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Design of stapled oxyntomodulin analogs containing functionalized biphenyl cross-linkers

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ABSTRACT

A panel of three lipid-modified, functionalized biphenyl cross-linkers (fBph) were synthesized and subsequently employed in the preparation of the stapled oxyntomodulin (OXM) analogs. In a luciferase-based reporter assay, these stapled OXM analogs showed varying degree of potency in activating GLP-1R and GCGR, presumably due to the disparate effect of the lipid chains on the local environment close to the ligand-receptor binding interface. In particular, the fBph-1 cross-linked peptide with the lipid chain attached to position-3 of the biphenyl cross-linker exhibited the highest dual agonist activity.

1. Introduction

Oxyntomodulin (OXM) is a natural 37-amino acid peptide hormone derived from proglucagon, and regulates glucose metabolism, insulin secretion, food intake, and energy expenditure [1,2]. As a GLP-1R/GCGR dual-agonist, OXM exhibits remarkable weight loss and glucose lowering effects that are superior to the GLP-1R-only agonists, making it an attractive therapeutic candidate for the concurrent treatment of obesity and type 2 diabetes mellitus (T2DM) [3]. However, the clinical application of OXM has not been realized because OXM undergoes rapid proteolytic degradation and renal clearance after administration, and thus requires more frequent injections [4].

Covalent side-chain cross-linking of bioactive peptides (also known as 'peptide stapling') to stabilize peptide α-helical conformation has proven to be a valuable strategy to increase peptide potency, stability and cell permeability [5–8]. Previously, we reported the design of a series of biaryl cross-linkers including 4,4′-bis(bromomethyl)biphenyl (Bph) and 6,6′-bis(bromomethyl)-3,3′-bipyridine (Bpy), which react with peptides containing cysteines located at *i* and *i*+7 positions to generate stapled peptides with improved proteolytic stability and cell permeability [9–13]. To extend this strategy to OXM, we designed a chimeric peptide, OXM-7, by incorporating some of the key residues of GLP-1 into the sequence along with two cysteines located at *i*, *i*+7 positions [14]. We then employed Bph and Bpy to cross-link OXM-7 to give rise to the stapled analogs, OXM-7-Bph and OXM-7-Bpy, respectively (Fig. 1A). The stapled OXMs showed greater plasma stability and sub-nanomolar dual agonist activities in activating GLP-1R and GCGR in the cell-based assays. The computational modeling of OXM-7-Bph in complex with the extracellular domain of GLP-1R reveals a binding mode in which the Bph cross-linker reinforces α-helical structure but projects away from the binding interface (Fig. 1B) [15]. Since the half-lives of these analogs were measured to be ~13 h in mouse serum *in vitro* and ~1–2 h in vivo after injection into mice, they are still not sufficiently long for once-weekly administration in clinical setting.

As a well-known human serum albumin binding motif, the PEGylated fatty acid has been used in modifying GLP-1 and other peptides to extend their circulatory half-lives [16–20]. Herein, we report the design and synthesis of three lipid-modified biphenyl cross-linkers, and their use in preparing the stapled OXM analogs

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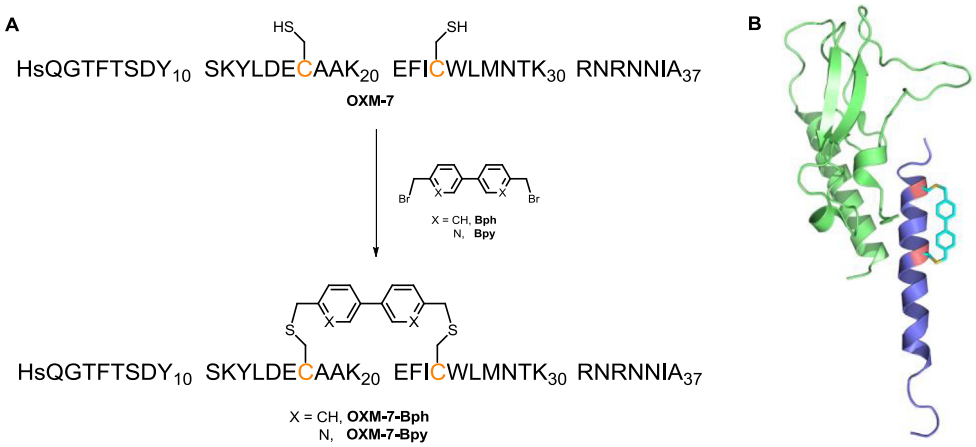

Fig. 1. (**A**) Scheme for preparing the Bph and Bpy cross-linked oxyntomodulin derivatives. (**B**) Structural model of the cross-linked oxyntomodulin, OXM-7-Bph, bound to the extracellular domain of GLP-1R (PDB code: 3C59): green, GLP-1R; purple, OXM-7-Bph; cyan, Bph; red, the cysteine cross-linking sites.

with enhanced pharmacokinetic properties.

2. Results and discussion

In designing functionalized cross-linkers for stapling with OXM-7, we decided to choose a long-chain lipid comprised of a PEG linker, glutamic acid, and C18 fatty diacid (octadecanedioic acid), which was reported to afford significantly improved half-life and glucose tolerance when attached to GLP-1 and excedin-4 (Fig. 2) [18,19]. To identify optimal attachment site, the lipid chain was introduced at three different positions of the Bph cross-linker: (i) position-3 on the biphenyl ring; (ii) position-2 on the biphenyl ring; and (iii) the benzylic position. The resulting cross-linkers were termed as fBph-1, fBph-2 and fBph-3, respectively.

Three parallel routes were chosen for the synthesis of the lipid-modified Bph cross-linkers (Schemes 1–3). In general, benzyl bromides were converted from benzyl alcohols in the final step because of their high reactivity. The Pd-catalyzed cross-coupling reaction was used to construct the biphenyl scaffold, and the lipid chain was attached through a series of condensation reactions. Several protecting groups were employed during the synthesis, including *tert*-butyldimethylsilyl (TBS) for the hydroxyl group, carboxybenzyl (Cbz) for the amino group, and *tert*-butyl for the carboxylic acid group. Five lipid chain building blocks, *N*-Cbz-prop-2-yn-1-amine (**L1**), *N*-Cbz-2-(2-(2-aminoethoxy)ethoxy)acetic acid (**L2**), *N*-Cbz-*O*-*tert*-butyl-glutamic acid (**L3**), octadecanedioic acid mono-*tert*-butyl ester (**L4**) and 2,2'-(ethane-1,2-diylbis(oxy)) diethanamine mono-Cbz carbamate (**L5**) were either commercially available or synthesized following the reported procedures [21–23].

For the synthesis of fBph-1 (Scheme 1), the Suzuki cross-coupling between methyl 4-bromo-2-hydroxybenzoate and (4-(methoxycarbonyl)phenyl)boronic acid was carried out to produce the biphenyl core bearing two carboxylates and one phenol group (**1**) in 94% yield [24]. After protecting the phenol with triflate (**2**), the carboxylates were reduced to the benzyl alcohols (**3**) by DIBAL followed by protection with TBS chloride to give compound **4**. Subsequent Sonogashira cross-coupling with *N*-Cbz-prop-2-yn-1-amine (**L1**) led to compound **5** [25]. Deprotection of the Cbz group together with reduction of alkyne to alkane under the hydrogenation condition afforded the propylamine intermediate, which was then coupled with *N*-Cbz-2-(2-(2-aminoethoxy)ethoxy) acetic acid (**L2**) to afford compound **6**. Then, three successive rounds of hydrogenation–coupling reactions were performed to conjugate **6** with **L2**, **L3**, and **L4** to give the fully protected compound **9** in 66% yield over 6 steps. After removal of the TBS group, treatment with PBr_3 converted the benzyl alcohols into the benzyl bromides and simultaneously hydrolyze the *tert*-butyl esters to afford the final lipid-modified cross-linker **10** (fBph-1) in 63% yield over two steps (Scheme 1).

For the synthesis of fBph-2 (**20**), the procedure was essentially the same as fBph-1 except the order of Sonogashira cross-coupling and DIBAL-mediated reduction was switched to increase the overall reaction yield (Scheme 2). For the synthesis of fBph-3 (**27**), 2-(4-bromophenyl)-2-hydroxyacetic acid was coupled with **L5** to give compound **21**. Then, the Suzuki cross-coupling between **21** and (4-(hydroxymethyl)phenyl) boronic acid was performed to generate biphenyl **22** in 59% yield. The hydroxyl groups in **22** were subsequently protected with the TBS group to give compound **23**. Then, three successive rounds of hydrogenation–coupling reactions were performed to conjugate **23** with **L2**, **L3** and **L4** to give rise to the fully protected lipid-modified cross-linker **26**. Subsequent deprotection followed by bromination with PBr_3 gave the lipid-modified cross-linker **27** (fBph-3) win 59% yield over two steps (Scheme 3).

For preparation of the cross-linked OXM peptides, the lipid-modified cross-linker (fBph-1/2/3) was allowed to react with OXM-7 in a mixed solvent containing 30 mM NH_4HCO_3 buffer/CH_3CN (3:2) at room temperature for three hours. The reaction mixtures were purified by preparative HPLC to give the corresponding cross-linked OXM peptides in 17–20% yield (last steps in Schemes 1–3). The purity and identity of the stapled OXM peptides were confirmed by analytical HPLC and mass spectrometry analysis, respectively (Fig. 3).

With the stapled OXM peptides in hand, we assessed their agonist activity toward the GLP-1R and GCGR using a luciferase reporter assay in HEK293 cells stably expressing human GLP-1R or GCGR and a cAMP response element (CRE)-driven luciferase

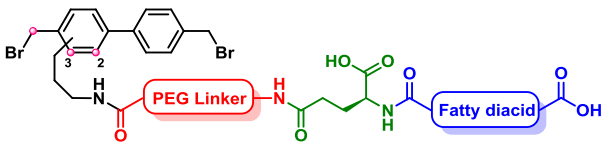

Fig. 2. Lipid-modified functionalized Bph cross-linkers.

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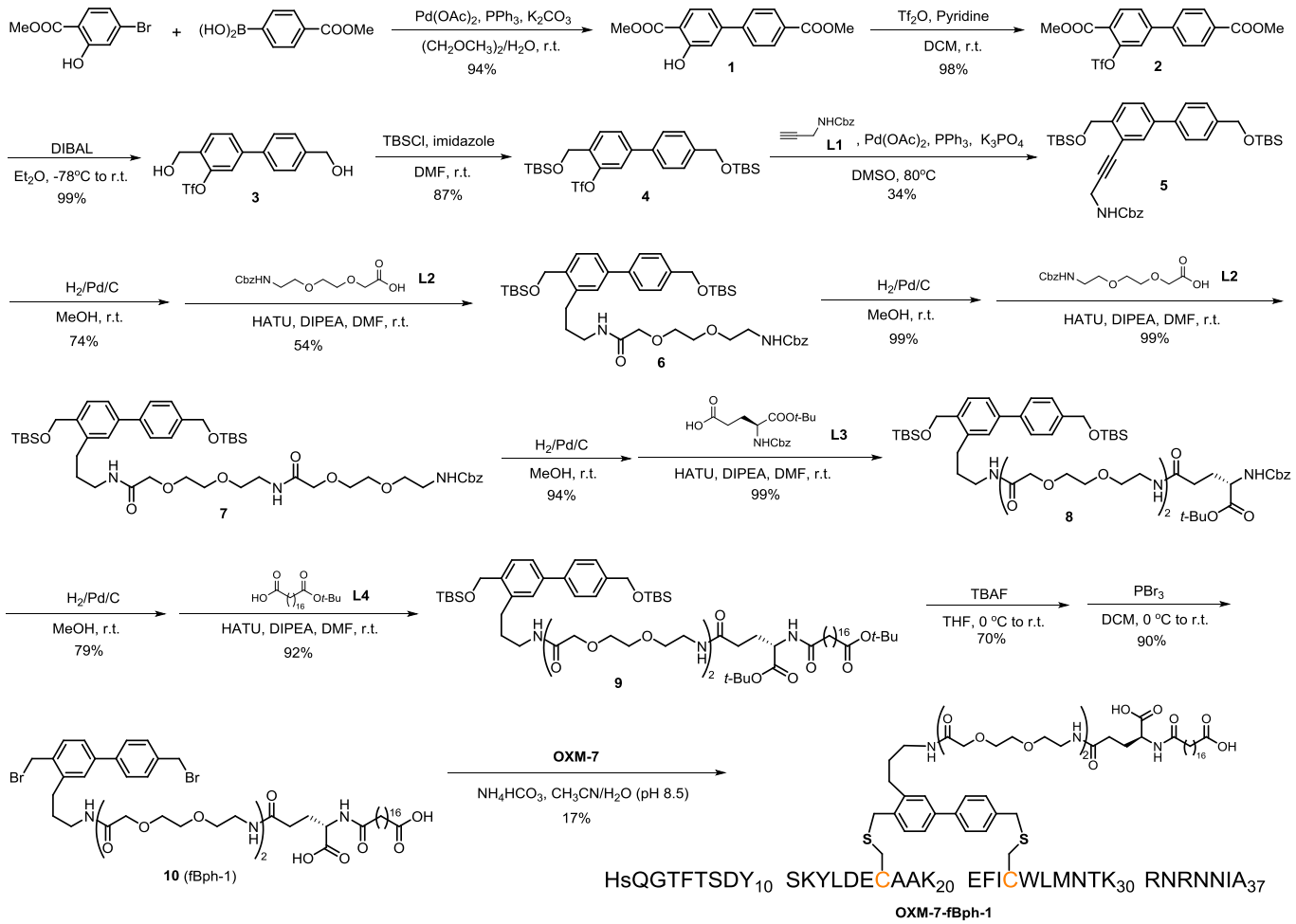

Scheme 1. Synthesis of OXM-7-fBph-1.

reporter (Fig. 4) [19,20]. The previously reported dual agonists, OXM-7-Bph and OXM-7-Bpy, were used as positive controls. All three fBph-stapled OXM-7 peptides showed decreased potency compared to the Bph- or Bpy-cross-linked peptides, particularly against GCGR (Table 1), which was also observed in the literature [26]. We hypothesize that the long lipid chain may affect the conformation of the biphenyl cross-linker to varying degree, which in turn affects local conformation of the middle region of the OXM peptide and subsequently the preferred binding mode towards GLP-1R and GCGR. OXM-7-fBph-1 with the lipid chain attached at position-3 of the biphenyl scaffold showed the highest agonist activity against both GLP-1R and GCGR (Table 1), suggesting that the lipid attachment to this position produces smallest perturbation to the local conformation of the OXM peptide. In contrast, OXM-7-fBph-2 with lipid chain attached to position-2 showed significant loss of potency against both GLP-1R and GCGR, suggesting that the lipid chain at the *ortho*-position may dramatically alter the orientation of the biphenyl ring and as a result the local conformation of the OXM peptide. When the lipid chain was introduced at the benzylic position in OXM-7-fBph-3, only 2-fold lower agonist activities were observed compared to OXM-7-fBph-1 (Table 1). We then compared the stability of OXM-7-fBph-1 in fresh mouse serum to that of parent peptide using the GLP-1R activation assay, and found that indeed the fatty diacid modified, crosslinked OXM peptide, OXM-7-fBph-1, exhibited longer half-life than the parent crosslinked peptide, OXM-7-Bph (Fig. 5).

3. Conclusion

In conclusion, we have designed and synthesized three lipid-chain modified, functionalized biphenyl cross-linkers, and employed them to prepare three stapled oxyntomodulin analogs containing cysteines at *i*, *i*+7 positions. The purity and identity of the stapled OXM peptides were confirmed by HPLC and mass spectrometric analyses, respectively. In the receptor activation assay, the stapled OXM analog with the lipid chain attached at position-3 of the biphenyl cross-linker showed the most potent dual agonist activity against GLP-1R and GCGR, most likely due to the smallest perturbation of the lipid chain to the local conformation of the OXM peptide. Our initial stability assay confirmed that the fatty diacid attachment extends serum half-life of the cross-linked OXM peptide. We are currently in the process of characterizing the in vivo pharmacokinetic properties of these lipid-modified crosslinked OXM analogs.

4. Experimental section

4.1. General information

Solvents and chemicals were purchased from commercial sources and used directly without further purification. Flash chromatography was performed with SiliCycle P60 silica gel (40–63 μm, 60 Å). ^{1}H and ^{13}C NMR spectra were recorded with Varian Mercury-

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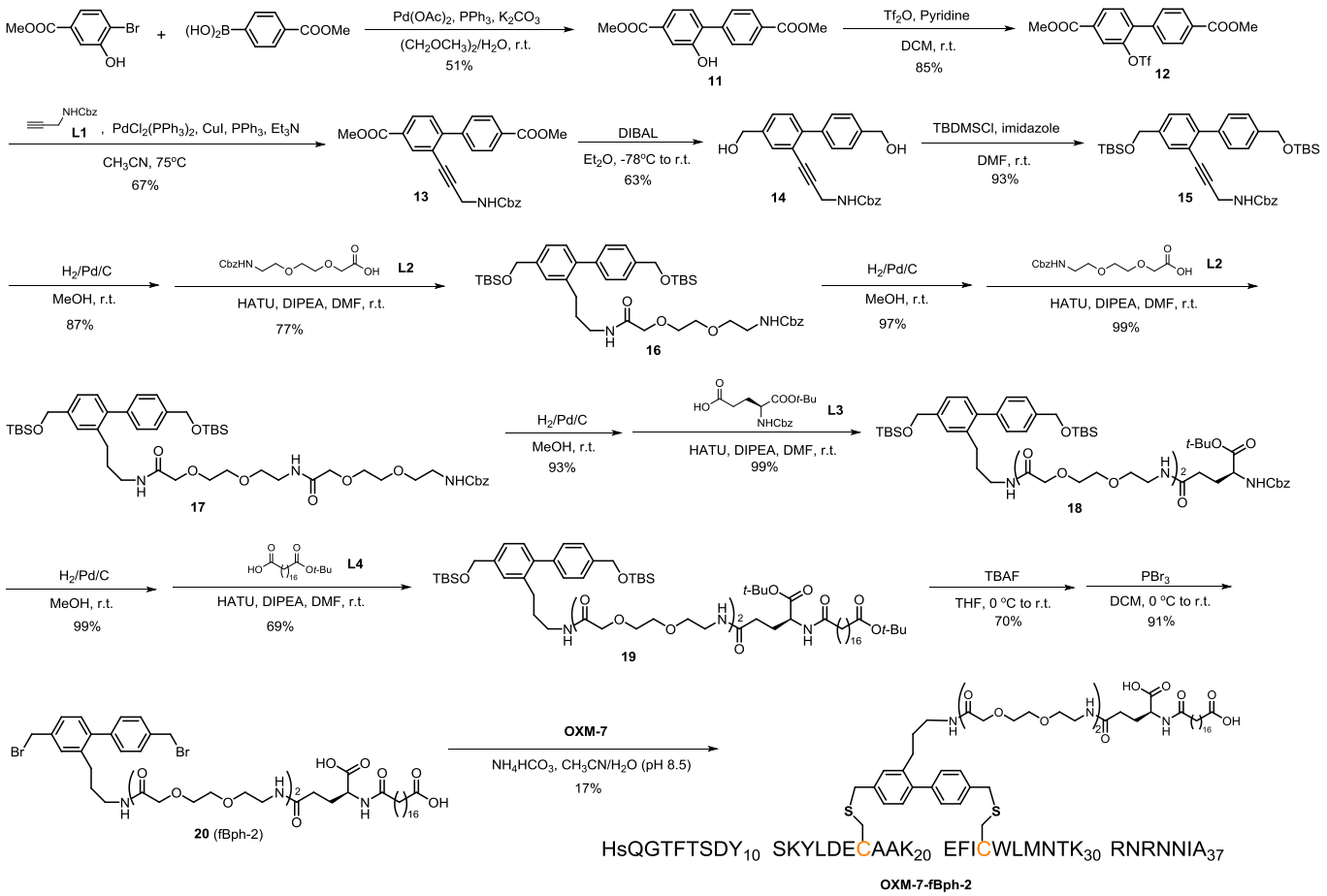

Scheme 2. Synthesis of OXM-7-fBph-2.

300 or Inova-500 MHz spectrometer. Chemical shifts were reported in ppm using either TMS or deuterated solvents as internal standards (TMS, 0.00; $CDCl_3$, 7.26; CD_3OD, 3.31; DMSO-d_6, 2.50). Multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs = broad. ^{13}C NMR spectra were recorded at 75 MHz or 126 MHz, and chemical shifts were reported in ppm using deuterated solvents as internal standards ($CDCl_3$). Electrospray LC-MS analysis was performed using a Finnigan LCQ Advantage IonTrap mass spectrometry coupled with a Surveyor HPLC system. Peptides were purified using a Gilson semi-preparative reverse-phase HPLC system equipped with Phenomenex C_{18} column with a flow rate of 5 mL/min and a gradient of 5–95% acetonitrile/H_2O while monitoring at 220 and 254 nm. Analytical HPLC was performed using Kinetex C_{18} column (250 × 4.6 mm) with a flow rate of 1.0 mL/min and a gradient of 5–95% acetonitrile/H_2O over 15 min with UV–vis detection wavelength set at 220 and 254 nm. The intact peptide masses were derived through deconvoluting charge ladders using ProMass software (Thermo Scientific). High resolution mass spectrometry was performed on a Bruker solariX XR Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS).

4.2. Synthesis of ***OXM-7-fBph-1***

4.2.1. Dimethyl 3-hydroxy-[1,1′-biphenyl]-4,4′-dicarboxylate (**1**)

To a solution of methyl 4-bromo-2-hydroxybenzoate (463 mg, 2 mmol) in $(CH_3OCH_2)_2/H_2O$ (10 mL, 1:1) was added (4-(methoxycarbonyl)phenyl)boronic acid (432 mg, 2.4 mmol), $Pd(OAc)_2$ (23 mg, 0.1 mmol), PPh_3 (26 mg, 0.1 mmol) and K_2CO_3 (829 mg, 6 mmol). The mixture was stirred at room temperature under argon protection for 12 h. The solution was neutralized with 1 N HCl and the aqueous phase was extracted with EtOAc (5 mL × 3). The combined organic layer was wash with H_2O, brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc = 5:1) to afford compound **1** (537 mg, 94%). Yellow solid; ^1H NMR (500 MHz, $CDCl_3$) δ 10.82 (s, 1H), 8.12 (d, J = 8.2 Hz, 2H), 7.92 (d, J = 8.3 Hz, 1H), 7.68 (d, J = 8.2 Hz, 2H), 7.24 (d, J = 1.3 Hz, 1H), 7.15 (dd, J = 8.3, 1.3 Hz, 1H), 3.98 (s, 3H), 3.95 (s, 3H); ESI-MS calcd for $C_{16}H_{15}O_5$ 287.1 [M + H^+], found 287.1.

4.2.2. Dimethyl 3-(((trifluoromethyl)sulfonyl)oxy)-[1,1′-biphenyl]-4,4′-dicarboxylate (**2**)

To a solution of **1** (440 mg, 1.5 mmol) in dichloromethane (10 mL) was added pyridine (593 mg, 7.5 mmol) and Tf_2O (650 mg, 2.3 mmol) at 0 °C. The mixture was stirred at room temperature for 12 h, and then concentrated. The residue was dissolved in EtOAc (10 mL), washed with 1 N HCl, brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc = 4:1) to afford compound **2** (614 mg, 98%). Colorless oil; ^1H NMR (300 MHz, $CDCl_3$) δ 8.22–8.11 (m, 3H), 7.71 (dd, J = 8.2, 1.7 Hz, 1H), 7.68–7.61 (m, 2H), 7.51 (d, J = 1.5 Hz, 1H), 4.00 (s, 3H), 3.96 (s, 3H); ESI-MS calcd for $C_{17}H_{14}F_3O_7S$ 419.0 [M + H^+], found 419.1.

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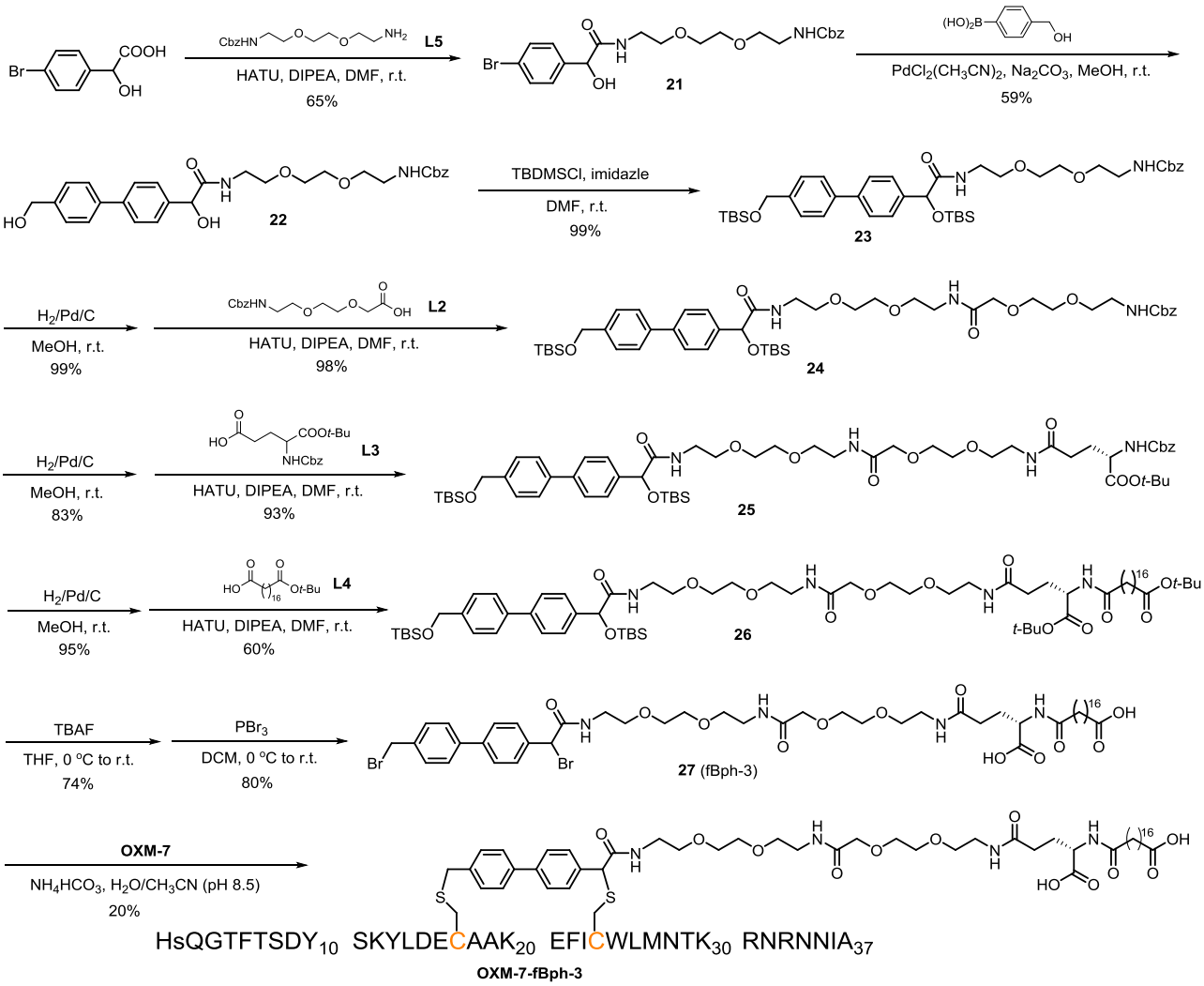

Scheme 3. Synthesis of OXM-7-fBph-3.

*4.2.3. 4,4′-Bis(hydroxymethyl)-[1,1′-biphenyl]-3-yl trifluoromethanesulfonate (**3**)*

To a solution of **2** (614 mg, 1.5 mmol) in Et_2O (10 mL) was added 1.2 M DIBAL in toluene (5 mL, 6 mmol) at −78 °C. The mixture was stirred at room temperature for 3 h. Then 10% potassium sodium tartrate solution was added, and the mixture was stirred for another 1 h. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc = 2:1) to afford compound **3** (530 mg, 99% yield). White solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71–7.60 (m, 2H), 7.60–7.53 (m, 2H), 7.51–7.45 (m, 3H), 4.84 (d, J = 5.6 Hz, 2H), 4.77 (d, J = 4.8 Hz, 2H); ESI-MS calcd for $C_{15}H_{13}F_3O_5SNa$ 385.0 [M + Na^+], found 385.0.

*4.2.4. 4,4′-Bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-3-yl trifluoromethanesulfonate (**4**)*

To a solution of **3** (530 mg, 1.5 mmol) in DMF (8 mL) was added imidazole (89 mg, 11.8 mmol) and TBSCl (888 mg, 5.9 mmol) at 0 °C. The mixture was stirred at room temperature for 1 h, saturated NH_4Cl was added and the aqueous phase was extracted with EtOAc (4 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc = 3:1) to afford compound **4** (754 mg, 87%). Colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (d, J = 8.1 Hz, 1H), 7.61 (dd, J = 8.0, 1.7 Hz, 1H), 7.56–7.48 (m, 2H), 7.44–7.40 (m, 3H), 4.86 (s, 2H), 4.79 (s, 2H), 0.96 (s, 18H), 0.13 (d, J = 4.5 Hz, 12H); ESI-MS calcd for $C_{27}H_{41}F_3O_5SSi_2Na$ 613.2 [M + Na^+], found 613.1.

*4.2.5. Benzyl (3-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-3-yl)prop-2-yn-1-yl)carbamate (**5**)*

To a solution of **4** (605 mg, 1 mmol) in DMSO (8 mL) was added *N*-Cbz-prop-2-yn-1-amine (**L1**) (291 mg, 1.5 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol), PPh_3 (32 mg, 0.12 mmol), K_3PO_4 (255 mg, 1.2 mmol). The mixture was stirred at 80 °C under argon protection for 16 h. Then H_2O was added and the aqueous phase was extracted with Et_2O (4 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc = 4:1) to afford compound **5** (220 mg, 34%). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (s, 1H), 7.57 (s, 2H), 7.53 (d, J = 8.1 Hz, 2H), 7.39–7.29 (m, 7H), 5.15 (s, 2H), 5.01 (s, 1H), 4.87 (s, 2H), 4.78 (s, 2H), 4.28 (s, 2H), 0.97 (s, 18H), 0.12 (s, 12H); ESI-MS calcd for $C_{37}H_{51}NO_4Si_2Na$ 652.3 [M + Na^+], found 652.3.

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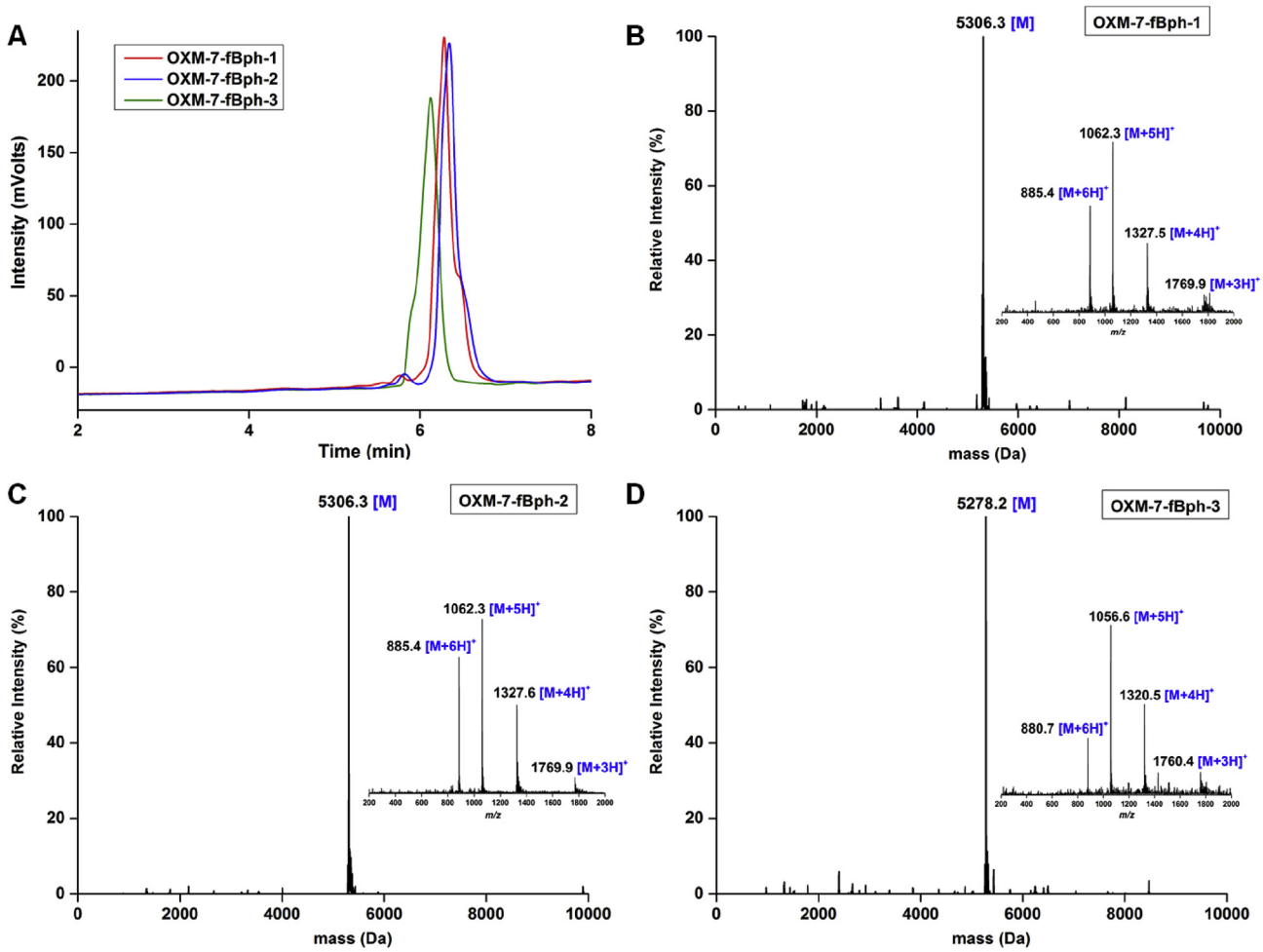

Fig. 3. Characterization of the purity and identity of the stapled OXM peptides. (**A**) HPLC traces of the lipid-modified Bph cross-linked oxyntomodulin analogs. (**B**) Deconvoluted mass spectrum of OXM-7-fBph-1. Calcd mass for $C_{241}H_{367}N_{59}O_{70}S_3$ 5306.6, found 5306.3. (**C**) Deconvoluted mass spectrum of OXM-7-fBph-2. Calcd mass for $C_{241}H_{367}N_{59}O_{70}S_3$ 5306.6, found 5306.3. (**D**) Deconvoluted mass spectrum of OXM-7-fBph-3. Calcd mass for $C_{239}H_{363}N_{59}O_{70}S_3$ 5278.6, found 5278.2.

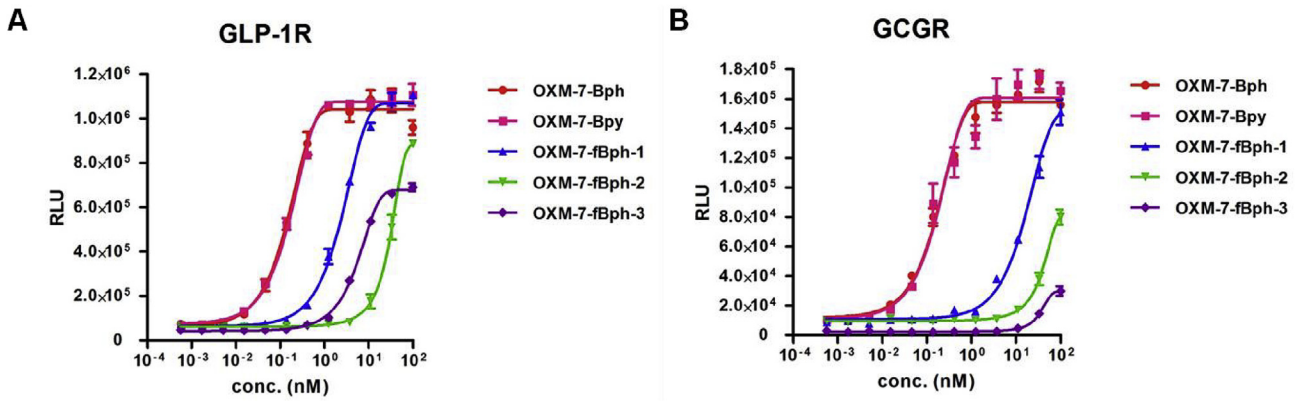

Fig. 4. Agonist activity of the cross-linked oxyntomodulin analogs against GLP-1R (A) and GCGR (B) in a cell-based luciferase reporter assay.

*4.2.6. Benzyl (2-(2-(2-((3-(4,4′-bis(((tert-butyldimethylsilyl)oxy) methyl)-[1,1′-biphenyl]-3-yl)propyl)amino)-2-oxoethoxy)ethoxy) ethyl)carbamate (**6**)*

To a solution of **5** (200 mg, 0.32 mmol) in MeOH (5 mL) was added Pd/C (40 mg, 20%). The mixture was stirred at room temperature under H_2 for 1 h, then filtered and concentrated to afford crude amine (118 mg, 74% yield) as colorless oil, which was directly used for the next step. To a solution of crude amine (118 mg, 0.24 mmol) in DMF (3 mL) was added *N*-Cbz-2-(2-(2-aminoethoxy) ethoxy)acetic acid (**L2**) (86 mg, 0.29 mmol), HATU (110 mg, 0.29 mmol) and DIPEA (124 mg, 0.96 mmol). The mixture was stirred at room temperature for 1 h. Then H_2O was added and the

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Table 1
Agonist activity of the cross-linked oxyntomodulin analogs in the activation of GLP-1R and GCGR.

Name	GLP-1R EC_{50} (nM)	GCGR EC_{50} (nM)
OMX-7-Bph	0.143	0.163
OMX-7-Bpy	0.162	0.186
OMX-7-fBph-1	2.498	24.47
OMX-7-fBph-2	36.71	>80
OMX-7-fBph-3	5.560	44.44

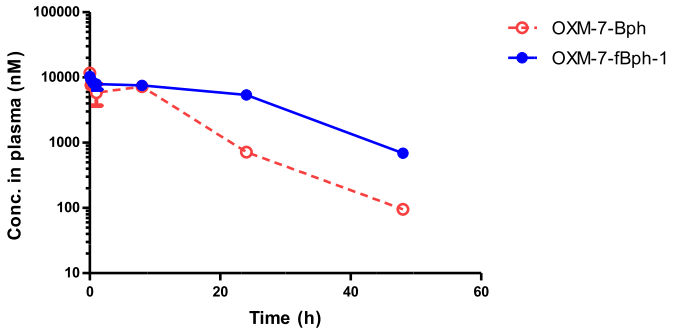

Fig. 5. Comparison of the *in vitro* stability of the lipid-modified OXM-7-fBph-1 vs. OXM-7-Bph in fresh mouse serum. The concentrations of the remaining intact OXM peptides in serum were determined using the GLP-1R activation assay.

aqueous phase was extracted with EtOAc (2 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc = 1:2) to afford compound **6** (100 mg, 54% yield). Colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.55 (d, J = 8.1 Hz, 2H), 7.48–7.41 (m, 2H), 7.41–7.35 (m, 3H), 7.34–7.30 (m, 5H), 6.79 (s, 1H), 5.07 (s, 2H), 4.78 (s, 2H), 4.76 (s, 2H), 3.97 (s, 2H), 3.61 (dd, J = 11.0, 5.5 Hz, 4H), 3.53 (t, J = 5.1 Hz, 2H), 3.42–3.31 (m, 4H), 2.73–2.64 (m, 2H), 1.94–1.81 (m, 2H), 0.95 (d, J = 3.2 Hz, 18H), 0.12 (s, 12H); ESI-MS calcd for $C_{43}H_{66}N_2O_7Si_2Na$ 801.4 [M + Na^+], found 801.4.

4.2.7. *Benzyl (21-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-3-yl)-8,17-dioxo-3,6,12,15-tetraoxa-9,18-diazahenicosyl)carbamate (**7**)*

To a solution of **6** (80 mg, 0.1 mmol) in MeOH (2 mL) was added Pd/C (16 mg, 20%). The mixture was stirred at room temperature under H_2 for 1 h, then filtered and concentrated to afford crude amine (64 mg, 99%) as colorless oil, which was directly used for the next step. To a solution of crude amine (64 mg, 0.1 mmol) in DMF (2 mL) was added *N*-Cbz-2-(2-(2-aminoethoxy)ethoxy)acetic acid (**L2**) (36 mg, 0.12 mmol), HATU (46 mg, 0.12 mmol) and DIPEA (52 mg, 0.4 mmol). The mixture was stirred at room temperature for 1 h. Then H_2O was added and the aqueous phase was extracted with EtOAc (2 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (DCM/MeOH = 15:1) to afford compound **7** (92 mg, 99% yield). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, J = 7.9 Hz, 2H), 7.47 (d, J = 7.7 Hz, 1H), 7.42 (d, J = 7.3 Hz, 1H), 7.38 (d, J = 6.7 Hz, 3H), 7.34–7.28 (m, 5H), 7.13 (s, 1H), 6.77 (s, 1H), 5.51 (s, 1H), 5.08 (s, 2H), 4.78 (s, 2H), 4.76 (s, 2H), 3.95 (s, 4H), 3.60–3.50 (m, 12H), 3.48–3.41 (m, 2H), 3.41–3.30 (m, 4H), 2.75–2.62 (m, 2H), 1.93–1.80 (m, 2H), 0.95 (d, J = 4.6 Hz, 18H), 0.12 (s, 12H); ESI-MS calcd for $C_{49}H_{77}N_3O_{10}Si_2Na$ 946.5 [M + Na^+], found 946.4.

4.2.8. *tert-Butyl 26-((((benzyloxy)carbonyl)amino)-1-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-3-yl)-5,14,23-trioxo-7,10,16,19-tetraoxa-4,13,22-triazaheptacosan-27-oate (**8**)*

To a solution of **7** (92 mg, 0.1 mmol) in MeOH (2 mL) was added Pd/C (18 mg, 20%). The mixture was stirred at room temperature under H_2 for 1 h, filtered and concentrated to afford crude amine (74 mg, 94% yield) as colorless oil, which was directly used for the next step. To a solution of crude amine (68 mg, 0.09 mmol) in DMF (1 mL) was added *N*-Cbz-*O*-*tert*-butyl-glutamic acid (**L3**) (35 mg, 0.1 mmol), HATU (38 mg, 0.1 mmol) and DIPEA (44 mg, 0.34 mmol). The mixture was stirred at room temperature for 1 h. Then H_2O was added and the aqueous phase was extracted with EtOAc (2 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (DCM/MeOH = 15:1) to afford compound **8** (95 mg, 99%). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, J = 8.1 Hz, 2H), 7.49–7.40 (m, 2H), 7.40–7.35 (m, 3H), 7.34–7.29 (m, 5H), 7.05 (s, 1H), 6.82 (s, 1H), 6.50 (s, 1H), 5.08 (s, 2H), 4.78 (s, 2H), 4.76 (s, 2H), 4.22–4.19 (m, 1H), 3.98 (s, 2H), 3.96 (s, 2H), 3.70–3.29 (m, 20H), 2.71 (t, J = 7.9 Hz, 2H), 2.30–2.10 (m, 2H), 1.93–1.88 (m, 2H), 1.56 (s, 9H), 0.95 (d, J = 4.4 Hz, 18H), 0.12 (s, 12H); ESI-MS calcd for $C_{58}H_{92}N_4O_{13}Si_2Na$ 1131.6 [M + Na^+], found 1131.5.

4.2.9. *tert-Butyl 1-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-3-yl)-26-(tert-butoxycarbonyl)-5,14,23,28-tetraoxo-7,10,16,19-tetraoxa-4,13,22,27-tetraazapentatetracontan-45-oate (**9**)*

To a solution of **8** (95 mg, 0.09 mmol) in MeOH (2 mL) was added Pd/C (19 mg, 20%). The mixture was stirred at room temperature under H_2 for 1 h, then filtered and concentrated to afford crude amine (66 mg, 79% yield) as colorless oil, which was directly used for the next step. To a solution of crude amine (65 mg, 0.07 mmol) in DMF (1 mL) was added octadecanedioic acid mono-*tert*-butyl ester (**L4**) (30 mg, 0.08 mmol), HATU (30 mg, 0.08 mmol) and DIPEA (26 mg, 0.2 mmol). The mixture was stirred at room temperature for 1 h. Then H_2O was added and the aqueous phase was extracted with EtOAc (2 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (DCM/MeOH = 15:1) to afford compound **9** (82 mg, 92% yield). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, J = 8.0 Hz, 2H), 7.49–7.41 (m, 2H), 7.40–7.35 (m, 3H), 7.05 (s, 1H), 6.85 (s, 1H), 6.75 (s, 1H), 6.49 (d, J = 7.2 Hz, 1H), 4.78 (s, 2H), 4.76 (s, 2H), 4.45–4.37 (m, 1H), 3.99 (s, 2H), 3.97 (s, 2H), 3.71–3.31 (m, 20H), 2.72 (t, J = 7.8 Hz, 2H), 2.32–2.24 (m, 2H), 2.19 (t, J = 7.4 Hz, 2H), 1.97–1.83 (m, 2H), 1.65–1.59 (m, 6H), 1.45 (s, 9H), 1.44 (s, 9H), 1.37–1.16 (m, 24H), 0.95 (d, J = 4.2 Hz,18H), 0.12 (s, 12H); ESI-MS calcd for $C_{72}H_{126}N_4O_{14}Si_2Na$ 1349.9 [M + Na^+], found 1349.8.

4.2.10. *1-(4,4′-Bis(bromomethyl)-[1,1′-biphenyl]-3-yl)-26-carboxy-5,14,23,28-tetraoxo-7,10,16,19-tetraoxa-4,13,22,27-tetraazapentatetracontan-45-oic acid (**10**, fBph-1)*

To a solution of **9** (69 mg, 0.05 mmol) in THF (1 mL) was added 1 M TBAF in THF (0.13 mL, 0.13 mmol) at 0 °C. The mixture was stirred at room temperature for 2 h. Then H_2O was added and the aqueous phase was extracted with EtOAc (2 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (DCM/MeOH = 10:1) to afford alcohol compound (40 mg, 70% yield) as colorless oil. To a solution of alcohol (40 mg, 0.036 mmol) in DCM (1 mL) was added PBr_3 (49 mg, 0.18 mmol) at 0 °C. The mixture was stirred at room temperature for 2 h, then H_2O was added . The organic layer was

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washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated to afford compound **10** (fBph-1) (36 mg, 90% yield). White solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, J = 8.0 Hz, 2H), 7.46 (d, J = 8.0 Hz, 2H), 7.44–7.37 (m, 3H), 7.17 (s, 1H), 7.12 (s, 1H), 7.04 (s, 1H), 4.59 (s, 2H), 4.55 (s, 2H), 4.52–4.47 (m, 1H), 4.08 (s, 2H), 4.02 (s, 2H), 3.76–3.31 (m, 18H), 2.88–2.78 (m, 2H), 2.53–2.33 (m, 2H), 2.32 (t, J = 7.4 Hz, 2H), 2.23 (t, J = 7.5 Hz, 2H), 2.18–2.04 (m, 2H), 2.05–1.97 (m, 2H), 1.68–1.55 (m, 4H), 1.28–1.22 (m, 24H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.48, 174.72, 173.92, 173.47, 170.84, 170.64, 141.18, 140.86, 140.45, 137.17, 134.67, 131.27, 129.55, 128.30, 127.45, 125.35, 70.79, 70.43, 70.41, 70.25, 70.16, 69.75, 69.73, 69.47, 52.21, 39.58, 38.89, 38.63, 36.26, 33.95, 33.24, 32.30, 31.56, 30.47, 29.68, 29.64, 29.58, 29.50, 29.43, 29.35, 29.30, 29.27, 29.23, 29.14, 29.06, 28.95, 28.22, 25.60, 24.74, 22.63; HRMS (ESI) calcd for $C_{52}H_{81}Br_2N_4O_{12}$ 1113.4197 [M + H^+], found 1113.4212.

4.2.11. OXM-7-fBph-1

To a solution of OXM-7 peptide (10 mg, 0.0023 mmol) in 30 mM NH_4HCO_3 buffer (0.5 mL) was added a solution of fBph-1 (2.5 mg, 0.0023 mmol) in CH_3CN (0.5 mL). The mixture was stirred at room temperature for 3 h. Under ice cooling, acetic acid was subsequently added dropwise to pH 5. Crude cross-linked peptide was purified by preparative reverse phase HPLC (RP-HPLC) to afford the title compound as a white powder after lyophilization (2.0 mg, 17% yield). Calcd mass for $C_{241}H_{367}N_{59}O_{70}S_3$ 5306.6 [M], ESI-MS m/z found 885.4 $[M+6H]^{6+}$, 1062.3 $[M+5H]^{5+}$, 1327.5 $[M+4H]^{4+}$, 1769.9 $[M+3H]^{3+}$; Deconvoluted mass found 5306.3.

4.3. Synthesis of ***OXM-7-fBph-2***

4.3.1. Dimethyl 2-hydroxy-[1,1′-biphenyl]-4,4′-dicarboxylate (*11*)

Compound **11** was prepared using the same procedure as that described for compound **1**. Yield: 51% (220 mg); Yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.16 (d, J = 8.1 Hz, 2H), 7.69 (dd, J = 7.9, 1.1 Hz, 1H), 7.66 (s, 1H), 7.61 (d, J = 8.1 Hz, 2H), 7.35 (d, J = 7.9 Hz, 1H), 3.96 (s, 3H), 3.94 (s, 3H); ESI-MS calcd for $C_{16}H_{15}O_5$ 287.1 [M + H^+], found 287.1.

4.3.2. Dimethyl 2-(((trifluoromethyl)sulfonyl)oxy)-[1,1′-biphenyl]-4,4′-dicarboxylate (*12*)

Compound **12** was prepared using the same procedure as that described for compound **2**. Yield: 85% (373 mg); White solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17–8.03 (m, 3H), 8.06 (s, 1H), 7.60–7.55 (m, 3H), 3.99 (s, 3H), 3.96 (s, 3H); ESI-MS calcd for $C_{17}H_{14}F_3O_7S$ 419.0 [M + H^+], found 419.1.

4.3.3. Dimethyl 2-(3-(((benzyloxy)carbonyl)amino)prop-1-yn-1-yl)-[1,1′-biphenyl]-4,4′-dicarboxylate (*13*)

To a solution of **12** (490 mg, 1.2 mmol) in CH_3CN (10 mL) was added *N*-Cbz-prop-2-yn-1-amine (**L1**) (443 mg, 2.3 mmol), $PdCl_2(PPh_3)_2$ (82 mg, 0.12 mmol), CuI (22 mg, 0.12 mmol), PPh_3 (61 mg, 0.23 mmol), Et_3N (607 mg, 6 mmol). The mixture was stirred at 75 °C under argon protection for 2 h, and then concentrated. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc = 2:1) to afford compound **13** (357 mg, 67% yield). Yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.21 (s, 1H), 8.10 (d, J = 7.9 Hz, 2H), 8.04 (d, J = 8.1 Hz, 1H), 7.64 (d, J = 7.7 Hz, 2H), 7.45 (d, J = 8.1 Hz, 1H), 7.39–7.29 (m, 5H), 5.13 (s, 2H), 4.86 (s, 1H), 4.10 (d, J = 5.0 Hz, 2H), 3.95 (s, 3H), 3.92 (s, 3H); ESI-MS calcd for $C_{27}H_{23}NO_6Na$ 480.2 [M + Na^+], found 480.1.

4.3.4. Benzyl (3-(4,4′-bis(hydroxymethyl)-[1,1′-biphenyl]-2-yl) prop-2-yn-1-yl)carbamate (*14*)

Compound **14** was prepared using the same procedure as that described for compound **3**. Yield: 63% (195 mg); White solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57–7.44 (m, 3H), 7.42–7.29 (m, 9H), 5.11 (s, 2H), 4.82 (s, 1H), 4.70 (s, 2H), 4.69 (s, 2H), 4.07 (d, J = 4.4 Hz, 2H); ESI-MS calcd for $C_{25}H_{23}NO_4Na$ 424.2 [M + Na^+], found 424.2.

4.3.5. Benzyl (3-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-2-yl)prop-2-yn-1-yl)carbamate (*15*)

Compound **15** was prepared using the same procedure as that described for compound **4**. Yield: 93% (287 mg); Brown oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 (d, J = 7.6 Hz, 2H), 7.45 (s, 1H), 7.38–7.29 (m, 9H), 5.12 (s, 2H), 4.79 (s, 1H), 4.76 (s, 2H), 4.73 (s, 2H), 4.10 (d, J = 4.3 Hz, 2H), 0.96 (d, J = 1.1 Hz, 18H), 0.12 (d, J = 4.8 Hz, 12H); ESI-MS calcd for $C_{37}H_{51}NO_4Si_2Na$ 652.3 [M + Na^+], found 652.3.

4.3.6. Benzyl (2-(2-(2-((3-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-2-yl)propyl)amino)-2-oxoethoxy)ethoxy)ethyl)carbamate (*16*)

Compound **16** was prepared using the same procedure as that described for compound **6**. Yield: 67% for 2 steps (240 mg); Yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.28 (m, 5H), 7.25–7.17 (m, 6H), 7.15 (d, J = 8.1 Hz, 1H), 6.60 (s, 1H), 5.07 (s, 2H), 4.78 (s, 2H), 4.75 (s, 2H), 3.90 (s, 2H), 3.60–3.54 (m, 4H), 3.54–3.46 (m, 2H), 3.39–3.29 (m, 2H), 3.18–3.08 (m, 2H), 2.66–2.56 (m, 2H), 1.72–1.60 (m, 2H), 0.96 (d, J = 3.0 Hz, 18H), 0.13 (d, J = 3.4 Hz, 12H); ESI-MS calcd for $C_{43}H_{66}N_2O_7Si_2Na$ 801.4 [M + Na^+], found 801.4.

4.3.7. Benzyl (21-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-2-yl)-8,17-dioxo-3,6,12,15-tetraoxa-9,18-diazahenicosyl)carbamate (*17*)

Compound **17** was prepared using the same procedure as that described for compound **7**. Yield: 96% for 2 steps (151 mg); Yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.27 (m, 6H), 7.23 (d, J = 8.0 Hz, 2H), 7.22–7.17 (m, 2H), 7.15 (d, J = 8.2 Hz, 2H), 6.59 (s, 1H), 5.08 (s, 2H), 4.78 (s, 2H), 4.75 (s, 2H), 3.95 (s, 2H), 3.88 (s, 2H), 3.68–3.32 (m, 16H), 3.15 (q, J = 6.8 Hz, 2H), 2.67–2.55 (m, 2H), 1.73–1.62 (m, 2H), 0.96 (d, J = 3.3 Hz, 18H), 0.13 (d, J = 3.6 Hz, 12H); ESI-MS calcd for $C_{49}H_{77}N_3O_{10}Si_2Na$ 946.5 [M + Na^+], found 946.4.

4.3.8. tert-Butyl 26-(((benzyloxy)carbonyl)amino)-1-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-2-yl)-5,14,23-trioxo-7,10,16,19-tetraoxa-4,13,22-triazaheptacosan-27-oate (*18*)

Compound **18** was prepared using the same procedure as that described for compound **8**. Yield: 92% for 2 steps (176 mg); Yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.28 (m, 7H), 7.24 (d, J = 7.9 Hz, 2H), 7.23–7.19 (m, 2H), 7.15 (d, J = 8.2 Hz, 1H), 7.05 (s, 1H), 6.64 (s, 1H), 6.55 (s, 1H), 5.08 (s, 2H), 4.79 (s, 2H), 4.75 (s, 2H), 4.24–4.16 (m, 1H), 3.97 (s, 2H), 3.91 (s, 2H), 3.68–3.34 (m, 18H), 3.15 (t, J = 6.6 Hz, 2H), 2.68–2.55 (m, 2H), 2.33–2.23 (m, 2H), 2.22–2.11 (m, 1H), 2.00–1.87 (m, 1H), 1.68 (s, 9H), 0.96 (d, J = 3.6 Hz, 18H), 0.13 (d, J = 4.1 Hz, 12H); ESI-MS calcd for $C_{58}H_{92}N_4O_{13}Si_2Na$ 1131.6 [M + Na^+], found 1131.5.

4.3.9. tert-Butyl 1-(4,4′-bis(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-2-yl)-26-(tert-butoxycarbonyl)-5,14,23,28-tetraoxo-7,10,16,19-tetraoxa-4,13,22,27-tetraazapentatetracontan-45-oate (*19*)

Compound **26** was prepared using the same procedure as that described for compound **9**. Yield: 68% for 2 steps (128 mg); Yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 (d, J = 7.5 Hz, 2H), 7.24 (d, J = 8.0 Hz, 2H), 7.20 (d, J = 6.5 Hz, 2H), 7.15 (d, J = 8.5 Hz, 1 H), 7.06 (s, 1H), 6.78 (s, 1H), 6.65 (s, 1H), 6.51 (d, J = 7.2 Hz, 1H), 4.79 (s, 2H), 4.76 (s, 2H), 4.43–4.35 (m, 1H), 3.98 (s, 2H), 3.92 (s, 2H), 3.68–3.41 (m, 20H), 3.20–3.13 (m, 2H), 2.63 (t, J = 7.8 Hz, 2H), 2.30–2.22 (m, 2H), 2.20 (t, J = 7.5 Hz, 4H), 1.71–1.52 (m, 4H), 1.45 (s, 9H), 1.44 (s, 9H), 1.34–1.21 (m, 24H), 0.96 (d, J = 3.5 Hz, 18H), 0.13 (d, J = 4.5 Hz,

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12H); ESI-MS calcd for $C_{72}H_{126}N_4O_{14}Si_2Na$ 1349.9 [M + Na^+], found 1349.8.

4.3.10. 1-(4,4′-Bis(bromomethyl)-[1,1′-biphenyl]-2-yl)-26-carboxy-5,14,23,28-tetraoxo-7,10,16,19-tetraoxa-4,13,22,27-tetraazapentatetracontan-45-oic acid (**20**, fBph-2)

Compound **20** was prepared using the same procedure as that described for compound **10**. Yield: 64% for 2 steps (50 mg); White Solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 (d, J = 7.9 Hz, 2H), 7.32 (s, 1H), 7.30–7.18 (m, 3H), 7.15 (d, J = 7.8 Hz, 1H), 6.85 (s, 1H), 4.56 (s, 2H), 4.52 (s, 2H), 4.50–4.44 (m, 1H), 4.02 (s, 2H), 3.99 (s, 2H), 3.71–3.34 (m, 16H), 3.19 (q, J = 6.5 Hz, 2H), 2.66–2.56 (m, 2H), 2.53–2.37 (m, 2H), 2.32 (t, J = 7.4 Hz, 2H), 2.25 (t, J = 7.5 Hz, 2H), 2.22–2.10 (m, 1H), 2.10–1.99 (m, 1H), 1.76–1.67 (m, 2H), 1.67–1.53 (m, 4H), 1.36–1.16 (m, 24H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.51, 174.70, 173.87, 173.54, 170.80, 170.41, 141.23, 141.16, 139.23, 137.21, 136.64, 130.63, 129.83, 129.49, 128.96, 126.74, 70.78, 70.72, 70.43, 70.31, 70.23, 70.10, 69.69, 69.49, 52.19, 39.55, 38.69, 38.60, 36.27, 34.02, 33.41, 33.31, 32.32, 31.56, 30.77, 30.19, 29.67, 29.43, 29.39, 29.37, 29.33, 29.26, 29.22, 29.20, 29.12, 29.00, 28.17, 25.62, 24.78, 22.63; HRMS (ESI) calcd for $C_{52}H_{81}Br_2N_4O_{12}$ 1113.4197 [M + H^+], found 1113.4384.

4.3.11. OXM-7-fBph-2

Peptide **OXM-7-fBph-2** was prepared using the same procedure as that described for **OXM-7-fBph-1**. Yield: 17% (2 mg); White solid; Calcd mass for $C_{241}H_{367}N_{59}O_{70}S_3$ 5306.6 [M], ESI-MS m/z found 885.4 $[M+6H]^{6+}$, 1062.3 $[M+5H]^{5+}$, 1327.6 $[M+4H]^{4+}$, 1769.9 $[M+3H]^{3+}$; Deconvoluted mass found 5306.3.

4.4. Synthesis of ***OXM-7-fBph-3***

4.4.1. Benzyl (2-(2-(2-(2-(4-bromophenyl)-2-hydroxyacetamido)ethoxy)ethoxy)ethyl)carbamate (**21**)

To a solution of 2-(4-bromophenyl)-2-hydroxyacetic acid (200 mg, 0.87 mmol) in DMF (6 mL) was added 2,2'-(ethane-1,2-diylbis(oxy))diethanamine mono-Cbz carbamate (**L5**) (282 mg, 1 mmol), HATU (329 mg, 0.87 mmol) and DIPEA (225 mg, 1.74 mmol). The mixture was stirred at room temperature for 1 h. Then H_2O was added and the aqueous phase was extracted with EtOAc (4 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (DCM/MeOH = 20:1) to afford compound **21** (280 mg, 65% yield). Light yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.50–7.27 (m, 9H), 6.82 (s, 1H), 5.28 (s, 1H), 5.12–5.03 (m, 3H), 3.66–3.44 (m, 9H), 3.43–3.31 (m, 4H); ESI-MS calcd for $C_{22}H_{28}BrN_2O_6$ 495.1 [M + H^+], found 495.0.

4.4.2. Benzyl (2-(2-(2-(2-hydroxy-2-(4'-(hydroxymethyl)-[1,1′-biphenyl]-4-yl)acetamido)ethoxy)ethoxy)ethyl)carbamate (**22**)

To a solution of **21** (180 mg, 0.36 mmol) in MeOH (5 mL) was added (4-(hydroxymethyl)phenyl)boronic acid (78 mg, 0.51 mmol), $PdCl_2(CH_3CN)_2$ (1 mg, 0.004 mmol), and Na_2CO_3 (76 mg, 0.72 mmol). The mixture was stirred at room temperature under argon protection for 2 h, then concentrated. The residue was purified by silica gel flash column chromatography (DCM/MeOH = 15:1) to afford compound **22** (110 mg, 59% yield). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58–7.50 (m, 4H), 7.44 (dd, J = 19.0, 7.5 Hz, 4H), 7.37–7.27 (m, 5H), 6.83 (s, 1H), 5.13–4.99 (m, 3H), 4.73 (s, 2H), 3.60–3.45 (m, 10H), 3.44–3.28 (m, 4H); ESI-MS calcd for $C_{29}H_{34}N_2O_7Na$ 545.2 [M + Na^+], found 545.3.

4.4.3. Benzyl (5-(4'-(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-4-yl)-2,2,3,3-tetramethyl-6-oxo-4,10,13-trioxa-7-aza-3-silapentadecan-15-yl)carbamate (**23**)

Compound **23** was prepared using the same procedure as that described for compound **4**. Yield: 99% (127 mg); Color oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56–7.48 (m, 6H), 7.39–7.29 (m, 7H), 7.22 (s, 1H), 5.23 (s, 1H), 5.12 (s, 1H), 5.10 (s, 2H), 4.78 (s, 2H), 3.61–3.49 (m, 8H), 3.43–3.30 (m, 4H), 0.95 (d, J = 5.0 Hz, 18H), 0.11 (d, J = 4.9 Hz, 12H); ESI-MS calcd for $C_{41}H_{63}N_2O_7Si_2$ 751.4 [M + H^+], found 751.1.

4.4.4. Benzyl (5-(4'-(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-4-yl)-2,2,3,3-tetramethyl-6,17-dioxo-4,10,13,19,22-pentaoxa-7,16-diaza-3-silatetracosan-24-yl)carbamate (**24**)

Compound **24** was prepared using the same procedure as that described for compound **7**. Yield: 97% for 2 steps (140 mg); Light yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56–7.48 (m, 6H), 7.40–7.29 (m, 7H), 7.19 (s, 1H), 5.54 (s, 1H), 5.12 (s, 1H), 5.10 (s, 2H), 4.78 (s, 2H), 3.98 (s, 2H), 3.67–3.43 (m, 16H), 3.42–3.29 (m, 4H), 0.95 (d, J = 3.4 Hz, 18H), 0.11 (d, J = 4.3 Hz, 12H); ESI-MS calcd for $C_{47}H_{73}N_3O_{10}Si_2Na$ 918.5 [M + Na^+], found 918.5.

4.4.5. tert-Butyl 29-(((benzyloxy)carbonyl)amino)-5-(4'-(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-4-yl)-2,2,3,3-tetramethyl-6,17,26-trioxo-4,10,13,19,22-pentaoxa-7,16,25-triaza-3-silatriacontan-30-oate (**25**)

Compound **25** was prepared using the same procedure as that described for compound **8**. Yield: 77% for 2 steps (110 mg); Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57–7.47 (m, 6H), 7.40–7.28 (m, 7H), 7.23 (s, 1H), 7.09 (s, 1H), 6.49 (s, 1H), 5.73 (d, J = 7.5 Hz, 1H), 5.12 (s, 1H), 5.09 (s, 2H), 4.78 (s, 2H), 4.25–4.17 (m, 1H), 4.00 (s, 2H), 3.67–3.46 (m, 16H), 3.45–3.30 (m, 4H), 2.31–2.23 (m, 2H), 2.23–2.11 (m, 1H), 1.99–1.88 (m, 1H), 1.45 (s, 9H), 0.95 (d, J = 2.3 Hz, 18H), 0.12 (d, J = 2.9 Hz, 12H); ESI-MS calcd for $C_{56}H_{88}N_4O_{13}Si_2Na$ 1103.6 [M + Na^+], found 1103.5.

4.4.6. tert-Butyl 29-(tert-butoxycarbonyl)-5-(4'-(((tert-butyldimethylsilyl)oxy)methyl)-[1,1′-biphenyl]-4-yl)-2,2,3,3-tetramethyl-6,17,26,31-tetraoxo-4,10,13,19,22-pentaoxa-7,16,25,30-tetraaza-3-silatritriacontan-33-oate (**26**)

Compound **26** was prepared using the same procedure as that described for compound **9**. Yield: 57% for 2 steps (43 mg); Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57–7.52 (m, 4H), 7.50 (d, J = 8.2 Hz, 2H), 7.38 (d, J = 8.3 Hz, 2H), 7.24 (s, 1H), 7.10 (s, 1H), 6.72 (s, 1H), 6.56–6.50 (m, 1H), 5.12 (s, 1H), 4.78 (s, 2H), 4.45–4.37 (m, 1H), 4.00 (s, 2H), 3.70–3.65 (m, 2H), 3.64–3.47 (m, 14H), 3.46–3.31 (m, 4H), 2.31–2.24 (m, 2H), 2.23–2.17 (m, 4H), 2.16–2.09 (m, 1H), 1.95–1.86 (m, 1H), 1.64–1.54 (m, 4H), 1.45 (s, 9H), 1.44 (s, 9H), 1.32–1.22 (m, 24H), 0.96 (s, 18H), 0.12 (s, 12H); ESI-MS calcd for $C_{70}H_{122}N_4O_{14}Si_2Na$ 1321.9 [M + Na^+], found 1321.9.

4.4.7. 1-Bromo-1-(4'-(bromomethyl)-[1,1′-biphenyl]-4-yl)-25-carboxy-2,13,22,27-tetraoxo-6,9,15,18-tetraoxa-3,12,21,26-tetraazanonacosan-29-oic acid (**27**, fBph-3)

Compound **27** was prepared using the same procedure as that described for compound **10**. Yield: 59% for 2 steps (20 mg); White Solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.60–7.49 (m, 6H), 7.49–7.40 (m, 2H), 7.22 (s, 1H), 7.16 (s, 1H), 6.16 (s, 1H), 5.73 (d, J = 7.5 Hz, 1H), 5.55 (s, 1H), 4.54 (s, 2H), 4.51–4.45 (m, 1H), 4.04 (s, 2H), 3.70–3.27 (m, 20H), 2.45–2.36 (m, 2H), 2.35–2.28 (m, 4H), 2.18–2.10 (m, 1H), 2.09–1.98 (m, 1H), 1.68–1.55 (m, 4H), 1.38–1.16 (m, 24H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.52, 173.78, 173.66, 170.87, 168.81, 167.59, 137.25, 137.10, 136.62, 129.58, 129.55, 129.00, 127.48, 127.45, 70.85, 70.50, 70.21, 70.14, 70.07, 69.74, 69.59, 69.36, 50.35, 40.10, 39.55, 38.75, 36.31, 34.00, 33.90, 33.17, 32.45, 29.69, 29.64, 29.35, 29.29, 29.21, 29.17, 29.14, 29.08, 29.04, 29.02, 28.91, 25.59, 24.74, 24.12,

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19.76; HRMS (ESI) calcd for $C_{50}H_{77}Br_2N_4O_{12}$ 1085.3884 [M + H^+], found 1085.4043.

4.4.8. OXM-7-fBph-3

Peptide **OXM-7-fBph-3** was prepared using the same procedure as that described for **OXM-7-fBph-1**. Yield: 20% (2.4 mg); White solid; Calcd mass for $C_{239}H_{363}N_{59}O_{70}S_3$ 5278.6 [M], ESI-MS *m/z* found 880.7 $[M+6H]^{6+}$, 1056.6 $[M+5H]^{5+}$, 1320.5 $[M+4H]^{4+}$, 1760.4 $[M+3H]^{3+}$; Deconvoluted mass found 5278.2.

4.5. Generation of CRE-Luc stable cell line overexpressing GLP-1R or GCGR

HEK293 cells were infected with lentivirus encoding firefly luciferase gene under the control of cAMP responsive element (CRE) promoter (Qiagen, The Netherlands) and then were selected using 1 μg/mL puromycin (Life Technologies, Carlsbad) for 1 week. The surviving cells (referred to as CRE-HEK293) were expanded and then transfected with a G418 selective mammalian expression plasmid encoding human GLP-1R or GCGR. In brief, GLP-1R or GCGR plasmid was transfected into CRE-HEK293 cells using Lipofectamine 2000 and selected with 400 μg/mL Geneticin (Life Technologies, Carlsbad, CA). Single colony stable cell line overexpressing CRE-luciferase and GLP-1R (HEK293-GLP-1R-CRE) or GCGR (HEK293-GCGR-CRE) was then established for *in vitro* activity assay.

4.6. In vitro GLP-1R or GCGR activation reporter assay

HEK293-GLP-1R-CRE cells or HEK293-GCGR-CRE cells were seeded in 384-well plates at a density of 5000 cells per well and cultured for 18 h in DMEM with 10% FBS at 37 °C and 5% CO_2. Cells were treated with peptides in a dose dependent manner for 16 h, and receptor activation was reported by luminescence intensities, using One-Glo (Promega, WI) luciferase reagent following manufacturer's instruction. The EC_{50} of each peptide was determined using GraphPad Prism 6 software (GraphPad, San Diego, CA). The assay was performed in triplicate, and the results were obtained with three independent experiments.

4.7. In vitro serum stability

To determine serum stability, peptides were incubated with fresh mouse serum at 37 °C. The incubations were initiated with the final peptide concentration of 10 μM. Aliquots of 20 μl mixture were taken at 0 min, 10 min, 30 min, 1 h, 8 h, 24 h and 48 h, and the amount of intact peptides was analyzed by the CRE reporter assay. Briefly, HEK293 cells expressing the GLP-1R were seeded overnight in a white 384-well plate at 5000 cells per well in 20 μl DMEM medium supplemented with 10% FBS in a humidified 37 °C, 5% CO_2 incubator. Then, 5 μl peptide (prepared as 5 × solution in OPTI-MEM) were added to each well and incubated at 37 °C. The following day, 10 μl/well Bright-Glo Luciferase reagent (Promega) were added and incubation was continued at room temperature for 10 min before luminescence acquisition using a luminometer (Envision). The measurements were performed in duplicate and the average concentrations were plotted using GraphPad Prism 6 software (GraphPad, San Diego, CA).

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References

[1] D.J. Drucker, Nat. Clin. Pract. Endocrinol. Metabol. 1 (2005) 22–31.
[2] X. Du, J.R. Kosinski, J. Lao, X. Shen, A. Petrov, G.G. Chicchi, G.J. Eiermann, A. Pocai, Am. J. Physiol. Endocrinol. Metab. 303 (2012) E265–E271.
[3] A. Pocai, Mol. Metabol. 3 (2014) 241–251.
[4] K. Fosgerau, T. Hoffmann, Drug Discov. Today 20 (2015) 122–128.
[5] V. Azzarito, K. Long, N.S. Murphy, A. Wilson, J. Nat. Chem. 5 (2013) 161–173.
[6] L.D. Walensky, G.H. Bird, J. Med. Chem. 57 (2014) 6275–6288.
[7] P.M. Cromm, J. Spiegel, T.N. Grossmann, ACS Chem. Biol. 10 (2015) 1362–1375.
[8] D.P. Fairlie, A. Dantas de Araujo, Biopolymers 106 (2016) 843–852.
[9] A. Muppidi, X. Li, J. Chen, Q. Lin, Bioorg. Med. Chem. Lett 21 (2011) 7412–7415.
[10] A. Muppidi, Z. Wang, X. Li, J. Chen, Q. Lin, Chem. Commun. 47 (2011) 9396–9398.
[11] A. Muppidi, K. Doi, S. Edwardraja, E.J. Drake, A.M. Gulick, H.G. Wang, Q. Lin, J. Am. Chem. Soc. 134 (2012) 14734–14737.
[12] A. Muppidi, K. Doi, C.P. Ramil, H.G. Wang, Q. Lin, Tetrahedron 70 (2014) 7740–7745.
[13] A. Muppidi, H. Zhang, F. Curreli, N. Li, A.K. Debnath, Q. Lin, Bioorg. Med. Chem. Lett 24 (2014) 1748–1751.
[14] A. Muppidi, H. Zou, P.Y. Yang, E. Chao, L. Sherwood, V. Nunez, A.K. Woods, P.G. Schultz, Q. Lin, W. Shen, ACS Chem. Biol. 11 (2016) 324–328.
[15] S. Runge, H. Thogersen, K. Madsen, J. Lau, R. Rudolph, J. Biol. Chem. 283 (2008) 11340–11347.
[16] C.Q. Pan, J.M. Buxton, S.L. Yung, I. Tom, L. Yang, H. Chen, M. MacDougall, A. Bell, T.H. Claus, K.B. Clairmont, J.P. Whelan, J. Biol. Chem. 281 (2006) 12506–12515.
[17] B.P. Ward, N.L. Ottaway, D. Perez-Tilve, D. Ma, V.M. Gelfanov, M.H. Tschop, R.D. Dimarchi, Mol. Metabol. 2 (2013) 468–479.
[18] J. Lau, P. Bloch, L. Schaffer, I. Pettersson, J. Spetzler, J. Kofoed, K. Madsen, L.B. Knudsen, J. McGuire, D.B. Steensgaard, H.M. Strauss, D.X. Gram, S.M. Knudsen, F.S. Nielsen, P. Thygesen, S. Reedtz-Runge, T. Kruse, J. Med. Chem. 58 (2015) 7370–7380.
[19] P.Y. Yang, H. Zou, E. Chao, L. Sherwood, V. Nunez, M. Keeney, E. Ghartey-Tagoe, Z. Ding, H. Quirino, X. Luo, G. Welzel, G. Chen, P. Singh, A.K. Woods, P.G. Schultz, W. Shen, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 4140–4145.
[20] P.Y. Yang, H. Zou, C. Lee, A. Muppidi, E. Chao, Q. Fu, X. Luo, D. Wang, P.G. Schultz, W. Shen, J. Med. Chem. 61 (2018) 3218–3223.
[21] H. Teller, M. Corbet, L. Mantilli, G. Gopakumar, R. Goddard, W. Thiel, A. Furstner, J. Am. Chem. Soc. 134 (2012) 15331–15342.
[22] M. Adamczyk, J.R. Fishpaugh, M. Thiruvazhi, Org. Prep. Proced. Int. 34 (2002) 326–331.
[23] M. Keller, S. Teng, G. Bernhardt, A. Buschauer, ChemMedChem 4 (2009) 1733–1745.
[24] G. Filippini, M. Nappi, P. Melchiorre, Tetrahedron 71 (2015) 4535–4542.
[25] R.H. Vekariya, R. Liu, J. Aube, Org. Lett. 16 (2014) 1844–1847.
[26] C.S. Fishburn, J. Pharmaceut. Sci. 97 (2008) 4167–4183.

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