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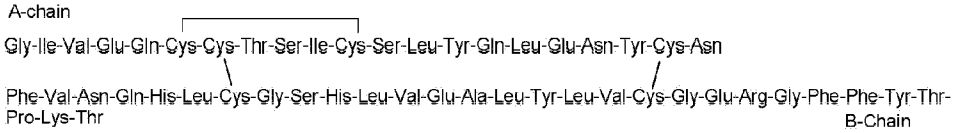
DESCRIPTION

[0001] The present disclosure relates to insulin analogues having therapeutic applications in the treatment of diabetes. More specifically, but not exclusively, it relates to single chain insulin analogues comprising the A-chain and the B-chain of insulin, where in addition to intermolecular disulfide bonds, the chains are connected together through the functional group of an amino acid side chain of an amino acid in the B-chain.

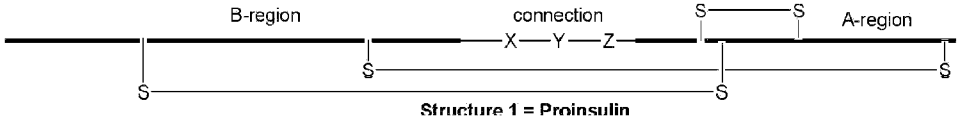
BACKGROUND TO THE INVENTION

[0002] Insulin is a peptide hormone secreted by the β-cells of the pancreas. It consists of two peptide chains, A and B, which are linked by two intermolecular disulphide bonds. The A-chain also contains an additional intramolecular disulfide bond. Human insulin has the structure 2.



Structure 2 = human Insulin

[0003] Insulin is produced as a single-chain precursor, preproinsulin, which consists of a propeptide of 24 amino acid followed by proinsulin containing 86 amino acids. The sequence of the prepropeptide is Prepeptide-[B-chain]-Arg-Arg-[connecting peptide]-Lys-Arg-[A-chain], wherein the connecting peptide consists of 31 amino acids. After the enzymatic removal of the prepeptide, the three disulfide bonds are formed and proinsulin is produced.



Structure 1 = Proinsulin

[0004] The mature insulin is then liberated by enzymatic cleavage of the connecting peptide at the Arg-Arg and Lys-Arg sites.

[0005] Proinsulin has a 100-fold lower affinity for the insulin receptor than native insulin because the essential residues for binding to the receptor, namely the N-terminal amino function of the A-chain and the C-terminal carboxyl function of the B-chain, are blocked.

[0006] The stability and solubility properties of insulin are important in the context of insulin therapeutics. A number of insulin analogues are known in the art.

[0007] By way of example, single-chain insulin analogues with insulin activity are disclosed in EP1193272. These single-chain insulins have a modified C-peptide of 5-18 amino acids and are reported to have up to 42% insulin activity.

[0008] US 5,597,796 discloses insulin analogues in which two or more amino acid residues are substituted by Glu and/or Asp. Similarly, US 20090069216 and WO 2007/096332 disclose fast acting single chain insulins containing a modified B-chain and a connecting peptide. The resulting analogues are particularly well suited for transdermal administration. Fibrillation-resistant insulin and insulin analogues are disclosed in US 8,192,957. Pegylated single chain insulins are disclosed in US 2010/0216690, whereas acylated single chain insulins are disclosed in WO 2007/104738.

[0009] WO 2005/054291 discloses single chain insulin analogues wherein the A-chain and B-chains are connected by a connecting peptide of 5-11 amino acids. Likewise, WO 95/16708 also discloses single chain insulin analogues wherein the A-chain and B-chains are connected by a connecting peptide of 1-15 amino acids, in which the C-terminal amino acid residue is other than Lys or Arg. EP0427296 dicloses human insulin precursors of the general formula B(1-29)-X_n-Y-A(1-21), wherein X_n is a peptide chain with n naturally occurring amino acid residues. Similarly, EP0741188 discloses single chain insulin derivatives of the formula b-BP-a having significant insulin activity; these single chain insulins are reported to have insulin activity but also a high affinity to the IGF-1 receptor.

[0010] Insulin derivatives containing D-amino acids at their A1 position have been shown to retain their biological activity [Geiger R, Geisen K, Regitz G, Summ HD, Langner D., Hoppe Seylers Z Physiol Chem. 1980 Apr;361(4):563-70]. Insulin derivatives containing D-Glu at the B21 position have been shown to be equipotent with natural insulin [Wang SH, Hu SQ, Burke GT, Katsoyannis PG. J Protein Chem. 1991 Jun;10(3):313-24.] WO 2011/031662 A1 discloses compositions and methods related to ester insulin or derivatives thereof. The compositions include Glu^{A4}-Thr^{B30} ester insulin, in which the side chains of Glu^{A4} and Thr^{B30} of native human insulin or an insulin analogue such as insulin lispro are covalently linked via a single ester bond.

[0011] The present invention seeks to provide new single chain insulin analogues that exhibit useful therapeutic properties, wherein the two insulin chains are connected through the amino acid side chain of an amino acid in the insulin B-chain. The invention is defined in the claims. Any subject-matter which is described herein, but which is not claimed, does not form part of the invention.

STATEMENT OF INVENTION

[0012] A first aspect of the invention relates to a single chain insulin analogue comprising:

1. (A) the A-chain of human or animal insulin, or an analogue thereof which is a variant wherein (a) one amino acid residue is replaced by a naturally or non-naturally occurring amino acid, (b) the order of two amino acid residues is reversed, or (c) both (a) and (b) are present together;

2. (B) the B-chain of human or animal insulin, or an analogue thereof which is a variant wherein (a) one amino acid residue is replaced by a naturally or non-naturally occurring amino acid, (b) the order of two amino acid residues is reversed, or (c) both (a) and (b) are present together;;
3. (C)
    1. (i) an intermolecular disulfide bond between the cysteine in position 7 of the insulin A-chain and the cysteine in position 7 of the insulin B-chain;
    2. (ii) an intermolecular disulfide bond between the cysteine in position 20 of the insulin A-chain and the cysteine in position 19 of the insulin B-chain;
    3. (iii) an intermolecular disulfide bond between the cysteine in position 6 and the cysteine in position 11 of the insulin A-chain; and
4. (D) a further covalent link, L, between the carboxyl terminal amino acid of said A-chain of human or animal insulin, or analogue thereof, with the side chain of the lysine residue in position 29 of said B-chain of human or animal insulin, or analogue thereof, wherein the further covalent link, L, is a direct bond;

wherein said single chain insulin analogue performs the same action as human insulin in terms of glycemic control.

[0013] The presently claimed insulin analogues differ from those known in the art by virtue of the fact that the two insulin chains are linked via the functional group of the side chain of an amino acid already forming part of the insulin B-chain. In view of this, they are distinct from conventional single chain peptides and cannot be considered as "proinsulin-like" peptides.

[0014] Proinsulin-like peptides, many of which are described in the literature and known in nature, contain a conventional connecting peptide, for example, where the N-terminal amino acid of one of the insulin chains is linked to the C-terminal amino acid of the other. Such peptides can be produced by conventional recombinant DNA techniques. However, is impossible to produce the presently claimed insulin analogues by recombinant DNA methodology or other biological methods. Accordingly, the insulin analogues described herein are the prototypes of a new single chain group of insulins not previously known in the art.

[0015] Incorporating a link between a functional group of an amino acid of one chain and the side chain functional group of an amino acid of the other chain means that at least one of the essential groups for biological activity, namely the N-terminal amino function of the A-chain or the C-terminal carboxyl function of the B-chain, remains free.

[0016] Advantageously, incorporating an additional link between the A-chain and the B-chain decreases the flexibility of the chains, which in turn reduces fibrillation and precipitation and increases the chemical and enzymatic stability. Single chain insulins where the A and B chains are connected through a C-peptide often exhibit higher chemical stability, but tend to show lower affinity to the insulin receptor because the N-terminal amino function of the A-chain and C-terminal carboxyl function of the B-chain are blocked. The presently claimed single chain insulin analogues retain the advantages of an additional link between the chains to decrease

flexibility, whilst at the same time alleviating the problem of low affinity by freeing up one or both of the terminal groups that are essential for activity.

DETAILED DESCRIPTION

[0017] The single-chain insulin analogues described herein encompass a group of structurally-related proteins wherein the A and B chains are covalently linked through at least the side chain functional group of an amino acid contained in the B-chain.

[0018] Thus, the present invention relates to single-chain insulin analogues comprising the A and B-chains of human or animal insulin, or analogues thereof, which are connected together, besides intermolecular disulfide bonds, by an additional linker which is formed between the carboxyl amino acid of the A-chain and the side chain of the lysine residue in position 29 of the B-chain.

[0019] As used herein, the term "insulin analogue" refers to an altered form of insulin, different from any occurring in nature, but still available to the human body for performing the same action as human insulin in terms of glycemic control. Through genetic engineering of the underlying DNA, the amino acid sequence of insulin can be changed to alter its absorption, distribution, metabolism, and excretion characteristics. Officially, the U.S. Food and Drug Administration (FDA) refers to these as "insulin receptor ligands", although they are more commonly referred to as insulin analogues. Modifications include insulin analogues that are more readily absorbed from the injection site and therefore act faster than natural insulin injected subcutaneously, intended to supply the bolus level of insulin needed at mealtime (prandial insulin); and those that are released slowly over a period of between 8 and 24 hours, intended to supply the basal level of insulin during the day and particularly at nighttime (basal insulin). Fast acting insulin analogues include insulin lispro (Eli Lilly and Company) and insulin aspart (Novo Nordisk), whereas long acting insulin analogues include NPH insulin, insulin glulisine (Sanofi-Aventis), insulin detemir (Novo Nordisk) and insulin glargine (Sanofi-Aventis).

[0020] Insulin analogues include variants of insulin. As used herein, the term "variant" includes any variation wherein wherein (a) one amino acid residue is replaced by a naturally or non-naturally occurring amino acid residue (b) the order of two amino acid residues is reversed, or (c) both (a) and (b) are present together. Preferably, the substitution is homologous.

[0021] Homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine, diaminobutyric acid ornithine, norleucine ornithine, pyridylalanine, thienylalanine, naphthylalanine and phenylglycine, a more detailed list of which appears below.

[0022] As used herein, amino acids are classified according to the following classes;

basic; H, K, R

acidic; D, E

non-polar; A, F, G, I, L, M, P, V, W

polar; C, N, Q, S, T, Y,

(using the internationally accepted single letter amino acid notation) and homologous and non-homologous substitution is defined using these classes. Thus, homologous substitution is used to refer to substitution from within the same class, whereas non-homologous substitution refers to substitution from a different class or by an unnatural amino acid.

[0023] Further variation may occur by virtue of reversing the sequence of a two amino acid residues within a sequence.

[0024] In one embodiment the replacement amino acid residue is selected from the residues of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

[0025] The replacement amino acid residue may additionally be selected from unnatural amino acids as described below.

[0026] As used herein, the term "derivative" refers to insulin that has undergone chemical modification, for example, to the amino acid side chains at the N-terminus and/or the C-terminus. Preferably, the chemical modification serves to alter the absorption, distribution, metabolism, and excretion characteristics of the analogue. Semisynthetic insulins were clinically used for some time based on chemical modification of animal insulins, for example Novo Nordisk enzymatically converted porcine insulin into semisynthetic 'human' insulin by removing the single amino acid that varies from the human variety, and chemically adding the human amino acid.

[0027] In one preferred embodiment, the insulin is chemically modified to alter its isoelectric point. Normal unmodified insulin is soluble at physiological pH. Modified derivatives of insulin have been created that have a shifted isoelectric point so that they exist in a solubility equilibrium in which most precipitates out but slowly dissolves in the bloodstream and is eventually excreted by the kidneys.

[0028] In one preferred embodiment, the single chain insulin analogue of the invention is derived from animal insulin.

[0029] The amino acid sequence of animal insulins in different mammals may be similar to human insulin (insulin human INN). However, there is considerable viability within vertebrate

species. Porcine insulin has only a single amino acid variation from the human variety, and bovine insulin varies by three amino acids. Both are active on the human receptor with approximately the same strength. Bovine insulin and porcine insulin were the first clinically used insulin analogues (naturally occurring, produced by extraction from animal pancreas), at the time when biosynthetic human insulin (insulin human rDNA) was not available. Insulin from sharks and some species of fish may be also effective.

[0030] The single chain insulin analogue of the invention comprises the A chain of human or animal insulin, or an analogue as defined above.

[0031] The single chain insulin analogue of the invention further comprises the B chain of human or animal insulin, or an analogue as defined above.

[0032] In a preferred embodiment, the single chain insulin analogue of the invention is biosynthetic insulin (insulin human rDNA).

[0033] In the present invention, the covalent link, L, is between the carboxyl terminal amino acid of the A-chain of human or animal insulin, or analogue thereof as defined above, with the side chain of the lysine residue in position 29 of the B-chain of human or animal insulin, or analogue thereof as defined above.

[0034] As used herein, the term "functional group" refers to specific groups of atoms or bonds within a molecule that are responsible for the characteristic chemical reactions of that molecule.

[0035] In the present invention, the covalent link, L, is a direct bond.

[0036] In one preferred embodiment, the covalent link L is a direct bond between the side chain of an aspartic acid residue or of a glutamic acid residue at the C-terminal amino acid of the A-chain and the side chain amino function of a Lys residue in the B-chain.

[0037] In one highly preferred embodiment, the two chains are connected directly through the side chain of an aspartic acid residue or of a glutamic acid residue contained as the C-terminal amino acid of the A-chain of insulin and the side chain amino function of the Lys residue contained as the C-terminal amino acid of B-chain of insulin.

[0038] As used herein, the term "alkyl" includes both saturated straight chain and branched alkyl groups which may be substituted (mono- or poly-) or unsubstituted. Preferably, the alkyl group is a C_{1-20} alkyl group, more preferably a C_{1-15}, more preferably still a C_{1-12} alkyl group, more preferably still, a C_{1-6} alkyl group, more preferably a C_{1-3} alkyl group. Particularly preferred alkyl groups include, for example, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, pentyl and hexyl. Suitable substituents include, for example, one or more groups selected from OH, O-alkyl, halogen, NH_2, NH-alkyl, N-$(alkyl)_2$, CF_3, NO_2, CN, COO-alkyl,

COOH, $CONH_2$, CO-NH-alkyl, CO-N(alkyl)$_2$, SO_2-alkyl, SO_2NH_2 and SO_2-NH-alkyl.

[0039] As used herein, the term "aryl" refers to a C_{6-12} aromatic group which may be substituted (mono- or poly-) or unsubstituted. Typical examples include phenyl and naphthyl etc. Suitable substituents include, for example, one or more groups selected from OH, O-alkyl, halogen, NH_2, NH-alkyl, N-(alkyl)$_2$, CF_3, NO_2, CN, COO-alkyl, COOH, $CONH_2$, CO-NH-alkyl, CO-N(alkyl)$_2$, SO_2-alkyl, SO_2NH_2 and SO_2-NH-alkyl.

[0040] The term "aralkyl" is used as a conjunction of the terms alkyl and aryl as given above.

[0041] Natural amino acids include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

[0042] As used herein, the term "non-natural amino acid" or "unnatural amino acid" includes alpha and alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid, halide derivatives of natural amino acids such as trifluorotyrosine, p-Cl-phenylalanine, p-F-phenylalanine, p-Br-phenylalanine, p-NO_2-phenylalanine, phenylglycine, sarcosine, penicillamine, D-2-methyltryptophan, phosphoserine, phosphothreonine, phosphotyrosine, p-I-phenylalanine, L-allyl-glycine, ß-alanine, ß-aspartic acid, ß-cyclohexylalanine, citrulline, homoserine, homocysteine, pyroglutamic acid, L-α-amino butyric acid, L-γ-amino butyric acid, L-α-amino isobutyric acid, α-cyclohexylglycine, diaminobutyric acid, diaminopimelic acid, N-ε-dinitrophenyl-lysine, L-1-naphthylalanine, L-2-naphthylalanine, 3-(2-pyridyl)-L-alanine, 3-(3-pyridyl)-L-alanine, 3-(4-pyridyl)-L-alanine, N-ε-methyl-lysine, N,N-ε-dimethyl-lysine, N,N,N-ε-trimethyl-lysine, 3-mercaptopropionic acid, L-ε-amino caproic acid, 7-amino heptanoic acid, 6-amino hexanoic acid L-methionine sulfone, ornithine, L-norleucine, L-norvaline, p-nitro-L-phenylalanine, L-hydroxyproline, γ-glutamic acid, γ-amino butyric acid L-thioproline, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe, pentamethyl-Phe, L-Phe (4-amino), L-Tyr (methyl), L-Phe (4-isopropyl), L-Tic (1,2,3,4tetrahydroisoquinoline-3-carboxyl acid), L-diaminopropionic acid and L-Phe (4-benzyl).

[0043] Advantageously, the introduction of unnatural amino acids leads to an increase in the enzymatic stability of the peptides.

[0044] The insulin analogues of the present invention may comprise amino acids in the L or D form, i.e. one or more residues, preferably all the residues may be in the L or D form. In the present invention, the linkage between the chains is formed between the C-terminal amino acid of the A-chain of human or animal insulin, or analogue thereof as defined above, and the side chain of the lysine residue in position 29 of the B-chain of human or animal insulin, or analogue thereof as defined above.

[0045] In one preferred embodiment, the A-chain comprises amino acids 1 to 21 of human insulin counting from the N-terminal end of the A chain.

[0046] In one preferred embodiment, the A-chain consists of amino acids 1 to 21 of human insulin counting from the N-terminal end of the A chain.

[0047] In another preferred embodiment, the A-chain comprises amino acids 1 to 20 of human insulin counting from the N-terminal end of the A chain.

[0048] In another preferred embodiment, the A-chain consists of amino acids 1 to 20 of human insulin counting from the N-terminal end of the A chain.

[0049] In one preferred embodiment, the B-chain comprises amino acids 1 to 29 of human insulin counting from the N-terminal end of the B chain.

[0050] In one preferred embodiment, the B-chain consists of amino acids 1 to 29 of human insulin counting from the N-terminal end of the B chain.

[0051] In one preferred embodiment, the B-chain of insulin further comprises up to 20 additional natural or unnatural amino acids at the C-terminal end. Preferably, the B-chain further comprises from 1 to 10, or more preferably from 1 to 5 additional natural or unnatural amino acids at the C-terminal end. In one highly preferred embodiment, the B-chain further comprises 1, 2 or 3 additional natural or unnatural amino acids at the C-terminal end. More preferably still, the B-chain further comprises 1, 2 or 3 additional natural amino acids at the C-terminal end.

[0052] In the present invention, the single chain insulin analogue further comprises:

1. (i) an intermolecular disulfide bond between the cysteine in position 7 of the insulin A-chain and the cysteine in position 7 of the insulin B-chain;
2. (ii) an intermolecular disulfide bond between the cysteine in position 20 of the insulin A-chain and the cysteine in position 19 of the insulin B-chain;
3. (iii) an intramolecular disulfide bond between the cysteine in position 6 and the cysteine in position 11 of the insulin A-chain.

[0053] In one preferred embodiment, the single chain insulin analogue is of formula (I),

(I)

wherein:

L is as defined above;

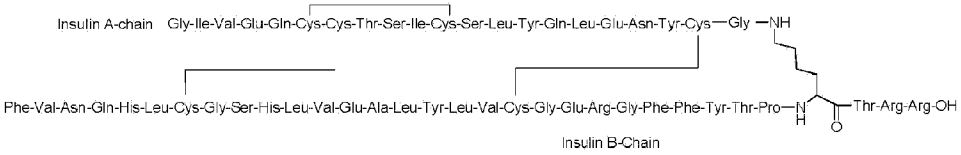
each Aaa_1 is independently a natural or unnatural amino acid;

f is an integer selected from 0 to 20;

R is OH or NH_2.

[0054] In one preferred embodiment, R is OH, f is 0, 1, 2 or 3 and each Aaa_1 is independently a natural amino acid. More preferably, the natural amino acid is selected from Arg and Thr.

[0055] In one preferred embodiment, the single chain insulin is selected from the following:



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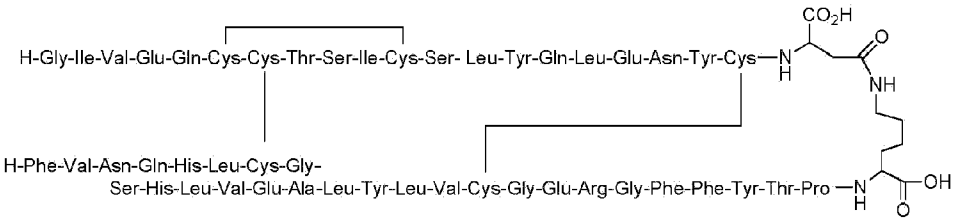


[2]

PHARMACEUTICAL COMPOSITIONS

[0056] One aspect of the invention relates to a pharmaceutical composition comprising an insulin analogue of the invention admixed with a pharmaceutically acceptable diluent, excipient or carrier, or a mixture thereof. Even though the analogues of the present invention (including their pharmaceutically acceptable salts, esters and pharmaceutically acceptable solvates) can be administered alone, they will generally be administered in admixture with a pharmaceutical carrier, excipient or diluent, particularly for human therapy. The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine.

[0057] Examples of such suitable excipients for the various different forms of pharmaceutical compositions described herein may be found in the "Handbook of Pharmaceutical Excipients, 2nd Edition, (1994), Edited by A Wade and PJ Weller.

[0058] Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985).

[0059] Examples of suitable carriers include lactose, starch, glucose, methyl cellulose, magnesium stearate, mannitol, sorbitol and the like. Examples of suitable diluents include ethanol, glycerol and water.

[0060] The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as, or in addition to, the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

[0061] Examples of suitable binders include starch, gelatin, natural sugars such as glucose, anhydrous lactose, free-flow lactose, beta-lactose, corn sweeteners, natural and synthetic gums, such as acacia, tragacanth or sodium alginate, carboxymethyl cellulose and polyethylene glycol.

[0062] Examples of suitable lubricants include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like.

[0063] Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

SALTS/ESTERS

[0064] The insulin analogues of the present invention can be present as salts or esters, in particular pharmaceutically acceptable salts or esters.

[0065] Pharmaceutically acceptable salts of the analogues of the invention include suitable acid addition or base salts thereof. A review of suitable pharmaceutical salts may be found in Berge et al, J Pharm Sci, 66, 1-19 (1977). Salts are formed, for example with strong inorganic acids such as mineral acids, e.g. sulphuric acid, phosphoric acid or hydrohalic acids; with strong organic carboxylic acids, such as alkanecarboxylic acids of 1 to 4 carbon atoms which are unsubstituted or substituted (e.g., by halogen), such as acetic acid; with saturated or unsaturated dicarboxylic acids, for example oxalic, malonic, succinic, maleic, fumaric, phthalic or tetraphthalic; with hydroxycarboxylic acids, for example ascorbic, glycolic, lactic, malic, tartaric or citric acid; with aminoacids, for example aspartic or glutamic acid; with benzoic acid; or with organic sulfonic acids, such as (C_1-C_4)-alkyl- or aryl-sulfonic acids which are unsubstituted or substituted (for example, by a halogen) such as methane- or p-toluene sulfonic acid.

[0066] Esters are formed either using organic acids or alcohols/hydroxides, depending on the functional group being esterified. Organic acids include carboxylic acids, such as alkanecarboxylic acids of 1 to 12 carbon atoms which are unsubstituted or substituted (e.g., by

halogen), such as acetic acid; with saturated or unsaturated dicarboxylic acid, for example oxalic, malonic, succinic, maleic, fumaric, phthalic or tetraphthalic; with hydroxycarboxylic acids, for example ascorbic, glycolic, lactic, malic, tartaric or citric acid; with aminoacids, for example aspartic or glutamic acid; with benzoic acid; or with organic sulfonic acids, such as (C_1-C_4)-alkyl- or aryl-sulfonic acids which are unsubstituted or substituted (for example, by a halogen) such as methane- or p-toluene sulfonic acid. Suitable hydroxides include inorganic hydroxides, such as sodium hydroxide, potassium hydroxide, calcium hydroxide, aluminium hydroxide. Alcohols include alkanealcohols of 1-12 carbon atoms which may be unsubstituted or substituted, e.g. by a halogen).

ENANTIOMERS/TAUTOMERS

[0067] In all aspects of the present invention previously discussed, the invention includes, where appropriate all enantiomers and tautomers of the analogues of the invention. The man skilled in the art will recognise compounds that possess an optical properties (one or more chiral carbon atoms) or tautomeric characteristics. The corresponding enantiomers and/or tautomers may be isolated/prepared by methods known in the art.

STEREO AND GEOMETRIC ISOMERS

[0068] Some of the analogues of the invention may exist as stereoisomers and/or geometric isomers - e.g. they may possess one or more asymmetric and/or geometric centres and so may exist in two or more stereoisomeric and/or geometric forms. The present invention contemplates the use of all the individual stereoisomers and geometric isomers of those analogues, and mixtures thereof. The terms used in the claims encompass these forms, provided said forms retain the appropriate functional activity (though not necessarily to the same degree).

[0069] The present invention also includes all suitable isotopic variations of the analogues or pharmaceutically acceptable salts thereof. An isotopic variation is defined as one in which at least one atom is replaced by an atom having the same atomic number but an atomic mass different from the atomic mass usually found in nature. Examples of isotopes that can be incorporated into the agent and pharmaceutically acceptable salts thereof include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorus, sulphur, fluorine and chlorine such as ^{2}H, ^{3}H, ^{13}C, ^{14}C, ^{15}N, ^{17}O, ^{18}O, ^{31}P, ^{32}P, ^{35}S, ^{18}F and ^{36}Cl, respectively. Certain isotopic variations, for example, those in which a radioactive isotope such as ^{3}H or ^{14}C is incorporated, are useful in drug and/or substrate tissue distribution studies. Tritiated, i.e., ^{3}H, and carbon-14, i.e., ^{14}C, isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with isotopes such as deuterium, i.e., ^{2}H, may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or

reduced dosage requirements and hence may be preferred in some circumstances. Isotopic variations of the analogues of the present invention and pharmaceutically acceptable salts thereof of this invention can generally be prepared by conventional procedures using appropriate isotopic variations of suitable reagents.

SOLVATES

[0070] The present invention also includes solvate forms of the analogues of the present invention. The terms used in the claims encompass these forms.

POLYMORPHS

[0071] The invention furthermore relates to analogues of the present invention in their various crystalline forms, polymorphic forms and (an)hydrous forms. It is well established within the pharmaceutical industry that chemical compounds may be isolated in any of such forms by slightly varying the method of purification and or isolation form the solvents used in the synthetic preparation of such compounds.

PRODRUGS

[0072] The invention further includes analogues of the present invention in prodrug form. Such prodrugs are generally analogues of the invention wherein one or more appropriate groups have been modified such that the modification may be reversed upon administration to a human or mammalian subject. Such reversion is usually performed by an enzyme naturally present in such subject, though it is possible for a second agent to be administered together with such a prodrug in order to perform the reversion in vivo. Examples of such modifications include ester (for example, any of those described above), wherein the reversion may be carried out be an esterase etc. Other such systems will be well known to those skilled in the art.

ADMINISTRATION

[0073] The pharmaceutical compositions of the present invention may be adapted for oral, rectal, vaginal, parenteral, intramuscular, intraperitoneal, intraarterial, intrathecal, intrabronchial, subcutaneous, intradermal, intravenous, nasal, buccal or sublingual routes of administration.

[0074] For oral administration, particular use is made of compressed tablets, pills, tablets, gellules, drops, and capsules. Preferably, these compositions contain from 1 to 250 mg and

more preferably from 10-100 mg, of active ingredient per dose.

[0075] Other forms of administration comprise solutions or emulsions which may be injected intravenously, intraarterially, intrathecally, subcutaneously, intradermally, intraperitoneally or intramuscularly, and which are prepared from sterile or sterilisable solutions. The pharmaceutical compositions of the present invention may also be in form of suppositories, pessaries, suspensions, emulsions, lotions, ointments, creams, gels, sprays, solutions or dusting powders.

[0076] An alternative means of transdermal administration is by use of a skin patch. For example, the active ingredient can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. The active ingredient can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.

[0077] Injectable forms may contain between 10 - 1000 mg, preferably between 10 - 250 mg, of active ingredient per dose.

[0078] Compositions may be formulated in unit dosage form, i.e., in the form of discrete portions containing a unit dose, or a multiple or sub-unit of a unit dose.

DOSAGE

[0079] A person of ordinary skill in the art can easily determine an appropriate dose of one of the instant compositions to administer to a subject without undue experimentation. Typically, a physician will determine the actual dosage which will be most suitable for an individual patient and it will depend on a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the individual undergoing therapy. The dosages disclosed herein are exemplary of the average case. There can of course be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

[0080] Depending upon the need, the agent may be administered at a dose of from 0.01 to 30 mg/kg body weight, such as from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

[0081] In an exemplary embodiment, one or more doses of 10 to 150 mg/day will be administered to the patient.

THERAPEUTIC USE

[0082] Another aspect of the invention relates to single chain insulin analogues as described above for use as a medicament.

[0083] Another aspect of the invention relates to single chain insulin analogues as described above for use in treating or preventing diabetes, or treating or preventing hyperglycemia.

[0084] The present invention is further described by way of the following non-limiting examples.

EXAMPLES

Abbreviations

[0085]

Boc
: t-butyloxycarbonyl

CTC
: chlorotrityl chloride

NMP
: N-methylpyrrolidone

DCM
: dichoromethane

TFA
: trifluoroacetic acid

RE
: rotary evaporator

DEE
: diethyl ether

DIC
: N,N'-diisopropylcarbodiimide

HOBt
: hydroxybenzotriazole

HOSu
: N-hydroxysuccinimide

DMF
: dimethylformamide

EDAC
: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

RT
: room temperature

DTT
: dithiothreitol

DMSO
: dimethylsulfoxide

MMt
: monomethoxytrityl

Trt
: trityl

DIPEA
: N,N-diisopropylethylamine

Fmoc
: fluorenylmethyloxycarbonyl

MeOH
: methanol

AcOH
: acetic acid

TFE
: trifluoroethyl alcohol

Dde
: N-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)

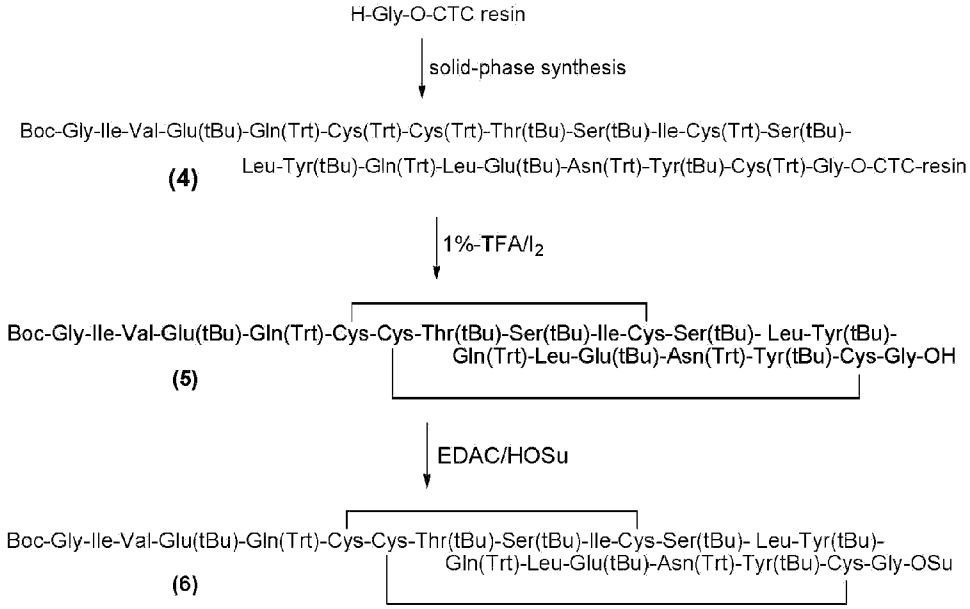
Example 1

***Synthesis of single chain insulin of structure* 10.**

(A) Synthesis of partially protected and carboxyl group activated insulin Gly(A21)-chain of structure 6.

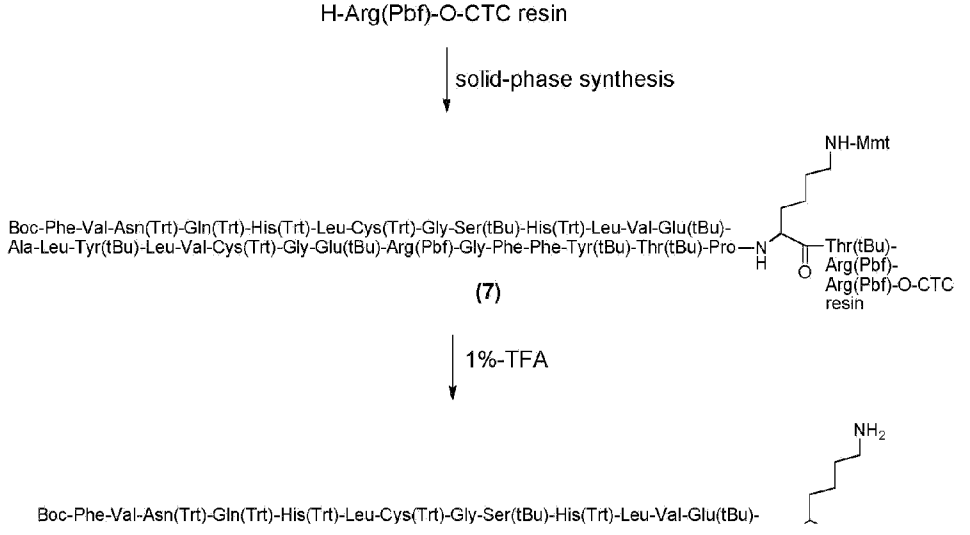
[0086] 3.0 g (1.0 mmol) of H-Gly-O-CTC resin (commercially available, Product of CBL-Patras) was elongated to the resin-bound protected Gly(A21)-chain using Fmoc-amino acids (Products of CBL-Patras) and DIC/HOBt for the activation of the amino acids except of the Gly(A1) residue which was introduced using Boc-Gly-OH. The resin was then washed 4X with NMP and 6X with DCM and then the protected peptide was cleaved from the resin and oxidized simultaneously by washing 8X with 1%-TFA in DCM containing 20,0 mmol of iodine. The filtrates from the 1% TFA washings were dropped into a 3% $Na_2S_2O_3$ solution, the DCM layer was washed with the $Na_2S_2O_3$ solution and water, concentrated in the RE and the protected

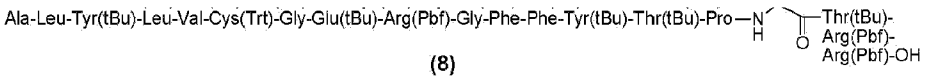
peptide was then precipitated with the addition of DEE, washed with DEE and dried in vacuum to constant weight. Yield 3,04g (90.0%).



(B) Solid-phase synthesis of selectively at the Lys^{29} deprotected 8(Arg31), B(Arg32) human insulin B chain of structure 8.

[0087] 4,0 g (1, 0 mmol) of H-Arg(Pbf)-O-CTC resin (commercially available from CBL-Patras), was elongated to the resin-bound protected Arg(B31), Arg(B32)-chain using Fmoc-amino acids and DIC/HOBt for the activation of the amino acids except of the Phe(B1) residue which was introduced using Boc-Phe-OH. The resin was then washed 4X with NMP and 6X with DCM and then the protected peptide was cleaved from the resin washing 8X with 1 %-TFA in DCM. The filtrates from the 1% TFA washings were dropped into a water solution of 1%-pyridine, the DCM layer was washed with water, concentrated in the RE and the protected peptide was then precipitated with the addition of DEE, washed with DEE and dried in vacuum to constant weight. Yield 5,61 g (90,2%).

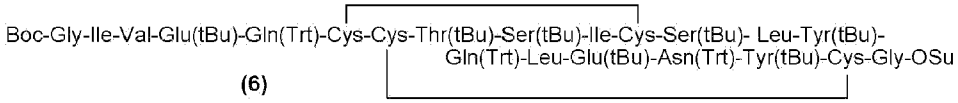




(8)

(C) Condensation in solution - Synthesis of single chain insulin Nr. 10

[0088] To a solution of 427,1 mg (0,1 mmol) protected A-chain Nr. **6** in 10,0 ml DMF 0,12 (0,1 mmol) HOSu and 0,17 g (0,1 mmol) EDAC were added and the mixture was stirred for 20 min at RT. Then 621,9 mg (0,1 mmol) of the selectively at the B-chain deprotected insulin B-chain Nr. **8** were added and the resulting mixture was stirred for additional 6 h at RT. The resulting solution was then dropped to 100 ml ice cold water and the resulting solid was filtered, washed with water and dried in vacuum. Then the solid was dissolved in 30 ml of a precooled at 0°C mixture of TFA/DTT/water (94:3:3) and stirred for 1 h and then the mixture was warmed to RT and stirred for additional 3 h at RT. The mixture was then concentrated in vacuum on a RE to ca 5 ml and 100 ml of ice cold DEE were added. The precipitated solid was filtered and washed with DEE and dried in vacuum to constant weight. Then the crude insulin was dissolved in 20% DMSO in a Na_2HPO_4 solution at pH = 7.8 and stirred for additional 48 h at RT. The obtained solution was then acidified and loaded to a C18 Chromasil column and purified by HPLC. The fractions which contained the main product were collected and lyophilized. Yield: 62,3 mg net peptide (10,7 %).



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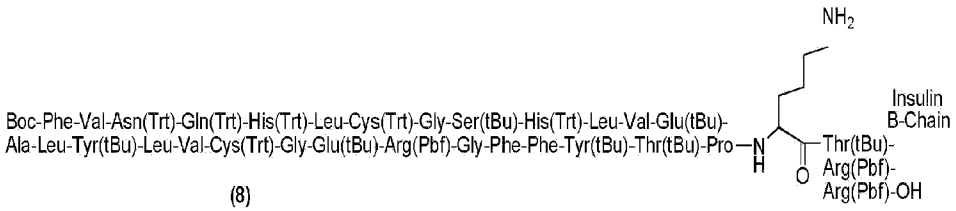
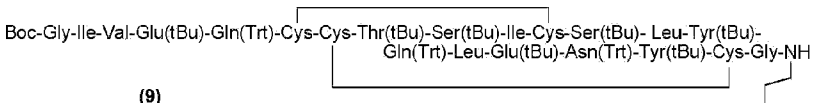


(8)

condensation



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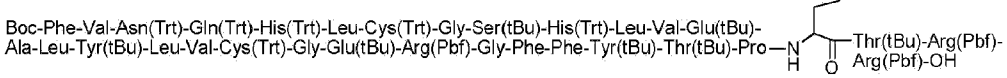
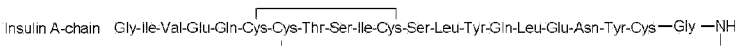
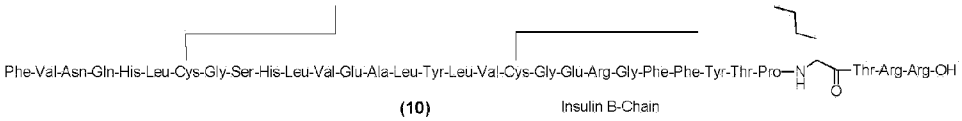


1.deprotection
2. DMSO



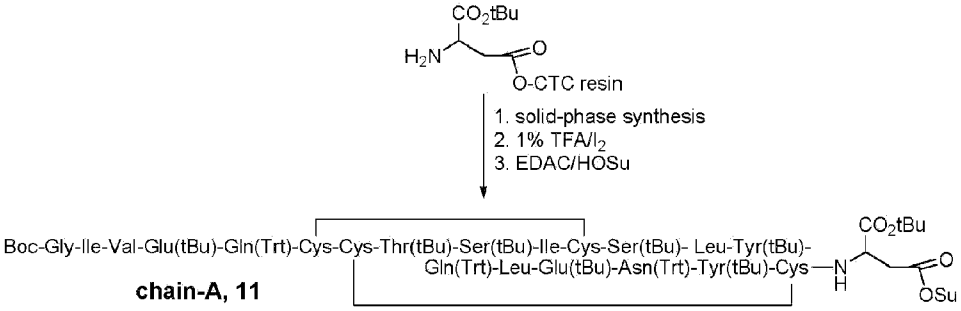


Example 2

***Synthesis of single chain insulin with structure* 15.**

(A) Synthesis of protected, carboxyl group activated and oxidized Asp^{21} insulin A-chain of structure 11.

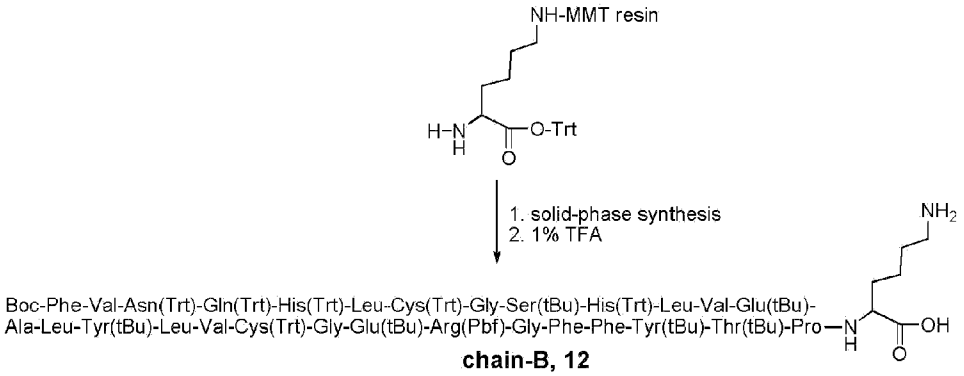
[0089] 4,0 g (1, 0 mmol) of H-Asp(CTC resin)-O^{t}Bu (commercially available from CBL-Patras) was elongated to the resin-bound protected Asp(A21)-chain using Fmoc-amino acids (Products of CBL-Patras) and DIC/HOBt for the activation of the amino acids except of the Gly(A1) residue which was introduced using Boc-Gly-OH. The resin was then washed 4X with NMP and 6X with DCM and then the protected peptide was cleaved from the resin and oxidized simultaneously by washing 8X with 1%-TFA in DCM which contained 20,0 mmol of iodine. The filtrates from the 1% TFA washings were dropped into a 3% $Na_2S_2O_3$ solution, the DCM layer was washed with the $Na_2S_2O_3$ solution and water, concentrated in the RE and the protected peptide was then precipitated with the addition of DEE, washed with DEE and dried in vacuum to constant weight. Yield 2,97 g (87,5%).



(B) Synthesis of selectively at the Lys^{29} side chain deprotected des- Thr^{30} human insulin of structure 12.

[0090] 3.2 g (1.0 mmol) of H-Lys(MMT resin)-OTrt (produced by reacting Fmoc-Lys-OH with MMt-chloride resin and DIPEA in DCM, followed by the esterification of the resin-bound Fmoc-Lys-OH obtained with Trt-chloride) was elongated to the resin-bound protected desThr(B30) human insulin B-chain using Fmoc-amino acids (Products of CBL-Patras) and DIC/HOBt for the

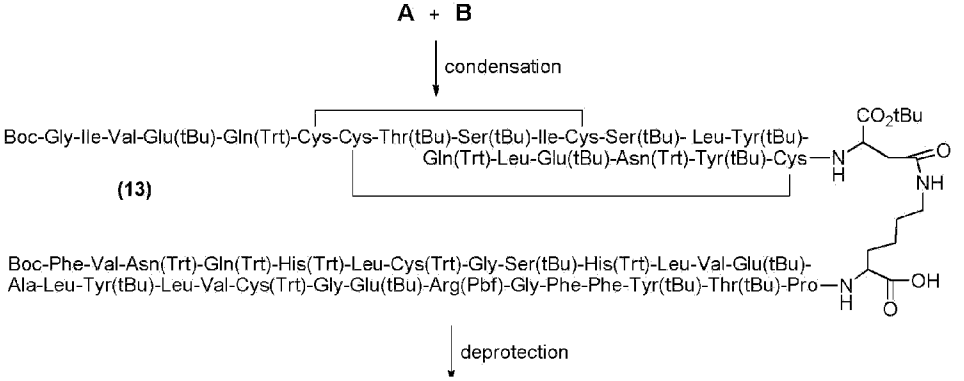
activation of the amino acids except of the Phe(B1) residue which was introduced using Boc-Phe-OH. The resin was then washed 4X with NMP and 6X with DCM and then the protected peptide was cleaved from the resin by washing 8X with 1%-TFA in DCM. The filtrates from the 1% TFA washings were dropped into a water solution of 1%-pyridine, the DCM layer was washed with water, concentrated in the RE and the protected peptide was then precipitated with the addition of DEE, washed with DEE and dried in vacuum to constant weight. Yield 4,93 g (94,1%).

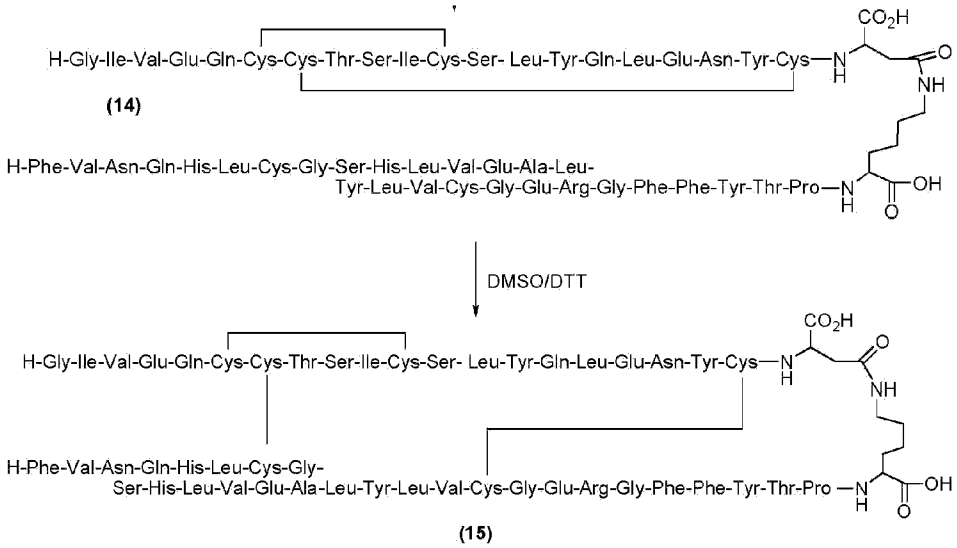


chain-B, 12

(C) Condensation of the partially protected insulin chains A and 6 in solution

[0091] To a solution of 339,6 mg (0,1 mmol) protected A-chain Nr. **11** in 10,0 ml DMF 0,12 (0,1 mmol) HOSu and 0,17 g (0,1 mmol) EDAC were added and the mixture was stirred for 20 min at RT. Then 524,5 mg (0,1 mmol) of the selectively at the B-chain deprotected insulin B-chain Nr. **12** were added and the resulting mixture was stirred for additional 6 h at RT. The resulting solution was then dropped to 100 ml ice cold water and the resulting solid was filtered, washed with water and dried in vacuum. Then the solid was dissolved in 30 ml of a precooled at 0°C mixture of TFA/DTT/water (94:3:3) and stirred for 1 h and then the mixture was warmed to RT and stirred for additional 3 h at RT. The mixture was then concentrated in vacuum on a RE to ca 5 ml and 100 ml of ice cold DEE were added. The precipitated solid was filtered and washed with DEE and dried in vacuum to constant weight. Then the crude insulin was dissolved in 20% DMSO in a Na_2HPO_4 solution at pH = 7.8 and stirred for additional 48 h at RT. The obtained solution was then acidified and loaded to a C18 Chromasil column and purified by HPLC. The fractions which contained the main product were collected and lyophilized. Yield: 78,9 mg net peptide (14,5 %).



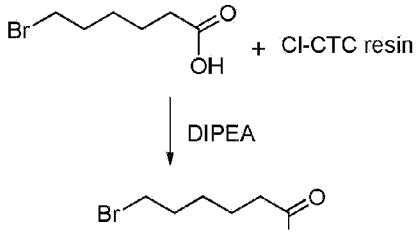


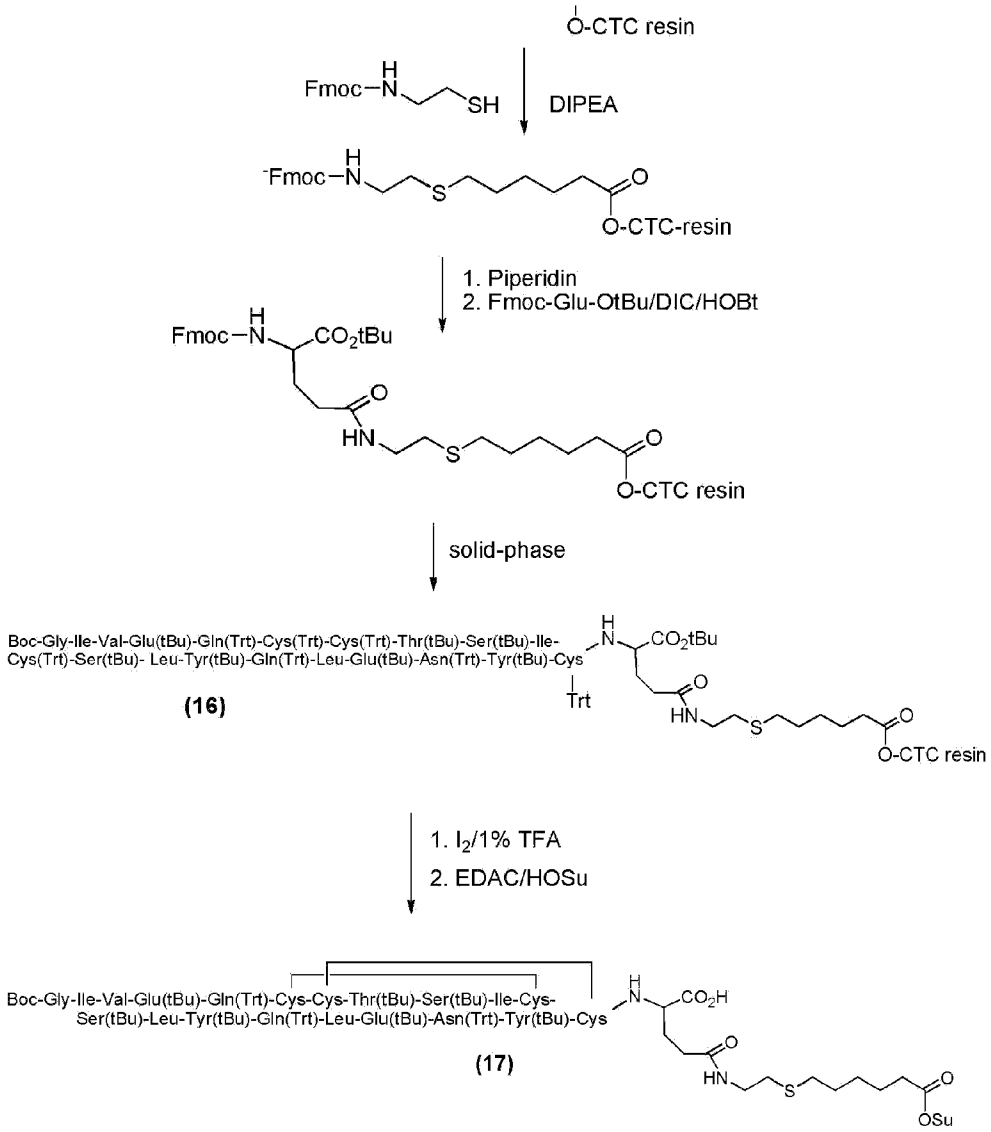
Example 3 (outside the scope of the present invention)

***Synthesis of single chain insulin with structure* 19.**

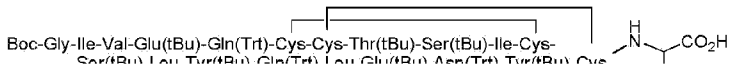
***(A) Synthesis of protected insulin chain-A with structure* 17.**

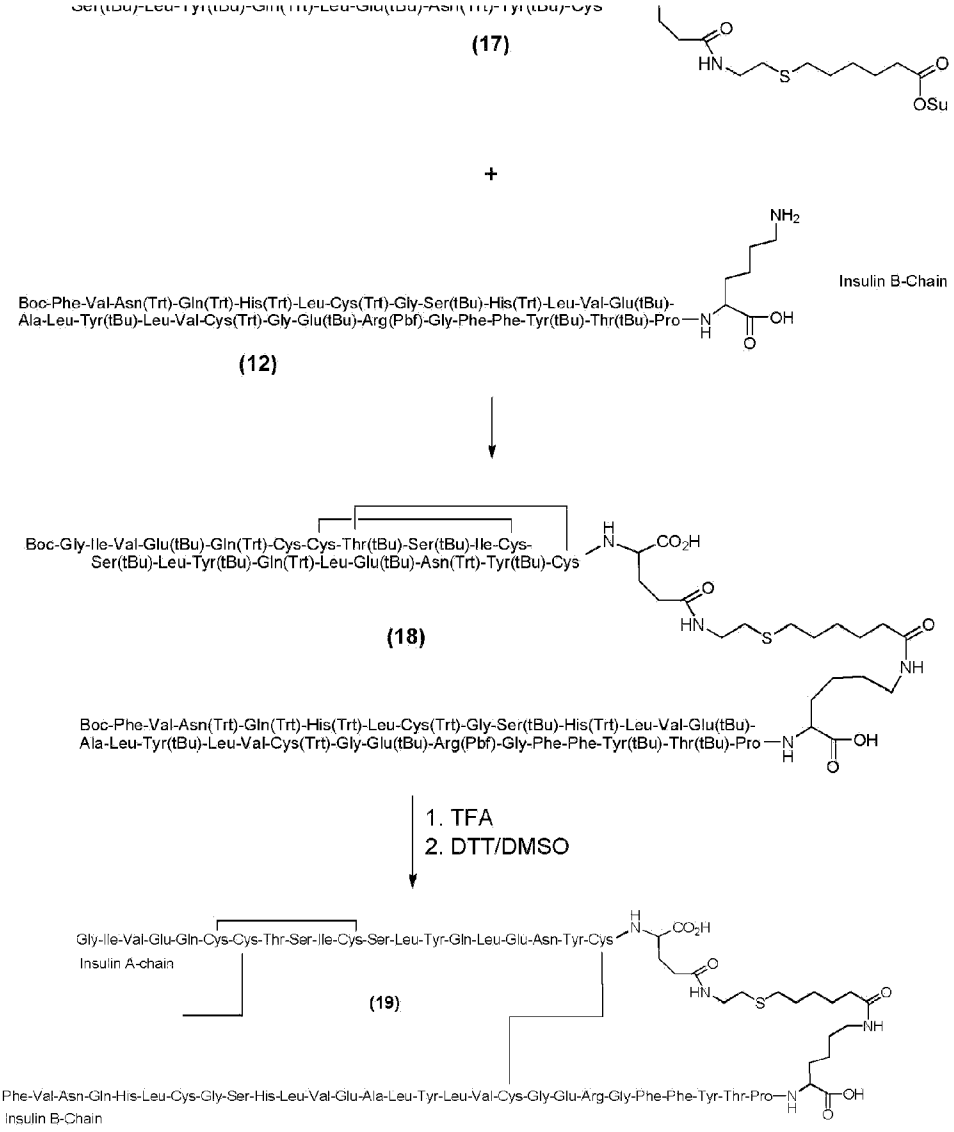
[0092] 4.13 g (10,0 mmol) of 6-Fmoc-amino(ethylthio)hexanoic acid, obtained by procedures well known in the art from Fmoc-cysteamine and 6-bromohexanoic acid, were dissolved in 200 ml DCM and then 20,0 g (1,6 mmol/g) CTC-chloride resin and 7,75 g (60 mmol) DIPEA were added and the mixture was shacked for 2 h at RT. Then 10,0 ml MeOH were added and the mixture was shacked for an additional h. The resin was filtered and washed 3X DCM/DIPEA/MeOH, 6X DCM. The obtained resin bound amino acid was then chain elongated with sequential couplings of Fmoc-amino acids and DIC/HOBt for their activation to the resin-bound protected peptide **16.** The resin **16** was then washed 4X with NMP and 6X with DCM and then the protected peptide was cleaved from the resin and oxidized simultaneously by washing 8X with 1 %-TFA in DCM which contained 200,0 mmol of iodine. The filtrates from the 1% TFA washings were dropped into a water solution of 1 %-pyridine, the DCM layer was washed with water, concentrated in the RE and the protected peptide was then precipitated with the addition of DEE, washed with DEE and dried in vacuum to constant weight. Yield 34,7 g (90,8%).





*(B) Condensation in solution of the partially protected and modified insulin chain A of structure **17** with the partially protected chain B of des Thr(B30) insulin of structure **12.*** To a solution of 382,3 mg (0,1 mmol) protected A-chain Nr. **17** in 10,0 ml DMF 0,12 (0,1 mmol) HOSu and 0,17 g (0,1 mmol) EDAC were added and the mixture was stirred for 20 min at RT. Then 524,5 mg (0,1 mmol) of the selectively at the side chain of Lys(B 29) deprotected des(B30) insulin B-chain Nr. **12** were added and the resulting mixture was stirred for additional 6 h at RT. The resulting solution was then dropped to 100 ml ice cold water and the resulting solid was filtered, washed with water and dried in vacuum. Then the solid was dissolved in 30 ml of a precooled at 0°C mixture of TFA/DTT/water (94:3:3) and stirred for 1 h and then the mixture was warmed to RT and stirred for additional 3 h at RT. The mixture was then concentrated in vacuum on a RE to ca 5 ml and 100 ml of ice cold DEE were added. The precipitated solid was filtered and washed with DEE and dried in vacuum to constant weight. Then the crude insulin was dissolved in 20% DMSO in a Na_2HPO_4 solution at pH = 7.8 and stirred for additional 48 h at RT. The obtained solution was then acidified and loaded to a C18 Chromasil column and purified by HPLC. The fractions which contained the main product were collected and lyophilized. Yield: 127,4 mg net peptide (22,8 %).





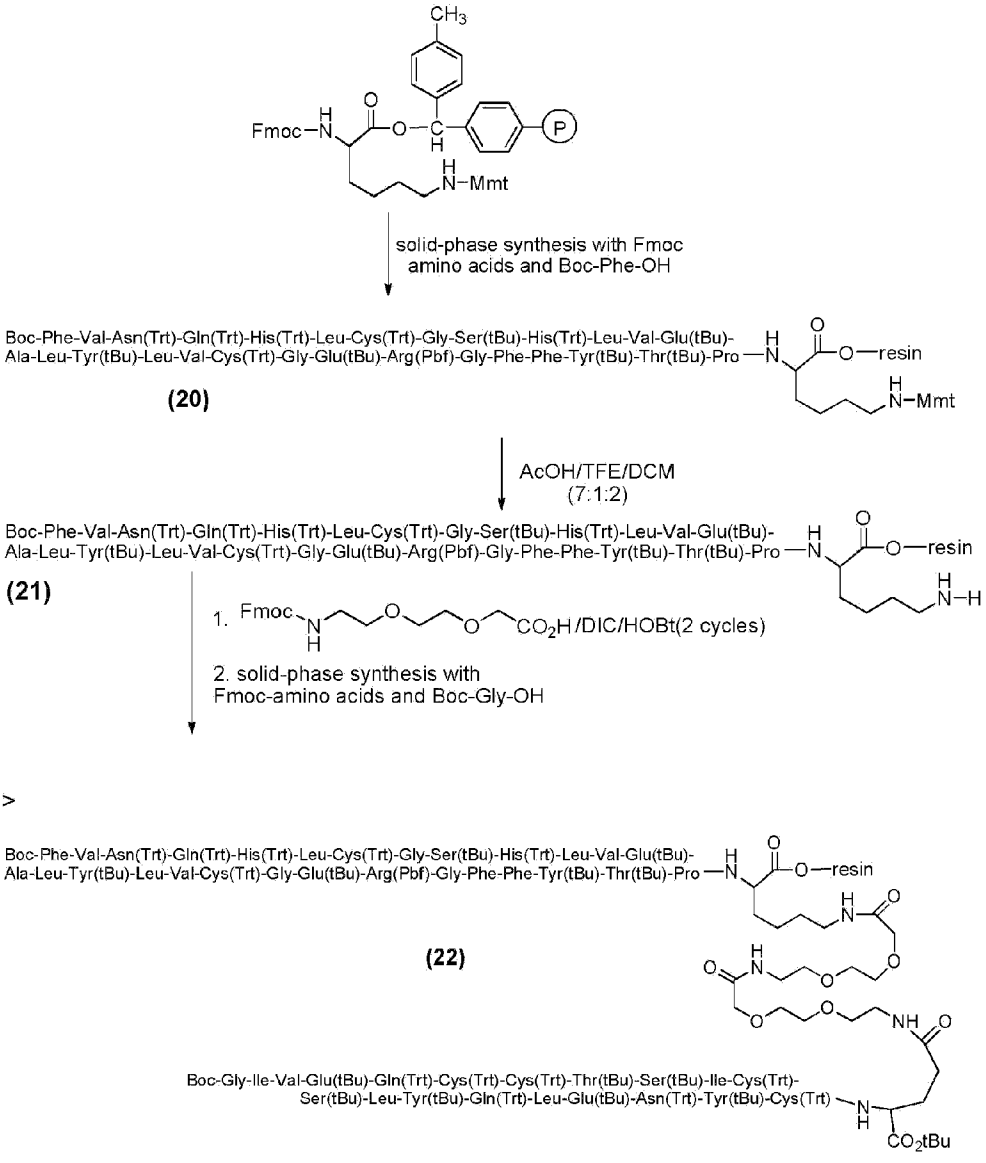
Example 4 (outside the scope of the present invention)

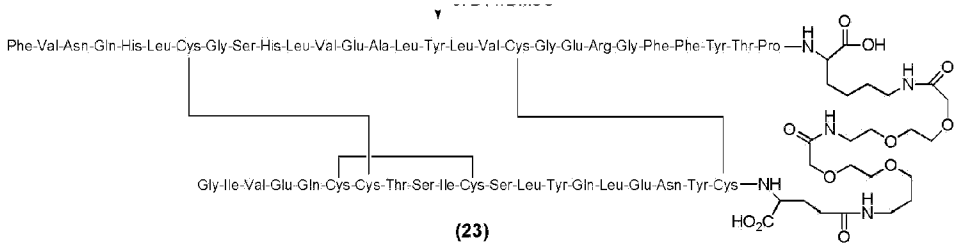
***Synthesis of single chain insulin of structure* 23.**

Solid-phase synthesis of single chain insulin Glu(A21), des Thr(B30) where the insulin chains are connected through the side chains of Glu(A21) and Lys(B29) and the oligoethylen glycol modifier.

[0093] The synthesis was started with the solid phase synthesis of the B-chain on 4-methyl-benzhydryl(diphenylmethyl) resin. After the completion of the synthesis the side chain Lys(B29) Mmt function was removed by the treatment of the resin with DCM/TFE/AcOH (7:2:1) for 3 h at

RT to provide the partially protected resin-bound peptide of structure **21.** Then on the Lys side chain two groups of 2-(2-(2-aminoethoxy)ethoxy)acetic acid were introduced using the corresponding Fmoc-derivatives followed by the coupling with Fmoc-Glu-OtBu. On the No-function of the Glu(A21) the rest of the A chain was assembled using Fmoc-amino acids. After the completion of the synthesis the resin-bound peptide was treated with 1% I_2 in DCM/TFE (9:1) in order to build the disulfide bonds. The resin was then washed with DCM and treated with TFA/DTT/H_2O (94:3:3) for 15 min at 0°C and filtered. The resin was then washed 3X with the TFA solution and the combined filtrates were left at DoC for additional 3h. The solution was concentrated on a RE and the remaining oil was dropped to ice cold DEE. The precipitated material was filtered, washed with DEE and dried in vacuum. Then the crude insulin was dissolved in 20% DMSO in a Na_2HPO_4 solution at pH = 7.8 and stirred for additional 48 h at RT. The obtained solution was then acidified and loaded to a C18 Chromasil column and purified by HPLC. The fractions which contained the main product **23** were collected and lyophilized. Yield: 82,4 mg net peptide (14,29 %).

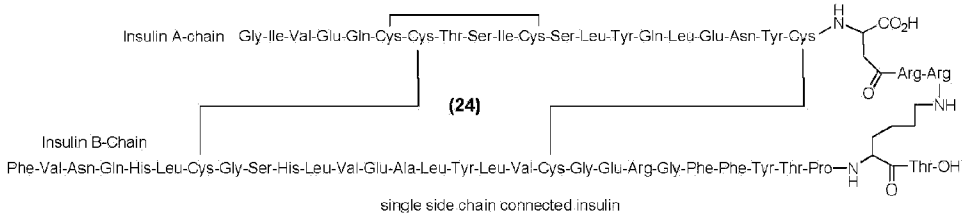




Example 5 (outside the scope of the present invention)

Synthesis of the single chain insulin synthesis of structure 24 on 2-chlorotrityl resin

[0094] The synthesis was started from 1,00 mmol H-Thr(tBu)-O-CTC resin and was performed as described in Example 3 with the exception that instead of Fmoc-Lys(Mmt)-OH for the introduction of Lys(B29) Fmoc-Lys(Dde)-OH was used and the selective removal of Dde was performed as usual with 2%-hydrazine in NMP. Yield: 94,3 mg (15,4%).



REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patentkrav

1. Enkeltkædet insulinanalog, der omfatter:
(A) A-kæden af human- eller dyreinsulin eller en analog dertil, som er en variant, hvor (a) én aminosyrerest er erstattet af en naturligt eller ikke-naturligt forekommende aminosyre, (b) rækkefølgen af to aminosyrerester er byttet om, eller (c) både (a) og (b) er til stede sammen;
(B) B-kæden af human- eller dyreinsulin eller en analog dertil, som er en variant, hvor (a) én aminosyrerest er erstattet af en naturligt eller ikke-naturligt forekommende aminosyre, (b) rækkefølgen af to aminosyrerester er byttet om, eller (c) både (a) og (b) er til stede sammen;
(C)
(i) en intermolekylær disulfidbinding mellem cysteinen i position 7 af insulin-A-kæden og cysteinen i position 7 af insulin-B-kæden;
(ii) en intermolekylær disulfidbinding mellem cysteinen i position 20 af insulin-A-kæden og cysteinen i position 19 af insulin-B-kæden;
(iii) en intramolekylær disulfidbinding mellem cysteinen i position 6 og cysteinen i position 11 af insulin-A-kæden; og
(D) en yderligere kovalent binding, L, mellem den carboxylterminale aminosyre af A-kæden af human- eller dyreinsulin eller en analog dertil og sidekæden af lysinresten i position 29 af B-kæden af human- eller dyreinsulin eller en analog dertil, hvor den yderligere kovalente binding, L, er en direkte binding;
hvor den enkeltkædede insulinanalog udfører den samme aktivitet som human insulin med hensyn til glykæmisk kontrol.

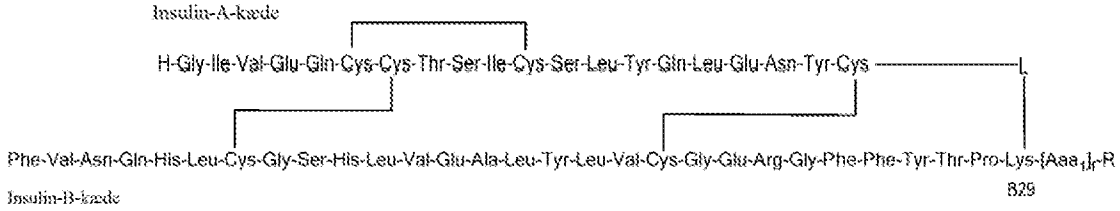
2. Enkeltkædet insulinanalog ifølge krav 1, hvor den yderligere kovalente binding, L, er en direkte binding en direkte binding mellem sidekæden af en asparaginsyrerest eller af en glutaminsyrerest i den C-terminale aminosyre af A-kæden og sidekædeaminofunktionen af en Lys-rest i B-kæden.

3. Enkeltkædet insulinanalog ifølge krav 1 eller krav 2, hvor A-kæden omfatter aminosyrerne 1 til 21 eller 1 til 20 af human insulin talt fra den N-terminale ende af A-kæden.

4. Enkeltkædet insulinanalog ifølge et hvilket som helst af ovennævnte krav, hvor B-kæden omfatter aminosyrerne 1 til 29 af human insulin talt fra den N-terminale ende af B-kæden.

5. Enkeltkædet insulinanalog ifølge krav 4, hvor B-kæden af insulin yderligere omfatter op til 20 yderligere naturlige eller unaturlige aminosyrer i den C-terminale ende.

6. Enkeltkædet insulinanalog ifølge et hvilket som helst af ovennævnte krav, som har formel (I)



(I)

hvor:
L er som defineret i krav 1;
hver Aaa_1 uafhængigt er en naturlig eller unaturlig aminosyre;
f er et heltal valgt blandt 0 til 20;
R er OH eller NH_2.

7. Enkeltkædet insulinanalog ifølge krav 6, hvor R er OH, f er 0, 1, 2 eller 3, og hver Aaa_1 uafhængigt er en naturlig aminosyre, mere fortrinsvis valgt blandt Arg og Thr.

8. Farmaceutisk sammensætning, der omfatter den enkeltkædede insulinanalog ifølge et hvilket som helst af kravene 1 til 7 og et farmaceutisk acceptabelt fortyndingsmiddel, excipiens eller bæremateriale.

9. Enkeltkædet insulinanalog ifølge et hvilket som helst af kravene 1 til 7 til anvendelse som et medikament.

10. Enkeltkædet insulinanalog ifølge et hvilket som helst af kravene 1 til 7 til anvendelse til behandling eller forebyggelse af diabetes.

11. Enkeltkædet insulinanalog ifølge et hvilket som helst af kravene 1 til 7 til anvendelse til behandling eller forebyggelse af hyperglykæmi.