Free Radical Based, Specific Desulfurization of Cysteine: A Powerful Advance in the Synthesis of Polypeptides and Glycopolypeptides

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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\(_2\)Cl\(_2\), 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 (\(^1\)H and \(^13\)C) were recorded on a Bruker AMX-400 MHz or Bruker Advance DRX-500 MHz, referenced to TMS or residual solvent. Low-resolution mass spectral analyses were performed with a JOEL JMS-DX-303-HF mass spectrometer or Waters Micromass ZQ mass spectrometer. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and flash column chromatography was performed on E. Merck silica gel 60 (40–63 mm). Yields refer to chromatographically pure compounds.

HPLC:

All separations involved a mobile phase of 0.05% TFA (v/v) in water (solvent A) /0.04% TFA in acetonitrile (solvent B). 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, X-Terra® MS C18 column (3.5 µm, 4.6 x 250.0 mm) at a flow rate of 0.6 mL/min, Varian Microsorb C4 column (4.6 x 250 mm) at a flow rate of 0.6 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.

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-Asn(Trt)-OH, Fmoc-Asp(OrBu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(tButhio)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(OrBu)-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(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-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 (60.0 mg of phenol, 0.2 ml of water, 0.15 ml of trisopropylsilane, and 3.0 ml TFA) for 2.0 hours. 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.

**General Procedure for Desulfurization**

The cysteiny1 peptide was dissolved in 200.0 μL of water (or buffer) under argon. To the solution, were added 200.0 μL of 0.5 M bond-breaker® TCEP solution (Pierce), 20.0 μL of thiol (ethanethiol or/and 2-methyl-2-propanethiol) and 10.0 μL of radical initiator (0.1 M in water). The reaction mixture was stirred at 37 °C. The desulfurization reactions were also performed at room temperature which required longer time. The reactions were monitored by LC-MS and purified directly by HPLC upon consumption of the starting material.

Note:

1. The bond-breaker® TCEP solution (Pierce) can be replaced with a solution of TCEP·HCl and NEt₃. The final pH range was 6.0-7.0.

2. In order to avoid undesired side-reactions, such as the intramolecular hydrogen abstraction, the peptide should be well soluble in the reaction conditions. Sometimes the addition of CH₃CN, PBS buffer, Guanidine buffer, DMF and MeOH was required.

3. Additional thiols (EtSH, t-BuSH) were used as hydrogen source. They can also prevent the undesired reaction mentioned above. While the desulfurization can occur without them, longer reaction time is required.
**Compound 1** Fmoc-RYKDSGCAHPRG-OH:

Fmoc-Gly-NovaSyn® TGT resin and Fmoc-Asp(OtBu)-Ser(ψMe, Me pro)-OH were used following the general SPPS procedure. Semiprep HPLC purification (20-45% CH₃CN/H₂O over 30 min, C4 column, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound 1 as a white powder.

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**Figure 1**: UV and MS traces from LC-MS analysis of compound 1: gradient 10-30% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

**Figure 2**: ESI-MS of compound 1

ESI calcd for C₇₀H₇₁N₂₁O₁₉S [M+H]⁺ m/z = 1568.70, [M+2H]²⁺ m/z = 784.85, found: 1568.71, 784.96
**Compound 2** Fmoc-RYKDSGAHPRG-OH:

1.0 mg of compound 1 was subjected to the desulfurization conditions as described in the general procedure. HPLC purification (10-30% CH$_3$CN/H$_2$O over 45 min, X-terra, 265 nm, 0.6 mL/min) followed by concentration at reduced pressure and lyophilization provided 0.8 mg of compound 2 as a white powder, 82% yield.

![UV traces from LC-MS analysis of the conversion of 1 to 2: gradient 10-30% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column.](image)

**Figure 3:** UV traces from LC-MS analysis of the conversion of 1 to 2: gradient 10-30% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column.

![ESI-MS of compound 2](image)

**Figure 4:** ESI-MS of compound 2
ESI calcd for C$_{70}$H$_{97}$N$_{21}$O$_{19}$ [M+H]$^+$ m/z = 1536.73, [M+2H]$^{2+}$ m/z = 768.87, found: 1536.53, 769.04.
Figure 5: UV and MS traces from LC-MS analysis of compound 2 after purification: gradient 10-30% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column

Compound 3 H-LRHKDSRWKITR-OH:

Fmoc-Arg(Pbf)-NovaSyn® TGT resin, Fmoc-Asp(OtBu)-Thr($\psi$Me, Mepro)-OH and Fmoc-Ile-Thr($\psi$Me, Mepro)-OH were used following the general SPPS procedure. Semiprep HPLC purification (5-25% CH$_3$CN/H$_2$O over 30 min, C4 column, 227 nm) followed by concentration at reduced pressure and lyophilization gave compound 3 as a white powder.

Figure 6: UV and MS traces from LC-MS analysis of compound 3: gradient 5-28% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-bridge column.
Figure 7: ESI-MS of compound 3
ESI calcd for C$_{73}$H$_{123}$N$_{27}$O$_{18}$S $[M+H]^+$ m/z = 1698.93, $[M+2H]^{2+}$ m/z = 849.96, found: 1698.74, 850.15.

Compound 4 H-LRHKDSARWKTR-OH:

1.0 mg of compound 3 was subjected to the desulfurization conditions as described in the general procedure. HPLC purification (5-28% CH$_3$CN/H$_2$O over 30 min, X-terra, 227 nm, 0.6 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.8 mg of compound 4 as a white powder, 81% yield.

Figure 8: UV traces from LC-MS analysis of the conversion of 3 to 4: gradient 5-28% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-bridge column
Figure 9: ESI-MS of compound 4
ESI calcd for C_{73}H_{123}N_{27}O_{18} [M+H]^+ m/z = 1666.95, [M+2H]^{2+} m/z = 833.98, found: 1666.84, 833.94.

Figure 10: UV and MS traces from LC-MS analysis of compound 4 after purification: gradient 5-28% CH_{3}CN/H_{2}O over 30 min at a flow rate of 0.2 mL/min, X-bridge column
Compound 5 H-VETRFP-CRNYEK-OH

Fmoc-Lys(Boc)-NovaSyn® TGT resin and Fmoc-Glu(OtBu)-Thr(ψMe, MePro)-OH were used following the general SPPS procedure. Semiprep HPLC purification (5-25% CH₃CN/H₂O over 30 min, C4 column, 227 nm) followed by concentration at reduced pressure and lyophilization provided compound 5 as a white powder.

Figure 11: UV and MS traces from LC-MS analysis of compound 5: gradient 5-28% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-terra column

Figure 12: ESI-MS of compound 5
ESI calcd for C₆₁H₁₀₅N₂₁O₁₉S [M+H]⁺ m/z = 1540.76, [M+2H]²⁺ m/z = 770.88, found: 1540.59, 770.99.
**Compound 6 H-VETRFARNYEK-OH**

1.0 mg of compound 5 was subjected to the desulfurization conditions as described in the general procedure. HPLC purification (5-28% CH$_3$CN/H$_2$O over 30 min, X-terra column, 227 nm, 0.6 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.7 mg of compound 6 as a white powder, 71% yield.

**Figure 13:** LC-MS analysis of conversion of 5 to 6: gradient 5-28% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, C4 column

**Figure 14:** ESI-MS of compound 6
ESI calcd for C$_{67}$H$_{105}$N$_{21}$O$_{19}$ [M+H]$^+$ m/z = 1508.79, [M+2H]$^{2+}$ m/z = 754.90, found: 1508.81, 755.03.
Compound 7 H-RFDSCRPMHWR:

Fmoc-Arg(Pbf)-NovaSyn® TGT resin and Fmoc-Asp(OtBu)-Ser(ψMe, M³pro)-OH were used following the general SPPS procedure. Semiprep HPLC purification (10-35% CH₃CN/H₂O over 30 min, C4 column, 280 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded compound 7 as a white powder.

Figure 15: UV and MS traces from LC-MS analysis of compound 6: gradient 5-28% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, C4 column

Figure 16: UV and MS traces from LC-MS analysis of compound 7: gradient 10-30% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-terra column
Figure 17: ESI-MS of compound 7
ESI calcd for C_{64}H_{95}N_{23}O_{15}S [M+H]^+ \text{m/z} = 1490.68 \quad [M+2H]^2+ \text{m/z} = 745.84, found: 1490.93, 746.02.

Compound 8 H-RFDSARPMHW:

1.1 mg of compound 7 was subjected to the desulfurization conditions as described in the general procedure. HPLC purification (5-30\% CH\textsubscript{3}CN/H\textsubscript{2}O over 30 min, X-bridge, 227 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.8 mg of compound 8 as a white powder, 74\% yield.

Figure 18: UV traces from LC-MS analysis of conversion of 7 to 8: gradient 5-30\% CH\textsubscript{3}CN/H\textsubscript{2}O over 30 min at a flow rate of 0.2 mL/min, X-bridge column.
Figure 19: ESI-MS of compound 8
ESI calcd for C_{64}H_{95}N_{23}O_{15} [M+H]^+ m/z = 1458.71  [M+2H]^2+ m/z = 729.86, found: 1458.76, 729.90.

Figure 20: UV and MS traces from LC-MS analysis of compound 8 after purification: gradient 5-30% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

Compound 9 H-VRYTCKLSCys(Acm)WR

Fmoc-Arg(Pbf)-NovaSyn® TGT resin, Fmoc-Leu-Ser($\psi^{Me}$, Me$^{pro}$)-OH and Fmoc-Tyr(OtBu)-Thr($\psi^{Me}$, Me$^{pro}$)-OH were used following the general SPPS procedure. Semiprep HPLC purification (10-40% CH$_3$CN/H$_2$O over 30 min, C4 column, 280 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded compound 9 as a white powder.
Figure 21: UV and MS traces from LC-MS analysis of compound 9: gradient 10-40% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, C4 column

Figure 22: ESI-MS of compound 9
ESI calcd for C$_{65}$H$_{102}$N$_{20}$O$_{10}$S$_2$ [M+H]$^+$ m/z = 1485.74 [M+2H]$^{2+}$ m/z = 743.37, found: 1485.92, 743.71.

**Compound 10 H-VRYTAKLSCys(Acm)WR**

1.5 mg of compound 9 (1.0 μmol) was subjected to the desulfurization conditions as described in the general procedure. HPLC purification (12-30% CH$_3$CN/H$_2$O over 30 min, X-terra, 280 nm, 0.6 mL/min) followed by concentration at reduced pressure and lyophilization afforded 1.3 mg of compound 8 (0.89 μmol) as a white powder, 89% yield.
Figure 23: UV traces from LC-MS analysis of conversion of 9 to 10:
gradient 12-35% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column

Figure 24: UV and MS traces from LC-MS analysis of compound 10 after purification:
gradient 12-35%, CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column
Figure 25: ESI-MS of compound 10
ESI calc for C_{65}H_{104}N_{20}O_{16}S [M+H]^+ m/z = 1453.77  [M+2H]^2+ m/z = 727.38, found: 1454.11, 727.67.

Compound 11 Fmoc-Thz-YTRGCAGK-OH

Fmoc-Gly-NovaSyn\textsuperscript{®} TGT resin and Fmoc-Tyr(OtBu)-Thr(ψMe, Me\textsubscript{pro})-OH were used following the general SPPS procedure. Semiprep HPLC purification (10-40% CH\textsubscript{3}CN/H\textsubscript{2}O over 30min, X-bridge column, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded compound 11 as a white powder.

Figure 26: UV and MS traces from LC-MS analysis of compound 11: gradient 15-45% CH\textsubscript{3}CN/H\textsubscript{2}O over 30 min at a flow rate of 0.2 mL/min, X-bridge column
Figure 27: ESI-MS of compound 11
calcd for C$_{54}$H$_{73}$N$_{13}$O$_{14}$S$_{2}$ [M+H]$^+$ m/z = 1192.48, [M+Na]$^+$ m/z = 1214.48, [M+2H]$^{2+}$ m/z =596.74, found: 1192.39, 1214.23, 596.62.

Compound 12 Fmoc-Thz-YTRGAAKG-OH

1.5 mg of compound 11 (1.26 μmol) was dissolved in 300.0 μL of 6.0 M Gn•HCl and 200.0 μL of 0.5 M bond-breaker$^{®}$ TCEP solution under argon. To the reaction mixture, were added 20.0 μL of EtSH, 10.0 μL of $t$-BuSH and 20.0 μL of VA-044 (32.0 mg/mL). The resulting solution was stirred at 37 °C. After 5 h, the reaction was diluted by the addition of 1.0 mL of water (0.05% TFA) and subjected directly to HPLC purification (15-45% CH$_3$CN/Water over 45 min, X-bridge column, 265 nm, and a flow rate of 16.0 mL/min). Compound 12 (1.1 mg, 0.95 μmol) was isolated in 75% yield as a white powder.
Figure 28: UV traces from LC-MS analysis of conversion of 11 to 12: gradient 15-45% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-bridge column.

Figure 29: ESI-MS of compound 12
calcd for C$_{54}$H$_{73}$N$_{13}$O$_{13}$S [M+H]$^+$ m/z = 1160.51, [M+2H]$^{2+}$ m/z = 580.76, found: 1160.32, 580.78.
Figure 30: UV and MS traces from LC-MS analysis of compound 12 after purification: gradient 15-45% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

**Compound 13** Biotin-KWRITNCEHR

Fmoc-Arg-NovaSyn® TGT resin, Fmoc-Lys(Biotin)-OH and Fmoc-Ile-Thr($\psi$Me, Me$_2$pro)-OH were used following the general SPPS procedure. Semiprep HPLC purification (10-40% CH$_3$CN/H$_2$O over 30 min, C4 column, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded compound 13 as a white powder.

Figure 31: UV and MS traces from LC-MS analysis of compound 13 after purification: gradient 10-30% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column
Compound 14 Biotin-KWRITNAEHR

1.0 mg of compound 13 (0.64 μmol) was dissolved in 200.0 μL of aqueous buffer (6 M Gn·HCl, 0.2 M Na₂HPO₄, 0.19 mM TCEP buffer at pH 6.3) and 200.0 μL of bond-breaker® TCEP solution (0.5 M). To the mixture, were added 20.0 μL of t-BuSH and 10.0 μL of VA-044 (32.0 mg/mL). The resulting solution was stirred at 37 °C. After 2 h, the reaction was diluted by the addition of 1.0 mL of water (0.05% TFA) and subjected directly to HPLC purification (10-30% CH₃CN/Water over 1h, X-terra column, 265 nm, and a flow rate of 0.6 mL/min). Compound 14 (0.9 mg, 0.59 μmol) was isolated in 92% yield as a white powder.
Figure 33: UV traces from LC-MS analysis of conversion of 13 to 14: gradient 10-30% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column.

Figure 34: ESI-MS of compound 14
calcd for C$_{67}$H$_{106}$N$_{23}$O$_{17}$S [M+H]$^+$ m/z = 1536.78, [M+2H]$^{2+}$ m/z = 768.89, found: 1536.63, 769.04.
**Figure 35:** UV and MS traces from LC-MS analysis of compound 14 after purification: gradient 10-30% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-terra column

**Compound 15**

**Scheme 1:** Synthesis of compound 15
To a solution of the phenol (149.0 mg, 0.8 mmol) and Boc-Gln-OH (246.0 mg, 1.0 mmol) in CH$_2$Cl$_2$ (2.5 mL) and THF (0.5 mL) were added EDCI (129.0 mg, 1.0 mmol) and DMAP (3.0 mg). The resulting solution was stirred at room temperature overnight at which point the volatile materials were removed in vacuo. The resulting oil was subjected to silica gel chromatography (1-5% methanol in CH$_2$Cl$_2$). 200.0 mg of phenol ester was isolated in 63% yield. 165.0 mg of phenol ester was dissolved in 4.0 M of HCl/dioxane (3.0 mL), and the resulting solution was stirred at room temperature for 1 h. The reaction was then concentrated in vacuo, leaving a slightly yellow oil. This material was treated with ether and subsequently concentrated. Compound B was obtained as a white solid which was used without further purification.

**Peptide A** was prepared by the general SPPS procedure. Fmoc-Gly-Novasyn® TGT resin and Fmoc-Asp(OtBu)-Ser($\psi$Me,Me-pro)-OH, Fmoc-Thz-OH were used. The peptide resin was washed into a peptide synthesis vessel with CH$_2$Cl$_2$. The resin was subjected to a cleavage cocktail (3.0 mL of CH$_2$Cl$_2$, 1.0 mL of trifluoroethanol, 1.0 mL of AcOH) for 2 hours. The resin was removed by filtration, and the resulting solution was concentrated. The starting carboxylic acid (peptide A, 43.0 mg of crude material) was dissolved in a solution of CH$_2$Cl$_2$ and DMF (1/1, 0.6 mL). To the resulting solution were added EDCI (10.6 mg) and HOBT (7.6 mg). The reaction mixture was stirred at room temperature for 2.0 min. Then compound B (19.6 mg) was added to the reaction mixture. The reaction was complete within 20.0 min at which point the solvents were removed in vacuo. The resulting oil was purified by silica gel chromatography (3-10% MeOH/CH$_2$Cl$_2$) to give protected peptide (32.6 mg). ESI-MS calcd for C$_{99}$H$_{134}$N$_{18}$O$_{24}$S$_{6}$ [M+H]$^+$ m/z = 2151.8, [M+Na]$^+$ m/z = 2173.8, found: 2151.9, 2173.9.

The side-chain protected peptide was treated with TFA/phenol/TES/H$_2$O (35/2/1/1) for 2.0 h. The majority of the solvents were removed by N$_2$ stream and the remaining residue was triturated with ether. Peptide C precipitated out and was washed with ether (3X). Further purification by preparative HPLC (20-70% CH$_3$CN/water over 30 min, 265 nm C4 column with a flow rate 16.0 mL/min) gave the pure peptide C as a white powder after lyophilization (20.0 mg). ESI-MS calcd for C$_{66}$H$_{90}$N$_{18}$O$_{16}$S$_{4}$ [M+H]$^+$ m/z = 1551.56, [M+2H]$^{2+}$ m/z = 776.57, found: 1551.74, 776.57.
Figure 36: UV and MS traces from LC-MS analysis of Peptide C: gradient 20-70% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, C4 column

Figure 37: ESI-MS of peptide C
ESI-MS calcd for C₆₆H₉₀N₁₈O₁₆S₄ [M+H]⁺ m/z = 1551.56, [M+2H]²⁺ m/z = 776.57, found: 1551.86, 776.70.

Peptide C (1.6 mg, 1.0 μmol) and disaccharide (1.3 mg, 3.0 μmol) were placed in a vial with a flea-sized stir-bar. Anhydrous DMSO (0.1 mL) was added to the mixture followed by HATU (1.1 mg, 3.0 μmol). To the reaction mixture was added 10.0 μL of a solution of
DIEA in DMF (35.0 μL DIEA/1.0 mL DMF). The reaction mixture turned yellow and was stirred at room temperature for 1.0 h. The reaction was quenched by water (0.5% TFA) and then subjected to HPLC purification (20-50% CH₃CN/water over 30 min with a flow rate 0.6 mL/min, X-terra column, 265 nm) to yield glycopeptide 15 (1.4 mg, 0.72 μmol, 72% yield) as a white solid. ESI calcd for C₈₂H₁₁₇N₂₁O₂₇S₄ [M+H]+ m/z = 1956.73 [M+2H]⁺⁺ m/z = 978.87, found: 979.22.

**Figure 38:** UV and MS traces from LC-MS analysis of conversion of Peptide C to 15: gradient 20-50% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-terra column.
**Figure 39:** UV and MS traces from LC-MS analysis of compound 15 after purification: gradient 20-50% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-terra column.

**Figure 40:** ESI-MS of compound 15
ESI calcd for C₈₂H₁₁₇N₂₁O₂₇S₄ [M+H]+ m/z = 1956.73 [M+2H]²⁺ m/z =978.87, found: 979.22.
**Compound 16** H-Cys(SrBu)GlyAspSerArgTrpMetArgGly-SEt

Scheme 2: Synthesis of Compound 16

Fmoc-Gly-NovaSyn® TGT resin, Boc-Cys(SrBu)-OH and Fmoc-Asp(OrBu)-Ser($\psi^{Mec}$, Me-pro)-OH were used following the general SPPS procedure. The peptide resin was washed into a peptide synthesis vessel with CH$_2$Cl$_2$. The resin was subjected to a cleavage cocktail (3.0 mL of CH$_2$Cl$_2$, 1.0 ml of trifluoroethanol, 1.0 ml of AcOH) for 2 hours. The resin was removed by filtration, and the resulting solution was concentrated. The starting carboxylic acid (30.0 mg of crude material) was dissolved in a solution of DMF (1.0 mL). To the resulting solution were added EtSH (24.0 $\mu$L, 0.32 mmol), EDCI (21.0 mg, 0.16 mmol) and HOBT (22.0 mg, 0.16 mmol). The reaction mixture was stirred at room temperature overnight at which point the solvents were removed in vacuo. The resulting oil was purified by silica gel chromatography (1-5% MeOH/CH$_2$Cl$_2$) to give the desired thiolester. The side-chain protected peptide was treated with TFA/phenol/TES/H$_2$O (35/2/1/1) for 2.0 h. A majority of the solvents were removed by N$_2$ stream and the remaining residue was triturated with ether. Compound 16 was precipitated out and was washed with ether (3X). Further purification by preparative HPLC (20-40% CH$_3$CN/water over 30 min, C4 column with a flow rate 16.0 mL/min, 280 nm) gave the pure compound 16 as a white powder after lyophilization. ESI-MS calcd for C$_{48}$H$_{78}$N$_{16}$O$_{12}$S$_4$ [M+H]$^+$ $m/z = 1199.49$, [M+2H]$^{2+} m/z = 600.24$, found: 1551.74, 776.57.
Figure 41: UV and MS traces from LC-MS analysis of compound 16 after purification: gradient 15-28%CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column

Figure 42: ESI-MS of compound 16
ESI calcd for C$_{48}$H$_{78}$N$_{16}$O$_{12}$S$_4$ [M+H]$^+$ m/z = 1199.49  [M+2H]$^{2+}$ m/z =600.24, found: 1199.52, 600.51.
Compound 19

Scheme 3 Synthesis of Compound 20

Compound 15 (0.7 mg, 0.36 μmol) and compound 16 (0.7 mg, 0.58 μmol) were dissolved in 150 μL of aqueous buffer (6.0 M Gn·HCl, 0.2 M Na₂HPO₄, 0.19 mM TCEP buffer at pH 6.3) and 10.0 μL of bond-breaker® TCEP solution (0.5 M). The resulting reaction mixture was stirred at room temperature. After 2.0 h, the reaction was quenched by the addition of 2.0 mL of water (0.05% TFA) and subjected directly to HPLC purification (10-35% CH₃CN/Water over 45.0 min, X-tterra column, 265 nm, and a flow rate of 0.6 mL/min). Compound 19 (0.7 mg, 0.24 μmol) was isolated with 67% yield as a white powder.

Figure 43: UV and MS traces from LC-MS analysis of synthesis of compound 19: gradient 10-35% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-tterra column
Figure 44: UV and MS traces from LC-MS analysis of compound 19 after purification: gradient 10-35% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column

Figure 45: ESI-MS of compound 19
calcd for $\text{C}_{119}\text{H}_{177}\text{N}_{35}\text{O}_{38}\text{S}_5$ [M+H]$^+$ $m/z$ = 2881.17, [M+2H]$^2+$ $m/z$ = 1441.09, [M+3H]$^3+$ $m/z$ = 961.06, [M+4H]$^4+$ $m/z$ = 721.04  found: 1441.61, 961.39 and 713.28.
Compound 20

Compound 19 (0.7 mg, 0.24 μmol) was dissolved in a solution of EtSH in water (200.0 μL, 2% v/v) under Argon. To the resulting solution were added a solution of TCEP (300.0 μL, 0.5 M) followed by t-BuSH (20.0 μL) and 10.0 μL of VA-044 (0.1 M). The reaction mixture was stirred at 37 °C for 2 h. The reaction was diluted by the addition of 2.0 mL of water (0.05% TFA) and subjected directly to HPLC purification (10-35% CH₃CN/Water over 45 min, X-terra column, 265 nm, and a flow rate of 0.6 mL/min). Compound 20 (0.6 mg, 0.21 μmol) was isolated with 87% yield as a white powder.

Note: To a solution of TCEP (73.0 mg TCEP⋅HCl were dissolved in 0.5 mL of water) was added 105.0 μL of Et₃N. The pH of the TCEP solution is close to neutral.

Figure 46: UV and MS traces from LC-MS analysis of synthesis of compound 20: gradient 10-35% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-terra column
Figure 47: UV and MS traces from LC-MS analysis of compound 20 after purification: gradient 10-35% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column.

Figure 48: ESI-MS of compound 20
calcd for C$_{118}$H$_{117}$N$_3$O$_{38}$S$_4$ [M+H]$^+$ m/z = 2849.19, [M+2H]$^{2+}$ m/z = 1425.10, [M+3H]$^{3+}$ m/z = 950.40, [M+4H]$^{4+}$ m/z = 713.05 found: 1425.28, 950.75 and 713.28.
Compound 23: Crotogossamide

Scheme 4: Synthesis of Crotogossamide 23

Fmoc-Gly-NovaSyn® TGT resin and Boc-Cys(SrBu)-OH were used following the general SPPS procedure. The peptide resin was washed into a peptide synthesis vessel with CH₂Cl₂. The resin was subjected to a cleavage cocktail (6.0 mL of CH₂Cl₂, 2.0 mL of TFE) for 12.0 hours. The resin was removed by filtration, and the resulting solution was concentrated. The starting carboxylic acid (132.0 mg of crude material) was dissolved in a solution of DMF (2.0 mL). To the resulting solution were added EtOOC₂H₄SH (320.0 μL), EDCI (80.0 mg, 0.42 mmol) and HOBT (82.0 mg, 0.61 mmol). The reaction was stirred at room temperature overnight at which point the solvents were removed in vacuo. The side-chain protected peptide was treated with TFA (3.0 mL), phenol (60.0 mg), triisopropylsilane (150.0 μL) and H₂O (200.0 μL) for 3.0 h. The majority of the solvents were removed by N₂ stream and the remaining residue was triturated with ether.

Compound 21 (135.0 mg, crude) was precipitated out of solution and was washed with ether (3X). Crude compound 21 (32.0 mg) was dissolved in DMF (400.0 μL) and water (150.0 μL). To the solution was added TCEP (150.0 μL, 0.5 M bond-breaker). The reaction mixture was stirred at room temperature for 1 hour, and then diluted with DMF and water. The resulting solution was subjected to HPLC purification (20-50% CH₂CN/Water over 30 min, C18 column, 218 nm, and a flow rate of 16.0 mL/min). Compound 22 (8.5 mg, 0.01 mmol) was isolated as a white powder.

Compound 22 (2.5 mg, 2.9 μmol) was dissolved in a solution of TCEP (500.0 μL, 0.5 M bond-breaker®) and MeOH (1.0 mL). To the resulting solution was added t-BuSH (50.0 μL) followed by V-50 (20.0 μL, 0.1 M in water). The reaction mixture was stirred at 37 °C for 3.0 hours, and another portion of TCEP (500.0 μL, 0.5 M bond-breaker®) and t-BuSH (50.0 μL) was added. 20.0 h later, the solvents were removed by nitrogen flow, and then the residue was dissolved in CH₂CN/H₂O (3.0 mL, 30%). The resulting solution was subjected to HPLC purification (15-35% CH₂CN/Water over 30 min, X-bridge column, 218 nm, and a flow rate of 16.0 mL/min). Compound 23 (1.5 mg, 1.8 μmol) was isolated with 62% as a white powder.
Figure 49: UV and MS traces from LC-MS analysis of synthesis of compound 21: gradient 10-60% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, C18 column

Figure 50: ESI-MS of compound 21
calcd for C$_{46}$H$_{74}$N$_{10}$O$_{13}$S$_3$ [M+H]$^+$ m/z = 1071.46, [M+Na]$^+$ m/z = 1093.46; found: 1071.28, and 1093.25.
Figure 51: UV and MS traces from LC-MS analysis of synthesis of compound 22: gradient 20-50% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, C18 column

Figure 52: UV and MS traces from LC-MS analysis of compound 22 after purification: gradient 20-50% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, C18 column
Figure 53: ESI-MS of compound 22
cald for C_{37}H_{56}N_{10}O_{11}S [M+H]^+ m/z = 849.39, [M+Na]^+ m/z = 871.39; found: 849.22, and 871.20.

Figure 54: UV and MS traces from LC-MS analysis of synthesis of compound 23:
gradient 20-50% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, C18 column
Figure 54: ESI-MS of compound 23
calcd for C_{37}H_{56}N_{10}O_{11} [M+H]^+ m/z = 817.41, [M+Na]^+ m/z = 832.41; found: 817.28, and 839.26.

Figure 55: UV and MS traces from LC-MS analysis of compound 23 after purification: gradient 20-50% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, C18 column
$^1$H-NMR (DMSO-$d_6$, 500 MHz): δ 8.51 (br, 1 H), 8.36 (br, 1 H), 8.32 (br, 1 H), 8.23 (d, 1 H, $J = 6.8$ Hz), 8.16 (t, 1 H, $J = 6.0$ Hz), 8.06 (t, 1 H, $J = 6.0$ Hz), 7.74 (d, 1 H, $J = 7.3$ Hz), 7.53 (d, 1 H, $J = 6.7$ Hz), 7.38 (m, 2 H), 7.26 (t, 2 H, $J = 7.4$ Hz), 7.19 (m, 3 H), 6.86 (s, 1 H), 5.03 (br, 1 H), 4.39 (m, 1 H), 4.30 (m, 3 H), 4.04 (q, 1 H, $J = 5.7$ Hz), 3.90 (m, 3 H), 3.76 (dd, 1 H, $J = 6.0$ and 17.0 Hz), 3.67 (m, 1 H), 3.41 (m, 2 H), 3.18 (dd, 1 H, $J = 4.5$ and 14.0 Hz), 2.98 (dd, 1 H, $J = 10.1$ and 14.0 Hz), 2.74 (dd, 1 H, $J = 5.2$ and 15.1 Hz), 2.54 (signal partially obscured), 1.76 (m, 1 H), 1.61 (m, 1 H), 1.53 (m, 1 H), 1.29 (m, 1 H), 1.28 (d, 3 H, $J = 7.0$ Hz), 1.03 (m, 1 H), 0.86 (d, 3 H, $J = 5.6$ Hz), 0.83 (d, 3 H, $J = 5.6$ Hz), 0.74 (t, 3 H, $J = 7.2$ Hz), 0.59 (d, 3 H, $J = 6.7$ Hz). ESI-MS of compound 23 calcd for C$_{37}$H$_{56}$N$_{10}$O$_{11}$ [M+H]$^+$ m/z = 817.41, [M+Na]$^+$ m/z = 839.41. Found 817.28 and 839.13.

**Compound 25**

Selenocystine (5.0 mg, 15.0 mmol) was dissolved in 200.0 μL of aqueous buffer (6.0 M Gn-HCl, 0.2 M Na$_2$HPO$_4$, 0.19 mM TCEP buffer at pH 6.3) and 100.0 μL of bond-breaker® TCEP solution (0.5 M). The solution was added to compound 15 (1.1 mg, 0.56 μmol), and the reaction mixture was stirred at room temperature. After 1 h, the ligation was complete and the desired products appeared as monomer, compound 24, and dimer. An additional 400.0 μL of bond-breaker® TCEP solution (0.5 M) and 0.5 mg of VA-044 were added. The reaction mixture was heated at 35 °C for 4.0 h after which time the reduction was complete. The reaction mixture was purified directly by HPLC (10-30% CH$_3$CN/Water over 30 min, X-bridge column, 265 nm, and a flow rate of 16.0 mL/min). Compound 25 (0.8 mg, 0.43 μmol) was isolated with 77% yield (two steps).

**Figure 56:** UV and MS traces from LC-MS analysis of synthesis of compound 24 and 25; gradient 10-35% CH$_3$CN/H$_2$O over 30 min at a flow rate of 0.2 mL/min, X-terra column
ESI-MS of compound 24
calcd for C_{77}H_{114}N_{22}O_{28}S_2Se [M+H]^+ m/z = 1938.68, [M+2H]^{2+} m/z = 970.34, found: 970.76.

ESI-MS of diselenide
calcd for C_{154}H_{226}N_{44}O_{56}S_4Se_2 [M+3H]^3+ m/z = 1292.78, [M+4H]^{4+} m/z = 969.83, found: 1292.84 and 970.15

**Figure 57:** UV and MS traces from LC-MS analysis of compound 25 after purification: gradient 10-35% CH₃CN/H₂O over 30 min at a flow rate of 0.2 mL/min, X-terra column

**Figure 58:** ESI-MS of compound 25
ESI calcd for C_{77}H_{114}N_{22}O_{28}S_2 [M+H]^+ m/z = 1859.76, [M+2H]^{2+} m/z = 930.38, found: 1859.60, 930.76.
Proton QWAN–V–Crotogossamide (4 1) DMSO August–30, 2007 Bruker AVII+ 500MHz
$^1$H NMR Spectrum of Crotogossamide

Provided by corresponding authors
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