accordingly, improved pharmaceuticals and methodologies are needed for treating diabetes that are less likely to induce hypoglycemia than current insulin therapies.

As described herein, high potency glucagon agonists are provided that exhibit enhanced aqueous solubility.

SUMMARY

As disclosed herein glucagon agonists are provided that have pharmacokinetic and pharmacodynamics profiles comparable to native hormone but with solubility at physiological pH and chemical stability, once commercially formulated, to constitute a ready-to-use medicine. In accordance with one embodiment glucagon peptides are provided that retain glucagon receptor activity and exhibit improved solubility relative to the native glucagon peptide (SEQ ID NO: 1). Native glucagon exhibits poor solubility in aqueous solution, particularly at physiological pH, with a tendency to aggregate and precipitate over time. In contrast, the glucagon peptides disclosed

-3-

herein exhibit at least 2-fold, 5-fold, or even higher solubility compared to native glucagon at a pH between 6 and 7.

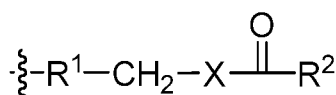
In one embodiment glucagon analogs having enhanced solubility and activity at the GLP-1 and/or GIP receptors are also provided. The enhanced solubility is derived from the discovery that single amide bonds within the glucagon amino acid sequence can be replaced by ester bonds that spontaneously rearrange, after administration to a patient and exposure to physiological conditions, to generate the parent native peptide sequence or a very similar structural analog. The ester-based analogs are biologically inactive but of significantly enhanced solubility relative to the native hormone in physiological buffers. In one embodiment the enhanced soluble glucagon analogs as disclosed herein can be further modified to delay the conversion of the ester bonds to amide bonds.

In one embodiment a glucagon agonist peptide is provided comprising the sequence $X_1X_2X_3GTFTSDYSX_{12}YLX_{15}SRRAQX_{21}FVX_{24}WLX_{27}X_{28}X_{29}$ (SEQ ID NO: 933) wherein one or more amino acids at a position selected from 5, 7, 8, 11, 13, 16, 17, 18, 19, 20, 21, 25 or 28 are linked to the preceding amino acid via an ester bond; wherein

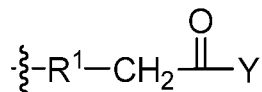
X_1 is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X_2 is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, amino iso-butyric acid (Aib), Val, or α -amino-N-butyric acid;

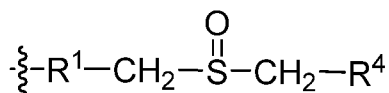
X_3 is an amino acid comprising a side chain of Structure I, II, or III:



Structure I



Structure II



Structure III

-4-

wherein R^1 is C_{0-3} alkyl or C_{0-3} heteroalkyl; R^2 is NHR^4 or C_{1-3} alkyl; R^3 is C_{1-3} alkyl; R^4 is H or C_{1-3} alkyl; X is NH, O, or S; and Y is NHR^4 , SR^3 , or OR^3 ;

X_{12} is Lys or Arg;

X_{15} is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid;

5 X_{21} is Asp, Lys, Cys, Orn, homocysteine or acetyl phenylalanine;

X_{24} is Gln, Lys, Cys, Orn, homocysteine or acetyl phenylalanine;

X_{27} is Met, Leu or Nle;

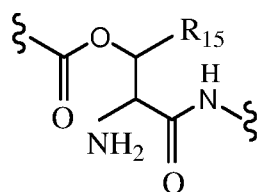
X_{28} is Asn, Lys, Arg, His, Asp or Glu; and

X_{29} is Thr, Lys, Arg, His, Gly, Asp or Glu, optionally wherein SEQ ID NO:

10 933 is further modified by one, two, three, or all of the amino acids at positions 16, 20, 21, and 24 being substituted with an α,α -disubstituted amino acid.

In accordance with one embodiment an enhanced soluble analog of glucagon is provided wherein the analog comprises one or more amino acids, located at a position selected from positions 5, 7, 8, 11 or 16, linked to the immediately preceding amino acid (i.e., at positions 4, 6, 7, 10 or 15, respectively) via an ester bond. In accordance with one embodiment an enhanced soluble analog of glucagon is provided wherein the analog comprises one or more amino acids, located at a position selected from positions 13, 17, 18, 19, 20, 21, 25 or 28, linked to the immediately preceding amino acid (i.e., at positions 12, 16, 17, 18, 19, 20, 24 or 27, respectively) via an ester bond. In one embodiment the side chain of a serine or threonine is linked via an ester bond with the alpha carboxy group of the immediate preceding amino acid in the glucagon amino acid sequence. In accordance with one embodiment a glucagon analog is provided comprising one or more ester linked amino acids comprising the structure of

25

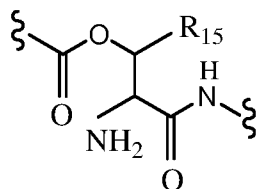


wherein

R_{15} is H or CH_3 and the ester linked amino acid is present at a position selected from 5, 7, 8, 11, 13, 16, 17, 18, 19, 20, 21, 25 or 28. In accordance with one embodiment a glucagon analog is provided comprising 1, 2, or 3 ester linked amino acids comprising the structure of

30

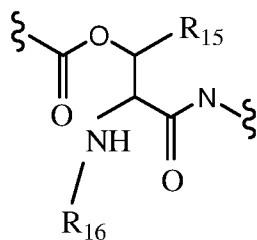
-5-



wherein

R₁₅ is H or CH₃ and the ester linked amino acid is present at a position selected from 5, 7, 8, 11, 13, 16, 17, 18, 19, 20, 21, 25 or 28, relative to the native glucagon sequence. In one embodiment R₁₅ is CH₃ and an ester linked amino acid is present at a position selected from 5, 7, 13, 17, 18, 19, 20, 21, 25 or 28, relative to the native glucagon sequence. In one embodiment R₁₅ is CH₃ and the ester linked amino acid is present at a position selected from 5 or 7. In one embodiment R₁₅ is H and the ester linked amino acid is present at a position selected from 8, 11 or 16, relative to the native glucagon sequence.

The enhanced soluble glucagon analogs of the present invention can be further modified to stabilize the ester bond configuration at physiological pH, so the ester bonds are not converted to amides until after administration to a patient. In accordance with one embodiment the alpha amine of the ester linked amino acid is covalently bound to a dipeptide that is either susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine. In this embodiment the glucagon analog is provided comprising one, two, three or more ester linked amino acids comprising the structure of

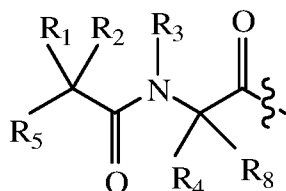


wherein

R₁₅ is H or CH₃; and

R₁₆ is an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine. In one embodiment R₁₆ is a dipeptide that is susceptible to cleavage by Dipeptidyl Peptidase IV (DPP-IV). In one embodiment R₁₆ is a dipeptide having the general structure of Formula I:

-6-



wherein

R₁ is C₁-C₄ alkyl or (C₁-C₄ alkyl)NH₂;

R₂ is H or is C₁-C₄ alkyl;

5 R₃ is selected from the group consisting of C₁-C₆ alkyl;

R₄ is selected from the group consisting of H, and C₁-C₄ alkyl;

R₈ is H; and

R₅ is NH₂ or OH. In another embodiment the dipeptide is selected from the group consisting of -Pro-Glu or -Azetidine-Lys.

10 In accordance with one embodiment the solubility of glucagon peptide analogs is further improved by amino acid substitutions and/or additions that introduce a charged amino acid into the C-terminal portion of the peptide, and in one embodiment at a position C-terminal to position 27 of SEQ ID NO: 1. Optionally, in addition to the introduction of ester bonds at an amino acid positions selected from positions 5, 7,
15 8, 11 or 16, one, two or three charged amino acids may be introduced within the C-terminal portion, and in one embodiment C-terminal to position 27 relative to native glucagon. In accordance with one embodiment the native amino acid(s) at positions 28 and/or 29 are substituted with a charged amino acid, and/or one to three charged amino acids are added to the C-terminus of the peptide, after position 29. In
20 exemplary embodiments, one, two or all of the charged amino acids are negatively charged. Additional modifications, e.g. conservative substitutions, may be made to the glucagon peptide that still allow it to retain glucagon activity.

The present invention further encompasses pharmaceutically acceptable salts of said glucagon agonists.

25 In some embodiments, modifications at position 1 and/or 2 of the glucagon peptide can increase the peptide's resistance to dipeptidyl peptidase IV (DPP IV) cleavage. For example, the amino acid at position 2 may be substituted with D-serine, D-alanine, valine, glycine, N-methyl serine, N-methyl alanine, or amino isobutyric acid. Alternatively, or in addition, the amino acid at position 1 may be substituted
30 with D-histidine (D-His), desaminohistidine, hydroxyl-histidine, acetyl-histidine,

-7-

homo-histidine, N-methyl histidine, alpha-methyl histidine, imidazole acetic acid, or alpha, alpha-dimethyl imidazole acetic acid (DMIA).

It was observed that modifications at position 2 (e.g. Aib at position 2) and in some cases modifications at position 1 (e.g. DMIA at position 1) may reduce glucagon activity, sometimes significantly. This reduction in glucagon activity can be restored by stabilization of the alpha-helix structure in the C-terminal portion of glucagon (around amino acids 12-29). In some embodiments, stabilization is via a covalent bond between amino acids at positions "i" and "i+4", wherein i is any integer from 12 to 25. In some specific embodiments, "i" and "i+4" are 12 and 16, 16 and 20, or 20 and 24, or 24 and 28. In some embodiments, this covalent bond is a lactam bridge between a glutamic acid at position 16 and a lysine at position 20. In exemplary embodiments, the bridge or linker is about 8 (or about 7-9) atoms in length. In other embodiments, stabilization is via a covalent bond between amino acids at positions "j" and "j+3," wherein j is any integer between 12 and 27. In exemplary embodiments, the bridge or linker is about 6 (or about 5-7) atoms in length. In yet other embodiments, stabilization is via a covalent bond between amino acids at positions "k" and "k+7," wherein k is any integer between 12 and 22. In some embodiments, this covalent bond is an intramolecular bridge other than a lactam bridge. For example, suitable covalent bonding methods (i.e., means of forming a covalent intramolecular bridge) include any one or more of olefin metathesis, lanthionine-based cyclization, disulfide bridge or modified sulfur-containing bridge formation, the use of α , ω -diaminoalkane tethers, the formation of metal-atom bridges, and other means of peptide cyclization.

In yet other embodiments, the helix is stabilized by non-covalent bonds (i.e., non-covalent intramolecular bridges), including but not limited to hydrogen-bonding, ionic interactions, and salt bridges.

In other embodiments of the invention, stabilization of the alpha-helix structure in the C-terminal portion of the glucagon peptide (around amino acids 12-29) is achieved through purposeful introduction of one or more α , α -disubstituted amino acids at positions that retain the desired activity. In some embodiments, one, two, three, four or more of positions 16, 17, 18, 19, 20, 21, 24 or 29 of a glucagon peptide is substituted with an α , α -disubstituted amino acid. For example, substitution of position 16 of a glucagon peptide with amino iso-butyric acid (Aib) provides a

-8-

stabilized alpha helix in the absence of a salt bridge or lactam. In specific aspects, stabilization of the alpha-helix is accomplished by introducing one or more α , α -disubstituted amino acids without introduction of a covalent intramolecular bridge, e.g., a lactam bridge, a disulfide bridge. Such peptides are considered herein as a peptide lacking a covalent intramolecular bridge. In some embodiments, one, two, three or more of positions 16, 20, 21 or 24 are substituted with Aib.

Thus, in some embodiments the present disclosure provides a glucagon peptide comprising the amino acid sequence:

X1-X2-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Z (SEQ ID NO: 940) wherein one or more amino acids at a position selected from 5, 7, 8, 11, 13, 16, 17, 18, 19, 20, 21, 25 or 28 are linked to the preceding amino acid via an ester bond, and optionally with 1 to 3 further amino acid modifications thereto (including for example an Aib substitution at position 16 and/or 20), wherein

X1 and/or X2 is a non-native amino acid that reduces susceptibility of (or increases resistance of) the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV). In one embodiment X1 is selected from the group consisting of His, D-His, (Des-amino)His, hydroxyl-His, acetyl-His, homo-His or alpha, alpha-dimethyl imidazole acetic acid (DMIA), N-methyl His, alpha-methyl His, and imidazole acetic acid and X2 is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Val, Gly, N-methyl Ser, Aib and N-methyl Ala; wherein Z is selected from the group consisting of -COOH (the naturally occurring C-terminal carboxylate), -Asn-COOH, Asn-Thr-COOH, and Y-COOH, wherein Y is 1 to 2 amino acids, optionally wherein an intramolecular bridge, preferably a covalent bond, connects the side chains of an amino acid at position i and an amino acid at position i+4, wherein i is 12, 16, 20 or 24. In one embodiment the glucagon peptide of SEQ ID NO: 940 is provided wherein one or more amino acids selected from amino acids at positions 7, 8, 11 or 16 are linked to the immediately preceding amino acid via an ester bond.

In some embodiments wherein the analog comprises an intramolecular bridge, the intramolecular bridge is a lactam bridge. In some embodiments, the amino acids at positions i and i+4 of SEQ ID NO: 940 are Lys and Glu, e.g., Glu16 and Lys20. In some embodiments, X1 is selected from the group consisting of: D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-

-9-

His, homo-His, and alpha, alpha-dimethyl imidazole acetic acid (DMIA). In other embodiments, X₂ is selected from the group consisting of: D-Ser, D-Ala, Gly, N-methyl-Ser, Val, and alpha, amino isobutyric acid (Aib).

In one embodiment a glucagon peptide is provided, optionally formulated with a pharmaceutically acceptable carrier, wherein the peptide comprises the amino acid sequence HSQGTFTSDYSX₁₂YLDSX₁₇X₁₈X₁₉X₂₀X₂₁FVX₂₄WLMX₂₈T (SEQ ID NO: 934), wherein

X₁₂ is Lys, Arg, Tyr or an ester linked amino acid;

X₁₇ is Arg or an ester linked amino acid;

10 X₁₈ is Arg or an ester linked amino acid;

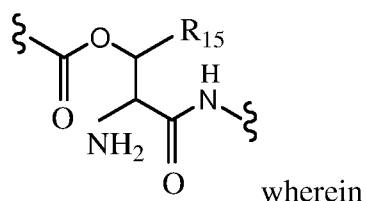
X₁₉ is Ala or an ester linked amino acid;

X₂₀ is Gln or an ester linked amino acid;

X₂₁ is Asp or an ester linked amino acid;

X₂₄ is Gln or an ester linked amino acid; and

15 X₂₈ is Asn or an ester linked amino acid. In accordance with one embodiment the ester linked amino acid comprises the structure



R₁₅ is H or CH₃.

20 In some embodiments, the glucagon peptide is covalently linked to a hydrophilic moiety at any of amino acid positions 16, 17, 20, 21, 24, 29, within a C-terminal extension (e.g., at position 40 of a C-terminal extension of SEQ ID NO: 26), or at the C-terminal amino acid. In exemplary embodiments, this hydrophilic moiety is covalently linked to a Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine residue

25 at any of these positions. Exemplary hydrophilic moieties include polyethylene glycol (PEG), for example, of a molecular weight of about 1,000 Daltons to about 40,000 Daltons, or about 20,000 Daltons to about 40,000 Daltons.

In other embodiments, the invention provides a glucagon peptide, comprising the amino acid sequence:

-10-

X1-X2-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Z (SEQ ID NO: 940),

wherein

one or more amino acids selected from amino acids at positions 7, 8, 11 or 16
5 are linked to the immediately preceding amino acid via an ester bond;

X1 and/or X2 is a non-native amino acid that reduces susceptibility of (or increases resistance of) the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),

wherein one, two, three, or four of positions 16, 20, 21, and 24 of the
10 glucagon peptide is substituted with an α , α -disubstituted amino acid, and

wherein Z is selected from the group consisting of -COOH (the naturally occurring C-terminal carboxylate), -Asn-COOH, Asn-Thr-COOH, and Y-COOH, wherein Y is 1 to 2 amino acids. In one embodiment X1 is selected from the group consisting of His, D-His, (Des-amino)His, hydroxyl-His, acetyl-His, homo-His or
15 alpha, alpha-dimethyl imidazole acetic acid (DMIA), N-methyl His, alpha-methyl His, and imidazole acetic acid and X2 is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Val, Gly, N-methyl Ser, Aib and N-methyl Ala; optionally in one embodiment X1 is His or DMIA and/or X2 is Ser, D-Ser or Aib.

Exemplary further amino acid modifications to the foregoing glucagon
20 peptides or analogs include substitution of Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile, optionally, in combination with substitution or addition of an amino acid comprising a side chain covalently attached (optionally, through a spacer) to an acyl or alkyl group, which acyl or alkyl group is non-native to a naturally-occurring amino acid, substitution of Lys at position 12 with
25 Arg; substitution of Asp at position 15 with Glu; substitution of Ser at position 16 with Thr or Aib; substitution of Gln at position 20 with Ser, Thr, Ala or Aib; substitution of Asp at position 21 with Glu; substitution of Gln at position 24 with Ser, Thr, Ala or Aib; substitution of Met at position 27 with Leu or Nle; substitution of Asn at position 28 with a charged amino acid; substitution of Asn at position 28
30 with a charged amino acid selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid; substitution at position 28 with Asn, Asp, or Glu; substitution at position 28 with Asp; substitution at position 28 with Glu; substitution of Thr at position 29 with a charged amino acid; substitution of Thr at

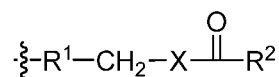
-11-

position 29 with a charged amino acid selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid; substitution at position 29 with Asp, Glu, or Lys; substitution at position 29 with Glu; insertion of 1-3 charged amino acids after position 29; insertion at position 30 (i.e., after position 29) of Glu or Lys; optionally with insertion at position 31 of Lys; addition of SEQ ID NO: 820 to the C-terminus, optionally, wherein the amino acid at position 29 is Thr or Gly; substitution or addition of an amino acid covalently attached to a hydrophilic moiety; or a combination thereof.

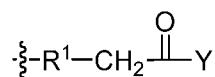
In yet further exemplary embodiments, any of the foregoing peptides can be further modified to improve stability by modifying the amino acid at position 15 of SEQ ID NO: 1 to reduce degradation of the peptide over time, especially in acidic or alkaline buffers. In exemplary embodiments, Asp at position 15 is substituted with a Glu, homo-Glu, cysteic acid, or homo-cysteic acid.

Alternatively, any of the glucagon peptides described herein can be further modified to improve stability by modifying the amino acid at position 16 of SEQ ID NO: 1. In exemplary embodiments, Ser at position 16 is substituted with Thr, or any of the amino acids substitutions described above which enhance potency at the glucagon receptor. Such modifications reduce cleavage of the peptide bond between Asp15-Ser16.

Maintained or enhanced activity at the glucagon receptor may be achieved by modifying the Gln at position 3 with a glutamine analog. For example, a glucagon peptide comprising a glutamine analog at position 3 may exhibit about 5%, about 10%, about 20%, about 50%, or about 85% or greater the activity of native glucagon (e.g. SEQ ID NO: 1) at the glucagon receptor. In some embodiments, the glutamine analog is a naturally occurring or a non-naturally occurring amino acid comprising a side chain of Structure I, II or III:

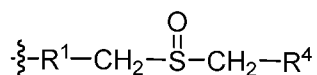


Structure I



Structure II

-12-



Structure III

wherein R¹ is C₀₋₃ alkyl or C₀₋₃ heteroalkyl; R² is NHR⁴ or C₁₋₃ alkyl; R³ is C₁₋₃ alkyl; R⁴ is H or C₁₋₃ alkyl; X is NH, O, or S; and Y is NHR⁴, SR³, or OR³. In some
 5 embodiments, X is NH or Y is NHR⁴. In some embodiments, R¹ is C₀₋₂ alkyl or C₁ heteroalkyl. In some embodiments, R² is NHR⁴ or C₁ alkyl. In some embodiments, R⁴ is H or C¹ alkyl.

Enhanced activity at the glucagon receptor of the glucagon peptide also may be achieved by covalently attaching an acyl or alkyl group, e.g., an acyl or alkyl group
 10 which is non-native to a naturally occurring amino acid (e.g., a C4 to C30 fatty acyl group, a C4 to C30 alkyl group), to the side chain of an amino acid of the glucagon peptide. In some embodiments, the acylated or alkylated glucagon peptides lack an intramolecular bridge, e.g., a covalent intramolecular bridge (e.g., a lactam). In certain aspects, the acyl or alkyl group is attached to the side chain of the amino acid
 15 of the glucagon peptide through a spacer, e.g., a spacer which is 3 to 10 atoms in length. In some embodiments, the acyl or alkyl group is attached to the side chain of the amino acid at position 10 of the glucagon peptide through a spacer. In specific embodiments, the acylated or alkylated glucagon peptides further comprise a modification which selectively decreases the activity of the peptide at the GLP-1
 20 receptor. For example, the acylated or alkylated glucagon peptide may comprise a C-terminal alpha carboxylate, a substitution of the Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile, a deletion of the amino acid(s) C-terminal to the amino acid at position 27 or 28, yielding a 27- or 28-amino acid peptide, or a combination thereof.

25 In some embodiments, any of the glucagon peptides described herein can be further modified to reduce degradation at various amino acid positions by modifying any one, two, three, or all four of positions 20, 21, 24, or 27. Exemplary embodiments include substitution of Gln at position 20 with Ala or Aib, substitution of Asp at position 21 with Glu, substitution of Gln at position 24 with Ala or Aib,
 30 substitution of Met at position 27 with Leu or Nle. Removal or substitution of methionine reduces degradation due to oxidation of the methionine. Removal or

-13-

substitution of Gln or Asn reduces degradation due to deamidation of Gln or Asn. Removal or substitution of Asp reduces degradation that occurs through dehydration of Asp to form a cyclic succinimide intermediate followed by isomerization to iso-aspartate.

5 The glucagon peptide may be part of a dimer, trimer or higher order multimer comprising at least two, three, or more peptides bound via a linker, wherein at least one or both peptides is a glucagon peptide. The dimer may be a homodimer or heterodimer. In some embodiments, the linker is selected from the group consisting of a bifunctional thiol crosslinker and a bi-functional amine crosslinker. In certain
10 embodiments, the linker is PEG, e.g., a 5 kDa PEG, 20 kDa PEG. In some embodiments, the linker is a disulfide bond. For example, each monomer of the dimer may comprise a Cys residue (e.g., a terminal or internally positioned Cys) and the sulfur atom of each Cys residue participates in the formation of the disulfide bond. In some aspects of the invention, the monomers are connected via terminal amino
15 acids (e.g., N-terminal or C-terminal), via internal amino acids, or via a terminal amino acid of at least one monomer and an internal amino acid of at least one other monomer. In specific aspects, the monomers are not connected via an N-terminal amino acid. In some aspects, the monomers of the multimer are attached together in a “tail-to-tail” orientation in which the C-terminal amino acids of each monomer are
20 attached together.

A conjugate moiety may be covalently linked to any of the glucagon peptides described herein, including a dimer, trimer or higher order multimer. Fusion peptides comprising the amino acid sequence of any of SEQ ID NOs: 820 to 822 are also contemplated.

25 Any of the modifications described above which increase glucagon receptor activity, retain partial glucagon receptor activity, improve solubility, increase stability, or reduce degradation can be applied to glucagon peptides individually or in combination.

30 In accordance with one embodiment a pharmaceutical composition is provided comprising any of the novel glucagon peptides disclosed herein, preferably sterile and preferably at a purity level of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, and a pharmaceutically acceptable diluent, carrier or excipient. Such compositions may contain a glucagon peptide at a concentration of at least A, wherein

-14-

A is 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 6 mg/ml, 7 mg/ml, 8 mg/ml, 9 mg/ml, 10 mg/ml, 11 mg/ml, 12 mg/ml, 13 mg/ml, 14 mg/ml, 15 mg/ml, 16 mg/ml, 17 mg/ml, 18 mg/ml, 19 mg/ml, 20 mg/ml, 21 mg/ml, 22 mg/ml, 23 mg/ml, 24 mg/ml, 25 mg/ml or higher. In other

5 embodiments, such compositions may contain a glucagon peptide at a concentration of at most B, wherein B is 30 mg/ml, 25 mg/ml, 24 mg/ml, 23 mg/ml, 22 mg/ml, 21 mg/ml, 20 mg/ml, 19 mg/ml, 18 mg/ml, 17 mg/ml, 16 mg/ml, 15 mg/ml, 14 mg/ml, 13 mg/ml, 12 mg/ml, 11 mg/ml, 10 mg/ml, 9 mg/ml, 8 mg/ml, 7 mg/ml, 6 mg/ml, 5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml, 1 mg/ml, or 0.1 mg/ml. In some embodiments,

10 the compositions may contain a glucagon peptide at a concentration range of A to B mg/ml, for example, 0.001 to 30.0 mg/ml. In one embodiment the pharmaceutical compositions comprise aqueous solutions that are sterilized and optionally stored within various containers. Such solutions can be used in accordance with one embodiment to prepare pre-formulated solutions ready for injection. In other

15 embodiments the pharmaceutical compositions comprise a lyophilized powder. The pharmaceutical compositions can be further packaged as part of a kit that includes a disposable device for administering the composition to a patient. Devices may include a syringe and needle, or a pre-filled syringe. The containers or kits may be labeled for storage at ambient room temperature or at refrigerated temperature.

20 In accordance with one embodiment a method of rapidly increasing glucose level, normalizing blood glucose level, stabilizing blood glucose level, or preventing or treating hypoglycemia using a pre-formulated aqueous composition of a glucagon peptide of the invention is provided. The method comprises the step of administering an effective amount of an aqueous solution comprising a novel modified glucagon

25 peptide of the present disclosure. In some embodiments, the aqueous composition is pre-packaged in a device that is used to administer the composition to the patient. In another embodiment a method is provided for inducing the temporary paralysis of the intestinal tract. The method comprises the step of administering one or more of the glucagon peptides disclosed herein to a patient in need thereof.

30 In yet another embodiment a method of reducing weight gain or inducing weight loss is provided, which involves administering an effective amount of an aqueous solution comprising a depsi glucagon peptide of the present disclosure. Methods for reducing weight gain or inducing weight loss are expected to be useful to

-15-

treat obesity of various causes, including drug-induced obesity, and reducing complications associated with obesity including vascular disease (coronary artery disease, stroke, peripheral vascular disease, ischemia reperfusion, etc.), hypertension, onset of diabetes type II, hyperlipidemia and musculoskeletal diseases.

5 In further embodiments, methods of treating hyperglycemia or diabetes involving co-administering insulin and a depsi glucagon peptide of the present disclosure are provided. Hyperglycemia includes diabetes, diabetes mellitus type I, diabetes mellitus type II, or gestational diabetes, either insulin-dependent or non-insulin-dependent, and reducing complications of diabetes including nephropathy,
10 retinopathy and vascular disease. Co-administration of insulin and a depsi glucagon peptide of the invention can reduce nocturnal hypoglycemia and/or buffer the hypoglycemic effects of insulin, allowing the same or higher doses of short-acting or long-acting insulin to be administered with fewer adverse hypoglycemic effects. Compositions comprising insulin together with a glucagon peptide of the invention
15 are also provided.

 In accordance with one embodiment an improved method of regulating blood glucose levels in insulin dependent patients is provided. The method comprises the steps of administering insulin in an amount therapeutically effective for the control of diabetes and administering a novel modified glucagon peptide of the present
20 disclosure in an amount therapeutically effective for the prevention of hypoglycemia, wherein said administering steps are conducted within twelve hours of each other. In one embodiment the glucagon peptide and the insulin are co-administered as a single composition.

 All therapeutic methods, pharmaceutical compositions, kits and other similar
25 embodiments described herein contemplate that the use of the term glucagon peptides, glucagon agonist analogs, glucagon agonists, or glucagon analogs includes all pharmaceutically acceptable salts or esters thereof.

 Oxyntomodulin is a 37 amino acid peptide that contains the 29 amino acid sequence of glucagon (i.e. SEQ ID NO: 1) followed by an 8 amino acid carboxy
30 terminal extension of SEQ ID NO: 821 (KRNRNNIA). While the present invention contemplates that glucagon analogs described herein may optionally be joined to this 8 amino acid carboxy terminal extension (SEQ ID NO: 821), the invention in some

-16-

embodiments also specifically contemplates glucagon analogs and uses of glucagon analogs lacking the 8 contiguous carboxy amino acids of SEQ ID NO: 821.

The foregoing summary is not intended to define every aspect of the invention, and additional embodiments are described in other sections, such as the Detailed Description. The entire document is intended to be related as a unified disclosure, and it should be understood that all possible combinations of features described herein may be contemplated, even if the combination of features are not found together in the same sentence, or paragraph, or section of this document. In particular, any known glucagon analog, including any of those disclosed herein, can be modified by the substitution of a depsi amino acid at a position selected from positions 16, 17, 20, 21, 24, 29, or within a C-terminal extension (e.g., at position 40) relative to the native glucagon sequence (SEQ ID NO: 1) to improve the solubility of the glucagon analog.

Moreover, the invention includes any one or all embodiments of the invention that are narrower in scope in any way than the variations defined by specific paragraphs herein. For example, where certain aspects of the invention are described as a genus, it should be understood that every member of a genus is, individually, an embodiment of the invention, and that combinations of two or more members of the genus are embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of a depsi-glucagon analog in accordance with one embodiment and its conversion to the native peptide structure at pH 7.4.

Fig. 2 is a schematic representation of the locations in native glucagon (SEQ ID NO: 1) where an amide bond can be substituted with an ester bond.

Figs. 3A & 3B represents the *in vitro* conversion of depsi glucagon analog **2** [D15:S16] (HSQGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT-R₁₃; SEQ ID NO: 1263) comprising a depsi-Ser at position 16 (Fig. 3A), and depsi glucagon analog **3** [D15:T16] (SEQ ID NO: 935) comprising a depsi-Thr at position 16 (Fig. 3B).

Fig. 4 represents data showing the *in vitro* kinetics of O-to-N acyl shift for depsi glucagon analogs **2** [D15:S16] (SEQ ID NO: 1263), **3** [D15:T16] (SEQ ID NO: 935), **4** [F6:T7] (SEQ ID NO: 1264), **5** [G4:T5] (SEQ ID NO: 1265), **6** [F15:T16] (SEQ ID NO: 1269) and **7** [D6:T7] (SEQ ID NO: 1270).

-17-

Fig. 5A and 5B are graphs demonstrating the ability of three depsi-glucagon analogs, **2** [D15:S16] (HSQGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT; SEQ ID NO: 1263, wherein a depsi-Ser is present at position 16); **5** [G4:T5] (HSQGX₅FTSDYSKYLDX₁₆RRAQDFVQWLMNT-R₁₃; SEQ ID NO: 936 comprising a depsi Thr at position 5); and **3** [D15:T16] (HSQGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT; SEQ ID NO: 935, comprising a depsi-Thr at position 16) to regulate blood glucose levels (Fig. 5A plots blood glucose levels; Fig. 5B plots the change in blood glucose levels). Analogs **2** [D15:S16] (SEQ ID NO: 1263), **3** [D15:T16] (SEQ ID NO: 935) and **5** [G4:T5] (SEQ ID NO: 1265) each increase blood glucose upon administration *in vivo*. The three depsi peptides show equivalent activity as native glucagon (SEQ ID NO: 1). Figure legend: vehicle (●), native glucagon (○), analog **3** (▲), analog **5** (◇) and analog **2** (□).

Fig. 6 is a schematic representation of modifications to the depsi-glucagon peptides to enhance the stability of the peptides at physiological pH. More particularly, a secondary trigger is added to the amino group that must first be removed before the depsi peptide can be converted.

Fig. 7A and 7B provide two separate synthetic schemes for preparation of depsi-peptides extended with an enzymatically susceptible substrate either on [G4:T5] (SEQ ID NO: 1265) (Fig. 7A), or an [F6:T7] (SEQ ID NO: 1264) (Fig. 7B) backbone.

Fig. 8 provides the structures of depsi-glucagon analogs **8** [D15:T16(Ac)] (SEQ ID NO: 1266), **10** [G4:T5(KZ)] (SEQ ID NO: 1267) and **15** [F6:T7(EP)] (SEQ ID NO: 1268) (Z = Azetidine).

Fig. 9 is a bar graph demonstrating the solubility of depsi-glucagon **10** [G4:T5(KZ)] (SEQ ID NO: 1267) at different buffers and pH ranging from 5 to 7.7. The solid line at concentration 1.0 reflects a target concentration for an injectable emergency glucagon formulation. The dotted line at concentration 0.2 represents the solubility of native glucagon in PBS. PBS = physiologically buffered saline, NaPB = sodium phosphate, CADSPB = 0.1M citric acid with 0.2M disodium phosphate buffer.

Fig. 10 is a bar graph representing the solubility of the glucagon and depsi-peptides **10-15** in PBS pH 7.4, at room temperature (see Example 4 for structures of

-18-

compounds 10-15). [black line represent pharmaceutically relevant concentration level for use of emergency glucagon treatment].

Fig. 11 is a bar graph of data demonstrating the temperature dependent aggregation of native glucagon (**1**) and depsi-peptides **8** [D15:T16(Ac)] (SEQ ID NO: 1266) and **10** [G4:T5(KZ)] (SEQ ID NO: 1267) incubated in pH 3 and pH 4 buffers, without agitation. Fluorescence was measured following binding of thioflavin-T.

Fig. 12 is a graph demonstrating the *in vitro* activity of glucagon depsi – peptides **5** [G4:T5] (SEQ ID NO: 1265), **8** [D15:T16(Ac)] (SEQ ID NO: 1266), **9** [G4:T5(Ac)] (SEQ ID NO: 1277), **10** [G4:T5(KZ)] (SEQ ID NO: 1267) and **15** [F6:T7(EP)] (SEQ ID NO: 1268) relative to native glucagon. The measured activity is an indirect function of cAMP synthesis as assessed in an engineered cell assay where the human glucagon receptor is over-expressed and coupled to a luminescence reporter.

Fig. 13 provides data demonstrating second trigger kinetics *in vivo* in rats administered four different depsi-glucagon analogs, three of which are further modified by attachment of a second trigger to the alpha amine of the depsi amino acid. **5** [G4:T5] (SEQ ID NO: 1265), **10** [G4:T5(KZ)] (SEQ ID NO: 1267), **16** [G4:T5(R)] (SEQ ID NO: 1275) and **17** [G4:T5(pE)] (SEQ ID NO: 1276) [each formulated in 0.01N HCl and dosage 10 nmol/kg]. Figure legend: vehicle (●), analog **5** (○), analog **17** (▲), analog **10** (■) and analog **16** (□).

Fig. 14 presents data representing the administration of native glucagon (**1**), depsi-peptide **5** [G4:T5] (SEQ ID NO: 1265) [formulated in 0.01N HCl] and **10** [G4:T5(KZ)] (SEQ ID NO: 1267) [formulated in 50 mM sodium phosphate with 150 mM sodium chloride pH 7.4; dose: 10 nmol/kg]. Vehicle (I) was 50 mM sodium phosphate with 150 mM sodium chloride pH 7.4 buffer and Vehicle (II) was 0.01N HCl.

Fig. 15 presents data representing the administration of native glucagon (**1**), depsi-peptides **13** [F6:T7(KZ)] (SEQ ID NO: 1273), **15** [F6:T7(EP)] (SEQ ID NO: 1268) and **4** [F6:T7] (SEQ ID NO: 1264) [all formulated in 0.01N HCl; dose: 10 nmol/kg]. Vehicle was 0.01N HCl.

Fig. 16 presents data representing the administration of depsi-peptide **15** [F6:T7(EP)] (SEQ ID NO: 1268) at 10 nmol/kg [formulated in PBS] in the presence of an orally administrated DPPIV inhibitor - Sitagliptin at 3, 10 and 30 mg/kg

[formulated in PBS and administrated by gavage]. Figure legend: Vehicle + **15** [F6:T7(EP)] (●), Sitagliptin (3 mg/kg) + **15** [F6:T7(EP)] (▲), Sitagliptin (30 mg/kg) + Vehicle (■), Sitagliptin (10 mg/kg) + **15** [F6:T7(EP)] (▼) and Sitagliptin (30 mg/kg) + **15** [F6:T7(EP)] (◆).

5 Fig. 17A & 17B are graphs demonstrating blood glucose level (Fig. 17A) and change in blood glucose level (Fig. 17B) in normal rats after administration of GLP-1 analogs with and without Sitagliptin challenge. The dose of Sitagliptin was 30 mg/kg (oral) and GLP-1 & GLP-1(Aib2) 10 nmol/kg (i.v.)

10 DETAILED DESCRIPTION

DEFINITIONS

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

15 As used herein, the term “pharmaceutically acceptable carrier” includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

20 As used herein the term "pharmaceutically acceptable salt" refers to salts of compounds that retain the biological activity of the parent compound, and which are not biologically or otherwise undesirable. Many of the compounds disclosed herein are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

25 Pharmaceutically acceptable base addition salts can be prepared from inorganic and organic bases. Salts derived from inorganic bases, include by way of example only, sodium, potassium, lithium, ammonium, calcium and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary and tertiary amines.

30 Pharmaceutically acceptable acid addition salts may be prepared from inorganic and organic acids. Salts derived from inorganic acids include hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like. Salts derived from organic acids include acetic acid, propionic acid, glycolic acid, pyruvic

acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluene-sulfonic acid, salicylic acid, and the like.

As used herein, the term "treating" includes prophylaxis of the specific disorder or condition, or alleviation of the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms. For example, as used herein the term "treating diabetes" will refer in general to altering glucose blood levels in the direction of normal levels and may include increasing or decreasing blood glucose levels depending on a given situation.

As used herein an "effective" amount or a "therapeutically effective amount" of a glucagon peptide refers to a nontoxic but sufficient amount of the peptide to provide the desired effect. For example one desired effect would be the prevention or treatment of hypoglycemia, as measured, for example, by an increase in blood glucose level. The amount that is "effective" will vary from subject to subject, depending on the age and general condition of the individual, mode of administration, and the like. Thus, it is not always possible to specify an exact "effective amount." However, an appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

The term, "parenteral" means not through the alimentary canal but by some other route such as subcutaneous, intramuscular, intraspinal, or intravenous.

As used herein, the term "purified" and like terms relate to the isolation of a molecule or compound in a form that is substantially free of contaminants normally associated with the molecule or compound in a native or natural environment.

As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. The term "purified polypeptide" is used herein to describe a polypeptide which has been separated from other compounds including, but not limited to nucleic acid molecules, lipids and carbohydrates.

The term "isolated" requires that the referenced material be removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide present in a living animal is not isolated, but the same polynucleotide, separated from some or all of the coexisting materials in the natural system, is isolated.

-21-

As used herein the term "native glucagon" refers to a peptide consisting of the sequence of SEQ ID NO: 1, and the term "native GLP-1" is a generic term that designates GLP-1(7-36)amide, GLP-1(7-37)acid or a mixture of those two compounds. As used herein, a general reference to "glucagon" or "GLP-1" in the
5 absence of any further designation is intended to mean native glucagon or native GLP-1, respectively.

A "glucagon peptide" as used herein includes any peptide comprising, either the amino acid sequence of SEQ ID NO: 1, or any analog of the amino acid sequence of SEQ ID NO: 1, including amino acid substitutions, additions, or deletions, or post
10 translational modifications (e.g. methylation, acylation, ubiquitination and the like) of the peptide, wherein the analog stimulates glucagon or GLP-1 receptor activity, e.g., as measured by cAMP production using the assay described in Example 3.

The term "glucagon agonist" refers to a complex comprising a glucagon peptide that stimulates glucagon receptor activity, e.g., as measured by cAMP
15 production using the assay described in Example 3. For example, a "glucagon agonist analog" may include a glucagon peptide comprising a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13 or analog of such a sequence that has been modified to include one or more conservative amino acid substitutions
20 at positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28 or 29.

As used herein an amino acid "modification" refers to a substitution, addition or deletion of an amino acid, and includes substitution with or addition of any of the 20 amino acids commonly found in human proteins, as well as atypical or non-naturally occurring amino acids. Throughout the application, all references to a
25 particular amino acid position by number (e.g. position 28) refer to the amino acid at that position in native glucagon (SEQ ID NO:1) or the corresponding amino acid position in any analogs thereof. For example, a reference herein to "position 28" would mean the corresponding position 27 for a glucagon analog in which the first amino acid of SEQ ID NO: 1 has been deleted. Similarly, a reference herein to
30 "position 28" would mean the corresponding position 29 for a glucagon analog in which one amino acid has been added before the N-terminus of SEQ ID NO: 1.

As used herein an amino acid "substitution" refers to the replacement of one amino acid residue by a different amino acid residue.

As used herein, the term "conservative amino acid substitution" is defined herein as exchanges within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:

Ala, Ser, Thr, Pro, Gly;

5 II. Polar, negatively charged residues and their amides:

Asp, Asn, Glu, Gln, cysteic acid and homocysteic acid;

III. Polar, positively charged residues:

His, Arg, Lys; Ornithine (Orn)

IV. Large, aliphatic, nonpolar residues:

10 Met, Leu, Ile, Val, Cys, Norleucine (Nle), homocysteine

V. Large, aromatic residues:

Phe, Tyr, Trp, acetyl phenylalanine

As used herein the general term "polyethylene glycol" or "PEG", refers to
15 mixtures of condensation polymers of ethylene oxide and water, in a branched or straight chain, represented by the general formula $H(OCH_2CH_2)_nOH$, wherein n is at least 9. Absent any further characterization, the term is intended to include polymers of ethylene glycol with an average total molecular weight selected from the range of 500 to 40,000 Daltons. "polyethylene glycol" or "PEG" is used in combination with a
20 numeric suffix to indicate the approximate average molecular weight thereof. For example, PEG-5,000 refers to polyethylene glycol having a total molecular weight average of about 5,000.

As used herein the term "pegylated" and like terms refers to a compound that has been modified from its native state by linking a polyethylene glycol polymer to
25 the compound. A "pegylated glucagon peptide" is a glucagon peptide that has a PEG chain covalently bound to the glucagon peptide.

As used herein a general reference to a peptide is intended to encompass peptides that have modified amino and carboxy termini. For example, an amino acid chain comprising an amide group in place of the terminal carboxylic acid is intended
30 to be encompassed by an amino acid sequence designating the standard amino acids.

As used herein a "linker" is a bond, molecule or group of molecules that binds two separate entities to one another. Linkers may provide for optimal spacing of the

two entities or may further supply a labile linkage that allows the two entities to be separated from each other. Labile linkages include photocleavable groups, acid-labile moieties, base-labile moieties and enzyme-cleavable groups.

As used herein a "dimer" is a complex comprising two subunits covalently bound to one another via a linker. The term dimer, when used absent any qualifying language, encompasses both homodimers and heterodimers. A homodimer comprises two identical subunits, whereas a heterodimer comprises two subunits that differ, although the two subunits are substantially similar to one another.

As used herein the term "pH stabilized glucagon peptide" refers to a glucagon agonist analog that exhibits superior stability and solubility, relative to native glucagon, in aqueous buffers in the broadest pH range used for pharmacological purposes.

As used herein the term "charged amino acid" refers to an amino acid that comprises a side chain that is negatively charged (i.e., de-protonated) or positively charged (i.e., protonated) in aqueous solution at physiological pH. For example negatively charged amino acids include aspartic acid, glutamic acid, cysteic acid, homocysteic acid, and homoglutamic acid, whereas positively charged amino acids include arginine, lysine and histidine. Charged amino acids include the charged amino acids among the 20 amino acids commonly found in human proteins, as well as atypical or non-naturally occurring amino acids.

Non-naturally occurring amino acids refer to amino acids that do not naturally occur *in vivo* but which, nevertheless, can be incorporated into the peptide structures described herein. Commercial sources of atypical amino acids include Sigma-Aldrich (Milwaukee, WI), ChemPep Inc. (Miami, FL), and Genzyme Pharmaceuticals (Cambridge, MA). Atypical amino acids may be purchased from commercial suppliers, synthesized de novo, or chemically modified or derivatized from other amino acids.

As used herein the term "acidic amino acid" refers to an amino acid that comprises a second acidic moiety, including for example, a carboxylic acid or sulfonic acid group.

As used herein the term "depsi-peptide" defines a peptide having an ester linked amino acid. A "depsi-glucagon" is a glucagon peptide that has been modified to comprise an ester linked amino acid within the peptide sequence, including for

example at a position selected from positions 5, 7, 8, 11, 13, 16, 17, 18, 19, 20, 21, 25 or 28 according to the numbering of native glucagon.

The term “alkyl” refers to a linear or branched hydrocarbon containing the indicated number of carbon atoms. Exemplary alkyls include methyl, ethyl, and linear propyl groups.

The term “heteroalkyl” refers to a linear or branched hydrocarbon containing the indicated number of carbon atoms and at least one heteroatom in the backbone of the structure. Suitable heteroatoms for purposes herein include but are not limited to N, S, and O.

EMBODIMENTS

As disclosed herein glucagon agonists are provided that have pharmacokinetic and pharmacodynamics profiles comparable to native hormone but with solubility at physiological pH and chemical stability, once commercially formulated, to constitute a ready-to-use medicine. In one embodiment glucagon analogs having activity at the GLP-1 and/or GIP receptors are also modified in accordance with the present disclosure to comprise one or more ester linked amino acids both as a means of enhancing their solubility and as a means of delaying their time of action and increasing their therapeutic index.

The enhanced solubility of the glucagon analogs is derived from the substitution of one or more native amide bonds with an ester bond. Applicants have discovered that single amide bonds within the glucagon amino acid sequence can be replaced by ester bonds to enhance solubility and that those ester bonds will spontaneously rearrange after administration to a patient and exposure to physiological conditions to generate the native amide bond. The ester-based analogs are biologically inactive but of significantly enhanced solubility relative to the native hormone in physiological buffers. Accordingly, one embodiment of the present invention is directed to a glucagon agonist that has been modified relative to the wild type peptide of His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr (SEQ ID NO: 1) to comprise one, two, three or more ester bonds replacing the native amide bonds (forming a “depsi-glucagon” analog) to improve the peptide's solubility in aqueous solutions, particularly at a pH ranging from about 5.5 to about 8.0.

In accordance with one embodiment the inactive depsi-glucagon analog is administered to patients, wherein the ester bonds are converted to amide bonds *in vivo* thus restoring activity to the glucagon analog. In one embodiment the ester linked amino acids are present at one or more of positions 5, 7, 8, 11, 13, 16, 17, 18, 19, 20, 21, 25 or 28 according to the numbering of native glucagon.

In accordance with one embodiment an enhanced soluble analog of glucagon is provided wherein the analog comprising one, two, three or more amino acids selected from positions 13, 17, 18, 19, 20, 21, 25 or 28 (according to the numbering of native glucagon) linked to the immediately preceding amino acid (i.e., positions 12, 16, 17, 18, 19, 20, 24 or 27 respectively) via an ester bond. In accordance with one embodiment an enhanced soluble analog of glucagon is provided wherein the analog comprising one, two, three or more amino acids selected from positions 5, 7, 8, 11 or 16 (according to the numbering of native glucagon) linked to the immediately preceding amino acid (i.e., positions 4, 6, 7, 10 or 15 respectively) via an ester bond. In one embodiment a single amino acid selected from amino acids at positions 7, 8, 11 or 16 is linked to the immediate preceding amino acid via an ester bond. In one embodiment a single amino acid selected from amino acids at positions 13, 17, 18, 19, or 25 is linked to the immediate preceding amino acid via an ester bond. In one embodiment a single amino acid selected from amino acids at positions 7, 8, or 11 is linked to the immediate preceding amino acid via an ester bond. In one embodiment a single amino acid selected from amino acids at positions 8, 11 or 16 is linked to the immediate preceding amino acid via an ester bond. In one embodiment a single amino acid selected from amino acids at positions 7, 11 or 16 is linked to the immediate preceding amino acid via an ester bond. In one embodiment a single amino acid selected from amino acids at positions 11 or 16 is linked to the immediate preceding amino acid via an ester bond. In one embodiment a single amino acid selected from amino acids at position 16 is linked to the immediate preceding amino acid via an ester bond.

In one embodiment the side chain of a serine or threonine is linked via an ester bond with the alpha carboxy group of the immediate preceding amino acid in the glucagon amino acid sequence. In one embodiment a glucagon analog is provided comprising a serine or threonine at one or more of positions 7, 8, 11 or 16 wherein the serine or threonine is linked to the immediate preceding amino acid via an ester bond.

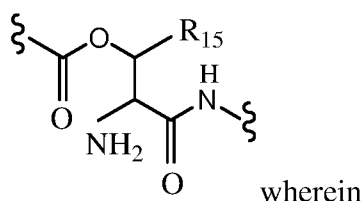
-26-

In one embodiment a serine or threonine at a single position selected from positions 8, 11 or 16 is linked to the immediate preceding amino acid via an ester bond. In one embodiment a serine or threonine at a single position selected from positions 11 or 16 is linked to the immediate preceding amino acid via an ester bond. In one

5 embodiment a serine or threonine at position 16 is linked to the immediate preceding amino acid via an ester bond. The glucagon peptide modified to comprise the ester linked amino acid can be selected from any known glucagon peptide as disclosed herein or known to those skilled in the art.

In accordance with one embodiment a glucagon peptide analog is provided

10 comprising one, two, three or more ester linked amino acids comprising the structure of Formula IV:



R_{15} is H or CH_3 . In one embodiment, the one, two, three or more ester linked

15 amino acids comprising the structure of Formula IV are located at position 5, 7, 8, 11, 13, 16, 17, 18, 19, 20, 21, 25 or 28; or at position 7, 8, 11, 13, 16, 17, 18, 19, 20, 21 or 25; or at position 7, 8, 11, 13, 17, 18, 19, or 25; or at position 13, 17, 18, 19, 20, 21, 25 or 28; or at positions 5, 7, 8, 11 or 16, or at position 8, 11 or 16, or at position 7 or 16, or at position 16, relative to native glucagon.

20 The enhanced soluble glucagon peptide analogs of the present invention can be further structurally modified to stabilize the ester bond configuration at physiological pH, so the ester bonds are not converted to amides until after administration to a patient and contact with serum. This enhanced stability allows for formulating the enhanced soluble glucagon peptide analog solutions at relatively

25 neutral pHs (e.g. pH of about 6.0 to about 8.0), which improves the long term stability of the glucagon peptide peptides. In accordance with one embodiment the alpha amine of the ester linked amino acid of the stabilized glucagon peptide analog is covalently bound to a compound that is metastable or cleavable upon contact with mammalian serum. In one embodiment the alpha amine of the ester linked amino

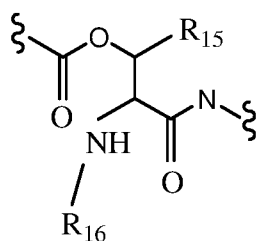
30 acid is covalently bound to an amino acid or a dipeptide that is susceptible to cleavage

-27-

by a peptidase present in mammalian serum, including for example, an aminopeptidase or Dipeptidyl Peptidase IV (DPP IV).

In one embodiment the improved soluble glucagon peptide analog of the present disclosure comprises one, two, three or more ester linked amino acids

5 comprising the structure of



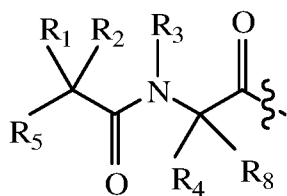
wherein

R₁₅ is H or CH₃ and

R₁₆ is an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a

10 diketopiperazine or diketomorpholine. In one embodiment R₁₆ is a dipeptide that is susceptible to cleavage by Dipeptidyl Peptidase IV. In accordance with one embodiment R₁₆ is a dipeptide of the general formula X-Pro, wherein X is any amino acid and the proline is linked to the primary amine of the ester linked amino acid. In one embodiment R₁₆ is selected from the group consisting of Gly-Pro, Lys-Pro and
15 Lys- Azetidine-2-carboxylic acid wherein the proline or Azetidine-2-carboxylic acid (Azetidine) is linked to the primary amine of the ester linked amino acid.

In an alternative embodiment R₁₆ is a dipeptide having the general structure of Formula I:



wherein

R₁ is H or C₁-C₁₈ alkyl;

R₂, R₄ and R₈ are independently selected from the group consisting of H, C₁-C₁₈ alkyl, C₂-C₁₈ alkenyl, (C₁-C₁₈ alkyl)OH, (C₁-C₁₈ alkyl)SH, (C₂-C₃ alkyl)SCH₃, (C₁-C₄ alkyl)CONH₂, (C₁-C₄ alkyl)COOH, (C₁-C₄ alkyl)NH₂, (C₁-C₄ alkyl)NHC(NH₂⁺)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₂-C₅ heterocyclic), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, (C₁-C₄ alkyl)(C₃-C₉ heteroaryl), and C₁-
25

-28-

C₁₂ alkyl(W₁)C₁-C₁₂ alkyl, wherein W₁ is a heteroatom selected from the group consisting of N, S and O, or R₁ and R₂ together with the atoms to which they are attached form a C₃-C₁₂ cycloalkyl; or R₄ and R₈ together with the atoms to which they are attached form a C₃-C₆ cycloalkyl;

5 R₃ is selected from the group consisting of C₁-C₁₈ alkyl, (C₁-C₁₈ alkyl)OH, (C₁-C₁₈ alkyl)NH₂, (C₁-C₁₈ alkyl)SH, (C₀-C₄ alkyl)(C₃-C₆)cycloalkyl, (C₀-C₄ alkyl)(C₂-C₅ heterocyclic), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and (C₁-C₄ alkyl)(C₃-C₉ heteroaryl) or R₄ and R₃ together with the atoms to which they are attached form a pyrrolidine ring;

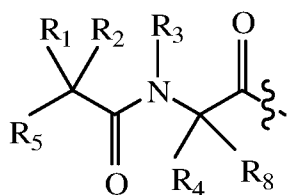
10 R₅ is NHR₆ or OH;

R₆ is H, C₁-C₈ alkyl; and

R₇ is selected from the group consisting of hydrogen and OH.

In one embodiment R₁₆ is a dipeptide having the general structure of Formula

I:



15

wherein

R₁ is selected from the group consisting of H and C₁-C₄ alkyl;

20 R₂ is selected from the group consisting of H, C₁-C₆ alkyl, C₂-C₈ alkenyl, (C₁-C₄ alkyl)OH, (C₁-C₄ alkyl)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and CH₂(C₅-C₉ heteroaryl);

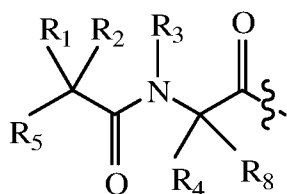
R₃ is C₁-C₆ alkyl;

R₄ is selected from the group consisting of H and C₁-C₄ alkyl or R₃ and R₄ together with the atoms to which they are attached form a pyrrolidine ring; and

R₇ is selected from the group consisting of hydrogen and OH

25 In one embodiment R₁₆ is a dipeptide having the general structure of Formula

I:

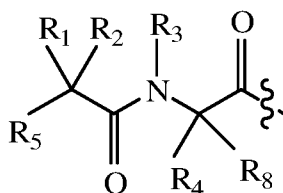


-29-

wherein

 R_1 is C_1 - C_4 alkyl or $(C_1$ - C_4 alkyl) NH_2 ; R_2 is H; R_3 is selected from the group consisting of C_1 - C_6 alkyl;5 R_4 is selected from the group consisting of H, and C_1 - C_4 alkyl; R_8 is H; and R_5 is NH_2 or OH, optionally wherein R_5 is NH_2 .In another embodiment R_{16} is a dipeptide having the general structure of

Formula I:

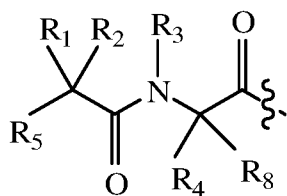


10

wherein

 R_1 is H, C_1 - C_4 alkyl or $(C_1$ - C_4 alkyl) NH_2 ; R_2 is H or is C_1 - C_4 alkyl; R_3 is selected from the group consisting of C_1 - C_6 alkyl;15 R_4 is selected from the group consisting of H, and C_1 - C_4 alkyl; R_8 is H; and R_5 is NH_2 or OH, optionally with the proviso that R_1 and R_2 are not both H.In one embodiment R_{16} is a dipeptide having the general structure of Formula

I:



20

wherein

 R_1 is C_1 - C_4 alkyl; R_2 is C_1 - C_4 alkyl; R_3 is C_1 - C_6 alkyl;25 R_4 is selected from the group consisting of H, and C_1 - C_4 alkyl; R_8 is H; and R_5 is NH_2 .

-30-

The glucagon peptides of the current disclosure can be further modified to enhance their solubility by introducing charge at its carboxy terminus. In one embodiment charge is added to the peptide by the substitution of native non-charged amino acids with charged amino acids selected from the group consisting of lysine, arginine, histidine, aspartic acid and glutamic acid, or by the addition of charged amino acids to the amino or carboxy terminus of the peptide. Surprisingly, applicants have discovered that substituting the normally occurring amino acid at position 28 and/or 29 with charged amino acids, and/or the addition of one to two charged amino acids at the carboxy terminus of the glucagon peptide, enhances the solubility and stability of the glucagon peptides in aqueous solutions at physiologically relevant pHs (i.e., a pH of about 6.5 to about 7.5) by at least 5-fold and by as much as 30-fold.

The solubility of glucagon or any known glucagon analog can be enhanced by the introduction of an ester bond for the native amide bond at any of positions 5, 7, 8, 11 or 16 relative to native glucagon. This includes introduction of an ester bond in any of the following Class 1, 2 or 3 glucagon related peptides:

Class 1 Glucagon Related Peptides

In certain embodiments, the glucagon related peptide is a Class 1 glucagon related peptide, which is described herein and in International Patent Publication No. WO 2009/155257 (published on December 23, 2009), International Patent Application Publication No. WO 2008/086086 (published on July 17, 2008), and International Patent Application Publication No. WO 2007/056362 (published on May 18, 2007), the contents of which are incorporated by reference in their entirety.

The biological sequences referenced in the following section (SEQ ID NOs: 801-915) relating to Class 1 glucagon related peptides correspond to SEQ ID NOs: 1-115 in International Patent Publication No. WO 2009/155257.

Activity

Class 1 glucagon peptides retain glucagon receptor activity relative to the native glucagon peptide (SEQ ID NO: 801). For example, the glucagon peptide can retain at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75% activity, 80% activity, 85% activity, or 90% of the activity of native glucagon (calculated as the inverse ratio of EC₅₀s for the glucagon peptide vs. glucagon, e.g., as measured by cAMP production using the assay generally described in Example 2). In some embodiments,

the Class 1 glucagon related peptides have the same or greater activity (used synonymously with the term “potency” herein) than glucagon.

In some embodiments, a Class 1 glucagon related peptide has been modified relative to the wild type peptide of His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-
5 Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr (SEQ ID NO: 801) to improve the peptide's solubility in aqueous solutions, particularly at a pH ranging from about 5.5 to about 8.0, while retaining the native peptide's biological activity.

For example, the solubility of any of the Class 1 glucagon related peptides
10 described herein can be further improved by attaching a hydrophilic moiety to the peptide. Introduction of such groups also increases duration of action, e.g. as measured by a prolonged half-life in circulation. Hydrophilic moieties are further described herein.

Modification with Charged Residues

15 In some embodiments, solubility is improved by adding charge to the Class 1 glucagon related peptide by the substitution of native non-charged amino acids with charged amino acids selected from the group consisting of lysine, arginine, histidine, aspartic acid and glutamic acid, or by the addition of charged amino acids to the amino or carboxy terminus of the peptide.

20 In accordance with some embodiments, the Class 1 glucagon related peptide is modified by amino acid substitutions and/or additions that introduce a charged amino acid into the C-terminal portion of the peptide, and in some embodiments at a position C-terminal to position 27 of SEQ ID NO: 801. Optionally, one, two or three charged amino acids may be introduced within the C-terminal portion, and in some
25 embodiments C-terminal to position 27. In accordance with some embodiments, the native amino acid(s) at positions 28 and/or 29 are substituted with a charged amino acid, and/or one to three charged amino acids are added to the C-terminus of the peptide, e.g. after position 27, 28 or 29. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. In other embodiments, one,
30 two, three or all of the charged amino acids are positively charged.

In specific exemplary embodiments, the Class 1 glucagon related peptide may comprise any one or two of the following modifications: substitution of N28 with E; substitution of N28 with D; substitution of T29 with D; substitution of T29 with E;

-32-

insertion of E after position 27, 28 or 29; insertion of D after position 27, 28 or 29. For example, D28E29, E28E29, E29E30, E28E30, D28E30.

Additional modifications, e.g. conservative substitutions, which modifications are further described herein, may be made to the Class 1 glucagon related peptide that
5 still allow it to retain glucagon activity.

Improved stability

Any of the Class 1 glucagon peptides may additionally exhibit improved stability and/or reduced degradation, for example, retaining at least 95% of the original peptide after 24 hours at 25 °C. Any of the Class 1 glucagon related peptides
10 disclosed herein may additionally exhibit improved stability at a pH within the range of 5.5 to 8, for example, retaining at least 75%, 80%, 90%, 95%, 96%, 97%, 98% or 99% of the original peptide after 24 hours at 25°C. In yet further exemplary embodiments, any of the foregoing Class 1 glucagon related peptides can be further modified to improve stability by modifying the amino acid at position 15 of SEQ ID
15 NO: 801 to reduce degradation of the peptide over time, especially in acidic or alkaline buffers. In exemplary embodiments, Asp at position 15 is substituted with a Glu, homo-Glu, cysteic acid, or homo-cysteic acid.

Alternatively, any of the Class 1 glucagon related peptides described herein can be further modified to improve stability by modifying the amino acid at position
20 16 of SEQ ID NO: 801. In exemplary embodiments, Ser at position 16 is substituted with Thr or Aib, or any of the amino acids substitutions described herein with regard to Class 1 glucagon related peptides which enhance potency at the glucagon receptor. Such modifications reduce cleavage of the peptide bond between Asp15-Ser16.

In some embodiments, any of the Class 1 glucagon related peptides described
25 herein can be further modified to reduce degradation at various amino acid positions by modifying any one, two, three, or all four of positions 20, 21, 24, or 27. Exemplary embodiments include substitution of Gln at position 20 with Ser, Thr, Ala or Aib, substitution of Asp at position 21 with Glu, substitution of Gln at position 24 with Ala or Aib, substitution of Met at position 27 with Leu or Nle. Removal or
30 substitution of methionine reduces degradation due to oxidation of the methionine. Removal or substitution of Gln or Asn reduces degradation due to deamidation of Gln or Asn. Removal or substitution of Asp reduces degradation that occurs through

-33-

dehydration of Asp to form a cyclic succinimide intermediate followed by isomerization to iso-aspartate.

Enhanced potency

In accordance with another embodiment, Class 1 glucagon related peptides are provided that have enhanced potency at the glucagon receptor, wherein the peptides comprise an amino acid modification at position 16 of native glucagon (SEQ ID NO: 801). By way of nonlimiting example, such enhanced potency can be provided by substituting the naturally occurring serine at position 16 with glutamic acid or with another negatively charged amino acid having a side chain with a length of 4 atoms, or alternatively with any one of glutamine, homoglutamic acid, or homocysteic acid, or a charged amino acid having a side chain containing at least one heteroatom, (e.g. N, O, S, P) and with a side chain length of about 4 (or 3-5) atoms. Substitution of serine at position 16 with glutamic acid enhances glucagon activity at least 2-fold, 4-fold, 5-fold and up to 10-fold greater at the glucagon receptor. In some embodiments, the Class 1 glucagon related peptide retains selectivity for the glucagon receptor relative to the GLP-1 receptors, e.g., at least 5-fold, 10-fold, or 15-fold selectivity.

DPP-IV Resistance

In some embodiments, the Class 1 glucagon peptides disclosed herein are further modified at position 1 or 2 to reduce susceptibility to cleavage by dipeptidyl peptidase IV. More particularly, in some embodiments, position 1 and/or position 2 of the Class 1 glucagon related peptide is substituted with the DPP-IV resistant amino acid(s) described herein. In some embodiments, position 2 of the analog peptide is substituted with an amino isobutyric acid. In some embodiments, position 2 of the analog peptide is substituted with an amino acid selected from the group consisting of D-serine, D-alanine, glycine, N-methyl serine, and ϵ -amino butyric acid. In another embodiment, position 2 of the Class 1 glucagon related peptide is substituted with an amino acid selected from the group consisting of D-serine, glycine, and aminoisobutyric acid. In some embodiments, the amino acid at position 2 is not D-serine.

Reduction in glucagon activity upon modification of the amino acids at position 1 and/or position 2 of the glucagon peptide can be restored by stabilization of the alpha-helix structure in the C-terminal portion of the glucagon peptide (around amino acids 12-29). The alpha helix structure can be stabilized by, e.g., formation of

-34-

a covalent or non-covalent intramolecular bridge (e.g., a lactam bridge between side chains of amino acids at positions “i” and “i+4”, wherein i is an integer from 12 to 25), substitution and/or insertion of amino acids around positions 12-29 with an alpha helix-stabilizing amino acid (e.g., an α,α -disubstituted amino acid), as further described herein.

Modifications at position 3

Glucagon receptor activity can be reduced by an amino acid modification at position 3 (according to the amino acid numbering of wild type glucagon), e.g. substitution of the naturally occurring glutamine at position 3, with an acidic, basic, or a hydrophobic amino acid. For example substitution at position 3 with glutamic acid, ornithine, or norleucine substantially reduces or destroys glucagon receptor activity.

Maintained or enhanced activity at the glucagon receptor may be achieved by modifying the Gln at position 3 with a glutamine analog as described herein. For example, glucagon agonists can comprise the amino acid sequence of SEQ ID NO: 863, SEQ ID NO: 869, SEQ ID NO: 870, SEQ ID NO: 871, SEQ ID NO: 872, SEQ ID NO: 873, and SEQ ID NO: 874.

Enhancing GLP-1 activity with C-terminal amides and esters

Enhanced activity at the GLP-1 receptor is provided by replacing the carboxylic acid of the C-terminal amino acid with a charge-neutral group, such as an amide or ester. Conversely, retaining the native carboxylic acid at the C-terminus of the peptide maintains the relatively greater selectivity of the Class 1 glucagon related peptide for the glucagon receptor vs. the GLP-1 receptor (e.g., greater than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20-fold).

Further modifications and combinations

Additional modifications may be made to the Class 1 glucagon related peptide which may further increase solubility and/or stability and/or glucagon activity. The Class 1 glucagon related peptide may alternatively comprise other modifications that do not substantially affect solubility or stability, and that do not substantially decrease glucagon activity. In exemplary embodiments, the Class 1 glucagon related peptide may comprise a total of up to 11, or up to 12, or up to 13, or up to 14 amino acid modifications relative to the native glucagon sequence. For example, conservative or non-conservative substitutions, additions or deletions may be carried out at any of positions 2, 5, 7, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 24, 27, 28 or 29.

Exemplary modifications of the Class 1 glucagon related peptide include but are not limited to:

(a) non-conservative substitutions, conservative substitutions, additions or deletions while retaining at least partial glucagon agonist activity, for example,

5 conservative substitutions at one or more of positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28 or 29, substitution of Tyr at position 10 with Val or Phe, substitution of Lys at position 12 with Arg, substitution of one or more of these positions with Ala;

10 (b) deletion of amino acids at positions 29 and/or 28, and optionally position 27, while retaining at least partial glucagon agonist activity;

(c) modification of the aspartic acid at position 15, for example, by substitution with glutamic acid, homoglutamic acid, cysteic acid or homocysteic acid, which may reduce degradation; or modification of the serine at position 16, for example, by substitution of threonine, Aib, glutamic acid or with another negatively
15 charged amino acid having a side chain with a length of 4 atoms, or alternatively with any one of glutamine, homoglutamic acid, or homocysteic acid, which likewise may reduce degradation due to cleavage of the Asp15-Ser16 bond;

(d) addition of a hydrophilic moiety such as the water soluble polymer polyethylene glycol, as described herein, e.g. at position 16, 17, 20, 21, 24, 29, 40 or
20 at the C-terminal amino acid, which may increase solubility and/or half-life;

(e) modification of the methionine at position 27, for example, by substitution with leucine or norleucine, to reduce oxidative degradation;

(f) modification of the Gln at position 20 or 24, e.g. by substitution with Ser, Thr, Ala or Aib, to reduce degradation that occurs through deamidation of Gln

25 (g) modification of Asp at position 21, e.g. by substitution with Glu, to reduce degradation that occurs through dehydration of Asp to form a cyclic succinimide intermediate followed by isomerization to iso-aspartate;

(h) modifications at position 1 or 2 as described herein that improve resistance to DPP-IV cleavage, optionally in combination with an intramolecular bridge such as
30 a lactam bridge between positions "i" and "i+4", wherein i is an integer from 12 to 25, e.g., 12, 16, 20, 24;

(i) acylating or alkylating the glucagon peptide as described herein, which may increase the activity at the glucagon receptor and/or the GLP-1 receptor, increase half-

-36-

life in circulation and/or extending the duration of action and/or delaying the onset of action, optionally combined with addition of a hydrophilic moiety, additionally or alternatively, optionally combined with a modification which selectively reduces activity at the GLP-1 peptide, e.g., a modification of the Thr at position 7, such as a substitution of the Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile; deleting amino acids C-terminal to the amino acid at position 27 (e.g., deleting one or both of the amino acids at positions 28 and 29, yielding a peptide 27 or 28 amino acids in length);

(j) C-terminal extensions as described herein;

(k) homodimerization or heterodimerization as described herein; and combinations of the (a) through (k).

In exemplary embodiments, Lys at position 12 is substituted with Arg. In other exemplary embodiments amino acids at positions 29 and/or 28, and optionally at position 27, are deleted.

In some specific embodiments, the glucagon peptide comprises (a) an amino acid modification at position 1 and/or 2 that confers DPP-IV resistance, e.g., substitution with DMIA at position 1, or Aib at position 2, (b) an intramolecular bridge within positions 12-29, e.g. at positions 16 and 20, or one or more substitutions of the amino acids at positions 16, 20, 21, and 24 with an α,α disubstituted amino acid, optionally (c) linked to a hydrophilic moiety such as PEG, e.g., through Cys at position 24, 29 or at the C-terminal amino acid, optionally (d) an amino acid modification at position 27 that substitutes Met with, e.g., Nle, optionally (e) amino acid modifications at positions 20, 21 and 24 that reduce degradation, and optionally (f) linked to SEQ ID NO: 820. When the glucagon peptide is linked to SEQ ID NO: 820, the amino acid at position 29 in certain embodiments is Thr or Gly. In other specific embodiments, the glucagon peptide comprises (a) Asp28Glu29, or Glu28Glu29, or Glu29Glu30, or Glu28Glu30 or Asp28Glu30, and optionally (b) an amino acid modification at position 16 that substitutes Ser with, e.g. Thr or Aib, and optionally (c) an amino acid modification at position 27 that substitutes Met with, e.g., Nle, and optionally (d) amino acid modifications at positions 20, 21 and 24 that reduce degradation. In a specific embodiment, the glucagon peptide is T16, A20, E21, A24, Nle27, D28, and E29.

-37-

In some embodiments, the Class 1 glucagon related peptide comprises the amino acid sequence:

X1-X2-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Z (SEQ ID NO: 940) with 1 to 3 amino acid

5 modifications thereto,

wherein X1 and/or X2 is a non-native amino acid that reduces susceptibility of (or increases resistance of) the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),

10 wherein Z is selected from the group consisting of -COOH (the naturally occurring C-terminal carboxylate), -Asn-COOH, Asn-Thr-COOH, and Y-COOH, wherein Y is 1 to 2 amino acids, and

wherein an intramolecular bridge, preferably a covalent bond, connects the side chains of an amino acid at position i and an amino acid at position i+4, wherein i is 12, 16, 20 or 24.

15 In some embodiments, the intramolecular bridge is a lactam bridge. In some embodiments, the amino acids at positions i and i+4 of SEQ ID NO: 839 are Lys and Glu, e.g., Glu16 and Lys20. In some embodiments, X1 is selected from the group consisting of: D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, and alpha, alpha-dimethyl imidazole acetic acid (DMIA). In other embodiments, X2 is selected from the group consisting of: D-Ser, D-Ala, Gly, N-methyl-Ser, Val, and alpha, amino isobutyric acid (Aib). In some embodiments, the glucagon peptide is covalently linked to a hydrophilic moiety at any of amino acid positions 16, 17, 20, 21, 24, 29, 40, within a C-terminal extension, or at the C-terminal amino acid. In exemplary embodiments, this

20 hydrophilic moiety is covalently linked to a Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine residue at any of these positions. Exemplary hydrophilic moieties include polyethylene glycol (PEG), for example, of a molecular weight of about 1,000 Daltons to about 40,000 Daltons, or about 20,000 Daltons to about 40,000 Daltons.

30 In other embodiments, the Class I glucagon related peptide comprises the amino acid sequence:

X1-X2-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Z (SEQ ID NO: 940),

-38-

wherein X1 and/or X2 is a non-native amino acid that reduces susceptibility of (or increases resistance of) the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV), wherein one, two, three, four or more of positions 16, 20, 21, and 24 of the glucagon peptide is substituted with an α , α -disubstituted amino acid, and

- 5 wherein Z is selected from the group consisting of –COOH (the naturally occurring C-terminal carboxylate), -Asn-COOH, Asn-Thr-COOH, and Y-COOH, wherein Y is 1 to 2 amino acids.

- Exemplary further amino acid modifications to the foregoing Class 1 glucagon related peptides or analogs include substitution of Thr at position 7 with an amino
10 acid lacking a hydroxyl group, e.g., aminobutyric acid (Abu), Ile, optionally, in combination with substitution or addition of an amino acid comprising a side chain covalently attached (optionally, through a spacer) to an acyl or alkyl group, which acyl or alkyl group is non-native to a naturally-occurring amino acid, substitution of Lys at position 12 with Arg; substitution of Asp at position 15 with Glu; substitution
15 of Ser at position 16 with Thr or Aib; substitution of Gln at position 20 with Ser, Thr, Ala or Aib; substitution of Asp at position 21 with Glu; substitution of Gln at position 24 with Ser, Thr, Ala or Aib; substitution of Met at position 27 with Leu or Nle; substitution of Asn at position 28 with a charged amino acid; substitution of Asn at position 28 with a charged amino acid selected from the group consisting of Lys, Arg,
20 His, Asp, Glu, cysteic acid, and homocysteic acid; substitution at position 28 with Asn, Asp, or Glu; substitution at position 28 with Asp; substitution at position 28 with Glu; substitution of Thr at position 29 with a charged amino acid; substitution of Thr at position 29 with a charged amino acid selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid; substitution at position 29
25 with Asp, Glu, or Lys; substitution at position 29 with Glu; insertion of 1-3 charged amino acids after position 29; insertion at position 30 (i.e., after position 29) of Glu or Lys; optionally with insertion at position 31 of Lys; addition of SEQ ID NO: 820 to the C-terminus, optionally, wherein the amino acid at position 29 is Thr or Gly; substitution or addition of an amino acid covalently attached to a hydrophilic moiety;
30 or a combination thereof.

Any of the modifications described above in reference to Class 1 glucagon agonists which increase glucagon receptor activity, retain partial glucagon receptor activity, improve solubility, increase stability, or reduce degradation can be applied to

-39-

Class 1 glucagon peptides individually or in combination. Thus, Class 1 glucagon related peptides can be prepared that retain at least 20% of the activity of native glucagon at the glucagon receptor, and which are soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, (e.g. pH 7), and optionally retain at least 95% of the original peptide (e.g. 5% or less of the original peptide is degraded or cleaved) after 24 hours at 25°C. Alternatively, high potency Class 1 glucagon peptides can be prepared that exhibit at least about 100%, 125%, 150%, 175%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 600%, 700%, 800%, 900% or 10-fold or more of the activity of native glucagon at the glucagon receptor, and optionally are soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, (e.g. pH 7), and optionally retains at least 95% of the original peptide (e.g. 5% or less of the original peptide is degraded or cleaved) after 24 hours at 25°C. In some embodiments, the Class 1 glucagon peptides described herein may exhibit at least any of the above indicated relative levels of activity at the glucagon receptor but no more than 1,000%, 5,000% or 10,000% of the activity of native glucagon at the glucagon receptor.

Examples of embodiments of Class 1 glucagon related peptides

In some embodiments a glucagon analog of SEQ ID NO: 833 is provided wherein 1 to 6 amino acids, selected from positions 1, 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21 or 24 of the analog differ from the corresponding amino acid of SEQ ID NO: 801. In accordance with another embodiment a glucagon analog of SEQ ID NO: 833 is provided wherein 1 to 3 amino acids selected from positions 1, 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21 or 24 of the analog differ from the corresponding amino acid of SEQ ID NO: 801. In another embodiment, a glucagon analog of SEQ ID NO: 807, SEQ ID NO: 808 or SEQ ID NO: 834 is provided wherein 1 to 2 amino acids selected from positions 1, 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21 or 24 of the analog differ from the corresponding amino acid of SEQ ID NO: 801, and in a further embodiment those one to two differing amino acids represent conservative amino acid substitutions relative to the amino acid present in the native sequence (SEQ ID NO: 801). In some embodiments a glucagon peptide of SEQ ID NO: 811 or SEQ ID NO: 813 is provided wherein the glucagon peptide further comprises one, two or three amino acid substitutions at positions selected from positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27 or 29. In some

-40-

embodiments the substitutions at positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 27 or 29 are conservative amino acid substitutions.

In some embodiments a glucagon agonist is provided comprising an analog peptide of SEQ ID NO: 801 wherein the analog differs from SEQ ID NO: 801 by
5 having an amino acid other than serine at position 2 and by having an acidic amino acid substituted for the native amino acid at position 28 or 29 or an acidic amino acid added to the carboxy terminus of the peptide of SEQ ID NO: 801. In some embodiments the acidic amino acid is aspartic acid or glutamic acid. In some
10 embodiments a glucagon analog of SEQ ID NO: 809, SEQ ID NO: 812, SEQ ID NO: 813 or SEQ ID NO: 832 is provided wherein the analog differs from the parent molecule by a substitution at position 2. More particularly, position 2 of the analog peptide is substituted with an amino acid selected from the group consisting of D-serine, alanine, D-alanine, glycine, n-methyl serine and amino isobutyric acid.

In another embodiment a glucagon agonist is provided comprising an analog
15 peptide of SEQ ID NO: 801 wherein the analog differs from SEQ ID NO: 801 by having an amino acid other than histidine at position 1 and by having an acidic amino acid substituted for the native amino acid at position 28 or 29 or an acidic amino acid added to the carboxy terminus of the peptide of SEQ ID NO: 801. In some
20 embodiments the acidic amino acid is aspartic acid or glutamic acid. In some embodiments a glucagon analog of SEQ ID NO: 809, SEQ ID NO: 812, SEQ ID NO: 813 or SEQ ID NO: 832 is provided wherein the analog differs from the parent molecule by a substitution at position 1. More particularly, position 1 of the analog
25 peptide is substituted with an amino acid selected from the group consisting of DMIA, D-histidine, desaminohistidine, hydroxyl-histidine, acetyl-histidine and homo-histidine.

In accordance with some embodiments the modified glucagon peptide comprises a sequence selected from the group consisting of SEQ ID NO: 809, SEQ ID NO: 812, SEQ ID NO: 813 and SEQ ID NO: 832. In a further embodiment a glucagon peptide is provided comprising a sequence of SEQ ID NO: 809, SEQ ID
30 NO: 812, SEQ ID NO: 813 or SEQ ID NO: 832 further comprising one to two amino acids, added to the C-terminus of SEQ ID NO: 809, SEQ ID NO: 812, SEQ ID NO: 813 or SEQ ID NO: 832, wherein the additional amino acids are independently selected from the group consisting of Lys, Arg, His, Asp Glu, cysteic acid or

-41-

homocysteic acid. In some embodiments the additional amino acids added to the carboxy terminus are selected from the group consisting of Lys, Arg, His, Asp or Glu or in a further embodiment the additional amino acids are Asp or Glu.

In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 807 or a glucagon agonist analog thereof. In some embodiments the peptide comprising a sequence selected from the group consisting of SEQ ID NO: 808, SEQ ID NO: 810, SEQ ID NO: 811, SEQ ID NO: 812 and SEQ ID NO: 813. In another embodiment the peptide comprising a sequence selected from the group consisting of SEQ ID NO: 808, SEQ ID NO: 810 and SEQ ID NO: 811. In some embodiments the glucagon peptide comprises the sequence of SEQ ID NO: 808, SEQ ID NO: 810 and SEQ ID NO: 811 further comprising an additional amino acid, selected from the group consisting of Asp and Glu, added to the C-terminus of the glucagon peptide. In some embodiments the glucagon peptide comprises the sequence of SEQ ID NO: 811 or SEQ ID NO: 813, and in a further embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 811.

In accordance with some embodiments a glucagon agonist is provided comprising a modified glucagon peptide selected from the group consisting of:

NH₂-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Xaa-Xaa-Xaa-R (SEQ ID NO: 834),
 NH₂-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asp-Thr-R (SEQ ID NO: 811) and
 NH₂-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Glu-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asp-Thr-R (SEQ ID NO: 813)
 wherein Xaa at position 15 is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid, the Xaa at position 28 is Asn or an acidic amino acid and the Xaa at position 29 is Thr or an acidic amino acid and R is an acidic amino acid, COOH or CONH₂, with the proviso that an acidic acid residue is present at one of positions 28, 29 or 30. In some embodiments R is COOH, and in another embodiment R is CONH₂.

The present disclosure also encompasses glucagon fusion peptides wherein a second peptide has been fused to the C-terminus of the glucagon peptide to enhance the stability and solubility of the glucagon peptide. More particularly, the fusion glucagon peptide may comprise a glucagon agonist analog comprising a glucagon

-42-

peptide NH₂-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Xaa-Xaa-Xaa-R (SEQ ID NO: 834), wherein R is an acidic amino acid or a bond and an amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) linked to the carboxy terminal amino acid of the glucagon peptide. In some embodiments the glucagon peptide is selected from the group consisting of SEQ ID NO: 833, SEQ ID NO: 807 or SEQ ID NO: 808 further comprising an amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) linked to the carboxy terminal amino acid of the glucagon peptide. In some embodiments the glucagon fusion peptide comprises SEQ ID NO: 802, SEQ ID NO: 803, SEQ ID NO: 804, SEQ ID NO: 805 and SEQ ID NO: 806 or a glucagon agonist analog thereof, further comprising an amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) linked to amino acid 29 of the glucagon peptide. In accordance with some embodiments the fusion peptide further comprises a PEG chain linked to an amino acid at position 16, 17, 21, 24, 29, within a C-terminal extension, or at the C-terminal amino acid, wherein the PEG chain is selected from the range of 500 to 40,000 Daltons. In some embodiments the amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) is bound to amino acid 29 of the glucagon peptide through a peptide bond. In some embodiments the glucagon peptide portion of the glucagon fusion peptide comprises a sequence selected from the group consisting of SEQ ID NO: 810, SEQ ID NO: 811 and SEQ ID NO: 813. In some embodiments the glucagon peptide portion of the glucagon fusion peptide comprises the sequence of SEQ ID NO: 811 or SEQ ID NO: 813, wherein a PEG chain is linked at position 21, 24, 29, within a C-terminal extension or at the C-terminal amino acid, respectively.

In another embodiment the glucagon peptide sequence of the fusion peptide comprises the sequence of SEQ ID NO: 811, further comprising an amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) linked to amino acid 29 of the glucagon peptide. In some embodiments the glucagon fusion peptide comprises a sequence selected from the group consisting of SEQ ID NO: 824, SEQ ID NO: 825 and SEQ ID NO: 826. Typically the fusion peptides of the present invention will have a C-terminal amino

-43-

acid with the standard carboxylic acid group. However, analogs of those sequences wherein the C-terminal amino acid has an amide substituted for the carboxylic acid are also encompassed as embodiments. In accordance with some embodiments the fusion glucagon peptide comprises a glucagon agonist analog selected from the group consisting of SEQ ID NO: 810, SEQ ID NO: 811 and SEQ ID NO: 813, further comprising an amino acid sequence of SEQ ID NO: 823 (GPSSGAPPPS-CONH₂) linked to amino acid 29 of the glucagon peptide.

In some embodiments the glucagon peptide of SEQ ID NO: 810, SEQ ID NO: 811, SEQ ID NO: 813, or SEQ ID NO: 832 is modified to comprise one or more hydrophilic groups covalently linked to the side chains of amino acids present at positions 21 and 24 of the glucagon peptide.

In accordance with some embodiments, the glucagon peptide of SEQ ID NO: 811 is modified to contain one or more amino acid substitution at positions 16, 17, 20, 21, 24 and/or 29, wherein the native amino acid is substituted with an amino acid having a side chain suitable for crosslinking with hydrophilic moieties, including for example, PEG. The native peptide can be substituted with a naturally occurring amino acid or a synthetic (non-naturally occurring) amino acid. Synthetic or non-naturally occurring amino acids refer to amino acids that do not naturally occur in vivo but which, nevertheless, can be incorporated into the peptide structures described herein.

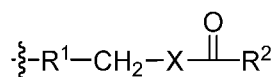
The polyethylene glycol chain may be in the form of a straight chain or it may be branched. In accordance with some embodiments the polyethylene glycol chain has an average molecular weight selected from the range of about 500 to about 40,000 Daltons. In some embodiments the polyethylene glycol chain has a molecular weight selected from the range of about 500 to about 5,000 Daltons. In another embodiment the polyethylene glycol chain has a molecular weight of about 20,000 to about 40,000 Daltons.

Any of the glucagon peptides described above may be further modified to include a covalent or non-covalent intramolecular bridge or an alpha helix-stabilizing amino acid within the C-terminal portion of the glucagon peptide (amino acid positions 12-29). In accordance with some embodiments, the glucagon peptide comprises any one or more of the modifications discussed above in addition to an amino acid substitution at positions 16, 20, 21, or 24 (or a combination thereof) with

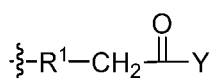
-44-

an α,α -disubstituted amino acid, e.g., Aib. In accordance with another embodiment, the glucagon peptide comprises any one or more modifications discussed above in addition to an intramolecular bridge, e.g., a lactam, between the side chains of the amino acids at positions 16 and 20 of the glucagon peptide.

- 5 In accordance with some embodiments, the glucagon peptide comprises the amino acid sequence of SEQ ID NO: 877, wherein the Xaa at position 3 is an amino acid comprising a side chain of Structure I, II, or III:

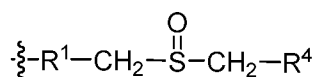


Structure I



10

Structure II



Structure III

- wherein R^1 is C_{0-3} alkyl or C_{0-3} heteroalkyl; R^2 is NHR^4 or C_{1-3} alkyl; R^3 is C_{1-3} alkyl; R^4 is H or C_{1-3} alkyl; X is NH, O, or S; and Y is NHR^4 , SR^3 , or OR^3 . In some
 15 embodiments, X is NH or Y is NHR^4 . In some embodiments, R^1 is C_{0-2} alkyl or C_1 heteroalkyl. In some embodiments, R^2 is NHR^4 or C_1 alkyl. In some embodiments, R^4 is H or C^1 alkyl. In exemplary embodiments an amino acid comprising a side chain of Structure I is provided wherein, R^1 is $\text{CH}_2\text{-S}$, X is NH, and R^2 is CH_3
 20 (acetamidomethyl-cysteine, C(Acm)); R^1 is CH_2 , X is NH, and R^2 is CH_3 (acetyldiaminobutanoic acid, Dab(Ac)); R^1 is C_0 alkyl, X is NH, R^2 is NHR^4 , and R^4 is H (carbamoyldiaminopropanoic acid, Dap(urea)); or R^1 is $\text{CH}_2\text{-CH}_2$, X is NH, and R^2 is CH_3 (acetylornithine, Orn(Ac)). In exemplary embodiments an amino acid comprising a side chain of Structure II is provided, wherein R^1 is CH_2 , Y is NHR^4 ,
 25 and R^4 is CH_3 (methylglutamine, Q(Me)); In exemplary embodiments an amino acid comprising a side chain of Structure III is provided wherein, R^1 is CH_2 and R^4 is H (methionine-sulfoxide, M(O)); In specific embodiments, the amino acid at position 3 is substituted with Dab(Ac). For example, glucagon agonists can comprise the amino acid sequence of SEQ ID NO: 863, SEQ ID NO: 869, SEQ ID NO: 871, SEQ
 30 ID NO: 872, SEQ ID NO: 873, and SEQ ID NO: 874.

In certain embodiments, the glucagon peptide is an analog of the glucagon peptide of SEQ ID NO: 877. In specific aspects, the analog comprises any of the amino acid modifications described herein, including, but not limited to: a substitution of Asn at position 28 with a charged amino acid; a substitution of Asn at position 28 with a charged amino acid selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid; a substitution at position 28 with Asn, Asp, or Glu; a substitution at position 28 with Asp; a substitution at position 28 with Glu; a substitution of Thr at position 29 with a charged amino acid; a substitution of Thr at position 29 with a charged amino acid selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid; a substitution at position 29 with Asp, Glu, or Lys; a substitution at position 29 with Glu; an insertion of 1-3 charged amino acids after position 29; an insertion after position 29 of Glu or Lys; an insertion after position 29 of Gly-Lys or Lys-Lys; and a combination thereof.

In certain embodiments, the analog of the glucagon peptide of SEQ ID NO: 877 comprises an α,α -disubstituted amino acid, such as Aib, at one, two, three, or all of positions 16, 20, 21, and 24.

In certain embodiments, the analog of the glucagon peptide of SEQ ID NO: 877 comprises one or more of the following: substitution of His at position 1 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV), substitution of Ser at position 2 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV), substitution of Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile; substitution of Tyr at position 10 with Phe or Val; substitution of Lys at position 12 with Arg; substitution of Asp at position 15 with Glu, substitution of Ser at position 16 with Thr or Aib; substitution of Gln at position 20 with Ala or Aib; substitution of Asp at position 21 with Glu; substitution of Gln at position 24 with Ala or Aib; substitution of Met at position 27 with Leu or Nle; deletion of amino acids at positions 27-29; deletion of amino acids at positions 28-29; deletion of the amino acid at positions 29; addition of the amino acid sequence of SEQ ID NO: 820 to the C-terminus, wherein the amino acid at position 29 is Thr or Gly, or a combination thereof.

In accordance with specific embodiments, the glucagon peptide comprises the amino acid sequence of any of SEQ ID NOs: 862-867 and 869-874.

In certain embodiments, the analog of the glucagon peptide comprising SEQ ID NO: 877 comprises a hydrophilic moiety, e.g., PEG, covalently linked to the amino acid at any of positions 16, 17, 20, 21, 24, and 29 or at the C-terminal amino acid.

5 In certain embodiments, the analog of the glucagon peptide comprising SEQ ID NO: 877 comprises an amino acid comprising a side chain covalently attached, optionally, through a spacer, to an acyl group or an alkyl group, which acyl group or alkyl group is non-native to a naturally-occurring amino acid. The acyl group in some embodiments is a C4 to C30 fatty acyl group. In other embodiments, the alkyl group
10 is a C4 to C30 alkyl. In specific aspects, the acyl group or alkyl group is covalently attached to the side chain of the amino acid at position 10. In some embodiments, the amino acid at position 7 is Ile or Abu.

The glucagon agonist may be a peptide comprising the amino acid sequence of any of the SEQ ID NOs: 801-919, optionally with up to 1, 2, 3, 4, or 5 further
15 modifications that retain glucagon agonist activity. In certain embodiments, the glucagon agonist comprises the amino acids of any of SEQ ID NOs: 859-919.

Class 2 Glucagon Related Peptides

In certain embodiments, the glucagon related peptide is a Class 2 glucagon
20 related peptide, which is described herein and in International Patent Publication No. WO 2010/011439, and U.S. Application No. 61/187,578, (filed on June 16, 2009) the contents of which are incorporated by reference in their entirety.

The biological sequences referenced in the following section (SEQ ID NOs: 1001-1262) relating to Class 2 glucagon related peptides correspond to SEQ ID NOs:
25 1-262 in International Patent Publication No. WO 2010/011439. SEQ ID NOs: 1284 to 1296 relating to Class 2 glucagon related peptides correspond to SEQ ID NOs: 657 to 669 in U.S. Application No. 61/187,578.

Activity

Native glucagon does not activate the GIP receptor, and normally has about
30 1% of the activity of native-GLP-1 at the GLP-1 receptor. Modifications to the native glucagon sequence described herein produce Class 2 glucagon related peptides that can exhibit potent glucagon activity equivalent to or better than the activity of native glucagon (SEQ ID NO: 1001), potent GIP activity equivalent to or better than the

-47-

activity of native GIP (SEQ ID NO: 1004), and/or potent GLP-1 activity equivalent to or better than the activity of native GLP-1. In this regard, the Class 2 glucagon related peptide may be one of a glucagon/GIP co-agonist, glucagon/GIP/GLP-1 tri-agonist, GIP/GLP-1 co-agonist, or a GIP agonist glucagon peptide, as further
5 described herein.

In some embodiments, the Class 2 glucagon related peptides described herein exhibit an EC₅₀ for GIP receptor activation activity of about 100 nM or less, or about 75, 50, 25, 10, 8, 6, 5, 4, 3, 2 or 1 nM or less. In some embodiments, the Class 2 glucagon related peptides exhibit an EC₅₀ for glucagon receptor activation of about
10 100 nM or less, or about 75, 50, 25, 10, 8, 6, 5, 4, 3, 2 or 1 nM or less. In some embodiments, the Class 2 glucagon related peptides exhibit an EC₅₀ for GLP-1 receptor activation of about 100 nM or less, or about 75, 50, 25, 10, 8, 6, 5, 4, 3, 2 or 1 nM or less. Receptor activation can be measured by in vitro assays measuring cAMP induction in HEK293 cells over-expressing the receptor, e.g. assaying
15 HEK293 cells co-transfected with DNA encoding the receptor and a luciferase gene linked to cAMP responsive element as described in Example 2.

In some embodiments, Class 2 glucagon related peptides exhibit at least about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175% or 200% or higher activity at the
20 GIP receptor relative to native GIP (GIP potency). In some embodiments, the glucagon peptides described herein exhibit no more than 1000%, 10,000%, 100,000%, or 1,000,000% activity at the GIP receptor relative to native GIP. In some embodiments, Class 2 glucagon related peptides exhibit at least about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175%, 200%, 250%, 300%,
25 350%, 400%, 450%, or 500% or higher activity at the glucagon receptor relative to native glucagon (glucagon potency). In some embodiments, the glucagon peptides described herein exhibit no more than 1000%, 10,000%, 100,000%, or 1,000,000% activity at the glucagon receptor relative to native glucagon. In some embodiments, Class 2 glucagon related peptides exhibit at least about 0.1%, 0.2%, 0.3%, 0.4%,
30 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175% or 200% or higher activity at the GLP-1 receptor relative to native GLP-1 (GLP-1 potency). In some embodiments, the glucagon peptides described herein exhibit no more than 1000%, 10,000%, 100,000%, or 1,000,000%

-48-

activity at the GLP-1 receptor relative to native GLP-1. A Class 2 glucagon related peptide's activity at a receptor relative to a native ligand of the receptor is calculated as the inverse ratio of EC_{50} s for the Class 2 glucagon related peptide vs. the native ligand.

5 In some embodiments, Class 2 glucagon related peptides exhibit activity at both the glucagon receptor and the GIP receptor ("glucagon/GIP co-agonists"). These Class 2 glucagon related peptides have lost native glucagon's selectivity for glucagon receptor compared to GIP receptor. In some embodiments, the EC_{50} of the Class 2 glucagon related peptide at the GIP receptor is less than about 50-fold, 40-fold, 30-
10 fold or 20-fold different (higher or lower) from its EC_{50} at the glucagon receptor. In some embodiments, the GIP potency of the Class 2 glucagon related peptide is less than about 500-, 450-, 400-, 350-, 300-, 250-, 200-, 150-, 100-, 75-, 50-, 25-, 20-, 15-, 10-, or 5-fold different (higher or lower) from its glucagon potency. In some
15 embodiments, the ratio of the EC_{50} of the Class 2 glucagon related peptide at the GIP receptor divided by the EC_{50} of the Class 2 glucagon related peptide at the glucagon receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some
embodiments, the ratio of the EC_{50} at the GIP receptor divided by the EC_{50} at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the GIP potency
20 of the Class 2 glucagon related peptide compared to the glucagon potency of the Class 2 glucagon related peptide is less than about 500, 450, 400, 350, 300, 250, 200, 150, 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the potency at the GIP receptor divided by the potency at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067,
25 0.1, 0.2). In some embodiments, GLP-1 activity have been significantly reduced or destroyed, e.g., by an amino acid modification at position 7, a deletion of the amino acid(s) C-terminal to the amino acid at position 27 or 28, yielding a 27- or 28-amino acid peptide, or a combination thereof.

30 In another aspect, Class 2 glucagon related peptides exhibit activity at the glucagon, GIP and GLP-1 receptors ("glucagon/GIP/GLP-1 tri-agonists"). These Class 2 glucagon related peptides have lost native glucagon's selectivity for the glucagon receptor compared to both the GLP-1 and GIP receptors. In some embodiments, the EC_{50} of the Class 2 glucagon related peptide at the GIP receptor is

less than about 50-fold, 40-fold, 30-fold or 20-fold different (higher or lower) from its respective EC_{50} s at the glucagon and GLP-1 receptors. In some embodiments, the GIP potency of the Class 2 glucagon related peptide is less than about 500-, 450-, 400-, 350-, 300-, 250-, 200-, 150-, 100-, 75-, 50-, 25-, 20-, 15-, 10-, or 5-fold different (higher or lower) from its glucagon and GLP-1 potencies. In some embodiments, the ratio of the EC_{50} of the tri-agonist at the GIP receptor divided by the EC_{50} of the tri-agonist at the GLP-1 receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the EC_{50} at the GIP receptor divided by the EC_{50} at the GLP-1 receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the GIP potency of the tri-agonist compared to the GLP-1 potency of the tri-agonist is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the potency at the GIP receptor divided by the potency at the GLP-1 receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In related embodiments, the ratio of the EC_{50} of the tri-agonist at the GIP receptor divided by the EC_{50} of the tri-agonist at the glucagon receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the EC_{50} at the GIP receptor divided by the EC_{50} at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the GIP potency of the tri-agonist compared to the glucagon potency of the tri-agonist is less than about 500, 450, 400, 350, 300, 250, 200, 150, 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the potency at the GIP receptor divided by the potency at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the EC_{50} of the tri-agonist at the GLP-1 receptor divided by the EC_{50} of the tri-agonist at the glucagon receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the EC_{50} at the GLP-1 receptor divided by the EC_{50} at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the GLP-1 potency of the tri-agonist compared to the glucagon potency of the tri-agonist is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the potency at the GLP-1 receptor divided by the potency at the glucagon receptor is

-50-

about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2).

In yet another aspect, Class 2 glucagon related peptides exhibit activity at the GLP-1 and GIP receptors, but in which the glucagon activity has been significantly reduced or destroyed (“GIP/GLP-1 co-agonists”), e.g., by an amino acid modification at position 3. For example, substitution at this position with an acidic, basic, or a hydrophobic amino acid (glutamic acid, ornithine, norleucine) reduces glucagon activity. In some embodiments, the EC_{50} of the glucagon peptide at the GIP receptor is less than about 50-fold, 40-fold, 30-fold or 20-fold different (higher or lower) from its EC_{50} at the GLP-1 receptor. In some embodiments, the GIP potency of the Class 2 glucagon related peptide is less than about 25-, 20-, 15-, 10-, or 5-fold different (higher or lower) from its GLP-1 potency. In some embodiments these Class 2 glucagon related peptides have about 10% or less of the activity of native glucagon at the glucagon receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1% but less than about 10%. In some embodiments, the ratio of the EC_{50} of the Class 2 glucagon related peptide at the GIP receptor divided by the EC_{50} of the Class 2 glucagon related peptide at the GLP-1 receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5, and no less than 1. In some embodiments, the ratio of the GIP potency of the Class 2 glucagon related peptide compared to the GLP-1 potency of the Class 2 glucagon related peptide is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5, and no less than 1.

In a further aspect, Class 2 glucagon related peptides exhibit activity at the GIP receptor, in which the glucagon and GLP-1 activity have been significantly reduced or destroyed (“GIP agonist glucagon peptides”), e.g., by amino acid modifications at positions 3 with Glu and 7 with Ile. In some embodiments, these Class 2 glucagon related peptides have about 10% or less of the activity of native glucagon at the glucagon receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1%, 0.5%, or 1% but less than about 1%, 5%, or 10%. In some embodiments these Class 2 glucagon related peptides also have about 10% or less of the activity of native GLP-1 at the GLP-1 receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1%, 0.5%, or 1% but less than about 1%, 5%, or 10%.

In some embodiments, when the Class 2 glucagon related peptide is not pegylated, the EC_{50} of the Class 2 glucagon related peptide for GIP receptor activation

-51-

is about 4, 2, 1 nM or less, or the analog has at least about 1%, 2%, 3%, 4% or 5% of the activity of native GIP at the GIP receptor. In related embodiments, the EC₅₀ of the unpegylated Class 2 glucagon related peptide for GLP-1 receptor activation is about 4, 2, 1 nM or less or has at least about 1%, 2%, 3%, 4% or 5% of the activity of native GLP-1 at the GLP-1 receptor. In yet other related embodiments, the EC₅₀ of the unpegylated Class 2 glucagon related peptide for glucagon receptor activation is about 4, 2, 1 nM or less, or at least about 5%, 10%, 15% or 20% of the activity of native glucagon at the glucagon receptor. In some embodiments, the unpegylated Class 2 glucagon related peptide has less than about 1% of the activity of native glucagon at the glucagon receptor. In other embodiments, the unpegylated Class 2 glucagon related peptide has less than about 10%, 5% or 1% of the activity of native GLP-1 at the GLP-1 receptor.

In embodiments where the Class 2 glucagon related peptides are linked to hydrophilic moieties such as PEG, the relative EC₅₀s at one or more receptors may be higher e.g., about 10-fold higher. For example, the EC₅₀ of a pegylated analog for GIP receptor activation is about 10 nM or less, or the Class 2 glucagon related peptide has at least about 0.1%, 0.2%, 0.3%, 0.4% or 0.5% of the activity of native GIP at the GIP receptor. In related embodiments, the EC₅₀ of a pegylated Class 2 glucagon related peptide for GLP-1 receptor activation is about 10 nM or less or has at least about 0.1%, 0.2%, 0.3%, 0.4% or 0.5% of the activity of native GLP-1 at the GLP-1 receptor. In yet other related embodiments, the EC₅₀ of a pegylated Class 2 glucagon related peptide for glucagon receptor activation is about 10 nM or less, or at least about 0.5%, 1%, 1.5% or 2% of the activity of native glucagon at the glucagon receptor. In some embodiments, the Class 2 glucagon related peptide has less than about 1% of the activity of native glucagon at the glucagon receptor. In other embodiments, the Class 2 glucagon related peptide has less than about 10%, 5% or 1% of the activity of native GLP-1 at the GLP-1 receptor.

Modifications

The modifications disclosed herein in reference to a Class 2 glucagon related peptide permit the manipulation of glucagon (SEQ ID NO: 1001) to create glucagon peptides that exhibit increased GIP activity, glucagon activity, and/or GLP-1 activity. Other modifications disclosed herein in reference to a Class 2 glucagon related peptide prolong the half-life, increase solubility, or increase stability of the resulting

-52-

peptide. Yet other modifications disclosed herein in reference to a Class 2 glucagon related peptide have no effect on activity, or can be made without destroying the desired activity or activities. Any of the combinations in reference to a Class 2 glucagon related peptide that serve the same purpose (e.g. increasing GIP activity) can be applied individually or in combination. Any of the single or sets of combinations in reference to a Class 2 glucagon related peptide that confer enhanced properties can be applied individually or in combination, e.g. increased GIP and/or GLP-1 activity can be combined with increased half-life. In related embodiments, 1, 2, 3, 4, 5, 6 or more of the amino acid modifications may be non-conservative substitutions, additions or deletions. In some embodiments, 1, 2, 3, 4, 5, 6 or more of the amino acid modifications may be conservative substitutions.

Modifications that affect GIP activity

Enhanced activity at the GIP receptor is provided by an amino acid modification at position 1. For example, His at position 1 is substituted with a large, aromatic amino acid, optionally Tyr, Phe, Trp, amino-Phe, nitro-Phe, chloro-Phe, sulfo-Phe, 4-pyridyl-Ala, methyl-Tyr, or 3-amino Tyr. The combination of Tyr at position 1 with stabilization of the alpha helix within the region corresponding to amino acids 12-29 provided a Class 2 glucagon related peptide that activates the GIP receptor as well as the GLP-1 receptor and the glucagon receptor. The alpha helix structure can be stabilized by, e.g., formation of a covalent or non-covalent intramolecular bridge, or substitution and/or insertion of amino acids around positions 12-29 with an alpha helix-stabilizing amino acid (e.g., an α,α -disubstituted amino acid).

Enhanced activity at the GIP receptor is also provided by amino acid modifications at positions 27 and/or 28, and optionally at position 29. For example, the Met at position 27 is substituted with a large aliphatic amino acid, optionally Leu, the Asn at position 28 is substituted with a small aliphatic amino acid, optionally Ala, and the Thr at position 29 is substituted with a small aliphatic amino acid, optionally Gly. Substitution with LAG at positions 27-29 provides increased GIP activity relative to the native MNT sequence at those positions.

Enhanced activity at the GIP receptor is also provided by an amino acid modification at position 12. For example, position 12 is substituted with a large, aliphatic, nonpolar amino acid, optionally Ile.

-53-

Enhanced activity at the GIP receptor is also provided by an amino acid modification at positions 17 and/or 18. For example, position 17 is substituted with a polar residue, optionally Gln, and position 18 is substituted with a small aliphatic amino acid, optionally Ala. A substitution with QA at positions 17 and 18 provides increased GIP activity relative to the native RR sequence at those positions.

Increased activity at the GIP receptor is provided by modifications that permit formation of an intramolecular bridge between amino acid side chains at positions from 12 to 29. For example, an intramolecular bridge can be formed by a covalent bond between the side chains of two amino acids at positions i and $i+4$ or between positions j and $j+3$, or between positions k and $k+7$. In exemplary embodiments, the bridge is between positions 12 and 16, 16 and 20, 20 and 24, 24 and 28, or 17 and 20. In other embodiments, non-covalent interactions such as salt bridges can be formed between positively and negatively charged amino acids at these positions.

Any of the modifications described above which increase GIP receptor activity can be applied individually or in combination. Combinations of the modifications that increase GIP receptor activity generally provide higher GIP activity than any of such modifications taken alone.

Modifications that affect glucagon activity

In some embodiments, enhanced glucagon potency is provided by an amino acid modification at position 16 of native glucagon (SEQ ID NO: 1001). By way of nonlimiting example, such enhanced potency can be provided by substituting the naturally occurring serine at position 16 with glutamic acid or with another negatively charged amino acid having a side chain with a length of 4 atoms, or alternatively with any one of glutamine, homoglutamic acid, or homocysteic acid, or a charged amino acid having a side chain containing at least one heteroatom, (e.g. N, O, S, P) and with a side chain length of about 4 (or 3-5) atoms. In some embodiments the glucagon peptide retains its original selectivity for the glucagon receptor relative to the GLP-1 receptors.

Glucagon receptor activity can be reduced by an amino acid modification at position 3, e.g. substitution of the naturally occurring glutamine at position 3, with an acidic, basic, or a hydrophobic amino acid. For example substitution at position 3 with glutamic acid, ornithine, or norleucine substantially reduces or destroys glucagon receptor activity.

Maintained or enhanced activity at the glucagon receptor may be achieved by modifying the Gln at position 3 with a glutamine analog, as described herein. For example, glucagon agonists can comprise the amino acid sequence of any of SEQ ID NOs: 1243-1248, 1250, 1251, and 1253-1256.

- 5 Restoration of glucagon activity which has been reduced by amino acid modifications at positions 1 and 2 is provided by modifications that stabilize the alpha helix structure of the C-terminal portion (amino acids 12-29) of the glucagon peptide or analog thereof. For example, an intramolecular bridge can be formed by a covalent bond between the side chains of two amino acids at positions i and i+4 or
10 between positions j and j+3, or between positions k and k+7. In other embodiments, non-covalent interactions such as salt bridges can be formed between positively and negatively charged amino acids at these positions. In yet other embodiments, one or more α , α -disubstituted amino acids are inserted or substituted into this C-terminal portion (amino acids 12-29) at positions that retain the desired activity. For example,
15 one, two, three or all of positions 16, 20, 21 or 24 are substituted with an α , α -disubstituted amino acid, e.g., Aib.

Modifications that affect GLP-1 activity

- Enhanced activity at the GLP-1 receptor is provided by replacing the carboxylic acid of the C-terminal amino acid with a charge-neutral group, such as an
20 amide or ester.

- Enhanced activity at the GLP-1 receptor is also provided by stabilizing the alpha-helix structure in the C-terminal portion of glucagon (around amino acids 12-29), e.g., through formation of an intramolecular bridge between the side chains of two amino acids, or substitution and/or insertion of amino acids around positions 12-
25 29 with an alpha helix-stabilizing amino acid (e.g., an α , α -disubstituted amino acid), as further described herein. In exemplary embodiments, the side chains of the amino acid pairs 12 and 16, 13 and 17, 16 and 20, 17 and 21, 20 and 24 or 24 and 28 (amino acid pairs in which i = 12, 16, 20, or 24) are linked to one another and thus stabilize the glucagon alpha helix. In some embodiments, the bridge or linker is about 8 (or
30 about 7-9) atoms in length, particularly when the bridge is between positions i and i+4. In some embodiments, the bridge or linker is about 6 (or about 5-7) atoms in length, particularly when the bridge is between positions j and j+3.

-55-

In some embodiments, intramolecular bridges are formed by (a) substituting the naturally occurring serine at position 16 with glutamic acid or with another negatively charged amino acid having a side chain with a length of 4 atoms, or alternatively with any one of glutamine, homoglutamic acid, or homocysteic acid, or a charged amino acid having a side chain containing at least one heteroatom, (e.g. N, O, S, P) and with a side chain length of about 4 (or 3-5) atoms, and (b) substituting the naturally occurring glutamine at position 20 with another hydrophilic amino acid having a side chain that is either charged or has an ability to hydrogen-bond, and is at least about 5 (or about 4-6) atoms in length, for example, lysine, citrulline, arginine, or ornithine. The side chains of such amino acids at positions 16 and 20 can form a salt bridge or can be covalently linked. In some embodiments the two amino acids are bound to one another to form a lactam ring.

In some embodiments, stabilization of the alpha helix structure in the C-terminal portion of the glucagon peptide is achieved through the formation of an intramolecular bridge other than a lactam bridge. For example, suitable covalent bonding methods include any one or more of olefin metathesis, lanthionine-based cyclization, disulfide bridge or modified sulfur-containing bridge formation, the use of α , ω -diaminoalkane tethers, the formation of metal-atom bridges, and other means of peptide cyclization are used to stabilize the alpha helix.

In yet other embodiments, one or more α , α -disubstituted amino acids are inserted or substituted into this C-terminal portion (amino acids 12-29) at positions that retain the desired activity. For example, one, two, three or all of positions 16, 20, 21 or 24 are substituted with an α , α -disubstituted amino acid, e.g., Aib.

Increased activity at the GLP-1 receptor is provided by an amino acid modification at position 20 as described herein.

Increased activity at the GLP-1 receptor is provided by adding GPSSGAPPPS (SEQ ID NO: 1095) or XGPSSGAPPPS (SEQ ID NO: 1096) to the C-terminus, wherein X is any amino acid. GLP-1 activity in such analogs can be further increased by modifying the amino acid at position 18, 28 or 29, or at position 18 and 29, as described herein.

A further modest increase in GLP-1 potency is provided by modifying the amino acid at position 10 to be a large, aromatic amino acid residue, optionally Trp.

-56-

Reduced activity at the GLP-1 receptor is provided, e.g., by an amino acid modification at position 7 as described herein.

Potency at the GLP-1 receptor can be further enhanced by an alanine substitution for the native arginine at position 18.

5 Any of the modifications described above in reference to a Class 2 glucagon related peptide which increase GLP-1 receptor activity can be applied individually or in combination. Combinations of the modifications that increase GLP-1 receptor activity generally provide higher GLP-1 activity than any of such modifications taken alone. For example, the invention provides glucagon peptides that comprise
10 modifications at position 16, at position 20, and at the C-terminal carboxylic acid group, optionally with a covalent bond between the amino acids at positions 16 and 20; glucagon peptides that comprise modifications at position 16 and at the C-terminal carboxylic acid group; glucagon peptides that comprise modifications at positions 16 and 20, optionally with a covalent bond between the amino acids at positions 16 and
15 20; and glucagon peptides that comprise modifications at position 20 and at the C-terminal carboxylic acid group.

Modifications that improve DPP-IV resistance

Modifications at position 1 and/or 2 can increase the peptide's resistance to dipeptidyl peptidase IV (DPP IV) cleavage. For example, position 1 and/or position 2
20 may be substituted with a DPP-IV resistant amino acid as described herein. In some embodiments, the amino acid at position 2 is substituted with N-methyl alanine.

It was observed that modifications at position 2 (e.g. Aib at position 2) and in some cases modifications at position 1 (e.g., DMIA at position 1) may reduce glucagon activity, sometimes significantly; surprisingly, this reduction in glucagon
25 activity can be restored by stabilizing the alpha-helix structure in the C-terminal portion of glucagon (around amino acids 12-29), e.g., through formation of a covalent bond between the side chains of two amino acids, as described herein. In some embodiments, the covalent bond is between amino acids at positions "i" and "i+4", or positions "j" and "j+3", e.g., between positions 12 and 16, 16 and 20, 20 and 24, 24
30 and 28, or 17 and 20. In exemplary embodiments, this covalent bond is a lactam bridge between a glutamic acid at position 16 and a lysine at position 20. In some embodiments, this covalent bond is an intramolecular bridge other than a lactam bridge, as described herein.

Modifications that reduce degradation

In yet further exemplary embodiments, any of the Class 2 glucagon related peptides can be further modified to improve stability by modifying the amino acid at position 15 and/or 16 of SEQ ID NO: 1001 to reduce degradation of the peptide over time, especially in acidic or alkaline buffers. Such modifications reduce cleavage of the Asp15-Ser16 peptide bond. In exemplary embodiments, the amino acid modification at position 15 is a deletion or substitution of Asp with glutamic acid, homoglutamic acid, cysteic acid or homocysteic acid. In other exemplary embodiments, the amino acid modification at position 16 is a deletion or substitution of Ser with Thr or Aib. In other exemplary embodiments, Ser at position 16 is substituted with glutamic acid or with another negatively charged amino acid having a side chain with a length of 4 atoms, or alternatively with any one of glutamine, homoglutamic acid, or homocysteic acid.

In some embodiments, the methionine residue present at position 27 of the native peptide is modified, e.g. by deletion or substitution. Such modifications may prevent oxidative degradation of the peptide. In some embodiments, the Met at position 27 is substituted with leucine, isoleucine or norleucine. In some specific embodiments, Met at position 27 is substituted with leucine or norleucine.

In some embodiments, the Gln at position 20 and/or 24 is modified, e.g. by deletion or substitution. Such modifications can reduce degradation that occurs through deamidation of Gln. In some embodiments, the Gln at position 20 and/or 24 is substituted with Ser, Thr, Ala or Aib. In some embodiments the Gln at position 20 and/or 24 is substituted with Lys, Arg, Orn, or Citrulline.

In some embodiments, the Asp at position 21 is modified, e.g. by deletion or substitution. Such modifications can reduce degradation that occurs through dehydration of Asp to form a cyclic succinimide intermediate followed by isomerization to iso-aspartate. In some embodiments, position 21 is substituted with Glu, homoglutamic acid or homocysteic acid. In some specific embodiments, position 21 is substituted with Glu.

30 Stabilization of the Alpha Helix Structure

Stabilization of the alpha-helix structure in the C-terminal portion of the Class 2 glucagon related peptide (around amino acids 12-29) provides enhanced GLP-1 and/or GIP activity and restores glucagon activity which has been reduced by amino

-58-

acid modifications at positions 1 and/or 2. The alpha helix structure can be stabilized by, e.g., formation of a covalent or non-covalent intramolecular bridge, or substitution and/or insertion of amino acids around positions 12-29 with an alpha helix-stabilizing amino acid (e.g., an α,α -disubstituted amino acid). Stabilization of the alpha-helix structure of a GIP agonist may be accomplished as described herein.

Acylation and alkylation

In accordance with some embodiments, the glucagon peptides disclosed herein are modified to comprise an acyl group or alkyl group, e.g., an acyl or alkyl group which is non-native to a naturally-occurring amino acid as described herein.

10 Acylation or alkylation can increase the half-life of the glucagon peptides in circulation. Acylation or alkylation can advantageously delay the onset of action and/or extend the duration of action at the glucagon and/or GLP-1 receptors and/or improve resistance to proteases such as DPP-IV and/or improve solubility. Activity at the glucagon and/or GLP-1 and/or GIP receptors of the glucagon peptide may be
15 maintained after acylation. In some embodiments, the potency of the acylated glucagon peptides is comparable to the unacylated versions of the glucagon peptides. Class 2 glucagon related peptides may be acylated or alkylated at the same amino acid position where a hydrophilic moiety is linked, or at a different amino acid position, as described herein.

20 In some embodiments, the invention provides a glucagon peptide modified to comprise an acyl group or alkyl group covalently linked to the amino acid at position 10 of the glucagon peptide. The glucagon peptide may further comprise a spacer between the amino acid at position 10 of the glucagon peptide and the acyl group or alkyl group. In some embodiments, the acyl group is a fatty acid or bile acid, or salt thereof, e.g. a C4 to C30 fatty acid, a C8 to C24 fatty acid, cholic acid, a C4 to C30
25 alkyl, a C8 to C24 alkyl, or an alkyl comprising a steroid moiety of a bile acid. The spacer is any moiety with suitable reactive groups for attaching acyl or alkyl groups. In exemplary embodiments, the spacer comprises an amino acid, a dipeptide, a tripeptide, a hydrophilic bifunctional, or a hydrophobic bifunctional spacer. In some
30 embodiments, the spacer is selected from the group consisting of: Trp, Glu, Asp, Cys and a spacer comprising $\text{NH}_2(\text{CH}_2\text{CH}_2\text{O})_n(\text{CH}_2)_m\text{COOH}$, wherein m is any integer from 1 to 6 and n is any integer from 2 to 12. Such acylated or alkylated glucagon peptides may also further comprise a hydrophilic moiety, optionally a polyethylene

glycol. Any of the foregoing glucagon peptides may comprise two acyl groups or two alkyl groups, or a combination thereof.

Conjugates and fusions

The GIP agonist can be linked, optionally via covalent bonding and optionally
5 via a linker, to a conjugate moiety as described herein.

In other embodiments, the second peptide is XGPSSGAPPPS (SEQ ID NO: 1096), wherein X is selected from one of the 20 common amino acids, e.g., glutamic acid, aspartic acid or glycine. In some embodiments X represents an amino acid, for example Cys, that further comprises a hydrophilic moiety covalently linked to the side
10 chain of that amino acid. Such C-terminal extensions improve solubility and also can improve GIP or GLP-1 activity. In some embodiments wherein the glucagon peptide further comprises a carboxy terminal extension, the carboxy terminal amino acid of the extension ends in an amide group or an ester group rather than a carboxylic acid.

In some embodiments, e.g., in glucagon peptides which comprise the C-
15 terminal extension, the threonine at position 29 of the native glucagon peptide is replaced with a glycine. For example, a glucagon peptide having a glycine substitution for threonine at position 29 and comprising the C-terminal extension of GPSSGAPPPS (SEQ ID NO: 1095) is four times as potent at the GLP-1 receptor as native glucagon modified to comprise the same C-terminal extension. This T29G
20 substitution can be used in conjunction with other modifications disclosed herein to enhance the affinity of the glucagon peptides for the GLP-1 receptor. For example, the T29G substitution can be combined with the S16E and N20K amino acid substitutions, optionally with a lactam bridge between amino acids 16 and 20, and optionally with addition of a PEG chain as described herein.

25 In some embodiments an amino acid is added to the C-terminus, and the additional amino acid is selected from the group consisting of glutamic acid, aspartic acid and glycine.

Modifications that enhance solubility

In another embodiment, the solubility of any of the glucagon peptides can be
30 improved by amino acid substitutions and/or additions that introduce a charged amino acid into the C-terminal portion of the peptide, preferably at a position C-terminal to position 27 of SEQ ID NO: 1001. Optionally, one, two or three charged amino acids may be introduced within the C-terminal portion, preferably C-terminal to position

-60-

27. In some embodiments the native amino acid(s) at positions 28 and/or 29 are substituted with one or two charged amino acids, and/or in a further embodiment one to three charged amino acids are also added to the C-terminus of the peptide. In exemplary embodiments, one, two or all of the charged amino acids are negatively charged. In some embodiments, the negatively charged (acidic amino acid) is aspartic acid or glutamic acid.

Additional modifications, e.g. conservative substitutions, may be made to the glucagon peptide that still allow it to retain GIP activity (and optionally GLP-1 activity and/or glucagon activity).

10 *Other modifications*

Any of the modifications described above in reference to a Class 2 peptide which increase or decrease GIP activity, which increase or decrease glucagon receptor activity, and which increase GLP-1 receptor activity can be applied individually or in combination. Any of the modifications described above in reference to a Class 2 glucagon related peptide can also be combined with other modifications that confer other desirable properties, such as increased solubility and/or stability and/or duration of action, as described herein with regard to Class 2 glucagon related peptides.

Alternatively, any of the modifications described above in reference to Class 2 glucagon related peptides can be combined with other modifications described herein in reference to Class 2 glucagon related peptides that do not substantially affect solubility or stability or activity. Exemplary modifications include but are not limited to:

(A) Improving solubility, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of native glucagon, preferably at a position C-terminal to position 27. Such a charged amino acid can be introduced by substituting a native amino acid with a charged amino acid, e.g. at positions 28 or 29, or alternatively by adding a charged amino acid, e.g. after position 27, 28 or 29. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. In other embodiments, one, two, three or all of the charged amino acids are positively charged. Such modifications increase solubility, e.g. provide at least 2-fold, 5-fold, 10-fold, 15-fold, 25-fold, 30-fold or greater solubility relative to native glucagon at a given pH between about 5.5 and 8, e.g., pH 7, when measured after 24 hours at 25°C.

-61-

(B) Increasing solubility and duration of action or half-life in circulation by addition of a hydrophilic moiety such as a polyethylene glycol chain, as described herein, e.g. at position 16, 17, 20, 21, 24 or 29, within a C-terminal extension, or at the C-terminal amino acid of the peptide,

5 (C) Increasing solubility and/or duration of action or half-life in circulation and/or delaying the onset of action by acylation or alkylation of the glucagon peptide, as described herein;

(D) Increasing duration of action or half-life in circulation through introducing resistance to dipeptidyl peptidase IV (DPP IV) cleavage by modification of the amino
10 acid at position 1 or 2 as described herein.

(E) Increasing stability by modification of the Asp at position 15, for example, by deletion or substitution with glutamic acid, homoglutamic acid, cysteic acid or homocysteic acid. Such modifications can reduce degradation or cleavage at a pH within the range of 5.5 to 8, for example, retaining at least 75%, 80%, 90%, 95%,
15 96%, 97%, 98% or 99%, up to 100% of the original peptide after 24 hours at 25°C. Such modifications reduce cleavage of the peptide bond between Asp15-Ser16.

(F) Increasing stability by modification of the Ser at position 16, for example by substitution with Thr or Aib. Such modifications also reduce cleavage of the peptide bond between Asp15-Ser16.

20 (G) Increasing stability by modification of the methionine at position 27, for example, by substitution with leucine or norleucine. Such modifications can reduce oxidative degradation. Stability can also be increased by modification of the Gln at position 20 or 24, e.g. by substitution with Ser, Thr, Ala or Aib. Such modifications can reduce degradation that occurs through deamidation of Gln. Stability can be increased by
25 modification of Asp at position 21, e.g. by substitution with Glu. Such modifications can reduce degradation that occurs through dehydration of Asp to form a cyclic succinimide intermediate followed by isomerization to iso-aspartate.

(H) Non-conservative or conservative substitutions, additions or deletions that do not substantially affect activity, for example, conservative substitutions at one or more of
30 positions 2, 5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 27, 28 or 29; substitution of one or more of these positions with Ala; deletion of amino acids at one or more of positions 27, 28 or 29; or deletion of amino acid 29 optionally combined with a C-terminal amide or ester in place of the C-terminal carboxylic acid group;

-62-

substitution of Lys at position 12 with Arg; substitution of Tyr at position 10 with Val or Phe;

Preservation of activity after pegylation is provided by the addition of GPSSGAPPPS (SEQ ID NO: 1095) to the C-terminus.

5 Some positions of the native glucagon peptide can be modified while retaining at least some of the activities of the parent peptide. Accordingly, applicants anticipate that one or more of the amino acids located at positions at positions 2, 5, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 24, 27, 28 or 29 can be substituted with an amino acid different from that present in the native glucagon peptide, and still retain activity at
10 the glucagon receptor.

 In some embodiments, position 18 is substituted with an amino acid selected from the group consisting of Ala, Ser, or Thr. In some embodiments the amino acid at position 20 is substituted with Ser, Thr, Lys, Arg, Orn, Citrulline or Aib. In some
15 embodiments, position 21 is substituted with Glu, homoglutamic acid or homocysteic acid. In some embodiments, the glucagon peptide comprises 1 to 10 amino acid modifications selected from positions 16, 17, 18, 20, 21, 23, 24, 27, 28 and 29. In exemplary embodiments, the modifications are one or more amino acid substitutions selected from the group consisting of Gln17, Ala18, Glu21, Ile23, Ala24, Val27 and Gly29. In some embodiments, 1 to 2 amino acids selected from positions 17-26 differ
20 from the parent peptide. In other embodiments, 1 to 2 amino acids selected from positions 17-22 differ from the parent peptide. In yet other embodiments, the modifications are Gln17, Ala18, Glu21, Ile23 and Ala24.

 In some embodiments, one or more amino acids are added to the carboxy terminus of the glucagon peptide. The amino acid is typically selected from one of
25 the 20 common amino acids, and in some embodiments the amino acid has an amide group in place of the carboxylic acid of the native amino acid. In exemplary embodiments the added amino acid is selected from the group consisting of glutamic acid and aspartic acid and glycine.

Other modifications that do not destroy activity include W10 or R20.

30 In some embodiments, the Class 2 glucagon related peptides disclosed herein are modified by truncation of the C-terminus by one or two amino acid residues yet retain similar activity and potency at the glucagon, GLP-1 and/or GIP receptors. In this regard, the amino acid at position 29 and/or 28 can be deleted.

Exemplary embodiments

In accordance with some embodiments of the invention, the analog of glucagon (SEQ ID NO: 1001) having GIP agonist activity comprises SEQ ID NO: 1001 with (a) an amino acid modification at position 1 that confers GIP agonist activity, (b) a modification which stabilizes the alpha helix structure of the C-terminal portion (amino acids 12-29) of the analog, and (c) optionally, 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) further amino acid modifications. In some embodiments, the analog exhibits at least about 1% activity of native GIP at the GIP receptor or any other activity level at the GIP receptor described herein.

In certain embodiments, the modification which stabilizes the alpha helix structure is one which provides or introduces an intramolecular bridge, including, for example, a covalent intramolecular bridge, such as any of those described herein. The covalent intramolecular bridge in some embodiments is a lactam bridge. The lactam bridge of the analog of these embodiments can be a lactam bridge as described herein. See, e.g., the teachings of lactam bridges under the section "Stabilization of the Alpha Helix Structure." For example, the lactam bridge may be one which is between the side chains of amino acids at positions i and i+4 or between the side chains of amino acids at positions j and j+3, wherein i is 12, 13, 16, 17, 20 or 24, and wherein j is 17. In certain embodiments, the lactam bridge can be between the amino acids at positions 16 and 20, wherein one of the amino acids at positions 16 and 20 is substituted with Glu and the other of the amino acids at positions 16 and 20 is substituted with Lys.

In alternative embodiments, the modification which stabilizes the alpha helix structure is the introduction of one, two, three, or four α,α -disubstituted amino acids at position(s) 16, 20, 21, and 24 of the analog. In some embodiments, the α,α -disubstituted amino acid is Aib. In certain aspects, the α,α -disubstituted amino acid (e.g., Aib) is at position 20 and the amino acid at position 16 is substituted with a positive-charged amino acid, such as, for example, an amino acid of Formula IV, which is described herein. The amino acid of Formula IV may be homoLys, Lys, Orn, or 2,4-diaminobutyric acid (Dab).

In specific aspects of the invention, the amino acid modification at position 1 is a substitution of His with an amino acid lacking an imidazole side chain, e.g. a large, aromatic amino acid (e.g., Tyr).

-64-

In certain aspects, the analog of glucagon comprises amino acid modifications at one, two or all of positions 27, 28 and 29. For example, the Met at position 27 can be substituted with a large aliphatic amino acid, optionally Leu, the Asn at position 28 can be substituted with a small aliphatic amino acid, optionally Ala, the Thr at
5 position 29 can be substituted with a small aliphatic amino acid, optionally Gly, or a combination of two or three of the foregoing. In specific embodiments, the analog of glucagon comprises Leu at position 27, Ala at position 28, and Gly or Thr at position 29.

In certain embodiments of the invention, the analog of glucagon comprises an
10 extension of 1 to 21 amino acids C-terminal to the amino acid at position 29. The extension can comprise the amino acid sequence of SEQ ID NO: 1095 or 1096, for instance. Additionally or alternatively, the analog of glucagon can comprise an extension of which 1-6 amino acids of the extension are positive-charged amino acids. The positive-charged amino acids may be amino acids of Formula IV, including, but
15 not limited to Lys, homoLys, Orn, and Dab.

The analog of glucagon in some embodiments is acylated or alkylated as described herein. For instance, the acyl or alkyl group may be attached to the analog of glucagon, with or without a spacer, at position 10 or 40 of the analog, as further described herein. The analog may additionally or alternatively be modified to
20 comprise a hydrophilic moiety as further described herein. Furthermore, in some embodiments, the analog comprises any one or a combination of the following modifications:

- (a) Ser at position 2 substituted with D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;
- 25 (b) Tyr at position 10 substituted with Trp, Lys, Orn, Glu, Phe, or Val;
- (c) Linkage of an acyl group to a Lys at position 10;
- (d) Lys at position 12 substituted with Arg or Ile;
- (e) Ser at position 16 substituted with Glu, Gln, homoglutamic acid, homocysteic acid, Thr, Gly, or Aib;
- 30 (f) Arg at position 17 substituted with Gln;
- (g) Arg at position 18 substituted with Ala, Ser, Thr, or Gly;
- (h) Gln at position 20 substituted with Ser, Thr, Ala, Lys, Citrulline, Arg, Orn, or Aib;

-65-

- (i) Asp at position 21 substituted with Glu, homoglutamic acid, homocysteic acid;
- (j) Val at position 23 substituted with Ile;
- (k) Gln at position 24 substituted with Asn, Ser, Thr, Ala, or Aib;
- 5 (l) and a conservative substitution at any of positions 2 5, 9, 10, 11, 12, 13, 14, 15, 16, 8 19 20, 21, 24, 27, 28, and 29.

In exemplary embodiments, the analog of glucagon (SEQ ID NO: 1001) having GIP agonist activity comprises the following modifications:

- 10 (a) an amino acid modification at position 1 that confers GIP agonist activity,
 - (b) a lactam bridge between the side chains of amino acids at positions i and i+4 or between the side chains of amino acids at positions j and j+3, wherein i is 12, 13, 16, 17, 20 or 24, and wherein j is 17,
 - (c) amino acid modifications at one, two or all of positions 27, 28 and 29,
 - 15 e.g., amino acid modifications at position 27 and/or 28, and
 - (d) 1-9 or 1-6 further amino acid modifications, e.g. 1, 2, 3, 4, 5, 6, 7, 8 or 9 further amino acid modifications,
- and the EC₅₀ of the analog for GIP receptor activation is about 10 nM or less.

The lactam bridge of the analog of these embodiments can be a lactam bridge
20 as described herein. See, e.g., the teachings of lactam bridges under the section “Stabilization of the Alpha Helix Structure.” For example, the lactam bridge can be between the amino acids at positions 16 and 20, wherein one of the amino acids at positions 16 and 20 is substituted with Glu and the other of the amino acids at positions 16 and 20 is substituted with Lys.

25 In accordance with these embodiments, the analog can comprise, for example, the amino acid sequence of any of SEQ ID NOs: 1005-1094.

In other exemplary embodiments, the analog of glucagon (SEQ ID NO: 1001) having GIP agonist activity comprises the following modifications:

- 30 (a) an amino acid modification at position 1 that confers GIP agonist activity,
- (b) one, two, three, or all of the amino acids at positions 16, 20, 21, and 24 of the analog is substituted with an α,α -disubstituted amino acid,

-66-

(c) amino acid modifications at one, two or all of positions 27, 28 and 29, e.g., amino acid modifications at position 27 and/or 28, and

(d) 1-9 or 1-6 further amino acid modifications, e.g. 1, 2, 3, 4, 5, 6, 7, 8 or 9 further amino acid modifications,

5 and the EC₅₀ of the analog for GIP receptor activation is about 10 nM or less.

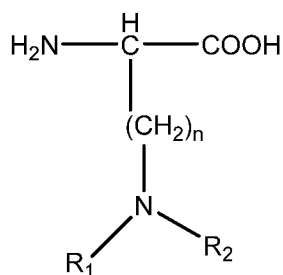
The α,α -disubstituted amino acid of the analog of these embodiments can be any α,α -disubstituted amino acid, including, but not limited to, amino iso-butyric acid (Aib), an amino acid disubstituted with the same or a different group selected from methyl, ethyl, propyl, and n-butyl, or with a cyclooctane or cycloheptane (e.g., 1-aminocyclooctane-1-carboxylic acid). In certain embodiments, the α,α -disubstituted amino acid is Aib. In certain embodiments, the amino acid at position 20 is substituted with an α,α -disubstituted amino acid, e.g., Aib.

In accordance with these embodiments, the analog can comprise, for example, the amino acid sequence of any of SEQ ID NOs: 1099-1141, 1144-1164, 1166-1169, and 1173-1178.

In yet other exemplary embodiments, the analog of glucagon (SEQ ID NO: 1001) having GIP agonist activity comprises the following modifications:

(a) an amino acid modification at position 1 that confers GIP agonist activity,

20 (b) an amino acid substitution of Ser at position 16 with an amino acid of Formula IV:



[Formula IV],

wherein n is 1 to 16, or 1 to 10, or 1 to 7, or 1 to 6, or 2 to 6, each of R₁ and R₂ is independently selected from the group consisting of H, C1-C18 alkyl, (C1-C18 alkyl)OH, (C1-C18 alkyl)NH₂, (C1-C18 alkyl)SH, (C0-C4 alkyl)(C3-C6)cycloalkyl, (C0-C4 alkyl)(C2-C5 heterocyclic), (C0-C4 alkyl)(C6-C10 aryl)R₇, and (C1-C4 alkyl)(C3-C9 heteroaryl), wherein R₇ is H or OH, and the side chain of the amino acid of Formula IV comprises a free amino group,

-67-

(c) an amino acid substitution of the Gln at position 20 with an alpha, alpha-disubstituted amino acid,

(d) amino acid modifications at one, two or all of positions 27, 28 and 29, e.g., amino acid modifications at position 27 and/or 28, and

5 (e) 1-9 or 1-6 further amino acid modifications, e.g. 1, 2, 3, 4, 5, 6, 7, 8 or 9 further amino acid modifications,

and the EC₅₀ of the analog for GIP receptor activation is about 10 nM or less.

The amino acid of Formula IV of the analog of these embodiments may be any amino acid, such as, for example, the amino acid of Formula IV, wherein n is 1, 2, 3,
10 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16. In certain embodiments, n is 2, 3, 4, or 5, in which case, the amino acid is Dab, Orn, Lys, or homoLys respectively.

The alpha, alpha-disubstituted amino acid of the analog of these embodiments may be any alpha, alpha-disubstituted amino acid, including, but not limited to, amino iso-butyric acid (Aib), an amino acid disubstituted with the same or a different group
15 selected from methyl, ethyl, propyl, and n-butyl, or with a cyclooctane or cycloheptane (e.g., 1-aminocyclooctane-1-carboxylic acid). In certain embodiments, the alpha, alpha-disubstituted amino acid is Aib.

In accordance with these embodiments, the analog can comprise, for example, the amino acid sequence of any of SEQ ID NOs: 1099-1165.

20 In yet other exemplary embodiments, the analog of glucagon (SEQ ID NO: 1001) having GIP agonist activity comprises:

(a) an amino acid modification at position 1 that confers GIP agonist activity, and

(b) an extension of about 1 to about 21 amino acids C-terminal to the amino acid at position 29, wherein at least one of the amino acids of the extension is acylated or
25 alkylated,

wherein the EC₅₀ of the analog for GIP receptor activation is about 10 nM or less.

In some embodiments, the acylated or alkylated amino acid is an amino acid of Formula I, II, or III. In more specific embodiments, the amino acid of Formula I is Dab, Orn, Lys, or homoLys. Also, in some embodiments, the extension of about 1 to
30 about 21 amino acids comprises the amino acid sequence of GPSSGAPPPS (SEQ ID NO: 1095) or XGPSSGAPPPS (SEQ ID NO: 1096), wherein X is any amino acid, or GPSSGAPPPK (SEQ ID NO: 1170) or XGPSSGAPPPK (SEQ ID NO: 1171) or XGPSSGAPPPSK (SEQ ID NO: 1172), wherein X is Gly or a small, aliphatic or non-

-68-

polar or slightly polar amino acid. In some embodiments, the about 1 to about 21 amino acids may comprise sequences containing one or more conservative substitutions relative to SEQ ID NO: 1095, 1096, 1170, 1171 or 1172. In some embodiments, the acylated or alkylated amino acid is located at position 37, 38, 39, 40, 41, 42, or 43 of the C-terminally-extended analog. In certain embodiments, the acylated or alkylated amino acid is located at position 40 of the C-terminally extended analog.

In some embodiments, the analog having GIP agonist activity further comprises amino acid modifications at one, two or all of positions 27, 28 and 29, e.g., amino acid modifications at position 27 and/or 28.

In any of the above exemplary embodiments, the amino acid modification at position 1 that confers GIP agonist activity can be a substitution of His with an amino acid lacking an imidazole side chain. The amino acid modification at position 1 can, for example, be a substitution of His with a large, aromatic amino acid. In some embodiments, the large, aromatic amino acid is any of those described herein, including, for example, Tyr.

In certain aspects, the analog does not comprise an amino acid modification at position 1 which modification confers GIP agonist activity. In some aspects, the amino acid at position 1 is not a large, aromatic amino acid, e.g., Tyr. In some embodiments, the amino acid at position 1 is an amino acid comprising an imidazole ring, e.g., His, analogs of His. In certain embodiments, the analog is not any of the compounds disclosed in International Patent Application Publication No. WO 2010/011439. In certain aspects, the analog comprises the amino acid sequence of any of SEQ ID NOs: 1284-1296.

Also, with regard to the above exemplary embodiments, amino acid modifications at one, two, or all of positions 27, 28, and 29 can be any of the modifications at these positions described herein. For example, the Met at position 27 can be substituted with a large aliphatic amino acid, optionally Leu, the Asn at position 28 can be substituted with a small aliphatic amino acid, optionally Ala, and/or the Thr at position 29 can be substituted with a small aliphatic amino acid, optionally Gly. Alternatively, the analog can comprise such amino acid modifications at position 27 and/or 28.

The analog of the above exemplary embodiments can further comprise 1-9 or 1-6 further, additional amino acid modifications, e.g. 1, 2, 3, 4, 5, 6, 7, 8 or 9 further amino acid modifications, such as, for example, any of the modifications described herein which increase or decrease the activity at any of the GIP, GLP-1, and glucagon receptors, improve solubility, improve duration of action or half-life in circulation, delay the onset of action, or increase stability. The analog can further comprise, for example, an amino acid modification at position 12, optionally, a substitution with Ile, and/or amino acid modifications at positions 17 and 18, optionally substitution with Q at position 17 and A at position 18, and/or an addition of GPSSGAPPPS (SEQ ID NO: 1095) or XGPSSGAPPPS (SEQ ID NO: 1096), or sequences containing one or more conservative substitutions relative to SEQ ID NO: 1095 or 1096, to the C-terminus, wherein X is any amino acid. The analog can comprise one or more of the following modifications:

- (i) Ser at position 2 substituted with D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;
- (ii) Tyr at position 10 substituted with Trp, Lys, Orn, Glu, Phe, or Val;
- (iii) Linkage of an acyl group to a Lys at position 10;
- (iv) Lys at position 12 substituted with Arg;
- (v) Ser at position 16 substituted with Glu, Gln, homoglutamic acid, homocysteic acid, Thr, Gly, or Aib;
- (vi) Arg at position 17 substituted with Gln;
- (vii) Arg at position 18 substituted with Ala, Ser, Thr, or Gly;
- (viii) Gln at position 20 substituted with Ala, Ser, Thr, Lys, Citrulline, Arg, Orn, or Aib;
- (ix) Asp at position 21 substituted with Glu, homoglutamic acid, homocysteic acid;
- (x) Val at position 23 substituted with Ile;
- (xi) Gln at position 24 substituted with Asn, Ala, Ser, Thr, or Aib; and
- (xii) a conservative substitution at any of positions 2, 5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 27, 28, and 29.

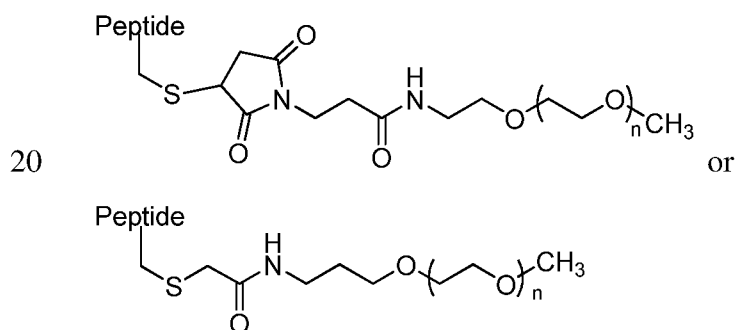
The analog in some embodiments comprise a combination of the modifications (i) through (xii). Alternatively or additionally, the analog can comprise an amino acid modification at position 3 (e.g., an amino acid substitution of Gln with

-70-

Glu), wherein the analog has less than 1% of the activity of glucagon at the glucagon receptor. Alternatively or additionally, the analog can comprise an amino acid modification at position 7 (e.g., an amino acid substitution of Thr with an amino acid lacking a hydroxyl group, e.g., Abu or Ile), wherein the analog has less than about 10% of the activity of GLP-1 at the GLP-1 receptor.

With regard to the exemplary embodiments, the analog can be covalently linked to a hydrophilic moiety. In some embodiments, the analog is covalently linked to the hydrophilic moiety at any of amino acid positions 16, 17, 20, 21, 24, 29, 40, or the C-terminus. In certain embodiments, the analog comprises a C-terminal extension (e.g., an amino acid sequence of SEQ ID NO: 1095) and an addition of an amino acid comprising the hydrophilic moiety, such that the hydrophilic moiety is covalently linked to the analog at position 40.

In some embodiments, the hydrophilic moiety is covalently linked to a Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine of the analog. The Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine may be an amino acid that is native to the glucagon sequence (SEQ ID NO: 1001) or it may be an amino acid which is replacing a native amino acid of SEQ ID NO: 1001. In some embodiments, wherein the hydrophilic moiety is attached to a Cys, the linkage to the hydrophilic moiety can comprise the structure



With regard to the analogs comprising a hydrophilic moiety, the hydrophilic moiety may be any of those described herein. See, e.g., the teachings under the section “Linkage of hydrophilic moieties.” In some embodiments, the hydrophilic moiety is a polyethylene glycol (PEG). The PEG in certain embodiments has a molecular weight of about 1,000 Daltons to about 40,000 Daltons, e.g., about 20,000 Daltons to about 40,000 Daltons.

-71-

With regard to the exemplary embodiments, the analog can comprise a modified amino acid in which the side chain is covalently linked to an acyl or alkyl group (e.g., an acyl or alkyl group which is non-native to a naturally-occurring amino acid). The acylated or alkylated analog can be in accordance with acylated or alkylated peptides described in the section "Acylation and alkylation." In some embodiments, the acyl group is a C4 to a C30 fatty acyl group, such as, for example, a C10 fatty acyl or alkyl group, a C12 fatty acyl or alkyl group, a C14 fatty acyl or alkyl group, a C16 fatty acyl or alkyl group, a C18 fatty acyl or alkyl group, a C20 acyl or alkyl group, or a C22 acyl or alkyl group. The acyl or alkyl group may be covalently attached to any amino acid of the analog, including, but not limited to the amino acid at position 10 or 40, or the C-terminal amino acid. In certain embodiments, the analog comprises a C-terminal extension (e.g., an amino acid sequence of SEQ ID NO: 1095) and an addition of an amino acid comprising the acyl or alkyl group, such that the acyl or alkyl group is covalently linked to the analog at position 40. In some embodiments, the acyl or alkyl group is covalently linked to the side chain of an amino acid of Formula I, II, or III, e.g., a Lys residue. The acyl or alkyl group may be covalently linked to an amino acid which is native to the glucagon sequence (SEQ ID NO: 1001) or may be linked to an amino acid which is added to the sequence of SEQ ID NO: 1001 or to the sequence of SEQ ID NO: 1001 followed by SEQ ID NO: 1095 (at the N- or C-terminus) or may be linked to an amino acid which replaces a native amino acid, e.g., the Tyr at position 10 of SEQ ID NO: 1001.

In the above exemplary embodiments, wherein the analog comprises an acyl or alkyl group, the analog may be attached to the acyl or alkyl group via a spacer, as described herein. The spacer, for example, may be 3 to 10 atoms in length and may be, for instance, an amino acid (e.g., 6-amino hexanoic acid, any amino acid described herein), a dipeptide (e.g., Ala-Ala, β Ala- β Ala, Leu-Leu, Pro-Pro, γ -Glu- γ -Glu), a tripeptide, or a hydrophilic or hydrophobic bifunctional spacer. In certain aspects, the total length of the spacer and the acyl or alkyl group is about 14 to about 28 atoms. In some embodiments, the amino acid spacer is not γ -Glu. In some embodiments, the dipeptide spacer is not γ -Glu- γ -Glu.

In still further exemplary embodiments, the analog of glucagon having GIP agonist activity comprises the amino acid sequence according to any one of SEQ ID NOs: 1227, 1228, 1229 or 1230 that further comprises the following modifications:

-72-

(a) optionally, an amino acid modification at position 1 that confers GIP agonist activity,

(b) an extension of about 1 to about 21 amino acids C-terminal to the amino acid at position 29, wherein at least one of the amino acids of the extension is
5 acylated or alkylated, and

(d) up to 6 further amino acid modifications,
wherein the EC₅₀ of the analog for GIP receptor activation is about 10 nM or less.

In some aspects, the acylated or alkylated amino acid is an amino acid of Formula I, II, or III. In more specific embodiments, the amino acid of Formula I is
10 Dab, Orn, Lys, or homoLys. Also, in some embodiments, the about 1 to about 21 amino acids comprises the amino acid sequence of GPSSGAPPPS (SEQ ID NO: 1095) or XGPSSGAPPPS (SEQ ID NO: 1096), wherein X is any amino acid, or GPSSGAPPPK (SEQ ID NO: 1170) or XGPSSGAPPPK (SEQ ID NO: 1171) or XGPSSGAPPPSK (SEQ ID NO: 1172), wherein X is Gly or a small, aliphatic or non-
15 polar or slightly polar amino acid. In some embodiments, the about 1 to about 21 amino acids may comprise sequences containing one or more conservative substitutions relative to SEQ ID NO: 1095, 1096, 1170, 1171 or 1172. In some embodiments, the acylated or alkylated amino acid is located at position 37, 38, 39, 40, 41, 42, or 43 of the C-terminally-extended analog. In certain embodiments, the
20 acylated or alkylated amino acid is located at position 40 of the C-terminally extended analog.

In any of the above exemplary embodiments, the amino acid at position 1 that confers GIP agonist activity can be an amino acid lacking an imidazole side chain. The amino acid at position 1 can, for example, be a large, aromatic amino acid. In
25 some embodiments, the large, aromatic amino acid is any of those described herein, including, for example, Tyr.

The analog of the above exemplary embodiments can further comprise 1-6 further amino acid modifications, such as, for example, any of the modifications described herein which increase or decrease the activity at any of the GIP, GLP-1, and
30 glucagon receptors, improve solubility, improve duration of action or half-life in circulation, delay the onset of action, or increase stability.

In certain aspects, glucagon analogs described in the above exemplary embodiment, comprise further amino acid modifications at one, two or all of positions

-73-

27, 28 and 29. Modifications at these positions can be any of the modifications described herein relative to these positions. For example, relative to SEQ ID NO: 1227, 1228, 1229 or 1230, position 27 can be substituted with a large aliphatic amino acid (e.g., Leu, Ile or norleucine) or Met, position 28 can be substituted with another small aliphatic amino acid (e.g., Gly or Ala) or Asn, and/or position 29 can be substituted with another small aliphatic amino acid (e.g., Ala or Gly) or Thr. Alternatively, the analog can comprise such amino acid modifications at position 27 and/or 28.

The analog can further comprise one or more of the following additional modifications:

- (i) the amino acid at position 2 is any one of D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;
- (ii) the amino acid at position 10 is Tyr, Trp, Lys, Orn, Glu, Phe, or Val;
- (iii) linkage of an acyl group to a Lys at position 10;
- (iv) the amino acid at position 12 is Ile, Lys or Arg;
- (v) the amino acid at position 16 is any one of Ser, Glu, Gln, homoglutamic acid, homocysteic acid, Thr, Gly, or Aib;
- (vi) the amino acid at position 17 is Gln or Arg;
- (vii) the amino acid at position 18 is any one of Ala, Arg, Ser, Thr, or Gly;
- (viii) the amino acid at position 20 is any one of Ala, Ser, Thr, Lys, Citrulline, Arg, Orn, or Aib or another alpha, alpha-disubstituted amino acid;
- (ix) the amino acid at position 21 is any one of Glu, Asp, homoglutamic acid, homocysteic acid;
- (x) the amino acid at position 23 is Val or Ile;
- (xi) the amino acid at position 24 is any one of Gln, Asn, Ala, Ser, Thr, or Aib; and
- (xii) one or more conservative substitutions at any of positions 2, 5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 27, 28, and 29.

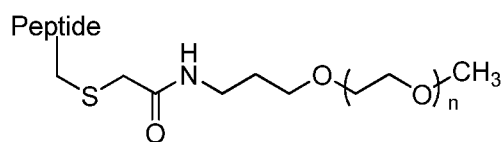
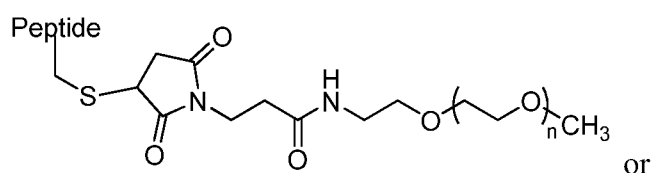
The analog in some embodiments comprise a combination of the modifications (i) through (xii). Alternatively or additionally, the analog can comprise an amino acid modification at position 3 (e.g., an amino acid substitution of Gln with Glu), wherein the analog has less than 1% of the activity of glucagon at the glucagon receptor. Alternatively or additionally, the analog can comprise an amino acid modification at position 7 (e.g., an amino acid substitution of Thr with an amino acid

-74-

lacking a hydroxyl group, e.g., Abu or Ile), wherein the analog has less than about 10% of the activity of GLP-1 at the GLP-1 receptor.

With regard to the exemplary embodiments, the analog can be covalently linked to a hydrophilic moiety. In some embodiments, the analog is covalently linked to the hydrophilic moiety at any of amino acid positions 16, 17, 20, 21, 24, 29, 40, or the C-terminus. In certain embodiments, the analog comprises a hydrophilic moiety covalently linked to the analog at position 24.

In some embodiments, the hydrophilic moiety is covalently linked to a Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine of the analog. The Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine may be an amino acid that is native to SEQ ID NO: 1001, 1227, 1228, 1229 or 1230 or it may be a substituted amino acid. In some embodiments, wherein the hydrophilic moiety is linked to a Cys, the linkage may comprise the structure



15

With regard to the analogs comprising a hydrophilic moiety, the hydrophilic moiety may be any of those described herein. See, e.g., the teachings under the section “Linkage of hydrophilic moieties.” In some embodiments, the hydrophilic moiety is a polyethylene glycol (PEG). The PEG in certain embodiments has a molecular weight of about 1,000 Daltons to about 40,000 Daltons, e.g., about 20,000 Daltons to about 40,000 Daltons.

With regard to the exemplary embodiments, the analog can comprise a modified amino acid within the C-terminal extension in which the side chain is covalently linked to an acyl or alkyl group. The acylated or alkylated analog can be in accordance with acylated or alkylated peptides described in the section “Acylation and alkylation.” In some embodiments, the acyl group is a C4 to a C30 fatty acyl group, such as, for example, a C10 fatty acyl or alkyl group, a C12 fatty acyl or alkyl group, a C14 fatty acyl or alkyl group, a C16 fatty acyl or alkyl group, a C18 fatty

25

-75-

acyl or alkyl group, a C20 acyl or alkyl group, or a C22 acyl or alkyl group. The acyl or alkyl group may be covalently attached to any amino acid of the analog, including, but not limited to the amino acid at position 10 or 40, or the C-terminal amino acid.

In some embodiments, the acyl or alkyl group is covalently linked to the side chain of an amino acid of Formula I, II, or III, e.g., a Lys residue. The acyl or alkyl group is covalently linked to an amino acid which is native to SEQ ID NO: 1001, 1227, 1228, 1229 or 1230 or it may be linked to a substituted amino acid. The acyl or alkyl group is covalently linked to an amino acid which is native to SEQ ID NO: 1095, 1096, 1171 or 1172, or it may be linked to a substituted amino acid.

In the above exemplary embodiments, wherein the analog comprises an acyl or alkyl group, the analog may be attached to the acyl or alkyl group via a spacer, as described herein. The spacer, for example, may be 3 to 10 atoms in length and may be, for instance, an amino acid (e.g., 6-amino hexanoic acid, any amino acid described herein), a dipeptide (e.g., Ala-Ala, β Ala- β Ala, Leu-Leu, Pro-Pro, γ -Glu- γ -Glu), a tripeptide, or a hydrophilic or hydrophobic bifunctional spacer. In certain aspects, the total length of the spacer and the acyl or alkyl group is about 14 to about 28 atoms. In some embodiments, the amino acid spacer is not γ -Glu. In some embodiments, the dipeptide spacer is not γ -Glu- γ -Glu.

In some very specific embodiments, an analog of the invention comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1099-1141, 1144-1164, 1166, 1192-1207, 1209-1221 and 1223 or selected from the group consisting of SEQ ID NOs: 1167-1169, 1173-1178 and 1225.

In still further exemplary embodiments, the analog of glucagon having GIP agonist activity comprises an acyl or alkyl group (e.g., an acyl or alkyl group which is non-native to a naturally occurring amino acid), wherein the acyl or alkyl group is attached to a spacer, wherein (i) the spacer is attached to the side chain of the amino acid at position 10 of the analog; or (ii) the analog comprises an extension of 1 to 21 amino acids C-terminal to the amino acid at position 29 and the spacer is attached to the side chain of an amino acid corresponding to one of positions 37-43 relative to SEQ ID NO: 1001, wherein the EC₅₀ of the analog for GIP receptor activation is about 10 nM or less.

In such embodiments, the analog may comprise an amino acid sequence of SEQ ID NO: 1001 with (i) an amino acid modification at position 1 that confers GIP

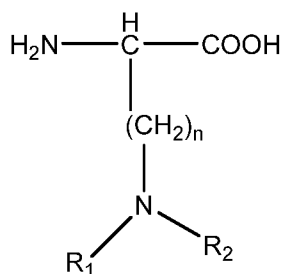
-76-

agonist activity, (ii) amino acid modifications at one, two, or all of positions 27, 28, and 29, (iii) at least one of:

(A) the analog comprises a lactam bridge between the side chains of amino acids at positions i and i+4 or between the side chains of amino acids at positions j and j+3, wherein i is 12, 13, 16, 17, 20 or 24, and wherein j is 17;

(B) one, two, three, or all of the amino acids at positions 16, 20, 21, and 24 of the analog is substituted with an α,α -disubstituted amino acid; or

(C) the analog comprises (i) an amino acid substitution of Ser at position 16 with an amino acid of Formula IV:



[Formula IV],

wherein n is 1 to 7, wherein each of R1 and R2 is independently selected from the group consisting of H, C₁-C₁₈ alkyl, (C₁-C₁₈ alkyl)OH, (C₁-C₁₈ alkyl)NH₂, (C₁-C₁₈ alkyl)SH, (C₀-C₄ alkyl)(C₃-C₆)cycloalkyl, (C₀-C₄ alkyl)(C₂-C₅ heterocyclic), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and (C₁-C₄ alkyl)(C₃-C₉ heteroaryl), wherein R₇ is H or OH, and the side chain of the amino acid of Formula IV comprises a free amino group; and (ii) an amino acid substitution of the Gln at position 20 with an alpha, alpha-disubstituted amino acid.

and (iv) up to 6 further amino acid modifications.

The alpha, alpha-disubstituted amino acid of the analog of these embodiments may be any alpha, alpha-disubstituted amino acid, including, but not limited to, amino iso-butyric acid (Aib), an amino acid disubstituted with the same or a different group selected from methyl, ethyl, propyl, and n-butyl, or with a cyclooctane or cycloheptane (e.g., 1-aminocyclooctane-1-carboxylic acid). In certain embodiments, the alpha, alpha-disubstituted amino acid is Aib.

The amino acid of Formula IV of the analog of these embodiments may be any amino acid, such as, for example, the amino acid of Formula IV, wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16. In certain embodiments, n is 2, 3, 4, or 5, in which case, the amino acid is Dab, Orn, Lys, or homoLys respectively.

-77-

In any of the above exemplary embodiments, the amino acid modification at position 1 that confers GIP agonist activity can be a substitution of His with an amino acid lacking an imidazole side chain. The amino acid modification at position 1 can, for example, be a substitution of His with a large, aromatic amino acid. In some
5 embodiments, the large, aromatic amino acid is any of those described herein, including, for example, Tyr.

Also, with regard to the above exemplary embodiments, amino acid modifications at one, two, or all of positions 27, 28, and 29 can be any of the modifications at these positions described herein. For example, the Met at position 27
10 can be substituted with a large aliphatic amino acid, optionally Leu, the Asn at position 28 can be substituted with a small aliphatic amino acid, optionally Ala, and/or the Thr at position 29 can be substituted with a small aliphatic amino acid, optionally Gly. Alternatively, the analog can comprise such amino acid modifications at position 27 and/or 28.

15 The analog of the above exemplary embodiments can further comprise 1-9 or 1-6 further, additional amino acid modifications, e.g. 1, 2, 3, 4, 5, 6, 7, 8 or 9 further amino acid modifications, such as, for example, any of the modifications described herein which increase or decrease the activity at any of the GIP, GLP-1, and glucagon receptors, improve solubility, improve duration of action or half-life in circulation,
20 delay the onset of action, or increase stability. The analog can further comprise, for example, an amino acid modification at position 12, optionally, a substitution with Ile, and/or amino acid modifications at positions 17 and 18, optionally substitution with Q at position 17 and A at position 18, and/or an addition of GPSSGAPPPS (SEQ ID NO: 1095) or XGPSSGAPPPS (SEQ ID NO: 1096), or sequences containing one or
25 more conservative substitutions relative to SEQ ID NO: 1095 or 1096, to the C-terminus, wherein X is any amino acid. The analog can comprise one or more of the following modifications:

- (i) Ser at position 2 substituted with D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;
- 30 (ii) Tyr at position 10 substituted with Trp, Lys, Orn, Glu, Phe, or Val;
- (iii) Linkage of an acyl group to a Lys at position 10;
- (iv) Lys at position 12 substituted with Arg;

-78-

- (v) Ser at position 16 substituted with Glu, Gln, homoglutamic acid, homocysteic acid, Thr, Gly, Lys, or Aib;
- (vi) Arg at position 17 substituted with Gln;
- (vii) Arg at position 18 substituted with Ala, Ser, Thr, or Gly;
- 5 (viii) Gln at position 20 substituted with Ala, Ser, Thr, Lys, Citrulline, Arg, Orn, or Aib;
- (ix) Asp at position 21 substituted with Glu, homoglutamic acid, homocysteic acid;
- (x) Val at position 23 substituted with Ile;
- (xi) Gln at position 24 substituted with Asn, Ala, Ser, Thr, or Aib; and
- 10 (xii) a conservative substitution at any of positions 2, 5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 27, 28, and 29.

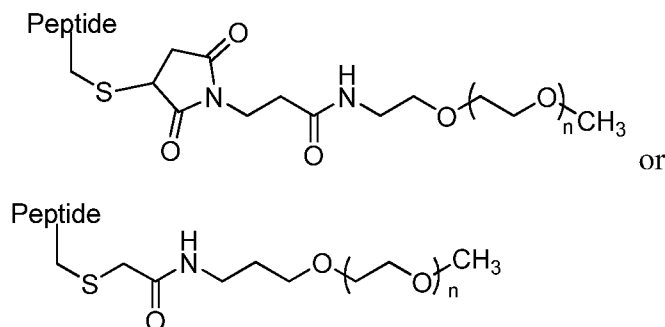
The analog in some embodiments comprise a combination of the modifications (i) through (xii). Alternatively or additionally, the analog can comprise an amino acid modification at position 3 (e.g., an amino acid substitution of Gln with
 15 Glu), wherein the analog has less than 1% of the activity of glucagon at the glucagon receptor. Alternatively or additionally, the analog can comprise an amino acid modification at position 7 (e.g., an amino acid substitution of Thr with an amino acid lacking a hydroxyl group, e.g., Abu or Ile), a deletion of the amino acid(s) C-terminal to the amino acid at position 27 or 28, yielding a 27- or 28-amino acid peptide, or a
 20 combination thereof, wherein the analog has less than about 10% of the activity of GLP-1 at the GLP-1 receptor.

With regard to the exemplary embodiments, the analog can be covalently linked to a hydrophilic moiety. In some embodiments, the analog is covalently linked to the hydrophilic moiety at any of amino acid positions 16, 17, 20, 21, 24, 29, 40, or
 25 the C-terminus. In certain embodiments, the analog comprises a C-terminal extension (e.g., an amino acid sequence of SEQ ID NO: 1095) and an addition of an amino acid comprising the hydrophilic moiety, such that the hydrophilic moiety is covalently linked to the analog at position 40.

In some embodiments, the hydrophilic moiety is covalently linked to a Lys,
 30 Cys, Orn, homocysteine, or acetyl-phenylalanine of the analog. The Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine may be an amino acid that is native to the glucagon sequence (SEQ ID NO: 1001) or it may be an amino acid which is replacing a native amino acid of SEQ ID NO: 1001. In some embodiments, wherein the

-79-

hydrophilic moiety is attached to a Cys, the linkage to the hydrophilic moiety can comprise the structure



5 With regard to the analogs comprising a hydrophilic moiety, the hydrophilic moiety may be any of those described herein. See, e.g., the teachings under the section “Linkage of hydrophilic moieties.” In some embodiments, the hydrophilic moiety is a polyethylene glycol (PEG). The PEG in certain embodiments has a molecular weight of about 1,000 Daltons to about 40,000 Daltons, e.g., about 20,000

10 Daltons to about 40,000 Daltons.

In the exemplary embodiments, wherein the analog comprises an acyl or alkyl group, which is attached to the analog via a spacer, the spacer can be any spacer as described herein. The spacer, for example, may be 3 to 10 atoms in length and may be, for instance, an amino acid (e.g., 6-amino hexanoic acid, any amino acid described

15 herein), a dipeptide (e.g., Ala-Ala, β Ala- β Ala, Leu-Leu, Pro-Pro, γ -Glu- γ -Glu), a tripeptide, or a hydrophilic or hydrophobic bifunctional spacer. In certain aspects, the total length of the spacer and the acyl or alkyl group is about 14 to about 28 atoms. In some embodiments, the amino acid spacer is not γ -Glu. In some embodiments, the dipeptide spacer is not γ -Glu- γ -Glu.

20 The acyl or alkyl group is any acyl or alkyl group as described herein, such as an acyl or alkyl group which is non-native to a naturally occurring amino acid. The acyl or alkyl group in some embodiments is a C4 to C30 fatty acyl group, such as, for example, a C10 fatty acyl or alkyl group, a C12 fatty acyl or alkyl group, a C14 fatty acyl or alkyl group, a C16 fatty acyl or alkyl group, a C18 fatty acyl or alkyl group, a

25 C20 acyl or alkyl group, or a C22 acyl or alkyl group, or a C4 to C30 alkyl group. In specific embodiments, the acyl group is a C12 to C18 fatty acyl group (e.g., a C14 or C16 fatty acyl group).

-80-

In some embodiments, the extension of about 1 to about 21 amino acids C-terminal to the amino acid at position 29 of the analog comprises the amino acid sequence of GPSSGAPPPS (SEQ ID NO: 1095) or XGPSSGAPPPS (SEQ ID NO: 1096), wherein X is any amino acid, or GPSSGAPPPK (SEQ ID NO: 1170) or XGPSSGAPPPK (SEQ ID NO: 1171) or XGPSSGAPPPSK (SEQ ID NO: 1172), wherein X is Gly or a small, aliphatic or non-polar or slightly polar amino acid. In some embodiments, the about 1 to about 21 amino acids may comprise sequences containing one or more conservative substitutions relative to SEQ ID NO: 1095, 1096, 1170, 1171 or 1172. In some embodiments, the acylated or alkylated amino acid is located at position 37, 38, 39, 40, 41, 42, or 43 of the C-terminally-extended analog. In certain embodiments, the acylated or alkylated amino acid is located at position 40 of the C-terminally extended analog.

The GIP agonist may be a peptide comprising the amino acid sequence of any of the amino acid sequences, e.g., SEQ ID NOs: 1005-1094, optionally with up to 1, 2, 3, 4, or 5 further modifications that retain GIP agonist activity. In certain embodiments, the GIP agonist comprises the amino acids of any of SEQ ID NOs: 1099-1275.

Class 3 Glucagon Related Peptides

In certain embodiments, the glucagon related peptide is a Class 3 glucagon related peptide, which is described herein and in International Patent Application Publication Nos. WO 2009/155258, WO 2008/101017, and U.S. Provisional Application No. 61/288,248 (filed on December 18, 2009) the contents of which are incorporated by reference in their entirety.

Some of the biological sequences referenced in the following section (SEQ ID NOs: 1-656) relating to Class 3 glucagon related peptides correspond to SEQ ID NOs: 1-656 in International Patent Application Publication No. WO 2009/155258.

Activity

The Class 3 glucagon related peptide can be a peptide that exhibits increased activity at the glucagon receptor, and in further embodiments exhibits enhanced biophysical stability and/or aqueous solubility. In addition, in some embodiments, the Class 3 glucagon related peptide has lost native glucagon's selectivity for the glucagon receptor versus the GLP-1 receptor, and thus represents co-agonists of those two

-81-

receptors. Selected amino acid modifications within the Class 3 glucagon related peptide can control the relative activity of the peptide at the GLP-1 receptor versus the glucagon receptor. Thus, the Class 3 glucagon related peptide can be a glucagon/GLP-1 co-agonist that has higher activity at the glucagon receptor versus the GLP-1 receptor, a glucagon/GLP-1 co-agonist that has approximately equivalent activity at both receptors, or a glucagon/GLP-1 co-agonist that has higher activity at the GLP-1 receptor versus the glucagon receptor. The latter category of co-agonist can be engineered to exhibit little or no activity at the glucagon receptor, and yet retain ability to activate the GLP-1 receptor with the same or better potency than native GLP-1. Any of these co-agonists may also include modifications that confer enhanced biophysical stability and/or aqueous solubility.

Modifications of the Class 3 glucagon related peptide can be made to produce a glucagon peptide having anywhere from at least about 1% (including at least about 1.5%, 2%, 5%, 7%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175%) to about 200% or higher activity at the GLP-1 receptor relative to native GLP-1 and anywhere from at least about 1% (including about 1.5%, 2%, 5%, 7%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175%, 200%, 250%, 300%, 350%, 400%, 450%) to about 500% or higher activity at the glucagon receptor relative to native glucagon. The amino acid sequence of native glucagon is SEQ ID NO: 1, the amino acid sequence of GLP-1(7-36)amide is SEQ ID NO: 52, and the amino acid sequence of GLP-1(7-37)acid is SEQ ID NO: 50. In exemplary embodiments, a Class 3 glucagon related peptide may exhibit at least 10% of the activity of native glucagon at the glucagon receptor and at least 50% of the activity of native GLP-1 at the GLP-1 receptor, or at least 40% of the activity of native glucagon at the glucagon receptor and at least 40% of the activity of native GLP-1 at the GLP-1 receptor, or at least 60% of the activity of native glucagon at the glucagon receptor and at least 60% of the activity of native GLP-1 at the GLP-1 receptor.

Selectivity of a Class 3 glucagon related peptide for the glucagon receptor versus the GLP-1 receptor can be described as the relative ratio of glucagon/GLP-1 activity (the peptide's activity at the glucagon receptor relative to native glucagon, divided by the peptide's activity at the GLP-1 receptor relative to native GLP-1). For example, a Class 3 glucagon related peptide that exhibits 60% of the activity of native glucagon at the glucagon receptor and 60% of the activity of native GLP-1 at the

-82-

GLP-1 receptor has a 1:1 ratio of glucagon/GLP-1 activity. Exemplary ratios of glucagon/GLP-1 activity include about 1:1, 1.5:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or 10:1, or about 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, or 1:1.5. As an example, a glucagon/GLP-1 activity ratio of 10:1 indicates a 10-fold selectivity for the glucagon receptor versus the GLP-1 receptor. Similarly, a GLP-1/glucagon activity ratio of 10:1 indicates a 10-fold selectivity for the GLP-1 receptor versus the glucagon receptor.

In some embodiments, the Class 3 glucagon related peptides have about 10% or less of the activity of native glucagon at the glucagon receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1% but less than about 10%, while exhibiting at least 20% of the activity of GLP-1 at the GLP-1 receptor. For example, exemplary Class 3 glucagon related peptides described herein have about 0.5%, about 1% or about 7% of the activity of native glucagon, while exhibiting at least 20% of the activity of GLP-1 at the GLP-1 receptor.

The Class 3 glucagon related peptide can be a glucagon peptide with increased or decreased activity at the glucagon receptor, or GLP-1 receptor, or both. The Class 3 glucagon related peptide can be a glucagon peptide with altered selectivity for the glucagon receptor versus the GLP-1 receptor.

Thus, as disclosed herein high potency Class 3 glucagon related peptides are provided that also exhibit improved solubility and/or stability. An exemplary high potency Class 3 glucagon related peptide exhibits at least about 200% of the activity of native glucagon at the glucagon receptor, and optionally is soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8, or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide (e.g. 5% or less of the original peptide is degraded or cleaved) after 24 hours at 25°C. As another example, an exemplary Class 3 glucagon related peptide exhibits greater than about 40% or greater than about 60% activity at both the glucagon and the GLP-1 receptors (at a ratio between about 1:3 and 3:1, or between about 1:2 and 2:1), is optionally soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide after 24 hours at 25°C. Another exemplary Class 3 glucagon related peptide exhibits about 175% or more of the activity of native glucagon at the glucagon receptor and about 20% or less of the activity of native GLP-1 at the GLP-1

-83-

receptor, is optionally soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide after 24 hours at 25°C. Yet another exemplary Class 3 glucagon related peptide exhibits about 10% or less of the activity of native glucagon at the glucagon receptor and at least about 20% of the activity of native GLP-1 at the GLP-1 receptor, is optionally soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide after 24 hours at 25°C. Yet another exemplary Class 3 glucagon related peptide exhibits about 10% or less but above 0.1% , 0.5% or 1% of the activity of native glucagon at the glucagon receptor and at least about 50%, 60%, 70%, 80%, 90% or 100% or more of the activity of native GLP-1 at the GLP-1 receptor, is optionally soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide after 24 hours at 25°C. In some embodiments, such Class 3 glucagon related peptides retain at least 22, 23, 24, 25, 26, 27 or 28 of the naturally occurring amino acids at the corresponding positions in native glucagon (e.g. have 1-7, 1-5 or 1-3 modifications relative to naturally occurring glucagon).

Modifications affecting glucagon activity

Increased activity at the glucagon receptor is provided by an amino acid modification at position 16 of native glucagon (SEQ ID NO: 1). In some embodiments, the Class 3 glucagon related peptide is a glucagon agonist that has been modified relative to the wild type peptide of His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser- Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu- Met-Asn-Thr (SEQ ID NO: 1) to enhance the peptide's potency at the glucagon receptor. The normally occurring serine at position 16 of native glucagon (SEQ ID NO: 1) can be substituted with select acidic amino acids to enhance the potency of glucagon, in terms of its ability to stimulate cAMP synthesis in a validated in vitro model assay (see Example 2). More particularly, this substitution enhances the potency of the analog at least 2-fold, 4-fold, 5-fold, and up to 10-fold greater at the glucagon receptor. This substitution also enhances the analog's activity at the GLP-1 receptor at least 5-fold, 10-fold, or 15-fold relative to native glucagon, but selectivity is maintained for the glucagon receptor over the GLP-1 receptor.

By way of nonlimiting example, such enhanced potency can be provided by substituting the naturally occurring serine at position 16 with glutamic acid or with another negatively charged amino acid having a side chain with a length of 4 atoms, or alternatively with any one of glutamine, homoglutamic acid, or homocysteic acid, or a charged amino acid having a side chain containing at least one heteroatom, (e.g. N, O, S, P) and with a side chain length of about 4 (or 3-5) atoms. In accordance with some embodiments, the serine residue at position 16 of native glucagon is substituted with an amino acid selected from the group consisting of glutamic acid, glutamine, homoglutamic acid, homocysteic acid, threonine, or glycine. In accordance with some embodiments, the serine residue at position 16 of native glucagon is substituted with an amino acid selected from the group consisting of glutamic acid, glutamine, homoglutamic acid and homocysteic acid, and in some embodiments the serine residue is substituted with glutamic acid.

In some embodiments, the enhanced potency Class 3 glucagon related peptide comprises a peptide of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or a glucagon agonist analog of SEQ ID NO: 5. In accordance with some embodiments, a Class 3 glucagon related peptide having enhanced potency at the glucagon receptor relative to wild type glucagon is provided wherein the peptide comprises the sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9 or SEQ ID NO: 10, wherein the glucagon peptide retains its selectivity for the glucagon receptor relative to the GLP-1 receptors. In some embodiments, the Class 3 glucagon related peptide having enhanced specificity for the glucagon receptor comprises the peptide of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 or a glucagon agonist analog thereof, wherein the carboxy terminal amino acid retains its native carboxylic acid group. In accordance with some embodiments, a Class 3 glucagon related peptide comprises the sequence of NH₂-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-COOH (SEQ ID NO: 10), wherein the peptide exhibits approximately fivefold enhanced potency at the glucagon receptor, relative to native glucagon as measured by the in vitro cAMP assay of Example 2.

Glucagon receptor activity can be reduced, maintained, or enhanced by an amino acid modification at position 3, e.g. substitution of the naturally occurring glutamine at position 3. In some embodiments, substitution of the amino acid at

-85-

position 3 with an acidic, basic, or hydrophobic amino acid (glutamic acid, ornithine, norleucine) has been shown to substantially reduce or destroy glucagon receptor activity. The analogs that are substituted with, for example, glutamic acid, ornithine, or norleucine have about 10% or less of the activity of native glucagon at the glucagon receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1% but less than about 10%, while exhibiting at least 20% of the activity of GLP-1 at the GLP-1 receptor. For example, exemplary analogs described herein have about 0.5%, about 1% or about 7% of the activity of native glucagon, while exhibiting at least 20% of the activity of GLP-1 at the GLP-1 receptor. In particular, any of the Class 3 glucagon related peptides, including glucagon analogs, glucagon agonist analogs, glucagon co-agonists, and glucagon/GLP-1 co-agonist molecules, described herein may be modified to contain a modification at position 3, e.g., Gln substituted with Glu, to produce a peptide with high selectivity, e.g., tenfold selectivity, for the GLP-1 receptor as compared to the selectivity for the glucagon receptor.

In another embodiment, the naturally occurring glutamine at position 3 of any of the Class 3 glucagon peptides can be substituted with a glutamine analog without a substantial loss of activity at the glucagon receptor, and in some cases, with an enhancement of glucagon receptor activity, as described herein. In specific embodiments, the amino acid at position 3 is substituted with Dab(Ac). For example, glucagon agonists can comprise the amino acid sequence of SEQ ID NO: 595, SEQ ID NO: 601, SEQ ID NO: 603, SEQ ID NO: 604, SEQ ID NO: 605, and SEQ ID NO: 606.

It was observed that modifications at position 2 (e.g. Aib at position 2) and in some cases modifications at position 1 may reduce glucagon activity. This reduction in glucagon activity can be restored by stabilizing the alpha-helix in the C-terminal portion of glucagon, e.g. through means described herein, for example, through a covalent bond between the side chains of the amino acids at positions “i” and “i+4”, e.g., 12 and 16, 16 and 20, or 20 and 24. In some embodiments, this covalent bond is a lactam bridge between a glutamic acid at position 16 and a lysine at position 20. In some embodiments, this covalent bond is an intramolecular bridge other than a lactam bridge. For example, suitable covalent bonding methods include any one or more of olefin metathesis, lanthionine-based cyclization, disulfide bridge or modified sulfur-

containing bridge formation, the use of α , ω -diaminoalkane tethers, the formation of metal-atom bridges, and other means of peptide cyclization.

Modifications affecting GLP-1 activity

Enhanced activity at the GLP-1 receptor is provided by replacing the
5 carboxylic acid of the C-terminal amino acid with a charge-neutral group, such as an amide or ester. In some embodiments, these Class 3 glucagon related peptides comprise a sequence of SEQ ID NO: 20, wherein the carboxy terminal amino acid has an amide group in place of the carboxylic acid group found on the native amino acid. These Class 3 glucagon related peptides have strong activity at both the glucagon and
10 GLP-1 receptors and thus act as co-agonists at both receptors. In accordance with some embodiments, the Class 3 glucagon related peptide is a glucagon and GLP-1 receptor co-agonist, wherein the peptide comprises the sequence of SEQ ID NO: 20, wherein the amino acid at position 28 is Asn or Lys and the amino acid at position 29 is Thr-amide.

15 Increased activity at the GLP-1 receptor is provided by modifications that stabilize the alpha helix in the C-terminal portion of glucagon (e.g. around residues 12-29).

In some embodiments, such modifications permit formation of an intramolecular bridge between the side chains of two amino acids that are separated
20 by three intervening amino acids (i.e., an amino acid at position "i" and an amino acid at position "i+4", wherein i is any integer between 12 and 25), by two intervening amino acids, i.e., an amino acid at position "j" and an amino acid at position "j+3," wherein j is any integer between 12 and 27, or by six intervening amino acids, i.e., an amino acid at position "k" and an amino acid at position "k+7," wherein k is any
25 integer between 12 and 22. In exemplary embodiments, the bridge or linker is about 8 (or about 7-9) atoms in length and forms between side chains of amino acids at positions 12 and 16, or at positions 16 and 20, or at positions 20 and 24, or at positions 24 and 28. The two amino acid side chains can be linked to one another through non-covalent bonds, e.g., hydrogen-bonding, ionic interactions, such as the
30 formation of salt bridges, or by covalent bonds.

In accordance with some embodiments, the Class 3 glucagon related peptide exhibits glucagon/GLP-1 receptor co-agonist activity and comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 11, 47, 48 and 49. In

some embodiments, the side chains are covalently bound to one another, and in some embodiments the two amino acids are bound to one another to form a lactam ring.

In accordance with some embodiments, the Class 3 glucagon related peptide

comprises SEQ ID NO: 45, wherein at least one lactam ring is formed between the

5 side chains of an amino acid pair selected from the group consisting of amino acid pairs 12 and 16, 16 and 20, 20 and 24 or 24 and 28. In some embodiments, the Class 3 glucagon related peptide comprises a glucagon peptide analog of SEQ ID NO: 20, wherein the peptide comprises an intramolecular lactam bridge formed between amino acid positions 12 and 16 or between amino acid positions 16 and 20. In some
10 embodiments, the Class 3 glucagon related peptide comprises the sequence of SEQ ID NO: 20, wherein an intramolecular lactam bridge is formed between amino acid positions 12 and 16, between amino acid positions 16 and 20, or between amino acid positions 20 and 24 and the amino acid at position 29 is glycine, wherein the sequence of SEQ ID NO: 29 is linked to the C-terminal amino acid of SEQ ID NO: 20. In a
15 further embodiment, the amino acid at position 28 is aspartic acid.

In some specific embodiments, stabilization of the alpha helix structure in the C-terminal portion of the Class 3 glucagon related peptide is achieved through the formation of an intramolecular bridge other than a lactam bridge. For example, suitable covalent bonding methods include any one or more of olefin metathesis,
20 lanthionine-based cyclization, disulfide bridge or modified sulfur-containing bridge formation, the use of α , ω -diaminoalkane tethers, the formation of metal-atom bridges, and other means of peptide cyclization are used to stabilize the alpha helix.

Furthermore, enhanced activity at the GLP-1 receptor may be achieved by stabilizing the alpha-helix structure in the C-terminal portion of the glucagon peptide
25 (around amino acids 12-29) through purposeful introduction of one or more α , α -disubstituted amino acids at positions that retain the desired activity. Such peptides may be considered herein as a peptide lacking an intramolecular bridge. In some aspects, stabilization of the alpha-helix is accomplished in this manner without introduction of an intramolecular bridge such as a salt bridge or covalent bond. In
30 some embodiments, one, two, three, four or more of positions 16, 17, 18, 19, 20, 21, 24 or 29 of a glucagon peptide is substituted with an α , α -disubstituted amino acid. For example, substitution of position 16 of the Class 3 glucagon related peptide with amino iso-butyric acid (Aib) enhances GLP-1 activity, in the absence of a salt bridge

-88-

or lactam. In some embodiments, one, two, three or more of positions 16, 20, 21 or 24 are substituted with Aib.

Enhanced activity at the GLP-1 receptor may be achieved by an amino acid modification at position 20. In some embodiments, the glutamine at position 20 is replaced with another hydrophilic amino acid having a side chain that is either charged or has an ability to hydrogen-bond, and is at least about 5 (or about 4-6) atoms in length, for example, lysine, citrulline, arginine, or ornithine.

Increased activity at the GLP-1 receptor is demonstrated in Class 3 glucagon related peptides comprising the C-terminal extension of SEQ ID NO: 26. GLP-1 activity in such Class 3 glucagon related peptides comprising SEQ ID NO: 26 can be further increased by modifying the amino acid at position 18, 28 or 29, or at position 18 and 29, as described herein.

A further modest increase in GLP-1 potency may be achieved by modifying the amino acid at position 10 to be Trp.

Combinations of the modifications that increase GLP-1 receptor activity may provide higher GLP-1 activity than any of such modifications taken alone. For example, the Class 3 glucagon related peptides can comprise modifications at position 16, at position 20, and at the C-terminal carboxylic acid group, optionally with a covalent bond between the amino acids at positions 16 and 20; can comprise modifications at position 16 and at the C-terminal carboxylic acid group; can comprise modifications at positions 16 and 20, optionally with a covalent bond between the amino acids at positions 16 and 20; or can comprise modifications at position 20 and at the C-terminal carboxylic acid group; optionally with the proviso that the amino acid at position 12 is not Arg; or optionally with the proviso that the amino acid at position 9 is not Glu.

Modifications affecting solubility

Addition of Hydrophilic moieties

The Class 3 glucagon related peptides can be further modified to improve the peptide's solubility and stability in aqueous solutions at physiological pH, while retaining the high biological activity relative to native glucagon. Hydrophilic moieties as discussed herein can be attached to the Class 3 glucagon related peptide as further discussed herein.

In accordance with some embodiments, introduction of hydrophilic groups at positions 17, 21, and 24 of the Class 3 glucagon related peptide comprising SEQ ID NO: 9 or SEQ ID NO: 10 are anticipated to improve the solubility and stability of the high potency glucagon analog in solutions having a physiological pH. Introduction of such groups also increases duration of action, e.g. as measured by a prolonged half-life in circulation.

In some embodiments, the Class 3 glucagon related peptide comprises a sequence selected from the group consisting of 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 and SEQ ID NO: 19, wherein the side chain of an amino acid residue at one of position 16, 17, 21 or 24 of said Class 3 glucagon related peptide further comprises a polyethylene glycol chain, having a molecular weight selected from the range of about 500 to about 40,000 Daltons. In some embodiments, the polyethylene glycol chain has a molecular weight selected from the range of about 500 to about 5,000 Daltons. In another embodiment, the polyethylene glycol chain has a molecular weight of about 10,000 to about 20,000 Daltons. In yet other exemplary embodiments the polyethylene glycol chain has a molecular weight of about 20,000 to about 40,000 Daltons.

Suitable hydrophilic moieties include any water soluble polymers known in the art, including the hydrophilic moieties described herein, homo- or co-polymers of PEG, and a monomethyl-substituted polymer of PEG (mPEG). In accordance with some embodiments the hydrophilic group comprises a polyethylene (PEG) chain. More particularly, in some embodiments, the Class 3 glucagon related peptide comprises the sequence of SEQ ID NO: 6 or SEQ ID NO: 7 wherein a PEG chain is covalently linked to the side chains of amino acids present at positions 21 and 24 of the Class 3 glucagon related peptide and the carboxy terminal amino acid of the Class 3 glucagon related peptide has the carboxylic acid group. In accordance with some embodiments, the polyethylene glycol chain has an average molecular weight selected from the range of about 500 to about 10,000 Daltons.

In accordance with some embodiments, the pegylated Class 3 glucagon related peptide comprises two or more polyethylene glycol chains covalently bound to the Class 3 glucagon related peptide wherein the total molecular weight of the glucagon chains is about 1,000 to about 5,000 Daltons. In some embodiments the pegylated

-90-

glucagon agonist comprises a peptide consisting of SEQ ID NO: 5 or a glucagon agonist analog of SEQ ID NO: 5, wherein a PEG chain is covalently linked to the amino acid residue at position 21 and at position 24, and wherein the combined molecular weight of the two PEG chains is about 1,000 to about 5,000 Daltons.

5 *Charged C-terminus*

The solubility of the Class 3 glucagon related peptide comprising SEQ ID NO: 20 can be further improved, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of glucagon peptide of SEQ ID NO: 20, preferably at a position C-terminal to position 27. Such a charged amino acid can be introduced by substituting a native amino acid with a charged amino acid, e.g. at positions 28 or 29, or alternatively by adding a charged amino acid, e.g. after position 27, 28 or 29. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. Additional modifications, e.g. conservative substitutions, may be made to the Class 3 glucagon related peptide that still allow it to retain glucagon activity. In some embodiments, an analog of the Class 3 glucagon related peptide of SEQ ID NO: 20 is provided wherein the analog differs from SEQ ID NO: 20 by 1 to 2 amino acid substitutions at positions 17-26, and, in some embodiments, the analog differs from the peptide of SEQ ID NO: 20 by an amino acid substitution at position 20.

20 *Acylation/Alkylation*

In accordance with some embodiments, the glucagon peptide is modified to comprise an acyl or alkyl group, e.g., a C4 to C30 acyl or alkyl group. In some aspects, the acyl group or alkyl group is not naturally occurring on an amino acid.. In specific aspects, the acyl or alkyl group is non-native to any naturally-occurring amino acid. Acylation or alkylation can increase the half-life in circulation and/or delay the onset of and/or extend the duration of action and/or improve resistance to proteases such as DPP-IV. The activity at the glucagon receptor and GLP-1 receptor of the Class 3 glucagon related peptides is maintained, if not substantially enhanced after acylation. Further, the potency of the acylated analogs were comparable to the unacylated versions of the Class 3 glucagon related peptides, if not substantially enhanced.

In some embodiments, the invention provides a Class 3 glucagon related peptide modified to comprise an acyl group or alkyl group covalently linked to the

amino acid at position 10 of the glucagon peptide. The glucagon peptide may further comprise a spacer between the amino acid at position 10 of the Class 3 glucagon related peptide and the acyl group or alkyl group. Any of the foregoing Class 3 glucagon related peptides may comprise two acyl groups or two alkyl groups, or a
5 combination thereof.

In a specific aspect of the invention, the acylated Class 3 glucagon related peptide comprises the amino acid sequence of any of SEQ ID NOs: 534-544 and 546-549.

C-terminal truncation

10 In some embodiments, the Class 3 glucagon related peptides described herein are further modified by truncation or deletion of one or two amino acids of the C-terminus of the glucagon peptide (i.e., position 29 and/or 28) without affecting activity and/or potency at the glucagon and GLP-1 receptors. In this regard, the Class 3 glucagon related peptide can comprise amino acids 1-27 or 1-28 of the native
15 glucagon peptide (SEQ ID NO: 1), optionally with one or more modifications described herein.

In some embodiments, the truncated Class 3 glucagon related peptide comprises SEQ ID NO: 550 or SEQ ID NO: 551. In another embodiment, the truncated glucagon agonist peptide comprises SEQ ID NO: 552 or SEQ ID NO: 553.

20 *C-terminal extension*

In accordance with some embodiments, the Class 3 glucagon related peptides disclosed herein are modified by the addition of a second peptide to the carboxy terminus of the glucagon peptide, for example, SEQ ID NO: 26, SEQ ID NO: 27 or SEQ ID NO: 28. In some embodiments, a Class 3 glucagon related peptide having a
25 sequence selected from the group consisting of 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 NO: 19, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, and SEQ ID NO: 69 is covalently bound through a peptide bond to a second peptide, wherein the second peptide comprises a sequence selected from the group consisting
30 of SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28. In a further embodiment, in Class 3 glucagon related peptides which comprise the C-terminal extension, the threonine at position 29 of the native glucagon peptide is replaced with a glycine. A Class 3 glucagon related peptide having a glycine substitution for threonine at

-92-

position 29 and comprising the carboxy terminal extension of SEQ ID NO: 26 is four times as potent at the GLP-1 receptor as native glucagon modified to comprise the carboxy terminal extension of SEQ ID NO: 26. Potency at the GLP-1 receptor can be further enhanced by an alanine substitution for the native arginine at position 18.

5 Accordingly, the Class 3 glucagon related peptide can have a carboxy terminal extension of SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28. In accordance with some embodiments, Class 3 glucagon related peptide comprising SEQ ID NO: 33 or SEQ ID NO: 20, further comprises the amino acid sequence of SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 linked to amino acid 29 of the glucagon peptide.

10 More particularly, the Class 3 glucagon related peptide comprises a sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13 SEQ ID NO: 14 and SEQ ID NO: 15, further comprising the amino acid sequence of SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 linked to amino acid 29 of the glucagon peptide. More particularly, the glucagon peptide comprises a sequence selected from
15 the group consisting of SEQ ID NO: 10, 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 NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 55 and SEQ ID NO: 56 further comprising the amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS) or SEQ ID NO: 821 linked to amino acid 29 of the Class 3 glucagon
20 related peptide. In some embodiments, the Class 3 glucagon related peptide comprises the sequence of SEQ ID NO: 64.

Other Modifications

Any of the modifications described above with regard to Class 3 glucagon related peptides which increase or decrease glucagon receptor activity and which
25 increase GLP-1 receptor activity can be applied individually or in combination. Combinations of the modifications that increase GLP-1 receptor activity generally provide higher GLP-1 activity than any of such modifications taken alone. Any of the modifications described above can also be combined with other modifications described herein in reference to Class 3 glucagon related peptides that confer other
30 desirable properties, such as increased solubility and/or stability and/or duration of action. Alternatively, any of the modifications described above can be combined with other modifications described herein in reference to Class 3 glucagon related peptides

-93-

that do not substantially affect solubility or stability or activity. Exemplary modifications include but are not limited to:

(A) Improving solubility, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of native glucagon, preferably at a position C-terminal to position 27. Such a charged amino acid can be introduced by substituting
5 a native amino acid with a charged amino acid, e.g. at positions 28 or 29, or alternatively by adding a charged amino acid, e.g. after position 27, 28 or 29. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. In other embodiments, one, two, three or all of the charged amino
10 acids are positively charged. Such modifications increase solubility, e.g. provide at least 2-fold, 5-fold, 10-fold, 15-fold, 25-fold, 30-fold or greater solubility relative to native glucagon at a given pH between about 5.5 and 8, e.g., pH 7, when measured after 24 hours at 25°C.

(B) Increasing solubility and duration of action or half-life in circulation by addition
15 of a hydrophilic moiety such as a polyethylene glycol chain, as described herein, e.g. at position 16, 17, 20, 21, 24 or 29, or at the C-terminal amino acid of the peptide.

(C) Increasing stability by modification of the aspartic acid at position 15, for example, by deletion or substitution with glutamic acid, homoglutamic acid, cysteic acid or homocysteic acid. Such modifications can reduce degradation or cleavage at a
20 pH within the range of 5.5 to 8, especially in acidic or alkaline buffers, for example, retaining at least 75%, 80%, 90%, 95%, 96%, 97%, 98% or 99% of the original peptide after 24 hours at 25°C.

(D) Increasing stability by modification of the methionine at position 27, for example, by substitution with leucine or norleucine. Such modifications can reduce oxidative
25 degradation. Stability can also be increased by modification of the Gln at position 20 or 24, e.g. by substitution with Ser, Thr, Ala or Aib. Such modifications can reduce degradation that occurs through deamidation of Gln. Stability can be increased by modification of Asp at position 21, e.g. by substitution with Glu. Such modifications can reduce degradation that occurs through dehydration of Asp to form a cyclic
30 succinimide intermediate followed by isomerization to iso-aspartate.

(E) Increasing resistance to dipeptidyl peptidase IV (DPP IV) cleavage by modification of the amino acid at position 1 or 2 with the DPP-IV resistant amino

acids described herein and including modification of the amino acid at position 2 with N-methyl-alanine.

(F) Conservative or non-conservative substitutions, additions or deletions that do not affect activity, for example, conservative substitutions at one or more of positions 2,

5 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28 or 29; deletions at one or more of positions 27, 28 or 29; or a deletion of amino acid 29 optionally combined with a C-terminal amide or ester in place of the C-terminal carboxylic acid group;

(G) Adding C-terminal extensions as described herein;

(H) Increasing half-life in circulation and/or extending the duration of action and/or
10 delaying the onset of action, for example, through acylation or alkylation of the glucagon peptide, as described herein;

(I) Homodimerization or heterodimerization as described herein.

Other modifications include substitution of His at position 1 with a large, aromatic amino acid (e.g., Tyr, Phe, Trp or amino-Phe); Ser at position 2 with Ala;
15 substitution of Tyr at position 10 with Val or Phe; substitution of Lys at position 12 with Arg; substitution of Asp at position 15 with Glu; substitution of Ser at position 16 with Thr or Aib.

Class 3 glucagon related peptides with GLP-1 activity that contain a non-conservative substitution of His at position 1 with a large, aromatic amino acid (e.g.,
20 Tyr) can retain GLP-1 activity provided that the alpha-helix is stabilized via an intramolecular bridge, e.g., such as any of those described herein.

Conjugates and fusions

The Class 3 glucagon related peptide can be linked, optionally via covalent bonding and optionally via a linker, to a conjugate moiety.

25 The Class 3 glucagon related peptide also can be part of a fusion peptide or protein wherein a second peptide or polypeptide has been fused to a terminus, e.g., the carboxy terminus of the Class 3 glucagon related peptide.

More particularly, the fusion Class 3 glucagon related peptide may comprise a glucagon agonist of SEQ ID NO: 55, SEQ ID NO: 9 or SEQ ID NO: 10 further
30 comprising an amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) linked to amino acid 29 of the glucagon peptide. In some embodiments, the amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR)

-95-

is bound to amino acid 29 of the Class 3 glucagon related peptide through a peptide bond. Applicants have discovered that in Class 3 glucagon related peptide fusion peptides comprising the C-terminal extension peptide of Exendin-4 (e.g., SEQ ID NO: 820 or SEQ ID NO: 821), substitution of the native threonine residue at position 29

5 with glycine dramatically increases GLP-1 receptor activity. This amino acid substitution can be used in conjunction with other modifications disclosed herein with regard to Class 3 glucagon related peptides to enhance the affinity of the glucagon analogs for the GLP-1 receptor. For example, the T29G substitution can be combined with the S16E and N20K amino acid substitutions, optionally with a lactam bridge

10 between amino acids 16 and 20, and optionally with addition of a PEG chain as described herein. In some embodiments, a Class 3 glucagon related peptide comprises the sequence of SEQ ID NO: 64. In some embodiments, the Class 3 glucagon related peptide portion of the glucagon fusion peptide is selected from the group consisting of SEQ ID NO: 55, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4,

15 and SEQ ID NO: 5 wherein a PEG chain, when present at positions 17, 21, 24, or the C-terminal amino acid, or at both 21 and 24, is selected from the range of 500 to 40,000 Daltons. More particularly, in some embodiments, the Class 3 glucagon related peptide segment is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 63, wherein the PEG chain is selected from the range of

20 500 to 5,000. In some embodiments, the Class 3 glucagon related peptide is a fusion peptide comprising the sequence of SEQ ID NO: 55 and SEQ ID NO: 65 wherein the peptide of SEQ ID NO: 65 is linked to the carboxy terminus of SEQ ID NO: 55.

In accordance with some embodiments, an additional chemical modification of the Class 3 glucagon related peptide of SEQ ID NO: 10 bestows increased GLP-1

25 receptor potency to a point where the relative activity at the glucagon and GLP-1 receptors is virtually equivalent. Accordingly, in some embodiments, a Class 3 glucagon related peptide comprises a terminal amino acid comprising an amide group in place of the carboxylic acid group that is present on the native amino acid. The relative activity of the Class 3 glucagon related peptide at the respective glucagon and

30 GLP-1 receptors can be adjusted by further modifications to the Class 3 glucagon related peptide to produce analogs demonstrating about 40% to about 500% or more of the activity of native glucagon at the glucagon receptor and about 20% to about 200% or more of the activity of native GLP-1 at the GLP-1 receptor, e.g. 50-fold,

-96-

100-fold or more increase relative to the normal activity of glucagon at the GLP-1 receptor. In some embodiments, the glucagon peptides described herein exhibit up to about 100%, 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native glucagon at the glucagon receptor. In some embodiments, the glucagon peptides
5 described herein exhibit up to about 100%, 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native GLP-1 at the GLP-1 receptor.

Exemplary Embodiments

In accordance with some embodiments, a glucagon analog is provided comprising the sequence of SEQ ID NO: 55, wherein said analog differs from SEQ
10 ID NO: 55 by 1 to 3 amino acids, selected from positions 1, 2, 3, 5, 7, 10, 11, 13, 14, 17, 18, 19, 21, 24, 27, 28, and 29, wherein said glucagon peptide exhibits at least 20% of the activity of native GLP-1 at the GLP-1 receptor.

In accordance with some embodiments a glucagon/GLP-1 receptor co-agonist is provided comprising the sequence:

15 NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Xaa-Arg-Arg-Ala-Xaa-Asp-Phe-Val-Xaa-Trp-Leu-Met-Xaa-Xaa-R (SEQ ID NO: 33) wherein the Xaa at position 15 is selected from the group of amino acids consisting of Asp, Glu, cysteic acid, homoglutamic acid and homocysteic acid, Xaa at position 16 is selected from the group of amino acids consisting of Ser, Glu, Gln, homoglutamic
20 acid and homocysteic acid, the Xaa at position 20 is Gln or Lys, the Xaa at position 24 is Gln or Glu, the Xaa at position 28 is Asn, Lys or an acidic amino acid, the Xaa at position 29 is Thr, Gly or an acidic amino acid, and R is COOH or CONH₂, with the proviso that when position 16 is serine, position 24 is Glu and either position 20 or position 28 is Lys. In some embodiments the glucagon/GLP-1 receptor co-agonist
25 comprises the sequence of SEQ ID NO: 33 wherein the amino acid at position 28 is aspartic acid and the amino acid at position 29 is glutamic acid. In another embodiment the amino acid at position 28 is the native asparagine, the amino acid at position 29 is glycine and the amino acid sequence of SEQ ID NO: 29 or SEQ ID NO: 65 is covalently linked to the carboxy terminus of SEQ ID NO: 33.

30 In some embodiments a co-agonist is provided comprising the sequence of SEQ ID NO: 33 wherein an additional acidic amino acid added to the carboxy terminus of the peptide. In a further embodiment the carboxy terminal amino acid of the glucagon analog has an amide in place of the carboxylic acid group of the natural

-97-

amino acid. In some embodiments the glucagon analog comprises a sequence selected from the group consisting of SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44.

In accordance with some embodiments a glucagon peptide analog of SEQ ID NO: 33 is provided, wherein said analog differs from SEQ ID NO: 33 by 1 to 3 amino acids, selected from positions 1, 2, 3, 5, 7, 10, 11, 13, 14, 17, 18, 19, 21 and 27, with the proviso that when the amino acid at position 16 is serine, either position 20 is lysine, or a lactam bridge is formed between the amino acid at position 24 and either the amino acid at position 20 or position 28. In accordance with some embodiments the analog differs from SEQ ID NO: 33 by 1 to 3 amino acids selected from positions 1, 2, 3, 21 and 27. In some embodiments the glucagon peptide analog of SEQ ID NO: 33 differs from that sequence by 1 to 2 amino acids, or in some embodiments by a single amino acid, selected from positions 1, 2, 3, 5, 7, 10, 11, 13, 14, 17, 18, 19, 21 and 27, with the proviso that when the amino acid at position 16 is serine, either position 20 is lysine, or a lactam bridge is formed between the amino acid at position 24 and either the amino acid at position 20 or position 28.

In accordance with another embodiment a relatively selective GLP-1 receptor agonist is provided comprising the sequence NH₂-His-Ser-Xaa-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Xaa-Arg-Arg-Ala-Xaa-Asp-Phe-Val-Xaa-Trp-Leu-Met-Xaa-Xaa-R (SEQ ID NO: 53) wherein the Xaa at position 3 is selected from the group of amino acids consisting of Glu, Orn or Nle, the Xaa at position 15 is selected from the group of amino acids consisting of Asp, Glu, cysteic acid, homoglutamic acid and homocysteic acid, Xaa at position 16 is selected from the group of amino acids consisting of Ser, Glu, Gln, homoglutamic acid and homocysteic acid, the Xaa at position 20 is Gln or Lys, the Xaa at position 24 is Gln or Glu, the Xaa at position 28 is Asn, Lys or an acidic amino acid, the Xaa at position 29 is Thr, Gly or an acidic amino acid, and R is COOH, CONH₂, SEQ ID NO: 26 or SEQ ID NO: 29, with the proviso that when position 16 is serine, position 20 is Lys, or alternatively when position 16 is serine the position 24 is Glu and either position 20 or position 28 is Lys. In some embodiments the amino acid at position 3 is glutamic acid. In some embodiments the acidic amino acid substituted at position 28 and/or 29 is aspartic acid or glutamic acid. In some embodiments the glucagon peptide, including a co-agonist peptide, comprises the sequence of SEQ ID NO: 33 further comprising an

-98-

additional acidic amino acid added to the carboxy terminus of the peptide. In a further embodiment the carboxy terminal amino acid of the glucagon analog has an amide in place of the carboxylic acid group of the natural amino acid.

5 In accordance with some embodiments a glucagon/GLP-1 receptor co-agonist is provided comprising a modified glucagon peptide selected from the group consisting of:

NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Xaa-Arg-Arg-Ala-Xaa-Asp-Phe-Val-Xaa-Trp-Leu-Xaa-Xaa-Xaa-R (SEQ ID NO: 34), wherein the Xaa at position 15 is selected from the group of amino acids consisting of
10 Asp, Glu, cysteic acid, homoglutamic acid and homocysteic acid, Xaa at position 16 is selected from the group of amino acids consisting of Ser, Glu, Gln, homoglutamic acid and homocysteic acid, the Xaa at position 20 is Gln or Lys, the Xaa at position 24 is Gln or Glu, the Xaa at position 27 is Met, Leu or Nle, and the Xaa at position 28 is Asn, Asp or Lys, R is COOH or CONH₂, the Xaa at position 29 is Thr or Gly, and
15 R is COOH, CONH₂, SEQ ID NO: 26 or SEQ ID NO: 29, with the proviso that when position 16 is serine, position 20 is Lys, or alternatively when position 16 is serine the position 24 is Glu and either position 20 or position 28 is Lys. In some embodiments R is CONH₂, the Xaa at position 15 is Asp, the Xaa at position 16 is selected from the group of amino acids consisting of Glu, Gln, homoglutamic acid and homocysteic
20 acid, the Xaas at positions 20 and 24 are each Gln the Xaa at position 28 is Asn or Asp and the Xaa at position 29 is Thr. In some embodiments the Xaas at positions 15 and 16 are each Glu, the Xaas at positions 20 and 24 are each Gln, the Xaa at position 28 is Asn or Asp, the Xaa at position 29 is Thr and R is CONH₂.

It has been reported that certain positions of the native glucagon peptide can
25 be modified while retaining at least some of the activity of the parent peptide. Accordingly, applicants anticipate that one or more of the amino acids located at positions at positions 2, 5, 7, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 24, 27, 28 or 29 of the peptide of SEQ ID NO: 11 can be substituted with an amino acid different from that present in the native glucagon peptide, and still retain activity at the glucagon
30 receptor. In some embodiments the methionine residue present at position 27 of the native peptide is changed to leucine or norleucine to prevent oxidative degradation of the peptide. In another embodiment the amino acid at position 20 is substituted with

-99-

Lys, Arg, Orn or Citrulline and/or position 21 is substituted with Glu, homoglutamic acid or homocysteic acid.

In some embodiments a glucagon analog of SEQ ID NO: 20 is provided wherein 1 to 6 amino acids, selected from positions 1, 2, 5, 7, 10, 11, 13, 14, 17, 18, 19, 21, 27, 28 or 29 of the analog differ from the corresponding amino acid of SEQ ID NO: 1, with the proviso that when the amino acid at position 16 is serine, position 20 is Lys, or alternatively when position 16 is serine the position 24 is Glu and either position 20 or position 28 is Lys. In accordance with another embodiment a glucagon analog of SEQ ID NO: 20 is provided wherein 1 to 3 amino acids selected from positions 1, 2, 5, 7, 10, 11, 13, 14, 17, 18, 19, 20, 21, 27, 28 or 29 of the analog differ from the corresponding amino acid of SEQ ID NO: 1. In another embodiment, a glucagon analog of SEQ ID NO: 8, SEQ ID NO: 9 or SEQ ID NO: 11 is provided wherein 1 to 2 amino acids selected from positions 1, 2, 5, 7, 10, 11, 13, 14, 17, 18, 19, 20 or 21 of the analog differ from the corresponding amino acid of SEQ ID NO: 1, and in a further embodiment the one to two differing amino acids represent conservative amino acid substitutions relative to the amino acid present in the native glucagon sequence (SEQ ID NO: 1). In some embodiments a glucagon peptide of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 is provided wherein the glucagon peptide further comprises one, two or three amino acid substitutions at positions selected from positions 2, 5, 7, 10, 11, 13, 14, 17, 18, 19, 20, 21, 27 or 29. In some embodiments the substitutions at positions 2, 5, 7, 10, 11, 13, 14, 16, 17, 18, 19, 20, 21, 27 or 29 are conservative amino acid substitutions.

In accordance with some embodiments a glucagon/GLP-1 receptor co-agonist is provided comprising a variant of the sequence of SEQ ID NO 33, wherein 1 to 10 amino acids selected from positions 16, 17, 18, 20, 21, 23, 24, 27, 28 and 29, respectively, of the variant differ from the corresponding amino acid of SEQ ID NO: 1. In accordance with some embodiments a variant of the sequence of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by one or more amino acid substitutions selected from the group consisting of Gln17, Ala18, Glu21, Ile23, Ala24, Val27 and Gly29. In accordance with some embodiments a glucagon/GLP-1 receptor co-agonist is provided comprising variants of the sequence of SEQ ID NO 33, wherein 1 to 2 amino acids selected from positions 17-26 of the variant differ from the corresponding amino acid of SEQ ID NO: 1. In accordance with some

-100-

embodiments a variant of the sequence of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by an amino acid substitution selected from the group consisting of Gln17, Ala18, Glu21, Ile23 and Ala24. In accordance with some embodiments a variant of the sequence of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by an amino acid substitution at position 18 wherein the substituted amino acid is selected from the group consisting of Ala, Ser, Thr, and Gly. In accordance with some embodiments a variant of the sequence of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by an amino acid substitution of Ala at position 18. Such variations are encompassed by SEQ ID NO: 55. In another embodiment a glucagon/GLP-1 receptor co-agonist is provided comprising variants of the sequence of SEQ ID NO 33, wherein 1 to 2 amino acids selected from positions 17-22 of the variant differ from the corresponding amino acid of SEQ ID NO: 1, and in a further embodiment a variant of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by 1 or 2 amino acid substitutions at positions 20 and 21. In accordance with some embodiments a glucagon/GLP-1 receptor co-agonist is provided comprising the sequence:
NH₂-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Xaa-Arg-Arg-Ala-Xaa-Xaa-Phe-Val-Xaa-Trp-Leu-Met-Xaa-Xaa-R (SEQ ID NO: 51), wherein the Xaa at position 15 is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid, the Xaa at position 16 is Ser, Glu, Gln, homoglutamic acid or homocysteic acid, the Xaa at position 20 is Gln, Lys, Arg, Orn or citrulline, the Xaa at position 21 is Asp, Glu, homoglutamic acid or homocysteic acid, the Xaa at position 24 is Gln or Glu, the Xaa at position 28 is Asn, Lys or an acidic amino acid, the Xaa at position 29 is Thr or an acid amino acid and R is COOH or CONH₂. In some embodiments R is CONH₂. In accordance with some embodiments a glucagon/GLP-1 receptor co-agonist is provided comprising a variant of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 47, SEQ ID NO: 48 or SEQ ID NO: 49, wherein the variant differs from said sequence by an amino acid substitution at position 20. In some embodiments the amino acid substitution is selected from the group consisting of Lys, Arg, Orn or citrulline for position 20.

In some embodiments a glucagon agonist is provided comprising an analog peptide of SEQ ID NO: 34 wherein the analog differs from SEQ ID NO: 34 by having an amino acid other than serine at position 2. In some embodiments the serine residue

-101-

is substituted with aminoisobutyric acid, D-alanine, and in some embodiments the serine residue is substituted with aminoisobutyric acid. Such modifications suppresses cleavage by dipeptidyl peptidase IV while retaining the inherent potency of the parent compound (e.g. at least 75, 80, 85, 90, 95% or more of the potency of the parent compound). In some embodiments the solubility of the analog is increased, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of native glucagon, preferably at a position C-terminal to position 27. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. In another embodiment the analog further comprises an acidic amino acid substituted for the native amino acid at position 28 or 29 or an acidic amino acid added to the carboxy terminus of the peptide of SEQ ID NO: 34.

In some embodiments the glucagon analogs disclosed herein are further modified at position 1 or 2 to reduce susceptibility to cleavage by dipeptidyl peptidase IV. In some embodiments a glucagon analog of SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 is provided wherein the analog differs from the parent molecule by a substitution at position 2 and exhibits reduced susceptibility (i.e., resistance) to cleavage by dipeptidyl peptidase IV. More particularly, in some embodiments, position 2 of the analog peptide is substituted with an amino acid selected from the group consisting of D-serine, D-alanine, valine, amino n-butyric acid, glycine, N-methyl serine and aminoisobutyric acid. In some embodiments, position 2 of the analog peptide is substituted with an amino acid selected from the group consisting of D-serine, D-alanine, glycine, N-methyl serine and aminoisobutyric acid. In another embodiment position 2 of the analog peptide is substituted with an amino acid selected from the group consisting of D-serine, glycine, N-methyl serine and aminoisobutyric acid. In some embodiments the amino acid at position 2 is not D-serine. In some embodiments the glucagon peptide comprises the sequence of SEQ ID NO: 21 or SEQ ID NO: 22.

In some embodiments a glucagon analog of SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 is provided wherein the analog differs from the parent molecule by a substitution at position 1 and exhibits reduced susceptibility (i.e., resistance) to cleavage by dipeptidyl peptidase IV. More particularly, position 1 of the analog peptide is substituted with an amino acid selected from the group consisting of D-histidine, alpha, alpha-dimethyl

-102-

imidazole acetic acid (DMIA), N-methyl histidine, alpha-methyl histidine, imidazole acetic acid, desaminohistidine, hydroxyl-histidine, acetyl-histidine and homo-histidine. In another embodiment a glucagon agonist is provided comprising an analog peptide of SEQ ID NO: 34 wherein the analog differs from SEQ ID NO: 34 by having an amino acid other than histidine at position 1. In some embodiments the solubility of the analog is increased, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of native glucagon, preferably at a position C-terminal to position 27. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. In another embodiment the analog further comprises an acidic amino acid substituted for the native amino acid at position 28 or 29 or an acidic amino acid added to the carboxy terminus of the peptide of SEQ ID NO: 34. In some embodiments the acidic amino acid is aspartic acid or glutamic acid.

In some embodiments the glucagon/GLP-1 receptor co-agonist comprises a sequence of SEQ ID NO: 20 further comprising an additional carboxy terminal extension of one amino acid or a peptide selected from the group consisting of SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28. In the embodiment wherein a single amino acid is added to the carboxy terminus of SEQ ID NO: 20, the amino acid is typically selected from one of the 20 common amino acids, and in some embodiments the additional carboxy terminus amino acid has an amide group in place of the carboxylic acid of the native amino acid. In some embodiments the additional amino acid is selected from the group consisting of glutamic acid, aspartic acid and glycine.

In an alternative embodiment a glucagon/GLP-1 receptor co-agonist is provided wherein the peptide comprises at least one lactam ring formed between the side chain of a glutamic acid residue and a lysine residue, wherein the glutamic acid residue and a lysine residue are separated by three amino acids. In some embodiments the carboxy terminal amino acid of the lactam bearing glucagon peptide has an amide group in place of the carboxylic acid of the native amino acid. More particularly, in some embodiments a glucagon and GLP-1 co-agonist is provided comprising a modified glucagon peptide selected from the group consisting of: NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu- Arg- Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu- Met-Xaa-Xaa-R (SEQ ID NO: 66)

-103-

NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu- Arg-
Arg-Ala-Lys-Asp-Phe-Val-Gln-Trp-Leu- Met-Xaa-Xaa-R (SEQ ID NO: 67)

NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser- Arg-
Arg-Ala-Lys-Asp-Phe-Val-Glu-Trp-Leu- Met-Xaa-Xaa-R (SEQ ID NO: 68)

5 NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser- Arg-
Arg-Ala-Gln-Asp-Phe-Val-Glu-Trp-Leu- Met-Lys-Xaa-R (SEQ ID NO: 69)

NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu- Arg-
Arg-Ala-Lys-Asp-Phe-Val-Glu-Trp-Leu- Met-Asn-Thr-R (SEQ ID NO: 16)

10 NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu- Arg-
Arg-Ala-Gln-Asp-Phe-Val-Glu-Trp-Leu- Met-Lys-Thr-R (SEQ ID NO: 17)

NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu- Arg-
Arg-Ala-Lys-Asp-Phe-Val-Glu-Trp-Leu- Met-Lys-Thr-R (SEQ ID NO: 18)

wherein Xaa at position 28 is Asp, or Asn, the Xaa at position 29 is Thr or Gly, R is

15 selected from the group consisting of COOH, CONH₂, glutamic acid, aspartic acid,
glycine, SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28, and a lactam bridge is
formed between Lys at position 12 and Glu at position 16 for SEQ ID NO: 66,

between Glu at position 16 and Lys at position 20 for SEQ ID NO: 67, between Lys at
position 20 and Glu at position 24 for SEQ ID NO: 68, between Glu at position 24
and Lys at position 28 for SEQ ID NO: 69, between Lys at position 12 and Glu at

20 position 16 and between Lys at position 20 and Glu at position 24 for SEQ ID NO:
16, between Lys at position 12 and Glu at position 16 and between Glu at position 24
and Lys at position 28 for SEQ ID NO: 17 and between Glu at position 16 and Lys at
position 20 and between Glu at position 24 and Lys at position 28 for SEQ ID NO:

18. In some embodiments R is selected from the group consisting of COOH, CONH₂,
25 glutamic acid, aspartic acid, glycine, the amino acid at position 28 is Asn, and the
amino acid at position 29 is threonine. In some embodiments R is CONH₂, the amino
acid at position 28 is Asn and the amino acid at position 29 is threonine. In another
embodiment R is selected from the group consisting of SEQ ID NO: 26, SEQ ID NO:
29 and SEQ ID NO: 65 and the amino acid at position 29 is glycine.

30 In a further embodiment the glucagon/GLP-1 receptor co-agonist is selected
from the group consisting of 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 and SEQ ID NO: 18,
wherein the peptide further comprises an additional carboxy terminal extension of one

-104-

amino acid or a peptide selected from the group consisting of SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28. In some embodiments the terminal extension comprises the sequence of SEQ ID NO: 26, SEQ ID NO: 29 or SEQ ID NO: 65 and the glucagon peptide comprises the sequence of SEQ ID NO: 55. In some

5 embodiments the glucagon/GLP-1 receptor co-agonist comprises the sequence of SEQ ID NO: 33 wherein the amino acid at position 16 is glutamic acid, the amino acid at position 20 is lysine, the amino acid at position 28 is asparagine and the amino acid sequence of SEQ ID No: 26 or SEQ ID NO: 29 is linked to the carboxy terminus of SEQ ID NO: 33.

10 In the embodiment wherein a single amino acid is added to the carboxy terminus of SEQ ID NO: 20, the amino acid is typically selected from one of the 20 common amino acids, and in some embodiments the amino acid has an amide group in place of the carboxylic acid of the native amino acid. In some embodiments the additional amino acid is selected from the group consisting of glutamic acid and
15 aspartic acid and glycine. In the embodiments wherein the glucagon agonist analog further comprises a carboxy terminal extension, the carboxy terminal amino acid of the extension, in some embodiments, ends in an amide group or an ester group rather than a carboxylic acid.

 In another embodiment the glucagon/GLP-1 receptor co-agonist comprises the
20 sequence: NH₂-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-Xaa-CONH₂ (SEQ ID NO: 19), wherein the Xaa at position 30 represents any amino acid. In some embodiments Xaa is selected from one of the 20 common amino acids, and in some embodiments the amino acid is glutamic acid, aspartic acid or glycine. The solubility
25 of this peptide can be further improved by covalently linking a PEG chain to the side chain of amino acid at position 17, 21, 24 or 30 of SEQ ID NO: 19. In a further embodiment the peptide comprises an additional carboxy terminal extension of a peptide selected from the group consisting of SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28. In accordance with some embodiments the glucagon/GLP-1
30 receptor co-agonist comprises the sequence of SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32.

 Additional site specific modifications internal to the glucagon sequence of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15,

-105-

SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 and SEQ ID NO: 64 can be made to yield a set of glucagon agonists that possess variable degrees of GLP-1 agonism. Accordingly, peptides that possess virtually identical in vitro potency at each receptor have been prepared and characterized. Similarly, peptides with tenfold selectively enhanced potency at each of the two receptors have been identified and characterized. As noted above substitution of the serine residue at position 16 with glutamic acid enhances the potency of native glucagon at both the Glucagon and GLP-1 receptors, but maintains approximately a tenfold selectivity for the glucagon receptor. In addition by substituting the native glutamine at position 3 with glutamic acid (SEQ ID NO: 22) generates a glucagon analog that exhibits approximately a tenfold selectivity for the GLP-1 receptor.

The solubility of the glucagon/GLP-1 co-agonist peptides can be further enhanced in aqueous solutions at physiological pH, while retaining the high biological activity relative to native glucagon by the introduction of hydrophilic groups at positions 16, 17, 21, and 24 of the peptide, or by the addition of a single modified amino acid (i.e., an amino acid modified to comprise a hydrophilic group) at the carboxy terminus of the glucagon/GLP-1 co-agonist peptide. In accordance with some embodiments the hydrophilic group comprises a polyethylene (PEG) chain. More particularly, in some embodiments the glucagon peptide comprises the sequence of 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 or SEQ ID NO: 18 wherein a PEG chain is covalently linked to the side chain of an amino acids at position 16, 17, 21, 24, 29 or the C-terminal amino acid of the glucagon peptide, with the proviso that when the peptide comprises SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 or SEQ ID NO: 13 the polyethylene glycol chain is covalently bound to an amino acid residue at position 17, 21 or 24, when the peptide comprises SEQ ID NO: 14 or SEQ ID NO: 15 the polyethylene glycol chain is covalently bound to an amino acid residue at position 16, 17 or 21, and when the peptide comprises SEQ ID NO: 16, SEQ ID NO: 17 or SEQ ID NO: 18 the polyethylene glycol chain is covalently bound to an amino acid residue at position 17 or 21.

In some embodiments the glucagon peptide comprises the sequence of SEQ ID NO: 11, SEQ ID NO: 12 or SEQ ID NO: 13, wherein a PEG chain is covalently linked to the side chain of an amino acids at position 17, 21, 24, or the C-terminal

-106-

amino acid of the glucagon peptide, and the carboxy terminal amino acid of the peptide has an amide group in place of the carboxylic acid group of the native amino acid. In some embodiments the glucagon/GLP-1 receptor co-agonist peptide comprises a sequence selected from the group consisting of 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 and SEQ ID NO: 19, wherein a PEG chain is covalently linked to the side chain of an amino acid at position 17, 21 or 24 of SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 19, or at position 16, 17 or 21 of SEQ ID NO: 14 and SEQ ID NO: 15 or at position 17 or 21 of SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18 of the glucagon peptide. In another embodiment the glucagon/GLP-1 receptor co-agonist peptide comprises the sequence of SEQ ID NO: 11 or SEQ ID NO: 19, wherein a PEG chain is covalently linked to the side chain of an amino acids at position 17, 21 or 24 or the C-terminal amino acid of the glucagon peptide.

In accordance with some embodiments, and subject to the proviso limitations described in the preceding paragraphs, the glucagon co-agonist peptide is modified to contain one or more amino acid substitution at positions 16, 17, 21, 24, or 29 or the C-terminal amino acid, wherein the native amino acid is substituted with an amino acid having a side chain suitable for crosslinking with hydrophilic moieties, including for example, PEG. The native peptide can be substituted with a naturally occurring amino acid or a synthetic (non-naturally occurring) amino acid. Synthetic or non-naturally occurring amino acids refer to amino acids that do not naturally occur in vivo but which, nevertheless, can be incorporated into the peptide structures described herein. Alternatively, the amino acid having a side chain suitable for crosslinking with hydrophilic moieties, including for example, PEG, can be added to the carboxy terminus of any of the glucagon analogs disclosed herein. In accordance with some embodiments an amino acid substitution is made in the glucagon/GLP-1 receptor co-agonist peptide at a position selected from the group consisting of 16, 17, 21, 24, or 29 replacing the native amino acid with an amino acid selected from the group consisting of lysine, cysteine, ornithine, homocysteine and acetyl phenylalanine, wherein the substituting amino acid further comprises a PEG chain covalently bound to the side chain of the amino acid. In some embodiments a glucagon peptide selected from the group consisting of 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, and SEQ ID NO: 19 is

-107-

further modified to comprise a PEG chain is covalently linked to the side chain of an amino acid at position 17 or 21 of the glucagon peptide. In some embodiments the pegylated glucagon/GLP-1 receptor co-agonist further comprises the sequence of SEQ ID NO: 26, SEQ ID NO: 27 or SEQ ID NO: 29.

5 In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 55 or SEQ ID NO: 56, further comprising a C-terminal extension of SEQ ID NO: 26, SEQ ID NO: 29 or SEQ ID NO: 65 linked to the C-terminal amino acid of SEQ ID NO: 55 or SEQ ID NO: 56, and optionally further comprising a PEG chain covalently linked to the side chain of an amino acids at position 17, 18, 21, 24 or 29
10 or the C-terminal amino acid of the peptide. In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 55 or SEQ ID NO: 56, wherein a PEG chain is covalently linked to the side chain of an amino acids at position 21 or 24 of the glucagon peptide and the peptide further comprises a C-terminal extension of SEQ ID NO: 26, or SEQ ID NO: 29.

15 In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 55, or SEQ ID NO: 33 or SEQ ID NO: 34, wherein an additional amino acid is added to the carboxy terminus of SEQ ID NO: 33 or SEQ ID NO: 34, and a PEG chain is covalently linked to the side chain of the added amino acid. In a further embodiment, the pegylated glucagon analog further comprises a C-terminal extension
20 of SEQ ID NO: 26 or SEQ ID NO: 29 linked to the C-terminal amino acid of SEQ ID NO: 33 or SEQ ID NO: 34. In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 19, wherein a PEG chain is covalently linked to the side chain of the amino acid at position 30 of the glucagon peptide and the peptide further comprises a C-terminal extension of SEQ ID NO: 26 or SEQ ID NO: 29 linked to the
25 C-terminal amino acid of SEQ ID NO: 19.

 The polyethylene glycol chain may be in the form of a straight chain or it may be branched. In accordance with some embodiments the polyethylene glycol chain has an average molecular weight selected from the range of about 500 to about 10,000 Daltons. In some embodiments the polyethylene glycol chain has an average
30 molecular weight selected from the range of about 1,000 to about 5,000 Daltons. In an alternative embodiment the polyethylene glycol chain has an average molecular weight selected from the range of about 10,000 to about 20,000 Daltons. In accordance with some embodiments the pegylated glucagon peptide comprises two or

-108-

more polyethylene glycol chains covalently bound to the glucagon peptide wherein the total molecular weight of the glucagon chains is about 1,000 to about 5,000 Daltons. In some embodiments the pegylated glucagon agonist comprises a peptide consisting of SEQ ID NO: 5 or a glucagon agonist analog of SEQ ID NO: 5, wherein
5 a PEG chain is covalently linked to the amino acid residue at position 21 and at position 24, and wherein the combined molecular weight of the two PEG chains is about 1,000 to about 5,000 Daltons.

In certain exemplary embodiments, the glucagon peptide comprises the amino acid sequence of SEQ ID NO: 1 with up to ten amino acid modifications and
10 comprises an amino acid at position 10 which is acylated or alkylated. In some embodiments, the amino acid at position 10 is acylated or alkylated with a C4 to C30 fatty acid. In certain aspects, the amino acid at position 10 comprises an acyl group or an alkyl group which is non-native to a naturally-occurring amino acid.

In certain embodiments, the glucagon peptide comprising an amino acid at
15 position 10 which is acylated or alkylated comprises a stabilized alpha helix. Accordingly, in certain aspects, the glucagon peptide comprises an acyl or alkyl group as described herein and an intramolecular bridge, e.g., a covalent intramolecular bridge (e.g., a lactam bridge) between the side chains of an amino acid at position *i* and an amino acid at position *i*+4, wherein *i* is 12, 16, 20, or 24. Alternatively or
20 additionally, the glucagon peptide comprises an acyl or alkyl group as described herein and one, two, three or more of positions 16, 20, 21 and/or 24 of the glucagon peptide are substituted with an α , α -disubstituted amino acid, e.g., Aib. In some instances, the non-native glucagon peptide comprises Glu at position 16 and Lys at position 20, wherein optionally a lactam bridge links the Glu and the Lys, and,
25 optionally, the glucagon peptide further comprises one or more modifications selected from the group consisting of: Gln at position 17, Ala at position 18, Glu at position 21, Ile at position 23, and Ala at position 24.

Also, in any of the embodiments, wherein the glucagon peptide comprises an amino acid at position 10 which is acylated or alkylated, the glucagon peptide can
30 further comprise a C-terminal amide in lieu of the C-terminal alpha carboxylate.

In some embodiments, the glucagon peptide comprising an acyl or alkyl group as described herein further comprises an amino acid substitution at position 1, at position 2, or at positions 1 and 2, wherein the amino acid substitution(s) achieve

-109-

DPP-IV protease resistance. For example, the His at position 1 may be substituted with an amino acid selected from the group consisting of: D-histidine, alpha, alpha-dimethyl imidazole acetic acid (DMIA), N-methyl histidine, alpha-methyl histidine, imidazole acetic acid, desaminohistidine, hydroxyl-histidine, acetyl-histidine and homo-histidine. Alternatively or additionally, the Ser at position 2 may be substituted with an amino acid selected from the group consisting of: D-serine, alanine, D-alanine, valine, glycine, N-methyl serine, N-methyl alanine, and amino isobutyric acid. In some embodiments the amino acid at position 2 is not D-serine.

The glucagon peptide comprising the amino acid at position 10 which is acylated or alkylated as described herein can comprise any amino acid sequence which is substantially related to SEQ ID NO: 1. For instance, the glucagon peptide comprises SEQ ID NO: 1 with up to 10 amino acid modifications (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 modifications). In certain embodiments, the amino acid sequence of the acylated or alkylated glucagon peptide is greater than 25% identical to SEQ ID NO: 1 (e.g., greater than 30%, 35%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or nearly 100% identical to SEQ ID NO: 1). In certain specific embodiments, the glucagon peptide is one which comprises SEQ ID NOs: 55 with an amino acid at position 10 acylated or alkylated as described herein. The glucagon peptide can be any of SEQ ID NOs: 55, 55 with 1 or 2 amino acid modifications, 2-4, 9-18, 20, 23-25, 33, 40-44, 53, 56, 61, 62, 64, 66-514, and 534.

The acyl or alkyl group of these embodiments may be any acyl or alkyl group described herein. For example, the acyl group may be a C4 to C30 (e.g., C8 to C24) fatty acyl group and the alkyl group may be a C4 to C30 (e.g., C8 to C24) alkyl group.

The amino acid to which the acyl or alkyl group is attached may be any of the amino acids described herein, e.g., an amino acid of any of Formula I (e.g., Lys), Formula II, and Formula III.

In some embodiments, the acyl group or alkyl group is directly attached to the amino acid at position 10. In some embodiments, the acyl or alkyl group is attached to the amino acid at position 10 via a spacer, such as, for example, a spacer which is 3 to 10 atoms in length, e.g., an amino acid or dipeptide. Suitable spacers for purposes of attaching an acyl or alkyl group are described herein.

In accordance with some embodiments, the Class 3 glucagon related peptide may be an analog of any of the foregoing Class 3 glucagon related peptides as

-110-

described herein, which analog exhibits agonist activity at the GIP receptor. The activity level of the analog at the glucagon receptor, the GLP-1 receptor, and the GIP receptor, the potency at each of these receptors, and the selectivity for each of these receptors may be in accordance with the teachings of Class 2 glucagon related peptides described herein. See, the teachings under the subsection of the Class 2 glucagon related peptide section entitled "Activity."

In some embodiments of the invention, an analog of a glucagon peptide, which analog exhibits agonist activity at the GIP receptor, is provided. The analog in certain embodiments comprises the amino acid sequence of SEQ ID NO: 1 with at least one amino acid modification (optionally, up to 15 amino acid modifications), and an extension of 1 to 21 amino acids C-terminal to the amino acid at position 29 of the analog.

In certain aspects, the analogs comprise at least one amino acid modification and up to 15 amino acid modifications (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 amino acid modifications, up to 10 amino acid modifications). In certain embodiments, the analogs comprise at least one amino acid modification at up to 10 amino acid modifications and additional conservative amino acid modifications. Conservative amino acid modifications are described herein.

In some aspects, at least one of the amino acid modifications confers a stabilized alpha helix structure in the C-terminal portion of the analog. Modifications which achieve a stabilized alpha helix structure are described herein. See, for example, the teachings under the section entitled Stabilization of the alpha helix/Intramolecular bridges. In some aspects, the analog comprises an intramolecular bridge (e.g., a covalent intramolecular bridge, a non-covalent intramolecular bridge) between the side chains of two amino acids of the analog. In certain aspects, an intramolecular bridge links the side chains of the amino acids at positions i and $i+4$, wherein i is 12, 13, 16, 17, 20, or 24. In other aspects, an intramolecular bridge connects the side chains of the amino acids at positions j and $j+3$, wherein j is 17, or at positions k and $k+7$ wherein k is any integer between 12 and 22. In certain embodiments, the intramolecular bridge is a covalent intramolecular bridge, e.g., a lactam bridge. In specific aspects, the lactam bridge connects the side chains of the amino acids at positions 16 and 20. In particular aspects, one of the amino acids at positions 16 and 20 is a positive-charged amino

-111-

acid and the other is a negative-charged amino acid. For example, the analog can comprise a lactam bridge connecting the side chains of a Glu at position 16 and a Lys at position 20. In other aspects, the negative-charged amino acid and the positive-charged amino acid form a salt bridge. In this instance, the intramolecular bridge is a non-covalent intramolecular bridge.

In particular aspects, the amino acid modification which confers a stabilized alpha helix is an insertion or substitution of an amino acid of SEQ ID NO: 1 with an α,α -disubstituted amino acid. Suitable α,α -disubstituted amino acids for purposes of stabilizing the alpha helix are described herein and include, for example, Aib. In some aspects, one, two, three, or more of the amino acids at positions 16, 20, 21, and 24 of SEQ ID NO: 1 are substituted with an α,α -disubstituted amino acid, e.g., Aib. In particular embodiments, the amino acid at position 16 is Aib.

The analog which exhibits agonist activity at the GIP receptor can comprise additional modifications, such as any of those described herein. For instance, the amino acid modifications may increase or decrease activity at one or both of the GLP-1 receptor and glucagon receptor. The amino acid modifications may increase stability of the peptide, e.g., increase resistance to DPP-IV protease degradation, stabilize the bond between amino acids 15 and 16. The amino acid modifications may increase the solubility of the peptide and/or alter the time of action of the analog at any of the GIP, glucagon, and GLP-1 receptors. A combination of any of these types of modifications may be present in the analogs which exhibit agonist activity at the GIP receptor.

Accordingly, in some aspects, the analog comprises the amino acid sequence of SEQ ID NO: 1 with one or more of: Gln at position 17, Ala at position 18, Glu at position 21, Ile at position 23, and Ala or Cys at position 24, or conservative amino acid substitutions thereof. In some aspects, the analog comprises a C-terminal amide in place of the C-terminal alpha carboxylate. In certain embodiments, the analog comprises an amino acid substitution at position 1, position 2, or positions 1 and 2, which substitution(s) achieve DPP-IV protease resistance. Suitable amino acid substitutions are described herein. For example, DMIA at position 1 and/or d-Ser or Aib at position 2. In some embodiments, the amino acid at position 2 is not D-serine.

Additionally or alternatively, the analog may comprise one or a combination of: (a) Ser at position 2 substituted with Ala; (b) Gln at position 3 substituted with Glu

-112-

or a glutamine analog; (c) Thr at position 7 substituted with a Ile; (d) Tyr at position 10 substituted with Trp or an amino acid comprising an acyl or alkyl group which is non-native to a naturally-occurring amino acid; (e) Lys at position 12 substituted with Ile; (f) Asp at position 15 substituted with Glu; (g) Ser at position 16 substituted with Glu; (h) Gln at position 20 substituted with Ser, Thr, Ala, Aib; (i) Gln at position 24 substituted with Ser, Thr, Ala, Aib; (j) Met at position 27 substituted with Leu or Nle; (k) Asn at position 29 substituted with a charged amino acid, optionally, Asp or Glu; and (l) Thr at position 29 substituted with Gly or a charged amino acid, optionally, Asp or Glu.

10 With regard to the analogs which exhibit agonist activity at the GIP receptor, the analog comprises an extension of 1-21 amino acids (e.g., 5-19, 7-15, 9-12 amino acids). The extension of the analog may comprise any amino acid sequence, provided that the extension is 1 to 21 amino acids. In some aspects, the extension is 7 to 15 amino acids and in other aspects, the extension is 9 to 12 amino acids. In some
15 embodiments, the extension comprises (i) the amino acid sequence of SEQ ID NO: 26 or 674, (ii) an amino acid sequence which has high sequence identity (e.g., at least 80%, 85%, 90%, 95%, 98%, 99%) with the amino acid sequence of SEQ ID NO: 26 or 674, or (iii) the amino acid sequence of (i) or (ii) with one or more conservative amino acid modifications.

20 In some embodiments, at least one of the amino acids of the extension is acylated or alkylated. The amino acid comprising the acyl or alkyl group may be located at any position of extension of the analog. In certain embodiments, the acylated or alkylated amino acid of the extension is located at one of positions 37, 38, 39, 40, 41, or 42 (according to the numbering of SEQ ID NO: 1) of the analog. In
25 certain embodiments, the acylated or alkylated amino acid is located at position 40 of the analog.

 In exemplary embodiments, the acyl or alkyl group is an acyl or alkyl group which is non-native to a naturally-occurring amino acid. For example, the acyl or alkyl group may be a C4 to C30 (e.g., C12 to C18) fatty acyl group or C4 to C30 (e.g.,
30 C12 to C18) alkyl. The acyl or alkyl group may be any of those discussed herein.

 In some embodiments, the acyl or alkyl group is attached directly to the amino acid, e.g., via the side chain of the amino acid. In other embodiments, the acyl or alkyl group is attached to the amino acid via a spacer (e.g., an amino acid, a dipeptide,

a tripeptide, a hydrophilic bifunctional spacer, a hydrophobic bifunctional spacer). In certain aspects, the spacer is 3 to 10 atoms in length. In some embodiments, the amino acid spacer is not γ -Glu. In some embodiments, the dipeptide spacer is not γ -Glu- γ -Glu.

5 Also, in exemplary embodiments, the amino acid to which the acyl or alkyl group is attached may be any of those described herein, including, for example, an amino acid of Formula I, II, or III. The amino acid which is acylated or alkylated may be a Lys, for example. Suitable amino acids comprising an acyl or alkyl group, as well as suitable acyl groups and alkyl groups, are described herein. See, for example,
10 the teachings under the sections entitled Acylation and Alkylation.

 In other embodiments, 1-6 amino acids (e.g., 1-2, 1-3, 1-4, 1-5 amino acids) of the extension are positive-charged amino acids, e.g., amino acids of Formula IV, such as, for example, Lys. As used herein, the term "positive-charged amino acid" refers to any amino acid, naturally-occurring or non-naturally occurring, comprising a
15 positive charge on an atom of its side chain at a physiological pH. In certain aspects, the positive-charged amino acids are located at any of positions 37, 38, 39, 40, 41, 42, and 43. In specific embodiments, a positive-charged amino acid is located at position 40.

 In other instances, the extension is acylated or alkylated as described herein
20 and comprises 1-6 positive charged amino acids as described herein.

 In yet other embodiments, the analogs which exhibit agonist activity at the GIP receptor comprises (i) SEQ ID NO: 1 with at least one amino acid modification, (ii) an extension of 1 to 21 amino acids (e.g., 5 to 18, 7 to 15, 9 to 12 amino acids) C-terminal to the amino acid at position 29 of the analog, and (iii) an amino acid
25 comprising an acyl or alkyl group which is non-native to a naturally-occurring amino acid which is located outside of the C-terminal extension (e.g., at any of positions 1-29). In some embodiments, the analog comprises an acylated or alkylated amino acid at position 10. In particular aspects, the acyl or alkyl group is a C4 to C30 fatty acyl or C4 to C30 alkyl group. In some embodiments, the acyl or alkyl group is attached
30 via a spacer, e.g., an amino acid, dipeptide, tripeptide, hydrophilic bifunctional spacer, hydrophobic bifunctional spacer). In certain aspects, the analog comprises an amino acid modification which stabilizes the alpha helix, such as a salt bridge between a Glu at position 16 and a Lys at position 20, or an alpha, alpha-disubstituted amino acid at

-114-

any one, two, three, or more of positions 16, 20, 21, and 24. In specific aspects, the analog additionally comprises amino acid modifications which confer DPP-IV protease resistance, e.g., DMIA at position 1, Aib at position 2. Analogs comprising further amino acid modifications are contemplated herein.

5 In certain embodiments, the analogs having GIP receptor activity exhibit at least 0.1% (e.g., at least 0.5%, 1%, 2%, 5%, 10%, 15%, or 20%) activity of native GIP at the GIP receptor. In some embodiments, the analogs exhibit more than 20% (e.g., more than 50%, more than 75%, more than 100%, more than 200%, more than 300%, more than 500%) activity of native GIP at the GIP receptor. In some
10 embodiments, the analog exhibits appreciable agonist activity at one or both of the GLP-1 and glucagon receptors. In some aspects, the selectivity for these receptors (GIP receptor and GLP-1 receptor and/or glucagon receptor) are within 1000-fold. For example, the selectivity for the GLP-1 receptor of the analogs having GIP
15 receptor activity can be less than 500-fold, 100-fold, within 50-fold, within 25 fold, within 15 fold, within 10 fold) the selectivity for the GIP receptor and/or the glucagon receptor.

In accordance with some embodiments, the Class 3 glucagon related peptide comprises the amino acid sequence of native glucagon (SEQ ID NO: 1) comprising the following modifications: Aib at position 2, Glu at position 3, Lys at position 10,
20 Glu at position 16, Gln at position 17, Ala at position 18, Lys at position 20, Glu at position 21, Ile at position 23, Ala at position 24; wherein Lys at position 10 is acylated with a C14 or C16 fatty acid, and wherein the C-terminal carboxylate is replaced with an amide. In a specific embodiment, this Class 3 glucagon related peptide is attached via a linker (L) to a NHR ligand (Y).

25 In accordance with some embodiments, the Class 3 glucagon related peptide comprises, consists essentially of, or consists of an amino acid sequence of any of SEQ ID NOs: 70-514, 517-534, or 554, optionally with up to 1, 2, 3, 4, or 5 further modifications that retain GLP-1 agonist and/or glucagon agonist activity. In certain
30 embodiments, the Class 3 glucagon related peptide comprises the amino acids of any of SEQ ID NOs: 562-760. In some embodiments, the Class 3 glucagon related peptide comprises the amino acid sequences of any of SEQ ID NOs: 1301-1421.

In accordance with one embodiment a glucagon analog having enhanced solubility relative to native glucagon is provided, wherein said analog comprises one

-115-

or more amino acids selected from positions 5, 7, 8, 11 or 16 linked via an ester bond and said glucagon analog comprises the sequence

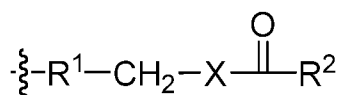
$X_1X_2X_3GTFTSDX_{10}SX_{12}YLX_{15}X_{16}X_{17}X_{18}AX_{20}X_{21}FVX_{24}WLX_{27}-Z$ (SEQ ID NO: 943)

5 wherein

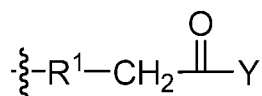
X_1 is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X_2 is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;

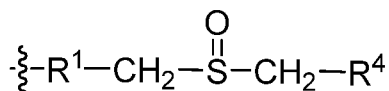
X_3 is an amino acid comprising a side chain of Structure I, II, or III:



Structure I



Structure II



Structure III

wherein R^1 is C_{0-3} alkyl or C_{0-3} heteroalkyl; R^2 is NHR^4 or C_{1-3} alkyl; R^3 is C_{1-3} alkyl; R^4 is H or C_{1-3} alkyl; X is NH, O, or S; and Y is NHR^4 , SR^3 , or OR^3 ;

20 X_{10} is selected from the group consisting of Tyr, Lys;

X_{12} is Lys, Ile or Arg;

X_{15} is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid;

X_{16} is Glu, Lys, Asp, Ser, glutamine, homoglutamic acid, homocysteic acid, Thr or Aib;

25 X_{17} is Arg or Gln;

X_{18} is Ala, Arg

X_{20} is Gln, Glu, Ser, Thr, Ala, Lys, Citrulline, Arg, Orn, or Aib;

X_{21} is Glu, Aib, Asp, Lys, Cys, Orn, homocysteine or acetyl phenylalanine;

X_{24} is Asn, Aib, Gln, Glu or Lys;

-116-

X₂₇ is Met, Leu or Nle;

X₂₈ is Asn or Ala;

X₂₉ is Thr or Gly; and

Z is selected from the group consisting of -COOH, -X₂₈-COOH, X₂₈-X₂₉-COOH, and X₂₈-X₂₉-GPSSGAPPPS (SEQ ID NO: 1299). Optionally, the sequence
 5 X₁X₂X₃GTFTSDX₁₀SX₁₂YLX₁₅X₁₆X₁₇X₁₈AX₂₀X₂₁FVX₂₄WLX₂₇-Z (SEQ ID NO: 943 further comprises

(i) a lactam bridge between the side chains of amino acids at positions i and i + 4, wherein i is 12, 16, 20 or 24, and

10 (ii) one, two, three, or all of the amino acids at positions 16, 20, 21, and 24 of the glucagon peptide is substituted with an α , α -disubstituted amino acid.

In accordance with one embodiment a glucagon analog having enhanced solubility relative to native glucagon is provided, wherein said analog comprises one or more amino acids selected from positions 5, 7, 8, 11 or 16 linked via an ester bond
 15 and said glucagon analog comprises the sequence
 YAQGTFTSDX₁₀SKYLDERAAQDFVQWLLEGGPSSGAPPPS-NH₂ (SEQ ID NO: 945) or

YX₂EGTFTSDX₁₀SIYLDKQAAX₂₀EFVNWLLAGGPSSGAPPPS (SEQ ID NO: 944)

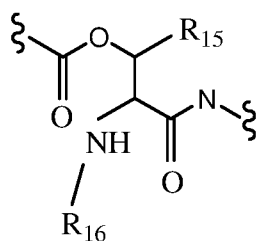
20 wherein

X₂ is Ser, D-Ser or Aib;

X₁₀ is Lys acylated with a C16 to C18 carbon atom chain, optionally via a γ Glu linker; and

X₂₀ is Gln or Aib. In one embodiment the sequence
 25 YAQGTFTSDX₁₀SKYLDERAAQDFVQWLLEGGPSSGAPPPS-NH₂ (SEQ ID NO: 945) or

YX₂EGTFTSDX₁₀SIYLDKQAAX₂₀EFVNWLLAGGPSSGAPPPS (SEQ ID NO: 944) comprises an ester linked amino acid comprising the structure of

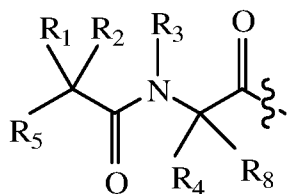


wherein

-117-

R₁₅ is H or CH₃ and

R₁₆ is an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine. In one embodiment the ester linked amino acid is at position 16. In one embodiment R₁₆ is a dipeptide that is susceptible to cleavage by Dipeptidyl Peptidase IV. In an alternative embodiment R₁₆ is a dipeptide having the general structure of Formula I:



wherein

- 10 R₁, R₂, R₄ and R₈ are independently selected from the group consisting of H, C₁-C₁₈ alkyl, C₂-C₁₈ alkenyl, (C₁-C₁₈ alkyl)OH, (C₁-C₁₈ alkyl)SH, (C₂-C₃ alkyl)SCH₃, (C₁-C₄ alkyl)CONH₂, (C₁-C₄ alkyl)COOH, (C₁-C₄ alkyl)NH₂, (C₁-C₄ alkyl)NHC(NH₂⁺)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₂-C₅ heterocyclic), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, (C₁-C₄ alkyl)(C₃-C₉ heteroaryl), and C₁-C₁₂ alkyl(W₁)C₁-C₁₂ alkyl, wherein W₁ is a heteroatom selected from the group consisting of N, S and O, or R₁ and R₂ together with the atoms to which they are attached form a C₃-C₁₂ cycloalkyl; or R₄ and R₈ together with the atoms to which they are attached form a C₃-C₆ cycloalkyl;

- 20 R₃ is selected from the group consisting of C₁-C₁₈ alkyl, (C₁-C₁₈ alkyl)OH, (C₁-C₁₈ alkyl)NH₂, (C₁-C₁₈ alkyl)SH, (C₀-C₄ alkyl)(C₃-C₆)cycloalkyl, (C₀-C₄ alkyl)(C₂-C₅ heterocyclic), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and (C₁-C₄ alkyl)(C₃-C₉ heteroaryl) or R₄ and R₃ together with the atoms to which they are attached form a pyrrolidine ring;

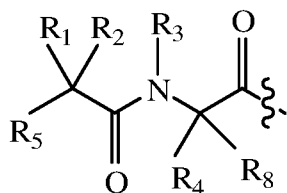
R₅ is NHR₆ or OH;

- 25 R₆ is H, C₁-C₈ alkyl; and

R₇ is selected from the group consisting of hydrogen and OH.

In one embodiment R₁₆ is a dipeptide having the general structure of Formula I:

-118-



wherein

R₁ is selected from the group consisting of H and C₁-C₄ alkyl;

- R₂ is selected from the group consisting of H, C₁-C₆ alkyl, C₂-C₈ alkenyl, (C₁-C₄ alkyl)OH, (C₁-C₄ alkyl)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and CH₂(C₅-C₉ heteroaryl) or R₂ and R₆ together with the atoms to which they are attached form a 5 member heterocyclic ring;

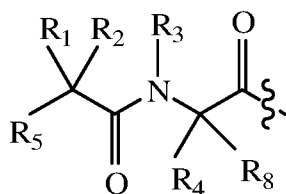
R₃ is C₁-C₆ alkyl;

- R₄ is selected from the group consisting of H and C₁-C₄ alkyl or R₃ and R₄ together with the atoms to which they are attached form a 5 member heterocyclic ring; and

R₇ is selected from the group consisting of hydrogen and OH.

In one embodiment R₁₆ is a dipeptide having the general structure of Formula

I:



wherein

R₁ is C₁-C₄ alkyl or (C₁-C₄ alkyl)NH₂;

R₂ is H or is C₁-C₄ alkyl;

R₃ is selected from the group consisting of C₁-C₆ alkyl;

- R₄ is selected from the group consisting of H, and C₁-C₄ alkyl;

R₈ is H; and

R₅ is NH₂ or OH.

- In accordance with one embodiment a glucagon analog having enhanced solubility relative to native glucagon is provided, wherein said analog comprises one or more amino acids selected from positions 5, 7, 8, 11 or 16 linked via an ester bond and said glucagon analog comprises the sequence

-119-

$X_1X_2QGTFTSDYSKYLX_{15}X_{16}RRAX_{20}DFVX_{24}WLMX_{28}T$ (SEQ ID NO: 946)

wherein

X_1 is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-
 5 His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X_2 is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;

X_{15} is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid;

X_{16} is Glu, Lys, Asp, Ser, glutamine, homoglutamic acid, homocysteic acid,
 10 Thr or Aib;

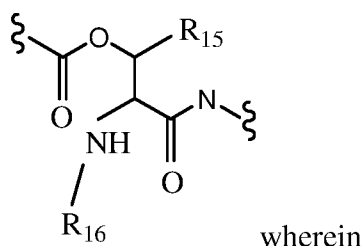
X_{20} is Gln or Lys;

X_{24} is Gln or Glu;

X_{28} is Asn, Asp or Lys;

or an analog of SEQ ID NO: 946 that differs from SEQ ID NO: 946 by 1 to 2 amino
 15 acid modifications, selected from positions 1, 2, 7, 10, 11, 13, 14, 17, 18, 19, 21, 27 and 29, wherein the glucagon peptide exhibits enhanced activity at the GLP-1 receptor as compared to native glucagon, optionally wherein the side chains of the amino acids at positions 12 and 16, positions 16 and 20, positions 20 and 24, or positions 24 and 28 of the glucagon peptide are linked by covalent bonds to provide
 20 the enhanced activity at the GLP-1 receptor, with the proviso that when the amino acid at position 16 is serine either position 20 is lysine, or the side chain of the amino acid at position 24 and the side chain of either the amino acid at position 20 or position 28 are linked by covalent bonds. In one embodiment the sequence
 $X_1X_2QGTFTSDYSKYLX_{15}X_{16}RRAX_{20}DFVX_{24}WLMX_{28}T$ (SEQ ID NO: 946)

25 comprises an ester linked amino acid comprising the structure of

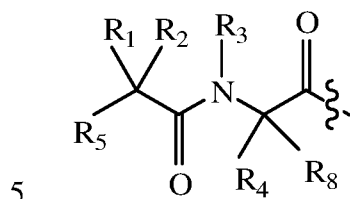


R_{15} is H or CH_3 and

R_{16} is an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or

-120-

diketomorpholine. In one embodiment the ester linked amino acid is located at position 16. In one embodiment R_{16} is a dipeptide that is susceptible to cleavage by Dipeptidyl Peptidase IV. In an alternative embodiment R_{16} is a dipeptide having the general structure of Formula I:



wherein

R_1 , R_2 , R_4 and R_8 are independently selected from the group consisting of H, C_1 - C_{18} alkyl, C_2 - C_{18} alkenyl, $(C_1$ - C_{18} alkyl)OH, $(C_1$ - C_{18} alkyl)SH, $(C_2$ - C_3 alkyl)SCH₃, $(C_1$ - C_4 alkyl)CONH₂, $(C_1$ - C_4 alkyl)COOH, $(C_1$ - C_4 alkyl)NH₂, $(C_1$ - C_4 alkyl)NHC(NH₂⁺)NH₂, $(C_0$ - C_4 alkyl)(C_3 - C_6 cycloalkyl), $(C_0$ - C_4 alkyl)(C_2 - C_5 heterocyclic), $(C_0$ - C_4 alkyl)(C_6 - C_{10} aryl)R₇, $(C_1$ - C_4 alkyl)(C_3 - C_9 heteroaryl), and C_1 - C_{12} alkyl(W₁) C_1 - C_{12} alkyl, wherein W₁ is a heteroatom selected from the group consisting of N, S and O, or R_1 and R_2 together with the atoms to which they are attached form a C_3 - C_{12} cycloalkyl; or R_4 and R_8 together with the atoms to which they are attached form a C_3 - C_6 cycloalkyl;

R_3 is selected from the group consisting of C_1 - C_{18} alkyl, $(C_1$ - C_{18} alkyl)OH, $(C_1$ - C_{18} alkyl)NH₂, $(C_1$ - C_{18} alkyl)SH, $(C_0$ - C_4 alkyl)(C_3 - C_6)cycloalkyl, $(C_0$ - C_4 alkyl)(C_2 - C_5 heterocyclic), $(C_0$ - C_4 alkyl)(C_6 - C_{10} aryl)R₇, and $(C_1$ - C_4 alkyl)(C_3 - C_9 heteroaryl) or R_4 and R_3 together with the atoms to which they are attached form a pyrrolidine ring;

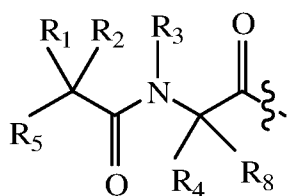
R_5 is NHR₆ or OH;

R_6 is H, C_1 - C_8 alkyl; and

R_7 is selected from the group consisting of hydrogen and OH.

In one embodiment R_{16} is a dipeptide having the general structure of Formula

25 I:



wherein

-121-

R₁ is selected from the group consisting of H and C₁-C₄ alkyl;

R₂ is selected from the group consisting of H, C₁-C₆ alkyl, C₂-C₈ alkenyl, (C₁-C₄ alkyl)OH, (C₁-C₄ alkyl)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and CH₂(C₅-C₉ heteroaryl) or R₂ and R₆ together with the

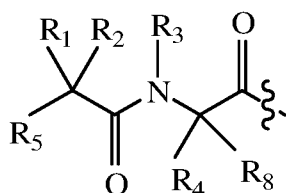
5 atoms to which they are attached form a 5 member heterocyclic ring;

R₃ is C₁-C₆ alkyl;

R₄ is selected from the group consisting of H and C₁-C₄ alkyl or R₃ and R₄ together with the atoms to which they are attached form a pyrrolidine ring; and

R₇ is selected from the group consisting of hydrogen and OH

10 In one embodiment R₁₆ is a dipeptide having the general structure of Formula I:



wherein

R₁ is C₁-C₄ alkyl or (C₁-C₄ alkyl)NH₂;

R₂ is H or is C₁-C₄ alkyl;

15 R₃ is selected from the group consisting of C₁-C₆ alkyl;

R₄ is selected from the group consisting of H, and C₁-C₄ alkyl;

R₈ is H; and

R₅ is NH₂ or OH.

In accordance with one embodiment a glucagon analog having enhanced
20 solubility relative to native glucagon is provided, wherein said analog comprises one or more amino acids selected from positions 5, 7, 8, 11 or 16 linked via an ester bond and said glucagon analog comprises the sequence

X₁X₂QGTFTSDYSKYLDX₁₆RRAX₂₀DFVX₂₄WLMX₂₈T (SEQ ID NO: 947)

X₁ is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);
25

X₂ is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α-amino-N-butyric acid;

X₁₆ is amino isobutyric acid (Aib);

30 X₂₀ is Gln, Glu, Ser, Thr, Ala, Lys, Citrulline, Arg, Orn, or Aib;

-122-

X₂₄ is Gln, Lys, Cys, Orn, homocysteine or acetyl phenylalanine; and

X₂₈ is a Asn, Arg, His, Glu, Lys or Asp,

or an analog thereof that comprises up to 10 amino acid modifications relative to the native sequence of SEQ ID NO: 947, said modifications selected from the group

5 consisting of

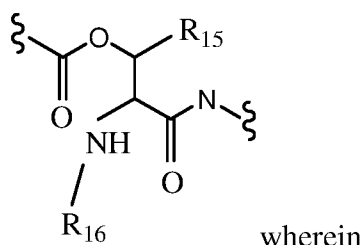
- a. substitution of Thr at position 7 with an amino acid lacking a hydroxyl group, optionally, Abu or Ile;
- b. substitution of Tyr at position 10 with Phe or Val;
- c. substitution of Lys at position 12 with Arg;
- 10 d. substitution of Asp at position 15 with Glu,
- e. substitution of Gln at position 20 with Ala or Aib;
- f. substitution of Asp at position 21 with Glu;
- g. substitution of Gln at position 24 with Ala or Aib;
- h. substitution of Met at position 27 with Leu or Nle;
- 15 i. addition of the amino acid sequence of GPSSGAPPPS (SEQ ID NO: 820) to the C-terminus, wherein the amino acid at position 29 is Thr or Gly,
- j. substitution or addition of an amino acid comprising a side chain covalently attached to an acyl or alkyl group which is non-native to a naturally-occurring amino acid;
- 20 k. a substitution of Asn at position 28 with a charged amino acid, optionally, wherein the charged amino acid is selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid;
- 25 l. an insertion of 1-3 charged amino acids after position 29; optionally, wherein the insertion comprises Glu or Lys;

or a combination thereof. In one embodiment the sequence

X₁X₂X₃GTFTSDYSKYLDX₁₆RRAX₂₀DFVX₂₄WLMX₂₈T (SEQ ID NO: 942)

30 comprises an ester linked amino acid comprising the structure of

-123-



R₁₅ is H or CH₃ and

R₁₆ is an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or

- 5 diketomorpholine. In one embodiment the ester linked amino acid is at position 16. In one embodiment R₁₆ is a dipeptide that is susceptible to cleavage by Dipeptidyl Peptidase IV.

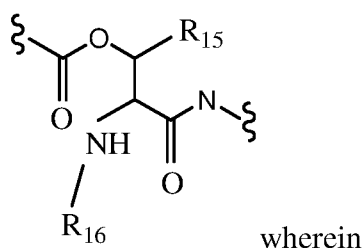
In accordance with one embodiment a glucagon analog having enhanced solubility relative to native glucagon is provided wherein, said analog comprising one
10 or more amino acids selected from positions 5, 7, 8, 11 or 16 linked via an ester bond and said glucagon analog comprises the sequence

HX₂QGTFTSDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 948) wherein

X₂ is selected from the group consisting of Ser, D-Ser, Ala, dAla, Gly, N-methyl-Ser, Aib, Val, or α-amino-N-butyric acid. In one embodiment

- 15 X₂ is Aib or D-Ser. In one embodiment X₂ is Aib. In accordance with one embodiment the ester linked amino acid is at position 7, 8, 11 or 16. In one embodiment the ester linked amino acid is at position 8, 11 or 16. In one embodiment the ester linked amino acid is at position 11 or 16. In one embodiment the ester linked amino acid is at 16.

- 20 In one embodiment the ester linked amino acid has the general structure of Formula V:



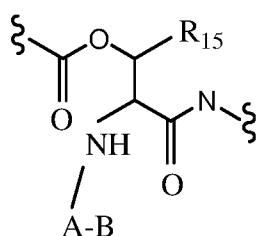
R₁₅ is H or CH₃; and

- 25 R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a

-124-

diketopiperazine or diketomorpholine. In accordance with one embodiment R_{16} is a dipeptide of the general formula X-Pro, wherein X is any amino acid and the proline is linked to the primary amine of the ester linked amino acid. In one embodiment R_{16} is selected from the group consisting of Gly-Pro, Lys-Pro and Lys- Azetidine-2-carboxylic acid wherein the proline or Azetidine-2-carboxylic acid (Azetidine) is linked to the primary amine of the ester linked amino acid.

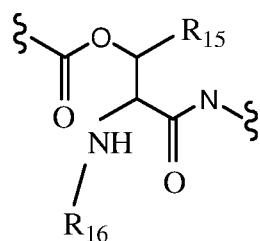
In one embodiment the ester linked amino acid has the general structure of Formula V:



wherein

- 10 R_{15} is H or CH_3 ;
 A is proline or azetidine and
 B is any amino acid, optionally selected from the group consisting of Lys, Glu, Asp, and Ala. In one embodiment A is an N-alkylated amino acid, optionally alkylated with a C_1 - C_4 alkyl and B is any amino acid, optionally lysine.
- 15 In one embodiment a glucagon analog having improved solubility relative to native glucagon is provided wherein the glucagon analog comprises an amino acid sequence of NH_2 -Xaa-Xaa-Gln-Gly-Xaa-Phe- Xaa-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Xaa-Arg-Arg-Ala-Xaa-Asp-Phe-Val-Xaa-Trp-Leu-Met-Xaa-Xaa-R (SEQ ID NO: 1298) wherein
- 20 the Xaa at position 1 is Tyr, His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA),
 the Xaa at position 2 is Ser, D-Ser or Aib,
 the Xaa at position 5 and 7 are independently Thr or an ester linked amino
 25 acid having the general structure of Formula V:

-125-



wherein

R_{15} is H or CH_3 with the proviso that at least one of the positions is an ester linked amino acid having the general structure of Formula V,

the Xaa at position 15 is selected from the group of amino acids consisting of
 5 Asp, Glu, cysteic acid, homoglutamic acid and homocysteic acid,

the Xaa at position 16 is selected from the group of amino acids consisting of Aib, Ser, Glu, Gln, homoglutamic acid, homocysteic acid and an amino acid of Formula V,

the Xaa at position 20 is Aib, Gln or Lys,

10 the Xaa at position 24 is Gln or Glu,

the Xaa at position 28 is Asn, Lys or an acidic amino acid,

the Xaa at position 29 is Thr, Gly or an acidic amino acid, and R is $COOH$ or $CONH_2$, with the proviso that when position 16 is serine, position 24 is Glu and either position 20 or position 28 is Lys. In one embodiment the Xaa at position 28 is Asn

15 and the Xaa at position 29 is Thr or Gly.

In one embodiment a glucagon analog having improved solubility relative to native glucagon is provided wherein the glucagon analog comprises an amino acid sequence selected from the group consisting of

$X_1X_2QGX_5FX_7SDYSKYLDSRRAQDFVQWLMNT$ (SEQ ID NO: 1279);

20 $HX_2QGX_5FX_7SDYSKYLDSRRAQDFVQWLMNT$ (SEQ ID NO: 1278);

$X_1X_2QGTFTSDYSKYLDX_{16}RRAQDFVQWLMNT$ (SEQ ID NO: 1281);

$HX_2QGTFTSDYSKYLDX_{16}RRAQDFVQWLMNT$ (SEQ ID NO: 1280);

and

$HSQGX_5FX_7SDYSKYLDSRRAQDFVQWLMNT$ (SEQ ID NO: 1282)

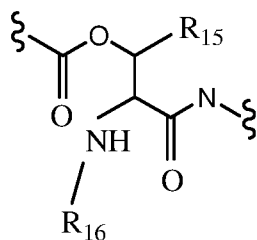
25 wherein

X_1 is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X_2 is selected from the group consisting of Ser, D-Ser, and Aib;

-126-

X₅ and X₇ are independently Thr or an ester linked amino acid having the general structure of Formula V:



wherein

R₁₅ is H or CH₃ with the proviso that at least one of X₅ and X₇ is an ester linked

5 amino acid having the general structure of Formula V; and

R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine; and

X₁₆ is an ester linked amino acid having the general structure of Formula V, wherein

10 R₁₅ is H or CH₃; and

R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine, with the proviso that at least one of X₅ and X₇ is an ester linked amino acid.

15 In one embodiment a glucagon analog having improved solubility relative to native glucagon is provided wherein the glucagon analog comprises an amino acid sequence selected from the group consisting of

HX₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1278);

HX₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1280);

20 and

HSQGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1282)

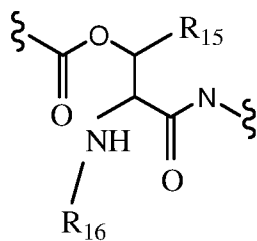
wherein

X₂ is selected from the group consisting of Ser, D-Ser, and Aib;

X₅ and X₇ are independently Thr or an ester linked amino acid having the

25 general structure of Formula V:

-127-



wherein

R₁₅ is H or CH₃ with the proviso that at least one of X₅ and X₇ is an ester linked amino acid having the general structure of Formula V; and

R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine; and

X₁₆ is an ester linked amino acid having the general structure of Formula V, wherein

R₁₅ is H or CH₃; and

R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine, with the proviso that at least one of X₅ and X₇ is an ester linked amino acid.

In one embodiment a glucagon analog having improved solubility relative to native glucagon is provided wherein the glucagon analog comprises an amino acid sequence selected from the group consisting of

HX₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1278);

HX₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1280);

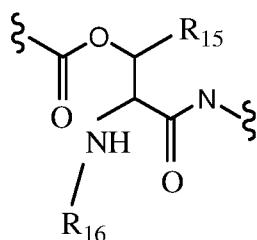
and

HSQGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1282)

wherein

X₂ is selected from the group consisting of Ser, D-Ser, and Aib;

X₅ and X₇ are independently Thr or an ester linked amino acid having the general structure of Formula V:



wherein

25

-128-

R₁₅ is H or CH₃; and

R₁₆ is a dipeptide selected from the group consisting of KZ, KP, EZ, AP, and EP, wherein Z is azetidine, with the proviso that one of X₅ and X₇ is an ester linked amino acid having the general structure of Formula V; and

5 X₁₆ is an ester linked amino acid having the general structure of Formula V, wherein R₁₅ is H or CH₃; and

R₁₆ is a dipeptide selected from the group consisting of KZ, KP, EZ, AP, and EP, wherein Z is azetidine, with the proviso that at least one of X₅ and X₇ is an ester linked amino acid.

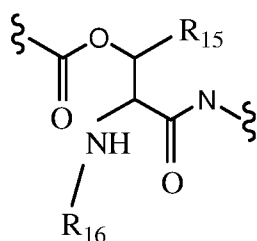
10 In a further embodiment the glucagon analog comprises an amino acid sequence selected from the group consisting of
HX₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1278);
and

HSQGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1282)

15 wherein

X₂ is selected from the group consisting of Ser, D-Ser, and Aib, optionally X₂ is Aib; optionally X₂ is D-Ser;

X₅ and X₇ are independently Thr or an ester linked amino acid having the general structure of Formula V:



20 wherein

R₁₅ is H or CH₃; and

R₁₆ is Lys-Pro or Lys- Azetidine-2-carboxylic acid, with the proviso that at least one of X₅ and X₇ is an ester linked amino acid, optionally wherein X₅ is threonine and the ester linked amino acid is at position 7 or alternatively, optionally
25 wherein X₇ is threonine and the ester linked amino acid is at position 5.

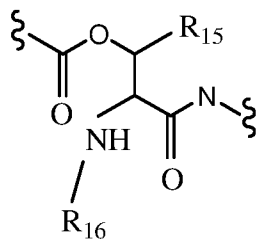
In a further embodiment the glucagon analog comprises an amino acid sequence selected from the group consisting of
HX₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1280); and
HSQGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1283)

-129-

wherein

X₂ is selected from the group consisting of Ser, D-Ser, and Aib, optionally X₂ is Aib; optionally X₂ is D-Ser; and

X₁₆ is an ester linked amino acid having the general structure of Formula V:



wherein

R₁₅ is H or CH₃; and

R₁₆ is Lys-Pro or Lys- Azetidine-2-carboxylic acid, optionally wherein X₅ is threonine. In one embodiment the glucagon analog is selected from the group consisting of SEQ Nos: 935, and 1263-1276.

10

Acylation

In accordance with one embodiment, the glucagon peptide is modified to comprise an acyl group, e.g., an acyl group which is not naturally-occurring on an amino acid (e.g., an acyl group which is non-native to a naturally-occurring amino acid). The addition of an acyl group causes the peptide to have one or more of a prolonged half-life in circulation, a delayed onset of action, an extended duration of action, an improved resistance to proteases, such as DPP-IV, and increased potency at the GLP-1 and glucagon receptors. As shown herein, acylation of the glucagon peptide does not lead to decreased activity at the glucagon and GLP-1 receptors. Rather, in some instances, acylation actually increases the activity at the GLP-1 and glucagon receptors. Accordingly, the potency of the acylated analogs is comparable to the unacylated versions of the glucagon co-agonist analogs, if not enhanced.

In accordance with one embodiment, the glucagon peptide is modified to comprise an acyl group which is attached to the glucagon peptide via an ester, thioester, or amide linkage for purposes of prolonging half-life in circulation and/or delaying the onset of and/or extending the duration of action and/or improving resistance to proteases such as DPP-IV.

Acylation can be carried out at any position within the glucagon peptide, including any of positions 1-29, a position within a C-terminal extension, or the C-

25

-130-

terminal amino acid, provided that glucagon and/or GLP-1 activity is retained, if not enhanced. Nonlimiting examples include positions 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28, or 29. In specific embodiments, acylation occurs at position 10 of the glucagon peptide and the glucagon peptide lacks an intramolecular bridge, e.g., a covalent intramolecular bridge (e.g., a lactam bridge). Such acylated peptides lacking an intramolecular bridge demonstrate enhanced activity at the GLP-1 and glucagon receptors as compared to the corresponding non-acylated peptides lacking a covalent intramolecular bridge and in comparison to the corresponding peptides lacking an intramolecular bridge acylated at a position other than position 10. As shown herein, acylation at position 10 can even transform a glucagon analog having little activity at the GLP-1 receptor to a glucagon analog having activity at both the glucagon and GLP-1 receptors. Accordingly, the position at which acylation occurs can alter the overall activity profile of the glucagon analog.

Glucagon peptides may be acylated at the same amino acid position where a hydrophilic moiety is linked, or at a different amino acid position. Nonlimiting examples include acylation at position 10 and pegylation at one or more positions in the C-terminal portion of the glucagon peptide, e.g., position 24, 28 or 29, within a C-terminal extension, or at the C-terminus (e.g., through adding a C-terminal Cys or Lys).

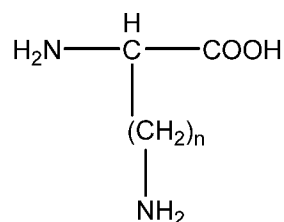
The acyl group can be covalently linked directly to an amino acid of the glucagon peptide, or indirectly to an amino acid of the glucagon peptide via a spacer, wherein the spacer is positioned between the amino acid of the glucagon peptide and the acyl group.

In a specific aspect of the invention, the glucagon peptide is modified to comprise an acyl group by direct acylation of an amine, hydroxyl, or thiol of a side chain of an amino acid of the glucagon peptide. In some embodiments, the glucagon peptide is directly acylated through the side chain amine, hydroxyl, or thiol of an amino acid. In some embodiments, acylation is at position 10, 20, 24, or 29. In this regard, the acylated glucagon peptide can comprise the amino acid sequence of SEQ ID NO : 1, or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, with at least one of the amino acids at positions 10, 20, 24, and 29 modified to any amino acid comprising a side chain amine, hydroxyl, or thiol. In some specific embodiments of the invention, the direct

-131-

acylation of the glucagon peptide occurs through the side chain amine, hydroxyl, or thiol of the amino acid at position 10.

In some embodiments, the amino acid comprising a side chain amine is an amino acid of Formula I:



5

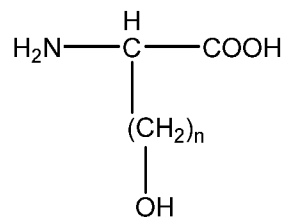
wherein $n = 1$ to 4

[Formula I]

In some exemplary embodiments, the amino acid of Formula I, is the amino acid wherein n is 4 (Lys) or n is 3 (Orn).

10

In other embodiments, the amino acid comprising a side chain hydroxyl is an amino acid of Formula II:



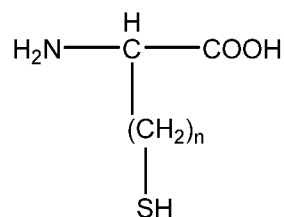
wherein $n = 1$ to 4

[Formula II]

15

In some exemplary embodiments, the amino acid of Formula II is the amino acid wherein n is 1 (Ser).

In yet other embodiments, the amino acid comprising a side chain thiol is an amino acid of Formula III:



20

wherein $n = 1$ to 4

[Formula III]

In some exemplary embodiments, the amino acid of Formula III is the amino acid wherein n is 1 (Cys).

-132-

In yet other embodiments, the amino acid comprising a side chain amine, hydroxyl, or thiol is a disubstituted amino acid comprising the same structure of Formula I, Formula II, or Formula III, except that the hydrogen bonded to the alpha carbon of the amino acid of Formula I, Formula II, or Formula III is replaced with a
5 second side chain.

In one embodiment of the invention, the acylated glucagon peptide comprises a spacer between the peptide and the acyl group. In some embodiments, the glucagon peptide is covalently bound to the spacer, which is covalently bound to the acyl group.

The amino acid to which the spacer is attached can be any amino acid (e.g., a
10 singly α -substituted amino acid or an α,α -disubstituted amino acid) comprising a moiety which permits linkage to the spacer. For example, an amino acid comprising a side chain NH_2 , $-\text{OH}$, or $-\text{COOH}$ (e.g., Lys, Orn, Ser, Asp, or Glu) is suitable. In this respect, the acylated glucagon peptide can comprise the amino acid sequence of SEQ ID NO: 1, or a modified amino acid sequence thereof comprising one or more of the
15 amino acid modifications described herein, with at least one of the amino acids at positions 10, 20, 24, and 29 modified to any amino acid comprising a side chain amine, hydroxyl, or carboxylate.

In some embodiments, the spacer is an amino acid comprising a side chain amine, hydroxyl, or thiol, or a dipeptide or tripeptide comprising an amino acid
20 comprising a side chain amine, hydroxyl, or thiol.

When acylation occurs through an amine group of a spacer, the acylation can occur through the alpha amine of the amino acid or a side chain amine. In the instance in which the alpha amine is acylated, the amino acid of the spacer can be any amino acid. For example, the amino acid of the spacer can be a hydrophobic amino
25 acid, e.g., Gly, Ala, Val, Leu, Ile, Trp, Met, Phe, Tyr, 6-amino hexanoic acid, 5-aminovaleric acid, 7-aminoheptanoic acid, 8-aminooctanoic acid. Alternatively, the amino acid of the spacer can be an acidic residue, e.g., Asp and Glu.

In the instance in which the side chain amine of the amino acid of the spacer is acylated, the amino acid of the spacer is an amino acid comprising a side chain amine,
30 e.g., an amino acid of Formula I (e.g., Lys or Orn). In this instance, it is possible for both the alpha amine and the side chain amine of the amino acid of the spacer to be acylated, such that the glucagon peptide is diacylated. Embodiments of the invention include such diacylated molecules.

-133-

The spacer (e.g., amino acid, dipeptide, tripeptide, hydrophilic or hydrophobic bifunctional spacer) in specific embodiments is 3 to 10 atoms (e.g., 6 to 10 atoms, (e.g., 6, 7, 8, 9, or 10 atoms) in length. In more specific embodiments, the spacer is about 3 to 10 atoms (e.g., 6 to 10 atoms) in length and the acyl group is a C12 to C18 fatty acyl group, e.g., C14 fatty acyl group, C16 fatty acyl group, such that the total length of the spacer and acyl group is 14 to 28 atoms, e.g., about 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 atoms. In some embodiments, the length of the spacer and acyl group is 17 to 28 (e.g., 19 to 26, 19 to 21) atoms.

The acyl group of the acylated glucagon peptide can be of any size, e.g., any length carbon chain, and can be linear or branched. In some specific embodiments of the invention, the acyl group is a C4 to C30 fatty acid. For example, the acyl group can be any of a C4 fatty acid, C6 fatty acid, C8 fatty acid, C10 fatty acid, C12 fatty acid, C14 fatty acid, C16 fatty acid, C18 fatty acid, C20 fatty acid, C22 fatty acid, C24 fatty acid, C26 fatty acid, C28 fatty acid, or a C30 fatty acid. In some embodiments, the acyl group is a C8 to C20 fatty acid, e.g., a C14 fatty acid or a C16 fatty acid.

In an alternative embodiment, the acyl group is a bile acid. The bile acid can be any suitable bile acid, including, but not limited to, cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, taurocholic acid, glycocholic acid, and cholesterol acid.

In some embodiments of the invention, the glucagon peptide is modified to comprise an acyl group by acylation of a long chain alkane by the glucagon peptide. In specific aspects, the long chain alkane comprises an amine, hydroxyl, or thiol group (e.g. octadecylamine, tetradecanol, and hexadecanethiol) which reacts with a carboxyl group, or activated form thereof, of the glucagon peptide. The carboxyl group, or activated form thereof, of the glucagon peptide can be part of a side chain of an amino acid (e.g., glutamic acid, aspartic acid) of the glucagon peptide or can be part of the peptide backbone.

With regard to these aspects of the invention, in which a long chain alkane is acylated by the glucagon peptide or the spacer, the long chain alkane may be of any size and can comprise any length of carbon chain. The long chain alkane can be linear or branched. In certain aspects, the long chain alkane is a C4 to C30 alkane. For example, the long chain alkane can be any of a C4 alkane, C6 alkane, C8 alkane,

-134-

C10 alkane, C12 alkane, C14 alkane, C16 alkane, C18 alkane, C20 alkane, C22 alkane, C24 alkane, C26 alkane, C28 alkane, or a C30 alkane. In some embodiments, the long chain alkane comprises a C8 to C20 alkane, e.g., a C14 alkane, C16 alkane, or a C18 alkane.

5 Also, in some embodiments, an amine, hydroxyl, or thiol group of the glucagon peptide is acylated with a cholesterol acid. In a specific embodiment, the glucagon peptide is linked to the cholesterol acid through an alkylated des-amino Cys spacer, i.e., an alkylated 3-mercaptopropionic acid spacer.

The acylated glucagon peptides described herein can be further modified to
10 comprise a hydrophilic moiety. In some specific embodiments the hydrophilic moiety can comprise a polyethylene glycol (PEG) chain. The incorporation of a hydrophilic moiety can be accomplished through any suitable means, such as any of the methods described herein. In this regard, the acylated glucagon peptide can comprise SEQ ID NO: 1, including any of the modifications described herein, in which at least one of
15 the amino acids at position 10, 20, 24, and 29 comprise an acyl group and at least one of the amino acids at position 16, 17, 21, 24, or 29, a position within a C-terminal extension, or the C-terminal amino acid are modified to a Cys, Lys, Orn, homo-Cys, or Ac-Phe, and the side chain of the amino acid is covalently bonded to a hydrophilic moiety (e.g., PEG). In some embodiments, the acyl group is attached to position 10,
20 optionally via a spacer comprising Cys, Lys, Orn, homo-Cys, or Ac-Phe, and the hydrophilic moiety is incorporated at a Cys residue at position 24.

Alternatively, the acylated glucagon peptide can comprise a spacer, wherein the spacer is both acylated and modified to comprise the hydrophilic moiety. Nonlimiting examples of suitable spacers include a spacer comprising one or more
25 amino acids selected from the group consisting of Cys, Lys, Orn, homo-Cys, and Ac-Phe.

Alkylation

In accordance with some embodiments, the glucagon peptide is modified to comprise an alkyl group, e.g., an alkyl group which is not naturally-occurring on an
30 amino acid (e.g., an alkyl group which is non-native to a naturally-occurring amino acid). Without being held to any particular theory, it is believed that alkylation of glucagon peptides will achieve similar, if not the same, effects as acylation of the glucagon peptides, e.g., a prolonged half-life in circulation, a delayed onset of action,

-135-

an extended duration of action, an improved resistance to proteases, such as DPP-IV, and increased potency at the GLP-1 and glucagon receptors.

Alkylation can be carried out at any position within the glucagon peptide, including any of positions 1-29, a position within a C-terminal extension, or the C-terminal amino acid, provided that the glucagon activity is retained. Nonlimiting examples include positions 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28, or 29. The alkyl group can be covalently linked directly to an amino acid of the glucagon peptide, or indirectly to an amino acid of the glucagon peptide via a spacer, wherein the spacer is positioned between the amino acid of the glucagon peptide and the alkyl group. Glucagon peptides may be alkylated at the same amino acid position where a hydrophilic moiety is linked, or at a different amino acid position.

Nonlimiting examples include alkylation at position 10 and pegylation at one or more positions in the C-terminal portion of the glucagon peptide, e.g., position 24, 28 or 29, within a C-terminal extension, or at the C-terminus (e.g., through adding a C-terminal Cys).

In a specific aspect of the invention, the glucagon peptide is modified to comprise an alkyl group by direct alkylation of an amine, hydroxyl, or thiol of a side chain of an amino acid of the glucagon peptide. In some embodiments, alkylation is at position 10, 20, 24, or 29. In this regard, the alkylated glucagon peptide can comprise the amino acid sequence of SEQ ID NO: 1, or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, with at least one of the amino acids at positions 10, 20, 24, and 29 modified to any amino acid comprising a side chain amine, hydroxyl, or thiol. In some specific embodiments of the invention, the direct alkylation of the glucagon peptide occurs through the side chain amine, hydroxyl, or thiol of the amino acid at position 10.

In one embodiment of the invention, the alkylated glucagon peptide comprises a spacer between the peptide and the alkyl group. In some embodiments, the glucagon peptide is covalently bound to the spacer, which is covalently bound to the alkyl group. In some exemplary embodiments, the glucagon peptide is modified to comprise an alkyl group by alkylation of an amine, hydroxyl, or thiol of a spacer, which spacer is attached to a side chain of an amino acid at position 10, 20, 24, or 29 of the glucagon peptide. The amino acid to which the spacer is attached can be any amino acid (e.g., a singly α -substituted amino acid or an α,α -disubstituted amino acid)

-136-

comprising a moiety which permits linkage to the spacer. For example, an amino acid comprising a side chain NH_2 , $-\text{OH}$, or $-\text{COOH}$ (e.g., Lys, Orn, Ser, Asp, or Glu) is suitable. In this respect, the alkylated glucagon peptide can comprise the amino acid sequence of SEQ ID NO: 1, or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, with at least one of the amino acids at positions 10, 20, 24, and 29 modified to any amino acid comprising a side chain amine, hydroxyl, or carboxylate.

In some embodiments, the spacer is an amino acid comprising a side chain amine, hydroxyl, or thiol or a dipeptide or tripeptide comprising an amino acid comprising a side chain amine, hydroxyl, or thiol.

When alkylation occurs through an amine group of a spacer, the alkylation can occur through the alpha amine of an amino acid or a side chain amine. In the instance in which the alpha amine is alkylated, the amino acid of the spacer can be any amino acid. For example, the amino acid of the spacer can be a hydrophobic amino acid, e.g., Gly, Ala, Val, Leu, Ile, Trp, Met, Phe, Tyr, 6-amino hexanoic acid, 5-aminovaleric acid, 7-aminoheptanoic acid, 8-aminooctanoic acid. Alternatively, the amino acid of the spacer can be an acidic residue, e.g., Asp and Glu, provided that the alkylation occurs on the alpha amine of the acidic residue. In the instance in which the side chain amine of the amino acid of the spacer is alkylated, the amino acid of the spacer is an amino acid comprising a side chain amine, e.g., an amino acid of Formula I (e.g., Lys or Orn). In this instance, it is possible for both the alpha amine and the side chain amine of the amino acid of the spacer to be alkylated, such that the glucagon peptide is dialkylated. Embodiments of the invention include such dialkylated molecules.

When alkylation occurs through a hydroxyl group of a spacer, the amino acid or one of the amino acids of the dipeptide or tripeptide can be an amino acid of Formula II. In a specific exemplary embodiment, the amino acid is Ser.

When alkylation occurs through a thiol group of spacer, the amino acid or one of the amino acids of the dipeptide or tripeptide can be an amino acid of Formula III.

In a specific exemplary embodiment, the amino acid is Cys.

The spacer (e.g., amino acid, dipeptide, tripeptide, hydrophilic or hydrophobic bifunctional spacer) in specific embodiments is 3 to 10 atoms (e.g., 6 to 10 atoms, (e.g., 6, 7, 8, 9, or 10 atoms)) in length. In more specific embodiments, the spacer is

-137-

about 3 to 10 atoms (e.g., 6 to 10 atoms) in length and the alkyl is a C12 to C18 alkyl group, e.g., C14 alkyl group, C16 alkyl group, such that the total length of the spacer and alkyl group is 14 to 28 atoms, e.g., about 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 atoms. In some embodiments, the length of the spacer and alkyl is
5 17 to 28 (e.g., 19 to 26, 19 to 21) atoms.

In accordance with certain foregoing embodiments, the bifunctional spacer can be a synthetic or non-naturally occurring amino acid comprising an amino acid backbone that is 3 to 10 atoms in length (e.g., 6-amino hexanoic acid, 5-aminovaleric acid, 7-aminoheptanoic acid, and 8-aminooctanoic acid). Alternatively, the spacer
10 can be a dipeptide or tripeptide spacer having a peptide backbone that is 3 to 10 atoms (e.g., 6 to 10 atoms) in length. The dipeptide or tripeptide spacer can be composed of naturally-occurring and/or non-naturally occurring amino acids, including, for example, any of the amino acids taught herein. In some embodiments, the spacer comprises an overall negative charge, e.g., comprises one or two negatively charged
15 amino acids. In some embodiments, the dipeptide spacer is selected from the group consisting of: Ala-Ala, β -Ala- β -Ala, Leu-Leu, Pro-Pro, γ -aminobutyric acid- γ -aminobutyric acid, and γ -Glu- γ -Glu.

Suitable methods of peptide alkylation via amines, hydroxyls, and thiols are known in the art. For example, a Williamson ether synthesis can be used to form an
20 ether linkage between a hydroxyl group of the glucagon peptide and the alkyl group. Also, a nucleophilic substitution reaction of the peptide with an alkyl halide can result in any of an ether, thioether, or amino linkage.

The alkyl group of the alkylated glucagon peptide can be of any size, e.g., any length carbon chain, and can be linear or branched. In some embodiments of the
25 invention, the alkyl group is a C4 to C30 alkyl. For example, the alkyl group can be any of a C4 alkyl, C6 alkyl, C8 alkyl, C10 alkyl, C12 alkyl, C14 alkyl, C16 alkyl, C18 alkyl, C20 alkyl, C22 alkyl, C24 alkyl, C26 alkyl, C28 alkyl, or a C30 alkyl. In some embodiments, the alkyl group is a C8 to C20 alkyl, e.g., a C14 alkyl or a C16 alkyl.

In some specific embodiments, the alkyl group comprises a steroid moiety of a
30 bile acid, e.g., cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, taurocholic acid, glycocholic acid, and cholesterol acid.

In some embodiments of the invention, the glucagon peptide is modified to comprise an alkyl group by reacting a nucleophilic, long chain alkane with the

-138-

glucagon peptide, wherein the glucagon peptide comprises a leaving group suitable for nucleophilic substitution. In specific aspects, the nucleophilic group of the long chain alkane comprises an amine, hydroxyl, or thiol group (e.g. octadecylamine, tetradecanol, and hexadecanethiol). The leaving group of the glucagon peptide can be part of a side chain of an amino acid or can be part of the peptide backbone. Suitable leaving groups include, for example, N-hydroxysuccinimide, halogens, and sulfonate esters.

In certain embodiments, the glucagon peptide is modified to comprise an alkyl group by reacting the nucleophilic, long chain alkane with a spacer which is attached to the glucagon peptide, wherein the spacer comprises the leaving group. In specific aspects, the long chain alkane comprises an amine, hydroxyl, or thiol group. In certain embodiments, the spacer comprising the leaving group can be any spacer discussed herein, e.g., amino acids, dipeptides, tripeptides, hydrophilic bifunctional spacers and hydrophobic bifunctional spacers further comprising a suitable leaving group.

With regard to these aspects of the invention, in which a long chain alkane is alkylated by the glucagon peptide or the spacer, the long chain alkane may be of any size and can comprise any length of carbon chain. The long chain alkane can be linear or branched. In certain aspects, the long chain alkane is a C₄ to C₃₀ alkane. For example, the long chain alkane can be any of a C₄ alkane, C₆ alkane, C₈ alkane, C₁₀ alkane, C₁₂ alkane, C₁₄ alkane, C₁₆ alkane, C₁₈ alkane, C₂₀ alkane, C₂₂ alkane, C₂₄ alkane, C₂₆ alkane, C₂₈ alkane, or a C₃₀ alkane. In some embodiments, the long chain alkane comprises a C₈ to C₂₀ alkane, e.g., a C₁₄ alkane, C₁₆ alkane, or a C₁₈ alkane.

Also, in some embodiments, alkylation can occur between the glucagon peptide and a cholesterol moiety. For example, the hydroxyl group of cholesterol can displace a leaving group on the long chain alkane to form a cholesterol-glucagon peptide product.

The alkylated glucagon peptides described herein can be further modified to comprise a hydrophilic moiety. In some specific embodiments the hydrophilic moiety can comprise a polyethylene glycol (PEG) chain. The incorporation of a hydrophilic moiety can be accomplished through any suitable means, such as any of the methods described herein. In this regard, the alkylated glucagon peptide can comprise SEQ ID

-139-

NO: 1 or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, in which at least one of the amino acids at position 10, 20, 24, and 29 comprise an alkyl group and at least one of the amino acids at position 16, 17, 21, 24, and 29, a position within a C-terminal extension or the C-terminal amino acid are modified to a Cys, Lys, Orn, homo-Cys, or Ac-Phe, and the side chain of the amino acid is covalently bonded to a hydrophilic moiety (e.g., PEG). In some embodiments, the alkyl group is attached to position 10, optionally via a spacer comprising Cys, Lys, Orn, homo-Cys, or Ac-Phe, and the hydrophilic moiety is incorporated at a Cys residue at position 24.

Alternatively, the alkylated glucagon peptide can comprise a spacer, wherein the spacer is both alkylated and modified to comprise the hydrophilic moiety. Nonlimiting examples of suitable spacers include a spacer comprising one or more amino acids selected from the group consisting of Cys, Lys, Orn, homo-Cys, and Ac-Phe.

Conjugates

The present disclosure also encompasses other conjugates in which glucagon peptides of the invention are linked, optionally via covalent bonding and optionally via a linker, to a conjugate moiety. Linkage can be accomplished by covalent chemical bonds, physical forces such electrostatic, hydrogen, ionic, van der Waals, or hydrophobic or hydrophilic interactions. A variety of non-covalent coupling systems may be used, including biotin-avidin, ligand/receptor, enzyme/substrate, nucleic acid/nucleic acid binding protein, lipid/lipid binding protein, cellular adhesion molecule partners; or any binding partners or fragments thereof which have affinity for each other.

The peptide can be linked to conjugate moieties via direct covalent linkage by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of these targeted amino acids. Reactive groups on the peptide or conjugate moiety include, e.g., an aldehyde, amino, ester, thiol, α -haloacetyl, maleimido or hydrazino group. Derivatizing agents include, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride or other agents known in the art. Alternatively, the conjugate moieties can be linked to the peptide indirectly through

-140-

intermediate carriers, such as polysaccharide or polypeptide carriers. Examples of polysaccharide carriers include aminodextran. Examples of suitable polypeptide carriers include polylysine, polyglutamic acid, polyaspartic acid, co-polymers thereof, and mixed polymers of these amino acids and others, e.g., serines, to confer desirable
5 solubility properties on the resultant loaded carrier.

Exemplary conjugate moieties that can be linked to any of the glucagon peptides described herein include but are not limited to a heterologous peptide or polypeptide (including for example, a plasma protein), a targeting agent, an immunoglobulin or portion thereof (e.g. variable region, CDR, or Fc region), a
10 diagnostic label such as a radioisotope, fluorophore or enzymatic label, a polymer including water soluble polymers, or other therapeutic or diagnostic agents. In one embodiment a conjugate is provided comprising a glucagon peptide of the present invention and a plasma protein, wherein the plasma protein is selected from the group consisting of albumin, transferrin, fibrinogen and globulins. In one embodiment the
15 plasma protein moiety of the conjugate is albumin or transferrin. In some embodiments, the linker comprises a chain of atoms from 1 to about 60, or 1 to 30 atoms or longer, 2 to 5 atoms, 2 to 10 atoms, 5 to 10 atoms, or 10 to 20 atoms long. In some embodiments, the chain atoms are all carbon atoms. In some embodiments, the chain atoms in the backbone of the linker are selected from the group consisting of
20 C, O, N, and S. Chain atoms and linkers may be selected according to their expected solubility (hydrophilicity) so as to provide a more soluble conjugate. In some embodiments, the linker provides a functional group that is subject to cleavage by an enzyme or other catalyst or hydrolytic conditions found in the target tissue or organ or cell. In some embodiments, the length of the linker is long enough to reduce the
25 potential for steric hindrance. If the linker is a covalent bond or a peptidyl bond and the conjugate is a polypeptide, the entire conjugate can be a fusion protein. Such peptidyl linkers may be any length. Exemplary linkers are from about 1 to 50 amino acids in length, 5 to 50, 3 to 5, 5 to 10, 5 to 15, or 10 to 30 amino acids in length. Such fusion proteins may alternatively be produced by recombinant genetic
30 engineering methods known to one of ordinary skill in the art.

As noted above, in some embodiments, the glucagon peptides are conjugated, e.g., fused to an immunoglobulin or portion thereof (e.g. variable region, CDR, or Fc region). Known types of immunoglobulins (Ig) include IgG, IgA, IgE, IgD or IgM.

-141-

The Fc region is a C-terminal region of an Ig heavy chain, which is responsible for binding to Fc receptors that carry out activities such as recycling (which results in prolonged half-life), antibody dependent cell-mediated cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC).

- 5 For example, according to some definitions the human IgG heavy chain Fc region stretches from Cys226 to the C-terminus of the heavy chain. The "hinge region" generally extends from Glu216 to Pro230 of human IgG1 (hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by aligning the cysteines involved in cysteine bonding). The Fc region of an IgG includes two constant
- 10 domains, CH2 and CH3. The CH2 domain of a human IgG Fc region usually extends from amino acids 231 to amino acid 341. The CH3 domain of a human IgG Fc region usually extends from amino acids 342 to 447. References made to amino acid numbering of immunoglobulins or immunoglobulin fragments, or regions, are all based on Kabat et al. 1991, Sequences of Proteins of Immunological Interest, U.S.
- 15 Department of Public Health, Bethesda, Md. In a related embodiment, the Fc region may comprise one or more native or modified constant regions from an immunoglobulin heavy chain, other than CH1, for example, the CH2 and CH3 regions of IgG and IgA, or the CH3 and CH4 regions of IgE.

- Suitable conjugate moieties include portions of immunoglobulin sequence that
- 20 include the FcRn binding site. FcRn, a salvage receptor, is responsible for recycling immunoglobulins and returning them to circulation in blood. The region of the Fc portion of IgG that binds to the FcRn receptor has been described based on X-ray crystallography (Burmeister et al. 1994, Nature 372:379). The major contact area of the Fc with the FcRn is near the junction of the CH2 and CH3 domains. Fc-FcRn
- 25 contacts are all within a single Ig heavy chain. The major contact sites include amino acid residues 248, 250-257, 272, 285, 288, 290-291, 308-311, and 314 of the CH₂ domain and amino acid residues 385-387, 428, and 433-436 of the CH3 domain.

- Some conjugate moieties may or may not include FcγR binding site(s). FcγR are responsible for ADCC and CDC. Examples of positions within the Fc region that
- 30 make a direct contact with FcγR are amino acids 234-239 (lower hinge region), amino acids 265-269 (B/C loop), amino acids 297-299 (C'/E loop), and amino acids 327-332 (F/G) loop (Sondermann et al., Nature 406: 267-273, 2000). The lower hinge region of IgE has also been implicated in the FcRI binding (Henry, et al., Biochemistry 36,

-142-

15568-15578, 1997). Residues involved in IgA receptor binding are described in Lewis et al., (J Immunol. 175:6694-701, 2005). Amino acid residues involved in IgE receptor binding are described in Sayers et al. (J Biol Chem. 279(34):35320-5, 2004).

Amino acid modifications may be made to the Fc region of an immunoglobulin. Such variant Fc regions comprise at least one amino acid modification in the CH3 domain of the Fc region (residues 342-447) and/or at least one amino acid modification in the CH2 domain of the Fc region (residues 231-341). Mutations believed to impart an increased affinity for FcRn include T256A, T307A, E380A, and N434A (Shields et al. 2001, J. Biol. Chem. 276:6591). Other mutations may reduce binding of the Fc region to FcγRI, FcγRIIA, FcγRIIB, and/or FcγRIIIA without significantly reducing affinity for FcRn. For example, substitution of the Asn at position 297 of the Fc region with Ala or another amino acid removes a highly conserved N-glycosylation site and may result in reduced immunogenicity with concomitant prolonged half-life of the Fc region, as well as reduced binding to FcγRs (Routledge et al. 1995, Transplantation 60:847; Friend et al. 1999, Transplantation 68:1632; Shields et al. 1995, J. Biol. Chem. 276:6591). Amino acid modifications at positions 233-236 of IgG1 have been made that reduce binding to FcγRs (Ward and Ghetie 1995, Therapeutic Immunology 2:77 and Armour et al. 1999, Eur. J. Immunol. 29:2613). Some exemplary amino acid substitutions are described in US Patents 7,355,008 and 7,381,408, each incorporated by reference herein in its entirety.

The present disclosure also encompasses glucagon fusion peptides or proteins wherein a second peptide or polypeptide has been fused to a terminus, e.g., the carboxy terminus of the glucagon peptide. Exemplary candidates for C-terminal fusion include SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) linked to amino acid 29 of the glucagon peptide.

In one embodiment a method of reducing weight gain or inducing weight loss in an individual comprises administering an effective amount of a composition comprising a glucagon agonist having an ester linked amino acid at position 5, 7, 8 11 or 16 and the sequence of SEQ ID NO: 33 wherein amino acid 29 of the glucagon peptide is optionally bound to a second peptide through a peptide bond, and said second peptide comprises the sequence of SEQ ID NO: 820 (GPSSGAPPPS) or SEQ ID NO: 823. In one embodiment the glucagon peptide is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID

-143-

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 and SEQ ID NO: 13 modified by the replacement of the amide bond at position 5, 7, 8 11 or 16 with an ester bond, further wherein amino acid 29 of the glucagon peptide is optionally bound to a second peptide through a peptide bond, and said second peptide comprises the sequence of SEQ ID NO: 820 (GPSSGAPPPS) or SEQ ID NO: 823. In one embodiment the glucagon peptide of the glucagon agonist is selected from the group consisting of SEQ ID NO: 11 and SEQ ID NO: 13, further modified to comprise 1, 2, or 3 ester linked amino acids.

The present disclosure also encompasses multimers of the modified glucagon peptides disclosed herein. Two or more of the modified glucagon peptides can be linked together using standard linking agents and procedures known to those skilled in the art. For example, dimers can be formed between two modified glucagon peptides through the use of bifunctional thiol crosslinkers and bi-functional amine crosslinkers, particularly for the glucagon peptides that have been substituted with cysteine, lysine ornithine, homocysteine or acetyl phenylalanine residues (e.g. SEQ ID NO: 4 and SEQ ID NO: 5). The dimer can be a homodimer or alternatively can be a heterodimer.

In accordance with one embodiment a pharmaceutical composition is provided wherein the composition comprises a glucagon agonist analog of the present disclosure, or pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier. The pharmaceutical composition can comprise any pharmaceutically acceptable ingredient, including, for example, acidifying agents, additives, adsorbents, aerosol propellants, air displacement agents, alkalizing agents, anticaking agents, anticoagulants, antimicrobial preservatives, antioxidants, antiseptics, bases, binders, buffering agents, chelating agents, coating agents, coloring agents, desiccants, detergents, diluents, disinfectants, disintegrants, dispersing agents, dissolution enhancing agents, dyes, emollients, emulsifying agents, emulsion stabilizers, fillers, film forming agents, flavor enhancers, flavoring agents, flow enhancers, gelling agents, granulating agents, humectants, lubricants, mucoadhesives, ointment bases, ointments, oleaginous vehicles, organic bases, pastille bases, pigments, plasticizers, polishing agents, preservatives, sequestering agents, skin penetrants, solubilizing agents, solvents, stabilizing agents, suppository bases, surface active agents, surfactants, suspending agents, sweetening agents, therapeutic agents,

thickening agents, tonicity agents, toxicity agents, viscosity-increasing agents, water-absorbing agents, water-miscible cosolvents, water softeners, or wetting agents.

In some embodiments, the pharmaceutical composition comprises any one or a combination of the following components: acacia, acesulfame potassium,

- 5 acetyltributyl citrate, acetyltriethyl citrate, agar, albumin, alcohol, dehydrated alcohol, denatured alcohol, dilute alcohol, aleuritic acid, alginic acid, aliphatic polyesters, alumina, aluminum hydroxide, aluminum stearate, amylopectin, α -amylase, ascorbic acid, ascorbyl palmitate, aspartame, bacteriostatic water for injection, bentonite, bentonite magma, benzalkonium chloride, benzethonium chloride, benzoic acid,
- 10 benzyl alcohol, benzyl benzoate, bronopol, butylated hydroxyanisole, butylated hydroxytoluene, butylparaben, butylparaben sodium, calcium alginate, calcium ascorbate, calcium carbonate, calcium cyclamate, dibasic anhydrous calcium phosphate, dibasic dehydrate calcium phosphate, tribasic calcium phosphate, calcium propionate, calcium silicate, calcium sorbate, calcium stearate, calcium sulfate,
- 15 calcium sulfate hemihydrate, canola oil, carbomer, carbon dioxide, carboxymethyl cellulose calcium, carboxymethyl cellulose sodium, β -carotene, carrageenan, castor oil, hydrogenated castor oil, cationic emulsifying wax, cellulose acetate, cellulose acetate phthalate, ethyl cellulose, microcrystalline cellulose, powdered cellulose, silicified microcrystalline cellulose, sodium carboxymethyl cellulose, cetostearyl
- 20 alcohol, cetrimide, cetyl alcohol, chlorhexidine, chlorobutanol, chlorocresol, cholesterol, chlorhexidine acetate, chlorhexidine gluconate, chlorhexidine hydrochloride, chlorodifluoroethane (HCFC), chlorodifluoromethane, chlorofluorocarbons (CFC) chlorophenoxyethanol, chloroxyleneol, corn syrup solids, anhydrous citric acid, citric acid monohydrate, cocoa butter, coloring agents, corn oil,
- 25 cottonseed oil, cresol, m-cresol, o-cresol, p-cresol, croscarmellose sodium, crospovidone, cyclamic acid, cyclodextrins, dextrans, dextrin, dextrose, dextrose anhydrous, diazolidinyl urea, dibutyl phthalate, dibutyl sebacate, diethanolamine, diethyl phthalate, difluoroethane (HFC), dimethyl- β -cyclodextrin, cyclodextrin-type compounds such as Captisol®, dimethyl ether, dimethyl phthalate, dipotassium
- 30 edentate, disodium edentate, disodium hydrogen phosphate, docusate calcium, docusate potassium, docusate sodium, dodecyl gallate, dodecyltrimethylammonium bromide, edentate calcium disodium, edtic acid, eglumine, ethyl alcohol, ethylcellulose, ethyl gallate, ethyl laurate, ethyl maltol, ethyl oleate, ethylparaben,

-145-

ethylparaben potassium, ethylparaben sodium, ethyl vanillin, fructose, fructose liquid, fructose milled, fructose pyrogen-free, powdered fructose, fumaric acid, gelatin, glucose, liquid glucose, glyceride mixtures of saturated vegetable fatty acids, glycerin, glyceryl behenate, glyceryl monooleate, glyceryl monostearate, self-emulsifying

5 glyceryl monostearate, glyceryl palmitostearate, glycine, glycols, glycofurol, guar gum, heptafluoropropane (HFC), hexadecyltrimethylammonium bromide, high fructose syrup, human serum albumin, hydrocarbons (HC), dilute hydrochloric acid, hydrogenated vegetable oil, type II, hydroxyethyl cellulose, 2-hydroxyethyl- β -cyclodextrin, hydroxypropyl cellulose, low-substituted hydroxypropyl cellulose, 2-

10 hydroxypropyl- β -cyclodextrin, hydroxypropyl methylcellulose, hydroxypropyl methylcellulose phthalate, imidurea, indigo carmine, ion exchangers, iron oxides, isopropyl alcohol, isopropyl myristate, isopropyl palmitate, isotonic saline, kaolin, lactic acid, lactitol, lactose, lanolin, lanolin alcohols, anhydrous lanolin, lecithin, magnesium aluminum silicate, magnesium carbonate, normal magnesium carbonate,

15 magnesium carbonate anhydrous, magnesium carbonate hydroxide, magnesium hydroxide, magnesium lauryl sulfate, magnesium oxide, magnesium silicate, magnesium stearate, magnesium trisilicate, magnesium trisilicate anhydrous, malic acid, malt, maltitol, maltitol solution, maltodextrin, maltol, maltose, mannitol, medium chain triglycerides, meglumine, menthol, methylcellulose, methyl

20 methacrylate, methyl oleate, methylparaben, methylparaben potassium, methylparaben sodium, microcrystalline cellulose and carboxymethylcellulose sodium, mineral oil, light mineral oil, mineral oil and lanolin alcohols, oil, olive oil, monoethanolamine, montmorillonite, octyl gallate, oleic acid, palmitic acid, paraffin, peanut oil, petrolatum, petrolatum and lanolin alcohols, pharmaceutical glaze, phenol,

25 liquified phenol, phenoxyethanol, phenoxypropanol, phenylethyl alcohol, phenylmercuric acetate, phenylmercuric borate, phenylmercuric nitrate, polacrillin, polacrillin potassium, poloxamer, polydextrose, polyethylene glycol, polyethylene oxide, polyacrylates, polyethylene-polyoxypropylene-block polymers, polymethacrylates, polyoxyethylene alkyl ethers, polyoxyethylene castor oil

30 derivatives, polyoxyethylene sorbitol fatty acid esters, polyoxyethylene stearates, polyvinyl alcohol, polyvinyl pyrrolidone, potassium alginate, potassium benzoate, potassium bicarbonate, potassium bisulfite, potassium chloride, potassium citrate, potassium citrate anhydrous, potassium hydrogen phosphate, potassium metabisulfite,

-146-

monobasic potassium phosphate, potassium propionate, potassium sorbate, povidone, propanol, propionic acid, propylene carbonate, propylene glycol, propylene glycol alginate, propyl gallate, propylparaben, propylparaben potassium, propylparaben sodium, protamine sulfate, rapeseed oil, Ringer's solution, saccharin, saccharin ammonium, saccharin calcium, saccharin sodium, safflower oil, saponite, serum

5 proteins, sesame oil, colloidal silica, colloidal silicon dioxide, sodium alginate, sodium ascorbate, sodium benzoate, sodium bicarbonate, sodium bisulfite, sodium chloride, anhydrous sodium citrate, sodium citrate dehydrate, sodium chloride, sodium cyclamate, sodium edentate, sodium dodecyl sulfate, sodium lauryl sulfate,

10 sodium metabisulfite, sodium phosphate, dibasic, sodium phosphate, monobasic, sodium phosphate, tribasic, anhydrous sodium propionate, sodium propionate, sodium sorbate, sodium starch glycolate, sodium stearyl fumarate, sodium sulfite, sorbic acid, sorbitan esters (sorbitan fatty esters), sorbitol, sorbitol solution 70%, soybean oil, spermaceti wax, starch, corn starch, potato starch, pregelatinized starch, sterilizable

15 maize starch, stearic acid, purified stearic acid, stearyl alcohol, sucrose, sugars, compressible sugar, confectioner's sugar, sugar spheres, invert sugar, *Sugartab*, Sunset Yellow FCF, synthetic paraffin, talc, tartaric acid, tartrazine, tetrafluoroethane (HFC), theobroma oil, thimerosal, titanium dioxide, alpha tocopherol, tocopheryl acetate, alpha tocopheryl acid succinate, beta-tocopherol, delta-tocopherol, gamma-

20 tocopherol, tragacanth, triacetin, tributyl citrate, triethanolamine, triethyl citrate, trimethyl- β -cyclodextrin, trimethyltetradecylammonium bromide, tris buffer, trisodium edentate, vanillin, type I hydrogenated vegetable oil, water, soft water, hard water, carbon dioxide-free water, pyrogen-free water, water for injection, sterile water for inhalation, sterile water for injection, sterile water for irrigation, waxes, anionic

25 emulsifying wax, carnauba wax, cationic emulsifying wax, cetyl ester wax, microcrystalline wax, nonionic emulsifying wax, suppository wax, white wax, yellow wax, white petrolatum, wool fat, xanthan gum, xylitol, zein, zinc propionate, zinc salts, zinc stearate, or any excipient in the *Handbook of Pharmaceutical Excipients*, Third Edition, A. H. Kibbe (Pharmaceutical Press, London, UK, 2000), which is

30 incorporated by reference in its entirety. *Remington's Pharmaceutical Sciences*, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980), which is incorporated by reference in its entirety, discloses various components used in formulating pharmaceutically acceptable compositions and known techniques for the

-147-

preparation thereof. Except insofar as any conventional agent is incompatible with the pharmaceutical compositions, its use in pharmaceutical compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

5 The pharmaceutical formulations disclosed herein may be designed to be short-acting, fast-releasing, long-acting, or sustained-releasing as described below. The pharmaceutical formulations may also be formulated for immediate release, controlled release or for slow release. The instant compositions may further comprise, for example, micelles or liposomes, or some other encapsulated form, or
10 may be administered in an extended release form to provide a prolonged storage and/or delivery effect. The disclosed pharmaceutical formulations may be administered according to any regime including, for example, daily (1 time per day, 2 times per day, 3 times per day, 4 times per day, 5 times per day, 6 times per day), every two days, every three days, every four days, every five days, every six days,
15 weekly, bi-weekly, every three weeks, monthly, or bi-monthly.

 The pharmaceutical compositions may be formulated to achieve a physiologically compatible pH. In some embodiments, the pH of the pharmaceutical composition may be at least 5, at least 5.5, at least 6, at least 6.5, at least 7, at least 7.5, at least 8, at least 8.5, at least 9, at least 9.5, at least 10, or at least 10.5 up to and
20 including pH 11, depending on the formulation and route of administration. In certain embodiments, the pharmaceutical compositions may comprise one or more buffering agents to achieve a physiological compatible pH. The buffering agents may include any compounds capable of buffering at the desired pH such as, for example, phosphate buffers (e.g. PBS), triethanolamine, Tris, bicine, TAPS, tricine, HEPES,
25 TES, MOPS, PIPES, cacodylate, MES, and others. In certain embodiments, the strength of the buffer is at least 0.5 mM, at least 1 mM, at least 5 mM, at least 10 mM, at least 20 mM, at least 30 mM, at least 40 mM, at least 50 mM, at least 60 mM, at least 70 mM, at least 80 mM, at least 90 mM, at least 100 mM, at least 120 mM, at least 150 mM, or at least 200 mM. In some embodiments, the strength of the buffer is
30 no more than 300 mM (e.g. at most 200 mM, at most 100 mM, at most 90 mM, at most 80 mM, at most 70 mM, at most 60 mM, at most 50 mM, at most 40 mM, at most 30 mM, at most 20 mM, at most 10 mM, at most 5 mM, at most 1 mM).

-148-

In one embodiment the pharmaceutical composition comprises a 1 mg/ml concentration of the glucagon peptide analog and 10-50 mM Triethanolamine at pH 7.0-8.5, or 6-9, or 7-9. In one embodiment the pharmaceutical composition comprises a 1 mg/ml concentration of the glucagon agonist analog and 20 mM Triethanolamine at pH 8.5.

Any of the modified glucagon peptides as disclosed herein can be provided in accordance with one embodiment as part of a kit. In one embodiment a kit for administering a glucagon agonist to a patient in need thereof is provided wherein the kit comprises any of the glucagon peptides of the invention in aqueous solution.

Exemplary glucagon peptides for inclusion in such kits include a glucagon peptide selected from the group consisting of 1) a glucagon peptide comprising the sequence of SEQ ID NO: 7, SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 13 or SEQ ID NO: 33; 2) a glucagon fusion peptide comprising a glucagon agonist analog of SEQ ID NO: 11 or SEQ ID NO: 13 or SEQ ID NO: 33, and an amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) linked to amino acid 29 of the glucagon peptide; and 3) a pegylated glucagon peptide of SEQ ID NO: 11 or SEQ ID NO: 13 or SEQ ID NO: 33, optionally further comprising an amino acid sequence of SEQ ID NO: 820 (GPSSGAPPPS), SEQ ID NO: 821 (KRNRNNIA) or SEQ ID NO: 822 (KRNR) linked to amino acid 29 of the glucagon peptide, wherein the PEG chain covalently bound to position 16, 17, 21, 24 or 29, within a C-terminal extension, or at the C-terminal amino acid has a molecular weight of about 500 to about 40,000 Daltons, wherein the glucagon peptide is modified to comprise one or more ester linked amino acids. In one embodiment the kit is provided with a device for administering the glucagon composition to a patient, e.g. syringe needle, pen device, jet injector or other needle-free injector. The kit may alternatively or in addition include one or more of a variety of containers, e.g., vials, tubes, bottles, single or multi-chambered pre-filled syringes, cartridges, infusion pumps (external or implantable), jet injectors, pre-filled pen devices and the like, optionally containing the glucagon peptide in a lyophilized form or in aqueous solution. Preferably, the kits will also include instructions for use. In accordance with one embodiment the device of the kit is an aerosol dispensing device, wherein the composition is prepackaged within the aerosol device. In another embodiment the kit comprises a syringe and a needle, and in one embodiment the

-149-

sterile glucagon composition is prepackaged within the syringe. In one embodiment a composition is provided comprising a glucagon peptide analog having enhance solubility as disclosed herein and an insulin peptide.

The compounds of this invention may be prepared by standard synthetic methods, recombinant DNA techniques, or any other methods of preparing peptides and fusion proteins. Although certain non-natural amino acids cannot be expressed by standard recombinant DNA techniques, techniques for their preparation are known in the art. Compounds of this invention that encompass non-peptide portions may be synthesized by standard organic chemistry reactions, in addition to standard peptide chemistry reactions when applicable.

Exemplary Embodiments

In embodiment 1 a glucagon peptide analog is provided having enhanced solubility relative to native glucagon, said analog comprising one or more amino acids selected from positions 5, 7, 8, 11 or 16 linked to the preceding amino acid in the peptide chain via an ester bond. Optionally, one or more amino acids selected from positions 5, 7 or 16 are linked to the preceding amino acid in the peptide chain via an ester bond. In one embodiment a glucagon analog having improved solubility is provided wherein the glucagon analog differs from SEQ ID NO: 1 by 1, 2, or 3 amino acids selected from positions 5, 7 or 16 being linked to the preceding amino acid in the peptide chain via an ester bond.

In accordance with embodiment 2 the analog of embodiment 1 is provided wherein an amino acid comprising a hydroxyl group bearing side chain is present at position 7, 8, 11 or 16, and an ester bond is formed between the hydroxyl group on the side chain of the amino acid at position 7, 8, 11 or 16 and the carboxylate of the amino acid at position 6, 7, 10 or 15, respectively.

In accordance with embodiment 3 an analog of any one of embodiments 1 or 2 is provided wherein the alpha amine of the ester linked amino acid is

a) covalently bound to an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase; or

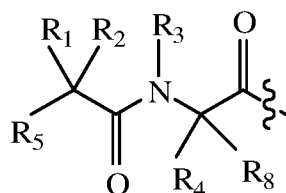
b) covalently linked to a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine.

-150-

In accordance with embodiment 4 an analog of any one of embodiments 1-3 is provided wherein the alpha amine of the ester linked amino acid is covalently bound to a dipeptide and said dipeptide is susceptible to cleavage by Dipeptidyl Peptidase IV.

5 In accordance with embodiment 5 an analog of embodiment 4 is provided wherein said dipeptide is Lys-Pro or Lys- Azetidine-2-carboxylic acid.

In accordance with embodiment 6 an analog of embodiment 4 is provided wherein said dipeptide has the general structure of Formula I:



10 wherein

R₁ is selected from the group consisting of H and C₁-C₄ alkyl;

R₂ and R₄ are independently selected from the group consisting of H, C₁-C₆ alkyl, C₂-C₈ alkenyl, (C₁-C₄ alkyl)OH, (C₁-C₄ alkyl)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and CH₂(C₅-C₉ heteroaryl);

15 R₃ is C₁-C₆ alkyl or R₃ and R₄ together with the atoms to which they are attached form a pyrrolidine ring; and

R₇ is selected from the group consisting of hydrogen and OH.

In accordance with embodiment 7 an analog of embodiments 6 is provided wherein

20 R₁ is C₁-C₄ alkyl or (C₁-C₄ alkyl)NH₂;

R₂ is H or is C₁-C₄ alkyl;

R₃ is C₁-C₆ alkyl;

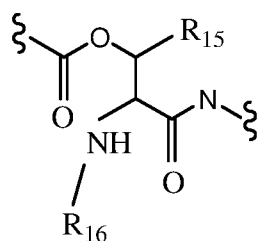
R₄ is H or C₁-C₄ alkyl;

R₈ is H; and

25 R₅ is NH₂ or OH.

In accordance with embodiment 8 an analog of any one of embodiments 1-7 is provided wherein said ester linked amino acid has the general structure of Formula V:

-151-



wherein

R₁₅ is H or CH₃; and

R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a

5 diketopiperazine or diketomorpholine.

In accordance with embodiment 9 an analog of any one of embodiments 1-8 is provided wherein the amino acid of Formula V is at position 16.

In accordance with embodiment 10 an analog of any one of embodiments 1-9 is provided wherein the analog comprises the sequence

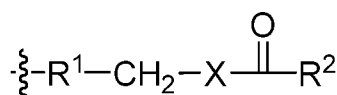
10 X₁X₂X₃GTFTSDX₁₀SX₁₂YLY₁₅X₁₆X₁₇X₁₈AX₂₀X₂₁FVX₂₄WLX₂₇-Z (SEQ ID NO: 943)

wherein

X₁ is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-
15 His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

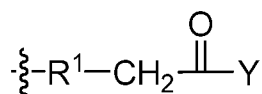
X₂ is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α-amino-N-butyric acid;

X₃ is an amino acid comprising a side chain of Structure I, II, or III:

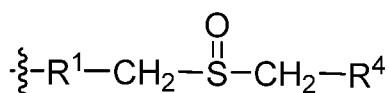


20

Structure I



Structure II



Structure III

25

wherein R¹ is C₀₋₃ alkyl or C₀₋₃ heteroalkyl; R² is NHR⁴ or C₁₋₃ alkyl; R³ is C₁₋₃ alkyl; R⁴ is H or C₁₋₃ alkyl; X is NH, O, or S; and Y is NHR⁴, SR³, or OR³;

-152-

X₁₀ is Tyr or Lys;

X₁₂ is Lys, Ile or Arg;

X₁₅ is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid;

X₁₆ is Glu, Lys, Asp, Ser, glutamine, homoglutamic acid, homocysteic acid,

5 Thr or Aib;

X₁₇ is Arg or Gln;

X₁₈ is Ala, Arg

X₂₀ is Gln, Glu, Ser, Thr, Ala, Lys, Citrulline, Arg, Orn, or Aib;

X₂₁ is Glu, Aib, Asp, Lys, Cys, Orn, homocysteine or acetyl phenylalanine;

10 X₂₄ is Asn, Aib, Gln, Glu or Lys;

X₂₇ is Met, Leu or Nle;

X₂₈ is Asn or Ala;

X₂₉ is Thr or Gly; and

Z is selected from the group consisting of -COOH, -X₂₈-COOH, X₂₈-X₂₉-

15 COOH, and X₂₈-X₂₉-GPSSGAPPPS SEQ ID NO: 1299).

In accordance with embodiment 11 an analog of any one of embodiments 1-10 is provided wherein the analog comprises

(i) a lactam bridge between the side chains of amino acids at positions i and i + 4, wherein i is 12, 16, 20 or 24, or

20 (ii) one, two, three, or all of the amino acids at positions 16, 20, 21, and 24 of the glucagon peptide is substituted with an α , α -disubstituted amino acid.

In accordance with embodiment 12 an analog of any one of embodiments 1-9 is provided wherein the analog comprises the sequence

YAQGTFTSDX₁₀SKYLDERAAQDFVQWLLEGGPSSGAPPPS-NH₂ (SEQ ID NO:

25 945) or

YX₂EGTFTSDX₁₀SIYLDKQAAX₂₀EFVNWLLAGGPSSGAPPPS (SEQ ID NO:

944)

wherein

X₂ is Ser, D-Ser or Aib;

30 X₁₀ is Lys acylated with a C16 to C18 carbon atom chain, optionally via a γ Glu linker; and

X₂₀ is Gln or Aib.

-153-

In accordance with embodiment 13 an analog of any one of embodiments 1-9 is provided wherein the analog comprises the sequence

$X_1X_2QGTFTSDYSKYLX_{15}X_{16}RRAX_{20}DFVX_{24}WLMX_{28}T$ (SEQ ID NO: 946)

wherein

5 X_1 is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X_2 is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;

10 X_{15} is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid;

X_{16} is Glu, Lys, Asp, Ser, glutamine, homoglutamic acid, homocysteic acid, Thr or Aib;

X_{20} is Gln or Lys;

X_{24} is Gln or Glu;

15 X_{28} is Asn, Asp or Lys;

or an analog of SEQ ID NO: 946 that differs from SEQ ID NO: 946 by 1 to 2 amino acid modifications, selected from positions 1, 2, 7, 10, 11, 13, 14, 17, 18, 19, 21, and 27, wherein the glucagon peptide exhibits enhanced activity at the GLP-1 receptor as compared to native glucagon,

20 wherein the side chains of the amino acids at positions 12 and 16, positions 16 and 20, positions 20 and 24, or positions 24 and 28 of the glucagon peptide are linked by covalent bonds to provide the enhanced activity at the GLP-1 receptor, with the proviso that when the amino acid at position 16 is serine either position 20 is lysine, or the side chain of the amino acid at position 24 and the side chain of either the
25 amino acid at position 20 or position 28 are linked by covalent bonds.

In accordance with embodiment 14 an analog of any one of embodiments 1-11 is provided wherein the analog comprises the sequence

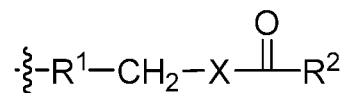
$X_1X_2X_3GTFTSDYSKYLDX_{16}RRAX_{20}DFVX_{24}WLMX_{28}T$ (SEQ ID NO: 942)

30 X_1 is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X_2 is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;

-154-

X₃ is an amino acid comprising a side chain of Structure I:



Structure I

wherein R¹ is C₀₋₃ alkyl or C₀₋₃ heteroalkyl; R² is NHR⁴ or C₁₋₃ alkyl;

5 R⁴ is H or C₁₋₃ alkyl; and X is NH, O, or S;

X₁₆ is amino isobutyric acid (Aib);

X₂₀ is selected from the group consisting of Asp, Lys, Cys, Orn, Aib, homocystein and acetyl phenylalanine;

10 X₂₄ is selected from the group consisting of Gln, Lys, Cys, Orn, homocysteine and acetyl phenylalanine; and

X₂₈ is selected from the group consisting of Asn, Arg, His, Glu, Lys and Asp, or an analog thereof that comprises up to 10 amino acid modifications relative to the native sequence of SEQ ID NO: 942, said modifications selected from the group consisting of

15 m. substitution of Thr at position 7 with an amino acid lacking a hydroxyl group, optionally, Abu or Ile;

n. substitution of Tyr at position 10 with Phe or Val;

o. substitution of Lys at position 12 with Arg;

p. substitution of Asp at position 15 with Glu,

20 q. substitution of Gln at position 20 with Ala or Aib;

r. substitution of Asp at position 21 with Glu;

s. substitution of Gln at position 24 with Ala or Aib;

t. substitution of Met at position 27 with Leu or Nle;

25 u. addition of the amino acid sequence of GPSSGAPPPS (SEQ ID NO: 820) to the C-terminus, wherein the amino acid at position 29 is Thr or Gly,

v. substitution or addition of an amino acid comprising a side chain covalently attached to an acyl or alkyl group which is non-native to a naturally-occurring amino acid;

30 w. a substitution of Asn at position 28 with a charged amino acid, optionally, wherein the charged amino acid is selected from the group

-155-

consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid;

x. an insertion of 1-3 charged amino acids after position 29; optionally, wherein the insertion comprises Glu or Lys;

5 or a combination thereof, wherein

one or more amino acids selected from positions 5 and 7 are linked to the preceding amino acid in the peptide chain via an ester bond.

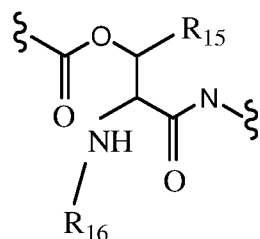
In accordance with embodiment 15 an analog of any one of embodiments 1-9 is provided wherein the analog comprises the sequence

10 HX₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1278)

wherein

X₂ is selected from the group consisting of Ser, D-Ser, and Aib;

X₅ and X₇ are independently Thr or an ester linked amino acid having the general structure of Formula V:



15 wherein

R₁₅ is H or CH₃; and

R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine, with the proviso that at least one of X₅ and X₇ is an ester linked amino acid.

20 In accordance with embodiment 16 an analog of any one of embodiments 1-15 is provided wherein

R₁₆ is Lys-Pro or Lys- Azetidine-2-carboxylic acid.

25 In accordance with embodiment 17 an analog of any one of embodiments 1-16 is provided wherein X₂ is Aib.

In accordance with embodiment 18 an analog of any one of embodiments 1-17 is provided wherein X₅ is threonine and the ester linked amino acid is at position 7.

-156-

In accordance with embodiment 19 an analog of any one of embodiments 1-18 is provided wherein the analog comprises an amino acid sequence selected from the group consisting of

X₁X₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1279);

5 HX₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1278);

X₁X₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1281);

HX₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1280);

and

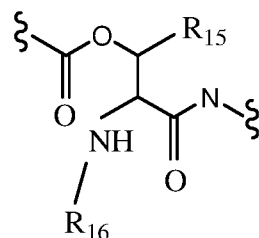
HSQGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1282)

10 wherein

X₁ is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X₂ is selected from the group consisting of Ser, D-Ser, and Aib;

15 X₅ and X₇ are independently Thr or an ester linked amino acid having the general structure of Formula V:



wherein

R₁₅ is H or CH₃ with the proviso that at least one of X₅ and X₇ is an ester linked amino acid having the general structure of Formula V; and

20 R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine; and

X₁₆ is an ester linked amino acid having the general structure of Formula V.

25 In accordance with embodiment 20 an analog of any one of embodiments 1-19 is provided wherein X₂ is Ser.

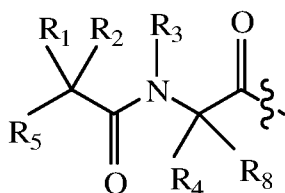
In accordance with embodiment 21 an analog of any one of embodiments 1-20 is provided wherein the analog comprises an amino acid sequence of

X₁X₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1281).

-157-

In accordance with embodiment 22 an analog of any one of embodiments 1-21 is provided wherein R₁₆ comprises a dipeptide selected from the group consisting of Lys-Pro and Lys-Azetidine-2-carboxylic acid.

In accordance with embodiment 23 an analog of any one of embodiments 1-21 is provided wherein R₁₆ comprises a dipeptide of Formula I:



wherein

R₁ is selected from the group consisting of H and C₁-C₄ alkyl;

R₂ and R₄ are independently selected from the group consisting of H, C₁-C₆ alkyl, C₂-C₈ alkenyl, (C₁-C₄ alkyl)OH, (C₁-C₄ alkyl)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and CH₂(C₅-C₉ heteroaryl);

R₃ is C₁-C₆ alkyl or R₃ and R₄ together with the atoms to which they are attached form a pyrrolidine ring; and

R₇ is selected from the group consisting of hydrogen and OH.

In accordance with embodiment 24 an analog of any one of embodiments 1-23 is provided, wherein the glucagon peptide is covalently linked to a hydrophilic moiety, optionally wherein the hydrophilic moiety is a polyethylene glycol (PEG).

In accordance with embodiment 25 an analog of any one of embodiments 1-24 is provided wherein the glucagon peptide is covalently linked to a non-native acyl or alkyl group.

In accordance with embodiment 26 a pharmaceutical composition comprising an analog of any one of embodiments 1-25 is provided comprising the analog and a pharmaceutically acceptable carrier.

In accordance with embodiment 27 a method of reducing weight gain or inducing weight loss in a patient in need thereof, or treating or preventing hypoglycemia in a patient in need thereof is provided wherein the method comprises administering to the patient a pharmaceutical composition of embodiment 27 in an amount effective to reduce weight gain or induce weight loss in the patient.

-158-

EXAMPLE 1

General Synthesis Protocol:

Glucagon analogs were synthesized using HBTU-activated "Fast Boc" single coupling starting from 0.2 mmole of Boc Thr(OBzl)Pam resin on a modified Applied Biosystem 430 A peptide synthesizer. Boc amino acids and HBTU were obtained from Midwest Biotech (Fishers, IN). Side chain protecting groups used were: Arg(Tos), Asn(Xan), Asp(OcHex), Cys(pMeBzl), His(Bom), Lys(2Cl-Z), Ser(OBzl), Thr(OBzl), Tyr(2Br-Z), and Trp(CHO). The side-chain protecting group on the N-terminal His was Boc.

Each completed peptidyl resin was treated with a solution of 20% piperidine in dimethylformamide to remove the formyl group from the tryptophan. Liquid hydrogen fluoride cleavages were performed in the presence of p-cresol and dimethyl sulfide. The cleavage was run for 1 hour in an ice bath using an HF apparatus (Penninsula Labs). After evaporation of the HF, the residue was suspended in diethyl ether and the solid materials were filtered. Each peptide was extracted into 30-70 ml aqueous acetic acid and a diluted aliquot was analyzed by HPLC [Beckman System Gold, 0.46x5cm Zorbax C8, 1ml/min, 45C, 214nm, A buffer =0.1%TFA, B=0.1%TFA/90%acetonitrile, gradient of 10% to 80%B over 10min].

Purification was done on a FPLC over a 2.2 x 25 cm Kromasil C18 column while monitoring the UV at 214nm and collecting 5 minute fractions. The homogeneous fractions were combined and lyophilized to give a product purity of >95%. The correct molecular mass and purity were confirmed using MALDI-mass spectral analysis.

General Pegylation Protocol: (Cys-maleimido)

Typically, the glucagon Cys analog is dissolved in phosphate buffered saline (5-10mg/ml) and 0.01M ethylenediamine tetraacetic acid is added (10-15% of total volume). Excess (2-fold) maleimido methoxyPEG reagent (Nektar) is added and the reaction stirred at room temp while monitoring reaction progress by HPLC. After 24hrs, the reaction mixture, is acidified and loaded onto a preparative reverse phase column for purification using 0.1%TFA/acetonitrile gradient. The appropriate fractions were combined and lyophilized to give the desired pegylated analogs.

-159-

EXAMPLE 2

Glucagon Receptor Binding Assay

The affinity of peptides to the glucagon receptor was measured in a competition binding assay utilizing scintillation proximity assay technology. Serial 3-fold dilutions of the peptides made in scintillation proximity assay buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% w/v bovine serum albumin) were mixed in 96 well white/clear bottom plate (Corning Inc., Acton, MA) with 0.05 nM (3-[¹²⁵I]-iodotyrosyl) Tyr10 glucagon (Amersham Biosciences, Piscataway, NJ), 1-6 micrograms per well, plasma membrane fragments prepared from cells over-expressing human glucagon receptor, and 1 mg/well polyethyleneimine-treated wheat germ agglutinin type A scintillation proximity assay beads (Amersham Biosciences, Piscataway, NJ). Upon 5 min shaking at 800 rpm on a rotary shaker, the plate was incubated 12h at room temperature and then read on MicroBeta1450 liquid scintillation counter (Perkin-Elmer, Wellesley, MA). Non-specifically bound (NSB) radioactivity was measured in the wells with 4 times greater concentration of “cold” native ligand than the highest concentration in test samples and total bound radioactivity was detected in the wells with no competitor. Percent specific binding was calculated as following: % Specific Binding = ((Bound-NSB)/(Total bound-NSB)) X 100. IC₅₀ values were determined by using Origin software (OriginLab, Northampton, MA).

EXAMPLE 3

Functional Assay- cAMP Synthesis

The ability of glucagon analogs to induce cAMP was measured in a firefly luciferase-based reporter assay. HEK293 cells co-transfected with either glucagon- or GLP-1 receptor and luciferase gene linked to cAMP responsive element were serum deprived by culturing 16h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 0.25% Bovine Growth Serum (HyClone, Logan, UT) and then incubated with serial dilutions of either glucagon, GLP-1 or novel glucagon analogs for 5 h at 37°C, 5% CO₂ in 96 well poly-D-Lysine-coated “Biocoat” plates (BD Biosciences, San Jose, CA). At the end of the incubation 100 microliters of LucLite luminescence substrate reagent (Perkin-Elmer, Wellesley, MA) were added to each well. The plate was shaken briefly, incubated 10 min in the dark and light output was measured on

-160-

MicroBeta-1450 liquid scintillation counter (Perkin-Elmer, Wellesley, MA). The luminescent light output indicates activation of the luciferase reporter gene, which in turn is a measure of the activation of the receptor. Effective 50% concentrations (“EC50”) were calculated by using Origin software (OriginLab, Northampton, MA).

5 EC50 is the concentration of the peptide that produces 50% of the peptide’s maximum activation response at the indicated receptor. A relatively lower EC50 indicates that a peptide is relatively more potent at that receptor, while a higher EC50 indicates that a peptide is less potent.

10 Example 4

Activities of Depsi--Glucagon Analogs

Analogues of glucagon were prepared to investigate whether depsi-bond pro-drugs can be designed that will convert within minutes at physiological pH to native hormone, and also possess sufficient solubility and stability as an aqueous, ready to
15 use formulation.

Of the seven alcohols in the glucagon sequence four are serine and three are threonine residues. Five of these seven amino acids are positioned in the midsection of the peptide. Our results illustrate that introduction of a single O-acyl isopeptide unit for either a serine or threonine amino acid produces a depsi-peptide that readily
20 converts *in vivo* to generate pharmacology that is virtually indistinguishable from the native hormone. Furthermore, the ester bond can be stabilized by judicious selection of chemical sequence and position such that a slightly acidic aqueous solution can be formulated and stored for extended periods. Enzymatically-labile protection of the depsi-peptide alpha-amine is also shown to provide much enhanced stability for those
25 formulations requiring extended or higher temperature stability, without any apparent change in pharmacodynamic action.

Methods

General peptide synthesis. The preparation of peptides (except **13-15**) were
30 conducted on automated peptide synthesizer CSBio (model CS336X) or ABI433 with standard Fmoc/*t*Bu solid-phase protocols (except the installation of depsi segments) utilizing DIC (Sigma-Aldrich) and 6-Cl-HOBt (Aapptec) coupling chemistry. Peptides **2-8**, **10-12** and **16-17** were synthesized as a C-terminus acid on pre-loaded

-161-

Fmoc-Thr(*t*Bu)-Wang resin (Aapptec, Louisville, KY) while peptide **9** was prepared as a C-terminus amide on H-Rink Amide-ChemMatrix resin (PCAS BioMatrix Saint-Jean-sur-Richelieu, Quebec Canada). The side chain protecting group scheme consisted of Arg(Pbf); Asp(O*t*Bu); Asn(Trt); Gln(Trt); Glu(O*t*Bu); His(Trt);
5 Lys(Boc); Ser(*t*Bu); Thr(*t*Bu); Tyr(*t*Bu); and Trp(Boc). All conventional amino acids were purchased from Midwest Biotech (Fisher, IN).

Depsi-peptide formation, (Peptides **2-7**): Amino acid residues up to Ser or Thr involved in the depsi bond were assembled as described above. The resulting resin
10 was treated with free side-chain-containing Boc-Ser-OH or Boc-Thr-OH (10 equivalents excess), DEPBT (10 equivalents excess) and DIPEA (10 equivalents excess) in DMF for 20 - 60 minutes followed by wash with DMF. In a separate vial, Fmoc-protected amino acids (Gly, Phe or Asp(O*t*Bu) - 20 equivalents excess) and DIC (20 equivalents excess) were mixed in DCM and DMF (4:1) for 5 minutes and
15 the resulting solution was transferred to the resin followed by the addition of DMAP (0.2 equivalent). The resin slurry was agitated at room temperature for 16-24 hours. Completion of reaction was confirmed by small scale TFA resin-cleavage. The remaining portion of peptide was assembled by automated procedure, as described above.

20

Depsi-peptide formation with secondary trigger, Method 1 [Fig.7A] (Peptides **8-12** and **16-17**): Amino acid residues up to Threonine at position 16 or 5 involved in depsi bond formation were assembled as described above in "General peptide synthesis" section with Fmoc-Thr(Trt) incorporated at the position of depsi-bond. The
25 resulting resin was treated with 20% piperidine in DMF for 20 minutes followed by DMF wash. For analogs **8** and **9**, N-terminus amine was capped by acetylation with acetic anhydride (4 mL) and pyridine (2 mL) in DMF for 1 hour. Alternatively for analogs **10-12**, two consecutive coupling steps utilizing described above DEPBT/DIPEA coupling chemistry was performed to obtain dipeptide extensions
30 Boc-Lys(Boc)-Aze, Boc-Lys(Boc)-Pro or Boc-Glu(O*t*Bu)-Aze. For analogs **16** and **17** single coupling of Boc-Arg(Pbf) or pGlu with DEPBT/DIPEA chemistry was performed. After DMF and DCM washes, resin was treated with 1% TFA in DCM

-162-

for 10-20 minutes (2 times). The subsequent depsi-bond formation was achieved as described above.

Depsi-peptide formation with secondary trigger, Method 2 [Fig. 7B] (Analog 13-

5 **15)**: Glucagon amino acid residues N28 to S8 were assembled on CSBio peptide synthesizer using Boc/Bzl protection scheme and DEPBT/DIPEA coupling chemistry on pre-loaded Boc-Thr(Bzl)-PAM resin (MidWest). The side chain protecting group scheme consisted of Arg(Tos); Asp(OBzl); Asn(Xan); Gln(Xan); Glu(OBzl); His(Tos); Lys(2-Cl-Z); Ser(Bzl); Thr(Bzl); Tyr(2-Br-Z); and Trp(Formyl). Upon N-terminus Boc-deprotection with TFA and subsequent DCM and DMF wash, the resulting resin was treated with Boc-Thr(Fmoc-Phe)-OH isoacyl dipeptide (Aapptech) (5 equivalents excess), DEPBT (5 equivalents excess) and DIPEA (5 equivalents excess) in DMF for 1-2 hours. N-terminus Boc protection from Thr7 was removed with neat TFA (2 time flash with slow drain ~ 2 minutes) followed by excessive DCM wash. Coupling of Boc-Aze or Boc-Pro was performed with 20 equivalents excess of amino acid and DEPBT and 5 equivalents excess DIPEA in DMF. Reaction was run for 10-30 minutes. Addition of following Boc-Lys(Boc), Boc-Ala or Boc-Glu(OBzl) was performed with standard 10 equivalent excess of amino acid, DEPBT and DIPEA for 30 minutes as described before. Segment H1-T5 was extended into Fmoc-F6-
20 depis-T7(aa1';aa2')S8-T29 fragment on CSBio peptide synthesizer using Fmoc-Thr(Bzl), Fmoc-Gly, Fmoc-Gln(Xan), Fmoc-Ser(Bzl) and Boc-His(Toc) with standard DEPBT/DIPEA chemistry and 20% piperidine in DMF used for Fmoc deprotection. The final resin was treated with neat TFA (2 times), washed with DCM and dried under vacuum prior to HF cleavage.

25

Peptides (except **13-15**) were cleaved from the resin and deprotected by treatment with TFA containing 3% TIS (Sigma-Aldrich), 3% water, 2.5% phenol, 1% DODT (Sigma-Aldrich) and 0.5% of Me₂S (Sigma-Aldrich) for 2 hours. Peptides **13-15** were cleaved in HF at 0 °C for 1 hour in presence of p-cresol as scavenger. Peptides were
30 purified by preparative RP-HPLC on an Amberchrom-XT20 (21.2 x 250 mm, DOW) and/or Kinetex C8 (AXIA packed, 21.2 x 250 mm, 5 µm, Phenomenex) column with 0.05% TFA/H₂O and 0.05% TFA/CH₃CN as elution buffers. Native glucagon (Eli Lilly and Co., Indianapolis, IN) was re-purified under the above conditions to ensure

-163-

identical counter-ion content. Purified peptides were analyzed and characterized by LC-MS (1260 Infinity-6120 Quadrupole LCMS, Agilent) on Kinetex C8 (4.6x75 mm, 2.6 μ m, Phenomenex) with 0.05% TFA/H₂O and 0.05% TFA/CH₃CN as eluents employing 5% B to 70% B in 15 minutes gradient with 2.5 minutes delay or 20% B to 50% B in 10 minutes. Peptide's concentration was assessed based on UV absorption at λ = 280 nm measured on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Extinction coefficients at λ = 280 nm were calculated using on-line Peptide Property Calculator (Innovagen, PepCalc.com).

- 10 **Kinetics of O-to-N acyl shift.** Approximately 0.5 mg of lyophilized peptide powder was treated with 500 μ L (2 μ L for analogs **4** and **6**) of PBS (pH 7.4) equilibrated at 37 $^{\circ}$ C. Solutions were kept at 37 $^{\circ}$ C intermittently vortexing. Aliquots of the reaction solutions were quenched into 1% TFA in water equilibrated at 0 $^{\circ}$ C. LC-MS analysis was performed employing 20% to 50% B gradient over ten minutes. Integrated area under the peak was determined and plotted as $\ln[(\text{Area of Depsi})/(\text{Area of Depsi} + \text{native})]$ against time. Linear regression was performed in Origin software and half-time of O-to-N acyl shift reaction was calculated based on equation $t_{1/2} = [\ln(2) / (\text{slope})] \times (-1)$. Experiment for analogs **2**, **3**, **4** and **5** was repeated at pH 5 (citric acid/disodium phosphate buffer) at room temperature. LC-MS and statistical analysis were as described in PBS, pH 7.4 at 37 $^{\circ}$ C experiment.
- 15
- 20

Stability study 1. ~1 mg of peptides was dissolved in ~1 mL of filter-sterilized PBS (pH 7.4 at room temperature). Peptides solutions were stored at room temperature in sterile cell culture hood and analyzed by LC-MS (20% to 50% B gradient over ten minutes) after quenching in 1% aq. TFA.

25

Stability study 2. Peptides were dissolved at high concentration (~5 mg/mL) with 0.1N HCl. The solution was filter sterilized and concentration was assessed by UV absorbance at λ = 280 nm as measured on NanoDrop spectrophotometer. Buffers solutions were prepared from 0.1M citric acid and 0.2 M Na₂HPO₄ mixed accordingly to obtain pH 3 (80/20) pH 4 (61/39), pH 5 (49/51), pH 6 (37/63) and pH 7 (18/82) buffers. Buffers were filter-sterilized through 0.22 μ m filter membrane prior to each experiment. Solutions of peptides in each buffer at 0.5 mg/mL was prepared and

30

-164-

divided into 3 portions ~1 mL/each in 1.5 mL non-silylated glass vial. Vials were tightly close with cap and paraffin film and incubated at 4 °C (cold room), room temperature (23 °C) and on hot-plate set at 40 °C with intermittent vortexing (1/day). Samples were analyzed by LC-MS with 5% to 70% B in 15 minutes linear after 2.5
5 minutes initial isocratic period.

Aggregation study. Fibrillation was measured according to modified Thioflavin-T fluorescence assay protocol. 8 mg of Thioflavin-T (ThT) was dissolved in 10 mL of phosphate buffer (50 mM sodium phosphate, 150 mM of sodium chloride, pH 7.4).
10 The solution was filtered through 0.22 mm syringe filter and stored at 4 °C in dark. Prior to experiment the 0.3 mL of ThT stock solution was further diluted in 15 mL of the phosphate buffer. 50 mL of peptide solution at 0.5 mg/mL derived from the pH-dependent stability experiment (Stability study 2) was added to 300 mL of working solution of ThT in phosphate buffer. The solution was incubated for 20 to 30 minutes.
15 The fluorescence intensity was measured on the Perkin-Elmer LS50B Luminescence Spectrometer (Perkin-Elmer, Waltham, MA) using following experimental parameters: 350 mL of peptide/ThT solution in sub-micro quartz cuvette [3 x 3 x 3 mm / Z = 9.85 (Hellna GmnG & Co. KG, Mullheim, DE)] excitation λ = 450 nm (slit width 5nm); emission λ = 482 nm (slit width 10 nm) integration 10 second, summed
20 from 60 seconds.

Solubility determination. To 1-2 mg of peptide in Eppendorf tube 200-500 mL of buffer was added. Buffers used were PBS (pH 7.4), 25 mM sodium phosphate (pH 7.7), 50 mM sodium phosphate with 150 mM sodium chloride (pH 7.7), 0.1 M citric
25 acid and 0.2 M disodium phosphate (pH 5, 6 and 7. Each was prepared according to the ratios presented in “Stability study 2” section). The amount of buffer added was kept below the volume needed to fully dissolve the peptides which resulted cloudy solutions with incompletely dissolved peptides. Samples were vortexed and sonicated for 5 minutes then equilibrated at room temperature for 1 hour, followed by
30 centrifugation at 10,000 rpm for 10 minutes. The concentration of dissolved peptide in supernatant was assessed based on UV absorbance at λ = 280 nm and calculated extinction coefficient.

-165-

Glucagon Receptor-mediated cAMP accumulation assay. The glucagon-induced cAMP production was measured in HEK293 cells over-expressing the glucagon receptor and a luciferase reporter gene linked to cAMP responsive element. The cells were serum deprived for 16 hours and then incubated with serial dilutions of glucagon analogs for 5 hours at 37 °C, 5% CO₂ in 96 well poly-D-Lysine-coated “Biocoat” plates (BD Biosciences, San Jose, CA). At the end of the incubation period 50 µL LucLite luminescence substrate reagent (Perkin-Elmer, Waltham, MA) was added to each well. The plate was shaken briefly, incubated for 10 minutes in the dark and light output was measured on MicroBeta-1450 liquid scintillation counter. Each peptide was run in duplicate. Origin software was used to calculate effective 50 % concentrations (EC₅₀) and standard deviation using sigmoidal fit with logistic function, Levenberg-Marguardt integration algorithm and statistical weight assigned to each data point.

Rat Studies. All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati. Male Wistar rats (Harlan, IN) were housed on a 12 : 12 hours light-dark cycle (8 am - 8 pm lights on) at 22 °C and constant humidity with free access to standard chow (Teklad LM-485) and water, except as noted. Animal’s age and body weight: Fig. 5A & 5B - 561.6 ± 40.1 g; Fig. 14 - 399.3 ± 36.8 g; Fig. 15 - 285.3 ± 16.1 g; Fig. 16 and 386.8 ± 28.7 g; Fig. 13 - 439.6 ± 39.3 g. The food was removed at the onset of the light phase, 3 hours prior the intraperitoneal administration of the compounds. The blood glucose level was determined at the intervals indicated using a handheld glucometer (Freestyle, Abbot). The statistical analysis of the results obtained in the *in vivo* experiments was performed using Prism 6.0 h (GraphPad Software, CA) applying One-way ANOVA followed by Dunnett’s tests, using the insulin group as control. P values lower than 0.05 were considered significant (** P<0.01; *** P<0.001). The results are presented as means ± SEM of eight replicates per group. Vehicle and dosage used in experiments: Fig. 5A – Vehicle: 0.01N HCl, peptides **2**, **3** and **5** at 10 nmol/kg; Fig. 14 – Vehicle (I): 50 mM sodium phosphate with 150mM sodium chloride pH 7.4, Vehicle (II): 0.01N HCl, peptides **1**, **5** and **10** at 10 nmol/kg; Fig. 15 - Vehicle: 0.01N HCl, peptides **1**, **4**, **13** and **15** at 10 nmol/kg; Fig. 13 - Vehicle: 0.01N HCl, peptides **5**, **10**, **16** and **17** at 10 nmol/kg; Fig. 16 - Vehicle: PBS,

-166-

analog **15** at 10 nmol/kg by s.c. injection, Sitagliptin (ApexBio) in PBS at 3, 10 and 30 mg/kg administered orally by gavage; Fig. 17 – Vehicle: PBS, GLP1 analogs at 10 nmol/kg by s.c. injection, Sitagliptin (ApexBio) in PBS at 30 mg/kg administered orally by gavage.

5

Abbreviations. Fmoc, fluorenylmethyloxycarbonyl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; OtBu, tert-butyl ester; tBu, tert-butyl ether; Trt, trityl; Boc, tert-butyloxycarbonyl; 2-Cl-Z, 2-chlorobenzyloxycarbonyl; 2-Br-Z, 2-bromobenzyloxycarbonyl; OBzl, benzyl ester; Bzl, benzyl ether; Tos, tosyl; Xan, 9-xanthenyl; DIC, N,N'-diisopropylcarbodiimide 6-Cl-HOBt, 1-Hydroxy-6-chloro-benzotriazole; DEPBT, (3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one); DIPEA, N,N-diisopropylethylamine; TFA, trifluoroacetic acid; pGlu, L-pyroglutamic acid; TIS, triisopropylsilane; DODT, [2,2'-(Ethylenedioxy)-diethenethiol]; Aze, azetidine.

- 15 Native Glucagon: SEQ ID NO: 1
 Cmp 2: [D15:S16], SEQ ID NO: 935;
 Cmp 3: [D15:T16], SEQ ID NO: 1263;
 Cmp 4: [F6:T7], SEQ ID NO: 1264;
 Cmp 5: [G4:T5], SEQ ID NO: 1265;
 20 Cmp 6: [F15:T16], SEQ ID NO: 1269;
 Cmp 7: [D6:T7], SEQ ID NO: 1270;
 Cmp 8: [D15:T16(Ac)], SEQ ID NO: 1266;
 Cmp 9: [G4:T5(Ac)-am], SEQ ID NO: 1277;
 Cmp 10: [G4:T5(KZ)], SEQ ID NO: 1267;
 25 Cmp 11: [G4:T5(KP)], SEQ ID NO: 1271;
 Cmp 12: [G4:T5(EZ)], SEQ ID NO: 1272;
 Cmp 13: [F6:T7(KZ)], SEQ ID NO: 1273;
 Cmp 14: [F6:T7(AP)], SEQ ID NO: 1274;
 Cmp 15: [F6:T7(EP)], SEQ ID NO: 1268;
 30 Cmp 16: [G4:T5(R)], SEQ ID NO: 1275; and
 Cmp 17: [G4:T5(pE)], SEQ ID NO: 1276.

Results & Discussion

-167-

A family of glucagon analogs possessing a single depsi-peptide bond was synthesized by automated Fmoc/tBu, or a combination of Boc/Bz and Fmoc/Bz solid-phase peptide chemistry [Table 1].

5 Table 1: Analytical data for glucagon analogs prepared and shown in Table

Compound	Abbreviation	R _t [min]	MW _{Tr}	MW _{Exp}	MS ions [m/z]
1	Gcg	11.69	3482.8	3483.3	871.7; 1161.8; 1741.9
2	D15:S16	11.25	3482.8	3483.4	871.7; 1161.7; 1742.1
3	D15:T16	11.15	3496.8	3497.4	875.1; 1166.4; 1749.3
4	F6:T7	11.21	3482.8	3483.2	871.6; 1161.7; 1742.0
5	G4:T5	11.27	3482.8	3483.3	871.7; 1161.8; 1742.0
6	F15:T16	11.51	3528.9	3529.4	883.1; 1177.1; 1765.3
7	D6:T7	11.05	3450.7	3451.3	863.6; 1151.1; 1726.1
8	D15:T16(Ac)	11.61	3538.8	3539.2	885.6; 1180.4; 1770.0
9	B2,G4:T5(Ac)-am	11.59	3521.8	3522.2	881.4; 1174.7; 1761.5
10	G4:T5(KZ)	11.30	3694.0	3695.0	924.4; 1232.3; 1848.3
11	G4:T5(KP)	11.34	3708.0	3709.0	927.9; 1236.8; 1855.5
12	G4:T5(EZ)	11.50	3695.0	3695.4	924.5; 1232.5; 1848.4
13	F6:T7(KZ)	11.23	3694.0	3694.7	924.4; 1232.2; 1847.9
14	F6:T7(AP)	11.46	3650.9	3651.5	913.7; 1217.8; 1826.2
15	F6:T7(EP)	11.48	3709.0	3709.6	928.1; 1237.0; 1855.7
16	G4:T5(R)	11.10	3638.9	3396.7	728.7; 1213.8; 1820.0
17	G4:T5(pE)	11.46	3593.9	3594.4	899.4; 1198.7; 1797.8

Peptides were analyzed and characterized by LC-MS (1260 Infinity-6120 Quadrupole LC-MS, Agilent) on a Kinetex C8 column (4.6 x 75 mm, 2.6 mm, Phenomenex) with 0.05 % TFA/H₂O and 0.05 % TFA/CH₃CN as eluents employing 5 % B to 70 % B in a 15 minutes gradient with 2.5 minutes initial delay; R_t, retentions time in LC chromatogram; UV detection at λ = 214 nm

The depsi-bond in analogs **2-12** and **16-17** was formed manually by coupling the subsequent Fmoc-protected amino acid with DIC and catalytic DMAP for 12 to 24 hours. In analogs **13-15** the isoacyl dipeptide (Boc-Thr[Fmoc-Phe]-OH) was used to ensure enantiomerically pure depsi constructs [details in Experimental Section].

As a first step we investigated the *O-to-N acyl* conversion of depsi-glucagon analogs to biologically active hormone under physiological conditions (PBS pH 7.4 and 37 °C) [Fig.4 and Table 2]. Three positions in the peptide sequence were assessed (T5, T7, S16), and a single comparison of serine vs threonine at position 16. Analysis was performed by quantitative LC-MS, as timed aliquots were quenched by dilution in ice-cold, 1% TFA. There was a nearly fiftyfold difference in the dynamic

-168-

reaction rate as peptide **5** [G4:T5] proved fastest with a recorded half-time of ~13 seconds and analog **2** [D15:S16] being slowest at 559 seconds.

5 Table 2: Determined half-time in the O-to-N acyl shift reaction

Peptide	$t_{1/2}$ (pH7; 37 °C) [sec]	$t_{1/2}$ (pH5; RT) [hours]
2	559.0	118.9
3	110.5	25.7
4	29.2	2.1
5	12.7	0.5
6	56.3	n.d.
7	52.4	n.d.

The nature of the hydroxylated amino acid at the depsi-bond substantially affected the reaction rate. This is clearly demonstrated when comparing peptide **3** with threonine at position 16 against the comparable analog **2** that differs only with serine at the same position. The presence of a methyl group at the side-chain beta-carbon of threonine accelerated the reaction by approximately fivefold to a half-time of slightly less than two minutes (~111 seconds). Furthermore, the specific location of the threonine depsi-bond exerted an even greater influence on the conversion rate than this single change from serine to threonine at position 16. The respective rates for T5, T7 and T16 were approximately 13, 29 and 111 seconds, which represents an 8.5x range difference in relative speed. This rate of conversion is consistent with the application of this chemistry to a glucagon pro-drug intended for emergency use. Of equal importance was the dynamic range as it suggested that the peptide stability could be fine-tuned through subtle changes in location of the depsi-bond and the choice of serine or threonine.

The differences in reaction rate between the three threonine analogs **3**, **4**, **5** may reflect dissimilarity in the nature of the specific depsi-dipeptides, or secondary structural differences in the N-terminal half of glucagon. Analogs **6** and **7** were prepared to investigate this question as they constitute single site changes to peptides **3** and **4**. In peptide **7** the depsi-bond is located at the 6-7 position in analogous fashion to peptide **4**, but phenylalanine 6 is replaced with aspartic acid, the amino acid native to position 15. The rate of reaction is slowed nearly twofold [Fig. 4, Table 2].

-169-

The opposite effect was observed in analog **6** where aspartic acid 15 was replaced with phenylalanine and the reaction rate nearly doubled, relative to **3**. These results collectively suggest that the difference in the speed of reaction for **3** and **4** can be explained by the nature of the dipeptides at positions 6-7 and 15-16, without any appreciable contribution from secondary structure.

The half-life of the *O-to-N acyl shift* of one minute or less satisfies the first criteria in a design of glucagon pro-drug. However, this conversion also represents a point of chemical instability that needs to be suppressed in a pre-formulated drug prior to its administration. Consequently, the degree to which the reaction is slowed by changes in pH and temperature needed to be determined. Peptides **2** and **5** represent the extremes in the rate of reaction at physiological conditions and their conversion to biologically active native glucagon was observed to be significantly reduced when studied at pH 5 and room temperature. Where peptide **5** converted with a half-life of ~13 seconds at physiological conditions it took thirty minutes when the pH and temperature were subtly reduced, a decrease of 140x [Table 2]. Peptide **2** exhibited an even greater relative response to change in temperature and pH as the rate of reaction slowed by more than 750x, now requiring five days for half conversion to native glucagon. While these changes represent dramatic differences in the speed of chemical reaction they also illustrate that such a depsi-peptide would require additional fortification in its stability for conventional use as a pre-formulated medicine.

The primary purpose of the depsi-bond is to render native glucagon sufficiently soluble in aqueous solution such that it could be used immediately as a pre-formulated drug at time of emergency use. The *O-to-N acyl shift* complicates the determination of inherent aqueous solubility for a depsi-peptide. This can be approximated by inhibiting the intramolecular rearrangement by acetylation in the alpha-amine of the depsi-bond, thus rendering it inert and the peptide suitable for biophysical study. Peptides **8** and **9** represent acetylated analogs of peptides **2** and **5**. Peptide **8** [D15:T16(Ac)] is stable in PBS demonstrating the importance of the single alpha-amine in the conversion of depi-peptide **2** to native glucagon. Its solubility in PBS is in excess of 2.5 mg/mL, which is more than tenfold relative to native glucagon (**1**) and sufficient to support formulation at 1 mg/mL. The aqueous solubility of the closely related peptide **9** [G4:T5(Ac)-am] where the depsi-bond is positioned at

-170-

residues 4 and 5 is also sizably enhanced relative to glucagon, but less so than analog **8**. These results demonstrate that a single depsi-bond can provide sufficient enhancement in aqueous solubility to constitute a concentration that would support medicinal use, without excessive change in the volume administered. Furthermore, the specific location of the depsi-bond exerts differential effects, with the mid-sequence placement providing enhanced solubility.

Peptides **8** and **9** were also used to evaluate the stability of the glucagon backbone, including hydrolytic cleavage of the single ester bond in PBS (pH 7.4, room temperature). The native sequence proved relatively inert under these conditions with the depsi-bond as expected being the most sensitive degradation point. Analog **8** demonstrated good stability through nearly a week of incubation, while in comparison the single depsi-bond in analog **9** resulted in almost 20% degradation. To further test the inherent stability of peptide **8** it was incubated at elevated temperature in a slightly modified physiological buffer of citric acid/phosphate (pH 7 at 40 °C) for five days. It exhibited a level of depsi-bond hydrolysis that was less than **9** when the latter was incubated at room temperature. The difference in stability to general aqueous hydrolysis reflects the propensity in *O-to-N acyl* rearrangement, which is a measure of the inherent stability of the ester bond. This positional difference in stability is an important consideration in the design of a depsi-peptide that meets all of the necessary criteria for medicinal application; speed to biological action, sufficient solubility and chemical stability as a prodrug in aqueous solution.

To further enhance the chemical stability of the depsi-peptide without compromising the rate at which it can generate active hormone a reversible means of protecting this ester bond was explored. Further lowering of pH in the formulated solution was envisioned as being undesirable as it would lead to increased acid-mediated hydrolytic degradation of the depsi-bond and other native amino acids, especially amides. Similarly, lowering the storage temperature to refrigerated conditions is unacceptable as an emergency use product should not be compromised by such a restriction. The acetylation of the depsi-peptide alpha-amine provides a sufficient increase in chemical stability without compromising aqueous solubility, but it is incapable of rearrangement to active hormone. The prospect of using an enzymatically-labile protection is attractive as it masks the nucleophile until that point when it is liberated by proteolysis [Fig. 6]. Several candidate substrates were

-171-

considered and a select number were assembled (**10-17**), across two different locations in the glucagon sequence.

An initial set of depsi-containing analogs was prepared in the G4:T5 sequence [peptides **10-12**]. The previous stability studies indicated this depsi-bond to be least stable and as such it provides the more stringent test for evaluating the integrity of the protection with putative, enzymatically susceptible substrates. Each of these three analogs (**10-12**) would be expected to generate native glucagon once cleaved by an enzyme, followed by an *O-to-N acyl shift*. This sequence site also constitutes a more straightforward synthetic target since the glycine at position four can be esterified to the threonine at residue five without fear of loss in stereochemical purity. The threonine at position 5 was incorporated as Thr(Trt) and the trityl side protection group was easily removed by treatment with 1% TFA. Esterification with Fmoc-Gly provided enantiomerically pure depsi-peptide which was further extended with the N-terminal glucagon tripeptide in standard Fmoc fashion [Fig. 7A].

Glucagon analog **10** [G4:T5(KZ)] represents a depsi-peptide that has been designed to rapidly cleave to **5** when exposed to the enzyme DPPIV. This exopeptidase is ubiquitous and demonstrates high substrate preference for dipeptides that include penultimate cyclic amino acids, such as proline. The dipeptide lysine-2,carboxyzetidine is an extremely favorable substrate that demonstrates a low K_m and high k_{cat} . Peptide **10** demonstrates excellent solubility (>2 mg/mL) in a variety of aqueous physiological buffers [Fig. 9]. Relative differences in solubility appear to be a function of buffer salt concentration and specific pH. It should be noted that the additional lysine in the substrate dipeptide increases the cationic nature of the peptide relative to native glucagon and this also assists aqueous solubility in the physiological pH range. The greatest concentration of **10**, (> 3 mg/mL) was achieved at pH of 7 in an aqueous buffer composed of 0.1M citric acid and 0.2M disodium phosphate (room temperature). This is 3x the concentration of native glucagon administered to patients once solubilized in dilute hydrochloric acid. Comparative solubility of analogs **10-15** was studied in PBS pH 7.4, at room temperature [Fig. 10]. All analogs are of much higher solubility than native glucagon, and the location of the depsi-bond and the specific nature of the added substrate subtly, but meaningfully influence the result. The solution stability of **8** and **10** was assessed to determine whether there might be subtle differences imposed by simple acetylation relative to an extended dipeptide that

-172-

terminates with a free alpha-amine. Both peptides were formulated in citric acid, phosphate buffer in a pH range of pH 3-7, and incubated for extended time at three different temperatures (4, 23, and 40 °C). The stability was determined with daily sampling and analysis by LC-MS. At 4 °C the peptide shows good stability in each of the three buffers, without slight apparent preference for lower pH. There is a variable degree of degradation observed at pH 7 (5 day), with complete loss at 40 °C and a fractional amount at 23 °C. The primary basis of chemical degradation was determined to be the hydrolytic cleavage of the ester bond to generate two peptide fragments. At pH 6, peptide **10** appeared comparably stable at 4 °C and room temperature to pH 7 buffer at refrigerated temperature. At 40 °C there was finite degradation (~40%) but much less than was observed at the elevated temperature and pH of 7. The analyses of **10** when incubated at pH 5 showed minor hydrolysis of the depsi-bond, and only when the temperature was elevated to 40 °C.

To test for biophysical stability an additional incubation study was conducted where native glucagon (**1**) was included as a positive control. Given the low aqueous solubility of glucagon at physiological pH the study included analysis at pH 3 and 4. Biophysical aggregation was measured by assistance of thioflavin-T mediated fluorescence [Fig. 11]. As expected the aggregation of native glucagon (**1**) and depsi analogs **8** and **10** stored at 4 °C was minimal, in this time period. At elevated temperatures (23 and 40 °C) **1** exhibited a strong fluorescence, indicative of biophysical aggregation. Peptides **8** and **10** show no evidence of such aggregation at 23 °C, and only a trace at 40 °C. The collective results demonstrate that the depsi-peptide bond G4:T5 once extended with a specific dipeptide (KZ) possesses high aqueous solubility at pH 5, and sizably enhanced chemical stability without propensity to form amyloid-like fibrils. Further comparative analysis of extended depsi-glucagon analogs (**10-15**) incubated in PBS at room temperature revealed no major difference in ester bond stability between the investigated peptides.

The assessment of *in vitro* potency at the glucagon receptor was conducted using an engineered cellular assay that measured cAMP production. The depsi-peptide **5** was observed to be equipotent to the glucagon standard, which was presumed to be a reflection of the rapid conversion to native sequence under the conditions of biochemical study. Analogs **8** and **9** represent acetylated, depsi-peptides differing in the location of the ester bond and they demonstrate activity less than 1%

-173-

of native glucagon. This validates that the insertion of a single ester bond is highly destructive to receptor signaling and subsequent biochemical action.

The biological activity of select depsi-peptide analogs was evaluated in non-diabetic rats. Peptides **1**, **2**, **3** and **5** were formulated in 0.01N HCl immediately prior to administration at a 10 nmol/kg dose. Elevation in blood glucose was followed through a two-hour period and each of the peptides exhibited a statistically significant increase separable from vehicle control, but without difference from one another [Fig. 5]. The pharmacodynamic response is consistent with historical glucagon action, peaking within 30 min and returning to initial glucose level at two hours. These results corroborate the rapid chemical conversion from an inactive precursor to a fully active hormone, as previously demonstrated by chromatography and biochemical assay. The absence of any biological difference across these three depsi-peptides was unexpected, as the *in vitro* rate in chemical rearrangement varies between one to eleven minutes. It is possible that a lower dose might demonstrate some subtle difference, but it is well-recognized that emergency glucagon therapy is administered at an excessively high dose to ensure full action.

In a subsequent test of *in vivo* action peptide **10** formulated in PBS was compared to **1** and **5** solubilized in dilute hydrochloric acid. The three peptide treatments demonstrated an increase in blood glucose relative to vehicle that was nearly identical to the first study [Fig. 14]. The rise in glucose peaked within thirty minutes at a magnitude that was approximately 30 mg/dL higher than identical vehicle treatment. The return to baseline was recorded within the two hours of peptide administration. Once again the unprotected depsi-peptide **5**, solubilized in dilute acid was identical to similarly administered glucagon. Most importantly, **10** was equally potent *in vivo*, despite the fact that it was no more than 3% the potency of the native hormone when studied *in vitro* [Fig. 12]. Changing the relative position of depsi bond (G4:T5 vs. F6:T7) as well the character of DPPIV substrate (KZ vs EP) was found to have no apparent effect on the biological activity of **13** [F6:T7(KZ)], **15** [F6:T7(EP)] and **4** [F6:T7] [Fig. 15], with recorded potency comparable to native glucagon (**1**).

The relative performance of peptide **10** [Fig. 14] and **13** [Fig. 15] is extremely encouraging as it indicates that a DPPIV substrate is capable of being rapidly removed, and presumably through the action of DPPIV. Two additional putative

-174-

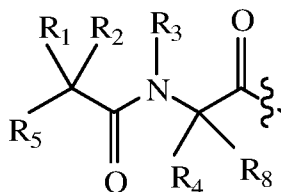
substrates for endogenous exopeptidases were also studied. Peptides **16** and **17** were designed for proteolysis by an N-terminal cationic aminopeptidase and a pyroglutamase, respectively. The test of these two depsi-peptides alongside peptide **5** and **10** is presented in Fig. 13. Peptides **5** and **10** perform in analogous fashion to what is reported in Fig. 14 while **16** and **17** elicit very little activity, indicating the superiority of DPPIV substrates. A potential dilemma in the use of a DPPIV substrate is the prospect of attenuated glucagon activity should a patient be using a DPPIV inhibitor, such as sitagliptin. Consequently, we explored whether sitagliptin (ApexBio) at a dose of 3, 10 or 30 mg/kg given orally 30 min prior to injection of 10 nmol/kg of analog **15** [F6:T7(PE)] would impair its ability to increase blood glucose [Fig. 16]. Treatment with the enzyme inhibitor alone had only an insignificant effect on blood glucose. All of the mice treated with **15** displayed a significant elevation in blood glucose while the presence of sitagliptin demonstrates a subtle dose response that reduces the effect by a small fractional amount that was nearly without consequence.

Whether sitagliptin is simply ineffective in inhibition of this DPPIV substrate under these experimental conditions or alternatively whether the substrate is being processed by an alternative endogenous enzyme is not possible to determine from the initial study. To further explore this question, we tested the ability of sitagliptin to inhibit GLP-1 proteolytic loss in potency. Paradoxically, exendin-4 has been reported to increase blood glucose upon acute administration to normal rats. When we compared the ability of an Aib2, DPPIV protected analog to change blood glucose it was apparent that there was a dramatic difference in potency relative to native hormone [Fig. 17A & 17B]. The addition of sitagliptin in comparable fashion to how it was used with depsi-peptides [Fig. 16] was highly effective in strengthening the biological effect, demonstrating its ability to inhibit endogenous DPPIV. These results support the prospect that a secondary enzyme is sufficient to rapidly initiate the conversion of peptides **10** [Fig. 14] and **13** [Fig. 15], and provide high potency glucagon agonism.

- 175 -

Claims:

1. A glucagon peptide analog having enhanced solubility relative to native glucagon, said analog comprising one or more amino acids selected from positions 5, 7, 8, 11 or 16 linked to the preceding amino acid in the peptide chain via an ester bond.
2. The analog of claim 1 wherein an amino acid comprising a hydroxyl group bearing side chain is present at position 7, 8, 11 or 16, and an ester bond is formed between the hydroxyl group on the side chain of the amino acid at position 7, 8, 11 or 16 and the carboxylate of the amino acid at position 6, 7, 10 or 15, respectively.
3. The analog of claim 1 or 2 wherein the alpha amine of the ester linked amino acid is
 - a) covalently bound to an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase; or
 - b) covalently linked to a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine.
4. The analog of claim 3 wherein the alpha amine of the ester linked amino acid is covalently bound to a dipeptide and said dipeptide is susceptible to cleavage by Dipeptidyl Peptidase IV.
5. The analog of claim 4 wherein said dipeptide is Lys-Pro or Lys-Azetidine-2-carboxylic acid.
6. The analog of claim 3 wherein said dipeptide has the general structure of Formula I:



wherein

- 176 -

R_1 is selected from the group consisting of H and C₁-C₄ alkyl;

R_2 and R_4 are independently selected from the group consisting of H, C₁-C₆ alkyl, C₂-C₈ alkenyl, (C₁-C₄ alkyl)OH, (C₁-C₄ alkyl)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₆-C₁₀ aryl) R_7 , and CH₂(C₅-C₉ heteroaryl);

R_3 is C₁-C₆ alkyl or R_3 and R_4 together with the atoms to which they are attached form a pyrrolidine ring; and

R_7 is selected from the group consisting of hydrogen and OH.

7. The analog of claim 6 wherein

R_1 is C₁-C₄ alkyl or (C₁-C₄ alkyl)NH₂;

R_2 is H or is C₁-C₄ alkyl;

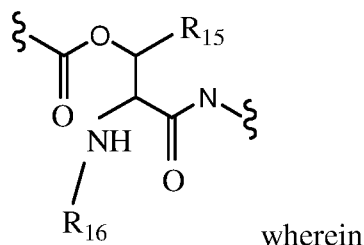
R_3 is C₁-C₆ alkyl;

R_4 is H or C₁-C₄ alkyl;

R_8 is H; and

R_5 is NH₂ or OH.

8. The analog of any one of claims 1-7 wherein said ester linked amino acid has the general structure of Formula V:



R_{15} is H or CH₃; and

R_{16} is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine.

9. The analog of claim 8 wherein the amino acid of Formula V is at position 16.

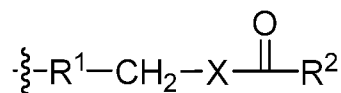
10. The analog of any one of claims 1-9 wherein the analog comprises the sequence $X_1X_2X_3GTFTSDX_{10}SX_{12}YLX_{15}X_{16}X_{17}X_{18}AX_{20}X_{21}FVX_{24}WLX_{27}-Z$ (SEQ ID NO: 943)

wherein

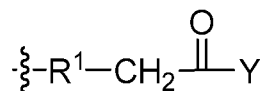
X_1 is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X_2 is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;

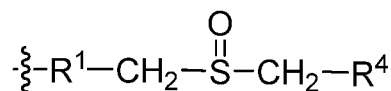
X_3 is an amino acid comprising a side chain of Structure I, II, or III:



Structure I



Structure II



Structure III

wherein R^1 is C_{0-3} alkyl or C_{0-3} heteroalkyl; R^2 is NHR^4 or C_{1-3} alkyl; R^3 is C_{1-3} alkyl; R^4 is H or C_{1-3} alkyl; X is NH, O, or S; and Y is NHR^4 , SR^3 , or OR^3 ;

X_{10} is Tyr or Lys;

X_{12} is Lys, Ile or Arg;

X_{15} is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid;

X_{16} is Glu, Lys, Asp, Ser, glutamine, homoglutamic acid, homocysteic acid, Thr or Aib;

X_{17} is Arg or Gln;

X_{18} is Ala, Arg

X_{20} is Gln, Glu, Ser, Thr, Ala, Lys, Citrulline, Arg, Orn, or Aib;

X_{21} is Glu, Aib, Asp, Lys, Cys, Orn, homocysteine or acetyl phenylalanine;

X_{24} is Asn, Aib, Gln, Glu or Lys;

X_{27} is Met, Leu or Nle;

- 178 -

X₂₈ is Asn or Ala;

X₂₉ is Thr or Gly; and

Z is selected from the group consisting of -COOH, -X₂₈-COOH, X₂₈-X₂₉-COOH, and X₂₈-X₂₉-GPSSGAPPPS SEQ ID NO: 1299).

11. The analog of claim 10 wherein the analog comprises

(i) a lactam bridge between the side chains of amino acids at positions i and i + 4, wherein i is 12, 16, 20 or 24, or

(ii) one, two, three, or all of the amino acids at positions 16, 20, 21, and 24 of the glucagon peptide is substituted with an α , α -disubstituted amino acid.

12. The analog of any one of claims 1-9 wherein the analog comprises the sequence

YAQGTFTSDX₁₀SKYLDERAAQDFVQWLLEGGPSSGAPPPS-NH₂ (SEQ ID NO: 945) or

YX₂EGTFTSDX₁₀SIYLDKQAAX₂₀EFVNWLLAGGPSSGAPPPS (SEQ ID NO: 944)

wherein

X₂ is Ser, D-Ser or Aib;

X₁₀ is Lys acylated with a C16 to C18 carbon atom chain, optionally via a γ Glu linker; and

X₂₀ is Gln or Aib.

13. The analog of any one of claims 1-9 wherein the analog comprises the sequence X₁X₂QGTFTSDYSKYLX₁₅X₁₆RRAX₂₀DFVX₂₄WLMX₂₈T (SEQ ID NO: 946) wherein

X₁ is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X₂ is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α -amino-N-butyric acid;

X₁₅ is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid;

- 179 -

X₁₆ is Glu, Lys, Asp, Ser, glutamine, homoglutamic acid, homocysteic acid, Thr or Aib;

X₂₀ is Gln or Lys;

X₂₄ is Gln or Glu;

X₂₈ is Asn, Asp or Lys;

or an analog of SEQ ID NO: 946 that differs from SEQ ID NO: 946 by 1 to 2 amino acid modifications, selected from positions 1, 2, 7, 10, 11, 13, 14, 17, 18, 19, 21, and 27, wherein the glucagon peptide exhibits enhanced activity at the GLP-1 receptor as compared to native glucagon,

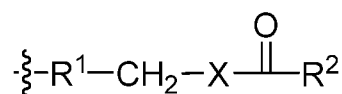
wherein the side chains of the amino acids at positions 12 and 16, positions 16 and 20, positions 20 and 24, or positions 24 and 28 of the glucagon peptide are linked by covalent bonds to provide the enhanced activity at the GLP-1 receptor, with the proviso that when the amino acid at position 16 is serine either position 20 is lysine, or the side chain of the amino acid at position 24 and the side chain of either the amino acid at position 20 or position 28 are linked by covalent bonds.

14. The analog of any one of claims 1-9 wherein the analog comprises the sequence X₁X₂X₃GTFTSDYSKYLDX₁₆RRAX₂₀DFVX₂₄WLMX₂₈T (SEQ ID NO: 942)

X₁ is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X₂ is selected from the group consisting of Ser, D-Ser, Ala, D-Ala, Gly, N-methyl-Ser, Aib, Val, or α-amino-N-butyric acid;

X₃ is an amino acid comprising a side chain of Structure I:



Structure I

wherein R¹ is C₀₋₃ alkyl or C₀₋₃ heteroalkyl; R² is NHR⁴ or C₁₋₃ alkyl; R⁴ is H or C₁₋₃ alkyl; and X is NH, O, or S;

X₁₆ is amino isobutyric acid (Aib);

- 180 -

X₂₀ is selected from the group consisting of Asp, Lys, Cys, Orn, Aib, homocystein and acetyl phenylalanine;

X₂₄ is selected from the group consisting of Gln, Lys, Cys, Orn, homocysteine and acetyl phenylalanine; and

X₂₈ is selected from the group consisting of Asn, Arg, His, Glu, Lys and Asp, or an analog thereof that comprises up to 10 amino acid modifications relative to the native sequence of SEQ ID NO: 942, said modifications selected from the group consisting of

- y. substitution of Thr at position 7 with an amino acid lacking a hydroxyl group, optionally, Abu or Ile;
 - z. substitution of Tyr at position 10 with Phe or Val;
 - aa. substitution of Lys at position 12 with Arg;
 - bb. substitution of Asp at position 15 with Glu,
 - cc. substitution of Gln at position 20 with Ala or Aib;
 - dd. substitution of Asp at position 21 with Glu;
 - ee. substitution of Gln at position 24 with Ala or Aib;
 - ff. substitution of Met at position 27 with Leu or Nle;
 - gg. addition of the amino acid sequence of GPSSGAPPPS (SEQ ID NO: 820) to the C-terminus, wherein the amino acid at position 29 is Thr or Gly,
 - hh. substitution or addition of an amino acid comprising a side chain covalently attached to an acyl or alkyl group which is non-native to a naturally-occurring amino acid;
 - ii. a substitution of Asn at position 28 with a charged amino acid, optionally, wherein the charged amino acid is selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid;
 - jj. an insertion of 1-3 charged amino acids after position 29; optionally, wherein the insertion comprises Glu or Lys;
- or a combination thereof, wherein

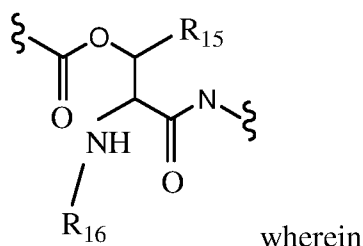
one or more amino acids selected from positions 5 and 7 are linked to the preceding amino acid in the peptide chain via an ester bond.

- 181 -

15. The analog of any one of claims 1-9 wherein the analog comprises the sequence HX₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1278) wherein

X₂ is selected from the group consisting of Ser, D-Ser, and Aib;

X₅ and X₇ are independently Thr or an ester linked amino acid having the general structure of Formula V:



R₁₅ is H or CH₃; and

R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine, with the proviso that at least one of X₅ and X₇ is an ester linked amino acid.

16. The analog of claim 15 wherein
R₁₆ is Lys-Pro or Lys- Azetidine-2-carboxylic acid.

17. The analog of claim 15 wherein X₂ is Aib.

18. The analog of claim 15 or 16 wherein X₅ is threonine and the ester linked amino acid is at position 7.

19. The analog of any one of claims 1-9 wherein the analog comprises an amino acid sequence selected from the group consisting of
X₁X₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1279);
HX₂QGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1278);
X₁X₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1281);

HX₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1280);

and

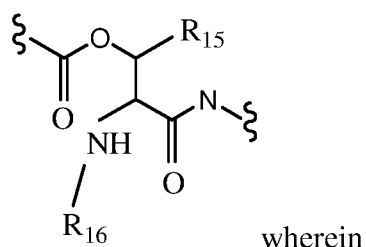
HSQGX₅FX₇SDYSKYLDSRRAQDFVQWLMNT (SEQ ID NO: 1282)

wherein

X₁ is selected from the group consisting of His, D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidazole acetic acid (DMIA);

X₂ is selected from the group consisting of Ser, D-Ser, and Aib;

X₅ and X₇ are independently Thr or an ester linked amino acid having the general structure of Formula V:



R₁₅ is H or CH₃ with the proviso that at least one of X₅ and X₇ is an ester linked amino acid having the general structure of Formula V; and

R₁₆ is H, an amino acid or dipeptide that is susceptible to cleavage by a serum peptidase, or a dipeptide that will chemically cleave by formation of a diketopiperazine or diketomorpholine; and

X₁₆ is an ester linked amino acid having the general structure of Formula V.

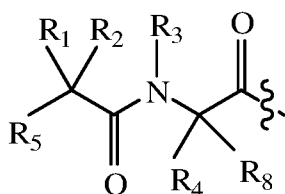
20. The analog of claim 19 wherein X₂ is Ser.

21. The analog of claim 19 wherein the analog comprises an amino acid sequence of X₁X₂QGTFTSDYSKYLDX₁₆RRAQDFVQWLMNT (SEQ ID NO: 1281).

22. The analog of any of claims 15-21 wherein R₁₆ comprises a dipeptide selected from the group consisting of Lys-Pro and Lys-Azetidine-2-carboxylic acid.

23. The analog of any of claims 15-21 wherein R₁₆ comprises a dipeptide of Formula I:

- 183 -



wherein

R₁ is selected from the group consisting of H and C₁-C₄ alkyl;

R₂ and R₄ are independently selected from the group consisting of H, C₁-C₆ alkyl, C₂-C₈ alkenyl, (C₁-C₄ alkyl)OH, (C₁-C₄ alkyl)NH₂, (C₀-C₄ alkyl)(C₃-C₆ cycloalkyl), (C₀-C₄ alkyl)(C₆-C₁₀ aryl)R₇, and CH₂(C₅-C₉ heteroaryl);

R₃ is C₁-C₆ alkyl or R₃ and R₄ together with the atoms to which they are attached form a pyrrolidine ring; and

R₇ is selected from the group consisting of hydrogen and OH.

24. The analog of any of the preceding claims, wherein the glucagon peptide is covalently linked to a hydrophilic moiety.

25. The analog of claim 24, wherein the hydrophilic moiety is a polyethylene glycol (PEG).

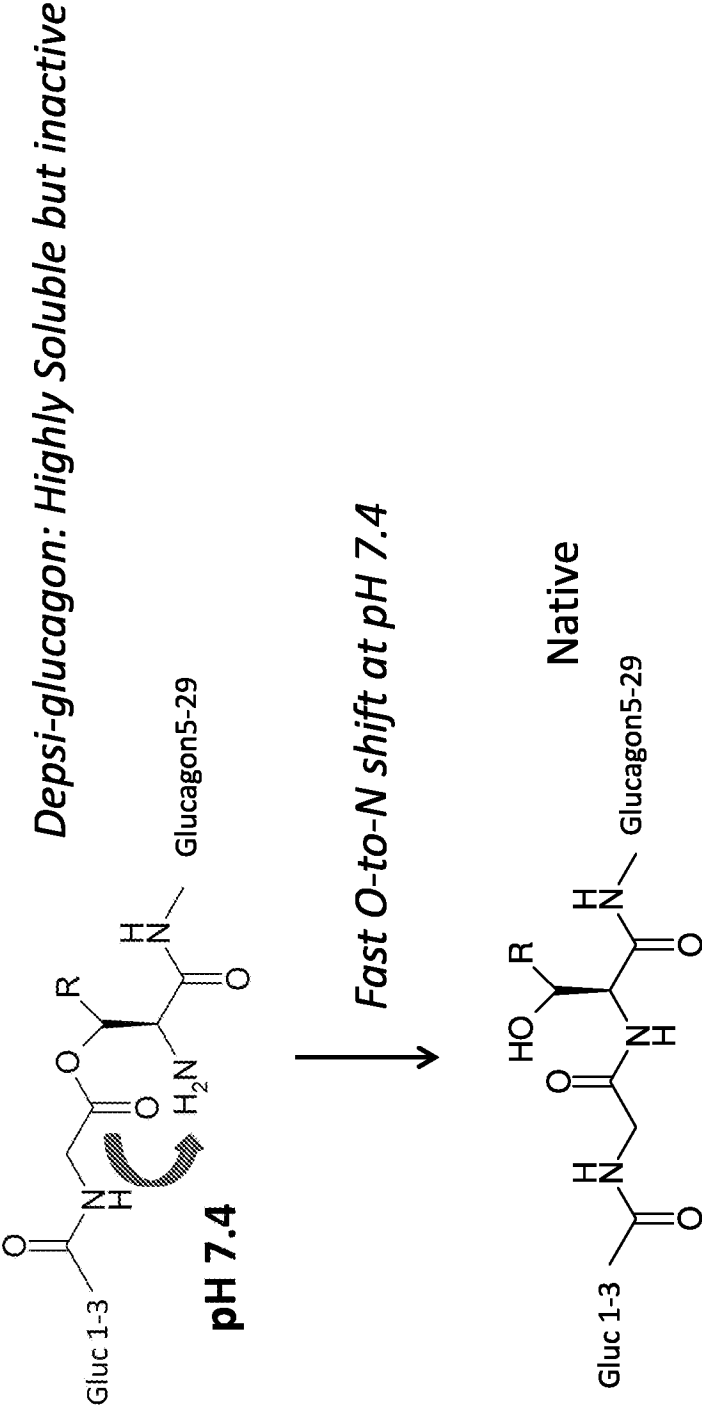
26. The analog of any of the preceding claims, wherein the glucagon peptide is covalently linked to a non-native acyl or alkyl group.

27. A pharmaceutical composition comprising an analog of any one of claims 1-26, and a pharmaceutically acceptable carrier.

28. A method of reducing weight gain or inducing weight loss in a patient in need thereof, said method comprising administering to the patient a pharmaceutical composition of claim 27 in an amount effective to reduce weight gain or induce weight loss in the patient.

29. A method of treating or preventing hypoglycemia in a patient in need thereof, comprising administering to the patient a pharmaceutical composition of claim 27 in an amount effective to treat or prevent hypoglycemia in the patient.

Fig. 1



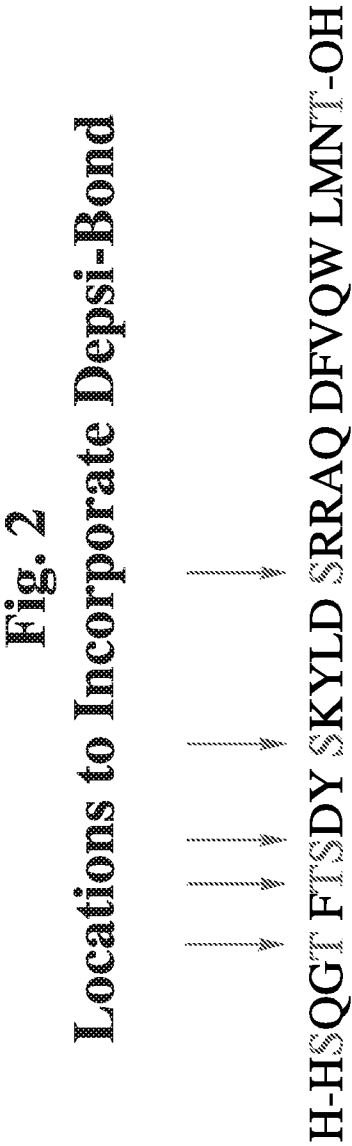


Fig. 3A

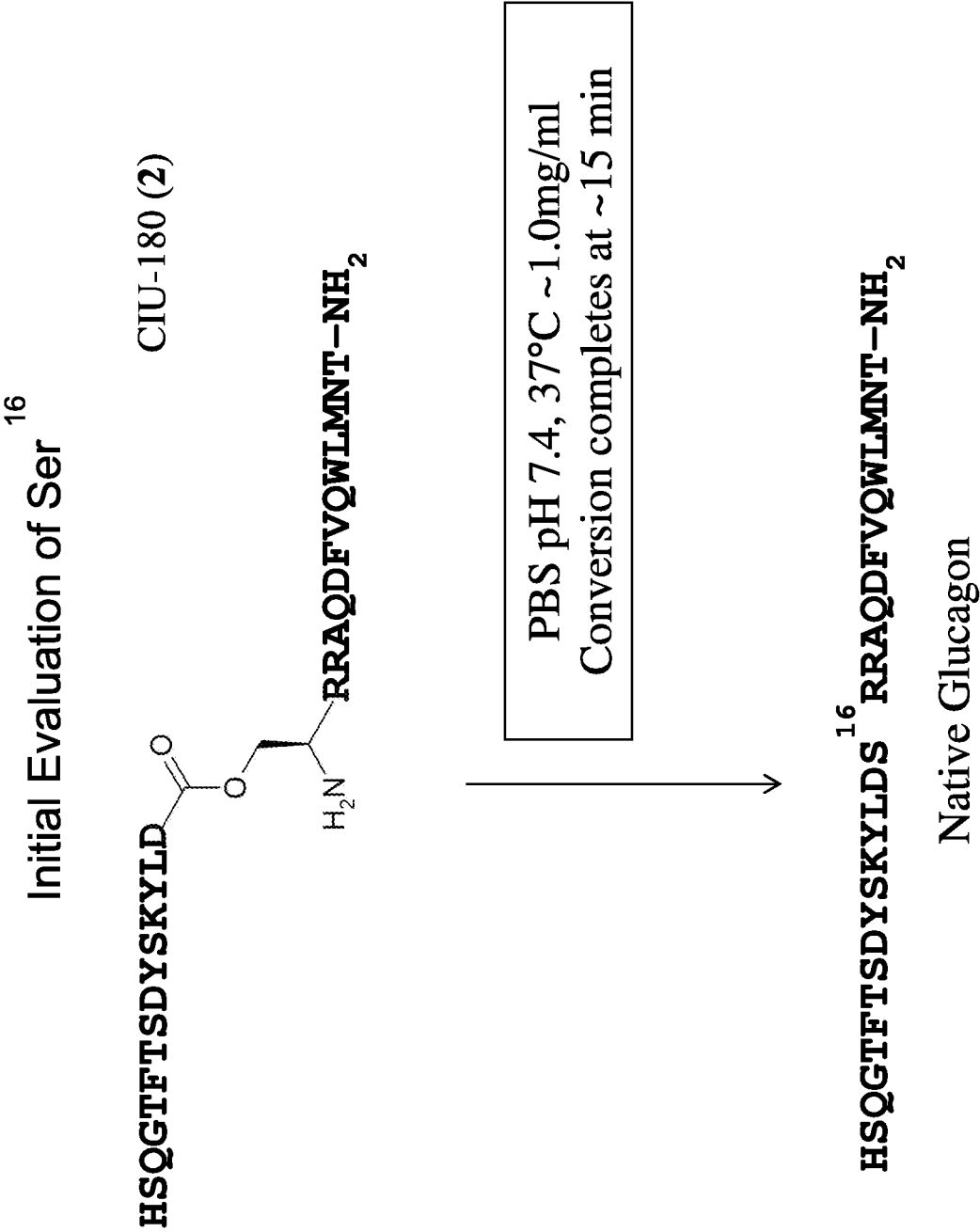
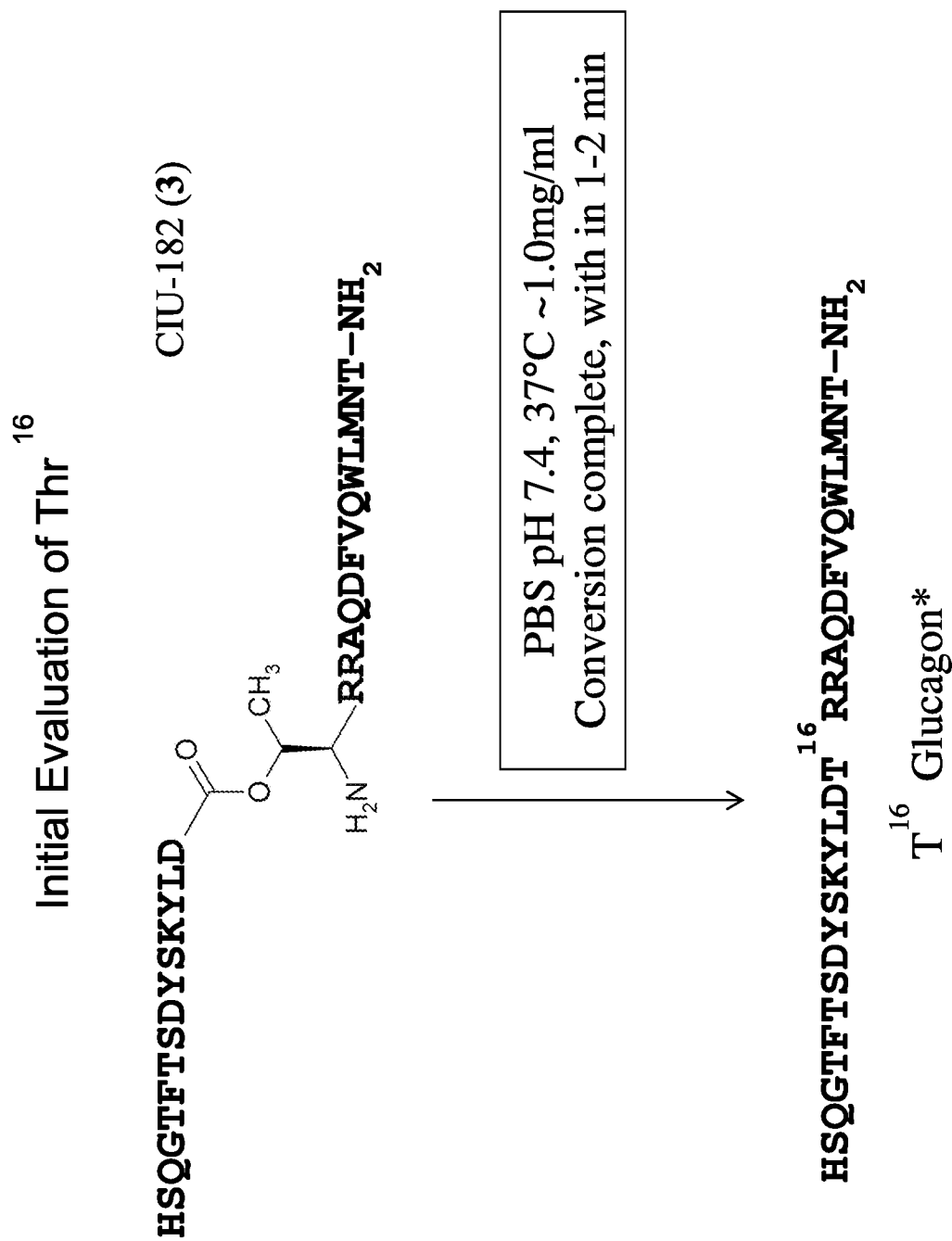
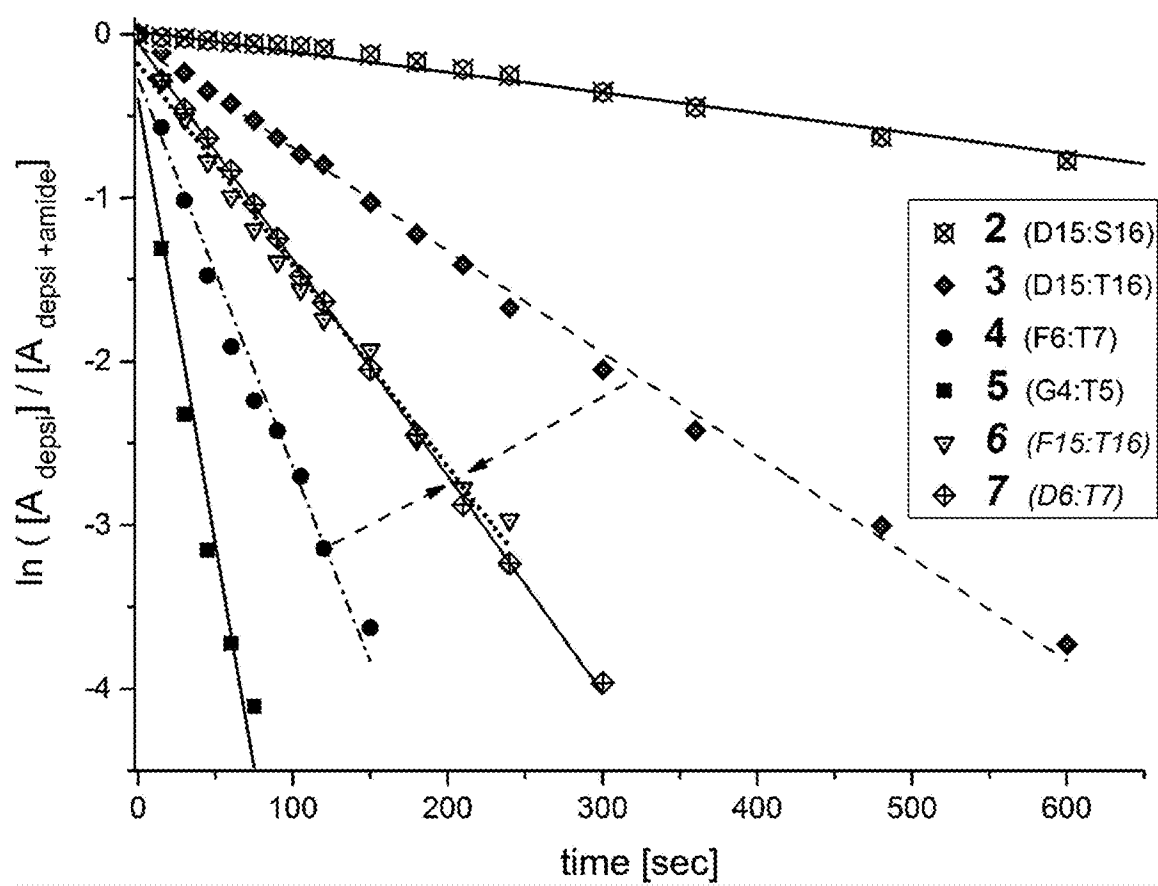


Fig. 3B



* Threonine at ¹⁶ is common to several species

Fig. 4



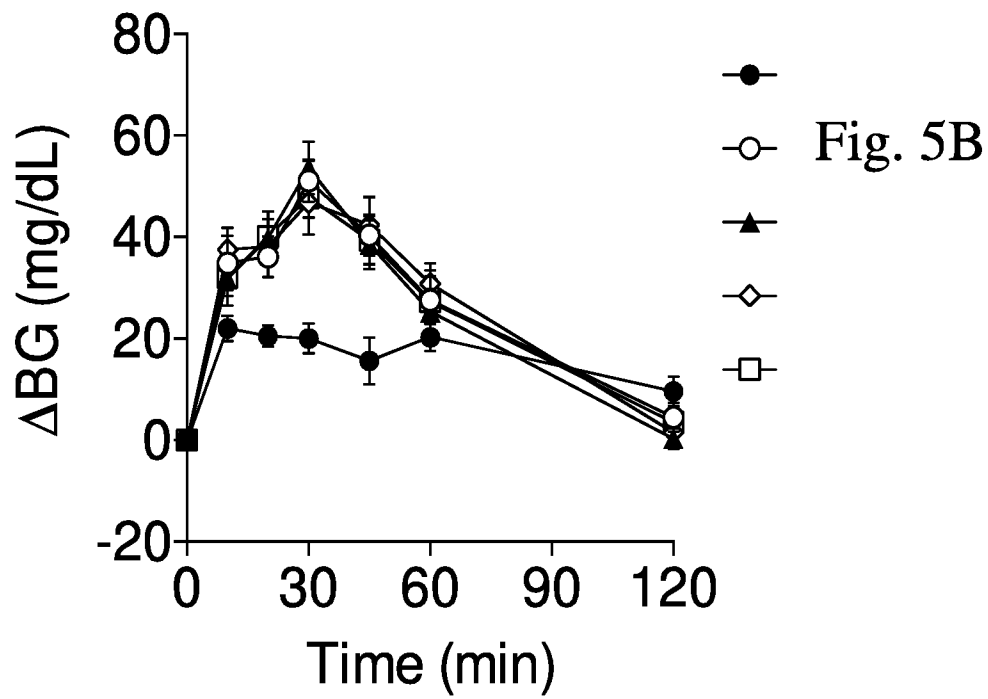
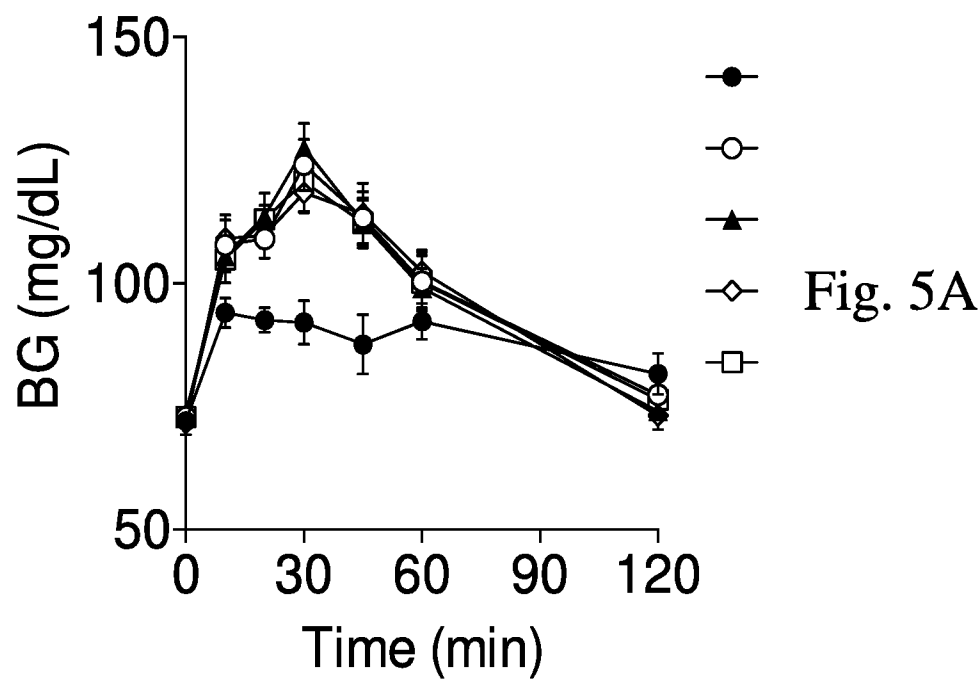


Fig. 6

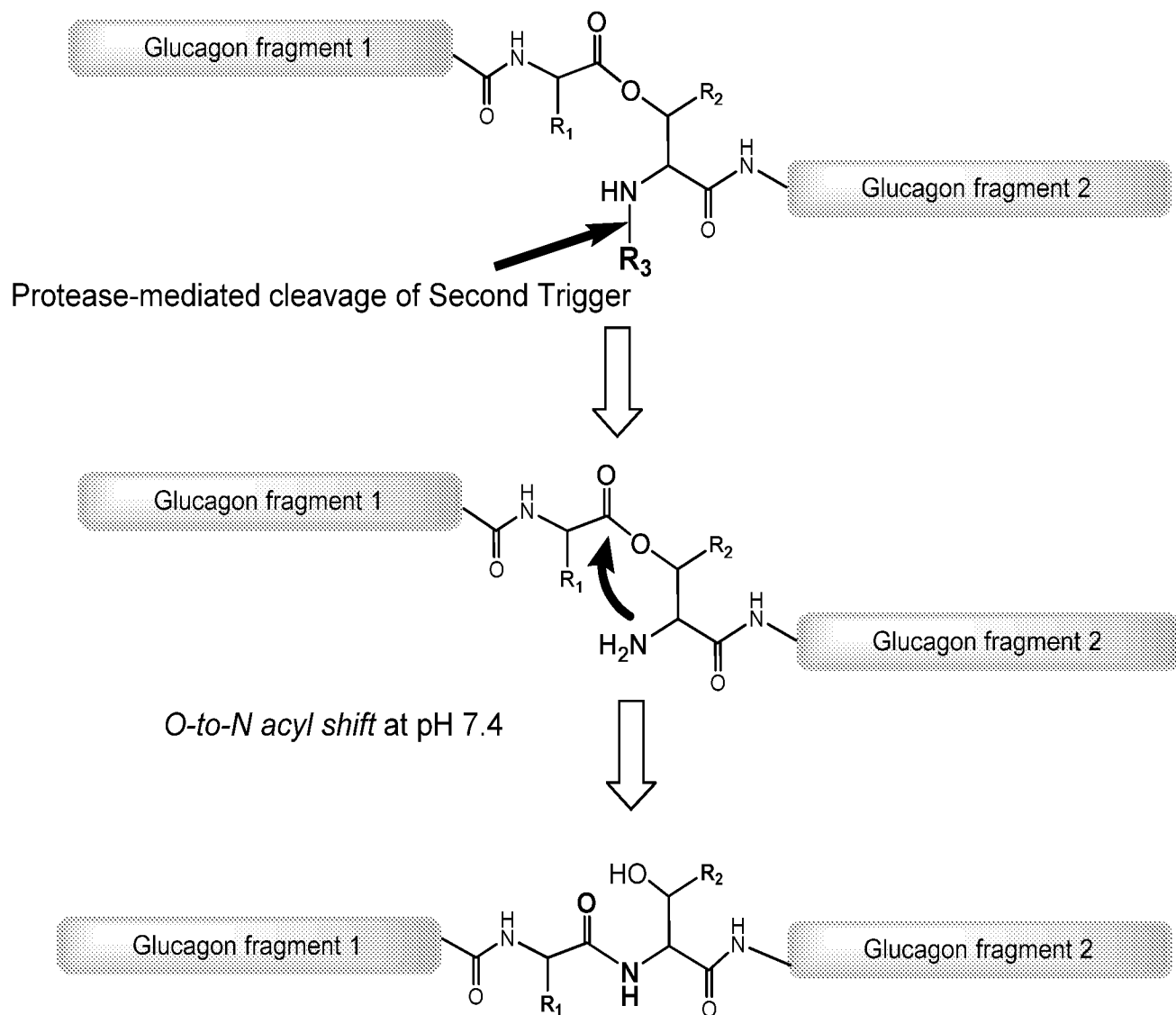
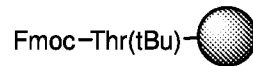
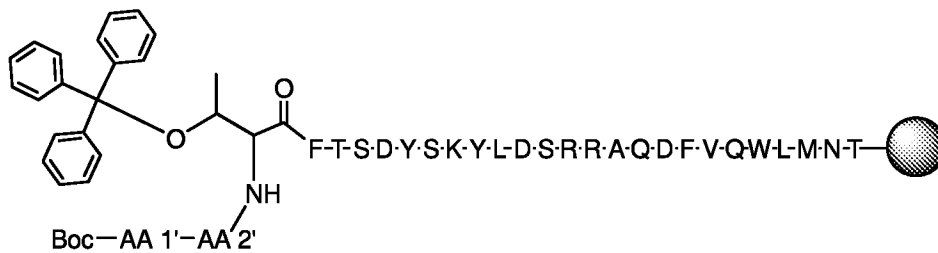


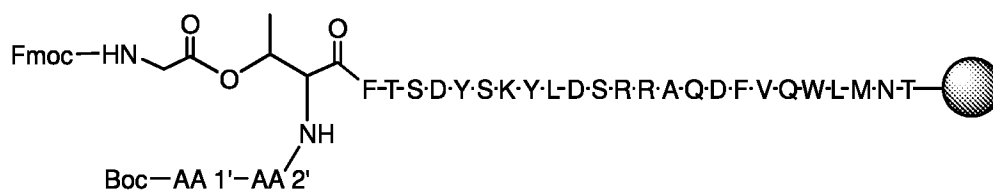
Fig. 7A



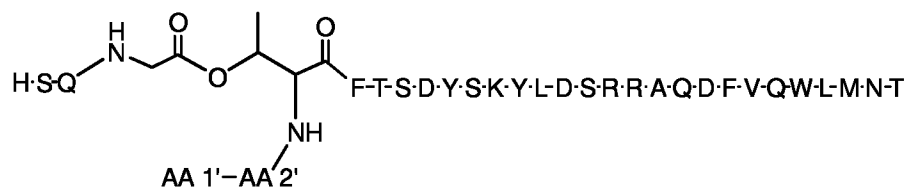
1. Fmoc synthesis on automated peptide synthesizer on Fmoc-Thr(tBu)-Wang resin with Fmoc-Thr(Trt) at position 5 and N-Boc-protected amino acid at position 1'.



2. Removal of O-Trityl protection with 1% TFA in DCM.
3. Formation of depsi bond with Fmoc-Gly/DIC/DMAP.

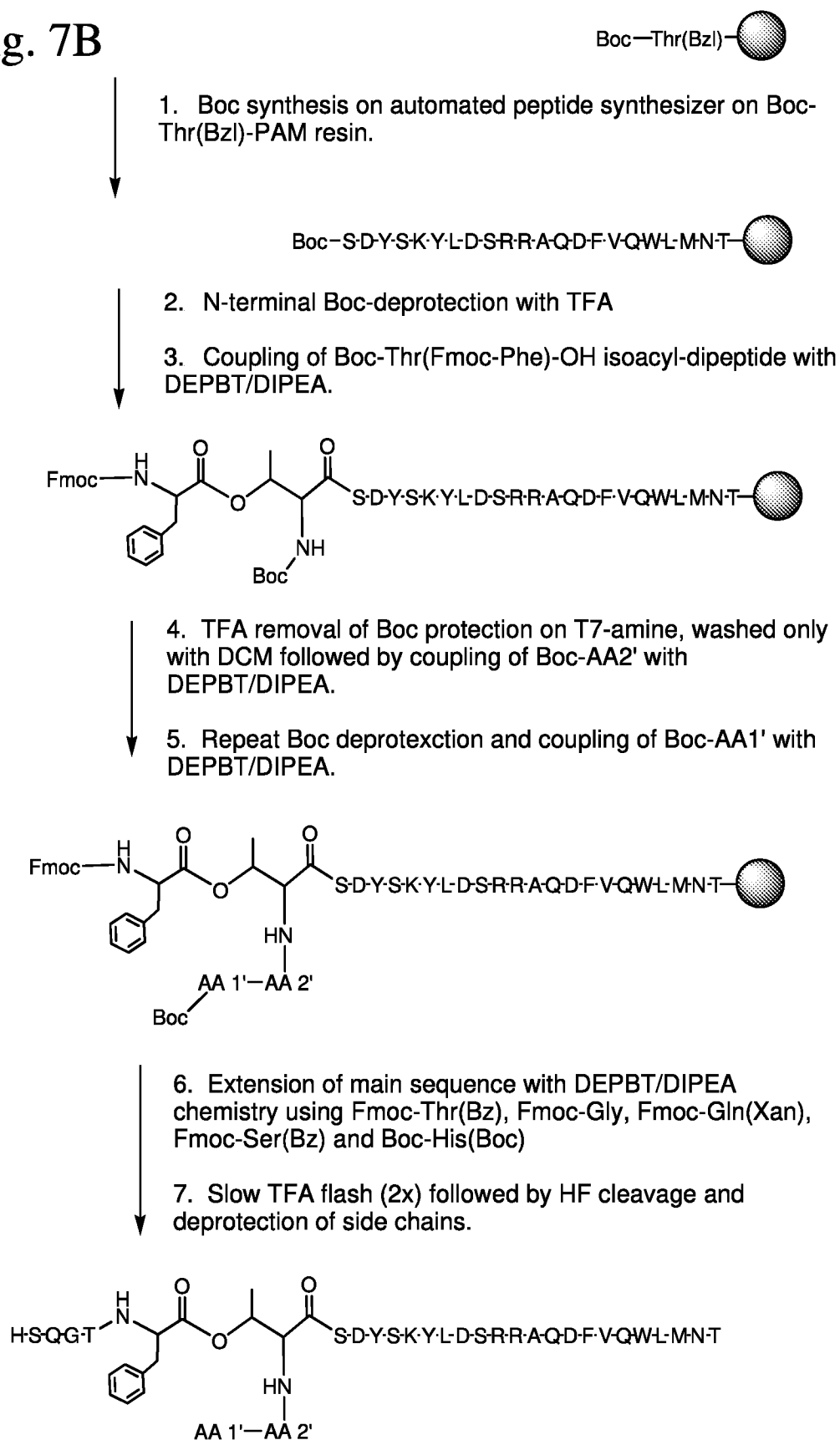


4. Extension to full sequence with standard Fmoc synthesis on automated peptide synthesizer
5. Final N-terminal-Fmoc deprotection followed by TFA cleavage and side chains protection removal.



9/17

Fig. 7B



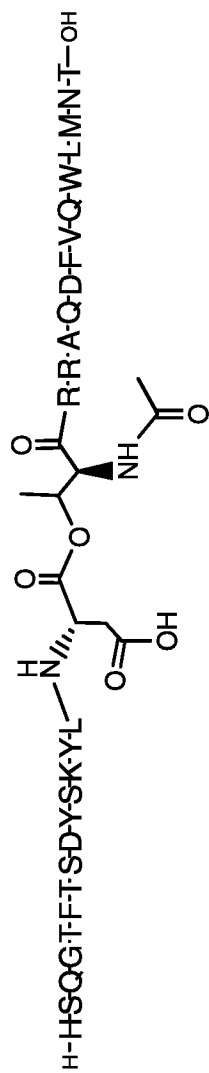


Fig. 8

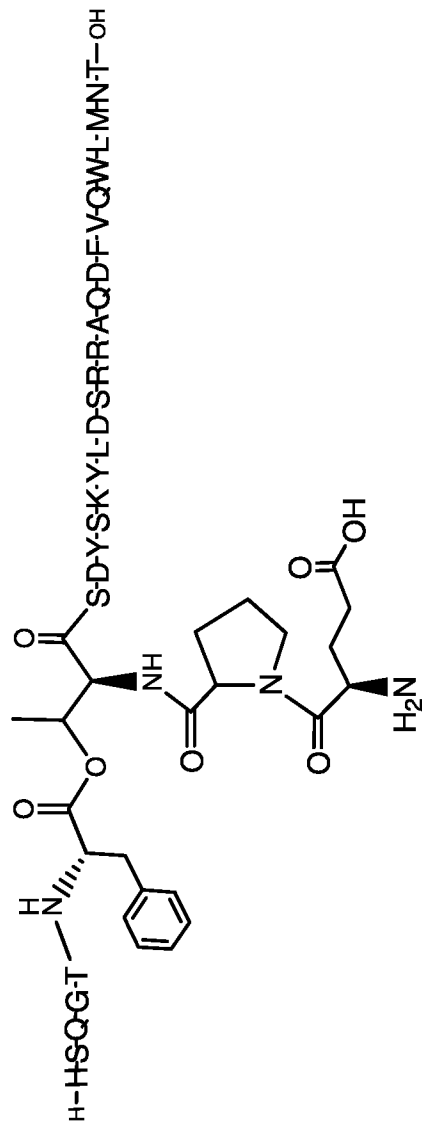
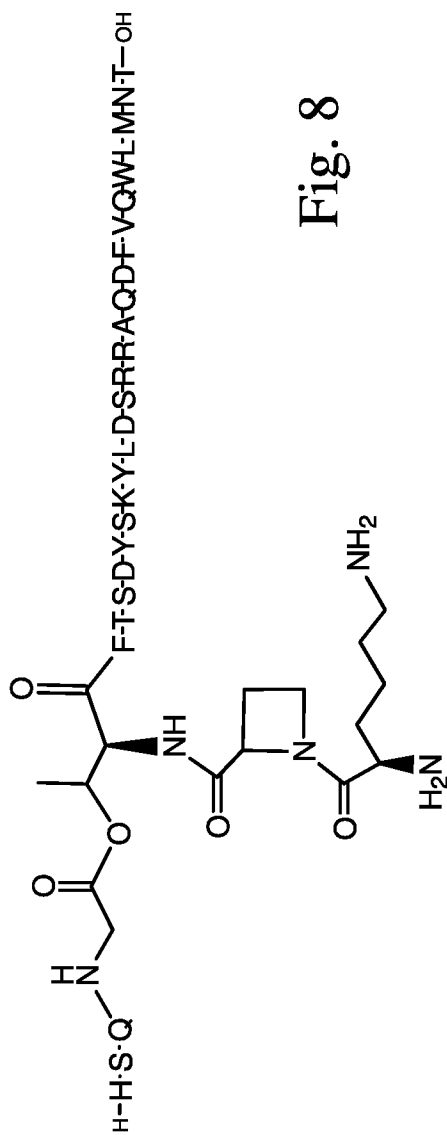


Fig. 9

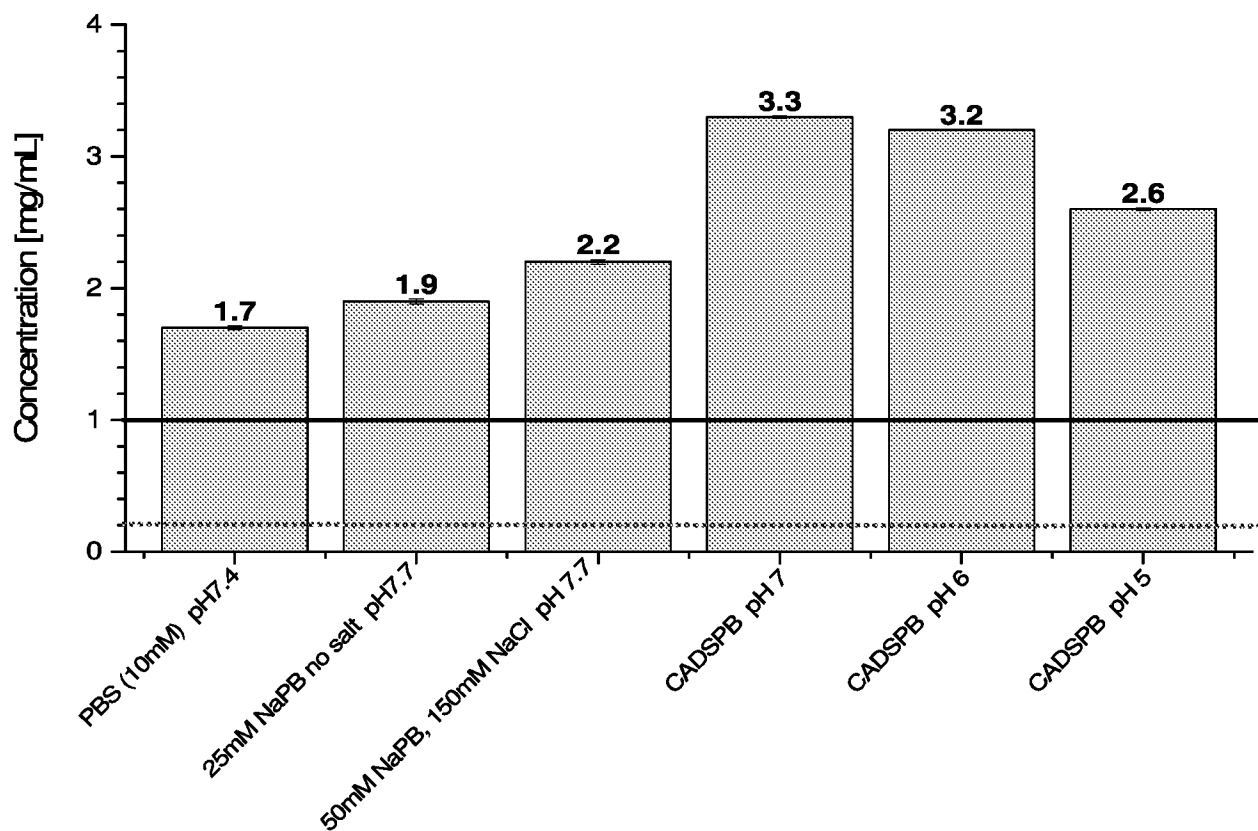


Fig. 10

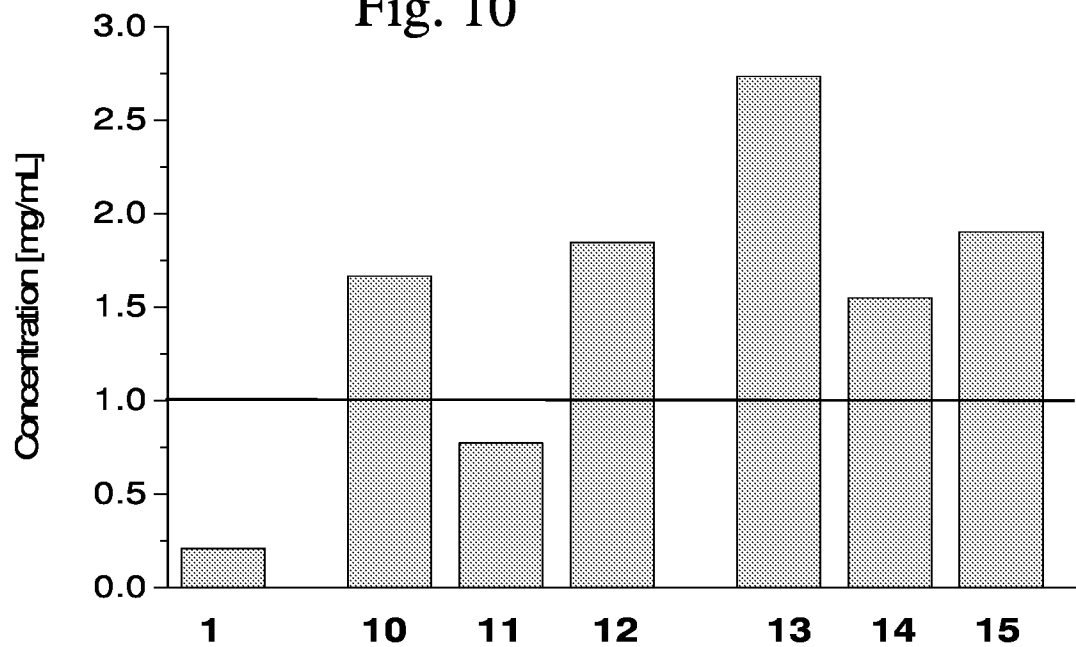


Fig. 11

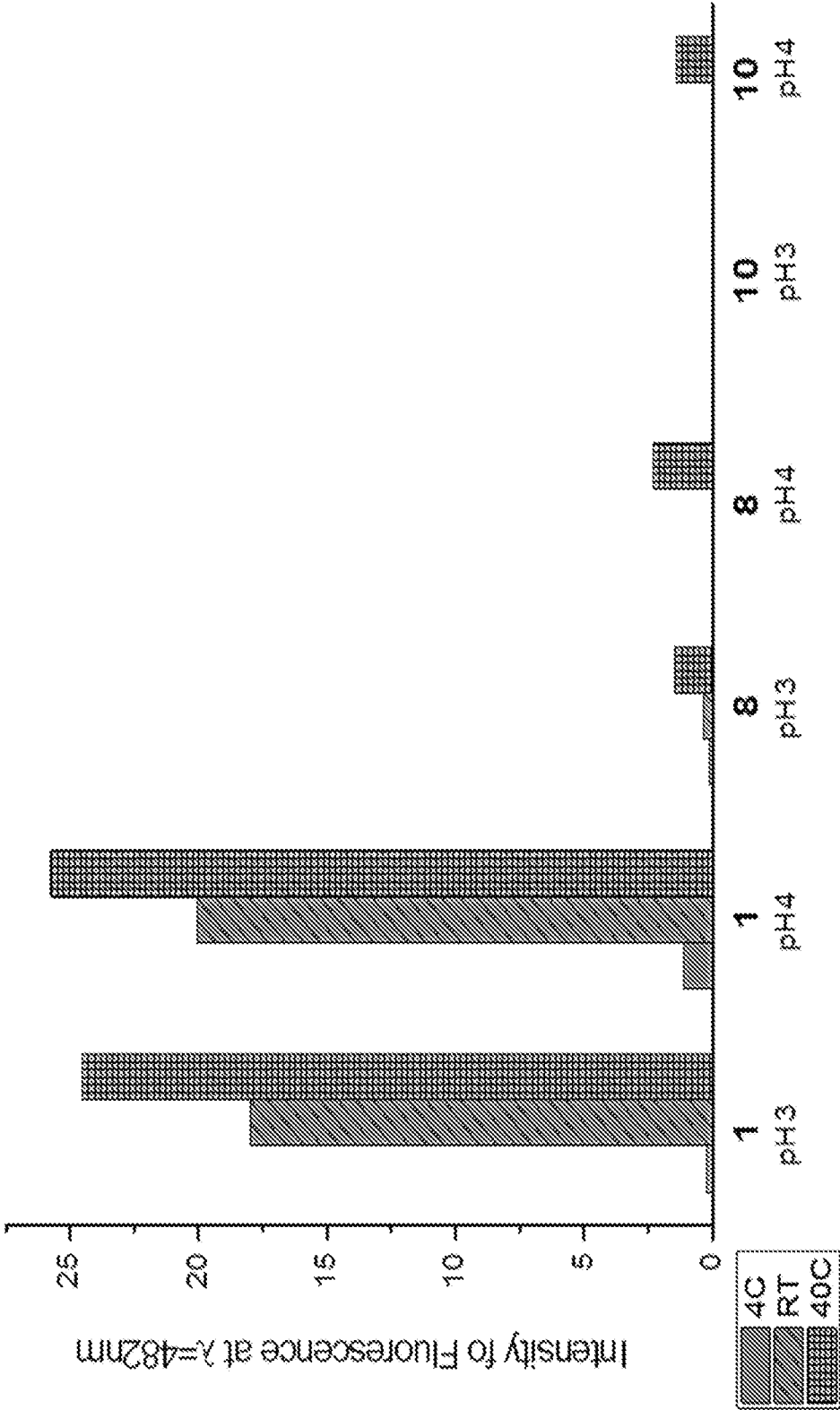


Fig. 12

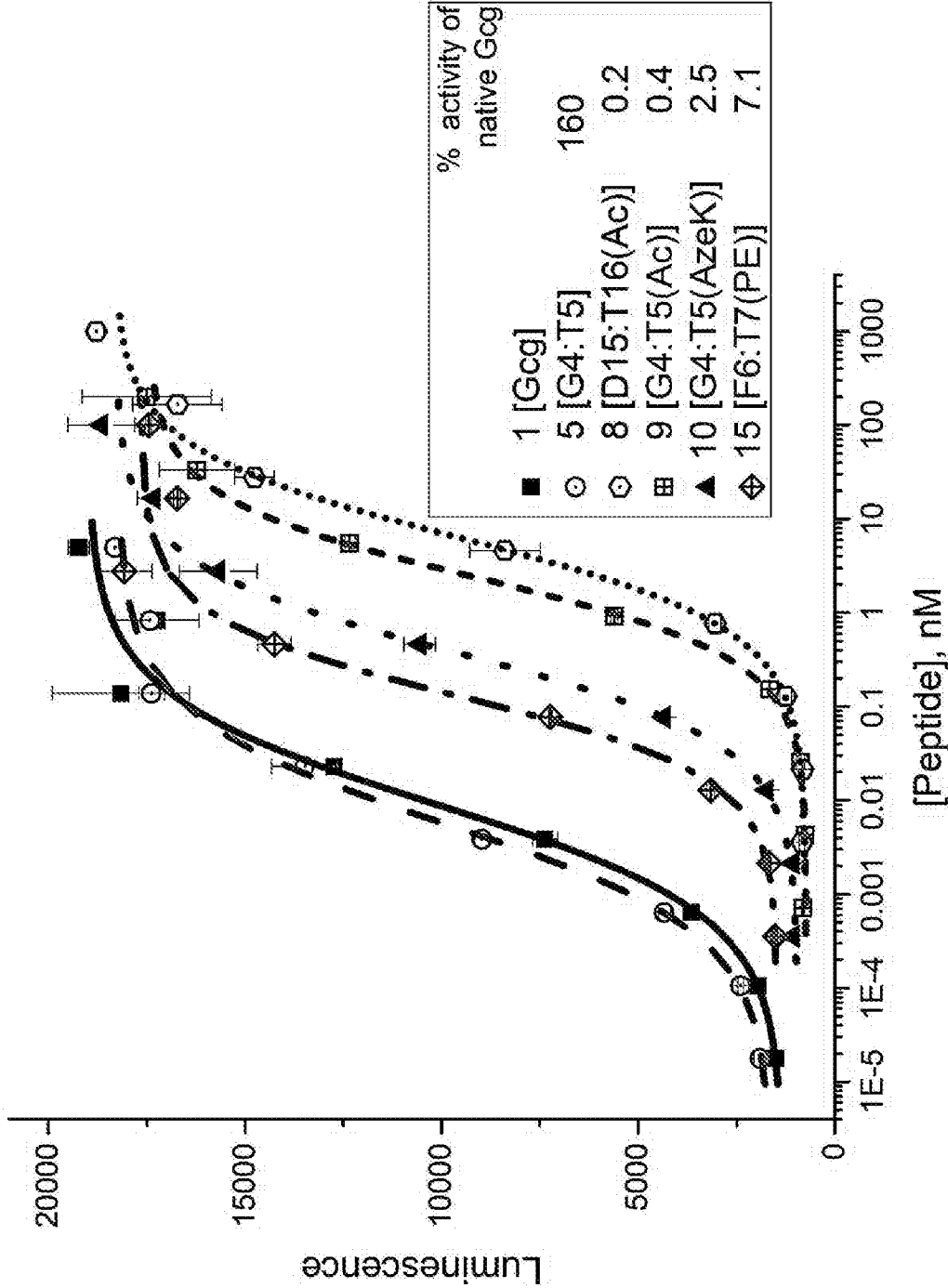
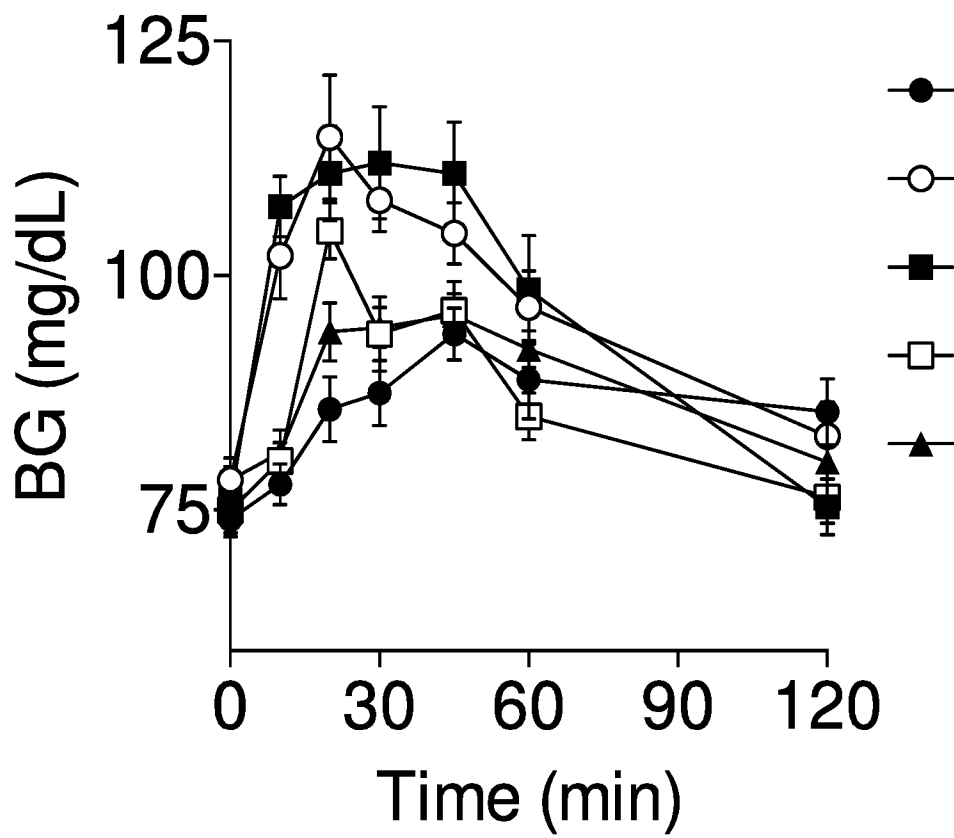


Fig. 13



15/17

Fig. 14

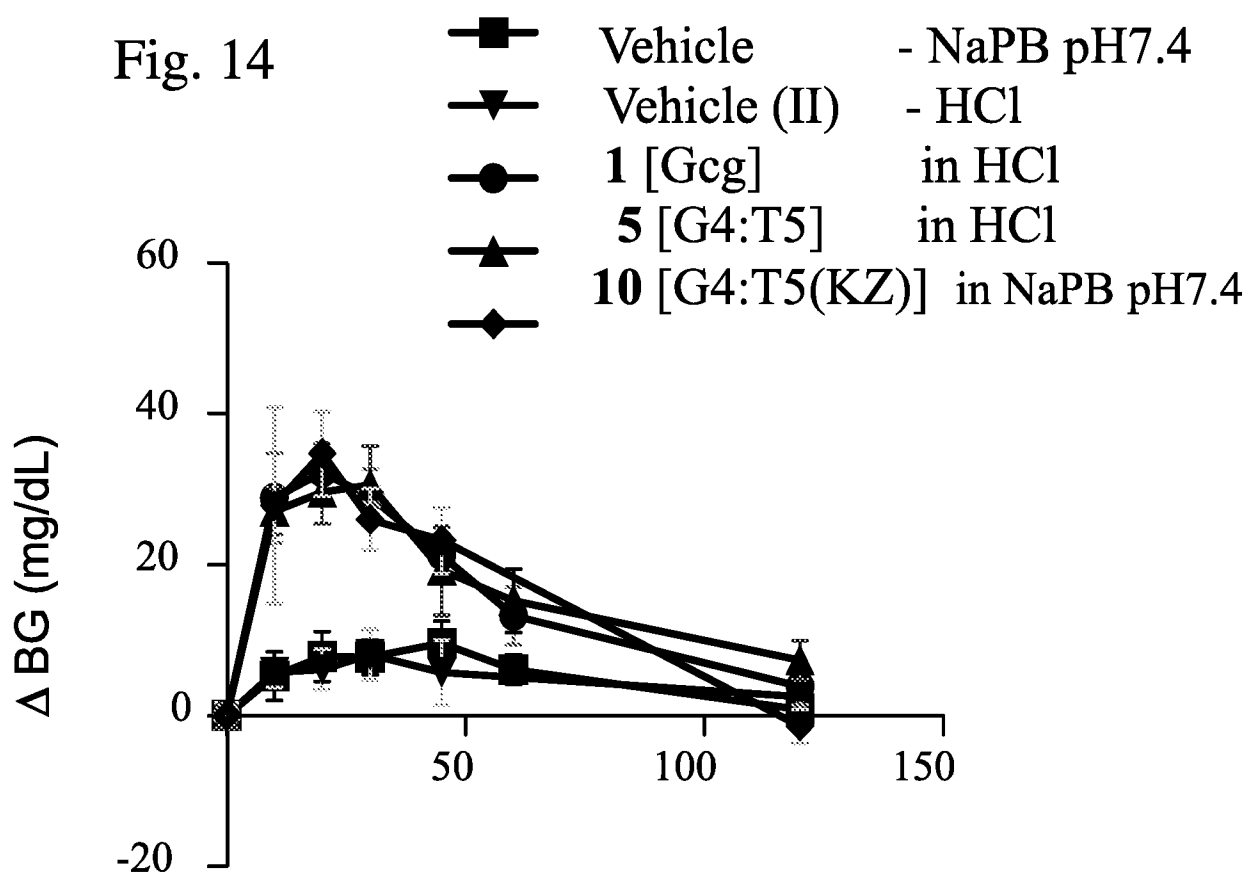
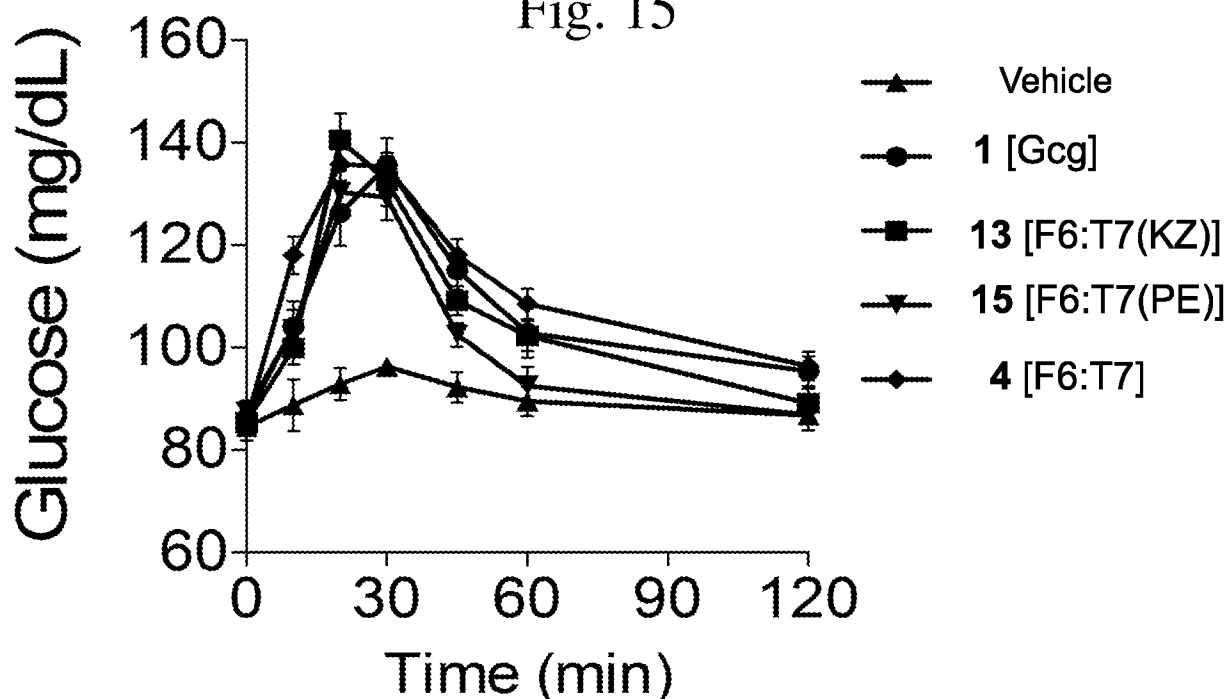


Fig. 15



16/17

Fig. 16

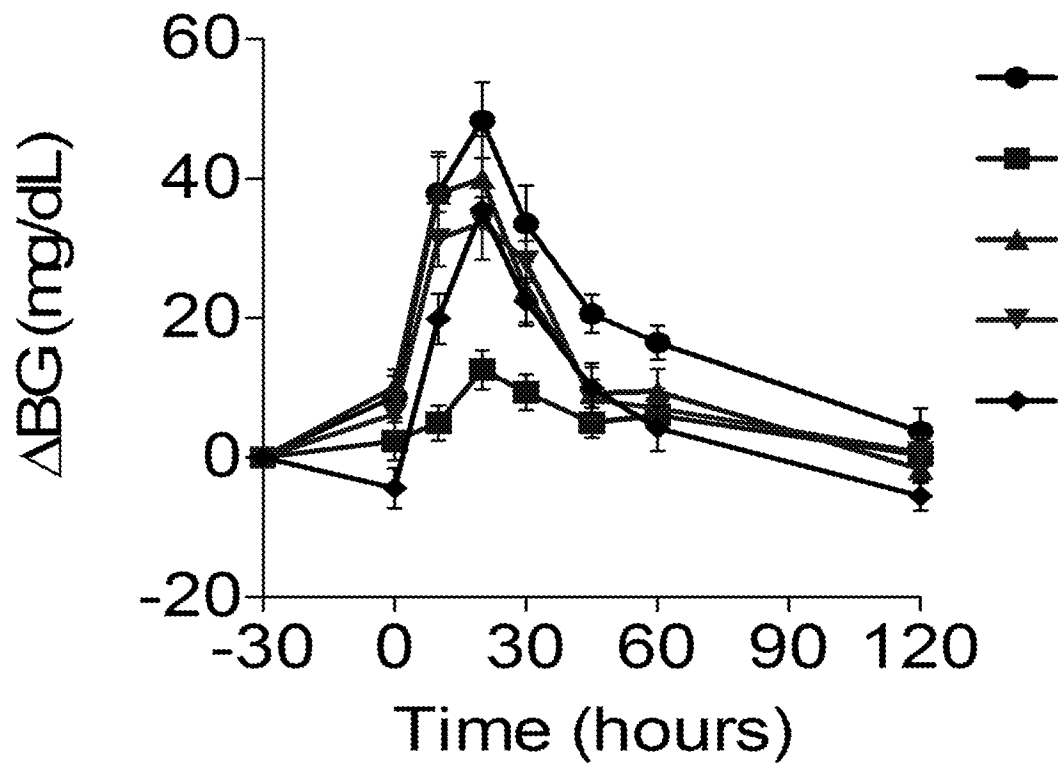


Fig. 17A

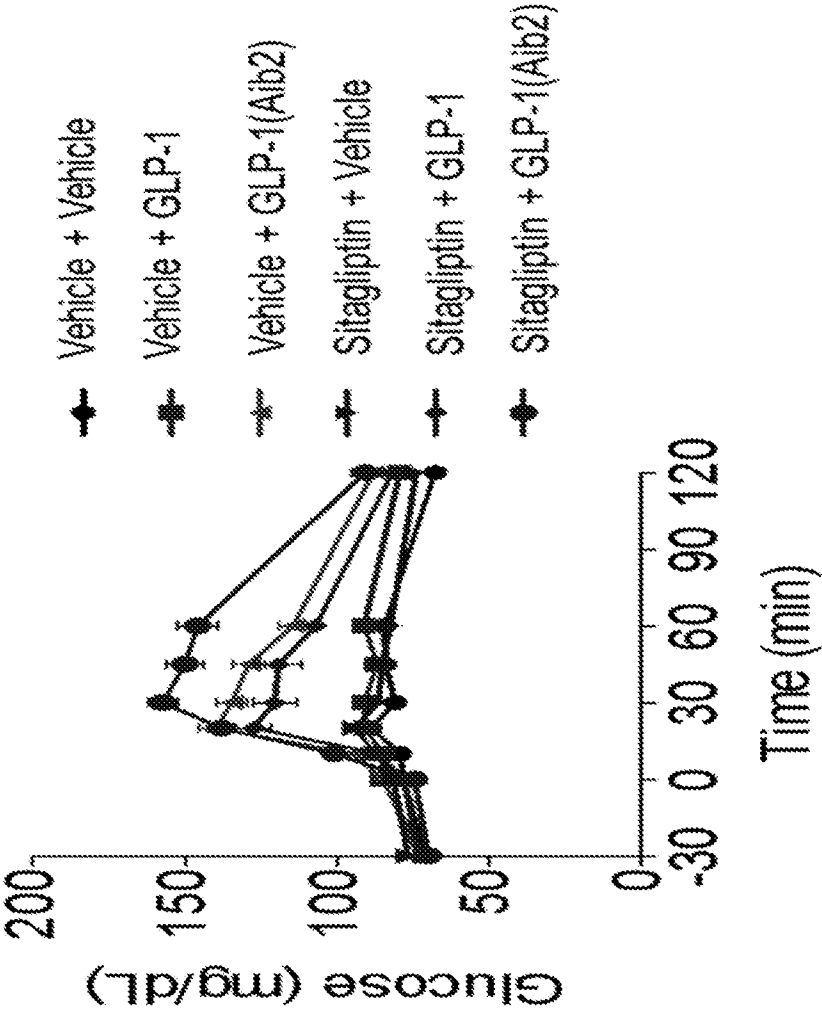
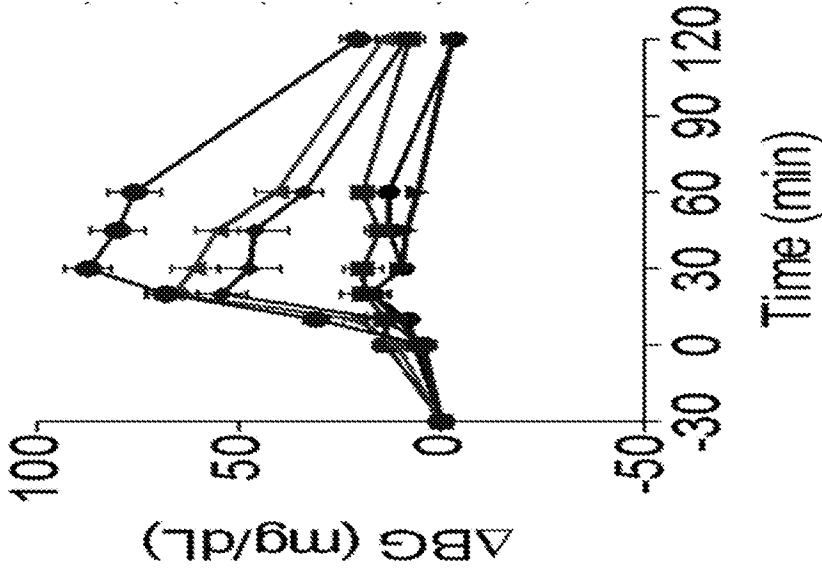


Fig. 17B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/34961

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/26; C07K 14/605 (2017.01)

CPC - A61K 38/26; C07K 14/605

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0098217 A1 (DIMARCHI, RD et al.) 28 April 2011; abstract; paragraph [0008]-[0009], [0014]-[0019], [0154], [0163]	1-2 ----- 3/1-2, 4/3/1-2, 5/4/3/1-2, 6/3/1-2, 7/6/3/1-2
Y	US 2011/0257076 A1 (DIMARCHI, RD et al.) 20 October 2011; paragraphs [0009]-[0010], [0012], [0014]-[0016]	3/1-2, 4/3/1-2, 5/4/3/1-2, 6/3/1-2, 7/6/3/1-2
Y	WO 2016/049190 A1 (INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION) 31 March 2016; page 75, lines 8-16	4/3/1-2, 5/4/3/1-2

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

31 July 2017 (31.07.2017)

Date of mailing of the international search report

25 AUG 2017

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/34961

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 8-29
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.