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Supporting information

Efficient Peptide Ligation between Allyl-protected Asp and Cys Followed by Palladium-mediated Deprotection

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Experimental

General methods and materials. MALDI-TOF mass spectra were recorded with microflex (BRUKER), using Protein Calibration Standard 2 as an external standard. Reversed-phase HPLC was performed on a 5C_{18}-AR-II and Protein-R column (Nacalai tesque, 4.6ID and 20ID × 250 mm for analysis and purification, respectively) with a PU-2080 plus Intelligent HPLC Pump (JASCO) and MD-2018 plus Photodiode Array Detector (JASCO) at 195 to 650 nm. All solvents and reagents were commercially available and used without further purification. All peptides were synthesized using Intavis ResPep SL (Intavis). Amino acids protected by 9-fluorenylmethoxycarbonyl (Fmoc) group were coupled with *O*-(1H-Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) as activator and 4-methylmorpholine (NMM) as base. The isolated yields of each peptide were estimated by using the molecular weights of TFA salt at Arg, Lys and His positions.

Synthesis of peptide fragment 1 and 2. To prepare the C-terminal hydrazide peptide, 2-Cl-(Trt)-Cl resin (10 μmol scale) was used. The preparation procedure was followed as described in previous report (J. Zheng, S. Tang, Y. Qi, Z. Wang, L. Liu, *Nat. Prot.*, 2014, **8**, 2483-2495). Briefly, the resin was swelled in 50% DCM/DMF for 30 min. After removing the solvent, 5% hydrazine in DMF (400 μL) was added to the resin and the mixture was agitated for 1 h at r.t. then the solvent was drained and washed by DMF. This operation was conducted again, and the resin was washed by DMF, DCM and DMF. Next, 5% MeOH/DMF (400 μL) was added and stirred for 10 min. After removing the solvent, the resin was washed by DMF, DCM and DMF. Immediately, to the resin were added Fmoc-Asp(tBu)-OH or Fmoc-Asp(OAllyl)-OH (4 equiv), HBTU (3.8 equiv) and DIEA (8 equiv). The mixture was stirred for 60 min, and then washed by DMF, DCM and DMF three times respectively. After automated SPPS, the peptide was cleaved by cleavage cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole). The mixture was rotated at room temperature for 2 h under nitrogen atmosphere, and filtered to remove the resin. Then ether (10 mL) was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. Obtained crude peptides were purified by RP-HPLC and identified by MALDI-TOF mass spectrometry. The isolated yields of peptide **1**, **2** were 19%, 27%, respectively.

Synthesis of peptide fragment 3. Fmoc-Gly-Alko resin (0.23 mmol/g, 10 μmol scale, Watanabe Chemical Industries.) was used for C-terminal carboxyl peptides. After automated SPPS, the peptide was cleaved by cleavage cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole). The mixture was rotated at r.t. for 2 h under nitrogen atmosphere, and filtered to remove the resin. Then ether (10 mL) was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. Obtained crude peptides were purified by RP-HPLC

and identified by MALDI-TOF mass spectrometry. The isolated yield of peptide **3** was 70%.

Native chemical ligation. To the solution of peptide **1** or **2** in 6 M Gn-HCl and 0.2 M NaH_2PO_4 (pH 3.0) was added the solution of $NaNO_2$ (10 equiv) in water under −15 °C, and the mixture was stirred for 15 min. To the mixture were added the solution of peptide **3** (1.2 equiv) and MPAA (50 equiv) in 6 M Gn-HCl and 0.2 M NaH_2PO_4 (pH 6.5) and the value of pH of the mixture was adjusted to pH 6.5−6.7 and the reaction mixture was stirred for 1 h at room temperature. The reaction was monitored by RP-HPLC, and the ligation products were identified by MALDI-TOF mass spectrometry.

Preparation of 100 mM Pd/TPPTS complex solution. To the solution of 15.9 μl $Pd(OAc)_2$ in degassed DMF (about 188 mM), 14.1 μL of 850 mM TPPTS aq. was added and the mixture was vigorously vortexed for 30 s under argon atmosphere. The solution can be stored for 1 day at −15 °C under argon atmosphere.

Deprotection of Asp(OAllyl) in NCL buffer. Peptide was dissolved in NCL buffer (6 M Gn-HCl, 0.2 M NaH_2PO_4, 100 mM MPAA, 40 mM TCEP and 20 mM $NaNO_2$) under argon atmosphere and the concentration of peptide was 2 mM. 100 mM Pd/TPPTS solution was added to the peptide solution and the mixture was stirred for 10 min under argon atmosphere at room temperature. The solutions of 6 M Gn aq. (15 μL) and 1 M DTT aq. (2 μL) were added to 2 μL of the reaction solution and the mixture was injected to RP-HPLC for the reaction tracking.

Synthesis of fluorescein-labeled PEP-19.

1) Synthesis of peptide fragments 6. To prepare the C-terminal hydrazide peptide, 2-Cl-(Trt)-Cl resin (20 μmol scale) was used. Briefly, the resin was swelled in 50% DCM/DMF for 30 min. After removing the solvent, 5% hydrazine in DMF (400 μL) was added to the resin and the mixture was agitated for 1 h at room temperature then the solvent was drained and washed by DMF. This operation was conducted again, and the resin was washed by DMF, DCM and DMF. Next, 5% MeOH/DMF (400 μL) was added and stirred for 10 min. After removing the solvent, the resin was washed by DMF, DCM and DMF. Immediately, to the resin were added Fmoc-Asp(OAllyl)-OH (4 equiv), HBTU (3.8 equiv) and DIEA (8 equiv). The mixture was stirred for 60 min, and then washed by DMF, DCM and DMF three times, respectively. As for Gly21, we incorporated Fmoc-(Dmb)Gly-OH instead of Fmoc-Gly-OH to avoid aspartimide formation during SPPS. After automated SPPS, coupling of alkyne derivatives to the N-terminal of the peptide was conducted with compounds TBS-COOH (4 equiv), HBTU (3.8 equiv) and DIEA (8 equiv). The mixture was rotated at room temperature for 1 h. Then the resin was washed by DMF and DCM. To the resin was added cleavage

cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole) and the mixture was gently stirred for 1.5 h. Then, 1% (w/v) of NBu_4I was added to the mixture to reduce oxidized Met and it was stirred for 30 min at room temperature and filtered to remove the resin. Then ether (10 mL) was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. Obtained crude peptides were purified by RP-HPLC and identified by MALDI-TOF mass spectrometry. The isolated yield of peptide **6** was 18%.

2) Synthesis of peptide fragment 7. Fmoc-Gly-Alko resin (0.23 mmol/g, 20 μmol scale, Watanabe Chemical Industries.) was used for C-terminal carboxyl peptides. After automated SPPS, the peptide was cleaved by a cleavage cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole). The mixture was rotated at room temperature for 2 h under nitrogen atmosphere, and filtered to remove the resin. Then ether (10 mL) was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. Obtained crude peptides were purified by RP-HPLC and identified by MALDI-TOF mass spectrometry. The isolated yield of peptide **7** was 26%.

3) Native chemical ligation and deprotection of Asp(OAllyl) in one pot. To the solution of peptide **6** in 6 M Gn-HCl and 0.2 M NaH_2PO_4 (pH 3.0) was added the solution of $NaNO_2$ (10 equiv) in water under −15 °C, and the mixture was stirred for 15 min. To the mixture were added the solution of peptide **7** (1.2 equiv) and MPAA (50 equiv) in 6 M Gn-HCl and 0.2 M NaH_2PO_4 (pH 6.5) and the value of pH of the mixture was adjusted to pH 6.5−6.7 and the reaction mixture was stirred under argon atmosphere for 1 h at RT. Then, 500 mM $MgCl_2$ and 6 N HCl were added to the reaction mixture for 5 min at r.t. To the mixture 100 mM Pd/TPPTS complex was added. Final concentration was peptide (2 mM), MPAA (100 mM), TCEP (40 mM), $MgCl_2$ (6 mM) and Pd/TPPTS (4 mM) in 6 M Gn-HCl and 0.2 M NaH_2PO_4 at pH 5.0. The mixture was stirred for 10 min under argon atmosphere at room temperature. The reaction was monitored by RP-HPLC, and the product was identified by MALDI-TOF mass spectrometry. After completion of the reaction, 1 M DTT was added to the peptide solution and the mixture was stirred for 10 min at room temperature. The reaction mixture was purified by RP-HPLC.

4) Desulfurization. Peptide **8** was dissolved in 500 mM TCEP in denaturing solution (6 M Gn-HCl, 0.2 M NaH_2PO_4 pH 6–7). To the solution were added *t*-BuSH and 100 mM VA-044 aq. Final concentration of the mixture was peptide **10** (1 mM), TCEP (460 mM), *t*-BuSH (5% v/v) and VA-044 (2 mM). The reaction mixture was placed at 37 °C. The product (peptide **9**) was confirmed by RP-HPLC and MALDI-TOF mass spectrometry.

5) TBS deprotection and fluorescein conjugation in one-pot. The solutions of 0.1 M KF aq. or 0.1 M $AgNO_3$ aq. were added to a solution of peptide **9** (0.10–0.20 μmol) in H_2O/*t*-BuOH (6:4) and the concentration was adjusted; peptide (1 mM), $AgNO_3$ (10 mM) and KF (10 mM). Then, the mixture was stirred at 37 °C for 2 h. The reaction was quenched by addition of sodium chloride and

aminoguanidine hydrochloride, which can trap silver ions as AgCl salt. To the supernatant were added fluorescein-azide (50 mM, Lumiprobe), $CuSO_4$ (50 mM), THPTA (100 mM). Then, the solution was degassed by argon bubbling. To the mixture solution was added sodium ascorbic acid (200 mM). The final concentration of the reaction mixture is as follows; peptide (0.5 mM), fluorescein-azide (2 mM), $CuSO_4$ (1 mM), THPTA (5 mM), sodium ascorbic acid (20 mM) and amino guanidine (20 mM). The reaction was conducted at 37 °C and the product (peptide **10**) was confirmed by RP-HPLC and MALDI-TOF mass spectrometry.

6) UV-Vis measurements of fluorescein-labeled PEP-19. UV-Vis spectrum was recorded on Shimadzu UV-2550 UV-Visible spectrophotometer. The spectrum was measured in denaturing buffer (Gn-HCl 6 M, NaH_2PO_4 0.2 M at pH 7.0) using a cell with a 1 cm path length.

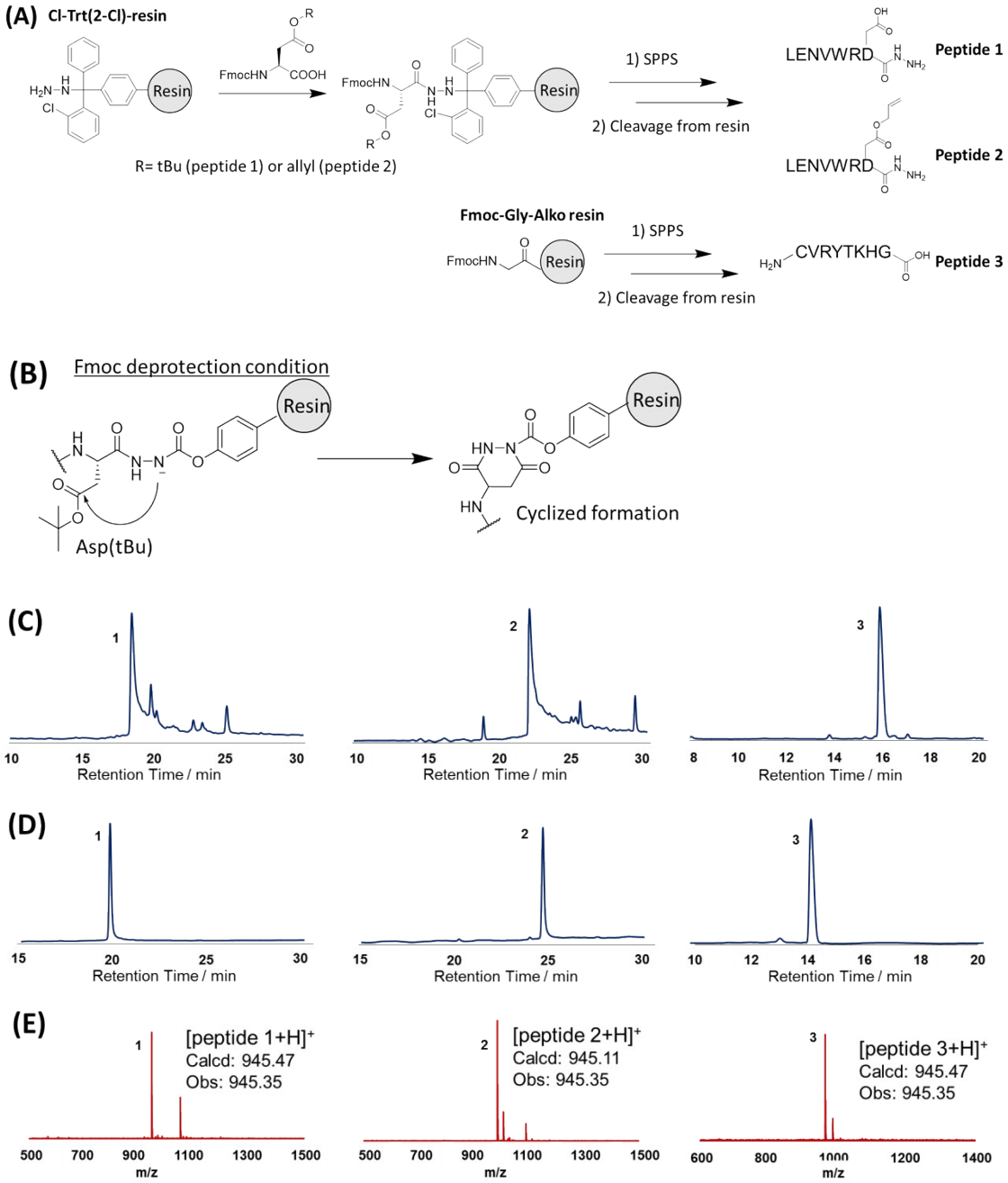


Figure S1. Synthesis of peptide **1**, **2** and **3**. (A) Synthetic scheme of peptide **1**, **2** and **3** using Cl-Trt(2-Cl) resin and Fmoc-Gly-Alko resin. (B) Proposed mechanism of cyclization for the synthesis of C-terminal Asp hydrazide during SPPS using Wang-resin. (C) HPLC charts of crude peptide **1**, **2** and 3. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10-40% for 30 min (**1**), 10-40% for 30 min (**2**), and 5-25% for 30 min (**3**). (D) HPLC charts of purified peptide **1**, **2** and **3**. Gradient: 5-35% for 30 min (**1**), 5-35% for 30 min (**2**), and 5-25% for 20 min (**3**). (E) MALDI-TOF mass spectra of peptide **1**, **2** and **3**.

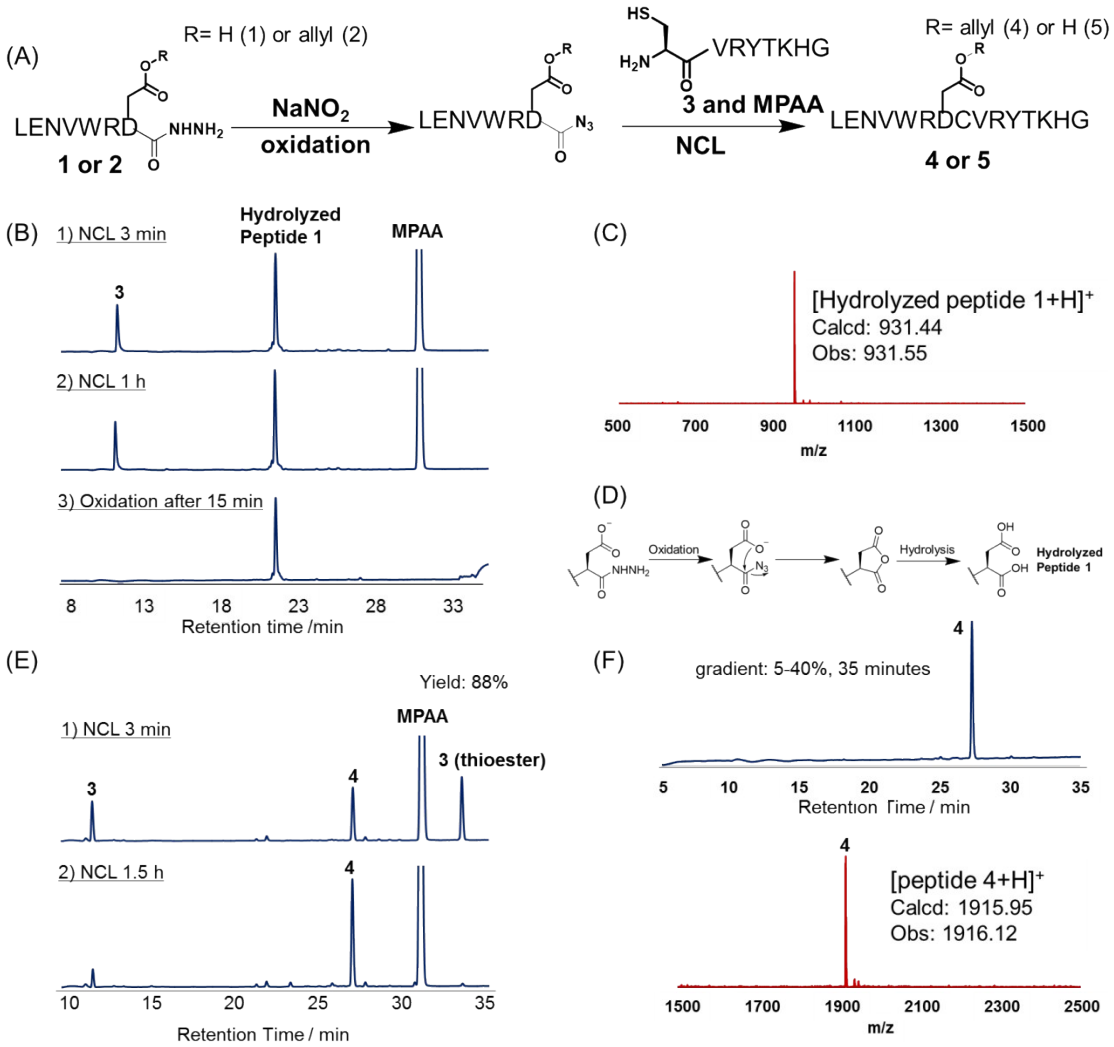
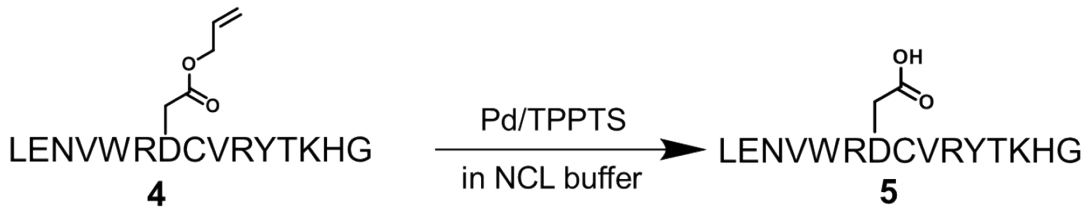


Figure S2. Native chemical ligation between peptide **1** or **2** and peptide **3**. (A) Synthetic scheme of peptide **4** (ligated product) or **5** (deprotected product). (B) Ligation between peptide **1** and peptide **3** after oxidation of hydrazide. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 5-40% for 35 min. (C) MALDI-TOF mass spectrum of hydrolyzed peptide **1**. (D) Proposed mechanism of hydrolysis of peptide **1** after oxidation of hydrazide. (E) Ligation between peptide **2** and peptide **3**. Gradient: 5-40% for 35 min. (F) HPLC chart of purified peptide **4** and MALDI-TOF mass spectrum of peptide **4**.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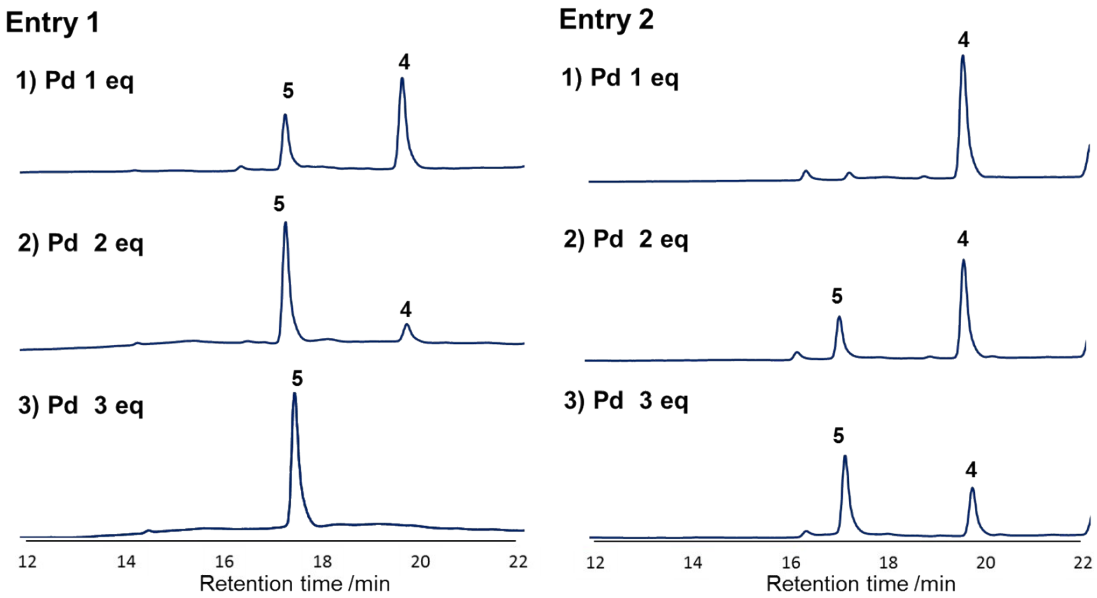
Pd/TPPTS
in NCL buffer
OH
LENVWRDCVRYTKHG
5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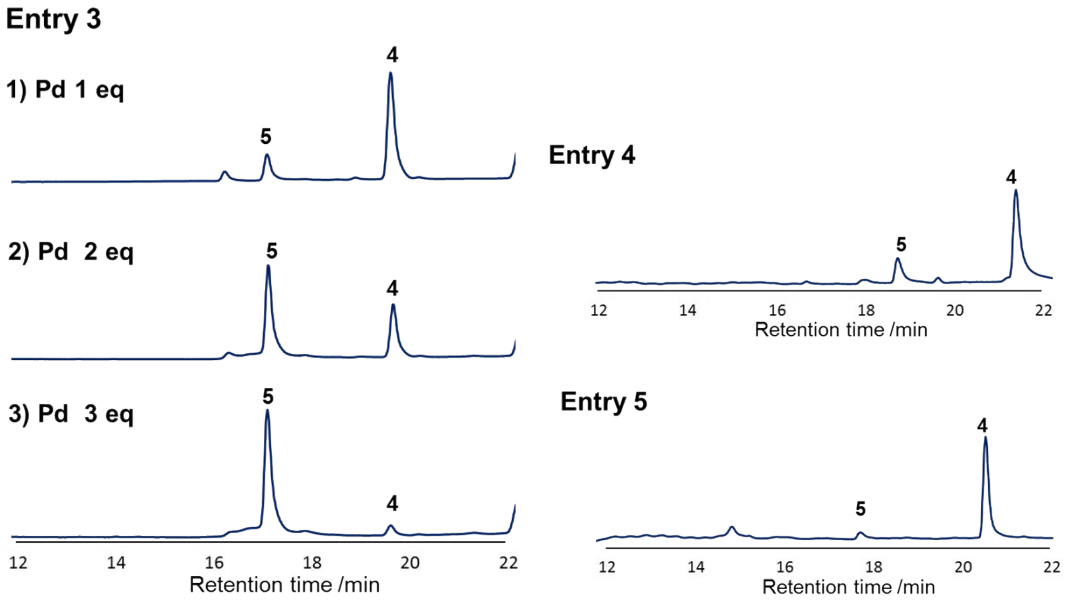
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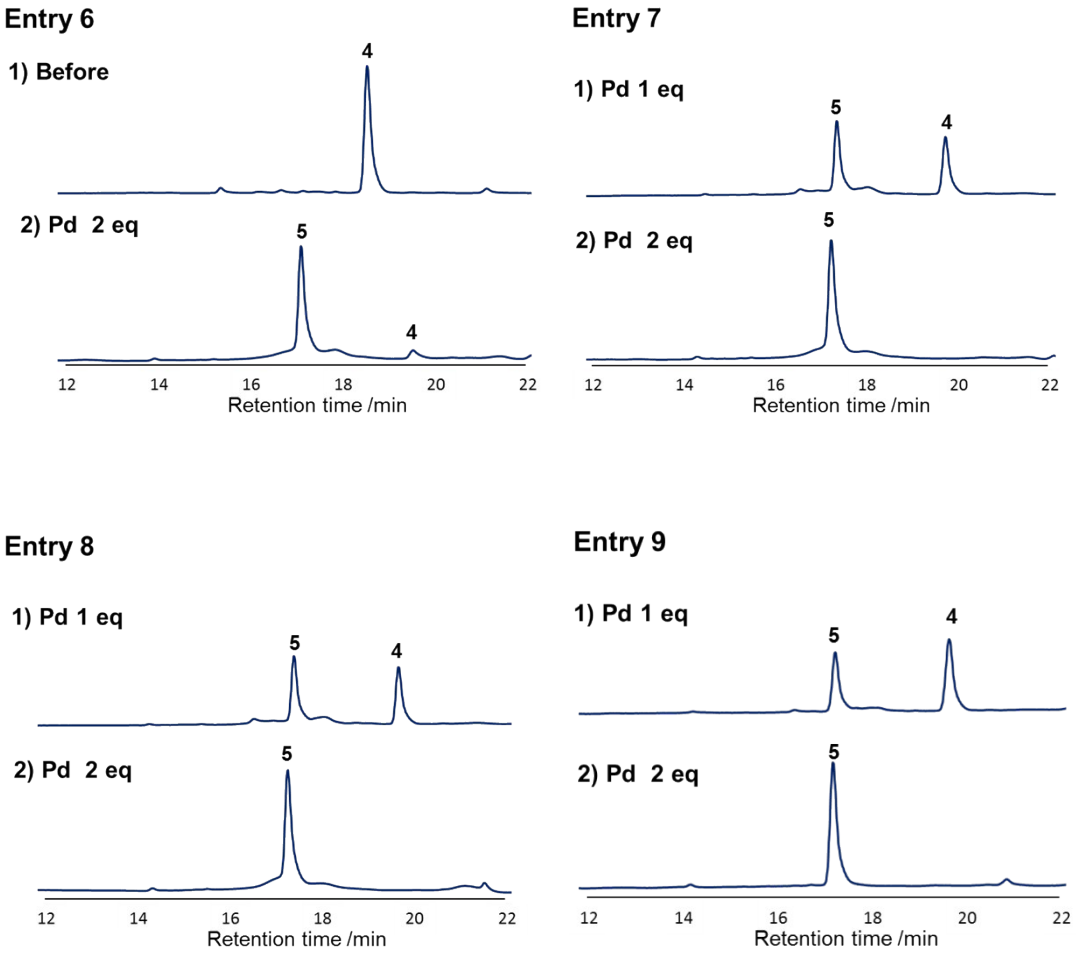

Figure S3. Comparison of reactions shown in Table 1. Each reaction was monitored at 220 nm by HPLC in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15−40% for 25 min.

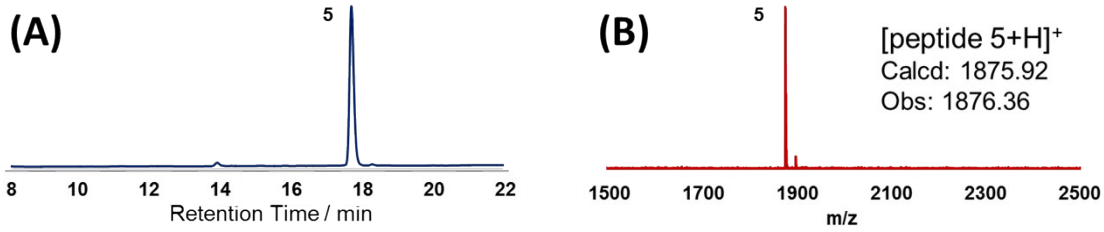

Figure S4. Identification of peptide **5**. (A) HPLC chart of purified peptide **5**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10-35% for 25 min. (B) MALDI-TOF mass spectrum of peptide **5**.

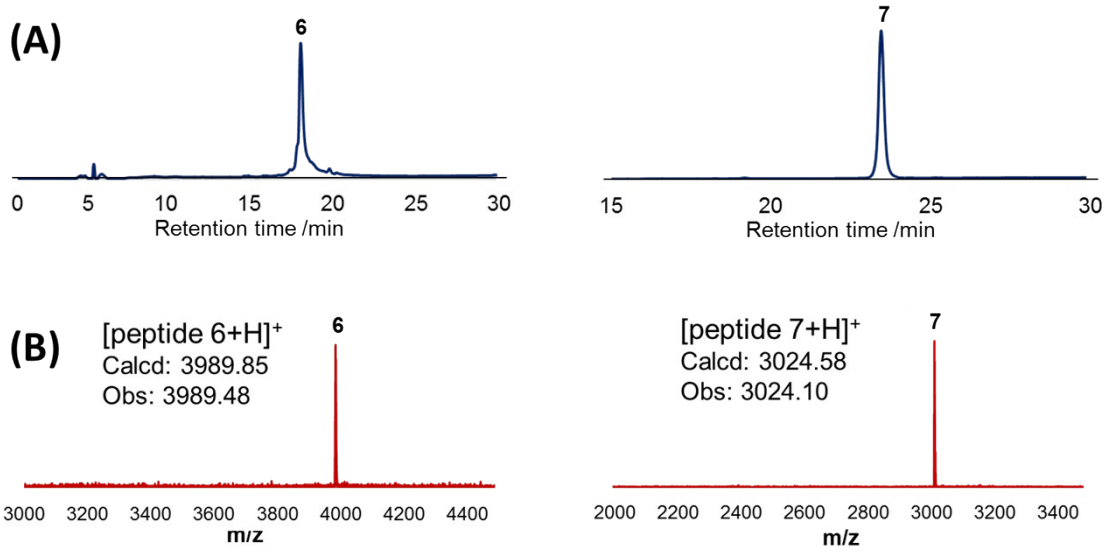

Figure S5. Identification of peptides **6** and **7**. (A) HPLC profiles of purified peptide **6** and **7**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10-70% for 30 min (**6**) and 10-40% for 30 min (**7**). (B) MALDI-TOF mass spectra of peptide **6** and **7**.

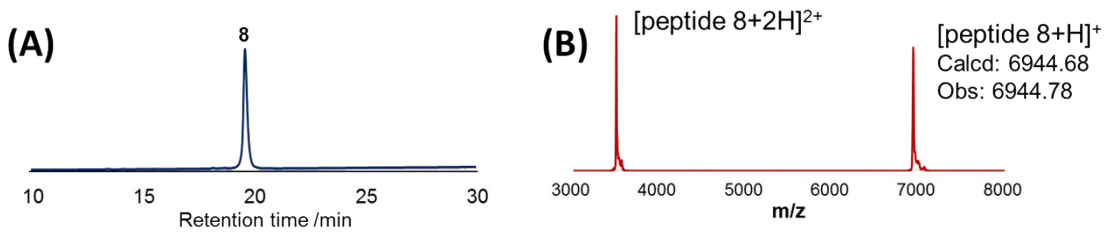

Figure S6. Identification of peptide **8**. (A) HPLC chart of purified peptide **8**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 20-45% for 25 min. (B) MALDI-TOF mass spectrum of peptide **8.**

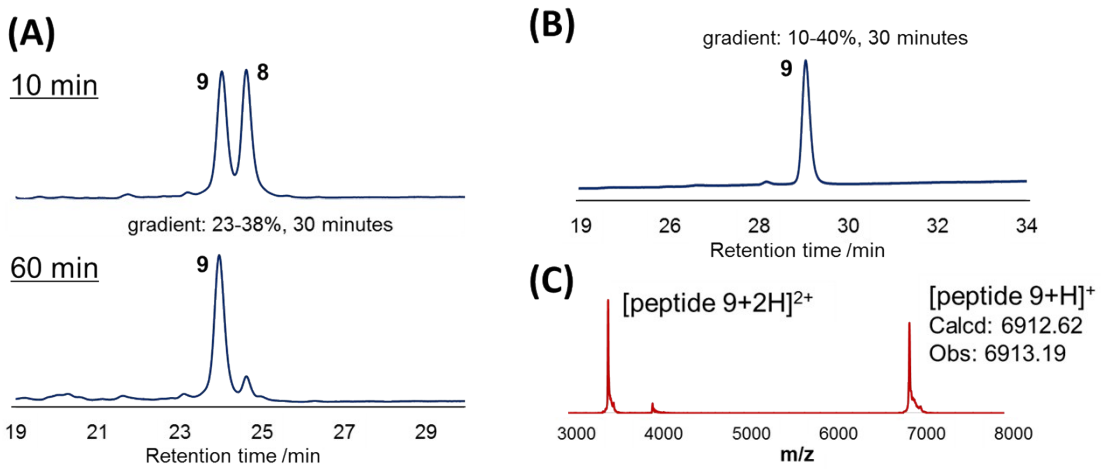

Figure S7. Desulfurization of peptide **8** to yield **9**. (A) Reaction monitoring by HPLC. (B) HPLC chart of purified peptide **9**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. (C) MALDI-TOF mass spectrum of peptide **9**.

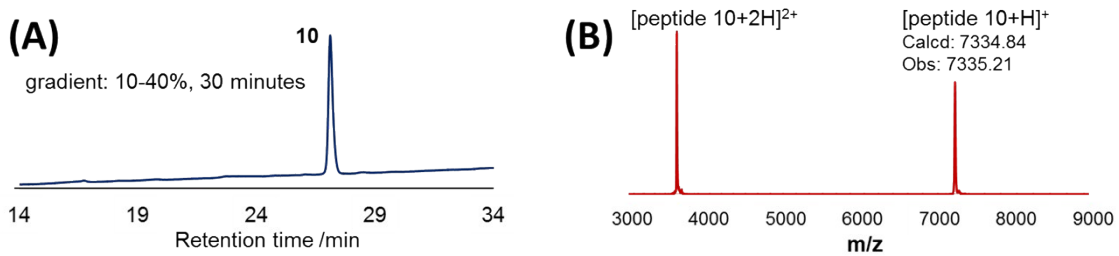

Figure S8. Identification of peptide **10**. (A) HPLC profile of purified peptide **10**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. (B) MALDI-TOF mass spectrum of peptide **10**.

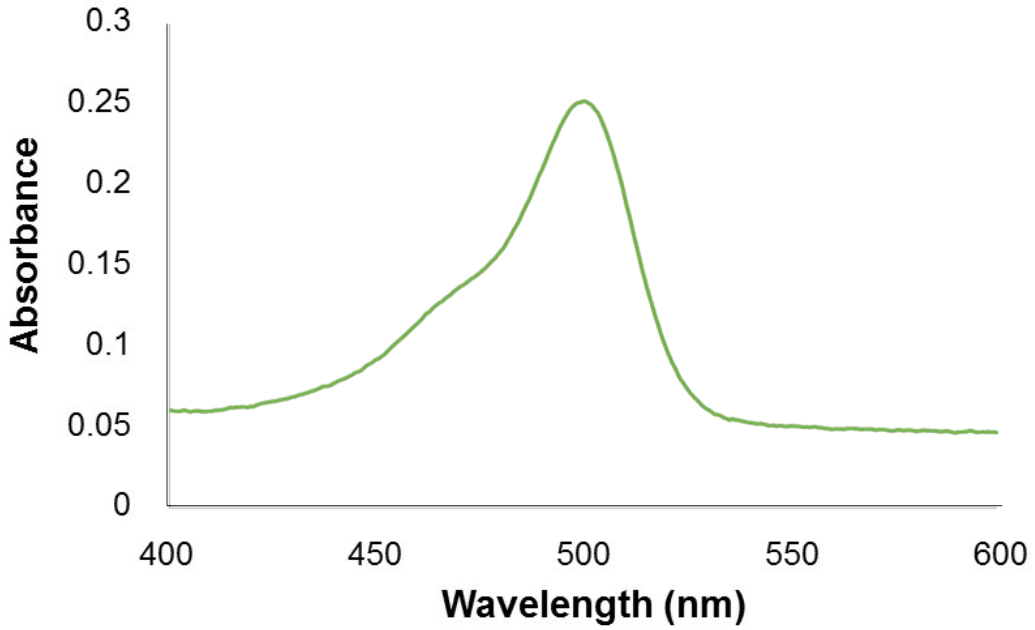

Figure S9. UV-vis spectrum of fluorescein-labeled PEP-19 (peptide **10**). The significant peak derived from fluorescein (~500 nm) was observed.