Insights into the Finer Issues of Native Chemical Ligation: An Approach to Cascade Ligations

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Content

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Materials and Methods

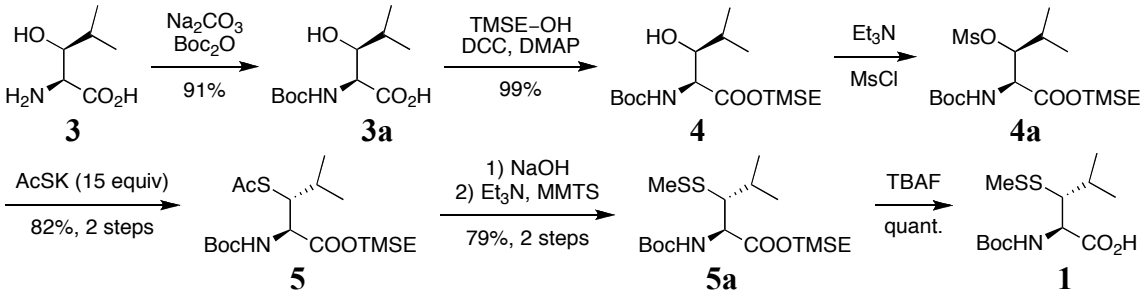
All commercial materials (Aldrich, Fluka, Nova) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher). Anhydrous THF, diethyl ether, CH_2Cl_2, toluene, and benzene were obtained from a dry solvent system (passed through column of alumina) and used without further 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 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.

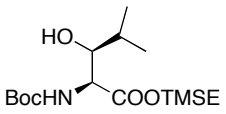
HPLC: All separations involved a mobile phase of 0.05% TFA (v/v) in water (solvent A)/0.04% TFA in acetonitrile (solvent B). LCMS analyses were performed using a Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector equipped with Varian Microsorb 100-5, C18 150x2.0mm and Varian Microsorb 300-5, C4 250x2.0mm columns at a flow rate of 0.2 mL/min. UPLC-MS analyses were performed using a Waters Acquity™ Ultra Preformance LC system equipped with Acquity UPLC® BEH C18, 1.7µl, 2.1 x 100 mm, Acquity UPLC® BEH C8, 1.7µl, 2.1 x 100 mm, Acquity UPLC® BEH 300 C4, 1.7µl, 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-Cys(Acm)-OH, Fmoc-Cys(EtThio)-OH, Fmoc-Cys(*t*Buthio)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH.

Preparation and Characterization of Compounds 1-8



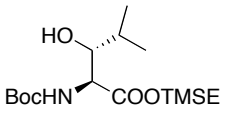


4

(2*S*,3*S*)-2-(trimethylsilyl)ethyl 2-(*tert*-butoxycarbonylamino)-3-hydroxy-4-methylpentanoate (4). In a 25 mL roundbottom flask, (2*S*,3*S*)-2-amino-3-hydroxy-4-methylpentanoic acid (**3**) (250.0 mg, 1.70 mmol, 1.0 equiv) was dissolved in H_2O (3.0 mL) and THF (3.0 mL). Solid Na_2CO_3 (216.1 mg, 2.04 mmol, 1.2 equiv) was added in one portion. The mixture was stirred at rt for 10 min, then Boc_2O (445.0 mg, 2.04 mmol, 1.2 equiv) was added at rt and the mixture was stirred at rt for 44 hrs. Water (4 mL) was added and the mixture was washed with Et_2O (2 × 4 mL). The aqueous layer was acidified with 1 N aq HCl to pH 4 and extracted with EtOAc (5 × 30 mL). The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated by rotary evaporation. Acid **3a** (381.2 mg, 91%, white solid) could be used in the next step without further purification.

In a 25 mL roundbottom flask, acid **3a** (288.2 mg, 1.17 mmol, 1.0 equiv), 2-trimethylsilylethanol (1.67 mL, 11.7 mmol, 10.0 equiv) and DMAP (14.2 mg, 0.12 mmol, 0.1 equiv) were dissolved in CH_2Cl_2 (5.8 mL). Dicyclohexylcarbodiimide (361.0 mg, 1.75 mmol, 1.5 equiv) was added to the above mixture and the solution was stirred at rt for 40 min. The mixture was filtered and concentrated by rotary evaporation. Purification by silica flash chromatography (10:1 hexanes/EtOAc) yielded **4** (405.0 mg, 99%) as a colorless oil.

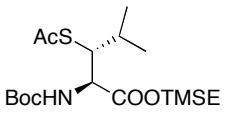
TLC: R_f 0.30 (5:1 hexanes/EtOAc). $[\alpha]_D^{20}$: +2.5° (*c* 0.6, $CDCl_3$). **IR** (ZnSe, film): 3452 (O–H st), 2959, 1718 (C=O st), 1511, 1392, 1367, 1252, 1169, 1048, 1004, 942, 862, 839, 780, 768. **^{1}H-NMR** (500 MHz): δ 5.45 (d, 1H, *J* = 6.2), 4.41 (m, 1H), 4.23 (t, 2H, *J* = 8.7), 3.45 (m, 1H), 2.57 (d, 1H, *J* = 7.4), 1.72 (m, 1H), 1.43 (s, 9H), 0.95–1.04 (m, 8H), 0.03 (s, 9H). **^{13}C-NMR** (150 MHz) δ 172.9, 157.2, 81.7, 80.4, 65.6, 57.9, 32.8, 29.9, 20.7, 20.3, 19.0, 0.1. **ESI-MS** *m/z* (rel int): (pos) 370.1 ($[M+Na]^+$, 100), 386.1 ($[M+K]^+$, 23).



7

(2*S*,3*R*)-2-(trimethylsilyl)ethyl 2-(*tert*-butoxycarbonylamino)-3-hydroxy-4-methylpentanoate (7). Prepared from (2*S*,3*R*)-2-amino-3-hydroxy-4-methylpentanoic acid (**6**) as above. Purification by silica flash chromatography (10:1 hexanes/EtOAc) yielded **7** as a colorless oil.

TLC: R_f 0.30 (5:1 hexanes/EtOAc). $[\alpha]_D^{20}$: −3.7° (*c* 0.2, $CDCl_3$). **IR** (ZnSe, film): 3440 (O–H st), 2958, 1716 (C=O st), 1501, 1391, 1367, 1252, 1164, 1056, 910, 860, 839, 734. **^{1}H-NMR** (500 MHz): δ 5.21 (d, 1H, *J* = 8.0), 4.41 (d, 1H, *J* = 9.5), 4.23 (t, 2H, *J* = 8.6), 3.65 (m, 1H), 1.95 (d, 1H, *J* = 4.8), 1.90 (m, 1H), 1.43 (s, 9H), 0.93–1.02 (m, 8H), 0.03 (s, 9H). **^{13}C-NMR** (150 MHz) δ 173.8, 157.5, 81.4, 79.2, 65.5, 57.2, 32.3, 29.8, 20.4, 20.3, 18.9, 0.1. **ESI-MS** *m/z* (rel int): (pos) 370.2 ($[M+Na]^+$, 100), 386.1 ($[M+K]^+$, 28).

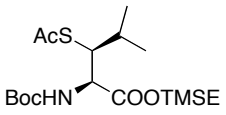


5

(2*R*,3*R*)-2-(trimethylsilyl)ethyl 3-(acetylthio)-2-(*tert*-butoxycarbonylamino)-4-methylpentanoate (5). In a 25 mL roundbottom flask, compound **5** (360.0 mg, 1.04 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (4.5 mL). Triethylamine (289.9 μL, 2.08 mmol, 2.0 equiv) and MsCl (120.9 μL, 1.56 mmol, 1.5 equiv) were added at 0 °C. The mixture was stirred at 0 °C for 40 min. Satd NH_4Cl was added and the aqueous layer was extracted with Et_2O. The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated by rotary evaporation. The crude material was used in the next step without further purification.

In a 25 mL roundbottom flask, the above crude material was dissolved in DMF (4.5 mL). Potassium thioacetate (1.78 g, 15.6 mmol, 15 equiv) was added in one portion to the above mixture and the solution was stirred at rt for 2.5 h, then warmed to 40 °C for 1 h, and 60 °C for 1.5 h. Satd. NH_4Cl was added and the aqueous layer was extracted with EtOAc. The combined organic extracts were washed with water and brine, dried ($MgSO_4$), filtered, and concentrated by rotary evaporation. Purification by silica flash chromatography (40:1 hexanes/EtOAc) yielded **5** (383.3 mg, 82%, 2 steps) as a brown oil.

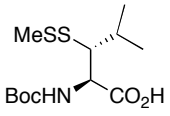
$[\alpha]_D^{20}$: +21.5° (*c* 1.5, $CDCl_3$). **IR** (ZnSe, film): 3433, 3366, 2960, 1720, 1498, 1367, 1250, 1161, 1055, 941, 860, 838, 772. **^{1}H-NMR** (500 MHz): δ 5.09 (d, 1H, *J* = 9.3), 4.63 (dd, 1H, *J* = 9.2, 3.6), 4.18 (m, 2H), 3.72 (t, 1H, *J* = 6.0), 2.31 (s, 3H), 1.92 (m, 1H), 1.41 (s, 9H), 0.96–1.06 (m, 8H), 0.03 (s, 9H). **^{13}C-NMR** (150 MHz) δ 195.9, 172.8, 157.0, 81.6, 65.6, 57.1, 55.1, 32.2, 29.8, 23.2, 22.5, 18.9, 0.1. **ESI-MS** *m/z* (rel int): (pos) 428.2 ($[M+Na]^+$, 100), 444.1 ($[M+K]^+$, 20).



8

(2*R*,3*S*)-2-(trimethylsilyl)ethyl 3-(acetylthio)-2-(*tert*-butoxycarbonylamino)-4-methylpentanoate (8). Prepared from (**7**) as above. Purification by silica flash chromatography (40:1 hexanes/EtOAc) yielded ***epi*-6** as a brown oil.

$[\alpha]_D^{20}$: −34.6° (*c* 0.3, $CDCl_3$). **IR** (ZnSe, film): 3443, 3374, 2960, 1717, 1498, 1367, 1251, 1164, 1056, 946, 861, 839, 766. **^{1}H-NMR** (500 MHz): δ 5.16 (d, 1H, *J* = 8.6), 4.63 (t, 1H, *J* = 6.5), 4.19 (t, 2H, *J* = 8.8), 3.70 (t, 1H, *J* = 6.3), 2.34 (s, 3H), 2.02 (m, 1H), 1.42 (s, 9H), 1.06 (d, 3H, *J* = 6.6), 1.00 (t, 2H, *J* = 8.8), 0.94 (d, 3H, *J* = 6.6), 0.03 (s, 9H). **^{13}C-NMR** (150 MHz) δ 196.0, 172.2, 156.5, 81.6, 65.5, 56.9, 54.7, 32.2, 29.8, 22.5, 21.5, 19.0, 0.1. **ESI-MS** *m/z* (rel int): (pos) 428.3 ($[M+Na]^+$, 100), 444.1 ($[M+K]^+$, 42).



1

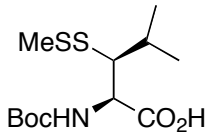
(2*R*,3*R*)-2-(*tert*-butoxycarbonylamino)-4-methyl-3-(methylsulfinothioyl)pentanoic acid (1). In a 25

mL roundbottom flask, compound **5** (157.0 mg, 0.39 mmol, 1.0 equiv) was dissolved in MeOH (7.8 mL) and treated with 1N NaOH (1.94 mL, 1.94 mmol, 5.0 equiv) at 0 °C for 30 min. The reaction mixture was carefully neutralized to pH 5 by the addition of 1N HCl at 0 °C, diluted with EtOAc and washed with water and brine. The organic extracts were dried ($MgSO_4$), filtered, and concentrated by rotary evaporation. The crude material was used in the next step without further purification.

In a 10 mL roundbottom flask, S-Methyl methanethiolsulfonate (128 μL, 1.36 mmol, 3.5 equiv) and Et_3N (64.8 μL, 0.47 mmol, 1.2 equiv) were dissolved in CH_2Cl_2 (3.9 mL). The above crude material was dropwise added to the above solution and stirred at rt for 30 min. The reaction mixture was concentrated by rotary evaporation. Purification by silica flash chromatography (40:1 hexanes/EtOAc) yielded **7** (124.1 mg, 79%, 2 steps) as a brown oil.

In a 10 mL roundbottom flask, compound **5a** (100 mg, 0.24 mmol, 1.0 equiv) was dissolved in THF (2 mL). TBAF (1M in THF) (0.73 mL, 0.73 mmol, 3.0 equiv) was added to the above mixture and the solution was stirred at rt for 30 min. The reaction mixture was acidified by the addition of 1N HCl (2 mL) and extracted with CH_2Cl_2. The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated by rotary evaporation. Purification by silica flash chromatography (40:1 hexanes/EtOAc → 40:1 hexanes/EtOAc + 2.5% AcOH → 30: 1 hexanes/EtOAc + 2.5% AcOH → 30/1 hexanes/EtOAc + 5% AcOH) yielded **7** (74.0 mg, 98%) as a brown solid.

$[\alpha]_D^{20}$: +117.9° (*c* 1.5, $CDCl_3$). **IR** (ZnSe, film): 2978, 2932, 1719, 1501, 1394, 1368, 1257, 1161, 1055, 1028, 953, 857, 778. **^{1}H-NMR** (500 MHz): δ 5.40 (d, 1H, *J* = 9.6), 4.73 (d, 1H, *J* = 9.2), 3.16 (d, 1H, *J* = 6.6), 2.38 (s, 3H), 1.90 (m, 1H), 1.43 (s, 9H), 1.11 (d, 3H, *J* = 6.6), 1.08 (d, 3H, *J* = 6.6). **^{13}C-NMR** (150 MHz) δ 176.8, 155.8, 80.5, 61.3, 55.3, 30.7, 28.3, 23.4, 20.7, 20.6. **ESI-MS** *m/z* (rel int): (pos) 332.0 ($[M+Na]^+$, 100), 348.1 ($[M+K]^+$, 30).



2

(2*R*,3*S*)-2-(*tert*-butoxycarbonylamino)-4-methyl-3-(methylsulfinothioyl)pentanoic acid (2). Prepared from (**7**) as above. Purification by silica flash chromatography (40:1 hexanes/EtOAc → 40:1 hexanes/EtOAc + 2.5% AcOH → 30: 1 hexanes/EtOAc + 2.5% AcOH → 30/1 hexanes/EtOAc + 5% AcOH) yielded **2** as a brown solid.

$[\alpha]_D^{20}$: –47.4° (*c* 1.5, $CDCl_3$). **IR** (ZnSe, film): 2979, 2932, 1716, 1658, 1510, 1396, 1368, 1240, 1162, 1055, 1011, 957, 888, 777. **^{1}H-NMR** (500 MHz): δ 5.38 (d, 1H, *J* = 7.7), 4.79 (m, 1H), 2.87 (d, 1H, *J* = 3.5), 2.39 (s, 3H), 2.05 (m, 1H), 1.44 (s, 9H), 1.14 (d, 3H, *J* = 5.6), 1.09 (d, 3H, *J* = 5.7). **^{13}C-NMR** (150 MHz) δ 175.4, 155.3, 80.5, 64.0, 55.3, 29.7, 28.3, 23.4, 21.2, 20.9. **ESI-MS** *m/z* (rel int): (pos) 332.0 ($[M+Na]^+$, 100), 348.1 ($[M+K]^+$, 35).

Preparation and Characterization of Peptide Precursors for Ligation

Unprotected Peptides

Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with DCM. The resin cleavage was effected by treatment with TFA/H_2O/TIS (95:2.5:2.5) for 45 min to yield the unprotected peptides. TFA was removed by N_2. The oily residue was triturated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was dissolved in MeCN/H_2O/AcOH (47.5:47.5:5) for HPLC purification.

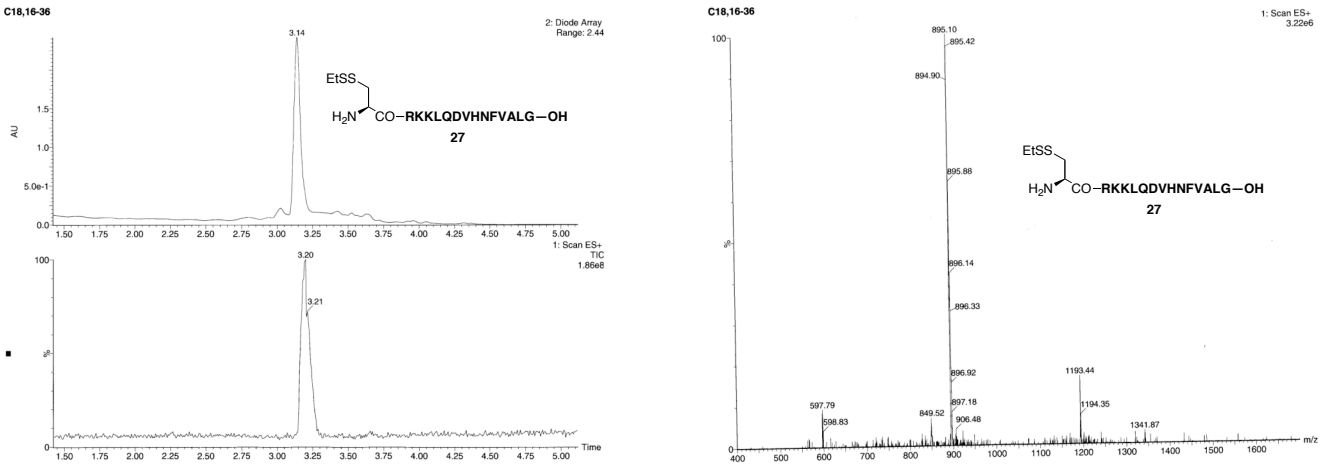


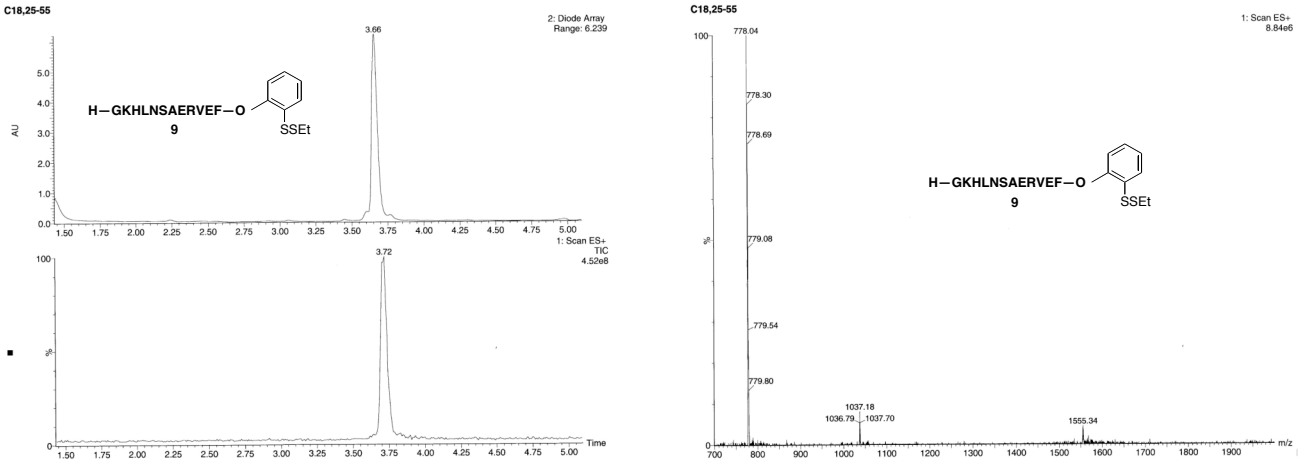
Figure S1. UPLC-MS analyses of purified unprotected peptides. Left: UV and mass traces of the purified product. Right: ESI mass spectrum of the desired product. **27** $C_{78}H_{130}N_{24}O_{20}S_2$ Exact Mass: 1786.93, $[M+2H]^{2+}$ *m/z* = 894.47, $[M+3H]^{3+}$ *m/z* = 596.64.

Fully Protected Peptidyl Acids

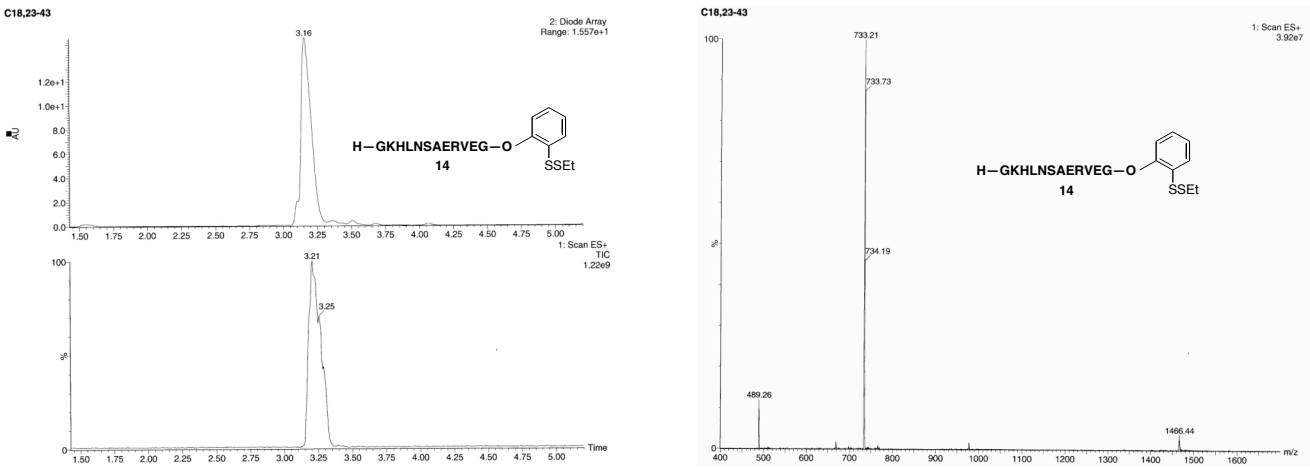
Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with DCM. The resin cleavage was effected by treatment with AcOH/TFE/DCM (1:1:8) for 2 × 1 hour to yield the fully protected peptidyl acids. The solvent was removed by N_2. The oily residue was triturated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was resuspended in MeCN/H_2O (1:1) and was lyophilized to dryness.

Peptide Phenolic Esters

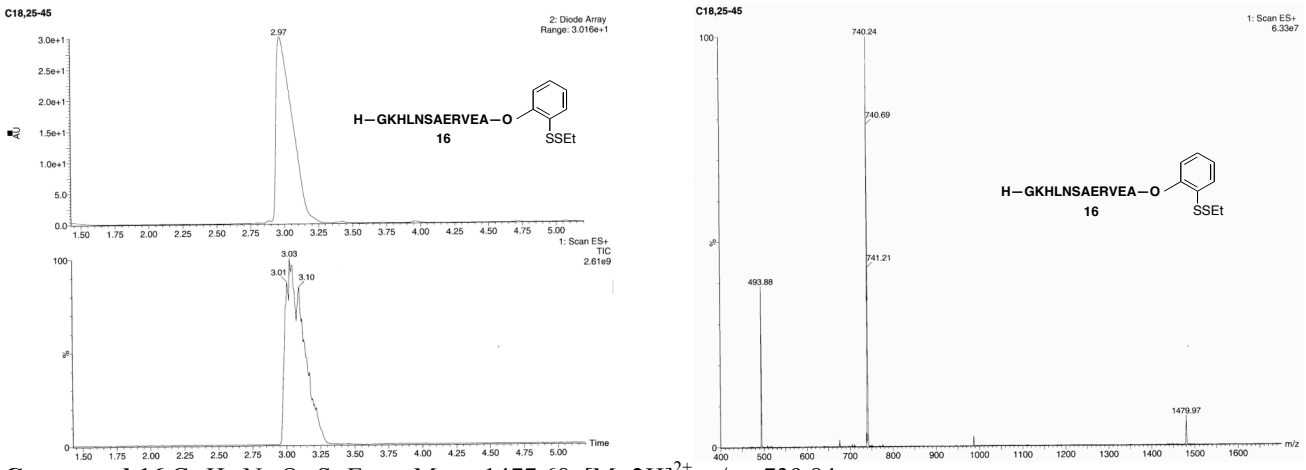
The fully protected peptidyl acid (29 mM, 1.1 equiv) and HCl·H-AA-O-(2-ethyldithio)-phenyl ester (26 mM, 1.0 equiv) in $CHCl_3$/TFE (v/v = 3/1) was cooled to -10 °C. HOOBt (1.1 equiv) and EDCI (1.1 equiv) were added. The reaction mixture was stirred at room temperature for 2 h. The solvent was then blown off under a gentle N_2 stream and TFA/H_2O/TIS (95:2.5:2.5) was added. After deprotection for 25 min, TFA was blown off and the oily residue was triturated with diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was dissolved in MeCN/H_2O/AcOH (47.5:47.5:5) for HPLC purification.



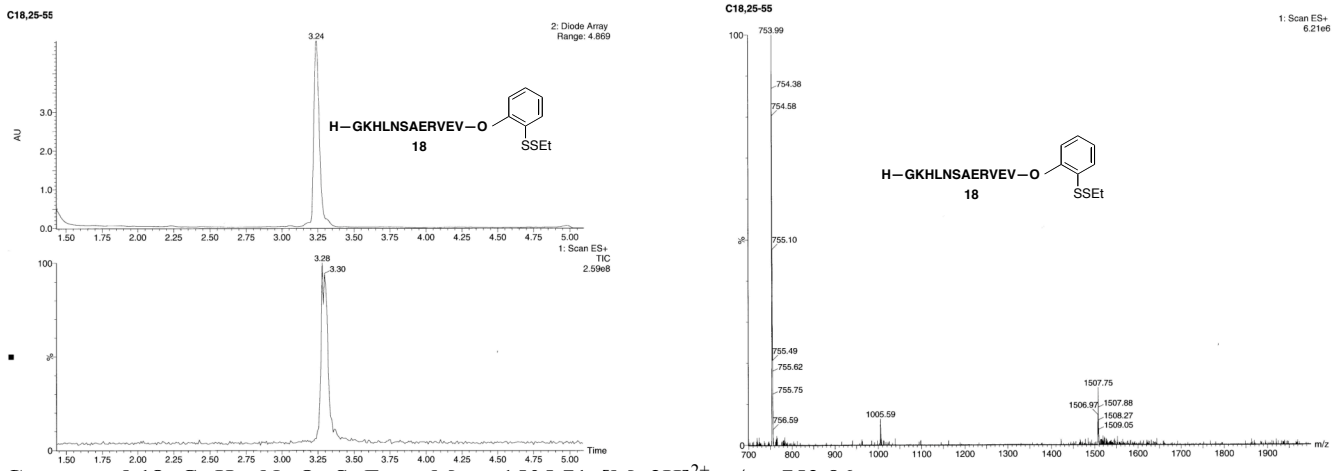
Compound 9: $C_{68}H_{103}N_{19}O_{19}S_2$ Exact Mass: 1553.71, $[M+2H]^{2+}$ *m/z* = 777.86.



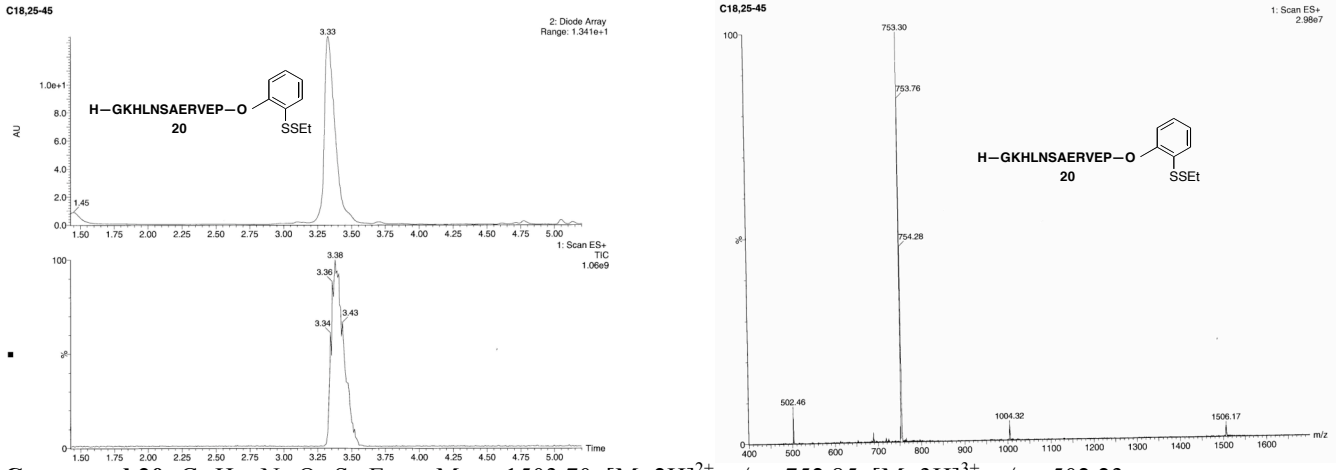
Compound 14: $C_{61}H_{97}N_{19}O_{19}S_2$ Exact Mass: 1463.66, $[M+2H]^{2+}$ *m/z* = 732.83.



Compound 16 $C_{62}H_{99}N_{19}O_{19}S_2$ Exact Mass: 1477.68, $[M+2H]^{2+}$ *m/z* = 739.84.



Compound 18: $C_{64}H_{103}N_{19}O_{19}S_2$ Exact Mass: 1505.71, $[M+2H]^{2+}$ *m/z* = 753.86.



Compound 20: $C_{64}H_{101}N_{19}O_{19}S_2$ Exact Mass: 1503.70, $[M+2H]^{2+}$ *m/z* = 752.85, $[M+3H]^{3+}$ *m/z* = 502.23.

Figure S2. UPLC-MS analyses of purified peptide phenolic esters. Left: UV and mass traces of the purified product. Right: ESI mass spectrum of the desired product.

1- or **2**-containing Peptides

The peptide resin from the Fmoc SPPS (0.05 mmol) was mixed with Boc-Leu(SSMe)-OH (1.0 equiv), HATU (3.0 equiv) and DIEA (6.0 equiv) in DMF 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 cleaved by treatment with AcOH/TFE/DCM (1:1:8) for 2 × 1 hour to yield the fully protected peptidyl acids or by treatment with TFA/H_2O/TIS (95:2.5:2.5) for 45 min to yield the unprotected peptides. The fully protected peptidyl acids can be further esterified with MeOH or with HCl·H-AA-O-(2-ethyldithio)-phenyl ester.

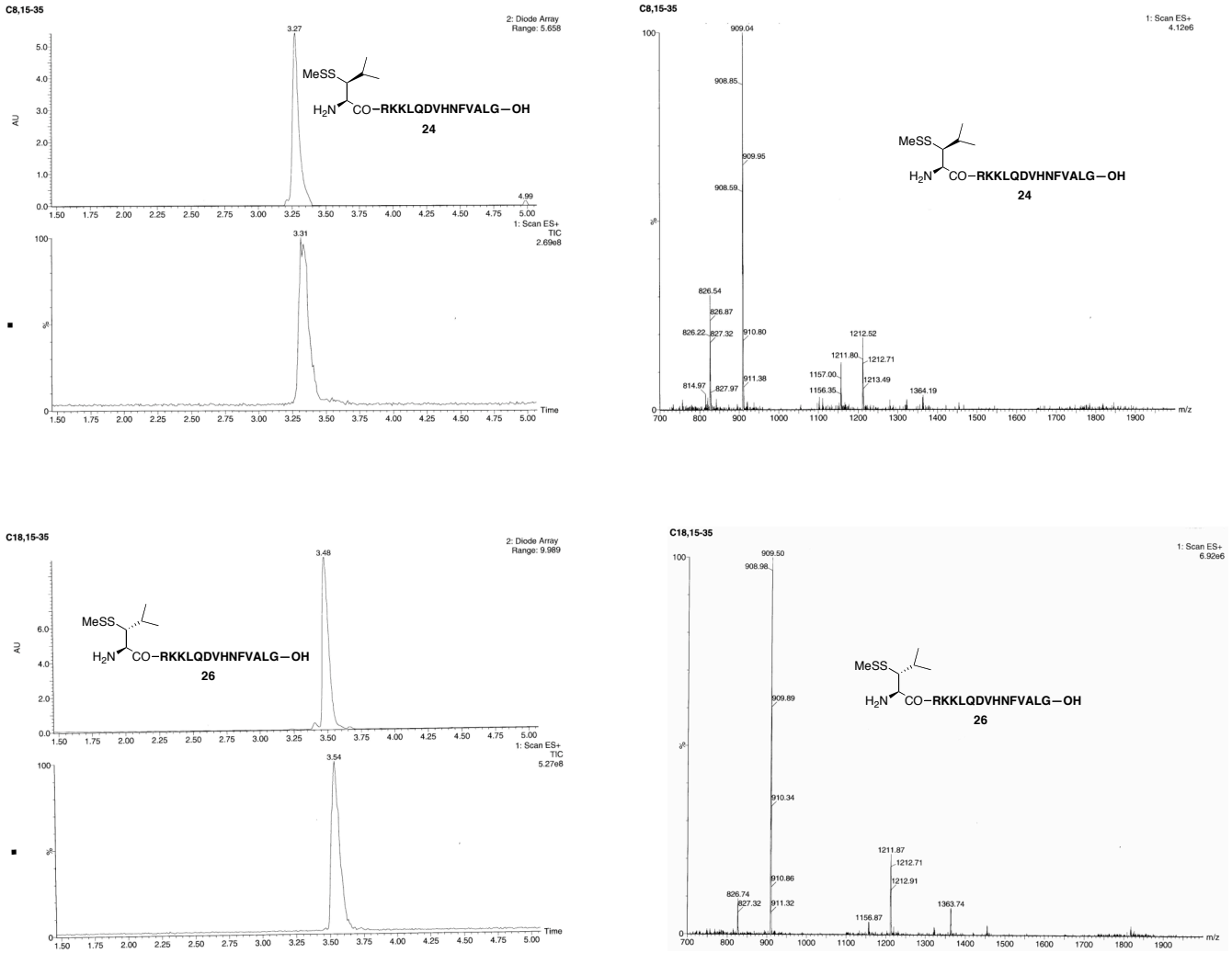


Figure S3. UPLC-MS analyses of purified **1**- or **2**-containing peptides. Left: UV and mass traces of the purified product. Right: ESI mass spectrum of the desired product. **24,** $C_{80}H_{134}N_{24}O_{20}S_2$ Exact Mass: 1814.96, $[M+2H]^{2+}$ *m/z* = 908.48; **26** $C_{80}H_{134}N_{24}O_{20}S_2$ Exact Mass: 1814.96, $[M+2H]^{2+}$ *m/z* = 908.48.

Preparation and Characterization of Ligation and Desulfurization Products

Peptide A (3.8 mM, 1.5 equiv) and peptide B (2.5 mM, 1.0 equiv) were dissolved in ligation buffer (6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, pH 7.5). The reaction mixture was stirred at room temperature. The reactions were monitored by UPLC-MS and purified directly by HPLC upon consumption of the starting material.

To a solution of the purified ligated peptide (0.6 mM) in degassed CH_3CN/H_2O (v/v = 1:1, 0.2 ml) were added 0.2 ml of 0.5 M bond-breaker® TCEP solution (Pierce), 0.02 ml of 2-methyl-2-propanethiol and 0.2 ml of radical initiator (0.1 M in H_2O). The reaction mixture was stirred at 37 °C. The reactions were monitored by UPLC-MS and purified directly by HPLC upon consumption of the starting material.

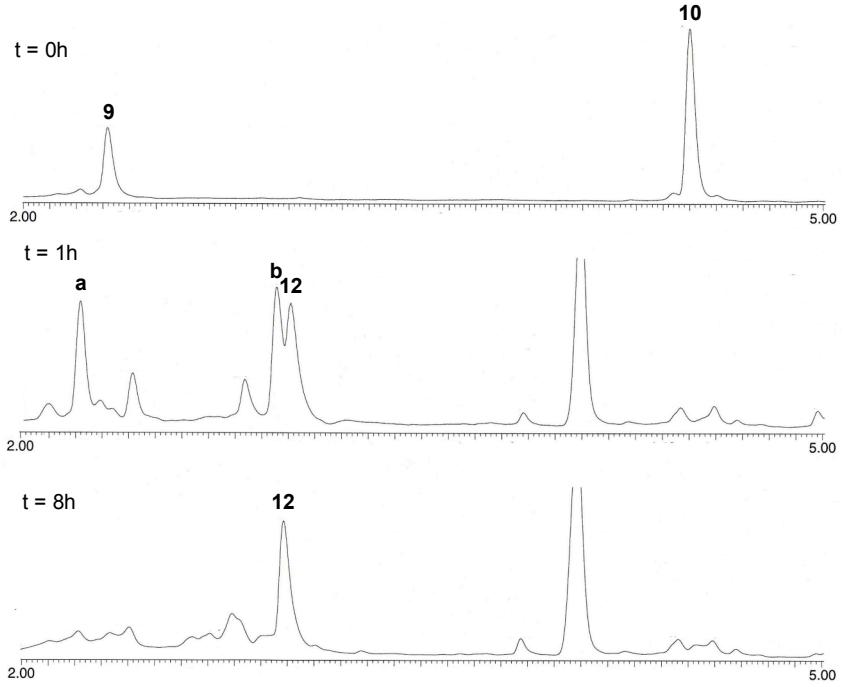
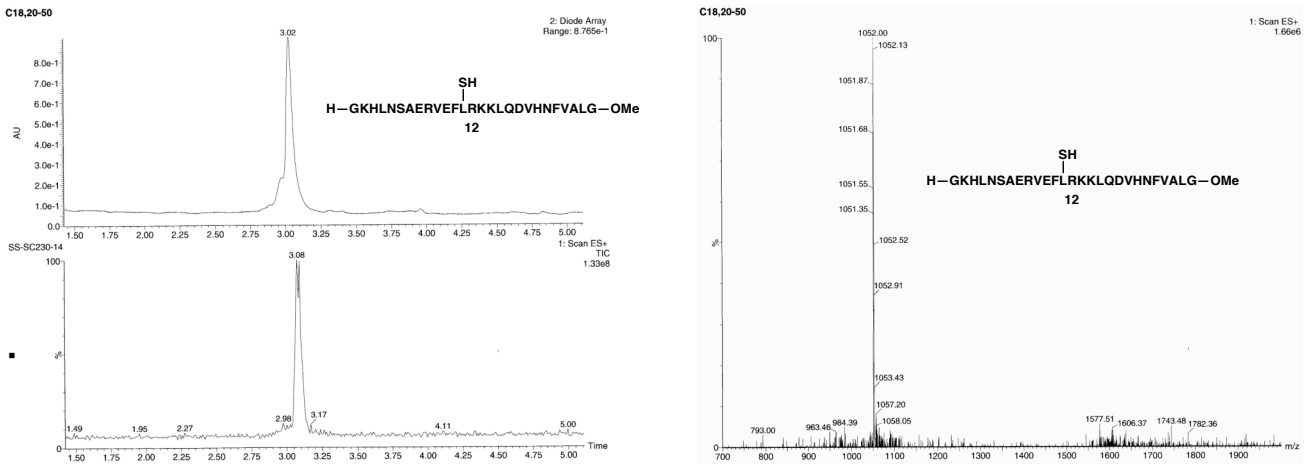
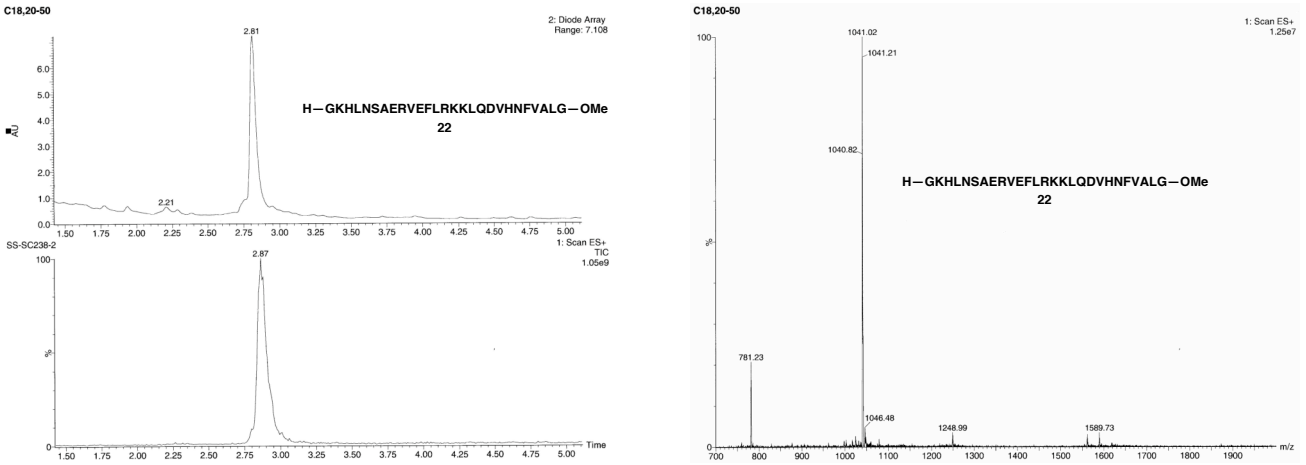


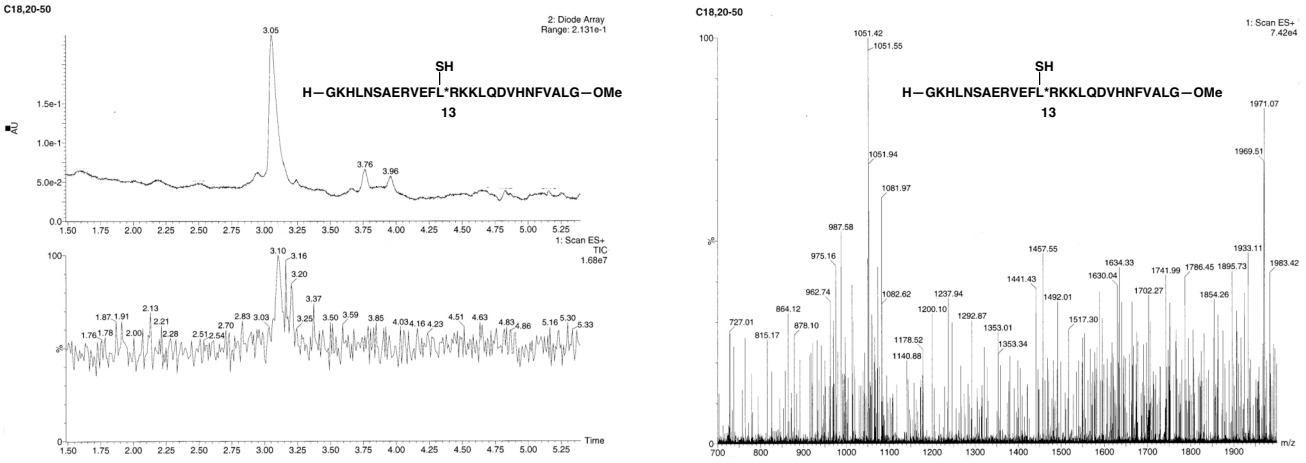
Figure S4. UPLC-MS analysis of model ligation between NH_2-GKHLNSAERVEF-O-(2-SSEt)-Ph **9** and NH_2-L(SSMe)RKKLQDVHNFVALG-CO_2Me **10** at t = 0h, 1h, and 8h. Peak a, NH_2-L(SH)RKKLQDVHNFVALG-CO_2Me; Peak b, NH_2-GKHLNSAERVEF-O-(2-SH)-Ph; **12**, NH_2-GKHLNSAERVEF-L(SH)RKKLQDVHNFVALG-CO_2Me.



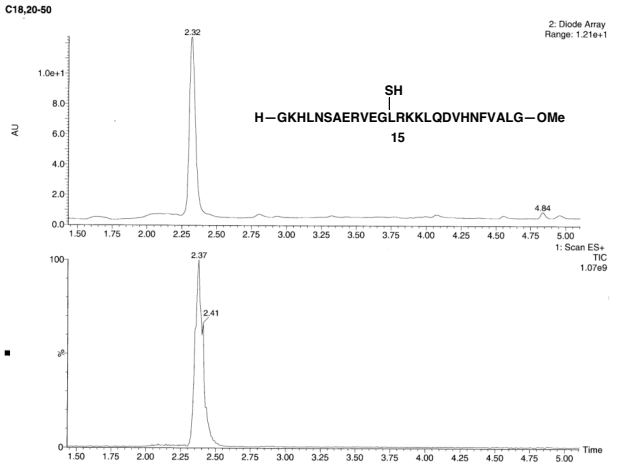
Compound 12: $C_{140}H_{227}N_{43}O_{38}S$ $[M+2H]^{2+}$ *m/z* = 1576.35, $[M+3H]^{3+}$ *m/z* = 1051.23, $[M+4H]^{4+}$ *m/z* = 788.67.

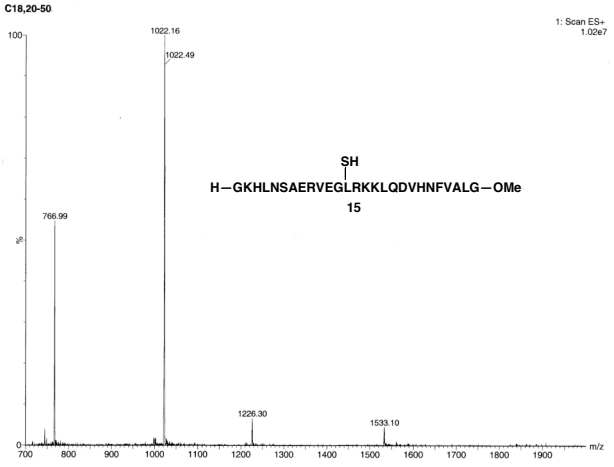


Compound 22: $C_{140}H_{227}N_{43}O_{38}$ $[M+2H]^{2+}$ *m/z* = 1560.36, $[M+3H]^{3+}$ *m/z* = 1040.57, $[M+4H]^{4+}$ *m/z* = 780.68.

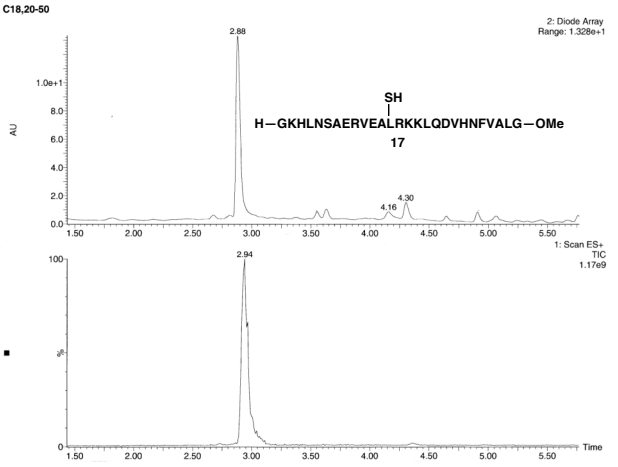


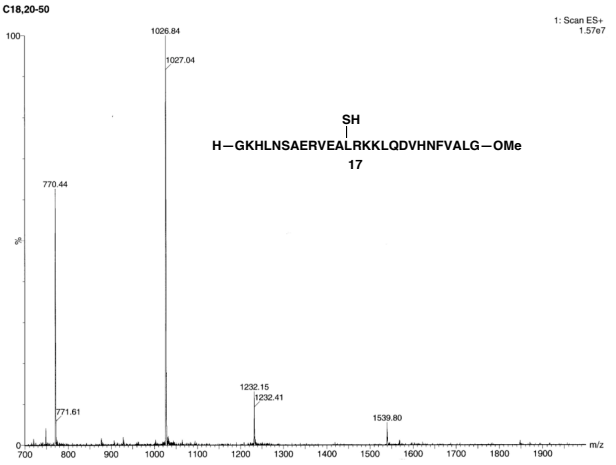
Compound 13: $C_{140}H_{227}N_{43}O_{38}S$ $[M+2H]^{2+}$ *m/z* = 1576.35, $[M+3H]^{3+}$ *m/z* = 1051.23, $[M+4H]^{4+}$ *m/z* = 788.67.



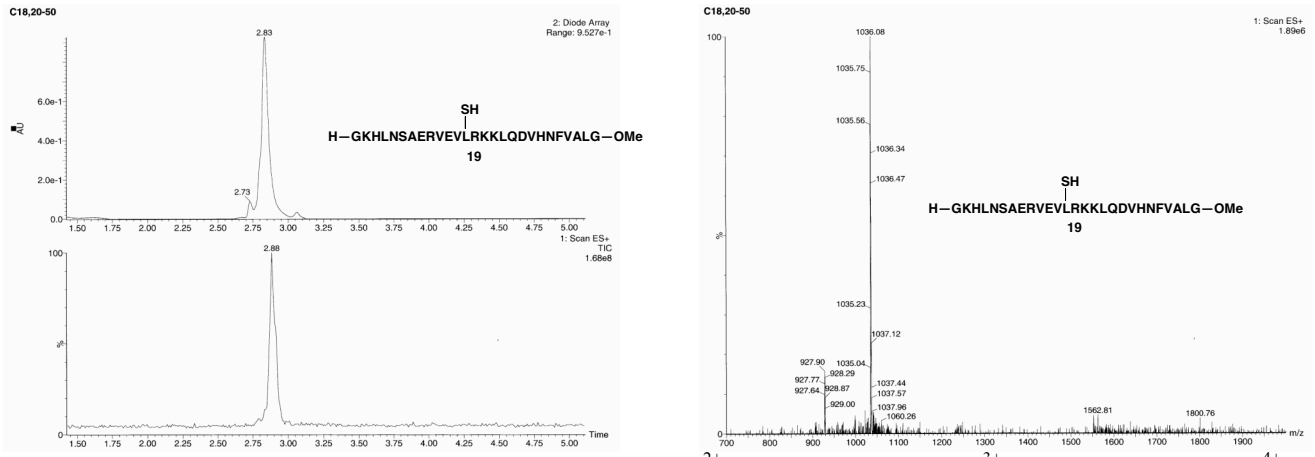


Compound 15: $C_{133}H_{221}N_{43}O_{38}S$ Exact Mass: 3060.64, $[M+2H]^{2+}$ *m/z* = 1531.32, $[M+3H]^{3+}$ *m/z* = 1021.21, $[M+4H]^{4+}$ *m/z* = 766.16.

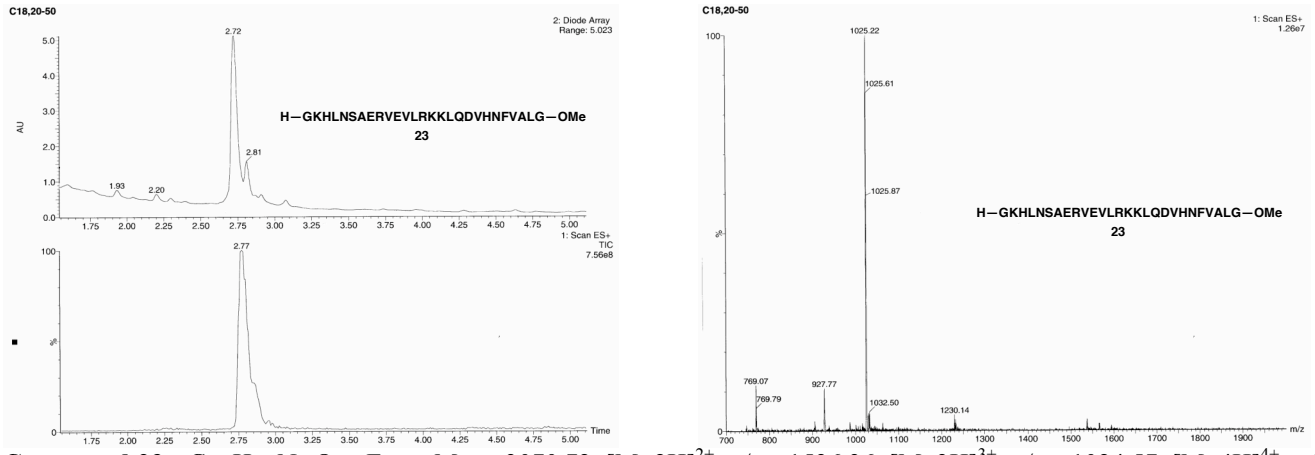




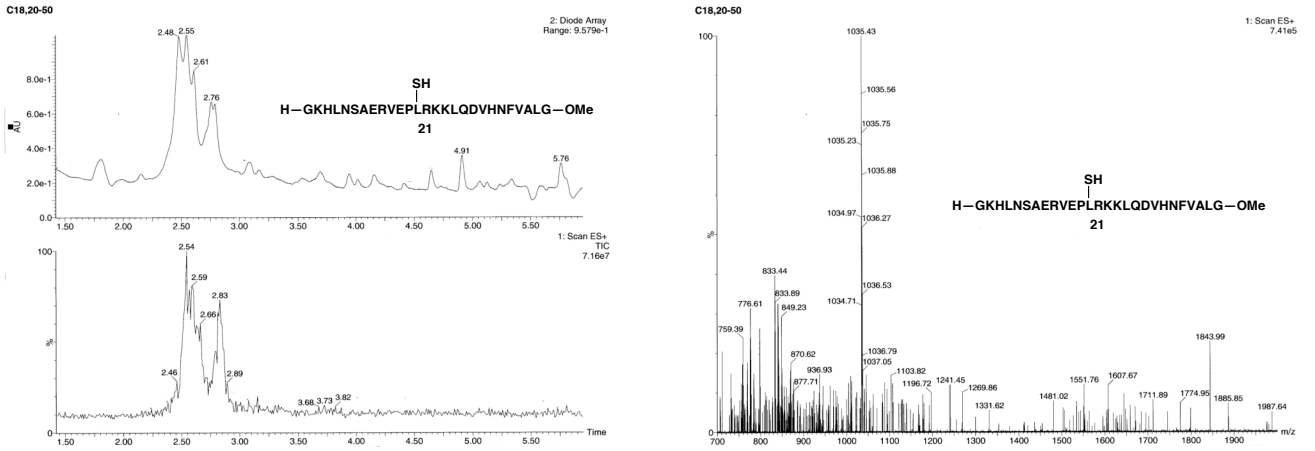
Compound 17: $C_{134}H_{223}N_{43}O_{38}S$ Exact Mass: 3074.66, $[M+2H]^{2+}$ *m/z* = 1538.33, $[M+3H]^{3+}$ *m/z* = 1025.89, $[M+4H]^{4+}$ *m/z* = 769.67.



Compound 19: $C_{136}H_{227}N_{43}O_{38}S$ Exact Mass: 3102.69, $[M+2H]^{2+}$ *m/z* = 1552.35, $[M+3H]^{3+}$ *m/z* = 1035.23, $[M+4H]^{4+}$ *m/z* = 776.67.



Compound 23: $C_{136}H_{227}N_{43}O_{38}$ Exact Mass: 3070.72, $[M+2H]^{2+}$ *m/z* = 1536.36, $[M+3H]^{3+}$ *m/z* = 1024.57, $[M+4H]^{4+}$ *m/z* = 768.68.



Compound 21 (contains inseparable impurity)**:** $C_{136}H_{225}N_{43}O_{38}S$ Exact Mass: 3100.67, $[M+2H]^{2+}$ *m/z* = 1551.34, $[M+3H]^{3+}$ *m/z* = 1034.56, $[M+4H]^{4+}$ *m/z* = 776.17.

Figure S5. UPLC-MS analysis of purified ligation and desulfurization products. Left: UV and mass traces of the purified product. Right: ESI mass spectrum of the desired product.

Procedure and Progress of Competition Reactions

Peptide **14** (1 mM, 0.8 equiv), Peptide A (2.5 mM, 1.0 equiv) and peptide B (2.5 mM, 1.0 equiv) were dissolved in ligation buffer (6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, pH 7.5). The reaction mixture was stirred at room temperature. The reactions were monitored by UPLC-MS.

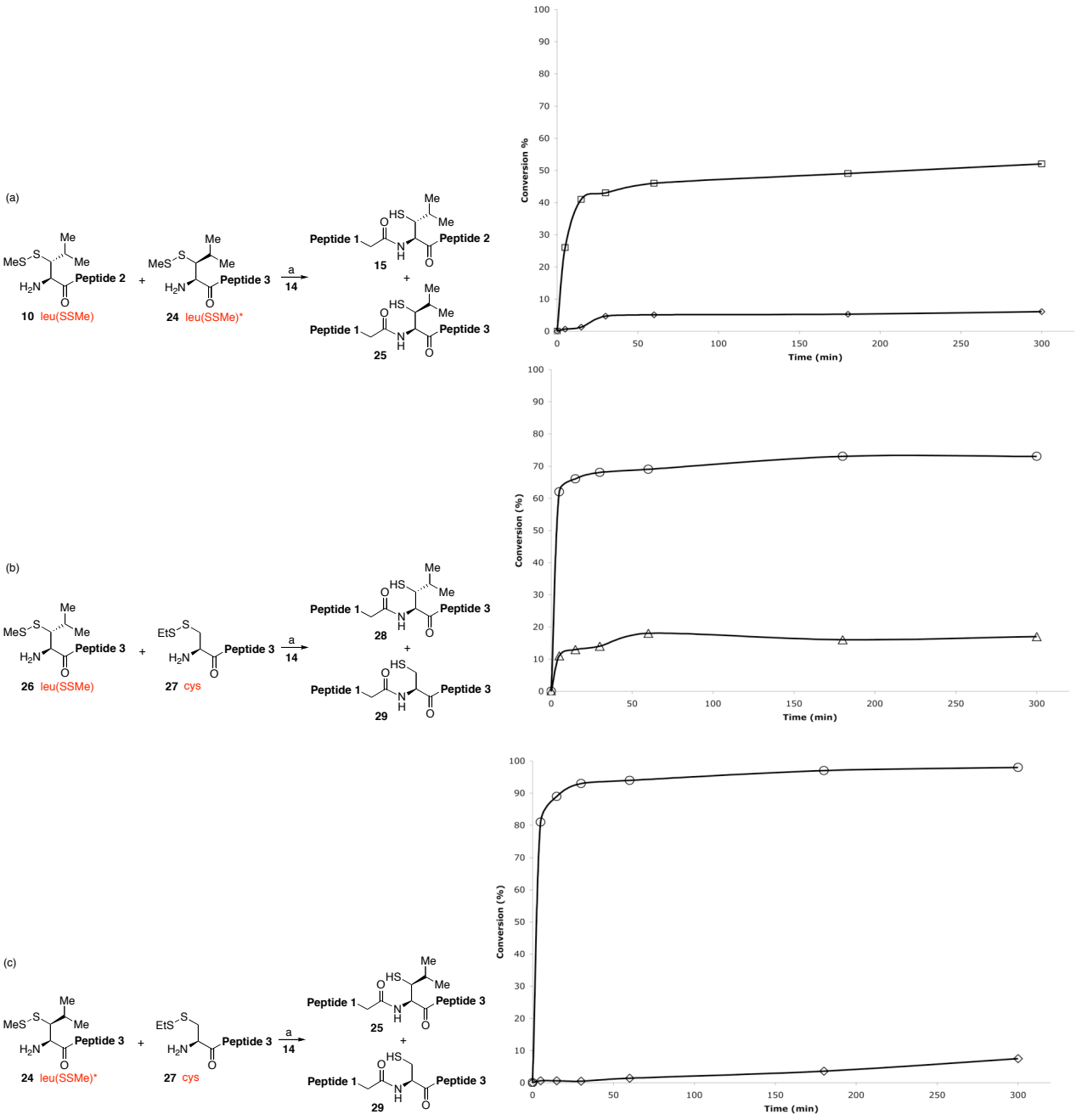


Figure S6. (a) Time course of the competition reaction between leu(SH) and leu(SH)* to form peptide-coupling products. (b) Time course of the competition reaction between leu(SH) and cys to form peptide-coupling products. (C) Time course of the competition reaction between leu(SH)* and cys to form peptide-coupling products. **15** (□), **25** (◇), **28** (△) and **29** (○). **Peptide 1**: GKHLNSAERVE–; **Peptide 2**: –RKKLQDVHNFVALG-OMe **Peptide 3**: –RKKLQDVHNFVALG–OH.

Preparation and Characterization of EPO(95-120)

Peptide **31** (1.0 mg, 1.1 equiv) and peptide **32** (0.5 mg, 1.0 equiv) were dissolved in ligation buffer (100 μL, 6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, pH 7.5). The reaction mixture was stirred at room temperature for 30 min. Peptide **30** (1.4 mg, 2.8 equiv) in ligation buffer (170 μL, 6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, pH 7.5) was added. The reaction mixture was stirred at room temperature for 30 min and quenched by 1.0 mL MESNa solution (10 mg MESNa in 1.0 mL MeCN:H_2O = 1:1). The ligation product was purified by HPLC to give 1.0 mg **33**, 61% yield.

To a solution of the above purified ligated peptide (0.36 mM) in degassed CH_3CN/H_2O (v/v = 1:1, 0.2 ml) were added 0.2 ml of 0.5 M bond-breaker® TCEP solution (Pierce), 20 μl of 2-methyl-2-propanethiol and 0.2 ml of radical initiator (0.1 M in H_2O). The reaction mixture was stirred at 37 °C for 1h and the desulfurization product was purified by HPLC to give 0.8 mg **34**, 82% yield.

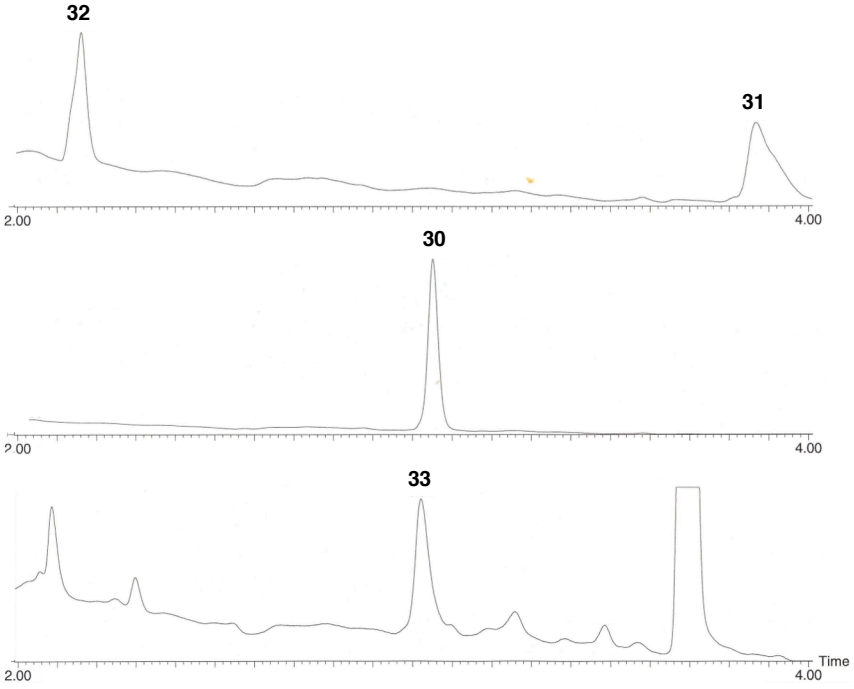


Figure S7. UPLC-MS analysis of the kinetically controlled one-pot synthesis of peptide **33**.

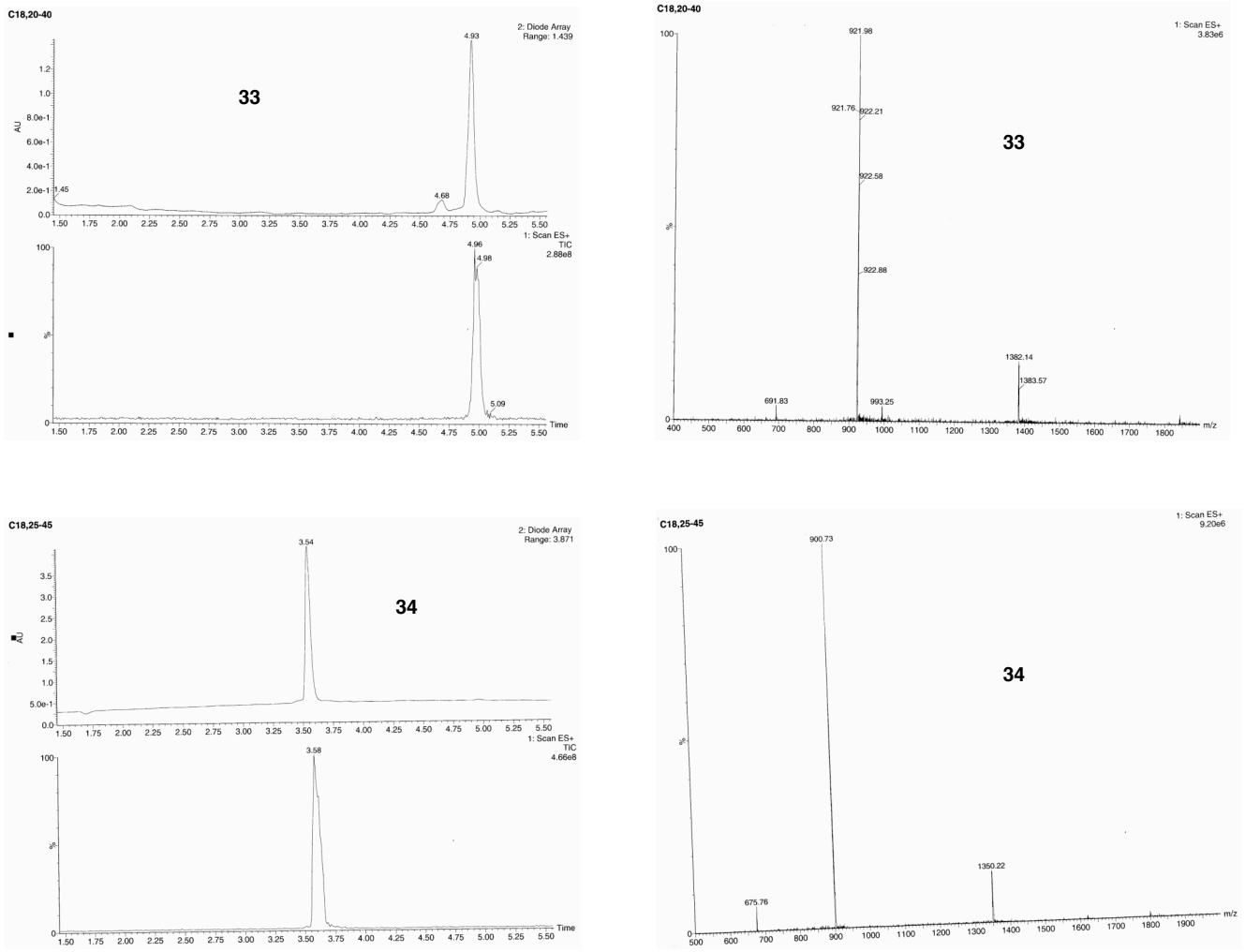


Figure S8. UPLC-MS analysis of purified **33** and its desulfurization product **34**. Left: UV and mass traces of the purified product. Right: ESI mass spectrum of the desired product. Ligation product **33** $C_{117}H_{209}N_{35}O_{37}S_2$ $[M+2H]^{2+}$ *m/z* = 1381.25, $[M+3H]^{3+}$ *m/z* = 921.16, $[M+4H]^{4+}$ *m/z* = 691.12; Desulfurization product **34** $C_{117}H_{209}N_{35}O_{37}$ $[M+2H]^{2+}$ *m/z* = 1349.27, $[M+3H]^{3+}$ *m/z* = 899.85, $[M+4H]^{4+}$ *m/z* = 675.13.

Preliminary Evidence for the Thiolactonization of Compound 31

In addition to the products shown above, we observed a sequence corresponding to initial cyclization of **31** with loss of the C-terminal ester. It is proposed that **31** undergoes transthiolactonization followed by S→N acyl transfer. This is then followed by transthioesterification with **30** to provide a product (~5%) whose mass is 1983.13 ($C_{86}H_{154}N_{26}O_{25}S$, $[M+2H]^{2+}$ m/z = 992.57). That **30** and **31** were joined through a thioester linkage was suggested by the fact that this compound disappeared, as expected, upon treatment with MESNa.

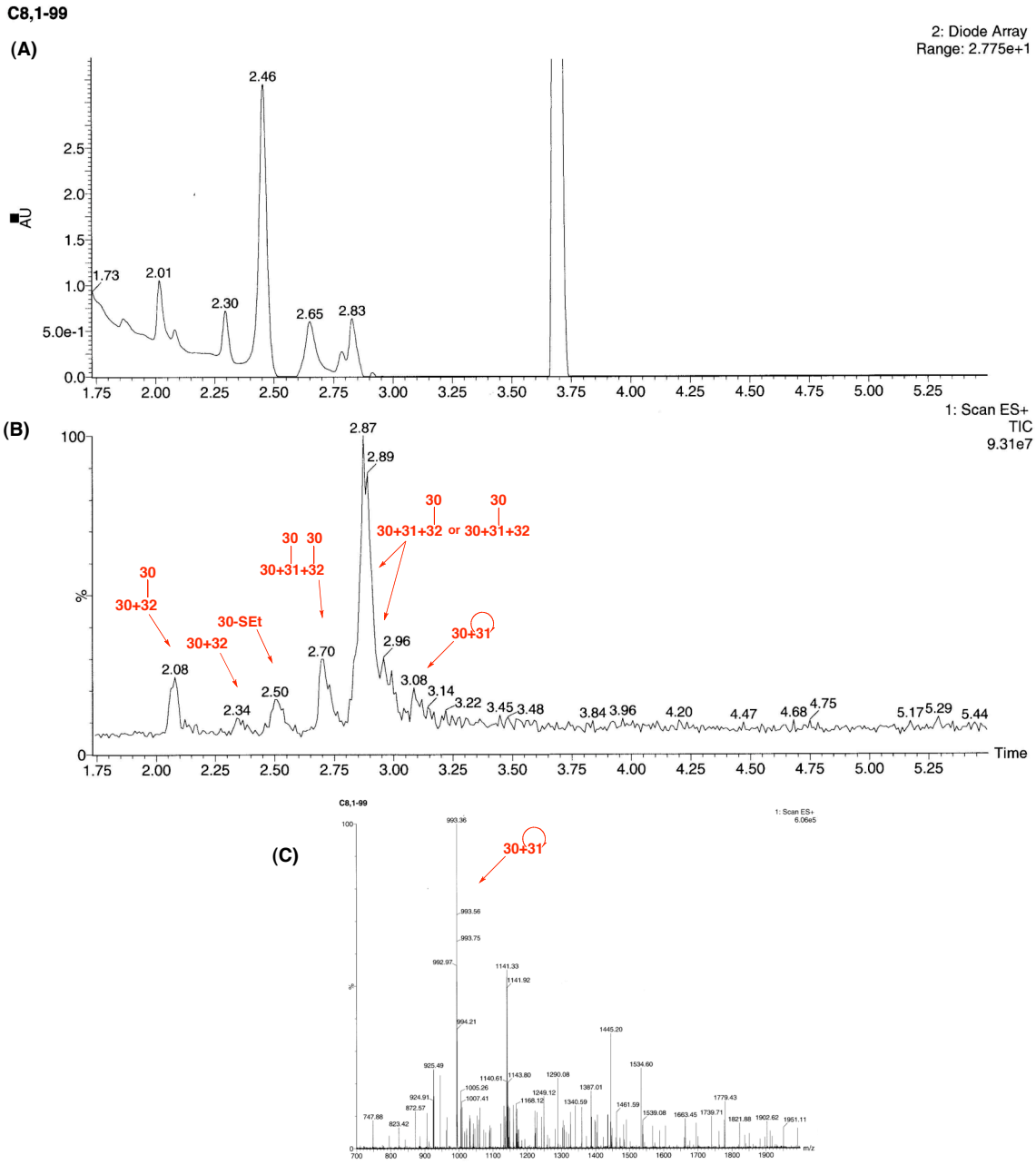


Figure S8. UPLC-MS analysis of crude mixture of kinetically controlled one-pot synthesis of peptide **33** before MESNa treatment. (A) UV trace of the reaction mixture. (B) Mass trace of the reaction mixture. (C) ESI mass spectrum of the side product.