Synthesis of Human GLP-1 (7-36) by Chemoselective α-Ketoacid Hydroxylamine Peptide Ligation of Unprotected Fragments

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Supporting Information

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1. General Methods.

Reagents: All reactions utilizing air- or moisture-sensitive reagents were performed in dried glassware under an atmosphere of dry Ar. CH_2Cl_2 was distilled over CaH_2. DMF and CH_3OH were dried by passage over molecular sieves under an Ar atmosphere. *N,N*-Diisopropylethylamine (DIPEA) and *N,N*-diethylamine were distilled from KOH. Other reagents were used without further purification. Oxone® was purchased from Alfa Aesar. Rink amide MBHA (0.72 mmol/g loading, 100–200 mesh) and 2-chlorotrityl chloride resin (1.4 mmol/g loading, 100–200 mesh) were purchased from Novabiochem. Thin layer chromatography (TLC) was performed on EMD precoated plates (silica gel 60 F254, Art 5715, 0.25 mm) and compounds visualized by fluorescence under UV light or by staining with phosphomolybdic acid. Column chromatography was performed on EMD Silica Gel 60 (230-400 mesh) using a forced flow of 0.5–1.0 bar.

Instruments: ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were measured on a Bruker Advance II 500 spectrometer or ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) were measured on a Varian Unity 400 spectrometer. Chemical shifts are expressed in parts per million (ppm) and are referenced to the internal solvent signals. Coupling constants are reported in Hertz (Hz). Splitting patterns are indicated as follows: a, apparent; br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Optical rotations were measured on a Jasco P–2000 polarimeter operating at the sodium D line with a 100 mm path length cell, and are reported as follows: $[\alpha]_D^T$ (concentration (g/100 ml), solvent). Infrared (IR) spectra were recorded on a JASCO FT/IR-430 spectrophotometer and are reported as wavenumbers (cm^{-1}). HPLC analyses were performed with a JASCO HPLC system composed of a degasser DG-2080-54, HPLC pump PU-2087, dynamic mixer MX-2080-32, and a UV detector UV-2077.

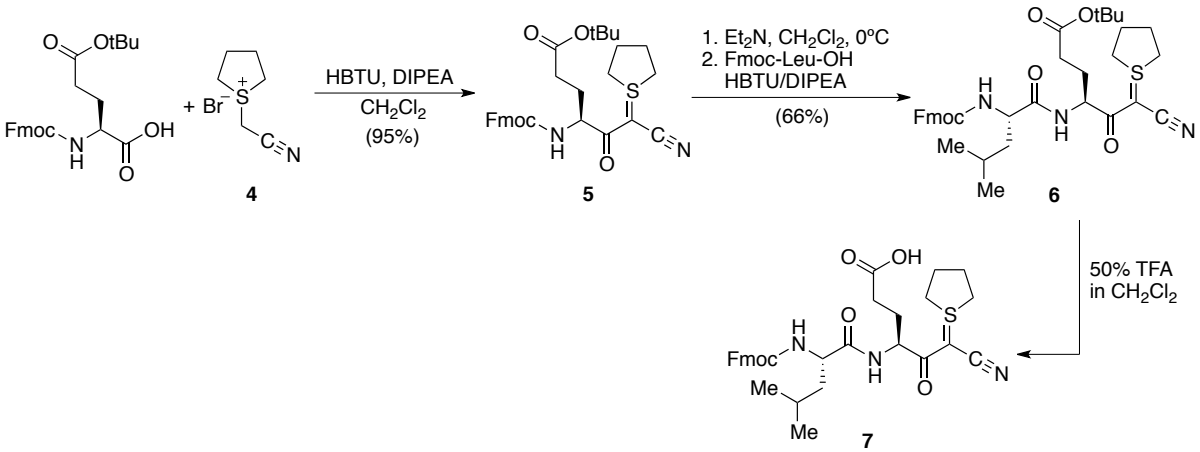
Analytical HPLC: Unless otherwise noted, analytical HPLC was carried out as follows:
Method A: Zorbax Eclipse XDB-C8 5μm 4.6 × 150 mm with a linear gradient of 5-60% buffer B over 25 min with a flow rate of 1 mL/min (buffer A = 0.1% TFA in H_2O; buffer B = 0.1% TFA in CH_3CN)
Method B: Shishedo CapcellPak C18 5μm 4.6 × 250 mm with a linear gradient of 5-95% buffer B over 30 min with a flow rate of 1 mL/min.
Method C: Phenomenex Luna C18 3μm 4.6 × 100 mm with a linear gradient of 5-80% buffer B over 25 min with a flow rate of 1 mL/min. The eluent was monitored at 220 nm, 280 nm and 300 nm.

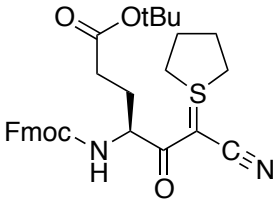
Preparative HPLC: Peptides were purified on Zorbax XDB-C8 PrepHT 21.2 × 250 mm column. Crude peptides (10-15 mg) were dissolved in 0-20% CH_3CN/80% (0.1% TFA in H_2O) to a concentration of 1 mg/mL and loaded onto the prep column by pumping at a flow rate of 10 mL/min. Peptides were eluted at a flow rate of 20 mL/min with a shallow gradient (e.g., 20%-40% B over 20 min) of increasing concentrations of buffer B (0.1% TFA in CH_3CN) in buffer A (0.1% TFA in H_2O). The exact gradient used was determined by the elution behavior of the desired peptide, as assessed

by prior analytical HPLC. Fractions containing the pure target peptide were identified by analytical LCMS and were combined and lyophilized.

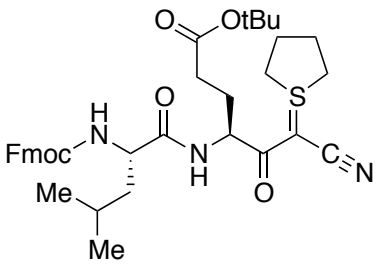
2. Experimental Procedures

2.1 Preparation of Fmoc-Leu-Glu-SY (7) for side chain attachment.



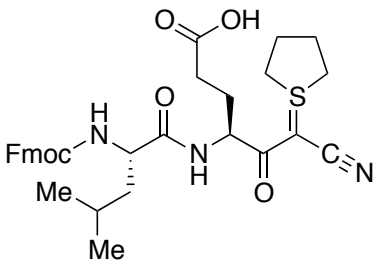


Fmoc-Glu(*t*Bu)-SY (5). Fmoc-Glu(*t*Bu)-OH (4.0 g, 9.4 mmol, 1.0 equiv) was dissolved in 40 mL CH_2Cl_2. HBTU (4.30 g, 11.3 mmol, 1.2 equiv), HOBt·H_2O (1.70 g, 11.3 mmol, 1.2 equiv), DIPEA (4.70 mL, 28.2 mmol, 3.0 equiv), and the bromide salt **4** (2.54 g, 12.2 mmol, 1.3 equiv) were added at 0 °C and the reaction stirred 2 h at rt. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed with 1M HCl solution (2 × 50 mL) and brine (2 × 50 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash column chromatography (EtOAc → 6:1 EtOAc:acetone) on silica gel to yield **5** as a white solid (4.8 g, 95%). R_f=0.40 (EtOAc/acetone 3:1); $[\alpha]_D^{23}$ +33.86 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.0 Hz, 2H), 7.38 (t, *J* = 7.0 Hz, 2H), 7.33-7.28 (m, 2H), 5.75 (d, *J* = 8.0 Hz, 1H), 4.68-4.63 (m, 1H), 4.33 (d, *J* = 7.0 Hz, 2H), 4.20-4.16 (m, 1H), 3.42-3.24 (m, 4H), 2.60-2.48 (m, 2H), 2.40-2.20 (m, 2H), 2.17–1.98 (m, 3H), 1.97–1.86 (m, 1H), 1.43 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 188.9, 172.5, 155.9, 144.2, 144.0, 141.4, 127.8, 127.2, 125.4, 120.1, 119.2, 80.6, 67.0, 55.6, 54.5, 47.3, 45.8, 45.2, 31.6, 28.9, 28.7, 28.6, 28.2; IR (thin film) v 3292.38, 2975.62, 2942.36, 2357.55, 2170.01, 1718.75, 1590.99, 1521.56, 1449.73, 1366.80, 1248.68, 1151.78; HRMS (ESI) (*m/z*) $[MNa]^+$ calcd for $C_{30}H_{34}N_2NaO_5S$, 557.2086, found 557.2063.



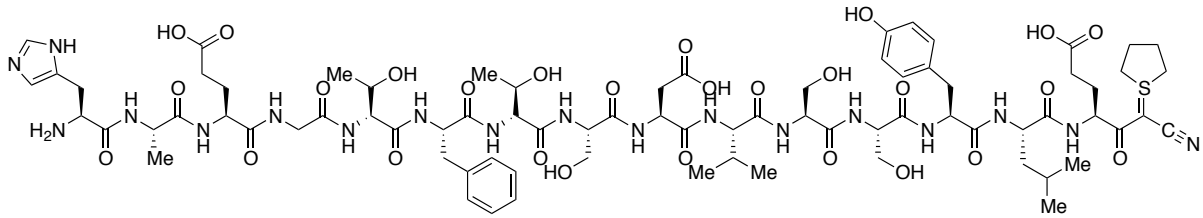
Fmoc-Leu-Glu(*t*Bu)-SY (6). To a stirred solution of Fmoc-Glu(*t*Bu)-SY (2.00 g, 3.73 mmol) in CH_2Cl_2 (3 mL) at 0 °C was added Et_2NH (3 mL) dropwise. The reaction was stirred at 0 °C for 3 h. Toluene (10 mL) was added to the reaction and the mixture was concentrated in vacuo to give the free amine as a colorless syrup, which was directly used for the coupling. In a separate flask,

Fmoc-Leu-OH (1.50 g, 4.47 mmol, 1.0 equiv) was dissolved in 15 mL CH_2Cl_2. HBTU (1.84 g, 4.85 mmol, 1.1 equiv), HOBt·H_2O (742 mg, 4.85 mmol, 1.1 equiv) and DIPEA (1.54 mL, 9.33 mmol, 2.0 equiv) were added at 0 °C and stirred 20 min. The deprotected sulfur ylide was added, and the solution was allowed to slowly warm to rt. After 3 h, the reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with 1M HCl solution (2 × 50 mL) and brine (2 × 50 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography (EtOAc → 10:1 EtOAc:acetone) on silica gel to yield **6** as a colorless syrup (2.8 g, 66%). R_f=0.39 (EtOAc/acetone 3:1); $[\alpha]_D^{23}$ +7.25 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.0 Hz, 2H), 7.60 (d, *J* = 7.0 Hz, 2H), 7.38 (d, *J* = 7.0 Hz, 2H), 7.36-7.28 (m, 2H), 6.91 (d, *J* = 6.5 Hz, 1H), 5.37-5.28 (m, 1H), 4.88-4.80 (m, 1H), 4.40-4.30 (m, 1H), 4.30-4.17 (m, 1H), 3.45-3.18 (m, 4H), 2.60-2.48 (m, 2H), 2.35-2.18 (m, 2H), 2.18-1.98 (m, 3H), 1.98-1.85 (m, 1H), 1.75-1.60 (m, 3H), 1.60-1.46 (m, 1H), 1.45-1.30 (s, 9H), 0.99-0.80 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 188.4, 172.6, 171.6, 156.2, 144.2, 143.9, 141.4, 127.8, 127.2, 125.4, 125.3, 120.1, 119.0, 80.6, 67.1, 54.7, 54.1, 53.7, 47.4, 45.8, 45.4, 42.4, 31.5, 28.7, 28.6, 28.5, 28.2, 24.8, 23.2, 22.1; IR (thin film) v 3296.23, 2955.86, 2171.45, 1720.19, 1665.71, 1584.24, 1507.10, 1450.21, 1367.28, 1253.50, 1151.78; HRMS (ESI) (*m/z*) $[MH]^+$ calcd for $C_{36}H_{46}N_3O_6S$, 648.3107, found 648.3121.



Fmoc-Leu-Glu-SY (7). Fmoc-Leu-Glu(tBu)-SY (2.50 g, 3.86 mmol) was dissolved in 2:3 TFA:CH_2Cl_2 (10 mL) with 5% Et_3SiH and allowed to stir 4 h. The mixture was concentrated in vacuo, the residue diluted with CH_2Cl_2 (100 mL) and washed with saturated aqueous NH_4Cl solution (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated to yield 7 as a white solid (2.1 g, 95%), which was directly used for solid phase synthesis without further purification. [α]D23 +5.50 (c = 1.0, $CHCl_3$); 1H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, J = 7.6 Hz, 2H), 7.58 (dd, J = 10.8, 7.0 Hz, 2H), 7.39-7.33 (m, 2H), 7.31-7.25 (m, 2H), 5.96 (d, J = 8.4 Hz, 1H), 4.98-4.90 (m, 1H), 4.45-4.35 (m, 1H), 4.35-4.24 (m, 2H), 4.22-4.17 (m, 2H), 3.41-3.30 (m, 2H), 3.25-3.10 (m, 2H), 2.44-2.18 (m, 4H), 2.18-2.05 (m, 2H), 2.05-1.78 (m, 3H), 1.65-1.42 (m, 3H), 0.99-0.80 (m, 6H); 13C NMR (100 MHz, CDCl3) δ 170.5, 158.2, 154.7, 138.4, 126.2, 125.9, 123.3, 109.9, 109.3, 107.5, 107.4, 102.1, 100.8, 49.2, 35.8, 35.6, 29.3, 28.1, 27.8, 24.3, 12.0, 10.7, 10.6, 10.4, 6.8, 5.3, 4.2; IR (thin film) v 3293.34, 2954.89, 2871.01, 2172.42, 1720.19, 1587.61, 1518.67, 1449.73, 1384.64, 1251.09, 1151.29; HRMS (ESI) (m/z) $[MH]^+$ calcd for $C_{32}H_{37}N_3NaO_6S$, 614.2301, found 614.2281.

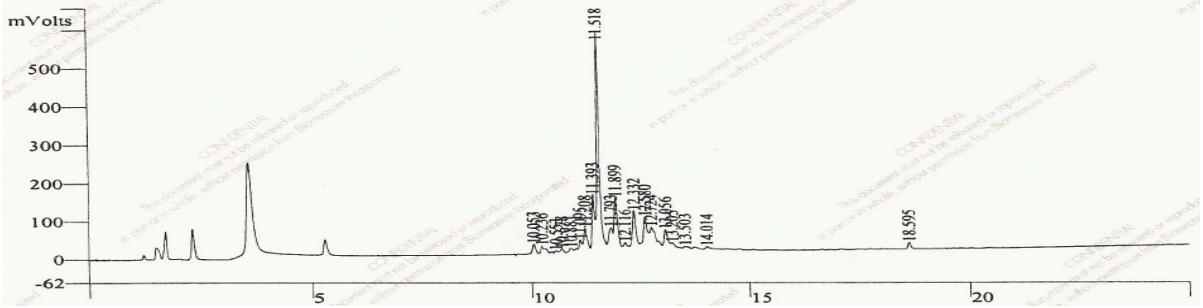
2.2 Preparation of GLP-1 (7-21) α-ketoacid (2).



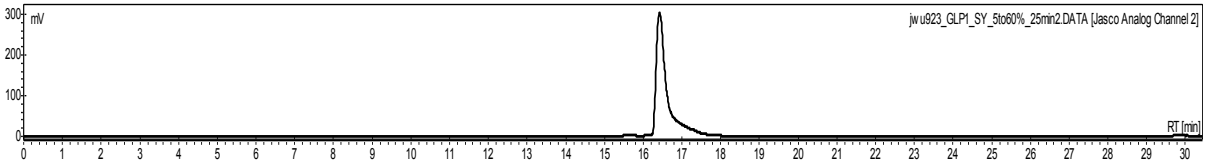
Chemical Formula: $C_{77}H_{110}N_{18}O_{27}S$
Exact Mass: 1750.75
Molecular Weight: 1751.87

GLP-1 (7-21) sulfur ylide (10). The peptide His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-SY was prepared on a 1 millimole scale using standard Fmoc SPPS protocols. 2-Chlorotrityl chloride resin (Nova Biochem., La Jolla, CA) with a substitution of 1.4 mmol/g was used to prepare the starting resin of Fmoc-Leu-Glu(Resin)-SY **8**. The 2-chlorotrityl chloride resin (714 mg, 1.00 mmol, 1.0 equiv) was preswollen using CH_2Cl_2. Fmoc-Leu-Glu-SY **7** (710 mg, 1.20 mmol, 1.2 equiv) and DIPEA (0.69 mL, 4.0 mmol, 4.0 equiv) were added to the mixture and shaken 3 h at rt. The resin was washed thoroughly with CH_2Cl_2. The rest of the sequence was synthesized on an Applied Biosystems (Foster City, CA) model 433A peptide synthesizer using standard Fmoc/HBTU/HOBt procedures. The Fmoc amino acids (AnaSpec, Inc, San Jose, CA) were used with the following side chain protection: Fmoc-Ala-OH, Fmoc-Asp(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-Glu(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ser(Trt)-OH, and Fmoc-Phe-OH. The Fmoc groups were removed by treatment with 25% piperidine in DMF. Fmoc amino acids (1.0 mmol) were pre-activated with HBTU/HOBt (0.95 mmol) and DIPEA (2M) and were coupled to the resin. The last step is the removal of the terminal Fmoc using 25% piperdine in DMF for 30 min. The resin was washed with DMF and then CH_2Cl_2. At the end of the assembly of the peptide chain, the peptide was cleaved using a solution of TFA/TIPS/H_2O (85/7.5/7.5, 22.3 mL) for 3 h. The TFA mixture is filtered into 80 mL Et_2O and centrifuged to give a crude pellet. The crude peptide was purified on preparative reverse-phase HPLC using a Phenomenex C18 Luna 5μ column (Phenomenex, Torrance, CA). The column was eluted with a linear gradient of 0-30% of buffer B over 60 min at a flow rate of 10 mL/min. The fractions were checked by analytical HPLC and those containing pure product were pooled and lyophilized to dryness to give 224 mg (13% from loading) of a white solid.

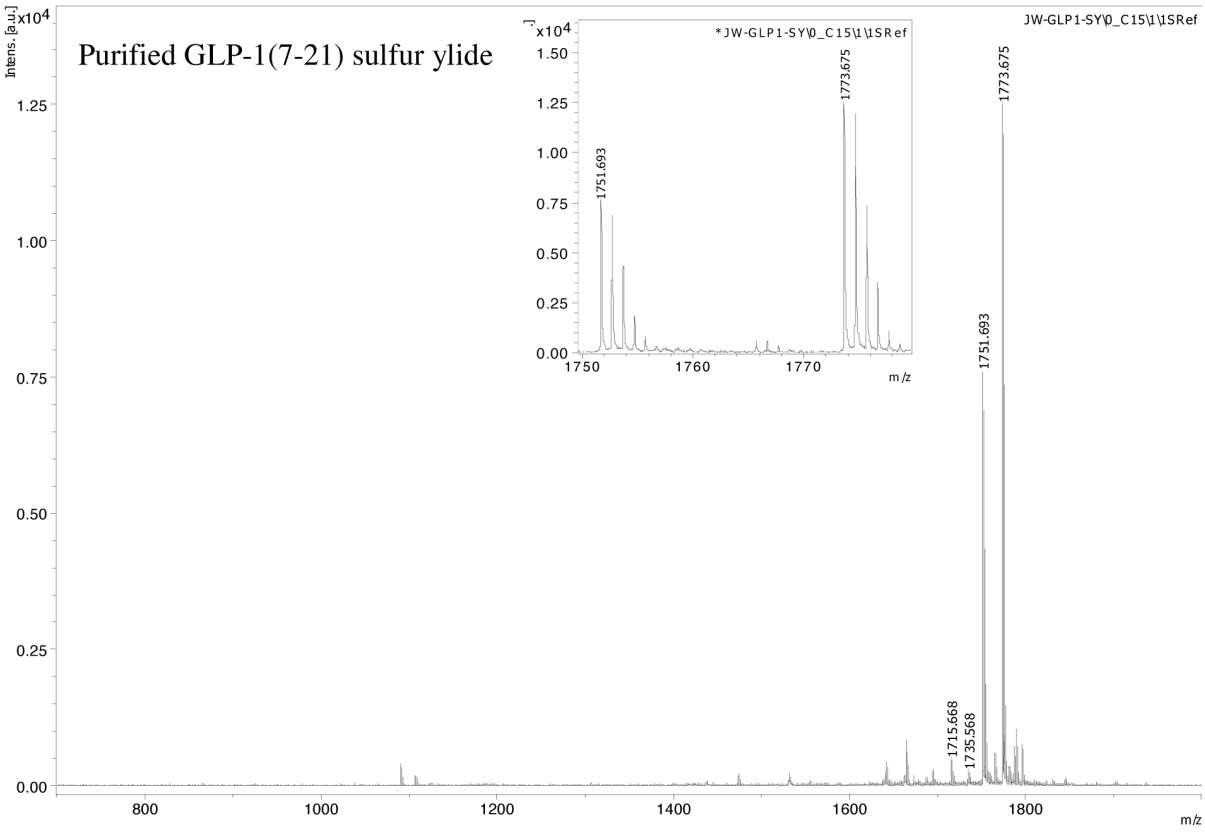
a) HPLC analysis of crude GLP-1(7-21) sulfur ylide from SPPS (Method C, 220 nm). t_r (**10**) = 11.5 min.

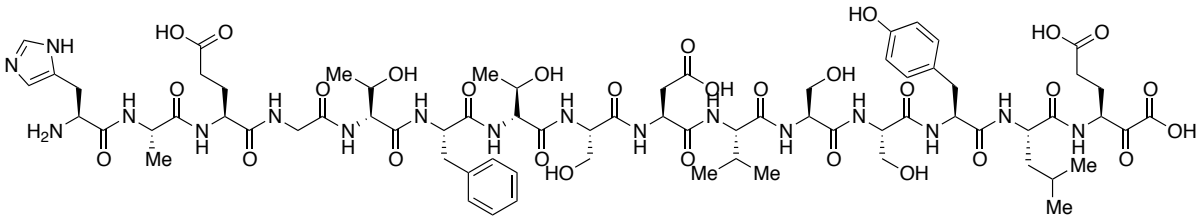


b) HPLC analysis of purified GLP-1(7-21) sulfur ylide (Method A, 280 nm).



c) MALDI MS (*m/z*) [MNa^+] calcd for $C_{77}H_{110}N_{18}NaO_{27}S$, 1773.74, found 1773.68; [MH^+] calcd for $C_{77}H_{111}N_{18}O_{27}S$, 1751.76, found 1751.69.





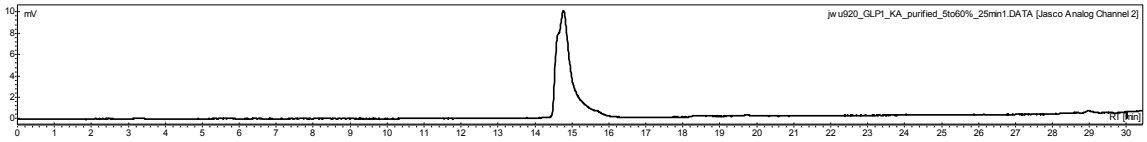
Chemical Formula: $C_{72}H_{103}N_{17}O_{29}$
Exact Mass: 1669.71
Molecular Weight: 1670.68

GLP-1(7-21) α-ketoacid (2). GLP-1(7-21) sulfur ylide **10** (10 mg, 0.0057 mmol, 1.0 equiv) was dissolved in 3:1 H_2O: DMF (0.28 mL, 20 mM). Oxone (7.0 mg, 0.011 mmol, 2.0 equiv) was added to

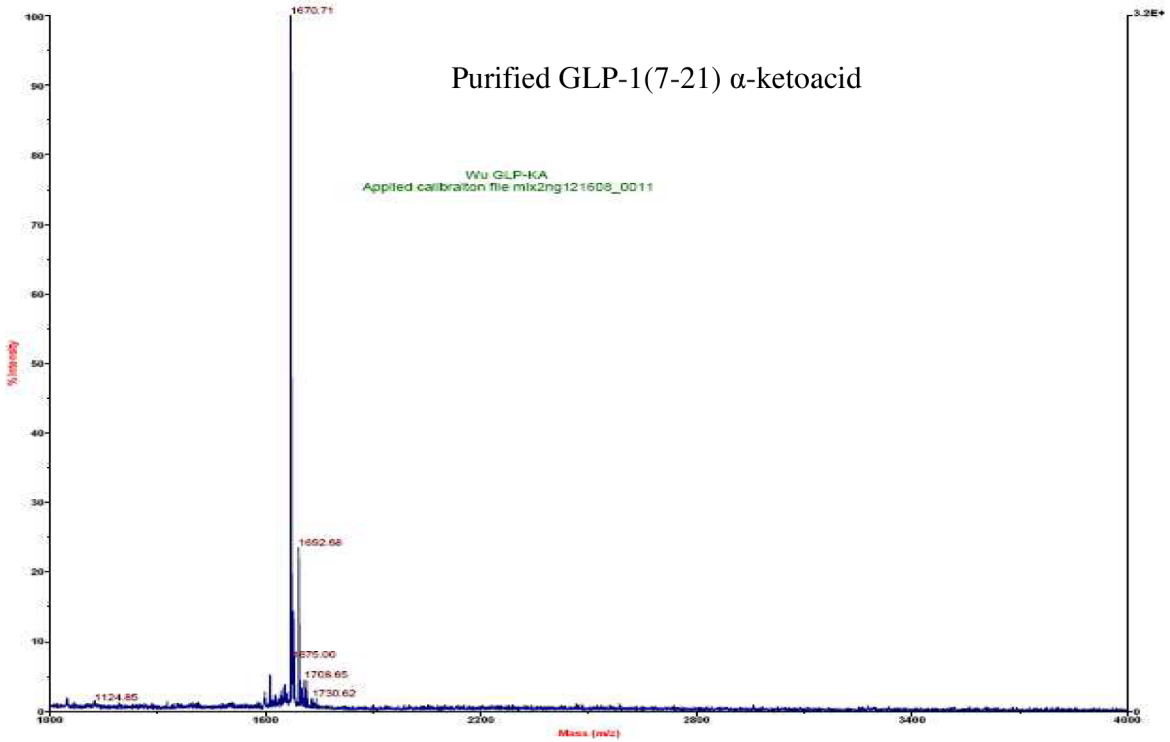
the reaction mixture in one portion. After stirring 10 min at rt, 0.1 mL Me_2S was added. After removing the Me_2S in vacuo, the crude α-ketoacid was purified by preparative reverse phase HPLC: Zorbax XDB-C8 21.2 × 250 mm column using a linear gradient of 20-40% buffer B over 20 min with a flow rate of 20 mL/min. t_r (α-ketoacid) = 14.8 min. After lyophilization, pure α-ketoacid **2** (6.2 mg, 65%) was obtained as a white solid.

Alternative procedure using dimethyldioxirane (DMDO): GLP-1(7-21) sulfur ylide **10** (6.0 mg, 0.0034 mmol, 1.0 equiv) was dissolved in 3:1 H_2O: DMF (0.2 mL, 0.02 M). DMDO (50 mM in acetone, 0.13 mL, 0.0068 mmol, 2.0 equiv) was added to the reaction mixture. After stirring 4 min at rt, 0.1 mL Me_2S was added. After removing the Me_2S in vacuo, the crude α-ketoacid was purified by preparative reverse phase HPLC: Zorbax XDB-C8 21.2 × 250 mm column using a linear gradient of 20-40% buffer B over 20 min with a flow rate of 20 mL/min. After lyophilization, pure α-ketoacid **2** (3.9 mg, 70%) was obtained as a white solid.

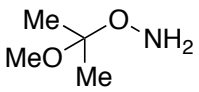
a) HPLC analysis of purified GLP-1(7-21) α-ketoacid (Method A, 280 nm).



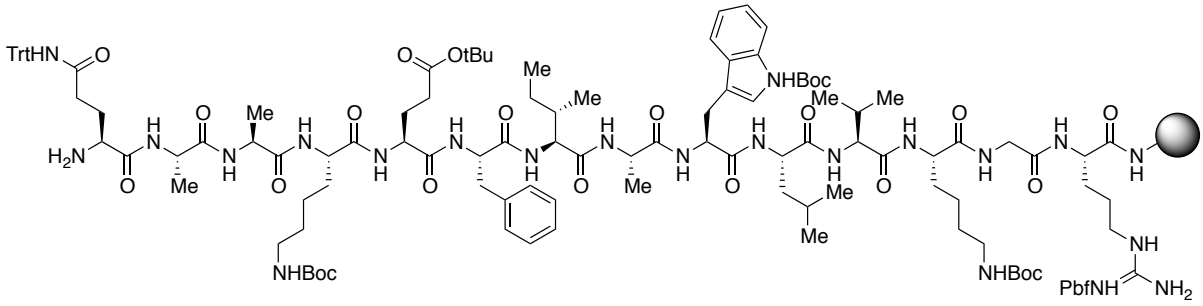
b) MALDI MS (*m/z*) [MH^+] calcd for $C_{72}H_{104}N_{17}O_{29}$, 1670.72, found 1670.71; [MNa^+] calcd for $C_{72}H_{103}N_{17}NaO_{29}$, 1692.70, found 1692.68.



2.3 Preparation of GLP-1 (22-36) hydroxylamine (3).



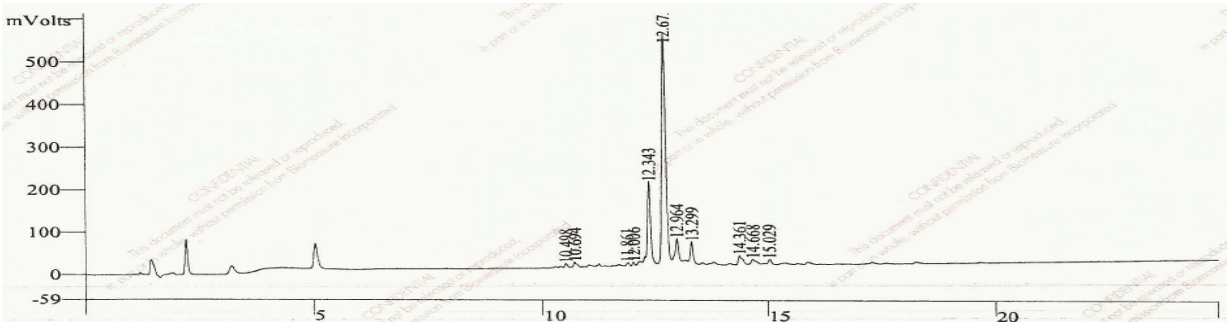
***O*-(2-Methoxypropyl)hydroxylamine (13)** [1] **.** A suspension of *N*-hydroxylphthalimide (15 g, 0.093 mol) and 2-methoxypropene (20 mL, 0.21 mol) in CH_3CN (300 mL) was stirred at rt for 6 days. The mixture was diluted with saturated aqueous $NaHCO_3$ solution and was concentrated in vacuo. The residue was extracted with EtOAc and the organic layer was washed with brine, dried and concentrated in vacuo. Recrystallization from Et_2O gave *O*-(2-methoxypropyl)-*N*-hydroxyphthalimide (15 g, 70%) as a yellow solid. This protected phthalimide (1.5 g, 6.4 mmol) was treated with hydrazine monohydrate (0.64 mL, 13 mmol) in MeOH (3.2 mL) and CH_2Cl_2 (8 mL). After stirring 2 h at rt, the reaction mixture was filtered, filtrate concentrated in vacuo, and 10% NaOH (13 mL) was added. The mixture was extracted with Et_2O and washed with brine, dried over Na_2SO_4 and concentrated in vacuo to give *O*-(2-methoxypropyl)hydroxylamine (0.38 g, 56%) as a colorless oil, which was directly used to the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.24 (s, 3H), 1.35 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 103.0, 49.0, 22.9. The analytical data was consistent with the literature report.

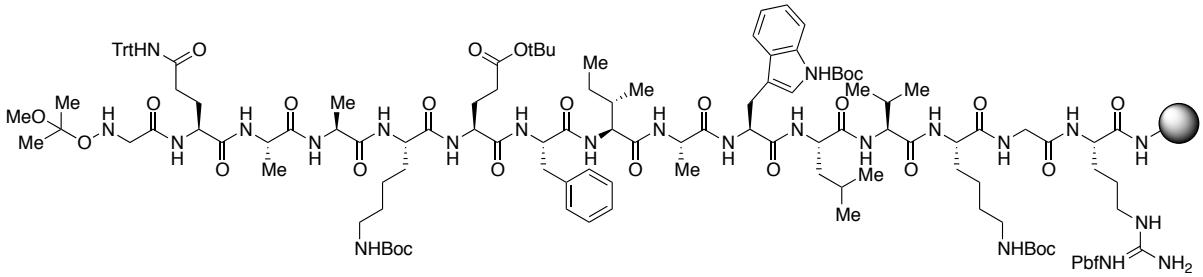


Resin-bound GLP-1 (23-36) hydroxylamine precursor (12). The title peptide was synthesized on an Applied Biosystems (Foster City, CA) model 433A peptide synthesizer on a 1 mmol scale using standard Fmoc/HBTU/HOBt procedures. Rink Amide MBHA resin (Nova Biochem., LaJolla, CA) with the substitution of 0.72 mmol/g was used. Fmoc amino acids (AnaSpec, Inc, San Jose, CA) were used with the following side chain protection: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Glu(tBu)-OH and Fmoc-Trp(Boc)-OH. The Fmoc groups were removed by treatment with 25% piperidine in DMF. Fmoc amino acids (1.0 mmol) were pre-activated with HBTU/HOBt (0.95 mmol) and DIPEA (2M) and were coupled to the resin. The last step was the removal of the terminal Fmoc using 25% piperidine in DMF for 30 min. The resin was washed with DMF and CH_2Cl_2 and dried. 3.3 g of resin bound hydroxylamine precursor was obtained from 1 mmol Rink Amide MBHA resin.

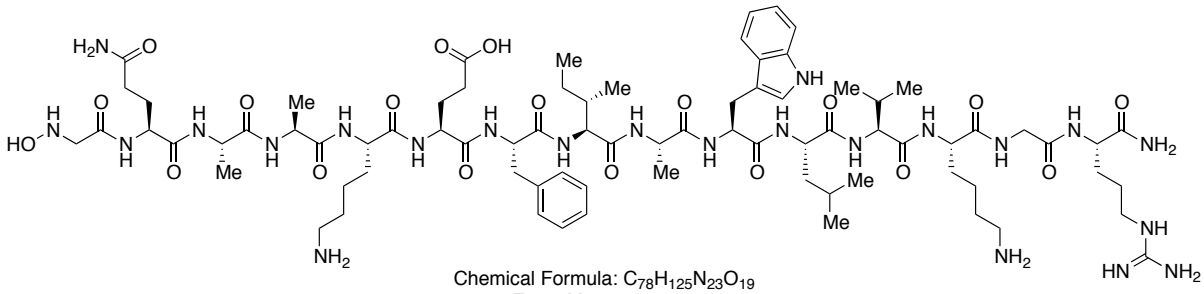
(1) Mori, K.; Koseki, K. *Tetrahedron* **1988**, 44, 6013-6020.

a) HPLC analysis of crude GLP-1(23-36) hydroxylamine precursor from SPPS (Method C, 220 nm), t_r (hydroxylamine precursor) = 12.6 min.





Resin-bound GLP-1 (22-36) hydroxylamine. A mixture of resin-bound peptide **12** (200 mg, 0.0660 mmol, 1.0 equiv), bromoacetic acid (46 mg, 0.33 mmol, 5.0 equiv) and DMAP (1 mg, 0.007 mmol, 0.1 equiv) was shaken in dry CH_2Cl_2 (2 mL) under an argon atmosphere at rt for 5 min, before DIC (0.05 mL, 0.33 mmol) was added dropwise. The mixture was shaken for 6 h and the solid material was filtered off and washed with CH_2Cl_2 (2 × 5 mL), DMF (2 × 10 mL), CH_2Cl_2 (2 × 5 mL). The resin was dried in vacuo to yield 220 mg of the coupled product. The resin bound bromide was mixed with *O*-(2-methoxypropyl)hydroxylamine (140 mg, 1.32 mmol, 20 equiv), DIPEA (0.050 mL, 0.33 mmol, 5.0 equiv) in dry DMF (2 mL), The reaction mixture was stirred at 60 °C for 20 h. Thereafter, the resin was washed with DMF (2 × 10 mL) and CH_2Cl_2 (2 × 10 mL), and dried in vacuo to give 210 mg of the product.

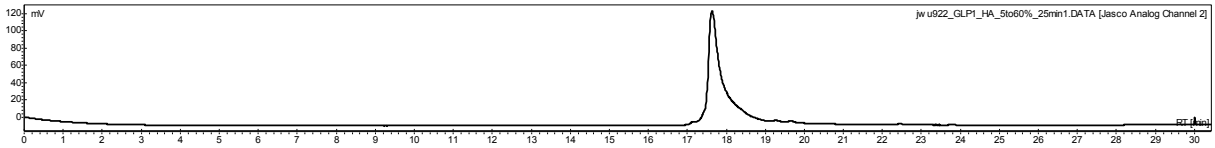


Chemical Formula: $C_{78}H_{125}N_{23}O_{19}$
Exact Mass: 1687.95
Molecular Weight: 1688.97

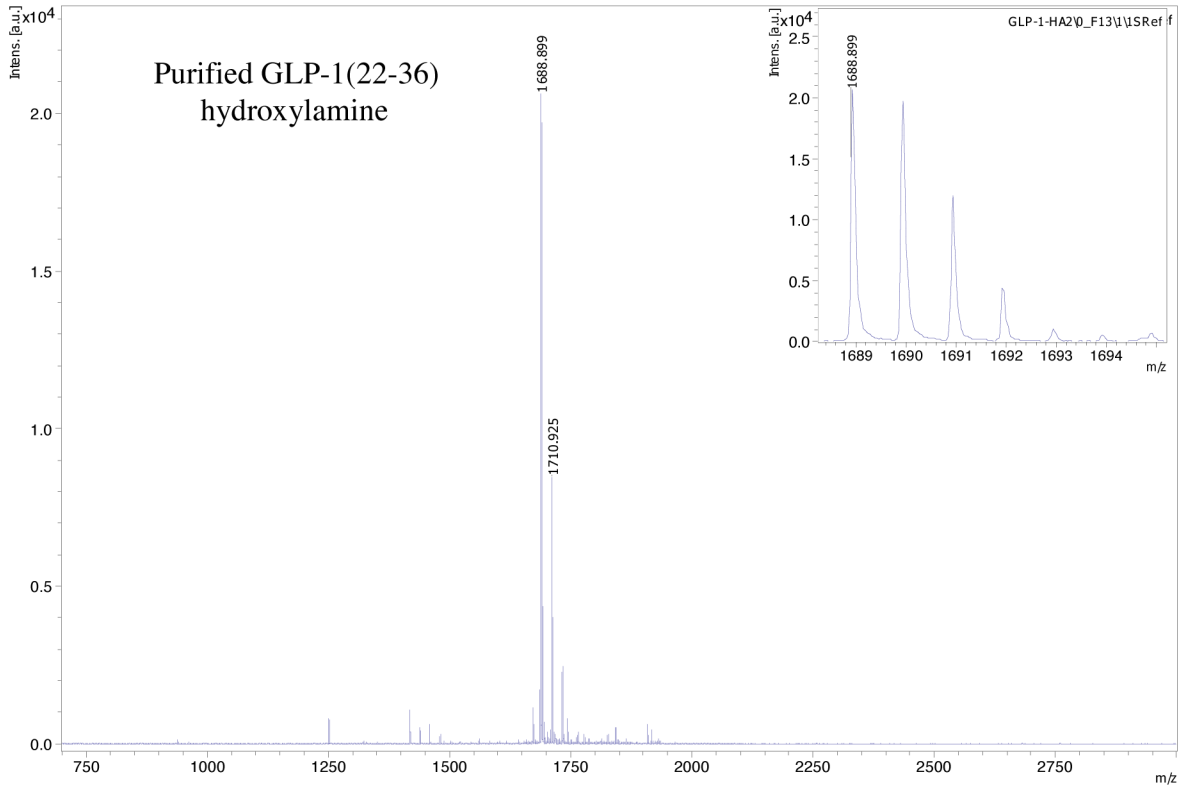
GLP-1 (22-36) hydroxylamine (3). The resin obtained above (120 mg) was mixed with a solution of TFA/TIPS/H_2O (95/2.5/2.5, 1.5 mL), and the resultant suspension was shaken under argon at rt for 2

h. The resin was filtered and rinsed with CH_2Cl_2 and TFA. After the combined filtrate and washing were concentrated in vacuo, the crude hydroxylamine was precipitated and triturated with chilled Et_2O. 50 mg of crude hydroxylamine was purified by preparative reverse phase HPLC using a linear gradient of 25-40% buffer B over 30 min with a flow rate of 20 mL/min. t_r (hydroxylamine) = 17.7 min. After lyophilization, pure hydroxylamine (8.5 mg, 15% from loading) was obtained as a white solid.

a) HPLC analysis of purified GLP-1(22-36) hydroxylamine (Method A, 280 nm).



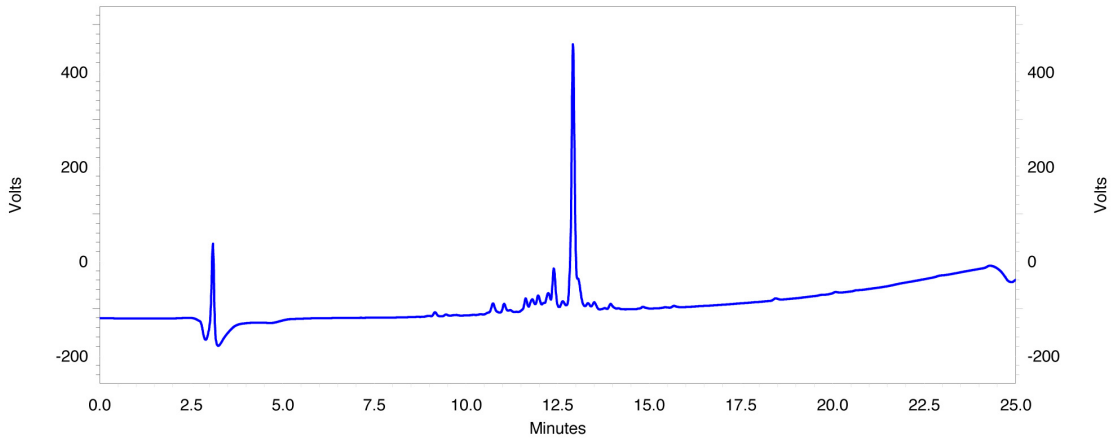
b) MALDI MS (*m/z*) [MH^+] calcd for $C_{78}H_{126}N_{23}O_{19}$, 1688.96, found 1688.90; [MNa^+] calcd for $C_{78}H_{125}N_{23}NaO_{19}$, 1710.94, found 1710.93.



Alternative procedure for the synthesis of ***3****.* DIPCDI (510 µL, 3.3 mmol, 10 equiv.) was added slowly to a solution of bromoacetic acid (917 mg, 6.6 mmol, 20 equiv.) in 2 mL of dichloromethane. The solution was stirred for 5 min and them was filtered from the urea. After that, 2,6-lutidine (384 µL, 3.3 mmol, 10 equiv.) was added and the resultant solution was mixed with the resin-bound peptide 12 (1.0 g, 0.33 mmol, 1 equiv.) and stirred until Kaiser test was negative (around 30 min.). The resin was washed with CH_2Cl_2, DMF and CH_2Cl_2 and the peptide was cleaved using a solution of TFA/TIPS/H_2O

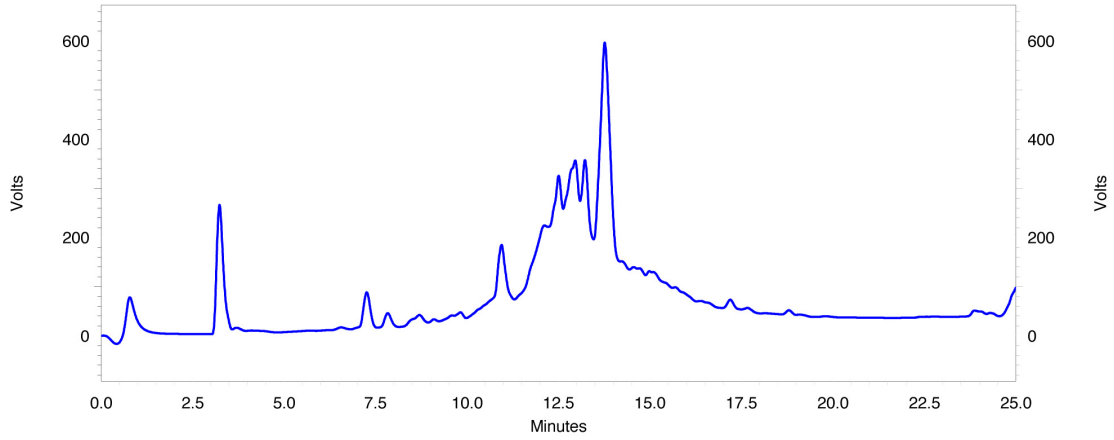
(95/2.5/2.5) for 1.5 h. The peptide was concentrated under vacuum and precipitated with Et_2O to afford 445 mg (78%) of crude peptide.

a) HPLC analysis of curde GLP-1 (22-36) bromo derivative.



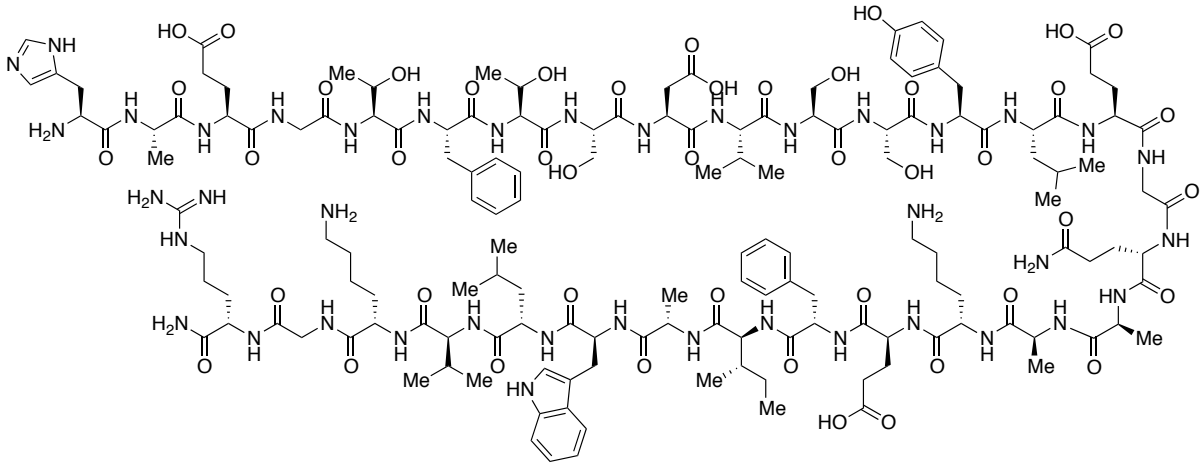
Hydroxylamine hydrochloride (403 mg, 5.8 mmol, 100 equiv.) was dissolved in 15 mL of absolute methanol and NaOMe (297 mg, 5.5 mmol, 95 equiv.) was added. The solution was stirred for 5 min, filtered and added to a solution of bromo derivative (100 mg, 0.058 mmol, 1 equiv.) in 10 mL of MeOH. After 1h, the solution was concentrated under vacuum to obtain the hydroxylamine derivative.

a) HPLC analysis of curde GLP-1 (22-36) hydroxylamine.



A small sample of 25 mg were purified by preparative HPLC using a linear gradient of 25-40% buffer B over 35 min with a flow rate of 15 mL/min. After lyophilization, pure hydroxylamine was obtained as a white solid (3 mg, 12%).

2.4 Synthesis of human GLP-1 (7-36) by α-ketoacid-hydroxylamine ligation.



Chemical Formula: $C_{149}H_{226}N_{40}O_{45}$
Exact Mass: 3295.66
Molecular Weight: 3297.63

Human GLP-1 (7-36) (1). C-terminal α-ketoacid **2** (3.0 mg, 0.0018 mmol, 1.0 equiv), N-terminal hydroxylamine **3** (3.4 mg, 0.0020 mmol, 1.05 equiv) and oxalic acid (0.5 mg, 0.004 mmol, 2.0 equiv) were dissolved in 3:1 degassed DMA: DMSO (0.18 mL, 10 mM) and stirred at 60 °C under argon. The reaction was monitored by analytical HPLC (Method A, 5-60% solvent B over 25 min). After 4 h, both α-ketoacid and hydroxylamine were almost consumed. After 20 h, the ligation reaction was directly applied to preparative reverse phase HPLC: Zorbax XDB-C8 21.2 × 250 mm column using a linear gradient of 25-55% buffer B over 30 min with a flow rate of 20 mL/min. t_r (GLP-1) = 19.8 min. After lyophilization, pure human GLP-1(7-36) (3.0 mg, 51%) was obtained as a white solid, along with D-Glu21 epimer (0.7 mg, 12%).

2.4.1 Monitoring of GLP-1 ligation reaction by HPLC.

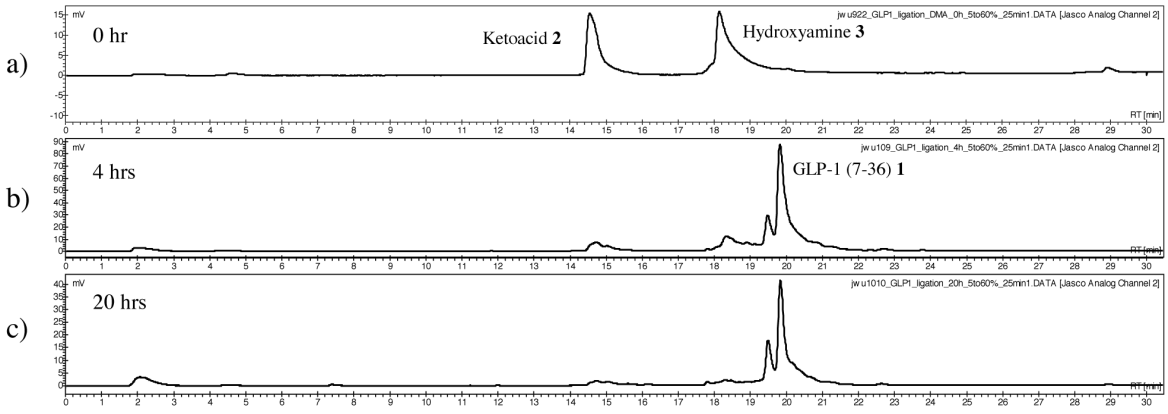
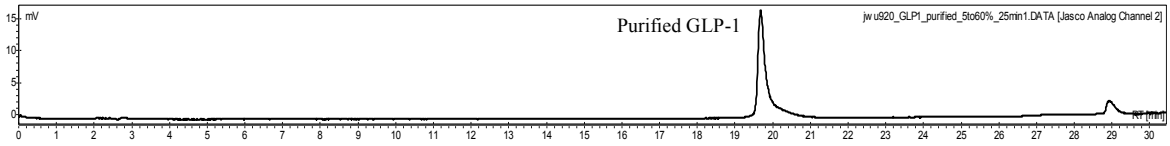


Figure 1. Monitoring of the GLP-1 ligation reaction by HPLC (samples taken directly from the reaction mixture without purification or workup). (a) Reaction at 0 hr. The first peak to elute is the α-ketoacid,

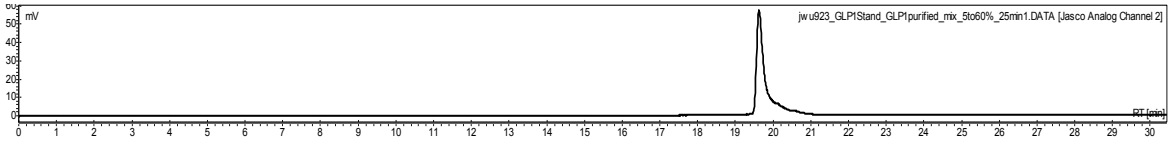
the second is the hydroxylamine. (b) Ligation reaction after 4 hrs. (c) Ligation reaction after 20 hrs. HPLC conditions: Method A, 280 nm.

2.4.2 Identification of the ligation product GLP-1 (7-36).

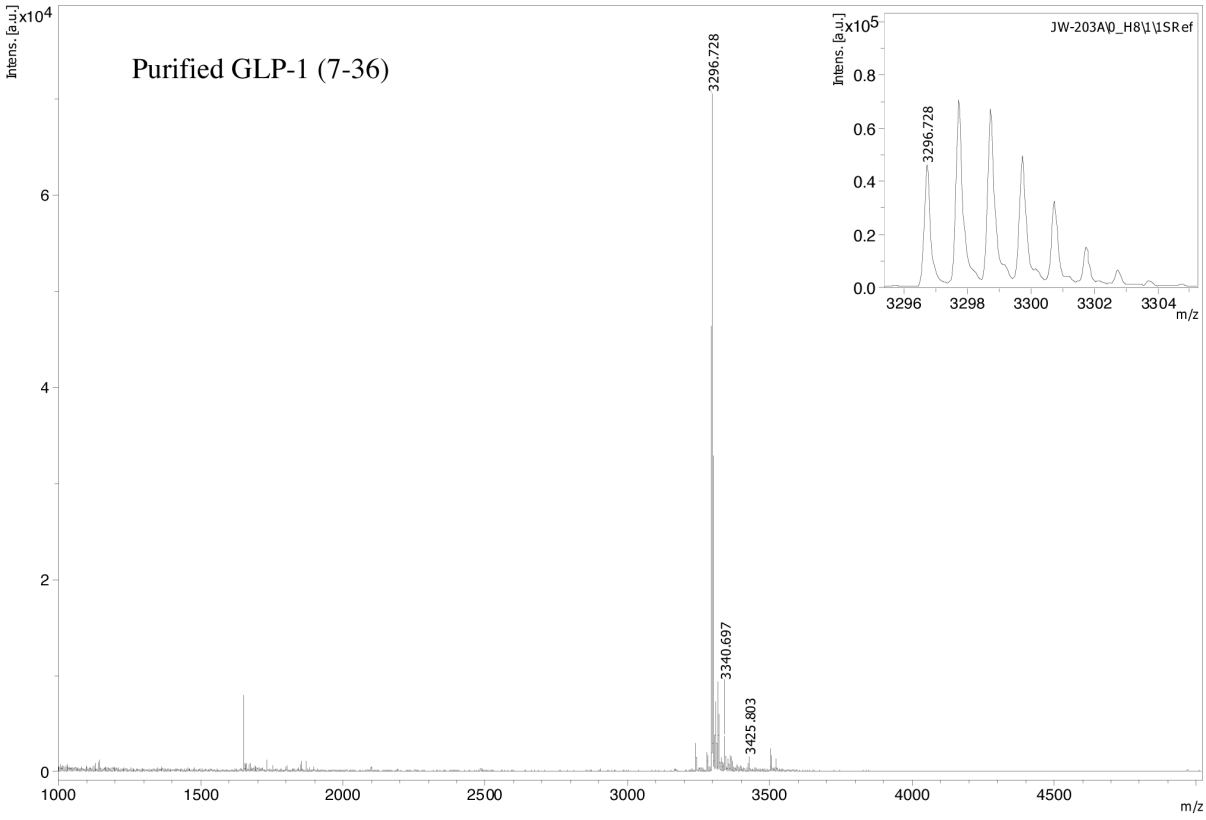
a) HPLC trace of purified GLP-1(7-36) (Method A, 280 nm).



b) HPLC trace of the mixture of purified GLP-1(7-36) and commercial GLP-1(7-36) sample.



c) MALDI MS (*m/z*) [MH^+] calcd for $C_{149}H_{227}N_{40}O_{45}$, 3296.67, found 3296.73.

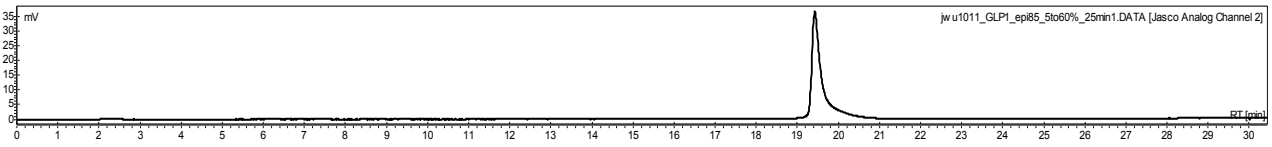


2.4.3 Identification of the byproduct from ligation reaction.

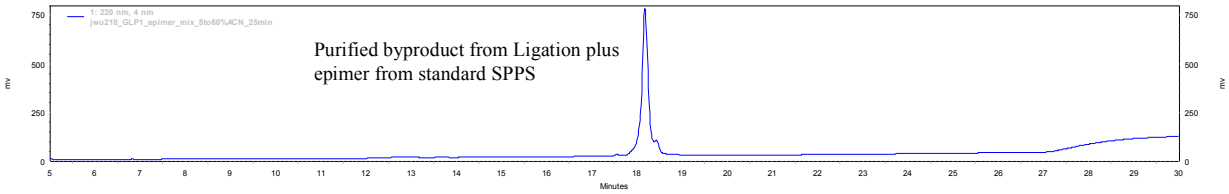
The ligation byproduct was isolated by preparative reverse phase HPLC: Zorbax XDB-C8 21.2 × 250 mm column using a linear gradient of 25-55% buffer B over 30 min with a flow rate of 20 mL/min. The D-Glu21 GLP-1 epimer was prepared on an Applied Biosystems (Foster City, CA) model 433A peptide synthesizer using standard Fmoc/HBTU/HOBt procedures. HPLC coinjection and MALDI

confirmed the byproduct from ligation is the D-Glu21 GLP-1 epimer.

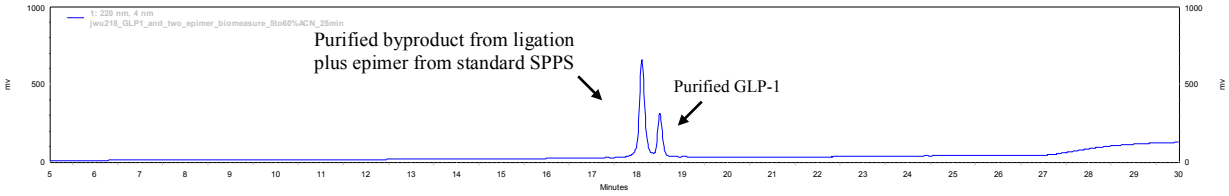
a) HPLC trace of the purified byproduct from ligation reaction (Method A, 280 nm).



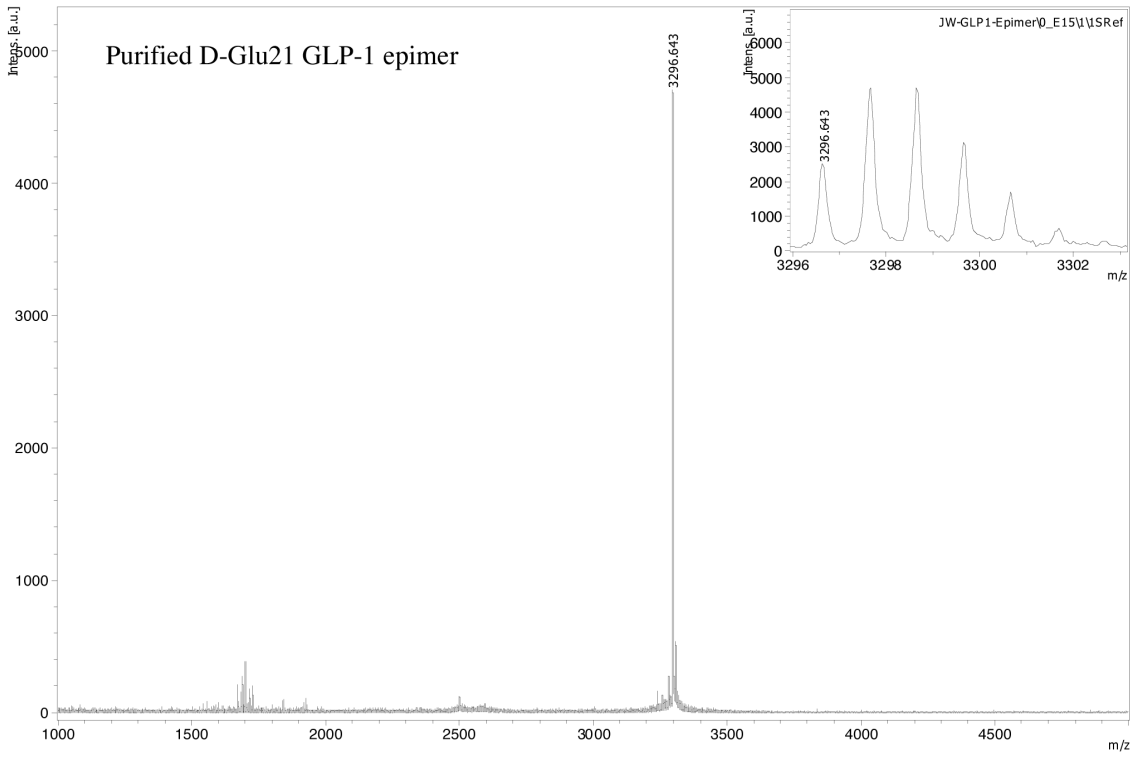
b) HPLC trace of the mixture of purified byproduct from ligation reaction and D-Glu21 GLP-1 epimer prepared by standard SPPS (Method B, 220 nm).



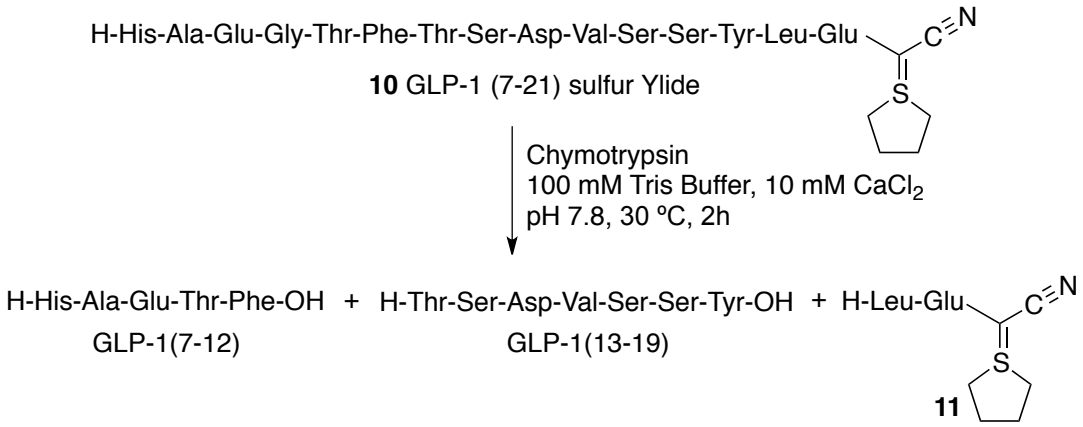
c) HPLC trace of the mixture of purified byproduct from ligation, D-Glu21 GLP-1 epimer prepared by standard SPPS and purified GLP-1 (Method B, 220 nm).



d) MALDI MS (*m/z*) [MH^+] calcd for $C_{149}H_{227}N_{40}O_{45}$, 3296.67, found 3296.64.



2.4.4 Investigation of the epimerization by enzymatic cleavage of GLP-1 (7-21) sulfur ylide 9.



Sulfur ylide **9** (1 mg) was digested by treatment with α-chymotrypsin (Sigma Chemical Co., St. Louis, MO) at a 1:60 enzyme/substrate ratio in 100 mM Tris HCl containing 10 mM $CaCl_2$, pH 7.8, at 30 ^{o}C. Aliquots of 0.1 mL were removed from the digest at specified intervals and checked by analytical HPLC. L-Leu-D-Glu-SY and L-Leu-L-Glu-SY were also synthesized and coinjected with the α-chymotrypsin treated reaction mixture.

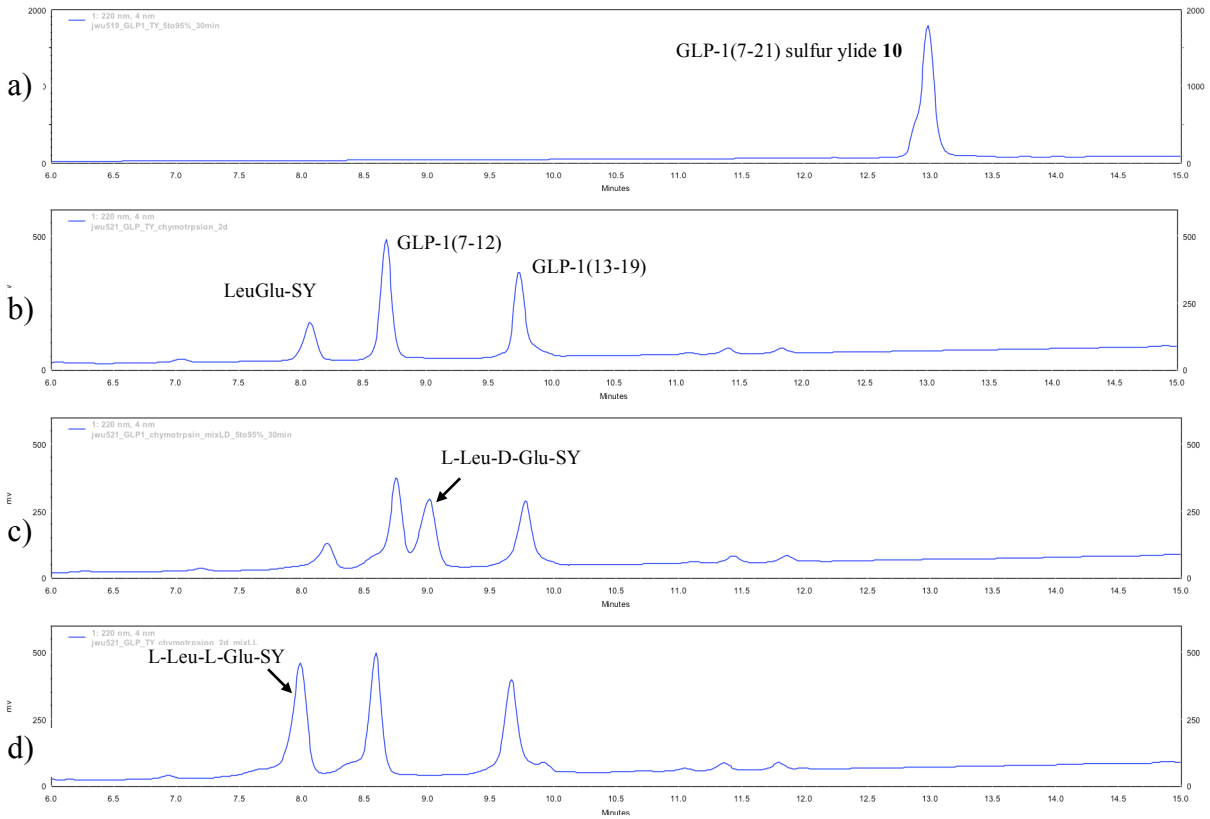


Figure 2. HPLC analysis of enzymatic cleavage of GLP-1(7-21) sulfur ylide **10** (samples taken directly from the reaction mixture without purification or workup). a) GLP-1 (7-21) sulfur ylide; b) Unpurified

mixture after digested with α-chymotrypsin for 2h; c) Coinjection of L-Leu-D-Glu-SY with the α-chymotrypsin digested reaction mixture (a new peak appeared); d) Coinjection of L-Leu-L-Glu-SY with the α-chymotrypsin digested reaction mixture (the peak height of dipeptide increased). HPLC conditions: Method B, 220 nm.

3. ^{1}H NMR and ^{13}C NMR Spectrum.