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Search for α-helical propensity in the receptor-bound conformation of glucagon-like peptide-1

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ABSTRACT

To elucidate the receptor-bound conformation of glucagon-like peptide-1 (GLP-1), a series of conformationally constrained GLP-1 analogues were synthesized by introducing lactam bridges between Lys^{i} and Glu^{i+4} to form α-helices at various positions. The activity and affinity of these analogues to GLP-1 receptors suggested that the receptor-bound conformation comprises two α-helical segments between residues 11-21 and 23-34. It is notable that the N-terminal α-helix is extended to Thr^{11}, and that Gly^{22} plays a pivotal role in arranging the two α-helices. Based on these findings, a highly potent bicyclic GLP-1 analogue was synthesized which is the most conformationally constrained GLP-1 analogue reported to date.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a 30 amino acid-containing peptide hormone released from intestinal L-cells.[1,2] Tissue-specific posttranslational processing of the glucagon precursor, proglucagon produces two equipotent endogenous forms of GLP-1(7–36)-NH_2 and GLP-1(7–37). It has been regarded as an important incretin due to its primary role in regulating postprandial insulin release in response to nutrient ingestion. It also induces pancreatic β-cell differentiation and proliferation, and inhibits apoptosis of the β-cells.[3–6] In addition, it inhibits glucagon secretion,[7,8] decelerates gastric emptying,[9–11] reduces appetite and induces satiety.[12–14] All of these regulatory functions have made GLP-1 and its agonists considered as potent therapeutic agents for treating diabetes.

Structure–activity relationship studies of GLP-1 have summarized that its N-terminal region is involved in receptor activation whereas the C-terminal part contributes to receptor binding.[15] A number of critical residues for receptor interaction were identified by an alanine scanning study, including His^{7}, Gly^{10}, Phe^{12}, Thr^{13}, Asp^{15}, Phe^{28} and Ile^{29} of which substitution with Ala resulted in significant loss in receptor binding or activation.[16] The structure of GLP-1 in solution was determined by 2D NMR in the presence of perdeuterated dodecylphosphocholine micelles, and was found to have two α-helical regions between Thr^{13}-Glu^{20} and Ala^{24}-Gly^{35}, a random coil between His^{7}-Thr^{13} and a linker region between Glu^{21}-Gln^{23}.[17,18] The C-terminal α-helix appears to be more stable compared to the N-terminal one as examined by an amide proton exchange experiment.[17]

Although the solution structure is of great help in comprehending the interaction of GLP-1 with its G-protein coupled receptor on membrane, the complex nature of their interaction cannot be easily mimicked by a simple micelle that was used to create a membrane-like environment in the previous 2D NMR studies. Upon recognizing each other, structures of a peptide and its receptor are likely to be mutually changed, thus the structure of a peptide when it binds to its receptor may be different from a solution structure when studied alone.[19] This suggests that the determination of the receptor-bound conformation of a peptide should be carried out in the presence of its receptor, however biophysical studies, such as X-ray crystallography and NMR spectroscopy, with a membrane protein has been found extremely challenging.

In order to elucidate the receptor-bound conformation of GLP-1, we have designed and synthesized a series of conformationally constrained GLP-1 analogues. A lactam bridge was introduced between Lys at the *i* position and Glu at the *i* + 4 position to induce and stabilize an α-helical structure as this conformational restriction has been widely employed to form α-helices in many peptides.[20–22] To survey the presence and locations of α-helices in the receptor-bound conformation, a lactam bridge was placed at various positions in the GLP-1 sequence (from the N- to C-terminus) and the interaction of the resulting cyclic peptides with the GLP-1 receptor was examined. If an α-helix formed and stabilized by a lactam bridge matches with one in the receptor-bound conformation, the corresponding cyclic peptide would exhibit high potency and affinity to the receptor since it can be easily

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recognized by the receptor. On the other hand, a mismatched α-helix will result in weak receptor interaction.

To examine the effects of the substitution with Lys/Glu and the lactam bridge formation, were also synthesized linear GLP-1 analogues that contain Lys(Ac) and Gln in place of Lys and Glu, respectively, to avoid creating unwanted electrostatic charges. In addition, amino acid residues that are known to be crucial for receptor binding and activation like His^7, Gly^{10}, Phe^{12}, Thr^{13}, Phe^{28}, and Ile^{29} were not substituted to retain full agonist activity. To evaluate the formation and stabilization of an α-helix by the installation of a lactam bridge, the synthesized cyclic GLP-1 analogues were analyzed by circular dichroism spectroscopy.

2. Results and discussion

A number of conformationally constrained GLP-1 analogues (**1C**–**8C** and **3CC** in Table 1) were designed and synthesized to search the presence and locations of α-helices in the receptor-bound conformation of GLP-1. All of the cyclic peptides as well as their linear counterparts were synthesized by using standard N-Fmoc/^{t}Bu solid-phase peptide chemistry. For the formation of a lactam bridge in a cyclic GLP-1 analogue, allyl protecting groups, such as an allyloxycarbonyl (Aloc) and an allyl ester (OAl) were used for Lys and Glu, respectively. These allyl protecting groups were orthogonally removed by the treatment of Pd^0 and an allyl scavenger, *N*,*N*′-dimethyl-barbituric acid (DMBA) while a peptide was still anchored to a polymer support.[23] Then, the released amine and carboxylic acid side chain groups of Lys and Glu were treated with a coupling reagent like BOP or PyBOP to create a lactam bridge on resin. In addition to the cyclic GLP-1 analogues (**1C**–**8C**), their linear counterparts (**1L**–**8L**) were also produced with Lys(Ac) at the *i* position and Gln at the *i* + *4* position to evaluate the effects of the substitution and cyclization. The cyclic and linear GLP-1 analogues were cleaved from resin and fully deprotected with TFA, followed by purification using reverse-phase HPLC. All of the peptides were characterized by analytical HPLC under various elution conditions and ESI-MS (Table 2 in Section 4).

To validate the formation and stabilization of α-helices by creating lactam bridges between Lys^i and Glu^{i+4}, the cyclic GLP-1 analogues (**1C**-**8C**) were examined by circular dichroism spectroscopy. A cyclic peptide was dissolved in aqueous TFE solutions (peptide concentration = 20 μM; 0, 10, 20, 35, and 50% TFE in water), and its CD spectra were acquired by scanning between 190–260 nm in wavelength at room temperature. Then, the helical content of the peptide was determined as previously reported.[24] As shown in Figure 1, all of the cyclic GLP-1 analogues were found to have similar or higher helical contents compared to GLP-1. However, the amount of the increased helicity by the lactam bridge formation appears to vary depending on their sequences.

The synthesized cyclic and linear GLP-1 analogues were then assessed for their receptor binding affinity and potency in receptor activation by using transfected HEK293 cells that overexpress human GLP-1 receptors. To determine receptor binding affinity, competitive binding assays were carried out with ^{125}I-exendin(9–39) as a radiotracer.[25] And, the activation of the receptor was examined by measuring luciferase activity after incubating the HEK293 cells with a peptide since a luciferase was linked to a cAMP responsive promoter in the transfected HEK293 cells.[26,27] The results of these biological assays were summarized in Table 1.

To examine a possibility of adopting an α-helical conformation at the N-terminus, a linear and a cyclic GLP-1 analogues (**1L** and **1C**, respectively) were synthesized by replacing Thr^{11} and Asp^{15} with Lys(Ac)/Gln (**1L**) or Lys/Glu (**1C**). When assessed with the GLP-1 receptor, both **1L** and **1C** showed lowered potency and binding affinity compared to GLP-1 presumably resulting from the substitution of Asp^{15}, one of the important residues for receptor interaction, to form a lactam bridge and create an α-helix at this N-terminal region. However, the cyclic peptide **1C** was found to have significantly higher potency (EC_{50} = 0.38 nM; 63-fold increase), compared to the linear peptide **1L** (EC_{50} = 24 nM; Table 1 and Fig. 2). Not only the increase in potency, the binding affinity of **1C** (IC_{50} = 110 nM) was also found to be higher than **1L** (IC_{50} = 440 nM). This indicates that the helix formed by the lactam bridge between residues 11–15 matches with one in the receptor-bound conformation and facilitated the stronger interaction with the receptor. It is notable that the previous 2D NMR studies[17,18] showed this N-terminal region as a random coil instead of the helical structure identified in this study. The flexible N-terminal chain in solution appears to coil and form an α-helix upon binding to the receptor. This finding clearly points out that the receptor-bound conformation of a flexible peptide can be different from a solution structure determined without the presence of its receptor.

Then, the lactam bridge was shifted toward the C-terminus by three residues substituting Ser^{14} and Ser^{18}, and the resulting linear and cyclic peptides (**2L** and **2C**, respectively) were examined for their potency and binding affinity. Surprisingly, both peptides showed weak receptor activation (EC_{50} = 16 and 8.4 nM for **2L** and **2C**, respectively) as well as poor receptor binding (IC_{50} = 230 nM and 210 nM for **2L** and **2C**, respectively) although no substantial effect by the substitution of both $Ser^{14,18}$ was observed in the previously reported alanine scanning study.[16] To check the insignificant role of $Ser^{14,18}$ in receptor interaction, [Ala^{14}]- and [Ala^{18}]GLP-1(7–36)-NH_2 were synthesized and examined for their receptor interaction. It is striking that the substitution of Ser^{14} with Ala resulted in 14-fold loss in potency whereas the substitution of Ser^{18} with Ala provided negligible effect (unpublished results). This finding suggests that Ser^{14} plays an important role in receptor recognition and presumably makes direct contact to the receptor in contrast to the previous alanine scanning study. It also explains the significantly reduced activity of **2L** and **2C**.

The next pair of a linear and a cyclic GLP-1 analogues (**3L** and **3C**, respectively) was synthesized by replacing Val^{16} and Leu^{20}. Remarkably, the cyclic peptide **3C** showed almost same potency and binding affinity (EC_{50} = 0.0075 nM, IC_{50} = 4.5 nM) as GLP-1. This indicates that the α-helical structure stabilized by the lactam bridge between Lys^{16} and Glu^{20} is a part of the receptor-bound conformation and accounts for the high potency of **3C**. This also confirms the presence of an α-helix in this N-terminal region revealed in the solution structure determined by 2D NMR.[17,18] In addition, it may be hypothesized that the hydrophobic α-helical face organized by Val^{16} and Leu^{20} does not make direct contact with the receptor since the bulge created by the lactam bridge did not prevent optimal receptor recognition.

Interestingly, the potency of the linear peptide **3L** (EC_{50} = 0.012 nM, IC_{50} = 6.6 nM) is quite comparable to the cyclic peptide **3C** although the cyclization still improved the potency by 1.6-fold. The high potency of **3L** may be caused by intrinsically high α-helical propensity in this region that could potentially make the linear peptide **3L** form an α-helix in the receptor without additional stabilization provided by the lactam bridge in **3C**. To validate this, another pair of a linear and a cyclic GLP-1 analogues (**3CL** and **3CC**, respectively) was synthesized by replacing Val^{16}/Leu^{20} with Cys^{16}/Cys^{20}. The disulfide bridge between Cys^{16}/Cys^{20} in **3CC** makes the ring smaller than one containing the lactam bridge between Lys^{16}/Glu^{20} in **3C**, thus disturbs the formation of the α-helical structure in this region. As anticipated, the cyclic peptide **3CC** (EC_{50} = 19 nM; 14-fold decrease) exhibited significantly lowered potency compared to its linear peptide **3CL** (EC_{50} = 1.4 nM), confirming the significance of the α-helix in this region for optimal receptor recognition.

Table 1
Receptor binding and activation by the conformationally constrained GLP-1 analogues

Entry	Peptide	Sequence	Receptor binding IC_{50} (nM) (pIC_{50} ± SEM)	Receptor activation EC_{50} (nM) (pEC_{50} ± SEM)
	GLP-1	HAEGTFTSDVSSYLEGQAAKEIFAWLVKGR	2.6 (8.58 ± 0.08)	0.0036 (11.44 ± 0.08)
1C	c[Lys^{11},Glu^{15}]GLP-1(7-36)-NH_2	----K---E----------	110 (6.97 ± 0.14)	0.38 (9.42 ± 0.02)
1L	[Lys(Ac)^{11},Gln^{15}]GLP-1(7-36)-NH_2	Ac ----K---Q----------	440 (6.36 ± 0.15)	24 (7.63 ± 0.02)
2C	c[Lys^{14},Glu^{18}]GLP-1(7-36)-NH_2	-------K---E----------	210 (6.67 ± 0.14)	8.4 (8.08 ± 0.10)
2L	[Lys(Ac)^{14},Gln^{18}]GLP-1(7-36)-NH_2	Ac -------K---Q----------	230 (6.64 ± 0.06)	16 (7.81 ± 0.09)
3C	c[Lys^{16},Glu^{20}]GLP-1(7-36)-NH_2	---------K---E----------	4.5 (8.35 ± 0.09)	0.0075 (11.12 ± 0.01)
3L	[Lys(Ac)^{16},Gln^{20}]GLP-1(7-36)-NH_2	Ac ---------K---Q----------	6.6 (8.18 ± 0.10)	0.012 (10.92 ± 0.05)
4C	c[Lys^{18},Glu^{22}]GLP-1(7-36)-NH_2	----------K---E----------	15.1 (7.82 ± 0.07)	0.0020 (11.70 ± 0.09)
4L	[Lys(Ac)^{18},Gln^{22}]GLP-1(7-36)-NH_2	Ac ----------K---Q----------	17 (7.77 ± 0.13)	0.0038 (11.42 ± 0.04)
5C	c[Lys^{20},Glu^{24}]GLP-1(7-36)-NH_2	----------K---E----------	3000 (5.52 ± 0.10)	0.36 (9.44 ± 0.10)
5L	[Lys(Ac)^{20},Gln^{24}]GLP-1(7-36)-NH_2	Ac ----------K---Q----------	1200 (5.91 ± 0.19)	0.10 (9.99 ± 0.06)
6C	c[Lys^{21},Glu^{25}]GLP-1(7-36)-NH_2	----------K---E----------	9200 (5.04 ± 0.17)	1.1 (8.96 ± 0.08)
6L	[Lys(Ac)^{21},Gln^{25}]GLP-1(7-36)-NH_2	Ac ----------K---Q----------	1600 (5.78 ± 0.16)	0.12 (9.91 ± 0.08)
7C	c[Lys^{23},Glu^{27}]GLP-1(7-36)-NH_2	----------K---E----------	3.3 (8.49 ± 0.05)	0.0029 (11.54 ± 0.03)
7L	[Lys(Ac)^{23},Gln^{27}]GLP-1(7-36)-NH_2	Ac ----------K---Q----------	2.6 (8.59 ± 0.07)	0.0028 (11.55 ± 0.10)
8C	c[Lys^{30},Glu^{34}]GLP-1(7-36)-NH_2	----------K---E--	1.5 (8.84 ± 0.09)	0.0021 (11.68 ± 0.08)
8L	[Gln^{30},Lys(Ac)^{34}]GLP-1(7-36)-NH_2	Ac ----------K---Q--	–[a]	0.0048 (11.32 ± 0.05)
3CC	c[Cys^{16,20}]GLP-1(7-36)-NH_2	---------C---C----------	–[a]	19 (7.73 ± 0.10)
3CL	[Cys^{16,20}]GLP-1(7-36)-NH_2	---------C---C----------	–[a]	1.4 (8.84 ± 0.10)
BC	c[Lys^{16},Glu^{20}]-c[Lys^{30},Glu^{34}]GLP-1(7-36)-NH_2	---------K---E---------K---E--	–[a]	0.0033 (11.48 ± 0.10)

[a] Not determined.

Table 2
Characterization of the synthesized GLP-1 analogues

Peptide	Retention time[a] (min)			Molecular mass	
	A	B	C	Calculated	Found
1C	17.9	13.0	13.0	3320.8	3320.4
1L	17.8	12.7	12.9	3379.8	3379.0
2C	18.7	13.7	13.6	3362.8	3362.9
2L	21.0	13.2	13.1	3421.9	3421.8
3C	18.0	12.8	11.6	3324.7	3324.5
3L	16.9	11.1	11.3	3383.7	3383.2
4C	17.8	12.7	12.9	3392.8	3392.4
4L	19.0	13.2	13.1	3451.8	3451.7
5C	16.0	11.1	11.4	3352.7	3352.3
5L	16.4	11.4	11.2	3411.8	3411.4
6C	19.1	13.3	13.3	3336.8	3336.4
6L	17.0	12.2	12.4	3395.8	3395.2
7C	18.3	13.0	13.1	3279.7	3279.6
7L	20.6	13.0	12.9	3338.8	3338.6
8C	19.1	13.0	13.8	3337.7	3337.2
8L	19.1	13.9	13.7	3396.8	3396.5
3CC	16.5	11.5	11.4	3289.7	3289.4
3CL	16.8	11.7	11.6	3291.7	3291.6
BC	17.6	12.2	12.4	3364.7	3364.6

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

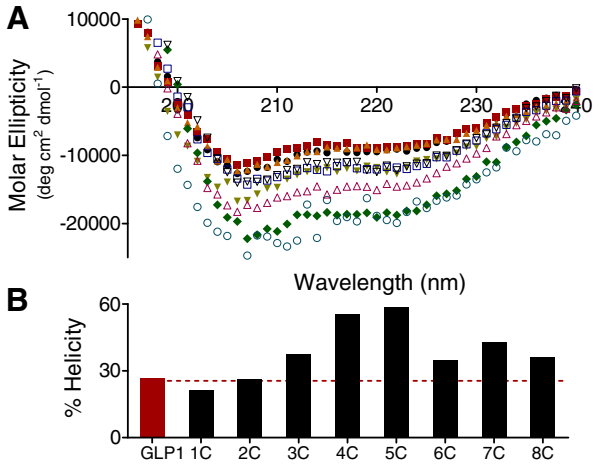

Figure 1. (A) CD spectra of cyclic GLP-1 analogues at 20% TFE (GLP-1 (●), 1C (■), 2C (▲), 3C (▼), 4C (♦), 5C (◇), 6C (○), 7C (□), 8C (△)); (B) % helicity determined.

Similar to the high potency and binding affinity of **3C**, the cyclic peptide **4C** that contains a lactam bridge between residues 18 and 22 showed a slightly higher potency (EC_{50} = 0.0020 nM) compared to GLP-1, indicating that the α-helical structure stabilized by the lactam bridge between residues 18–22 is a part of the receptor-bound conformation. The comparable potency of the linear peptide **4L** (EC_{50} = 0.0038 nM; 1.9-fold decrease compared to **4C**) also suggests the intrinsically high α-helical propensity in this region.

We have also introduced lactam bridges in the linker region by substituting Leu^{20}/Ala^{24} (**5L** and **5C**) and Glu^{21}/Lys^{25} (**6L** and **6C**) to survey α-helical conformations. In contrast to the higher potency of **3C** and **4C** compared to **3L** and **4L**, both cyclic peptides **5C** and **6C** exhibited significantly lower potency and binding affinity (EC_{50} = 0.36 and 1.1 nM, respectively) compared to their linear counterparts **5L** and **6L** (EC_{50} = 0.10 and 0.12 nM, respectively). The significantly reduced potency of **5C** (3-fold decrease compared to **5L**) and **6C** (9-fold decrease compared to **6L**) suggests that the α-helical structures formed by the lactam bridges in the linker region are not compatible to the receptor.

In contrast to the weak receptor interaction of **5C** and **6C**, cyclic GLP-1 analogues containing lactam bridges at the C-terminal region (**7C** and **8C**) were well accepted by the receptor. The placement of a lactam bridge between residues 23–27 in **7C** resulted in a comparable receptor binding and activation (EC_{50} = 0.0029 nM, IC_{50} = 3.3 nM) compared to GLP-1. When the lactam bridge was shifted further to the C-terminus by replacing Ala^{30} and Lys^{34}, the resulting cyclic peptide **8C** showed even higher receptor interaction (EC_{50} = 0.0021 nM, IC_{50} = 1.5 nM) compared to GLP-1. The increased potency of **8C** (about 1.7-fold compared to GLP-1) appears to result from the additional stabilization of an α-helix at the C-terminal region by the lactam bridge, and indicates that the GLP-1 receptor clearly prefers an α-helical structure at the C-terminal region. As demonstrated in the solution structure determined by 2D NMR,[17,18] the intrinsically high α-helical propensity in the C-terminal region provided the linear peptides **7L** and **8L** with high potency and binding affinity although slightly stronger receptor interaction was still observed by their cyclic peptides. In addition, it may be proposed that the helical face organized by Ala^{30} and Lys^{34} does not make direct contact with the receptor since the presence of the lactam bridge between residues 30–34 did not deter receptor binding.

In summary, the high and increased receptor interaction of the cyclic GLP-1 analogues **1C**, **3C**, **4C**, **7C**, and **8C** suggests that the receptor-bound conformation of GLP-1 mainly comprises of two α-helices. The N-terminal α-helix appears to be organized by residues 11–21, while the C-terminal one spans residues 23–34. These two α-helices were connected by a flexible amino acid Gly^{22}, which was found to be highly important for playing a pivotal role since the efforts to consolidate the two N- and C-terminal α-helices by making the linker region α-helical led to the severe rejection by the receptor as shown by **5C** and **6C**. In addition, the uncompromised receptor recognition by **3C** and **8C** may indicate that the α-helical faces organized by Val^{16}/Leu^{20} and Ala^{30}/Lys^{34} do not make direct contact to the receptor, and these residues may be further exploited to achieve higher potency in future.

Based on the strong receptor interaction of **3C** and **8C**, we have synthesized a bicyclic GLP-1 analogue containing two lactam bridges, one between residues 16–20 as shown in **3C** and the other between residues 30–34 as found in **8C**. These two lactam bridges were installed to stabilize the two α-helices at the N- and C-terminal regions, and indeed facilitated high potency (EC_{50} = 0.0033 nM) as anticipated. This is the most conformationally constrained GLP-1 analogue with high potency reported until now and will be of great value to further elucidate the receptor-bound conformation of GLP-1 in future.

3. Conclusion

In order to elucidate the receptor-bound conformation of GLP-1, a series of GLP-1 analogues containing lactam bridges between Lys^{i} and Glu^{i+4} were synthesized. The lactam bridges were introduced at various positions in search for the presence and location of α-helices in GLP-1 when it binds to its receptor. Together with their linear counterparts (**1L**–**8L**), the cyclic peptides (**1C**–**8C**) were examined for their receptor binding and activation. The high potency of the cyclic peptides **3C**, **4C**, **7C**, and **8C** indicates the presence of two α-helices as displayed in the solution structure determined by 2D NMR. However, the substantially stronger receptor interaction of **1C** compared to **1L** suggests that the N-terminal α-helix extends over residues 11–21 in the receptor-bound conformation. The C-terminal α-helix was found to span residues 23–34, and its stabilization by a lactam bridge between residues

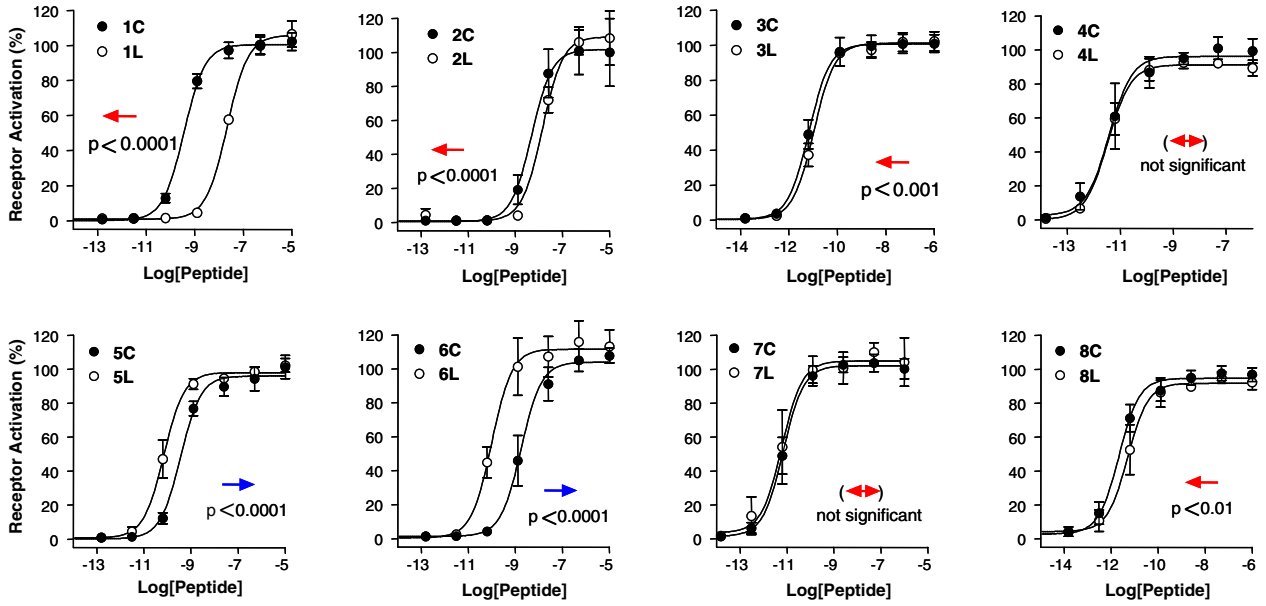

Figure 2. Concentration–activity plots of the cyclic and linear GLP-1 analogues in receptor activation.

30–34 increased receptor binding affinity and potency compared to GLP-1. The weak receptor interaction of **5C** and **6C** proposed that the linker region do not prefer forming an α-helical structure, and the flexible residue of Gly at position 22 may play a pivotal role to arrange the two α-helices upon binding to the receptor. Based on these findings, a bicyclic GLP-1 analogue was synthesized and it is the most constrained GLP-1 analogue with high potency reported until now.

4. Experimental

4.1. Materials and general procedures

N^{α}-Fmoc protected amino acids, aminomethylated polystyrene resin, Rink amide linker, and Rink amide resin were purchased from EMD Bioscience (San Diego, CA) and Senn Chemicals (Dielsdorf, Switzerland). All amino acids used were of L-configuration unless otherwise stated. Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid (TFA), anisole, acetic anhydride, ninhydrin, and methylsulfide (Acros Organic, Morris Plains, NJ); *N*,*N*-diisopropylethylamine (DIEA), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroni-um hexafluorophosphate (HBTU), and 1-hydroxybenzo-triazole (HOBt) (Advance ChemTech, Louisville, KY); *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), and acetonitrile (ACN) (Fischer Scientific, Pittsburgh, PA); 1,2-ethanedithiol, 2,2,2-trifluoroethanol (TFE), and Amberlite IRA-67 ion exchange resin (Alfa Aesar, Ward Hill, MA); tetrakis(triphenyphosphine)palladium (0), triisopropylsilane, *N*,*N*′-dimethylbarbituric acid (DMBA), piperidine, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Aldrich, Milwaukee, WI). The solvents for peptide synthesis and purification were used without purification. The purity of each peptide was checked by HPLC (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 × 250 mm, 10 μm) and (B) a phenyl-bonded silica column (Zorbax SB-Phenyl, 4.6 × 250 mm, 5 μm). The molecular mass of the peptides were confirmed by ESI-MS (LCQ Deca XP Plus ESI-ion trap mass spectrometer, Thermo Electron Corporation, West Palm Beach, FL).

4.2. Chemistry

4.2.1. General procedure for peptide synthesis

All of the GLP-1 analogues were synthesized by using standard N^{α}-Fmoc/^{t}Bu solid-phase peptide synthesis protocol. They were constructed either by manual synthesis, by using an ABI-433A automated peptide synthesizer, or by using a CEM Discover SPS manual microwave peptide synthesizer.

For the manual synthesis, 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,[28,29] 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 by treating 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 which 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 carried out 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 test and TNBS tests, the resin was washed with DMF (3 × 1 min) and the unreacted amines were capped by treating 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 in vacuo. A growing peptide was frequently characterized by cleaving small amount of resin and analyzing the released peptide with analytical HPLC and ESI-MS.

For the automated peptide synthesis, an ABI-433A automated peptide synthesizer was used with a modified Fmoc synthesis protocol. Rink amide resin (0.25 mmol, 0.6–0.75 mmol/g) was placed

in a reaction vessel and the Fmoc protecting group on the resin was removed with piperidine (20% in DMF, 1 × 5 min and 1 × 30 min) followed by washing with DMF (4 × 1 min). Then, were delivered and reacted for 2 h a preactivated Fmoc-amino acid which was prepared by mixing a Fmoc-amino acid (4 equiv), HBTU (4 equiv), HOBt (4 equiv), and DIEA (8 equiv) in DMF for 30 min. The deprotection and coupling steps were repeated for each amino acid in the sequence. After the synthesis was finished, the resin was washed with DCM (4 × 1 min) and dried in vacuo. While most of the automated peptide synthesis was followed as described above, some adjustments (e.g., prolonged coupling reaction time, double coupling of a Fmoc-amino acid, and acetylation of unreacted amines) were employed to achieve higher synthetic efficiency.

For the microwave-assisted peptide synthesis, a CEM Discover SPS manual microwave peptide synthesizer was used. After swelling Rink amide resin (0.25 mmol, 0.6–0.75 mmol/g) in DMF for 10 min, the resin was washed with DMF (3 × 1 min). To remove Fmoc protecting groups, piperidine (4 mL, 20% in DMF containing 0.5 M HOBt) was added to the resin and the mixture was irradiated (50 W maximum power, 75 °C maximum temperature) for 5 min. The resin was washed with DMF (3 × 1 min). To couple a Fmoc-amino acid, the mixture of the resin and a preactivated Fmoc-amino acid (vide supra) was irradiated (25 W maximum power, 75 °C maximum temperature) for 10 min. Then, the resin was washed with DMF (3 × 1 min) and the coupling reaction was checked with Kaiser ninhydrin and TNBS tests. These deprotection and coupling steps were repeated until all of the amino acids in the sequence were coupled. All of the microwave irradiation were carried out at atmospheric pressure using an open vessel setting. After the synthesis was completed, the resin was washed with DCM (5 × 1 min) and dried in vacuo.

4.2.2. General procedure for the formation of lactam bridges

For the selective removal of allyl protecting groups of Lys and Glu, were placed a fully protected peptide still bound on resin (0.25 mmol), $Pd(PPh_3)_4$ (30 mg, 0.1 equiv), and DMBA (390 mg, 10 equiv) 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, degassed DCM/DMF (4 mL, 3:1) was delivered by a syringe and the reaction mixture was kept under nitrogen for 30 min with occasional shaking. The resin was washed with DMF (3 × 1 min) and the reaction was repeated again. Then, the resin was treated with BOP/HOBt/DIEA (6, 6, and 12 equiv) or PyBOP/HOBt/ DIEA (6, 6, and 12 equiv) for 2–6 h until the reaction was complete. The resin was washed with DMF (3 × 1 min).

4.2.3. Synthesis of a disulfide-containing GLP-1 analogue (3CC)

The purified peptide with free sulfhydryl groups (**3CL**, 0.25 mmol) was dissolved in 50% aqueous acetonitrile (25 mL), and slowly added with a syringe pump (infusion rate of 0.5 mL/h) to a solution of potassium ferricyanide that was prepared by mixing $K_3Fe(CN)_6$ (1 mmol), water (100 mL), acetonitrile (20 mL), and ammonium acetate (1 mL, 1 M) and adjusting the pH to 8.5 with concentrated ammonium hydroxide.[30] After the addition was completed, the reaction mixture was acidified to pH 4 with acetic acid and a weakly basic anion-exchange resin (Amberlite IRA-67, approximately 5–10 mL) was added to the solution. The anion-exchange resin was filtered and the volatiles were removed to get the cyclized peptide (**3CC**) which were further purified by HPLC.

4.2.4. Synthesis of a bicyclic GLP-1 analogue (BC)

For the synthesis of a bicyclic GLP-1 analogue containing two lactam bridges, a partially completed GLP-1 analogue (residues 30–36) was first constructed on Rink amide resin using the synthetic procedures described above. After the selective removal of the allyl protecting groups on Lys(Aloc)[30] and Glu(OAl)[34], the C-terminal lactam bridge was created using BOP. Then, the sequence up to position 16 was grown from the monocyclic peptide. Another selective removal of the allyl groups on Lys(Aloc)[16]/Glu(OAl)[20] and an amide bond formation introduced the second, N-terminal lactam bridge. Then, the remaining amino acids were coupled to His[7] to complete the synthesis of the bicyclic GLP-1 analogue.

4.2.5. General procedure for cleavage and final deprotection of peptides

A cleavage mixture of trifluoroacetic acid, dimethylsulfide, 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-polystyrene tube, and the mixture was stirred for 90 min at room temperature in the dark. Then, the TFA solution was filtered, and the resin was washed with trifluoroacetic acid (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 then the ether solution was decanted to remove the scavengers. Washing with cold diethyl ether was repeated and the peptide was dried in vacuo.

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 in vacuo followed by HPLC and ESI-MS analysis.

4.2.6. 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 peptide was purified with HPLC using a reverse-phase semi-preparative Vydac column (C_4-bonded, 214TP1010, 10 × 250 mm, 10 μm) or Zorbax column (Ph-bonded, SB-Phenyl, 9.4 × 250 mm, 5 μm) with gradient elution at a flow rate of 3.0 mL/min (see Table 3). 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 higher than 95%. The molecular mass of the purified peptides was confirmed by ESI-MS (see Table 2).

4.3. Circular dichroism spectroscopy

GLP-1 and its analogues were dissolved in water to make stock solutions of 50 μM which was determined by UV absorbance at 280 nm ($\varepsilon = 6760\ M^{-1}\ cm^{-1}$). These stock solutions were subsequently used to prepared 20 μM peptide solutions containing various concentrations of TFE (0%, 10%, 20%, 35%, and 50%). CD spectra of the peptide solutions were acquired by using an Aviv circular dichroism spectrometer (model 202, Lakewood, NJ) at room temperature using a cell with a path length of 0.1 cm under constant nitrogen flush. Each peptide solution was scanned between the wavelength of 190–260 nm, and the absorption spectra were averaged and corrected by subtracting absorption spectra of blank. The CD data was expressed in terms of mean residue ellipticity in deg cm^2 $dmol^{-1}$. % Helicity of each peptide was determined as previously reported.[24]

4.4. Biology

4.4.1. Receptor binding assay

HEK293 cells were grown in 96-well plates in DMEM containing 10% fetal bovine serum to a density of about 5,000 cells/well (37 °C, 5% CO_2 humidified atmosphere). Transfection was

Table 3
Purification of the synthesized GLP-1 analogues

Peptide	HPLC Gradient	Retention time[a] (min)
1C	E1	12.4
1L	F	11.0
2C	E1	13.7
2L	E1	13.0
3C	E1	13.4
3L	E1	12.9
4C	E1	12.7
4L	E1	13.0
5C	E1	12.7
5L	E1	13.3
6C	E2	13.0
6L	E1	10.4
7C	E2	12.7
7L	E1	9.6
8C	E1	12.9
8L	G	11.8
3CC	F	11.9
3CL	H	13.4
BC	F	12.4

[a] HPLC conditions: (E1) 32–32–44% acetonitrile in aqueous trifluoroacetic acid (0.1%) over 3 and 12 min, respectively, flow rate of 3.0 mL/min (C4-bonded column, Vydac 214TP1010, 4.6 × 250 mm, 10 μm); (E2) 32–32–44% acetonitrile in aqueous trifluoroacetic acid (0.1%) over 3 and 12 min, respectively, flow rate of 3.0 mL/min (Zorbax SB-Phenyl, 9.4 × 250 mm, 5 μm); (F) 30–30–42% acetonitrile in aqueous trifluoroacetic acid (0.1%) over 3 and 12 min, respectively, flow rate of 3.0 mL/min (C4-bonded column, Vydac 214TP1010, 4.6 × 250 mm, 10 μm); (G) 34–34–46% acetonitrile in aqueous trifluoroacetic acid (0.1%) over 3 and 12 min, respectively, flow rate of 3.0 mL/min (C4-bonded column, Vydac 214TP1010, 4.6 × 250 mm, 10 μm); (H) 28–28–40% acetonitrile in aqueous trifluoroacetic acid (0.1%) over 3 and 12 min, respectively, flow rate of 3.0 mL/min (C4-bonded column, Vydac 214TP1010, 4.6 × 250 mm, 10 μm).

performed in 50 μL of serum-free DMEM per well containing 0.35 μL Lipofectamine® reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Three cDNAs (5 ng/well each) encoding either the GLP-1 receptor or vector control (pcDNA1) in addition to a multimerized cAMP responsive promoter linked to luciferase and beta galactosidase were present in the transfection mix.[26,27]

Using the transfected HEK293 cells that overexpress human GLP-1 receptors, competition binding assays were performed in Hanks' balanced salt solution, supplemented with 25 mM HEPES, pH 7.3, 0.2% bovine serum albumin and 0.15 mM phenymethylsulfonyl fluoride. Also included was 0.4 μM dipeptidyl-peptidase 4 inhibitor ([1-(2-pyrrolodinylcarbonyl)-2-pyrrolidinyl]boronic acid) that delays enzymatic degradation of GLP-1 and its analogues.[25] Competition binding experiments of GLP-1 analogues were carried out using 15 pM ^{125}I-exendin(9–39) as a radioligand. Cell monolayers were washed three times with Hanks' balanced salt solution and then hydrolyzed in 1 N NaOH for γ counting (Beckman Gamma 5500B).

4.4.2. Receptor activation assay

To measure receptor-mediated transcriptional activity, GLP-1 or its agonists were added at the desired final concentrations to the transfected HEK293 cells that overexpress human GLP-1 receptors, and the incubation was continued for 4 h. The medium was then aspirated and 50 μL of Steady Light Plus® reagent (Perkin Elmer Life and Analytical Sciences, Waltham MA) was added. Luciferase activity was determined in a Topcount® plate reader (Perkin Elmer Life and Analytical Sciences). The potency of the compounds (EC_{50} values) was determined by sigmoidal curve fitting using the GraphPad Prizm program (version 3.0, San Diego, CA).

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