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(54) **Biologically active fragments of glucagon-like insulinotropic peptide**

Biologisch aktive Fragmente des Glucagon ähnlichen, insulinotropen Peptides

Fragments biologiquement actifs de peptide insalinotrope de type glucagon

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(56) References cited:
**WO-A-93/25579**

- **ENDOCRINOLOGY (BALTIMORE) (1989), 125(6), 3109-14 CODEN: ENDOAO;ISSN: 0013-7227, 1989, XP000574778 SUZUKI, SEIJI ET AL: "Comparison of the effects of various C-terminal and N-terminal fragment peptides of glucagon-like peptide-1 on insulin and glucagon release from the isolated perfused rat pancreas"**
- **BIOMED. RES. (1988), 9(SUPPL. 3), 213-17 CODEN: BRESD5;ISSN: 0388-6107, 1988, XP000574772 KAWAI, KOICHI ET AL: "The biological effects of glucagon-like peptide-1 (GLP-1) and its structure-activity relationship"**
- **INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH, vol. 40, no. 3 / 04, 1 September 1992, pages 333-343, XP000311244 SVETLANA MOJSOV: "STRUCTURAL REQUIREMENTS FOR BIOLOGICAL ACTIVITY OF GLUCAGON-LIKE PEPTIDE-I"**
- **ENDOCRINOLOGY (BALTIMORE) (1990), 126(4), 2164-8 CODEN: ENDOAO;ISSN: 0013-7227, 1990, XP000574862 GEFEL, DOV ET AL: "Glucagon-like peptide-I analogs: effects on insulin secretion and adenosine 3',5'-monophosphate formation"**
- **J. CLIN. ENDOCRINOL. METAB. (1995), 80(3), 952-7 CODEN: JCEMAZ;ISSN: 0021-972X, 1995, XP000574780 DEACON, CAROLYN F. ET AL: "Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo"**
- **ENDOCRINOLOGY (1995), 136(8), 3585-97 CODEN: ENDOAO;ISSN: 0013-7227, 1995, XP000574863 KIEFFER, TIMOTHY J. ET AL: "Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV"**



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## Description

### Field of Invention

**[0001]** The present invention relates to medicinal chemistry and provides novel peptides and compositions thereof that are useful for treating diabetes.

### Background of the Invention

**[0002]** Endocrine secretions of pancreatic islets are regulated by complex control mechanisms driven not only by blood-borne metabolites such as glucose, amino acids, and catecholamines, but also by local paracrine influences. The major pancreatic islet hormones, glucagon, insulin and somatostatin, interact with specific pancreatic cell types (A, B, and D cells, respectively) to modulate the secretory response. Although insulin secretion is predominantly controlled by blood glucose levels, somatostatin inhibits glucose-mediated insulin secretion.

**[0003]** The human hormone glucagon is a 29-amino acid hormone produced in pancreatic A-cells. The hormone belongs to a multi-gene family of structurally related peptides that include secretin, gastric inhibitory peptide, vasoactive intestinal peptide and glicentin. These peptides variously regulate carbohydrate metabolism, gastrointestinal motility and secretory processing. However, the principal recognized actions of pancreatic glucagon are to promote hepatic glycogenolysis and glyconeogenesis, resulting in an elevation of blood sugar levels. In this regard, the actions of glucagon are counter regulatory to those of insulin and may contribute to the hyperglycemia that accompanies Diabetes mellitus (Lund, P.K., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.,* 79:345-349 (1982)).

**[0004]** When glucagon binds to its receptor on insulin producing cells, cAMP production increases which in turn stimulates insulin expression (Korman, L.Y., *et al.*, *Diabetes,* 34:717-722 (1985)). Moreover, high levels of insulin down-regulate glucagon synthesis by a feedback inhibition mechanism (Ganong, W.F., *Review of Medical Physiology,* Lange Publications, Los Altos, California, p. 273 (1979)). Thus, the expression of glucagon is carefully regulated by insulin, and ultimately by serum glucose levels.

**[0005]** Preproglucagon, the zymogen form of glucagon, is translated from a 360 base pair gene and is processed to form proglucagon (Lund, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 79:345-349 (1982)). Patzelt, *et al.* (*Nature*, 282:260-266 (1979)) demonstrated that proglucagon is further processed into glucagon and a second peptide. Later experiments demonstrated that proglucagon is cleaved carboxyl to Lys-Arg or Arg-Arg residues (Lund, P.K., et *al.,* Lopez L.C., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.,* 80:5485-5489 (1983), and Bell, G.I., *et al.*, *Nature* 302:716-718 (1983)). Bell, G.I., *et al.,* also discovered that proglucagon contained three discrete and highly homologous peptide regions which were designated glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2). Lopez, *et al.*, demonstrated that GLP-1 was a 37 amino acid peptide and that GLP-2 was a 34 amino acid peptide. Analogous studies on the structure of rat preproglucagon revealed a similar pattern of proteolytic cleavage at Lys-Arg or Arg-Arg residues, resulting in the formation of glucagon, GLP-1, and GLP-2 (Heinrich, G., *et al.*, *Endocrinol.,* 115:2176-2181 (1984)). Finally, human, rat, bovine, and hamster sequences of GLP-1 have been found to be identical (Ghiglione, M., *et al.*, *Diabetologia,* 27:599-600 (1984)).

**[0006]** The conclusion reached by Lopez, *et al.,* regarding the size of GLP-1 was confirmed by studying the molecular forms of GLP-1 found in the human pancreas (Uttenthal, L.O., *et al. J. Clin. Endocrinol. Metabol.*, 61:472-479 (1985)). Their research showed that GLP-1 and GLP-2 are present in the pancreas as 37 and 34 amino acid peptides respectively.

**[0007]** The similarity between GLP-1 and glucagon suggested to early investigators that GLP-1 might have biological activity. Although some investigators found that GLP-1 could induce rat brain cells to synthesize cAMP (Hoosein, N. M., *et al.*, *Febs Lett.* 178:83-86 (1984)), other investigators failed to identify any physiological role for GLP-1 (Lopez, L.C., *et al. supra*). However, GLP-1 is now known to stimulate insulin secretion (insulinotropic action) causing glucose uptake by cells which decreases serum glucose levels (*see,* e g., Mojsov, S., *Int. J. Peptide Protein Research,* 40: 333-343 (1992)).

**[0008]** Numerous GLP-1 analogs demonstrating insulinotropic action are known in the art. These variants and analogs include, for example, GLP-1(7-36), $Gln^{9}$-GLP-1(7-37), D-$Gln^{9}$-GLP-1(7-37), acetyl-$Lys^{9}$-GLP-1(7-37), $Thr^{16}$-$Lys^{18}$-GLP-1(7-37), and $Lys^{18}$-GLP-1(7-37). Derivatives of GLP-1 include, for example, acid addition salts, carboxylate salts, lower alkyl esters, and amides (*see,* e.g., WO91/11457 (1991)). More importantly, it was demonstrated using GLP-1(8-37)OH that the histidine residue at position 7 is very important to insulinotropic activity of GLP-1 (Suzuki, S., *et al. Diabetes Res.*; *Clinical Practice* 5 (Supp. 1):S30 (1988).

**[0009]** *Endocrinology, Volume 125, Number 6, Issued 1989, Suzuki et al.*, "Comparison of the Effects of Various C Terminal and N-Terminal Fragment Peptides of Glucagen-Like Peptide-1 on Insulin and Glucagon Release from the Isolated Perfused Rat Pancreas", pages 3109-3114 teaches that truncated glucagon-like peptide-1 (GLP-1) posseses a potent stimulatory activity for insulin production. The activities of N and C-terminal fragments were examined.

**[0010]** In view of the above, it was most surprising when the present inventors discovered that administering N-terminal deletion mutants of GLP-1 to experimental animals caused an increase in serum glucose uptake in the absence of any insulinotropic activity. This discovery suggests that an entirely new mechanism for lowering elevated blood glucose levels may exist and directly lead to the present invention.

**[0011]** Accordingly, the primary object of this invention is to provide novel, C-terminal GLP-1 fragments having no insulinotropic action but which are nonetheless useful for treating diabetes and hyperglycemic conditions. Further objects of the present invention are pharmaceutical compositions that contain biologically-active GLP-1 fragments, as well as methods for using such compounds to treat diabetes.

## Summary of the Invention

**[0012]** According to a first aspect of the present invention there is provided a protected, unprotected, or partially protected GLP-1 fragment selected from the following formulae:

$H_2N$-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys- Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-$R^1$;

$H_2N$-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys- Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-$R^1$;

$H_2N$-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-$R^1$; and

$H_2N$-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys- Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-$R^2$;

wherein $R^1$ is selected from $NH_2$, OH, Gly-$NH_2$, and Gly-OH;1 $R^2$ is selected from OH and $NH_2$.

**[0013]** Preferably, the formula of the GLP-1 fragment is:

$H_2N$-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys- Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-$R^1$ wherein $R^1$ is selected from $NH_2$, OH, Gly-$NH_2$, and Gly-OH;

$H_2N$-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys- Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-$R^1$ wherein $R^1$ is selected from $NH_2$, OH, Gly-$NH_2$, and Gly-OH;

$H_2N$-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys- Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-$R^1$

wherein $R^1$ is selected from $NH_2$, OH, Gly-$NH_2$, and Gly-OH;

$H_2N$-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys- Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-$R^1$

wherein $R^2$ is selected from $NH_2$ and OH.

**[0014]** The GLP-1 fragment is preferably protected or partially protected. In particular, the side chain of the amino acid Lys may be protected. The side chain of the amino acid Lys may be protected by acetyl or C1-Z.

**[0015]** According to a second aspect of the present invention, there is provided a GLP-1 fragment of the present invention, for use in treating diabetes or hyperglycemia in a mammal.

**[0016]** According to a third aspect of the present invention, there is provided a pharmaceutical composition comprising a GLP-1 fragment of the present invention, in combination with a pharmaceutical carrier, diluent or excipient.

**[0017]** The composition may be used for use in treating diabetes or hyperglycemia in a mammal.

**[0018]** According to a further aspect of the present invention, there is provided the use of a GLP-1 fragment according to the present invention in the preparation of a medicament for the treatment of diabetes.

## Detailed Description of the Invention

**[0019]** In one embodiment, the present invention provides novel, biologically-active, C-terminal fragments of GLP-1. For purposes of this specification, the term "biologically-active" refers to the ability of a substance to lower elevated levels of blood glucose in a mammal without stimulating insulin secretion.

**[0020]** Given the sequence information herein disclosed and the state of the art in solid phase protein synthesis, biologically-active GLP-1 fragments can be obtained via chemical synthesis. However, it also is possible to obtain a biologically-active GLP-1 fragment by fragmenting proglucagon using, for example, proteolytic enzymes. Moreover, recombinant DNA techniques may be used to express biologically-active GLP-1 fragments.

**[0021]** The principles of solid phase chemical synthesis of polypeptides are well known in the art and may be found in general texts in the area such as Dugas, H. and Penney, C., Bioorganic Chemistry (1981) Springer-Verlag, New York, pgs. 54-92, Merrifield, J.M., *Chem. Soc.,* 85:2149 (1962), and Stewart and Young, *Solid Phase Peptide Synthesis,* pp. 24-66, Freeman (San Francisco, 1969).

**[0022]** For example, a biologically-active GLP-1 fragment may be synthesized by solid-phase methodology utilizing an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404) and synthesis cycles supplied by Applied Biosystems. Boc amino acids and other reagents are commercially available from Applied Biosystems and other chemical supply houses. Sequential Boc chemistry using double couple protocols are applied to the starting p-methyl benzhydryl amine resins for the production of C-terminal carboxamides. For the production of C-terminal acids, the corresponding PAM resin is used. Asp, Gln, and Arg are coupled using preformed hydroxy benzotriazole esters. The following side chain protecting groups may be used:

Arg, Tosyl
Asp, cyclohexyl
Glu, cyclohexyl
Ser, Benzyl
Thr, Benzyl
Tyr, 4-bromo carbobenzoxy

**[0023]** Boc deprotection may be accomplished with trifluoroacetic acid in methylene chloride. Following completion of the synthesis the peptides may be deprotected and cleaved from the resin with anhydrous hydrogen fluoride (HF) containing 10% meta-cresol. Cleavage of the side chain protecting group(s) and of the peptide from the resin is carried

out at zero degrees centigrade or below, preferably -20°C for thirty minutes followed by thirty minutes at 0°C. After removal of the HF, the peptide/resin is washed with ether, and the peptide extracted with glacial acetic acid and lyophilized.

**[0024]** The preparation of protected, unprotected, and partially protected GLP-1 has been described in the art. See U.S. Pat. No. 5,120,712 and 5,118,666, herein incorporated by reference, and Orskov, C., *et al*., *J. Biol. Chem.,* 264 (22):12826-12829 (1989) and WO 91/11457 (Buckley, D.I., *et al*., published August 8, 1991).

**[0025]** Likewise, the state of the art in molecular biology provides the ordinarily skilled artisan another means by which biologically-active GLP-1 fragments can be obtained. Although GLP-1 fragments may be produced by solid phase peptide synthesis, recombinant methods, or by fragmenting glucagon, recombinant methods may be preferable because higher yields are possible. The basic steps in the recombinant production of a biologically-active GLP-1 fragment are:

a) isolating a natural DNA sequence encoding GLP-1 or constructing a synthetic or semi-synthetic DNA coding sequence for GLP-1,

b) placing the coding sequence into an expression vector in a manner suitable for expressing proteins either alone or as a fusion proteins,

c) transforming an appropriate eukaryotic or prokaryotic host cell with the expression vector,

d) culturing the transformed host cell under conditions that will permit expression of a GLP-1 intermediate, and

e) recovering and purifying the recombinantly produced protein.

**[0026]** As previously stated, the coding sequences for GLP-1 fragments may be wholly synthetic or the result of modifications to the larger, native glucagon-encoding DNA. A DNA sequence that encodes preproglucagon is presented in Lund, *et al., Proc. Natl. Acad. Sci. U.S.A.* 79:345-349 (1982) and may be used as starting material in the recombinant production of a biologically-active GLP-1 fragment by altering the native sequence to achieve the desired results.

**[0027]** Synthetic genes, the *in vitro* or *in vivo* transcription and translation of which results in the production of a biologically-active GLP-1 fragment, may be constructed by techniques well known in the art. Owing to the natural degeneracy of the genetic code, the skilled artisan will recognize that a sizable yet definite number of DNA sequences may be constructed which encode GLP-1 intermediates.

**[0028]** The methodology of synthetic gene construction is well known in the art. *See* Brown, *et al.* (1979) *Methods in Enzymology,* Academic Press, N.Y., Vol. 68, pgs. 109-151. DNA sequences that encode GLP-1 intermediates can be designed based on the amino acid sequences herein disclosed. Once designed, the sequence itself may be generated using conventional DNA synthesizing apparatus such as the Applied Biosystems Model 380A or 380B DNA synthesizers (Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404).

**[0029]** To effect the expression of a biologically-active GPL-1 fragment, one inserts the engineered synthetic DNA sequence in any one of many appropriate recombinant DNA expression vectors through the use of appropriate restriction endonucleases. *See generally* Maniatis *et al.* (1989) *Molecular Cloning*; *A Laboratory Manual,* Cold Springs Harbor Laboratory Press, N.Y., Vol. 1-3. Restriction endonuclease cleavage sites are engineered into either end of the DNA encoding the GLP-1 fragment to facilitate isolation from, and integration into, known amplification and expression vectors. The particular endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the parent expression vector to be employed. The choice of restriction sites are chosen so as to properly orient the coding sequence with control sequences to achieve proper in-frame reading and expression of the protein of interest. The coding sequence must be positioned so as to be in proper reading frame with the promoter and ribosome binding site of the expression vector, both of which are functional in the host cell in which the protein is to be expressed.

**[0030]** To achieve efficient transcription of the coding region, it must be operably associated with a promoter-operator region. Therefore, the promoter-operator region of the gene is placed in the same sequential orientation with respect to the ATG start codon of the coding region.

**[0031]** A variety of expression vectors useful for transforming prokaryotic and eukaryotic cells are well known in the art. *See The Promega Biological Research Products Catalogue* (1992) (Promega Corp., 2800 Woods Hollow Road, Madison, WI, 53711-5399); and *The Stratagene Cloning Systems Catalogue* (1992) (Stratagene Corp., 11011 North Torrey Pines Road, La Jolla, CA, 92037). Also, U.S. Patent No. 4,710,473 describes circular DNA plasmid transformation vectors useful for expression of exogenous genes in *E. coli* at high levels. These plasmids are useful as transformation vectors in recombinant DNA procedures and:

(a) confer on the plasmid the capacity for autonomous replication in a host cell;

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