Supporting Information

Total Synthesis of the α-Subunit of the Human Glycoprotein Hormones (hGPH): Toward Fully Synthetic Homogeneous Human Follicle-Stimulating Hormone (hFSH)

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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 diethyl ether, dichlormethane were obtained from a dry solvent system (passed through column of alumina) and used without further drying. NMR spectra (^{1}H and ^{13}C) were recorded on a Bruker AMX-400 MHz or Bruker Advance DRX-500 MHz, referenced to TMS or residual solvent. 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 E. Merck silica gel 60 (40–63 mm). Yields refer to chromatographically pure compounds.

HPLC

All separations involved a mobile phase of 0.05% TFA (v/v) in water (solvent A) /0.04% TFA in acetonitrile (solvent B).
HPLC LC-MS chromatographic separations were performed using a Waters 2695 Separations Module and a Waters 2996 Photodiode Array Detector equipped with Varian Microsorb C18 column (150 x 2 mm) or Waters C8 X-Bridge column (150 x 2.1 mm) or Varian 300-5 C4 column (250 x 2 mm) at a flow rate of 0.2 mL/min.
UPLC LC-MS chromatographic separations were performed using a Waters Acquity system equipped with an Acquity UPLC BEH C18 column (100 x 2.1 mm).
HPLC separations were performed using a Rainin HPXL solvent delivery system equipped with a Rainin UV-1 detector using either Microsorb 100-5 C18 column (250 x 21.4 mm) at a flow rate of 16 mL/min or Waters C8 X-Bridge column (150 x 19 mm) at a flow rate of 16 mL/min or Varian 300-5 C4 column (250 x 4.6 mm) at a flow rate of 1.4 mL/min.

I - General procedures

Solid-phase peptide synthesis by Fmoc-strategy

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous S3 flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc protocols. The deblock mixture was a mixture of 100:2:2 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-Cys(*t*Buthio)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gln(Dmcp)-OH, Fmoc-Glu(OAll)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(O*t*Bu)-OH, Fmoc-Thr(O*t*Bu)-OH, Fmoc-Thz-OH Fmoc-Tyr(O*t*Bu)-OH, Fmoc-Val-OH. The following didpeptide from NovaBiochem were used: Fmoc-Phe-Ser($\psi^{Me,Me}$Pro)-OH, Fmoc-Val-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Gly-(Dmb)Gly-OH. Upon completion of automated synthesis on a 0.1 mmol scale, the peptide resin was washed into a peptide synthesis vessel with MeOH. After drying the resin was subjected to a cleavage cocktail (1:1:3 of acetic acid/trifluoroethanol/methylene chloride) for 3 times 30 min (4 mL each) after filtration, the resin was infused with trifluoroethanol/chloroform (1:4, 4 mL) over night. The resulting cleavage and infusion solution were pooled and concentrated. The oily residue was resuspended in minimum amount of trifluoroethanol and precipitated with water. The resulting mixture was immediately lyophilized.

Acido labile protecting group removal: Cocktail B

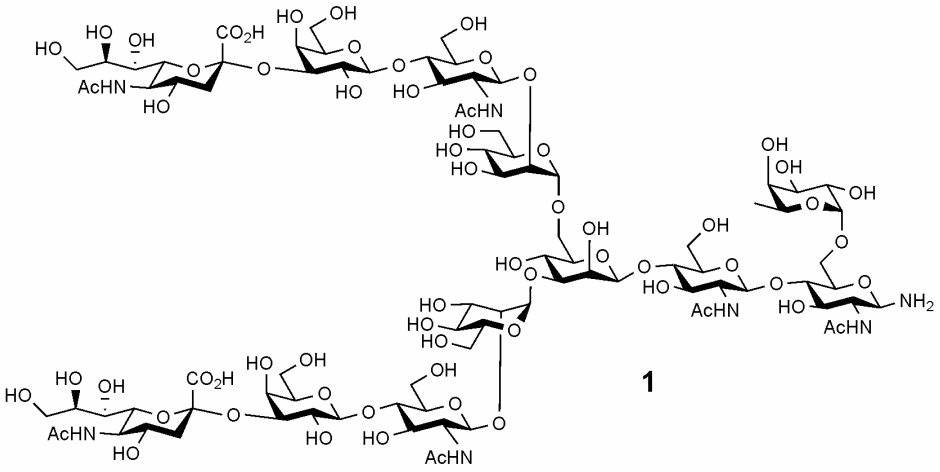
Peptides were subjected to cocktail B (3 mL / 100 mg of peptide) consisting of trifluoroacetic acid (88% by volume), water (5% by volume), phenol (5% by weight), and *i*Pr$_3$SiH (2% by volume). The resulting solution was concentrated and the oily residue was triturated in ice-cold diethyl ether (3 x 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 resulting solid was purified by HPLC.

Native chemical ligation buffer

The buffer required for native chemical ligation (NCL) was freshly prepared prior to the reaction. Na_2HPO_4 (56.6 mg, 0.4 mmol) was solubilized in water (1 mL), Guanidine·HCl (1.146 g, 12 mmol), and TCEP·HCl (10.8 mg, 0.04 mmol) were then added, solubilzed, the volume adjusted to 2 mL and the pH was brought to 7 with a solution of NaOH (5 M, 20 µL). After 15 min degassing with argon, 4-mercaptophenylacetic acid (MPAA) (67 mg, 0.4 mmol) was added and the pH was brought to 7.2 with a solution of NaOH (5 M, 125 µL). After 15 min degassing the solution was ready for use.

Glycan anomeric amine instalation (Kochetkov reaction)

Dodecasaccharide **2**[1] (11 mg, 4.23 μmol) was dissolved in water (5 mL) and added to $(NH_4)HCO_3$ (6g, BioUltra, 99.5% (T), Cat. No. 09830 Fluka). The resultant slurry was warmed to 40 °C and stirred very slowly at this temperature for three days. After three days, the clear supernatant was filtered through a plug of cotton. The remaining material was rinced with the same amount of cold water (2 x 5mL), filtered, pooled with the clear supernatant, immediately frozen and lyophilized. The remaining material was finally dissolved in water (5 mL), filtered through a plug of cotton, frozen and lyophilized. The lyophilization was deemed complete until the mass of the product remained constant. This provided a white solid, the dodecasaccharide glycosylamine **1** (11 mg, quantitative). **1** was kept at room temperature on the lyophilizer and used within 48 h after the completion of the lyophilization.

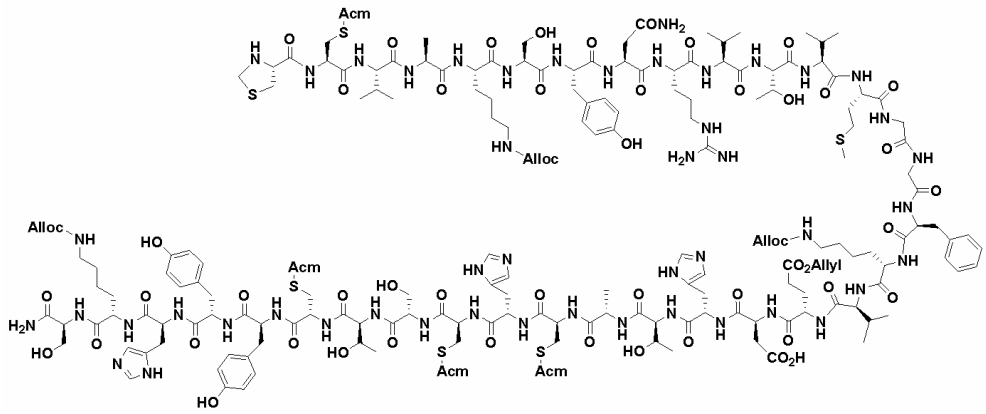


Exact mass calcd. for $C_{90}H_{149}N_7O_{65}$ $[M-2H]^{2-}$ 1182.9, Found 1182.8. MALDI-TOF calcd. for $C_{90}H_{149}N_7O_{65}$ $[M+Na]^+$ 2391.85, Found 2391.44.

[1] For characterization see: Nagorny, P.; Fasching, B.; Li, X.; Chen, G.; Aussedat, B.; Danishefsky, S. *J. Am. Chem. Soc.* **2009**, *131*, 5792-5799.

II - Compounds related to the first synthetic strategy for α-hGPH featuring chitobiose at glycosylation sites.

Compound 10, α-GPH[59-92]



Fmoc-PAL-PEG-PS resin (0.1 mmol) was used following the general SPPS procedure using dipeptides Fmoc-Gly-(Dmb)Gly-OH and Fmoc-Val-Thr($\psi^{Me,Me}$Pro)-OH at positions 73 and 69 respectively. The peptide-resin was subjected to cleavage with cocktail B (9 mL) for 90 min and then filtered. The resulting solution was concentrated and the oily residue was triturated with 45 ml ice-cold diethyl ether to give a white precipitate, which was centrifugated. This step was repeated twice. 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 15 min. Lyophilization of the collected fractions provided peptide **10** (74 mg, 17%) as a white solid.

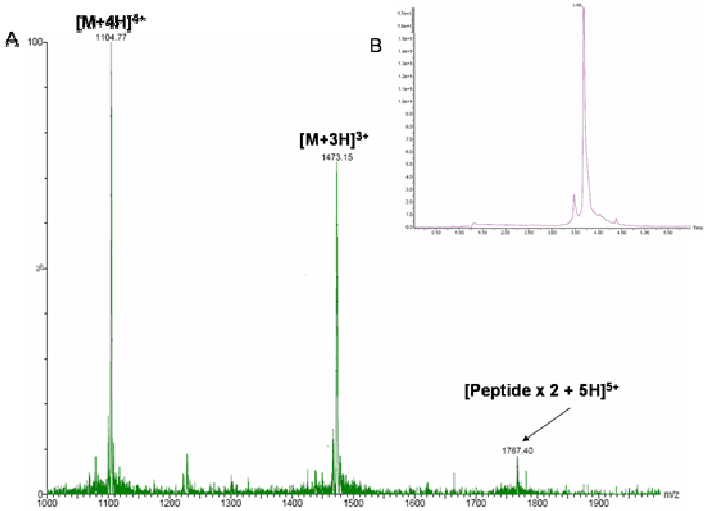
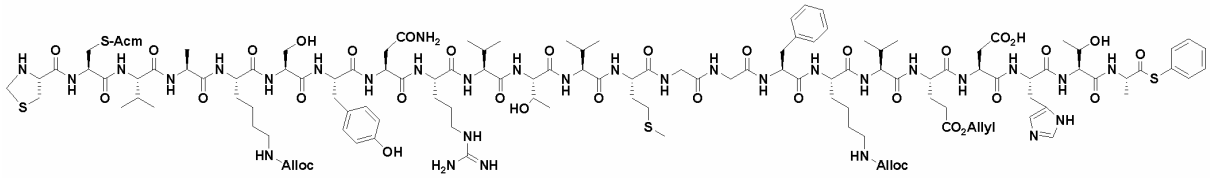


Figure S1: A - ESI-MS of compound **10**. ESI calculated for $C_{190}H_{284}N_{52}O_{58}S_6$ $[M+3H]^{3+}$ *m/z*: 1473.33, found: 1473.15; $[M+4H]^{4+}$ *m/z*:1105.24, found: 1104.77. **B** - UV traces from UPLC analysis of purified compound **10**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 15, α-GPH[59-81]



Peptide **13** was prepared from 0.2 mmol of Fmoc-Thr(*t*Bu)-NovaSyn® TGT resin following the general SPPS procedure using dipeptides Fmoc-Gly-(Dmb)Gly-OH and Fmoc-Val-Thr($\psi^{Me,Me}$Pro)-OH at positions 73 and 69 respectively, followed by cleavage from resin. The cleaved peptide was then purified on SiO_2 from 5% to 10% methanol in dichloromethane with 0.5% acetic acid. The fractions were concentrated and the resulting oil was solubilized in minimum amount of trifluoroethanol, precipitated with water and lyophilized to afford **13** (245 mg, 31%). To a solution of peptide **13** (84 mg, 21 µmol) and H-Ala-SPh·HCl **14** (18.3 mg, 84 µmol) in dichloromethane/chloroform (1:4, 6 mL) was added EDC (20.2 µL, 92 µmol) and HOOBt (20.5 mg, 84 µmol). The mixture was sonicated for 2 h. After concentration, the crude was treated with cocktail B (7 mL) following the general procedure. The crude peptide was resuspended in minimal amount of isopropanol and diluted in water/acetonitrile (1:1, 0.05% trifluoroacetic acid). The resulting solution was purified by RP-HPLC (C18 semiprep, 30% to 90% Acetonitrile/Water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 14 min. Lyophilization of the collected fractions provided peptide **15** (12 mg, 22%) as a white solid.

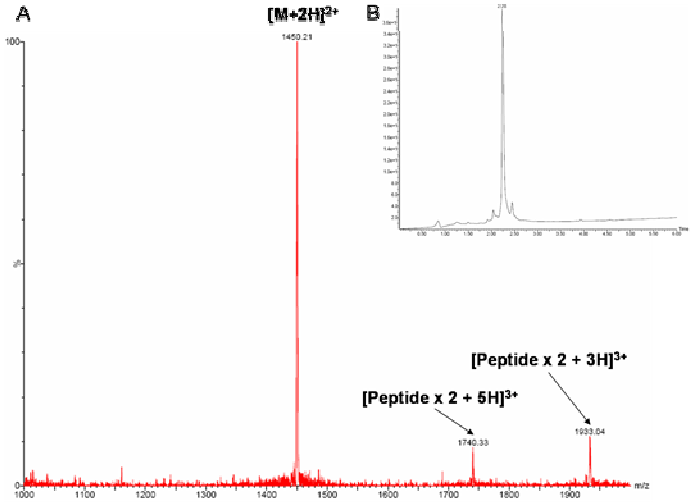
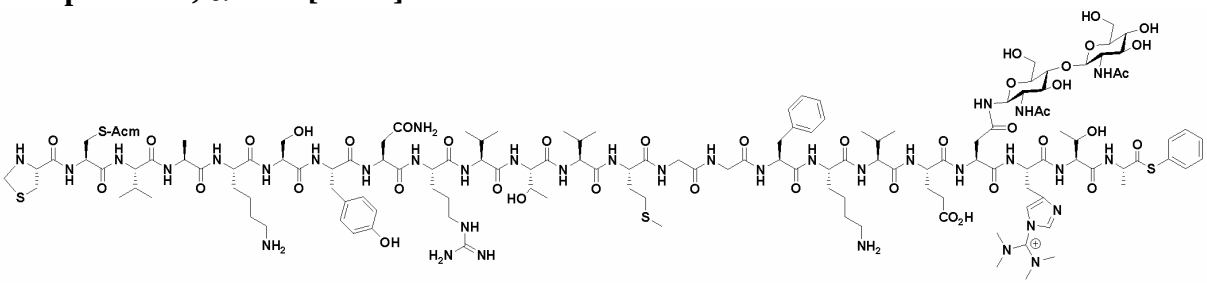


Figure S2: A - ESI-MS of compound **15**. ESI calculated for $C_{128}H_{192}N_{32}O_{37}S_4$ $[M+2H]^{2+}$ *m/z*: 1450.67, found:1450.21. **B** - UV traces from UPLC analysis of purified compound **15**; gradient: 30% to 90% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 16, α-GPH[59-81]



Peptide **15** (3.97 mg, 1.37 µmol) and chitobiose amine **4** (1.75 mg, 4.13 µmol) were combined and solubilized in anhydrous DMSO (125 µL). To this mixture, a freshly prepared solution of HATU in anhydrous DMSO (0.5mg/µL, 2.9 µL, 3.8 µmol)was added followed by DIEA (0.5 µL, 2. 87µmol). The solution immediately turned into a deep, golden-yellow color and was stired for 80 min. Once the reaction had reached completion, the mixture was diluted with DMSO (390 µL). To this was added Pd(dppf)Cl_2 (1.23 mg, 1.68 µmol) followed by phenylsilane (7 µL, 56.8 µmol). The reaction was stirred in the dark for 1 h and then quenched with ice-cold water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1.5 mL). The resulting supsension was immediately purified by RP-HPLC (C18 semiprep, 10% to 80% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 14 min. Lyophilization of the collected fractions provided peptide **16** (1.2 mg, 29%) as a white solid.

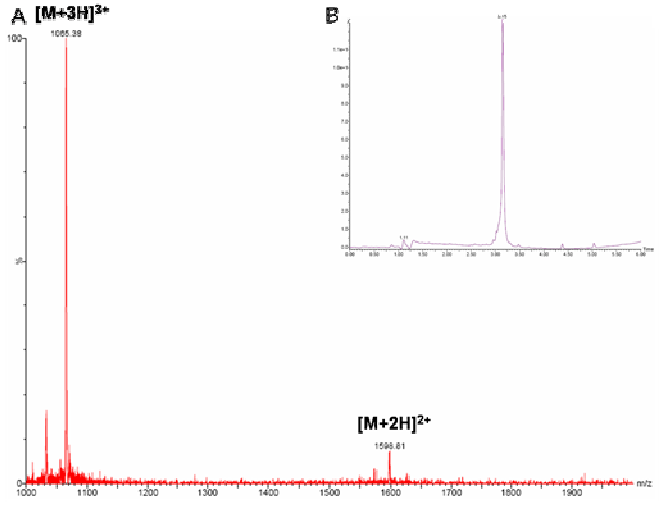
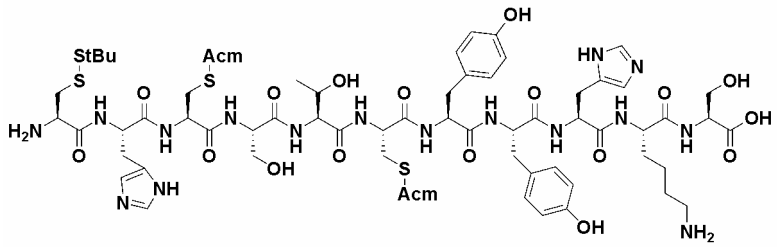


Figure S3: A - ESI-MS of compound **16**. ESI calculated for peptide + tetramethyluronium $C_{138}H_{218}N_{37}O_{42}S_4$ $[M+2H]^{2+}$ *m/z*: 1598.84, found: 1598.81, $[M+3H]^{3+}$ *m/z*: 1066.23, found: 1065.38. **B** - UV traces from UPLC analysis of purified compound **16**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 17, α-GPH[82-92]



Peptide **17** was prepared from 0.1 mmol of Fmoc-Ser(*t*Bu)-NovaSyn® TGT resin following the general procedure for SPPS. After cleavage from the resin, the peptide (130 mg, 53 µmol) was subjected to a deprotection with cocktail B (3.5 mL) for 90 min and treated as described in the general procedure. The resulting solid was purified by RP-HPLC (C18 semiprep, 10% to 80% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 10 min. Lyophilization of the collected fractions provided peptide **17** (59 mg, 71%) as a white solid.

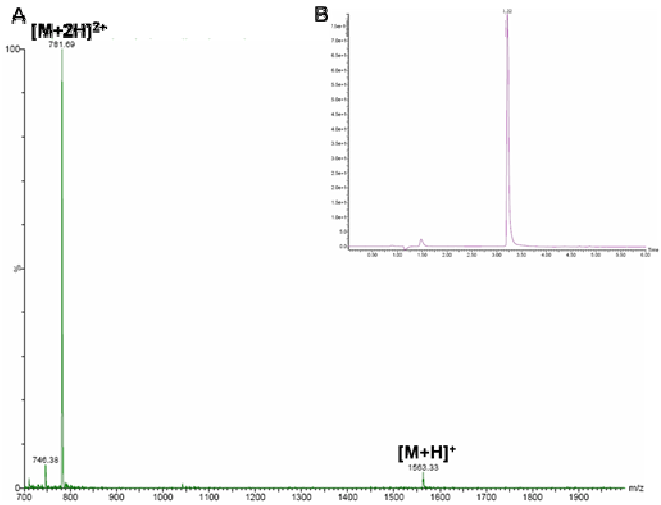
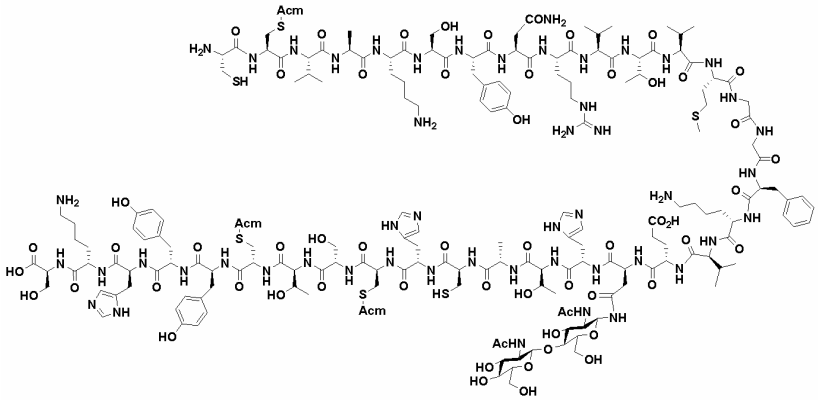


Figure S4: A - ESI-MS of compound **17**. ESI calculated for $C_{65}H_{96}N_{18}O_{19}S_4$ [M+H]+ *m/z*:1562.83, found: 1563.33; $[M+2H]^{2+}$ *m/z*:781.91, found:781.69. **B** - UV traces from UPLC analysis of purified compound **17**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 18, α-GPH[59-92]



Freshly purified peptides **17** (25.7 mg, 16.46 µmol) and **16** (26.3 mg, 8.23 µmol) were combined and solubilized into NCL buffer (1 mL), prepared as described in the general procedure. To this mixture was added neutral TCEP solution (0.5 M, 100 µL). The reaction was stirred for 3 h. After completion of the reaction, a solution of $MeONH_2$·HCl (0.6 M, 1.25 mL) and a neutral TCEP solution (0.5 M, 100 µL) were added, the pH was brought to 4-4.5 with HCl solution (2 M, 200 µL). The turbid mixture was stirred for 4h and then diluted dropwise with water/acetonitrile (1 mL, 1:1, 0.05% trifluoroacetic acid) and purified by RP-HPLC (C18 semiprep, 10% to 60% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 14 min. Lyophilization of the collected fractions provided peptide **18** (11.5 mg, 31% over 2 steps) as a white solid.

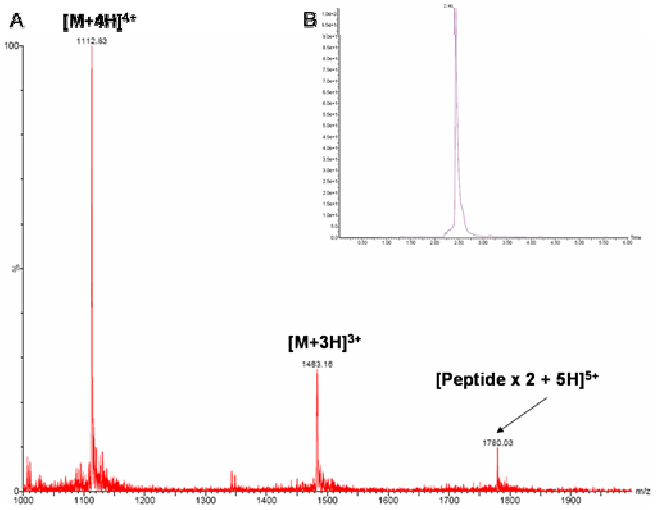
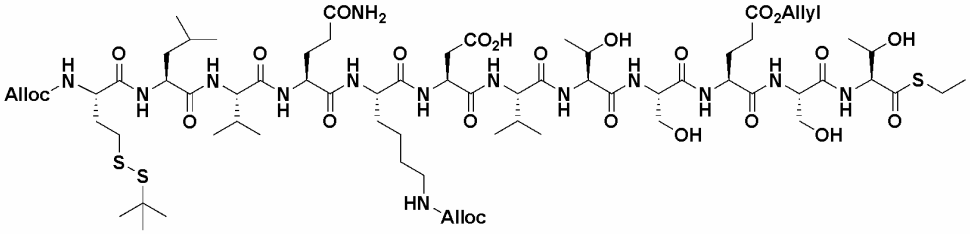


Figure S5: A - ESI-MS of compound **18**. ESI calculated for $C_{187}H_{289}N_{53}O_{61}S_6$ $[M+3H]^{3+}$ *m/z*: 1483.66, found: 1483.16; $[M+4H]^{4+}$ *m/z*: 1113.0, found: 1112.83. **B** - UV traces from UPLC analysis of purified compound **18**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 22, α-GPH[47-58]

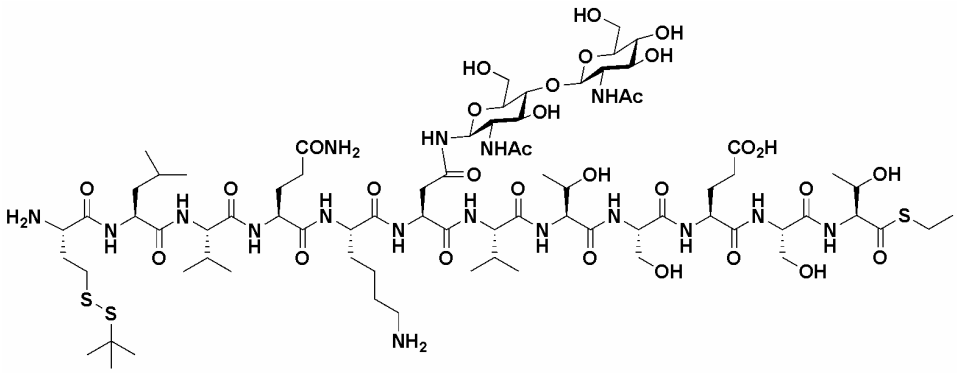


Peptide **22** was prepared from 0.1 mmol of Fmoc-Ser(*t*Bu)-NovaSyn® TGT resin following the general SPPS procedure, using Alloc-hCys(S*t*Bu)-OH **23** (146 mg, 74%) followed by cleavage from resin. To a solution of the resulting peptide (67 mg, 34 µmol) in trifluoroethanol/chloroform (1:4, 1 mL) was added EDC (13.2 µL, 84 µmol) and HOOBt

(13.8 mg, 84 µmol) and finally H-Thr-SEt·HCl **24** (20.2 mg, 101 µmol). The mixture was stirred for 2 h. After concentration the crude was treated with cocktail B (3.5 mL) for 80 min following the general procedure. The crude peptide was resuspended in minimal amount of DMSO and precipitated with ice-cold water with 0.05% trifluoroacetic acid. Centrifugation and lyophilization afforded **22** (44 mg) used as such in the following step.

ESI calculated for $C_{71}H_{118}N_{14}O_{25}S_3$ $[M+H]^+$ *m/z*: 1663.97, found: 1664.18

Compound 20, α-GPH[47-58]



Peptide **22** (13.2 mg, 7.9 µmol) and chitobiose amine **4** (10 mg, 23.6 µmol) were combined and solubilized in anhydrous DMSO (740 µL). To this mixture, a freshly prepared solution of HATU in anhydrous DMSO (0.5 mg/µL, 15.2 µL, 20 µmol) was added, followed by DIEA (2.8 µL, 16 µmol). The solution immediately turned into a deep, golden-yellow color and was stired for 130 min. Once the reaction had reached completion, the glycopeptide was precipitated with cold water with 0.05% trifluoroacetic acid and centrifuged (process repeated twice) and the precipitate was lyophilized. The crude glycopeptide was diluted with DMF (3 mL) then $Pd(dppf)Cl_2$ (1.54 mg, 2.1 µmol) was added and followed by phenylsilane (140 µL, 1.13 mmol). The reaction was stirred in the dark for 2 h and then quenched with ice-cold water/acetonitrile (2 mL, 9:1, 0.05% trifluoroacetic acid). The resulting supsension was filtered and immediately purified by RP-HPLC (C18 semiprep, 10% to 60% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 17 min. Lyophilization of the collected fractions provided peptide **20** (5.1 mg, 35% over two steps) as a white solid.

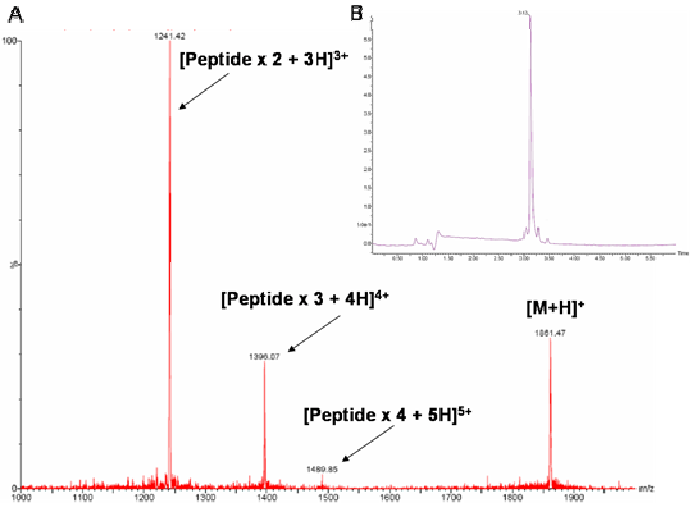
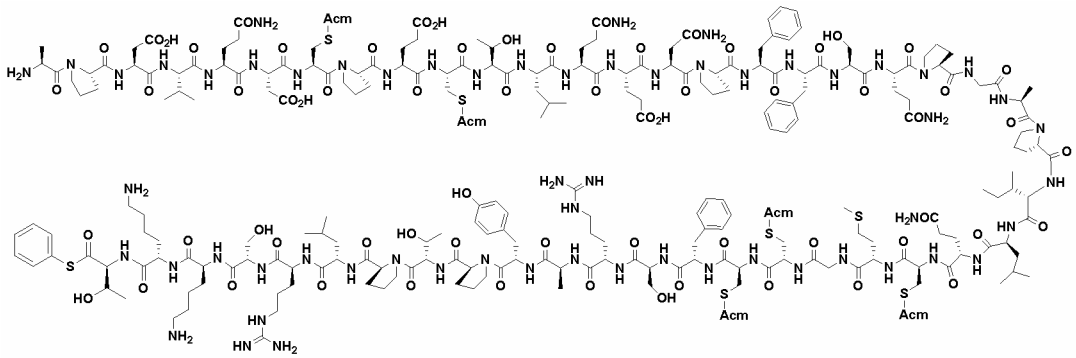


Figure S6: A - ESI-MS of compound **20**. ESI calculated for $C_{76}H_{133}N_{17}O_{30}S_3$ $[M+H]^+$ *m/z*: 1861.16 , found: 1861.47. **B** - UV traces from UPLC analysis of purified compound **20**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 19, α-GPH[1-46]



Peptide **19** was prepared from 0.1 mmol of Fmoc-Lys(Boc)-NovaSyn® TGT resin following the general SPPS procedure using dipeptides Fmoc-Phe-Ser($\psi^{Me,Me}$Pro)-OH at position 34 to afford the fully protected off-resin peptide (525 mg, 67%). To a solution of this peptide (104 mg, 13.2 µmol) in dichloromethane (630 µL) was added EDC (5.9 µL, 33 µmol) and HOOBt (5.4 mg, 33 µmol) and finally H-Thr-SPh·HCl **21** (9.6 mg, 39 µmol). The mixture was stirred for 105 min. After concentration the crude was treated for 80 min with cocktail B (3 mL) following the general procedure. The crude peptide was purified by RP-HPLC (C18 semiprep, 20% to 65% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 14 min. Lyophilization of the collected fractions provided peptide **19** (41 mg, 54%) as a white solid.

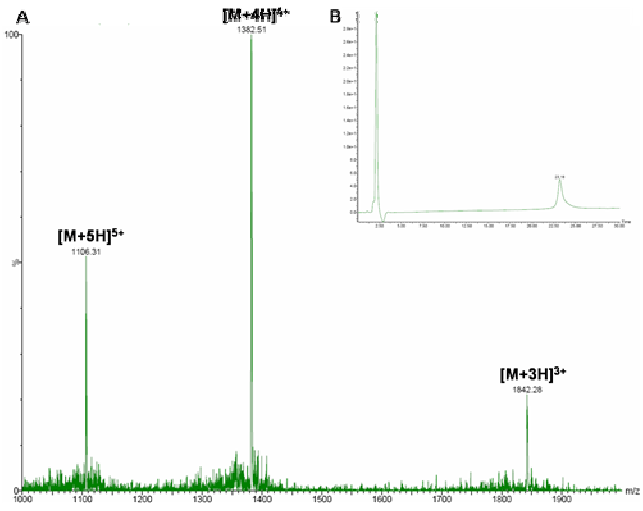
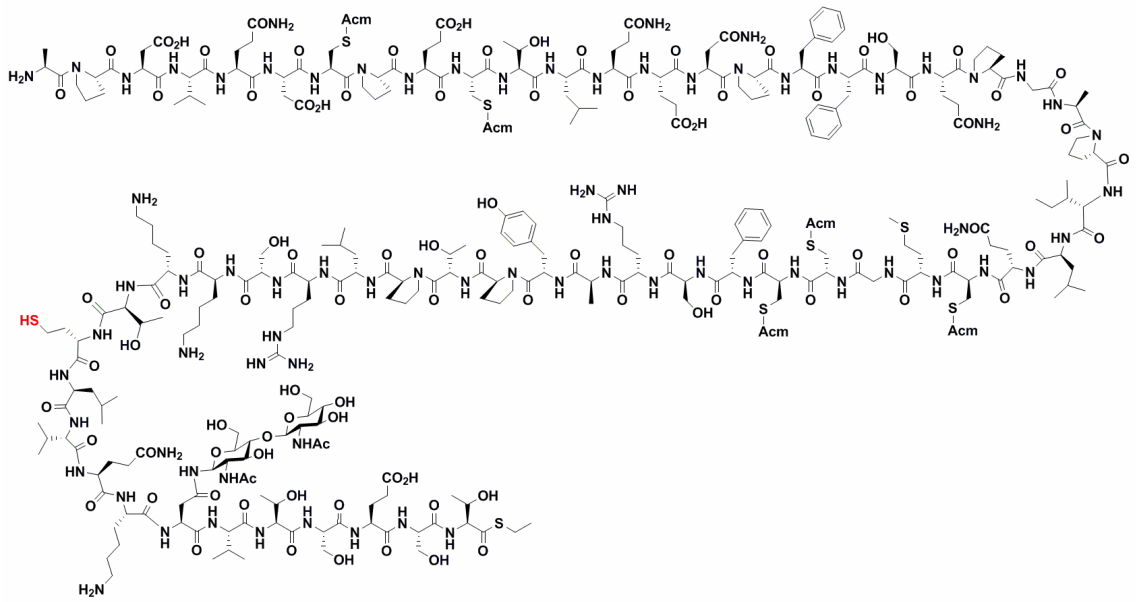


Figure S7: A - ESI-MS of compound **19**. ESI calculated for $C_{241}H_{370}N_{64}O_{71}S_7$ $[M+3H]^{3+}$ *m/z*: 1842.45, found: 1842.77; $[M+4H]^{4+}$ *m/z*: 1382.09, found: 1382.69; $[M+5H]^{5+}$ *m/z*: 1105.87, found: 1106.24. **B** - UV traces from HPLC analysis of purified compound **19**; gradient: 10% to 60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

Compound 25, α-GPH[1-58]



Peptide **20** (9.4 mg, 5 µmol) and **19** (27.3 mg, 5 µmol) freshly purified were combined and solubilized into NCL buffer (642 µL), prepared as described in the general procedure. To this mixture was added neutral TCEP solution (0.5 M, 72 µL). After 5 h the mixture was diluted dropwise with water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1.5 mL) and neutral TCEP solution (0.5 M, 100 µL). This mixture was stirred for 10 min and prepurified by RP-HPLC (C18 semiprep, 20% to 50% acetonitrile/water over 45 min, 16 mL/min, λ = 230 nm). After lyophilization, the resulting peptide was solubilized in DMSO (400 µL) to which was added ethanethiol (100µL, 1.38 mmol) followed by DIEA (10 µL, 57.5 µmol) for 4 h. The mixture was diluted dropwise with cold water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1.5 mL) and purified by RP-HPLC (C18 semiprep, 20% to 50% acetonitrile/water over 45 min, 16

mL/min, λ = 230 nm). Product eluted at 27 min. Lyophilization of the collected fractions provided peptide **25** (13 mg, 36%) as a white solid.

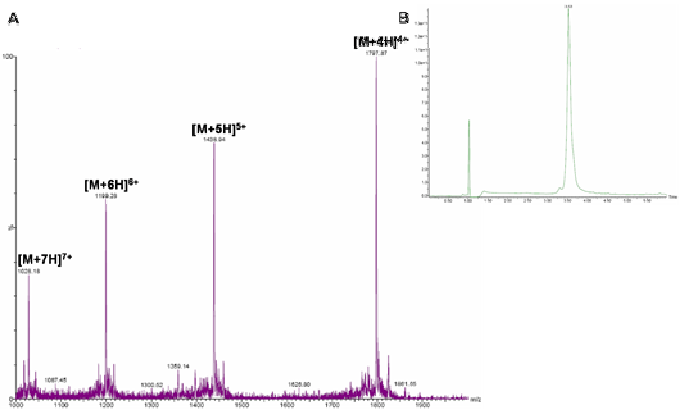
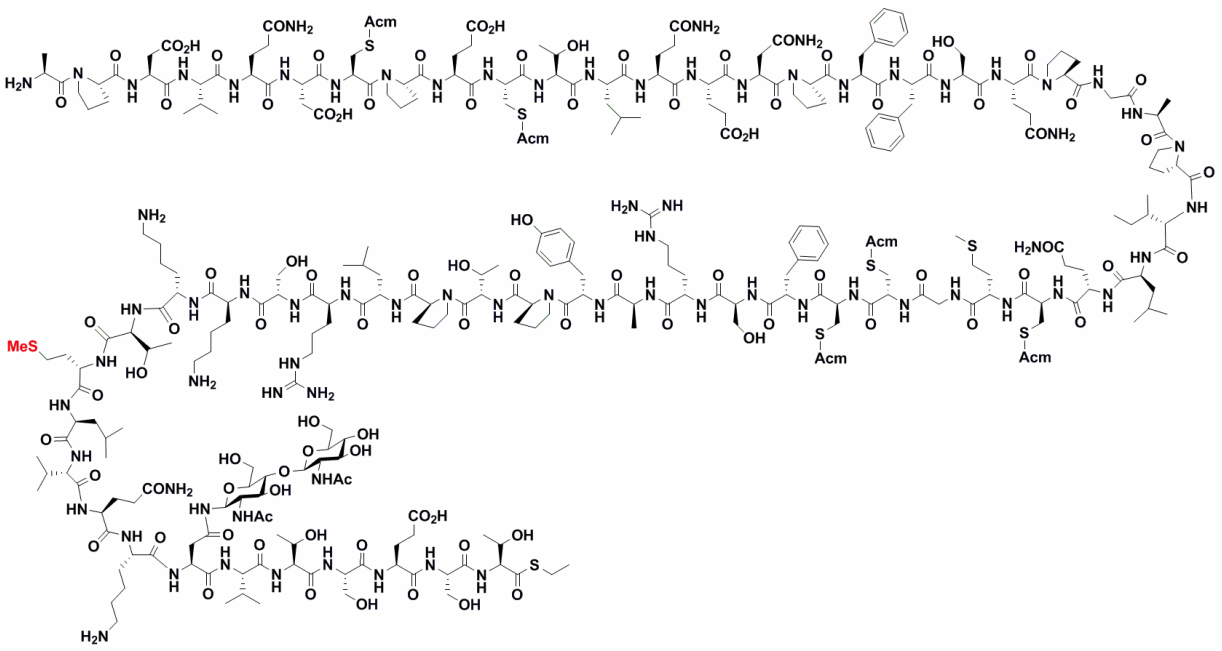


Figure S8: A - ESI-MS of compound **25**. ESI calculated for $C_{307}H_{489}N_{81}O_{101}S_8$ $[M+3H]^{3+}$ *m/z*: 2396.72, found: 2396.52; $[M+4H]^{4+}$ *m/z*: 1797.79, found: 1797.87 ; $[M+5H]^{5+}$ *m/z*: 1438.43, found: 1438.94; $[M+6H]^{6+}$ *m/z*: 1198.86, found: 1199.28; $[M+7H]^{7+}$ *m/z*: 1027.73, found: 1028.18. **B** - UV traces from HPLC analysis of purified compound **25**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 6, α-GPH[1-58]



Peptide **25** (4 mg, 0.55 µmol) was solubilized in buffer (100 mM $NaHCO_3$, 5 mM TCEP·HCl, pH = 8.6, 802 µL) after 20 sec of vigorous stirring a soultion of MeI in DMF was added (10 M, 43 µL) and the stirring maintained for 2 min. A solution of ethanethiol in DMF (2 M, 305 µL) was then added and stirred for 15 sec to quench the reaction. The mixture was then diluted with ice-cold water/acetonitrile (1:1, 1% trifluoroacetic acid, 2.2 mL) and immediately purified by RP-HPLC (C18 semiprep, 10% to 60% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 21 min. Lyophilization of the collected fractions provided peptide **6** (2 mg, 51%) as a white solid.

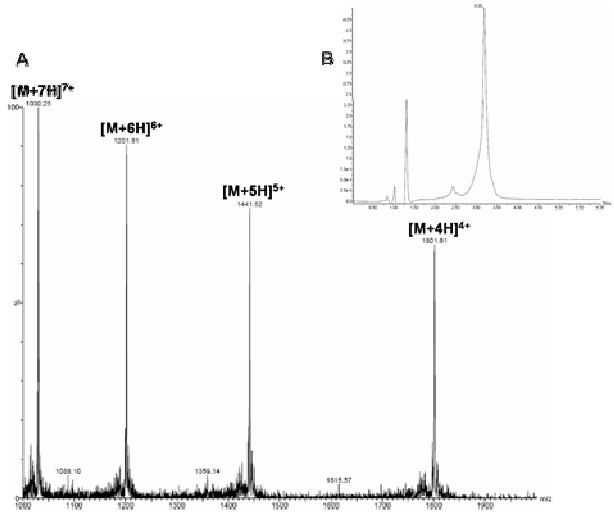
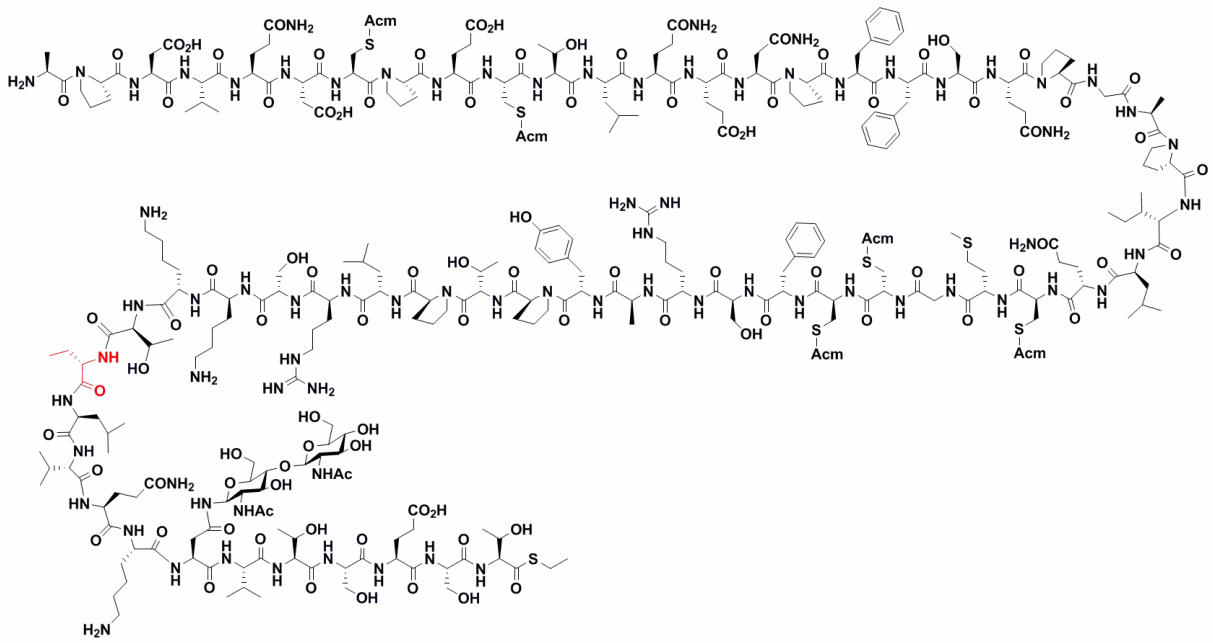


Figure S9: A - ESI-MS of compound **6**. ESI calculated for $C_{308}H_{491}N_{81}O_{101}S_8$ $[M+4H]^{4+}$ *m/z*: 1801.30, found: 1801.62; $[M+5H]^{5+}$ *m/z*: 1441.24, found: 1441.52; $[M+6H]^{6+}$ *m/z*: 1201.20, found: 1201.51; $[M+7H]^{7+}$ *m/z*: 1029.74, found: 1030.26. **B** - UV traces from UPLC analysis of purified compound **6**; gradient: 25% to 45% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 28, α-GPH[1-58]



Peptide **25** (0.3 mg, 0.04 µmol) was solubilized in water/acetonitrile/DMF/EtSH (22:44:33:1, 150 µL). To this mixture was added neutral TCEP solution (0.5 M, 72 µL), tBuSH (5µL) and VA-044 (0.1 M, 7.5 µL). This mixture was warmed to 37 °C and checked for product formation by LC-MS. After 18h no starting material was detected. The LC-MS trace showed an additional peak corresponding to [M+16], wich is likely to be the product of the Met29 side chain (**28(Ox)**).

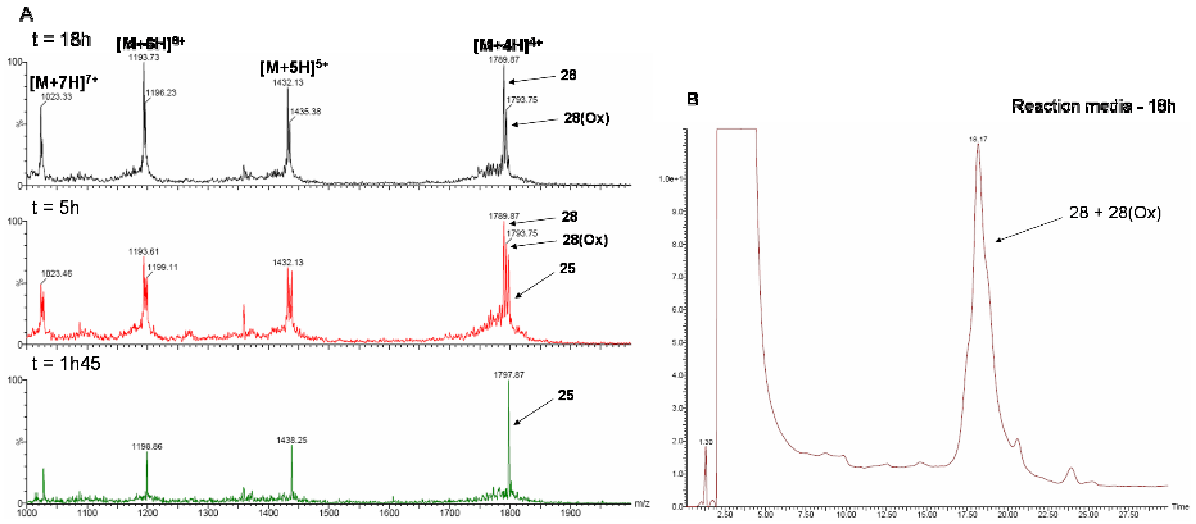


Figure S10: A - ESI-MS of the desulfurization reaction. ESI calculated for **28** $C_{307}H_{489}N_{81}O_{101}S_7$ $[M+4H]^{4+}$ *m/z*: 1789.7,7 found: 1789.87; $[M+5H]^{5+}$ *m/z*: 1432.02, found: 1432.13; $[M+6H]^{6+}$ *m/z*: 1193.51, found: 1193.61; $[M+7H]^{7+}$ *m/z*: 1023.15, found: 1023.33. ESI calculated for **28(Ox)** $C_{307}H_{489}N_{81}O_{102}S_7$ $[M+4H]^{4+}$ *m/z*: 1793.77, found: 1793.75; $[M+5H]^{5+}$ *m/z*: 1435.22, found: 1435.38; $[M+6H]^{6+}$ *m/z*: 1196.18, found: 1196.23; $[M+7H]^{7+}$ *m/z*: 1025.44, found: 1023.58.**B** - UV traces from HPLC analysis of reaction media; gradient: 25% to 35% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

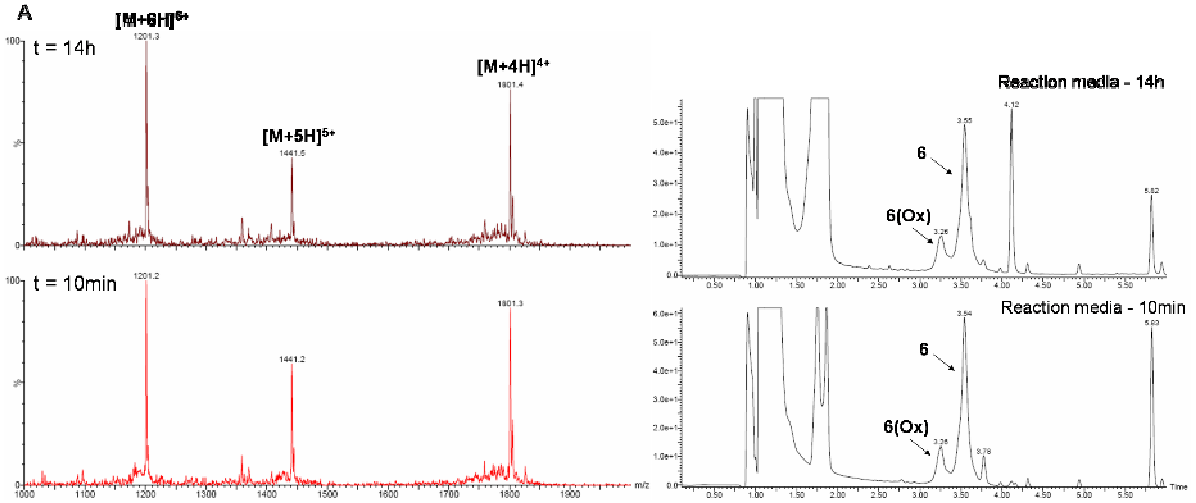
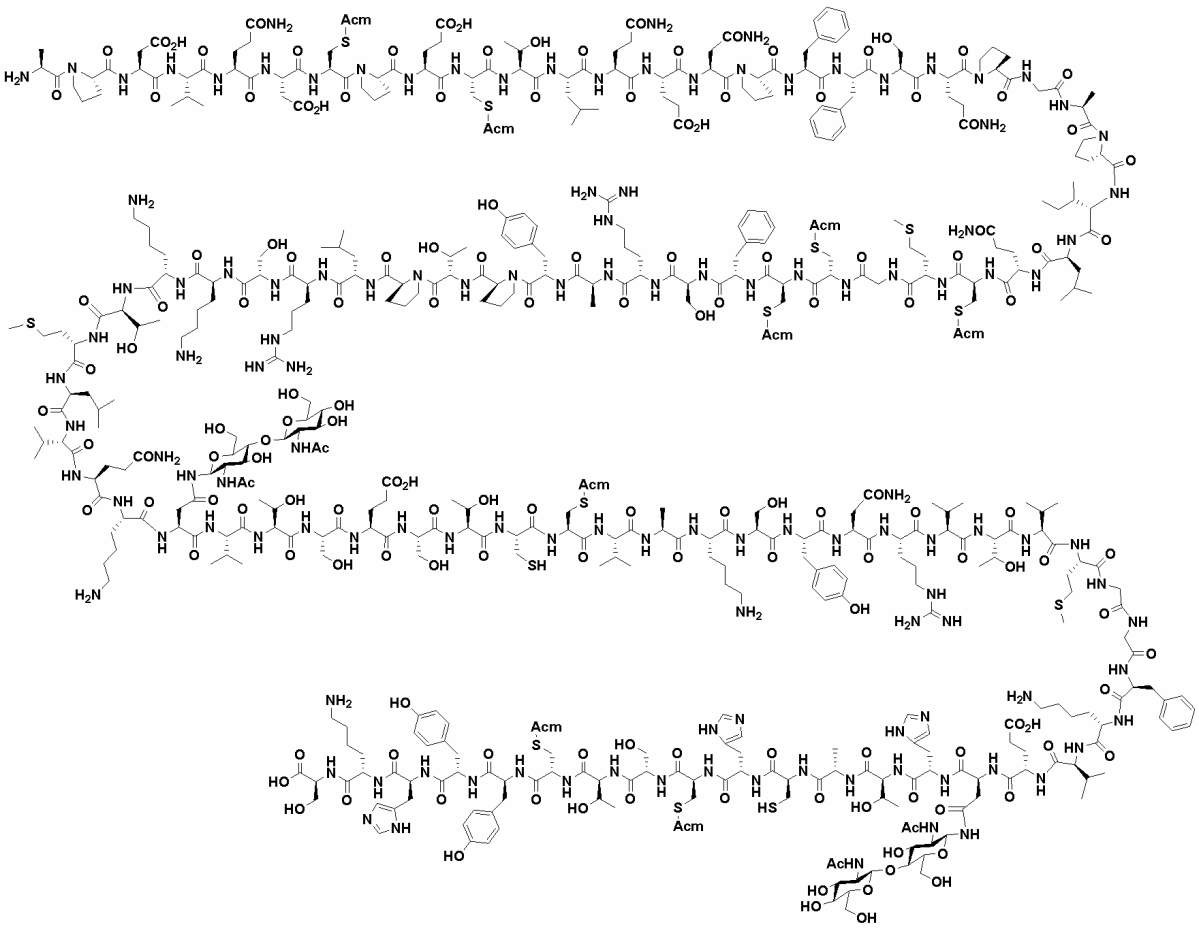


Figure S11: A - ESI-MS of compound **6** after 10min and 14h. ESI calculated for $C_{308}H_{491}N_{81}O_{101}S_8$ $[M+4H]^{4+}$ *m/z*: 1801.30, found: 1801.40; $[M+5H]^{5+}$ *m/z*: 1441.24, found: 1441.50; $[M+6H]^{6+}$ *m/z*: 1201.20, found: 1201.3. - UV traces from UPLC analysis of reaction media after 10min and 14h; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Peptide **6** (0.3 mg, 0.04 µmol) was solubilized in water/acetonitrile/DMF/EtSH (22:44:33:1, 150 µL). To this mixture was added neutral TCEP solution (0.5 M, 72 µL), tBuSH (5µL) and VA-044 (0.1 M, 7.5 µL). This mixture was warmed to 37 °C and checked for product formation by LC-MS. Besides **6** and **6(Ox)** (corresponding to [M+16], wich is likely to be the product of the Met29 side chain), no mass were detected for the other peaks.

Compound 29, α-GPH[1-92]



Peptide **18** (1.3 mg, 0.29 µmol) and **6** (1.9 mg, 0.26 µmol) freshly purified were combined and solubilized into NCL buffer (36 µL) prepared as described in the general procedure. To this mixture was added neutral TCEP solution (0.5 M, 4 µL). After 14 h, another portion of neutral TCEP solution (0.5 M, 2 µL) was added and the reaction was stirred for another 4 h. After completion of the reaction, the mixture was diluted dropwise with water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 0.5 mL) and neutral TCEP solution (0.5 M, 20 µL). This mixture was stirred for 10 min and desalted on biogel (Bio-Rad P4 Medium) equilibrated with water/acetonitrile (4:1, 0.05% trifluoroacetic acid) and then purified by RP-HPLC (C18 semiprep, 20% to 55% acetonitrile/water over 45 min, 16 mL/min, λ = 230 nm). Product eluted at 20 min. Lyophilization of the collected fractions provided peptide **29** (0.6 mg, 20%) as a white solid.

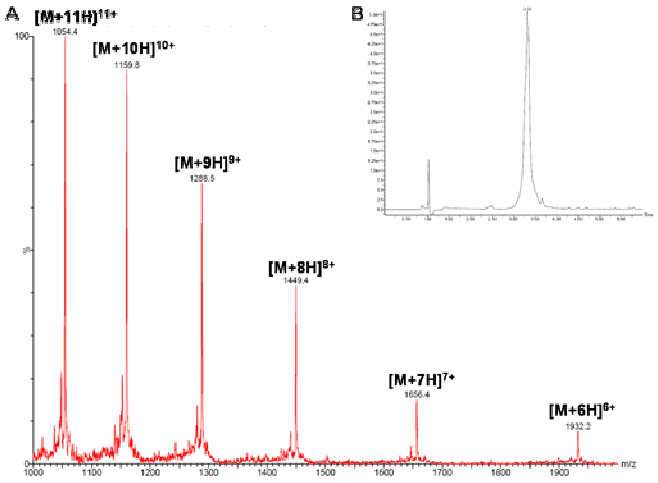
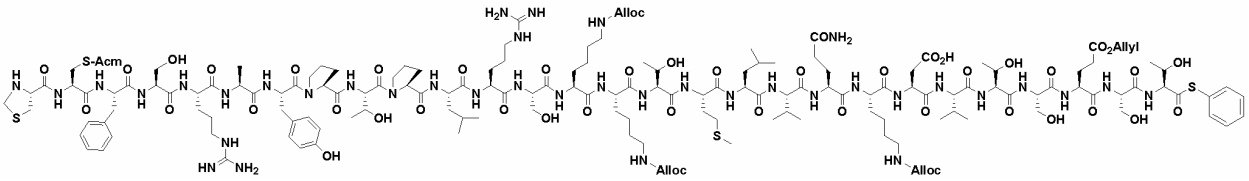


Figure S12: ESI-MS of compound **29**. ESI calculated for $C_{493}H_{774}N_{134}O_{162}S_{13}$ $[M+6H]^{6+}$ *m/z*: 1932.17, found: 1932.2; $[M+7H]^{7+}$ *m/z*: 1656.29, found: 1656.40, $[M+8H]^{8+}$ *m/z*: 1449.38, found: 1449.4; $[M+9H]^{9+}$ *m/z*: 1288.45, found: 1288.50; $[M+10H]^{10+}$ *m/z*: 1159.70, found: 1159.80, $[M+11H]^{11+}$ *m/z*: 1054.37, found: 1054.40.
UV traces from HPLC analysis of purified compound **29**; gradient: 30% to 80% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C8 X-Bridge column.

III - Compounds related to the second synthetic strategy for α-hGPH featuring chitobiose at glycosylation sites.

Compound 33, α-GPH[31-58]



Peptide **33** was prepared from 0.2 mmol of Fmoc-Ser(*t*Bu)-NovaSyn® TGT resin following the general SPPS procedure using Fmoc-Gln(Dmcp)-OH and dipeptides Fmoc-Phe-Ser($\psi^{Me,Me}$Pro)-OH at positions 50 and 34 respectively, to afford the fully protected off-resin peptide (714 mg, 75%). To a solution of this peptide (238 mg, 49.5 µmol) in trifluoroethanol/chloroform (1:9, 2.2 mL) precooled to 0°C was added EDC (21.5 µL, 121 µmol) and HOOBt (19.7 mg, 121 µmol) and finally H-Thr-SPh·HCl **21** (35.7 mg, 144 µmol). The mixture was stirred for 75 min at room temperature. After concentration the crude was treated for 90 min with cocktail B (6 mL) following the general procedure. The crude peptide was prepurified by RP-HPLC (C18 semiprep, 30% to 80% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 14 min. The product was then repurified on RP-HPLC (C8 X-Bridge semiprep, 30% to80% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 16 min. Lyophilization of the collected fractions provided peptide **33** (23 mg, 13%) as a white solid. (The low yield obtained is due to the double purification).

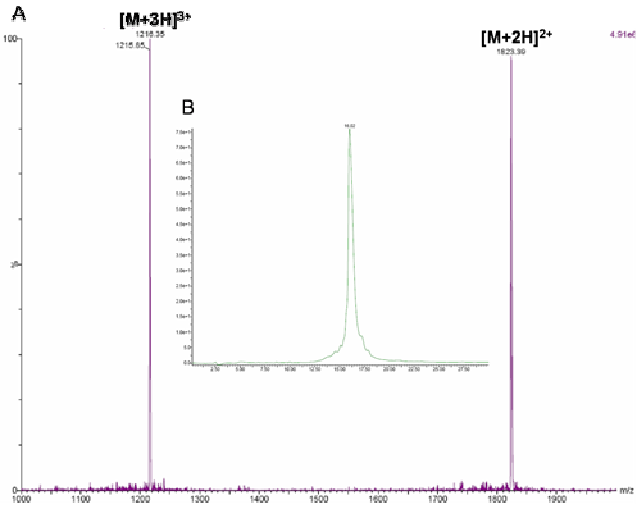
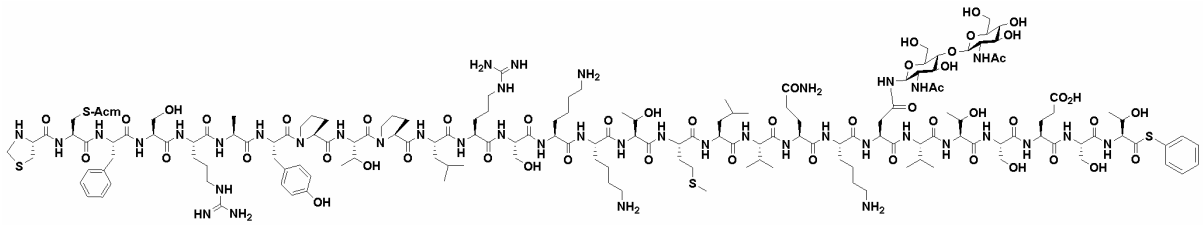


Figure S13: A - ESI-MS of compound **33**. ESI calculated for $C_{161}H_{251}N_{39}O_{49}S_4$ $[M+2H]^{2+}$ *m/z*: 1823.60, found: 1823.39; $[M+3H]^{3+}$ *m/z*: 1216.07, found: 1216.35. **B** - UV traces from HPLC analysis of purified compound **33**; gradient: 30% to 80% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C8 X-Bridge column.

Compound 31, α-GPH[31-58]



Peptide **33** (5mg, 1.37 µmol) and chitobiose amine **4** (1.75 mg, 4.13 µmol) were combined and solubilized in anhydrous DMSO (125 µL). To this mixture, a freshly prepared solution of HATU in anhydrous DMSO (0.5mg/µL, 2.9 µL, 3.8 µmol) was added, followed by DIEA (0.5 µL, 2.87 µmol). The solution immediately turned into a deep, golden-yellow color and was stired for 1h. Once the reaction had reached completion, the mixture was diluted with DMSO (390 µL). $Pd(dppf)Cl_2$ (1.23 mg, 1.68 µmol) was added, followed by phenylsilane (7 µL, 56.8 µmol). The reaction was stirred in the dark for 30 min and then quenched with ice-cold water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1 mL). The resulting supsension was filtered and immediately purified by RP-HPLC (C18 semiprep, 10% to 75% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 13 min. Lyophilization of the collected fractions provided peptide **31** (2.9 mg, 56%) as a white solid.

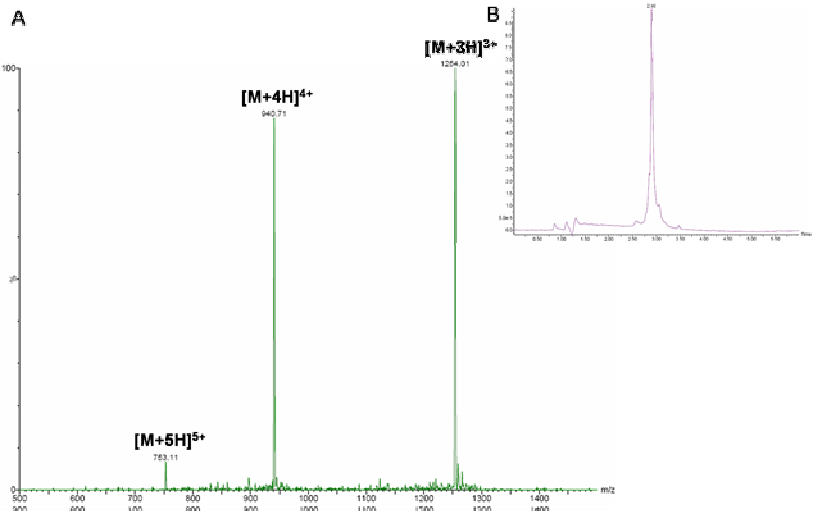
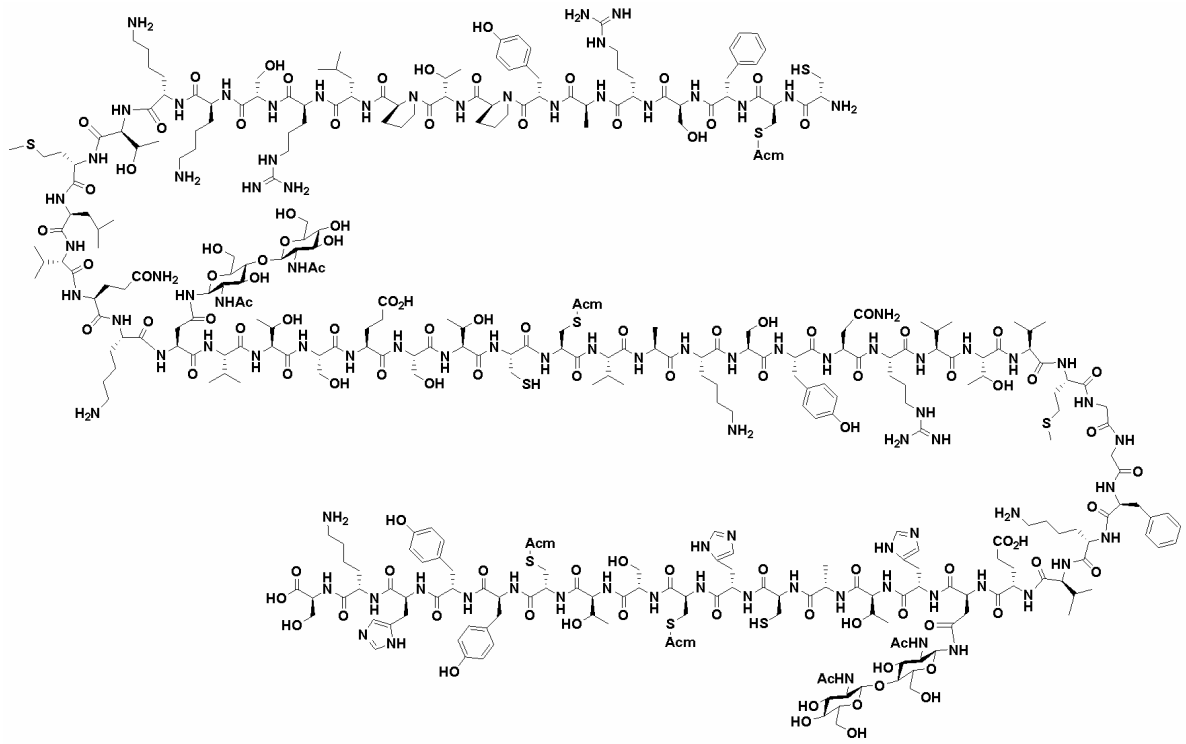


Figure S14: A - ESI-MS of compound **31**. ESI calculated for $C_{162}H_{262}N_{42}O_{52}S_4$ $[M+3H]^{3+}$ *m/z*: 1253.77, found: 1254.01; $[M+4H]^{4+}$ *m/z*: 940.58, found: 940.71; $[M+5H]^{5+}$ *m/z*: 752.66, found: 753.11. **B** - UV traces from UPLC analysis of purified compound **31**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 34, α-GPH[31-92]



Freshly purified peptides **18** (4.1 mg, 1.09 µmol) and **31** (4.8 mg, 1.085 µmol) were combined and solubilized into NCL buffer (149 µL), prepared as described in the general procedure. To this mixture was added neutral TCEP solution (0.5 M, 16 µL). After 225 min reaction another portion of neutral TCEP solution (0.5 M, 16 µL) was added and the raction was stirred for total 260 min. After completion of the reaction, a solution of $MeONH_2$·HCl (0.6 M, 200 µL) and neutral TCEP solution (0.5 M, 16 µL) were added, the pH was brought to 4-4.5 with HCl solution (2 M, 25 µL). The turbid mixture was stirred for 4 h and diluted dropwise with water/acetonitrile (1.5 mL, 1:1, 0.05% trifluoroacetic acid) and neutral TCEP solution (0.5 M, 100 µL). This mixture was stirred for 10 min and purified by RP-HPLC (C18 semiprep, 20% to 50% acetonitrile/water over 45 min, 16 mL/min, λ = 230 nm). Product eluted at 13 min. Lyophilization of the collected fractions provided peptide **34** (3.4 mg, 39% over 2 steps) as a white solid.

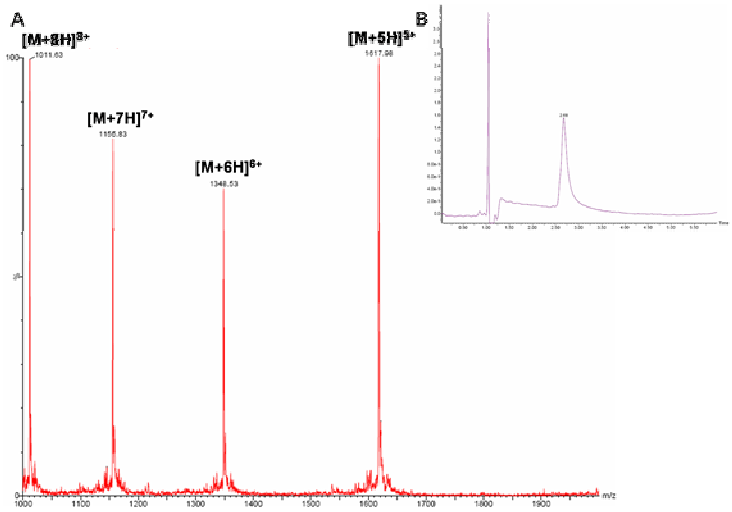
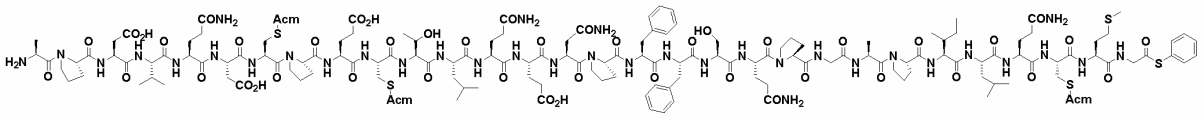


Figure S15: A - ESI-MS of compound **34**. ESI calculated for $C_{342}H_{545}N_{95}O_{113}S_9$ $[M+5H]^{5+}$ *m/z*: 1617.82, found: 1617.98; $[M+6H]^{6+}$ *m/z*: 1348.35, found: 1348.53; $[M+7H]^{7+}$ *m/z*: 1155.87, found: 1155.83; $[M+8H]^{8+}$ *m/z*: 1011.51, found: 1011.63. **B** - UV traces from UPLC analysis of purified compound **34**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 32, α-GPH[1-30]



Peptide **32** was prepared from 0.1 mmol of Fmoc-Gly-NovaSyn® TGT resin following the general SPPS procedure using dipeptide Fmoc-Phe-Ser($\psi^{Me,Me}$Pro)-OH at position 19. After cleavage, the peptide (170 mg, 34 µmol) was solubilized in dichloromethane (1.5 mL). PyBOP (174 mg, 340 µmol), DIEA (58 µL, 340 µmol) and thiophenol (102 µL, 1 mmol) were added. The reaction mixture was stirred for 105 min. After concentration, the resulting peptide was subjected to deprotection with cocktail B (3.5 mL) for 90 min and treated as described in the general procedure. The crude peptide was purified by RP-HPLC (C18 semiprep, 30% to 55% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 16 min. Lyophilization of the collected fractions provided peptide **32** (44 mg, 38% from the solid support synthesis) as a white solid.

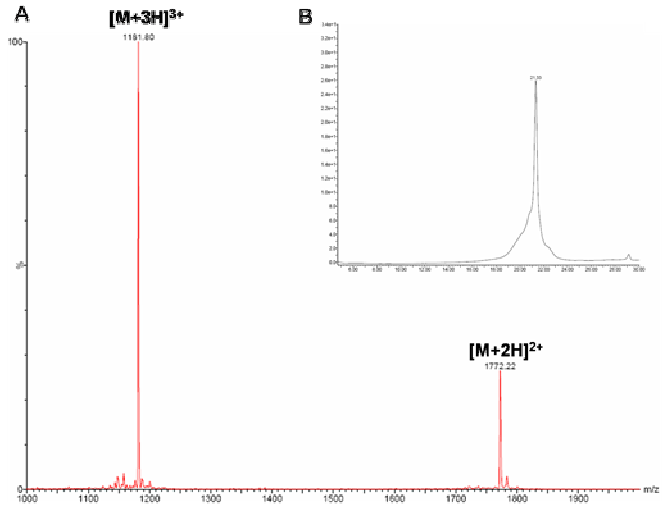
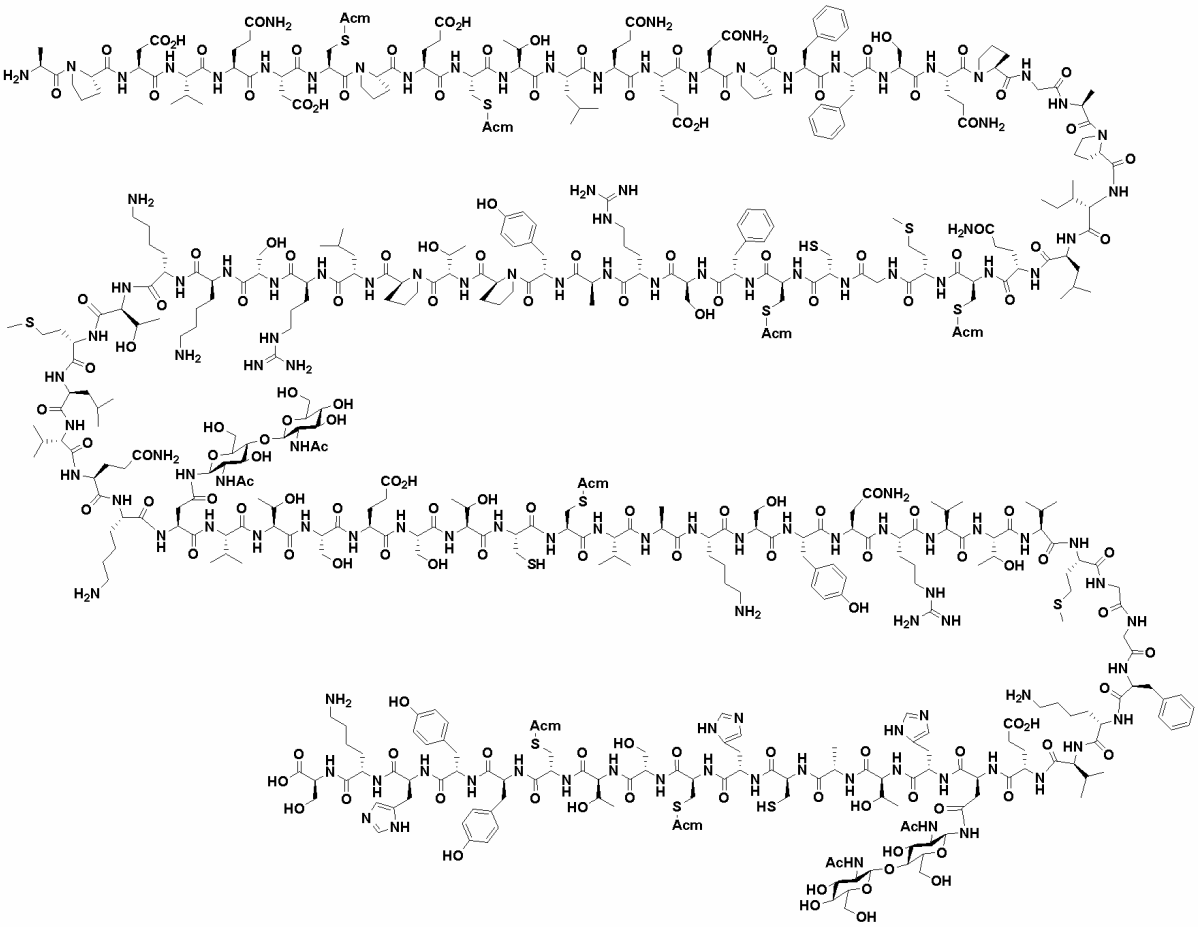


Figure S16: A - ESI-MS of compound **32**. ESI calculated for $C_{154}H_{230}N_{38}O_{48}S_5$ $[M+2H]^{2+}$ *m/z*: 1772.01, found: 1772.22 $[M+3H]^{3+}$ *m/z*: 1181.67, found: 1181.80. **B** - UV traces from HPLC analysis of purified compound **32**; gradient: 20% to 60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

Compound 30, α-GPH[1-92]



Freshly purified peptides **34** (3.5 mg, 0.432 µmol) and **32** (1.7 mg, 0.476 µmol) were combined and solubilized into NCL buffer (90 µL), prepared as described in the general procedure. To this mixture was added neutral TCEP solution (0.5 M, 10 µL). After 2 h

another portion of neutral TCEP solution (0.5 M, 10 µL) was added and the reaction was stirred for total 4 h. After completion of the reaction, the mixture was diluted dropwise with water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1.5 mL) and desalted on biogel (Bio-Rad P6 fine) equilibrated with water/acetonitrile (4:1, 0.05% trifluoroacetic acid) the resulting 5 mg were then purified by RP-HPLC (C18 semiprep, 20% to 50% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 15 min. Lyophilization of the collected fractions provided peptide **30** (1.56 mg, 31%) as a white solid.

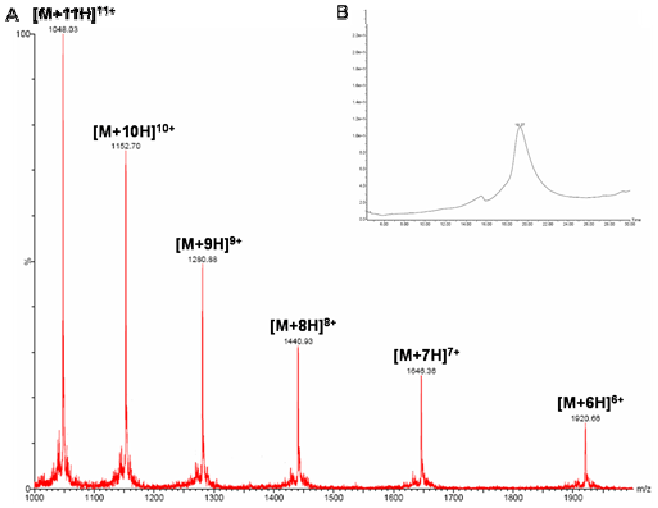
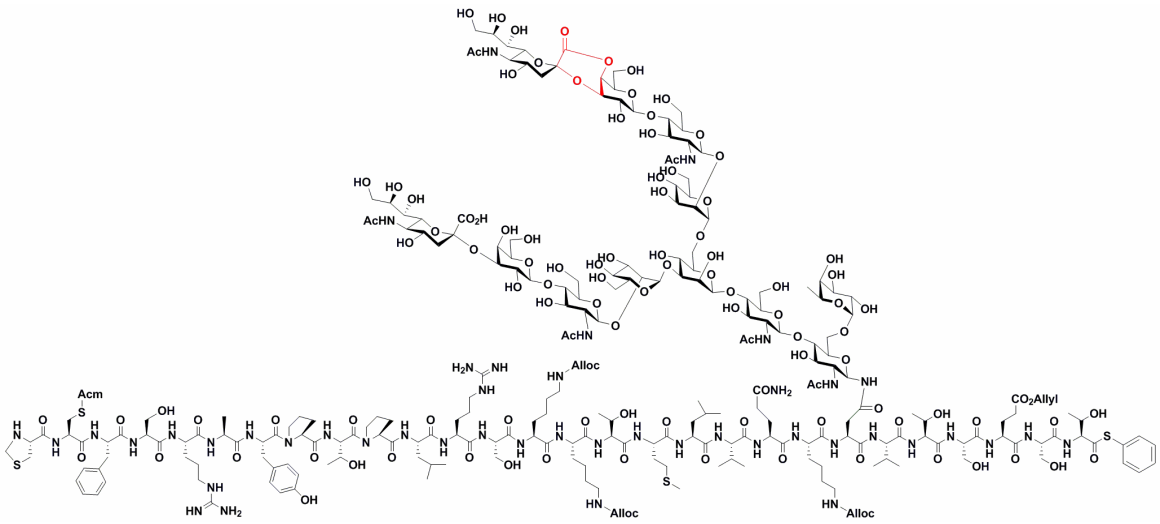


Figure S17: A - ESI-MS of compound **30**. ESI calculated for $C_{490}H_{769}N_{133}O_{161}S_{13}$ $[M+6H]^{6+}$ *m/z*: 1920.33, found: 1920.66; $[M+7H]^{7+}$ *m/z*: 1646.14, found: 1646.36; $[M+8H]^{8+}$ *m/z*: 1440.49, found: 1440.93; $[M+9H]^{9+}$ *m/z*: 1280.88, found: 1280.88; $[M+10H]^{10+}$ *m/z*:1152.59, found: 1152.70; $[M+11H]^{11+}$ *m/z*: 1047.90, found: 1148.03. **B** - UV traces from HPLC analysis of purified compound **30**; gradient: 20% to 50% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

IV - Compounds related to the synthetic strategy for α-hGPH featuring dodecasaccharide at glycosylation sites.

Compound 35, α-GPH[31-58]



Peptide **33** (6.4 mg, 1.75 µmol) and glycan amine **1** (1.4 mg, 0.59 µmol) were combined and solubilized in anhydrous DMSO (60 µL). To this mixture, a freshly prepared solution of HATU in anhydrous DMSO (0.5 mg/µL, 4.48 µL, 5.9 µmd) was added, followed by DIEA (1.32 µL, 7.6 µmol). The solution turned into a deep, golden-yellow color and this was stired for 1 h. Once the reaction had reached completion, the reaction mixture was diluted with ice-cold water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1.5 mL) and purified by RP-HPLC (C18 semiprep, 30% to 70% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 10 min. Lyophilization of the collected fractions provided peptide **35** as a white solid and as a mixture of monolactone and dilactone (1.45 mg, 42%, 42% average of five reactions, based on the amount of glycan amine used).

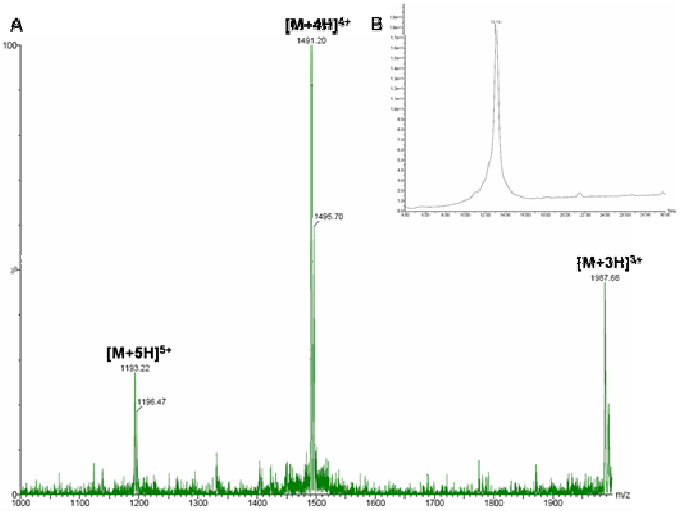
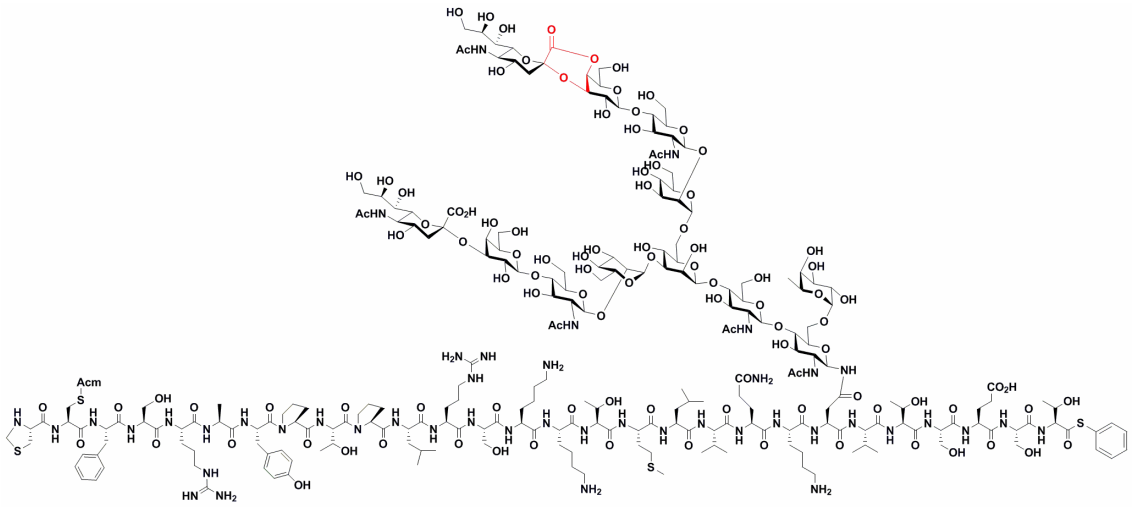


Figure S18: A - ESI-MS of compound **35**. ESI calculated for monolactone $C_{251}H_{396}N_{46}O_{112}S_4$ $[M+4H]^{4+}$ *m/z*: 1495.58, found: 1495.70; $[M+5H]^{5+}$ *m/z*:1196.66, found: 1196.47. ESI calculated for dilactone $C_{251}H_{394}N_{46}O_{111}S_4$ $[M+3H]^{3+}$ *m/z*: 1987.77, found: 1987.66; $[M+4H]^{4+}$ *m/z*: 1491.08, found: 1491.20; $[M+5H]^{5+}$ *m/z*:1193.06, found: 1193.22. **B** - UV traces from HPLC analysis of purified compound **35**; gradient: 30% to 70% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

Compound 36, α-GPH[31-58]



Glycopeptide **35** (2.3 mg, 0.38 µmol) was solubilized in DMSO (275 µL). A freshly prepared solution of $Pd(dppf)Cl_2$ in DMSO (10 mg/mL, 17 µL, 0.26 µmol) was added followed by phenylsilane (18.8 µL, 13.6 µmol). The reaction was stirred in the dark for 45 min and then quenched with ice-cold water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1 mL). The resulting supsension was prepurified on biogel (Bio-Rad P4 medium) equilibrated with water/acetonitrile (4:1, 0.05% trifluoroacetic acid) and the resulting desalted peptide was then purified by RP-HPLC (C18 semiprep, 10% to 60% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 16 min. Lyophilization of the collected fractions provided peptide **36** (0.65 mg, 30%) as a white solid.

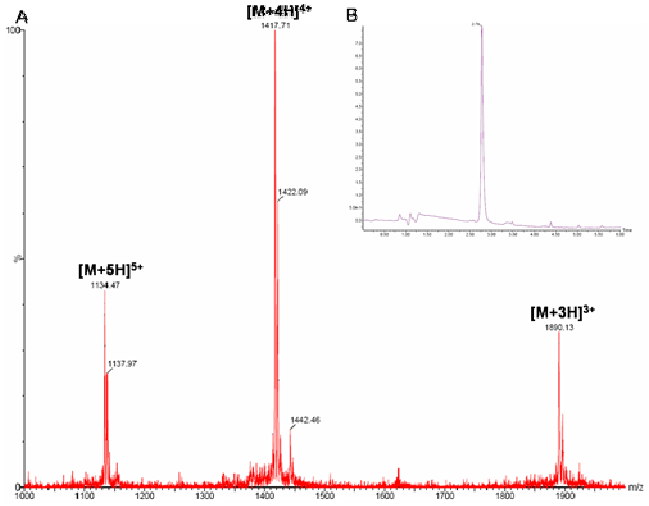
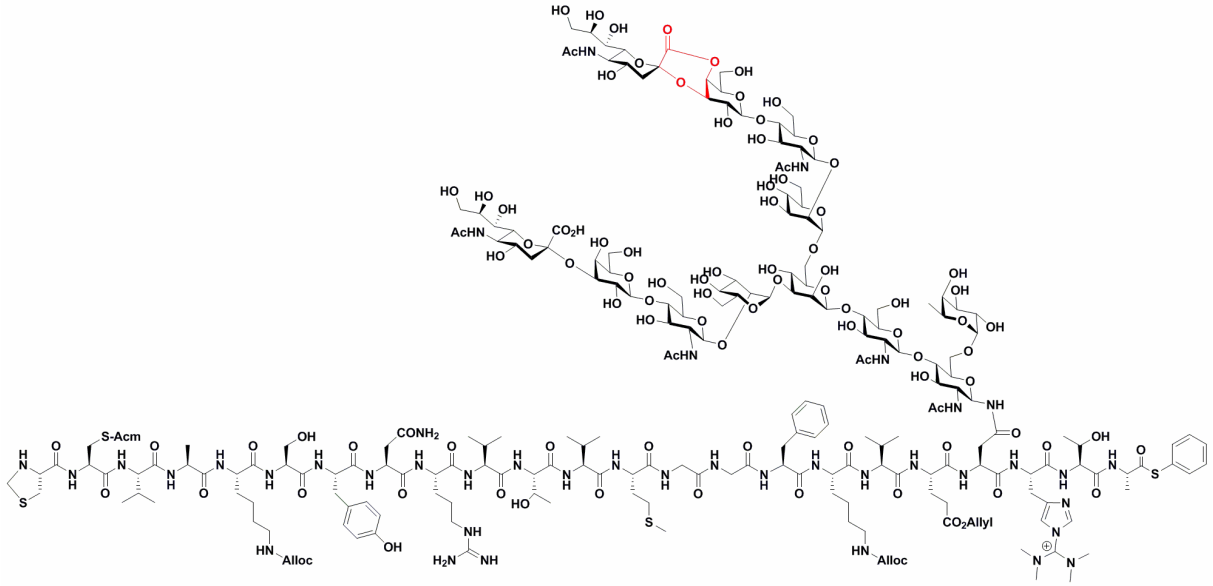


Figure S19: A - ESI-MS of compound **36**. ESI calculated for monolactone $C_{236}H_{380}N_{46}O_{106}S_4$ $[M+3H]^{3+}$ *m/z*: 1896.35, found: 1896.26; $[M+4H]^{4+}$ *m/z*: 1422.51, found: 1422.34 $[M+5H]^{5+}$ *m/z*: 1138.21, found: 1138.22. ESI calculated for dilactone $C_{236}H_{378}N_{46}O_{105}S_4$ $[M+3H]^{3+}$ *m/z*: 1890.34, found: 1890.38; $[M+4H]^{4+}$ *m/z*: 1418.00, found: 1418.09 $[M+5H]^{5+}$ *m/z*: 1134.60, found: 1134.35. **B** - UV traces from UPLC analysis of purified compound **36**; gradient: 10% to 60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

Compound 37, α-GPH[59-82]



Peptide **15** (5.1 mg, 1.77 µmol) and glycan amine **1** (1.4 mg, 0.59 µmol) were combined and solubilized in anhydrous DMSO (70 µL). To this mixture, a freshly prepared solution of HATU in anhydrous DMSO (0.5 mg/µL, 4.48 µL, 5.9 µmol) was added followed by DIEA (1.03 µL, 5.9 µmol). The solution turned into a deep, golden-yellow color and was stired for 1h. Once the reaction had reached completion, the reaction mixture was diluted with ice-cold water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1.5 mL) and purified by RP-HPLC (C18 semiprep, 30% to 90% acetonitrile/water over 30 min, 16 mL/min, λ = 265 nm). Product eluted at 7 min. Lyophilization of the collected fractions provided peptide **37** as a white solid and as a mixture of monolactone and dilactone (0.8 mg, 26% based on the amount of glycan amine used).

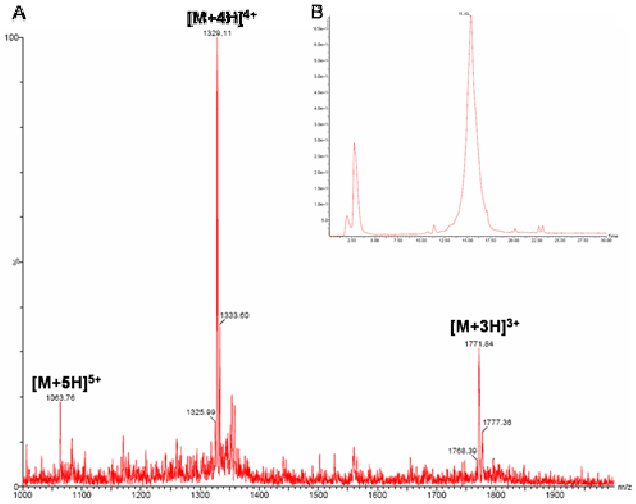
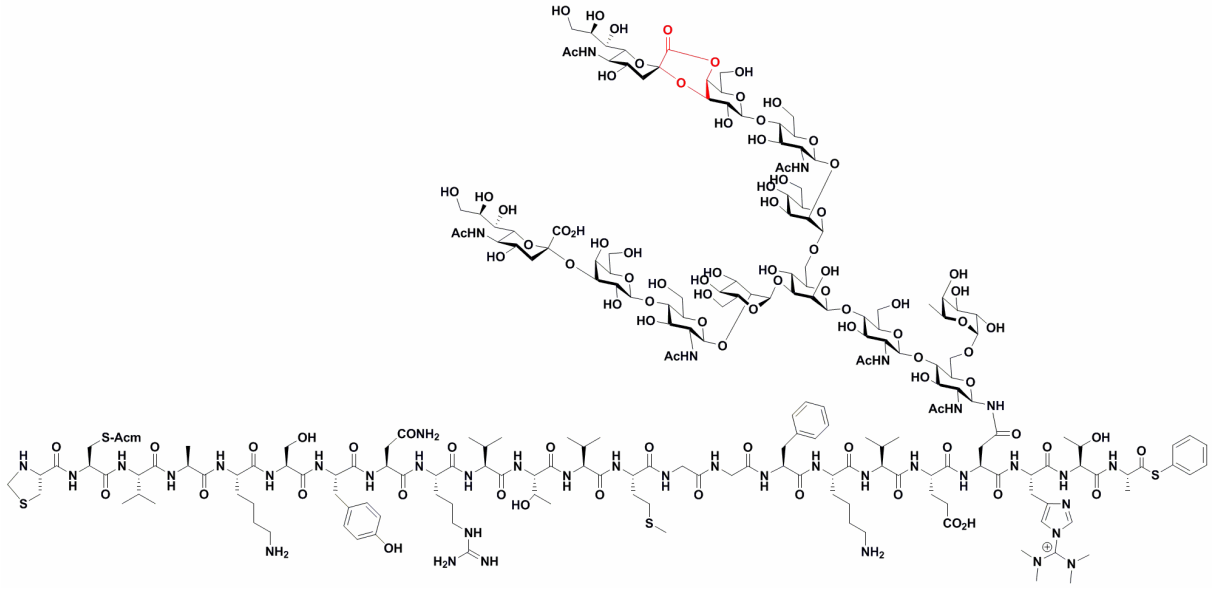


Figure S20: A - ESI-MS of compound **37**. ESI calculated for monolactone + tetramethyluronium $C_{223}H_{348}N_{41}O_{100}S_4$ $[M+3H]^{3+}$ *m/z*: 1778.20, found: 1777.36; $[M+4H]^{4+}$ *m/z*:1333.90, found: 1333.60. ESI calculated for dilactone + tetramethyluronium $C_{223}H_{346}N_{41}O_{99}S_4$ $[M+3H]^{3+}$ *m/z*:1772.20, found: 1771.84; $[M+4H]^{4+}$ *m/z*: 1329.40, found: 1329.11; $[M+5H]^{5+}$ *m/z*: 1063.72, found: 1063.76. **B** - UV traces from HPLC analysis of purified compound **37**; gradient: 20% to 70% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

Compound 38, α-GPH[59-82]



Glycopeptide **37** (2.4 mg, 0.45 µmol) was solubilized in anhydrous DMSO (300 µL). A freshly prepared solution of $Pd(dppf)Cl_2$ in anhydrous DMSO (10 mg/mL, 17.3 µL, 0.236 µmol) was added and followed by phenylsilane (17.3 µL,140 µmol). The reaction was stirred in the dark for 45 min and then quenched with ice-cold water/acetonitrile (1.5 mL, 1:1, 0.05% trifluoroacetic acid). The resulting supsension was isolated on biogel (Bio-Rad P4 medium) equilibrated with water/acetonitrile (4:1, 0.05% trifluoroacetic acid). Lyophilization of the collected fractions provided peptide **38** (2.3 mg) as a white solid, used in the next step with no further purification.

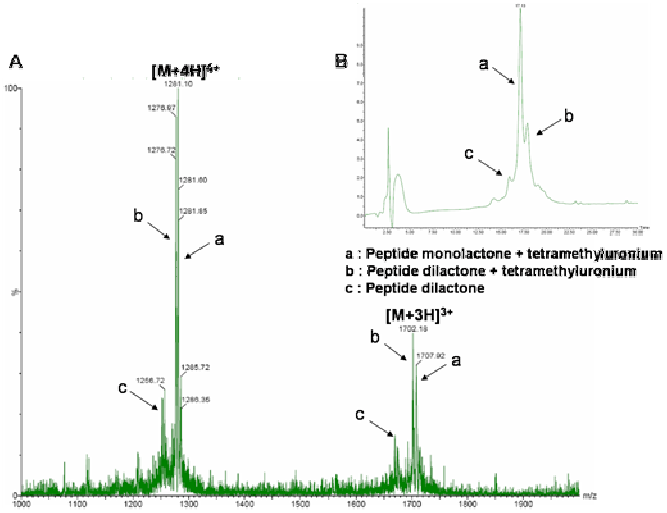
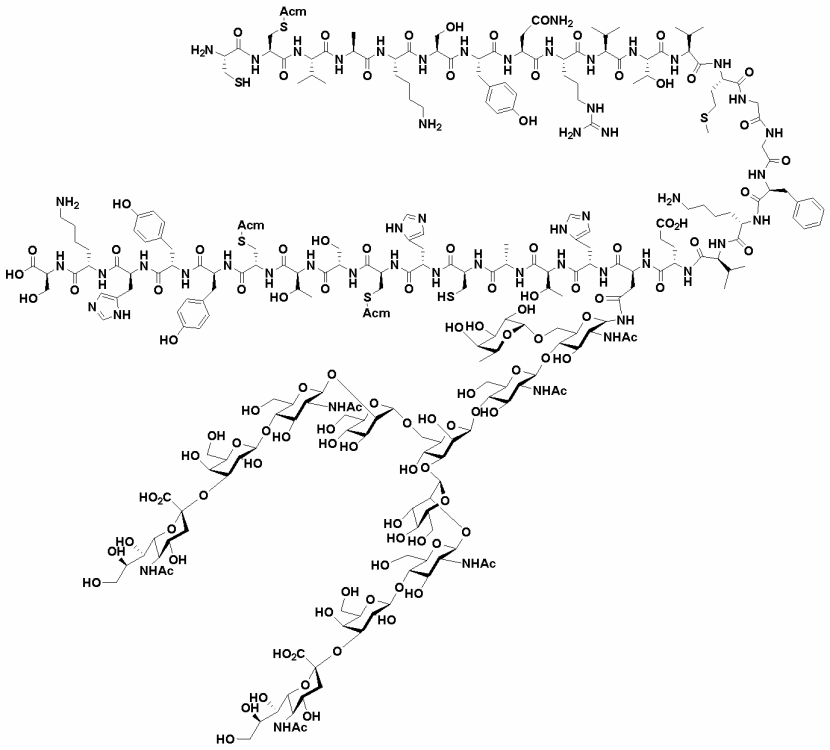


Figure S21: A - ESI-MS of compound **38**. ESI calculated for monolactone + tetramethyluronium $C_{212}H_{336}N_{41}O_{96}S_4$ $[M+3H]^{3+}$ *m/z*: 1708.80, found: 1708.67; $[M+4H]^{4+}$ *m/z*: 1281.85, found: 1281.10. ESI calculated for dilactone + tetramethyluronium $C_{212}H_{334}N_{41}O_{95}S_4$ $[M+3H]^{3+}$ *m/z*: 1702.80, found: 1702.18; $[M+4H]^{4+}$ *m/z*: 1277.35, found: 1276.72. ESI calculated for dilactone $C_{212}H_{334}N_{41}O_{95}S_4$ $[M+3H]^{3+}$ *m/z*: 1669.75, found: 1669.56; $[M+4H]^{4+}$ *m/z*: 1252.56, found: 1252.10. **B** - UV traces from HPLC analysis of purified compound **38**; gradient: 10% to 60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

Compound 39, α-GPH[59-92]



Freshly purified peptides **17** (4.4 mg, 2.8 µmol) and **38** (4.6 mg) were combined and solubilized into NCL buffer (130 µL), prepared as described in the general procedure. To this mixture was added neutral TCEP solution (0.5 M, 14 µL). After 2 h, another portion of neutral TCEP solution (0.5 M, 14 µL) was added and the reaction stirred for total 4 h. After completion of the ligation a solution of $MeONH_2 \cdot HCl$ (0.6 M, 144 µL) and neutral TCEP solution (0.5 M, 14 µL) were added, the pH was brought to 4-4.5 with HCl solution (2 M, 18 µL). The turbid mixture was stirred for 270 min and diluted dropwise with water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1.5 mL) and neutral TCEP solution (0.5 M, 20 µL). The resulting supsension was prepurified on biogel (Bio-Rad P4 medium) equilibrated with water/acetonitrile (4:1, 0.05% trifluoroacetic acid). The crude peptide was purified by RP-HPLC (C8 X-bridge semiprep, 15% to 35% acetonitrile/water over 30 min, 16 mL/min, λ = 230 nm). Product eluted at 10 min. Lyophilization of the collected fractions provided peptide **39** (1.1 mg, 21% over 3 steps) as a white solid.

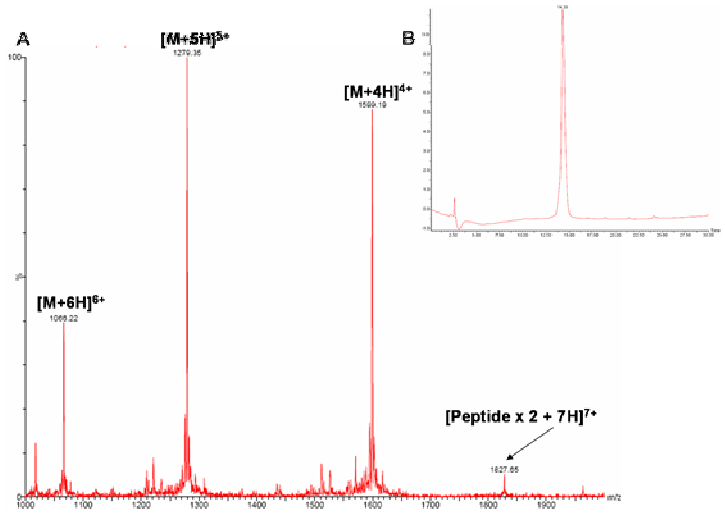
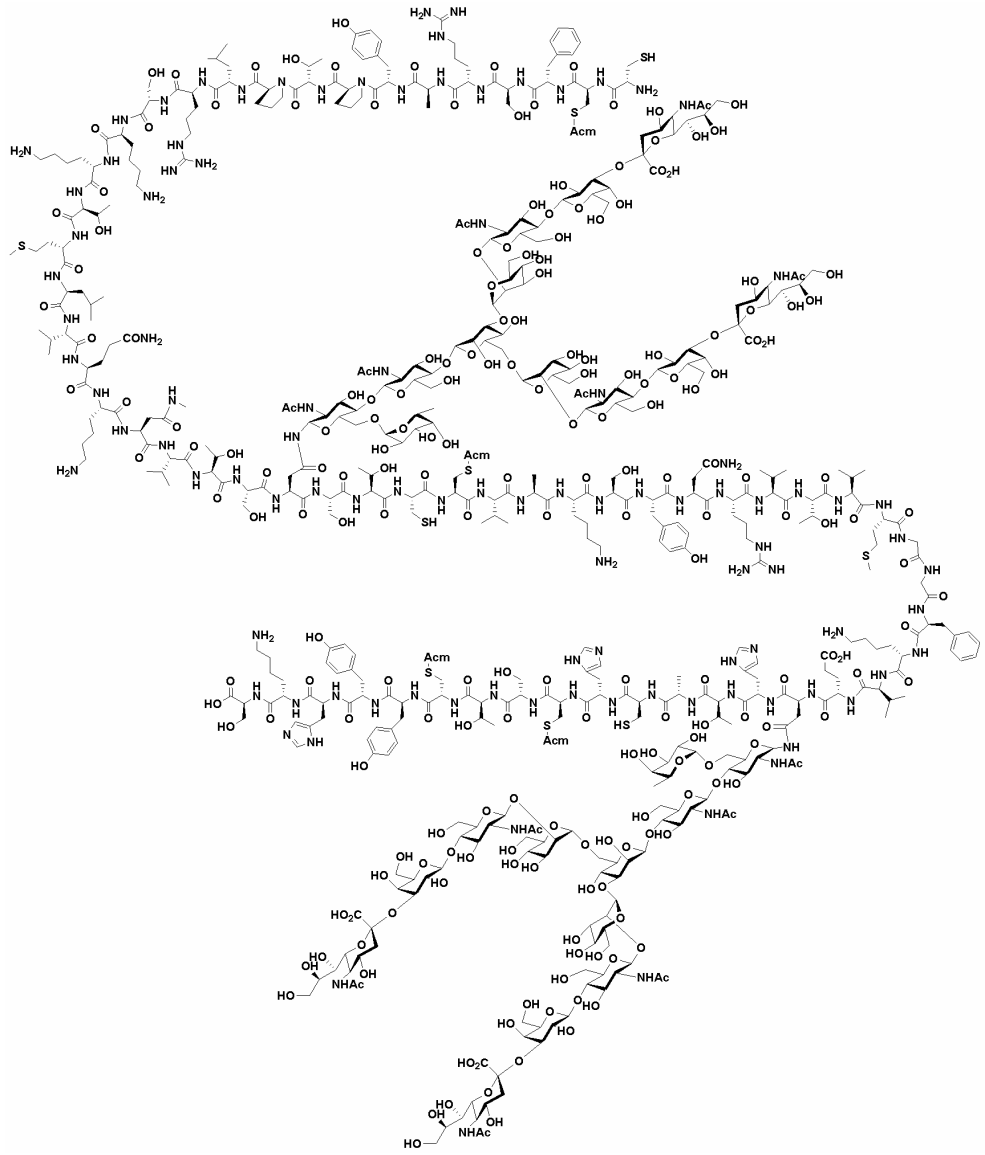


Figure S22: A - ESI-MS of compound **39**. ESI calculated for $C_{261}H_{409}N_{57}O_{116}S_6$ $[M+4H]^{4+}$ *m/z*: 1599.43, found: 1599.19; $[M+5H]^{5+}$ *m/z*: 1279.74, found: 1279.35; $[M+6H]^{6+}$ *m/z*: 1066.62, found: 1066.22. **B** - UV traces from HPLC analysis of purified compound **39**; gradient: 10% to 60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

Compound 40, α-GPH[31-92]



Freshly purified peptides **39** (2 mg, 3.12 µmol) and **36** (2.14 mg, 3.75 µmol) were combined and solubilized into NCL buffer (45 µL), prepared as described in the general procedure. To this mixture was immediatly added neutral TCEP solution (0.5 M, 5 µL). After 2 h reaction, another portion of neutral TCEP solution (0.5 M, 5 µL) was added and the reaction was stirred for total 8 h. After completion of the ligation a solution of $MeONH_2 \cdot HCl$ (0.6 M, 45 µL) and neutral TCEP solution (0.5 M, 5 µL) were added, the pH was brought to 4-4.5 with HCl solution (2 M, 5 µL). The turbid mixture was stirred for 270 min and diluted dropwise with water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1 mL). The resulting mixture was prepurified on biogel (Bio-Rad P4 medium) equilibrated with water/acetonitrile (4:1, 0.05% trifluoroacetic acid) and purified by RP-HPLC (C4 semiprep, 10% to 30% acetonitrile/water over 30 min, 1.4 mL/min, λ = 230 nm). Product eluted at 18 min. Lyophilization of the collected fractions provided peptide **40** (1.1 mg, 33% over 2 steps) as a white solid.

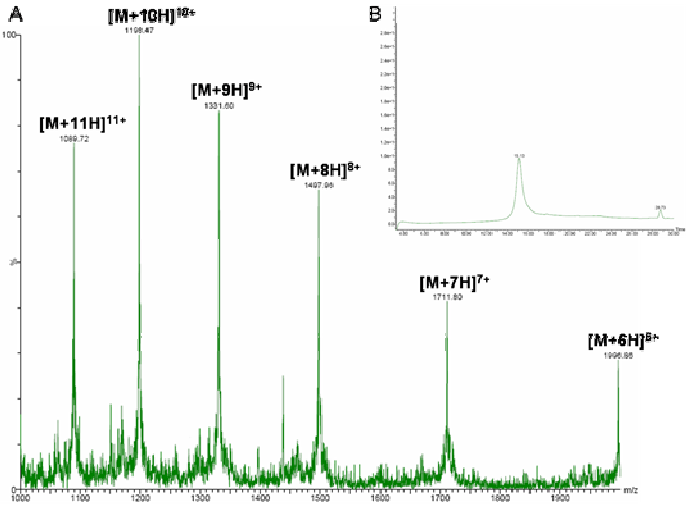
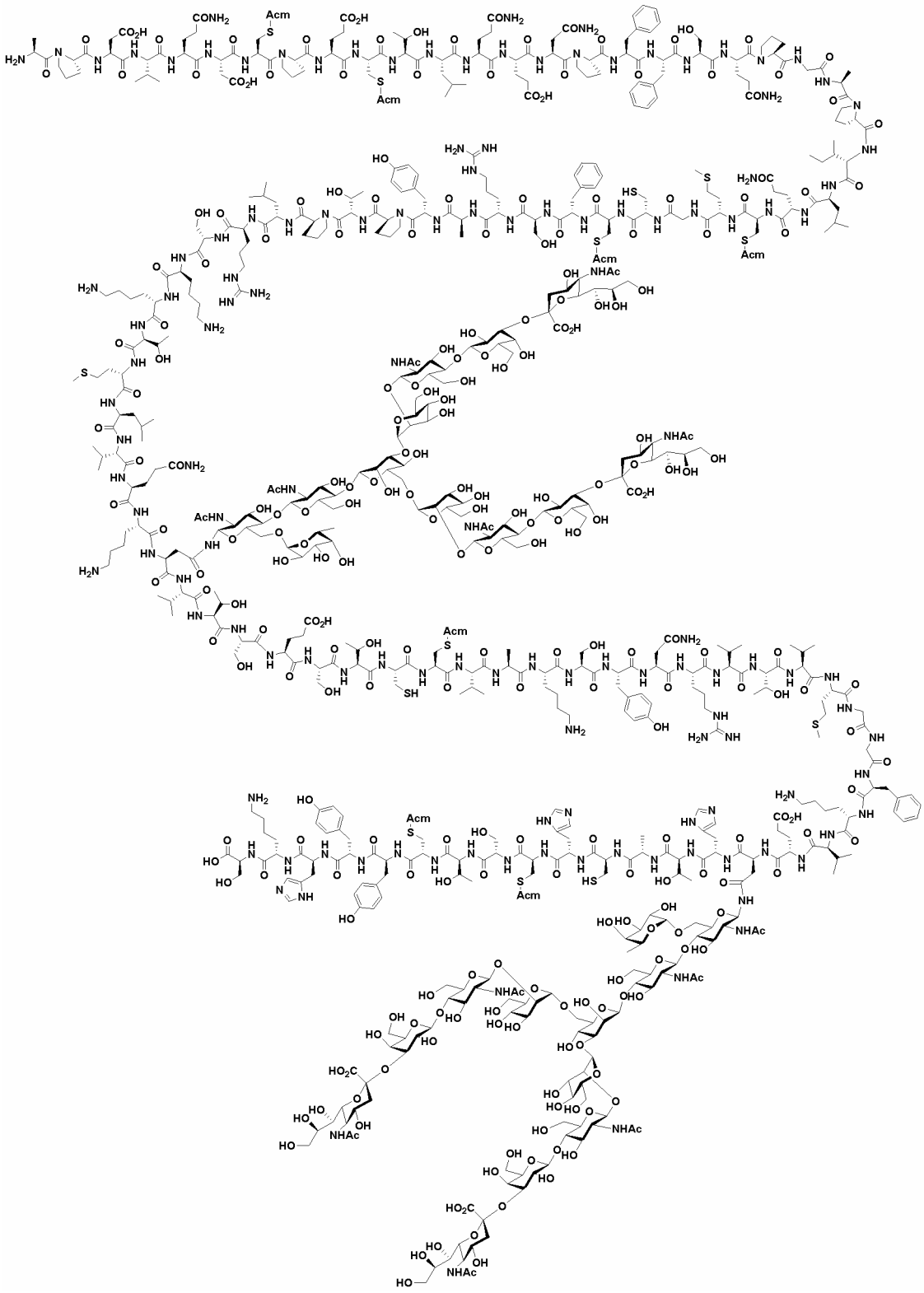


Figure S23: A - ESI-MS of compound **40**. ESI calculated for $C_{490}H_{786}N_{104}O_{222}S_9$ $[M+6H]^{6+}$ *m/z*: 1996.77, found: 1996.86; $[M+7H]^{7+}$ *m/z*: 1711.66, found: 1711.80; $[M+8H]^{8+}$ *m/z*:1497.82, found: 1497.96; $[M+9H]^{9+}$ *m/z*: 1331.51, found: 1331.60; $[M+10H]^{10+}$ *m/z*: 1198.46, found: 1198.47; $[M+11H]^{11+}$ *m/z*: 1089.60, found: 1089.72. **B** - UV traces from HPLC analysis of purified compound **40**; gradient: 10% to 60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min, Varian C18 column.

Compound 41, α-GPH[1-92]



Freshly purified peptides **40** (1.1 mg, 0.918 µmol) and **32** (0.35 mg, 1 µmol) were combined and solubilized into NCL buffer (18 µL), prepared as described in the general procedure. To this mixture was added neutral TCEP solution (0.5 M, 2 µL). After 2 h another portion of

neutral TCEP solution (0.5 M, 2 µL) was added and the reaction was stirred for total 5 h. After completion of the ligation, the mixture was diluted dropwise with water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 1 mL) and desalted on biogel (Bio-Rad P6 Medium) equilibrated with water/acetonitrile (4:1, 0.05% trifluoroacetic acid) then purified by RP-HPLC (C4 semiprep, 10% to 60% acetonitrile/water over 30 min, 1.4 mL/min, λ = 230 nm). Product eluted at 15 min. Lyophilization of the collected fractions provided peptide **41** (0.4 mg, 28%) as a white solid.

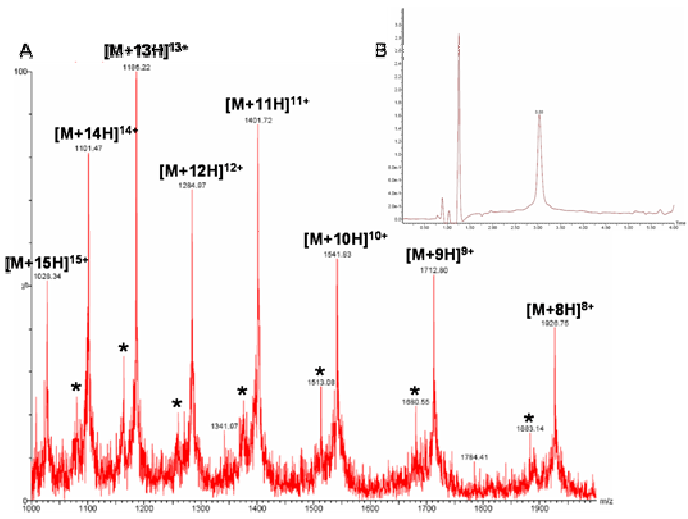
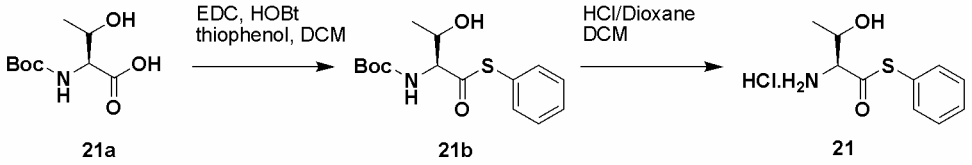


Figure S24: A - ESI-MS of compound **41**. ESI calculated for $C_{638}H_{1009}N_{141}O_{271}S_{13}$ $[M+8H]^{8+}$ *m/z*: 1926.93, found: 1926.75; $[M+9H]^{9+}$ *m/z*: 1712.94, found: 1712.80; $[M+10H]^{10+}$ *m/z*: 1541.74, found: 1541.83; $[M+11H]^{11+}$ *m/z*: 1401.67, found: 1401.72; $[M+12H]^{12+}$ *m/z*: 1284.95, found: 1284.97; $[M+13H]^{13+}$ *m/z*: 1186.19, found: 1186.22; $[M+14H]^{14+}$ *m/z*: 1101.53, found: 1101.47; $[M+15H]^{15+}$ *m/z*: 1028.16, found: 1028.34. The star (*) indicates the product missing one unit of scialic acid, wich is very likely lost during the ionisation process. **B** - UV traces from UPLC analysis of purified compound **41**; gradient: 20% to 50% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min, BEH C18 column.

V – Amino acid derivatives.

Compound 21, H-Thr-SPh·HCl



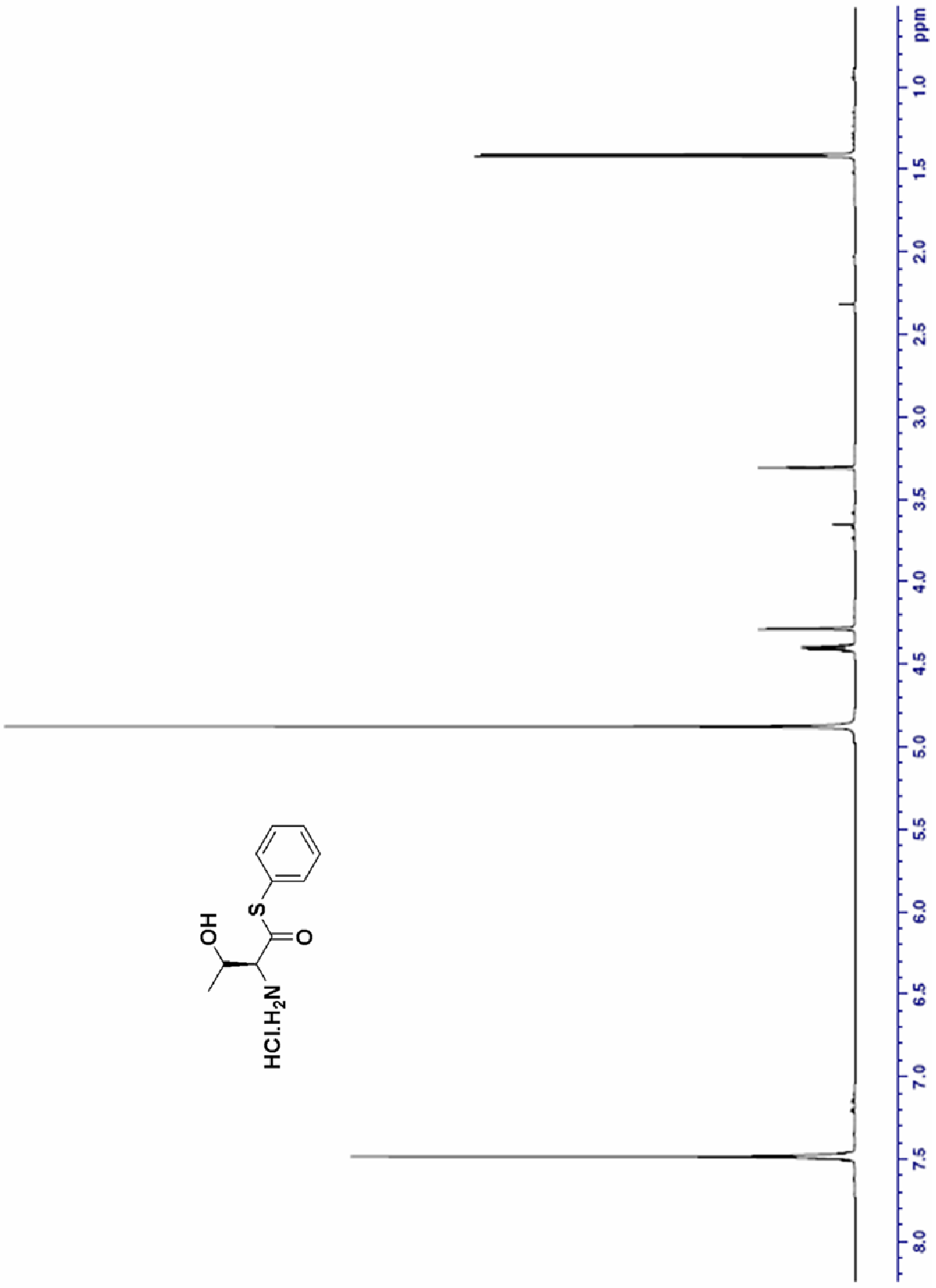
21a 21b 21

Boc-Thr-OH **21a** (2.19 g, 10 mmol) was solubilized in dichloromethane (50 mL). To this solution EDC (1.77 mL, 10 mmol), HOBt (4.05 g, 30 mmol) and thiophenol (5.11 mL, 50 mmol) were added. The mixture was stirred for 5 h, concentrated *in vacuo* and purified by flash chromatography (silica gel, 10% to 40% ethyl acetate/hexane). **21b** (2.9 g, 9.3 mmol) was directly solubilized in an ice-cold mixture of dichloromethane (5 mL) and HCl in dioxane (4 M, 5 mL) at 0°C. After 3 h at room temperature, the solution was concentrated *in vacuo*, azeotroped with toluene, resuspended in water/acetonitrile (1:1, 0.05% trifluoroacetic acid, 4 mL) and lyophilized. To afford **21** as white solid (2.25 g, 91% over two steps).

^{1}H NMR (600MHz, CD_3OD): δ 7.46-7.51 (m, 5H), 4.38 (dq, 1H, *J* = 6.5, 4.1 Hz), 4.28 (d, 1H, *J* = 4.0 Hz), 1.41 (d, 3H, *J* = 6.5 Hz).

^{13}C NMR (150MHz, CD_3OD): δ 194.5, 135.8, 131.3, 130.6, 126.7, 67.2, 65.7, 20.6.

MS (ESI): calculated for $C_{10}H_{13}NO_2S$ $[M+H]^+$ *m/z*: 211.28, found: 211.49; $[M+Na]^+$ *m/z*: 234.06, found: 233.79.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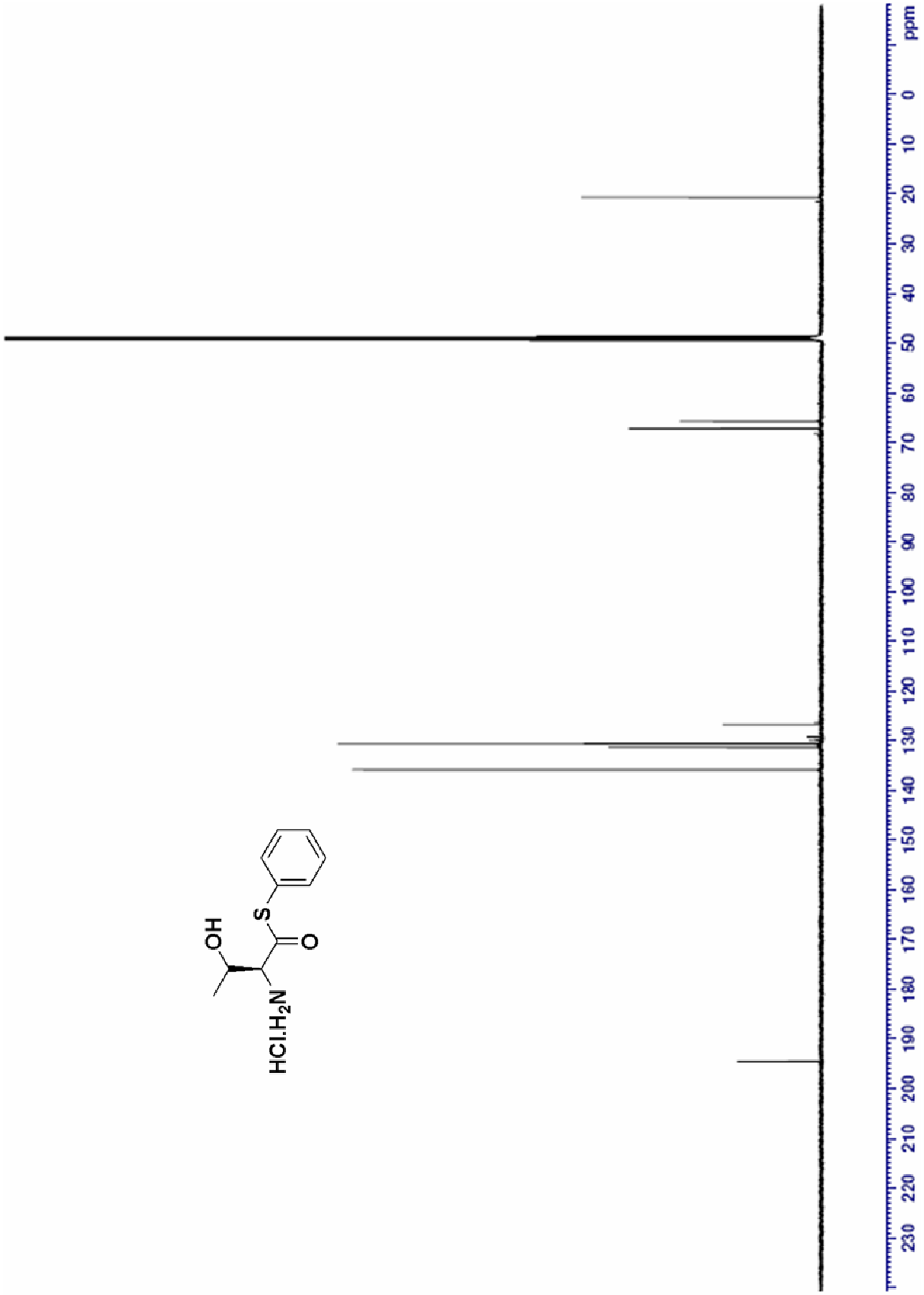
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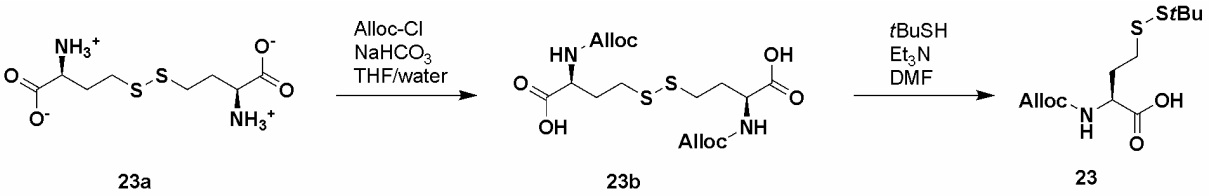
Compound 23, Alloc-hCys(S*t*Bu)-OH

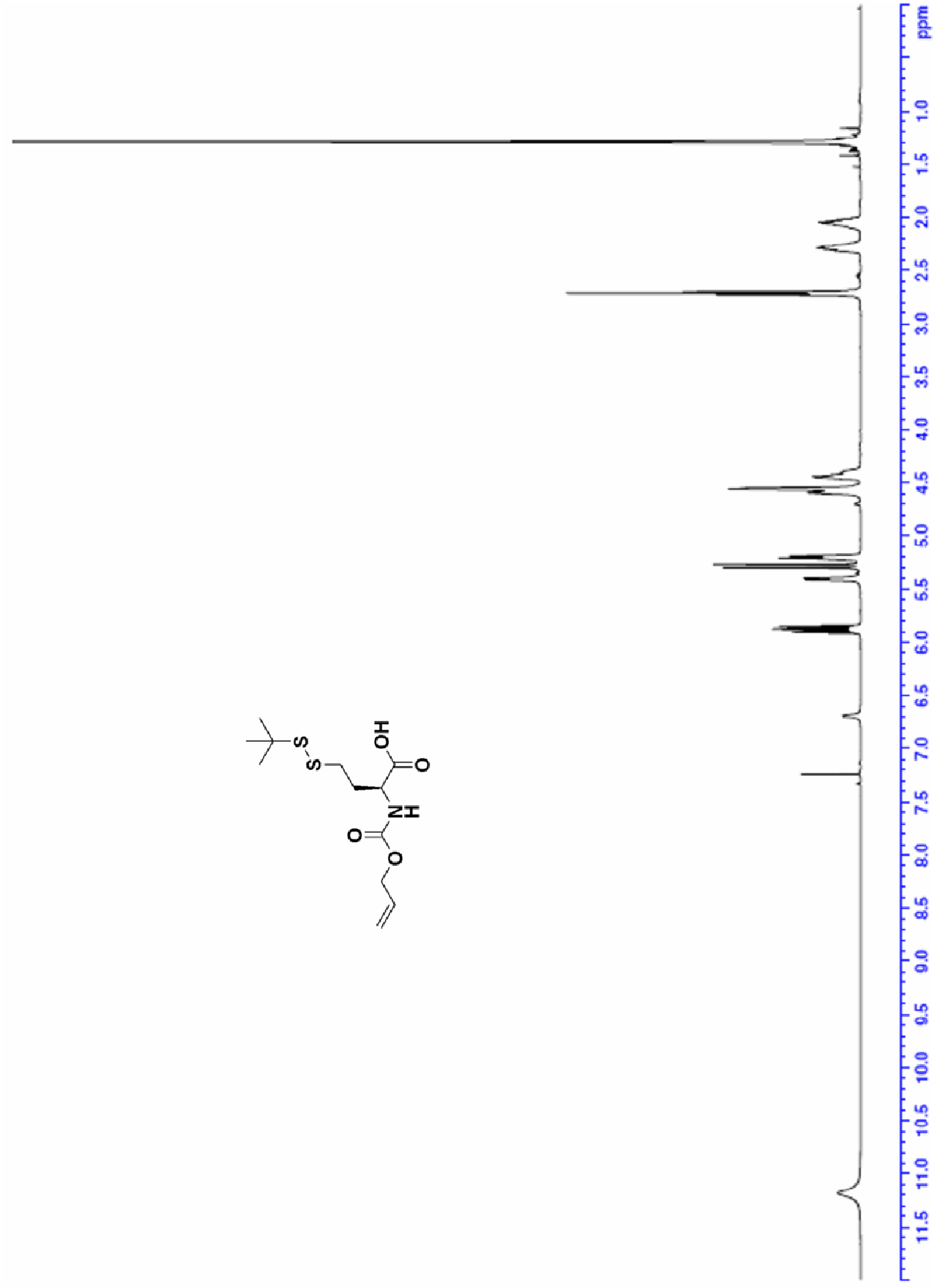


Homocystine **23a** (1.1 g, 4.1 mmol) was dissolved in water/THF (1:1, 100 mL). $NaHCO_3$ (3.13 g, 37 mmol) was added and the mixture cooled to 0°C. Allyl chloroformate (1.3 mL, 12.2 mmol) was added dropwise and the suspension was stirred over night at room temperature. The pH was brought to 2 with a HCl solution (6 M) and the solution was extracted 3 times with EtOAc (3 x 25 mL). The pooled organic phases were dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford **23b** as a white solid. Without further purification, **23b** was solubilized in DMF (20 mL). Et_3N (1.42 mL, 10.3 mmol) and tert-butyl thiol (4.6 mL, 41.2 mmol) were added and the mixture was heated to 60°C for 2 h. After water dilution, the pH was brought to 2 with a HCl solution (6 M) and the mixture was extracted with ethyl acetate (3 x 50mL). The combined organic phases were washed with brine, dryed over Na_2SO_4, filtered and concentrated *in vacuo*. The product was purified by flash chromatography (silica gel, 0.5 to 1% methanol/dichloromethane with 0.1% acetic acid) to afford **23** as a white solid (575 mg, 46% over 2 steps).

^{1}H NMR (500MHz, $CDCl_3$): δ 11.2 (br s, 1H), 5.84 (ddt, 1H, *J* = 17.8, 10.6, 6.3 Hz), 5.3 (d, 1H, *J* = 18 Hz), 5.2 (d, 1H, *J* = 10.1 Hz), 4.5 (m, 2H), 4.4 (m, 1H), 2.7 (t, 2H, *J* = 8 Hz), 2.2 (m, 1H), 2.0 (m, 1H), 1.3 (s, 9H).

^{13}C NMR (125MHz, $CDCl_3$): δ 176.5, 156.0, 132.3, 118.1, 66.1, 53.4, 47.9, 35.8, 32.1, 29.9.

MS (ESI): calculated for $C_{12}H_{21}NO_4S_2$ $[M+H]^+$ *m/z*: 307.43, found: 307.10.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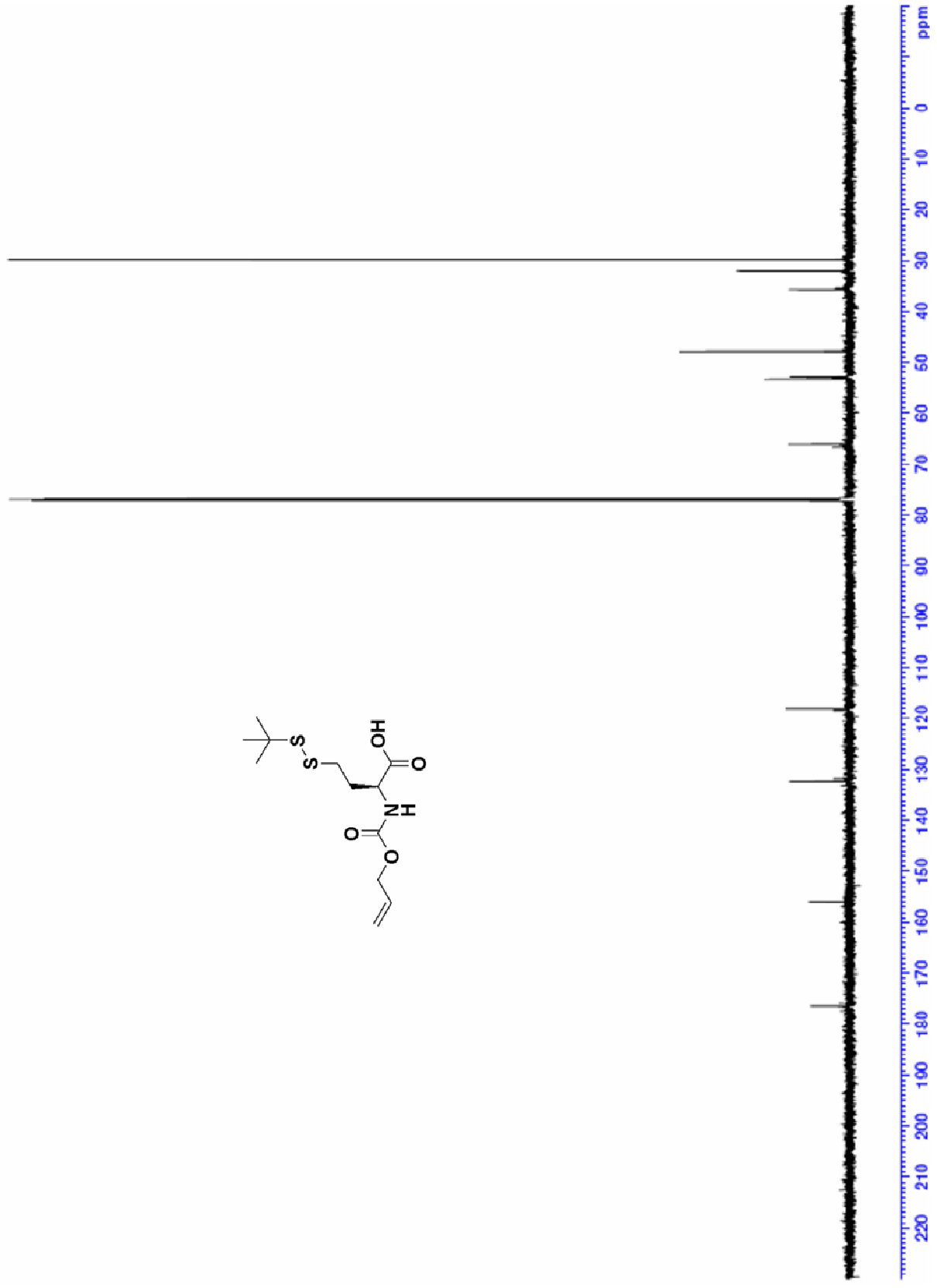
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NH
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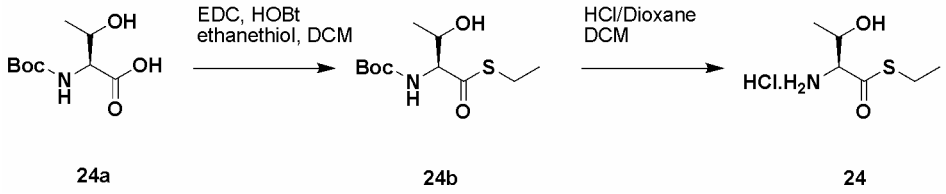
O
S
S

Compound 24, H-Thr-SEt·HCl



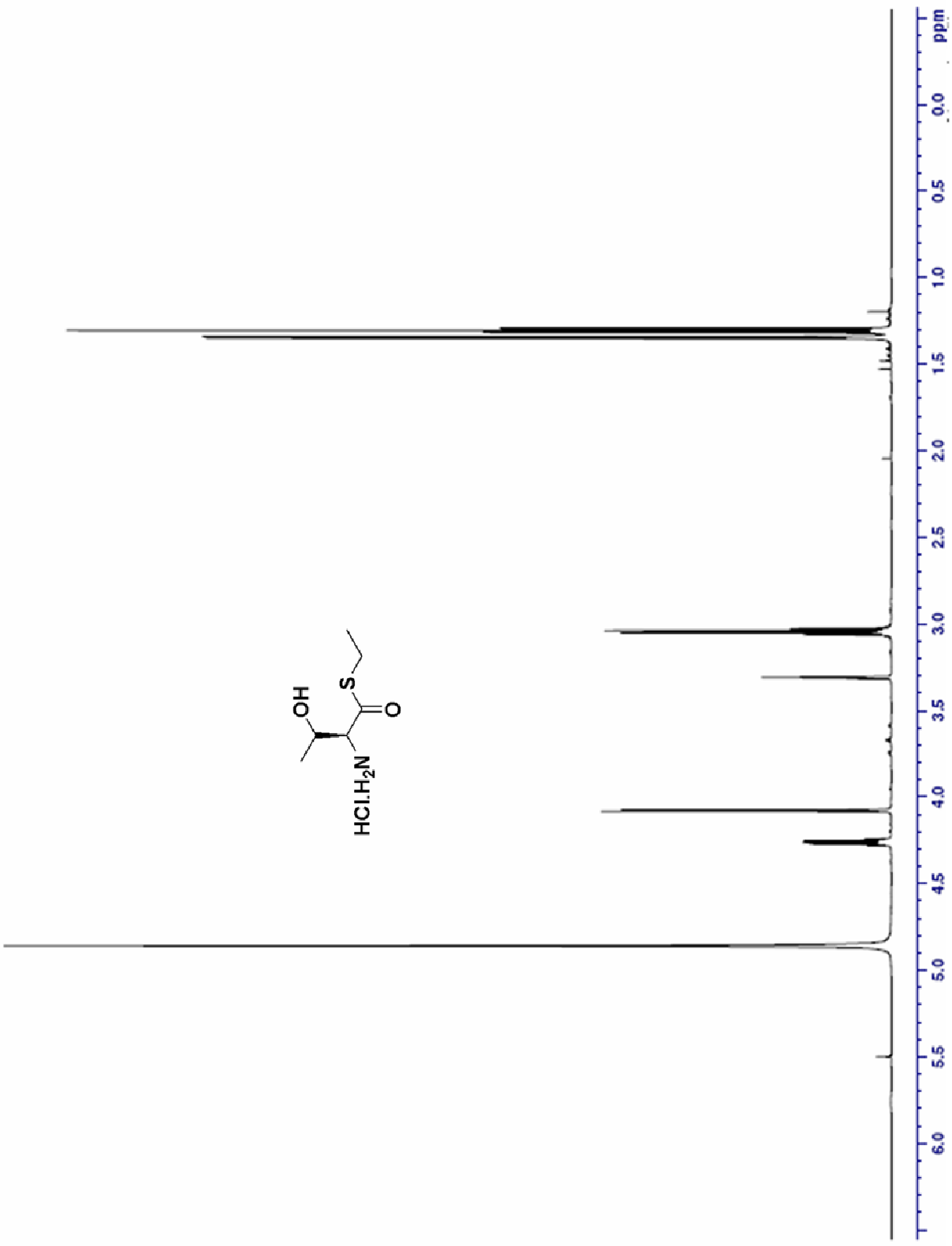
24a 24b 24

Boc-Thr-OH **24a** (2.19 g, 10 mmol) was solubilized in dichloromethane (50 mL). To this solution EDC (1.77 mL, 10 mmol), HOBt (4.05 g, 30 mmol) and ethanthiol (3.7 mL, 50 mmol) were added. The mixture was stirred for 5 h, concentrated *in vacuo* and purified by flash chromatography (silica gel, 10% to 40% ethyl acetate/hexane). **24b** (2.37 g, 9 mmol) was directly solubilized in an ice-cold mixture of dichloromethane (5 mL) and HCl in dioxane (4 M, 5 mL) at 0°C. After 3 h at room temperature the solution was concentrated *in vacuo*, azeotroped with toluene, resuspended in water/acetonitrile (4 mL, 1:1, 0.05% trifluoroacetic acid) and lyophilized. To afford **24** as a colorless oil (1.66 g, 83% over two steps).

^{1}H NMR (600MHz, CD_3OD): δ 4.24 (dq, 1H, *J* = 6.4, 4.4 Hz), 4.07 (d, 1H, *J* = 4.4 Hz), 3.02 (q, 2H, *J* = 7.4 Hz), 1.34 (d, 3H, *J* = 6.5 Hz), 1.29 (t, 3H, *J* = 7.4 Hz).

^{13}C NMR (150MHz, CD_3OD): δ 196.2, 67.3, 66.0, 24.7, 20.7, 14.8.

MS (ESI): calculated for $C_{10}H_{13}NO_2S$ $[M+H]^+$ *m/z*: 163.24, found: 163.84; $[M+Na]^+$ *m/z*: 186.23, found: 185.75.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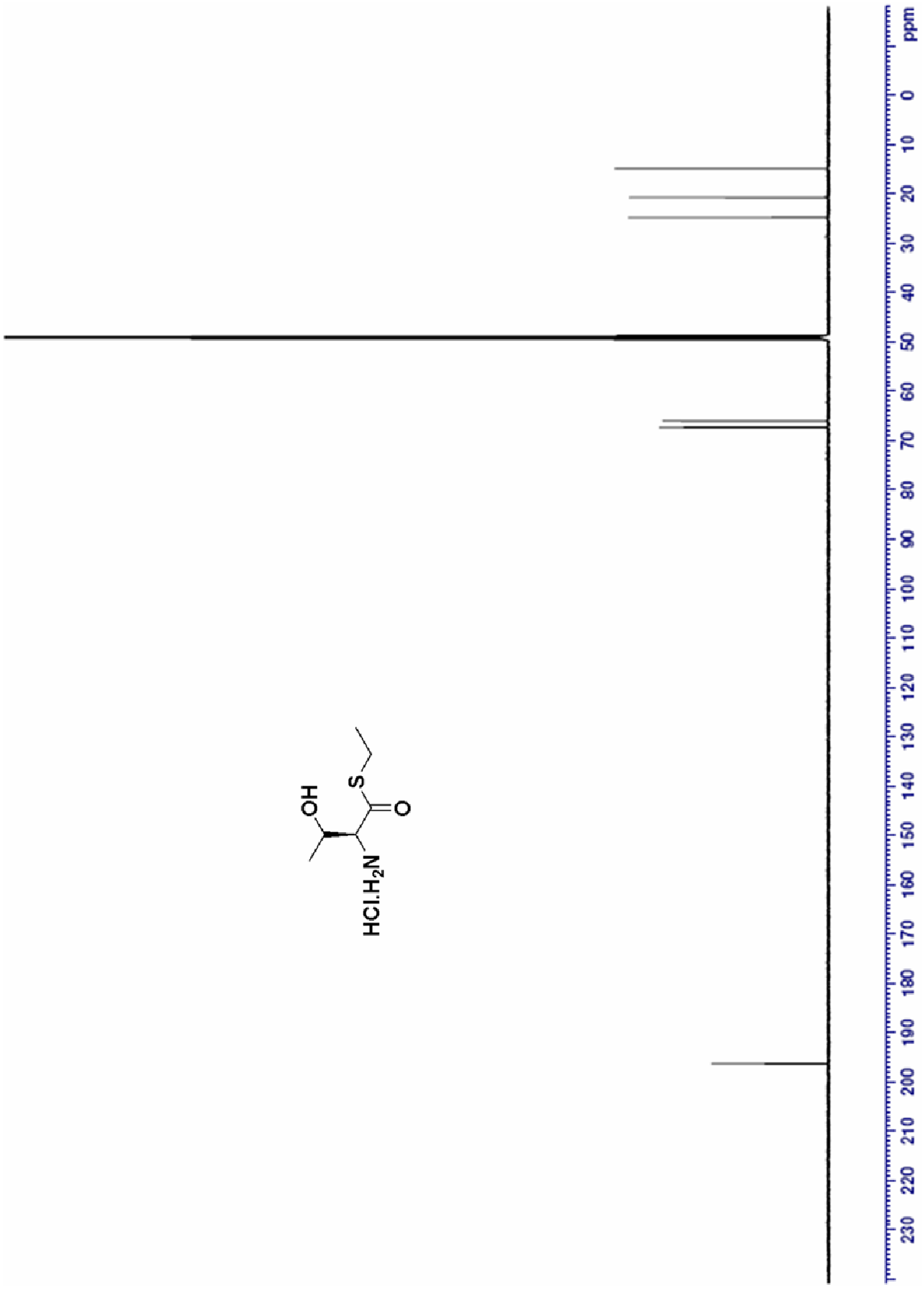
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