Structure−Activity and Protraction Relationship of Long-Acting Glucagon-like Peptide-1 Derivatives: Importance of Fatty Acid Length, Polarity, and Bulkiness

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We here report a series of derivatives describing the structure−activity relationship around liraglutide, a once-daily human glucagon-like peptide-1 fatty acid derivative, with respect to potency as well as protraction in vivo. The spacer region between the fatty acid and the peptide is mostly important for potency, whereas the fatty acid or fatty acid mimetic is important for both potency and protraction. The length of the fatty acid is the most important parameter for protraction.

Introduction

Glucagon-like peptide-1 (GLP-1[a])-based therapeutics are rapidly becoming an important treatment class.[1] GLP-1 is an incretin hormone that simultaneously lowers blood glucose and body weight,[2] in contrast to most other glucose-lowering drugs that lead to an indirect increase in body weight. With obesity being the major underlying cause of type-2 diabetes and the spreading of both diabetes and obesity being referred to as a pandemic, a drug class that has a simultaneous lowering of both blood glucose and body weight seems highly warranted. The mechanism of action of GLP-1 is to stimulate insulin secretion, stimulate beta-cell function, increase beta-cell mass, and to decrease glucagon secretion, gastric emptying, and appetite.[3,4] Physiologically, GLP-1 is secreted from the L-cells in the intestine as a response of meal ingestion.

Two very diverse drug discovery approaches are being pursued. One is to design inhibitors of the enzyme known to be responsible for degradation of endogenous GLP-1, the dipeptidyl peptidase-IV (DPP-IV) enzyme, and thus prolong the effect of the physiologically released GLP-1. This approach has led to orally available compounds with glucose-lowering capacity.[5−7] However, the levels of GLP-1 obtained by this approach are only in the supraphysiological range,[8] and this has not led to a significant lowering of body weight in clinical or preclinical studies. The glucose-lowering efficacy is significant and clinically relevant but somewhat modest.[1,9,10] The other approach has been to administer by injection pharmacological levels of GLP-1 analogues. GLP-1 itself is very short-acting, with a half-life after intravenous administration of less than 2 min in humans.[11] Exenatide is an analogue of GLP-1 with 53% homology. Exenatide was originally isolated from the saliva of the Gila monster *Heloderma suspectum*[12] but is marketed as a synthetic peptide in a twice-daily injection regime.[13] Exenatide has a half-life of 26 min after intravenous administration.[14] We have earlier reported that derivatization of GLP-1 analogues with fatty acids led to half-lives after subcutaneous administration that is compatible with once-daily administration in humans.[15,16] One such analogue is liraglutide,[17] which is currently in large-scale phase-3 clinical trials. In contrast to the DPP-IV inhibitors, analogues of GLP-1 have demonstrated a significant body weight lowering in both preclinical models and in humans.[13] Other analogues or conjugates have been reported but are less progressed in clinical trials.[18]

Only very few actual structure−activity studies have been published, and none of them have led to drug selection.[19,20] Previously, we published an alanine scan of GLP-1,[21] a series of position-8-substituted analogues,[22] and the series of long-acting fatty-acid-derived analogues that led to the discovery of liraglutide.[15] Reports exist on selected analogues or derivatives of GLP-1,[23−28] but other drug discovery efforts and systematic structure−activity studies are not available in the scientific literature. We here report a series of analogues describing the structure−activity relationship around liraglutide with respect to potency as well as protraction in vivo. All of the analogues described are derivatized in position 26 of GLP-1(7−37).

Results and Discussion

Figure 1 illustrates the structural overview of all compounds. The amino acid sequence is very close to that of native GLP-1, with only one substitution of Lys[34] to Arg. This substitution was chosen with the original selection of liraglutide to allow for monoacylation.[15] Table 1 describes all of the compounds synthesized as well as their potency for the cloned human GLP-1 receptor and their in vivo protraction after subcutaneous administration to pigs.

Compounds Derivatized with Single-Chain Fatty Acids with an Altered Chain Length. Compounds **1**−**6** were all derived with a γ-glutamic acid spacer and a single-chain fatty acid, where only the length of the alkyl chain was altered. Compound **5** is liraglutide. The long half-life after subcutaneous administration for liraglutide has been proven in numerous studies in humans where liraglutide has a half-life of 11−15 h and a true once-daily profile in pharmacodynamic studies.[29,30]

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[a] Abbreviations: β-Ala, 3-aminopropanoic acid; BHK, baby hamster kidney; Boc, tert-butyloxycarbonyl; DDE, 1-(4,4-dimethyl-2,6-dioxocyclohex-1ylidene)ethyl-; DPP-IV, dipeptidyl peptidase IV; DIPEA, *N*,*N*-diisopropylethylamine; Gaba, 4-aminobutyric acid; Fmoc, 9-fluorenylmethyloxocarbonyl GLP-1, glucagon-like peptide-1; HPLC, high-performance liquid chromatography; MALDI-TOF; matrix-assisted laser desorption/ionization time-of-flight mass spectrometer; NMP, *N*-methyl pyrrolidone; RT, retention time; SAR, structure−activity relationship; SD, standard deviation; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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Figure 1. Structural overview of compounds. All compounds have the amino acid sequence of native GLP-1 with one modification, Arg^{34}, and are derivatized at position 26 with a spacer and an acyl group.

A long half-life resulting in pharmacological levels of GLP-1 throughout the day is important for optimal efficacy.[31] This has been shown for native GLP-1, where fasting blood glucose is lowered by 4 mM,[2] and is also evident when comparing the clinical efficacy of exenatide administered twice daily, resulting in a lowering of fasting blood glucose around 1−1.5 mM,[32,33] to that of liraglutide administered once daily, where fasting blood glucose is lowered by 3−4 mM.[17,34] The mechanism of protraction of acylated peptides is based on binding to the fatty acid binding sites on albumin.[35] When comparing liraglutide to the closely related compounds **1**−**4** and **6**, potency for the cloned human receptor was only negatively effected if the chain length of the fatty acid was longer than 16 carbon atoms (**6**, 170 pM, vs **1**−**5**, 39, 66, 29, 27 and 61 pM, respectively). The protraction in vivo, however, was closely related to the length of the fatty acid, starting with a half-life of 0.8 h (**1**, C10 fatty acid) through 5.1 h (**2**, C11 fatty acid), 7.6 h (**3**, C12 fatty acid), 9 h (**4**, C14 fatty acid), and 16 h (**5**, C16 fatty acid) to 21 h (**6**, C18 fatty acid). Figure 2 shows the pharmacokinetic profiles in pigs of compounds **2**, **3**, **5**, and **6**. Compounds **7**−**10** describe a similar series for the shorter-length fatty acids; only the spacer is different (γ-aminobutyric acid). Again, a simple relationship was shown between fatty acid chain length and protraction in vivo with half-lives of 1.6, 1.7, 2.4, and 4.6 h for compounds **7**−**10** with C10, C11, C12, and C14 fatty acids. The potency was not negatively effected for any of these compounds. Compounds **11**−**13** describe a third similar series with β-alanine as the spacer. Again, potency was not effected, and there was a simple relationship between fatty acid chain length and in vivo protraction (1.2, 2.8, and 6.5 h for fatty acids C11, C12, and C14). For the same length fatty acid, the γ-Glu spacer resulted in the longest half-life in vivo (**2**, 5.1 h, vs **8**, 1.7 h, and **11**, 1.2 h).

Compounds Exploring the Importance of the Spacer Region. The spacer in liraglutide is γ-Glu. Previously, we reported that other spacers like β-Ala and Gaba combined with a C16 fatty acid resulted in similar potent and protracted compounds.[15] Compounds **14**−**18** explored the importance of the spacer region. As γ-Glu is asymmetric, we investigated whether changing the chirality had any importance. Neither α-Glu nor D-γ-Glu as the spacer changed potency or in vivo protraction significantly, even though the half-life of compound **14** was somewhat longer (**14**, 22 h, **15**, 12 h, vs **5**, 16 h). Omitting the spacer completely resulted in markedly destroyed potency for the receptor (**16**, 4440 pM, vs **5**, 61 pM), whereas protraction was not effected (**16**, 16 h, vs **5**, 16 h). Potency may have been destroyed as a result of the derivatized peptide not being able to bind to the receptor because the conformation was constrained. With compounds **17**−**18**, we explored if the potency could be restored by using triethylenglycol as the spacer (**17**) or replacing the spacer with a sulfonamide as a carboxylic acid mimetic (**18**). In both cases, this only led to a partial restoration of potency (**17**, 1570 pM, and **18**, 2110 pM, vs **16**, 4440 pM), whereas protraction was maintained (**17**, 13 h, **18**, 15 h, vs **5**, 16 h). The potency could be almost fully restored and protraction maintained if a spacer was reintroduced together with a shorter-chain-length sulfonamide (**19**, 350 pM, 9.4 h).

Compounds with Altered Omega Terminals. The length and composition of the fatty acid is important for binding to the fatty acid sites on albumin and, thus, for protraction. Compounds **20**−**23** explored whether varying the omega terminal in relatively short fatty-acid-derived analogues could increase the protraction. Inserting an unsaturated double bond into the omega terminal resulted in a slightly improved protraction for compounds with 10 carbon atoms in the fatty acid chain (**20**, 1.9 h, vs **1**, 0.8 h). Potency was not effected. Inserting a terminal amine or alkylamine (**21**, **22**) destroyed protraction for the same number carbon atoms (**21**, 2.0 h, and **22**, 1.1 h, vs **2**, 5.1 h) and slightly decreased potency (**21**, 160 pM, and **22**, 140 pM, vs **2**, 66 pM) when a γ-Glu spacer was used, whereas when a β-Ala spacer was used, the negative effect on protraction seemed slightly smaller (**23**, 2.0 h, vs **12**, 2.8 h), but potency was still decreased (**23**, 110 pM, vs **12**, 27 pM). Inserting a hydroxyl group into the omega terminal destroyed protraction somewhat when a γ-Glu spacer and a relative short fatty acid were used (**24**, 2.6h, vs **3**, 7.6 h), as well as with a β-Ala spacer and a longer fatty acid (**25**, 4.6 h vs 8.8 h for a β-Ala-C16, reported previously).[15] Increasing the size and charge in the omega terminal by incorporating an alkylsulfonic acid did not change protraction (**26**, 2.8 h, vs **11** and **12**, 1.2 and 2.8 h, respectively) but again decreased potency slightly (**26**, 110 pM, vs **11** and **12**, 37 and 27 pM, respectively).

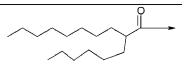
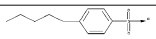
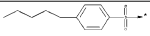
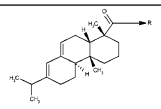
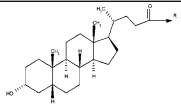
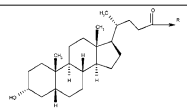
Compounds with Polar Elements in the Fatty Acid Side Chain. Introducing one ether group in the fatty acid close to the spacer (**27**, **28**) had little effect on potency but destroyed protraction (**27**, 1.7 h, vs **3**, 7.6 h, and **28**, 3.1 h, vs **4**, 9 h). Potency was slightly decreased (**27**, 81, vs **3**, 29 pM, and **28**, 70, vs **4**, 27 pM). Introducing three ether groups into the chain (**29**, 320 pM, 0.2 h) completely destroyed protraction and decreased potency compared to the matched C14 fatty acid chain (**4**, 27 pM, 9.0 h). The additional hydrophilicity within the fatty acid chain may prevent binding to the fatty acid sites on albumin. In compound **30**, three hydroxyl groups were introduced, two in the middle of the fatty acid chain and one in the omega terminal. Again, potency and protraction were decreased but were not completely destroyed (**30**, 210 pM, 2.3 h). Compounds **31**−**35** explored positioning amide groups within the fatty acid chain. Compounds **31**−**33** have one amide group positioned similar to the backbone peptide, with the omega terminal fatty acid increasing in chain length from seven to eight and nine carbon atoms. Protraction increased slightly with fatty acid chain length, but none of the compounds were equally protracted with the same length fatty acid without the amide group introduced (**31**−**33**, 2.5, 4.5, and 4.3 h, respectively, vs **3**, 7.6 h, and **4**, 9 h). Compound **34** has a longer spacer and an extra amide group added within the fatty acid chain, resulting in only little protraction (2.0 h), but retained potency (75 pM). Compound **35** has a β-Ala spacer and an amide group added with a relative long fatty acid. Protraction was partially destroyed (**35**, 3.4 h, vs **13**, 6.5 h), but potency was retained (**35**, 48 pM, vs **13**, 55 pM).

Compounds with Bulkiness in the Fatty Acid. Compound **36** (2-hexyldecanoyl as the acyl group) is derivatized with a fatty acid equivalent to 16 carbon atoms as that in liraglutide, except that a bulky fatty acid was used. Bulkiness did lead to comparable protraction (**36**, 18 h, vs **5**, 16 h); however, potency was lowered considerably (**36**, 400 pM, vs **5**, 61 pM). The increased bulkiness in the fatty acid may lead to a conformation of compound **36**, which limits optimal interaction with the

Table 1. List of All Compounds and Their Potency on the Cloned Human GLP-1 Receptor as Well as Their in Vivo Protraction in Pigs[a,b]

Compound	Acyl group	Spacer	Potency (pmol/L)	Half-life (h)
GLP-1	-	-	55 ± 19	1.2
1	-CO-$(CH_2)_8CH_3$	γ-Glu	39 ± 17	0.8
2	-CO-$(CH_2)_9CH_3$	γ-Glu	66 ± 23	5.1
3	-CO-$(CH_2)_{10}CH_3$	γ-Glu	29 ± 7	7.6
4	-CO-$(CH_2)_{12}CH_3$	γ-Glu	27 ± 2	9.0
5	-CO-$(CH_2)_{14}CH_3$ (liraglutide)	γ-Glu	61 ± 7	16
6	-CO-$(CH_2)_{16}CH_3$	γ-Glu	170 ± 40	21
7	-CO-$(CH_2)_8CH_3$	Gaba	28 ± 5	1.6
8	-CO-$(CH_2)_9CH_3$	Gaba	8.6 ± 3.6	1.7
9	-CO-$(CH_2)_{10}CH_3$	Gaba	19 ± 1	2.4
10	-CO-$(CH_2)_{12}CH_3$	Gaba	35 ± 11	4.6
11	-CO-$(CH_2)_9CH_3$	β-Ala	37 ± 6	1.2
12	-CO-$(CH_2)_{10}CH_3$	β-Ala	27 ± 3	2.8
13	-CO-$(CH_2)_{12}CH_3$	β-Ala	55 ± 15	6.5
14	-CO-$(CH_2)_{14}CH_3$	D-γ-Glu	74 ± 32	22
15	-CO-$(CH_2)_{14}CH_3$	α-Glu	76 ± 1	12
16	-CO-$(CH_2)_{14}CH_3$	-	4440 ± 440	16
17	-CO-$(CH_2)_{14}CH_3$	Triethylenglycol	1570 ± 60	13
18	-SO_2-$(CH_2)_{11}CH_3$	-	2110 ± 210	15
19	-SO_2-$(CH_2)_{11}CH_3$	β-Ala	350 ± 20	9.4
20	-CO-$(CH_2)_7CH{=}CH_2$	γ-Glu	36 ± 0.4	1.9
21	-CO-$(CH_2)_{10}NHCO$-CH_3	γ-Glu	160 ± 30	2.0
22	-CO-$(CH_2)_{10}NH_2$	γ-Glu	140 ± 30	1.1
23	-CO-$(CH_2)_{11}NH_2$	β-Ala	110 ± 20	2.0
24	-CO-$(CH_2)_{11}OH$	γ-Glu	45 ± 8	2.6
25	-CO-$(CH_2)_{15}OH$	β-Ala	65 ± 7	4.6
26	-CO-$(CH_2)_{10}SO_3H$	β-Ala	110 ± 50	2.5
27	-CO-$CH_2O(CH_2)_8CH_3$	γ-Glu	81 ± 19	1.7
28	-CO-$(CH_2)_2O(CH_2)_9CH_3$	γ-Glu	70 ± 20	3.1
29	-CO-$CH_2O(CH_2)_2O(CH_2)_2O(CH_2)_2CH_3$	γ-Glu	320 ± 150	0.2
30	-CO-$(CH_2)_8(OH)CH_2(OH)(CH_2)_6OH$	γ-Glu	210 ± 20	2.3
31	-CO-$(CH_2)_6CH_3$	8-Aminooctanoyl	48 ± 4	2.5
32	-CO-$(CH_2)_6CH_3$	9-Aminononanoyl	64 ± 12	4.5
33	-CO-$(CH_2)_6CH_3$	10-Aminodecanoyl	39 ± 12	4.3
34	-CO-$(CH_2)_4NHCO$-$(CH_2)_4CH_3$	5-Aminopentanoyl	74 ± 35	2.0

Table 1. (Continued)

35	-CO-$(CH_2)_{10}$NHCO-$(CH_2)_2CH_3$	β-Ala	48 ± 2	3.4
36		γ-Glu	400 ± 100	18
37	-CO-$(CH_2)_9$Phenyl	γ-Glu	31 ± 14	2.4
38	-CO-$(CH_2)_4$Cyclohexyl	γ-Glu	73 ± 1	2.0
39		γ-Glu	26 ± 2	3.0
40		-	380	14
41		γ-Glu	260 ± 80	6.0
42		γ-Glu	18 ± 10	6.8
43		β-Ala	360 ± 150	8.8

[a] The receptor potency data are given as mean ± SD of two or three individual experiments with triplicate samples. Protraction is expressed as the terminal half-life after subcutaneous administration. [b] A few compounds (**3**−**6**) as well as their receptor potency was reported earlier.[15] The data herein are all new, carried out in this series of experiments, except for compound **5**, where the receptor data were previously reported as well.

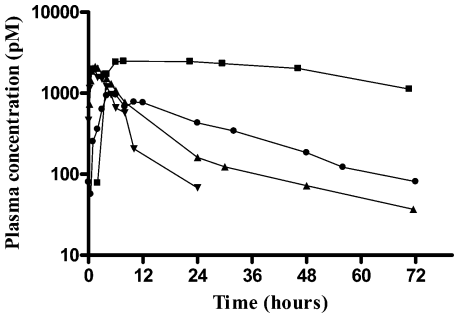


Figure 2. In vivo protraction in pigs. Protraction increased with increasing length of fatty acid side chain for compounds 2 (▼), 3 (▲), 5 (●), and 6 (■).

human GLP-1 receptor. Compounds **37** and **38** were used to investigate whether benzyl and hexyl rings could be used to add bulkiness and maintain protraction without destroying potency. Compound **37**, with a phenyl ring in the omega terminal, is equivalent to a total number of 16 carbon atoms. Potency was retained (**37**, 31 pM, vs **5**, 61 pM), but protraction was destroyed (**37**, 2.4 h, vs **5**, 16 h). Compound **38**, with a hexyl ring in the omega terminal, was synthesized with an lower equivalent number of carbon atoms (11). Potency tended to be lower (**38**, 73 pM, vs **37**, 31 pM), and protraction was similarly destroyed (**38**, 2 h, vs **5**, 16 h). Inserting bulkiness in the form of a pentylbenzenesulfonyl closer to the spacer resulted in retained potency but, again, partially destroyed protraction (**39**, 26 pM, 3.0 h). When the pentylbenzenesulfonyl was used without a spacer, potency was decreased (**40**, 380 pM, vs **39**, 26 pM), but protraction was regained (**40**, 14 h, vs **39**, 3.0 h). Retained protraction was also observed with the sulfonylamides, as described above (**18** and **19**). Finally, we explored if protraction and potency could be obtained by structurally diverse substituents of a nonfatty acid nature. Compound **41** was derivatized with an abietoyl moiety. Protraction was partially maintained (**41**, 6.0 h, vs **5**, 16 h), but potency was decreased (**41**, 260 pM, vs **5**, 61 pM). Compounds **42** and **43** were derivatized with a litocholyl and two different spacers. In both cases, protraction was partially maintained (**42**, 6.8 h, and **43**, 8.8 h). Potency was maintained when the γ-Glu was used (**42**, 18 pM) but decreased more than 10 fold when the β-Ala spacer was used (**43**, 380 pM).

Conclusion

We have explored the structure−activity relationship around analogues of liraglutide with respect to potency on the cloned human GLP-1 receptor as well as protraction in vivo in pigs. With respect to simple fatty acids, there was a straightforward relationship between structure and potency, with only very long fatty acids decreasing potency slightly, whereas protraction correlated directly to fatty acid length. The spacer region between the peptide backbone and the fatty acid was important for potency but had little impact on protraction in pigs. Inserting hydrophilicity into the omega terminal of the fatty acid moiety had little effect on protraction but tended to decrease potency. Adding polar elements or heteroatoms generally was acceptable for potency but not optimal for protraction. Bulkiness in the fatty acid decreased both potency and protraction, but a simple structure−activity or protraction relationship was not present with bulkiness as a parameter. Long-acting human GLP-1 analogues aimed for once-daily administration may be highly desirable compounds for the treatment of type-2 diabetes and, potentially, also obesity. We have described the further structure−activity studies around analogues of liraglutide, explaining the importance of the different regions for potency and protraction.

Experimental Section

Synthesis. GLP-1 Analogue Starting Peptides. Analogues were synthesized by the solid-phase peptide synthesis (SPPS) methodology using Fmoc strategy on an Applied Biosystems 431A peptide

synthesizer on the 0.25 mmol scale using manufacturer-supplied FastMoc UV protocols starting with a Fmoc−Gly−Wang resin (NovaBiochem). Protected amino acid derivatives used were, Fmoc−Ala−OH, Fmoc−Arg(Pmc)−OH, Fmoc−Asp(OBut)−OH, Fmoc−His(Trt)−OH, Boc−His(Boc)−OH, Fmoc−Gln(Trt)−OH, Fmoc−Glu(OBut)−OH, Fmoc−Gly−OH, Fmoc−Ile−OH, Fmoc−Leu−OH, Fmoc−Lys(DDE)−OH, Fmoc−Lys(Boc)−OH, Fmoc−Phe−OH, Fmoc−Ser(But)−OH, Fmoc−Thr(But)−OH, Fmoc−Trp(Boc)−OH, Fmoc−Tyr(But)−OH, Fmoc−Val−OH, Fmoc−Glu(OH)−OBut, Fmoc−D-Glu(OH)−OBut, Fmoc−5−aminopentanoic acid, Fmoc−8−aminooctanoyl−OH, Fmoc−9−aminononanoyl−OH, Fmoc−10−aminodecanoyl−OH, Fmoc−11−aminoundecanoic acid, Fmoc−β−alanine−OH, and Fmoc−Gaba−OH. Further materials used (Aldrich, Milwaukee, WI) were acetic acid, butyric acid, hexanoic acid, octanoic acid, decanoic acid, undecanoic acid, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, octanoic acid, (2-hexyl)decanoic acid, 9,10,16-trihydroxyhexanoic acid, 10-phenyldecanoic acid, 5-cyclohexylpentanoic acid, 9-enedecanoic acid, lithocholic acid, abietic acid, dodecanoylsulfonylchloride, and 4-pentylbenzenesulfonylchloride.

Synthesis of Acylated GLP-1 Analogues 1−43. All acyl-modified peptides, except **18**, **19**, **32**, **33**, **34**, **38**, **39**m and **40**, were synthesized as previously described.[15] In brief, the starting peptide was cleaved from the resin, and the side chain was deprotected in TFA/phenol/thioanisole/water/ethanedithiol (83.25:6.25:4.25:4.25:2.0) for 180 min. The cleavage mixture was filtered, and the filtrate was concentrated in a stream of nitrogen. The crude peptide was precipitated from the residual oil with diethyl ether and washed twice with diethyl ether. After drying, the crude peptide was 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 C18 silica. The column was eluted with a gradient of acetonitrile against 0.05 mol/L $(NH_4)_2SO_4$, 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.

Synthesis of Intermediates. All acyl intermediates acylated on lysine[26] were prepared as monosuccinimidyl esters, as previously described.[15]

Acylation. The starting peptide was dissolved in a mixture of NMP and water with DIPEA in surplus. To the mixture was added the acyl intermediate monosuccinimidyl ester, and the reaction was allowed for 2.5 h. The reaction was quenched by the addition of a solution of glycine.

Synthesis of Acylated GLP-1 Analogues 18, 19, 32, 33, 34, 38, 39, and 40. All of these analogues were synthesized as described above, with the exception that they were acylated on the side chain of the lysine in position 26 with the peptide still attached on the resin and fully protected on the side chain groups. except the epsilon-amine on Lys[26]. The Lys[26] was incorporated with the use of Fmoc−Lys(DDE)−OH and, subsequently, the histidine in the N-terminus as Boc−His(Boc)−OH to be compatible with full protection after deprotection of the DDE on Lys.[26] After completion of the synthesis of the native peptide chain, the DDE protecting group was selectively removed. To the protected peptidylresin, NMP was added, and after swelling, a freshly prepared solution of hydrazine hydrate, 2% in NMP, was added. The reaction mixture was stirred for 3 min at room temperature and then filtered on a glass filter. More hydrazine solution was added on the filter, and the hydrazine was left to react for 15 min and then filtered off by applying vacuum. The resin was then washed extensively with NMP and DCM and was ready for acylation on resin. The analogues **32**, **33**, **34**, and **38** were then synthesized to completion on the Applied Biosystems 431A peptide synthesizer, as described above. The analogues **19** and **39** were first added to the spacer Fmoc−β-Ala−OH or Fmoc−Glu(OH)−OBut on the Applied Biosystems 431A peptide synthesizer with removal of the Fmoc group. Analogues **18**, **19**, **39**, and **40** were then acylated with the either dodecanoylsulfonylchloride or 4-pentylbenzenesulfonylchloride (8 equiv) and DIPEA (8 equiv) in NMP and gently shaken at 60 °C for 20 h. All of the analogues **18**, **19**, **32**, **33**, **34**, **38**, **39**, and **40** were cleaved from the protected peptidyl resins by treatment with TFA/TIS/H_2O (95/2.5/2.5) v/v/v) for 90 min at room temperature. The cleavage mixtures were filtered, and the filtrates were concentrated to oils with a stream of nitrogen. The crude peptides were precipitated from this oil with excess diethyl ether, washed three times, and dried to white powders.

Purification and Characterization. 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. HPLC analysis was performed using a ZORBAX 300 SB-CN column (4.6 × 150 mm) (Rockland Technologies, DE) and a TFA/acetonitrile system. The column was run at 65 °C, and the gradient was 0−100% B over 60 min, where buffer A was 0.1% TFA and buffer B was 0.07% TFA in acetonitrile. Mass spectrometric analysis was performed on a Voyager RP MALDI-TOF instrument (Perseptive Biosystems Inc., Framingham, 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. Calibration was performed using GLP-1(7−36) amide as the external standard. The resulting accuracy was within 0.1%.

Receptor Experiments. Baby hamster kidney (BHK) cells expressing the cloned human GLP-1 receptor were grown, and plasma membranes were prepared as previously described.[15] The receptor assay was carried out by measuring cAMP as a response to stimulation by GLP-1 or derivatives. Incubations were in 96 well microtiter plates in a total volume of 140 μL and with the following final concentrations: 50 mmol/L tris-HCl, 1 mmol/L glycol-bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.5 mmol/L $MgSO_4$, 1.7 mmol/L adenosine 5′-triphosphate (ATP), 20 mmol/L guanosine triphosphate (GTP), 2 mmol/L 3-isobutyl-1-methylxanthine (IBMX), and 0.01% (w/v) tween-20, pH 7.4. Compounds were dissolved and diluted in buffer. A 2.5 μg plasma membrane was added to each well. Incubation was for 90 min at room temperature in the dark with shaking. The reaction was stopped by the addition of 25 μL of 0.5 mmol/L 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 of the GLP-1 analogues were administered subcutaneously at a dose of 0.5 nmol/kg; GLP-1 was administered at a dose of 5 nmol/kg. To 200 μL of peptide in 5 mM phosphate with 35 mg/mL mannitol, pH 7.4 (buffer), was added 5 μL of 1 mol/L NH_4OH, after which more buffer was added to 50 nmol/mL, pH 7.4, except for compounds **18** and **39**, which were dissolved in buffer, pH 7.4, to which was added 10% ethanol. Blood samples were collected by means of a catheter placed in the jugular vein, according to the following schedule: before dosing and 2, 4, 6, 8, 24, 36, 48, and 72 h after dosing. The 1 mL blood samples were stabilized with 35 μL of 0.18 M EDTA, pH 7.4, with the addition of 15000 KIE/mL aprotinin (Novo Nordisk A/S) and 3% (w/v) bacitracin (Sigma). Plasma samples were measured in a series of immunoassays, with antibodies directed against the N-terminal, the midsection, or the C-terminal of both native GLP-1 and modified GLP-1. Samples were tested without prior extraction. The relevant peptide added to normal pig plasma was used as a calibrator (concentrations from 5000 to 39 pmol/L). Concentrations were calculated from the calibrators using a four-parameter logistic curve fit using the MultiCalc software from Wallac. Data were analyzed for the individual pigs in each group by use of noncompartmental methods using the PC-based software WinNonlin (version 2.1, Scientific Consulting Inc., U.S.A.).

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Supporting Information Available: Supporting Table 1. List of all compounds with analytical details and supporting data: tracings for key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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