Supporting Information for

Resin-bound crypto-thioester for native chemical ligation

Naoto Naruse, Kento Ohkawachi, Tsubasa Inokuma, Akira Shigenaga, Akira Otaka*

Institute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences, Tokushima University, Tokushima 770-8505, Japan.

E-mail:
aotaka@tokushima-u.ac.jp

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General Methods

Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ (ESI-TOF). For HPLC separation, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil 5C_{18}-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min), or a Cosmosil 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B), was used for HPLC elution.

Preparation of peptide thioester using resin-bound SEAlide peptide.

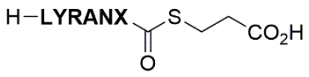
Synthesis of resin-bound SEAlide peptides 4a-j.

4a-j

Typical procedure: On aminomethyl ChemMatrix® resin (~1.0 mmol amine/g, 5.0 g, 5.0 mmol) was coupled of Fmoc-Phe-OH (9.7 g, 25 mmol) with the aid of DIPCDI (3.9 mL, 25 mmol) and HOBt·H_2O (3.4 g, 25 mmol) in DMF at room temperature for 24 h. Unreacted amino group of the resin was capping by the use of 10 equivalents of acetic anhydride and pyridine. Then Fmoc removal by 20% (v/v) piperidine in DMF gave Phe-incorporated resin (0.43 mmol amine/g, 7.2 g, 3.1 mmol).* To vary the initial amine content of the resin, reaction conditions (equivalent of amino acid and reaction temperature) was changed. For the preparation of 0.27 mmol amine/g resin, 0.5 equivalents of Fmoc Phe-OH was coupled for 24 h. Coupling of 5 equivalents of Fmoc-Phe-OH for 48 h afforded 0.58 mmol amine/g resin. The resulting resin (0.43 mmol amine/g, 233 mg, 0.1 mmol) was treated with **5a** (288 mg, 0.40 mmol), HATU (137 mg, 0.36 mmol) and DIPEA (63 μL, 0.36 mmol) to yield the anilide-linked resin. On this resin, standard Fmoc SPPS (Acylation: Fmoc amino acid (4.0 equiv.), DIPCDI (4.0 equiv.) and HOBt·H_2O (4.0 equiv.) in DMF for 2 h; Fmoc removal: 20% (v/v) piperidine in DMF for 10 min) was performed for chain elongation to give the protected peptide resin as the precursor of the peptide thioester. This protected resin was treated with TFA–TES–H_2O (95:2.5:2.5 (v/v)) at room temperature for 2 h to give the deprotected peptide resin **4a**.

*Content of amine of the internal standard (Phe)-incorporated resin was determined by quantitative spectrophotometric monitoring following piperidine deprotection of the Fmoc-Phe-resin according to literature (ref. 1).

Preparation of peptide thioesters 6a-j.



6a-j

Typical procedure: Resin-bound SEAlide peptide **4a** (20 mg, 6.3 μmol) was reacted with 40 mM TCEP·HCl, 50 mM sodium ascorbate, 5% (v/v) MPA in 0.2 M sodium phosphate buffer (pH 4.0), and the mixture was stirred at 50 ºC for 12 h. After the resin in the reaction mixture was filtered off, the filtrate was purified by semi-preparative HPLC to give **6a** (3.4 mg, 3.4 μmol, 53% isolated yield).

6a (**X** = Ala): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 18.2 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 21.6 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 795.4, found 795.4.

6b (**X** = Gly) (4.0 mg, 4.0 μmol, 57% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 15.6 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 20.0 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 781.4, found 781.4.

6c (**X** = Lys) (4.4 mg, 3.6 μmol, 58% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 14.4 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 17.7 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 852.4, found 852.4.

6d (**X** = Arg) (4.3 mg, 3.5 μmol, 63% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 16.0 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 18.3 min. MS (ESI-TOF) *m/z* calcd for ($[M+2H]^{2+}$) 440.7, found 440.7.

6e (**X** = Ser) (4.0 mg, 3.8 μmol, 72% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 15.5 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 19.3 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 811.4, found 811.4.

6f (**X** = Asn) (2.2 mg, 2.0 μmol, 33% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 14.8 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 18.9 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 838.4, found 838.4.

6g (**X** = His) (2.0 mg, 1.7 μmol, 33% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 14.3 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 19.1 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 861.4, found 861.4.

6h (**X** = Leu) (2.6 mg, 2.4 μmol, 43% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 23.3 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 10 to 35% over 30 min, retention time = 25.7 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 837.4, found 837.4.

6i (**X** = Phe) (3.0 mg, 2.7 μmol, 52% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 25.9 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 10 to 35% over 30 min, retention time = 27.1 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 871.4, found 871.4.

6j (**X** = Val) (1.9 mg, 2.3 μmol, 43% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 21.9 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 10 to 35% over 30 min, retention time = 22.9 min. MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 823.4, found 823.4.

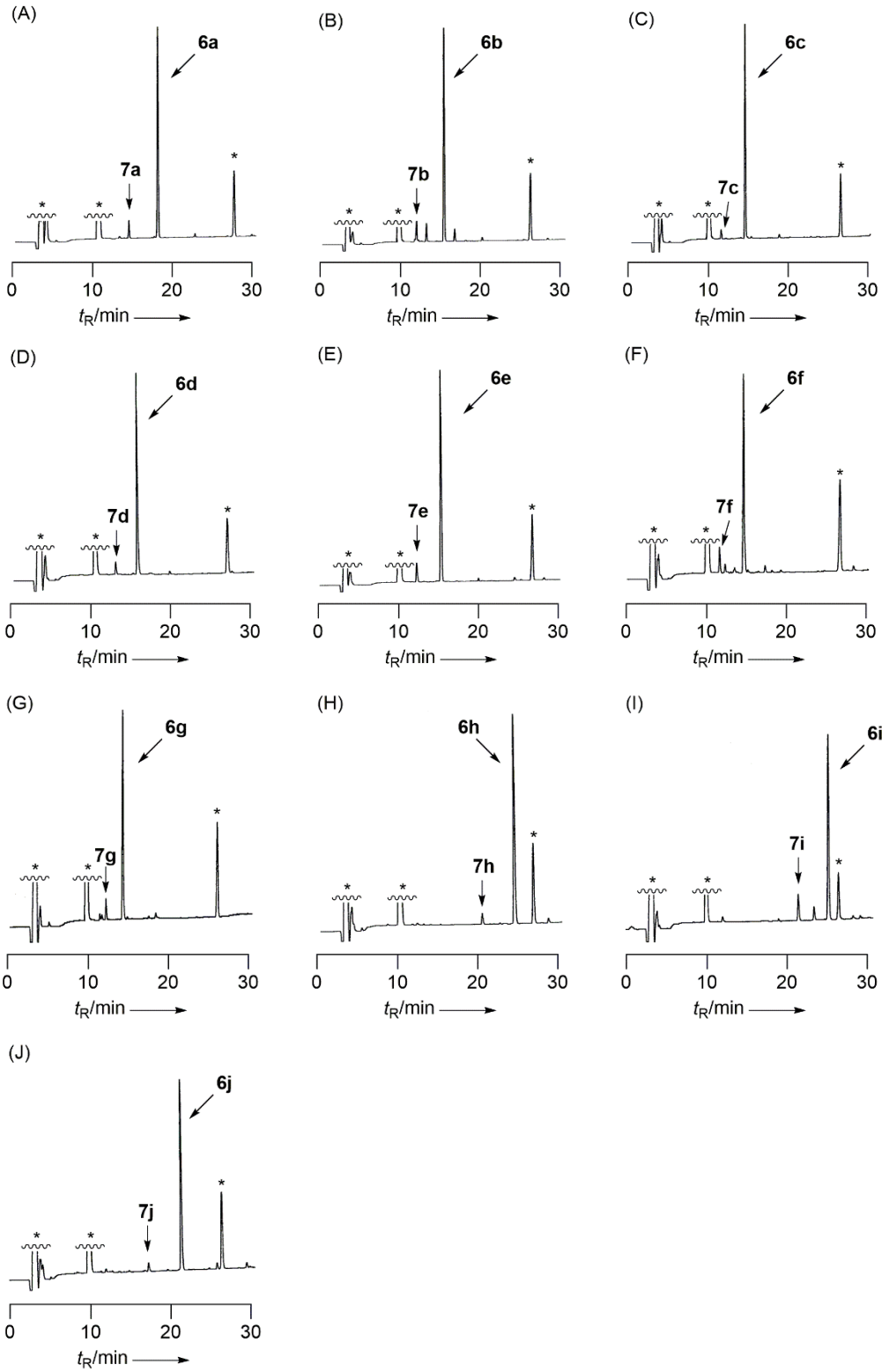
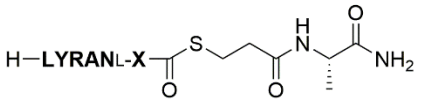
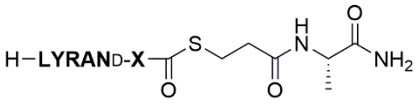


Figure S1. HPLC charts of crude reaction material. (A) **X** = Ala, *t* = 12 h; (B) **X** = Gly, *t* = 6 h; (C) **X** = Lys, *t* = 12 h; (D) **X** = Arg, *t* = 12 h; (E) **X** = Ser, *t* = 12 h; (F) **X** = Asn, *t* = 12 h; (G) **X** = His, *t* = 1 h; (H) **X** = Leu, *t* = 24 h; (I) **X** = Phe, *t* = 24 h; (J) **X** = Val, *t* = 72 h. *Non- peptide impurity.

Preparation of peptide thioesters S1a, e, g and S2a, e, g.



S1a, e, g



S2a, e, g

Typical procedure: On 4-methylbenzhydrylamine (MBHA) resin (0.70 mmol amine/g, 0.13 g, 0.09 mmol), introduction of Boc-Ala-OH (5.0 equiv.) in the presence of DIPCDI (5.0 equiv.), HOBt·H_2O (5.0 equiv.) and DIEA (2.0 equiv.) in DMF at room temperature for 2 h followed by Boc removal by TFA–anisole–toluene (50:2:48 (v/v), 30 min) afforded the Boc-Ala-incorporated resin. Next, treatment of the resulting resin with S-Trt mercaptopropionic acid (5.0 equiv.), DIPCDI (5.0 equiv.), HOBt·H_2O (5.0 equiv.) and DIEA (2.0 equiv.) in DMF at room temperature for 2 h followed by Trt removal by TFA–TES (95:5 (v/v), 30 min) gave $HSCH_2CH_2CO$-Ala-MBHA resin. Activated Boc-L-Ala-OH (5.0 equiv.) with DIPCDI (5.0 equiv.), HOBt·H_2O (5.0 equiv.) and DIPEA (2.0 equiv.) in DMF was coupled with $HSCH_2CH_2CO$-Ala-MBHA resin for 2 h, and the resin was subsequently subjected to Boc removal by TFA–anisole–toluene (50:2:48 (v/v), 30 min). On the resulting resin, standard in situ neutralization Boc SPPS (Acylation: Boc amino acid (5.0 equiv.), DIPCDI (5.0 equiv.), HOBt·H_2O (5.0 equiv.) and DIEA (2.0 equiv.) in DMF at room temperature for 2 h; Boc removal: TFA–anisole–toluene (50:2:48 (v/v), 30 min)) was performed for chain elongation to give a protected peptide resin. The resulting completed resin (50 mg, 25.0 μmol) was treated with 1 M TMSOTf–thioanisole in TFA/*m*-cresol/1, 2-ethaneditiol (100/5/5 (v/v)) at 4 °C for 2 h. The resin was filtered off and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide thioester. The crude peptide thioester was purified by preparative HPLC to give the purified peptide **S1a** (4.0 mg, 3.3 μmol, 13% isolated yield).

S1a (X = L-Ala): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 15.6 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 8 to 18% over 30 min, retention time = 27.0 min. MS (ESI-TOF) m/z calcd for ($[M+2H]^{2+}$) 433.2, found 433.3.

S1e (X = L-Ser) (5.2 mg, 4.2 μmol, 16% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 14.5 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 8 to 18% over 30 min, retention time = 16.2 min. MS (ESI-TOF) m/z calcd for ($[M+H]^{+}$) 881.4, found 881.3.

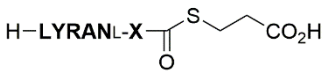
S1g (X = L-His) (1.1 mg, 0.9 μmol, 3% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 14.1 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 15% over 30 min, retention time = 19.7 min. MS (ESI-TOF) m/z calcd for ($[M+2H]^{2+}$) 466.2, found 466.3.

S2a (X = D-Ala) (4.2 mg, 3.5 μmol, 13% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 17.2 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 8 to 18% over 30 min, retention time = 26.2 min. MS (ESI-TOF) m/z calcd for ($[M+2H]^{2+}$) 433.2, found 433.3.

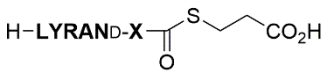
S2e (X = D-Ser) (4.3 mg, 3.5 μmol, 13% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 14.6 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 8 to 18% over 30 min, retention time = 15.5 min. MS (ESI-TOF) m/z calcd for ($[M+H]^{+}$) 881.4, found 881.3.

S2g (X = D-His) (6.0 mg, 4.7 μmol, 18% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 14.0 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 5 to 15% over 30 min, retention time = 16.5 min. MS (ESI-TOF) m/z calcd for ($[M+2H]^{2+}$) 466.2, found 466.3.

Preparation of peptide thioesters S3a, g and S4a, g.



S3a, g



S4a, g

Typical procedure: Peptide thioester **S1a** was dissolved in 6 M guanidine·HCl–0.1 M sodium phosphate buffer containing 2% (v/v) MPA (pH 7.3). The reaction mixture was incubated at 37 °C for 1 h and reaction progress was monitored by analytical HPLC.

S3a (**X** = L-Ala): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 18.4 min, MS (ESI-TOF) *m/z* calcd for ($[M+H]^{+}$) 795.4, found 795.4.

S3g (**X** = L-His): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 16.0 min, MS (ESI-TOF) *m/z* calcd for ($[M+H]^{+}$) 861.4, found 861.4.

S4a (**x** = D-Ala): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 18.7 min, MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 795.4, found 795.4.

S4g (**x** = D-His): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 16.3 min, MS (ESI-TOF) *m/z* calcd for ($[M+H]^+$) 861.4, found 861.4.

Preparation of peptide S5and S6.

H−**LYRAN**L-**S**−**CYRANK**−NH_2 **S5**

H−**LYRAN**D-**S**−**CYRANK**−NH_2 **S6**

NCL between **S1e** or **S2e** and N-terminal cysteinyl peptide (H-**CYRANK**-NH_2) was performed in 6 M Gn·HCl–0.1 M HEPPS buffer (pH6.8), 40 mM MPAA and 40 mM TCEP·HCl at 37 °C. After incubation for 2 h, the crude product was purified by semi-preparative HPLC to give the ligated product **S5** or **S6**.

Ligation product **S5**: Analytical HPLC conditions, linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 15.8 min, MS (ESI-TOF) m/z calcd for ($[M+2H]^{2+}$) 729.4, found 729.3.

Ligation product **S6**: Analytical HPLC conditions, linear gradient of solvent B in solvent A, 5 to 35% over 30 min, retention time = 15.8 min, MS (ESI-TOF) m/z calcd for ($[M+2H]^{2+}$) 729.4, found 729.3.

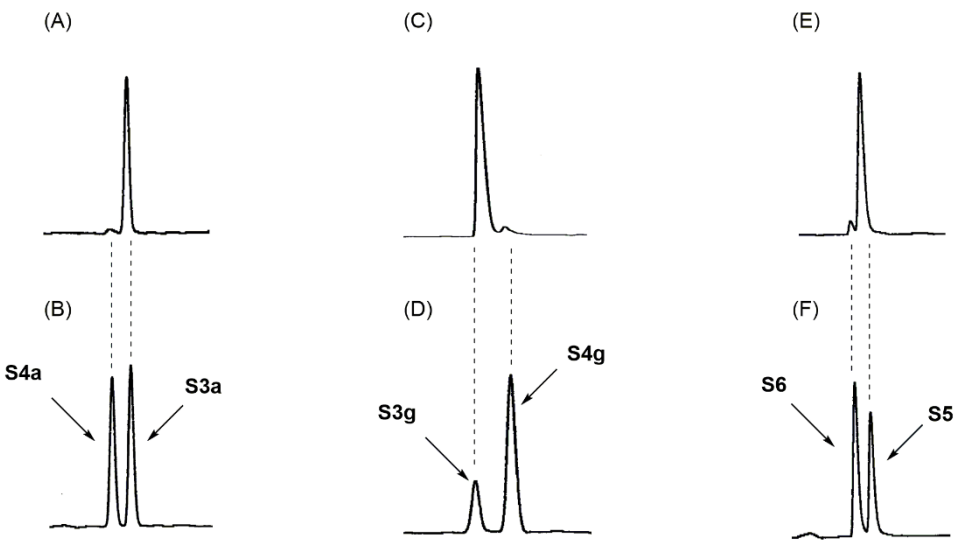
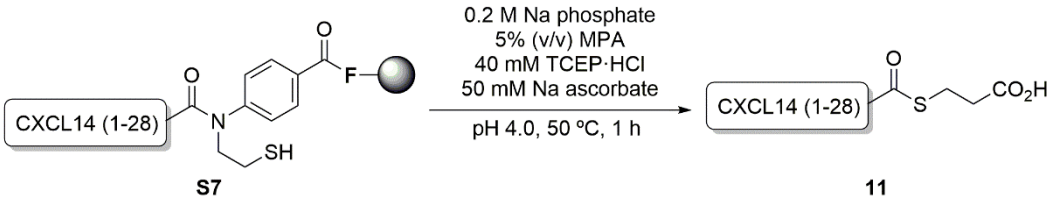


Figure S2. Verification of epimerization of C-terminal chiral amino acids during N–S acyl transfer mediated thioesterification. (A) Crude reaction material (**X** = Ala), *t* = 12 h; (B) Co-injection **S3a** with **S4a**. (C) Crude reaction material (**X** = His), *t* = 1 h; (D) Co-inkection **S3g** with **S4g**. (E) Crude reaction material (**X** = Ser), *t* = 12 h; (F) Co-injection **S5** with **S6**. Analytical HPLC conditions of chart (A), (B): linear gradient of 0.1% (v/v) TFA–MeCN in 0.1% (v/v) TFA aq. (11:89–15:85 over 60 min) at a flow rate 1.0 mL/min, detection at 220 nm. Analytical HPLC conditions of chart (C), (D): linear gradient of 0.1% (v/v) TFA–MeCN in 0.1% (v/v) TFA aq. (7:93–10:90 over 30 min) at a flow rate 1.0 mL/min, detection at 220 nm. Analytical HPLC conditions of chart (E), (F): linear gradient of 0.1% (v/v) TFA–MeCN in 0.1% (v/v) TFA aq. (7:93–10:90 over 30 min) at a flow rate 1.0 mL/min, detection at 220 nm. Only a critical retention time region of the HPLC charts was enlarged.

Application of the resin-bound SEAlide peptide to synthesis of CXCL14.

Preparation of N-terminal fragment 11.

Scheme S1. Preparation of N-terminal fragment **11**.



Resin-bound SEAlide peptide **S7** (30 mg, 4.1 μmol) was reacted with 40 mM TCEP·HCl, 50 mM sodium ascorbate, 5% (v/v) MPA in 0.2 M sodium phosphate buffer (pH 4.0), and the mixture was stirred at 50 °C for 1 h. After the resin in the reaction mixture was filtered off, the filtrate was purified by semi-preparative HPLC to give **11** (3.3 mg, 0.7 μmol, 17% isolated yield).

11: Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 17.5 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 10 to 30% over 30 min, retention time = 24.2 min. MS (ESI-TOF) *m/z* calcd for ($[M+3H]^{3+}$) 856.5, found 856.4.

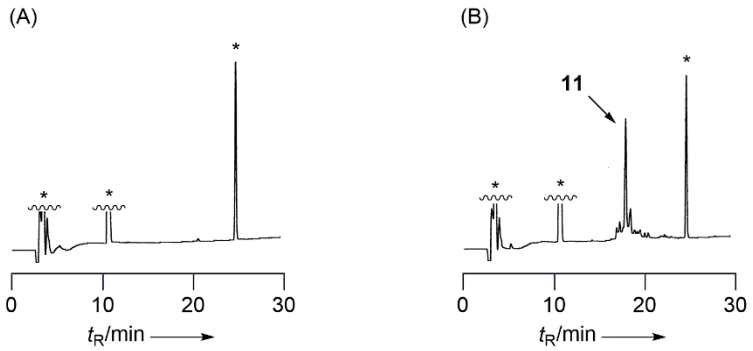


Figure S3. HPLC monitoring of preparation of N-terminal fragment **11**. (A) *t* = 0 h; (B) *t* = 1 h. *Non-peptide impurity.

Preparation of CXCL14 using one-pot/three-fragment ligation.

The first NCL between alkyl thioester peptide **11** (4.0 mg, 0.84 μmol) and N-Cys-SEAlide peptide **12** (2.9 mg, 0.84 μmol) was performed in 6 M Gn·HCl–0.2 M HEPPS buffer containing 50 mM MPAA and 30 mM TCEP·HCl (pH 6.7, 0.84 mL) at 37 ºC. The reaction was completed within 3 h. Then, N-Cys peptide **14** (3.9 mg, 0.84 μmol) solution in 1.0 M sodium phosphate buffer (pH 6.6, 0.84 mL) was added to the reaction mixture. The second NCL proceeded at 37 ºC within 24 h, and then the crude material was purified by semi-preparative HPLC to give CXCL14 (4-Cys) (3.2 mg, 0.26 μmol, 31% isolated yield). The purified CXCL14 (4-Cys) (3.2 mg, 0.26 μmol) was oxidized with air in 3 M Gn·HCl–0.1 M sodium phosphate buffer (pH 7.7, 5.2 mL) at 37 ºC for h. After oxidative folding followed by purification using semi-preparative HPLC, human CXCL14 (1.6 mg, 0.13 μmol) was obtained in 50% isolated yield.

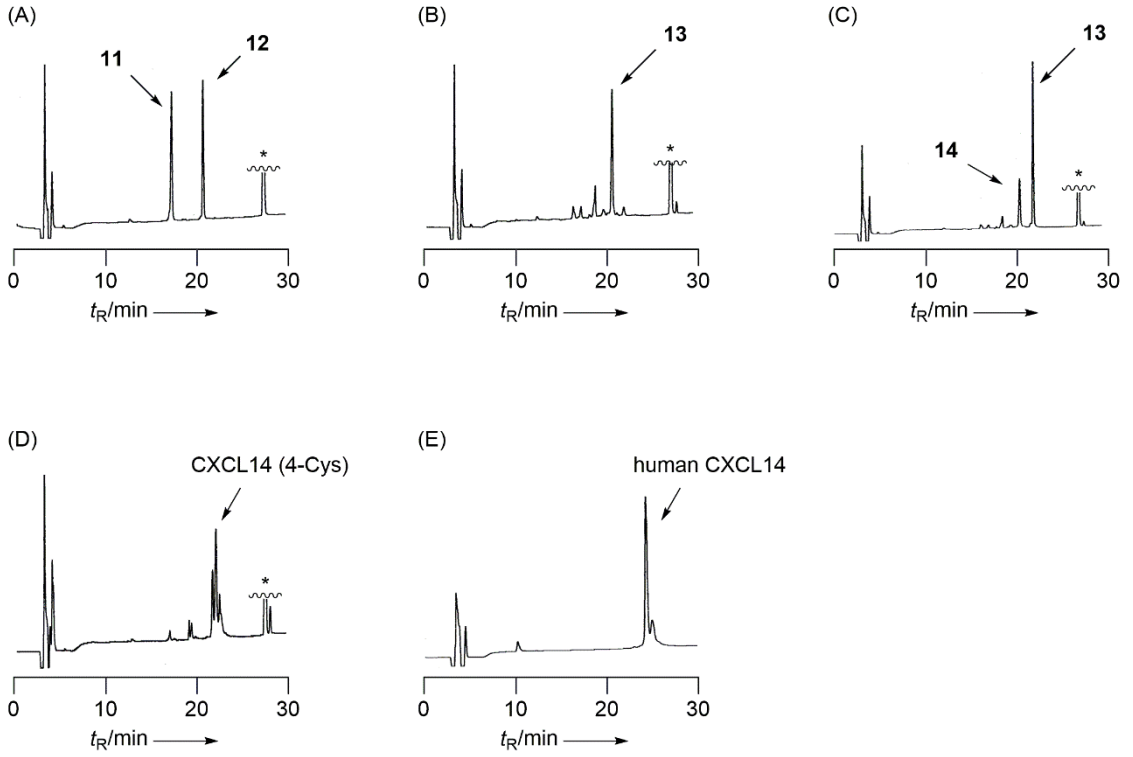
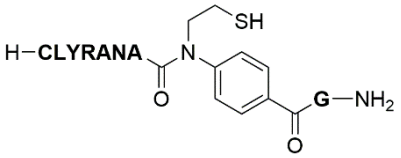


Figure S4. HPLC monitoring of one-pot/three-fragment ligation for synthesis of human CXCL14. (A) First NCL (t < 1 min); (B) First NCL (t = 3 h); (C) Second NCL, (t < 1 min); (D) Second NCL (t = 24 h); (E) Oxidation with air (t = 24 h). Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA–MeCN in 0.1% (v/v) TFA aq. (5:95–45:55 over 30 min) at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA.

Application of the resin-bound SEAlide peptide to one-pot three fragment ligation.

Preparation of N-Cys-SEAlide peptide 16.



16

The protected peptide resin was constructed on NovaSyn® TGR resin (loading: 0.25 mmol/g) using Fmoc SPPS (Acylation: Fmoc amino acid (4.0 equiv.), DIPCDI (4.0 equiv.) and HOBt·H_2O (4.0 equiv.) in DMF or **5a** (2.0 equiv.), HATU (1.9 equiv.) and DIEA (1.9 equiv.) in DMF for 2 h; Fmoc removal: 20% (v/v) piperidine in DMF for 10 min). The completed resin (300 mg, 53.1 μmol) was treated with TFA/*m*-cresol/1, 2-ethaneditiol/thioanisole/H_2O (80/5/5/5/5 (v/v)) at room temperature for 2 h. The resin was filtered off and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide thioester. The crude peptide thioester was purified by preparative HPLC to give the purified peptide **16** (29.0 mg, 22.8 μmol, 43% isolated yield).

16: Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 20.8 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 13 to 28% over 30 min, retention time = 19.1 min. MS (ESI-TOF) *m/z* calcd for ($[M+2H]^{2+}$) 523.2, found 523.3.

Preparation of peptide 19 using one-pot/three-fragment ligation.

The first NCL between resin-bound SEAlide peptide **15** (16.5 mg, 5.0 μmol) and N-Cys-SEAlide peptide **16** (1.3 mg, 1.0 μmol) was performed in 6 M Gn·HCl-0.1 M HEPPS buffer containing 40 mM MPAA and 40 mM TCEP·HCl (pH 6.9, 1 mL) at 37 ºC. The reaction was completed within 12 h. Then, N-Cys peptide **17** (0.95 mg, 1.0 μmol) solution in 0.5 M sodium phosphate buffer containing 40 mM TCEP·HCl (pH 7.4, 1 mL) was added to the reaction mixture. The second NCL proceeded at 37 ºC within 24 h, and then the crude material was purified by semi-preparative HPLC to give **19** (1.1 mg, 0.4 μmol, 40% isolated yield).

19: Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 55% over 30 min, retention time = 17.4 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 10 to 30% over 30 min, retention time = 28.7 min. MS (ESI-TOF) *m/z* calcd for ($[M+3H]^{3+}$) 697.0, found 697.0.

Recycling of the resin-bound SEAlide peptide.

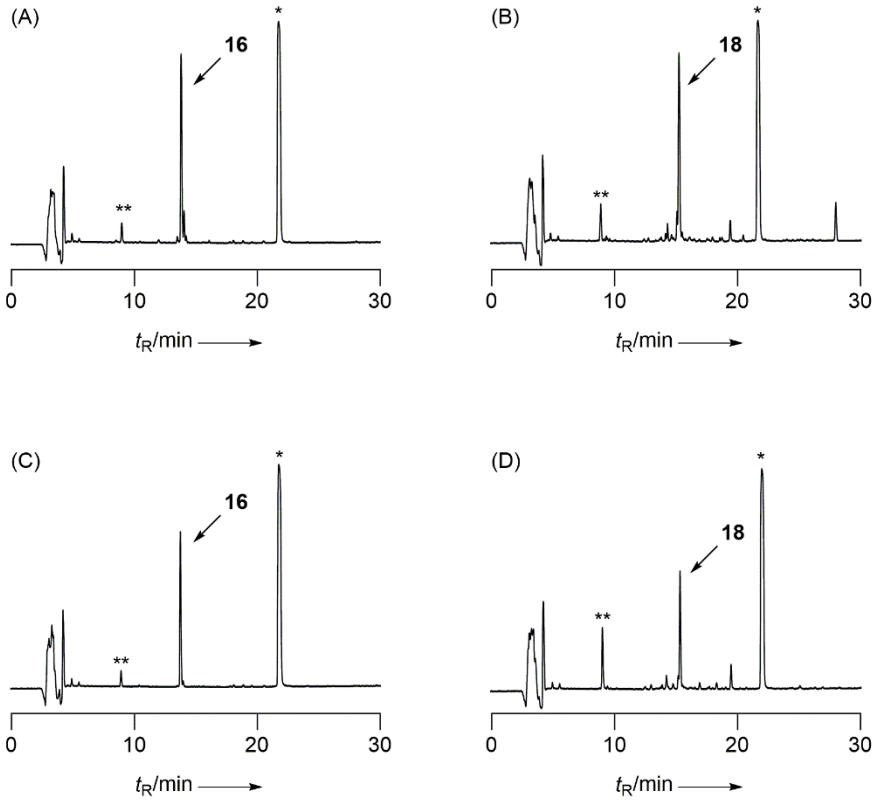


Figure S5. HPLC monitoring of recycling of resin-bound SEAlide peptide to NCL. (A) First round NCL (t < 1 min) in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 6.9), 40 mM MPAA and 40 mM TCEP·HCl; (B) First round NCL (t = 12 h); (C) Second round NCL (t < 1 min) in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 6.9), 40 mM MPAA and 40 mM TCEP·HCl; (D) Second round NCL (t = 42 h). Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA–MeCN in 0.1% (v/v) TFA aq. (5:95–55:45 over 30 min) at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA; **Non-peptide impurity.

Reference

1. Fields, G. B.; Tian, Z.; Barany, G. In *Synthetic Peptides, A User's Guide*; Grant, G.A. Eds.; W. H. Freeman and Company: New York, 1992; pp 119.