Electronic Supplementary Information

Examination of native chemical ligation using peptidyl prolyl thioester

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General Methods
Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™. For HPLC separations, a Cosmosil 5C18-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil 5C18-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) or a Cosmosil 5C18-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 Peptidyl Prolyl Thioesters 1a-d, f, h-s

\[ \text{H-LYRXP-S(CH}_2\text{)\text{2CO-L-NH}_2} \]

General procedure: Peptidyl prolyl thioesters 1 were prepared by Boc SPPS using \textit{in situ} neutralization protocol\(^{[S1]}\) on HSCH\(_2\)CH\(_2\)CO-Leu-4-methylbenzhydrylamine (MBHA) resin (0.70 mmol amine/g, 0.10 g, 0.070 mmol). For the incorporation of amino acids on the prolyl thioester, preactivated Boc amino acid (Boc amino acid, diisopropylcarbodiimide, and HOBt\(\cdot\)H\(_2\)O, 4 equiv. each in DMF for 30 min) was added to the resin. Then, 1 equiv. of diisopropylethylamine was added to the reaction mixture in four times every 30 minutes. Other amino acids were condensed according to the standard \textit{in situ} neutralization protocol. The resulting completed resin was treated with 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)-thioanisole in TFA (50 μL/1 mg resin)/m-cresol (100/5, (v/v)) at 4 °C for 2 h, and then the resin was filtrated off. To the filtrate was added cooled Et\(_2\)O to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et\(_2\)O to afford crude peptidyl prolyl thioesters 1. The crude peptides were purified by preparative HPLC to give the purified peptidyl prolyl thioesters 1.

\(1\text{a (X = Ala)}\): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.2 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 25% over 30 min. MS (ESI-TOF) \(m/z\) calcd ([M + H]\(^+\)) 819.5, found 819.3.

\(1\text{b (X = Val)}\): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 20.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 32% over 30 min. MS (ESI-TOF) \(m/z\) calcd ([M + H]\(^+\)) 847.5, found 847.3.
1c (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 27% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 805.4, found 805.2.

1d (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 16.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 21% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 863.4, found 863.2.

1f (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 23% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 877.5, found 877.3.

1h (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 16.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 21% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 835.5, found 835.3.

1i (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 21% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 849.5, found 849.3.

1j (X = Leu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 22.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 26% to 32% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 861.5, found 861.3.

1k (X = Ile): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 26% to 30% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 861.5, found 861.3.

1l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 21.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 25% to 33% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 879.5, found 879.2.

1m (X = Pro): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 18.2 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 25% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 845.5, found 845.3.
1n (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.2 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 26% to 32% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 895.5, found 895.2.

1o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 20.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 24% to 27% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 911.5, found 911.2.

1p (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 24.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 25% to 33% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 934.5, found 934.2.

1q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 14.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 19% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 885.5, found 885.2.

1r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 14.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 25% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 876.5, found 876.3.

1s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 13.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 25% over 30 min. MS (ESI-TOF) m/z calcd ([M + H]+) 904.5, found 904.3.
Preparation of Peptidyl Prolyl Thioesters 1e (X = Asn) and 1g (X = Gln)

\[ \text{H-LRANKLYRXP-S(CH}_2\text{)}_2\text{CO-L-NH}_2 \]

Peptidyl prolyl thioesters 1e and 1g were prepared by Boc SPPS as similar to that of 1a.

1e (X = Asn): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 16.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 16% to 22% over 30 min. MS (ESI-TOF) \(m/z\) calcd ([M + 2H]\(^2+\)) 722.9, found 722.9.

1g (X = Gln): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 15.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 22% over 30 min. MS (ESI-TOF) \(m/z\) calcd ([M + 2H]\(^2+\)) 729.9, found 729.9.

Preparation of N-Terminal Cysteinyl Peptide 2

\[ \text{H-CYRANK-NH}_2 \]

The peptide was elongated on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.60 g, 0.13 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-m-cresol-thioanisole-H\(_2\)O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 \(\mu\)L/1 mg resin), 2 h, at room temperature) followed by HPLC purification afforded the desired N-terminal cysteinyl peptide 2.

2: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 15% over 30 min, retention time = 14.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 13% over 30 min. MS (ESI-TOF) \(m/z\) calcd ([M + H]\(^+\)) 753.4, found 753.2.
Optimization of NCL Conditions for Peptidyl Prolyl Thioester

Peptidyl prolyl thioester 1b (0.11 mg, 0.10 µmol) and N-terminal cysteinyl peptide 2 (0.11 mg, 0.10 µmol) were dissolved in 94 µL of various ligation buffers. After addition of 6 µL benzamide as internal standard, the reaction mixture was incubated at 25, 37, or 50 °C and the reaction progress was monitored by analytical HPLC (a linear gradient of solvent B in solvent A, 1% to 50% over 30 min). According to report by Kent, reaction rates were estimated based on peak integration of the HPLC at reaction time = 0, 1, 2, 3 and 6 h. Second order rate constants (k) were derived from equation 1/[2] = kt + 1/[2]₀.

3b: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 16.2 min. MS (ESI-TOF) m/z calcd ([M + 2H]^2+) 691.4, found 691.3.

MPAA ester of 1b: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 23.0 min. MS (ESI-TOF) m/z calcd ([M + H]^+) 797.4, found 797.3.
Representative HPLC charts of NCL Reactions of Peptidyl Prolyl Thioesters
*Internal standard (benzamide)

**Figure S1.** HPLC monitoring of NCL reaction of 1b with 2 in entry 3 of Table 1.

**Figure S2.** HPLC monitoring of NCL reaction of 1b with 2 in entry 4 of Table 1.
Figure S3. HPLC monitoring of NCL reaction of 1b with 2 in entry 5 of Table 1.

Figure S4. HPLC monitoring of NCL reaction of 1b with 2 in entry 6 of Table 1.
**Figure S5.** HPLC monitoring of NCL reaction of 1b with 2 in entry 7 of Table 1.

**Figure S6.** HPLC monitoring of NCL reaction of 1b with 2 in entry 9 of Table 1.
Figure S7. HPLC monitoring of NCL reaction of 1b with 2 in entry 10 of Table 1.
Figure S8. NCL reaction rates with 2 in conditions of Table 1.
Preparation of Diastereomers of Peptide 3b

These peptides were synthesized on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.10 g, 0.022 mmol) using standard Fmoc SPPS protocols, respectively. After TFA cleavage (TFA-m-cresol-thioanisole-H₂O-1,2-ethanedithiol (80:5:5:5 (v/v), 50 μL/1 mg resin), 2 h, at room temperature), crude materials were used for following HPLC experiment.

3b (L-Pro): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 17.1 min. MS (ESI-TOF) m/z calcd ([M + 2H]²⁺) 691.4, found 691.3.

3b (D-Pro): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 18.3 min. MS (ESI-TOF) m/z calcd ([M + 2H]²⁺) 691.4, found 691.3.

Evaluation of Racemization of Pro at Ligation Site

![Analytical HPLC chart of mixture of crude 3b (L-Pro) and 3b (D-Pro). HPLC chart after 24 h of NCL reaction of 1b with 2 in entry 10 of Table 1.](image)
Preparation of Peptide Thioesters 7 (Fr 1) and 8 (Fr 2)

\[
\text{H-GVEINVK-S(CH}_2\text{)}_2\text{CO-L-NH}_2 \quad \text{H-CSGSPQCLKP-S(CH}_2\text{)}_2\text{CO-L-NH}_2
\]

7 and 8 were prepared by Boc SPPS as similar to preparation of 1.

7: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 23% over 30 min. MS (ESI-TOF) \( m/z \) calcd ([M + H\(^+\)]\(^+)\) 958.5, found 958.2.

8: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 16.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 23% over 30 min. MS (ESI-TOF) \( m/z \) calcd ([M + H\(^+\)]\(^+)\) 1219.6, found 1219.0.

Preparation of Peptide 9 (Fr 3)

\[
\text{H-CKDAGMRFGKCMNRKCHCTP-OH}
\]

The peptide was elongated on Fmoc-Pro-Wang resin (1.1 mmol amine/g, 0.20 g, 0.22 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-m-cresol-thioanisole-H\(2\)O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 \( \mu \)L/1 mg resin), 2 h, at room temperature) followed by HPLC purification afforded the desired peptide 9.

9: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 13.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 16% to 22% over 30 min. MS (ESI-TOF) \( m/z \) calcd ([M + 3H\(^3+\)]\(^3+\)) 762.7, found 762.5.
One-pot/Sequential NCL for the Synthesis of 6Cys-SH Kaliotoxin[53] 10

Protocol 1
Kinetically controlled ligation of peptide thioester 7 (1.78 mg, 1.50 μmol) and peptide thioester 8 (2.17 mg, 1.50 μmol) was performed in 6 M Gn·HCl-0.4 M Na phosphate buffer containing 167 mM TCEP and 250 mM MPAA (pH 7.0, 1.5 mL, 1.0 mM each peptide) at 25 °C. The reaction was completed within 3 h. After addition of peptide 9 (4.46 mg, 1.50 μmol) to the reaction mixture, temperature was elevated to 50 °C. The second NCL proceeded smoothly within 24 h. The crude material was purified by semi-preparative HPLC to give the purified 6Cys-SH kaliotoxin 10 (2.23 mg, 0.451 μmol, 30%).

Protocol 2
Ligation of peptide thioester 7 (1.78 mg, 1.50 μmol) and peptide thioester 8 (2.17 mg, 1.50 μmol) was performed in 6 M Gn·HCl-0.4 M Na phosphate buffer containing 20 mM TCEP and 30 mM MPAA (pH 7.0, 0.75 mL, 2.0 mM each peptide) at 37 °C. The reaction was completed within 3 h. After confirmation of the completion of the first NCL by HPLC analysis, peptide 9 (4.46 mg, 1.50 μmol) in 6 M Gn·HCl-0.4 M Na phosphate buffer containing 246 mM TCEP, 370 mM MPAA (pH 7.0, 0.75 mL) was added to the reaction mixture to yield the 6Cys-SH kaliotoxin 10 in one-pot manner. The second NCL proceeded smoothly within 24 h. The crude material was purified by semi-preparative HPLC to give the purified 6Cys-SH kaliotoxin 10 (2.17 mg, 0.439 μmol, 29%).

7 + 8: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 18.0 min. MS (ESI-TOF) m/z calcd ([M + 2H]2+) 980.0, found 979.7.

MPAA ester of 7: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 18.4 min. MS (ESI-TOF) m/z calcd ([M + H]+) 908.5, found 908.2.

Intramolecular thioester of (7 + 8): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 9.8 min. MS (ESI-TOF) m/z calcd ([M + 2H]2+) 870.9, found 870.7.

Cyclic peptide of 8: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 11.5 min. MS (ESI-TOF) m/z calcd ([M + H]+) 1001.5, found 1001.2.
**MPAA ester of (7 + 8):** Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 20.7 min. MS (ESI-TOF) m/z calcd ([M + 2H]$^{2+}$) 955.0, found 954.7.

**10:** Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 15.0 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min. MS (ESI-TOF) calcd (average isotopes) 4027.8, found 4027.3.
Folding for Preparation of Kaliotoxin 6

A modified method of previously reported one\textsuperscript{[S4]} was used to the folding. The 6Cys-SH kaliotoxin 10 (1.33 mg) was dissolved in 6 M Gdn·HCl-0.1 M Na phosphate buffer (pH 8.0, 0.66 mL), and the resulting solution was added to 50 mM Tris·HCl buffer containing 2 mM reduced form glutathione, 0.2 mM oxidized form glutathione and 0.003% (v/v) Tween 20 (pH 8.0, 3.44 mL, final concentration of protein 0.324 mg/mL). After storage at room temperature for one day, the solution was diluted with 0.1% TFA aq (1.0 mL). The crude material was purified by semi-preparative HPLC to give kaliotoxin 6 (0.60 mg, 0.12 µmol, 48%).

6: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 40% over 30 min, retention time = 17.3 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 40% over 30 min. MS (ESI-TOF) calcd (average isotopes) 4021.8, found 4021.1.

**Figure S10.** HPLC monitoring of the folding of 6Cys-SH kaliotoxin 10 ($t = 20$ h).

**Figure S11.** HPLC chart of kaliotoxin 10 after purification.
Influence of X Residue on Formation of Deleted Peptide

General procedure: Peptidyl prolyl thioester 1 (0.11 mg, 0.10 μmol) and N-terminal cysteinyl peptide 2 (0.11 mg, 0.10 μmol) were dissolved in 6 M Gn-HCl-0.4 M Na phosphate buffer containing 167 mM TCEP and 250 mM MPAA. The reaction mixture was incubated at 50 °C and the reaction progress was monitored by analytical HPLC.

3a (X = Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 14.9 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 677.4, found 677.3.

3b (X = Val): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 16.1 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 691.4, found 691.3.

3c (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.4 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 670.4, found 670.3.

3d (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 14.7 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 699.4, found 699.3.

3e (X = Asn): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.7 min. MS (ESI-TOF) m/z calcd ([M + 3H]^{3+}) 660.4, found 660.4.

3f (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 14.0 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 706.4, found 706.2.

3g (X = Gln): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.2 min. MS (ESI-TOF) m/z calcd ([M + 3H]^{3+}) 665.0, found 665.4.

3h (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.1 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 685.4, found 685.3.

3i (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.4 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 692.4, found 692.3.

3j (X = Leu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 17.7 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 698.4, found 698.3.
3k (X = Ile): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 17.2 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 698.4, found 698.3.

3l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 17.4 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 707.4, found 707.3.

3m (X = Pro): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.6 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 690.4, found 690.3.

3n (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 18.2 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 715.4, found 715.3.

3o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 16.2 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 723.4, found 723.2.

3p (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 18.5 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 734.9, found 734.8.

3q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 13.5 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 710.4, found 710.3.

3r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 13.1 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 705.9, found 705.8.

3s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 13.8 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 719.9, found 719.8.

4: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 14.2 min. MS (ESI-TOF) m/z calcd ([M + 2H]^{2+}) 593.3, found 593.3.

Deletion peptide derived from 1e or 1g (H-LRANKLYR-CYRANK-NH2):
Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.9 min. MS (ESI-TOF) m/z calcd ([M + 3H]^{3+}) 590.0, found 590.0.

Hydrolyzed peptide (H-LYR-OH): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 13.2 min. MS (ESI-TOF) m/z calcd ([M + H]^+) 451.3, found 451.4.
**Figure S12.** HPLC monitoring of NCL reaction of $1c$ ($X = $ Gly) with $2$.
*Non-peptidic impurity.*

**Figure S13.** HPLC monitoring of NCL reaction of $1h$ ($X = $ Ser) with $2$.
*Non-peptidic impurity.*
References for Supporting Information


