Supplementary Information

Synthesis of homogeneous antifreeze glycopeptides via a ligation-desulfurisation strategy

James Garner,[a] Katrina A. Jolliffe,[b] Margaret M. Harding,*[a] and Richard J. Payne*[b]

[a]School of Chemistry, University of New South Wales, Australia, NSW 2052
harding@unsw.edu.au

[b]School of Chemistry, University of Sydney, Australia, NSW 2006
r.payne@chem.usyd.edu.au

Materials

Commercial reagants were used as supplied unless otherwise noted. Wang resin preloaded with Fmoc-Ala-OH (resin loading 0.50 mmol/g), Fmoc-Thr[α-GalNAc$(OAc)_3$]-OH, Fmoc-Ala-OH, Fmoc-thiazolidine carboxylic acid, Fmoc-Cys(Trt)-OH and PyBOP were purchased from Novabiochem. Water was obtained from a Milli-Q ultra pure water purification system (Millipore Corp.). Peptide synthesis grade DMF was purchased from Labscan Asia Co. Ltd. (Bangkok, Thailand). Ethyl 3-mercaptopropionate was purchased from TCI. VA-044 was purchased from Wako Inc. and was recrystallised from methanol before use. All other reagents were purchased from Sigma-Aldrich.

Methods

Analytical reverse-phase (RP-) HPLC was performed on a Waters System 2695 separations module with an Alliance series column heater at 30 °C and 2996 photodiode array detector and employed a Waters Sunfire C18 column (2.1 x 150 mm column, 5 μm particle size, flow rate of 0.2 mL min^{-1}). Preparative and Semi-preparative RP-HPLC was performed using a Waters 600 Multisolvent Delivery System and Waters 500 pump with a 2996 photodiode array detector or Waters 490E Programmable wavelength detector operating at 230 and 214 nm. Preparative RP-HPLC employed a Waters Sunfire Prep C18 OBD column (19 x 150 mm, 5 μm particle size, flow rate 7 mL min^{-1}). Semi-preparative RP-HPLC employed a Grace Vydac "Protein and Peptide" C18 column (10 x 250 mm, 10 μm particle size, flow rate 4 mL min^{-1}). The mobile phase consisted of eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) for all HPLC runs.

LC-MS was performed on a Thermo Separation Products: Spectra System consisting of a P400 Pump and UV6000LP Photodiode array detector coupled to a Thermoquest Finnigan LCQ Deca ion trap MS (ESI) operating in positive mode. The system employed a Waters Sunfire C18 column (2.1 x 150 mm, 5 μm particle size, flow rate 0.2 mL min^{-1}, 30 °C). The mobile phase consisted of eluents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile).

Glycopeptides were weighed on a Mettler Toledo XS105 DualRange Analytical Balance (accuracy ± 0.03 mg).

Iterative Peptide Assembly: Amino acids were coupled manually in polypropylene syringes with sintered discs (Torviq) using the following sequence of steps: deprotection, washing, coupling (method A or method B), washing, capping, washing. *Deprotection*: The resin was treated with 10% piperidine/DMF (2 x 3 min). Resin loading was determined by measuring the absorbance of piperidine-fulvene adduct at λ = 301 nm. *Washing*: The resin was sequentially washed with DMF (5x), CH_2Cl_2 (5x) and DMF (5x) using 3 mL of solvent per 1 mL of swollen resin. *Coupling method (A)*: A double coupling method was used for the addition of Fmoc-Ala-OH, Fmoc-thiazolidine carboxylic acid or Fmoc-Cys(Trt)-OH. The resin was agitated on an orbital shaker (175 rpm) for 2 h with a pre-activated solution (5 min) containing the Fmoc-amino acid (4 eq), PyBOP (4 eq) and

NMM (8 eq) in DMF (2 mL) (reagent equivalents are with respect to the original resin loading). The solution was discarded and a further pre-activated solution was added as previously prepared and the resin was agitated on an orbital shaker (175 rpm) for 2 h. *Coupling method (B)*: Coupling of Fmoc-Thr[α-GalNAc(OAc)$_3$]-OH was performed by adding pre-activated amino acid (1.2 eq), PyBOP (1.2 eq) and NMM (2.4 eq) in DMF (2 mL) and agitated on an orbital shaker (175 rpm) for 20 h. *Capping*: Unreacted amine sites were capped by shaking with 10% Ac_2O/pyridine (1 x 3 min) using 3 mL of solution per 1 mL of swollen resin.

Native chemical ligation: Glycopeptide thioester **7** (1.25-1.5 eq) was dissolved in 1:1 v:v *N*-methylpyrrolidinone : 6 M Gn.HCl, 1 M HEPES Buffer (pH 8.5) (110 μL) which had been gently degassed with argon (2 min). The solution was added to glycopeptide **4** (or **9**) (1 eq.). Thiophenol (2 vol%) was added to the solution and the reaction gently agitated. The final pH of the solution was measured to be 7.2-7.4. The solution was incubated at 37 ºC for 24 h. LC-MS analysis indicated complete consumption of **4** (or **9**) after 24 h by which time the pH had decreased to 6.8-6.9.

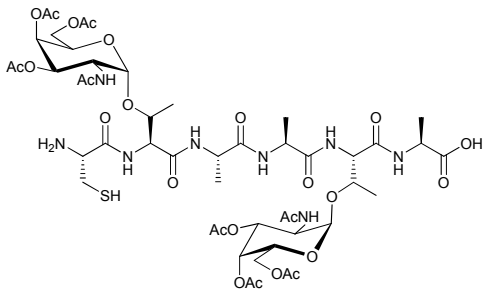
Thiazolidine demasking: Methoxyamine.HCl (0.2 M, ~70 μL) was added to the ligation solution until the pH reached ~4.2. The solution was incubated at 37 ºC for 17 h after which LC-MS analysis indicated complete conversion of the ligation product.

Acetate Deprotection: Hydrazine hydrate was added to the above solution such that the solution was 5 vol% N_2H_4. The solution was left to stand at rt for 40 min. LC-MS indicated global deprotection of the acetate groups, and the product was immediately purified by RP-HPLC.

Desulfurisation: Water and buffer (6 M Gn.HCl/0.1 M NaH_2PO_4 adjusted to pH 6.5) were degassed with argon for 10 min before use to prepare peptide and reagent stock solutions. The peptide (~1 mg) was dissolved in water (7 μL). Stock solutions of glutathione (40 mM) and VA-044 (200 mM) in water were prepared. A stock solution of 0.5 M TCEP in the buffer solution was prepared. The peptide and stock solutions were flushed further with argon before use. Aliquots of glutathione (6.5 μL), VA-044 (6.5 μL) and TCEP (20 μL) were added to the peptide solution and the vessel flushed with argon. The reaction vessel was incubated at 65 ºC with occasional agitation until LC-MS analysis showed no further consumption of the starting material (6 h). The reaction was quenched with 0.1% TFA in water (100 μL).

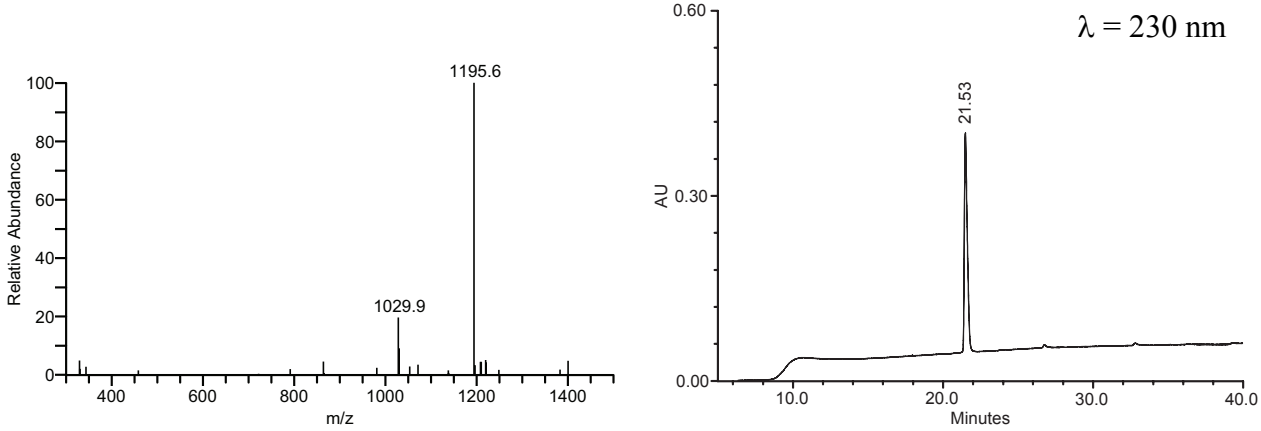
Analytical Data

H_2N-CT[α-GalNAc(OAc)$_3$]AAT[α-GalNAc(OAc)$_3$]A-OH (4)

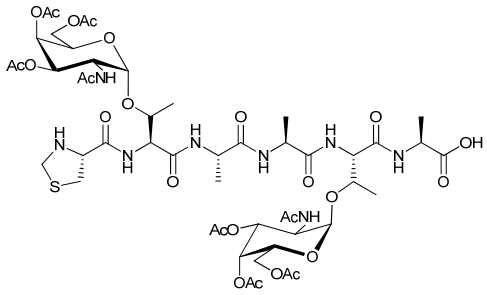


The peptide was assembled on preloaded Fmoc-Ala-Wang resin following the *iterative peptide assembly* (23 μmol scale). *Cleavage from Resin*: The resin was washed with CH_2Cl_2 (10x). The product was cleaved from the resin by agitating with 85:5:5:5 v/v/v/v TFA:triisopropylsilane:thioanisole:H_2O (3 mL) for 90 min. The solution was collected in a flask and the resin washed with TFA (3x). The combined solutions were removed *in vacuo* and the product isolated as a pale yellow oil. The product was purified by preparative RP-HPLC (10 to 70% B over 60 min). Glycopeptide **4** was obtained, after lyophilisation, as a fluffy white solid (15.8 mg, 57% over 6 steps).

analytical HPLC: t_R 21.53 min (Gradient 0 to 75% B over 40 min); ESI (*m*/*z*): 1195.6 $[M+H]^+$, calcd $C_{48}H_{75}N_8O_{25}S$ (1195.5).

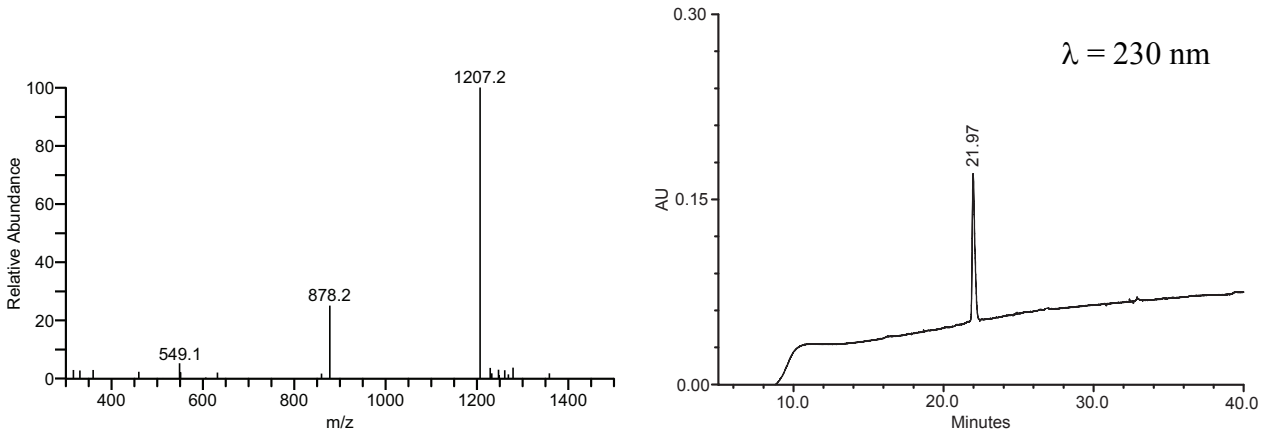


Thiaz-T[α-GalNAc(OAc)$_3$]AAT[α-GalNAc(OAc)$_3$]A-OH (6)

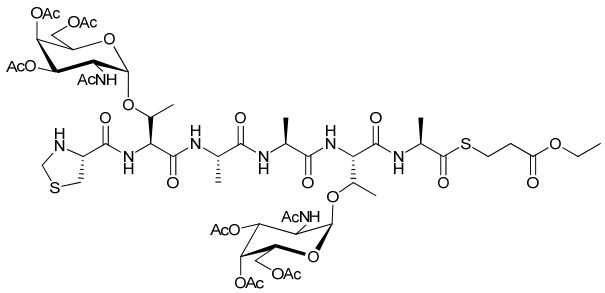


The peptide was assembled on preloaded Fmoc-Ala-Wang resin following the *iterative peptide assembly* (10 μmol scale). *Cleavage from Resin*: The resin was washed with CH_2Cl_2 (10x). The product was cleaved from the resin by agitating with 85:5:5:5 v/v/v/v TFA:triisopropylsilane:thioanisole:H_2O (3 mL) for 90 min. The solution was collected in a flask and the resin washed with TFA (3x). The combined solutions were removed *in vacuo* and the product isolated as a pale yellow oil. The product was purified by preparative RP-HPLC (0 to 100% B over 60 min). Glycopeptide **6** was obtained, after lyophilisation, as a fluffy white solid (8.6 mg, 71% over 6 steps).

analytical HPLC: t_R 21.97 min (Gradient 0 to 75% B over 40 min); ESI (*m*/*z*): 1207.2 $[M+H]^+$, calcd $C_{48}H_{75}N_8O_{25}S$ (1207.6).

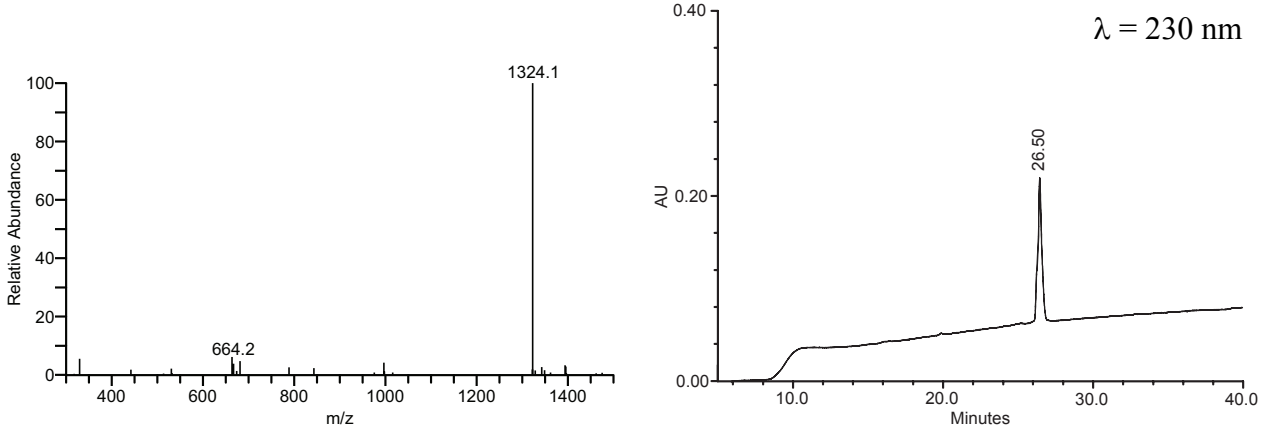


Thiaz-T[α-GalNAc(OAc)$_3$]AAT[α-GalNAc(OAc)$_3$]A-thioester (7)

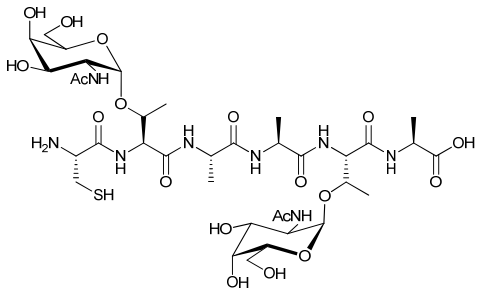


Glycopeptide **6** (7.5 mg, 6.21 μmol) was dissolved in anhydrous THF (150 μL) and cooled to 0 °C. Ethyl 3-mercaptopropionate (0.81 μL, 1 eq) was added, followed by HOBt (1 mg, 1.2 eq) and DIPEA (4.3 μL, 4 eq). The solution was stirred at 0 °C for 15 min before DIC (0.96 μL, 1.2 eq) was added. The reaction was agitated on an orbital shaker at rt for 15 h at which point LC-MS indicated complete conversion of the starting material. The solution was purified by preparative RP-HPLC (0 to 80% B over 45 min). Glycopeptide thioester **7** was obtained, after lyophilisation, as a fluffy white solid (6.57 mg, 80%).

analytical HPLC: t_R 26.50 min (Gradient 0 to 75% B over 40 min); ESI (*m*/*z*): 1324.1 $[M+H]^+$, calcd $C_{54}H_{83}N_8O_{26}S_2$ (1324.4).

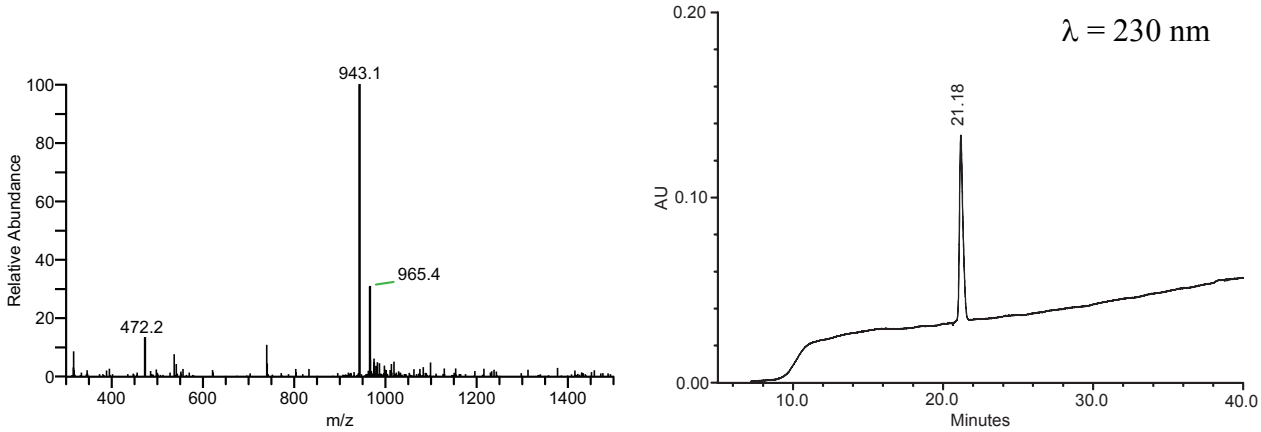


H_2N-CT(α-GalNAc)AAT(α-GalNAc)A-OH (8)

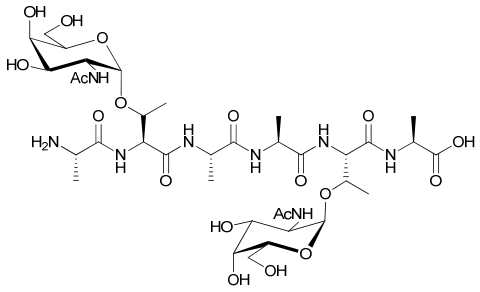


Glycopeptide **4** (1.63 mg, 1.37 μM) was treated with hydrazine according to the general acetate deprotection procedure. The product was purified by semi-preparative RP-HPLC (0% B for 10 min, then 0 to 25% B over 35 min). Glycopeptide **8** was obtained, after lyophilisation, as a white fluffy solid (1.03 mg, 80%).

analytical HPLC: t_R 21.18 min (Gradient 0 to 25% B over 40 min); ESI (*m*/*z*): 943.1 $[M+H]^+$, calcd $C_{36}H_{63}N_8O_{19}S$ (943.4).

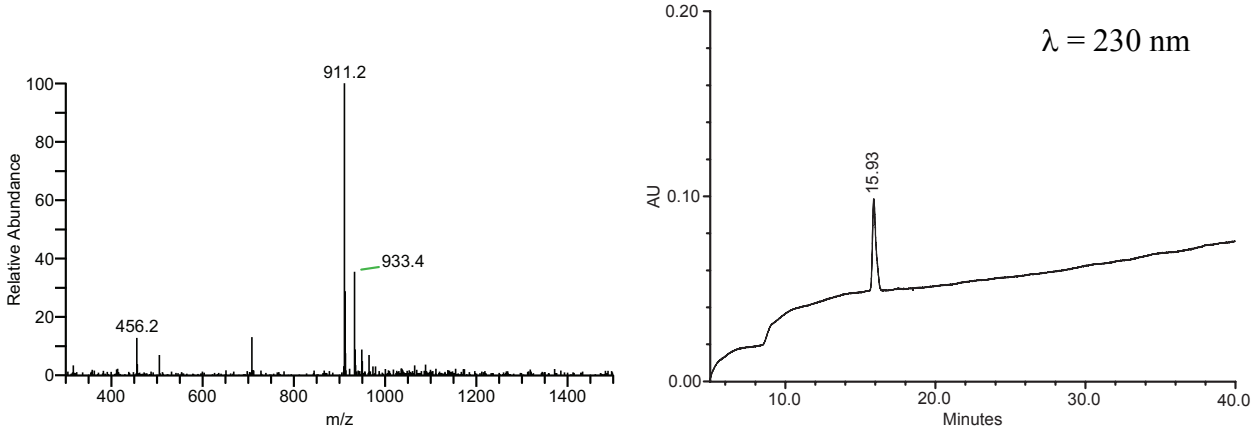


H_2N-AT(α-GalNAc)AAT(α-GalNAc)A-OH (1)

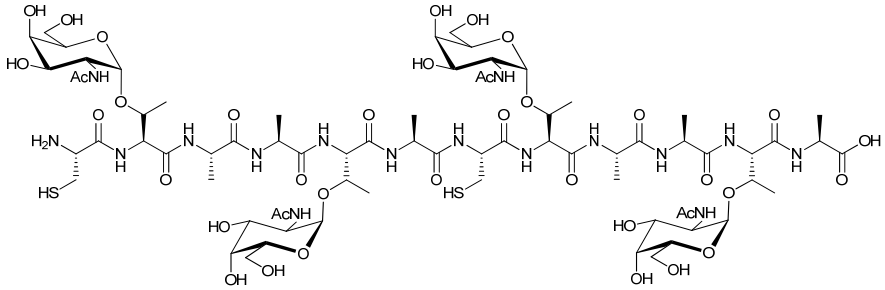


Glycopeptide **8** (1.03 mg, 1.09 μmol) was treated according to the general desulfurisation procedure. The product was purified by semi-preparative RP-HPLC (0% B for 10 min, then 0 to 25% B over 35 min). Glycopeptide **1** was obtained, after lyophilisation, as a fluffy white solid (0.90 mg, 90%).

analytical HPLC: t_R 15.93 min (Gradient 0 to 25% B over 40 min); ESI (*m*/*z*): 911.2 $[M+H]^+$, calcd $C_{36}H_{63}N_8O_{19}$ (911.4).

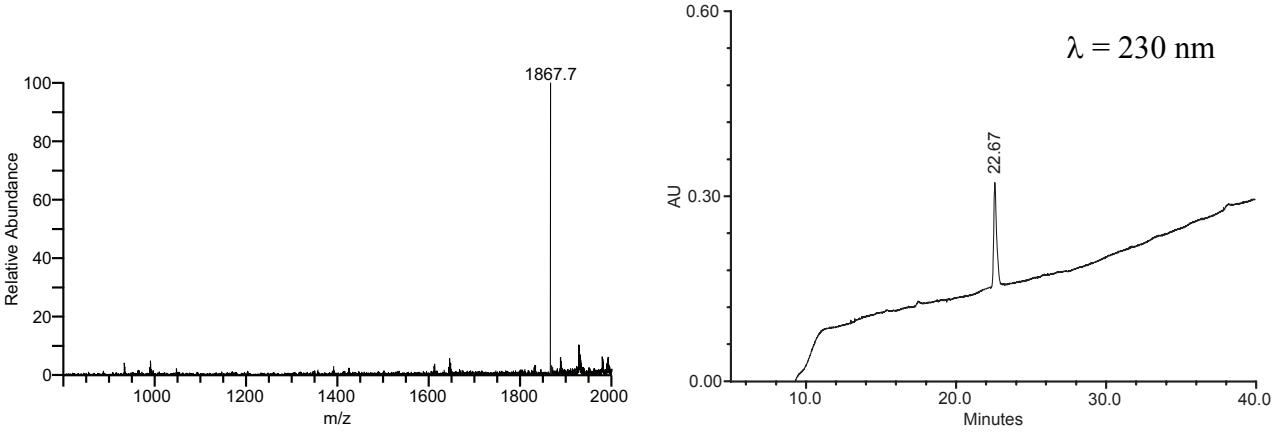


H_2N-CT(α-GalNAc)AAT(α-GalNAc)ACT(α-GalNAc)AAT(α-GalNAc)A-OH (9)

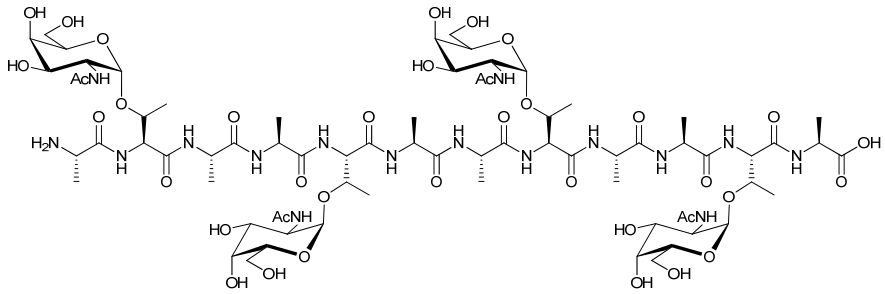


Glycopeptide thioester **7** (2.90 mg, 2.19 μmol) was ligated with with glycopeptide acid **4** (1.73 mg, 1.45 μmol) according to the general native chemical ligation procedure described above. The pH started at 7.4 and decreased to 6.9 by reaction completion (24 h). The product was used in the next step without further purification. The ligation product was demasked according to the general procedure and then treated with hydrazine according to the general acetate deprotection procedure. The product was purified by semi-preparative RP-HPLC (0% B for 10 min then 0 to 25% B over 35 min). Glycopeptide **9** was obtained, after lyophilisation, as a white fluffy solid (1.20 mg, 44% over 3 steps).

analytical HPLC: t_R 22.67 min (Gradient 0 to 25% B over 40 min); ESI (*m*/*z*): 1867.24 $[M+H]^+$, calcd $C_{72}H_{123}N_{16}O_{37}S_2$ (1867.8).

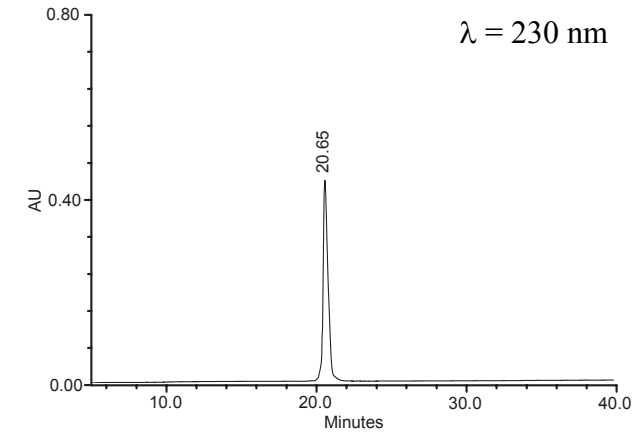


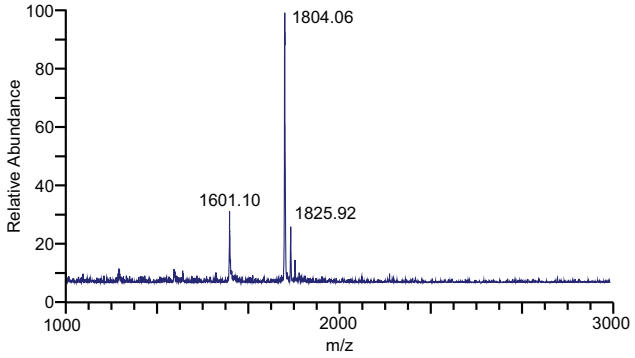
H_2N-AT(α-GalNAc)AAT(α-GalNAc)ACT(α-GalNAc)AAT(α-GalNAc)A-OH (2)



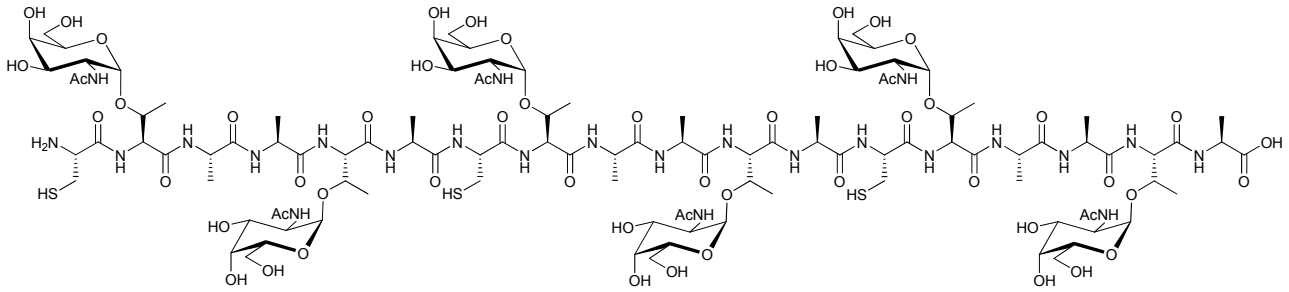
Glycopeptide **9** (1.02 mg, 0.55 μmol) was treated according to the general desulfurisation procedure. The product was purified by semi-preparative RP-HPLC (0% B for 10 min, then 0 to 25% B over 35 min). Glycopeptide **2** was obtained, after lyophilisation, as a fluffy white solid (0.91 mg, 92%).

MALDI-TOF (*m*/*z*): 1804.10 ($[M+H]^+$, theor. 1803.82); analytical HPLC: t_R 20.65 min (Gradient 0 to 25% B over 40 min).



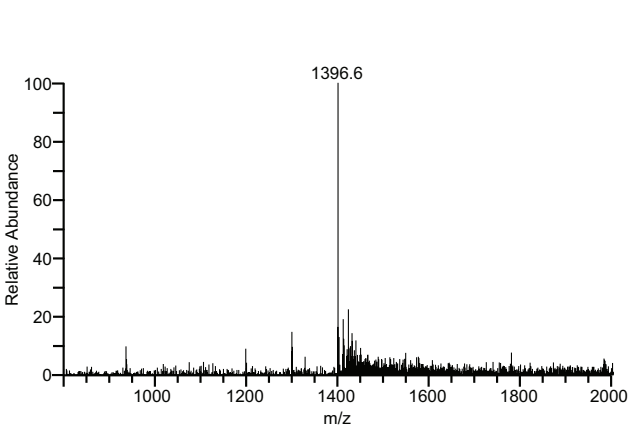


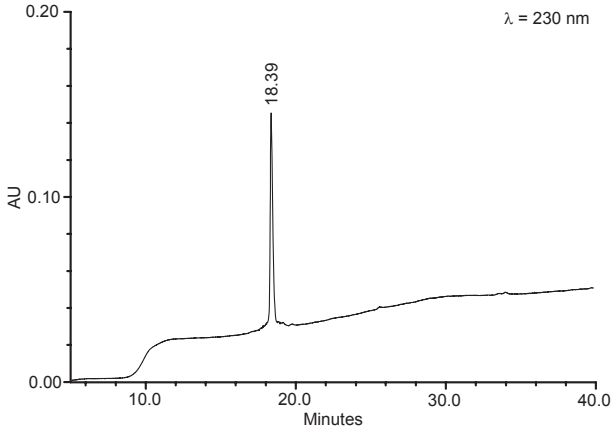
H_2N-CT(α-GalNAc)AAT(α-GalNAc)ACT(α-GalNAc)AAT(α-GalNAc)ACT(α-GalNAc)AAT(α-GalNAc)A-OH (10)



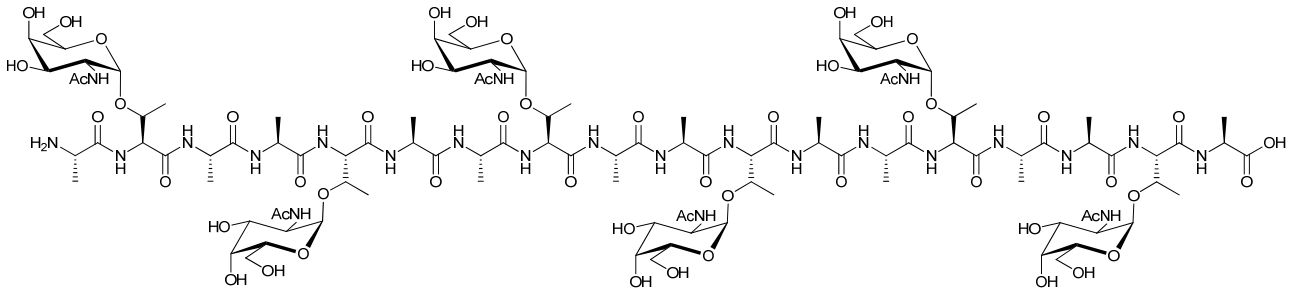
Glycopeptide thioester **7** (2.80 mg, 2.12 μmol) was ligated with with glycopeptide **9** (2.87 mg, 1.54 μmol) according to the general ligation procedure. The pH started at 7.2 and decreased to 6.8 by reaction completion (24 h). The product was used in the next step without further purification. The ligation product was demasked according to the general procedure and then treated with hydrazine according to the general acetate deprotection procedure. The product was purified by semi-preparative RP-HPLC (0% B for 10 min, then 0 to 25% B over 35 min). Glycopeptide **10** was obtained, after lyophilisation, as a white fluffy solid (1.35 mg, 32% over 3 steps).

analytical HPLC: t_R 18.39 min (Gradient 0 to 50% B over 40 min); ESI (*m*/*z*): calcd for $C_{108}H_{183}N_{24}O_{55}S_3$ $[M+2H]^{2+}$ 1396.6, found, 1396.6.





H_2N-AT(α-GalNAc)AAT(α-GalNAc)AAT(α-GalNAc)AAT(α-GalNAc)AAT(α-GalNAc)AAT(α-GalNAc)A-OH (3)



Glycopeptide **10** (1.01 mg, 0.36 μmol) was treated according to the general desulfurisation procedure. The product was purified by semi-preparative RP-HPLC (0% B for 10 min, then 0 to 55% B over 35 min). Glycopeptide **3** was obtained, after lyophilisation, as a fluffy white solid (0.85 mg, 88%).

analytical HPLC: t_R 16.26 min (Gradient 0 to 50% B over 40 min); ESI (*m*/*z*): calcd for $C_{108}H_{183}N_{24}O_{55}$ $[M+2H]^{2+}$ 1348.6, found, 1348.9.