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Supporting Information

Development of a Chemical Methodology for the Preparation of Peptide Thioesters Applicable to Naturally Occurring Peptides Using a Sequential Quadruple Acyl Transfer System

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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 Peptides **1** and **3**

H-**AKLRFGCPSRHWKFL**-NH_2 **1**

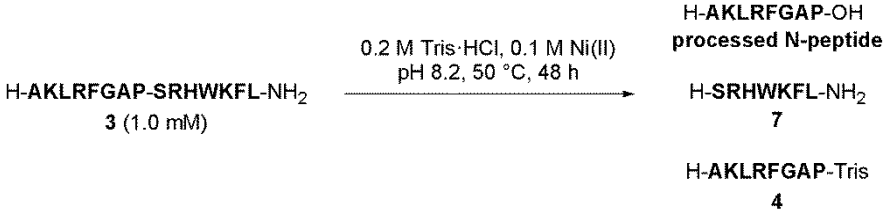
H-**AKLRFGAPSRHWKFL**-NH_2 **3**

General procedure: Protected peptide resin corresponding to peptide **1** or **3** was prepared by Fmoc SPPS on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.23 g, 0.05 mmol). The resulting completed resin was treated with TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin) at room temperature 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 peptide **1** or **3**. The crude peptide was purified by preparative HPLC to give the purified peptide **1** or **3**.

1: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 22.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 19% to 29% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{87}H_{133}N_{27}O_{16}S$ ([*M*+3H]$^{3+}$) 615.7, found 615.7.

3: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 22.2 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 19% to 29% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{87}H_{133}N_{27}O_{16}$ ([*M*+2H]$^{2+}$) 907.0, found 906.7.

Ni(II)-mediated Hydrolysis of Peptide **3**



Scheme S1. Ni(II)-mediated hydrolysis of peptide **3**.

Peptide **3** (0.25 mg, 0.1 μmol) was dissolved in 0.2 M Tris·HCl buffer containing 0.1 M $NiCl_2 \cdot 6H_2O$ (pH 8.2, 0.1 mL, 1.0 mM peptide). The reaction mixture was incubated at 50 °C and the reaction progress was monitored by analytical HPLC.

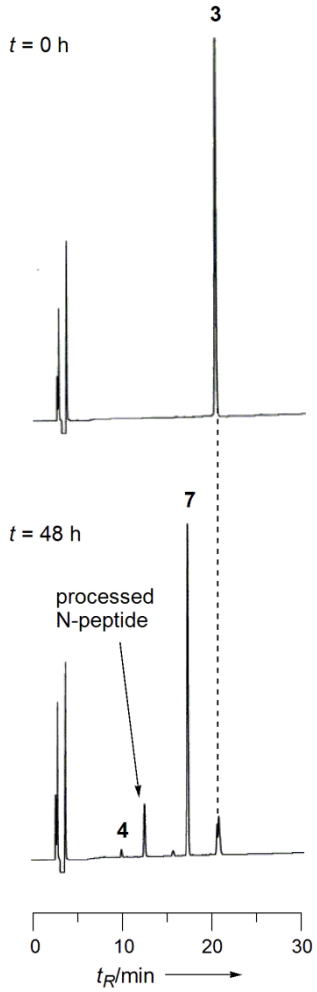


Figure S1. HPLC monitoring of Ni(II)-mediated hydrolysis of peptide **3**.

Processed N-peptide (H-AKLRFGAP-OH): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.4 min. MS (ESI-TOF) *m*/*z* calcd for $C_{40}H_{66}N_{12}O_9$ ($[M+H]^+$) 859.5, found 859.3.

7 (H-SRHWKFL-NH_2): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 17.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{47}H_{69}N_{15}O_8$ ($[M+H]^+$) 972.6, found 972.2.

4 (H-AKLRFGAP-Tris): 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 for $C_{44}H_{75}N_{13}O_{11}$ ($[M+2H]^{2+}$) 481.8, found 481.9.

Preparation of Peptides **5a-l**, **n-t**, and **u** for Their Ni(II)-mediated Conversion

Ac-**LYRAXSRHWKFL**-NH_2

5a-l, **n-u**

Protected peptide resins corresponding to the title peptides were constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

5a (X = Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 25.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{76}H_{113}N_{23}O_{15}$ ($[M+3H]^{3+}$) 530.3, found 530.6.

5b (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{75}H_{111}N_{23}O_{15}$ ($[M+2H]^{2+}$) 787.9, found 787.8.

5c (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 17.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 31% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{77}H_{113}N_{23}O_{17}$ ($[M+2H]^{2+}$) 816.9, found 816.8.

5d (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 17.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 31% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{78}H_{115}N_{23}O_{17}$ ($[M+2H]^{2+}$) 823.9, found 823.8.

5e (X = Asn): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 31% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{77}H_{114}N_{24}O_{16}$ ($[M+2H]^{2+}$) 816.4, found 816.3.

5f (X = Gln): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50%

over 30 min, retention time = 16.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{78}H_{116}N_{24}O_{16}$ ($[M+2H]^{2+}$) 823.5, found 823.3.

5g (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{76}H_{113}N_{23}O_{16}$ ($[M+2H]^{2+}$) 802.9, found 802.8.

5h (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 17.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 31% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{77}H_{115}N_{23}O_{16}$ ($[M+2H]^{2+}$) 809.9, found 809.8.

5i (X = Cys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 18.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{76}H_{113}N_{23}O_{15}S$ ($[M+2H]^{2+}$) 810.9, found 810.8.

5j (X = Pro): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{78}H_{115}N_{23}O_{15}$ ($[M+2H]^{2+}$) 807.9, found 807.8.

5k (X = Val): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 21.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 25% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{78}H_{117}N_{23}O_{15}$ ($[M+2H]^{2+}$) 809.0, found 808.8.

5l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 19.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 28% to 36% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{78}H_{117}N_{23}O_{15}S$ ($[M+2H]^{2+}$) 824.9, found 824.8.

5n (X = Ile): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 45% over 30 min, retention time = 23.4 min. Preparative HPLC conditions: A linear gradient of solvent B

in solvent A, 27% to 37% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{79}H_{119}N_{23}O_{15}$ ($[M+2H]^{2+}$) 816.0, found 815.8.

5o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 18.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{82}H_{117}N_{23}O_{16}$ ($[M+2H]^{2+}$) 841.0, found 840.8.

5p (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 21.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 27% to 37% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{82}H_{117}N_{23}O_{15}$ ($[M+2H]^{2+}$) 833.0, found 832.8.

5q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 15.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 28% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{79}H_{115}N_{25}O_{15}$ ($[M+2H]^{2+}$) 828.0, found 827.8.

5r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 15.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 29% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{79}H_{120}N_{24}O_{15}$ ($[M+2H]^{2+}$) 823.5, found 823.3.

5s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 15.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 29% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{79}H_{120}N_{26}O_{15}$ ($[M+2H]^{2+}$) 837.5, found 837.3.

5t (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 21.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 27% to 37% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{84}H_{118}N_{24}O_{15}$ ($[M + 2H]^{2+}$) 852.5, found 852.2.

5u (X = D-Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 24.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{76}H_{113}N_{23}O_{15}$

([*M*+2H]$^{2+}$) 794.9, found 794.8.

Preparation of Peptide **5m**

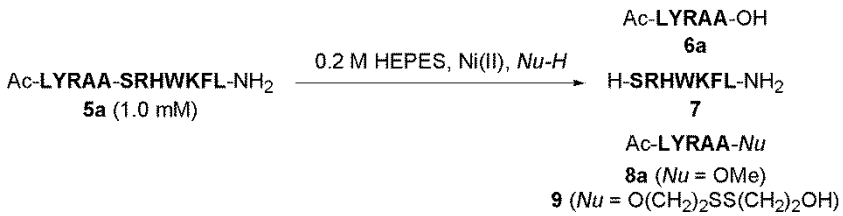
Ac-**KLYRALSRHWKFL**-NH_2

5m

Protected peptide resin corresponding to **5m** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

5m: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 19.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 25% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{85}H_{131}N_{25}O_{16}$ ([*M*+3H]$^{3+}$) 880.0, found 879.8.

Ni(II)-mediated Conversion of Peptide **5a** to Oxyesters **8a** and **9**



Scheme S2. Ni(II)-mediated conversion of peptide **5a** to oxyesters **8a** and **9**.

Peptide **5a** (0.20 mg, 0.10 μmol) was treated with various concentration of $NiCl_2$ (1~10 mM) in 0.2 M HEPES-alcohol (TFE, *i*-PrOH, MeOH, DTDE) buffer (96 μL, pH 7.8~8.2) in the presence of 0.05% (w/v) *p*-toluenesulfonamide in H_2O (4 μL) aq. as an internal standard, the reaction progress was monitored by analytical HPLC (a linear gradient of solvent B in solvent A, 15% to 35% over 30 min). Fraction converted was determined by HPLC separation and integration of **8a** (or **9**) (integ. **8a** (or **9**)) as a fraction of the sum of the unreacted **5a** (integ. **5a**) + hydrolyzed **6a** (integ. **6a**) + integ. **8a** (or **9**).

*Internal standard (*p*-toluenesulfonamide)

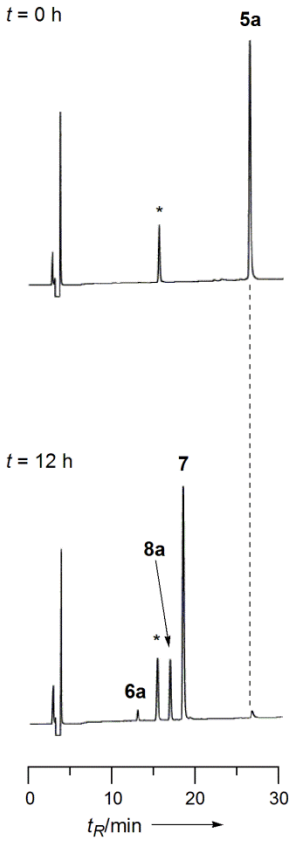


Figure S2. HPLC monitoring of Ni(II) mediated conversion of peptide **5a** to methyl ester **8a** in entry **5** of Table 1.

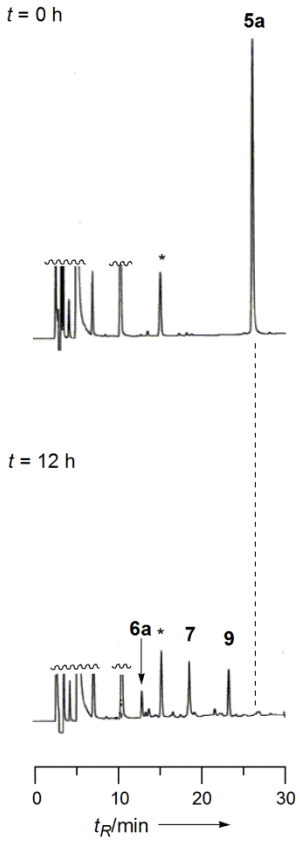


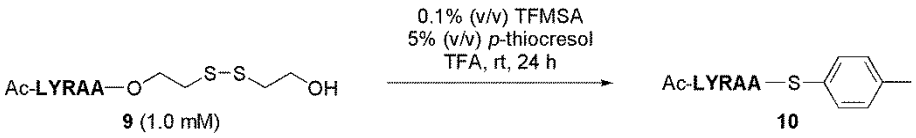
Figure S3. HPLC monitoring of Ni(II) mediated conversion of peptide **5a** to DTDE ester **9** in entry **12** of Table 1.

6a: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.6 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{46}N_8O_8$ ($[M+H]^+$) 635.3, found 635.3.

8a: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 16.4 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{48}N_8O_8$ ($[M+H]^+$) 649.4, found 649.2.

9: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 23.0 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{33}H_{54}N_8O_9S_2$ ($[M+H]^+$) 771.3, found 771.2.

Conversion of DTDE Ester Peptide **9** to Methylphenyl Thioester Peptide **10**



Scheme S3. Conversion of DTDE ester peptide **9** to methylphenyl thioester peptide **10**.

DTDE ester peptide **9** (0.09 mg, 0.1 μmol) was dissolved in TFA containing 0.1% (v/v) TFMSA, 5% (v/v) *p*-thiocresol (100 μL, 1.0 mM peptide). The reaction mixture was incubated at room temperature for 24 h. The reaction progress was monitored by analytical HPLC (a linear gradient of solvent B in solvent A, 10% to 50% over 30 min).

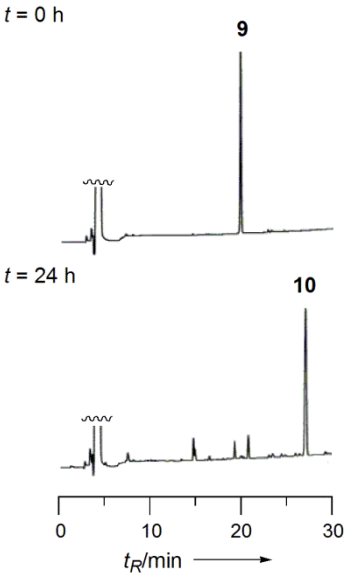


Figure S4. HPLC monitoring of methylphenyl thioesterification of peptide **9** to peptide **10**.

Methylphenyl thioester 10: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 50% over 30 min, retention time = 27.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{36}H_{52}N_8O_7S$ ($[M+H]^+$) 771.3, found 771.2.

Because the resulting thioester **10** was eluted as a single peak on HPLC analysis, risk of epimerization during the converting step could not be verified by the use of peptide **5a** and alternative peptide therefore was synthesized.

Examination of Epimerization During the Conversion of a SRHW-tagged Parent Peptide to the Corresponding DTDE and Methylthiophenyl Esters

As mentioned above, the parent peptide **5a** was unsuitable for the validation of epimerization, alternative parent peptide, Ac-**LYRASRHWKFL**-NH_2 **S28**, was synthesized in a manner similar to those employed for **5a**.

1. Preparation of Peptide **S28**

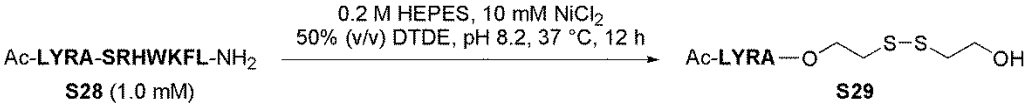
Ac-**LYRASRHWKFL**-NH_2

S28

Protected peptide resin corresponding to **S28** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **S28**.

S28: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 25.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{73}H_{108}N_{22}O_{14}$ ($[M+3H]^{3+}$) 759.4, found 759.3.

2. Conversion of Peptide **S28** to DTDE Ester Peptide **S29** (**L-Ala**)



Scheme S4. Conversion of peptide **S28** to DTDE ester peptide **S29** (**L-Ala**).

Peptide **S28** (3.95 mg, 2.0 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) DTDE (pH 8.2, 2.0 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 12 h. After confirmation of the completion of the reaction by HPLC analysis, the solution was diluted with 0.1% TFA aq (2.0 mL). HPLC analysis of the crude material clearly indicated that product corresponding to the DTDE ester appeared as single peak on HPLC chart (Figure 5S (A)). As mentioned later, obtained DTDE ester had **L-Ala** configuration. The crude material was purified by semi-preparative HPLC to give the purified DTDE ester **S29** (0.73 mg, 0.90 μmol, 45%).

S29: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30

min, retention time = 22.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{49}N_7O_8S_2$ ([*M*+H]$^+$) 700.3, found 700.3.

3. Preparation of Stereo-defined DTDE Ester Peptides **S29** (**L-Ala**) and **S30** (**D-Ala**)

Ac-**LYR-L-Ala**-DTDE Ac-**LYR-D-Ala**-DTDE

S29 (**L-Ala**) **S30** (**D-Ala**)

2-Chlorotrityl resin 50 mg (1.57 mmol/g) was swollen in DMF for 30 min. To the resin were added dithiodiethanol (0.1 mL, 10 equiv. for resin) and pyridine (0.07 mL, 10 equiv. for resin) in DMF. Then, incorporation of the C-terminal AA was performed using Fmoc-**L-Ala**-OH (or Fmoc-**D-Ala**-OH) and DIPCDI (10 equiv. each for resin) in the presence of DMAP (0.1 equiv. for resin). Standard elongation steps by Fmoc protocol followed by deprotection and subsequent HPLC purification afforded the desired reference peptide **S29** (**L-Ala**) or **S30** (**D-Ala**). Peptides **S29** and **S30** were well resolved each other on HPLC analysis (Figure S5 (C)). Based on the HPLC analysis in Figure S5, we concluded that no epimerization occurred during oxyesterification step.

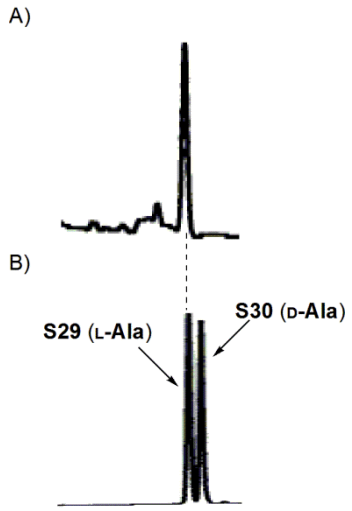


Figure S5. (A) HPLC chart after 12 h of DTDE esterification of **S28**. (B) HPLC chart of synthesized **L-Ala** (**S29**) and **D-Ala** (**S30**) containing DTDE ester peptide.

S29 (**L-Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 23.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{49}N_7O_8S_2$ ([*M*+H]$^+$) 700.3, found 700.2.

S30 (**D-Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50%

over 30 min, retention time = 24.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{49}N_7O_8S_2$ ($[M+H]^+$) 700.3, found 700.1.

4. Conversion of Stereo-defined DTDE Ester Peptide **S29** (L-**Ala**) to Methylphenyl Thioester Peptides **S31** (L-**Ala**) and **S32** (D-**Ala**)

Stereo-defined DTDE ester peptide **S29** (L-**Ala**) (2.4 mg, 3.0 μmol) was converted to the methylphenyl thioester by the action of TFA containing 0.1% (v/v) TFMSA, 5% (v/v) thiocresol (3 mL, 1.0 mM peptide) for 24 h at room temperature. HPLC analysis of the crude material indicated that two components corresponding to the methylthiophenyl esters appeared as separable peaks (Figure S6). Although the procedure for characterization of the configuration of the thioester part was described later, the resulting two components were homogeneously purified to give L-**Ala**-containing methylphenyl thioester **S31** (L-**Ala**) (0.50 mg, 0.64 μmol, 21%) and D-**Ala**-containing methylphenyl thioester **S32** (D-**Ala**) (0.15 mg, 0.19 μmol, 6%).

L-Ala-containing methylphenyl thioester S31 (L-**Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 23.4 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 60% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{33}H_{47}N_7O_6S$ ($[M+H]^+$) 670.3, found 670.2.

D-Ala-containing methylphenyl thioester S31 (D-**Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 23.8 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 60% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{33}H_{47}N_7O_6S$ ($[M+H]^+$) 670.3, found 670.2.

Hydrolyzed peptide of S29: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 15.2 min. MS (ESI-TOF) *m*/*z* calcd for $C_{26}H_{41}N_7O_7$ ($[M+H]^+$) 564.3, found 564.3.

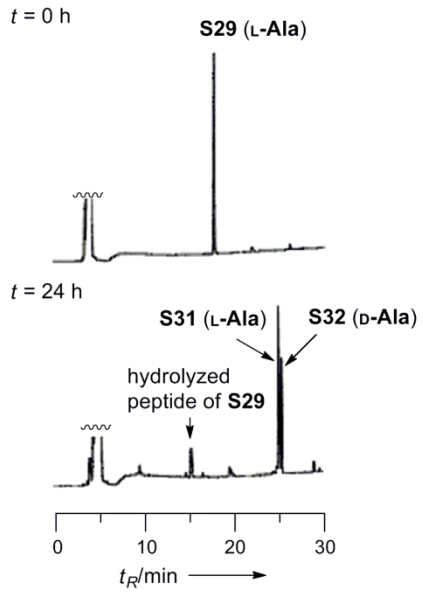
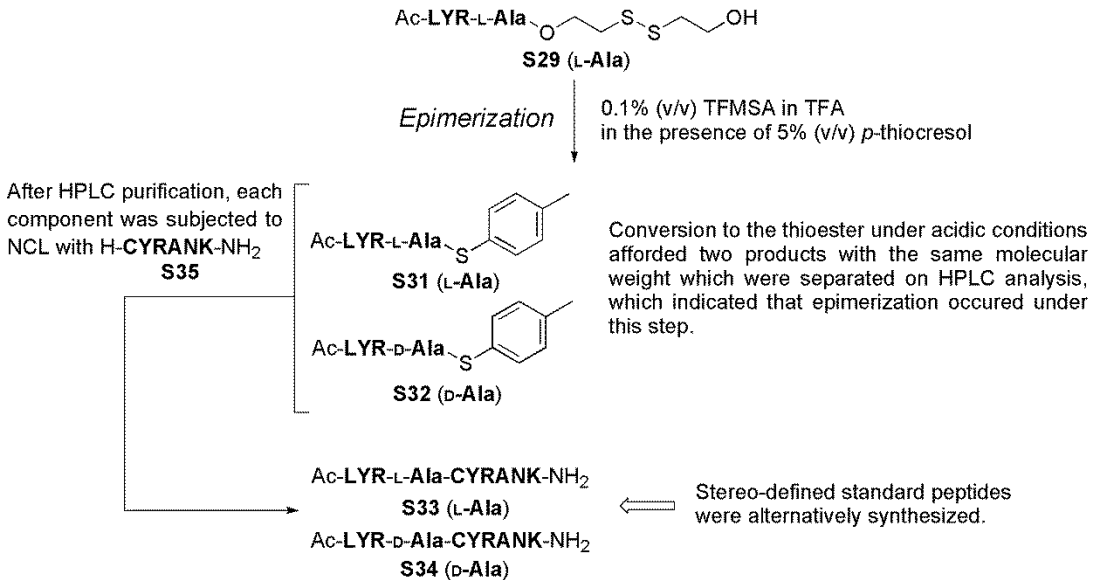


Figure S6. HPLC monitoring of thioesterification of synthesized L-**Ala**-containing DTDE ester peptide **S29**.

5. Preparation of Stereo-defined Ligated Products **S33** (L-**Ala**) and **S34** (D-**Ala**)



Scheme S5. Preparation of stereo-defined ligated products **S33** (L-**Ala**) and **S34** (D-**Ala**).

In order to determine the configuration of alanine of **S31** and **S32**, resulting methylthiophenyl ester **S31** and **S32** were subjected to NCL with N-terminal cysteinyl peptide, H-**CYRANK**-NH_2 **S35**, and then, resulting ligated peptides were analyzed by HPLC using stereo-defined Ac-**LYR**-L-**Ala**-**CYRANK**-NH_2 **S33** and Ac-**LYR**-D-**Ala**-**CYRANK**-NH_2 **S34** as authentic samples.

Ac-**LYR**-L-**Ala**-**CYRANK**-NH_2 **S33** (L-**Ala**)

Ac-**LYR**-D-**Ala**-**CYRANK**-NH_2 **S34** (D-**Ala**)

Protected peptide resins corresponding to the title peptides were constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

L-Ala-containing ligated product S33 (**L-Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 19.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 14% to 24% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{57}H_{91}N_{19}O_{14}S$ ($[M+2H]^{2+}$) 649.8, found 649.8.

D-Ala-containing ligated product S34 (**D-Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 18.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 14% to 24% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{57}H_{91}N_{19}O_{14}S$ ($[M+2H]^{2+}$) 649.8, found 649.8.

6. Preparation of N-terminal Cysteinyl Peptide **S35**

H-**CYRANK**-NH_2

S35

Protected peptide resin corresponding to **S35** was constructed NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

N-terminal cysteinyl peptide S35: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 11.6 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 for $C_{31}H_{52}N_{12}O_8S$ ($[M+H]^+$) 753.4, found 753.2.

7. NCL of Methylphenyl Thioester Peptides **S31** (**L-Ala**), **S32** (**D-Ala**) with N-terminal Cysteinyl Peptide **S35**

Methylphenyl thioester peptide **S31** (**L-Ala**) or **S32** (**D-Ala**) (0.08 mg, 0.1 μmol) and N-terminal cysteinyl peptide **S35** (0.14 mg, 0.13 μmol) were dissolved in 6 M Gn·HCl-0.2 M Na phosphate buffer containing 20 mM TCEP and 50 mM sodium ascorbate (pH 7.0, 0.1 mL) to perform NCL reaction. One hour reaction at 37 °C followed by HPLC purifications gave ligated peptides. Analysis of each obtained peptide by HPLC using authentic stereo-defined samples **S33** (**L-Ala**) and **S34**

(**D-Ala**) showed that thioester peptides **S31** and **S32** had C-terminal **L-Ala** and **D-Ala** residues, respectively.

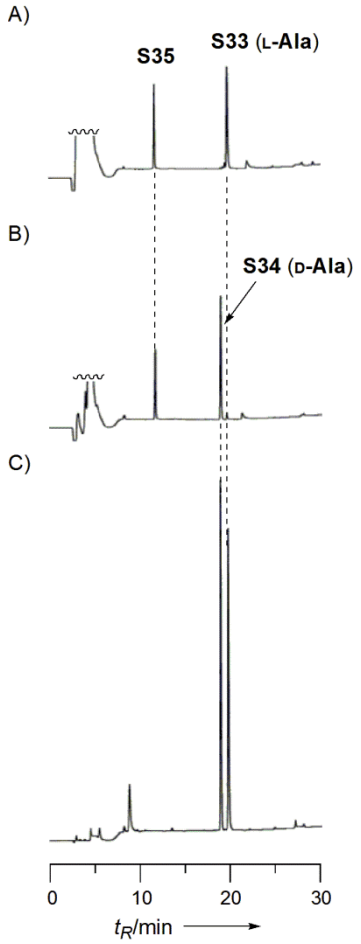
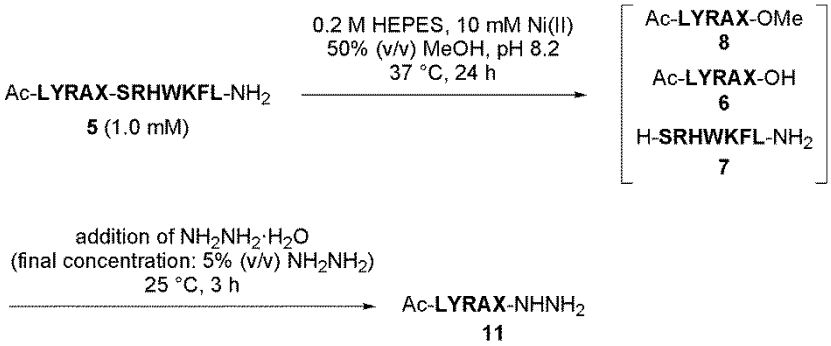


Figure S7. A) HPLC chart after 1 h of NCL reaction of **S31** (**L-Ala**) with **S35**. B) HPLC chart after 1 h of NCL reaction of **S32** (**D-Ala**) with **S35**. C) Analytical HPLC chart of mixture of synthesized ligated product **S33** (**L-Ala**) and **S34** (**D-Ala**).

Conversion of Peptides **5** to Peptide Hydrazides **11**



Scheme S6. Conversion of peptides **5** to peptide hydrazides **11**.

General procedure: Peptide **5** (0.20 mg, 0.1 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) MeOH (pH 8.2, 0.1 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 24 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (4.9 μL) into the reaction mixture (final concentration: 5% (v/v) NH_2NH_2). And then, additional reaction at 25 °C for 3 h gave

peptide hydrazide **11**. The reaction progress was monitored by analytical HPLC. Fraction converted was determined by HPLC separation and integration of **11** (integ. **11**) as a fraction of the sum of the unreacted **5** (integ. **5**) + hydrolyzed **6** (integ. **6**) + integ. **8** + integ. **11**.

11a (X = Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.5 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{48}N_{10}O_7$ ($[M+H]^+$) 649.4, found 649.3.

6b (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.2 min. MS (ESI-TOF) *m*/*z* calcd for $C_{28}H_{44}N_8O_8$ ($[M+H]^+$) 621.4, found 621.3.

8b (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 13.1 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{46}N_8O_8$ ($[M+H]^+$) 635.3, found 635.3.

11b (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 7.7 min. MS (ESI-TOF) *m*/*z* calcd for $C_{28}H_{46}N_{10}O_7$ ($[M+H]^+$) 635.4, found 635.4.

6c (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.1 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{46}N_8O_{10}$ ($[M+H]^+$) 679.3, found 679.3.

8c (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.9 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{48}N_8O_{10}$ ($[M+H]^+$) 693.3, found 693.3.

11c (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.1 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{48}N_{10}O_9$ ($[M+H]^+$) 693.4, found 693.3.

6d (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.8 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{48}N_8O_{10}$ ($[M+H]^+$) 693.3, found 693.3.

8d (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 14.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{50}N_8O_{10}$ ([*M*+H]$^+$) 707.4, found 707.2.

11d (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.4 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{50}N_{10}O_9$ ([*M*+H]$^+$) 707.4, found 707.3.

6g (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 9.6 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{46}N_8O_9$ ([*M*+H]$^+$) 651.3, found 651.3.

8g (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{48}N_8O_9$ ([*M*+H]$^+$) 665.4, found 665.3.

11g (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 7.7 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{48}N_{10}O_8$ ([*M*+H]$^+$) 665.4, found 665.3.

6h (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.5 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{48}N_8O_9$ ([*M*+H]$^+$) 665.4, found 665.3.

8h (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 13.3 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{50}N_8O_9$ ([*M*+H]$^+$) 679.4, found 679.3.

11h (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.4 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{50}N_{10}O_8$ ([*M*+H]$^+$) 679.4, found 679.3.

11j (X = Pro): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.5 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{50}N_{10}O_7$ ([*M*+H]$^+$) 675.4, found 675.4.

8k (X = Val): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 19.4 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{52}N_8O_8$ ([*M*+H]$^+$) 677.4, found 677.4.

11k (X = Val): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 9.3 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{52}N_{10}O_7$ ([*M*+H]$^+$) 677.4, found 677.4.

6l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 18.2 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{50}N_8O_8S$ ([*M*+H]$^+$) 695.3, found 695.3.

8l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 22.9 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{52}N_8O_8S$ ([*M*+H]$^+$) 709.4, found 709.2.

11l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 14.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{52}N_{10}O_7S$ ([*M*+H]$^+$) 709.4, found 709.3.

6m (X = Leu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 15.5 min. MS (ESI-TOF) *m*/*z* calcd for $C_{38}H_{64}N_{10}O_9$ ([*M*+H]$^+$) 805.5, found 805.3.

8m (X = Leu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 20.1 min. MS (ESI-TOF) *m*/*z* calcd for $C_{39}H_{66}N_{10}O_9$ ([*M*+H]$^+$) 819.5, found 819.3.

11m (X = Leu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 11.6 min. MS (ESI-TOF) *m*/*z* calcd for $C_{38}H_{66}N_{12}O_8$ ([*M*+H]$^+$) 819.5, found 819.3.

6o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 16.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{35}H_{50}N_8O_9$ ([*M*+H]$^+$) 727.4, found 727.3.

8o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 20.4 min. MS (ESI-TOF) *m*/*z* calcd for $C_{36}H_{52}N_8O_9$ ([*M*+H]$^+$) 741.4, found 741.3.

11o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 11.9 min. MS (ESI-TOF) *m*/*z* calcd for $C_{35}H_{52}N_{10}O_8$ ([*M*+H]$^+$) 741.4, found 741.3.

6p (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 20.9 min. MS (ESI-TOF) *m*/*z* calcd for $C_{35}H_{50}N_8O_8$ ([*M*+H]$^+$) 711.4, found 711.3.

8p (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 25.8 min. MS (ESI-TOF) *m*/*z* calcd for $C_{36}H_{52}N_8O_8$ ([*M*+H]$^+$) 725.4, found 725.3

11p (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 16.7 min. MS (ESI-TOF) *m*/*z* calcd for $C_{35}H_{52}N_{10}O_7$ ([*M*+H]$^+$) 725.4, found 725.3.

6q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 11.2 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{48}N_{10}O_8$ ([*M*+H]$^+$) 701.4, found 701.3.

8q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 13.5 min. MS (ESI-TOF) *m*/*z* calcd for $C_{33}H_{50}N_{10}O_8$ ([*M*+H]$^+$) 715.4, found 715.3.

11q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 9.8 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{50}N_{12}O_7$ ([*M*+H]$^+$) 715.4, found 715.3.

6r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 10.5 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{53}N_9O_8$ ([*M*+H]$^+$) 692.4, found 692.3.

8r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 13.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{33}H_{55}N_9O_8$ ([*M*+H]$^+$) 706.4, found 706.3.

11r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 8.7 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{55}N_{11}O_7$ ([*M*+H]$^+$) 706.4, found 706.3.

6s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 11.3 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{53}N_{11}O_8$ ([*M*+H]$^+$) 720.4, found 720.3.

8s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 14.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{33}H_{55}N_{11}O_8$ ([*M*+H]$^+$) 734.4, found 734.3.

11s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 9.6 min. MS (ESI-TOF) *m*/*z* calcd for $C_{32}H_{55}N_{13}O_7$ ([*M*+H]$^+$) 734.4, found 734.3.

6t (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 21.5 min. MS (ESI-TOF) *m*/*z* calcd for $C_{37}H_{51}N_9O_8$ ([*M*+H]$^+$) 750.4, found 750.3.

8t (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 26.6 min. MS (ESI-TOF) *m*/*z* calcd for $C_{38}H_{53}N_9O_8$ ([*M*+H]$^+$) 764.4, found 764.3.

11t (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 15.9 min. MS (ESI-TOF) *m*/*z* calcd for $C_{37}H_{53}N_{11}O_7$ ([*M*+H]$^+$) 764.4, found 764.3.

6u (X = D-Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.2 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{46}N_8O_8$ ([*M*+H]$^+$) 635.3, found 635.3.

8u (X = D-Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 16.2 min. MS (ESI-TOF) *m*/*z* calcd for $C_{30}H_{48}N_8O_8$ ([*M*+H]$^+$) 649.4, found 649.3.

11u (X = D-Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.7 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{48}N_{10}O_7$ ([*M*+H]$^+$) 649.4, found 649.3.

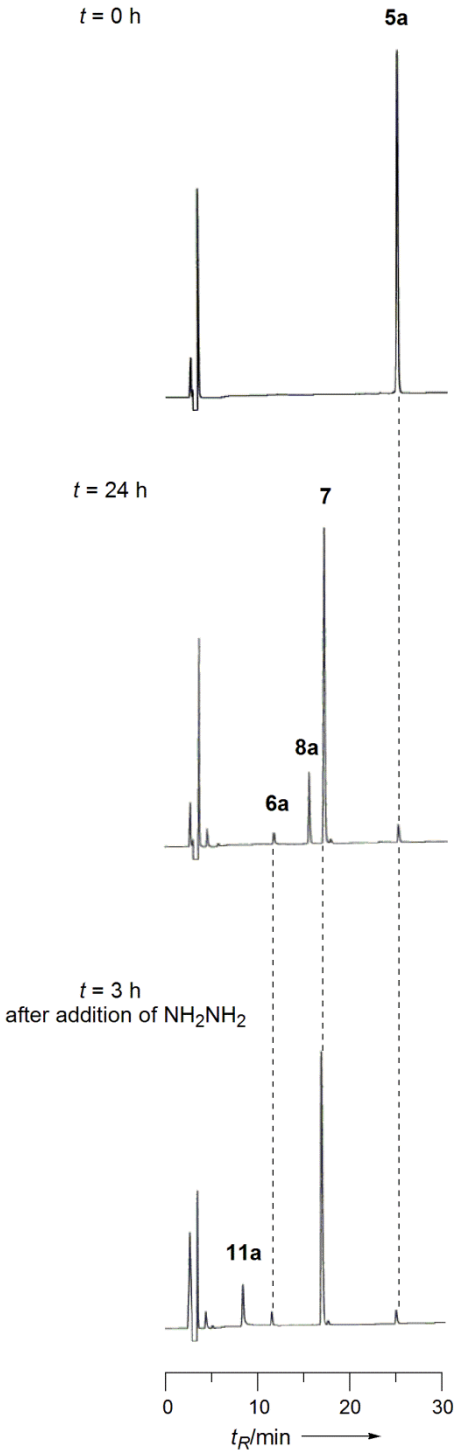


Figure S8. HPLC monitoring of conversion of peptide **5a (X = Ala)** to peptide hydrazide **11a** in entry 1 of Table 2.

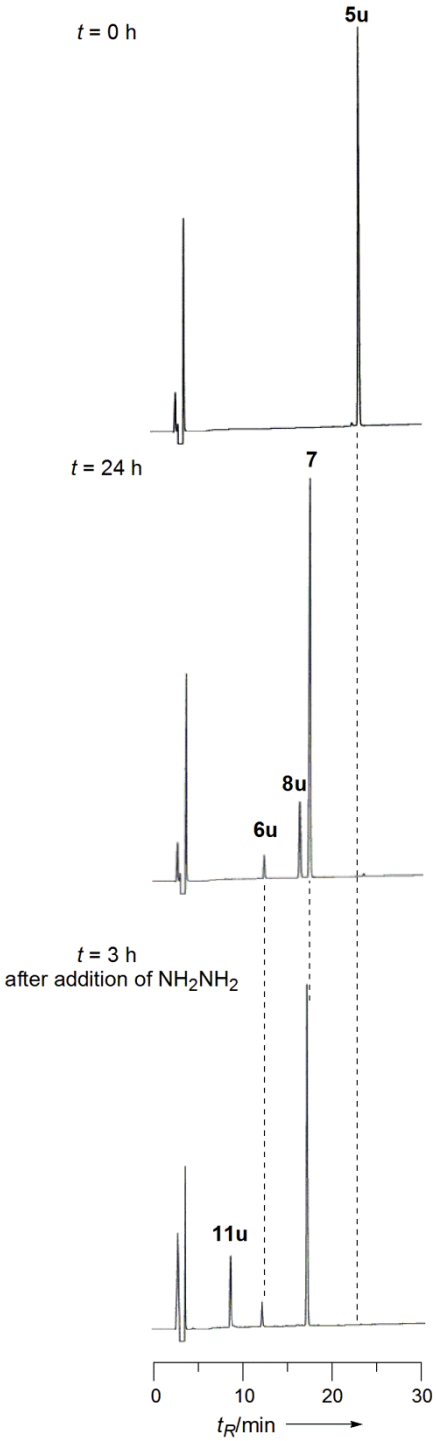
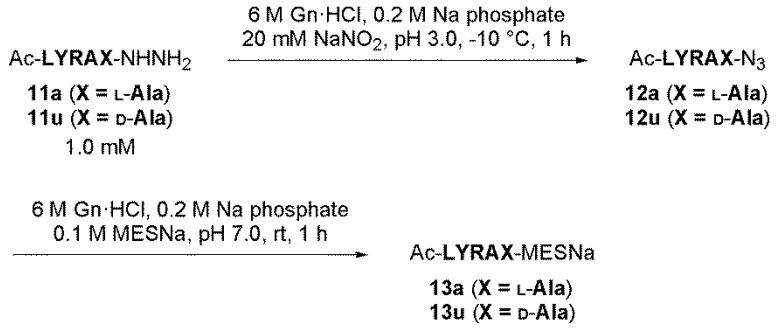


Figure S9. HPLC monitoring of conversion of peptide **5u (X = D-Ala)** to peptide hydrazide **11u**.

Preparation of Peptide Hydrazides **11a** and **11u**

Peptide **5a** (8.18 mg, 4.0 μmol) (or peptide **5u** (8.18 mg, 4.0 μmol)) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) MeOH (pH 8.2, 4.0 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 12 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (0.2 mL) into the reaction mixture (final concentration: 5% (v/v) NH_2NH_2). And then, additional reaction for 1 h at 25 °C gave peptide hydrazide **11a** (or peptide hydrazide **11u**). After confirmation of the completion of the reaction by HPLC analysis, the solution was diluted with 0.1% TFA aq. (4.0 mL). The crude material was purified by semi-preparative HPLC to give the purified peptide hydrazide **11a** (2.81 mg, 3.20 μmol, 80%) (or peptide **11u** (3.14 mg, 3.58 μmol, 90%)).

Conversion of Peptide Hydrazide **11a** (or **11u**) to MESNa Ester **13a** (or **13u**)



Scheme S7. Conversion of peptide hydrazide **11a** (or **11u**) to MESNa ester **13a** (or **13u**).

Peptide **11a** (0.087 mg, 0.1 μmol) (or Peptide **11u** (0.087 mg, 0.1 μmol)) was dissolved in 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 0.1 mL, 3 mM peptide). The reaction mixture was stored at -10 °C (Fig. S10 (A)). Then. 10 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was stored at -10 °C for 1 h (Fig. S10 (B)). After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M MESNa (0.1 mL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 2.0 M NaOH aq.. The reaction mixture was stored at room temperature for 1 h (Fig. S10 (C)). The reaction was monitored by analytical HPLC.

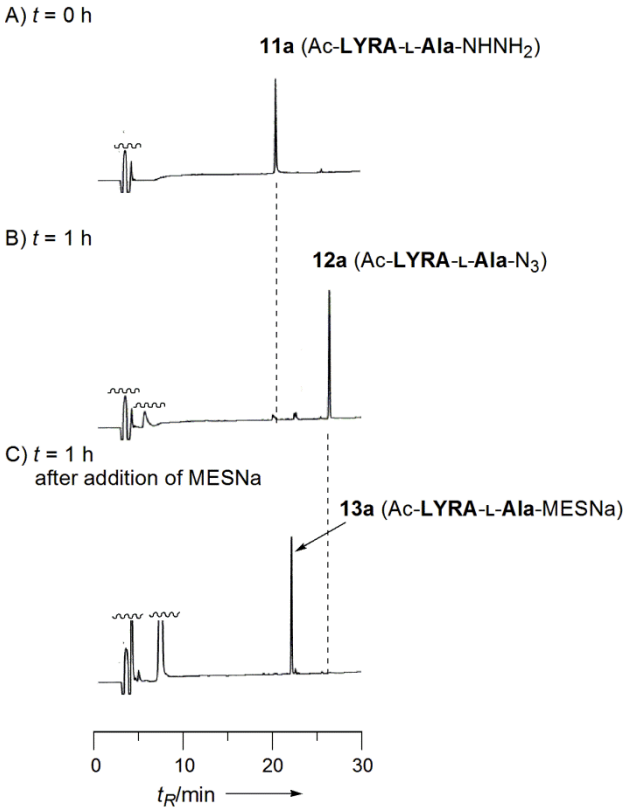


Figure S10. A) HPLC chart after 0 h of azidation of peptide **5a (X = Ala)** to peptide azide **12a**. B) HPLC chart after 1 h of azidation of peptide **5a (X = Ala)** to peptide azide **12a**. C) HPLC chart after 1 h of thioesterification of peptide **12a** to peptide thioester **13a**.

12a (Ac-**LYRA-L-Ala**-N_3): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 26.5 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{45}N_{11}O_7$ ($[M+H]^+$) 660.4, found 660.3.

13a (Ac-**LYRA-L-Ala**-MESNa): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 22.0 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{50}N_8O_{10}S_2$ ($[M+H]^+$) 759.3, found 759.1.

12u (Ac-**LYRA-D-Ala**-N_3): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 27.7 min. MS (ESI-TOF) *m*/*z* calcd for $C_{29}H_{45}N_{11}O_7$ ($[M+H]^+$) 660.4, found 660.3.

13u (Ac-**LYRA-D-Ala**-MESNa): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 23.1 min. MS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{50}N_8O_{10}S_2$ ($[M+H]^+$) 759.3, found 759.1.

Evaluation of Epimerization of Ala at Thioesterification Site

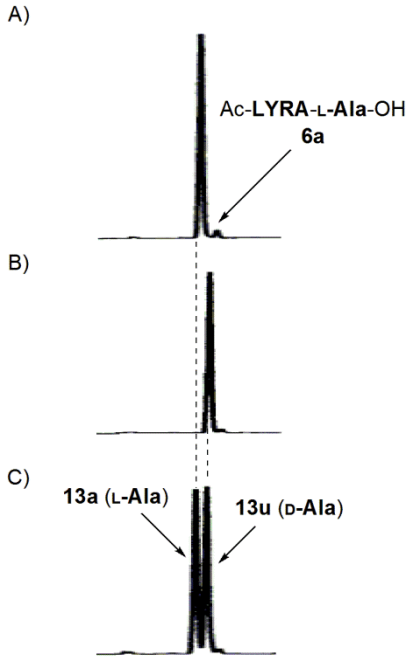
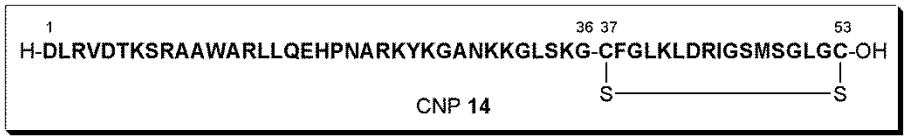


Figure S11. A) Analytical HPLC chart of crude **13a** (**L-Ala**). B) Analytical HPLC chart of crude **13u** (**D-Ala**). C) Analytical HPLC chart of mixture of crude **13a** (**L- Ala**) and **13u** (**D-Ala**).

Preparation of 43-residue CNP Peptide **16** and N-terminal Cysteinyl CNP Fragment **20**



H-**DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKG-SRHWKFL**-NH_2

16

Protected peptide resin corresponding to **16** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.5g, 0.11 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **16**.

16: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 50% over 30 min, retention time = 16.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 19% to 29% over 30 min. HRMS (ESI-TOF) m/z calcd for $C_{224}H_{363}N_{75}O_{57}$ ($[M+H]^+$) 5018.7, found 5018.6.

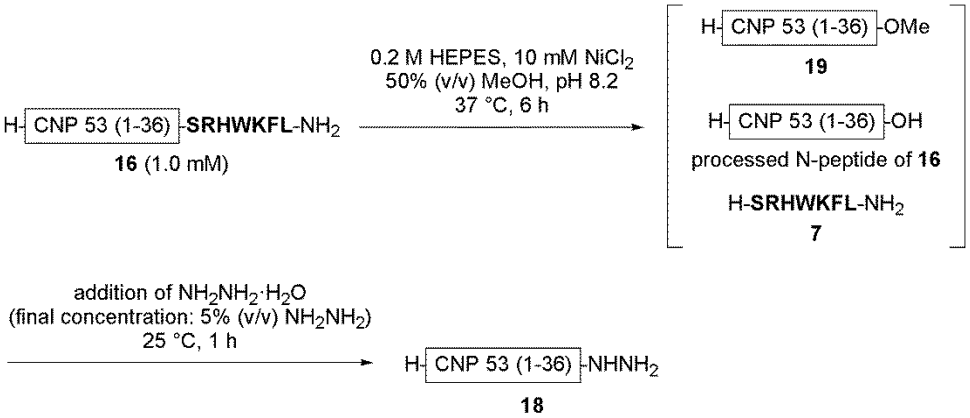
H-**CFGLKLDRIGSMSGLGC**-OH

20

Protected peptide resin corresponding to **20** were constructed on Wang resin (1.1 mmol amine/g, 0.09 g, 0.099 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **20**.

20: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 23.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{74}H_{125}N_{21}O_{22}S_3$ ($[M+2H]^{2+}$) 878.9, found 878.7.

Conversion of 43-residue CNP Peptide **16** to 36-residue Peptide Hydrazide **18**



Scheme S8. Conversion of 43-residue CNP peptide **16** to 36-residue peptide hydrazide **18**.

Peptide **16** (6.73 mg, 1.0 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) MeOH (pH 8.2, 1.0 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 6 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (0.053 mL) into the reaction mixture (final concentration: 5% (v/v) NH_2NH_2). And then, additional reaction at 25 °C for 1 h gave peptide hydrazide **18**. After confirmation of the completion of the reaction by HPLC analysis, the solution was diluted with 0.1% TFA aq. (4.0 mL). The crude material was purified by semi-preparative HPLC to give the purified peptide hydrazide **18** (2.80 mg, 0.69 μmol, 69%).

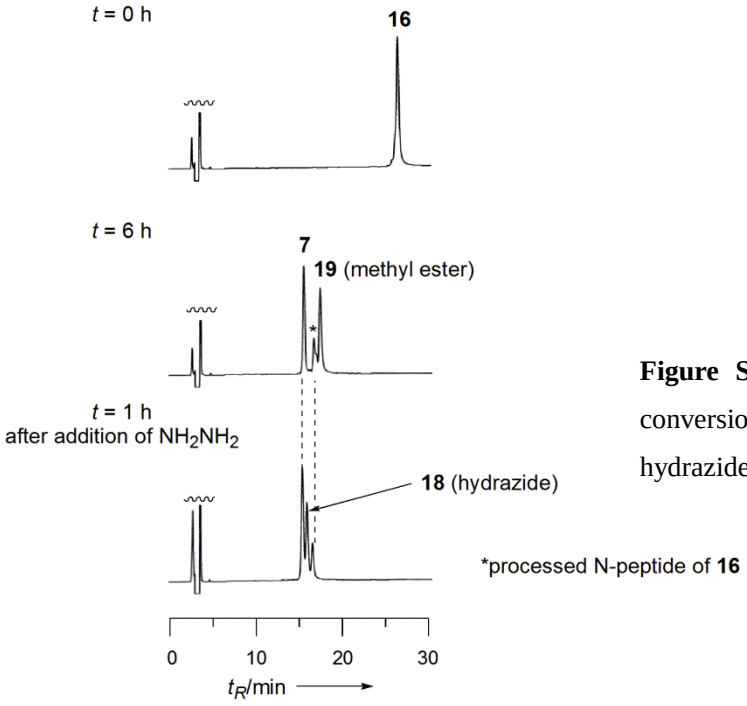


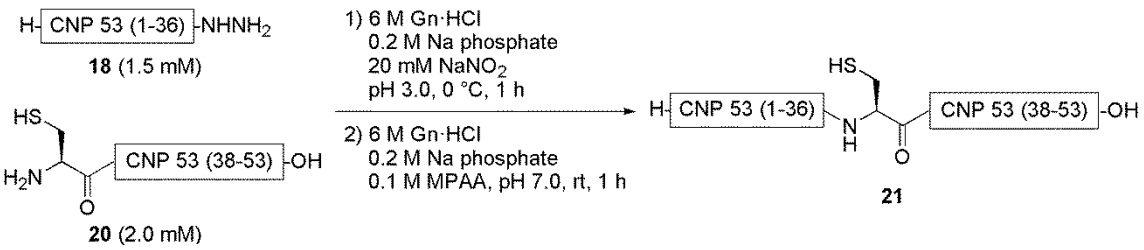
Figure S12. HPLC monitoring of conversion of peptide **16** to peptide hydrazide **18**.

Processed N-peptide of 16: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min, retention time = 16.6 min. HRMS (ESI-TOF) m/z calcd for $C_{177}H_{296}N_{60}O_{50}$ ($[M+H]^+$) 4064.6, found 4064.5.

Methylester 19: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min, retention time = 17.3 min. HRMS (ESI-TOF) m/z calcd for $C_{178}H_{298}N_{60}O_{50}$ ($[M+H]^+$) 4078.6, found 4078.3.

Hydrazide 18: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min, retention time = 15.8 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min. HRMS (ESI-TOF) m/z calcd for $C_{177}H_{298}N_{62}O_{49}$ ($[M+H]^+$) 4078.7, found 4078.4.

NCL for the Synthesis of Reduced Form CNP 53 **21**



Scheme S9. NCL for the synthesis of reduced form CNP 53 **21**.

Peptides **18** (3.22 mg, 0.58 μmol) and **20** (1.57 mg, 0.75 μmol) were dissolved in 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 0.19 mL, 3 mM or 4 mM each peptides). The reaction mixture was stored at 0 °C. Then. 19 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was stored at 0 °C for 1 h. After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M MPAA (0.19 mL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 2.0 M NaOH aq.. The reaction mixture was stored at room temperature for 1 h. After completion of the reaction, the solution was diluted with 30 mM TCEP aq. (pH7.0, 0.4 mL). The crude material was purified by semi-preparative HPLC to give the reduced form CNP 53 **21** (2.00 mg, 0.27 μmol, 47%).

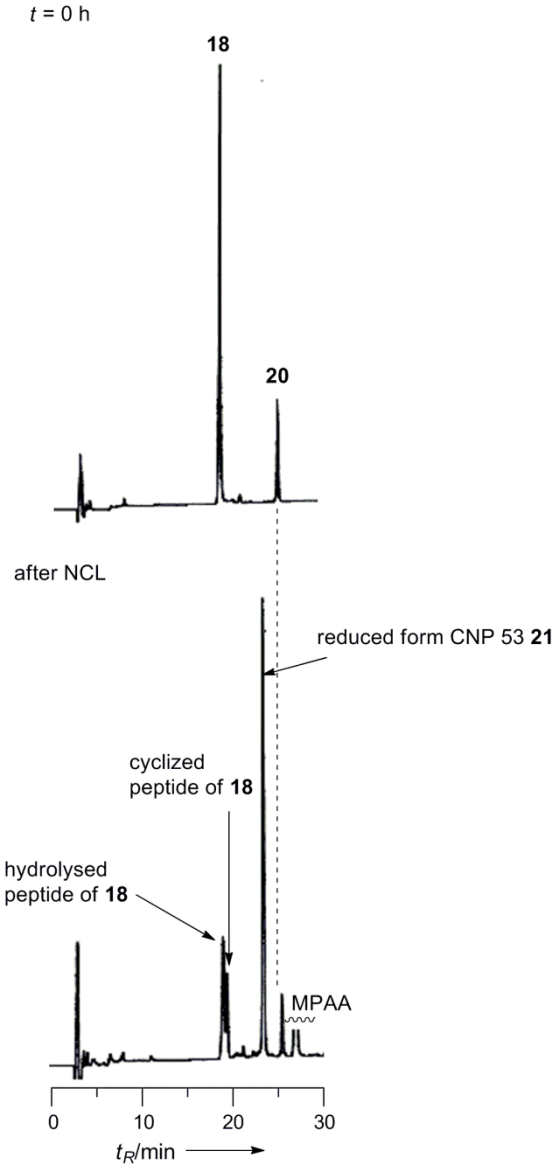


Figure S13. HPLC monitoring of NCL of peptide hydrazide **18** with N-terminal Cysteinyl CNP Fragment **20**.

Reduced form CNP 53 21: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.1 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min. HRMS (ESI-TOF) calcd for $C_{251}H_{419}N_{81}O_{71}S_3$ ([*M*+H]$^+$) 5803.7, found 5803.3.

Cyclized peptide of 18: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 19.8 min. HRMS (ESI-TOF) calcd for $C_{251}H_{417}N_{81}O_{70}S_3$ ([*M*+H]$^+$) 4046.6, found 4046.3.

Folding for Preparation of CNP 53 **14**

Reduced form CNP 53 **21** (1.94 mg, 0.26 μmol) was dissolved in 0.1 M Na phosphate buffer containing 6 M Gn·HCl, (pH 7.3, 0.79 mL) and DMSO (0.09 mL). The reaction mixture was incubated at 37 °C for 24 h. The crude material was purified by semi-preparative HPLC to give the purified folded CNP 53 **14** (1.28 mg, 0.17 μmol, 66%).

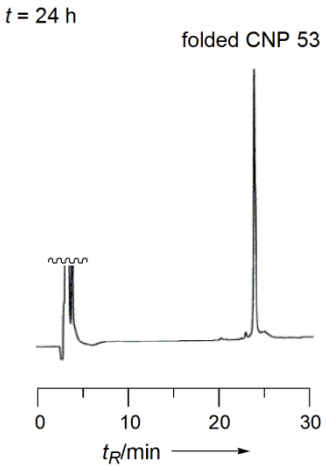


Figure S14. HPLC monitoring of the folding of reduced form CNP 53 **21** (*t* = 24 h).

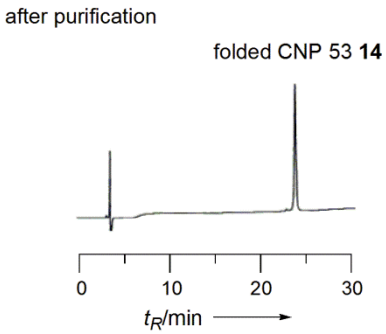
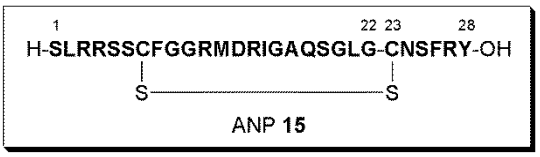


Figure S15. HPLC chart of folded CNP 53 **14** after purification.

Folded CNP 53 14: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.4 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min. HRMS (ESI-TOF) calcd for $C_{251}H_{417}N_{81}O_{71}S_3$ ([*M*+H]$^+$) 5801.7, found 5801.6.

Preparation of 29-residue ANP Peptide **17** and N-terminal Cysteinyl ANP Fragment **24**



H- **SLRRSSCFGGRMDRIGAQSGLG-SRHWKFL**-NH_2

17

Protected peptide resin corresponding to **17** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.5g, 0.11 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **17**.

17: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 50% over 30 min, retention time = 19.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{140}H_{226}N_{50}O_{37}S_2$ ($[M+3H]^{3+}$) 1088.9, found 1088.8.

H- **CNSFRY**-OH

24

Protected peptide resin corresponding to **24** were constructed on Wang resin (1.1 mmol amine/g, 0.09 g, 0.099 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), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **24**.

24: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 50% over 30 min, retention time = 14.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 22% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{34}H_{48}N_{10}O_{10}S$ ($[M+H]^+$) 789.3, found 789.2.

Conversion of 29-residue ANP Peptide **17** to 22-residue Peptide Hydrazide **22**

Peptide **17** (4.60 mg, 1.1 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) MeOH (pH 8.2, 1.1 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 3 h, followed by addition of 0.2 M HEPES buffer containing 0.1 M EDTA (pH 8.2, 1.1 mL). And then, $NH_2NH_2 \cdot H_2O$ (0.116 mL) was added into the reaction mixture (final

concentration: 5% (v/v) NH_2NH_2). Additional reaction at 25 °C for 3 h gave peptide hydrazide **22**. After confirmation of the completion of the reaction by HPLC analysis, the solution was diluted with 30 mM TCEP aq. (pH7.0, 2.2 mL). The crude material was purified by semi-preparative HPLC to give the purified peptide hydrazide **22** (2.30 mg, 0.76 μmol, 71%).

Methylester 25: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 23.2 min. MS (ESI-TOF) *m*/*z* calcd for $C_{94}H_{161}N_{35}O_{30}S_2$ ([*M*+H]$^+$) 775.7, found 775.9.

Hydrazide 22: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 20.7 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{93}H_{161}N_{37}O_{29}S_2$ ([*M*+H]$^+$) 775.7, found 775.9.

NCL for the Synthesis of Reduced Form ANP **27**

Peptide **22** (0.90 mg, 0.30 μmol) was dissolved in 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 0.1 mL, 3 mM peptide). The reaction mixture was stored at -10 °C. Then. 10 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was stored at -10 °C for 1 h. After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M MESNa (0.1 mL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 2.0 M NaOH aq.. The reaction mixture was stored at room temperature for 1 h. And then, peptide **24** (0.40 mg. 0.40 μmol) and thiophenol (0.01 mL) were added into the reaction mixture (final concentration: 5% (v/v) thiophenol). After completion of the reaction, the solution was diluted with 30 mM TCEP aq. (pH7.0, 0.2 mL). The crude material was purified by semi-preparative HPLC to give the reduced form ANP **27** (0.78 mg, 0.21 μmol, 70%).

Peptidyl azide 26: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 24.8 min. MS (ESI-TOF) *m*/*z* calcd for $C_{93}H_{158}N_{38}O_{29}S_2$ ([*M*+3H]$^{3+}$) 789.0, found 788.9.

MESNa ester 23: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 22.1 min. MS (ESI-TOF) *m*/*z* calcd for $C_{95}H_{163}N_{35}O_{32}S_4$ ([*M*+3H]$^{3+}$) 812.4, found 812.2.

Reduced form ANP 27: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 25.3 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for

$C_{127}H_{205}N_{45}O_{39}S_3$ ([*M*+3H]$^{3+}$) 1027.8, found 1027.8.

Folding for Preparation of ANP **15**

Reduced form ANP **27** (0.78 mg, 0.21 μmol) was dissolved in 0.1 M Na phosphate buffer containing 6 M Gn·HCl, (pH 7.3, 0.63 mL) and DMSO (0.07 mL). The reaction mixture was incubated at 37 °C for 24 h. The crude material was purified by semi-preparative HPLC to give the purified folded ANP **15** (0.67 mg, 0.18 μmol, 86%).

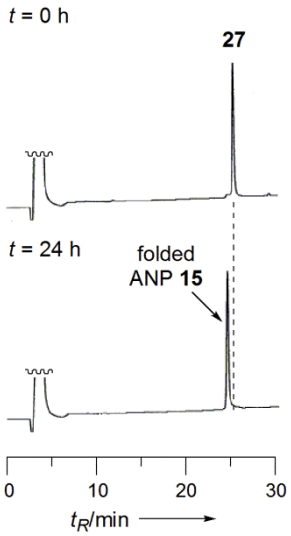


Figure S16. HPLC monitoring of the folding of reduced form ANP **27**.

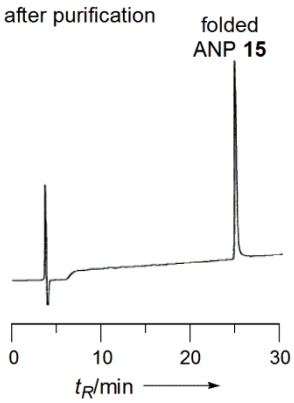


Figure S17. HPLC chart of folded ANP **15** after purification.

Folded ANP 15: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 24.3 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for $C_{127}H_{203}N_{45}O_{39}S_3$ ([*M*+3H]$^{3+}$) 1027.1, found 1027.1.

Preparation of Peptide **S36**

H-**CYRANK**(biotin)-NH_2

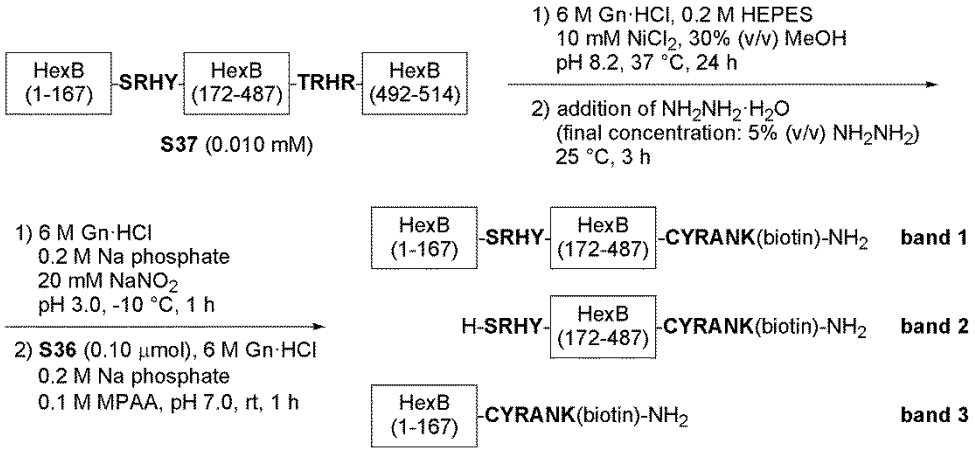
S36

Protected peptide resin corresponding to **S36** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.2 g, 0.044 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), at room

temperature for 2 h) followed by HPLC purification afforded the desired peptide.

S36: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 8% to 18% over 30 min. MS (ESI-TOF) *m/z* calcd for $C_{41}H_{66}N_{14}O_{10}S_2$ ($[M+H]^+$) 979.5, found 979.1.

Application of SQAT System to Expressed Protein



Scheme S10. Application of SQAT system to expressed protein.

HexB **S37** (0.057 mg, 0.01 μmol) was dissolved in 0.2 M HEPES buffer containing 6 M Gn·HCl, 10 mM $NiCl_2 \cdot 6H_2O$ and 30% (v/v) MeOH (pH 8.2, 0.1 mL, 0.01 mM protein). The reaction mixture was incubated at 37 °C for 24 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (5.3 μL) into the reaction mixture (final concentration: 5% (v/v) NH_2NH_2). And then, the reaction mixture was incubated at 25 °C for 3 h. The protein was then buffer-exchanged, by use of a centrifugal filter equipped with a 10 kDa molecular weight cut off, into 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 0.4 mL) by repeated dilution/concentration and ultimately obtained in the original reaction volume of ligation buffer (0.1 mL). The reaction mixture was stored at -10 °C. Then. 10 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was stored at -10 °C for 1 h. After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M MPAA (0.1 mL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 2.0 M NaOH aq. followed by addition of peptide **S36** (0.12 mg, 0.1 μmol). The reaction mixture was stored at room temperature for 12 h, followed by addition of 30 mM TCEP aq. (pH 7.0, 0.2 mL). And then, the reaction mixture was exchanged into 0.1% TFA aqueous solution by use of a centrifugal filter equipped with a 10 kDa molecular weight cut off followed by silver staining and streptavidin-HRP blotting.

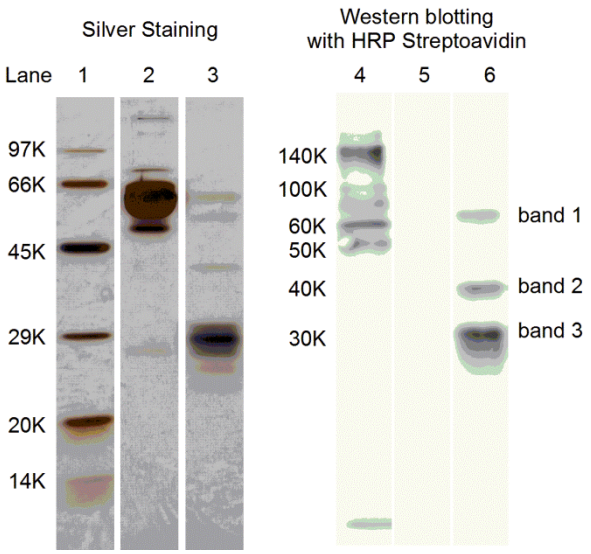


Figure S18. SDS-page analyses of SQAT-mediated editing of Hex B. Lane 1, standard; Lanes 2 and 5, intact Hex B; Lanes 3 and 6, fragments tagged with biotinylated peptide; Lane 4, biotinylated standard.