Supporting Information

Reducible Solubilizing Tags (RST) Compatible with Peptide Ligation for Chemical Protein Synthesis and Semi-synthesis

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1 General information

1.1 General information for reagents

All solvents in reagent grade (RCI) or HPLC grade (DUKSAN) were used without purification. All commercially available amino acids and coupling reagents (purchased from Aldrich and GL Biochem) were used without further purification. Anhydrous DCM was distilled from calcium hydride (CaH_2). Trifluoroethanol (TFE), trifluoroacetic acid (TFA) and hexafluoro-isopropanol (HFIP) were purchased from J&K Scientific. N-Diisopropylethylamine (DIPEA), Triisopropylsilane (TIPS) and 4-Dimethylaminopyridine (DMAP) were purchased from Energy-chemical. 2-chlorotrityl chloride resin was purchased from GL Biochem.

1.2 General information for LC-MS methods

Analytical reversed-phased (RP) high-performance liquid chromatograph (HPLC) was performed with a Waters system equipped with a photodiode array detector (Waters 2996) and a Vydac 218TP C18 column (5 μm, 300 Å, 4.6 × 250mm) involving a mobile phase of ACN containing 0.1%TFA and water containing 0.1%TFA at a flow rate of 0.6 mL/min. UPLC H-class was performed with a Waters system equipped with an ACQUITY UPLC photodiode array eλ detector and a Waters ACQUITY BEH C18 column (1.7 μm, 130 Å, 2.1 × 50mm) involving a mobile phase of ACN containing 0.1%TFA and water containing 0.1%TFA at a flow rate of 0.4 mL/min. Preparative HPLC was performed with a Waters system and used a Vydac 218TP C18 column (10 μm, 22 × 250 mm) involving a mobile phase of ACN containing 0.1%TFA and water containing 0.1%TFA at a flow rate of 10 mL/min or a YMC C18 column (20 μm, 30 × 250 mm) at a flow rate of 20 mL/min. Mass analysis was performed with a Waters 3100 mass spectrometer equipped with an electrospray ionization source (ESI).

2 General procedures

2.1 Fmoc-based solid phase peptide synthesis (SPPS)

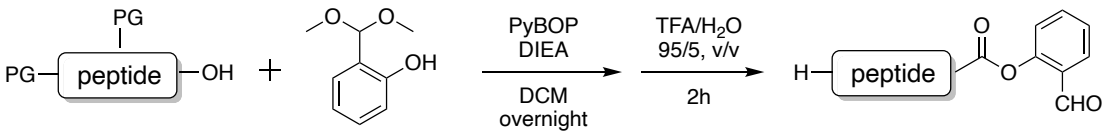
Peptides were synthesized manually by Fmoc-SPPS on 2-chlorotrityl chloride resin

(GL Biochem or CSBio, resin loading: 0.5 mmol/g). Firstly, the resin was swollen in the dry DCM for 30min, then the solvent was washed away. The first amino acid FmocHN-Xaa-COOH (4.0 equiv.) and DIEA (8.0 equiv.) was dissolved in DCM and added into the resin and shaking for 2~3 h to load the amino acid. The resin was then washed with DCM (5 mL × 3 times), DMF (5 mL × 3 times) and DCM (5 mL × 3 times). Subsequently, a mixture of MeOH/DIEA/DCM (2:1:17, v/v/v) was added into the resin and shaking for 30 min to cap the resin. After capping, the resin was submitted to iterative peptide assembly (Fmoc-SPPS). The deblock solution was a mixture of piperidine/DMF (20:80, v/v). FmocHN-Trp(Boc)-COOH, FmocHN-Arg(Pbf)-COOH, FmocHN-Lys(Boc)-COOH, FmocHN-Glu(O^{t}Bu)-COOH, FmocHN-Gln(Trt)-COOH, FmocHN-Thr(^{t}Bu)-COOH, FmocHN-Ser(^{t}Bu)-COOH, FmocHN-Pro-COOH, FmocHN-Phe-COOH, FmocHN-Leu-COOH, FmocHN-Ile-COOH, FmocHN-Tyr(^{t}Bu)-COOH, FmocHN-Asp(O^{t}Bu)-COOH, FmocHN-Val-COOH, FmocHN-Gly-COOH, FmocHN-Met-COOH, FmocHN-Ala-COOH, FmocHN-Cys(SMe)-COOH, FmocHN-Cys(S^{t}Bu)-COOH, FmocHN-Cys(Trt)-COOH, FmocHN-His(Trt)-COOH, FmocHN-Asn(Trt)-COOH, BocHN-Ser(^{t}Bu)-COOH, BocHN-Thz-COOH, BocHN-Thr(^{t}Bu)-COOH were used for coupling. For the coupling step, a solution of Fmoc protected amino acid or Boc protected amino acid (4.0 equiv. according to the resin capacity), HATU (4.0 equiv.) and DIEA (10 equiv.) in DMF was gently agitated with the resin at room temperature for 1h.

2.2 Cleavage of fully protected crude peptide from resin

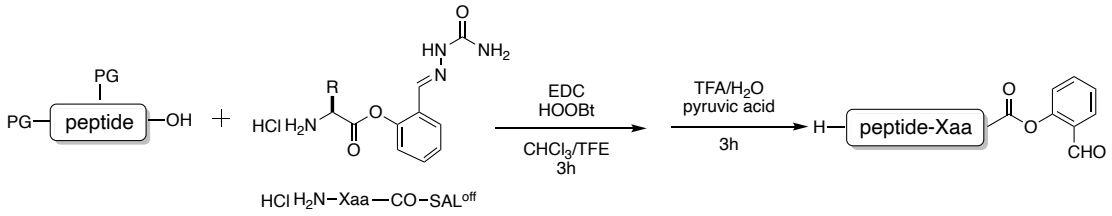
The fully protected peptide on resin was treated with a cocktail of DCM/AcOH/TFE=8:1:1 (v/v/v, 5-10 mL), 2 times and 1.5 h for each time. After cleavage, the resulting cleavage solutions were combined and concentrated to give crude protected peptide bearing the free carboxylic acid at the C-terminus.

2.3 Preparation of peptide salicylaldehyde ester by direct coupling strategy



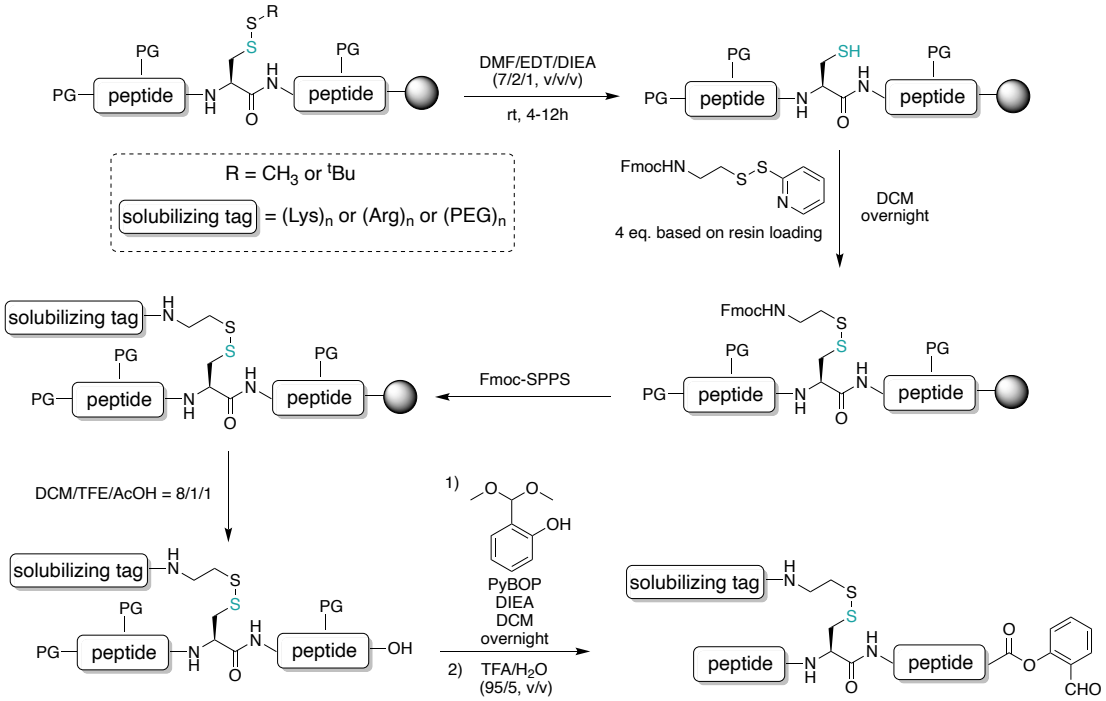
Fully protected crude peptide (1.0 equiv.) was dissolved in dry DCM at a concentration of 10 mM. DIEA (6.0 equiv.) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (3.0 equiv.) was added, followed by α, α-dimethoxy-salicylaldehyde (30.0 equiv.). The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed by evaporation at reduced pressure. Subsequently, TFA/H_2O (95/5, v/v) was added, and the resulting mixture was stirred at room temperature for 2 h. After global deprotection, TFA was blown off and the residues was triturated with diethyl ether (50 mL, 3 times) and centrifuged. The crude peptide salicylaldehyde ester was dried under vacuum and purified by HPLC. This strategy only suitable for the preparation of C-terminal Gly or Pro peptide salicylaldehyde ester.

2.4 Preparation of peptide salicylaldehyde ester by "N+1" strategy



Fully protected crude peptide (1.0 equiv., peptide concentration = 10 mM) and the corresponding amino L-Amino salicylaldehyde semicarbazone ester hydrochloride (HCl·H_2N-Xaa-CO-SAL^{off}, 6 equiv.) was dissolved in $CHCl_3$/TFE (3:1, v/v) and then Hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOOBt) (3.0 equiv.) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) (3.0 equiv.) were added. The reaction mixture was stirred at room temperature for 3h. The solvent was removed under vacuo. Then TFA/H_2O (95/5, v/v) and pyruvic acid (100 equiv.) were added into the resulting residue, and the resulting mixture was stirred for 3 h at room temperature. After global protection, TFA was blown off and the residues was triturated with diethyl ether (50 mL, 3 times) and centrifuged. The crude peptide salicylaldehyde ester was dried under vacuum and purified by HPLC.

2.5 Synthesis of peptide salicylaldehyde ester with reducible solubilizing tags at internal Cys

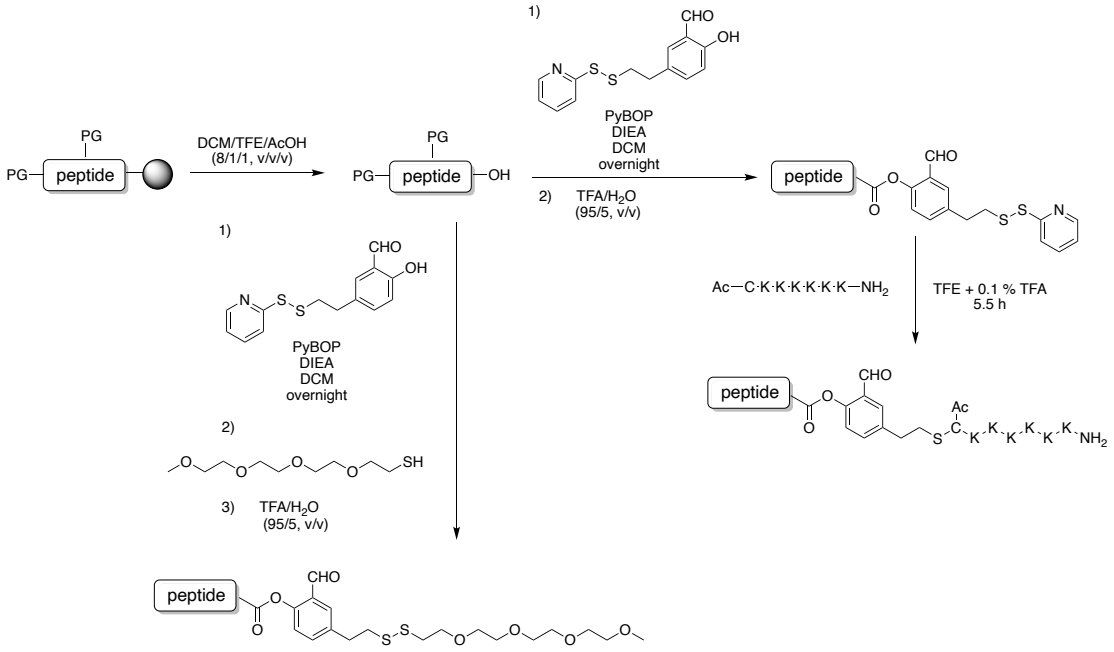


Generally, we used FmocHN-Cys(S^{t}Bu)-COOH for Fmoc-SPPS and after peptide assembly, 5 mL EDT/DIEA/DMF (2/1/7, v/v/v) was added into the resin and shaked for 4 h-12 h to selectively remove the *tert*-butylthio (S^{t}Bu) protecting group . However, it was found *tert*-butylthio was hard to be removed on resin in some cases. In this context, FmocHN-Cys(SMe)-COOH was synthesized instead of FmocHN-Cys(S^{t}Bu)-COOH in Fmoc-SPPS. After selectively removing the S^{t}Bu or SMe protecting group, the resin was treated with a solution of (9H-fluoren-9-yl)methyl 2-(pyridin-2-yldisulfanyl)ethylcarbamate in DCM for 4-12 h, the linker was generated via disulfide formation. After removing the Fmoc group on the linker, poly-Arg-tag, poly-Lys-tag or PEG-tag were attached by Fmoc-SPPS. Finally, peptide salicylaldehyde ester with reducible solubilizing tags at internal Cys was prepared according to the **General procedure 2.3**.

The reducible solubilizing tags were detached with 0.2 M TCEP in pH ~ 7 PB buffer (0.2 M PB solution, containing 6 M Gn·HCl) or ACN/H_2O for 30 min ~ 1 h. It was noted that the solubilizing tag could be removed during free radical based desulfurization step, highlighting the powerfulness of this method by combining with

CPL-desulfurization.

2.6 Synthesis of peptide salicylaldehyde ester with reducible solubilizing tags at salicylaldehyde



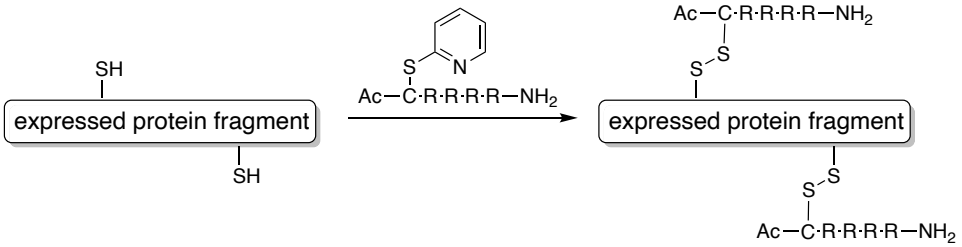
For peptide salicylaldehyde ester with PEG_4 solubilizing tag, fully protected crude peptide (1.0 equiv., peptide concentration = 10 mM) was dissolved in dry DCM. DIEA (6.0 equiv.) and PyBOP (3.0 equiv.) was added, followed by 2-hydroxy-5-(2-(pyridin-2-yldisulfaneyl)ethyl)benzaldehyde (10.0 equiv.). The reaction mixture was stirred at room temperature for overnight. Then, tetraethylene glycol monomethyl ether thiol was added into the reaction mixture and reacted for 5.5 h. After that, solvent was removed by vacuo. TFA/H_2O (95/5, v/v) was added and reacted for 2 h. After global deprotection, TFA was blown off and the residues was precipitated with diethyl ether (50 mL, 3 times) and centrifuged. The crude peptide salicylaldehyde ester with PEG_4 solubilizing tag was dried under vacuum and purified by HPLC.

For peptide salicylaldehyde ester with Lys_6 solubilizing tag, fully protected crude peptide (1.0 equiv., peptide concentration = 10 mM) was dissolved in dry DCM. DIEA (6.0 equiv.) and PyBOP (3.0 equiv.) was added, followed by 2-hydroxy-5-(2-(pyridin-2-yldisulfaneyl)ethyl)benzaldehyde (10.0 equiv.). The reaction mixture was stirred at room temperature for overnight. After that, solvent was removed by vacuo. TFA/H_2O

(95/5, v/v) was added and reacted for 2 h. After global deprotection, TFA was blown off and the residues was precipitated with diethyl ether (50 mL, 3 times) and centrifuged. Then the crude peptide salicylaldehyde ester (1.0 equiv., crude peptide concentration = 10 mM) and solubilizing tag Ac-CKKKKKK-NH_2 (5 equiv.) were dissolved in TFE (containing 0.1% TFA) and reacted for 5.5 h. After that, solvent was blown off and the residues was precipitated with diethyl ether (50 mL, 3 times) and centrifuged. The crude peptide salicylaldehyde ester with Lys_6 solubilizing tag was dried under vacuum and purified by HPLC.

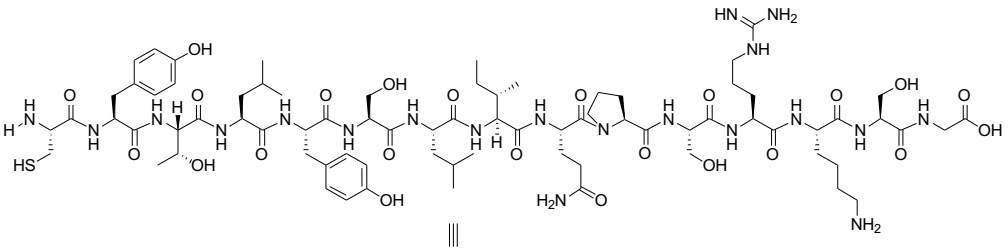
The solubilizing tags attached via salicylaldehyde ester could be removed during the acidolysis step after STL or CPL.

2.7 Synthesis of expressed protein segment with solubilizing tags



Expressed protein fragment and solubilizing tag Ac-C(Spyr)RRRR-$CONH_2$ (30 equiv.) were dissolved in 75% ACN/H_2O and reacted for overnight at room temperature. After that, the excess solubilizing tags were removed by ultra-filtration. The expressed protein fragment with Arg_4 tag was afforded after lyophilization.

3 Synthesis of 2a



H-CYTLYSLIQPSRKSG-OH

2a

N-terminus model peptide H-CYTLYSLIQPSRKSG-OH (**2a**) was assembled by Fmoc-SPPS at 0.1 mol scale. After Fmoc-SPPS, 10 mL TFA/H_2O/TIPS (90/5/5, v/v/v) was

added to cleavage the peptide from resin and globally deprotect for 2 h at room temperature. The crude peptide was purified by preparative reverse-phase HPLC (10 to 50% ACN/H_2O over 30 min, 0.1% TFA). After lyophilization, 104.92 mg H-CYTLYSLIQPSRKSG-OH (**2a**) was obtained as a white solid (Isolated yield: 61.14 %).

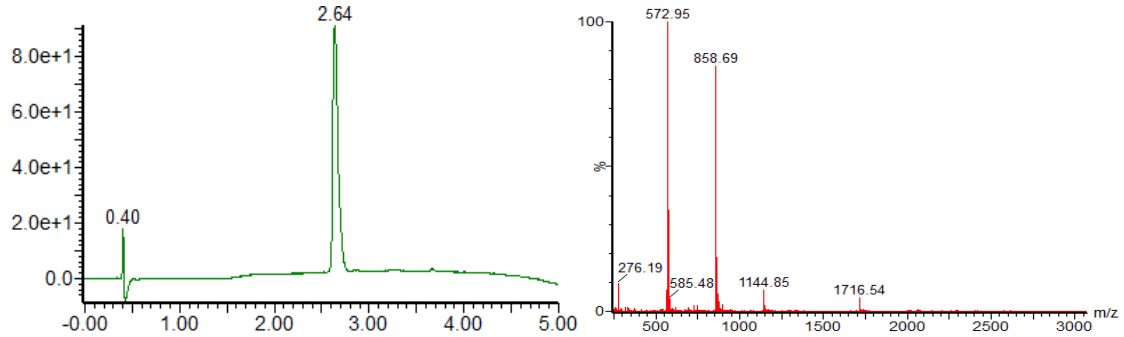
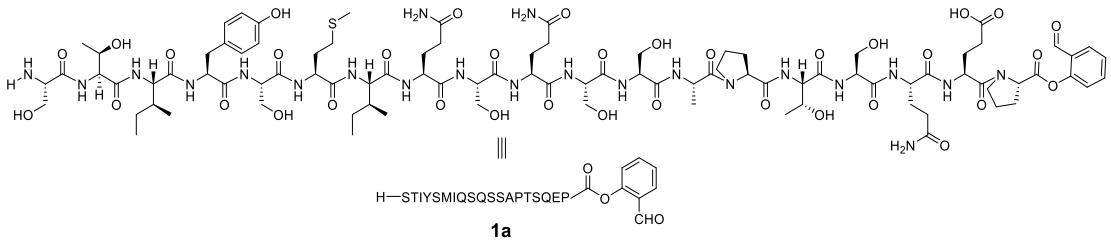


Figure S1. UV trace from analytical RP-UPLC and its ESI-MS of purified H-CYTLYSLIQPSRKSG-OH (**2a**). Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{76}H_{122}N_{20}O_{23}S$ $[M+H]^+$ m/z = 1716.98, found 1716.54, $[M+2H]^{2+}$ m/z = 858.99, found 858.69, $[M+3H]^{3+}$ m/z = 573.00, found 572.95.

4 Synthesis of peptide salicylaldehyde ester (1a-e)

4.1 Synthesis of 1a



1a

0.02 mmol C-terminus peptide salicylaldehyde ester **1a** was synthesized according to **General procedure 2.3**. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 2.49 mg peptide salicylaldehyde ester **1a** was obtained in an isolated yield of 5.80%.

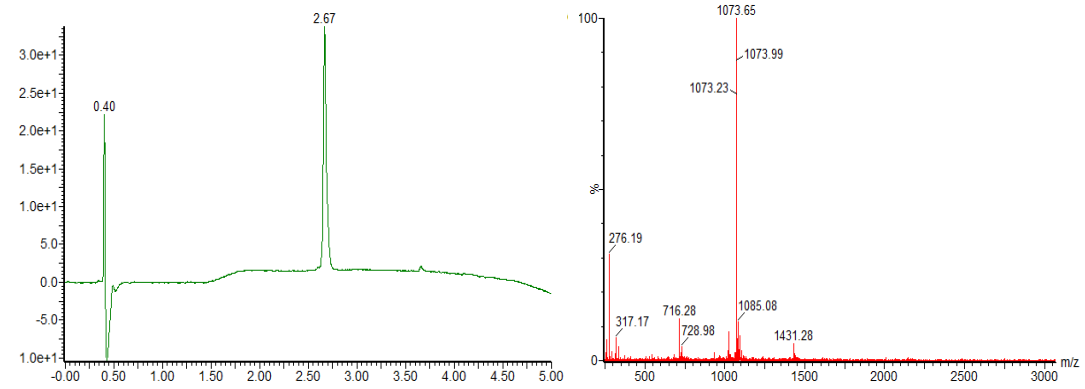
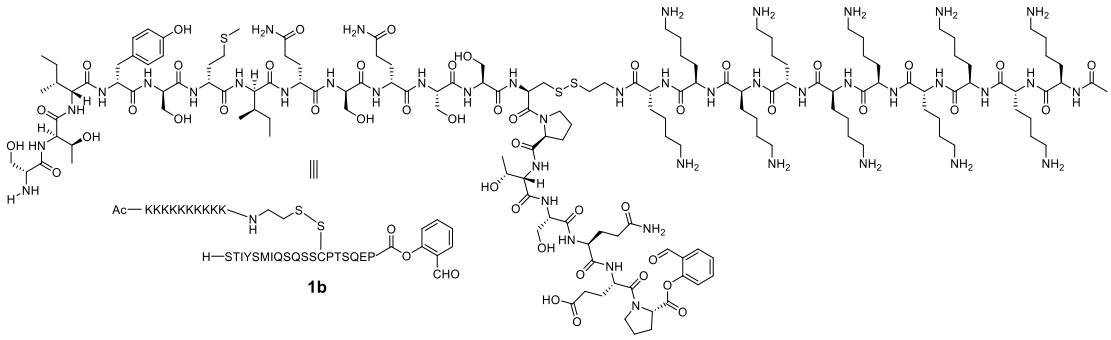


Figure S2. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **1a**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{92}H_{140}N_{22}O_{35}S$ $[M+2H]^{2+}$ m/z = 1074.16, found 1073.65, $[M+3H]^{3+}$ m/z = 716.44, found 716.28.

4.2 Synthesis of 1b



0.02 mmol C-terminus peptide salicylaldehyde ester **1b** was synthesized according to **General procedure 2.5**. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 40% ACN/H_2O over 30 min. After lyophilization, 17.25 mg peptide salicylaldehyde ester **1b** was obtained in an isolated yield of 24.11 %.

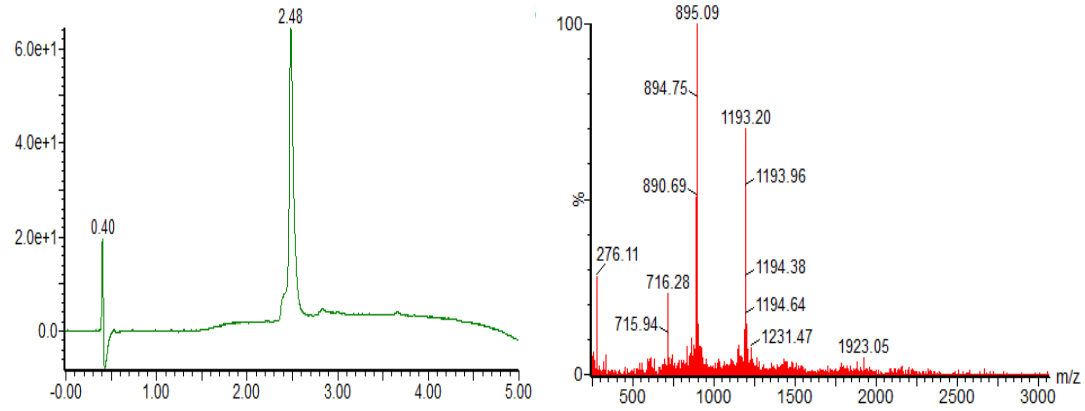
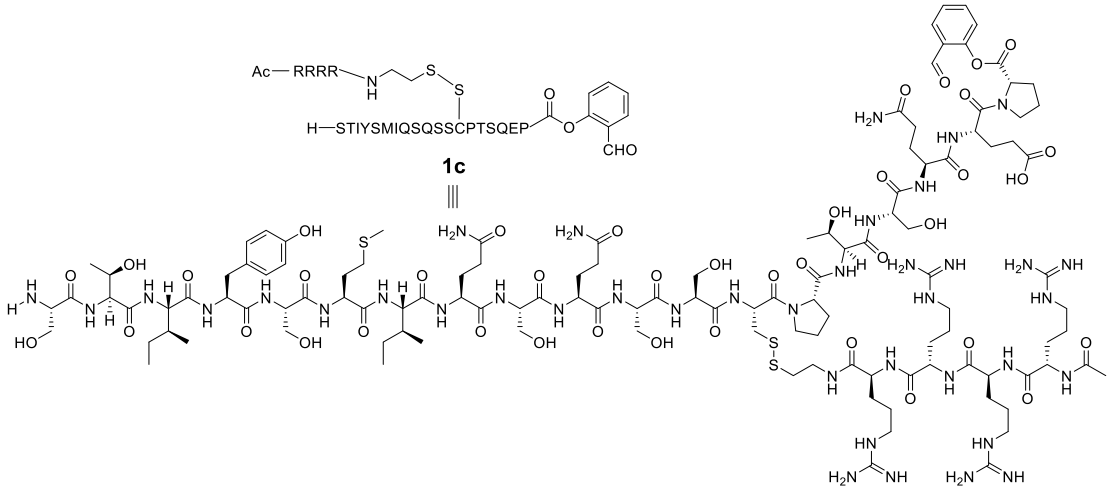


Figure S3. UV trace from analytical RP-UPLC and its ESI-MS of peptide

salicylaldehyde ester **1b**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{156}H_{267}N_{43}O_{46}S_3$ $[M+3H]^{3+}$ m/z = 1193.42, found 1193.20, $[M+4H]^{4+}$ m/z = 895.32, found 895.09, $[M+5H]^{5+}$ m/z = 716.46, found 716.28.

4.3 Synthesis of 1c



0.02 mmol C-terminus peptide salicylaldehyde ester **1c** was synthesized according to **General procedure 2.5**. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 11.33 mg peptide salicylaldehyde ester **1c** was obtained in an isolated yield of 19.40%.

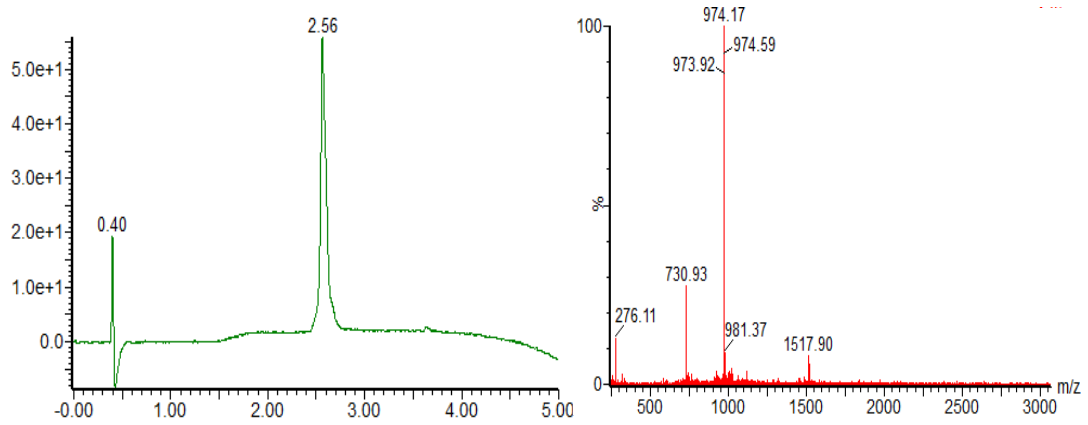
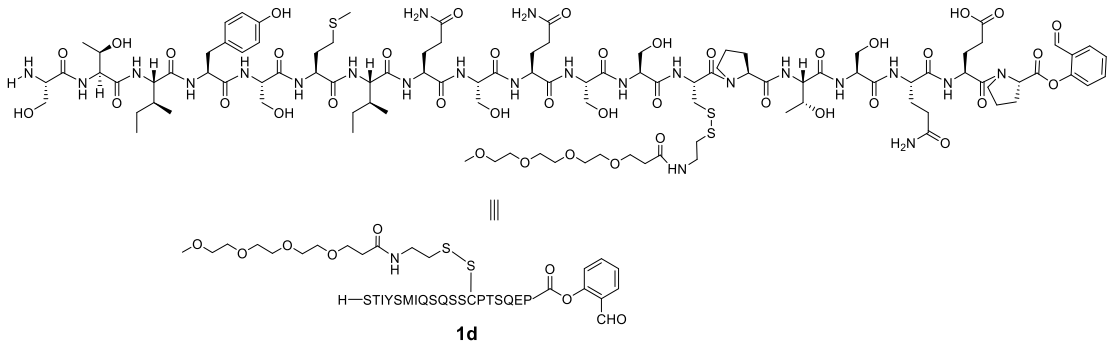


Figure S4. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **1c**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{120}H_{195}N_{39}O_{40}S_3$ $[M+3H]^{3+}$ m/z = 974.43, found 974.17, $[M+4H]^{4+}$ m/z = 731.07, found 730.93.

4.4 Synthesis of 1d



1d

0.04 mmol C-terminus peptide salicylaldehyde ester **1d** was synthesized according to **General procedure 2.5**. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 14.48 mg peptide salicylaldehyde ester **1d** was obtained in an isolated yield of 14.65%.

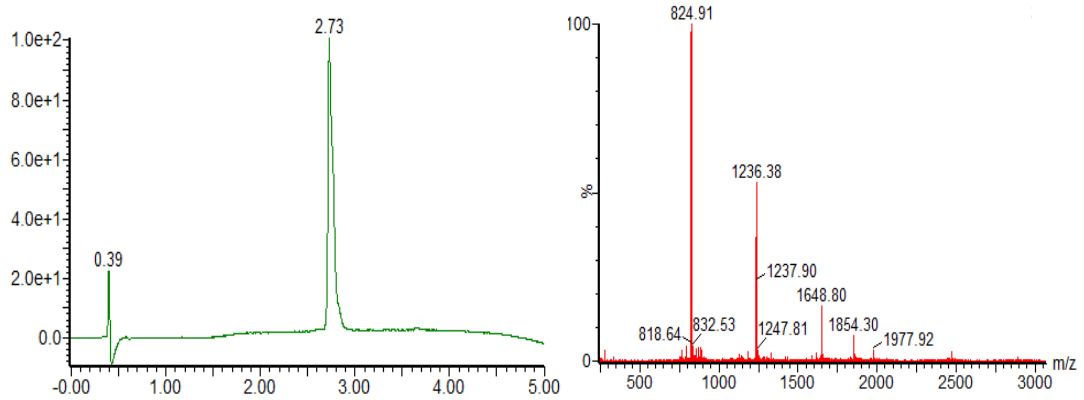
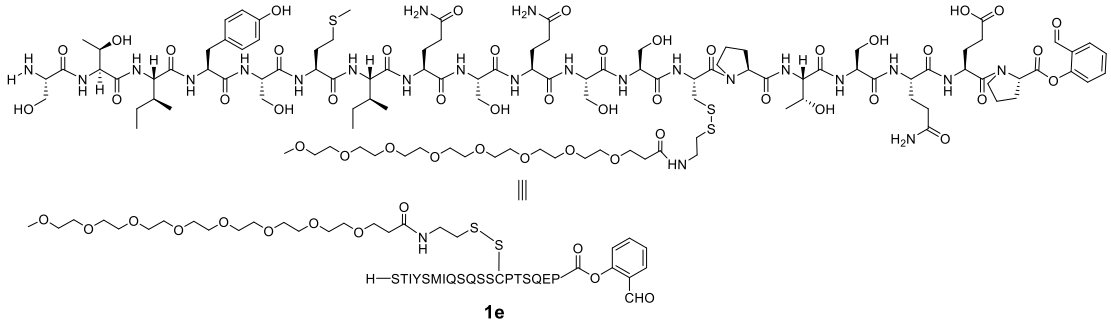


Figure S5. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **1d**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{104}H_{163}N_{23}O_{40}S_3$ $[M+2H]^{2+}$ m/z = 1236.87, found 1236.38, $[M+3H]^{3+}$ m/z = 824.92, found 824.91.

4.5 Synthesis of 1e



1e

0.04 mmol C-terminus peptide salicylaldehyde ester **1e** was synthesized according to **General procedure 2.5**. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O

over 30 min. After lyophilization, 21.98 mg peptide salicylaldehyde ester **1e** was obtained in an isolated yield of 20.76%.

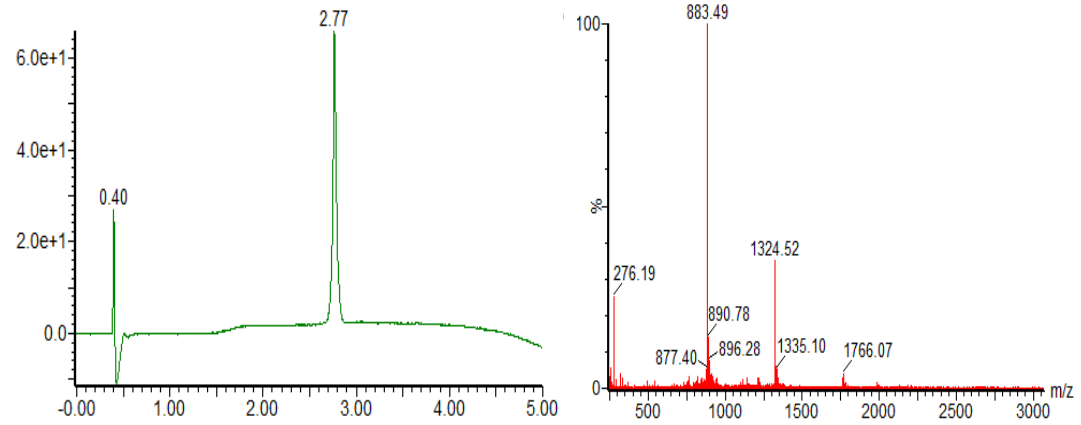
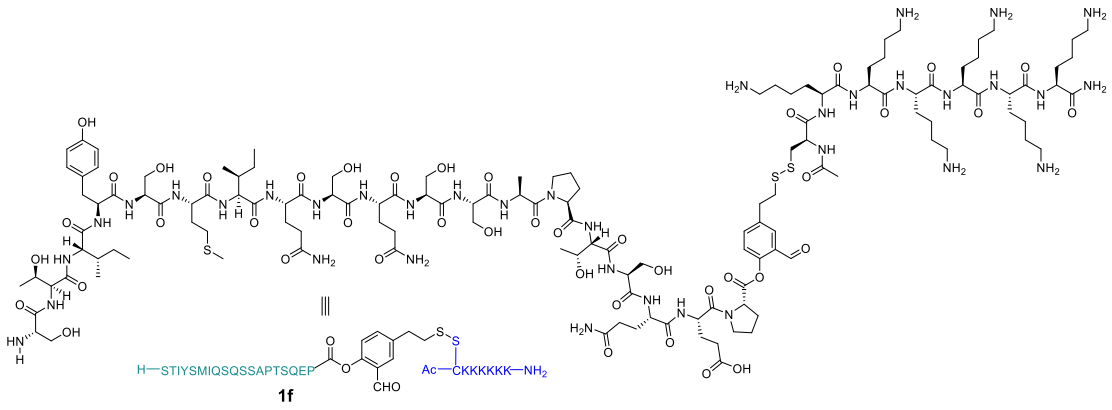


Figure S6. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **1e**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{112}H_{179}N_{23}O_{44}S_3$ $[M+2H]^{2+}$ m/z = 1324.98, found 1324.52, $[M+3H]^{3+}$ m/z = 883.66, found 883.49.

5 Synthesis of peptide salicylaldehyde ester (1f-g)

5.1 Synthesis of 1f



0.02 mmol C-terminus peptide salicylaldehyde ester **1f** was synthesized according to **General procedure 2.6**. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 12.22 mg peptide salicylaldehyde ester **1f** was obtained in an isolated yield of 19.49%.

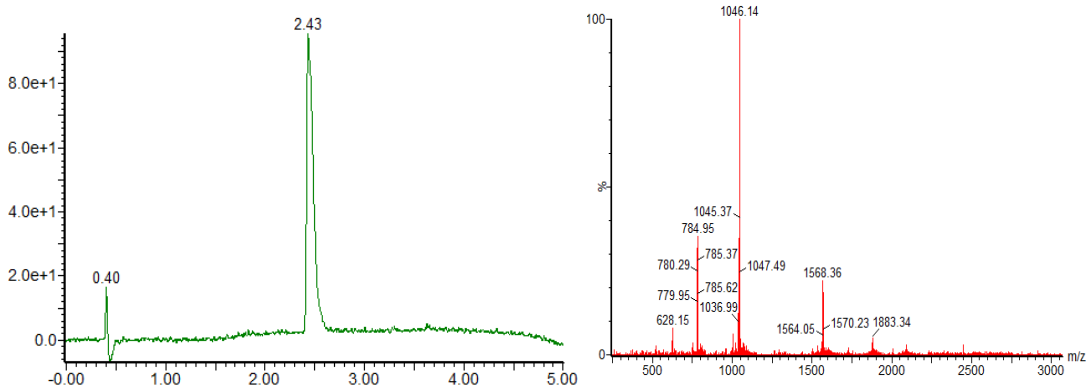
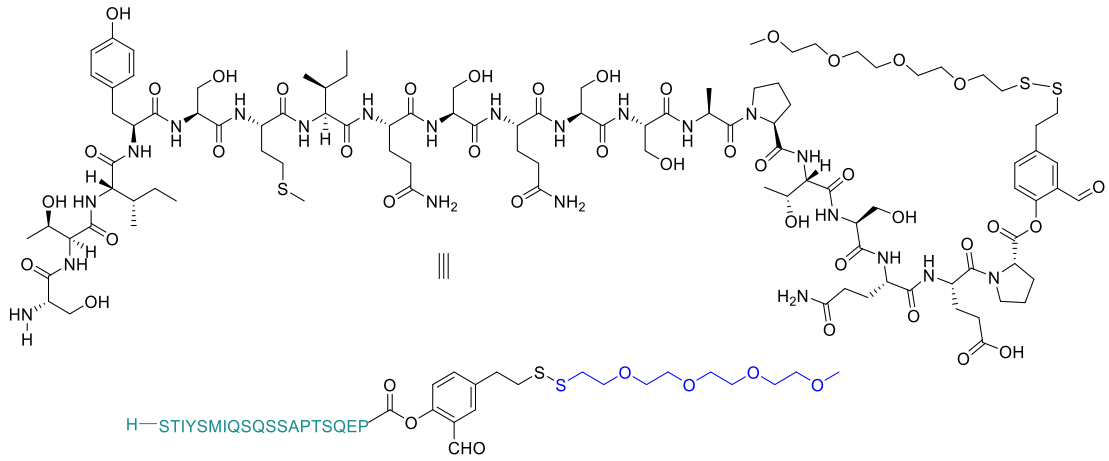


Figure S7. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **1f**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{135}H_{224}N_{36}O_{43}S_3$ $[M+2H]^{2+}$ m/z = 1568.82, found 1568.36, $[M+3H]^{3+}$ m/z = 1046.22, found 1046.14, $[M+4H]^{4+}$ m/z = 784.92, found 784.95, $[M+5H]^{5+}$ m/z = 628.13, found 628.15.

5.2 Synthesis of 1g



1g

0.02 mmol C-terminus peptide salicylaldehyde ester **1g** was synthesized according to **General procedure 2.6**. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 8.07 mg peptide salicylaldehyde ester **1g** was obtained in an isolated yield of 16.61%.

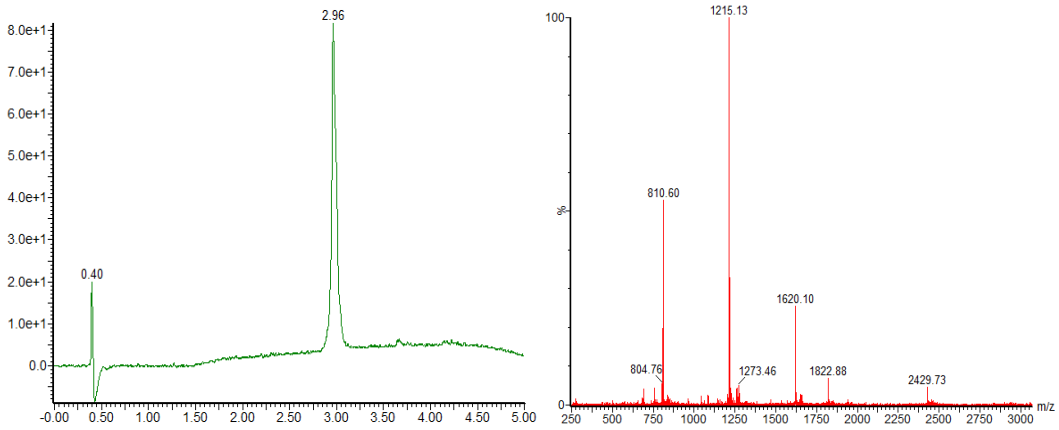
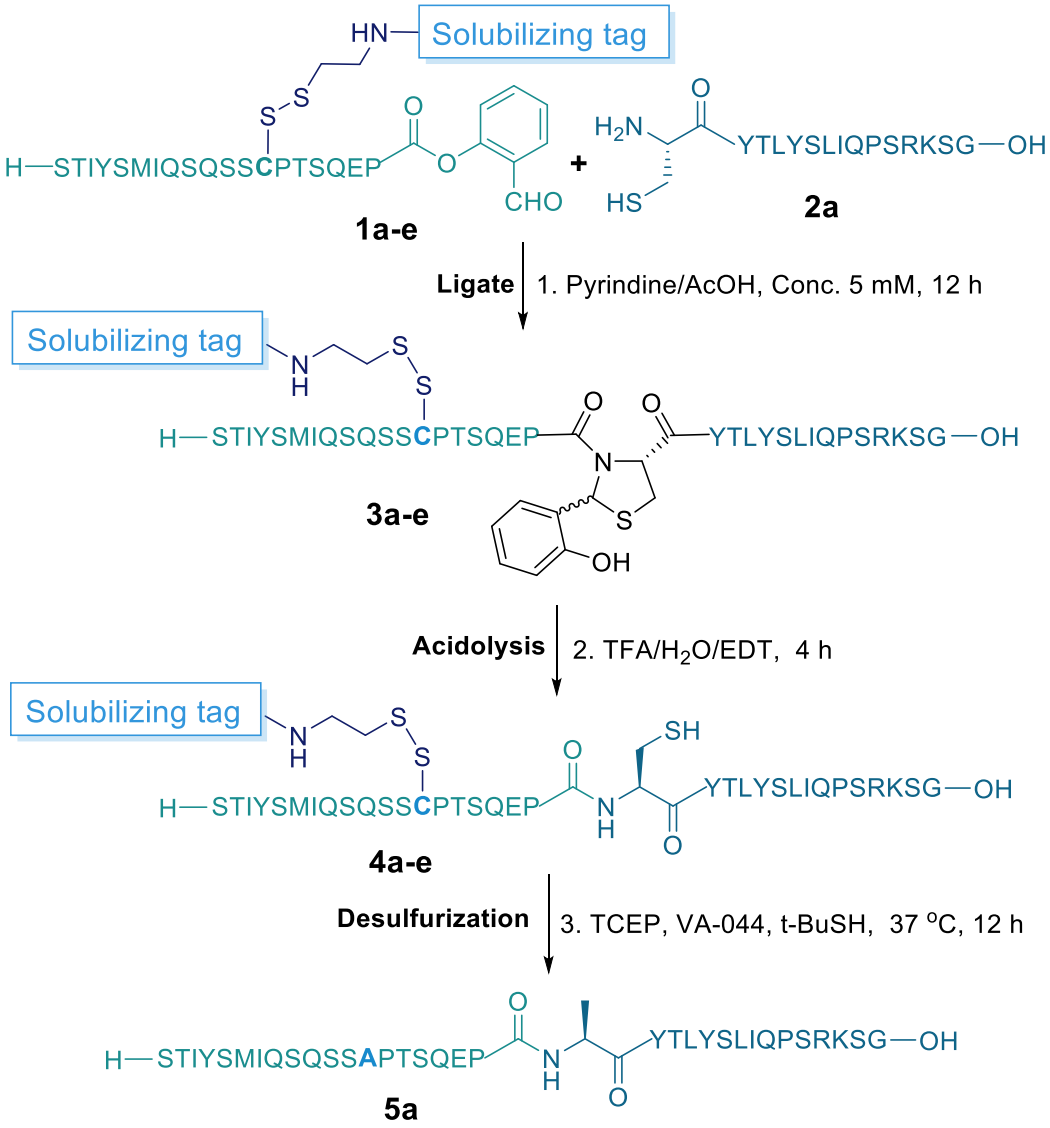


Figure S8. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **1g**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{103}H_{162}N_{39}O_{39}S_3$ $[M+H]^+$ m/z = 2429.72, found 2429.73, $[M+2H]^{2+}$ m/z = 1215.36, found 1215.13, $[M+3H]^{3+}$ m/z = 810.58, found 810.60.

6 CPL/CPL-desulfurization between 2a and 1a-e



6.1 CPL between 2a and 1a (no tag)

Peptide H-CYTLYSLIQPSRKSG-OH (**2a**) (0.4 mg, 0.23 μmol) and peptide salicylaldehyde ester **1a** (0.5 mg, 0.23 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The residue was treated with 100 μL TFA/H_2O/EDT (90/5/5, v/v/v) for 4 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged.

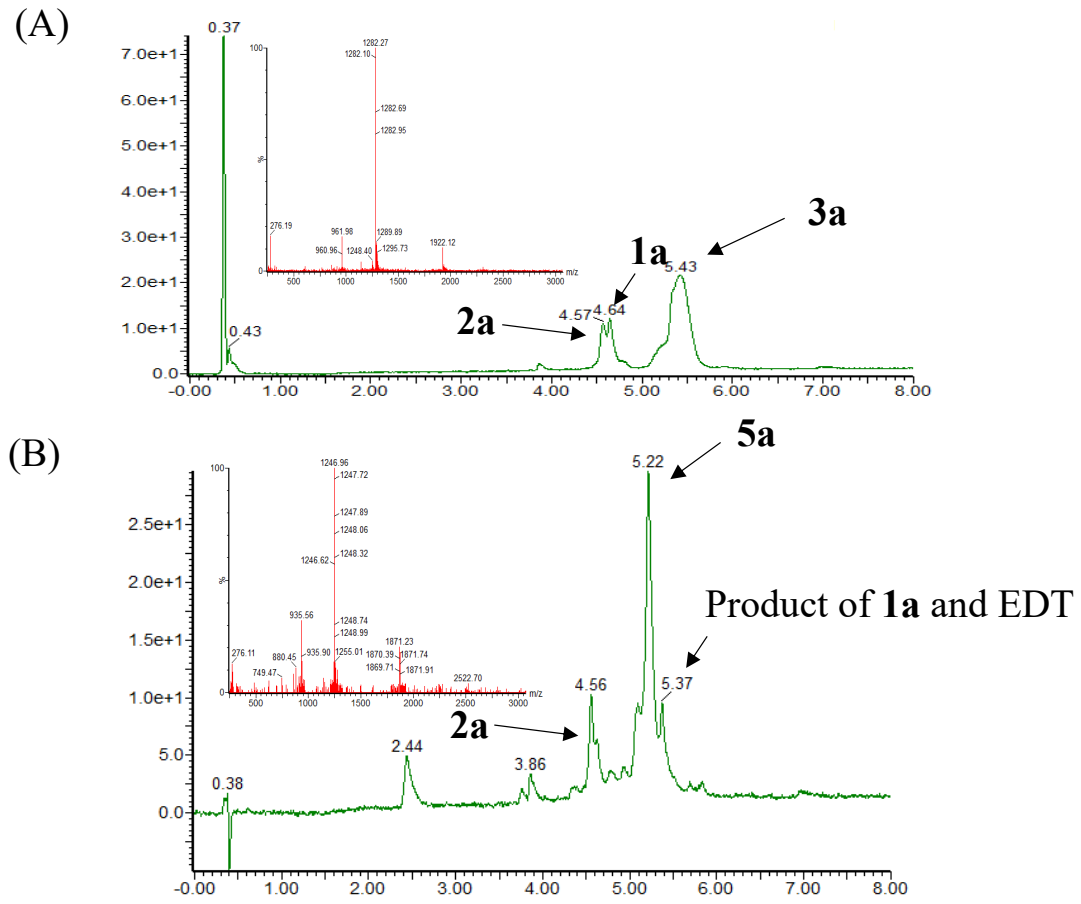


Figure S9. UV trace from analytical RP-UPLC of cysteine ligation reaction mixture between model N-terminus peptide and model C-terminus peptide salicylaldehyde ester with no tag. (A) Cysteine ligation at 12h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 4h. Gradient: 10-50 % ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

6.2 CPL-desulfurization between 2a and 1b (K_{10} tag)

Peptide H-CYTLYSLIQPSRKSG-OH (**2a**) (1.44 mg, 0.84 μmol) and peptide salicylaldehyde ester **1b** (3.00 mg, 0.84 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The residue was treated with 383 μL TFA/H_2O/EDT (90/5/5, v/v/v) for 4 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. The residue was dissolved in 0.9 mL PB buffer (pH ~ 7), 0.9 mL TCEP solution (0.5 M in PB buffer, pH ~ 7), 90 μL ^{t}BuSH and 0.9 mL VA-044 solution (0.1 M in PB buffer, pH ~ 7) were added. The

reaction mixture was stirred at 37°C for overnight. Finally, the product was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 1.01 mg peptide **5a** was obtained in an isolated yield of 32.48%.

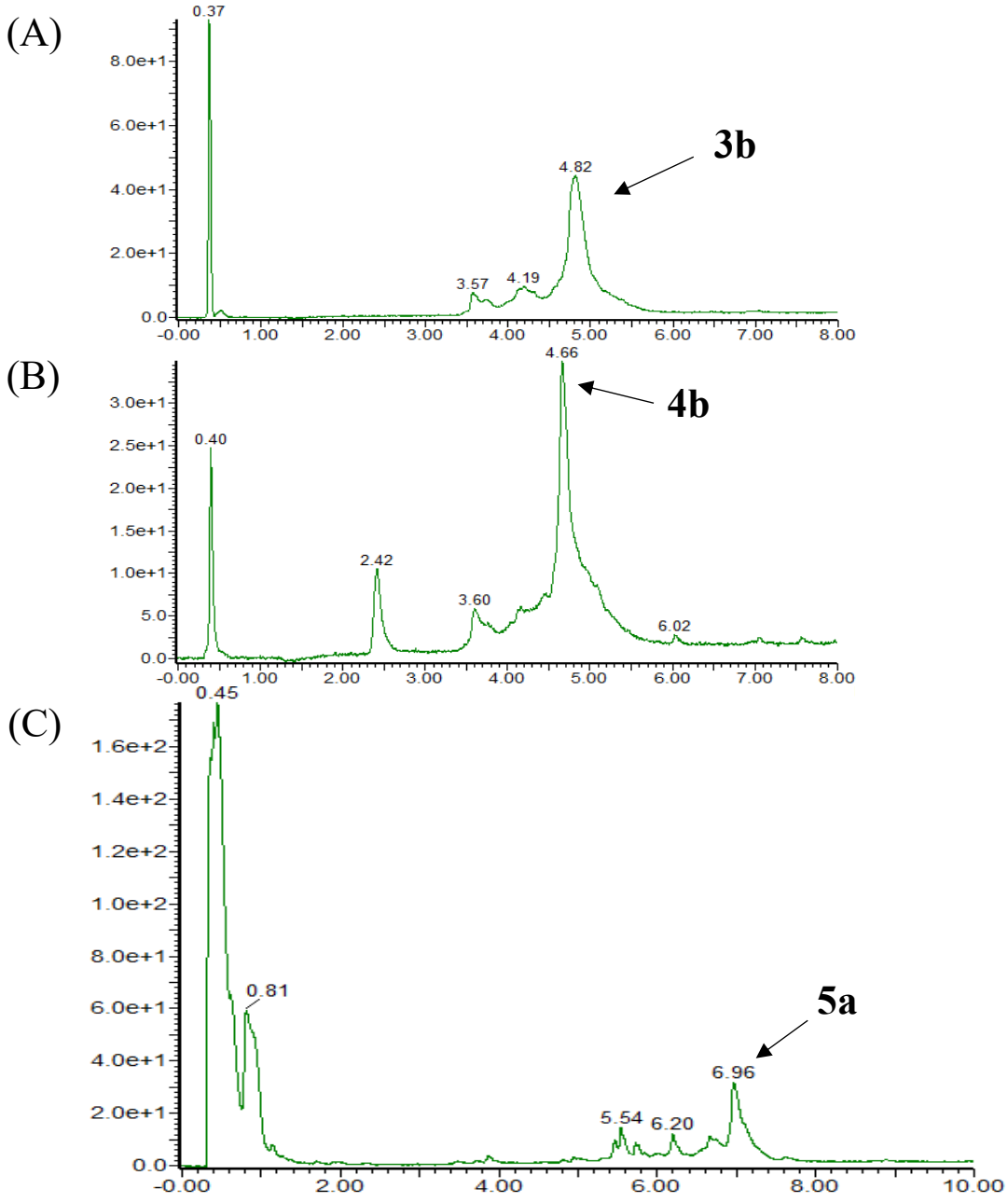


Figure S10. UV trace from analytical RP-UPLC of cysteine ligation reaction mixture between model N-terminus peptide and model C-terminus peptide salicylaldehyde ester with Lys_{10} tag at internal Cys. (A) Cysteine ligation at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 4 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (C) Desulfurization at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1%

TFA over 10 min at a flow rate of 0.4 mL/min.

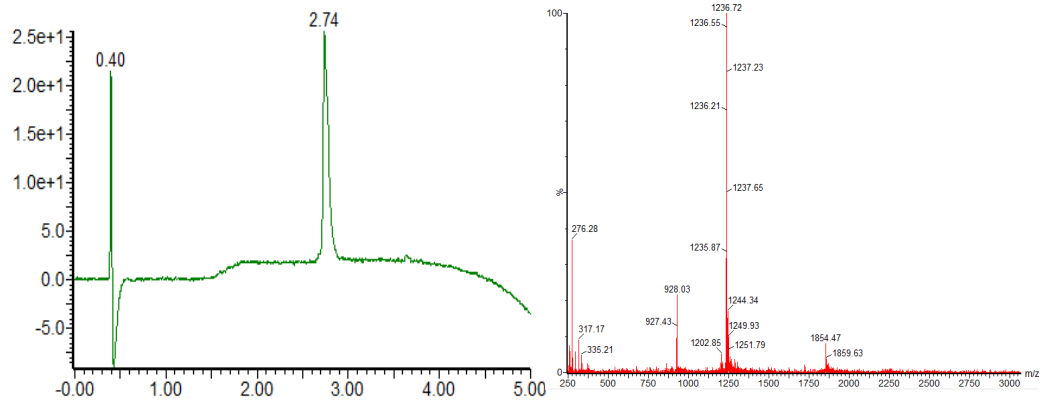


Figure S11. UV trace from analytical RP-UPLC and its ESI-MS of **5a**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{161}H_{256}N_{42}O_{56}S$ $[M+2H]^{2+}$ m/z = 1855.04, found 1854.47, $[M+3H]^{3+}$ m/z = 1237.03, found 1236.72, $[M+4H]^{4+}$ m/z = 928.03, found 928.03.

6.3 CPL-desulfurization between 2a and 1c (R_4 tag)

Peptide H-CYTLYSLIQPSRKSG-OH (**2a**) (2.05 mg, 1.2 μmol) and peptide salicylaldehyde ester **1c** (3.50 mg, 1.2 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The residue was treated with 500 μL TFA/H_2O/EDT (90/5/5, v/v/v) for 4 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. The residue was dissolved in 1.3 mL PB buffer (pH ~ 7), 1.3 mL TCEP solution (0.5 M in PB buffer, pH ~ 7), 130 μL ^{t}BuSH and 1.3 mL VA-044 solution (0.1 M in PB buffer, pH ~ 7) were added. The reaction mixture was stirred at 37°C for overnight. Finally, the product was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 1.12 mg peptide **5a** was obtained in an isolated yield of 25.23%.

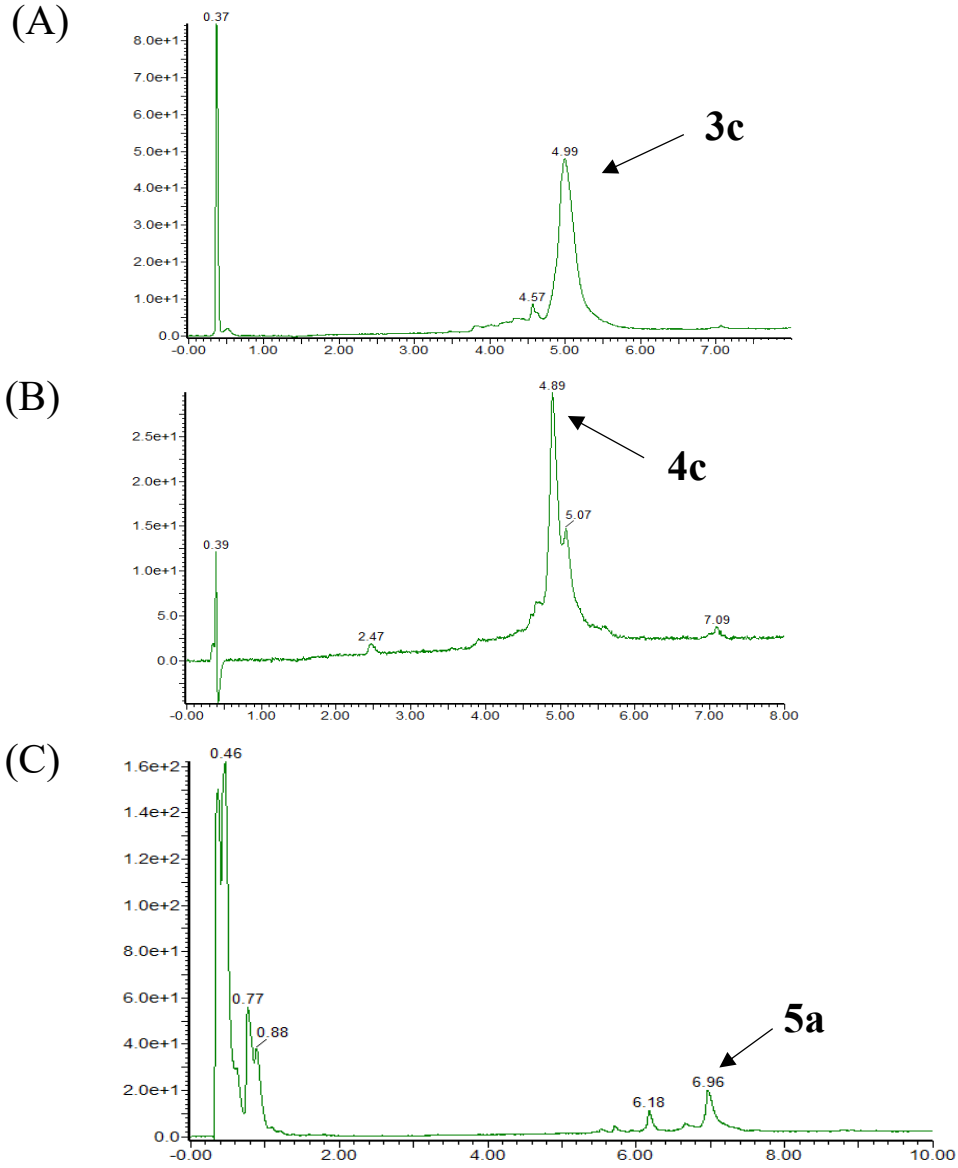


Figure S12. UV trace from analytical RP-UPLC of cysteine ligation reaction mixture between model N-terminus peptide and model C-terminus peptide salicylaldehyde ester with Arg_4 tag at internal Cys. (A) Cysteine ligation at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 4 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (C) Desulfurization at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

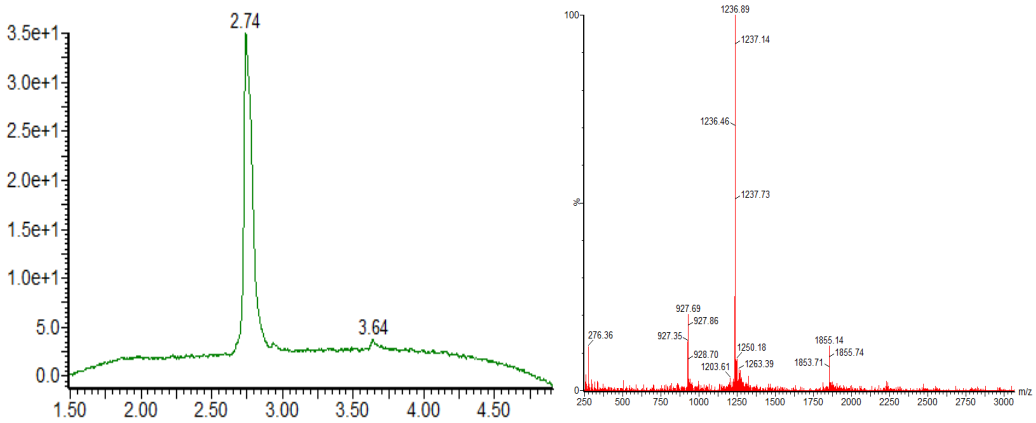


Figure S13. UV trace from analytical RP-UPLC and its ESI-MS of **5a**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{161}H_{256}N_{42}O_{56}S$ $[M+2H]^{2+}$ m/z = 1855.04, found 1855.14, $[M+3H]^{3+}$ m/z = 1237.03, found 1236.89, $[M+4H]^{4+}$ m/z = 928.03, found 927.69.

6.4 CPL-desulfurization between 2a and 1d (PEG_3 tag)

Peptide H-CYTLYSLIQPSRKSG-OH (**2a**) (2.08 mg, 1.2 μmol) and peptide salicylaldehyde ester **1d** (3.00 mg, 1.2 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The residue was treated with 600 μL TFA/H_2O/EDT (90/5/5, v/v/v) for 4 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. The residue was dissolved in 1.3 mL PB buffer (pH~7), 1.3 mL TCEP solution (0.5 M in PB buffer, pH~7), 130 μL ^{t}BuSH and 1.3 mL VA-044 solution (0.1 M in PB buffer, pH~7) were added. The reaction mixture was stirred at 37 °C for overnight. Finally, the product was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 2.22 mg peptide **5a** was obtained in an isolated yield of 48.33%.

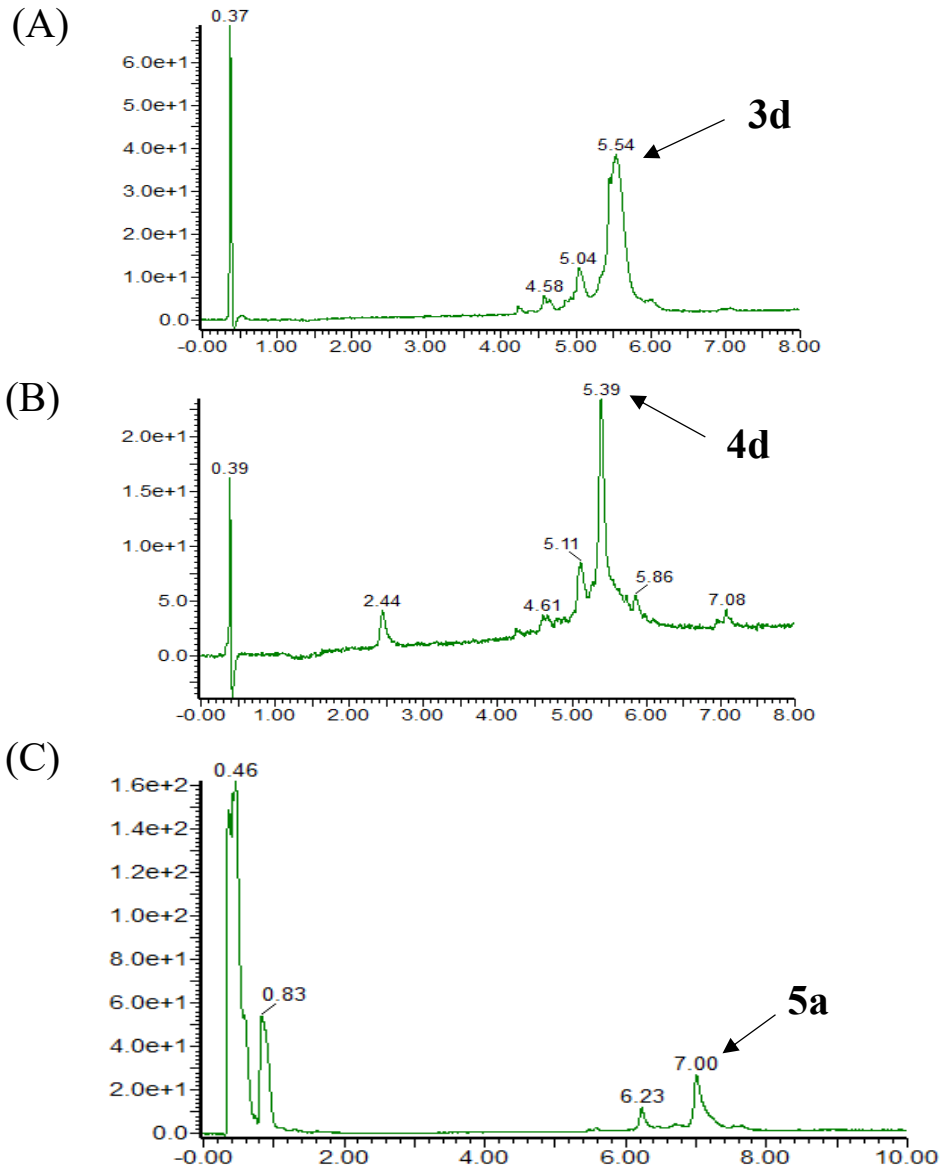


Figure S14. UV trace from analytical RP-UPLC of cysteine ligation reaction mixture between model N-terminus peptide and model C-terminus peptide salicylaldehyde ester with PEG_4 tag at internal Cys. (A) Cysteine ligation at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 4 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (C) Desulfurization at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

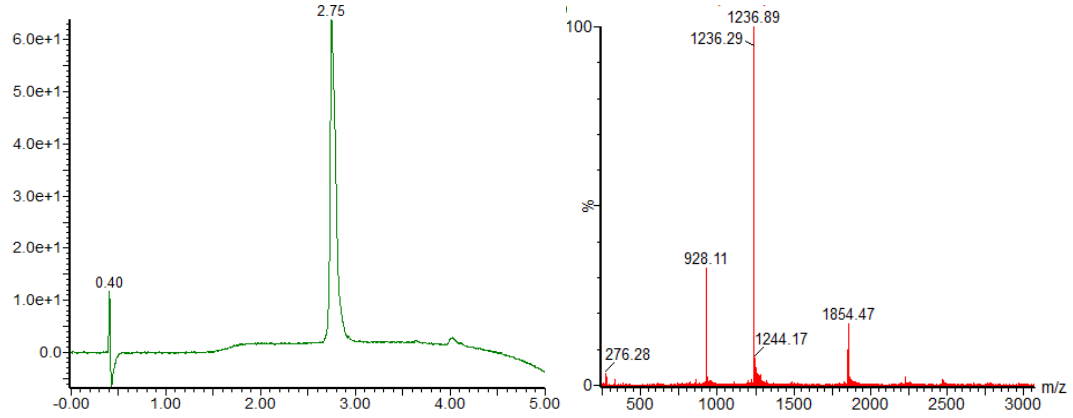


Figure S15. UV trace from analytical RP-UPLC and its ESI-MS of **5a**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{161}H_{256}N_{42}O_{56}S$ $[M+2H]^{2+}$ m/z = 1855.04, found 1854.47, $[M+3H]^{3+}$ m/z = 1237.03, found 1236.89, $[M+4H]^{4+}$ m/z = 928.03, found 928.11.

6.5 CPL-desulfurization between 2a and 1e (PEG_7 tag)

Peptide H-CYTLYSLIQPSRKSG-OH (**2a**) (2.04 mg, 1.2 μmol) and peptide salicylaldehyde ester **1e** (3.16 mg, 1.2 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The residue was treated with 550 μL TFA/H_2O/EDT (90/5/5, v/v/v) for 4 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. The residue was dissolved in 1.3 mL PB buffer (pH~7), 1.3 mL TCEP solution (0.5 M in PB buffer, pH~7), 130 μL ^{t}BuSH and 1.3 mL VA-044 solution (0.1 M in PB buffer, pH~7) were added. The reaction mixture was stirred at 37 °C for overnight. Finally, the product was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 1.48 mg peptide **5a** was obtained in an isolated yield of 33.45%.

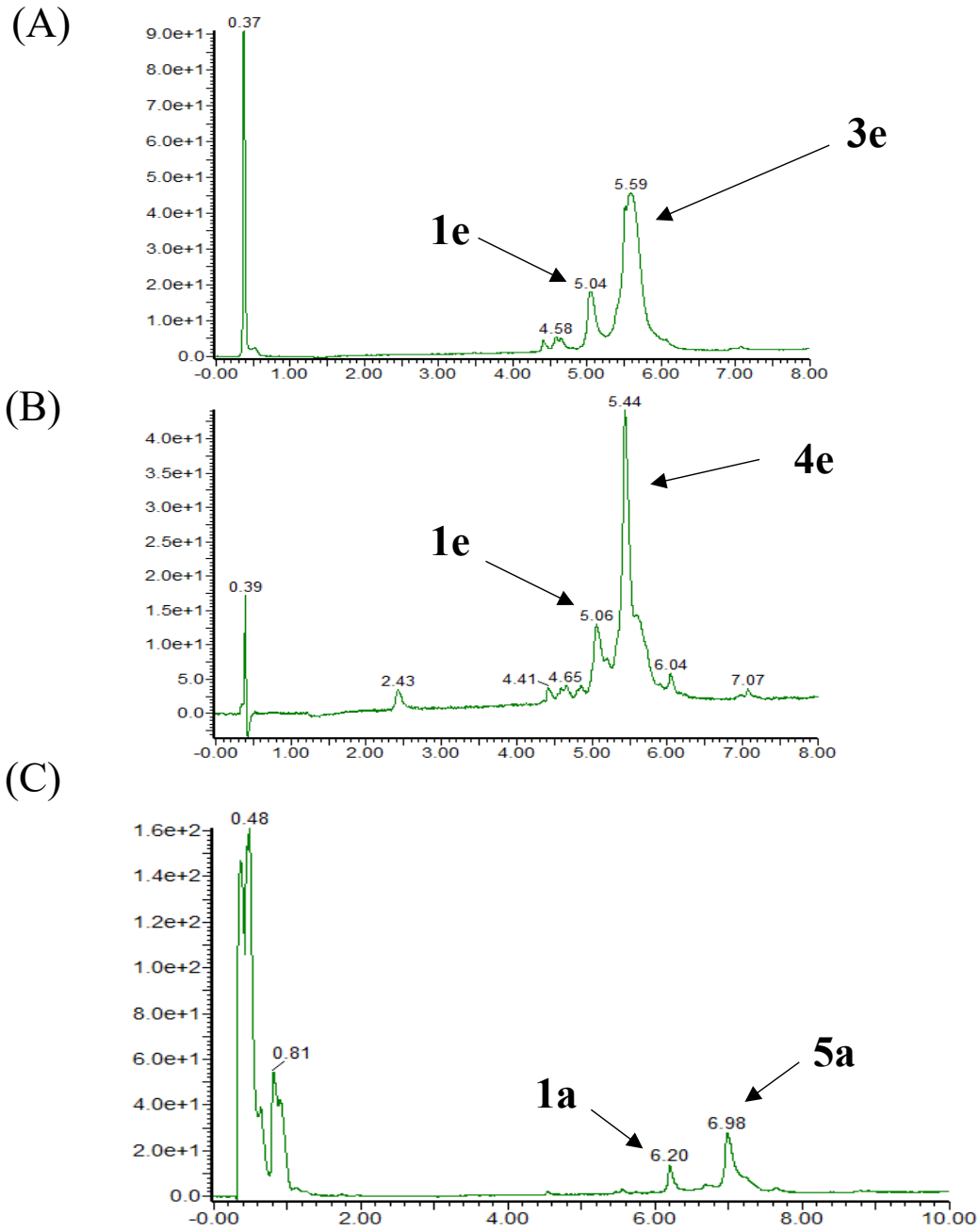


Figure S16. UV trace from analytical RP-UPLC of cysteine ligation reaction mixture between model N-terminus peptide and model C-terminus peptide salicylaldehyde ester with PEG_8 tag at cysteine site. (A) Cysteine ligation at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 4 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (C) Desulfurization at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

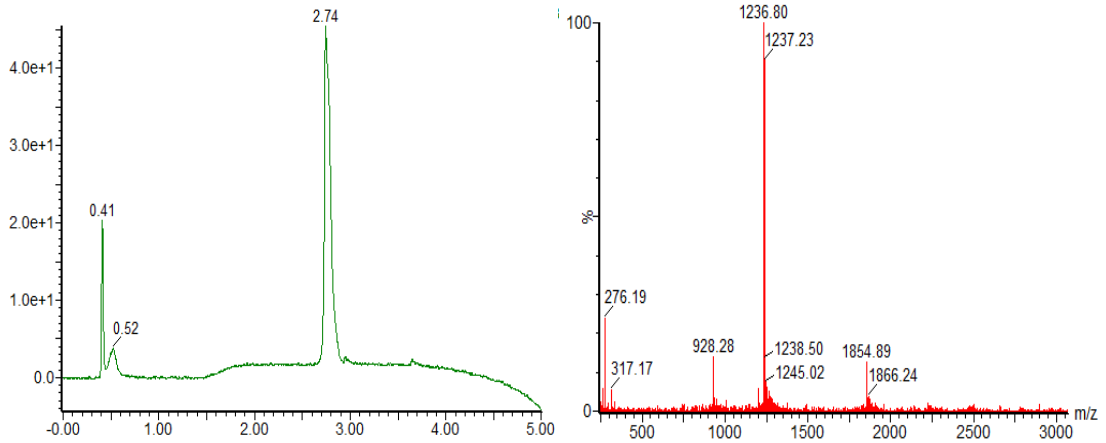
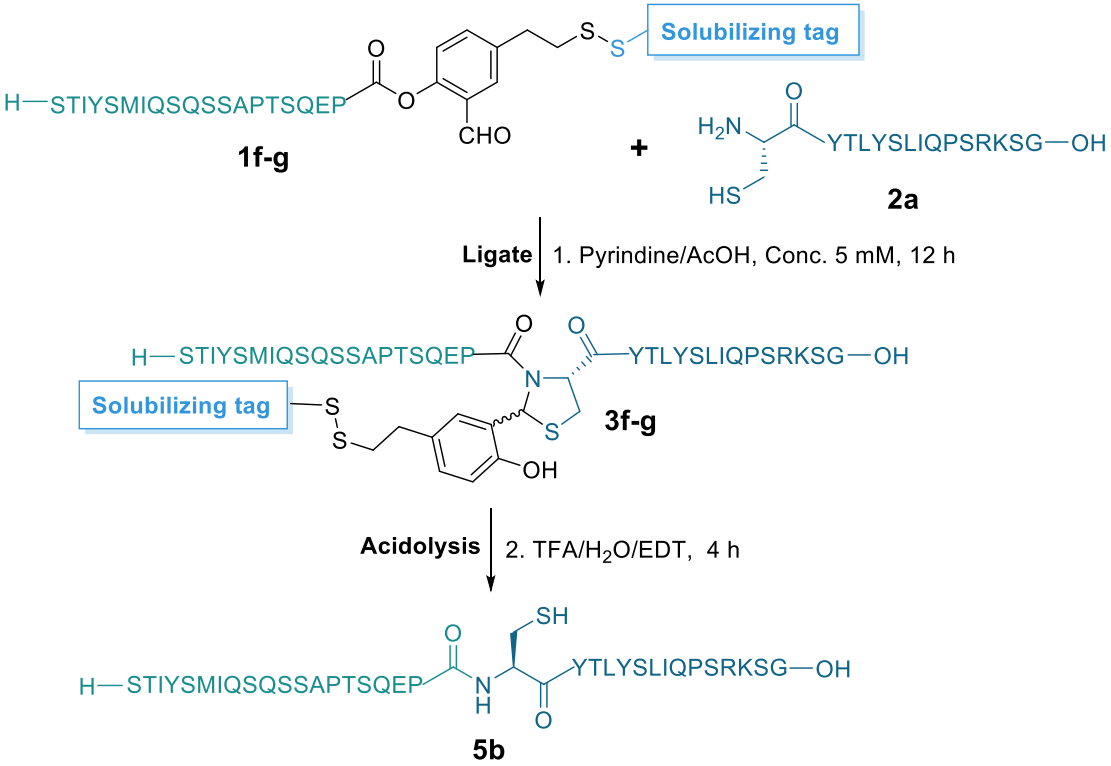


Figure S17. UV trace from analytical RP-UPLC and its ESI-MS of **5a**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{161}H_{256}N_{42}O_{56}S$ $[M+2H]^{2+}$ m/z = 1855.04, found 1854.89, $[M+3H]^{3+}$ m/z = 1237.03, found 1236.80, $[M+4H]^{4+}$ m/z = 928.03, found 928.28.

7 CPL between 2a and 1f-g



7.1 CPL between 2a and 1f (K_6 tag)

Peptide H-CYTLYSLIQPSRKSG-OH (**2a**) (1.64 mg, 0.96 μmol) and peptide salicylaldehyde ester **1f** (3.00 mg, 0.96 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture

was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The residue was treated with 900 μL TFA/H_2O/EDT (90/5/5, v/v/v) for 4 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. Finally, the product was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 1.14 mg peptide **5b** was obtained in an isolated yield of 31.90%.

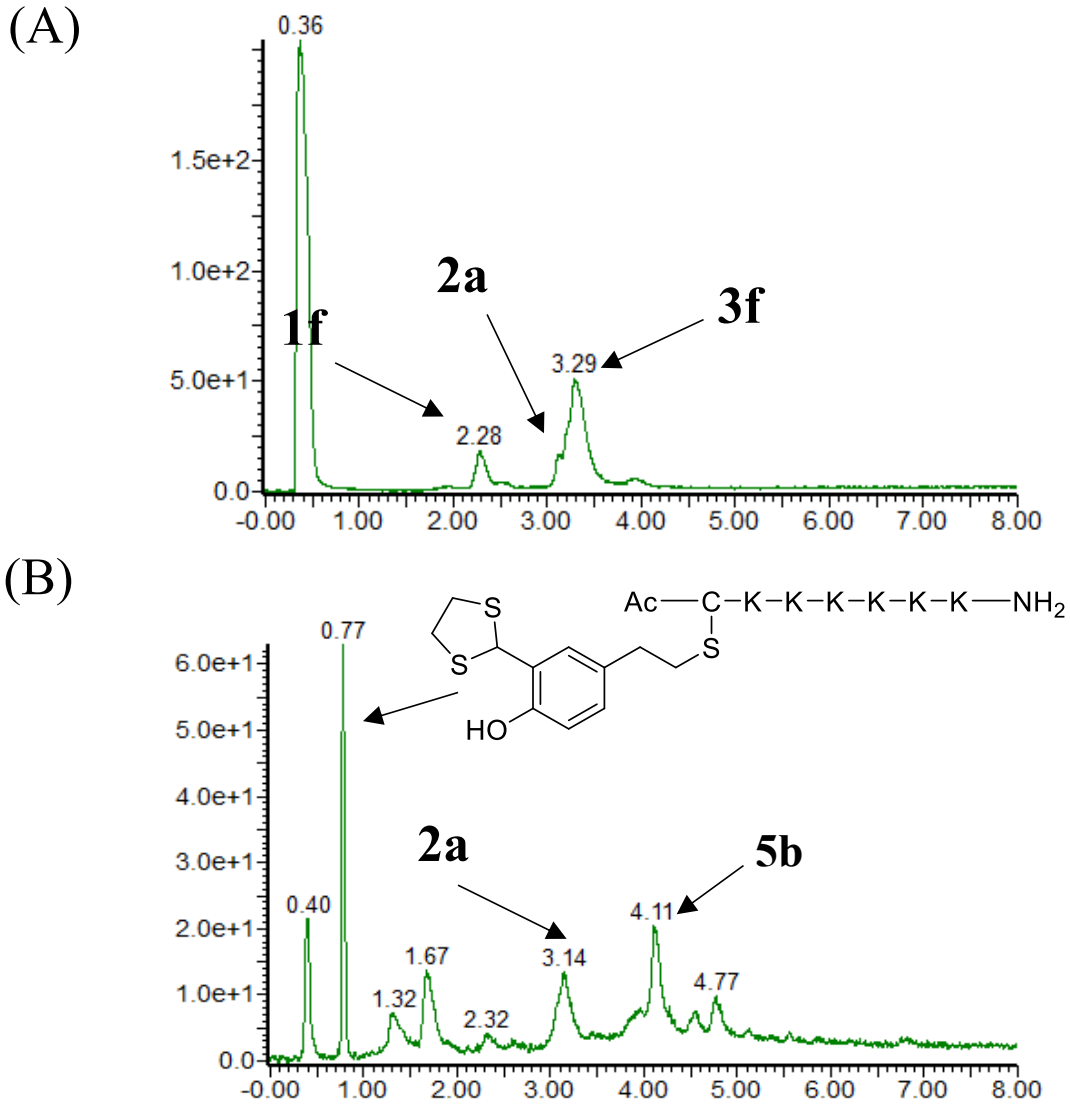


Figure S18. UV trace from analytical RP-UPLC of cysteine ligation reaction mixture between model N-terminus peptide and model C-terminus peptide salicylaldehyde ester with Lys_6 tag via salicylaldehyde ester. (A) Cysteine ligation at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 4 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

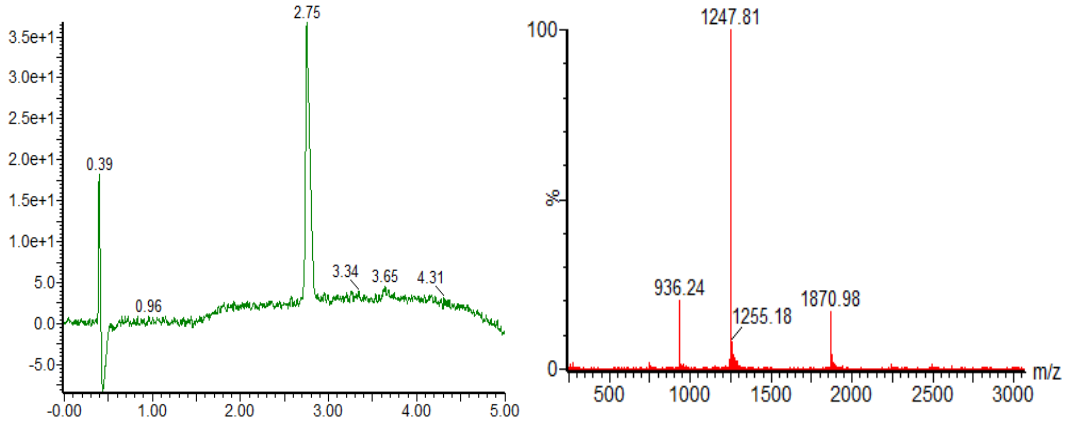


Figure S19. UV trace from analytical RP-UPLC and its ESI-MS of **5b**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{161}H_{256}N_{42}O_{56}S_2$ $[M+2H]^{2+}$ m/z = 1871.09, found 1870.98, $[M+3H]^{3+}$ m/z = 1247.73, found 1247.81, $[M+4H]^{4+}$ m/z = 936.05, found 936.24.

7.2 CPL between 2a and 1g (PEG_4 tag)

Peptide H-CYTLYSLIQPSRKSG-OH (**2a**) (2.12 mg, 1.2 μmol) and peptide salicylaldehyde ester **1g** (3.00 mg, 1.2 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The residue was treated with 1 mL TFA/H_2O/EDT (90/5/5, v/v/v) for 4 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. Finally, the product was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 1.57 mg peptide **5b** was obtained in an isolated yield of 35.00%.

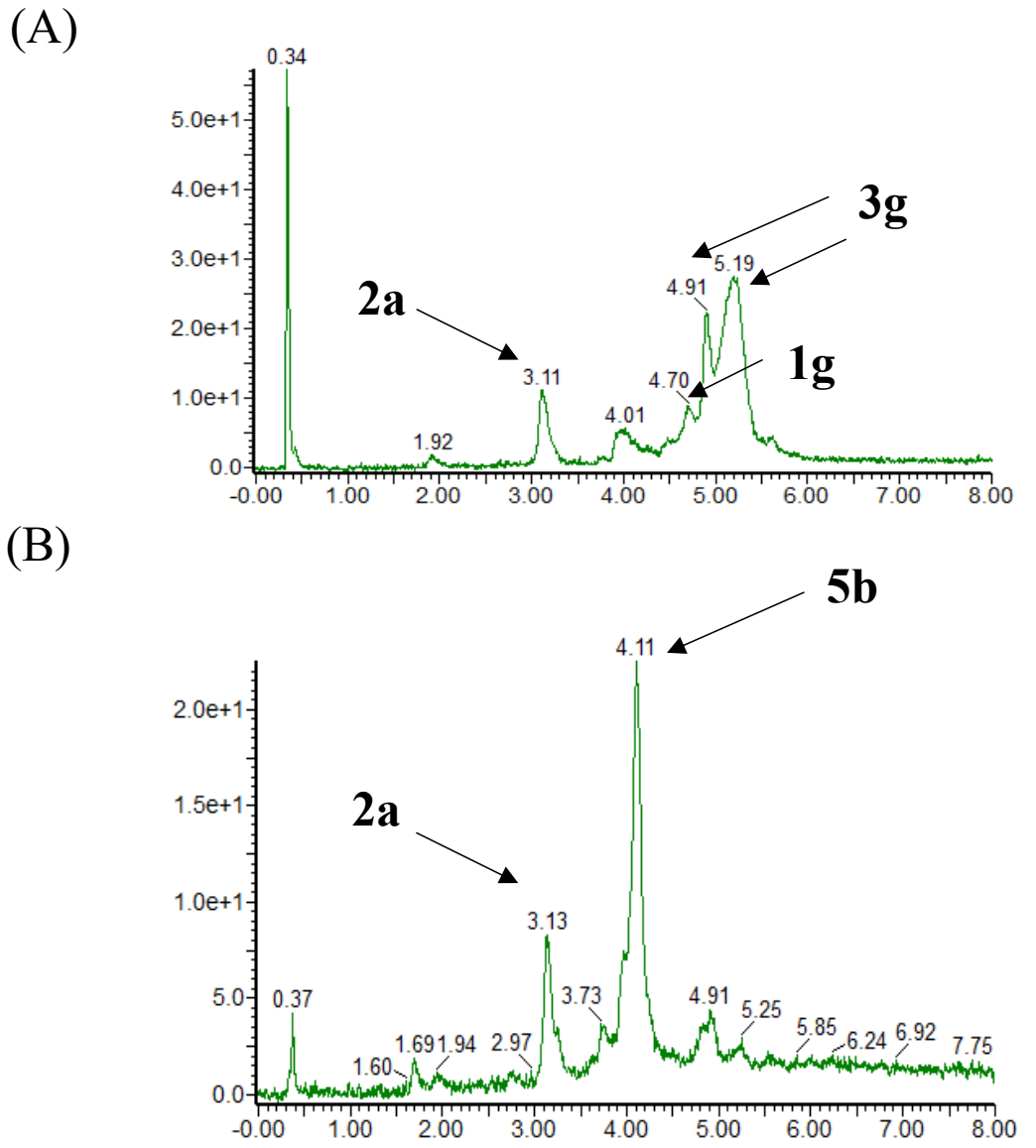


Figure S20. UV trace from analytical RP-UPLC of cysteine ligation reaction mixture between model N-terminus peptide and model C-terminus peptide salicylaldehyde ester with PEG_4 tag via salicylaldehyde ester. (A) Cysteine ligation at 12 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 4 h. Gradient: 10-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

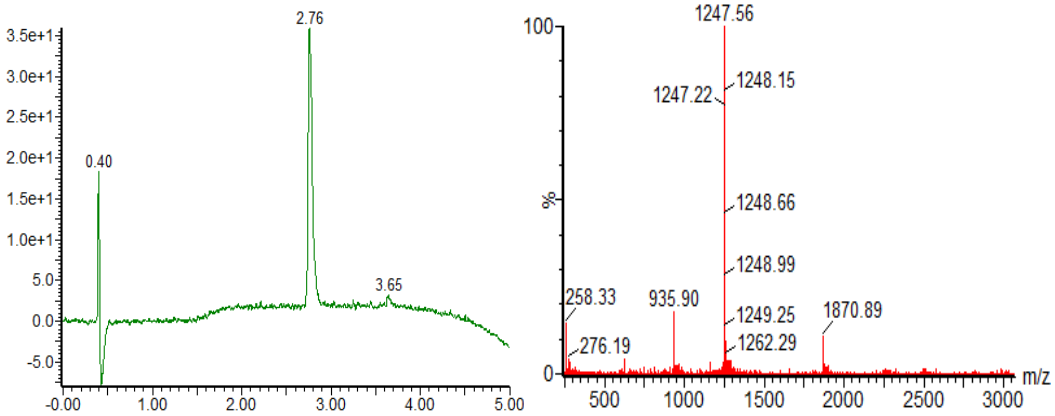
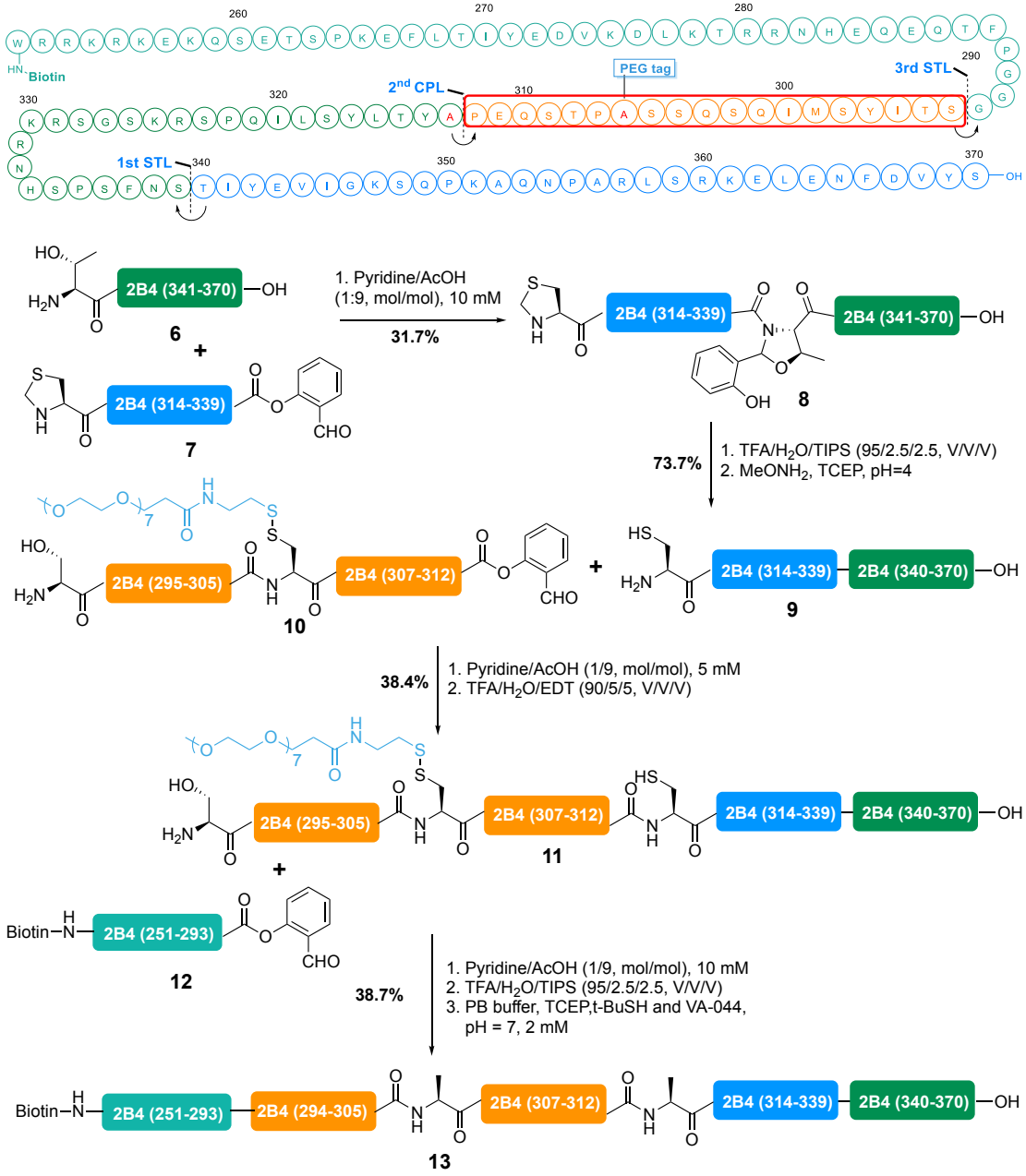
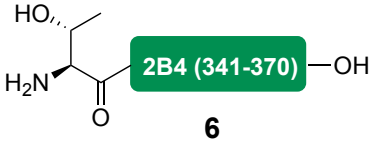


Figure S21. UV trace from analytical RP-UPLC and its ESI-MS of **5b**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{161}H_{256}N_{42}O_{56}S_2$ $[M+2H]^{2+}$ m/z = 1871.09, found 1870.89, $[M+3H]^{3+}$ m/z = 1247.73, found 1247.56, $[M+4H]^{4+}$ m/z = 936.05, found 936.90.

8 Synthesis of 2B4 tail (251-370)



8.1 Synthesis of 6



0.1 mol peptide **6** was assembled by Fmoc-SPPS general procedure. After Fmoc-SPPS, 10 mL TFA/H_2O/TIPS (95/2.5/2.5, v/v/v) was added to cleavage the peptide from resin and globally deprotect for 2 h at room temperature. The crude peptide was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 40% ACN/H_2O over 30 min. After lyophilization, 180.72 mg **6** was obtained in an isolated yield of 50.43%.

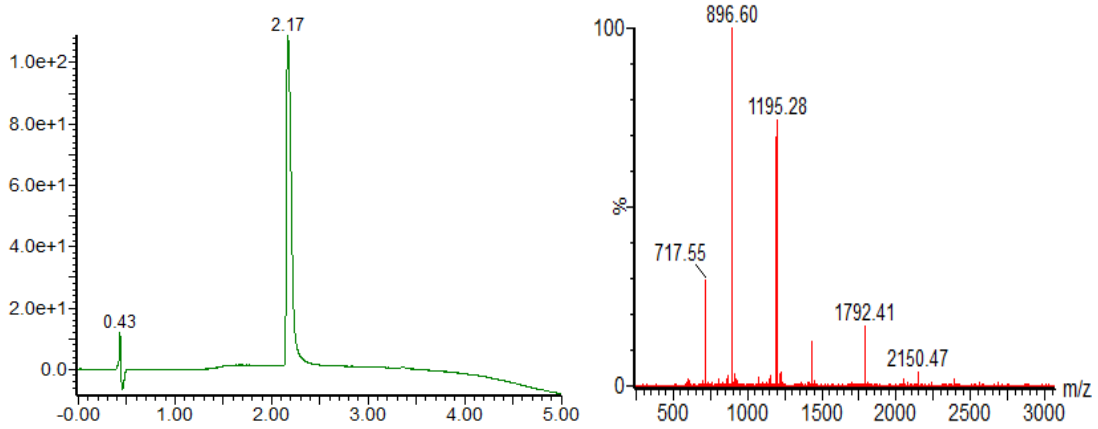


Figure S22. UV trace from analytical RP-UPLC and its ESI-MS of **6**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{159}H_{254}N_{44}O_{50}$ $[M+2H]^{2+}$ m/z = 1792.00, found 1792.41, $[M+3H]^{3+}$ m/z = 1195.00, found 1195.28, $[M+4H]^{4+}$ m/z = 896.50, found 896.60, $[M+5H]^{5+}$ m/z = 717.40, found 717.55.

8.2 Synthesis of 7

7

0.04 mmol C-terminus peptide salicylaldehyde ester **7** was synthesized by salicylaldehyde ester preparation general procedure. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 64.45 mg peptide salicylaldehyde ester **7** was obtained in an isolated yield of 49.88%.

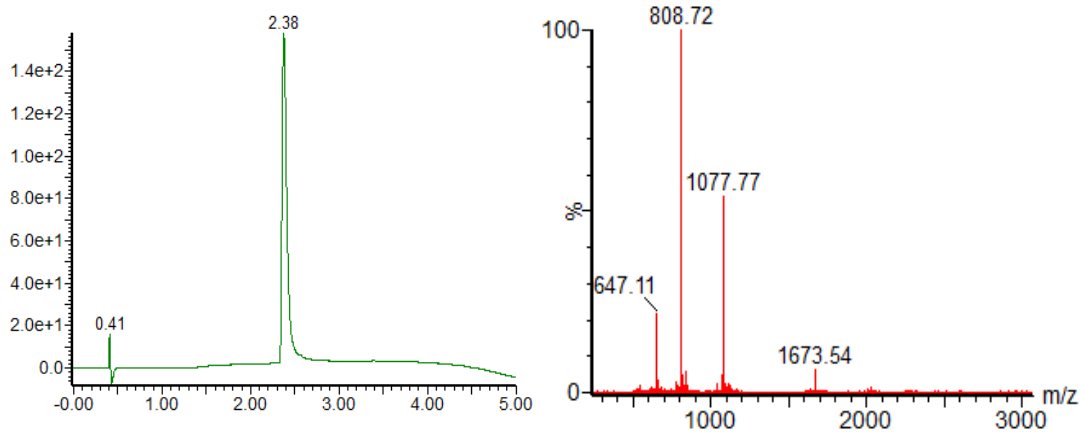
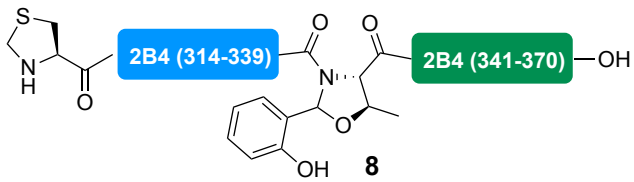


Figure S23. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **7**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{142}H_{217}N_{43}O_{42}S$ $[M+3H]^{3+}$ m/z = 1077.87, found 1077.77, $[M+4H]^{4+}$ m/z = 808.65, found 808.72, $[M+5H]^{5+}$ m/z = 647.12, found 647.11.

8.3 Synthesis of 8



6 (33.26 mg, 9.3 μmol) and **7** (20 mg, 6.2 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The product was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 13.33 mg **8** was obtained in an isolated yield of 31.65%.

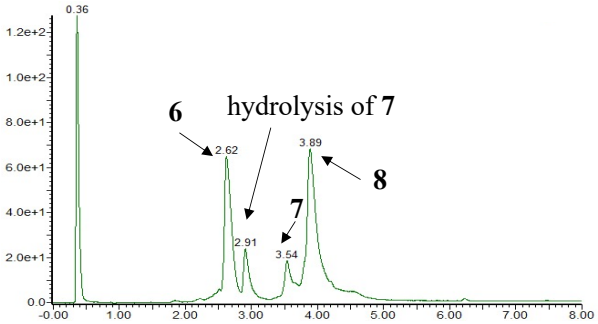


Figure S24. UV trace from analytical RP-UPLC of Ser/Thr ligation reaction mixture between **6** and **7** at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

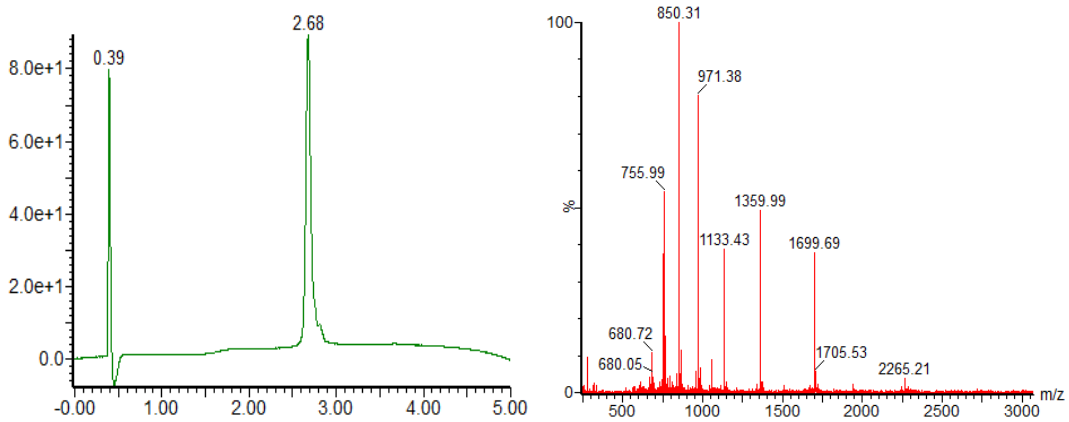
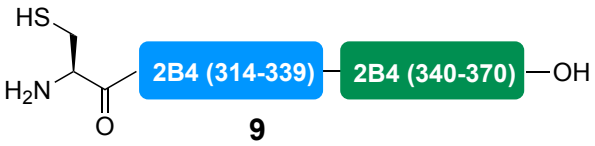


Figure S25. UV trace from analytical RP-UPLC and its ESI-MS of **8**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{301}H_{469}N_{87}O_{91}S$ $[M+3H]^{3+}$ m/z = 2265.88, found 2265.21, $[M+4H]^{4+}$ m/z = 1699.66, found 1699.69, $[M+5H]^{5+}$ m/z = 1359.93, found 1359.99, $[M+6H]^{6+}$ m/z = 1133.44, found 1133.43, $[M+7H]^{7+}$ m/z = 971.66, found 971.38, $[M+8H]^{8+}$ m/z = 850.33, found 850.31, $[M+9H]^{9+}$ m/z = 755.96, found 755.99, $[M+10H]^{10+}$ m/z = 680.46, found 680.72.

8.4 Synthesis of 9



9

8 (13.33 mg, 1.96 μmol) was treated with 1 mL TFA/H_2O/TIPS (95/2.5/2.5, v/v/v) for 30 min. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. The residue was dissolved in 470 μL thiazolidine-opening (Thz-opening) buffer (0.2 M PB buffer, pH~4, containing 6 M Gn·HCl, 0.05 M TCEP·HCl and 0.3 M $MeONH_2$·HCl) and reacted for 12 h. Finally, the product was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 9.65 mg peptide salicylaldehyde ester **9** was obtained in an isolated yield of 73.68%.

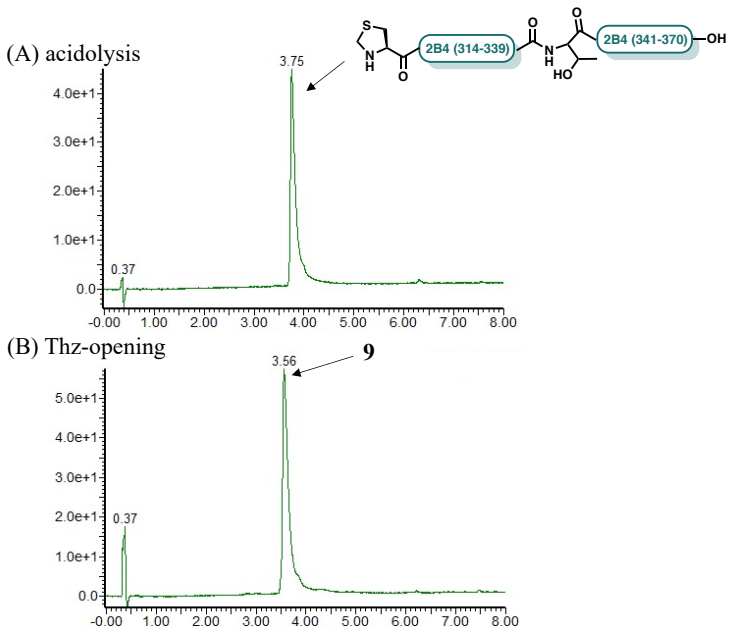


Figure S26. UV trace from analytical RP-UPLC of acidolysis and Thz-opening of **8**. (A) Acidolysis at 30 min. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Thz-opening at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

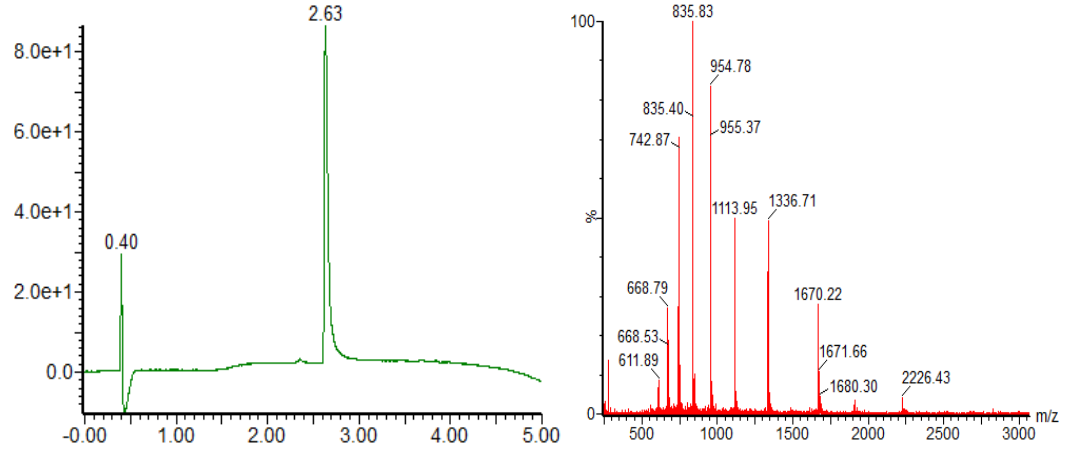
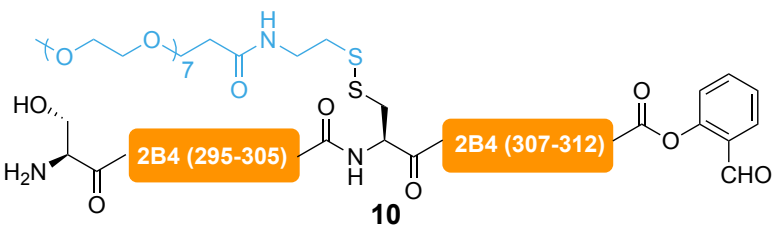


Figure S27. UV trace from analytical RP-UPLC and its ESI-MS of **9**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{293}H_{465}N_{87}O_{90}S$ $[M+3H]^{3+}$ m/z = 2227.17, found 2226.43, $[M+4H]^{4+}$ m/z = 1670.63, found 1670.22, $[M+5H]^{5+}$ m/z = 1336.70, found 1336.71, $[M+6H]^{6+}$ m/z = 1114.09, found 1113.95, $[M+7H]^{7+}$ m/z = 955.07, found 954.78, $[M+8H]^{8+}$ m/z = 835.82, found 835.83, $[M+9H]^{9+}$ m/z = 743.06, found 742.87, $[M+10H]^{10+}$ m/z = 668.85, found 668.79.

8.5 Synthesis of 10



0.02 mmol C-terminus peptide salicylaldehyde ester **10** was synthesized by attachment of reducible solubilizing tags at internal Cys and salicylaldehyde ester preparation general procedure. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 21.98 mg peptide salicylaldehyde ester **10** was obtained in an isolated yield of 20.76%.

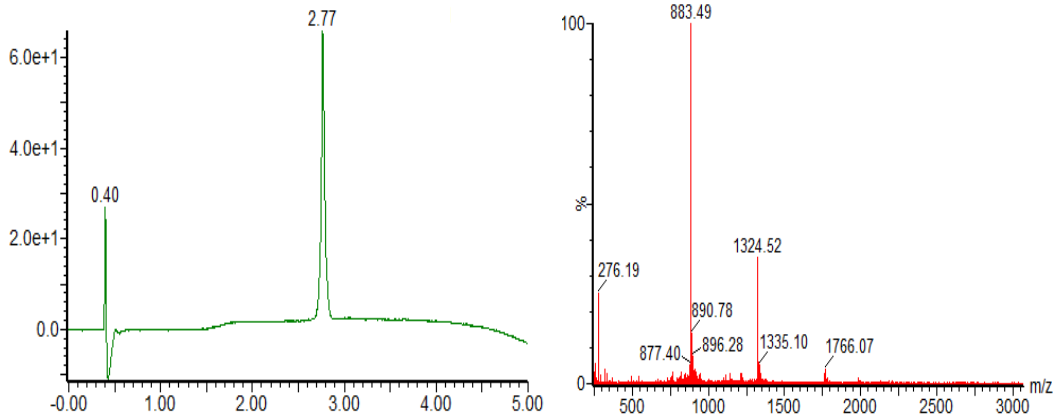
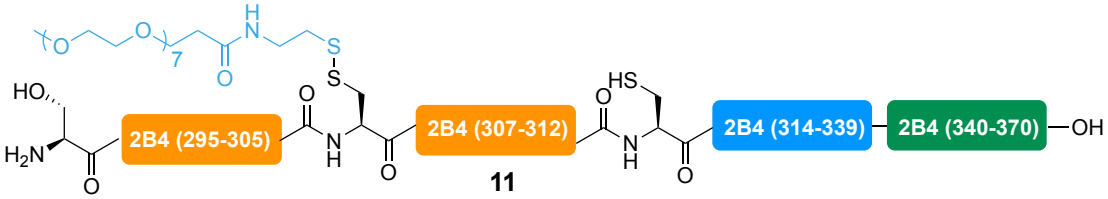


Figure S28. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **10**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{112}H_{179}N_{23}O_{44}S_3$ $[M+2H]^{2+}$ m/z = 1324.98, found 1324.52, $[M+3H]^{3+}$ m/z = 883.66, found 883.49.

8.6 Synthesis of 11



9 (9.00 mg, 1.3 μmol) and **10** (3.57 mg, 1.3 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The residue was treated with 1.3 mL TFA/H_2O/EDT (90/5/5, v/v/v) for 4 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. Finally, product was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 4.59 mg **11** was obtained in an isolated yield of 38.36%.

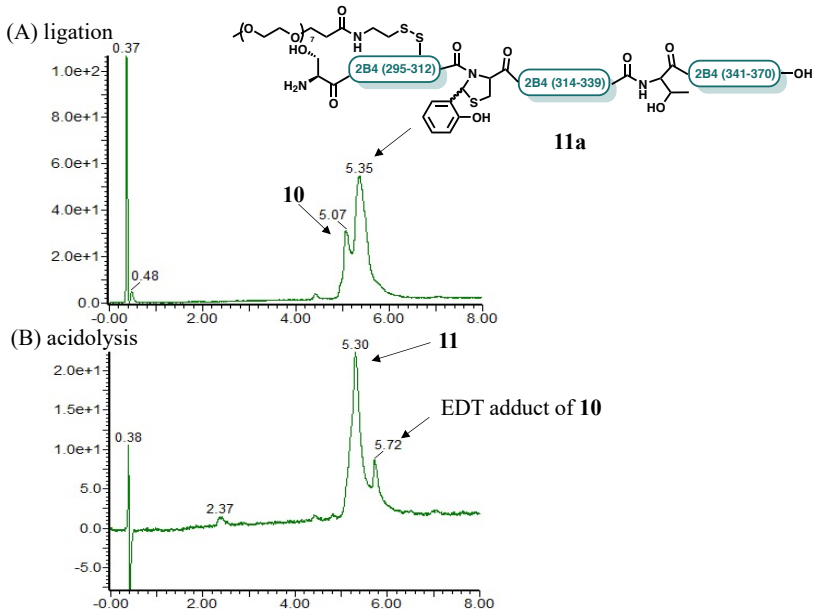


Figure S29. UV trace from analytical RP-UPLC of cysteine ligation reaction mixture between **9** and **10**. (A) Cysteine ligation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 4 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

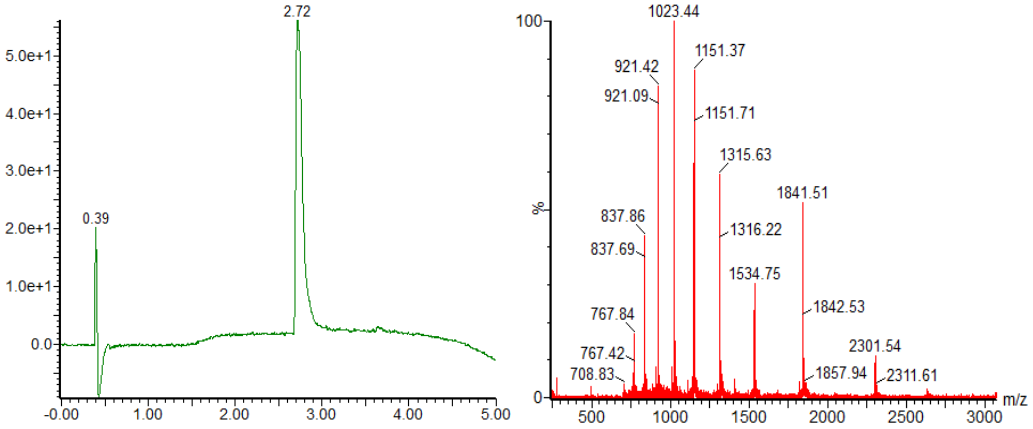
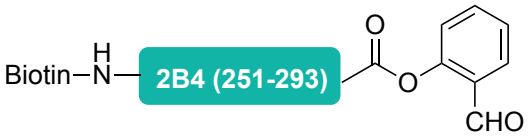


Figure S30. UV trace from analytical RP-UPLC and its ESI-MS of **11**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{398}H_{638}N_{110}O_{132}S_4$ $[M+4H]^{4+}$ m/z = 2302.09, found 2301.54, $[M+5H]^{5+}$ m/z = 1841.87, found 1841.51, $[M+6H]^{6+}$ m/z = 1535.06, found 1534.75, $[M+7H]^{7+}$ m/z = 1315.91, found 1315.63, $[M+8H]^{8+}$ m/z = 1151.55, found 1151.37, $[M+9H]^{9+}$ m/z = 1023.71, found 1023.44, $[M+10H]^{10+}$ m/z = 921.44, found 921.42, $[M+11H]^{11+}$ m/z = 837.76, found 837.86, $[M+12H]^{12+}$ m/z = 768.03, found 767.84, $[M+13H]^{13+}$ m/z = 709.03, found 708.83.

8.7 Synthesis of 12



12

0.02 mmol C-terminus peptide salicylaldehyde ester **12** was synthesized by salicylaldehyde ester preparation general procedure. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 24.55 mg peptide salicylaldehyde ester **12** was obtained in an isolated yield of 22.06%.

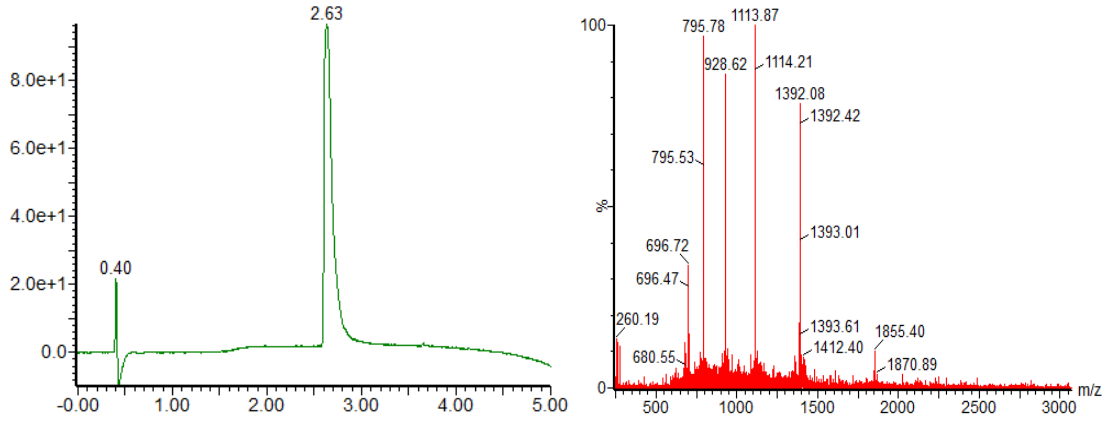


Figure S31. UV trace from analytical RP-UPLC and its ESI-MS of **12**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{245}H_{381}N_{73}O_{74}S$ $[M+3H]^{3+}$ m/z = 1856.06, found 1855.40, $[M+4H]^{4+}$ m/z = 1392.30, found 1392.08, $[M+5H]^{5+}$ m/z = 1114.04, found 1113.87, $[M+6H]^{6+}$ m/z = 928.53, found 928.62, $[M+7H]^{7+}$ m/z = 796.03, found 795.78, $[M+8H]^{8+}$ m/z = 696.65, found 696.72.

8.8 Synthesis of 13

13

11 (3 mg, 0.33 μmol) and **12** (3.6 mg, 0.65 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The crude solid was treated with 309 μL TFA/H_2O/TIPS (95/2.5/2.5, v/v/v) for 30 min. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. The residue was dissolved in 323 μL PB buffer (pH~7), 323 μL TCEP solution (0.5 M in PB buffer,

pH~7), 32.3 μL ^{t}BuSH and 323 μL VA-044 solution (0.1 M in PB buffer, pH~7) were added. The reaction mixture was stirred at 37°C for overnight. Finally, product was purified by preparative reverse-phase HPLC using gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 1.80 mg **13** was obtained in an isolated yield of 38.65%.

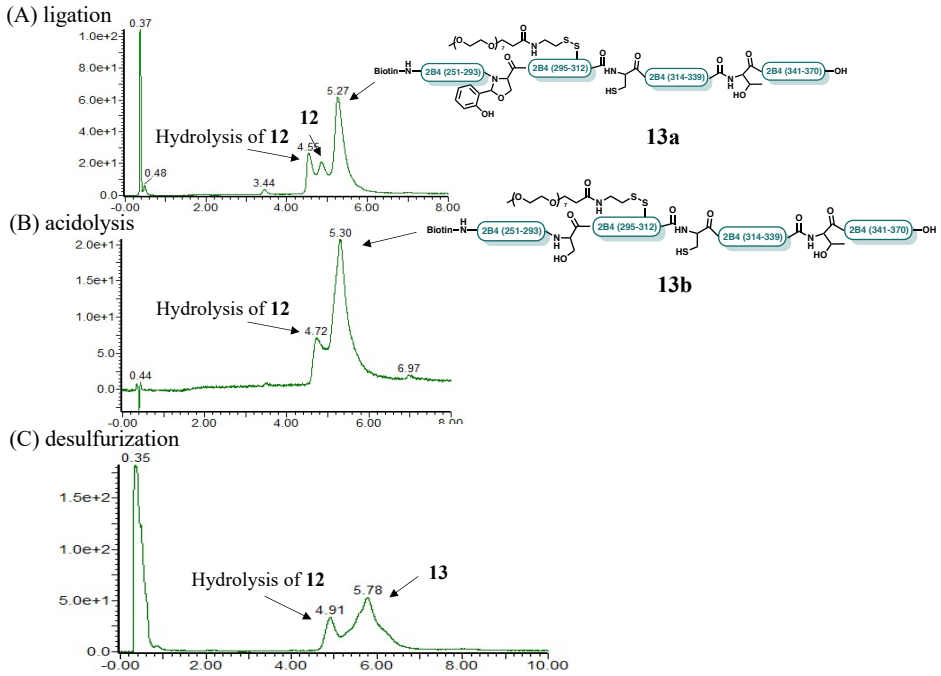
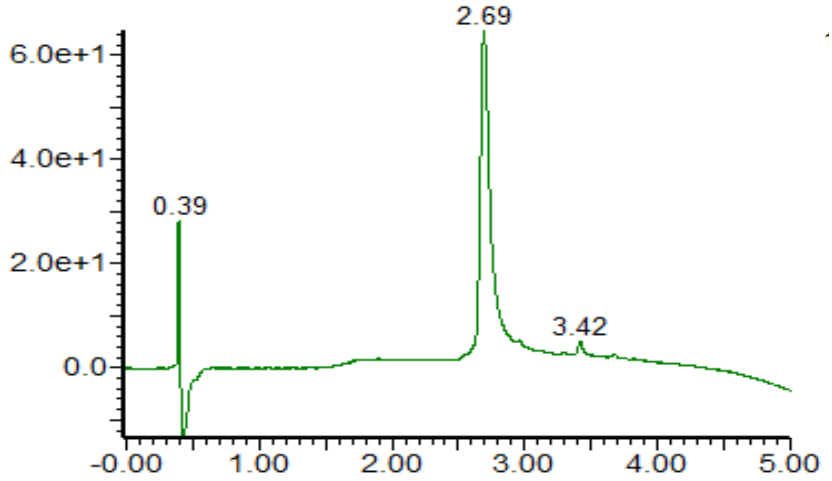


Figure S32. UV trace from analytical RP-UPLC of Ser/Thr ligation reaction mixture between **11** and **12**. (A) Ser/Thr ligation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 30 min. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (C) Desulfurization at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.



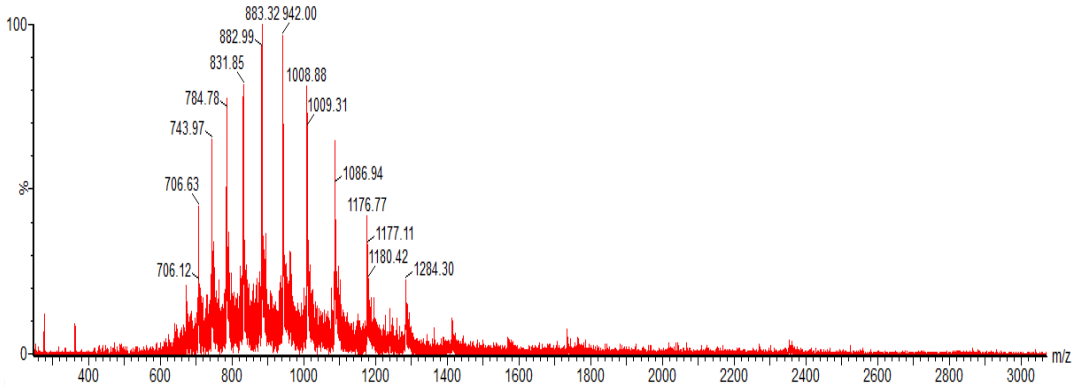
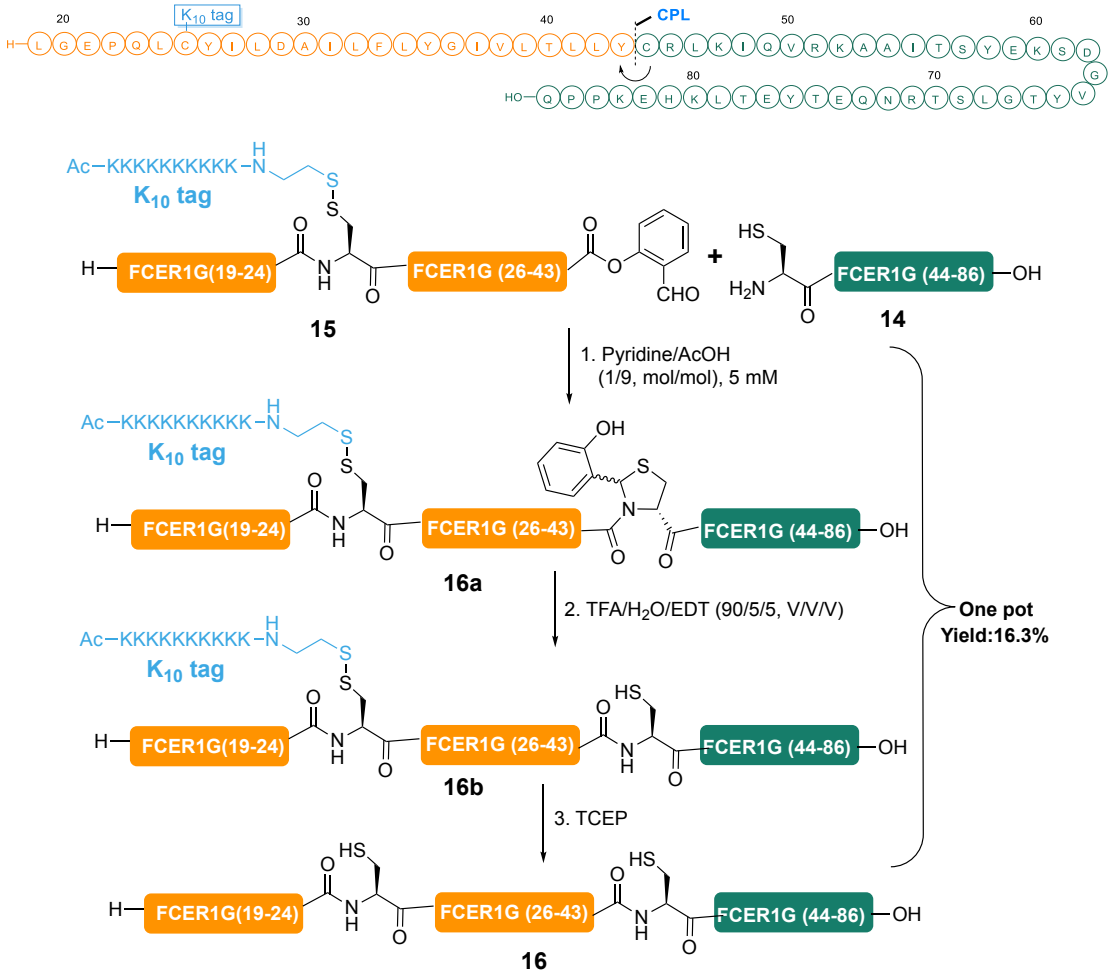


Figure S33. UV trace from analytical RP-UPLC and its ESI-MS of **13**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{616}H_{974}N_{182}O_{195}S_2$ $[M+11H]^{11+}$ m/z = 1284.07, found 1284.30, $[M+12H]^{12+}$ m/z = 1177.15, found 1176.77, $[M+13H]^{13+}$ m/z = 1086.67, found 1086.94, $[M+14H]^{14+}$ m/z = 1009.13, found 1008.88, $[M+15H]^{15+}$ m/z = 941.92, found 942.00, $[M+16H]^{16+}$ m/z = 883.11, found 883.32, $[M+17H]^{17+}$ m/z = 831.22, found 831.85, $[M+18H]^{18+}$ m/z = 785.10, found 784.78, $[M+19H]^{19+}$ m/z = 743.83, found 784.78, $[M+20H]^{20+}$ m/z = 706.69, found 706.63.

9 Synthesis of FCER1G



9.1 Synthesis of 14

14

0.1 mol peptide **14** was assembled by Fmoc-SPPS general procedure. After Fmoc-SPPS, 10 mL TFA/H_2O/EDT (90/5/5, v/v/v) was added to cleavage the peptide from resin and globally deprotect for 2 h at room temperature. The crude peptide was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 40% ACN/H_2O over 30 min. After lyophilization, 61.18 mg **14** was obtained in an isolated yield of 12.31%.

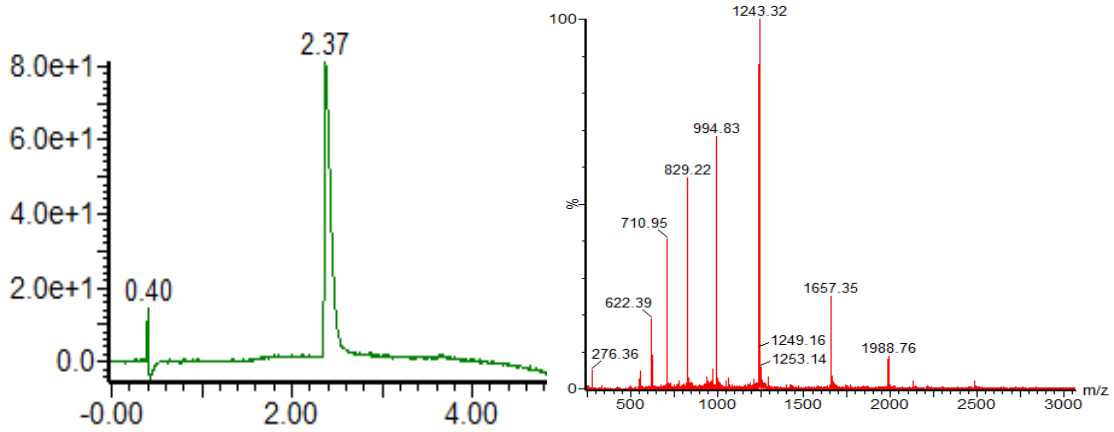
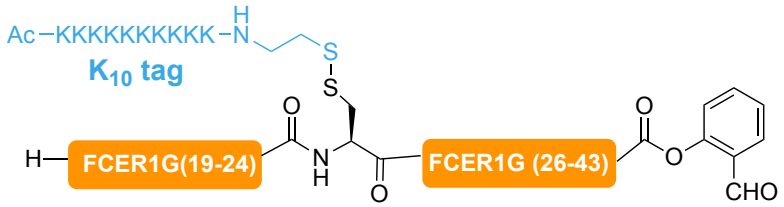


Figure S34. UV trace from analytical RP-UPLC and its ESI-MS of **14**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{216}H_{353}N_{63}O_{69}S$ $[M+3H]^{3+}$ m/z = 1657.19, found 1657.35, $[M+4H]^{4+}$ m/z = 1243.15, found 1243.32, $[M+5H]^{5+}$ m/z = 994.72, found 994.83, $[M+6H]^{6+}$ m/z = 829.10, found 829.22, $[M+7H]^{7+}$ m/z = 710.80, found 710.95, $[M+8H]^{8+}$ m/z = 622.08, found 622.39.

9.2 Synthesis of 15



15

0.01 mmol C-terminus peptide salicylaldehyde ester **15** was synthesized by attachment of reducible solubilizing tags at internal Cys and salicylaldehyde ester preparation general procedure. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 50% ACN/H_2O to 95% ACN/H_2O over 30 min.

After lyophilization, 3.19 mg peptide salicylaldehyde ester **15** was obtained in an isolated yield of 7.31%.

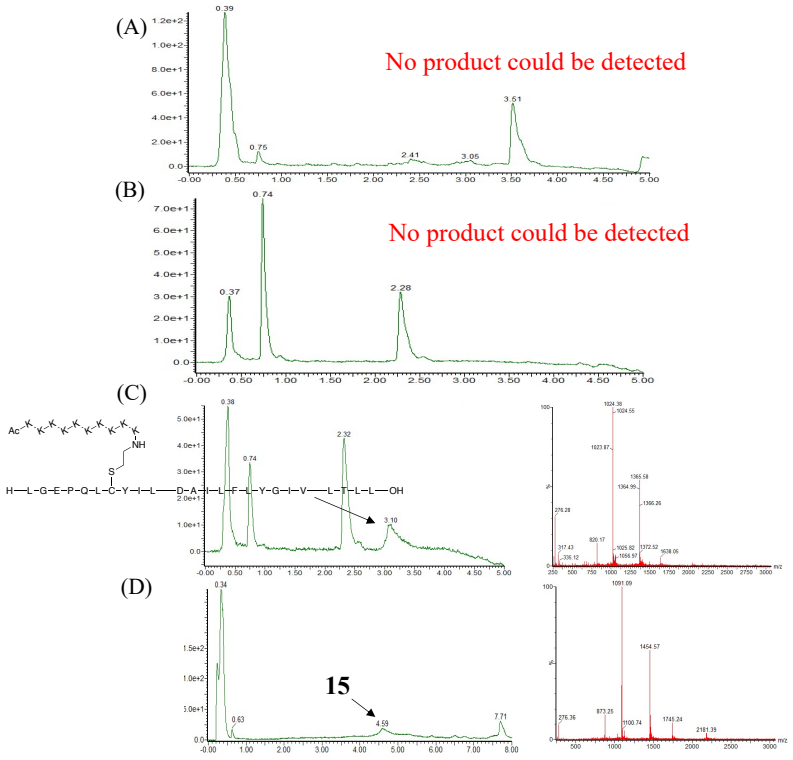


Figure S35. UV trace from analytical RP-UPLC of peptide salicylaldehyde ester **15** preparation check. (A) SPPS with selectively deprotection of S^{t}Bu protecting group of Cys. Gradient: 50-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. (B) Disulfide formation between (9H-fluoren-9-yl)methyl 2-(pyridin-2-yldisulfanyl)ethylcarbamate and peptide on resin at internal Cys. Gradient: 50-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. (C) Attachment of K_{10} solubilizing tag on resin at internal Cys. Gradient: 50-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. (D) peptide salicylaldehyde ester **15** preparation. Gradient: 50-95% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

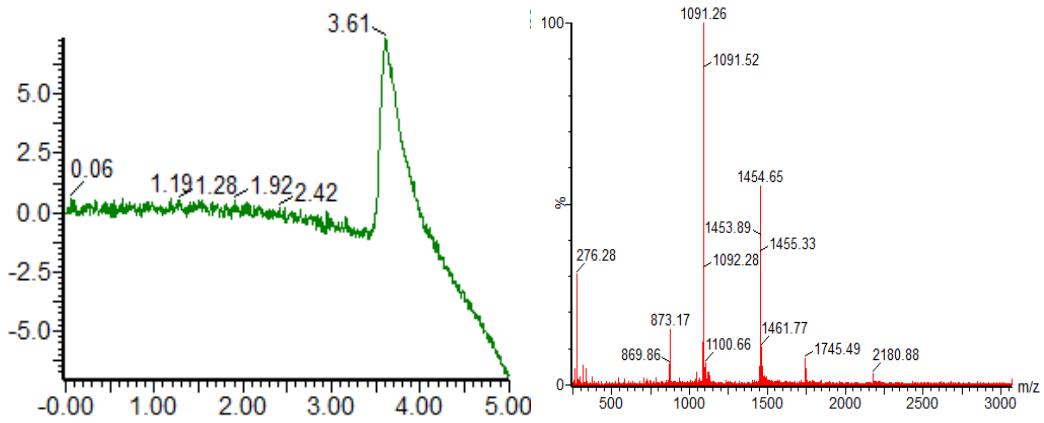


Figure S36. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **15**. Gradient: 50-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{211}H_{349}N_{47}O_{47}S_2$ $[M+2H]^{2+}$ m/z = 2181.26, found 2180.88, $[M+3H]^{3+}$ m/z = 1454.50, found 1454.65, $[M+4H]^{4+}$ m/z = 1091.13, found 1091.26, $[M+5H]^{5+}$ m/z = 873.10, found 873.17.

9.3 Synthesis of 16

16

14 (2.00 mg, 0.40 μmol) and **15** (1.76 mg, 0.40 μmol) were dissolved in pyridine/acetic acid (1/9, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The crude solid was treated with 400 μL TFA/H_2O/EDT (90/5/5, v/v/v) for 1 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. The residue was dissolved in 400 μL TCEP solution (0.5 M in 50 % ACN/H_2O, pH~7). The reaction mixture was stirred at room temperature for 30 min. Finally, product was purified by preparative reverse-phase HPLC using gradient of 50% ACN/H_2O to 95% ACN/H_2O over 30 min. After lyophilization, 0.51 mg **16** was obtained in an isolated yield of 16.33%.

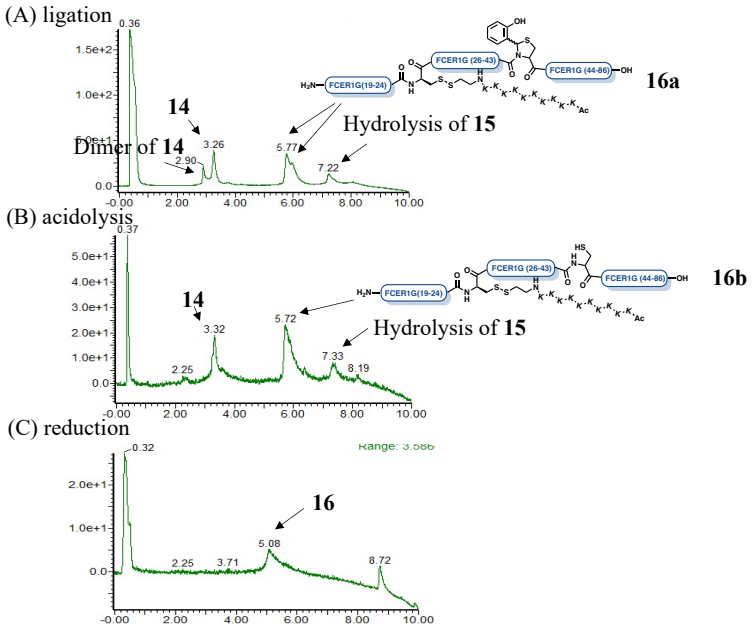


Figure S37. UV trace from analytical RP-UPLC of Cys ligation reaction mixture between **14** and **15**. (A) Cys ligation at 12 h. Gradient: 10-95% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 1 h. Gradient: 10-95% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. (C) Solubilizing tag detachment with TCEP at 30 min. Gradient: 50-95% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

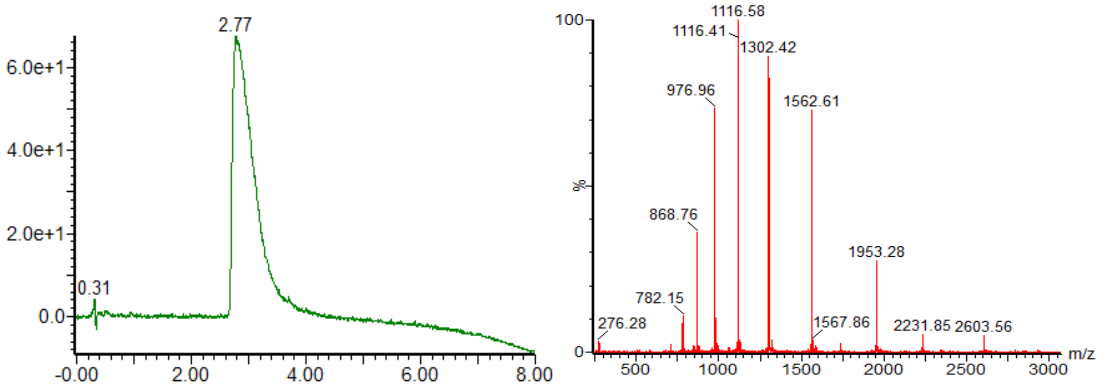
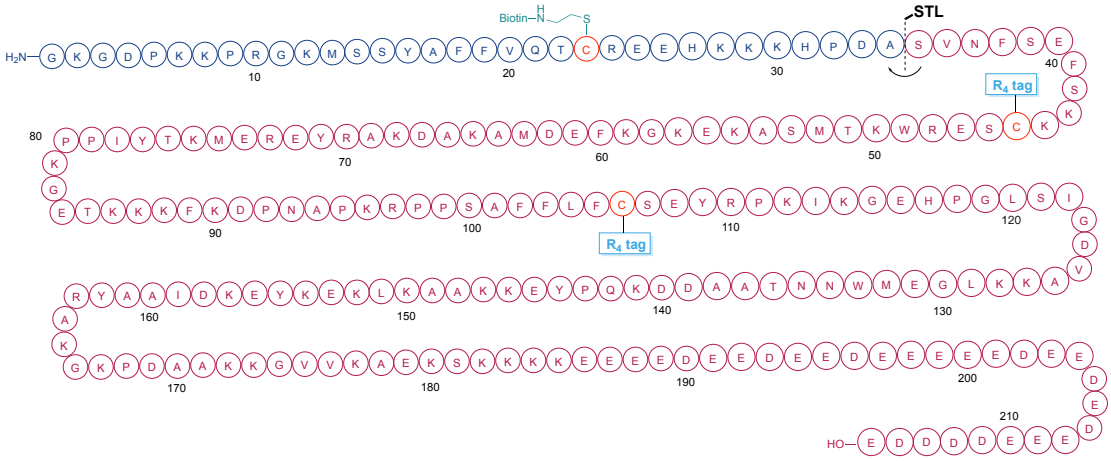
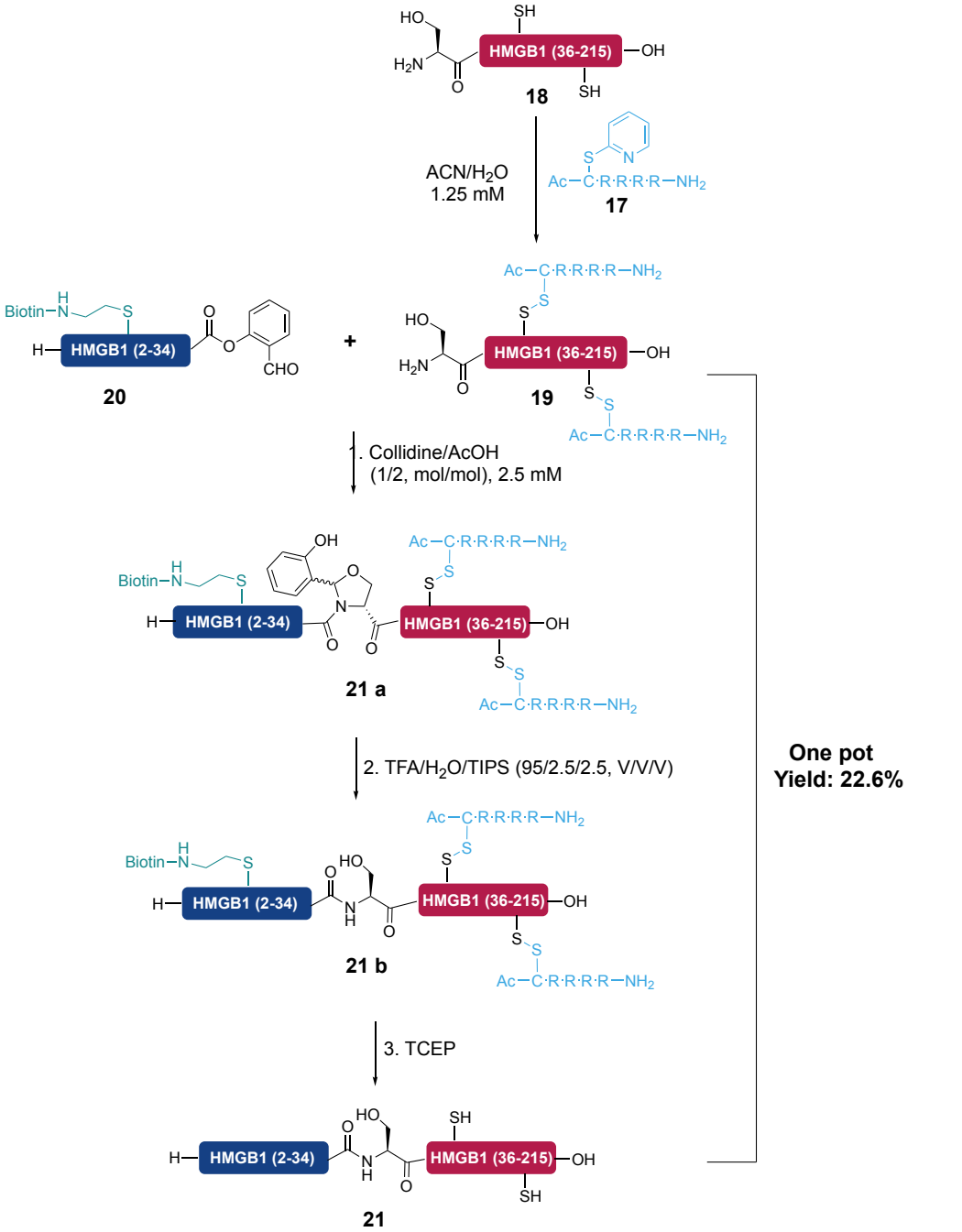


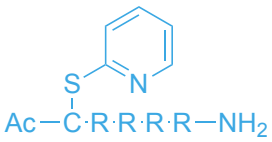
Figure S38. UV trace from analytical RP-UPLC and its ESI-MS of **16**. Gradient: 50-95% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{356}H_{569}N_{89}O_{103}S_2$ $[M+3H]^{3+}$ m/z = 2603.70, found 2603.56, $[M+4H]^{4+}$ m/z = 1953.03, found 1953.28, $[M+5H]^{5+}$ m/z = 1562.62, found 1562.61, $[M+6H]^{6+}$ m/z = 1302.35, found 1302.42, $[M+7H]^{7+}$ m/z = 1116.44, found 1116.58, $[M+8H]^{8+}$ m/z = 977.01, found 976.96, $[M+9H]^{9+}$ m/z = 868.57, found 868.76, $[M+10H]^{10+}$ m/z = 781.81, found 782.15.

10 Semi-synthesis of HMGB1


Biotin
STL
R4 tag
R4 tag


HMGB1 (36-215)
18
ACN/H2O
1.25 mM
17
Biotin
HMGB1 (2-34)
20
HMGB1 (36-215)
19
1. Collidine/AcOH
(1/2, mol/mol), 2.5 mM
HMGB1 (2-34)
HMGB1 (36-215)
21 a
2. TFA/H2O/TIPS (95/2.5/2.5, V/V/V)
HMGB1 (2-34)
HMGB1 (36-215)
21 b
3. TCEP
HMGB1 (2-34)
HMGB1 (36-215)
21
One pot
Yield: 22.6%

10.1 Synthesis of 17



17

0.2 mol peptide **17** was assembled by Fmoc-SPPS general procedure. After Fmoc-SPPS, 10 mL TFA/H_2O/TIPS (90/5/5, v/v/v) was added to cleavage the peptide from resin and globally deprotect for 2 h at room temperature. The crude peptide was purified by preparative reverse-phase HPLC using gradient of 5% ACN/H_2O to 40% ACN/H_2O over 30 min. After lyophilization, 93.79 mg **17** was obtained in an isolated yield of 52.33%.

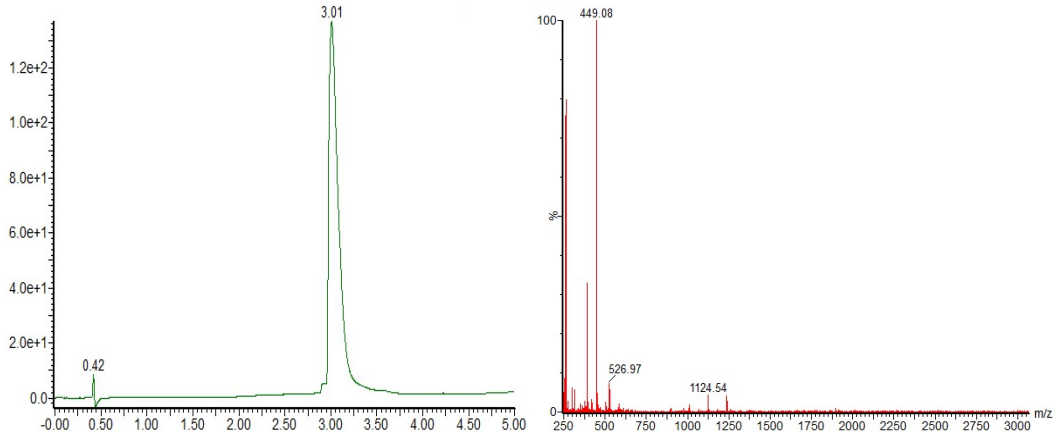
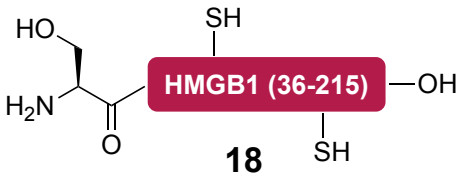


Figure S39. UV trace from analytical RP-UPLC and its ESI-MS of **17**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{34}H_{61}N_{19}O_6S_2$ $[M+2H]^{2+}$ m/z = 449.06, found 449.08.

10.2 Synthesis of 18



18

HMGB1 DNA sequence was optimized for *E. coli* codon and inserted in pET28a plasmid with an N-terminal TEV recognition sequence (TEVs) and C-terminal intein-His_6 tag to generate pET28a-TEVs-HMGB1(35-215)-intein-His plasmid for HMGB1(35-215) expression. The fusion protein sequence was shown at below:

TEVs HMGB1(35-215)

MENLYFQSVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPK

GETKKKFKDPNAPKRPPSAFFLFCSEYRPKIKGEHPGLSIGDVAKKLGEMWNNTAAD

DKQPYEKKAAKLKEKYEKDIAAYRAKGKPDAAKKGVVKAEKSKKKKEEEEDEEDE

Intein tag

EDEEEEEDEEDEDEEEDDDDECITGDALVALPEGESVRIADIVPGARPNSDNAIDLKV

LDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLVDVAGVPTLLWKL

IDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYTVGVPGLVRFLEAHHRDPDAQ

AIADELTDGRFYYAKVASVTDAGVQPVYSLRVDTADHAFITNGFVSHATGLTGLNS

GLTTNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ

His tag

KLAAALEHHHHHH

pET28a plasmid was transformed into BL21. The expression was induced by including 0.2 mM IPTG when OD_{600} reached 0.8, and the culture was grown at 16 ℃ for overnight. The bacteria were collected by centrifugation then sonicated in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM imidazole, 1mM PMSF). After centrifugation (20000 g, 1 h, 4 ℃), the supernatant was loaded onto the Histrap HP column, followed by thoroughly washing with 50 mL lysis buffer. The HMGB1 protein was eluted by 10 mL elution buffer (50 mM Tris-HCl, pH 8.5, 500 mM NaCl, 500 mM imidazole). After digestion by TEV to release the N-terminal Ser, 100 mM Mesna was included to trigger the intein splicing. After 24 h, the solution was directly injected into HPLC for HMGB1(35-215) purification (20-50% CH_3CN/H_2O over 30 min). The eluted fractions were checked by LC-MS. The fractions containing HMGB1 were combined and lyophilized.

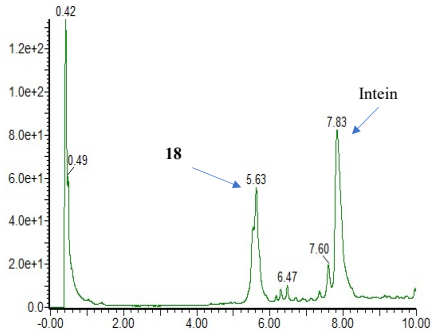


Figure S40. UV trace from analytical RP-UPLC of eluted fractions containing **18**. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

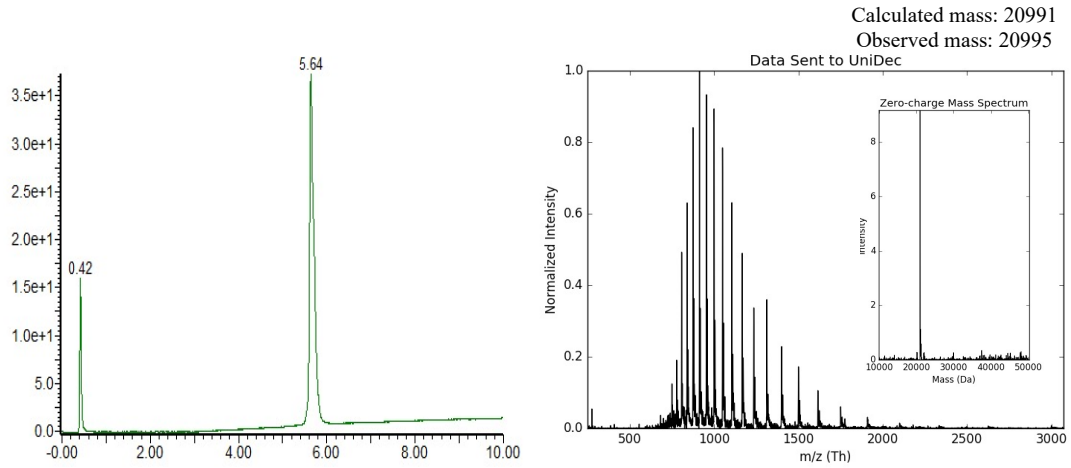
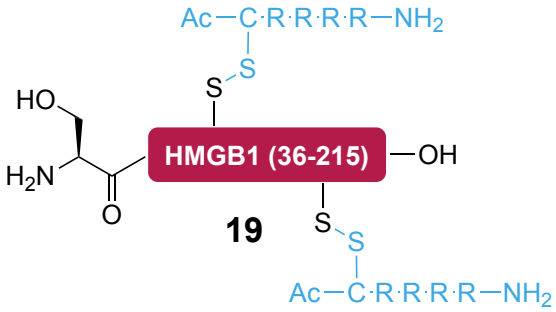


Figure S41. UV trace from analytical RP-UPLC, its ESI-MS and its deconvolution of mass spectra of **18**. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{914}H_{1432}N_{244}O_{310}S_6$ $[M+11H]^{11+}$ m/z = 1909.28, found 1909.17, $[M+12H]^{12+}$ m/z = 1750.26, found 1751.68, $[M+13H]^{13+}$ m/z = 1615.70, found 1615.10, $[M+14H]^{14+}$ m/z = 1500.36, found 1500.20, $[M+15H]^{15+}$ m/z = 1400.41, found 1400.04, $[M+16H]^{16+}$ m/z = 1312.95, found 1313.26, $[M+17H]^{17+}$ m/z = 1235.77, found 1236.13, $[M+18H]^{18+}$ m/z = 1167.17, found 1167.38, $[M+19H]^{19+}$ m/z = 1105.80, found 1105.91, $[M+20H]^{20+}$ m/z = 1050.56, found 1050.54, $[M+21H]^{21+}$ m/z = 1000.58, found 1000.67, $[M+22H]^{22+}$ m/z = 955.14, found 955.29, $[M+23H]^{23+}$ m/z = 913.14, found 913.89, $[M+24H]^{24+}$ m/z = 875.63, found 875.45, $[M+25H]^{25+}$ m/z = 840.65, found 840.74, $[M+26H]^{26+}$ m/z = 808.35, found 808.31, $[M+27H]^{27+}$ m/z = 778.45, found 779.02, $[M+28H]^{28+}$ m/z = 750.69, found 750.57.

10.3 Synthesis of 19



Expressed HMGB1 fragment **18** (5.00 mg, 0.24 μmol) and peptide **17** (6.45 mg, 7.2 μmol) were dissolved in ACN/H_2O (3/1, v/v) at a concentration of 1.25 mM at room temperature. The reaction mixture was stirred at room temperature for overnight. Then, product was purified by ultra-filtration using 3kDa Millipore Ultra centrifugal filter. After lyophilization, 5.18 mg **19** was obtained in quantitively yield.

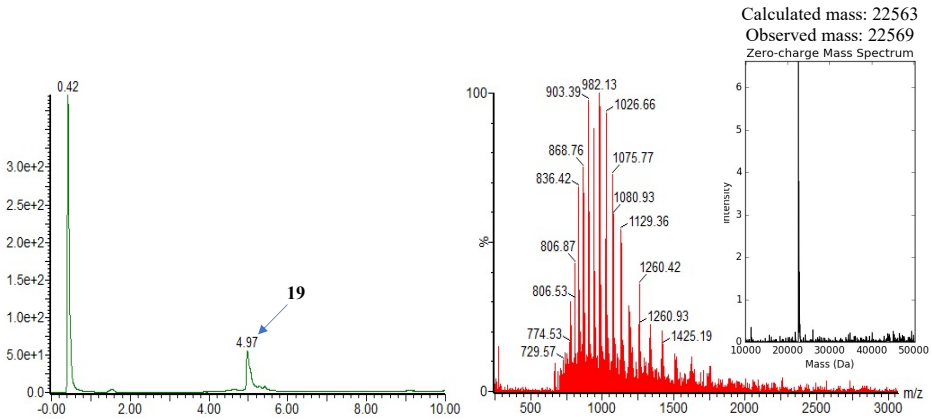
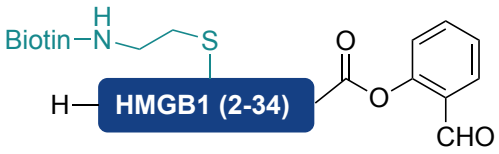


Figure S42. UV trace from analytical RP-UPLC, its ESI-MS and its deconvolution of mass spectra of **19**. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{972}H_{1546}N_{280}O_{322}S_8$ $[M+15H]^{15+}$ m/z = 1505.20, found 1505.71, $[M+16H]^{16+}$ m/z = 1411.19, found 1411.30, $[M+17H]^{17+}$ m/z = 1328.24, found 1328.07, $[M+18H]^{18+}$ m/z = 1254.50, found 1254.16, $[M+19H]^{19+}$ m/z = 1188.53, found 1188.71, $[M+20H]^{20+}$ m/z = 1129.15, found 1129.36, $[M+21H]^{21+}$ m/z = 1075.43, found 1075.77, $[M+22H]^{22+}$ m/z = 1026.59, found 1026.66, $[M+23H]^{23+}$ m/z = 982.00, found 982.13, $[M+24H]^{24+}$ m/z = 941.13, found 941.07, $[M+25H]^{25+}$ m/z = 903.52, found 903.39, $[M+26H]^{26+}$ m/z = 868.81, found 868.76, $[M+27H]^{27+}$ m/z = 836.67, found 836.42, $[M+28H]^{28+}$ m/z = 806.83, found 806.87, $[M+29H]^{29+}$ m/z = 779.04, found 778.76, $[M+30H]^{30+}$ m/z = 753.10, found 753.62, $[M+31H]^{31+}$ m/z = 728.84, found 729.57.

10.4 Synthesis of 20



20

0.04 mmol C-terminus peptide salicylaldehyde ester **20** was synthesized by attachment of Biotin tag at internal Cys and salicylaldehyde ester preparation general procedure. The crude peptide salicylaldehyde ester was purified by preparative reverse-phase HPLC using gradient of 10% ACN/H_2O to 40% ACN/H_2O over 30 min. After lyophilization, 67.15 mg peptide salicylaldehyde ester **20** was obtained in an isolated yield of 40.02%.

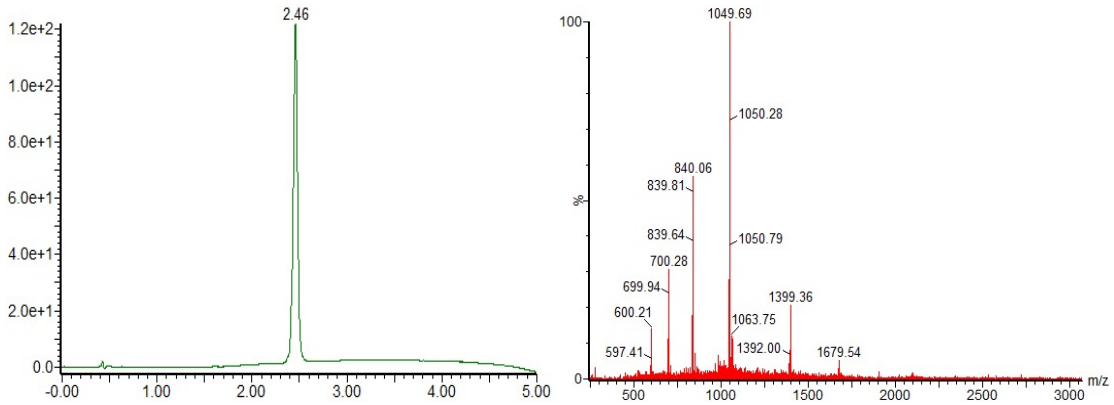
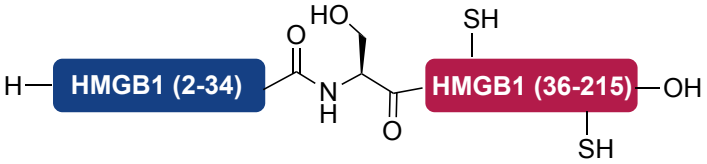


Figure S43. UV trace from analytical RP-UPLC and its ESI-MS of peptide salicylaldehyde ester **20**. Gradient: 5-95% ACN/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{185}H_{286}N_{54}O_{50}S_4$ $[M+3H]^{3+}$ m/z = 1399.29, found 1399.36, $[M+4H]^{4+}$ m/z = 1049.72, found 1049.69, $[M+5H]^{5+}$ m/z = 839.98, found 840.06, $[M+6H]^{6+}$ m/z = 700.15, found 700.28, $[M+7H]^{7+}$ m/z = 600.27, found 600.21.

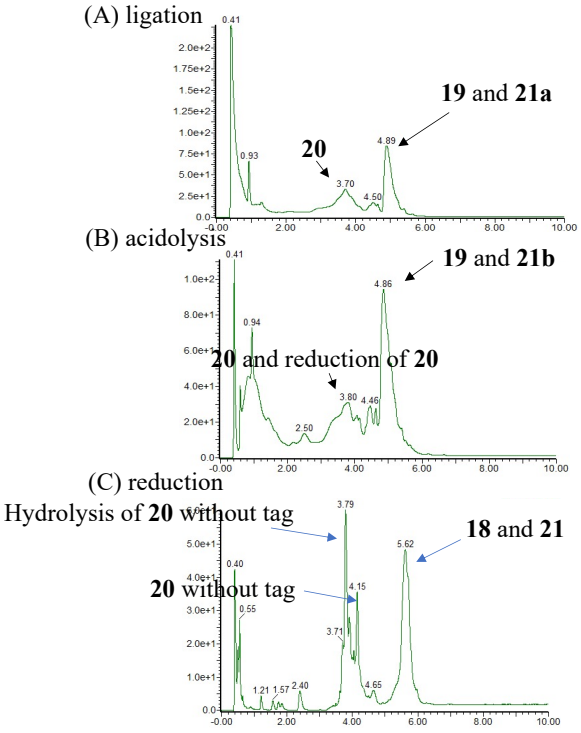
10.5 Synthesis of 21



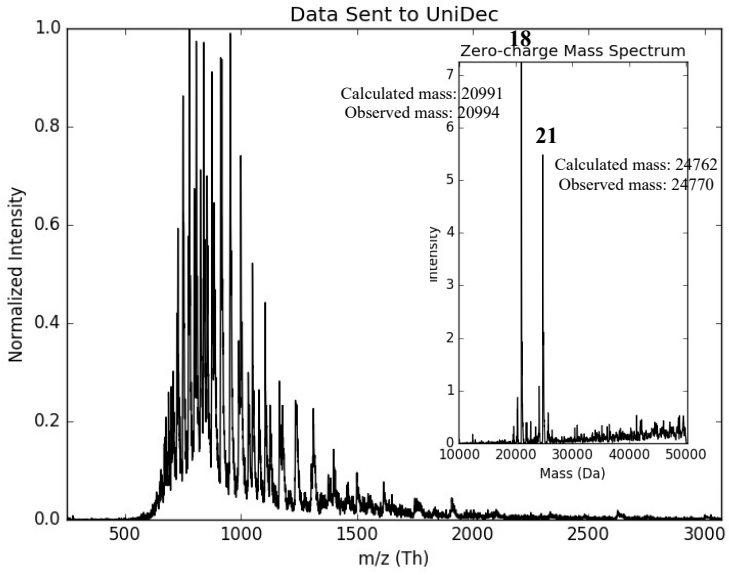
21

19 (2.00 mg, 0.089 μmol) and **20** (0.94 mg, 0.45 μmol) were dissolved in collidine/acetic acid (1/2, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for 6 h. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The crude solid was treated with 200 μL TFA/H_2O/TIPS (95/2.5/2.5, v/v/v) for 0.5 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. Trace residue was checked after treating with 100 mM TCEP (pH=7, in the PB buffer) for 1 h by LC-MS to calculate the reaction conversion. After that, the solid was dissolved in 6 M guanidine, followed by 10 times dilution in refolding buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl). After centrifugation, the supernatant was concentrated and loaded onto the size exclusion column. The according HMGB1 monomer fractions were collected and further purified by streptavidin resin: 100 μL resin slurry was added into 2 mg protein solution and incubated for 1 h at 4 ℃. After centrifugation (1000 g, 2 min), the supernatant was discarded and the resin was resuspended by adding another 1 mL

refolding buffer. After washing by repeating these steps twice, the protein was eluted by directly treating resin with 20 mM TCEP in refolding buffer for 1 h at room temperature. The elution was checked by LC-MS. The co-eluted tags can be removed by following concentration step. The concentration of final product solution was measured by the absorbance at 280 nm and corrected by extinction coefficients. After calculation, the overall isolation yield is 22.73%.



(D) deconvolution of mass spectrum



(E) Size exclusion chromatography analysis of recombinant and synthetic HMGB1

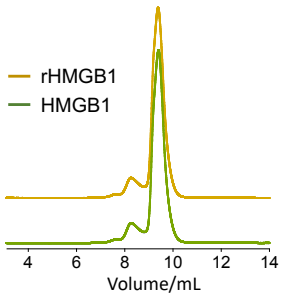


Figure S44. UV trace from analytical RP-UPLC of Ser ligation reaction mixture between **19** and **20**. (A) Ser ligation at 6 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. (B) Acidolysis at 0.5 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. (C) Solubilizing tag detachment with TCEP at 1 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. (D) Deconvolution of mass spectrum of reaction mixture after treating with TCEP. (E) Size exclusion chromatography analysis of recombinant and synthetic HMGB1. rHMGB1: recombinant full-length HMGB1, which was purified under native condition.

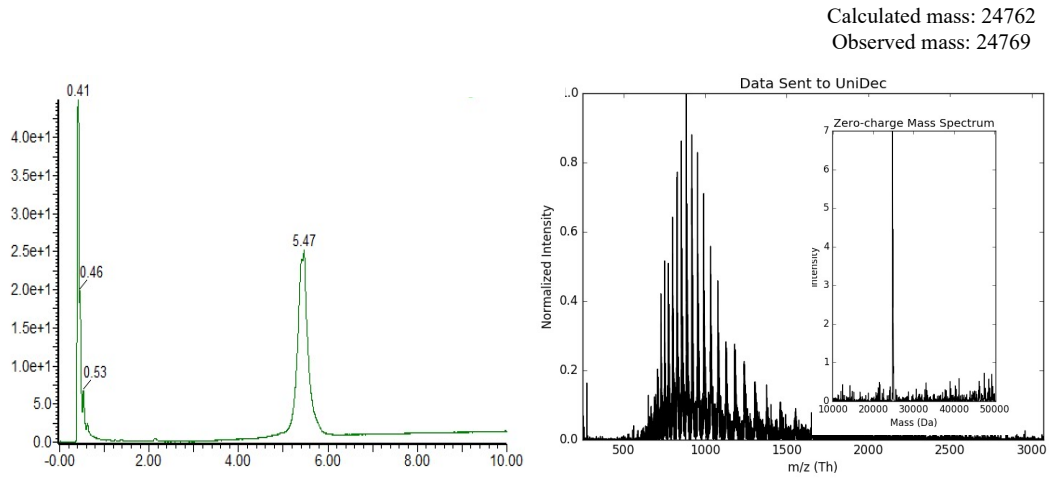


Figure S45. UV trace from analytical RP-UPLC its ESI-MS and its deconvolution of mass spectra of protein **21**. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{1080}H_{1693}N_{295}O_{356}S_8$ $[M+15H]^{15+}$ m/z = 1651.83, found 1652.02, $[M+16H]^{16+}$ m/z = 1548.65, found 1548.72, $[M+17H]^{17+}$ m/z = 1457.61, found 1457.70, $[M+18H]^{18+}$ m/z = 1376.69, found 1376.76, $[M+19H]^{19+}$ m/z = 1304.29, found 1304.11, $[M+20H]^{20+}$ m/z = 1239.12, found 1239.68, $[M+21H]^{21+}$ m/z = 1180.16, found 1180.08, $[M+22H]^{22+}$ m/z = 1126.57, found 1127.75, $[M+23H]^{23+}$ m/z = 1077.63, found 1078.22, $[M+24H]^{24+}$ m/z = 1032.77, found 1032.67, $[M+25H]^{25+}$ m/z = 991.50, found 992.37, $[M+26H]^{26+}$ m/z = 953.40, found 953.43, $[M+27H]^{27+}$ m/z = 918.13, found 918.63, $[M+28H]^{28+}$ m/z = 885.38, found 885.95, $[M+29H]^{29+}$ m/z = 854.88, found 854.96, $[M+30H]^{30+}$ m/z = 826.42, found 826.77, $[M+31H]^{31+}$ m/z = 799.79, found 799.93, $[M+32H]^{32+}$ m/z = 774.83, found 774.87, $[M+33H]^{33+}$ m/z = 751.38, found 752.10, $[M+34H]^{34+}$ m/z = 729.31, found 729.57, $[M+35H]^{35+}$ m/z = 708.50, found 709.17.

10.6 Control experiment for synthesis of 21

18 (0.50 mg, 0.024 μmol) and **20** (0.50 mg, 0.12 μmol) were dissolved in collidine/acetic acid (1/2, mol/mol) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for 6 h. Then, the solvent was removed under condensed air and washed with diethyl ether and centrifuged. The crude solid was treated with 50 μL TFA/H_2O/TIPS (95/2.5/2.5, v/v/v) for 0.5 h. Then, TFA was blown off and the reside was washed with diethyl ether and centrifuged. Finally, the solid was treated with 100 mM TCEP in PB buffer (pH=7) for 1 h, following by checking with LC-MS.

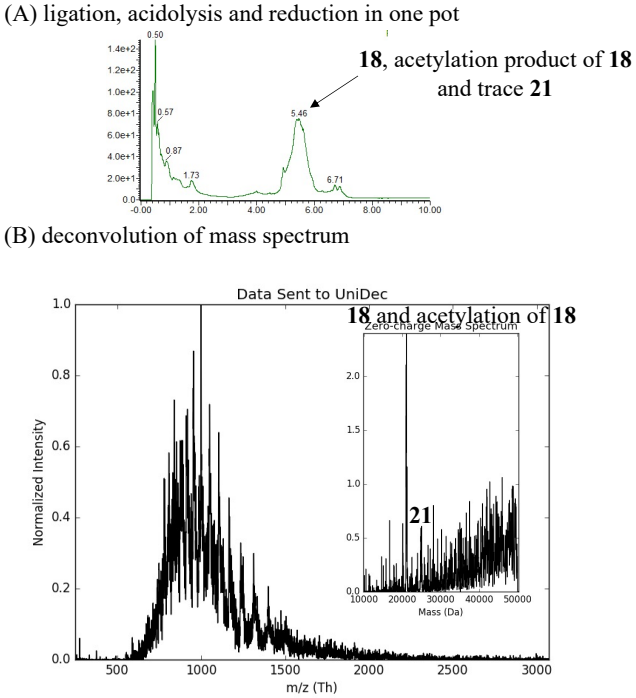
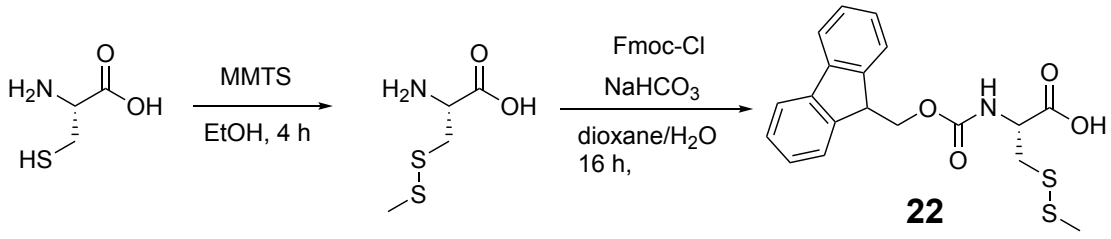


Figure S46. UV trace from analytical RP-UPLC of Ser ligation reaction mixture between **18** and **20**. (A) Ser ligation after 6 h, acidolysis after 0.5 h and Biotin tag detachment with TCEP after 1 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. (B) Deconvolution of mass spectrum of reaction mixture after treating with TCEP.

11 Synthesis of N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(methylthio)-L-cysteine (22)



To a solution of L-cysteine (242.32 mg, 2 mmol) in 15 mL of degassed EtOH was added methyl methanethiosulfonate (MMTS, 0.2 mL, 2.0 mmol) dropwise. The mixture was

stirred at room temperature for 4 h. Then, the reaction was concentrated in vacuo to afford crude S-(methylthio)-L-cysteine, which without further purification was used in the next step directly. This crude product was subsequently dissolved in a 35 mL $NaHCO_3$ solution. To this crude S-(methylthio)-L-cysteine) in 100 mL round flask, $NaHCO_3$ (506 mg, 6 mmol) and H_2O (35 mL) was added. After stirring for 5 min, a solution of 9-Fluorenylmethyl chloroformate (621 mg, 2.4 mmol) in 18 mL dioxane was added dropwise to the above reaction mixture. The resulting reaction mixture was stirred at room temperature for 16 h and subsequently washed with diethyl ether (2 X 50 mL). The reaction mixture was then acidified with HCl (2M) to adjust the pH to 1 and extracted with EtOAc (2 X 50 mL) The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified using silica gel column chromatography eluting with Hex/EtOAc (1:1) containing 1% of HOAc to afford compound **22** as a white solid (570.7 mg, 73.3 % over two steps). HRMS-ESI (m/z): calcd for $C_{19}H_{19}NO_4S_2$ $[M+H]^+$: 390.0834, found: 390.0828. ^1H NMR ($CDCl_3$, 400 MHz,) δ 9.34 (s, 1H), 7.79 (d, J = 7.5 Hz, 2H), 7.69 – 7.53 (m, 2H), 7.43 (t, J = 7.4 Hz, 2H), 7.34 (t, J = 7.4 Hz, 2H), 5.89 (d, J = 8.0 Hz, 1H), 4.79 (dd, J = 12.1, 6.5 Hz,1H), 4.52 – 4.36 (m, 2H), 4.27 (t, J = 6.9 Hz. 1H), 3.24 (ddd, J = 20.6, 14.1, 5.3 Hz, 2H), 2.42 (s, 3H).

^{13}C NMR ($CDCl_3$, 101 MHz) δ 174.93, 156.18, 143.76, 141.35, 127.84, 127.18, 125.18, 120.08, 67.50, 53.35, 47.08, 39.49, 23.09

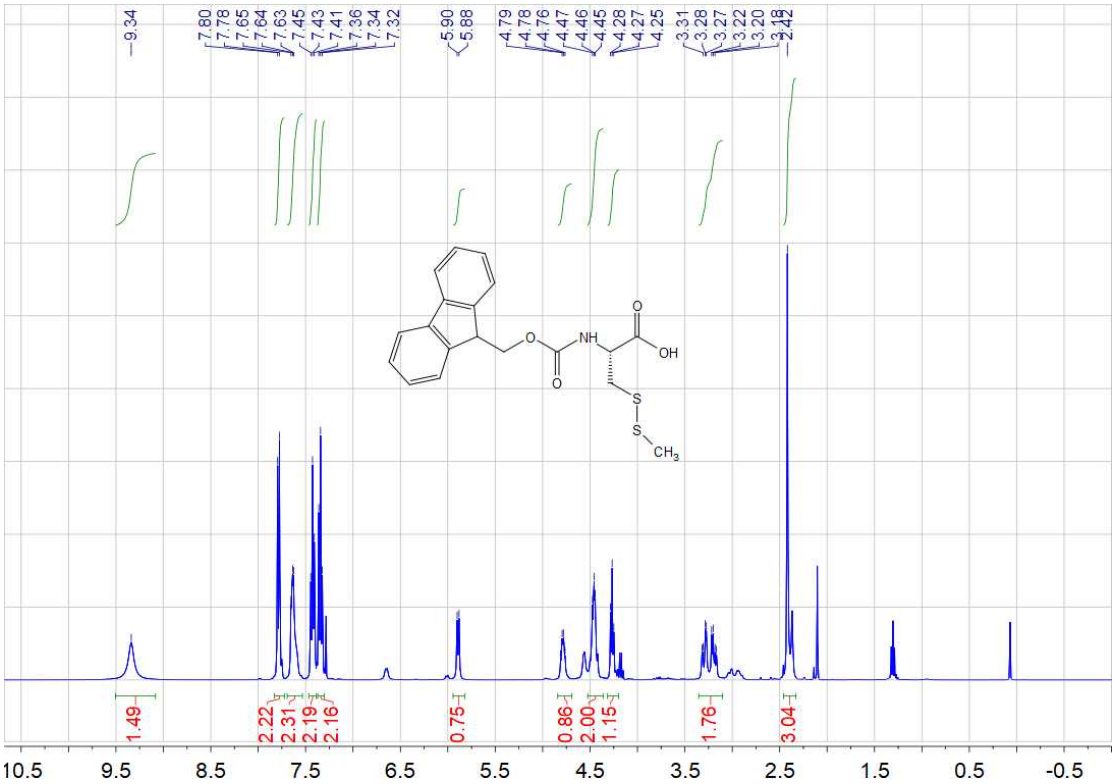


Figure S47. ^1H NMR spectrum of compound **22**.

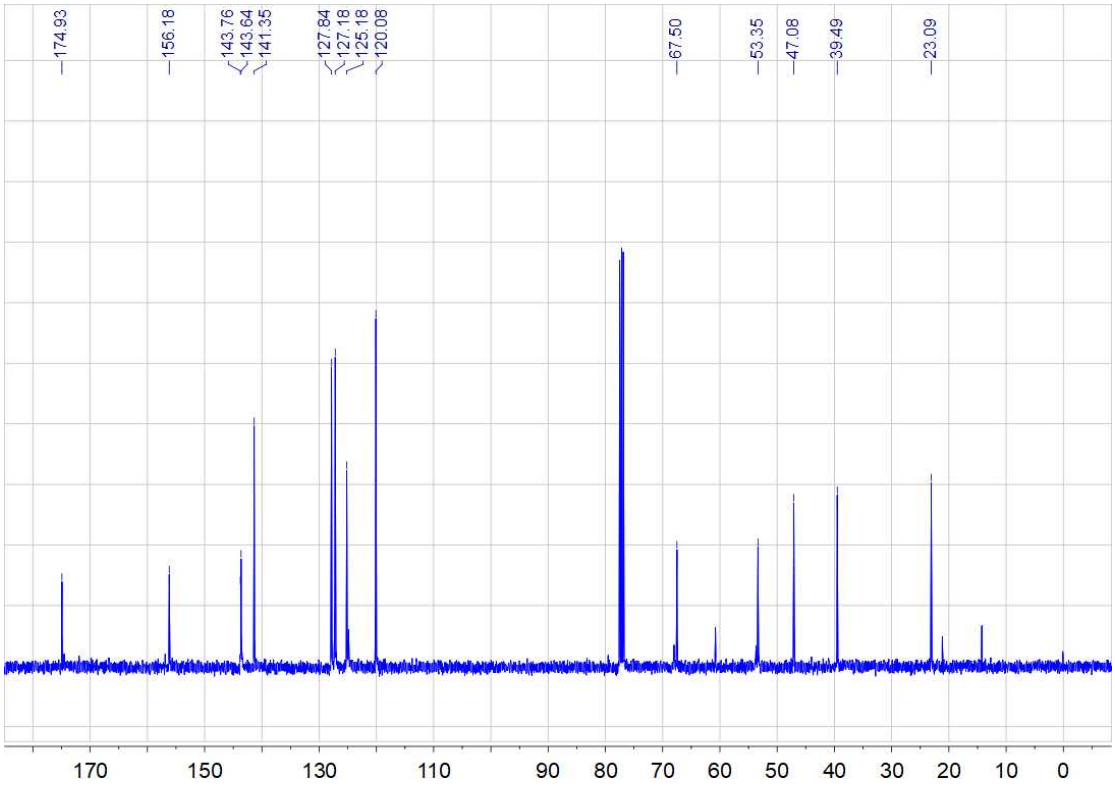
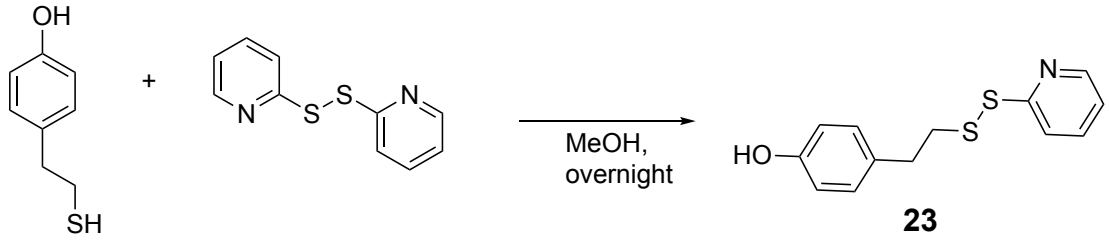


Figure S48. ^{13}C NMR spectrum of compound **22**.

12 Synthesis of 4-(2-(pyridin-2-yldisulfanyl)ethyl)phenol (23)



23

4-(2-mercaptoethyl)phenol was synthesized by reported procedure.[54] 2,2'-Dipyridyldisulfide (6.14 g, 27.8 mmol) was dissolved in 40 mL methanol, then a solution of 4-(2-mercaptoethyl)phenol (2.86 g, 18.5 mmol) in 40 mL methanol was added dropwise. The reaction was stirred at room temperature overnight. The solvent was removed in vacuum and the resulting residue was purified by silica gel column chromatography (ethyl acetate/hexane = 1/5) to give product **23** (3.8 g, 78% yield). HRMS-ESI (m/z): calcd for $C_{13}H_{13}NO_2S_2$ $[M+H]^+$: 264.0517, found: 264.0511. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.45 (s, 1H), 7.76 (t, J = 5.9 Hz, 1H), 7.67 (t, J = 7.7 Hz, 1H), 7.21 – 6.76 (m, 6H), 3.01 (dd, J = 14.9, 7.4 Hz, 2H), 2.96 – 2.85 (m, 2H). ^{13}C NMR ($CDCl_3$,101 MHz) δ 160.48, 155.2, 149.06, 137.92, 130.87, 129.79, 121.07, 120.17, 115.77, 40.49, 34.49.

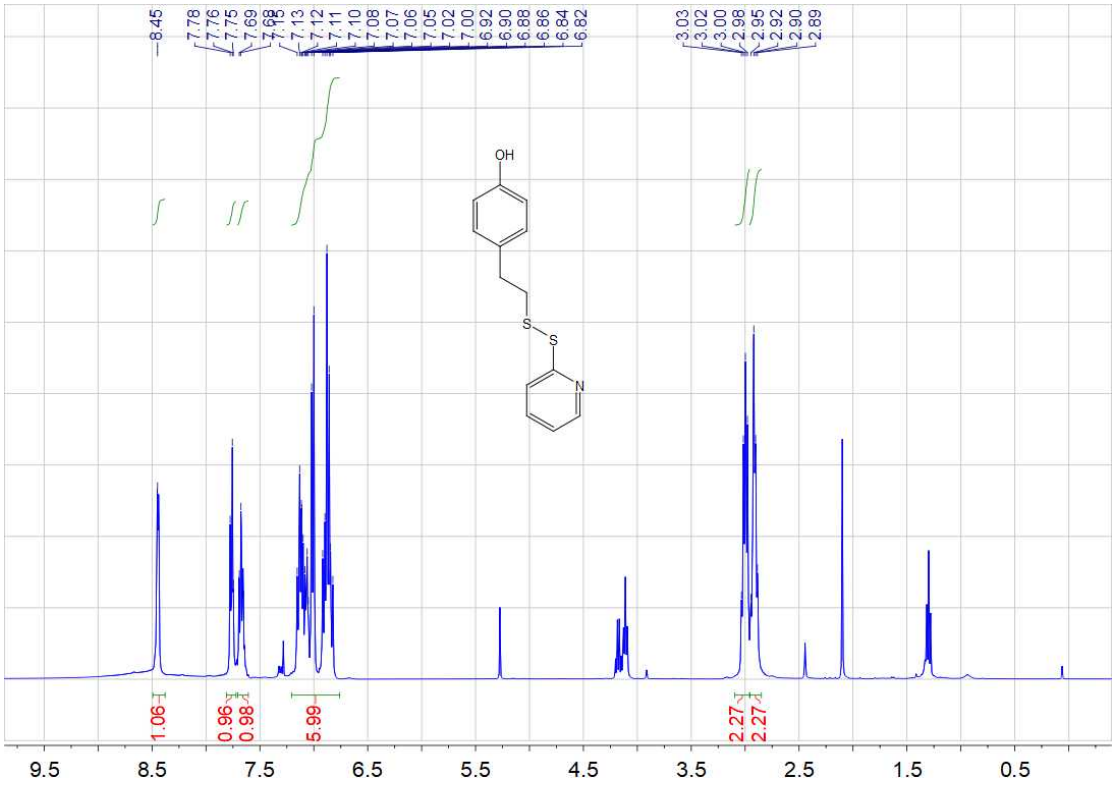


Figure S49. ^{1}H NMR spectrum of compound **23**.

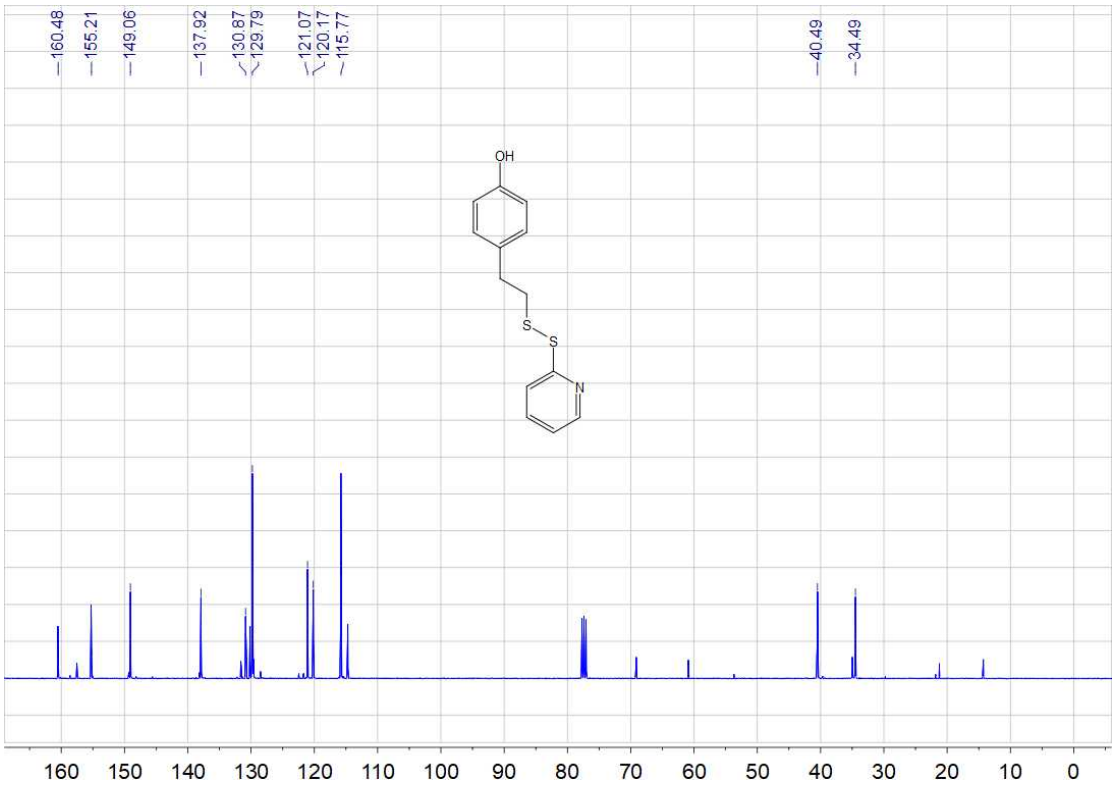
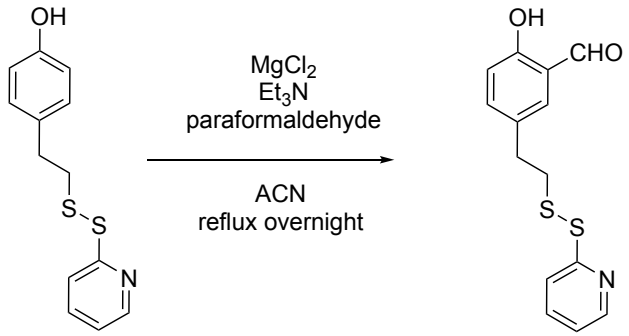


Figure S50. ^{13}C NMR spectrum of compound **23**.

13 Synthesis of 2-hydroxy-5-(2-(pyridin-2-yldisulfanyl)ethyl)benzaldehyde (24)



23 **24**

To the compound **23** (3.8 g, 14.4 mmol) in 250 mL round flask under nitrogen atmosphere was added dry acetonitrile (73 mL), Et_3N (7.65 mL), and $MgCl_2$ (2.06 g, 21.7 mmol). The resulting mixture was stirred at room temperature for 15 min. After that, dry paraformaldehyde (3.03 g, 101 mmol) was added. The reaction mixture was heated at reflux temperature under nitrogen atmosphere for 12 h. The reaction mixture was allowed to cool to room temperature. Then the solvent was removed under vacuo and the resulting residue was acidified with 250 mL HCl (1 M), extracted with Et_2O (3 X 100 mL) The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified using silica gel column chromatography eluting with Hex/EtOAc (6:1) to afford compound **24** (2.16 g, 51%). HRMS-ESI (m/z): calcd for $C_{14}H_{13}NO_2S_2$ $[M+H]^+$: 292.0466, found: 292.0460. ^1H NMR ($CDCl_3$, 400 MHz) δ 10.77 (s, 1H), 9.62 (s, 1H), 8.26 (d, J = 4.8 Hz, 1H), 7.45 (ddd, J = 12.9, 9.9, 4.5 Hz, 2H), 7.20 – 7.05 (m, 2H), 6.94 – 6.85 (m, 1H), 6.69 (d, J = 8.5 Hz, 1H), 2.90 – 2.69 (m, 4H). ^{13}C NMR ($CDCl_3$, 101 MHz) δ 196.41, 159.97, 159.87 149.45, 137.39, 137.16, 133.29, 130.96, 120.80, 120.35, 119.60, 117.52, 39.61, 33.77.

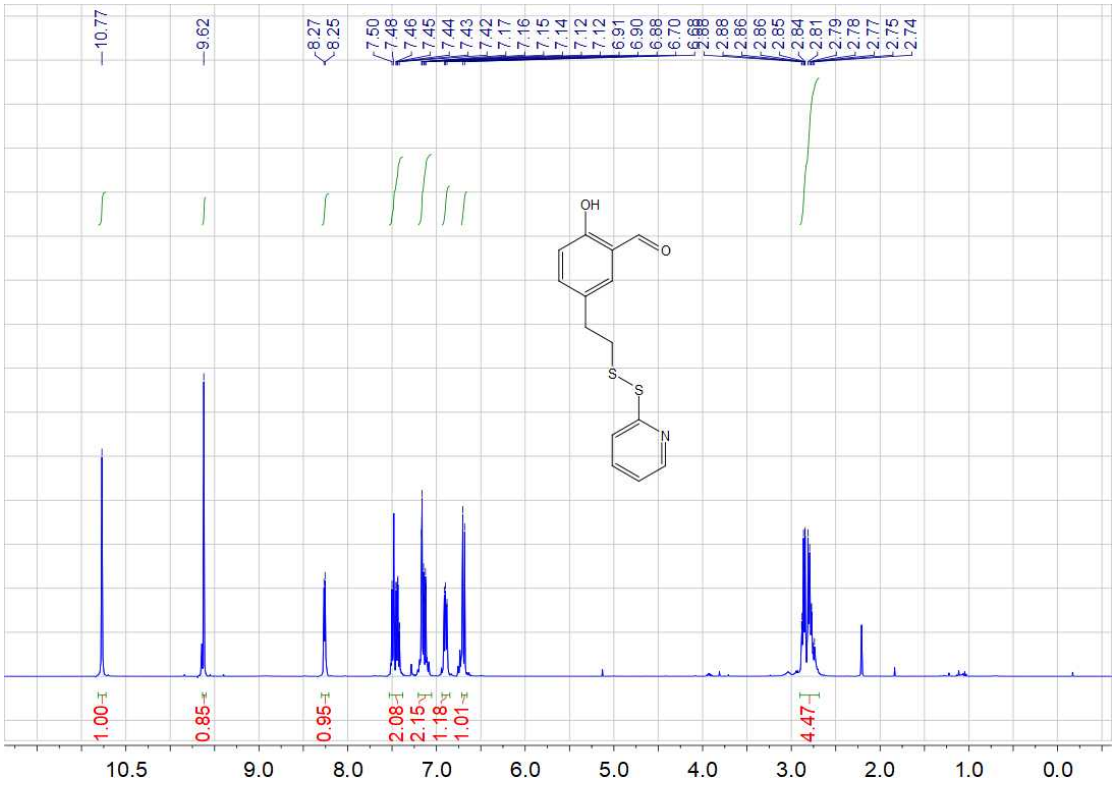


Figure S51. ^{1}H NMR spectrum of compound **24**.

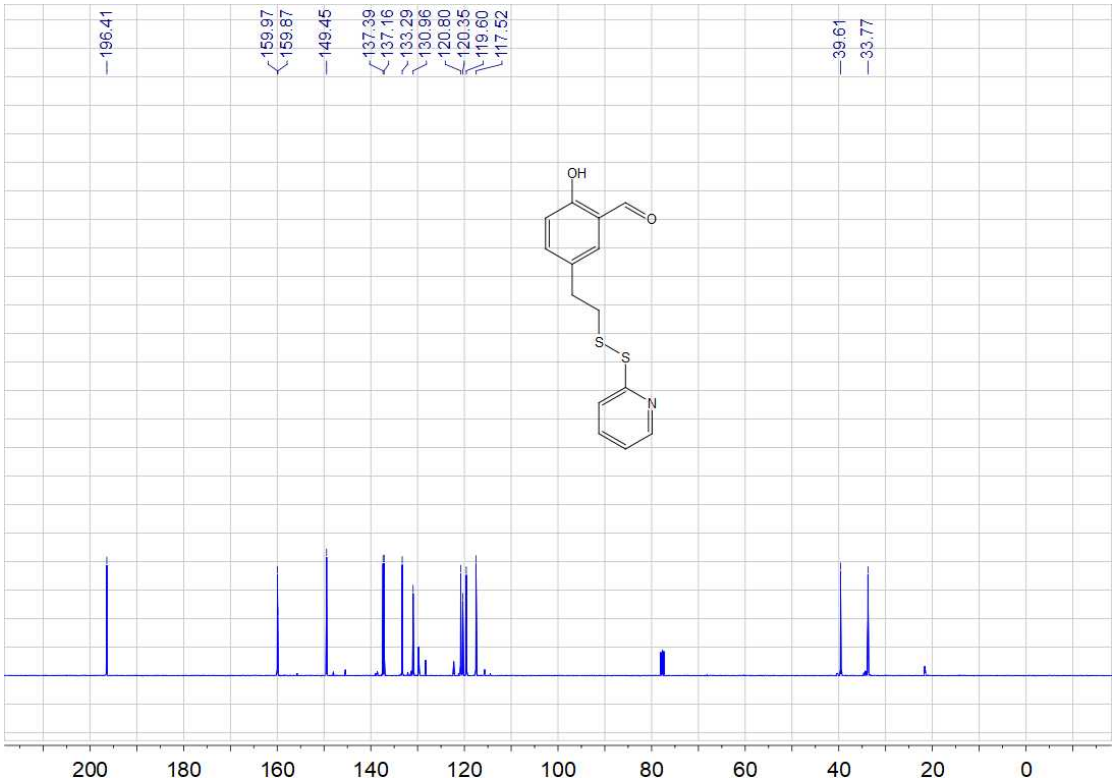


Figure S52. ^{13}C NMR spectrum of compound **24**.

14 References

1. Zhao, H.; Sen, S.; Udayabhaskararao, T.; Sawczyk, M.; Kučanda, K.; Manna, D.; Kundu, P. K.; Lee, J.-W.; Král, P.; Klajn, R., Reversible trapping and reaction acceleration within dynamically self-assembling nanoflasks. *Nature Nanotechnology* **2016,** *11* (1), 82-88.