Development of a *o*-Aminoanilides-Mediated Native Chemical Ligation Assisted DADA Strategy for the Synthesis of Disulfide Surrogate Peptides

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List of contents

1.General information

1.1 Materials and Reagents

Rink amide AM resin was purchased from Tianjin Nankai HECHENG (TianJin, China). All of Fmoc-amino acids, HCTU, HATU, HBTU, HOBt, HOAt, PyAOP were purchased from GL Biochem (Shanghai, China), CS Bio. NH_2-Tyr(O*t*Bu)-O*t*Bu and Boc-Gly-OH were purchased from Bidepharm (Shanghai, China). N,N-Diisopropylethylamine (DIEA), 4-methylmorpholine (NMM), trifluoroacetic acid (TFA), trifluoroacetic acid (TFA, HPLC grade), $SnCl_2$, $Pd(PPh_3)_4$, phenylsilane, thioanisole, TIPS, 3,4-diaminobenzoic acid and glutathione reduced (GSG) were purchased from energy-chemical (Shanghai, China). Glutathione oxidized (GSSG) were purchased from Macklin. Dichloromethane (DCM), 1,2-ethanedithiol, dimethylformamide (DMF), $NaNO_2$ and anhydrous diethyl ether were purchased from Sinopharm Chemical Reagent. 4-Mercaptophenylacetic acid (MPAA) was purchased from Alfa Aesar.

1.2 HPLC

Analytical and semi-preparative HPLC was performed on a SHIMADZU (Prominence LC-20AT) (mobile phases were ddH_2O (solution B, 0.1 % HPLC grade trifluoroacetic acid added) and acetonitrile (solution A, 0.08 % HPLC grade trifluoroacetic acid added)). The flow rate of analytical HPLC flow rate was 1.0 mL/min. The model of the analytical column is Welch "Ultimate XB-C18", 250 × 4.6 mm, 5 μm particle size, the program is 5-70% 30min 1 mL; the model of the semi-preparative column is Welch "Ultimate XB-C18", 250 × 10 mm, 10 μm particle size. Simultaneously monitor analytical samples at 214 nm and 254 nm. The flow rate of Semi-preparative HPLC was 4.0 mL/min.

1.3 Mass spectrometry

ESI-MS spectra were collected using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Fisher Scientific. USA) equipped with a standard ESI ion source.

2. Synthesis of disulfide surrogate peptides

2.1 Protocol of Fmoc-based solid phase peptide synthesis (35 ℃)

156 mg of Rink amide AM resin (0.05 mmol, 0.32 mmol g^{-1}) was weighed and swollen in DCM/DMF (1/1, V/V) for 30 minutes, and then the resin was washed in the order of DMF, DCM, DMF (each 3 times).

The Fmoc group was removed in a 20% piperidine/DMF solution supplemented with 0.1 M Oxyma (twice, 5 min and 10 min).

In a 5 mL test tube, 2 mL of DMF was added to dissolve 0.2 mmol of Fmoc-protected amino acid, HCTU (0.2 mmol) and DIEA (0.4 mmol). Subsequently, the amino acids were successfully activated by vortexing the solution for 30 seconds at room temperature. The activated amino acid was then added to the resin and reacted for 30 minutes. The amino acids were secondary coupled by adding a mixed solution of Fmoc-amino acid (0.2 mmol), HATU (0.2 mmol), HOAT (0.2 mmol) and DIEA (0.4 mmol) in DMF to the resin and reacted for 30 minutes.

Protocol of peptide cleavage

After the peptide sequence was coupled, the resin was drained and the cleavage cocktails (TFA/ thioanisole/1,2-ethanedithiol/TIPS/H_2O/Phenol, 77.5/5/2.5/5/5/5) was added for removal of peptide side chain protecting groups and final cleavage (3 h). Subsequently, the cleavage solution was concentrated by blowing N_2, and the crude peptide was obtained by precipitation with ice ether and centrifugation. Finally, the crude peptides were purified and identified by semi-RP HPLC and ESI-MS, respectively.

Removal of the allyl protecting group

$Pd(PPh_3)_4$ (1 equiv.) and $PhSiH_3$ (10 equiv.) were dissolved in DCM, then added to the resin and reacted with shaking at room temperature for 1.5 h (twice). After the reaction, the palladium-washing reagent was dissolved in DMF and added to the resin to react for 5 min (3 times).

Removal of the PNZ protecting group

$SnCl_2$ (6 M) and HCl/CH_3OH (5 mM) were dissolved in DMF, then added to the resin and reacted with shaking at room temperature for 1 h (twice). After the reaction, the resin was washed five times in the order of DMF, H_2O, CH_3OH, DCM, and DMF.

2.2 Synthesis and characterization of conotoxin vil14a disulfide surrogate

2.2.1 Synthesis of conotoxin vil14a disulfide surrogate by on-resin cyclization strategy

After removing the Fmoc protecting group on the resin, Fmoc-Tyr(*t*Bu)-OH was assembled onto the resin by standard Fmoc-based SPPS. Subsequently, diaminodiacid 1 (**DADA1**, 0.1 mmol, prepared according to ref. 1) was coupled under PyAOP (0.2 mmol), HOAt (0.2 mmol), NMM (0.4 mmol) conditions. Fmoc-Met-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Met-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(*t*Bu)-OH and Fmoc-Ile -OH were coupled to the peptide sequence by standard Fmoc-based SPPS. Subsequently, the allyl protecting group of

DADA1 was removed through $Pd(PPh_3)_4/PhSiH_3$, and then the Fmoc protecting group of Fmoc-Ile-OH was removed. Cyclization was then performed overnight at PyAOP (0.2 mmol), HOAt (0.2 mmol), NMM (0.4 mmol) conditions. Finally, the peptide was cleaved from the resin by TFA cleavage reagent to obtained **1**. However, HPLC and ESI-MS analysis demonstrated that the correct cyclization product was not obtained.

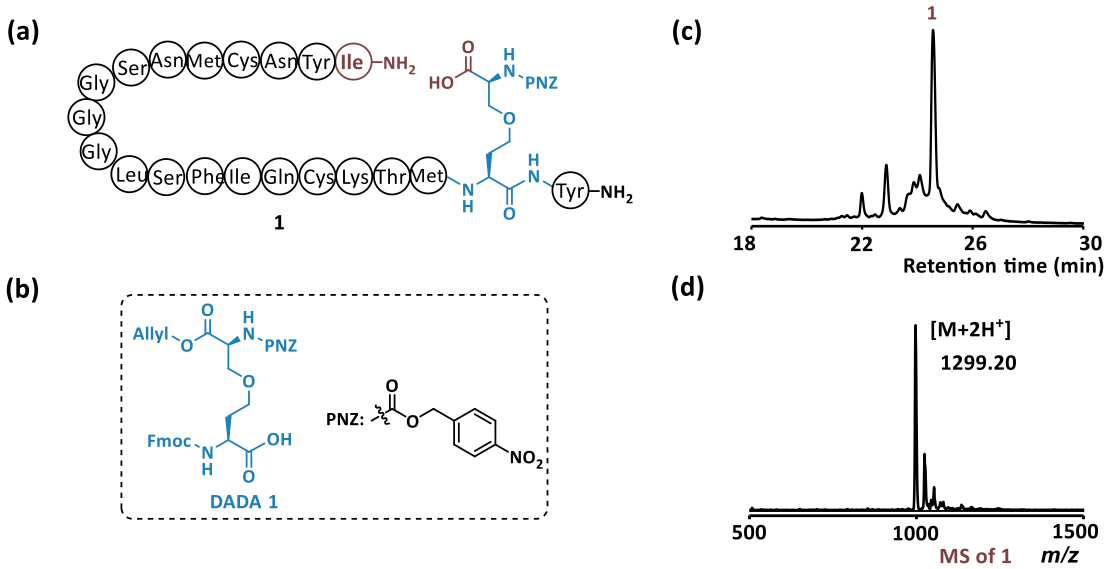


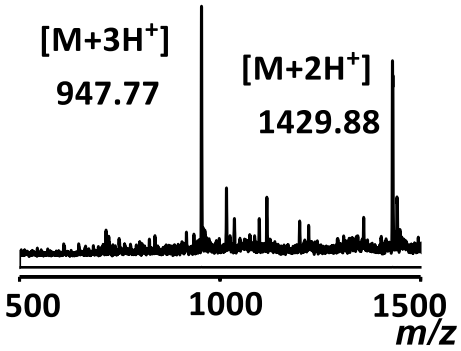
Figure S1. (a) Sequence of product **1**; (b) Structure of **DADA1**; (c) HPLC traces of **1**; (d) ESI-MS of **1**.

2.2.2 Synthesis of conotoxin vil14a disulfide surrogate by *o*-aminoanilides-mediated native chemical ligation assisted DADA strategy

After removing the Fmoc protecting group on the resin, Fmoc-Ala-OH was assembled onto the resin by standard Fmoc-based SPPS. Fmoc-Dbz-OH (4 equiv., prepared according to ref. 2) and Fmoc-Gln(Trt)-OH were coupled under HBTU (0.2 mmol), HOBt (0.2 mmol), DIEA (0.4 mmol) conditions for 1h×2. Subsequently, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Met-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(*t*Bu)-OH and Fmoc-Ile-OH were coupled to the peptide sequence by standard Fmoc-based SPPS. Then, **DADA1** (0.1 mmol) was coupled under PyAOP (0.2 mmol), HOAt (0.2 mmol), NMM (0.4 mmol) conditions. Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Gly-OH and Boc-Gly-OH were coupled to the peptide sequence. Subsequently, the allyl protecting group of **DADA1** was removed through $Pd(PPh_3)_4/PhSiH_3$, and then NH_2-Tyr(*t*Bu)-O*t*Bu was coupled to the peptide sequence at HATU, HOAt, DIEA conditions. Next, the PNZ protecting group of **DADA1** was removed through $SnCl_2$. Fmoc-Met-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Cys(Trt)-OH were coupled. Finally, the peptide was cleaved from the resin by TFA cleavage reagent. HPLC and ESI-MS analysis demonstrated that the correct precursor of NCL product was obtained. (Linear gradient from 5% to 70% acetonitrile in 0.1% trifluoroacetic acid, 30 min, 4 mL/min).

After purification of precursor of NCL product, *o*-aminoanilides-mediated native chemical ligation was performed. Firstly, 3.0 mg of precursor of NCL product (0.1 mM) was dissolved in 5

mL and pre-cooled in an ice-salt bath at -15 ℃. Then 34.5 mg $NaNO_2$ was dissolved in 1 mL pH 3.0 0.2 M phosphate buffer (6 M Gn·HCl) solution and pre-cooled for 7 min. Subsequently, 13.7 μL of $NaNO_2$ (0.7 mM) stock solution was added to the peptide solution to oxidize *o*-aminoanilide for 25 min. Next, MPAA (9.88 mg, 6 mM) dissolved in 4 mL of pH 7.0 0.2 M phosphate buffer (6 M Gn·HCl) was added to the mixture at room temperature to obtain the corresponding thioester. The reaction was carried out at pH 6.5 for 1 hour. RP-HPLC and ESI-MS analysis indicated that the correct ligation product was obtained. (Linear gradient from 5% to 70% acetonitrile in 0.1% trifluoroacetic acid, 30 min, 1 mL/min).



MS of the hydrolysis product of the Gln thioester to the acid

Figure S2. ESI-MS of the hydrolysis product of the Gln thioester to the acid.

2.2.3 The disulfide bond formation of conotoxin vil14a disulfide surrogate

The correct ligation product (2.84 mg) was dissolved in 28.4 mL of water, followed by the addition of oxidized glutathione (6.12 mg, 10 equiv.)/reduced glutathione (30.7 mg, 100 equiv.). Folding was completed after adjusting the pH to 7.5 for 2 h. RP-HPLC and ESI-MS analysis indicated that the folding product was obtained. (Linear gradient from 5% to 70% acetonitrile in 0.1% trifluoroacetic acid, 30 min, 1 mL/min).

2.3. Synthesis and characterization of κ-Hefutoxin 1

2.3.1 Synthesis of κ-Hefutoxin 1 disulfide surrogate by on-resin cyclization strategy

After removing the Fmoc protecting group on the resin, **DADA1** (0.1 mmol) was assembled onto the resin under PyAOP (0.2 mmol), HOAt (0.2 mmol), NMM (0.4 mmol) conditions. Subsequently, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gly-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Tyr(*t*Bu)-OH were coupled to the peptide sequence by standard Fmoc-based SPPS. Next, the allyl protecting group of **DADA2** was removed through $Pd(PPh_3)_4/PhSiH_3$, and then the Fmoc protecting group of Fmoc-Tyr(*t*Bu)-OH was removed. Cyclization was then performed overnight under PyAOP (0.2 mmol), HOAt (0.2 mmol), NMM (0.4 mmol) conditions. Finally, the peptide was cleaved from the resin by using TFA cleavage reagent. HPLC and ESI-MS analysis demonstrated that the correct cyclization product **3** was not obtained.

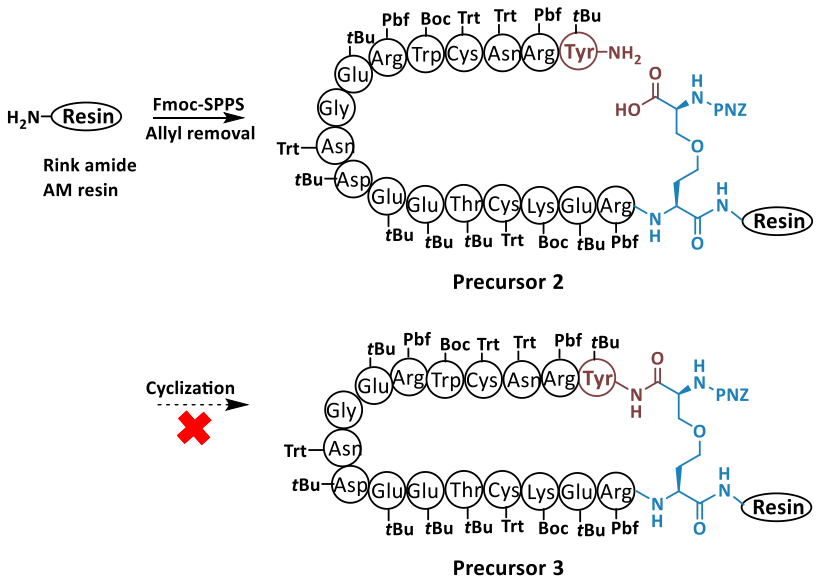


Figure S3. Synthetic route for the synthesis of κ-Hefutoxin 1 disulfide bond surrogate by on-resin cyclization.

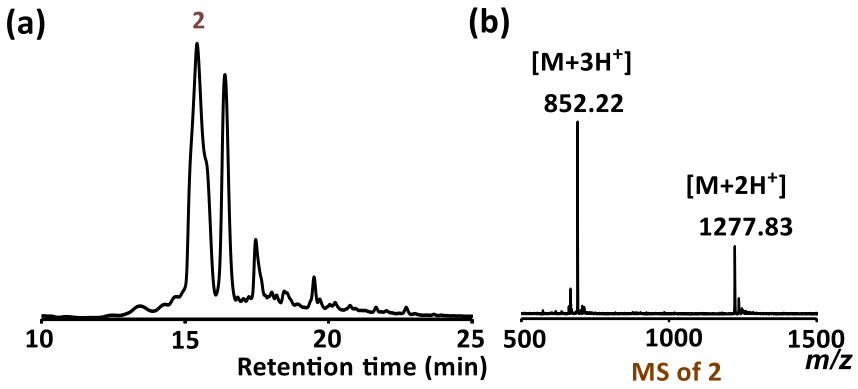


Figure S4. (a) HPLC traces of **2**; (b) ESI-MS of **2**.

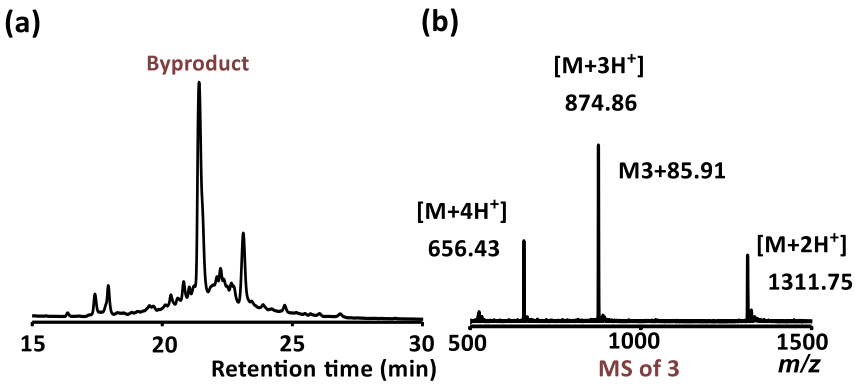


Figure S5. (a) HPLC traces of **3**; (b) ESI-MS of **3**.

2.3.2 Synthesis of κ-Hefutoxin 1 disulfide surrogate by *o*-aminoanilides-mediated native chemical ligation assisted DADA strategy

After removing the Fmoc protecting group on the resin, Fmoc-Ala-OH was assembled onto the resin by standard Fmoc-based SPPS. Fmoc-Dbz-OH (4 equiv., prepared according to ref. 2) and Fmoc-Asn(Trt)-OH were coupled under HBTU (0.2 mmol), HOBt (0.2 mmol), DIEA (0.4 mmol) conditions for 1h×2. Subsequently, Fmoc-Arg(Pbf)-OH and Fmoc-Tyr(*t*Bu)-OH were coupled to the peptide sequence by standard Fmoc-based SPPS. Then, **DADA2** (0.1 mmol) was coupled under PyAOP (0.2 mmol), HOAt (0.2 mmol), NMM (0.4 mmol) conditions. Fmoc-Ala-OH, Fmoc-His(Trt)-OH and Boc-Gly-OH were coupled to the peptide sequence. Next, the PNZ protecting group of **DADA1** was removed through $SnCl_2$. Fmoc-Arg(Pbf)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gly-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Trp(Boc)-OH and Fmoc-Cys(Trt)-OH were coupled. Finally, the peptide was cleaved from the resin by using TFA cleavage reagent. HPLC and ESI-MS analysis demonstrated that the correct precursor of NCL product was obtained. (Linear gradient from 5% to 70% acetonitrile in 0.1% trifluoroacetic acid, 30 min, 4 mL/min).

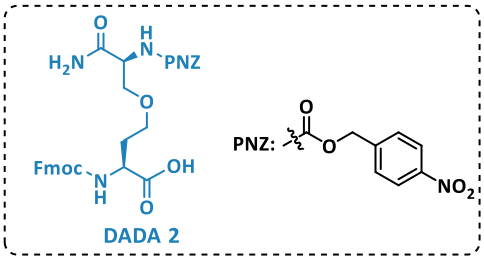


Figure S6. Structure of **DADA2**.

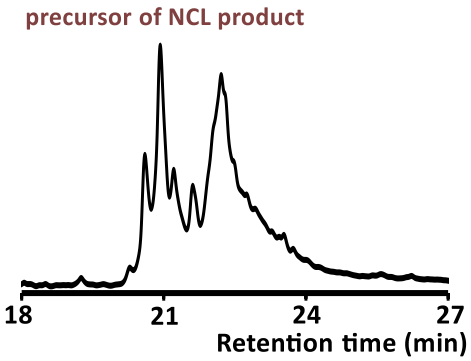
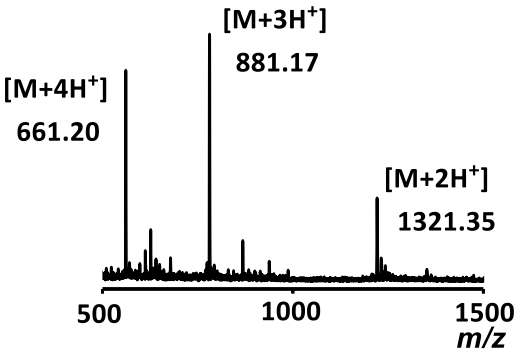


Figure S7. HPLC traces of precursor of NCL product.

After purification of precursor of NCL product, *o*-aminoanilides-mediated native chemical ligation was performed. Firstly, 3.0 mg of precursor of NCL product (0.1 mM) was dissolved in 5 mL and pre-cooled in an ice-salt bath at -15 ℃. Then 34.5 mg $NaNO_2$ was dissolved in 1 mL pH 3.0, 0.2 M phosphate buffer (6 M Gn·HCl) solution and pre-cooed for 7 min. Subsequently, 14 μL of $NaNO_2$ (0.7 mM) stock solution was added to the peptide solution to oxidize *o*-aminoanilide for 23 min. Next, MPAA (10.09 mg, 6 mM) dissolved in 5 mL of pH 7.0 0.2 M phosphate buffer (6 M

Gn·HCl) was added to the mixture at room temperature to obtain the corresponding thioester. The reaction was carried out at pH 6.5 for 1 hour. RP-HPLC and ESI-MS analysis indicated that the correct ligation product was obtained. (Linear gradient from 5% to 70% acetonitrile in 0.1% trifluoroacetic acid, 30 min, 1 mL/min).



MS of the hydrolysis product of the Asn thioester to the acid

Figure S8. MS of the hydrolysis product of the Asn thioester to the acid.

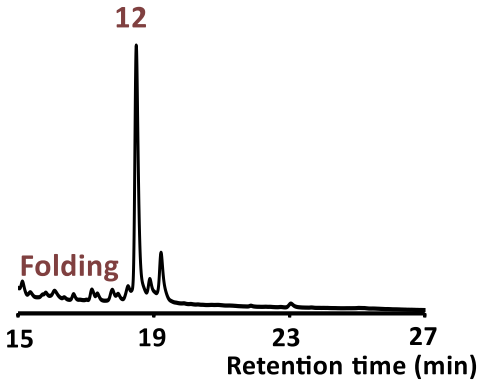


Figure S9. HPLC traces of **12**.

2.3.3 The disulfide bond formation of κ-Hefutoxin 1 disulfide surrogate

The correct ligation product (2.6 mg) was dissolved in 26 mL of water, followed by the addition of oxidized glutathione (5.97 mg, 10 equiv.)/reduced glutathione (30.0 mg, 100 equiv.). Folding was completed after adjusting the pH to 7.5 for 3 h. RP-HPLC and ESI-MS analysis indicated that the correct folding product was obtained. (Linear gradient from 5% to 70% acetonitrile in 0.1% trifluoroacetic acid, 30 min, 1 mL/min).

References

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2. J. X. Wang, G. M. Fang, Y. He, D. L. Qu, M. Yu, Z. Y. Hong and L. Liu, *Angew. Chem. Int. Ed.*, 2015, **54**, 2194-2198.