Table of contents

1. Materials

Fmoc-D-Thr(t-Bu)-Wang resin was purchased from GL Biochem Co., Ltd (Shanghai, China). 2-Chlorotrityl chloride resin was purchased from Tianjin Nankai Hecheng Science & Technology Co., Ltd (Tianjin, China). 9-Fluorenylmethyl carbazate (Fmoc-$NHNH_2$) was purchased from Adamas Reagent Co., Ltd (Shanghai, China). Fmoc-D-amino acids were purchased from GL Biochem Co., Ltd (Shanghai, China). Trifluoroacetic acid (TFA), N, N-dimethylformamide (DMF), thioanisole, triisopropylsilane (TIPS), 2,2′-azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044), Acetylacetone (acac), $PdCl_2$, Silver acetate were purchased from J&K Scientific Ltd (Beijing, China). 4-Mercaptophenylacetic acid (MPAA) was purchased from Alfa Aesar Chemicals Co., Ltd (Shanghai, China). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl) was purchased from Adamas-beta, Titan Scientific Co., Ltd (Shanghai). Tert-butyl mercaptan (t-BuSH), and 1,2-Ethanedithiol (EDT) was purchased from TCI Development Co., Ltd (Shanghai, China). Piperidine, $Na_2HPO_4·12H_2O$, and Et_2O were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Guanidine hydrochloride (Gn·HCl) and glycerin was purchased from GENERAL-REAGENT, Titan Scientific Co., Ltd (Shanghai). NaOH, $NaH_2PO_4·2H_2O$, hydrochloric acid, acetic acid, and NaCl were purchased from Sinopharm Chemical Reagent (Beijing, China). Dichloromethane (DCM) and $NaNO_2$ were purchased from Beijing Chemical Works (Beijing, China). Ethyl cyanoglyoxylate-2-oxime (Oxyma), N, N′-diisopropylcarbodiimide (DIC), ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl) aminomethane (Tris), and 2- [4-(2 hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) were purchased from Adamas Reagent Co., Ltd (Shanghai, China). Acetonitrile (HPLC grade) was purchase from J. T. Baker (Phillipsburg, NJ, USA). L-deoxynucleoside phosphoramidites and L-dNTPs were purchased from WuXi AppTec (Tianjin, China). 4S Gelred was purchased from Sangon Biotech Co., Ltd (shanghai, China). DNase I was purchased from Takara Bio Inc (Beijing, China)

2. Sequence alignment of wt Dpo4 and synthetic D-Dpo4

Except the five mutations for ligation, all the methionines are replaced by norleucines. All the mutation sites in D-Dpo4 are highlighted in the sequence.

```
wt Dpo4   M IVLFVDFDY FYAQVEEVLN PSLKGKPVVV CVFSGRFEDS GAVATANYEA RKFGVKAGIP
D-Dpo4    (Nle)IVLFVDFDY FYAQVEEVLN PSLKGKPVVV SVFSGRFEDS GAVATANYEA RKFGVKAGIP

          IVEAKKILPN AVYLP M RKEV YQQVSSRI M N LLREYSEKIE IASIDEAYLD ISDKVRDYRE
          IVEAKKILPN AVYLP(Nle)RKEV YQQVSCRI(Nle)N LLREYSEKIE IASIDEAYLD ISDKVRDYRE

          AYNLGLEIKN KILEKEKITV TVGISKNKVF AKIAAD M AKP NGIKVIDDEE VKRLIRELDI
          AYALGLEIKN KILEKEKITV TVGISKNKVF AKIAAD(Nle)AKP NGIKVIDDEE VKRLIRELDI

          ADVPGIGNIT AEKLKKLGIN KLVDTLSIEF DKLKG M IGEA KAKYLISLAR DEYNEPIRTR
          ADVPGIGNIT AEKLKKLGIN KLVDTLAIEF DKLKG(Nle)IGEA KAKYLISLAR DEYNEPIRTR

          VRKSIGRIVT  M KRNSRNLEE IKPYLFRAIE ESYYKLDKRI PKAIHVVAVT EDLDIVSRGR
          VRKSIGRIVT (Nle)KRNSRNLEE IKPYLFRAIE ESYYKLDKRI PKAIHVVAVT EDLDIVSRGR

          TFPHGISKET AYSESVKLLQ KILEEDERKI RRIGVRFSKF IEAIGLDKFF DT
          TFPHGISKET AYAESVKLLQ KILEEDERKI RRIGVRFSKF IEAIGLDKFF DT
```

3. Methods

3.1 Synthesis of Fmoc-hydrazine 2-Cl-(Trt)-Cl resin

The resin was prepared as previously described[1]. First, 50 mmol 2-Cl-(Trt)-Cl resin (0.5 mmol/g, 100 g) was swollen in 1 L dichloromethane (DCM). After 10 min, the solution containing 200 mmol Fmoc NHNH2·HCl (51 g) and 500 mmol diisopropylethylamine (DIEA, 85 mL) was added dropwise into the resin suspension at 0 ℃. The mixture was gently shaken at 37 ℃ overnight. Finally, the resin was capped by the 3 addition of 30 mL MeOH, and washed successively with N, N-dimethylformamide (DMF), H_2O, DMF, DCM, MeOH, and diethyl ether. The dried resin can be stored for a long time (over a year) at 4 ℃.

3.2 Fmoc based solid phase peptide synthesis

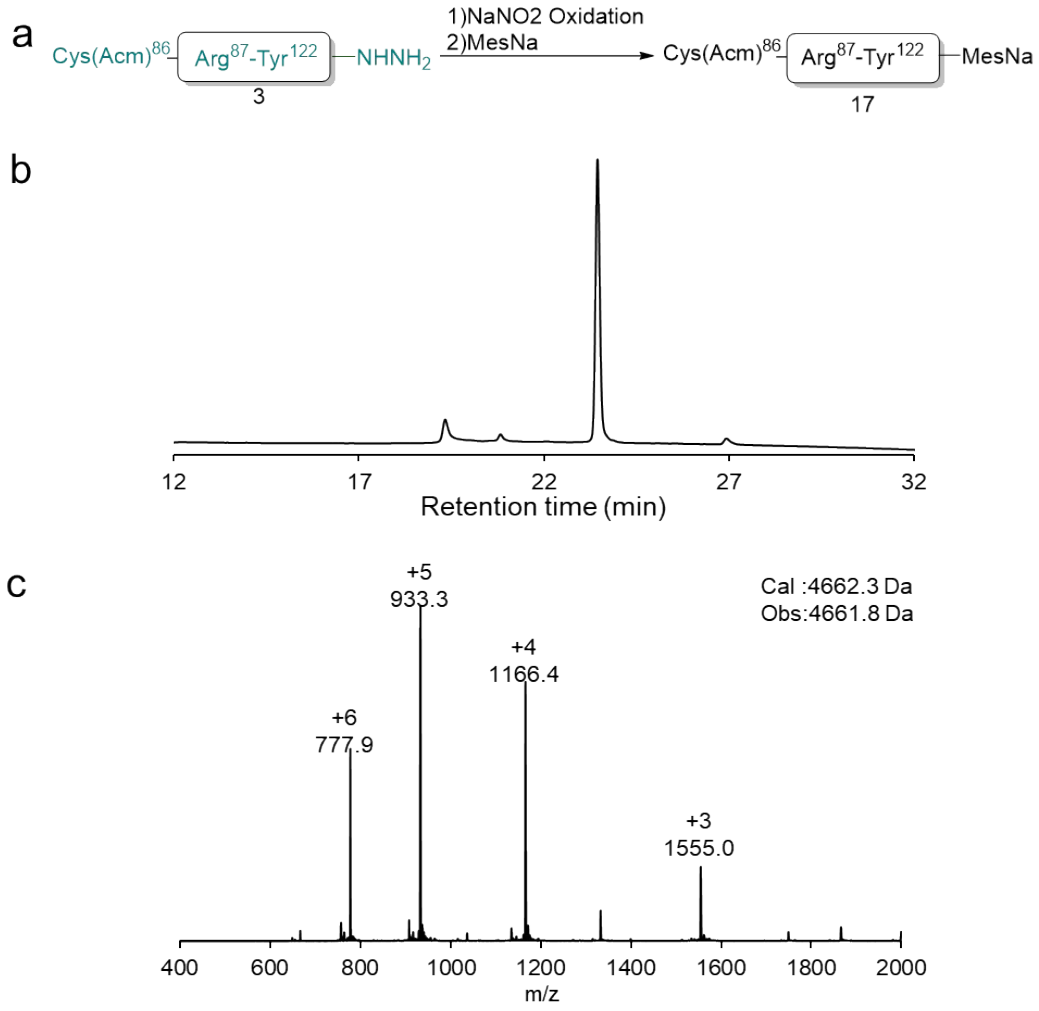
All peptides were synthesized by using the Liberty blue microwave peptide synthesizer (CEM Corporation). To achieve an optimal synthesis, we modified the standard synthesis procedure. The first step of every standard procedure was Fmoc deprotection by using 20% piperidine and 0.1 M Oxyma in DMF at 90 ℃ for 1 min. Then, a DMF washing step was carried out to clean the resin (4 times). Resin (0.25 mmol) and 4 eq. of the Fmoc amino acids (dissolved in DMF, 0.2 mM, 5 mL), 4 eq. Oxyma (dissolved in DMF, 1 mM, 1 mL), 4 eq. DIC (dissolved in DMF, 0.5 mM, 2 mL) were mixed and heated to 90 ℃ by microwave for 2 min. The standard synthesis procedure ended with DMF washing steps (3 times). After synthesis, the peptides were cleaved from resin using TFA cleavage cocktails (TFA/TIPS/water/thioanisole/EDT, 82.5:5:5:5:2.5, vol/vol/vol/vol/vol) for 3 h. Next, the TFA solution was collected and then concentrated by nitrogen blowing. The peptides were precipitated by cold ethyl ether. The mixture was centrifuged and the supernatant was discarded. This process was repeated 3 times, and the resulting peptide was purified by RP-HPLC to provide the target peptide.

3.3 RP-HPLC and ESI-MS

RP-HPLC analysis was carried out on Ultimate XB-C4 column (Welch, 5 μm, 4.6× 250 mm) at a flow rate of 1 mL/min. RP-HPLC purification was carried out on Ultimate XB-C4 column (for the ligation products, Welch, 5 μm, 10 × 250 mm) or Ultimate XB-C4 column (for the crude peptides, Welch, 5 μm, 21.2 × 150 mm) at a flow rate of 4-6 mL/min. The purified products were characterized by ESI-MS on a Shimadzu LC/MS-2020 system.

*3.4 Preparation ligation of peptide **17***

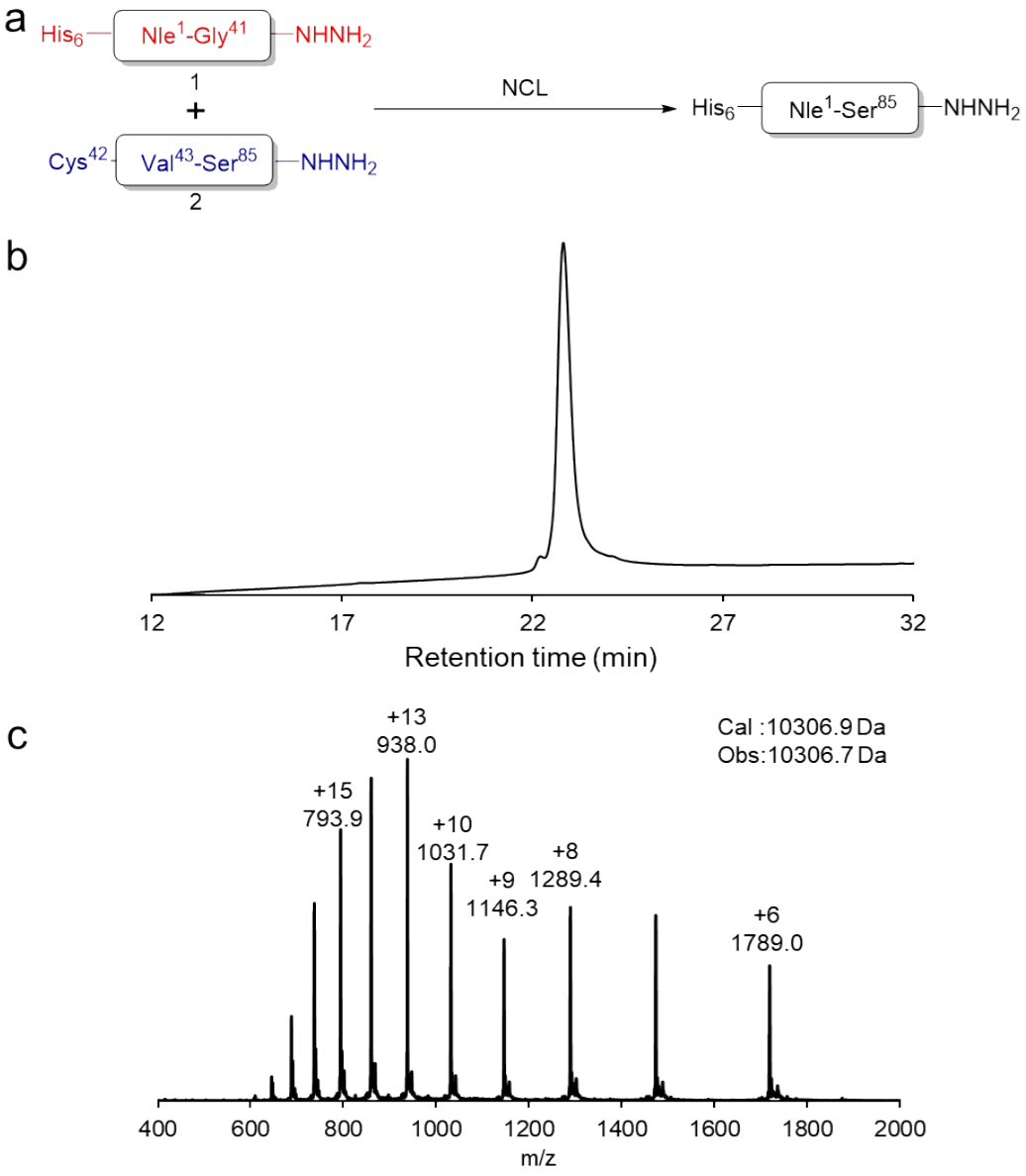
a) D-Dpo4-seg **3**-NHNH2(20 mg) was dissolved in 3 ml acidified ligation buffer (6 M Gn·HCl, 0.1 M Na_2HPO_4, pH 2.4). The mixture was cooled in ice-salt bath, and 88 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min. After which 50 mg MesNa was added, the pH of the reaction mixture was adjusted to 5.0 with NaOH solution at room temperature. After 1 h, the reaction mixture was purified by HPLC (purification conditions: 20-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). Peptide **17** was obtained with a yield of 60% (12 mg). b) Analytical HPLC chromatogram of **17** (λ=214 nm). Column: Welch C4. Gradient: 20%-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of **17**.



Supplementary Figure S1 Analytical HPLC chromatogram and ESI-MS spectrum of **17**

*3.5 Preparation ligation of peptide **1** and **2***

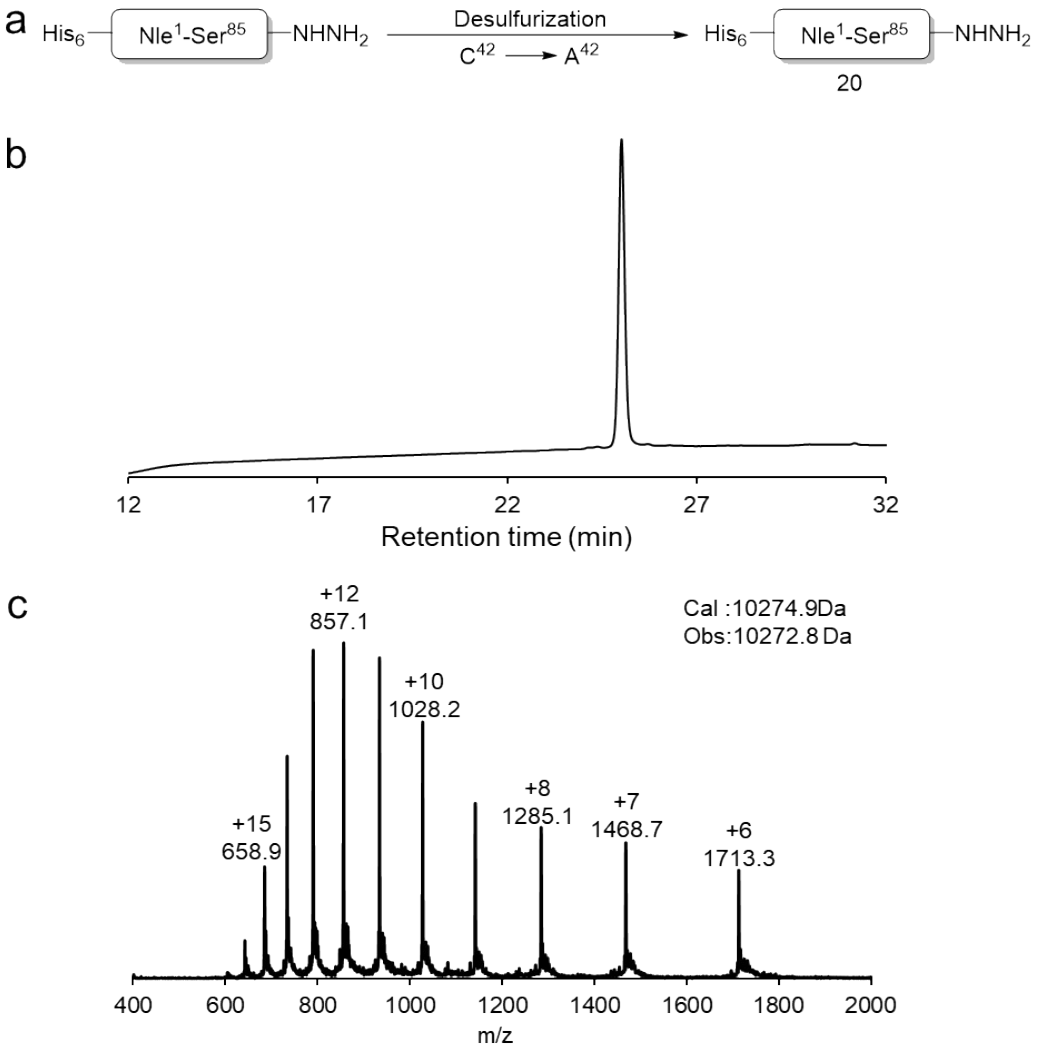
a) D-Dpo4-seg **1**-NHNH2(15mg) was dissolved in 2 ml acidified ligation buffer (6 M Gn·HCl, 0.1 M Na_2HPO_4, pH 2.4). The mixture was cooled in ice-salt bath, and 55 µl 0.5 M NaNO2 (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 23 mg MPAA was added. After the addition of D-Dpo4-**2** (12 mg), the pH of the reaction mixture was adjusted to 6.6 with NaOH solution at room temperature. After 15 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 20-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch CN column). The ligation product of **1** and **2** was obtained with a yield of 37.0% (9.34 mg). b) Analytical HPLC chromatogram of ligation product of **1** and **2** (λ=214 nm). Column: Welch C4. Gradient: 20%-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of the ligation product.



Supplementary Figure S2 Analytical HPLC chromatogram and ESI-MS spectrum of ligation product of **1** and **2**.

*3.6 Desulfurization of ligation product of **1** and **2***

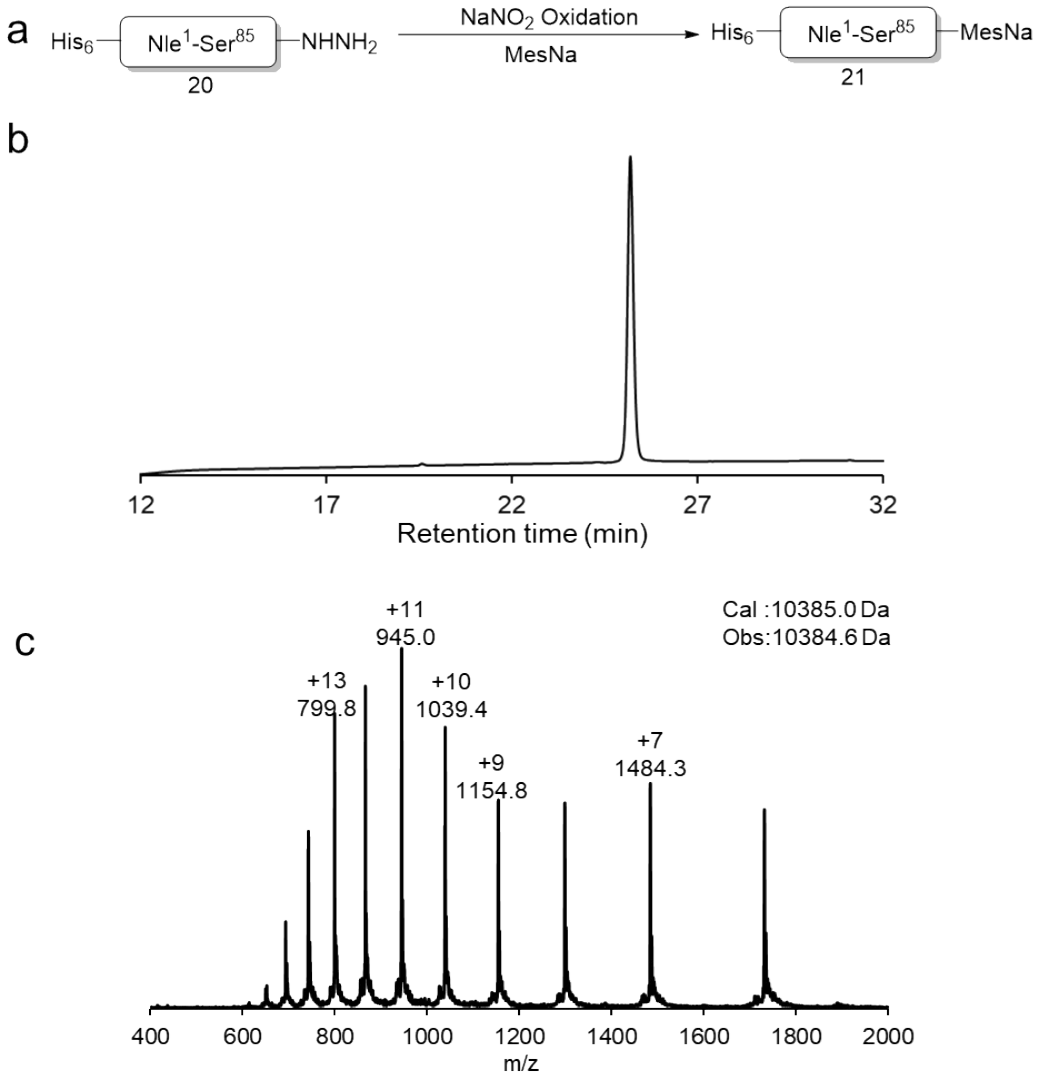
a) 9.34mg ligation product of **1** and **2** was dissolved in 3 ml 200 mM TCEP solution (6 M Gn·HCl and 0.1 Na_2HPO_4, pH 6.8), with 0.06 mmol (19.2 mg) VA-044 and 0.3 ml t-BuSH added. The reaction was under stirring overnight at 37 °C. The desulfurization product Dpo4-10 was analyzed by HPLC and ESI-MS and purified by semi-preparative HPLC (purification conditions: 20-80% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch CN column). After lyophilization, D-Dpo4-**20** was obtained with a yield of 71.0% (6.63mg). b) Analytical HPLC chromatogram of **20** (λ=214 nm). Column: Welch C4. Gradient: 20%-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of **20**.



Supplementary Figure S3 Analytical HPLC chromatogram and ESI-MS spectrum of 20

*3.7 Preparation of peptide **21***

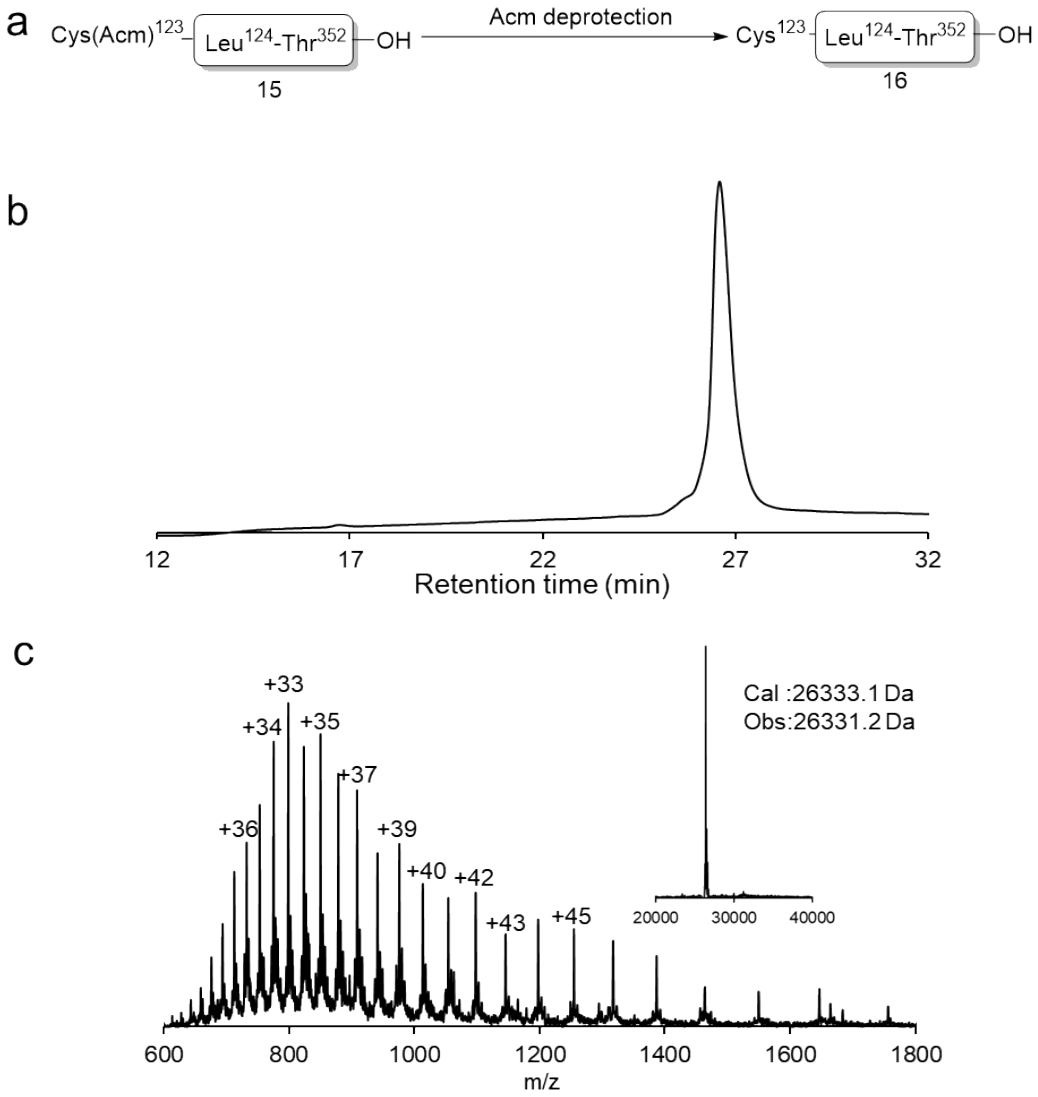
a) D-Dpo4-**10** (6 mg) was dissolved in 0.5 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH2PO4, pH 3.0). The mixture was cooled in ice-salt bath, and 12 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 8.4 mg MesNa was added. The pH of the reaction mixture was adjusted to 5.0 with NaOH solution at room temperature. After 1 h, the products were purified by HPLC (purification conditions: 20-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Dpo4-**21** was obtained with a yield of 74% (4.44 mg). b) Analytical HPLC chromatogram of **21** (λ=214 nm). Column: Welch C4. Gradient: 20%-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of **21**.



Supplementary Figure S4 Analytical HPLC chromatogram and ESI-MS spectrum of **21**

*3.8 Removal Acm protection of peptide **15***

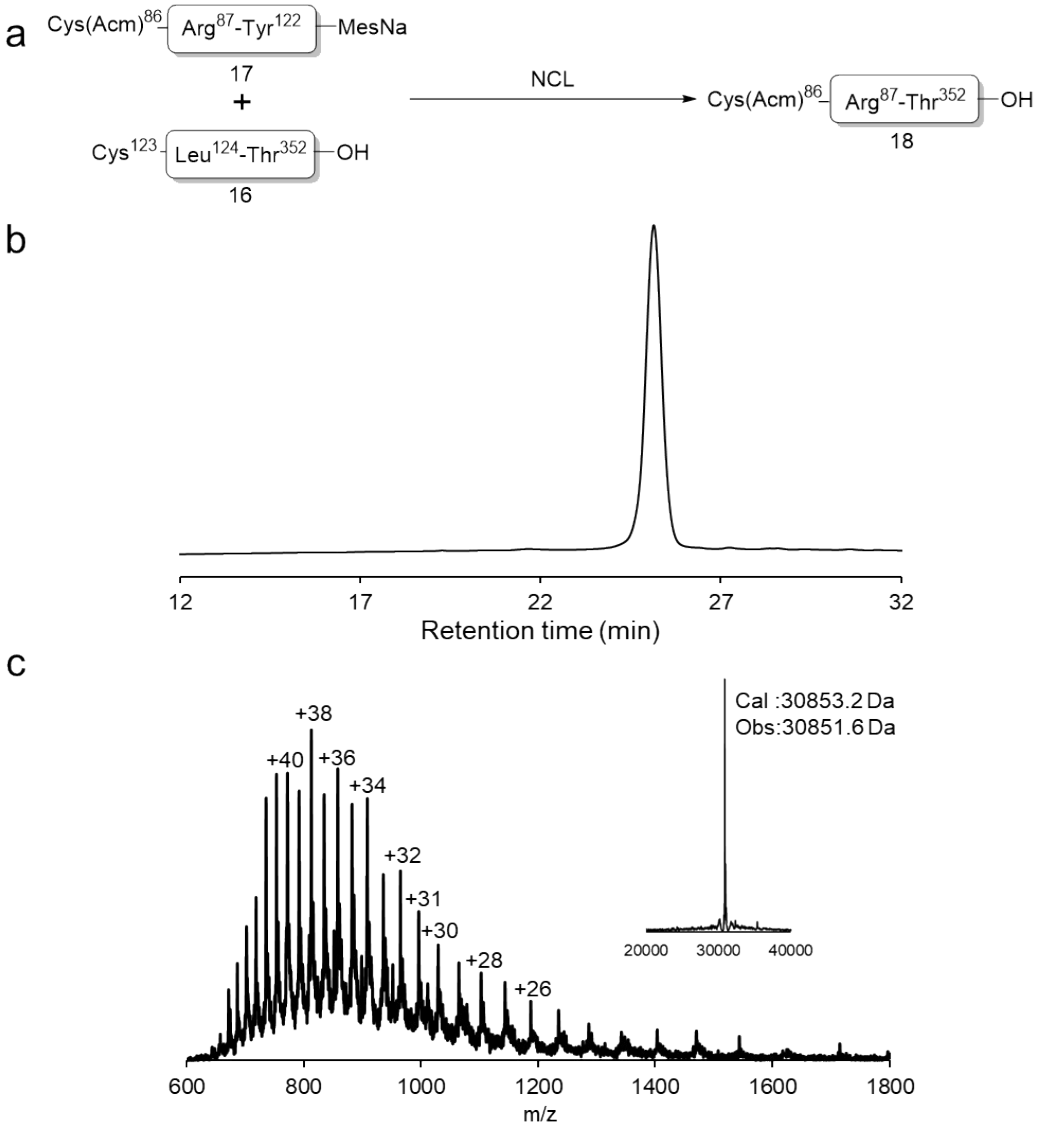
a) D-Dpo4-**15** (15 mg) was dissolved in a 550 µl Gn HCl buffer (containing 6 M Gn·HCl, 0.1 M Na_2HPO_4, 40 mM TCEP, pH 7). 2.9 mg $PdCl_2$ was dissolved in a 550 µl Gn·HCl buffer (6 M Gn·HCl, 0.1 M Na_2HPO_4, 40 mM TCEP, pH 7) and added to the peptide solution. After 16 h, 2 ml 1 M DTT (in an aqueous solution of 6 M Gn·HCl and 0.1 M Na_2HPO_4) was added. The reaction mixture was under stirring for 30 min and purified by HPLC (purification conditions: 20-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Dpo4-**16** was obtained with a yield of 72.7% (10.9 mg). b) Analytical HPLC chromatogram of **16** (λ=214 nm). Column: Welch C4. Gradient: 20%-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of **16**.



Supplementary Figure S5 Analytical HPLC chromatogram and ESI-MS and deconvoluted spectrum of **16**

*3.9 Ligation of **17** and **16***

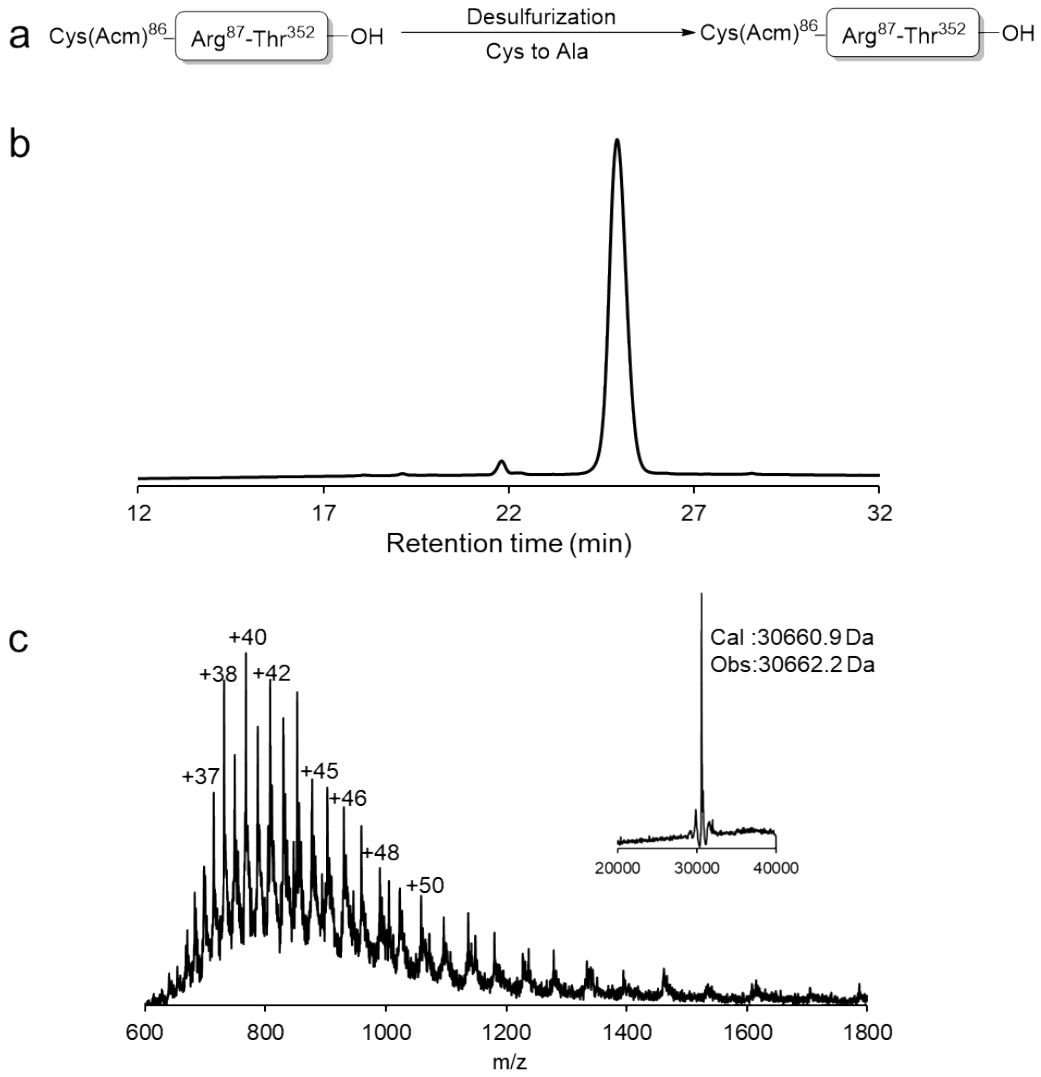
a) D-Dpo4-**17** (10.2mg) and D-Dpo4-**16** (23 mg) were dissolved in 850 μl ligation buffer (containing 6 M Gn·HCl, 0.1 M Na_2HPO_4, 100 mM MPAA, 40 mM TCEP, pH 7). the pH of the reaction mixture was adjusted to 6.6 with NaOH solution at room temperature. The reaction was carried out at 37 ℃. After 15h, the products were analyzed and purified by HPLC (purification conditions: 20-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Dpo4-**17** was obtained with a yield of 78.1% (21 mg). b) Analytical HPLC chromatogram of **18** (λ=214 nm). Column: Welch C4. Gradient: 20%-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of **18**.



Supplementary Figure S6 Analytical HPLC chromatogram and ESI-MS and deconvoluted spectrum of **18**

*3.10 Desulfurization of peptide **18***

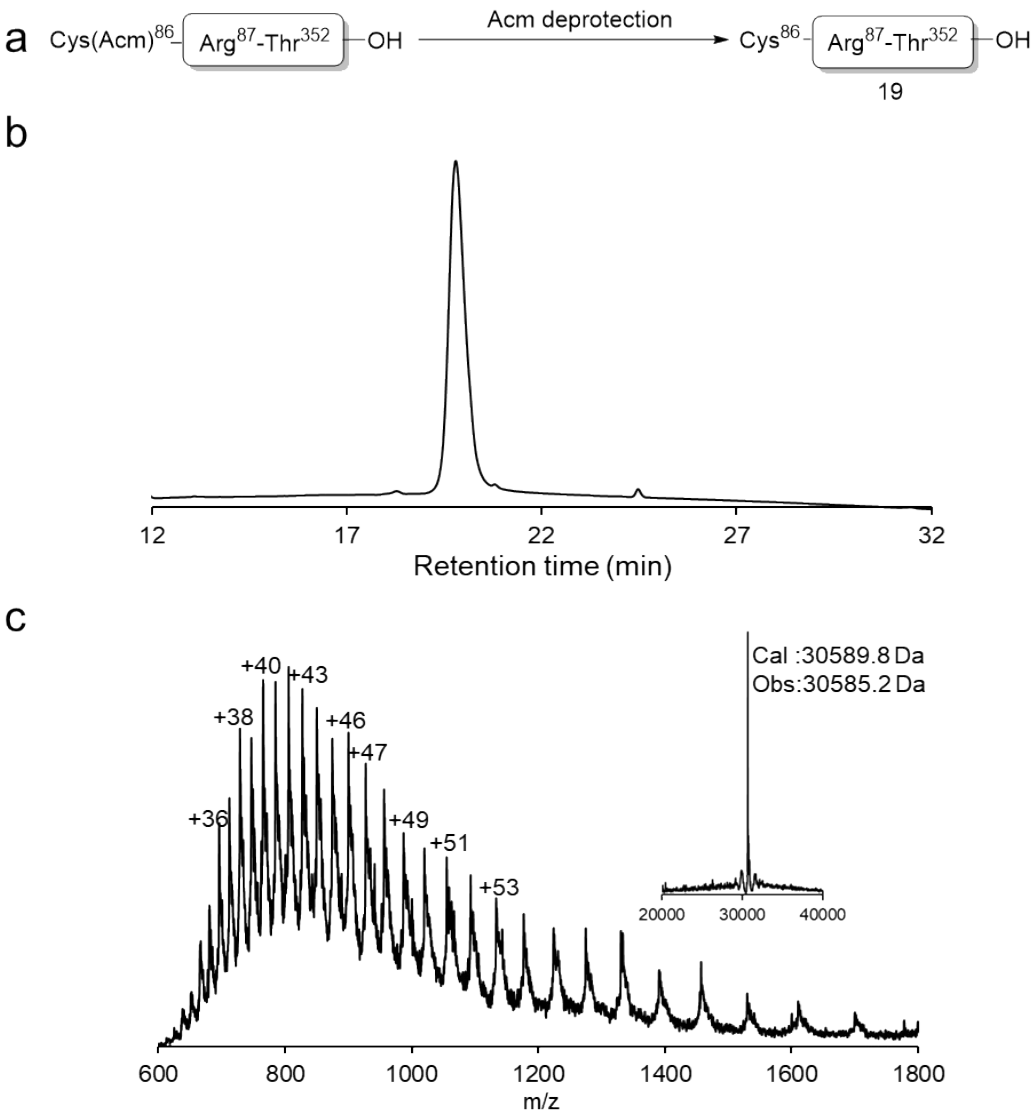
a) D-Dpo4-**18** (21 mg) was dissolved in 5.5 ml aqueous solution of 6 M Gn·HCl, 0.1 M Na_2HPO_4, 300 mM TCEP, pH 7.0. After 0.6 ml t-BuSH and 33 mg VA-044 were added, the reaction was under stirring overnight at 37 °C. The desulfurization product of D-Dpo4-**18** was analyzed by HPLC and ESI-MS, and purified by semi-preparative HPLC (purification conditions: 20%-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). After lyophilization, the desulfurization product was obtained with a yield of 79.0% (16.6 mg). b) Analytical HPLC chromatogram of the desulfurization product (λ=214 nm). Column: Welch C4. Gradient: 20%-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of of the desulfurization product.



Supplementary figure S7 Analytical HPLC chromatogram and ESI-MS and deconvoluted spectrum of the desulfurization product

*3.11 Removal Acm protection of peptide **18***

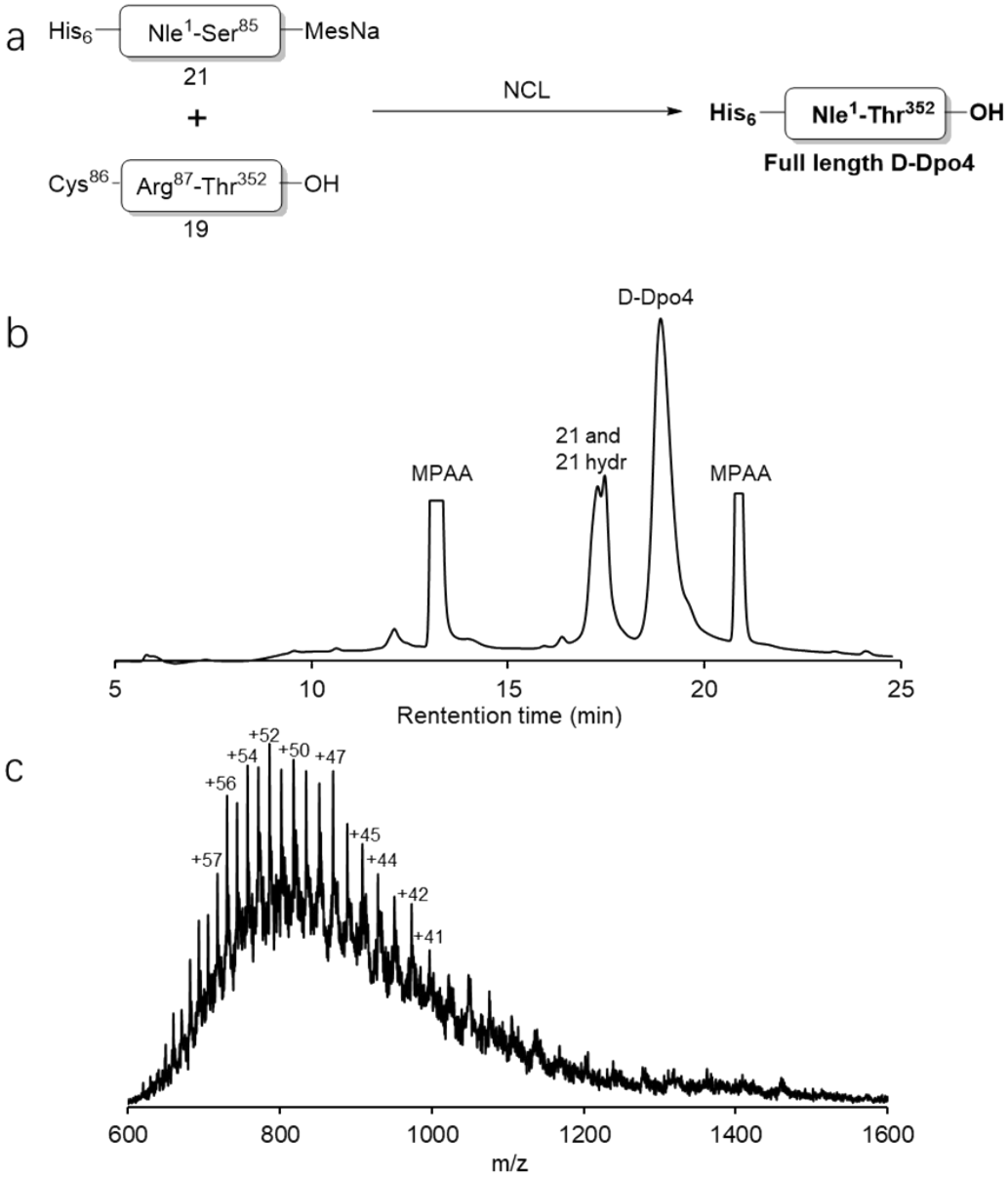
a) The desulfurization product of D-Dpo4-**18** (13 mg) was dissolved in a 0.9 ml 50% acetic acid aqueous solution. Subsequently, 2.3 mg silver acetate was added to the solution. The reaction was under stirring overnight. After 12 h, 1 ml 4 M DTT (in aqueous solution of 6 M Gn·HCl and 0.1 M Na_2HPO_4) was added. The system was diluted with 2 ml ligation buffer (6 M Gn·HCl, 0.1 M Na_2HPO_4, pH=7.4). After centrifugation, the supernatant was purified by semi-preparative HPLC. The precipitant was washed thoroughly and purified (purification conditions: 20%-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). After lyophilization, the Acm-removed product D-Dpo4-**19** was obtained with a yield of 75.4% (9.8 mg). b) Analytical HPLC chromatogram of 19 (λ=214 nm). Column: Welch C4. Gradient: 20%-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of **19**.



Supplementary Figure S8 Analytical HPLC chromatogram and ESI-MS and deconvoluted spectrum of **19**

*3.12 Ligation of **19** and **21***

a) D-Dpo4-**19** (9.1 mg) and D-Dpo4-21 (6.5 mg) were dissolved in a 0.3 ml ligation buffer (containing 6 M Gn·HCl, 0.1 M Na_2HPO_4, 100 mM MPAA, 40 mM TCEP, pH 7). After adjusting pH to 6.5, the reaction was under 37 °C stirring overnight. After 18h, the products were analyzed and purified by HPLC. (purification conditions: 20%-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). After lyophilization, the full-length D-Dpo4 was obtained with a yield of 70% (8.5 mg). b) Analytical HPLC trace of ligation (λ=214 nm). Column: Welch C4. Gradient: 30%-80% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. c) ESI-MS spectrum of D-Dpo4.



Supplementary Figure S9 Analytical HPLC trace of D-Dpo4 and ESI-MS spectrum

3.13 Protein Refolding and purification

Lyophilized D-Dpo4 was dissolved in a denaturation buffer containing 6 M Gn·HCl, and dialyzed against a series of renaturation buffers which contained 4 M, 2 M, 1 M, 0.5 M, 0.25 M and 0 M Gn·HCl, respectively. Each step of the dialysis was carried out at 4 °C for 10 h with gentle stirring. The denaturation and renaturation buffers also contained 50 mM Tris-acetate (pH 7.5), 50 mM NaOAc, 1 mM DTT, 0.5 mM EDTA and 16% glycerol. After renaturation, the enzyme was dialyzed against a buffer containing 10 mM potassium phosphate (pH 7.0), 50 mM NaCl, 10 mM $MgOAc_2$, 10% glycerol and 0.1% 2-Mercaptoethanol. The folded polymerase was incubated at 78 °C for 10 min to precipitate the thermolabile peptides, which were subsequently removed by ultracentrifugation at 19 000 rpm. for 40 min at 4 °C. The supernatant was incubated in Ni-NTA Superflow resin (Qiagen, Venlo, Netherlands) overnight at 4 °C, and purified according to previously described methods but without the use of Mono S column[2, 3]. The concentration of the purified Dpo4- 5m was measured spectrophotometrically at 280 nm using an extinction coefficient of 24 058 $M^{-1}cm^{-1}$ and M.W. of 40.8 kDa.

3.14 miPCR by synthetic D-Dpo4-5m

The Single-stranded 128 nt L-DNA template, primers and oligonucleotides were synthesized by WuXi AppTec. The PCR reactions were performed in 25 μl reaction systems, including 25 mM HEPES (pH 7.5), 5 mM MgCl2, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10% glycerol, 3% DMSO, 0.1 mg/ml BSA, 160 μM (each) L-dNTPs, 0.2 μM (each) L-primers, 60 nM L-ssDNA template and ~1 μM D-Dpo4 polymerase. Considering that the mirror-image DNA polymerization was less efficient than the corresponding natural system, the PCR reaction was performed in two phases of 15 cycles each, with the addition of fresh enzyme between the phases. The PCR program settings were 86 °C for 30 s (initial denaturation); 86 °C for 15 s (denaturation), 54 °C for 15 s (annealing) and 65 °C for 2min (extension) for 30 cycles; 65 °C for 5 min (final extension). The products were analyzed by 3% agarose gel electrophoresis and stained by 4S Gelred (Sangon Biotech, China). Both the products of miPCR and PCR were digested by 5U DNase I (Takara，China) at 37 °C for 30 min, and analyzed by 3% agarose gel electrophoresis.

Supplementary Table S1 | DNA oligo sequences

D/L-DNA Oligo	DNA sequence
D/L-5S rRNA(rrfB)-T	5’CCTAACCGATATCACACTCACTCGCGGGATC GAGATCTCGATCCTCTACGCCGGACGCATCGT GGCCGGCATCACCGGCGCCACAGGTGCGGTT GCTGGCGCCTATAGTTGGTCGTCATTGGAGTATC-3’
D/L-5S rRNA(rrfB)-F	5’-CCTAACCGATATCACACTCAC-3’
D/L-5S rRNA(rrfB)-R	5’-GATACTCCAATGACGACCAAC-3’

Supplementary Refernces

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2. W. Jiang, B. Zhang, C. Fan, M. Wang, J. Wang, Q. Deng, X. Liu, J. Chen, J. Zheng, L. Liu and T. F. Zhu, *Cell Discov.*, 2017, **3**, 17037.
3. K. A. Fiala and Z. Suo, *Biochemistry*, 2004, **43**, 2106-2115.