

COMMUNICATION

Traceless synthesis of protein thioesters using enzyme-mediated hydrazinolysis and subsequent self-editing of cysteinyl prolyl sequence

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A traceless thioester-producing protocol featuring carboxypeptidase Y-mediated hydrazinolysis of cysteinyl prolyl leucine-tagged peptides has been developed. The hydrazinolysis followed by thioesterification affords cysteinyl prolyl thioesters. Self-editing of the tag and subsequent trans-thioesterification yields peptide thioesters. The developed protocol was successfully applied to conversion of recombinant proteins to thioesters.

Proteins possessing various modifications or unnatural amino acids have served as molecular probes in the investigation of the biological significance of proteins. Access to such proteins is achieved mainly by native chemical ligation (NCL) with peptide/protein thioesters as key synthetic intermediates.^{1–3} Because NCL produces proteins *via* a chemoselective amide forming reaction between a thioester and N-terminal cysteine units, protein thioesters available from expressed proteins could function as indispensable intermediates in the preparation of semi-synthetic proteins.³ Among semi-synthetic protocols using recombinant-protein-derived thioesters, expressed protein ligation has gained wide popularity.⁴ This technique features the use of intein-fused recombinant proteins for production of the requisite thioesters. Such intein-fused proteins produce protein thioesters by the action of intein as a self-processing element. Application of the intein-mediated protocol however, does not always produce the desired thioesters efficiently. This is due partly to incomplete folding or the less soluble nature of some intein fusions.⁵ Thus, other recombinant protein-compatible protocols are highly desired. Transpeptidase-mediated methods using sortase A (SrtA)⁶ or butelase 1⁷ were developed as alternative protocols and have enjoyed success in producing thioesters. A potential limitation of these protocols is that enzyme-recognizing sequences

required for trans-peptidylation remain in the resulting thioesters. Consequently, a traceless synthesis of protein thioesters has been required.⁸ In this context, we focused on carboxypeptidase Y (CPaseY) (EC 3.4.16.5),⁹ which is easily obtained from commercial sources or baker's yeast,¹⁰ for C-terminal selective thioesterification of an appropriate recombinant protein. CPaseY, a serine protease, exhibits exopeptidase activity and can hydrolyze a peptide bond at the C-terminal end of a peptide or protein through an O-acyl enzyme intermediate. Instead of hydrolysis of the O-acyl enzyme intermediate affording peptide/protein acids, it was envisioned that hydrazinolysis of the intermediate could yield a peptide/protein hydrazide which works as a thioester precursor *via* the corresponding acyl azide.^{11,12} Herein, we report a novel traceless thioester-producing system using the CPaseY-mediated hydrazinolysis of a C-terminal tagged peptide. Initially, we attempted the CPaseY-mediated hydrazinolysis of a model peptide (**1**) in the presence of hydrazine in aqueous solution (Figure 1A). Although the desired peptide hydrazide (**2**) was observed in the reaction, over-reaction products (**3**, **4**) were generated (Figure 1B).

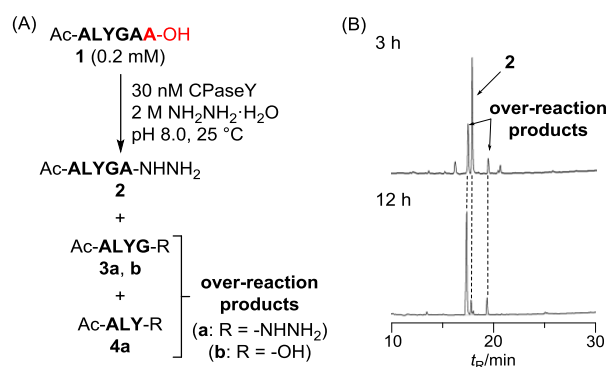


Figure 1. (A) CPaseY-mediated digestion of model peptide **1** in the presence of hydrazine; (B) HPLC monitoring of CPY-mediated hydrazinolysis. HPLC conditions: see Table S2 in ESI.

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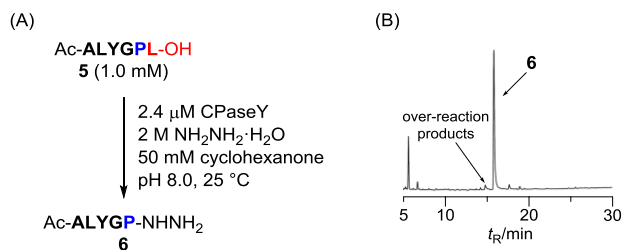
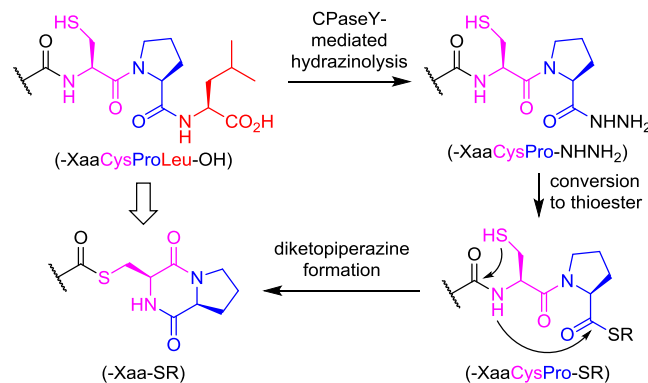


Figure 2. (A) Suppression of over-reaction by the use of substrate preference of CPaseY in the presence of cyclohexanone; (B) HPLC monitoring of CPaseY-mediated hydrazinolysis of peptide 5. HPLC conditions: see ESI.

This result is consistent with the fact that CPaseY also hydrolyzes C-terminal amide peptides.^{13,14} Applicability of other nucleophiles, including thiols, to the CPaseY-mediated reaction was examined, but attempts failed to yield the desired products (Table S3 in ESI). We next attempted to suppress further degradation of the resulting hydrazide (**2**) by hydrazone formation using carbonyl compounds (Figure S1 in ESI). Although cyclohexanone suppresses the CPaseY-induced over-reaction with the yield of **2** approaching 70%, no further significant improvement of the yield by tuning the reaction conditions including the use of aniline catalyst¹⁵ was achieved. We next examined the applicability of the substrate preference of CPaseY to suppression of the over-reaction. CPaseY shows strong preference for hydrophobic amino acids as the C-terminal residue with such residues being quickly cleaved.¹⁶ On the other hand, hydrophilic and prolyl residues are disfavored and are removed slowly. We reasoned that a peptide possessing a C-terminal “–(disfavored amino acid)–(favored amino acid)–OH” sequence should resist the CPaseY-induced over-reaction to yield the corresponding C-terminal disfavored amino acyl hydrazide preferentially. To test this hypothesis, Ac-ALYGPL-OH (**5**) (1.0 mM), which has Pro and Leu as disfavored and favored residues, respectively, was subjected to the hydrazinolysis protocol (2.4 μM CPaseY, 2 M NH₂NH₂·H₂O, 50 mM cyclohexanone, pH 8.0, 25 °C) as shown in Figure 2A. After a 12 h reaction of **5**, the desired Ac-ALYGP-NHNH₂ (**6**) was formed as the major product (Figure 2B). Proline was proven to be the best choice for the disfavored residue because the reaction of Ac-ALYGL-OH (**X** = R or K) under the aforementioned conditions produced over-reaction products (Figure S2 in ESI). The resulting hydrazide (**6**) should be efficiently converted to the corresponding prolyl thioester *via* the peptidyl azide according to Liu’s protocol.¹² However, the sequence of reactions affords only the prolyl thioester which is not a versatile thioester in the standard NCL protocol.¹⁷ Consequently, we next examined the conversion of the prolyl thioester to other amino acyl thioesters. Our previous research concerned the use of the prolyl thioesters under a specific NCL conditions optimized for the prolyl thioesters. NCL of a prolyl thioester peptide tended to produce two-residue (Xaa-Pro)-deleted ligation products, by diketopiperazine formation.¹⁸ The cysteinyl prolyl ester (CPE) system was developed by Kawakami and Aimoto¹⁹ for preparation of peptide thioesters. Under weakly basic conditions (pH > 7.8), CPE peptides (-XaaCysPro-OR) are converted to thioesters (-Xaa-SR) *via* its self-editing through an N–S acyl transfer followed by diketopiperazine

formation. We envisioned that an amino acyl cysteinyl prolyl thioester (-XaaCysPro-SR) resulting from the hydrazinolysis of a C-terminal CysProLeu-OH-tagged peptide (-XaaCysProLeu-OH), followed by thioesterification, should be converted to the Xaa thioester (Xaa-SR: R = cysteinyl proline diketopiperazine) (Scheme 1).



Scheme 1. Envisioned strategy for synthesis of thioester other than prolyl thioester.

In order to confirm the versatility of such a protocol in preparation of proteogenic amino acid thioesters, conversion of a model -XaaCysPro-SR peptide to the corresponding Xaa-SR peptide followed by NCL was conducted. A model peptide, H-LYRAACP-NHNH₂, (1.0 mM) (**7**) was treated with NaNO₂ in 6 M guanidine-HCl–50 mM Na phosphate (pH 3.0) at -10 °C for 30 min and then the reaction was followed by the addition of 4-mercaptophenylacetic acid (MPAA) and an N-terminal cysteinyl peptide (**8**) (pH 6.5) (Figure 3). After 3 min of the addition, three types of possible thioesters including **9** (-AlaCysPro-SAr: Ar = -C₆H₄CH₂COOH), **10** (-Ala-SDKP: SDKP = cysteinyl prolyl diketopiperazine) and **11** (-Ala-SAr) as the major thioester were detected. After 12 h reaction, the thioester (**11** (or **10**)) underwent NCL with **8** to afford the ligated peptide (**12**) as shown in Figure 3B. Although the conversion of prolyl ester in the CPE system to the thioester requires weakly basic conditions (pH > 7.8),¹⁹ the prolyl thioester (**9**) was converted to the thioester (**11**) under slightly acidic conditions without any accompanying hydrolysis products. This result clearly indicates that the C-terminal-XaaCysPro-NHNH₂ peptides function as precursors to the Xaa-SR peptide.

Thus, we optimized the reaction conditions for efficient formation of the -XaaCysPro-NHNH₂ peptide. As summarized in Table S6 in ESI, a C-terminally CysProLeu-OH-tagged model peptide H-LYRAACPL-OH (**13a**) was subjected to the hydrazinolysis reaction under various reaction conditions (concentration of CPaseY, hydrazine and cyclohexanone and pH). On the basis of these results, the reaction conditions shown in entry 3 in Table S6 (1.2 μM CPaseY in the presence of 0.2 M NH₂NH₂·H₂O, 60 mM cyclohexanone, pH 6.4, at 25 °C) were tentatively adopted as optimal. We then re-evaluated the effectiveness of carbonyl compounds in suppression of the over-reaction as summarized in Table S7 in ESI. Some carbonyl compounds clearly suppressed the over-reaction and cyclohexanone was the most effective and economical additive among carbonyl compounds examined.

stained gel analysis to be about 70%. The generality of the developed protocol was further confirmed by successful chemical modification of DsRED-express protein (**S8** in ESI, Figure S5 in ESI).²⁴

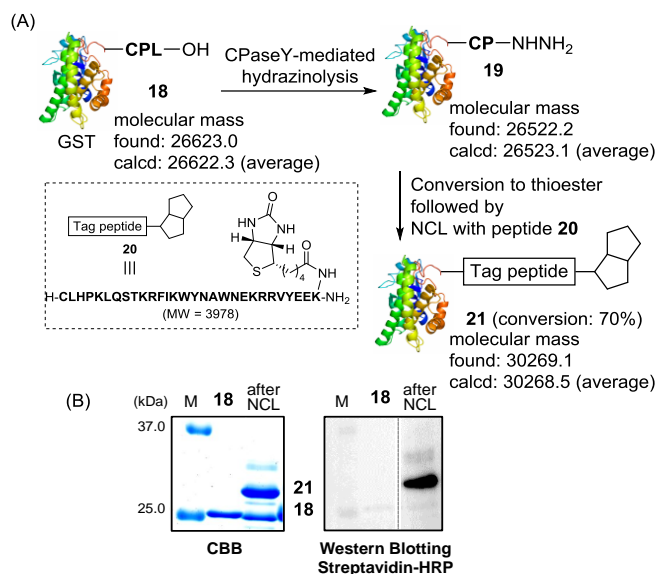


Figure 4. (A) Scheme for the application of CPY-mediated protocol to C-terminally CysProLeu-OH-tagged recombinant GST protein (**18**); (B) SDS-page analysis of protein samples

Conclusions

A traceless thioester-producing protocol applicable to naturally occurring sequences has been developed. The protocol features a sequence of enzymatic and chemical reactions. The enzymatic reaction of C-terminally CysProLeu-OH-tagged peptides with CPaseY in the presence of NH₂NH₂ and cyclohexanone affords the -CysPro-NHNH₂ peptides. Chemical conversion of the hydrazide to -CysPro-SR results in the formation of various peptide thioesters *via* self-editing process. Chemical modification of recombinant proteins is also achieved with the developed protocol.

Conflicts of interest

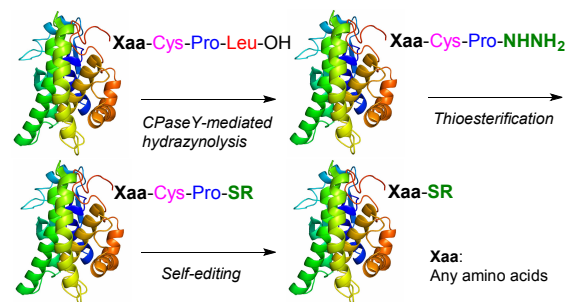
There are no conflicts to declare.

Acknowledgements

We are grateful to Dr. Mai Makino (Nara Medical University) and Prof. Hiroshi Ueno (Nara Women's Univ.) for providing information about CPaseY. And we acknowledge Prof. Toru Kawakami and Prof. Hironobu Hojo (Osaka Univ.) for providing comments about the CPE system. We are grateful to Prof. Toshifumi Takao (Osaka Univ.) for measuring ESI-Q-TOF MS. This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI). C.K. is grateful for a Scholarship from the Yoshida Scholarship Foundation. And A.S. is grateful for the Research Foundation for Pharmaceutical Sciences.

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Thioester-producing protocol featuring carboxypeptidase Y (CPaseY)-mediated hydrazinolysis and subsequent self-editing of tag affords protein thioesters in a traceless manner.

Supplementary Information

Traceless synthesis of protein thioesters using enzyme-mediated hydrazinolysis and subsequent self-editing of cysteinyl prolyl sequence

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General Information

All commercial reagents were used without further purification. CPaseY^{S1} was purchased from Worthington Industries. CPaseY was stored as 0.5 mg/mL (7.7 μ M) of aqueous solution (pure water) at -78 °C. Each peptide or protein was characterized by MS analyses as mentioned below. Mass spectra (for peptide samples) were recorded on Waters MICROMASS[®] LCT PREMIER[™] by electrospray ionization time-of-flight (ESI-TOF) reflection experiments or LC-MS (Shimadzu, Japan, Prominence-I LC-2030, LCMS-2020, a Cosmosil 5C18-AR-II analytical column (Nacalai Tesque, Japan, 4.6 \times 250 mm, flow rate 1 mL min⁻¹, eluting products were detected by UV at 250 nm and MS). ESI mass spectra (for protein samples) were obtained using a QTOF mass spectrometer, which is a hybrid quadrupole orthogonal acceleration tandem mass spectrometer fitted with a Z-spray[™] nanoflow electrospray ion source (Waters, MA). Samples were dissolved in 0.3 % aqueous formic acid/acetonitrile (1:1, v/v) at the concentration of 20 pmol/ μ L, and loaded into a borosilicate nanoflow tip (Thermo Fisher Scientific, MA). Calibration was performed using cluster ions derived from NaI as the external standard, and allowed for the measurements with a mass accuracy of about 50 ppm. MS data were processed by the maximum entropy data enhancement program, MaxEnt 1[™] (Waters), which is capable of deconvoluting a spectrum with peaks in a variety of charge states to the singly charge-state spectrum. For HPLC separation, Cosmosil 5C₁₈-AR-II analytical column (Nacalai Tesque, 4.6 \times 250 mm, flow rate 1.0 mL/min), Cosmosil 5C₁₈-AR-II semi-preparative column (Nacalai Tesque, 10 \times 250 mm, flow rate 3.0 mL/min), or Cosmosil 5C₁₈-AR-II preparative column (Nacalai Tesque, 20 \times 250 mm, flow rate 10.0 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.

1. General Procedure for Peptide Synthesis

Unless otherwise noted, peptides used in this work were synthesized by Fmoc solid-phase peptide synthesis (Fmoc SPPS) on NovaSyn[®] TGR resin (Rink amide type: 0.25 mmol amine/g), Rink Amide AM resin (0.62 mmol amine/g), Wang resin (0.80 mmol alcohol/g) or HMPB-ChemMatrix resin (0.5 mmol alcohol/g). Fmoc SPPS was performed according to the following protocol.

1. Removal of Fmoc groups was carried out using 20% (v/v) piperidine in DMF for 10 min at room temperature.
2. The resin was washed with DMF (10 times)
3. A standard Fmoc-protected amino acid (4 equiv.) was coupled with the aid of *N,N*-diisopropylcarbodiimide (DIPCI) (4 equiv.) and 1-hydroxybenzotriazole monohydrate (HOBt·H₂O) (4 equiv.) or *N,N*-diisopropylethylamine (DIPEA) (4.0 equiv.) and *N,N,N',N'*-tetramethyl-*O*-(benzotriazole-1-yl)uronium hexafluorophosphate (HBTU, 3.9 equiv.) in DMF for 1.5 h. Completion of the coupling reaction was checked by the Kaiser ninhydrin test. The coupling reaction was repeated until the Kaiser test became negative.
4. The resin was washed with DMF (5 times).
5. The cycle of steps 1 to 4 was repeated.

Deprotection of acid-labile protecting groups with concomitant release of peptides from a resin was achieved using a cocktail of TFA-*m*-cresol-thioanisole-H₂O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μ L/1 mg resin) at room temperature for 2 h. The resin was filtered off and the filtrate was concentrated by N₂ stream. Then cooled diethyl ether (Et₂O) was added to the concentrate and the formed precipitate was collected by centrifugation. The obtained precipitate was thoroughly washed with cooled Et₂O, and purified by preparative HPLC.

2. Attempt of CPaseY-mediated Hydrazinolysis of Peptide Acids (1 and 5)

2-1. Preparation of peptides acids (1 and 5)

Peptides were synthesized on Fmoc-Ala-O-Wang (for **1**) or Fmoc-Leu-O-Wang (for **5**) resin (see below) by Fmoc SPPS (see general procedure for peptide synthesis).

Requisite Fmoc-amino acid-loaded resins were prepared as follows. On Wang resin (0.80 mmol/g), Fmoc-Xaa-OH (10 equiv.) was coupled with the aid of HBTU (9.9 equiv.), DIPEA (10 equiv.) and DMAP (0.05 equiv.) in DMF at room temperature for 3 h. The resulting resin was treated with Ac₂O (10 equiv.) and pyridine (10 equiv.) in DMF at room temperature for 30 min to mask the unreacted hydroxyl groups with Ac group. After the capping, the loading of Xaa was checked by quantification of the Fmoc group. Using Fmoc-Xaa resins, peptide acids (**1** and **5**) were synthesized as mentioned above. Characterization data of synthetic peptides are shown in Table S1.

Table S1. Characterization data of synthetic peptides (**1** and **5**).

Peptide	Analytical HPLC ^a		Preparative HPLC ^b	<i>m/z</i>		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
1	20.4	1–40	13–23	607.3 [M + H] ⁺	607.2	45
5	25.7	5–40	22–32	675.4 [M + H] ⁺	675.3	56

2-2. Initial attempt of CPaseY-mediated hydrazinolysis of peptide acid (1)

Peptide substrate Ac-**ALYGAA**-OH (**1**) (0.050 μmol) was incubated in 250 μL of aqueous solution containing 30 nM CPaseY and 2 M $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ (pH 8.0) at 25 $^\circ\text{C}$ for 12 h. The reaction was monitored and analyzed by HPLC. Before analysis, the reaction solution was diluted two-fold by quenching buffer^a and incubated at 37 $^\circ\text{C}$ for 10 min. Data of the resulting peptides including over-reaction products from reaction are summarized in Table S2.

^aQuenching buffer: 50 mM Na phosphate, 100 mM TCEP, pH 6.8.

Table S2. Characterization data of peptides.

Peptide	Analytical HPLC ^a		<i>m/z</i>	
	Retention time (min)		Calcd	Found
Ac- ALYGAA -OH (1)	20.5		607.3 [M + H] ⁺	607.4
Ac- ALYGA -NHNH ₂ (2)	17.8		550.3 [M + H] ⁺	550.3
Ac- ALYG -NHNH ₂ (3a)	17.5		479.3 [M + H] ⁺	479.4
Ac- ALYG -OH (3b)	19.4		465.2 [M + H] ⁺	465.4
Ac- ALY -NHNH ₂ (4a)	17.5		422.2 [M + H] ⁺	422.2
Ac- ALYGA -OH ^b	19.4		536.3 [M + H] ⁺	536.3

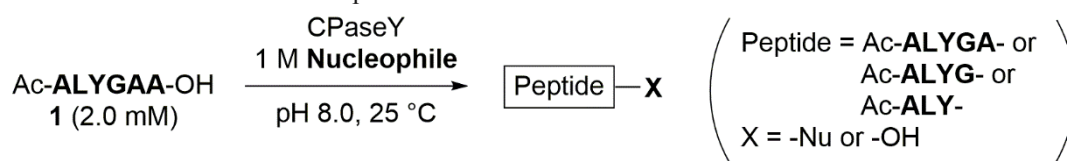
^aCosmosil 5C₁₈-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min.; HPLC conditions: a linear gradient of 0.1% TFA-MeCN (1–40% over 30 min) in 0.1 % TFA aq. ^bTrace amount of hydrolysis product of **1** was detected.

2-3. Attempt of the direct conversion of peptide substrate (1) to the corresponding thioester

For the attempt of the direct conversion to the thioester, **1** (0.050 μmol) was incubated in 250 μL of 50 mM HEPES buffer containing 100 nM CPaseY and 1 M sodium 2-mercaptoethanesulfonate (MESNa) (pH 6.0, 7.0 and 8.0) at 25 $^\circ\text{C}$ for 24 h. And the attempted reactions were monitored and analyzed by HPLC. Before analysis, the reaction solution was diluted two-fold by quenching buffer^a and incubated at 37 $^\circ\text{C}$ for 10 min. Furthermore, other nucleophiles indicated in Table S3 were also examined. Results are summarized in Table S3.

^aThe quenching buffer: 50 mM Na phosphate, 100 mM TCEP, pH 6.8.

Table S3. Examination of other nucleophiles in the CPaseY-mediated reaction.



Entry	CPaseY (nM)	Nucleophile	X = Nu (%)	X = OH (%)
1	100	MESNa (pH 6.0)	0	100
2	100	MESNa (pH 7.0)	0	100
3	100	MESNa (pH 8.0)	0	100
4	60	$\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$	84	16
5	60	NH_2NHMe	62	38
6	60	NH_2NMe_2	26	74
7	60	NH_2OH	29	70
8	60	NaN_3	0	100

2-4. Suppression of the over-reactions by the addition of cyclohexanone

To suppress the CPaseY-mediated over-reaction, **1** (0.050 μmol) was incubated in 250 μL of aqueous solution containing 30 nM CPaseY, 2 M $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$ and 50 mM cyclohexanone (pH 8.0) at 25 $^\circ\text{C}$ for 12 h. The reaction was monitored and analyzed by HPLC. Before analysis, the reaction solution was diluted two-fold by quenching buffer^a and incubated at 37 $^\circ\text{C}$ for 10 min.

^aThe quenching buffer: 50 mM Na phosphate, 200 mM $\text{MeONH}_2 \cdot \text{HCl}$, 100 mM TCEP, pH 6.8.

The HPLC yield was calculated as described in Figure S1.

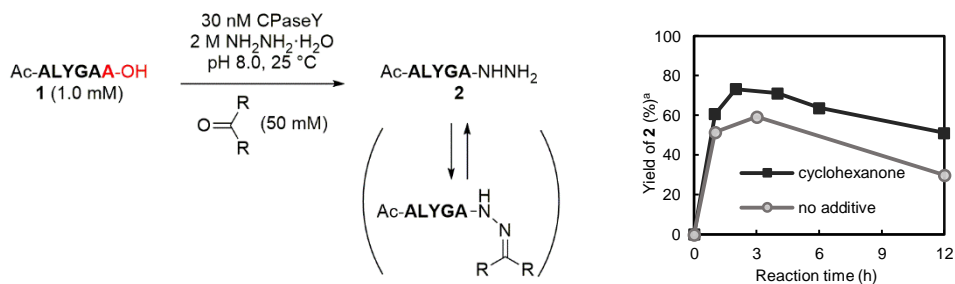


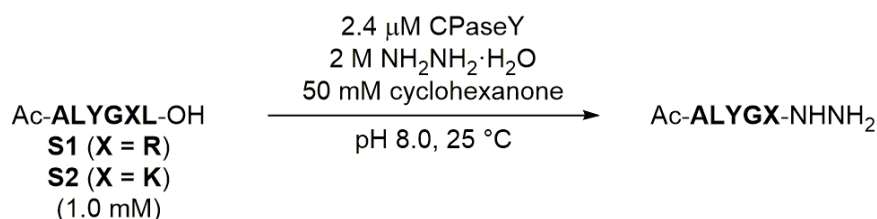
Figure S1. Suppression of over-reaction with cyclohexanone by formation of hydrazone. ^aYield (%) was determined by HPLC separation of product **2** (integ. **2**) as a fraction of the sum of remaining substrate **1** (integ. **1**) + over-reaction products (integ. **over**) + integ. **2**.

2-5. CPaseY-mediated hydrazinolysis of peptide **5** and other C-terminal -XaaLeu-OH (Xaa = Arg (S1) or Lys(S2))

Ac-ALYGPL-OH (**5**) (0.050 μmol) was incubated in 50 μL of aqueous solution containing 2.4 μM CPaseY and 2 M $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$ with 50 mM cyclohexanone (pH 8.0) at 25 $^\circ\text{C}$ for 12 h. The reaction was monitored and analyzed by LC-MS. Before analysis, the reaction solution was diluted two-fold by quenching buffer^a and incubated at 37 $^\circ\text{C}$ for 10 min. Ac-ALYGP-NHNH₂ (**6**): retention time = 15.8 min (analytical HPLC conditions: linear gradient of solvent B in solvent A, 5–40% over 30 min); The observed m/z was 576.2 (calculated m/z : 576.3 $[\text{M} + \text{H}]^+$).

^aThe quenching buffer: 50 mM Na phosphate, 200 mM $\text{MeONH}_2 \cdot \text{HCl}$, 100 mM TCEP, pH 6.8.

Ac-ALYGXL-OH (**S1** (X = R) and **S2** (X = K)) was subjected to CPaseY-mediated hydrazinolysis in a manner similar to that for peptide **5** (Figure S2).



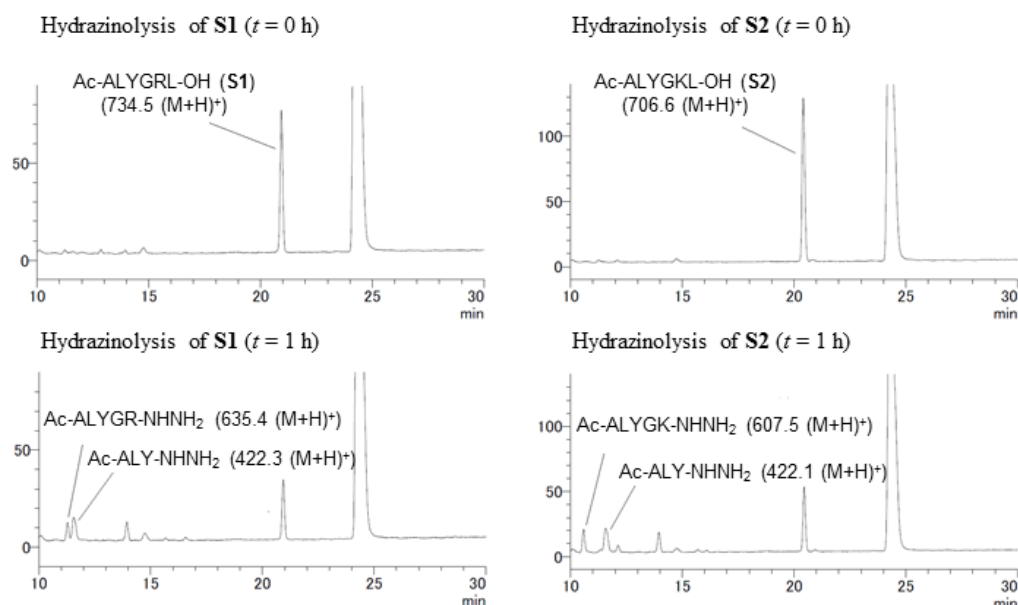


Figure S2. CPaseY-mediated hydrazinolysis of C-terminal -XaaLeu-OH peptide

3. CPE-like Thioesterification of Peptide Hydrazide (7) and Subsequent NCL with N-Terminal Cys Peptide (8)

3-1. Synthesis of peptide hydrazide (7) and N-terminal Cys peptide (8)

H-LYRAACP-NHNH₂ (7) was synthesized on hydrazine 2-Cl Trt resin by Fmoc SPPS (see general methods). The hydrazine 2-Cl Trt resin was prepared according to the protocol described by Liu. LC-MS conditions: A linear gradient of solvent B in solvent A, 5–35% over 30 min, retention time = 14.8 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 5–18% over 30 min. MS (ESI-TOF) *m/z* calcd ([M+H]⁺) 807.4, found 807.4.

H-CFGRK-NH₂ (8) was synthesized on NovaSyn[®] TGR resin by Fmoc SPPS according to the protocol described by Otaka.^{S2}

3-2. Thioesterification of 7 and subsequent NCL with 8^{S3}

Peptide hydrazide (7) sample obtained by chemical synthesis (mentioned above) or by CPaseY-mediated protocol (mentioned below) (0.100 μmol) was subjected to NCL with N-terminal Cys peptide (8). Experimental procedure was described in the following section (Typical experimental procedure for conversion of C-terminally CysProLeu-OH-tagged peptide to the corresponding hydrazide followed by the NCL protocol: Page S9). Data of peptides formed in the reaction were summarized in Table S4.

Table S4. Characterization data of substrate peptide and observed peptide sample in the reaction.

Peptide	Analytical HPLC ^a		<i>m/z</i>	
	Retention time (min)		Calcd	Found
7	14.8		807.4 [M + H] ⁺	807.7
8	11.0		606.3 [M + H] ⁺	606.5
9	26.8		943.4 [M + H] ⁺	943.6
10	15.7		775.4 [M + H] ⁺	775.7
11	22.8		743.4 [M + H] ⁺	743.7
12	17.7		592.4 [M + 2H] ²⁺	592.5

^aCosmosil 5C₁₈-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min.; HPLC conditions: a linear gradient of 0.1% TFA-MeCN (5–40% over 30 min) in 0.1 % TFA aq.

4. Evaluation of CPaseY-mediated Hydrazinolysis under Several Reaction Conditions

4-1. Preparation of C-terminal –CPX–OH substrate peptides (13)

All peptides were prepared according to the protocol described for preparation of peptide acids **1** and **5**.

Characterization data of peptides are shown in Table S5.

Table S5. Characterization data of synthesized peptides.

Peptide	Analytical HPLC ^a		Preparative HPLC ^b	<i>m/z</i>		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
13a	25.9	5–30	17–24	905.5 [M + H] ⁺	905.4	52
13b	22.8	5–30	13–23	923.4 [M + H] ⁺	923.2	27
13c	25.5	5–30	15–25	905.5 [M + H] ⁺	905.6	49
13d	22.6	5–30	13–23	939.5 [M + H] ⁺	939.7	57
13e	18.1	5–30	10–20	863.4 [M + H] ⁺	863.6	52
13f	28.2	5–30	16–26	939.5 [M + H] ⁺	939.7	52
13g	22.5	5–30	13–23	955.5 [M + H] ⁺	955.7	61
13h	28.8	5–30	15–26	978.5 [M + H] ⁺	978.7	20
13i	17.2	5–30	8–18	849.4 [M + H] ⁺	849.6	13
13j	15.6	5–30	8–18	920.5 [M + H] ⁺	920.6	36
13k	17.2	5–30	9–19	907.4 [M + H] ⁺	907.7	48
13l	15.3	5–30	8–13	906.4 [M + H] ⁺	906.6	17

^aCosmosil 5C₁₈-AR-II analytical column and ^bCosmosil 5C₁₈-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

4-2. Experiments for optimization, re-evaluation of carbonyl additives, and evaluation of influence of the C-terminal amino acids shown in Table S6, S7 and S8

To optimize the reaction conditions, the concentrations of NH₂NH₂·H₂O, cyclohexanone and CPaseY and the pH were separately changed from the standard procedure. HPLC yields were summarized in Table S6.

Standard procedure (Table S6, entry 3): H-LYRAACPL-OH (**13a**) (0.050 μmol) was incubated in 50 μL (1 mM peptide) of aqueous solution of 1.2 μM CPaseY, 0.2 M NH₂NH₂·H₂O, 60 mM cyclohexanone, pH 6.4 at 25 °C for 1 h. The reaction was monitored and analyzed by LC-MS. Before analysis, the reaction solution was diluted two-fold by quenching buffer^a and incubated at 37 °C for 10 min. ^aThe quenching buffer: 50 mM Na phosphate, 200 mM MeONH₂·HCl, 200 mM TCEP, pH 6.8.

To re-evaluate carbonyl compounds as additives for this reaction, each carbonyl compound was used instead of cyclohexanone. HPLC yields were summarized in Table S7.

To evaluate the influence of C-terminal amino acid of substrate, instead of peptide substrate (**13a**), each peptide **13b–k** was subjected to the optimal reaction conditions shown in Table S6 (entry 3). HPLC yields were summarized in Table S8.

Table S6. Optimization of CPaseY-mediated hydrazinolysis.

$\text{H-LYRAACPL-OH} \xrightarrow[\text{pH, 25 } ^\circ\text{C, 1 h}]{\text{CPaseY, NH}_2\text{NH}_2 \cdot \text{H}_2\text{O, cyclohexanone}} \text{H-LYRAACP-NHNH}_2$ 13a (1.0 mM) 7						
Entry	CPY (μM)	NH_2NH_2 (M)	cyclohexanone (mM)	pH	Remaining Substrate 13a (%)	Yield of 7 (%) ^a
1	1.2	1.0	60	6.4	0	>97
2	1.2	0.5	60	6.4	0	>97
3	1.2	0.2	60	6.4	0	>97 (76) ^b
4	1.2	0.05	60	6.4	0	89
5	1.2	0.2	90	6.4	0	>97
6	1.2	0.2	30	6.4	0	93
7	1.2	0.2	60	7.4	0	>97
8	1.2	0.2	60	5.4	0	>97
9	1.2	0.2	60	4.4	0	83
10	0.1	0.2	60	6.4	39	60

^aYield (%) was determined by HPLC separation and integration of product **7** (integ. **7**) as a fraction of the sum of the remaining **13a** (integ. **13**) + over-reaction products (integ. **over**) + integ. **7**. ^bIsolated yield.

Table S7. Re-evaluation of carbonyl additives for CPaseY-mediated hydrazinolysis

$\text{H-LYRAACPL-OH} \xrightarrow[\text{pH 6.4, 25 } ^\circ\text{C, 1 h}]{\text{1.2 } \mu\text{M CPaseY, 1.0 M NH}_2\text{NH}_2 \cdot \text{H}_2\text{O, 60 mM additive}} \text{H-LYRAACP-NHNH}_2$ 13a (1.0 mM) 7			
Entry	Additive	Yield of 7 (%)	Over-reaction products (%)
1	No	48	52
2	2-formylpyridine	25	75
3	3-formylpyridine	54	46
4	acetone	27	73
5	isobutylaldehyde	64	36
6	pivalaldehyde	27	73
7	3-pentanone	87	13
8	cyclopentanone	94	6
9	cyclohexanone	97	3
10	cycloheptanone	97	3
11	4-piperidone·HCl	86	14
12	2,2,6,6-tetramethyl-4-piperidone·HCl	85	15
13	1,4-cyclohexanedione	76	24

^aYield (%) was determined by HPLC separation and integration of product **7** (integ. **7**) as a fraction of the sum of product **7** (integ. **7**) + over-reaction products (integ. **over**).

Table S8. Evaluation of C-terminal amino acids of a substrate for CPY-mediated hydrazinolysis.

$\text{H-LYRAACP-X-OH (13, 1.0 mM)} \xrightarrow[\text{pH 6.4, 25 }^\circ\text{C, 1 h}]{\substack{1.2 \mu\text{M CPaseY} \\ 0.2 \text{ M NH}_2\text{NH}_2 \cdot \text{H}_2\text{O} \\ 60 \text{ mM cyclohexanone}}} \text{H-LYRAACP-NHNH}_2 \text{ (7)}$				
Entry	X	Remaining Substrate 13 (%)	Hydrazide 7 (%) ^a	Over-reaction products (%)
1	Leu (13a)	0	>97	<3
2	Met (13b)	0	>97	<3
3	Ile (13c)	0	>97	<3
4	Val (13d)	0	>97	<3
5	Phe (13e)	0	95	5
6	Tyr (13f)	4	91	5
7	Trp (13g)	73	16	11
8	Gly (13h)	90	9	– ^b
9	Lys (13i)	71	29	– ^b
10	Asp (13j)	88	12	– ^b
11	Asn (13k)	77	23	– ^b

^aYield (%) was determined by HPLC separation and integration of product 7 (integ. 7) as a fraction of the sum of the remaining substrate (integ. substrate) + over-reaction products (integ. over). ^b Not observed.

5. Application to Preparation of Various Amino Acyl Thioester

5-1. Preparation of the C-terminal -XaaCysProLeu-OH peptides (14)

All peptides were prepared according to the protocol described for preparation of peptide acids 1 and 5. Characterization data of synthetic peptides are summarized in Table S9

Table S9. Characterization data of synthetic peptides (14).

Peptide (14) Xaa	Analytical HPLC ^a		Preparative HPLC ^b	<i>m/z</i>		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
14a (Gly)	25.0	5–30	14–24	892.5 [M + H] ⁺	892.6	39
14b (Arg)	24.2	5–30	16–26	991.5 [M + H] ⁺	991.5	44
14c (Lys)	23.5	5–30	12–22	963.5 [M + H] ⁺	963.6	35
14d (His)	23.8	5–30	13–23	972.5 [M + H] ⁺	971.6	26
14e (Ser)	25.0	5–30	16–26	922.5 [M + H] ⁺	922.6	43
14f (Thr)	25.8	5–30	16–26	936.5 [M + H] ⁺	936.5	41
14g (Cys)	27.9	5–30	16–36	938.5 [M + H] ⁺	938.6	35
14h (Asn)	24.4	5–30	14–24	949.5 [M + H] ⁺	949.6	49
14i (Gln)	25.2	5–30	15–25	963.5 [M + H] ⁺	963.5	50
14j (Glu)	26.0	5–30	14–24	964.5 [M + H] ⁺	964.5	44
14k (Met)	26.0	5–35	18–28	967.2 [M + H] ⁺	9675	54
14l (Trp)	22.4	5–40	22–32	1021.5 [M + H] ⁺	1021.4	6
14m (Tyr)	25.2	5–35	16–26	998.5 [M + H] ⁺	998.5	31
14n (Phe)	25.5	5–40	22–32	982.5 [M + H] ⁺	982.6	49
14o (Leu)	27.5	5–35	18–28	948.5 [M + H] ⁺	948.6	52
14p (Ile)	18.6	5–55	20–30	948.5 [M + H] ⁺	948.3	42
14q (Val)	29.2	5–35	18–28	934.5 [M + H] ⁺	934.7	28

^aCosmosil 5C₁₈-AR-II analytical column ^bCosmosil 5C₁₈-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

5-2. CPaseY-mediated hydrazinolysis of C-terminal -XaaCysProLeu -OH peptides (14)

CPaseY-mediated conversion of various substrate peptides (**14**) to the corresponding hydrazide (**15**) were performed as follows. Each peptide (**14**) (per 1 mM peptide) was treated with aqueous solution containing 1.2 μ M CPaseY, 0.2 M $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ and 60 mM cyclohexanone, pH 6.4 at 25 °C for 1 h. And the yield (%) of **15** was determined by HPLC separation and integration of product (integ. **product**) as a fraction of the sum of the remaining substrate (integ. **substrate**) + other products derived from substrate (integ. **others**) + integ. **product**. The HPLC yields were summarized in the column Step 1 of Table 1. Furthermore, characterization data of the obtained hydrazide were summarized in Table S10.

5-3. CPE-like thioesterification of peptide hydrazide (15) and subsequent NCL with N-terminal Cys peptide (8)

Each peptide hydrazide (**15**) was subjected to NCL with N-terminal Cys peptide (**8**) as shown in below. Each obtained hydrazide peptide (**15**) (per 1.5 mM peptide) was treated with 50 mM Na phosphate buffer containing 6 M $\text{Gn}\cdot\text{HCl}$ and 7.5 mM NaNO_2 , pH 3.0 at -10 °C for 30 min. Then 50 mM Na phosphate buffer containing 6 M $\text{Gn}\cdot\text{HCl}$, 50 mM MPAA and 2.0 mM H-CFGRK-NH₂ (**8**) was added to the reaction mixture and the pH was adjusted to pH 6.5. the reaction mixture was incubated at rt for 12 h. And the yield (%) of **17** was determined by HPLC separation and integration of product (integ. **product**) as a fraction of the sum of the remaining substrate (integ. **substrate**) + other products derived from substrate (integ. **others**) + integ. **product**. The HPLC yields were summarized in the column Step 2 of Table 1. Data of peptides formed in the reaction were summarized in Table S11.

Table S10. Characterization data of obtained peptide hydrazide (**15**).

Peptide	Analytical HPLC ^a		<i>m/z</i>	
	Retention time (min)	Gradient (%)	Calcd	Found
15a (Gly)	14.0	5–30	793.4 [M + H] ⁺	793.5
15b (Arg)	14.2	5–30	892.5 [M + H] ⁺	892.6
15c (Lys)	14.0	5–30	864.5 [M + H] ⁺	864.5
15d (His)	14.2	5–30	873.5 [M + H] ⁺	873.6
15e (Ser)	14.1	5–30	823.4 [M + H] ⁺	823.4
15f (Thr)	14.8	5–30	837.4 [M + H] ⁺	837.5
15g (Cys)	16.5	5–30	839.4 [M + H] ⁺	839.4
15h (Asn)	13.6	5–30	850.4 [M + H] ⁺	850.6
15i (Gln)	14.6	5–30	864.4 [M + H] ⁺	864.4
15j (Glu)	15.2	5–30	865.4 [M + H] ⁺	865.4
15k (Met)	16.5	5–35	867.4 [M + H] ⁺	867.4
15l (Trp)	20.5	5–35	922.5 [M + H] ⁺	922.7
15m (Tyr)	18.2	5–30	899.5 [M + H] ⁺	899.5
15n (Phe)	19.7	5–35	883.5 [M + H] ⁺	883.6
15o (Leu)	18.5	5–35	849.5 [M + H] ⁺	849.6
15p (Ile)	17.4	5–35	849.5 [M + H] ⁺	849.5
15q (Val)	15.8	5–35	835.5 [M + H] ⁺	835.5

^aCosmosil 5C₁₈-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min.

5-4. Detail experimental procedure for conversion of C-terminally CysProLeu-OH-tagged peptides (13a, 14) to the corresponding hydrazides (7, 15) followed by the NCL protocol.

C-Terminally CysProLeu-OH-tagged peptide substrate (H-LYRAACPL-OH (**13a**)) (2.0 μ mol) was incubated in 0.4 mL (5.0 mM peptide) of hydrazinolysis solution [0.3 μ M CPaseY, 0.2 M $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$, 60 mM cyclohexanone, pH 6.4] at 25 °C for 1 h. To prepare hydrazinolysis

solution, aqueous solution containing $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ (0.2 M) and cyclohexanone (60 mM) was prepared and the pH was adjusted to pH 6.4 with 6 M HCl. To the resulting solution was added the stock solution of CPaseY in H_2O (7.7 μM : 0.5 mg protein/mL) (final concentration: 0.3 μM CPaseY, 0.2 M $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$, 60 mM cyclohexanone, pH 6.4). The solution was used for the hydrazinolysis reaction. After completion of the reaction, the reaction solution was diluted two-fold by the quenching buffer (50 mM Na phosphate, 200 mM $\text{MeONH}_2\cdot\text{HCl}$, 200 mM TCEP, pH 6.8) with additional incubation at 37 °C for 10 min. The crude material was analyzed by LC-MS and purified by semi-preparative HPLC to give the hydrazide (**7**) (1.2 mg, 1.50 μmol , 76%). Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 5–18% over 30 min. MS (ESI-TOF) m/z calcd for $\text{C}_{35}\text{H}_{59}\text{N}_{12}\text{O}_8\text{S}$ $[\text{M}+\text{H}]^+$ 807.4; Found 807.4. The resulting hydrazide (**7**) (0.10 μmol) was dissolved in 65 μL of 50 mM Na phosphate buffer containing 6 M $\text{Gn}\cdot\text{HCl}$ (pH 3.0) and the reaction mixture was stored at -10 °C. Then, 2.5 μL of 0.2 M NaNO_2 aq. was added to the solution and the reaction mixture was stored at -10 °C for 30 min. After that, 33 μL of 50 mM Na phosphate containing 6 M $\text{Gn}\cdot\text{HCl}$ and 150 mM MPAA was added to the mixture, and the pH of the mixed solution was adjusted to pH 6.5 with 1.0 M NaOH aq.. To the mixture was added N-terminal cysteinyl peptide (**8**) (0.20 μmol) and the mixed solution was incubated at room temperature for 12 h. Resulting solution was analyzed by LC-MS and HPLC yield of NCL products (**12**) was calculated according to the equation as described in section 5-3. ESI-TOF MS, found: 592.5, m/z calcd for m/z $[\text{M}+2\text{H}]^{2+}$ ($\text{C}_{53}\text{H}_{88}\text{N}_{18}\text{O}_{11}\text{S}$): 592.4. HPLC yields of other NCL products (**17**) were also similarly obtained.

Table S11. Characterization data of substrate peptide and observed peptide sample in the reaction.

Peptide	Analytical HPLC ^a		m/z	
	Retention time (min)	Gradient (%)	Calcd	Found
17a (Gly)	17.1	5–35	585.3 $[\text{M} + 2\text{H}]^{2+}$	585.6
17b (Arg)	16.5	5–35	634.9 $[\text{M} + 2\text{H}]^{2+}$	635.2
17c (Lys)	16.7	5–35	620.8 $[\text{M} + 2\text{H}]^{2+}$	621.2
17d (His)	16.3	5–35	625.3 $[\text{M} + 2\text{H}]^{2+}$	625.7
17e (Ser)	17.4	5–35	600.3 $[\text{M} + 2\text{H}]^{2+}$	600.6
17f (Thr)	17.9	5–35	607.3 $[\text{M} + 2\text{H}]^{2+}$	607.7
17g (Cys)	18.9	5–35	608.3 $[\text{M} + 2\text{H}]^{2+}$	608.6
17h (Asn)	17.2	5–35	613.8 $[\text{M} + 2\text{H}]^{2+}$	614.1
17i (Gln)	17.5	5–35	620.8 $[\text{M} + 2\text{H}]^{2+}$	621.2
17j (Glu)	17.8	5–35	621.3 $[\text{M} + 2\text{H}]^{2+}$	621.7
17k (Met)	20.1	5–35	622.3 $[\text{M} + 2\text{H}]^{2+}$	622.6
17l (Trp)	20.7	5–40	649.9 $[\text{M} + 2\text{H}]^{2+}$	650.2
17m (Tyr)	17.5	5–35	638.3 $[\text{M} + 2\text{H}]^{2+}$	638.7
17n (Phe)	20.3	5–40	630.3 $[\text{M} + 2\text{H}]^{2+}$	630.6
17o (Leu)	19.1	5–40	613.4 $[\text{M} + 2\text{H}]^{2+}$	613.7
17p (Ile)	18.7	5–40	613.4 $[\text{M} + 2\text{H}]^{2+}$	613.6
17q (Val)	17.5	5–40	606.4 $[\text{M} + 2\text{H}]^{2+}$	606.7

^aCosmosil 5C₁₈-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min.

6. Chemical Synthesis of Reduced Form CNP53 (S3)

6-1. Preparation of peptide fragments S5 and S6

Both peptides were prepared according to the protocol described for the preparation of peptide acids **1** and **5**.

Characterization data of peptides are shown in Table S12.

Table S12. Characterization data of synthetic peptides required for the preparation of reduced form CNP 53

Peptide	Analytical HPLC ^a		Preparative HPLC ^b	<i>m/z</i>		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
S6	22.1	5–35	14–20	547.9 [M + 8H] ⁸⁺	547.8	7
S5	23.9	5–30	22–32	878.9 [M + 2H] ²⁺	878.7	16

^aCosmosil 5C₁₈-AR-II analytical column and ^bCosmosil 5C₁₈-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

6-2. CPaseY-mediated hydrazinolysis of C-terminally CysProLeu-tagged peptide (S6)

Peptide **S6** (0.85 μmol) was incubated in 1.0 mL (0.85 mM peptide) of aqueous solution containing 0.6 μM CPaseY, 0.2 M NH₂NH₂·H₂O and 60 mM cyclohexanone (pH 6.4) and incubated at 25 °C for 1 h. After completion of the reaction, the solution was diluted two-fold by the quenching buffer^a. The crude material was analyzed by HPLC and purified by semi-preparative HPLC to give hydrazide **S7** (2.6 mg, 0.60 μmol, 70%). Characterization data of peptides are shown in Table S13.

^aThe quenching buffer: 50 mM Na phosphate, 200 mM MeONH₂·HCl, 200 mM TCEP, pH 6.8.

6-3. CPE-like Thioesterification followed by NCL using peptide S7 and S5

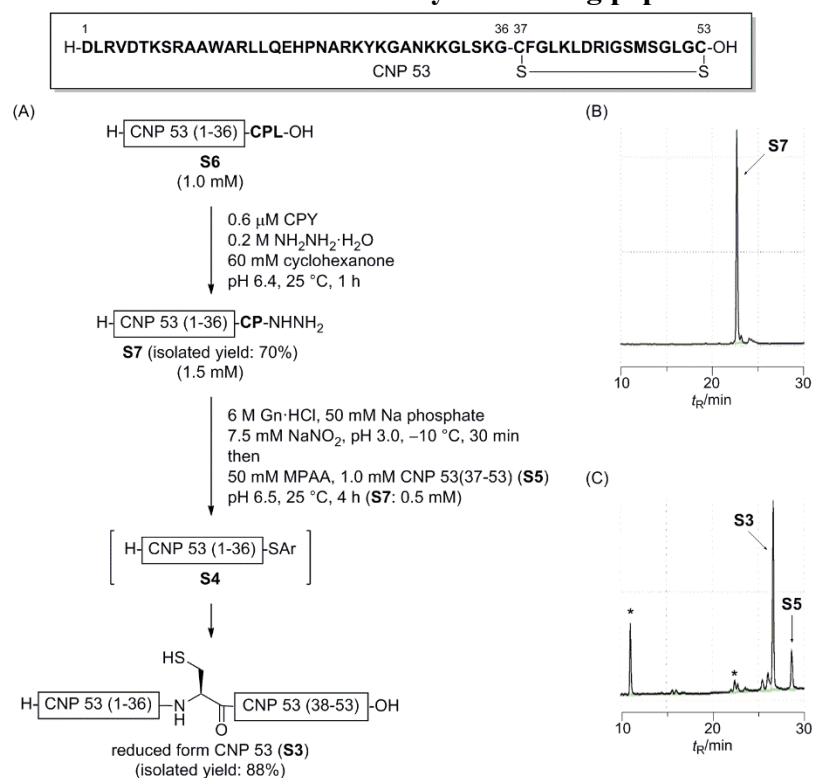


Figure S3. Preparation of reduced form CNP 53 (**S3**) using the CPaseY-mediated synthesis of thioester fragment (**S4**) followed by NCL with N-terminal cysteine peptide (**S5**). (A) Synthetic scheme for the preparation of reduced form CNP 53 (**S3**). (B) CPaseY-mediated hydrazinolysis reaction of the C-terminally CysProLeu-tagged substrate (**S6**) (after 1 h of reaction). (C) NCL of 0.5 mM of *in-situ* formed thioester (**S4**) with 1.0 mM of N-terminal cysteinyl peptide (**S5**) (after 4 h of NCL). HPLC conditions: a linear gradient of 0.1% TFA-MeCN (5–35% over 30 min) in 0.1 % TFA aq. *Non-peptidic impurity.

Purified hydrazide (**S7**) (0.60 μmol) was dissolved in 390 μL of 50 mM Na phosphate buffer containing 6 M Gn·HCl (pH 3.0) and the reaction mixture was stored at $-10\text{ }^\circ\text{C}$. Then, 15 μL of 0.2 M NaNO_2 aq. was added to the solution, and the reaction mixture was stored at $-10\text{ }^\circ\text{C}$ for 1 h. After that, 200 μL of 50 mM Na phosphate containing 6 M Gn·HCl and 150 mM MPAA was added to the solution, and the pH of the mixed solution was adjusted to pH 6.5 with 1.0 M NaOH aq.. To the mixture was added N-terminal Cys peptide **S5** (1.2 μmol) in 600 μL of 50 mM Na phosphate containing 6 M Gn·HCl and 50 mM MPAA (pH6.5) and the reaction mixture was incubated at room temperature for 4 h. The reaction was monitored and analyzed by HPLC. Before analysis, the reaction solution was diluted twice with quenching buffer^a and incubated at $37\text{ }^\circ\text{C}$ for 10 min. After completion of the reaction (4 h), the solution was diluted twice with the quenching buffer. The crude material was purified by semi-preparative HPLC to give reduced form CNP 53 **S3** (3.0 mg, 0.522 μmol , 88%).

^aThe quenching buffer: 6 M Gn·HCl, 50 mM Na phosphate, 100 mM TCEP, pH 6.8.

Table S13. Characterization data of the resulting hydrazide (**S7**) and reduced form CNP 53 (**S3**).

Peptide	Analytical HPLC ^a		Semi-preparative HPLC ^b	<i>m/z</i>		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
S7	22.8	5–35	13–23	1070.1 [M+4H] ⁴⁺	1070.2	70
S3	23.9	5–35	14–24	1161.0 [M+5H] ⁵⁺	1161.1	88

^aCosmosil 5C₁₈-AR-II analytical column and ^bCosmosil 5C₁₈-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

7. Application of CPaseY-mediated Protocol to Expressed Proteins

7-1. Expression and purification of recombinant C-terminally tagged GST (**18**) and DsRED-express (**S8**)

Amino acid sequence of C-terminally CysProLeu-tagged GST (**18**)

MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPY
YIDGDVKLQSMARIYIADKHNMLGGCPKERAIEISMLEGAVLDIRYGVSRVIAYSKDFE
TLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLD
AFPKLVCFFKKRIEAIQIDKYLKSSKYIAWPLQGWQATFGGGDHPKSDLVPRAACPL

Amino acid sequence of C-terminally CysProLeu-tagged DsRED-express (**S8**)

GMASSEDVIKEFMRFKVRMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPF
AWDILSPQFQYGSKVYVKHPADIPDYKLSFPEGFKWERVMNFEDGGVVTVTQDSSL
QDGSFIYKVKFIGVNFPSDGPVMQKKTMGWEASTERLYPRDGVLLKGEIHKALKLKDG
GHYLVFEFKSIYMAKKPVQLPGYYYVDSKLDITSHNEDYTIVEQYERAEGRHHLFLYR
AACPL

cDNAs of C-terminally CysProLeu-tagged GST (**18**) and C-terminally CysProLeu-tagged DsRED-express (**S8**)^{S4} were cloned into the pEV3b^{S5} and the pGEX-2T vectors, respectively (pEV3b and pGEX-2T constructs were designed for affording the tagged GST (**18**) itself and GST-tagged DsRED-express fusion (**S8**), respectively). The expressions of the proteins were done by transforming the constructed plasmids in to *E. coli* Rosetta 2 DE3 (Merck) competent cells. The transformed cells were grown in 1 L of LB-ampicillin (+) medium at $37\text{ }^\circ\text{C}$ until the optical density became 0.6 before the addition of isopropyl- α -D-thiogalactopyranoside (IPTG). After 24 h growth at $25\text{ }^\circ\text{C}$ in the presence of 0.1 mM IPTG, cells were harvested by centrifugation and suspended in phosphate-buffered saline (PBS). The cells were then lysed in

PBS (pH 7.4) by sonication, and a supernatant was obtained by centrifugation during which Triton X-100 was added to a final concentration of 1%. Each supernatant containing the tagged GST (**18**) or the GST-tagged DsRED-express fusion (**S8**) proteins was applied to a GSH-Sepharose (GE Healthcare, Uppsala, Sweden) affinity chromatography column equilibrated with PBS (pH 7.4) containing 1% TritonX-100. For the elution of **18**, the adsorbed protein was eluted with PBS (pH 7.4) containing 20 mM reduced L-glutathione (GSH). The eluted protein was concentrated by using centrifugal filters (10000 MW cut off). For the elution of the tagged DsRED-express (**S8**), cleavage of the thrombin-recognition sequence located between the GST and tagged DsRED-express (**S8**) infusion proteins was carried out by adding 1 mL of a thrombin solution comprising 1 U/mL in PBS, and then incubating the column for 16 h at 25 °C. The eluted **S8** was concentrated by using centrifugal filters (10000 MW cut off).

7-2. Application to expressed CysProLeu-OH-tagged GST protein (**18**).

Expressed CysProLeu-OH-tagged GST protein (**18**) (9.7 nmol) was incubated in 90 μ L (108 μ M) of aqueous solution containing 1.2 μ M CPaseY, 0.2 M $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$, 60 mM cyclohexanone (pH 6.4) at 25 °C for 1 h to convert to the CysPro-NHNH₂ protein (**19**). The reaction mixture was quenched with 50 mM Na phosphate containing 200 mM DTT, 200 mM $\text{MeONH}_2\cdot\text{HCl}$ (90 μ L). The mixture was incubated at 25 °C for 10 min and then buffer-exchanged, by use of a desalting column and centrifugal filter equipped with a 10 kDa molecular weight cut off, into 50 mM Na phosphate (pH 6.8, 120 μ L). A part of the protein mixture consisting of **18** and **19** (3.0 nmol, 34 μ L) was mixed with 4.5 μ L of 50 mM Na phosphate containing 6 M $\text{Gn}\cdot\text{HCl}$ and the pH was adjusted to 3.0 with 1 M HCl aq. To the mixture, 25 mM NaNO_2 aq. (4.5 μ L) was added at 4 °C and the reaction mixture was stored at 4 °C for 30 min (75 mM). Then excess biotinylated N-terminal Cys peptide^{S6} (**20**) (12 nmol) in 50 mM Na phosphate containing 6 M $\text{Gn}\cdot\text{HCl}$ and 150 mM MPAA (24 μ L) was added to the reaction mixture and the mixture was incubated at rt. Subsequently, the crude proteins including the desired biotinylated protein (**21**) was obtained by trichloroacetic acid (TCA) precipitation to remove small molecules. All samples including starting protein (**18**) was analyzed by SDS-PAGE (CBB stain and Western blot analysis using streptavidin-HRP) (Row CBB stained gel image Fig S4. For Fig. 4, lanes 1–3 were used). Characterization of protein samples was performed by ESI-QTOF MS analyses of samples desalted by Protein R HPLC column. The conversion yield (~ 70%) was estimated by quantitation of the ligated protein from CBB-stained gel image using image J software. C-Terminally CysProLeu-OH-tagged protein (**18**): ESI-QTOF MS, found: 26623.0, calcd for molecular mass (M) ($\text{C}_{1218}\text{H}_{1881}\text{N}_{305}\text{O}_{336}\text{S}_{14}$) 26622.3 (average). C-Terminally CysPro-NHNH₂ protein (**19**): ESI-QTOF MS, found: 26522.2, calcd for M ($\text{C}_{1212}\text{H}_{1873}\text{N}_{306}\text{O}_{334}\text{S}_{14}$) 26523.1 (average). C-Terminally biotin-peptide-modified protein (**21**): ESI-QTOF MS, found: 30269.1, calcd for M ($\text{C}_{1386}\text{H}_{2135}\text{N}_{355}\text{O}_{376}\text{S}_{15}$) 30268.5 (average).

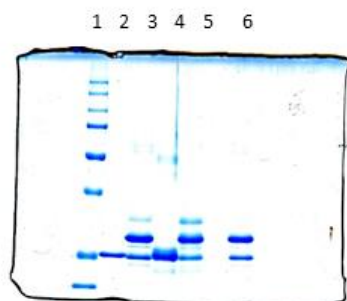


Figure S4. SDS-page analysis of protein samples followed by CBB stain: Lane 1: standard; Lanes 2 and 4: tagged GST protein (**18**); Lanes 3, 5, and 6: crude mixture after CPaseY-mediated thioesterification followed by NCL with **20**.

7-3. Application to the C-terminally-CysProLeu-tagged DsRED-express protein (S8)

Recombinant DsRED-express protein (**S8**) (14.5 nmol) was incubated in 266 μ L (55 μ M) of aqueous solution containing 1.2 μ M CPY, 0.2 M $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$, 60 mM cyclohexanone (pH 6.4) at 25 $^\circ\text{C}$ for 1 h. The reaction mixture was quenched with 266 μ L of 50 mM Na phosphate containing 200 mM DTT, 200 mM $\text{MeONH}_2\cdot\text{HCl}$. The mixture was incubated at 25 $^\circ\text{C}$ for 10 min and then buffer-exchanged, by use of a centrifugal filter equipped with a 10 kDa molecular weight cut off, into 50 mM Na phosphate (pH 6.8, 150 μ L) by repeated dilution/concentration about 10 times. A part of the protein mixture (3.0 nmol, 53 μ L) was mixed with 40 μ L of 50 mM Na phosphate containing 6 M $\text{Gn}\cdot\text{HCl}$ and the pH was adjusted to 3.0 with 1 M HCl aq.. To the mixture, 10 μ L of 1.48 mM NaNO_2 aq. was added at 4 $^\circ\text{C}$ and the reaction mixture was stored at 4 $^\circ\text{C}$ for 30 min. Then biotinylated N-terminal Cys peptide (**20**) (96.3 nmol) in 50 μ L of 50 mM Na phosphate containing 6 M $\text{Gn}\cdot\text{HCl}$ and 30 mM MPAA was added to the reaction mixture and the mixture was incubated at room temperature. After that the crude protein was obtained by trichloroacetic acid (TCA) precipitation to remove small molecules and analyzed by SDS-PAGE (CBB stain and Western blot analysis using streptavidin-HRP). The conversion yield was estimated by quantitation of the ligated protein from CBB-stained gel image using imageJ software (Figure S5).

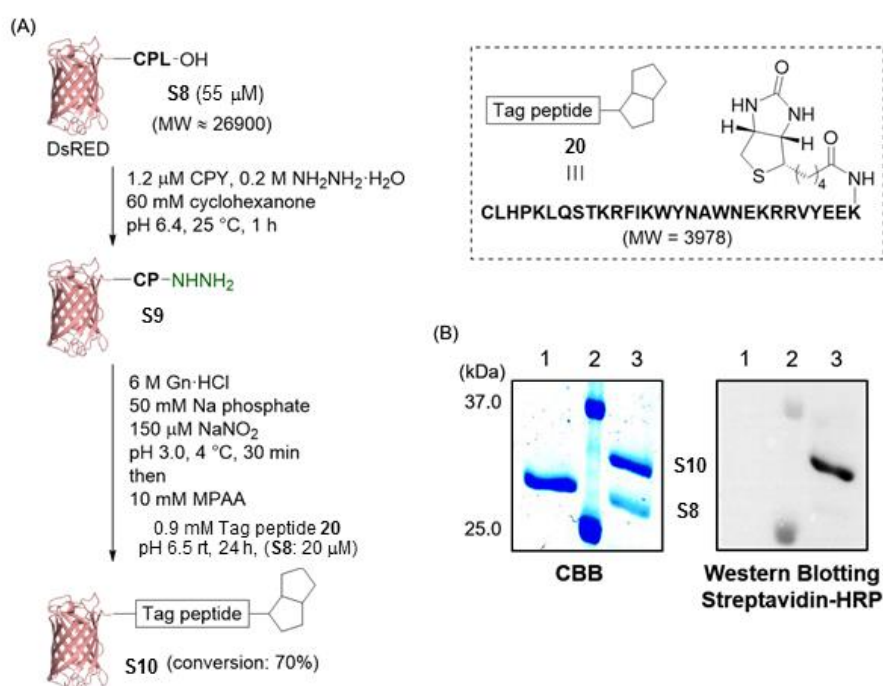


Figure S5. (A) Scheme for the application of CPY-mediated protocol to C-terminally CysProLeu-OH-tagged recombinant DsRED-express protein (**S8**); (B) SDS-page analysis of protein samples: Lane 1: DsRED-express protein (**S8**); Lane 2: standard; Lane 3 crude mixture after CPaseY-mediated thioesterification followed by NCL with **20**.

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