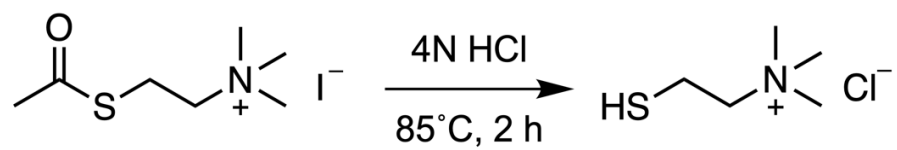


Thiocholine-Mediated One-Pot Peptide Ligation and Desulfurization

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Scheme S1. Synthetic scheme of thiocholine chloride.

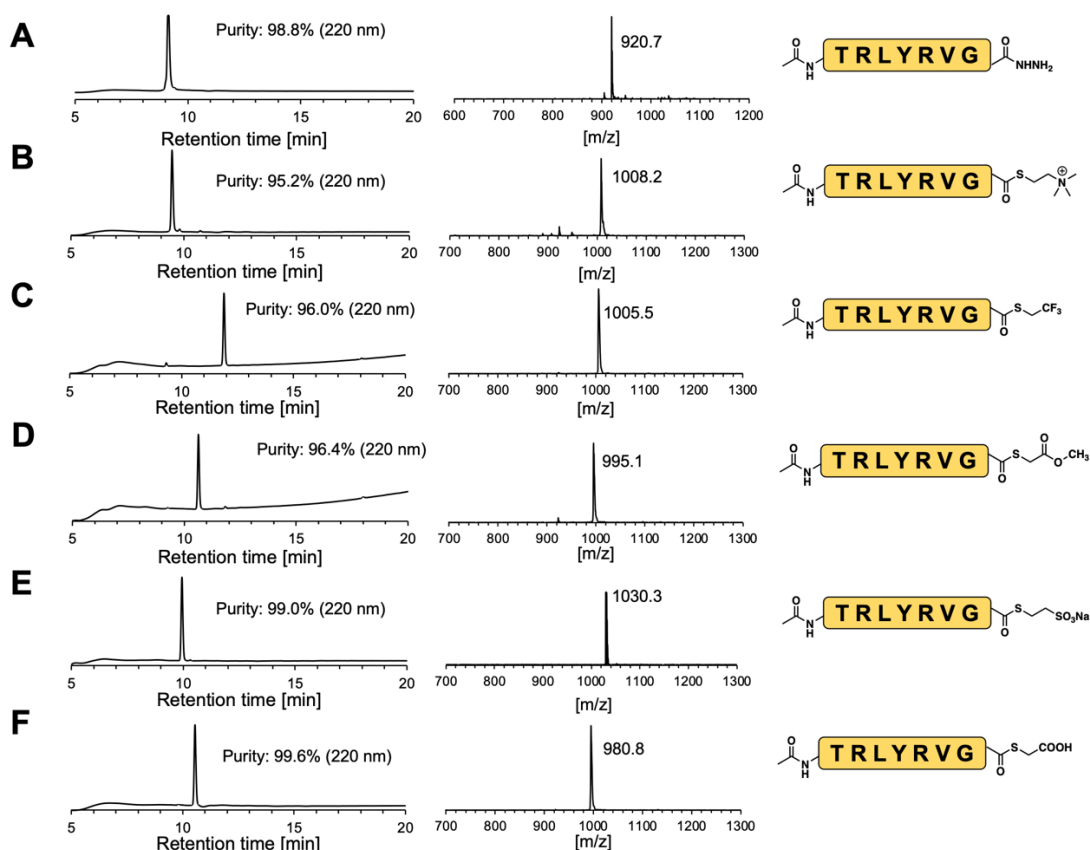


Figure S1. Synthesis of model peptide thioesters. HPLC charts and MALDI-TOF mass spectra of purified model peptide thioesters are shown. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10–70% for 20 min with 5C18-AR-II column. (A) **Peptide hydrazide**; Calculated mass $[M+H]^+$: 920.5; Mass Found $[M+H]^+$: 920.7. (B) **Peptide thiocholine thioester 1**; Calculated mass $[M]^+$: 1007.6; Mass Found $[M]^+$: 1008.2. (C) **Peptide TFET thioester**; Calculated mass $[M+H]^+$: 1005.1; Mass Found $[M+H]^+$: 1005.5. (D) **Peptide MTG thioester**; Calculated mass $[M+H]^+$: 994.5; Mass Found $[M+H]^+$: 995.1. (E) **Peptide MESNa thioester**; Calculated mass $[M+H]^+$: 1030.5; Mass Found $[M+H]^+$: 1030.3. (F) **Peptide TGA thioester**; Calculated mass $[M+H]^+$: 981.1; Mass Found $[M+H]^+$: 980.8.

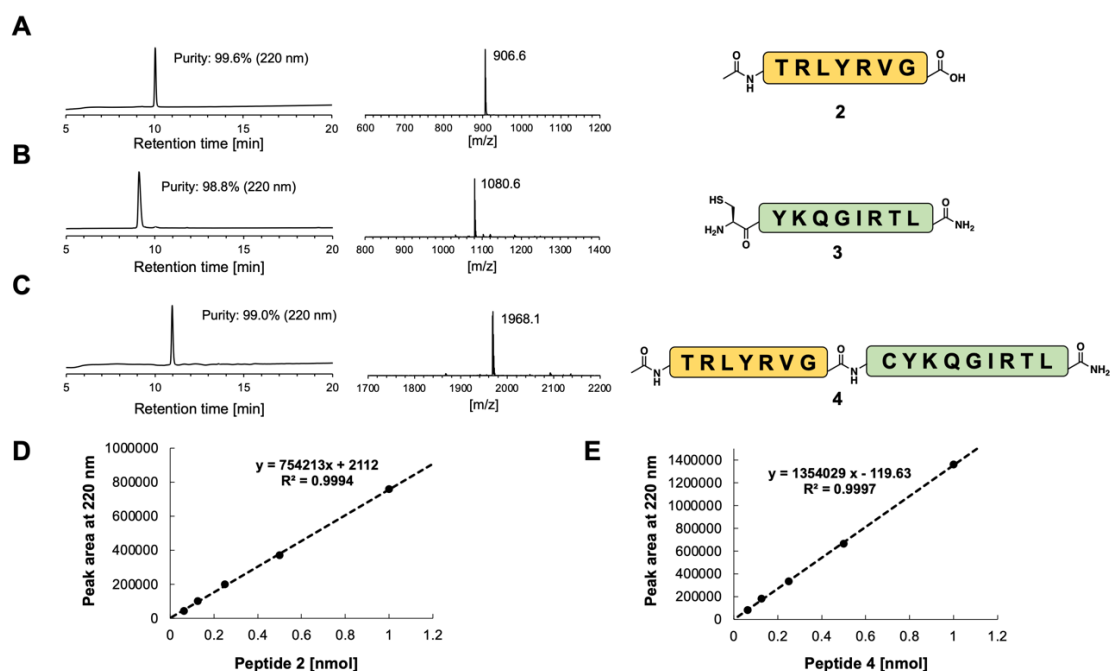


Figure S2. Synthesis of model peptides **2**, **3**, and **4**. HPLC charts and MALDI-TOF mass spectra of purified peptides **2**, **3**, and **4**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10–70% for 20 min with 5C18-AR-II column. (A) **2**; Calculated mass $[M+H]^+$: 906.5; Mass Found $[M+H]^+$: 906.6. (B) **3**; Calculated mass $[M+H]^+$: 1080.6; Mass Found $[M+H]^+$: 1080.6. (C) **4**; Calculated mass $[M+H]^+$: 1968.1; Mass Found $[M+H]^+$: 1968.1. (D) Calibration curve of peptide **2**. (E) Calibration curve of peptide **4**.

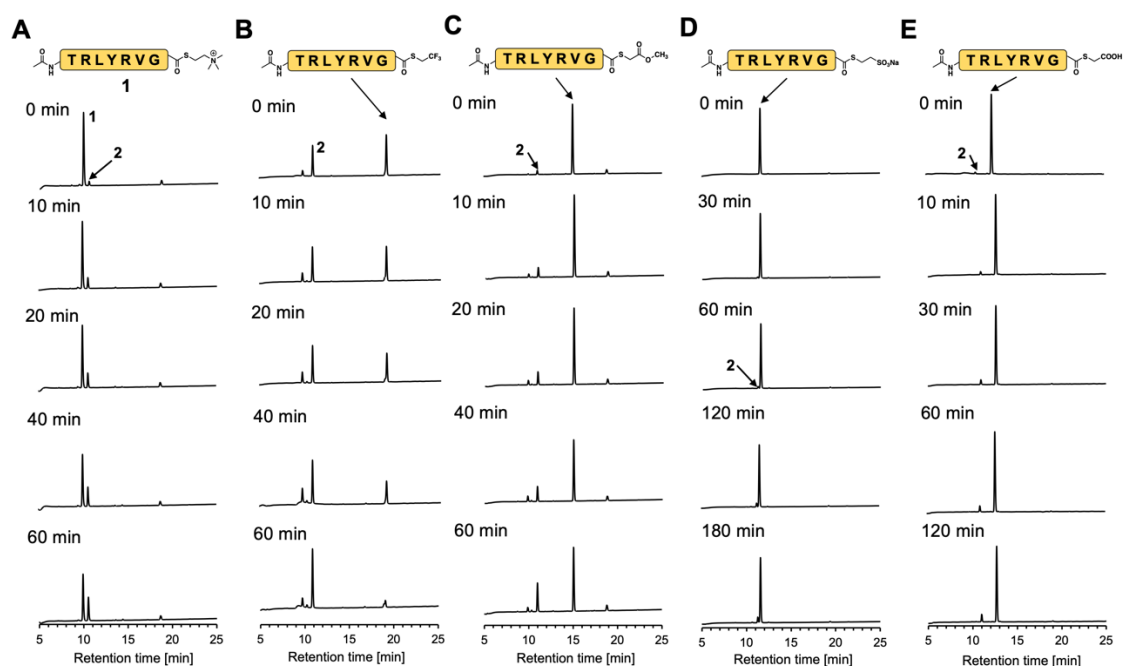


Figure S3. Time course HPLC analyses of hydrolysis reaction with each peptide thioester. Each peptide (1 mM) of (A) thiocholine thioester **1**, (B) TFET thioester, (C) MTG thioester, (D) MESNa thioester, or (E) TGA thioester was incubated in denaturing buffer (0.2 M NaH_2PO_4 , 6 M $\text{Gn}\cdot\text{HCl}$, 40 mM TCEP) at pH 7.0, 25°C. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–50% for 30 min with 5C18-AR-II column.

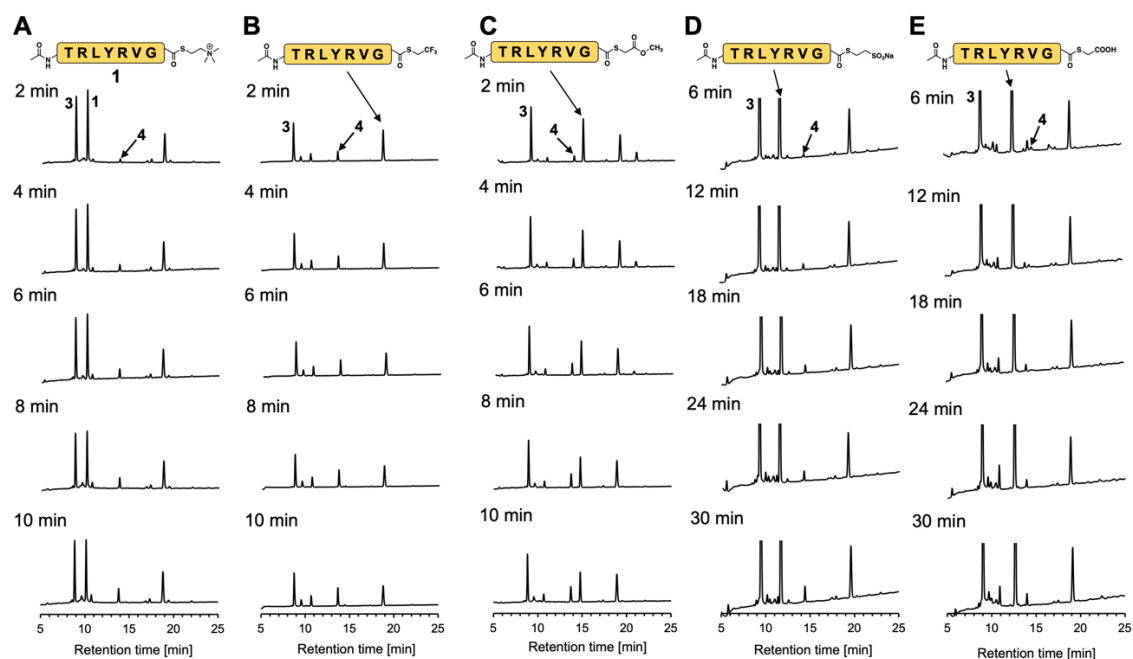


Figure S4. Time course HPLC analyses of NCL reaction between each model peptide thioester and peptide 3. Each model peptide (0.1 mM) of (A) thiocholine thioester **1**, (B) TFET thioester, (C) MTG thioester, (D) MESNa thioester, or (E) TGA thioester, and peptide **3** were incubated in denaturing buffer (0.2 M NaH_2PO_4 , 6 M $\text{Gn}\cdot\text{HCl}$, 40 mM TCEP) at pH 7.0, 25°C. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–50% for 30 min with 5C18-AR-II column.

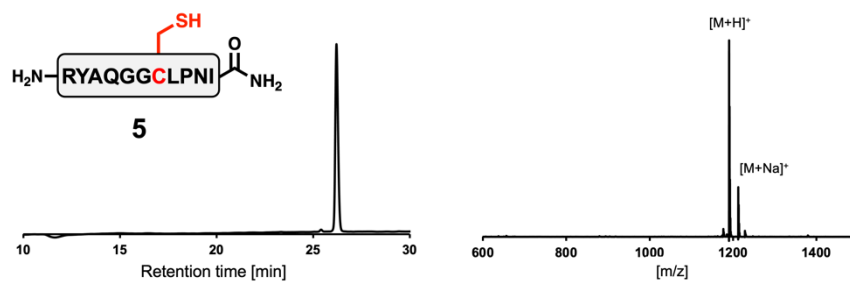


Figure S5. Synthesis of peptides 5. A HPLC chart and MALDI-TOF mass spectrum of purified peptide 5. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 5–30% for 30 min with 5C18-AR-II column. Calculated mass $[\text{M}+\text{H}]^+$: 1190.6; Mass Found $[\text{M}+\text{H}]^+$: 1190.6.

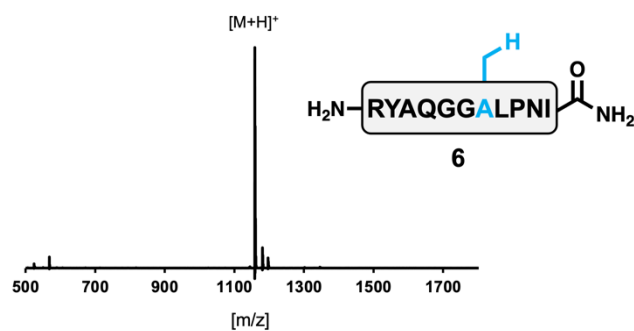


Figure S6. MALDI-TOF mass spectrum of peptide 6. Calculated mass $[M+H]^+$: 1158.6; Mass Found $[M+H]^+$: 1158.7.

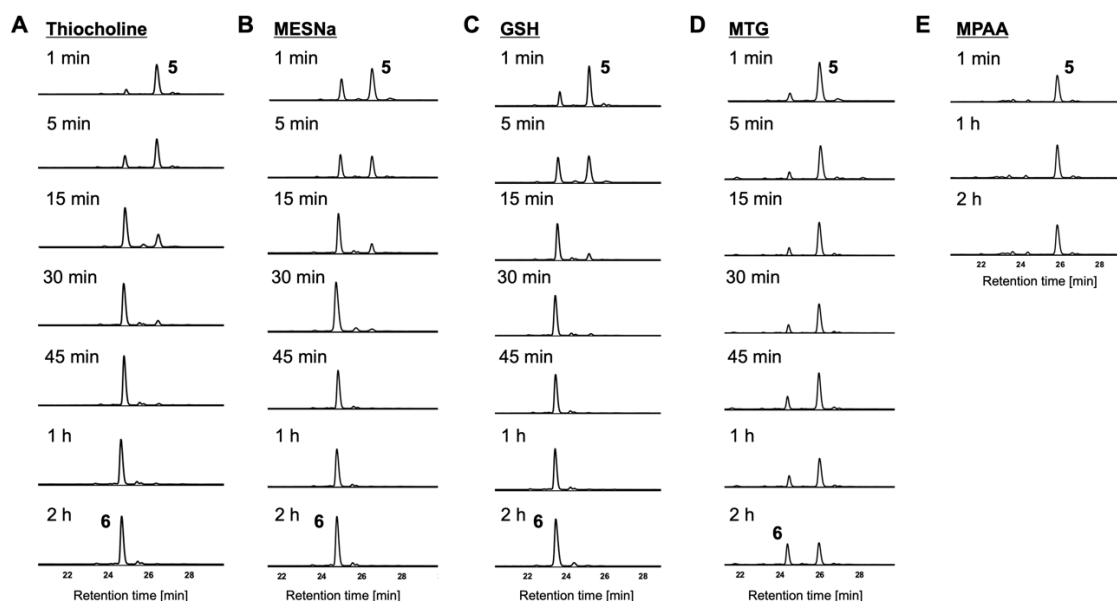


Figure S7. Time course HPLC analyses of model peptide desulfurization with different thiol additives. Peptide **5** (0.8 mM) and 80 mM thiol additives, (A) thiocholine, (B) MESNa, (C) GSH, (D) MTG, or (E) MPAA (80 mM), TCEP (300 mM), and VA-044 (20 mM) were reacted in denaturing buffer (0.2 M NaH_2PO_4 , 6 M $\text{Gn}\cdot\text{HCl}$) at pH 7.0, 37°C. The reaction was quenched with 100 mM ascorbic acid before HPLC analyses. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 5–30% for 30 min with 5C18-AR-II column.

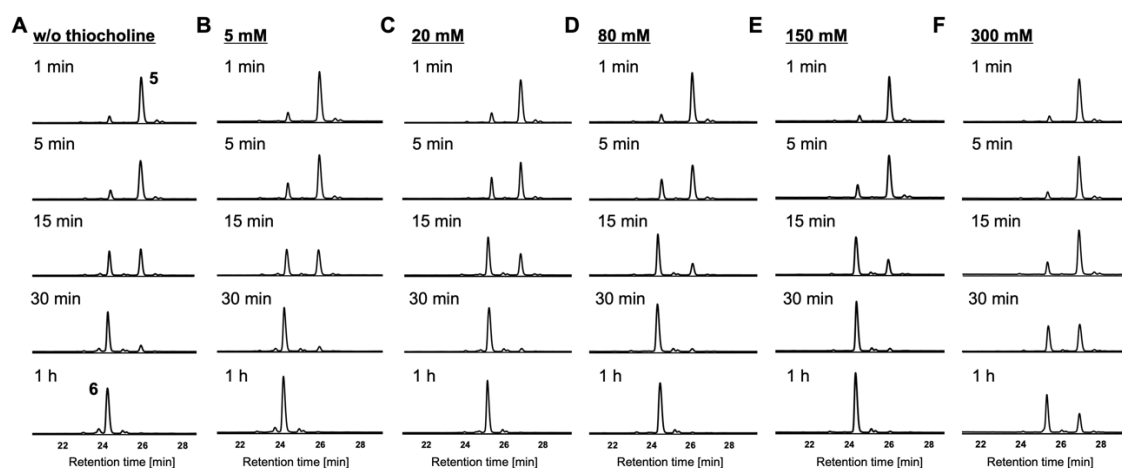


Figure S8. Time course HPLC analyses of model peptide desulfurization with different concentration of thiocholine. Peptide **5** (0.8 mM) and thiocholine (A) 0 mM, (B) 5 mM, (C) 20 mM, (D) 80 mM, (E) 150 mM, or (F) 300 mM, TCEP (300 mM), and VA-044 (20 mM) were reacted in denaturing buffer (0.2 M NaH_2PO_4 , 6 M $\text{Gn}\cdot\text{HCl}$) at pH 7.0, 37°C. The reaction was quenched with 100 mM ascorbic acid before HPLC analyses. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 5–30% for 30 min with 5C18 AR-II.

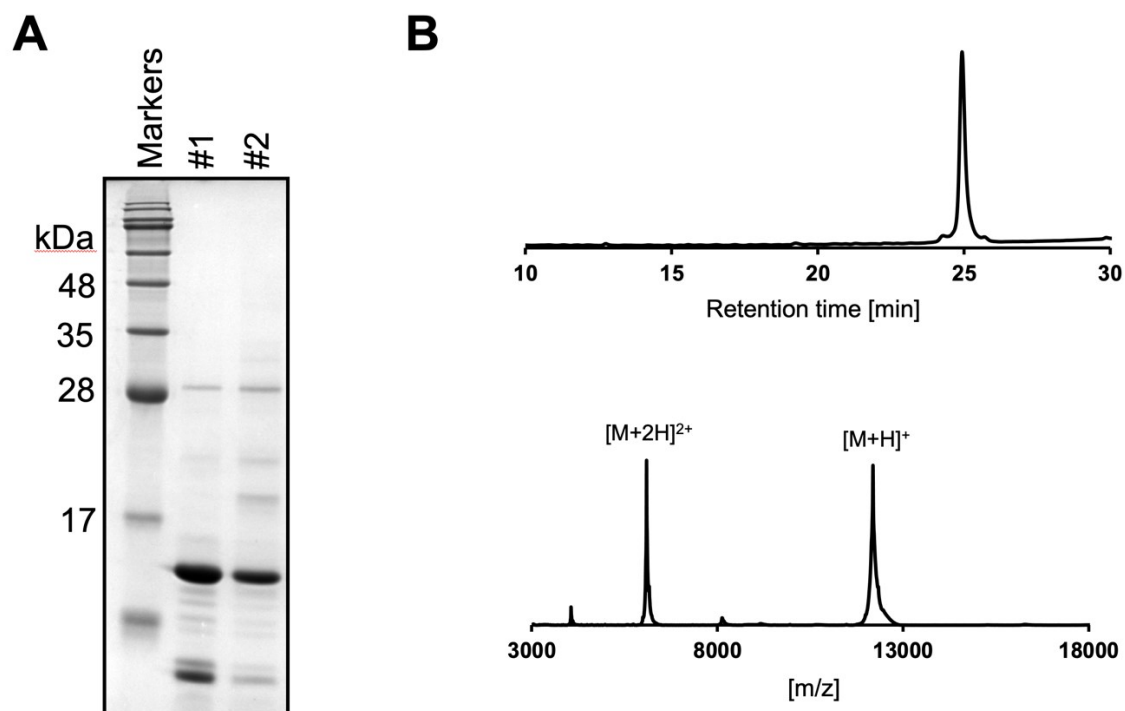


Figure S10. Preparation of H3 C-terminal peptide **9**. (A) SDS-PAGE of peptide **9** before HPLC purification. (B) HPLC chart and MALDI-TOF mass spectrum of peptide **9** after HPLC purification. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 20–70% for 30 min with Jupiter 5C4 column. Calculated mass of **9** $[M+H]^+$: 12190.1; Mass Found $[M+H]^+$: 12190.2.

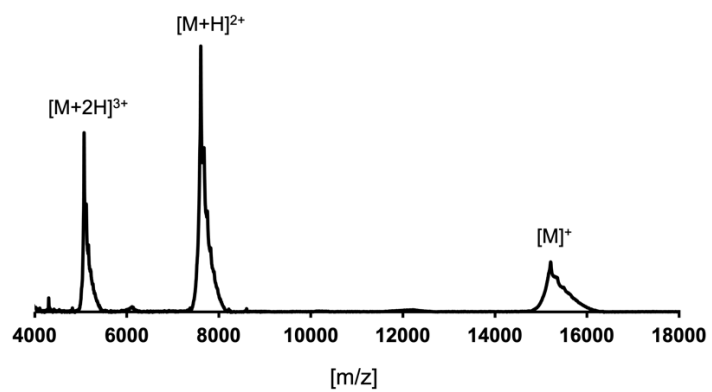


Figure S11. MALDI-TOF mass spectrum of peptide **10**. Calculated mass of **10** $[M]^+$: 15211.7; Mass Found $[M]^+$: 15210.7.

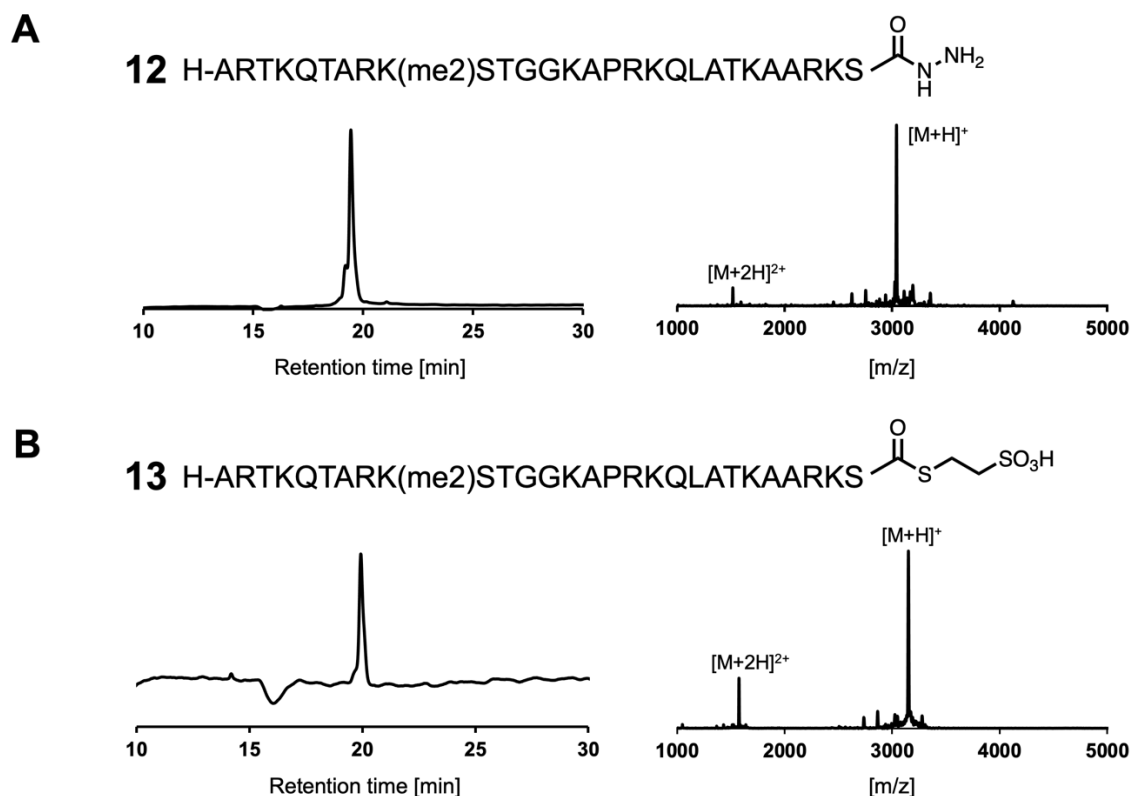


Figure S12. Synthesis of H3K9me₂ N-terminal peptide **13**. HPLC charts and MALDI-TOF mass spectra of purified peptide **12** (A) and peptide **13** (B). HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. (A) Gradient: 2–25% for 30 min with 5C18-AR-II column. (B) Gradient: 5–30% for 30 min with 5C18-AR-II column. Calculated mass of **12** [M+H]⁺: 3040.6; Mass Found [M]⁺: 3040.2. Calculated mass of **13** [M+H]⁺: 3150.7; Mass Found [M]⁺: 3150.9.

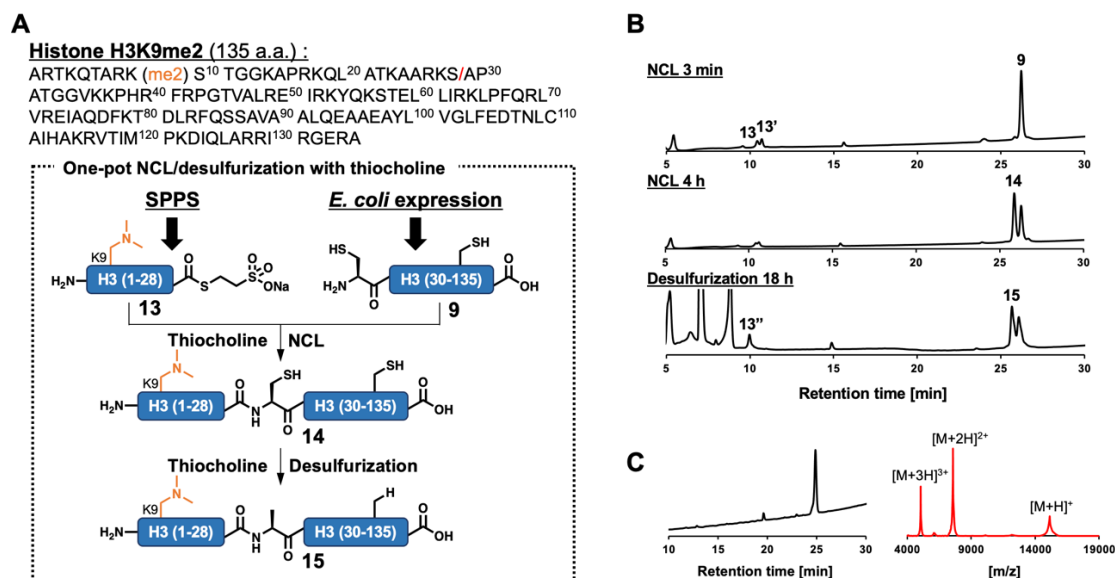


Figure S13. Semisynthesis of Arabidopsis H3K9me2 via thiocholine-mediated one-pot NCL/desulfurization. (A) Amino-acid sequence and synthetic scheme of H3K9me2. A red slash represents the ligation junction. (B) Reaction tracking of the one-pot NCL/desulfurization by analytical HPLC (gradient: 5–75% for 30 min) at 220 nm. Compounds **13'** and **13''** are thiocholine thioester and hydrolysis of peptides **13**, respectively. HPLC profiles at NCL at 3 min, NCL at 4 h and desulfurization at 18 h were shown. (C) HPLC profile (left, gradient 20–70% for 30 min) and MALDI-TOF mass spectrum (right) of purified peptide **15**. Calculated mass of **15** $[M+H]^+$: 15133.5; mass found $[M+H]^+$: 15132.8.