Advances in Proline Ligation

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Content

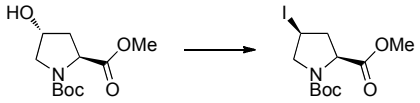
Materials and methods:

All commercial materials (Aldrich, Fluka, Nova) were used without further purification. All solvents were reagent or HPLC grade (Fisher). Anhydrous DMF, THF, Et_2O, 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 DRX-500 MHz, referenced to residual solvent. High-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.

Solid Phase Peptide Synthesis: Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized using the standard automated Fmoc protocol (HATU, DIEA, DMF). The deblocking solution was a mixture of DMF/piperidine/DBU (100/5/5). The NovaSyn® TGT resins from EMD Biosciences were employed for the synthesis. The following amino acids from Sigma Aldrich, EMD Biosciences, Chem-Impex International and PolyPeptide Laboratories were employed: Boc-Gly-OH, Boc-Ala-OH, Boc-Phe-OH, Boc-Val-OH, Boc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Val-OH. Boc-Selenoproline-OH (Dimer) was synthesized. 0.55 eq of selenoproline was used along with an extended 2-hour reaction time for the amide bond-forming step.

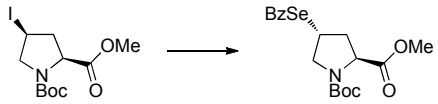
HPLC: All separations of peptides involved a mobile phase of 0.05% TFA (v/v) in water (solvent A) / 0.04% TFA in acetonitrile (solvent B). Preparative and analytical HPLC separations were performed using a Rainin HPXL solvent delivery system equipped with a Rainin UV-1 detector. LC-MS chromatographic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

Preparation and Characterization of compounds 34-36



33 **34**

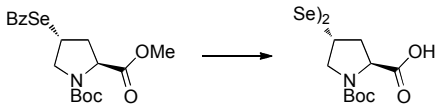
(2*S*,4*S*)-1-*tert*-butyl 2-methyl 4-iodopyrrolidine-1,2-dicarboxylate (34). To a stirred solution of *N*-Boc-*trans*-4-hydroxy-L-proline methyl ester **33** (2.45 g, 10.0 mmol) in THF (40 mL) at 0°C was added diisopropyl azodicarboxylate (2.45 g, 12.1 mmol), triphenylphosphine (3.90 g, 14.9 mmol) and methyl iodide (1.70 g, 12.0 mmol). After warming to ambient temperature and stirring for 3 hours, the reaction mixture was filtered and the filtrate concentrated *in vacuo*. The crude residue was purified by flash column chromatography (4:1, Hexanes:EtOAc) to provide **34** (3.08 g, 8.70 mmol, 87%), as a white solid: spectral data consistent with reported values[1]



34 **35**

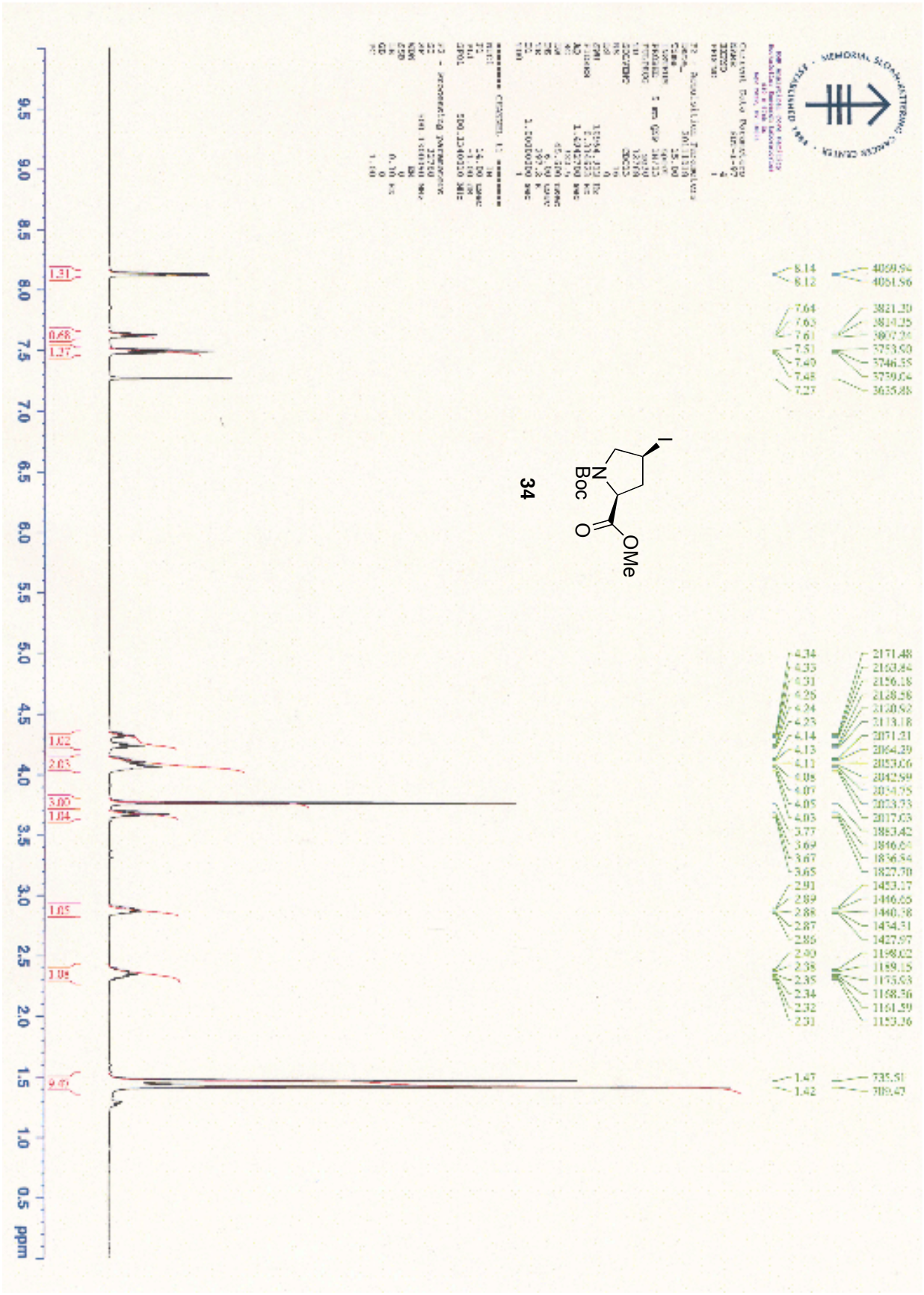
(2*S*,4*R*)-1-*tert*-butyl 2-methyl-4-(benzoylselanyl)pyrrolidine-1,2 dicarboxylate (35). To a solution of benzoic acid (278 mg, 2.28 mmol) in toluene (1 mL) was added Woollins Reagent (404 mg, 0.76 mmol) and the reaction was warmed to reflux. After 2 hours the reaction was cooled to 50°C. **34** (135 mg, 0.38 mmol) was dissolved in DMF (1 mL) and added dropwise to the reaction mixture followed by the addition of diisopropylethylamine (500 μL, 2.85 mmol). After 2 hours the reaction mixture was diluted with water (5 mL) and extracted with EtOAc (3 x 5 mL). The organics were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude residue was purified by flash

column chromatography (gradient 10:1 to 4:1, Hexanes:EtOAc) to provide **35** (132 mg, o.32 mmol, 84%) as a white solid: $[\alpha]_D^{25}$ = -18.8 (*c* = 0.03, $CHCl_3$); mp 92-94°C; R_f 0.4 (4:1, Hex:EtOAc); IR (cm^{-1}): 2950, 2250, 1752, 1694, 1644, 1396; ^1H NMR ($CDCl_3$): δ7.86 (d, 2H, *J* = 5.35 Hz), 7.63-7.59 (m, 1H), 7.48-7.45 (m, 2H), 4.48 (ddd, 1H, *J* = 6.90, 5.85, 4.40 Hz), 4.24 (t, 1H, *J* = 6.50 Hz), 4.11 (ddd, 1H, *J* = 6.90, 6.85, 4.45 Hz), 3.78 (s, 3H), 3.69 (ddd, 1H, *J* = 6.50, 5.45, 4.55 Hz), 2.61 (m, 2H), 1.47-1.43 (s, 9H); ^{13}C NMR ($CDCl_3$): δ195.3, 174.4, 174.1, 155.5, 154.8, 140.0, 135.4, 129.8, 128.7, 81.9, 60.3, 59.9, 53.8, 53.6, 39.5, 37.9, 29.8, 29.7; ESMS calcd for $C_{18}H_{23}NO_5SeNa$ (M + Na^+): 436.06, Found (M + Na^+): 436.1.



35 **36**

(2*S*,4*R*)-1-(*tert*-butoxycarbonyl)-4-hydroselenopyrrolidine-2-carboxylic acid (36). To a solution of **35** (103 mg, 0.25 mmol) in 10% aq. MeOH (2.5 mL) at 0°C is added K_2CO_3 (96 mg, 0.50 mmol). After 2 hours, AcOH (500 μL) was added, the reaction mixture diluted with brine (5 mL) and extracted with CH_2Cl_2:*n*BuOH (10:1, 3 x 5 mL). The organics were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (EtOAc:AcOH, 95:5) to provide **36** (117 mg, 0.20 mmol, 79%) as a pale yellow foam: $[\alpha]_D^{25}$ = -102.8 (*c* = 0.03, $CHCl_3$); R_f 0.2 (EtOAc); IR (cm^{-1}): 3395, 2980, 2253, 1688, 1402, 1162; ^1H NMR ($CDCl_3$): δ4.49 (m, 1H), 3.96 (m, 1H), 3.77 (m, 1H), 3.54 (m, 1H), 2.59 (m, 2H), 1.52-1.45 (s, 9H); ^{13}C NMR ($CDCl_3$): 179.2, 178.3, 156.9, 83.5, 82.5, 60.2, 55.0, 39.6, 37.8, 29.7, 29.6, 22.1; ESMS calcd for $C_{20}H_{32}N_2O_8Se_2Na$ (M + Na^+): 611.03, Found (M + Na^+): 611.2.


34
N
Boc
O
OMe
I
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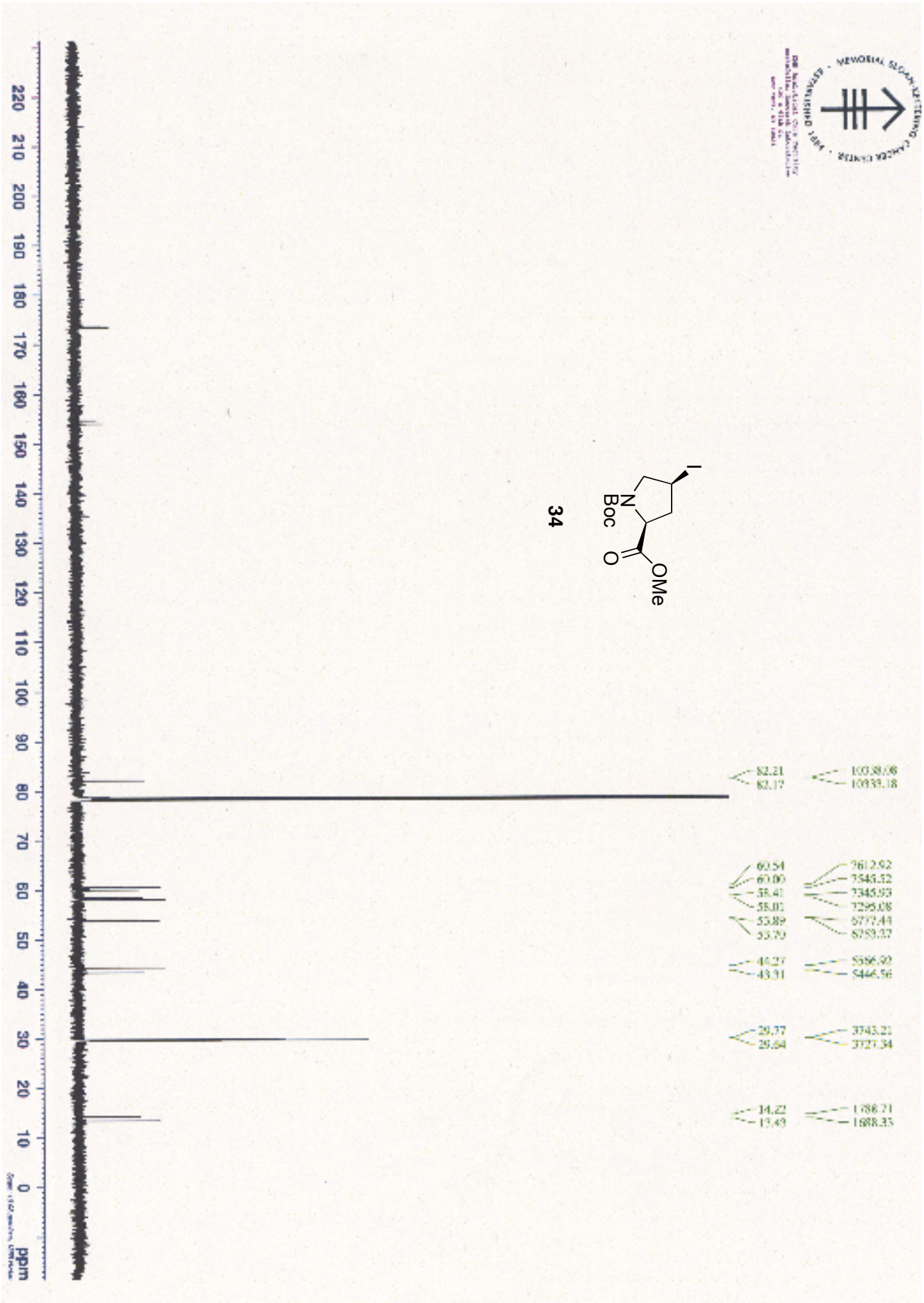
0.68
1.37
1.02
2.03
3.00
1.04
1.05
1.08
9.07


34
N
Boc
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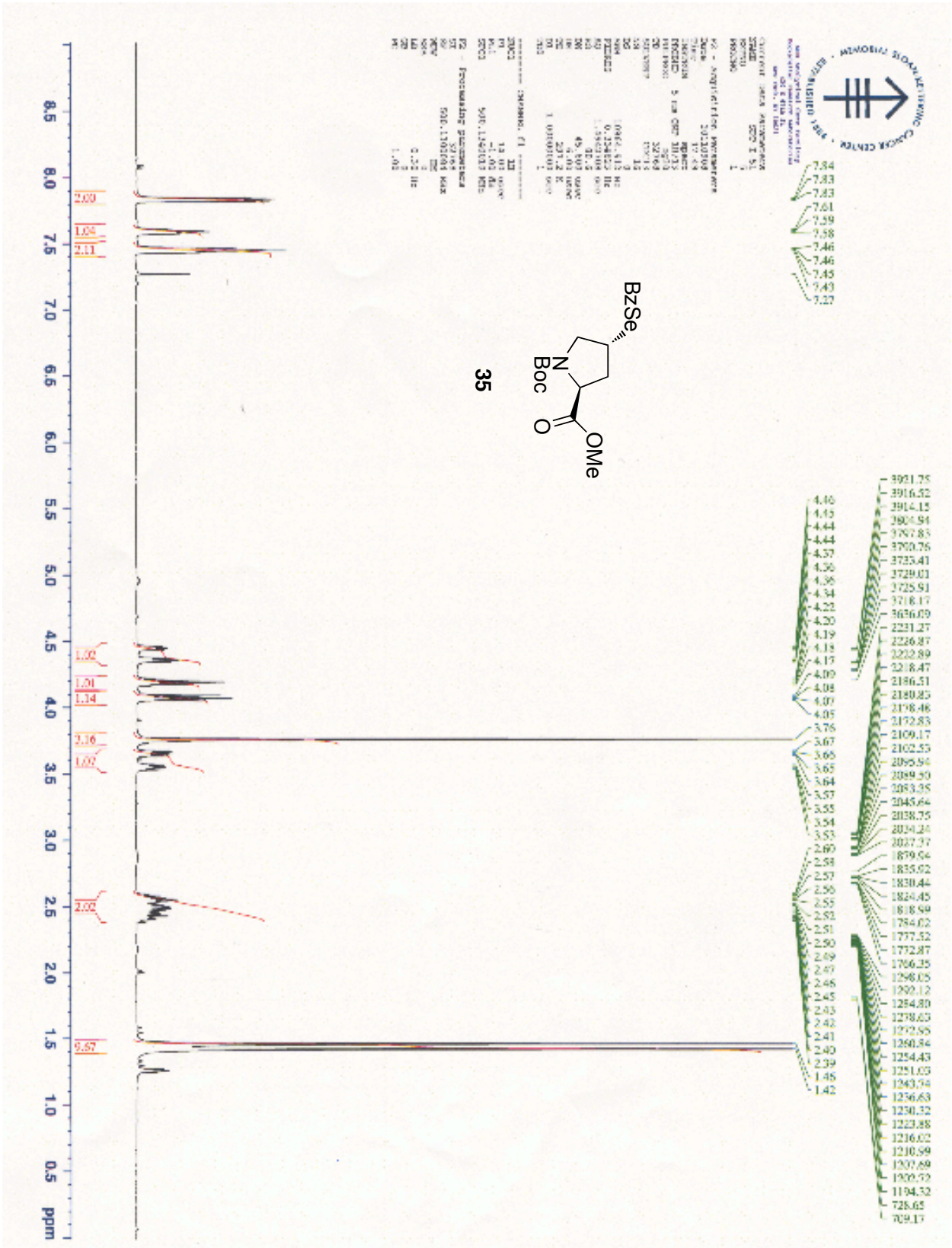
BzSe
N
Boc
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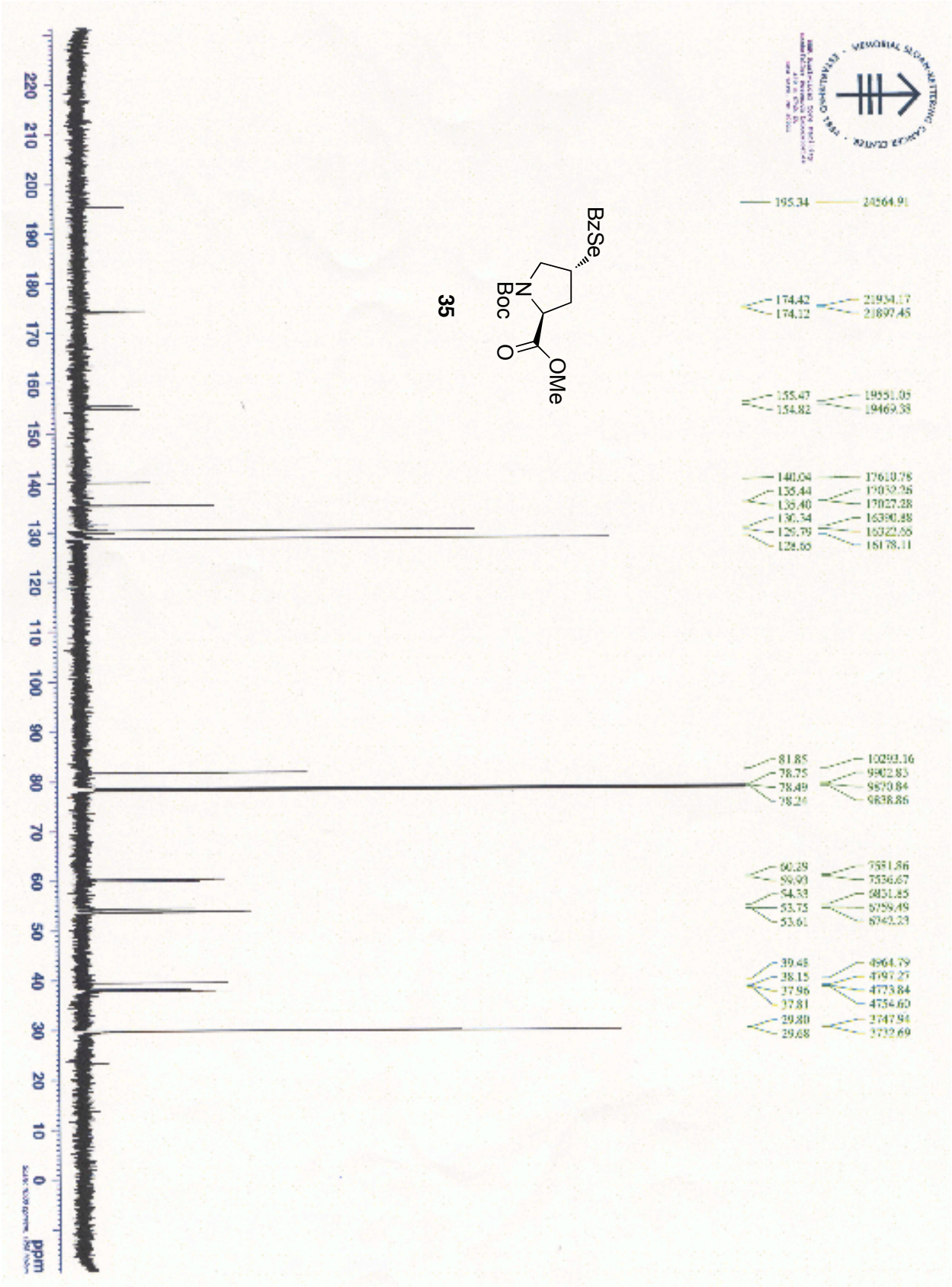
OMe
35


BzSe
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Boc
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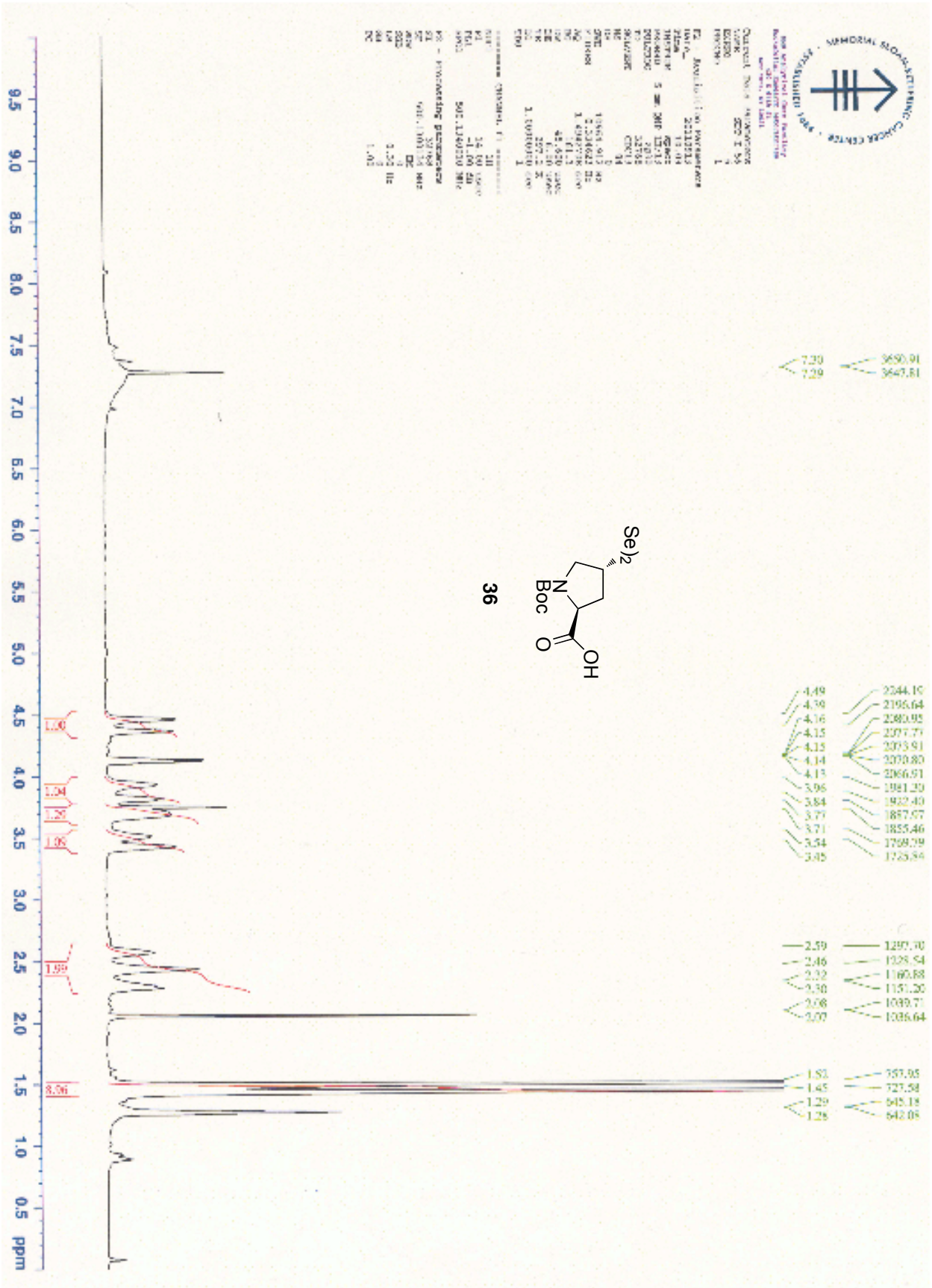
35
ppm


36
Se)2
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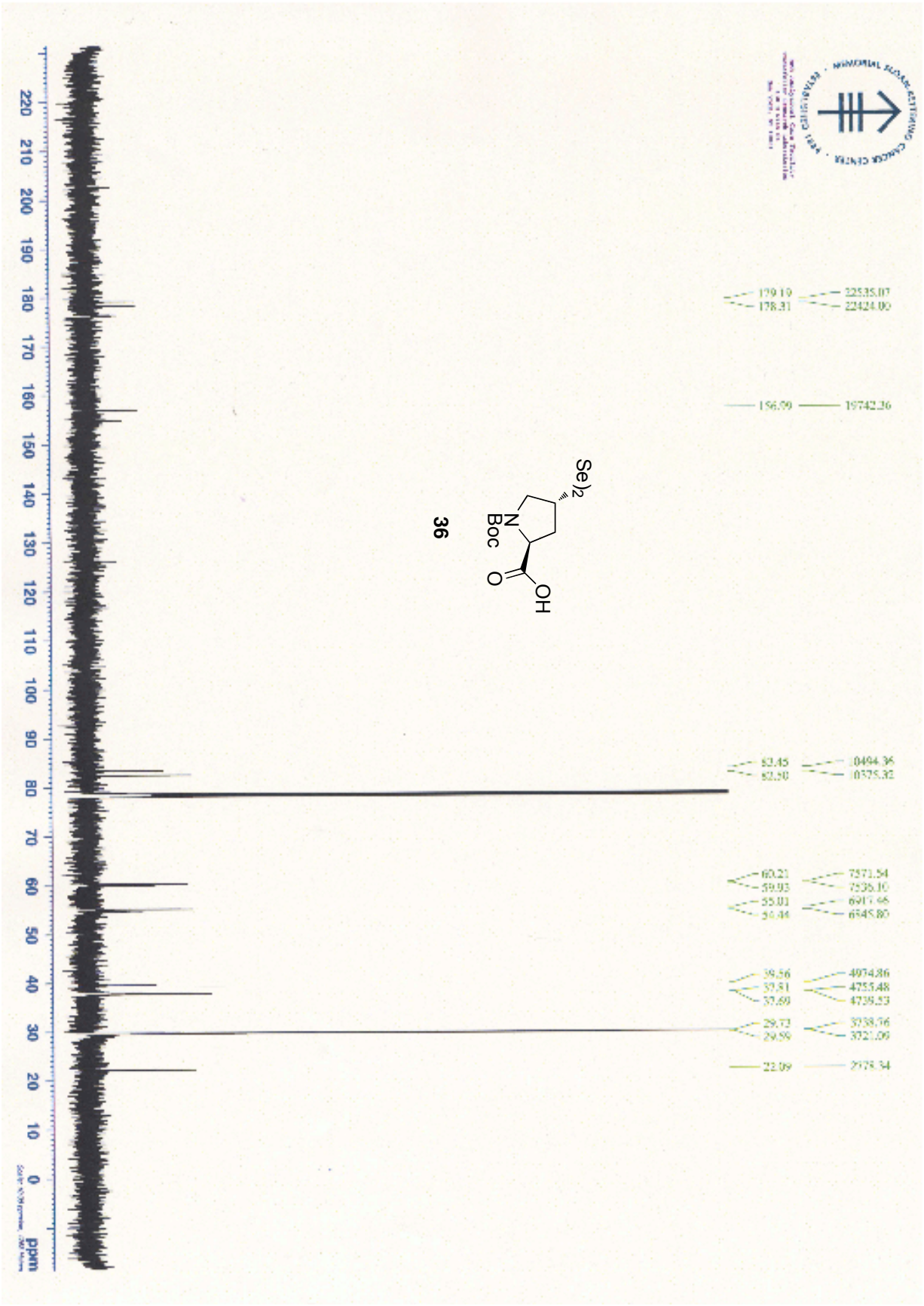
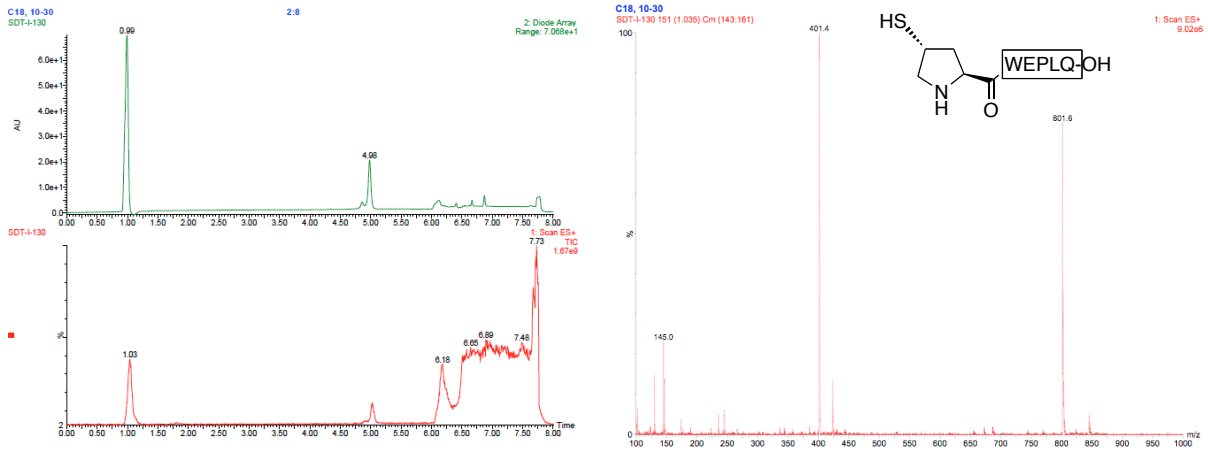
Boc
O
OH
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
1.00
1.04
1.25
1.05
1.99
8.56


36
Se)2
N
Boc
O
OH
ppm

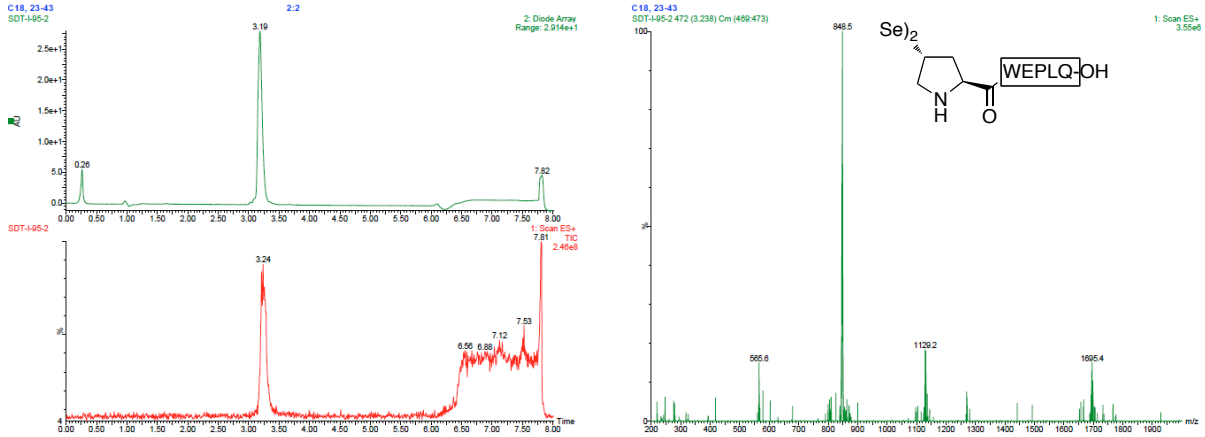
Preparation and Characterization of Peptide Precursors for Ligation

Unprotected Peptides: Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with CH_2Cl_2. The resin cleavage was affected by treatment with TFA/H_2O/TIS (95:2.5:2.5) for 45 min to provide the unprotected peptides. The solvent was removed by a stream of N_2 and the residue triturated with Et_2O and centrifuged to give a white pellet. After the Et_2O was decanted, the solid was dissolved in 30% aq. CH_3CN (5% AcOH) for HPLC purification.

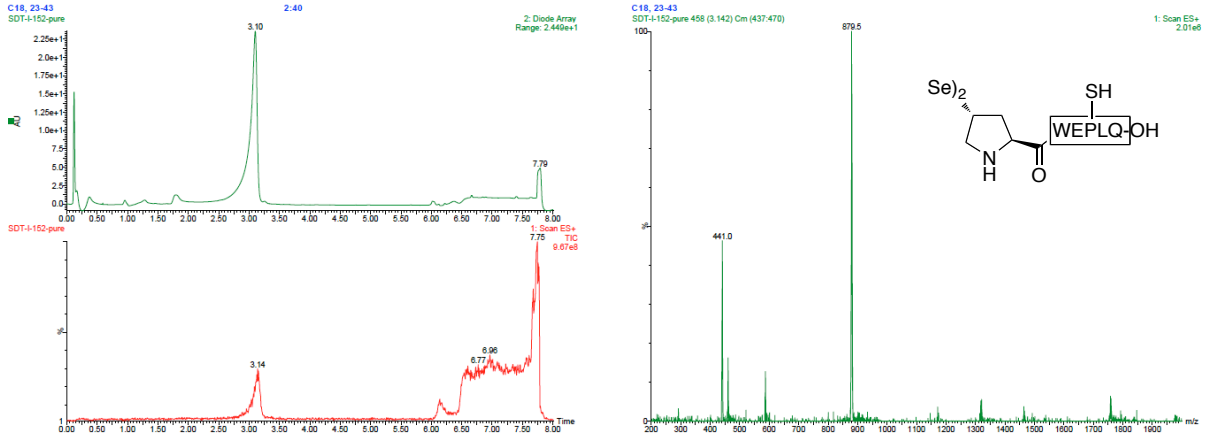


41: $C_{37}H_{52}N_8O_{10}S$, Exact Mass: 800.35, $[M+H]^+$ *m/z* = 801.35, $[M+2H]^{2+}$ *m/z* = 401.18.

Found: $[M+H]^+$ *m/z* = 801.6, $[M+2H]^{2+}$ *m/z* = 401.4.



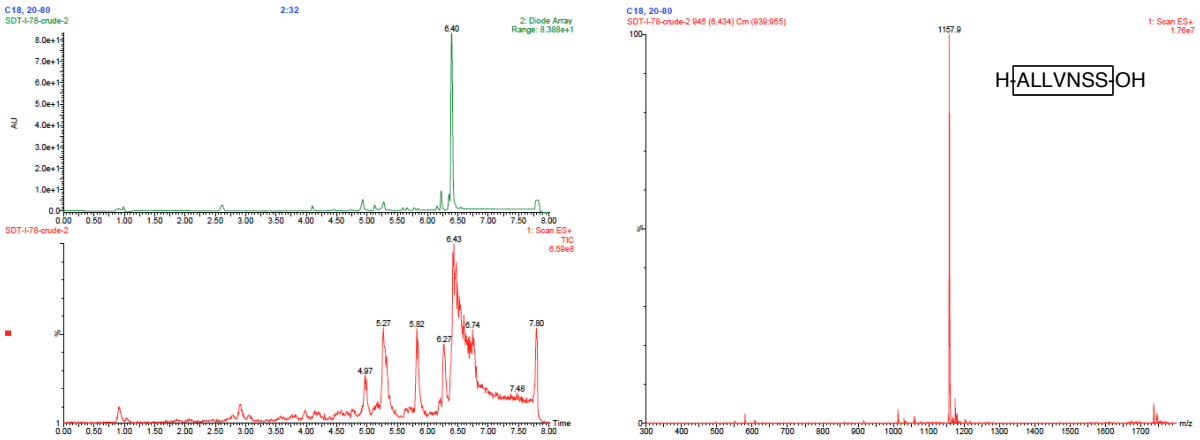
43: $C_{74}H_{102}N_{16}O_{20}Se_2$, Exact Mass: 1694.57, $[M+H]^+$ *m/z* = 1695.57, $[M+2H]^{2+}$ *m/z* = 848.29. Found: $[M+H]^+$ *m/z* = 1695.4, $[M+2H]^{2+}$ *m/z* = 848.5.



45: $C_{74}H_{102}N_{16}O_{20}S_2Se_2$, Exact Mass: 1759.52, $[M+2H]^{2+}$ *m/z* = 880.26, $[M+4H]^{4+}$ *m/z* = 440.88. Found: $[M+2H]^{2+}$ *m/z* = 879.5, $[M+4H]^{4+}$ *m/z* = 441.0.

Fully Protected Peptidyl Acid: Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with CH_2Cl_2. The resin cleavage was affected by treatment with AcOH/TFE/DCM (1:1:3) for 2 x 1

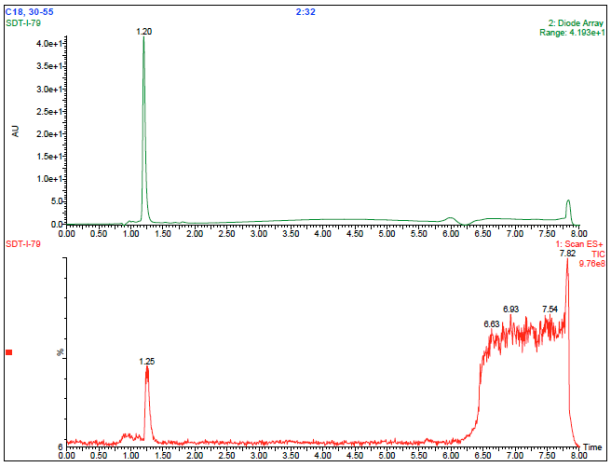
hour to provide the peptidyl acids. The solvent was removed by a stream of N_2. The residue was triturated with Et_2O and centrifuged to give a white pellet. After the Et_2O was decanted, the solid was dissolved in 50% aq. CH_3CN and lyophilized to dryness.

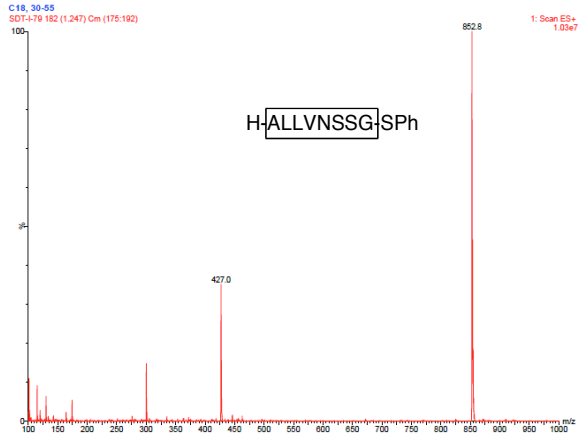


Peptidyl Acid: $C_{62}H_{91}N_8O_{13}$, Exact Mass: 1155.67, $[M+H]^+$ *m/z* = 1156.67.

Found: $[M+H]^+$ *m/z* = 1157.9

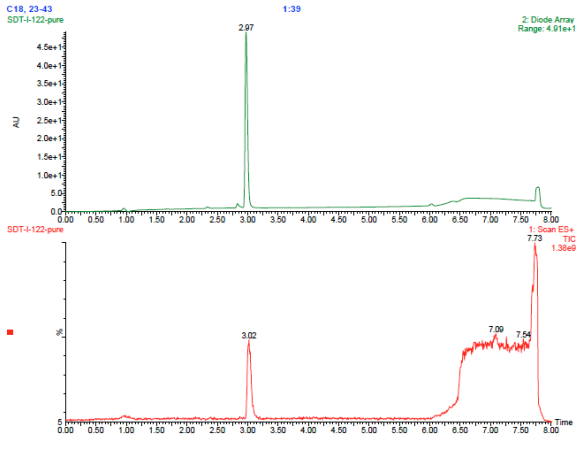
Peptide Thiophenolic Esters: The peptidyl acid (0.043 mmol) and HCl□H-AA-SPh (0.13 mmol) were dissolved in $CHCl_3$:TFE (3:1, 4 mL) and cooled to -10 °C. HOOBt (0.13 mmol) and EDCI (0.13 mmol) were added and the reaction stirred at ambient temperature for 2.5 hours. The solvent was removed by a stream of N_2 and the oil dissolved in CH_2Cl_2 (10 mL). The organics were washed with H_2O (3 x 5 mL) and concentrated by a stream of N_2. TFA/H_2O/TIS (95:2.5:2.5, 5 ml) was added and the reaction stirred 20 minutes. The solvent was removed by a stream of N_2 and the residue triturated with Et_2O and centrifuged to give a white pellet. After the Et_2O was decanted, the solid was dissolved in 30% aq. CH_3CN (5% AcOH) for HPLC purification.

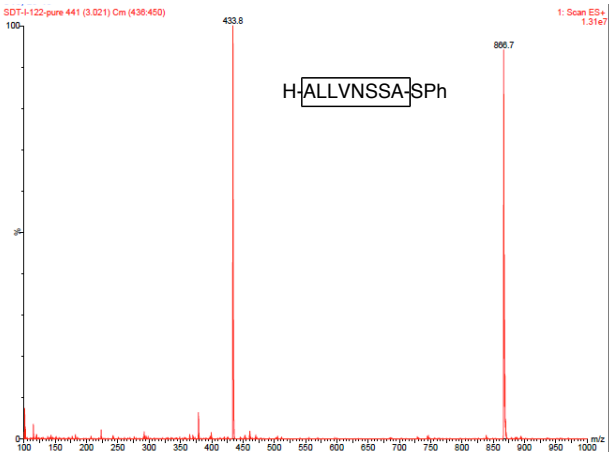




38: $C_{38}H_{61}N_9O_{11}S$, Exact Mass: 851.42, $[M+H]^+$ *m/z* = 852.42, $[M+2H]^{2+}$ *m/z* 426.71.

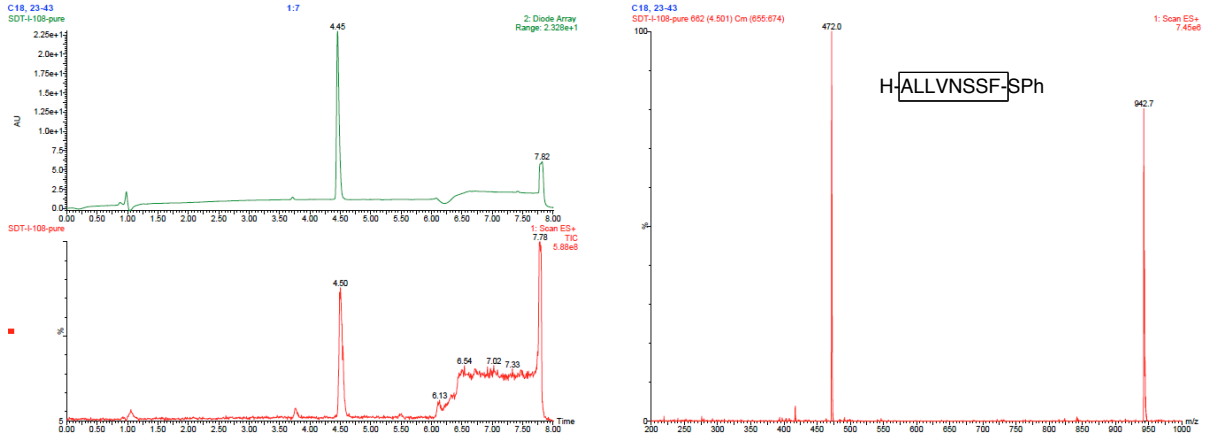
Found: $[M+H]^+$ *m/z* = 852.8, $[M+2H]^{2+}$ *m/z* 427.0.





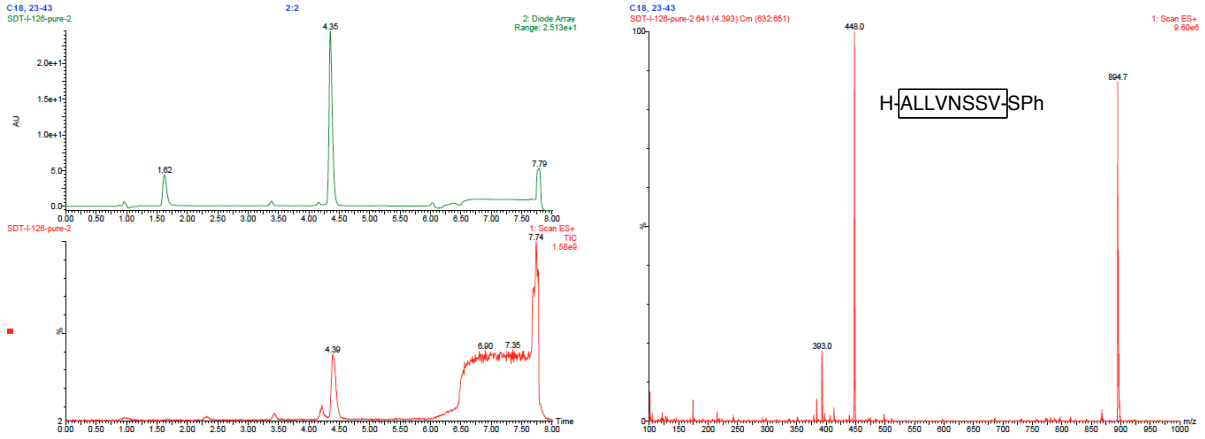
48: $C_{39}H_{63}N_9O_{11}S$, Exact Mass: 865.44, $[M+H]^+$ *m/z* = 866.40, $[M+2H]^{2+}$ *m/z* 433.72.

Found: $[M+H]^+$ *m/z* = 866.7, $[M+2H]^{2+}$ *m/z* 433.8.



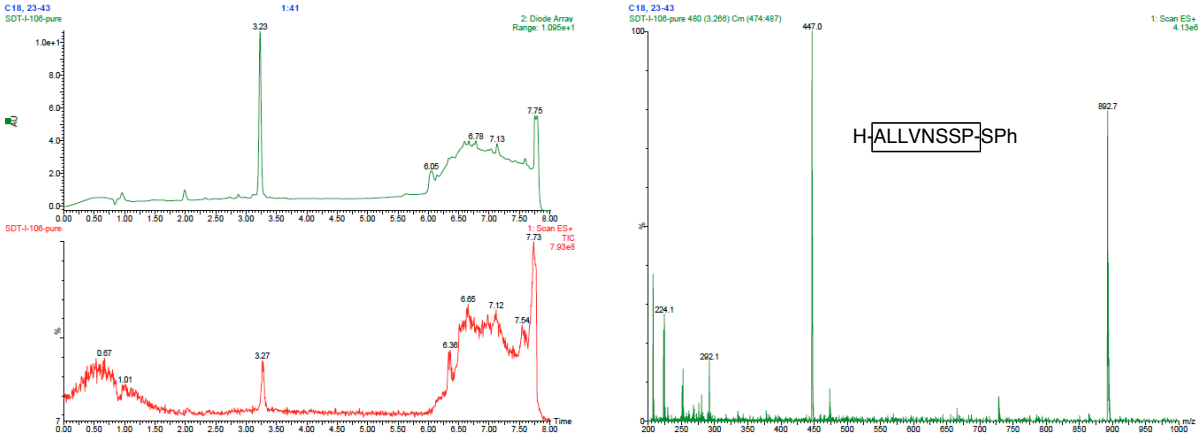
50: $C_{45}H_{67}N_9O_{11}S$, Exact Mass: 941.47, $[M+H]^+$ *m/z* = 942.5, $[M+2H]^{2+}$ *m/z* 471.74.

Found: $[M+H]^+$ *m/z* = 942.7, $[M+2H]^{2+}$ *m/z* 472.0.



52: $C_{41}H_{67}N_9O_{13}S$, Exact Mass: 893.47, $[M+H]^+$ *m/z* = 894.47, $[M+2H]^{2+}$ *m/z* 447.7.

Found: $[M+H]^+$ *m/z* = 894.7, $[M+2H]^{2+}$ *m/z* 448.0.

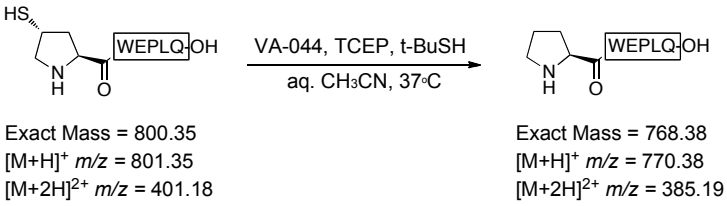


54: $C_{45}H_{71}N_{11}O_{11}S$, Exact Mass: 891.45, $[M+H]^+$ *m/z* = 892.45, $[M+2H]^{2+}$ *m/z* 446.73.

Found: $[M+H]^+$ *m/z* = 892.7, $[M+2H]^{2+}$ *m/z* 447.0.

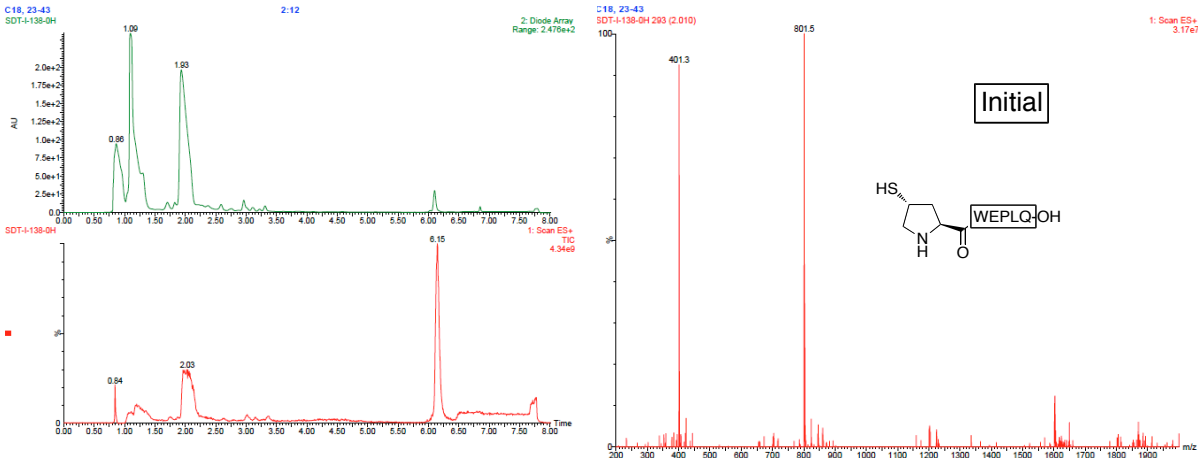
Preparation and Characterization of Dethiolation Products

To a solution of thiopeptide (2 μmol) in degassed 50% aq. CH_3CN (200 μl) was added TCEP bond-breaker® solution (Pierce, 200 μl, 0.5 M), t-BuSH (200 μl) and VA-044 radical initiator (200 μl, 0.1 M in H_2O) and the reaction mixture was stirred at 37°C. The reaction was monitored by UPLC-MS and purified by HPLC.

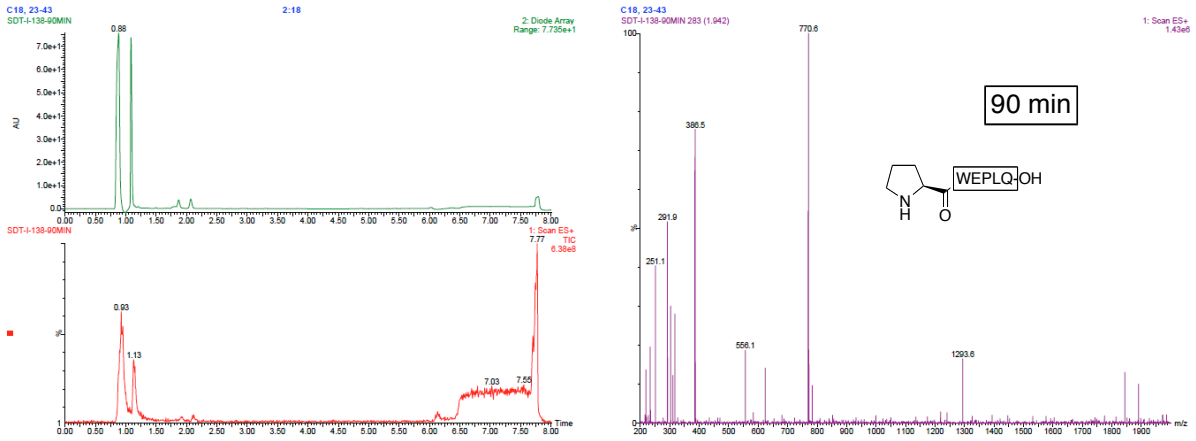


Exact Mass = 800.35
$[M+H]^+$ *m/z* = 801.35
$[M+2H]^{2+}$ *m/z* = 401.18

Exact Mass = 768.38
$[M+H]^+$ *m/z* = 770.38
$[M+2H]^{2+}$ *m/z* = 385.19

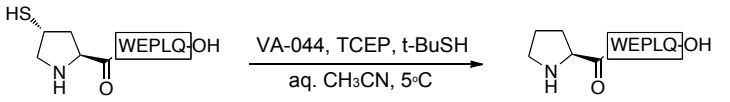


Found: $[M+H]^+$ *m/z* = 801.5, $[M+2H]^{2+}$ *m/z* 401.3.



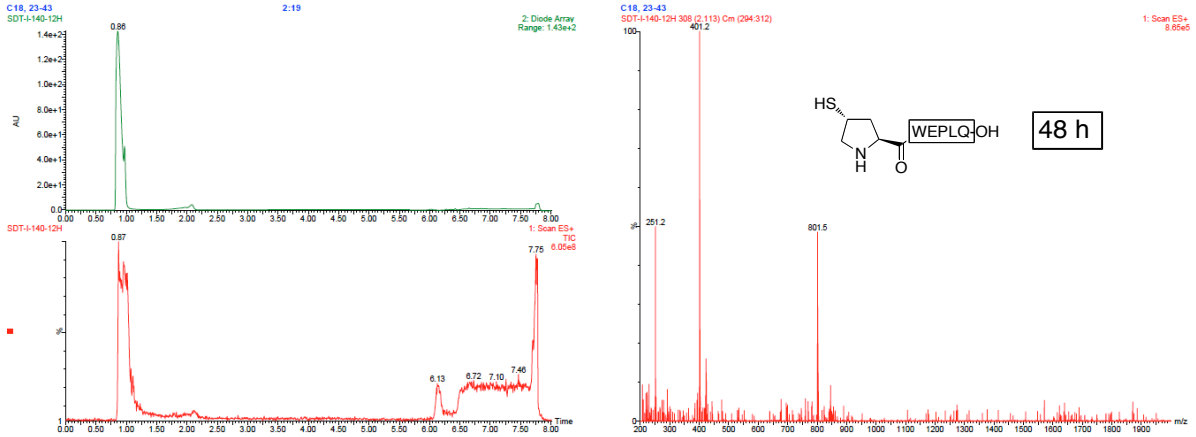
Found: $[M+H]^+$ *m/z* = 770.6, $[M+2H]^{2+}$ *m/z* 386.5, 83%

To a solution of thiopeptide (2 μmol) in 50% aq. CH_3CN (200 μl) was added TCEP (200 μl, 0.5 M), t-BuSH (200 μl) and VA-044 radical initiator (200 μl, 0.1 M in H_2O) and the reaction mixture was stirred at 5°C. After 48 h, the reaction was warmed to ambient temperature to observe slow dethiolation. The reaction was monitored by UPLC-MS.

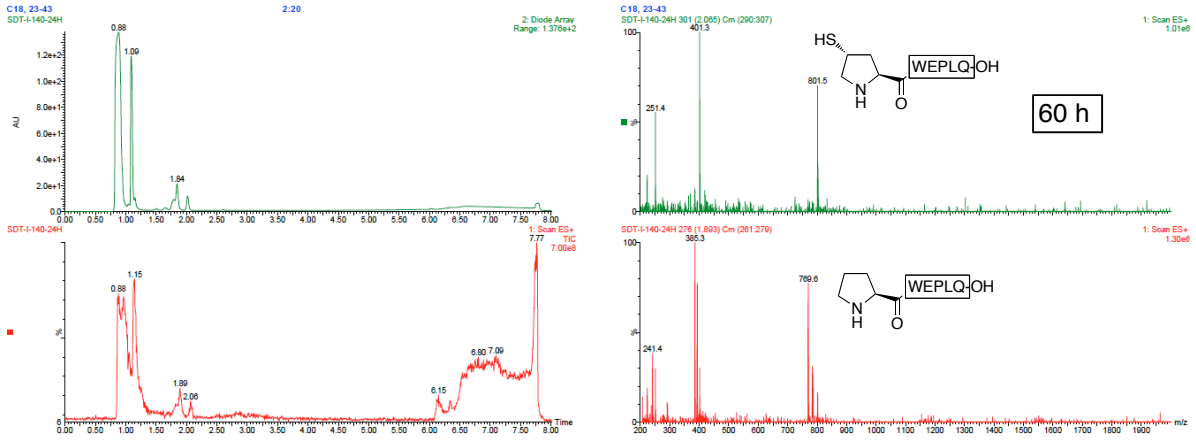


Exact Mass = 800.35
$[M+H]^+$ *m/z* = 801.35
$[M+2H]^{2+}$ *m/z* = 401.18

Exact Mass = 768.38
$[M+H]^+$ *m/z* = 770.38
$[M+2H]^{2+}$ *m/z* = 385.19



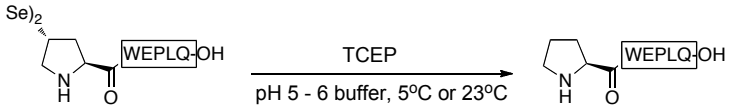
Found: $[M+H]^+$ *m/z* = 801.5, $[M+2H]^{2+}$ *m/z* 401.3.



Found: $[M+H]^+$ *m/z* = 801.5, $[M+2H]^{2+}$ *m/z* 401.3 and $[M+H]^+$ *m/z* = 769.6, $[M+2H]^{2+}$ *m/z* 385.3.

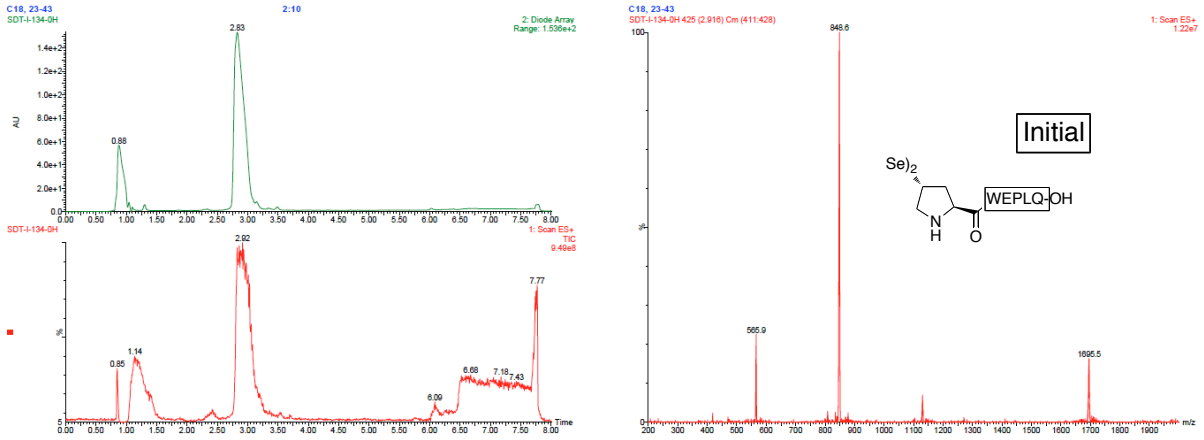
Preparation and Characterization of Deselenation Products

To a solution of selenopeptide (2μmol) in 500 μl of ligation buffer (6 M Gdm•HCl, 200 mM Na_2HPO_4) was added TCEP (200 μl, 0.5 M) and the pH adjusted to 5-6 by 1 M HCl. The reaction mixture was stirred at 23°C, monitored by UPLC-MS and purified directly by HPLC.

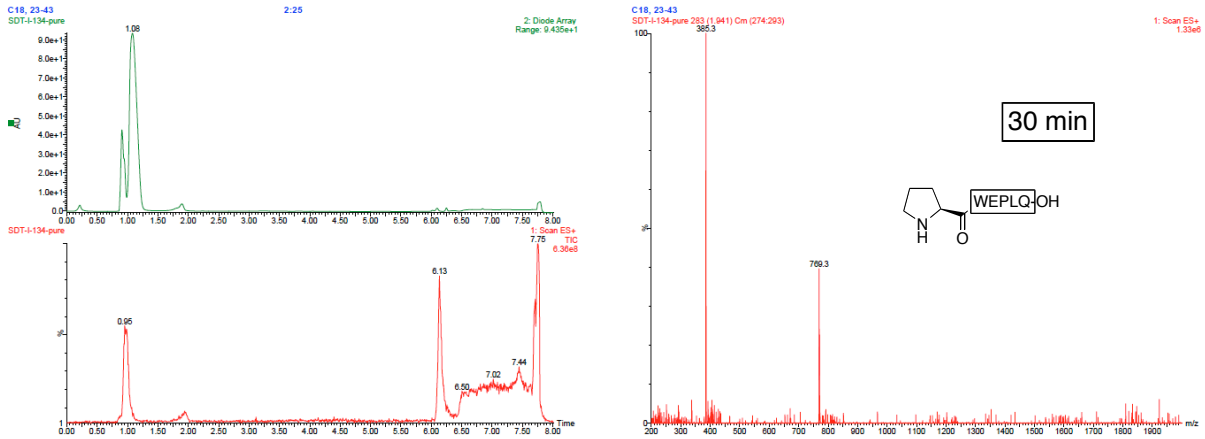


Exact Mass = 1694.57
$[M+H]^+$ *m/z* = 1695.57
$[M+2H]^{2+}$ *m/z* = 848.29

Exact Mass = 768.38
$[M+H]^+$ *m/z* = 770.38
$[M+2H]^{2+}$ *m/z* = 385.19

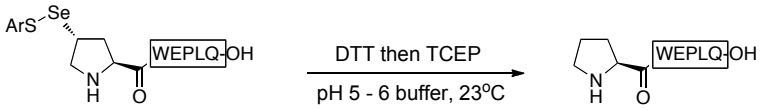


Found: $[M+H]^+$ *m/z* = 1695.5, $[M+2H]^{2+}$ *m/z* 848.6.



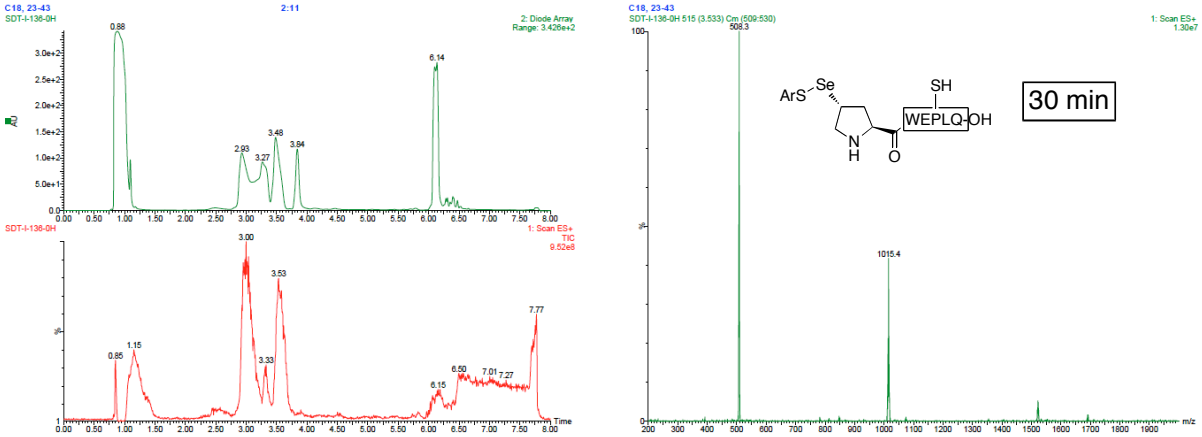
Found: $[M+H]^+$ *m/z* = 769.3, $[M+2H]^{2+}$ *m/z* 385.3, 92%

To a solution of selenopeptide (2 μmol) in 500 μl of ligation buffer (6 M Gdm•HCl, 200 mM Na_2HPO_4) was added 4-mercaptophenylacetic acid (MPAA, 1.6 mg, μmol) to generate the selenylsulfide. After stirring 30 min, Dithiothreitol (DTT, 3 mg, 20 μmol) was added and the reaction continued stirring 30 min. TCEP (200 μl, 0.5 M) was added and the pH adjusted to 5-6 by 1 M HCl. The reaction mixture was stirred at 23°C, monitored by UPLC-MS and purified by HPLC.

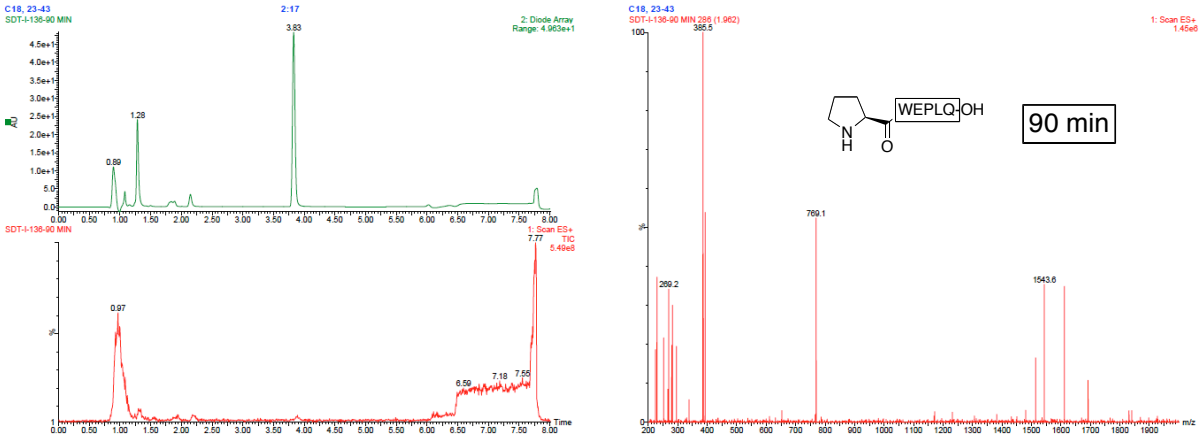


Exact Mass = 1014.30
$[M+H]^+$ *m/z* = 1015.30
$[M+2H]^{2+}$ *m/z* = 508.15

Exact Mass = 768.38
$[M+H]^+$ *m/z* = 770.38
$[M+2H]^{2+}$ *m/z* = 385.19



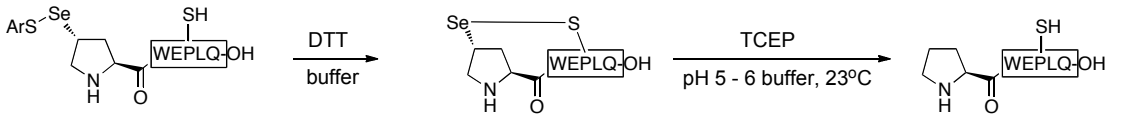
Found: $[M+H]^+$ *m/z* = 1015.4, $[M+2H]^{2+}$ *m/z* 508.3.



Found: $[M+H]^+$ *m/z* = 769.1, $[M+2H]^{2+}$ *m/z* 385.5, 91%

To a solution of selenopeptide (2 μmol) in 500 μl of ligation buffer (6 M Gdm•HCl, 200 mM Na_2HPO_4) was added MPAA (1.6 mg, μmol) to generate the selenylsulfide. DTT was added and the reaction stirred 30 min. TCEP (200 μl, 0.5 M) was added and the pH

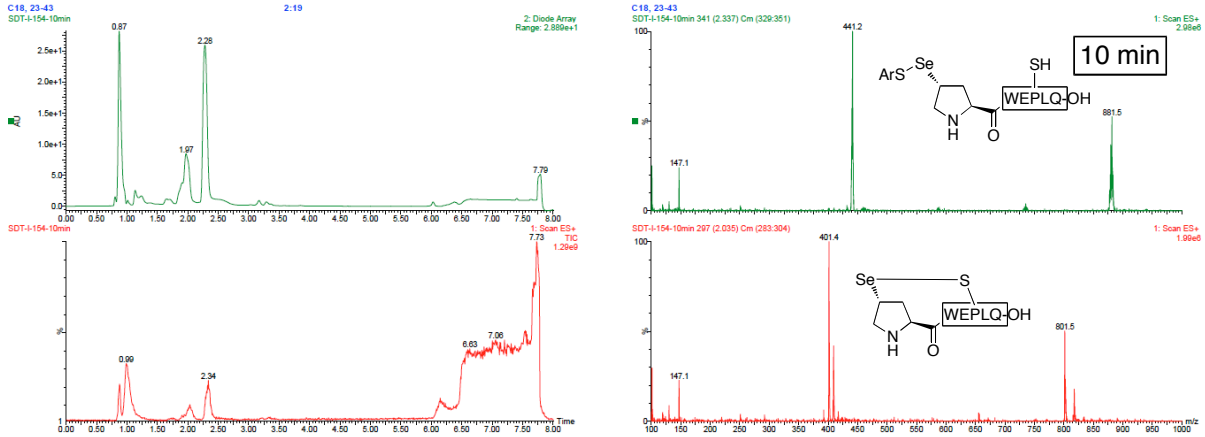
adjusted to 5-6 by 1 M HCl. The reaction mixture was stirred at 23°C, monitored by UPLC-MS and purified by HPLC.



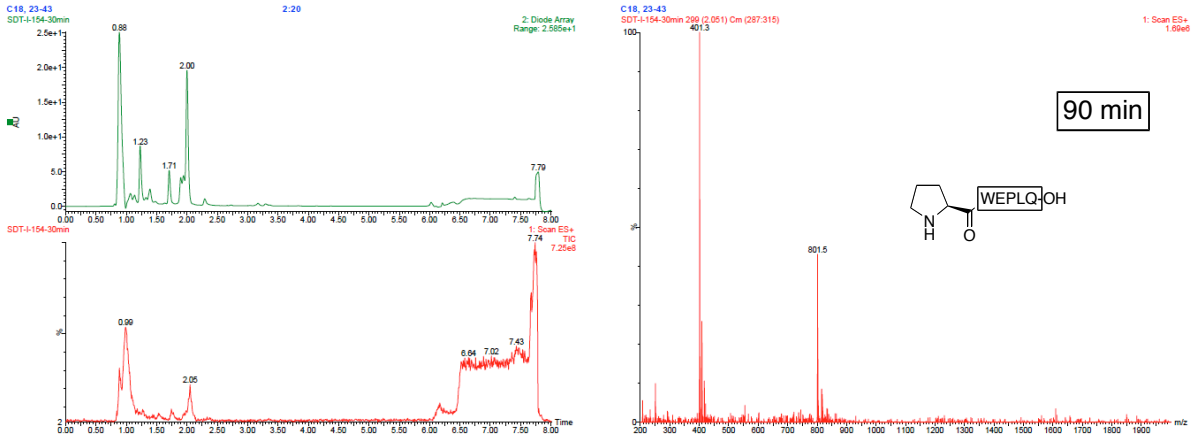
Exact Mass = 1014.30
$[M+H]^+$ *m/z* = 1015.30
$[M+2H]^{2+}$ *m/z* = 508.15

Exact Mass = 878.25
$[M+H]^+$ *m/z* = 879.25
$[M+2H]^{2+}$ *m/z* = 440.13

Exact Mass = 800.35
$[M+H]^+$ *m/z* = 801.35
$[M+2H]^{2+}$ *m/z* = 401.18



Found: $[M+H]^+$ *m/z* = 881.5, $[M+2H]^{2+}$ *m/z* 441.5. $[M+H]^+$ *m/z* = 801.5, $[M+2H]^{2+}$ *m/z* 401.4.



Found: $[M+H]^+$ *m/z* = 801.5, $[M+2H]^{2+}$ *m/z* 401.3, 90%

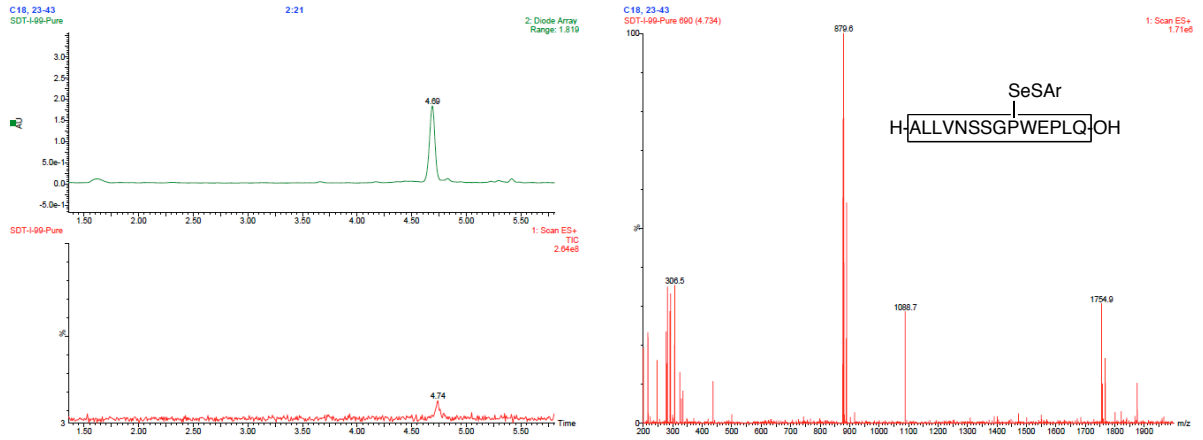
Preparation and Characterization of Ligation/Deselenation Products

H-ALLVNSS"AA"-SPh (2 mg, 2.36 μmol) and diselenide peptide H-***P***WEPLQ-OH **22** (2 mg, 1.18 μmol) were dissolved in 1 mL of degassed ligation buffer (6 M Gdm•HCl, 200 mM Na_2HPO_4). MPAA (34 mg, 200 mM) was added and the pH of the reaction adjusted to 7.4 to 7.6 by 1 M NaOH. The reaction was degassed (freeze-pump-thaw), allowed to stir under an argon atmosphere at ambient temperature and monitored by UPLC-MS. After consumption of the thioester the reaction was diluted with 50% aq. CH_3CN to 3 mL and purified by HPLC. The pure selenopeptide was dissolved in 1mL of ligation buffer (6 M Gdm•HCl, 200 mM Na_2HPO_4) and treated with DTT (3.6 mg, 23.6 μmol). After 30 min, TCEP (500 μl, 0.5 M) was added, the pH adjusted to 5-6 by 1 M HCl, and the reaction monitored by UPLC-MS. After removal of the selenide, the reaction mixture was diluted with 50% aq. CH_3CN to 3 mL and purified by HPLC.

Note: *Separation of the ligation adduct from unreacted N-terminal selenopeptide was difficult. An alternative, one pot protocol was developed.*

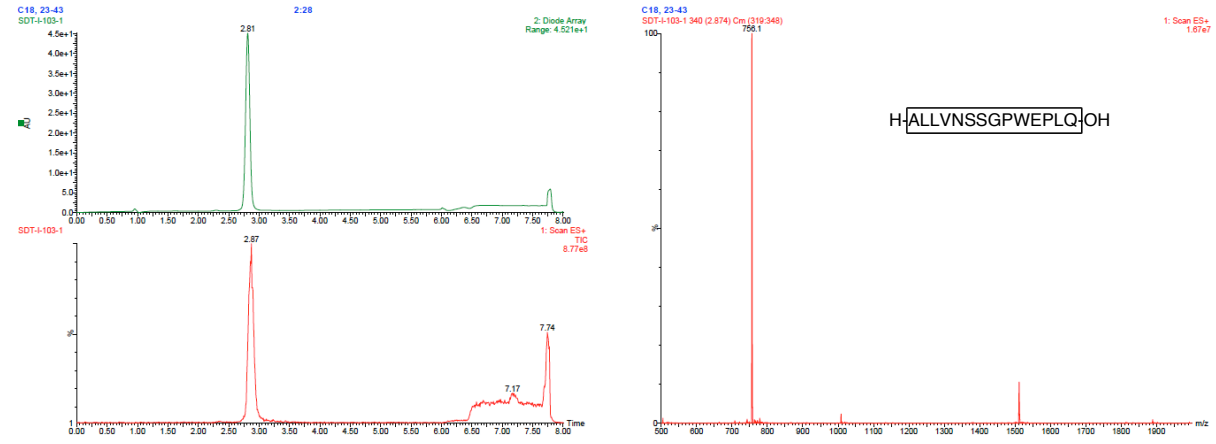
H-ALLVNSS"AA"-SPh (2 mg, 2.36 μmol) and diselenide peptide H-***P***WEPLQ-OH **22** (2 mg, 1.18 μmol) were dissolved in 1 mL of degassed ligation buffer (6 M Gdm•HCl, 200 mM Na_2HPO_4). MPAA (34 mg, 200 mM) was added and the pH of the reaction adjusted to 7.4 to 7.6 by 1 M NaOH. The reaction was degassed (freeze-pump-thaw) and allowed to stir under an argon atmosphere at ambient temperature. After consumption of the thioester, the crude selenopeptide was treated with DTT (68 mg, 400 mM). After

30 min, TCEP (500 μl, 0.5 M) was added and the pH adjusted to 5-6 by 1 M HCl. After removal of the selenide, the reaction mixture was diluted with 50% aq. CH_3CN to 3mL and purified by HPLC.



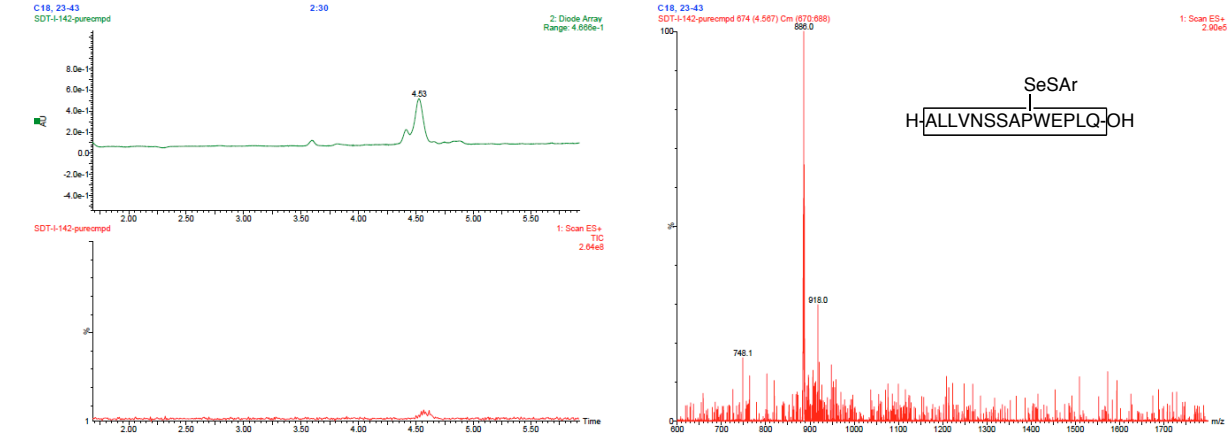
$C_{77}H_{113}N_{17}O_{23}SSe$, Exact Mass: 1755.71, $[M+H]^+$ *m/z* = 1756.71, $[M+2H]^{2+}$ *m/z* 878.86.

Found: $[M+H]^+$ *m/z* = 1754.9, $[M+2H]^{2+}$ *m/z* 879.6.



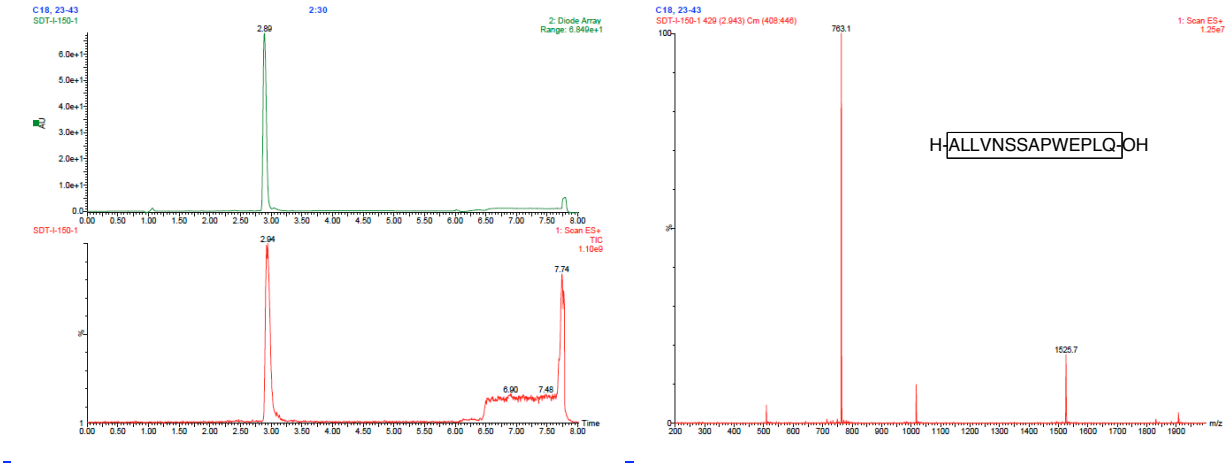
47: $C_{69}H_{107}N_{17}O_{21}$, Exact Mass: 1509.78, $[M+H]^+$ *m/z* = 1510.78, $[M+2H]^{2+}$ *m/z* 755.89.

Found: $[M+H]^+$ *m/z* = 1510.9, $[M+2H]^{2+}$ *m/z* 756.1, 84%



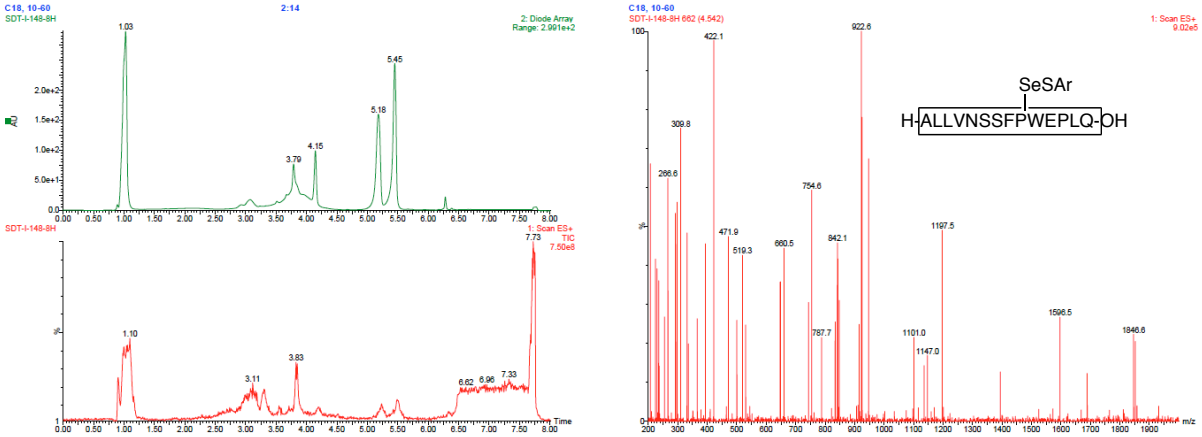
$C_{78}H_{115}N_{17}O_{23}SSe$, Exact Mass: 1769.72, $[M+2H]^{2+}$ *m/z* 885.86.

Found: $[M+2H]^{2+}$ *m/z* 886.0.



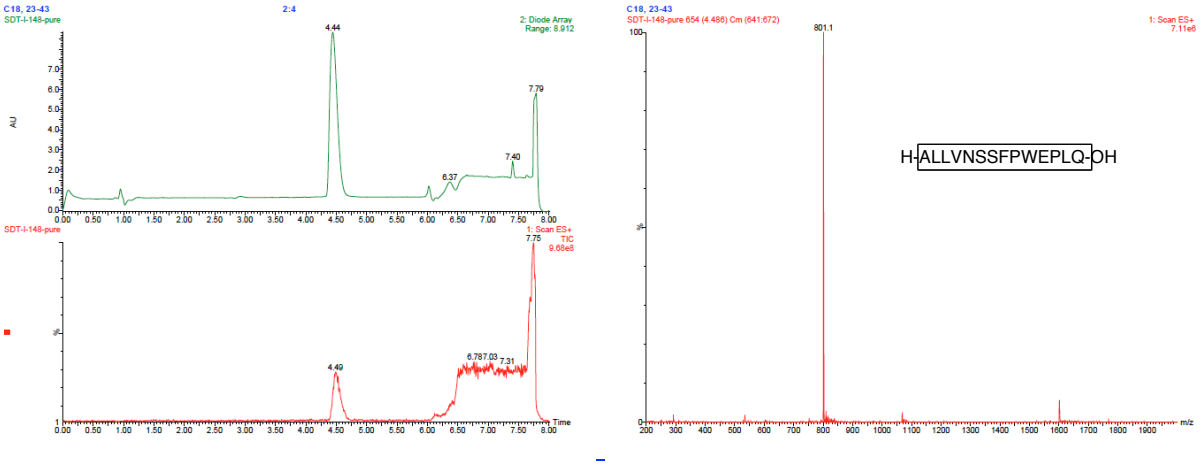
49: $C_{70}H_{109}N_{17}O_{21}$, Exact Mass: 1523.80, $[M+H]^{+}$ *m/z* = 1524.80, $[M+2H]^{2+}$ *m/z* 762.90.

Found: $[M+H]^{+}$ *m/z* = 1525.7, $[M+2H]^{2+}$ *m/z* 763.1, 88%



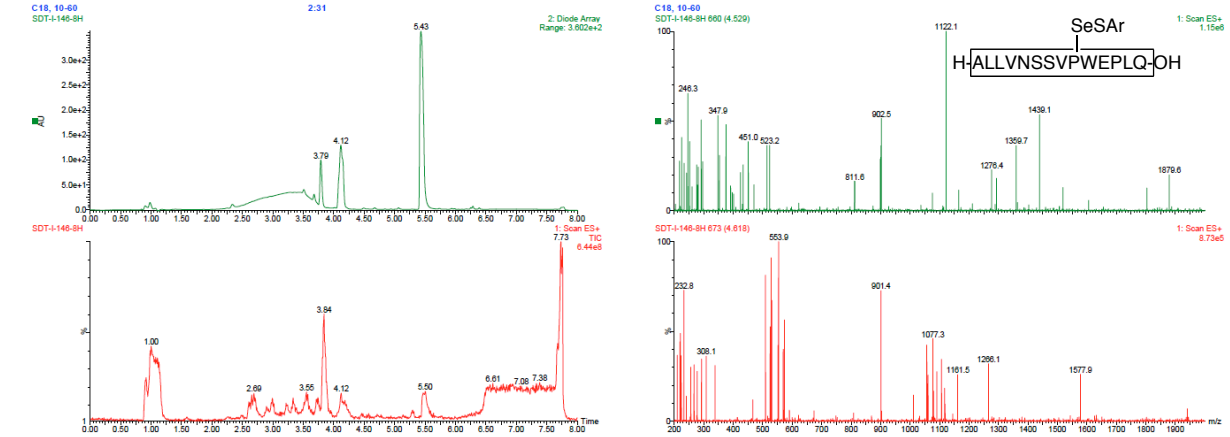
Crude: $C_{84}H_{119}N_{17}O_{23}SSe$, Exact Mass: 1845.76, $[M+H]^+$ *m/z* = 1846.76, $[M+2H]^{2+}$ *m/z* 923.88.

Found: $[M+H]^+$ *m/z* = 1846.6, $[M+2H]^{2+}$ *m/z* 922.6.



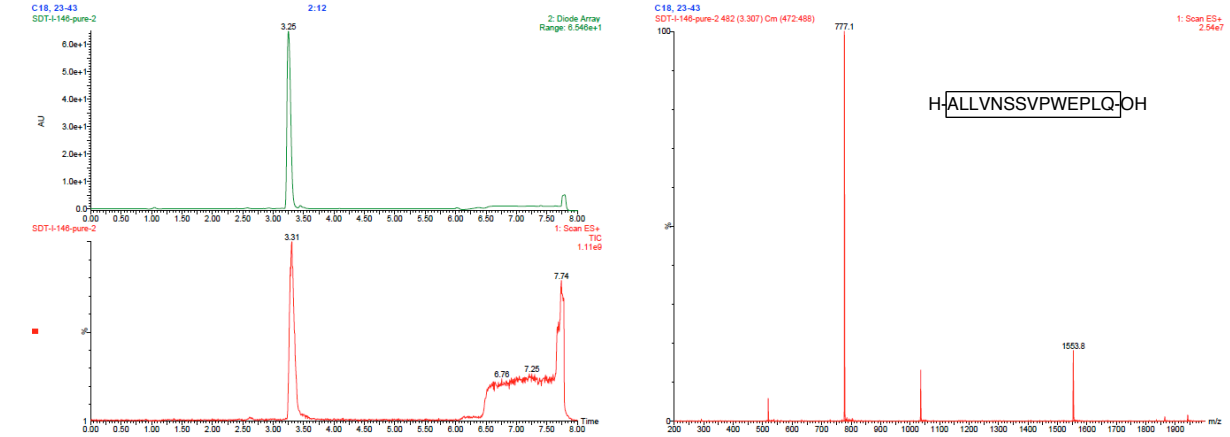
51: $C_{76}H_{113}N_{17}O_{21}$, Exact Mass: 1599.83, $[M+2H]^{2+}$ *m/z* 800.92.

Found: $[M+2H]^{2+}$ *m/z* 801.1, 80%



Crude: $C_{80}H_{119}N_{17}O_{23}SSe$, Exact Mass: 1797.76, $[M+2H]^{2+}$ *m/z* 900.38, $[M+4H]^{4+}$ *m/z* 450.44.

Found: $[M+2H]^{2+}$ *m/z* = 902.5, $[M+4H]^{4+}$ *m/z* 451.0.



53: $C_{72}H_{113}N_{17}O_{21}$, Exact Mass: 1551.83, $[M+H]^{+}$ *m/z* = 1552.83, $[M+2H]^{2+}$ *m/z* 776.92.

Found: $[M+H]^{+}$ *m/z* = 1553.8, $[M+2H]^{2+}$ *m/z* 777.1, 66%

Notes and Reference

(1) Shangguan, N.; Joullie, M. *Tetrahedron Lett* **2009**, *50*, 6748.