Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting information: Examination of mercaptobenzyl sulfonates as catalysts for native chemical ligation: Application to the assembly of a glycosylated Glucagon-Like Peptide 1 (GLP-1) analogue

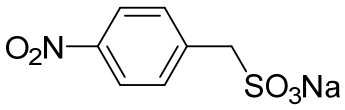
Ben Cowper, Tsz Mei Sze, Bhavesh Premdjee, Aileen Bongat-White, Andrew Hacking, and Derek Macmillan*

Department of Chemistry, University College London, 20 Gordon Street, London, WC1H 0AJ, UK.

General Experimental details

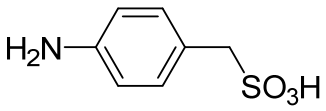
^{1}H NMR spectra were recorded at 500 MHz, 400 MHz, or 300 MHz and ^{13}C NMR spectra at 125 MHz or 100 MHz on Bruker 500 MHz and 400 MHz instruments respectively. Chemical shifts (δ) were reported in ppm and coupling constants (J) in Hz, signals were sharp unless stated as broad (br), s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet. Automated Peptide synthesis was performed on an Applied Biosystems 433A peptide synthesiser employing the FastMoc^{TM} protocol. Mass spectra were generally obtained on a Waters uPLC/SQD-LC series electrospray mass spectrometer. LC-MS was performed using a gradient of 5-95% acetonitrile containing 0.1% formic acid over 10 minutes (flow rate of 0.6 mL/min). Analytical HPLC was performed on a Dionex Ultimate 3000 series instrument using a Phenomenex Sphereclone ODS(C_{18}) column (250 x 4.5 mm) with a gradient of 5-95% acetonitrile containing 0.1% TFA over 55 minutes (flow rate of 1.0 mL/min). Semi-preparative HPLC was also performed on Dionex systems using either Phenomenex Jupiter Proteo (250 x 10mm, **method A**) or Phenomenex Jupiter Proteo (250 x 21.2 mm, **method B**) columns operating with a gradient of 5-60% acetonitrile containing 0.1% TFA over 45 minutes and at flow rates of 4.0 mL/min or 10.0 mL/min respectively.

Sodium *p*-nitrobenzyl sulfonate **9**



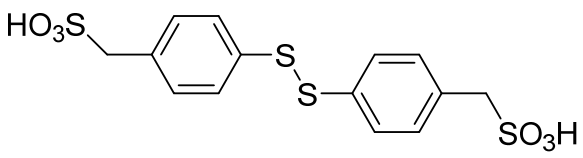
p-nitrobenzyl chloride (47.0 g, 0.27 mol) was added to a solution of sodium sulfite (34.03 g, 0.27 mol) in 138 mL of water. The mixture was stirred vigorously and boiled under reflux overnight. The reaction mixture was then cooled to 20 °C. The precipitate was filtered, washed with ether and dried in the oven to give yellow crystals (60.97 g, 94 % yield). 295-299 °C (decomp.). IR (cm^{-1}): 1537 (N-O), 1358 (N-O), ^{1}H NMR (500 MHz, D_2O) δ: 8.22 (2H, d, J= 8.8 Hz, 2x ArH), 7.62 (2H, d, J= 8.7 Hz, 2x ArH), 4.31 (2H, s, CH_2). ^{13}C NMR (125 MHz, D_2O) δ: 147.7, 140.1, 131.8, 124.1, 56.9 ppm. m/z (ESI-MS): $C_7H_6NO_5SNa$ required m/z= 239, found m/z= 216 $[M-Na]^-$.

p-aminobenzylsulfonic acid **11**



10 % Palladium on carbon (0.5 g) was added to sodium *p*-nitrobenzyl sulfonate (5.0 g, 0.02 mol) dissolved in 50 mL of water. The reaction mixture was hydrogenated at ambient pressure for 22 h. The mixture was filtered through celite to remove the catalyst and the filtrate was acidified with 12M hydrochloric acid to pH 6 to form the *p*-aminobenzylsulfonate zwitterion. The white precipitate was filtered and air dried (3.07 g, 83%). m.p.299 °C (decomp.). IR (cm^{-1}): 3450 (N-H), 3051 (Ar-H). ^{1}H NMR (500 MHz, D_2O) δ: 7.20 (2H, d, J = 8.2 Hz, 2x ArH), 6.81 (2H, d, J= 8.2 Hz, 2x ArH), 4.04 (2H, s, CH_2) ^{13}C NMR (125 MHz, D_2O) δ: 146.4 (Ar qC), 131.7 (Ar CH), 122.8 (Ar qC), 116.8 (Ar CH), 56.7 (CH_2). m/z (ESI-MS): $C_7H_8NO_3SNa$ required m/z= 187.0, found m/z= 186 $[M-H]^-$

4,4'-dithiobis(benzylsufonic acid) **13**



p-aminobenzyl sulfonic acid (10.0 g, 0.05 mol) was dissolved in a solution of sodium hydroxide (2.0 g, 0.05 mol) in 50 mL of water, followed by addition of 10 mL of concentrated hydrochloric acid.

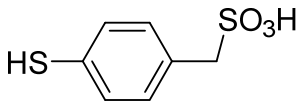
A solution of sodium nitrite (3.5 g, 0.05 mol), in 10 mL of water was added to the resulting suspension gradually at 0 °C. The reaction mixture was poured into a solution of sodium disulfide, prepared by co-melting sulfur (1.6 g, 0.05 mol) and sodium sulfide nonahydrate (12.0 g, 0.05 mol) in 50 mL of water at 65 °C.

The reaction mixture was warmed until gas ceased to evolve and then cooled to room temperature. The insoluble material was filtered off.

A solution of aniline (9.34 g, 0.1 mol) and concentrated hydrochloric acid (8.5 mL) in 20 mL of water was added to the filtrate to afford a thick yellow precipitate. The precipitate was filtered and washed with hexane to give dianilinium 4,4'-dithiobis(benzylsulfonate) (9.45 g, 64%). m.p. 264 °C (decomp.). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.31-7.46 (8H, m, 8x ArH), 3.81 (4H, s, 2x CH_2). m/z (ES): $C_{26}H_{28}O_6S_4N_2$ required m/z= 592, found m/z= 404 $[M\text{-}2ArNH_3^+]^-$

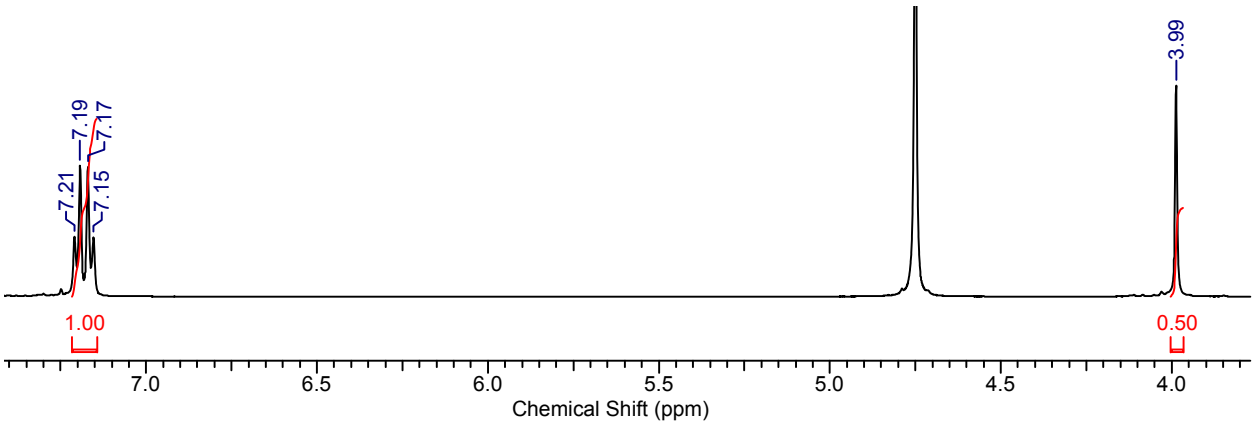
The salt (1.0 g, 1.69 mmol) was dissolved in hot methanol-water (2:1) and subjected to ion exchange column chromatography (Dowex 50X2-100, H-form, methanol/water (2:1) as eluent) to exchange the anilinium ions for protons. Acidic fractions (pH 2) were collected and evaporated *in vacuo.* to afford a brown solid (0.68 g, 99%). m.p. 255- 258 °C (decomp.). ^{1}H NMR (500 MHz, D_2O) δ: 7.52 (4H, d, J = 8.4 Hz, 4x ArH), 7.32 (4H, d, J= 8.4 Hz, 4x ArH), 4.09 (4H, s, 2x CH_2). ^{13}C NMR (125 MHz, D_2O) δ: 136.5 (qC), 131.8 (C-H), 131.6 (qC), 128.5 (CH), 56.8 (CH_2) ppm.

4-mercaptobenzyl sulfonic acid **5**

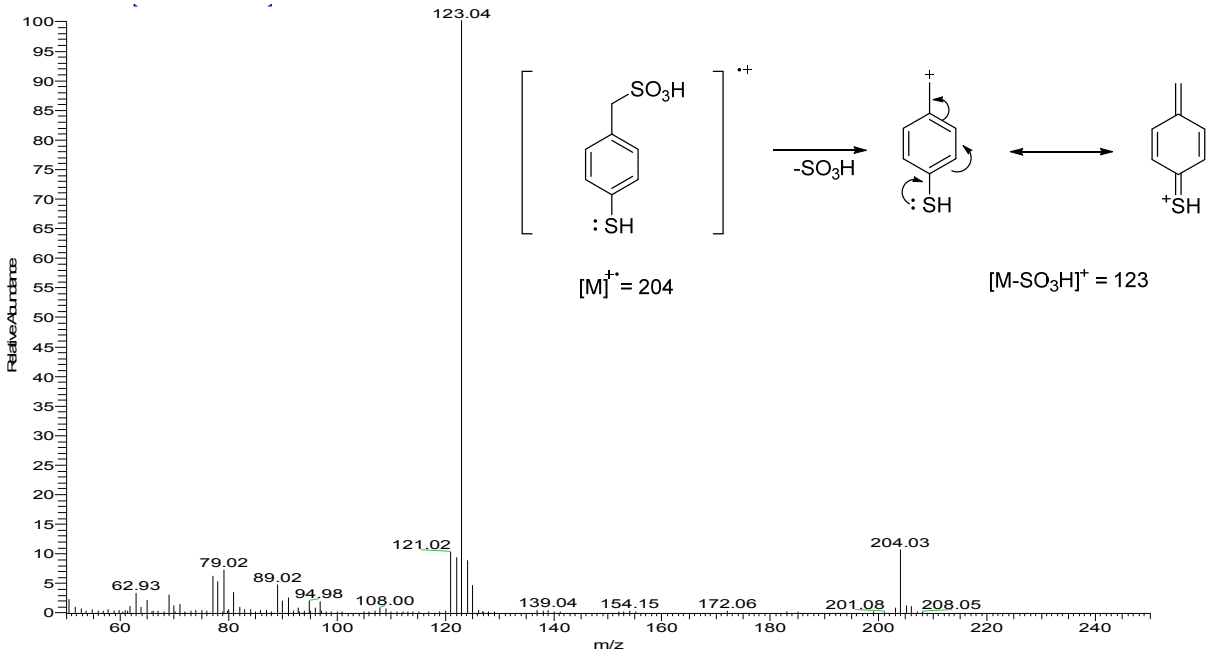


4,4'-dithiobis(benzylsulfonic acid) (2.26 g, 5.6 mmol) was dissolved in 40 mL of methanol. Triphenylphosphine (3.20 g, 12 mmol) and 1 mL of water were added to the resulting solution. The mixture was stirred overnight at room temperature. The reaction mixture was evaporated *in vacuo.* and the residue was dissolved in 40 mL of dichloromethane and extracted with water. The extract was washed with dichloromethane and evaporated in *vacuo* to give *p*-mercaptobenzylsulfonic acid as a brown solid (1.44 g, 64 %). m.p.105-109 °C. ^{1}H NMR (500 MHz, D_2O) δ: 7.20 (2H, d, J= 8.0 Hz, 2x ArH), 7.16(2H, d, J= 8.0 Hz, 2x ArH), 3.99 (2H, s, CH_2). ^{13}C NMR (125 Hz, D_2O) δ: 131.4 (ArCH), 131.0 (qC), 129.4 (qC), 129.2 (ArCH), 56.7 (CH_2) ppm. m/z (EI): $C_7H_8O_3S_2$ required m/z= 204, found m/z= 204 $[M]^+$

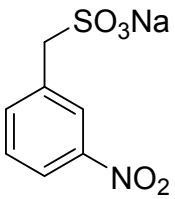
^{1}H NMR of 4-MBSA (**5**) in D_2O (solvent peak at 4.75ppm)



EI-MS spectrum of 4-MBSA (**5**)



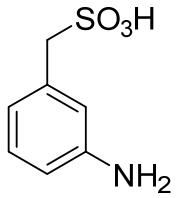
Sodium 3-nitrobenzyl sulfonate **10**



3-nitrobenzyl chloride (10.0 g, 0.058 mol) was added to a solution of sodium sulfite (8.0 g, 0.063 mol) in 25 mL of water. The suspension was stirred vigorously and

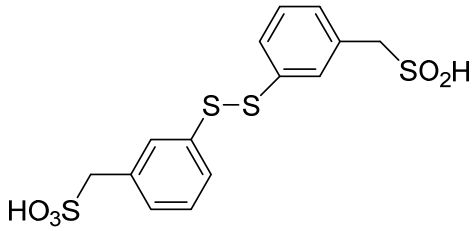
boiled under reflux overnight. The reaction mixture was then cooled to 20 °C. The precipitate was then filtered-off and washed with ether to afford the products as a pale brown solid (12.5 g, 90%), ^{1}H NMR (300 MHz, D_2O) δ: 8.29 (1H, s, 1x ArH), 8.24 (1H, d, = 8.3 Hz 1x ArH), 7.82 (1H, d, J= 7.8 Hz, 1 x ArH), 7.63 (1H, t, J= 8.0 Hz, 1 x ArH), 4.31 (2H, s, CH_2) ppm. ^{13}C NMR (75 MHz, D_2O) δ: 148.4 (qC), 137.7 (CH), 134.3 (qC), 130.3 (CH), 125.8 (CH), 123.6 (CH), 56.7 (CH_2) ppm.

3-aminobenzyl sulfonic acid **12**



10% Palladium on carbon (1.0 g) was added to sodium *m*-nitrobenzyl sulfonate (10.0 g, 42 mmol) dissolved in 75 mL of water. The reaction mixture was hydrogenated at ambient pressure for 24 h. The mixture was filtered through celite to remove the catalyst and the filtrate was acidified with 12 M hydrochloric acid to pH 5. The product failed to precipitate and so the reaction mixture was concentrated to approx. ¼ of the original volume and stored at 4 ^{o}C overnight. A crystalline product was subsequently formed, filtered-off and washed with cold water and finally dried under vacuum (6.1 g, 78%). ^{1}H NMR (300 MHz, D_2O) δ: 7.57-7.49 (2H, m, 2x ArH), 7.41-7,37 (2H, m, 2x ArH), 4.28 (2H, s, CH_2) ppm. ^{13}C NMR (75 MHz, D_2O) δ: 137.7 (qC), 134.6 (qC), 131.7 (CH), 130.9 (CH), 125.2 (CH) 123.1 (CH), 56.9 (CH_2) ppm.

3,3’-dithiobis(benzylsulfonic acid) **14**



m-aminobenzyl sulfonic acid (6.0 g, 0.032 mol) was dissolved in a solution of sodium hydroxide (1.23 g, 0.032 mol) in 32 mL of water, followed by addition of 6.4 mL of concentrated hydrochloric acid.

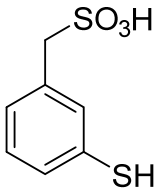
A solution of sodium nitrite (2.2 g, 0.032 mol), in 6.4 mL of water was added to the resulting suspension gradually at 0 °C. The reaction mixture was poured into a solution of sodium disulfide, prepared by co-melting sulfur (1.02 g, 0.032 mol) and sodium sulfide nonahydrate (7.68 g, 0.032 mol) in 32 mL of water at 65 °C.

The reaction mixture was allowed to warm to room temperature until gas ceased to evolve and then the insoluble material was filtered off.

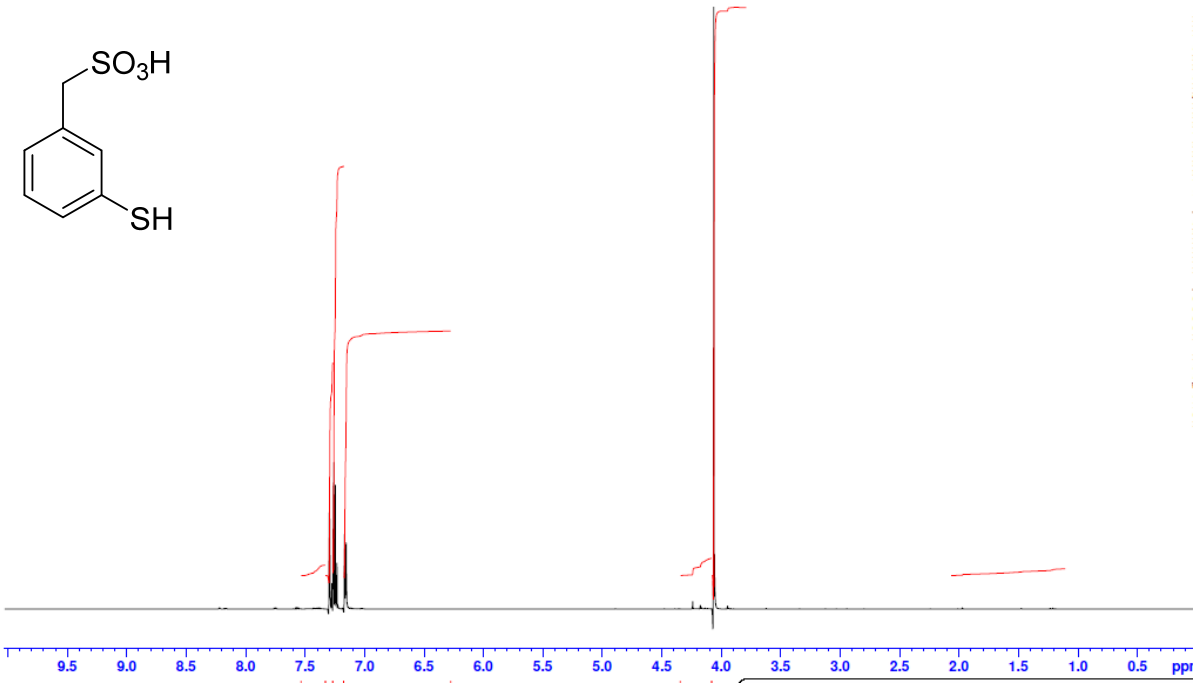
A solution of aniline (5.95 g, 0.064 mol) and concentrated hydrochloric acid (5.5 mL) in 10 mL of water was added to the filtrate, forming a yellow precipitate. The precipitate was filtered and washed with hexane to give dianilinium 3,3’-dithiobis(benzylsulfonate) (6.01 g, 64%) which was progressed to the free acid form without further purification.

The salt (3.0 g, 1.69 mmol) was dissolved in hot methanol-water (2:1) and subjected to ion exchange column chromatography (Dowex 50X2-100, H-form, methanol/water (2:1) as eluent) to exchange the anilinium ions for protons. Acidic fractions were collected and evaporated *in vacuo.* to afford brown solid (1.8 g, 88%). ^{1}H NMR (500 MHz, D_2O) δ: 7.58-7.25 (8H, m, 8x ArH), 4.11 (4H, s, 2x CH_2) ppm.
[Note: the salt has poor solubility in 2:1 MeOH/H_2O]

3-mercaptobenzyl sulfonic acid **6**

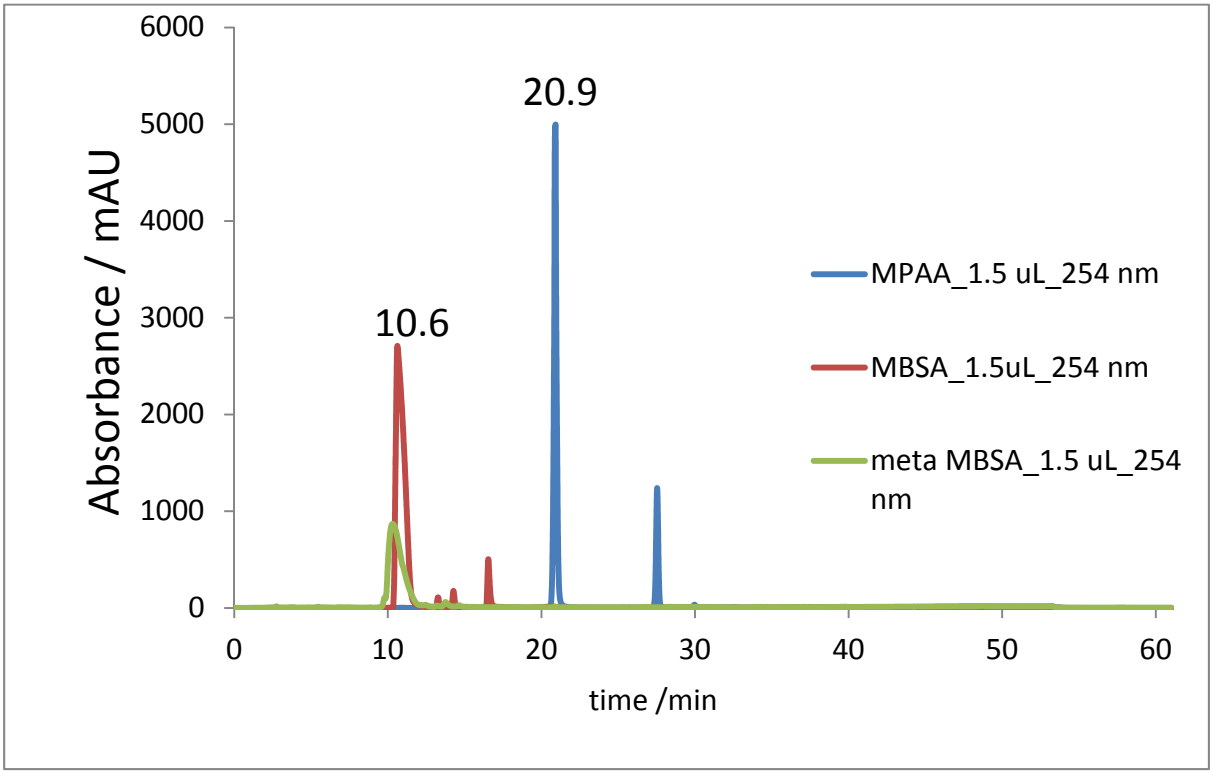


3,3'-dithiobis(benzylsulfonic acid) (2.23 g, 6 mmol) was dissolved in 40 mL of methanol. Triphenylphosphine (3.20 g, 12 mmol) and 1 mL of water were added to the resulting solution. The mixture was stirred overnight at room temperature. The reaction mixture was evaporated *in vacuo.* and the residue was dissolved in 40 mL of dichloromethane and extracted with water. The aqueous extract was washed with dichloromethane (20 mL) and evaporated in *vacuo.* to give *3*-mercaptobenzyl sulfonic acid as a waxy pale brown solid (1.48 g, 64 %). ^{1}H NMR (300 MHz, D_2O) δ: 7.34-7.24 (3H, m, 3x ArH), 7.21-7.17(1H, m, ArH), 4.09 (2H, s, CH_2) ppm. ^{13}C NMR (75 MHz, D_2O) δ: 133.4 (qC), 131.7 (qC), 131.1 (CH), 130.0 (CH), 129.0 (CH), 128.1 (CH), 57.1 ($\underline{C}H_2$) ppm.



Analytical HPLC analysis of **5**, **6** and MPAA.

Analytical HPLC was performed on a Dionex Ultimate 3000 series instrument using a Phenomenex Sphereclone ODS(C_{18}) column (250 x 4.5 mm) with a gradient of 5-95% acetonitrile containing 0.1% TFA over 55 minutes (flow rate of 1.0 mL/min).

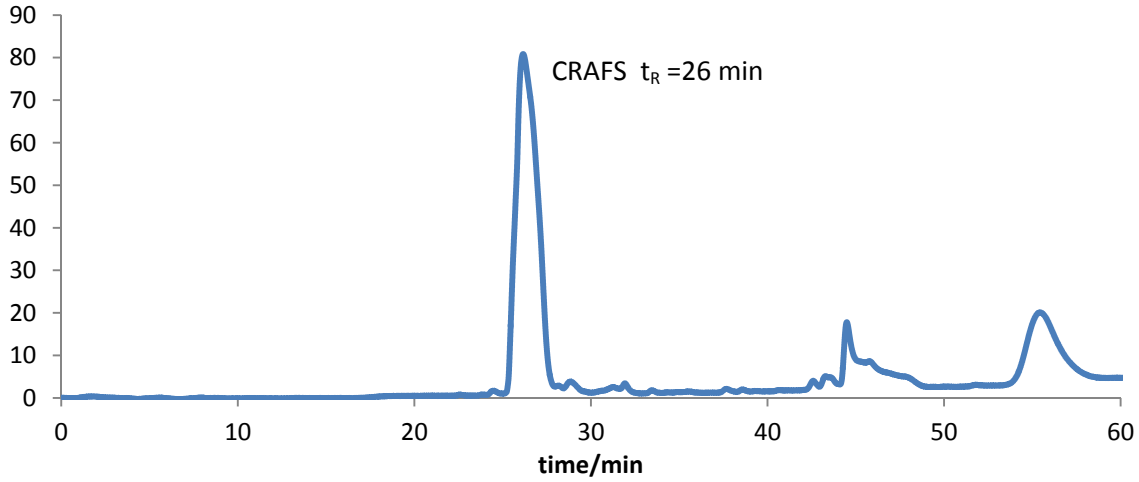


Synthesis of model peptide H-CRAFS-OH (**16**)

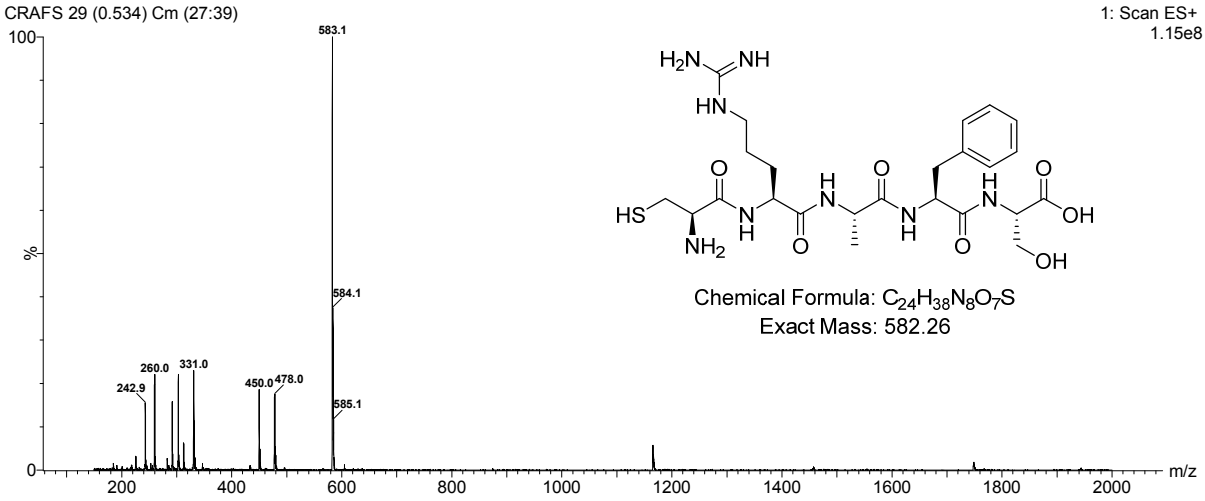
Serine pre-loaded NovaSynTGT resin (250 mg, 0.05 mmol) was treated with 20% piperidine (v/v) in DMF (2mL, 15 min). The solution was then filtered and the resin washed with DMF and DCM. The resin was then coupled to Fmoc-Phe-OH (0.5 mmol) with 0.45 M HBTU/HOBt (1 mL) and DIPEA (0.15 mL) in dry DMF (1 mL), on a horizontal shaker for 2 h at room temperature. The resin was then filtered off and washed exhaustively with DMF and DCM. The dried resin was then treated with 20% piperidine (v/v) in DMF (2 mL, 15 min), filtered, washed with DMF and DCM, and dried. The process was repeated for coupling reactions with Fmoc-Ala-OH, Fmoc-Arg(Pmc)-OH and Fmoc-Cys(Trt)-OH. Following chain assembly, the resin was treated with a cleavage cocktail (4.0 mL) comprised of TFA (95 %), water (2.5%) and EDT (2.5 % v/v) for 4 h at room temperature with stirring.

The resin was then filtered-off and the filtrate was added to diethyl ether to induce precipitation. The precipitate was collected by centrifugation (3000rpm, 4°C, 15 min), dissolved in water and purified by HPLC (Method B). The collected fractions were freeze-dried overnight and the pure peptide was obtained as a white fluffy solid (21 mg, 70 %). m/z (LC-MS): calculated m/z= 582.3, measured m/z= 583.1 $[M+H]^+$.

Semi-prep HPLC purification of H-CRAFS-OH **16**:



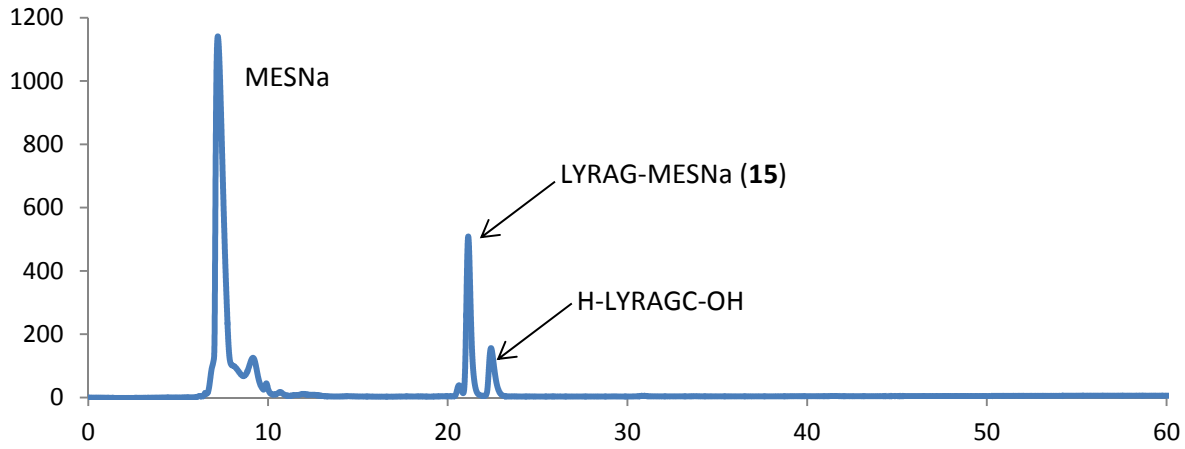
MASS SPECTRUM of HPLC purified H-CRAFS-OH



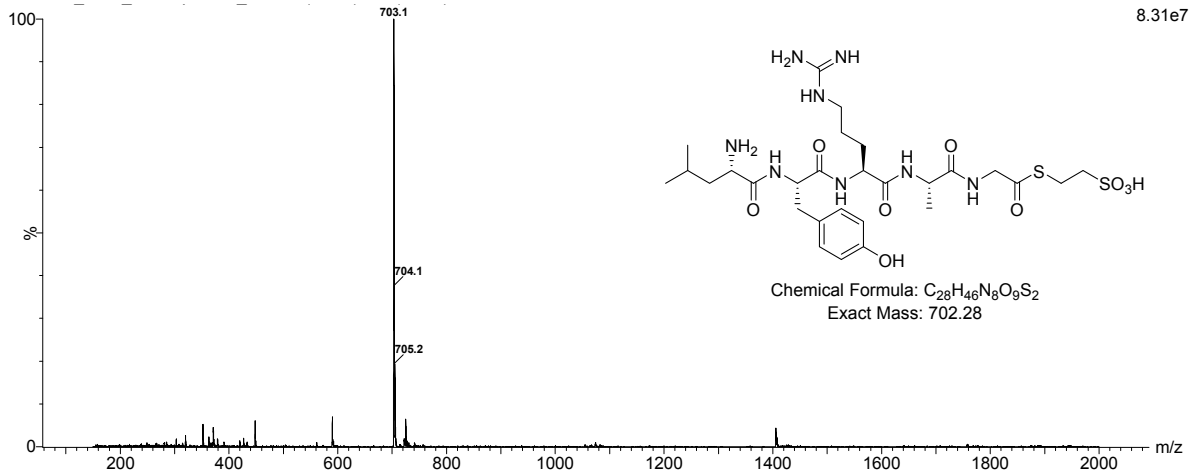
Synthesis of LYRAG-MESNa thioester **15**

H-LYRAGC-OH (5 mg) was dissolved in 5 mL of 0.1 M sodium phosphate buffer (pH 5.8) containing TCEP (25 mg), 10 % MESNa (0.5 g). The reaction mixture was shaken on an Eppendorf thermomixer at 55 °C for 26 h then purified by HPLC (Method B). Fractions containing the product were identified by LC-MS and then lyophilised to obtain the product as a fluffy white solid (4.5 mg, 79 %). m/z (LC-MS): calculated m/z= 702.3, measured m/z= 703.1 $[M+H]^+$.

HPLC purification OF LYRAG-MESNa thioester **15**:

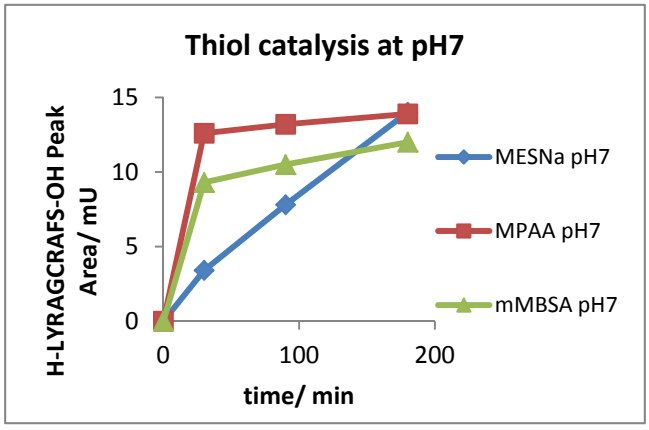


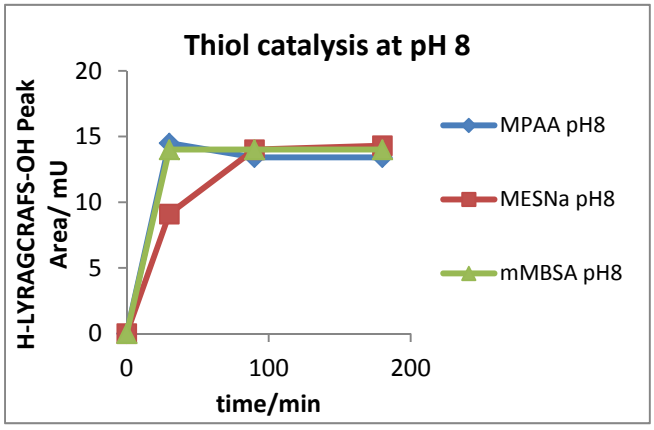
MASS SPECTRUM OF purified LYRAG-MESNa thioester **15**:



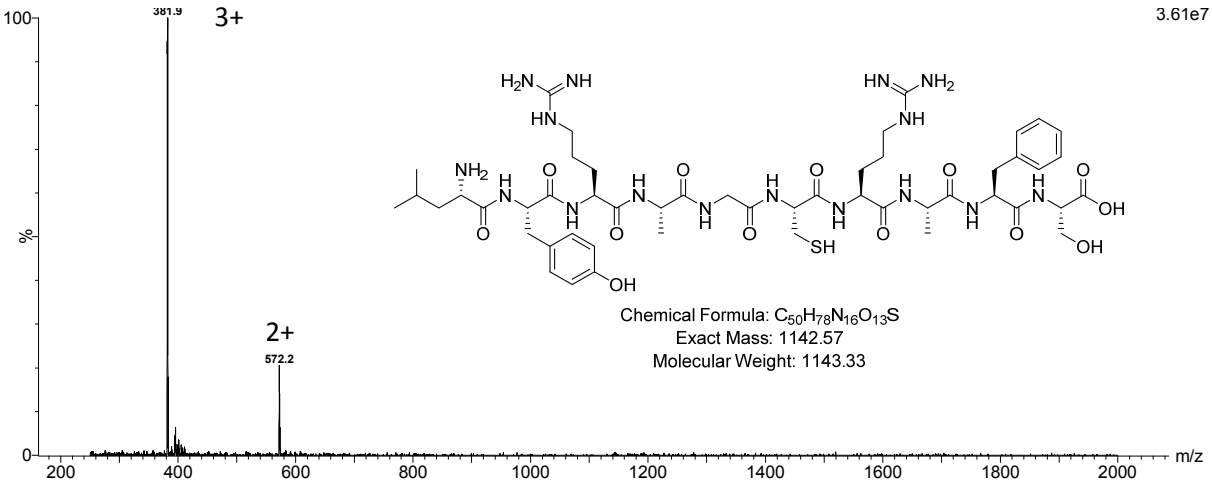
Ligation reactions between LYRAG-MESNa thioester (**15**) and CRAFS (**16**):

Where possible, thiol additives were prepared as 1 M stock solutions and used at 0.1 M final concentration in ligation reactions. Final peptide concentrations were approximately 1 mM for CRAFS (1.02 mM) and LYRAG thioester (0.8 mM). Typical 0.5 mL reactions were prepared as follows: CRAFS (2 $mgmL^{-1}$ in water (3.4 mM), 150 µL) and LYRAG-MESNa thioester (2 $mgmL^{-1}$ in water (2.8 mM), 150 µL) were added to sodium phosphate buffer at the appropriate pH (1 M, 100 µL, final concentration = 200 mM). Thiol additive (1 M, 50 µL) was then added, followed by TCEP (1 M, 10 µL) and water (40 µL). Each 0.5 mL reaction was shaken gently on an Eppendorf thermomixer at 21 ^{o}C for 3 h. Reaction progress was monitored by analytical HPLC at 0.5, 1.5, and 3 h by removing a 20 µL aliquot from the reaction mixture, quenching through the addition of 10% aqueous TFA (20 µL) and 20 µL samples were analysed.





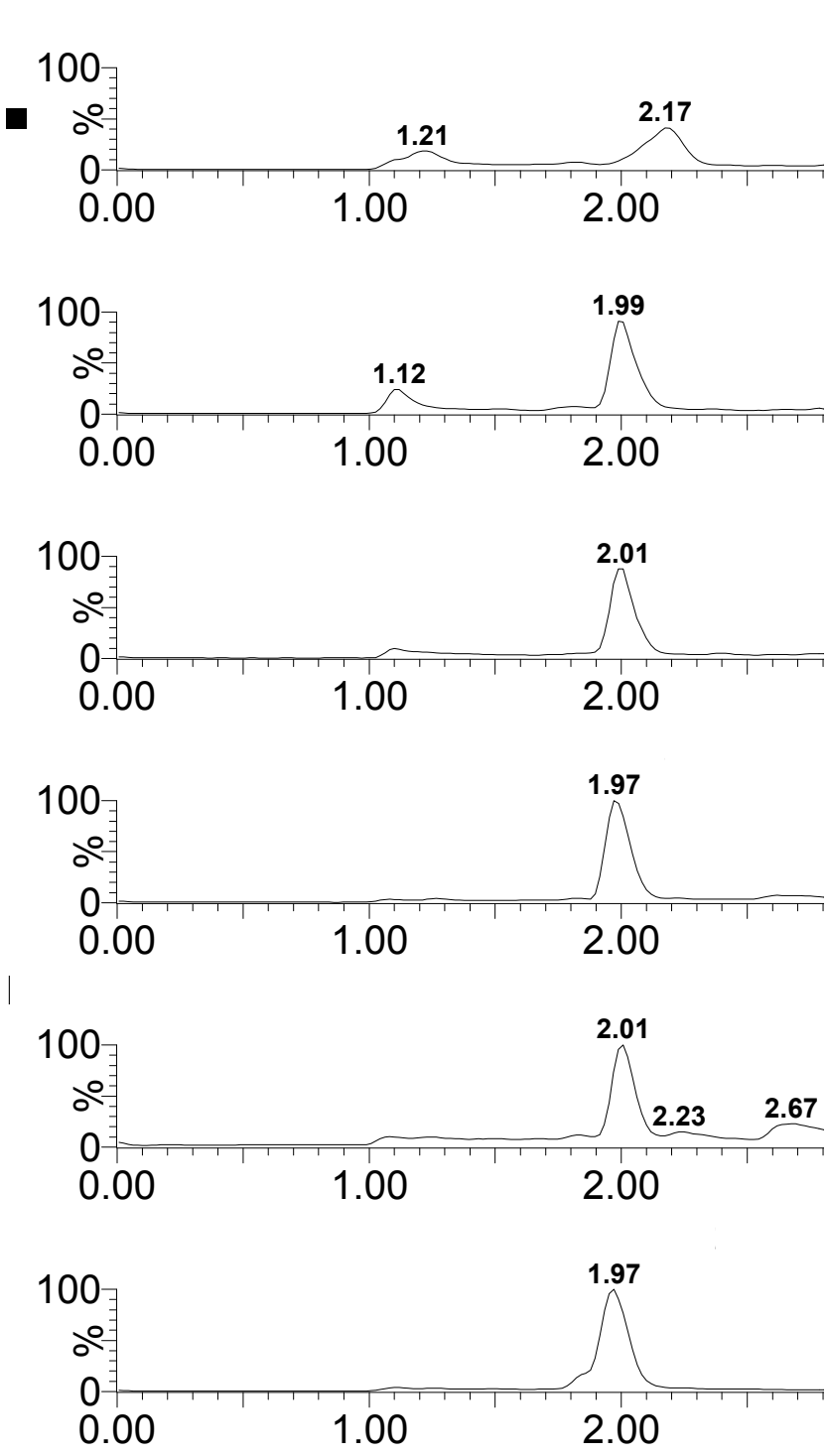
MASS SPECTRUM OF H-LYRAGCRAFS-OH (LIGATION PRODUCT):



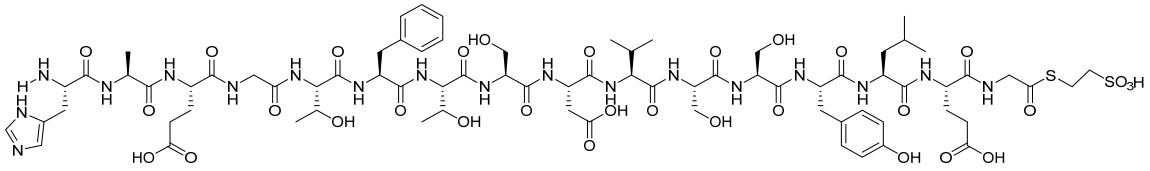
SELECTED **TIC** TRACES FOR MODEL LIGATION REACTIONS

NOTE: LC-MS analysis was conducted after 3 h (0.1 M catalyst concentration). Neither H-LYRAG-MESNa thioester nor H-CRAFS-OH were retained on the LC-MS column and both eluted with buffer salts. All components with t_R <1 min are diverted to waste and so not observed in the TIC.

The ligation product (see spectrum above) has t_R ~2.0 min

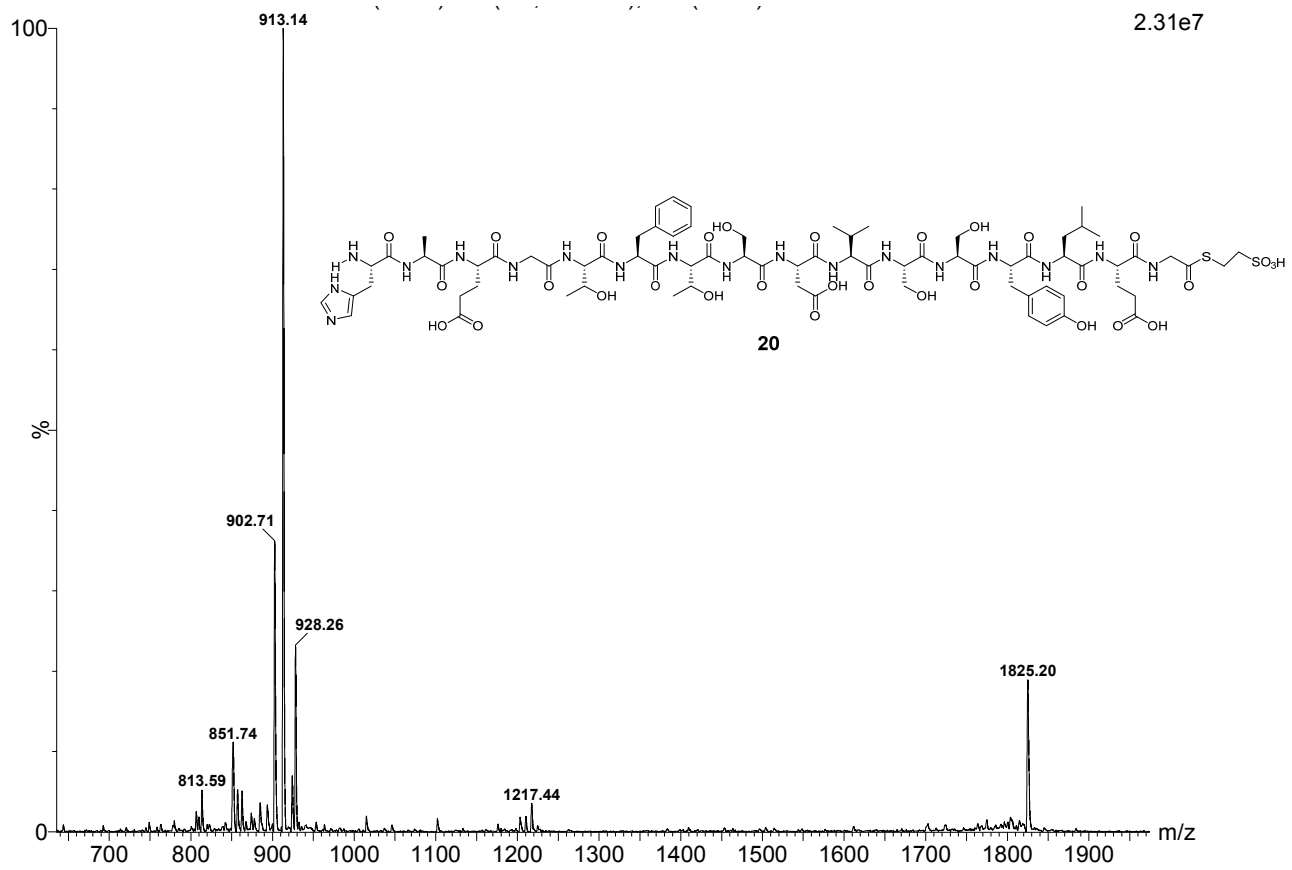


HAEGTFTSDVSSYLEG-$CH_2CH_2SO_3H$ [GLP-1 (7-22)] thioester 20

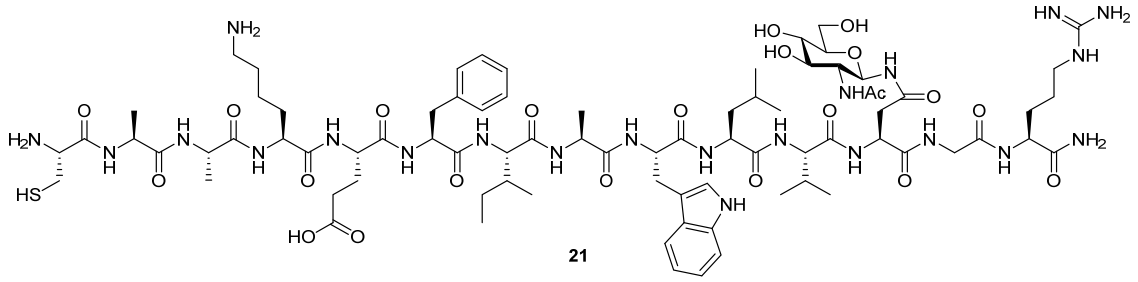


20

Peptide H-HAEGTFTSDVSSYLEGC-OH (6 mg) was treated with MESNa (600 mg) in 6 M guanidinium hydrochloride containing Na Phosphate buffer (0.1 M; pH 5.8, 6.0 mL), and TCEP.HCl (30 mg) was added. The reaction mixture was heated in an Eppendorf thermomixer at 55^{o}C with shaking (800 rpm) for 72 hours. The thioester was then and purified by preparative (RP)HPLC (Method A) to afford the desired peptide **20** (23%). ESI^{+} (*m/z*) calculated 1823.9 found 1825.2 $[MH]^{+}$

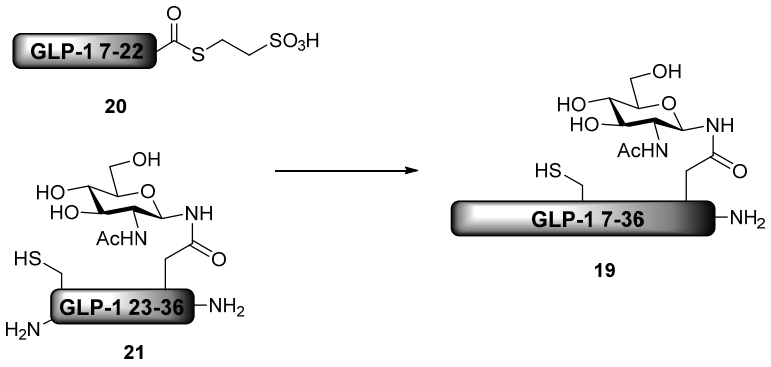


CAAKEFIAWLVN(GlcNAc)GR-NH_2 [GLP-1 (23-36)] **21**



Glycopeptide **21** was manually assembled on Rink amide MBHA resin using the standard protocols. An exception to this was the glycoamino acid coupling, for which only 3 equivalents were employed using HATU (3 equivalents) and DIPEA (6 equivalents). The peptide was cleaved with Reagent K (10 mL) for 4 hours, and precipitated in ether. The crude peptide was treated with aqueous hydrazine hydrate (5%, 15 mL) containing DTT (50 mM). The glycopeptide was purified by preparative (RP)HPLC (Method A, t_r 36.5 min) yielding 39 mg of the desired peptide **21** (22%). ESI^+ (*m/z*) calculated for $C_{80}H_{126}N_{22}O_{22}S$ 1778.91 found $[MH]^+$ 1779.68.

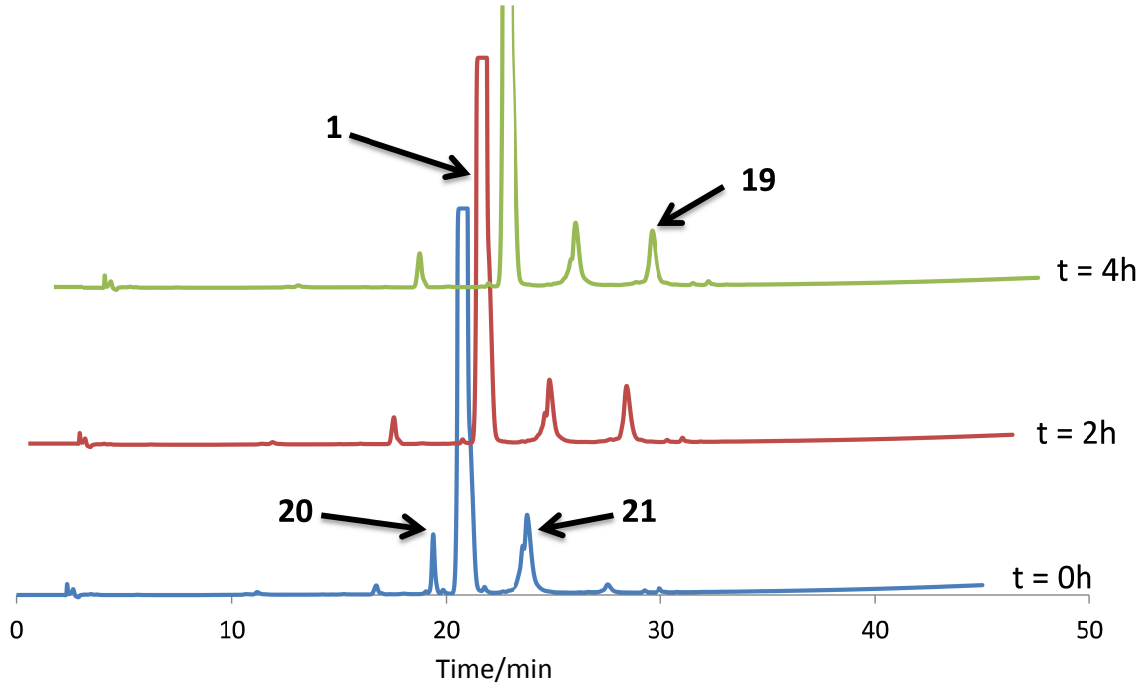
HAEGTFTSDVSSYLEGCAAKEFIAWLVN(GlcNAc)GR-NH_2 [GLP-1 (7-36)] **19**



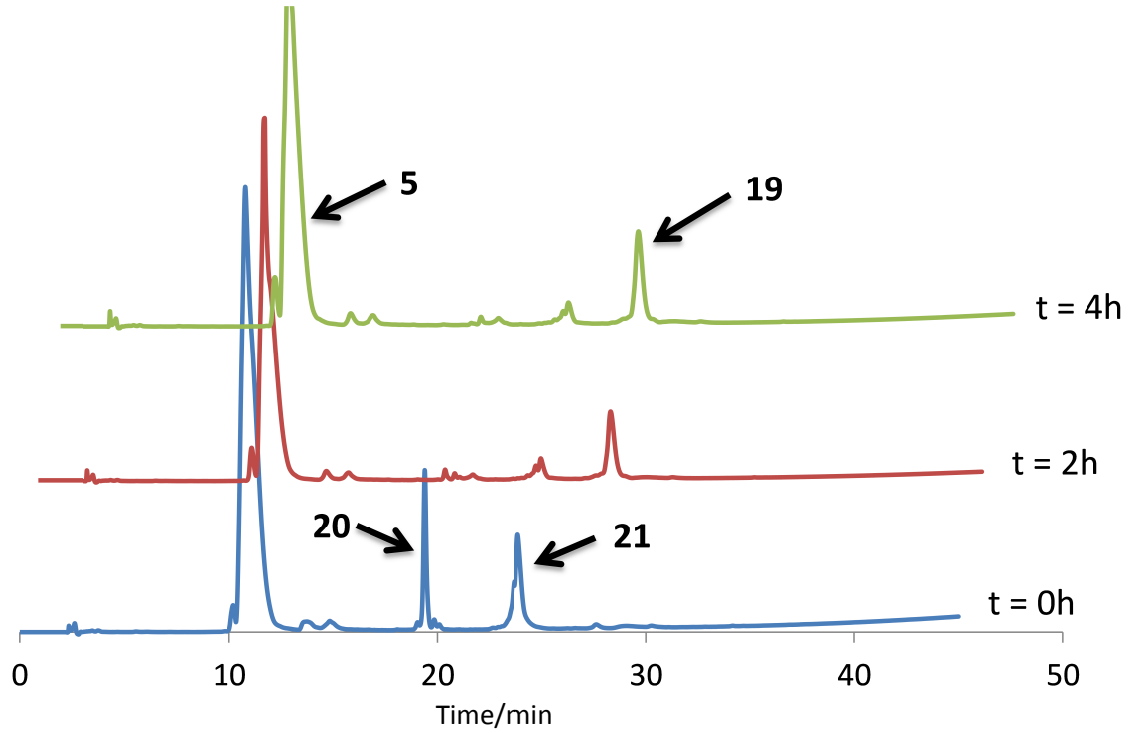
The GLP-1 peptide thioester **20** (1.2 mg) was dissolved in 0.1 mL ligation buffer (8M urea, 0.3 M sodium phosphate buffer; pH 7, 25 mM TCEP) and added to the C-terminal glycopeptide **21** (1.6 mg) dissolved in ligation buffer (0.1 mL). **4-MBSA (or MPAA**) was added to a final concentration of 50 mM and the pH of the final reaction mixture was adjusted to 7.0. The final concentrations of **20** and **21** were approximately 3 mM and 4 mM respectively. The reaction was followed by analytical

HPLC and LC-MS over the course of 4 h. The product glycopeptide (**19**) was then purified by preparative (RP)HPLC (Method B) t_r 40.7 min). In the case of the MPAA mediated ligation the product co-eluted with MPAA yielding a white solid. ESI^+ LC-MS (*m/z*) calculated for $C_{153}H_{230}N_{40}O_{50}S$ 3459.64 found $[MH_4]^{4+}$ 866.27.

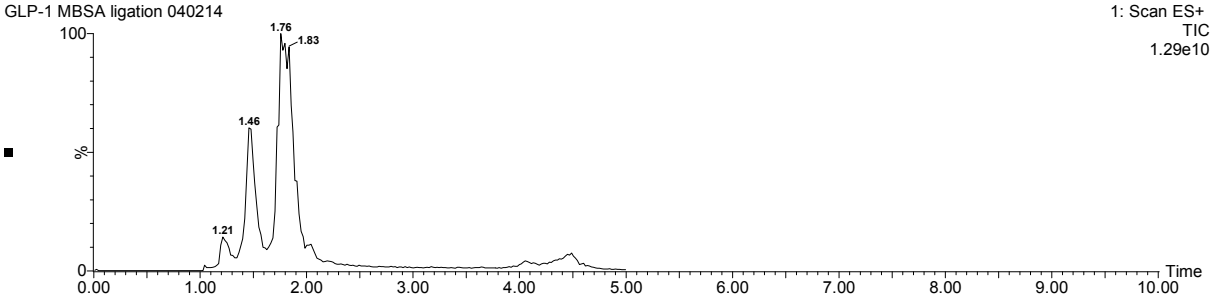
Analytical HPLC analysis of 50 mM MPAA catalysed synthesis of GLP1 analogue **19**



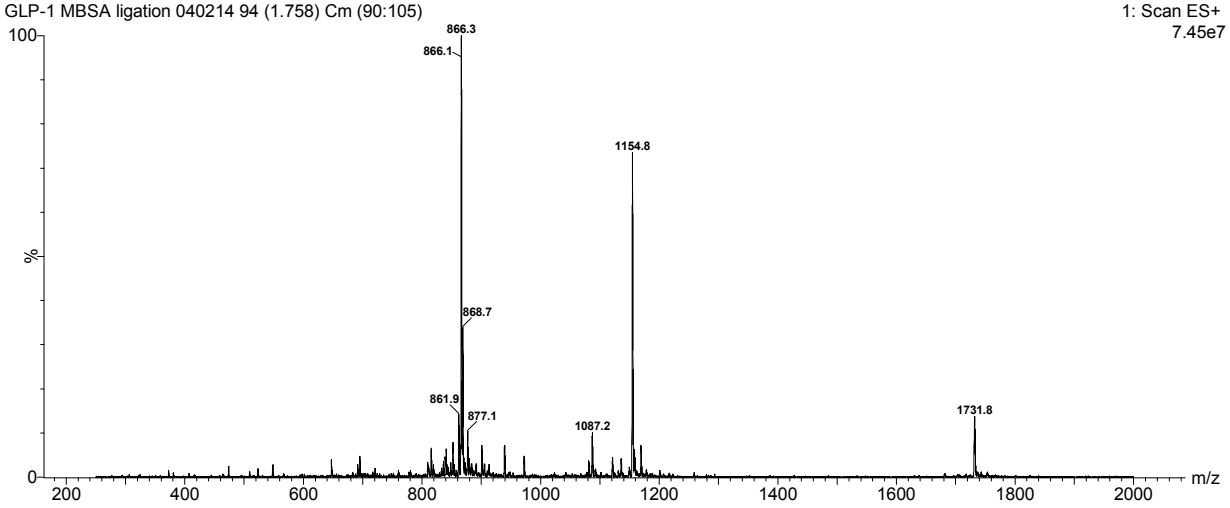
Analytical HPLC analysis of 50 mM 4-MBSA catalysed synthesis of GLP1 analogue **19**



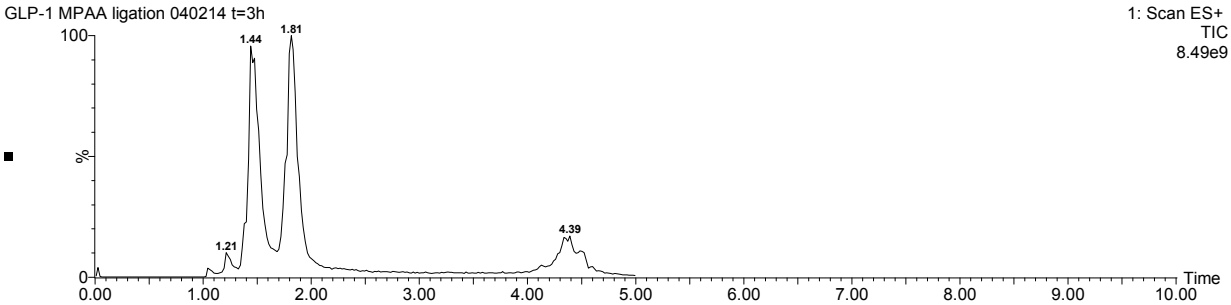
LC-MS ANALYSIS OF GLP-1 LIGATION USING 50 mM MBSA after 3 h: (The major species at 1.78 min is GLP-1. The minor species (1.48 min) is excess C-terminal "half" added in excess).



MASS SPECTRUM OF GLP-1 (The major species at 1.78 min).



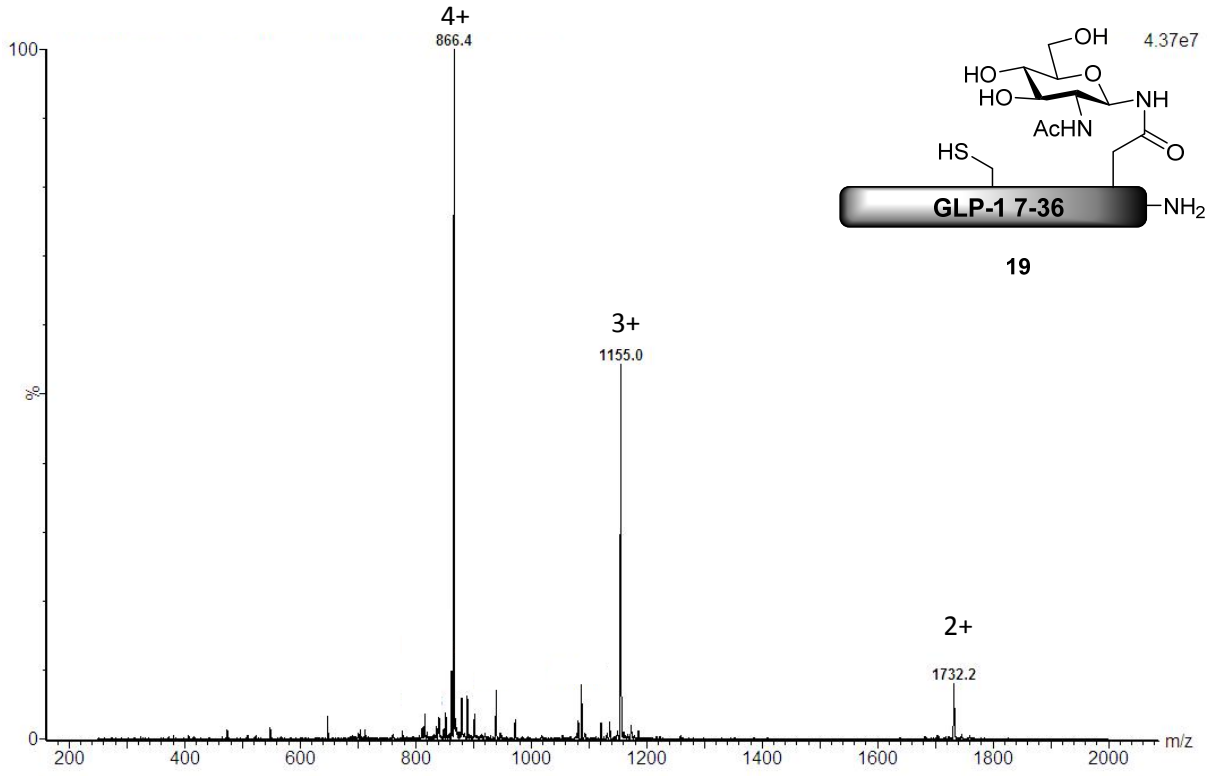
LC-MS ANALYSIS OF GLP-1 LIGATION USING 50 mM MPAA after 3 h:



Synthesis of [GLP-1 (7-36)] employing 3-MBSA.

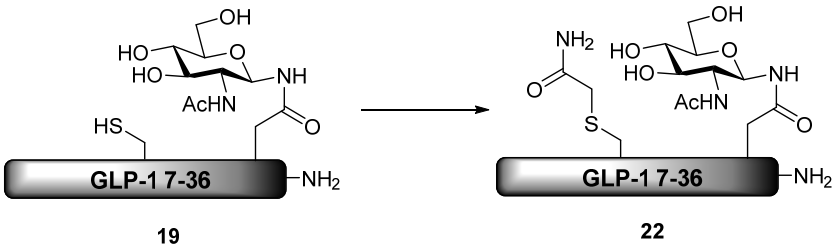
peptide thioester **20** (0.75 mg) was dissolved in 0.35 mL ligation buffer (6 M guanidine hydrochloride, 0.1 M sodium phosphate buffer; pH 7, 35 mM TCEP) and added to the C-terminal glycopeptide **21** (1.3 mg) dissolved in ligation buffer (0.1 mL). **3-MBSA** (50 µL) was added to a final concentration of 100 mM and the pH of the final reaction mixture was adjusted to 7.0. The reaction was followed by analytical HPLC and LC-MS over the course of 4 h. The product glycopeptide (**19**) was then purified by preparative (RP)HPLC (Method B, t_r 40.7 min). ESI^+ LC-MS (*m/z*) calculated for $C_{153}H_{230}N_{40}O_{50}S$ 3459.64 found $[MH_4]^{4+}$ 866.27.

Mass spectrum of HPLC purified **19**



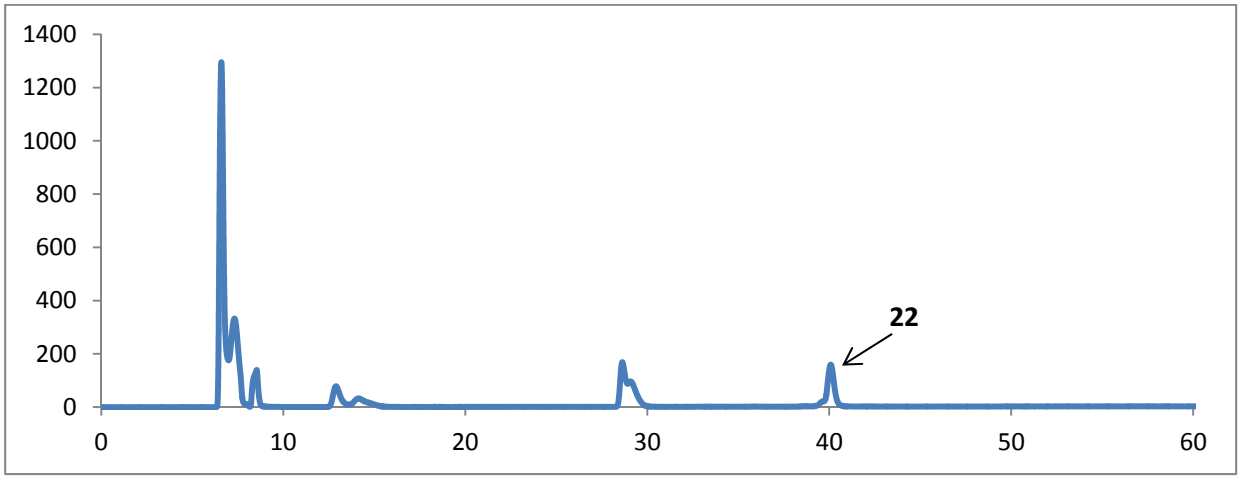
19

HAEGTFTSDVSSYLEG”Q”AAKEFIAWLVN(GlcNAc)GR-NH_2 [GLP-1(7-36)] **22**

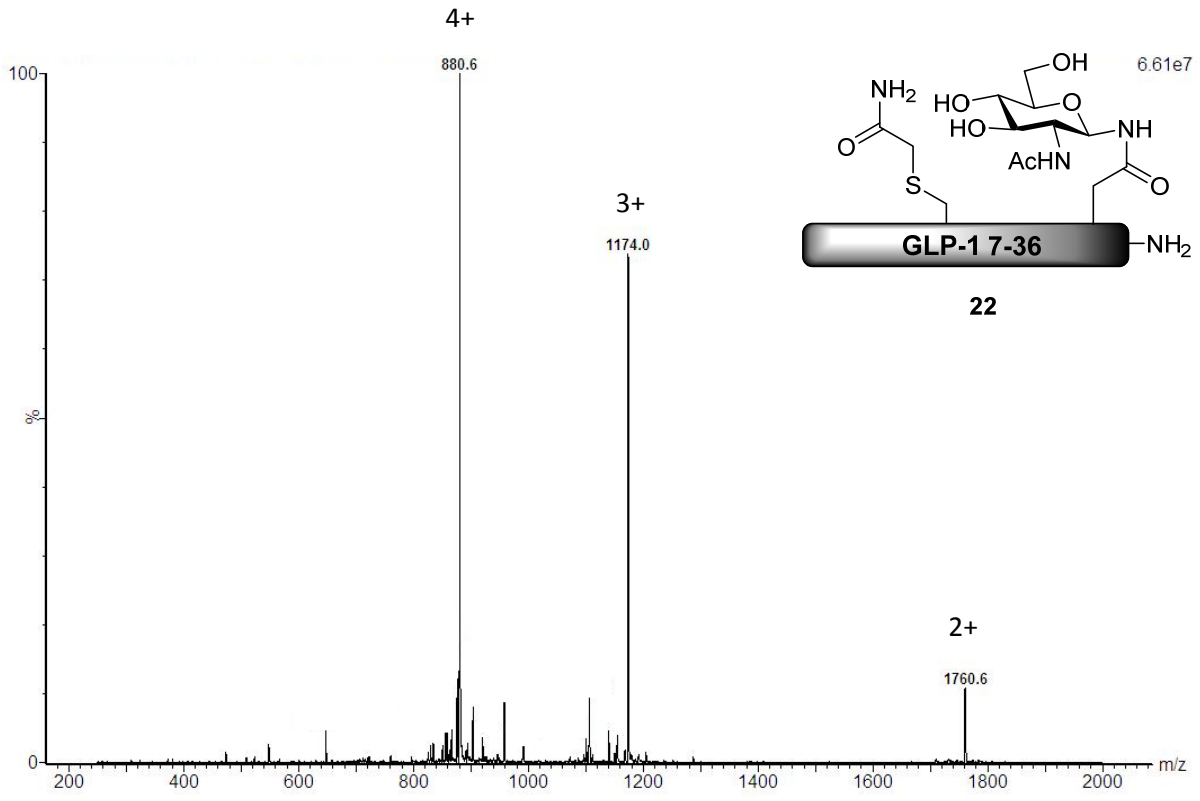


Glycopeptide **19** (8.4 mg) was stirred in NH_4HCO_3 (50 mM, 6 mL) and treated with TCEP (1 M) to a final concentration of 10 mM. The mixture was stirred under nitrogen and after 5 minutes, a solution of freshly prepared iodoacetamide (100 mM, 0.6 mL) was added. The mixture was stirred at room temperature for 90 min, after which LC-MS analysis suggested complete conversion to the product **22**. The product was purified by preparative (RP)HPLC (Method B, t_r 40.7 min) to yield 5.8 mg of the capped glycopeptide. ESI^+ LC-MS (*m/z*) calculated for $C_{155}H_{233}N_{41}O_{51}S$ 3516.66 found $[MH_4]^{4+}$ 880.62.

HPLC Purification of **22**



MASS SPECTRUM of HPLC purified **22**

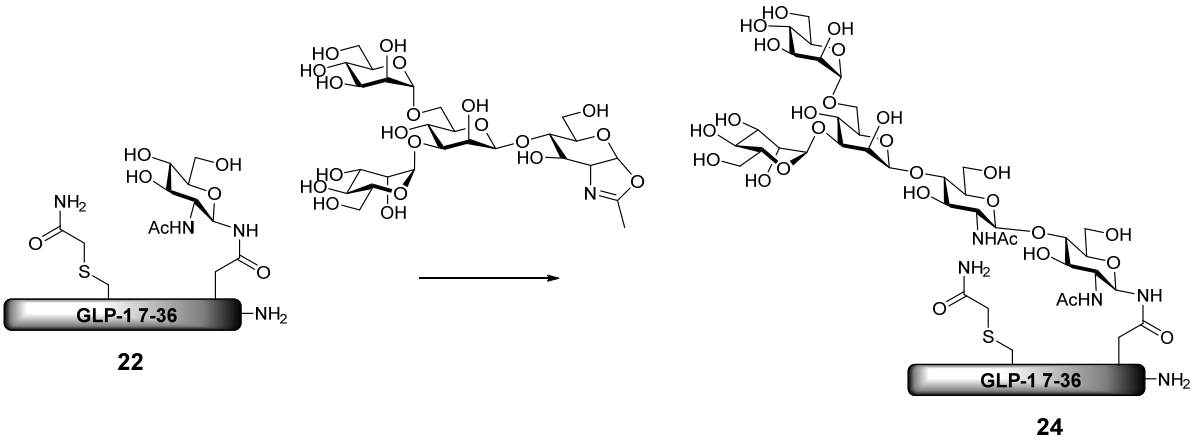


Expression of Endo A (from K. Fujita, N. Tanaka, M. Sano, I. Kato, Y. Asada and K. Takegawa, *Biochem. Biophys. Res. Commun*. 2000, 267, 134-138.)

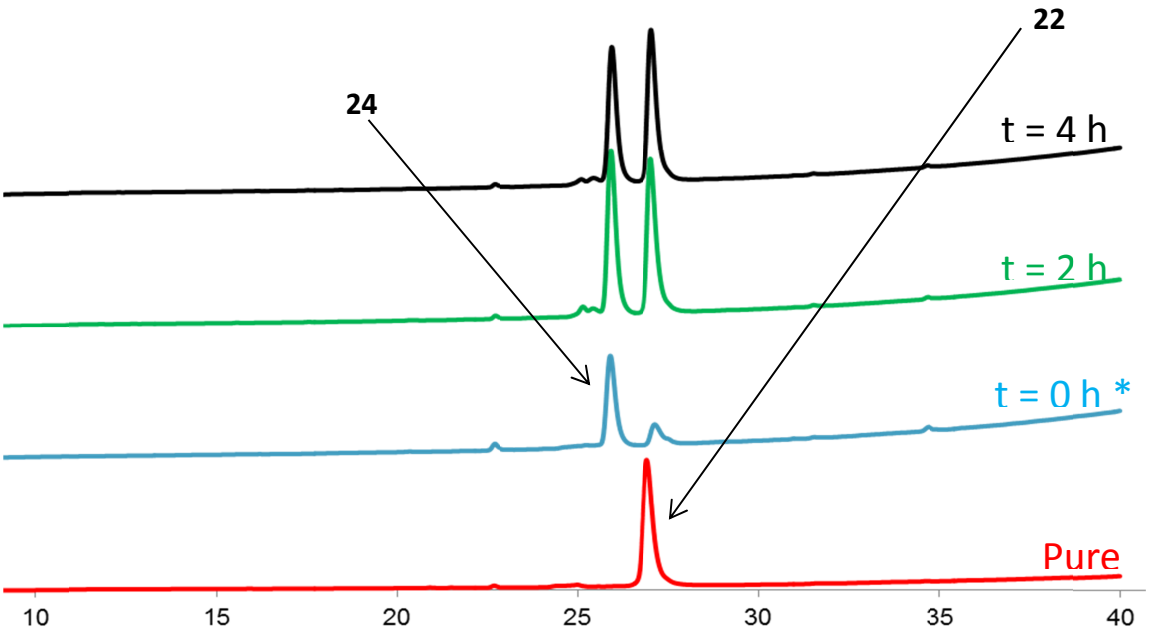
Endo A pGEX2T was transformed into BL21(DE3) cells and a colony was used to seed LB medium (10 mL) containing ampicillin (100 µg/ml) and grown at 37 °C overnight. The following day, the overnight culture was used to seed fresh LB medium (500 mL) containing ampicillin (100 µg/ml) and grown to an optical density (OD_{600}) of ~ 0.6. At this point, protein expression was induced by the addition of IPTG at a final concentration of 1 mM. Expression was carried out at 25 °C for 16 hours. Cells were harvested by centrifugation at 8000 RPM for 15 minutes at 4 °C (Beckman Coulter Avanti J-26XP). The combined cell pellets were resuspended in binding buffer (PBS, 1 mM EDTA, 1 mM DTT, 20 mL) and sonicated on ice for 5 bursts of 30 seconds, with 30 seconds of cooling time in between. Cell debris was pelleted at 10000 RPM for 15 minutes at 4 °C and the supernatant was loaded onto

a glutathione Superflow resin (3.0 mL, Qiagen) which had been pre-equilibrated with 10 volumes of binding buffer). The columns were subsequently washed with a further 10 volumes of binding buffer, before the proteins were eluted with elution buffer (50 mM Tris pH 8.0, 100 mM NaCl, 50 mM reduced glutathione, and 1 mM DTT). The eluted proteins were subsequently concentrated in an Amicon Ultra molecular weight cut off device (10 kDa, Millipore) to the required concentration.

Glycosidation assays with GLP-1 (7-36) and Endo A.



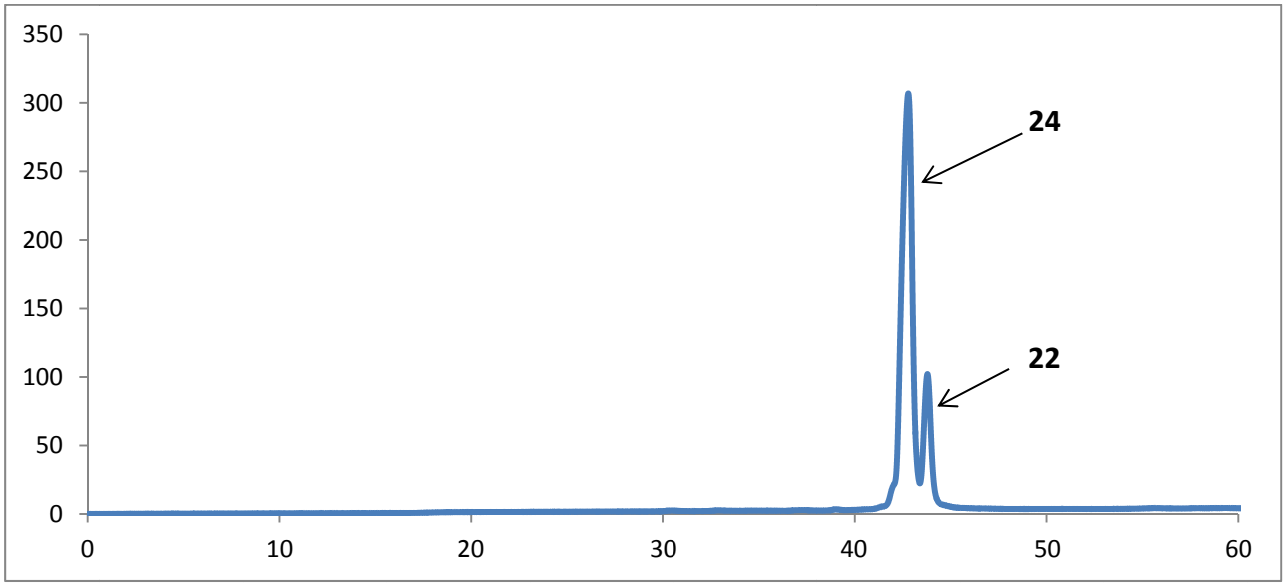
GLP-1 (7-36) **22** (700 µg) and $Man_3GlcNAc$ oxazoline (1.5 mg, 10 equivalents) were dissolved in potassium phosphate buffer (50 mM, pH 6.4, 375 µL). PMSF (100 mM in isopropanol) was added to a final concentration of 1 mM and Endo A was added from a 100 µM stock to a final concentration of 1 µM. The reaction was incubated at 23 °C and monitored by analytical (RP)HPLC at various time points. In the case of the wild type Endo A, product formation was confirmed by LC-MS and the GLP-1 pentasaccharide **24** was purified by preparative (RP)HPLC using a Phenomenex Jupiter 4 µm Proteo 90 Å, LC Column (diameter = 250 mm × 21.2 mm): flow rate 7.0 mL/min, detection at 230, 254 and 280 nm using a gradient of 95% water (0.1% TFA)/5% acetonitrile (0.1% TFA), to 40% water (0.1% TFA)/60% acetonitrile (0.1% TFA), over 45 min (t_r 42.8 min). ESI^+ LC-MS (*m/z*) calculated for $C_{181}H_{276}N_{42}O_{71}S$ 4205.90 found $[MH_4]^{4+}$ 1053.08.



* Mixture injected into analytical HPLC approximately 3 minutes after addition of Endo A

For preparative reactions **22** was incubated with **23** and WT EndoA for 10 minutes prior to purification.

Semi-Pep HPLC purification of **24**.



Mass spectrum of HPLC purified **24**.