An Advance in the Chemical Synthesis of Homogeneous N-Linked Glycopolypeptides by Convergent Aspartylation.

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Materials and methods

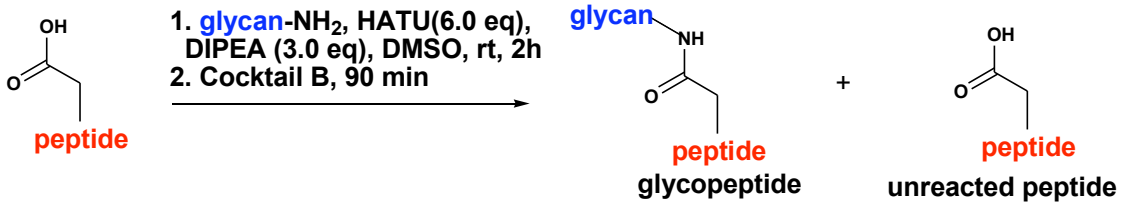
All commercial materials (Aldrich, Fluka, Nova) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher). Anhydrous THF, diethyl ether, CH_2Cl_2, toluene, and benzene were obtained from a dry solvent system (passed through column of alumina) and used without further drying. Low-resolution mass spectral analyses were performed with a JOEL JMS-DX-303-HF mass spectrometer or Waters Micromass ZQ mass spectrometer. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and flash column chromatography was performed on Silicycle Siliaflash P60 gel (40–63 um). Yields refer to chromatographically pure compounds.

HPLC: All separations of peptides and glycopeptides involved a mobile phase of 0.05% TFA (v/v) in water (solvent A), 0.04% TFA in acetonitrile (solvent B). Preparative and analytical HPLC separations were performed using a Rainin HPXL solvent delivery system equipped with a Rainin UV-1 detector. LC-MS chromatographic separations were performed using a Waters Acquity Ultra Performance LC system equipped with acquity UPLC®BEN C18 column (1.7 µm, 2.1 x 100.0 mm) or acquity UPLC®BEN C8 column (1.7 µm, 2.1 x 100.0 mm) at a flow rate of 0.3 mL/min, Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector equipped with XBridge™ C18 column (5.0 µm, 2.1 x 150 mm), X-Terra™ MS C18 column (3.5 µm, 2.1 x 100.0 mm) or Varian Microsorb C18 column (2 x 150 mm) at a flow rate of 0.2 mL/min**.** HPLC separations were performed using: X-Bridge™ Prep C18 column OBDTM (5.0 µm, 19 x 150 mm) at a flow rate of 16 mL/min, Microsorb 100-5 C18 column at a flow rate of 16.0 mL/min, Microsorb 300-5 C8 column at a flow rate of 16.0 mL/min, or Microsorb 300-5 C4 column at a flow rate of 16.0 mL/min.

Solid-phase peptide synthesis according to Fmoc-strategy

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc protocols. The deblock mixture was a mixture of 100/5/5 of DMF/piperidine/DBU. The following Fmoc amino acids from NovaBiochem were employed: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-(Dmb)Gly-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Ile-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Ser(*t*Bu)-Ser($\psi^{Me,Me}$Pro)-OH, Fmoc-Asp(*t*Bu)-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Val-(Dmb)Gly-OH, Fmoc-Leu-Ser($\psi^{Me,Me}$Pro)-OH. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide synthesis vessel with DCM. The resin cleavage was effected by treatment with AcOH/TFE/DCM (1:1:4) for 3 x 25 min to yield peptidyl acids in good yield.

General procedure for one flask aspartylation/deprotection:

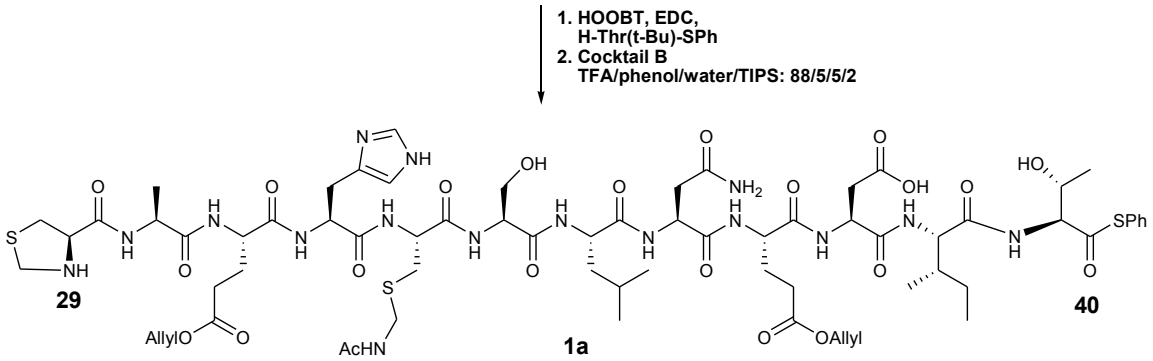


To a mixture of Peptide (0.002 mmol, 1.0 eq), Glycan-NH_2 (0.004 mmol, 2.0 eq) and HATU (0.012 mmol, 6.0 eq) was added 120 uL DMSO (containing 0.006 mmol, 3.0 eq DIPEA). The reaction mixture was stirred at room temperature for 2 h. Next the solution was treated with cocktail TFA (60.0 mg of phenol, 0.2 ml of water, 0.15 ml of triisopropylsilane, and 3.0 ml TFA) for 90 mins. The reaction was concentrated under a stream of argon and the residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The mixture was purified directly by HPLC.

LC-MS and MS characterization of peptides

Glycopeptide **1a**

Boc-Thz-Ala-Glu(OAll)-His(Trt)-Cys(Acm)-Ser(t-Bu)-Leu-Asn(Trt)-Glu(OAll)-Asp(Ot-Bu)-Ile-OH



1a

$C_{68}H_{100}N_{16}O_{22}S_3$
Exact Mass: 1588.64
Mol. Wt.: 1589.81

Following the general procedure for SPPS, peptide (0.10 mmol) was synthesized by automated Applied Biosystems Pioneer continuous flow peptide synthesizer, employing Fmoc-Ile-NovaSyn® TGT resin.

To a solution of peptide (170 mg, 0.081 mmol) and H-Thr(*t*Bu)-SPh (60 mg, 0.20 mmol) in anhydrous $CHCl_3$/TFE (2 mL, 3:1) was added EDC (45 uL) and HOOBT (37 mg) at rt. The reaction mixture was stirred at rt for 2 h. The reaction was concentrated under a stream of nitrogen. Next the mixture was treated with degassed cocktail TFA (120.0 mg of phenol, 0.40 ml of water, 0.30 ml of triisopropylsilane, and 6.0 ml TFA) for 90 mins. The reaction was concentrated under a stream of argon. The residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The resulting solid was purified by HPLC (30-60% MeCN/H_2O over 30 min, C18 column, 16 mL/min, 230 nm) to give peptide **1a** (51 mg, 40% over two steps).

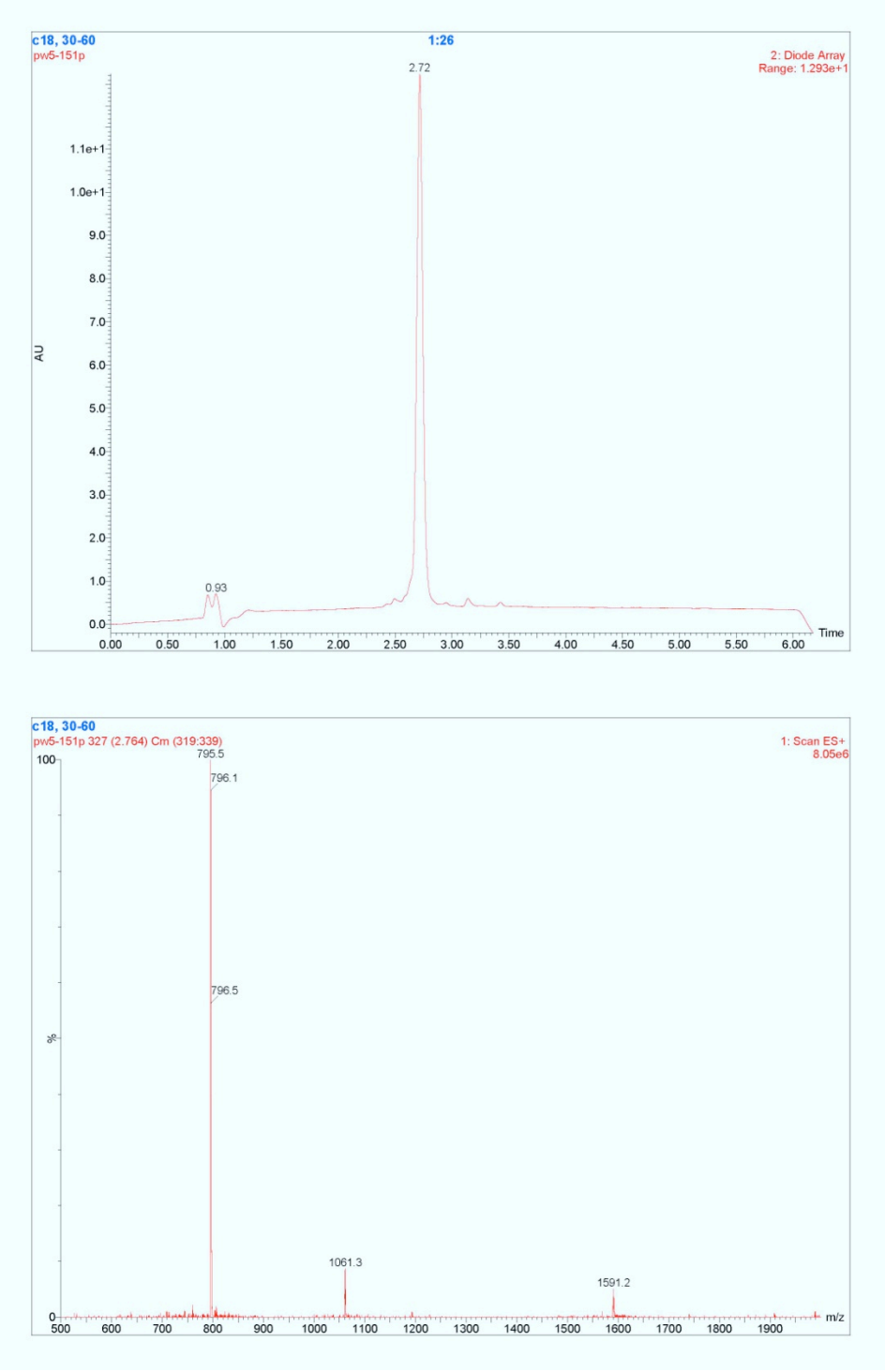
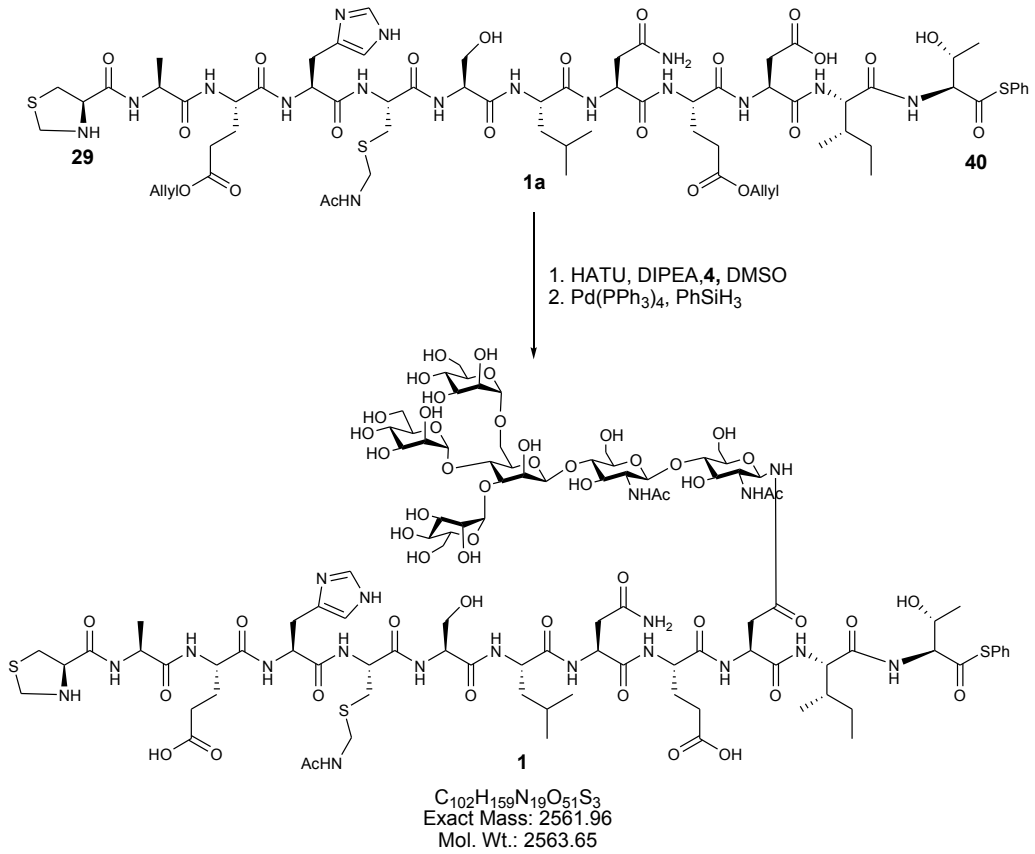


Figure 1. UV and MS traces from UPLC-MS analysis of peptide **1a**: gradient 30-60% CH_3CN/H_2O over 6 min at a flow rate of 0.3 mL/min, C18 column. (b) ESI-MS of peptide **1a.** ESI calcd for $C_{68}H_{100}N_{16}O_{22}S_3$ $[M+H]^+$ *m/z* = 1590.81 da, $[M+2H]^{2+}$ *m/z* = 795.91 da, found: 1591.23 da, 795.99 da.

Glycopeptide **1**



To an oven-dried vial were charged peptide **1a** (4.0 mg, 2.52 μmol), hexasaccharide **4** (1.6 mg, 1.5 μmol). A stock solution of HATU (5.0 mg HATU in 200 uL DMSO) 20 μL and a stock solution of diisopropylethyl amine (10 μL in 200 uL DMSO) 20 μL were added to the vial sequentially. The solution was stirred at room temperature for 1 h.

To the solution of reaction mixture, $Pd(PPh_3)_4$ (1.5 mg) in DMSO/NMP (1:1 100 uL) and $PhSiH_3$ (3 μL) was added. The light yellow, clear solution was stirred at rt for 60 minutes and quenched by addition of 2 mL MeCN/H_2O =1/1. The quenched reaction mixture was subject to HPLC purification (20-50% MeCN/H_2O over 30 min, Microsorb 100-5 C18 column, 16 mL/min, 230 nm) and lyophilization afforded glycopeptide **1** as a white powder (0.77 mg, 20%).

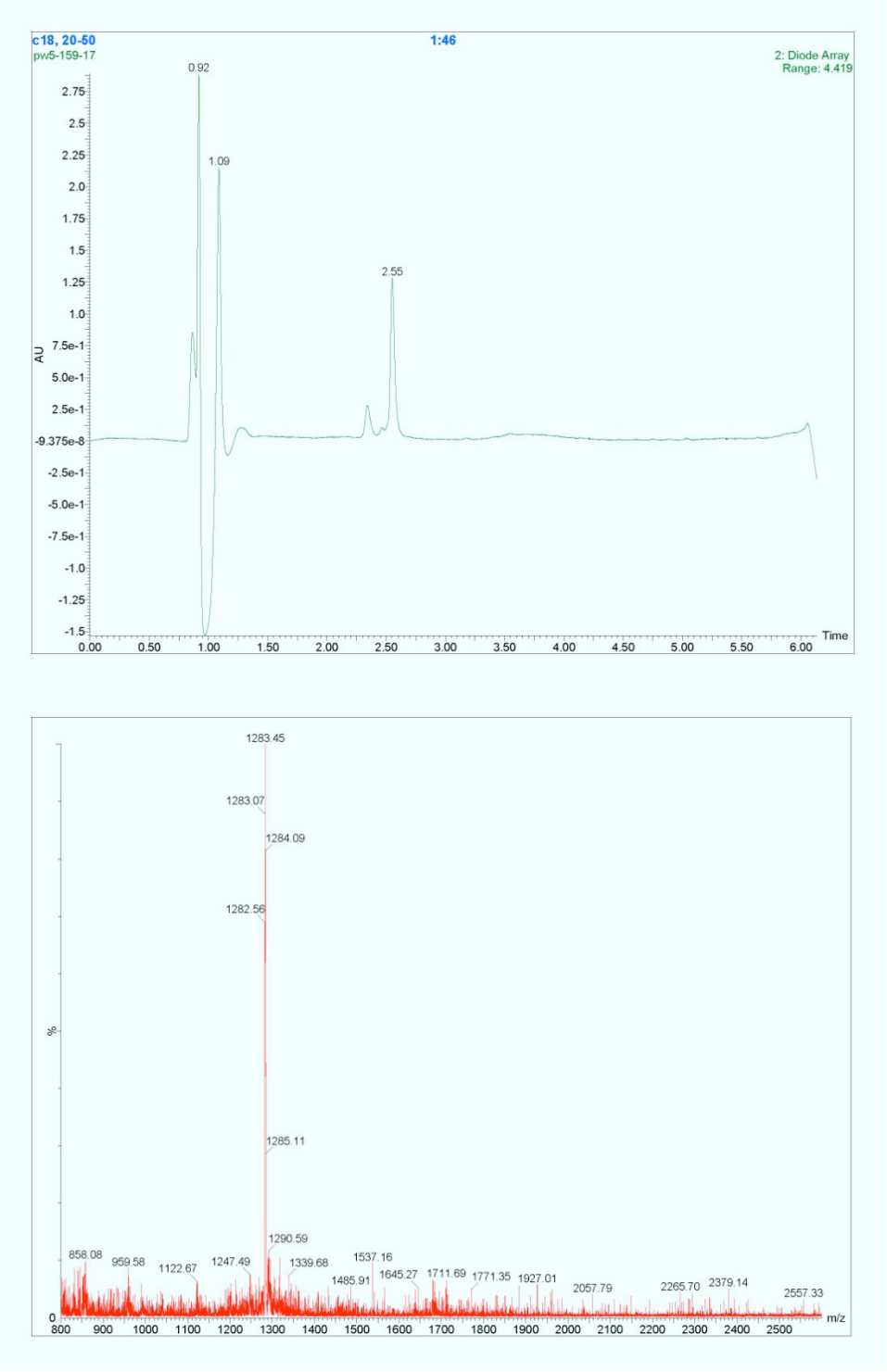
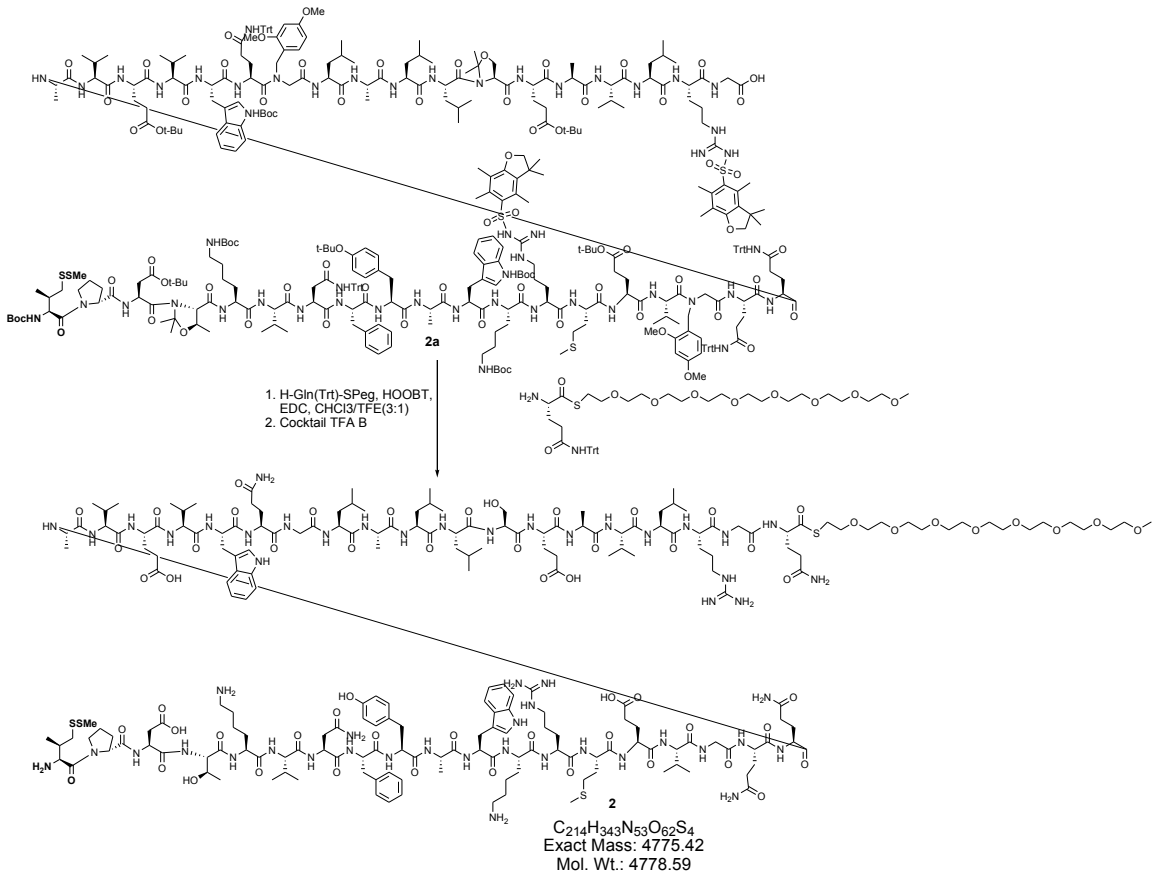


Figure 2. UV and MS traces from LC-MS analysis of glycopeptides **1**: gradient 20-50% CH_3CN/H_2O over 6 min at a flow rate of 0.3 mL/min, C18 column. (b) ESI-MS of glycopeptides **1.** ESI calcd for $C_{102}H_{160}N_{19}O_{51}S_3$ $[M+H]^+$ *m/z* = 2564.65 da, $[M+2H]^{2+}$ *m/z* = 1282.83 da, found: 1283.07 da.

Peptide **2**



Following the general procedure for SPPS, peptide was synthesized by automated Applied Biosystems Pioneer continuous flow peptide synthesizer, employing Fmoc-Gly-NovaSyn® TGT resin and other standard Fmoc amino acids.

To a solution of **2a** (60 mg, 8.7 umol) and H-Gln(Trt)-SPeg (20 mg, 26 umol) in anhydrous $CHCl_3$/TFE (1.2 mL, 3:1) was added EDC (3 uL) and HOOBT (3 mg) at rt. The reaction mixture was stirred at rt for 5 h. The solution was concentratedand treated with cocktail TFA (60.0 mg of phenol, 0.2 ml of water, 0.15 ml of triisopropylsilane, and 3.0 ml TFA) for one hour. The reaction was concentrated under a stream of argon and the residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The resulting solid was purified by HPLC (38-48% MeCN/H_2O over 30 min, X-BridgeTM Prep C18 column OBDTM, 16 mL/min, 230 nm) to give peptide **2** (6.9 mg, 18% over two steps).

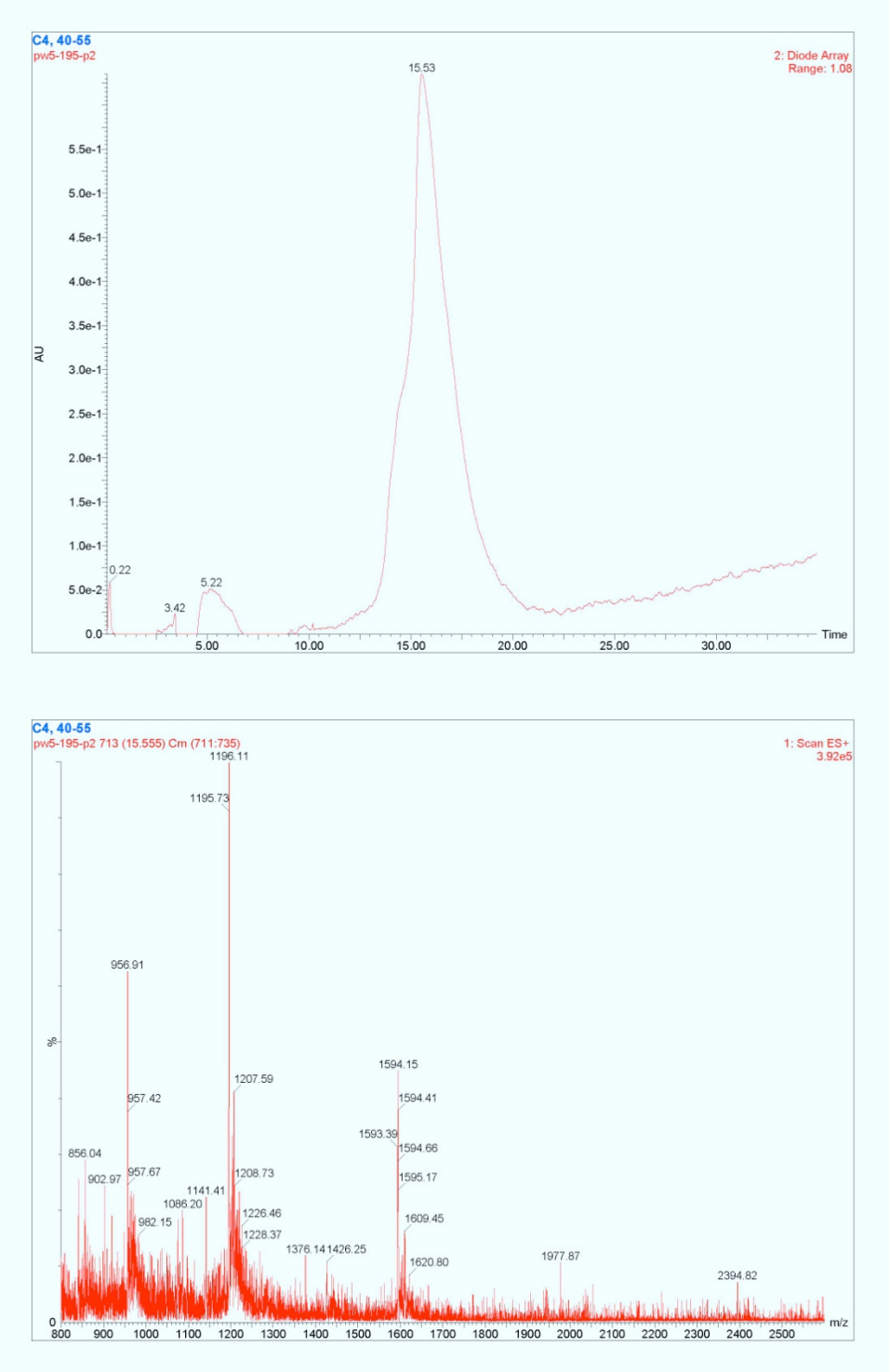
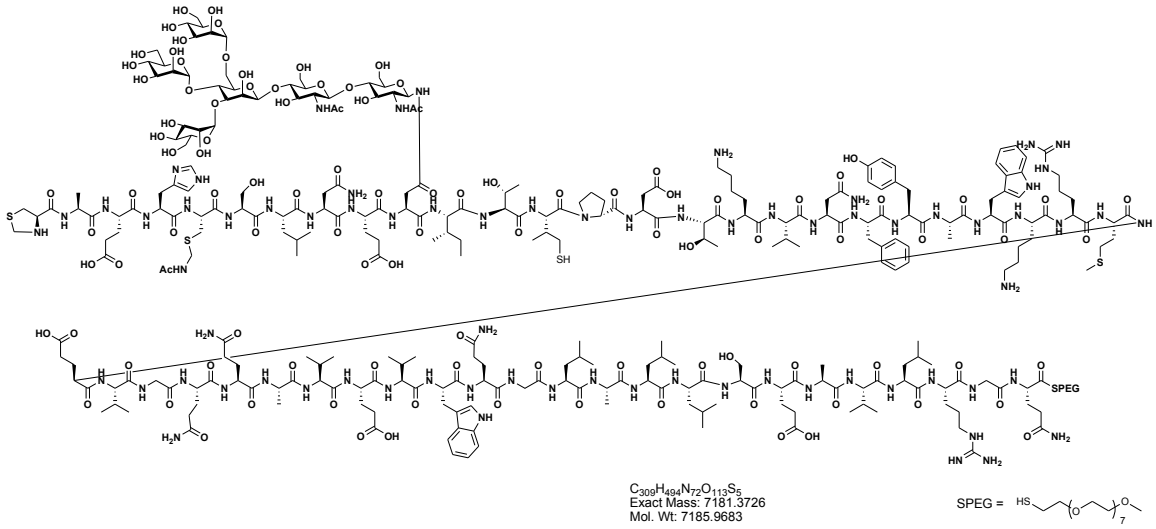


Figure 3. UV and MS traces from LC-MS analysis of peptide **2**: gradient 40-55% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C4 column. (b) ESI-MS of peptide **2.** ESI calcd for $C_{214}H_{344}N_{53}O_{62}S_4$ $[M+H]^+$ *m/z* = 4779.59 da, $[M+2H]^{2+}$ *m/z* =

2390.30 da, $[M+3H]^{3+}$ *m/z* = 1593.86 da, $[M+4H]^{4+}$ *m/z* = 1195.66 da , $[M+5H]^{5+}$ *m/z* = 956.72 da, found: 1594.15 da, 1195.73 da, 956.91 da.

Glycopeptide **3**



Peptide **2** (1.5 mg, 0.32 μmol) and glycopeptide **1** (0.7 mg, 0.27 μmol) were dissolved in 60 uL solution of 6 M GND·HCl, 0.1 M Na_2HPO_4 and 50 mM TCEP·HCl (pH 6.8). The reaction was stirred at rt for 24 hours. The reaction was quenched by addition of 2 mL MeCN/H_2O =1/1. The quenched reaction mixture was subject to HPLC purification (40-46% MeCN/H_2O over 30 min, Microsorb 100-5 C8 column, 16 mL/min, 230 nm) and lyophilization afforded glycopeptide **3** as a white powder (0.20 mg, 10%).

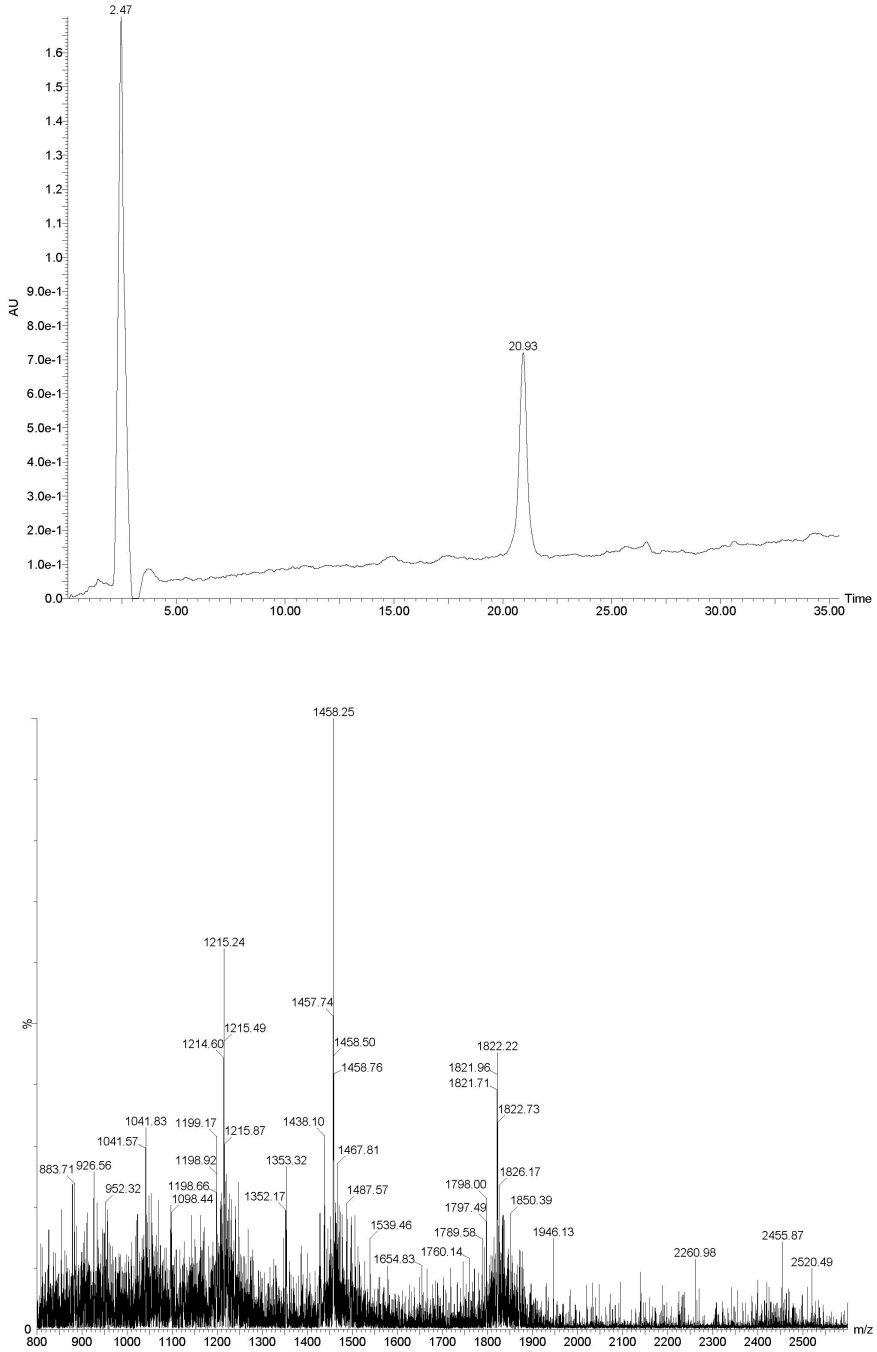
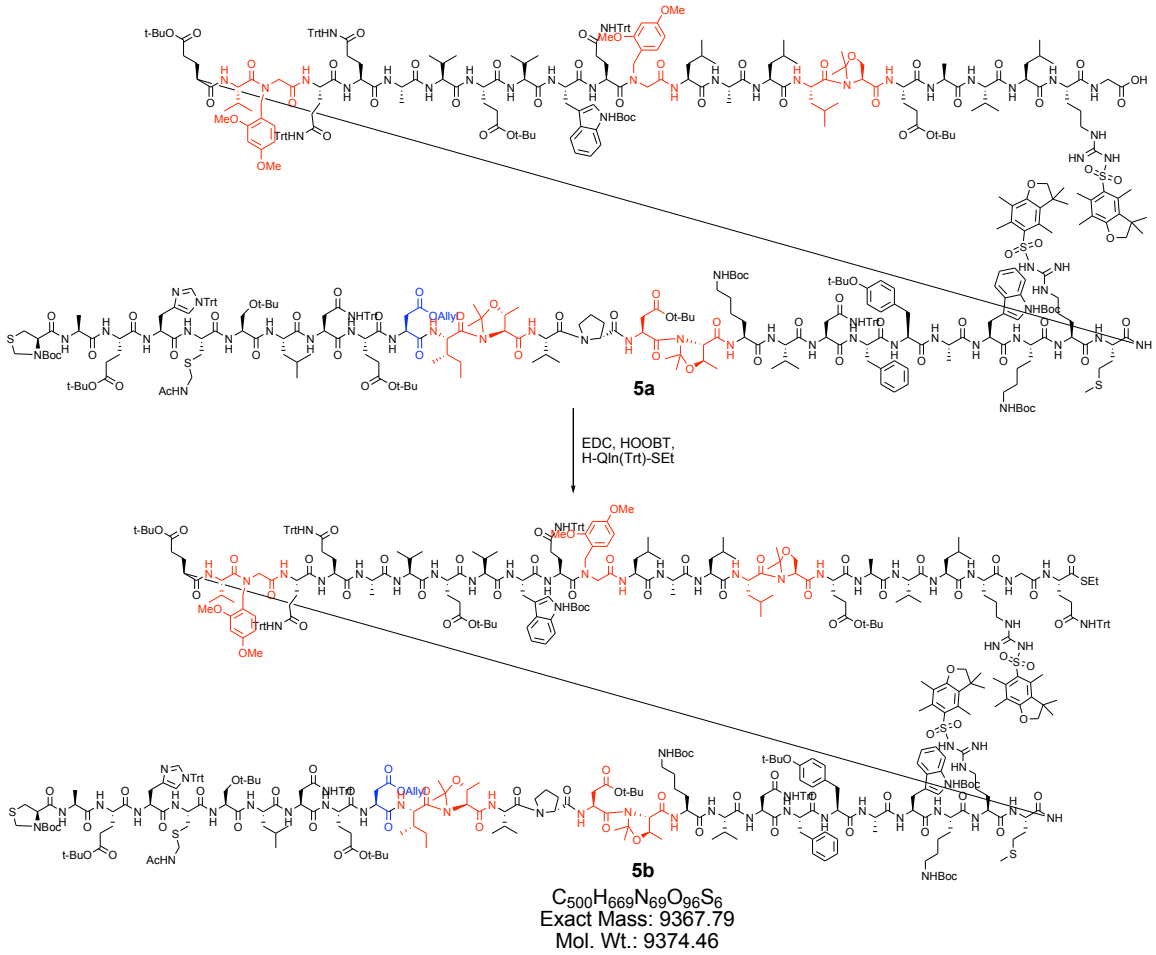


Figure 4. UV and MS traces from LC-MS analysis of glycopeptides **3**: gradient 40-48% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C4 column. (b) ESI-MS of glycopeptides **3.** ESI calcd for $C_{309}H_{494}N_{72}O_{113}S_5$ $[M+H]^+$ *m/z* = 7186.97 da, $[M+3H]^{3+}$

m/z = 2396.32 da, $[M+4H]^{4+}$ *m/z* = 1797.49.88 da, $[M+5H]^{5+}$ *m/z* = 1438.19 da, $[M+4H]^{6+}$ *m/z* = 1198.66 da , found: 1215.24 da, 1458.25 da, 1822.22 da.

Sometimes, mixture of glycopeptides EPO 29-40 with HATU will afford a complex, which possessed [M+99] Mol weight. [1]

Peptide **5b**



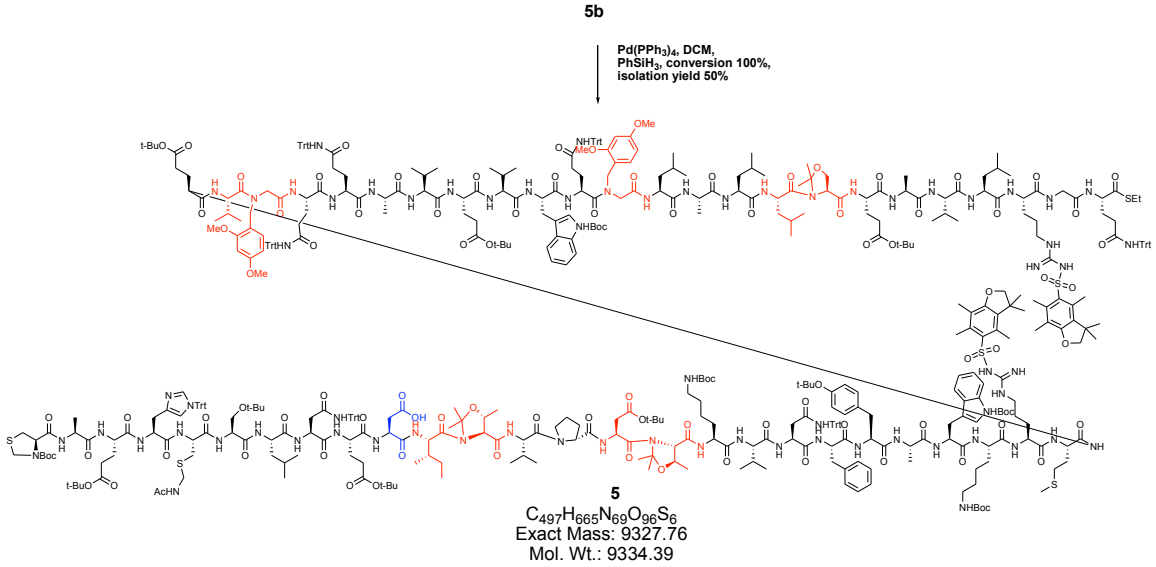
Following the general procedure for SPPS, peptide was synthesized by automated Applied Biosystems Pioneer continuous flow peptide synthesizer, employing Fmoc-Gly-NovaSyn® TGT resin, Boc-Thz-OH and other standard Fmoc amino acids.

To a solution of peptide **5a** (120 mg, 0.013 mmol) and H-Gln(Trt)-SEt (24 mg, 0.056 mmol) in anhydrous $CHCl_3$/TFE (1.2 mL, 3:1) was added EDC (12 uL, 0.056 ummol) and HOOBT (10 mg, 0.061 ummol) at rt. The reaction mixture was stirred at rt for 5 h. The reaction was concentrated under a stream of argon and the residue was

passed through short silica gel column (2%-5% MeOH/CH_2Cl_2) to give a white solid **5b** (61 mg, 50%).

ESI calcd for $C_{500}H_{670}N_{69}O_{96}S_6$ $[M+H]^+$ *m/z* = 9375.46 da, $[M+5H]^{5+}$ *m/z* = 1874.89 da, found: 1874.90 da.

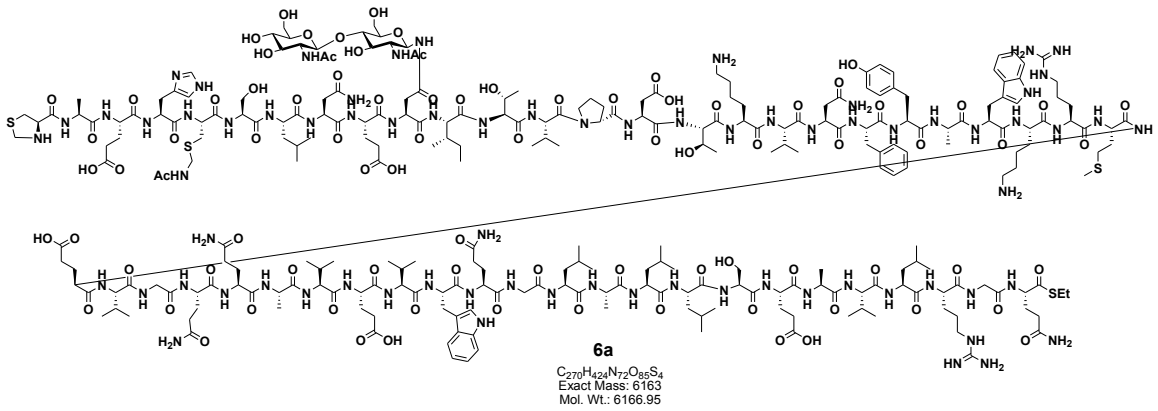
Peptide **5**



To a solution of peptide **5b** (80 mg, 8.5 μmol) and $Pd(PPh_3)_4$ (2.0 mg, 1.7 umol) in DCM (1.0 mL) was added $PhSiH_3$ (21 μL, 170 umol). The light yellow, clear solution was stirred at rt for 20 minutes. The reaction was concentrated under a stream of nitrogen and the residue was passed through short silica gel column (2%-8% MeOH/CH_2Cl_2), the faction was concentrated and lyophilized to give a white solid **5** (40 mg, 50%).

ESI calcd for $C_{497}H_{666}N_{69}O_{96}S_6$ $[M+H]^+$ *m/z* = 9335.39 da, $[M+5H]^{5+}$ *m/z* = 1867.88 da, found: 1867.92 da.

Glycopeptide **6a**.



To a mixture of peptide **5** (19 mg, 0.002 mmol), disaccharide **7** (1.7 mg, 0.004 mmol) and HATU (4.5 mg, 0.012 mmol) was added DMSO (120 uL) and DIPEA (1.1 uL, 0.006 mmol). The reaction mixture was stirred at room temperature for 2 h. Next the solution was treated with cocktail TFA (60.0 mg of phenol, 0.2 ml of water, 0.15 ml of triisopropylsilane, and 3.0 ml TFA) for 90 mins. The reaction was concentrated under a stream of argon and the residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The mixture was purified via RP-HPLC (42-50% MeCN/H_2O over 30 min, Microsorb 300-5 C8 column, 16 mL/min, 230 nm). Product eluted at 19-22 min. The fractions were collected, concentrated, and lyophilized to provide glycopeptide **6a** (6.2 mg, 52%) as a white solid.

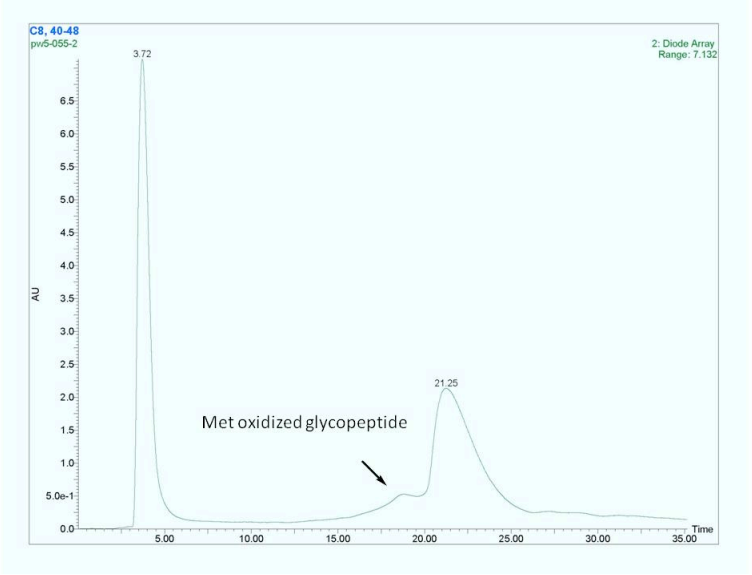


Figure 5. Crude LC-MS traces of reaction after deprotection: gradient 40-48% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C8 column.

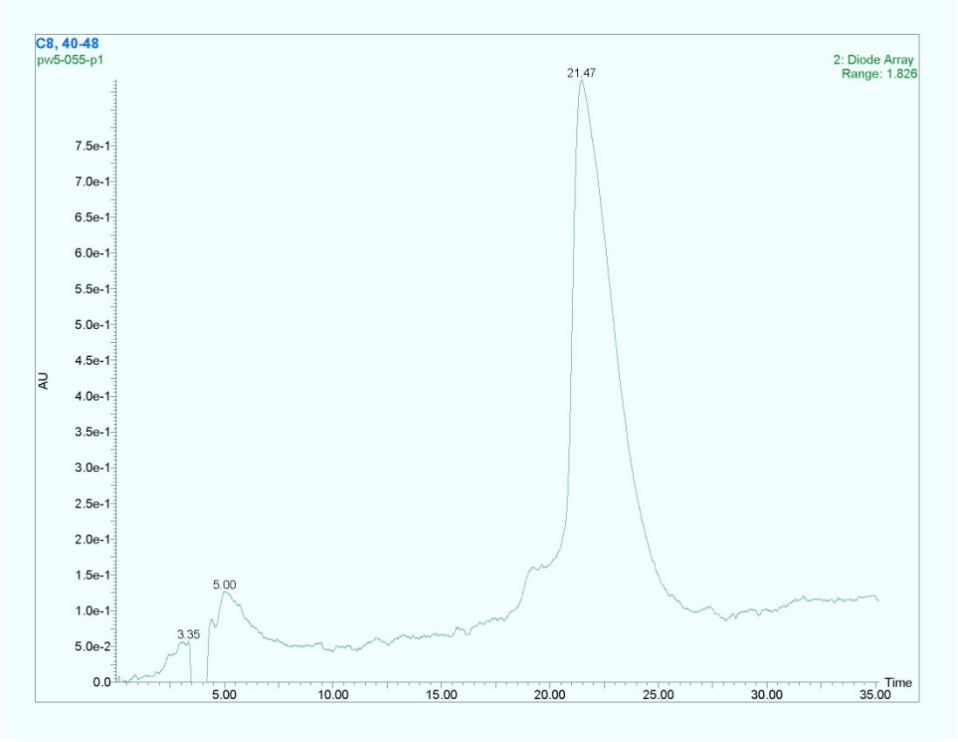
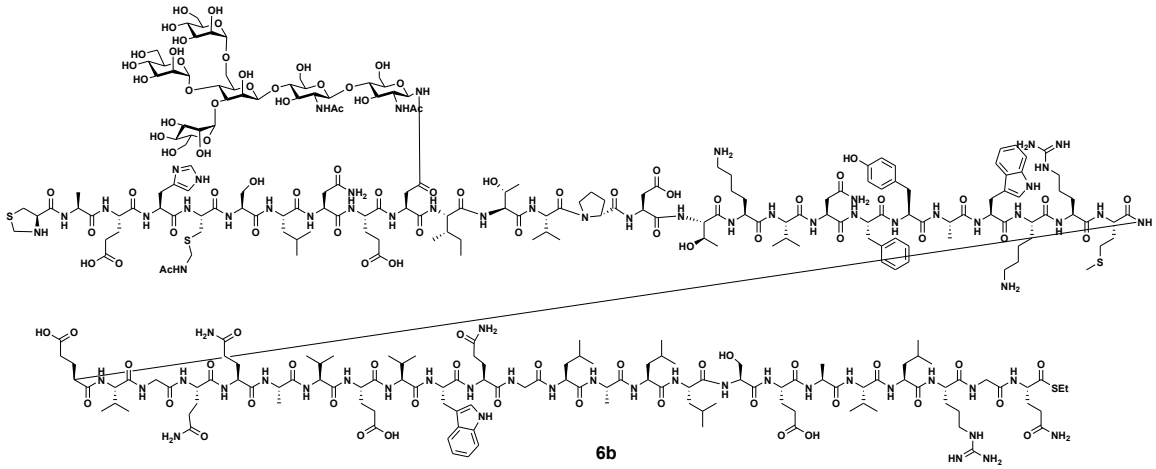


Figure 6. UV and MS traces from LC-MS analysis of glycopeptide **6a**: gradient 40-48% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C8 column. (b) ESI-MS of glycopeptide **6a.** ESI calcd for $C_{270}H_{425}N_{72}O_{85}S_4$ $[M+H]^+$ *m/z* = 6166.95 da, $[M+2H]^{2+}$ *m/z* = 3084.48 da, $[M+3H]^{3+}$ *m/z* = 2056.65 da, $[M+4H]^{4+}$ *m/z* = 1542.74 da, $[M+5H]^{5+}$

m/z = 1234.39 da, $[M+6H]^{6+}$ *m/z* = 1028.83 da, found: 2056.90 da, 1542.77 da, 1234.36 da, 1029.33 da.

Glycopeptide **6b**



To a mixture of peptide **5** (6 mg, 0.64 umol), hexasaccharide **4** (0.9 mg, 0.84 umol) and HATU (1.5 mg, 0.004 mmol) was added DMSO (35 uL) and DIPEA (0.3 uL). The reaction mixture was stirred at room temperature for 2 h. Next the solution was treated with cocktail TFA (30.0 mg of phenol, 0.1 ml of water, 0.07 ml of triisopropylsilane, and 1.5 ml TFA) for 90 mins. The reaction was concentrated under a stream of argon and the residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The mixture was purified via RP-HPLC (42-50% MeCN/H_2O over 30 min, Microsorb 300-5 C8 column, 16 mL/min, 230 nm). Product eluted at 20-23 min. The fractions were collected, concentrated, and lyophilized to provide glycopeptide **6b** (1.4 mg, 32%) as a white solid.

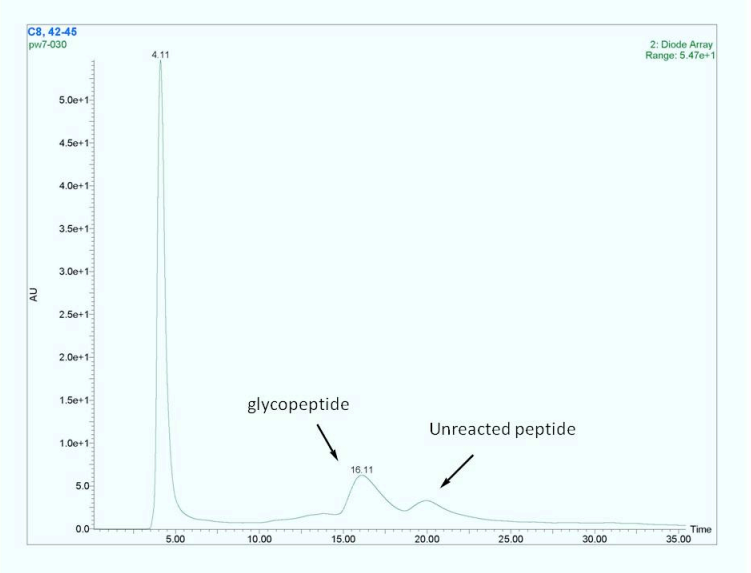


Figure 7. Crude LC-MS traces of reaction after deprotection: gradient 42-45% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C8 column.

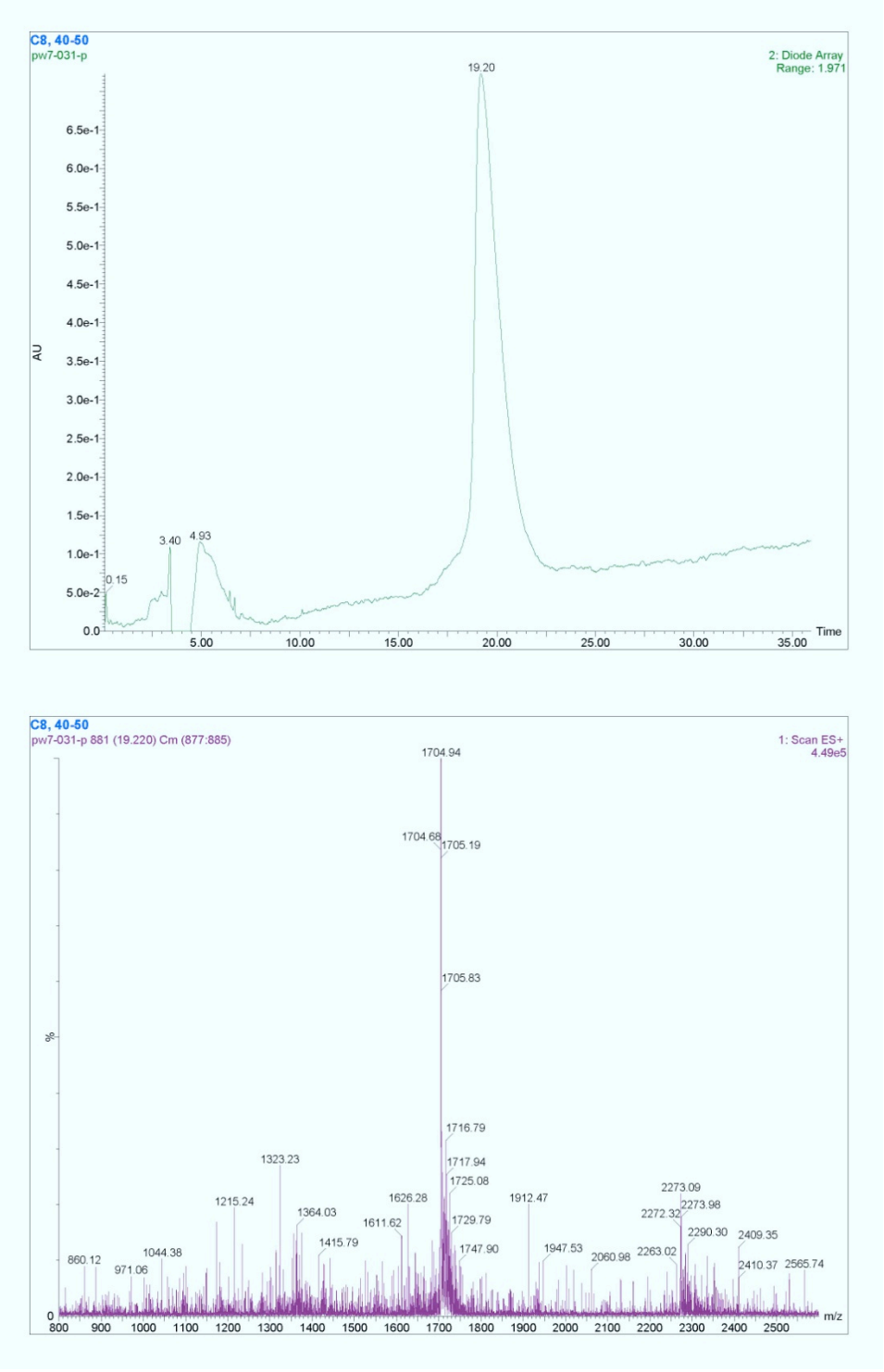
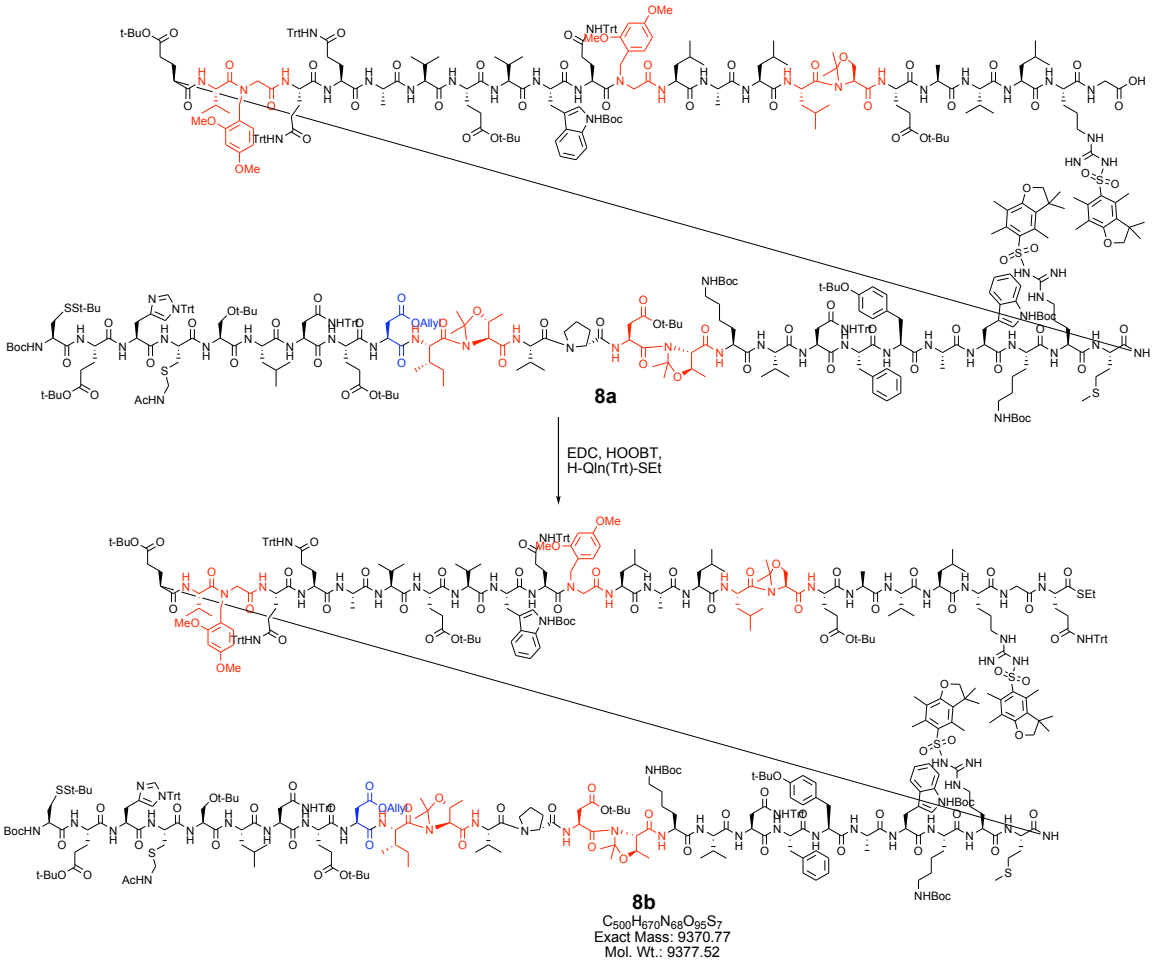


Figure 8. UV and MS traces from LC-MS analysis of glycopeptide **6b**: gradient 40-48% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C8 column. (b) ESI-MS of glycopeptide **6b.** ESI calcd for $C_{294}H_{465}N_{72}O_{105}S_4$ $[M+H]^+$ *m/z* = 6816.51 da, $[M+2H]^{2+}$

m/z = 3408.76 da, $[M+3H]^{3+}$ *m/z* = 2272.84 da, $[M+4H]^{4+}$ *m/z* = 1704.88 da, $[M+5H]^{5+}$ *m/z* = 1364.10 da, found: 2273.09 da, 1704.94 da.

Peptide 8b

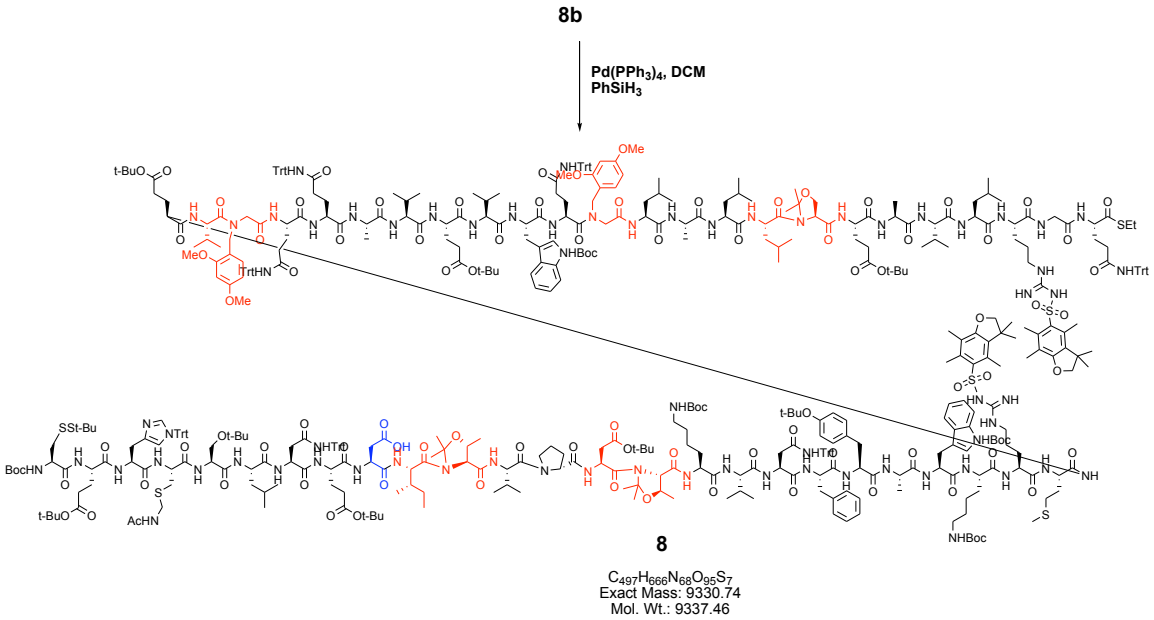


Following the general procedure for SPPS, peptide was synthesized by automated Applied Biosystems Pioneer continuous flow peptide synthesizer, employing Fmoc-Gly-NovaSyn® TGT resin, Boc-Cys(Acm)-OH and other standard Fmoc amino acids.

To a solution of peptide **8a** (166 mg, 0.019 mmol) and H-Gln(Trt)-SEt (28 mg, 0.06 mmol) in anhydrous $CHCl_3$/TFE (1.6 mL, 3:1) was added EDC (17 uL) and HOOBT (11 mg) at rt. The reaction mixture was stirred at rt for 5 h. The reaction was concentrated under a stream of nitrogen and the residue was passed through short silica gel column (2%-8% MeOH/CH_2Cl_2) to give a white solid **8b** (100 mg, 58%).

ESI calcd for $C_{500}H_{670}N_{68}O_{95}S_7$ $[M+H]^+$ *m/z* = 9378.52 da, $[M+5H]^{5+}$ *m/z* = 1876.50 da, found: 1876.90 da.

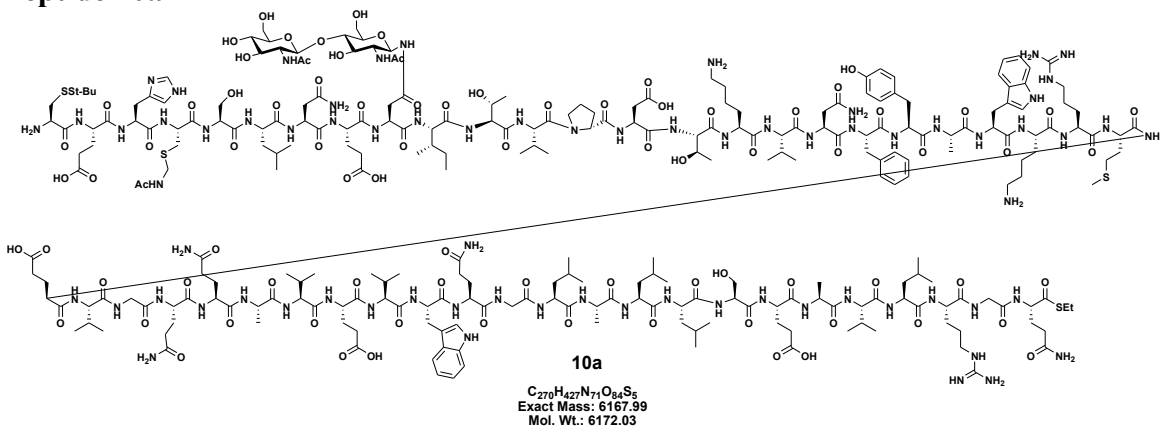
Peptide 8



To a solution of peptide 8**b** (100 mg, 0.011 mmol) and $Pd(PPh_3)_4$ (2.0 mg, 0.0017 mmol) in DCM (2.5 mL) was added $PhSiH_3$ (23 µL, 0.18 mmol). The light yellow, clear solution was stirred at rt for 20 minutes. The reaction was concentrated under a stream of nitrogen and the residue was passed through short silica gel column (3%-12% MeOH/CH_2Cl_2), the faction was concentrated and lyophilized to give a white solid **8** (42 mg, 42%).

ESI calcd for $C_{497}H_{666}N_{68}O_{95}S_7$ $[M+H]^+$ *m/z* = 9338.46 da, $[M+5H]^{5+}$ *m/z* = 1868.49 da, found: 1868.7 da.

Peptide 10a



To a mixture of peptide **8** (40 mg, 0.0043 mmol), chitobiose **7** (5.2 mg, 0.012 mmol) and HATU (9.7 mg, 0.025 mmol) was added DMSO (200 uL) and DIPEA (2.2 uL, 0.012 mmol). The reaction mixture was stirred at room temperature for 2 h. Next the solution was treated with cocktail TFA (120.0 mg of phenol, 0.4 ml of water, 0.3 ml of triisopropylsilane, and 6.0 ml TFA) for 90 min. The reaction was concentrated under a stream of argon and the residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The mixture was purified via RP-HPLC (32-42% MeCN/H_2O over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm). Product eluted at 16-19 min. The fractions were collected, concentrated, and lyophilized to provide glycopeptide **10a** (14.4 mg, 54%) as a white solid.

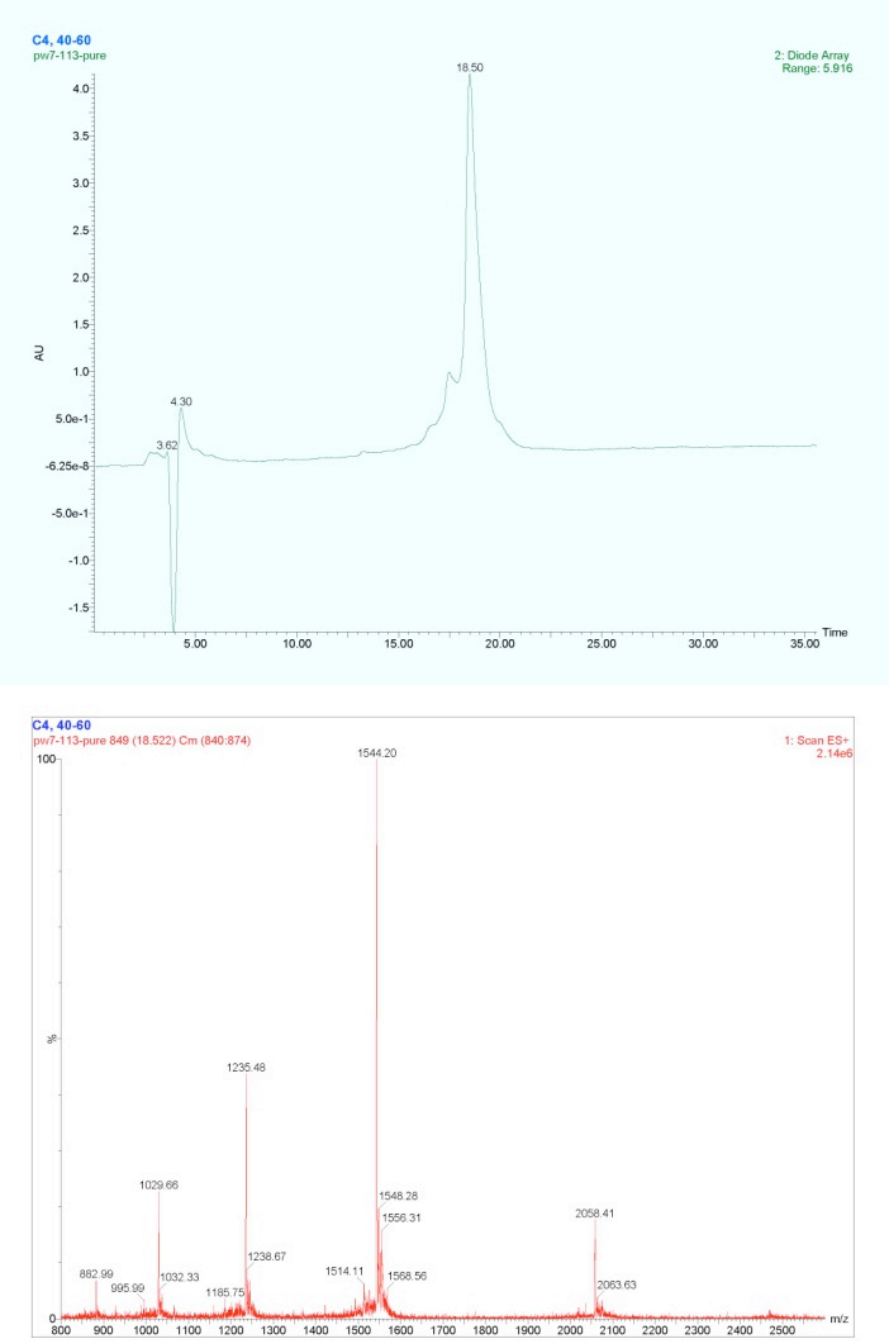
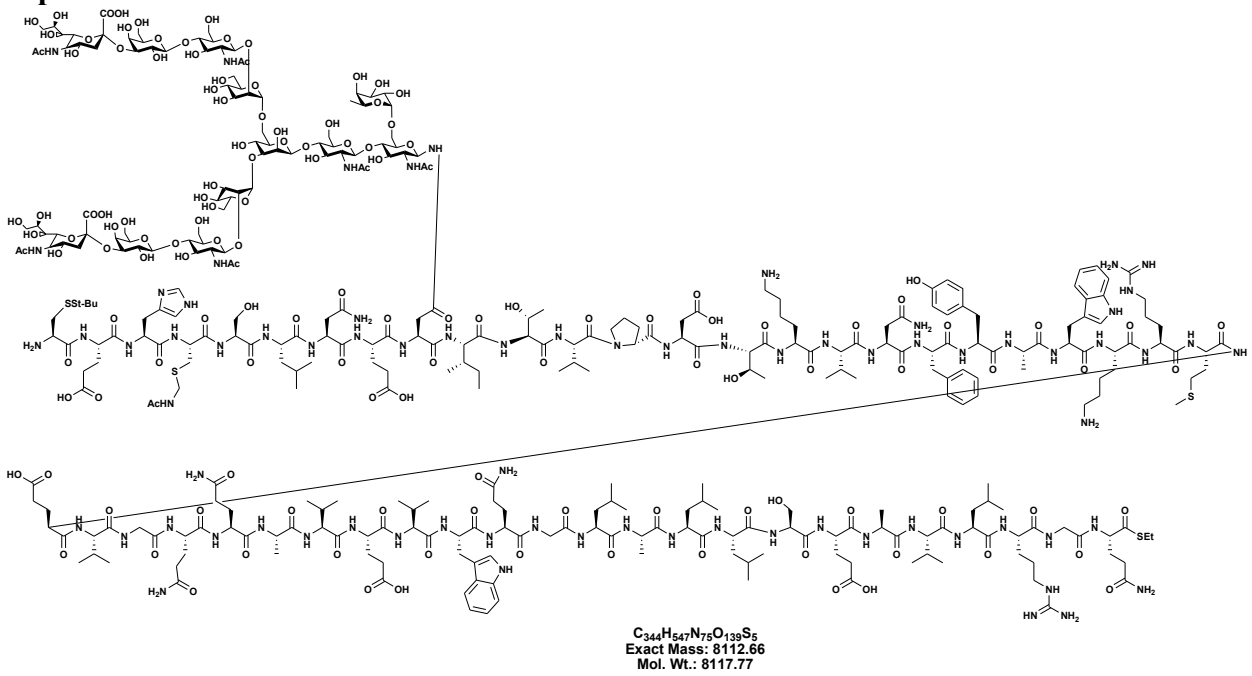
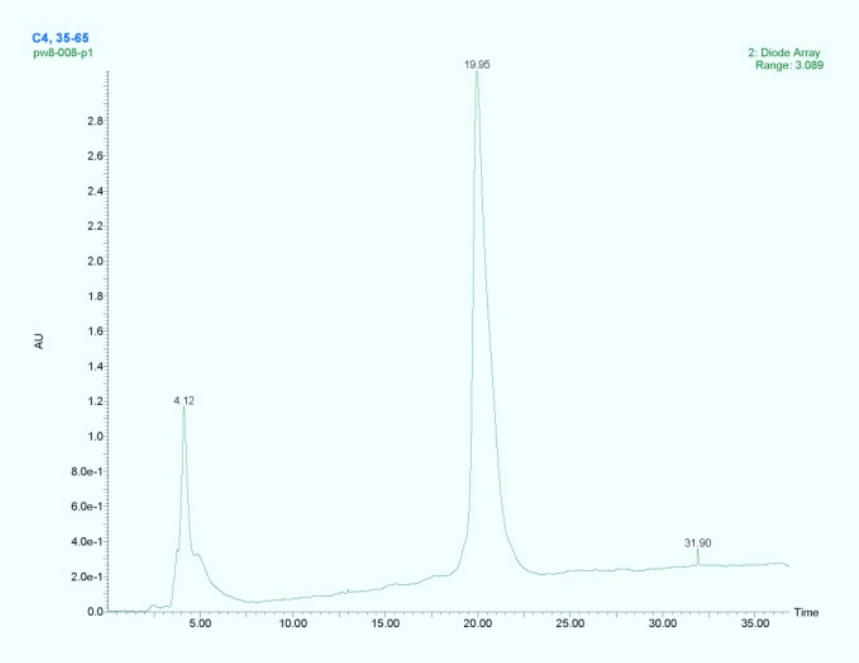


Figure 9. (a) UV and MS traces from LC-MS analysis of glycopeptide **10a**: gradient 40-60% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C4 column. (b) ESI-MS of glycopeptide **21.** ESI calcd for $C_{270}H_{427}N_{71}O_{84}S_5$ $[M+H]^+$ *m/z* = 6173.03 da, $[M+3H]^{3+}$ *m/z* = 2058.34 da, $[M+4H]^{4+}$ *m/z* = 1544.01 da, $[M+5H]^{5+}$ *m/z* = 1235.41 da, $[M+6H]^{6+}$ *m/z* = 1029.67 da, $[M+7H]^{7+}$ *m/z* = 882.72, found: 2058.41 da, 1544.20 da, 1235.48 da, 1029.66 da, 882.99 da.

Peptide 10b



To a mixture of peptide **8** (20 mg, 0.0021 mmol), dodecasaccharide **9** (7.1 mg, 0.030 mmol) and HATU (6.5 mg, 0.017 mmol) was added DMSO (110 uL) and DIPEA (1.8 uL, 0.010 mmol). The reaction mixture was stirred at room temperature for 2 h. Next the solution was treated with cocktail TFA (60.0 mg of phenol, 0.2 ml of water, 0.15 ml of triisopropylsilane, and 3.0 ml TFA) for 90 min. The reaction was concentrated under a stream of argon and the residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The peptide was dissolved in 3.0 mL solution of 6 M GND·HCl, 0.1 M Na_2HPO_4, pH 6.8. The mixture was stirred at rt for 48 hours to open lactone ester bond. The mixture was purified via RP-HPLC (40-47% MeCN/H_2O over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm). Product eluted at 20-22 min. The fractions were collected, concentrated, and lyophilized to provide glycopeptide **10b** (5.6 mg, 32%) as a white solid.



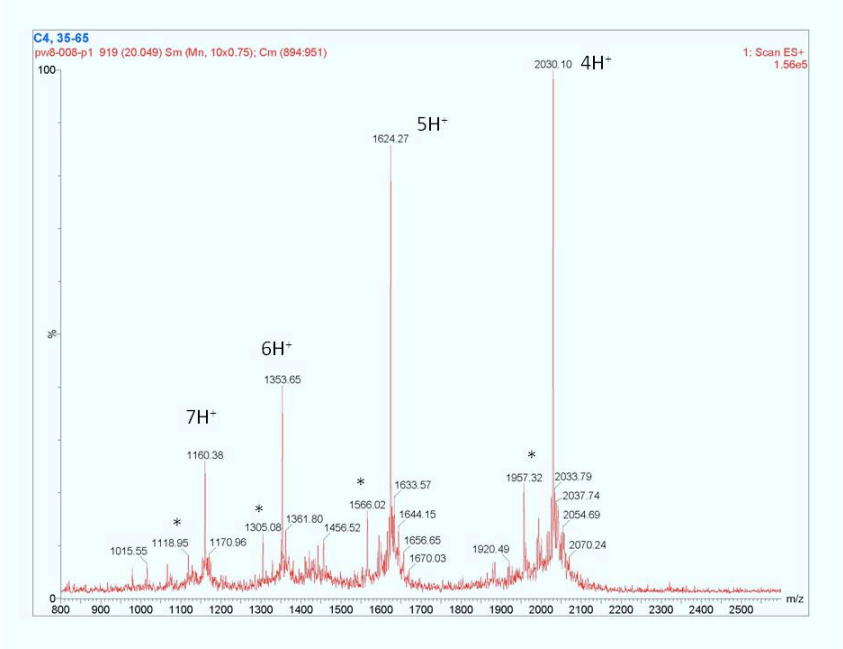
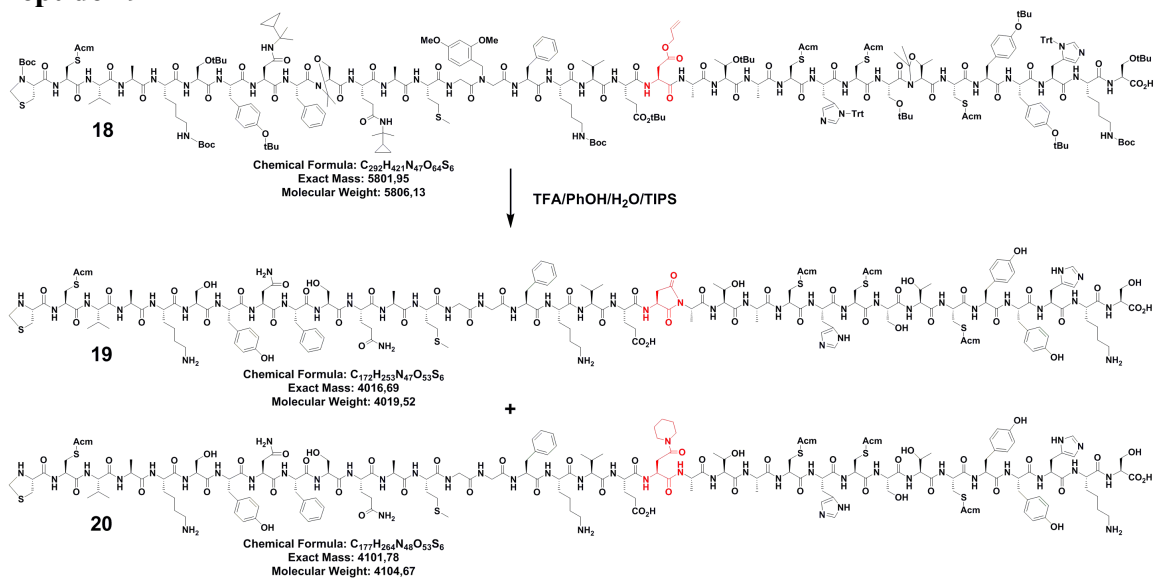


Figure 10 (a) UV and MS traces from LC-MS analysis of glycopeptide: gradient 35-65% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C4 column. (b) ESI-MS of glycopeptide**.** ESI calcd for $C_{344}H_{547}N_{75}O_{139}S_5$ $[M+H]^+$ *m/z* = 8118.77 da, $[M+4H]^{4+}$ *m/z* = 2030.44 da, $[M+5H]^{5+}$ *m/z* = 1624.55 da, $[M+6H]^{6+}$ *m/z* = 1353.96 da, $[M+7H]^{7+}$ *m/z* = 1060.68 da, $[M+8H]^{8+}$ *m/z* = 1015.72, found: 2030.48 da, 1624.91 da, 1353.65 da, 1060.64 da, 1015.55 da.

*One sialic acid was cleave during lc-ms ionized.

Peptide 19



A small portion of off-resin peptidyl acid **18** was subjected to cleavage with cocktail B for 90 min following the general procedure. The resulting solid was analyzed by UPLC to determine the amount of aspartimide formed during SPPS.

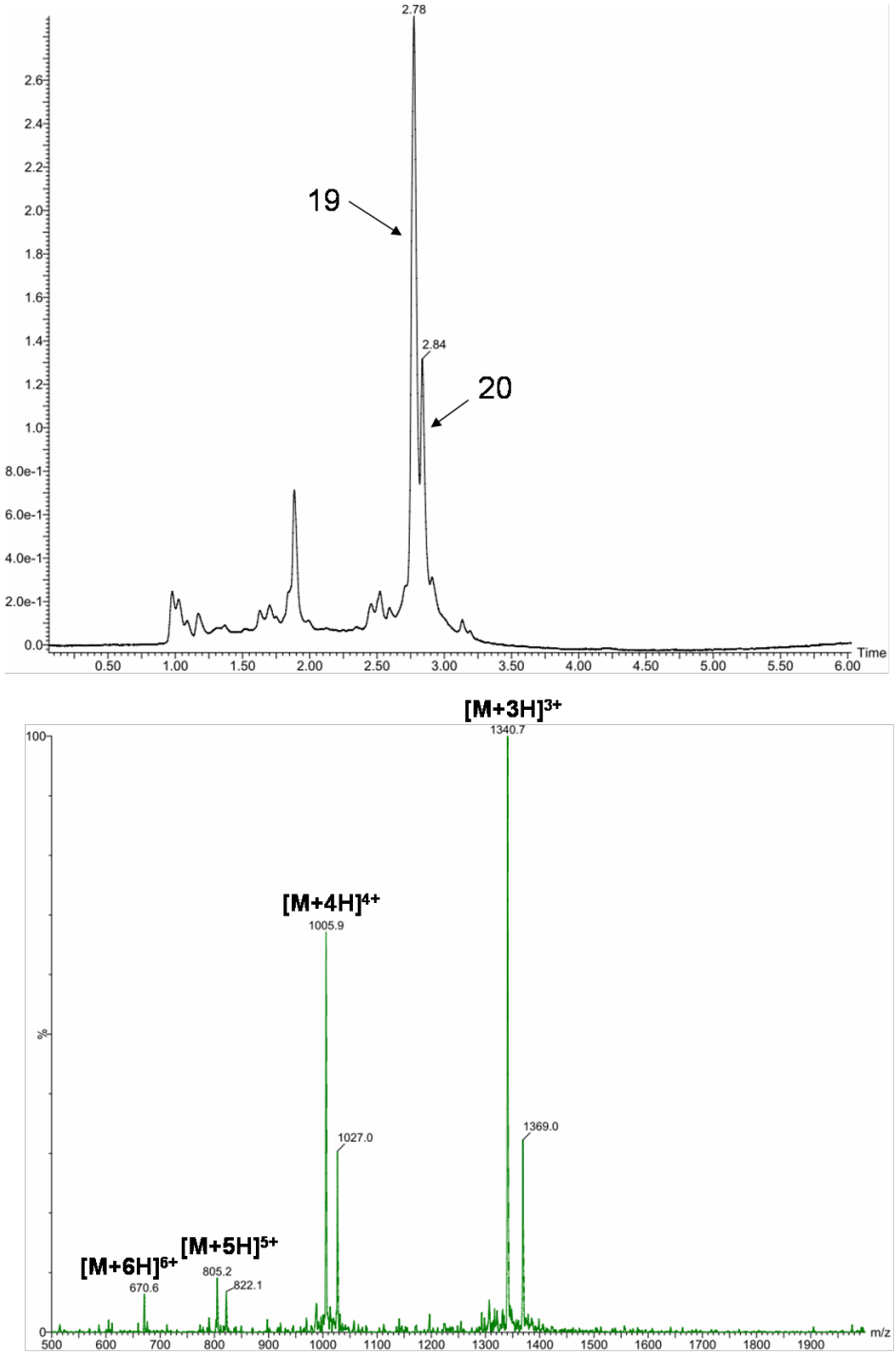
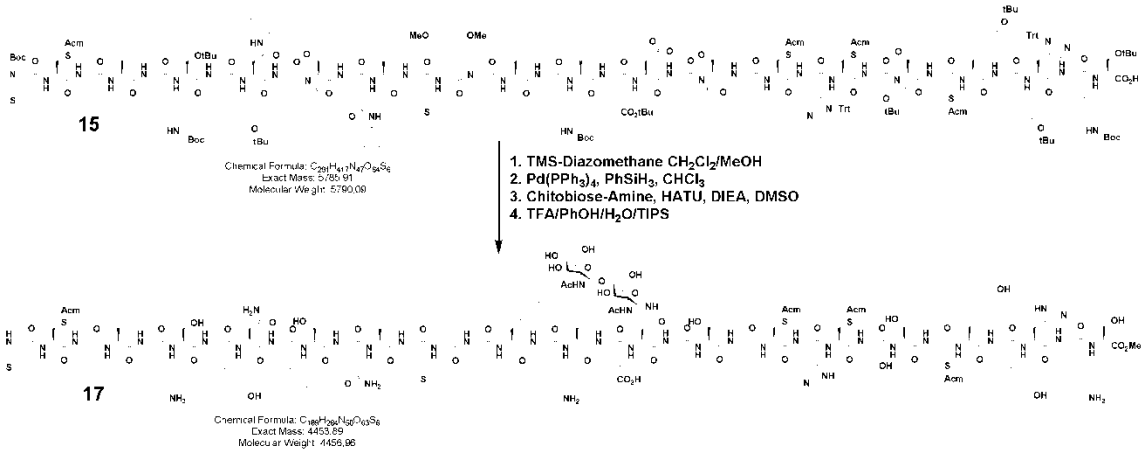


Figure 11: UV and MS traces from LC-MS analysis of peptide **19** and **20**: gradient: 10% to 60% CH_3CN/H_2O over 6 min at a flow rate of 0.3 mL/min, BEH C4 column. (b) ESI-MS of peptide **19**.

ESI calculated for **19** $C_{172}H_{253}N_{47}O_{53}S_6$ $[M+3H]^{3+}$ *m/z*: 1340.8 da, $[M+4H]^{4+}$ *m/z*: 1005.9 da, $[M+5H]^{5+}$ *m/z*: 804.9 da, $[M+6H]^{6+}$ *m/z*: 670.6 da, found: 1340.7 da, 1005.9 da, 805.2 da, 670.9 da.

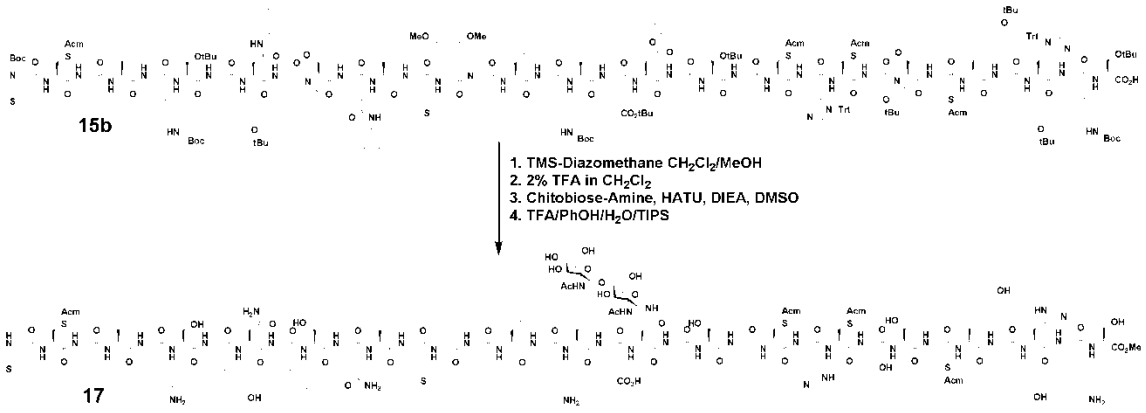
ESI calculated for **20** $C_{177}H_{264}N_{48}O_{53}S_6$ $[M+3H]^{3+}$ *m/z*: 1369.2 da, $[M+4H]^{4+}$ *m/z*: 1027.2 da, $[M+5H]^{5+}$ *m/z*:821.9 da, found: 1369.0 da, 1027.0 da,822.1 da.

Glycopeptide 17



The peptidyl acid **15** (45 mg, 7.8 µmol) was solubilized in DCM/MeOH (9:1, 1.5 mL), cooled at 0 °C and TMS-Diazomethane (2 M solution in Et_2O, 40 µL, 80 µmol) was added. The solution was stirred at room temperature for 30 min and the peptide was precipited by ice-cold diethyl ether (3 x 45 mL) to give a white precipitate, which was centrifugated. The precipitate was solubilized in water/acetonitrile (1:1, 0.05% trifluoroacetic acid) and lyophilized. The crude peptide was solubilized in chloroform (1 mL). To this solution was added $Pd(PPh_3)_4$ (11.3 mg, 9.7 µmol) followed by phenylsilane (19 µL, 155 µmol). The reaction was stirred in the dark for 20 min and then quenched by ice-cold diethyl ether (3 x 45 mL) to give a white precipitate, which was centrifugated. The precipitate was solubilized in water/acetonitrile (1:1, 0.05% trifluoroacetic acid) and lyophilized. 45 mg of peptide **16** were recovered after lyophilization. Peptide (20 mg, 3.46 µmol) and chitobiose amine **7** (4 mg, 9.45 µmol) were combined and solubilized in anhydrous DMSO (288 µL). To this mixture, a freshly prepared solution of HATU in anhydrous DMSO (0.5mg/µL, 7 µL, 9.3 µmol) was added followed by DIEA (1.2 µL, 6.9 µmol). The solution immediately turned into a deep, golden-yellow color and was stired

for 30 min and quenched by ice-cold water/trifluoroacetic acid (99.95:0.05). The precipitated peptide was centrifugated, resuspended in water/acetonitrile (1:1, 0.05% trifluoroacetic acid) and lyophilized. The peptide was subjected to cleavage with cocktail B (3 mL) for 90 min following the general procedure. The resulting solid was purified by RP-HPLC (C18 semiprep, 10% to 60% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 18 min. Lyophilization of the collected fractions provided glycopeptide **17** (7.2 mg, 45%) as a white solid.



The peptidyl acid **15b** (45 mg, 7.8 μmol) was solubilized in dichloromethane/methanol (9:1, 1.5 mL), cooled at 0 °C and TMS-Diazomethane (2 M solution in Et_2O, 40 μL, 80 μmol) was added. The solution was stirred at room temperature for 30 min and the peptide was precipited by ice-cold diethyl ether (3 x 45 mL) to give a white precipitate, which was centrifugated. The precipitate was solubilized in water/acetonitrile (1:1, 0.05% trifluoroacetic acid) and lyophilized. Resulting peptide was then solubilized in dichloromethane (2 mL). To this solution was added dichloromethane/trifluoroacetic acid (98:2, 5 mL). The reaction was stirred for 60 min and then quenched by ice-cold diethyl ether (1 x 45 mL) to give a white precipitate, which was centrifugated. The precipitate was solubilized in water/acetonitrile (1:1, 0.05% trifluoroacetic acid) and lyophilized. 45 mg of peptide were recovered after lyophilization. Peptide (20 mg, 3.46 μmol) and chitobiose amine (4 mg, 9.45 μmol) were combined and solubilized in anhydrous DMSO (288 μL). To this mixture, a freshly prepared solution of HATU in anhydrous DMSO (0.5mg/μL, 7 μL, 9.3 μmol) was added followed by DIEA (1.2 μL, 6.9 μmol). The

solution immediately turned into a deep, golden-yellow color and was stired for 30 min and quenched by ice-cold water/trifluoroacetic acid (99.95:0.05). The precipitated peptide was centrifugated, resuspended in water/acetonitrile (1:1, 0.05% trifluoroacetic acid) and lyophilized. The peptide was subjected to cleavage with cocktail B (3 mL) for 90 min following the general procedure. The resulting solid was purified by RP-HPLC (C18 semiprep, 10% to 60% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 18 min. Lyophilization of the collected fractions provided peptide **17** (1.8 mg, 11%) as a white solid.

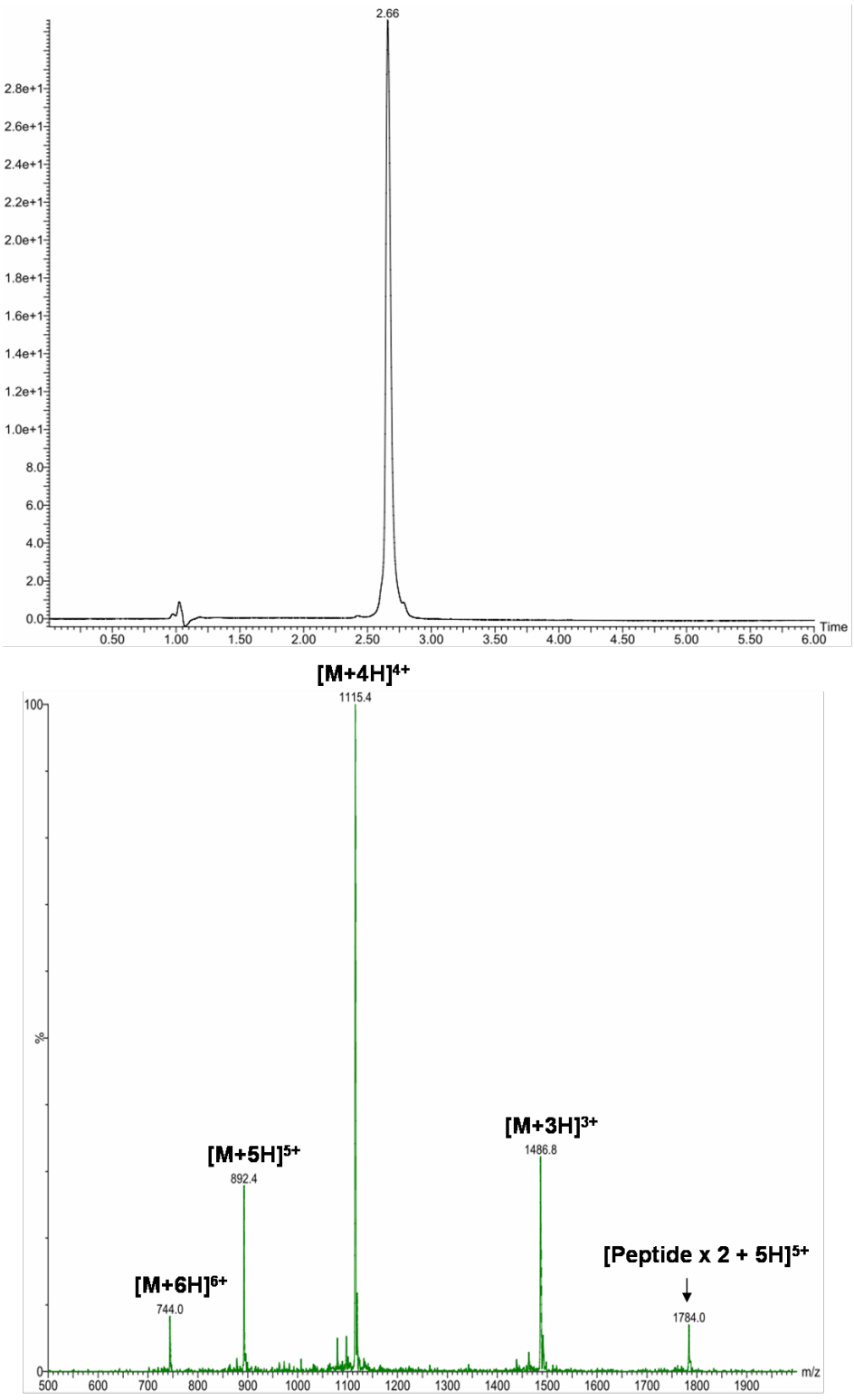
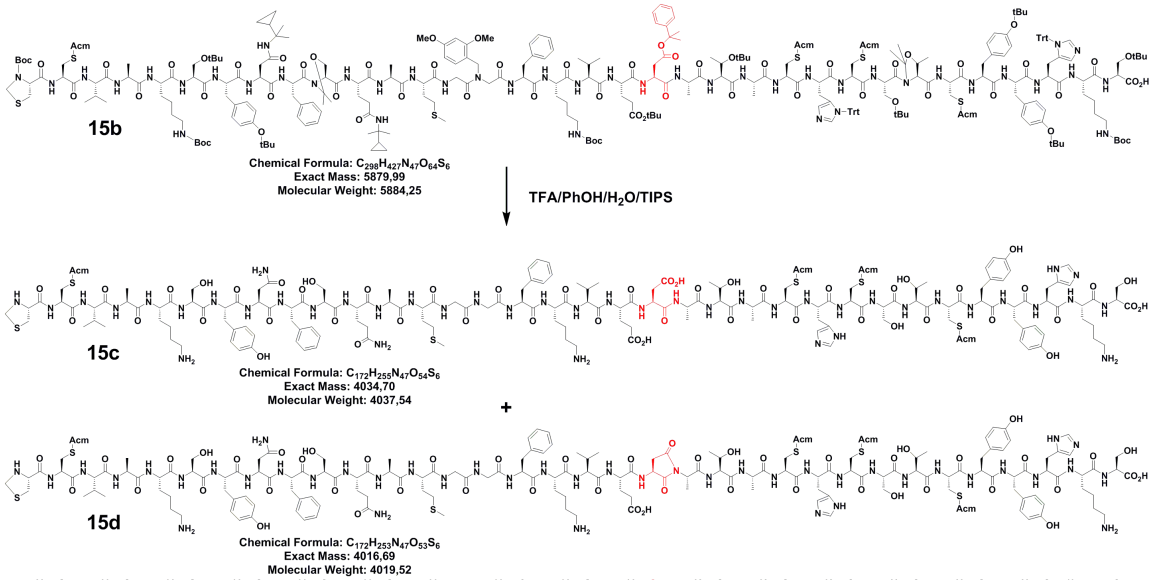


Figure 12: UV and MS traces from LC-MS analysis of compound **17**: gradient: 10% to 60% CH_3CN/H_2O over 6 min at a flow rate of 0.3 mL/min, BEH C4 column. (b) ESI-MS of compound **17**.

ESI calculated for $C_{189}H_{284}N_{50}O_{63}S_6$ $[M+3H]^{3+}$ *m/z*: 1486.6 da, $[M+4H]^{4+}$ *m/z*: 1115.2 da, $[M+5H]^{5+}$ *m/z*: 892.4 da, $[M+6H]^{6+}$ *m/z*: 743.8 da, found: 1486.8 da, 1115.4 da, 892.4 da, 744.0 da.

To check if aspartimide was formed during SPPS, a small portion of off-resin peptidyl acid **15b** was subjected to cleavage with cocktail B for 90 min following the general procedure. The resulting solid was analyzed by UPLC to determine the amount of aspartimide formed during SPPS.



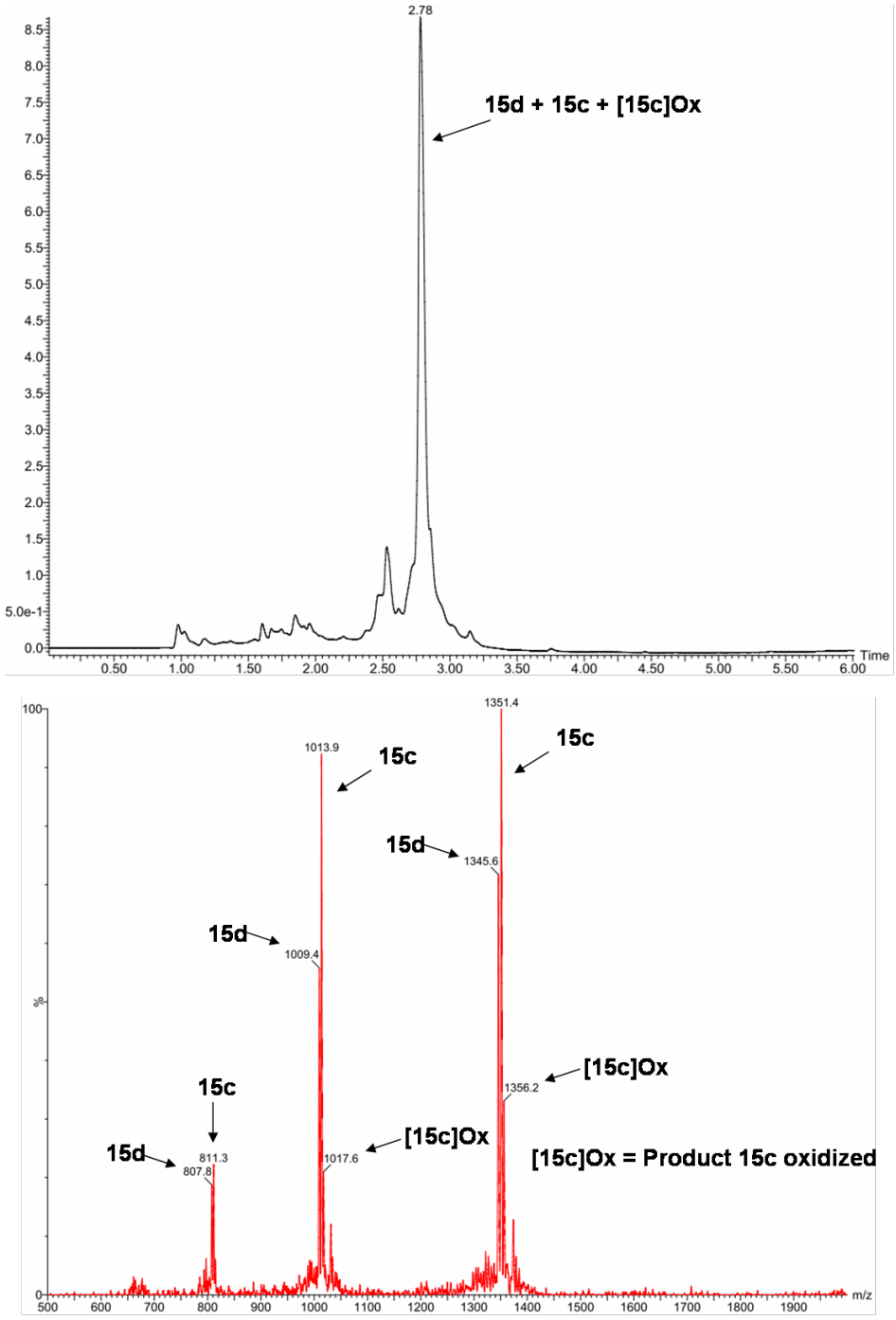
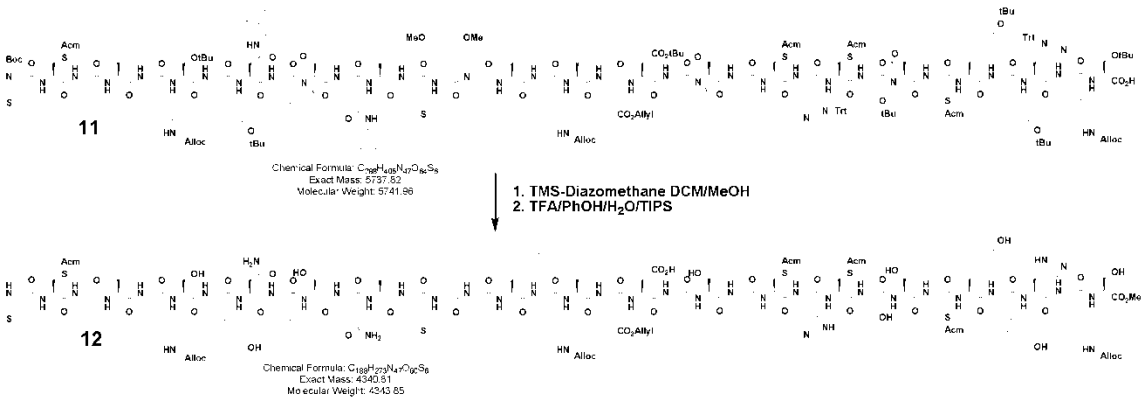


Figure 13: UV and MS traces from LC-MS analysis of compound **15c** and **15d**: gradient: 10% to 60% CH_3CN/H_2O over 6 min at a flow rate of 0.3 mL/min, BEH C4 column. (b) ESI-MS of compound **15c** and **15d**.

Along with compounds **15c** and **15d** some oxidized product of **15c** (**[15c]Ox**) was observed, most likely on the methionine residue.

ESI calculated for **15c** $C_{173}H_{257}N_{47}O_{54}S_6$ $[M+3H]^{3+}$ *m/z*: 1351.5 da, $[M+4H]^{4+}$ *m/z*: 1013.9 da, $[M+5H]^{5+}$ *m/z*: 811.3 da, found: 1351.4 da, 1013.9 da, 811.3 da.
ESI calculated for **15d** $C_{173}H_{255}N_{47}O_{53}S_6$ $[M+3H]^{3+}$ *m/z*: 1345.5, $[M+4H]^{4+}$ *m/z*: 1009.4, $[M+5H]^{5+}$ *m/z*: 807.7, found: 1345.6 da, 1009.4 da, 807.7 da.

Peptide 12



The peptidyl acid **11** (45 mg, 7.8 μmol) was solubilized in dichloromethane/methanol (9:1, 1.5 mL), cooled at 0 °C and TMS-Diazomethane (2 M solution in Et_2O, 40 μL, 80 μmol) was added. The solution was stirred at room temperature for 30 min and the peptide was precipited by ice-cold diethyl ether (3 x 45 mL) to give a white precipitate, which was centrifugated. The precipitate was solubilized in water/acetonitrile (1:1, 0.05% trifluoroacetic acid) and lyophilized. The peptide was subjected to cleavage with cocktail B (3 mL) for 90 min following the general procedure. The resulting solid was purified by RP-HPLC (C18 semiprep, 20% to 70% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 19.5 min. Lyophilization of the collected fractions provided peptide **12** (3 mg, 12%) as a white solid.

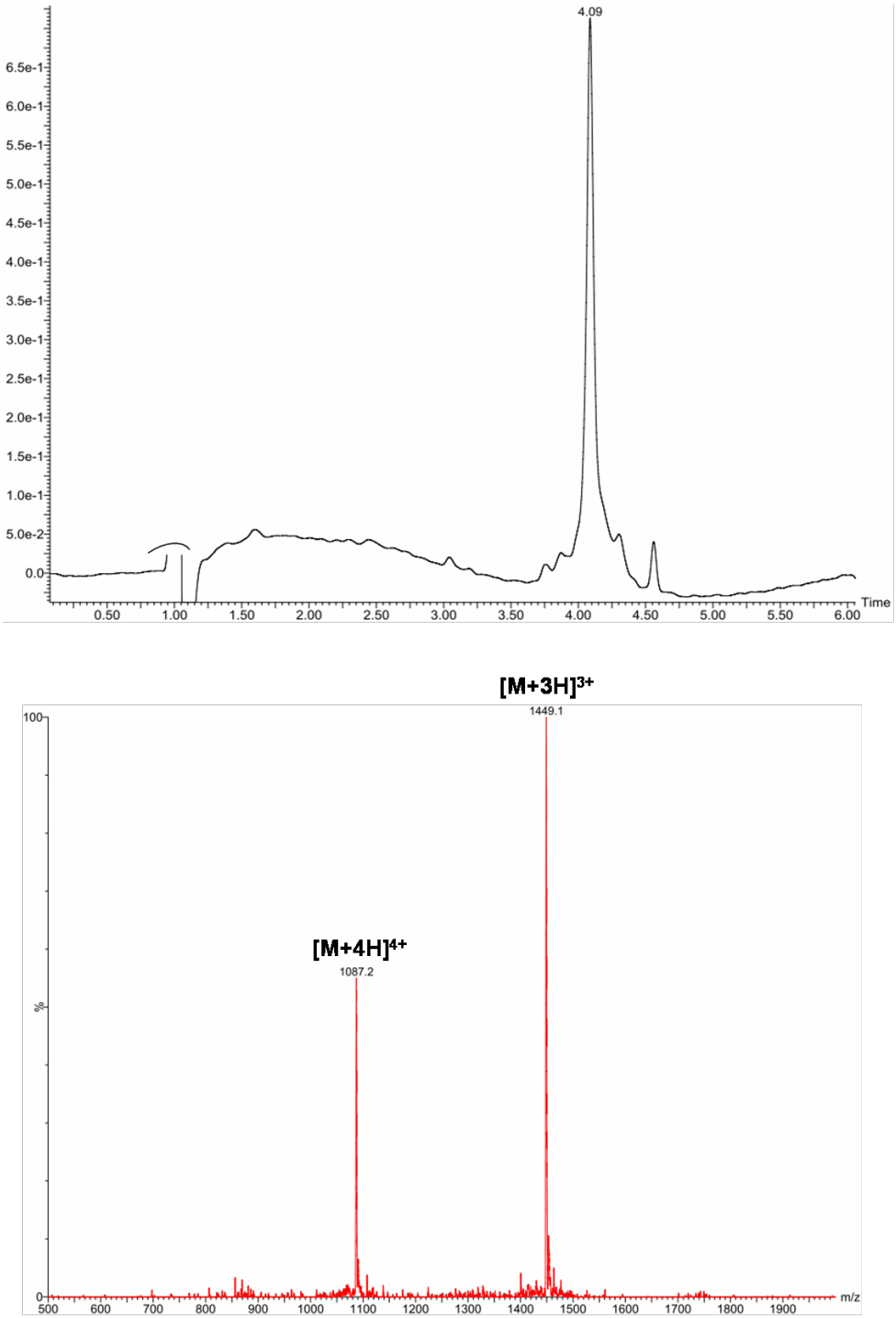


Figure 14: UV and MS traces from LC-MS analysis of peptide **12**: gradient: 10% to 60% CH_3CN/H_2O over 6 min at a flow rate of 0.3 mL/min, BEH C4 column. (b) ESI-MS of peptide **12.**

ESI calculated for $C_{188}H_{273}N_{47}O_{60}S_6$ $[M+3H]^{3+}$ *m/z*: 1449.0, $[M+4H]^{4+}$ *m/z*: 1087.0, found: 1449.1da, 1087.2 da.

Reference:

1. (a) Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678. (b) Yuan, Y.; Chen, J.; Wan, Q.; Tan, Z.; Chen, G.; Kan, C.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2009**, *131*, 5432.