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

Yusuke Tsuda, Akira Shigenaga, Kohei Tsuji, Masaya Denda, Kohei Sato, Keisuke Kitakaze, Takahiro Nakamura, Tsubasa Inokuma, Kohji Itoh, and Akira Otaka*}[a]

open_201500086_sm_misellaneous_information.pdf
Table of Contents

General Methods--------------------------------------------------------------- S2
Preparation of Peptides 1 and 3 ------------------------------------------------ S2
Ni(II)-mediated Hydrolysis of Peptide 3 ---------------------------------------- S2
Preparation of Peptides 5a-l, n-t, u for Their Ni(II)-mediated Conversion ------ S4
Preparation of Peptide 5m ----------------------------------------------------- S7
Ni(II)-mediated Conversion of Peptide 5a to Oxyster 8a and 9------------------ S7
Conversion of DTDE Ester Peptide 9 to Methylthiophenyl Ester Peptide 10------- S9
Examination of Epimerization During the Conversion of a SRHW-tagged Parent Peptide to the Corresponding DTDE and Methylphenyl Thioesters ------------------------------ S10
  1. Preparation of Peptide S28 ----------------------------------------------- S10
  2. Conversion of Peptide S28 to DTDE Ester Peptide S29 (l-Ala) -------------- S10
  3. Preparation of Stereo-defined DTDE Ester Peptides S29 (l-Ala) and S30 (n-Ala) --- S11
  4. Conversion of Stereo-defined DTDE Ester Peptide S29 (l-Ala) to Methylphenyl Thioester Peptides S31 (l-Ala) and S32 (n-Ala) ----------------- S12
  5. Preparation of Stereo-defined Ligated Products S33 (l-Ala) and S34 (n-Ala) ---- S13
  6. Preparation of N-terminal Cysteinyl Peptide S35 -------------------------------- S14
  7. NCL of Methylphenyl Thioester Peptides S31 (l-Ala), S32 (n-Ala) with N-terminal Cysteinyl Peptide S35 ----------------------------------- S14
Conversion of Peptides 5 to Peptide Hydrazides 11 -------------------------------- S15
Preparation of Peptide Hydrazides 11a and 11u -------------------------------- S23
Conversion of Peptide Hydrazide 11a (or 11u) to MESNa Ester 13a (or 13u) ------- S23
Evaluation of Racemization of Ala at Thioesterification Site --------------------- S25
Preparation of 43-residue CNP Peptide 16 and N-terminal Cysteinyl CNP Fragment 20---- S25
Conversion of 43-residue CNP Peptide 16 to 36-residue Peptide Hydrazide 18-------- S26
NCL for the Synthesis of Reduced Form CNP 53 21 ---------------------------------- S27
Folding for Preparation of CNP 53 14 ------------------------------------------ S29
Preparation of 29-residue ANP Peptide 17 and N-terminal Cysteinyl ANP Fragment 24---- S30
Conversion of 29-residue ANP Peptide 17 to 22-residue Peptide Hydrazide 22--------- S30
NCL for the Synthesis of Reduced Form ANP 27 ------------------------------------- S31
Folding for Preparation of ANP 15 --------------------------------------------- S32
Preparation of Peptide S36 ------------------------------------------------------ S32
Application of SQAT System to Expressed Protein ------------------------------------ S33
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-NH₂ 1
H-AKLRFGAPSRHWKFL-NH₂ 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-H₂O-1,2-ethanediathiol (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 Et₂O to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et₂O 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 C₈₇H₁₃₃N₂₇O₁₆S ([M+3H]⁺) 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 C₈₇H₁₃₃N₂₇O₁₆ ([M+2H]⁺2) 907.0, found 906.7.

Ni(II)-mediated Hydrolysis of Peptide 3

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 NiCl₂·6H₂O (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.

**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 C₄₇H₆₉N₁₂O₉ ([M+H]⁺) 859.5, found 859.3.

**7 (H-SRHWKFL-NH₂):** 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 C₄₇H₆₉N₁₅O₈ ([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 C₄₄H₇₅N₁₃O₁₁ ([M+2H]²⁺) 481.8, found 481.9.
Preparation of Peptides 5a-l, n-t, and u for Their Ni(II)-mediated Conversion

Ac-LYRAXSRHKFL-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$_2$O-1,2-ethanediethiol (80:5:5:5 (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\(_{78}H_{116}N_{22}O_{16}\) ([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\(_{76}H_{113}N_{22}O_{16}\) ([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\(_{77}H_{115}N_{22}O_{16}\) ([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\(_{76}H_{113}N_{22}O_{16}S\) ([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\(_{78}H_{115}N_{22}O_{15}\) ([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\(_{78}H_{115}N_{22}O_{15}\) ([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\(_{79}H_{117}N_{23}O_{16}S\) ([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_{118}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_{83}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_{23}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_{80}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_{79}H_{113}N_{23}O_{15}
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$_2$O-1,2-ethanedithiol (80:5:5:5 (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]$_{3}^{3+}$) 880.0, found 879.8.

**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$_2$O (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)

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_{8}O_{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_{8}O_{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_{8}O_{9}S_{2} ([M+H]^+) 771.3, found 771.2.
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_{8}O_{7}S ([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-NH₂ S28, was synthesized in a manner similar to those employed for 5a.

1. Preparation of Peptide S28

Ac-LYRASRHWKFL-NH₂ 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-H₂O-1,2-ethanedithiol (80:5:5:5 (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 C₇₃H₁₀₈N₂₂O₁₄ ([M+3H]⁺) 759.4, found 759.3.

2. 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 NiCl₂-6H₂O 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_{7}O_{8}S_{2} ([M+H]^+) 700.3, found 700.3.

3. Preparation of Stereo-defined DTDE Ester Peptides S29 (l-Ala) and 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.

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_{7}O_{8}S_{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 calcld for C_{30}H_{49}N_{7}O_{8}S_{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 calcld for C_{33}H_{47}N_{7}O_{6}S ([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 calcld for C_{33}H_{47}N_{7}O_{6}S ([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 calcld for C_{26}H_{41}N_{7}O_{7} ([M+H]^+) 564.3, found 564.3.
5. Preparation of Stereo-defined Ligated Products S33 (L-Ala) and S34 (D-Ala)

After HPLC purification, each component was subjected to NCL with H-CYRANK-NH₂ S35. Conversion to the thioester under acidic conditions afforded two products with the same molecular weight which were separated on HPLC analysis, which indicated that epimerization occurred under this step.

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 cysteinyi peptide, H-CYRANK-NH₂ S35, and then, resulting ligated peptides were analyzed by HPLC using stereo-defined Ac-LYR-L-Ala-CYRANK-NH₂ S33 and Ac-LYR-D-Ala-CYRANK-NH₂ S34 as authentic samples.

Ac-LYR-L-Ala-CYRANK-NH₂ S33 (L-Ala)  Ac-LYR-D-Ala-CYRANK-NH₂ 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-H₂O-1,2-ethanedithiol (80:5:5:5 v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

**1-Ala-containing ligated product S33** (1-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 calcld for C₅₇H₉₁N₁₉O₁₄S ([M+2H]⁺) 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 calcld for C₅₇H₉₁N₁₉O₁₄S ([M+2H]⁺) 649.8, found 649.8.

6. Preparation of N-terminal Cysteinyl Peptide S35

H-CYRANK-NH₂

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-H₂O-1,2-ethanedithiol (80:5:5:5 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 calcld for C₃₁H₅₂N₁₂O₆S ([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 Gdn-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...
showed that thioester peptides S31 and S32 had C-terminal L-Ala and D-Ala residues, respectively.

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 NiCl₂·6H₂O 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 NH₂NH₂·H₂O (4.9 µL) into the reaction mixture (final concentration: 5% (v/v) NH₂NH₂). 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_{8}O_{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_{10}O_{7} ([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_{30}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_{10}O_{7} ([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_{8}O_{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_{8}O_{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_{8}O_{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_{48}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_{8}O_{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_{8}O_{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_{8}O_{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_{8}O_{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_{48}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_{6}O_{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_{6}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_{8}O_{8}S ([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_{8}O_{8}S ([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_{7}S ([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_{39}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_{8}O_{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_{8}O_{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_{50}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_{8}O_{8} ([M+H]^+) 725.4, found 725.3.

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_{8}O_{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_{50}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_{52}N_{9}O_{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_{9}O_{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_{53}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_{53}N_{13}O_{7} ([M+H]^+) 734.3, 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_{9}O_{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_{11}O_{7} ([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_{8}O_{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_{8}O_{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 NiCl₂·6H₂O 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 NH₂NH₂·H₂O (0.2 mL) into the reaction mixture (final concentration: 5% (v/v) NH₂NH₂). 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 NaN₂ 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.
12a (Ac-LYRA-L-Ala-N₃): 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 C₂₉H₄₅N₁₁O₇ ([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 C₃₁H₅₀N₈O₁₀S₂ ([M+H]⁺) 759.3, found 759.1.

12u (Ac-LYRA-D-Ala-N₃): 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 C₂₉H₄₅N₁₁O₇ ([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 C₃₁H₅₀N₈O₁₀S₂ ([M+H]⁺) 759.3, found 759.1.

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.
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-DRVDTKSRAAWARLQLRPNARKYKGANKKGLSKG-SRHWKFL-NH₂

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-H₂O-1,2-ethanedithiol (80:5:5:5 (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 C₂₂₄H₃₆₃N₇₅O₅₇ ([M+H]⁺) 5018.7, found 5018.6.
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-H₂O-1,2-ethanedithiol (80:5:5:5 (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 C₇₄H₁₂₅N₂₁O₂₂S₃ ([M+2H]⁺) 878.9, found 878.7.

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 NiCl₂·6H₂O 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 NH₂NH₂·H₂O (0.053 mL) into the reaction mixture (final concentration: 5% (v/v) NH₂NH₂). 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%).
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 NaNO₂ 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%).

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 Cysteinyln ANP Fragment 24

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-H2O-1,2-ethanedithiol (80:5:5:5 (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 C140H226N50O37S2 ([M+3H]+) 1088.9, found 1088.8.

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-H2O-1,2-ethanedithiol (80:5:5:5 (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 C34H48N10O10S ([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 NiCl2-6H2O 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, NH2NH2·H2O (0.116 mL) was added into the reaction mixture (final
concentration: 5% (v/v) NH$_2$NH$_2$). 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
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_{205}N_{45}O_{39}S_{3} ([M+3H]^3+) 1027.8, found 1027.8.

**Preparation of Peptide S36**

H-CYRANK (biotin)-NH₂

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-H₂O-1,2-ethanedithiol (80:5:5:5 (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) \( m/z \) calcld 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

HexB (1-167) SRHY HexB (172-487) TRHR HexB (492-514) S37 (0.010 mM)

1) \( 6 \) M Gn·HCl, 0.2 M HEPES 10 mM NiCl\(_2\), 30% (v/v) MeOH pH 8.2, 37 °C, 24 h
2) addition of NH\(_2\)NH\(_2\)·H\(_2\)O (final concentration: 5% (v/v) NH\(_2\)NH\(_2\)H\(_2\)) 25 °C, 3 h

HexB (1-167) SRHY HexB (172-487) CYRANK(biotin)-NH\(_2\) band 1

HexB (1-167) H-SRHY HexB (172-487) CYRANK(biotin)-NH\(_2\) band 2

HexB (1-167) CYRANK(biotin)-NH\(_2\) band 3

Scheme S10. Application of SQAT system to expressed protein.

HexB S37 (0.057 mg, 0.01 \( \mu \)mol) was dissolved in 0.2 M HEPES buffer containing 6 M Gn·HCl, 10 mM NiCl\(_2\)·6H\(_2\)O 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\(_2\)NH\(_2\)·H\(_2\)O (5.3 \( \mu \)L) into the reaction mixture (final concentration: 5% (v/v) NH\(_2\)NH\(_2\)H\(_2\)). 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 \( \mu \)L 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 \( \mu \)mol). 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.