Supporting Information

3-Nitro-2-pyridinesulfenyl-mediated Solid-phase Disulfide Ligation in the Synthesis of Disulfide Bond-containing Cyclic Peptides

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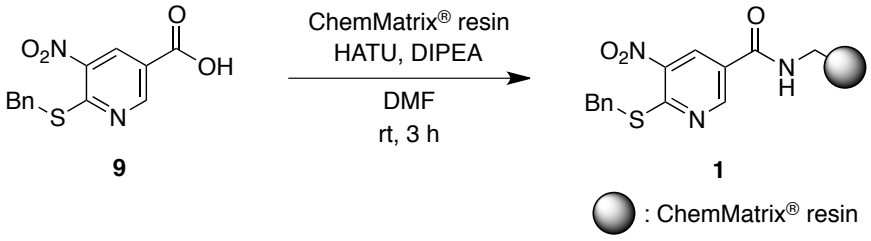
1. General information

All reaction mixtures were stirred magnetically. ^{1}H NMR spectra were measured in $CDCl_3$ solutions using Bruker AVANCE-III (400 MHz) spectrophotometers and TMS (0.00 ppm) as a reference. ^{13}C NMR spectra were measured in $CDCl_3$ solutions using Bruker AVANCE-III (400 MHz) spectrophotometers and $CDCl_3$ (77.05 ppm) as a reference. Chemical shifts were reported in ppm from TMS. Mass spectra were obtained on Waters MICRO MASS LCT-premier. Column chromatography was performed on silicagel 60N (spherical, neutral) (4-50 μm or 63-210 μm), thin layer chromatography (TLC) was performed on precoated plates (0.25 mm, silica gel Merk Kieselgel $60F_{254}$), and compounds were visualized with UV light, phosphomolybdic acid stain, and ninhydrin stain. Preparative HPLC was performed using a C18 reversed-phase column (19 x 150 mm; SunFire™ Prep C18 OBD™ 5 μm) with a binary solvent system. Analytical HPLC was performed using a C18 reversed-phase column (4.6 x 150 mm; SunFire™ C18 5 μm) with a binary solvent system. Solvents and reagents were purchased from Kanto Chemical Co., Inc., Kokusan Chemical Co., Ltd., Wako Pure Chemical Industries, Ltd., and Watanabe Chemical Industries, Ltd.

2. Synthesis of resin 1



To a solution of 6-(benzylthio)-5-nitronicotinic acid **9** (406 mg, 1.40 mmol) in DMF (12 mL) was added *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU, 420 mg, 1.37 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 201 μL, 1.40 mmol) at room temperature. The reaction mixture was voltex stirred for 1.5 min at room temperature. Then aminomethyl ChemMatrix® resin (500 mg, 0.70 mmol/g, 0.35 mmol) was added into the reaction vessel with vortex stirring for 3 h at room temperature. Reaction completion was monitored by the Kaiser test. After the mixture was filtered off, the resin was sequentially washed with DMF (x 5) and MeOH

(x 3). This operation was repeated thrice and the resin was dried in *vacuo* to obtain the resin **1** (547 mg). Elementary analysis of resin **1** revealed was $C_{156.62}H_{249.24}N_6O_{68.31}S_2$: (Calcd C, 55.30; H, 8.21; N, 2.47. Found: C, 54.62; H, 8.54; N, 2.36).

3. Synthesis of the peptide fragments

3-1. H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH_2 (11)

A 20% piperidine/DMF solution was added to the Fmoc-Rink amide resin (500 mg, 0.290 mmol) in a reaction vessel. After removing the 20% piperidine/DMF solution, the peptide chain was elongated by the Fmoc-SPPS method using the Fmoc amino acid (0.870 mmol, 3 equiv.), HOBt·H_2O (0.870 mmol, 3 equiv.) and DIPCI (0.870 mmol, 3 equiv.). H-Asn(Trt)-Cys(*t*-Bu)-Pro-Leu-Gly-NH-resin (673 mg) was treated with a TFA cocktail (TFA : H_2O : triisopropylsilane : 1,2-ethaneditiol = 94 : 2.5 : 1.0 : 2.5, 25 mL), and the mixture was stirred for 3 h at room temperature. After filtration, TFA was removed under reduced pressure. The residue was precipitated with ether, washed twice with ether, and dried in *vacuo*. The crude was purified by reversed-phase HPLC to give peptide **11** (55.7 mg, 82.9 μmol, 29% yield). HRMS (ES+) calcd for $C_{24}H_{44}N_7O_6S$ $[M+H]^+$ 558.3074, found 558.3074.

3-2. Fmoc-Cys-Tyr-Ile-Gln-OH (12a)

N,*N*'-Dicyclohexylcarbodiimide (94.9 mg, 0.460 mmol) was added to a solution of Fmoc-Gln(Trt)-OH (401 mg, 0.657 mmol) in CH_2Cl_2 (12 mL) in flask. After stirring for 10 min at room temperature, the mixture was filtered and concentrated under reduced pressure. The residue dissolved in DMF and *N*,*N*-dimethyl-4-aminopyridine (DMAP, 8.03 mg, 65.7 μmol) were added to Wang-resin (300 mg, 0.219 mmol) in the reaction vessel. After stirring for 3 h, the peptide chain was elongated by the Fmoc-SPPS method using the Fmoc amino acid (0.219 mmol, 3 equiv.), HOBt·H_2O (0.219 mmol, 3 equiv.) and DIPCI (0.219 mmol, 3 equiv.). Fmoc-Cys(Trt)-Tyr(*t*-Bu)-Ile-Gln(Trt)-OCH_2-resin (670 mg) was treated with a TFA cocktail (TFA : H_2O : triisopropylsilane : 1,2-ethaneditiol = 94 : 2.5 : 1.0 : 2.5, 23.8 mL), and the mixture was stirred for 3 h at room temperature. After filtration, TFA was removed under reduced pressure. The residue was precipitated with ether, washed twice with ether, and dried in *vacuo*. The crude was purified by reversed-phase HPLC to give peptide **12a** (23.5 mg, 31.5 μmol, 31% yield). HRMS (ES+) calcd for $C_{38}H_{46}N_5O_9S$

$[M+H]^+$ 748.3016, found 748.3016.

3-3. Fmoc-Cys-Tyr-Ile-Gln-$SCH_2CH_2CO_2H$ (12b)

Thioester peptide **12b** was synthesized by the method of Hojo *et al*.[1] Fmoc-Rink amide resin (100 mg, 58 μmol) was treated with 20% piperidine/DMF solution for 25 min (x 2). Then, Fmoc-Arg(Pbf)-OH (124 mg, 174 μmol), HOBt·H_2O (26.6 mg, 174 μmol) and DIPCI (27.0 μL, 174 μmol) were added to the resin. Following the same procedure, another Arg residue was introduced to give the Fmoc-[Arg(Pbf)]$_2$-NH-resin. After removal of the Fmoc group, a solution of Fmoc-Gln(Trt)-N(Et)Cys(Trt)-OH[1] (114 mg, 116 μmol), DIPCI (27.0 μL, 174 μmol and HOBt·H_2O (26.6 mg, 174 μmol) in 1,2-dichloroethane (1,2-DCE) was added to the resin and the mixture was vortex stirred overnight at room temperature. The peptide chain from Fmoc-Gln(Trt)-(Et)Cys(Trt)-[Arg(Pbf)]$_2$-NH-resin was elongated by the Fmoc-SPPS method, yielding the Fmoc-Cys(Trt)-Tyr(*t*-Bu)-Ile-Gln(Trt)-[Arg(Pbf)]$_2$-NH-resin (183 mg). Part of the resin (117 mg) was treated with a TFA cocktail (TFA : H_2O : triisopropylsilane : 1,2-ethaneditiol = 94 : 2.5 : 1.0 : 2.5, 2 mL), and the mixture was stirred for 2 h at room temperature. After filtration, the TFA was removed by N_2 stream. The residue was precipitated with ether, washed twice with ether, and dried in *vacuo*. The crude (66.7 mg) was dissolved in a solution (3 mL) of 50% CH_3CN aq. containing 6 M urea and 5% (v/v) 3-mercaptopropionic acid. The mixture was stirred for 3 days at room temperature. Subsequent purification using reversed-phase HPLC gave peptide thioester **12b** (3.06 mg, 3.66 μmol, 10%). HRMS (ES+) calcd for $C_{41}H_{50}N_5O_{10}S_2$ $[M+H]^+$ 836.2999, found 836.3020.

4. Solid-phase disulfide ligation

4-1. Synthesis of the Npys chloride resin 2

Resin **1** (12.6 mg, 7.45 μmol) was added to a solution of 2% SO_2Cl_2 in 1,2-DCE (1.25 mL) in the presence of pyridine (3.02 μL, 37.3 μmol) at 0 ºC. After vortex stirring for 20 min at 0 ºC, the solution was filtered under a gentle stream of nitrogen. Then, the resin was sequentially washed with ice-chilled CH_2Cl_2 five times and ice-chilled 90% aqueous formic acid thrice under a gentle stream of nitrogen to give the Npys chloride resin **2**. Elemental analysis of this resin **2** indicated that the formula was $C_{142.62}H_{263.24}Cl_2N_6O_{68.31}S_2$ (Calcd C, 52.06; H, 8.06; Cl, 2.16; N, 2.55. Found C, 46.45;

H, 7.81; Cl, 1.42; N, 2.41). The chlorine content was 66%. Resin **2** was immediately used in the reaction with the peptide **11**

4-2. Synthesis of disulfide peptide 14a

A solution of H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH_2 (**11**) (1.00 mg, 1.49 μmol) in ice-chilled 90% formic acid aq. (161 μL) was added to Npys chloride resin **2** at room temperature. After vortex stirring for 1 h at room temperature, the reaction mixture was filtered. Resulting peptide-resin **13** was washed with H_2O ten times. Then, a solution of Fmoc-Cys-Tyr-Ile-Gln-OH (**12a**) (0.93 mg, 1.24 μmol) in DMF/H_2O (2:1, 270 μL) was added to the resin at room temperature. After vortex stirring for 30 min at room temperature, the mixture was filtered and the resin was washed with DMF five times. The filtrate and DMF used for the wash were collected and condensed in *vacuo* to give disulfide peptide **14a** (1.19 mg, 0.886 μmol, 71% yield). HRMS (ES+) calcd for $C_{58}H_{79}N_{12}O_{15}S_2$ $[M+H]^+$ 1247.5229, found 1247.5229.

4-3. Synthesis of disulfide peptide 14b

Disulfide peptide **14b** was synthesized in a similar manner as disulfide peptide **14a** using resin **1** (12.2 mg, 7.18 μmol), H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH_2 (**11**) (0.96 mg, 1.44 μmol), and Fmoc-Cys-Tyr-Ile-Gln-$SCH_2CH_2CO_2H$ (**12b**) (1.00 mg, 1.20 μmol in DMF/H_2O (2:1, 261 μL)). Yield: 63% (1.01 mg, 0.757 μmol). HRMS (ES+) calcd for $C_{61}H_{83}N_{12}O_{16}S_3$ [M+H]+ 1335.5212, found 1335.5200.

5. Intramolecular amide formation (synthesis of peptide 15)

5-1. Conventional method from disulfide peptide 14a

To a solution of disulfide peptide **14a** (1.19 mg, 0.886 μmol) in DMF (886 μL) was added DIPEA (0.377 μL, 2.22 μmol) and HATU (0.409 mg, 1.33 μmol) at 0 ºC. After stirring for 5 min at room temperature, reversed-phase HPLC gave **15** (0.70 mg, 0.570 μmol, 64% yield) as a white solid. HRMS (ES+) calcd for $C_{58}H_{77}N_{12}O_{14}S_2$ $[M+H]^+$ 1229.5124, found 1229.5129.

5-2. Silver-mediated chemical ligation[2] from thioester-containing disulfide peptide 14b

To a solution of HOOBt (1.25 mg, 7.67 μmol) and DIPEA (0.652 μL, 3.83 μmol) in DMSO (50 μL) was added peptide **14b** at room temperature. Then a tiny portion of AgCl was added, and the mixture was vigorously stirred for 2 days at room temperature in the dark. After removing AgCl by filtration, reversed-phase HPLC gave **15** (0.57 mg, 0.464 μmol, 91% yield) as a white solid. HRMS (ES+) calcd for $C_{58}H_{77}N_{12}O_{14}S_2$ $[M+H]^+$ 1229.5124, found 1229.5112.

6. Synthesis of oxytocin (10)

Peptide **15** (0.70 mg, 0.570 μmol) was treated with 20% piperidine/DMF (216 μL) at room temperature. After stirring for 5 min, reversed-phase HPLC gave **10** (0.20 mg, 0.178 μmol, 31% yield) as a white solid. HRMS (ES+) calcd for $C_{43}H_{67}N_{12}O_{14}S_2$ $[M+H]^+$ 1007.4443, found 1007.4418.

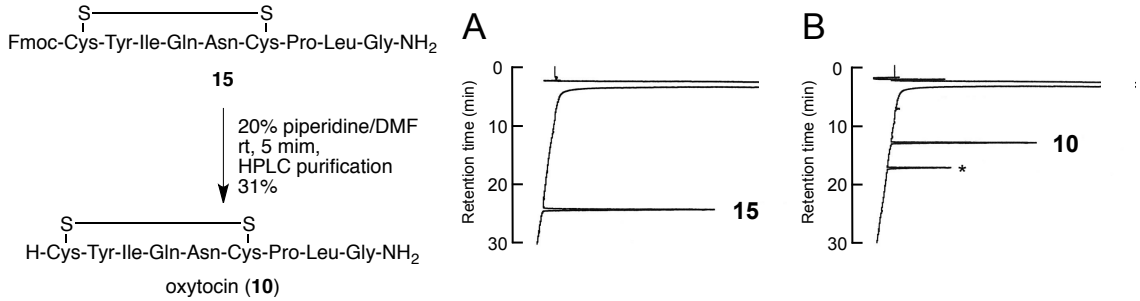


Fig. S1 Deprotection of the Fmoc group at the N-terminal of **15**. A) t = 0 min, B) t = 5 min. HPLC conditions: a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 65% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 0.9 mL/min with detection at 230 nm. Asterisk denotes the non-peptide peak.

7. References

[1] H. Hojo, Y. Onuma, Y. Akimoto, Y. Nakahara and Y. Nakahara, *Tetrahedron Lett.*, 2007, **48**, 25.

[2] Y. Asahina, S. Kamitori, T. Takao, N. Nishi and H. Hojo, *Angew. Chem. Int. Ed.*, 2013, **52**, 9733.