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 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 Peptidyl Prolyl Thioesters **1a-d**, **f**, **h-s**

H-**LYRXP**-S$(CH_2)_2$CO-**L**-NH_2

1a-d, **f**, **h-s**

General procedure: Peptidyl prolyl thioesters **1** were prepared by Boc SPPS using *in situ* neutralization protocol[S1] on $HSCH_2CH_2CO$-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·H_2O, 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 *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_2O to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptidyl prolyl thioesters **1**. The crude peptides were purified by preparative HPLC to give the purified peptidyl prolyl thioesters **1**.

1a (**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.

1b (**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)

H-**LRANKLYRXP**-S$(CH_2)_2$CO-**L**-NH_2

1e and **1g**

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**

H-**CYRANK**-NH_2

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_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), 2 h, at room temperature) followed by HPLC purification afforded the desired N-terminal cystenyl 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,[S2] 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 $1/[\mathbf{2}] = kt + 1/[\mathbf{2}]_0$.

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)

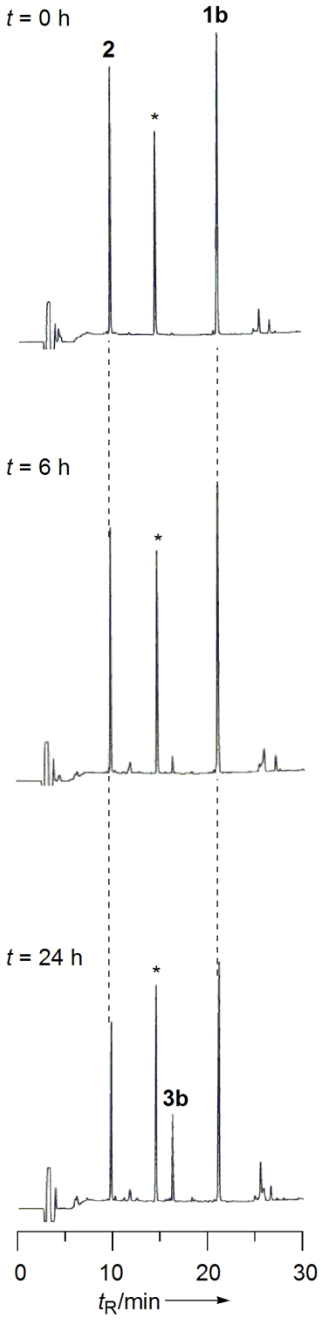

Figure S1. HPLC monitoring of NCL reaction of **1b** with **2** in entry **3** of Table 1.

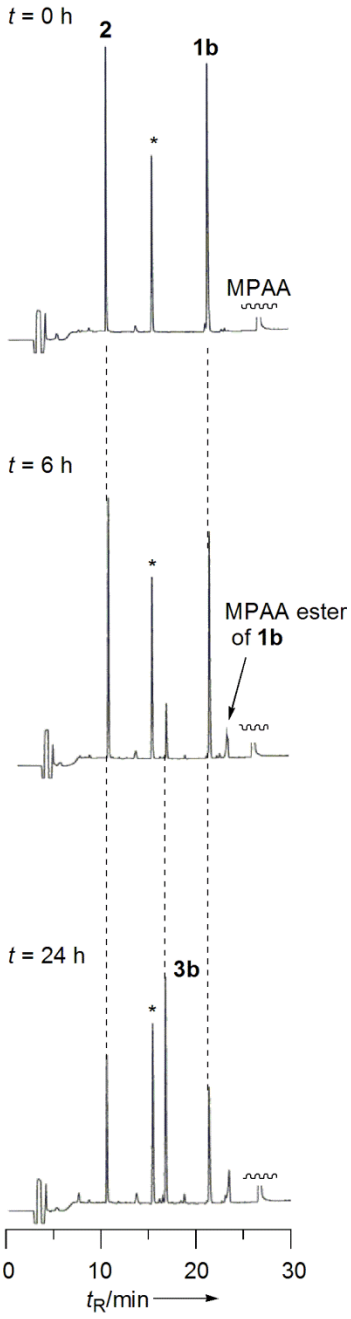

Figure S2. HPLC monitoring of NCL reaction of **1b** with **2** in entry **4** of Table 1.

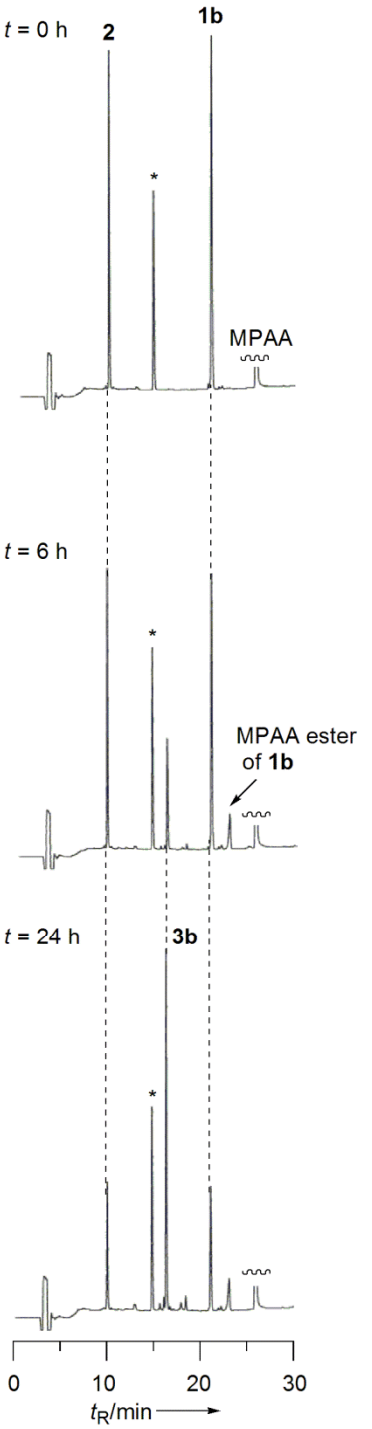

Figure S3. HPLC monitoring of NCL reaction of **1b** with **2** in entry **5** of Table 1.

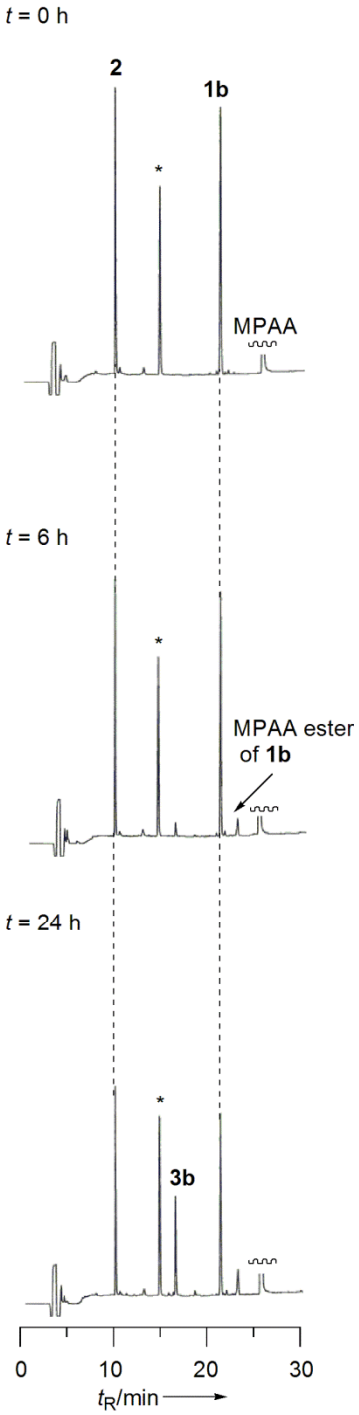

Figure S4. HPLC monitoring of NCL reaction of **1b** with **2** in entry **6** of Table 1.

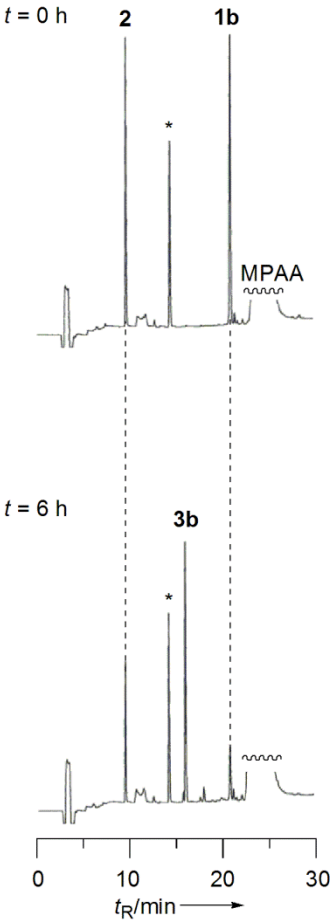

Figure S5. HPLC monitoring of NCL reaction of **1b** with **2** in entry **7** of Table 1.

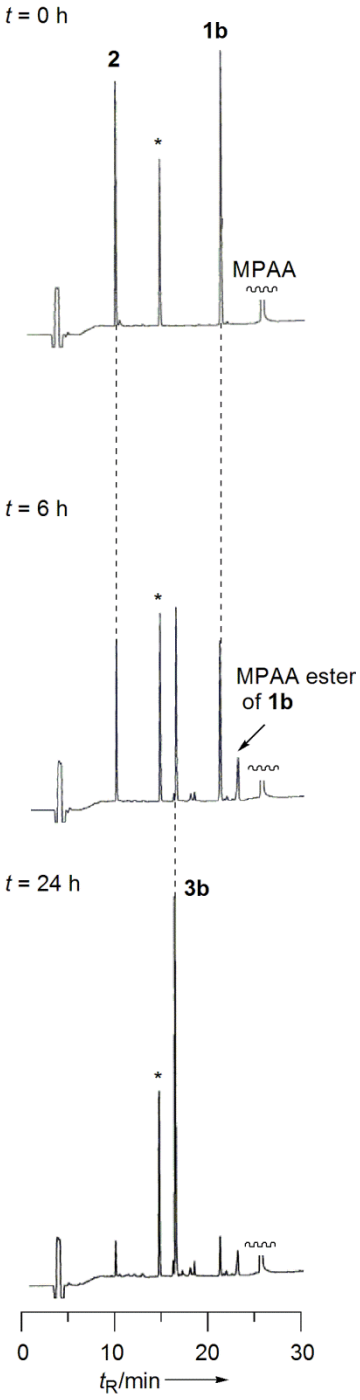

Figure S6. HPLC monitoring of NCL reaction of **1b** with **2** in entry **9** of Table 1.

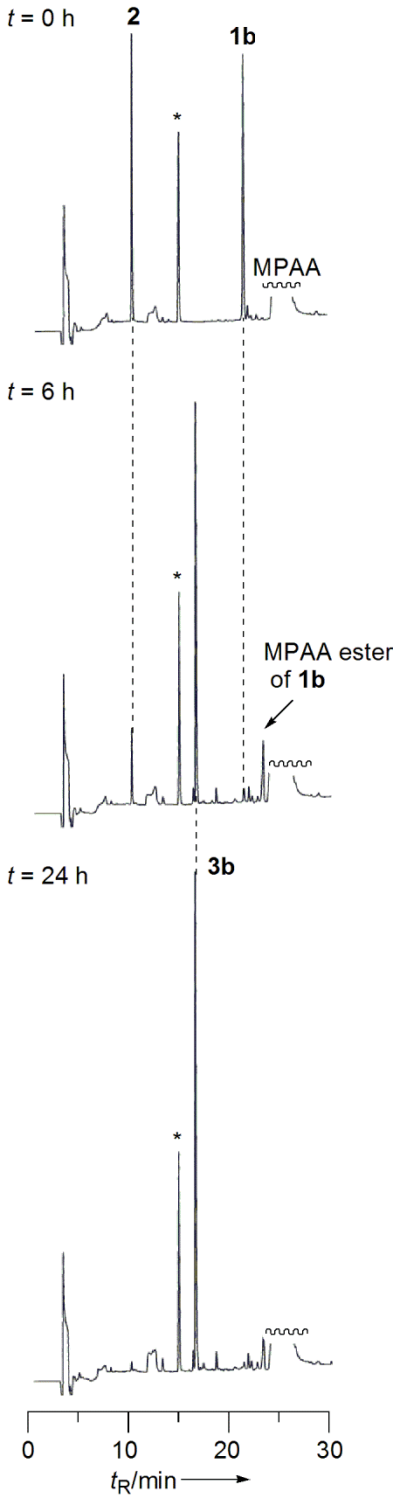

Figure S7. HPLC monitoring of NCL reaction of **1b** with **2** in entry **10** of Table 1.

NCL Reaction Rates with N-Terminal Cysteinyl Peptide 2

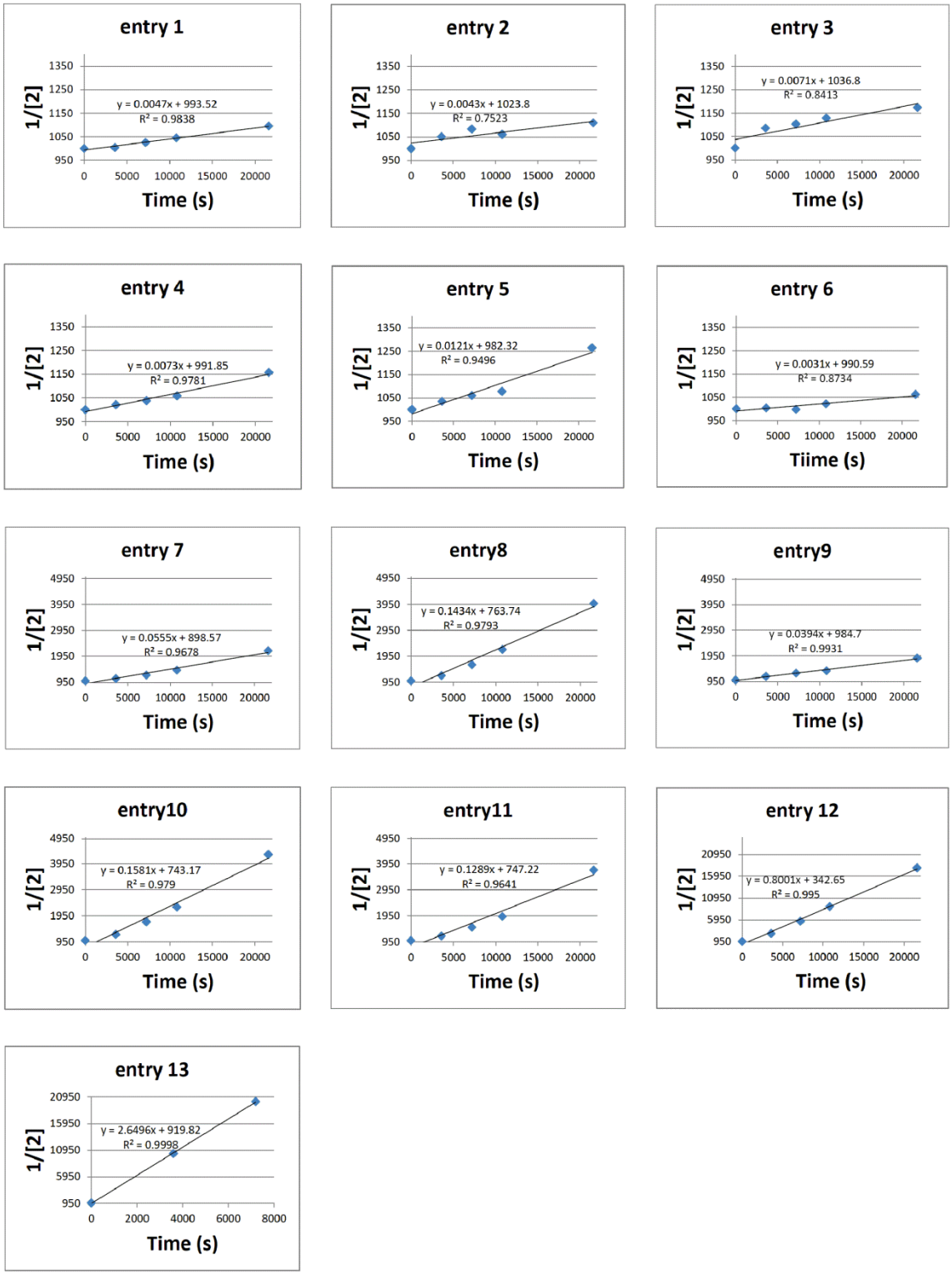

Figure S8. NCL reaction rates with **2** in conditions of Table 1.

Preparation of Diastereomers of Peptide **3b**

H-**LYRV-L-Pro-CYRANK**-NH_2 H-**LYRV-D-Pro-CYRANK**-NH_2

3b (L-Pro) **3b (D-Pro)**

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-H_2O-1,2-ethanedithiol (80:5:5:5:5 (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

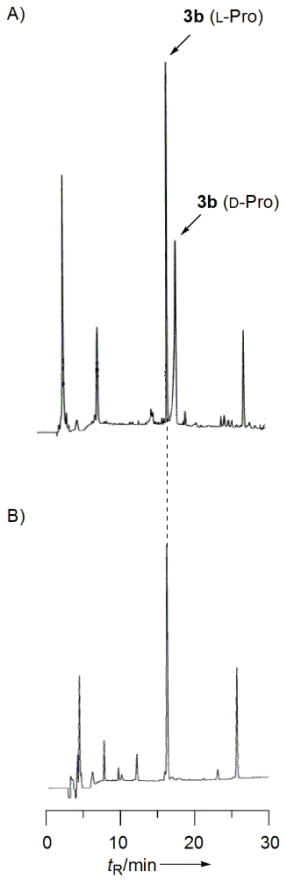

Figure S9. A) Analytical HPLC chart of mixture of crude **3b** (L-Pro) and **3b** (D-Pro). B) HPLC chart after 24 h of NCL reaction of **1b** with **2** in entry **10** of Table 1.

Preparation of Peptide Thioesters **7** (Fr 1) and **8** (Fr 2)

H-**GVEINVK**-S$(CH_2)_2$CO-**L**-NH_2 H-**CSGSPQCLKP**-S$(CH_2)_2$CO-**L**-NH_2

7 **8**

Peptide thioesters **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)

H-**CKDAGMRFGKCMNRKCHCTP**-OH

9

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_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/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 ($[M + 3H]^{3+}$) 762.7, found 762.5.

One-pot/Sequential NCL for the Synthesis of 6Cys-SH Kaliotoxin[S3] **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[S4] was used to the folding. The 6Cys-SH kaliotoxin **10** (1.33 mg) was dissolved in 6 M Gn·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.

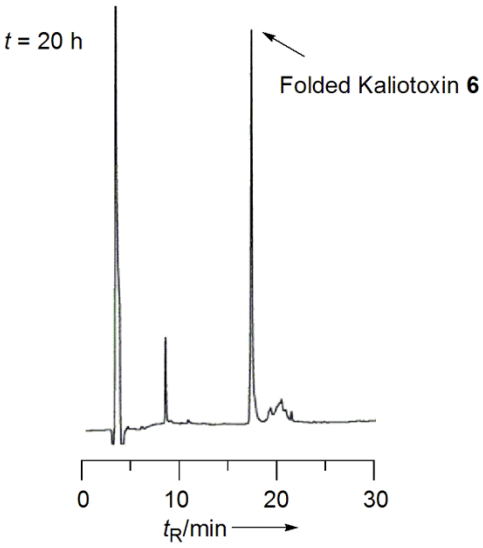

Figure S10. HPLC monitoring of the folding of 6Cys-SH kaliotoxin **10** (t = 20 h).

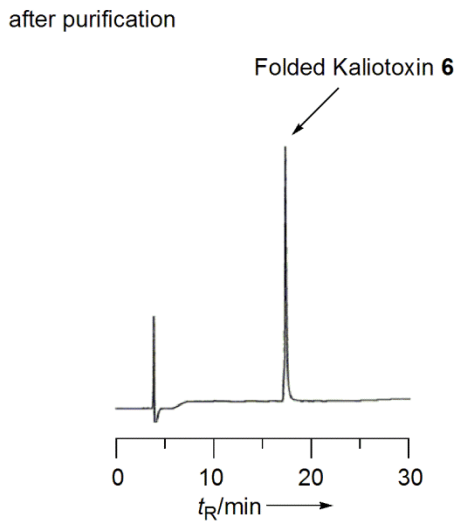

Figure S11. HPLC chart of kaliotoxin **10** after purification.

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-NH_2):
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.

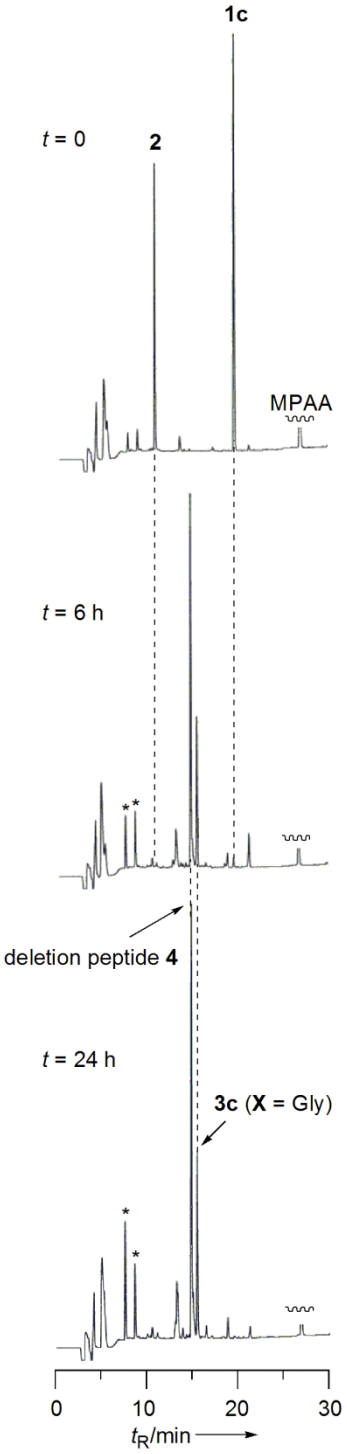

Figure S12. HPLC monitoring of NCL reaction of **1c** (**X** = Gly) with **2**. *Non-peptidic impurity.

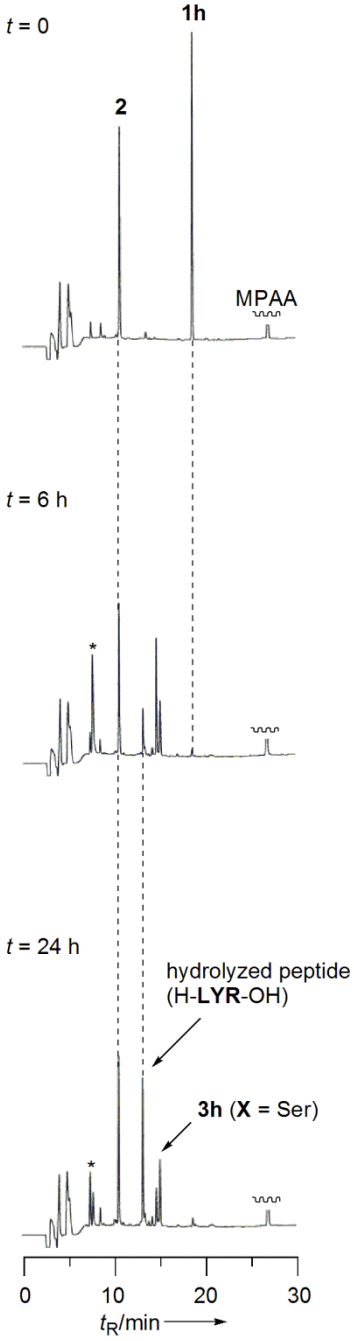

Figure S13. HPLC monitoring of NCL reaction of **1h** (**X** = Ser) with **2**. *Non-peptidic impurity.

References for Supporting Information

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