Novel insulin derivatives having an extremely delayed time-action profile

The invention relates to novel insulin analogues with basal time/action profile, their preparation and use.

The incidence of diabetes has increased in recent years to an almost epidemic extent. The disorder may result in a serious shortening of life expectancy. People with diabetes must frequently supply their body with insulin from outside. It is sensible to optimize the treatment with insulin. Different insulins with specific pharmacological properties are now available. In practice, the different insulins are differentiated according to their duration of action into short-acting insulins, fast-acting insulins, long-acting insulins and mixed insulins. Designations used synonymously for long-acting insulins are slow insulins, depot insulin or else basal insulin. The active ingredients in many of these insulin products are so-called insulin analogues which have been derived from human insulin by substitution, deletion and/or addition of one or more amino acids. The terms "insulin analogues" and "insulins" are used synonymously herein.

The policy of intensified insulin therapy attempts to diminish the health risk by aiming at a stable control of the blood glucose level by early administration of basal insulins. One example of a current basal insulin is the medicament Lantus® (active ingredient: insulin glargine = Gly (A21), Arg (B31), Arg (B32) human insulin). The general aim of developing novel, improved basal insulins is to minimize the number of hypoglycemic events. An ideal basal insulin in this connection is one acting reliably for at least 24 hours in each patient. The insulin effect ideally has a delayed onset and a time/action profile which is as shallow as possible, so that the risk of brief hypoglycemia is distinctly minimized and administration is even possible without previous intake of foodstuffs. There is a good supply of basal insulin when the insulin effect persists at the same level for as long as possible, i.e. the body is supplied with a constant amount of insulin. The risk of hypoglycemic events is thus low and a patient- and a day-specific variability is minimized. The pharmacokinetic profile of an ideal basal insulin should thus be characterized by a delayed onset of action and by a delayed, i.e. long-lasting and uniform, action.

However – despite the therapeutic advantages already achieved – none of the slow insulins described to date shows the pharmacokinetic properties of an ideal basal insulin. Desirable insulins have such a shallow and long-lasting time/action profile that the risk of hypoglycemic events and of the day-dependent variations in the patient is further minimized and the duration of action is further delayed, so that it is no longer necessary in some circumstances to administer insulin daily. This would make simplified treatment of diabetics possible, especially of elderly diabetics and those in need of care, who are no longer able to inject insulin themselves, and would thus also be of great economic benefit. Such basal insulins would additionally be beneficial in the early phase of type 2 diabetes. Clinicians report that the injection phobia present in many people deters them from starting insulin therapy in good time. As a consequence, the control of blood glucose is poor, leading to the late sequelae of diabetes. A basal insulin which reduces the number of insulin doses given by injection might have the effect of making insulin therapy more acceptable to patients.

Kohn et al. (Peptides 28 (2007) 935-948) describe how it is possible to optimize the pharmacodynamics of insulin by preparing insulin analogues whose isoelectric point (pI) is shifted, by addition of lysine or arginine at the B chain end or at the N terminus of the A and B chain, in the direction of the alkaline range compared with the isoelectric point of human insulin (pI = 5.6), so that the solubility under physiological conditions is reduced and a prolonged time/action profile results. Compound 18 from Kohn et al. (Arg (A0), Gly (A21), Arg (B31), Arg (B32) human insulin (experimentally determined pI = 7.3; calculated pI = 7.58) is described in this connection as the best compound in the context of the idea. Kohn et al. therefore regard the main aim in designing novel insulin analogues as being the addition of positively charged amino acids to the amino acid sequence of human insulin for the purpose of increasing the isoelectric point from pI = 5.6 into the neutral range.

However, it has now surprisingly been found that the described desirable basal time/action profile is obtained with insulin analogues which are characterized by the features that

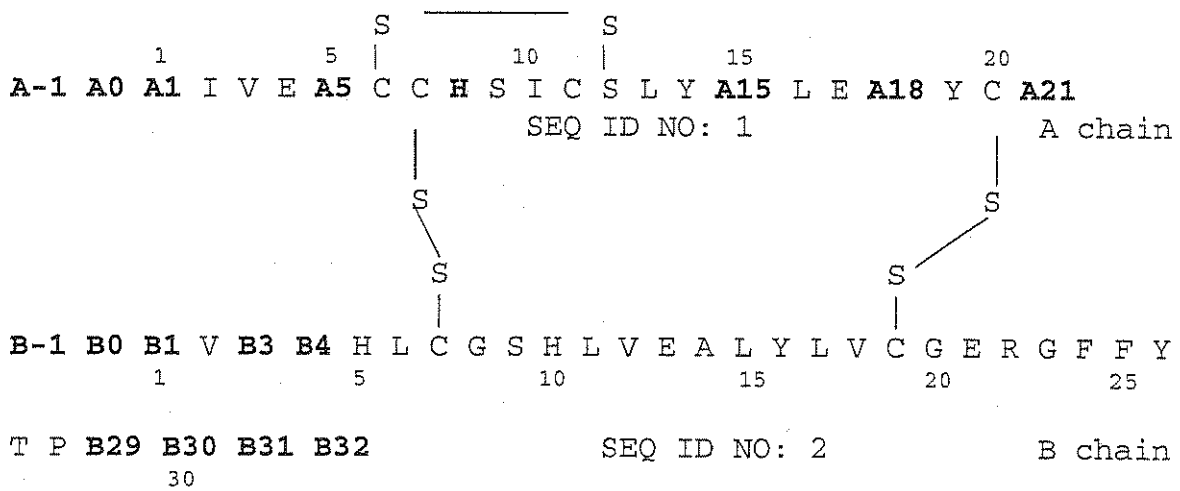
• the B chain end consists of an amidated basic amino acid residue such as lysine or argininamide, i.e. in the amidated basic amino acid residue at the B chain end the

carboxyl group of the terminal amino acid is present in its amidated form, and

- the A8 amino acid position is occupied by a histidine residue, and
- the A21 amino acid position is occupied by a glycine residue, alanine residue, serine residue or threonine residue, and
- no more than one amino acid residue of the group comprising A5, A15, A18, B-1, B0, B1, B2, B3 and B4 corresponds to Asp or Glu.

Surprisingly, precisely the insulin analogues described have the desired advantageous time/action profiles approaching the ideal, i.e. a delayed shallow onset of action and a longer duration of action. The risk of hypoglycemic events is thus distinctly minimized. The delay is so marked that it is surprisingly possible to detect the effect even in model experiments on rats. The delayed action of insulin glargine cannot by contrast be unambiguously observed in rats, as shown in figure 2. Figure 1 shows the hypoglycemic effect of the compound YKL202 and YKL203 of the invention. The results obtained in the experiment on rats are confirmed in an experiment on dogs. Once again, the duration of action is observed to be distinctly longer than that of insulin glargine. Thus, novel basal insulins which need to be administered distinctly less frequently have been provided. Besides these pharmacokinetic advantages described, the analogues of the invention show better properties compared with insulin glargine in pharmacological respects. Moreover, the claimed insulins also show advantages in physicochemical respects.

It has now surprisingly been found that insulin analogues of the formula I



where

A-1 corresponds to Lys, Arg or an amino group;

A0 corresponds to Lys, Arg or a chemical bond;

A1 corresponds to Arg or Gly;

A5 corresponds to Asp, Glu or Gln;

A15 corresponds to Asp, Glu or Gln;

A18 corresponds to Asp, Glu or Asn;

A21 corresponds to Ala, Ser, Thr or Gly;

B-1 corresponds to Asp, Glu or an amino group;

B0 corresponds to Asp, Glu or a chemical bond;

B1 corresponds to Asp, Glu, Phe or a chemical bond;

B3 corresponds to Asp, Glu or Asn;

B4 corresponds to Asp, Glu or Gln;

B29 corresponds to Arg, Lys or an amino acid selected from the group comprising the amino acids Phe, Ala, Thr, Ser, Val, Leu, Glu or Asp, or a chemical bond;

B30 corresponds to Thr or a chemical bond;

B31 corresponds to Arg, Lys or a chemical bond;

B32 corresponds to Arg-amide or Lys-amide.

where no more than one amino acid residue of the group comprising A5, A15, A18, B-1, B0, B1, B2, B3 and B4 corresponds to Asp or Glu, have the desired pharmacological profile, i.e. a delayed onset of action and by a longer-lasting and uniform effect. The invention therefore relates to these insulin analogues.

The invention further relates to an insulin analogue as described above, where one amino acid residue of the group comprising A5, A15, A18, B-1, B0, B1, B2, B3 and B4 corresponds to Asp or Glu.

The invention further relates to an insulin analogue as described above, where, preferably, A-1 corresponds to Arg, A0 corresponds to Arg, A5 corresponds to Glu, A15 corresponds to Glu, A18 corresponds to Asp, A8 corresponds to His, A21 corresponds to Gly, B0 corresponds to Glu, B3 corresponds to Asp, B4 corresponds to Glu, B30 corresponds to Arg or B30 corresponds to Lys.

The invention further relates to an insulin analogue as described above, where this is selected from a group comprising

Arg (A-1), Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), Glu (A5), His (A8), Gly (A21), Lys (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), Glu (A15), His (A8), Gly (A21), Lys (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B0), Lys (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Asp (B3), Lys (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B30) – NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B4), Lys (B30) – NH_2 human insulin,
Arg (A0), His (A8), Gly (A21), Arg (B31), Arg (B32) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Arg (B31), Lys (B32) – NH_2 – human insulin,
Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B31), Arg (B32) – NH_2 – human insulin,
Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B31), Lys (B32) – NH_2 – human insulin,

Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B31), Arg (B32) – NH_2 – human insulin,
Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B31), Lys (B32) – NH_2 – human insulin,
Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B31), Arg (B32) – NH_2 – human insulin,
Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B31), Lys (B32) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B31), Arg (B32) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B31), Lys (B32) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B31), Arg (B32) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B31), Lys (B32) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B31), Arg (B32) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B31), Lys (B32) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Arg (B30) – NH_2 – human insulin,
Arg (A0), His (A8), Gly (A21), Lys (B30) – NH_2 – human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Arg (B30) – NH_2 – human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Lys (B30) – NH_2 – human insulin,
Arg (A0), Arg (A1), His (A8), Gly (A21), Arg (B30) – NH_2 – human insulin,
Arg (A0), Arg (A1), His (A8), Gly (A21), Lys (B30) – NH_2 – human insulin,
His (A8), Gly (A21), Arg (B31), Arg (B32) – NH_2 – human insulin.

Specification of the term "human insulin" in the designations of the insulin analogues mentioned makes reference to the amino acid sequences of the A chain and B chain of human insulin, and all deviations (additions, substitutions, deletions) therefrom are indicated in a given designation of an insulin analogue.

The invention further relates to a process for preparing an insulin analogue as described above, where a precursor of the insulin analogue is prepared recombinantly, the precursor is processed enzymatically to two-chain insulin, and a coupling with argininamide is carried out in the presence of an enzyme having trypsin activity, and the insulin analogue is isolated.

The invention further relates to the use of an insulin analogue as described above for the manufacture of a medicament for the treatment of diabetes mellitus, in particular of diabetes mellitus of type I or type II.
The invention further relates to a pharmaceutical comprising an insulin analogue as described above and/or physiologically acceptable salts thereof.

The invention further relates to a pharmaceutical comprising an insulin analogue as described above which is employed therapeutically for cartilage regeneration.
The invention further relates to a pharmaceutical comprising an insulin analogue as described above which is employed therapeutically for assisting beta cell regeneration.

The invention further relates to a formulation of the insulin analogue as described above, where the formulation is in aqueous form comprising the dissolved insulin analogue.

The invention further relates to a formulation of the insulin analogue as described above, where the formulation is in the form of powder, in particular in crystalline and/or amorphous form.

The invention further relates to a formulation of the insulin analogue as described above, where the formulation is in the form of a suspension.

The invention further relates to a formulation of the insulin analogue as described above, where the formulation additionally comprises a chemical chaperone.

The invention further relates to a DNA coding for a precursor of an insulin analogue as described above.

The invention further relates to a DNA coding for the A chain of an insulin analogue as described above.

The invention further relates to a DNA coding for the B chain of an insulin analogue as described above.

The invention further relates to a vector comprising a DNA as described above.

The invention further relates to a host organism comprising a DNA as described above or a vector as described above.

The invention further relates to a preproinsulin analogue, wherein the C peptide carries the amino acid residue arginine at its N terminus and its C terminus is characterized by the form Arg Arg, Arg Lys or Lys Arg Arg.

The invention further relates to a formulation as described above which additionally comprises also a glucagon-like peptide-1 (GLP1) or an analogue or derivative thereof, or exendin-3 or -4 or an analogue or derivative thereof, preferably exendin-4.

The invention further relates to a formulation as described above in which an analogue of exendin-4 is selected from a group comprising
H-desPro^{36}-exendin-4-Lys$_6$-NH$_2$,
H-des(Pro^{36,37})-exendin-4-Lys$_4$-NH$_2$ and
H-des(Pro^{36,37})-exendin-4-Lys$_5$-NH$_2$,
or a pharmacologically tolerable salt thereof.

The invention further relates to a formulation as described above in which an analogue of exendin-4 is selected from a group comprising
desPro^{36} [Asp^{28}]exendin-4 (1-39),
desPro^{36} [IsoAsp^{28}]exendin-4 (1-39),
desPro^{36} [Met(O)^{14}, Asp^{28}]exendin-4 (1-39),
desPro^{36} [Met(O)^{14}, IsoAsp^{28}]exendin-4 (1-39),
desPro^{36} [Trp(O$_2$)^{25}, Asp^{28}]exendin-2 (1-39),
desPro^{36} [Trp(O$_2$)^{25}, IsoAsp^{28}]exendin-2 (1-39),
desPro^{36} [Met(O)^{14}Trp(O$_2$)^{25}, Asp^{28}]exendin-4 (1-39) and
desPro^{36} [Met(O)^{14}Trp(O$_2$)^{25}, IsoAsp^{28}]exendin-4 (1-39),
or a pharmacologically tolerable salt thereof.

The invention further relates to a formulation as described in the preceding paragraph, in which the peptide -Lys$_6$-NH$_2$ is attached to the C termini of the analogues of exendin-4.

The invention further relates to a formulation as described above in which an analogue of exendin-4 is selected from a group comprising
H-(Lys)$_6$- des Pro^{36} [Asp^{28}]exendin-4(1-39)-Lys$_6$-NH$_2$

des $Asp^{28}Pro^{36}$, Pro^{37}, Pro_{38} exendin-4(1-39) -NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39) -NH_2,
H-Asn-$(Glu)_5$ des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39) -NH_2,
des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-Asn-$(Glu)_5$- des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,

H-$(Lys)_6$- des Pro^{36} [$Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-Lys_6-NH_2,
H- des Asp^{28} Pro^{36}, Pro^{37}, Pro^{38} [$Trp(O_2)^{25}$]exendin-4(1-39) -NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [$Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39) -NH_2,
H-Asn-$(Glu)_5$- des Pro^{36}, Pro^{37}, Pro^{38} [$Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39) -NH_2,
des Pro^{36}, Pro^{37}, Pro^{38} [$Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [$Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-Asn-$(Glu)_5$- des Pro^{36}, Pro^{37}, Pro^{38} [$Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-$(Lys)_6$- des Pro^{36} [$Met(O)^{14}$, Asp^{28}]exendin-4(1-39)-Lys_6-NH_2,
des $Met(O)^{14}$ Asp^{28} Pro ^{36}, Pro^{37}, Pro^{38} exendin-4(1-39) -NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro ^{37}, Pro^{38} [$Met(O)^{14}$, Asp^{28}]exendin-4(1-39) -NH_2,
H-Asn-$(Glu)_5$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, Asp^{28}] exendin-4(1-39) -NH_2,
des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, Asp^{28}]exendin-4(1-39)-Lys_6-NH_2,
H-Asn-$(Glu)_5$ des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, Asp^{28}] exendin-4(1-39)-$(Lys)_6$-NH_2,

H-$(Lys)_6$- des Pro^{36} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-Lys_6-NH_2,
des Asp^{28} Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$]exendin-4(1-39) -NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39) -NH_2,
H-Asn-$(Glu)_5$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, Asp^{28}] exendin-4(1-39) -NH_2,
des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-Asn-$(Glu)_5$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}] exendin-4(1-39)-$(Lys)_6$-NH_2,
or a pharmacologically tolerable salt thereof.

The invention further relates to a formulation as described above which additionally

comprises Arg^{34}, Lys^{26} (N^{ε}(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37) [liraglutide] or a pharmacologically tolerable salt thereof.

It is clear to a skilled worker in this connection that the insulins of the invention may be item of a pharmaceutical formulation which has an advantageous effect after administration. Aqueous solutions are the starting point in this connection. Further components must accordingly be miscible. The risk of viral animal contamination is minimized in that the preparation ought not to comprise any components derived from animal sources. It is further advantageous to prevent microbial contamination by adding preservatives. It is possible by adding isotonic agents to compensate for a possible negative effect of the formulation on the physiology of the tissue cells at the administration site. The addition of protamine may have a stabilizing effect, so that substantially salt-free insulin preparation can be obtained by adding protamine to the formulation. Addition of a phenolic component may lead to stabilization of the structure of the insulin analogue used and thus additionally bring about inter alia the delaying effect on the onset of action. It is also possible to add to the formulation substances which stabilize the spatial structure of the slow insulins of the invention and lead to better thermal stability. Such chemical chaperones may be for example short synthetic peptides, which may also comprise amino acid analogues or include for example peptide sequences derived from the C peptide of insulin.

The insulins of the invention can be incorporated into nanoparticles for developing depot forms. Also conceivable are so-called slow release formulations in which the slow insulin of the invention is present reversibly bound to a polymer carrier.

The insulins of the invention can be administered in parallel with fast-acting insulin such as Apidra®, NovoRapid®, Humalog ® or insulin derivatives undergoing development or formulations with an appropriate time/action profile or inhalable insulin or nasally or orally administered insulins which are undergoing development. It will be clear to a skilled worker in this connection that appropriately formulated mixtures of fast-acting and slow insulin of the invention can also be used for this purpose. The insulin analogues of the invention can further be used in pharmaceutical preparations which comprise peptides which are described by an activity comparable to GLP-1 (glucagon like Peptide-1) or exendin-4 or exendin-3.

GLP-1 (7-37), exenatide (Byetta®) or peptides whose preparation is described in the patent applications WO 2006/058620, WO 2001/04156, WO 2004/005342 and WO 98/08871 represent examples of such peptides. Formulations particularly advantageous in this connection are those comprising a depot formulation of these peptides. Types of therapy advantageous especially in the initial phase of type II diabetes are those which provide in parallel with the administration of the pharmaceuticals of the invention, which increase the effect of insulin, such as, for example, metformin. Combination therapies with dipeptidyl peptidase-4 inhibitors which increase the level of incretins are, like combinations with sulfonylureas which increase insulin secretion in the pancreas, likewise possible. The slow insulins of the invention can be employed particularly advantageously when regeneration of beta cells from appropriate stem cells is initiated by administration of differentiation factors. All these applications are mentioned by way of example for the therapy of diabetes, and the invention likewise relates thereto. The invention thus further relates to the use of the insulins of the invention in combination with other active ingredients for the treatment of diabetes, especially diabetes of type I or type II diabetes.

The invention also relates further to the use of the insulins of the invention in regeneration processes which relate to the skeleton, such as, for example, cartilage regeneration. It is important in this use to make controlled use of the mitogenic potential of insulins without at the same time eliciting a strong metabolic reaction.

The invention further relates to a pharmaceutical which comprises an analogue of the invention which represents in particular an aqueous formulation or a powder.

The pharmaceutical is a pharmaceutical preparation which is preferably a solution or suspension for injection purposes; it is characterized by a content of at least one insulin analogue of the invention, and/or at least one of the physiologically tolerated salts thereof in dissolved, amorphous and/or crystalline – preferably in dissolved - form.

The preparation preferably has a pH of between about 2.5 and 8.5, in particular between 4.0 and 8.5, comprises a suitable tonicity agent, a suitable preservative and, where appropriate, a suitable buffer, and preferably also a particular zinc ion

concentration, in sterile aqueous solution. The totality of the preparation ingredients apart from the active ingredient forms the preparation carrier. Suitable tonicity agents are for example glycerol, glucose, mannitol, NaCl, calcium or magnesium compounds such as $CaCl_2$ etc. The solubility of the insulins of the invention or the physiologically tolerated salts thereof at weakly acidic pH values is influenced by the choice of the tonicity agent and/or preservative.

Examples of suitable preservatives are phenol, m-cresol, benzyl alcohol and/or p-hydroxybenzoic esters.

Buffer substances which can be used in particular for adjusting a pH between about 4.0 and 8.5 are for example sodium acetate, sodium citrate, sodium phosphate etc. Otherwise, physiologically acceptable dilute acids (typically HCl) or alkalis (typically NaOH) are also suitable for adjusting the pH.

If the preparation has a zinc content, preference is given to one of from 1 µg/ml to 2 mg/ml, in particular from 1 µg/ml to 200 µg zinc/ml. The action profile of the insulin analogues of the invention can surprisingly be influenced satisfactorily by addition of zinc. This allows the production of preparations which differ in relation to the total duration of action, the speed of onset of action and the profile of the effect curve, thus allowing an individual stabilization of the patient.

For the purpose of varying the active ingredient profile of the preparation of the invention it is also possible to admix unmodified insulin, preferably bovine, porcine or human insulin, especially human insulin, or insulin analogues and derivatives thereof. It is likewise possible to admix one or more exendin-4 derivatives or peptides which are characterized by an activity comparable to GLP-1 (glucagon like peptide-1). The invention likewise relates to such pharmaceuticals (preparations).

Preferred active ingredient concentrations are those corresponding to about 1-1500, more preferably about 5-1000 and in particular about 40-400 international units/ml.

The insulin analogues of the invention are initially prepared biotechnologically as precursor which does not yet include the amide. The skilled worker is familiar with a

large number of possibilities for preparing insulins. Host cell systems used in this connection are bacteria, yeasts and higher plants or plant cells for cultivation by fermentation. If cost considerations permit, expression systems which use animal cells as host system are also conceivable. However, the precondition therefor is reliable freedom from animal viruses. It is thus clear that the expression systems described by way of example represent only a small segment of the host/vector systems developed for the recombinant preparation of proteins. For example, biotechnological processes based on yeast or plant systems such as mosses, algae or higher plants such as tobacco, pea, safflower, barley, corn or oilseed rape are not described in the application. Nevertheless, the invention likewise includes host/vector systems and coding DNA sequences which allow the target peptides to be prepared in appropriate biotechnological expression systems. Host organisms can thus be selected in particular from the plant kingdom from organisms of the first division Schizophyta comprising Schizomycetes, bacteria or blue algae, organisms of the 2nd division Phycophyta class V Chlorophyceae, organisms of the 2nd division Phycophyta class VII Rhodophyceae, organisms of the 3rd division Mycophyta, organisms of the 5th division Bryophyta and organisms of the 7th division Spermatophyta.

European patent application EP-A 1 222 207 describes a plasmid pINT358d which codes for a preproinsulin which includes a modified C peptide. It is now possible with the aid of the polymerase chain reaction (PCR) to modify the proinsulin-encoding sequence specifically so that it is possible to express preproinsulins which can serve as precursors of the insulins of the invention. Corresponding fusion proteins need not necessarily be prepared intracellularly. It is clear to the skilled worker that such proteins can also be prepared by bacterial expression with subsequent secretion into the periplasm and/or into the culture supernatant. European patent application EP-A 1 364 029 describes this by way of example. The invention likewise relates to the proinsulin precursors which lead to the analogues of the invention.

The proinsulins prepared in this way can in principle be converted into an insulin analogue precursor which includes lysine or arginine in position A0 and carries lysine or arginine at the C-terminal end of the B chain. It is alternatively possible to add a positively charged amino acid semisynthetically to the N terminus of the A chain if

lysine or arginine are not already present.

If the proinsulins of the invention are in the form of inclusion bodies or soluble form after intracellular expression in bacteria, these precursors must be folded by in vitro folding into the correct conformation before the processing and biochemical modification can be undertaken. In this connection, the described fusion protein allows direct folding after denaturation by means of urea or guanidinium hydrochloride, and the invention likewise relates to folding intermediates.

Biochemical methods are used to concentrate the individual intermediates, especially separation processes whose underlying principles are published and in fact the subject of textbooks. It is clear to the skilled worker that such principles can consequently be combined and thus may lead to processes which have not previously been published in their sequence. The invention thus likewise relates to processes which lead to purification of the analogues of the invention.

The invention further relates to a process for preparing the insulin analogues of the invention, where a precursor of the insulin analogue is prepared recombinantly and converted enzymatically into a two-chain insulin precursor which carries arginine or lysine N-terminally in relation to amino acid 1 of the A chain, and has at the C-terminal end of the B chain a lysine or arginine residue which is converted with argininamide or lysinamide in the presence of an enzyme having trypsin activity into the amide and thus into the slow insulin of the invention, and is prepared with high purity by a biochemical purification process.

Proteins which differ through substitution of at least one naturally occurring amino acid residue by other amino acid residues and/or addition and/or deletion of at least one amino acid residue from the corresponding, otherwise identical naturally occurring protein are referred to as "analogues" of proteins. It is also possible in this connection for the added and/or replaced amino acid residues to be ones which do not occur naturally.

Proteins which are obtained by chemical modification of certain amino acid residues of initial proteins are referred to as "derivatives" of proteins. The chemical

modification may consist for example of addition of one or more particular chemical groups to one or more amino acids.

Key to figures:

Fig. 1: Blood glucose-lowering effect of insulin analogues of the invention in rats

Fig. 2 Blood glucose-lowering effect of insulin glargine in rats

The following examples are intended to illustrate the concept of the invention without having a restrictive effect in this connection.

Example 1: Preparation of the vector derivative pINT3580 which codes for Gly (A21)-insulin and a modified C peptide which carries Arg Arg at the C/A chain boundary.

European patent application EP-A 1 222 207 describes the plasmids pINT358d, pINT91d and the primer sequence Tir. DNA of these products is used to construct the plasmid pINT3580. The plasmid pINT358d is moreover characterized by a gene sequence which codes for a modified C peptide having particular properties. Three primer sequences are synthesized:

pint3580_glya21rev

5′- CAAAGGTCGACTATTA**GCC**GCAGTAGTTCTCCAGCTGG-3′ (SEQ ID NO: 3)

This primer serves after working up to introduce glycine (bold print, underlined) instead of asparagin in position 21 of the A chain of the proinsulin sequence encoded by pINT358d.

arg_cjuncf

5′-GTCCCTGCAG**CGT**CGCGGCATCGTGGAGCAG-3′ (SEQ ID NO: 4)

This primer serves like the primer arg_cjunc_rev for introducing arginine instead of lysine at the insulin A/B chain boundary.

arg_cjunc_rev

5′- CCACGATGCC GCG**ACG**CTGC AGGGACCCCT CCAGCG-3′ (SEQ ID NO: 5)

The codon for the arginine to be introduced is in bold print in both primers.
A PCR is carried out in accordance with the European patent application EP-A 1 222 207 with each of the primer pairs Tir / arg_cjunc_rev and arg_cjuncf / pint3580_glya21rev and with DNA of the plasmid pINT358d as template. Aliquots of the products of the two reactions are combined and employed together with the primer pair Tir / pint3580_glya21rev in a third PCR. The product of this reaction is purified after fractionation of the reaction mixture by gel electrophoresis and is digested with the restriction enzymes Sal1 / Nco1 in accordance with the manufacturer's instructions in one and the same reaction, the reaction mixture is fractionated by gel electrophoresis, and the DNA fragment encoding the proinsulin sequence is isolated. The fragment is then inserted by a DNA ligase reaction into the Nco1 / Sal1-opened pINT91d vector DNA.

The ligation mixture is used to transform competent E. coli bacterial cells. The transformation mixture is taken out on selection plates which contain 25 mg/l ampicillin. Plasmid DNA is isolated from colonies and characterized by DNA sequence analysis. Correct plasmids are called pINT3580.

Example 2: Construction of the plasmid pINT3581 coding for His (A8), Gly (A21)-preproinsulin

The construction takes place as described in example 1 by 3 polymerase chain reactions. The product of the third reaction is inserted after Nco1 / Sal1 cleavage into the Nco1 / Sal1-opened pINT91d vector DNA. The primers Tir and pint3580_glya21rev are used. Two further primers are synthesized:

pint3580_Ha8f

5'-AGCAGTGCTGC**CAC**AGCATCTGCTCCCTCTAC-3' (SEQ ID NO: 6)

pint3580_Ha8rev

5'-GAG CAGATGCT **GTG** GCAGCACTG CTCCACGATG-3' (SEQ ID NO: 7)

The codon which codes for histidine in position 8 of the A chain is emphasized by emboldening in each case. The construction is carried out as described in example 1. Template for PCR1 and 2 is DNA of the plasmid pINT3580. PCR1 is carried out with the primer pair Tir/ pint3580_Ha8rev and PCR2 is carried out with the primer pair pint3580_Ha8f/ pint3580_glya21rev. With the primer pair Tir / pint3580_glya21rev is employed in PCR 3. Template in this case is a mixture of the reaction products of PCR1 and PCR2. Correct plasmids are called pINT3581.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202, which is characterized as Arg (A0), His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin, and which results after amidation with argininamide.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202b, which is characterized as Arg (A0), His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-human insulin, and which results after amidation with lysinamide.

Example 3: Construction of the plasmid pINT3582 coding for His (A8), Glu (A5), Gly (A21) – preproinsulin

The construction takes place as described in example 1 and 2 by 3 polymerase chain reactions. The product of the third reaction is inserted after Nco1/ Sal1 cleavage into the Nco1 / Sal1-opened pINT91d vector DNA. The primers Tir and pint3580_glya21rev are used. Two further primers are synthesized.

pint3581_Ea5f

5´GCATCGTGGAG**GAG**TGCTGCCACAGCATCTG 3´ (SEQ ID NO: 8)

pint3581_Ea5rev

5'-CTGT GGCAGCA**CTC** CTCCACGATG CCGCGACG-3' (SEQ ID NO: 9)

The codon which codes for glutamic acid in position 5 of the A chain is emphasized by emboldening in each case. The construction is carried out as described in example 1. Template is DNA of the plasmid pINT3581. Correct plasmids are called pINT3582.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-1, which is characterized as Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin, and which results after amidation with argininamide.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-1b, which is characterized as Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-human insulin, and which results after amidation with lysinamide.

Example 4: Construction of the pl;asmid pINT3583 coding for His (A8), Asp (A18), Gly(A21) – preproinsulin

The construction differs from example 1 by taking place by only one polymerase chain reaction. The product of this reaction is inserted after Nco1 / Sal1 cleavage into the Nco1 / Sal1-opened pINT91d vector DNA. The primer Tir is used. One further primer is synthesized:

pint3580_Da18rev

5′ CAAAGGTCGACTATTAGCCGCAGTA**GTC**CTCCAGCTGGTAGAGGGAG 3′ (SEQ ID NO: 10)

The codon which codes for aspartic acid in position 18 of the A chain is emphasized by emboldening. Template is DNA of the plasmd pINT3581. Correct plasmids are called pINT3583.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202, which is characterized as Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin, and which results after amidation with argininamide.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-2b, which is characterized as Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-human insulin, and which results after amidation with lysinamide.

Example 5: Construction of the plasmid pINT3585 coding for His (A8), Glu (A15), Gly (A21) – preproinsulin

The construction differs from example 1 by taking place by only one polymerase chain reaction. The product of this reaction is inserted after Nco1/ Sal1 cleavage into the Nco1 / Sal1-opened pINT91d vector DNA. The primer Tir is used. One further primer is synthesized:

pint3580_Ea15rev

5´- CAAAGGTCGA CTATTAGCCG CAGTAGTTCTCCAG**CTC**GTA GAGGGAGCAG ATGCTG -3´ (SEQ ID NO: 11)

The codon which codes for glutamic acid in position 15 of the A chain is emphasized by emboldening. Template is DNA of the plasmid pINT3581. Correct plasmids are called pINT3585.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-3, which is characterized as Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin, and which results after amidation with argininamide.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-3b, which is characterized as Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-human insulin, and which results after amidation with lysinamide.

Example 6: Construction of the plasmid pINT3588 coding for His (A8), Gly (A21), Asp (B3)- preproinsulin

Construction takes place as described in example 1 and 2 by 3 polymerase chain reactions. The product of the third reaction is inserted after Nco1/ Sal1 cleavage into the Nco1 / Sal1-opened pINT91d vector DNA. The primers Tir and pint3580_glya21rev are used. Two further primers are synthesized:

pint3581_Db3f

5´- GCACGATTTGTG**GAC**CAGCACCTGTGCGGC -3´ (SEQ ID NO: 12)

pint3581_Db3rev

5´- CACAGG TGCTG**GTC**CA CAAATCGTGC CGAATTTC -3´ (SEQ ID NO: 13)

The codon which codes for asparagine in position 3 of the insulin B chain is emphasized by emboldening in each case. Construction is carried out as described in example 1. Template is DNA of the plasmid pINT3581. Correct plasmids are called pINT3588.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-4, which is characterized as Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B31), Arg (B32)-NH_2-human insulin, and which results after amidation with argininamide.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-4b, which is characterized as Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B31), Lys (B32)-NH_2-human insulin, and which results after amidation with lysinamide.

Example 7: Construction of the plasmid pINT3593 coding for His (A8), Gly (A21), Glu (B4) preproinsulin

Construction takes place as described in example 1 and 2 by 3 polymerase chain reactions. The product of the third reaction is inserted after Nco1/ Sal1 cleavage into

the Nco1 / Sal1-opened pINT91d vector DNA. The primers Tir and pint3580_glya21rev are used. Two further primers are synthesized:

pint3581_Eb4f

5′- ACGATTTGTGAAC**GAG**CACCTGTGCGGCTC -3′ (SEQ ID NO: 14)

pint3581_Eb4rev

5′- CGCACAGG TG**CTC**GTTCA CAAATCGTGC CGAATTTC -3′ (SEQ ID NO: 15)

The codon which codes for glutamine in position 4 of the insulin B chain is emphasized by emboldening. The construction is carried out as described in example 1. Template is DNA of the plasmid pINT3581. Correct plasmids are called pINT3593.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-5, which is characterized as Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B31), Arg (B32)-NH_2-human insulin, and which results after amidation with argininamide.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-5b, which is characterized as Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B31), Lys (B32)-NH_2-human insulin, and which results after amidation with lysinamide.

Example 8: Construction of the plasmid pINT3597 coding for His (A8), Gly (A21), Glu (B0) - preproinsulin

Construction takes place by 2 polymerase chain reactions. The primer pint3580_glya21rev is used. Two further primers are synthesized:

pint3581_Eb0f1

5′- CAA**C**AGGAA ATTCGGCACG A**GAG**TTTGTG AACCAGCACC TGTG-3' (SEQ ID NO: 16)

pint3581_Eb01f2

5´- TATCGA CCAT GG CAACAACA TCAA**C**AGGAA ATTCGGCACG A**GAG**-3'
(SEQ ID NO: 17)

There is partial overlap of the two primers in this case. Pint3581_Eb0f2 contains an NcoI recognition sequence. This is depicted underlined. The codon which codes for glutamic acid in position 0 at the start of the B chain is emphasized by emboldening in each case. Template for PCR1 is DNA of the plasmid pINT3581.

PCR1 is carried out with the primer pair pint3581_Eb-1f2 / pint3580_glya21rev. Template for PCR2 is the product from PCR1. PCR2 is carried out with the primer pair pint3581_Eb-1f2 / pint3580_glya21rev. The product from PCR2 covers the complete preproinsulin sequence. The product of the second reaction is inserted after Nco1/ Sal1 cleavage into the Nco1 / Sal1-opened pINT91d vector DNA. Correct plasmids are called pINT3597.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-6, which is characterized as Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B31), Arg (B32)-NH_2-human insulin, and which results after amidation with argininamide.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-6b, which is characterized as Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B31), Lys (B32)-NH_2-human insulin, and which results after amidation with lysinamide.

Replacement of the codon for glutamic acid in position B0 by the codon of aspartic acid and following the example results in plasmids which have aspartic acid instead of glutamic acid in position B0.

Example 9: Construction of the plasmid pINT3700 coding for His (A8), Gly (A21), Lys (B0)-preproinsulin

The construction serves to introduce the codon for lysine instead of the codon for

arginine at the boundary between presequence and start of the B chain. Construction takes place by two polymerase chain reactions. The primer pint3580_glya21rev is used. Two further primers are synthsized:

pint3581_Eb0f1

5´- CAA**C**AGGAA ATTCGGCA **AAG**TTTGTG AACCAGCACC TGTG – 3' (SEQ ID NO: 18)

pint3581_Eb01f2

5´- TATCGA CCAT GG CAACAACA TCAA**C**AGGAA ATTCGGCA**GAG** – 3' (SEQ ID NO: 19)

There is partial overlap of the two primers in this case, and pint3581_Eb0f2 contains an NcoI recognition sequence. This is depicted underlined. The codon which codes for glutamic acid in position 0 at the start of the B chain is emphasized by emboldening in each case. Template for PCR1 is DNA of the plasmid pINT3581. PCR1 is carried out with the primer pair pint3581_Eb-1f2 / pint3580_glya21rev. Template for PCR2 is the product from PCR1. PCR2 is carried out with the primer pair pint3581_Eb-1f2 / pint3580_glya21rev. The product from PCR2 covers the complete preproinsulin sequence. The product of the second reaction is inserted after Nco1/ Sal1 cleavage into the Nco1 / Sal1-opened pINT91d vector DNA. Correct plasmids are called pINT3700.

The preproinsulin encoded by the plasmid is precursor for the compound Arg (A0), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin. The compound is obtained by initially preparing the intermediate Arg (A0), His (A8), Gly (A21) of the Thr (B30)-human insulin by reaction with trypsin and lysyl endopeptidase C, which intermediate is subsequently converted by amidation with argininamide into the desired compound YKL202-7, which is characterized as Arg (A0), His(A8), Gly(A21), Arg (B30)-NH_2-human insulin.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-7b,

which is characterized as Arg (A0), His (A8), Gly (A21), Lys (B30)-NH_2-human insulin, and which results after amidation with lysinamide.

Example 10: Construction of the plasmid pINT3701 coding for Gly (A21) Lys (B0)-preproinsulin

The construction serves to introduce the codon for lysine instead of the codon for arginine at the boundary between presequence and start of the B chain. Following the construction strategy from Example 9 but using DNA of the plasmid pINT358d as template for PCR1 results in the plasmid pINT3701 which, like the original plasmid pINT358d, is characterized by the boundary between C peptide and A chain being formed by the amino acid sequence Lys-Arg.

Example 11: Construction of the plasmid pINT3702 coding for His (A8), Gly (A21), Lys (B0)-preproinsulin with the C peptide according to Example 10

Following the construction plan described in Example 2 but using DNA of the plasmid pINT3701 as template results in the plasmid pINT3702.

The preproinsulin encoded by the plasmid is precursor for the compound Arg (A0), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin. The compound is obtained by initially preparing the intermediate Arg (A0), His (A8), Gly (A21) of the Thr (B30)-human insulin by reaction only with lysyl endopeptidase C, which intermediate is subsequently converted by amidation with argininamide into the desired compound YKL202-8, which is characterized as Arg (A0), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin.

The preproinsulin encoded by the plasmid is precursor for the compound YKL202-8b, which is characterized as Arg (A0), His (A8), Gly (A21), Lys (B30)-NH_2-human insulin, and which results after amidation with lysinamide.

Example 12: Preparation of the vector derivative pINT3703 which codes for His (A8), Gly (A21), Lys (B0)-preproinsulin and a modified C peptide which carries the tripeptide Lys-Arg-Arg at the C/A chain boundary.

DNA of the plasmid pINT3702 is used as template, and the primers pint3580_glya21rev and Tir are used. Two primer sequences are synthesized.

3702_arg_cjuncf

5′ - TGC AGAAGCGC**AGA**GGCATCGTG GAGCAGTGC – 3' (SEQ ID NO: 20)

This primer serves like the primer arg_cjunc_rev for introducing arginine at the insulin A/B chain boundary.

3702_cjunc_rev

5′ - TCC ACGATGCC**TCT**GCGCTTCTG CAGGGACCC – 3' (SEQ ID NO: 21)

The codon for the arginine to be introduced is in bold print in both primers. A PCR is carried out in accordance with the European Patent Application EP-A 1 222 207 with each of the primer pairs Tir/3702_cjunc_rev and 3702_arg_cjuncf/pint3580_glya21rev and with DNA of the plasmid pINT3702 as template. Aliquots of the products of the two reactions are combined and employed together with the primer pair Tir / pint3580_glya21rev in a third PCR. The product of this reaction is purified after fractionation of the reaction mixture by gel electrophoresis and is digested with the restriction enzymes Sal1 / Nco1 in accordance with the manufacturer's instructions in one and the same reaction, the reaction mixture is fractionated by gel electrophoresis, and the DNA fragment encoding the proinsulin sequence is isolated. The fragment is then inserted by a DNA ligase reaction into the Nco1 / Sal1-opened pINT91d vector DNA.

The ligation mixture is used to transform competent E. coli bacterial cells. The transformation mixture is plated out on selection plates which contain 25 mg/l ampicillin. Plasmid DNA is isolated from colonies and characterized by DNA sequence analysis. Correct plasmids are called pINT3702.

The preproinsulin encoded by the plasmid is precursor for the compound Arg (A-1),

Arg (A0), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin. The compound is obtained by initially preparing the intermediate Arg (A-1), Arg (A0), His (A8), Gly (A21) of the Thr (B30)-human insulin by reaction with lysyl endopeptidase C, and the intermediate is subsequently converted by amidation with argininamide into the desired compound.

The preproinsulin encoded by the plasmid is precursor for the compound Arg (A-1), Arg (A0), His (A8), Gly (A21), Lys (B30)-NH_2-human insulin, which results after amidation with lysinamide.

It is clear to the skilled worker that aspartic acid or glutamic acid can be introduced into the appropriate positions of the A or B chain in accordance with Examples 3-8. These proinsulins are precursor for the compounds

YKL202-10a	Arg (A-1), Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin
YKL202-10b	Arg (A-1), Arg (A0), Glu (A5), His (A8), Gly (A21), Lys (B30)-NH_2-human insulin
YKL202-11a	Arg (A-1), Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin
YKL202-11b	Arg (A-1), Arg (A0), Glu (A15), His (A8), Gly (A21), Lys (B30)-NH_2-human insulin
YKL202-12a	Arg (A-1), Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin
YKL202-12b	Arg (A-1), Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin
YKL202-13a	Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B30)-NH_2-human insulin
YKL202-13b	Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B0), Lys (B30)-NH_2-human insulin
YKL202-14a	Arg (A-1), Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B30)-NH_2-human insulin
YKL202-14b	Arg (A-1), Arg (A0), His (A8), Gly (A21), Asp (B3),

	Lys (B30)-NH_2-human insulin
YKL202-15a	Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B30)-NH_2-human insulin
YKL202-15b	Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B4), Lys (B30)-NH_2-human insulin

Example 13: Preparation of the vector derivative pINT3704 which codes for His (A8), Gly (A21), Lys (B0)-preproinsulin

This example describes the preparation of the vector derivative pINT3704 which codes for His (A8), Gly (A21), Lys (B0)-preproinsulin, and a modified C peptide which carries the tripeptide Lys-Arg-Arg at the C/A chain boundary and in which the amino acid glycine which occurs in position A21 in human insulin is deleted.

Following the synthesis scheme described in Example 12 and replacing the primers 3702_arg_cjuncf and 3702_arg_cjuncfrev by the primers 3703_Δ Ga1f and 3703_Δ Ga1rev and using DNA of the plasmid pINT3703 as template results in the plasmid pINT3704 wherein the amino acid glycine in position 1 of the A chain is deleted in the encoded preproinsulin, and the remaining course of the sequence corresponds to that of the sequence encoded by pINT3703.

3703_Δ Ga1f

5' - TGC AGAAGCGC**AGA**ATCGTG GAGCAGTGCTGC – 3' (SEQ ID NO: 22)

3703_Δ Ga1rev

5´ - TGCTCC ACGAT**TCT**GCGCTTCTG CAGGGACCC - 3´ (SEQ ID NO: 23)

The preproinsulin encoded by the plasmid is precursor for the compound Arg (A0), Arg (A1), His (A8), Gly (A21), Arg (B30)-NH_2-human insulin. The compound is obtained by initially preparing the intermediate Arg (A0), Arg (A1), His (A8), Gly (A21), des Thr (B30)-human insulin by reaction with lysyl endopeptidase C, and the intermediate is then converted by amidation with argininamide into the desired

compound.

The preproinsulin encoded by the plasmid is precursor for the compound Arg (A0), Arg (A1), His (A8), Gly (A21), Lys (B30)-NH_2-human insulin which results after amidation with lysinamide.

It is clear to the skilled worker that negatively charged amino acids can be introduced into the preproinsulin sequence in accordance with Example 12. It is also possible in analogy to Example 13 to prepare from the plasmid pINT3702 a plasmid that codes for a proinsulin sequence which permits the preparation of insulin derivatives which carry an arginine as N-terminal amino acid in position 1 of the A chain.

Example 14: Expression of the proinsulin derivatives

The expression is carried out in accordance with example 1 of European patent application EP-A 1 222 207.

Example 15: Folding of the proinsulin derivatives

The folding takes place in principle by the method described in EP-A 0 668 282

Example 16: Processing of the proinsulins from Examples 2-8 with trypsin

The folded preproinsulin is enzymatically processed to give the two-chain Arg (A0)-insulin precursor whose C-terminal B chain end is characterized by lysine or arginine. The enzymatic processing of the folded preproinsulin precursor takes place as described for example in example 4 of WO91/03550. It proves to be particularly advantageous in this case to employ the trypsin variant described in WO 2007/031187 A1.

Example 17: Processing of the proinsulins from Examples 9-13 with endoproteinase Lys-C

Endoprotease Lys-C from Achromobacter lyticus is commercially available

(Merck/Calbiochem). The reaction is carried out with slight modification as described by Jekel, P.A. et al. [Anal. Biochem. 134, 347-354, (1983)]. A pH of 9.5 is set, and the reaction temperature is 30°C.

In the one-pot reaction, the presequence is eliminated and the C peptide starting with Thr (B30) is cleaved out, so that the C-terminal end of the B chain is formed by lysine which is present as reactive site for the enzyme-catalysed coupling reaction.

Example 18: Preparation of the Arg-NH_2 or Lys-NH_2 insulin compound from the two-chain precursor by coupling with arginin- or lysinamide

Irrespective of the positioning of the additional acidic amino acids, a standard reaction is carried out as follows: 100 mg of Arg (A0), Gly (A21), Arg (B31) – insulin analogue are dissolved in 0.95 ml of argininamide solution (446 g/L), and 0.13 mL of M Na acetate buffer (pH 5.8) and 2 ml of DMF are added. The reaction mixture is cooled to 12°C and started by adding 0.094 ml of trypsin (0.075 mg, Roche Diagnostics). The reaction is stopped after 8 h by adding TFA to pH 2.5 and analyzed by HPLC. There is formation of >60% Arg (A0), Gly (A21), Arg (B31), Arg (B32)-NH_2-insulin analogue. Addition of trypsin inhibitor solution is followed by purification of the amidated analogue in analogy to US 5,656,722.

Preparation of the corresponding lysinamide compound takes place analogously. However, an aqueous lysinamide stock solution containing 366g/L lysinamide in solution forms the starting material.

Example 19: Semisynthetic preparation of Lys (A-1), Arg (A0), His (A8), Gly (A21), Arg (B30)-insulin amide

The compound YKL202-8 described in Example 11, which corresponds to an insulin derivative of the structure Arg (A0), His (A8), Gly (A21), Arg (B30)-insulin amide serves as starting material for preparing the compound YKL202-11, Lys (A-1), Arg (A0), His (A8), Gly (A21), Arg (B30)-insulin amide. 200 mg of the compound are dissolved in 40 ml of a 100 mM $Na_2 HP0_4$ / CH_3CN (50:50) mixture and a pH of 7.7-8.2 is set. As further material, a Boc-Lys (Boc)-NHS ester is prepared by mixing

0.3 mM Boc-Lys(Boc)-OH, 0.4 mM N-hydroxysuccinimide (NHS) and 0.4 mM dicyclohexylcarbodiimide (DCC) in dichloromethane for 40 minutes. The reaction mixture is concentrated to dryness in a rotary vacuum evaporator. The mixture is then taken up in 5 ml of methanol and added to the dissolved initial insulin. The reaction is stirred at room temperature for at least 60, but at most 120, minutes. The reaction is stopped by adding 200 µl of TFA. The mass of the reaction products is characterized by LC-MS mass spectroscopy in order to demonstrate that monoacylated product consisting of three components is present. The components are separated by HPLC. During this, diacylated and triacylated byproducts are also removed. The fractions from the HPLC separation are analysed by mass spectroscopy. The 3 fractions each containing monoacylated product are subjected to an amino acid sequence analysis, interpretion of which allows identification of the HPLC fraction which contains the desired compound, from which the Boc protective groups are subsequently removed by hydrolysis. After renewed HPLC purification, the desired product is tested for its biological properties.

Example 20: Preparation of the plasmid pINT358_ha8 which codes for His (A8), Gly (A21) proinsulin.

Following the preparation scheme from Example 2 and using DNA of the plasmid pINT358d as template for PCR1 and PCR2 results in the plasmid pINT358_ha8 which codes for His (A8), Gly (A21) proinsulin. The proinsulin encoded by the plasmid serves as precursor for the compound of the form His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin. Processing of the preproinsulin to give His (A8), Gly (A21), Arg (B31) as intermediate before amidation takes place as described in WO91/03550 in this case. Conventional trypsin is employed in this case.

Example 21: Formulation of the amidated insulin derivatives

In order to test the insulin derivatives of the invention for their biopharmacological and physicochemical properties, a solution of the compounds was prepared as follows: the insulin derivative of the invention was dissolved with a target concentration of 240 ± 5 µM in 1 mM hydrochloric acid with 80 µg/mL zinc (as zinc

chloride). For this purpose, initially an amount of the freeze-dried material which is about 30% higher than required on the basis of the molecular weight and the desired concentration was weighed out. The concentration present was then determined by analytical HPLC and the solution was subsequently made up to the volume necessary to achieve the target concentration with 5 mM hydrochloric acid with 80 µg/mL zinc. If necessary, the pH was readjusted to 3.5 ± 0.1. After the final analysis by HPLC to verify the target concentration of 240 ± 5 µM, the finished solution was transferred by means of a syringe with a 0.2 µm filter attachment into a sterile vial which was closed with a septum and a crimped cap. No optimization of the formulations, e.g. in relation to addition of isotonic agents, preservatives or buffer substances, was carried out for the short-term single testing of the insulin derivatives of the invention.

Example 22: Preparation of His (A8), Gly (A21), Arg(B31) human insulin by yeast expression

EP-A 1 364 032 describes the expression and secretion of a fusion protein of hirudin and miniproinsulin by yeast. Yeast host organisms which are mentioned as particularly advantageous in this connection are S. cerevisiae, K. lactis, H. polymorpha and P. pastoris. Example 4 of said patent application describes the construction of a plasmid which permits preparation of the fusion protein in P. pastoris and whose DNA serves as template for preparing a miniproinsulin whose amino acid sequence in positions A8, A15, A18 and A21 in the A chain is characterized by the amino acids histidine (A8), glutamic acid (A15), aspartic acid (A18) and glycine (A21). The primer pichia_H_if1 is used. The following primers are newly synthesized and purified after synthesis:

pichia_G21_rev

5′-TTTTTTGGCG CCGAATTCAC TATTA**GCC**AC *AGTAGTTTTC CAGCTGGTA*-3′ (SEQ ID NO: 24)

The codon emphasized by emboldening marks the A21 positions.

pichia_H8_f with the sequence

5´-GAACAATGTTGT**CAT**AGTATCTGTTCTTTG*TACCAGCTGGAAAACTACTG*-3´ (SEQ ID NO: 25)

The codon emphasized by emboldening marks the A8 positions.

pichia-a1-12rev

5´- GAACAGATACT**ATG**ACAACATTGTTCAACGATACC -3´ (SEQ ID NO: 25)

The codon emphasized by emboldening marks the A8 positions.

Equimolar amounts (1 μg) of primer pichia_G21_rev and primer pichia_H8f, which partially overlap, are denatured at 95°C in the presence of a thermophilic polymerase and polymerase buffer which contains the 4 deoxynucleotides dATP, dCTP, dGTP and dTTP 5´, and then incubated at 56°C for 10-15 minutes. The reaction volume of this reaction 1 is 25 μl. In a standard polymerase chain reaction (reaction 2), the primers pichia_H_if1 and pichia-a1-12rev are reacted in parallel with the described template DNA. In a further polymerase chain reaction, the reaction products of reactions 1 and 2 are reacted as template with the primers pichia_H_if1 / pichia_G21rev. The reaction product of this reaction is purified by gel electrophoresis. After reaction with the restriction enzymes XhoI and SacII, the DNA fragment which covers the fusion product is inserted into the described vector pPICZαA in analogy to Example 4. The result is the plasmid pPIC_ins202. Fusion protein encoded by the plasmid is expressed using the commercially available expression kit described. Preparation of the His (A8), Gly (A21), Arg (B31)-human insulin precursor is followed by the coupling of arginine amide in accordance with the description on arginine (B31). The result is the compound His (A8), Gly (A21), Arg (B31), Arg (B32)-NH2-insulin (YKL203) which itself represents a novel slow insulin corresponding to Example 20 and is tested in the rat experiment. Coupling with lysine amide leads analogously to the compound YKL203b which is characterized as His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-insulin.

The compound can, however, also serve as intermediate for preparing the compound Arg (A0), His (A8), Gly (A21), Arg (B31) Arg(B32)-NH_2-insulin whose

preparation is based on Kohn et al. [Peptides 28 (2007) 935-948]. For this purpose, 200 mg of the compound His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin are dissolved in 40 ml of a mixture of 100 mM Na_2HP0_4 / CH_3CN (50:50) and a pH of 7.7-8.2 is set.

As further material, a Boc-Arg-NHS ester is prepared by mixing 0.3 mM Boc-Arg-OH, 0.4 mM N-hydroxysuccinimide (NHS) and 0.4 mM dicyclohexylcarbodiimide (DCC) in dichloromethane for 40 minutes. The reaction mixture is concentrated to dryness in a rotary vacuum evaporator. The mixture is then taken up in 5 ml of methanol and added to the dissolved initial insulin. The reaction is stirred at room temperature for at least 60, but at most 120, minutes. The reaction is stopped by adding 200 μl of TFA. The mass of the reaction products is characterized by LC-MS mass spectroscopy in order to demonstrate that monoacylated product consisting of three components is present. The components are separated by HPLC. During this, diacylated and triacylated byproducts are also removed. The fractions from the HPLC separation are analysed by mass spectroscopy. The three fractions each containing monoacylated product are subjected to an amino acid sequence analysis, interpretion of which allows identification of the HPLC fraction which contains the desired compound, from which the Boc protective groups are subsequently removed by gentle hydrolysis. After renewed HPLC purification, the desired product is tested for its biological properties.

Example 23: Evaluation of the blood glucose-lowering effect of novel insulin analogues in rats

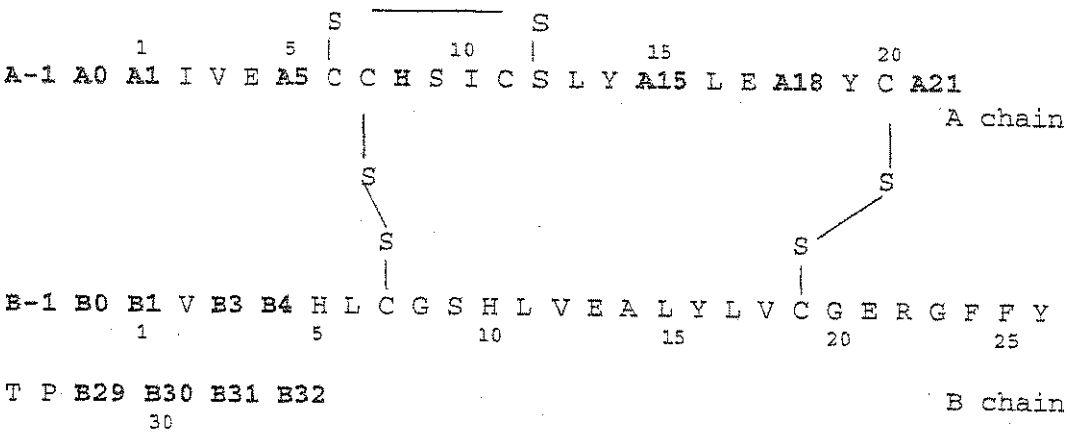
The blood glucose-lowering effect of selected novel insulin analogues is tested in healthy male normal glycemic Wistar rats. Male rats receive subcutaneous injection of a dose of 9 nmol/kg of an insulin analogue. Blood samples are taken from the animals immediately before the injection of the insulin analogue and at regular intervals up to eight hours after the injection, and the blood glucose content therein is determined. The experiment shows clearly (cf. fig. 1) that the insulin analogue of the invention leads to a distinctly delayed onset of action and a longer, uniform duration of action.

Example 24: Evaluation of the blood glucose-lowering effect of novel insulin analogues in dogs

The blood glucose-lowering effect of selected novel insulin analogues is tested in healthy male normoglycemic beagle dogs. Male animals receive subcutaneous injection of a dose of 6 nmol/kg of an insulin analogue. Blood samples are taken from the animals immediately before the injection of the insulin analogue and at regular intervals up to 48 hours after the injection, and the blood glucose content therein is determined. The experiment shows clearly that the insulin analogue of the invention leads to a distinctly delayed shallow onset of action and a longer, uniform duration of action.

Claims:

1. An insulin analogue of the formula I



where

A-1 corresponds to Lys, Arg or an amino group;

A0 corresponds to Lys, Arg or a chemical bond;

A1 corresponds to Arg or Gly;

A5 corresponds to Asp, Glu or Gln;

A15 corresponds to Asp, Glu or Gln;

A18 corresponds to Asp, Glu or Asn;

A21 corresponds to Ala, Ser, Thr or Gly;

B-1 corresponds to Asp, Glu or an amino group;

B0 corresponds to Asp, Glu or a chemical bond;

B1 corresponds to Asp, Glu, Phe or a chemical bond;

B3 corresponds to Asp, Glu or Asn;

B4 corresponds to Asp, Glu or Gln;

B29 corresponds to Arg, Lys or an amino acid selected from the group comprising the amino acids Phe, Ala, Thr, Ser, Val, Leu, Glu or Asp, or a chemical bond;

B30 corresponds to Thr or a chemical bond;

B31 corresponds to Arg, Lys or a chemical bond;

B32 corresponds to Arg-amide or Lys-amide.

where no more than one amino acid residue of the group comprising A5, A15, A18, B-1, B0, B1, B2, B3 and B4 corresond simultaneously and independently of one another to Asp or Glu.

2. The insulin analogue as claimed in claim 1, where A-1 corresponds to Arg.

3. The insulin analogue as claimed in one or more of the preceding claims, where A0 corresponds to Arg.

4. The insulin analogue as claimed in one or more of the preceding claims, where A5 corresponds to Glu.

5. The insulin analogue as claimed in one or more of the preceding claims, where A15 corresponds to Glu.

6. The insulin analogue as claimed in one or more of the preceding claims, where

A18 corresponds to Asp.

7. The insulin analogue as claimed in one or more of the preceding claims, where A21 corresponds to Gly.

8. The insulin analogue as claimed in one or more of the preceding claims, where B0 corresponds to Glu.

9. The insulin analogue as claimed in one or more of the preceding claims, where B3 corresponds to Asp.

10. The insulin analogue as claimed in one or more of the preceding claims, where B4 corresponds to Glu.

11. The insulin analogue as claimed in one or more of the preceding claims, where B30 corresponds to a chemical bond.

12. The insulin analogue as claimed in one or more of the preceding claims, where B31 corresponds to Arg or Lys.

13. The insulin analogue as claimed in one or more of claims 1 to 12, selected from a group comprising:

Arg (A-1), Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), Glu (A5), His (A8), Gly (A21), Lys (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), Glu (A15), His (A8), Gly (A21), Lys (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B31)-NH_2 human insulin,

Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B0), Lys (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Asp (B3), Lys (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B31)-NH_2 human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Glu (B4), Lys (B31)-NH_2 human insulin,
Arg (A0), His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-human insulin,
Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin,
Arg (A0), Glu (A5), His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-human insulin,
Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin,
Arg (A0), Asp (A18), His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-human insulin,
Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin,
Arg (A0), Glu (A15), His (A8), Gly (A21), Arg (B31), Lys (B32)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B31), Arg (B32)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Asp (B3), Arg (B31), Lys (B32)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B31), Arg (B32)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Glu (B4), Arg (B31), Lys (B32)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B31), Arg (B32)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Glu (B0), Arg (B31), Lys (B32)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Arg (B31)-NH_2-human insulin,
Arg (A0), His (A8), Gly (A21), Lys (B31)-NH_2-human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Arg (B31)-NH_2-human insulin,
Arg (A-1), Arg (A0), His (A8), Gly (A21), Lys (B31)-NH_2-human insulin,
Arg (A0), Arg (A1), His (A8), Gly (A21), Arg (B31)-NH_2-human insulin,
Arg (A0), Arg (A1), His (A8), Gly (A21), Lys (B31)-NH_2-human insulin,
His (A8), Gly (A21), Arg (B31), Arg (B32)-NH_2-human insulin.

14. A process for preparing an insulin analogue as claimed in any of claims 1 to13, where a precursor of the insulin analogue is prepared recombinantly, the precursor is processed enzymatically to two-chain insulin, and a coupling with argininamide is carried out in the presence of an enzyme having trypsin activity, and the insulin analogue is isolated.

15. The use of an insulin analogue as claimed in any of claims 1 to 13 for the manufacture of a medicament for treating diabetes mellitus.

16. The use as claimed in claim 15 in a process for the manufacture of a medicament for the treatment of diabetes mellitus of type I or type II, for cartilage regeneration or for assisting beta cell regeneration.

17. A pharmaceutical comprising an insulin analogue as claimed in any of claims 1 to 13 and/or physiologically acceptable salts thereof.

18. A formulation of the insulin analogue as claimed in any of claims 1 to 13, where the formulation is in aqueous form comprising the dissolved insulin analogue.

19. A formulation of the insulin analogue as claimed in any one of claims 1 to 13, where the formulation is in the form of powder.

20. The formulation as claimed in claim 19, where the insulin analogue as claimed in any of claims 1 to 13 is present in crystalline and/or amorphous form.

21. A formulation of the insulin analogue as claimed in any of claims 1 to 13, where the formulation is in the form of a suspension.

22. A formulation of the insulin analogue as claimed in any of claims 1 to 13, where the formulation additionally comprises a chemical chaperone.

23. DNA coding for the A chain of an insulin analogue as claimed in any of claims 1 to 13.

24. DNA coding for the B chain of an insulin analogue as claimed in any of claims 1

to 13.

25. A vector comprising a DNA as claimed in one or more of claims 23 to 24.

26. A host organism comprising a DNA as claimed in one or more of claims 23 to 24.

27. A host organism comprising a DNA as claimed in one or more of claims 23 to 24 or a vector as claimed in claim 25.

28. The formulation as claimed in one or more of claims 18 to 22, which additionally comprises a glucagon-like peptide-1 (GLP1) or an analogue or derivative thereof, or exendin-3 or -4 or an analogue or derivative thereof.

29. The formulation as claimed in claim 28, which additionally comprises exendin-4.

30. The formulation as claimed in claim 28, wherein an analogue of exendin-4 is selected from a group comprising
H-desPro^{36}-exendin-4-Lys$_6$-NH$_2$,
H-des(Pro^{36,37})-exendin-4-Lys$_4$-NH$_2$ and
H-des(Pro^{36,37})-exendin-4-Lys$_5$-NH$_2$,
or a pharmacologically tolerable salt thereof.

31. The formulation as claimed in claim 28, wherein an analogue of exendin-4 is selected from a group comprising
desPro^{36} [Asp^{28}]exendin-4 (1-39),
desPro^{36} [IsoAsp^{28}]exendin-4 (1-39),
desPro^{36} [Met(O)^{14}, Asp^{28}]exendin-4 (1-39),
desPro^{36} [Met(O)^{14}, IsoAsp^{28}]exendin-4 (1-39),
desPro^{36} [Trp(O$_2$)^{25}, Asp^{28}]exendin-2 (1-39),

desPro^{36} [Trp(O$_2$)^{25}, IsoAsp^{28}]exendin-2 (1-39),
desPro^{36} [Met(O)^{14}Trp(O$_2$)^{25}, Asp^{28}]exendin-4 (1-39) and
desPro^{36} [Met(O)^{14}Trp(O$_2$)^{25}, IsoAsp^{28}]exendin-4 (1-39),
or a pharmacologically tolerable salt thereof.

32. The formulation as claimed in claim 28, where the peptide -Lys$_6$-NH$_2$ is attached to the C termini of the analogues of exendin-4.

33. The formulation as claimed in claim 28, wherein an analogue of exendin-4 is selected from a group comprising
H-(Lys)$_6$- des Pro^{36} [Asp^{28}]exendin-4(1-39)-Lys$_6$-NH$_2$
des Asp^{28}Pro^{36}, Pro^{37}, Pro$_{38}$ exendin-4(1-39) -NH$_2$,
H-(Lys)$_6$- des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39) -NH$_2$,
H-Asn-(Glu)$_5$ des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39) -NH$_2$,
des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39)-(Lys)$_6$-NH$_2$,
H-(Lys)$_6$- des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39)-(Lys)$_6$-NH$_2$,
H-Asn-(Glu)$_5$- des Pro^{36}, Pro^{37}, Pro^{38} [Asp^{28}]exendin-4(1-39)-(Lys)$_6$-NH$_2$,

H-(Lys)$_6$- des Pro^{36} [Trp(O$_2$)^{25}, Asp^{28}]exendin-4(1-39)-Lys$_6$-NH$_2$,
H- des Asp^{28} Pro^{36}, Pro^{37}, Pro^{38} [Trp(O$_2$)^{25}]exendin-4(1-39) -NH$_2$,
H-(Lys)$_6$- des Pro^{36}, Pro^{37}, Pro^{38} [Trp(O$_2$)^{25}, Asp^{28}]exendin-4(1-39) -NH$_2$,
H-Asn-(Glu)$_5$- des Pro^{36}, Pro^{37}, Pro^{38} [Trp(O$_2$)^{25}, Asp^{28}]exendin-4(1-39) -NH$_2$,
des Pro^{36}, Pro^{37}, Pro^{38} [Trp(O$_2$)^{25}, Asp^{28}]exendin-4(1-39)-(Lys)$_6$-NH$_2$,
H-(Lys)$_6$- des Pro^{36}, Pro^{37}, Pro^{38} [Trp(O$_2$)^{25}, Asp^{28}]exendin-4(1-39)-(Lys)$_6$-NH$_2$,
H-Asn-(Glu)$_5$- des Pro^{36}, Pro^{37}, Pro^{38} [Trp(O$_2$)^{25}, Asp^{28}]exendin-4(1-39)-(Lys)$_6$-NH$_2$,
H-(Lys)$_6$- des Pro^{36} [Met(O)^{14}, Asp^{28}]exendin-4(1-39)-Lys$_6$-NH$_2$,
des Met(O)^{14} Asp^{28} Pro ^{36}, Pro^{37}, Pro^{38} exendin-4(1-39) -NH$_2$,
H-(Lys)$_6$- des Pro^{36}, Pro ^{37}, Pro^{38} [Met(O)^{14}, Asp^{28}]exendin-4(1-39) -NH$_2$,
H-Asn-(Glu)$_5$- des Pro^{36}, Pro^{37}, Pro^{38} [Met(O)^{14}, Asp^{28}] exendin-4(1-39) -NH$_2$,
des Pro^{36}, Pro^{37}, Pro^{38} [Met(O)^{14}, Asp^{28}]exendin-4(1-39)-(Lys)$_6$-NH$_2$,
H-(Lys)$_6$- des Pro^{36}, Pro^{37}, Pro^{38} [Met(O)^{14}, Asp^{28}]exendin-4(1-39)-Lys$_6$-NH$_2$,
H-Asn-(Glu)$_5$ des Pro^{36}, Pro^{37}, Pro^{38} [Met(O)^{14}, Asp^{28}] exendin-4(1-39)-(Lys)$_6$-NH$_2$,

H-(Lys)$_6$- des Pro^{36} [Met(O)^{14}, Trp(O$_2$)^{25}, Asp^{28}]exendin-4(1-39)-Lys$_6$-NH$_2$,

des Asp^{28} Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$]exendin-4(1-39) -NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39) -NH_2,
H-Asn-$(Glu)_5$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, Asp^{28}] exendin-4(1-39) -NH_2,
des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-$(Lys)_6$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}]exendin-4(1-39)-$(Lys)_6$-NH_2,
H-Asn-$(Glu)_5$- des Pro^{36}, Pro^{37}, Pro^{38} [$Met(O)^{14}$, $Trp(O_2)^{25}$, Asp^{28}] exendin-4(1-39)-$(Lys)_6$-NH_2,
or a pharmacologically tolerable salt thereof.

34. The formulation as claimed in claim 28, which additionally comprises Arg^{34}, Lys^{26} (N^{ε}(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37) [liraglutide].

SEQUENCE LISTING

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<120> Novel insulin derivatives with extremely delayed time/action profile

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