Supporting Information

Robust Synthesis of C-terminal Cysteine-Containing Peptide Acids through A Peptide Hydrazide-Based Strategy

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Content

1. General Information

Materials

O-(6-Chlorobenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HCTU), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HATU), and Fmoc-protected amino acids were purchased from GL Biochem (Shanghai, China). 2-Chlorotrityl chloride resin was purchased from Tianjin Nankai Hecheng Science & Technology Co., Ltd (China). 4-Mercaptophenylacetic acid(MPAA), sodium 2-mercaptoethanesulflnate (MESNa), 2-mercaptoethanol (β-ME), and iodine (I_2) were purchased from Alfa Aesar. N, N-Dimethylformamide (DMF), thioanisole, trifluoroacetic acid (TFA), phenylsilane, acetic acid (HPLC grade), 1,2-ethanedithiol (EDT), reduced glutathione (GSH), oxidized glutathione (GSSG), methanol (MeOH) were purchased from J&K Chemical Ltd. Acetonitrile (HPLC grade) was purchased from J. T. Baker. $Na_2HPO_4 \cdot 12H_2O$, guanidine hydrochloride (Gn•HCl), and diethyl ether were purchased from Sinopharm Chemical Reagent Co., Ltd. Dichloromethane (DCM) and sodium nitrite ($NaNO_2$) were purchased from Beijing Chemical Industy Group Co., Ltd. CS136XT synthesize r(automated peptie synthesizer) was purchased from CS Bio Co., Ltd.

HPLC

Peptide fragments were analyzed by RP-HPLC using analytical columns (welch XB-C4, 250mm× 4.6 mm, 5 μm particle size, flow rate 1.0 mL/min, rt) on SHIMADZU instruments (Prominence LC-20AT). They were purified by RP-HPLC using semi preparative column (welch XB-C4, 150 mm× 21.2 mm, 5 μm particle size, flow rate 5.0 mL/min, rt) on SHIMADZU instruments (Prominence LC-20AT). Analytical injections were monitored at 214 nm and 254 nm wavelength. **Solvent A**: acetonitrile (containing 0.1% TFA), **Solvent B**: deionized distilled water (containing 0.1% TFA). Both solvents were sonicated for 10 min before use.

Mass Spectrometry (MS)

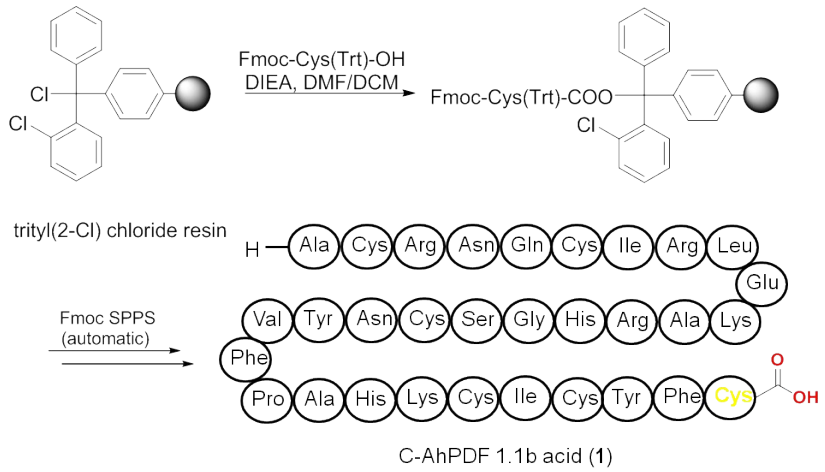
Products were identified by electrospray ionization mass spectrometry (ESI-MS). ESI-MS was measured on an Agilent 1200/6340 mass spectrometer in Center of Biomedical Analysis. The buffers for MS analysis were 50% CH3CN/H_2O (v/v) containing 0.1% formic acid. MALDI-TOF mass spectra were measured on an Applied Biosystems 4700 Proteomics Analyzer 283.

Fmoc-based solid-phase peptide synthesis

All peptides were synthesized by running the CS136XT synthesizer on a scale of 0.1 mmol using standard Fmoc-based SPPS protocol. 2-Chlorotrityl chloride resin was used to synthesize C-terminal cysteine-containing peptide acids, and hydrazine 2-chlorotrityl resin was used to synthesize C-terminal cysteine-containing peptide hydrazides. Prior to coupling Fmoc-protected amino acid, the resin was swollen in DMF for 10 min . Coupling of Fmoc-protected amino acid was performed by HATU

(or HCTU)-based method. For most amino acids, single coupling using HATU or HCTU for 30 min is enough, as shown in Table 2A. However, for sterically hindered amino acids, including Fmoc-Thr(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, and Fmoc-Val-OH, double coupling was carried out, as shown in Table 2B. For Fmoc deprotection, a solution of 20% piperidine in DMF was used (twice, 10 min+10min). After assembly of amino acids, the resin was treated by TFA cocktail (TFA/thioanisole/EDT/phenol/H_2O, 85/5/5/2.5/2.5) for 2.5 h at room temperature. The combined TFA solutions was concentrated by N_2 blowing. Crude peptides were precipitated with cold diethyl ether, and obtained as a white powder after centrifugation. The crude peptides were dissolved in a mixed solution containing water (containing 0.1% TFA) and acetonitrile (containing 0.1% TFA). After analyzing by HPLC and MS, the desired peptides were purified by preparative HPLC, and lyophilized to a white powder.

2. Fmoc SPPS of C-AhPDF 1.1b acid (1) using trityl(2-Cl) chloride resin



Scheme S1. Fmoc SPPS of C-AhPDF 1.1b acid (**1**) using trityl(2-Cl) chloride resin

The linear C-AhPDF 1.1b acid (**1**) was assembled by running the CS136XT synthesizer on a scale of 0.1 mmol using trityl(2-Cl) chloride resin and standard Fmoc-based SPPS protocol. After the assembly of C-AhPDF 1.1b on the solid support, C-AhPDF 1.1b acid (**1**) was cleaved from the resin by TFA cocktail (TFA/thioanisole/EDT/phenol/H_2O, 85/5/5/2.5/2.5) for 2.5 h at room temperature. The crude C-AhPDF 1.1b acid (**1**) was precipitated by cold ether, and obtained as a white powder. Crude C-AhPDF 1.1b acid (**1**) was analyzed by analytic HPLC and ESI-MS, as shown in Figure S1.

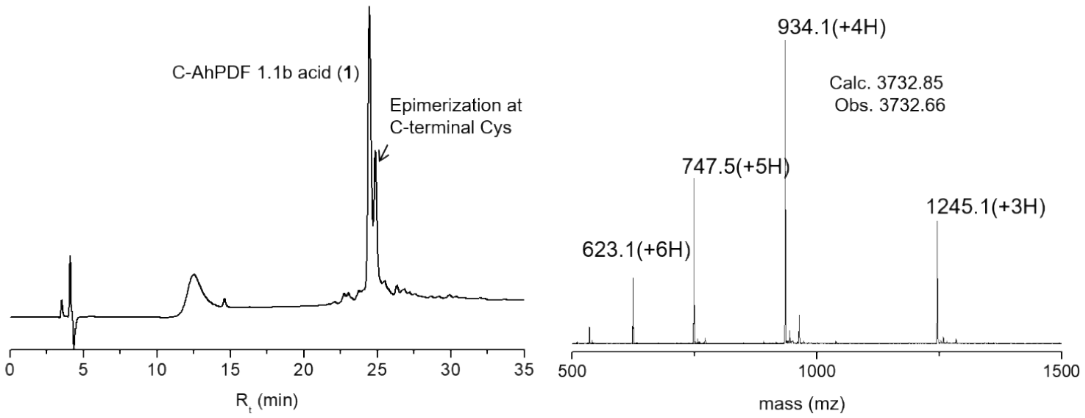


Figure S1. Analytic HPLC trace (210 nm) and ESI-MS of crude C-AhPDF 1.1b acid (**1**) prepared by using trityl(2-Cl) resin. (gradient: 5-5% **Solvent A** in 2 min, then 20-40% **Solvent A** in 30 min).

3. Peptide hydrazide-based preparation of C-AhPDF 1.1b acid (1)

Preparation of hydrazine-trityl(2-Cl) resin

Scheme S2. Preparation of hydrazine-trityl(2-Cl) resin.

250 mg trityl(2-Cl) chloride resin (0.1 mmol, 0.40 mmol/g) was swollen in DMF (10 mL) for 15 min, then was treated by a solution of 5% NH_2NH_2 in N, N-dimethyformamide (0.5 mL 80% hydrazine in 7.5 ml DMF, 30 min x2) to afford hydrazine trityl(2-Cl) resin, as shown in Scheme S2. Then, 10 mL methanol was added to the resin to quench unreacted trityl(2-Cl) chloride, 20 min, RT. After washing by DMF/DCM/DMF, the obtained resin was directly used for the preparation of C-terminal Cys-containing peptide hydrazides.

Preparation of C-AhPDF1.1b hydrazide (**2**)

C-AhPDF 1.1b hydrazide (**2**) was synthesized on 0.1 mmol scale. Isolation yield: (195 mg, 52 μmol, 52 %). HPLC: t_R= 22.6 min (gradient: 5-5% B in 2 min, then 20-40% B in 30 min). ESI-MS: obs. 3746.17 m/z (deconv.), calc. 3746.41.

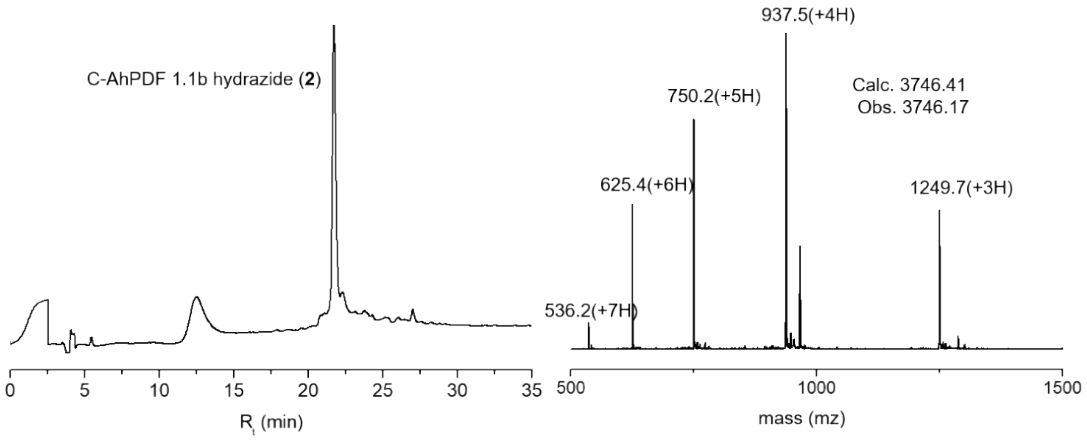


Figure S2. Analytic HPLC trace (210 nm) and ESI-MS of crude C-AhPDF 1.1b hydrazide (**2**) (gradient: 5-5% **Solvent A** in 2 min, then 20-40% **Solvent A** in 30 min).

Hydrolysis of C-AhPDF1.1b MESNa-thioester (pH 8)

C-AhPDF 1.1b hydrazide (3.75 mg, 1.0 μmol) was added to 0.25 mL of PBS buffer (0.2 M Na_2HPO_4, 6 M Gn·HCl, pH 3). At -10 ^oC, 10 equivalents of $NaNO_2$ (20 μL, 0.01 mM in pH3 PBS) were added dropwise. After 20 min, 200 equivalents of MESNa (0.25 mL, 800 mM in pH 8 PBS) was added. Then, the pH value of the solution was adjusted to 8 with NaOH (0.2 M) at room temperature. After 1 h, the reaction mixture was monitored by HPLC and analyzed by ESI-MS, as shown in Figure S3.

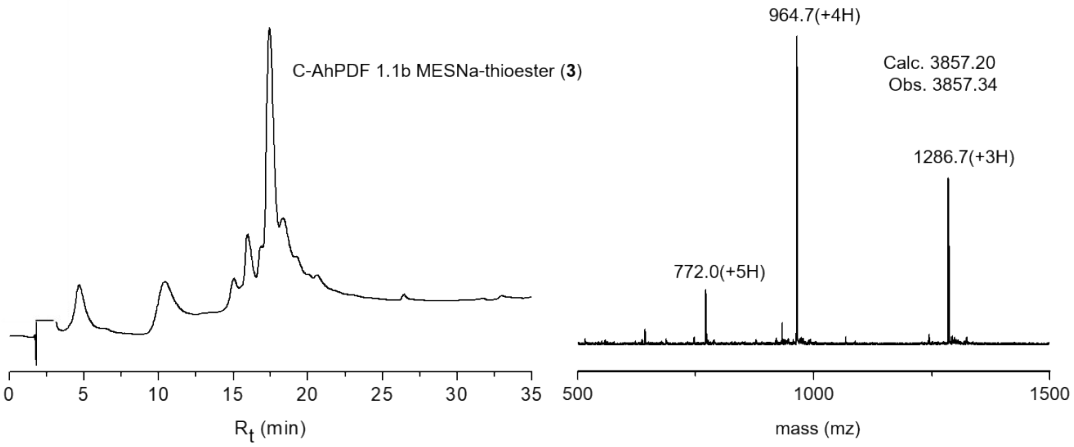


Figure S3. HPLC analysis (210nm) of reaction of hydrolysis of C-AhPDF 1.1b MESNa-thioester (**3**) and ESI-MS of **3** (gradient: 5-5% **Solvent A** in 2 min, then 20-

50% **Solvent A** in 30 min).

Hydrolysis of C-AhPDF1.1b MPAA-thioester (pH 8)

C-AhPDF 1.1b hydrazide (3.75 mg, 1.0 μmol) was added to 0.25 mL of PBS buffer (0.2 M Na_2HPO_4, 6 M Gn·HCl, pH 3). At -10 °C, 10 equivalents of $NaNO_2$ (20 μL, 0.01 mM in pH3 PBS) were added dropwise. After 20 min, 200 equivalents of MPAA (0.25 mL, 800 mM in pH8 PBS) was added. Then, the pH value of the solution was adjusted to 8 with NaOH (0.2 M) at room temperature. After 1 h, the reaction mixture was monitored by HPLC and analyzed by ESI-MS, as shown in Figure S4.

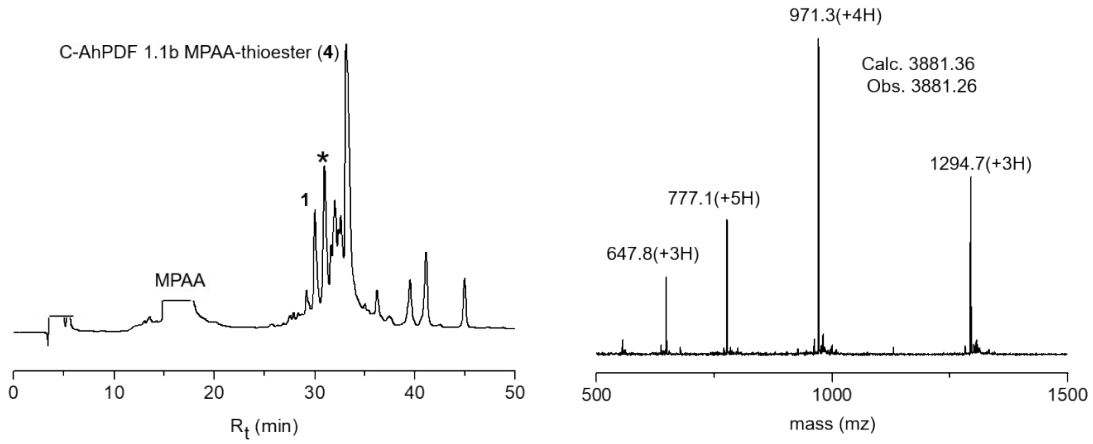


Figure S4. HPLC analysis (210nm) of reaction of hydrolysis of C-AhPDF 1.1b MPAA-thioester (1 h, 400 mM MPAA**)** and ESI-MS of **4**. ∗ denotes the remaining C-AhPDF 1.1b hydrazide (**2**). (gradient: 5-5% **Solvent A** in 2 min, 20-20% **Solvent A** in 10 min, then 20-50% **Solvent A** in 30 min).

Hydrolysis of C-AhPDF1.1b MESNa-thioester by MPAA (pH 8)

C-AhPDF1.1b hydrazide (3.75 mg, 1.0 μmol) was added to 0.25 mL of PBS buffer (0.2 M Na_2HPO_4, 6 M Gn·HCl, pH 3). At -10 °C, 10 equivalents of $NaNO_2$ (20 μL, 0.01 mM in pH 3 PBS) were added. After 20 min, 20 equivalents of MPAA (125 μl, 0.16 M in PBS) was added at room temperature. After 5 min, 200 equivalents of MESNa (125 ul, 1.6 M in pH 8) was added into the reaction mixture, and pH value of the solution was adjusted to 8. After 1 h, the reaction mixture was monitored by HPLC and analyzed by MS, as shown in Figure S5.

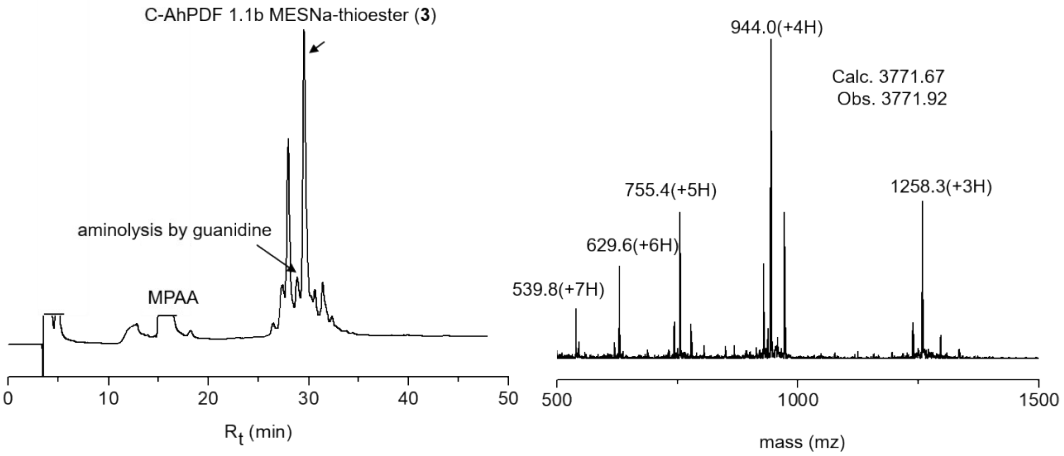


Figure S5. HPLC analysis (210 nm) of reaction of hydrolysis of C-AhPDF 1.1b MPAA-thioester (1 h, 400 mM MESNa) and ESI-MS of the side product of aminolysis of thioester by guanidine. (gradient: 5-5% **Solvent A** in 2 min, 20-20% **Solvent A** in 10 min, then 20-50% **Solvent A** in 30 min).

Mercaptoethanol-mediated hydrolysis of C-AhPDF1.1b thioester (pH 8)

C-AhPDF1.1b hydrazide (3.75 mg, 1.0 μmol) was added to 0.25 mL of PBS buffer (0.2 M Na_2HPO_4, 6 M Gn·HCl, pH 3). At -10 °C, 10 equivalents of $NaNO_2$ (20 μL, 0.01 mM in pH 3 PBS) were added. After 20 min, 200 equivalents of 2-mercaptoethanol (250 μl, 0.8 M in pH 8 PBS) was added into the reaction mixture, and pH value of the solution was adjusted to 8. After 1 h, the reaction mixture was monitored by HPLC and analyzed by MS, as shown in Figure S6.

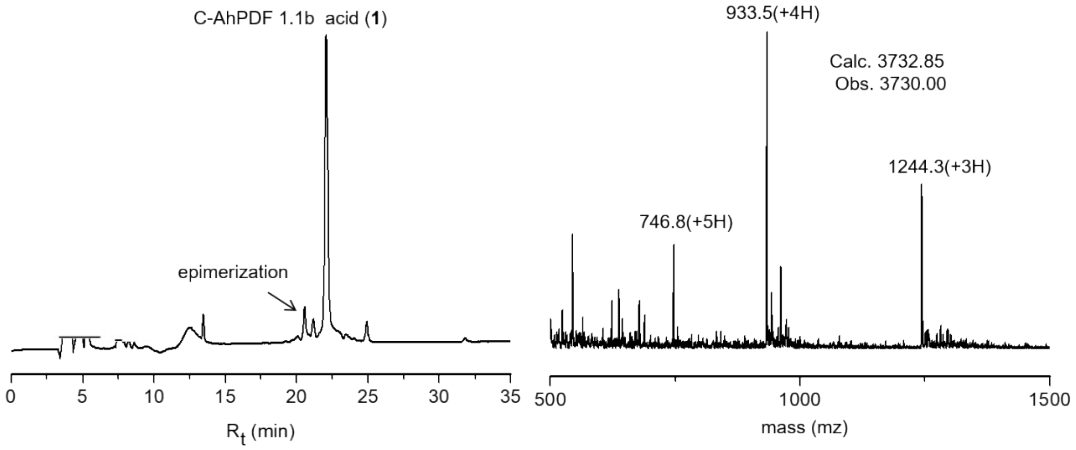


Figure S6. HPLC analysis (210 nm) of reaction of mercaptoethanol-mediated hydrolysis of C-AhPDF 1.1b thioester (1 h, 400 mM mercaptoethanol) and ESI-MS of racemized C-AhPDF 1.1b acid. (gradient: 5-5% **Solvent A** in 2 min, then 20-50% **Solvent A** in 30 min)

C-AhPDF1.1b hydrazide (3.75 mg, 1.0 μmol) was added to 0.25 mL of PBS buffer (0.2 M Na_2HPO_4, 6 M Gn·HCl, pH 3). At -10 °C, 10 equivalents of $NaNO_2$ (20 μL, 0.01 mM in pH 3 PBS) were added. After 20 min, 20 equivalents of MPAA (125 μl, 0.16 M in PBS) was added at room temperature. After 5 min, 200 equivalents of 2-mercaptoethanol (125 μl, 1.6 M in pH8 PBS) was added into the reaction mixture, and pH value of the solution was adjusted to 8. After 1 h, the reaction mixture was monitored by HPLC and analyzed by MS, as shown in Figure S7. By HPLC, the conversion yield was determined to be 98%.

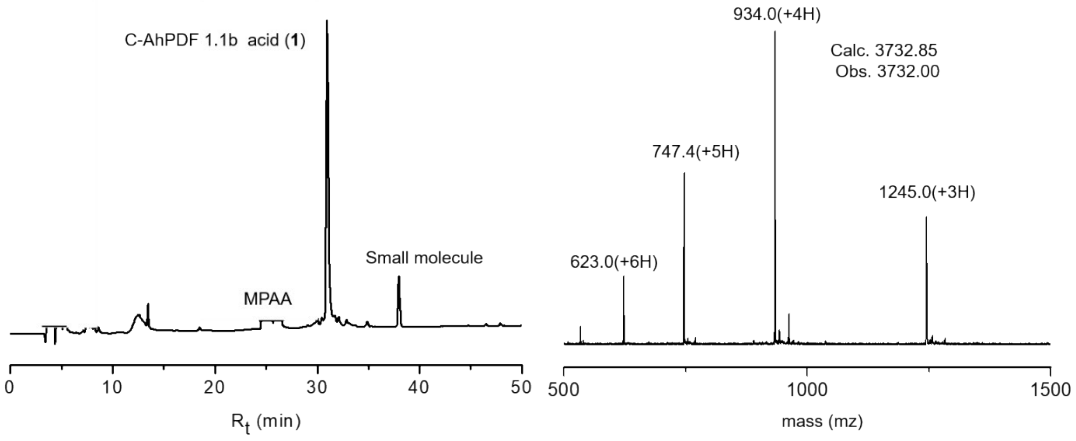


Figure S7. HPLC analysis (210 nm) of reaction of mercaptoethanol-mediated hydrolysis of C-AhPDF 1.1b MPAA thioester (1 h, 40 mM MPAA, 400 mM mercaptoethanol) and ESI-MS of the desired C-AhPDF 1.1b acid (**1**). (gradient: 5-5% **Solvent A** in 2 min, 20-20% **Solvent A** in 10 min, then 20-50% **Solvent A** in 30 min).

4. Peptide hydrazide-based preparation of somatostatin

Preparation of somatostatin hydrazide (**6**)

Somatostatin hydrazide (**6**) was synthesized on 0.1 mol scale. Isolation yield: (79 mg, 48 μmol, 48 %). HPLC: t_R= 17.6 min (gradient: 5-5%B in 2 min, then 20-40%B in 30 min). ESI-MS: obs. 1653.72 m/z (deconv.), calc. 1653.90.

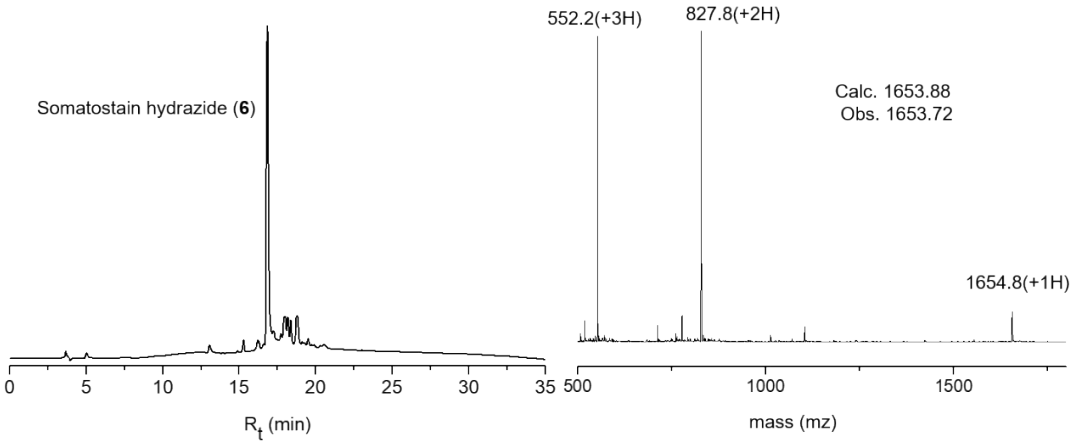


Figure S8. Analytic HPLC trace (210 nm) and ESI-MS of crude somatostatin hydrazide (**6**). (gradient: 5-5% **Solvent A** in 2 min, then 5-95% **Solvent A** in 30 min).

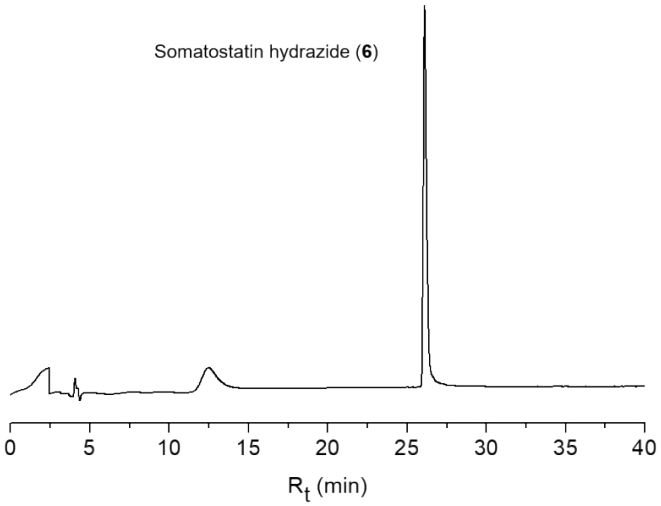


Figure S9. Analytic HPLC trace (210 nm) of purified somatostatin hydrazide (**6**) (gradient: 5-5% **Solvent A** in 2 min, then 20-40% **Solvent A** in 30 min).

Mercaptoethanol-mediated synthesis of somatostatin acid (**7**)

Somatostatin hydrazide (**6**) (1.7 mg, 1.0 μmol) was added to 0.25 mL of PBS buffer (0.2 M Na_2HPO_4, 6 M Gn·HCl, pH 3). At -10 °C, 10 equivalents of $NaNO_2$ (20 μL, 0.01 mM in pH 3 PBS) were added. After 20 min, 20 equivalents of MPAA (125μL, 0.16 M in PBS, with final pH value of 5) were added at room temperature. After 5 min, 200 equivalents of 2-mercaptoethanol (125 μl, 1.6 M in PBS) was added, and pH value of the solution was adjusted to 8. After 1 h, the reaction mixture was monitored by HPLC and analyzed by MS. By analytic HPLC, the conversion yield was determined to be 98%. The somatostatin acid (**7**) was isolated by semi-preparative HPLC and lyophilized to a white powder, giving an isolation yield of ~72% (1.18 mg, 0.72 μmol).

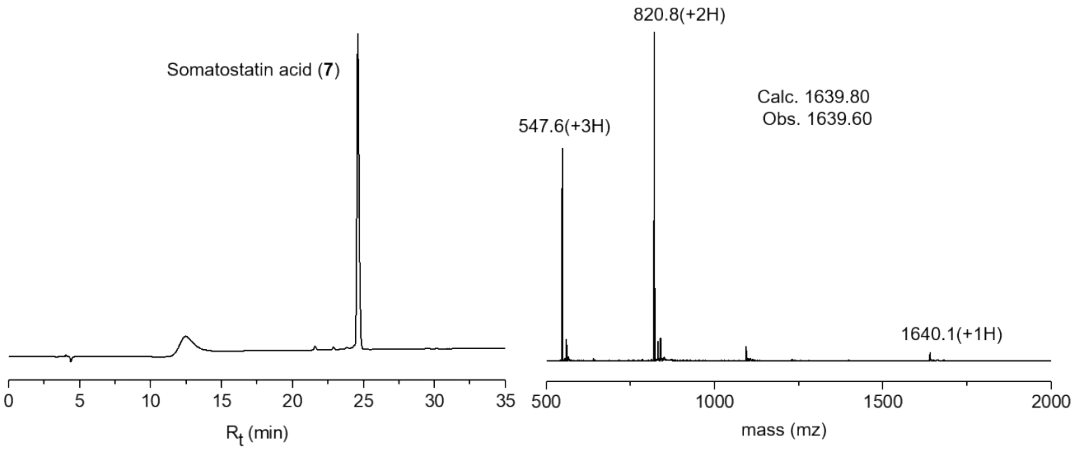


Figure S10. Analytic HPLC trace (210 nm) and ESI-MS of purified somatostatin acid (**7**) (gradient: 5-5% **Solvent A** in 2 min, then 20-50% **Solvent A** in 30 min).

I_2-mediated folding of somatostatin acid (**7**)

Somatostatin acid (**7**) (1.0 mg, 0.6 μmol) was dissolved into a mixture solution (500 μL AcOH and 100 μL H_2O). Then, 10 equivalents of I_2 in 15 μL MeOH were added, and the reaction was incubated at room temperature for 2 h and the reaction quenched by addition of 1 M ascorbic acid until the mixture became colorless. The folded somatostain was isolated by semi-preparative HPLC and lyophilized to a white powder, giving an isolation yield of ~68% (0.67 mg, 0.41 μmol).

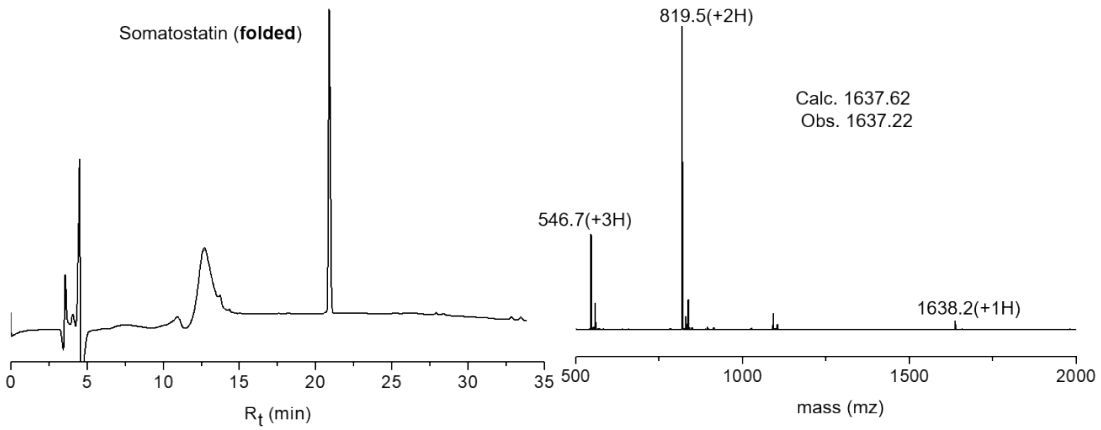


Figure S11. Analytic HPLC trace (210 nm) and ESI-MS of purified somatostatin (gradient: 5-5% **Solvent A** in 2 min, then 20-70% **Solvent A** in 30 min).

5. Peptide hydrazide-based preparation of Riparin 1.1b acid

Preparation of Riparin 1.1b hydrazide

Riparin 1.1b hydrazide was synthesized on 0.1 mol scale. Isolation yield: (68 mg, 56 µmol, 56 %). HPLC: t_R= 14.5 min (gradient: 5-5% in 2 min, then 5-95% B in 30 min). ESI-MS: obs. 1212.72 m/z (deconv.), calc. 1212.45.

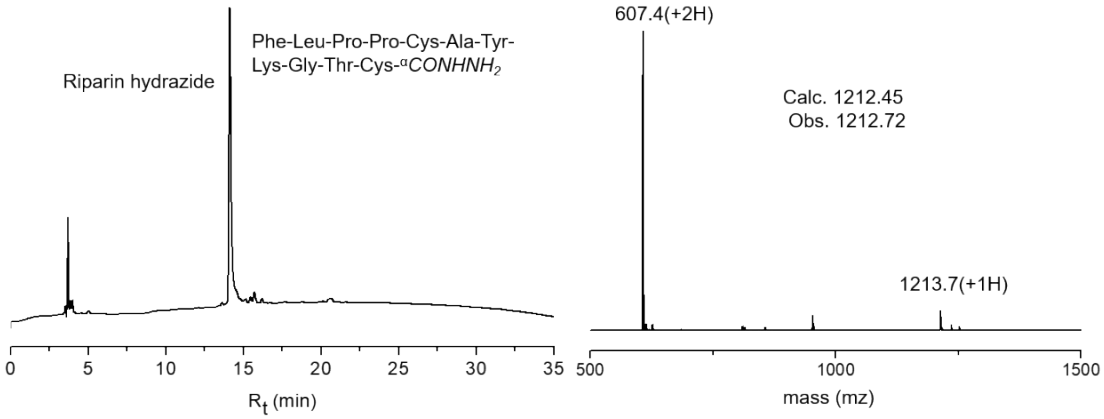


Figure S12. Analytic HPLC trace (210 nm) and ESI-MS of crude Riparin 1.1b hydrazide (gradient: 5-5% **Solvent A** in 2 min, then 5-95% **Solvent A** in 30 min).

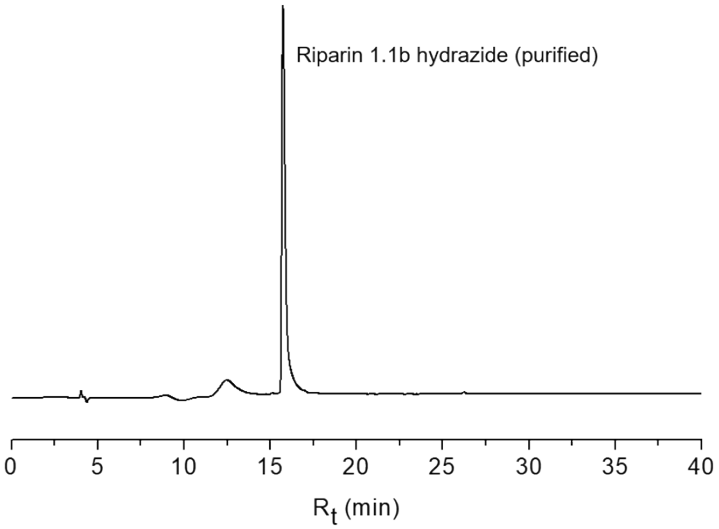


Figure S13. Analytic HPLC trace (210nm) of purified Riparin hydrazide (gradient: 5-5% **Solvent A** in 2 min, then 20-40% **Solvent A** in 30 min).

Mercaptoethanol-mediated synthesis of Riparin 1.1b

Riparin 1.1b hydrazide (1.2 mg, 1.0 μmol) was added to 0.25 mL of PBS buffer (0.2 M Na_2HPO_4, 6 M Gn·HCl, pH 3). At -10 °C, 10 equivalents of $NaNO_2$ (20 μL, 0.01 mM in pH 3 PBS) were added. After 20 min, 20 equivalents of MPAA (125 μL, 0.16 M in PBS, with final pH value of 5) were added at room temperature. After 5 min, 200 equivalents of 2-mercaptoethanol (125 μl, 1.6 M in PBS) was added, and pH value of the solution was adjusted to 8. After 1 h, the reaction mixture was monitored by HPLC and analyzed by MS. By analytic HPLC, 98% of Riparin 1.1b hydrazide has been converted to Riparin 1.1b acid. The Riparin 1.1b acid was isolated by semi-preparative HPLC and lyophilized to a white powder, giving an isolation yield of ~71% (0.9 mg, 0.7 μmol).

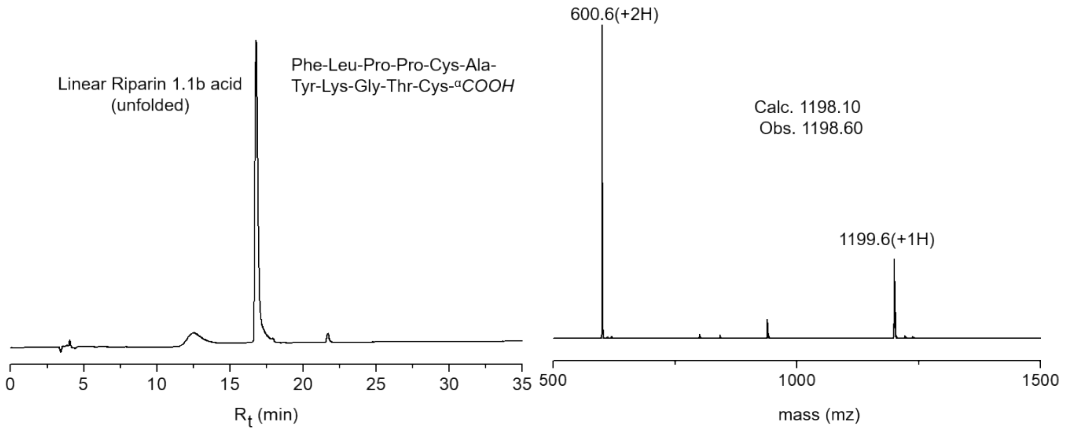


Figure S14. Analytic HPLC trace (210nm) of purified Riparin 1.1 b acid (gradient: 5-5% **Solvent A** in 2 min, then 20-50% **Solvent A** in 30 min).

Folding of somatostatin acid Riparin 1.1b acid

The Riparin 1.1b acid (1.2 mg, 1.0 μmol) was dissolved in 12 mL PBS buffer (50 mM Na_2HPO_4, 8 mM GSSG, 1 mM GSH, pH 7.5), with a protein concentration of 0.1 mg/mL. Then, the reaction mixture was stirred at room temperature for 6 hours. After HPLC purification and lyophilization, corrected folded Riparin 1.1b was obtained as a white powder, giving an isolation yield of ~65% (0.8 mg, 0.65 μmol).

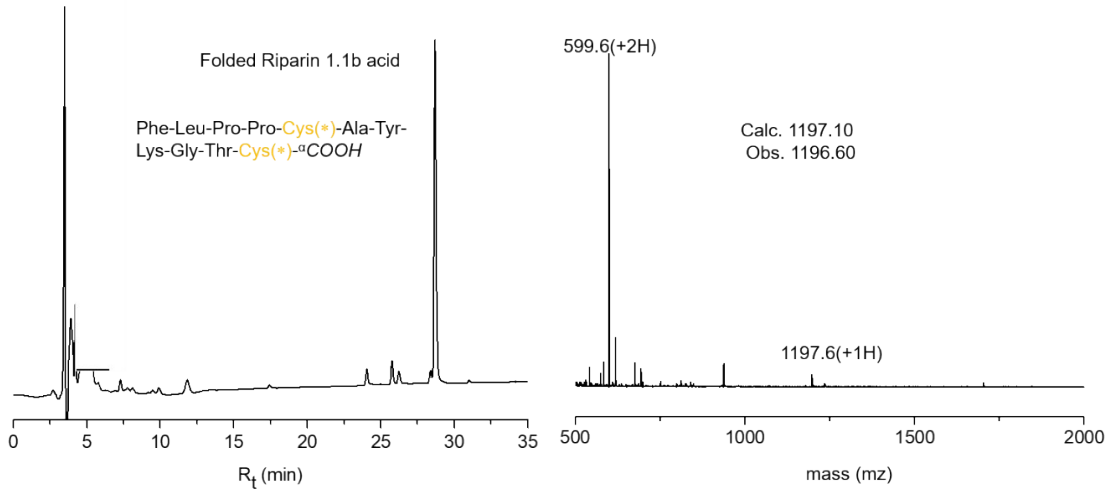


Figure S15. HPLC analysis (210 nm) and ESI-MS of the folded Riparin 1.1b acid (6 h, 8 Mm GSSG, 1 Mm GSH), with a disulfide bond formed between ^{5}Cys(*) and ^{11}Cys(*) (gradient: 5-5% **Solvent A** in 2 min, then 20-40% **Solvent A** in 30 min).

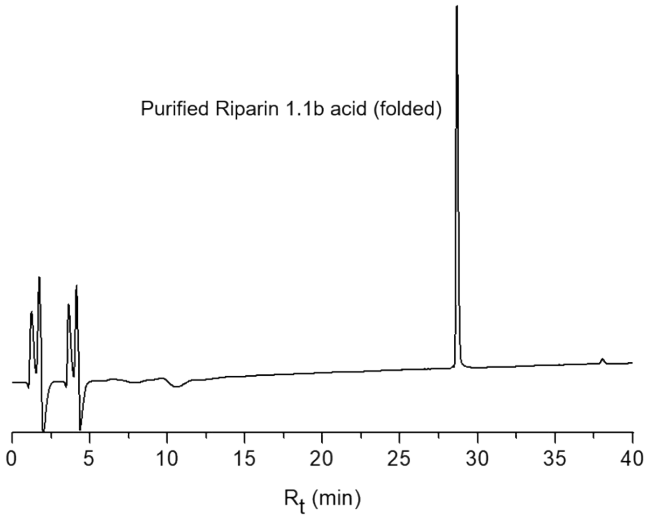


Figure S16. Analytic HPLC trace (210 nm) of purified Riparin 1.1b (gradient: 5-5% **Solvent A** in 2 min, then 20-40% **Solvent A** in 30 min).

6. Peptide hydrazide-based preparation of Vc 1.1 acid

Preparation of Vc 1.1 hydrazide (**8**)

α-conotoxin Vc 1.1 hydrazide (**8**) was synthesized on 0.1 mol scale. Isolation yield: (104 mg, 53 μmol, 53 %). HPLC: t_R= 13.5 min (gradient: 5-5% in 2 min, then 20-70%B in 30 min). ESI-MS: obs. 1967.18 m/z (deconv.), calc. 1968.20.

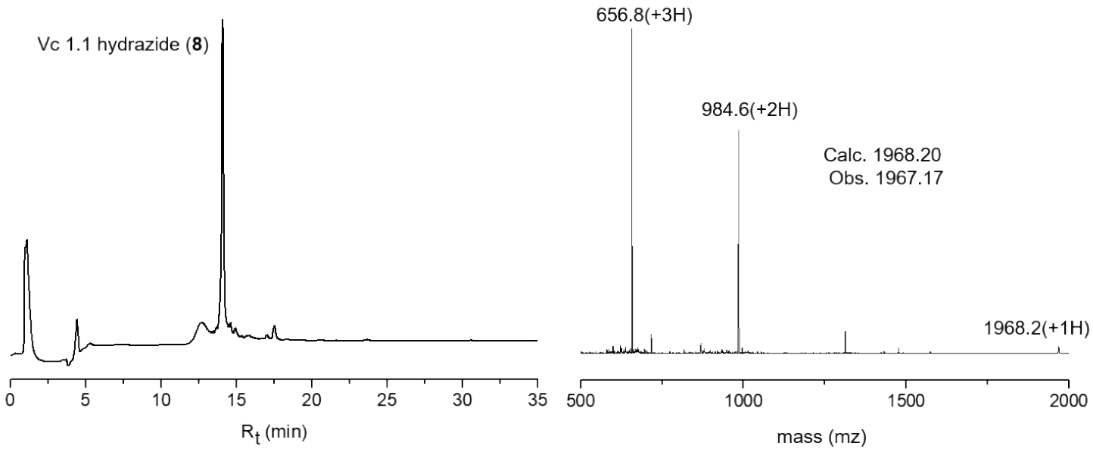


Figure S17. Analytic HPLC trace (210 nm) and ESI-MS of crude Vc 1.1 hydrazide (**8**) (gradient: 5-5% **Solvent A** in 2 min, then 20-70% **Solvent A** in 30 min).

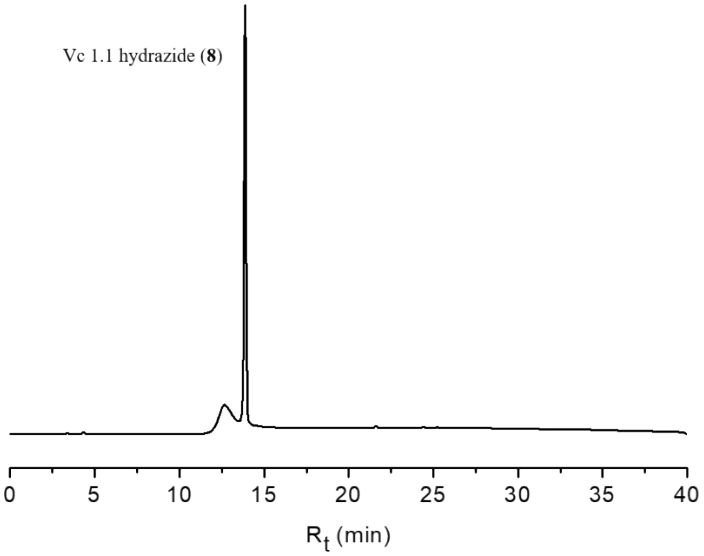


Figure S18. Analytic HPLC trace (210nm) of purified Vc 1.1 hydrazide (**8**) (gradient: 5-5% **Solvent A** in 2 min, then 20-70% **Solvent A** in 30 min).

Mercaptoethanol-mediated synthesis of α-conotoxin Vc1.1 acid

Vc1.1 hydrazide (**8**) (2.0 mg, 1.0 μmol) was added to 0.25 mL of PBS buffer (0.2 M Na_2HPO_4, 6 M Gn·HCl, pH 3). At -10 °C, 10 equivalents of $NaNO_2$ (20 μL, 0.01 mM in pH3 PBS) were added. After 20 min, 20 equivalents of MPAA (125 μL, 0.16 M in PBS, with final pH value of 5) were added at room temperature. After 5 min, 200 equivalents of 2-mercaptoethanol (125 μl, 1.6 M in PBS) was added, and pH value of the solution was adjusted to 8. After 1 h, the reaction mixture was monitored by HPLC and analyzed by MS. By analytic HPLC, the conversion yield was determined to be 98%. The Vc 1.1 acid (**9**) was isolated by semi-preparative HPLC and lyophilized to a white powder, giving an isolation yield of ~68% (1.3 mg, 0.68 μmol).

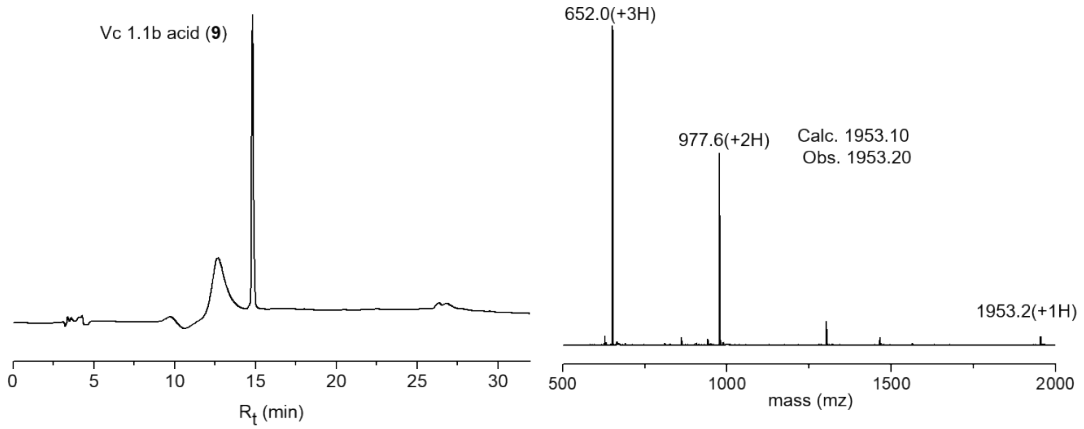


Figure S19. Analytic HPLC trace (210nm) and ESI-MS of purified Vc 1.1 acid (**9**) (gradient: 5-5% **Solvent A** in 2 min, then 20-70% **Solvent A** in 30 min).

The Vc 1.1 acid (**9**) (2.0 mg, 1.0 μmol) was dissolved in PBS buffer (50 mM Na_2HPO_4, 8 mM GSSG, 1 mM GSH, pH 7.5), with a protein concentration of 0.1 mg/mL. Then, the reaction mixture was stirred at room temperature. After 6 h, the reaction mixture was monitored by HPLC and analyzed by MS. The conversion yield of this step was determined to be 82%. After HPLC purification and lyophilization, partially folded Vc 1.1 (**10**) was obtained as a white powder, giving an isolation yield of ~51% (1.0 mg, 0.5 μmol).

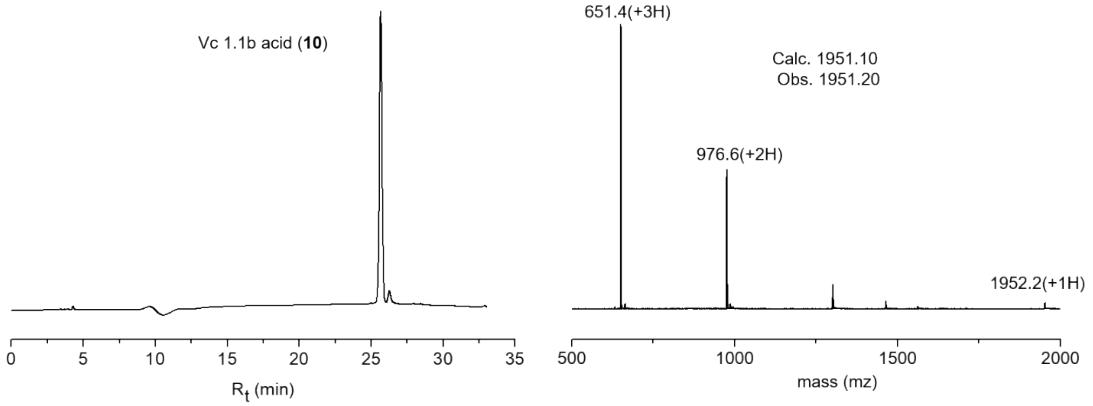


Figure S20. Analytic HPLC trace (210nm) and ESI-MS of purified Vc 1.1 acid (**10**) (gradient: 5-5% **Solvent A** in 2 min, then 20-40% **Solvent A** in 30 min).

The partially folded Vc 1.1 (**10**) (1.0 mg, 0.5 μmol) was dissolved into a mixture solution (500 μL AcOH and 100 μL H_2O). Then, 10 equivalents of I_2 in 15 μL MeOH were added, and the reaction was incubated at room temperature for 2 h and the reaction quenched by addition of 1 M ascorbic acid until the mixture became colorless. The folded Vc 1.1 was isolated by semi-preparative HPLC and lyophilized to a white powder, giving an isolation yield of ~71% (0.64 mg, 0.36 μmol).

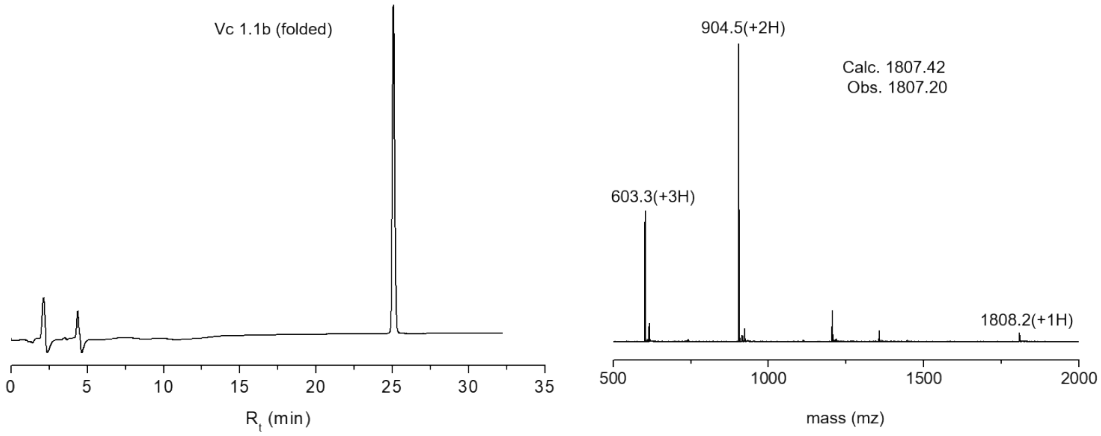


Figure S21. Analytic HPLC trace (210 nm) and ESI-MS of purified Vc 1.1 (gradient: 5-5% **Solvent A** in 2 min, then 20-40% **Solvent A** in 30 min).