COO–b2–, or –b1–COO–b2–, and a1, a2, b1, and b2 are each independently a straight or branched-chain C_1–C_6 alkylene;

[00205] n is from 10 to 2400; and

[00206] R is any one selected from the group consisting of 2,5-dioxopyrrolidinyl, 2,5-dioxopyrrolyl, aldehyde, maleimide, C_6–C_{20} aryl disulfide, C_5–C_{20} heteroaryl disulfide, vinyl sulfone, thiol, halogenated acetamide, succinimide, *p*-nitrophenyl carbonate, thioester, and derivatives thereof, without being limited thereto.

[00207] More specifically,

[00208] the preparation method may include: preparing a mono-PEGylated immunoglobulin Fc region by linking a linker of Formula 3 above to the N-terminus of an immunoglobulin Fc region; and preparing a conjugate by linking the linker of the mono-PEGylated immunoglobulin Fc region prepared in the above-described step to a physiologically active polypeptide, or

[00209] the preparation method may include: preparing a mono-PEGylated immunoglobulin Fc region by linking a linker of Formula 3 to the N-terminus of an immunoglobulin Fc region; purifying the mono-PEGylated immunoglobulin Fc region prepared in the above-described step by anion-exchange chromatography in a buffer solution with a pH of 6.0 to 8.5, a pH of 6.0 to 8.0, a pH of 6.0 to 7.5, a pH of 6.0 to 7.0, a pH of 6.1 to 6.9, a pH of 6.2 to 6.8, or a pH of 6.3 to 6.7; and preparing a conjugate by linking the linker of the mono-PEGylated immunoglobulin Fc region purified in the above-described step to a physiologically active polypeptide, without being limited thereto.

[00210] In addition, in the preparation method of the present invention,

[00211] (i) the mono-PEGylated immunoglobulin Fc region may be prepared by linking the linker of Formula 3 to the N-terminus of the immunoglobulin Fc region in the presence of a reducing agent at a pH of 4.0 to 8.0, a pH of 4.5 to 7.5, a pH of 5.5 to 7.5, a pH of 5.6 to 7.4, a pH of 5.7 to 7.3 or a pH of 5.8 to 7.2; and/or

[00212] (ii) the conjugate may be prepared by linking the linker of the mono-PEGylated immunoglobulin Fc region to the physiologically active polypeptide at a pH of 5.5 to 8.0, a pH of 6.0 to 7.5, or a pH of 6.5 to 7.5, without being limited thereto.

[00213] In addition, the step of preparing the conjugate according to the preparation method of the present invention may be performed by reacting the physiologically

[00275] Specifically, the reaction was performed in a composition including a 5 mM Bis-Tris buffer (pH 6.5) and potassium phosphate, and 10 mM $NaCNBH_3$ (sodium cyanoborohydride) was added thereto as a reducing agent. In order to obtain a mono-PEGylated immunoglobulin Fc region, the reaction solution was diluted with the Bis-Tris buffer and purified.

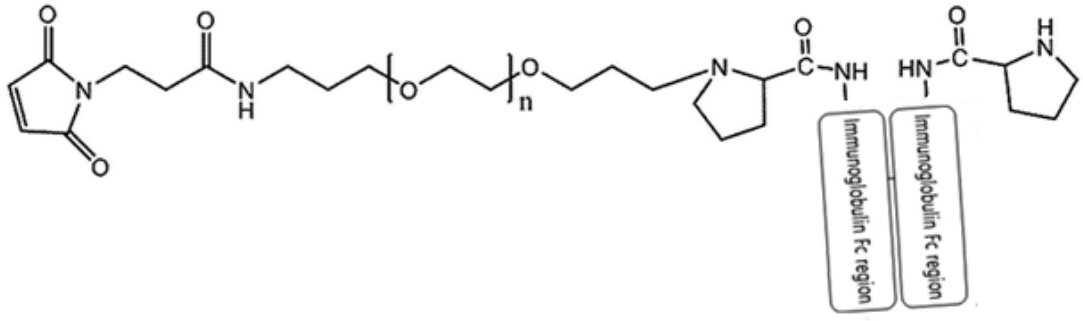
[00276] Unlike the preparation method of the above-described Comparative Examples in which the mono-PEGylated GLP-1/GIP/Glucagon trigonal agonist analog and glucagon analog were purified by cation-exchange chromatography, the mono-PEGylated immunoglobulin Fc region was purified using a CaptoQ ImpRes column (GE Healthcare, anion-exchange chromatography) using a Bis-Tris buffer and a sodium chloride concentration gradient.

[00277] Example 1-2. Analysis of Structure of Mono-PEGylated Immunoglobulin Fc Region

[00278] The mono-PEGylated immunoglobulin Fc region prepared in Example 1-1 was structurally analyzed by MALDI-TOF and Peptide mapping. As a result of MALDI-TOF, the resultant was identical to an expected molecular weight of the mono-PEGylated immunoglobulin Fc region (FIG. 1), and as a result of Peptide mapping, it was confirmed that over 90% of PEG was PEGylated at the N-terminus of the immunoglobulin Fc region.

[00279] Meanwhile, as a result of analyzing the mono-PEGylated immunoglobulin Fc region (Formula 2) prepared in Example 1-1 above using SE-HPLC, RP-HPLC, and IE-HPLC assays, the purity was confirmed to be 90% or more in SE-HPLC, 90% or more in RP-HPLC, and 80% or more in IE-HPLC.

[00280] [Formula 2]



[00281] Example 2: Preparation of Conjugate by Linking PEGylated Immunoglobulin Fc Region to Physiologically Active Polypeptide

[CLAIMS]

[Claim 1]

A compound having a structure of Formula 1 below or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof:

[Formula 1]

$X–L1–(OCH_2CH_2)_nO–L2–R$

wherein in Formula 1 above,

X comprises, at the N-terminus, a hinge sequence that comprises an amino acid sequence of SEQ ID NO: 9 (Pro–Ser–Cys–Pro);

L1 is a straight or branched-chain C_1–C_6 alkylene;

L2 is –a1–CONH–, –a1–NHCO–, –a1–NHCO–a2–, –COO–, –b1–COO–, –COO–b2–, or –b1–COO–b2–, in which a1, a2, b1, and b2 are each independently a straight or branched-chain C_1–C_6 alkylene;

n is from 10 to 2,400; and;

R is any one selected from the group consisting of 2,5-dioxopyrrolidinyl, 2,5-dioxopyrrolyl, aldehyde, maleimide, C_6–C_{20} aryl disulfide, C_5–C_{20} heteroaryl disulfide, vinyl sulfone, thiol, halogenated acetamide, succinimide, *p*-nitrophenyl carbonate, and thioester.

[Claim 2]

The compound or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof according to claim 1, wherein in Formula 1,

L1 is a straight or branched-chain C_1–C_6 alkylene;

L2 is –a1–NHCO– or –a1–NHCO–a2–, in which a1 and a2 are each independently a straight or branched-chain C_1–C_6 alkylene;

n is from 200 to 250; and

R is maleimide.

[Claim 3]

The compound or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof according to claim 1, wherein the L1 is linked to a proline residue (Pro) of SEQ

ID NO: 9.

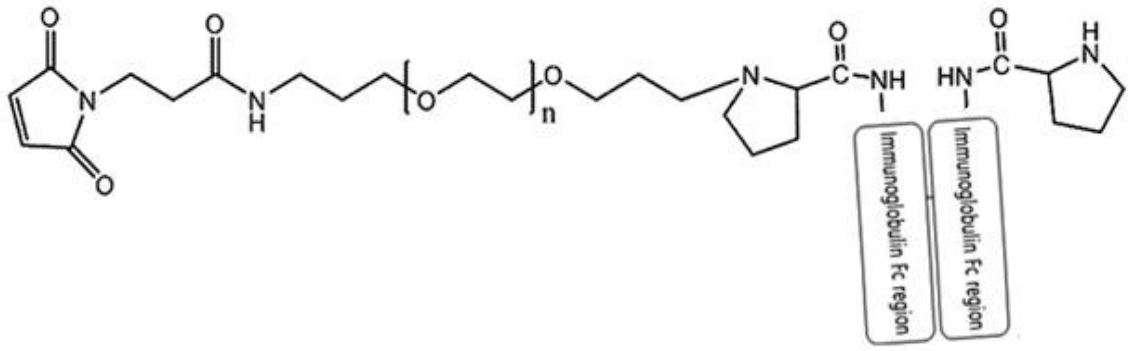
[Claim 4]

The compound or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof according to claim 1, wherein the L1 is linked to an amine reactive group located at one end of X.

[Claim 5]

A compound having a structure of Formula 2 below or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof:

[Formula 2]



wherein in Formula 2 above, n is from 200 to 250.

[Claim 6]

The compound or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof according to claim 1, wherein the X is an immunoglobulin Fc region derived from IgG, IgA, IgD, IgE, or IgM.

[Claim 7]

The compound or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof according to claim 6, wherein the X is an immunoglobulin Fc region derived from IgG1, IgG2, IgG3, or IgG4.

[Claim 8]

The compound or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof according to claim 1, wherein the X is an immunoglobulin Fc region in a dimeric form.

[Claim 9]

The compound or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof according to claim 1, wherein the X is an immunoglobulin Fc region comprising an amino acid sequence of SEQ ID NO: 10.

[Claim 10]

The compound or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof according to claim 1, wherein the compound has a size of 40 kDa to 250 kDa.

[Claim 11]

A composition for preparing a long-acting drug conjugate comprising a compound having a structure of Formula 1 below, or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof,

wherein the drug is a physiologically active polypeptide:

[Formula 1]

$X–L1–(OCH_2CH_2)_nO–L2–R$

in Formula 1 above,

X comprises, at the N-terminus, a hinge sequence that comprises an amino acid sequence of SEQ ID NO: 9 (Pro–Ser–Cys–Pro);

L1 is a straight or branched-chain C_1–C_6 alkylene;

L2 is –a1–CONH–, –a1–NHCO–, –a1–NHCO–a2–, –COO–, –b1–COO–, –COO–b2–, or –b1–COO–b2–, in which a1, a2, b1, and b2 are each independently a straight or branched-chain C_1–C_6 alkylene;

n is from 10 to 2,400; and

R is any one selected from the group consisting of 2,5-dioxopyrrolidinyl, 2,5-dioxopyrrolyl, aldehyde, maleimide, C_6–C_{20} aryl disulfide, C_5–C_{20} heteroaryl disulfide, vinyl sulfone, thiol, halogenated acetamide, succinimide, *p*-nitrophenyl carbonate, and

thioester.

[Claim 12]

The composition according to claim 11, wherein in Formula 1 above,

L1 is a straight or branched-chain C_1–C_6 alkylene;

L2 is –a1–NHCO– or –a1–NHCO–a2–, in which a1 and a2 are each independently a straight or branched-chain C_1–C_6 alkylene;

n is from 200 to 250; and

R is maleimide.

[Claim 13]

The composition according to claim 11, wherein the physiologically active polypeptide is selected from the group consisting of glucagon-like peptide-1 (GLP-1), granulocyte colony stimulating factor (G-CSF), human growth hormone (hGH), erythropoietin (EPO), glucagon, insulin, growth hormone releasing hormone, growth hormone releasing peptide, interferons, interferon receptors, G-protein-coupled receptors, interleukins, interleukin receptors, enzymes, interleukin-binding protein, cytokine-binding protein, macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoprotein, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressor, metastasis growth factor, α-1 antitrypsin, albumin, α-lactalbumin, apolipoprotein-E, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, blood factors VII, VIIa, VIII, IX, and XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, lenin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factor, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, incretins, gastric inhibitory polypeptide (GIP), GLP-1/GIP dual agonist, GLP-1/GIP/Glucagon trigonal agonist, cell surface antigens, virus derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

[Claim 14]

The composition according to claim 11, wherein R of the compound of the composition is linked to cysteine of the drug.

[Claim 15]

The composition according to claim 11, wherein the X is an immunoglobulin Fc region derived from IgG1, IgG2, IgG3, or IgG4.

[Claim 16]

The composition according to claim 11, wherein the composition is used to prepare a long-acting drug conjugate without performing ultrafiltration/diafiltration in preparation of the long-acting drug conjugate.

[Claim 17]

A method for preparing a long-acting conjugate of a physiologically active polypeptide, the method comprising:

preparing a conjugate by linking a mono-PEGylated immunoglobulin Fc region, prepared by linking a linker of Formula 3 below to the N-terminus of an immunoglobulin Fc region comprising a hinge sequence that comprises an amino acid sequence of SEQ ID NO: 9 (Pro–Ser–Cys–Pro), to a physiologically active polypeptide:

[Formula 3]

$CHO–L1–(OCH_2CH_2)_nO–L2–R$

wherein in Formula 3 above,

L1 is a straight or branched-chain C_1–C_6 alkylene;

L2 is –a1–CONH–, –a1–NHCO–, –a1–NHCO–a2–, –COO–, –b1–COO–, –COO–b2–, or –b1–COO–b2–, in which a1, a2, b1, and b2 are each independently a straight or branched-chain C_1–C_6 alkylene;

n is from 10 to 2400; and;

R is any one selected from the group consisting of 2,5-dioxopyrrolidinyl, 2,5-dioxopyrrolyl, aldehyde, maleimide, C_6–C_{20} aryl disulfide, C_5–C_{20} heteroaryl disulfide, vinyl sulfone, thiol, halogenated acetamide, succinimide, *p*-nitrophenyl carbonate, and thioester.

[Claim 18]

The method according to claim 17, wherein the mono-PEGylated immunoglobulin Fc region is prepared by linking the linker of Formula 3 above to the N-terminus of the immunoglobulin Fc region at a pH of 4.0 to 8.0 in the presence of a reducing agent.

[Claim 19]

The method according to claim 17, wherein the conjugate is prepared by linking the linker of the mono-PEGylated immunoglobulin Fc region to the physiologically active polypeptide at a pH of 5.5 to 8.0.

[Claim 20]

The method according to claim 17, wherein the preparing of the conjugate is performed by reacting the mono-PEGylated immunoglobulin Fc region with the physiologically active polypeptide in a molar ratio of 1:1 to 1:3.

[Claim 21]

The method according to claim 17, wherein the method comprises:

preparing a mono-PEGylated immunoglobulin Fc region by linking a linker of Formula 3 to the N-terminus of the immunoglobulin Fc region; and

preparing a conjugate by linking the linker of the mono-PEGylated immunoglobulin Fc region prepared in the previous step to a physiologically active polypeptide.

[Claim 22]

The method according to claim 21, wherein the linker of the mono-PEGylated immunoglobulin Fc region is linked to a cysteine residue of the physiologically active polypeptide.

[Claim 23]

The method according to claim 21, wherein the method comprises:

preparing a mono-PEGylated immunoglobulin Fc region by linking a linker of Formula

3 to the N-terminus of an immunoglobulin Fc region; purifying the mono-PEGylated immunoglobulin Fc region prepared in the previous step by anion-exchange chromatography in a buffer solution with a pH of 6.0 to 8.5; and

preparing a conjugate by linking the linker of the mono-PEGylated immunoglobulin Fc region purified in the previous step to a physiologically active polypeptide.

[Claim 24]

The method according to claim 21, wherein the method is performed without ultrafiltration/diafiltration after preparing the mono-PEGylated immunoglobulin Fc region.

[Claim 25]

The method according to claim 17, further comprising purifying the conjugate by hydrophobic interaction chromatography.

[Claim 26]

The method according to claim 17, wherein, in Formula 3,

L1 is a straight or branched-chain C_1–C_6 alkylene; L2 is –a1–NHCO– or –a1–NHCO–a2–, in which a1 and a2 are each independently a straight or branched-chain C_1–C_6 alkylene;

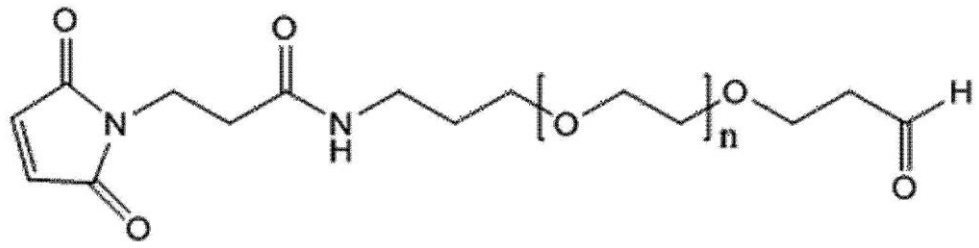
n is from 200 to 250; and

R is maleimide.

[Claim 27]

The method according to claim 17, wherein the linker has a structure of Formula 4 below:

[Formula 4]



wherein in Formula 4 above, n is from 200 to 250.

[Claim 28]

The method according to claim 17, wherein the linker has a size of 1 kDa to 100 kDa.

[Claim 29]

The method according to claim 17, wherein the physiologically active polypeptide is selected from the group consisting of glucagon-like peptide-1 (GLP-1), granulocyte colony stimulating factor (G-CSF), human growth hormone (hGH), erythropoietin (EPO), glucagon, insulin, growth hormone releasing hormone, growth hormone releasing peptide, interferons, interferon receptors, G-protein-coupled receptors, interleukins, interleukin receptors, enzymes, interleukin-binding protein, cytokine-binding protein, macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoprotein, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressor, metastasis growth factor, α-1 antitrypsin, albumin, α-lactalbumin, apolipoprotein-E, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, blood factors VII, VIIa, VIII, IX, and XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, lenin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factor, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, incretins, GLP-1/GIP dual agonist, gastric inhibitory polypeptide (GIP), GLP-1/GIP/Glucagon trigonal agonist, cell surface antigens, virus derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

[Claim 30]

The method according to claim 29, wherein the physiologically active polypeptide is a GLP-1/GIP/Glucagon trigonal agonist, glucagon, or an analog thereof.

[Claim 31]

The method according to claim 30, wherein the physiologically active polypeptide includes any one amino acid sequence of SEQ ID NOS: 1 to 6.

[Claim 32]

The method according to claim 17, wherein the immunoglobulin Fc region is derived from IgG1, IgG2, IgG3, or IgG4.

[Claim 33]

A long-acting drug conjugate prepared using the method according to any one of the methods of claims 17 to 32.

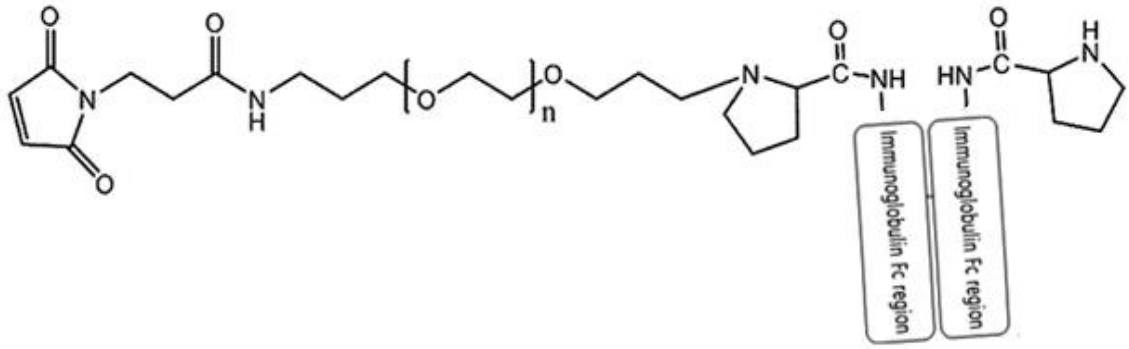
[Claim 34]

A long-acting drug conjugate prepared by reacting the composition according to any one of claims 11 to 16 with a physiologically active polypeptide.

[Claim 35]

A composition for preparing a long-acting drug conjugate compound having a structure of Formula 2 below or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof:wherein the drug is a physiologically active polypeptide:

[Formula 2]



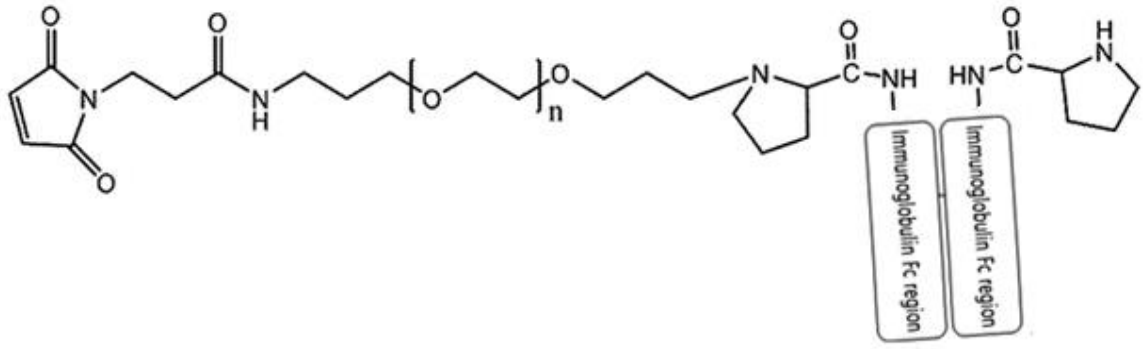
wherein in Formula 2 above, n is from 200 to 250.

[Claim 36]

A method for preparing a long-acting conjugate of a physiologically active polypeptide, the method comprising:

preparing a conjugate by linking a compound having a structure of Formula 2 below or a stereoisomer, a solvate, or a pharmaceutically acceptable salt thereof, to a physiologically active polypeptide:

[Formula 2]



wherein in Formula 2 above, n is from 200 to 250.

[Claim 37]

A long-acting drug conjugate prepared by reacting the composition of claim 35 with a physiologically active polypeptide.

[Claim 38]

A long-acting drug conjugate prepared by the method of claim 36.