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(54) Title: NOVEL DERIVATIVE OF AN INSULIN ANALOGUE

(57) Abstract: The present invention provides a novel derivative of an analogue of human insulin, useful for the treatment of diabetes.

NOVEL DERIVATIVE OF AN INSULIN ANALOGUE

FIELD OF THE INVENTION

The present invention provides a novel derivative of an analogue of human insulin, useful for the treatment of diabetes.

BACKGROUND OF THE INVENTION

Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

Insulin is a polypeptide hormone secreted by β-cells of the pancreas. Insulin consists of two polypeptide chains designated the A and B chains which are linked together by two inter-chain disulphide bridges. In human, porcine and bovine insulin, the A and B chains contains 21 and 30 amino acid residues, respectively. However, from species to species, there are variations among the amino acid residues present in the different positions in the two chains. The widespread use of genetic engineering has made it possible to prepare analogues of natural occurring insulins by exchanging, deleting and adding one or more of the amino acid residues. Insulin is used for the treatment of diabetes and diseases connected therewith or resulting from it.

For decades, insulin preparations with different duration of action have been developed and put on the market and general examples of such preparations are long-acting insulin preparations, medium acting insulin preparations and fast acting insulin preparations. Many patients take 2-4 injections per day, every week, every month, and every year, optionally for decades. No basal insulin products have to date been approved for administration less often than by daily subcutaneous injection. The discomfort of a large number of daily injections can, for example, be diminished by using insulin derivatives having an extremely long duration of action.

Various patent applications including WO 2010/049488 and WO 2011/161125 mention the possibility of administering insulin derivatives with long intervals. WO 2009/115469 relates to certain acylated protease stabilised insulins wherein at least one hydrophobic amino acid has been substituted with hydrophilic amino acids.

It would be very desirable for diabetic patients, if basal insulin preparations for administration approximately once weekly were available.

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OBJECTS OF THE INVENTION

The object of this invention is to overcome or ameliorate at least one of the

disadvantages of the prior art, or to provide a useful alternative.

Another aspect of this invention relates to the furnishing of insulin derivatives with long pharmacokinetic (hereinafter PK) profiles, e.g. so that a subcutaneous treatment once a week or more seldom will be a satisfactory treatment of the diabetic patient's need for basal insulin treatment.

Another aspect of this invention relates to the furnishing of insulin derivatives with long PK profiles, e.g. PK profiles being longer than the PK profile of human insulin, after subcutaneous administration. In this connection, the PK profile can be determined as explained in Examples 5 and 6 herein.

Another aspect of this invention relates to the furnishing of insulin derivatives having a high solubility in an aqueous medium optionally containing zinc, e.g. a solubility which is higher than the solubility of human insulin. In this connection, the solubility can be determined as explained in Example 7 herein.

Another aspect of this invention relates to the furnishing of insulin derivatives which are soluble in an aqueous medium containing zinc, such as at least 5 zinc ions per insulin hexamer, when measured after storage at least 4 weeks at 37°C or below after preparation. In this connection, the solubility may e.g. be determined as explained in Example 7 herein.

Another aspect of this invention relates to the furnishing of insulin derivatives which are soluble in an aqueous medium containing zinc, such as at least 5 zinc ions per insulin hexamer, when measured within 24-48 hours after preparation. In this connection, the solubility may be determined as explained in Example 7 herein.

Another aspect of this invention relates to the furnishing of insulin derivatives having good stability against enzymes, e.g. proteolytic enzymes, e.g. proteolytic enzymes present in the human stomach, e.g. pepsin, chymotrypsin and carboxypeptidase A. In this connection, the stability against enzymes can be determined as explained in Example 1 of WO 2008/034881.

Another aspect of this invention relates to the furnishing of insulin derivatives having good stability, especially chemical stability and physical stability, at storage, e.g. storage at 5°C and at 30°C, for e.g. 2 years and for 2 weeks, respectively. In this connection, the chemical stability can be determined as explained in Examples 9 and 10 herein and the physical stability can be determined as explained in Examples 9 and 10 herein.

Another aspect of this invention relates to the furnishing of insulin derivatives which can efficiently be administered orally, e.g. once daily, to diabetic patients. Also, or alternatively, this invention relates to the furnishing of insulin derivatives which have a high

oral bioavailability.

Another aspect of this invention relates to the furnishing of insulin derivatives having reduced daily fluctuations, e.g. variations between plasma concentrations (C_{max} and C_{min}) following, e.g. once weekly subcutaneously administration.

Another aspect of this invention relates to the furnishing of insulin derivatives having reduced influence on the day-to-day variation in bioavailability following oral administration.

Another aspect of this invention relates to the furnishing of insulin derivatives having high potency, i.e. evoke a large response at low drug concentration, (drug activity expressed in terms of the amount required to produce an effect of given intensity).

Another aspect of this invention relates to the furnishing of insulin derivatives that bind very well to the insulin receptor. In this connection, the insulin receptor affinity can be determined as explained in Example 2 herein.

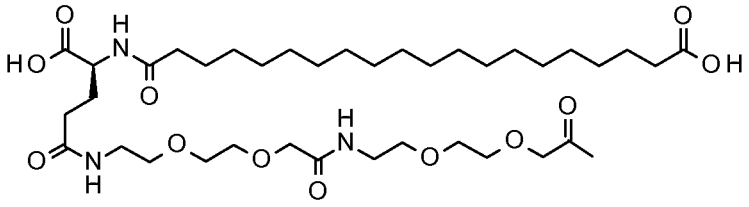
Another aspect of this invention relates to the furnishing of insulin derivatives having a low insulin receptor affinity. In this connection, the insulin receptor affinity can be determined as explained in Example 2 herein.

DEFINITIONS

The term “diabetes” or “diabetes mellitus” includes type 1 diabetes, type 2 diabetes, gestational diabetes (during pregnancy) and other states that cause hyperglycaemia. The term is used for a metabolic disorder in which the pancreas produces insufficient amounts of insulin, or in which the cells of the body fail to respond appropriately to insulin thus preventing cells from absorbing glucose. As a result, glucose builds up in the blood.

Type 1 diabetes, also called insulin-dependent diabetes mellitus (IDDM) and juvenile-onset diabetes, is caused by B-cell destruction, usually leading to absolute insulin deficiency. Type 2 diabetes, also known as non-insulin-dependent diabetes mellitus (NIDDM) and adult-onset diabetes, is associated with predominant insulin resistance and thus relative insulin deficiency and/or a predominantly insulin secretory defect with insulin resistance.

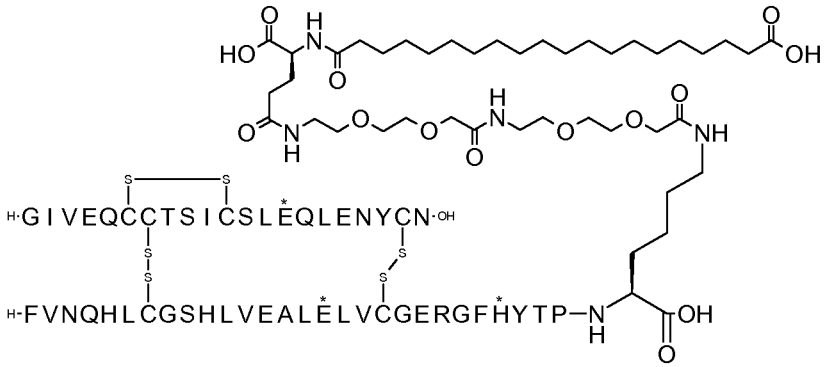
Herein, the naming of the insulins is done according to the following principles: The names are given as mutations and modifications (acylations) relative to human insulin. For the naming of the acyl moiety, the naming is done according to IUPAC nomenclature and in other cases as peptide nomenclature. For example, naming the acyl moiety:



can for example be named "eicosanedioyl-γGlu-OEG-OEG", "eicosanedioyl-γGlu-2xOEG" or, "eicosanedioyl-gGlu-2xOEG" or "19-carboxynonadecanoyl-γGlu-OEG-OEG", wherein OEG is short hand notation for the amino acid $NH_2(CH_2)_2O(CH_2)_2OCH_2CO_2H$, [2-(2-aminoethoxy)ethoxy]acetic acid and γGlu (and gGlu) is short hand notation for the amino acid gamma glutamic acid in the L-configuration. Alternatively, the acyl moiety may be named according to IUPAC nomenclature (OpenEye, IUPAC style). According to this nomenclature, the above acyl moiety of the invention is assigned the following name: [2-[2-[2-[[2-[2-[2-[[(4S)-4-carboxy-4-(19-carboxynonadecanoylamino)butanoyl]amino]ethoxy]-ethoxy]acetyl]amino]ethoxy]ethoxy]acetyl].

For example, the insulin of Example 1 (with the sequence/structure given below) is named "A14E, B16E, B25H, B29K(*N*$^{\varepsilon}$Eicosanedioyl-gGlu-2xOEG), desB30 human insulin" to indicate that the amino acid in position A14, Y in human insulin, has been mutated to E, the amino acid in position B16, Y in human insulin, has been mutated to E, the amino acid in position B25, F in human insulin, has been mutated to H, the amino acid in position B29, K as in human insulin, has been modified by acylation on the epsilon nitrogen in the lysine residue of B29, denoted *N*$^{\varepsilon}$, by the residue eicosanedioyl-gGlu-2xOEG, and the amino acid in position B30, T in human insulin, has been deleted.

Asterisks in the formula below indicate that the residue in question is different (i.e. mutated) as compared to human insulin.



SEQ ID Nos: 1 and 2

Alternatively, the insulins of the invention may be named according to IUPAC nomenclature (OpenEye, IUPAC style). According to this nomenclature, the insulin of

Example 1 (i.e. Compound 1) is assigned the following name: N{Epsilon-B29}-[2-[2-[2-[[2-[2-[2-[[(4S)-4-carboxy-4-(19-carboxynonadecanoyl amino)butanoyl]amino]ethoxy]-ethoxy]acetyl]amino]ethoxy]ethoxy]acetyl]-[GluA14,GluB16,HisB25],des-ThrB30-Insulin(human).

SUMMARY OF THE INVENTION

This invention relates to a derivative of an insulin analogue, i.e. A14E, B16E, B25H, B29K(N(eps)eicosanedioyl-gGlu-2xOEG), desB30 human insulin (Compound 1).

According to a first aspect, the present invention provides A14E, B16E, B25H, B29K(N(eps)-Eicosanedioyl-gGlu-2xOEG), desB30 human insulin (Compound 1).

According to a second aspect, the present invention provides a method of treating or preventing diabetes comprising administering to a subject an effective amount of the compound of the first aspect

According to a third aspect, the present invention provides the use of the compound of the first aspect in the manufacture of a medicament for the treatment or prevention of diabetes.

According to a fourth aspect, the present invention provides an aqueous solution comprising the compound of the first aspect.

According to a fifth aspect, the present invention provides a pharmaceutical composition comprising the compound of the first aspect, and one or more excipients.

Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

DETAILED DESCRIPTION OF THIS INVENTION

It has, surprisingly, been found that A14E, B16E, B25H, B29K(N(eps)eicosanedioyl-gGlu-2xOEG), desB30 human insulin relates to the above objects to a sufficient degree. For example, a subcutaneous treatment with Compound 1 once a week or more seldom will be a satisfactory treatment of the diabetic patient’s need for basal insulin treatment. Furthermore, Compound 1 has a high solubility in an aqueous medium optionally containing zinc. In one aspect, Compound 1 has a solubility which is higher than the solubility of human insulin.

In one aspect, Compound 1 is soluble in an aqueous medium containing zinc such as at least 5 zinc ions per insulin hexamer, at least 6 zinc ions per insulin hexamer, at least 7 zinc ions per insulin hexamer, at least 8 zinc ions per insulin hexamer or at least 9 zinc ions per

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insulin hexamer, wherein the solubility is measured after storage at least 4 weeks at 37°C or below after preparation.

In one aspect of the invention, Compound 1 is soluble in an aqueous medium containing zinc such as at least 5 zinc ions per insulin hexamer, at least 6 zinc ions per insulin hexamer, at least 7 zinc ions per insulin hexamer, at least 8 zinc ions per insulin hexamer, at least 9 zinc ions per insulin hexamer, at least 10 zinc ions per insulin hexamer, at least 11 zinc ions per insulin hexamer or at least 12 zinc ions per insulin hexamer, wherein the solubility is measured within 24-48 hours after preparation.

In one aspect, the solubility is determined as explained in Example 7 herein.

Pharmaceutical compositions containing Compound 1 can be prepared in a manner known *per se*, i.e., by using the excipients usually used in similar insulin compositions. Injectable pharmaceutical compositions containing Compound 1 can be prepared using conventional techniques of the pharmaceutical industry which involve dissolving and mixing the ingredients as appropriate to give the desired end product. Thus, according to one

procedure, Compound 1 is dissolved in an amount of water which is somewhat less than the final volume of the pharmaceutical composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted, if necessary, using an acid, for example, hydrochloric acid, or a base, for example, aqueous sodium hydroxide, as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

More precisely, an insulin preparation of this invention, for example a solution, may be prepared by dissolving Compound 1 in an aqueous medium at slightly acidic conditions. The aqueous medium is e.g. made isotonic by addition of a tonicity regulating agent. Furthermore, the aqueous medium may contain e.g. buffers, preservatives and zinc ions. The pH value of the solution is adjusted towards neutrality without getting too close to the isoelectric point of the compound of this invention in order to avoid potential precipitation. The pH value of the final insulin preparation depends upon the concentration of zinc ions, and the concentration of the compound of this invention. The insulin preparation is made sterile, for example, by sterile filtration.

A pharmaceutical composition may contain one or more excipients.

The term "excipient" broadly refers to any component other than the active therapeutic ingredient(s). The excipient may be an inert substance, an inactive substance, and/or a not medicinally active substance.

The excipient may serve various purposes depending on the pharmaceutical composition, e.g. as a carrier, vehicle, diluent, tablet aid, and/or to improve administration, and/or absorption of the active substance. Examples of excipients include, but is not limited to, diluents, buffers, preservatives, tonicity regulating agents (also known as tonicity agents or isotonic agents), chelating agents, surfactants, protease inhibitors, wetting agents, emulsifiers, antioxidants, bulking agents, metal ions, oily vehicles, proteins and/or a zwitterion and stabilisers.

The pharmaceutical composition of pharmaceutically active ingredients with various excipients is known in the art, see e.g. Remington: The Science and Practice of Pharmacy (e.g. 19th edition (1995), and any later editions).

The insulin compositions are administered to the patients in a manner known *per se*, e.g. according to the general knowledge of the patient combined with the general knowledge of the physician. This invention is best used at the convenience of the patient. Therefore, specific administration intervals will be explored for each patient where dosages are administered less than daily. The final mode of use thus depends both on the product's capabilities and on the disposition and preference of the patient. This is due to the fact that the effect of any insulin

product depends on the insulin need of the individual patient and the sensitivity to the pharmacodynamic actions of said insulin and lastly also to the preferences of the patient in a given situation. These conditions may change over time, both in terms of longer periods (years) and from day to day. The optimal dose level for any patient will depend on a variety of factors including the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the state to be treated. It is recommended that the dosage regimen be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions, however taking into consideration the present teachings concerning dosage intervals.

For the convenience of the patients, it is presumed that they prefer that the time interval (time lag) from the administration of Compound 1 to the next administration of Compound 1 has the same length, or approximately the same length, counted in number of days. It can even be expected that the patients will prefer that the administration of Compound 1 takes place once weekly, i.e., on the same day in the week, e.g. every Sunday. This will be an administration of Compound 1 every 7^{th} day and not more frequently on an average calculated for a period of time of 1 month, 6 months or 1 year. For some patients, it may be desirable to administer Compound 1 every 6^{th} day or approximately every 6^{th} day and not more frequently on an average calculated for a period of time of 1 month, 6 months or 1 year. For other patients, it may be desirable to administer Compound 1 every 5^{th} day or approximately every 5^{th} day and not more frequently on an average calculated for a period of time of 1 month, 6 months or 1 year. For other patients, it may be desirable to administer Compound 1 every 4^{th} day or approximately every 4^{th} day and not more frequently on an average calculated for a period of time of 1 month, 6 months or 1 year. Even other patients may find it advantageous to administer Compound 1 twice weekly, e.g. with an interval of about 3-4 days between each administration on an average calculated for a period of time of 1 month, 6 months or 1 year. For some patients, it may be desirable to administer Compound 1 every 3^{rd} day or approximately every 3^{th} day and not more frequently on an average calculated for a period of time of 1 month, 6 months or 1 year. For other patients, it may be desirable to administer Compound 1 every 2^{nd} day or approximately every 2^{nd} day and not more frequently on an average calculated for a period of time of 1 month, 6 months or 1 year. For some patients, it may be desirable to administer Compound 1 every 8^{th} day or approximately every 8^{th} day and not more frequently on an average calculated for a period of time of 1 month, 6 months or 1 year. Even other patients may not administer Compound 1 with a time interval of precisely the same length (counted in days), week after week, month after month or year after year. Some patients may administer Compound 1 sometime in the time interval from every 6^{th}

to every 8th day on an average calculated for a period of time of 1 month, 6 months or 1 year and not more frequently. Other patients may administer Compound 1 sometime in the time interval from every 5th to every 7th day on an average calculated for a period of time of 1 month, 6 months or 1 year and not more frequently. Even other patients may administer Compound 1 sometime in the time interval from every 4th to every 8th day on an average calculated for a period of time of 1 month, 6 months or 1 year and not more frequently. The time intervals mentioned here are to be understood as average time intervals within a period of time of say weeks, months or years. Here, it is intended that the term "day" covers 24 hours (i.e., a day and night) and, for the sake of easiness, a number of hours which is not divisible by 24 is to be rounded up to a whole number of days. Hence, e.g. 30 hours corresponds to 1 day and 40 hours corresponds to 2 days. The above mentioned administrations are parenterally.

The patients may have a daily basal insulin requirement of above about 0.2 IU/kg body weight/day and below about 1 IU/kg body weight/day and, furthermore, the patients may have a total (i.e., basal plus prandial) daily insulin requirement of above about 1 IU/kg body weight/day. However, these ranges may vary considerably from patient to patient and may for several patients be somewhat outside the ranges mentioned here.

Diseases and conditions which are the primary targets for this invention are diabetes mellitus (type 1 or 2) or other conditions characterized by hyperglycaemia, but also metabolic diseases and conditions in general where the metabolic effects of insulin has a clinical relevance or are of interest, such as pre-diabetes, impaired glucose tolerance, metabolic syndrome, obesity, cachexia, *in vivo* beta-cell loss/death, excessive appetite, and inflammation. All these types of conditions are known to or believed to benefit from a stable metabolic state in the subject who has the disease or condition. At any rate, any therapeutic regimen where administration of insulin is included may be modified by implementing the current teachings, meaning that such therapies will include administration of prolonged-profile-of-action insulins according to the teachings provided herein.

In order to exercise this invention, Compound 1 may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. Further options are to administer the insulin composition orally, nasally or pulmonary, preferably in pharmaceutical compositions, powders or liquids, specifically designed for the purpose in question.

Alternatively, in order to exercise this invention, Compound 1 may be administered orally to patients in need of such a treatment. Oral administration may be performed by orally administering solid, semi-solid or liquid pharmaceutical compositions.

Embodiments of the method of this invention include those wherein administration of Compound 1 is supplemented with more frequent administrations of a fast-acting naturally occurring insulin, insulin analogue or insulin derivative and/or administration of a non-insulin anti-diabetic drug. In one embodiment of this invention, administration of Compound 1 is supplemented with administration of a non-insulin anti-diabetic drug, such as metformin.

PREFERRED FEATURES OF THIS INVENTION

To sum up and supplement the above statements, the features and clauses of this invention are as follows:

1. A14E, B16E, B25H, B29K(N(eps)eicosanedioyl-gGlu-2xOEG), desB30 human insulin (Compound 1).
2. A pharmaceutical composition comprising Compound 1.
3. Compound 1 for use as a medicament.
4. Compound 1 for use in the preparation of a pharmaceutical composition for the treatment or prevention of diabetes.
5. Compound 1 for use in the preparation of a pharmaceutical composition for the treatment or prevention of diabetes Type 1 and/or Type 2.
6. Compound 1 for use in the treatment of diabetes, wherein the compound is administered to the same patient every 2nd day or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.
7. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 3rd day or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.
8. Compound 1 for use in the treatment of diabetes, wherein the compound is administered twice a week or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.
9. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 4th day or less frequently, and, on average, during a period of time of at

least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

10. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 5th day or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

11. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 6th day or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

12. Compound 1 for use in the treatment of diabetes, wherein the compound is administered once weekly or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

13. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 8th day or more frequently.

14. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 9th day or more frequently.

15. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 10th day or more frequently.

16. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 11th day or more frequently.

17. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 12th day or more frequently.

18. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 14th day or more frequently.

19. Compound 1 for use in the treatment of diabetes, wherein the compound is administered every 21st day or more frequently.

20. Compound 1 according to any one of clauses 6-19, wherein the currently or repeatedly treatment lasts for more than 1 month.

21. Compound 1 according to any one of clauses 6-19, wherein the currently or repeatedly treatment lasts for more than 2 month.

22. Compound 1 according to any one of clauses 6-19, wherein the currently or repeatedly treatment lasts for more than 3 month.

23. Compound 1 according to any one of clauses 6-19, wherein the currently or repeatedly treatment lasts for more than 1 year (one year).

24. Compound 1 according to any one of clauses 2-23, wherein the compound is administered parenteral, preferably subcutaneous, intramuscular or intravenous.

25. Compound 1 according to any one of clauses 2-23, wherein the compound is administered orally.

26. A method of treatment or prevention of diabetes, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1.

27. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 2nd day or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

28. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 3rd day or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

29. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient twice a week or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

30. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 4th day or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

31. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 5th day or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

32. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 6th day or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

33. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient

once weekly or less frequently and, on average, during a period of time of at least 1 month, 6 months or 1 year, said compound is not administered more frequently to the same patient.

34. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 8th day or more frequently.

35. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 9th day or more frequently.

36. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 10th day or more frequently.

37. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 11th day or more frequently.

38. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 12th day or more frequently.

39. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 14th day or more frequently.

40. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient every 21st day or more frequently.

41. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient wherein the currently or repeatedly treatment for diabetes with Compound 1 lasts for more than 1 month.

42. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient wherein the currently or repeatedly treatment for diabetes with Compound 1 lasts for more than 2 months.

43. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient wherein the currently or repeatedly treatment for diabetes with Compound 1 lasts for more than 3 months.

44. The method according to clause 26, which method comprises administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient wherein the currently or repeatedly treatment for diabetes with Compound 1 lasts for more than 1 year (one year).

45. The method according to clause 26, which method comprises parenteral, preferably subcutaneous, intramuscular or intravenous, administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient.

46. The method according to clause 26, which method comprises orally administering to a subject in need thereof a therapeutically effective amount of Compound 1 to the same patient.

47. An aqueous solution comprising Compound 1.

48. An aqueous solution comprising Compound 1 and at least 5 zinc ions per insulin hexamer.

49. An aqueous solution comprising Compound 1 and at least 6 zinc ions per insulin hexamer.

50. An aqueous solution comprising Compound 1 and at least 7 zinc ions per insulin hexamer.

51. An aqueous solution comprising Compound 1 and at least 8 zinc ions per insulin hexamer.

52. An aqueous solution comprising Compound 1 and at least 9 zinc ions per insulin hexamer.

53. An aqueous solution comprising Compound 1 and at least 10 zinc ions per insulin hexamer.

54. An aqueous solution comprising Compound 1 and at least 11 zinc ions per insulin hexamer.

55. An aqueous solution comprising Compound 1 and at least 12 zinc ions per insulin hexamer.

56. The aqueous solution according to any one of clauses 47-55, wherein the pH is in the range of from 7 to 8.

57. The aqueous solution according to any one of clauses 47-55, wherein the pH is about 7.4.

58. A pharmaceutical composition comprising Compound 1, and one more excipients.

59. A pharmaceutical composition comprising Compound 1, and one more excipients selected from the group consisting of diluents, buffers, preservatives, tonicity

regulating agents, chelating agents, surfactants, protease inhibitors, wetting agents, emulsifiers, antioxidants, bulking agents, metal ions, oily vehicles, proteins and/or a zwitterion and stabilisers.

60. The pharmaceutical composition according to clause 59, comprising at least 4.5 zinc ions per insulin hexamer.

61. The pharmaceutical composition according to clause 59, comprising at least 5 zinc ions per insulin hexamer.

62. The pharmaceutical composition according to clause 59, comprising at least 6 zinc ions per insulin hexamer.

63. The pharmaceutical composition according to clause 59, comprising at least 7 zinc ions per insulin hexamer.

64. The pharmaceutical composition according to clause 59, comprising at least 8 zinc ions per insulin hexamer.

65. The pharmaceutical composition according to clause 59, comprising at least 9 zinc ions per insulin hexamer.

66. The pharmaceutical composition according to clause 59, comprising at least 10 zinc ions per insulin hexamer.

67. The pharmaceutical composition according to clause 59, comprising at least 11 zinc ions per insulin hexamer.

68. The pharmaceutical composition according to clause 59, comprising at least 12 zinc ions per insulin hexamer.

69. The pharmaceutical composition according to any one of clauses 59-68, wherein the pH is in the range of from 7 to 8.

70. The pharmaceutical composition according to any one of clauses 59-68, wherein the pH is about 7.4.

71. The pharmaceutical composition according to any one of clauses 59-68, which is in the form of an aqueous solution.

72. The pharmaceutical composition according to any one of clauses 59-68, which is in the form of a tablet.

73. The pharmaceutical composition according to any one of clauses 59-68, which is in the form of a solid, semi-solid or liquid preparation, contained in a capsule such as a soft or a hard capsule.

74. Any novel product, apparatus, method or use defined by a feature and or a claim and/or a combination of features and/or claims described herein.

Any combination of two or more of the embodiments described herein is considered within the scope of the present invention.

EXAMPLES

The invention is further illustrated with reference to the following examples, which are not intended to be in any way limiting to the scope of the invention as claimed.

The following abbreviations are used herein:

βAla is beta-alanyl;
Aoc is 8-aminooctanoic acid;
tBu is *tert*-butyl;
DCM is dichloromethane;
DIC is diisopropylcarbodiimide;
DIPEA = DIEA is *N*,*N*-disopropylethylamine;
DMF is *N*,*N*-dmethylformamide;
DMSO is dimethyl sulphoxide;
EtOAc is ethyl acetate;
Fmoc is 9-fluorenylmethyloxy-carbonyl;
γGlu (gGlu) is gamma L-glutamyl;
DγGlu (DgGlu) is gamma D-glutamyl;
HCl is hydrochloric acid;
HOAc is acetic acid;
HOBt is 1-hydroxybenzotriazole;
NMP is *N*-methylpyrrolidone;
MeCN is acetonitrile;
OEG is [2-(2-aminoethoxy)ethoxy]ethylcarbonyl;
Su is succinimidyl-1-yl = 2,5-dioxo-pyrrolidin-1-yl;
OSu is succinimidyl-1-yloxy= 2,5-dioxo-pyrrolidin-1-yloxy;
RPC is reverse phase chromatography;
RT is room temperature;
TFA is trifluoroacetic acid;
THF is tetrahydrofuran;
TNBS is 2,4,6-trinitrobenzene-sulfonic acid;
TRIS is tris(hydroxymethyl)-aminomethane; and
TSTU is *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

The following examples and general procedures refer to intermediate compounds and final products identified in the specification and in the synthesis schemes. The preparation of the compound of the present invention is described in detail using the following examples, but the chemical reactions described are disclosed in terms of their general applicability to the preparation of compound of the invention. Occasionally, the reaction may not be applicable as described to each compound included within the disclosed scope of the invention. The compounds for which this occurs will be readily recognised by those skilled in the art. In these cases, the reactions can be successfully performed by conventional modifications known to those skilled in the art, which is, by

appropriate protection of interfering groups, by changing to other conventional reagents, or by routine modification of reaction conditions. Alternatively, other reactions disclosed herein or otherwise conventional will be applicable to the preparation of the corresponding compounds of the invention. In all preparative methods, all starting materials are known or may easily be prepared from known starting materials. All temperatures are set forth in degrees Celsius and unless otherwise indicated, all parts and percentages are by weight when referring to yields and all parts are by volume when referring to solvents and eluents.

Construction of vectors, yeast expression, processing and purification of insulin analogues can be done using the standard techniques readily recognised by those skilled in the art. One non-limiting example of preparation of insulin analogues was described previously (Glendorf T, Sørensen AR, Nishimura E, Pettersson I, & Kjeldsen T: Importance of the Solvent-Exposed Residues of the Insulin B Chain α-Helix for Receptor Binding; *Biochemistry* 2008 **47** 4743-4751). Briefly, mutations are introduced to insulin coding vectors using overlap extension PCR. Insulin analogues are expressed as proinsulin-like fusion proteins, with an Ala-Ala-Lys mini C-peptide in Saccharomyces cerevisiae strain MT663. The single-chain precursors are enzymatically converted into two-chain desB30 analogues using A. lyticus endoprotease. Full conversion to the two-chain desB30 analogue is verified by MALDI-TOF MS, and its purity is measured by RP-HPLC at both acidic and neutral pH.

The compound of the invention can be purified by employing one or more of the following procedures which are typical within the art. These procedures can - if needed - be modified with regard to gradients, pH, salts, concentrations, flow, columns and so forth. Depending on factors such as impurity profile, solubility of the insulin derivative in question etcetera, these modifications can readily be recognised and made by a person skilled in the art.

After acidic HPLC or desalting, the compounds are isolated by lyophilisation of the pure fractions. After neutral HPLC or anion exchange chromatography, the compounds are desalted, precipitated at isoelectrical pH, or purified by acidic HPLC.

Typical purification procedures

The HPLC system is a Gilson system consisting of the following: Model 215 Liquid handler, Model 322-H2 Pump and a Model 155 UV Dector. Detection is typically at 210 nm and 280 nm. The Âkta Purifier FPLC system (Amersham Biosciences) consists of the following: Model P-900 Pump, Model UV-900 UV detector, Model pH/C-900 pH and conductivity detector, Model Frac-950 Frction collector. UV detection is typically at 214 nm, 254 nm and 276 nm. Äkta Explorer Air FPLC system (Amersham BioGE Health

Caresciences) consists of the following: Model P-900 Pump, Model UV-900 UV detector, Model pH/C-900 pH and conductivity detector, Model Frac-950 Fraction collector. UV detection is typically at 214 nm, 254 nm and 276 nm.

Acidic HPLC

Column: Phenomenex, Gemini, 5µ, C18, 110 Å, 250x30 cm
Flow: 20 mL/min
Eluent: A: 0.1% TFA in water,
B: 0.1% TFA in CH_3CN
Gradient: 0-7.5 min: 10% B
7.5-87.5 min: 10% B to 60% B
87.5-92.5 min: 60% B
92.5-97.5 min: 60% B to 100% B

Neutral HPLC

Column: Phenomenex, Gemini, C18, 5µm 250 x 30.00 mm, 110 Å
Flow: 20 mL/min
Eluent: A: 20% CH_3CN in aqueous 10mM TRIS + 15mM $(NH_4)SO_4$ pH = 7.3
B: 80% CH_3CN, 20% water
Gradient: 0-7.5 min: 0% B
7.5-52.5 min: 0% B to 60% B
52.5-57.5 min: 60% B
57.5-58 min: 60% B to 100% B
58-60 min: 100% B
60-63 min: 10% B

Anion exchange chromatography

Column: 150 mL (2.6x28cm) Poros 50HQ
Flow: 25 mL/min
Eluent: A buffer: 15 mM TRIS, 50 mM Ammoniumacetat in 50% Ethanol, pH 7.5 (1.6 mS/cm)
B buffer: 15 mM TRIS, 500 mM Ammoniumacetat in 50% Ethanol, pH 7.5 (14 mS/cm)
Gradient: 0-80% B over 20CV

Solid phase synthesis

19-((S)-1-carboxy-3-{2-[2-({2-[2-(2,5-dioxopyrrolidin-1-yloxycarbonylmethoxy)ethoxy]ethyl-carbamoyl}methoxy)ethoxy]ethylcarbamoyl}propylcarbamoyl)nonadecanoic acid; (Alternative name: Eicosanedioyl-gGlu-OEG-OEG-OSu)

19-((S)-1-carboxy-3-{2-[2-({2-[2-(2,5-dioxopyrrolidin-1-yloxycarbonylmethoxy)-ethoxy]ethylcarbamoyl}methoxy)ethoxy]ethylcarbamoyl}propylcarbamoyl)nonadecanoic acid can be synthesised on solid support using procedures well known to skilled persons in the art of solid phase peptide synthesis. This procedure e.g. comprises attachment of a Fmoc protected amino acid to a polystyrene 2-chlorotritylchloride resin. The attachment can, e.g. be accomplished using the free N-protected amino acid in the presence of a tertiary amine, like triethyl amine or *N,N*-diisopropylethylamine (see references below). The C-terminal end (which is attached to the resin) of this amino acid is at the end of the synthetic sequence being coupled to the parent insulins of the invention. After attachment of the Fmoc amino acid to the resin, the Fmoc group is deprotected using, e.g. secondary amines, like piperidine or diethyl amine, followed by coupling of another (or the same) Fmoc protected amino acid and deprotection. The synthetic sequence is terminated by coupling of a mono-*tert*-butyl protected fatty (α, ω) diacid, namely eicosanedioic acid mono-*tert*-butyl ester. Cleavage of the compounds from the resin is accomplished using diluted acid like 0.5-5% TFA/DCM (trifluoroacetic acid in dichloromethane), acetic acid (e.g. 10% in DCM, or HOAc/triflouroethanol/DCM 1:1:8), or hecafluoroisopropanol in DCM (see e.g. "*Organic Synthesis on Solid Phase*", F.Z. Dörwald, Wiley-VCH, 2000. ISBN 3-527-29950-5; "*Peptides: Chemistry and Biology*", N. Sewald & H.-D. Jakubke, Wiley-VCH, 2002, ISBN 3-527-30405-3; and "*The Combinatorial Cheemistry Catalog*" 1999, Novabiochem AG; and references cited therein). This ensures that the *tert*-butyl ester present in the compound as carboxylic acid protecting groups is not deprotected. Finally, the C-terminal carboxy group (liberated from the resin) is activated, e.g. as the *N*-hydroxysuccinimide ester (OSu) and used either directly or after purification as coupling reagent, or after deprotection in attachment to A14E, B16E, B25H, desB30 human insulin.

Alternatively, the acylation reagents 19-((S)-1-carboxy-3-{2-[2-({2-[2-(2,5-dioxo-pyrrolidin-1-yloxycarbonylmethoxy)ethoxy]ethylcarbamoyl}methoxy)ethoxy]ethylcarbamoyl}-propylcarbamoyl)nonadecanoic acid can be prepared by solution phase synthesis:

The mono-*tert*-butyl protected fatty diacid, eicosanedioic acid mono-*tert*-butyl ester, is activated, e.g. as OSu-ester as described below or as any other activated ester known to those skilled in the art, such as HOBt- or HOAt-ester. This active ester is coupled with glutamic acid α-*tert*-butyl ester in a suitable solvent such as THF, DMF, NMP (or a solvent

mixture) in the presence of a suitable base, such as DIPEA or triethylamine. The intermediate is isolated, e.g. by extractive procedures or by chromatographic procedures. The resulting intermediate is again subjected to activation (as described above) and to coupling with OEG-OEG ([2-(2-{2-[2-(2-Amino-ethoxy)-ethoxy]-acetylamino}-ethoxy)-ethoxy]-acetic acid) as described above followed by activation with TSTU to afford the acylation reagent 19-((S)-1-carboxy-3-{2-[2-({2-[2-(2,5-dioxopyrrolidin-1-yloxycarbonyl-methoxy)ethoxy]ethylcarbamoyl}methoxy)ethoxy]ethylcarbamoyl}propylcarbamoyl)-nonadecanoic acid.

The acylation reagent prepared by the above described methods may be *tert*-butyl de-protected after activation as OSu ester. This may be done by TFA treatment of the OSu-activated *tert*-butyl protected acylation reagent. After acylation of A14E,B16E,B25H,desB30 human insulin, the resulting unprotected acylated A14E,B16E,B25H,desB30 human insulin is obtained, e.g. as described in Example 1.

If the reagent prepared by any of the above methods is not *tert*-butyl de-protected after activation as OSu ester, acylation of A14E,B16E,B25H,desB30 human insulin affords the corresponding *tert*-butyl protected acylated A14E,B16E,B25H,desB30 human insulin. In order to obtain unprotected acylated A14E,B16E,B25H,desB30 human insulin, the protected insulin is to be de-protected. This can be done by TFA treatment to afford unprotected acylated A14E,B16E,B25H,desB30 human insulin.

Alternatively, the acylation reagent can be synthesised in solution using benzyl protection of the carboxylic acid groups as illustrated below.

19-((S)-1-Carboxy-3-{2-[2-({2-[2-(2,5-dioxo-pyrrolidin-1-yloxycarbonylmethoxy)ethoxy]-ethylcarbamoyl}methoxy)ethoxy]ethylcarbamoyl}propylcarbamoyl)nonadecanoic acid; (Alternative name: Eicosanedioyl-gGlu-OEG-OEG-OSu)

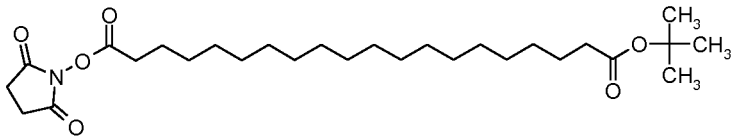
LCMS Method (LCMS)

A Waters Micromass ZQ mass spectrometer was used to identify the mass of the sample after elution from a Waters Alliance HT HPLC system.

Eluents:	A: 0.1% Trifluoroacetic acid in water
	B: 0.1% Trifluoroacetic acid in acetonitrile
Column:	Phenomenex, Jupiter C4 50 X 4.60 mm, id: 5 µm
Gradient:	10%-90% B over 7.5 min at 1.0 mL/min
Column:	Phenomenex, Jupiter 5µ C4 300Å 50 x 4.60 mm
LC method:	10-90% B 10min: A: 0.1% CH_3CN B: CH_3CN:

0-7.5 min: 10-90% B
7.5-8.5 min: 90-10% B
8.5-9.5 min 10% B
Flow: 1 mL/min
9.5 – 10.00 min 10% B
Flow: 0.1 mL/min

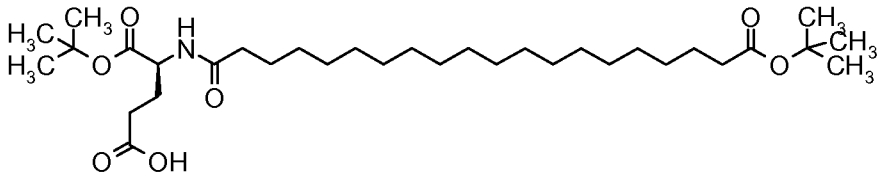
Eicosanedioic acid *tert*-butyl ester *N*-hydroxysuccinimide ester



Eicosanedioic acid mono-*tert*-butyl ester (5 g, 12.54 mmol) and TSTU (4.53g, 15.05 mmol) were mixed in THF (50 mL), DIPEA (2.62 mL) was added and the resulting cloudy mixture was stirred at RT for 2h, then DMF (30 mL) was added resulting in a clear solution which was further stirred overnight. The resulting mixture was evaporated to almost dryness and the residue was mixed with cold acetonitrile resulting in the precipitation of a precipitate. This was filtered off and dried *in vacuo* overnight, affording 6.01 g (97%) of eicosanedioic acid *tert*-butyl ester *N*-hydroxysuccinimide ester.

MS (electrospray): m/z: 440 (M-56 (tBu)).

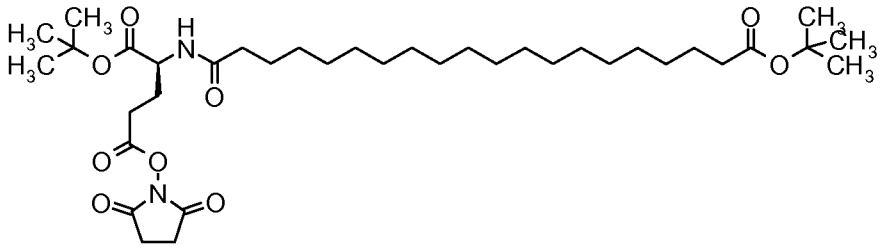
(S)-2-(19-*tert*-Butoxycarbonylnonadecanoylamino)pentanedioic acid 1-*tert*-butyl ester



Eicosanedioic acid *tert*-butyl ester 2,5-dioxo-pyrrolidin-1-yl ester (6.01g, 12.124 mmol) was dissolved in THF (150 mL) and mixed with a slurry of H-Glu-OtBu (2.71 g, 13.33 mmol) in DMF/water (1/1, 40 mL). This resulted in a gel-like solution which was heated to give a clear solution that was stirred at RT for 3 hours. Then the solution was evaporated, 100 mL of water was added and the mixture was heated to 60°C which resulted in a solution which crystallised on cooling. The precipitate was re-crystallised from acetonitrile and the crystals were dried in vacuum. Yield 6.82 g (96%).

MS (electrospray): m/z 584 (M+1).

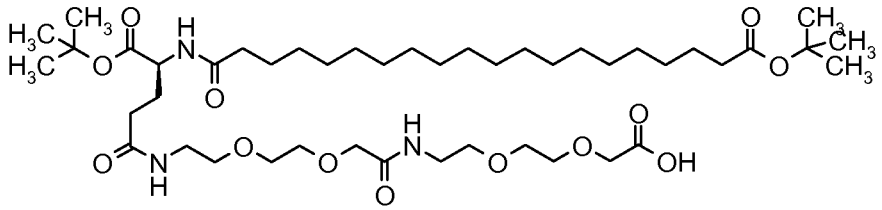
(S)-2-(19-*tert*-Butoxycarbonylnonadecanoylamino)pentanedioic acid 1-*tert*-butyl ester 5-(2,5-dioxopyrrolidin-1-yl) ester



(S)-2-(19-*tert*-Butoxycarbonylnonadecanoylamino)pentanedioic acid 1-*tert*-butyl ester (6.52g, 11.17 mmol) was dissolved in THF (100 mL), DIPEA (2.14 mL) was added followed by a solution of TSTU (3.70 g, 12.29 mmol) in acetonitrile (25 mL). The mixture was stirred overnight at RT, then it was evaporated, resulting in a brownish residue which was re-crystallised from acetonitrile. After cooling overnight at 5°C a powder was formed. This was dissolved in THF and dried with $MgSO_4$, filtered and evaporated to dryness to afford 6.17 g (81%) of the title compound.

MS (electrospray): m/z: 681 (M+1).

19-{(S)-1-*tert*-Butoxycarbonyl-3-[2-(2-{[2-(2-carboxymethoxyethoxy)ethylcarbamoyl]-methoxy}ethoxy)ethylcarbamoyl]propylcarbamoyl}nonadecanoic acid *tert*-butyl ester; (Alternative name: ^{t}Bu-Eicosanedioyl-gGlu(O^{t}Bu)-OEG-OEG-OH)

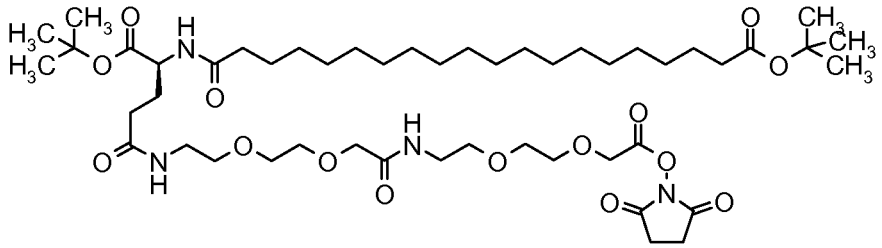


To a solution of 2-(19-*tert*-Butoxycarbonylnonadecanoylamino)pentanedioic acid 1-*tert*-butyl ester 5-(2,5-dioxopyrrolidin-1-yl) ester (2.50 g) and [2-(2-{2-[2-(2-aminoethoxy)-ethoxy]acetylamino}ethoxy)ethoxy]acetic acid (alternative name: H-OEG-OEG-OH)(1.47 g) in ethanol (40 mL) was added DIPEA (1.26 mL). The mixture was stirred at room temperature overnight and then concentrated *in vacuo*. To the residue was added aqueous 0.1 N HCl (150 mL) and ethyl acetate (200 mL). The layers were separated and the aqueous layer was extracted with ethyl acetate (100 mL). The combined organic layers were washed with water and brine, dried (magnesium sulphate) and concentrated *in vacuo* to give an oil, which crystallised on standing.

Yield 96% (3.1 g). LCMS: Theoretical mass: 874.2. Found: 874.49.

19-((S)-1-*tert*-Butoxycarbonyl-3-{2-[2-({2-[2-(2,5-dioxo-pyrrolidin-1-yloxycarbonylmethoxy)-ethoxy]ethylcarbamoyl}methoxy)ethoxy]ethylcarbamoyl}propylcarbamoyl)nonadecanoic acid *tert*-butyl ester;

(Alternative name: ^{t}Bu-Eicosanedioyl-gGlu(O^{t}Bu)-OEG-OEG-OSu)

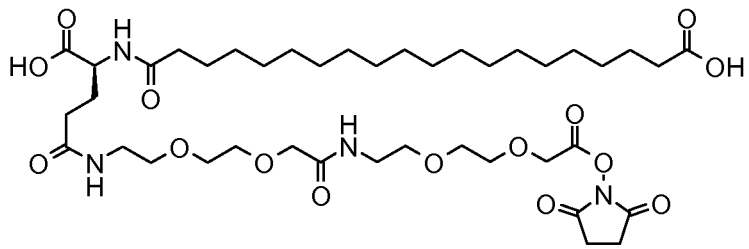


To a solution of 19-{(S)-1-tert-Butoxycarbonyl-3-[2-(2-{[2-(2-carboxymethoxy-ethoxy)ethylcarbamoyl]methoxy}ethoxy)ethylcarbamoyl]propylcarbamoyl}nonadecanoic acid *tert*-butyl ester (3.1 g) in acetonitrile (50 mL) was added TSTU (1.39 g) and DIPEA (0.91 mL). The mixture was stirred at room temperature overnight and then concentrated in vacuo. To the residue was added aqueous 0.1 N HCl (100 mL) and ethyl acetate (200 mL). The layers were separated and the aqueous layer was extracted with ethyl acetate (50 mL). The combined organic layers were washed with water and brine, dried (magnesium sulphate) and concentrated *in vacuo* to give an oil.

Yield 99% (3.4 g). LCMS: Theoretical mass: 971.2 Found: 971.8.

19-((S)-1-Carboxy-3-{2-[2-({2-[2-(2,5-dioxo-pyrrolidin-1-yloxycarbonylmethoxy)ethoxy]ethyl-carbamoyl}methoxy)ethoxy]ethylcarbamoyl}propylcarbamoyl)nonadecanoic acid;

(Alternative name: Eicosanedioyl-gGlu-OEG-OEG-OSu)



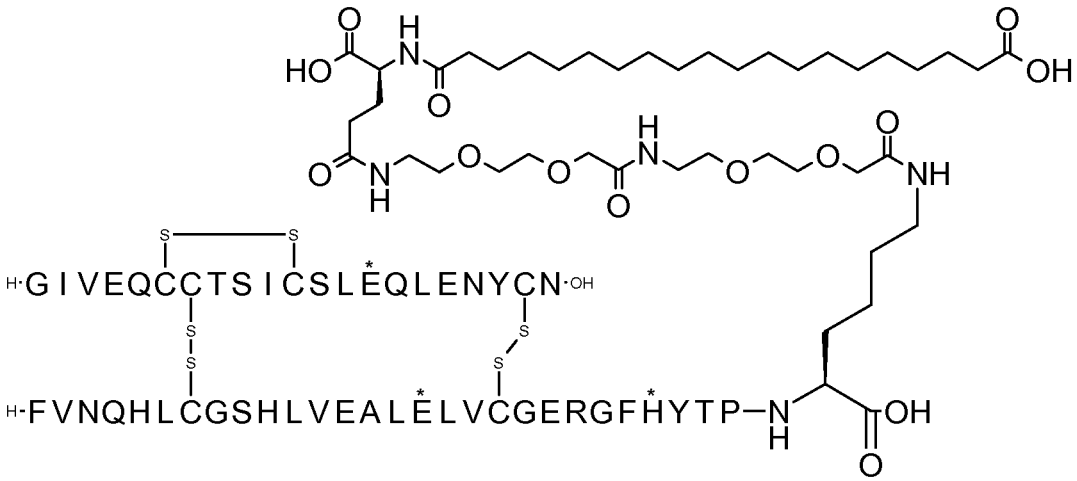
19-((S)-1-*tert*-Butoxycarbonyl-3-{2-[2-({2-[2-(2,5-dioxo-pyrrolidin-1-yloxycarbonylmethoxy)-ethoxy]ethylcarbamoyl}-methoxy)ethoxy]ethylcarbamoyl}propylcarbamoyl)nonadecanoic acid *tert*-butyl ester (3.4 g) was stirred in TFA (75 mL) for 45 min and then concentrated *in vacuo*. The residue was co-concentrated with toluene 3 times to give a solid. The residue was crystallised in 2-propanol and filtered to give a white crystalline compound.

Yield 80% (2.4 g). LCMS: Theoretical mass: 859.03 Found: 859.44.

For acylation of the lysine residue inposition B29 (in the epsilon position) of A14E, B16E, B25H, desB30 human insulin, acylation is preferably performed at alkaline pH (e.g. at pH 10, 10.5, or 11). This is illustrated in Example 1 herein.

Example 1

N{Epsilon-B29}-[2-[2-[2-[[2-[2-[2-[[(4S)-4-carboxy-4-(19-carboxynonadecanoyl amino)butanoyl]amino]ethoxy]ethoxy]acetyl]amino]ethoxy]ethoxy]acetyl]-[GluA14,GluB16,HisB25],des-ThrB30-Insulin(human);
(Alternative name: A14E, B16E, B25H, B29K(N^{ε}eicosanedioyl-gGlu-2xOEG), desB30 human insulin; Compound 1)



A14E, B16E, B25H, desB30 human insulin (3.0 g, 0.53 mmol) was dissolved in 150 mM aqueous Na_2CO_3 (40 mL) and 5 mL THF was added. The pH value was adjusted to 11.0 with 1M aqueous NaOH. Under vigorous stirring, 19-((S)-1-carboxy-3-{2-[2-({2-[2-(2,5-dioxopyrrolidin-1-yloxycarbonylmethoxy)ethoxy]ethylcarbamoyl}methoxy)ethoxy]-ethylcarbamoyl}propylcarbamoyl)nonadecanoic acid (641 mg, 0.75 mmol, prepared as described above) dissolved in a mixture of 1.5 mL THF and 1.5 mL DMF during one minute. While adding, pH was kept constant at 10.5–11 with addition of 1N aqueous NaOH. The mixture was stirred for one hour.

The pH value was adjusted to 7.5 with 1M HCl and 50% ethanol was added to a volume of 500 mL. The pH value was adjusted to 7.5. The conductivity was measured to 1.6 mS/Cm.

Purification was performed by anion exchange chromatography on an Äkta Explorer:

Column: 150 mL (2.6x28 cm) Poros 50HQ

A buffer: 15 mM TRIS, 50 mM ammonium acetate in 50% ethanol, pH 7.5 (1.6 mS/cm)

B buffer: 15 mM TRIS, 500mM ammonium acetate in 50% ethanol, pH 7.5 (14 mS/cm)
Gradient: 0-80%B over 20CV
Flow: 25mL/min.

The product pool, 700 mL, was diluted with 700 mL of 50% ethanol and purified once more:
Column: 150 mL (2.6x28 cm) Poros 50HQ
A buffer: 15 mM TRIS, 50 mM ammonium acetate in 50% ethanol, pH 7.5 (1.6 mS/cm)
B buffer: 15 mM TRIS, 500mM ammonium acetate in 50% ethanol, pH 7.5 (14 mS/cm)
Gradient: 0-100% B over 12CV
Flow: 25 mL/min.

The product pool, 300 mL, was diluted with 300 mL water and desalted on a C18 column:
Column: 30x250 mm (Daiso_200_15um_FEFgel304_ODDMS_30x250 mm), CV=177 mL
A Buffer: 10% acetonitrile in milli-Q water + 0.1% TFA
B Buffer: 80% acetonitrile in milli-Q water + 0.1% TFA
Gradient: 25-80%B over 20 min.
Flow: 35 mL/min.

The product fraction was freeze dried to afford the TFA salt, which was dissolved in 50 mL water plus 10 mL acetonitrile and pH was adjusted to 8.0 with 0.5M aqueous NaOH and freeze dried to afford 1.25 g (36%) of the title insulin.

LC-MS (electrospray): m/z = 1593.1 (M+4)/4. Calculated: 1594.1.

Example 2

Insulin receptor affinity

The affinity of the acylated insulin analogues of this invention for the human insulin receptor is determined by a SPA assay (Scintillation Proximity Assay) microtiterplate antibody capture assay. SPA-PVT antibody-binding beads, anti-mouse reagent (Amersham Biosciences, Cat No. PRNQ0017) are mixed with 25 mL of binding buffer (100 mM HEPES pH 7.8; 100 mM sodium chloride, 10 mM $MgSO_4$, 0.025% Tween-20). Reagent mix for a single Packard Optiplate (Packard No. 6005190) is composed of 2.4 µl of a 1:5000 diluted purified recombinant human insulin receptor (either with or without exon 11), an amount of a stock solution of A14Tyr[^{125}I]-human insulin corresponding to 5000 cpm per 100 µl of

reagent mix, 12 µl of a 1:1000 dilution of F12 antibody, 3 mL of SPA-beads and binding buffer to a total of 12 mL. A total of 100 µl reagent mix is then added to each well in the Packard Optiplate and a dilution series of the insulin derivative is made in the Optiplate from appropriate samples. The samples are then incubated for 16 hours while gently shaken. The phases are the then separated by centrifugation for 1 min and the plates counted in a Topcounter. The binding data were fitted using the nonlinear regression algorithm in the GraphPad Prism 2.01 (GraphPad Software, San Diego, CA) and affinities are expressed relative (in percentage (%)) to the affinity of human insulin.

A related assay is also used wherein the binding buffer also contains 1.5%HSA in order to mimic physiological conditions.

Table 1

Insulin receptor affinities of selected insulins of the invention

Test compound	Relative IR-A affinity (0% HSA) (%)	Relative IR-A affinity (1.5% HSA) (%)
Compound 1	0.1	0.01

Example 3

Hydrophobicity of the insulin derivatives of the invention

The hydrophobicity of an insulin derivative is found by reverse phase HPLC run under isocratic conditions. The elution time of the insulin derivative is compared to that of human insulin (herein designated HI) or another derivative with a known hydrophobicity under the same conditions. The hydrophobicity, k'rel, is calculated as: $k'rel_{deriv} = ((t_{deriv}-t_0)/(t_{ref}-t_0))*k'rel_{ref}$. Using HI as reference: $k'rel_{ref} = k'rel_{HI} = 1$. The void time of the HPLC system, t_0, is determined by injecting 5 µl of 0.1 mM $NaNO_3$.

Running conditions:

Column: Lichrosorb RP-C18, 5µm, 4 x 250 mm

Buffer A: 0.1 M natrium phosphate pH 7.3, 10 vol% CH_3CN

Buffer B: 50 vol% CH_3CN

Injection volume: 5 µl

Run time: Maximum 60 minutes

After running an initial gradient, the isocratic level for running the derivative and reference (for example HI) is chosen, and the elution times of the derivative and reference under isocratic conditions are used in the above equation to calculate $k'rel_{deriv}$.

Table 2

Hydrophobicity of the insulin derivatives of the invention

Test compound	Relative hydrophobicity $k'rel_{deriv}$
Compound 1	0.6

Example 4

Degradation of insulin analogues using duodenum lumen enzymes

Degradation of insulin analogues using duodenum lumen enzymes (prepared by filtration of duodenum lumen content) from SPD rats. The assay is performed by a robot in a 96 well plate (2mL) with 16 wells available for insulin analogues and standards. Insulin analogues ~15 µM are incubated with duodenum enzymes in 100 mM Hepes, pH=7.4 at 37°C, samples are taken after 1, 15, 30, 60, 120 and 240 min and reaction quenched by addition of TFA. Intact insulin analogues at each point are determined by RP-HPLC. Degradation half time is determined by exponential fitting of the data and normalized to half time determined for the reference insulins, A14E, B25H, desB30 human insulin or human insulin in each assay. The amount of enzymes added for the degradation is such that the half time for degradation of the reference insulin is between 60 minutes and 180 minutes. The result is given as the degradation half time for the insulin analogue in rat duodenum divided by the degradation half time of the reference insulin from the same experiment (relative degradation rate).

Table 3

Degradation

Test compound	Duodenum degradation Relative stability vs. A14E, B25H, desB30 human insulin

Compound 1	0.7

Example 5

Intravenous rat PK

Anaesthetized rats are dosed intravenously (*i.v.*) with insulin analogues at various doses and plasma concentrations of the test compound is measured using immunoassays or mass spectrometry at specified intervals for 4 hours or more post-dose. Pharmacokinetic parameters are subsequently calculated using WinNonLin Professional (Pharsight Inc., Mountain View, CA, USA).

Non-fasted male Wistar rats (Taconic) weighing approximately 200 gram are used. Body weight is measured and rats are subsequently anaesthetized with Hypnorm/Dormicum (each compound is separately diluted 1:1 in sterile water and then mixed; prepared freshly on the experimental day). Anaesthesia is initiated by 2 mL/kg Hypnorm/Doricum mixture sc followed by two maintenance doses of 1 mL/kg sc at 30 minutes intervals, and two maintenance doses of 1 mL/kg sc with 45 minutes intervals. If required in order to keep the rats lightly anaesthetised throughout a further dose(s) 1-2 mL/kg sc is supplied. Weighing and initial anaesthesia is performed in the rat holding room in order to avoid stressing the animals by moving them from one room to another.

Table 4

Rat PK

Test compound	Rat PK i.v. MRT (h)
Compound 1	24.5

Example 6

Dog intravenous pharmacokinetic (PK) profiles

The objective of this protocol is to obtain pharmacokinetic (PK) data from plasma concentration-time profiles of different insulin analogues after intravenous administration to beagle dogs, and to calculate relevant pharmacokinetic parameters for the analogues.

The animals had free access to domestic quality drinking water. The animals were weighed on each day of dosing. Each test substance was given to 3 animals. Consideration

had been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal. A full plasma concentration-time profile was obtained from each animal. During blood sampling, the dogs were placed on a table and fixated by an animal technician sitting beside. This procedure was trained during the acclimatization period. Blood samples, 0.5 mL, were collected into EDTA tubes according to the following schedule:

Predose (-10, 0), and 5, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 300, 480, 600, 720, 960, 1440, 1920, 2880, 4320, 5760, 7200, 8640, 10080 minutes.

During periods of frequent sampling, the blood samples were taken from Venflon catheters in cephalic veins kept open with Heparin.saline. The other blood samples were taken from a jugular vein.

Blood samples were kept on ice for max 20 minutes before centrifugation at 4°C for 4 minutes at 1,300 g.

Plasma was immediately transferred to two micronic tubes, 80 µl plasma in each from each blood sample and placed according to the rack outline. The plasma was stored at -20°C until assayed.

Plasma concentration-time profiles were analyzed by a non-compartmental pharmacokinetics analysis using WinNonlin Professional (Pharsight Inc., Mountain View, CA, USA).

Calculations were performed using individual concentration-time values from each animal.

Table 5

Dog PK

Test compound	Dog intravenous $T_{½}$ ± SD (hours	Dog intravenous MRT ± SD (hours) (Mean retention time)
Compound 1	92 ± 22	121 ± 28

Example 7

Initial solubility of Compound 1 and comparison Compound A in the presence of zinc

Compound 1 and comparison Compound A (i.e. N{Epsilon-B29}-[2-[2-[2-[[2-[2-[2-[[(4S)-4-carboxy-4-(19-carboxynonadecanoylamino)butanoyl]amino]ethoxy]ethoxy]acetyl]-

amino]ethoxy]ethoxy]acetyl]-[GluA14,HisB16,HisB25],des-ThrB30-Insulin(human); Alternative name: A14E, B16H, B25H, B29K(*N*$^{\varepsilon}$eicosanedioyl-gGlu-2xOEG), desB30 human insulin, respectively, were dissolved in milli-Q water at a pH value of about 8. Phenol, cresol, zinc acetate (Zn), sodium chloride and glycerol were added in the mentioned order resulting in a final pharmaceutical composition containing: 4.2-5 mM insulin, 1.6% glycerol, 25 mM phenol, 25 mM cresol, pH 7.4 and the zinc and sodium chloride concentration stated in the table below. Pharmaceutical compositions were stored for 24 hours at 22°C and centrifuged at 15,000 x g for 15 minutes. 100 µl of the supernatant was transferred to HPLC vials and concentration determined using acidic gelfiltration as described in Eur. Pharm. NovoRapid. The amount of soluble insulin was determined in percentage of the starting concentration. The accuracy of the measurement was +/- 2%.

Table 6

Solubility of Compound 1 and comparison Compound A, respectively, in presence of Zn

Zn/hexamer	0 mM NaCl Compound A % soluble insulin	20 mM NaCl Compound A % soluble insulin	0 mM NaCl Compound 1 % soluble insulin	20 mM NaCl Compound 1 % soluble insulin
4.5	100	100		100
5.5	101	100		100
5.9			100	
6.5	100	100		100
6.6			100	
7.4			100	
7.5	100	100		100
8.3			100	
8.5	100	100		100
9.2			100	
9.5	96	100		103
10.0			100	
10.5	100	99		100
10.9			100	

Zn/hexamer	0 mM NaCl Compound A % soluble insulin	20 mM NaCl Compound A % soluble insulin	0 mM NaCl Compound 1 % soluble insulin	20 mM NaCl Compound 1 % soluble insulin
11.5	100	91		100
11.8			100	
12.5	99	85		100
12.7			100	
13.5	82	67		100
13.6			100	
14.4			100	
14.5	64	23		100
15.3			100	
15.5	47	5		91
16.2			92	
16.5	19	1		79
17.1			13	
17.5	19	1		60
18.5	19	1		9

Conclusion

The comparison compound, in a composition without NaCl, is soluble under the tested conditions in presence of up to 12.5 zinc molecules per hexamer. Comparison Compound A, in a composition with 20 mM NaCl, is soluble under the tested conditions in the presence of up to 10.5 molecules of zinc/hexamer.

Compound 1 is soluble in a composition without NaCl under the tested conditions up to about 15.3 zinc hexamer insulin. Furthermore, Compound 1 is soluble with 20 mM NaCl under the tested conditions up to about 14.5 molecules of zinc per hexamer insulin.

Example 8

Initial solubility of Human insulin in the presence of zinc

Human insulin was dissolved in milli-Q water at a pH value of about 8. Phenol, cresol, zinc acetate (Zn), sodium chloride and glycerol were added in the mentioned order

resulting in a final formulation containing: 4.2-5 mM insulin 1.6% glycerol, 25 mM phenol, 25 mM cresol, pH, 7.4 and the zinc and sodium chloride concentration stated in the table below. The formulations were stored 24 hours at 22°C and then centrifuged at 15 000 x g for 15 min. 100 µl of the supernatant was transferred to HPLC vials and concentration determined on using acidic gelfiltration described in Eur. Pharm. NovoRapid. Amount of soluble insulin was determined in percent of starting concentration.

Accuracy of measurement is +/- 2%.

Table 7

Solubility of human insulin in the presence of zinc

Zn/hexamer	0 mM NaCl % soluble insulin	20 mM NaCl % soluble insulin
2	100	100
4	100	100
6	83	100
8	15	19

Conclusion

Human insulin is soluble in formulations containing up to 6 Zn/insulin hexamer when the formulation contains NaCl and up to 4 Zn/insulin hexamer when the formulation contains close to no NaCl.

Example 9

Chemical and physical stability as a function of zinc and sodium chloride content

The aim of this experiment was to measure the chemical and physical stability of a formulation within the zinc/hexamer window determined by SEC experiments. Furthermore to test if the presence of sodium chloride affected the chemical and/or physical stability.

Formulations

Formulations contained: 3.6 mM of Compound 1, 25 mM phenol, 25 mM cresol, pH 7.4. Zinc and sodium chloride as specified below.

Table 8

Zinc containing formulations of Compound 1

Zinc per hexamer	Sodium chloride mM	Glycerol % w/w
5.8	20	1.6
5.8	75	0.7
5.8	120	0
8.1	20	1.6
8.1	75	0.7
8.1	120	0
10.5	20	1.6
10.5	75	0.7
10.5	120	0

The formulation was prepared as follows:

Compound 1 powder was dissolved in milli-Q water in a stock solution in about the double amount as the final concentration in the formulation. Phenol, cresol, zinc acetate, sodium chloride and glycerol was added in the mentioned order. The resulting solution had a pH about 7.8 and was adjusted to pH 7.4 using 0.2 N HCl, resulting in a final increase in chloride concentration of 1.45 mM Chloride.

The formulation was sterile filtered and filled in 3 ml Cartridges with stoppers.

Physical stability was measured as follows:

Fibrillation tendency was measured in Thioflavin T (THT) assay. Potential precipitation leading to visible particle formation was measured as potential increase in turbidity. Particle formation below 2 µm was measured by dynamic light scattering (DLS). Particle formation above 2 µm was measured by Micro Flow Imaging (MFI).

Chemical stability was measured as increase in High Molecular Weight Particles (HMWP) in percent and decrease in purity as measured by reverse phase UPLC.

Fibrillation tencency in Thioflavin T assay

Concentration of Compound 1 was determined according to the method described in WO 2013/153000.

Table 9

Lagtimes measured in hours in Thioflavin T assay. Lag time to fibrillation increase as a function of zinc content in the formulation. Formulations containing more than 5.8 zn/ hexamer do not fibrillate and has thus a lag time higher than 45 hours.

Zn/ hexamer / mM NaCl	Lag time in hours	Insulin concentration in mM before ThT assay	Insulin concentration in % of starting concentration after ThT assay
5.8 Zn/ hexamer / 20 mM NaCl	15	4.3	91%
5.8 Zn/ hexamer / 75 mM NaCl	14	4.2	90%
5.8 Zn/ hexamer / 120 mM NaCl	15	4.2	88%
8.1 Zn/ hexamer / 20 mM NaCl	45	4.3	100%
8.1 Zn/ hexamer / 75 mM NaCl	45	4.2	100%
8.1 Zn/ hexamer / 120 mM NaCl	45	4.1	100%
10.5 Zn/ hexamer / 20 mM NaCl	45	4.0	100%
10.5 Zn/ hexamer / 75 mM NaCl	45	4.2	100%
10.5 Zn / hexamer / 120 mM NaCl	45	4.2	100%

Quiescent stability of Compound 1 probed by DLS

The physical stability of Compound 1 formulated with varying concentrations of NaCl and Zn-acetate stored at 4°C, 37°C and 45°C was probed by dynamic light scattering (DLS).

Method

Each sample was measured in triplets on a DynaPro plate reader at 25°C by recording 20 acquisitions of 10 seconds; data are reported as an average of the three measurements. The samples were not subjected to filtering, but instead they were centrifuged at 15 000 x g for 20 min to remove only the very largest flocculates and aggregates, which would otherwise block the measurements. Further, paraffin oil was used for sealing the wells of the DLS microtiter plate instead of the more commonly used plastic foil.

Table 10

Protein oligomer average size measured as hydrodynamic diameter (HD) in nm for the different formulations incubated for 2-8 weeks at 4°C, 30°C, 37°C or 45°C

Formulation Zn NaCl	**2 weeks 4˚C**		**8 weeks 4˚C**		**2 weeks 37˚C**		**2 weeks 45˚C**		**8 weeks 30˚C**		**8 weeks 37˚C**	
	HR Diam /nm	St.D./ nm	HR Diam /nm	St.D./ nm	HR Diam /nm	St.D./ nm	HR Diam /nm	St.D./ nm	HR Diam /nm	St.D./ nm	HR Diam /nm	St.D./ nm
5.8 Zn/hexamer / 20 mM NaCl	4.05	0.10	3.99	0.06	3.8	0.01	3.84	0.02	4.08	0.04	4.17	0.05
5.8 Zn/hexamer / 75 mM NaCl	5.25	0.09	5.02	0.02	4.96	0.02	4.97	0.04	5.12	0.09	5.14	0.06
8.1 Zn/hexamer / 20 mM NaCl	3.88	0.03	3.88	0.02	3.87	0.01	3.87	0.03	3.94	0.01	4.14	0.03

Formulation Zn NaCl	2 weeks 4˚C		8 weeks 4˚C		2 weeks 37˚C		2 weeks 45˚C		8 weeks 30˚C		8 weeks 37˚C	
	H R Diam /nm	St.D./ nm	H R Diam /nm	St.D./ nm	H R Diam /nm	St.D./ nm	H R Diam /nm	St.D./ nm	H R Diam /nm	St.D./ nm	H R Diam /nm	St.D./ nm
8.1 Zn/hexamer / 75 mM NaCl	5.21	0.01	5.24	0.04	5.17	0.02	5.11	0.03	5.20	0.06	5.32	0.12
10.5 Zn/ hexamer/ 20 mM NaCl	4.34	0.02	4.28	0.04	4.2	0.05	4.17	0.01	4.23	0.03	4.29	0.02
10.5 Zn/hexamer / 75 mM NaCl	5.95	0.03	5.93	0.04	5.68	0.05	5.63	0.05	5.65	0.04	5.71	0.04

H R: Hydrodynamic radius (nm)

Diam: Diameter (nm)

St.D: Standard Deviation

The protein oligomer average size determined with DLS ranges from 3.8 nm (for a formulation with 5,8Zn/insulin hexamer, 20mM NaCl, at 37°C, after 2 weeks) to 5.95 nm (for a formulation with 10.5 Zn/insulin hexamer, 75 mM NaCl at 4°C, after 2 weeks). For the samples stored at 4°C the hydrodynamic diameter decreases 1% on average whereas it increased 1 and 4% for the samples stored at 37 and 45°C, respectively. Moreover, all of the recorded auto-correlation functions were compatible with unimodal particle distributions, indicating rather narrow size distributions absent of any large aggregates.

Conclusion

Although the different formulation conditions exhibited noticeable different average oligomer sizes, the change over time was extraordinarily small if present at all, and all formulations appeared to be physically stable at 4°C, 37°C as well as 45°C within the 8 week period tested. No aggregates were formed during the period.

Particle measurement above 2 µm using MFI

The formulations were analyzed for sub-visible particle formation in the micrometer range using Micro Flow Imaging (MFI™). Particle counts were generally low, and a large fraction of particles had a dark spherical appearance expected for silicone-oil droplets. However, large translucent flake-like particles appeared in formulations containing 10.5 Zn/hexamer and 150mM or 75mM after 2 weeks incubation at 45°C and 8 weeks at 37°C, respectively.

Table 11

Particle concentrations in mL for the different formulations incubated for 2-20 weeks at 4°C, 30°C, 37°C or 45°C. Particles with Circularity*Aspect Ratio*Intensity STD >75 and ECD <3µm were rejected from the analysis as potentially representing silicone oil.

Time		2 weeks			4 weeks		8 weeks			20 weeks	
Zn/Hex	[NaCl] (mM)	4°C	37°C	45°C	4°C	37°C	4°C	30°C	37°C	4°C	25°C
		89.9	91.8	224.1	202.5	179.7	15.3	80.3	162.4	24.9	5.7
5.8	20	38.2	24.9	57.4	28.7	108.9	21.0	22.9	28.7	1.9	49.7
5.8	75	78.6	80.3	45.9	47.8	86.0	7.7	21.0	19.1	32.5	38.2
5.8	120	19.1	30.6	40.1	7.6	147.2					
8.1	20	105.1	87.9	137.6	26.8	273.2	93.6	82.2	34.4	53.5	370.5
8.1	75	17.2	65.0	57.3	118.5	210.2	59.3	105.1	51.6	72.6	57.3
8.1	120	74.6	44.0	23.0	21.0	191.1					
10.5	20	158.7	78.4	152.9	230.0	326.7	137.6	290.4	131.8	13.4	17.2
10.5	75	63.1	203.2	154.9	225.5	221.7	120.7	126.1	586.6	28.7	44.0
10.5	120	80.3	343.9	1807.6	267.5	279.0					

Table 12

Particle volume fractions (nL particles per mL sample volume) for the different formulations incubated for 2-20 weeks at 4°C, 30°C, 37°C or 45°C. Particles with Circularity*AspectRatio*IntensitySTD >75 and ECD <3µm were rejected from the analysis as potentially representing silicone oil.

Time		2 weeks			4 weeks		8 weeks			20 weeks	
Zn/Hex	[NaCl] (mM)	4°C	37°C	45°C	4°C	37°C	4°C	30°C	37°C	4°C	25°C
		0.01	0.01	0.5	0.06	0.04	0	0.03	0.02	0.01	0
5.8	20	0	0	0.12	0.03	0.03	0	0	0.01	0	0.04
5.8	75	0	0.01	0.02	0	0.03	0.05	0	0	0.05	0.19
5.8	120	0	0	0	0	0.06					
8.1	20	0.01	0	0.01	0	0.02	0	0.01	0	0.02	0.22
8.1	75	0	0.01	0.06	0.01	0.01	0.03	0.02	0.01	0.01	0.01
8.1	120	0.02	0.01	0.03	0	0.03					
10.5	20	0.01	0.01	0.02	0.02	0.08	0.09	0.06	0.03	0.06	0
10.5	75	0.02	0.11	0.04	0.01	0.03	0.01	0.02	0.36	0.01	0.01
10.5	120	0.02	0.26	1.32	0.12	0.08					

Physical stability conclusion

The physical stability was measured as lag time in ThT assay as a function of zinc/hexamer increase with increasing zinc content from 5.8 to 8.1 Zn/insulin hexamer. Average oligomer size change as measured by DLS revealed no change in oligomer size and no aggregate formation in any of the formulations. Particle measurement as determined by MFI showed increase in particle formation in formulations containing 10.5 Zn/ hexamer and 75 mM NaCl.

The physical stability was thus optimal in a formulations containing above 5.8 and below 10.5 Zn/insulin hexamer.

Chemical stability

HMWP formation was measured using gelfiltration column in acetic acid free eluent as described in WO 2013/153000. HMWP for samples stored at 4°C was subtracted HMPW for samples stored at 30°C or 37°C.

Table 13

HMWP development for the different formulations incubated for 2-8 weeks at 4°C, 30°C or 37°C.

	2w 37˚C-2w 4˚C	8w 30˚C-8w 4˚C	4w 30˚C-4W 4˚C	8w 37˚C-8w 4˚C
5.8 Zn/hexamer / 20 mM NaCl	0.35	0.32	0.52	1.01
5.8 Zn/hexamer / 75 mM NaCl	0.41	0.35	0.58	1.17
5.8 Zn/hexamer / 120 mM NaCl	0.40	0.34		
8.1 Zn/hexamer / 20 mM NaCl	0.25	0.22	0.43	0.75
8.1 Zn/ hexamer / 75 mM NaCl	0.26	0.23	0.43	0.73
8.1 Zn/examer / 120 mM NaCl	0.28	0.31		
10.5 Zn/hexamer / 20 mM NaCl	0.28	0.32	0.45	0.76
10.5 Zn/hexamer / 75 mM NaCl	0.25	0.26	0.39	0.72
10.5 Zn/hexamer / 120 mM NaCl	0.28	0.23		

Conclusion

Formulations containing 5.8 Zn/ insulin hexamer has more HMWP development than formulations containing 8.1 Zn/ insulin hexamer or above.

Purity loss

Loss in purity was measured relative to start. The purity measured by reverse phase chromatography for samples stored at 4°C was subtracted purity measured for samples stored at 30°C or 37°C. A UPLC purity method slightly modified relative to method described in WO 2013/153000 was used. In the present instance Waters CSH, C18 column was used which in this case improves the separation and numbers of injections allowed on the column before it has to be changed.

Table 14

Loss in purity in **%** for the different formulations incubated for 2-8 weeks at 4°C, 30°C or 37°C.

	2w 37˚C-2w 4˚C	8w 30˚C-8w 4˚C	4w 30˚C-4W 4˚C	8w 37˚C-8w 4˚C
5.8 Zn/hexamer / 20 mM NaCl	1.800	1.700	3.29	6.12
5.8 Zn/hexamer / 75 mM NaCl	1.500	1.300	2.86	5.44
8.1 Zn/hexamer / 20 mM NaCl	1.200	1.000	1.92	3.78
8.1 Zn/hexamer / 75 mM NaCl	1.000	1.000	1.90	3.24
10.5 Zn/hexamer / 20 mM NaCl	1.000	1.100	1.91	3.45
10.5 Zn/hexamer / 75 mM NaCl	0.900	0.800	1.58	3.10

Conclusion

The formulations containing 5.8 Zn/insulin hexamer have the highest degradation. Formulations containing 8.1 Zn/insulin hexamer or more have lower degradation. The chemical stability is thus optimal in formulations with 8.1.zinc/hexamer or more. The stability is higher in formulations containing 75 mM NaCl than in formulations containing 20 mM NaCl.

Example 10

The aim of this experiment was to investigate the oligomerisation by size exclusion chromatography as a function of NaCl content in the formulation containing comparative Compound A (i.e. N{Epsilon-B29}-[2-[2-[2-[[2-[2-[2-[[(4S)-4-carboxy-4-(19-carboxy-nonadecanoylamino)butanoyl]amino]ethoxy]ethoxy]acetyl]amino]ethoxy]ethoxy]acetyl]-[GluA14,HisB16,HisB25],des-ThrB30-Insulin(human); Alternative name: A14E, B16H, B25H, B29K(N^{ε}eicosanedioyl-gGlu-2xOEG), desB30 human insulin at 4.2 mM insulin and

fixed zinc/insulin hexamer. Furthermore, the aim was to measure the physical and chemical stability.

Formulation

Compound A was dissolved in milli-Q water at a pH value of about 8. Phenol, cresol, zinc acetate (Zn) and glycerol were added in the mentioned order resulting in a final formulation containing: 4.5 Zn/ 6 insulins, 25 mM phenol, 25 mM cresol, pH 7.4 an insulin concentration of 4.2 mM and sodium chloride (NaCl), zinc acetate and glycerol as stated in the table below.

Physical stability was assessed by measurement of

1. Fibrillation tendency. Measured by Thioflavin T assay. Fibrillation tendency was measured in Thioflavin T (THT) assay as lagtime to fibrillation. THT assay was measured as described on freshly prepared samples; and
2. Oligomer radii in nm and aggregate formation below 4 µm by Dynamic light scattering.

Chemical stability of the formulations were measured as increase in High Molecular Weight Protein (HMWP) increase in insulin related impurities after storage for four weeks (4w) at 37°C relatively to the amount of HMWP after storage at 4°C.

HMWP was measured using HMWP Method 2 as described in WO 2013/153000.

Formation of insulin related impurities like deamidation compounds was measured using reverse phase chromatography (UPLC).

Amount of monomer was measured in native gel filtration using Method 2 as described in WO 2013/153000 in eluent without phenol.

Table 15

HMWP formation and lag time to fibrillation in THT assay of Compound A

Zink/ins hexamer, NaCl and glycerol content	% monomer SEC Without phenol	% monomer SEC With phenol	HMWP formation (%) 4w 37˚C	THT lag times (hours)	HMWP Formation (%) 4w 37˚C
4 Zn/hexamer 20 mM NaCl, 1.6% glycerol	61	48	0.4	15.6	0.89
4 Zn/hexamer 50 mM NaCl, 1.1%	49	33	0.39	19.2	0.8

Zink/ins hexamer, NaCl and glycerol content	% monomer SEC Without phenol	% monomer SEC With phenol	HMWP formation (%) 4w 37˚C	THT lag times (hours)	HMWP Formation (%) 4w 37˚C
glycerol					
4 Zn/hexamer 75 mM NaCl, 0.7% glycerol	46	30	0.43	22.0	0.81
4 Zn/hexamer 120 mM NaCl	45	29	0.49	23.0	0.87
5 Zn/hexamer, 20 mM NaCl, 1.6% glycerol	78	48	0.52	22.0	0.85
5 Zn/hexamer 50 mM NaCl 1.1% glycerol	68	36	0.41	27.7	0.84
5 Zn/hexamer 75 mM NaCl 0.7% glycerol	62	32	0.40	30.9	0.79
5 Zn/hexamer, 120 mM NaCl	64	32	0.35	29.6	0.77
6 Zn/hexamer 20 mM NaCl 1.6% glycerol	86	44	0.35	34.2	0.8
6 Zn/hexamer 50 mM NaCl 1.1% glycerol	77	37	0.28	40.4	0.73
6 Zn/hexamer 75 mM NaCl 0.7% glycerol	77	35	0.33	45.0	0.73
6 Zn/hexamer 120 mM NaCl	62	28	0.40	45.0	0.73
7 Zn/hexamer 20 mM NaCl 1.6% glycerol	58	34	0.45	45.0	0.95

Conclusion

The amount of Compound A monomer decreases as a function of sodium chloride concentration with a large effect of addition of just up to 50 mM NaCl. Chemical degradation measured as HMWP formation and impurity formation is low in all formulations despite the monomeric content. THT lag times increase with zinc content and sodium chloride content.

Table 16

Average hydrodynamic radii R_h avg. in nm and normalized intensity I_{norm} avg. in 10^6 count/sec (4°C). Note: Samples were not measured at t=0.

Insulin	Zink/hexamer, NaCl content and glycerol content	R_h avg. (nm)		I_{norm} avg. (10^6 cts)	
		2w	4w	2 w	4 w
Degludec		1.14	1.15	1.44	1.76
NovoRapid		2.49	2.49	1.94	2.27
Compound A	4 Zn/hexamer 20 mM NaCl, 1.6% glycerol	2.35	2.32	7.52	7.53
	4 Zn/hexamer 50 mM NaCl, 1.1% glycerol	2.96	3.02	14.7	16.1
	4 Zn/hexamer 75 mM NaCl, 0.7% glycerol	3.41	3.49	18.0	19.5
	4 Zn/hexamer 120 mM NaCl	4.11	4.16	21.7	23.4
	5 Zn/hexamer 50 mM NaCl 1.1% glycerol	3.07	3.11	13.3	14.8
	5 Zn/hexamer 75 mM NaCl 0.7% glycerol	3.39	3.49	20.0	20.1
	5 Zn/hexamer 120 mM NaCl	3.79	3.94	21.9	22.2

Insulin	Zink/hexamer, NaCl content and glycerol content	R_h avg. (nm)		I_{norm} avg. (10^6 cts)	
		2w	4w	2 w	4 w
	6 Zn/hexamer 50 mM NaCl 1.6% glycerol	2.90	3.03	15.6	16.7
	6 Zn/hexamer 75 mM NaCl 1.1% glycerol	3.23	3.41	17.9	19.8
	6 Zn/hexamer 120 mM NaCl 0.7% glycerol	3.88	3.85	24.3	23.1
	7 Zn/hexamer 20 mM NaCl 1.6% glycerol	2.52	2.14	18.0	8.24
	5 Zn/hexamer 20 mM NaCl, 1.6% glycerol	2.18	2.28	7.85	6.56
	6 Zn/hexamer 20 mM NaCl, 1.6% glycerol	2.04	1.99	5.64	4.65

R_h avg. (nm): Average hydrodynamic radii in nm

I_{norm} avg. (10^6 cts): Normalized intensity in 10^6 count/sec (37°C)

Table 17

Average hydrodynamic radii R_h avg. in nm and normalized intensity I_{norm} avg. in 10^6 count/sec (37°C). Note: Samples were not measured at t=0.

Insulin	Zn/hexamer, NaCl content and glycerol content	R_h avg. (nm)		I_{norm} avg. (10^6 cts)	
		2 w	4 w	2 w	4 w
Degludec		1.14	1.14	1.44	1.50
NovoRapid		2.49	2.46	1.94	1.94

Insulin	Zn/hexamer, NaCl content and glycerol content	R_h avg. (nm)		I_{norm} avg. (10^6 cts)	
		2 w	4 w	2 w	4 w
Compound A	4 Zn/hexamer 20 mM NaCl, 1.6% glycerol	2.35	2.26	7.52	10.6
	4 Zn/hexamer 50 mM NaCl, 1.1% glycerol	2.96	2.99	14.7	15.6
	4 Zn/hexamer 75 mM NaCl, 0.7% glycerol	3.41	3.43	18.0	18.9
	4 Zn/hexamer 120 mM NaCl	4.11	4.03	21.7	23.0
	5 Zn/hexamer 50 mM NaCl 1.1% glycerol	3.07	3.02	13.3	16.4
	5 Zn/hexamer 75 mM NaCl 0.7% glycerol	3.39	3.47	20.0	19.6
	5 Zn/hexamer 120 mM NaCl	3.79	3.88	21.9	21.5
	6 Zn/hexamer 50 mM NaCl 1.6% glycerol	2.90	2.90	15.6	15.7
	6 Zn/hexamer 75 mM NaCl 1.1% glycerol	3.23	3.23	17.9	18.1
	6 Zn/hexamer 120 mM NaCl 0.7% glycerol	3.88	3.87	24.3	22.4
	7 Zn/hexamer 20 mM NaCl 1.6% glycerol	2.52	2.40	18.0	12.7
	5 Zn/hexamer 20 mM NaCl, 1.6% glycerol	2.18	2.11	7.85	10.7

Insulin	Zn/hexamer, NaCl content and glycerol content	R_h avg. (nm)		I_{norm} avg. (10^6 cts)	
		2 w	4 w	2 w	4 w
	6 Zn/hexamer 20 mM NaCl, 1.6% glycerol	2.04	1.96	5.64	9.73

R_h avg. (nm): Average hydrodynamic radii in nm

I_{norm} avg. (10^6 cts): Normalized intensity in 10^6 count/sec (37°C)

Conclusion

The hydrodynamic radius increases with increasing salt concentration. Zn concentration has a minor impact on size except at 7 Zn per insulin hexamer. No significant effect on oligomer size and physical stability from incubation temperature.

2014333979 15 Jan 2018

CLAIMS:

1. A14E, B16E, B25H, B29K(N(eps)-Eicosanedioyl-gGlu-2xOEG), desB30 human insulin (Compound 1).

2. The compound of claim 1, when used as a medicament.

3. A method of treating or preventing diabetes comprising administering to a subject an effective amount of the compound of claim 1.

4. Use of the compound of claim 1 in the manufacture of a medicament for the treatment or prevention of diabetes.

5. The method of claim 3, wherein said compound is administered to the same patient every 2nd day or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, and wherein said compound is not administered more frequently to the same patient.

6. The use of claim 4, wherein said compound is to be administered to the same patient every 2nd day or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, and wherein said compound is not administered more frequently to the same patient.

7. The method of claim 3, wherein said compound is administered twice a week, or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, and wherein said compound is not administered more frequently to the same patient.

8. The use of claim 4, wherein said compound is to be administered twice a week, or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, and wherein said compound is not administered more frequently to the same patient.

9. The method of claim 3, wherein said compound is administered once weekly or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, and wherein said compound is not administered more frequently to the same patient.

10. The use of claim 4, wherein said compound is to be administered once weekly or less frequently, and, on average, during a period of time of at least 1 month, 6 months or 1 year, and wherein said compound is not administered more frequently to the same patient.

11. An aqueous solution comprising the compound of claim 1.

12. The aqueous solution according to claim 11 comprising at least 5 zinc ions per insulin hexamer.

13. The aqueous solution according to claim 11 or claim 12, wherein the pH is in the range of from 7 to 8.

14. A pharmaceutical composition comprising the compound of claim 1, and one or more excipients.

15. The pharmaceutical composition according to claim 14, comprising at least 4.5 zinc ions per insulin hexamer.

eolf-seql

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