Expedited Articles

Potent Derivatives of Glucagon-like Peptide-1 with Pharmacokinetic Properties Suitable for Once Daily Administration

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A series of very potent derivatives of the 30-amino acid peptide hormone glucagon-like peptide-1 (GLP-1) is described. The compounds were all derivatized with fatty acids in order to protract their action by facilitating binding to serum albumin. GLP-1 had a potency (EC_{50}) of 55 pM for the cloned human GLP-1 receptor. Many of the compounds had similar or even higher potencies, despite quite large substituents. All compounds derivatized with fatty acids equal to or longer than 12 carbon atoms were very protracted compared to GLP-1 and thus seem suitable for once daily administration to type 2 diabetic patients. A structure−activity relationship was obtained. GLP-1 could be derivatized with linear fatty acids up to the length of 16 carbon atoms, sometimes longer, almost anywhere in the C-terminal part without considerable loss of potency. Derivatization with two fatty acid substituents led to a considerable loss of potency. A structure−activity relationship on derivatization of specific amino acids generally was obtained. It was found that the longer the fatty acid, the more potency was lost. Simultaneous modification of the N-terminus (in order to obtain better metabolic stability) interfered with fatty acid derivatization and led to loss of potency.

Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin and is produced by the L-cells of the intestine. Since its discovery in 1984, GLP-1 has received much attention as a possibly new treatment for type 2 diabetes.[1−7] GLP-1 stimulates insulin secretion and biosynthesis and inhibits glucagon release, each very favorable effects in the treatment of hyperglycemia. Most importantly, both these effects are glucose-dependent[8,9] and therefore represent a very safe way of lowering increased blood glucose. In addition, GLP-1 inhibits gastric emptying,[10−12] thereby decreasing postprandial glucose excursions. Gastric acid secretion is inhibited, too,[10] and thus, GLP-1 compounds may provide protection against gastric ulcers. Also, GLP-1 has been shown to be a potent appetite suppressant,[13−15] although effects on body weight in humans still need to be shown. An effect on body weight in clinical use would make the GLP-1 receptor an even more attractive target, since the majority of type 2 diabetic patients are obese. Lately, GLP-1 has been shown be able to stimulate growth and proliferation of pancreatic β-cells.[16] Overt type 2 diabetics are, among other things, characterized as having insufficient β-cell capacity. No existing drugs for type 2 diabetes approach this problem. With this long list of beneficial effects, GLP-1 compounds have a large potential as a new class of drugs for the treatment of type 2 diabetes and impaired glucose tolerance (IGT).

GLP-1 is a 30-amino acid peptide hormone. Generally, peptide hormones this size are not orally available and thus need to be administered by injection or through an alternative way feasible for peptides (pulmonal, buccal). Due to its strong tendency to fibrillate, GLP-1, like its close analogue glucagon, is a very difficult molecule to handle in solution. In a recent publication, it was described how problematic it is to formulate a GLP-1 compound and thus how difficult it may become to ever make a drug out of GLP-1.[17] The metabolic and pharmacokinetic properties of GLP-1 add to these problems. Dipeptidyl peptidase IV (DPP-IV) rapidly degrades GLP-1(7−36)amide,[18,19] rendering the rest of the molecule, GLP-1(9−36)amide, inactive. Indeed, GLP-1(9−36)amide may even act as an antagonist.[20] Simultaneously, the kidneys clear GLP-1 quickly. The half-life of GLP-1(7−36)amide in humans has been determined to be 1.5 min after iv administration and 1.5 h after sc administration.[3] N-Terminally modified, DPP-IV-resistant analogues are of course still subject to renal clearance, and as a result of this, they have half-lives of only 4−5 min.[21]

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Table 1. List of All Compounds and Their Potency Measured Using the Cloned Human GLP-1 Receptor Expressed in BHK Cells[a]

compd	parent peptide	acyl site	acyl substituent	potency (EC_{50}, pM)
1	GLP-1(7–37)		none	55 ± 19
2	$K^{8}R^{26,34}$-GLP-1(7–37)	K^{8}	γ-Glu-C16	1260 ± 210
3	$K^{18}R^{26,34}$-GLP-1(7–37)	K^{18}	γ-Glu-C16	35.2 ± 6.2
4	$K^{23}R^{26,34}$-GLP-1(7–37)	K^{23}	γ-Glu-C16	30.1 ± 3.3
5	R^{34}-GLP-1(7–37)	K^{26}	γ-Glu-C16	61.0 ± 7.1
6	$K^{27}R^{26,34}$-GLP-1(7–37)	K^{27}	γ-Glu-C16	36.3 ± 0.3
7	R^{26}-GLP-1(7–37)	K^{34}	γ-Glu-C16	121 ± 26
8	$K^{36}R^{26,34}$-GLP-1(7–36)	K^{36}	γ-Glu-C16	36.4 ± 2.1
9	$R^{26,34}$-GLP-1(7–38)	K^{38}	γ-Glu-C16	53.0 ± 2.8
10	GLP-1(7–37)	$K^{26,34}$	bis-C16-diacid	7000 ± 7
11	GLP-1(7–37)	$K^{26,34}$	bis-γ-Glu-C16	16700 ± 3700
12	GLP-1(7–37)	$K^{26,34}$	bis-γ-Glu-C14	3050 ± 350
13	GLP-1(7–37)	$K^{26,34}$	bis-C12-diacid	177 ± 52
14	R^{34}-GLP-1(7–37)	K^{26}	C16-diacid	154 ± 66
15	R^{34}-GLP-1(7–37)	K^{26}	C14-diacid	72 ± 0.7
16	R^{34}-GLP-1(7–37)	K^{26}	γ-Glu-C18	194 ± 24
17	R^{34}-GLP-1(7–37)	K^{26}	γ-Glu-C14	22.0 ± 7.1
18	R^{34}-GLP-1(7–37)	K^{26}	γ-Glu-C12	27.3 ± 8.4
19	desamino-$H^{7}R^{34}$-GLP-1(7–37)	K^{26}	γ-Glu-C16	687 ± 129
20	R^{34}-GLP-1(7–37)	K^{26}	GABA-C16	84.4 ± 22.1
21	R^{34}-GLP-1(7–37)	K^{26}	β-Ala-C16	113 ± 3
22	R^{34}-GLP-1(7–37)	K^{26}	Iso-Nip-C16	410 ± 120
23	desamino-$H^{7}R^{26}$-GLP-1(7–37)	K^{34}	γ-Glu-C16	2360 ± 370
24	desamino-$H^{7}R^{26}$-GLP-1(7–37)	K^{34}	C8	236 ± 66
25	desamino-$H^{7}R^{26}$-GLP-1(7–37)	K^{34}	γ-Glu-C8	169 ± 1
26	$K^{36}R^{26,34}$-GLP-1(7–36)	K^{36}	C20-diacid	210 ± 14
27	$K^{36}R^{26,34}$-GLP-1(7–36)	K^{36}	C16-diacid	7.89 ± 1.21
28	$K^{36}R^{26,34}$-GLP-1(7–36)	K^{36}	γ-Glu-C18	116 ± 3
29	$R^{26,34}$-GLP-1(7–38)	K^{38}	C16-diacid	5.60 ± 3.5
30	$R^{26,34}$-GLP-1(7–38)	K^{38}	C12-diacid	4.19 ± 0.98
31	$R^{26,34}$-GLP-1(7–38)	K^{38}	γ-Glu-C18	115 ± 21
32	$R^{26,34}$-GLP-1(7–38)	K^{38}	γ-Glu-C14	54 ± 1
33	$G^{8}R^{26,34}$-GLP-1(7–38)	K^{38}	γ-Glu-C16	328 ± 14
34	$E^{37}R^{26,34}$-GLP-1(7–38)	K^{38}	γ-Glu-C16	27.2 ± 0.1
35	$E^{37}G^{8}R^{26,34}$-GLP-1(7–38)	K^{38}	γ-Glu-C16	135 ± 7
36	$E^{37}G^{8}R^{26,34}$-GLP-1(7–38)	K^{38}	γ-Glu-C18	213 ± 30

[a] Abbreviations used for acyl groups in lysine N^{ϵ}-acylated peptides: γ-Glu-C8 = γ-L-glutamoyl(N^{α}-octanoyl); γ-Glu-C14 = γ-L-glutamoyl(N^{α}-tetradecanoyl); γ-Glu-C16 = γ-L-glutamoyl(N^{α}-hexadecanoyl); γ-Glu-C18 = γ-L-glutamoyl(N^{α}-octadecanoyl); C8 = octanoyl; C12-diacid = ω-carboxyundecanoyl; C16-diacid = ω-carboxypentadecanoyl; C20-diacid = ω-carboxynonadecanoyl; GABA-C16 = γ-aminobutyroyl(N^{γ}-hexadecanoyl); Iso-Nip-C16 = 1-(hexadecanoyl)piperidyl-4-carboxy. Data are given as mean ± SD of two individual experiments with triplicate samples.

The principle of fatty acid derivatization has been used to protract the action of insulin by facilitating binding to serum albumin.[22−25] In this article we describe the structure−activity relationship (SAR) of a large number of fatty acid derivatives of GLP-1. We show that these compounds have pharmacokinetic properties in pigs suitable for once daily administration. In the absence of a nonpeptide agonist these compounds may prove to be the best drug candidates for the treatment of type 2 diabetes via the GLP-1 receptor target.

Discussion

The acylated compounds were all synthesized as part of a program aimed at protracting the action of the peptide hormone GLP-1. Fatty acids or fatty diacids, optionally extended with a "spacer" between the ε-amino group of the lysine side chain and the carboxyl group of the fatty acid, were used. Acylation with mono activated esters of symmetrical diacids presented a potential synthesis problem, which was solved by using an excess of diacid versus *N*-hydroxysuccinimide in the activation step. Acylation with simple fatty acids increases the net negative charge of the resulting molecule by 1, whereas peptides acylated with a L-glutamoyl spacer or with diacids provide a net increase of the negative charge by 2. In the two latter cases, an enhanced effect on binding to albumin is predicted.[22,26] This extra negative charge added to the acylated molecule is also expected

His^{7}-Ala-Glu-Gly^{10}-Thr-Phe-Thr-Ser-Asp^{15}-Val-Ser Ser-Tyr-Leu^{20}-Glu-Gly-Gln-Ala-Ala^{25}-Lys-Glu-Phe-Ile-Ala^{30}-Trp-Leu-Val-Lys-Gly-Arg-Gly^{37}

Figure 1. Amino acid sequence of GLP-1.

The amino acid sequence of GLP-1 can be seen in Figure 1. GLP-1(7–37) and close analogues thereof were derivatized on position 8, 18, 23, 26, 27, 34, 36, or 38 with fatty acids and optionally a spacer (Table 1). The SAR of the compounds was investigated using a functional assay employing the cloned human GLP-1 receptor expressed in baby hamster kidney (BHK) cells. Figure 2 shows examples of the dose−response curves for a few selected compounds. All compounds tested were full agonists and were shown to activate the GLP-1 receptor selectively (by using the cloned human glucagon receptor, data not shown). Fatty acids have been shown to interact with cell membranes as well as albumin. However, the selective activation of the GLP-1 receptor evidences that this phenomenon is not important for these compounds.

All compounds acylated with a fatty acid equal to or longer than 12 carbon atoms were considerable protracted compared to GLP-1, which had a half-life after sc administration of only 1.2 h. Table 2 shows plasma half-lives after sc administration to pigs for a selection of very potent compounds (**4**, **5**, **7**, **8**, **18**, **20**, **21**, **27**, **35**). All had half-lives equal to or longer than 9 h. Bioavailability was measured for selected compounds only and

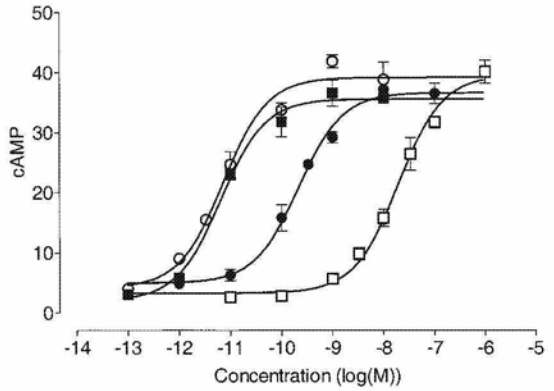

Figure 2. Dose−response curves of GLP-1 and selected compounds using the cloned human GLP-1 receptor expressed in BHK cells: **1** (GLP-1) (■), **11** (□), **13** (●), **27** (○). Data are from one representative experiment with triplicate samples and are shown as mean ± SD.

Table 2. Plasma Half-Lives in Pigs of GLP-1 and Selected Potent Acylated Compounds[a]

compd	plasma $t_{1/2}$ (h)	compd	plasma $t_{1/2}$ (h)
1 (GLP-1)	1.2	**18**	15 ± 3
4	20 ± 2	**20**	31 ± 4
5	14 ± 2	**21**	8.8 ± 1
7	13	**27**	13 ± 4
8	12 ± 1	**35**	11 ± 1

[a] The half-lives were calculated from individual pigs after a single sc injection. Each compound was injected in two pigs. Data are shown as mean ± SD. For **7**, half-life could only be calculated from one pig.

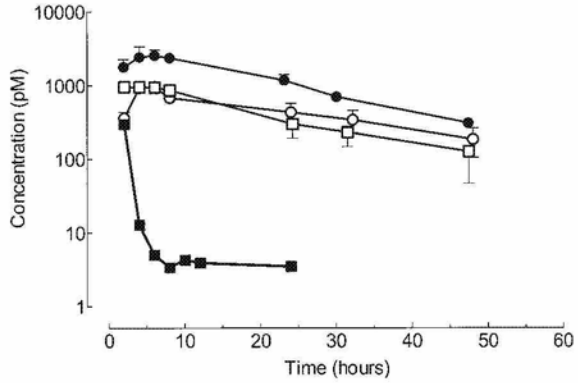

Figure 3. Pharmacokinetic profile of selected compounds after sc administration to pigs: **1** (GLP-1) (■), **5** (○), **7** (●), **8** (□). Two pigs were dosed with compounds, and the following immunoassay was performed in duplicate. Data are expressed as mean ± SD.

not shown). Figure 3 illustrates the dramatic difference in plasma half-lives between GLP-1 and three potent acylated compounds (**5**, **7**, **8**).

Site of Acylation. Many different positions in the C-terminal part of GLP-1 could be derivatized with quite long fatty acids (**3**−**9**, 30−121 pM) without affecting the potency. Binding affinity was not measured for these compounds as they all bind to albumin as part of their mechanism of protraction and it has not been possible to set up a reproducible binding assay without albumin. Derivatizing amino acids in the N-terminal part of the peptide, as exemplified in **2** (1260 pM), led to a substantial loss of potency. Earlier findings from this group showed that positions 7, 10, 12, 13, and 15 in the N-terminal part were important for binding to and 29 were important in the C-terminus.[27] Even though only position 8 in the N-terminus was acylated and this compound found has a poor potency, the present results nicely support those earlier reported from this group.[27]

Number of Fatty Acid Substituents. The natural sequence of GLP-1 has two lysines. When using an analogue of a naturally occurring hormone, there is always a risk of introducing a change in the amino acid sequence, which can lead to an immune response to the analogue. In order not to have to use analogues of GLP-1, derivatization with two fatty acids was examined (**10**−**13**). Only **13** with a C12 fatty acid had an acceptable potency (177 pM). Derivatization with C14 or C16 fatty acids, either as diacids or as monoacids with a γ-Glu spacer, resulted in compounds with a marked loss of activity (**10**−**12**).

Compounds Derivatized on Lysine 26. The potencies of the compounds were comparable when looking at a series of different length diacids (**14**, **15**) or fatty acids with the same spacer (**5**, **16**−**18**). Within the γ-Glu spacer monoacid series (**5**, **16**−**18**), derivatization with a C18 acid (**16**, 194 pM) led to a significant loss of activity compared to C16 (**5**, 61 pM), C14 (**17**, 22 pM), and C12 (**18**, 27 pM). Within the diacid series (**14**, **15**), the diacid could be no longer than a C14 (**15**, 72 pM) before a loss in potency (**14**, 154 pM), compared to the γ-Glu spacer monoacid series (**17**, **18**, 22−27 pM), was seen. Earlier, this group and others have tried to modify the amino terminus of GLP-1 in order to make the molecule more resistant to enzymatic breakdown.[21,28,29] Desaminohistidine[7] represents one of the more potent suggestions to a modification giving metabolic stability.[29] Nevertheless, as seen when comparing **19** (687 pM) to **5** (61 pM), considerably more potent compounds could be obtained by not modifying the N-terminus when a combination with acylation was desired. This could of course be caused both by the position of the fatty acid and because of the modified histidine. However as shown below, further data from the position 34 acylation series showed that acylation with a short chain fatty acid (C8) led to a considerable more potent compound than a corresponding C16 fatty acid.

Compounds Derivatized with Different Spacers. The γ-Glu spacer is optically active. Thus, it presents a greater analytical challenge when upscaling the compounds for good manufacturing production guidelines. We therefore investigated other spacers without optical activity. A GABA spacer (**20**, 84 pM) gave a compound with equal affinity to the γ-Glu spacer (**5**, 61 pM). A β-alanine spacer reduced the activity slightly (**21**, 113 pM) and a piperidyl-4-carboxy spacer resulted in a 6-fold lower activity (**22**, 410 pM).

Compounds Derivatized on Lysine 34. Acylation of lysine 34 (**7**, **23**−**25**) generally led to compounds a little less potent than those on lysine 26. Comparing **7** (121 pM) with **5** (61 pM) showed a 2-fold difference in the potency when acylating with a γ-Glu spacer and a C16 fatty acid. This difference can of course also be explained by the respective arginine substitutions. Acylating with a γ-Glu spacer and a C16 fatty acid when combined with the above-discussed N-terminal modification led to a 20-fold loss of potency (**23**, 2360 pM vs **7**). Using a short chain fatty acid, the combination could

pM vs **25**, 169 pM). However, the C8 fatty acid will not lead to much protraction (data not shown).

Compounds Derivatized on Lysine 36. Lysine 36 could be derivatized with quite long fatty acids (**26**, 210 pM) without too much loss of activity. The longer the fatty acid, irrespective of whether diacids or γ-Glu-spaced monoacids were used, the more loss of activity was observed. Going from a C16 diacid to a C20 shifts the potency from 7.9 to 210 pM (**27** vs **26**). A C16 γ-Glu-spaced monoacid and the corresponding C18 decreases potency from 36 to 116 pM (**8** vs **28**). Acylation of lysine 36 with a γ-Glu-spaced C16 acid (**8**, 36 pM) led to a more potent compound than the same modification on position 26 (**5**, 61 pM) or 34 (**7**, 121 pM).

Compounds Derivatized on Lysine 38. The compounds derivatized on lysine 38 (**9**, **29**–**36**) were also very potent. In this series we tried some further modifications. Substitution of glycine instead of alanine at position 8 gives stability toward enzymatic breakdown.[21] However, as expected from earlier studies,[21] this also leads to loss of activity (**33**, 328 pM vs **9**, 53 pM). Substitution of glycine in position 37 with glutamic acid was investigated because adding a negative charge adjacent to the fatty acid may increase binding of fatty acid-derived peptides to albumin.[22,26] This substitution was possible without loss of activity; in fact, it actually seemed to increase activity (**34**, 27 pM vs **9**), but when combined with the glycine substitution, again some activity was lost (**35**, 135 pM).

Conclusion

We found that the peptide hormone GLP-1 could be derivatized almost anywhere in the C-terminal part of the peptide. Derivatization with both short and long fatty acids and amino acid-derived spacers led to compounds that were highly potent. Several compounds were both very potent and had plasma half-lives above 10 h, making them suitable as drugs for treatment of type 2 diabetes using once daily administration.

Experimental Section

Instrumentation. Analytical HPLC analysis of acylated GLP-1 analogues was performed using UV detection at 214 nm and a Vydac 214TP54 4.6- × 250-mm, 5-μm C-4 silica column at 42 °C. The column was eluted at 1 mL/min with a gradient of 0–100% acetonitrile, 0.07% TFA against 0.1% TFA in water.

Mass spectral data were obtained using a Voyager RP MALDI-TOF (matrix assisted laser desorption ionization time-of-flight) instrument (Perseptive Biosystems Inc., MA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction and the accelerating voltage in the ion source was 25 kV. Sinapinic acid was used as matrix and calibration was performed using external standards with a resulting mass accuracy of 0.1%.

Synthesis of GLP-1 Analogue Starting Peptides. Solid-phase methodology was used to synthesize these analogues. The peptides were synthesized according to the Fmoc strategy on an Applied Biosystems 431A peptide synthesizer in 0.25-mmol scale using the manufacturer supplied FastMoc UV protocols starting with either a Fmoc-Gly-Wang resin, Fmoc-Lys(Boc)-Wang resin or Rink-amide resin (NovaBiochem). The protected amino acid derivatives used were commercially obtained Fmoc amino acids. The derivatives used, where side chain protection was needed, were: Fmoc-Arg(Pmc), Fmoc-Trp(Boc), Fmoc-Glu(OBut), Fmoc-Lys(Boc), Fmoc-Gln(Trt), Fmoc-Tyr(But), Fmoc-Ser(But), Fmoc-Thr(But), Fmoc-His(Trt),

The peptides were cleaved from the resin and side chain deprotected in TFA/phenol/thioanisole/water/ethanedithiol (83.25:6.25:4.25:4.25:2.0) for 180 min. The cleavage mixtures were filtered and the filtrates were concentrated in a stream of nitrogen. The crude peptides were precipitated from the residual oil with diethyl ether and washed twice with diethyl ether. After drying, the crude peptides were dissolved in 50% aqueous acetic acid, diluted to 10% with water and purified by semipreparative HPLC (Waters, Millipore) on a 25- × 250-mm column packed with 7-μm C-18 silica. The column was eluted with a gradient of acetonitrile against 0.05 M $(NH_4)_2SO_4$, 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% aqueous TFA. The peptide was eluted from the Sep-Pak cartridge with 70% acetonitrile/0.1% TFA in water and isolated from the eluate by lyophilization after dilution with water. The final product obtained was characterized by amino acid analysis, analytical RP-HPLC and MALDI-MS. Amino acid analysis and mass spectrometry data agreed with the expected structure within the experimental error of the methods (mass spectrometry ± 3 mass units, amino acid analysis ± 10%). RP-HPLC showed a peptide purity >95%.

The RP-HPLC analyses were performed using UV detection at 214 nm and a Vydac 218TP54 4.6- × 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) a gradient of 5–60% acetonitrile against 0.1 M ammonium sulfate in water, pH 2.5 and (B) a gradient of 5–60% acetonitrile, 0.1% TFA against 0.1% TFA in water. In this manner the following starting peptides were prepared:

R^{26}-GLP-1(7–37)	$E^{37}G^{8}R^{26,34}$-GLP-1(7–38)
R^{34}-GLP-1(7–37)	$K^{27}R^{26,34}$-GLP-1(7–37)
$K^{36}R^{26,34}$-GLP-1(7–36)	$K^{23}R^{26,34}$-GLP-1(7–37)
$R^{26,34}$-GLP-1(7–38)	$K^{18}R^{26,34}$-GLP-1(7–37)
desamino-$H^{7}R^{26}$-GLP-1(7–37)	$K^{8}R^{26,34}$-GLP-1(7–37)
desamino-$H^{7}R^{34}$-GLP-1(7–37)	$E^{37}R^{26,34}$-GLP-1(7–38)
$G^{8}R^{26,34}$-GLP-1(7–38)	

Synthesis of Intermediates. Alkanedioic Acid Monosuccinimidyl Ester. The alkanedioic acid monoesters were prepared from the corresponding alkanedioic acids according to the procedure described in the literature.[30]

N^{α}-Alkanoyl-L-glutamic Acid α-*tert*-Butyl Ester γ-Succinimidyl Ester. The N^{α}-alkanoyl-L-glutamic acid α-*tert*-butyl ester γ- succinimidyl esters were prepared form L-glutamic acid α-*tert*-butyl ester and the corresponding alkanoic acids according to the procedure described in the literature.[31]

γ-(N^{γ}-Hexadecanoylamino)butyric Acid Succinimidyl Ester. A mixture of hexadecanoic acid succinimidyl ester (3 g, 8.48 mmol), prepared as described in the literature,[32] and 4-aminobutyric acid (0.87 g, 8.48 mmol) in DMF (200 mL) was stirred at 25 °C for 60 h. The reaction mixture was filtered and the filtrate was added drop by drop to a 10% aqueous solution of citric acid (500 mL). The precipitate was collected and dried in vacuo. To a suspension of the residue in DMF (35 mL) was added a solution of DCC (1.45 g, 7 mmol) and HONSu (0.89 g, 7.7 mmol) in dichloromethane (20 mL). The resulting mixture was stirred at 25 °C for 20 h and then filtered. The solvent was evaporated to give a solid residue, which after recrystallization from a mixture of *n*-heptane (50 mL) and 2-propanol (2.5 mL) gave the title compound (2.5 g, 75%).

Synthesis of Acylated GLP-1 Analogues 2–36. All acyl-modified peptides, except **20** and **24**, were synthesized according to general Methods A and B. Peptide sequence, site of acylation, acyl substituent, yields, and physical data are given in Tables 1 and 3.

Method A. A mixture of starting peptide (1 equiv), DIPEA (28 equiv), NMP (70 μl/μmol starting peptide) and water (66 μL/μmol starting peptide) was gently shaken for 5 min at 25 °C. To the resulting mixture was added a solution of al-

Table 3. List of All Compounds with Method of Synthesis, Purification Yield, and Estimated Purity as Determined by Analytical HPLC and Measured and Theoretical Molecular Weight

		yield			MW	
compd	method	%	mg	HPLC purity (%)	calcd	found
1						
2	B	28	3.2	>95	3837	3835
3	B	15	1.9	>95	3820	3823
4	B	24	3.1	>90	3779	3779
5	B	6	0.2	>95	3751	3751
6	B	16	2.1	>95	3778	3780
7	B	30	6.0	>90	3751	3751
8	B	23	3.1	>95	3694	3695
9	B	47	7.2	>80	3880	3880
10	A	14	4.8	>90	3893	3893
11	B	20	2.4	>90	4091	4091
12	B	9	27.3	>90	4035	4035
13	A	9	1.0	>90	3780	3780
14	A	12	0.6	>90	3653	3653
15	A	43	11.5	>90	3624	3626
16	B	34	15.5	>80	3779	3780
17	B	29	6.1	>90	3723	3722
18	B	36	8.2	>85	3695	3693
19	B	41	9.1	>95	3737	3738
20	*a*	35	15.8	>90	3707	3705
21	A	55	11.6	>95	3694	3694
22	A	44	12	>95	3733	3734
23	B	35	5.6	>95	3737	3737
24	*a*	45	9.2	>95	3496	3496
25	B	27	4.1	>90	3625	3625
26	A	8	0.5	>90	3651	3651
27	A	11	0.6	>90	3595	3595
28	B	27	3.7	>95	3722	3722
29	A	26	3.9	>90	3808	3808
30	A	16	0.9	>90	3752	3752
31	B	11	3.6	>90	3935	3933
32	B	26	3.9	>90	3880	3880
33	B	38	5.0	>95	3894	3894
34	B	26	5.1	>95	3981	3980
35	B	29	2.9	>90	3967	3966
36	B	32	3.2	>90	3995	3994

[a] Specific method described below.

starting peptide); the reaction mixture was gently shaken for 5 min at 25 °C and then allowed to stand at 25 °C for an additional 2.5 h. The reaction was quenched by the addition of a solution of glycine (22 equiv) in 50% aqueous ethanol (165 μL/μmol starting peptide). The reaction mixture was purified by preparative 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 min. Fractions containing the product were isolated and lyophilized to give the final product. Characterization was performed by HPLC and mass spectral analysis (Table 3).

Method B. A mixture of starting peptide (1 equiv), DIPEA (28 equiv), NMP (245 μl/μmol starting peptide) and water (245 μL/μmol starting peptide) was gently shaken at 25 °C for 5 min. To the resulting mixture was added a solution of N^{α}-alkanoyl-L-glutamic acid α-*tert*-butyl ester γ-succinimidyl ester (5.9 equiv), prepared as described above, in NMP (75 μL/μmol starting peptide); the reaction mixture was gently shaken at 25 °C for 5 min and then allowed to stand at 25 °C for an additional 2 h. The reaction was quenched by the addition of a solution of glycine (22 equiv) in 50% aqueous ethanol (165 μL/μmol starting peptide). A 0.5% aqueous solution of ammonium acetate (12.5 mL/μmol starting peptide) was added, and the resulting mixture was eluted onto a preequilibrated Varian C8 Mega Bond Elut cartridge. The immobilized compound was washed with 5% aqueous acetonitrile (3.75 mL/μmol starting peptide) and finally liberated from the cartridge by elution with TFA (1.5 mL/μmol starting peptide). The eluate was allowed to stand at 25 °C for 1.75 h and then evaporated. The residue was purified by preparative chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitrile/TFA system. The column was heated to 65 °C and containing the product were isolated, and lyophilized to give the final product. Characterization was performed by HPLC and mass spectral analysis (Table 3).

$R^{26}K^{34}$-(N^{ϵ}-(γ-Aminobutyroyl(N^{γ}-hexadecanoyl)))-GLP-1(7−37)-OH, 20. To a mixture of R^{26}-GLP-1(7−37)-OH (41.1 mg, 12.2 μmol), DIPEA (44 mg, 341 μmol), NMP (5.76 mL) and water (2.88 mL) was added a solution of γ-(N^{γ}-hexadecanoyl)aminobutyric acid succinimidyl ester (16 mg, 36.5 μmol), prepared as described above, in NMP (400 μL). The reaction mixture was gently shaken at 25 °C for 5 min and then allowed to stand at 25 °C for an additional 1.5 h. The reaction was quenched by the addition of a solution of glycine (20 mg, 268 μmol) in water (200 μL). The solvent was evaporated and the residue purified by preparative 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 min. Fractions containing the product were isolated and lyophilized to provide **20** (15.8 mg, 35%). Characterization was performed by HPLC and mass spectral analysis (Table 3).

Desamino-$H^{7}R^{26}K^{34}$-(N^{ϵ}-octanoyl)-GLP-1(7−37)-OH, 24. To a mixture of desamino-$H^{7}R^{26}$-GLP-1(7−37)-OH (19.8 mg, 5.89 μmol), DIPEA (21.2 mg, 164 μmol), NMP (1.38 mL) and water (1.38 mL) was added a solution of octanoic acid succinimidyl ester (20.7 mg, 36.5 μmol), prepared as described in the literature,[33] in NMP (106 μL). The reaction mixture was gently shaken for 5 min at 25 °C and then allowed to stand for an additional 1.5 h at 25 °C. The reaction was quenched by the addition of a solution of glycine (9.7 mg, 129 μmol) in water (97 μL. The solvent was evaporated and the residue purified by preparative 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 min. Fractions containing the product were isolated and lyophilized to provide **24** (9.2 mg, 45%). Characterization was performed by HPLC and mass spectral analysis (Table 3).

Receptor Experiments. Baby hamster kidney (BHK) cells expressing the cloned human GLP-1 receptor (BHK 467-12A) were grown in DMEM media with the addition of 100 IU/mL penicillin,100 μL/mL streptomycin, 10% fetal calf serum and 1 mg/mL Geneticin G-418 (Life Technologies). Plasma membranes were prepared by homogenization in buffer (10 mM Tris-HCl, 30 mM NaCl and 1 mM dithiothreitol, pH 7.4, containing, in addition, 5 mg/L leupeptin (Sigma), 5 mg/L pepstatin (Sigma), 100 mg/L bacitracin (Sigma), and 16 mg/L aprotinin (Calbiochem-Novabiochem, La Jolla, CA)). 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 functional receptor assay was carried out by measuring cAMP as a response to stimulation by GLP-1 or derivatives. Incubations were carried out in 96-well microtiter plates in a total volume of 140 mL and with the following final concentrations: 50 mM Tris-HCl, 1 mM EGTA, 1.5 mM $MgSO_4$, 1.7 mM ATP, 20 mM GTP, 2 mM 3-isobutyl-1-methylxanthine (IBMX), 0.01% w/v Tween-20, pH 7.4. Compounds were dissolved and diluted in buffer. GTP was freshly prepared for each experiment; 2.5 μg of membrane was added to each well and the mixture was incubated for 90 min at room temperature in the dark with shaking. The reaction was stopped by the addition of 25 mL of 0.5 M HCl. Formed cAMP was measured by a scintillation proximity assay (RPA 542, Amersham, UK). Dose−response curves were plotted for the individual compounds and EC_{50} values calculated using GraphPad Prism software.

Pharmacokinetic Experiments. The experiments were performed in Landrace × Duroc × Yorkshire pigs weighing between 25 and 50 kg. All the GLP-1 analogues were administered sc at a dose of 0.5 nmol/kg; GLP-1 was administered at a dose of 15 nmol/kg. Blood samples were collected by means of a catheter placed in the jugular vein, according to the

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