Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Azole reagents enabled ligation of peptide acyl pyrazoles for chemical protein synthesis

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1 Representative additives for native chemical ligation or related methods

Table S1. Representative additives for native chemical ligation or related methods.

Entry	Additive	pK_a[a]	Precursors	Acyl donor	Post-Ligation treatment	Refs
			Thioester			
1	2-mercaptoethanesulfonate, sodium salt (MESNA)	9.2	MPA-thioester; acyl azide or N-hydroxysuccinimide (NHS) ester	MESNA thioester	Desulfurization or None	1-3
2	4-mercaptophenylacetic acid (MPAA)	6.6	MPA-thioester; MESNA-thioester; SEAlide thioester acyl-azide; acyl-pyrazole or N-hydroxysuccinimide (NHS) ester	MPAA- thioester	None	4-9
3	(4-hydroxy)-thiophenol (MPOH)	6.8	MeNbz	MPOH- thioester	None	10
4	Trifluoroethanethiol (TFET)	7.3	MPA-thioester or acyl-azide; MESNA-thioester	TFET- thioester	Desulfurization; Thiazolidines deprotection-ligation	11, 12
5	Methyl thioglycolate (MTG)	7.9	MESNA-thioester or acyl-azide;	MTG- thioester	Tfa-thiazolidines deprotection-ligation; desulfurization	13, 14
6	2-sulfanylmethyl-4-dimethylaminopyridine (SMDMAP)	6.15	MPAL-thioester	SMDMAP- thioester	Desulfurization	15
7	Thiocholine	7.8	MESNA-thioester or acyl-azide	Thiocholine thioester	Desulfurization	16
			Acyl azole			
8	Imidazole	7.0	MPA-thioester or MPAA-thioester	Acyl imidazole	Desulfurization	17, 18
9	2-methylimidazole (2-MIM)	7.85	MPA-thioester or TFET- thioester	Acyl methylimidazole	Desulfurization	19, 20
10	1,2,4-triazole	10.2	MeNbz	Acyl triazole	Desulfurization; Refolding reaction	21
11	3-methylpyrazole or imidazole	3.54[a]; 7.0	Acyl pyrazole	Acyl methylpyrazole; acyl imidazole	Refolding reaction; Sequential ligation-desulfurization	**This work**
			Selenoester			
12	Diphenyldiselenide (DPDS)	5.9	Acyl pyrazole	Aryl selenoester	Deselenization	22-24
13	N-Alkyl bis(2-selanylethyl)amine	4.5	SEA thioester	Alkyl selenoester	None	25

[a]pK_a of 3-methylpyrazole was obtained from ref.[26] The other compound's pK_a values were obtained from "experimental properties" for each compound in SciFinder Scholar.

2 General reagents and methods

Commercially available materials were obtained from Adamas, Energy Chemicals, Bidepharm (Shanghai) or Sigma-Aldrich. Standard Fmoc-amino acids, Fmoc-Asp(OtBu)-(Dmb)Gly-OH, 2-Cl-(Trt)-Cl resins, Rink amide MBHA resin, 1-hydroxybenzotriazole (HOBt), 2-(1h-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were purchased from GL Biochem (Shanghai). The reagents acetylacetone (acac) were purchased from Aladdin. Sodium ascorbate, ethylene diamine tetraacetic acid (EDTA) and 2,2′-(ethylenedioxy)diethanethiol (DODT) were purchased from TCI. 2,2'-Azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044) were obtained from J&K Scientific. N,N-diisopropylethylamine (DIPEA), N,N'-diisopropylcarbodiimide (DIC), dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), 2-methyl-2-propanethiol (*t*-BuSH), *L*-Cystine, *L*-Cysteine, ammonium hydrogen carbonate (NH_4HCO_3), ammonium acetate (CH_3COONH_4) and guanidine hydrochloride (Gdn•HCl) were obtained from Adamas. Fmoc-$NHNH_2$ was obtained from Innochem. Trifluoroacetic acid (TFA) and triisopropylsilane (TIPS) were purchased from Energy Chemicals. 3-methylpyrazole, imidazole, 1,2,4-triazole, pyrazole and Fmoc-*D*-Ala-OH were obtained from Bidepharm (Shanghai). L-arginine hydrochloride (Arg•HCl), tris(hydroxymethyl)aminomethane (Tris), glutathione reduced (GSH), trypsin (from bovine pancreas) and

glutathione oxidized (GSSG) were purchased from Sangon. The reagents N,N-dimethylformamide (DMF) and dichloromethane (DCM) were purchased from GHTCH (Guangdong). 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 4-mercaptophenylacetic acid (MPAA) were purchased from Alfa Aesar. Acetonitrile (MeCN) used in analytical HPLC was obtained from Fisher. Acetonitrile (MeCN) used in preparative HPLC were obtained from Sigma-Aldrich. Analytical HPLC (Agilent 1260) was performed on a Welch Ultimate XB-C4 (4.6 × 250 mm, 300 Å, 5 μm particle size) or Daisogel C18 column (120 Å, 5 μm, 4.6 × 250 mm) running at a flow rate of 1 mL/min with UV detection at 214 and 254 nm. Semi-preparative HPLC (Shimadzu AR-20) was performed using Waters a XBridge® peptide BEH C18 OBD™ Prep column (300 Å, 5 μm, 10 × 250 mm) or a Welch Ultimate XB-C4 Prep column (300 Å, 5 μm, 10 × 250 mm) running at a flow rate of 4.7 mL/min with UV detection at 214 and 254 nm. Preparative HPLC (Ruihe® Tech) was performed using a Welch Ultimate XB-C4 column Prep column (300 Å, 5 μm, 30 × 250 mm) running at a flow rate of 40 mL/min or a Daisogel C18 Prep column (120 Å, 5 μm, 30 × 250 mm) running at a flow rate of 35 mL/min with UV detection at 214 and 254 nm. Solvent A: 0.1 % TFA in water; Solvent B: 0.1 % TFA in MeCN. LC-MS was performed on an Agilent LC/MSD (ESI) system on ACE 5 C4 column (150 × 4.6 mm) running at a flow rate of 0.3 mL/min with UV detection at 214 nm (with a gradient of 5 to 90% MeCN (with 0.1% FA) in 10 min) at 40 °C. LCMS separations involved a mobile phase of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B).

3 General synthesis procedures

3.1 Resin loading

3.1.1 Preloading of 2-Cl-(Trt)-$NHNH_2$ resin

2-Chlorotrityl chloride resin (0.9 mmol/g, 1 g) was swollen in DMF for 20 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2× 5 mL). The resin was treated with freshly prepared Fmoc-$NHNH_2$ (1.4 mmol/g) in DMF (8 mL) for 90 min at 25 °C and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL). The resin was treated with freshly prepared 5% MeOH in DMF (20 mL) for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL). [27, 28] The resin was subsequently submitted to iterative peptide assembly (Fmoc-SPPS).

3.1.2 Preloading of Rink amide MBHA resin

Rink amide MBHA resin (1.2 mmol/g, 0.5 g) was swollen in DMF for 20 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2× 5 mL). The resin was treated with 20% piperidine in DMF (5 mL, 2 × 10 min) at 25 °C and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL). DIPEA (1.2 mmol) was added to a solution of Fmoc-AA-OH (0.6 mmol) and TBTU (0.6 mmol) in DMF (5 mL). After 3 min of pre-activation, the mixture was added to the resin, which was then shaken for 2 h at 25 °C. The resin was washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL), and then capped with 20% acetic anhydride in DMF (10 mL) for 20 min, and washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL) again.

3.2 Estimation of amino acid loading

The resin (10 mg) loaded with the first amino acid was treated with 2% DBU/DMF (2 mL) for 30 min at 25 °C to remove the Fmoc group. The blank group was set up. The deprotection solution (2 mL) was diluted to 10 ml with MeCN, and then 0.8 mL was further diluted to 10 mL with MeCN. The UV absorbance of the resulting piperidine-fulvene adduct solution was measured (λ = 304 nm) to estimate the amino acid loading on the resin.

3.3 Fmoc deprotection

The resin was treated with 20% piperidine in DMF (5 mL, 2 × 10 min) at 25 °C and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

3.4 Capping

The resin was treated with 20% Acetic anhydride in DMF (10 mL) at 25 °C. After 15 min the resin was filtered and washed with DMF (2 × 5 mL), CH_2Cl_2 (2 × 5 mL) and DMF (2 × 5 mL).

3.5 Coupling of general amino acids

Peptides were synthesized on a CS Bio 136XT synthesizer using Fmoc solid phase peptide synthesis (SPPS) chemistry. The following Fmoc amino acids with side-chain protecting groups were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-L-Met-OH, Fmoc-L-His(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, etc. SPPS was performed on 2-Cl-(Trt)-Cl or Rink amide MBHA resins. Fmoc deprotections were performed with 20% piperidine in DMF (10 min × 2). Couplings were performed with Fmoc amino acid (4.0 equiv to resin substitution), TBTU (3.9 equiv) and DIPEA (8.0 equiv) in DMF for 60 min (45 °C). After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

3.6 Coupling of Fmoc-Asp(OtBu)-(Dmb)Gly-OH

A solution of Fmoc-Asp(OtBu)-(Dmb)Gly-OH (2 equiv), HOBT (1.96 equiv), and DIC (4 equiv) in DMF was added to the resin. The reaction was shaken for 4 h at 25 °C. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

3.7 Coupling of Fmoc-Cys(Trt)-OH

A solution of Fmoc-Cys(Trt)-OH (4 equiv), HOBT (3.9 equiv), and DIC (8 equiv) in DMF was added to the resin. The reaction was shaken for 2 h at 25 °C. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

3.8 Coupling of Fmoc-Tyr(SO_3nP)-OH

A solution of Fmoc-Tyr(SO_3nP)-OH [29] (1.5 equiv), HOBT (1.45 equiv), and DIC (3 equiv) in DMF was added to the resin. The reaction was shaken for overnight at 25 °C. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

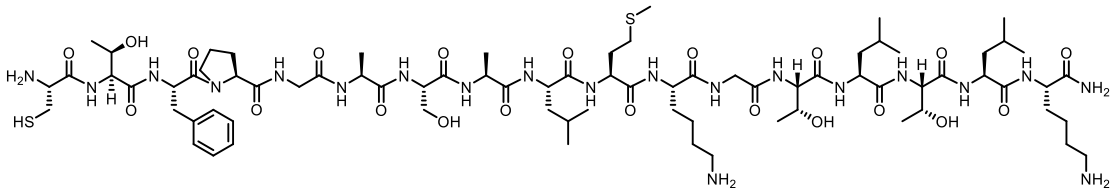
3.9 Cleavage from the resin

The resin was washed with CH_2Cl_2 (6 × 5 mL) and dried in vacuo. An acidic cocktail containing either TFA/DODT/H_2O (95:2.5:2.5, v/v/v) (3 mL per 100 mg of resin) as indicated was then added to the resin which was gently agitated for 120 min. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide.

4 Synthesis of model peptide and peptide hydrazides

4.1 Model Cys-peptide 1 and other peptides

Peptide **1** was synthesized on Rink Amide MBHA resin (theoretical loading: 0.9 mmol/g) using Fmoc-Lys(Boc)-OH with 0.5 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide **1**. Purification of the crude peptide by preparative HPLC (25 to 50% MeCN over 20 min, 0.1% TFA) afforded peptide **1** as a fluffy white solid after lyophilization (0.5 g resin; 90 mg, 20.7%). The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively.



CTFPGASALMKGTLTLK-NH_2 (1)

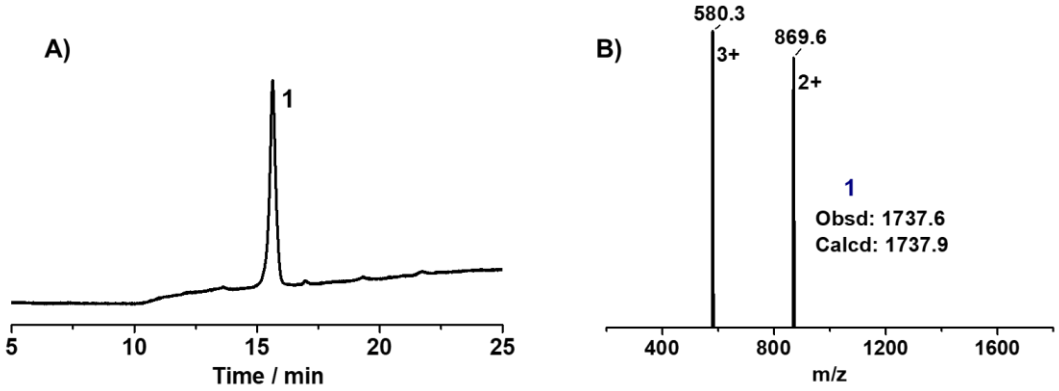
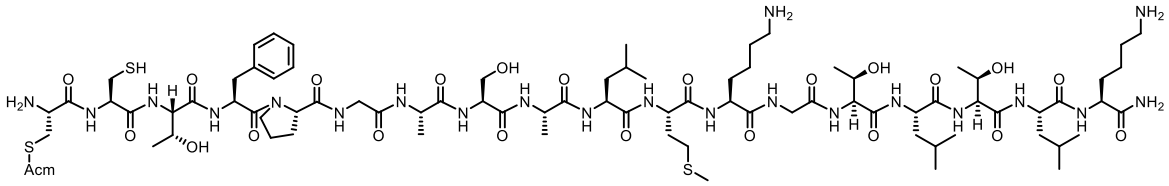


Figure S1. (A) Analytical HPLC trace (25 to 50% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) of purified peptide **1**. (B) ESI-MS analysis of **1** with the observed mass 1737.6 Da, calculated mass 1737.9 Da.

Peptide **1a** was synthesized on Rink Amide MBHA resin (theoretical loading: 0.9 mmol/g) using Fmoc-Lys(Boc)-OH with 0.5 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide **1a**. Purification of the crude peptide by preparative HPLC (25 to 50% MeCN over 20 min, 0.1% TFA) afforded peptide **1a** as a fluffy white solid after lyophilization (0.1 g resin; 28.7 mg, 30.0%). The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively.



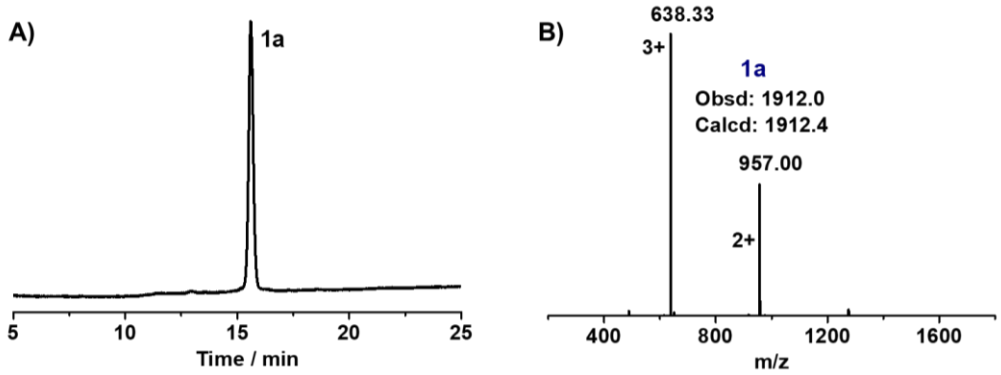
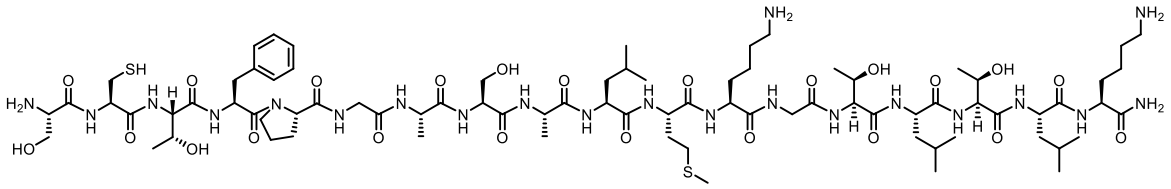


Figure S2. (A) Analytical HPLC trace (25 to 50% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) of purified peptide **1a**. (B) ESI-MS analysis of **1a** with the observed mass 1912.0 Da, calculated mass 1912.4 Da.

Peptide **1b** was synthesized on Rink Amide MBHA resin (theoretical loading: 0.9 mmol/g) using Fmoc-Lys(Boc)-OH with 0.5 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide **1b**. Purification of the crude peptide by preparative HPLC (25 to 50% MeCN over 20 min, 0.1% TFA) afforded peptide **1b** as a fluffy white solid after lyophilization (0.1 g resin; 26.4 mg, 29.0%). The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively.



SCTFPGASALMKGTLTLK-NH_2 (1b)

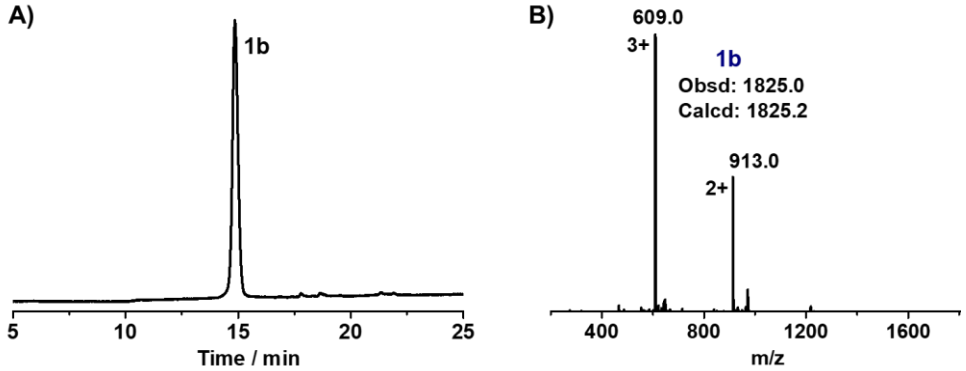
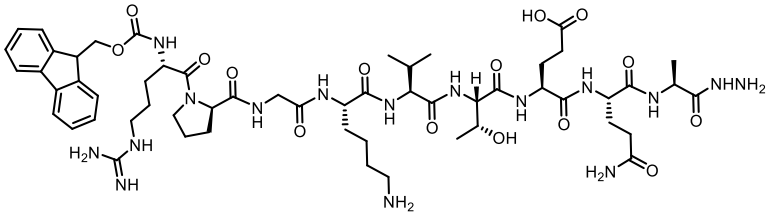


Figure S3. (A) Analytical HPLC trace (25 to 50% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) of purified peptide **1b**. (B) ESI-MS analysis of **1b** shows the observed mass 1825.0 Da, calculated mass 1825.2 Da.

4.2 Model peptide hydrazides

Peptide hydrazides (**2-14)** were synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-$NHNH_2$ with 0.4~0.5 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptides. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the peptide hydrazides (**2-14**). The purity and mass of the model peptides were confirmed using analytical HPLC and ESI-MS, respectively.



Fmoc-RPGKVTEQA-$NHNH_2$ (2)

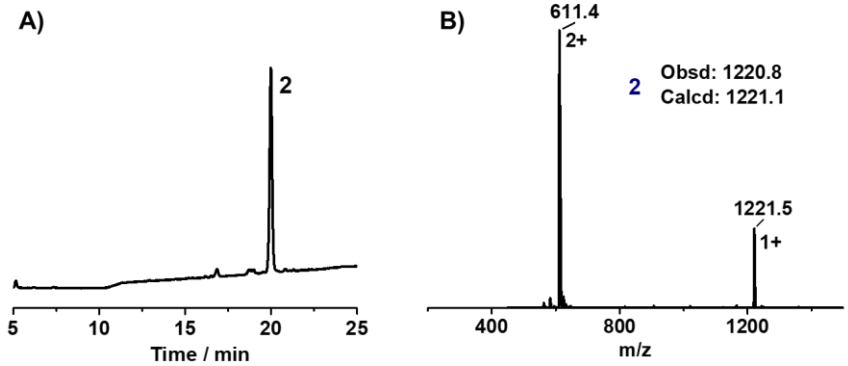
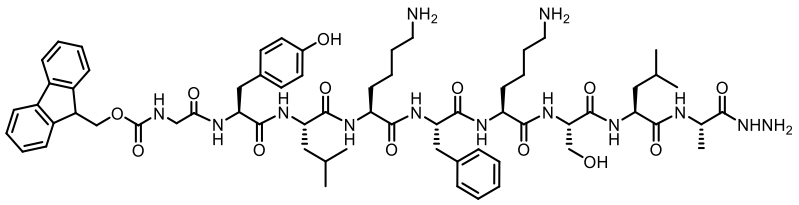


Figure S4. (A) Analytical HPLC trace (25 to 50% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) of peptide hydrazide **2**. (B) ESI-MS analysis of **2** shows the observed mass 1220.8 Da, calculated mass 1221.1 Da.



Fmoc-GYLKFKSLA-$NHNH_2$ (3)

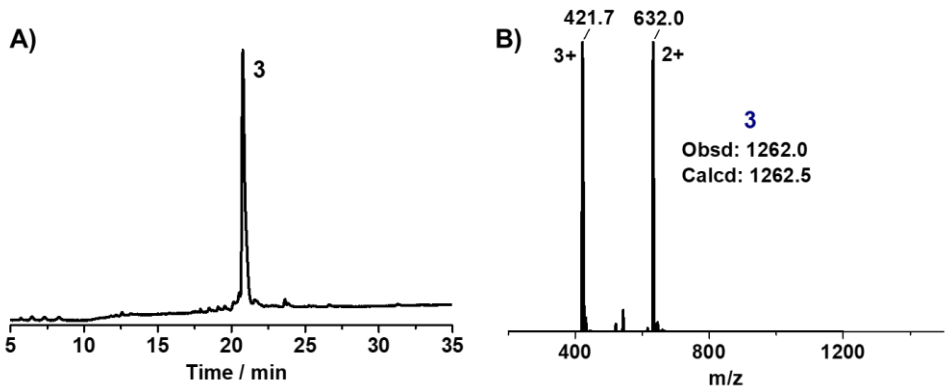
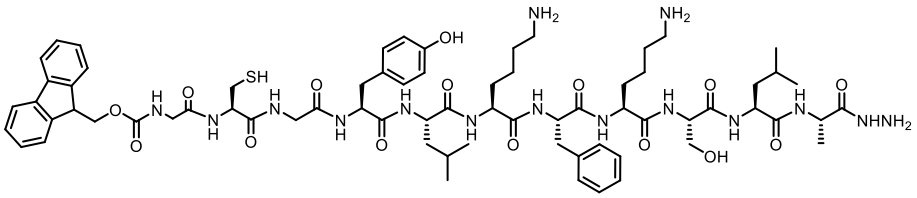


Figure S5. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 60% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **3**. (B) ESI-MS analysis of **3** shows the observed mass 1262.0 Da, calculated mass 1262.5 Da.



Fmoc-GCGYLKFKSLA-NHNH$_2$ (3*)

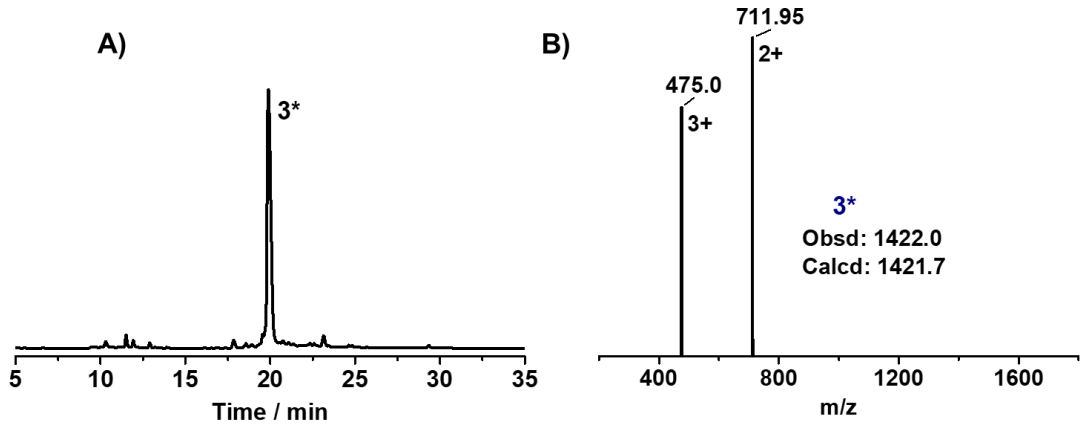
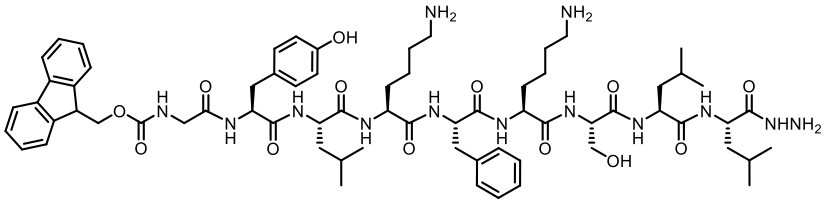


Figure S6. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 60% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **3***. (B) ESI-MS analysis of **3*** shows the observed mass 1422.0 Da, calculated mass 1421.7 Da.



Fmoc-GYLKFKSLL-NHNH$_2$ (4)

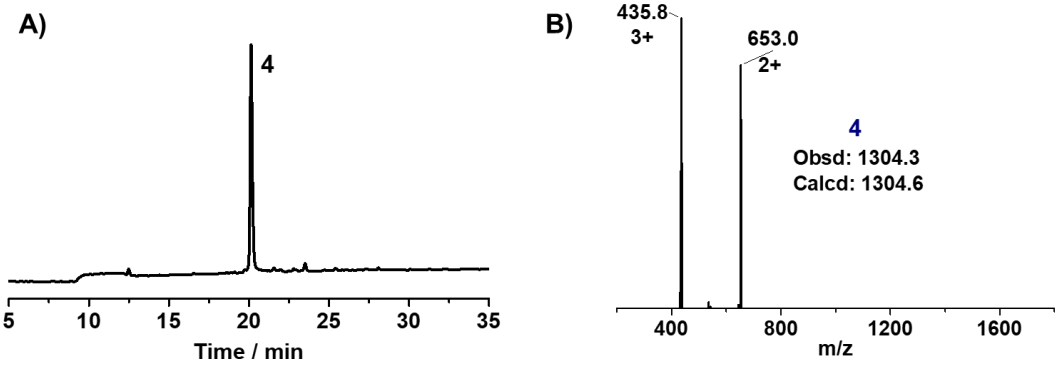
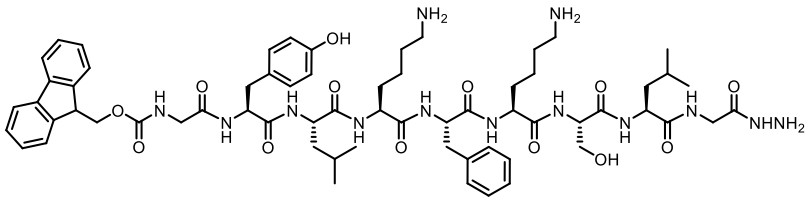


Figure S7. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 60% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **4**. (B) ESI-MS analysis of **4** shows the observed mass 1304.3 Da, calculated mass 1304.6 Da.



Fmoc-GYLKFKSLG-NHNH$_2$ (5)

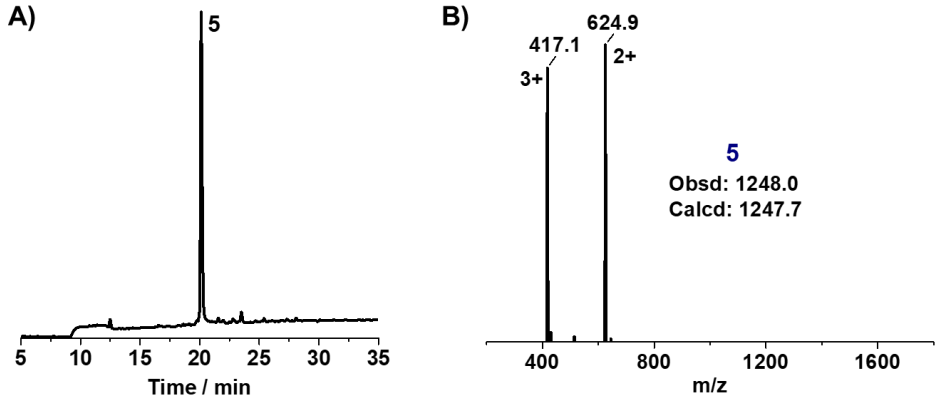
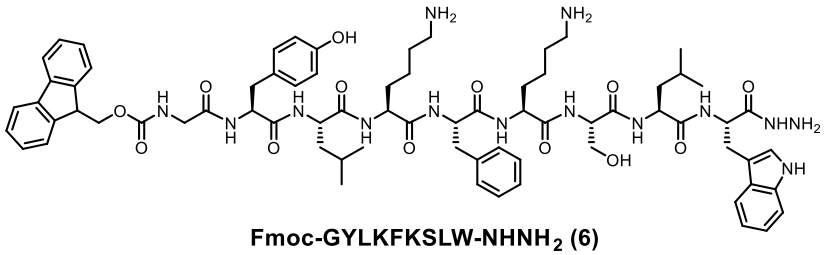


Figure S8. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 56% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **5**. (B) ESI-MS analysis of **5** shows the observed mass 1248.0 Da, calculated mass 1247.7 Da.



Fmoc-GYLKFKSLW-NHNH$_2$ (6)

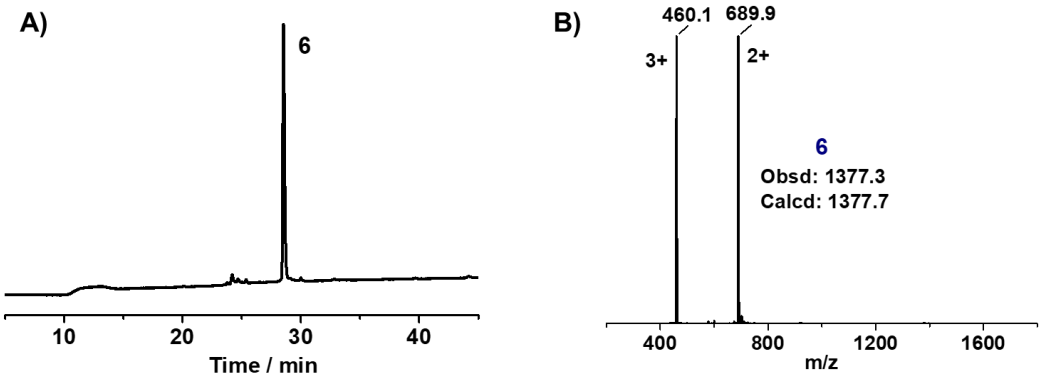
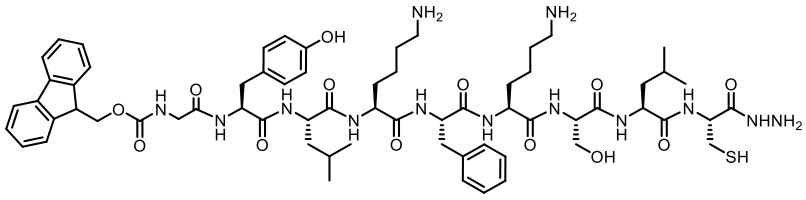


Figure S9. (A) Analytical HPLC trace (20% to 30% MeCN in 3 min then to 56% MeCN in 35 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **6**. (B) ESI-MS analysis of **6** shows the observed mass 1377.3 Da, calculated mass 1377.7 Da.



Fmoc-GYLKFKSLC-NHNH$_2$ (7)

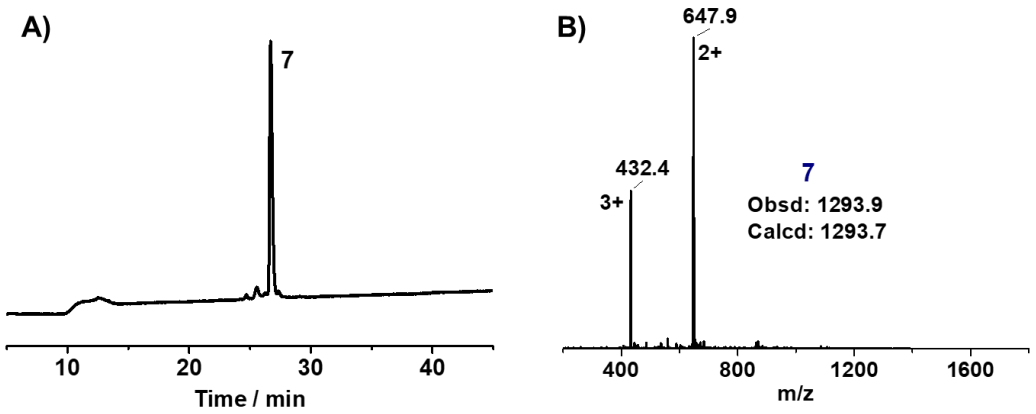
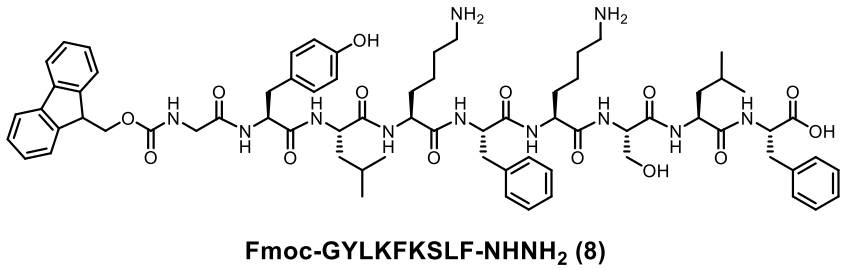


Figure S10. (A) Analytical HPLC trace (20% to 30% MeCN in 3 min then to 52% MeCN in 35 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **7**. (B) ESI-MS analysis of **7** shows the observed mass 1293.9 Da, calculated mass 1293.7 Da.



Fmoc-GYLKFKSLF-NHNH$_2$ (8)

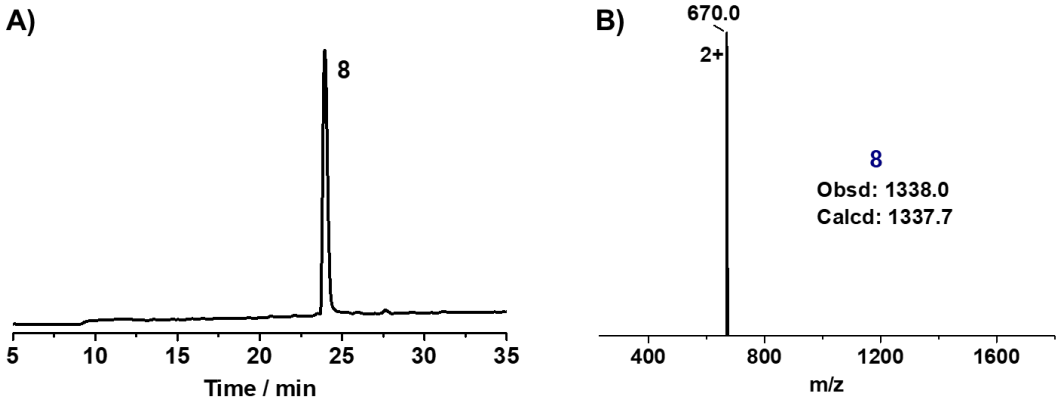
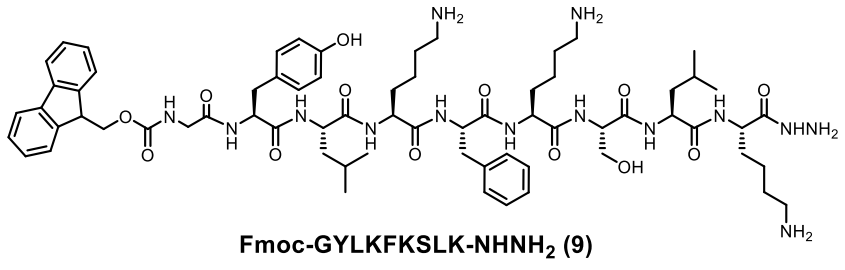


Figure S11. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 56% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **8**. (B) ESI-MS analysis of **8** shows the observed mass 1338.0 Da, calculated mass 1337.7 Da.



Fmoc-GYLKFKSLK-NHNH$_2$ (9)

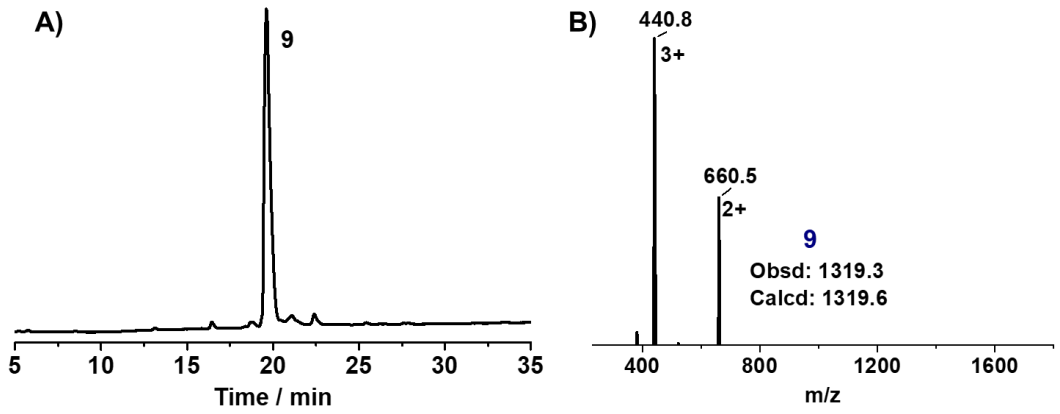
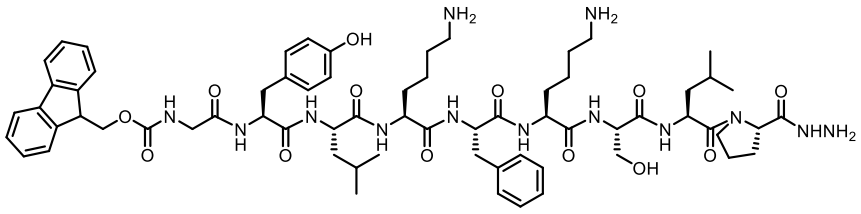


Figure S12. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **9**. (B) ESI-MS analysis of **9** shows the observed mass 1319.3 Da, calculated mass 1319.6 Da.



Fmoc-GYLKFKSLP-NHNH$_2$ (10)

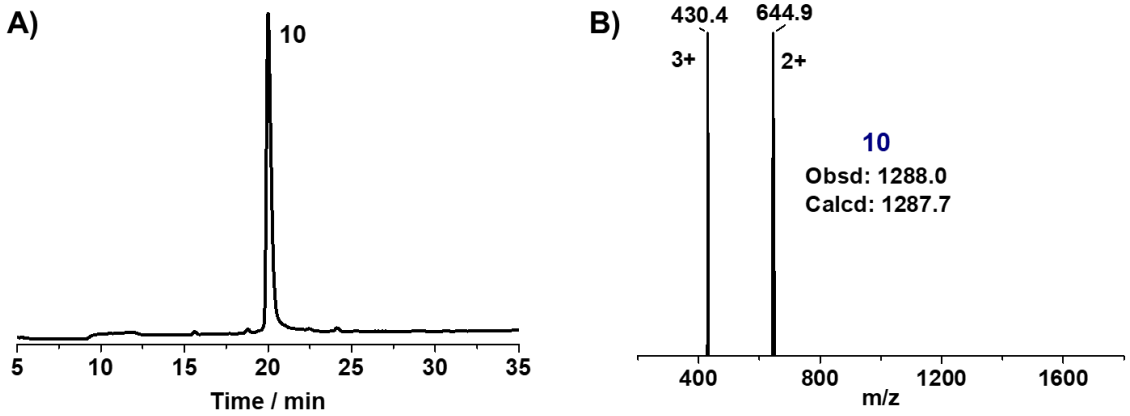
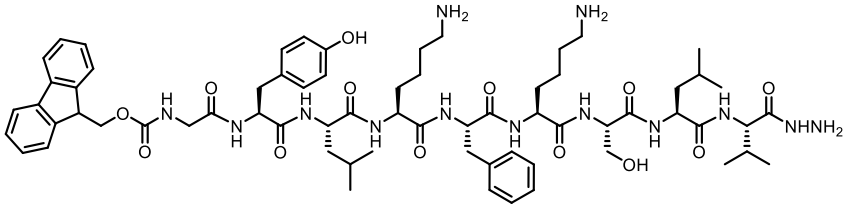


Figure S13. (A) Analytical HPLC trace (20% to 30% MeCN in 3 min then to 51% MeCN in 30 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **10**. (B) ESI-MS analysis of **10** shows the observed mass 1288.0 Da, calculated mass 1287.7 Da.



Fmoc-GYLKFKSLV-NHNH$_2$ (11)

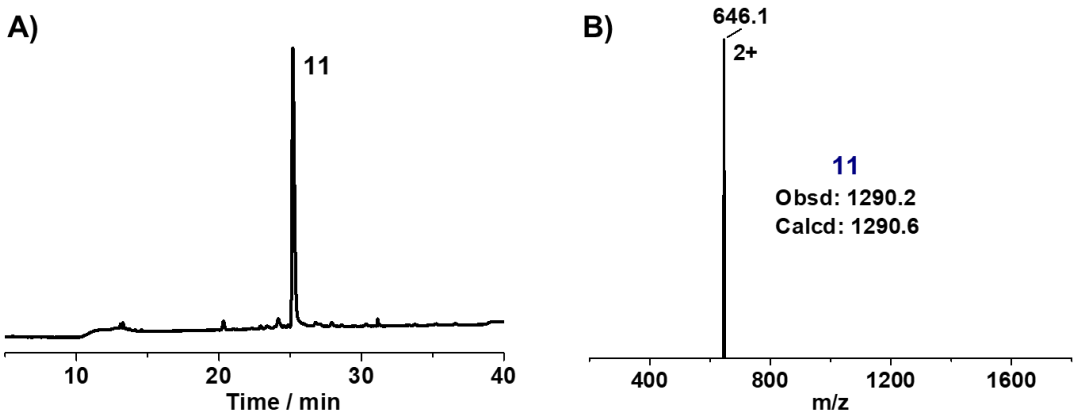
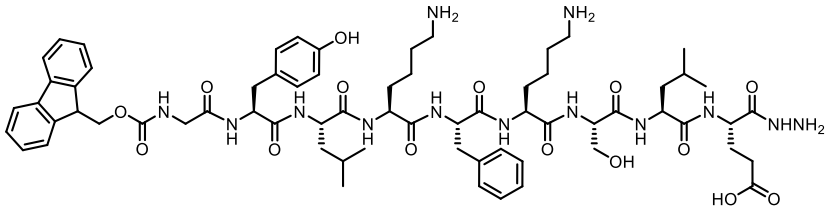


Figure S14. (A) Analytical HPLC trace (20% to 30% MeCN in 3 min then to 57% MeCN in 35 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **11**. (B) ESI-MS analysis of **11** shows the observed mass 1290.2 Da, calculated mass 1290.6 Da.



Fmoc-GYLKFKSLE-NHNH$_2$ (12)

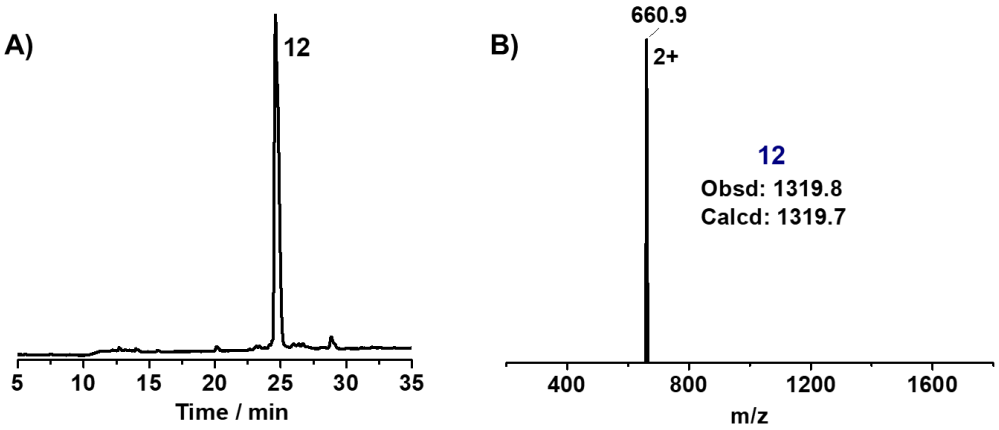
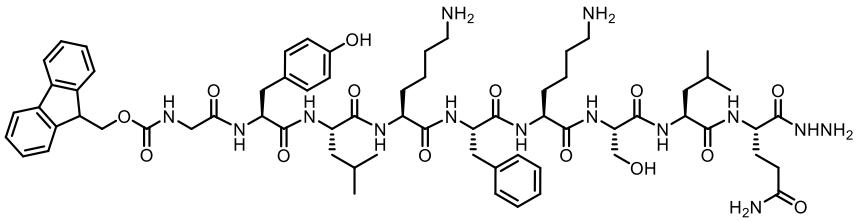


Figure S15. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **12**. (B) ESI-MS analysis of **12** shows the observed mass 1319.8 Da, calculated mass 1319.7 Da.



Fmoc-GYLKFKSLQ-NHNH$_2$ (13)

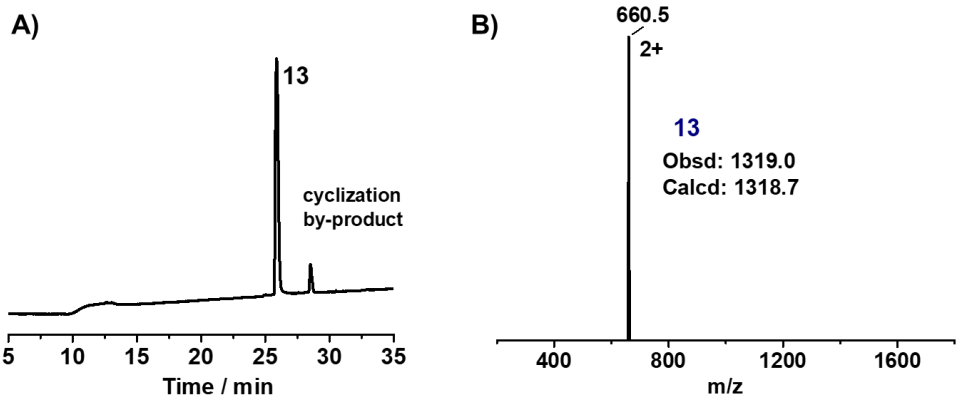
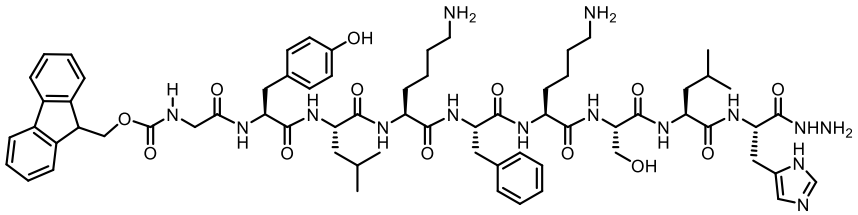


Figure S16. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **13**. (B) ESI-MS analysis of **13** shows the observed mass 1319.0 Da, calculated mass 1318.7 Da.



Fmoc-GYLKFKSLH-NHNH$_2$ (14)

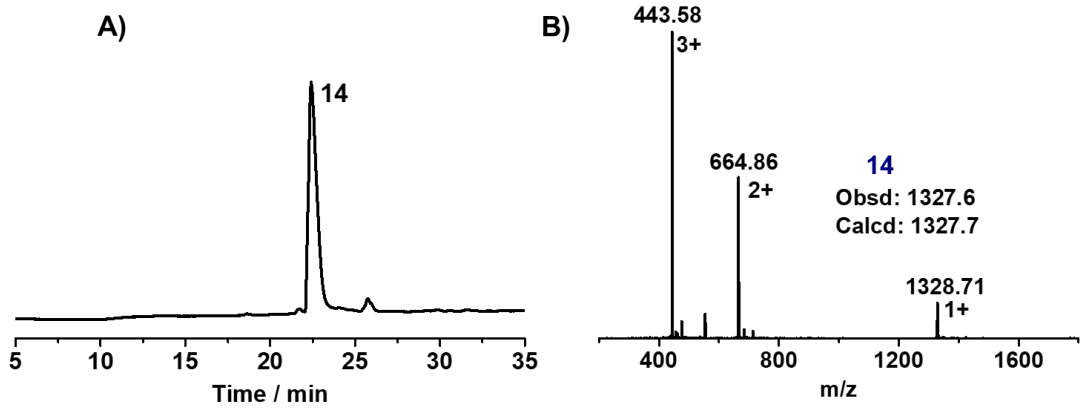
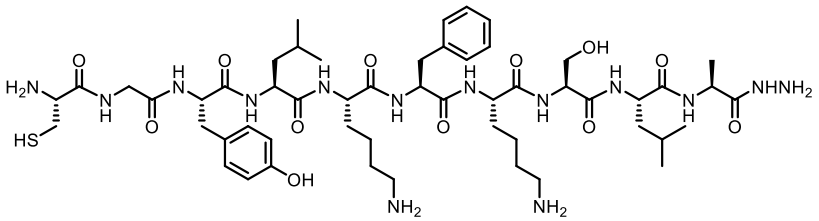


Figure S17. (A) Analytical HPLC trace (20% to 28% MeCN in 3 min then to 52% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **14**. (B) ESI-MS analysis of **14** shows the observed mass 1422.0 Da, calculated mass 1421.7 Da.



CGYLKFKSLA-NHNH$_2$ (15)

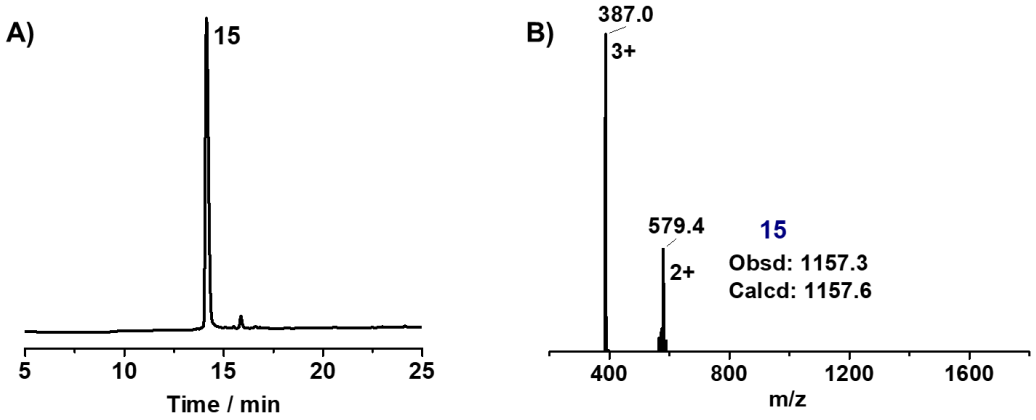
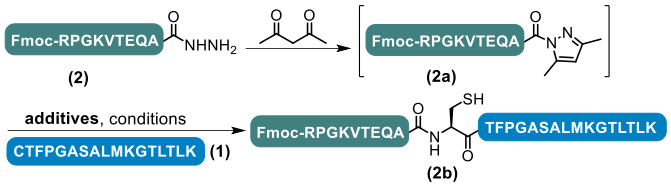


Figure S18. (A) Analytical HPLC trace (15% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of peptide hydrazide **15**. (B) ESI-MS analysis of **15** shows the observed mass 1157.3 Da, calculated mass 1157.6 Da.

5 Optimization of azole reagents to promote ligation of peptide acyl-pyrazoles

5.1 Optimization of different azole reagents

Table S2. Optimization of the ligation conditions.[a b]



Entry	pK_a[d]	Additive[e]	pH of ligation	Yield [%][f]
1		No additive	3.0	3
2		No additive	4.0	6
3		No additive	5.0	24
4		No additive	6.5	41
5	6.6	MPAA	5.0	66
6		MPAA	6.5	83
7	10.2	1,2,4-triazole	5.0	66
8		1,2,4-triazole	6.5	88
9	2.49	Pyrazole	5.0	81
10		Pyrazole	6.5	57
11	4.37	3,5-dimethylpyrazole	5.0	40
12		3,5-dimethylpyrazole	6.5	56
13	3.54	3-methylpyrazole	5.0	93 (87)[c]
14		3-methylpyrazole	6.5	58
15	6.99	Imidazole	5.0	43
16		Imidazole	6.5	93 (85)[c]

[a]The acyl-pyrazole formation step was conducted with 2.5 eq acac at pH 3.0 and 37 °C in 6 M Gdn•HCl, 0.2 M Na_2HPO_4 aqueous solution for 40 min. [b] Ligation condition: 6 M Gdn•HCl, 0.2 Na_2HPO_4, 20 mM TCEP, no additives (pH 3.0–6.5) or additives (pH 5.0 and 6.5), 37 °C, 3 hours. [c] Same as in b, but without TCEP. [d] pK_a values characteristics were predicted from "experimental properties" for each compound in SciFinder Scholar. [e] MPAA concentration: 100 mM, other additives concentration: 2.5 M. [f] The HPLC yield of the ligation of **1**.

5.1.1 The ligation of peptide acyl-pyrazole and N-terminal cysteine peptide without additive at different pH

The peptide hydrazide **2** (1.48 μmol, 1 equiv, 1.8 mg) was dissolved in 200 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 3.75 μL of the 1 M acetylacetone stock solution (3.7 μmol, 2.5 equiv) was added into the mixture and incubated at 37 °C for 40 min. The Cys-peptide **1** (1.04 μmol, 0.7 equiv, 1.8 mg) was dissolved in 200 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into four portions, and their pH were adjusted to 3.0, 4.0, 5.0 and 6.5, respectively. Each reaction mixture was stirred at 37 °C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min. When the pH increased from 3.0 to 6.5, the ligation rate increased in a pH dependent manner. The results showed that N-acylpyrazole could be used as an acyl donor for ligation.

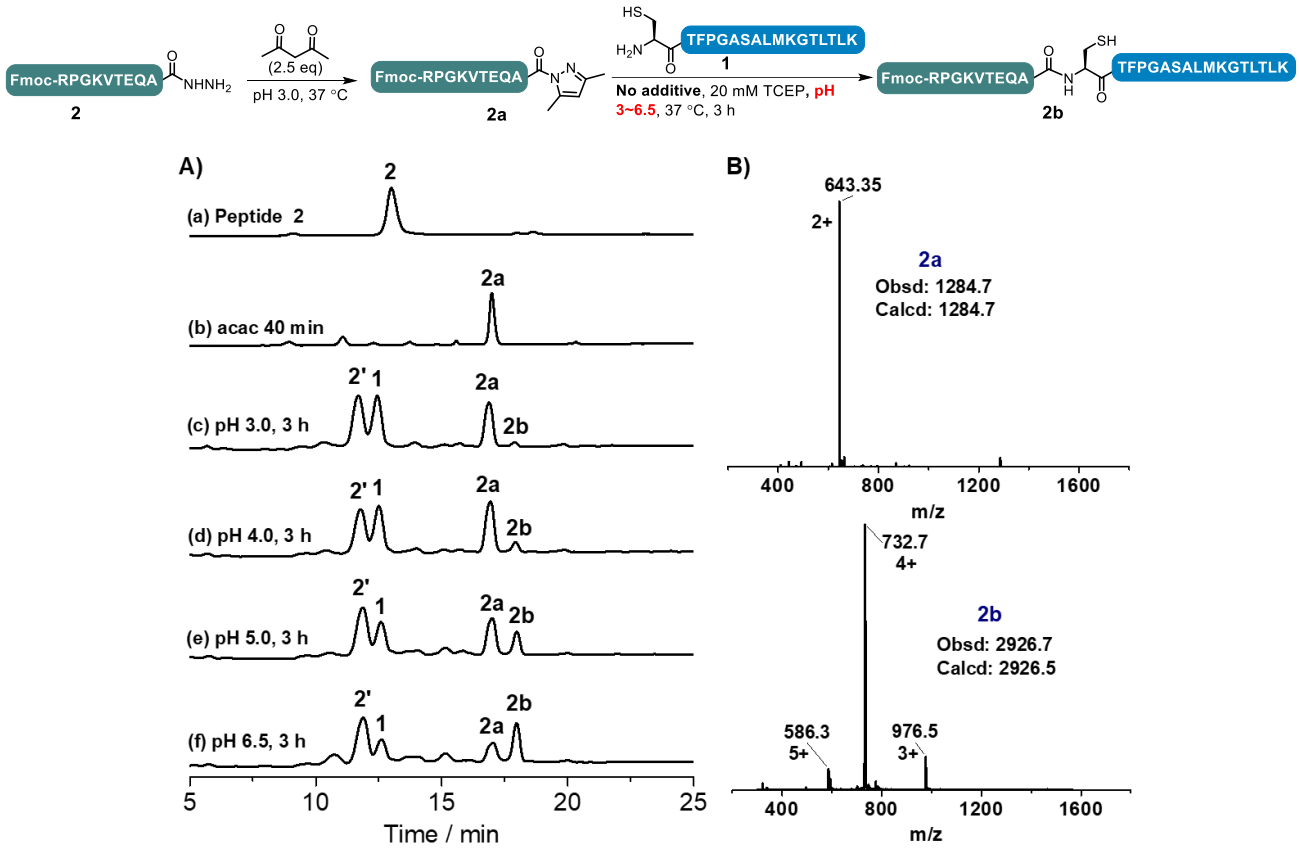


Figure S19. (A) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide hydrazide **2** without additive at different pH conditions. The peak **2'** was hydrolysis by-product. (B) ESI-MS analysis of peptide acyl-pyrazole **2a** and the product **2b,** respectively. **2a**: observed mass 1284.7 Da, calculated mass 1284.7 Da; **2b**: observed mass 2926.7 Da, calculated mass 2926.6 Da.

5.1.2 The ligation of peptide acyl-pyrazole and N-terminal cysteine peptide with different additives

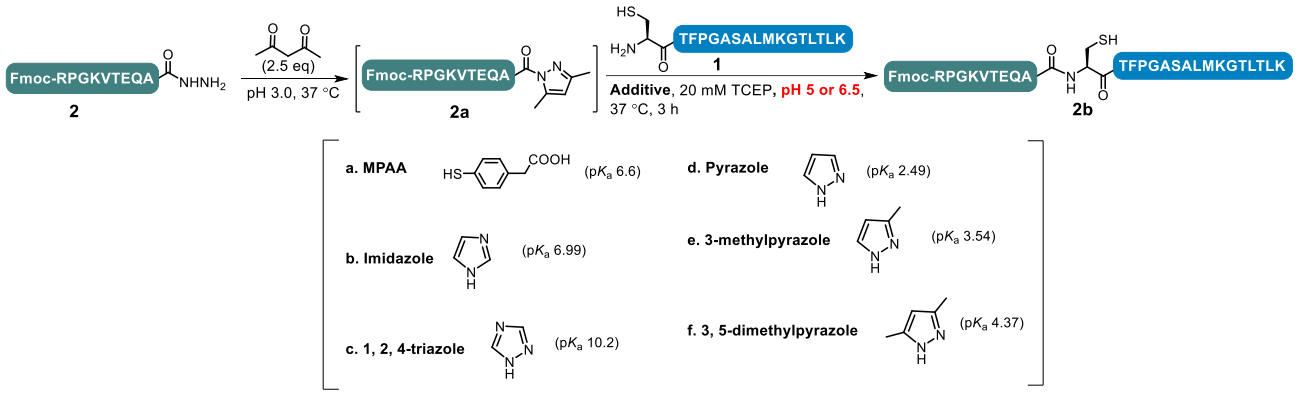


Figure S20. Ligation reactions between Cys-peptide **1** and peptide hydrazide **2** using a variety of additives.

(1) Additive was MPAA

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 60 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. After that, the Cys-peptide **1** (0.26 μmol, 0.7 equiv, 0.45 mg) was dissolved in 65 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 40 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 3.72 mg of MPAA (100 mM, final conc.) was added. The mixed solution was divided into two portions and then adjusted to pH 5.0 and 6.5, respectively, with NaOH (6.0 M). Each reaction mixture was stirred at 37°C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min.

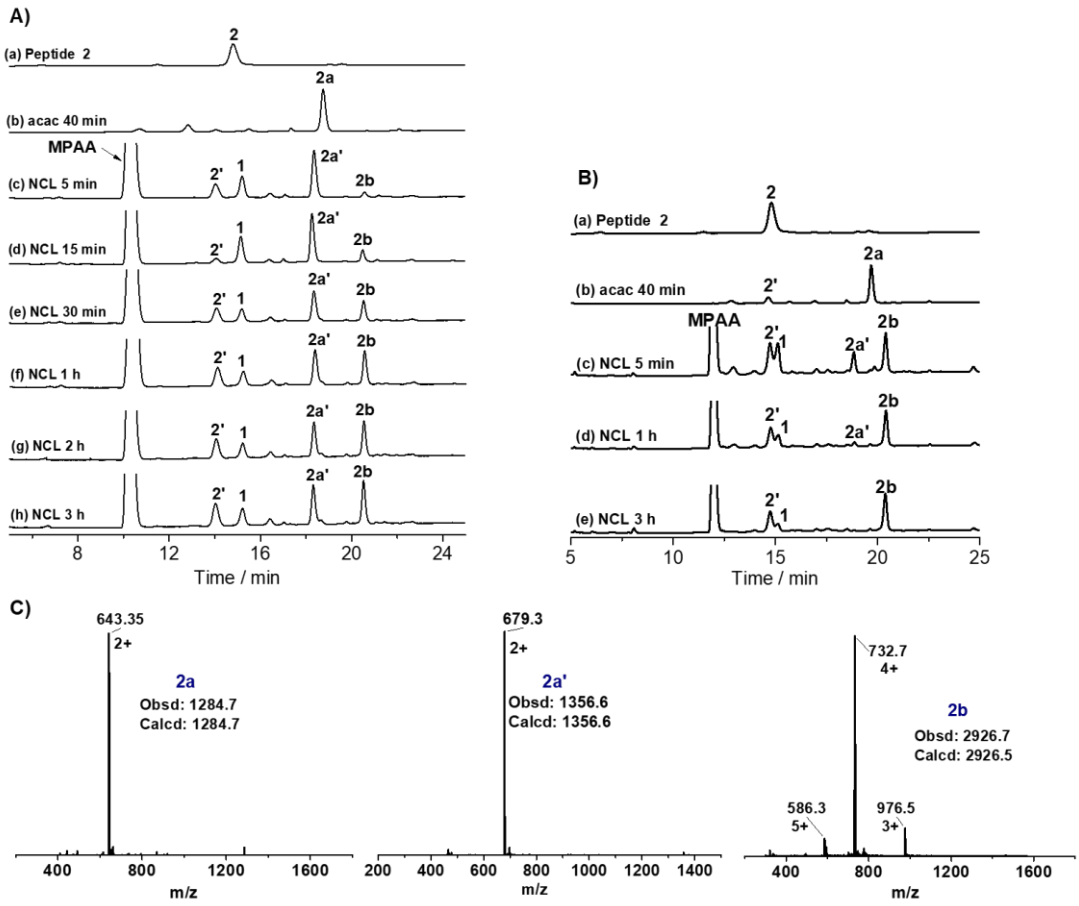


Figure S21. (A-B) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide hydrazide **2** using MPAA at pH 5.0 (A) and pH 6.5 (B) for 3 h, respectively. The peak **2'** is hydrolysis by-product. (C) ESI-MS analysis of peptide acyl-pyrazole **2a**, peptide thioester **2a'** and the ligation product **2b**, respectively. **2a**: observed mass 1284.7 Da, calculated mass 1284.7 Da; **2a'**: observed mass 1356.6 Da, calculated mass 1356.6 Da; **2b**: observed mass 2926.7 Da, calculated mass 2926.5 Da.

(2) Additive was imidazole

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 43 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. After that, the Cys-peptide **1** (0.26 μmol, 0.7 equiv, 0.45 mg) was dissolved in 65 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 40 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 22.1 μL of imidazole solution (final conc. in 2.5 M, prepared as 1 mg/μL stock solution) was added. The mixed solution was divided into two portions and then adjusted to pH 5.0 and 6.5, respectively, with NaOH (2.0 M). Each reaction mixture was stirred at 37°C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min.

Note: In order to avoid the severe hydrolysis of the peptide acyl-pyrazole caused by the direct addition of solid imidazole. The imidazole was pre-dissolved in 12 M HCl to prepare a 1 mg/μL stock solution and then diluted in the appropriate volume of buffer A before being added to the ligation mixture.

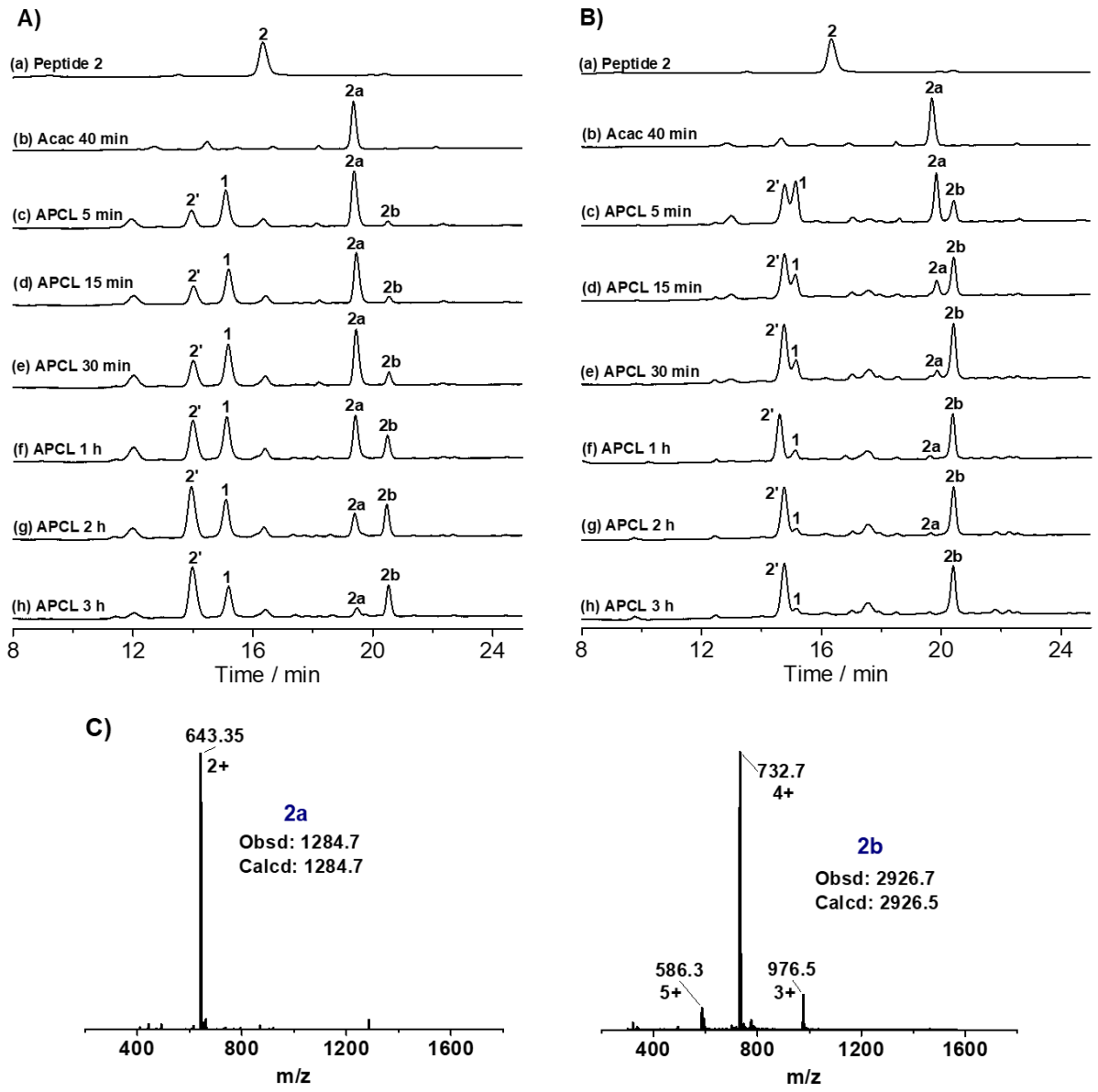


Figure S22. (A-B) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide hydrazide **2** using imidazole at pH 5.0 (A) and pH 6.5 (B) for 3 h, respectively. The peak **2'** is hydrolysis by-product. (C) ESI-MS analysis of peptide acylpyrazole **2a** and the ligation product **2b**. **2a**: observed mass 1284.7 Da, calculated mass 1284.7 Da; **2b**: observed mass 2926.7 Da, calculated mass 2926.5 Da.

(3) Additive was 1,2,4-triazole

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 43 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. After that, the Cys-peptide **1** (0.26 μmol, 0.7 equiv, 0.45 mg) was dissolved in 65 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 40 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 22.4 μL of 1,2,4-triazole solution (final conc. in 2.5 M, prepared as 1 mg/μL stock solution) was added. The mixed solution was divided into two portions and then adjusted to pH 5.0 and 6.5, respectively, with NaOH (2.0 M). Each reaction mixture was stirred at 37°C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min.

Note: 1,2,4-triazole was pre-dissolved in 6 M HCl to prepare a 1 mg/μL stock solution.

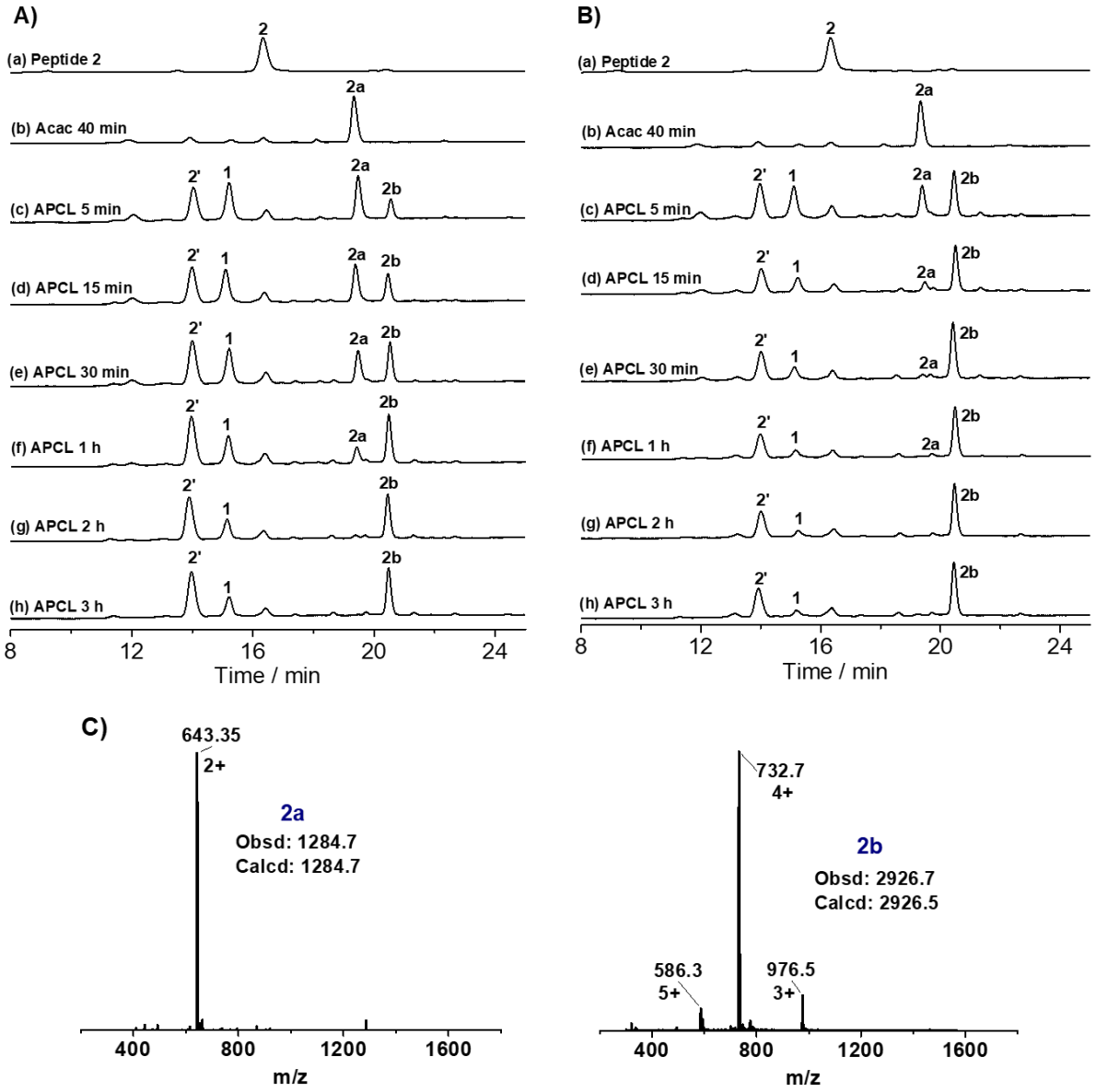


Figure S23. (A-B) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide hydrazide **2** using 1,2,4-triazole at pH 5.0 (A) and pH 6.5 (B) for 3 h, respectively. The peak **2'** is hydrolysis by-product. (C) ESI-MS analysis of peptide acyl-pyrazole **2a** and the ligation product **2b**, respectively. **2a**: observed mass 1284.7 Da, calculated mass 1284.7 Da; **2b**: observed mass 2926.7 Da, calculated mass 2926.5 Da.

(4) Additive was pyrazole

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 60 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. After that, the Cys-peptide **1** (0.26 μmol, 0.7 equiv, 0.45 mg) was dissolved in 65 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 40 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 22.1 mg of pyrazole (2.5 M, final conc.) was added. The mixed solution was divided into two portions and then adjusted to pH 5.0 and 6.5, respectively, with NaOH (2.0 M). Each reaction mixture was stirred at 37°C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min.

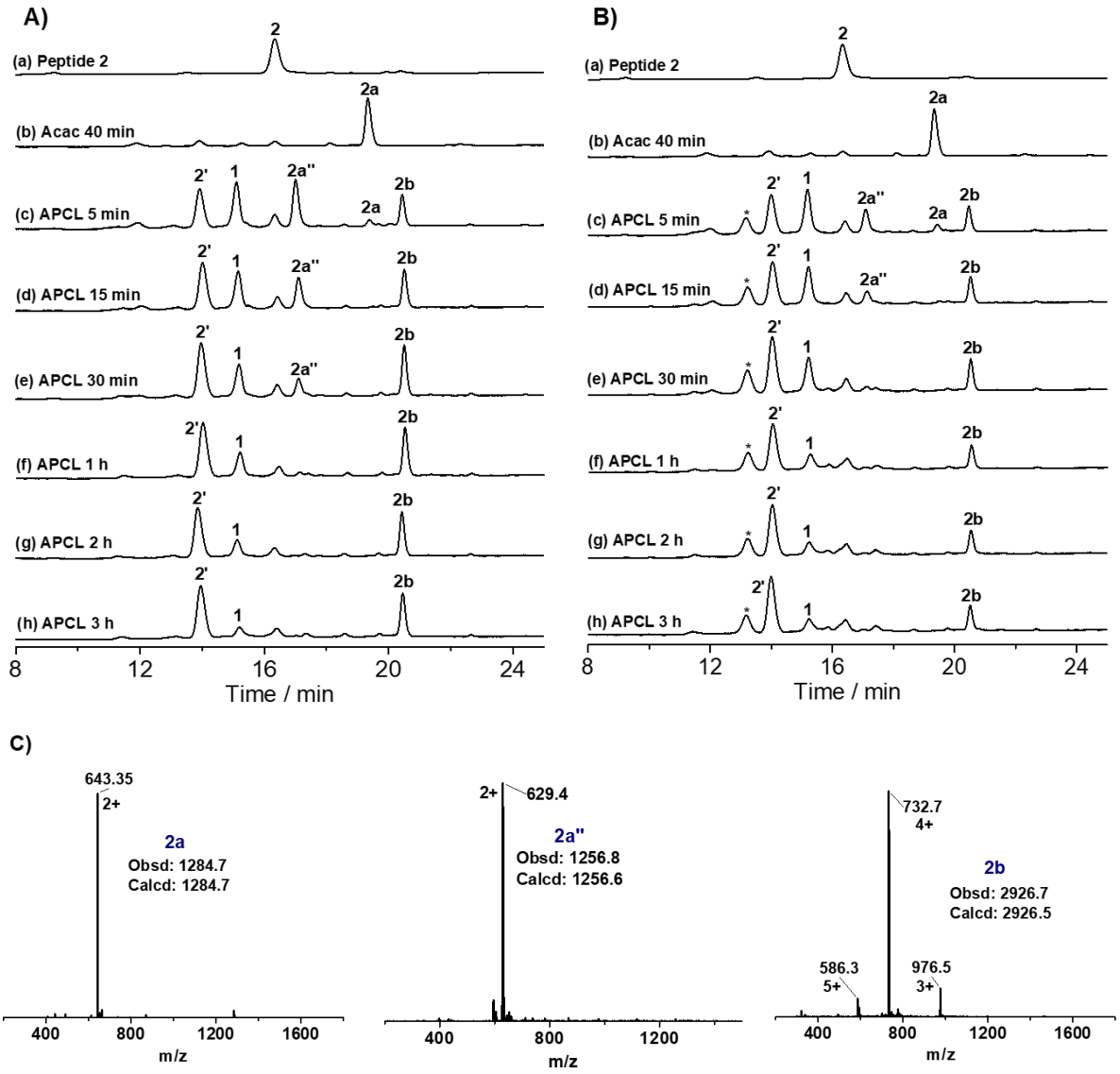


Figure S24. (A-B) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide hydrazide **2** using pyrazole at pH 5.0 (A) and pH 6.5 (B) for 3 h, respectively. Note: * denotes unknown by-product with the observed mass of 1255 Da and peak **2'** is hydrolysis by-product. (C) ESI-MS analysis of peptide acyl-pyrazole **2a**, the pyrazole substituted intermediate **2a''** and ligation product **2b**, respectively. **2a**: observed mass 1284.7 Da, calculated mass 1284.7 Da; **2a''**: observed mass 1256.8 Da, calculated mass 1256.6 Da; **2b**: observed mass 2926.7 Da, calculated mass 2926.5 Da.

(5) Additive was 3-methylpyrazole

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 50 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. The Cys-peptide **1** (0.26 μmol, 0.7 equiv, 0.45 mg) was dissolved in 50 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 26.7 μL of 3-methylpyrazole (2.5 M, final conc.) was added. The mixed solution was divided into two portions and then adjusted to pH 5.0 and 6.5, respectively, with NaOH (2.0 M). Each reaction mixture was stirred at 37°C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min.

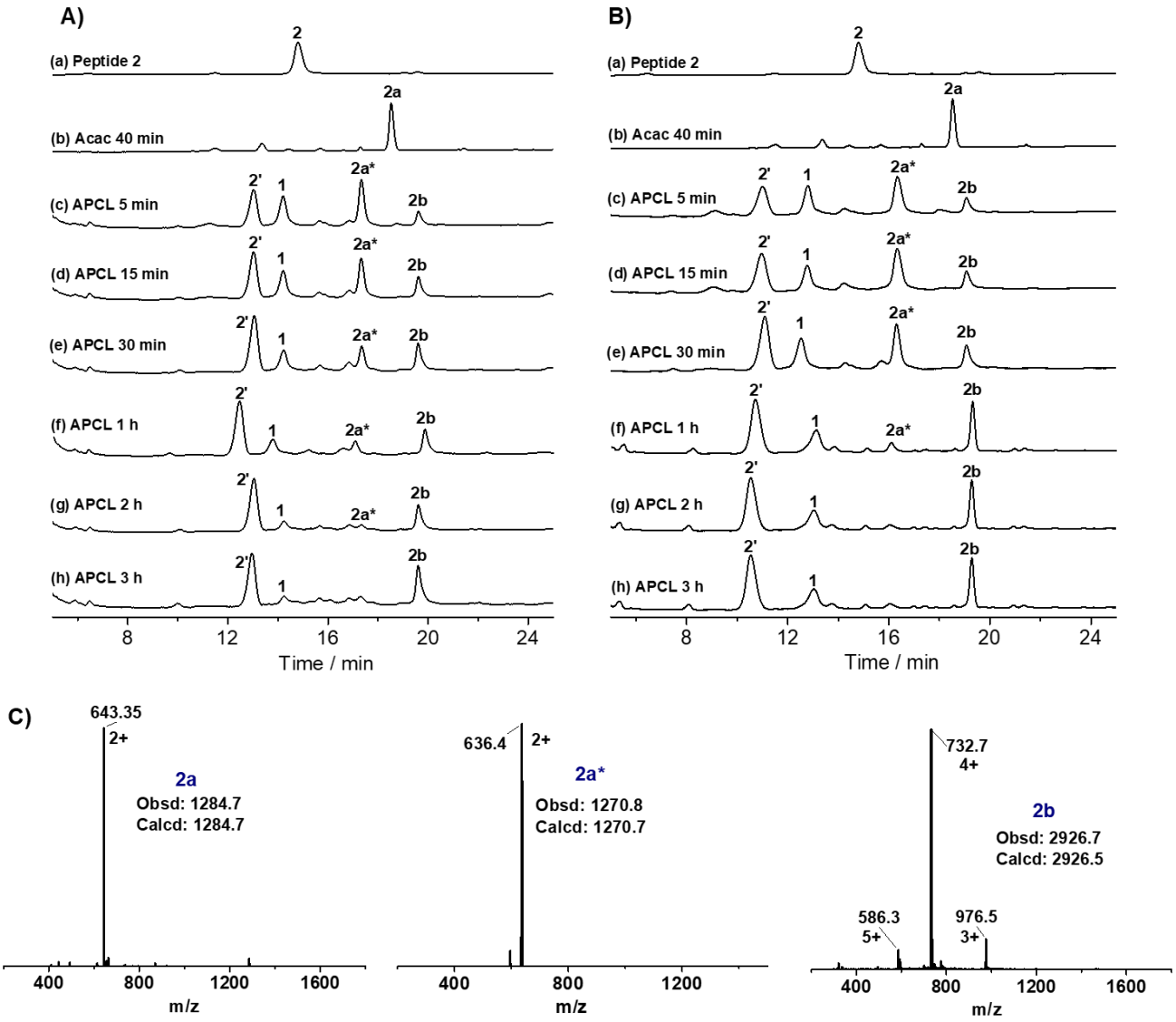


Figure S25. (A-B) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide hydrazide **2** using 3-methylpyrazole at pH 5.0 (A) and pH 6.5 (B) for 3 h, respectively. The peak **2'** is hydrolysis by-product. (C) ESI-MS analysis of peptide acyl-pyrazole **2a**, the 3-methylpyrazole substituted intermediate **2a*** and the ligation product **2b**, respectively. **2a**: observed mass 1284.7 Da, calculated mass 1284.7 Da; **2a***: observed mass 1270.8 Da, calculated mass 1270.7 Da; **2b**: observed mass 2926.7 Da, calculated mass 2926.5 Da.

(6) Additive was 3,5-dimethylpyrazole

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 60 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. After that, the Cys-peptide **1** (0.26 μmol, 0.7 equiv, 0.45 mg) was dissolved in 65 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 40 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 31.2 mg of 3,5-dimethylpyrazole (2.5 M, final conc.) was added. The mixed solution was divided into two portions and then adjusted to pH 5.0 and 6.5, respectively, with NaOH (2.0 M). Each reaction mixture was stirred at 37°C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min.

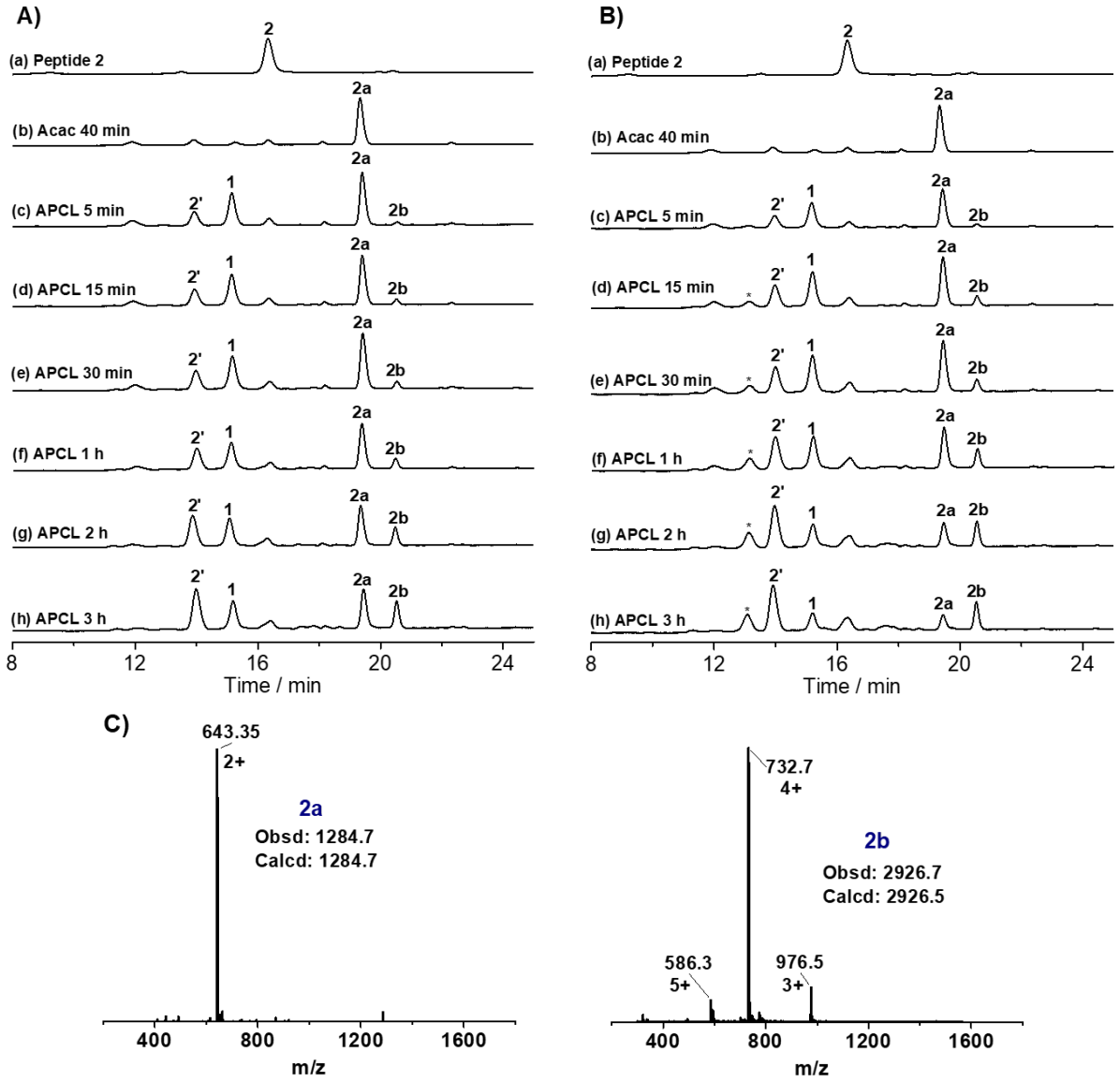


Figure S26. (A-B) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide hydrazide **2** using 3,5-dimethylpyrazole at pH 5.0 (A) and pH 6.5 (B) for 3 h, respectively. The peak **2'** is hydrolysis by-product. (C) ESI-MS analysis of peptide acyl-pyrazole **2a** and the ligation product **2b**, respectively. **2a**: observed mass 1284.7 Da, calculated mass 1284.7 Da; **2b**: observed mass 2926.7 Da, calculated mass 2926.5 Da.

(7) Ligations without reductant TCEP

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 60 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. After that, the Cys-peptide **1** (0.26 μmol, 0.7 equiv, 0.45 mg) was dissolved in 65 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0) and added into the resulting mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 3-methylpyrazole (final conc. in 2.5 M), pH 5.0, 37 °C, 3 h; (b) imidazole (final conc. in 2.5 M, prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 3 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min.

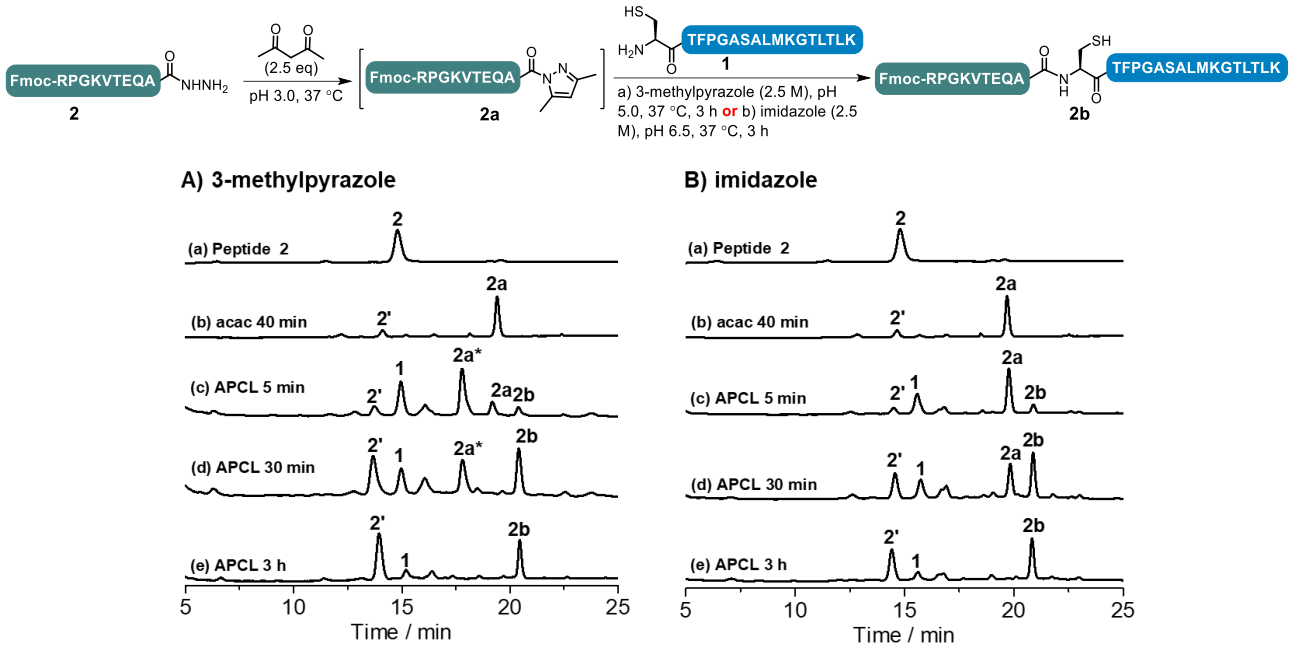
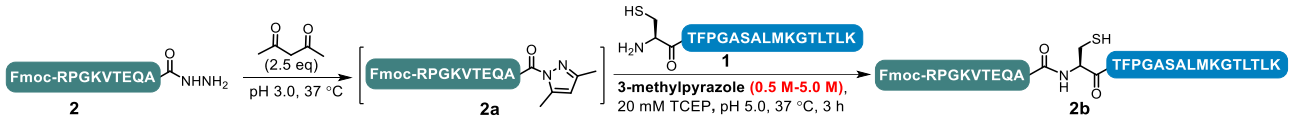


Figure S27. Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide hydrazide **2** using 3-methylpyrazole at pH 5.0 (A) and imidazole at pH 6.5 (B), but without TCEP in each condition. The peak **2'** is hydrolysis by-product, **2a** is peptide acyl-pyrazole **2a*** is the 3-methylpyrazole substituted intermediate and **2b** is the ligation product.

5.1.3 Kinetics of ligation reactions with azole reagents

Ligation time-courses were plotted for the reaction of Cys-peptide (**1**) with peptide hydrazide (**2**) in the presence of the above additives: MPAA (100 mM), imidazole (2.5 M), 1,2,4-triazole (2.5 M), pyrazole (2.5 M), 3-methylpyrazole (2.5 M), 3,5-dimethylpyrazole (2.5 M) and no additive. The reactions were analyzed by analytical HPLC (25 to 50% MeCN (with 0.1% TFA) in 20min) at 5 min, 15 min, 30 min, 1 h, 2 h and 3 h respectively. Conversion estimations were based upon the relative peak areas of the Cys-peptide starting material (**1**) and the desired ligation product (**2b**) at λ = 214 nm. Results have been summarized in **Figure 2**.

5.2 Optimization of 3-methylpyrazole ligation concentration



The peptide hydrazide **2** (1.85 μmol, 1 equiv, 2,25 mg) was dissolved in 250 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 4.7 μL of the 1 M acetylacetone stock solution (2.5 equiv, 4.63 μmol) was added into the mixture and incubated at 37 °C for 1 h. The Cys-peptide **1** (1.3 μmol, 0.7 equiv, 2.25 mg) was dissolved in 250 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into four portions, and 0.5 M, 1.0 M, 2.5 M and 5.0 M of 3-methylpyrazole (final concentration) were added, respectively. Each mixed solution was adjusted the pH to 5.0 and incubated at 37 °C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min. When the concentration of 3-methylpyrazole was increased from 0.5 M to 2.5 M, the ligation rate increased in a concentration dependent manner. However, when the concentration was continued to increase to 5.0 M, the conversion of ligation dropped to 25%. Consequently, the optimum concentration of 3-methylpyrazole was 2.5 M.

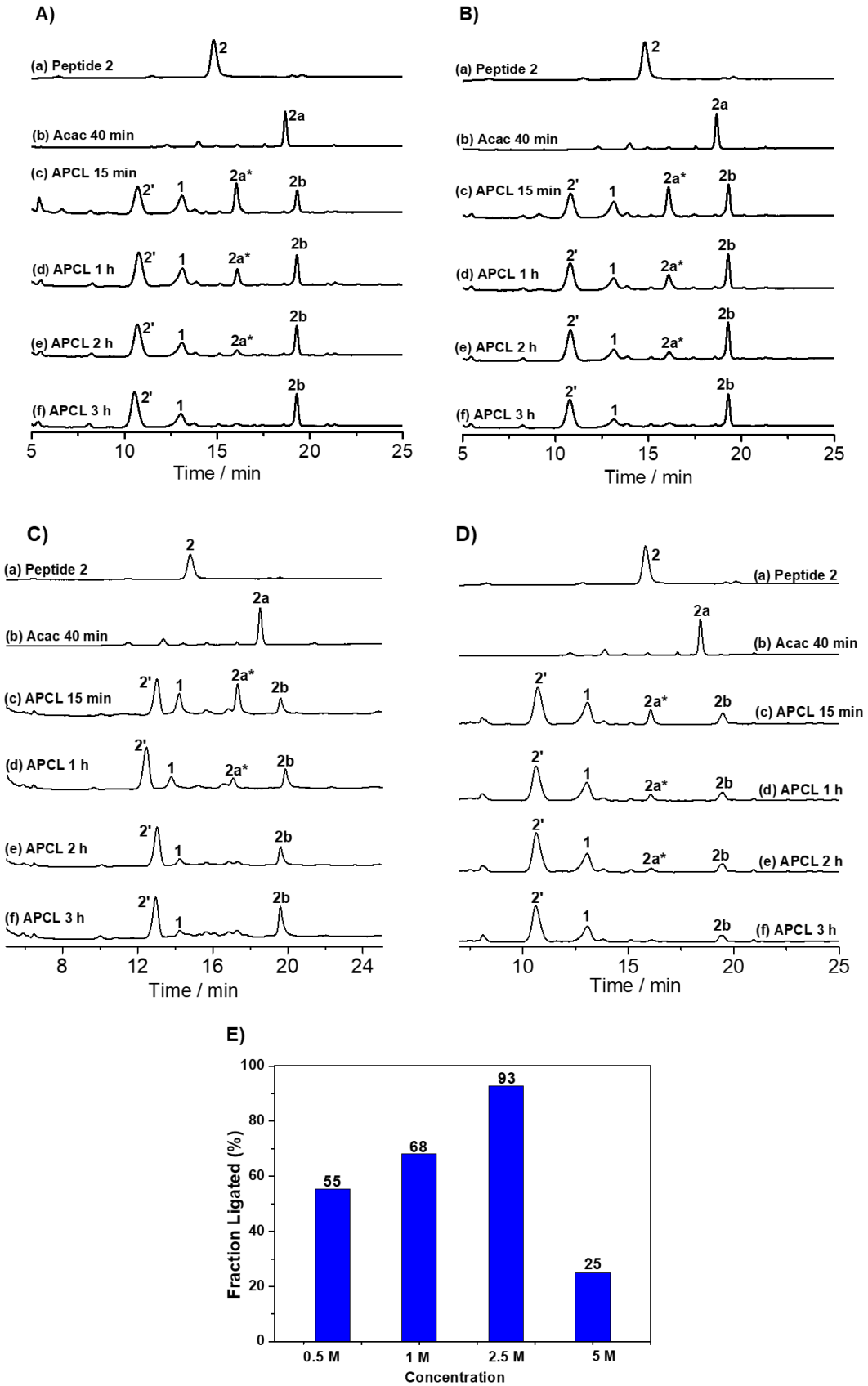
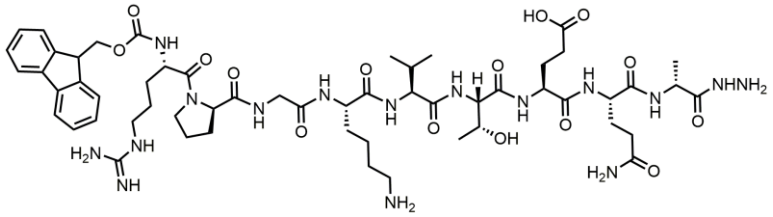


Figure S28. (A-D) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and peptide acylpyrazole **2a** in the presence of different concentration of 3-methylpyrazole: (A) 0.5 M; (B) 1.0 M; (C) 2.5 M; (D) 5.0 M. (E) Histogram statistics of ligation conversion for different concentrations of 3-methylpyrazole.

5.3 Investigation of possible racemization during the ligation

5.3.1 Preparation of the model peptide Ala$_D$-hydrazide ***2$_D$***

Peptide hydrazide (**2$_D$**) was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-*D*-Ala-OH with 0.4 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the *D*-peptide hydrazides (**2$_D$**) (50 mg resin; 15 mg, 61%). The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively.



Fmoc-RPGKVTEQA$_D$-NHNH$_2$ (2$_D$)

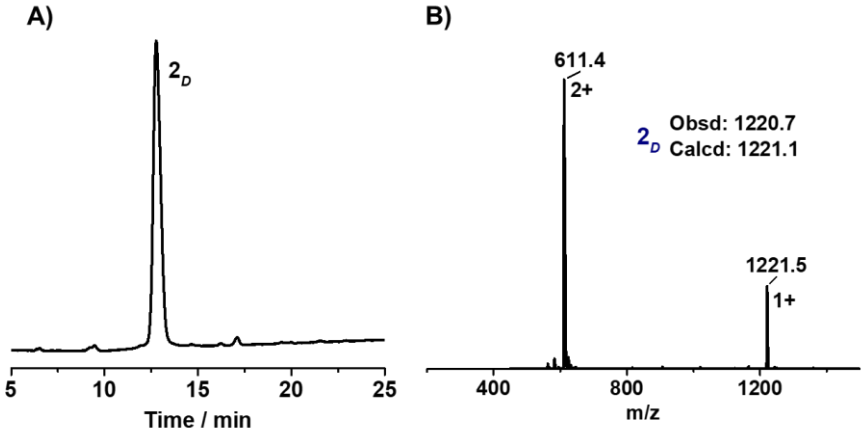
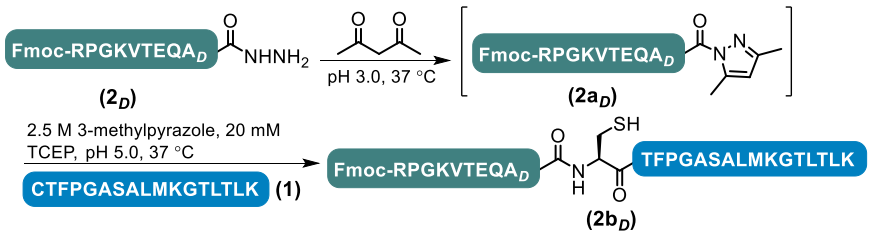


Figure S29. (A) Analytical HPLC trace (25 to 50% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) of *D*-peptide hydrazide **2$_D$**. (B) ESI-MS analysis of **2$_D$** shows the observed mass 1220.7 Da, calculated mass 1221.1 Da.

5.3.2 Preparation of the ligation product ***2b$_D$*** *of peptide Ala$_D$-hydrazides* ***2$_D$*** *and Cys-peptide* ***1***



The peptide hydrazide **2$_D$** (1.85 μmol, 1 equiv, 2,25 mg) was dissolved in 250 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 4.7 μL of the 1 M acetylacetone stock solution (2.5 equiv, 4.63 μmol) was added into the mixture and incubated at 37 °C for 1 h. The Cys-peptide **1** (1.3 μmol, 0.7 equiv, 2.25 mg) was dissolved in 250 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 133.2 μL of 3-methylpyrazole (2.5 M, final conc.) was added. The pH of mixed solution was adjusted to 5.0 with 1 M NaOH, and the resulting mixture was incubated at 37 °C for 3 h (confirmed by HPLC monitoring). The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min. After 3 hours incubation, the mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min to obtain 2.4 mg of *D*-configuration product (**2b$_D$**) (isolated yield 63.1%). The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively.

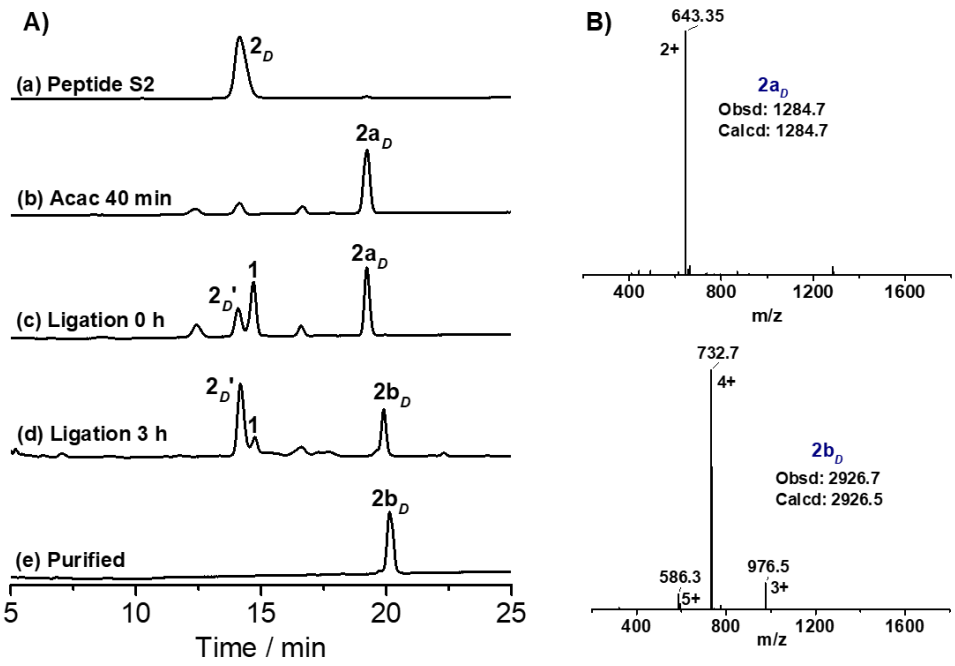


Figure S30. (A) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between Cys-peptide **1** and D-peptide hydrazide $\mathbf{2}_D$ using 3-methylpyrazole additive for 3 h. The peak $\mathbf{2}_D$' is hydrolysis by-product. (B) ESI-MS analysis of peptide acyl-pyrazole $\mathbf{2a}_D$ and the ligation product $\mathbf{2b}_D$.

*5.3.3 Co-injection experiment of peptide **2b** and **2b$_D$***

The APCL processes were performed as previously described to afford ligation product **2b** (*L*-configuration). The potential racemization of the ligation with 3-methylpyrazole or imidazole was tested by co-injection of the ligation product with the *D*-product $\mathbf{2b}_D$, and it indicated that no epimerization was observed in both cases.

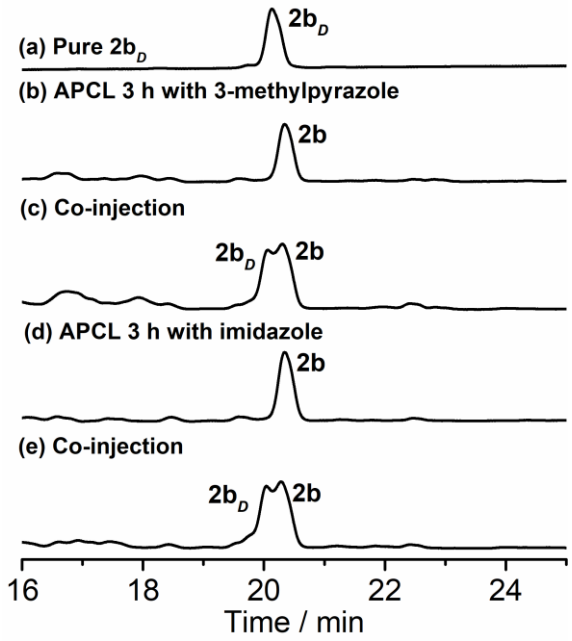


Figure S31. Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of the rate of epimerization. (a) The purified *D*-product $\mathbf{2b}_D$; (b) the 3 h ligation mixture of Cys-peptide **1** and peptide hydrazide **2** with 3-methylpyrazole; (c) co-injection of purified ligation *D*-product $\mathbf{2b}_D$ and the 3 h ligation mixtures of Cys-peptide **1** and peptide hydrazide **2** with 3-methylpyrazole; (d) the 3 h ligation mixture of Cys-peptide **1** and peptide hydrazide **2** with imidazole; (e) co-injection of purified ligation *D*-product $\mathbf{2b}_D$ and the 3 h ligation mixtures of Cys-peptide **1** and peptide hydrazide **2** with imidazole.

5.4 Mechanistic studies

Table S3. Ligation experiment between peptide **2a** and different N-terminal peptides.

Entry	C-terminal peptide	N-terminal peptide	Conversion (%)[c]
1	Fmoc-RPGKVTEQA **2a**	Acm: CTFPGASALMKGTLTLK **1a**	ND[a] ND[b]
2	Fmoc-RPGKVTEQA **2a**	CTFPGASALMKGTLTLK **1b**	ND[a] ND[b]

[a] Reaction conditions: **1a** or **1b** (2 mM), **2a** (2.85 mM), in buffer (6 M Gn·HCl, 200 mM Na_2HPO_4, 20 mM TCEP, 2.5 M 3-methylpyrazole, pH 5.0), 37 °C, 3 h.

[b] Reaction conditions: Same as in a, but with 2.5 M imidazole, pH 6.5, 37 °C, 3 h. [c] Product not detected on HPLC-MS.

*5.4.1 Ligation between **2a** and **1a***

(1) Additive was 3-methylpyrazole

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 50 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. The peptide **1a** (0.26 μmol, 0.7 equiv, 0.5 mg) was dissolved in 50 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 26.7 μL of 3-methylpyrazole (2.5 M, final conc.) was added. The pH of the mixture was adjusted to 5.0 with 1 M NaOH, and the resulting mixture was stirred at 37°C for 3 h. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min. The mass of the peptide was confirmed using ESI-MS. No ligation product was observed.

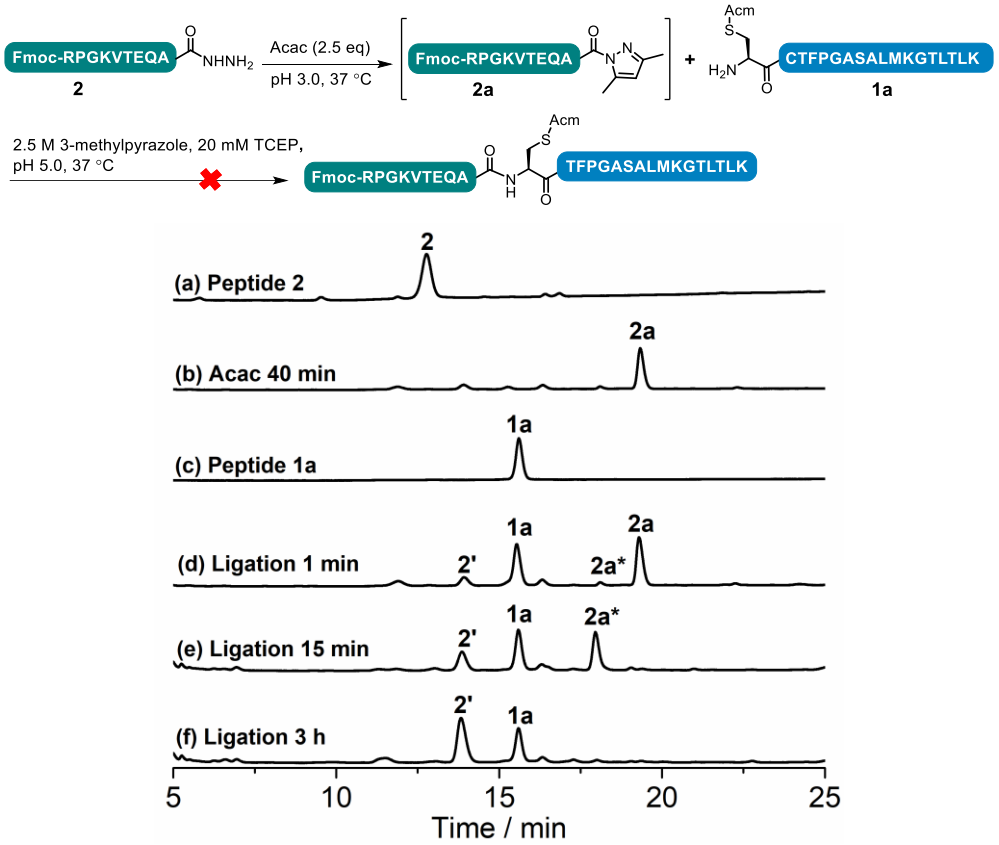


Figure S32. Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between peptide **1a** and peptide acylpyrazole **2a** using 3-methylpyrazole for 3 h. The peak **2'** denotes hydrolysis by-product and **2a*** denotes 3-methylpyrazole substituted intermediate.

(2) Additive was imidazole

The peptide hydrazide **2** (0.37 µmol, 1 equiv, 0.45 mg) was dissolved in 43 µL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 µL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 µmol) was added into the mixture and incubated at 37 °C for 40 min. After that, the peptide **1a** (0.26 µmol, 0.7 equiv, 0.5 mg) was dissolved in 65 µL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 40 mM TCEP, pH 3.0) and was added into the resulting mixture. Subsequently, 22.1 µL of imidazole stock solution (final conc. in 2.5 M, prepared as 1 mg/µL stock solution) was added. The pH of the mixture was adjusted to 6.5 with 2 M NaOH, and the resulting mixture was stirred at 37°C for 3 h. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min. The mass of the peptide was confirmed using ESI-MS. No ligation product was observed.

Note: In order to avoid the severe hydrolysis of the peptide acyl-pyrazole caused by the direct addition of solid imidazole. The imidazole was pre-dissolved in 12 M HCl to prepare a 1 mg/µL stock solution and then diluted in the appropriate volume of buffer A before being added to the ligation mixture.

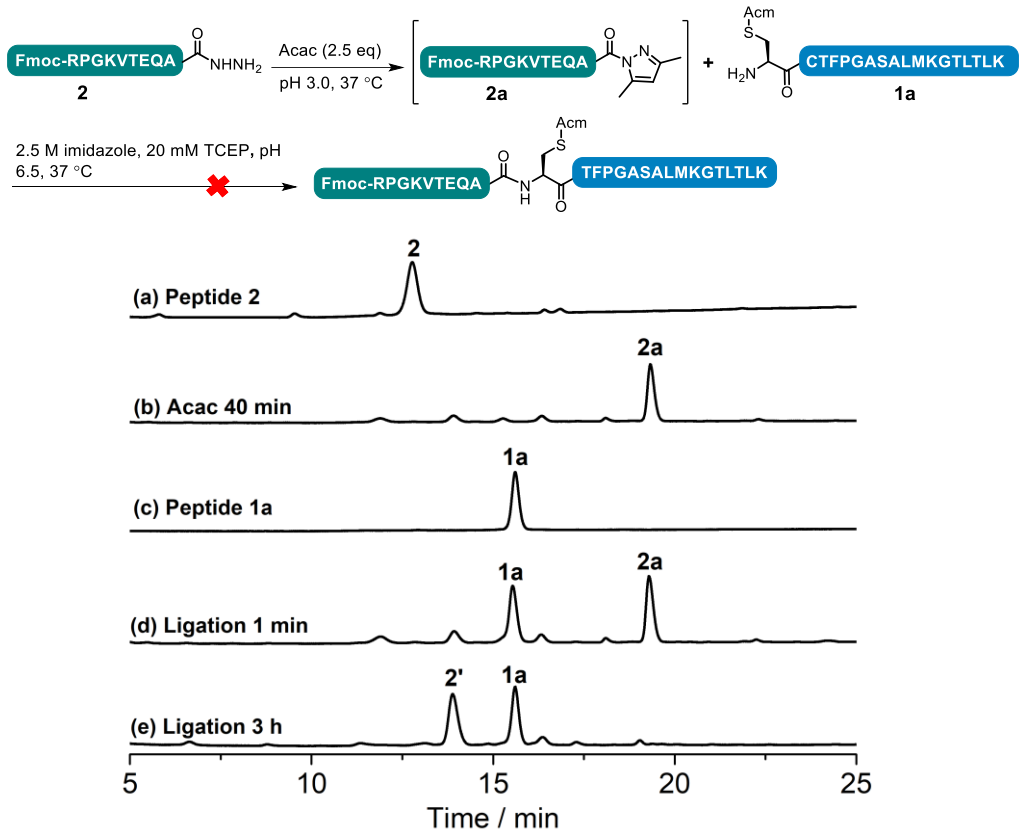


Figure S33. Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between peptide **1a** and peptide acylpyrazole **2a** using imidazole for 3 h. The peak **2'** denotes hydrolysis by-product.

*5.4.2 Ligation between **2a** and **1b***

(1) Additive was 3-methylpyrazole

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 50 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. The peptide **1b** (0.26 μmol, 0.7 equiv, 0.47 mg, 2 mM) was dissolved in 50 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 26.7 μL of 3-methylpyrazole (2.5 M, final conc.) was added. The pH of the mixture was adjusted to 5.0 with 1 M NaOH, and the resulting mixture was stirred at 37°C for 3 h. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min. The mass of the peptide was confirmed using ESI-MS. No ligation product was observed.

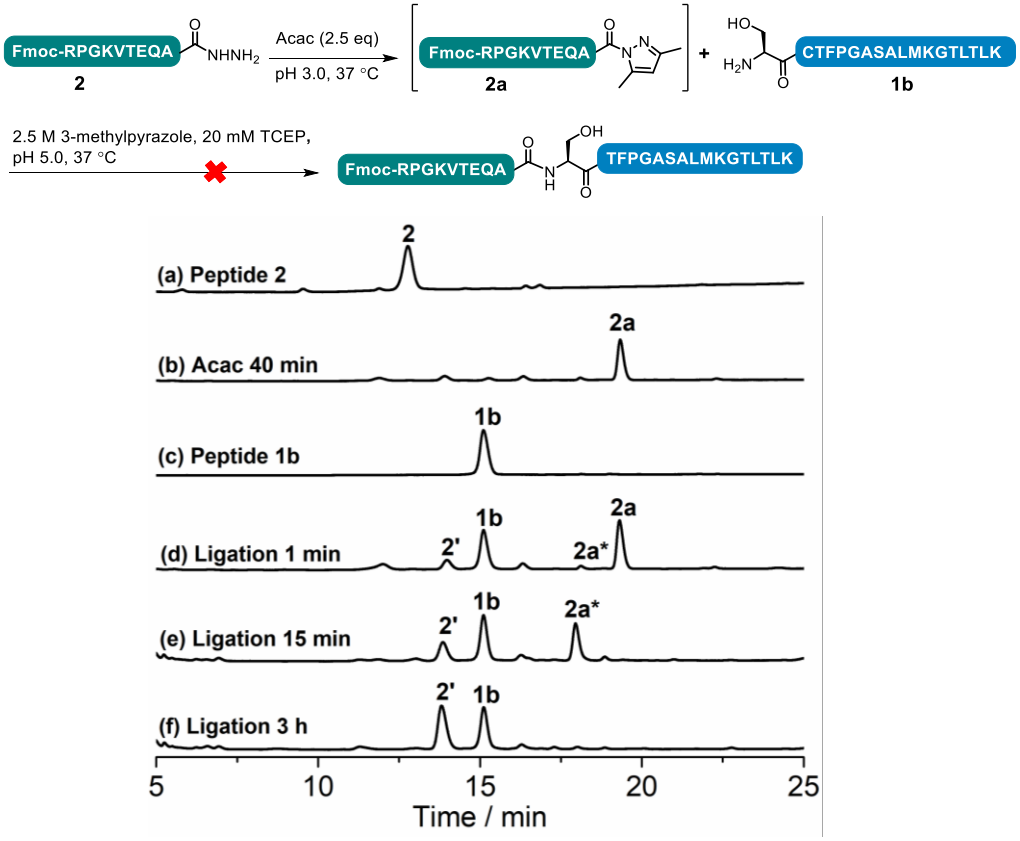


Figure S34. Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between peptide **1b** and peptide acylpyrazole **2a** using 3-methylpyrazole for 3 h. The peak **2'** denotes hydrolysis by-product and **2a*** denotes 3-methylpyrazole substituted intermediate.

(2) Additive was imidazole

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 43 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (2.5 equiv, 0.92 μmol) was added into the mixture and incubated at 37 °C for 40 min. Then, the peptide **1b** (0.26 μmol, 0.7 equiv, 0.47 mg, 2 mM) was dissolved in 65 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 40 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 22.1 μL of imidazole stock solution (final conc. in 2.5 M, prepared as 1 mg/μL stock solution) was added. The pH of the mixture was adjusted to 6.5 with 2 M NaOH, and the resulting mixture was stirred at 37°C for 3 h. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min. The mass of the peptide was confirmed using ESI-MS. No ligation product was observed.

Note: The imidazole was pre-dissolved in 12 M HCl to prepare a 1 mg/μL stock solution and then diluted in the appropriate volume of buffer A before being added to the ligation mixture.

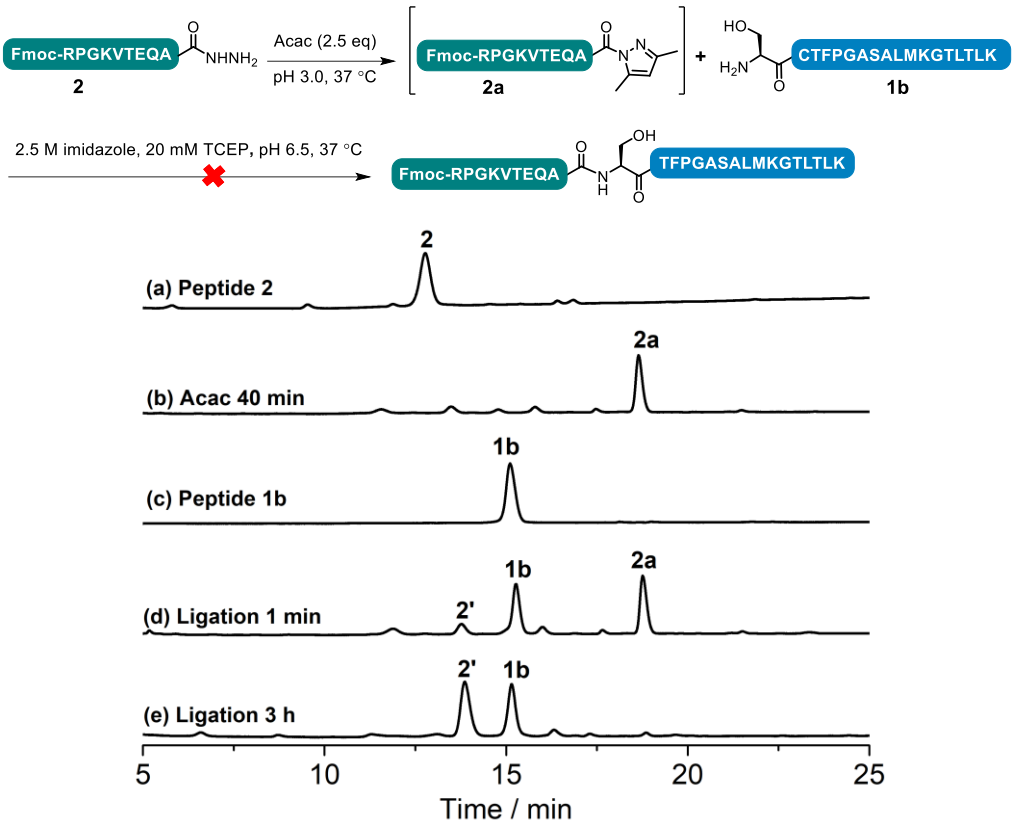


Figure S35. Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of ligation between peptide **1b** and peptide acylpyrazole **2a** using imidazole for 3 h. The peak **2'** denotes hydrolysis by-product.

6 3-methylpyrazole or imidazole to promote ligation of different model hydrazides with Cys-peptide

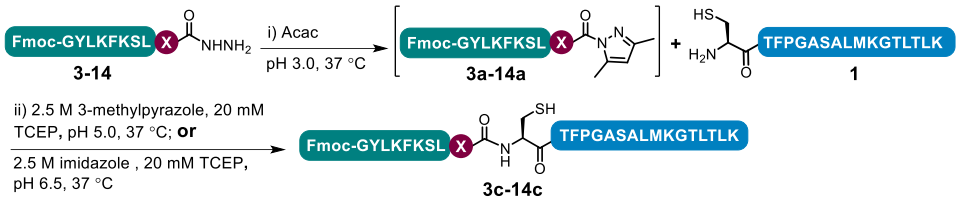


Table S4. Ligation yields of different peptide hydrazides.[a]

Entry	Ligation site (X)	Ligation time (h)	HPLC yield (%)		Isolated yield (%)[d]	
			3-methylpyrazole	Imidazole	3-methylpyrazole	Imidazole
1	Ala (**3**)	3	92	54	48	32
2	Leu (**4**)	3	82	80	47	43
3	Gly (**5**)	3	93	84	57	52
4	Trp (**6**)	4[b] (6)[c]	82	30	69	15
5	Cys (**7**)	3[b] (6) [c]	75	19	48	11
6	Phe (**8**)	3	86	37	61	22
7	Lys (**9**)	2	93	80	67	53
8	Pro (**10**)	10[b] (15)[c]	3	37	1.0	14
9	Val (**11**)	6	29	24	26	13
10	Glu (**12**)	2	10	5	5	2
11	Gln (**13**)	2	76	87	41	54
12	His (**14**)	0.5	56	72	30	47
13	(**3***)[e]	2	74	56	65	37

[a]Conditions: i) 6 M Gdn•HCl, 0.2 M Na_2HPO_4, 1.5–2.5 equiv of acac, 37 °C, pH 3.0; ii) 6 M Gdn•HCl, 0.2 M Na_2HPO_4, 20 mM TCEP, 2.5 M azoles, pH 5.0 or 6.5, 37 °C. [b]3-methylpyrazole additive, pH 5.0. [c]Imidazole additive, pH 6.5. [d]The weighed isolated yield of the ligation product. [e]Sequence of the model peptide hydrazide **3***: Fmoc-GCGYLKFKSLA-$NHNH_2$.

(1) **X** = Ala

The peptide hydrazide **3** (4.76 μmol, 1 equiv, 6.0 mg) was dissolved in 0.66 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.22 μL of acetylacetone (2.5 equiv, 11.88 μmol) was added into the mixture and incubated at 37 °C for 1.5 h. The Cys-peptide **1** (3.33 μmol, 0.7 equiv, 5.79 mg, 2 mM) was dissolved in 0.66 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 3-methylpyrazole (final conc. in 2.5 M), pH 5.0, 37 °C, 3 h; (b) imidazole (final conc. in 2.5 M, prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 3 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 60% MeCN (with 0.1% TFA) in 25 min. After 3 h incubation, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 2.38 mg of ligation product (**3c**) (3-methylpyrazole, yield 48%) and 1.55 mg of ligation product (**3c**) (imidazole, yield 32%), respectively. The product **3c** was analysed by ESI-MS with the observed mass 2967.6 Da, calculated mass 2967.6 Da.

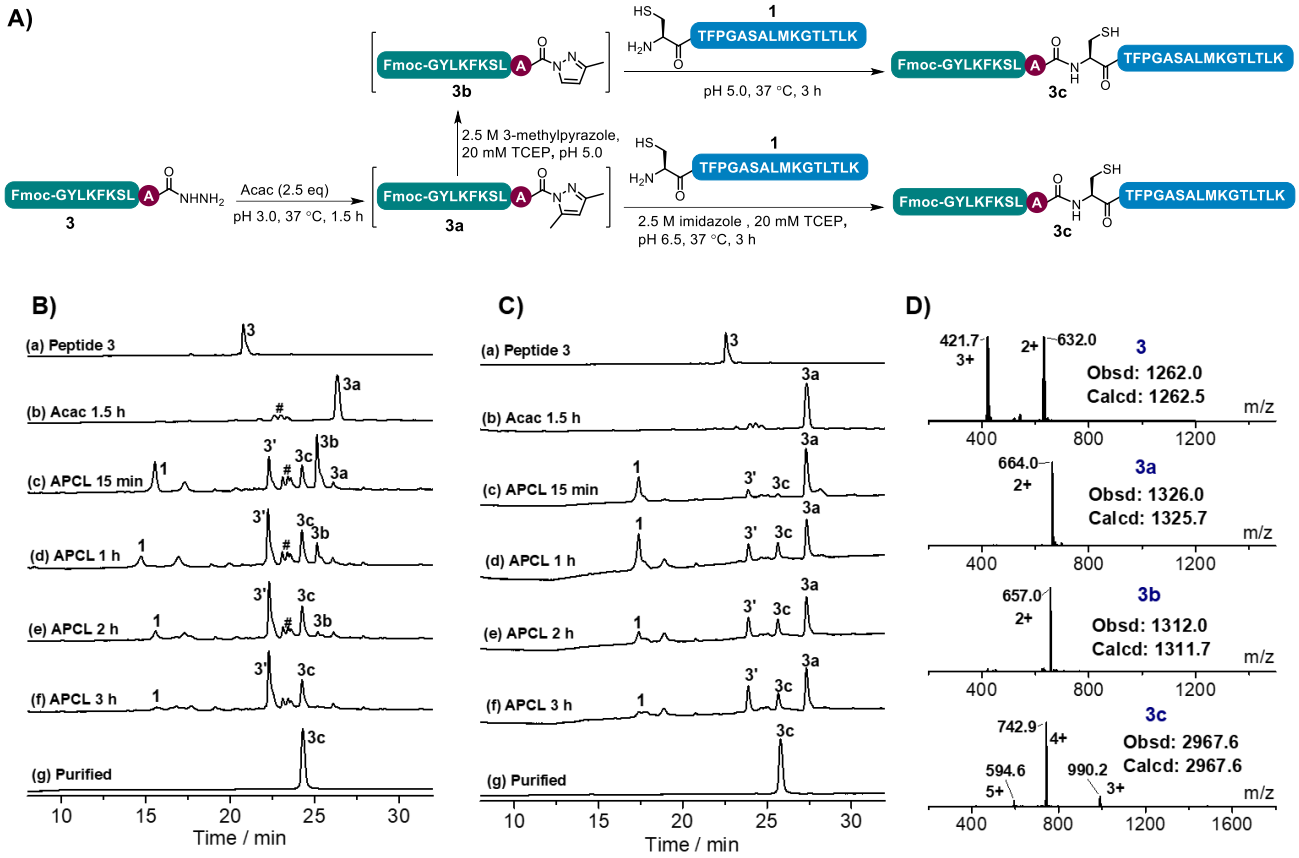


Figure S36. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **3** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 60% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C) for 3 h. Note: **3a** is acylpyrazole, **3b** is 3-methylpyrazole substituted intermediate, **3c** is ligation product and **3'** is hydrolysis by-product. (D) ESI-MS traces of peptide **3**, **3a**, **3b** and the ligation product **3c**, respectively. **3**: observed mass 1262.0 Da, calculated mass 1262.5 Da; **3a**: observed mass 1326.0 Da, calculated mass 1325.7 Da; **3b**: observed mass 1312.0 Da, calculated mass 1311.7 Da; **3c**: observed mass 2967.6 Da, calculated mass 2967.6 Da.

(2) **X** = Leu

The peptide hydrazide **4** (4.6 μmol, 1 equiv, 6.0 mg) was dissolved in 0.64 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.18 μL of acetylacetone (11.5 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 105 min. The Cys-peptide **1** (3.22 μmol, 0.7 equiv, 5.6 mg, 2 mM) was dissolved in 0.64 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 3 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 3 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 60% MeCN (with 0.1% TFA) in 25 min. After 3 h incubation, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 2.4 mg of ligation product (**4c**) (3-methylpyrazole, yield 47%) and 2.1 mg of ligation product (**4c**) (imidazole, yield 43%), respectively. The product **4c** was analysed by ESI-MS with the observed mass 3010.3 Da, calculated mass 3010.6 Da.

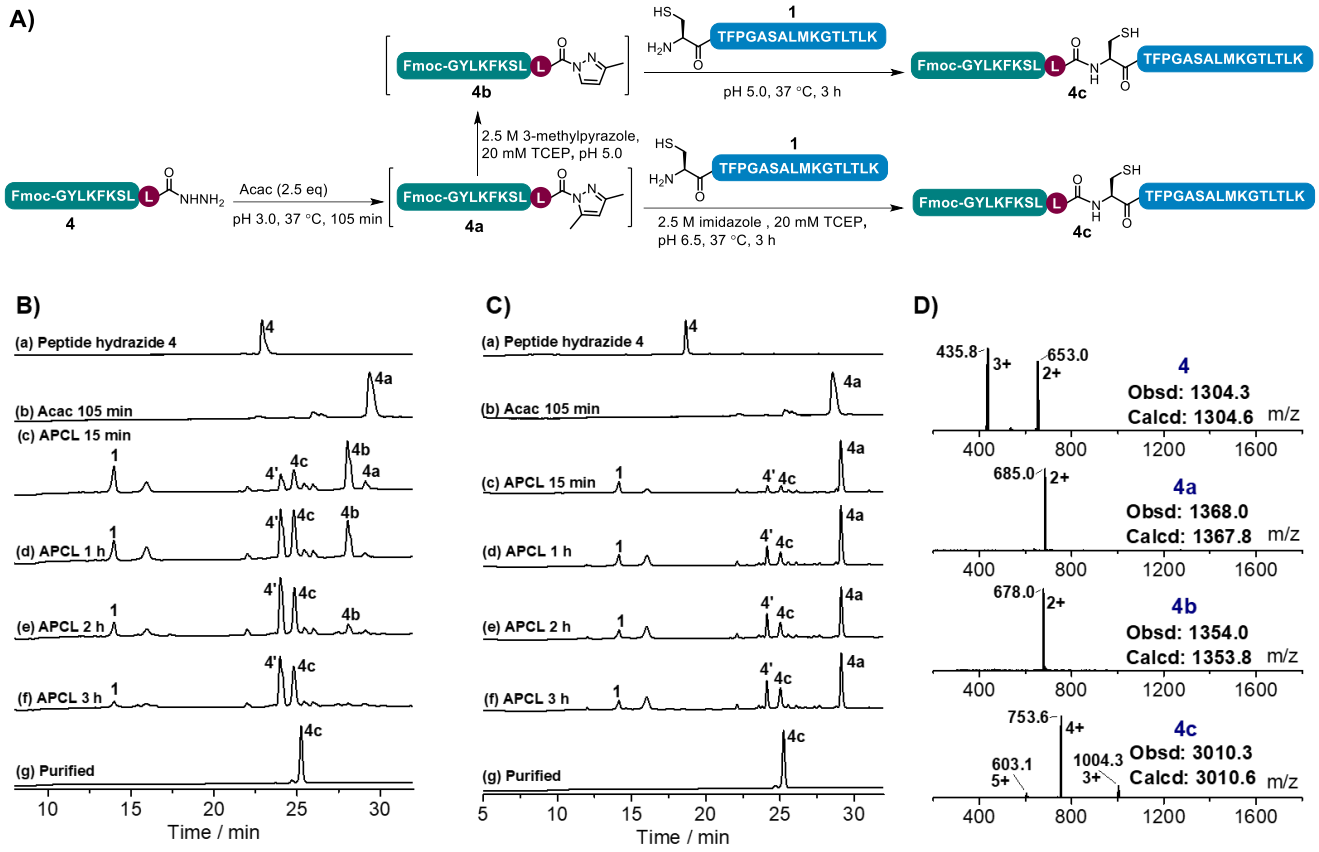


Figure S37. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **4** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 60% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C) for 3 h. Note: **4a** is peptide acylpyrazole, **4b** is 3-methylpyrazole substituted intermediate, **4c** is ligation product and **4'** is hydrolysis by-product. (D) ESI-MS traces of **4**, **4a**, **4b** and the ligation product **4c**, respectively. **4**: observed mass 1304.3 Da, calculated mass 1304.6 Da; **4a**: observed mass 1368.0 Da, calculated mass 1367.8 Da, **4b**: observed mass 1354.0 Da, calculated mass 1353.8 Da; **4c**: observed mass 3010.3 Da, calculated mass 3010.6 Da.

(3) **X** = Gly

The peptide hydrazide **5** (4.8 μmol, 1 equiv, 6.0 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.23 μL of acetylacetone (12.0 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 105 min. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 3 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 3 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 56% MeCN (with 0.1% TFA) in 25 min. After 3 h incubation, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 2.82 mg of ligation product (**5c**) (3-methylpyrazole, yield 57%) and 2.58 mg of ligation product (**5c**) (imidazole, yield 52%), respectively. The product **5c** was analysed by ESI-MS with the observed mass 2953.8 Da, calculated mass 2953.6 Da.

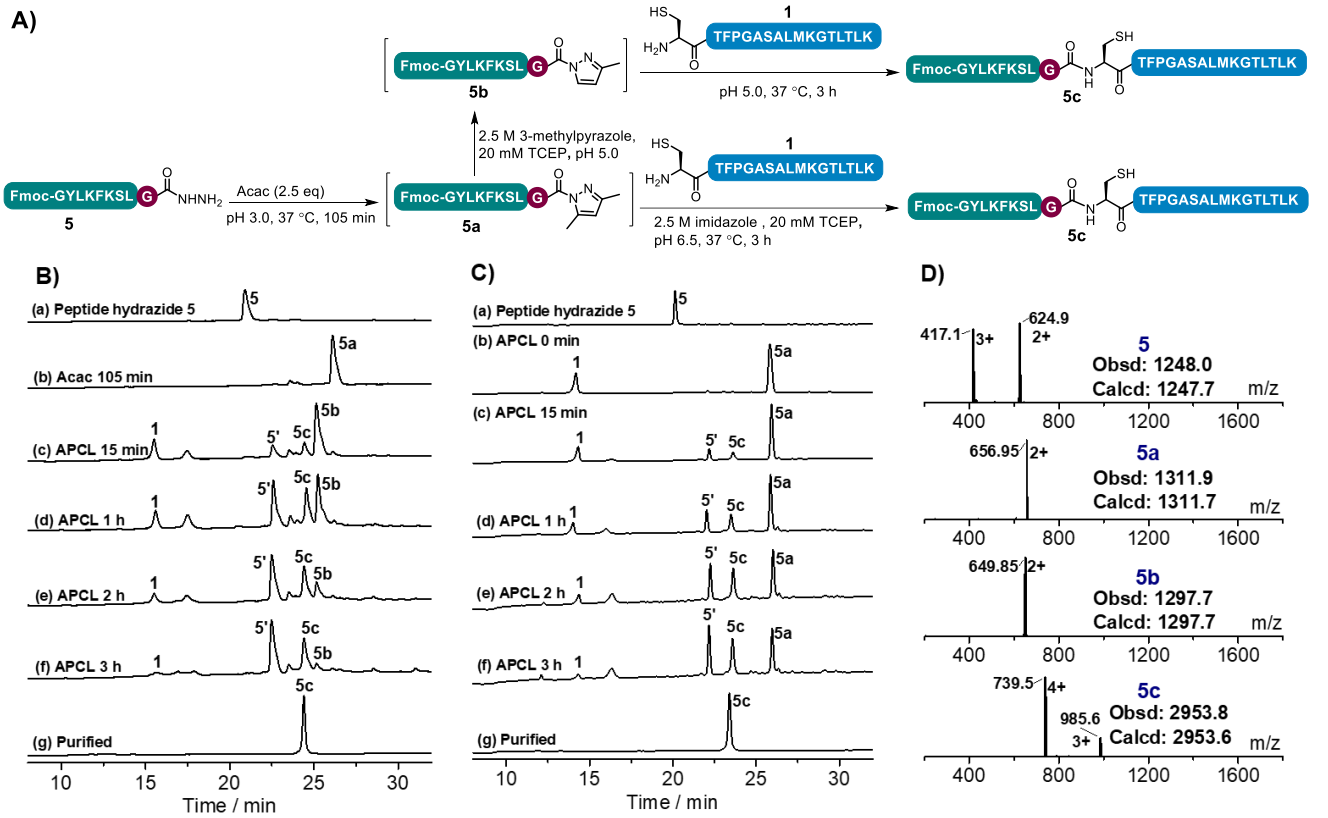


Figure S38. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **5** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 56% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C) for 3 h. Note: **5a** is peptide acylpyrazole, **5b** is 3-methylpyrazole substituted intermediate, **5c** is ligation product and **5'** is hydrolysis by-product. (D) ESI-MS traces of **5**, **5a**, **5b** and the ligation product **5c**, respectively. **5**: observed mass 1248.0 Da, calculated mass 1247.7 Da; **5a**: observed mass 1311.9 Da, calculated mass 1311.7 Da; **5b**: observed mass 1297.7 Da, calculated mass 1297.7 Da; **5c**: observed mass 2953.8 Da, calculated mass 2953.6 Da.

(4) **X** = Trp

The peptide hydrazide **6** (4.8 μmol, 1 equiv, 6.6 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.23 μL of acetylacetone (12.0 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 140 min. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 4 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 6 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 30 % in 3 min, then to 50% MeCN (with 0.1% TFA) in 35 min. Then, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 3.6 mg of ligation product (**6c**) (3-methylpyrazole, yield 69%) and 0.78 mg of ligation product (**6c**) (imidazole, yield 15%), respectively. The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **6c** with the observed mass 3084.0 Da, calculated mass 3083.8 Da.

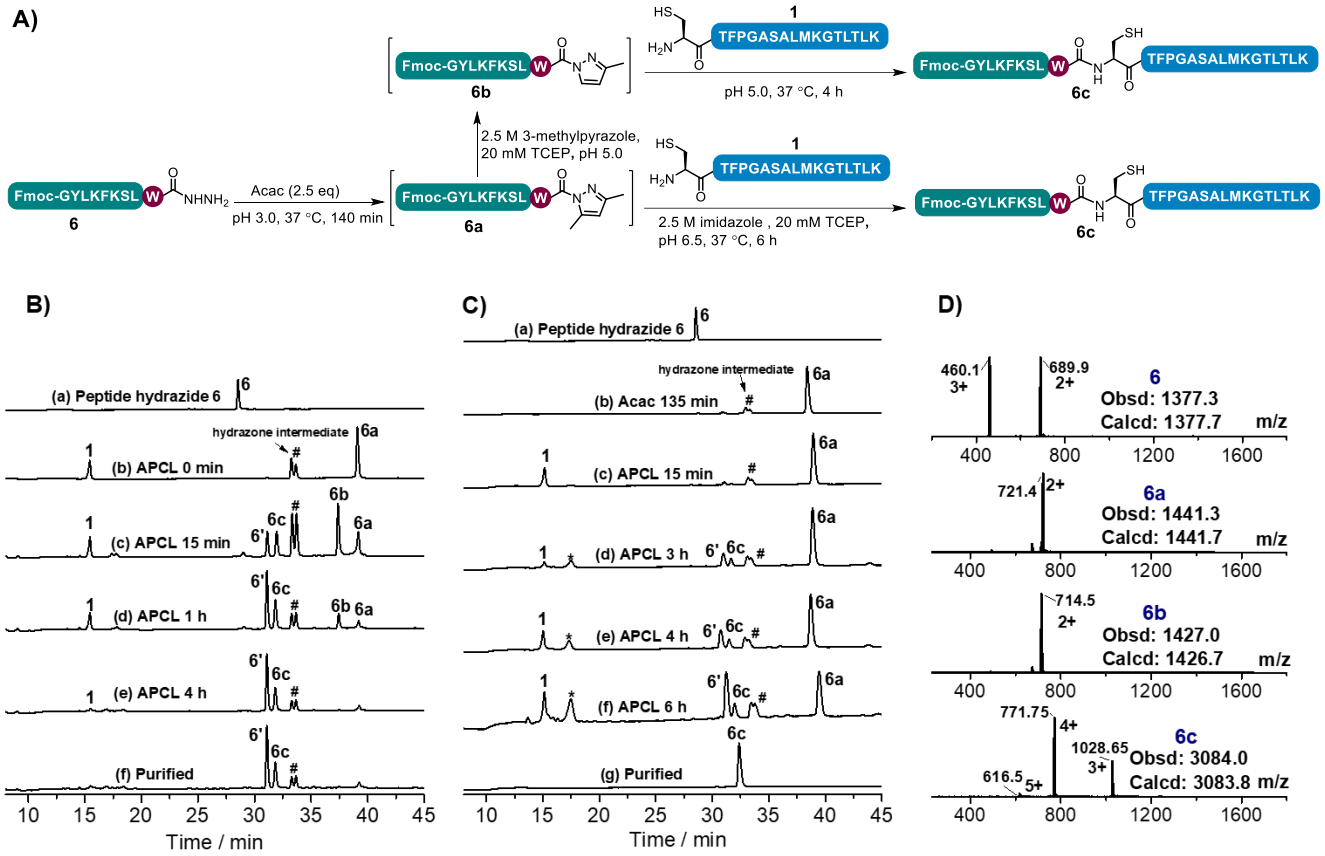


Figure S39. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **6** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 30% in 3 min, then to 50% MeCN in 35 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **6a** is peptide acylpyrazole, **6b** is 3-methylpyrazole substituted intermediate, **6c** is ligation product and **6'** is hydrolysis by-product. The peak * of (C) corresponds to +82 Da adduct to the remaining peptide **1**. (D) ESI-MS traces of **6**, **6a**, **6b** and the ligation product **6c**, respectively. **6**: observed mass 1377.3 Da, calculated mass 1377.7 Da; **6a**: observed mass 1441.3 Da, calculated mass 1441.7 Da; **6b**: observed mass 1427.0 Da, calculated mass 1426.7 Da; **6c**: observed mass 3084.0 Da, calculated mass 3083.8 Da.

(5) **X** = Cys

The peptide hydrazide **7** (4.8 μmol, 1 equiv, 6.2 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.23 μL of acetylacetone (12.0 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 105 min. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 3 h; (b) 2.5 M imidazole (prepared as a 1 mg/μL stock solution), pH 6.5, 37 °C, 6 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 30 % in 3 min, then to 52% MeCN (with 0.1% TFA) in 35 min. Then, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 2.4 mg of ligation product (**7c**) (3-methylpyrazole, yield 48%) and 0.55 mg of ligation product (**7c**) (imidazole, yield 11%), respectively. The product **7c** was analysed by ESI-MS with the observed mass 2999.5 Da, calculated mass 2999.6 Da.

Note: Peak * may be formed by the reaction between excess acetylacetone and the N-terminal Cys of peptide **1**, leading to the formation of a thiazolidine by-product (+82 Da of **1**). This side reaction is usually slow, so we chose to add 2.5 equivalents of acetylacetone in order to promote the formation of the N-acylpyrazole peptide at a faster rate. To avoid this * byproduct, we suggest that the addition of acetylacetone can be reduced to 1 to 1.1 equivalents, since the use of stoichiometric acetylacetone converts the peptidyl hydrazides to the acylpyrazoles.

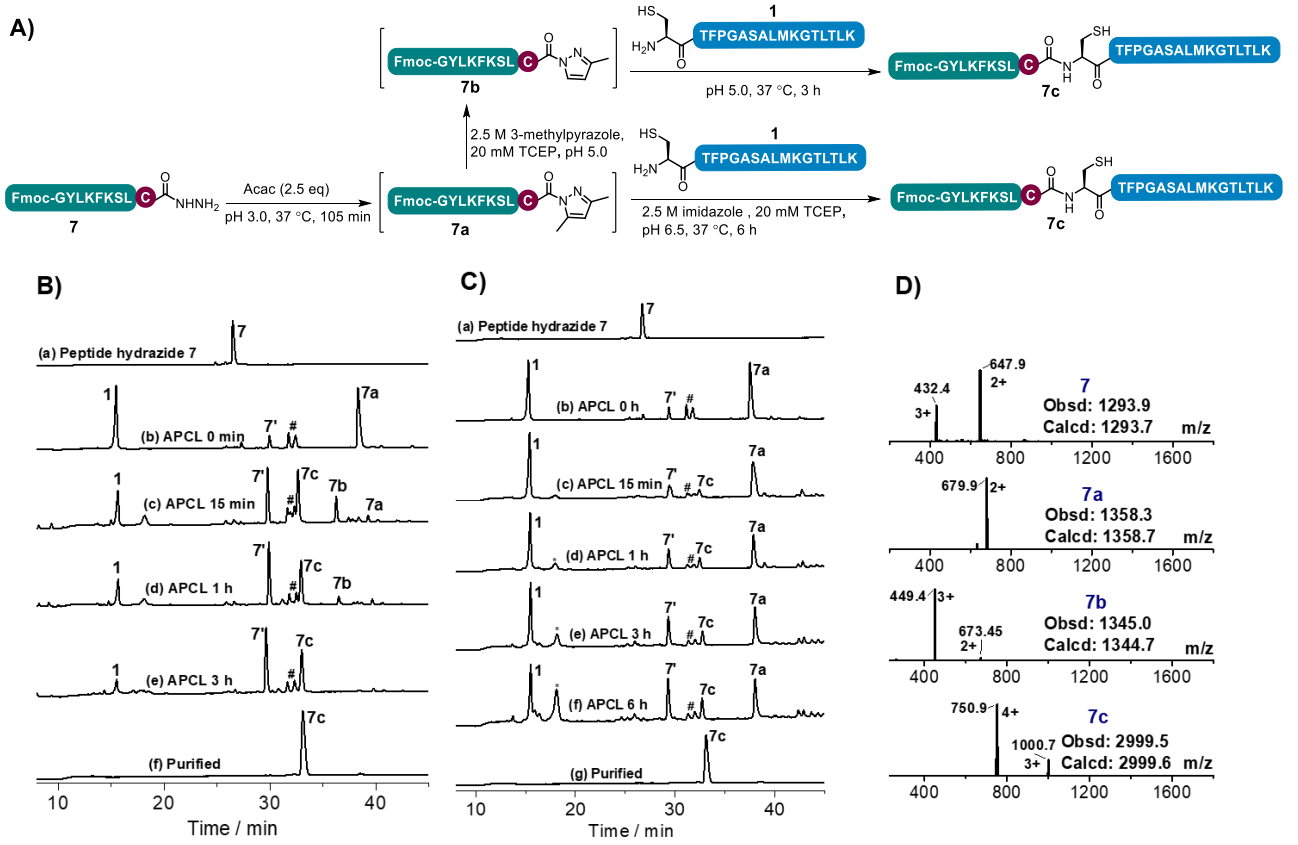


Figure S40. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **7** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 30% in 3 min, then to 52% MeCN in 35 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **7a** is peptide acylpyrazole, **7b** is 3-methylpyrazole substituted intermediate, **7c** is ligation product and **7'** is hydrolysis by-product. The peak # denotes hydrazone intermediate and peak * of (C) corresponds to a +82 Da adduct to the remaining peptide **1**. (D) ESI-MS traces of **7**, **7a**, **7b** and the ligation product **7c**, respectively. **7**: observed mass 1293.9 Da, calculated mass 1293.7 Da; **7a**: observed mass 1358.3 Da, calculated mass 1358.7 Da; **7b**: observed mass 1345.0 Da, calculated mass 1344.7 Da; **7c**: observed mass 2999.5 Da, calculated mass 2999.6 Da.

(6) **X** = Phe

The peptide hydrazide **8** (4.8 μmol, 1 equiv, 6.7 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.23 μL of acetylacetone (12.0 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37°C for 150 min. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 3 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 3 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 56% MeCN (with 0.1% TFA) in 25 min. Then, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 3.13 mg of ligation product (**8c**) (3-methylpyrazole, yield 61%) and 1.13 mg of ligation product (**8c**) (imidazole, yield 22%), respectively. The product **8c** was analysed by ESI-MS with the observed mass 3043.9 Da, calculated mass 3043.6 Da.

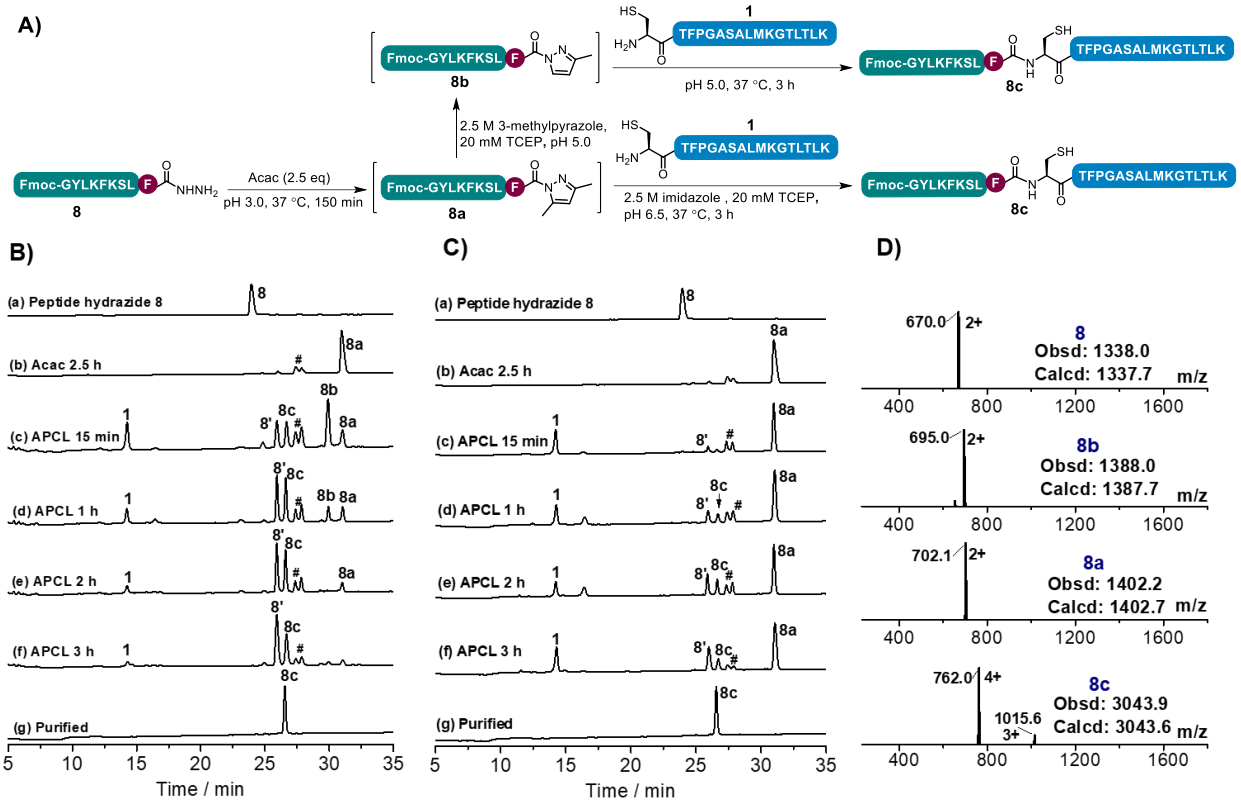


Figure S41. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **8** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 56% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **8a** is peptide acylpyrazole, **8b** is 3-methylpyrazole substituted intermediate and **8c** is ligation product. **8'** is hydrolysis by-product and peak # denotes hydrazone intermediate. (D) ESI-MS traces of **8**, **8a**, **8b** and the ligation product **8c**, respectively. **8**: observed mass 1338.0 Da, calculated mass 1337.7 Da; **8a**: observed mass 1402.2 Da, calculated mass 1402.7 Da; **8b**: observed mass 1388.0 Da, calculated mass 1387.7 Da; **8c**: observed mass 3043.9 Da, calculated mass 3043.6 Da.

(7) **X** = Lys

The peptide hydrazide **9** (4.8 μmol, 1 equiv, 6.4 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.23 μL of acetylacetone (12.0 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 15 min. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 2 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 2 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 50% MeCN (with 0.1% TFA) in 25 min. Then, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 3.4 mg of ligation product (**9c**) (3-methylpyrazole, yield 67%) and 2.7 mg of ligation product (**9c**) (imidazole, yield 53%), respectively. The product **9c** was analysed by ESI-MS with the observed mass 3024.8 Da, calculated mass 3024.6 Da.

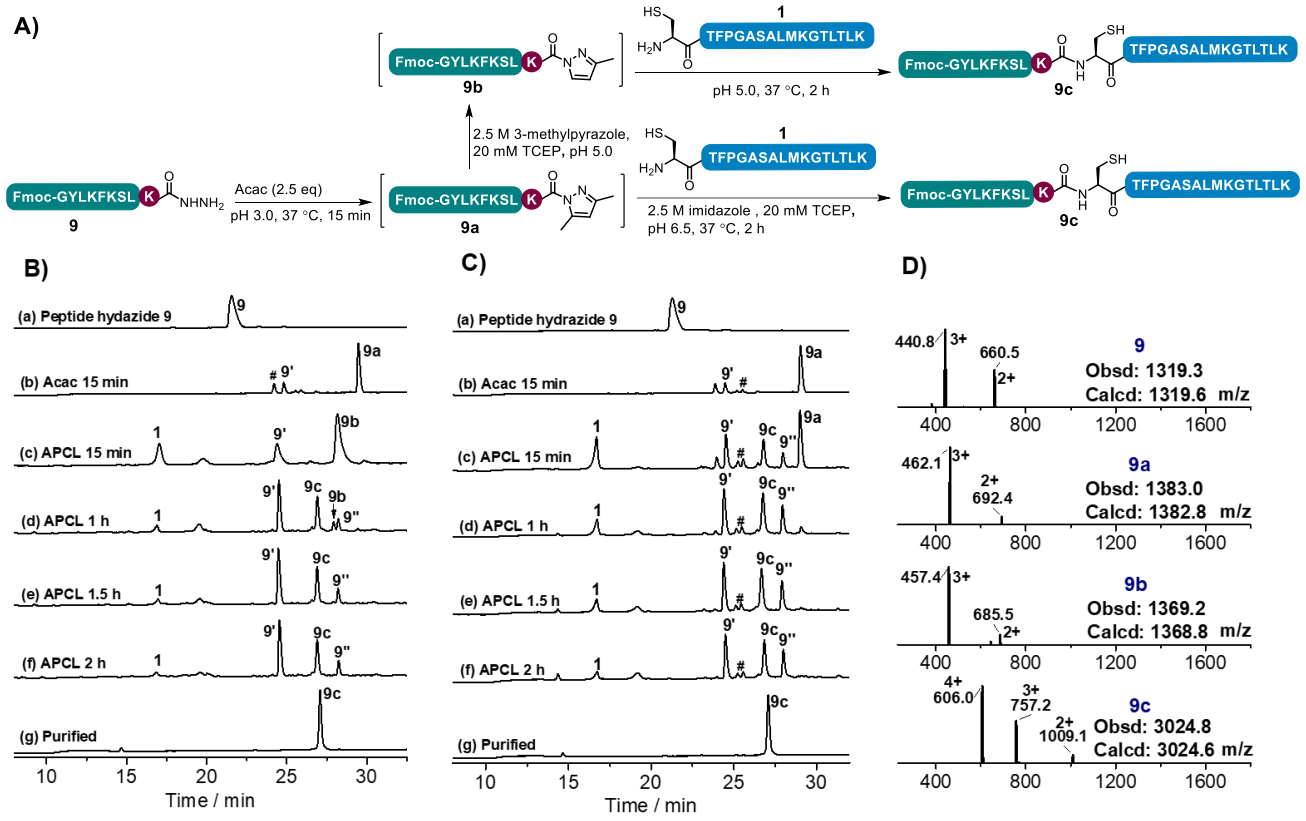


Figure S42. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **9** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **9a** is peptide acylpyrazole, **9b** is 3-methylpyrazole substituted intermediate, **9c** is ligation product and **9'** is hydrolysis by-product. The peak # denotes hydrazone intermediate and **9''** denotes C-terminal side-chain lactamization of Lys. (D) ESI-MS traces of **9**, **9a**, **9b** and the ligation product **9c**, respectively. **9**: observed mass 1319.3 Da, calculated mass 1319.6 Da; **9a**: observed mass 1383.0 Da, calculated mass 1382.8 Da; **9b**: observed mass 1369.2 Da, calculated mass 1368.8 Da; **9c**: observed mass 3024.8 Da, calculated mass 3024.6 Da.

(8) **X** = Pro

The peptide hydrazide **10** (4.8 μmol, 1 equiv, 6.2 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.23 μL of acetylacetone (12.0 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 3 h. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 10 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 15 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 30 % in 3 min, then to 51% MeCN (with 0.1% TFA) in 30 min. It was found that difficult to generate the ligation product (yield~1.0 %) of Pro acyl-pyrazole with Cys-peptide **1** using 3-methylpyrazole. The mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 0.7 mg of ligation product (**10c**) (imidazole, yield 14%). The product **10c** was analysed by ESI-MS with the observed mass 2994.3 Da, calculated mass 2994.7 Da.

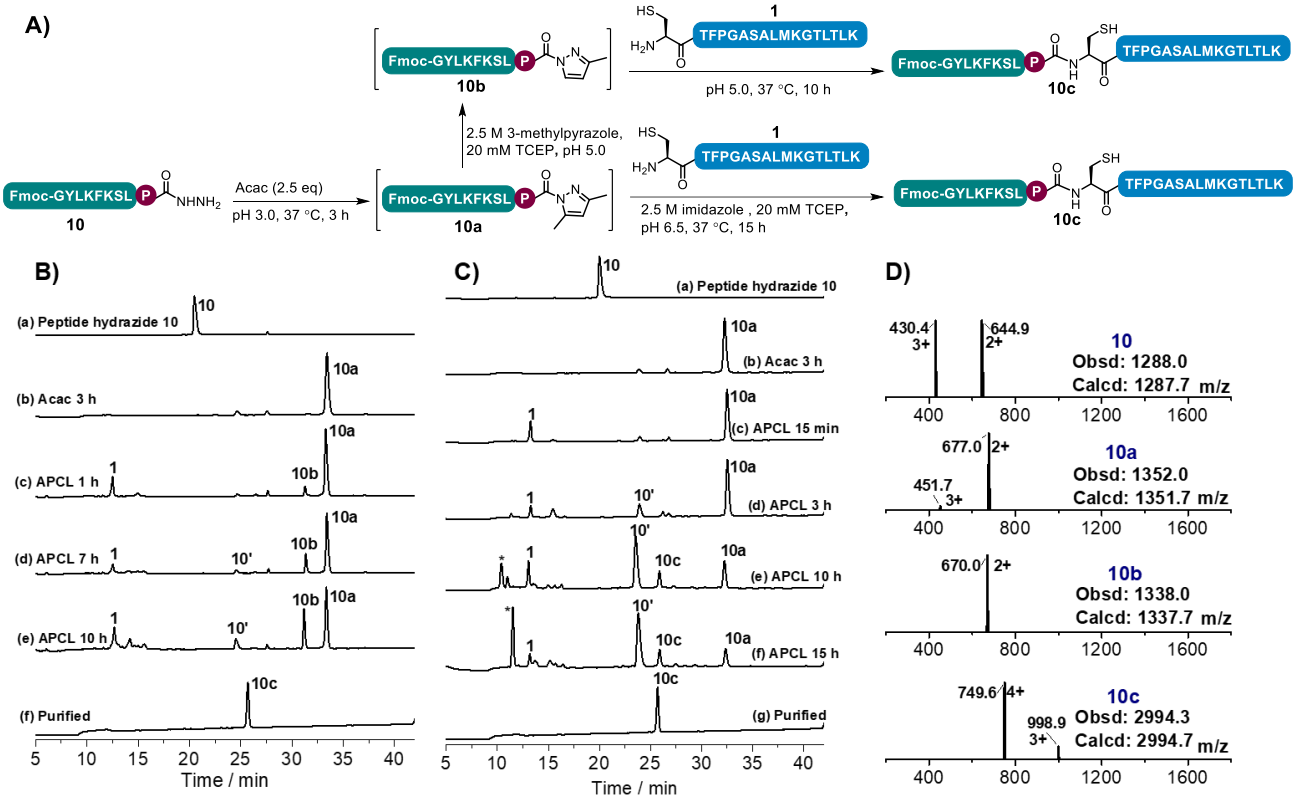


Figure S43. (A**)** Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **10** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 30% in 3 min, then to 51% MeCN in 30 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **10a** is peptide acylpyrazole, **10b** is 3-methylpyrazole substituted intermediate, **10c** is ligation product and **10'** is hydrolysis by-product. The peak * is non peptide ununified by-product. (D) ESI-MS traces of **10**, **10a**, **10b** and the ligation product **10c**, respectively. **10**: observed mass 1288.0 Da, calculated mass 1287.7 Da; **10a**: observed mass 1352.0 Da, calculated mass 1351.7 Da; **10b**: observed mass 1338.0 Da, calculated mass 1337.7 Da; **10c**: observed mass 2994.3 Da, calculated mass 2994.7 Da.

(9) **X** = Val

The peptide hydrazide **11** (4.8 μmol, 1 equiv, 6.2 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.23 μL of acetylacetone (12.0 μmol, 2.5 equiv) was added into the reaction mixture was incubated at 37 °C for 2 h. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 6 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 6 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 30 % in 3 min, then to 57% MeCN (with 0.1% TFA) in 35 min. Then, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 1.31 mg of ligation product (**11c**) (3-methylpyrazole, yield 26%) and 0.65 mg of ligation product (**11c**) (imidazole, yield 13%), respectively. The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively. The product **11c** was analysed by ESI-MS with the observed mass 2995.8 Da, calculated mass 2995.7 Da.

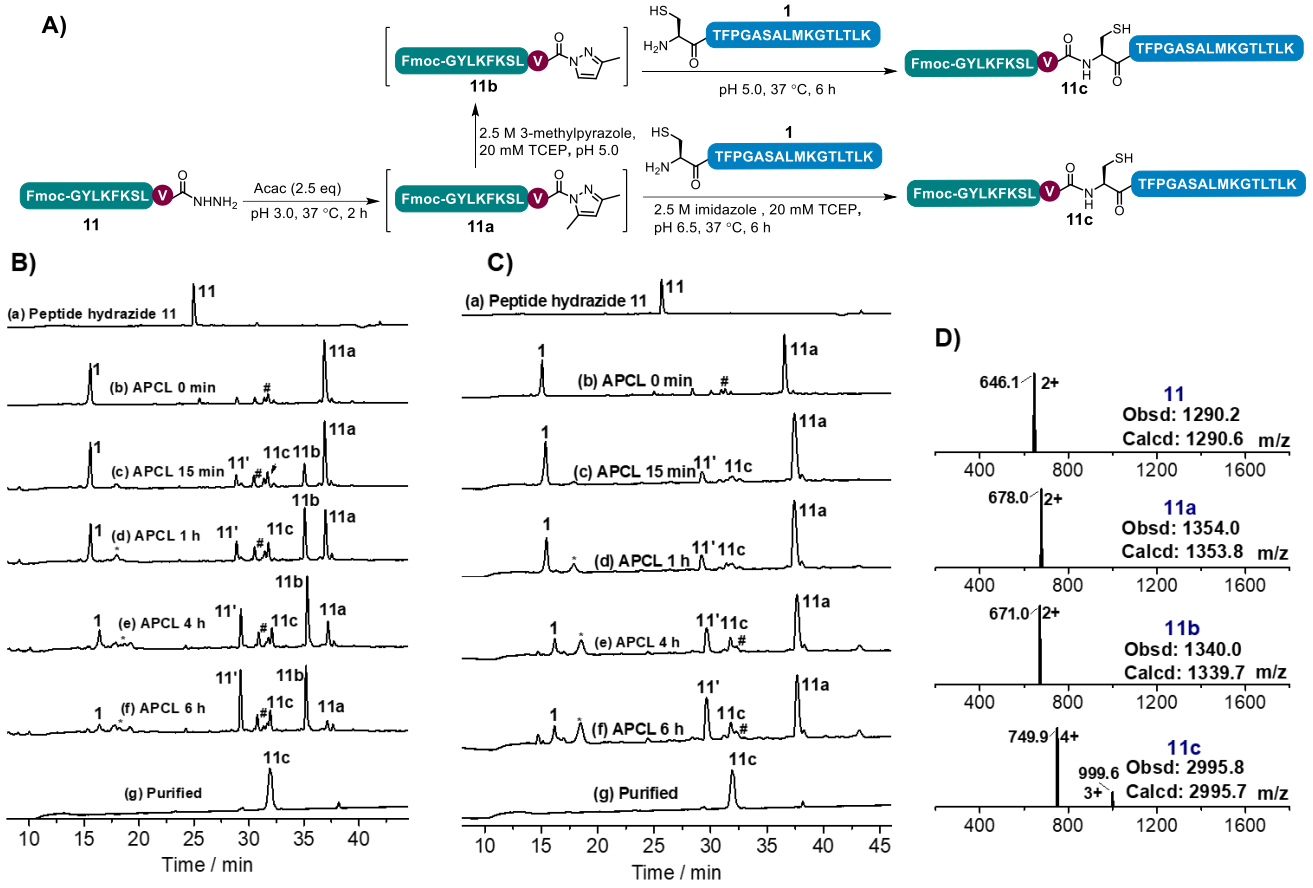


Figure S44. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **11** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 30% in 3 min, then to 57% MeCN in 35 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **11a** is peptide acylpyrazole, **11b** is 3-methylpyrazole substituted intermediate, **11c** is ligation product and **11'** is hydrolysis by-product. The peak # denotes hydrazone intermediate and peak * corresponds to a +82 Da adduct to the remaining peptide **1**. (D) ESI-MS traces of **11**, **11a**, **11b** and the ligation product **11c**, respectively. **11**: observed mass 1290.2 Da, calculated mass 1290.6 Da; **11a**: observed mass 1354.0 Da, calculated mass 1353.8 Da; **11b**: observed mass 1340.0 Da, calculated mass 1339.7 Da; **11c**: observed mass 2995.8 Da, calculated mass 2995.7 Da.

(10) **X** = Glu

Other thioester methods have reported that the C-terminal Asp or Glu may be isomerized or hydrolyzed during thioester formation and ligation.[30] We first followed the standard procedure of acetylacetonate activation of Glu peptide hydrazide in an attempt to obtain the peptide pyrazole product, which failed and was entirely a hydrolysis by-product (data not shown). Next, we tried the addition of acetylacetone mixed with 3-methylpyrazole or imidazole for subsequent experiments. The peptide hydrazide **12** (4.8 μmol, 1 equiv, 6.3 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0) and divided into two portions, which were put in the following conditions: (a) acetylacetone (0.62 μL, 6.0 μmol, 2.5 equiv), 2.5 M 3-methylpyrazole, pH 3.0, 37 °C, 20 min; (b) acetylacetone (0.62 μL, 6.0 μmol, 2.5 equiv), 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 3.0, 37 °C, 20 min, respectively. Then the Cys-peptide **1** (1.68 μmol, 0.7 equiv, 2.9 mg, 2 mM) was dissolved in 0.335 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into each portion. Then the mixed solution (a) was put in the conditions of pH 5.0, 37 °C, 2 h (confirmed by HPLC monitoring) and the mixed solution (b) was put in the conditions of pH 6.5, 37 °C, 2 h (confirmed by HPLC monitoring). The reaction mixtures a and b were analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 50% MeCN (with 0.1% TFA) in 25 min, respectively. Finally, each milted solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 0.25 mg of ligation product (**12c**) (3-methylpyrazole, yield 5%) and 0.1 mg of ligation product (**12c**) (imidazole, yield 2%), respectively. The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively. The product **12c** was analysed by ESI-MS with the observed mass 3025.6 Da, calculated mass 3025.6 Da. The results were the same as those reported in the literature above in that the hydrolysis reaction still occurred readily and the ligation yield was extremely low (< 6%).

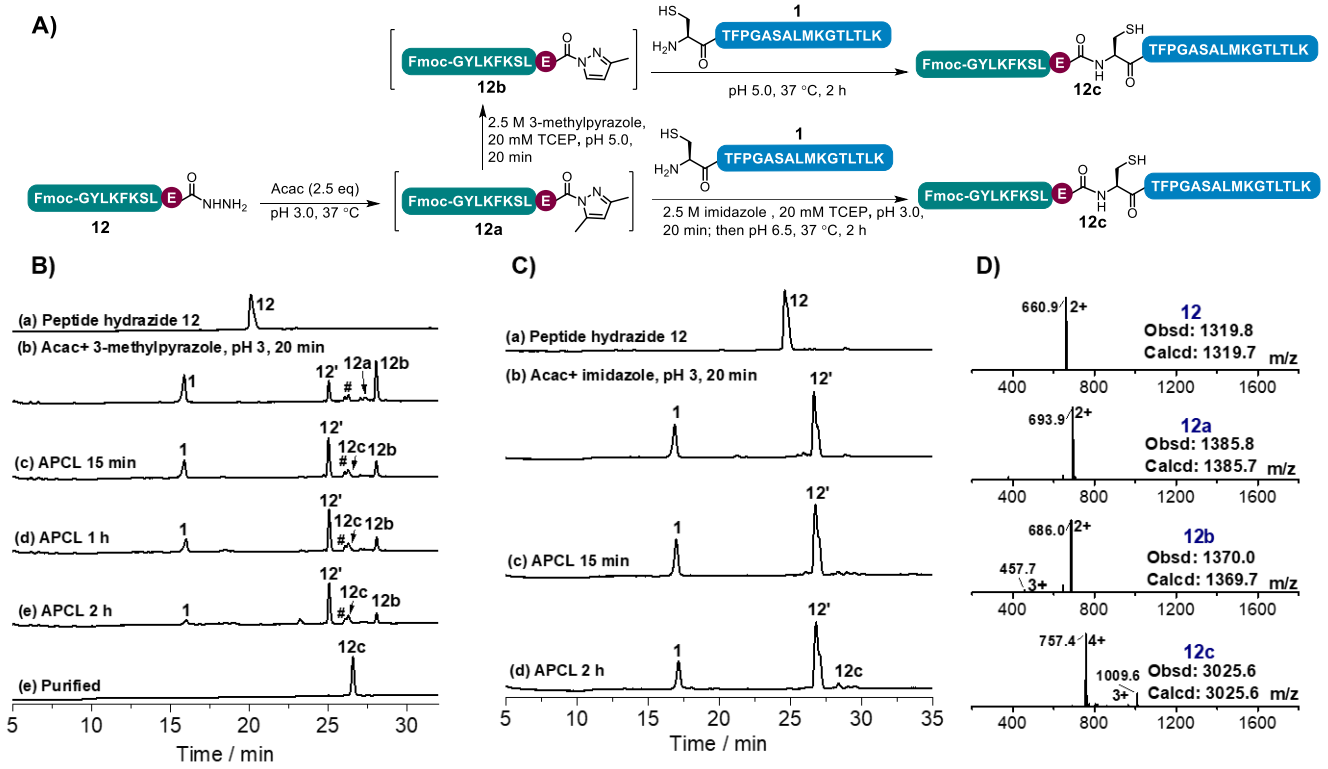


Figure S45. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **12** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **12a** is peptide acylpyrazole, **12b** is 3-methylpyrazole substituted intermediate, **12c** is ligation product and **12'** is hydrolysis by-product. The peak # denotes hydrazone intermediate. (D) ESI-MS traces of **12**, **12a**, **12b** and the ligation product **12c**, respectively. **12**: observed mass 1319.8 Da, calculated mass 1319.7 Da; **12a**: observed mass 1385.8 Da, calculated mass 1385.7 Da; **12b**: observed mass 1370.0 Da, calculated mass 1369.7 Da; **12c**: observed mass 3025.6 Da, calculated mass 3025.6 Da.

(11) **X** = Gln

The peptide hydrazide **13** (4.8 μmol, 1 equiv, 6.33 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.23 μL of acetylacetone (12.0 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 1 h. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 2 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 2 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 50% MeCN (with 0.1% TFA) in 25 min. Finally, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 2.1 mg of ligation product (**13c**) (3-methylpyrazole, yield 41 %) and 2.74 mg of ligation product (**13c**) (imidazole, yield 54%), respectively. The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively. The product **13c** was analysed by ESI-MS with the observed mass 3025.0 Da, calculated mass 3024.6 Da.

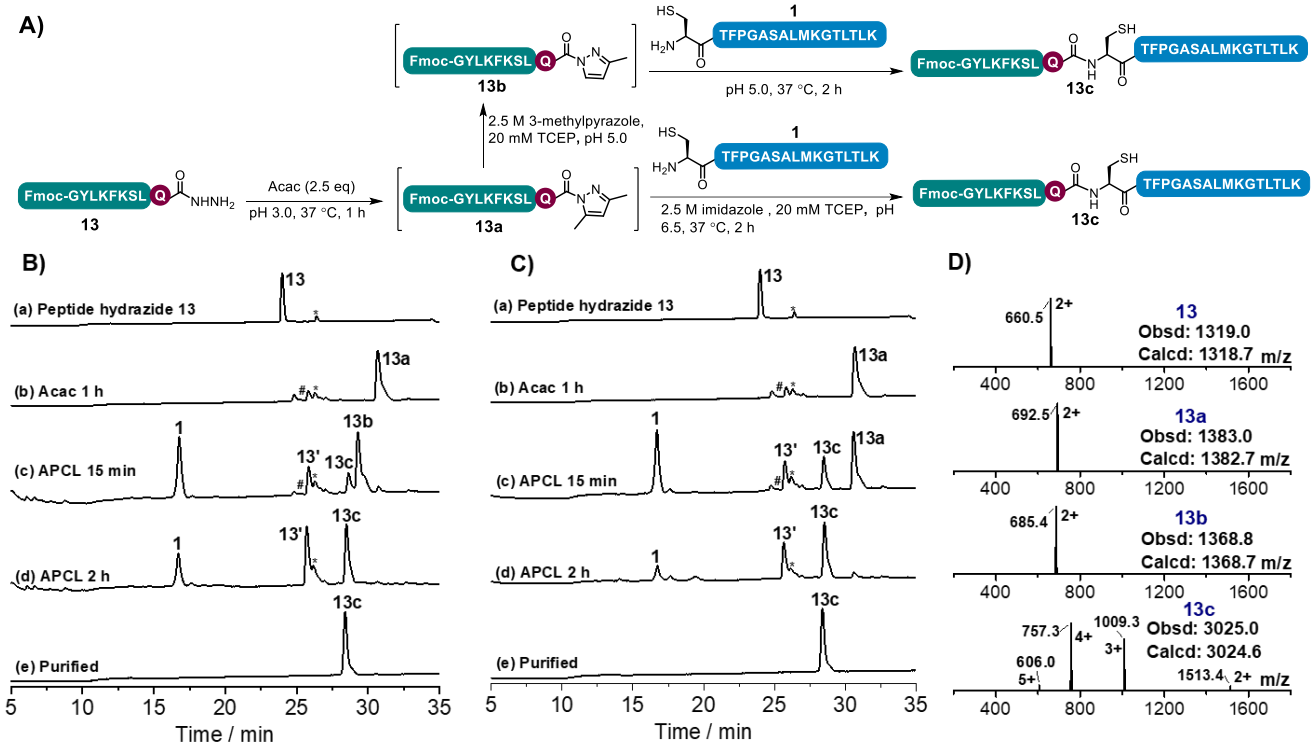


Figure S46. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **13** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **13a** is peptide acylpyrazole, **13b** is 3-methylpyrazole substituted intermediate, **13c** is ligation product and **13'** is hydrolysis by-product. The peak **#** denotes hydrazone intermediate and ***** denotes the cyclization by-product of the hydrazide with Gln side chain amide group[7]. (D) ESI-MS traces of **13**, **13a**, **13b** and the ligation product **13c**, respectively. **13**: observed mass 1319.0 Da, calculated mass 1318.7 Da; **13a**: observed mass 1383.0 Da, calculated mass 1382.7 Da; **13b**: observed mass 1368.8 Da, calculated mass 1368.7 Da; **13c**: observed mass 3025.0 Da, calculated mass 3024.6 Da.

(12) **X** = His

The peptide hydrazide **14** (4.8 μmol, 1 equiv, 6.37 mg) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 7.2 μL of 1 M acetylacetone solution (7.2 μmol, 1.5 equiv) was added into the reaction mixture and incubated at 37 °C for 0.5 h. The Cys-peptide **1** (3.36 μmol, 0.7 equiv, 5.8 mg, 2 mM) was dissolved in 0.67 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 0.5 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 0.5 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 52% MeCN (with 0.1% TFA) in 25 min. Finally, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 1.53 mg of ligation product (**14c**) (3-methylpyrazole, yield 30 %) and 2.4 mg of ligation product (**14c**) (imidazole, yield 47%), respectively. The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively. The product **14c** was analysed by ESI-MS with the observed mass 3034.70 Da, calculated mass 3034.59 Da.

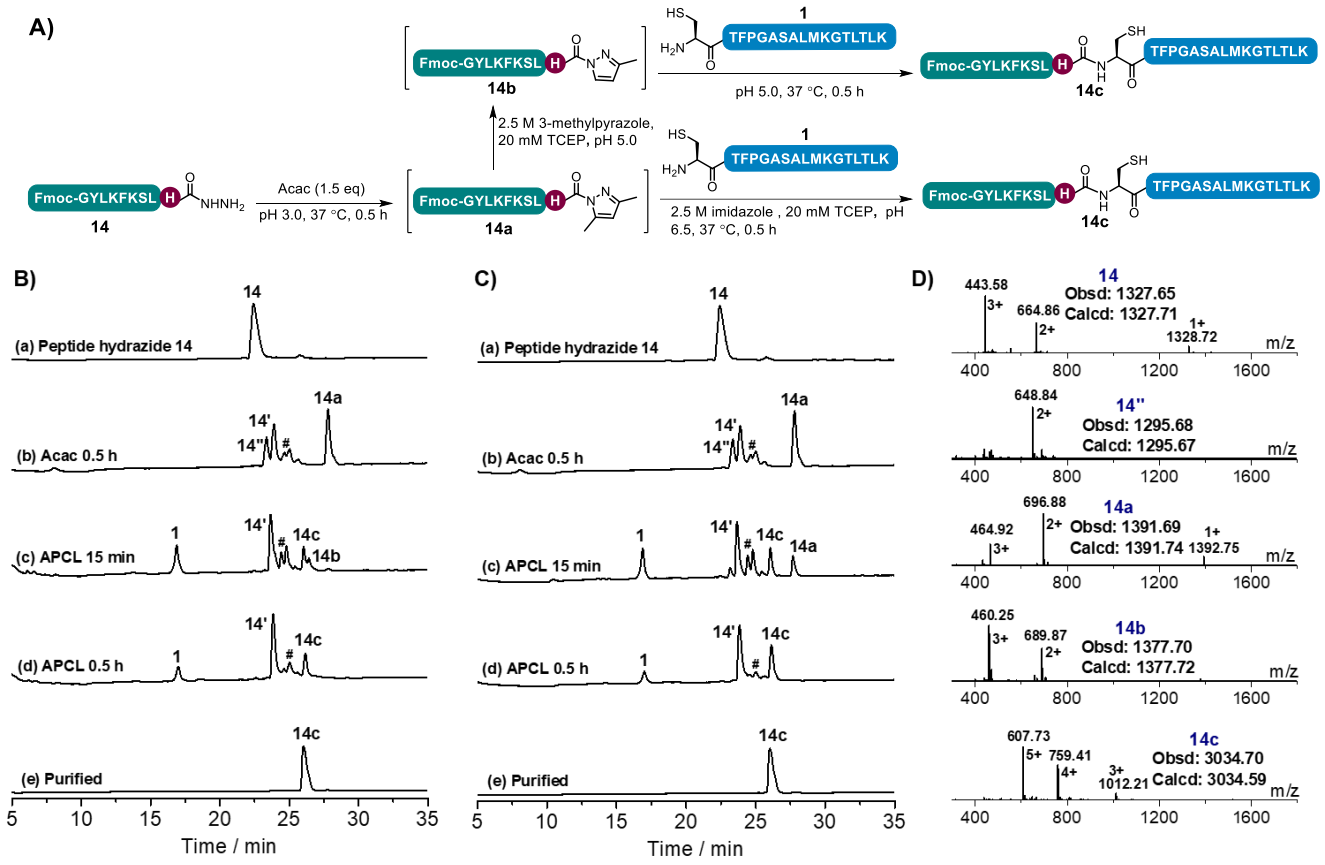


Figure S47. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **14** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 52% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C). Note: **14a** is peptide acylpyrazole, **14b** is 3-methylpyrazole substituted intermediate, **14c** is ligation product and **14'** is hydrolysis by-product. The peak # denotes hydrazone intermediate and **14''** denotes the side-chain cyclized intermediate after the substitution of the acyl pyrazole with the imidazole group from the C-terminal His. (D) ESI-MS traces of **14**, **14''**, **14a**, **14b** and the ligation product **14c**, respectively. **14**: observed mass 1327.65 Da, calculated mass 1327.71 Da; **14''**: observed mass 1295.68 Da, calculated mass 1295.67 Da; **14a**: observed mass 1391.69 Da, calculated mass 1391.74 Da; **14b**: observed mass 1377.70 Da, calculated mass 1377.72 Da; **14c**: observed mass 3034.70 Da, calculated mass 3034.59 Da.

(13) **X** =Ala (with internal Cys)

The peptide hydrazide **3***(4.76 μmol, 1 equiv, 6.8 mg) was dissolved in 0.66 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.22 μL of acetylacetone (11.88 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 1.5 h. The Cys-peptide **1** (3.33 μmol, 0.7 equiv, 5.79 mg, 2 mM) was dissolved in 0.66 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the mixture. Subsequently, the mixed solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, pH 5.0, 37 °C, 3 h; (b) 2.5 M imidazole (prepared as 1 mg/μL stock solution), pH 6.5, 37 °C, 3 h, respectively. The reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 20 to 28 % in 3 min, then to 60% MeCN (with 0.1% TFA) in 25 min. After 3 hours incubation, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 30 to 50% MeCN (with 0.1% TFA) in 30 min to obtain 3.4 mg of ligation product (**3c***) (3-methylpyrazole, yield 65%) and 1.9 mg of ligation product (**3c***) (imidazole, yield 37%), respectively. The purity and mass of the peptide were confirmed using analytical HPLC and ESI-MS, respectively. The product **3c*** was analysed by ESI-MS with the observed mass 3127.7 Da, calculated mass 3127.6 Da.

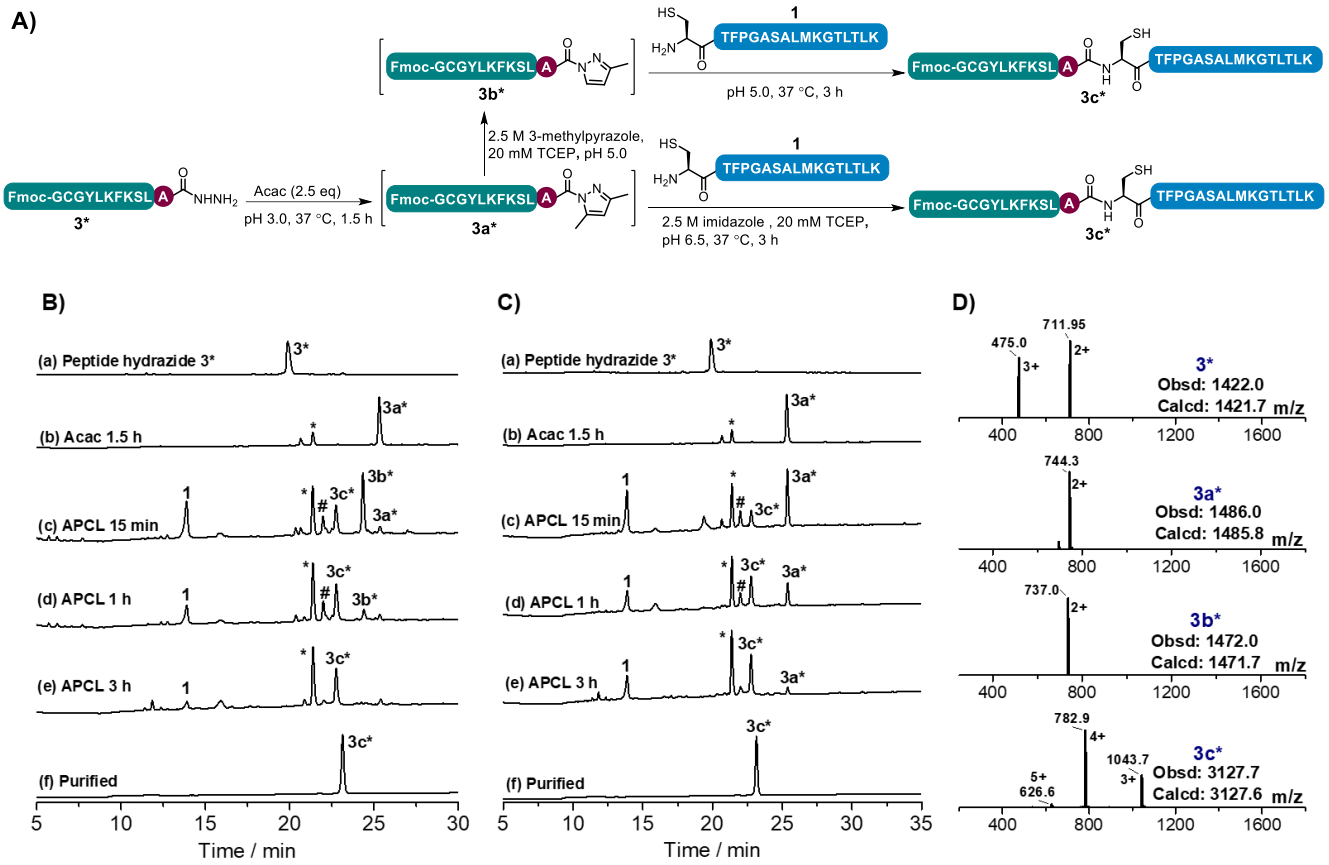


Figure S48. (A) Schematic representation of the ligation between Cys-peptide **1** and peptide hydrazide **3*** with 3-methylpyrazole and imidazole, respectively. (B-C) Analytical HPLC traces (20% to 28% in 3 min, then to 60% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of ligation using 3-methylpyrazole (B) and imidazole (C) for 3 h. Note: **3a*** is peptide acylpyrazole, **3b*** is the 3-methylpyrazole substituted intermediate, **3c*** is ligation product and * is hydrolysis by-product. The peak # denotes thiolactone. (D) ESI-MS traces of **3***, **3a***, **3b*** and the ligation product **3c***, respectively. **3***: observed mass 1422.0 Da, calculated mass 1421.7 Da; **3a***: observed mass 1486.0 Da, calculated mass 1485.8 Da; **3b***: observed mass 1472.0 Da, calculated mass 1471.7 Da; **3c***: observed mass 3127.7 Da, calculated mass 3127.6 Da.

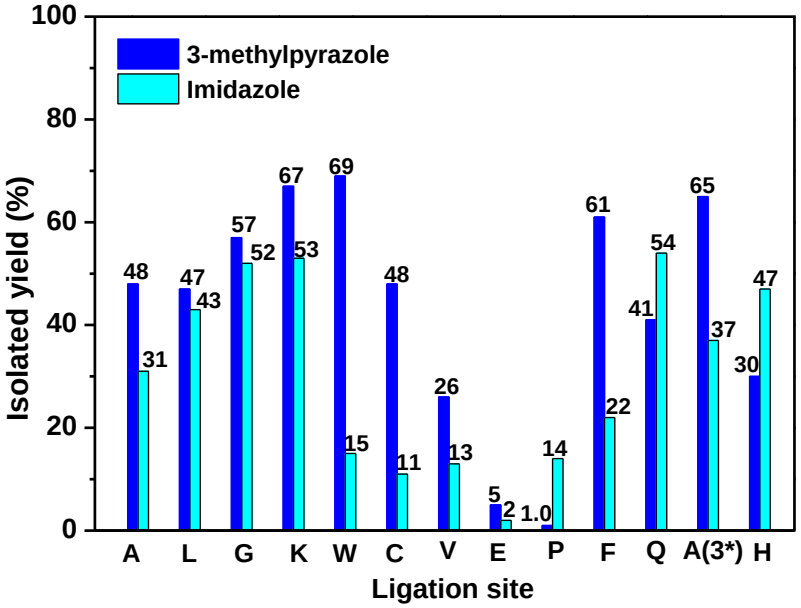


Figure S49. Comparison of ligation yield for different peptide hydrazides (**3**–**14**) with Cys-peptide **1** using 3-methylpyrazole or imidazole.

7 One-pot ligation–desulfurization

The peptide hydrazide **2** (0.37 μmol, 1 equiv, 0.45 mg) was dissolved in 43 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 0.93 μL of the 1 M acetylacetone stock solution (0.93 μmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 50 min. After that, the Cys-peptide **1** (0.26 μmol, 0.7 equiv, 0.45 mg, 2 mM) was dissolved in 65 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 40 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 22.1 μL of imidazole stock solution (prepared as 1 mg/μL stock solution) was added. The pH of mixed solution was adjusted to 6.5 with 2 M NaOH, and the resulting mixture was stirred at 37°C for 3 h (confirmed by HPLC monitoring). Upon completion of the ligation, to the mixture was added t-BuSH (13 μL, 5%, v/v), a neutral solution (52 μL) of TCEP (0.2 M (final conc.), 13.4 mg) and an aqueous solution (65 μL) of 0.1 M VA-044 (25 equiv, 6.5 μmol). The mixture solution (**2b** concentration: 1 mM) was incubated at 37 °C (the reaction was monitored by HPLC). After the desulfurization was completed (8 h), the reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 20 min.

Note: In order to avoid the severe hydrolysis of the peptidyl pyrazole caused by the direct addition of solid imidazole. The imidazole was pre-dissolved in 12 M HCl to prepare a 1 mg/μL stock solution and then diluted in the appropriate volume of buffer A before being added to the ligation mixture. (2) VA-044 was pre-dissolved in buffer (6 M Gdn•HCl, 2.5 M imidazole, pH 6.5) to prepare a 0.1 M stock solution. (3) 14.3 mg of TCEP was pre-dissolved in 52 μL of buffer (6 M Gdn•HCl, 2.5 M imidazole, pH 6.5).

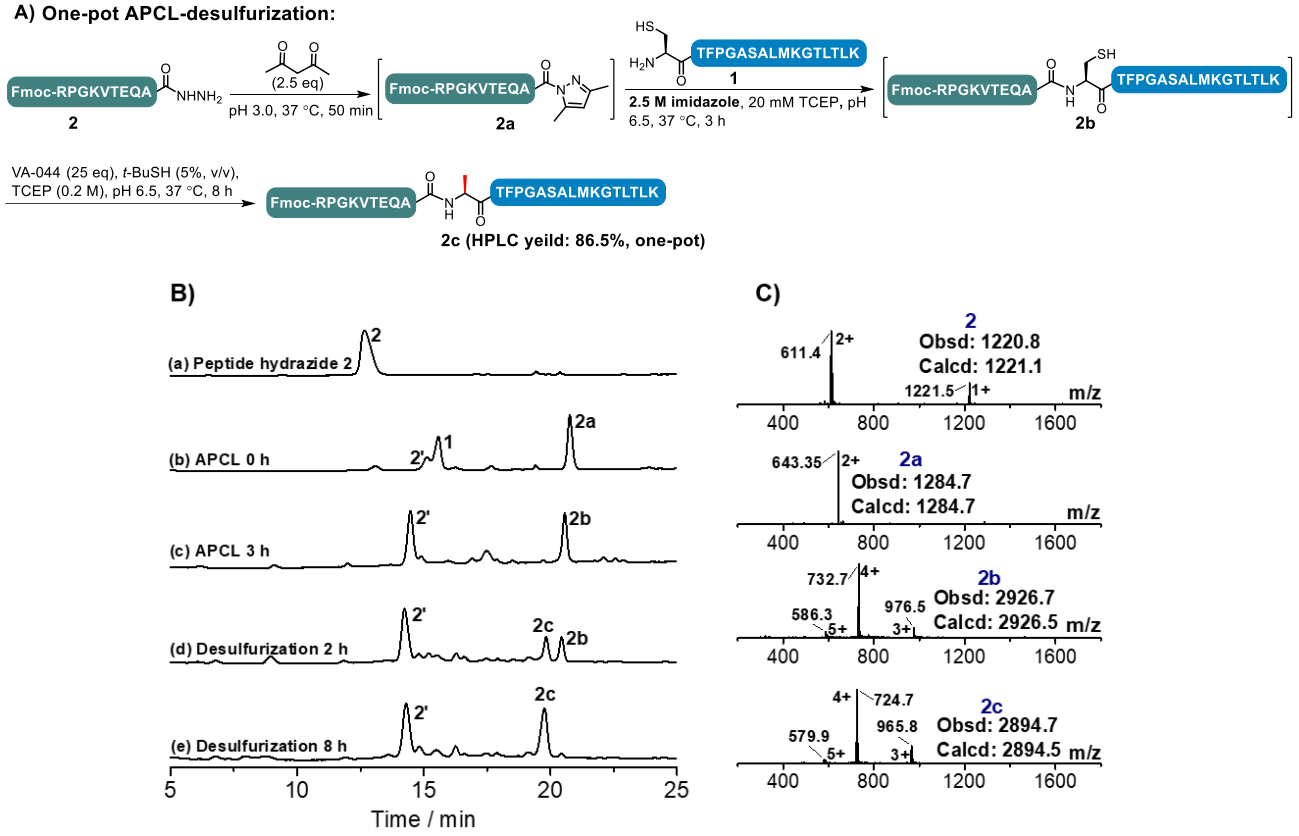


Figure S50. (A) Schematic representation of the ligation and desulfurization in one pot between Cys-peptide **1** and peptide hydrazide **2**. (B) Analytical HPLC traces (25% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of the ligation and desulfurization in one pot. Note: **2a** is peptide acylpyrazole, **2b** is ligation product, **2c** is desulfurization product and **2'** is hydrolysis by-product. (C) ESI-MS traces of **2**, **2a**, **2b** and **2c**, respectively. **2**: observed mass 1220.8 Da, calculated mass 1221.1 Da; **2a**: observed mass 1284.7 Da, calculated mass 1284.7 Da; **2b**: observed mass 2926.7 Da, calculated mass 2926.5 Da; **2c**: observed mass 2894.7 Da, calculated mass 2894.5 Da.

8 Total synthesis of ubiquitin via a one-pot sequential ligation–desulfurization

8.1 Synthesis of the peptide fragments

*8.1.1 Synthesis of ubiquitin(1–27)-NHNH$_2$ fragment **16***

Peptide hydrazide **16** was synthesized on 2-Cl-(Trt)-Cl resin (0.5 g, theoretical loading: 0.9 mmol/g) using Fmoc-NHNH$_2$ with 0.5 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H$_2$O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. The precooled diethyl ether was added to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide hydrazide.

The 208 mg of crude peptide hydrazide was dissolved in 15 mL of buffer (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 20 mM TCEP, pH 3). The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 20 to 45% MeCN (with 0.1% TFA) in 25 min to afford the desired fragment **16** as a white amorphous powder (21 mg, 10.1% isolated yield). Overall, 41 mg of segment **16** was obtained. The purity and mass of the peptide were confirmed using analytical HPLC (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) and ESI-MS, respectively.

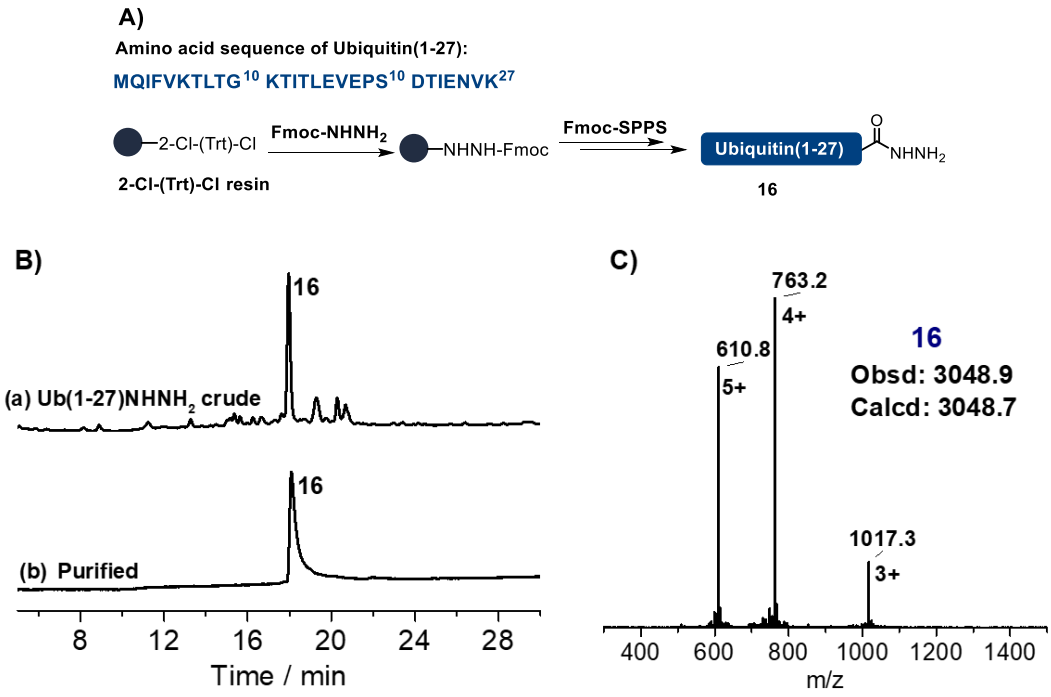


Figure S51. (A) Schematic representation of synthesis of ubiquitin(1–27)-NHNH$_2$ fragments **16**. (B) Analytical HPLC traces (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of crude and purified ubiquitin(1–27)-NHNH$_2$ **16**. (C) ESI-MS analysis of **16** shows the observed mass 3048.9 Da, calculated mass 3048.7 Da.

*8.1.2 Synthesis of ubiquitin(28–45)-NHNH$_2$ fragment **17***

Peptide hydrazide (**17)** was synthesized on 2-Cl-(Trt)-Cl resin (0.25 g, theoretical loading: 0.9 mmol/g) using Fmoc-NHNH$_2$ with 0.45 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H$_2$O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. The precooled diethyl ether was added to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide hydrazide (**17**). The 241 mg of crude peptide hydrazide was dissolved in 15 mL of buffer (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 20 mM TCEP, pH 3). The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 20 to 45% MeCN (with 0.1% TFA) in 25 min to afford the desired fragment **17** as a white amorphous powder (40.5 mg, 16.8% isolated yield). The purity and mass of the peptide were confirmed using analytical HPLC (20 to 70% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) and ESI-MS, respectively.

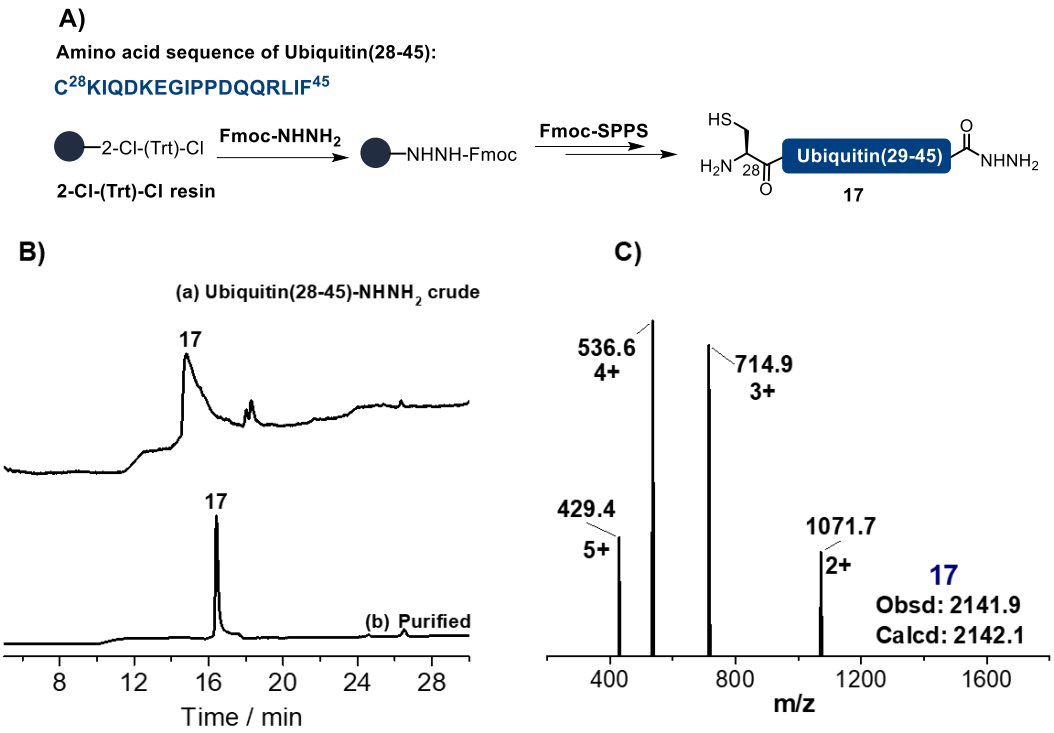


Figure S52. (A) Schematic representation of synthesis of ubiquitin(28–45) fragment **17**. (B) Analytical HPLC traces (20 to 70% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of crude and purified ubiquitin(28–45)-$NHNH_2$ **17**. (C) ESI-MS analysis of **17** shows the observed mass 2141.9 Da, calculated mass 2142.1 Da.

*8.1.3 Synthesis of ubiquitin(46–76) fragment **18***

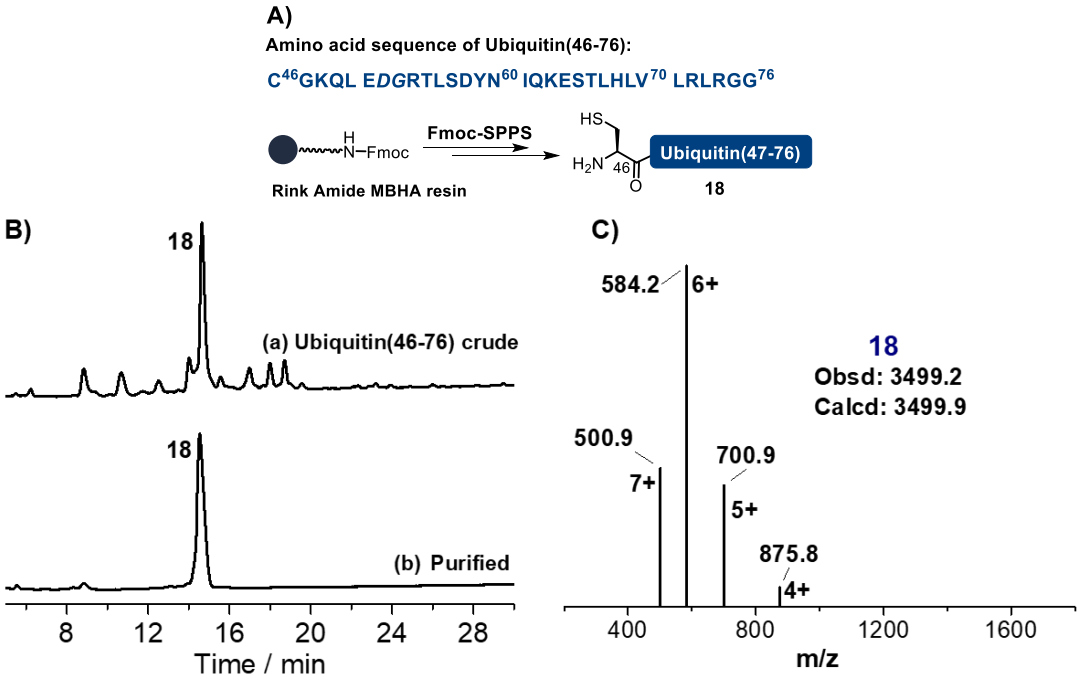


Figure S53. (A) Schematic representation of synthesis of ubiquitin(46–76) fragment **18**, and the dipeptide building blocks used during SPPS are shown in italic. (B) Analytical HPLC traces (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of crude and purified ubiquitin(46–76) fragment **18**. (C) ESI-MS analysis of **18** shows the observed mass 3499.2 Da, calculated mass 3499.9 Da.

The peptide (**18**) was synthesized using Fmoc-Gly-OH on Rink amide MBHA resin (0.8 g, theoretical loading: 1.2 mmol/g) with a loading of 0.46 mmol/g. It was elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2.5 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. The precooled diethyl ether was added to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide (**18**). The 468 mg of crude peptide was dissolved in 15 mL of buffer (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 20 mM TCEP, pH 3). The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-

preparative HPLC at 25 °C with a gradient of 25 to 45% MeCN (with 0.1% TFA) in 25 min to afford the desired fragment **18** as a white amorphous powder (35.1 mg, 7.5% isolated yield). The purity and mass of the peptide were confirmed using analytical HPLC (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) and ESI-MS, respectively.

8.2 Synthesis of ubiquitin via a one-pot sequential ligation-desulfurization (on a 1 μmol scale)

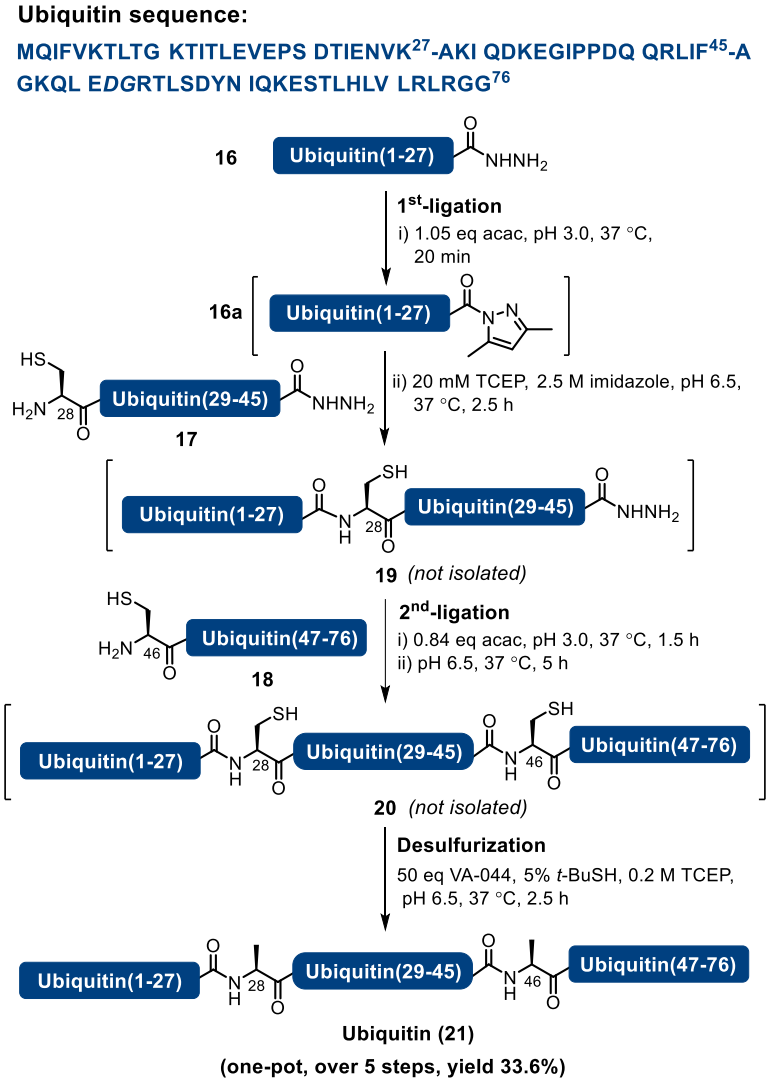


Figure S54. (A) Schematic representation of one-pot synthesis of ubiquitin (**21)** in the N-to-C direction. The isolated yield is calculated based on the limiting Cys-peptide starting material (**18**).

The ubiquitin(1–27)-$NHNH_2$ (**16**) (1 μmol, 1 equiv, 3.11 mg) was dissolved in 100 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then acetylacetone (1.05 equiv, 1.05 μmol, prepared as 1 M stock solution in water, 1.05 μL) was added into the mixture and incubated at 37 °C for 20 min (confirmed by HPLC monitoring). Then, Cys-peptide of ubiquitin(28–45)-$NHNH_2$ (**17**) (0.7 μmol, 0.7 equiv, 1.5 mg, 3 mM) was dissolved in 93 μL of buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, pH 3.0) and added into the resulting mixture. Subsequently, 39.6 μL of the imidazole stock solution (prepared as 1 mg/μL stock solution) was added. The pH of the mixture was adjusted to 6.5 with 2 M NaOH, and the resulting mixture was stirred at 37 °C for 2.5 h (confirmed by HPLC monitoring). After the 1st-ligation was completed, the pH of mixture was adjusted to 3.0 and 0.84 equiv of acetylacetone (1.2 eq of **17**, prepared as 0.1 M stock in water) was added. The reaction mixture was incubated at 37 °C for 1.5 h (confirmed by HPLC monitoring). Then, the Cys-peptide ubiquitin(46–76) **18** (0.63 μmol, 0.63 equiv, 2.21 mg, 2.5 mM) was added into the above reaction mixture and gently agitated to aid dissolution. Then the mixed solution was adjusted to pH 6.5 and stirred at 37 °C for 5 h (confirmed by HPLC monitoring). Upon completion of the 2nd-ligation, the *t*-BuSH (35 μL, 5%, v/v), a solution (65 μL) of TCEP (0.2 M (final conc.), 36.1 mg) and an aqueous solution (350 μL) of 0.1 M VA-044 (50 equiv, for two Cys, 35 μmol) were sequentially added into the mixture (final concentration of substrate: 1 mM). The mixture solution was incubated at 37 °C (the reaction was monitored by HPLC). After the desulfurization was completed (2.5 h), the reaction mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 25 to 43% MeCN (with 0.1% TFA) in 35 min to collect the desired fractions and lyophilized, affording desired ubiquitin (**21)** as a white powder (1.81 mg, isolated yield 33.6%, over 5 steps in one-pot). The isolated yield was calculated based on the Cys-peptide starting material (**18**) and the desired product (**21**).The reaction traces and mass of

the peptide were confirmed using analytical HPLC (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) and ESI-MS, respectively.

Note: In order to avoid the severe hydrolysis of the peptidyl pyrazole caused by the direct addition of solid imidazole. The imidazole was pre-dissolved in 12 M HCl to prepare a stock solution (1 mg/μL). (2) VA-044 was pre-dissolved in buffer (6 M Gdn•HCl, 2.5 M imidazole, pH 6.5) to prepare a 0.1 M stock solution. (3) 36.1 mg of TCEP was pre-dissolved in 52 μL of buffer (6 M Gdn•HCl, 2.5 M imidazole, pH 6.5).

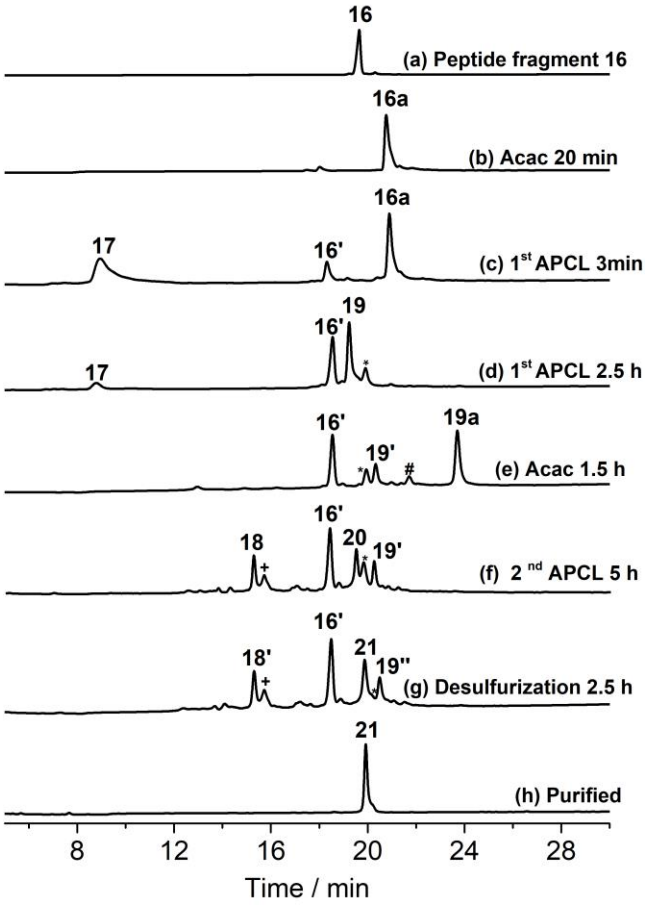


Figure S55. Analytical HPLC traces (20% to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of one-pot synthesis of ubiquitin (**21**) in the N-to-C direction. (a-d) 1st-ligation: **16** is ubiquitin(1–27)-$NHNH_2$, **16a** is ubiquitin(1–27)-acylpyrazole, **17** is ubiquitin(28–45)-hydrazide, **19** is 1st-ligation product and **16'** is hydrolysis by-product. The peak ***** denotes lactamiation of C-terminus of the Lys27 by-product. (e) acac activation: **19a** is ubiquitin(1–45)-acylpyrazole, **19'** denotes hydrolysis by-product and peak **#** denotes hydrazone intermediate. (f) 2nd-ligation at 5 h: **18** is ubiquitin(46–76), **20** is 2nd-ligation product and the peak **+** denotes a +82 Da adduct of the remaining peptide **18**. (g) desulfurization: **18'** is desulfurized versions from the remaining **18**, **19''** is desulfurized version from **19'** and **21** is the desulfurization product. (h) The purified **21**.

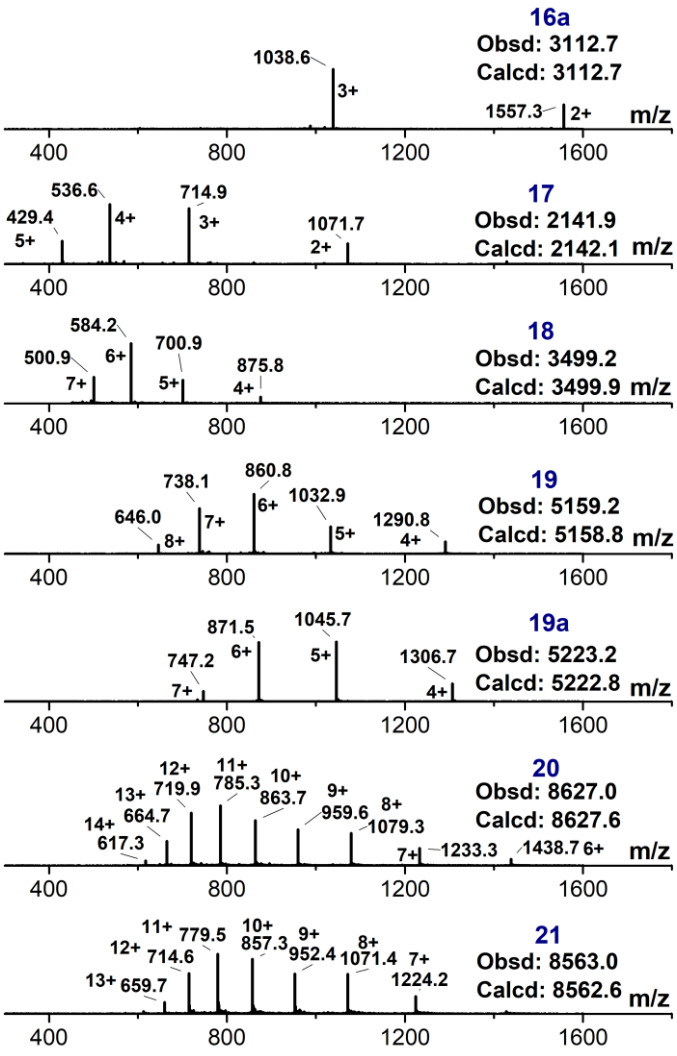


Figure S56. ESI-MS analysis of **16a**, **17**, **18**, 1st-ligation product **19,** ubiquitin(1–45)-acylpyrazole **19a**, 2nd -ligation product **20** and desulfurization product **21**.

9 Synthesis of azurin via an expression protein ligation using 3-methylpyrazole additive

9.1 Synthesis of the peptide fragments

*9.1.1 Recombinant expression and purification of of azurin(1–111) hydrazide fragment **22***

The gene encoding for the azurin(1–111)-intein-CBD fusion protein was synthesized and codon-optimized for *E. Coli* expression (GenScript Inc., Nanjing). The synthetic gene was cloned upstream of the Mycobacterium xenopi DNA Gyrase A (Mxe GyrA) intein and a chitin-binding domain (CBD) into a pTXB1 expression vector via *NdeI* and *SpeI* restriction sites. The vector pTXB1-azurin(1–111)-intein-CBD required for the expression of the desired Azurin(1–111)-intein-CBD can be obtained from GL Biochem. The cleavage site between Phe and Cys is marked in bold. The full amino acid sequence of azurin(1–111)-intein-CBD was:
AECSVDIQGNDQMQFNTNAITVDKSCKQFTVNLSHPGNLPKNVMGHNWVLSTAADMQGVVTDGMASGLDKDYLKPDDSRVIAHTKLI
GSGEKDSVTFDVSKLKEGEQYMF**FC**ITGDALVALPEGESVRIADIVPGARPNSDNAIDLKVLDRHGNPVLADRLFHSGEHPVYTVRTVE
GLRVTGTANHPLLCLVDVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYTVGVPGLVRFLEAHHRDPDAQAIAD
ELTDGRFYYAKVASVTDAGVQPVYSLRVDTADHAFITNGFVSHATGLTGLNSGLTTNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQP
HTSLAGWEPSNVPALWQLQ

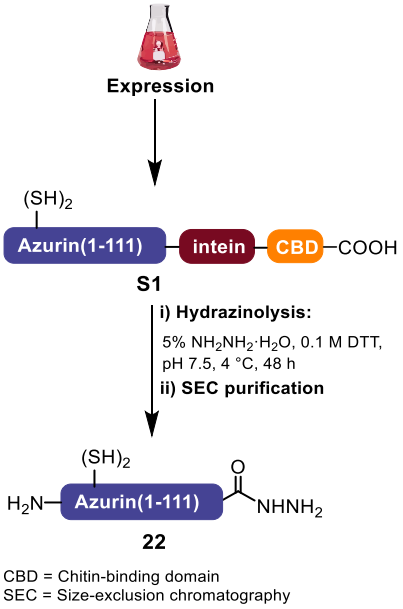


Figure S57. Expression and purification of azurin(1–111) fragment **22**.

Some incubation solutions and buffer used for the expression and purification of azurin (1-111)-$NHNH_2$ include the following:
(a) LB (2 L): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7
(b) Cell lysis buffer: 0.1 M NaH_2PO_4, 0.25 M NaCl, 1 M Urea, 1 mM EDTA, 0.1% Triton-X100, pH 7.5
(c) SEC eluting buffer: 6 M Gdn•HCl, 0.1 M NaH_2PO_4, 2 mM TCEP, 0.1 M NaCl, pH 5
(d) Dialysis buffer: 10 mM CH_3COONH_4, pH 5, 4 times, these dialysis buffers were changed every 12 h

The plasmid was firstly transformed into BL21(DE3) *E. coli* cells chemically. An overnight culture of the cells harboring an expression vector was inoculated (1:50 dilution) in a 4 L flask containing 80 μg/mL Ampicillin in 2 L LB at 37 °C. After reaching an OD_{600} of 0.4–0.6 expression of azurin(1–111)-intein-CBD (**S1**) was induced by the addition of 1 mL 1 M IPTG stock solution (final conc. 0.5 mM) at 18 °C for 18 h. Cells (9 g) were harvested by centrifugation (8000 rpm, 4 °C, 15 min). Typically, 7 g cell precipitates were resuspended in 40 mL of cell lysis buffer and lysed by ultrasonication (30–40 % power, 3 s on 5 s off, 25 min). The crude lysate was centrifuged (16000 rpm, 4 °C, 20 min, 3 times) and the supernatant was retained.

The 7.75 mL of 43.5% $NH_2NH_2 \cdot H_2O$ solution (80% $NH_2NH_2 \cdot H_2O$: 12 M HCl = 2:3 (v/v), mixed and pre-cooled) (5%, final conc.) was added dropwise to a 40 mL of supernatant at 4 °C in 15 min. Then, DTT (650 mg, 0.1 M, final conc.) was added into the mixture and gently agitated to aid dissolution. The mixed solution was adjusted to pH 7.5 by HCl (6 M) and stirred at 4°C for 48 h to produce the azurin(1–111) hydrazide (confirmed by SDS-PAGE monitoring). After cleavage, the mixture was centrifuged (16000 rpm, 40 min, 4 °C) to retain the precipitates. The 20 mL of SEC eluting buffer was added into precipitates and gently agitated to aid dissolution. The supernatant was centrifuged (16000 rpm, 40 min, 4 °C, 4 times), filtered and purified by size-exclusion chromatography (SEC) on a 16/600 column (120 mL) at 0.5 mL/min with a AKTA pure chromatography system. Absorption was monitored at 280 nm. The column was washed with 140 mL of SEC eluting buffer to collect the fractions of #18–20 (15 mL). In all, 60 mL of azurin(1–111)-$NHNH_2$ (**22**) fraction was collected. The 60 mL of fraction were gradually dialyzed to the 2 L dialysis buffer. After dialysis, it was lyophilized to obtain the white powder of azurin(1–111)-$NHNH_2$ (**22**).(20 mg/L LB). The purity and mass of the peptide were confirmed using analytical HPLC (20% to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) and ESI-MS, respectively.

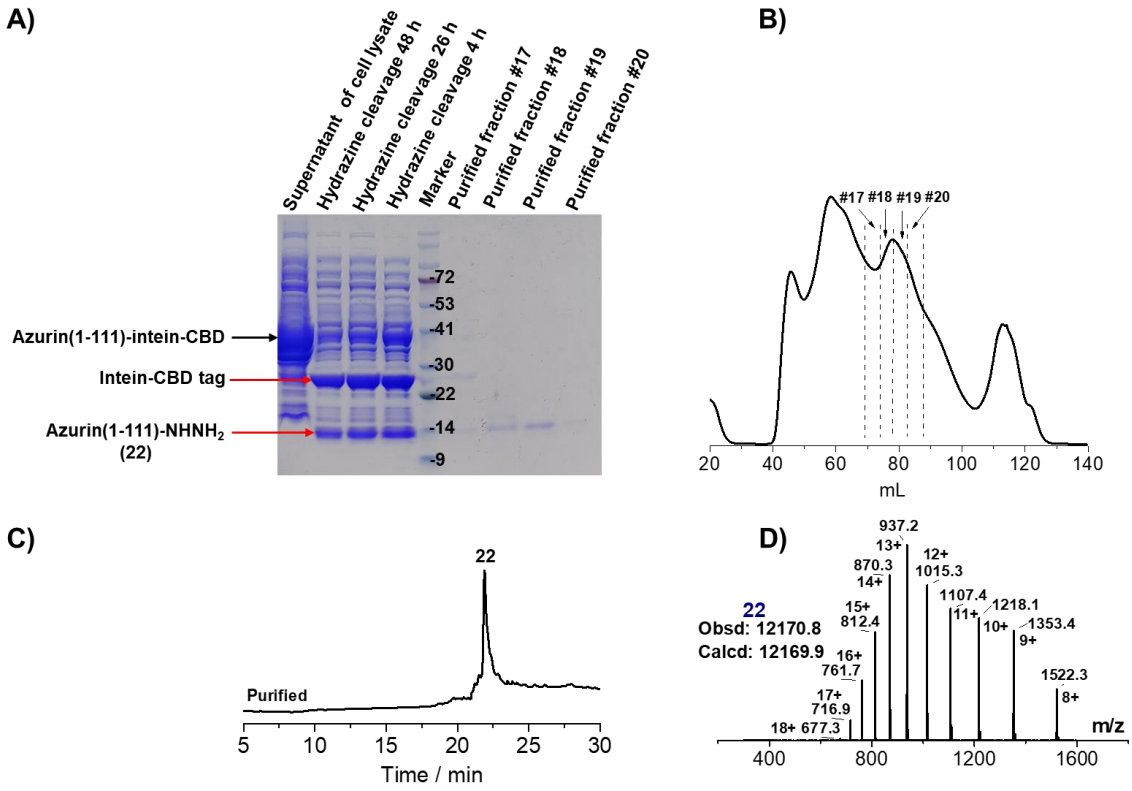


Figure S58. (A) SDS-PAGE traces of the azurin(1–111)-intein-CBD cell lysis, hydrazine cleavage the intein-CBD tag for 48 h and SEC purification of **22**. Lane 1: supernatant of cell lysate; lane 2: hydrazine cleavage 48 h; lane 3: hydrazine cleavage 26 h; lane 4: hydrazine cleavage 4 h; lane 5: molecular weight standard; lane 6: fraction #17 after SEC purification; lane 7: fraction #18 after SEC purification; lane 8: fraction #19 after SEC purification; lane 9: fraction #20 after SEC purification. (B) Purification of azurin(1–111)-$NHNH_2$ by SEC16/60HiLoad column (λ = 280 nm). (C) Analytical HPLC trace (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of the purified azurin(1–111)-$NHNH_2$ **22**. (D) ESI-MS analysis of **22** shows the observed mass 12170.8 Da, calculated mass 12169.9 Da.

*9.1.2 Synthesis of azurin(112–128) fragment **23***

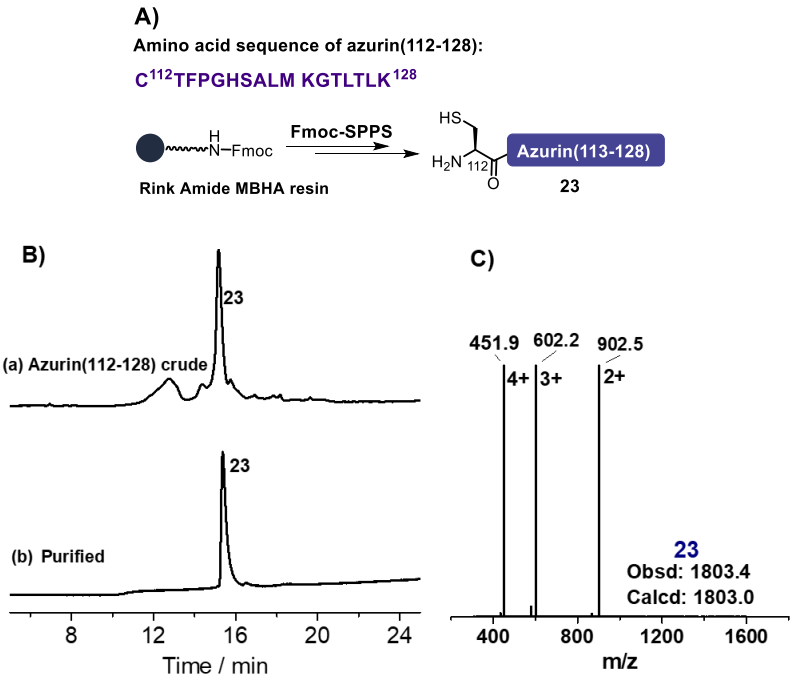


Figure S59. (A) Schematic representation of synthesis of azurin(112–128) fragment **23**. (B) Analytical HPLC traces (20 to 50% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) of crude and purified azurin(112–128) fragment **23**. (C) ESI-MS analysis of **23** shows the observed mass 1803.4 Da, calculated mass 1803.0 Da.

Peptide (**23)** was synthesized on Rink amide MBHA resin (0.5 g, theoretical loading: 1.2 mmol/g) using Fmoc-Lys(Boc)-OH with 0.55 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2.5 h at 25 °C. After filtration, the filtrate was

concentrated by blowing with a gentle flow of N_2. The precooled diethyl ether was added to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide. The 248 mg of crude peptide was dissolved in 15 mL of buffer (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 20 mM TCEP, pH 3) and centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25 °C with a gradient of 20 to 50% MeCN (with 0.1% TFA) in 20 min to obtain 45.2 mg of peptide **23** (0.25 g resin, 18.2%). The purity and exact mass of the peptide **23** were confirmed using analytical HPLC (20 to 50% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) and ESI-MS, respectively.

9.2 Synthesis of azurin 25 via a one-pot ligation–refolding (on a 1 μmol scale)

The peptide azurin(1–111)-$NHNH_2$ (**22)** (1 μmol, 1 equiv, 12.2 mg) was dissolved in 0.2 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 2.5 μL of the 1 M acetylacetone stock solution (2.5 equiv, 2.5 μmol) was added into the mixture and incubated at 37 °C for 1.5 h (confirmed by HPLC monitoring). The Cys-peptide azurin(112-128) (**23**) (1.8 μmol, 1.8 equiv, 3.25 mg) was dissolved in 0.2 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0) and added into mixture. Subsequently, 102.5 μL of 3-methylpyrazole (2.5 M, final conc) was added. The pH of the mixture was adjusted to 5.0 with 1 M NaOH, and the resulting mixture was stirred at 37 °C. After the ligation was completed (2 h), the resulting mixture was reduced by the addition of 0.2 equiv of TCEP buffer (prepared as 1 M stock solution, 0.2 μL) and incubated for 10 min. The resulting mixture (0.5 mL) was added dropwise to a 15 mL refolding buffer (0.4 M Arg•HCl, 0.2 M Tris, 0.1 M $(NH_4)_2SO_4$, 2 mM EDTA, 0.2 mM GSSG, 1 mM GSH, pH 8.2) (final protein conc. was ~0.3 mg/mL) at 4 °C in 30 min, and was stirred in a refrigerator at 4 °C for 24 h. The resulting mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC with a gradient: 25 to 45% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and immediately lyophilized, affording the desired folded protein azurin (**24a**) as a white amorphous powder (4.34 mg, 31.2% isolated yield, over 3 steps in one-pot). The reaction traces and mass of the peptide were confirmed using analytical HPLC (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) and ESI-MS, respectively.

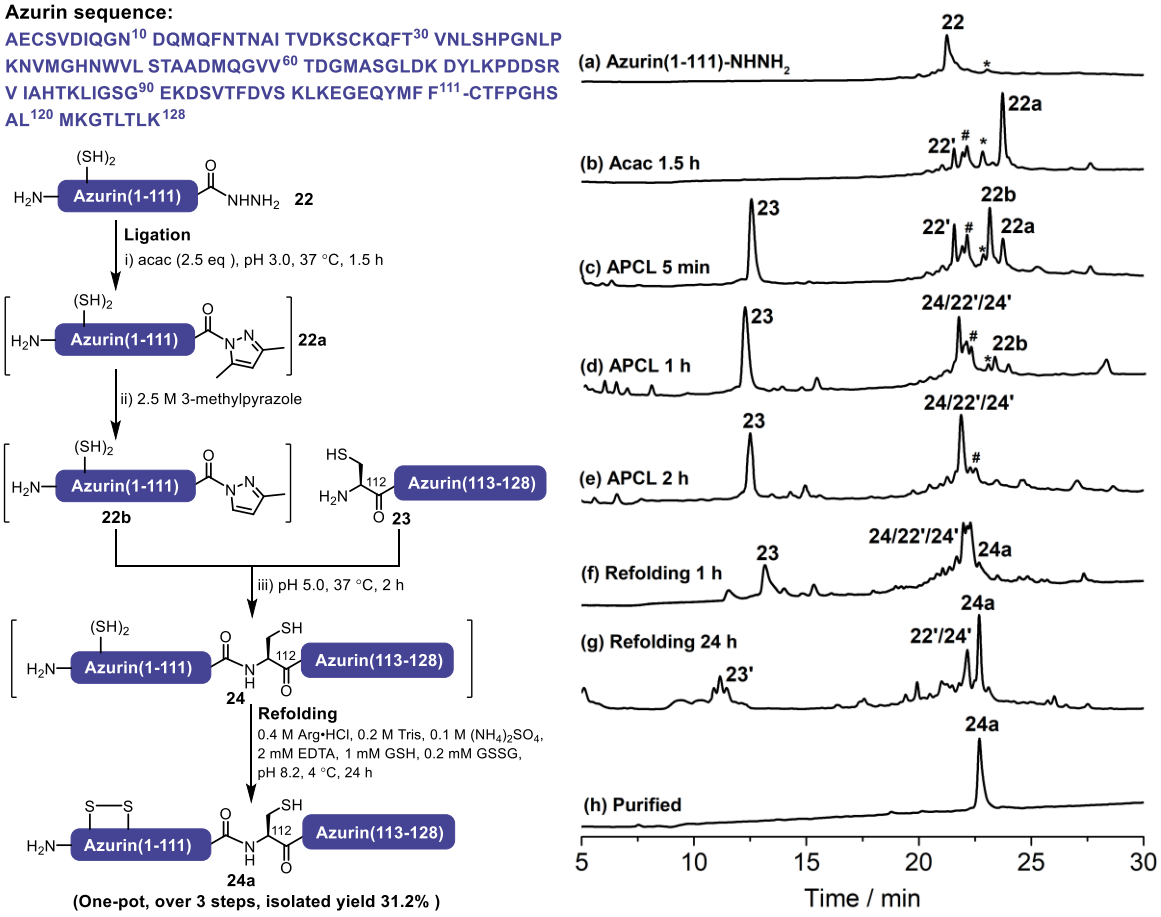
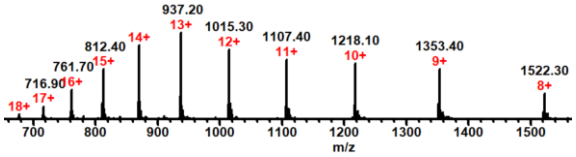
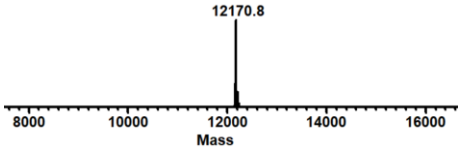
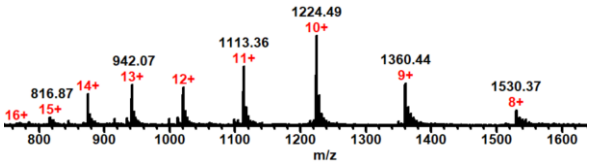
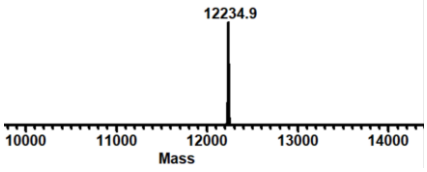
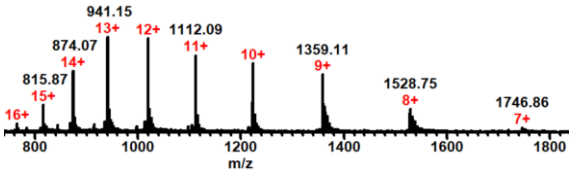
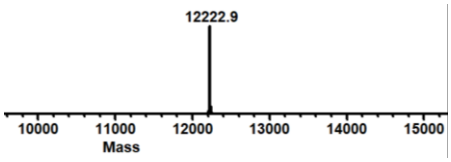
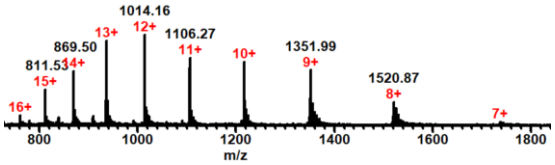
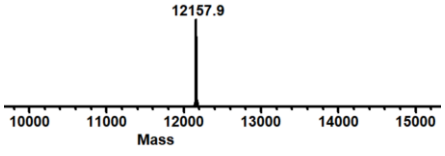
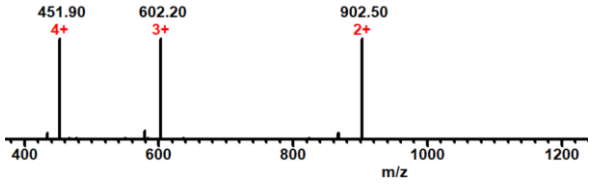
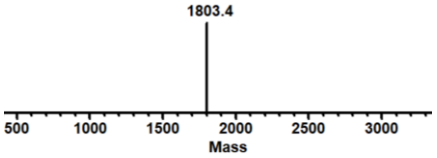
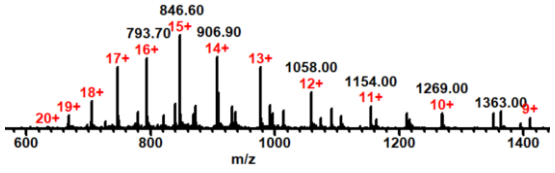
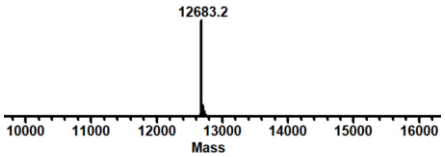
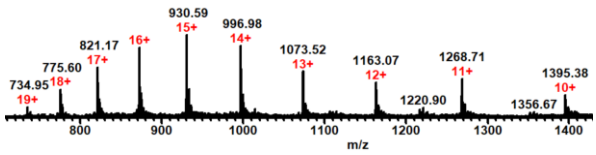
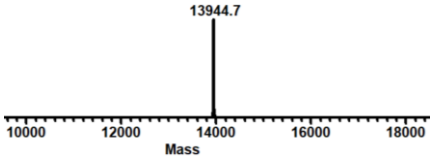
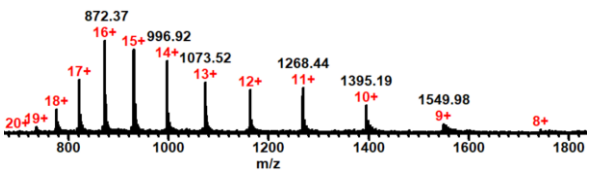
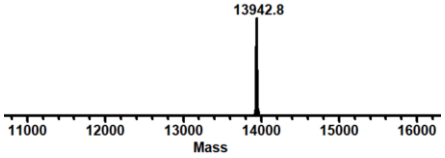


Figure S60. Analytical HPLC traces (20% to 50% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of synthesis of azurin (**24a**) via one-pot ligation-refolding. Note: (a-b) Acac activation: **22a** is azurin(1–111)-acylpyrazole and **22'** is hydrolysis by-product. The peak ***** is non protein and peak # denotes hydrazone intermediate. (c-e) The APCL at 5 min, 1 h and 2 h, respectively. **22b** is 3-methylpyrazole substituted intermediate, **24** is ligation product and **24'** is truncated versions of **24** at Met13. (f-g) The refolding at 24 h: **23'** is the oxidized by-products of the remaining **23** and **24a** is the folded product. (h) The purified folded protein **24a**.

Table S5. Characterization of the reaction from the initial substrate **22** and **23** to the final folded product **24a** through ES-IMS and corresponding deconvolution results.

Protein	ESI-MS	Deconvolution (Da)	Calcd. (Da)
22			12169.9
22a			12233.9
22b			12222.8
22'			12158.7
23			1803.0
24'			12684.6
24			13944.7
24a			13942.8

9.3 Fluorescence emission spectra of azurin

It has been reported in the literature that the single tryptophan (Trp48) residue in azurin was deeply buried in the interior of the protein. As a consequence, the fluorescence emission maximum was strongly blue-shifted, from about 350 nm for tryptophan exposed to water to about 310 nm and this characteristic shift was used as a basis for judging the refolding of azurin.[31] Here, the folded azurin(1-128) (**24a**) was dissolved in a buffer A (50 mM CH_3COONH_4, pH 5.1) (10 μM, 400 μL). And the linear azurin(1–128) (**24**) was dissolved in a buffer B (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 5.0) (10 μM, 400 μL). The characteristic shift of the synthetic folded azurin(1–128) (**24a**) and linear azurin(1-128) (**24**) were used Hitachi F-7000 spectrofluorometer with an excitation wavelength of 295 nm at room temperature, while the excitation and emission band-passes were 5 and 2.5 nm, respectively. The emission spectra were recorded at 200–700 nm with the scan speed of 1200 nm/min. The fluorescence emission results showed that linear to folded azurin were detected with a characteristic blue shift from 350 nm to 308 nm, which was consistent with the literature.[31]

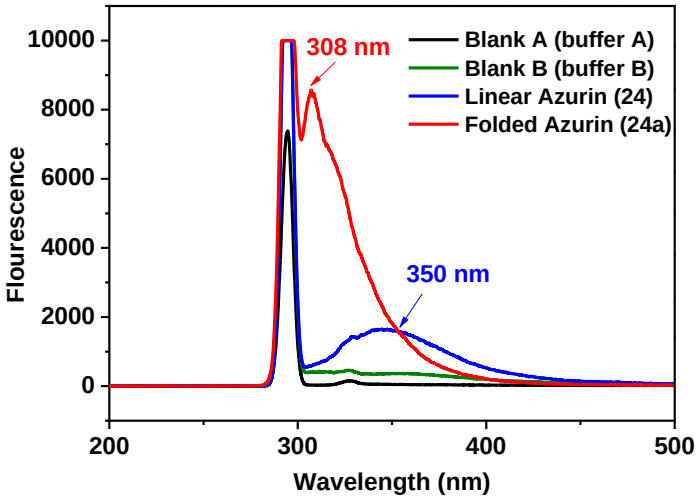


Figure S61. Fluorescence emission spectra of the linear azurin (**24**) and folded azurin (**24a**).

9.4 Enzyme digestion

The folded azurin (**24a**) was dissolved in a solution of 25 mM NH_4HCO_3, pH 8.0 (1 mg/mL, 50 µL). Trypsin (0.5 mg/mL, 5 µL) was firstly added to the mixture to digest the protein. The reaction was carried out at 37 °C for 2 h. Finally, the enzymatic reaction was analysed by LC-MS. As results showed that, after trypsin treatment of the protein azurin (**24a**), a peptide fragment bearing the Cys3–Cys26 disulfide bond can be clearly observed with mass 2975.3 Da (calculated mass 2975.3 Da); another peptide fragment containing Cys112 can be clearly observed a mass of 2223.0 Da (calculated mass 2223.0 Da), which agrees well with the reported structure of wild type azurin protein.

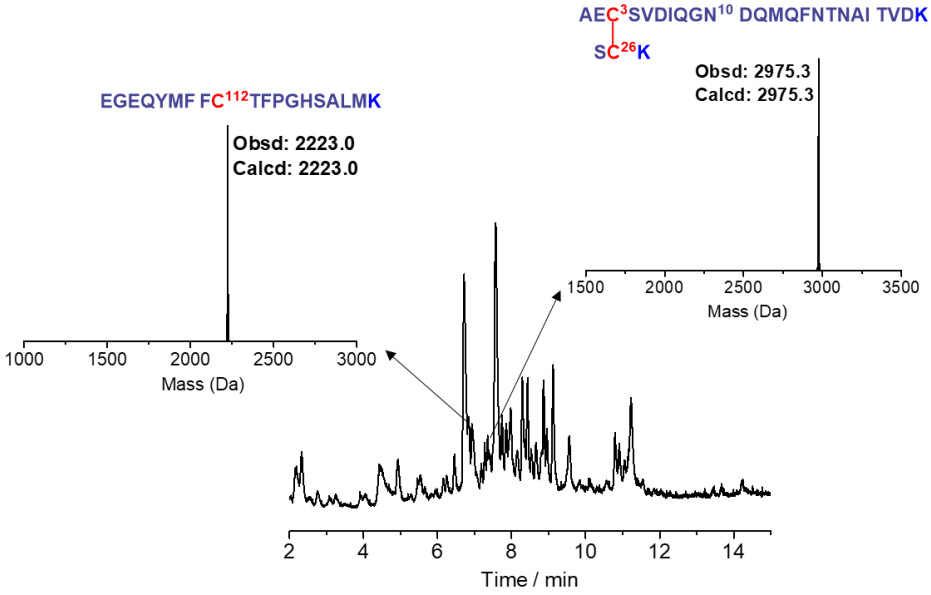


Figure S62. LC-MS analysis of the trypsin digest of folded azurin (**24a**).

9.5 The assay of Cu^{II} incorporation of azurin

The folded protein of azurin **24a** (0.12 mg, 0.0108 µmol) was dissolved in 0.4 mL of buffer (50 mM CH_3COONH_4, pH 5.1), followed by incremental addition of 1 mM $CuSO_4$, shaken for 5 min at 4 °C and monitored by UV-vis spectroscopy until saturation was measured near 625 nm. The UV-vis spectroscopy results showed that the characteristic Cu^{II} absorption peak at 627 nm (λ_{max} = 627 nm) was monitored, which was consistent with the literature [32, 33]. The mass of the azurin (**24a**) and after Cu^{II} incorporation of azurin **25** was confirmed using ESI-MS.

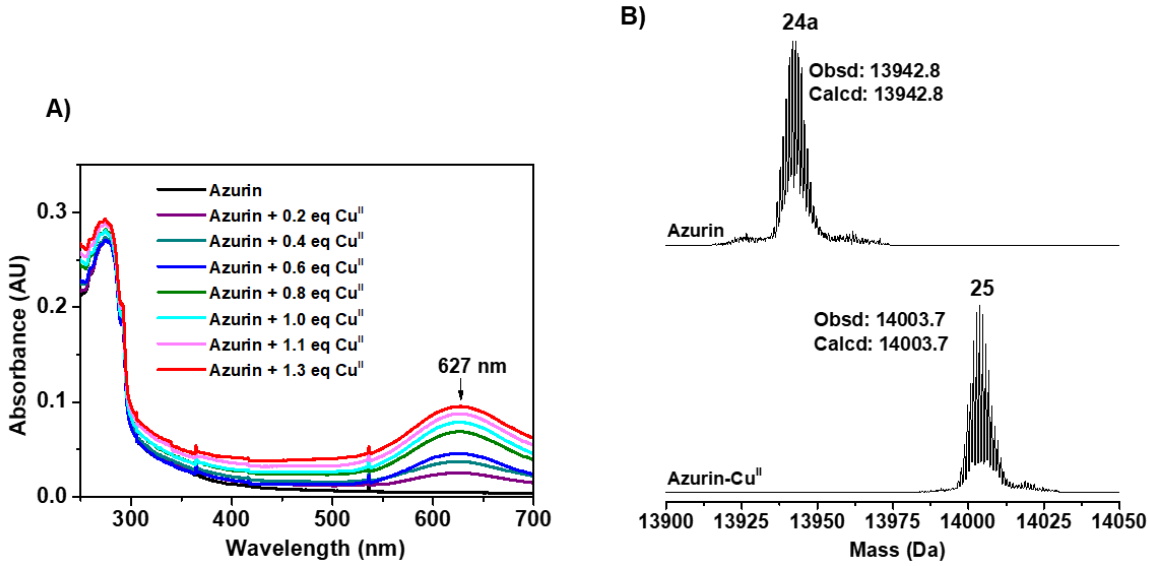


Figure S63. (A) The UV-visible absorption spectra traces of Cu^{II} incorporation of azurin. (B) Deconvolution ESI-MS analysis of the apo-protein **24a** and azurin-Cu^{II} **25**.

9.6 Circular dichroism (CD)

The secondary structure content of the synthetic azurin (**24a**) and azurin-Cu^{II} (**25**) using far-UV CD spectroscopy (190 to 260 nm). Spectra were recorded on a Chirascan™-Plus Circular Dichroism Spectrometer (Applied Photophysics Ltd, U.K.), using a quartz cuvette with a path length of 0.1 cm, and obtained by averaging 3 wavelength scans in 1 nm steps, with a signal averaging time of 1 s and a bandwidth of 1 nm. Each purified protein was dissolved separately in CH_3COONH_4 buffer. Measuring conditions: azurin (**24a**) conc.: ~20 µM; azurin-Cu^{II} (**25**) conc.: ~18 µM; Buffer: 50 mM CH_3COONH_4, pH 5.1. The proper folding of **24a** and **25** was confirmed by CD spectrum, which agrees well of reported literature.[31, 34]

Table S6. Secondary structure of the synthetic azurin (**24a**) and azurin-Cu^{II} (**25**).

Protein	α (%)	Antiparallel β (%)	Parallel β (%)	β-turn (%)	Other (%)
Wild-type azurin[a]	10	33	11	22	24
Wild-type azurin[b]	13	28	10	18	31
Synthetic azurin (**24a**)	12.5	31	9.6	15.2	31.7
Synthetic azurin-Cu^{II} (**25**)	12.4	31	9.6	15.3	31.7

[a] Values from the crystal structure,[34] b from the CD spectral data.[31]

10 Synthesis of the sulfated hirudin variant-1 (sY^{63}-HV1) via ligation-refolding using imidazole additive

10.1 Synthesis of the peptide fragments

*10.1.1 Synthesis and purification of of HV1(1–27) hydrazide fragment **26***

Peptide hydrazide (**26)** was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-NHNH$_2$ with 0.41 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H$_2$O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N$_2$. The precooled diethyl ether was added to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide hydrazide (**26**). The 200 mg of crude peptide hydrazide was dissolved in 20 mL of buffer (6 M Gdn•HCl, 0.2 M Na$_2$HPO$_4$, 20 mM TCEP, pH 3.0). The mixture was centrifuged (8000 rpm, 5 min, 4 °C) filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 20 to 40% MeCN (with 0.1% TFA) in 25 min to afford the desired fragment **26** as a white amorphous powder (25 mg, 6.1% isolated yield). The purity and mass of the peptide were confirmed using analytical HPLC (15 to 65% MeCN (with 0.1% TFA) in 15 min, λ = 214 nm) and ESI-MS, respectively. ESI-MS analysis of **26** shows the observed mass 2835.2 Da, calculated mass 2835.2 Da.

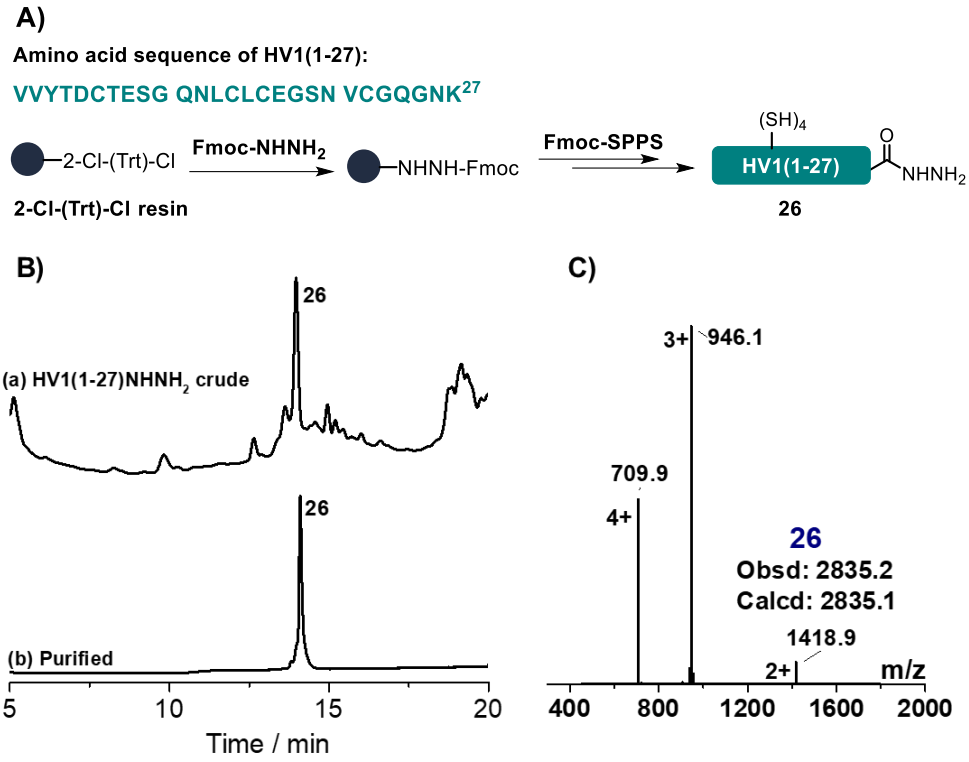


Figure S64. (A) Schematic representation of synthesis of HV1(1–27)-NHNH$_2$ fragment **26**. (B) Analytical HPLC traces (15 to 65% MeCN (with 0.1% TFA) in 15 min, λ = 214 nm) of crude and purified HV1(1–27)-NHNH$_2$ **26** (C) ESI-MS analysis of **26** shows the observed mass 2835.2 Da, calculated mass 2835.2 Da.

*10.1.2 Synthesis of HV1(28-65)-sY^{63} fragment **27***

Peptide fragment (**27)** was synthesized on Rink amide MBHA resin (theoretical loading: 1.2 mmol/g) using Fmoc-Gln(Trt)-OH with 0.47 mmol/g loading and elongated according to above standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H$_2$O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2.5 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N$_2$. The precooled diethyl ether was added to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide. The 500 mg of crude peptide was dissolved in 15 mL of buffer (6 M Gdn•HCl, 0.2 M Na$_2$HPO$_4$, 20 mM TCEP, pH 3.0). The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 15 to 50% MeCN (with 0.1% TFA) in 30 min to afford the desired fragment **27** as a white amorphous powder (25 mg, 4.9% isolated yield). The purity and mass of the peptide **27** were confirmed using analytical HPLC (15 to 50% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) and ESI-MS, respectively.

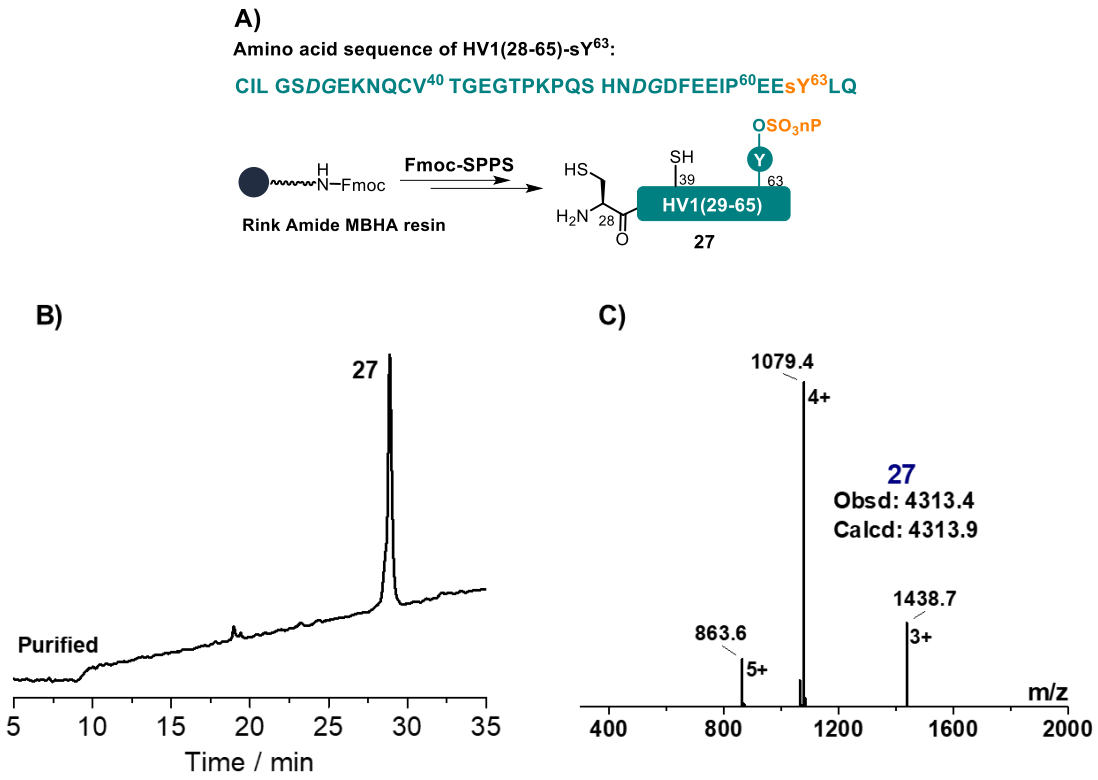


Figure S65. (A) Schematic representation of synthesis of HV1(28–65)-sY^{63} fragment **27**. (B) Analytical HPLC trace (15 to 50% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) of the purified HV1(28–65)-sY^{63} fragment **27**. (C) ESI-MS analysis of **27** with the observed mass 4313.4 Da, calculated mass 4313.9 Da.

10.2 Synthesis of sY^{63}-HV1 29 via one-pot ligation–refolding using imidazole additive (on a 1 μmol scale)

The peptide hydrazide HV1(1–27) (**26)** (1 μmol, 1 equiv, 2.84 mg) was dissolved in 0.2 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 20 mM TCEP, pH 3.0). Then, 1.2 μL of the 1 M acetylacetone stock solution (1.2 equiv, 1.2 μmol) was added into the mixture and incubated at 37 °C for 15 min (confirmed by HPLC monitoring). The Cys-peptide HV1(28-65)-sY^{63} (**27**) (0.7 μmol, 0.7 equiv, 3.02 mg, final conc. 2 mM) was dissolved in 0.15 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 20 mM TCEP, pH 3.0) and added into the mixture. Subsequently, 59.5 μL of imidazole stock solution (final conc. in 2.5 M, prepared as 1 mg/μL stock solution) was added. The pH of the mixture solution was adjusted to pH 6.5 and stirred at 37 °C (confirmed by HPLC monitoring). During ligation, nP-protected group of peptide **27** was auto-removed in ligation buffer and the de-nP peptide **27'** was formed.[29] At the beginning of ligation reaction, the nP-protected ligation product **28a** was formed, and then the nP was also auto-removed to obtain de-nP ligation product **28**.

After the ligation was completed (9 h), the reaction mixture was reduced by the addition of 2 equiv of TCEP buffer (prepared as 1 M stock solution, 2 μL) and incubated for 10 min. Then, 350 μL of the ligation mixture was exchanged into a 500 μL buffer containing 6 M Gdn•HCl, 0.2 M Na_2HPO_4 at pH 6 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 11000 rpm, 4 °C, 3 times). The resulting ligation mixture (~120 μL) was added dropwise to a 10 mL refolding buffer (0.2 M Tris, 4 mM *L*-Cysteine, 2 mM *L*-Cystine, 4 M NaCl, pH 8.5) (final protein conc. was ~0.25 mg/mL) at 4 °C in 30 min, and was stirred in a refrigerator at RT for 16 h. The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC with a two-step gradient: 10 to 20% MeCN in 3 min, then 20 to 40% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and lyophilized, affording the desired folded sY^{63}-HV1 (**29**) as a white powder (1.92 mg, 39.0% isolated yield over 3 steps). The purity and mass of the sY^{63}-HV1 (**29**) were confirmed using analytical HPLC (15 to 50% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) and ESI-MS, respectively.

Note: The imidazole was pre-dissolved in 12 M HCl to prepare a 1 mg/μL stock solution and then diluted in the appropriate volume of buffer A before being added to the ligation mixture.

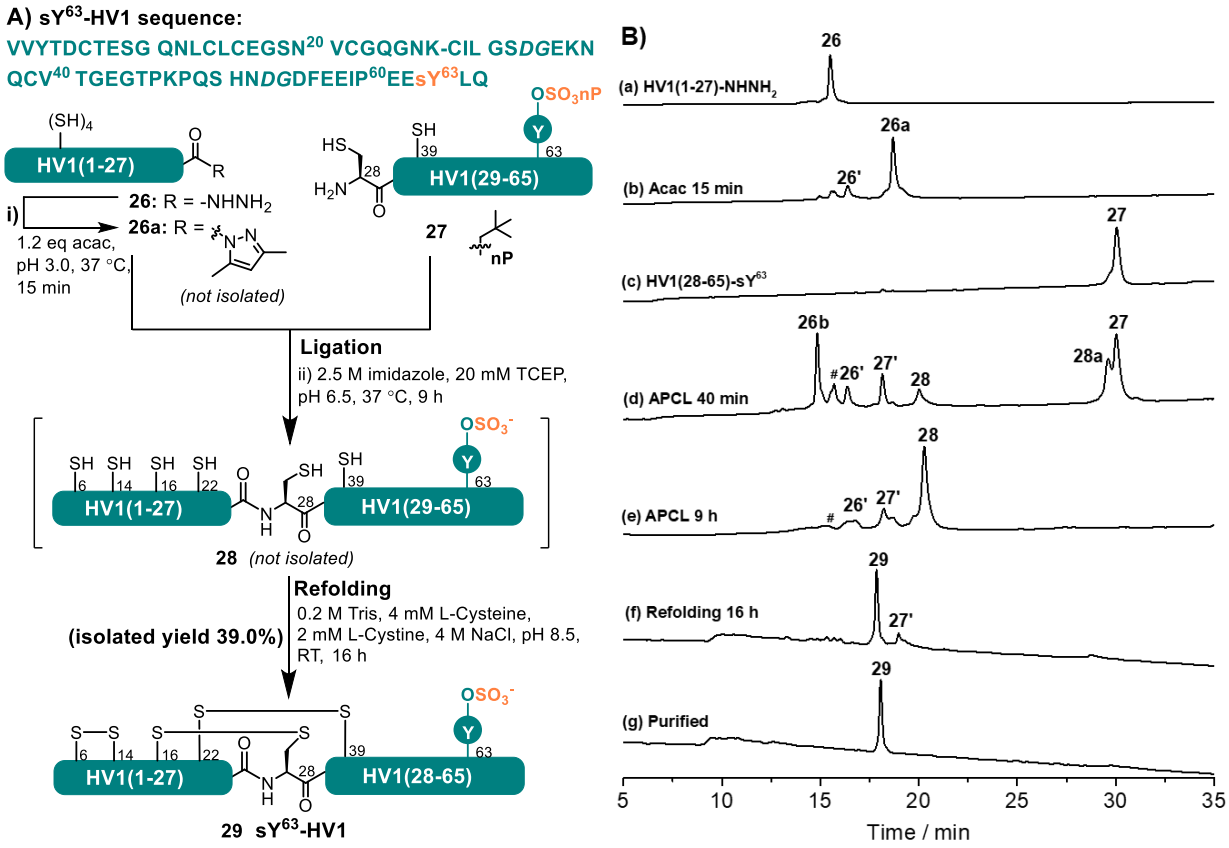


Figure S66. (A) Sequence and synthetic scheme for the sY^{63}-HV1 **29**. The ligation site is indicated with a dash and any dipeptides used are in italic. (B) Analytical HPLC traces (15% to 50% MeCN in 30 min (with 0.1% TFA), λ = 214 nm) of synthesis of folded sY^{63}-HV1 (**29**) via one-pot ligation and refolding. Note: (a-b) acac activation: **26a** is HV1(1-27)-acylpyrazole and **26'** is hydrolysis by-product. (c) The purified Cys-peptide HV1(28–65)-sY^{63} **27**. (d-e) APCL at 40 min and 9 h, respectively. **26b** is thiolactone resulted from the thiol-exchange between the C-acylpyrazole and thiol side-chains of **26a**. **27'** corresponds to nP removed derivative of **27**, **28a** is ligation product and **28** is nP removed product. The peak # is unknown by-products (observed mass: 2922.0 and 3048.0). (f) The refolding at 16 h: **29** is the folded product. (g) The purified folded protein **29**.

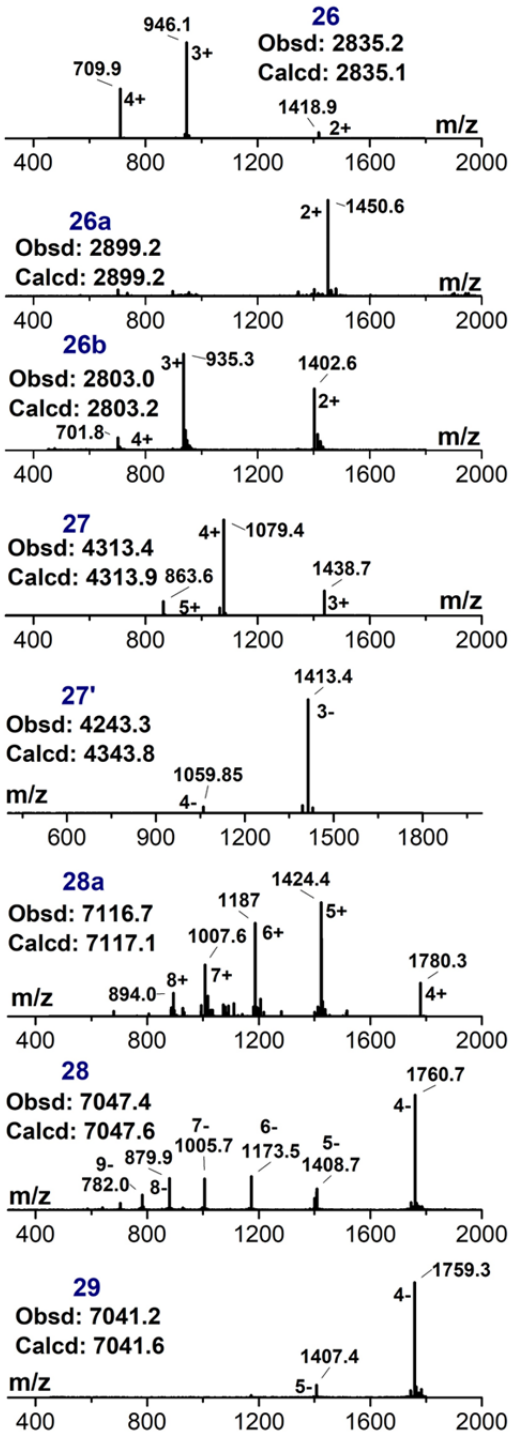


Figure S67. Characterization of the reaction from the initial substrate **26** and **27** to the final folded product **29** through ESI-MS.

10.3 Synthesis of sY^{63}-HV1 29 via ligation–refolding using MPAA method (on a 1 μmol scale)

The peptide hydrazide HV1(1–27) (**26)** (1 μmol, 1 equiv, 2.84 mg) was dissolved in 0.2 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 0.2 M MPAA, 40 mM TCEP, pH 3.0). Then, acetylacetone (2.5 equiv, 2.5 μmol, prepared as 1 M stock solution, 2.5 μL) was added into the reaction mixture and incubated at 37 °C for 2 h (confirmed by HPLC monitoring). The Cys-peptide HV1(28–65)-sY^{63} (**27**) (0.7 μmol, 0.7 equiv, 3.02 mg, 2 mM) was dissolved in 0.15 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 0.2 M MPAA, 40 mM TCEP, pH 6.5) and added into the mixture. The pH of the mixture solution was adjusted to pH 6.5, and the mixture was stirred at 37°C (confirmed by HPLC monitoring). During ligation, nP-protected group of **27** was auto-removed in ligation buffer and the de-nP peptide **27'** was formed. At the beginning of ligation reaction, the nP-protected ligation product **28a** was formed, and then the nP was also auto-removed to obtain de-nP ligation product **28**.

After the ligation was completed (14 h), the resulted mixture was reduced by the addition of 2 equiv of TCEP buffer (prepared as 1 M stock solution, 2 μL) and incubated for 10 min. Then, 350 μL of the ligation mixture was exchanged into a 500 μL buffer containing 6 M Gdn•HCl, 0.2 M Na_2HPO_4 at pH 6 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 11000 rpm, 4 °C, ultrafiltration ≥ 8 times). The resulting ligation mixture (~150 μL) was added dropwise to a 10 mL refolding buffer (0.2 M Tris, 4 mM *L*-Cysteine, 2 mM *L*-Cystine, 4 M NaCl, pH 8.5) (final protein conc. was ~0.25 mg/mL) at 4 °C in 30 min, and was stirred in a refrigerator at RT for 16 h. The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC with a two-step gradient: 10 to 20% MeCN in 3 min, then 20 to 40% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and lyophilized, affording the folded sY^{63}-HV1 (**29**) as a white powder (1.35 mg, 27.5% isolated yield over 3 steps). The purity and mass of the sY^{63}-HV1 (**29**) were confirmed using analytical HPLC (15 to 50% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) and ESI-MS, respectively.

Compared to the imidazole-assisted one-pot ligation-refolding synthesis of the protein sY^{63}-HV1, the MPAA method was more time-consuming in the thioester conversion and ligation process, and the removal of the large amounts of remaining MPAA and TCEP from the mixture requires more than eight solution changes by high-speed ultracentrifugation, resulting in a complex operation and lower isolated yield.

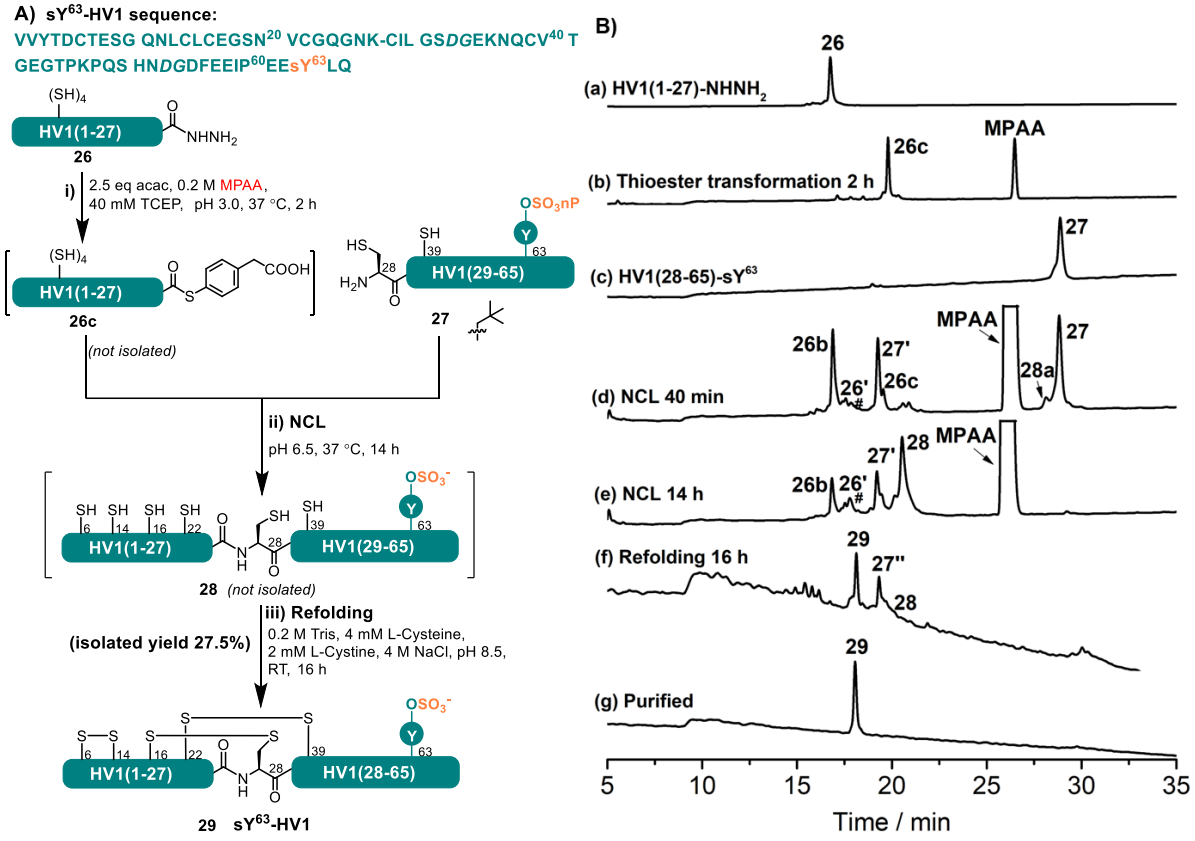


Figure S68. (A) Schematic representation of synthesis of folded sY^{63}-HV1 (**29**) using MPAA method. (B) Analytical HPLC traces (15% to 50% MeCN in 30 min (with 0.1% TFA), λ = 214 nm) of synthesis of sY^{63}-HV1 (**29**) via one-pot ligation and refolding. Note: (a-b) acac activation: **26** is HV1(1-27)-$NHNH_2$, **26c** is HV1(1–27)-thioester; (c-e) ligation reaction: **26b** is thiolactone derived from the thiol-exchange between the C-thioester and thiol side-chains of **26c**. **27'** corresponds to nP removed derivative of **27**, **28a** is the ligation product and **28** is nP removed product. The peak **#** is unknown by-products (observed mass: 2922.0 and 3048.0); (f) refolding reaction: **29** is folded product and **27''** is SO_3np group removed derivative of **27'**; (g) the purified folded **29**.

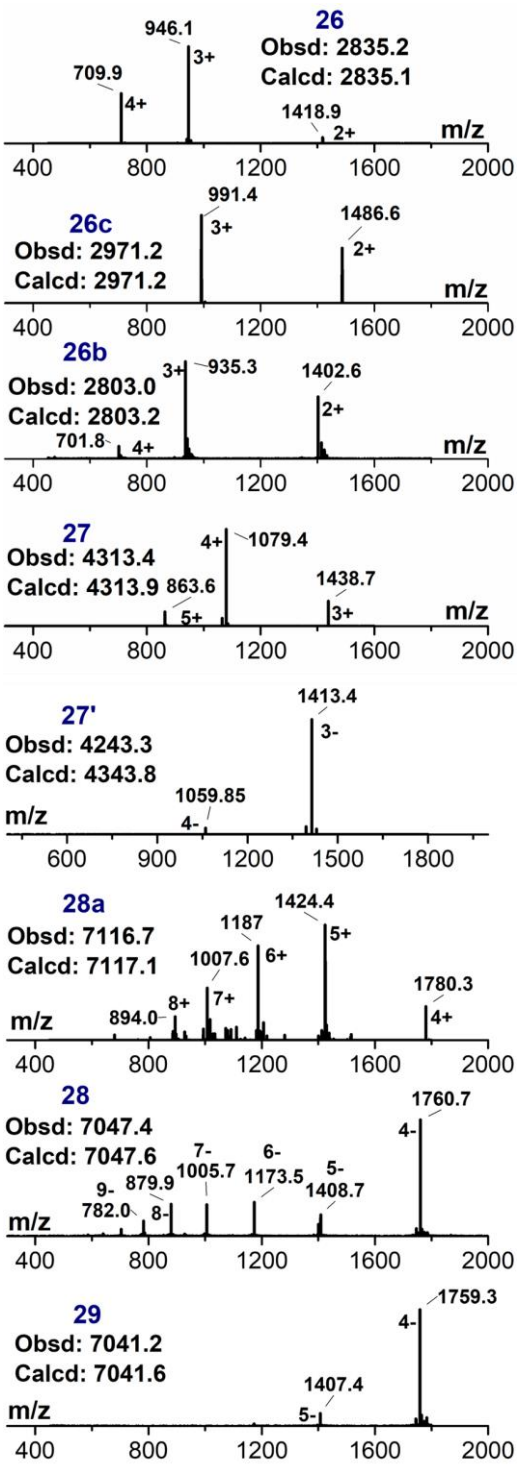


Figure S69. Characterization of the reaction from the initial substrate **26** and **27** to the final folded product **29** through ESI-MS.

11 Synthesis of the cyclic protein kalata B1 (kB1) via one-pot cyclization-refolding using 3-methylpyrazole additive

11.1 Cyclization of model peptide hydrazide 15

The peptide hydrazide **15** (7.2 µmol, 1 equiv, 8.33 mg) was dissolved in 2.85 mL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 1.85 µL of acetylacetone (18.0 µmol, 2.5 equiv) was added into the reaction mixture and incubated at 37 °C for 20 min. The resulting solution was divided into two portions, which were put in the following conditions: (a) 2.5 M 3-methylpyrazole, 20 mM TCEP, 2 mM peptide **15a** (final cyclization conc.), pH 5.0, 37 °C, 2 h; (b) 2.5 M imidazole (prepared as 1 mg/µL stock solution), 20 mM TCEP, 2 mM peptide **15a** (final cyclization conc.), pH 6.5, 37 °C, 2 h, respectively. The each of reaction mixture was analyzed by analytical HPLC at 25 °C with a gradient of 15 to 50% MeCN (with 0.1% TFA) in 20 min. Finally, each mixed solution was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 20 to 50% MeCN (with 0.1% TFA) in 25 min to obtain 2.06 mg of ligation product (**15c**) (condition (a), yield 77.4%) and 2.03 mg of ligation product (**15c**) (condition (b), yield 76.0%), respectively. The purity and mass of the peptides **15c** were confirmed using analytical HPLC and ESI-MS, respectively.

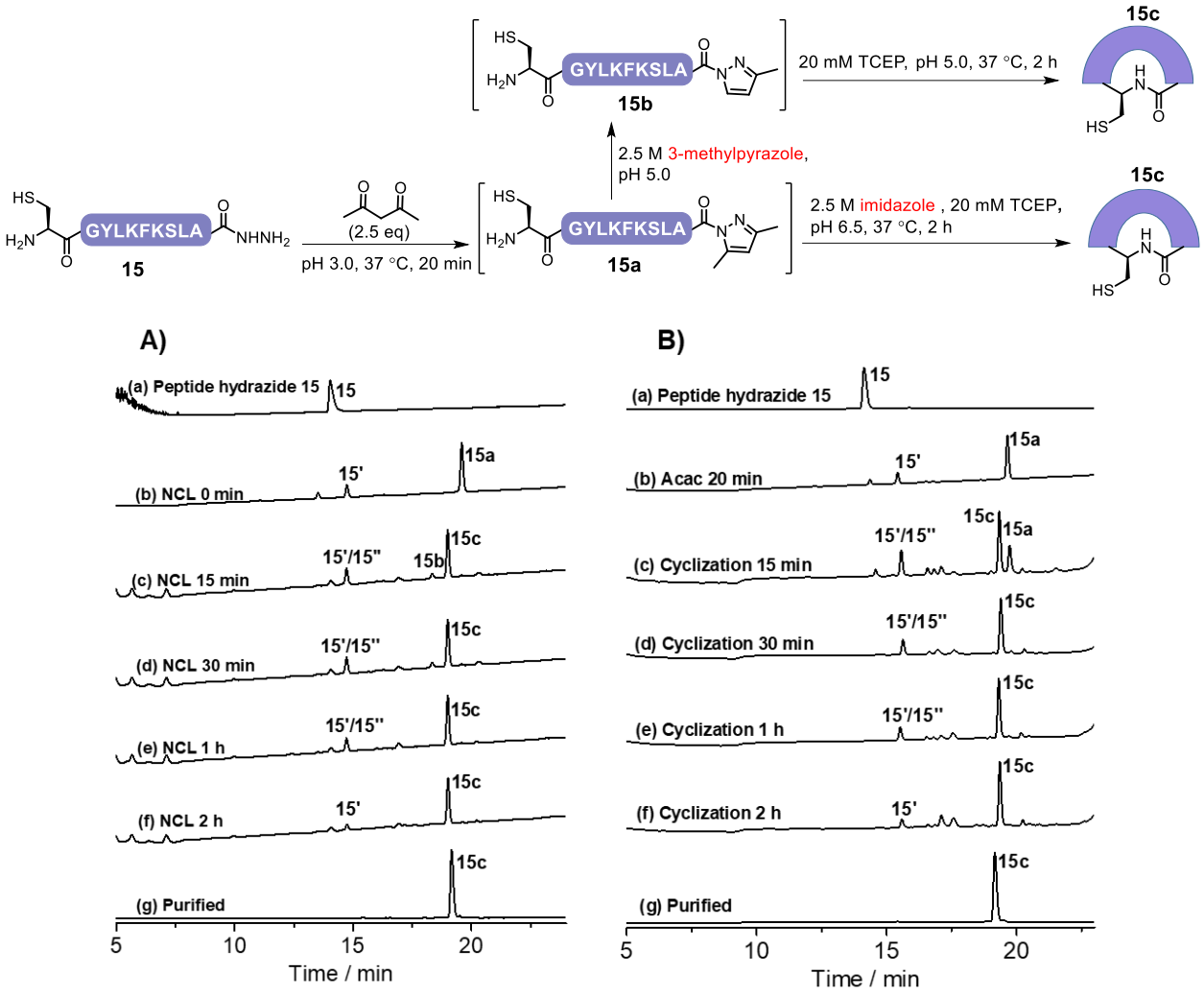


Figure S70. (A-B) Analytical HPLC traces (15% to 50% MeCN in 20 min (with 0.1% TFA), λ = 214 nm) of cyclization reactions of model peptide **15** using 3-methylpyrazole (A) and imidazole (B). **15a** is peptide-acylpyrazole and **15b** is 3-methylpyrazole substituted intermediate. **15c** is ligation product, **15'** is hydrolysis by-product and **15''** denotes thiolactone.

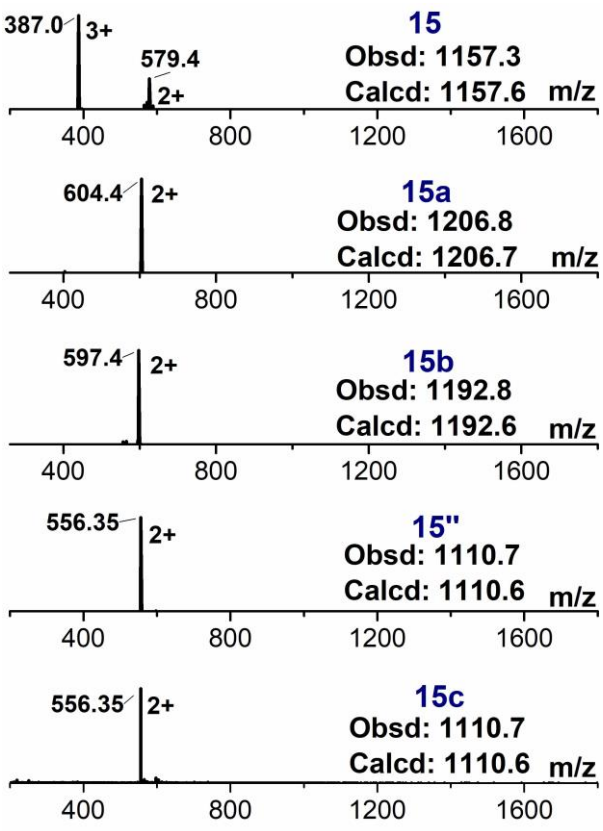


Figure S71. Characterization of the reaction from the initial substrate **15** to the final ligation product **15c** through ES-IMS.

11.2 Synthesis of cyclic protein kB1 via a one-pot cyclization–refolding

11.2.1 Synthesis of the peptide hydrazide kB1-NHNH$_2$ ***30***

Peptide hydrazide (**30)** was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-NHNH$_2$ with 0.3 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H$_2$O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. The precooled diethyl ether was added to precipitate crude peptide. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide hydrazide (**30**). The 180 mg of crude peptide hydrazide was dissolved in 20 mL of buffer (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 20 mM TCEP, pH 3.0). The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC at 25 °C with a gradient of 20 to 45% MeCN (with 0.1% TFA) in 25 min to afford the desired peptide **30** as a white amorphous powder (15 mg, 4.7% isolated yield). The purity and mass of the peptide were confirmed using analytical HPLC (20 to 51% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) and ESI-MS, respectively.

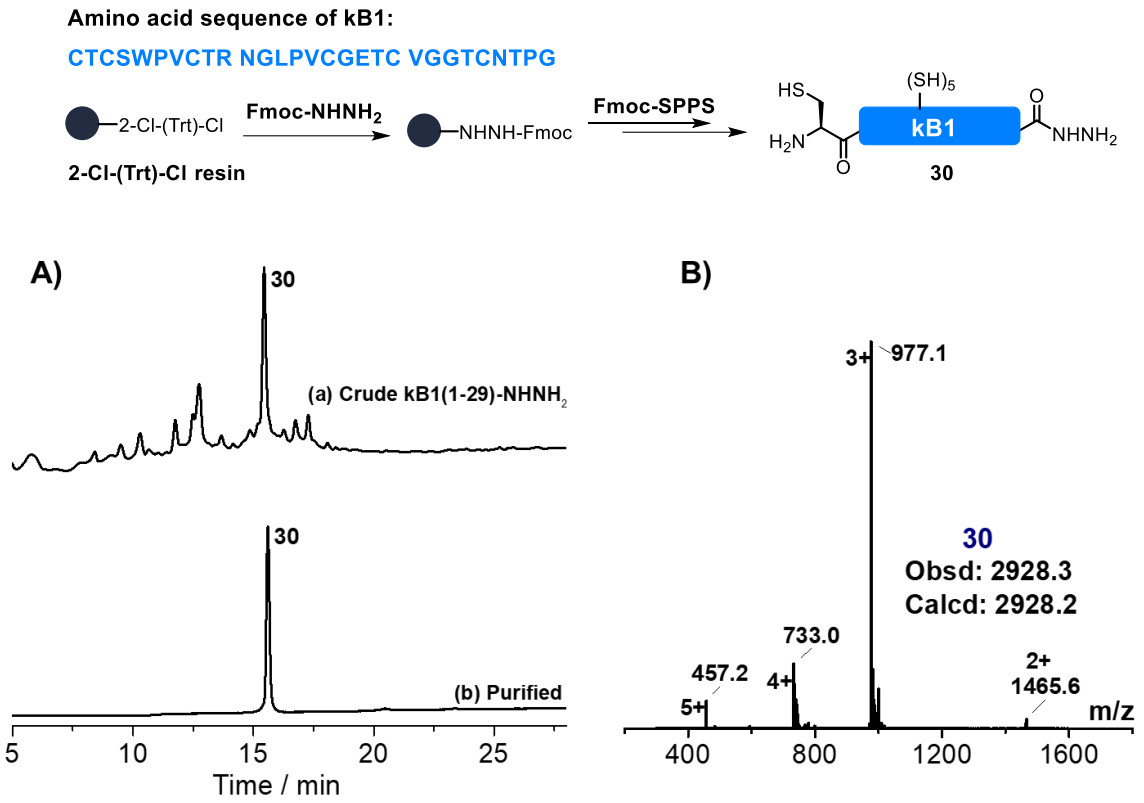


Figure S72. (A) Analytical HPLC traces (20 to 51% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of the crude and purified kB1(1-29)-NHNH$_2$ **30**. (B) ESI-MS analysis of **30** shows the observed mass 2928.3 Da, calculated mass 2928.2 Da.

11.2.2 Synthesis of cyclic peptide kB1 via a one-pot cyclization–refolding using 3-methylpyrazole without TCEP

The peptide kB1-NHNH$_2$ (**30)** (1 μmol, 1 equiv, 2.95 mg) was dissolved in 380 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 3.0). Then, 15 μL of 0.1 M acetylacetone solution (1.5 μmol, 1.5 equiv) was added into the mixture and incubated at 37 °C for 2 h. Subsequently, 102.5 μL of 3-methylpyrazole (2.5 M, final conc.) was added. After adjusting the pH to 5.0 by using 1 M NaOH, the mixture was incubated at 37 °C for 3 h (confirmed by HPLC monitoring).

After the cyclization was completed (3 h), the resulting mixture (0.5 mL) was added dropwise to a 8.3 mL refolding buffer[35] (50 mM NH_4HCO_3, 50 %(v/v) *i*-PrOH, 1 mM GSH, pH 7.5) (final protein conc. was ~0.35 mg/mL) at 4 °C in 30 min, and was stirred in a refrigerator at 4 °C for 15 h. The resulting mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC with a gradient: 20 to 51% MeCN (with 0.1% TFA) in 25 min to collect the desired fractions and lyophilized, affording desired folded kB1 (**32**) as a white powder (1.98 mg, 68.5% isolated yield, over 3 steps in one-pot). The reaction traces and mass of the peptide were confirmed using analytical HPLC (20 to 51% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) and ESI-MS, respectively.

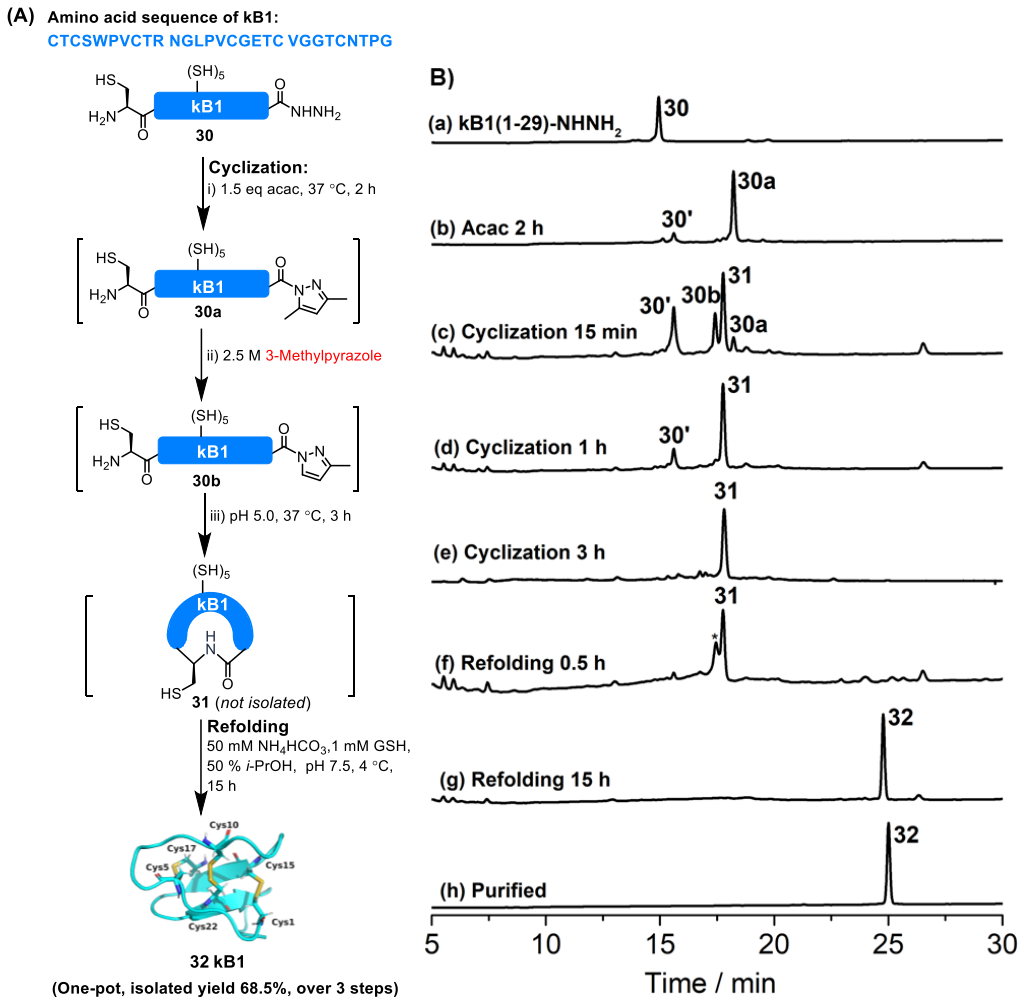


Figure S73. (A) Sequence and synthetic scheme for cyclic kB1 (**32**) via one-pot ligation–refolding using 3-methylpyrazole additive without TCEP. (B) Analytical HPLC traces (20% to 51% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of reaction from the initial substrate **30** to the final product kB1 **32**. Note: (a-b) acac activation: **30** is kB1-$NHNH_2$, **30a** is kB1-acylpyrazole and **30'** is thiolactone. (c-e) Cyclization at 15 min, 1 h and 3 h, respectively. **30b** is 3-methylpyrazole substituted intermediate and **31** is the cyclization product. (f-g) The refolding at 0.5 h and 15 h, respectively. The peak * denotes the refolding intermediates and **32** is the folded product. (h)The purified folded product **32**.

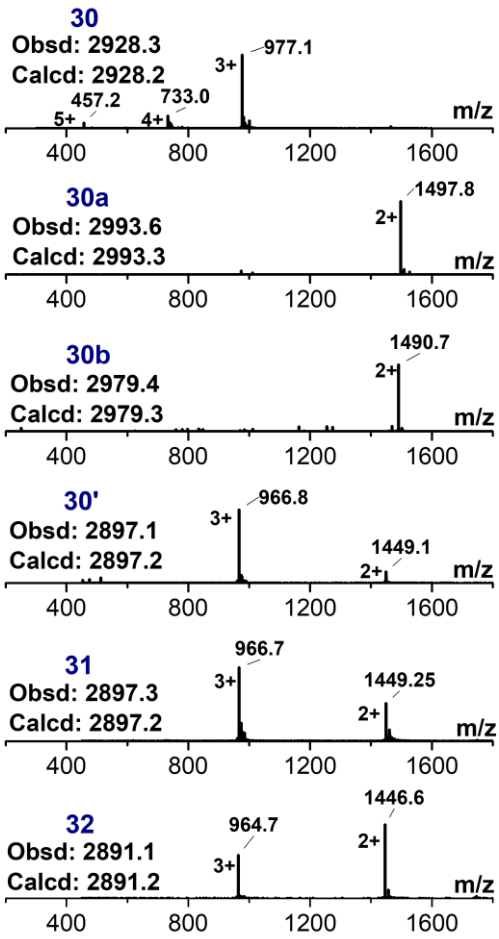


Figure S74. Characterization of the reaction from the initial substrate **30** to the final folded product kB1 **32** through ESI-MS.

11.3 Synthesis of cyclic peptide kB1 using MPAA method

The peptide kB1-$NHNH_2$ (**30)** (1 μmol, 1 equiv, 2.95 mg) was dissolved in 480 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, 0.2 M MPAA, 40 mM TCEP, pH 3.0). Then, acetylacetone (1.5 μmol, 1.5 equiv, a 0.1 M stock solution, 15 μL) was added into the reaction mixture and incubated at 37 °C for 3 h. Subsequently, the pH of the peptide thioester mixture was adjusted to 6.5 by using 1 M NaOH, and the resulting mixture was incubated at 37 °C for 3 h (confirmed by HPLC monitoring). After the cyclization was completed (3 h), the resulting mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC with a gradient: 20 to 51% MeCN (with 0.1% TFA) in 25 min to collect the desired fractions and lyophilized, affording the desired cyclization kB1 (**31**) as white powder (1.82 mg, 62.8% isolated yield, over 2 steps)

The purified cyclic peptide kB1 (**31**) powder was dissolved in 100 μL of buffer A (6 M Gdn•HCl, 0.2 M Na_2HPO_4, pH 6.0) and added dropwise to a 5.2 mL refolding buffer (50 mM NH_4HCO_3, 50 %(v/v) *i*-PrOH, 1 mM GSH, pH 7.5) (final protein conc. was ~0.35 mg/mL) at 4 °C in 30 min. Then, the resulting mixture was stirred in a refrigerator at 4 °C for 30 h. After the refolding reaction was completed, the resulting mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC with a gradient: 20 to 51% MeCN (with 0.1% TFA) in 25 min to collect the desired fractions and lyophilized, affording the desired folded kB1 (**32**) as a white powder (0.8 mg, 43.8% isolated yield). The reaction traces and mass of the peptide were confirmed using analytical HPLC (20 to 51% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) and ESI-MS, respectively.

It was found that the modified 3-methylpyrazole method has great advantages over the conventional MPAA method for the one-pot synthesis of Cys-rich cyclic peptide kB1, with simple operation (no thioester required), fewer purification steps, and a greatly improved overall yield (total yield of 68.5% vs. 27.5% for the MPAA method).

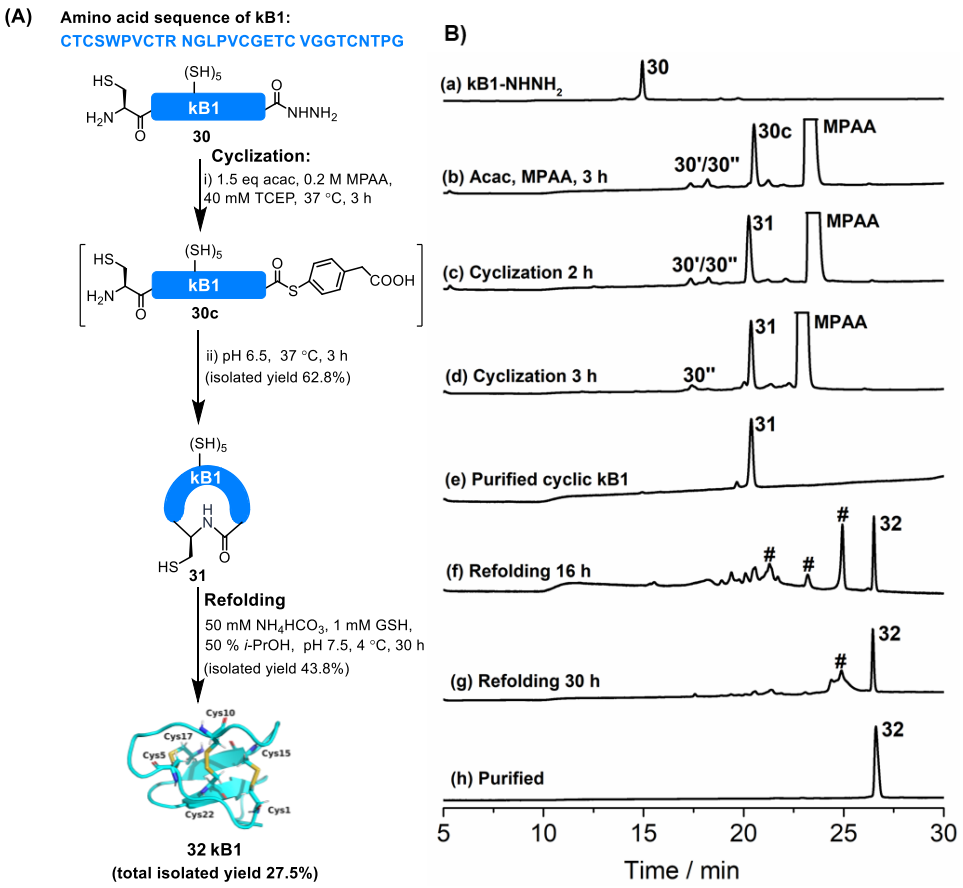


Figure S75. (A) Schematic representation of synthesis of kB1 (**32**) via ligation–purification-refolding using MPAA method. (B) Analytical HPLC traces (20% to 51% MeCN in 25 min (with 0.1% TFA), λ = 214 nm) of reaction from the initial substrate **30** to the final product kB1 **32**. Note: (a-b) thioester transformation: **30** is kB1-$NHNH_2$, **30c** is peptide thioester and **30''** is hydrolysis by-product. (c-d) Cyclization reaction: **30'** is thiolactone and **31** is the cyclization product. (e) The purified cyclization product **31**. (f-g) Refolding reaction: peak # denotes the refolding intermediate and **32** is the folded product. (h) The purified folded product **32**.

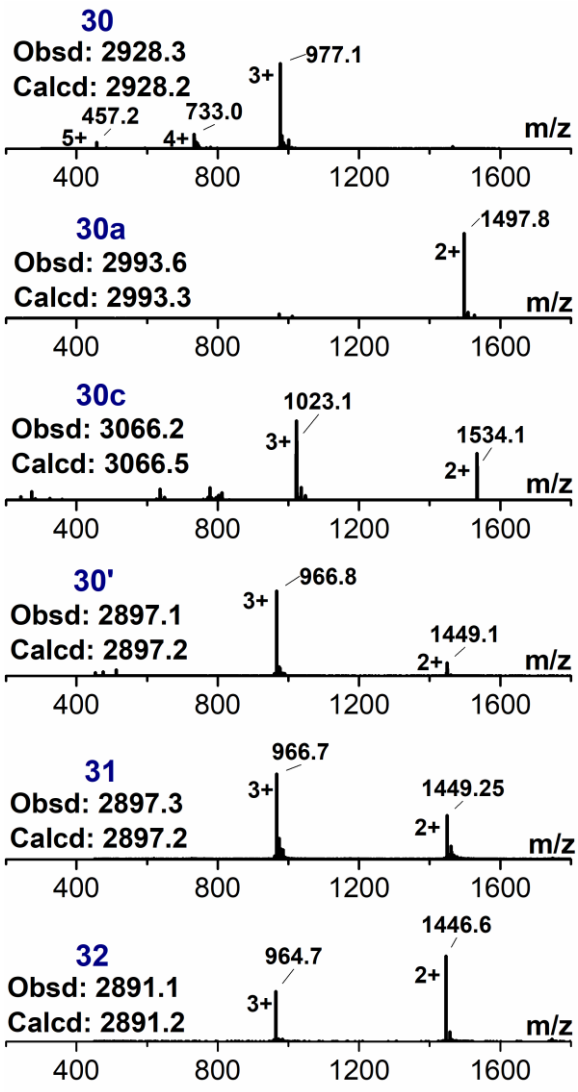


Figure S76. Characterization of the reaction from the initial substrate **30** to the final folded product kB1 **32** through ESI-MS.

11.4 Circular dichroism (CD)

The secondary structure content of the synthetic kB1 (**32**), obtained by APCL method, using far-UV CD spectroscopy (190 to 260 nm). Spectra were recorded on a Chirascan™-Plus Circular Dichroism Spectrometer (Applied Photophysics Ltd, U.K.), using a quartz cuvette with a path length of 0.1 cm, and obtained by averaging 3 wavelength scans in 1 nm steps, with a signal averaging time of 1 s and a bandwidth of 1 nm. Each purified protein was dissolved separately in NH_4HCO_3 buffer. Measuring conditions: kB1 (**32**) conc.: ~17 µM; Buffer: 25 mM NH_4HCO_3, pH 8. The proper folding of **32** was confirmed by CD spectrum, which agrees well of reported literature.[35]

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