Supporting Information

A Photo-Cleavable Purification/Protection Handle Assisted Synthesis of Giant Modified Proteins with Tandem Repeats

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1. General information

1.1 Materials and instruments

All chemicals were obtained from commercial sources: Adamas, Macklin, Energy Chemicals, Sigma Aldrich, and were used without further purification unless otherwise stated. Amino acids, HATU, HOAt and 2-Cl(Trt)-Cl resins were acquired from GL Biochem (Shanghai). Ni-NTA resins were acquired from Smart-Lifesciences. Dry solvents were acquired from solvent purification system (Vigor YJC-7). Reactions were monitored by TLC plates and visualizations were performed with UV light (254 nm). UV irradiation was performed under UV Lamps from Gongben company (20 watt, LED, 365 nm). The flash chromatography was carried out on Teledyne ISCO CombiFlash Rf+. 1H NMR spectra were recorded on Bruker AVANCE III HD 400 MHz; 13C NMR spectra were recorded on Bruker AVANCE III HD 101 and 126 MHz. HRMS was recorded on Waters 2D H class & VION IMS QTOF MS. Analytical LC-MS: Shimadzu LC-MS 2020; Column: ReproSil-Pur (C4/C18, 3 μm, 2.1 × 100 mm); Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile. Preparative reverse-phase HPLC: Shimadzu HPLC SPD-20A; Column: ReproSil (C4/C18, 5 μm, 10 × 150 mm); Solvent A: 0.05% TFA in water; Solvent B: 0.04% TFA in acetonitrile.

2. Synthesis of building blocks for SPPS

2.1 Synthesis of the photo-cleavable moiety of PPH

Preparation of compound S1

![Reaction Scheme]

To the solution of compound 1^1 (2.3 g, 6.7 mmol, 1.0 equiv.) in TFA (2 mL), triisopropylsilane (i-Pr3SiH) (20 μL) was added at 0 °C. Then the solution was stirred at room temperature for 3 h. After concentrated to ~200 μL with a nitrogen flow, the residue was precipitated with ice-cold Et2O. The white solid was filtered and the residue solvent was removed in vacuo to afford compound S1 as a white powder (1.5 g, 5.2 mmol, 77%). 1H NMR (400 MHz, DMSO-d6) δ 7.67 (d, J = 8.7 Hz, 1H), 7.58 (d, J = 2.7 Hz, 1H), 7.32 (dd, J = 8.6, 2.8 Hz, 1H), 4.89 (s, 2H), 4.86 (s, 2H). 13C NMR (126 MHz, DMSO-d6) δ 170.04, 158.73, 149.09, 134.40, 124.96, 120.52, 111.67, 65.37, 30.36. HRMS (ESI) calculated for C9H8BrNO5: [M-H] m/z 287.9508, Found: 287.9505.
Preparation of compound 3

To the solution of compound S1 (1.5 g, 5.2 mmol, 1.0 equiv.) in dry DCM (20 mL), oxalyl chloride (2 mL, 26.0 mmol, 5.0 equiv.) was added. After the addition of DMF (5 mL), the mixture was stirred at 0 °C for 30 min, and then the solvent was removed in vacuo to afford the crude compound as yellow oil. The crude product was dissolved in dry DCM (15 mL), and DIPEA (893 μL, 5.2 mmol, 1.0 equiv.) was added subsequently. Then the solution of trifluoroacetate salt 2^2 (2.9 g, 10.4 mmol, 2.0 equiv.) in dry DMF (15 mL) was added. After 1 h, the solution was diluted with DCM, washed with water and brine. The organic phase was combined and dried with anhydrous Na₂SO₄. After filtration and concentration under reduced pressure, the residue was purified by flash chromatography to afford compound 3 as a white powder (1.4 g, 2.5 mmol, 47% over two steps). ¹H NMR (400 MHz, DMSO-d₆) δ 8.27 (d, J = 6.8 Hz, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.45 - 7.29 (m, 6H), 4.97 (s, 2H), 4.62 (s, 2H), 4.31 (d, J = 6.9 Hz, 2H), 4.21 (t, J = 6.9 Hz, 1H), 3.23 - 3.16 (m, 2H), 3.10 (t, J = 6.2 Hz, 2H). ¹³C NMR (101 MHz, DMSO-d₆) δ 167.49, 158.63, 156.74, 149.03, 144.34, 141.19, 134.00, 128.06, 125.58, 124.68, 120.57, 120.40, 112.16, 67.73, 65.81, 47.18, 42.93, 36.41. HRMS (ESI) calculated for C₂₆H₂₄BrN₃O₆ [M+H]^+ 554.0942, Found: 554.0927.

Preparation of compound 4

To the solution of compound 3 (1.4 g, 2.5 mmol, 1.0 equiv.) in DMSO (20 mL), NH₂-Cys-COOH (600 mg, 5.0 mmol, 2.0 equiv.) and Et₃N (1 mL, 7.4 mmol, 3.0 equiv.) were added. The mixture was stirred vigorously for 2.5 h, followed by the addition of (Boc)₂O (2 mL, 15.0 mmol, 6.0 equiv.) and DIPEA (1 mL, 7.5 mmol, 3.0 equiv.). Then, the mixture was stirred at room temperature for another 3.5 h. The solution was directly purified by flash reverse phase chromatography. After lyophilization, compound 4 was obtained as a white powder (1.1 g, 1.6 mmol, 64% over 2 steps). ¹H NMR (400 MHz, DMSO-d₆) δ 8.23 (t, J = 5.8 Hz, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.68 (d, J = 7.4 Hz, 2H), 7.60 (d, J = 2.6 Hz, 1H), 7.51 (d, J = 8.6 Hz, 1H), 7.47 - 7.23 (m, 6H), 7.12 (d, J = 8.4 Hz, 1H), 4.58 (s, 2H), 4.31 (d, J = 6.9 Hz, 2H), 4.21 (t, J = 6.9 Hz, 1H), 4.11 - 3.93 (m, 3H), 3.21 (q, J = 6.4 Hz, 2H), 3.10 (q, J = 6.3 Hz, 2H), 2.78 (dd, J = 13.6, 4.8 Hz, 1H), 2.64 (dd, J = 13.6, 9.4 Hz, 1H), 1.39 (s, 9H). ¹³C NMR (101 MHz, DMSO-d₆) δ 172.33, 167.11, 156.97, 156.74, 149.03, 144.34, 141.19, 134.00, 128.06, 125.31, 124.68, 123.84, 140.68, 133.06, 127.55, 127.00, 125.91, 125.08, 120.07, 119.55, 111.67, 78.19, 67.23, 65.31, 53.30, 46.68, 38.46, 32.71, 32.11, 28.13,
2.2 Synthesis of Fmoc-Thr(α-GalNAc)-OH

Preparation of compound S4

To the solution of S23 (7.8 g, 23.5 mmol, 1.0 equiv.) and S34 (7.4 g, 30.6 mmol, 1.3 equiv.) in dry DCM (150 mL), EDCI (9.0 g, 47.0 mmol, 2.0 equiv.) and DMAP (584 mg, 4.7 mmol, 0.2 equiv.) were added. Then the solution was stirred at room temperature for 4 h. Subsequently, the solution was diluted with DCM and washed with saturated NaHCO₃ solution twice. And then, the organic phase was concentrated under reduced pressure and purified by flash chromatography to afford compound S4 as yellow oil (10.9 g, 21.0 mmol, α/β = 1:5, 89%).

S4α: ¹H NMR (400 MHz, Chloroform-d) δ 7.96 (d, J = 8.0 Hz, 1H), 7.56 (d, J = 7.8 Hz, 1H), 7.52 - 7.45 (m, 1H), 7.36 (t, J = 7.6 Hz, 1H), 6.60 (d, J = 3.6 Hz, 1H), 5.53 (d, J = 3.4 Hz, 1H), 5.42 (dd, J = 10.9, 3.2 Hz, 1H), 4.47 (t, J = 6.8 Hz, 1H), 4.16 (dd, J = 11.3, 7.3 Hz, 1H), 4.13 - 4.01 (m, 2H), 2.59 - 2.46 (m, 2H), 2.18 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H), 1.62 (s, 2H), 1.51 - 1.46 (m, 2H), 0.95 (t, J = 7.3 Hz, 3H).


S4β: ¹H NMR (400 MHz, Chloroform-d) δ 8.02 (d, J = 8.0 Hz, 1H), 7.55 (d, J = 7.8 Hz, 1H), 7.36 - 7.44 (m, 1H), 7.37 - 7.31 (m, 1H), 5.79 (d, J = 8.5 Hz, 1H), 5.41 (d, J = 3.3 Hz, 1H), 4.96 (dd, J = 10.8, 3.3 Hz, 1H), 4.19 - 4.07 (m, 3H), 3.98 (dd, J = 10.8, 8.5 Hz, 1H), 2.50 (t, J = 7.1 Hz, 2H), 2.17 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.67 - 1.60 (m, 2H), 1.54 - 1.46 (m, 2H), 0.95 (t, J = 7.3 Hz, 3H).¹³C NMR (101 MHz, Chloroform-d) δ 170.35, 169.97, 169.65, 163.27, 162.61, 159.41, 127.18, 125.85, 97.34, 93.27, 79.01, 71.71, 71.49, 66.22, 60.89, 59.96, 30.66, 22.09, 20.66, 20.62, 20.60, 19.56, 13.67. HRMS (ESI) calculated for C₂₅H₂₉N₃O₉Na: [M+Na]+ 538.1802, found 538.1816.

Preparation of compound S6

A solution of donor S4 (10.9 g, 21.0 mmol, 1.0 equiv.) and acceptor S53 (12.5 g, 31.5 mmol, 1.5 equiv.) in dry DCM (300 mL) was stirred for 30 min at 0 °C with 4 Å molecular sieves under argon atmosphere.
To the solution Ph$_3$PAuNTf$_2$ (2.3 g, 3.2 mmol, 0.15 equiv.) was added. Then the solution was stirred at 0 °C for 1 h. Subsequently, the 4 Å molecular sieves was filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography to afford S6 as a white solid (6.5 g, 9.7 mmol, 46%). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.77 (d, $J = 7.6$ Hz, 2H), 7.63 (d, $J = 7.7$ Hz, 2H), 7.43 - 7.37 (m, 2H), 7.32 (dt, $J = 11.2$, 5.7 Hz, 2H), 5.67 (d, $J = 9.5$ Hz, 1H), 5.47 (d, $J = 1.8$ Hz, 1H), 5.34 (dd, $J = 11.2$, 3.3 Hz, 1H), 5.11 (d, $J = 3.7$ Hz, 1H), 4.46 - 4.25 (m, 6H), 4.10 (d, $J = 6.5$ Hz, 2H), 3.64 (dd, $J = 11.2$, 3.7 Hz, 1H), 2.15 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 1.50 (s, 9H), 1.39 - 1.32 (m, 3H).

Preparation of compound S7

To the solution of S6 (6.5 g, 9.7 mmol, 1.0 equiv.) in THF/AcOH/Ac$_2$O (3/2/1, v/v/v, 20 mL), activated zinc dust (7.6 g, 116.4 mmol, 12 equiv.) was added. Then the solution was stirred at room temperature for 3 h. Subsequently, the zinc dust was filtered off and the filtrate was concentrated. The residue solution was diluted with DCM and washed with saturated NaHCO$_3$ solution. And then, the residue was concentrated and purified by flash chromatography to afford S7 as a white solid (5.3 g, 7.2 mmol, 74%).

$^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.78 (d, $J = 7.7$ Hz, 2H), 7.64 (d, $J = 7.4$ Hz, 2H), 7.44 - 7.31 (m, 4H), 5.96 (d, $J = 9.9$ Hz, 1H), 5.52 (d, $J = 9.6$ Hz, 1H), 5.42 - 5.36 (m, 1H), 5.09 (dd, $J = 11.5$, 2.9 Hz, 1H), 4.92 - 4.86 (m, 1H), 4.62 (t, $J = 10.8$ Hz, 1H), 4.45 (q, $J = 10.5$, 9.3 Hz, 2H), 4.34 - 4.03 (m, 8H), 2.16 (s, 3H), 2.04 (s, 3H), 2.00 (s, 6H), 1.46 (s, 9H), 1.32 (d, $J = 6.3$ Hz, 3H).

Preparation of compound S8

To the solution of Fmoc-Thr-(α-Ac$_3$GalNAc)-OtBu S7 (5.3 g, 7.2 mmol, 1.0 equiv.) in TFA (20 mL), triisopropylsilane (i-Pr$_3$SiH) (200 μL) and water (1 mL) were added. Then the solution was stirred at room temperature for 4 h. After concentrated to ~2 mL with a nitrogen flow, the residue solvent was azeotropically distilled with toluene. The residue was then purified by flash chromatography to afford compound S8 as a white solid (4.0 g, 6.0 mmol, 83%). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.75 (d, $J = 11.5$ Hz, 2H), 7.61 (s, 2H), 7.35 (d, $J = 30.3$ Hz, 4H), 6.29 (d, $J = 10.2$ Hz, 1H), 5.94 (d, $J = 9.7$ Hz, 1H), 5.40 - 5.32 (m, 1H), 5.14 (d, $J = 11.9$ Hz, 1H), 5.02 (d, $J = 16.9$ Hz, 2H), 4.40 (d, $J = 22.4$ Hz, 2H), 4.26 - 4.02 (m, 6H), 2.16 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.94 (s, 3H), 1.27 (d, $J = 7.1$ Hz, 3H).
3. Solid-phase peptide synthesis (SPPS)

**Loading of hydrazine resin with Fmoc-Thr(tBu)-OH and Fmoc-Ala-OH**

2-Cl(Trt)-Cl resin was swollen in dry DCM for 30 min and treated with 10% hydrazine monohydrate in DMF for another 30 min. After that, the resin was washed with DMF, DCM. Then the resin was capped with MeOH by treating with a solution of DIPEA/MeOH/DCM (1/1/17, v/v/v) for 1 h. The resin was then washed with DCM, DMF, DCM and the excessive hydrazine on the resin was capped with a solution of Ac$_2$O/DIPEA/DMF (1/2/2, v/v/v) for another 30 min. The resin was washed with DMF, DCM, MeOH, Et$_2$O and the residue solvent was removed in vacuo to give the hydrazide resin $S_9$ (0.42 mmol/g) and $S_{10}$ (0.33 mmol/g). The initial loading was measured by the UV-absorbance of the piperidine-fulvene adduct ($\lambda = 301$ nm, $\varepsilon = 7800$ M$^{-1}$cm$^{-1}$).

**General procedures for SPPS**

**Fmoc-Deprotection:** The hydrazine resin was deprotected by treating with 20% piperidine in DMF at 40 °C for 10 min twice. Then the resin was wash with DMF (3 times), DCM (3 times), DMF (3 times).

**Coupling with normal amino acids:** After Fmoc-deprotection, the hydrazine resin was treated with a solution of 8.0 equiv. Fmoc-protected amino acids [Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Ser(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Val-OH, Fmoc-Asp(OrBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(SrBu)-OH, Fmoc-His(Boc)-OH], HATU, 16.0 equiv. DIPEA in DMF (the concentration of amino acid: 0.25 M) at 40 °C for 30 min.

**Coupling with Glycosylated amino acid S8/photo-cleavable moiety 4:** After deprotection, the hydrazine resin was treated with a solution of compound S8/4 (1.6 equiv.), HATU (1.6 equiv.), HOAt (1.6 equiv.) and DIPEA (3.2 equiv.) in DMF$^3$ (the concentration of amino acid: 0.2 M) at 40 °C for 30 min, and then added a solution of compound S8/4 (0.4 equiv.), HATU (0.4 equiv.), HOAt (0.4 equiv.) and DIPEA (0.8 equiv.) in DMF (the concentration of amino acid: 0.2 M) to repeat the procedure.

**Capping:** The resin was washed by DMF (3 times) after coupling, and then treated with a solution of Ac$_2$O/lutidine/DMF (5/6/89, v/v/v) at 40 °C for 5 min to cap N-terminal residual amine. Then the resin was wash with DMF (3 times), DCM (3 times), DMF (3 times).

**Solid phase synthesis of 5a on hydrazine resin S9 (0.05 mmol)**

The peptide 5a was synthesized according to the Fmoc-based SPPS outlined in the general procedures.
above. After the last SPPS cycle, the resin was washed with DMF (3 x 5 mL), DCM (3 x 5 mL), MeOH (3 x 5 mL), Et2O (3 x 5 mL) and dried. The resin bound peptides was cleaved with Reagent H6 (2 mL) at room temperature for 2 h. The mixture was filtered and the resin was washed with Reagent H twice, and the filtrates were combined and concentrated to ~200 μL with a nitrogen flow. Then the residue was precipitated with ice-cold Et2O. The mixture was filtered and the white filter cake was collected and dried in vacuo to give crude peptide 5a. The crude product was purified by HPLC and lyophilized to afford a white powder (12.6 mg, 4.2 x 10⁻³ mmol, 8 % isolated yield).

**Figure S1.** Analytical LCMS trace of the purified 5a; A) Analytical HPLC: tR = 5.3 min (2-30% solvent B in solvent A over 10 min, λ = 214 nm, C4 column); B) ESI-MS calculated for 5a (C127H183N48O37S): [M + 5H]⁺ m/z = 1504.1, [M + 3H]³⁺ m/z = 1003.1, [M + 4H]⁴⁺ m/z = 752.1, [M + 5H]⁵⁺ m/z = 602.2, found: 1504.1, 1003.5, 753.1, 602.6.

**Solid phase synthesis of 5b on hydrazine resin S9 (0.05 mmol)**

The peptide 5b was synthesized according to the Fmoc-based SPPS outlined in the general procedures above. After the last SPPS cycle, the resin was washed with DMF (3 x 5 mL), DCM (3 x 5 mL), MeOH (3 x 5 mL), Et2O (3 x 5 mL) and dried. The resin bound peptides was cleaved from the resin by treating with cocktail B (TFA/Phenol/H2O/i-Pr3SiH =88/5/5/2, v/v/v/v, 2mL) at room temperature for 2 h. The mixture was filtered and the resin was washed with cocktail B twice, and the filtrates were combined and concentrated to ~200 μL with a nitrogen flow. Then the residue was precipitated with ice-cold Et2O. The mixture was filtered and the white filter cake was collected and dried in vacuo to give crude peptide 5b. The crude product was purified by HPLC and lyophilized to afford a white powder (10.5 mg, 5.2 x 10⁻³ mmol, 10% isolated yield).
**Figure S2.** Analytical LCMS trace of the purified 5b; A) Analytical HPLC: $t_R = 7.1$ min (2-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C18 column); B) ESI-MS calculated for 5b ($C_{84}H_{137}N_{27}O_{27}S_2$): $[M + 2H]^{2+} \text{m/z} = 1011.7$, $[M + 3H]^{3+} \text{m/z} = 674.8$, found: 1011.6, 674.8.

**Solid phase synthesis of 8a on hydrazine resin S10 (0.132 mmol)**

The peptide 8a was synthesized according to the Fmoc-based SPPS outlined in the general procedures above. After the last SPPS cycle, the resin was washed with DMF (3 × 5 mL) and then treated with 10% hydrazine monohydrate in DMF to remove the acetyl protection on the saccharides. After 10 h then the resin was washed with DMF (3 × 5 mL), DCM (3 × 5 mL), MeOH (3 × 5 mL), EtO (3 × 5 mL) and dried. Subsequently, the resin bound peptide was cleaved with Reagent H (3 mL) from resin at room temperature for 2 h. The mixture was filtered and the resin was washed with Reagent H twice, and the filtrates were combined and concentrated to ~200 μL with a nitrogen flow. Then the residue was precipitated with ice-cold EtO. The mixture was filtered and the white filter cake was collected and dried in vacuo to give crude peptide 8a. The crude product was purified by HPLC and lyophilized to afford a white powder (279 mg, 0.06 mmol, 45% isolated yield).
Figure S3. Analytical LCMS trace of the purified 8a; A) Analytical HPLC: $t_R = 4.7$ min (5-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C4 column); B) ESI-MS calculated for 8a ($C_{227}H_{359}N_{63}O_{100}S$): $[M + 3H]^{3+} / m/z = 1868.6$, $[2M + 7H]^{7+} / m/z = 1601.8$, $[M + 4H]^{4+} / m/z = 1401.7$, $[M + 5H]^{5+} / m/z = 1121.6$, $[M + 6H]^{6+} / m/z = 934.8$, found: 1869.1, 1602.4, 1402.0, 1121.9, 935.2.

Solid phase synthesis of 8b on hydrazine resin S10 (0.132 mmol)

The peptide 8b was synthesized according to the Fmoc-based SPPS outlined in the general procedures above. After the last SPPS cycle, the resin was washed with DMF (3 × 5 mL) and then treated with 10% hydrazine monohydrate in DMF to remove the acetyl protection on the saccharides. After 10 h, the resin was washed with DMF (3 × 5 mL), DCM (3 × 5 mL), MeOH (3 × 5 mL), Et2O (3 × 5 mL) and dried. Subsequently, the resin bound peptide was cleaved from the resin by treating with cocktail B (TFA/Phenol/H2O/i-Pr3SiH =8 8/5/5/2, v/v/v/v, 3 mL) at room temperature for 2 h. The mixture was filtered and the resin was washed with cocktail B twice, and the filtrates were combined and concentrated to ~200 μL with a nitrogen flow. Then the residue was precipitated with ice-cold Et2O. The mixture was filtered and the white filter cake was collected and dried in vacuo to give crude peptide 8b. The crude product was purified by HPLC and lyophilized to afford a white powder (279 mg, 0.06 mmol, 45% isolated yield).
Figure S4. Analytical LCMS trace of the purified 8b; A) Analytical HPLC: $t_R = 7.1$ min (5-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C4 column); B) ESI-MS calculated for 8b ($C_{184}H_{312}N_{42}O_{90}S_2$): $[2M + 5H]^5+ m/z = 1849.0$, $[M + 3H]^3+ m/z = 1540.0$, $[M + 4H]^4+ m/z = 1155.2$, found: 1848.3, 1540.4, 1155.6.

**Solid phase synthesis of 13c on hydrazine resin S10 (0.012 mmol)**

The AFGP mimic 13c was synthesized on a preloaded resin S10 (0.012 mmol) according to Fmoc-strategy SPPS protocol (See general procedures for SPPS). After the last SPPS cycle, the resin was washed with DMF ($3 \times 5$ mL) and then treated with 10% hydrazine monohydrate in DMF to remove the acetyl protection on the saccharides. After 10 h, the resin was washed with DMF ($3 \times 3$ mL), MeOH ($3 \times 3$ mL), EtO ($3 \times 3$ mL) and dried. The resin was treated with Cocktail B (TFA/Phenol/H$_2$O/i-Pr$_3$SiH =8 8/5/5/2, v/v/v/v, 3 mL) at room temperature for 2 h. The mixture was filtered and the resin was washed with cocktail B twice, and the filtrates were combined and concentrated to ~100 µL with a nitrogen flow, and the residue was precipitated with ice-cold EtO. The white solid was filtered and dried in vacuo to give crude peptide 13c. The crude peptide was purified by HPLC and lyophilized to afford a white powder (12 mg, 0.005 mmol, 42% isolated yield).
Figure S5. Analytical LCMS trace of the purified 13c; A) Analytical HPLC: $t_R = 6.4$ min (2-20% Solvent B in solvent A over 10 min, $\lambda = 214$ nm, C18 column); B) ESI-MS calculated for 13c ($C_{90}H_{154}N_{22}O_{45}$): $[M + 2H]^{2+}$/m/z = 1133.2, $[M + 3H]^{3+}$/m/z = 755.8, found: 1133.6, 756.1.

4. The assembly of peptide segments

4.1 General procedures for peptide assembly

Activation of peptide hydrazide to thioester and the subsequent NCL: To a solution (6 M Gnd-HCl, 0.2 M phosphate, 0.2 M MPAA, pH 3) of peptide hydrazide 5a-10a (1.8 mM, 1.0 equiv.), a solution of acetylacetonate (150 mM, 2.0 equiv.) was added. After vigorously stirring at room temperature for 5-10 h, the reaction was monitored by LC-MS. After completion, the solution was adjusted to pH 6.5 and mixed with the solution (6 M Gnd-HCl, 0.2 M phosphate, 20 mM TCEP, pH 6.5, 1/3 volume of thioester solution) of another peptide segment 5b-10b with a free N-terminal cysteine (7 mM, 1.2-1.5 equiv.). The mixture was degassed by sparging with argon, and then stirred at 37 °C for 1 h.

Purification of NCL product with Ni-NTA resin: Ni-NTA resin (50% suspension, 3.3 × 10⁻⁴ mmol/mL, 1.0 equiv.) was washed and equilibrated with washing buffer (6 M Gnd-HCl, 0.2 M phosphate, pH 7) for 5 min. Then the NCL reaction solution was added to the beads. A gentle shaking was performed until no ligation product left in the solution monitored by LC-MS. The Ni-NTA resin was washed three times with washing buffer (6 M Gnd-HCl, 0.2 M phosphate, pH 7) and distilled water, respectively. Subsequently, the peptide hydrazide containing His₆ tag was eluted in an acidic solution (6 M Gnd-HCl, 0.2 M phosphate, pH 3) which was directly used in the next thioesterification cycle or PPH removal.

Removal of PPH: To the elution of Ni-NTA resin from last procedure, the semicarbazide hydrochloride
(50 mM) and DTT (50 mM) were added. After degassed with a steady flow of argon for 10 min, the solution was irradiated with UV light (365 nm, 20 W). The reaction was completed in 15 min, and then the product was purified with HPLC and lyophilized to yield a white solid.

4.2 Synthetic Details and Analytical data

4.2.1 Activation of peptide hydrazide 5a to thioester 5SR:
To a solution (6 M Gnd-HCl, 0.2 M phosphate, 0.2 M MPAA, pH 3, 1.6 mL) of peptide hydrazide 5a (10 mg, 2.9 × 10⁻³ mmol, 1.0 equiv.), a solution of acetylacetonate (150 mM, 2.0 equiv., 39 μL) was added. The resulting mixture was vigorously stirred at room temperature for 5 h to give 5SR. After completion monitored by LC-MS, the yield was calculated by peak-integration of the HPLC profile (λ = 214 nm) (2.9 × 10⁻³ mmol, > 99% HPLC yield).

Figure S6. Analytical LCMS trace of the crude 5SR; A) Analytical HPLC: t_R = 5.7 min (2-30% solvent B in solvent A over 10 min, λ = 214 nm, C4 column); B) ESI-MS calculated for 5SR (C₁₃₅H₁₈₇N₆₄O₉₉S₂): [M + 2H]²⁺ m/z = 1572.2, [M + 3H]³⁺ m/z = 1048.5, [M + 4H]⁴⁺ m/z = 786.6, [M + 5H]⁵⁺ m/z = 629.5, found: 1574.8, 1049.0, 787.2, 629.9.

4.2.2 Synthesis of 6a via ligation of 5SR and 5b and the subsequent Ni-NTA purification
The solution (from 4.2.1) containing 5SR (2.9 × 10⁻³ mmol, 1.0 equiv.) was adjusted to pH 6.5 and mixed with the solution (6 M Gnd-HCl, 0.2 M phosphate, 20 mM TCEP, pH 6.5, 500 μL) of peptide 5b (3.5 × 10⁻³ mmol, 7.0 mM, 1.2 equiv., 7.1 mg). The mixture was degassed by sparging with argon and stirred at 37 °C for 1 h. Subsequently, the crude product 6a was purified with Ni-NTA resin as stated in general procedures for peptide assembly. The acidic elution was then directly used in the next thioesterification cycle or PPH removal. The yield was calculated by peak-integration of the HPLC profile (λ = 214 nm) (2.6 × 10⁻³ mmol, 89% HPLC yield).
Figure S7. Analytical LCMS trace of 6a; A) and B): Analytical HPLC of crude and Ni-NTA resin purified 6a, tR = 5.8 min (2-30% solvent B in solvent A over 10 min, λ = 214 nm, C4 column); C) ESI-MS calculated for 6a (C207H308N73O64S2): [M + 3H]^{3+} m/z = 1636.8, [M + 4H]^{4+} m/z = 1227.8, [M + 5H]^{5+} m/z = 982.5, [M + 6H]^{6+} m/z = 818.9, [M + 7H]^{7+} m/z = 702.0, [M + 8H]^{8+} m/z = 614.4, found: 1637.3, 1228.2, 982.2, 819.3, 702.4, 614.8.

4.2.3 PPH removal of 6a to yield 40mer 6b
To the acidic solution (5 mL, from 4.2.2) with eluted 6a (1.9 × 10^{-3} mmol) from Ni-NTA resin, semicarbazide hydrochloride (50 mM, 0.25 mmol, 28 mg) and DTT (50 mM, 0.25 mmol, 39 mg) were added. After degassed with a steady flow of argon for 10 min, the solution was irradiated with UV light (365 nm, 20 W). The reaction was completed in 15 min, and then the product was purified with HPLC and lyophilized to yield a white solid 6b (4.0 mg, 1.0 × 10^{-3} mmol, 52% isolated yield from 5a).
Figure S8. Analytical LCMS trace of 6b; A) and B): Analytical HPLC of crude and HPLC purified 6b, t<sub>R</sub> = 6.6 min (2-30% solvent B in solvent A over 10 min, λ = 214 nm, C4 column); C) ESI-MS calculated for 6b (C<sub>207</sub>H<sub>308</sub>N<sub>73</sub>O<sub>64</sub>S<sub>2</sub>): [M + 3H]<sup>+</sup> m/z = 1279.1, [M + 4H]<sup>+</sup> m/z = 959.6, [M + 5H]<sup>+</sup> m/z = 767.9, [M + 6H]<sup>+</sup> m/z = 640.1, found: 1279.1, 959.6, 767.9, 640.1.

4.2.4 Activation of peptide hydrazide 6a to thioester 6SR:
The solution (from 4.2.2) with of eluted 6a (7.0 × 10<sup>-4</sup> mmol) from the Ni-NTA resin was concentrated to 380 μL by ultrafiltration (Millipore MWCO 3000), MPAA (0.2 M, 12 mg, 0.076 mmol) and a solution of acetylacetone (150 mM, 2.0 equiv., 9 μL) were then added. The mixture was vigorously stirred after adjustment of pH to 3.0, and the reaction was monitored by LC-MS. The conversion was completed in 5 h to give 6SR. The yield was calculated by peak-integration of the HPLC profile (λ = 214 nm) (7.0 × 10<sup>-4</sup> mmol, > 99% HPLC yield).
Figure S9. Analytical LCMS trace of the crude 6SR: A) Analytical HPLC: $t_R = 6.6$ min (2-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C4 column); B) ESI-MS calculated for 6SR (C_{215}H_{312}N_{71}O_{66}S_{3}): $[M + 3H]^3^+$ m/z = 1682.2, $[M + 4H]^4^+$ m/z = 1261.9, $[M + 5H]^5^+$ m/z = 1009.7, $[M + 6H]^6^+$ m/z = 841.6, $[M + 7H]^7^+$ m/z = 721.5, $[M + 8H]^8^+$ m/z = 631.4, found: 1682.3, 1262.0, 1009.9, 841.8, 721.8, 631.6.

4.2.5 Synthesis of 7a via ligation of 6SR and 6b and the subsequent Ni-NTA purification
The solution (from 4.2.4) containing 6SR (7.0 × 10^{-4} mmol, 1.0 equiv.) was adjusted to pH 6.5 and mixed with the solution (6 M Gnd-HCl, 0.2 M phosphate, 20 mM TCEP, pH 6.5, 6 mL) of peptide 6b (1.0 × 10^{-3} mmol, 7.0 mM, 1.5 equiv., 4.0 mg). The mixture was degassed by sparging with argon and stirred at 37 ℃ for 1 h. Subsequently, the crude product 7a was purified with Ni-NTA resin as stated in general procedures for peptide assembly. The acidic elution was then directly used in the next thioesterification cycle or PPH removal. The yield was calculated by peak-integration of the HPLC profile ($\lambda = 214$ nm) (5.6 × 10^{-4} mmol, 80% HPLC yield).
**Figure S10.** Analytical LCMS trace of 7a; A) and B): Analytical HPLC of crude and Ni-NTA resin purified 7a, \( t_R = 6.9 \) min (2-30% solvent B in solvent A over 10 min, \( \lambda = 214 \) nm, C4 column); C) ESI-MS calculated for 7a (C\(_{36}\)H\(_{55}\)N\(_{12}\)O\(_{11}\)S\(_{4}\)): [M + 5H]\(^{+}\) m/z = 1742.9, [M + 6H]\(^{+}\) m/z = 1452.6, [M + 7H]\(^{+}\) m/z = 1245.2, [M + 8H]\(^{+}\) m/z = 1089.7, [M + 9H]\(^{+}\) m/z = 968.7, [M + 10H]\(^{+}\) m/z = 872.0, [M + 11H]\(^{11+}\) m/z = 792.8, [M + 12H]\(^{12+}\) m/z = 726.8, [M + 13H]\(^{13+}\) m/z = 671.0, [M + 14H]\(^{14+}\) m/z = 623.1, found: 1742.8, 1452.5, 1245.4, 1089.8, 968.9, 872.1, 793.0, 727.0, 671.2, 623.3.

### 4.2.6 PPH removal of 7a to yield 80mer 7b

To the acidic solution (1 mL, from 4.2.5) with eluted 7a (5.6 × 10\(^{-4}\) mmol) from Ni-NTA resin, semicarbazide hydrochloride (50 mM, 0.25 mmol, 5.6 mg) and DTT (50 mM, 0.25 mmol, 7.8 mg) were added. After degassed with a steady flow of argon for 10 min, the solution was irradiated with UV light (365 nm, 20 W). The reaction was completed in 15 min, and then the product was purified with HPLC and lyophilized to yield a white solid 7b (2.2 mg, 2.5 × 10\(^{-3}\) mmol, 30% isolated yield from 5a).
Figure S11. Analytical LCMS trace of 6b; A) and B): Analytical HPLC of crude and HPLC purified 6b, t\text{R} = 7.5 min (2-30% solvent B in solvent A over 10 min, \( \lambda = 214 \) nm, C4 column); C) ESI-MS calculated for 6b (C\(_{320}\)H\(_{504}\)N\(_{102}\)O\(_{108}\)S\(_{4}\)): [M + 5H]\(^+\) m/z = 1528.3, [M + 6H]\(^+\) m/z = 1273.7, [M + 7H]\(^+\) m/z = 1091.9, [M + 8H]\(^+\) m/z = 955.6, [M + 9H]\(^+\) m/z = 849.5, [M + 10H]\(^+\) m/z = 764.6, [M + 11H]\(^+\) m/z = 695.2, found: 1528.2, 1273.7, 1092.0, 955.6, 849.6, 764.7, 695.4.

4.2.7 Activation of peptide hydrazide 8a to thioester 8Sr:

To a solution (6 M Gnd-HCl, 0.2 M phosphate, 0.2 M MPAA, pH 3, 19 mL) of peptide hydrazide 8a (196 mg, 0.035 mmol, 1.0 equiv.), a solution of acetylacetonate (150 mM, 2.0 equiv., 470 \( \mu \)L) was added. The resulting mixture was vigorously stirred at room temperature for 10 h. After completion monitored by LC-MS, the yield was calculated by peak-integration of the HPLC profile (\( \lambda = 214 \) nm) (0.035 mmol, > 99% HPLC yield).
A) Figure S12. Analytical LCMS trace of the crude 8SR: A) Analytical HPLC: $t_R = 5.5$ min (5-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C4 column); B) ESI-MS calculated for 8SR (C_{235}H_{363}N_{61}O_{102}S_{2}): [M + 3H]$^+$ m/z = 1914.0, [2M + 7H]$^+$ m/z = 1640.7, [M + 4H]$^+$ m/z = 1435.7, [M + 5H]$^+$ m/z = 1148.8, [M + 6H]$^+$ m/z = 957.5, found: 1914.8, 1641.3, 1436.3, 1149.3, 958.0.

4.2.8 Synthesis of 9a via ligation of 8SR and 8b and the subsequent Ni-NTA purification

The solution (from 4.2.7) containing 8SR (0.035 mmol, 1.0 equiv.) was adjusted to pH 6.5 and mixed with the solution (6 M Gnd-HCl, 0.2 M phosphate, 20 mM TCEP, pH 6.5, 6 mL) of peptide 8b (0.042 mmol, 7.0 mM, 1.2 equiv., 194 mg). The mixture was degassed by sparging with argon and stirred at 37 °C for 1 h. Subsequently, the crude product 9a was purified with Ni-NTA resin as stated in general procedures for peptide assembly. The acidic elution was then directly used in the next thioesterification cycle or PPH removal. The yield was calculated by peak-integration of the HPLC profile ($\lambda = 214$ nm) (0.035 mmol, > 99% HPLC yield).
**Figure S13.** Analytical LCMS trace of 9a; A) and B): Analytical HPLC of crude and Ni-NTA resin purified 9a, $t_R = 5.2$ min (5-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C4 column); C) ESI-MS calculated for 9a ($C_{40}H_{69}N_{10}O_{19}S_2$): [M + 6H]$^6+$ m/z = 1684.2, [M + 7H]$^7+$ m/z = 1443.8, [M + 8H]$^8+$ m/z = 1263.4, [M + 9H]$^9+$ m/z = 1123.4, [M + 10H]$^{10+}$ m/z = 1010.9, found: 1684.6, 1444.2, 1263.8, 1123.4, 1011.1.

**4.2.9 PPH removal of 9a to yield 60mer 9b**

To the solution (100 mL, from 4.2.8) with eluted 9a (0.028 mmol) from Ni-NTA resin, semicarbazide hydrochloride (50 mM, 5 mmol, 558 mg) and DTT (50 mM, 5 mmol, 771 mg) were added. After degassed with a steady flow of argon for 10 min, the solution was irradiated with UV light (365 nm, 20 W). The reaction was completed in 15 min, and then the product was purified with HPLC and lyophilized to yield a white solid 9b (86 mg, $9.5 \times 10^{-3}$ mmol, 34% isolated yield from 8a).
**Figure S14.** Analytical LCMS trace of 9b; A) and B): Analytical HPLC of crude and HPLC purified 9b, \( t_R = 5.8 \text{ min} \) (5-30\% solvent B in solvent A over 10 min, \( \lambda = 214 \text{ nm, C4 column} \)); C) ESI-MS calculated for 9b (C\(_{360}\)H\(_{604}\)N\(_{82}\)O\(_{180}\)S\(_{2}\)): [M + 5H]\(^{5+}\) m/z = 1806.1, [M + 6H]\(^{6+}\) m/z = 1505.2, [M + 7H]\(^{7+}\) m/z = 1290.3, found: 1806.6, 1505.8, 1290.9.

4.2.10 Activation of peptide hydrazide 9a to thioester 9SR:

The solution (from 4.2.8) with of eluted 9a (6.6 \( \times \) 10\(^{-3}\) mmol) from the Ni-NTA resin was concentrated to 3.0 mL by ultrafiltration (Millipore MWCO 3000). After that, MPAA (0.2 M, 101 mg, 0.6 mmol) and a solution of acetylacetone (150 mM, 2.0 equiv., 88 \( \mu \text{L} \)) were then added. The mixture was vigorously stirred after adjustment of pH to 3.0, and the reaction was monitored by LC-MS. The conversion was completed in 10 h. The yield was calculated by peak-integration of the HPLC profile (\( \lambda = 214 \text{ nm} \)) (6.6 \( \times \) 10\(^{-3}\) mmol, \( > 99\% \) HPLC yield).
**Figure S15.** Analytical LCMS trace of the crude 9SR: A) Analytical HPLC: $t_R = 6.3$ min (5-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C4 column); B) ESI-MS calculated for 9SR ($C_{415}H_{663}N_{101}O_{192}S_3$): $[M + 5H]^{5+}$ m/z = 1706.9, $[M + 6H]^{6+}$ m/z = 1463.2, $[M + 7H]^{7+}$ m/z = 1280.4, $[M + 8H]^{8+}$ m/z = 1138.3, found: 1707.7, 1463.9, 1281.2, 1138.9.

**4.2.11 Synthesis of 8a via ligation of 9SR and 9b and the subsequent Ni-NTA purification**

The solution (from 4.2.10) containing 9SR ($6.6 \times 10^{-3}$ mmol, 1.0 equiv.) was adjusted to pH 7 and mixed with the solution (6 M Gnd-HCl, 0.2 M phosphate, 20 mM TCEP, pH 6.5, 1 mL) of peptide 9b ($7.9 \times 10^{-3}$ mmol 7.9 mM, 1.2 equiv., 71 mg). The mixture was degassed by sparging with argon and stirred at 37 ºC for 1 h. Subsequently, the crude product 10a was purified with Ni-NTA resin as stated in general procedures for peptide assembly. The acidic elution was then directly used in the next thioesterification cycle or PPH removal. The yield was calculated by peak-integration of the HPLC profile ($\lambda = 214$ nm) ($4.0 \times 10^{-3}$ mmol, 61% HPLC yield).
**Figure S16.** Analytical LCMS trace of 10a; A) and B): Analytical HPLC of crude and Ni-NTA resin purified 10a; A) and B): Analytical HPLC of crude and Ni-NTA resin purified 10a, $t_r = 6.2$ min (5-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C4 column); C) ESI-MS calculated for 10a (C70H1239N183O370S4): [M + 10H]$^{10+}$ m/z = 1910.3, [M + 11H]$^{11+}$ m/z = 1736.7, [M + 12H]$^{12+}$ m/z = 1592.1, [M + 13H]$^{13+}$ m/z = 1470.0, [M + 14H]$^{14+}$ m/z = 1364.8, [M + 15H]$^{15+}$ m/z = 1274.5, [M + 16H]$^{16+}$ m/z = 1194.3, [M + 17H]$^{17+}$ m/z = 1124.1, [M + 18H]$^{18+}$ m/z = 1061.7, [M + 19H]$^{19+}$ m/z = 1005.9, [M + 20H]$^{20+}$ m/z = 955.6, found: 1911.3, 1737.4, 1592.7, 1470.2, 1365.4, 1274.5, 1195.0, 1124.6, 1062.1, 1006.2, 956.1.

4.2.12 Removal of PPH to yield 120mer peptide 10b

To the solution (17 mL, from 4.2.11) of eluted 10a (2.65 × 10^{-3} mmol) from Ni-NTA resin, the semicarbazide hydrochloride (50 mM, 0.85 mmol, 94 mg) and DTT (50 mM, 0.85 mmol, 131 mg) were added. After degassed with a steady flow of argon for 10 min, the solution was irradiated with UV light (365 nm, 20 W). The reaction was completed in 15 min, and then the product was purified with HPLC and lyophilized to yield a white solid 10b (15 mg, 8.4 × 10^{-4} mmol, 19% isolated yield from 8a).
Figure S17. Analytical LCMS trace of 10b; A) and B): Analytical HPLC of crude and HPLC purified 10b, \( t_R = 6.5 \) min (5-30% solvent B in solvent A over 10 min, \( \lambda = 214 \) nm, C4 column); C) ESI-MS calculated for 10b (C\(_{720}H_{1204}N_{162}O_{360}S_4\)): \([M + 10H]^{10+} \) m/z = 1802.9, \([M + 11H]^{11+} \) m/z = 1639.1, \([M + 12H]^{12+} \) m/z = 1502.5, \([M + 13H]^{13+} \) m/z = 1387.0, \([M + 14H]^{14+} \) m/z = 1288.0, \([M + 15H]^{15+} \) m/z = 1202.2, found: 1803.5, 1639.7, 1503.2, 1387.6, 1288.5, 1202.6.

4.2.13 Activation of peptide hydrazide 10a to thioester 10Sr:

The solution (from 4.2.11) of eluted 10a (1.14 \( \times 10^{-3} \) mmol, 1.0 equiv.) from the Ni-NTA resin was concentrated to 200 \( \mu \)L by ultrafiltration (Millipore MWCO 10000). And then, MPAA (0.2 M, 6.7 mg, 0.2 mmol) and a solution of acetylacetonate (150 mM, 2.0 equiv., 15.2 \( \mu \)L) were added. After vigorously stirred at room temperature for 10 h after adjustment of pH to 3.0, the reaction was monitored by LC-MS and the conversion was completed. The yield was calculated by peak-integration of the HPLC profile (\( \lambda = 214 \) nm) (9.2 \( \times 10^{-4} \) mmol, 80% HPLC yield).
Figure S18. Analytical LCMS trace of the crude 10SR: A) Analytical HPLC: tR = 6.8 min (5-30% solvent B in solvent A over 10 min, \( \lambda = 214 \) nm, C4 column); B) ESI-MS calculated for 10SR (C\(_{775}\)H\(_{1263}\)N\(_{181}\)O\(_{372}\)S\(_{5}\)): [M + 10H]\(^{10+}\) m/z = 1923.9, [M + 11H]\(^{11+}\) m/z = 1749.1, [M + 12H]\(^{12+}\) m/z = 1603.4, [M + 13H]\(^{13+}\) m/z = 1480.1, [M + 14H]\(^{14+}\) m/z = 1374.5, [M + 15H]\(^{15+}\) m/z = 1282.9, [M + 16H]\(^{16+}\) m/z = 1202.8, [M + 17H]\(^{17+}\) m/z = 1132.1, [M + 18H]\(^{18+}\) m/z = 1069.3, [M + 19H]\(^{19+}\) m/z = 1013.1, [M + 20H]\(^{20+}\) m/z = 962.4, found: 1925.0, 1749.9, 1604.1, 1480.9, 1375.2, 1283.6, 1203.7, 1132.7, 1070.0, 1013.5, 963.2.

4.2.14 Synthesis of 11a via ligation of 10SR and 10b and the subsequent Ni-NTA purification

The solution (from 4.2.13) containing 10SR (4.6 \times 10^{-4} \text{ mmol}, 1.0 equiv.) was adjusted to pH 6.5 and mixed with the solution (6 M Gnd-HCl, 0.2 M phosphate, 20 mM TCEP, pH 6.5, 80 \text{ \mu L}) of peptide 10b (5.5 \times 10^{-4} \text{ mmol}, 6.9 mM, 1.2 equiv., 9.9 mg). The mixture was degassed by sparging with argon and stirred at 37 \textdegree C for 1 h. Subsequently, the crude product 11a was purified with Ni-NTA resin as stated in general procedures for peptide assembly. The acidic elution was then directly used in the removal of PPH. The yield was calculated by peak-integration of the HPLC profile (\( \lambda = 214 \) nm) (2.1 \times 10^{-4} \text{ mmol}, 75\% HPLC yield).
Figure S19. Analytical LCMS trace of 11a; A) and B): Analytical HPLC of crude and Ni-NTA resin purified 11a, \( t_R = 6.6 \text{ min} \) (5-30% solvent B in solvent A over 10 min, \( \lambda = 214 \text{ nm} \), C4 column); C) ESI-MS calculated for 11a (C\(_{1487}\)H\(_{2459}\)N\(_{343}\)O\(_{730}\)S\(_{8}\)): [M + 19H]\(^{19+}\) m/z = 1952.5, [M + 21H]\(^{21+}\) m/z = 1766.7, [M + 23H]\(^{23+}\) m/z = 1613.1, [M + 25H]\(^{25+}\) m/z = 1484.2, [M + 27H]\(^{27+}\) m/z = 1374.3, [M + 29H]\(^{29+}\) m/z = 1279.6, [M + 31H]\(^{31+}\) m/z = 1197.1, [M + 33H]\(^{33+}\) m/z = 1126.1 [M + 35H]\(^{35+}\) m/z = 1060.4, [M + 37H]\(^{37+}\) m/z = 1003.1, found: 1953.6, 1767.3, 1613.8, 1485.1, 1374.8, 1280.3, 1197.8, 1126.1, 1060.8, 1003.6.

4.2.15 Removal of PPH to yield 240mer peptide 11b

To the solution (2.5 mL, from 4.2.14) with eluted 11a (2.1 \times 10^{-4} \text{ mmol}) from Ni-NTA resin, the semicarbazide hydrochloride (50 mM, 0.125 mmol, 14 mg) and DTT (50 mM, 0.125 mmol, 19 mg) were added. After degassed with a steady flow of argon for 10 min, the solution was irradiated with UV light (365 nm, 20 W). The reaction was completed in 15 min, and then the product was purified with HPLC and lyophilized to yield a white solid 11b (3.6 mg, 9.9 \times 10^{-5} \text{ mmol}, 9% isolated yield from 8a).
Figure S20. Analytical LCMS trace of the purified 11b; A) and B): Analytical HPLC of crude and HPLC purified 11b, t_R = 6.9 min (5-30% solvent B in solvent A over 10 min, λ = 214 nm, C4 column); C) ESI-MS calculated for 11b (C_{1440}H_{2404}N_{322}O_{720}S_{8}): [M + 19H]^{19+} m/z = 1896.0, [M + 20H]^{20+} m/z = 1801.3, [M + 21H]^{21+} m/z = 1715.5, [M + 22H]^{22+} m/z = 1637.6, [M + 23H]^{23+} m/z = 1566.4, [M + 24H]^{24+} m/z = 1501.2, [M + 25H]^{25+} m/z = 1441.2, [M + 26H]^{26+} m/z = 1385.8, [M + 27H]^{27+} m/z = 1335.3, [M + 28H]^{28+} m/z = 1286.9, [M + 29H]^{29+} m/z = 1242.5, [M + 30H]^{30+} m/z = 1201.1, found: 1896.1, 1802.0, 1715.9, 1638.3, 1567.0, 1502.1, 1441.8, 1386.4, 1335.3, 1287.6, 1243.2, 1201.9.

4.2.16 Synthesis of 10a via ligation of 10SR and 9b and the subsequent Ni-NTA purification
The solution (from 4.2.13) containing 10SR (4.6 × 10^{-4} mmol, 1.0 equiv.) was adjusted to pH 6.5 and mixed with the solution (6 M Gnd-HCl, 0.2 M phosphate, 20 mM TCEP, pH 6.5, 80 µL) of peptide 9b (5.5 × 10^{-4} mmol, 6.9 mM, 1.2 equiv., 5.0 mg). The mixture was degassed by sparging with argon and stirred at 37 °C for 1 h. Subsequently, the crude product 12a was purified with Ni-NTA resin as stated in general procedures for peptide assembly. The acidic elution was then directly used in the removal of PPH. The yield was calculated by peak-integration of the HPLC profile (λ = 214 nm) (2.8 × 10^{-4} mmol, 61% HPLC yield).
Figure S21. Analytical LCMS trace of 12a; A) and B): Analytical HPLC of crude and Ni-NTA resin purified 10a, t_R = 6.4 min (5-30% solvent B in solvent A over 10 min, λ = 214 nm, C4 column) c) ESI-MS calculated for 12a (C_{1127}H_{1859}N_{263}O_{550}S_{6}): [M + 15H]^{15+} m/z = 1873.4, [M + 16H]^{16+} m/z = 1756.4, [M + 17H]^{17+} m/z = 1653.1, [M + 18H]^{18+} m/z = 1561.3, [M + 19H]^{19+} m/z = 1479.2, [M + 20H]^{20+} m/z = 1405.3, [M + 21H]^{21+} m/z = 1338.4, [M + 22H]^{22+} m/z = 1277.6, [M + 23H]^{23+} m/z = 1222.1, [M + 24H]^{24+} m/z = 1171.2, [M + 25H]^{25+} m/z = 1124.4, [M + 26H]^{26+} m/z = 1081.2, [M + 27H]^{27+} m/z = 1041.2, [M + 28H]^{28+} m/z = 1004.1, found: 1874.5, 1757.2, 1653.5, 1562.0, 1479.7, 1405.9, 1339.0, 1278.2, 1222.7, 1171.8, 1124.7, 1081.8, 1041.3, 1004.7.

4.2.17 Removal of PPH to yield 180mer peptide 12b
To the solution (2.7 mL, from 4.2.16) with eluted 12a (2.8 × 10^{-4} mmol), the semicarbazide hydrochloride (50 mM, 0.135 mmol, 15 mg) and DTT (50 mM, 0.135 mmol, 21 mg) were added. After degassed with a steady flow of argon for 10 min, the solution was irradiated with UV light (365 nm, 20 W). The reaction was complete in 15 min, and then the product was purified with HPLC and lyophilized to yield a white solid 12b (2.7 mg, 1.0 × 10^{-4} mmol, 11% isolated yield from 8a).
Figure S22. Analytical LCMS trace of the purified 12b; A) and B): Analytical HPLC of crude and HPLC purified 12b, tR = 6.7 min (5-30% solvent B in solvent A over 10 min, λ = 214 nm, C4 column); C) ESI-MS calculated for 12b (C₁₀₈₀H₁₈₀₄N₂₄₂O₅₄₄S₆): [M + 14H]⁺ \( m/z = 1930.4 \), [M + 15H]⁺ \( m/z = 1801.8 \), [M + 16H]⁺ \( m/z = 1689.2 \), [M + 17H]⁺ \( m/z = 1590.0 \), [M + 18H]⁺ \( m/z = 1501.7 \), [M + 19H]⁺ \( m/z = 1422.7 \), [M + 20H]⁺ \( m/z = 1351.6 \), [M + 21H]⁺ \( m/z = 1287.3 \), found: 1931.1, 1802.7, 1698.8, 1590.5, 1502.3, 1423.2, 1352.3, 1288.0.

5. Metal-free desulfurization

80mer Muc1 7c:

To a solution (6 M Gnd-HCl, 0.2 M phosphate, 0.3 M TCEP, pH 7.0, 170 μL) of peptide 7b (0.9 mg, 1.2 \( \times 10^{-5} \) mmol, 1.0 equiv.), a mixed solution of reduced glutathione (200 mM, 17 μL) and VA-044 (50 mM, 2.0 equiv., 9 μL) in water was added. After degassed with a steady flow of argon for 10 min, the desulfurization was carried out at 65 °C for 3 h, followed by the addition of the mixed solution (26 μL) of reduced glutathione and VA-044 in water every 3 h. After 9 h, the product was purified with HPLC and lyophilized to obtain a white solid 7c (0.45 mg, 6.0 \( \times 10^{-5} \) mmol, 50% isolated yield)
**Figure S23.** Analytical LCMS trace of the purified 7c; A) Analytical HPLC: $t_R = 7.2$ min (2-30% solvent B in solvent A over 10 min, $\lambda = 214$ nm, C4 column); B) ESI-MS calculated for 7c ($C_{320}H_{504}N_{102}O_{108}$):

- $[M + 5H]^5+ m/z = 1502.6$,
- $[M + 6H]^6+ m/z = 1252.4$,
- $[M + 7H]^7+ m/z = 1073.6$,
- $[M + 8H]^8+ m/z = 939.5$,
- $[M + 9H]^9+ m/z = 835.2$,
- $[M + 10H]^{10+} m/z = 751.8$,
- $[M + 11H]^{11+} m/z = 683.6$,

found: 1502.7, 1252.3, 1073.6, 939.6, 835.4, 752.0, 683.7.

**30mer AFGP mimic 8c:**

To a solution (6 M Gnd⋅HCl, 0.2 M phosphate, 0.3 M TCEP, pH 7.0, 923 μL) of peptide 8b (10mg, 2.2 × 10⁻³ mmol, 1 equiv.), a mixed solution of reduced glutathione (200 mM, 183 μL) and VA-044 (50 mM, 2.0 equiv., 44 μL) in water was added. After degassed with a steady flow of argon for 10 min, the desulfurization was carried out at 65 °C for 3 h, followed by the addition of the mixed solution (450 μL) of reduced glutathione and VA-044 in water every 3 h. After 9 h, the product was purified with HPLC and lyophilized to obtain a white solid 8c (4.7 mg, 1.0 × 10⁻³ mmol, 45% isolated yield).
Figure S24. Analytical LCMS trace of the purified 8c; A) Analytical HPLC: \( t_R = 4.4 \) min (5-30% solvent B in solvent A over 10 min, \( \lambda = 214 \) nm, C4 column); B) ESI-MS calculated for 8c (C_{180}H_{304}N_{42}O_{90}): [2M + 5H]^{5+} m/z = 1799.8, [M + 3H]^{3+} m/z = 1500.0, [M + 4H]^{4+} m/z = 1125.2, found: 1800.3, 1500.4, 1125.5.

60mer AFGP mimic 9c:

To a solution (6 M Gnd-HCl, 0.2 M phosphate, 0.3 M TCEP, pH 7.0, 1 mL) of peptide 9b (5mg, \( 5.5 \times 10^{-4} \) mmol, 1.0 equiv.), a mixed solution of reduced glutathione (200 mM, 100 \( \mu \)L) and VA-044 (50 mM, 2.0 equiv., 22 \( \mu \)L) in water was added. After degassed with a steady flow of argon for 10 min, the desulfurization was carried out at 65 °C for 3 h, followed by the addition of the mixed solution (500 \( \mu \)L) of reduced glutathione and VA-044 in water every 3 h. After 9 h, the product was purified with HPLC and lyophilized to obtain a white solid 9c (2.5 mg, \( 2.8 \times 10^{-4} \) mmol, 50% isolated yield).
Figure S25. Analytical LCMS trace of the purified 9c; A) Analytical HPLC: t_R = 5.7 min (5-30% solvent B in solvent A over 10 min, λ = 214 nm, C4 column); B) ESI-MS calculated for 9c (C_{360}H_{604}N_{82}O_{180}): [M + 5H]^5+ m/z = 1793.2, [M + 6H]^6+ m/z = 1494.5, found: 1793.9, 1495.0.

120mer AFGP mimic 10c:
To a solution (6 M Gdn-HCl, 0.2 M phosphate, 0.3 M TCEP, pH 7.0, 800 μL) of peptide 10b (4mg, 2.2 × 10^{-4} mmol, 1.0 equiv.), a mixed solution of reduced glutathione (200 mM, 80 μL) and VA-044 (50 mM, 2.0 equiv., 18 μL) in water was added. After degassing with a steady flow of argon for 10 min, the desulfurization was carried out at 65 °C for 3 h, followed by the addition of the mixed solution (400 μL) of reduced glutathione and VA-044 in water every 3 h. After 9 h, the product was purified with HPLC and lyophilized to obtain a white solid 10c (2.3 mg, 1.3 × 10^{-4} mmol, 58% isolated yield)
Figure S26. Analytical LCMS trace of the purified 10c; A) Analytical HPLC: \( t_R = 6.2 \) min (5-30\% solvent B in solvent A over 10 min, \( \lambda = 214 \) nm, C4 column); B) ESI-MS calculated for 10c (C\(_{720}\)H\(_{1204}\)N\(_{162}\)O\(_{360}\)): [M + 9H]\(^9+\) m/z = 1989.0, [M + 10H]\(^{10+}\) m/z = 1790.0, [M + 11H]\(^{11+}\) m/z = 1627.4, [M + 12H]\(^{12+}\) m/z = 1491.9, [M + 13H]\(^{13+}\) m/z = 1377.2, [M + 14H]\(^{14+}\) m/z = 1278.9, found: 1989.7, 1790.4, 1627.9, 1492.5, 1377.9, 1279.6.

240mer AFGP mimic 11c:

To a solution (6 M Gnd-HCl, 0.2 M phosphate, 0.3 M TCEP, pH 7.0, 500 \( \mu \)L) of peptide 11b (2.5mg, 7.0 \( \times \) 10\(^{-5}\) mmol, 1.0 equiv.), a mixed solution of reduced glutathione (200 mM, 50 \( \mu \)L) and VA-044 (50 mM, 2.0 equiv., 11 \( \mu \)L) in water was added. After degassing with a steady flow of argon for 10 min, the desulfurization was carried out at 65 °C for 3 h, followed by the addition of the mixed solution (250 \( \mu \)L) of reduced glutathione and VA-044 in water every 3 h. After 9 h, the product was purified with HPLC and lyophilized to obtain a white solid 11c (1.8 mg, 4.9 \( \times \) 10\(^{-5}\) mmol, 70\% isolated yield)
**Figure S27.** Analytical LCMS trace of the purified 11c; A) Analytical HPLC: \( t_R = 6.8 \) min (5-30% solvent B in solvent A over 10 min, \( \lambda = 214 \) nm, C4 column); B) ESI-MS calculated for 11c (C\(_{1440}\)H\(_{2404}\)N\(_{322}\)O\(_{720}\)):

- \([M + 19H]^{19+}\) m/z = 1987.0,
- \([M + 20H]^{20+}\) m/z = 1882.5,
- \([M + 21H]^{21+}\) m/z = 1788.4,
- \([M + 22H]^{22+}\) m/z = 1703.3,
- \([M + 23H]^{23+}\) m/z = 1625.9,
- \([M + 24H]^{24+}\) m/z = 1555.3,
- \([M + 25H]^{25+}\) m/z = 1490.5,
- \([M + 26H]^{26+}\) m/z = 1430.9,
- \([M + 28H]^{28+}\) m/z = 1325.0,


**180mer AFGP mimic 12c:**

To a solution (6 M Gnd-HCl, 0.2 M phosphate, 0.3 M TCEP, pH 7.0, 800 \( \mu\)L) of peptide 12b (2.7 mg, \( 1.0 \times 10^{-4} \) mmol, 1.0 equiv.), a mixed solution of reduced glutathione (200 mM, 80 \( \mu\)L) and VA-044 (50 mM, 2.0 equiv., 18 \( \mu\)L) in water was added. After degassing with a steady flow of argon for 10 min, the desulfurization was carried out at 65 °C for 3 h, followed by the addition of the mixed solution (400 \( \mu\)L) of reduced glutathione and VA-044 in water every 3 h. After 9 h, the product was purified with HPLC and lyophilized to obtain a white solid 12c (1.5 mg, \( 5.7 \times 10^{-5} \) mmol, 57% isolated yield)
A) Analytical LCMS trace of the purified 12c; A) Analytical HPLC: \( t_R = 7.4 \) min (5-30% solvent B in solvent A over 10 min, \( \lambda = 214 \) nm, C4 column); B) ESI-MS calculated for 12c (C\(_{1080}\)H\(_{1804}\)N\(_{242}\)O\(_{540}\)): [M + 14H]+ m/z = 1916.7, [M + 15H]+ m/z = 1789.0, [M + 16H]+ m/z = 1677.2, [M + 17H]+ m/z = 1578.6, [M + 18H]+ m/z = 1491.0, [M + 19H]+ m/z = 1412.6, [M + 20H]+ m/z = 1342.0, found: 1917.7, 1791.3, 1678.8, 1580.0, 1491.6, 1413.6, 1342.5.

6. Circular dichroism spectropolarimetry

All synthetic AFGP mimics were dissolved in Milli-Q water (0.1 mg/mL) and incubated at 0, 10, 20, 30 and 40 °C. Far-UV CD spectra were carried out by means of Jasco J-815 Spectropolarimeter\(^7\) with cell length of 1 mm and the scan wavelength of from 190 nm to 240 nm.
Figure S29. Circular dichroism spectra of synthetic AFGP mimic 13c, 8c, 9c, 10c, 12c, 11c at various temperature ranging from 0 °C to 40 °C.

7. Evaluation of antifreeze activity

The measurement of thermal hysteresis activity (THA) to evaluate antifreeze activity was performed by means of Otago Nanoliter Osmometer (Otago Osmometers Ltd., Dunedin, New Zealand) according to the procedures reported previously. TH (TH = |Tm-Tf|) is defined to be the difference between melting point and freezing point of AF(G)P solution.

Figure S30. Ice crystal morphology in the presence of synthetic AFGP mimic at 10 mg/mL: a) 15mer AFGP mimic 13c, b) 30mer AFGP mimic 8c, c) 60mer AFGP mimic 9c.
8. NMR-spectra of novel compounds

Figure S31. $^1$H NMR spectrum of compound S1.

Figure S32. $^{13}$C NMR spectrum of compound S1.
Figure S33. $^1$H NMR spectrum of compound 3.

Figure S34. $^{13}$C NMR spectrum of compound 3
Figure S35. $^1$H NMR spectrum of compound 4.

Figure S36. $^{13}$C NMR spectrum of compound 4.
$^1$H NMR (400 MHz, Chloroform-$d$)

Figure S37. $^1$H NMR spectrum of compound S4α.

$^{13}$C NMR (101 MHz, Chloroform-$d$)

Figure S38. $^{13}$C NMR spectrum of compound S4α.
\(^1\)H NMR (400 MHz, Chloroform-\(d\))

Figure S39. \(^1\)H NMR spectrum of compound S4\(\beta\).

\(^{13}\)C NMR (101 MHz, Chloroform-\(d\))

Figure S40. \(^{13}\)C NMR spectrum of compound S4\(\beta\).
9. High-resolution mass spectra of AFGP mimic 10c, 12c, 11c.

**Figure S41.** HRMS (ESI) of antifreeze segment 10c. Molecular mass (Average): calculated for C_{720}H_{1204}N_{162}O_{360}: 17881.09587 Da; found: 17881.28700 Da, error: 10.689 ppm.

**Figure S42.** HRMS (ESI) of antifreeze segment 12c. Molecular mass (Average): calculated for C_{1080}H_{1804}N_{242}O_{540}: 26819.46600 Da; found: 26819.00000 Da, error: 17.380 ppm.
Figure S43. HRMS (ESI) of antifreeze segment 11c. Molecular mass (Average): calculated for $C_{1440}H_{2404}N_{322}O_{720}$: 35748.60600 Da; found: 35748.00000 Da, error: 16.950 ppm.
10. Reference