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Chemoenzymatic Synthesis of Glycosylated Glucagon-like Peptide 1: Effect of Glycosylation on Proteolytic Resistance and in Vivo Blood Glucose-Lowering Activity

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Abstract: Glucagon-like peptide 1 (7−36) amide (GLP-1) has been attracting considerable attention as a therapeutic agent for the treatment of type 2 diabetes. In this study, we applied a glycoengineering strategy to GLP-1 to improve its proteolytic stability and in vivo blood glucose-lowering activity. Glycosylated analogues with *N*-acetylglucosamine (GlcNAc), *N*-acetyllactosamine (LacNAc), and α2,6-sialyl *N*-acetyllactosamine (sialyl LacNAc) were prepared by chemoenzymatic approaches. We assessed the receptor binding affinity and cAMP production activity in vitro, the proteolytic resistance against dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase (NEP) 24.11, and the blood glucose-lowering activity in diabetic db/db mice. Addition of sialyl LacNAc to GLP-1 greatly improved stability against DPP-IV and NEP 24.11 as compared to the native type. Also, the sialyl LacNAc moiety extended the blood glucose-lowering activity in vivo. Kinetic analysis of the degradation reactions suggested that the sialic acid component played an important role in decreasing the affinity of peptide to DPP-IV. In addition, the stability of GLP-1 against both DPP-IV and NEP24.11 incrementally improved with an increase in the content of sialyl LacNAc in the peptide. The di- and triglycosylated analogues with sialyl LacNAc showed greatly prolonged blood glucose-lowering activity of up to 5 h after administration (100 nmol/kg), although native GLP-1 showed only a brief duration. This study is the first attempt to thoroughly examine the effect of glycosylation on proteolytic resistance by using synthetic glycopeptides having homogeneous glycoforms. This information should be useful for the design of glycosylated analogues of other bioactive peptides as desirable pharmaceuticals.

Introduction

GLP-1 is a peptide hormone secreted from enteroendocrine L-cells of the intestine in response to nutrient absorption. It stimulates insulin secretion from β-cells in a glucose-dependent manner. GLP-1 also acts to suppress glucagon secretion, gastric emptying, and appetite and to improve pancreatic β-cell functions. It is now attracting considerable attention for its therapeutic benefits in type 2 diabetes.[1−5] However, the use of native GLP-1 as a therapeutic agent is limited by its short half-life in plasma and low in vivo activity. GLP-1 is rapidly inactivated by proteolytic enzymes such as DPP-IV[6−8] and NEP 24.11[9−11] under physiological conditions, requiring continuous administration for adequate efficacy.[12,13] GLP-1 also suffers from physical instability. It is known that aggregation propensity exists for GLP-1.[14−16] In addition, GLP-1 is potentially immunogenic.[4] Thus, formation of antibodies might become problematic with long-term administration of GLP-1.

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To improve the pharmacokinetic property and in vivo activity of GLP-1, a number of studies have been conducted. DPP-IV-resistant analogues were designed by attaching chemical groups[17−19] or by substituting amino acids at the N-terminus of the peptide.[20−25] Conjugation with polyethylene glycol (PEG) was shown to increase the in vivo activity of GLP-1, as well as other therapeutic peptides and proteins, by improving the proteolytic stability.[26−28] Also proposed as an effective method to increase the plasma half-life and in vivo activity of GLP-1 were attaching a fatty acid chain to facilitate binding to plasma protein[29−31] and conjugation with albumin.[32−34] The GLP-1 analogue exendin-4, isolated from the saliva of the Gila monster *Heloderma suspectrum*, has a 53% amino acid sequence identity with GLP-1.[35] Exendin-4, which is resistant to degradation by DPP-IV and NEP 24.11,[36] has a longer extended half-life and greater in vivo activity than does native GLP-1.[37,38]

As another approach to developing novel long-acting analogues, we applied glycoengineering strategy to GLP-1. It has been reported that N-linked carbohydrates improve and prolong in vivo activity of protein by serum half-life extension.[39−41] A notable example involves mutating human erythropoietin to incorporate two additional N-linked glycosylation sites, and this results in a protein with a longer plasma half-life and an increased in vivo activity.[39,40] In addition, carbohydrate can increase solubility and reduce immunogenicity of peptides and proteins.[42] These findings suggest that the introduction of a sialylated carbohydrate moiety to GLP-1 should enhance its therapeutic utility by improving its pharmacokinetic property, in vivo biological activity, and solubility and by reducing its immunogenicity.

A study by Meurer et al. showed that glycosylation contributes to improving the inhibitory effect of the GLP-1 receptor antagonist exendin(9−39) in vivo.[43] However, no actual study on enhancing the therapeutic potential of GLP-1 by glycosylation has been reported. The aim of this study was to design and synthesize glycosylated GLP-1 analogues and to evaluate the effect of glycosylation on the proteolytic resistance and the efficacy in vivo. Glycosylated GLP-1 analogues were prepared by a chemoenzymatic approach using glycosyltransferases. We characterized the receptor binding affinity and cAMP production activity in vitro, stability against proteolytic enzymes such as DPP-IV and NEP 24.11, and blood glucose-lowering activity in diabetic db/db mice on the basis of the degree and sites of glycosylation and types of glycan.

Results and Discussion

1. Molecular Design and Preparation of Glycosylated GLP-1 Analogues. To thoroughly examine the effects of the degree and sites of glycosylation and types of glycan, preparation of homogeneous glycopeptides was required. To date, several methods have been reported for the synthesis of glycopeptides having a homogeneous complex type N-linked glycan.[44,45] However, these methods are difficult to apply to the synthesis of various glycopeptides for structure−activity relationship study due to the use of precious and complicated glycan derivatives as materials. What is known is that the nonreducing terminal structure of N-linked glycans is responsible for the half-life of glycoprotein. Terminal sialic acid protects the protein from degradation by proteolytic enzymes[46] and reduces glomerular filtration by increasing the charge of the molecules.[47] It has also been reported that hepatic asialoglycoprotein receptor removes glycoprotein from circulation by binding to the terminal galactose residue.[48] Therefore, we prepared glycosylated analogues containing a nonreducing terminal carbohydrate of complex type N-linked glycan such as GlcNAc, LacNAc, or sialyl LacNAc. Glycopeptides having these carbohydrates can be easily prepared by a chemoenzymatic

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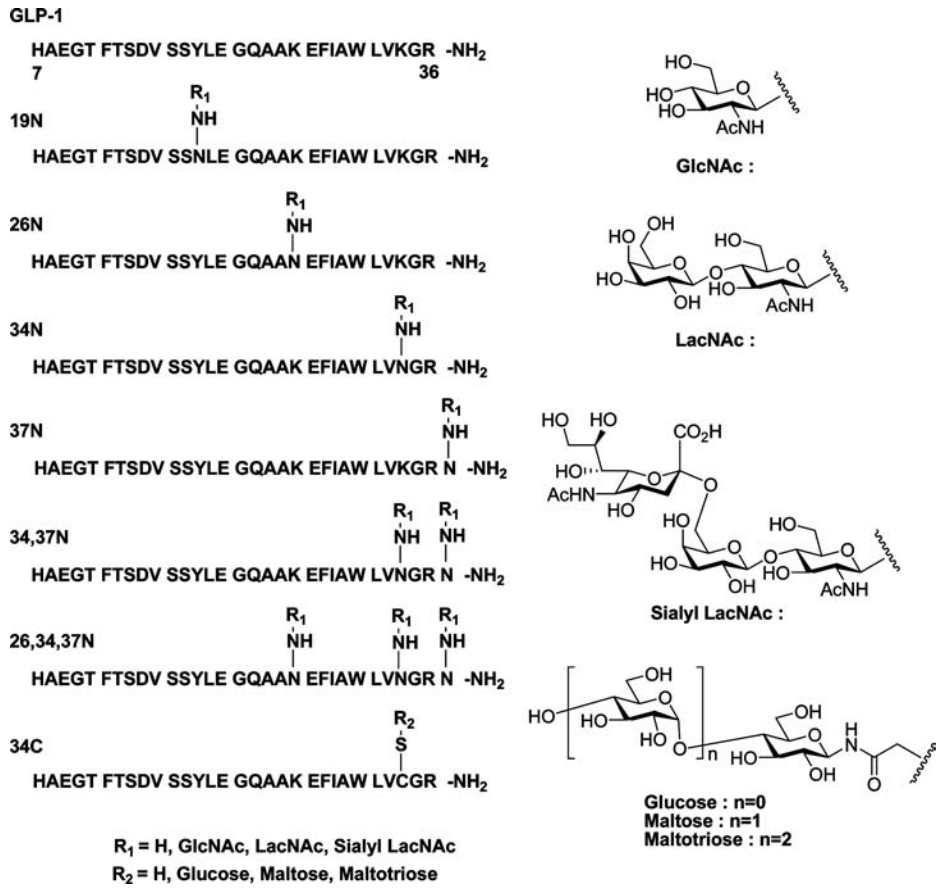

Figure 1. Peptide sequence and glycan structure of GLP-1 analogues.

approach, which is applicable to the preparation of various homogeneous glycopeptides for structure−activity relationship study.[49,50]

To retain the receptor binding and agonistic activity of glycosylated GLP-1 analogues, we chose glycosylation sites according to previous structure−activity studies of GLP-1. The conformation has been studied in the presence of micelles, which adopt an α-helical structure from the residue Thr13 to the nearby Lys34 with a random coil region at the N-terminus.[51,52] The helical structure was required for GLP-1 receptor binding, and one face of the helix composed of amino acids such as Ser17, Glu21, Ala24, Phe28, Trp31, and Leu32 interacted with the receptor.[53] The N-terminal random coil region is important for both receptor binding and agonistic activity.[53] Accordingly, glycosylation of residues located on the opposite side of the receptor binding face in the helical region or the C-terminus was thought to have a low influence on receptor binding and agonistic activity. Therefore, we chose positions 19, 26, 34, and the C-terminus (37) as glycosylation sites (Figure 1, Table 1).

Glycopeptides having GlcNAc were prepared by solid-phase peptide synthesis (SPPS) methodology using fluorenylmethoxycarbonyl (Fmoc) amino acids and Fmoc−Asn(Ac_3GlcNAcβ)−OH as the building blocks. The crude products were purified by reverse-phase high-performance liquid chromatography (RP-HPLC). Unglycosylated analogues with substitution to Asn were prepared in the same manner. Glycopeptides containing LacNAc or sialyl LacNAc were synthesized by enzymatic carbohydrate elongation using glycosyltransferases (Scheme 1). The galactose residue was transferred from uridine-5′-diphosphogalactose (UDP-galactose) to the GlcNAc moiety in the peptide by β1,4-galactosyltransferase. Sialylation of the LacNAc moiety was performed by transfer of sialic acid from cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-NANA) by α2,6-sialyltransferase.

To understand the effect of the charge and size of the carbohydrate on stability against DPP-IV, GLP-1 analogues having carbohydrates such as glucose, maltose, and maltotriose were prepared. These glycosylated analogues were synthesized by alkylation of a cysteine residue in peptide with the *N*-iodoacetyl glycosylamines (Scheme 2).[54,55] An analogue substituted with Cys was prepared by the Fmoc SPPS method. β-Glycosylamines were prepared by reactions of the corresponding reducing carbohydrates in an aqueous solution of ammonia.[56] Treatment of the β-glycosylamines with iodoacetic anhydride produced these *N*-iodoacetyl glycosylamine derivatives (Scheme 3).[57] The reactions of enzymatic carbohydrate

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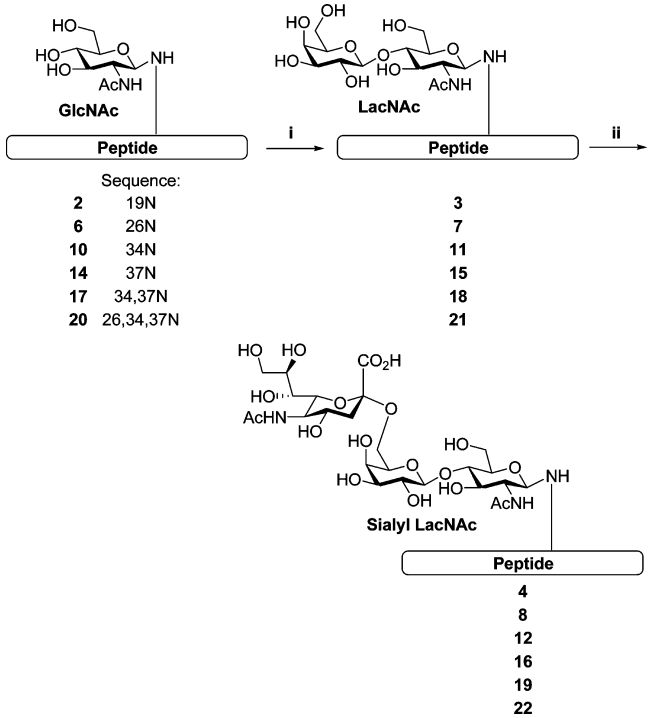
Table 1. Summary of Synthesized GLP-1 Analogues, Theoretical and Observed Molecular Weights, Purity, and in Vitro Activities

			MALDI-TOF MS analysis		HPLC analysis	receptor binding assay	cAMP production
no.	peptide sequence	glycan group	calcd mass[a]	observed[b]	purity (%)[c]	IC_{50} (nM)[d]	EC_{50} (nM)[d]
1	19N	H	3248.6	3249.4	97.3	11	0.92
2		GlcNAc	3451.8	3452.7	93.8	41	8.1
3		LacNAc	3613.9	3614.5	93.7	38	8.3
4		sialyl LacNAc	3905.2	3906.1	89.5	>100	50
5	26N	H	3283.6	3284.5	97.2	1.4	0.09
6		GlcNAc	3486.8	3487.9	96.2	2.8	0.28
7		LacNAc	3648.9	3649.9	91.6	2.0	0.33
8		sialyl LacNAc	3940.2	3940.8	91.0	2.9	0.92
9	34N	H	3283.6	3284.8	90.1	1.00	0.11
10		GlcNAc	3486.8	3487.8	93.1	0.85	0.17
11		LacNAc	3648.9	3650.0	86.2	0.76	0.11
12		sialyl LacNAc	3940.2	3941.1	93.3	0.70	0.16
13	37N	H	3411.7	3412.8	90.9	0.72	0.09
14		GlcNAc	3614.9	3616.1	90.1	0.53	0.11
15		LacNAc	3777.1	3778.7	85.2	0.47	0.12
16		sialyl LacNAc	4068.3	4069.4	90.9	0.71	0.11
17	34,37N	GlcNAc	3804.1	3804.9	98.5	N.T.	N.T.
18		LacNAc	4128.3	4129.6	99.5	N.T.	N.T.
19		sialyl LacNAc	4710.8	4711.9	98.1	3.0	0.20
20	26,34,37N	GlcNAc	3993.2	3993.9	94.0	N.T.	N.T.
21		LacNAc	4479.6	4480.5	86.0	N.T.	N.T.
22		sialyl LacNAc	5353.4	5354.5	92.7	51	6.1
23	34C	H	3272.6	3273.4	94.9	N.T.	N.T.
24		glucose	3491.8	3492.5	97.0	N.T.	N.T.
25		maltose	3653.9	3654.9	93.3	N.T.	N.T.
26		maltotriose	3816.1	3817.0	95.9	N.T.	N.T.
	GLP-1[e]					0.41	0.11

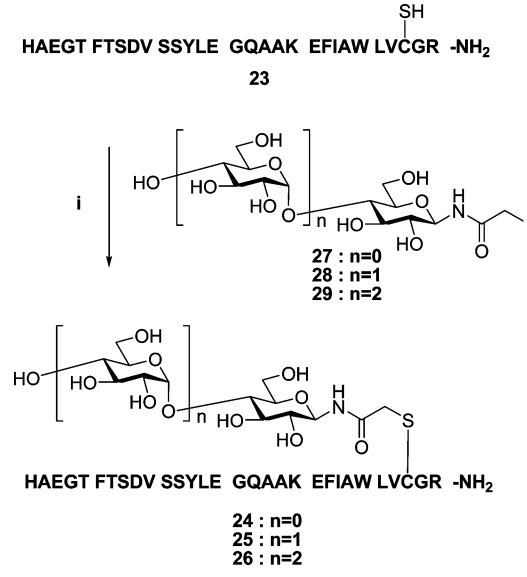
[a] Molecular weight of free base. [b] $[M + H]^+$. [c] The purity was determined by analytical RP-HPLC with UV detection at 220 nm. [d] N.T.: not tested. [e] Human GLP-1 receptor expressing CHO cell membrane.

Scheme 1. Enzymatic Carbohydrate Elongation Using Glycosyltransferases[a]



[a] Reagents and conditions: (i) UDP-galactose, β1,4-galactosyltransferase, 25 mM HEPES buffer containing 10 mM $MnCl_2$ and 0.01% Triton X-100 (pH 7.5), 25 °C; (ii) CMP-NANA, α2,6-sialyltransferase, 25 mM HEPES buffer containing 0.01% Triton X-100 (pH 7.5), 37 °C.

Scheme 2. Alkylation of the Cysteine Residue in Peptide with the *N*-Iodoacetyl Glycosylamines[a]

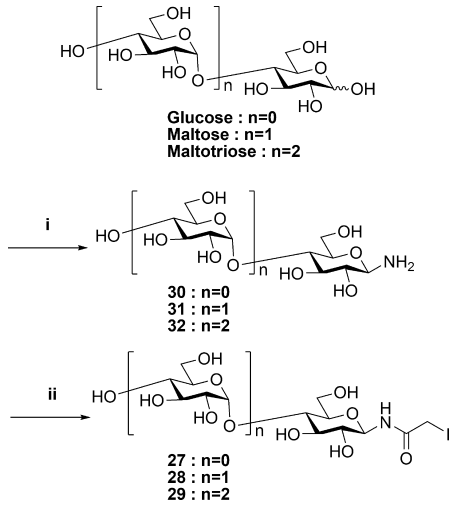


[a] Reagents and conditions: (i) *N*-Iodoacetyl glycosylamine (**27**, **28**, or **29**), 100 mM phosphate buffer (pH 8.0), 37 °C.

elongation and alkylation were monitored by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). After the reactions were completed, the products were isolated by RP-HPLC. The purity and identity of the synthesized GLP-1 analogues were verified by RP-HPLC and matrix-associated laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), respectively (Table 1).

2. Receptor Binding and Agonistic Activity of Glycosylated GLP-1 Analogues. The receptor binding and agonistic activities[58] of the GLP-1 analogues were investigated by competitive binding assay and cAMP production assay, respectively (Table 1). As expected, the receptor binding and agonistic activity of monoglycosylated analogues **6**−**8**, **10**−**12**, and **14**−**16** at

Scheme 3. Synthesis of *N*-Iodoacetyl Glycosylamines[a]



[a] Reagents and Conditions: (i) NH_3, NH_4HCO_3, H_2O, 40−50 °C; (ii) iodoacetic anhydride, $NaHCO_3$, $MeCN-H_2O$, room temperature.

positions such as 26, 34, and 37 were not significantly altered from those of the native GLP-1. In particular, analogues **10**−**12** and **14**−**16** glycosylated at positions 34 and 37 were found to have activity nearly equal to that of the native GLP-1. However, GLP-1 analogues **2**−**4** glycosylated at position 19 showed greatly reduced receptor binding and agonistic activity as compared to the native GLP-1. Analogue **4**, in particular, having sialyl LacNAc at position 19 showed little agonistic activity. It is well-known that the N-terminal region of GLP-1 plays an important role in receptor binding and agonistic activity.[53] Thus, glycosylation at a position near the N-terminus may result in low activity due to high hindrance to the binding of GLP-1 receptors.

The diglycosylated analogue **19** having sialyl LacNAc moieties at positions 34 and 37 had reduced agonistic activity, as compared to the corresponding monoglycosylated analogues **12** and **16**. In addition, the activity of the triglycosylated analogue **22** was much lower than that of mono- or diglycosylated analogues **8**, **12**, **16**, and **19**. Binding affinity and activity decreased with an increase in the content of carbohydrate having sialic acid, in agreement with the findings of a previous study on the glycosylation of therapeutic protein.[59]

3. Effect of Glycosylation on Proteolytic Stability against Recombinant Human DPP-IV and NEP 24.11. To evaluate the effect of glycosylation on proteolytic stability, the degradation of GLP-1 analogues by DPP-IV and NEP 24.11 was observed. Initially, degradation reactions of GLP-1 analogues by recombinant human DPP-IV were kinetically analyzed with the Michaelis−Menten kinetic equation (Figure 2).[60] DPP-IV is found in great abundance in the kidney and plasma, which releases the N-terminal dipeptide His7−Ala8.[6−8] The native GLP-1 was easily degraded by DPP-IV with k_{cat}/K_M and K_M values similar to those in the previous study.[60] In contrast, k_{cat}/K_M values of monoglycosylated analogues **2**−**4**, **6**−**8**, **10**−**12**, and **14**−**16** were lower than those of GLP-1 and unglycosylated analogues **1**, **5**, **9**, and **13**. Proteolytic resistance of GLP-1 against DPP-IV was improved by carbohydrate addition. As compared to k_{cat}/K_M values of analogues having the same type of carbohydrate at a different position, values of analogues **14**−**16** glycosylated at position 37 away from the cleavage site were slightly higher. However, analogues **2**−**4**, **6**−**8**, and **10**−**12** glycosylated at position 19, 26, or 34 had nearly equal k_{cat}/K_M values. It is thought that resistance to DPP-IV would not be significantly influenced by the glycosylation site. In contrast, the in vitro activity of analogues **10**−**12** glycosylated at position 34 was greater than that of analogues **2**−**4** and **6**−**8** glycosylated at positions 19 and 26 (Table 1). Therefore, position 34 was found to be the glycosylation site with the best combination of in vitro agonistic activity and proteolytic stability against DPP-IV.

Comparison of k_{cat}/K_M values of analogues having another type of carbohydrate at the same position showed that the values of analogues **4**, **8**, and **12** having sialyl LacNAc were lower than those of analogues **2**, **3**, **6**, **7**, **10**, and **11** having GlcNAc or LacNAc. Moreover, analogues **4**, **8**, and **12** had significantly greater K_M values than did analogues **2**, **3**, **6**, **7**, **10**, and **11**. This indicated that addition of sialyl LacNAc would improve the stability of peptide by decreasing the affinity to DPP-IV. On the other hand, the k_{cat}/K_M and K_M values of analogues **26** having maltotriose were nearly equal to those of analogues **24** and **25** having glucose or maltose. Although glucose, maltose, and maltotriose are of comparable size to GlcNAc, LacNAc, and sialyl LacNAc, maltotriose is not negatively charged, while sialyl LacNAc is. These results suggest that the negative charge of sialic acid plays an important role in decreasing the affinity of peptide for DPP-IV, although other structural components of the sialic acid, such as the glycerol side chain, have not been eliminated as being benign changes from the triglucoside model glycopeptide. Also, the k_{cat}/K_M and K_M values of the diglycosylated analogue **19** and the triglycosylated analogue **22** having sialyl LacNAc moieties were considerably altered, as compared to the monoglycosylated analogues. The stability of GLP-1 against DPP-IV was incrementally improved by increasing the content of sialic acid.

The stability of GLP-1 analogues against NEP 24.11 was evaluated by quantification of the remaining intact GLP-1 analogue after incubation with recombinant human NEP 24.11 (Figure 3). NEP 24.11 cleaves GLP-1 at the N-terminal side of hydrophobic amino acids such as Val16, Tyr19, Leu20, Phe28, Ile29, and Leu32.[9,10] Native GLP-1 was rapidly degraded by NEP 24.11. In contrast, glycosylated analogues **10**−**12**, **19**, and **22** were degraded more slowly. When monoglycosylated analogues **10**−**12** were compared, analogue **12** having the sialyl LacNAc moiety showed higher stability against NEP 24.11. Moreover, the diglycosylated analogue **19** and the triglycosylated analogue **22** showed little degradation. Addition of sialyl LacNAc improved the stability of GLP-1 against NEP 24.11 as well as DPP-IV.

4. Effect of Glycosylation on Blood Glucose-Lowering Activity in Vivo. The blood glucose-lowering activity of glycosylated GLP-1 analogues was evaluated in diabetic db/db mice. To investigate the effect of glycosylation on the efficacy in vivo based on types of glycan and the degree of glycosylation, compounds **10**−**12**, **19**, and **22** were candidates for in vivo study. Although addition of sialyl LacNAc improved proteolytic resistance of GLP-1 in vitro, improving its pharmacological action in vivo is important for practical purpose. After subcutaneous administration of GLP-1 analogues at equivalent doses (100 nmol/kg) to mice, blood glucose levels were monitored

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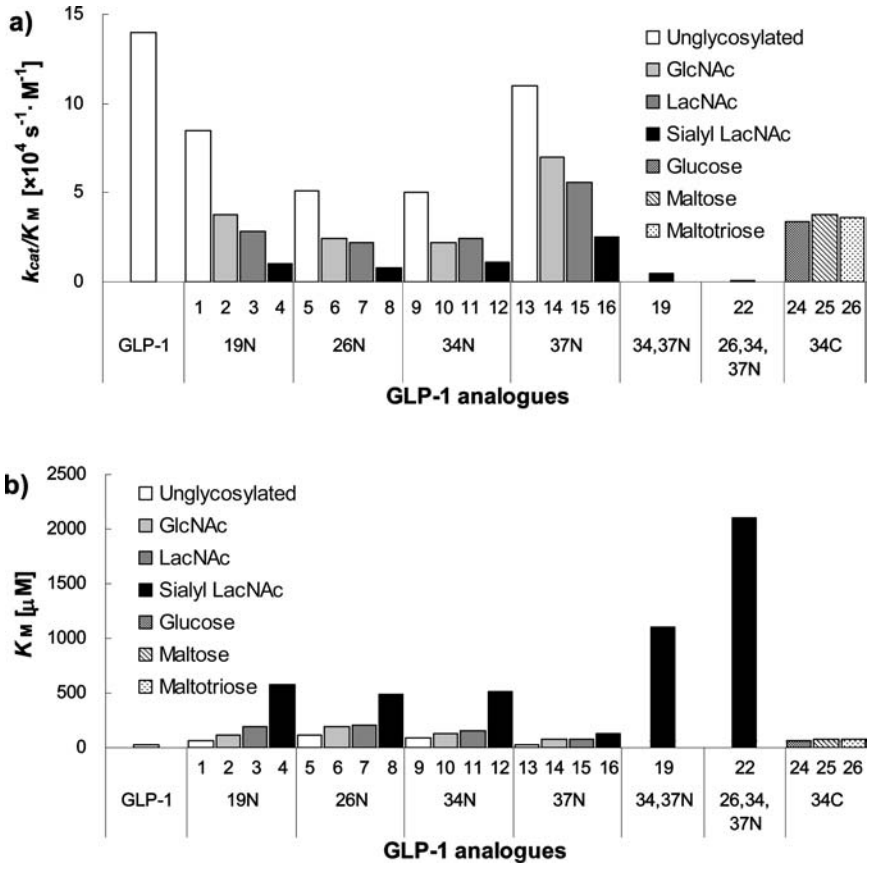

Figure 2. (a) k_{cat}/K_M and (b) K_M of glycosylated GLP-1 analogues to recombinant human DPP-IV.

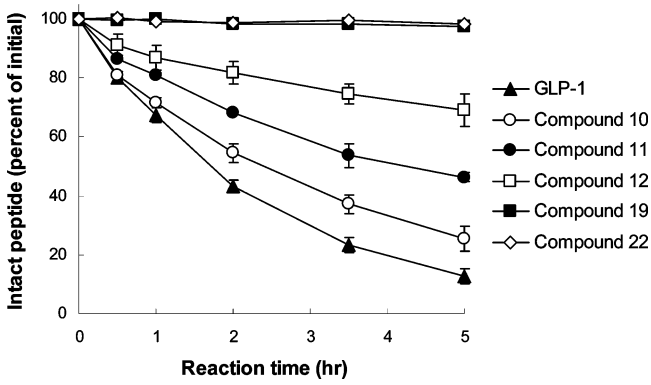

Figure 3. Degradation profiles of glycosylated GLP-1 analogues incubated with recombinant human NEP 24.11. The quantity of the peptide (peak area) at $t = 0$ was adjusted to 100%. Values are mean ± SEM ($n = 3$).

(Figure 4). Administration of native GLP-1 showed a brief duration of the blood glucose-lowering effect because GLP-1 has a short circulating half-life due to rapid inactivation by proteolytic enzymes. In contrast, monoglycosylated GLP-1 analogues **10**−**12** reduced blood glucose levels for a longer period of time than did the native GLP-1. In particular, analogue **12** glycosylated with sialyl LacNAc showed significantly improved in vivo activity with prolonged glucose-lowering activity of up to 4 h after administration (Figure 4a). Prolongation of the in vivo activity of glycosylated analogue **10**−**12** was consistent with its proteolytic stability. The marked improvement of the in vivo activity of analogue **12** was associated with its great proteolytic stability caused by the sialic acid component. Addition of sialyl LacNAc can improve the pharmacokinetic property of GLP-1 by increasing proteolytic stability and therefore prolong the in vivo activity. The pharmacokinetic properties of glycosylated GLP-1 analogues were also evaluated in rats. Analogue **12** exhibited a higher plasma concentration as compared to native GLP-1 after intravenous administration at equivalent doses in rats (data not shown). It has been reported that binding with the terminal galactose residue to the asialoglycoprotein receptor results in rapid clearance of glycoprotein.[48] However, galactose-terminal analogue **11** reduced blood glucose levels as long as GlcNAc-terminal analogue **10**. The analogue **11** having only one galactose residue was thought to have a low affinity for the asialoglycoprotein receptor. The affinity depended on the number and the spatial arrangement of the terminal galactose moieties.[61]

Comparison of analogues **12**, **19**, and **22** by considering the number of sialyl LacNAc moieties showed that the diglycosylated analogue **19** and the triglycosylated analogue **22** exhibited more prolonged glucose-lowering activity than did the monoglycosylated analogue **12** for up to 5 h (Figure 4b), while the analogues **19** and **22** had reduced in vitro agonistic activity. On the other hand, although the stability against DPP-IV of glycosylated analogue **4** having sialyl LacNAc at position 19 was comparable to that of analogue **12**, analogue **4** showed rapid recovery of blood glucose levels due to its low in vitro agonistic activity (data not shown). It is thought that the balance between proteolytic stability and in vitro activity is important for improvement of in vivo activity. Addition of sialyl LacNAc moieties at appropriate sites such as position 26, 34, or 37 should

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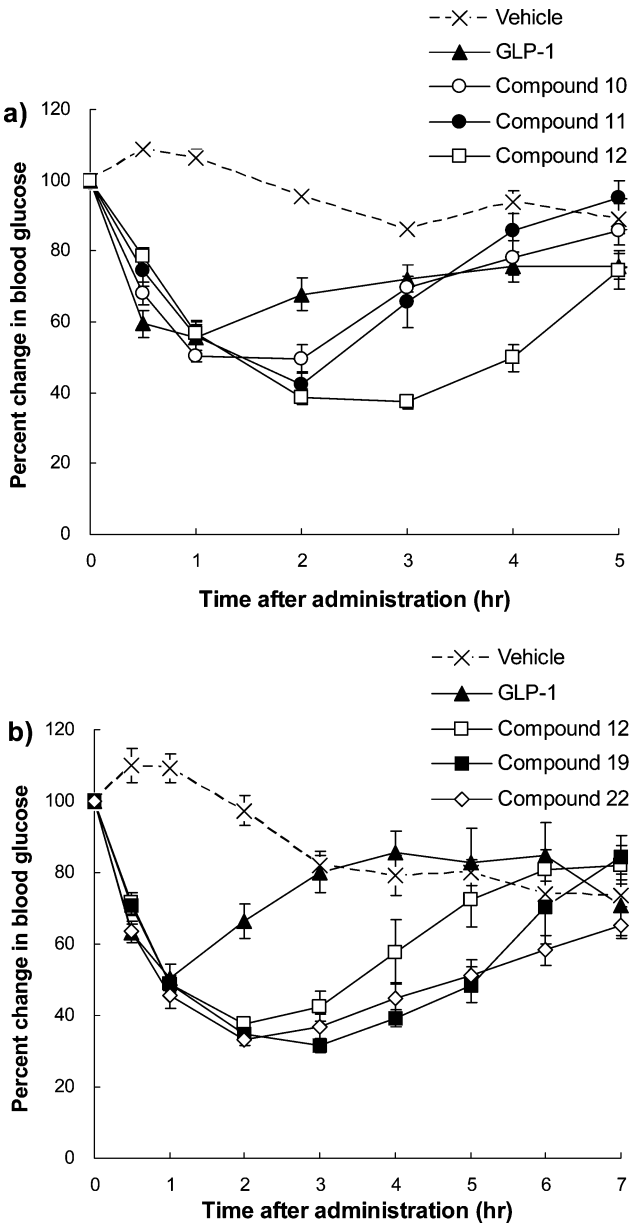

Figure 4. Glucose-lowering activities of glycosylated GLP-1 analogues (100 nmol/kg) in diabetic db/db mice. (a) Comparing compounds **10**−**12** and GLP-1; (b) comparing compounds **12**, **19**, **22** and GLP-1. The level blood glucose at $t = 0$ was assisted to 100%. Values are mean ± SEM ($n = 6-7$).

extend in vivo activity by considerably increasing circulation time in plasma while retaining the ability to bind to and activate the receptor.

Conclusions

The present study suggests that the addition of sialyl LacNAc is a prospective strategy for enhancing the therapeutic utility of GLP-1 by improving its proteolytic stability and in vivo activity. We designed analogues di- and triglycosylated with sialyl LacNAc having greatly improved proteolytic stability against DPP-IV and NEP 24.11 and prolonged blood glucose-lowering activity. Kinetic analysis of the degradation reaction suggested that the sialic acid component can play an important role in decreasing the affinity of GLP-1 to DPP-IV. Sialyl LacNAc can improve the pharmacokinetic property of GLP-1 by increasing its proteolytic stability and therefore prolong in vivo activity.

This study is the first attempt to thoroughly examine the effect of glycosylation on proteolytic stability by using synthetic glycopeptides having homogeneous glycoforms. This information should be useful and available for designing glycosylated analogues of not only GLP-1 but also other bioactive peptides as desirable pharmaceuticals.

Experimental Section

Materials and General Procedures. Unless otherwise stated, all commercially available solvents and reagents were used without purification. GLP-1 and GLP-1 analogues (**2**, **6**, **14**, and **17**) were purchased from the Peptide Institute, Inc. TentaGel SRAM resin was purchased from Hipep Laboratories, and Fmoc amino acid derivatives were purchased from Nova Biochem Co. Ltd. β1,4-Galactosyltransferase was purchased from Toyobo Co. Ltd. α2,6-Sialyltransferase was purchased from Toyobo Co. Ltd. or Japan Tobacco Inc. UDP-Galactose and CMP-NANA were purchased from Yamasa Shoyu Co. Recombinant human DPP-IV and NEP 24.11 were purchased from R&D Systems, Inc. BKS.Cg-+ Lepr^{db}/+ Lepr^{db} mice were purchased from Clea Japan, Inc. ^{1}H spectra were recorded on a Bruker AV400 (400 MHz) spectrometer. MALDI-TOF MS data were recorded by a Bruker Ultraflex mass spectrometer. LC-ESI-MS data were recorded using an Agilent 1100 series. Semipreparative RP-HPLC was performed on a Shimadzu LC-8A series or a Waters HPLC system. Analytical RP-HPLC was performed on a Shimadzu LC-10A series or an Agilent 1100 series. The purity of compounds **1**−**26** was determined by analytical RP-HPLC on an Inertsil ODS-3 4.6 × 250 mm at 25 °C. The column was eluted with a linear gradient of 5−25% solvent B (acetonitrile containing 0.1% TFA) against solvent A (0.1% aqueous TFA) in 10 min, and then 25−45% solvent B against solvent A in 35 min at 1 mL/min. The eluate was monitored with UV absorption at 220 nm. The purity is estimated from percentage of peak area of compounds relative to the combined areas of all peaks present.

General Procedures of Synthesis of Glycosylamine (30−32). Glycosylamines of glucose, maltose, and maltotriose were prepared according to a previously described method.[56]

***N*-Iodoacetyl-β-D-glucopyranosylamine (27).** To a solution of **30** (540 mg, 3.0 mmol) in 1 M $NaHCO_3$ aq (20 mL) and acetonitrile (5 mL) was added iodoacetic anhydride (1.62 mg, 4.58 mmol). The reaction mixture was stirred at room temperature for 2.5 h and then acidified to pH 5.0 by adding AcOH. After evaporation, the crude product was purified by RP-HPLC using Develosil Combi-RP-5 20 × 150 mm. The column was eluted with 2% aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA) for 12 min at 10 mL/min. The fraction was lyophilized to give **27** (301 mg, 29%): ^{1}H NMR (D_2O) δ 4.93 (d, 1H, $J_{1,2} = 9.1$ Hz, H-1), 3.88 (br d, 1H, $J_{6a,6b} = 12.6$ Hz, H-6a), 3.82 (s, 2H, CH_2ICO), 3.72 (dd, 1H, $J_{5,6b} = 5.3$ Hz and $J_{6a,6b} = 12.4$ Hz, H-6b), 3.54 (t, 2H, $J_{2,3} = J_{3,4} = 9.1$ Hz, H-3), 3.51 (m, 1H, H-5), 3.41 (t, 1H, $J_{3,4} = J_{4,5} = 9.1$ Hz, H-4), 3.38 (t, 1H, $J_{1,2} = J_{2,3} = 9.1$ Hz, H-2).

4-*O*-(α-D-Glucopyranosyl)-1-*N*-iodoacetyl-β-D-glucopyranosylamine (28). To a solution of **31** (683 mg, 2.0 mmol) in 1 M $NaHCO_3$ aq (15 mL) and acetonitrile (5 mL) was added iodoacetic anhydride (1.20 mg, 3.4 mmol). The reaction mixture was stirred at room temperature for 3.5 h and then acidified to pH 5.0 by adding AcOH. After evaporation, the crude was purified by RP-HPLC using Develosil Combi-RP-5 20 × 150 mm. The column was eluted with a linear gradient of 0−5% acetonitrile containing 0.1% formic acid (FA) against 0.1% aqueous FA for 12 min at 10 mL/min. The fraction was lyophilized to give **28** (350 mg, 34%): ^{1}H NMR (D_2O) δ 5.42 (d, 1H, $J_{1',2'} = 3.5$ Hz, H-1′), 4.95 (d, 1H, $J_{1,2} = 9.1$ Hz, H-1), 3.86−3.56 (m, 10H, H-3, H-4, H-5, H-6a, H-6b, H-3′, H-4′, H-5′, H-6′a, H-6′b), 3.82 (s, 2H, CH_2ICO), 3.57 (dd, 1H, $J_{1',2'} = 3.5$ Hz, and $J_{2',3'} = 9.9$ Hz, H-2′), 3.41 (br t, 2H, $J_{1,2} = J_{3',4'} = 9.4$ Hz, H-2, H-4′).

4-*O*-[4-*O*-(α-D-Glucopyranosyl)-α-D-glucopyranosyl]-1-*N*-iodoacetyl-β-D-glucopyranosylamine (29). To a solution of **32** (1.05 g, 2.1 mmol) in 1 M $NaHCO_3$ aq (15 mL) and acetonitrile (5 mL) was added iodoacetic anhydride (1.42 g, 4.0 mmol). The reaction mixture was stirred at room temperature for 2 h and then acidified to pH 5.0 by adding AcOH. After evaporation, the crude was purified by RP-HPLC using Develosil Combi-RP-5 20 × 150 mm. The column was eluted with a linear gradient of 0−5% acetonitrile containing 0.1% FA against 0.1% aqueous FA for 14 min at 10 mL/min. The fraction was lyophilized to give **29** (350 mg, 25%): ^{1}H NMR (D_2O) δ 5.41 (d, 1H, $J_{1',2'} = 4.0$ Hz, H-1′), 5.39 (d, 1H, $J_{1'',2''} = 4.0$ Hz, H-1″), 4.95 (d, 1H, $J_{1,2} = 9.1$ Hz, H-1), 3.99−3.55 (m, 16H, H-3, H-4, H-5, H-6a, H-6b, H-2′, H-3′, H-4′, H-5′, H-6′a, H-6′b, H-2″, H-3″, H-5″, H-6″a, H-6″b), 3.82 (s, 2H, CH_2ICO), 3.41 (br t, 2H, $J_{1,2} = J_{3'',4''} = 9.1$ Hz, H-2, H-4″).

Synthesis of Unglycosylated Analogues (1, 5, 9, 13, and 23). All of these GLP-1 analogues were synthesized by SPPS methodology using Fmoc strategy on an automated peptide synthesizer (AAPPTEC, APEX396) starting with TentaGel S RAM resin (0.24 mmol/g). The protected amino acid derivatives used were Fmoc−Ala−OH, Fmoc−Asn(Trt)−OH, Fmoc−Arg(Pbf)−OH, Fmoc−Asp(OBut)−OH, Fmoc−Cys(Trt)−OPfp, Fmoc−Gly−OH, Fmoc−Gln(Trt)−OH, Fmoc−Glu(OBut)−OH, Fmoc−His(Trt)−OH, Fmoc−Ile−OH,Fmoc−Leu−OH,Fmoc−Lys(Boc)−OH,Fmoc−Met−OH, Fmoc−Phe−OH, Fmoc−Ser(But)−OH, Fmoc−Thr(But)−OH, Fmoc−Trp(Boc)−OH, Fmoc−Tyr(But)−OH, and Fmoc−Val−OH. A cycle of the automated peptide synthesizer was defined as follows. The resin was mixed with 20% (v/v) piperidine in *N*,*N*′-dimethylformamide (DMF), and the reaction mixture was stirred for 5 min at ambient temperature. This process was repeated but with stirring for 15 min. The resin was filtered and washed with *N*-methylpyrrolidone (NMP) and DMF and then added to a solution of 5 equiv of Fmoc amino acid, 5 equiv of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 5 equiv of *N*-hydroxybenzotriazole (HOBt), and 10 equiv of diisopropylethylamine (DIEA) in NMP and DMF. The reaction mixture was then stirred for 60 min at ambient temperature. This coupling process was performed again for 60 min. The reaction mixture was filtered, and the residual resin was washed with NMP and DMF. The resin was subjected to deprotection of the Fmoc group in the same manner as described above. In the case of Fmoc−Cys(Trt)−OPfp, couplings were performed with a solution of 5 equiv of Fmoc−Cys(Trt)−OPfp in DMF and NMP with 5 equiv of HOBt. After completion of the peptide synthesis, the resin was washed with NMP and MeOH, dried in vacuo, and treated with TFA/phenol/thioanisole/water/1,2-ethanedithiol (82.5:5:5:5:2.5) for 2 h at ambient temperature to release the peptide from the resin and for concurrent deprotection. The mixture containing peptide was filtrated, and the resin was washed with TFA. The combined filtrate was precipitated from diethyl ether to give a crude peptide as an amorphous solid and washed with diethyl ester. After being dried, the crude peptide was dissolved in 10% aqueous acetic acid and purified by semipreparative RP-HPLC. The column was eluted with a gradient of 0.1% TFA acetonitrile against 0.1% aqueous TFA. The peptide-containing fraction was collected and then lyophilized to give **1** (3.2 mg, 18% yield), **5** (2.3 mg, 14% yield), **9** (1.4 mg, 9% yield), **13** (1.5 mg, 9% yield), or **23** (33 mg, 14% yield). Purities determined by analytical RP-HPLC and MALDI-TOF MS data of the compounds are given in Table 1.

Synthesis of Analogues Having GlcNAc Moiety (10 and 20). All of these analogues were synthesized as described above, with the exception that GlcNAc−Asn was incorporated using Fmoc−Asn(Ac_3GlcNAcβ)−OH. Coupling was performed with a solution of 3 equiv of Fmoc−Asn(Ac_3GlcNAcβ)−OH with 3 equiv of HBTU, 3 equiv of HOBt, and 6 equiv of DIEA in NMP and DMF. The reaction mixture was then stirred for 24 h at ambient temperature. After completion of the synthesis of the peptide, the resin was washed with NMP and MeOH, dried in vacuo, and treated with TFA/phenol/thioanisole/water/1,2-ethanedithiol (82.5:5:5:5:2.5) for 2 h at ambient temperature to release the peptide from the resin and for concurrent deprotections. The mixture containing the peptide was filtered, and the resin was washed with TFA. The combined filtrate was precipitated from diethyl ether to give crude peptide as an amorphous solid and washed with diethyl ester. The crude peptide was dissolved in methanol, and 1 N NaOH aq was added to adjust the pH to 12.3. The mixture was shaken for 1 h and then neutralized with 1 N AcOH aq. After concentration under a stream of nitrogen, the crude peptide was dissolved in 10% aqueous acetic acid and purified by semipreparative RP-HPLC. The column was eluted with a gradient of 0.1% TFA acetonitrile against 0.1% aqueous TFA. The peptide-containing fraction was collected and then lyophilized to give **10** (73 mg, 29% yield) or **20** (21 mg, 7% yield). Purities determined by analytical RP-HPLC and MALDI-TOF MS data of the compounds are presented in Table 1.

Enzymatic Carbohydrate Elongation by β1,4-Galactosyltransferase. Synthesis of GLP-1 Analogues Having LacNAc Moieties (3, 7, 11, 15, 18, and 21). A solution of GLP-1 analogues having GlcNAc **2**, **6**, **10**, **14**, **17**, or **20** (2 mM) in 25 mM HEPES buffer (pH 7.5) containing 5 mM (**2**, **6**, **10**, or **14**), 6 mM (**17**), or 9 mM (**20**) UDP-galactose, 0.2 U/mL β1,4-galactosyltransferase, 10 mM $MnCl_2$, 0.01% Triton X-100 was incubated at 25 °C. The reaction procedure was monitored by LC-ESI-MS analysis. After the reaction had been completed, the crude peptide was purified by semipreparative RP-HPLC. The column was eluted with a gradient of 0.1% TFA acetonitrile against 0.1% aqueous TFA. The peptide-containing fraction was collected and then lyophilized to give **3** (5.0 mg, quantitative yield), **7** (2.5 mg, 65% yield), **11** (23 mg, 70% yield), **15** (2.9 mg, 79% yield), **18** (12 mg, 71% yield), or **21** (24 mg, 86% yield). Purities determined by analytical RP-HPLC and MALDI-TOF MS data of the compounds are given in Table 1.

Enzymatic Carbohydrate Elongation by α2,6-Sialyltransferase. Synthesis of GLP-1 Analogues Having Sialyl LacNAc Moieties (4, 8, 12, 16, 19, and 22). A solution of GLP-1 analogues having LacNAc **3**, **7**, **11**, **15**, **18**, or **21** (1 mM) in 25 mM HEPES buffer (pH 7.5) containing 5 mM (**3**, **7**, **11**, or **15**), 10 mM (**18**), or 15 mM (**21**) CMP-NANA, 0.05 U/mL (**3**, **7**, **11**, or **15**), 0.1 U/mL (**18**), or 0.15 U/mL (**21**) α2,6-sialyltransferase, 0.01% Triton X-100 was incubated at 37 °C. The reaction procedure was monitored by LC-ESI-MS analysis. After the reaction had been completed, the crude peptide was purified by semipreparative RP-HPLC. The column was eluted with a gradient of acetonitrile against 25 mM ammonium acetate buffer (pH 4.0). The peptide-containing fraction was collected and then lyophilized to give **4** (2.3 mg, 47% yield), **8** (4.5 mg, quantitative yield), **12** (11 mg, 64% yield), **16** (4.5 mg, quantitative yield), **19** (1.7 mg, 65% yield), or **22** (12 mg, 64% yield). Purities determined by analytical RP-HPLC and MALDI-TOF MS data of the compounds are given in Table 1.

Alkylation of Thiol Group in Peptide. Synthesis of GLP-1 Analogues Having Oligomaltose Moieties (24, 25, and 26). A solution of GLP-1 analogues **23** (1 mM) in 100 mM phosphate buffer (pH 8.0) containing 4 mM iodoacetylated glycan **27**, **28**, or **29** was incubated at 37 °C. The reaction procedure was monitored by LC-ESI-MS analysis. After the reaction had been completed, the crude peptide was purified by semipreparative RP-HPLC. The column was eluted with a gradient of 0.1% TFA acetonitrile against 0.1% aqueous TFA. The peptide-containing fraction was collected and then lyophilized to give **24** (1.7 mg, 80% yield), **25** (1.6 mg, 74% yield), or **26** (1.6 mg, 74% yield). Purities determined by analytical RP-HPLC and MALDI-TOF MS data of the compounds are given in Table 1.

Measurement of Binding Affinity and cAMP Production. Binding affinity was assessed by measuring the inhibition of radiolabeled GLP-1 binding to human GLP-1 receptor-expressing chinese hamster ovary (CHO) cell membrane. Cell membrane fractions (5 μg) were incubated with 62 pM [^{125}I]GLP-1 and GLP-1 analogue (final conc. 10^{-11} to 10^{-6} M) in 25 mM HEPES (pH 7.4) containing 5 mM MgCl, 1 mM $CaCl_2$, 0.25 mg/mL bacitracin, and

0.1% bovine serum albumin (BSA) at room temperature for 2 h (100 μL). Membranes were filtered onto a 96-well GF/C plate (PerkinElmer, Inc.) that had been presoaked in 1% polyethylenimine containing 0.5% BSA, and then washed with 25 mM HEPES buffer containing 0.5% BSA (pH 7.4). Radioactivity associated with the lysates was determined using a gamma counter. Nonspecific binding was determined by the amount of binding in the presence of 1 μM unlabeled GLP-1. Dose−response curves were plotted for the individual compounds. IC_{50} values were calculated using XLfit software (IDBS Inc.).

For measurement of cAMP production, human GLP-1 receptor-expressing CHO cells were passaged into multiwell plates (4000 cells/well) and cultured for an additional 48 h. The cells were washed with assay buffer (Hanks balanced salt solution containing 20 mM HEPES, 0.1% BSA, pH 7.4) and then exposed to GLP-1 analogues (final conc. 10^{-12} to 10^{-6} M) in assay buffer containing 0.33 mM isobutylmethylxanthine and 0.67 mM RO20−1724 at room temperature for 1 h. The cells were lysed with 1% Triton X-100, and the cAMP formed was measured using a cAMP femtomolar kit (Cis Bio international). Dose−response curves were plotted for the individual compounds. EC_{50} values were calculated using XLfit software.

Characterization of Stability against Recombinant Human DPP-IV. GLP-1 or GLP-1 analogue (20−500 μM) was incubated at 37 °C in 100 mM HEPES buffer containing 0.05% Tween80 and 1 mM EDTA−2Na (pH 7.5) with 0.33 μg/mL (**1**−**16**, **24**−**26**, or GLP-1), 0.66 μg/mL (**19**), or 1.32 μg/mL (**22**) recombinant human DPP-IV (60 μL). At 5 or 10 min intervals, 7 μL was removed from the reaction mixture, and the reaction was terminated by the addition of 28 μL of 8 M GuHCl solution. The reaction products were subjected to RP-HPLC on a Develosil RPAQUEOUS-AR-3 2.0 × 100 mm at 30 °C, and the C-terminal degradation product was quantified by using UV absorption at 210 nm. The initial rate of the degradation reaction was determined from the slope of the linear part obtained by plotting product concentration versus time. The resulting initial rates were plotted versus peptide concentration, and kinetic parameters (K_M and K_M/k_{cat}) were determined using XLfit software based on the Michaelis−Menten kinetic equation.

Characterization of Stability against Recombinant Human NEP 24.11. The 125 μM GLP-1 or GLP-1 analogue (**10**−**12**, **19**, and **22**) was incubated at 37 °C in 50 mM HEPES buffer containing 50 mM NaCl, and 0.05% Tween 80 with 4 μg/mL recombinant human NEP 24.11 (pH 7.4, 84 μL). After 0.5, 1, 2, 3.5, and 5 h, 8 μL was removed from the reaction mixture, and the reaction was terminated by addition of 32 μL of 8 M GuHCl solution. The reaction products were subjected to RP-HPLC on a Develosil ODS-HG-5 4.6 × 150 mm at 30 °C, and the area of intact GLP-1 analogue was measured using UV absorption at 210 nm.

Blood Glucose-Lowering Activity in Obese Diabetic db/db Mice. Male BKS.Cg-+ $Lepr^{db}$/+ $Lepr^{db}$ mice (13−15 weeks of age) were allowed ad libitum access to food and water until the start of the experiment. At $t = -2$ h, access to food was restricted, and the tip of the tail was cut. At $t = 0$ min, a 1 μL blood sample was collected. Immediately thereafter, each mouse was injected subcutaneously with test sample (100 nmol/kg) or vehicle, and additional blood samples were collected. The vehicle was saline containing 1% BSA. Blood glucose levels were measured with a glucose oxidase biosensor (DIAMETERα; Arkray, Inc.). The effects of the test samples on blood glucose were expressed as % change relative to the respective pretreatment ($t = 0$ min) level. The number of mice tested was 6−7 for each group. Data are presented as means ± SEM. Statistical differences were analyzed using the Dunnett's multiple comparison test, and P values less than 0.05 were regarded as significant.

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Supporting Information Available: RP-HPLC profiles and MALDI-TOF MS spectra for compounds **1**−**26**, ^{1}H NMR spectra for compounds **27**−**29**, the tabulated values of k_{cat}, K_M, and k_{cat}/K_M for the data shown in Figure 2, and complete ref 39. This material is available free of charge via the Internet at http://pubs.acs.org.

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