Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Pyrazolone Ligation-Mediated Versatile Sequential Bioconjugations

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1. General Remarks on Materials and Methods

1.1 Abbreviations

DCM, dichloromethane; DIC, *N, N'*-diisopropylcarbodiimide; DMF, dimethylformamide, ESI-MS, electrospray ionization mass spectrometry; ESI-TOP, electrospray ionization time-of-flight; equiv, equivalent; HCl, hydrochloric acid; HFIP, hexafluoro-2-propanol; HOSu, *N*-hydroxysuccinimide; H_2O, water; LC-MS, liquid chromatography mass spectrometry; MBHA, 4-Methylbenzhydrylamine; MeCN, acetonitrile; MeOH, methanol; MS, mass spectrometry; $NaIO_4$, sodium periodate; Na_2SO_4, sodium sulfate; Oxyma, ethyl cyanohydroxyiminoacetate; RP-HPLC, reverse-phase high performance liquid chromatography; rt, room temperature; SPPS, solid-phase peptide synthesis; *t*Bu, *tert*-butyl; TIS, triisopropylsilane; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; UPLC-MS, Ultra performance liquid chromatography-mass spectrometry.

1.2 Materials and Methods

Reagents and solvents were obtained and used without purification unless specified from the commercial sources*: Sigma-Aldrich, Acros Organics, TCI America, 1-Click Chemistry, Fisher Scientific, VWR Internationals, Santa Cruz Biotechnology, Combi Blocks, BDH and Alfa Aesar*. Orthogonally protected Fmoc-amino acids were purchased from Iris Biotech as pre-weighed cartridges and SPPS resins were purchased from NovaBioChem. Aqueous buffers were prepared freshly using Millipore Grade I water. Mettler Toledo pH meter was used to adjust the final pH. The reaction mixtures for small molecule was heated and stirred using IKA IKAMAG RET Basic Stainless-Steel Hotplate Stirrer. The reactions with peptide were vortexed (200 rpm, rt) and was incubated at 25 °C or 37 °C (VWR Gravity Convection Incubator, Basics 120V). TLC was performed on silica gel coated with aluminum TLC plates (Merck, TLC Silica gel 60 F254). The compounds were visualized using a UV lamp (254 nm) and Potassium Permanganate stain. Organic solvents were removed by BUCHI rotavapor R-210/215, whereas aqueous samples were lyophilized by CHRiST APLHA 3-4 LSC Basic. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Aeon 400 MHz NMR spectrometer. ^{1}H NMR spectra were referenced to TMS (0 ppm), $CDCl_3$ (7.26 ppm) whereas ^{13}C NMR spectra referenced to $CDCl_3$ (77.16 ppm). Peak multiplicities are designated by the following abbreviations: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. Chemical shifts (δ) are reported in ppm relative to solvent residual peaks and coupling constants are reported in Hz. All the NMR spectra were recorded at 298 K. For LC-MS and HR-MS analysis, MS detection was obtained using Waters Xevo G2-XS QToF mass spectrometer in ESI+ mode. Preparative HPLC was performed using a Waters Prep 150 LC system equipped with a Waters XSelect CSH C18 column (19 x 250 mm, 5 µm) at a flow rate of 20 mL/min (Buffer A: 89.9% water / 10% acetonitrile with 0.1% TFA; Buffer B: 89.9% acetonitrile / 10% water with 0.1% TFA).

A 50 mM sodium acetate buffer pH 4.5 or a 50 mM sodium phosphate buffer pH 6.0 or a 50 mM sodium phosphate buffer pH 7.4 was prepared directly before each experiment.

1.3 Automated Solid-Phase Peptide Synthesis

The automated SPPS was performed on a Symphony X peptide synthesizer (Gyros Biotechnologies). Peptides were synthesized using Fmoc-SPPS chemistry on rink amide

MBHA resin (100-200 mesh, ~0.34 mmol/g loading). Couplings were carried out for 2-4 h using Fmoc amino acid (1.2 mmol/g loading, 12 equiv.), DIC (12 equiv.), Oxyma (12 equiv.). Fmoc protecting group was removed using 20% piperidine in DMF.

Peptides were cleaved from the resin by treatment with freshly made solution of TFA/TIS/H_2O (95:2.5:2.5, v/v/v, 15 mL) or TFA/H_2O (97.5:2.5, v/v, 15 mL) for 3-4 h at room temperature. The eluted cleavage solution was precipitated in cold diethyl ether. The crude peptide samples were agitated on a vortex and spun in a centrifuge. The supernatant was removed and the crude peptide pellet was washed with cold ether twice. The crude peptide pellet was dissolved in 10% MeCN/H_2O and then filtered via a 0.2 μm filter unit. The resulting solution was loaded onto a preparative RP-HPLC column and resolved using one of the following methods: Method A (0-40 min, 15-35% B, flow rate 20 mL/min), Method B (0-40 min, 18-38% B, flow rate 20 mL/min) Method C (0-40 min, 20-40% B, flow rate 20 mL/min), Method D (0-40 min, 10-40% B, flow rate 5 mL/min), and Method E (0-40 min, 20-50% B, flow rate 5 mL/min). Buffer A: 89.9% water / 10% acetonitrile with 0.1% TFA; Buffer B: 89.9% acetonitrile / 10% water with 0.1% TFA.

2. Reaction Optimization

2.1 General procedure for screening β-ketoester with hydrazine (Figure 2a). A stock solution of hydrazine (2.3 μL, 75.00 μmol, 200 μL buffer pH 4.5) was prepared before the experiment. A 4 mL vial equipped with a stir bar was charged with β-ketoester (5.00 μmol, 1.0 equiv.), MeOH (700 μL), 50 mM buffer solution (330 μL, pH 4.5) and 20 μL stock solution of hydrazine (7.5 μmol, 1.5 equiv.) respectively. The reaction mixture, with final concentration of β-keto ester (4.8 mM) and hydrazine (7.1 mM), was stirred (150 rmp) at 30 °C. Aliquots were taken at 2 h and 24 h for LC-MS analysis.

2.2 Procedure for the reaction of methylhydrazine or (1-phenylethyl)hydrazine with methyl-3-oxo-5-phenylpentanoate (Figure S1). A stock solution hydrazine (15.00 μmol, 400 μL MeOH) was prepared before the experiment. A 4 mL vial was equipped with a stir bar was charged with methyl-3-oxo-5-phenylpentanoate (1.0 mg, 5.00 μmol, 1.0 equiv.), MeOH (500 μL), 50 mM buffer solution (350 μL, pH 4.5 or pH 7.4) and 200 μL stock solution of hydrazine (7.5 μmol, 1.5 equiv) respectively. The reaction mixture, with final concentration of methyl-3-oxo-5-phenylpentanoate (4.8 mM) and hydrazine (7.2 mM), was stirred (150 rmp) at rt. Aliquots were taken at the indicated time points for LC-MS analysis.

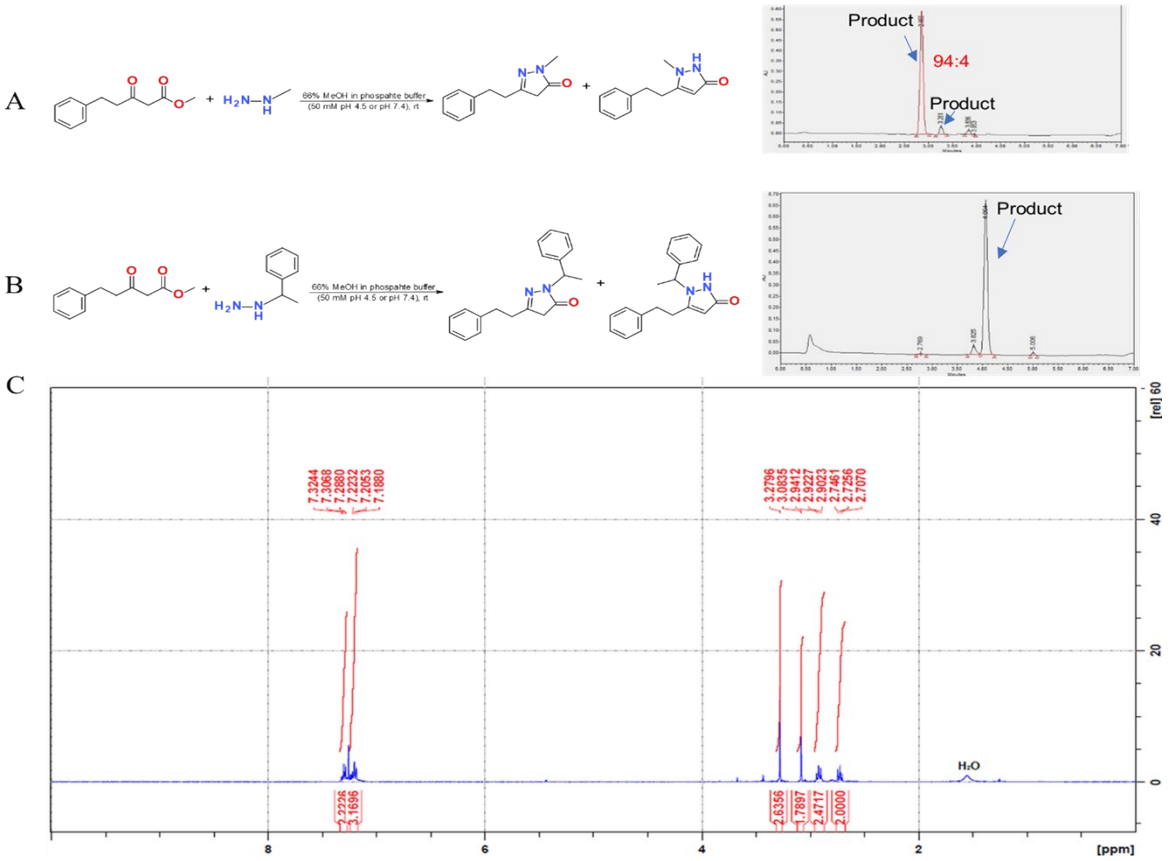
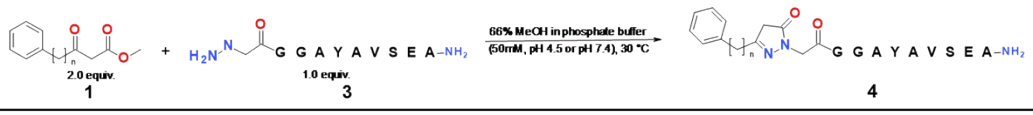
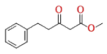
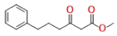
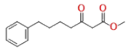


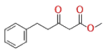
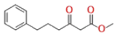
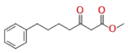
Figure S1. A. Reaction of methylhydrazine with methyl-3-oxo-5-phenylpentanoate. **B.** Reaction of (1-phenylethyl)hydrazine with methyl-3-oxo-5-phenylpentanoate. **C.** NMR trace of the crude reaction extract of methylhydrazine with methyl-3-oxo-5-phenylpentanoate.

2.3 General procedure for the optimization of β-ketoester with hydrazine-bearing peptide **3** (Table S1 and Figure S2). A stock solution of β-ketoester (9.0 µmol, 750 uL MeOH) was prepared before the experiment. A 4 mL vial equipped with a stir bar was charged with hydrazine-bearing peptide **3** (1.3 mg, 1.50 µmol, 1.0 equiv.), 50 mM buffer solution (350 µL, pH 4.5 or pH 7.4), MeOH (450 µL), and 250 µL stock solution of β-ketoester (3.0 µmol, 2.0 equiv.) respectively. The reaction mixture, with final concentration of **3** (1.4 mM) and β-keto ester (2.9 mM), was stirred (150 rpm) at 30 °C. Aliquots were taken at the indicated time points for LC-MS analysis.

Table S1. Optimization of β-ketoester with hydrazine-bearing peptide **3.**



Entry	pH	R^1	20min Yield	40min Yield	60min Yield
A	4.5		48%	70%	74%
B	4.5		52%	62%	65%
C	4.5		42%	49%	62%
D	4.5		45%	51%	60%

Entry	pH	R^1	3h Yield	6h Yield	30h Yield
E	7.4		17%	30%	64%
F	7.4		15%	28%	61%
G	7.4		19%	27%	60%
H	7.4		16%	22%	56%

All reactions were performed in 1.50 μmol scale. Yields were determined by LC-MS of the crude reaction mixture.

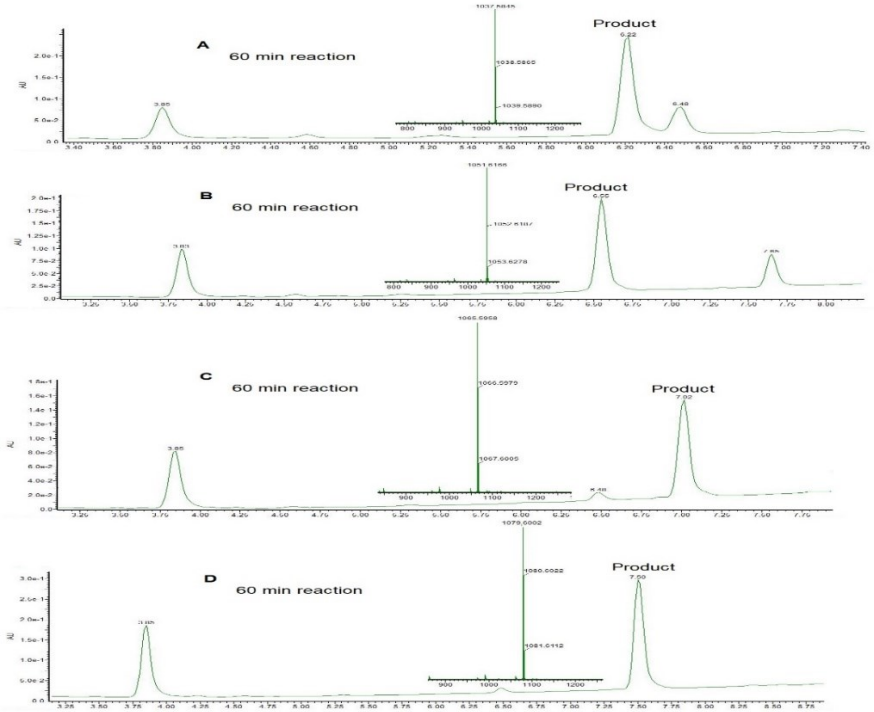


Figure S2. UPLC-MS trace for the reaction at pH 4.5 on Table S1 (the peak at 3.85 min was the starting hydrazine peptide, the 3rd peak (minor) possessed different mass from the desired pyrazolone product).

2.4 General procedure for the optimization of hydrazine structure with β-ketoester-bearing peptide **5** (Table S2 and Figure S3). A stock solution of hydrazine-bearing peptide (7.2 mg, 7.50 µmol, 100 µL DMSO) and β-ketoester-bearing peptide **5** (3.00 µmol, 40 µL DMSO) were prepared before the experiment. A 4 mL vial equipped with a stir bar was charged with 20 µL stock solution of hydrazine-bearing peptide (1.50 µmol, 1.0 equiv.), MeOH (710 µL), 50 mM buffer solution (350 µL, pH 6.0) and 20 µL stock solution β-ketoester-bearing peptide **5** (1.50 µmol, 1.0 equiv.) respectively. The reaction mixture, with final concentration of hydrazine-bearing peptide (1.4 mM) and **5** (1.4 mM), was stirred (150 rpm) at 30 °C. Aliquots were taken at the indicated time points for LC-MS analysis.

Table S2. Optimization of hydrazine structure with β-keto ester-bearing peptide **5.**

5 **7**

Entry	pH	Hydrazine	1h Yield	2hYield	4h Yield	24h Yield
A	6.0	G G A Y A V S E A–NH2 **3**	17%	25%	37%	61%
B	6.0	G G A Y A V S E A–NH2 **S-1**	4%	7%	12%	31%
C	6.0	G G A Y A V S E A–NH2 **S-2**	13%	14%	14%	36%

All reactions were performed in 1.5 µmol scale. The yield was determined LC-MS of the crude reaction mixture.

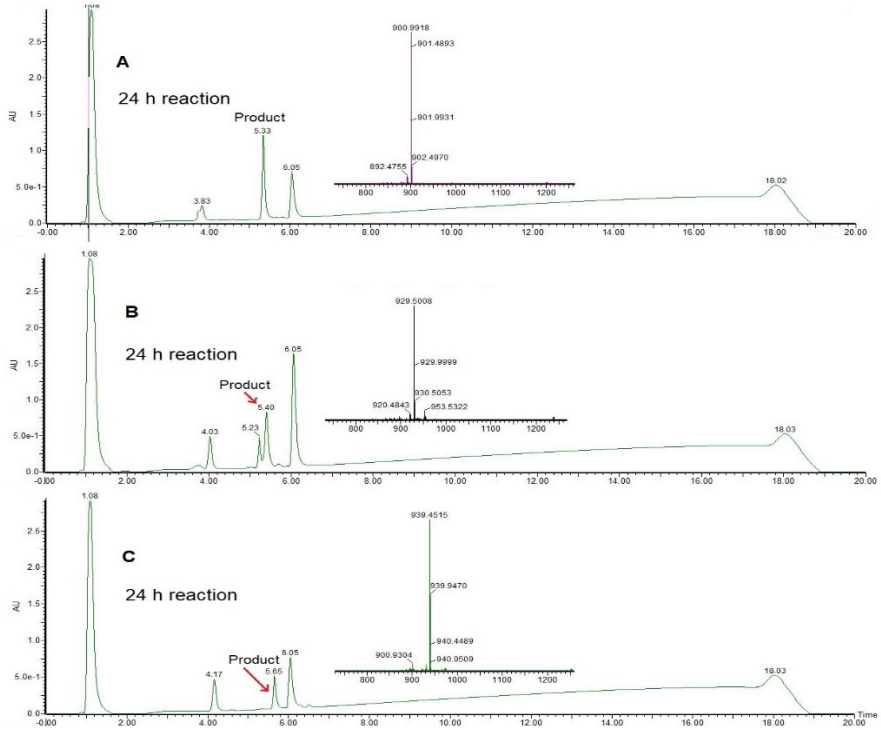
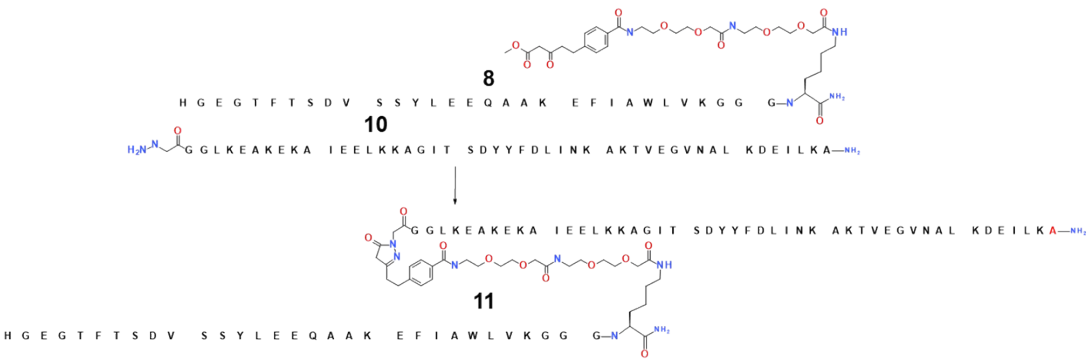


Figure S3. UPLC-MS trace for the reaction on Table S2 (The peaks at 3.83 min (trace A), 4.03 min (trace B) and 4.17 min (trace C) were starting hydrazine peptide; the peak at 6.05 min was the starting β-ketoester).

2.5 General procedure for the optimization of pyrazolone ligation of GLP-1 analog **8** and ABDCon **10** (Table S3)**.** A 4 ml dram vial was charged with ABDCon **10** (1.0 equiv.), 50 mM buffer solution, GLP-1 analog **8** (1.0 equiv.) and catalyst respectively. The final concentration of both peptides was 400 µM. The reaction mixture was placed on a vortex for 2 seconds and was incubated at the indicated temperature. Aliquots were taken at the indicated time points for LC-MS analysis.

Table S3. Optimization of pyrazolone ligation of GLP-1 analog **8** and ABDCon **10**.



Entry	Changes from the standard conditions	Yield
1.	none	66%
2.	50 mM pH 4.5, catalyst A (20 equiv.), 8 M Urea, rt	26%
3.	50 mM pH 7.4, catalyst A (20 equiv.), 8 M Urea, rt	37%
4.	50 mM pH 4.5, catalyst A (20 equiv.), 8 M Urea, 60 °C	47%
5.	50 mM pH 4.5, catalyst A (50 equiv.), rt	26%
6.	50 mM pH 4.5, catalyst A (20 equiv), rt	13%
7.	50 mM pH 4.5, catalyst B (20 equiv), rt	10%
8.	50 mM pH 4.5, catalyst C (20 equiv.), rt	10%
9.	50 mM pH 7.4, 50 mM catalyst A, 3 M NaCl, rt	28%
10.	50 mM pH 7.4, 50 mM catalyst A, 8 M Urea, rt	42%
11.	50 mM pH 7.4, 50 mM 2-(1H-imidazol-2-yl)-methylamine, 150 mM NaCl, rt	0%
12.	50 mM pH 7.4, 50 mM *N,N*-Dimethylethylenediamine, 150 mM NaCl, rt	0%
13. .	50 mM pH 7.4, 50 mM *p*-Phenylenediamine, 150 mM NaCl, rt	34%

All reactions were performed in 0.20 µmol scale. Yields were determined after 1 d by LC-MS of the crude reaction mixture. Catalyst A = 2-amino-5-methoxybenzoic acid, Catalyst B = aniline, Catalyst C = 3,5-diaminobenzoic acid.

Table S4. Pyrazolone ligation of GLP1-1 analog **8** (400 µM) and ABDCon **10** (400 µM) in 50 mM 2-amino-5-methoxybenzoic acid, 50 mM phosphate buffer solution pH 7.4 and 8 M urea at different temperatures**.**

Time (h)	2 h	6 h	21 h	48 h
25 °C	25%	40%	66%	74%
30 °C	37%	54%	74%	
40 °C	55%	84%		

2.6 General procedure for stability test (Figure S4 and Figure S5). A 4 mL vial was charged with 1.5 mg of peptide and 50 mM acetate buffer solution (3 mL, pH 4.5). The solution was mixed by repeatedly pipetting up and down before aliquoting into three 1.5 mL vial. The samples were incubated in dark according to the indicated temperatures (4 °C, 25 °C, and 37 °C). Aliquots were taken at 30 mins, 1 d, 2 d 4 d, and 7 d. This procedure was repeated using 50 mM phosphate buffer pH 7.4.

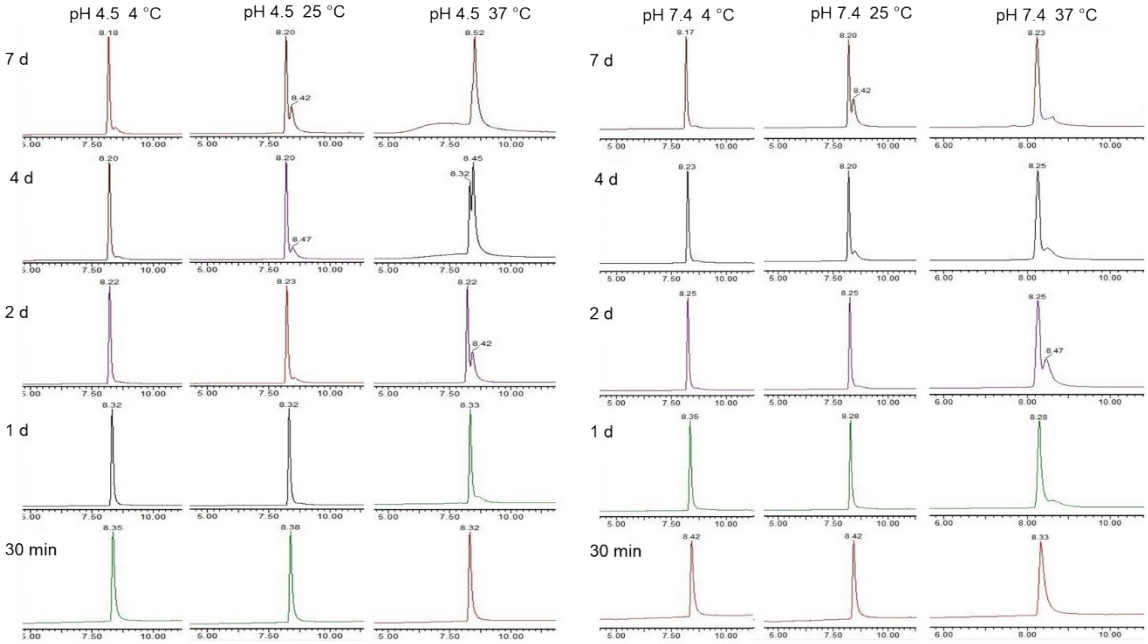


Figure S4. UPLC traces of the stability test sample of peptide GLP-1-ABDCon **11**.

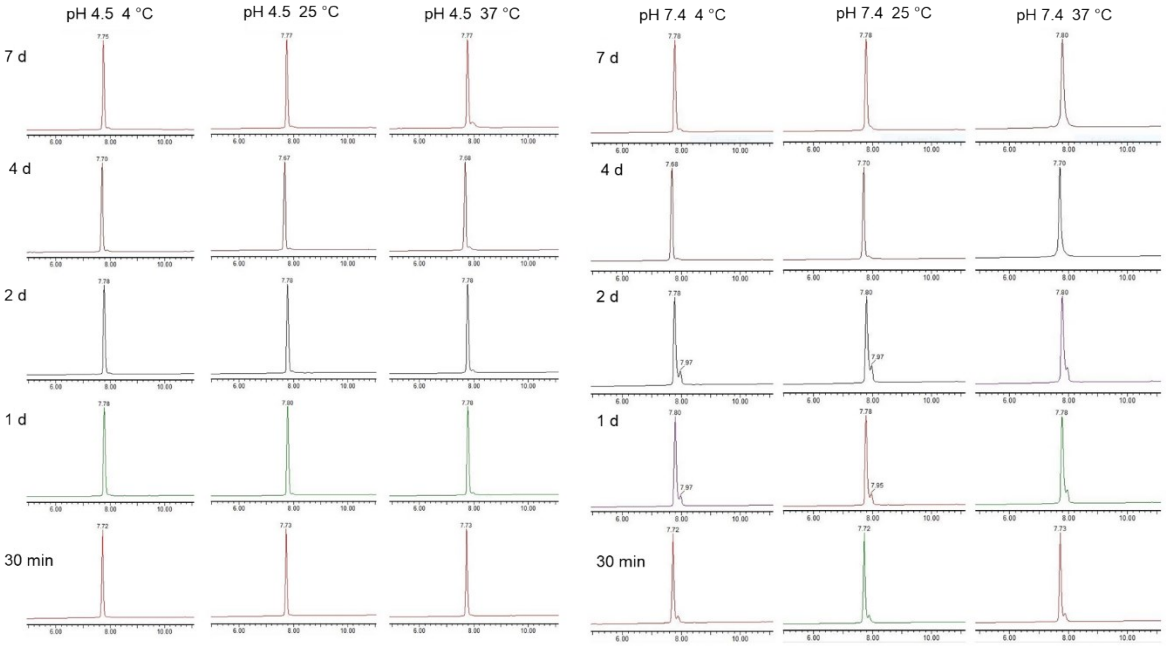


Figure S5. UPLC traces of the stability test sample of peptide ABDCon-sCT **12**.

3. Pyrazolone Ligation

3.1 Pyrazolone ligation of GLP-1 analog **8** with ABDCon **10** (Figure 3a & 3b). A scintillation vial was charged with GLP-1 analog **8** (15.9 mg, 4.00 µmol, 1.0 equiv.) and 50 mM buffer solution (4 mL, pH 7.4). To this solution was added a solution of ABDCon **10** (20.9 mg, 4.00 µmol, 1.0 equiv.) in 50 mM buffer solution pH 7.4 (1 mL). The reaction mixture, with a final concentration of **8** (800 µM) and **10** (800 µM), was placed on a vortex for 2 seconds and was incubated at 25 °C. Aliquots were taken at 2 h, 6 h, 24 h, and 48 h for LC-MS analysis. After 48 h, the reaction mixture was diluted with 5 mL Milli Q water before loading to RP-HPLC for purification. Compound **11** was isolated as a lyophilized white solid (20.0 mg, 2.19 µmol, 54%). MS (ESI-TOF) calculated for $C_{413}H_{651}N_{103}O_{130}$ [M+8H$^+$/8] 1143.4, found 1143.4.

3.2 Pyrazolone ligation of sCT analog **9** with ABDCon **10** (Figure 3a & 3c). A scintillation vial was charged with sCT analog **9** (7.9 mg, 2.00 µmol, 1.0 equiv.) and 50 mM buffer solution (2 mL, pH 4.5). To this solution was added a solution of ABDCon **10** (10.4 mg, 2.00 µmol, 1.0 equiv.) in 50 mM buffer solution pH 4.5 (2 mL). The reaction mixture, with final concentration of **9** (500 µM) and **10** (500 µM), was placed on a vortex for 2 seconds and was incubated at 37 °C. Aliquots were taken at 2 h, 5 h and 24 h for LC-MS analysis. After 24 h, the reaction mixture was diluted with 5 mL Milli Q water before loading to RP-HPLC for purification. Compound **12** was isolated as a lyophilized white solid (11.8 mg, 1.29 µmol, 64%). MS (ESI-TOF) calculated for $C_{403}H_{657}N_{107}O_{129}S_2$ [M+8H$^+$/8] 1142.2, found 1142.1.

4. Pyrazolone-Mediated Sequential Conjugation

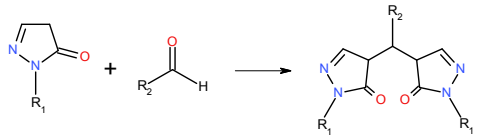


Figure S6. The formation of bis-pyrazolone product.

4.1 Pyrazolone-mediated sequential ligation of GLP-1-ABDCon **11**, formaldehyde and CCK8 **14** (Figure 4a & 4b). A stock solution of formaldehyde (1.5 µL, 20.7 µmol, 200 µL 50 mM buffer solution pH 7.4) and CCK8 **14** (4.2 mg, 2.90 µmol, 1000 µL 50 mM phosphate buffer solution pH 7.4) were prepared respectively before the experiment. A scintillation vial was charged with GLP-1-ABDcon **11** (6.6 mg, 1.38 µmol, 1.0 equiv.) and 50 mM buffer solution (4 mL, pH 7.4). To this solution was added 20 µL of stock solution of formaldehyde (2.07 µmol, 1.5 equiv.). The reaction mixture was mixed by repeatedly pipetting up and down before incubating at 37 °C. After 10 mins, an aliquot was taken for LC-MS. A 500 µL stock solution of CCK8 **14** (1.45 µmol, 1.05 equiv.) and 50 mM buffer solution (480 µL, pH 7.4) were added respectively. The reaction mixture, with final concentration of **11** (276 µM), formaldehyde (414 µM) and **14** (290 µM), was placed on a vortex for 2 seconds and was incubated at 37 °C. After 2 h, an aliquot was taken for LC-MS analysis before purification. The reaction mixture

was diluted with 5 mL Milli Q water before loading to RP-HPLC for purification. Compound **15** was isolated as a lyophilized white solid (9.5 mg, 0.89 μmol, 65%). MS (ESI-TOF) calculated for $C_{480}H_{742}N_{116}O_{151}S_3$ [M+8H$^+$/8] 1332.2, found 1332.2

4.2 Pyrazolone-mediated sequential ligation of ABDCon-sCT **12**, GLP-1 analog **13** and CCK8 **14** (Figure 4a & 4c). A stock solution of GLP-1 analog **13** (6.00 mg, 1.36 μmol, 1000 μL buffer solution pH 7.4) and CCK8 **14** (2.0 mg, 1.36 μmol, 1000 μL buffer solution pH 7.4) were prepared respectively before the experiment. A scintillation vial was charged with ABDCon-sCT **12** (6.0 mg, 0.65 μmol, 1.0 equiv.) and 50 mM buffer solution (4 mL, pH 7.4). To this solution was added a 500 μL of stock solution of GLP-1 analog **13** (0.68 μmol, 1.05 equiv.). The reaction mixture was mixed by repeatedly pipetting up and down before incubating at 37 °C. After 10 mins, an aliquot was taken for LC-MS. A 500 μL stock solution of CCK8 **14** (0.68 μmol, 1.05 equiv.) was added. The reaction mixture, with final concentration of **12** (130 μM), **13** (136 μM) and **14** (136 μM), was placed on a vortex for 2 seconds and was incubated at 37 °C. After 6 h, the reaction mixture was diluted with 5 mL Milli Q water before loading to RP-HPLC for purification. Compound **16** was isolated as a lyophilized white solid (5.8 mg, 0.39 μmol, 60%). MS (ESI-TOF) calculated for $C_{664}H_{1032}N_{172}O_{211}S_5$ [M+8H$^+$/8] 1871.1, found 1871.0.

5. Pyrazolone-Mediated Macrocyclization

5.1 Pyrazolone-mediated macrocyclization of GLP-1 analog **19**, formaldehyde and peptide **17** (Figure S7). A stock solution of formaldehyde (3.35 μL, 45.00 μmol, 500 μL buffer solution pH 7.4) and peptide **17** (2.9 mg, 3.20 μmol, 1000 μL buffer solution pH 7.4) were prepared respectively before the experiment. A scintillation vial was charged with GLP-1 analog **19** (6.6 mg, 1.5 μmol, 1.0 equiv.) and 50 mM buffer solution (4 mL, pH 7.4). To this solution was added a 25 μL stock solution of formaldehyde (2.25 μmol, 1.5 equiv.), 500 μL of peptide **17** (1.58 μmol, 1.05 equiv.) and 50 mM buffer solution (475 μL, pH 7.4) respectively. The reaction mixture, with final concentration of **19** (300 μM), formaldehyde (450 μM) and **17** (316 μM), was placed on a vortex for 2 seconds and was incubated at 37 °C. After 1 h, an aliquot was taken for LC-MS analysis before purification. The reaction mixture was diluted with 5 mL Milli Q water before loading to RP-HPLC for purification. Compound **S-3** was isolated as a lyophilized white solid (5.6 mg, 1.1 μmol, 70%). MS (ESI-TOF) calculated for $C_{240}H_{359}N_{61}O_{75}S$ [M+3H$^+$/3] 1777.9, found 1777.7

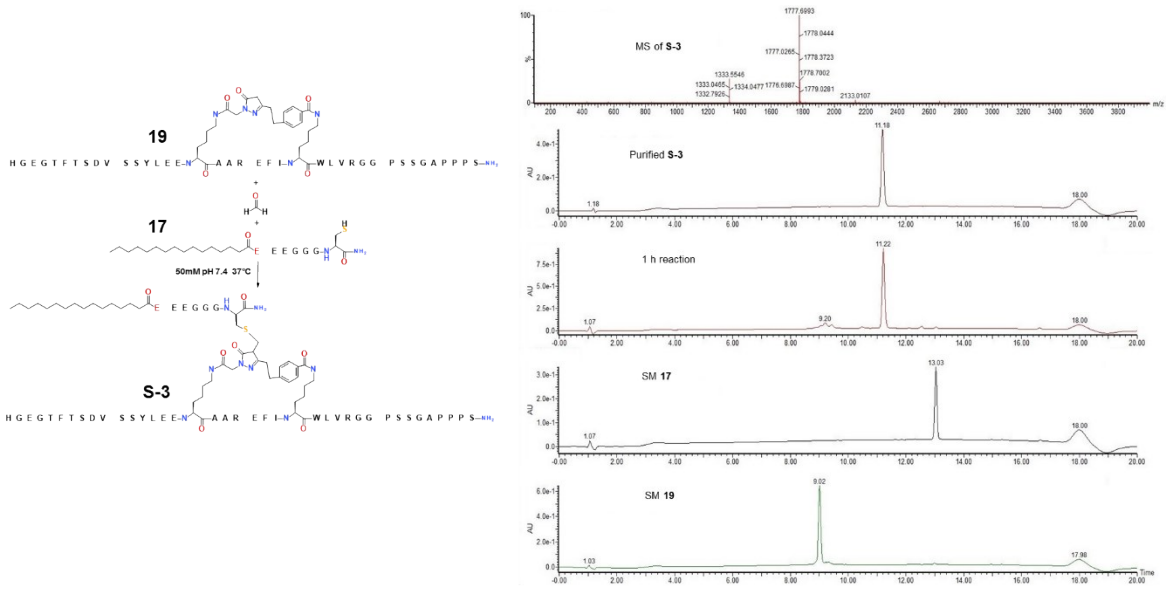


Figure S7. Pyrazolone-mediated macrocyclization of GLP-1 analog **19**, formaldehyde and peptide **17**.

5.2 Pyrazolone-mediated macrocyclization of GLP-1 analog **19**, peptide **17** and peptide **18** (Figure 5a & S8). A stock solution of CCK8 peptide **18** (6.5 mg, 4.40 µmol, 1000 µL buffer pH 7.4) and peptide **17** (4.1 mg, 4.34 µmol, 1000 µL buffer solution pH 7.4) were prepared respectively before the experiment. A scintillation vial was charged with GLP-1 analog **19** (8.8 mg, 2.00 µmol, 1.0 equiv.) and 50 mM buffer solution (3 mL, pH 7.4). To this solution was added a 500 µL stock solution of CCK8 peptide **18** (2.2 µmol, 1.1 equiv.) and 500 µL of peptide **17** (2.2 µmol, 1.1 equiv.) respectively. The reaction mixture, with final concentration of **19** (500 µM), **18** (550 µM) and **17** (550 µM), was placed on a vortex for 2 seconds and was incubated at 37 °C. After 1 h, an aliquot was taken for LC-MS analysis before purification. The reaction mixture was diluted with 5 mL Milli Q water before loading to RP-HPLC for purification. Compound **20** was isolated as a lyophilized white solid (6.3 mg, 0.93 µmol, 62%). MS (ESI-TOF) calculated for $C_{308}H_{445}N_{73}O_{95}S_3$ [M+4H$^+$/4] 1697.6, found 1697.4. Note: The bis-pyrazolone product was detected by LC-MS at retention time of 9.07 min (Figure 5d, 1 h trace). Compound **S-4** was isolated as a lyophilized white solid (<1 mg). MS (ESI-TOF) calculated for $C_{467}H_{668}N_{118}O_{142}S_2$ [M+5H$^+$/5] 2054.2, found 2054.6.

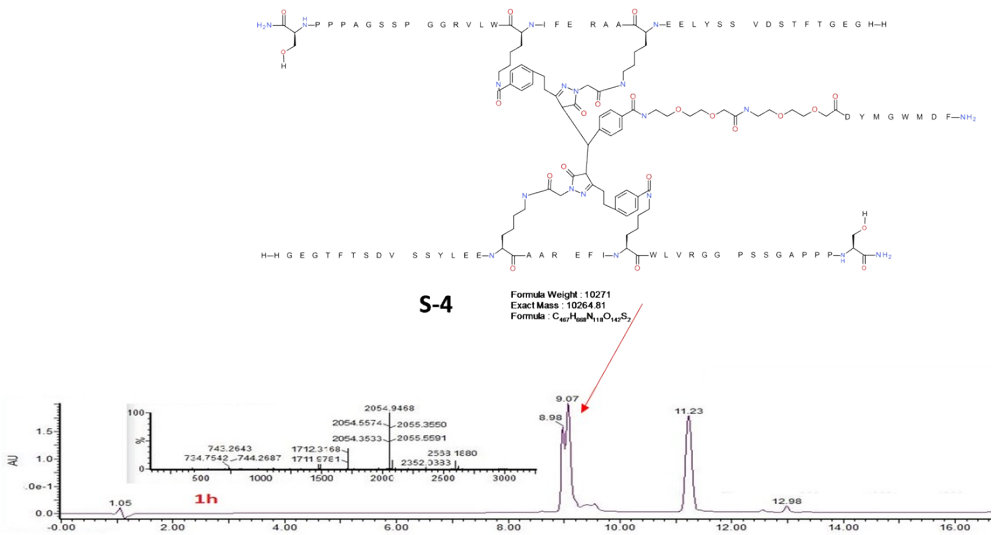


Figure S8. The bis-pyrazolone product **S-4** in the ligation of GLP-1 analog **19**, peptide **17** and peptide **18**.

5.3 Pyrazolone-mediated macrocyclization of GLP-1 analog **21** and formaldehyde (Figure S9). A stock solution of formaldehyde (3 µL, 45 µmol, 500 µL buffer pH 7.4) was prepared before the experiment. A scintillation vial was charged with GLP1-analog **21** (6.9 mg, 1.50 µmol, 1.0 equiv.) and 50 mM buffer solution (50 mL, pH 7.4). To this solution was added a 25 µL stock solution of formaldehyde (2.25 µmol, 1.5 equiv.) and 50 mM buffer solution (975 µL, pH 7.4). The reaction mixture, with final concentration of **21** (29 µM) and formaldehyde (44 µM), was placed on a vortex for 2 seconds and was incubated at 37 °C. After 1 h, an aliquot was taken for LC-MS analysis before purification. The reaction mixture was loaded to RP-HPLC for purification. Compound **S-5** was isolated as a lyophilized white solid (5.0 mg, 1.10 µmol, 72%). MS (ESI-TOF) calculated for $C_{207}H_{302}N_{56}O_{64}S$ [M+3H$^+$/3] 1544.7, found 1544.4.

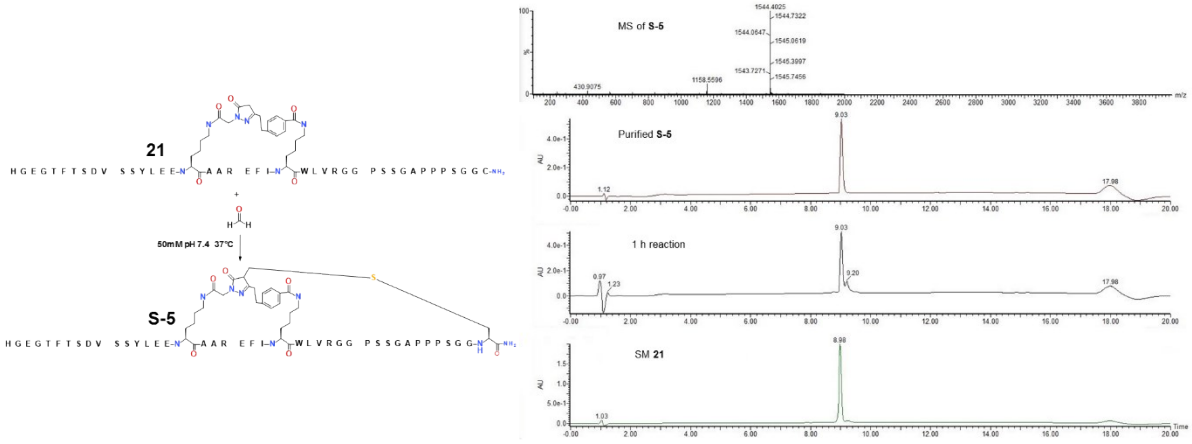


Figure S9. Pyrazolone-mediated macrocyclization of GLP-1 analog **21** and formaldehyde.

5.4 Pyrazolone-mediated macrocyclization of GLP-1 analog **21** and CCK8 peptide **18** (Figure 5b). A stock solution of CCK8 peptide **18** (4.9 mg, 4.5 µmol, 1000 µL buffer solution pH 7.4)

was prepared before the experiment. A scintillation vial was charged with GLP-1 analog **21** (6.9 mg, 1.5 µmol, 1.0 equiv.), 50 mM buffer solution (4.5 mL, pH 7.4) and a 500 µL stock solution of CCK8 peptide **18** (1.65 µmol, 1.1 equiv.). The reaction mixture, with final concentration of **21** (330 µM) and **18** (330 µM), was placed on a vortex for 2 seconds and was incubated at 37 °C. After 1 h, an aliquot was taken for LC-MS analysis before purification. The reaction mixture was diluted with 5 mL Milli Q water before loading to RP-HPLC for purification. Compound **22** was isolated as a lyophilized white solid (6.2 mg, 1.0 µmol, 68%). MS (ESI-TOF) calculated for $C_{275}H_{388}N_{68}O_{84}S_3$ [M+4H$^+$/4] 1522.7 found 1522.6.

5.5 Pyrazolone-mediated macrocyclization of GLP-1 analog **23** (Figures 5c & S10). A stock solution of 1.0 mM $NaIO_4$ (1.1 mg, 5.0 µmol, 5 mL buffer solution pH 7.4) was prepared and placed on an ice bath. A scintillation vial charged with GLP-1 analog **23** (5.5 mg, 1.20 µmol, 1.0 equiv.) and 50 mM buffer solution (4 mL, pH 7.4) was placed on an ice bath. To this solution was added a 1.8 mL cold stock solution of $NaIO_4$ (1.80 µmol, 1.5 equiv.) and a cold 50 mM buffer solution (200 µL, pH 7.4). The reaction mixture, with final concentration of **23** (200 µM) and $NaIO_4$ (300 µM), was placed on a vortex for 2 seconds, put back on an ice bath and was kept in dark. After 30 mins, an aliquot was taken for LC-MS analysis before quenching the reaction with sodium thiosulfate with the final concentration two times of that $NaIO_4$. The quenched reaction was taken out of the ice bath and left it sit at room temperature for 4 h. After 4 h, aliquot was taken for LC-MS before purification. The reaction mixture was diluted with 5 mL Milli Q water before loading to RP-HPLC for purification. Compound **24** was isolated as a lyophilized white solid (2.9 mg, 0.5 µmol, 53%). MS (ESI-TOF) calculated for $C_{205}H_{299}N_{53}O_{66}$ [M+3H$^+$/3] 1521.6, found 1521.4.

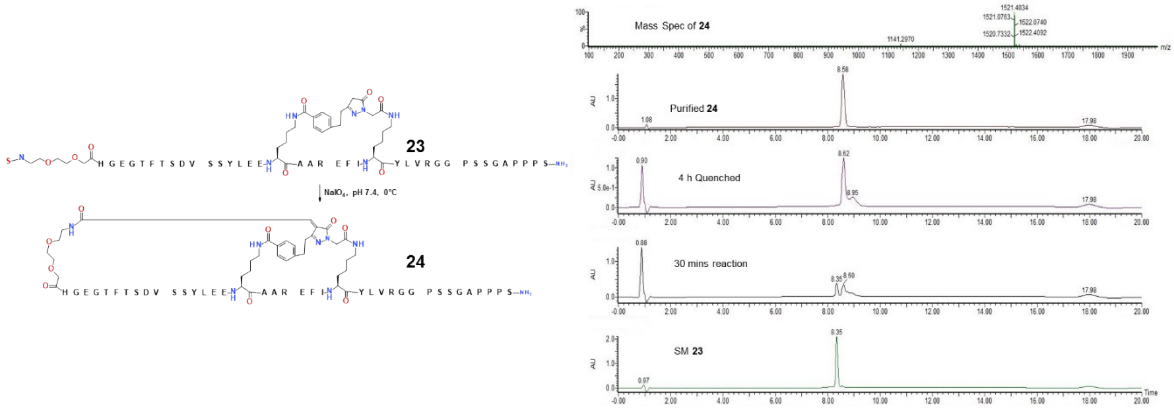


Figure S10. Pyrazolone-mediated macrocyclization of GLP-1 analog **23**.

5.6 Pyrazolone-mediated macrocyclization of GLP-1 analog **S-6** (Figure S11). A stock solution of 1.0 mM $NaIO_4$ (1.1 mg, 5.0 µmol, 5 mL buffer solution pH 7.4) was prepared and placed on an ice bath. A scintillation vial charged with GLP-1 analog **S-6** (1.0 mg, 0.20 µmol, 1.0 equiv.) and 50 mM buffer solution (720 µL, pH 7.4) was placed on an ice bath. To this solution was added 200 µL cold stock solution of $NaIO_4$ (0.20 µmol, 1.0 equiv.) and a cold 50 mM buffer solution (80 µL, pH 7.4). The reaction mixture, with final concentration of **S-6** (200 µM) and $NaIO_4$ (200 µM) was placed on a vortex for 2 seconds, put back on an ice bath and was kept in dark. After 30 mins, an aliquot was taken for LC-MS analysis before quenching the reaction with sodium thiosulfate with the final concentration two times of that $NaIO_4$. The quenched

reaction was taken out of the ice bath and let it sit at room temperature. Aliquots were taken for LC-MS. MS (ESI-TOF) calculated for **S-7** $C_{212}H_{310}N_{56}O_{71}S$ [M+3H$^+$/3] 1604.6, found 1604.4.

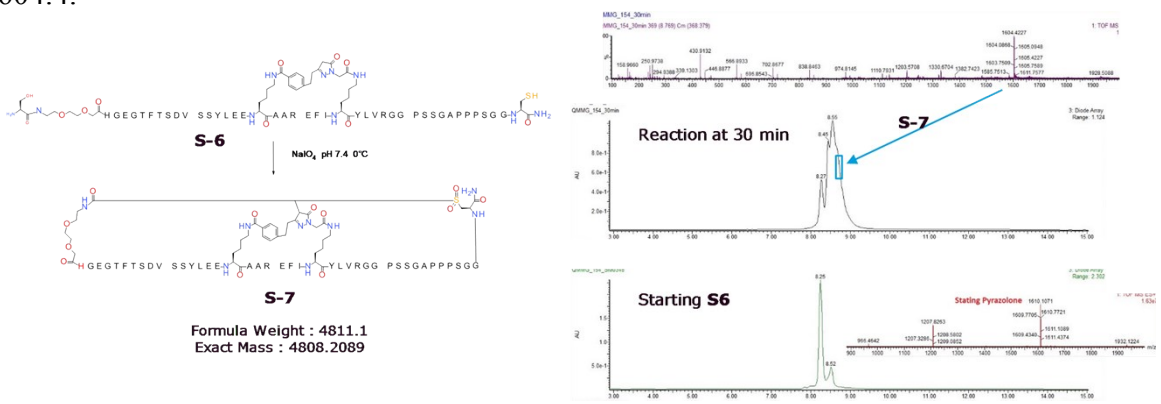


Figure S11. Pyrazolone-mediated macrocyclization of GLP-1 analog **S-6**.

6. General Procedure of Peptide Synthesis and Characterization

6.1 General Procedure for small molecule and characterization of unmodified short peptide

4-(5-methoxy-3,5-dioxopentyl)benzoic acid **S-8** was prepared according to a known procedure.[1] A reaction flask charged with stir bar was flame-dried under vacuum and allowed to cool under nitrogen. The flask was then charged with diisopropyl amine (3.2 mL, 23.00 mmol, 2.3 equiv.) and THF (60 mL). The reaction mixture was cooled to 0 °C with an ice bath. To the cooled reaction mixture was added 2.5 M *n*-butyllithium solution in hexane (9.2 mL, 23.00 mmol, 2.3 equiv.) dropwise. The reaction mixture was stirred at 0 °C for 30 min, before adding the solution of methyl acetoacetate (1.1 mL, 10.00 mmol, 1.0 equiv.) dropwise. The reaction mixture was stirred at 0 °C for 60 min before cooling to -78 °C. To this reaction mixture was added the solution of *tert*-Butyl-4-(bromomethyl)benzoate (2.8 mg, 10.50 mmol, 1.05 equiv.) in THF dropwise. The reaction was allowed to warm up to room temperature and left stirring overnight. The reaction mixture was diluted with diethyl ether and quenched with 1 M HCl. The aqueous phase was extracted three times and the combined organic phase was dried with Na_2SO_4 and concentrated under reduced pressure. The crude material was purified on silica gel column. A scintillation vial was charged with the purified material and solution of TFA (2 mL) in DCM (2 mL). The reaction mixture was left shaking for 3 h. The reaction mixture was concentrated under reduced pressure and purified on a silica gel column. Compound **S-8** was isolated as a white powder (850 mg, 3.4 mmol, 34% for two steps). MS (ESI-TOF) calculated for $C_{12}H_{14}O_3$ [M+H$^+$] 251.02, found 251.08. ^{1}H NMR (400 MHz, $CDCl_3$) 2.92 (t, J = 6.7 Hz, 2 H), 3.00 (t, J = 7.08 Hz, 2 H), 3.45 (s, 2 H), 3.72 (s, 3 H), 7.29 (d, J = 8.24 Hz, 2 H), 8.02 (d, J = 8.24 Hz, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) 201.27, 171.83, 167.58, 147.17, 130.66, 128.70, 127.54, 52.56, 49.23, 43.97, 29.53.

[1] Adeel, M.; Rashid, M.; Rasool, N.; Ahmad, R.; Villinger, A.; Reinke, H.; Fischer, C.; Langer, P. *Synthesis,* 2009, **2,** 243-250.

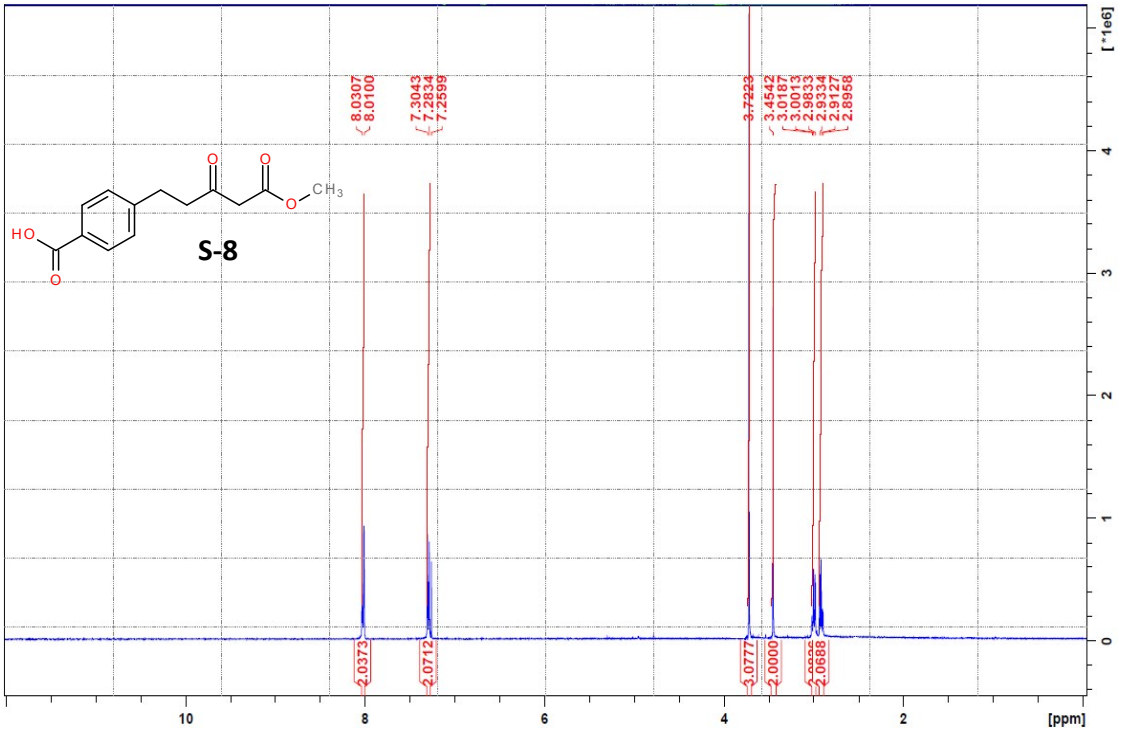


Figure S12. ^{1}H NMR of compound **S-8**.

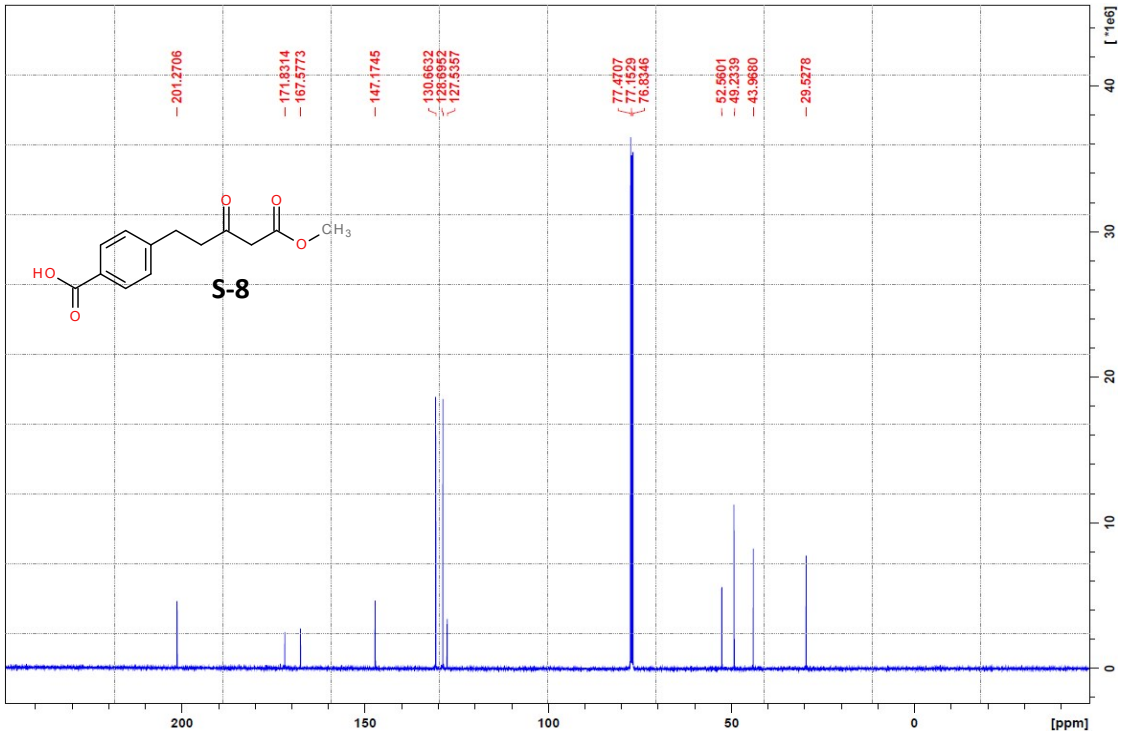


Figure S13. ^{13}C NMR of compound **S-8**.

CCK8 **14** was synthesized at 200 µmol scale using the general procedure for SPPS. Purification by preparative RP-HPLC afforded **14** (64.5 mg, 43.05 µmol, 22%). MS (ESI-TOF) calculated for $C_{66}H_{91}N_{13}O_{21}S_3$ [M+H$^+$] 1498.5, found 1498.4.

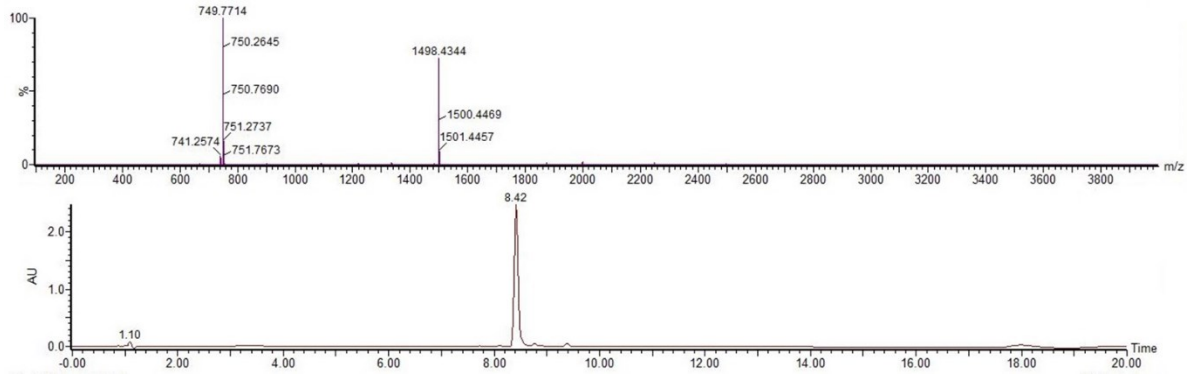


Figure S14. LC-MS of CCK8 **14**.

Peptide **17** was synthesized at 200 μmol scale using the general procedure for SPPS. Purification by preparative RP-HPLC afforded **17** (18.00 mg, 19.62 μmol, 10%). MS (ESI-TOF) calculated for $C_{40}H_{68}N_8O_{14}S_1$ [M+H$^+$] 917.5, found 917.4.

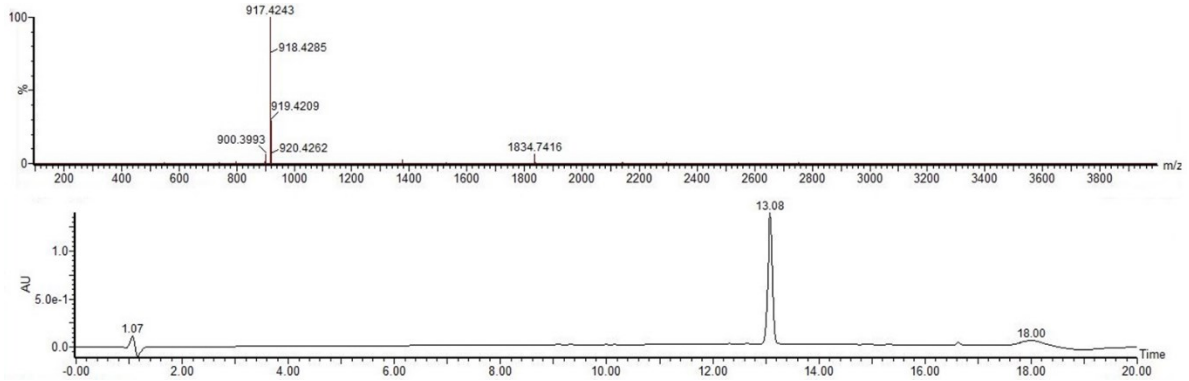


Figure S15. LC-MS of peptide **17**.

6.2 General procedure for β-ketoester-bearing peptide.

The resin-bound peptide with unprotected *N*-terminal (from the automated solid-phase peptide synthesizer) was washed successively with DCM (3 x 10 mL) and DMF (3 x 5 min). A solution of 4-(5-methoxy-3,5-dioxopentyl)benzoic acid **S-8** (10.0 equiv.), DIC (10.0 equiv.) and Oxyma (10.0 equiv.) in DMF (5 mL) was added to the resin and left shaking for 5 h. The excess reagent was removed by washing the resin with DCM (3 x 10 mL). The β-ketoester-bearing peptide was cleaved from the resin using TFA/H_2O (97.5:2.5, v/v, 15 mL) for 3 h at room temperature and purified using the general method mentioned above.

β-ketoester-bearing peptide **5** was synthesized at 100 μmol scale using the general procedure for β ketoester-bearing peptide. Purification by preparative RP-HPLC afforded **5** (20.0 mg, 20.9 μmol, 21%). MS (ESI-TOF) calculated for $C_{43}H_{57}N_9O_{16}$ [M+H$^+$] 956.4, found 956.4.

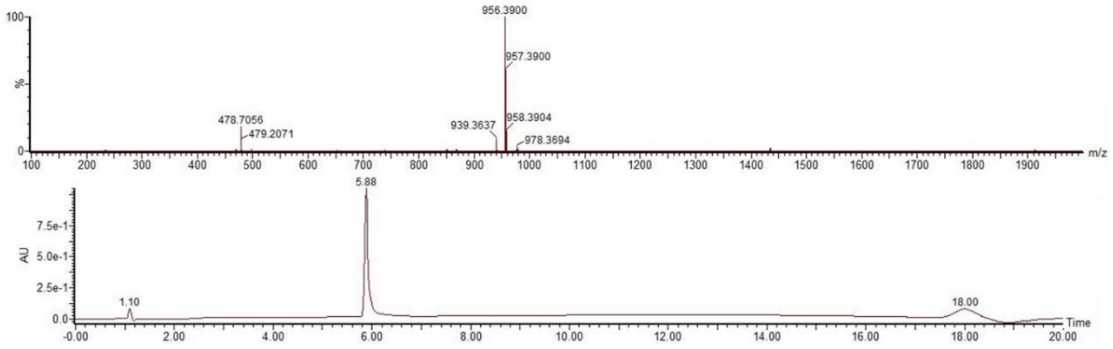


Figure S16. LC-MS of peptide **5**.

GLP-1 analog **8** was synthesized at 100 μmol scale using the general procedure for β-ketoester-bearing peptide. Purification by preparative RP-HPLC afforded **8** (50.0 mg, 12.6 μmol, 13%). MS (ESI-TOF) calculated for $C_{180}H_{268}N_{42}O_{59}$ [M+3H$^+$/3] 1322.4, found 1322.3.

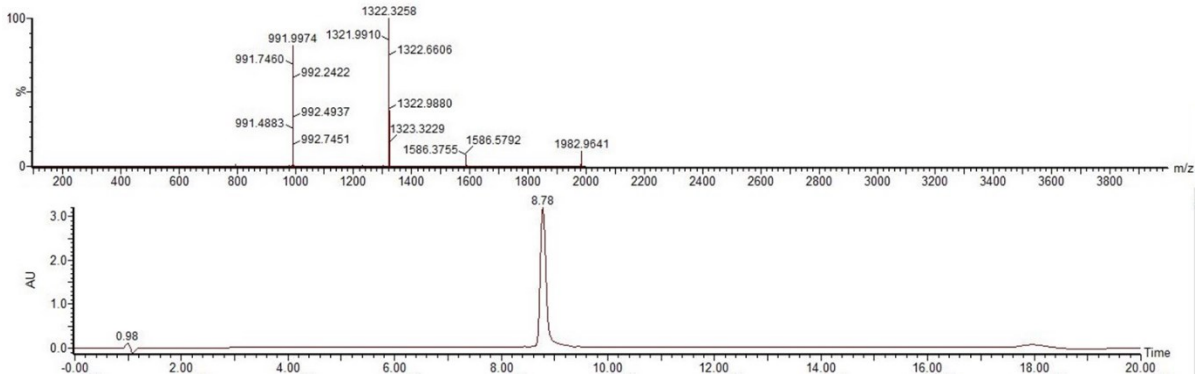


Figure S17. LC-MS of GLP-1 analog **8**.

sCT analog **9** was synthesized at 100 μmol scale using the general procedure for β-ketoester-bearing peptide. The crude peptide pellet was treated with I_2 (254 mg) in AcOH/H_2O (8 mL/2 mL) for 10 min, then quenched by sat. aq. solution of ascorbic acid until the purple color disappeared. The resulting solution was purified by preparative RP-HPLC afforded **9** (75.0 mg, 19.0 μmol, 19%). MS (ESI-TOF) calculated for $C_{170}H_{274}N_{46}O_{58}S_2$ [M+3H$^+$/3] 1319.1, found 1319.0.

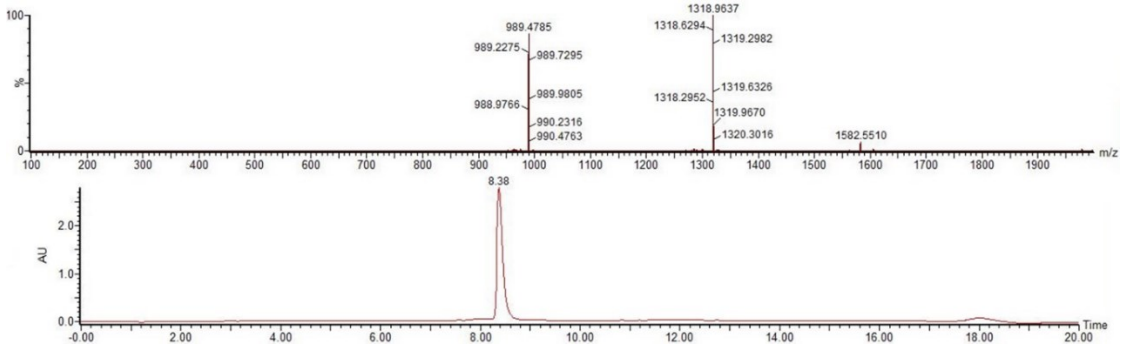


Figure S18. LC-MS of sCT **9**.

6.3 General procedure for hydrazine-bearing peptide

The resin bound peptide with unprotected *N*-terminal (from the automated solid-phase peptide synthesizer) was washed successively with DCM (3 x 10 mL) and DMF (3 x 5 min). A solution of bromo acetic acid (20.0 equiv.) and DIC (10.0 equiv.) in DMF (5 mL) was added to the resin and left shaking for 45 min. This procedure was repeated for two more times and then the resin was washed successively with DCM (3 x 10 mL) and DMSO (3 x 5 min). For peptide **S-1** and **S-2** the resin was treated with 6-bromohexanoic acid (10.0 equiv.) and 4-(chloromethyl)benzoic acid (10.0 equiv.) respectively for 16 h instead of bromo acetic acid. A solution of 2% anhydrous hydrazine in DMSO (5 mL) was added to the resin and left shaking for 90 min. The excess reagent was removed by washing the resin with DCM (3 x 10 mL). The hydrazine-bearing peptide was cleaved from the resin using TFA/TIS/H_2O (95:2.5:2.5, v/v/v, 15 mL) for 3 h at room temperature and purified using the general method mentioned above.

Hydrazine-bearing peptide **3** was synthesized at 100 µmol scale using the general procedure for hydrazine-bearing peptide. Purification by preparative RP-HPLC afforded **3** (30.0 mg, 33.5 µmol, 34%). MS (ESI-TOF) calculated for C_{37} H_{58} N_{12} O_{14} [M+H^+] 895.4, found 895.4.

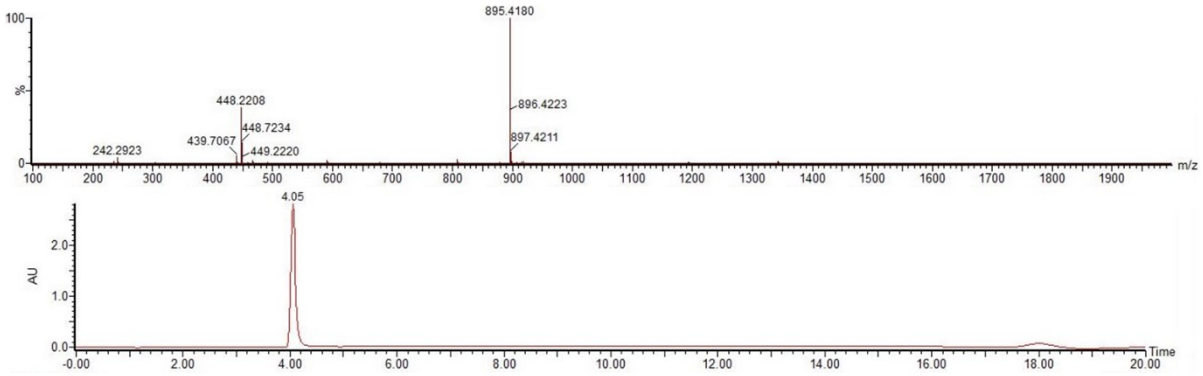


Figure S19. LC-MS of peptide **3**.

Hydrazine-bearing peptide **S-1** was synthesized at 100 µmol scale using the general procedure for hydrazine-bearing peptide. Purification by preparative RP-HPLC afforded **S-1** (33.0 mg, 34.7 µmol, 35%). MS (ESI-TOF) calculated for $C_{41}H_{66}N_{12}O_{14}$ [M+H^+] 951.5, found 951.5.

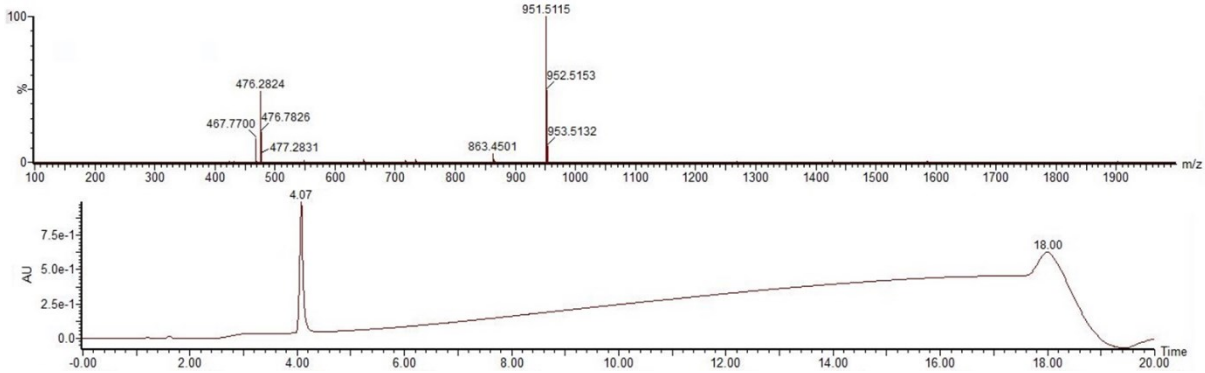


Figure S20. LC-MS of peptide **S-1**.

Hydrazine-bearing peptide **S-2** was synthesized at 100 µmol scale using the general procedure for hydrazine-bearing peptide. Purification by preparative RP-HPLC afforded **S-2** (50.0 mg, 51.5 µmol, 51%). MS (ESI-TOF) calculated for $C_{43}H_{62}N_{12}O_{14}$ [M+H$^+$] 971.5, found 971.4.

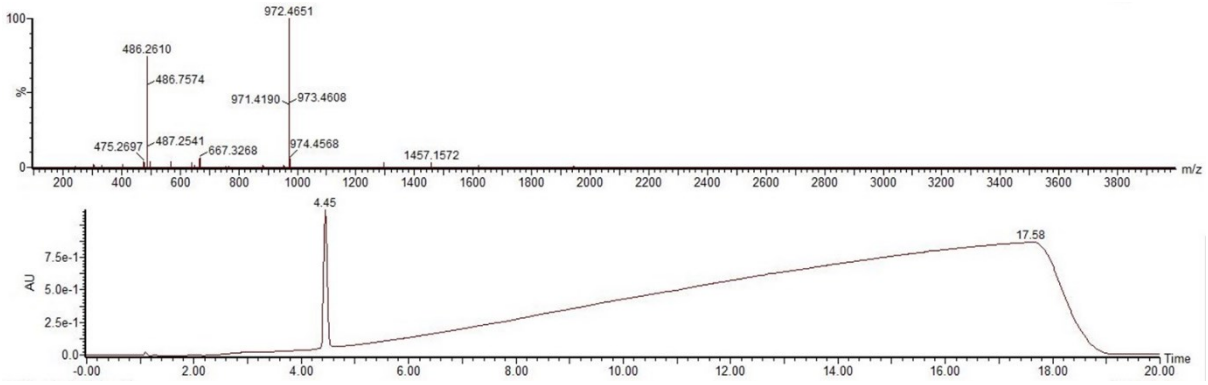


Figure S21. LC-MS of peptide **S-2**.

ABDCon **10** was synthesized at 100 µmol scale using the general procedure for hydrazine-bearing peptide. Purification by preparative RP-HPLC afforded **10** (132.0 mg, 25.3 µmol, 28%). MS (ESI-TOF) calculated for $C_{234}H_{389}N_{61}O_{73}$ [M+4H$^+$/4] 1307.3, found 1307.2.

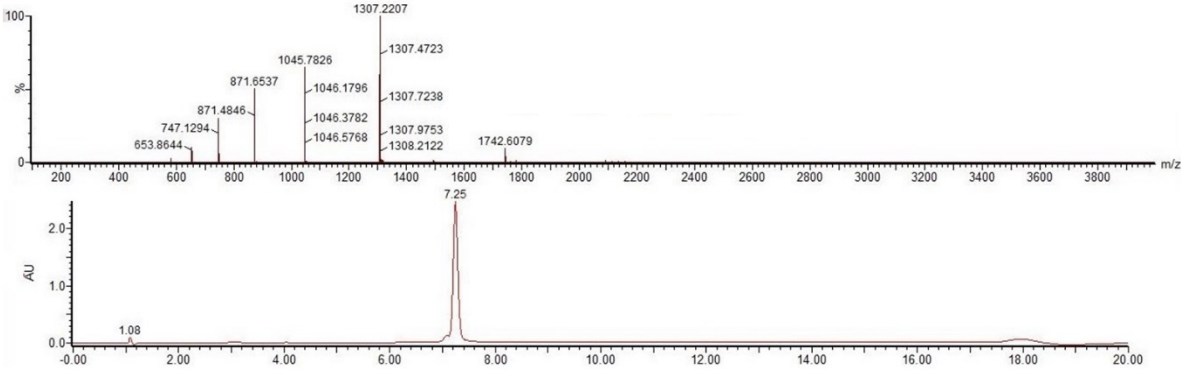


Figure S22. LC-MS of ABDCon **10**.

6.4 General Procedure for aldehyde-bearing peptide

A 50 mM phosphate buffer solution at pH 10.5 was prepared directly before the experiment. A scintillation vial was charged with the purified peptide and 50 mM buffer solution (10.0 mL, pH 10.5). To this solution was added 2.0 equiv. solution of 200 mM 4-carboxybenzaldehye, HOSu and DIC in DMF and left shaking for 30 min. An aliquot was taken for LC-MS before purification. The reaction mixture was diluted with 30 mL Milli Q water and a drop of TFA. The reaction mixture was filtered via a 0.2 um filter unit before loading to RP-HPLC for purification.

GLP-1 analog **13** was synthesized at 100 µmol scale using the general procedure for aldehyde-bearing peptide. Purification by preparative RP-HPLC afforded **13** (142.8 mg, 32.8 µmol, 33%). MS (ESI-TOF) calculated for $C_{195}H_{286}N_{52}O_{62}$ [M+3H$^+$/3] 1451.2, found 1451.0.

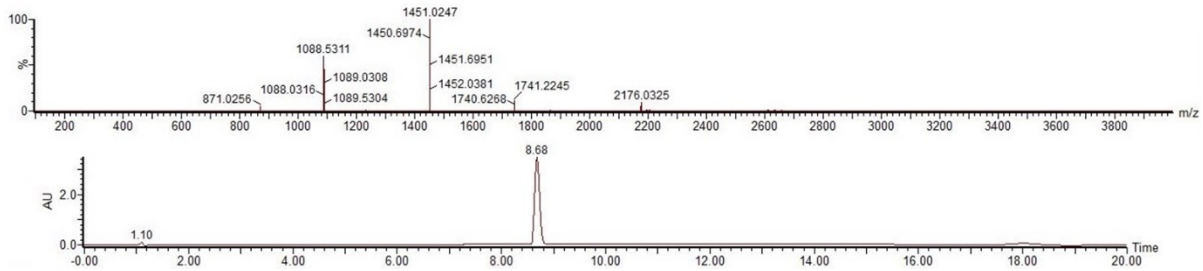


Figure S23. LC-MS of GLP-1 analog **13**.

CCK8 **18** was synthesized at 100 μmol scale using the general procedure for aldehyde-bearing peptide. Purification by preparative RP-HPLC afforded **18** (24.0 mg, 16.2 μmol, 8%). MS (ESI-TOF) calculated for $C_{69}H_{88}N_{12}O_{21}S_2$ [M+H$^+$] 1486.6, found 1486.5.

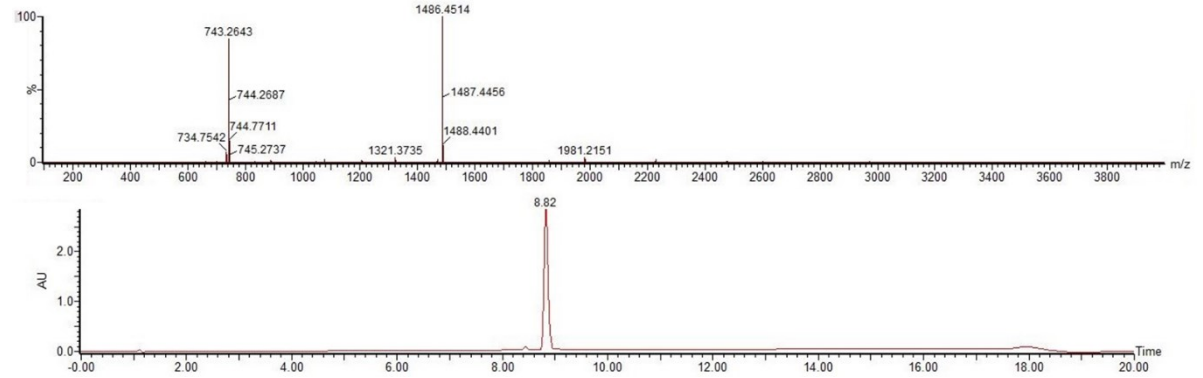


Figure S24. LC-MS of GLP-1 CCK8 **18**.

6.5 General procedure for the synthesis of pyrazolone stapled peptides

The resin-bound peptide with Boc-His1, Lys17(ivDde) and Lys24(Mtt) was washed successively with DCM (3 x 10 mL) and DMF (3 x 5 min). A solution of 10% hydrate hydrazine in DMF (5 mL) was added to the resin and left shaking for 16 h. The resin was washed successively with DCM (3 x 10 mL) and DMF (3 x 5 min) and then a solution of 4-(5-methoxy-3,5-dioxopentyl)benzoic acid **S-8** (10.0 equiv.), DIC (10.0 equiv.) and Oxyma (10.0 equiv.) in DMF (5 mL) was added to the resin and left shaking for 5 h. The resin was washed successively with DCM (3 x 10 mL). A solution of 40% HFIP in DCM (10 mL) was added to the resin and left shaking for 90 min. This procedure was repeated for two more times and then the resin was washed successively DCM (3 x 10 mL) and DMF (3 x 5 min). A solution of tri-boc-hydrazinoacetic acid (10.0 equiv.), DIC (10.0 equiv.) and oxyma (10.0 equiv.) in DMF (5mL) was added to the resin and left shaking for 5 h. The excess reagent was removed by washing the resin with DCM (3 x 10 mL). The modified peptide was cleaved from the resin using TFA/H_2O (97.5:2.5, v/v, 15 mL) for 3-4 h at room temperature and purified using the general method mentioned above.

GLP-1 analog **19** was synthesized at 100 µmol scale using the general procedure for the synthesis of pyrazolone stapled peptides. Purification by preparative RP-HPLC afforded **19** (16.0 mg, 3.6 µmol, 4%). MS (ESI-TOF) calculated for $C_{199}H_{291}N_{53}O_{61}$ [M+3H$^+$/3] 1468.2, found 1467.9.

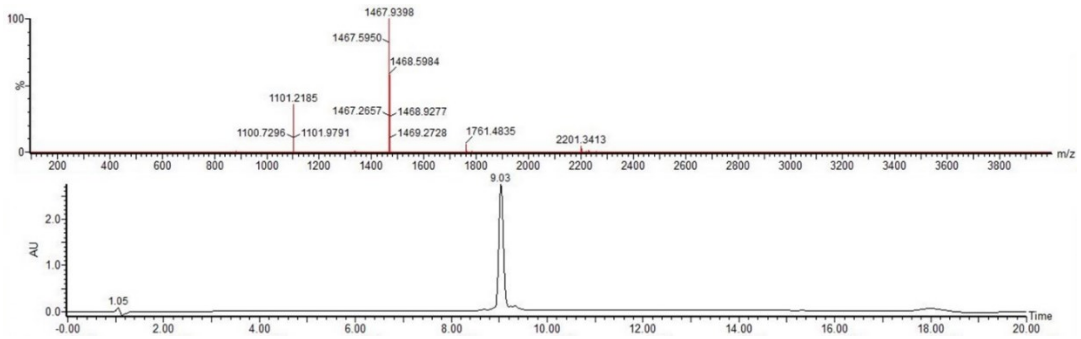


Figure S25. 4LC-MS of GLP-1 analog **19**.

GLP-1 analog **21** was synthesized at 100 µmol scale using the general procedure for the synthesis of pyrazolone stapled peptides. Purification by preparative RP-HPLC afforded **21** (8.0 mg, 1.7 µmol, 2%). MS (ESI-TOF) calculated for $C_{206}H_{302}N_{56}O_{64}S_1$ [M+3H$^+$/3] 1540.7, found 1549.3.

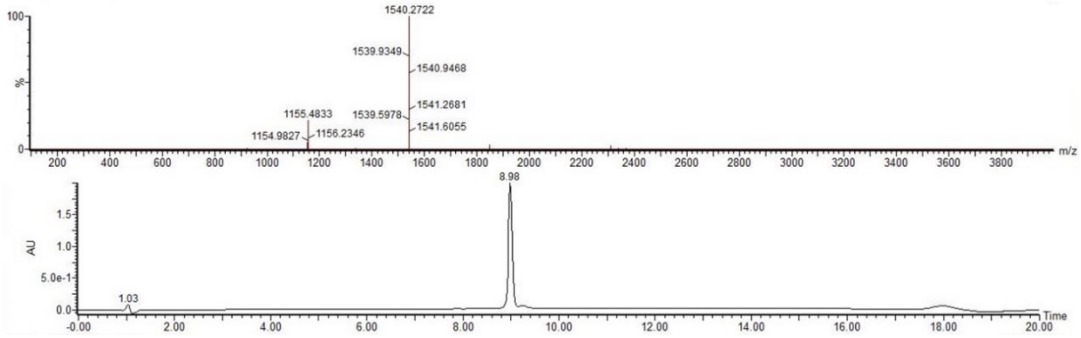


Figure S26. LC-MS of GLP-1 GLP-1 analog **21**.

GLP-1 analog **23** was synthesized at 100 µmol scale using the general procedure for the synthesis of pyrazolone stapled peptides. Purification by preparative RP-HPLC afforded **23** (7.5 mg, 1.6 µmol, 2%). MS (ESI-TOF) calculated for $C_{206}H_{306}N_{54}O_{67}$ [M+3H$^+$/3] 1538.0, found 1537.8.

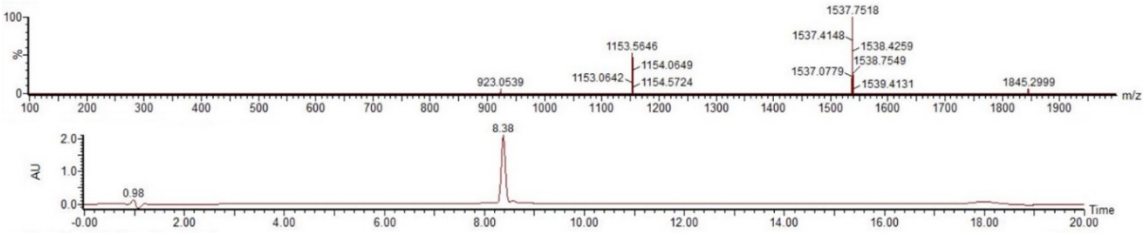


Figure S27. LC-MS of GLP-1 GLP-1 analog **23**.

GLP-1 analog **S-6** was synthesized at 50 µmol scale using the general procedure for the synthesis of pyrazolone stapled peptides. Purification by preparative RP-HPLC afforded **S-6** (9.2 mg, 1.9 µmol, 4%). MS (ESI-TOF) calculated for $C_{213}H_{317}N_{57}O_{70}S_1$ [M+3H$^+$/3] 1610.4, found 1610.1.

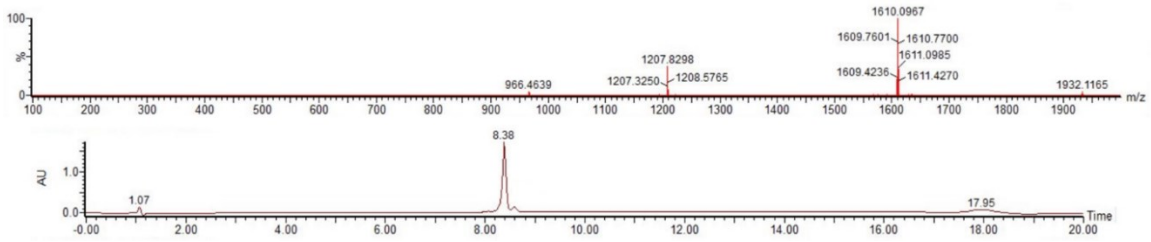


Figure S28. LC-MS of GLP-1 GLP-1 analog **S-6**.