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Development of Potent Glucagon-like Peptide-1 Agonists with High Enzyme Stability via Introduction of Multiple Lactam Bridges

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Glucagon-like peptide-1 (GLP-1) has the ability to lower the blood glucose level, and its regulatory functions make it an attractive therapeutic agent for the treatment of type 2 diabetes. However, its rapid degradation by enzymes like dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase (NEP) 24.11 severely compromises its effective clinical use. Whereas specific DPP-IV inhibitors have been developed, NEP 24.11 targets multiple sites in the GLP-1 sequence, which makes it difficult to block. To address this drawback, we have designed and synthesized conformationally constrained GLP-1 analogues by introducing multiple lactam bridges that stabilized both α-helices in the N- and C-terminal regions simultaneously. In addition to improving the receptor activation capability (up to 5-fold) by fixing the α-helical conformations required for optimal receptor interaction, the introduced lactam bridges provided outstanding shielding over NEP 24.11 (half-life of >96 h). These highly constrained peptides are the first examples of NEP 24.11-resistant GLP-1 analogues.

Introduction

Glucagon-like peptide-1 (GLP-1)[a] is an incretin released from intestinal L-cells in response to nutrient ingestion[1] and endogenously exists as two equipotent forms, GLP-1(7−36)-NH_2 and GLP-1(7−37), that are produced by post-translational processing of preproglucagon.[2,3] It interacts with GLP-1 receptors in many organs, including pancreatic β-cells,[4] and exerts a variety of biological responses, such as glucose-dependent insulin secretion,[5,6] promotion of insulin gene transcription,[7−9] stimulation of β-cell proliferation and neogenesis, inhibition of β-cell apoptosis,[10,11] and suppression of glucagon secretion.[12] It also inhibits gastric emptying and induces satiety, leading to body weight decrease.[13−15] These unique physiological functions have led to GLP-1 receiving significant attention as a promising therapeutic agent for treating diabetes, especially type 2.

However, GLP-1 is found to have a very short circulation in vivo (a plasma half-life of approximately 2 min) resulting from its high susceptibility to enzymatic degradation,[6,16,17] and this rapid proteolysis significantly compromises its effective clinical applications.[18] Two enzymes mainly responsible for the cleavage of GLP-1 are dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase (NEP) 24.11.[19,20] DPP-IV is a ubiquitous serine protease and cleaves substrates with Pro or Ala at the penultimate N-terminal position.[21] It cleaves a peptide bond between Ala^8 and Glu^9 of GLP-1, and the resulting metabolite, GLP-1(9−36)-NH_2, is found to lose binding affinity and the ability to activate the GLP-1 receptor nearly completely.[22,23] To prolong the activity of the endogenous GLP-1, DPP-IV inhibitors like sitagliptin have been developed as therapeutic agents for the treatment of type 2 diabetes.[24,25] By blocking GLP-1 degradation, DPP-IV inhibitors have shown the potential to decrease the risk of hypoglycemia and restore pancreatic β-cells.[25] However, DPP-IV inhibitors have been found to interact with DPP8 and DPP9 which are closely related to DPP-IV.[26,27] The inhibition of these two DPP enzymes has been linked to several side effects, including alopecia, thrombocytopenia, splenomegaly, reticulopenia, and gastrointestinal toxicity in animal models.[26,28] Whereas a DPP-IV specific inhibitor may be difficult to create, highly selective DPP-IV inhibitors like sitagliptin have shown significant benefits in the management of diabetes.[24,25,29]

On the other hand, NEP 24.11 is a membrane-bound zinc metallopeptidase and cleaves substrates at a peptide bond immediately N-terminal to a hydrophobic residue.[30] It cleaves GLP-1 at multiple sites, in particular Asp^{15}−Val^{16}, Ser^{18}−Tyr^{19}, Tyr^{19}−Leu^{20}, Glu^{27}−Phe^{28}, Phe^{28}−Ile^{29}, and Trp^{31}−Leu^{32} bonds.[20] Although the significance of GLP-1 degradation by NEP 24.11 was evidenced in a recent study with a NEP inhibitor candoxatril,[31] much less research effort has been spent on blocking NEP 24.11 compared to DPP-IV.[32,33] The multiple cleavage sites widely dispersed in the GLP-1 sequence make the inhibition of NEP 24.11 difficult to achieve by simple amino acid substitutions, whereas this strategy was effective in blocking DPP-IV.[34,35] Long-acting GLP-1 agonists should ideally have significant stability against both enzymes, DPP-IV and NEP 24.11.

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[a] Abbreviations: GLP-1, glucagon-like peptide-1; DPP-IV, dipeptidyl peptidase-IV; NEP 24.11, neutral endopeptidase 24.11; CD, circular dichroism; ACN, acetonitrile; DIEA, *N*,*N*-diisopropylethylamine; DMBA, *N*,*N*′-dimethylbarbituric acid; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; IBMX, 3-isobutyl-1-methylxanthine; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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Among stable GLP-1 analogues developed, liraglutide is a FDA-approved GLP-1 agonist that has an Arg34Lys mutation and a C16 acyl chain via a glutamoyl spacer added to Lys^{26}.[36−38] This GLP-1 analogue showed a maximum concentration after 10−14 h and an increased plasma half-life between 10 and 14 h.[37] On the other hand, taspoglutide is another GLP-1 analogue currently being investigated in clinical trials.[39,40] It has substitutions of Ala^8 and Gly^{35} with α-aminoisobutyric acids that greatly improved the half-life to 9.8 h.[41]

Structure−activity relationship studies of GLP-1 have found that the N-terminal region of the peptide is critical for receptor activation whereas receptor binding is more strongly contributed by the C-terminal region.[34,42,43] A number of amino acid residues that are important for receptor binding or activation, such as His^7, Gly^{10}, Phe^{12}, Thr^{13}, Asp^{15}, Phe^{28}, and Ile^{29}, were identified by alanine scanning studies.[44,45] Its solution structure was determined by two-dimensional (2D) NMR in the presence of a dodecylphosphocholine micelle[46−49] which was used to provide a membrane-like environment.[50,51] In solution, GLP-1 is found to have two α-helical segments between residues 13−20 and 24−35, separated by a short linker region comprising residues 21−23.[50] Although the micelle used in the 2D NMR studies offers a water/lipid interface, specific interactions between amino acid residues in the peptide and the receptor cannot be properly mimicked by the simple structure of the micelle.

To investigate the significance of the two α-helices found in the solution structures of GLP-1 on receptor interaction, we have conducted a cyclization scanning study.[52,53] A lactam bridge was introduced between Lys^i and Glu^{i+4} to fix an α-helical structure as this conformational restriction has been widely employed to form α-helices in many peptides.[52−55] To survey the presence and locations of α-helices in the receptor-bound conformation, we introduced a lactam bridge at various positions in the GLP-1 sequence (from the N- to C-terminus) and examined the interaction of the resulting cyclic peptide with the GLP-1 receptor. If an α-helix formed by a lactam bridge matches one in the receptor-bound conformation, the cyclic peptide would exhibit high binding affinity and efficacy because it can be easily recognized by the receptor. On the other hand, a mismatched α-helix would result in weak receptor interaction. Whereas the N-terminal α-helical segment was observed to extend to Thr^{11}, the receptor-bound conformation suggested by this cyclization scanning study appears to be similar to the solution structures determined by 2D NMR.[50,51] It was found that two α-helices in the N- and C-terminal regions (between residues 11−21 and 23−34) are connected by a kink region containing Gly^{22} that plays a pivotal role in the arrangement of the two α-helical segments. The recently elucidated crystal structure of GLP-1 in complex with a truncated N-terminal domain of the receptor confirms the significance of the helical structures in receptor interaction.[56] Via stabilization of the α-helical segments with a lactam bridge, several cyclic GLP-1 analogues acquired enhanced potency in receptor activation.[53]

On the basis of these findings and a hypothesis that strategically placed lactam bridges would deter enzyme degradation, we have designed and synthesized a series of GLP-1 analogues containing two lactam bridges between either Lys^i and Glu^{i+4} or between Glu^i and Lys^{i+4}. Two lactam bridges in the bicyclic GLP-1 analogues were introduced to stabilize both α-helices in the N- and C-terminal regions simultaneously and in turn to promote stronger receptor interaction that would result in higher efficacy. On the other hand, these lactam bridges were also thought to protect the cyclic peptides from proteases. Whereas the cleavage of GLP-1 by DPP-IV can be easily inhibited via substitution of Ala^8 with D-Ala or Aib,[34,35,43] the degradation by NEP 24.11 is difficult to prevent via a similar approach because NEP 24.11 targets multiple sites in the GLP-1 sequence (vide supra). Thus, multiple lactam bridges placed in both the N- and C-terminal regions of GLP-1 would provide significant protection from NEP 24.11 and result in potent and long-acting GLP-1 agonists. In particular, highly stable GLP-1 analogues with stronger receptor binding affinity would be of great value for the development of effective molecular probes for imaging pancreatic β-cells. Although the GLP-1 receptor is one of the β-cell specific biomarkers, it has not been successfully used for β-cell imaging because GLP-1 analogues are quickly degraded in vivo. In addition, these conformational restrictions introduced into the N- and C-terminal regions of GLP-1 would help to elucidate the structure of the N-terminal region that is found to be critical for receptor activation. The N-terminal structure of GLP-1 would allow the long-pursued rational design of nonpeptidic GLP-1 mimetics; however, all of the previously reported 2D NMR studies of GLP-1 could not clearly determine the N-terminal region due to the high degree of motion as a linear peptide.[50,51]

Results and Discussion

Chemical Synthesis and CD Spectroscopy. All of the GLP-1 analogues in this study (Table 1) were synthesized by following standard solid-phase peptide synthesis protocol with *N*-Fmoc/^{t}Bu chemistry. Rink amide resin was used as a polymer support to yield peptides with C-terminal carboxamides. While all *N*-Fmoc-amino acids used for the synthesis had acid-labile protecting groups for their side chains (e.g., ^{t}Bu, Boc, Trt, and Pbf), the side chain functional groups of Glu and Lys that would form lactam bridges were orthogonally protected with allyl protecting groups.[57,58] The allyl groups were selectively removed with $Pd(PPh_3)_4$ and an allyl scavenger like *N*,*N*′-dimethylbarbituric acid (DMBA) or $PhSiH_3$, and then a lactam bridge was formed with a coupling reagent like PyBOP while the peptide chain was still bound on the solid support. As illustrated in Figure 1, GLP-1 analogues containing more than two lactam bridges were synthesized by repeating the steps of growing a partial sequence on the solid support, selectively removing the allyl protecting groups, and forming a lactam bridge. This synthetic strategy allowed us to construct the multicycle GLP-1 analogues with nonoverlapping lactam bridges listed in Table 1. The completed cyclic peptides were cleaved from the resin and fully deprotected with TFA. All peptides were characterized by analytical HPLC and ESI-MS after being purified via semipreparative HPLC.

We studied the cyclic GLP-1 analogues by circular dichroism (CD) spectroscopy to examine the stability of α-helical conformations with lactam bridges. CD spectra of a peptide (20 μM) in aqueous TFE (0, 10, 20, and 50%) were recorded by scanning between 190 and 260 nm in wavelength.[53] Compared to GLP-1, all of the cyclic GLP-1 analogues were found to be more helical, indicating that lactam bridges indeed induced and stabilized α-helical conformations (Figure 2).

Receptor Activation Assays. The cyclic GLP-1 analogues were examined for their ability to interact with the GLP-1

Table 1. Biological Activity and Enzyme Stability of the GLP-1 Analogues Containing Multiple Lactam Bridges

Peptide	Sequence		EC_{50} (nM) (pEC_{50}±SEM)[a]	Relative Potency (%)	DPP-IV ($t_{1/2}$±SEM)[a] (h)	NEP 24.11 ($t_{1/2}$±SEM)[a] (h)
	GLP-1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR	4.6 (8.3±0.08)	100	2.0±0.2	4.6±0.7
1	c[E^{16}, K^{20}]GLP-1(7-36)-NH_2	---------E---K----------	3.8 (8.4±0.06)	120	1.8±0.3	3.2±0.4
2	c[E^{18}, K^{22}]GLP-1(7-36)-NH_2	----------E---K----------	0.6 (9.2±0.11)	770	2.1±0.4	10±3.5
3	c[E^{22}, K^{26}]GLP-1(7-36)-NH_2	----------E---K----------	2.8 (8.3±0.03)	160	2.9±0.8	6.9±0.5
4	c[E^{30}, K^{34}]GLP-1(7-36)-NH_2	----------E---K--	5.3 (8.6±0.13)	90	3.3±0.4	6.9±0.1
5	c[E^{16}, K^{20}]-c[E^{30}, K^{34}]GLP-1(7-36)-NH_2	---------E---K---------E---K--	3.3 (8.5±0.04)	140	3.1±0.1	6.6±1.9
6	c[K^{16}, E^{20}]-c[K^{30}, E^{34}]GLP-1(7-36)-NH_2	---------K---E---------K---E--	7.0 (8.2±0.11)	70	2.9±0.1	17±5.0
7	c[E^{18}, K^{22}]-c[E^{30}, K^{34}]GLP-1(7-36)-NH_2	----------E---K-------E---K--	1.9 (8.7±0.14)	240	3.6±0.2	> 96
8	c[K^{18}, E^{22}]-c[K^{30}, E^{34}]GLP-1(7-36)-NH_2	----------K---E-------K---E--	1.0 (9.0±0.09)	460	4.1±0.2	18±2.2
9	c[E^{22}, K^{26}]-c[E^{30}, K^{34}]GLP-1(7-36)-NH_2	----------E---K---E---K--	1.6 (8.8±0.11)	290	6.0±1.8	3.5±0.9
10	c[K^{22}, E^{26}]-c[K^{30}, E^{34}]GLP-1(7-36)-NH_2	----------K---E---K---E--	2.2 (8.7±0.14)	210	7.3±0.4	1.2±0.3
11	c[E^{16}, K^{20}]-c[E^{22}, K^{26}]-c[E^{30}, K^{34}]GLP-1 (7-36)-NH_2	---------E---K-E---K---E---K--	1.8 (8.7±0.09)	260	> 24	> 96

[a] All experiments were performed in triplicate and repeated at least three times ($n \geq 3$).

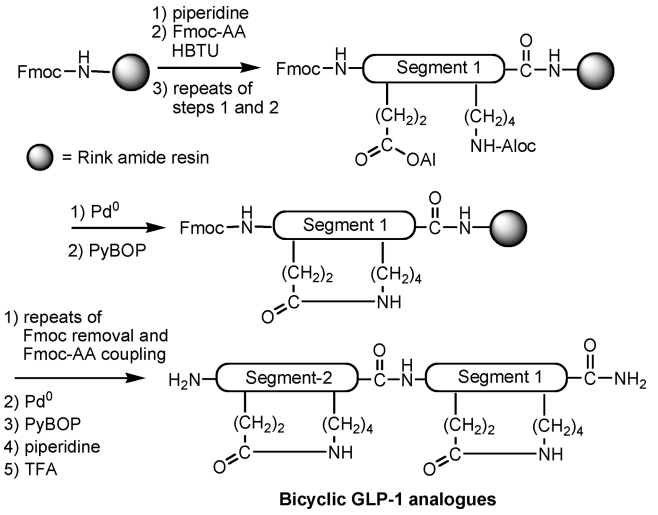

Figure 1. Solid-phase synthesis of GLP-1 analogues containing multiple lactam bridges.

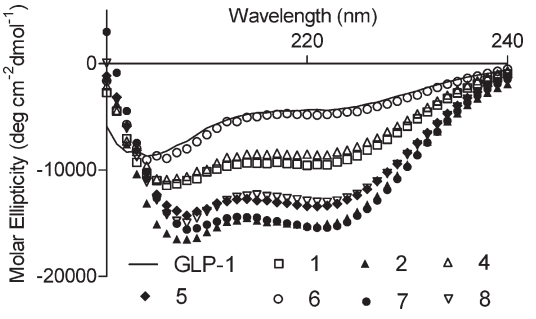

Figure 2. CD spectra of the cyclic GLP-1 analogues.

receptor to evaluate the effect of the lactam bridges on fixing the helical structures required for receptor recognition. Their potency in receptor activation was determined by using stably transfected HEK293 cells overexpressing human GLP-1 receptors.[59] Cyclic AMP accumulation by the GLP-1 analogues was measured in subconfluent cultures of the HEK293 cells in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), as previously reported.[59] For dose−response experiments, the HEK293 cells were treated with a peptide at various concentrations for 20 min at 37 °C. After the reaction had been quenched with trichloroacetic acid, the produced cAMP was isolated by a two-column chromatographic method (Figure 3).[60]

As summarized in Table 1, all of the GLP-1 analogues containing one lactam bridge between Glu^{i} and Lys^{i+4} (**1**−**4**) showed high efficacy in receptor activation presumably

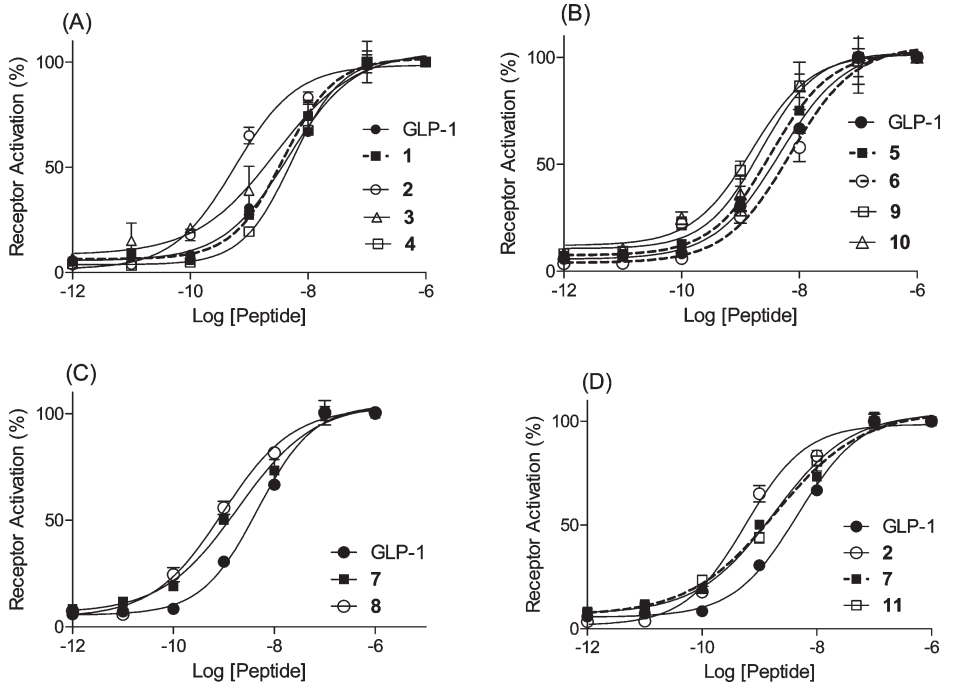

Figure 3. Concentration response of the cyclic GLP-1 analogues in receptor activation.

resulting from the stabilized helical segments by the introduced lactam bridges. In particular, peptide **2** with a lactam bridge between Glu^{18} and Lys^{22} was found to be remarkably potent (7-fold increase), indicating the significance of the N-terminal helix in receptor activation. The enhanced and extended N-terminal helix by the lactam bridge between Glu^{18} and Lys^{22} was also evidenced in the CD spectrum of peptide **2** (Figure 2). In addition, this suggests that Ser^{18} and Gly^{22} do not directly make contact with the receptor. Similarly, the lactam bridges in peptides **1**, **3**, and **4** were found to stabilize helical segments in the N- or C-terminal region, resulting in comparable potency in receptor activation.

To achieve stronger interaction with the receptor, two lactam bridges were simultaneously installed in the N- and C-terminal regions, creating a series of bicyclic GLP-1 analogues (**5**−**10**). For synthetic convenience (vide supra), only nonoverlapping lactam bridges were combined together to make bicyclic peptides. As expected, the simultaneous introduction of two lactam bridges led to a comparable or modest increase in potency resulting from the stabilization of the receptor-bound conformation comprising two helical segments. For instance, the combination of the lactam bridges in monocyclic peptides **1** and **4** (EC_{50} = 3.8 and 5.8 nM, respectively) showed a potency similar to that of the resulting bicyclic peptide **5** (EC_{50} = 3.3 nM). The combination of the lactam bridges in monocyclic peptides **3** and **4** (EC_{50} = 2.8 and 5.3 nM, respectively) also led to bicyclic peptide **9** with a marginally increased potency (EC_{50} = 1.6 nM). However, this synergistic increase was not observed for bicyclic peptide **7** (EC_{50} = 1.9 nM) that was made of the lactam bridges from monocyclic peptides **2** and **4** (EC_{50} = 0.6 and 5.3 nM, respectively).

To investigate the orientation of the lactam bridges upon helix stabilization, we have synthesized bicyclic GLP-1 analogues with lactam bridges between Glu^{i} and Lys^{i+4} (**5**, **7**, and **9**) or between Lys^{i} and Glu^{i+4} (**6**, **8**, and **10**). However, the orientation of the lactam bridges does not appear to significantly affect the efficacy, although a subtle preference was observed. As a previous study by Houston and co-workers suggested that lactam bridges oriented from Glu^{i} to Lys^{i+4} tend to be more helix-stabilizing than those oriented from Lys^{i} to Glu^{i+4} by having the amide bonds of the lactam bridges aligned with the helix dipole created by the amide bonds of helical peptide backbones,[61] the CD spectra of peptides **5** and **7** exhibited higher helix contents compared to those of peptides **6** and **8**, respectively, in agreement with this speculation (Figure 2). However, bicyclic peptides **5**, **7**, and **9** that have two lactam bridges between Glu^{i} and Lys^{i+4} were found to be comparably effective compared to bicyclic peptides **6**, **8**, and **10** containing two lactam bridges between Lys^{i} and Glu^{i+4}, respectively.

Nevertheless, all of the bicyclic GLP-1 analogues except peptide **6** were found to have their helical structures significantly stabilized by introduction of two lactam bridges as shown in the CD spectra (Figure 2). They were also found to interact with the receptor more strongly than GLP-1 (1.4−4.6-fold increase). On the basis of these exciting results, we have designed a GLP-1 analogue with three lactam bridges between residues 16 and 20, 22 and 26, and 30 and 34. With the three lactam bridges fixing the required helical structures, tricyclic peptide **11** yielded an efficacy comparable to that of GLP-1.

DPP-IV Stability. In addition to the improved receptor interaction, the introduced lactam bridges were thought to increase enzyme stability by hindering the enzymes in recognizing the cyclic peptides as substrates. Because DPP-IV is one of the main enzymes that degrade GLP-1, all of the cyclic GLP-1 analogues were evaluated in terms of their stability to DPP-IV. A GLP-1 analogue (100 μM) was incubated with recombinant DPP-IV (0.2 ng/mL) over 24 h,[19] and aliquots collected during the incubation were analyzed by HPLC to assess the DPP-IV degradation. Because all of the cyclic GLP-1 analogues possess the native N-terminal sequence (i.e., Ala^{8}-Glu^{9}), no substantial stability to DPP-IV was expected. However, it was interesting that the cyclic peptides with a lactam bridge placed in the C-terminal region (**3** and **4**) were found to be slightly more resistant to DPP-IV (1.5-fold increase) than those with N-terminal cyclization (**1** and **2**),

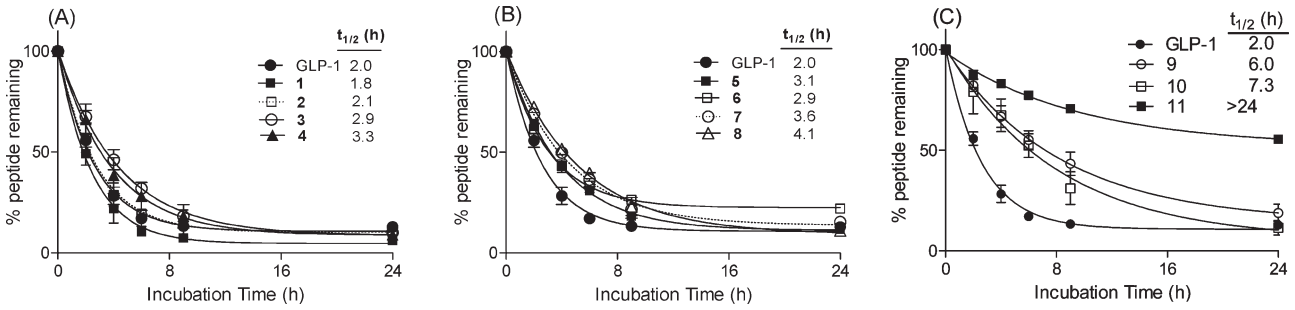

Figure 4. Degradation of the cyclic GLP-1 analogues by DPP-IV.

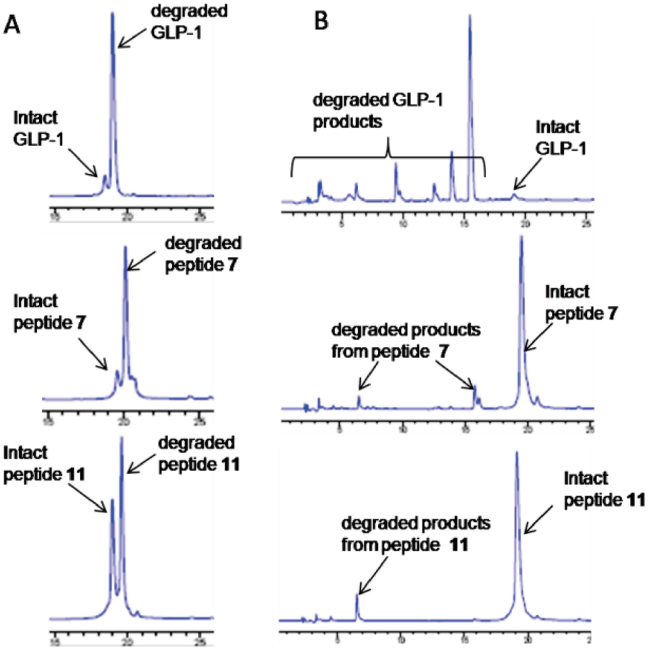

Figure 5. HPLC chromatograms of GLP-1 analogues after a 24 h incubation with (A) DPP-IV or (B) NEP 24.11.

although the difference is not significant (Figure 4A). The DPP-IV stability by the C-terminal cyclization became more evident (more than 3-fold increase) when two lactam bridges were simultaneously placed in the C-terminal region, which is inconsistent with our initial expectation that the N-terminal cyclization would result in steric hindrance on the enzyme's recognition of the Ala^8–Glu^9 bond at the N-terminus (Figure 4B). Remarkably, tricyclic peptide **11** was found to be exceptionally stable, exhibiting a half-life of much longer than 24 h, although no direct modification was made on the enzyme recognition site (Figures 4C and 5A).

NEP 24.11 Stability. Whereas the degradation of GLP-1 by DPP-IV can be blocked by DPP-IV inhibitors[24,25] or replacement of Ala^8 with D-Ala or Aib,[34,35,43] the cleavage of the peptide by NEP 24.11 has been studied less. However, it has been suggested that up to 50% of GLP-1 degradation may be contributed by NEP 24.11.[62] Because NEP 24.11 targets multiple sites in the sequence of GLP-1 resulting in all inactive fragments,[20] this would not be easily achieved by simple amino acid substitutions that were found to be effective with DPP-IV. However, we have hypothesized that the lactam bridges placed in the sequence of GLP-1 would provide significant protection against NEP 24.11 in addition to the increase in potency. To examine NEP 24.11 stability, we incubated all of the GLP-1 analogues (100 μM) with recombinant NEP 24.11 (1.0 μg/mL) over 24–96 h, and aliquots collected during the incubation were analyzed by HPLC.

Monocyclic GLP-1 analogues (**2**–**4**) exhibited moderately longer half-lives than GLP-1 (approximately 2-fold increase), and this small enhancement of NEP 24.11 stability can be attributed to the protection of a specific region where a lactam bridge was introduced (Figure 6A). Because one lactam bridge covers only a small area of the sequence, the monocyclic GLP-1 analogues cannot obtain significant protection from NEP 24.11. However, introduction of two lactam bridges was found to be quite effective in blocking enzyme degradation. Two lactam bridges located in the N- and C-terminal regions in bicyclic peptides **5**–**8** shielded a larger area of the sequence over NEP 24.11, yielding a high stability [$t_{1/2} > 17$ h (Figure 6B,C)]. In particular, bicyclic peptide **7** that has two lactam bridges between residues 18 and 22 and residues 30 and 34 was found to be extremely resistant to the enzyme. and only a small extent of the degradation was observed even after a 96 h incubation (Figures 5B and 6C).

Interestingly, the orientation of the lactam bridges in the bicyclic GLP-1 analogues appears to influence NEP 24.11 stability. For instance, bicyclic peptide **6** that has two lactam bridges between Lys^i and Glu^{i+4} was found to be more resistant to the degradation (approximately 3-fold increase) compared to bicyclic peptide **5** in which two lactam bridges were placed at the same positions but consisting of Glu^i and Lys^{i+4}. In contrast, bicyclic peptide **7** that has two lactam bridges between Glu^i and Lys^{i+4} exhibited a superior stability over NEP 24.11 compared to bicyclic peptide **8** with two lactam bridges between Lys^i and Glu^{i+4} at the same positions. Compared to the other bicyclic peptides, bicyclic peptides **9** and **10** were found to be degraded as rapidly as GLP-1 because both lactam bridges were placed only in the C-terminal region, leaving the N-terminal segment unshielded.

Remarkably, tricyclic GLP-1 analogue **11** that has three lactam bridges between residues 16 and 20, 22 and 26, and 30 and 34 was found to be extremely stable versus NEP 24.11. The three lactam bridges cover more than half of the length of the peptide and allowed the tricyclic peptide to remain almost completely intact even after a 96 h incubation (Figures 5B and 6C). In addition to the exceptionally high NEP 24.11 stability, tricyclic peptide **11** also has outstanding stability versus DPP-IV. This is the most highly constrained GLP-1 analogue with the highest enzyme stability and potency reported to date.

This exceptionally high enzyme stability of the bicyclic and tricyclic GLP-1 analogues is important for the achievement of long-acting in vivo activity. Together with the stronger

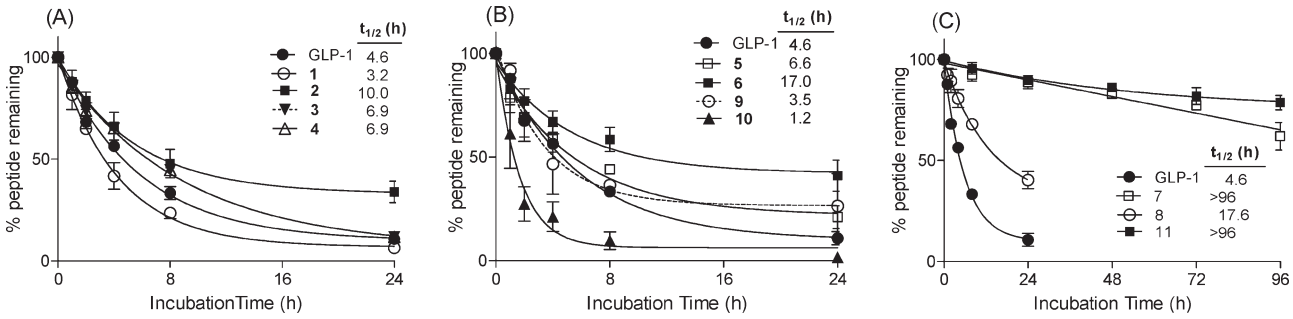

Figure 6. Degradation of the cyclic GLP-1 analogues by NEP 24.11.

binding affinity, this remarkable stability can lead to the development of effective molecular probes for imaging pancreatic β-cells. As the β-cell loss has a correlation with the progression of the disease, it is important to determine the functional β-cell mass for early diagnosis of diabetes and evaluation of diabetic therapies, including islet transplantation. Toward these ends, these highly stable GLP-1 analogues with improved potency would be of great value and are currently being investigated.

Experimental Section

Materials and General Procedures. N^{α}-Fmoc-protected amino acids, aminomethylated polystyrene resin, and Rink amide linker were purchased from EMD Chemicals (Gibbstown, NJ) and Senn Chemicals (Dielsdorf, Switzerland). All amino acids used were of the L-configuration unless otherwise stated. Other chemicals and solvents were purchased from the following sources: *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, and acetonitrile (ACN) from Fischer Scientific (Pittsburgh, PA); *N*,*N*-diisopropylethylamine (DIEA), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBt) from Advance ChemTech (Louisville, KY); benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and trifluoroacetic acid (TFA) from Oakwood Products (West Columbia, SC); piperidine, anisole, acetic anhydride, ninhydrin, methyl sulfide, 1,2-ethanedithiol, tetrakis(triphenylphosphine)palladium, triisopropylsilane, *N*,*N*′-dimethylbarbituric acid (DMBA), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) from Aldrich (Milwaukee, WI); and recombinant human dipeptidyl peptidase-IV and recombinant human neprilysin (neutral endopeptidase 24.11, EC 3.4.24.11) from R&D Systems (Minneapolis, MN). The solvents for peptide synthesis and purification were used without purification. The purity of each peptide was checked by an HPLC system (1100 series, Agilent Technologies, Foster City, CA) equipped with a diode array UV detector using two reverse-phase analytical HPLC columns: (A) a C_{18}-bonded silica column (Vydac 218TP104, 4.6 mm × 250 mm, 10 μm) and (B) a phenyl-bonded silica column (Zorbax SB-Phenyl, 4.6 mm × 250 mm, 5 μm). The molecular masses of the peptides were confirmed by ESI-MS (LCQ Deca XP Plus ESI-ion trap mass spectrometer, Thermo Electron Corp., West Palm Beach, FL).

General Procedure for Peptide Synthesis. All of the GLP-1 analogues were synthesized manually using standard *N*-Fmoc/^{t}Bu solid-phase peptide synthesis protocol. Aminomethylated polystyrene resin (0.25 mmol, 0.4 mmol/g) was swollen in DMF for 10 min and washed with DMF (3 × 1 min). Fmoc-Rink amide linker (203 mg, 1.5 equiv), HBTU (379 mg, 4 equiv), HOBt (135 mg, 4 equiv), and DIEA (0.35 mL, 8 equiv) were dissolved in DMF (3 mL). Then, the solution was added to the resin and shaken for 2 h. The coupling reaction was followed by Kaiser ninhydrin and TNBS tests,[63,64] and unreacted amines were capped by using acetic anhydride (0.5 mL, 20 equiv) in DMF (3 mL) for 30 min. The Fmoc protecting group of the Rink amide linker was removed via treatment with piperidine (20% in DMF, 1 × 5 min and 1 × 30 min) and washed with DMF (3 × 1 min). The first amino acid was introduced by using a preactivated Fmoc amino acid that was prepared by mixing a Fmoc amino acid (4 equiv), HBTU (4 equiv), HOBt (4 equiv), and DIEA (8 equiv) in DMF (3 mL) for 30 min. The coupling reaction was conducted for 2−4 h or until Kaiser ninhydrin and TNBS tests became negative. When a coupling reaction was found to be incomplete, the resin was washed with DMF (3 × 1 min) and the amino acid was coupled again with a freshly prepared preactivated Fmoc amino acid. When the second coupling reaction did not result in negative Kaiser ninhydrin and TNBS tests, the resin was washed with DMF (3 × 1 min) and the unreacted amines were capped by being treated with acetic anhydride (20 equiv) in DMF for 5−10 min. These steps (removal of a Fmoc group and coupling of a Fmoc amino acid) were repeated until all amino acids in the sequence of a peptide were coupled. Then, the resin was washed with DCM (5 × 1 min) and dried under vacuum. A growing peptide was frequently characterized via cleavage of a small amount of resin and analysis of the released peptide via RP-HPLC and ESI-MS.

General Procedure for the Formation of Lactam Bridges. For the selective removal of allyl protecting groups of Lys and Glu, a fully protected peptide still bound on resin (0.25 mmol) was placed in a 12 mL polypropylene reaction vessel. The reaction vessel was then sealed with a rubber septum and flushed with nitrogen for 10 min. To the vessel were added via a syringe $Pd(PPh_3)_4$ (30 mg, 0.1 equiv) and DMBA (390 mg, 10 equiv) dissolved in a degassed DCM/DMF mixture (4 mL, 3:1), and the reaction mixture was kept under nitrogen for 30 min with occasional shaking. The resin was washed with a LiCl/DMF mixture (0.8 M, 3 × 1 min), and the reaction was repeated again. Then, the resin was treated with a PyBOP (6 equiv)/HOBt (6 equiv)/DIEA (12 equiv) or BOP (6 equiv)/HOBt (6 equiv)/DIEA (12 equiv) mixture dissolved in a DCM/DMF/NMP mixture (1:1:1, 1% Triton X-100) for 6−8 h until the reaction was complete. The resin was washed with DMF (3 × 1 min).

General Procedure for the Synthesis of Bicyclic GLP-1 Analogues. For the synthesis of a GLP-1 analogue containing two lactam bridges, a partially completed GLP-1 analogue (residues 30−36) was first constructed on Rink amide resin by following the synthetic procedures described above. After the selective removal of the allyl protecting groups on Lys(Aloc) and Glu(OAl), the C-terminal lactam bridge was created with PyBOP. Then, the peptide was grown up to the position of the N-terminal lactam bridge, and another selective removal of the allyl groups on Lys(Aloc) and Glu(OAl) was conducted followed by the formation of the N-terminal lactam bridge. Then, the remaining amino acids were coupled to complete the synthesis of a bicyclic GLP-1 analogue.

General Procedure for Cleavage and Final Deprotection of Peptides. A cleavage mixture of trifluoroacetic acid (TFA), dimethyl sulfide, 1,2-ethanedithiol, and anisole (20 mL, 36:1:1:2) was added to a peptide on dried resin (0.25 mmol) in a disposable 50 mL polypropylene tube, and the mixture was stirred for 90 min

Table 2. Characterization of the GLP-1 Analogues Containing Multiple Lactam Bridges[a]

peptide	retention time			molecular mass	
	A	B	C	calculated	found
1	21.2	11.9	11.8	3324.68	3324.60
2	18.4	13.4	13.4	3392.84	3392.80
3	19.1	14.1	14.0	3351.75	3352.00
4	19.0	13.9	13.6	3337.72	3337.50
5	17.8	12.7	12.6	3364.70	3363.94
6	17.6	12.6	12.5	3364.70	3364.80
7	19.2	14.2	14.2	3432.86	3432.80
8	19.6	14.6	14.3	3432.86	3432.80
9	20.0	15.1	14.9	3391.80	3391.70
10	20.1	15.5	15.2	3391.80	3391.70
11	18.7	13.7	13.9	3418.75	3418.71

[a] HPLC conditions: (A) 10 to 90% ACN in aqueous trifluoroacetic acid (0.1%) over 40 min with a flow rate of 1.0 mL/min (C_{18}-bonded column, Vydac 218TP104, 4.6 mm × 250 mm, 10 μm), (B) 20 to 60% ACN in aqueous trifluoroacetic acid (0.1%) over 20 min with a flow rate of 1.0 mL/min (C_{18}-bonded column, Vydac 218TP104, 4.6 mm × 250 mm, 10 μm), and (C) 20 to 60% ACN in aqueous trifluoroacetic acid (0.1%) over 20 min at a flow rate of 1.0 mL/min (Zorbax SB-Phenyl, 4.6 mm × 250 mm, 5 μm).

at room temperature in the dark. Then, the TFA solution was filtered, and the resin was washed with TFA (2 mL) and DCM (2 mL). The combined TFA solution was concentrated to a volume of approximately 3 mL with a gentle stream of nitrogen, and the peptide was precipitated with cold diethyl ether (40 mL). The precipitated peptide was centrifuged, and the ether solution was decanted to remove the scavengers. Washing with cold diethyl ether was repeated, and the precipitated peptide was centrifuged, decanted, and dried under vacuum.

To monitor the progress of peptide synthesis, a small amount of resin (approximately 20 mg) was collected and treated with the cleavage mixture (2 mL) for 90 min at room temperature in the dark. The TFA solution was filtered and concentrated to a volume of approximately 0.5 mL with a gentle stream of nitrogen. The peptide was precipitated with cold ether (10 mL), and the centrifuged peptide was washed with ether again. The peptide was dried under vacuum followed by HPLC and ESI-MS analysis.

General Procedure for the Purification of Peptides. A crude peptide was dissolved in 50% aqueous acetic acid, and the insoluble was removed by centrifugation. The acetic acid solution containing peptide was purified with HPLC by using a reverse-phase semipreparative Vydac column (C_4-bonded, 214TP1010, 10 mm × 250 mm, 10 μm) with gradient elution at a flow rate of 3.0 mL/min. A fraction containing the peptide was collected and lyophilized. The purity of all of the synthesized peptides was checked by analytical HPLC and found to be greater than 95%. The molecular mass of the purified peptides was confirmed by ESI-MS (Table 2).

Circular Dichroism Spectroscopy. A stock solution of a GLP-1 analogue (50 μM) was prepared by dissolving the peptide in water based on the UV absorbance at 280 nm (ε = 6760 M^{-1} cm^{-1}). A series of peptide solutions (20 μM) were then prepared with various TFE concentrations (0, 10, 20, and 50%), and their CD spectra were recorded with an Aviv (Lakewood, NJ) circular dichroism spectrometer (model 202) at room temperature by using a cell with a path length of 1.0 cm under constant nitrogen flush. Each peptide was scanned between wavelengths of 190 and 260 nm, and the absorption spectra were averaged and corrected by subtraction of the spectra of the blank. The CD data were expressed in terms of mean residue ellipticity in degrees per square centimeter per decimole.[53]

GLP-1 Receptor Activation Assay. Cyclic AMP accumulation was assessed in subconfluent cultures of HEK293 cells stably expressing the human GLP-1 receptor in the presence of a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), as described previously.[59] For dose−response experiments, cells were treated with a peptide for 20 min at 37 °C. In all cases, reactions were stopped with trichloroacetic acid (1.2 M), and accumulated cAMP was isolated by the two-column chromatographic method.[60] The potency of the peptides (EC_{50} values) was determined by sigmoidal curve fitting using GraphPad Prism version 5.0 (GraphPad, San Diego, CA).

Degradation of Cyclic GLP-1 Analogues by Dipeptidyl Peptidase-IV. A GLP-1 analogue (100 μM) was incubated with the recombinant human DPP-IV enzyme (0.2 ng/mL) in Tris buffer (25 mM, pH 8.0) at 37 °C. To quantify the remaining peptide, an internal standard with an HPLC retention time different from that of the peptide and the degraded peptide were incubated together with the peptide; 0, 2, 4, 6, 9, and 24 h after the incubation, an aliquot of the solution (100 μL) was quenched with aqueous TFA (10%, 20 μL) and analyzed via HPLC [elution gradient, 10 to 90% ACN in aqueous trifluoroacetic acid (0.1%) over 40 min; C_{18}-bonded column, Zorbax C-18, 4.6 mm × 250 mm]. The concentrations of intact and degraded peptides were determined on the basis of their peak areas relative to that of an internal standard, and their half-lives were calculated by nonlinear regression via GraphPad Prism version 5.0.

Degradation of Cyclic GLP-1 Analogues by Neutral Endopeptidase 24.11. A GLP-1 analogue (100 μM) was incubated with the recombinant human NEP 24.11 enzyme (1.0 μg/mL) in HEPES buffer (50 mM, pH 7.4, 50 mM NaCl) at 37 °C;[65] 0, 1, 2, 4, 8, 24, and 96 h after the incubation, an aliquot of the solution (100 μL) was quenched with aqueous TFA (10%, 20 μL) and analyzed via HPLC [elution gradient, 10 to 90% ACN in aqueous trifluoroacetic acid (0.1%) over 40 min; C_{18}-bonded column, Zorbax C-18, 4.6 mm × 250 mm]. Similarly, the concentrations of intact peptides were determined on the basis of their peak areas relative to that of an internal standard, and their half-lives were calculated by nonlinear regression via GraphPad Prism version 5.0.

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