Electronic Supplementary Information

Direct synthesis of N-terminal thiazolidine-containing peptide thioesters from peptide hydrazides

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General methods

All commercially available reagents and protected amino acids were purchased and used without further purification. All peptides were synthesized manually. NCL reactions were carried out under an atmosphere of argon. LCMS analyses were carried on a Waters Alliance HPLC system with Acquity SQ Detector (ESI-single quadrupole (SQ)). Mass spectrum of the synthesized LacZα was recorded on a Bruker Compact (ESI-Q-TOF). For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a YMC-Triart C18 analytical column (YMC, 4.6 × 250 mm, flow rate 1.0 mL/min), a YMC-Triart C18 semi-preparative column (YMC, 10 × 250 mm, flow rate 3.0 mL/min) or a YMC-Actus Triart C18 preparative column (YMC, 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.

Synthesis and reactions of peptide hydrazide 1a

Hydrazine-incorporated resin was prepared by a previously reported method.[S1] On 2-chlorotritylchloride resin (1.52 mmol/g, 197 mg, 0.30 mmol) was reacted hydrazine hydrate (2 equiv.) with trimethylamine (3 equiv.) in dry DMF at room temperature. After 1 h reaction, MeOH (0.1 mL) was added. After 10 min reaction, the resulting resin was successively washed with DMF, H_2O and MeOH. The hydrazine incorporated resin was reacted with Fmoc-Gly-OH (1.5 mmol) with *N*,*N'*-diisopropylcarbodiimide (DIPCI, 1.5 mmol) and HOBt·H_2O (1.5 mmol) in DMF at room temperature. After 2 h, the loading was checked by quantification of the Fmoc group (0.51 mmol/g). Then Fmoc removal was performed with 20% (*v*/*v*) piperidine in DMF to give a Gly-incorporated resin. On the resin, peptide was elongated using standard Fmoc-based protocols (5.0 equiv. each of amino acid using HOBt·H_2O (5 equiv.) and DIPCI (5 equiv.) in DMF (2 hour) and Fmoc removal with 20% piperidine in DMF (10 min)). For synthesis of H-Thz-**LYRAG**-NHNH-incorporated resin, Boc-Thz-OH was coupled using standard SPPS conditions at the N-terminus.

The resulting resin (5 mg) was treated under global deprotection conditions (250 μL/5 mg resin) at room temperature. After 2 h, 5 μL of 10% (*w*/*w*) $NaNO_2$ aq was added to the mixture at -10 °C. Stored at -10 °C for 20 min, cold Et_2O was added to the reaction mixture to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide azide **2a**. To the crude product was added 300 μL of 3% (*w*/*w*) MESNa in buffer (6 M Gn·HCl, 0.2 M Na phosphate, pH 7.3).

After 30 min at room temperature, 0.1% TFA aq as much as the volume of the buffer was added to quench the thiolysis, and then the solution was analysed by LCMS.

Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min.

Peptide hydrazide **1a**: Retention time = 9.6 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$), 593.4 found 593.7.

Peptide thioester **3a**: Retention time = 11.3 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$), 703.3 found 703.8 (8.8 mg from 46 mg protected resin (0.77 mmol/g loading), 69% isolated yield).

Nitrated thioester **4a**: Retention time = 16.1 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$), 748.3 found 748.8.

H-Thz-**LYRAG**-S$(CH_2)_2SO_3H$: Retention time = 15.0 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$), 818.3 found 818.6 ((23.5 mg from 100 mg protected resin (0.77 mmol/g loading), 68% isolated yield)).

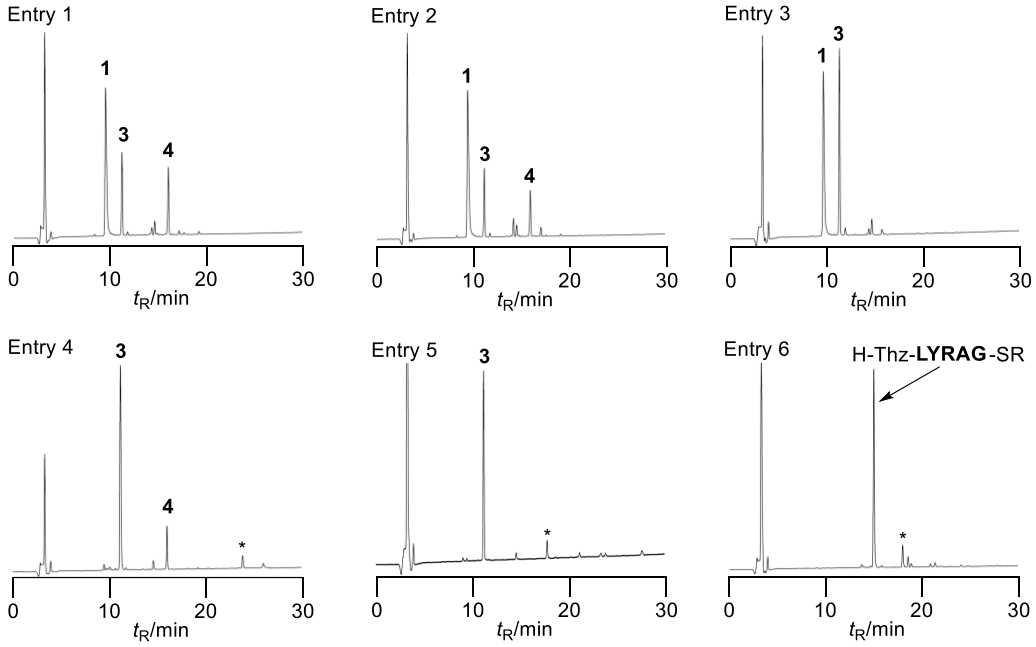

Fig. S1 HPLC analyses of conversion of peptide hydrazide **1a** to peptide thioester **3a**. Global deprotection conditions: TFA-triisopropylsilane (TIS)-H_2O = 95:2.5:2.5 (entry 1); TFA-TIS-H_2O = 95:2.5:2.5 at 0 °C (entry 2); TFA-TIS-H_2O-*m*-cresol = 90:2.5:2.5:5 (entry 3); TFA-TIS-H_2O-thioanisole = 90:2.5:2.5:5 (entry 4); TFA-TIS-H_2O-*m*-cresol-thioanisole = 80:2.5:2.5:5:10 (entry 5); TFA-TIS-H_2O-*m*-cresol-thioanisole = 80:2.5:2.5:5:10, H-Thz-**LYRAG**-NHNH-resin was used (entry 6) *Non-peptidic compounds derived from additives for global deprotections. R = $(CH_2)_2SO_3H$

Scope of the conversion of peptide hydrazides to peptide thioesters

Peptide hydrazides **1a–q** (H-**LYRA**-Xaa-$NHNH_2$) were synthesized and converted to the corresponding thioesters **3a–q** as described above. For a peptide resin with Ser or His residue at C-terminus, thiolysis was conducted with 10% (*w*/*w*) MESNa in 6 M Gn·HCl, 0.2 M Na phosphate buffer (pH 4.0). A peptide resin with Met residue at C-terminus was treated with a cocktail (TFA-TIS-H_2O-*m*-cresol-thioanisole = 75:2.5:2.5:5:15) as global deprotection conditions.

Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min except for **3n**, 5% to 15% over 60 min for **3n**.

Xaa = Ala (**3b**): Retention time = 11.8 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 717.3, found 717.6.

Xaa = Val (**3c**): Retention time = 16.5 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 745.3, found 745.5.

Xaa = Ile (**3d**): Retention time = 18.4 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 759.4, found 759.6.

Xaa = Leu (**3e**): Retention time = 18.9 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 759.4, found 759.7.

Xaa = Phe (**3f**): Retention time = 20.8 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 793.3, found 793.6.

Xaa = Pro (**3g**): Retention time = 14.2 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 743.3, found 743.6.

Xaa = Ser (**3h**): Retention time = 10.1 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 733.3, found 733.7.

Xaa = Thr (**3i**): Retention time = 11.5 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 747.3, found 747.5.

Xaa = Glu (**3j**): Retention time = 10.9 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 775.3, found 775.6.

Xaa = Cys (**3k**): Retention time = 13.4 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 749.3, found 749.5.

Xaa = Met (**3l**): Retention time = 16.8 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 777.3, found 777.5.

Xaa = Tyr (**3m**): Retention time = 15.6 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 809.3, found 809.6.

Xaa = His (**3n**): Retention time = 9.7 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 783.3, found 783.6.

Xaa = Lys (**3o**): Retention time = 9.3 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 774.4, found 774.6.

Xaa = Arg (**3p**): Retention time = 9.9 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 802.4, found 802.6.

Xaa = Trp (**3q**): Retention time = 21.2 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 832.3, found 832.7.

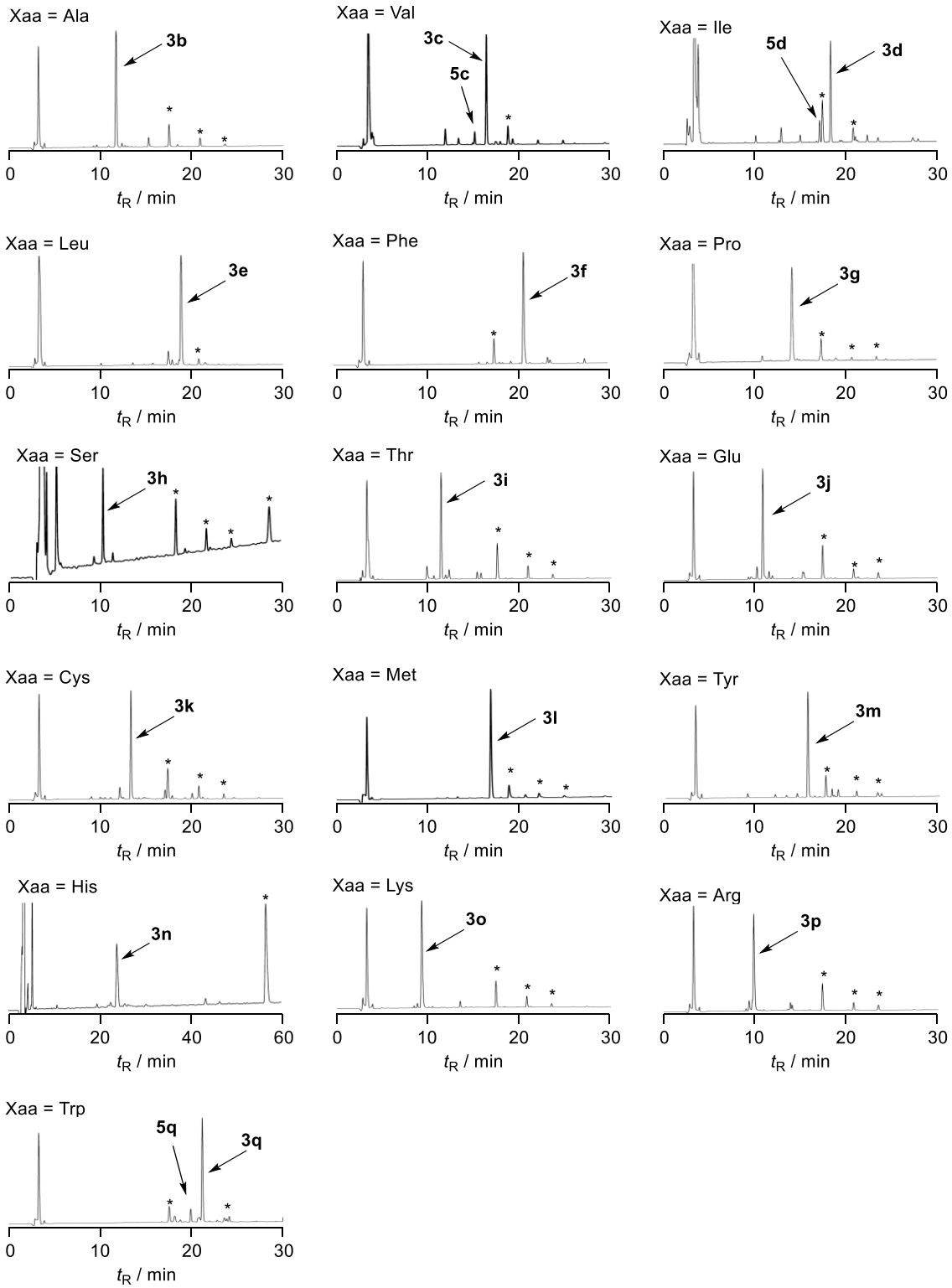

Fig. S2 HPLC charts of crude materials after conversion of hydrazides to thioesters (H-**LYRA**-**Xaa**-$S(CH_2)_2SO_3H$). HPLC conditions were described above. *Non-peptidic compounds derived from additives for global deprotections.

Check of epimerization of C-terminal amino acids during conversion of hydrazides

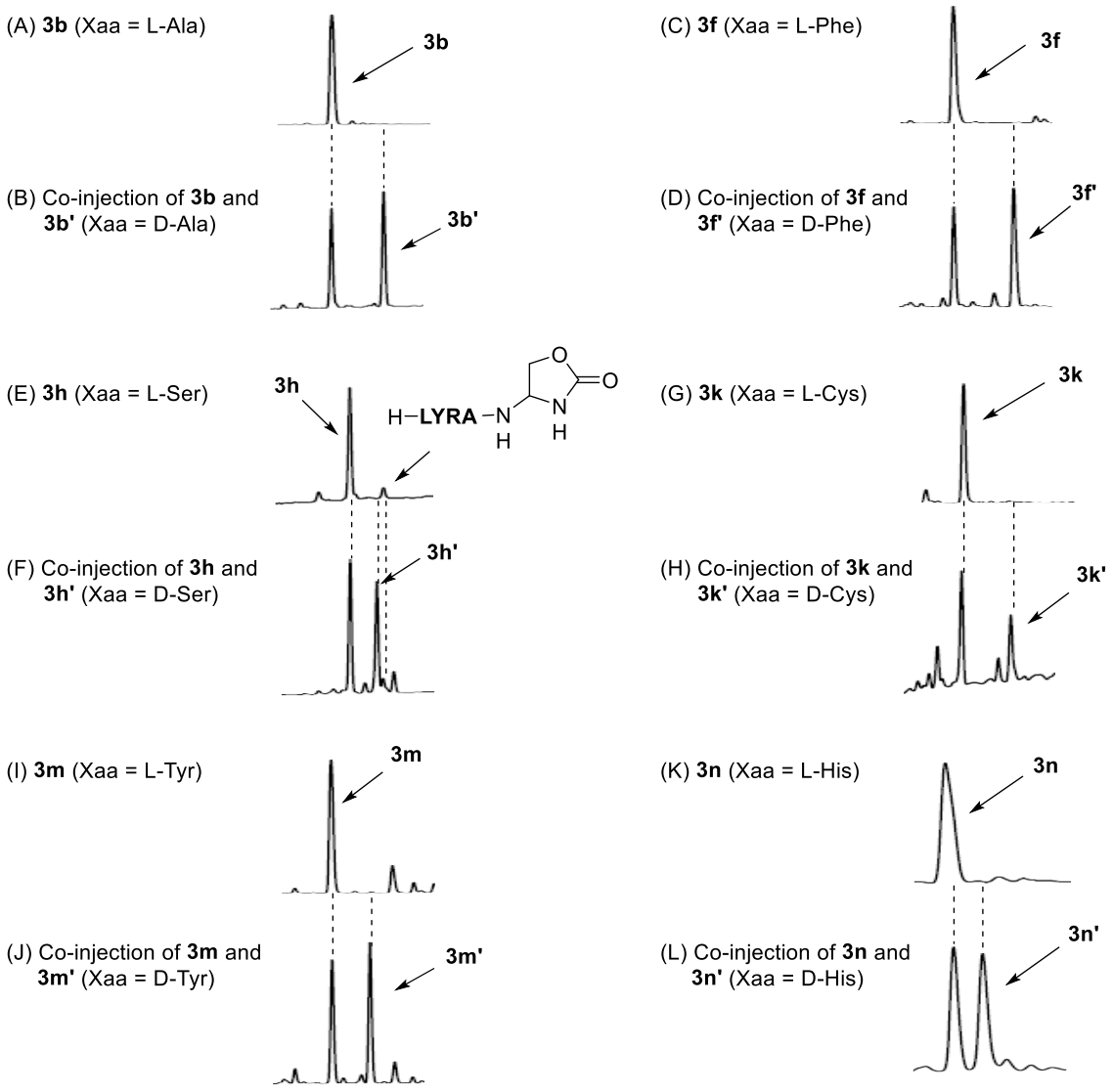

Fig. S3 HPLC examination of epimerization of C-terminal amino acids during conversion of hydrazides to thioesters. Only a critical retention time region of the HPLC charts was enlarged. D-Xaa containing diastereomer peptide **3b'**, **3f'**, **3h'**, **3k'**, **3m'** and **3n'** was synthesized by standard Fmoc SPPS protocols using Fmoc-D-Xaa-OH.

Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent in solvent A, 5% to 35% over 30 min for Xaa = Ala, Phe, Ser, Cys, and Tyr, 5% to 15% over 60 min for Xaa = His.

3b: Retention time = 12.0 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 717.3, found 717.6.

3b': Retention time = 13.7 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 717.3, found 717.6.

3f: Retention time = 20.8 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 793.3, found 793.5.

3f': Retention time = 22.7 min, MS (ESI ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 793.3, found 793.6.
3h: Retention time = 10.2 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 733.3, found 733.6.
3h': Retention time = 11.0 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 733.3, found 733.5.
3k: Retention time = 11.8 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 749.3, found 749.7.
3k': Retention time = 15.4 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 749.3, found 749.5.
3m: Retention time = 15.9 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 809.3, found 809.6.
3m': Retention time = 17.1 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 809.3, found 809.7.
3n: Retention time = 24.5 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 783.3, found 783.4.
3n': Retention time = 25.5 min, MS (ESI-SQ) *m*/*z* calcd ($[M + H]^+$) 783.3, found 783.4.

Syntheses of peptides for synthesis of LacZα

Syntheses of thioester 6, 7 and 8: For the preparations of peptide thioester **6**, **7** and **8**, NH_2NH-Trt(2-Cl)-resin (0.77 mmol/g loading) was used. On this resin, standard Fmoc SPPS (coupling: 5.0 equiv. of amino acid using DIPCDI (5.0 equiv.) and HOBt·H_2O (5.0 equiv.) in DMF, 2 h; Fmoc removal: 20% (*v*/*v*) piperidine/DMF, 10 min) was performed for the chain elongation to give a protected peptide resin. The resulting completed resin was converted to the corresponding thioesters using TFA-TIS-H_2O-*m*-cresol-thioanisole (80:2.5:2.5:5:10, (*v*/*v*), 50 μL/1 mg resin) as described above. The crude peptide thioester was purified by preparative HPLC (YMC-Actus Triart C18 with a linear gradient of solvent B in solvent A over 30 min: 19% to 29% for **6** (5.5 mg from 46 mg of protected resin, 31% isolated yield), 25% to 30% for **7** (16.3 mg from 100 mg of protected resin, 34% isolated yield) or 20% to 24% for **8** (28.9 mg from 100 mg peptidyl resin, 64% isolated yield)).

Analytical HPLC conditions for **6**: YMC-Triart C18 analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 20.5 min, MS (ESI-SQ) *m*/*z* calcd ($[M+H]^+$) 1035.4, found 1035.6.

Analytical HPLC conditions for **7**: YMC-Triart C18 analytical column with a linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 14.3 min, MS (ESI-SQ) *m*/*z* calcd ($[M+3H]^{3+}$) 844.7, found 845.3.

Analytical HPLC conditions for **8**: YMC-Triart C18 analytical column with a linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 11.8 min, MS (ESI-SQ) *m*/*z* calcd ($[M+2H]^{2+}$) 883.8, found 884.3.

Synthesis of peptide acid 9: For the preparation of peptide **9**, Fmoc-Arg(Pbf)-Aloc PEG resin (0.22 mmol/g, 0.45 g, 0.10 mmol) was used. On this resin, the peptide was elongated using standard Fmoc SPPS to give a protected peptide. The resulting resin was treated with TFA-TIS-H_2O (95:2.5:2.5, (*v*/*v*), 50 μL/1 mg resin) at room temperature for 2 h. Then the resin in the reaction mixture was filtrated off. To the resulting filtrate was added cooled Et_2O to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide **9**. The peptide was purified by preparative HPLC (YMC-Actus Triart C18 with a linear gradient of solvent B in solvent A over 30 min: 17% to 27%) to give the purified peptide **9** (2.3 mg from 51 mg of protected resin, 18% isolated yield)

Analytical HPLC conditions: YMC-Triart C18 analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 18.1 min, MS (ESI-SQ) *m*/*z* calcd ([M + 3H]$^{3+}$) 734.7, found 735.1.

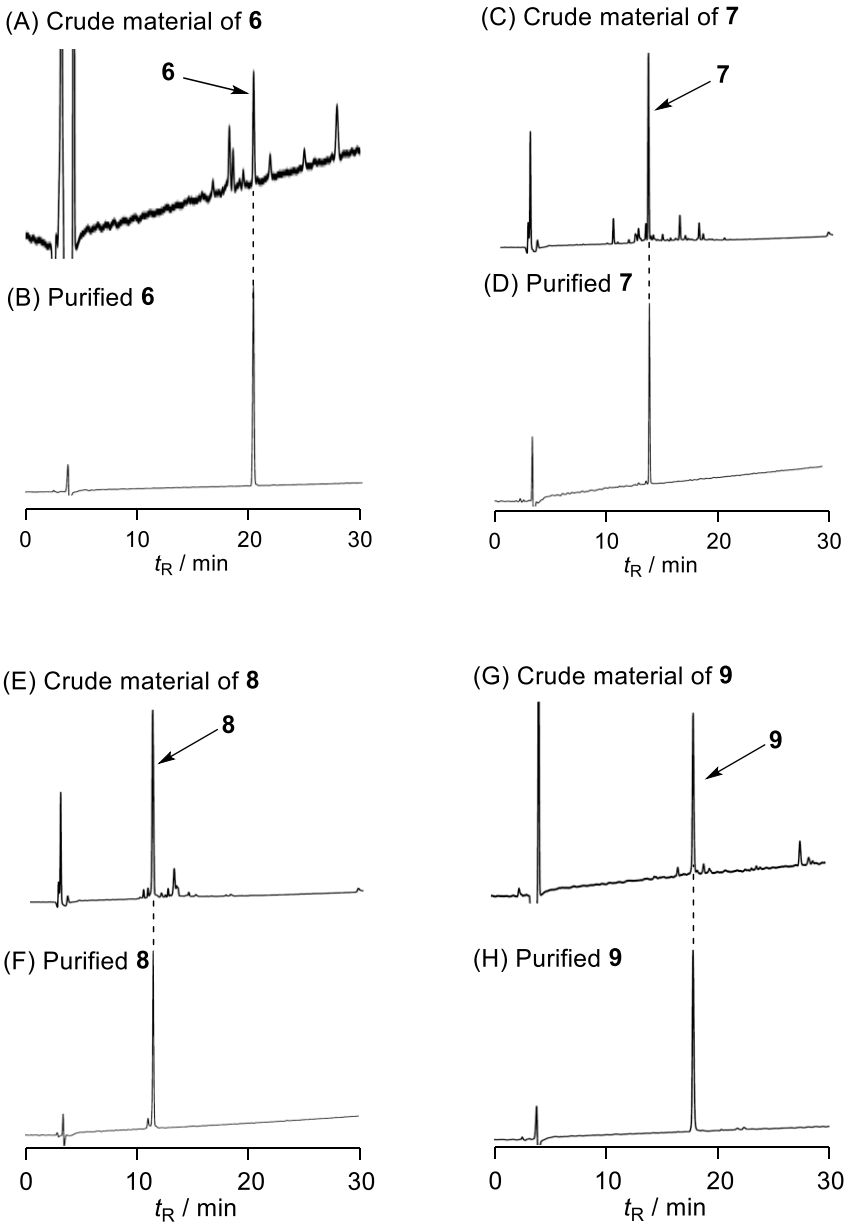

Fig. S4 HPLC analyses of synthesized peptides for LacZα: (A) crude material of **6**; (B) purified **6**; (C) crude material of **7**; (D) purified **7**; (E) crude material of **8**; (F) purified **8**; (G) crude material of **9**; purified **9**. HPLC conditions were described above.

Assemblies of peptides for LacZα synthesis

First NCL for synthesis of 10 (Fig. S5): Peptide thioester **8** (3.0 μmol) and N-Cys peptide **9** (3.0 μmol) were dissolved in 1.5 ml of ligation buffer (6 M Gn·HCl, 0.2 M Na phosphate, 100 mM 4-mercaptophenylacetic acid (MPAA), 50 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), pH 7.3), and the solution was incubated at 37 °C. The reaction was monitored by LC-MS and completed within 7 h. To the reaction mixture was added $MeONH_2$·HCl (25 mg, final concentration 0.2 M) and incubated at 37°C for additional 11 h. The crude peptide was purified by preparative HPLC (YMC-Actus Triart C18 with a linear gradient of solvent B in solvent A over 30 min: 20% to 30%) to give the purified peptide **10** (6.6 mg, 1.4 μmol, 48% isolated yield). Analytical HPLC conditions for **10**: YMC-Triart C18 analytical column with a linear gradient of solvent B in solvent A, 15% to 45% over 30 min, retention time = 16.5 min, MS (ESI) *m*/*z* calcd ($[M+3H]^{3+}$) 1271.9, found 1272.7.

Second NCL for synthesis of 11 (Fig. S6): Peptide thioester **7** (0.96 μmol) and N-Cys peptide **10** (0.92 μmol) were dissolved in 0.50 mL of ligation buffer (6 M Gn·HCl, 0.2 M Na phosphate, 40 mM MPAA, 20 mM TCEP·HCl, pH 7.3), and the solution was incubated at 37 °C. The reaction was monitored by LC-MS and completed within 5 h. To the reaction mixture was added $MeONH_2$·HCl (9.5 mg, final concentration 0.2 M) and incubated at 37 °C for 3 h. The crude peptide was purified by preparative HPLC (YMC-Actus Triart C18 with a linear gradient of solvent B in solvent A over 30 min: 24% to 34%) to give the purified peptide **11** (3.7 mg, 0.50 μmol, 54% isolated yield).
Analytical HPLC conditions for **11**: YMC-Triart C18 analytical column with a linear gradient of solvent B in solvent A, 15% to 45% over 30 min, retention time = 20.4 min, MS (ESI) *m*/*z* calcd ($[M+4H]^{4+}$) 1549.2, found 1549.1.

Third NCL followed by desulfurization for synthesis of 12 (Fig. S7): Peptide thioester **6** (0.89 μmol) and N-Cys peptide **11** (0.41 μmol) were dissolved in 0.40 mL of ligation buffer (6 M Gn·HCl, 2.5 M imidazole, 30 mM TCEP·HCl, pH 7.1),[S2] and the solution was incubated at 37 °C. After completion of the ligation after 11 h, to the reaction mixture was added desulfurization buffer (6 M Gn·HCl, 0.1 M Na phosphate, 450 mM TCEP·HCl, 80 mM glutathione reduced form, pH 4.4, 0.4 mL) and aqueous solution of VA-044 (1 M, 45 μL). Then, the solution (final pH 6.4) was incubated at 37 °C. After the desulfurization of three cysteine residues was completed within 16 h, the reaction mixture was adjusted to pH 1-2 by addition of TFA. The crude peptide was purified by preparative HPLC (YMC-Triart C18 semi-preparative column with a linear gradient of solvent B in solvent

A over 30 min: 31% to 37%) to give the purified LacZα (**12**) (2.8 mg, 0.35 μmol, 85% isolated yield).

Analytical HPLC conditions for **12**: YMC-Triart C18 with a linear gradient of solvent B in solvent A, 15% to 45% over 30 min, retention time = 23.7 min, MS (ESI-Q-TOF) *m*/*z* calcd (average isotope composition) 6991.8415, found 6991.9812.

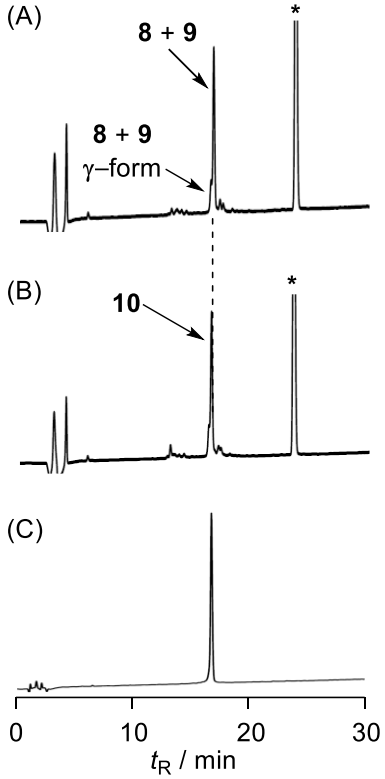

Fig. S5 HPLC monitoring of NCL between **8** and **9**. (A) NCL (*t* = 7 h). (B) Deprotection of Thz (*t* = 11 h). (C) Peptide **10** after purification. * MPAA.

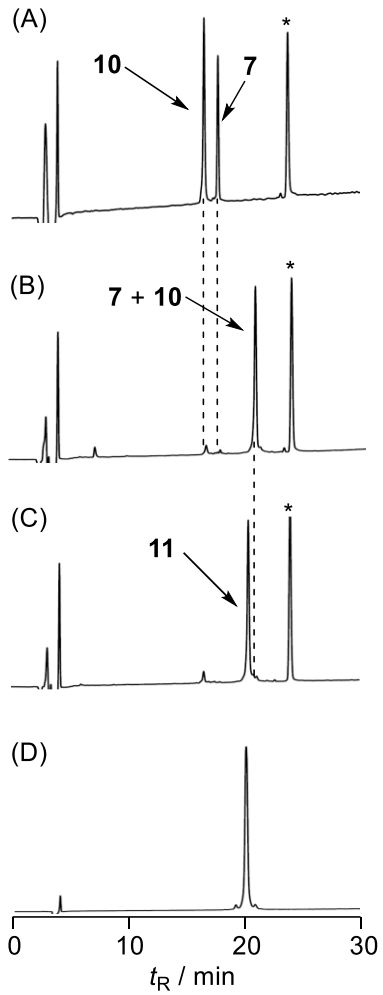

Fig. S6 HPLC monitoring of NCL between peptide **7** and **10**. (A) NCL (t = <1 min). (B) NCL (t = 3 h). (C) Deprotection of Thz (t = 3 h). (D) Peptide **11** after purification. * MPAA.

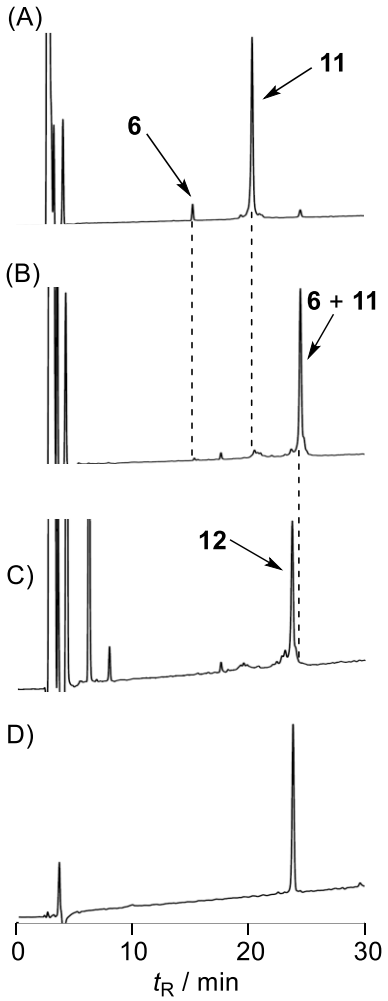

Fig. S7 HPLC monitoring of NCL between **6** and **11** followed by desulfurization. (A) NCL (t = <1 min). (B) NCL (t = 11 h). (C) One-pot desulfurization (t = 16 h). (D) Peptide **12** after purification.

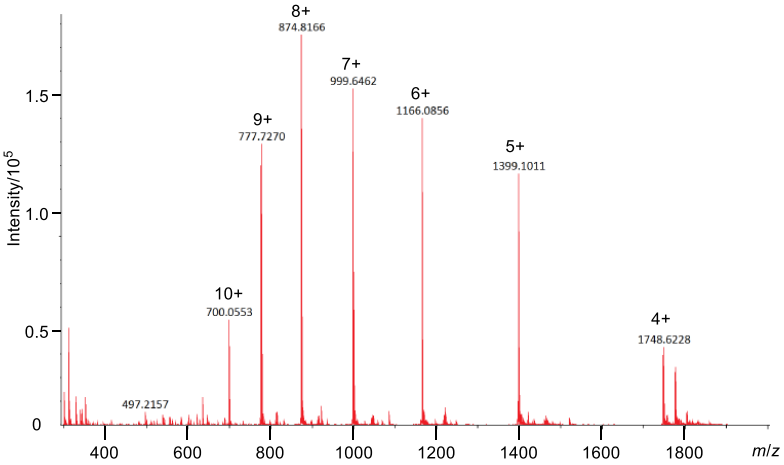

Fig. S8 Mass spectrum of synthesized LacZα.

α-Complementation assay of synthesized LacZα

The α-complementation was examined by β-D-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (ONPG). *Escherichia coli* DH5α (Takara), which expresses LacZα, and *E. coli* W3110[S3], which expressing full length of LacZ, were used in this study. Cells were grown in LB until the early stationary phase and then harvested by centrifugation. The pellet was resuspended in phosphate buffered saline (PBS) and homogenized on ice. Unbroken cells were removed by centrifugation and 0.22-μm PVDF filter membrane, and lysed protein concentration was measured by Bradford assay[S4]. Bovine serum albumin was used as a reference standard. For β-D-galactosidase assay, 1 mM ONPG was reacted with 200 μg/mL lysed *E. coli* protein in PBS at 45 °C for 30 min. Synthesized peptide was added at the final concentration of 100 μg/mL.

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