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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 μM) 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, 4.6×250 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/μL, 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 $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), Cosmosil $5C_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min), or Cosmosil $5C_{18}$-AR-II preparative column (Nacalai Tesque, 20 × 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·H_2O) (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-lable protecting groups with concomitant release of peptides from a resin was achieved using a cocktail of TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin) at room temperature for 2 h. The resin was filtered off and the filtrate was concentrated by N_2 stream. Then cooled diethyl ether (Et_2O) was added to the concentrate and the formed precipitate was collected by centrifugation. The obtained precipitate was thoroughly washed with cooled Et_2O, 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 Ac_2O (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]	*m/z*		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 $NH_2NH_2{\cdot}H_2O$ (pH 8.0) at 25 ˚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 ˚C for 10 min. Data of the resulting peptides including over-reaction products from reaction are summarized in Table S2.

[a]Quenching buffer: 50 mM Na phosphate, 100 mM TCEP, pH 6.8.

Table S2. Characterization data of peptides.

Peptide	Analytical HPLC[a]	*m/z*	
	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

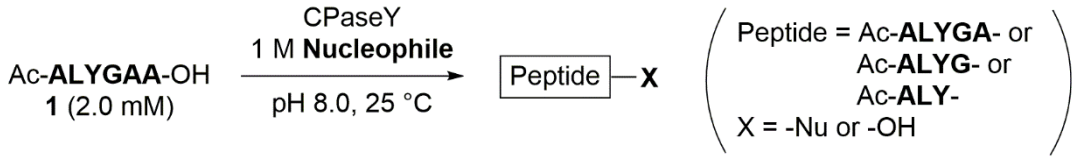
[a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min.; HPLC conditions: a linear gradient of 0.1% TFA-MeCN (1–40% over 30 min) in 0.1 % TFA aq. [b]Trace amount of hydorolysis product of **1** was detected.

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 ˚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 ˚C for 10 min. Furthermore, other nucleophiles indicated in Table S3 were also examined. Results are summarized in Table S3.

[a]The 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	$NH_2NH_2{\cdot}H_2O$	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 $NH_2NH_2 \cdot H_2O$ and 50 mM cyclohexanone (pH 8.0) at 25 ˚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 ˚C for 10 min. [a]The quenching buffer: 50 mM Na phosphate, 200 mM $MeONH_2 \cdot HCl$, 100 mM TCEP, pH 6.8. The HPLC yield was calculated as described in Figure S1.

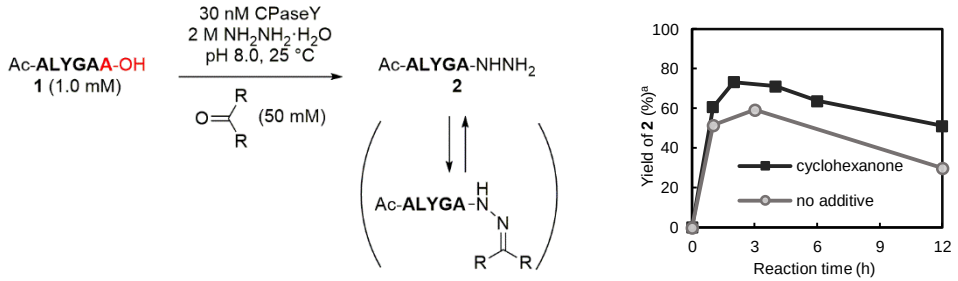
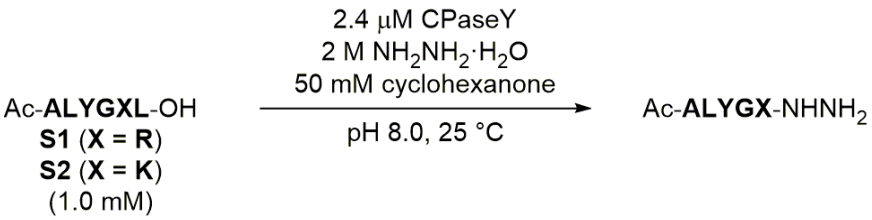


Figure S1. Suppression of over-reaction with cyclohexanone by formation of hydrazone. [a]Yield (%) was determined by HPLC separation of product **2** (integ. **2**) as a fraction of the sum of remaining substrate **1** (integ. **1**) + over-reaction products (integ. **over**) + integ. **2**.

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 $NH_2NH_2 \cdot H_2O$ with 50 mM cyclohexanone (pH 8.0) at 25 ˚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 ˚C for 10 min. Ac-**ALYGP**-$NHNH_2$ (**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 $[M + H]^+$). [a]The quenching buffer: 50 mM Na phosphate, 200 mM $MeONH_2 \cdot HCl$, 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).



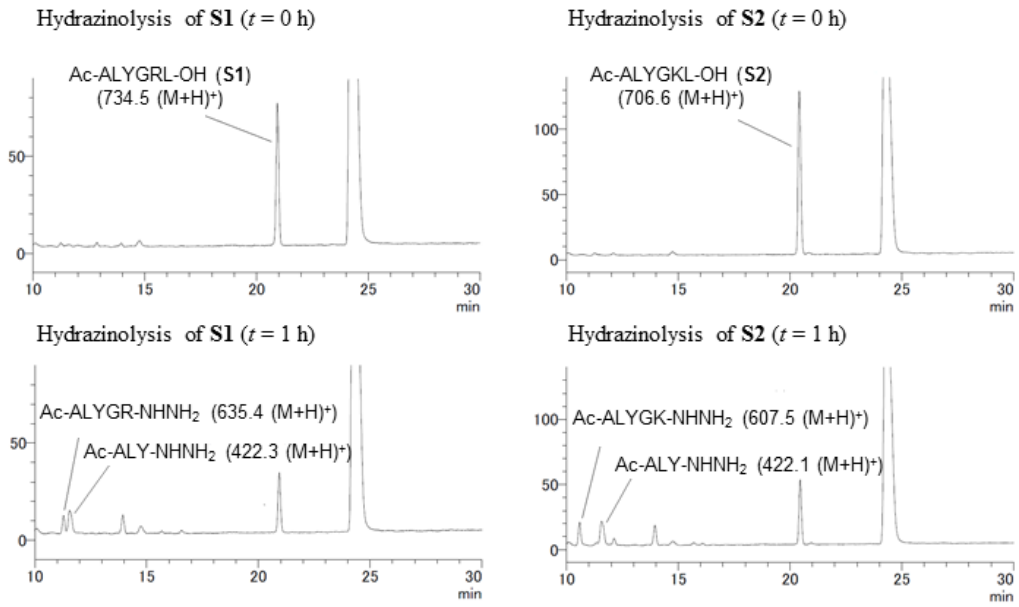


Figure S2. CPaseY-mediated hydrazinolysis of C-terminal -XaaLeu-OH peptide

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**-$NHNH_2$ (**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 condtions: 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**-NH_2 (**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]	*m/z*	
	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

[a]Cosmosil $5C_{18}$-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]	*m/z*		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

[a]Cosmosil $5C_{18}$-AR-II analytical column and [b]Cosmosil $5C_{18}$-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

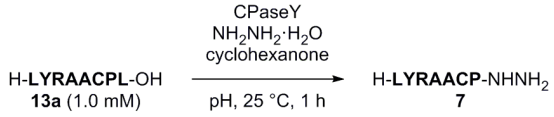
To optimize the reaction conditions, the concentrations of $NH_2NH_2{\cdot}H_2O$, cyclohexanone and CPaseY and the pH were separately changed from the standard procedure. HPLC yields were summarized in Table S6.

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 $NH_2NH_2{\cdot}H_2O$, 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. [a]The quenching buffer: 50 mM Na phosphate, 200 mM $MeONH_2{\cdot}HCl$, 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.

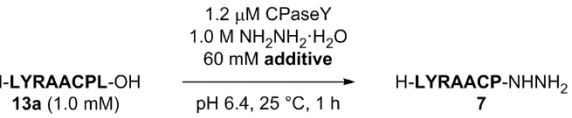
Table S6. Optimization of CPaseY-mediated hydrazinolysis.



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

[a]Yield (%) 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**. [b]Isolated yield.

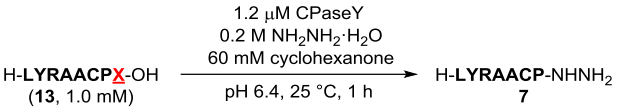
Table S7. Re-evaluation of carbonyl additives for CPaseY-mediated hydrazinolysis



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

[a]Yield (%) 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.



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]

[a]Yield (%) 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]	*m/z*		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

[a]Cosmosil $5C_{18}$-AR-II analytical column [b]Cosmosil $5C_{18}$-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 μM CPaseY, 0.2 M $NH_2NH_2 \cdot H_2O$ 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 Gn·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 Gn·HCl, 50 mM MPAA and 2.0 mM H-**CFGRK**-NH_2 (**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]		*m/z*	
	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

[a]Cosmosil $5C_{18}$-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 μmol) was incubated in 0.4 mL (5.0 mM peptide) of hydrazinolysis solution [0.3 μM CPaseY, 0.2 M $NH_2NH_2 \cdot H_2O$, 60 mM cyclohexanone, pH 6.4] at 25 ˚C for 1 h. To prepare hydrazinolysis

solution, aqueous solution containing $NH_2NH_2{\cdot}H_2O$ (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 $NH_2NH_2{\cdot}H_2O$, 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 $MeONH_2{\cdot}HCl$, 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 $C_{35}H_{59}N_{12}O_8S$ $[M+H]^+$ 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 Gn·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 Gn·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* $[M+2H]^{2+}$ ($C_{53}H_{88}N_{18}O_{11}S$): 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 $[M + 2H]^{2+}$	585.6
17b (Arg)	16.5	5–35	634.9 $[M + 2H]^{2+}$	635.2
17c (Lys)	16.7	5–35	620.8 $[M + 2H]^{2+}$	621.2
17d (His)	16.3	5–35	625.3 $[M + 2H]^{2+}$	625.7
17e (Ser)	17.4	5–35	600.3 $[M + 2H]^{2+}$	600.6
17f (Thr)	17.9	5–35	607.3 $[M + 2H]^{2+}$	607.7
17g (Cys)	18.9	5–35	608.3 $[M + 2H]^{2+}$	608.6
17h (Asn)	17.2	5–35	613.8 $[M + 2H]^{2+}$	614.1
17i (Gln)	17.5	5–35	620.8 $[M + 2H]^{2+}$	621.2
17j (Glu)	17.8	5–35	621.3 $[M + 2H]^{2+}$	621.7
17k (Met)	20.1	5–35	622.3 $[M + 2H]^{2+}$	622.6
17l (Trp)	20.7	5–40	649.9 $[M + 2H]^{2+}$	650.2
17m (Tyr)	17.5	5–35	638.3 $[M + 2H]^{2+}$	638.7
17n (Phe)	20.3	5–40	630.3 $[M + 2H]^{2+}$	630.6
17o (Leu)	19.1	5–40	613.4 $[M + 2H]^{2+}$	613.7
17p (Ile)	18.7	5–40	613.4 $[M + 2H]^{2+}$	613.6
17q (Val)	17.5	5–40	606.4 $[M + 2H]^{2+}$	606.7

[a]Cosmosil $5C_{18}$-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]	*m/z*		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

[a]Cosmosil $5C_{18}$-AR-II analytical column and [b]Cosmosil $5C_{18}$-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 $NH_2NH_2{\cdot}H_2O$ 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.

[a]The quenching buffer: 50 mM Na phosphate, 200 mM $MeONH_2{\cdot}HCl$, 200 mM TCEP, pH 6.8.

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

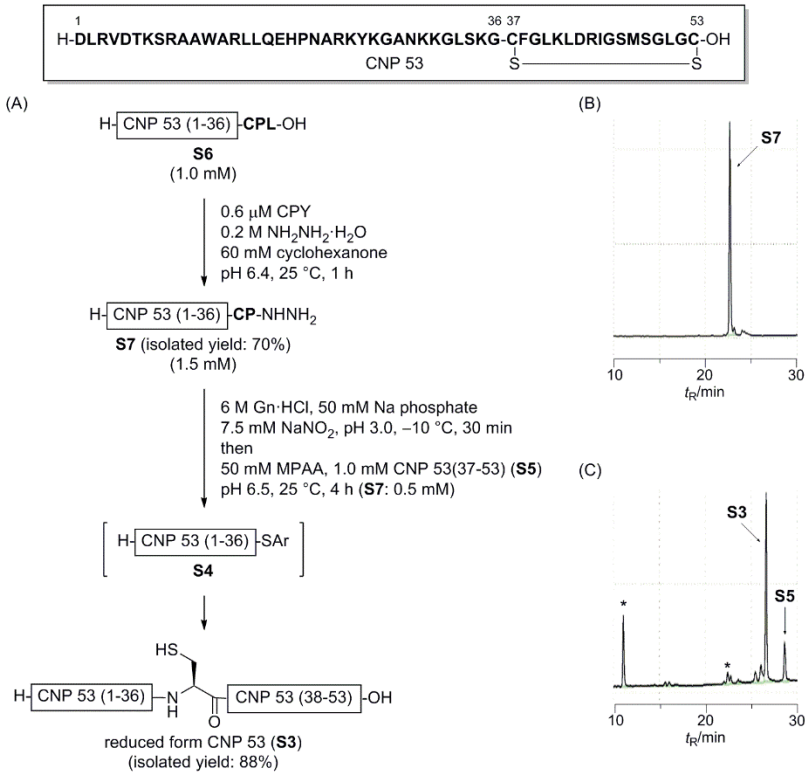


Figure S3. Preparation of reduced form CNP 53 (**S3**) using the CPaseY-mediated synthesis of thioester fragment (**S4**) followed by NCL with N-terminal cysteine peptide (**S5**). (A) Synthetic scheme for the preparation of reduced form CNP 53 (**S3**). (B) CPaseY-mediated hydrazinolysis reaction of 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 ˚C. Then, 15 μL of 0.2 M $NaNO_2$ aq. was added to the solution, and the reaction mixture was stored at -10 ˚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 ˚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%).
[a]The 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]	*m/z*		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

[a]Cosmosil $5C_{18}$-AR-II analytical column and [b]Cosmosil $5C_{18}$-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
YIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFE
TLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLD
AFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLVPRAA**CPL**

Amino acid sequence of C-terminally CysProLeu-tagged DsRED-express (**S8**)
GMASSEDVIKEFMRFKVRMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPF
AWDILSPQFQYGSKVYVKHPADIPDYKKLSFPEGFKWERVMNFEDGGVVTVTQDSSL
QDGSFIYKVKFIGVNFPSDGPVMQKKTMGWEASTERLYPRDGVLKGEIHKALKLKDG
GHYLVEFKSIYMAKKPVQLPGYYYVDSKLDITSHNEDYTIVEQYERAEGRHHLFLYR
AA**CPL**

cDNAs of C-terminally CysProLeu-tagged GST (**18**) and C-terminally CysProLeu-tagged Ds-RED-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 °C until the optical density became 0.6 before the addition of isopropyl-α-D-thiogalactopyranoside(IPTG). After 24 h growth at 25 °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 μL (108 μM) of aqueous solution containing 1.2 μM CPaseY, 0.2 M $NH_2NH_2{\cdot}H_2O$, 60 mM cyclohexanone (pH 6.4) at 25 ˚C for 1 h to convert to the CysPro-$NHNH_2$ protein (**19**). The reaction mixture was quenched with 50 mM Na phosphate containing 200 mM DTT, 200 mM $MeONH_2{\cdot}HCl$ (90 μL). 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 μL). A part of the protein mixture consisting of **18** and **19** (3.0 nmol, 34 μL) was mixed with 4.5 μL of 50 mM Na phosphate containing 6 M Gn·HCl and the pH was adjusted to 3.0 with 1 M HCl aq. To the mixture, 25 mM $NaNO_2$ aq. (4.5 μL) 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 Gn·HCl and 150 mM MPAA (24 μL) 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 strained 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-Q-TOF MS, found: 26623.0, calcd for molecular mass (M) ($C_{1218}H_{1881}N_{305}O_{336}S_{14}$) 26622.3 (average). C-Terminal CysPro-$NHNH_2$ protein (**19**): ESI-QTOF MS, found: 26522.2, calcd for M ($C_{1212}H_{1873}N_{306}O_{334}S_{14}$) 26523.1 (average). C-Terminally biotin-peptide-modified protein (**21**): ESI-QTOF MS, found: 30269.1, calcd for M ($C_{1386}H_{2135}N_{355}O_{376}S_{15}$) 30268.5 (average).

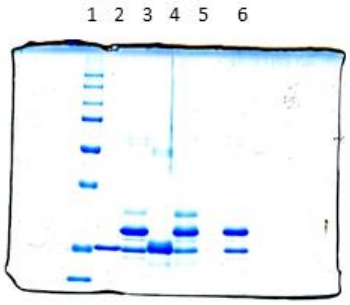


Figure S4. SDS-page analysis of protein samples followed by CBB strain: Lane 1: standard; Lanes 2 and 4: tagged GST protein (**18**); Lanes 3, 5, and 6; crude mixture after CPaseY-mediated thioesterification followed by NCL with **20**.

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 μL (55 μM) of aqueous solution containing 1.2 μM CPY, 0.2 M $NH_2NH_2{\cdot}H_2O$, 60 mM cyclohexanone (pH 6.4) at 25 ˚C for 1 h. The reaction mixture was quenched with 266 μL of 50 mM Na phosphate containing 200 mM DTT, 200 mM $MeONH_2{\cdot}HCl$. The mixture was incubated at 25 ˚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 μL) by repeated dilution/concentration about 10 times. A part of the protein mixture (3.0 nmol, 53 μL) was mixed with 40 μL of 50 mM Na phosphate containing 6 M Gn·HCl and the pH was adjusted to 3.0 with 1 M HCl aq.. To the mixture, 10 μL of 1.48 mM $NaNO_2$ aq. was added at 4 ˚C and the reaction mixture was stored at 4 ˚C for 30 min. Then biotinylated N-terminal Cys peptide (**20**) (96.3 nmol) in 50 μL of 50 mM Na phosphate containing 6 M Gn·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).

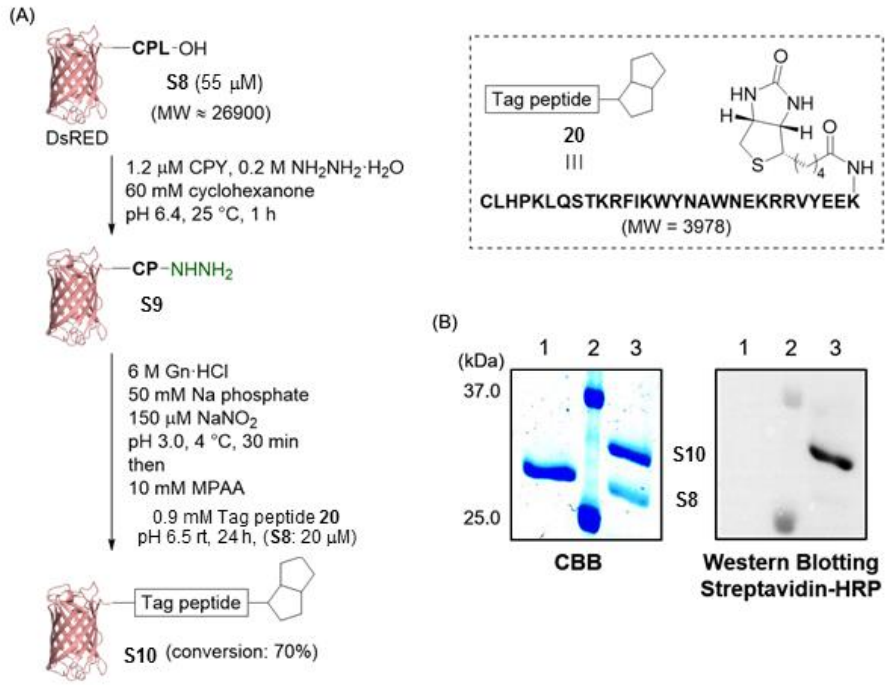


Figure S5. (A) Scheme for the application of CPY-mediated protocol to C-terminally CysProLeu-OH-tagged recombinant DsRED-express protein (**S8**); (B) SDS-page analysis of protein samples: Lane 1: DsRED-express protein (**S8**); Lane 2: standard; Lane 3 crude mixture after CPaseY-mediated thioesterification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

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