Supporting Information

Toward Homogeneous Erythropoietin: Chemical Synthesis of the Ala^{1}-Gly^{28} Glycopeptide Domain by "Alanine" Ligation

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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. All reactions were performed under an atmosphere of pre-purified dry Ar(g). NMR spectra (^1H 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

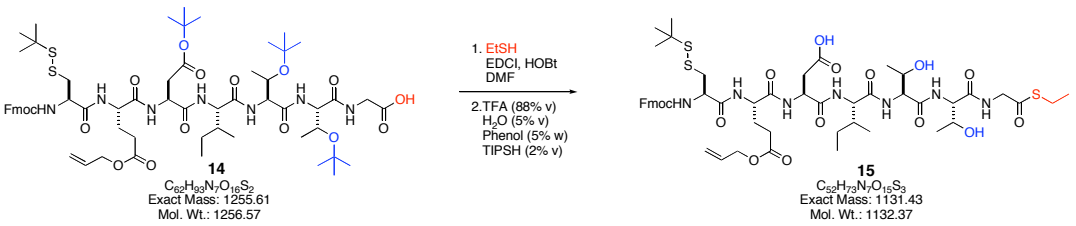
All separations involved a mobile phase of 0.05% TFA (v/v) in water (solvent A) /0.04% TFA in acetonitrile (solvent B). Preparative and analytical HPLC separations were performed using a Rainin HPXL solvent delivery system equipped with a Rainin UV-1 detector. LC-MS chromatographic separations were performed using a Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector equipped with Varian Microsorb C18 column (2 x 150 mm) at a flow rate of 0.2 mL/min or Varian Microsorb C4 column (2 x 250 mm) at a flow rate of 0.2 mL/min. HPLC separations were performed using either Microsorb 100-5 C18 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/t-Bu 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-Asp(O*t*Bu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(*t*Buthio)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Pro-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH. The following didpeptide from NovaBiochem was used: Fmoc-Asp(OtBu)-Ser($\psi^{Me,\ Me}$-pro)-OH. Other amino acids were supplied by Chem Impex, including Fmoc-Glu(OAl)-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 was subjected to a cleavage cocktail (1:1:8 acetic acid/trifluoroethanol/methylene chloride) for 1 hour (7 mL x 3). The resin was removed by filtration and the resulting solution was concentrated. The oily residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The resulting solid was ready for HPLC purification.



Peptide **14** was prepared from Fmoc-Gly-NovaSyn® TGT resin following the general SPPS procedure. To a solution of peptide **14** (40 mg, 0.032 mmol) and ethanethiol (70 μL, 0.95 mmol) in DMF (2.0 mL) at rt were added EDCI (61 mg, 0.32 mmol) and HOBt (43 mg, 0.32 mmol). The reaction was stirred at rt 3.5 hours. The reaction was concentrated under a stream of air and the residue was filtered through a plug of SiO_2 (10% MeOH/CH_2Cl_2) to give a white solid.

The solid was taken up in a deprotection cocktail (2.0 mL) consisting of trifluoroacetic acid (88% by volume), water (5% by volume), phenol (5% by weight), and iPr$_3$SiH (2% by volume). The clear, pale yellow solution was stirred at rt for 2 hours. The reaction was transferred to a polypropylene vial and concentrated under a stream of air. The resulting residue was triturated with cold Et_2O (3 x 7 mL), centrifuged, and the Et_2O carefully decanted. The remaining white solid was purified by RP-HPLC (C18 semiprep, 45% → 75% MeCN/H_2O, 0 → 30 min, 16 mL/min, λ = 265 nm). Product eluted from 21-23 minutes. The fractions were collected, concentrated, and lyophilized to provide peptide **15** (14.6 mg, 40% over two steps) as a white solid.

ESI calcd for $C_{52}H_{73}N_7O_{15}S_3$ $[M+H]^+$ m/z: 1132.44, found: 1132.75

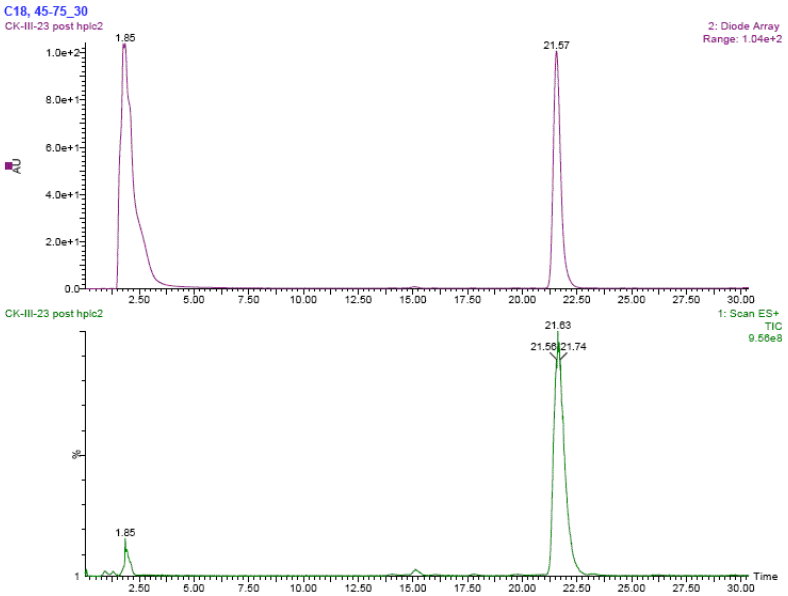


Figure S1: UV and MS traces from LC-MS analysis of compound **15**; gradient: 45-75% MeCN/H_2O over 30 min at a flow rate of 0.2 mL/min, Varian Microsorb C18 column

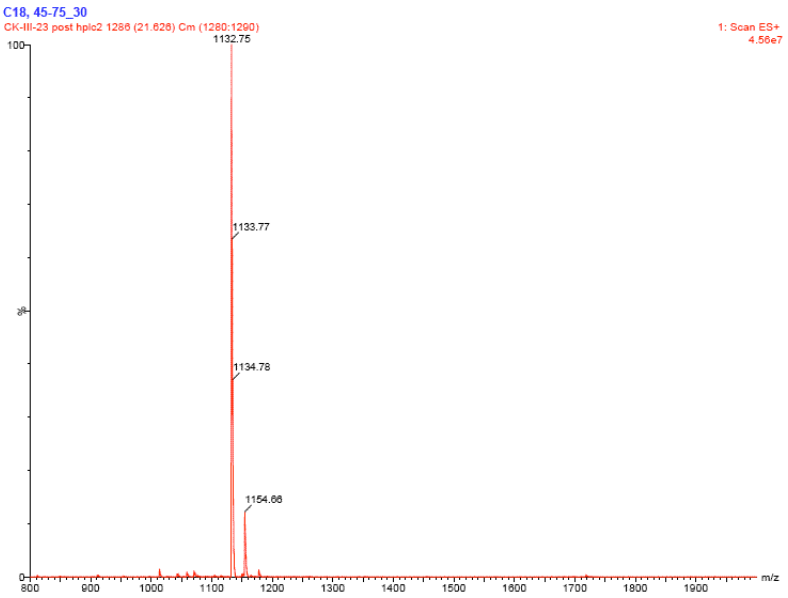
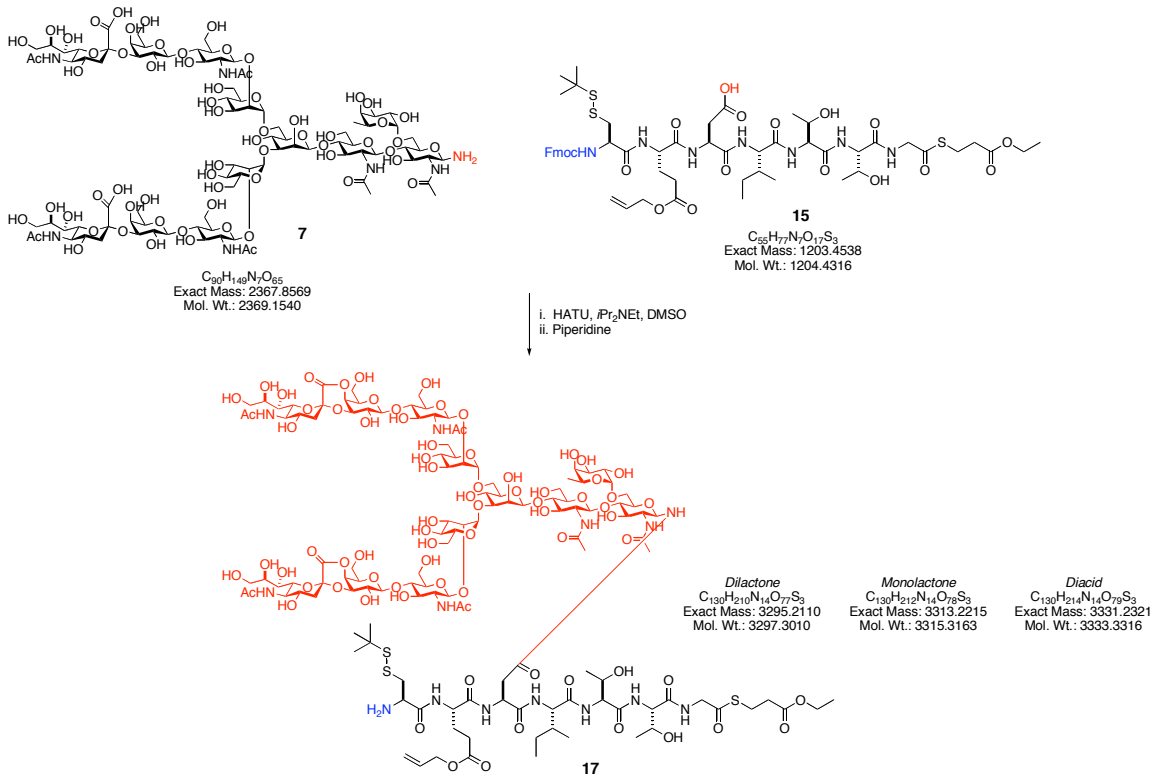


Figure S2: ESI-MS of compound **15**



To a vial containing peptide **15** (2.2 mg, 1.8 μmol), dodecasaccharide **7** (2.2 mg, 0.93 μmol), and HATU (2.8 mg, 7.4 μmol) were added DMSO (200 μL) and *i*Pr$_2$NEt (0.7 μL, 3.7 μmol). The bright yellow, clear solution was stirred at rt for 1 hour, 5 minutes. Piperidine (2.0 μL, 19 μmol) was then added to the reaction mixture. The bright yellow color became a deep, golden-yellow color. The reaction was stirred at rt for 1 hour, at which point the reaction was quenched by addition of MeCN/H_2O (1 mL, 1:1, 0.05% TFA). The mixture was purified via RP-HPLC (C18 semiprep, 15% → 45% MeCN/H_2O, 0 → 30 min, 16 mL/min, λ = 230 nm). Product eluted at 20-23 min. The fractions were collected, concentrated, and lyophilized to provide **17** (2.0 mg, 65% over two steps) as a mixture of dilactone, monolactone, and diacid products as a white solid.

Dilactone: ESI calcd for $C_{130}H_{210}N_{14}O_{77}S_3$ $[M+2H]^{2+}$ *m/z:* 1648.61, found: 1648.66

Monolactone: ESI calcd for $C_{130}H_{212}N_{14}O_{78}S_3$ $[M+2H]^{2+}$ *m/z:* 1657.62, found:1658.05

Diacid: ESI calcd for $C_{130}H_{214}N_{14}O_{79}S_3$ $[M+2H]^{2+}$ *m/z:* 1666.62, found: 1666.90

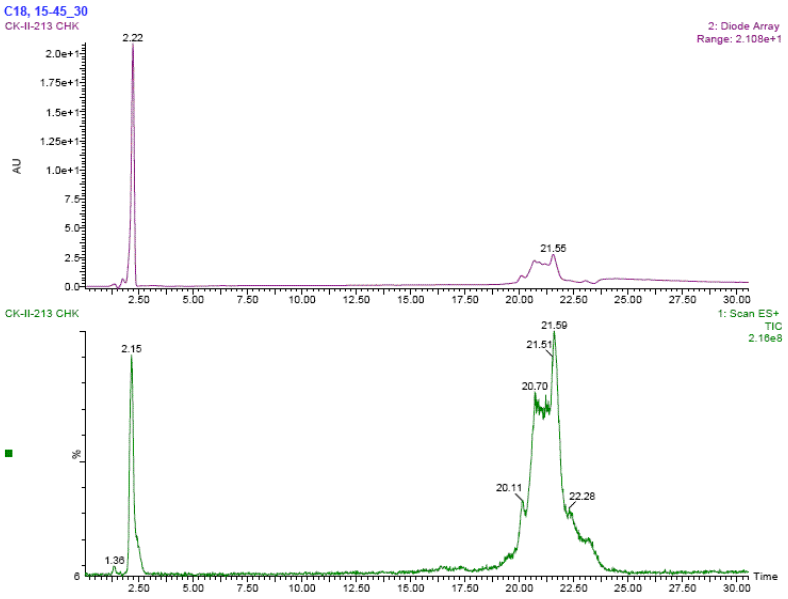


Figure S3: UV and MS traces from LC-MS analysis of compound **17**; gradient: 15-45% MeCN/H_2O over 30 min at a flow rate of 0.2 mL/min, Varian Microsorb C18 column

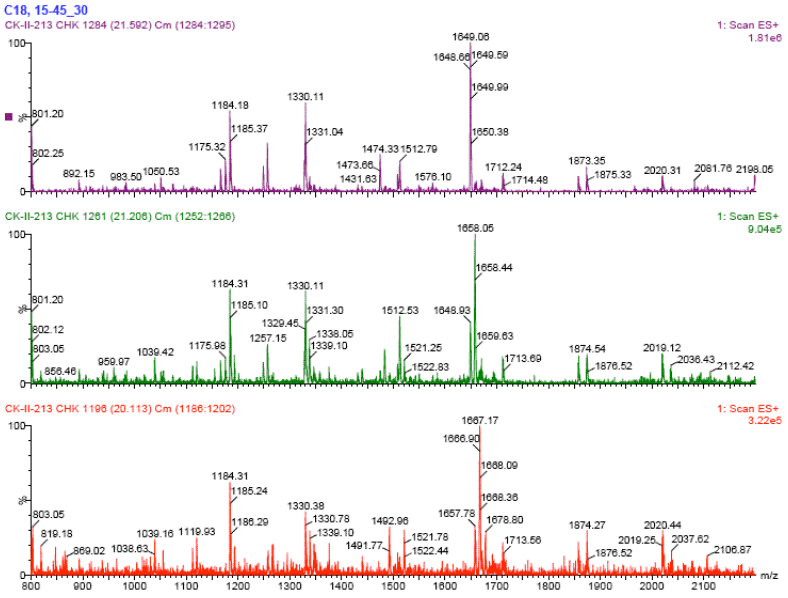
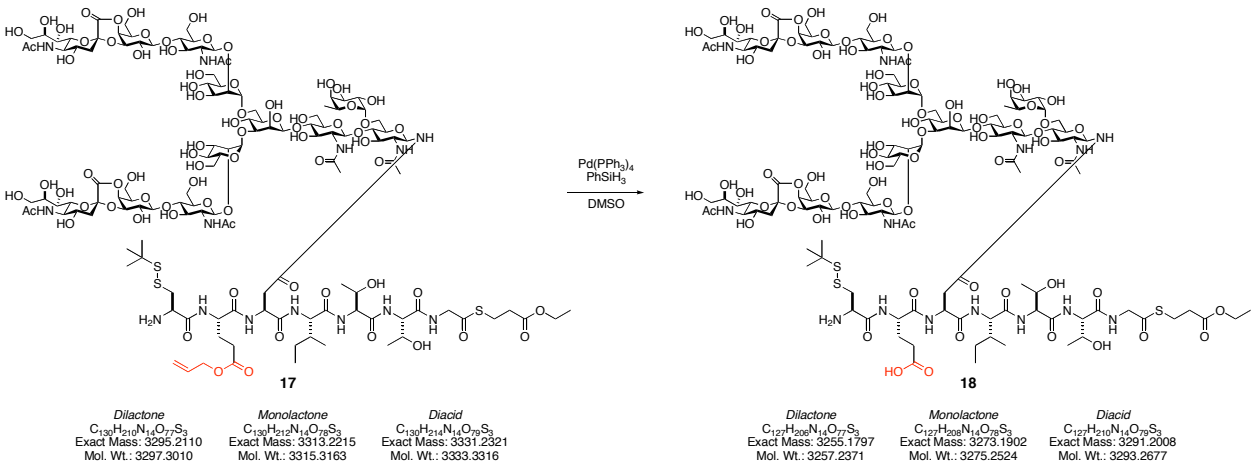


Figure S4: ESI-MS of compound **17**



To a solution of peptide **17** (2.0 mg, 0.61 μmol) and $Pd(PPh_3)_4$ (~0.4 mg, 0.35 μmol) in DMSO (200 μL) was added $PhSiH_3$ (1.5 μL, 12 μmol). The light yellow, clear solution was stirred at rt for 8 minutes. The reaction was quenched by addition of MeCN/H_2O (1 mL, 1:1, 0.05% TFA). The mixture was purified via RP-HPLC (C18 semiprep, 10% → 40% MeCN/H_2O, 0 → 30 min, 16 mL/min, λ = 232 nm). Product eluted at 20-22 min. The fractions were collected, concentrated, and lyophilized to provide **18** (1.8 mg, 90%) as a mixture of dilactone, monolactone, and diacid products as a white solid.

Dilactone: ESI calcd for $C_{127}H_{206}N_{14}O_{77}S_3$ $[M+2H]^{2+}$ *m/z:* 1628.60, found: 1628.70

Monolactone: ESI calcd for $C_{127}H_{208}N_{14}O_{78}S_3$ $[M+2H]^{2+}$ *m/z:* 1637.60, found:1637.69

Diacid: ESI calcd for $C_{127}H_{210}N_{14}O_{79}S_3$ $[M+2H]^{2+}$ *m/z:* 1646.61, found: 1646.68

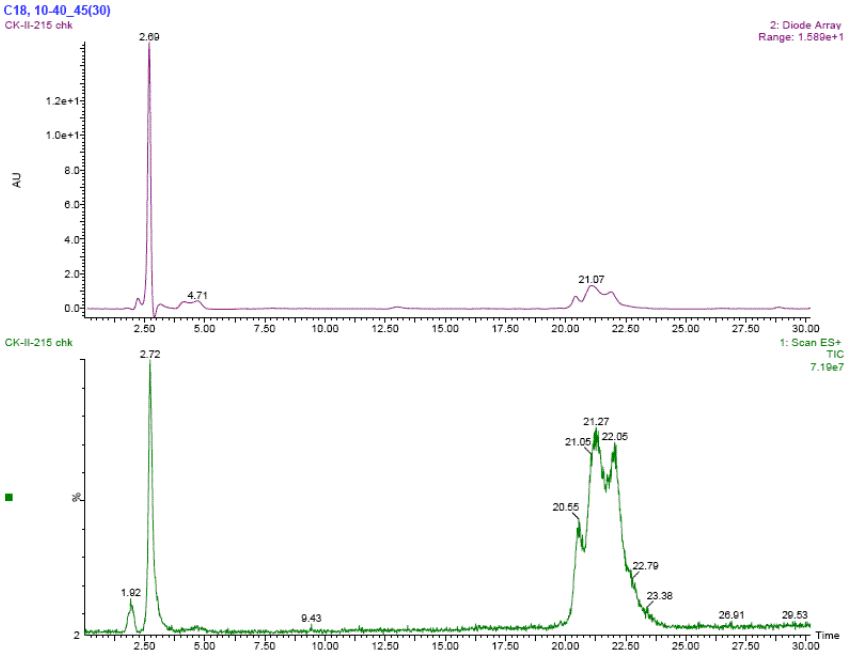


Figure S5: UV and MS traces from LC-MS analysis of compound **18**; gradient: 10-40% MeCN/H_2O over 30 min at a flow rate of 0.2 mL/min, Varian Microsorb C18 column

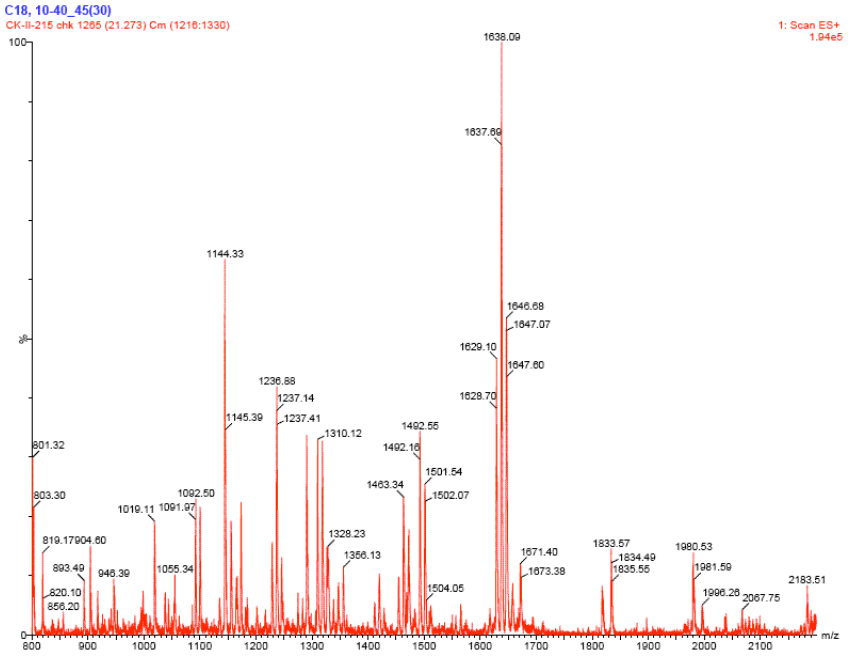
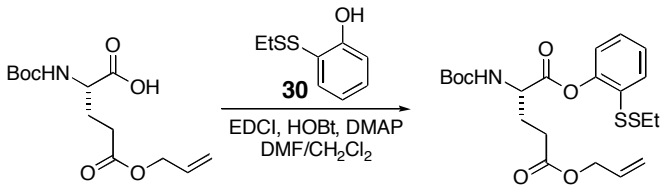


Figure S6: ESI-MS of compound **18**



29

$C_{13}H_{21}NO_6$
Exact Mass: 287.1369
Mol. Wt.: 287.3089

31

$C_{21}H_{29}NO_6S_2$
Exact Mass: 455.1436
Mol. Wt.: 455.5881

To a solution of *o*-SSEt-phenol (**30**) (100 mg, 0.54 mmol), Boc-Glu(OAl)-OH (**29**) (231 mg, 0.81 mmol) in DMF/CH_2Cl_2 (3 mL, 1:1) were added EDCI (154 mg, 0.81m mmol), HOBt (109 mg, 0.81 mmol), and DMAP (3 mg, 25 μmol). The colorless reaction was stirred at rt for 21 hours. The reaction was concentrated in vacuo and azeotroped with toluene (4 x 3mL). Purification by flash chromatography (silica gel, 12% EtOAc/Hexanes) provided the desired product **31** (194 mg, 79%) as a colorless oil.

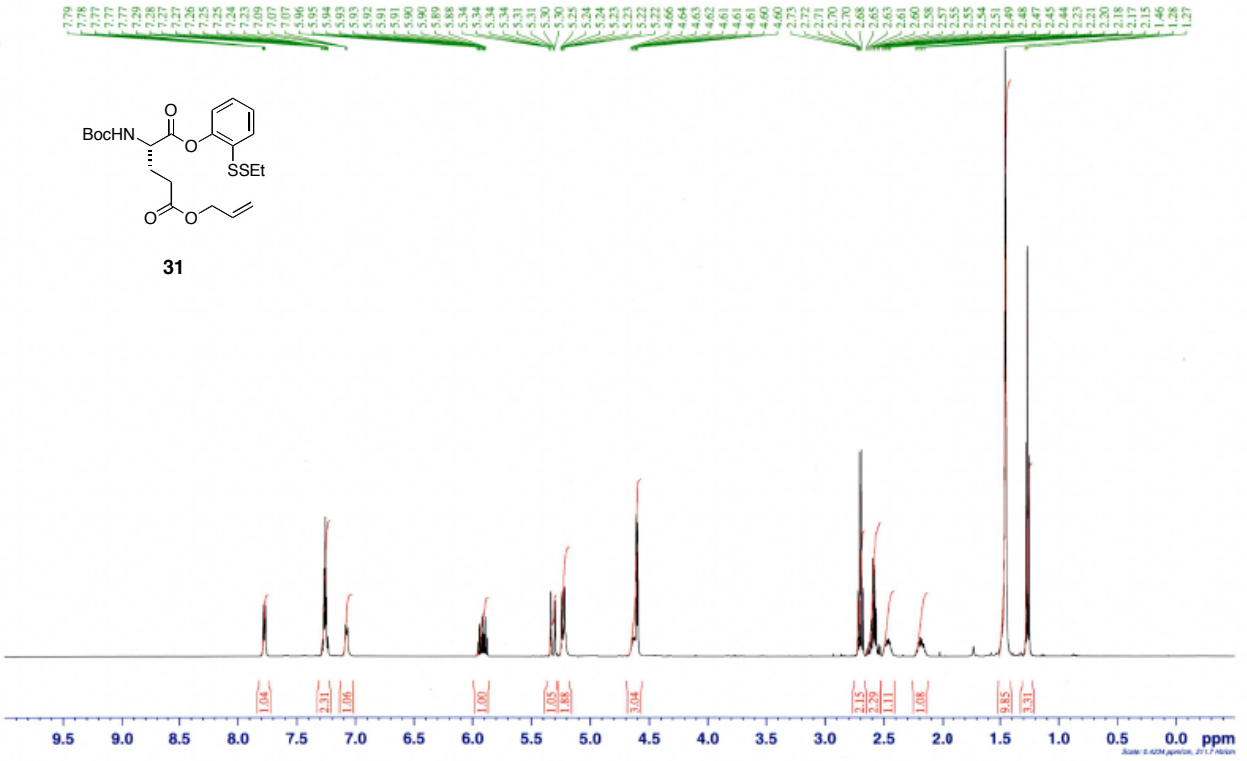
IR (liquid film): υ 3365 (br), 2977, 2930, 1770, 1734, 1716 cm^{-1}.

$[\alpha]_D^{20.0}$ = –29.1° (*c* 1.02, CH_2Cl_2)

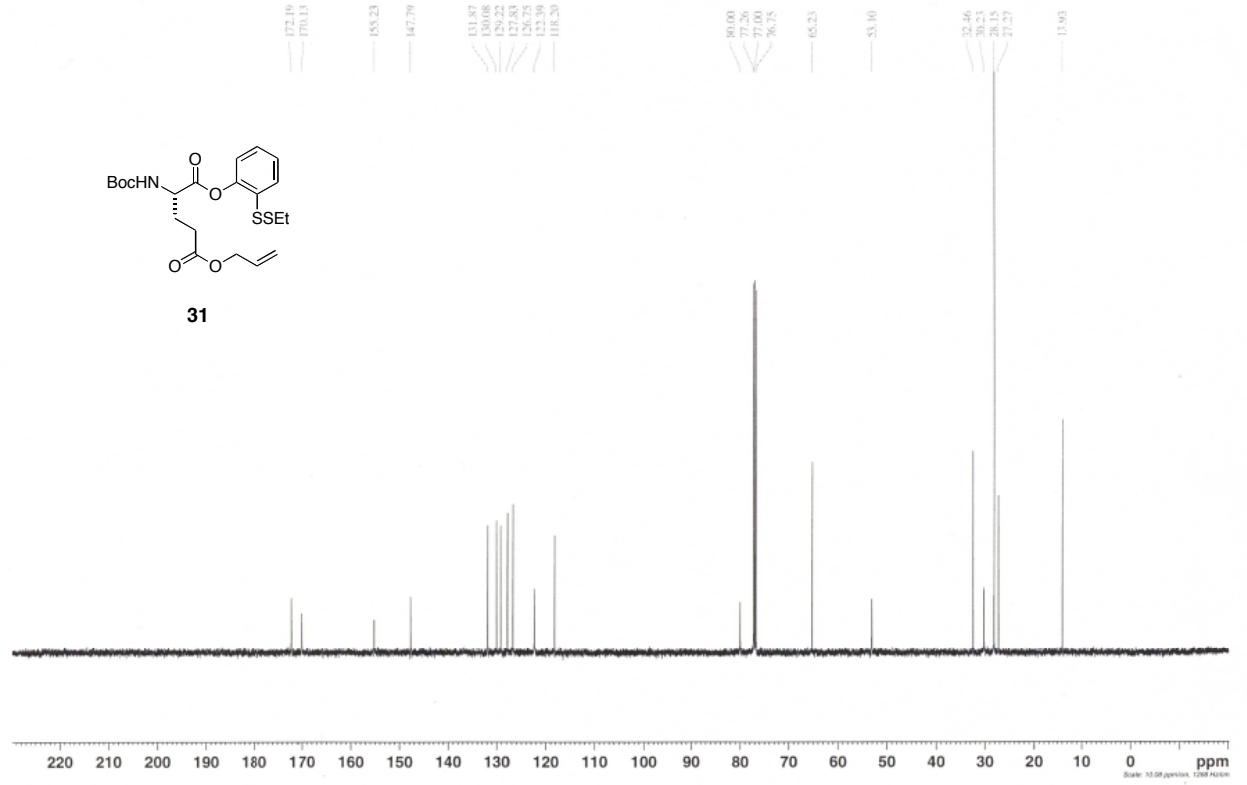
^{1}H NMR (500MHz, $CDCl_3$): δ 7.77 (m, 1H), 7.23-7.29 (m, 2H), 7.08 (m, 1H), 5.92 (ddt, 1H, *J* = 17.1, 10.5, 5.7 Hz), 5.32 (ddt, 1H, *J* = 17.2, 3.0, 1.3 Hz), 5.20-5.25 (m, 2H), 4.61-4.67 (m, 1H), 4.61 (dt, 2H, *J* = 5.7, 1.3 Hz), 2.70 (q, 2H, *J* = 7.3 Hz), 2.53-2.65 (m, 2H), 2.47 (m, 1H), 2.19 (m, 1H), 1.46 (s, 9H), 1.27 (t, 3H, *J* = 7.3 Hz).

^{13}C NMR (125MHz, $CDCl_3$): δ 172.2, 170.1, 155.2, 147.8, 131.9, 130.1, 129.2, 127.8, 126.7, 122.4, 118.2, 80.0, 65.2, 53.1, 32.5, 30.2, 28.1 (3C), 27.3, 13.9.

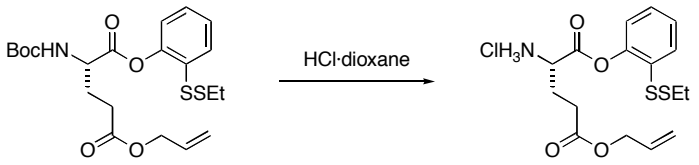
HRMS (ESI+ TOF): Calcd for $C_{21}H_{30}NO_6S_2$ (M+H): 456.1509. Found: 456.1515


BocHN
O
O
SSEt
O
O
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm

31


BocHN
O
O
SSEt
O
O
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

31



31

$C_{21}H_{29}NO_6S_2$
Exact Mass: 455.1436
Mol. Wt.: 455.5881

32

$C_{16}H_{22}ClNO_4S_2$
Exact Mass: 391.0679
Mol. Wt.: 391.9332

31 (269 mg, 0.59 mmol) was taken up in HCl·dioxane (7 mL, 4.0 M solution). The clear solution was stirred at rt for 1 hour. The solvent was removed under a stream of nitrogen and the residue triturated with Et_2O. The Et_2O was removed under a stream of nitrogen to give **32** as a pale-yellow oil, which solidified upon placing under high vacuum (218 mg, 94%).

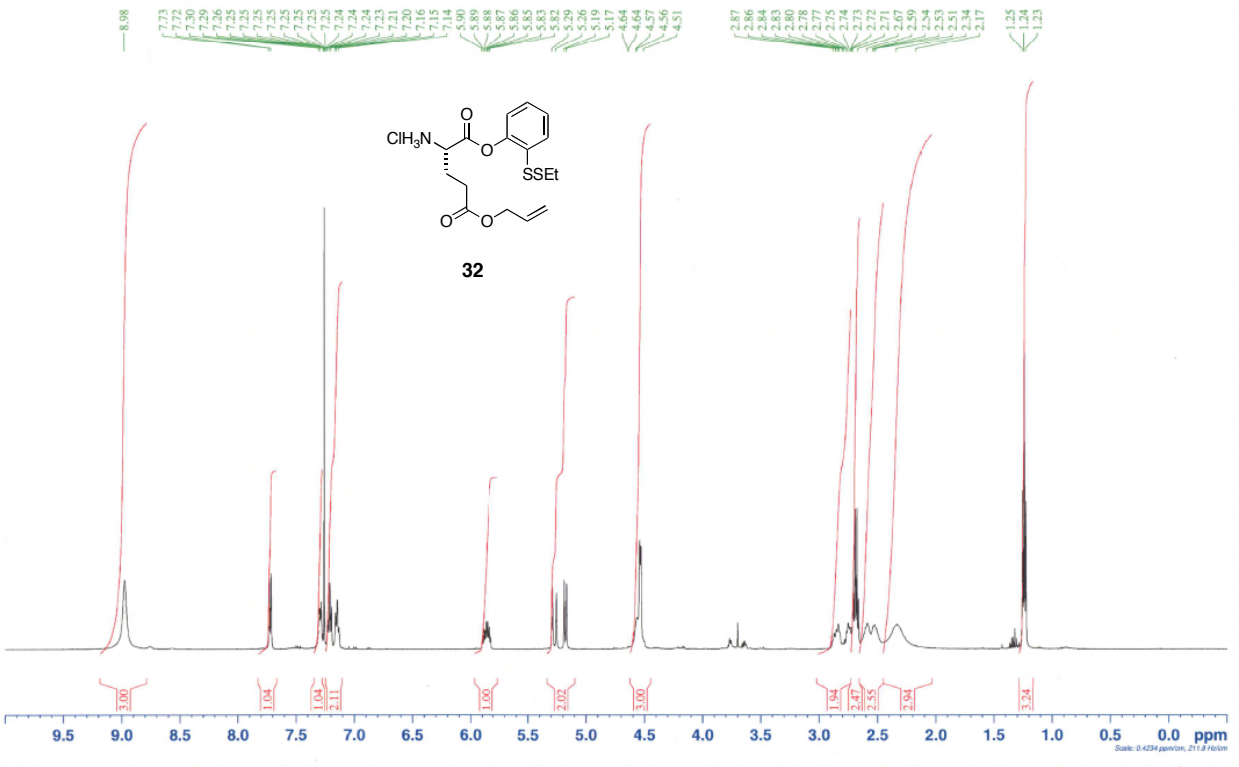
IR (liquid film): υ 2927 (br), 1767, 1738 cm^{-1}.

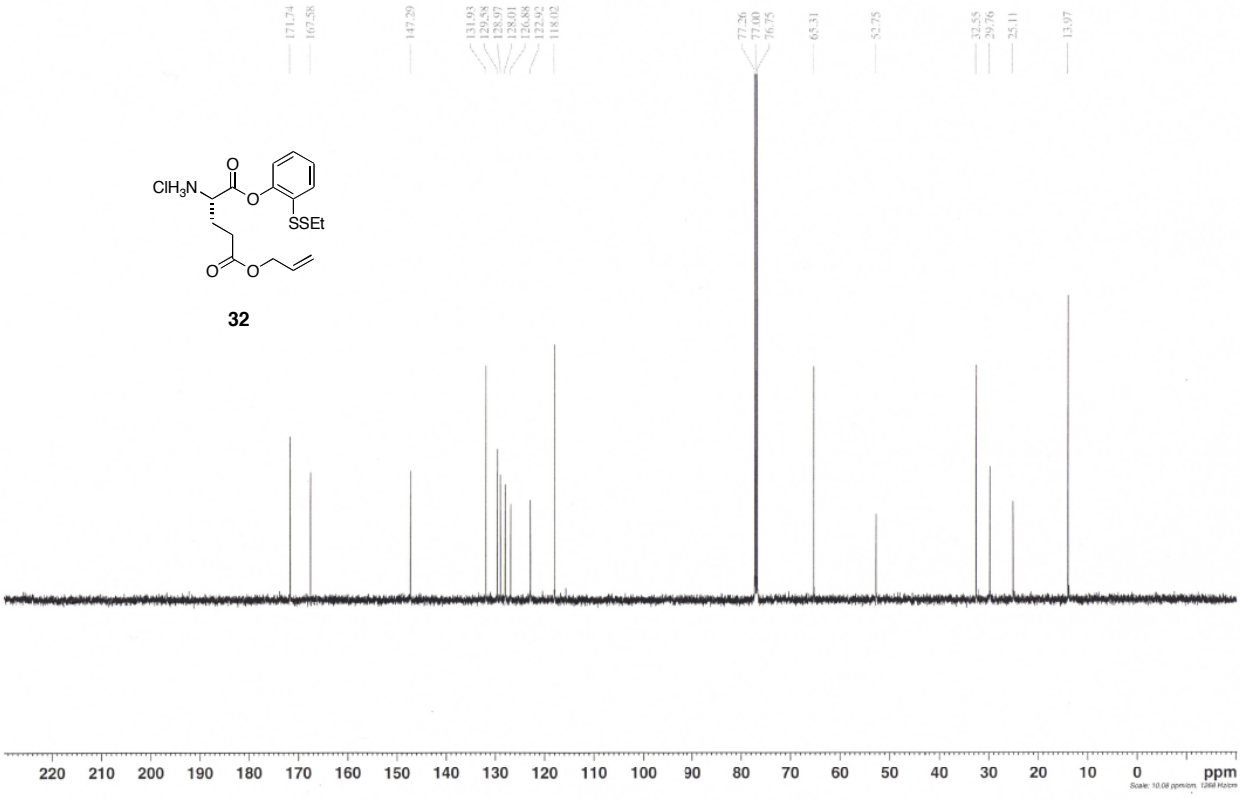
$[\alpha]_D^{20.0} = -42.9°$ (*c* 0.79 , CH_2Cl_2)

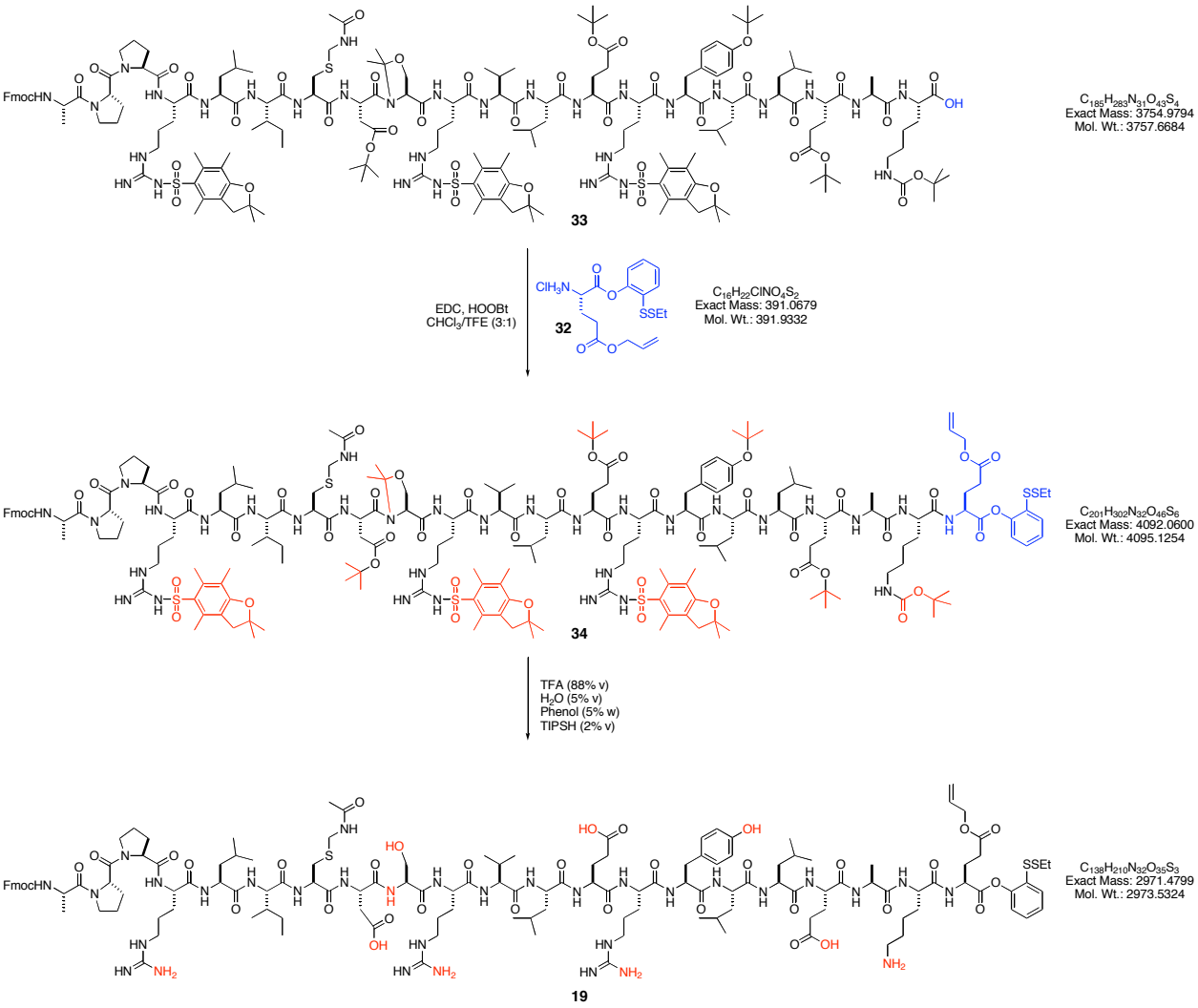
^{1}H NMR (500MHz, $CDCl_3$): δ 8.98 (br s, 3H), 7.72 (d, 1H, *J* = 7.7 Hz), 7.28-7.31 (m, 1H), 7.19-7.24 (m, 1H), 7.13-7.18 (m, 1H), 5.87 (ddt, 1H, *J* = 17.0, 10.5, 5.6 Hz), 5.27 (d, 1H, *J* = 17.2 Hz), 5.18 (d, 1H, *J* = 10.4 Hz), 4.53-4.60 (m, 1H), 4.53 (br d, 2H, *J* = 5.3 Hz), 2.81-2.90 (m, 1H), 2.70-2.77 (m, 1H), 2.68 (q, 2H, *J* = 7.3 Hz), 2.55-2.65 (m, 1H), 2.47-2.55 (m, 1H), 1.24 (t, 3H, *J* = 7.3 Hz).

^{13}C NMR (125MHz, $CDCl_3$): δ 171.7, 167.6, 147.3, 131.9, 129.6, 129.0, 128.0, 126.9, 122.9, 118.0, 65.3, 52.7, 32.5, 29.8, 25.1, 14.0.

HRMS (ESI+ TOF): Calcd for $C_{16}H_{22}NO_4S_2$ (M-Cl): 356.0985. Found: 356.0909


ClH3N
O
O
SSEt
O
O
32


ClH3N
O
O
SSEt
O
O
32



Peptide **33** was prepared using Fmoc-Lys(Boc)-NovaSyn® TGT resin and Fmoc-Asp(OtBu)-Ser ($\psi^{Me,Me}$pro)-OH following the general SPPS procedure. To a solution of peptide **33** (245 mg, 0.35 mmol) and H-Glu(OAll)-O(*o*-SSEt-phenyl) **32** (10 mg, 0.024 mmol) in $CHCl_3$/CF_3CH_2OH (1.2 mL, 3:1) at rt was added EDC (3.5 μL, 0.020 mmol) and HOOBt (3.3 mg, 0.020 mmol). The clear, golden-yellow solution was stirred at rt 3 hours. The reaction was concentrated under a stream of air to give a yellow oil, which was placed under high vacuum for 1 hour.

The crude oil was then taken up in a deprotection cocktail (2.0 mL) consisting of trifluoroacetic acid (88% by volume), water (5% by volume), phenol (5% by weight), and iPr$_3$SiH (2% by volume). The clear orange solution was stirred at rt for 2.5 hours. The reaction

was transferred to a polypropylene vial and concentrated under a stream of air. The resulting red residue was triturated with cold Et_2O (3 x 8 mL), centrifuged, and the Et_2O carefully decanted. The remaining peach-colored solid was purified by RP-HPLC (C4 semiprep, 46% MeCN/H2O, 16 mL/min, 265 nm). Product eluted from 18-22 minutes. The fractions were collected, concentrated, and lyophilized to provide **19** (8.6 mg, 36% over two steps) as a white solid.

ESI calcd for $C_{138}H_{210}N_{32}O_{35}S_3$ $[M+2H]^{2+}$ *m/z:* 1486.75, $[M+3H]^{3+}$ *m/z:* 991.50, found: 1487.53, 992.14

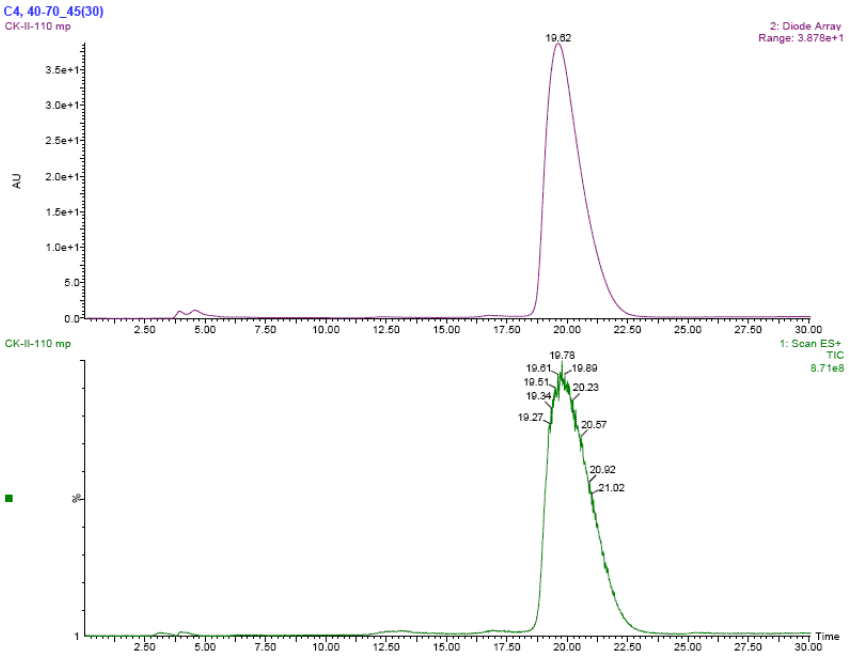


Figure S7: UV and MS traces from LC-MS analysis of compound **19**; gradient: 40-70% MeCN/H_2O over 30 min at a flow rate of 0.2 mL/min, Varian Microsorb C4 column

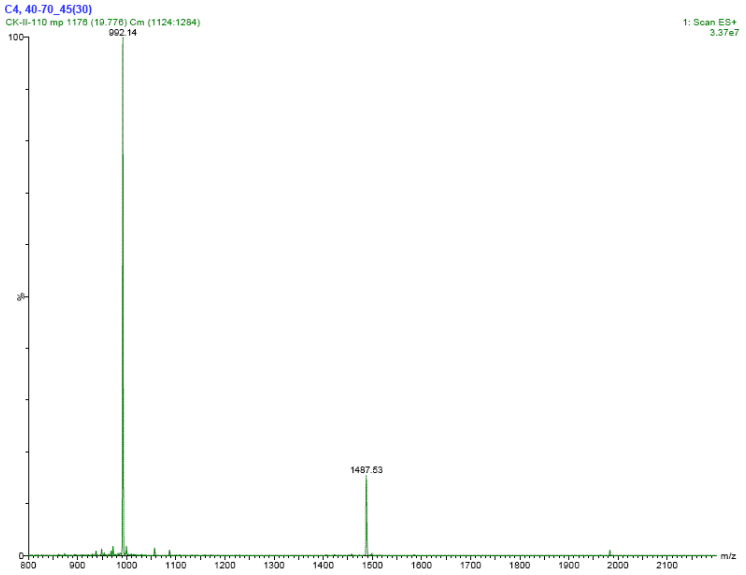
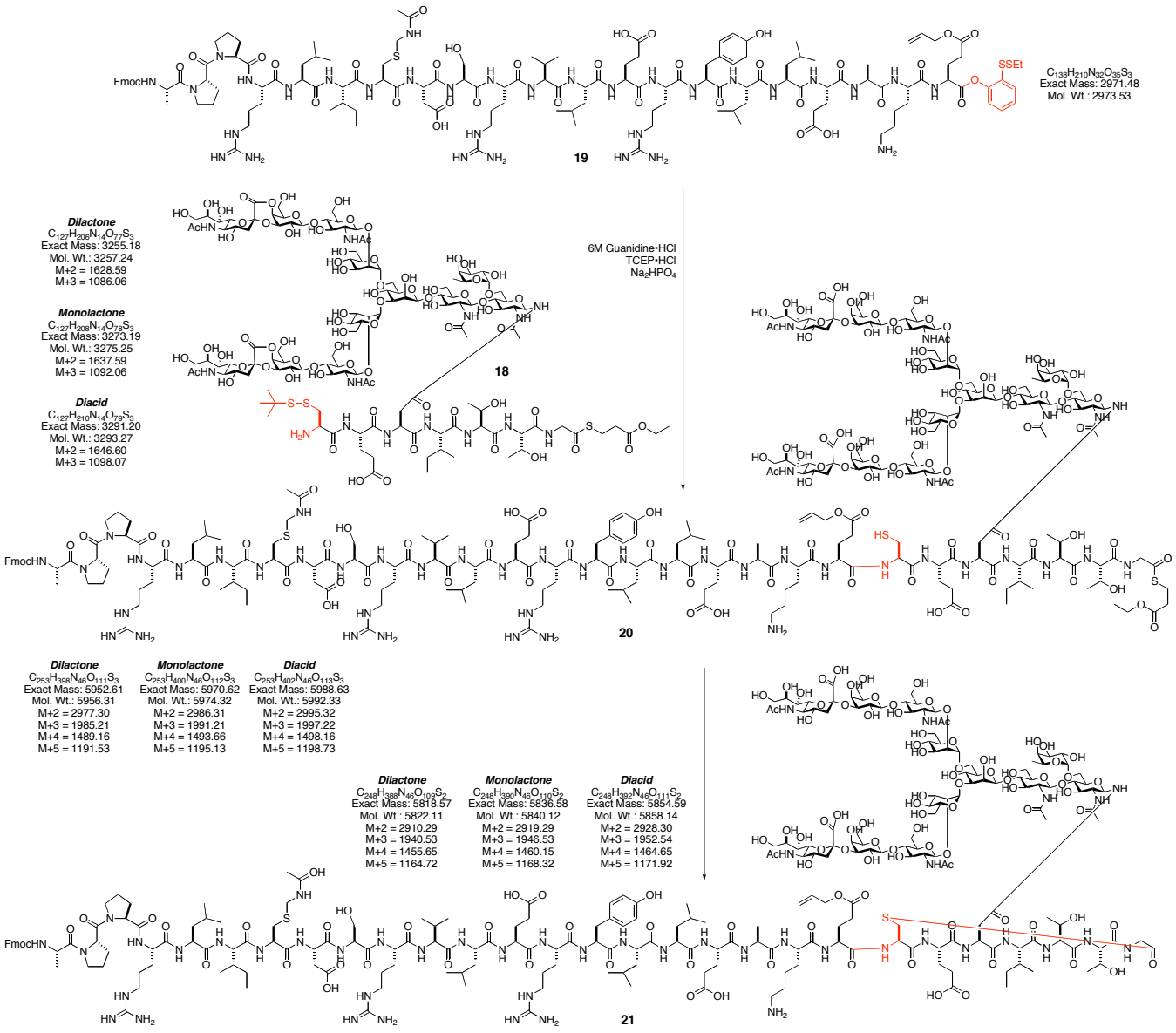


Figure S8: ESI-MS of compound **19**



A stock solution of 6M guanidine·HCl was first prepared by dissolving guanidine·HCl (2.865 g, 30 mmol) in water until a final volume of 5 mL was reached. A second stock solution was prepared by dissolving Na_2HPO_4 (28.4 mg, 0.20 mmol) and TCEP·HCl (5.4 mg, 0.019 mmol) in an aliquot of the 6M guanidine·HCL (1 mL).

Peptide **19** (3.5 mg, 1.2 μmol) and glycopeptide **18** (1.8 mg, 0.55 μmol) were dissolved in an aliquot of the guanidine·HCl/Na_2HPO_4/TCEP·HCl solution (300 μL, 1.8 mM). Then TCEP (20 μL, 0.5M solution) was added. The reaction was stirred at rt for 24 hours. The reaction was

quenched by addition of MeCN/H_2O (1 mL, 1:1, 0.05% TFA). The mixture was purified via RP-HPLC (C18 semiprep, 25% → 55% MeCN/H_2O, 0 → 30 min, 16 mL/min, λ = 265 nm). Product eluted at 25-27 min. The fractions were collected, concentrated, and lyophilized to provide **21** (0.7 mg, 20%) as a white solid.

Diacid: ESI calcd for $C_{248}H_{392}N_{46}O_{111}S_2$ $[M+3H]^{3+}$ *m/z:* 1952.54, $[M+4H]^{4+}$ *m/z:* 1464.65, found: 1953.00, 1465.71.

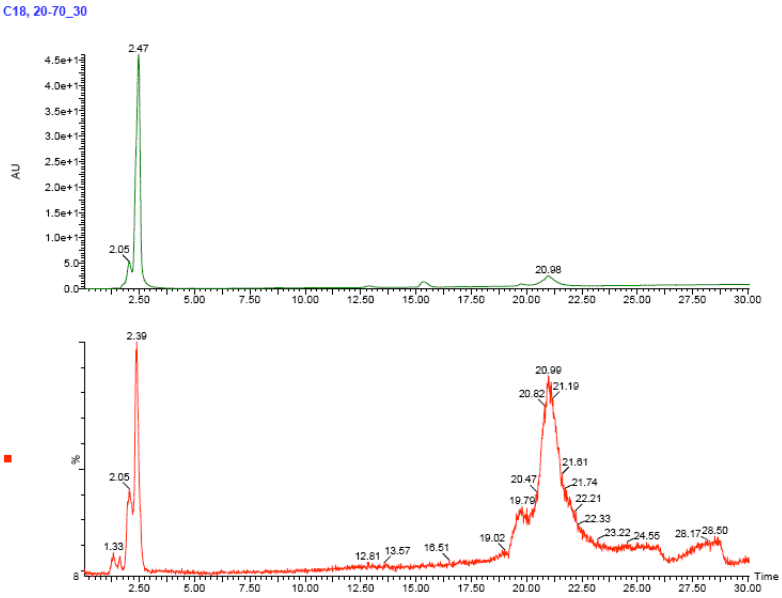


Figure S9: UV and MS traces from LC-MS analysis of compound **21**; gradient: 20-70% MeCN/H_2O over 30 min at a flow rate of 0.2 mL/min, Varian Microsorb C18 column

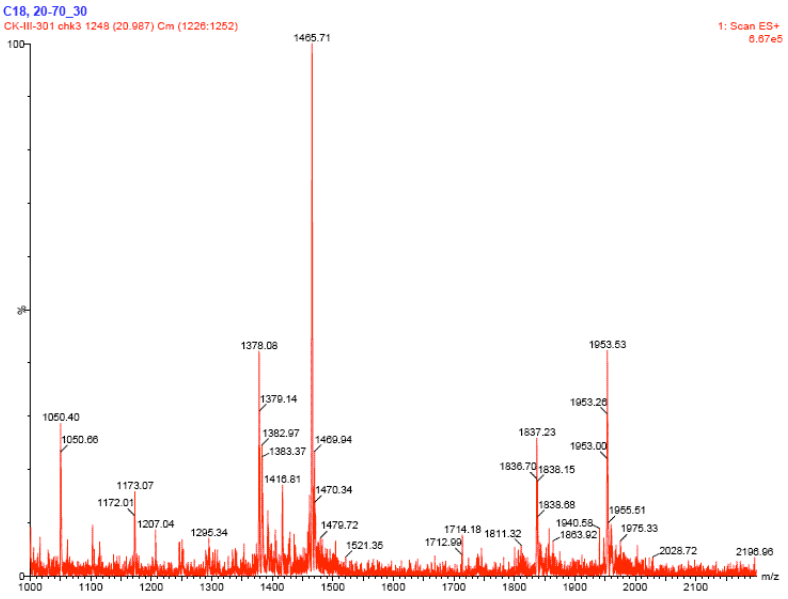
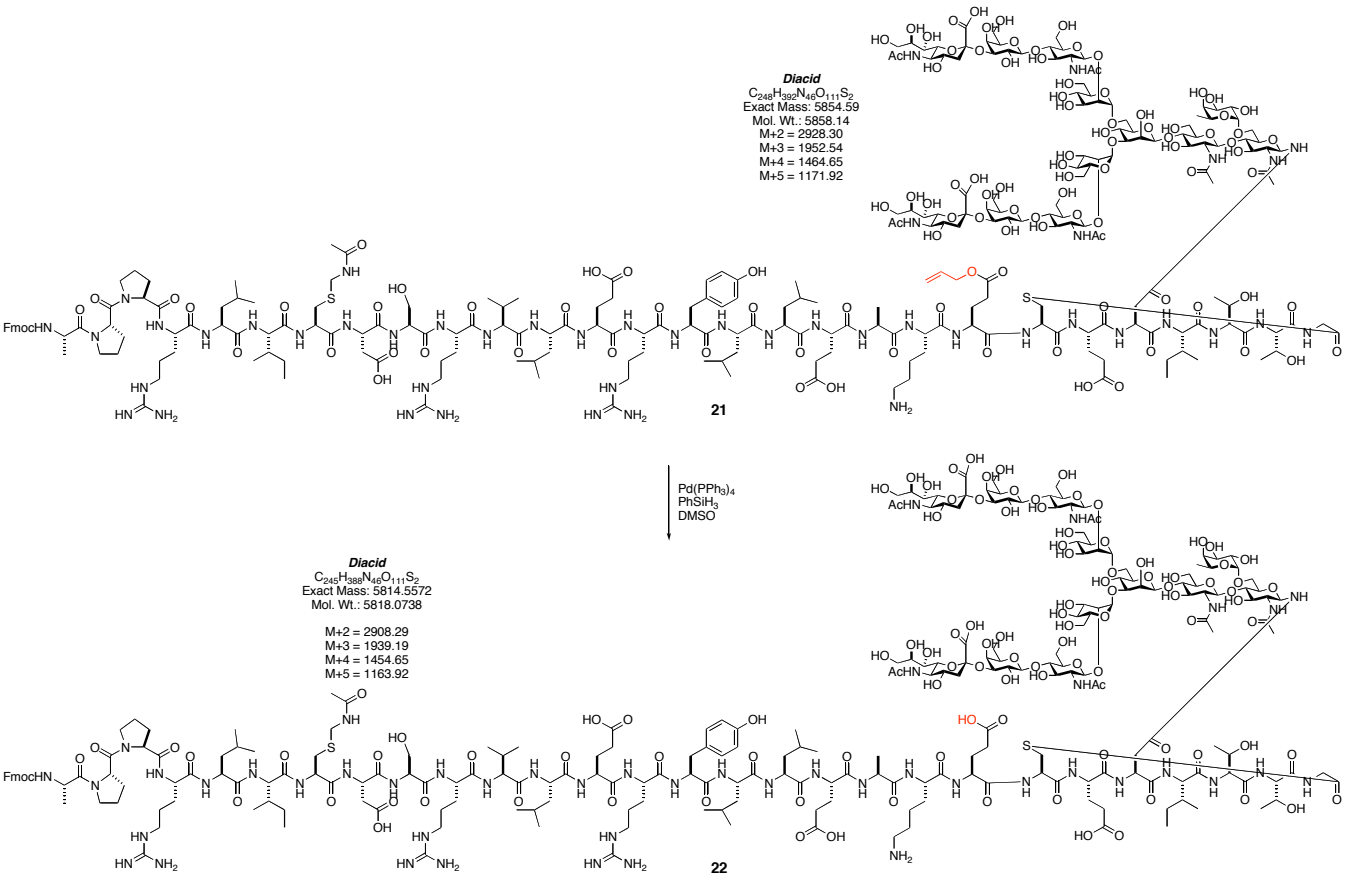


Figure S10: ESI-MS of compound **21**



To a solution of peptide **21** (0.7 mg, 0.12 μmol) and $Pd(PPh_3)_4$ (~0.2 mg, 0.12 μmol) in DMSO (200 μL) was added $PhSiH_3$ (0.6 μL, 4.8 μmol). The light yellow, clear solution was stirred at rt for 8 minutes. The reaction was quenched by addition of MeCN/H_2O (1 mL, 1:1, 0.05% TFA). The mixture was purified via RP-HPLC (C4 semiprep, 30% → 50% MeCN/H_2O, 0 → 30 min, 16 mL/min, λ = 265 nm). Product eluted at 16-18 min. The fractions were collected, concentrated, and lyophilized to provide **22** (0.7 mg, >99%) as a white solid.

Diacid: ESI calcd for $C_{245}H_{388}N_{46}O_{111}S_2$ $[M+3H]^{3+}$ *m/z:* 1939.19, $[M+4H]^{4+}$ *m/z:* 1454.65, found: 1939.69, 1455.14.

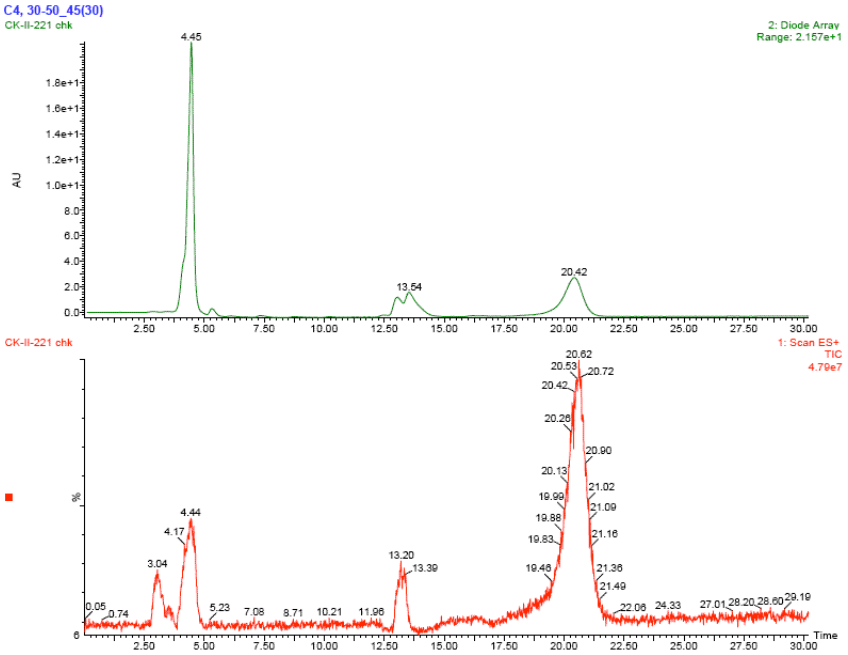


Figure S11: UV and MS traces from LC-MS analysis of compound **22**; gradient: 30-50% MeCN/H_2O over 30 min at a flow rate of 0.2 mL/min, Varian Microsorb C4 column

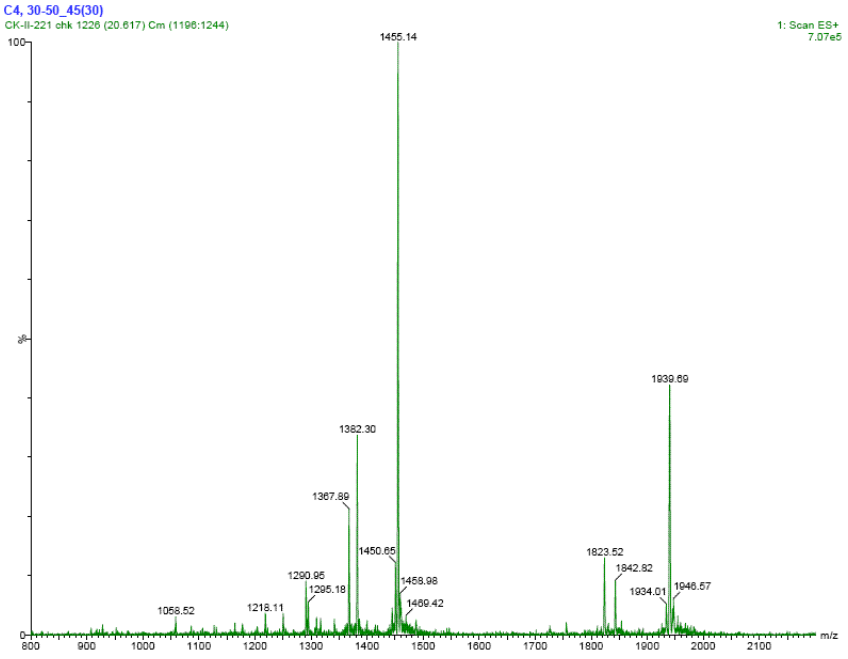
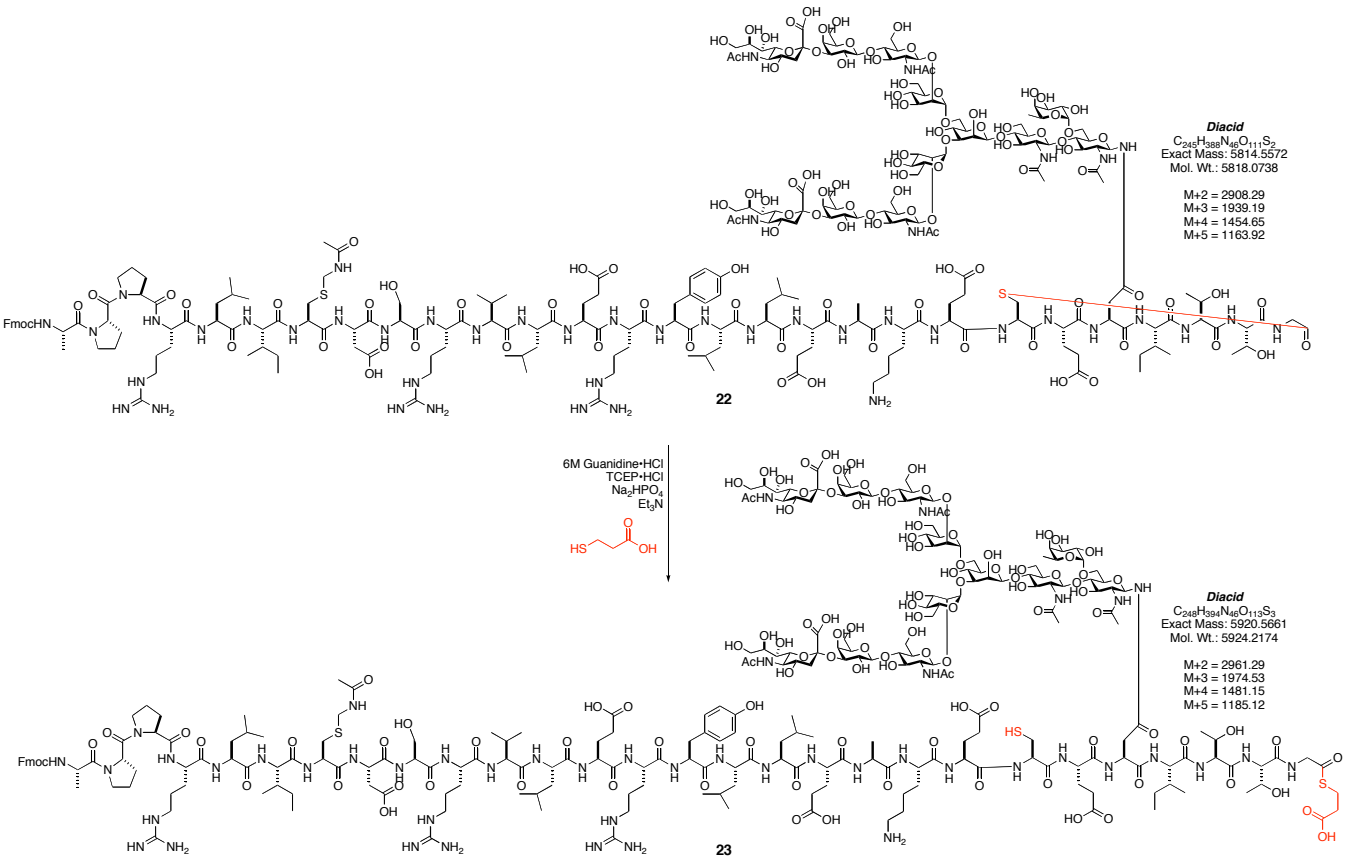


Figure S12: ESI-MS of compound **22**



A stock solution of 6M guanidine·HCl was first prepared by dissolving guanidine·HCl (2.865 g, 30 mmol) in water until a final volume of 5 mL was reached. A second stock solution was prepared by dissolving Na_2HPO_4 (28.4 mg, 0.20 mmol) and TCEP·HCl (5.4 mg, 0.019 mmol) in an aliquot of the 6M guanidine·HCL (1 mL).

Glycopeptide **22** (0.7 mg, 0.12 μmol) was dissolved in an aliquot of the guanidine·HCl/Na_2HPO_4/TCEP·HCl solution (150 μL, 0.8 mM). Then thiopropionic acid (3 μL, 0.034 mmol) and triethylamine (4 μL, 0.029 mmol) were added. The reaction was stirred at rt for 18 hours. The reaction was quenched by addition of MeCN/H_2O (1 mL, 1:1, 0.05% TFA). The mixture was purified via RP-HPLC (C4 semiprep, 30% → 50% MeCN/H_2O, 0 → 30 min, 16 mL/min, λ = 265 nm). Product eluted at 16-18 min. The fractions were collected, concentrated, and lyophilized to provide **23** (0.5 mg, 70%) as a white solid.

Diacid: ESI calcd for $C_{248}H_{394}N_{46}O_{113}S_3$ $[M+3H]^{3+}$ *m/z:* 1974.53, $[M+4H]^{4+}$ *m/z:* 1481.15, found: 1975.38, 1481.85.

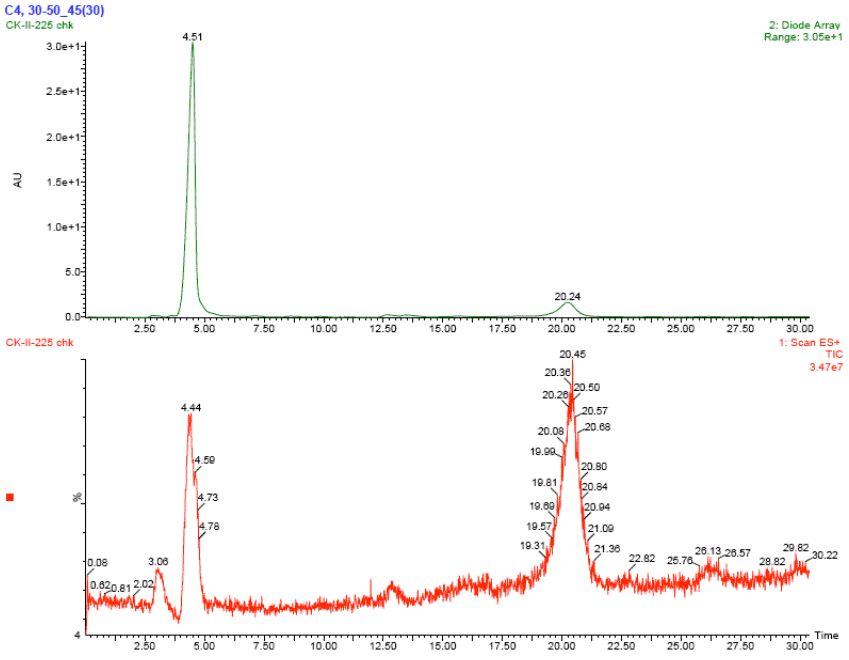


Figure S13: UV and MS traces from LC-MS analysis of compound **23**; gradient: 30-50% MeCN/H_2O over 30 min at a flow rate of 0.2 mL/min, Varian Microsorb C4 column

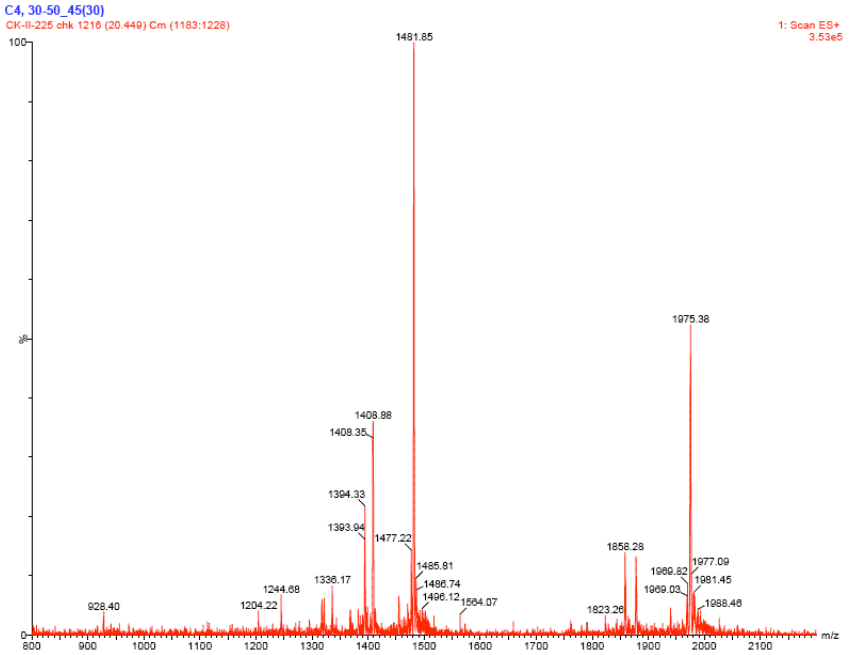
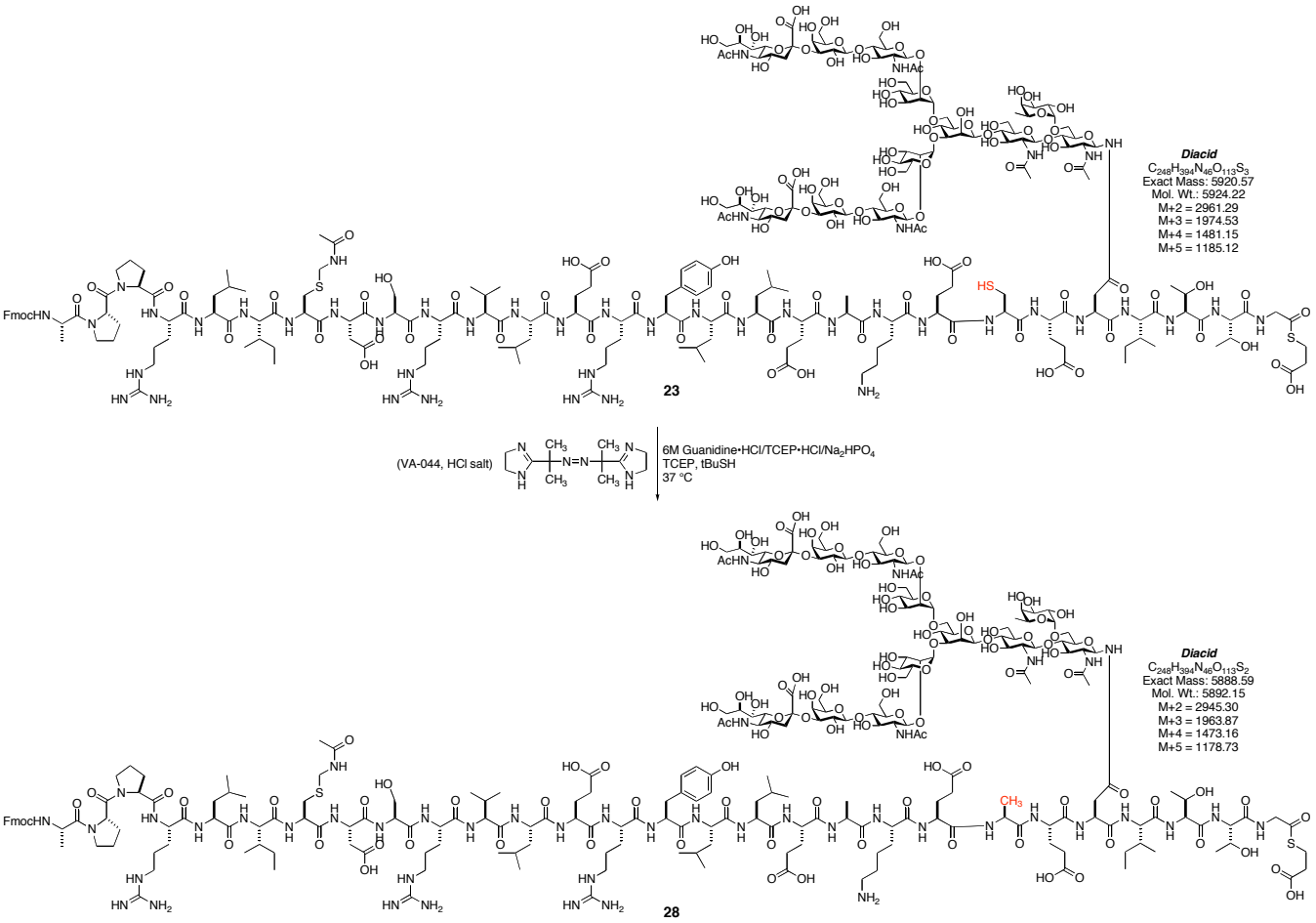


Figure S14: ESI-MS of compound **23**



A stock solution of 6M guanidine·HCl was first prepared by dissolving guanidine·HCl (2.865 g, 30 mmol) in water until a final volume of 5 mL was reached. A second stock solution was prepared by dissolving Na_2HPO_4 (28.4 mg, 0.20 mmol) and TCEP·HCl (5.4 mg, 0.019 mmol) in an aliquot of the 6M guanidine·HCL (1 mL). The solution was degassed under argon. A stock solution of the radical initiator, VA-044, was prepared by dissolving VA-044 (32 mg, 0.13 mmol) in water (1 mL). The solution was degassed under argon.

Glycopeptide **23** (~0.45 mg, 0.08 μmol) was dissolved in an aliquot of the guanidine·HCl/Na_2HPO_4/TCEP·HCl solution (100 μL). Then TCEP (50 μL, 0.5M solution), an

aliquot of the VA-044 solution (50 μL), and *tert*-butylthiol (20 μL) were added. The reaction was heated at 37 °C for 3.5 hours. Thiopropionic acid (1.5 μL, 0.017 mmol), triethylamine (2.5 μL, 0.018 mmol), and additional VA-044 solution (50 μL) were added. The reaction continued to stir at 37 °C for 13.5 hours. The reaction was quenched by addition of MeCN/H_2O (1 mL, 1:1, 0.05% TFA). The mixture was purified via RP-HPLC (C18 semiprep, 25% → 60% MeCN/H_2O, 0 → 30 min, 16 mL/min, λ = 265 nm). Product eluted at 20 min. The fractions were collected, concentrated, and lyophilized to provide **28** (0.3 mg, 67%) as a white solid.

Diacid: ESI calcd for $C_{248}H_{394}N_{46}O_{113}S_2$ $[M+3H]^{3+}$ *m/z:* 1963.87, $[M+4H]^{4+}$ *m/z:* 1473.16, found: 1964.35, 1473.73.

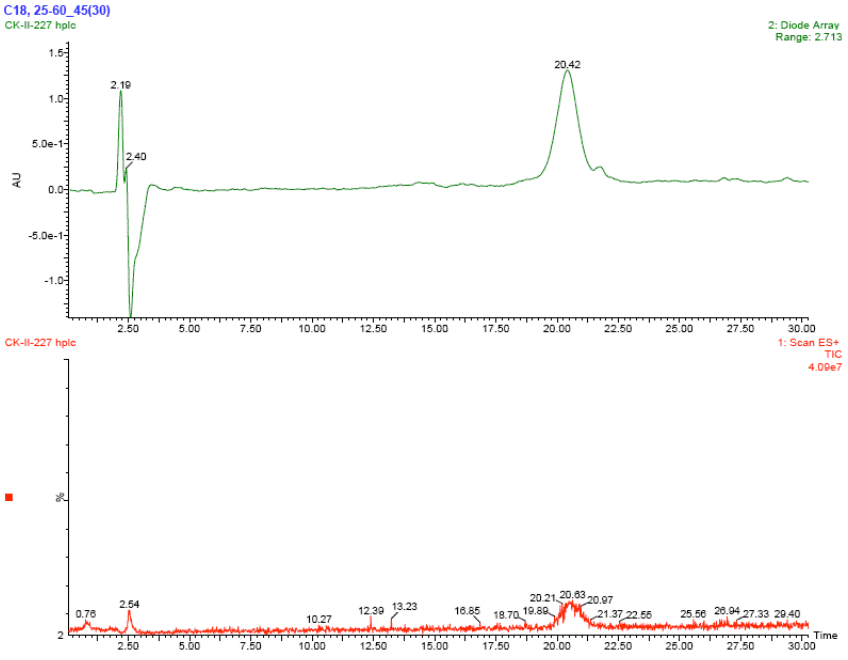


Figure S15: UV and MS traces from LC-MS analysis of compound **28**; gradient: 25-60% MeCN/H_2O over 30 min at a flow rate of 0.2 mL/min, Varian Microsorb C18 column

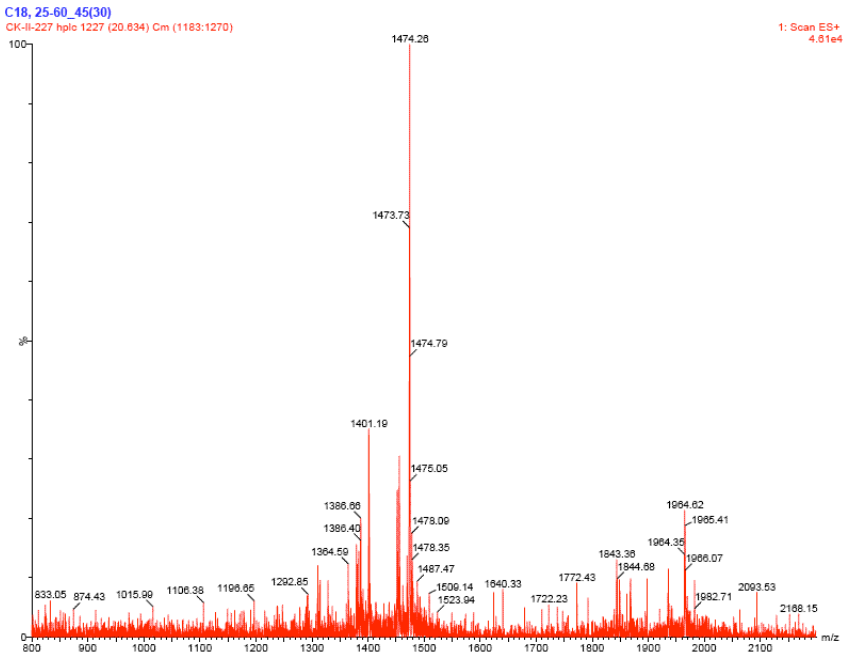


Figure S16: ESI-MS of compound **28**

Complete Citation for Reference 6:

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