











































Val<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,36</sup>-bis-(GAB-GLit) GLP-1(7-36);  
 Val<sup>8</sup>Arg<sup>34</sup>Lys<sup>26, 36</sup>-bis-(GAB-GLit) GLP-1(7-36);  
 Val<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,36</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Val<sup>8</sup>Arg<sup>34</sup>Lys<sup>26, 36</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Val<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,37</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Val<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,37</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Val<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Val<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Val<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Val<sup>8</sup>Arg<sup>26</sup>Lys<sup>34, 39</sup>-bis-(GAB-GLit) GLP-1(7-39);  
 Val<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,39</sup>-bis-(GAB-GLit) GLP-1(7-39);  
 Val<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36,39</sup>-bis-(GAB-GLit) GLP-1(7-39);  
 Ser<sup>8</sup>Lys<sup>26, 34</sup>-bis-(GAB-GLit) GLP-1(7-36); Ser<sup>8</sup>Lys<sup>26, 34</sup>-bis-(GAB-GLit) GLP-1(7-37); Ser<sup>8</sup>Lys<sup>26, 34</sup>-bis-(GAB-GLit) GLP-1(7-38); Ser<sup>8</sup>Lys<sup>26, 34</sup>-bis-(GAB-GLit) GLP-1(7-39)  
 Ser<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,36</sup>-bis-(GAB-GLit) GLP-1(7-36);  
 Ser<sup>8</sup>Arg<sup>34</sup>Lys<sup>26, 36</sup>-bis-(GAB-GLit) GLP-1(7-36);  
 Ser<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,36</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Ser<sup>8</sup>Arg<sup>34</sup>Lys<sup>26, 36</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Ser<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,37</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Ser<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,37</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Ser<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Ser<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Ser<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Ser<sup>8</sup>Arg<sup>26</sup>Lys<sup>34, 39</sup>-bis-(GAB-GLit) GLP-1(7-39);  
 Ser<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,39</sup>-bis-(GAB-GLit) GLP-1(7-39);  
 Ser<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36,39</sup>-bis-(GAB-GLit) GLP-1(7-39);  
 Thr<sup>8</sup>Lys<sup>26, 34</sup>-bis-(GAB-GLit) GLP-1(7-36); Thr<sup>8</sup>Lys<sup>26, 34</sup>-bis-(GAB-GLit) GLP-1(7-37); Thr<sup>8</sup>Lys<sup>26, 34</sup>-bis-(GAB-GLit) GLP-1(7-38); Thr<sup>8</sup>Lys<sup>26, 34</sup>-bis-(GAB-GLit) GLP-1(7-39)  
 Thr<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,36</sup>-bis-(GAB-GLit) GLP-1(7-36);  
 Thr<sup>8</sup>Arg<sup>34</sup>Lys<sup>26, 36</sup>-bis-(GAB-GLit) GLP-1(7-36);  
 Thr<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,36</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Thr<sup>8</sup>Arg<sup>34</sup>Lys<sup>26, 36</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Thr<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,37</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Thr<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,37</sup>-bis-(GAB-GLit) GLP-1(7-37);  
 Thr<sup>8</sup>Arg<sup>26</sup>Lys<sup>34,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Thr<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Thr<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36,38</sup>-bis-(GAB-GLit) GLP-1(7-38);  
 Thr<sup>8</sup>Arg<sup>26</sup>Lys<sup>34, 39</sup>-bis-(GAB-GLit) GLP-1(7-39);  
 Thr<sup>8</sup>Arg<sup>34</sup>Lys<sup>26,39</sup>-bis-(GAB-GLit) GLP-1(7-39);  
 Thr<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36,39</sup>-bis-(GAB-GLit) GLP-1(7-39).

#### Pharmaceutical Compositions

The present invention also relates to pharmaceutical compositions comprising a GLP-1 derivative of the present invention and a pharmaceutically acceptable vehicle or carrier.

Preferably, the pharmaceutical compositions comprise an isotonic agent, a preservative and a buffer. Examples of isotonic agents are sodium chloride, mannitol and glycerol. Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol. Suitable buffers include sodium acetate and sodium phosphate.

The pharmaceutical compositions preferably further comprise a surfactant in order to improve the solubility and/or the stability of the GLP-1 derivative. Individual embodiments of the surfactant, such as a detergent, for use in the pharmaceutical composition of the invention include ethoxylated castor oil, polyglycolized glycerides, acetylated monoglycerides, sorbitan fatty acid esters, poloxamers, such as 188 and 407, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene derivatives such as alkylated and alkoxy-

ethylene derivatives thereof, glycerol, cholic acid or derivatives thereof, lecithins, alcohols and phospholipids, glycerophospholipids (lecithins, cephalins, phosphatidyl serine), glyceroglycolipids (galactopyransoide), sphingophospholipids (sphingomyelin), and sphingoglycolipids (ceramides, gangliosides), DSS (docusate sodium, CAS registry no [577-11-7]), docusate calcium, CAS registry no [128-49-4]), docusate potassium, CAS registry no [7491-09-0]), SDS (sodium dodecyl sulfate or sodium lauryl sulfate), dipalmitoyl phosphatidic acid, sodium caprylate, bile acids and salts thereof and glycine or taurine conjugates, ursodeoxycholic acid, sodium cholate, sodium deoxycholate, sodium taurocholate, sodium glycocholate, N-Hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, anionic (alkylaryl-sulphonates) monovalent surfactants, palmitoyl lysophosphatidyl-L-serine, lysophospholipids (e.g. 1-acyl-sn-glycero-3-phosphate esters of ethanolamine, choline, serine or threonine), alkyl, alkoxy (alkyl ester), alkoxy (alkyl ether)-derivatives of lysophosphatidyl and phosphatidylcholines, e.g. lauroyl and myristoyl derivatives of lysophosphatidylcholine, and modifications of the polar head group, that is cholines, ethanolamines, phosphatidic acid, serines, threonines, glycerol, inositol, and the positively charged DODAC, DOTMA, DCP, BISHOP, lysophosphatidylserine and lysophosphatidylthreonine, zwitterionic surfactants (e.g. N-alkyl-N,N-dimethylammonio-1-propanesulfonates, 3-cholamido-1-propyldimethylammonio-1-propanesulfonate, dodecylphosphocholine, myristoyl lysophosphatidylcholine, hen egg lysolecithin), cationic surfactants (quaternary ammonium bases) (e.g. cetyltrimethylammonium bromide, cetylpyridinium chloride), non-ionic surfactants, polyethyleneoxide/polypropyleneoxide block copolymers (Pluronic/Tetronics, Triton X-100, Dodecyl β-D-glucopyranoside) or polymeric surfactants (Tween-40, Tween-80, Brij-35). Other preferred surfactants include fusidic acid derivatives-(e.g. sodium tauro-dihydrofusidate etc.), long-chain fatty acids and salts thereof C6-C12(e.g. oleic acid and caprylic acid), acylcarnitines and derivatives, N<sup>α</sup>-acylated derivatives of lysine, arginine or histidine, or side-chain acylated derivatives of lysine or arginine, N<sup>α</sup>-acylated derivatives of dipeptides comprising any combination of lysine, arginine or histidine and a neutral or acidic amino acid, N<sup>α</sup>-acylated derivative of a tripeptide comprising any combination of a neutral amino acid and two charged amino acids, or the surfactant may be selected from the group of imidazoline derivatives.

One group of preferred surfactants consists of zwitterionic surfactants, cationic surfactants, non-ionic surfactants and polymeric surfactants.

Another group of preferred surfactants consists of SDS, sodium caprylate, sodium cholate, sodium deoxycholate, sodium taurocholate and sodium glycocholate.

A further group of preferred surfactants consists of lauroyl lysophosphatidylcholine, palmitoyl lysophosphatidyl-L-serine, myristoyl lysophosphatidylcholine and N-Hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate.

The pharmaceutical compositions preferably also comprise zinc.

The pharmaceutical compositions preferably further comprise another antidiabetic agent. The term "antidiabetic agent" includes compounds for the treatment and/or prophylaxis of insulin resistance and diseases wherein insulin resistance is the pathophysiological mechanism.

In one embodiment of this invention, the antidiabetic agent is an insulin, more preferably human insulin.

In another embodiment the antidiabetic agent is a hypoglycaemic agent, preferably an oral hypoglycaemic agent. Oral hypoglycaemic agents are preferably selected from the group consisting of sulfonylureas, biguanides, thiazolidinediones, glucosidase inhibitors, glucagon antagonists, GLP-1 agonists, potassium channel openers, insulin sensitizers, hepatic enzyme inhibitors, glucose uptake modulators, compounds modifying the lipid metabolism, compounds lowering food intake, and agents acting on the ATP-dependent potassium channel of the  $\beta$ -cells.

Preferred sulfonylureas are tolbutamide, glibenclamide, glipizide and gliclazide. A preferred biguanide is metformin. Preferred thiazolidinediones are troglitazone and ciglitazone. A preferred glucosidase inhibitor is acarbose. Preferred agents acting on the ATP-dependent potassium channel of the  $\beta$ -cells are: glibenclamide, glipizide, gliclazide, and repaglinide.

The pharmaceutical compositions of the present invention may further comprise another antiobesity agent.

In one embodiment of this invention, the antiobesity agent is leptin.

In another embodiment the antiobesity agent is amphetamine.

In another embodiment the antiobesity agent is dexfenfluramine.

In another embodiment the antiobesity agent is sibutramine.

In another embodiment the antiobesity agent is orlistat.

In another embodiment the antiobesity agent is selected from a group of CART agonists, NPY antagonists, orexin antagonists, H3-antagonists, TNF agonists, CRF agonists, CRF BP antagonists, urocortin agonists,  $\beta$ 3 agonists, MSH agonists, CCK agonists, serotonin re-uptake inhibitors, mixed serotonin and noradrenergic compounds, 5HT agonists, bombesin agonists, galanin antagonists, growth hormone, growth hormone releasing compounds, glucagon, TRH agonists, uncoupling protein 2 or 3 modulators, leptin agonists, DA agonists (Bromocriptin, Doprexin), lipase/amylase inhibitors, PPAR modulators, PXR modulators or TR P agonists.

The present invention also relates to pharmaceutical compositions comprising water and a GLP-1 derivative which has a helix content as measured by CD at 222 nm in H<sub>2</sub>O at 22 $\pm$ 2° C. exceeding 25%, preferably in the range of 25% to 50%, at a peptide concentration of about 10  $\mu$ M. The size of the partially helical, micelle-like aggregates may be estimated by size-exclusion chromatography. Similarly, the apparent (critical micelle concentrations) CMC's of the peptides may be estimated from the concentration dependent fluorescence in the presence of appropriate dyes (e.g. Brito, R. & Vaz, W. (1986) Anal. Biochem. 152, 250-255).

That the derivatives have a partially structured micellar-like aggregate conformation in aqueous solutions makes them more soluble and stable in solution as compared to the native peptide. The increased solubility and stability can be seen by comparing the solubility after 9 days of standing for a derivative and native GLP-1(7-37) in a pharmaceutical formulation, e.g. 5 mM phosphate buffer, pH 6.9 added 0.1 M NaCl.

Circular Dichroism (CD) can be used to show that the GLP-1 derivatives have a certain partially structured conformation independent of their concentration. In contrast, for

native GLP-1(7-37) an increase in the helix content is seen with increasing concentration, from 10-15% to 30-35% (at 500  $\mu$ M concentration) in parallel with peptide self-association. For the GLP-1 derivatives forming partially structured micellar-like aggregates in aqueous solution the helix content remains constant above 30% at concentrations of 10  $\mu$ M. The aggregated structured conformation is an inherent property of the derivative present in water or dilute aqueous buffer without the need for any additional structure-inducing components.

The pharmaceutical compositions of the present invention may be prepared by conventional techniques, e.g. as described in Remington's *Pharmaceutical Sciences*, 1985 or in Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

For example, injectable compositions of the GLP-1 derivative of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

A composition for nasal administration of certain peptides may, for example, be prepared as described in European Patent No. 272097(to Novo Nordisk A/S) or in WO 93/18785.

In a preferred embodiment of the present invention, the GLP-1 derivative is provided in the form of a composition suitable for administration by injection. Such a composition can either be an injectable solution ready for use or it can be an amount of a solid composition, e.g. a lyophilised product, which has to be dissolved in a solvent before it can be injected. The injectable solution preferably contains not less than about 2 mg/ml, preferably not less than about 5 mg/ml, more preferred not less than about 10 mg/ml of the GLP-1 derivative and, preferably, not more than about 100 mg/ml of the GLP-1 derivative.

#### Uses

The present invention also relates to the use of a GLP-1 derivative of the invention for the preparation of a medicament which has a protracted profile of action relative to GLP-1(7-37).

The present invention relates also to the use of a GLP-1 derivative of the invention for the preparation of a medicament with protracted effect for the treatment of non-insulin dependent diabetes mellitus.

The present invention also relates to the use of a GLP-1 derivative of the invention for the preparation of a medicament with protracted effect for the treatment of insulin dependent diabetes mellitus.

The present invention also relates to the use of a GLP-1 derivative of the invention for the preparation of a medicament with protracted effect for the treatment of obesity.

The present invention also relates to the use of a GLP-1 derivative of the present invention for treating insulin resistance.

The present invention also relates to the use of a GLP-1 derivative of the present invention for the preparation of a medicament with protracted effect for the treatment of obesity.

The present invention relates to a method of treating insulin dependent or non-insulin dependent diabetes mellitus in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of a GLP-1 derivative of the present invention together with a pharmaceutically acceptable carrier.

The present invention relates to a method of treating obesity in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of a GLP-1 derivative of the present invention together with a pharmaceutically acceptable carrier.

The particular GLP-1 derivative to be used and the optimal dose level for any patient will depend on the disease to be treated and on a variety of factors including the efficacy of the specific peptide derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case.

The pharmaceutical compositions of the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the GLP-1 derivative in the form of a nasal or pulmonary spray. As a still further option, the GLP-1 derivatives of the invention can also be administered transdermally, e.g. from a patch, optionally a iontophoretic patch, or transmucosally, e.g. buccally.

#### Methods of Production

The parent peptide can be produced by a method which comprises culturing a host cell containing a DNA sequence encoding the polypeptide and capable of expressing the polypeptide in a suitable nutrient medium under conditions permitting the expression of the peptide, after which the resulting peptide is recovered from the culture.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared of published recipes (e.g. in catalogues of the American Type Culture Collection). The peptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like, dependent on the type of peptide in question.

The DNA sequence encoding the parent peptide may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the peptide by hybridisation using synthetic oligonucleotide probes in accordance with standard techniques (see, for example, Sambrook, J, Fritsch, EF and Maniatis, T, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989). The DNA sequence encoding the peptide may also be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage and Caruthers, *Tetrahedron Letters* 22(1981), 1859-1869, or the method described by Matthes et al., *EMBO Journal* 3(1984), 801-805. The DNA sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in U.S. Pat. No. 4,683,202 or Saiki et al., *Science* 239(1988), 487-491.

The DNA sequence may be inserted into any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the peptide is operably linked to additional segments required for transcription of the DNA, such as a promoter. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the peptide of the invention in a variety of host cells are well known in the art, cf. for instance Sambrook et al., supra.

The DNA sequence encoding the peptide may also, if necessary, be operably connected to a suitable terminator, polyadenylation signals, transcriptional enhancer sequences, and translational enhancer sequences. The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate.

To direct a parent peptide of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the peptide in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that normally associated with the peptide or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the present peptide, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., supra).

The host cell into which the DNA sequence or the recombinant vector is introduced may be any cell which is capable of producing the present peptide and includes bacteria, yeast, fungi and higher eukaryotic cells. Examples of suitable host cells well known and used in the art are, without limitation, *E. coli*, *Saccharomyces cerevisiae*, or mammalian BHK or CHO cell lines.

The GLP-1 derivatives of this invention can be used in the treatment of various diseases.

The particular GLP-1 derivative to be used and the optimal dose level for any patient will depend on the disease to be treated and on a variety of factors including the efficacy of the specific peptide derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity

of the case. It is recommended that the dosage of the GLP-1 derivative of this invention be determined for each individual patient by those skilled in the art.

In particular, it is envisaged that the GLP-1 derivative will be useful for the preparation of a medicament with a protracted profile of action for the treatment of non-insulin dependent diabetes mellitus and/or for the treatment of obesity.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

#### EXAMPLES

The following acronyms for commercially available chemicals are used:

DMF: N,N-Dimethylformamide

DCC: N,N-Dicyclohexylcarbodiimide

NMP: N-Methyl-2-pyrrolidone

EDPA: N-Ethyl-N,N-diisopropylamine

EGTA: Ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid

GTP: Guanosine 5'-triphosphate

TFA: Trifluoroacetic acid

THF: Tetrahydrofuran

H-Glu(OH)-OBu<sup>t</sup>: L-Glutamic acid  $\alpha$ -tert-butyl ester

Cae-ONSu: Decanoic acid 2,5-dioxopyrrolidin-1-yl ester

Cap-ONSu: Octanoic acid 2,5-dioxopyrrolidin-1-yl ester

Lau-ONSu: Dodecanoic acid 2,5-dioxopyrrolidin-1-yl ester

Myr-ONSu: Tetradecanoic acid 2,5-dioxopyrrolidin-1-yl ester

Pal-ONSu: Hexadecanoic acid 2,5-dioxopyrrolidin-1-yl ester

Ste-ONSu: Octadecanoic acid 2,5-dioxopyrrolidin-1-yl ester

Abbreviations:

PDMS: Plasma Desorption Mass Spectrometry

MALDI-MS: Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry

HPLC: High Performance Liquid Chromatography

amu: atomic mass units

Lit-Glu(ONSu)-OBu<sup>t</sup>: N <sup>$\alpha$</sup> -Lithochoyl-L-glutamic acid  $\alpha$ -t-butyl ester  $\gamma$ -2,5-dioxopyrrolidin-1-yl ester

Cap-Glu(ONSu)-OBu<sup>t</sup>: N <sup>$\alpha$</sup> -Octanoyl-L-glutamic acid  $\alpha$ -t-butyl ester  $\gamma$ -2,5-dioxopyrrolidin-1-yl ester

Cae-Glu(ONSu)-OBu<sup>t</sup>: N <sup>$\alpha$</sup> -Decanoyl-L-glutamic acid  $\alpha$ -t-butyl ester  $\gamma$ -2,5-dioxopyrrolidin-1-yl ester

Lau-Glu(ONSu)-OBu<sup>t</sup>: N <sup>$\alpha$</sup> -Dodecanoyl-L-glutamic acid  $\alpha$ -t-butyl ester  $\gamma$ -2,5-dioxopyrrolidin-1-yl ester

Myr-Glu(ONSu)-OBu<sup>t</sup>: N <sup>$\alpha$</sup> -Tetradecanoyl-L-glutamic acid  $\alpha$ -t-butyl ester  $\gamma$ -2,5-dioxopyrrolidin-1-yl ester

Pal-Glu(ONSu)-OBu<sup>t</sup>: N <sup>$\alpha$</sup> -Hexadecanoyl-(L)-glutamic acid  $\alpha$ -t-butyl- $\gamma$ -2,5-dioxopyrrolidin-1-yl diester

Ste-Glu(ONSu)-OBu<sup>t</sup>: N <sup>$\alpha$</sup> -Octadecanoyl-(L)-glutamic acid  $\alpha$ -t-butyl- $\gamma$ -2,5-dioxopyrrolidin-1-yl diester

Lau- $\beta$ -Ala-ONSu: N <sup>$\beta$</sup> -Dodecanoyl- $\beta$ -alanine 2,5-dioxopyrrolidin-1-yl ester

Pal- $\beta$ -Ala-ONSu: N <sup>$\beta$</sup> -Hexadecanoyl- $\beta$ -alanine 2,5-dioxopyrrolidin-1-yl ester

Lau-GABA-ONSu: N <sup>$\gamma$</sup> -Dodecanoyl- $\gamma$ -aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Myr-GABA-ONSu: N <sup>$\gamma$</sup> -Tetradecanoyl- $\gamma$ -aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Pal-GABA-ONSu: N <sup>$\gamma$</sup> -Hexadecanoyl- $\gamma$ -aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Ste-GABA-ONSu: N <sup>$\gamma$</sup> -Octadecanoyl- $\gamma$ -aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Pal-Isonip-ONSu: N-Hexadecanoyl-piperidine-4-carboxylic acid 2,5-dioxopyrrolidin-1-yl ester

Pal-Glu(OBu<sup>t</sup>)-ONSu: N <sup>$\alpha$</sup> -Hexadecanoyl-L-glutamic acid  $\alpha$ -2,5-dioxopyrrolidin-1-yl ester  $\gamma$ -t-butyl ester

HOOC-(CH<sub>2</sub>)<sub>6</sub>-COONSu:  $\omega$ -Carboxyheptanoic acid 2,5-dioxopyrrolidin-1-yl ester

HOOC-(CH<sub>2</sub>)<sub>10</sub>-COONSu:  $\omega$ -Carboxyundecanoic acid 2,5-dioxopyrrolidin-1-yl ester

HOOC-(CH<sub>2</sub>)<sub>12</sub>-COONSu:  $\omega$ -Carboxytridecanoic acid 2,5-dioxopyrrolidin-1-yl ester

HOOC-(CH<sub>2</sub>)<sub>14</sub>-COONSu:  $\omega$ -Carboxypentadecanoic acid 2,5-dioxopyrrolidin-1-yl ester

HOOC-(CH<sub>2</sub>)<sub>16</sub>-COONSu:  $\omega$ -Carboxyheptadecanoic acid 2,5-dioxopyrrolidin-1-yl ester

HOOC-(CH<sub>2</sub>)<sub>18</sub>-COONSu:  $\omega$ -Carboxynonadecanoic acid 2,5-dioxopyrrolidin-1-yl ester

N <sup>$\alpha$</sup> -alkanoyl-Glu(ONSu)-OBu<sup>t</sup>: N <sup>$\alpha$</sup> -Alkanoyl-(L)-glutamic acid  $\alpha$ -t-butyl- $\gamma$ -2,5-dioxopyrrolidin-1-yl diester

#### Analytical

#### Plasma Desorption Mass Spectrometry

##### Sample Preparation:

The sample is dissolved in 0.1% TFA/EtOH (1:1) at a concentration of 1  $\mu$ g/ $\mu$ l. The sample solution (5–10  $\mu$ l) is placed on a nitrocellulose target (Bio-ion AB, Uppsala, Sweden) and allowed to adsorb to the target surface for 2 minutes. The target is subsequently rinsed with 2 $\times$ 25  $\mu$ l 0.1% TFA and spin-dried. Finally, the nitrocellulose target is placed in a target carousel and introduced into the mass spectrometer.

##### MS Analysis:

PDMS analysis was carried out using a Bio-ion 20 time-of-flight instrument (Bio-ion Nordic AB, Uppsala, Sweden). An acceleration voltage of 15 kV was applied and molecular ions formed by bombardment of the nitrocellulose surface with 252-Cf fission fragments were accelerated towards a stop detector. The resulting time-of-flight spectrum was calibrated into a true mass spectrum using the H<sup>+</sup> and NO<sup>+</sup> ions at m/z 1 and 30, respectively. Mass spectra were generally accumulated for 1.0 $\times$ 10<sup>6</sup> fission events corresponding to 15–20 minutes. Resulting assigned masses all correspond to isotopically averaged molecular masses. The accuracy of mass assignment is generally better than 0.1%.

#### MALDI-MS

MALDI-TOF MS analysis was carried out using a Voyager RP instrument (PerSeptive Biosystems Inc., Framingham, Mass.) equipped with delayed extraction and operated in linear mode. Alpha-cyano-4-hydroxy-cinnamic acid was used as matrix, and mass assignments were based on external calibration.

#### Example 1

##### Synthesis of Lys<sup>26</sup>(N <sup>$\epsilon$</sup> -tetradecanoyl) GLP-1(7-37)

The title compound was synthesised from GLP-1(7-37). A mixture of GLP-1(7-37) (25 mg, 7.45  $\mu$ m), EDPA (26.7 mg, 208  $\mu$ m), NMP (520  $\mu$ l) and water (260  $\mu$ l) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of Myr-ONSu (2.5 mg, 7.67  $\mu$ m) in NMP (62.5  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature and then allowed to stand for 20 min. An additional amount of Myr-ONSu (2.5 mg, 7.67  $\mu$ m) in NMP (62.5  $\mu$ l) was added and the resulting mixture gently shaken for 5 min. After a total reaction time of 40 min. the reaction was quenched by the addition of a

## 161

solution of glycine (12.5 mg, 166  $\mu\text{mol}$ ) in 50% aqueous ethanol (12.5 ml). The title compound was isolated from the reaction mixture by HPLC using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system, yield: 1.3 mg (corresponding to 4.9% of the theoretical yield). The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The isolated product was analysed by PDMS and the m/z value for the protonated molecular ion was found to be 3567.9 $\pm$ 3. The resulting molecular weight was thus 3566.9 $\pm$ 3 amu (theoretical value: 3565.9 amu). The position of acylation (Lys26) was verified by enzymatic cleavage of the title compound with *Staphylococcus aureus* V8 protease and subsequent mass determination of the peptide fragments by PDMS.

In addition to the title compound two other GLP-1 derivatives were isolated from the reaction mixture by using the same chromatographic column and a more shallow gradient (35–38% acetonitrile in 60 minutes), see Examples 2 and 3.

## Example 2

Synthesis of Lys<sup>34</sup>(N<sup>ε</sup>-tetradecanoyl) GLP-1(7-37)

The title compound was isolated by HPLC from the reaction mixture described in Example 1. PDMS analysis yielded a protonated molecular ion at m/z 3567.7 $\pm$ 3. The molecular weight was found to be 3566.7 $\pm$ 3 amu (theoretical value: 3565.9 amu). The acylation site was determined on the basis of the fragmentation pattern.

## Example 3

Synthesis of Lys<sup>26, 34</sup>-bis(N<sup>ε</sup>-tetradecanoyl) GLP-1(7-37)

The title compound was isolated by HPLC from the reaction mixture described in Example 1. PDMS analysis yielded a protonated molecular ion at m/z 3778.4 $\pm$ 3. The molecular weight was found to be 3777.4 $\pm$ 3 amu (theoretical value: 3776.1 amu).

## Example 4

Synthesis of Lys<sup>26</sup>(N<sup>ε</sup>-tetradecanoyl)Arg<sup>34</sup>GLP-1(7-37)

The title compound was synthesised from Arg<sup>34</sup>GLP-1(7-37). A mixture of Arg<sup>34</sup>GLP-1(7-37) (5 mg, 1.47  $\mu\text{mol}$ ), EDPA (5.3 mg, 41.1  $\mu\text{mol}$ ), NMP (105  $\mu\text{l}$ ) and water (50  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of Myr-ONSu (0.71 mg, 2.2  $\mu\text{mol}$ ) in NMP (17.8  $\mu\text{l}$ ), the reaction mixture was gently shaken for 5 min. at room temperature and then allowed to stand for 20 min. After a total reaction time of 30 min. the reaction was quenched by the addition of a solution of glycine (25 mg, 33.3  $\mu\text{mol}$ ) in 50% aqueous ethanol (2.5 ml). The reaction mixture was purified by HPLC as described in Example 1. PDMS analysis yielded a protonated molecular ion at m/z 3594.9 $\pm$ 3. The molecular weight was found to be 3593.9 $\pm$ 3 amu (theoretical value: 3593.9 amu).

## Example 5

Synthesis of Gly<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-tetradecanoyl) GLP-1(7-37)

The title compound was synthesised from Gly<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-37) which was purchased from QCB. A

## 162

mixture of Gly<sup>8</sup>Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-37) (1.3 mg, 0.39  $\mu\text{mol}$ ), EDPA (1.3 mg, 10  $\mu\text{mol}$ ), NMP (125  $\mu\text{l}$ ) and water (30  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of Myr-ONSu (0.14 mg, 0.44  $\mu\text{mol}$ ) in NMP (3.6 ml), the reaction mixture was gently shaken for 15 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (0.1 mg, 1.33  $\mu\text{mol}$ ) in 50% aqueous ethanol (10  $\mu\text{l}$ ). The reaction mixture was purified by HPLC, and the title compound (60  $\mu\text{g}$ , 4%) was isolated.

## Example 6

Synthesis of Arg<sup>26, 34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-tetradecanoyl) GLP-1(7-37)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-37)-OH (5.0 mg, 1.477  $\mu\text{mol}$ ), EDPA (5.4 mg, 41.78  $\mu\text{mol}$ ), NMP (105  $\mu\text{l}$ ) and water (50  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of Myr-ONSu (0.721 mg, 2.215  $\mu\text{mol}$ ) in NMP (18  $\mu\text{l}$ ). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 45 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.5 mg, 33.3  $\mu\text{mol}$ ) in 50% aqueous ethanol (250  $\mu\text{l}$ ). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (1.49 mg, 28%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3595 $\pm$ 3. The resulting molecular weight was thus 3594 $\pm$ 3 amu (theoretical value 3594 amu).

## Example 7

Synthesis of Lys<sup>26,34</sup>bis(N<sup>ε</sup>-( $\omega$ -carboxynonadecanoyl)) GLP-1(7-37)-OH

A mixture of GLP-1(7-37)-OH (70 mg, 20.85  $\mu\text{mol}$ ), EDPA (75.71 mg, 585.8  $\mu\text{mol}$ ), NMP (1.47 ml) and water (700  $\mu\text{l}$ ) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution of HOOC—(CH<sub>2</sub>)<sub>18</sub>—COONSu (27.44 mg, 62.42  $\mu\text{mol}$ ) in NMP (686  $\mu\text{l}$ ), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 50 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (34.43 mg, 458.7  $\mu\text{mol}$ ) in 50% aqueous ethanol (3.44 ml). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (8.6 mg, 10%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 4006 $\pm$ 3. The resulting molecular weight was thus 4005 $\pm$ 3 amu (theoretical value 4005 amu).

## Example 8

Synthesis of Arg<sup>26, 34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-( $\omega$ -carboxynonadecanoyl)) GLP-1(7-36)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-36)-OH (5.06 mg, 1.52  $\mu\text{mol}$ ), EDPA (5.5 mg, 42.58  $\mu\text{mol}$ ), NMP (106  $\mu\text{l}$ ) and water (100  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution



## 163

of HOOC—(CH<sub>2</sub>)<sub>18</sub>—COONSu (1.33 mg, 3.04 μmol) in NMP (33.2 μl), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2.5 h at room temperature. The reaction was quenched by the addition of a solution of glycine (2.50 mg, 33.34 μmol) in 50% aqueous ethanol (250 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.46 mg, 8%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3652±3. The resulting molecular weight was thus 3651±3 amu (theoretical value 3651 amu).

## Example 9

Synthesis of Arg<sup>26, 34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-(ω-carboxynonadecanoyl)) GLP-1(7-38)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (5.556 mg, 1.57 μmol), EDPA (5.68 mg, 43.96 μmol), NMP (116.6 μl) and water (50 μl) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>18</sub>—COONSu (1.38 mg, 3.14 μmol) in NMP (34.5 μl), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2.5 h at room temperature. The reaction was quenched by the addition of a solution of glycine (2.5 mg, 33.3 μmol) in 50% aqueous ethanol (250 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.7 mg, 12%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3866±3. The resulting molecular weight was thus 3865±3 amu (theoretical value 3865 amu).

## Example 10

Synthesis of Arg<sup>34</sup>Lys<sup>26</sup>(N<sup>ε</sup>-(ω-carboxynonadecanoyl)) GLP-1(7-37)-OH

A mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (5.04 mg, 1.489 μmol), EDPA (5.39 mg, 41.70 μmol), NMP (105 μl) and water (50 μl) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>18</sub>—COONSu (1.31 mg, 2.97 μmol) in NMP (32.8 μl), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 30 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.46 mg, 32.75 μmol) in 50% aqueous ethanol (246 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (1.2 mg, 22%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3709±3. The resulting molecular weight was thus 3708±3 amu (theoretical value 3708 amu).

## Example 11

Synthesis of Arg<sup>34</sup>Lys<sup>26</sup>(N<sup>ε</sup>-(ω-carboxyheptadecanoyl)) GLP-1(7-37)-OH

A mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (5.8 mg, 1.714 μmol), EDPA (6.20 mg, 47.99 μmol), NMP (121.8 μl) and

## 164

water (58 μl) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>16</sub>—COONSu (2.11 mg, 5.142 μmol) in NMP (52.8 μl), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (2.83 mg, 37.70 μmol) in 50% aqueous ethanol (283 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.81 mg, 13%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3681±3. The resulting molecular weight was thus 3680±3 amu (theoretical value 3680 amu).

## Example 12

Synthesis of Arg<sup>26, 34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-(ω-carboxyheptadecanoyl)) GLP-1(7-37)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-37)-OH (3.51 mg, 1.036 μmol), EDPA (3.75 mg, 29.03 μmol), NMP (73.8 μl) and water (35 μl) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>16</sub>—COONSu (1.27 mg, 3.10 μmol) in NMP (31.8 μl), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h and 10 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (1.71 mg, 22.79 μmol) in 50% aqueous ethanol (171 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.8 mg, 21%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3682±3. The resulting molecular weight was thus 3681±3 amu (theoretical value 3681 amu).

## Example 13

Synthesis of Arg<sup>26, 34</sup>Lys<sup>38</sup>(N<sup>68</sup>-(ω-carboxylheptadecanoyl)) GLP-1(7-38)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (5.168 mg, 1.459 μmol), EDPA (5.28 mg, 40.85 μmol), NMP (108.6 μl) and water (51.8 μl) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>16</sub>—COONSu (1.80 mg, 4.37 μmol) in NMP (45 μl), the reaction mixture was gently shaken for 10 min. at room temperature, and then allowed to stand for an additional 2 h and 15 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.41 mg, 32.09 μmol) in 50% aqueous ethanol (241 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.8 mg, 14%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3838±3. The resulting molecular weight was thus 3837±3 amu (theoretical value 3837 amu).

## Example 14

Synthesis of Arg<sup>26, 34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-(ω-carboxyheptadecanoyl)) GLP-1(7-36)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-36)-OH (24.44 mg, 7.34 μmol), EDPA (26.56 mg, 205.52 μmol), NMP (513 μl)

## 165

and water (244.4  $\mu$ l) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>16</sub>—COONSu (9.06 mg, 22.02  $\mu$ mol) in NMP (1.21 ml), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 30 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.12 mg, 161.48  $\mu$ mol) in 50% aqueous ethanol (1.21 ml). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (7.5 mg, 28%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3625 $\pm$ 3. The resulting molecular weight was thus 3624 $\pm$ 3 amu (theoretical value 3624 amu).

## Example 15

Synthesis of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-( $\omega$ -carboxyundecanoyl)) GLP-1(7-37)-OH

A mixture of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>36</sup>GLP-1(7-37)-OH (4.2 mg, 1.24  $\mu$ mol), EDPA (4.49 mg, 34.72  $\mu$ mol), NMP (88.2  $\mu$ l) and water (42  $\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>10</sub>—COONSu (1.21 mg, 3.72  $\mu$ mol) in NMP (30.25  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 40 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.04 mg, 27.28  $\mu$ mol) in 50% aqueous ethanol (204  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.8 mg, 18%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3598 $\pm$ 3. The resulting molecular weight was thus 3597 $\pm$ 3 amu (theoretical value 3597 amu).

## Example 16

Synthesis of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-( $\omega$ -carboxyundecanoyl)) GLP-1(7-38)-OH

A mixture of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (5.168 mg, 1.46  $\mu$ mol), EDPA (5.28 mg, 40.88  $\mu$ mol), NMP (108.6  $\mu$ l) and water (51.7  $\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>10</sub>—COONSu (1.43 mg, 4.38  $\mu$ mol) in NMP (35.8  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 50 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.41 mg, 32.12  $\mu$ mol) in 50% aqueous ethanol (241  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.85 mg, 16%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3753 $\pm$ 3. The resulting molecular weight was thus 3752 $\pm$ 3 amu (theoretical value 3752 amu).

## Example 17

Synthesis of Lys<sup>26,34</sup>bis(N<sup>ε</sup>-( $\omega$ -carboxyundecanoyl)) GLP-1(7-37)-OH

A mixture of GLP-1(7-37)-OH (10.0 mg, 2.98  $\mu$ mol), EDPA (10.8 mg, 83.43  $\mu$ mol), NMP (210  $\mu$ l) and water (100

## 166

$\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>10</sub>—COONSu (2.92 mg, 8.94  $\mu$ mol) in NMP (73  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 50 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (4.92 mg, 65.56  $\mu$ mol) in 50% aqueous ethanol (492  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (1.0 mg, 9%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3781 $\pm$ 3. The resulting molecular weight was thus 3780 $\pm$ 3 amu (theoretical value 3780 amu).

## Example 18

Synthesis of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>36</sup>(N<sup>68</sup>-( $\omega$ -carboxyundecanoyl)) GLP-1(7-36)-OH

A mixture of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>36</sup>GLP-1(7-36)-OH (15.04 mg, 4.52  $\mu$ mol), EDPA (16.35 mg, 126.56  $\mu$ mol), NMP (315.8  $\mu$ l) and water (150.4  $\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>10</sub>—COONSu (4.44 mg, 13.56  $\mu$ mol) in NMP (111  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 40 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (7.5 mg, 99.44  $\mu$ mol) in 50% aqueous ethanol (750  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (3.45 mg, 22%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3540 $\pm$ 3. The resulting molecular weight was thus 3539 $\pm$ 3 amu (theoretical value 3539 amu).

## Example 19

Synthesis of Arg<sup>34</sup>Lys<sup>26</sup>(N<sup>ε</sup>-( $\omega$ -carboxyundecanoyl)) GLP-1(7-37)-OH

A mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (5.87 mg, 1.73  $\mu$ mol), EDPA (6.27 mg, 48.57  $\mu$ mol), NMP (123.3  $\mu$ l) and water (58.7  $\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>10</sub>—COONSu (1.70 mg, 5.20  $\mu$ mol) in NMP (42.5  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 40 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.86 mg, 286  $\mu$ mol) in 50% aqueous ethanol (286  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C., and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (1.27 mg, 20%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3597 $\pm$ 3. The resulting molecular weight was thus 3596 $\pm$ 3 amu (theoretical value 3596 amu).

## Example 20

Synthesis of Arg<sup>34</sup>Lys<sup>26</sup>(N<sup>ε</sup>-( $\omega$ -carboxyheptanoyl)) GLP-1(7-37)-OH

A mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (4.472 mg, 1.32  $\mu$ mol), EDPA (4.78 mg, 36.96  $\mu$ mol), NMP (94  $\mu$ l) and water

## 167

(44.8  $\mu$ l) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>6</sub>—COONSu (1.07 mg, 3.96  $\mu$ mol) in NMP (26.8  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 1 h and 50 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.18 mg, 29.04  $\mu$ mol) in 50% aqueous ethanol (218  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.5 mg, 11%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3540 $\pm$ 3. The resulting molecular weight was thus 3539 $\pm$ 3 amu (theoretical value 3539 amu).

## Example 21

Synthesis of Arg<sup>26, 34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-( $\omega$ -carboxyheptanoyl)) GLP-1(7-38)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (5.168 mg, 1.459  $\mu$ mol), EDPA (5.28 mg, 40.85  $\mu$ mol), NMP (108.6  $\mu$ l) and water (51.6  $\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>6</sub>—COONSu (1.18 mg, 4.37  $\mu$ mol) in NMP (29.5  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 1 h and 50 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.40 mg, 32.09  $\mu$ mol) in 50% aqueous ethanol (240  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.5 mg, 9%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3697 $\pm$ 3. The resulting molecular weight was thus 3695 $\pm$ 3 amu (theoretical value 3695 amu).

## Example 22

Synthesis of Arg<sup>26, 34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-( $\omega$ -carboxyheptanoyl)) GLP-1(7-37)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-37)-OH (5.00 mg, 1.47  $\mu$ mol), EDPA (5.32 mg, 41.16  $\mu$ mol), NMP (105  $\mu$ l) and water (50  $\mu$ l) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>6</sub>—COONSu (1.19 mg, 4.41  $\mu$ mol) in NMP (29.8  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (2.42 mg, 32.34  $\mu$ mol) in 50% aqueous ethanol (242  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.78 mg, 15%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3542 $\pm$ 3. The resulting molecular weight was thus 3541 $\pm$ 3 amu (theoretical value 3541 amu).

## Example 23

Synthesis of Arg<sup>26, 34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-carboxyheptanoyl)) GLP-1(7-36)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-36)-OH (5.00 mg, 1.50  $\mu$ mol), EDPA (5.44 mg, 42.08  $\mu$ mol), NMP (210  $\mu$ l) and

## 168

water (50  $\mu$ l) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>6</sub>—COONSu (1.22 mg, 4.5  $\mu$ mol) in NMP (30.5  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (2.47 mg, 33.0  $\mu$ mol) in 50% aqueous ethanol (247  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.71 mg, 14%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3484 $\pm$ 3. The resulting molecular weight was thus 3483 $\pm$ 3 amu (theoretical value 3483 amu).

## Example 24

Synthesis of Lys<sup>26, 34</sup>bis(N<sup>ε</sup>-( $\omega$ -carboxyheptanoyl)) GLP-1(7-37)-OH

A mixture of GLP-1(7-37)-OH (10 mg, 2.5  $\mu$ mol), EDPA (10.8 mg, 83.56  $\mu$ mol), NMP (210  $\mu$ l) and water (100  $\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>6</sub>—COONSu (2.42 mg, 8.92  $\mu$ mol) in NMP (60.5  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h and 35 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (4.92 mg, 65.54  $\mu$ mol) in 50% aqueous ethanol (492  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (2.16 mg, 24%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3669 $\pm$ 3. The resulting molecular weight was thus 3668 $\pm$ 3 amu (theoretical value 3668 amu).

## Example 25

Synthesis of Arg<sup>34</sup>Lys<sup>26</sup>(N<sup>ε</sup>-( $\omega$ -carboxypentadecanoyl)) GLP-1(7-37)-OH

A mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (4.472 mg, 1.321  $\mu$ mol), EDPA (4.78 mg, 36.99  $\mu$ mol), NMP (93.9  $\mu$ l) and water (44.7  $\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>14</sub>—COONSu (1.519 mg, 3.963  $\mu$ mol) in NMP (38  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 1 h at room temperature. The reaction was quenched by the addition of a solution of glycine (2.18 mg, 29.06  $\mu$ mol) in 50% aqueous ethanol (218  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.58 mg, 12%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3654 $\pm$ 3. The resulting molecular weight was thus 3653 $\pm$ 3 amu (theoretical value 3653 amu).

## Example 26

Synthesis of Arg<sup>26, 34</sup>Lys<sup>36</sup>(N<sup>ε</sup>-( $\omega$ -carboxyheptanoyl)) GLP-1(7-36)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>36</sup>GLP-1(7-36)-OH (5.00 mg, 1.50  $\mu$ mol), EDPA (5.44 mg, 42.08  $\mu$ mol), NMP (210  $\mu$ l) and

## 169

water (50  $\mu$ l) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution HOOC-(CH<sub>2</sub>)<sub>14</sub>-COONSu (1.72 mg, 4.5  $\mu$ mol) in NMP (43  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 1 h at room temperature. The reaction was quenched by the addition of a solution of glycine (2.48 mg, 33  $\mu$ mol) in 50% aqueous ethanol (248  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.58 mg, 11%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3596 $\pm$ 3. The resulting molecular weight was thus 3595 $\pm$ 3 amu (theoretical value 3595 amu).

## Example 27

## Synthesis of Lithocholic Acid 2,5-dioxo-pyrrolidin-1-yl Ester

To a mixture of lithocholic acid (5.44 g, 14.34 mmol), N-hydroxysuccinimide (1.78 g, 15.0 mmol), anhydrous THF (120 ml) and anhydrous acetonitrile (30 ml), kept at to 10° C., was added a solution of N,N'-dicyclohexylcarbodiimide (3.44 g, 16.67 mmol) in anhydrous THF. The reaction mixture was stirred at ambient temperature for 16 h, filtered and concentrated in vacuo. The residue was dissolved in dichloromethane (450 ml), washed with a 10% aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 $\times$ 150 ml) and water (2 $\times$ 150 ml), and dried (MgSO<sub>4</sub>). Filtered and the filtrate concentrated in vacuo to give a crystalline residue. The residue was recrystallised from a mixture of dichloromethane (30 ml) and n-heptane (30 ml) to give the title compound (3.46 g, 51%) as a crystalline solid.

## Example 28

Synthesis of Arg<sup>34</sup>Lys<sup>26</sup>(N<sup>ε</sup>-lithocholyl) GLP-1(7-37)-OH

A mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (4.472 mg, 1.32  $\mu$ mol), EDPA (4.78 mg, 36.96  $\mu$ mol), NMP (94  $\mu$ l) and water (44.8  $\mu$ l) was gently shaken for 10 min. at room temperature. To the resulting mixture was added a solution of lithocholic acid 2,5-dioxo-pyrrolidin-1-yl ester (1.87 mg, 3.96  $\mu$ mol) in NMP (46.8  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 1 h at room temperature. The reaction was quenched by the addition of a solution of glycine (2.18 mg, 29.04  $\mu$ mol) in 50% aqueous ethanol (218  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (1.25 mg, 25%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3744 $\pm$ 3. The resulting molecular weight was thus 3743 $\pm$ 3 amu (theoretical value 3743 amu).

## Example 29

Synthesis of N<sup>ε</sup>-tetradecanoyl-Glu(ONSu)-OBu<sup>t</sup>

To a suspension of H-Glu(OH)-OBu<sup>t</sup> (2.5 g, 12.3  $\mu$ mmol), DMF (283 ml) and EDPA (1.58 g, 12.3 mmol) was added

## 170

drop by drop a solution of Myr-ONSu (4.0 g, 12.3 mmol) in DMF (59 ml). The reaction mixture was stirred for 16 h at room temperature and then concentrated in vacuo to a total volume of 20 ml. The residue was partitioned between 5% aqueous citric acid (250 ml) and ethyl acetate (150 ml), and the phases were separated. The organic phase was concentrated in vacuo and the residue dissolved in DMF (40 ml). The resulting solution was added drop by drop to a 10% aqueous solution of citric acid (300 ml) kept at 0° C. The precipitated compound was collected and washed with iced water and dried in a vacuum drying oven. The dried compound was dissolved in DMF (23 ml) and HONSu (1.5 g, 13 mmol) was added. To the resulting mixture was added a solution of N,N'-dicyclohexylcarbodiimide (2.44 g, 11.9 mmol) in dichloromethane (47 ml). The reaction mixture was stirred for 16 h at room temperature, and the precipitated compound was filtered off. The precipitate was recrystallised from n-heptane/2-propanol to give the title compound (3.03 g, 50%).

## Example 30

Synthesis of Glu<sup>22,23,30</sup>Arg<sup>26</sup>, <sup>34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-tetradecanoyl))) GLP-1(7-38)-OH

A mixture of Glu<sup>22,23,30</sup>Arg<sup>26</sup>, <sup>34</sup>Lys<sup>38</sup>-GLP1(7-38)-OH (1.0 mg, 0.272  $\mu$ mol), EDPA, (0.98 mg, 7.62  $\mu$ mol), NMP (70  $\mu$ l) and water (70  $\mu$ l) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>α</sup>-tetradecanoyl-Glu(ONSu)-OBu<sup>t</sup>, prepared as described in Example 29, (0.41 mg, 0.816  $\mu$ mol) in NMP (10.4  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 45 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (0.448 mg, 5.98  $\mu$ mol) in 50% aqueous ethanol (45  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (0.9 ml) was added, and the resulting mixture was immobilised on a Varian 500 mg C8 Mega Bond Elut® cartridge, the immobilised compound washed with 5% aqueous acetonitrile (10 ml), and finally liberated from the cartridge by elution with TFA (10 ml). The eluate was concentrated in vacuo, and the reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.35 mg, 32%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 4012 $\pm$ 3. The resulting molecular weight was thus 4011 $\pm$ 3 amu (theoretical value 4011 amu).

## Example 31

Synthesis of Glu<sup>23,26</sup>Arg<sup>34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-tetradecanoyl))) GLP-1(7-38)-OH

A mixture of Glu<sup>23,26</sup>Arg<sup>34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (6.07 mg, 1.727  $\mu$ mol), EDPA (6.25 mg, 48.36  $\mu$ mol), NMP (425  $\mu$ l) and water (425  $\mu$ l) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>α</sup>-tetradecanoyl-Glu(ONSu)-OBu<sup>t</sup>, prepared as described in example 29, (2.65 mg, 5.18  $\mu$ mol) in NMP (66.3  $\mu$ l), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 45 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.85 mg, 38.0  $\mu$ mol) in 50% aqueous ethanol (285  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (5.4 ml) was added,

## 171

and the resulting mixture was immobilised on a Varian 500 mg C8 Mega Bond Elut® cartridge, the immobilised compound washed with 5% aqueous acetonitrile (10 ml), and finally liberated from the cartridge by elution with TFA (10 ml). The eluate was concentrated in vacuo, and the reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.78 mg, 12%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3854±3. The resulting molecular weight was thus 3853±3 amu (theoretical value 3853 amu).

## Example 32

Synthesis of Lys<sup>26, 34</sup>-bis(N<sup>ε</sup>-(ω-carboxytridecanoyl)) GLP-1(7-37)-OH

A mixture of GLP-1(7-37)-OH (30 mg, 8.9 μmol), EDPA (32.3 mg, 250 μmol), NMP (2.1 ml) and water (2.1 ml) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>12</sub>—COONSu (12.7 mg, 35.8 μmol) in NMP (318 μl), the reaction mixture was gently shaken for 1 h and 40 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (3.4 mg, 44.7 μmol) in 50% aqueous ethanol (335 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (10 mg, 29%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3840±3. The resulting molecular weight was thus 3839±3 amu (theoretical value 3839 amu).

## Example 33

Synthesis of Lys<sup>26, 34</sup>-bis(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-tetradecanoyl))) GLP-1(7-37)-OH

A mixture of GLP-1(7-37)-OH (300 mg, 79.8 μmol), EDPA (288.9 mg, 2.24 mmol), NMP (21 ml) and water (21 ml) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>α</sup>-tetradecanoyl-Glu(ONSu)-OBu<sup>t</sup>, prepared as described in Example 29, (163 mg, 319.3 μmol) in NMP (4.08 ml), the reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 1 h at room temperature. The reaction was quenched by the addition of a solution of glycine (131.8 mg, 1.76 mmol) in 50% aqueous ethanol (13.2 ml). A 0.5% aqueous solution of ammonium acetate (250 ml) was added, and the resulting mixture was divided into four equal portions. Each portion was eluted onto a Varian 500 mg C8 Mega Bond Elut® cartridge, the immobilised compound washed with 0.1% aqueous TFA (3.5 ml), and finally liberated from the cartridge by elution with 70% aqueous acetonitrile (4 ml). The combined eluates were diluted with 0.1% aqueous TFA (300 ml). The precipitated compound was collected by centrifugation, washed with 0.1% aqueous TFA (50 ml), and finally isolated by centrifugation. To the precipitate was added TFA (60 ml), and the resulting reaction mixture was stirred for 1 h and 30 min. at room temperature. Excess TFA was removed in vacuo, and the residue was poured into water (50 ml). The precipitated compound was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and

## 172

a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (27.3 mg, 8%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 4036±3. The resulting molecular weight was thus 4035±3 amu (theoretical value 4035 amu).

## Example 34

Synthesis of Arg<sup>26, 34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-(ω-carboxypentadecanoyl)) GLP-1(7-38)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (30 mg, 8.9 μmol), EDPA (32.3 mg, 250 μmol), NMP (2.1 ml) and water (2.1 ml) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution HOOC—(CH<sub>2</sub>)<sub>14</sub>—COONSu (13.7 mg, 35.8 μmol) in NMP (343 μl), the reaction mixture was gently shaken for 1 h at room temperature. The reaction was quenched by the addition of a solution of glycine (3.4 mg, 44.7 μmol) in 50% aqueous ethanol (335 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (4.8 mg, 14%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3894±3. The resulting molecular weight was thus 3893±3 amu (theoretical value 3893 amu).

## Example 35

Synthesis of N<sup>α</sup>-hexadecanoyl-Glu(ONSu)-OBu<sup>t</sup>

To a suspension of H-Glu(OH)-OBu<sup>t</sup> (4.2 g, 20.6 mmol), DMF (500 ml) and EDPA (2.65 g, 20.6 mmol) was added drop by drop a solution of Pal-ONSu (7.3 g, 20.6 mmol) in DMF (100 ml). The reaction mixture was stirred for 64 h at room temperature and then concentrated in vacuo to a total volume of 20 ml. The residue was partitioned between 10% aqueous citric acid (300 ml) and ethyl acetate (250 ml), and the phases were separated. The organic phase was concentrated in vacuo and the residue dissolved in DMF (50 ml). The resulting solution was added drop by drop to a 10% aqueous solution of citric acid (500 ml) kept at 0° C. The precipitated compound was collected and washed with iced water and dried in a vacuum drying oven. The dried compound was dissolved in DMF (45 ml) and HONSu (2.15 g, 18.7 mmol) was added. To the resulting mixture was added a solution of N,N'-dicyclohexylcarbodiimide (3.5 g, 17 mmol) in dichloromethane (67 ml). The reaction mixture was stirred for 16 h at room temperature, and the precipitated compound was filtered off. The precipitate was recrystallised from n-heptane/2-propanol to give the title compound (6.6 g, 72%).

## Example 36

Synthesis of Lys<sup>26, 34</sup>-bis(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)-OH

A mixture of GLP-1(7-37)-OH (10 mg, 2.9 μmol), EDPA (10.8 mg, 83.4 μmol), NMP (0.7 ml) and water (0.7 ml) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>α</sup>-hexadecanoyl-Glu(ONSu)-OBu<sup>t</sup>, prepared as described in Example 33, (163 mg, 319.3 μmol) in NMP (4.08 ml), the reaction mixture was gently shaken 1 h and 20 min. at room

temperature. The reaction was quenched by the addition of a solution of glycine (4.9 mg, 65.6  $\mu\text{mol}$ ) in 50% aqueous ethanol (492  $\mu\text{l}$ ). A 0.5% aqueous solution of ammonium-acetate (9 ml) was added, and the resulting mixture eluted onto a Varian 1 g C8 Mega Bond Elut® cartridge, the immobilised compound washed with 5% aqueous acetonitrile (10 ml), and finally liberated from the cartridge by elution with TFA (10 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (2.4 mg, 20%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 4092 $\pm$ 3. The resulting molecular weight was thus 4091 $\pm$ 3 amu (theoretical value 4091 amu).

#### Example 37

##### Synthesis of Arg<sup>34</sup>Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)-OH

A mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (3.7 mg, 1.1  $\mu\text{mol}$ ), EDPA (4.0 mg, 30.8  $\mu\text{mol}$ ), acetonitrile (260  $\mu\text{l}$ ) and water (260  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>α</sup>-hexadecanoyl-Glu(ONSu)-OBu<sup>t</sup>, prepared as described in Example 35, (1.8 mg, 3.3  $\mu\text{mol}$ ) in acetonitrile (44.2  $\mu\text{l}$ ), and the reaction mixture was gently shaken for 1 h and 20 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (1.8 mg, 24.2  $\mu\text{mol}$ ) in 50% aqueous ethanol (181  $\mu\text{l}$ ). A 0.5% aqueous solution of ammonium-acetate (12 ml) and NMP (300  $\mu\text{l}$ ) were added, and the resulting mixture eluted onto a Varian 1 g C8 Mega Bond Elut® cartridge, the immobilised compound washed with 5% aqueous acetonitrile (10 ml), and finally liberated from the cartridge by elution with TFA (6 ml). The eluate was allowed to stand for 2 h at room temperature and then concentrated in vacuo. The residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.23 mg, 6%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3752 $\pm$ 3. The resulting molecular weight was thus 3751 $\pm$ 3 amu (theoretical value 3751 amu).

#### Example 38

##### Synthesis of Arg<sup>26, 34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-tetradecanoyl))) GLP-1(7-38)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (14 mg, 4.0  $\mu\text{mol}$ ), EDPA (14.3 mg, 110.6  $\mu\text{mol}$ ), NMP (980  $\mu\text{l}$ ) and water (980  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>α</sup>-tetradecanoyl-Glu(ONSu)-OBu<sup>t</sup>, prepared as described in Example 29, (12.1 mg, 23.7  $\mu\text{mol}$ ) in NMP (303  $\mu\text{l}$ ), and the reaction mixture was gently shaken for 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (6.5 mg, 86.9  $\mu\text{mol}$ ) in 50% aqueous ethanol (652  $\mu\text{l}$ ). A 0.5% aqueous solution of ammonium-acetate (50 ml) was added, and the resulting mixture eluted onto a Varian 1 g C8 Mega Bond Elut® cartridge, the immobilised compound washed with 5% aqueous acetonitrile (15 ml), and finally liberated from the cartridge by elution with TFA (6 ml). The eluate was allowed

to stand for 1 h and 45 min. at room temperature and then concentrated in vacuo. The residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (3.9 mg, 26%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3881 $\pm$ 3. The resulting molecular weight was thus 3880 $\pm$ 3 amu (theoretical value 3880 amu).

#### Example 39

##### Synthesis of Arg<sup>26, 34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-( $\omega$ -carboxypentadecanoyl)) GLP-1(7-38)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (14 mg, 4.0  $\mu\text{mol}$ ), EDPA (14.3 mg, 111  $\mu\text{mol}$ ), NMP (980  $\mu\text{l}$ ) and water (980  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of HOOC-(CH<sub>2</sub>)<sub>14</sub>-COONSu (4.5 mg, 11.9  $\mu\text{mol}$ ) in NMP (114  $\mu\text{l}$ ), the reaction mixture was gently shaken for 1 h and 45 min. at room temperature. An additional solution of HOOC-(CH<sub>2</sub>)<sub>14</sub>-COONSu (4.0 mg, 10.4  $\mu\text{mol}$ ) in NMP (100  $\mu\text{l}$ ) was added, and the resulting mixture was gently shaken for an additional 1 h and 30 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (1.5 mg, 19.8  $\mu\text{mol}$ ) in 50% aqueous ethanol (148  $\mu\text{l}$ ). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (3.9 mg, 26%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3809 $\pm$ 3. The resulting molecular weight was thus 3808 $\pm$ 3 amu (theoretical value 3808 amu).

#### Example 40

##### Synthesis of Arg<sup>26, 34</sup>Lys<sup>38</sup>(N<sup>6S</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-38)-OH

A mixture of Arg<sup>26, 34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (14 mg, 4.0  $\mu\text{mol}$ ), EDPA (14.3 mg, 110.6  $\mu\text{mol}$ ), NMP (980  $\mu\text{l}$ ) and water (980  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>α</sup>-hexadecanoyl-Glu(ONSu)-OBu<sup>t</sup>, prepared as described in Example 35, (6.4 mg, 11.9  $\mu\text{mol}$ ) in NMP (160  $\mu\text{l}$ ), and the reaction mixture was gently shaken for 1 h and 20 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (6.5 mg, 87  $\mu\text{mol}$ ) in 50% aqueous ethanol (653  $\mu\text{l}$ ). A 0.5% aqueous solution of ammonium-acetate (50 ml) was added, and the resulting mixture eluted onto a Varian 1 g C8 Mega Bond Elut® cartridge, the immobilised compound washed with 5% aqueous acetonitrile (10 ml), and finally liberated from the cartridge by elution with TFA (6 ml). The eluate was allowed to stand for 1 h and 30 min. at room temperature and then concentrated in vacuo. The residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (7.2 mg, 47%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3881 $\pm$ 3. The resulting molecular weight was thus 3880 $\pm$ 3 amu (theoretical value 3880 amu).

#### Example 41

##### Synthesis of Arg<sup>18,23,26,30,34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-hexadecanoyl) GLP-1(7-38)-OH

A mixture of Arg<sup>18,23,26,30,34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (1.0 mg, 0.27  $\mu\text{mol}$ ), EDPA (0.34 mg, 2.7  $\mu\text{mol}$ ) and DMSO (600

## 175

$\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of Pal-ONSu (0.28 mg, 0.8  $\mu\text{mol}$ ) in NMP (7  $\mu\text{l}$ ). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 6 h at room temperature. The reaction was quenched by the addition of a solution of glycine (1.6 mg, 21.7  $\mu\text{mol}$ ) in 50% aqueous ethanol (163  $\mu\text{l}$ ). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (0.17 mg, 16%) was isolated, and the product was analysed by MALDI-MS. The m/z value for the protonated molecular ion was found to be 3961 $\pm$ 3. The resulting molecular weight was thus 3960 $\pm$ 3 amu (theoretical value 3960 amu).

## Example 42

Synthesis of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-( $\omega$ -carboxytridecanoyl)) GLP-1(7-38)-OH

A mixture of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>38</sup>GLP-1(7-38)-OH (14 mg, 4.0  $\mu\text{mol}$ ), EDPA (14.3 mg, 111  $\mu\text{mol}$ ), NMP (980  $\mu\text{l}$ ) and water (980  $\mu\text{l}$ ) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of HOOC—(CH<sub>2</sub>)<sub>12</sub>—COONSu (4.2 mg, 11.9  $\mu\text{mol}$ ) in NMP (105  $\mu\text{l}$ ), the reaction mixture was gently shaken for 1 h and 50 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (6.5 mg, 87  $\mu\text{mol}$ ) in 50% aqueous ethanol (652  $\mu\text{l}$ ). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (5.8 mg, 39%) was isolated, and the product was analysed by MALDI-MS. The m/z value for the protonated molecular ion was found to be 3780 $\pm$ 3. The resulting molecular weight was thus 3779 $\pm$ 3 amu (theoretical value 3781 amu).

## Example 43

Synthesis of Arg<sup>34</sup>Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>ε</sup>-tetradecanoyl))) GLP-1(7-37)-OH

A mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (15 mg, 4.4  $\mu\text{mol}$ ), EDPA (16 mg, 124  $\mu\text{mol}$ ), NMP (2 ml) and water (4.8 ml) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>ε</sup>-tetradecanoyl-Glu(ONSu)-OBU<sup>t</sup>, prepared as described in Example 29, (12.1 mg, 23.7  $\mu\text{mol}$ ) in NMP (303  $\mu\text{l}$ ), and the reaction mixture was gently shaken for 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (6.5 mg, 86.9  $\mu\text{mol}$ ) in 50% aqueous ethanol (652  $\mu\text{l}$ ). A 0.5% aqueous solution of ammonium-acetate (50 ml) was added, and the resulting mixture eluted onto a Varian 1 g C8 Mega Bond Elut® cartridge, the immobilised compound washed with 5% aqueous acetonitrile (15 ml), and finally liberated from the cartridge by elution with TFA (6 ml). The eluate was allowed to stand for 1 h and 45 min. at room temperature and then concentrated in vacuo. The residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (3.9 mg, 26%) was isolated, and the product was analysed by MALDI-MS. The m/z value for the protonated molecular ion was found to be 3723 $\pm$ 3. The resulting molecular weight was thus 3722 $\pm$ 3 amu (theoretical value 3723 amu).

## 176

## Example 44

Synthesis of N<sup>ε</sup>-octadecanoyl-Glu(ONSu)-OBU<sup>t</sup>

To a suspension of H-Glu(OH)-OBU<sup>t</sup> (2.82 g, 13.9 mmol), DMF (370 ml) and EDPA (1.79 g, 13.9 mmol) was added drop by drop a solution of Ste-ONSu (5.3 g, 13.9 mmol) in DMF (60 ml). Dichloromethane (35 ml) was added, and the reaction mixture was stirred for 24 h at room temperature and then concentrated in vacuo. The residue was partitioned between 10% aqueous citric acid (330 ml) and ethyl acetate (200 ml), and the phases were separated. The organic phase was concentrated in vacuo and the residue dissolved in DMF (60 ml). The resulting solution was added drop by drop to a 10% aqueous solution of citric acid (400 ml) kept at 0° C. The precipitated compound was collected and washed with iced water and dried in a vacuum drying oven. The dried compound was dissolved in DMF (40 ml) and HONSu (1.63 g, 14.2 mmol) was added. To the resulting mixture was added a solution of DCC (2.66 g, 12.9 mmol) in dichloromethane (51 ml). The reaction mixture was stirred for 64 h at room temperature, and the precipitated compound was filtered off. The precipitate was recrystallised from n-heptane/2-propanol to give the title compound (4.96 g, 68%).

## Example 45

Synthesis of Arg<sup>26</sup>, <sup>34</sup>Lys<sup>38</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>ε</sup>-octadecanoyl))) GLP-1(7-38)-OH

A mixture of Arg<sup>26</sup>, <sup>34</sup>GLP-1(7-38)-OH (28 mg, 7.9  $\mu\text{mol}$ ), EDPA (28.6 mg, 221.5  $\mu\text{mol}$ ), NMP (1.96 ml) and water (1.96 ml) was gently shaken for 5 min. at room temperature. To the resulting mixture was added a solution of N<sup>ε</sup>-octadecanoyl-Glu(ONSu)-OBU<sup>t</sup> (17.93 g, 31.6  $\mu\text{mol}$ ), prepared as described in Example 44, in NMP (448  $\mu\text{l}$ ), and the reaction mixture was gently shaken for 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (13.1 mg, 174  $\mu\text{mol}$ ) in 50% aqueous ethanol (1.3 ml). A 0.5% aqueous solution of ammonium-acetate (120 ml) was added, and the resulting mixture was divided into two equal portions. Each portion was eluted onto a Varian 5 g C8 Mega Bond Elut® cartridge, the immobilised compound washed with 5% aqueous acetonitrile (25 ml), and finally liberated from the cartridge by elution TFA (25 ml). The combined eluates were allowed to stand for 1 h and 25 min. at room temperature and then concentrated in vacuo. The residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65° C. and the acetonitrile gradient was 0–100% in 60 minutes. The title compound (3.6 mg, 11%) was isolated, and the product was analysed by MALDI-MS. The m/z value for the protonated molecular ion was found to be 3940 $\pm$ 3. The resulting molecular weight was thus 3939 $\pm$ 3 amu (theoretical value 3937 amu).

## BIOLOGICAL FINDINGS

## Protraction of GLP-1 Derivatives After s.c. Administration

The protraction of a number GLP-1 derivatives of the invention was determined by monitoring the concentration thereof in plasma after sc administration to healthy pigs, using the method described below. For comparison also the concentration in plasma of GLP-1(7-37) after sc. administration was followed. The results are given in Table 1. The

protraction of other GLP-1 derivatives of the invention can be determined in the same way.

Pigs (50% Duroc, 25% Yorkshire, 25% Danish Landrace, app 40 kg) were fasted from the beginning of the experiment. To each pig 0.5 nmol of test compound per kg body weight was administered in a 50  $\mu$ M isotonic solution (5 mM phosphate, pH 7.4, 0.02% Tween®-20(Merck), 45 mg/ml mannitol (pyrogen free, Novo Nordisk). Blood samples were drawn from a catheter in vena jugularis at the hours indicated in Table 1. 5 ml of the blood samples were poured into

activated carbon (Merck) in 40 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 0.6 mM thiomersal, pH 7.5, was added to each tube. Before use, the suspension was mixed and allowed to stand for 2 h at 4° C. All samples were incubated for 1 h at 4° C. and then centrifuged at 3400\*g for 25 min. Immediately after the centrifugation, the supernatant was decanted and counted in a  $\gamma$ -counter. The concentration in the samples was calculated from individual standard curves. The following plasma concentrations were found, calculated as % of the maximum concentration for the individual compounds (n=2):

TABLE 1

Test compound*)	Hours after sc. Administration								
	0.75	1	2	4	6	8	10	12	24
GLP-1(7-37)		100	9	1					
Example 25	73	92	100	98	82	24	16	16	16
Example 17	76	71	91	100	84	68	30		9
Example 43		39	71	93	100	91	59	50	17
Example 37		26	38	97	100	71	81	80	45
Example 11	24	47	59	71	100	94	100		94
Example 12	36	54	65	94	80	100	85		93
Example 32	55	53	90	83	88	70	98	100	100
Example 14	18	25	32	47	98	83	97		100
Example 13	15	22	38	59	97	85	100		76
Example 38	60	53	100	66	48	39	25	29	0
Example 39	38	100	70	47	33	33	18	27	14
Example 40	47	19	50	100	51	56	34	14	0
Example 34	19	32	44	84	59	66	83	84	100

\*)The test compounds are the title compounds of the examples with the numbers given

chilled glasses containing 175  $\mu$ l of the following solution: 0.18 M EDTA, 1500 KIE/ml aprotinin (Novo Nordisk) and 3% bacitracin (Sigma), pH 7.4. Within 30 min, the samples were centrifuged for 10 min at 5–6000\*g. Temperature was kept at 4° C. The supernatant was pipetted into different glasses and kept at minus 20 ° C. until use.

The plasma concentrations of the peptides were determined by RIA using a monoclonal antibody specific for the N-terminal region of GLP-1(7-37). The cross reactivities were less than 1% with GLP-1(1-37) and GLP-1(8-36)amide and <0.1% with GLP-1(9-37), GLP-1(10-36)amide and GLP-1(11-36)amide. The entire procedure was carried out at 4° C.

The assay was carried out as follows: 100  $\mu$ l plasma was mixed with 271  $\mu$ l 96% ethanol, mixed using a vortex mixer and centrifuged at 2600\*g for 30 min. The supernatant was decanted into Minisorp tubes and evaporated completely (Savant Speedvac AS290). The evaporation residue was reconstituted in the assay buffer consisting of 80 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 0.1% HSA (Orpha 20/21, Behring), 10 mM EDTA, 0.6 mM thiomersal (Sigma), pH 7.5. Samples were reconstituted in volumes suitable for their expected concentrations, and were allowed to reconstitute for 30 min. To 300  $\mu$ l sample, 100  $\mu$ l antibody solution in dilution buffer containing 40 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 0.1% HSA, 0.6 mM thiomersal, pH 7.5, was added. A non-specific sample was prepared by mixing 300  $\mu$ l buffer with 100  $\mu$ l dilution buffer. Individual standards were prepared from freeze dried stocks, dissolved in 300  $\mu$ l assay buffer. All samples were pre-incubated in Minisorp tubes with antibody as described above for 72 h. 200  $\mu$ l tracer in dilution buffer containing 6-7000 CPM was added, samples were mixed and incubated for 48 h. 1.5 ml of a suspension of 200 ml per liter of heparin-stabilised bovine plasma and 18 g per liter of

Table 1 shows that the GLP-1 derivatives of the invention have a protracted profile of action relative to GLP-1(7-37) and are much more persistent in plasma than GLP-1(7-37). It also appears from Table 1 that the time at which the peak concentration in plasma is achieved varies within wide limits, depending on the particular GLP-1 derivative selected.

#### Stimulation of cAMP Formation in a Cell Line Expressing the Cloned Human GLP-1 Receptor

In order to demonstrate efficacy of the GLP-1 derivatives, their ability to stimulate formation of cAMP in a cell line expressing the cloned human GLP-1 receptor was tested. An  $\text{EC}_{50}$  was calculated from the dose-response curve.

Baby hamster kidney (BHK) cells expressing the human pancreatic GLP-1 receptor were used (Knudsen and Pridal, 1996, Eur. J. Pharm. 318, 429–435). Plasma membranes were prepared (Adelhorst et al, 1994, J. Biol. Chem. 269, 6275) by homogenisation in buffer (10 mmol/l Tris-HCl and 30 mmol/l NaCl pH 7.4, containing, in addition, 1 mmol/l dithiothreitol, 5 mg/l leupeptin (Sigma, St. Louis, Mo., USA), 5 mg/l pepstatin (Sigma, St. Louis, Mo., USA), 100 mg/l bacitracin (Sigma, St. Louis, Mo., USA), and 16 mg/l aprotinin (Novo Nordisk A/S, Bagsvaerd, Denmark)). The homogenate was centrifuged on top of a layer of 41 w/v % sucrose. The white band between the two layers was diluted in buffer and centrifuged. Plasma membranes were stored at –80° C. until used.

The assay was carried out in 96-well microtiter plates in a total volume of 140  $\mu$ l. The buffer used was 50 mmol/l Tris-HCl, pH 7.4 with the addition of 1 mmol/l EGTA, 1.5 mmol/l  $\text{MgSO}_4$ , 1.7 mmol/l ATP, 20 mM GTP, 2 mmol/l 3-isobutyl-1-methylxanthine, 0.01% Tween-20 and 0.1% human serum albumin (Reinst, Behringwerke AG, Marburg, Germany). Compounds to be tested for agonist activity were dissolved and diluted in buffer, added to the membrane



179

preparation and the mixture was incubated for 2 h at 37° C. The reaction was stopped by the addition of 25  $\mu$ l of 0.05 mol/l HCl. Samples were diluted 10 fold before analysis for cAMP by a scintillation proximity assay (RPA 538, Amersham, UK). The following results were obtained:

Test Compound <sup>a)</sup>	EC <sub>50</sub> , pM	Test Compound <sup>a)</sup>	EC <sub>50</sub> , pM
GLP-1(7-37)	61	Example 31	96
Example 45	120	Example 30	41
Example 43	24	Example 26	8.8
Example 40	55	Example 25	99
Example 39	5.1	Example 19	79
Example 38	54	Example 16	3.5
Example 37	60		

<sup>a)</sup>The test compounds are the title compounds of the examples with the numbers given.

#### Example 46

##### Synthesis of Arg<sup>26,34</sup>,Lys<sup>36</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-36)-OH

To a mixture of Arg<sup>26,34</sup>,Lys<sup>36</sup>GLP-1(7-36)-OH (12.2 mg, 3.67  $\mu$ mol), EDPA (13.3 mg, 103  $\mu$ mol), NMP (1.71 ml) and water (855  $\mu$ l) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (5.94 mg, 11  $\mu$ mol), prepared as described in PCT application no. PCT/DK97/00340, in NMP (148  $\mu$ l). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (6 mg, 81  $\mu$ mol) in water (0.6 ml). A 0.5% aqueous solution of ammonium-acetate (38 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (20 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (3.1 mg, 23%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3695 $\pm$ 3. The resulting molecular weight was thus 3694 $\pm$ 3 amu (theoretical value 3694 amu).

#### Example 47

##### Synthesis of Arg<sup>26,34</sup>,Lys<sup>36</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-octadecanoyl))) GLP-1(7-36)-OH

To a mixture of Arg<sup>26,34</sup>,Lys<sup>36</sup>GLP-1(7-36)-OH (12.2 mg, 3.7  $\mu$ mol), EDPA (13.3 mg, 103  $\mu$ mol), NMP (1.71 ml) and water (855  $\mu$ l) was added a solution of Ste-Glu(ONSu)-OBu<sup>t</sup> (6.25 mg, 11  $\mu$ mol), prepared as described in PCT application no. PCT/DK97/00340, in NMP (1 ml). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (6 mg, 81  $\mu$ mol) in water (0.6 ml). A 0.5% aqueous solution of ammonium acetate (54 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (20 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax

180

300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (3.7 mg, 27%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3723 $\pm$ 3. The resulting molecular weight was thus 3722 $\pm$ 3 amu (theoretical value 3722 amu).

#### Example 48

##### Synthesis of Lithocholic Acid 2,5-dioxopyrrolidin-1-yl Ester

To a solution of lithocholic acid (5.44 g, 14.3 mmol) in a mixture of anhydrous THF (120 ml) and anhydrous acetonitril (30 ml) was added N-hydroxysuccinimide (1.78 g, 15 mmol). The mixture was cooled to 10° C., a solution of DCC (3.44 g, 16.7 mmol) in anhydrous THF (30 ml) was added drop wise, and the resulting reaction mixture stirred for 16 h at room temperature. The reaction mixture was filtered and partitioned between dichloromethane (450 ml) and 10% aqueous Na<sub>2</sub>CO<sub>3</sub>(150 ml). The phases were separated, and the organic phase washed with 10% aqueous Na<sub>2</sub>CO<sub>3</sub>(150 ml), water (2 $\times$ 150 ml), and dried (MgSO<sub>4</sub>). The solvent was concentrated in vacuo. The residue was crystallised from a mixture of dichloromethane (30 ml) and n-heptane (30 ml). The precipitate was dried in a vacuum drying oven for 36 h to give the title compound (3.46 g, 51%).

#### Example 49

##### Synthesis of Lit-Glu(ONSu)-OBu<sup>t</sup>

A suspension of H-Glu(OH)-OBu<sup>t</sup> (1.28 g, 6.33 mmol), DMF (88 ml) and EDPA (0.82 g, 6.33 mmol) and lithocholic acid 2,5-dioxopyrrolidin-1-yl ester, prepared as described in example 48, was stirred for 16 h at room temperature. The reaction mixture was concentrated in vacuo and the residue dissolved in ethyl acetate (40 ml). The resulting solution was washed with 5% aqueous citric acid (2 $\times$ 25 ml), brine (10 ml), and filtered). The solvent was concentrated in vacuo and the residue dissolved in DMF (12 ml). The resulting solution was added drop wise to a 10% aqueous solution of citric acid whereby the product precipitates. The precipitate was collected and washed with iced water, and dried in vacuo. The crude product was recrystallised from a mixture of n-heptane (40 ml) and 2-propanol (17 ml). The precipitate was dried in a vacuum drying oven for 4 h to give the free acid intermediate.

To a solution of the free acid intermediate in DMF (18 ml) was added hydroxysuccinimide (0.45 g, 3.91 mmol), followed by a solution of DCC (0.73 g, 3.56 mmol) in dichloromethane (18 ml). The resulting mixture was stirred at ambient temperature for 18 h, and then filtered. The filtrate was concentrated in vacuo to a solid, and the residue was dissolved in dichloromethane (25 ml), and the filtration repeated, the solvent removed in vacuo to give a foam. The residue was dissolved in refluxing n-heptane (35 ml), and the product crystallised b addition of 2-propanol. The precipitate was collected, washed with cold n-heptane, dried at 35° C. in vacuo to give the title compound (1.34 g, 57%).

#### Example 50

##### Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-lithochoyl))) GLP-1(7-37)-OH

To a mixture of Arg<sup>34</sup>,Lys<sup>26</sup>GLP-1(7-37)-OH (41.1 mg, 12.2  $\mu$ mol), EDPA (44 mg, 340  $\mu$ mol), NMP (5.76 ml) and

181

water (2.88 ml) was added a solution of Lit-Glu(ONSu)-OBu<sup>t</sup> (24 mg, 37 μmol), prepared as described in example 49, in NMP (600 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 75 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20 mg, 268 μmol) in water (2 ml). A 0.5% aqueous solution of ammonium acetate (128 ml) was added, and the resulting mixture divided into two equal portions, and each portion eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (2x25 ml), and finally liberated from the cartridge by elution with TFA (2x25 ml). The combined eluates were concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (5 mg, 11%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3872±3. The resulting molecular weight was thus 3871±3 amu (theoretical value 3871 amu).

## Example 51

Synthesis of Arg<sup>26</sup>,Lys<sup>34</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)-OH

To a mixture of Arg<sup>26</sup>,Lys<sup>34</sup>GLP-1(7-37)-OH (18 mg, 5.3 μmol), EDPA (19.3 mg, 149 μmol), NMP (2.52 ml) and water (1.26 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (8.6 mg, 16 μmol) in NMP (215 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (8.8 mg, 117 μmol) in water (0.88 ml). A 0.5% aqueous solution of ammonium acetate (50 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (6 mg, 30%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3752±3. The resulting molecular weight was thus 3751±3 amu (theoretical value 3751 amu).

## Example 52

Synthesis of Desamino-His<sup>7</sup>,Arg<sup>26</sup>,Lys<sup>34</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)-OH

To a mixture of desamino-His<sup>7</sup>,Arg<sup>26</sup>,Lys<sup>34</sup>GLP-1(7-37)-OH (14.3 mg, 4.2 μmol), EDPA (15.3 mg, 119 μmol), NMP (2 ml) and water (1 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (6.84 mg, 12.7 μmol) in NMP (171 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 50 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (7 mg, 99 μmol) in water (700 μl). A 0.5% aqueous solution of ammonium acetate (42 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25

182

ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (5.6 mg, 35%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3738±3. The resulting molecular weight was thus 3737±3 amu (theoretical value 3737 amu).

## Example 53

Synthesis of Gly<sup>8</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-38)-OH

To a mixture of Gly<sup>8</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>GLP-1(7-38)-OH (11.8 mg, 3.4 μmol), EDPA (12.1 mg, 94 μmol), NMP (1.65 ml) and water (0.83 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (5.4 mg, 10 μmol) in NMP (135 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 75 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.5 mg, 73.7 μmol) in water (553 μl). A 0.5% aqueous solution of ammonium acetate (36 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (5 mg, 38%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3895±3. The resulting molecular weight was thus 3894±3 amu (theoretical value 3894 amu).

## Example 54

Synthesis of Gly<sup>8</sup>,Glu<sup>37</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-38)-OH

To a mixture of Gly<sup>8</sup>,Glu<sup>37</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>GLP-1(7-38)-OH (9 mg, 2.48 μmol), EDPA (9 mg, 69.4 μmol), NMP (1.25 ml) and water (0.63 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (4 mg, 7.4 μmol) in NMP (100 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 105 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (4.1 mg, 54.6 μmol) in water (410 μl). A 0.5% aqueous solution of ammonium acetate (27 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by elution with TFA (15 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (2.9 mg, 29%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3967±3. The resulting molecular weight was thus 3966±3 amu (theoretical value 3967 amu).

## Example 55

Synthesis of Gly<sup>8</sup>,Glu<sup>37</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-octadecanoyl))) GLP-1(7-38)-OH

To a mixture of Gly<sup>8</sup>,Glu<sup>37</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>GLP-1(7-38)-OH (9 mg, 2.5 μmol), EDPA (9 mg, 69.4 μmol), NMP (1.25

183

ml) and water (0.63 ml) was added a solution of Ste-Glu (ONSu)-OBu' (4.2 mg, 7.4  $\mu$ mol in NMP (105  $\mu$ l). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 105 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (4.1 mg, 54.6  $\mu$ mol) in water (409  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (27 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by elution with TFA (15 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (3.2 mg, 32%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3995 $\pm$ 3. The resulting molecular weight was thus 3994 $\pm$ 3 amu (theoretical value 3995 amu).

## Example 56

## Synthesis of Cap-Glu(ONSu)-OBu'

To a solution of octanoic acid (5 g, 34.7 mmol) and N-hydroxysuccinimide (4 g, 34.7 mmol) in anhydrous acetonitril (10 ml) was added a solution of DCC (7.15 g, 34.7 mmol) in anhydrous dichloromethane (15 ml), and the resulting reaction mixture stirred for 16 h at room temperature. The precipitated solid was filtered off and recrystallised from a mixture of n-heptane (40 ml) and 2-propanol (2 ml). The precipitate was dried in a vacuum drying oven for 16 h to give the intermediate Cap-ONSu. A suspension of the crude ester intermediate (3.9 g, 16.2 mmol), (L)-H-Glu (OH)-OBu' (3.28 g, 16.2 mmol), DMF (268 ml) and EDPA (2.1 g, 16.2 mmol) was stirred for 64 h at room temperature. The reaction mixture was concentrated in vacuo and the residue dissolved in ethyl acetate (50 ml). The resulting solution was washed with 5% aqueous citric acid (2 $\times$ 25 ml). The solvent was concentrated in vacuo and the residue dissolved in DMF (36 ml). The resulting solution was added drop wise to a 10% aqueous solution of citric acid (357 ml) and extracted with ethyl acetate (200 ml), and dried (MgSO<sub>4</sub>). The solvent was concentrated in vacuo to give the crude glutamic acid intermediate. To a mixture of the crude glutamic acid intermediate, N-hydroxysuccinimide (1.85 g, 16.1 mmol) and DMF (25 ml) was added a solution of DCC (3.32 g, 16.1 mmol) in dichloromethane (15 ml). The resulting mixture was stirred at ambient temperature for 20 h. The reaction mixture was filtered and the solvent concentrated in vacuo. The residue was purified on a silica gel column (40–63 $\mu$ ), eluted with a mixture of dichloromethane and acetonitril (1:1) to give the title compound (0.63 g, 6% over all).

## Example 57

Synthesis of Desamino-His<sup>7</sup>,Arg<sup>26</sup>,Lys<sup>34</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-octanoyl))) GLP-1(7-37)-OH

To a mixture of desamino-His<sup>7</sup>,Arg<sup>26</sup>,Lys<sup>34</sup>GLP-1(7-37)-OH (14.3 mg, 4.2  $\mu$ mol), EDPA (15.3 mg, 119  $\mu$ mol), NMP (2 ml) and water (1 ml) was added a solution of Cap-Glu (ONSu)-OBu' (6.8 mg, 12.7  $\mu$ mol), prepared as described in example 56, in NMP (135  $\mu$ l). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of

184

glycine (7 mg, 93  $\mu$ mol) in water (698  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (42 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (4.1 mg, 27%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3626 $\pm$ 3. The resulting molecular weight was thus 3625 $\pm$ 3 amu (theoretical value 3625 amu).

## Example 58

Synthesis of Glu<sup>37</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-38)-OH

To a mixture of Glu<sup>37</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>GLP-1(7-38)-OH (17.6 mg, 4.9  $\mu$ mol), EDPA (17.6 mg, 136  $\mu$ mol), NMP (1.23 ml) and water (2.46 ml) was added a solution of Pal-Glu (ONSu)OBu' (7.9 mg, 14.6  $\mu$ mol) in NMP (197  $\mu$ l). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (8 mg, 107  $\mu$ mol) in water (804  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (49 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (5.1 mg, 26%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3981 $\pm$ 3. The resulting molecular weight was thus 3980 $\pm$ 3 amu (theoretical value 3981 amu).

## Example 59

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-octadecanoyl))) GLP-1(7-37)-OH

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (41.1 mg, 12.2  $\mu$ mol), EDPA (44 mg, 341  $\mu$ mol), NMP (5.76 ml) and water (2.88 ml) was added a solution of Ste-Glu(ONSu)-OBu' (20.7 mg, 36.5  $\mu$ mol) in NMP (517  $\mu$ l). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (20.1 mg, 268  $\mu$ mol) in water (2.01 ml). A 0.5% aqueous solution of ammonium-acetate (120 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (15.4 mg, 34%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be

## 185

3781±3. The resulting molecular weight was thus 3780±3 amu (theoretical value 3779 amu).

## Example 60

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-decanoyl) GLP-1(7-37)

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (20 mg, 5.9 μmol), EDPA (21.4 mg, 165 μmol), NMP (2.8 ml) and water (1.4 ml) was added a solution of Cac-ONSu (4.8 mg, 17.7 μmol) in NMP (119 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (9.8 mg, 130 μmol) in water (98 μl). The resulting mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (7.4 mg, 35%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3539.6±3. The resulting molecular weight was thus 3538.6±3 amu (theoretical value 3538 amu).

## Example 61

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-(hexadecanoyl)) GLP-1(7-37)-OH

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (41.1 mg, 12.2 μmol), EDPA (44 mg, 340 μmol), NMP (2.88 ml) and water (2.88 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (5.5 mg, 10.2 μmol) in NMP (137 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.6 mg, 74.6 μmol) in water (560 μl). A 0.5% aqueous solution of ammonium acetate (34 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The solvent was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (15 mg, 34%) was isolated, and the product was analysed by PDMS.

## Example 62

Synthesis of Arg<sup>26,34</sup>,Lys<sup>27</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)-OH

To a mixture of Arg<sup>26,34</sup>,Lys<sup>27</sup>GLP-1(7-37)-OH (11.6 mg, 3.4 μmol), EDPA (12.3 mg, 94.9 μmol), NMP (1.6 ml) and water (0.8 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (5.5 mg, 10.2 μmol) in NMP (137 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.6 mg, 74.6 μmol) in water (560 μl). A 0.5% aqueous solution of ammonium acetate (34 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The solvent was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (2.1 mg, 16%) was isolated, and the product was analysed by PDMS.

## Example 63

Synthesis of Arg<sup>26,34</sup>,Lys<sup>23</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)-OH

To a mixture of Arg<sup>26,34</sup>,Lys<sup>23</sup>GLP-1(7-37)-OH (11.6 mg, 3.4 μmol), EDPA (12.3 mg, 94.9 μmol), NMP (1.6 ml)

## 186

and water (0.8 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (5.5 mg, 10.2 μmol) in NMP (137 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.6 mg, 74.6 μmol) in water (560 μl). A 0.5% aqueous solution of ammonium acetate (34 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The solvent was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (3.1 mg, 24%) was isolated, and the product was analysed by PDMS.

## Example 64

Synthesis of Arg<sup>26,34</sup>,Lys<sup>18</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)-OH

To a mixture of Arg<sup>26,34</sup>,Lys<sup>18</sup>GLP-1(7-37)-OH (11.7 mg, 3.4 μmol), EDPA (12.2 mg, 94.6 μmol), NMP (1.6 ml) and water (0.8 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (5.5 mg, 10.2 μmol) in NMP (137 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.6 mg, 74.6 μmol) in water (560 μl). A 0.5% aqueous solution of ammonium acetate (34 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The solvent was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (1.9 mg, 15%) was isolated, and the product was analysed by PDMS.

## Example 65

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-(octanoyl)) GLP-1(7-37)-OH

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (41.1 mg, 12.2 μmol), EDPA (44 mg, 341 μmol), NMP (5.76 ml) and water (2.88 ml) was added a solution of Cap-ONSu (8.8 mg, 36.5 μmol), prepared as described in example 56, in NMP (106 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 115 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20 mg, 268 μmol) in water (200 μl). The solvent was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (18.8 mg, 44%) was isolated, and the product was analysed by PDMS.

## Example 66

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-(dodecanoyl)) GLP-1(7-37)-OH

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (41.1 mg, 12.2 μmol), EDPA (44 mg, 341 μmol), NMP (5.76 ml) and water

## 187

(2.88 ml) was added a solution of Lau-ONSu (8.8 mg, 36.5  $\mu$ mol), prepared in a similar manner as described for Cap-ONSu in example 56), in NMP (271  $\mu$ l). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 100 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20.1 mg, 268  $\mu$ mol) in water (200  $\mu$ l). The solvent was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (18 mg, 42%) was isolated, and the product was analysed by PDMS.

## Example 67

## Synthesis of Pal-GABA-ONSu

A mixture of Pal-ONSu (3 g, 8.48 mmol),  $\gamma$ -aminobutyric acid (0.87 g, 8.48 mmol) in DMF (200 ml) was stirred at room temperature for 60 h. The reaction mixture was filtered and the filtrate was added drop wise to 10% aqueous citric acid (500 ml). The precipitated N-acylated intermediate was collected and dried in vacuo. To a suspension of the dried intermediate in DMF (35 ml) was added a solution of DCC (1.45 g, 7.0 mmol) in dichloromethane (20 ml). The resulting mixture was stirred at room temperature for 20 h, and then filtered. The solvent was removed in vacuo to give a solid residue. The residue was recrystallised from a mixture of n-heptane (50 ml) and 2-propanol (2.5 ml) to give the title compound (2.5 g, 75%).

## Example 68

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -aminobutyroyl(N<sup>ε</sup>-hexadecanoyl))) GLP-1(7-37)-OH

To a mixture of Arg<sup>34</sup>, Lys<sup>26</sup>GLP-1(7-37)-OH (41.1 mg, 12.2  $\mu$ mol), EDPA (44 mg, 341  $\mu$ mol), NMP (5.76 ml) and water (2.88 ml) was added a solution of Pal-GABA-ONSu (16 mg, 36.5  $\mu$ mol), prepared as described in example 67) in NMP (400  $\mu$ l). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 100 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20 mg, 268  $\mu$ mol) in water (200  $\mu$ l). The solvent was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (15.8 mg, 35%) was isolated, and the product was analysed by PDMS.

## Example 69

Synthesis of N<sup>ε</sup>-hexadecanoyl-D-glutamic acid  $\alpha$ -t-butyl ester- $\gamma$ -2,5-dioxopyrrolidin-1-yl ester

A mixture of Pal-ONSu (6.64 g, 18.8 mmol), D-glutamic acid  $\alpha$ -tert-butyl ester (4.5 g, 18.8 mmol) and EDPA (4.85 g, 37.5 mmol) in DMF (538 ml) was stirred at room temperature for 60 h. The solvent was removed and the residue dissolved in ethyl acetate (175 ml). The resulting solution was extracted with 10% aqueous citric acid (2 $\times$ 125 ml), and the organic phase concentrated in vacuo. The residue was dissolved in DMF (60 ml), and the resulting mixture slowly added to 10% aqueous citric acid (500 ml). The precipitated compound was collected and dried in vacuo, to give the

## 188

crude N-acylated glutamic acid intermediate. The crude intermediate was dissolved in DMF (35 ml), and a solution of DCC (3.5 g, 17 mmol) in dichloromethane (70 ml) was added. The resulting mixture was stirred at room temperature for 20 h, and then filtered. The filtrate was concentrated in vacuo, and the solid residue recrystallised from a mixture of n-heptane (75 ml) and 2-propanol (5 ml), to give the title compound (5.2 g, 50%).

## Example 70

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -D-glutamyl(N<sup>ε</sup>-hexadecanoyl))) GLP-1(7-37)-OH

To a mixture of Arg<sup>34</sup>, Lys<sup>26</sup>GLP-1(7-37)-OH (41.1 mg, 12.2  $\mu$ mol), EDPA (44 mg, 341  $\mu$ mol), NMP (5.76 ml) and water (2.88 ml) was added a solution of N<sup>ε</sup>-hexadecanoyl-D-glutamic acid  $\alpha$ -t-butyl ester- $\gamma$ -2,5-dioxopyrrolidin-1-yl ester (19.7 mg, 36.5  $\mu$ mol) in NMP (491  $\mu$ l). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 95 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20 mg, 268  $\mu$ mol) in water (2 ml). A 0.5% aqueous solution of ammonium acetate (120 ml) was added, and the resulting mixture divided into to equal portions, and each portion eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The combined eluates were concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (10.5 mg, 23%) was isolated, and the product was analysed by PDMS.

## Example 71

Synthesis of Lys<sup>34</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>ε</sup>-tetradecanoyl))) GLP-1(7-37)

To a mixture of GLP-1(7-37)-OH (33.6 mg, 8.9  $\mu$ mol), EDPA (32.4 mg, 250  $\mu$ mol), NMP (2.1 ml) and water (2.1 ml) was added a solution of Myr-Glu(ONSu)-OBu<sup>t</sup> (9.1 mg, 17.9  $\mu$ mol), prepared as described above, in NMP (228  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 80 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (14.8 mg, 197  $\mu$ mol) in water (1.47 ml). A 0.5% aqueous solution of ammonium acetate (100 ml) was added, and the resulting mixture divided into two equal portions, and each portion eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (2 $\times$ 25 ml), and finally liberated from the cartridge by elution with TFA (2 $\times$ 25 ml). The combined eluates were concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (0.19 mg, 0.6%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3693 $\pm$ 3. The resulting molecular weight was thus 3692 $\pm$ 3 amu (theoretical value 3695 amu).

## Example 72

Synthesis of Arg<sup>26,34</sup>,Lys<sup>8</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>ε</sup>-hexadecanoyl))) GLP-1(7-37)

To a mixture of Arg<sup>26,34</sup>Lys<sup>8</sup>GLP-1(7-37)-OH (10.3 mg, 3  $\mu$ mol), EDPA (10.8 mg, 83  $\mu$ mol), NMP (1.44 ml) and

## 189

water (0.72 ml) was added a solution of Pal-Glu(ONSu)-OBu<sup>t</sup> (4.8 mg, 8.9  $\mu$ mol), prepared as described above, in NMP (120  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 70 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (4.9 mg, 65.3  $\mu$ mol) in water (490  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (30 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (3.2 mg, 28%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3836 $\pm$ 3. The resulting molecular weight was thus 3835 $\pm$ 3 AMU (theoretical value 3836 AMU).

## Example 73

Synthesis of Lau-Glu(ONSu)-OBu<sup>t</sup>

To a solution of H-Glu-OBu<sup>t</sup> (3 g, 15 mmol) in DMF (344 ml) was added EDPA (2.58 ml, 15 mmol) and a solution of Lau-ONSu (4.5 g, 15 mmol), prepared in a similar manner as described for Cap-ONSu in example 56, in DMF (74 ml). The resulting mixture was stirred at ambient temperature for 18 h, and the solvent removed in vacuo. The oily residue was partitioned between ethyl acetate (150 ml) and 5% aqueous citric acid (250 ml). The organic phase was concentrated in vacuo. The residue was dissolved in DMF (40 ml) and the solution added drop by drop to a 10% aqueous citric acid solution (350 ml). The precipitated product was collected, washed with water and dried in vacuo for 18 h to give the intermediate free acid. To solution of the free acid intermediate in DMF (25 ml) was added N-hydroxysuccinimide (1.7 g, 14.8 mmol) and a solution of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (2.58 g, 13.5 mmol) in dichloromethane (52 ml). The resulting mixture was stirred at room temperature for 18 h, and the solvents removed in vacuo. The oily residue was partitioned between dichloromethane (80 ml) and water (80 ml). The organic phase was washed with 5% aqueous citric acid, dried (MgSO<sub>4</sub>), and concentrated in vacuo to a solid. The solid residue was crystallised from a mixture of n-heptane (77 ml) and 2-propanol (50 ml), and finally recrystallised from n-heptane (76 ml) to give the title compound (2.96 g, 46%).

## Example 74

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N <sup>$\epsilon$</sup> -( $\gamma$ -glutamyl(N <sup>$\alpha$</sup> -dodecanoyl))) GLP-1(7-37)

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (20.6 mg, 6.1  $\mu$ mol), EDPA (22 mg, 171  $\mu$ mol), NMP (2.88 ml) and water (1.44 ml) was added a solution Lau-Glu(ONSu)-OBu<sup>t</sup> (10.2 mg, 21.2  $\mu$ mol), prepared as described in example 73, in NMP (255  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 75 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (10 mg, 134  $\mu$ mol) in water (100  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (61 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25

## 190

ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (8.2 mg, 36%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3693 $\pm$ 3. The resulting molecular weight is 3692 $\pm$ 3 AMU (theoretical value 3693 AMU).

## Example 75

Synthesis of Lau- $\beta$ -Ala-ONSu

To a solution of Lau-ONSu (4.25 g, 14.3 mmol), prepared in a similar manner to in DMF (400 ml) was added EDPA (1.84 g, 14.3 mmol) and  $\beta$ -alanine (1.27 g, 14.3 mmol). The resulting mixture was stirred at ambient temperature for 18 h. Water (250 ml) and DMF (50 ml) were added and the solution stirred for 1 h at room temperature. The solvents were removed in vacuo to give a solid. The solid residue was dissolved in DMF (50 ml) and the solution added drop by drop to a 5% aqueous solution of citric acid (200 ml). The precipitate collected, washed with water (50 ml) and dried in vacuo to give the title compound (3.6 g, 93%).

## Example 76

Synthesis of Pal- $\beta$ -Ala-ONSu

To a solution of Pal-ONSu (4.25 g, 14.3 mmol) in DMF (400 ml) was added EDPA (1.84 g, 14.3 mmol) and  $\beta$ -alanine (1.27 g, 14.3 mmol). The resulting mixture was stirred at ambient temperature for 18 h. Water (250 ml) and DMF (50 ml) were added and the solution stirred for 1 h at room temperature. The solvents were removed in vacuo to give a solid. The solid residue was dissolved in DMF (50 ml) and the solution added drop by drop to a 5% aqueous solution of citric acid (200 ml). The precipitate collected, washed with water (50 ml) and dried in vacuo to give the title compound (3.6 g, 93%).

## Example 77

## Synthesis of Myr-GABA-ONSu

To a solution of Myr-ONSu (4 g, 12.3 mmol) in DMF (350 ml) was added EDPA (1.58 g, 12.3 mmol) and  $\gamma$ -aminobutyric acid (1.26 g, 12.3 mmol). The resulting mixture was stirred at ambient temperature for 18 h. Water (50 ml) was added and the solution stirred for 1 h at room temperature. The solvents were removed in vacuo to give a solid. The solid residue was dissolved in DMF (75 ml) and the solution added drop by drop to a 5% aqueous solution of citric acid (250 ml). The precipitate collected, washed with water (100 ml) and dried in vacuo to give the free acid intermediate (3.65 g, 95%). To a solution of the free acid intermediate (3 g, 9.6 mmol), N-hydroxysuccinimide (1.65 g, 14.4 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (3.67 g, 19.1 mmol) in DMF (330 ml) was stirred for 18 h at room temperature, and the solvent removed in vacuo to give a solid. The solid residue was dissolved in dichloromethane (100 ml) and washed with brine (100 ml). The organic phase was dried (MgSO<sub>4</sub>) and concentrated in vacuo to give a solid. The solid residue was recrystallised from n-heptane (75 ml) to give the title compound (2.8 g, 71%).

## Example 78

Synthesis of Pal- $\beta$ -Ala-ONSu

To a solution of Pal-ONSu (0.9 g, 2.8 mmol) in DMF (100 ml) were added N-hydroxysuccinimide (0.35 g, 3 mmol) and

## 191

N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (0.79 g, 4.1 mmol). The resulting mixture was stirred at ambient temperature for 40 h, and the solvent removed in vacuo. The solid residue was partitioned between water (50 ml) and dichloromethane (50 ml). The organic phase was separated, dried (MgSO<sub>4</sub>) and the solvent removed in vacuo to give the title compound (1.1 g, 94%)

## Example 79

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>68</sup>-(β-alanyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (19.2 mg, 5.7 μmol), EDPA (20.5 mg, 159 μmol), NMP (2.7 ml) and water (1.35 ml) was added a solution Pal-β-Ala-ONSu (7.2 mg, 17 μmol), prepared as described in example 79, in NMP (181 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (9.3 mg, 125 μmol) in water (93 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (11.6 mg, 55%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3694±3. The resulting molecular weight was thus 3693±3 AMU (theoretical value 3693 AMU).

## Example 80

Synthesis of Pal-Glu(OBu<sup>t</sup>)-ONSu

To a solution of H-Glu(OH)-OBu<sup>t</sup> (2.7 g, 11.3 mmol) and Pal-ONSu (3.98 g, 11.3 mmol) in DMF (300 ml) was added EDPA (3.2 g, 24.8 mmol). The resulting mixture was stirred at ambient temperature for 18 h, and the solvent concentrated in vacuo to give an oil. The oily residue was dissolved in DMF (60 ml) and the solution added drop by drop to a 10% aqueous solution of citric acid (300 ml) whereby a precipitate was formed. The precipitate was collected, washed with cold water (25 ml), and dried in vacuo to give free acid intermediate (4.44 g, 89%). The free acid intermediate (4 g, 9.1 mmol) was dissolved in DMF (50 ml) and N-hydroxysuccinimide (1.15 g, 10 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.6 g, 13.6 mmol) were added. The resulting mixture was stirred at room temperature for 60 h, the solvent concentrated in vacuo to give the crude title compound (8.2 g).

## Example 81

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-(α-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (25.6 mg, 7.6 μmol), EDPA (27.4 mg, 212 μmol), NMP (3.5 ml) and water (1.75 ml) was added a solution of Pal-Glu(OBu<sup>t</sup>)-ONSu (12.2 mg, 22.7 μmol), prepared as described in example 80, in NMP (305 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 100 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.5 mg, 168 μmol) in water (125 μl). A 0.5% aqueous solution of ammonium acetate (72.5 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and

## 192

finally liberated from the cartridge by elution with TFA (30 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (6.1 mg, 22%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3751±3. The resulting molecular weight was thus 3750±3 AMU (theoretical value 3751 AMU).

## Example 82

Synthesis of Ste-GABA-ONSu

To a solution of Ste-ONSu (3 g, 7.9 mmol) in DMF (270 ml) was added EDPA (1 g, 7.9 mmol) and a solution of γ-aminobutyric acid (0.81 g, 7.9 mmol) in water (40 ml). The resulting suspension was stirred at ambient temperature for 18 h, and then concentrated in vacuo to a final volume of 50 ml. The resulting suspension was added to a 5% aqueous solution of citric acid (500 ml) whereby a precipitate is formed. The precipitate was collected and washed with water (50 ml), and dried in vacuo for 4 h to give the free acid intermediate (2.8 g, 97%). To a mixture of the free acid intermediate (2.6 g, 7 mmol), N-hydroxysuccinimide (1.21 g, 10.5 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.69 g, 14 mmol) in NMP (300 ml) was stirred for 70 h, and the solvent removed in vacuo to give a solid. The solid residue was dissolved in dichloromethane (100 ml) and washed with brine (2×100 ml). The organic phase was dried (MgSO<sub>4</sub>) and concentrated in vacuo to give a solid. The solid residue was recrystallised from n-heptane (75 ml) to give the title compound (2.2 g, 67%).

## Example 83

Synthesis of Pal-Isonip-ONSu

To a suspension of 1-hexadecanoylbenzotriazole (3 g, 8.4 mmol), prepared as described in the literature (Kreutzberger; van der Goot, Arch. Pharm., 307, 1974), in DMF (350 ml) were added EDPA (1.08 g, 8.4 mmol) and a solution of piperidine-4-carboxylic acid in water (20 ml). The resulting suspension was stirred at room temperature for 12d, and then concentrated in vacuo to an oil. The oily residue was added drop by drop to a 5% aqueous solution of citric acid (300 ml) whereby a precipitate was formed. The precipitate was collected and washed with water (50 ml), dried in vacuo for 2 h to give the free acid intermediate (3 g, 97%). To a solution of the free acid intermediate (2.8 g, 7.6 mmol), N-hydroxysuccinimide (1.31 g, 11.4 mmol) in DMF (250 ml) was added N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.92 g, 15.2 mmol). The resulting mixture was stirred at ambient temperature for 18 h, and the solvent removed in vacuo to give an oil. The oily residue was dissolved in dichloromethane (100 ml), washed with brine (50 ml), dried (MgSO<sub>4</sub>) and concentrated in vacuo to give the crude title compound (4.1 g, quant.).

## Example 84

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-(piperidinyl-4-carbonyl(N-hexadecanoyl))) GLP-1(7-37)

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (25 mg, 7.4 μmol), EDPA (26.7 mg, 207 μmol), NMP (3.5 ml) and water (1.75 ml) was added a solution Pal-Isonip-ONSu (13.7 mg,

## 193

30  $\mu\text{mol}$ ), prepared as described in example 83 in NMP (343  $\mu\text{l}$ ). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.2 mg, 163  $\mu\text{mol}$ ) in water (122  $\mu\text{l}$ ). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (12 mg, 44%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3734 $\pm$ 3. The resulting molecular weight was thus 3733 $\pm$ 3 amu (theoretical value 3733 amu).

## Example 85

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>6</sup>-( $\gamma$ -glutamyl(N <sup>$\alpha$</sup> -decanoyl))) GLP-1(7-37)

To a mixture of Arg<sup>34</sup>GLP-1(7-37)-OH (25 mg, 7.4  $\mu\text{mol}$ ), EDPA (26.7 mg, 207  $\mu\text{mol}$ ), NMP (3.5 ml) and water (1.75 ml) was added a solution of Cac-Glu(ONSu)-OBu<sup>t</sup> (10 mg, 22.1  $\mu\text{mol}$ ) in NMP (252  $\mu\text{l}$ ). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 140 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.2 mg, 162  $\mu\text{mol}$ ) in water (122  $\mu\text{l}$ ). A 0.5% aqueous solution of ammonium acetate (73 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (12.2 mg, 45%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3669.7 $\pm$ 3. The resulting molecular weight was thus 3668.7 $\pm$ 3 amu (theoretical value 3667 amu).

## Example 86

## General Method A

## Synthesis of Alkanoic Acid 2,5-dioxopyrrolidin-1-yl Ester

To a solution of the alkanolic acid (34.7 mmol) and N-hydroxysuccinimide (4 g, 34.7 mmol) in anhydrous acetonitril (10 ml) was added a solution of DCC (7.15 g, 34.7 mmol) in anhydrous dichloromethane (15 ml), and the resulting reaction mixture was stirred for 16 h at room temperature. The precipitated solid was filtered off and recrystallised from a mixture of n-heptane and 2-propanol. The precipitate was dried in vacuo for 16 h to give the title compound.

Synthesis of Lys(N<sup>6</sup>-alkanoyl)-peptide

To a mixture of the peptide (5.9  $\mu\text{mol}$ ), EDPA (21 mg, 164  $\mu\text{mol}$ ), NMP (5.8 ml) and water (2.9 ml) was added a solution of the alkanolic acid 2,5-dioxopyrrolidin-1-yl ester (37  $\mu\text{mol}$ ), prepared as described above, in NMP (0.5 ml). The reaction mixture was gently shaken for 5 min at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the

## 194

addition of a solution of glycine (9.7 mg, 129  $\mu\text{mol}$ ) in water (97  $\mu\text{l}$ ). The solvent was removed in vacuo, and the residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% for 60 minutes.

Coupling of a desired group A comprising the 5- or 6-membered ring system Y to the N-terminal end of the peptide may be performed using solid phase protein synthesis techniques as explained above.

## Example 87

## General Method B

Synthesis of N <sup>$\alpha$</sup> -alkanoyl-(L)-glutamic acid  $\alpha$ -tert-butyl- $\gamma$ -(2,5-dioxopyrrolidin-1-yl) Diester

A suspension of the alkanolic acid 2,5-dioxopyrrolidin-1-yl ester (16.2 mmol), prepared as described under General method A, (L)-glutamic acid  $\alpha$ -tert-butyl ester (3.28 g, 16.2 mmol), DMF (268 ml) and EDPA (2.1 g, 16.2 mmol) was stirred for 64 h at room temperature. The reaction mixture was concentrated in vacuo and the residue was dissolved in ethyl acetate (50 ml). The resulting solution was washed with 5% aqueous citric acid (2 $\times$ 25 ml). The solvent was concentrated removed in vacuo and the residue dissolved in DMF (36 ml). The resulting solution was carefully added to a 10% aqueous solution of citric acid (357 ml) and extracted with ethyl acetate (200 ml) and dried (MgSO<sub>4</sub>). The solvent was concentrated removed in vacuo to give the crude glutamic diester intermediate. To a mixture of the crude diester, N-hydroxysuccinimide (1.85 g, 16.1 mmol) and anhydrous DMF (25 ml) was added a solution of DCC (3.32 g, 16.1 mmol) in anhydrous dichloromethane (15 ml). The resulting mixture was stirred at ambient temperature for 20 h. The reaction mixture was filtered and the solvent was concentrated removed in vacuo. The residue was purified on a silica gel column (40–63  $\mu\text{M}$ ) and eluted with a mixture of dichloromethane and acetonitril (1:1) to give the title compound.

Synthesis of Lys(N<sup>6</sup>-( $\gamma$ -glutamyl(N <sup>$\alpha$</sup> -alkanoyl))) peptide

To a mixture of the peptide (4.2  $\mu\text{mol}$ ), EDPA (15.3 mg, 119  $\mu\text{mol}$ ), NMP (2 ml) and water (1 ml) was added a solution of N <sup>$\alpha$</sup> -alkanoyl-(L)-glutamic acid  $\alpha$ -tert-butyl- $\gamma$ -(2,5-dioxopyrrolidin-1-yl) diester (12.7  $\mu\text{mol}$ ), prepared as described above, in NMP (135 ml). The reaction mixture was gently shaken for 5 min at room temperature and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (7 mg, 93  $\mu\text{mol}$ ) in water (698  $\mu\text{l}$ ). A 0.5% aqueous solution of ammonium acetate (42 ml) was added, and the resulting mixture was eluted onto a Varian 5 g C8 Mega Bond Elut® cartridge, the immobilised compound was washed with 5% aqueous acetonitril (25 ml) and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% for 60 minutes.

Coupling of a desired group A comprising the 5- or 6-membered ring system Y to the N-terminal end of the peptide may be performed using solid phase protein synthesis techniques as explained above.



## Synthesis of N-terminal Modified Peptides

Peptides were synthesised according to the Fmoc strategy on an Applied Biosystems 431A peptide synthesiser in 0.25 mmol scale using the manufacturer supplied FastMoc UV protocols starting with a Fmoc-Gly-Wang resin (NovaBiochem). The protected amino acid derivatives used were commercially obtained Fmoc amino acids, and Adoc-Imidazolylpropionic acid. The derivatives used where side chain protection was needed were: Fmoc-Arg(Pmc), Fmoc-Trp(Boc), Fmoc-Glu(OBu<sup>t</sup>), Fmoc-Lys(Boc), Fmoc-Gln(Trt), Fmoc-Tyr(Bu<sup>t</sup>), Fmoc-Ser(Bu<sup>t</sup>), Fmoc-Thr(Bu<sup>t</sup>), Fmoc-His(Trt) and Fmoc-Asp(OBu<sup>t</sup>), and Adoc-Imidazolylpropionic acid.

The peptides were cleaved from the resin and side chain deprotected in TFA/phenol/thioanisole/water/ethanedithiol (83.25:6.25:4.25:4.25:2.00) for 180 min. The cleavage mixture was filtered and the filtrate was concentrated by a stream of nitrogen. The crude peptide was precipitated from this oil with diethyl ether and washed twice with diethyl ether. After drying the crude peptide was dissolved in 50% aqueous acetic acid and diluted to 10% acetic acid with water and purified by semipreparative HPLC on a 25 mm×250 mm column packed with 7 μm C-18 silica. The column was eluted with a gradient of acetonitril against 0.05 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 2.5 at 10 ml/min. at 40° C. The peptide-containing fractions were collected, diluted with 3 volumes of water and applied to a Sep-Pak® C18 cartridge (Waters part. 51910) which was equilibrated with 0.1% TFA. The peptide was eluted from the Sep-Pak® cartridge with 70% acetonitrile/0.1% TFA, water and isolated from the eluate by lyophilization after dilution with water. The final product obtained was characterised by amino acid analysis, analytical RP-HPLC and by PDMS. Amino acid analysis and mass spectrometry agreed with the expected structure within the experimental error of the method (mass spectrometry +/-2 amu, amino acid analysis +/- 10%, RP-HPLC showed a peptide purity >95%).

The RP-HPLC analysis was performed using UV detection at 214 nm and a Vydac 218TP54 4.6 mm×250 mm, 5 μm C-18 silica column which was eluted at 1 ml/min. at 42° C. Two different elution conditions were used: a gradient of 5–60% acetonitrile/0.1 M ammonium sulfate, water pH 2.5; and a gradient of 5–60% acetonitrile, 0.1% TFA/0.1% TFA, water.

## Example 89

Synthesis of Cap-Glu(ONSu)-OBu<sup>t</sup>

To a solution of octanoic acid (5 g, 34.7 mmol) and N-hydroxysuccinimide (4 g, 34.7 mmol) in anhydrous acetonitril (10 ml) was added a solution of DCC (7.15 g, 34.7 mmol) in anhydrous dichloromethane (15 ml), and the resulting reaction mixture stirred for 16 h at room temperature. The precipitated solid was filtered off and recrystallised from a mixture of n-heptane (40 ml) and 2-propanol (2 ml). The precipitate was dried in a vacuum drying oven for 16 h to give the intermediate Cap-ONSu. A suspension of the crude ester intermediate (3.9 g, 16.2 mmol), (L)-H-Glu(OH)-OBu<sup>t</sup> (3.28 g, 16.2 mmol), DMF (268 ml) and EDPA (2.1 g, 16.2 mmol) was stirred for 64 h at room temperature. The reaction mixture was concentrated in vacuo and the residue dissolved in ethyl acetate (50 ml). The resulting solution was washed with 5% aqueous citric acid (2×25 ml). The solvent was concentrated in vacuo and the residue

dissolved in DMF (36 ml). The resulting solution was added drop wise to a 10% aqueous solution of citric acid (357 ml) and extracted with ethyl acetate (200 ml), and dried (MgSO<sub>4</sub>). The solvent was concentrated in vacuo to give the crude glutamic acid intermediate. To a mixture of the crude glutamic acid intermediate, N-hydroxysuccinimide (1.85 g, 16.1 mmol) and DMF (25 ml) was added a solution of DCC (3.32 g, 16.1 mmol) in dichloromethane (15 ml). The resulting mixture was stirred at ambient temperature for 20 h. The reaction mixture was filtered and the solvent concentrated in vacuo. The residue was purified on a silica gel column (40–63 μm), eluted with a mixture of dichloromethane and acetonitril (1:1) to give the title compound (0.63 g, 6% over all).

## Example 90

Synthesis of Desamino-His<sup>7</sup>,Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-(γ-glutamyl(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37)

To a mixture of desamino-His<sup>7</sup>,Arg<sup>34</sup>GLP-1(7-37)-OH (20 mg, 5.9 μmol), EDPA (21.5 mg, 166 μmol), NMP (2.8 ml) and water (1.4 ml) was added a solution Pal-Glu(ONSu)-OBu<sup>t</sup> (9.6 mg, 17.8 μmol in NMP (240 μl)). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 75 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (9.8 mg, 130 μmol) in water (979 μl). A 0.5% aqueous solution of ammonium acetate (58 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (9.1 mg, 41%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3739±3. The resulting molecular weight was thus 3738±3 amu (theoretical value 3736 amu).

## Example 91

## Synthesis of Myr-GABA-ONSu

To a solution of Myr-ONSu (4 g, 12.3 mmol) in DMF (350 ml) was added EDPA (1.58 g, 12.3 mmol) and γ-aminobutyric acid (1.26 g, 12.3 mmol). The resulting mixture was stirred at ambient temperature for 18 h. Water (50 ml) was added and the solution stirred for 1 h at room temperature. The solvents were removed in vacuo to give a solid. The solid residue was dissolved in DMF (75 ml) and the solution added drop by drop to a 5% aqueous solution of citric acid (250 ml). The precipitate collected, washed with water (100 ml) and dried in vacuo to give the free acid intermediate (3.65 g, 95%). To a solution of the free acid intermediate (3 g, 9.6 mmol), N-hydroxysuccinimide (1.65 g, 14.4 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (3.67 g, 19.1 mmol) in DMF (330 ml) was stirred for 18 h at room temperature, and the solvent removed in vacuo to give a solid. The solid residue was dissolved in dichloromethane (100 ml) and washed with brine (100 ml). The organic phase was dried (MgSO<sub>4</sub>) and concentrated in vacuo to give a solid. The solid residue was recrystallised from n-heptane (75 ml) to give the title compound (2.8 g, 71%).

## Example 92

Synthesis of Desamino-His<sup>7</sup>,Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-(γ-aminobutyroyl(N<sup>γ</sup>-hexadecanoyl))) GLP-1(7-37)

To a mixture of desamino-His<sup>7</sup>,Arg<sup>34</sup>GLP-1(7-37)-OH (20 mg, 8.9 μmol), EDPA (21.5 mg, 166 μmol), NMP (2.8

## 197

ml) and water (1.4 ml) was added a solution Pal-GABA-ONSu (7.8 mg, 17.8  $\mu$ mol) in NMP (181  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (9.3 mg, 125  $\mu$ mol) in water (93  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (11.6 mg, 55%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3692 $\pm$ 3. The resulting molecular weight was thus 3691 $\pm$ 3 amu (theoretical value 3693 amu).

## Example 93

Synthesis of Desamino-His<sup>7</sup>,Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\beta$ -alanyl(N<sup>γ</sup>-hexadecanoyl))) GLP-1(7-37)

To a mixture of desamino-His<sup>7</sup>,Arg<sup>34</sup>GLP-1(7-37)-OH (25 mg, 7.4  $\mu$ mol), EDPA (26.8 mg, 208  $\mu$ mol), NMP (3.5 ml) and water (1.75 ml) was added a solution Pal- $\beta$ -Ala-ONSu (9.4 mg, 22.2  $\mu$ mol) in NMP (236  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 130 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.2 mg, 163  $\mu$ mol) in water (122  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (13.4 mg, 49%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3681 $\pm$ 3. The resulting molecular weight was thus 3680 $\pm$ 3 amu (theoretical value 3678 amu).

## Example 94

Synthesis of Ste-GABA-ONSu

To a solution of Ste-ONSu (3 g, 7.9 mmol) in DMF (270 ml) was added EDPA (1 g, 7.9 mmol) and a solution of  $\gamma$ -aminobutyric acid (0.81 g, 7.9 mmol) in water (40 ml). The resulting suspension was stirred at ambient temperature for 18 h, and then concentrated in vacuo to a final volume of 50 ml. The resulting suspension was added to a 5% aqueous solution of citric acid (500 ml) whereby a precipitate is formed. The precipitate was collected and washed with water (50 ml), and dried in vacuo for 4 h to give the free acid intermediate (2.8 g, 97%). To a mixture of the free acid intermediate (2.6 g, 7 mmol), N-hydroxysuccinimide (1.21 g, 10.5 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.69 g, 14 mmol) in NMP (300 ml) was stirred for 70 h, and the solvent removed in vacuo to give a solid. The solid residue was dissolved in dichloromethane (100 ml) and washed with brine (2 $\times$ 100 ml). The organic phase was dried (MgSO<sub>4</sub>) and concentrated in vacuo to give a solid. The solid residue was recrystallised from n-heptane (75 ml) to give the title compound (2.2 g, 67%).

## Example 95

Synthesis of Arg<sup>34</sup>,Ala<sup>8</sup>(N<sup>α</sup>-(imidazol-4-ylprop-2-enoyl),Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -aminobutyroyl(N<sup>γ</sup>-hexadecanoyl))) GLP-1(8-37)

To a mixture of Arg<sup>34</sup>,Ala<sup>8</sup>(N<sup>α</sup>-(imidazol-4-ylprop-2-enoyl) GLP-1(8-37)-OH (5.6 mg, 1.7  $\mu$ mol), EDPA (6 mg,

## 198

46.2  $\mu$ mol), NMP (0.78 ml) and water (0.39 ml) was added a solution Pal-GABA-ONSu (2.2 mg, 5  $\mu$ mol) in NMP (54  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 80 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.7 mg, 36  $\mu$ mol) in water (27  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (1.9 mg, 31%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3690 $\pm$ 3. The resulting molecular weight was thus 3689 $\pm$ 3 amu (theoretical value 3690 amu).

## Example 96

Synthesis of Arg<sup>34</sup>,Ala<sup>8</sup>(N<sup>α</sup>-(imidazol-4-ylacetyl),Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -aminobutyroyl(N<sup>γ</sup>-hexadecanoyl))) GLP-1(8-37)

To a mixture of Arg<sup>34</sup>,Ala<sup>8</sup>(N<sup>α</sup>-(imidazol-4-ylacetyl) GLP-1(8-37)-OH (5.3 mg, 1.6  $\mu$ mol), EDPA (5.7 mg, 43.9  $\mu$ mol), NMP (0.74 ml) and water (0.37 ml) was added a solution Pal-GABA-ONSu (2 mg, 4.7  $\mu$ mol) in NMP (52  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 80 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.6 mg, 34  $\mu$ mol) in water (26  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (2.2 mg, 38%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3676 $\pm$ 3. The resulting molecular weight was thus 3675 $\pm$ 3 amu (theoretical value 3678 amu).

## Example 97

Synthesis of Desamino-His<sup>7</sup>,Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -aminobutyroyl(N<sup>γ</sup>-tetradecanoyl))) GLP-1(7-37)

To a mixture of desamino-His<sup>7</sup>,Arg<sup>34</sup>GLP-1(7-37)-OH (25 mg, 7.4  $\mu$ mol), EDPA (26.9 mg, 208  $\mu$ mol), NMP (3.5 ml) and water (1.75 ml) was added a solution Myr-GABA-ONSu (9.1 mg, 22.2  $\mu$ mol), prepared as described in example 91, in NMP (228  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.2 mg, 163  $\mu$ mol) in water (122  $\mu$ l). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (10.5 mg, 39%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3667 $\pm$ 3. The resulting molecular weight was thus 3664 $\pm$ 3 amu (theoretical value 3662 amu).

## Example 98

Synthesis of Desamino-His<sup>7</sup>,Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -aminobutyroyl(N<sup>γ</sup>-octadecanoyl))) GLP-1(7-37)

To a mixture of desamino-His<sup>7</sup>,Arg<sup>34</sup>GLP-1(7-37)-OH (25 mg, 7.4  $\mu$ mol), EDPA (26.8 mg, 207  $\mu$ mol), NMP (3.5

199

ml) and water (1.75 ml) was added a solution Ste-GABA-ONSu (10.4 mg, 22.2  $\mu$ mol), prepared as described in example 94, in NMP (259  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 170 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.2 mg, 163  $\mu$ mol) in water (122  $\mu$ l) and the reaction mixture purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (7 mg, 25%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3719 $\pm$ 3. The resulting molecular weight was thus 3718 $\pm$ 3 amu (theoretical value 3720 amu).

## Example 99

## General Method A

## Synthesis of Alkanoic Acid 2,5-dioxopyrrolidin-1-yl Ester

To a solution of the alkanolic acid (34.7 mmol) and N-hydroxysuccinimide (4 g, 34.7 mmol) in anhydrous acetonitril (10 ml) was added a solution of DCC (7.15 g, 34.7 mmol) in anhydrous dichloromethane (15 ml), and the resulting reaction mixture was stirred for 16 h at room temperature. The precipitated solid was filtered off and recrystallised from a mixture of n-heptane and 2-propanol. The precipitate was dried in vacuo for 16 h to give the title compound.

Synthesis of Lys(N<sup>ε</sup>-alkanoyl)-peptide

To a mixture of the desired parent peptide (5.9  $\mu$ mol), EDPA (21 mg, 164  $\mu$ mol), NMP (5.8 ml) and water (2.9 ml) was added a solution of the alkanolic acid 2,5-dioxopyrrolidin-1-yl ester (37  $\mu$ mol), prepared as described above, in NMP (0.5 ml). The reaction mixture was gently shaken for 5 min at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (9.7 mg, 129  $\mu$ mol) in water (97  $\mu$ l). The solvent was removed in vacuo, and the residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient is 0–100% for 60 minutes.

## General Method B

Synthesis of N<sup>α</sup>-alkanoyl-(L)-glutamic Acid  $\alpha$ -tert-butyl- $\gamma$ -(2,5-dioxopyrrolidin-1-yl) Diester

A suspension of the alkanolic acid 2,5-dioxopyrrolidin-1-yl ester (16.2 mmol), prepared as described under General method A, (L)-glutamic acid  $\alpha$ -tert-butyl ester (3.28 g, 16.2 mmol), DMF (268 ml) and EDPA (2.1 g, 16.2 mmol) was stirred for 64 h at room temperature. The reaction mixture was concentrated in vacuo and the residue was dissolved in ethyl acetate (50 ml). The resulting solution was washed with 5% aqueous citric acid (2 $\times$ 25 ml). The solvent was removed in vacuo and the residue dissolved in DMF (36 ml). The resulting solution was carefully added to a 10% aqueous solution of citric acid (357 ml) and extracted with ethyl acetate (200 ml) and dried (MgSO<sub>4</sub>). The solvent was removed in vacuo to give the crude glutamic diester intermediate. To a mixture of the crude diester,

200

N-hydroxysuccinimide (1.85 g, 16.1 mmol) and anhydrous DMF (25 ml) was added a solution of DCC (3.32 g, 16.1 mmol) in anhydrous dichloromethane (15 ml). The resulting mixture was stirred at ambient temperature for 20 h. The reaction mixture was filtered and the solvent was removed in vacuo. The residue was purified on a silica gel column (40–63  $\mu$ m) and eluted with a mixture of dichloromethane and acetonitril (1:1) to give the title compound.

Synthesis of Lys(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-alkanoyl))) peptide

To a mixture of the desired parent peptide (4.2  $\mu$ mol), EDPA (15.3 mg, 119  $\mu$ mol), NMP (2 ml) and water (1 ml) was added a solution of N<sup>α</sup>-alkanoyl-(L)-glutamic acid  $\alpha$ -tert-butyl- $\gamma$ -(2,5-dioxopyrrolidin-1-yl) diester (12.7  $\mu$ mol), prepared as described above, in NMP (135 ml). The reaction mixture was gently shaken for 5 min at room temperature and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (7 mg, 93  $\mu$ mol) in water (698  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (42 ml) was added, and the resulting mixture was eluted onto a Varian 5 g C8 Mega Bond Elut® cartridge, the immobilised compound was washed with 5% aqueous acetonitril (25 ml) and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient is 0–100% for 60 minutes.

## Example 100

Synthesis of Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -glutamyl(N<sup>α</sup>-tetradecanoyl))) GLP-1(9-37)

To a mixture of Arg<sup>34</sup>GLP-1(9-37)-OH (22.4 mg, 7.1  $\mu$ mol), EDPA (25.5 mg, 197  $\mu$ mol), NMP (3.14 ml) and water (1.57 ml) was added a solution of Myr-Glu(ONSu)-OBu<sup>t</sup> (10.8 mg, 21.2  $\mu$ mol) in NMP (270  $\mu$ l). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (11.6 mg, 155  $\mu$ mol) in water (116  $\mu$ l). A 0.5% aqueous solution of ammonium acetate (67 ml) was added, and the resulting mixture eluted onto a Varian 5 g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated in vacuo, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65° C. and the acetonitril gradient was 0–100% in 60 minutes. The title compound (2.3 mg, 9.2%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3516.0 $\pm$ 3. The resulting molecular weight was thus 3515.0 $\pm$ 3 amu (theoretical value 3515 amu).

## Example 101

In this example and examples 102 and 103, the phrase “8 mM phosphate buffer of pH 7.4” means 4 mM NaH<sub>2</sub>PO<sub>4</sub>, 2H<sub>2</sub>O and 4 mM Na<sub>2</sub>HPO<sub>4</sub>, 2H<sub>2</sub>O pH adjusted to 7.4 (using Sodium Hydroxide and/or Hydrochloric acid).

the term “Compound 1” means Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-( $\gamma$ -Glu(N<sup>α</sup>-tetradecanoyl))) GLP-1(7-37).

201

the term "Compound 2" means Arg<sup>34</sup>,Lys<sup>26</sup>(N<sup>ε</sup>-(γ-Glu(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37).  
 the term "Compound 3" means Arg<sup>26,34</sup>,Lys<sup>36</sup>(N<sup>ε</sup>-(γ-Glu(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-36).  
 the term "Compound 4" means Arg<sup>26</sup>,Lys<sup>34</sup>(N<sup>ε</sup>-(γ-Glu(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-37).  
 the term "Compound 5" means Gly<sup>8</sup>,Glu<sup>37</sup>,Arg<sup>26,34</sup>,Lys<sup>38</sup>(N<sup>ε</sup>-(γ-Glu(N<sup>α</sup>-hexadecanoyl))) GLP-1(7-38).

General Example 101	
Compound	2-7.5 mg/ml
Mannitol	34-50 mg/ml
Phenol	5-7.5 mg/ml
8 mM phosphate buffer of pH 7.4	

Mannitol and phenol were dissolved in the phosphate buffer preadjusted to pH 7.4. The compound was then dissolved under slow stirring. The pH was adjusted to 7.4 using sodium hydroxide and/or hydrochloric acid. Finally, the formulation was sterilised by filtration through an appropriate filter.

The following specific formulations were produced using this procedure:

<u>Example 101-A</u>		
Compound 1	2.0 mg/ml	
Mannitol	38 mg/ml	
Phenol	5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-B</u>		
Compound 1	5 mg/ml	
Mannitol	36.9 mg/ml	
Phenol	5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-C</u>		
Compound 1	7.5 mg/ml	
Mannitol	34 mg/ml	
Phenol	7.5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-D</u>		
Compound 2	2.0 mg/ml	
Mannitol	38 mg/ml	
Phenol	5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-E</u>		
Compound 2	5 mg/ml	
Mannitol	36.9 mg/ml	
Phenol	5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-F</u>		
Compound 2	7.5 mg/ml	
Mannitol	34 mg/ml	
Phenol	7.5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-G</u>		
Compound 3	2.0 mg/ml	
Mannitol	38 mg/ml	
Phenol	5 mg/ml	

202

-continued

8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-H</u>		
Compound 3	5 mg/ml	
Mannitol	36.9 mg/ml	
Phenol	5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-I</u>		
Compound 3	7.5 mg/ml	
Mannitol	34 mg/ml	
Phenol	7.5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-J</u>		
Compound 4	2.0 mg/ml	
Mannitol	38 mg/ml	
Phenol	5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-K</u>		
Compound 4	5 mg/ml	
Mannitol	36.9 mg/ml	
Phenol	5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 101-L</u>		
Compound 4	7.5 mg/ml	
Mannitol	34 mg/ml	
Phenol	7.5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 102</u>		
Compound	2-7.5 mg/ml	
Mannitol	19-25 mg/ml	
Benzyl Alcohol	14-18 mg/ml	
8 mM phosphate buffer of pH 7.4		
Mannitol and benzyl alcohol were dissolved in the phosphate buffer preadjusted to pH 7.4. The compound was then dissolved under slow stirring. The pH was adjusted to 7.4 using sodium hydroxide and/or hydrochloric acid. Finally, the formulation was sterilised by filtration through an appropriate filter.		
The following specific formulations were produced using this procedure:		
<u>Example 102-A</u>		
Compound 1	2.0 mg/ml	
Mannitol	25 mg/ml	
Benzyl alcohol	14 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 102-B</u>		
Compound 1	7.5 mg/ml	
Mannitol	19 mg/ml	
Benzyl alcohol	18 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 102-C</u>		
Compound 2	2.0 mg/ml	
Mannitol	25 mg/ml	

-continued

-continued

Benzyl alcohol	14 mg/ml	5
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 102-D</u>		
Compound 2	7.5 mg/ml	10
Mannitol	19 mg/ml	
Benzyl alcohol	18 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 102-E</u>		
Compound 3	2.0 mg/ml	15
Mannitol	25 mg/ml	
Benzyl alcohol	14 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 102-F</u>		
Compound 3	7.5 mg/ml	20
Mannitol	19 mg/ml	
Benzyl alcohol	18 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 102-G</u>		
Compound 5	2.0 mg/ml	25
Mannitol	25 mg/ml	
Benzyl alcohol	14 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 102-H</u>		
Compound 5	7.5 mg/ml	30
Mannitol	19 mg/ml	
Benzyl alcohol	18 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 103</u>		
Compound	2-7.5 mg/ml	35
Mannitol	42-44 mg/ml	
Metacresol	2.5-4.0 mg/ml	
8 mM phosphate buffer of pH 7.4		

8 mM phosphate buffer of pH 7.4	ad 100 ml	5
<u>Example 103-D</u>		
Compound 2	7.5 mg/ml	10
Mannitol	42 mg/ml	
Metacresol	4 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 103-E</u>		
Compound 3	2.0 mg/ml	15
Mannitol	44 mg/ml	
Metacresol	2.5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 103-F</u>		
Compound 3	7.5 mg/ml	20
Mannitol	42 mg/ml	
Metacresol	4 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 103-G</u>		
Compound 5	2.0 mg/ml	25
Mannitol	44 mg/ml	
Metacresol	2.5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 103-H</u>		
Compound 5	7.5 mg/ml	30
Mannitol	42 mg/ml	
Metacresol	4 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	

Mannitol and metacresol were dissolved in the phosphate buffer preadjusted to pH 7.4. The compound was then dissolved under slow stirring. The pH was adjusted to 7.4 using sodium hydroxide and/or hydrochloric acid. Finally, the formulation was sterilised by filtration through an appropriate filter.

The following specific formulations were produced using this:

<u>Example 103-A</u>		
Compound 1	2.0 mg/ml	55
Mannitol	44 mg/ml	
Metacresol	2.5 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 103-B</u>		
Compound 1	7.5 mg/ml	60
Mannitol	42 mg/ml	
Metacresol	4 mg/ml	
8 mM phosphate buffer of pH 7.4	ad 100 ml	
<u>Example 103-C</u>		
Compound 2	2.0 mg/ml	65
Mannitol	44 mg/ml	
Metacresol	2.5 mg/ml	

**Example 104**

Circular Dichroism (CD) at 222 nm as a function of peptide concentration for peptides dissolved in 10 mM tris buffer, pH 8, and 23° C. was measured for native GLP-1(7-37) and the following eight GLP-1 derivatives of the present invention:

- (a) Example 37
- (b) Example 50
- (c) Example 63
- (d) Example 51
- (e) Example 55
- (f) Example 61
- (g) Example 68
- (h) Example 64

The results are shown in FIG. 1. Note that the CD signal is proportional to the average content of  $\alpha$ -helix in the peptides, i.e., a CD value of -1 corresponds to 10%  $\alpha$ -helix content under these conditions. The figure shows that, as the concentration of native GLP-1(7-37) is raised between 25 and 1000  $\mu$ M, the content of  $\alpha$ -helix increases from about 15% to about 30-35% in parallel with the formation of higher oligomers. In contrast with this concentration dependent behaviour, the figure shows that the helix content remains high and essentially independent of the concentration in the 1-200  $\mu$ M range for the GLP-1 derivatives of the present invention forming partially structured micelle-like aggregates under the same conditions.

**Example 105**

**Equilibrium Solubility**

For pH-solubility profiles, solutions containing the peptide and additives (surfactant and, where indicated, other

## 205

excipients) at the appropriate concentrations were prepared at pH 9–10. The solutions were filtered, samples were withdrawn, and the pH was adjusted to the desired value in the range of 3–8. The samples were left for 24 hours at 23° C. to attain solubility equilibrium. After centrifugation (20, 000 g for 20 minutes, 23° C.) of each sample, the pH was measured, and the solubility was determined from measurement of the absorbance at 276 nm of the supernatant.

## Long Term Physical Stability

acylated GLP-1(7-37) analogues were dissolved at twice the desired final concentration and incubated briefly (<10 minutes) at pH 11.5, 23° C. before filtration and mixing with an equal volume of a filtered solution containing all the excipients in twice the desired final concentration. The pH was then measured and adjusted as needed. The solution was transferred to pen-fill cartridges containing a small glass ball (to allow visual determination of changes in solution

## 206

viscosity). The containers were sealed and incubated at the desired temperature between 4° C. and 37° C. At appropriate time intervals, the samples were gently turned and visually examined using a light box. When physical changes were apparent (precipitation, crystallization or gelation), the sample was centrifuged and the absorbance was measured in the supernatant to determine whether the component coming out of solution was the peptide or not.

Equilibrium solubility was determined as a function of pH for 1 mg/ml solutions of the acylated GLP-1(7-37) analogue N<sup>6</sup>-hexadecanoyl- $\gamma$ -glutamyl-Lys26,Art34GLP-1(7-37) in the absence of additives and in the presence of increasing amounts of the surfactant LPCL (lauroyl lysophosphatidyl choline). The results show that 1 mM and 2 mM LPCL enhance solubility relative to the reference composition, while full solubility is obtained in the presence of 5 mM LPCL.

## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 96

<210> SEQ ID NO 1

<211> LENGTH: 31

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: derivatives of human GLP-1

<400> SEQUENCE: 1

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly  
20 25 30

<210> SEQ ID NO 2

<211> LENGTH: 31

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 2

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
20 25 30

<210> SEQ ID NO 3

<211> LENGTH: 31

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 3

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Lys Gly  
20 25 30

<210> SEQ ID NO 4

<211> LENGTH: 31

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

-continued

---

```

<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 4

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

```

```

<210> SEQ ID NO 5
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 5

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

```

```

<210> SEQ ID NO 6
<211> LENGTH: 33
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 6

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

```

Lys

```

<210> SEQ ID NO 7
<211> LENGTH: 34
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 7

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

```

Arg Lys

```

<210> SEQ ID NO 8
<211> LENGTH: 31
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 8

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

```

-continued

<210> SEQ ID NO 9  
 <211> LENGTH: 31  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 9

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

<210> SEQ ID NO 10  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 10

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

Lys

<210> SEQ ID NO 11  
 <211> LENGTH: 34  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 11

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

Arg Lys

<210> SEQ ID NO 12  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 12

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

Lys

<210> SEQ ID NO 13  
 <211> LENGTH: 34  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 13



-continued

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Lys Gly Arg  
 20 25 30

Arg Lys

<210> SEQ ID NO 14  
 <211> LENGTH: 31  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

&lt;400&gt; SEQUENCE: 14

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly  
 20 25 30

<210> SEQ ID NO 15  
 <211> LENGTH: 31  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

&lt;400&gt; SEQUENCE: 15

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
 20 25 30

<210> SEQ ID NO 16  
 <211> LENGTH: 31  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

&lt;400&gt; SEQUENCE: 16

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Lys Gly  
 20 25 30

<210> SEQ ID NO 17  
 <211> LENGTH: 31  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

&lt;400&gt; SEQUENCE: 17

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Lys Gly  
 20 25 30

<210> SEQ ID NO 18  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:

-continued

---

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 18

His	Gly	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly
1				5					10					15	

Gln	Ala	Ala	Arg	Glu	Phe	Ile	Ala	Trp	Leu	Val	Arg	Gly	Arg	Gly	Arg
			20					25					30		

Lys

<210> SEQ ID NO 19

<211> LENGTH: 34

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 19

His	Gly	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly
1				5					10					15	

Gln	Ala	Ala	Arg	Glu	Phe	Ile	Ala	Trp	Leu	Val	Arg	Gly	Arg	Gly	Arg
			20					25					30		

Arg Lys

<210> SEQ ID NO 20

<211> LENGTH: 31

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 20

His	Gly	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly
1				5					10					15	

Gln	Ala	Ala	Arg	Glu	Phe	Ile	Ala	Trp	Leu	Val	Lys	Gly	Lys	Gly
			20					25					30	

<210> SEQ ID NO 21

<211> LENGTH: 31

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 21

His	Gly	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly
1				5					10					15	

Gln	Ala	Ala	Lys	Glu	Phe	Ile	Ala	Trp	Leu	Val	Arg	Gly	Lys	Gly
			20					25					30	

<210> SEQ ID NO 22

<211> LENGTH: 33

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 22

His	Gly	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly
1				5					10					15	

Gln	Ala	Ala	Arg	Glu	Phe	Ile	Ala	Trp	Leu	Val	Lys	Gly	Arg	Gly	Arg
			20					25					30		

Lys

-continued

<210> SEQ ID NO 23  
 <211> LENGTH: 34  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen  
  
 <400> SEQUENCE: 23

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
 20 25 30

Arg Lys

<210> SEQ ID NO 24  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 24

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Lys Gly Arg  
 20 25 30

Lys

<210> SEQ ID NO 25  
 <211> LENGTH: 34  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 25

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Lys Gly Arg  
 20 25 30

Arg Lys

<210> SEQ ID NO 26  
 <211> LENGTH: 32  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 26

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Lys  
 20 25 30

<210> SEQ ID NO 27  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

-continued

&lt;400&gt; SEQUENCE: 27

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
 20 25 30

Lys

&lt;210&gt; SEQ ID NO 28

&lt;211&gt; LENGTH: 34

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: mutagen

&lt;400&gt; SEQUENCE: 28

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
 20 25 30

Arg Lys

&lt;210&gt; SEQ ID NO 29

&lt;211&gt; LENGTH: 35

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: mutagen

&lt;400&gt; SEQUENCE: 29

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
 20 25 30

Arg Glu Lys  
35

&lt;210&gt; SEQ ID NO 30

&lt;211&gt; LENGTH: 36

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: mutagen

&lt;400&gt; SEQUENCE: 30

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
 20 25 30

Arg Glu Phe Lys  
35

&lt;210&gt; SEQ ID NO 31

&lt;211&gt; LENGTH: 37

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: mutagen

&lt;400&gt; SEQUENCE: 31

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

-continued

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
 20 25 30

Arg Glu Phe Pro Lys  
 35

<210> SEQ ID NO 32  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 32

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
 20 25 30

Arg Glu Phe Pro Glu Lys  
 35

<210> SEQ ID NO 33  
 <211> LENGTH: 39  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 33

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
 1 5 10 15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
 20 25 30

Arg Glu Phe Pro Glu Lys  
 35

<210> SEQ ID NO 34  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 34

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
 1 5 10 15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
 20 25 30

Val Arg Gly Arg Gly Lys  
 35

<210> SEQ ID NO 35  
 <211> LENGTH: 39  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 35

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
 1 5 10 15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
 20 25 30

-continued

---

Val Arg Gly Arg Gly Arg Lys  
35

<210> SEQ ID NO 36  
<211> LENGTH: 40  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 36

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
1 5 10 15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
20 25 30

Val Arg Gly Arg Gly Arg Arg Lys  
35 40

<210> SEQ ID NO 37  
<211> LENGTH: 41  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 37

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
1 5 10 15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
20 25 30

Val Arg Gly Arg Gly Arg Arg Glu Lys  
35 40

<210> SEQ ID NO 38  
<211> LENGTH: 42  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 38

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
1 5 10 15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
20 25 30

Val Arg Gly Arg Gly Arg Arg Glu Phe Lys  
35 40

<210> SEQ ID NO 39  
<211> LENGTH: 43  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 39

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
1 5 10 15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
20 25 30

Val Arg Gly Arg Gly Arg Arg Glu Phe Pro Lys  
35 40

-continued

<210> SEQ ID NO 40  
 <211> LENGTH: 44  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 40

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
 1 5 10 15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
 20 25 30

Val Arg Gly Arg Gly Arg Arg Glu Phe Pro Glu Lys  
 35 40

<210> SEQ ID NO 41  
 <211> LENGTH: 45  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 41

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
 1 5 10 15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
 20 25 30

Val Arg Gly Arg Gly Arg Arg Glu Phe Pro Glu Glu Lys  
 35 40 45

<210> SEQ ID NO 42  
 <211> LENGTH: 37  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 42

Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser  
 1 5 10 15

Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val  
 20 25 30

Arg Gly Arg Gly Lys  
 35

<210> SEQ ID NO 43  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 43

Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser  
 1 5 10 15

Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val  
 20 25 30

Arg Gly Arg Gly Arg Lys  
 35

<210> SEQ ID NO 44  
 <211> LENGTH: 39

-continued

---

```

<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 44

Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser
 1             5             10
Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val
                20             25             30
Arg Gly Arg Gly Arg Arg Lys
          35

```

```

<210> SEQ ID NO 45
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 45

Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser
 1             5             10
Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val
                20             25             30
Arg Gly Arg Gly Arg Arg Glu Lys
          35             40

```

```

<210> SEQ ID NO 46
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 46

Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser
 1             5             10
Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val
                20             25             30
Arg Gly Arg Gly Arg Arg Glu Phe Lys
          35             40

```

```

<210> SEQ ID NO 47
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 47

Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser
 1             5             10
Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val
                20             25             30
Arg Gly Arg Gly Arg Arg Glu Phe Pro Lys
          35             40

```

```

<210> SEQ ID NO 48
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

```



-continued

---

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 48

Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser  
1 5 10 15

Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val  
20 25 30

Arg Gly Arg Gly Arg Arg Glu Phe Pro Glu Lys  
35 40

<210> SEQ ID NO 49

<211> LENGTH: 44

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 49

Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser  
1 5 10 15

Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val  
20 25 30

Arg Gly Arg Gly Arg Arg Glu Phe Pro Glu Glu Lys  
35 40

<210> SEQ ID NO 50

<211> LENGTH: 36

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 50

Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser  
1 5 10 15

Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg  
20 25 30

Gly Arg Gly Lys  
35

<210> SEQ ID NO 51

<211> LENGTH: 37

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 51

Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser  
1 5 10 15

Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg  
20 25 30

Gly Arg Gly Arg Lys  
35

<210> SEQ ID NO 52

<211> LENGTH: 38

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 52

-continued

---

Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser  
 1 5 10 15  
 Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg  
 20 25 30  
 Gly Arg Gly Arg Arg Lys  
 35

<210> SEQ ID NO 53  
 <211> LENGTH: 39  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 53

Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser  
 1 5 10 15  
 Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg  
 20 25 30  
 Gly Arg Gly Arg Arg Glu Lys  
 35

<210> SEQ ID NO 54  
 <211> LENGTH: 40  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 54

Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser  
 1 5 10 15  
 Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg  
 20 25 30  
 Gly Arg Gly Arg Arg Glu Phe Lys  
 35 40

<210> SEQ ID NO 55  
 <211> LENGTH: 41  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 55

Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser  
 1 5 10 15  
 Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg  
 20 25 30  
 Gly Arg Gly Arg Arg Glu Phe Pro Lys  
 35 40

<210> SEQ ID NO 56  
 <211> LENGTH: 42  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 56

Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser  
 1 5 10 15

-continued

Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg  
 20 25 30

Gly Arg Gly Arg Arg Glu Phe Pro Glu Lys  
 35 40

<210> SEQ ID NO 57  
 <211> LENGTH: 43  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 57

Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser  
 1 5 10 15

Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg  
 20 25 30

Gly Arg Gly Arg Arg Glu Phe Pro Glu Glu Lys  
 35 40

<210> SEQ ID NO 58  
 <211> LENGTH: 35  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 58

Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr  
 1 5 10 15

Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly  
 20 25 30

Arg Gly Lys  
 35

<210> SEQ ID NO 59  
 <211> LENGTH: 36  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 59

Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr  
 1 5 10 15

Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly  
 20 25 30

Arg Gly Arg Lys  
 35

<210> SEQ ID NO 60  
 <211> LENGTH: 37  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 60

Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr  
 1 5 10 15

Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly  
 20 25 30

-continued

Arg Gly Arg Arg Lys  
35

<210> SEQ ID NO 61  
<211> LENGTH: 38  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 61

Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr  
1 5 10 15

Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly  
20 25 30

Arg Gly Arg Arg Glu Lys  
35

<210> SEQ ID NO 62  
<211> LENGTH: 39  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 62

Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr  
1 5 10 15

Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly  
20 25 30

Arg Gly Arg Arg Glu Phe Lys  
35

<210> SEQ ID NO 63  
<211> LENGTH: 40  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 63

Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr  
1 5 10 15

Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly  
20 25 30

Arg Gly Arg Arg Glu Phe Pro Lys  
35 40

<210> SEQ ID NO 64  
<211> LENGTH: 41  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 64

Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr  
1 5 10 15

Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly  
20 25 30

Arg Gly Arg Arg Glu Phe Pro Glu Lys  
35 40

-continued

<210> SEQ ID NO 65  
 <211> LENGTH: 42  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 65

Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr  
 1 5 10 15  
 Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly  
 20 25 30  
 Arg Gly Arg Arg Glu Phe Pro Glu Glu Lys  
 35 40

<210> SEQ ID NO 66  
 <211> LENGTH: 34  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 66

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

<210> SEQ ID NO 67  
 <211> LENGTH: 35  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 67

Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu  
 1 5 10 15  
 Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg  
 20 25 30  
 Gly Arg Lys  
 35

<210> SEQ ID NO 68  
 <211> LENGTH: 36  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 68

Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu  
 1 5 10 15  
 Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg  
 20 25 30  
 Gly Arg Arg Lys  
 35

<210> SEQ ID NO 69  
 <211> LENGTH: 37

-continued

---

```

<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 69

Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu
 1           5           10           15
Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg
          20           25           30
Gly Arg Arg Glu Lys
          35

```

```

<210> SEQ ID NO 70
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 70

Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu
 1           5           10           15
Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg
          20           25           30
Gly Arg Arg Glu Phe Lys
          35

```

```

<210> SEQ ID NO 71
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 71

Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu
 1           5           10           15
Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg
          20           25           30
Gly Arg Arg Glu Phe Pro Lys
          35

```

```

<210> SEQ ID NO 72
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 72

Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu
 1           5           10           15
Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg
          20           25           30
Gly Arg Arg Glu Phe Pro Glu Lys
          35           40

```

```

<210> SEQ ID NO 73
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

```

-continued

---

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 73

Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu  
 1 5 10 15

Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg  
 20 25 30

Gly Arg Arg Glu Phe Pro Glu Glu Lys  
 35 40

<210> SEQ ID NO 74

<211> LENGTH: 33

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 74

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu  
 1 5 10 15

Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
 20 25 30

Lys

<210> SEQ ID NO 75

<211> LENGTH: 34

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 75

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu  
 1 5 10 15

Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
 20 25 30

Arg Lys

<210> SEQ ID NO 76

<211> LENGTH: 35

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 76

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu  
 1 5 10 15

Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
 20 25 30

Arg Arg Lys  
35

<210> SEQ ID NO 77

<211> LENGTH: 36

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 77

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu

-continued

---

1                    5                    10                    15  
 Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
                   20                    25                    30

Arg Arg Glu Lys  
           35

<210> SEQ ID NO 78  
 <211> LENGTH: 37  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 78

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu  
 1                    5                    10                    15

Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
                   20                    25                    30

Arg Arg Glu Phe Lys  
           35

<210> SEQ ID NO 79  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 79

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu  
 1                    5                    10                    15

Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
                   20                    25                    30

Arg Arg Glu Phe Pro Lys  
           35

<210> SEQ ID NO 80  
 <211> LENGTH: 39  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 80

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu  
 1                    5                    10                    15

Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly  
                   20                    25                    30

Arg Arg Glu Phe Pro Glu Lys  
           35

<210> SEQ ID NO 81  
 <211> LENGTH: 40  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 81

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu  
 1                    5                    10                    15

Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly



-continued

---

20	25	30
Arg Arg Glu Phe Pro Glu Glu Lys		
35	40	

<210> SEQ ID NO 82  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 82

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

Val Lys Gly Arg Gly Lys
35

<210> SEQ ID NO 83  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 83

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

Val Arg Gly Arg Gly Lys
35

<210> SEQ ID NO 84  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 84

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

Val Arg Gly Lys Gly Lys
35

<210> SEQ ID NO 85  
 <211> LENGTH: 32  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 85

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

-continued

---

```

<210> SEQ ID NO 86
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 86

```

```

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

```

```

<210> SEQ ID NO 87
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 87

```

```

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

```

```

<210> SEQ ID NO 88
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 88

```

```

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

```

```

<210> SEQ ID NO 89
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 89

```

```

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val
 1             5             10             15
Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu
      20             25             30
Val Lys Gly Arg Gly Arg Lys
      35

```

```

<210> SEQ ID NO 90
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

```

```

<400> SEQUENCE: 90

```

```

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val
 1             5             10             15

```

-continued

---

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu  
                   20                                  25                                  30

Val Arg Gly Arg Gly Arg Lys  
           35

<210> SEQ ID NO 91  
 <211> LENGTH: 39  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 91

His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val  
   1                  5                                  10                                  15

Ser Ser Tyr Leu Glu Gly Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu  
                   20                                  25                                  30

Val Arg Gly Lys Gly Arg Lys  
           35

<210> SEQ ID NO 92  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 92

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
   1                  5                                  10                                  15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly Arg  
                   20                                  25                                  30

Lys

<210> SEQ ID NO 93  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 93

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
   1                  5                                  10                                  15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly Arg  
                   20                                  25                                  30

Lys

<210> SEQ ID NO 94  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 94

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly  
   1                  5                                  10                                  15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Lys Gly Arg  
                   20                                  25                                  30

Lys

-continued

```

<210> SEQ ID NO 95
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 95

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
 1             5             10             15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly Lys
      20             25             30
    
```

```

<210> SEQ ID NO 96
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutagen

<400> SEQUENCE: 96

His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
 1             5             10             15

Gln Ala Ala Arg Glu Phe Ile Ala Trp Leu Val Arg Gly Lys Gly Lys
      20             25             30
    
```

What is claimed is:

1. A glucagon-like peptide-1 (GLP-1) derivative of formula I (SEQ ID NO:2):

```

 7  8  9 10 11 12 13 14 15 16 17
His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-
18 19 20 21 22 23 24 25 26 27 28
Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-
29 30 31 32 33 34 35 36 37
Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Glu
    
```

wherein

- (a) the  $\epsilon$ -amino group of Lys at position 26 is substituted with a lipophilic substituent optionally via a spacer, and
  - (b) the lipophilic substituent is (i)  $\text{CH}_3(\text{CH}_2)_n\text{CO}$ — wherein n is 6, 8, 10, 12, 14, 16, 18, 20 or 22 (ii)  $\text{HOOC}(\text{CH}_2)_m\text{CO}$ — wherein m is 10, 12, 14, 16, 18, 20 or 22, or (iii) lithocholyl, and
  - (c) the spacer is an amino acid residue except Cys, or the spacer is  $\gamma$ -aminobutanoyl.
2. The GLP-1 derivative of claim 1, wherein the lipophilic substituent is linked to the  $\epsilon$ -amino group of Lys via a spacer.
3. The GLP-1 derivative of claim 2, wherein the spacer is  $\gamma$ -glutamyl.
4. The GLP-1 derivative of claim 2, wherein the spacer is  $\beta$ -asparagyl.
5. The GLP-1 derivative of claim 2, wherein the spacer is glycylyl.
6. The GLP-1 derivative of claim 2, wherein the spacer is  $\gamma$ -aminobutanoyl.
7. The GLP-1 derivative of claim 2, wherein the spacer is  $\beta$ -alanyl.

30

8. A pharmaceutical composition comprising a GLP-1 derivative of claim 1 and a pharmaceutically acceptable vehicle or carrier.

9. The pharmaceutical composition of claim 8, further comprising an isotonic agent, a preservative and a buffer.

10. The pharmaceutical composition of claim 9, wherein the isotonic agent is sodium chloride, mannitol and glycerol.

11. The pharmaceutical composition of claim 9, wherein the preservative is phenol, m-cresol, methyl p-hydroxybenzoate or benzyl alcohol.

12. The pharmaceutical composition of claim 9, wherein the buffer is sodium acetate or sodium phosphate.

13. The pharmaceutical composition of claim 8, further comprising a surfactant.

14. The pharmaceutical composition of claim 8, further comprising zinc.

15. The pharmaceutical composition of claim 8, further comprising an antidiabetic agent.

16. The pharmaceutical composition of claim 15, wherein the antidiabetic agent is human insulin.

17. The pharmaceutical composition of claim 15, wherein the antidiabetic agent is a hypoglycemic agent.

18. The pharmaceutical composition of claim 8, further comprising an antiobesity agent.

19. A method of treating diabetes, comprising administering to a patient a therapeutically effective amount of a GLP-1 derivative of claim 1.

20. A method of treating obesity, comprising administering to a patient a therapeutically effective amount of a GLP-1 derivative of claim 1.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 6,458,924 B2  
APPLICATION NO. : 09/398111  
DATED : October 1, 2002  
INVENTOR(S) : Knudsen et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In claim 1, Column 249, Line 41: "Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Glu"  
should read --Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly--.

Signed and Sealed this

First Day of July, 2008



JON W. DUDAS

*Director of the United States Patent and Trademark Office*