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(54) **NOUVEAUX ANALOGUES DE L'INSULINE PRESENTANT UN  
POUVOIR DE LIAISON ACCRU AVEC LE ZINC**

(54) **NOVEL INSULIN ANALOGS WITH ENHANCED ZINC  
BINDING**

(57) L'invention concerne des analogues de l'insuline présentant un pouvoir de liaison accru avec le zinc. Elle concerne également des complexes zinc-analogues d'insuline stables qui présentent un profil d'activité retardé par rapport à l'insuline humaine. L'invention concerne en outre un procédé de production desdits analogues ainsi que l'utilisation de ces derniers notamment dans des préparations pharmaceutiques pour le traitement du diabète sucré de type I mais aussi de type II.

(57) The invention relates to insulin analogs exhibiting enhanced zinc binding capacity and to stable zinc complexes thereof having a retarded activity in comparison with human insulin. The invention further relates to a method for the production of said insulin analogs and to their use, particularly in pharmaceutical preparations for therapy of type I and type II diabetes mellitus.

**PCT**

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<p>(21) International application number: PCT/EP99/03490</p> <p>(22) International filing date: 21 May 1999 (21.05.99)</p> <p>(30) Data relating to the priority: 198 25 447.4 6 June 1998 (06.06.98) DE</p> <p>(71) Applicant (for all designated States except US): HOECHST MARION ROUSSEL DEUTSCHLAND GMBH [DE/DE]; Brüningstrasse 50, D-65929 Frankfurt am Main (DE).</p> <p>(72) Inventors; and (75) Inventors/Applicants (US only): ERTL, Johann [DE/DE]; Ginsterweg 10, D-65817 Bremthal (DE). HABERMANN, Paul [DE/DE]; Rossertstrasse 35, D-65817 Eppstein (DE). GEISEN, Karl [DE/DE]; Jahnstrasse 43, D-60318 Frankfurt (DE). SEIPKE, Gerhard [DE/DE]; Wiesenstrasse 44, D-65719 Hofheim (DE). WOLLMER, Axel [DE/DE]; Hans Böckler Allee 31, D-52074 Aachen (DE).</p>	<p>(81) Designated states: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>Without the International Search Report and to be republished once the report has been received.</i></p>	

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(54) Bezeichnung: NEUE INSULINANALOGA MIT ERHÖHTER ZINKBINDUNG

(57) Abstract

The invention relates to insulin analogs exhibiting enhanced zinc binding capacity and to stable zinc complexes thereof having a retarded activity in comparison with human insulin. The invention further relates to a method for the production of said insulin analogs and to their use, particularly in pharmaceutical preparations for therapy of type I and type II diabetes mellitus.

(57) Zusammenfassung

Die vorliegende Erfindung betrifft Insulinanaloga, welche ein erhöhtes Zinkbindungsvermögen aufweisen, sowie stabile Zinkkomplexe derselben, die im Vergleich zu Humaninsulin ein verzögertes Wirkprofil aufweisen, ein Verfahren zu deren Herstellung und deren Verwendung insbesondere in pharmazeutischen Zubereitungen zur Therapie des Diabetes mellitus vom Typ I wie auch Typ II.

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## Description

## Novel insulin analogs with increased zinc binding

The present invention relates to insulin analogs which have an increased zinc binding ability, and to stable zinc complexes thereof which, in comparison with human insulin, have a delayed profile of action, to a process for their preparation and to their use, in particular in pharmaceutical preparations for the therapy of diabetes mellitus of type I and also type II.

Worldwide, approximately 120 million people suffer from diabetes mellitus. Among these, approximately 12 million are type I diabetics, for whom the substitution of the lacking endocrine insulin secretion is the only possible therapy at present. Those affected are prescribed insulin injections, as a rule several times daily, for life. Unlike type I diabetes, in type II diabetes there is not fundamentally a lack of insulin, but in a large number of cases, especially in the advanced stage, treatment with insulin, if appropriate in combination with an oral antidiabetic, is regarded as the most favorable form of therapy.

In healthy people, the release of insulin by the pancreas is strictly coupled to the concentration of the blood glucose. Increased blood glucose levels, such as occur after meals, are rapidly compensated by a corresponding increase in insulin secretion. In the fasting state, the plasma insulin level drops to a basal value which is sufficient to guarantee a continuous supply of insulin-sensitive organs and tissues with glucose and to keep the hepatic glucose production low during the night. The replacement of the endogenous insulin secretion by exogenous, mostly subcutaneous, administration of insulin as a rule does not nearly achieve the quality of the physiological regulation of the blood glucose described above. Frequently, there are losses of control of the blood glucose upward or downward, which in their most severe forms can be life-threatening. In addition, blood glucose levels which have been raised for years without initial symptoms, however, also represent a considerable risk to health. The large-scale DCCT study in the USA (The Diabetes Control and Complications Trial Research Group (1993) *N, Engl. J. Med.* **329**, 977-986) clearly demonstrated that chronically raised blood glucose levels are largely responsible for the development of diabetic late damage. Diabetic late damage is micro- and macrovascular damage which is manifested, under certain circumstances, as retinopathy, nephropathy, or neuropathy and leads to blindness, kidney failure

and the loss of extremities and is moreover accompanied by a high risk of cardiovascular diseases. It can be derived from this that an improved therapy of diabetes must primarily aim to keep the blood glucose as closely as possible in the physiological range. According to the concept of intensified insulin therapy, this should be achieved by a number of daily injections of rapid- and slow-acting insulin preparations. Rapid-acting formulations are given at mealtimes in order to level out the post-prandial increase in blood glucose. Slow-acting basal insulins should ensure the basic supply of insulin, in particular during the night, without leading to hypoglycemia.

The basal insulins available at present fulfill this requirement only inadequately. The frequently used NPH insulins especially have a too strongly pronounced maximum action and have too short an overall action. In the case of administration in the evening, this involves the risk of nightly hypoglycemia and morning hyperglycemia.

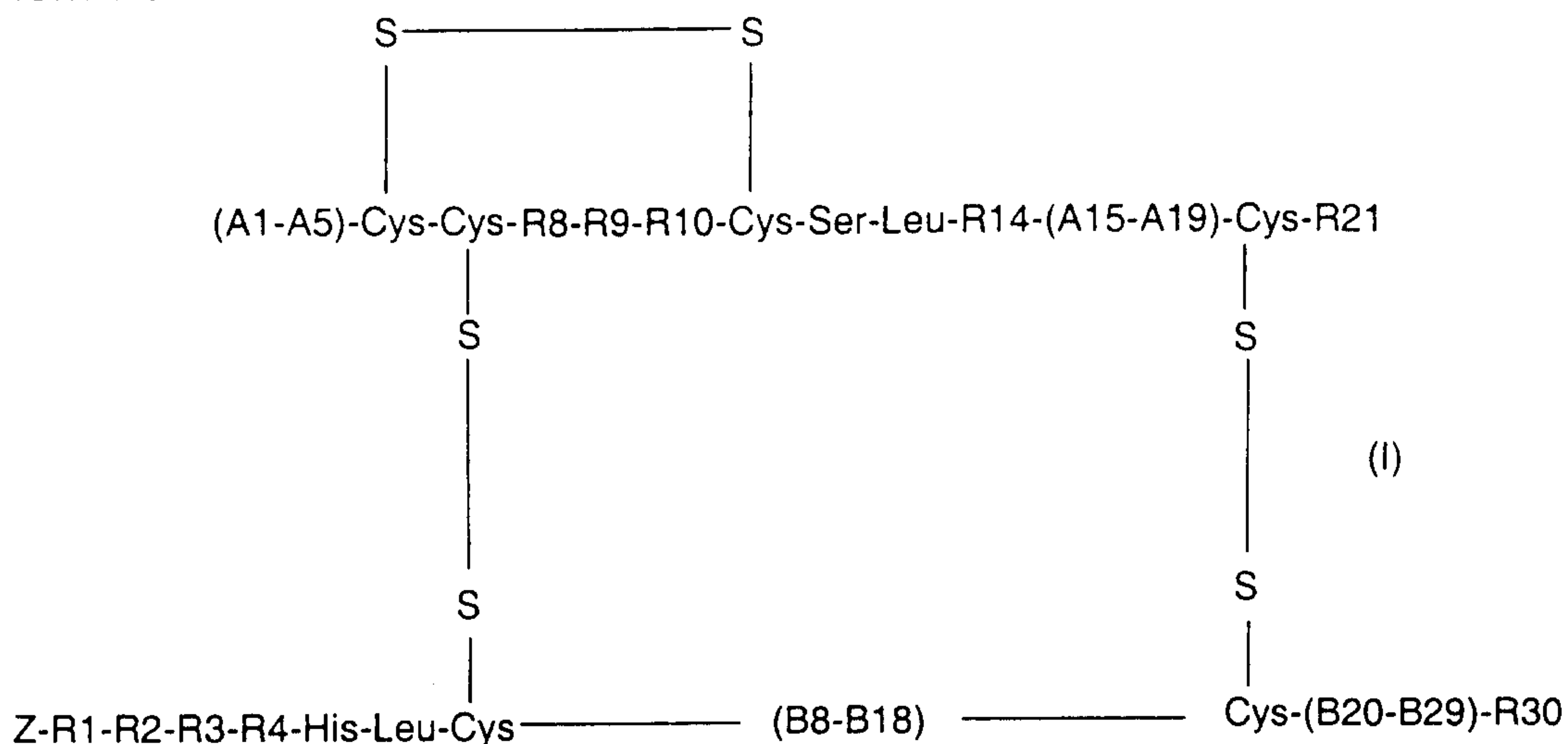
EP 0 821 006 discloses insulin analogs having increased zinc binding ability, which in combination with zinc have a delayed profile of action compared with human insulin. These analogs differ from human insulin essentially by variation of the amino acid in position A21 of the A chain and by addition of a histidine residue or of a peptide having 2 to 35 amino acid residues, which contains 1 to 5 histidine residues, in position B30 of the B chain.

It is the object of the present invention to provide further insulin analogs (analogues of human or animal insulin) which have an increased zinc binding ability, form a stable complex comprising a hexamer of the insulin analog and zinc, and, in a suitable preparation, make possible an improved therapy of diabetes mellitus of type I and of type II on subcutaneous injection as a result of the profile of action, which is delayed in comparison with human insulin.

Insulin analogs are derived from naturally occurring insulins, namely human insulin (see SEQ ID NO: 1: A chain of human insulin and SEQ ID NO: 2: B chain of human insulin) or animal insulins by substitution or absence of at least one naturally occurring amino acid residue and/or addition of at least one amino acid residue to the A and/or B chain of the naturally occurring insulin.

The object is achieved by

1. an insulin analog or a physiologically tolerable salt thereof of the formula I



in which

- (A1-A5) are the amino acid residues in the positions A1 to A5 of the A chain of human insulin (cf. SEQ ID NO: 1) or animal insulin,
- (A15-A19) are the amino acid residues in the positions A15 to A19 of the A chain of human insulin (cf. SEQ ID NO: 1) or animal insulin,
- (B8-B18) are the amino acid residues in the positions B8 to B18 of the B chain of human insulin (cf. SEQ ID NO: 2) or animal insulin,
- (B20-B29) are the amino acid residues in the positions B20 to B29 of the B chain of human insulin (cf. SEQ ID NO: 2) or animal insulin,
- R8 is Thr or Ala,
- R9 is Ser or Gly,
- R10 is Ile or Val,
- R14 is Tyr, His, Asp or Glu,
- R21 is Asn, Asp, Gly, Ser, Thr, Ala, Glu or Gln,
- R1 is any desired genetically encodable amino acid residue, absent or a hydrogen atom,
- R2 is Val, Ala or Gly,
- R3 is Asn, His, Glu or Asp,
- R4 is Ala, Ser, Thr, Asn, Asp, Gln, Gly or Glu,
- R30 is any desired genetically encodable amino acid residue or -OH,
- Z is a hydrogen atom or a peptide residue having 1 to 4 genetically encodable amino acid residues, comprising 1 to 4 histidine residues (His),

with the proviso that in the case in which Z is a hydrogen atom, R1 or R3 is His, Glu or Asp, where R3 is His, if R1 is a neutral or negatively charged amino acid residue, or with the proviso that in the case in which Z is a hydrogen atom, R14

is His, Asp or Glu, and furthermore with the proviso that the insulin analog or the physiologically tolerable salts thereof of the formula I differs from human insulin not only by variation of the amino acid residues in the positions R3 or R3 in combination with R21 or R3 in combination with R4 in formula I (cf. SEQ ID NO: 1 and SEQ ID NO: 2).

Preferably, the insulin analog or the physiologically tolerable salt thereof is one wherein

2. R8 is Thr, R9 is Ser and R10 is Ile,
3. R1 is Phe, His, Asn, Asp or Gly,
4. R30 is Thr, Ala or Ser or
5. wherein R21 is Asn and R1 is Phe.
6. A preferred embodiment of the present invention is an insulin analog or a physiologically tolerable salt thereof of the formula I, wherein R2 is Val, R3 is Asn and R4 is Gln.

An insulin analog or a physiologically tolerable salt thereof of the formula I is furthermore preferred which is distinguished in that R14 is

7. Tyr,
8. His,
9. Asp or
10. Glu.

An insulin analog or a physiologically tolerable salt thereof of the formula I is furthermore preferred which is distinguished in that R30 is

11. Thr,
12. Ala,

13. Ser or

14. -OH.

An insulin analog or a physiologically tolerable salt thereof of the formula I is furthermore preferred which is distinguished in that Z is

15. His,

16. His-Ala- or

17. His-Ala-Ala-.

Examples of insulin analogs according to the present invention are

18. an insulin analog or a physiologically tolerable salt thereof of the formula I, which is distinguished in that the B chain has the sequence

His Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr  
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys

(SEQ ID NO: 3), for example His(B0), des(B30) human insulin,

19. an insulin analog or a physiologically tolerable salt thereof of the formula I, which is distinguished in that the B chain has the sequence

His Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr  
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 4), for example His(B0)-human insulin,

20. an insulin analog or a physiologically tolerable salt thereof of the formula I, which is distinguished in that the B chain has the sequence

His Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu  
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 5), for example His(B-1), Ala(B0) human insulin or

21. an insulin analog or a physiologically tolerable salt thereof of the formula I, which is distinguished in that the B chain has the sequence

His Ala Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala  
Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 6), for example His(B-2), Ala(B-1), Ala(B0)-human insulin.

The present invention furthermore relates to a process for the preparation of the insulin analog or of a physiologically tolerable salt thereof according to the present invention, comprising the construction of a replicable expression vehicle which contains a DNA sequence which codes for a precursor of the insulin analog having the amino acid sequence II

Met- $X^2_m$ -(Arg)<sub>p</sub>-Z-R1-R2-R3-R4-His-Leu-Cys-(B8-B18)-Cys-(B20-B29)-R30-  
 $X^1_n$ -Arg-(A1-A5)-Cys-Cys-R8-R9-R10-Cys-Ser-Leu-R14-(A15-A19)-Cys-R21  
II,

in which

$X^1_n$  is a peptide chain having n amino acid residues, where n is an integer from 0 to 34,

$X^2_m$  is a peptide chain having m amino acid residues, where m is an integer from 0 to 20,

p is 0, 1 or 2,

R30 is any desired genetically encodable amino acid residue or is absent and

Z is absent or is a peptide residue having 1 to 4 genetically encodable amino acid residues, comprising 1 to 4 histidine residues (His)

and the other variables have the meanings mentioned above under No. 1, where the abovementioned provisos also apply, expression in a host cell and release of the insulin analog from its precursor using chemical and/or enzymatic methods.

The host cell is preferably a bacterium, particularly preferably the bacterium *E. coli*.

The host cell is preferably a yeast, particularly preferably *Saccharomyces cerevisiae*.

During expression in *E. coli*, the fusion proteins mentioned (SEQ ID NO: 7 to 9) as a rule form insoluble inclusion bodies, which can be isolated by centrifugation after cell disruption and are dissolved again using chaotropic additives (e.g. 8 M urea or 6 M guanidinium chloride). The dissolved fusion protein can be subjected to sulfitolysis, in which SH radicals are converted into S-sulfonates (e.g. R.C. Marshall and A.S. Iglis in 'Practical Protein Chemistry – A Handbook', edited by A. Darbre (1986), pages 49-53). The solubility of the fusion protein is thereby improved and purification, for example by means of anion-exchange or gel permeation chromatography, is facilitated.

The conversion of the derivatized fusion protein into preproinsulin with a native spatial structure and correctly formed disulfide bridges (folding) is carried out in dilute aqueous solution by addition of a limited amount of an SH reagent such as mercaptoethanol, cysteine or glutathione and subsequent aerial oxidation. Alternatively, the dissolved, underivatized fusion protein can also be directly folded under similar conditions (EP-A-0 600 372; EP-A-0 668 292).

Preproinsulin is then converted into biologically active insulin by limited proteolytic cleavage. For this, it is possible to use trypsin which removes the presequence indicated in formula II by  $\text{Met-X}_m^2\text{-(Arg)}_p$  and cleaves at the peptide chain indicated by  $\text{X}_n^1\text{-Arg}$  and thus separates the B and A chain. As a rule, the sequence  $\text{X}_n^1$  begins with Arg, Arg<sub>2</sub> or it is not present ( $n=0$ ), so that after the cleavage an insulin derivative is present which is prolonged by Arg or Arg<sub>2</sub> at the C terminus of the B chain. These amino acids can be removed using carboxypeptidase B. The tryptic cleavage can also be carried out by increasing the trypsin concentration or prolonging the reaction time such that cleavage additionally takes place at lysine(B29). In this case, a des(B30) insulin derivative results.

The insulin analog formed during the cleavage can be purified by standard chromatographic procedures (e.g. ion-exchange and reversed phase chromatography) and finally isolated by precipitation, crystallization or simple freeze-drying.

The precursor of the insulin analog preferably has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg  
 His Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val  
 Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly  
 Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg  
 Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr  
 Cys Asn

(SEQ ID NO: 7), for example the sequence of His(B0)-preproinsulin, or the  
 sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg  
 His Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu  
 Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr  
 Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly  
 Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg  
 Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr  
 Cys Asn

(SEQ ID NO: 8), for example the sequence of His(B-1), Ala(B0) preproinsulin,  
 or the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg  
 His Ala Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr  
 Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr  
 Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly  
 Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg  
 Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys  
 Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn

(SEQ ID NO: 9), for example the sequence of His(B-2), Ala(B-1), Ala(B0)-  
 preproinsulin.

The present invention also relates to the abovementioned precursors of the  
 insulin analogs according to the present invention, in particular the  
 preproinsulins, the DNA sequences which code for a precursor of the insulin  
 analog according to the present invention, the expression vehicles which  
 contain a DNA sequence which codes for a novel precursor of the insulin  
 analog according to the present invention, and a host cell which is transformed  
 using such an expression vehicle.

The present invention furthermore relates to a pharmaceutical preparation comprising at least one insulin analog and/or at least one physiologically tolerable salt according to the present invention.

Preferably, the pharmaceutical preparation is distinguished in that it contains the insulin analog according to the invention and/or the physiologically tolerable salt thereof in dissolved, amorphous and/or crystalline form.

The pharmaceutical preparation alternatively furthermore contains a depot auxiliary, preferably protamine sulfate, the insulin analog and/or the physiologically tolerable salt thereof preferably being present in a cocrystallizate with the protamine sulfate.

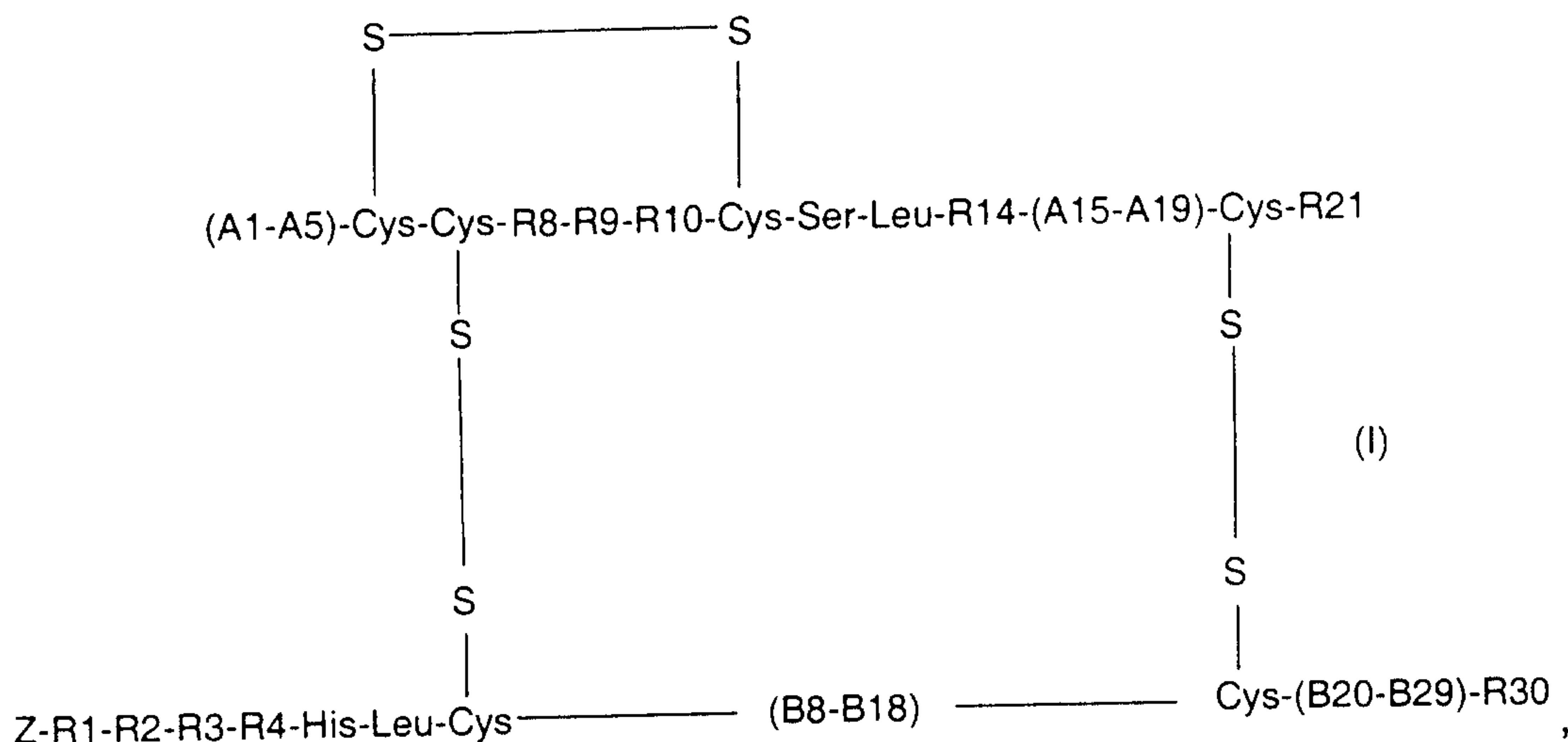
The pharmaceutical preparation according to the present invention can alternatively additionally contain unmodified human insulin and/or a further insulin analog, preferably Gly(A21)-Arg(B31)-Arg(B32)-human insulin.

The present invention furthermore relates to an injectable solution having insulin activity, which contains the pharmaceutical preparation according to the present invention in dissolved form, preferably containing 1  $\mu\text{g}$  to 2 mg of zinc per ml, particularly preferably containing 5  $\mu\text{g}$  to 200  $\mu\text{g}$  of zinc per ml.

The present invention furthermore relates to the use of the insulin analog and/or its physiologically tolerable salt according to the present invention for the production of a pharmaceutical preparation which has an insulin activity having a delayed onset of action.

The object set at the outset is furthermore achieved by an insulin-zinc complex, comprising an insulin hexamer and 4 to 10 zinc ions per insulin hexamer, wherein the insulin hexamer consists of six molecules of an insulin analog of the formula I

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in which

- (A1-A5) are the amino acid residues in the positions A1 to A5 of the A chain of human insulin or animal insulin,
- (A15-A19) are the amino acid residues in the positions A15 to A19 of the A chain of human insulin or animal insulin,
- (B8-B18) are the amino acid residues in the positions B8 to B18 of the B chain of human insulin or animal insulin,
- (B20-B29) are the amino acid residues in the positions B20 to B29 of the B chain of human insulin or animal insulin,
- R8 is Thr or Ala,
- R9 is Ser or Gly,
- R10 is Ile or Val,
- R14 is Tyr, His, Asp or Glu,
- R21 is Asn, Asp, Gly, Ser, Thr, Ala, Glu or Gln,
- R1 is any desired genetically encodable amino acid residue, absent or a hydrogen atom,
- R2 is Val, Ala or Gly,
- R3 is Asn, His, Glu or Asp,
- R4 is Ala, Ser, Thr, Asn, Asp, Gln, Gly or Glu,
- R30 is any desired genetically encodable amino acid residue or -OH,
- Z is a hydrogen atom or a peptide residue having 1 to 4 genetically encodable amino acid residues, comprising 1 to 4 histidine residues (His).

The insulin-zinc complex preferably contains 5 to 8 zinc ions per insulin hexamer.

The insulin-zinc complex preferably contains an insulin hexamer which consists of six molecules of the insulin analog of the formula I described above according to the present invention.

The insulin-zinc complex according to the present invention is preferably also one wherein the B chain of the insulin analog of the formula I has the sequence

Phe Val His Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys  
Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 10), for example His(B3)-human insulin, or wherein the B chain of the insulin analog of the formula I has the sequence

Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys  
Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 11), for example Asp(B3)-human insulin.

The present invention also relates to a pharmaceutical preparation, comprising at least one insulin-zinc complex according to the invention and a pharmaceutical preparation comprising an acidic solution of at least one insulin analog and/or a physiologically tolerable salt thereof with an appropriate amount of zinc ions, which makes possible the formation of an insulin-zinc complex according to the present invention, the insulin analog and/or the physiologically tolerable salt preferably containing the insulin analog of the formula I according to the present invention described above or an insulin analog of the formula I whose B chain has the sequence with the number SEQ ID NO's.: 3, 4, 5, 10 or 11.

the pharmaceutical preparation is preferably one which comprises the insulin-zinc complex in dissolved, amorphous and/or crystalline form.

The present invention also relates to an injectable solution having insulin activity, comprising the pharmaceutical preparation in dissolved form and preferably contains 1  $\mu$ g to 2 mg of zinc per ml, particularly preferably contains 5  $\mu$ g to 200  $\mu$ g of zinc per ml.

The present invention also relates to the use of the insulin-zinc complex for the production of a pharmaceutical preparation which has an insulin activity having a delayed onset of action.

The insulin analogs according to the present invention are biologically active and exhibit a strongly delayed action after subcutaneous administration as a weakly acidic, clear solution containing  $80 \mu\text{g}$  of  $\text{Zn}^{++}$ /ml (zinc/ml) in the dog. In the case of the insulin analog which is prolonged at the N-terminus of the B chain by histidine, His(B0), des(B30) human insulin (see SEQ ID NO.: 3), the profile of action depends, for example, very strongly on the amount of added zinc ions. A zinc-free preparation has no depot effect at all (total action 6 - 8 h, Example 8) and hardly differs in its pharmacodynamics from human insulin, while after addition of zinc ions ( $80 \mu\text{g}/\text{ml}$ ), a strong delay in action is found (total action approximately 16 h, Example 8). The observed depot effect is thus significantly more marked than that of NPH-insulin. Moreover, this analog has the advantage that the pharmacodynamics can be controlled by prespecification of the zinc content within a range which is not possible with human insulin. Formulations having a rapid onset of action can be prepared just like those having a moderately or strongly delayed action with an active substance just by varying the zinc content. Thus the profile of action can be individually adapted to the needs of the patient, either using a preparation having an appropriately preset zinc content or by mixing of preparations having a high and low zinc content by the physician or the patients themselves.

The analogs described here are furthermore those which, in comparison to human insulin, have an increased affinity for zinc ions.

In aqueous neutral solution, human insulin forms hexamers which in each case complex two zinc ions via the His(B10) side chains. These zinc ions cannot be removed by dialysis against aqueous buffers in neutral solution. Under the same conditions, the analogs described here bind more than 4 zinc ions. In the case of the His(B0)-des(B30)- and His(B3)-insulin according to the invention, these are approximately 7 zinc ions/hexamer; in the case of Asp(B3) insulin 4.2 zinc ions/hexamer were measured (Example 9).

It is known that in neutral solutions zinc leads to the formation of relatively high molecular weight associates and to the precipitation of the insulin. After the injection of a weakly acidic zinc-containing preparation which contains insulin which is dissolved to give a clear solution, the formation of insulin-zinc

complexes and, as a result, the precipitation of the insulin occur in the subcutaneous tissue due to neutralization. Insulin goes into solution again from this depot and then passes into the blood stream and to the site of action with a delay. This delay in action is only slight in the case of human insulin, but strongly developed in the case of the analogs described here on account of the increased affinity for zinc. The increased zinc binding is therefore the basis of the zinc-dependent prolongation of action described above.

The present invention therefore not only relates to the insulin analogs described but also to the associated insulin-zinc complexes. These complexes differ from the corresponding human insulin-zinc complexes in that they have a higher content of firmly bound zinc. It is therefore evident that in addition to zinc other transition metal ions such as, for example, cobalt or copper can also be employed for the formation of corresponding complexes.

Example 1: Construction of the plasmid pINT345d coding for the variant His(B3)-preproinsulin.

The US patent having the patent No. 5358857 describes the plasmid pINT90d. DNA of this plasmid is used as a starting material for the construction of the plasmid pINT345d, which is characterized by two new properties compared with pINT90d. On the one hand, it codes for a preproinsulin analog which contains the amino acid histidine instead of asparagine in position 3 of the B chain and on the other hand it carries a recognition sequence for the restriction enzyme BssH2 immediately before the start of the sequence coding for this preproinsulin variant, so that the sequence coding for the N-terminal 10 amino acids of the preproinsulin analog can be easily manipulated if the Dra3 cleavage site in the course of the preproinsulin sequence is taken into account. For the construction of the plasmid pINT345d, DNA of the plasmid pINT90d is cleaved in position 284bp by the restriction enzyme Nco1 and in position 351 bp by the restriction enzyme Dra3 in a double digestion mixture so that two fragments are formed. After separation of the cleavage mixture by gel electrophoresis, the large residual plasmid DNA fragment is isolated.

This DNA fragment is then reacted with the synthetic DNA fragment of the form

½ Nco1	BssH2	B1	B2	His	B4	B5	B6										
5' - C	ATG	GCA	ACA	ACA	TCA	ACA	GGA	AAT	TCG	GCG	CGC	TTT	GTG	CAC	CAG	CAC	CTG
3' -		CGT	TGT	TGT	AGT	TGT	CCT	TTA	AGC	CGC	GCG	AAA	CAC	GTG	GTC	GTG	GAC

B7 B8 B9 ½ Dra3

TGC GGC TCC CAC CTA - 3'  
 ACG CCG AGG GTG -5'

in a T4-DNA ligase reaction. Competent E.coli K12 cells are transformed with the ligation mixture and the transformation mixture is plated out onto NA plates which contain 20mg/l ampicillin. The plates are incubated at 37° C overnight. Plasmid DNA is isolated from resulting colonies and cleaved using the restriction enzyme BssH2. The desired plasmid DNA is linearized in the course of this and thus differs from pINT90d DNA which contains no BssH2 cleavage site and accordingly is not cleaved.

The plasmid DNA of a clone which behaves correctly is designated by pINT345d.

It is used as a starting material for the construction of the preproinsulin variants described below.

Example 2: Construction of the plasmid pINT342d coding for the variant His(B0)-preproinsulin

DNA of the plasmid pINT345d is double-digested with the enzymes BssH2 and Dra3 and the large residual plasmid fragment is isolated after separation by gel electrophoresis. This DNA fragment is reacted with the synthetic DNA fragment of the form

	His	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10				
5'-	CG	CGC	CAC	TTT	GTT	AAC	CAG	CAC	CTG	TGC	GGC	TCC	CAC	CTA	- 3'
3'-		G	GTG	AAA	CAA	TTG	GTC	GTG	GAC	ACG	CCG	AGG	GTG		- 5'
	½ BssH2				Hpa1										½ Dra3

in a T4 DNA ligase reaction. The plasmid pINT342d is formed, which comprises an additional Hpa1 cleavage site compared with the starting plasmid. The plasmid codes for a preproinsulin variant which has a histidine in position B0.

Example 3: Construction of the plasmid pINT343d coding for the variant His(B-1), Ala(B0)-preproinsulin

DNA of the residual plasmid fragment described in Example b is reacted with a synthetic DNA fragment of the form

15

```

          His Ala B1  B2  B3  B4  B5  B6  B7  B8  B9  B10 B11
5'- CG CGC CAC GCT TTT GTT AAC CAG CAC CTG TGC GGC TCC CAC CTA - 3'
3'-      G GTG CGA AAA CAA TTG GTC GTG GAC ACG CCG AGG GTG      - 5'
      ½ BssH2                Hpa1                                ½ Dra3

```

in a T4 DNA ligase reaction. The plasmid pINT343d is formed, which, like pINT342d, also contains an additional Hpa1 cleavage site compared with the starting vector.

Example 4: Construction of the plasmid pINT344d coding for the variant His(B-2),Ala(B-1),Ala(B0)-preproinsulin

DNA of the residual plasmid fragment described in Example b is reacted with a synthetic DNA fragment of the form

```

          His Ala Ala B1  B2  B3  B4  B5  B6  B7  B8  B9  B10 B11
5'- CG CGC CAC GCT GCT TTT GTT AAC CAG CAC CTG TGC GGC TCC CAC CTA - 3'
3'-      G GTG CGA CGA AAA CAA TTG GTC GTG GAC ACG CCG AGG GTG      - 5'
      ½ BssH2                Hpa1                                ½ Dra3

```

in a T4 DNA ligase reaction. The plasmid pINT344d results, which comprises an additional Hpa1 cleavage site compared with the starting vector.

Example 5: Expression of the constructed insulin variants

The plasmids pINT 342d, 343d and 344d are, by way of example, in each case transformed into E.coli K12 W3110. Recombinant bacteria which contain the plasmids for the respective variant are then fermented according to Example 4 of the US patent having the patent No. 5227293 and the desired raw material for the production of the respective insulin variant is thus produced.

Example 6: Preparation of His(B0),des(B30)-insulin

According to Example 5, the preproinsulin variant is expressed in E.coli and isolated in the form of inclusion bodies after cell disruption by centrifugation. The inclusion bodies are dissolved in urea (8 mol/l), subjected to sulfitolysis and purified by anion exchange (Q-Sepharose) and gel permeation chromatography (Sephacryl S 200). The buffers employed in the chromatography contain 4 M urea and 50 mM Tris/HCl

(tris(hydroxymethyl)aminomethane/HCl) pH 8.5. The fractional elution on the anion exchanger is carried out by applying a gradient of 0 to 0.5 M NaCl. The concentration of the urea is then reduced to < 1 M by ultrafiltration and dilution and the preproinsulin-S-sulfonate is isolated by precipitation at pH 4 and finally dried.

For the formation of the correct disulfide bridges, as are present in natural proinsulin, the preproinsulin-S-sulfonate is dissolved at pH 10.8, in a buffer which contains 20 mM glycine, at a concentration of 0.3 g/l, treated with mercaptoethanol (approximately 25-50 mol/mol of preproinsulin) and stirred overnight at 4°C. The batch is then adjusted to pH 3.5 with phosphoric acid and centrifuged. The preproinsulin contained in the supernatant is adjusted to pH 8.2 for conversion into insulin after addition of tris (25 mM) and treated with trypsin (1.5 mg/g of preproinsulin). The course of the proteolytic cleavage is monitored by means of reversed phase HPLC. After approximately 6 hours, the batch contains a high content of His(B0),des(B30)-insulin. The reaction is ended by acidification to pH 3.5. The insulin analog is purified by ion-exchange chromatography (S-Hyper-D, Sepracor) and reversed phase chromatography (PLRP-S RP300, Polymer Laboratories). The ion-exchange chromatography is carried out in a buffer which contains 30% 2-propanol and 50 mM lactic acid (pH 3.5). The bound insulin is eluted by a linear gradient of 0 to 0.5 M NaCl. The reversed phase chromatography is carried out in 0.1% trifluoroacetic acid, to which increasing amounts of acetonitrile are admixed for elution. The product is isolated by precipitation at pH 5.4 and lyophilized.

#### Example 7: Formulation of insulin analogs for parenteral administration

The preparations contain, per ml, 40 or 100 IU of insulin (1 IU corresponds to approximately 6.2 nmol), 20 mg of 85% glycerol, 2.7 mg of m-cresol and, if appropriate, zinc<sup>++</sup> (as zinc chloride) in aqueous, sterile solution at pH 4.

#### Example 8: Profile of action of His(B0),des(B30)-insulin in the dog

6 dogs (beagles) in each case received subcutaneous administrations of a preparation containing 40 U/ml (Example 7) and the indicated content of zinc. The dose was 0.3 IU/kg. In the further course of the experiment, the concentration of the blood glucose was measured after the times indicated. The values were standardized percentagewise on the respective starting value and averaged.

<i>time (hours)</i>	0	1	2	3	4	5	6	7	8	9	10	12
zinc-free	100	59	50	61	75	84	89	98	103	97	104	100
80 $\mu$ g zinc <sup>++</sup> /ml	100	97	83	75	65	56	51	58	68	72	78	82

#### Example 9: Zinc binding of insulin analogs

A preparation of insulin (0.3 mM insulin, 0.13 M NaCl, 0.1% phenol, 100  $\mu$ g/ml zinc<sup>++</sup> (as zinc chloride), 25 mM tris/HCl, pH 7.4) was extensively dialyzed against zinc-free neutral buffer (3h against 0.15 M NaCl, 10 mM tris/HCl pH 7.4 at room temperature, 72 hours against 10 mM tris/HCl pH 7.4 at 15°C and again 16 h against 10 mM tris/HCl pH 7.4 at 15°C). The dialyzates were then acidified and analyzed. The concentration of insulin was determined by reversed phase HPLC and that of the zinc by atomic absorption spectroscopy. The zinc values were corrected using the zinc content of a control batch which contained no insulin.

#### Zinc binding

<i>Insulin</i>	<i>mol of zinc/mol of hexamer</i>
human insulin	2.5
His(B3)-insulin	6.9
Asp(B3)-insulin	4.2
His(B0),des(B30)-insulin	6.8



2

## (2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 Amino acids  
 (B) TYPE: Amino acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGIE: linear

(ii) MOLECULE TYPE: Protein

- (ix) FEATURES:  
 (A) NAME/KEY: Protein  
 (B) LOCATION: 1..30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr
1				5					10					15	
Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr		
			20					25					30		

## (2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 Amino acids  
 (B) TYPE: Amino acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGIE: linear

(ii) MOLECULE TYPE: Protein

- (ix) FEATURES:  
 (A) NAME/KEY: Protein  
 (B) LOCATION: 1..30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

His	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu
1				5					10					15	
Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys		
			20					25					30		

## (2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 31 Amino acids  
 (B) TYPE: Amino acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGIE: linear

(ii) MOLECULE TYPE: Protein

## 3

## (ix) FEATURES:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..31

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

His	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu
1				5					10					15	
Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr	
			20					25					30		

## (2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 Amino acids
  - (B) TYPE: Amino acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGIE: linear

## (ii) MOLECULE TYPE: Protein

## (ix) FEATURES:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..32

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

His	Ala	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala
1				5					10					15	
Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr
			20					25					30		

## (2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 Amino acids
  - (B) TYPE: Amino acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGIE: linear

## (ii) MOLECULE TYPE: Protein



5

## (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 99 Amino acids  
 (B) TYPE: Amino acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGIE: linear

(ii) MOLECULE TYPE: Protein

- (ix) FEATURES:  
 (A) NAME/KEY: Protein  
 (B) LOCATION: 1..99

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met	Ala	Thr	Thr	Ser	Thr	Gly	Asn	Ser	Ala	Arg	His	Ala	Phe	Val	Asn
1				5					10					15	
Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys
			20					25					30		
Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr	Arg	Arg	Glu	Ala	Glu
		35					40					45			
Asp	Pro	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly
	50					55					60				
Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln	Lys	Arg	Gly	Ile
65					70					75					80
Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn
				85					90					95	
Tyr	Cys	Asn													

## (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 100 Amino acids  
 (B) TYPE: Amino acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGIE: linear

(ii) MOLECULE TYPE: Protein

- (ix) FEATURES:  
 (A) NAME/KEY: Protein  
 (B) LOCATION: 1..100



## (ix) FEATURES:

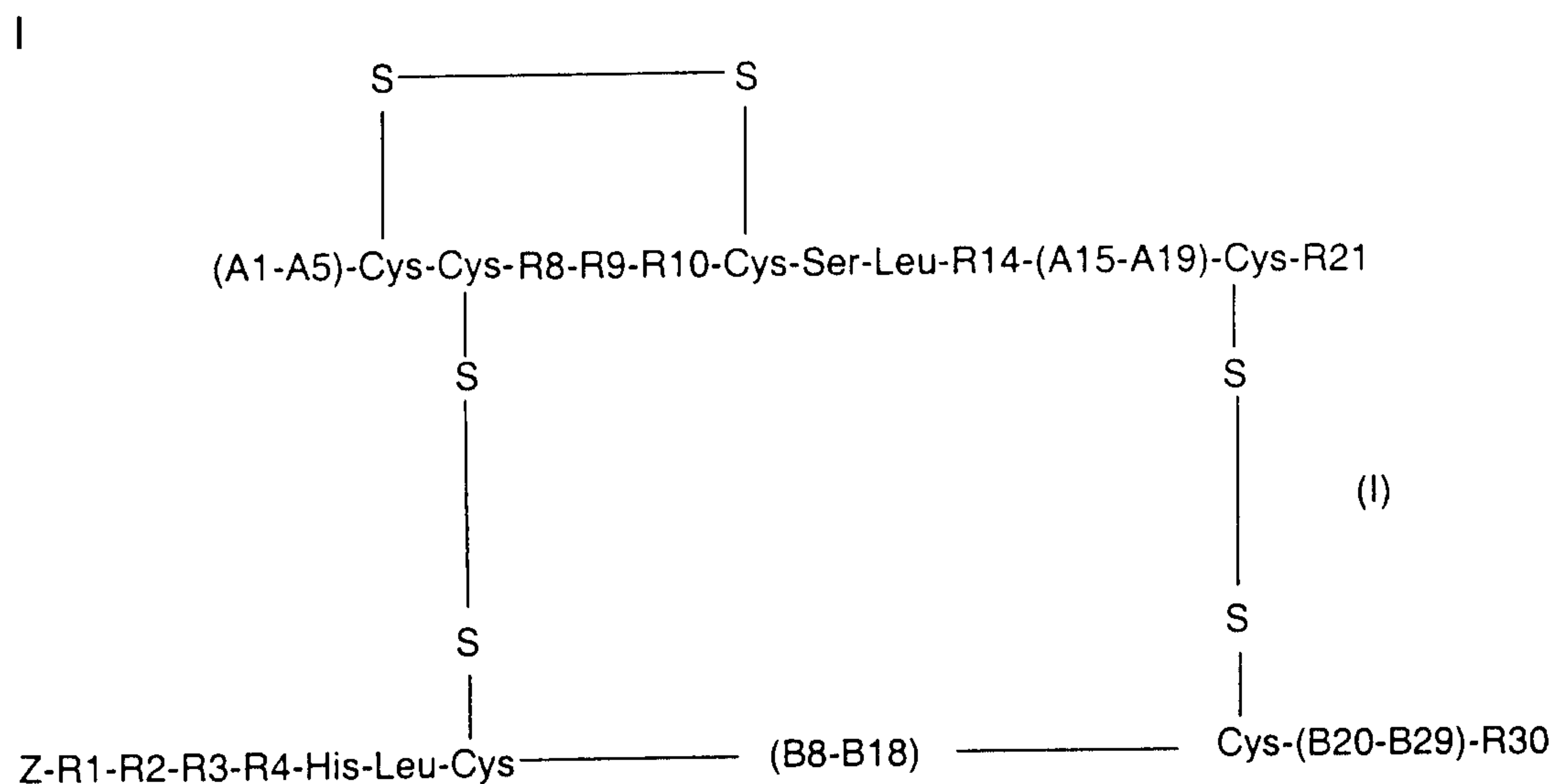
- (A) NAME/KEY: Protein
- (B) LOCATION: 1..30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

Phe	Val	Asp	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr
1				5					10					15	
Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr		
			20					25					30		

## Patent claims

1. An insulin analog or a physiologically tolerable salt thereof of the formula



in which

- (A1-A5) are the amino acid residues in the positions A1 to A5 of the A chain of human insulin or animal insulin,
- (A15-A19) are the amino acid residues in the positions A15 to A19 of the A chain of human insulin or animal insulin,
- (B8-B18) are the amino acid residues in the positions B8 to B18 of the B chain of human insulin or animal insulin,
- (B20-B29) are the amino acid residues in the positions B20 to B29 of the B chain of human insulin or animal insulin,
- R8 is Thr or Ala,
- R9 is Ser or Gly,
- R10 is Ile or Val,
- R14 is Tyr, His, Asp or Glu,
- R21 is Asn, Asp, Gly, Ser, Thr, Ala, Glu or Gln,
- R1 is any desired genetically encodable amino acid residue, absent or a hydrogen atom,
- R2 is Val, Ala or Gly,
- R3 is Asn, His, Glu or Asp,
- R4 is Ala, Ser, Thr, Asn, Asp, Gln, Gly or Glu,
- R30 is any desired genetically encodable amino acid residue or -OH,
- Z is a hydrogen atom or a peptide residue having 1 to 4 genetically encodable amino acid residues, comprising 1 to 4 histidine residues (His),

with the proviso that in the case in which Z is a hydrogen atom, R1 or R3 is His, Glu or Asp, where R3 is His, if R1 is a neutral or negatively charged amino acid residue, or with the proviso that in the case in which Z is a hydrogen atom, R14 is His, Asp or Glu, and furthermore with the proviso that the insulin analog or the physiologically tolerable salt thereof of the formula I differs from human insulin not only by variation of the amino acid residues in the positions R3 or R3 in combination with R21 or R3 in combination with R4 in formula I.

2. An insulin analog or a physiologically tolerable salt thereof as claimed in claim 1, wherein R8 is Thr, R9 is Ser and R10 is Ile.
3. An insulin analog or a physiologically tolerable salt thereof as claimed in claim 1 or 2, wherein R1 is Phe, His, Asn, Asp or Gly.
4. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 3, wherein R30 is Thr, Ala or Ser.
5. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 4, wherein R21 is Asn and R1 is Phe.
6. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 5, wherein R2 is Val, R3 is Asn and R4 is Gln.
7. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 6, wherein R14 is Tyr.
8. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 6, wherein R14 is His.
9. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 6, wherein R14 is Asp.
10. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 6, wherein R14 is Glu.
11. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 10, wherein R30 is Thr.

12. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 10, wherein R30 is Ala.

13. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 10, wherein R30 is Ser.

14. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 10, wherein R30 is -OH.

15. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 14, wherein Z is His.

16. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 14, wherein Z is His-Ala-.

17. An insulin analog or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 14, wherein Z is His-Ala-Ala-.

18. An insulin analog or a physiologically tolerable salt thereof as claimed in claim 15, wherein the B chain has the sequence

His Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val  
Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys

(SEQ ID NO: 3).

19. An insulin analog or a physiologically tolerable salt thereof as claimed in claim 15, wherein the B chain has the sequence

His Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val  
Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 4).

20. An insulin analog or a physiologically tolerable salt thereof as claimed in claim 16, wherein the B chain has the sequence

His Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu  
Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 5).

21. An insulin analog or a physiologically tolerable salt thereof as claimed in claim 17, wherein the B chain has the sequence

His Ala Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr  
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 6).

22. A process for the preparation of an insulin analog or of a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 21, comprising the construction of a replicable expression vehicle which contains a DNA sequence which codes for a precursor of the insulin analog having the amino acid sequence II

Met- $X^2_m$ -(Arg)<sub>p</sub>-Z-R1-R2-R3-R4-His-Leu-Cys-(B8-B18)-Cys-(B20-B29)-R30-  
 $X^1_n$ -Arg-(A1-A5)-Cys-Cys-R8-Ser-R10-Cys-Ser-Leu-R14-(A15-A19)-Cys-R21  
II,

in which

$X^1_n$  is a peptide chain having n amino acid residues, where n is an integer from 0 to 34,  
 $X^2_m$  is a peptide chain having m amino acid residues, where m is an integer from 0 to 20,  
p is 0, 1 or 2,  
R30 is any desired genetically encodable amino acid residue or is absent and  
Z is absent or is a peptide residue having 1 to 4 genetically encodable amino acid residues, comprising 1 to 4 histidine residues (His)

and the other variables have the meanings mentioned in claim 1, where the provisos mentioned in claim 1 also apply, expression in a host cell and release of the insulin analog from its precursor using chemical and/or enzymatic methods.

23. A process as claimed in claim 22, wherein the host cell is a bacterium.

24. The process as claimed in claim 23, wherein the bacterium is *E. coli*.
25. The process as claimed in claim 22, wherein the host cell is a yeast.
26. The process as claimed in claim 25, wherein the yeast is *Saccharomyces cerevisiae*.
27. The process as claimed in one of claims 22 to 26 for the preparation of an insulin analog as claimed in claim 19, wherein the precursor of the insulin analog has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg  
 His Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val  
 Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr  
 Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly  
 Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg  
 Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr  
 Cys Asn

(SEQ ID NO: 7).

28. The process as claimed in one of claims 22 to 26 for the preparation of an insulin analog as claimed in claim 20, wherein the precursor of the insulin analog has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg  
 His Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu  
 Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr  
 Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly  
 Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg  
 Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr  
 Cys Asn

(SEQ ID NO: 8).

29. The process as claimed in one of claims 22 to 26 for the preparation of an insulin analog as claimed in claim 21, wherein the precursor of the insulin analog has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg  
His Ala Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr  
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr  
Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly  
Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg  
Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys  
Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn

(SEQ ID NO: 9).

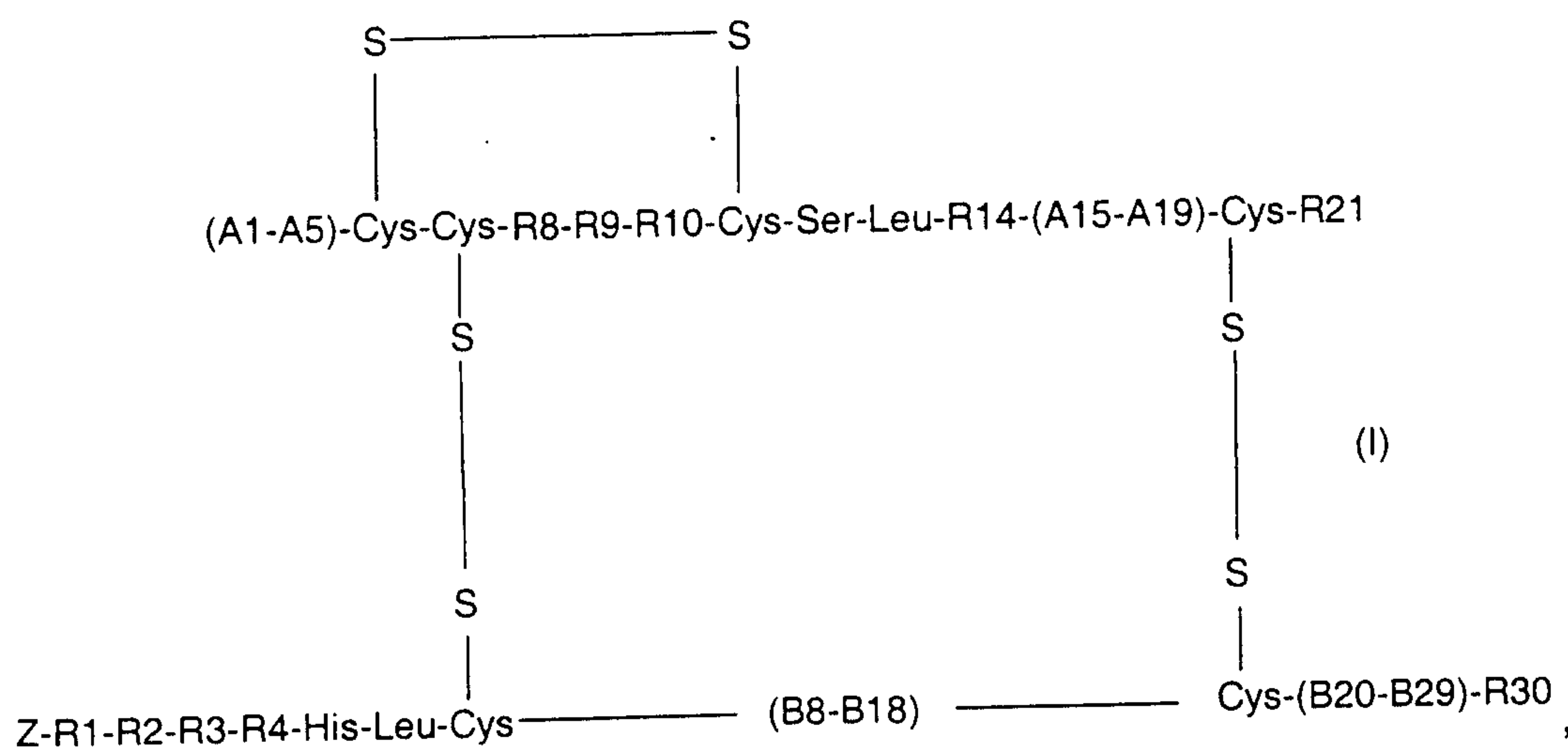
30. A precursor of the insulin analog as set forth in claim 22.
31. A precursor of the insulin analog as set forth in claim 27.
32. A precursor of the insulin analog as set forth in claim 28.
33. A precursor of the insulin analog as set forth in claim 29.
34. A DNA sequence which codes for a precursor of the insulin analog as claimed in claim 30.
35. A DNA sequence which codes for a precursor of the insulin analog as claimed in claim 31.
36. A DNA sequence which codes for a precursor of the insulin analog as claimed in claim 32.
37. A DNA sequence which codes for a precursor of the insulin analog as claimed in claim 33.
38. An expression vehicle comprising a DNA sequence as claimed in claim 34.
39. An expression vehicle comprising a DNA sequence as claimed in claim 35.
40. An expression vehicle comprising a DNA sequence as claimed in claim 36.

41. An expression vehicle comprising a DNA sequence as claimed in claim 37.
42. A host cell which is transformed using an expression vehicle as claimed in one of claims 38 to 41.
43. A pharmaceutical preparation comprising at least one insulin analog and/or at least one physiologically tolerable salt thereof as claimed in one or more of claims 1 to 21.
44. The pharmaceutical preparation as claimed in claim 43, which contains the insulin analog and/or the physiologically tolerable salt thereof in dissolved, amorphous and/or crystalline form.
45. The pharmaceutical preparation as claimed in claim 43 or 44, which furthermore contains a depot auxiliary.
46. The pharmaceutical preparation as claimed in claim 45, wherein the depot auxiliary is protamine sulfate, the insulin analog and/or the physiologically tolerable salt thereof being present in a cocrystallizate with the protamine sulfate.
47. The pharmaceutical preparation as claimed in one or more of claims 43 to 46, additionally containing unmodified human insulin.
48. The pharmaceutical preparation as claimed in one or more of claims 43 to 47, additionally containing an insulin analog.
49. The pharmaceutical preparation as claimed in claim 48, wherein the insulin analog is Gly(A21)-Arg(B31)-Arg(B32)-human insulin.
50. An injectable solution having insulin activity, comprising the pharmaceutical preparation as claimed in one or more of claims 43 to 49 in dissolved form.
51. The injectable solution as claimed in claim 50, containing 1  $\mu$ g to 2 mg of zinc per ml.

52. The injectable solution as claimed in claim 51, containing 5 µg to 200 µg of zinc per ml.

53. The use of the insulin analog and/or its physiologically tolerable salt as claimed in one or more of claims 1 to 21 for the production of a pharmaceutical preparation which has an insulin activity with a delayed onset of action.

54. An insulin-zinc complex comprising an insulin hexamer and 4 to 10 zinc ions per insulin hexamer, wherein the insulin hexamer consists of six molecules of an insulin analog of the formula I



in which

- (A1-A5) are the amino acid residues in the positions A1 to A5 of the A chain of human insulin or animal insulin,
- (A15-A19) are the amino acid residues in the positions A15 to A19 of the A chain of human insulin or animal insulin,
- (B8-B18) are the amino acid residues in the positions B8 to B18 of the B chain of human insulin or animal insulin,
- (B20-B29) are the amino acid residues in the positions B20 to B29 of the B chain of human insulin or animal insulin,
- R8 is Thr or Ala,
- R9 is Ser or Gly,
- R10 is Ile or Val,
- R14 is Tyr, His, Asp or Glu,
- R21 is Asn, Asp, Gly, Ser, Thr, Ala, Glu or Gln,
- R1 is any desired genetically encodable amino acid residue, absent or a hydrogen atom,

- R2 is Val, Ala or Gly,  
 R3 is Asn, His, Glu or Asp,  
 R4 is Ala, Ser, Thr, Asn, Asp, Gln, Gly or Glu,  
 R30 is any desired genetically encodable amino acid residue or -OH,  
 Z is a hydrogen atom or a peptide residue having 1 to 4 genetically encodable amino acid residues, comprising 1 to 4 histidine residues (His).

55. The insulin-zinc complex as claimed in claim 54, comprising 5 to 8 zinc ions per insulin hexamer.

56. The insulin-zinc complex as claimed in claim 54 or 55, wherein the insulin hexamer consists of six molecules of the insulin analog of the formula I as claimed in one or more of claims 1 to 21.

57. The insulin-zinc complex as claimed in claim 54 or 55, wherein the B chain of the insulin analog of the formula I has the sequence

Phe Val His Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys  
 Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 10).

58. The insulin-zinc complex as claimed in claim 54 or 55, wherein the B chain of the insulin analog of the formula I has the sequence

Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys  
 Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr

(SEQ ID NO: 11).

59. A pharmaceutical preparation comprising at least one insulin-zinc complex as claimed in one or more of claims 54 to 58.

60. A pharmaceutical preparation comprising an acidic solution of at least one insulin analog as claimed in one or more of claims 54 to 58 and/or a physiologically tolerable salt thereof with an appropriate amount of zinc ions, which makes possible the formation of an insulin-zinc complex as claimed in one or more of claims 54 to 58.

61. The pharmaceutical preparation as claimed in claim 60, comprising an acidic solution of the insulin analog and/or the physiologically tolerable salt thereof as claimed in one or more of claims 1 to 21 with an appropriate amount of zinc ions which makes possible the formation of an insulin-zinc complex as claimed in claim 56.

62. The pharmaceutical preparation as claimed in claim 60, comprising an acidic solution of at least one insulin analog as claimed in one of claims 57 or 58 and/or a physiologically tolerable salt thereof with an appropriate amount of zinc ions, which makes possible the formation of an insulin-zinc complex as claimed in one of claims 57 or 58.

63. The pharmaceutical preparation as claimed in one or more of claims 59 to 62, which contains the insulin-zinc complex in dissolved, amorphous and/or crystalline form.

64. An injectable solution having insulin activity, comprising the pharmaceutical preparation as claimed in claim 63 in dissolved form.

65. The injectable solution as claimed in claim 64, containing from 1  $\mu\text{g}$  to 2 mg of zinc per ml.

66. The injectable solution as claimed in claim 65, containing from 5  $\mu\text{g}$  to 200  $\mu\text{g}$  of zinc per ml.

67. The use of the insulin-zinc complex as claimed in one or more of claims 54 to 58 for the production of a pharmaceutical preparation which has an insulin activity having a delayed onset of action.