Chemistry as an Expanding Resource in Protein Science: Fully Synthetic and Fully Active Human Parathyroid Hormone-Related Protein (1-141)**

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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, CH2Cl2, 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 13C) were recorded on a Bruker Advance II 600 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). LCMS analyses were performed using a Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector equipped with Varian Microsorb 100-5, C18 150x2.0mm and Varian Microsorb 300-5, C4 250x2.0mm columns at a flow rate of 0.2 mL/min. UPLC-MS analyses were performed using a Waters Acquity^{TM} Ultra Preformance LC system equipped with Acquity UPLC$^{®}$ BEH C18, 1.7μl, 2.1 x 100 mm, Acquity UPLC® BEH C8, 1.7 μl, 2.1 x 100 mm, Acquity UPLC® BEH 300 C4, 1.7 μl, 2.1 x 100 mm columns at a flow rate of 0.3 mL/min. Preparative separations were performed using a Ranin HPLC solvent delivery system equipped with a Rainin UV-1 detector and Varian Dynamax using Varian Microsorb 100-5, C18 250x21.4mm and Varian Microsorb 300-5, C4 250x21.4mm columns at a flow rate of 16.0 mL/min.

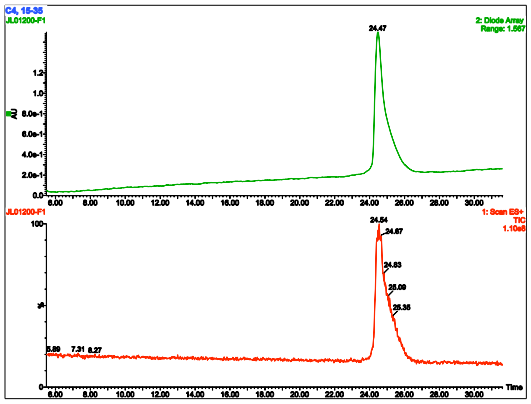
Solid Phase Peptide Synthesis

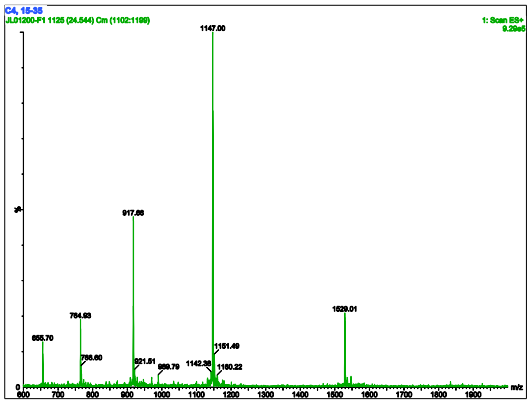
Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc protocols. The deblock mixture was a mixture of 100/5/5 of DMF/piperidine/DBU. The following Fmoc amino acids from NovaBiochem were employed: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Val-OH. Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH. Pseudoproline dipeptides were employed at position of Val^{2}-Ser^{3}, Lys^{13}-Ser^{14}, Asp^{113}-Ser^{114}, Asp^{128}-Thr^{129}, Asp^{137}-Ser^{138}.[1] Aspartimide preventing reagent Fmoc-Asp(Die)-OH was prepared by following literature procedure[2] and used at positions of Asp^{10}, Asp^{17}, Asp^{62}, Asp^{63}.

Synthesis of 2

2

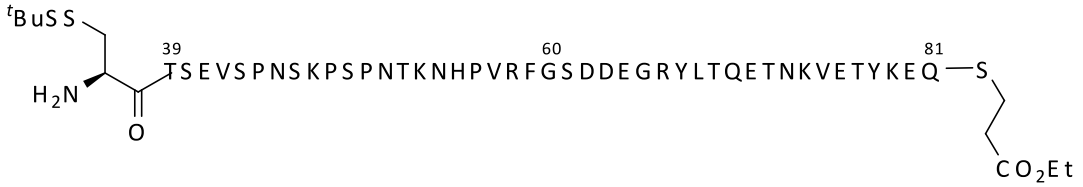
The fully protected peptide **6** (510. 00 mg, 67.6 μmol, 1.0 eq) was mixed with (2S)-1-(2-(ethylsulfinothioyl)phenoxy)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-guanidino)pentan-2-aminium chloride (85.37 mg, 2.0 eq) and HOOBt (22.06 mg, 2.0 eq) in the solvent (1.5 ml, $CHCl_3$/TFE= 3:1 v/v) and then cooled down to -10 ^oC. To the mixture was added slowly EDC (23.9 μl, 2.0 eq). The mixture was subsequently allowed to warm to 23 ^oC and stirred for 3 h, monitored with UPLC. The resulting mixture was treated with 5% HOAc (2.0 ml) in water and the organic layer was separated. The organic layer then was injected in a cocktail B solution (TFA/PhOH/*i*Pr_3SiH/H_2O 88:2:6:4 v/v, 30.0 ml) and stirred for 1.5 h. After that, the solution was then concentrated under N_2 stream and the crude product was precipitated by pouring in cold diethyl ether (30.0 ml). The suspension was centrifuged and the upper ether layer was decanted. The precipitated was purged with diethyl ether (2 x 30.0 ml) and the precipitated was dissolved in aq. MeCN (20.0 ml) and lypholized. The resulting crude product was further purified with preparative RP-HPLC (linear gradient 31-41% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 21.45 min) to afford 33.12 mg of peptide **2** (11% yield).





Peptide 2: Calcd for $C_{205}H_{325}N_{63}O_{53}S_2$: 4581.41 Da (average isotopes), $[M+3H]^{3+}$ *m/z* = 1528.14, $[M+4H]^{4+}$ *m/z* = 1146.35, $[M+5H]^{5+}$ *m/z* = 917.28, $[M+6H]^{6+}$ *m/z* = 764.57, $[M+7H]^{7+}$ *m/z* = 655.49.

Synthesis of 3

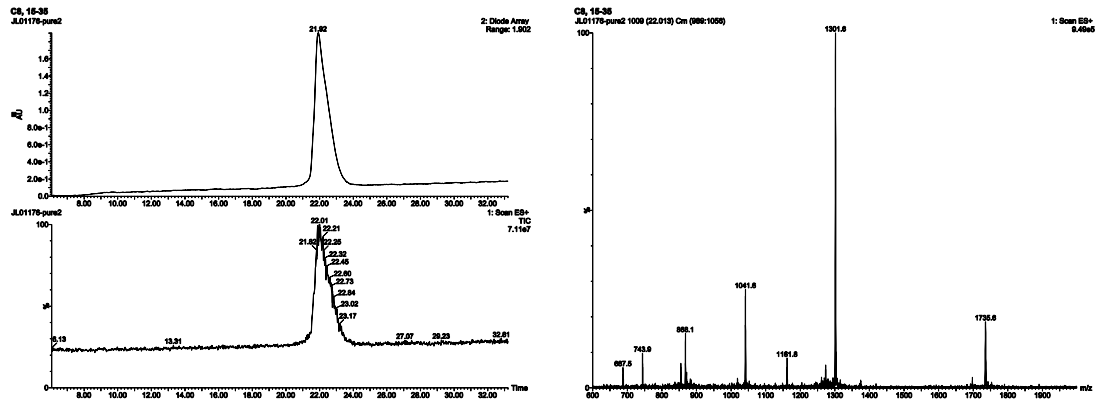


3

The crude fully protected peptide **8** (61.35 mg, 15.3 μmmol, 1.0 eq) from the Fmoc SPPS was mixed with H_2N-Gln(Trt)-S$(CH_2)_2$COOEt•HCl (12.4 mg, 1.5 eq), and HOOBt (3.74 mg, 1.5 eq) in a mixed solvent of TFE (150 μl) and $CHCl_3$ (450 μl) at -10 ^oC. To this mixture was added EDC (4.1 μl, 1.5 eq). The mixture was then allowed to warm up to 23 ^oC and stirred for 3 h. The reaction was monitored with UPLC and quenched with $CHCl_3$ (5.0 ml) and 5% HOAc/H_2O(5.0 ml). The organic layer was separated, dried (Na_2SO_4) and concentrated under reduced pressure.

The residue was dissolved in DMF (1.0 ml) and treated with piperidine (20% in DMF, 5.0 ml). The mixture was stirred for 7 min. and was then quenched with $CHCl_3$ (10 ml) and sat. $NaHCO_3$ solution (10 ml). The organic layer was separated , dried (Na_2SO_4) and concentrated under reduced pressure.

The resulting residue was then was combined with crude fully protected peptide **10**(68.53 mg, 1.0 eq, prepared from SPPS) and HOOBt (12.46 mg, 5.0 eq). The mixture was dissolved in $CHCl_3$ (300 μl). To this mixture was added EDC (13.5 μl, 5.0 eq) at -10 ^oC. The mixture was then allowed to warm up to 23 ^oC and stirred for 2.5 h. The reaction was monitored with UPLC and quenched with $CHCl_3$ (2.0 ml) and 5% HOAc/H_2O (2.0 ml). The organic layer was separated, dried (Na_2SO_4) and concentrated under reduced pressure. The residue was dissolved in cocktail B (20 ml) and stirred for 1.5 h. The resulting solution was concentrated with N_2 stream. The residue was triturate with cold Et_2O (2 X 20 ml) and purified with preparative RP-HPLC (linear gradient 12-25% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 19.55 min). 40.8 mg pure product **3** was obtained, overall yield 51%.

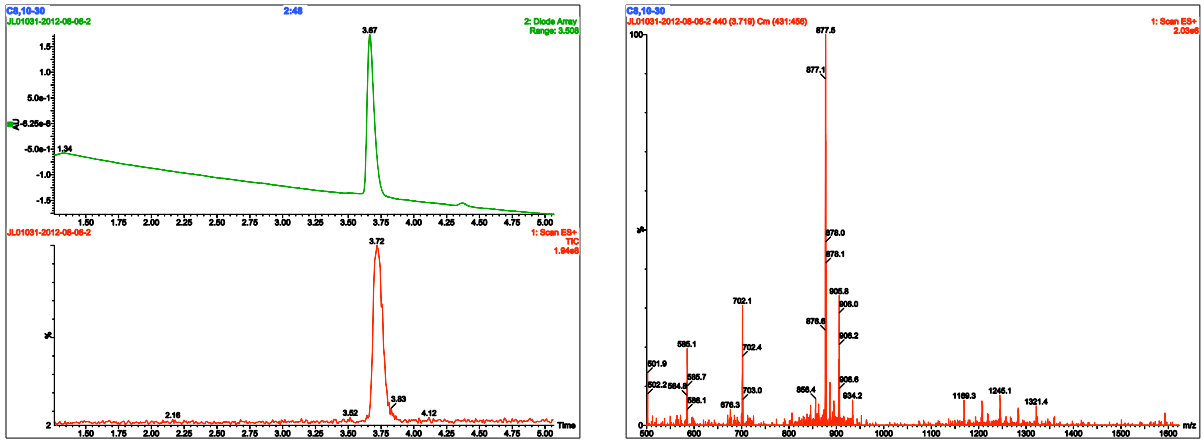


Peptide 3: Calcd for $C_{220}H_{346}N_{62}O_{78}S_3$: 5200.42 Da (average isotopes), $[M+3H]^{3+}$ *m/z* = 1734.47, $[M+4H]^{4+}$ *m/z* = 1301.11, $[M+5H]^{5+}$ *m/z* = 1041.08, $[M+6H]^{6+}$ *m/z* = 867.74, $[M+7H]^{7+}$ *m/z* = 743.92, $[M+8H]^{8+}$ *m/z* = 651.05.

Synthesis of 4

4

The crude fully protected peptide **7** (91.30 mg, 14.80 μmmol, 1.0 eq) from the Fmoc SPPS was mixed with H_2N-Ser(tBu)-O-(2-SSEt)Ph•HCl (8.12 mg, 1.5 eq), and HOOBt (3.62 mg, 1.5 eq) in a mixed solvent of TFE (185 μl) and $CHCl_3$ (555 μl) at -10 ^oC. To this mixture was added EDC (3.9 μl, 1.5 eq). The mixture was then allowed to warm up to 23 ^oC and stirred for 3 h. The reaction was monitored with UPLC and quenched with $CHCl_3$ (5.0 ml) and 5% HOAc/H_2O(5.0 ml). The organic layer was separated, dried (Na_2SO_4) and concentrated under reduced pressure. The residue was then dissolved in cocktail B (3.0 ml) and stirred for 40 min and then the solution was concentrated by N_2 stream. The residue was then triturated with Et_2O (2x 5 ml) and then purified with preparative RP-HPLC (linear gradient 10-30% solvent B over 30 min, Microsorb 100-8 C8 column, 16 ml/min, 230 nm, product eluted at about 16.24 min). 11.80 mg pure product **4** was obtained in 23% yield.

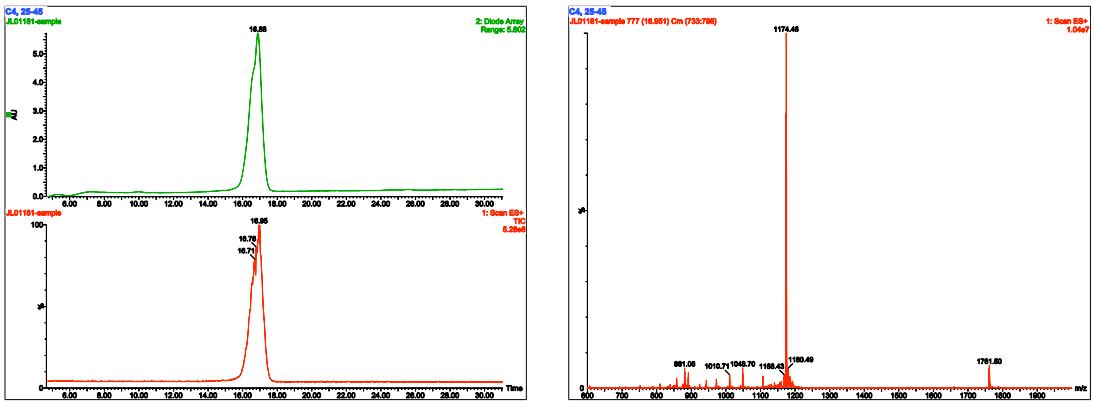


Peptide 4, Calcd for $C_{151}H_{272}N_{52}O_{37}S_3$: 3502.02 Da (average isotopes), $[M+3H]^{3+}$ m/z = 1168.34, $[M+4H]^{4+}$ m/z = 876.51, $[M+5H]^{5+}$ m/z = 701.40, $[M+6H]^{6+}$ m/z = 584.67.

Synthesis of 5

5

The synthesis of **5** was directly accomplished via Fmoc-SPPS (0.05 mmol scale). The crude product was purified with preparative RP-HPLC (linear gradient 20-40% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at ~ 20.33 min). 62.1 mg product was obtained (34% yield).

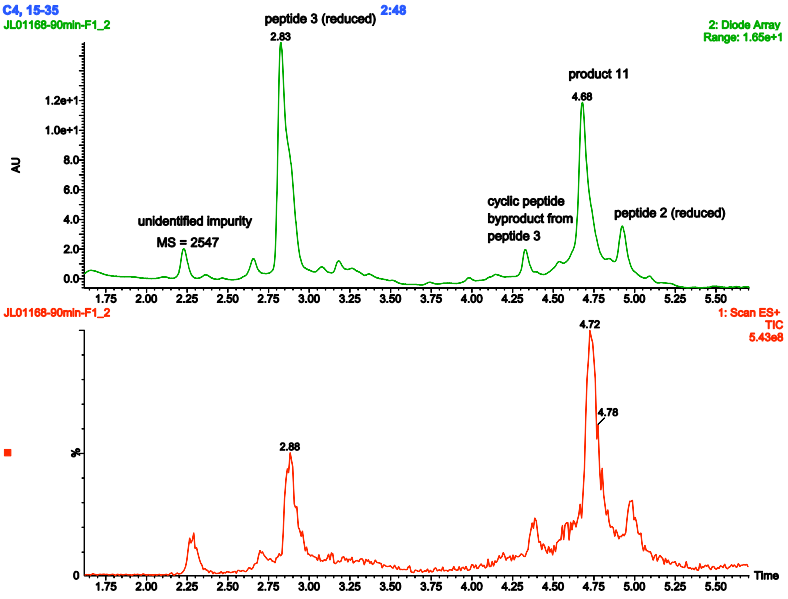


Peptide 5. Calcd for $C_{145}H_{231}N_{43}O_{55}S_2$: 3518.60 Da (average isotopes), $[M+2H]^{2+}$ m/z = 1760.30, $[M+3H]^{3+}$ m/z = 1173.87, $[M+4H]^{4+}$ m/z = 880.65.

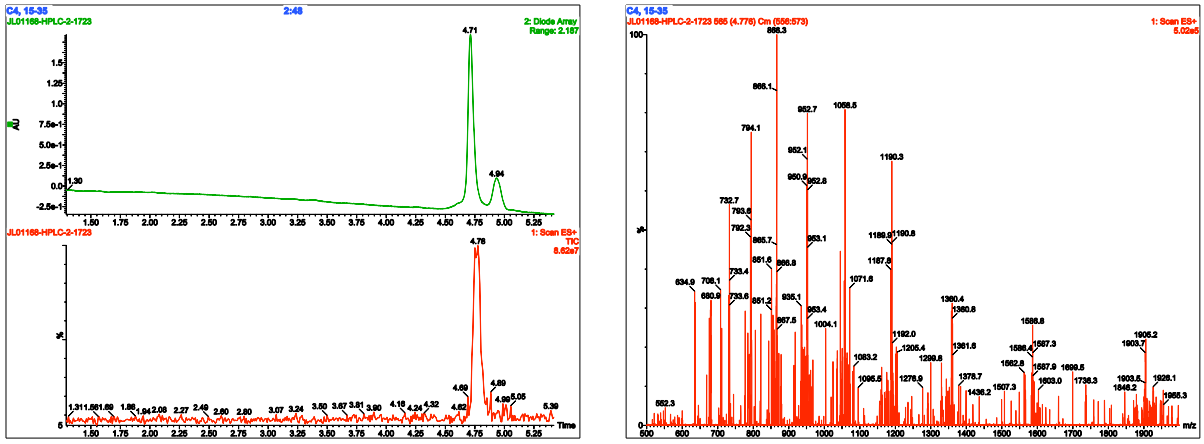
Synthesis of 11

11

Peptide **2** (3.02 mg, 0.66 μmol) and peptide **3** (4.29 mg, 1.25 eq) were dissolved in aq MeCN and lyophilized. To the resulting starting materials was added ligation buffer (329 μl, 6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, pH 7.2). The mixture was stirred under argon at 23 ^{o}C for 2 h, monitored with UPLC and then purified with preparative RP-HPLC (linear gradient 19-37% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 16.85 min) to afford 2.50 mg peptide **11** (40% yield).



UPLC monitoring spectrum of the NCL reaction between peptide **2** and **3**.

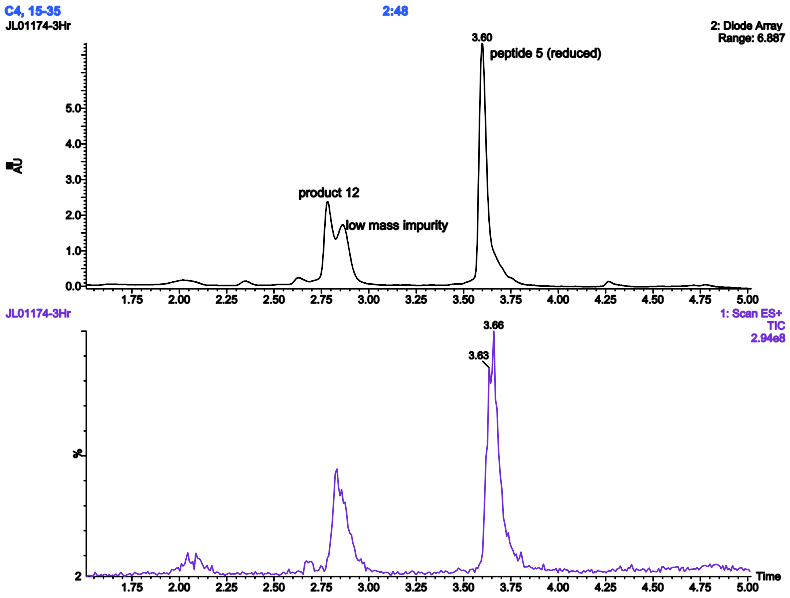


Peptide 11. Calcd for $C_{413}H_{653}N_{125}O_{130}S_2$, Expected Mass 9507.78, $[M+6H]^{6+}$ *m/z* = 1585.63, $[M+7H]^{7+}$ *m/z* = 1359.25, $[M+8H]^{8+}$ *m/z* = 1189.47, $[M+9H]^{9+}$ *m/z* = 1057.42, $[M+10H]^{10+}$ *m/z* = 951.78, $[M+11H]^{11+}$ *m/z* = 865.34, $[M+12H]^{12+}$ *m/z* = 793.32, $[M+13H]^{13+}$ *m/z* = 732.37, $[M+14H]^{14+}$ *m/z* = 680.13.

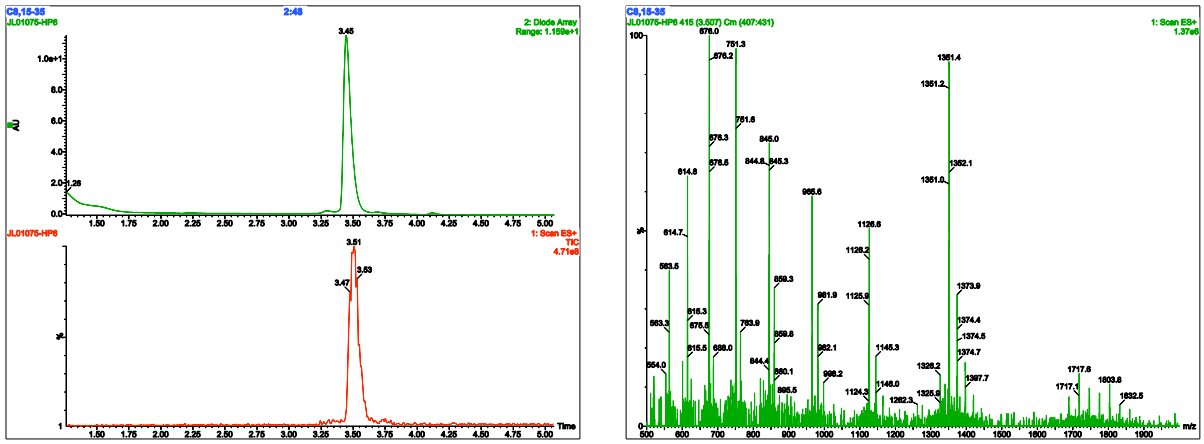
Synthesis of 12

12

Peptide **4** (1.80 mg, 0.51 μmol) and peptide **5** (2.64 mg, 1.50 eq) were dissolved in aq MeCN and lyophilized. To the resulting starting materials was added ligation buffer (400 μl, 6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, pH 7.2). The mixture was stirred under argon at 23 ^oC for 3 h, monitored with UPLC and then purified with preparative RP-HPLC (linear gradient 24-44% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 23.37 min). to afford 1.67 mg peptide **12** (49% yield).



UPLC monitoring spectrum of the NCL reaction between peptide **4** and **5**.

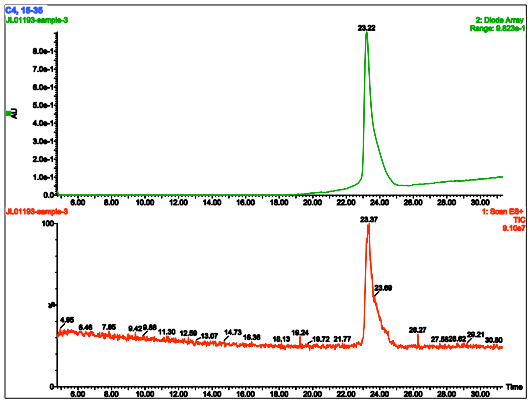


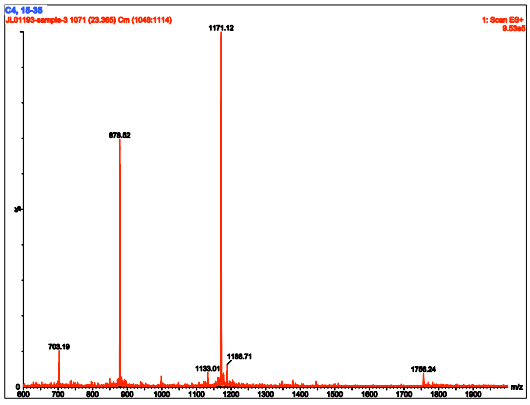
Peptide 12. Calcd for $C_{284}H_{485}N_{95}O_{91}S_2$, Expected Mass 6746.57, $[M+4H]^{4+}$ m/z = 1687.64, $[M+5H]^{5+}$ m/z = 1350.31, $[M+6H]^{6+}$ m/z = 1125.43, $[M+7H]^{7+}$ m/z = 964.8, $[M+8H]^{8+}$ m/z = 844.32, $[M+9H]^{9+}$ m/z = 750.62, $[M+10H]^{10+}$ m/z = 675.66, $[M+11H]^{11+}$ m/z = 614.32, $[M+12H]^{12+}$ m/z = 563.21.

Synthesis of 13

13

The fully protected peptide **15** (147.0 mg, 25.6 μmol, 1.0 eq) was mixed with (S)-ethyl 3-((2-amino-3-(4-(tert-butoxy)phenyl)propanoyl)thio)propanoate (18.12 mg, 2.0 eq) and HOOBt (7.96 mg, 2.0 eq) in the solvent (0.25 ml, $CHCl_3$/TFE= 3:1 v/v) and then cooled down to -10 ^oC. To the mixture was added slowly EDC (9.1 μl, 2.0 eq). The mixture was subsequently allowed to warm to 23 ^oC and stirred for 3 h, monitored with UPLC. The resulting mixture was treated with 5% HOAc (0.5 ml) in water and the organic layer was separated. The organic layer then was injected in a cocktail B solution (20.0 ml) and stirred for 1.5 h. After that, the solution was then concentrated under N_2 stream and the crude product was precipitated by pouring in cold diethyl ether (20.0 ml). The suspension was centrifuged and the upper ether layer was decanted. The precipitated was purged with diethyl ether (2 x 20.0 ml) and the precipitated was dissolved in aq. MeCN (15.0 ml) and lypholized. The resulting crude product was further purified with preparative RP-HPLC (linear gradient 20-30% solvent B over 30 min, Microsorb 100-8 C8 column, 16 ml/min, 230 nm, product eluted at about 20.01 min) to afford 25.34 mg of peptide **13** (29% yield).





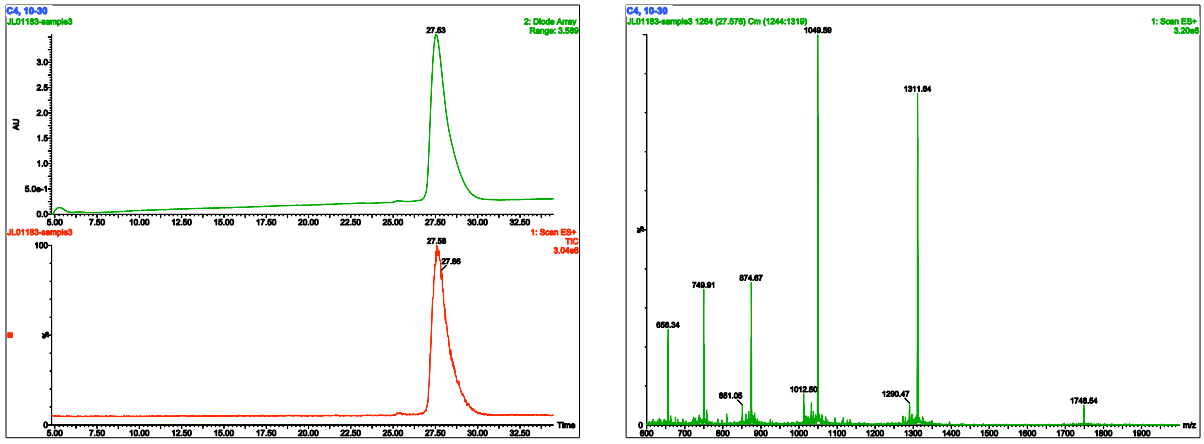
Peptide 13. Calcd for $C_{147}H_{229}N_{43}O_{51}S_3$: 3508.58, $[M+2H]^{2+}$ *m/z* = 1755.29, $[M+3H]^{3+}$ *m/z* = 1170.53, $[M+4H]^{4+}$ *m/z* = 878.15, $[M+5H]^{5+}$ *m/z* = 702.72.

Synthesis of 14

14

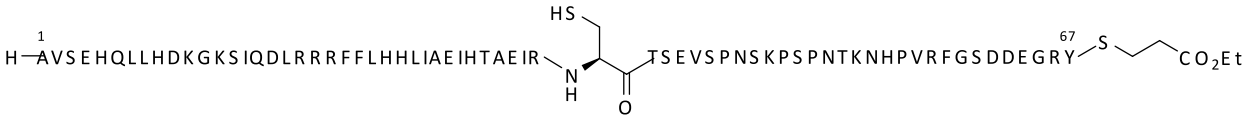
The peptide resin **16** (0.10 mmol, 1.0 eq) from the Fmoc SPPS was mixed with Boc-Leu(SSMe)-OH (31.91 mg, 1.0 eq), HATU (114.02 mg, 3.0 eq), and DIEA (104 μl, 6.0 eq) in DMF (1.0 ml) and stirred at 23 ^{o}C for 10 min. The reasin was washed with DMF, DCM, and MeOH several times and dried under vacuum. The resin was cleaved by treatment with AcOH/TFE/DCM (1:1:8) for 2 x 1 hour to yield the crude fully protected peptide (266.80 mg) .

The fully protected peptide (266.80 mg, 29.7 μmol, 1.0 eq) and (2*S*)-2-(ethylsulfinothioyl)phenyl 2-amino-3-(tert-butoxy)propanoate (19.58 mg, 2.0 eq) was dissolved in solvents (594 μl, $CHCl_3$/TFE= 3:1 v/v). To this mixture was added HOOBt (9.69 mg, 2.0 eq). The mixture was then sonicated and cooled to -10 ^{o}C. To the mixture was added slowly EDC (11.0 μl, 2.0 eq) with stirring. The mixture was subsequently allowed to warm to 23 ^{o}C and stirred for 3 h, monitored with UPLC. The resulting mixture was treated with 5% HOAc in water (1.0 ml) and the organic layer was separated. The organic layer then was injected in a cocktail B solution (30.0 ml) and stirred for 1.5 h. After that, the solution was then concentrated under N_2 stream and the crude product was precipitated by pouring in cold diethyl ether (30.0 ml). The suspension was centrifuged and the upper ether layer was decanted. The precipitated was purged with diethyl ether twice (30.0 ml each) and the precipitated was dissolved in aq. MeCN (1:1 v/v, 20 ml) and lypholized. The resulting crude product was further purified with preparative RP-HPLC (linear gradient 5-15% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 19.15 min) to afford 46.46 mg of peptide **14** (9% overall yield).



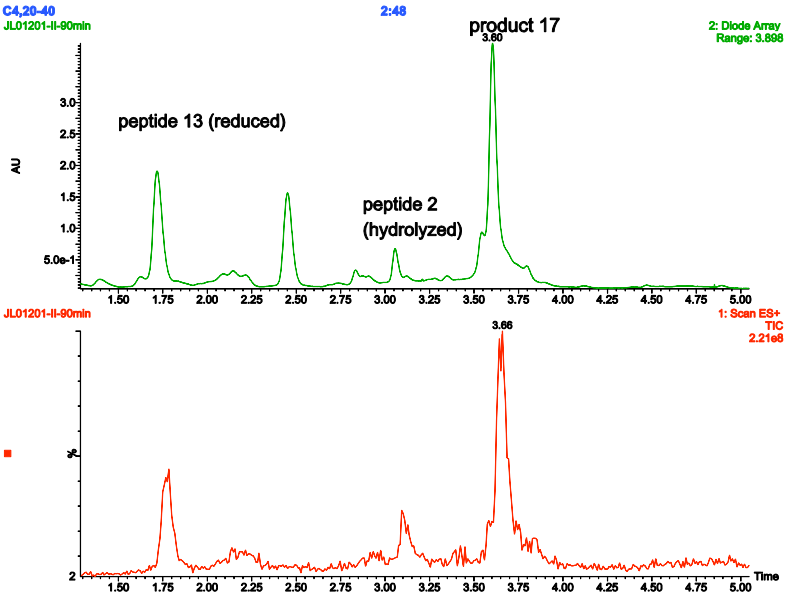
Peptide 14. Calcd for $C_{225}H_{391}N_{71}O_{64}S_4$: 5239.84, $[M+3H]^{3+}$ *m/z* = 1747.61, $[M+4H]^{4+}$ *m/z* = 1310.96, $[M+5H]^{5+}$ *m/z* = 1048.97, $[M+6H]^{6+}$ *m/z* = 874.31, $[M+7H]^{7+}$ *m/z* = 749.55, $[M+8H]^{8+}$ *m/z* = 655.98.

Synthesis of 17

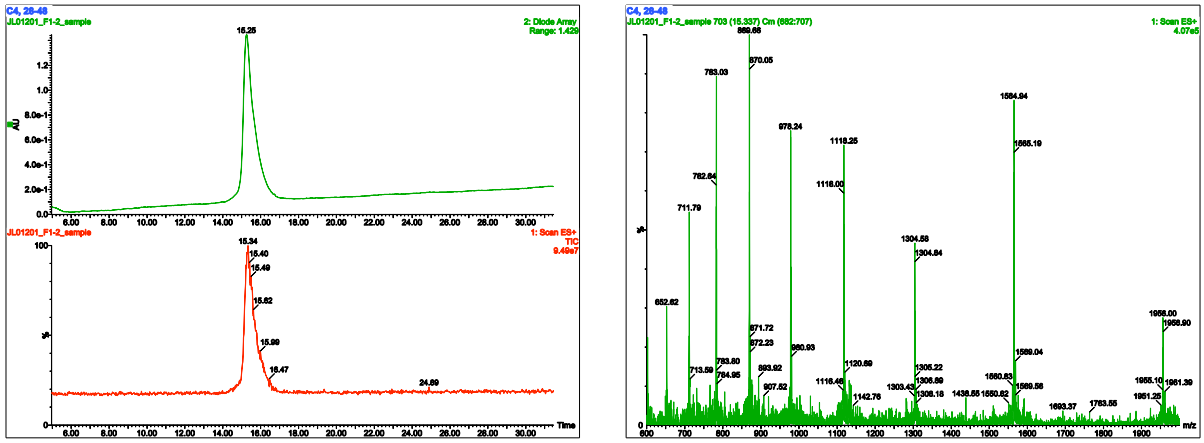


17

Peptide **2** (2.5 mg, 0.39 μmol, 1.00 eq) and peptide **13** (1.54 mg, 0.44 μmol, 1.12 eq) were dissolved in aq MeCN and lyophilized. To the resulting starting materials was added ligation buffer (300 μl, 6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, pH 7.2). The mixture was stirred under argon at 23 ^oC for 3 h, monitored with UPLC and then purified with preparative RP-HPLC (linear gradient 24-44% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 23.91 min). to afford 1.63 mg peptide **17** (49% yield).

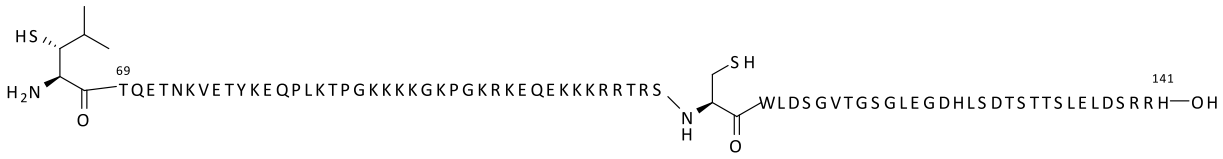


UPLC monitoring spectrum of the NCL reaction between peptide **2** and **13**.



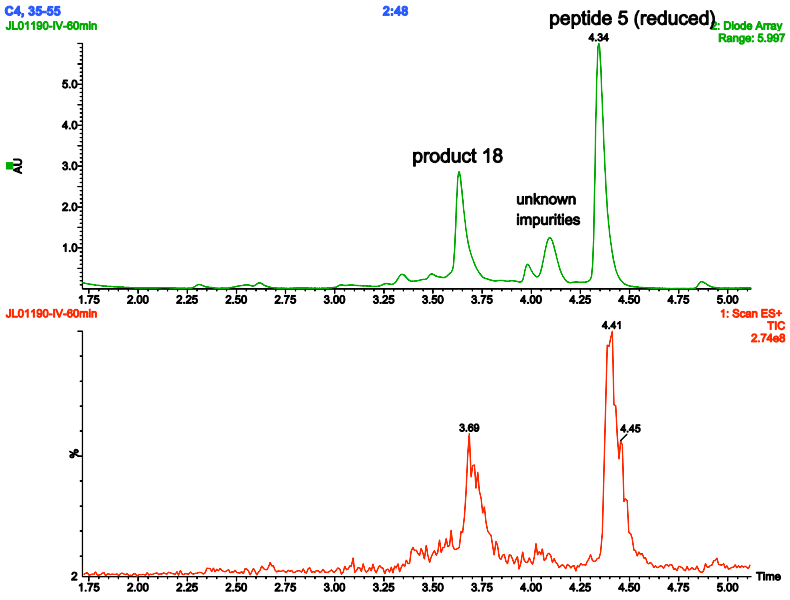
Peptide 17. Calcd for $C_{340}H_{536}N_{106}O_{103}S_2$, Expected Mass 7815.94, $[M+4H]^{4+}$ m/z = 1954.99, $[M+5H]^{5+}$ m/z = 1564.19, $[M+6H]^{6+}$ m/z = 1303.66, $[M+7H]^{7+}$ m/z = 1117.56, $[M+8H]^{8+}$ m/z = 977.99, $[M+9H]^{9+}$ m/z = 869.44, $[M+10H]^{10+}$ m/z = 782.59, $[M+11H]^{11+}$ m/z = 711.54, $[M+12H]^{12+}$ m/z =652.33.

Synthesis of 18

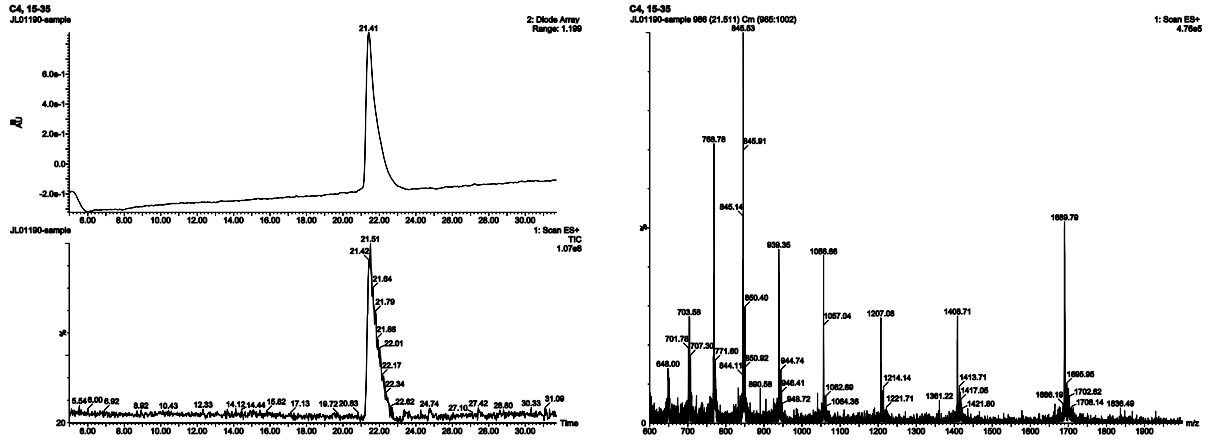


18

Peptide **14** (3.53 mg, 0.77 μmol, 1.0 eq) and peptide **5** (2.70 mg, 0.77 μmol, 1.0 eq) were dissolved in ligation buffer (350 μl, 6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, pH 7.2). The mixture was stirred under argon at 23 °C for 3 h, monitored with UPLC and then purified with preparative RP-HPLC (linear gradient 12-32% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 10.28 min) to afford 3.35 mg peptide **18** (56% yield).

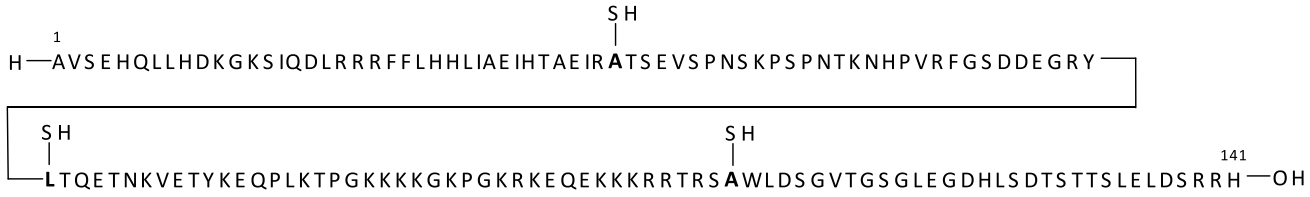


UPLC monitoring spectrum of the NCL reaction between peptide **14** and **5**.



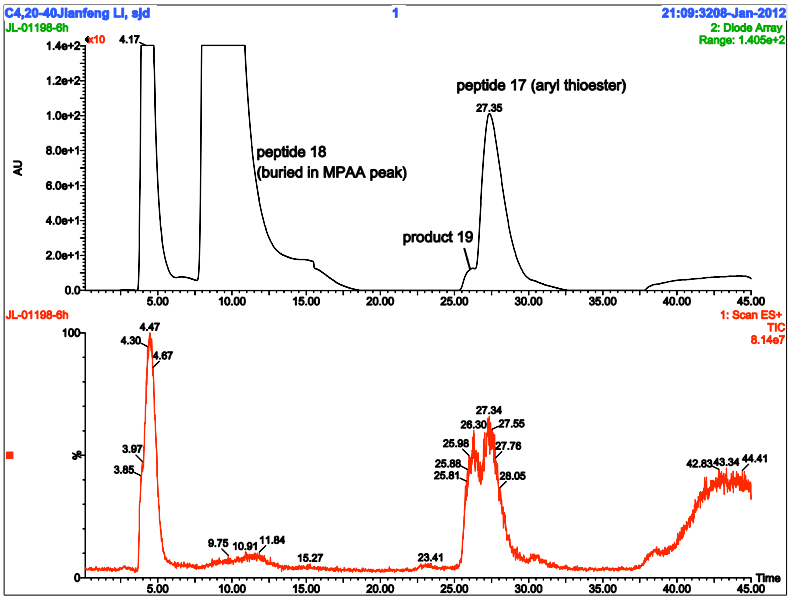
Peptide 18. Calcd for $C_{357}H_{602}N_{114}O_{118}S_2$, Expected Mass 8438.41, $[M+5H]^{5+}$ *m/z* = 1688.68, $[M+6H]^{6+}$ *m/z* = 1407.40, $[M+7H]^{7+}$ *m/z* = 1206.49, $[M+8H]^{8+}$ *m/z* = 1055.80, $[M+9H]^{9+}$ *m/z* = 938.60, $[M+10H]^{10+}$ *m/z* = 844.84, $[M+11H]^{11+}$ *m/z* = 768.13, $[M+12H]^{12+}$ *m/z* = 704.20, $[M+13H]^{13+}$ *m/z* = 650.11.

Synthesis of 19

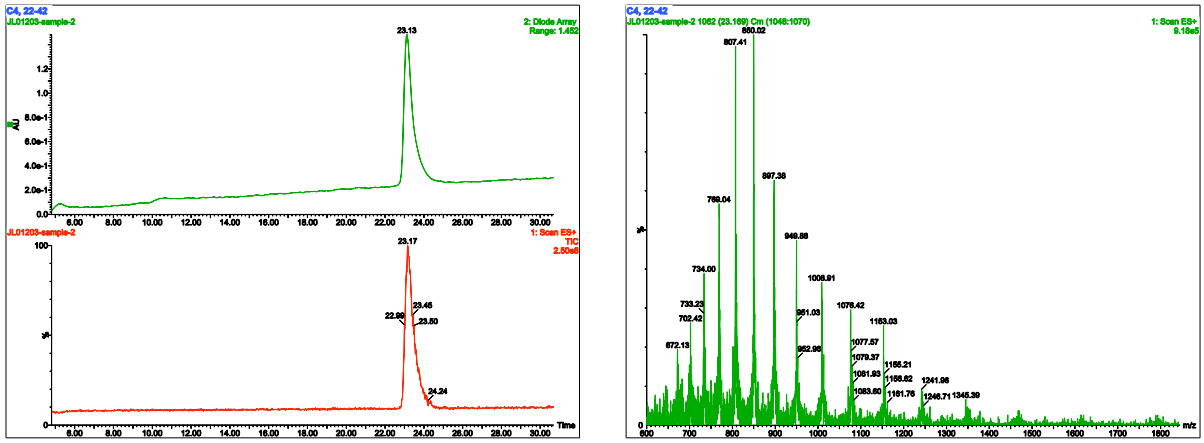


19

Ligation of peptide **17** and peptide **18** was conducted under the kinetically controlled conditions. Peptide **17** (4.24 mg, 0.54 μmol, 1.0 eq) and peptide **18** (5.29 mg, 0.62 μmol, 1.2 eq) were dissolved in ligation buffer (226 μl, 6 M Gdn·HCl, 300 mM Na_2HPO_4, 20 mM TCEP, 200 mM MPAA, pH 7.2). The mixture was stirred under argon at 23 ^oC for 20 h. The reaction was monitored with LC-MS and then purified with preparative RP-HPLC (linear gradient 20-40% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 23.55 min) to afford 4.00 mg (46% yield) peptide **19**.

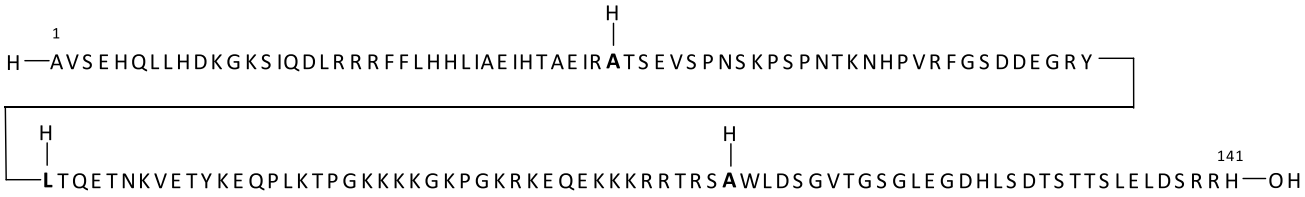


UPLC monitoring spectrum of the NCL reaction between peptide **17** and **18**.



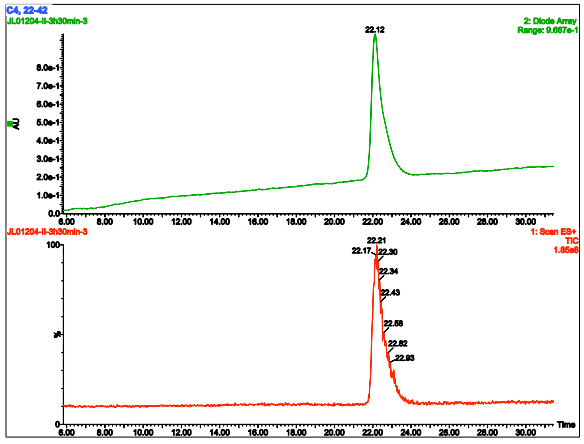
Peptide 19. Calcd for $C_{692}H_{1128}N_{220}O_{219}S_3$, Expected Mass 16120.31, $[M+12H]^{12+}$ m/z = 1344.36, $[M+13H]^{13+}$ m/z =1241.02, $[M+14H]^{14+}$ m/z = 1152.45, $[M+15H]^{15+}$ m/z = 1075.69, $[M+16H]^{16+}$ m/z = 1008.52, $[M+17H]^{17+}$ m/z = 949.25, $[M+18H]^{18+}$ m/z = 897.13. $[M+19H]^{19+}$ m/z = 849.44, $[M+20H]^{20+}$ m/z = 807.02, $[M+21H]^{21+}$ m/z = 768.63, $[M+22H]^{22+}$ m/z = 733.74, $[M+23H]^{23+}$ m/z = 701.88, $[M+24H]^{24+}$ m/z = 672.68.

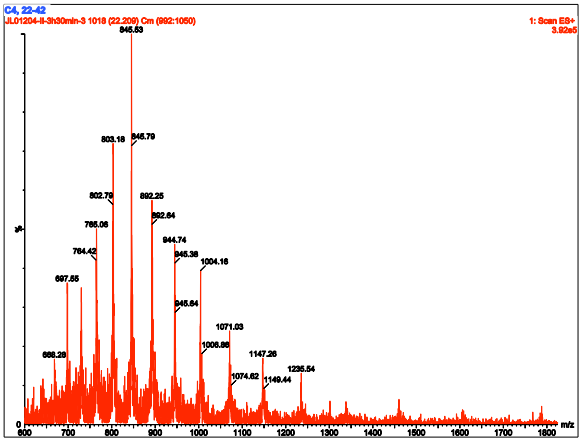
Synthesis of 1



1

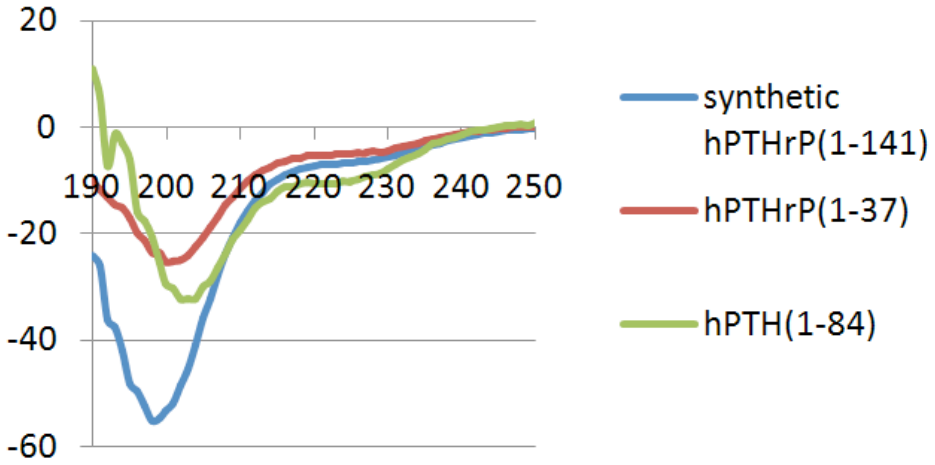
Peptide 19 (4.00 mg, 0.25 μmol) was dissolved in aq MeCN (0.67 ml). To this buffer was added VA-044 (21.8 mg) and Bond Breaker (675 μl, 0.5 M solution of TCEP) and *t*BuSH (67 μl). The system was stirred under argon atmosphere at 37 ^{o}C for 2 h. Additional VA-044 (21.8 mg in 1.0 ml water) and *t*BuSH (67 μl) were added to the mixture and the mixture was stirred for additional 1 h. The reaction was monitored with LC-MS. The product was directly purified with preparative HP preparative RP-HPLC (linear gradient 20-40% solvent B over 30 min, Microsorb 100-8 C4 column, 16 ml/min, 230 nm, product eluted at about 22.15 min) to afford 2.77 mg **1** (70% yield).





Peptide 1. Calcd for $C_{692}H_{1128}N_{220}O_{219}$, Expected Mass 16024.39, $[M+13H]^{13+}$ *m/z* =1233.65, $[M+14H]^{14+}$ *m/z* = 1145.60, $[M+15H]^{15+}$ *m/z* = 1069.29, $[M+16H]^{16+}$ *m/z* = 1002.52, $[M+17H]^{17+}$ *m/z* = 943.61, $[M+18H]^{18+}$ *m/z* = 891.24. $[M+19H]^{19+}$ *m/z* = 844.39, $[M+20H]^{20+}$ *m/z* = 802.22, $[M+21H]^{21+}$ *m/z* = 764.07, $[M+22H]^{22+}$ *m/z* = 729.38, $[M+23H]^{23+}$ *m/z* = 697.71, $[M+24H]^{24+}$ *m/z* = 668.68.

The CD spectrum of hPTHrP(1-141), compared with those of hPTHP(1-37) and hPTH(1-84).



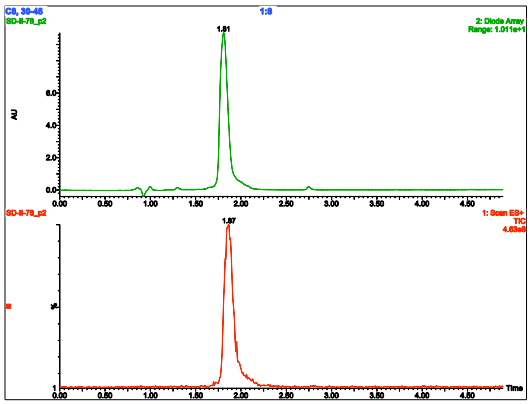
Synthesis of hPTHrP(1–37)

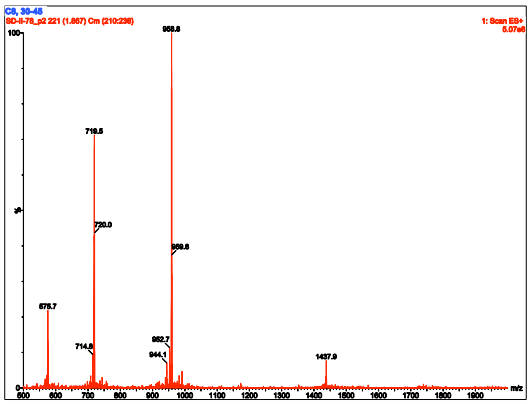
Peptidyl thiophenol ester 20

H–AVSEHQLLHDKGKSIQDLRRRFF–SPh
1 20 23

The fully protected peptidyl acid (1-22) was prepared on a 0.05 mol scale by SPPS according to the general procedure using standard Fmoc amino acids, and Fmoc-Asp(ODie)-OH to suppress the aspartimide formation. After cleavage, 201.1 mg crude peptide was obtained (80% yield).

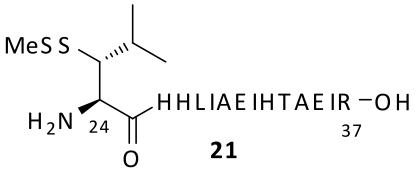
The fully protected peptidyl acid (62.8 mg, 12.4 μmol, 1.0 equiv) and HCl·H-Phe-SPh (10.9 mg, 37.3 μmol, 3.0 equiv) in $CHCl_3$/TFE (v/v = 3:1, 1.5 mL) was cooled to –10 °C. HOOBt (6.1 mg, 37.3 μmol, 3.0 equiv) and EDCI (6.60 μL, 37.3 μmol, 3.0 equiv) were added. The reaction mixture was stirred at room temperature for 3 h. The solvent was then blown off under a gentle N_2 stream and 10 mL of TFA/H_2O/TIS (95:2.5:2.5) was added. After deprotection for 45 min, TFA was blown off and the oily residue was triturated with 6 mL of diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 35-55% solvent B over 30 min, Microsorb 100-8 C18 column, 16 mL/min, 230 nm), and the product eluted at 22.5-25 min. The fractions were collected, and concentrated via lyophilization to provide peptide **20 (**15.2 mg, 43%) as a white solid.



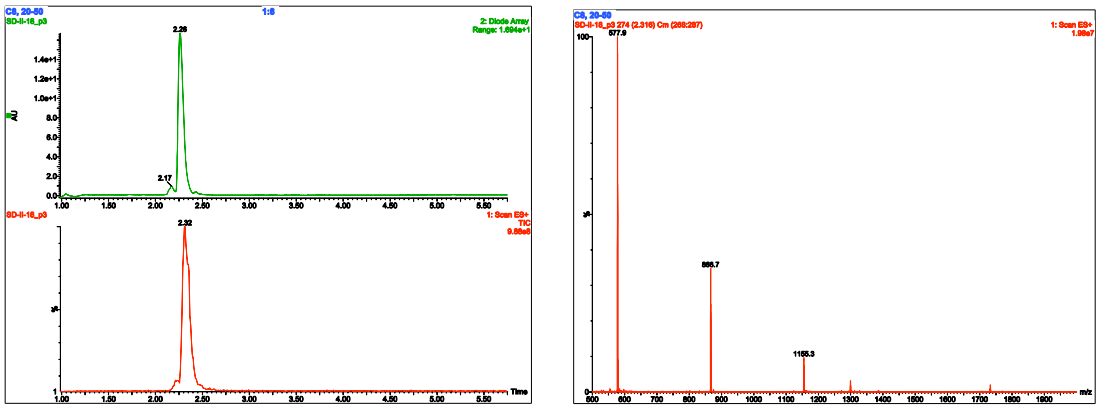


Peptide 20: Calcd for $C_{129}H_{202}N_{40}O_{33}S$: 2873.30 Da(average isotopes), $[M+3H]^{3+}$ *m/z* = 958.77, $[M+4H]^{4+}$ *m/z* = 719.32, $[M+5H]^{5+}$ *m/z* = 575.66; observed: $[M+3H]^{3+}$ *m/z* = 958.7, $[M+4H]^{4+}$ *m/z* = 719.4, $[M+5H]^{5+}$ *m/z* = 575.8.

Thioleucine-containing Peptide 21



The peptide resin from the Fmoc SPPS (13.01 μmol, 1.0 equiv) was mixed with Boc-Leu(SSMe)-OH (6.03 mg, 19.52 μmol, 1.5 equiv), HATU (24.7 mg, 65.05 μmol, 5.0 equiv) and DIEA (18.13 μL, 104.1 μmol, 8.0 equiv) in DMF (600 μL) and stirred at room temperature for 10 min. The resin was washed with DMF, DCM and MeOH several times and dried under vacuum. The dried resin was treated with TFA/TIS/H_2O (95:2.5:2.5) for 40 min, TFA was blown off by N_2 and the oily residue was triturated with diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 18-33% solvent B over 30 min, Microsorb 100-8 C18 column, 16 mL/min, 230 nm), and the product eluted at 18.5-23 min. The fractions were collected, and concentrated via lyophilization to afford peptide **21** (11.98 mg, 53%) as a white solid.



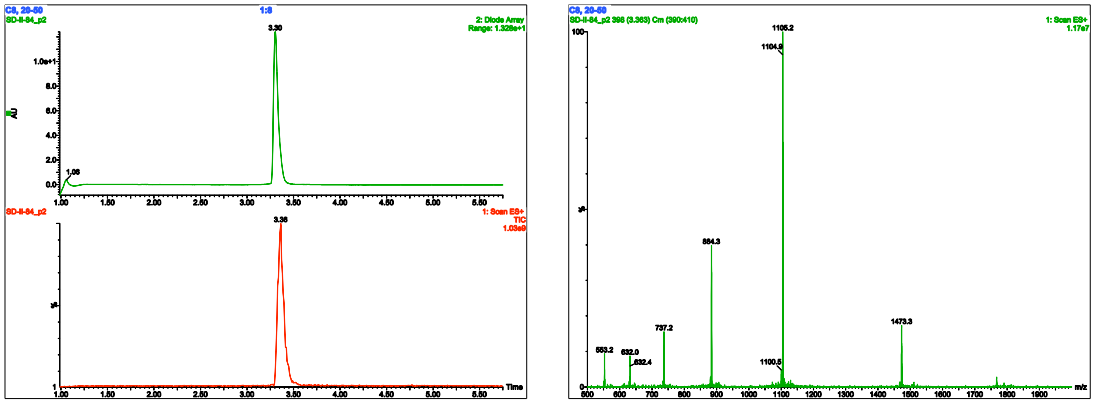
Peptide 21: Calcd for $C_{75}H_{123}N_{23}O_{20}S_2$: 1731.05 Da(average isotopes), $[2M+3H]^{3+}$ *m/z* = 1155.03, $[M+2H]^{2+}$ *m/z* = 866.52, $[M+3H]^{3+}$ *m/z* = 578.02; observed: $[2M+3H]^{3+}$ *m/z* = 1155.3, $[M+2H]^{2+}$ *m/z* = 866.7, $[M+3H]^{3+}$ *m/z* = 577.9.

hPTHrP(1-37) (22)

H–AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTAEIR–OH
1 37
22

Peptide **20** (2.83 mg, 0.985 μmol, 1.1 equiv) and peptide **21** (1.55 mg, 0.895 μmol, 1.0 equiv) were dissolved in 500 μL of ligation buffer (6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP·HCl, pH 7.2~7.3). The reaction mixture was stirred at room temperature for 18 h and monitored by LC-MS. Upon completion, the reaction mixture was diluted with 3 mL of guanidine buffer (6 M Gn·HCl, 200 mM Na_2HPO_4), and then concentrated using an Amicon® ultra-4 centrifugal filter (ultracel-3 membrane, MWCO 3000). The dilution/centrifugation process was repeated two more times to remove unreacted peptide 21 and other low molecular weight byproduct (i.e. thiophenol). The resulting buffer solution (~300 μL) containing ligated peptide was transferred to a small reaction vessel, degassed for 15 min using an argon stream, followed by the addition of 0.3 mL of 0.5 M bond-breaker® TCEP solution (Pierce), 30 μL of 2-methyl-2-propanethiol and 0.2 mL of radical initiator VA-044 (0.1 M in degassed H_2O). The reaction was stirred at 37 °C under an argon atmosphere and monitored by LC-MS. Upon completion, the reaction was diluted with 2 mL of CH_3CN/H_2O/AcOH (25:75:5). The crude mixture was purified using RP-HPLC (linear gradient 25-40% solvent B over 30 min, Microsorb 300-5 C8 column, 16 mL/min, 230

nm), and the product eluted at 19.5-21 min. The fractions were collected, and concentrated via lyophilization to afford hPTHrP(1-37) (1.86 mg, 47%) as a white solid.



Peptide 22: Calcd for $C_{197}H_{317}N_{63}O_{53}$: 4416.02 Da(average isotopes), $[M+3H]^{3+}$ *m/z* = 1473.01, $[M+4H]^{4+}$ *m/z* = 1105.01, $[M+5H]^{5+}$ *m/z* = 884.20, $[M+6H]^{6+}$ *m/z* = 737.00, $[M+7H]^{7+}$ *m/z* = 631.86, $[M+8H]^{8+}$ *m/z* = 553.00; observed: $[M+3H]^{3+}$ *m/z* = 1473.3, $[M+4H]^{4+}$ *m/z* = 1105.2, $[M+5H]^{5+}$ *m/z* = 884.2, $[M+6H]^{6+}$ *m/z* = 737.2, $[M+7H]^{7+}$ *m/z* = 632.0, $[M+8H]^{8+}$ *m/z* = 553.2.

(1) Haack, T.; Mutter, M. *Tetrahedron Lett*. **1992**, *33*, 1589.

(2) Mergler, M.; Dick, F. *J. Peptide Sci.* **2005**, *11*, 650-657.