Supporting information

Organoruthenium-Catalyzed Chemical Protein Synthesis to Elucidate the Functions of Epigenetic Modifications on Heterochromatin Factors

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Experimental section

General methods and materials. MALDI-TOF mass spectra were recorded with microflex or autoflex max (BRUKER), using peptide calibration standard II, protein calibration standard I, or protein calibration standard II as external standards. For the analysis, α-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA) was employed as a matrix substance. NMR spectra were recorded with an AVENCE 600 instrument (Bruker), AVANCE III HD 300MHz-1, or 500MHz-1 (Bruker). ESI mass spectra were recoded with JMS-T100CS (JEOL). Reversed-phase HPLC was performed on a $5C_{18}$-AR-II, Protein-R column, or $5C_{18}$-AR-300 (Nacalai tesque, 4.6 ID and 10 ID × 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.

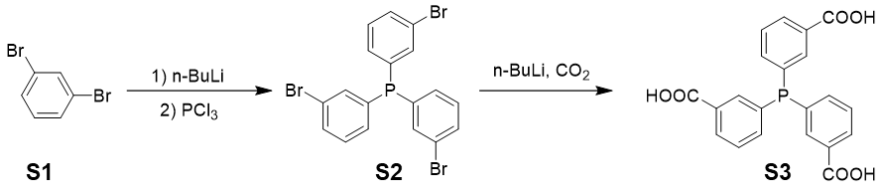
For organic synthesis, anhydrous THF was purchased from Kanto Chemical. $[CpRu(MeCN)_3]PF_6$ and allyl alcohol were purchased from Tokyo Chemical Industry. $[Cp^*Ru(MeCN)_3]PF_6$, Cp*Ru(cod)Cl, and were purchased from Sigma-aldrich.

Peptides were prepared by using Fmoc-Gly-Alko-Peg resin (Watanabe Chemical Industries) for peptide **1**, Fmoc-Lys-Alko-Peg resin (Watanabe Chemical Industries) for peptide **3** and **3a**, Fmoc-Ser-Alko-Peg resin (Watanabe Chemical Industries) for peptide **13** and **22**, Fmoc-NH-SAL Resin (0.40 mmol/g, Watanabe Chemical Industries) for peptides bearing amide groups at C-terminus, and Cl-Trt(2-Cl)-resin (Watanabe Chemical Industries) for peptides bearing acyl hyrazides. Fmoc-protected amino acids were purchased from Watanabe Chemical Industries or Novabiochem. *N,N*-dimethylformamide (DMF) was purchased from Kanto Chemical. *N*-methlpyrrolidone was purchased from Nacalai tesque. [(2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBt), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), 1-Hydroxy-7-azabenzotriazole (HOAt) were purchased from Watanabe Chemical Industries. *N*,*N*′-Diisopropylcarbodiimide was purchased from Wako Pure Chemical Industries. The isolated yields of each peptide were estimated by using the molecular weights of TFA salt at Arg, Lys, and His positions and N-terminal amine groups.

For native chemical ligation (NCL), 4-mercaprophenylacetic acid (MPAA) and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Wako Pure Chemical Industries.

Organic Synthesis

Synthesis of tris(3-carboxyphenyl)phosphine (TPPTC) (S3)



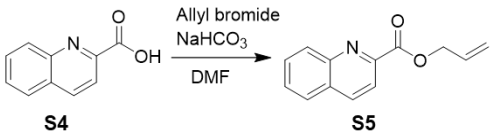
1st step

1,3-Dibromobenzene (**S1**) (2.21 g, 9.0 mmol) was dissolved in anhydrous THF (18 mL) under argon atmosphere. The solution was cooled to -78 °C and *n*-BuLi (1.6 M in hexane, 5.62 mL, 9.0 mmol) was added dropwise. The reaction solution was stirred for 30 min at -78 °C. Then, phosphorus trichloride (PCl_3) (0.41 mg, 3.0 mmol) was added to the reaction mixture, the whole solution was stirred for 1 h -78 °C, and then warmed to room temperature and stirred for 1 h. The reaction was quenched with water, and the organic layer was extracted by diethyl ether three times, dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (hexane/dichloromethane = 1/1) to afford tris(3-bromophenyl)phosphine (**S2**) (0.41 g, 27% yield) as a solid. **^{1}H NMR** ($CDCl_3$, 600 MHz) δ = 7.54 (3 H, m), 7.43 (3 H, m), 7.25 (6 H, m). **^{31}P NMR** (243 MHz, $CDCl_3$) d -2.62.

2nd step

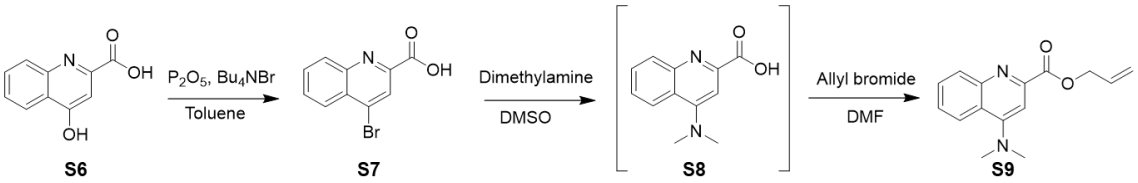
To a solution of compound **S2** (0.26 mg, 0.52 mmol) in anhydrous THF (4.0 mL) at -78 °C, was added dropwise n-butyllithium (1.6 M in hexane, 1.08 mL, 1.73 mmol) under argon atmosphere. The solution was stirred for 2 h at -78 °C, then excess crushed dry ice was added, and the reaction mixture was stirred for 20 min at room temperature. The reaction was quenched with water, and dichloromethane was added. The mixture was acidify with 1 N HCl aq, and the organic layer was extracted with a mixture of dichloromethane and methanol (5:1), dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (chloroform/methanol = 10/1) to afford TPPTC (**S3**) (98 mg, 48% yield) as a solid. **^{1}H NMR** (DMSO, 600 MHz) δ = 8.01 (3 H, d, *J* = 8.2 Hz), 7.80 (3 H, d, *J* = 6.9 Hz), 7.61 (3 H, t, *J* = 6.7 Hz), 7.54 (3 H, t, *J* = 7.3 Hz). **^{31}P NMR** (243 MHz, DMSO) δ = -5.74.

Synthesis of compound S5



2-Quinolinecarboxylic acid (**S4**) (0.20 g, 1.15 mmol) was dissolved in anhydrous DMF (6.0 mL) under argon atmosphere, and sodium bicarbonate (0.16 g, 1.84 mmol) was added to the solution. To the reaction solution, was added allyl bromide (0.21 mg, 1.73 mmol) dropwise, and the whole mixture was stirred at 50 °C overnight. The reaction was quenched with water, and the organic layer was extracted by dichloromethane, dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (hexane/ethyl acetate = 5/1) to afford compound **S5** (0.24 g, 1.11 mmol, 97% yield). **^{1}H NMR** ($CDCl_3$, 600 MHz) δ = 8.34 (2 H, m), 8.22 (1 H, d, *J* = 8.8 Hz), 7.91 (1 H, d, *J* = 8.2 Hz), 7.82 (1 H, t, *J* = 7.9 Hz), 7.68 (1 H, t, *J* = 9.4 Hz), 6.15 (1 H, m), 5.51 (1 H, d, *J* = 17.0 Hz), 5.37 (1 H, d, *J* = 10.3 Hz) 5.01 (1 H, d, *J* = 5.3 Hz).

Synthesis of compound S9



1st step

To a flask containing kynurenic acid hydrate (**S6**) (0.92 g, 4.86 mmol), diphosphorus pentoxide (1.66 g, 11.7 mmol) and tetrabutylammonium bromide (1.88 mg, 5.83 mmol), was added anhydrous toluene (20.0 mL), and the reaction mixture was stirred at 100 °C for 2 h. The organic layer was collected, and the flask was washed with toluene. The whole solution was dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (chloroform/methanol = 20/1) to afford compound **S7** (0.19 g, 16% yield). **^{1}H NMR** ($CDCl_3$, 600 MHz) δ = 8.52 (2 H, m), 8.30 (1 H, m), 7.90 (1 H, m), 7.81 (1 H, m).

2nd step

To a solution of compound **S7** (35 mg, 0.14 mmol) in DMSO (1.0 mL), was added diethylamine solution (50% in water) (5 mL), and the whole reaction mixture was refluxed at 100 °C overnight. Then, the solution was concentrated *in vacuo*, and the resulting oil (**S8**) (30 mg) was employed for the next reaction without further purification.

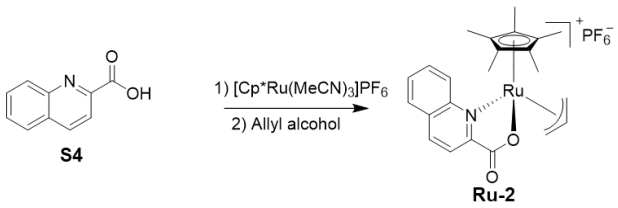
3rd step

Compound **S8** (30 mg) was dissolved in anhydrous DMF (1.0 mL) under argon atmosphere, and sodium bicarbonate (30 mg, 0.36 mmol) was added to the solution. To the reaction solution, was added allyl bromide (44 mg, 0.36 mmol) dropwise, and the whole mixture was stirred at 50 °C overnight. The reaction was quenched with water, and the organic layer was extracted by dichloromethane, dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column

chromatography (hexane/ethyl acetate = 2/1) to afford compound **S9** (21 mg, 82 μmol, 59% yield from compound **S7**). **^{1}H NMR** ($CDCl_3$, 600 MHz) δ = 8.25 (1 H, d, *J* = 8.2 Hz), 8.11 (1 H, d, *J* = 8.2 Hz), 7.71 (1 H, t, *J* = 7.9 Hz), 7.60 (1 H, s), 7.56 (1 H, t, *J* = 7.6 Hz), 6.16 (1 H, m), 5.49 (1 H, d, *J* = 17.3 Hz), 5.35 (1 H, d, *J* = 10.3 Hz), 5.01 (1 H, d, *J* = 5.9 Hz), 3.14 (6 H, s).

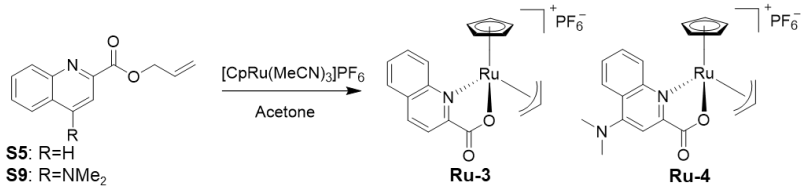
Synthesis of half-sandwich Ru complexes coordinated by N,O-ligands

Synthesis of **Ru-2**



Under argon atmosphere, $[Cp^*Ru(MeCN)_3]PF_6$ (25 mg, 50 μmol) and quinoline carboxylic acid (**S4**) (8.7 mg, 50 μmol) was dissolved in dry degassed acetone (1 mL) and mixed at room temperature. After stirring for 1 h, allyl alcohol (2.9 mg, 50 μmol) was added, and the whole reaction mixture was stirred at room temperature for 1 h. The solution was concentrated *in vacuo*, and the resulting oil was dissolved with a minimal amount of dichloromethane, followed by the addition of hexane to cause precipitation. The solvent was removed *in vacuo*, and the resulting solid was dried under vacuum overnight. The desired compound (**Ru-2**) was obtained (30 mg, quantitatively). **^{1}H NMR** (acetone-d_6, 300 MHz) δ = 8.93 (1 H, d, *J* = 8.3 Hz), 8.36 (1 H, dd, *J* = 8.7 Hz, *J* = 1.4 Hz), 8.20 (2 H, m), 7.95 (2 H, m), 5.19 (1 H, m), 4.51 (1 H, dd, *J* = 6.6 Hz, *J* = 3.0 Hz), 4.43 (1 H, m), 4.26 (1 H, m), 3.78 (2 H, m), 1.85 (15 H, m).

Synthesis of **Ru-3** and **Ru-4**



Under argon atmosphere, $[CpRu(MeCN)_3]PF_6$ (1.00 eq) was dissolved in anhydrous degassed acetone (1.0 mL) and mixed at room temperature with a solution of each allyl ester (1.0 eq) in anhydrous acetone (1.0 mL). After stirring for 30 min, the reaction mixture was concentrated in vacuo to remove solvent. The resulting precipitate was dried under vacuum overnight to give the desired products.

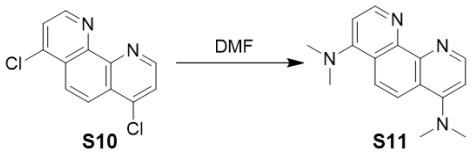
Ru-3

Compound **S5** (20 mg, 92 μmol) was employed an allyl ester for the synthesis of **Ru-3** (33 mg, 67% yield). **^{1}H NMR** (CD_3CN, 600 MHz) δ = 8.81 (1 H, d, *J* = 8.5 Hz), 8.23 (1 H, d, *J* = 8.8 Hz), 8.14 (2 H, m), 7.97 (1 H, t, *J* = 7.6 Hz), 7.90 (1 H, d, *J* = 9.4 Hz), 6.20 (5 H, s), 4.71 (2 H, m), 4.48 (1 H, d, *J* = 10.3 Hz), 4.39 (1 H, m), 4.19 (1 H, m).

Ru-4

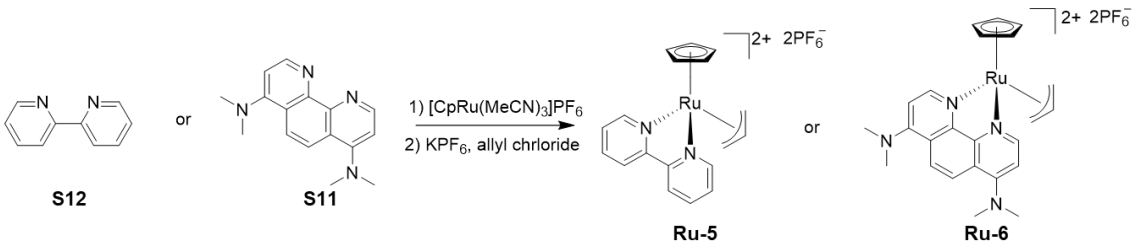
Compound **S9** (20 mg, 92 μmol) was employed an allyl ester for the synthesis of **Ru-4** (47 mg, quantitatively). **^{1}H NMR** (CD_3CN, 600 MHz) δ = 8.27 (1 H, d, *J* = 8.5 Hz), 7.93 (1 H, t, *J* = 8.2 Hz), 7.68 (2 H, m), 7.34 (1 H, s), 6.13 (5 H, m), 4.56 (2 H, m), 4.35 (1 H, d, *J* = 9.9 Hz), 4.29 (1 H, m), 4.07 (1 H, m).

Synthesis of compound S11



Under argon atmosphere, compound **S10** (0.40 mg, 1.61 mmol) was dissolved in anhydrous DMF (15 mL), and the reaction solution was refluxed at 160 °C overnight. The solvents were removed under reduced pressure, and the resulting oil was redissolved in THF. To the organic solvent, was added brine containing 0.5 N NaOH aq, and the water layer was extracted with THF three times. The integrated organic layer was dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (chloroform/methanol = 10/1) to afford compound **S11** (0.16 g, 0.60 mmol, 37% yield). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ = 8.95 (2 H, d, *J* = 5.4 Hz), 7.96 (2 H, s), 7.00 (2 H, d, *J* = 5.4 Hz), 3.13 (12 H, s).

Synthesis of half-sandwich Ru complexes coordinated by N,N-ligands



$[CpRu(MeCN)_3]PF_6$ (25 mg, 58 μmol) was dissolved in degassed CH_3CN (1.0 mL) under argonatmosphere, and 2,2'-bipyridine (**S12**) (9.0 mg, 58 μmol) or compound **S11** (15 mg, 58 μmol) was added to the reaction solution, respectively. The mixture was stirred at room temperature for 30 min, and potassium hexafluorophosphate (12 mg, 63 μmol) was added to the

reaction solution, followed by the addition of allyl chloride (5.1 mg, 66 μmol) dissolved in CH_3CN (1.0 mL). After stirring at room temperature for 1 h, the solvent was removed under reduced pressure. A mixture of ethanol and water (1.0 mL: 1.0 mL) was added to the resulting solid to wash, and the solvent was removed either by filtration or decantation. CH_3CN (1.0 mL) was added to redissolve the solid, followed by the addition of diethyl ether (4.0 mL) to cause precipitation. After decantation, the resulting solid was dried in vacuo overnight to afford the desired products.

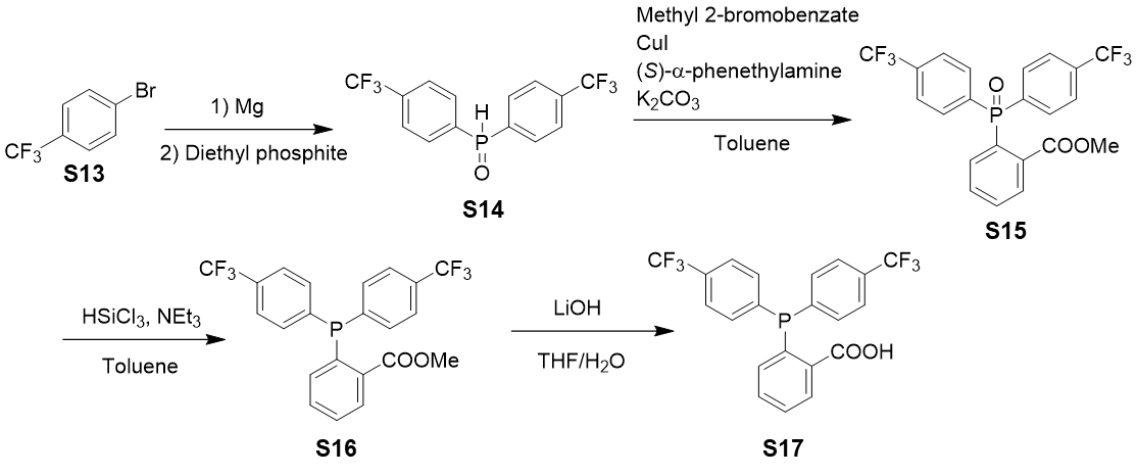
Ru-5 (19 mg, 24 μmol, 42% yield)

^{1}H NMR (acetone-d_6, 300 MHz) δ = 9.34 (2 H, dd, *J* = 5.8 Hz, *J* = 0.6 Hz), 8.82 (2 H, dd, *J* = 8.3 Hz, *J* = 0.9 Hz), 8.51 (2 H, td, *J* = 8.3 Hz, *J* = 1.7 Hz), 7.91 (2 H, m), 6.71 (5 H, s), 5.31 (1 H, m), 4.95 (2 H, d, *J* = 11.1 Hz), 4.65 (2 H, d, *J* = 6.4 Hz). HRMS(ESI): m/z calcd for $C_{18}H_{18}N_2Ru$ ($[M]^+$): 364.051; found: 364.029, ($[M\text{-allyl}]^+$): 323.012; found: 323.000.

Ru-6 (27 mg, 32 μmol, 55% yield)

^{1}H NMR (acetone-d_6, 300 MHz) δ = 8.85 (2 H, d, *J* = 6.9 Hz), 8.20 (2 H, s), 7.20 (2 H, d, *J* = 6.9 Hz), 6.55 (5 H, s), 4.85 (1 H, m), 4.73 (2 H, m), 4.44 (2 H, d, *J* = 6.0 Hz), 3.50 (12 H, s). **^{13}C NMR** (acetone-d_6, 126 MHz) δ = 157.6, 156.1, 152.2, 150.3, 149.6, 122.4, 121.4, 110.3, 109.8, 109.4, 97.6, 96.9, 89.9, 87.9, 83.3, 80.6, 66.4, 65.2, 43.1. HRMS(ESI): m/z calcd for $C_{24}H_{28}N_4S$ ($[M]^+$): 474.136; found: 474.116, ($[M\text{-allyl}]^+$): 433.097; found: 433.101.

Synthesis of compound S17



1st step (**S14**)

4-Bromotrifluoride (**S13**) (2.18 g, 9.69 mmol) was dissolved in anhydrous THF (3.5 mL) under nitrogen atmosphere. The solution was transferred dropwise at 4 °C to a flask containing Mg (240 mg, 9.88 mmol) suspended in anhydrous THF (10 mL). The mixture was stirred at 4 °C for 10 min and stirred at room temperature for 5 h (Grignard solution). In a different flask, diethyl

phosphite (0.28 mg, 2.00 mmol) was dissolved in anhydrous THF (3.0 mL) under nitrogen atmosphere, and Grignard solution was added to this flask dropwise at 4 °C. The whole mixture was stirred for 10 min at 4 °C and stirred at room temperature for 2 h. 1 N HCl aq (6 mL) was added dropwise at 4 °C to quench the reaction, and the organic layer was extracted with ethyl acetate. The combined organic phase was washed with brine, dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (hexane/ethyl acetate = 1/4) to afford compound **S14** (0.39 g, 1.16 mmol, 58% yield). **^{1}H NMR** ($CDCl_3$, 300 MHz) d 9.01 (0.5 H, s), 7.84 (8 H, m), 7.38 (0.5 H, s). **^{31}P NMR** ($CDCl_3$, 122 MHz) d 17.8.

2nd step (**S15**)

To a test tube, was added CuI (11 mg, 60 μmol). The tube was substituted with nitrogen atmosphere, (*S*)-α-phenethylamine (7.3 mg, 60 μmol) was added, followed by methyl 2-bromobenzoate (65 mg, 0.30 mmol) and anhydrous toluene (1.5 mL). The mixture was stirred for 5 min at room temperature, and compound **S14** (0.10 g, 0.30 mmol) was added. After stirring for 5 min at room temperature, K_2CO_3 (83 mg, 0.60 mmol) was added and the reaction mixture was refluxed at 110 °C overnight. The reaction was tracked by ^{31}P NMR measurement. After reaction completion, the reaction solution was diluted by ethyl acetate, and quenched with saturated NH_4Cl aq. The combined organic layer was dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (hexane/ethyl acetate = 2/1) to afford compound **S15** (0.39 g, 1.16 mmol, 58% yield). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ = 8.03 (1 H, m), 7.73 (11 H, m), 3.54 (3 H, s). **^{19}F NMR** ($CDCl_3$, 282 MHz) d -63.2. **^{31}P NMR** ($CDCl_3$, 122 MHz) δ = 29.0.

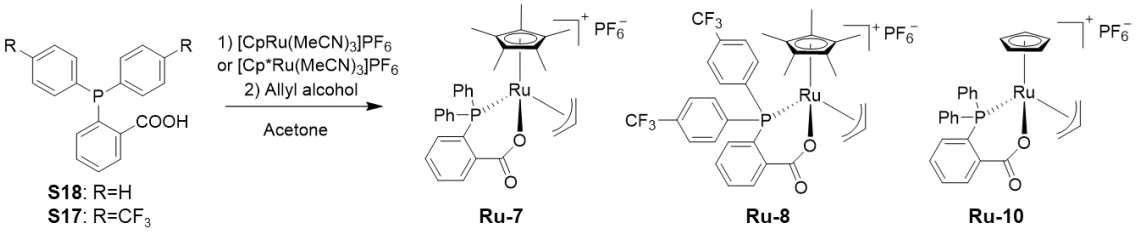
3rd step (**S16**)

To a flask containing compound **S15** (76 mg, 0.16 mmol) was added anhydrous toluene (6.0 mL) and triethylamine (84 mg, 0.83 mmol). The flask was substituted argon gas and cooled to 4 °C. Trichlorosilane (0.11 mg, 0.80 mmol) was added dropwise with a micro-syringe, and the whole mixture was refluxed 110 °C overnight. The mixture was diluted by ethyl acetate, and the reaction was quenched with saturated $NaHCO_3$ aq, and water layer was extracted with ethyl acetate. After filtration to remove insoluble byproducts, the combined organic layer was washed with brine, dried under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (hexane/ethyl acetate = 5/1) to afford compound **S16** (49 mg, 0.11 mmol, 69% yield). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ = 8.14 (1 H, m), 7.59 (4 H, d, J = 8.1 Hz), 7.47 (2 H, m), 7.36 (4 H, t, J = 7.6 Hz), 6.89 (1 H, m), 3.82 (3 H, s). **^{19}F NMR** ($CDCl_3$, 282 MHz) δ = -62.8. **^{31}P NMR** ($CDCl_3$, 122 MHz) δ = -5.3.

4th step (**S17**)

Compound **S16** (48 mg, 0.10 mmol) was added to a test tube, and nitrogen gas was purged. Compound **S16** was dissolved in degassed THF (1.0 mL), and the tube was cooled to 4 °C. To the solution was added LiOH aq (48 mg, 2.00 mmol) dissolved in degassed H_2O (1.0 mL) dropwise, and the whole reaction solution was stirred at 70 °C overnight. The solution was acidified with 1 N HCl (2.5 mL), and the water layer was extracted with ethyl acetate. The combined organic layer was washed with brine, dried under Na_2SO_4, filtered, concentrated *in vacuo* to afford compound **S17** (42 mg, 95 μmol, 95% yield). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ = 8.22 (1 H, m), 7.59 (4 H, d, *J* = 8.3 Hz), 7.50 (2 H, m), 7.36 (4 H, t, *J* = 7.3 Hz), 6.91 (1 H, m). **^{13}C NMR** ($CDCl_3$, 126 MHz) δ = 170.7, 142.2, 142.1, 139.8, 139.6, 134.3, 134.2, 134.0, 133.3, 132.6, 132.5, 132.0, 131.4, 131.1, 130.9, 130.6, 129.1, 125.4, 125.3, 125.1, 122.9. **^{19}F NMR** ($CDCl_3$, 282 MHz) δ = -62.8. **^{31}P NMR** ($CDCl_3$, 122 MHz) δ = -4.8. HRMS(ESI): m/z calcd for $C_{21}H_{13}F_6O_2P$ ($[M-H]^-$): 441.056; found: 441.064.

Synthesis of half-sandwich Ru complexes coordinated by P,O-ligands



$[CpRu(MeCN)_3]PF_6$ or $[Cp^*Ru(MeCN)_3]PF_6$ (1.0 equiv.) was dissolved in anhydrous degassed acetone (2.0 mL) under argon atmosphere. Compound **S18** or **S17** (1.0 equiv.) was added, and the solution turned into dark red color. After stirring for 30 min at room temperature, allyl alcohol (2.0 equiv.) dissolved in degassed acetone (1.0 mL) was added, and the reaction solution turned into orange-yellow color. After stirring for 3 h at room temperature, the solvent was removed under reduced pressure. The resulting oil was redissolved with a minimal amount of dichloromethane, and hexane was added to cause precipitation. After cooling at -15 °C for 2 h, the solvent was removed by decantation, and the resulting solid was dried under vacuum overnight to afford the desired products.

Ru-7

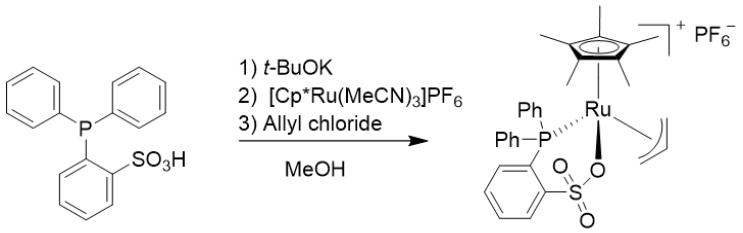
$[Cp^*Ru(MeCN)_3]PF_6$ (35 mg, 69 μmol) and compound **S18** (21 mg, 69 μmol) were employed for the synthesis of **Ru-7** (47 mg, 62 μmol, 90% yield). **^{1}H NMR** (CD_2Cl_2, 300 MHz) δ = 8.35 (1 H, m), 7.87-7.30 (13 H, m), 4.39 (1 H, m), 4.29 (1 H, m), 3.84 (1 H, dd, *J* = 11.1 Hz *J* = 3.1 Hz), 3.76 (1 H, td, *J* = 6.6 Hz, *J* = 2.1 Hz), 2.26 (1 H, m).

Ru-8

$[Cp^*Ru(MeCN)_3]PF_6$ (15 mg, 30 μmol) and compound **S17** (13 mg, 30 μmol) were employed for the synthesis of **Ru-8** (21 mg, 22 μmol, 72% yield). **^{1}H NMR** (acetone-d_6, 300 MHz) δ = 8.39 (1 H, m), 8.15-8.11 (2 H, m), 7.92-7.82 (6 H, m), 7.72 (1 H, m), 7.58 (1 H, m), 7.44(1 H, m), 4.49 (2 H, m), 3.91 (1 H, dd, *J* = 7.4 Hz, *J* = 3.3 Hz), 3.84 (1 H, m), 2.35 (1 H, m), 1.71 (15 H, d, *J* = 2.0 Hz). **^{13}C NMR** (acetone-d_6, 126 MHz) δ = 168.1, 138.8, 138.4, 137.5, 137.4, 133.9, 133.8, 133.7, 133.6, 133.2, 131.8, 131.7, 130.8, 126.7, 126.6, 126.2, 126.1, 125.1, 125.0, 124.9, 124.8, 123.8, 108.4, 100.3, 80.6, 56.0, 9.1. **^{31}P NMR** (acetone-d_6, 122 MHz) δ = 27.8, -144.3. **^{19}F NMR** (acetone-d_6, 282 MHz) δ = -63.8, -64.0, -71.8, -73.3. HRMS(ESI): m/z calcd for $C_{34}H_{32}F_6O_4PRu$ ($[M\text{-allyl}+H]^+$): 679.0775; found: 679.0761.

Ru-10

$[CpRu(MeCN)_3]PF_6$ (30 mg, 69 μmol) and compound **S18** (21 mg, 69 μmol) were employed for the synthesis of **Ru-10** (47 mg, 62 μmol, 90% yield). **^{1}H NMR** (acetone-d_6, 300 MHz) δ = 8.10 (1 H, m), 7.79-7.41 (10 H, m), 7.27 (2 H, m), 7.02 (1 H, m), 5.99 (5 H, s), 5.01 (1 H, dd, *J* = 8.2 Hz, *J* = 2.8 Hz), 4.52 (1 H, m), 3.99 (1 H, m), 3.60 (1 H, m), 2.52 (1 H, d, *J* = 18.2 Hz). **^{13}C NMR** (acetone-d_6, 126 MHz) δ =169.8, 137.4, 137.4, 136.5, 136.4, 133.5, 133.5, 133.1, 133.1, 133.0, 132.9, 132.4, 132.4, 132.0, 131.9, 131.8, 131.7, 130.0, 129.9, 129.8, 129.4, 129.3, 129.0, 128.8, 128.7, 128.0, 127.6, 127.5, 127.0, 126.5, 126.1, 100.8, 95.5, 83.9, 82.1, 80.6, 71.6, 54.6, 53.3. **^{31}P NMR** ($CDCl_3$, 122 MHz) δ = 27.6, -144.2. HRMS(ESI): m/z calcd for $C_{27}H_{24}O_2PRu$ ($[M\text{-allyl}+H]^+$): 473.0244; found: 473.0211.

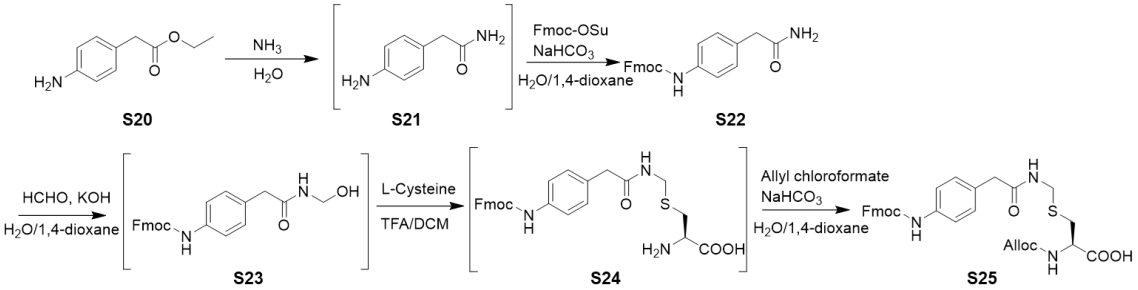


S19 **Ru-8**

Compound **S19** (30 mg, 59 μmol) and *t*-BuOK (6.7 mg, 59 μmol) were dissolved in degassed anhydrous MeOH (1.5 mL) under argon atmosphere. After stirring for 30 min at room temperature, allyl chloride (14 mg, 0.12 mmol) dissolved in degassed MeOH (0.5 mL) was added dropwise, and the whole reaction solution was stirred overnight at room temperature. After filtration to remove inorganic salts, the solvent was removed under reduced pressure. The resulting oil was dissolved with a minimal amount of dichloromethane and was covered with hexane to cause precipitation. This reprecipitation was repeated three times, and the resulting solid was dried *in vacuo* overnight to afford **Ru-8** (27 mg, 31 μmol, 53% yield). **^{1}H NMR** (CD_2Cl_2, 300 MHz) δ =

8.18 (1 H, m), 7.74-7.46 (12 H, m), 7.12 (1 H, m), 4.98 (1 H, m), 3.91 (2 H, m), 3.69 (1 H, m), 2.35 (1 H, d, J = 10.5 Hz), 1.70-1.51 (15 H, m). **^{31}P NMR** (CD_2Cl_2, 122 MHz) δ = 45.7.

Synthesis of a solubilizing tag bearing phenylacetamidomethyl (Phacm) linker [Alloc-Cys(Phacm-Fmoc)-OH] (S25)



1st step (**S21**)

To a flask containing compound **S20** (1.00 g, 5.58 mmol), was added NH_4OH aq (30% (w/w)) (4.5 mL) and distilled water (3.0 mL), and the mixture was stirred overnight at room temperature. The organic solvent was removed under reduced pressure, and excess amount of NaCl was added to the residual solution. Then, the water layer was extracted with THF at least five times. The combined organic layer was dried under Na_2SO_4, filtered, concentrated *in vacuo* to afford compound **S21**. This compound was used for the next reaction without further purification.

2nd step (**S22**)

Compound **S21** was dissolved in a mixture of water and 1,4-dioxane (40 mL, 1:1), and $NaHCO_3$ (1.13 g, 13.4 mmol) and Fmoc-OSu (2.26 g, 6.70 mmol) were added. After stirring overnight at room temperature, the aqueous layer was extracted with THF. The combined organic layer was drier under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (hexane/ethyl acetate = 20/80 → hexane/ethyl acetate = 0/100 → ethyl acetate/MeOH = 95/5) to afford compound **S22** (0.96 g, 2.59 mmol, 46% yield). **^{1}H NMR** (DMSO-d_6, 300 MHz) δ = 9.64 (1 H, s), 7.91 (2 H, d, J = 7.6 Hz), 7.76 (2 H, d, J = 7.2 Hz), 7.41 (7 H, m), 7.15 (2 H, J = 8.2 Hz), 6.83 (1 H, s), 4.47 (2 H, d, J = 6.5 Hz), 4.31 (1 H, t, J = 6.4 Hz), 3.29 (2 H, s). **^{13}C NMR** (DMSO-d_6, 126 MHz) δ = 172.9, 153.9, 144.3, 141.3, 137.8, 131.0, 129.8, 128.2, 127.6, 125.6, 120.6, 118.7, 66.0, 47.1, 42.0. HRMS(ESI): m/z calcd for $C_{23}H_{20}N_2O_3$ ($[M+Na]^+$): 395.137; found: 395.131.

3rd step (**S23**)

To a test tube containing compound **S22** (0.92 g, 2.47 mmol), was added 1,4-dioxane (8.0 mL) and 1.5 M $CaCl_2$ aq (4.0 mL), and the whole mixture was heated to 70 °C for 5 min to dissolve

compound **S22**. Formaldehyde (35% in water) (0.42 mL, 4.94 mmol) and a small amount of KOH (14 mg, 0.25 mmol) were added, and the reaction solution was stirred overnight at 35 °C. The reaction was tracked by ^{1}H NMR. The reaction was quenched by saturated NH_4Cl aq, and the aqueous layer was extracted with THF three times. The combined organic layer was drier under Na_2SO_4, filtered, concentrated *in vacuo* to afford compound **S23**, which was used for the next reaction without further purification.

4th step (**S24**)

Compound **S23** was dissolved in a mixture of dichloromethane and TFA (8.0 mL, 1:1) at 4 °C. L-Cysteine (0.30 mg, 2.46 mmol) was added to the solution, and the mixture was stirred for 1 h at room temperature. After the removal of the solvent under reduced pressure, the resulting oil (**S24**) was employed for the next reaction without further purification.

5th step (**S25**)

To flask containing compound **S24**, was added a mixture of 1,4-dioxane and water (20 mL, 1:1), and the solution was neutralized with solid $NaHCO_3$ to adjust the pH around 8.0. Then, allylchloroformate (0.35 g, 2.95 mmol) was added to the reaction solution, and the mixture was stirred at room temperature until the starting material disappeared on TLC stained by ninhydrin. The solution was acidified with 1 N HCl aq, and the water layer was extracted with ethyl acetate. The combined organic layer was drier under Na_2SO_4, filtered, concentrated *in vacuo*, and purified by column chromatography (hexane/ethyl acetate = 20/80) to afford Alloc-Cys(Phacm-Fmoc)-OH (**S25)** (0.46 g, 0.78 mmol, 32% yield). **^{1}H NMR** (DMSO-d_6, 300 MHz) δ = 12.8 (1 H, br), 9.65 (1 H, s), 8.64 (1 H, t, *J* = 6.3 Hz), 7.91 (2 H, d, *J* = 7.1 Hz), 7.75 (2 H, *J* = 7.4 Hz), 7.59 (1 H, d, *J* = 8.6 Hz), 7.38 (6 H, m), 7.14 (2 H, d, *J* = 8.3 Hz), 5.90 (1 H, m), 5.30 (1 H, dd, *J* = 17.2 Hz, *J* = 1.9 Hz), 5.19 (1H, dd, *J* = 10.4 Hz, *J* = 1.7 Hz), 4.48 (4 H, m), 4.25 (4 H, m), 3.37 (2 H, s), 3.01 (1 H, dd, *J* = 13.7 Hz, *J* = 4.6 Hz), 2.78 (1 H, dd, *J* = 13.6 Hz, *J* = 9.8 Hz). **^{13}C NMR** (DMSO-d_6, 126 MHz) δ = 172.8, 171.0, 156.3, 153.9, 144.3, 141.3, 137.9, 133.9, 130.5, 129.8, 128.2, 127.6, 125.6, 120.6, 118.7, 117.5, 66.0, 65.0, 54.4, 47.1, 42.0, 40.9, 32.2. HRMS(ESI): m/z calcd for $C_{31}H_{31}N_3O_7S$ ($[M-H]^-$): 588.1883; found: 588.1877.

Part 1. Exploring of Metal Complexes for the Alloc Deprotection.

Removal of the alloc group of peptide 1 with Pd/phosphine complexes. To the solution of $Pd(OAc)_2$ in degassed *N*,*N*-dimethylformamide (200 mM), was added 4.0 equiv. of phosphine ligands (TPPTS, σ-Danphos, TPPTC, TPPMS, and DBPPS) dissolved or suspended (as for TPPMS) in degassed water and the mixture was vigorously vortexed for 30 s at room temperature under argon atmosphere to afford Pd/phosphine complexes (100 mM). Peptide **1** was prepared by following our previous report.[1] To peptide **1** solution in 15.0 μL denaturing buffer (6 M Gn·HCl aq containing 0.2 M NaH_2PO_4 at pH 7.0) (4.0 mM) were added denaturing buffer 5.0 μL, 2.4 μL TCEP aq (500 mM), 0.4 μL $MgCl_2$ (500 mM), and 6.0 μL MPAA aq (500 mM). Then, to the mixture was added 1.2 μL Pd/phosphine solution (100 mM) and the reaction mixture were stirred for 10 min at room temperature under argon atmosphere. The final concentration of the reaction mixture was peptide **1** (2 mM), TCEP (40 mM), $MgCl_2$ (6 mM), MPAA (100 mM), and palladium complexes (4 mM). For analysis of each reaction, aliquot from each reaction mixture was treated 15.0 μL MESNa aq (1.0 M) and 2.0 μL TCEP (500 mM), followed by stirring for 10 s and injection into analytical HPLC.

Deprotection of the alloc group of peptide 1 with Ru complexes. To peptide **1** solution in 15.0 μL denaturing buffer (4 mM) were added denaturing buffer (7.0 μL for 10 mol% reaction or 8.0 μL for 5 mol% reaction) and 6.0 μL MPAA aq (500 mM). Then, to the mixture was added each Ru complex solution (3 mM, 2.0 μL for 10 mol% reaction or 1.0 μL for 5 mol% reaction) dissolved in acetonitrile. The final concentration of the reaction mixture was peptide **1** (2 mM), MPAA (100 mM), Ru complexes (0.2 mM or 0.1 mM). The reaction solution was stirred for each time at room temperature under air conditions. For analysis of each reaction, 2.0 μL of aliquot from each reaction mixture was treated 15.0 μL denaturing buffer and 2.0 μL TCEP (500 mM), followed by stirring for 10 s to quench the reaction, and the solution was injected to analytical HPLC.

Evaluation of the formation of MPAA disulfide dimer. To 240 μL denaturing buffer (pH 7.0) was added 60 μL MPAA (500 mM), and the mixture containing 100 mM MPAA was stirred under argon atmosphere for 3 h at 37 °C. To reduce the disulfide bond, 0.3 μL or 0.6 μL TCEP (500 mM) was added to 30 μL MPAA solution, and the whole mixture was stirred for 10 min at room temperature. To 20 μL solution containing 100 mM MPAA and 5 mM TCEP oxide were added

powdered peptide **1** (0.08 mg) and **Ru-4** (10 mM, 0.2 μL) successively. The reaction mixture was stirred for 10 min at room temperature. For analysis of each reaction, 1.0 μL of aliquot from MPAA solution or 2.0 μL of aliquot from peptide solution was treated 15.0 μL denaturing buffer and 2.0 μL TCEP (500 mM), followed by stirring for 10 s to quench the reaction, and the solution was injected to analytical HPLC.

Calculation of TON of Ru-3, Ru-4, and Ru-10. To peptide 1 solution in 15.0 μL denaturing buffer (4 mM) were added 8.4 μL denaturing buffer and 6.0 μL MPAA aq (500 mM). Then, to the mixture was added each 0.6 μL **Ru-3**, **Ru-4**, or **Ru-10** solution (1 mM) dissolved in acetonitrile. The final concentration of the reaction mixture was peptide 1 (2 mM), MPAA (100 mM), Ru complexes (0.02 mM). The reaction solution was stirred for each time at room temperature. For analysis of each reaction, 2.0 μL of aliquot from each reaction mixture was treated 15.0 μL denaturing buffer and 2.0 μL TCEP (500 mM), followed by stirring for 10 s to quench the reaction, and the solution was injected to analytical HPLC.

Reaction between peptide 1 and Ru complexes in the absence of MPAA. To peptide **1** solution in 15.0 μL denaturing buffer (4 mM) were 13.0 μL denaturing buffer and each 2.0 μL **Ru-4** or **Ru-10** solution (3 mM) dissolved in acetonitrile. The final concentration of the reaction mixture was peptide **1** (2 mM), Ru complexes (0.2 mM). The reaction solution was stirred for 1 h at room temperature. For analysis of each reaction, 2.0 μL of aliquot from each reaction mixture was treated 15.0 μL denaturing buffer and 2.0 μL TCEP (500 mM), followed by stirring for 10 s to quench the reaction. The solution was injected to analytical HPLC. Each collected peak was analyzed by MALDI LIFT-TOF/TOF and biotools (Bruker).

Deactivation test to examine the stability of Ru complexes toward MPAA. The stability of Pd/TPPTS complexes toward MPAA was examined in our previous experiments.[1] To check the stability of **Ru-4** and **Ru-10** against MPAA, 79.0 μL denaturing buffer, 20.0 μL MPAA aq (500 mM), and 1.0 μL Ru complex solution (20 mM) were mixed, and the solution was stirred at room temperature under argon atmosphere. At each time point (3, 10, or 30 min), each Ru solution was picked and added to powdered peptide **1**. The final concentration of every reaction mixture was peptide **1** (2 mM), MPAA (100 mM), **Ru-4** or **Ru-10** (0.2 mM). The mixtures were stirred at room temperature for 10 min under air, and 2.0 μL of aliquot from each reaction mixture was treated 15.0 μL denaturing buffer and 2.0 μL TCEP (500 mM), followed by stirring for 10 s to quench the reaction. The solution was injected to analytical HPLC.

Elucidation of deactivation mechanism of Ru complexes by MPAA. To a tube containing **Ru-**

3 (2.4 mg, 4.5 μmol) was added MPAA (3.7 mg, 22 μmol) solution in acetone-d_6 (600 μL). DIEA (3.8 μL, 22 μmol) was added to the **Ru-3** solution, and the whole mixture was stirred for 1 h at room temperature. The resulting precipitation was spined down by centrifugation, and supernatant was collected by decantation and analyzed by ^{1}H NMR. The precipitation was dissolved in a mixture of CD_3CN (300 μL) and D_2O (300 μL) and analyzed by ^{1}H NMR and MALDI-TOF mass.

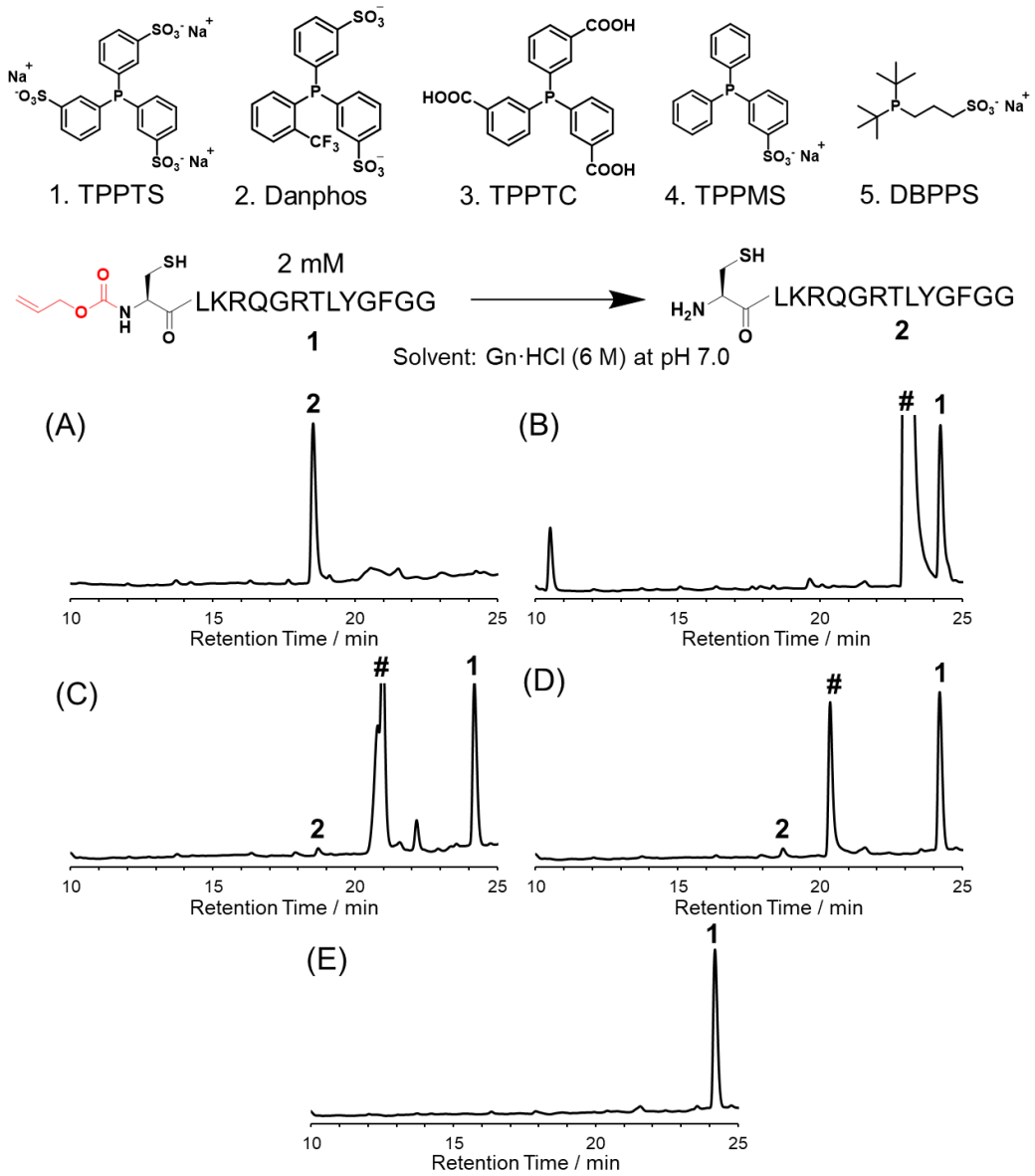
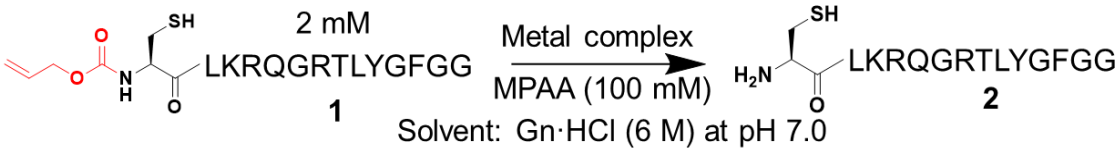


Figure S1. HPLC analysis of the removal of the alloc group of peptide **1** with Pd/phosphine complexes (2.0 equiv.). HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10–35% for 25 min. (A) Pd/TPPTS, (B) Pd/Danphos, (C) Pd/TPPTC, (D)Pd/TPPMS, (E)Pd/DBPPS. # = Each phosphine ligand.

Notes: We screened bis(3-sulfonatophenyl)(2-trifluoromethylphenyl)phosphine (σ-Danphos),[2] 3-phosphanetriymono(benzenesulfonic acid) monosodium salt (TPPMS), and triphenylphosphan-3,3′,3″-tricarboxylate (TPPTC)[3] and 3-(di-*tert*-butylphosphonium)propane sulfonate (DBPPS) as three-valent water-soluble phosphine ligands to change the electron density of Pd. However, none of these Pd complexes did not show any catalytic activity for the alloc remocal under NCL conditions.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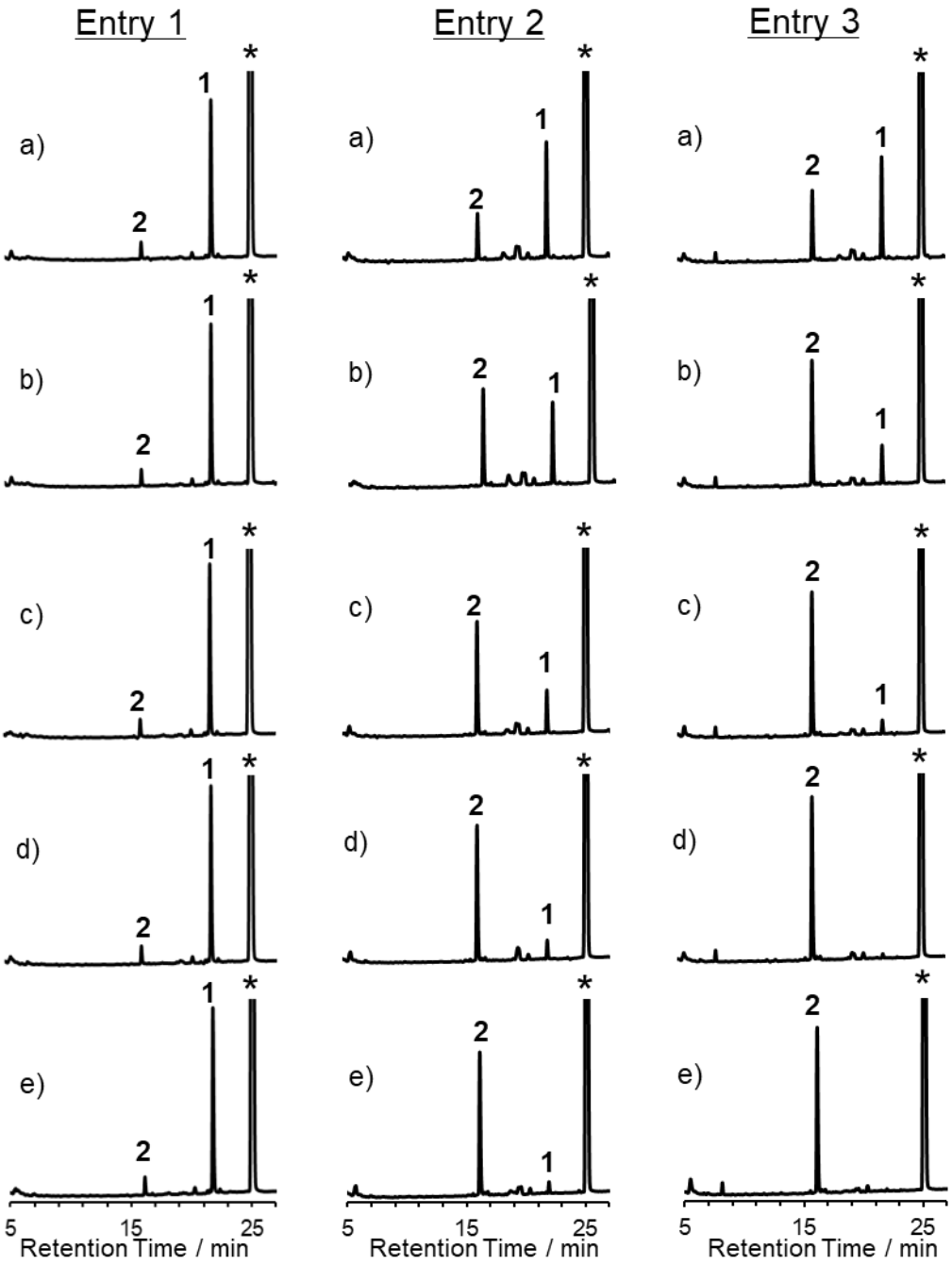
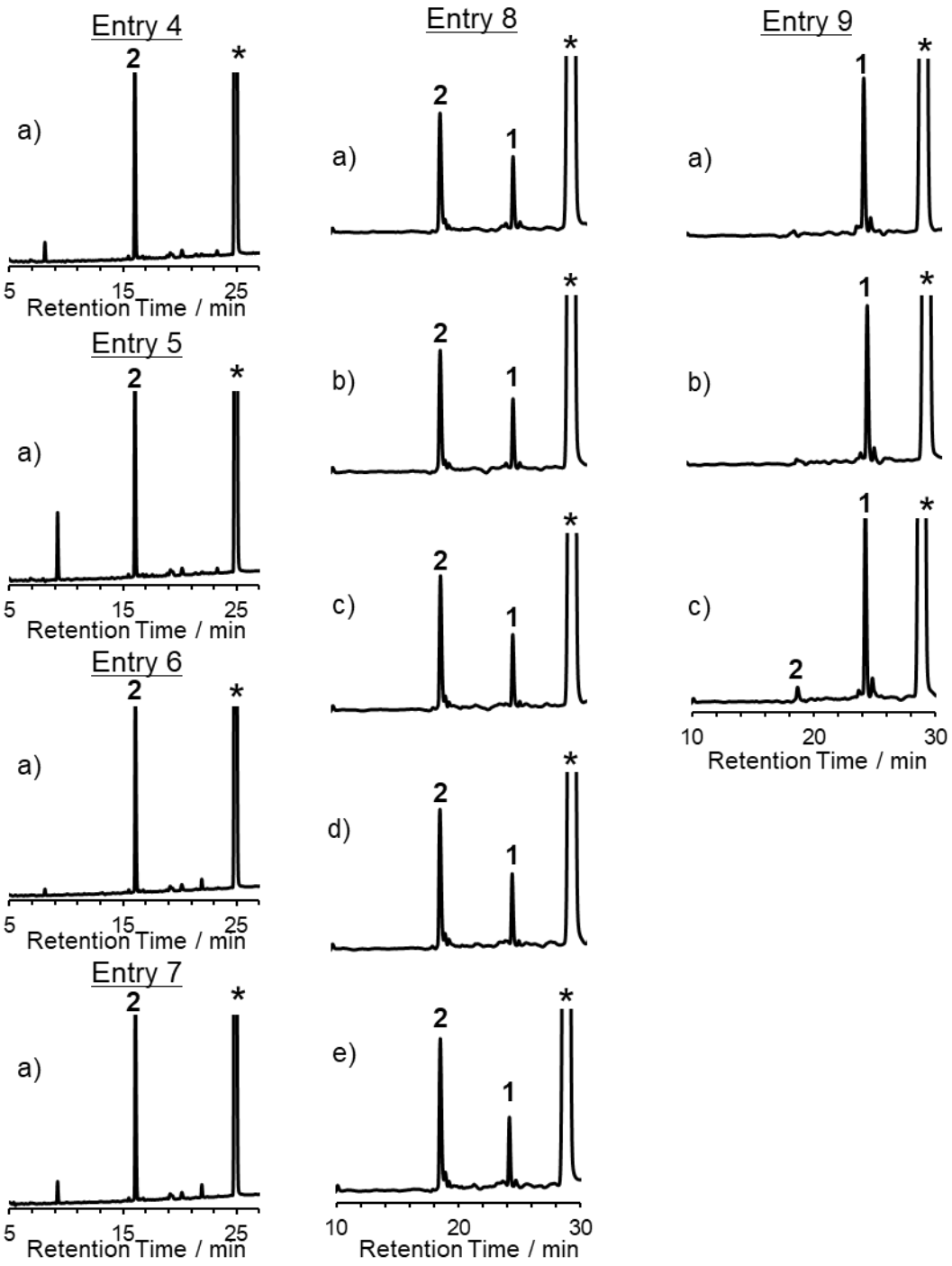
LKRQGRTLYGFGG
1
Metal complex
MPAA (100 mM)
Solvent: Gn·HCl (6 M) at pH 7.0
LKRQGRTLYGFGG
2


Entry 1
Entry 2
Entry 3
a)
b)
c)
d)
e)
1
2
*
5
15
25
Retention Time / min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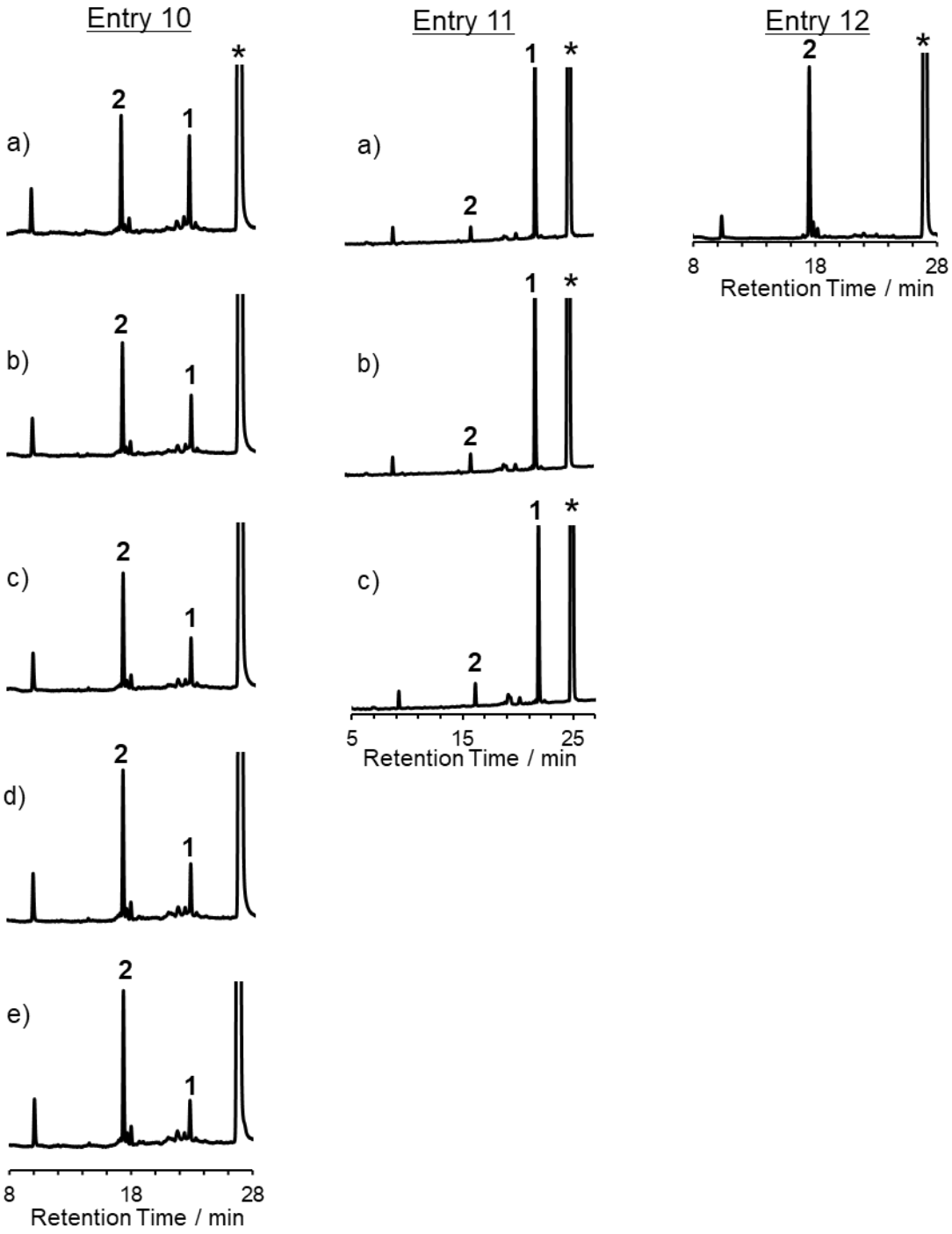
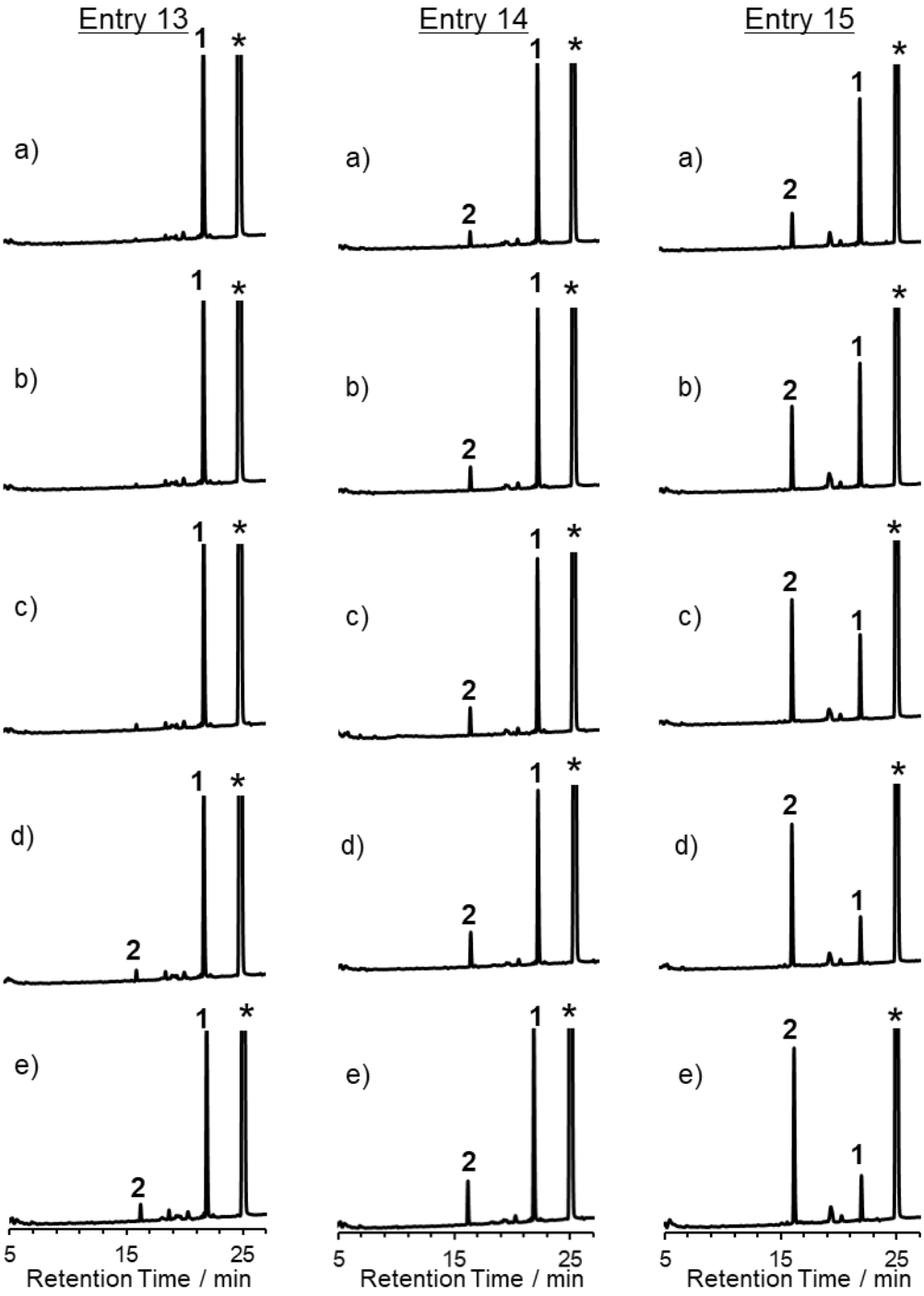
Entry 5
Entry 6
Entry 7
Entry 8
Entry 9
a)
b)
c)
d)
e)
1
2
*
5
15
25
10
20
30
Retention Time / min


Entry 10
Entry 11
Entry 12
a)
b)
c)
d)
e)
1
2
*
8
18
28
5
15
25
Retention Time / min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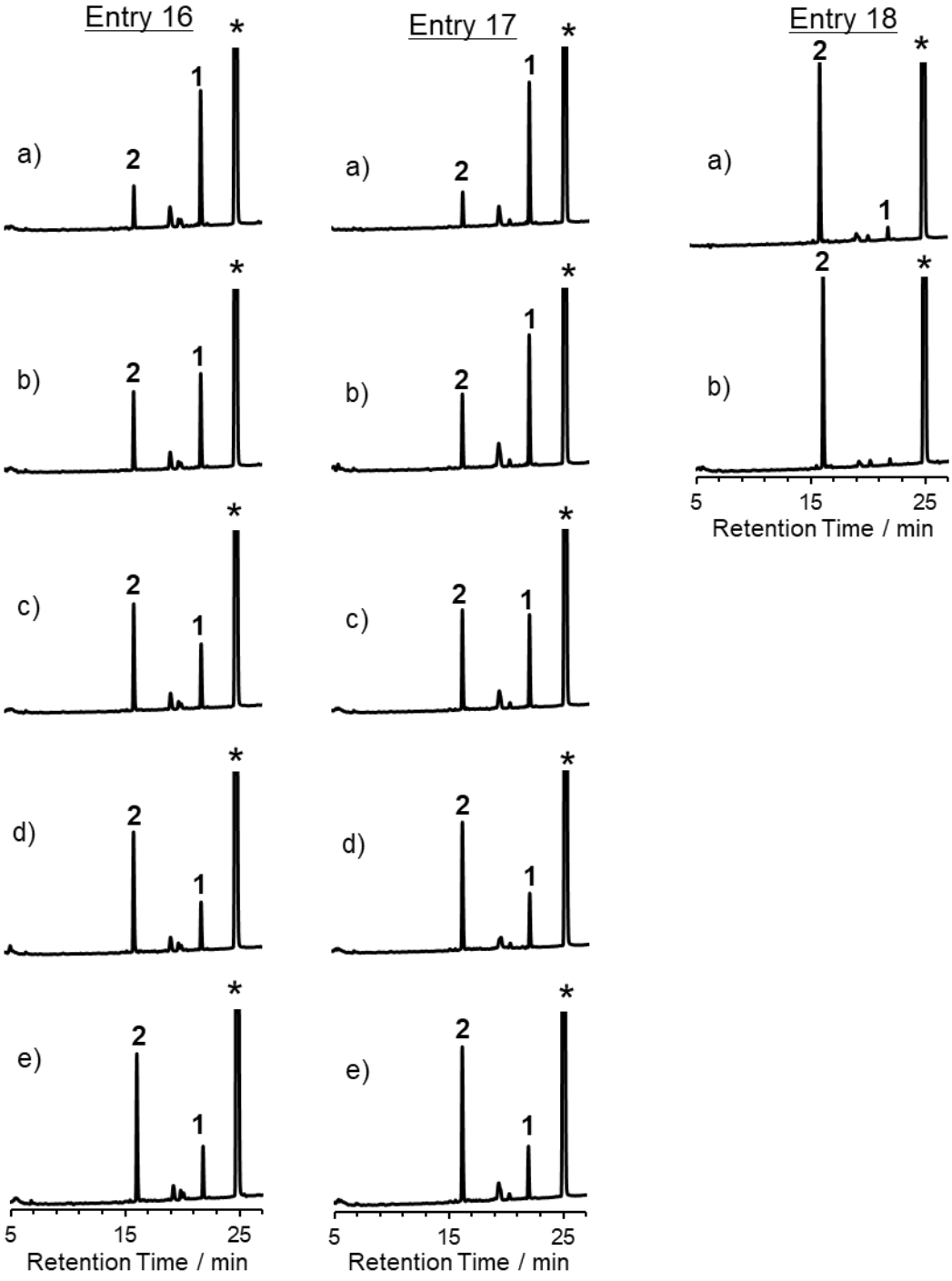
Entry 14
Entry 15
a)
b)
c)
d)
e)
1
2
*
5
15
25
Retention Time / min


Entry 16
Entry 17
Entry 18
a)
b)
c)
d)
e)
1
2
*
5
15
25
Retention Time / min

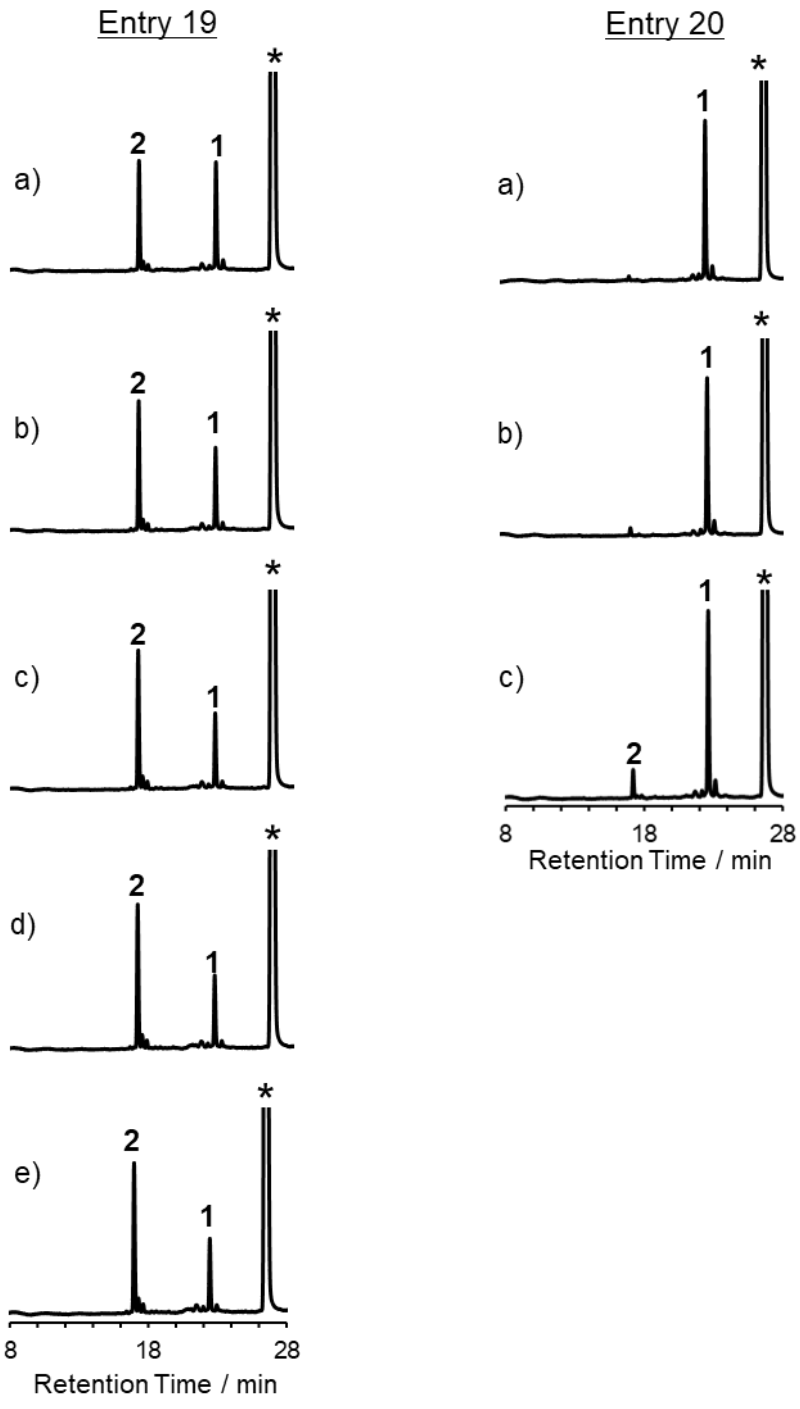

Entry 19
Entry 20
a)
b)
c)
d)
e)
1
2
*
8
18
28
Retention Time / min

Figure S2. Removal of Alloc groups with Ru complexes. Peptide **1** was reacted with each metal complex. Entry 1: Pd/TPPTS complex (10 mol%), entry 2: **Ru-1** (10 mol%), entry 3: **Ru-2** (10 mol%), entry 4: **Ru-3** (10 mol%), entry 5: **Ru-4** (10 mol%), entry 6: **Ru-3** (5 mol%), entry 7: **Ru-4** (5 mol%), entry 8: **Ru-3** (5 mol%) + TCEP 3 mM, entry 9: **Ru-3** (5 mol%) + TCEP 10 mM, entry 10: **Ru-4** (5 mol%) + TCEP 3 mM, entry 11: **Ru-4** (5 mol%) + TCEP 10 mM, entry 12: **Ru-4** (5 mol%) + TCEP 5 mM (that was completely consumed before the addition of **Ru-4** as shown in Figure S3), entry 13: **Ru-5** (5 mol%), entry 14: **Ru-6** (5 mol%), entry 15: **Ru-7** (5 mol%), entry 16: **Ru-8** (5 mol%), entry 17: **Ru-9** (5 mol%), entry 18: **Ru-10** (5 mol%), entry 19: **Ru-10** (5 mol%) + TCEP 3 mM, entry 20: **Ru-10** (5 mol%) + TCEP 10 mM. Reaction time: a) 10 min, b) 30 min, c) 1 h, d) 2 h, e) 3 h. *=MPAA.

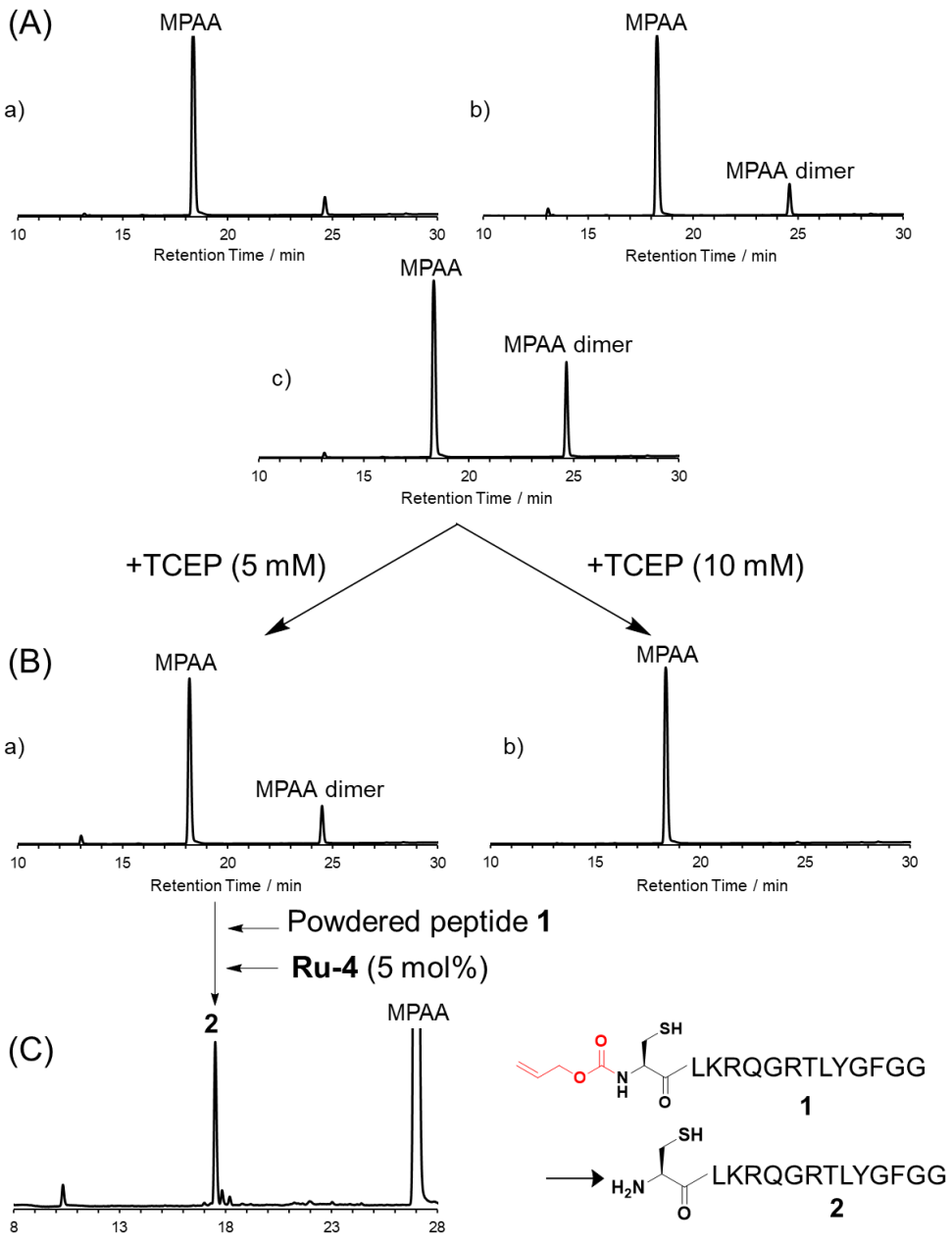
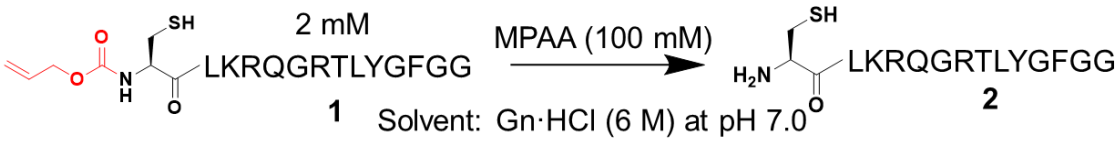
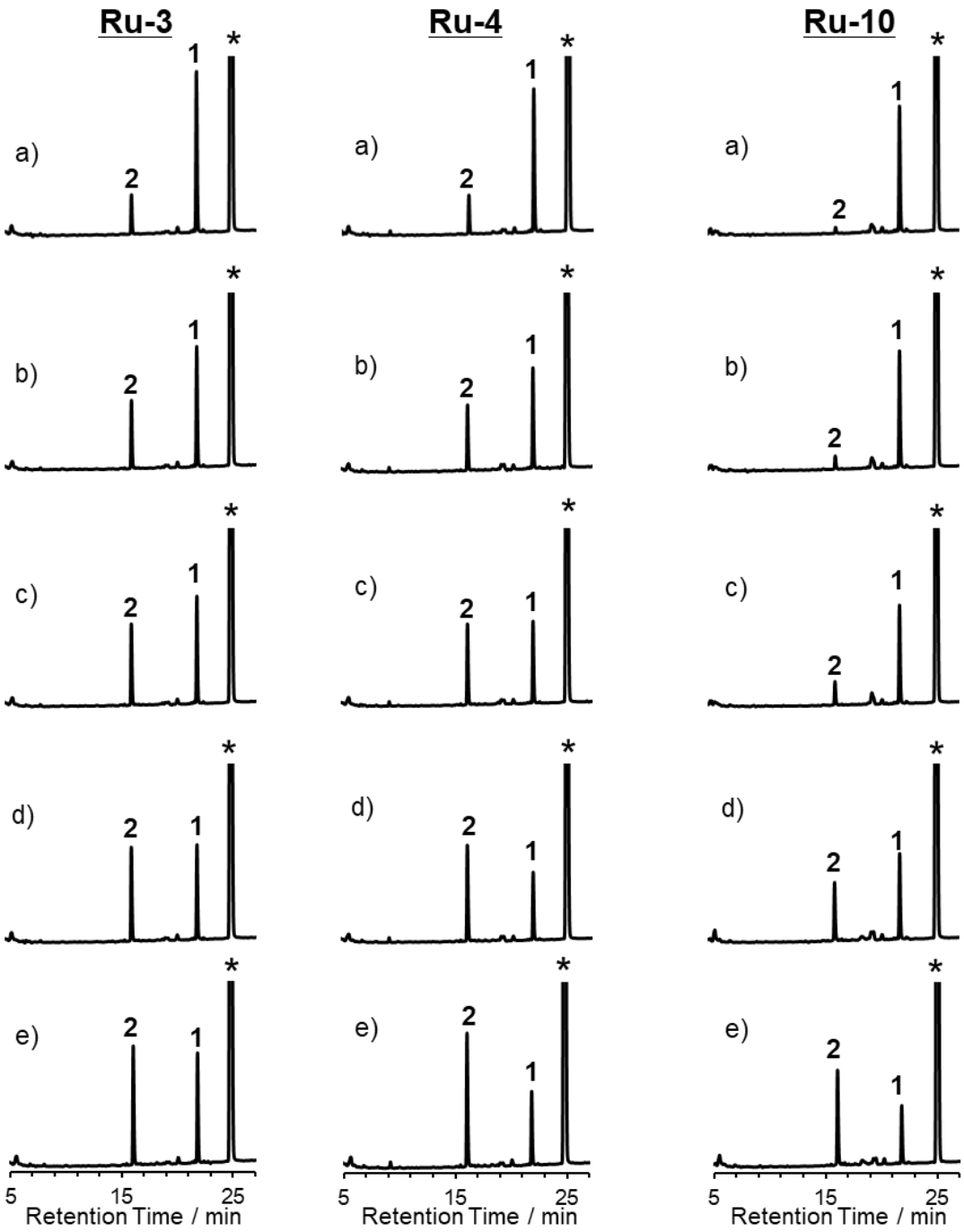


Figure S3. Evaluation of the formation of MPAA disulfide dimer. (A) HPLC analysis of the formation of the disulfide bond in 100 mM MPAA solution. a) 1 min, b) 1 h, c) 3 h. Gradient: 10–70% for 30 min. (B) Reduction of disulfide bond of MPAA dimer with the treatment a) of TCEP (5 mM) or b) of TCEP (10 mM). Gradient: 10–70% for 30 min. (C) Removal of the alloc group of peptide **1** with **Ru-4**. Peptide 1 (2 mM), MPAA (100 mM), TCEP oxide (5 mM), **Ru-4** (0.1 mM) in denaturing buffer at pH 7.0 under air atmosphere. Gradient: 10–35% for 25 min.


2 mM
LKRQGRTLYGFGG
1
MPAA (100 mM)
Solvent: Gn·HCl (6 M) at pH 7.0
LKRQGRTLYGFGG
2


Ru-3
Ru-4
Ru-10
a)
b)
c)
d)
e)
1
2
*
5
15
25
Retention Time / min

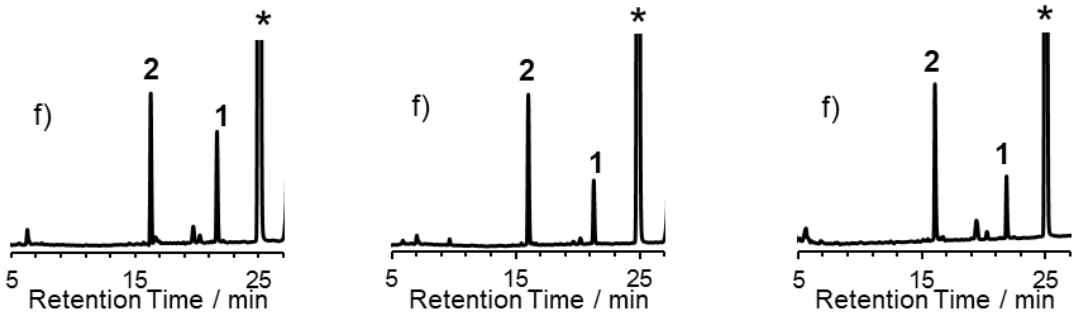


Figure S4. HPLC analysis for calculation of TON and TOF of **Ru-3**, **Ru-4**, and **Ru-10** under NCL conditions. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10–35% for 25 min. Peptide **1** was reacted with each metal complex (1.0 mol%). Reaction time: a) 1 min, b) 3 min, c) 5 min, d) 10 min, e) 30 min, f) 2 h. * = MPAA.

Table S1. Comparison of the efficiency of each Ru complex for the alloc deprotection.

			Conversion yields [%]					
Entry	Metal complexes	Quantity (mol%)	1 min	3 min	5 min	10 min	30 min	2 h
1	**Ru-3**	1	19	35	44	52	52	56
2	**Ru-4**	1	20	41	51	60	65	70
3	**Ru-10**	1	6	11	21	47	65	72

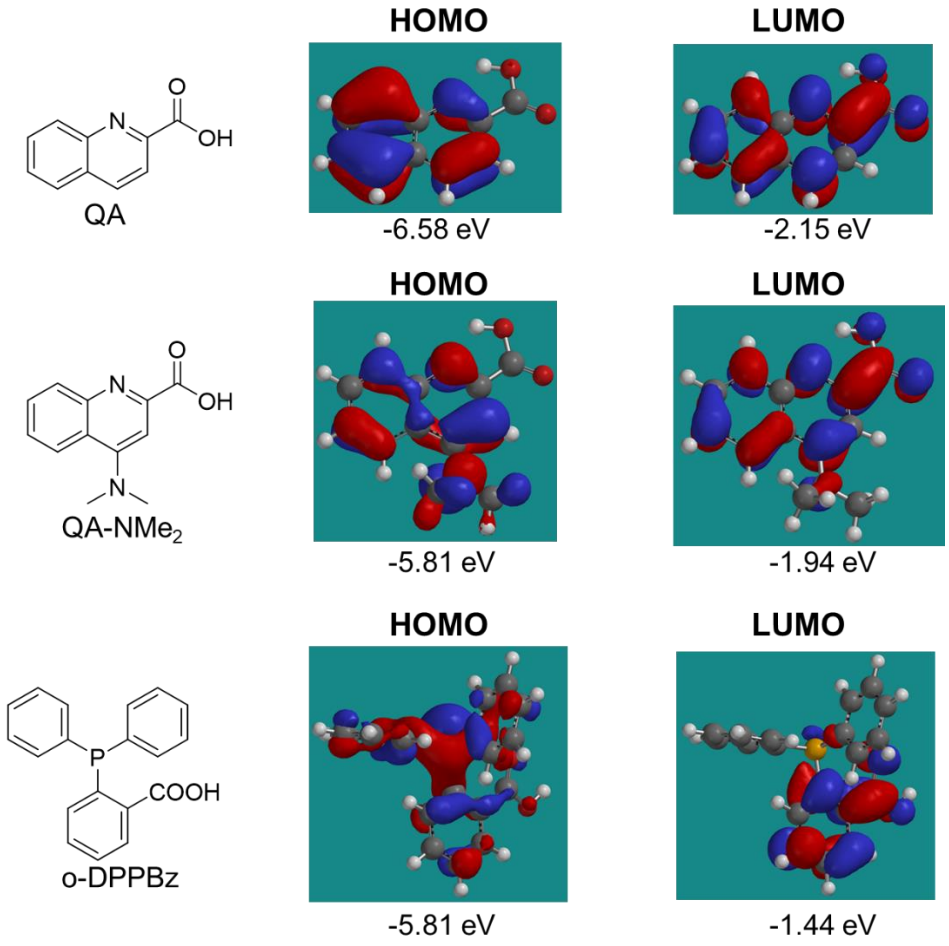


Figure S5. Density functional theory (DFT) calculations of bidentate ligands of **Ru-3**, **Ru-4**, or **Ru-10**. Each data was calculated by SPARTAN 10' for windows software from Wavefunction Inc. at 6-31G** level.

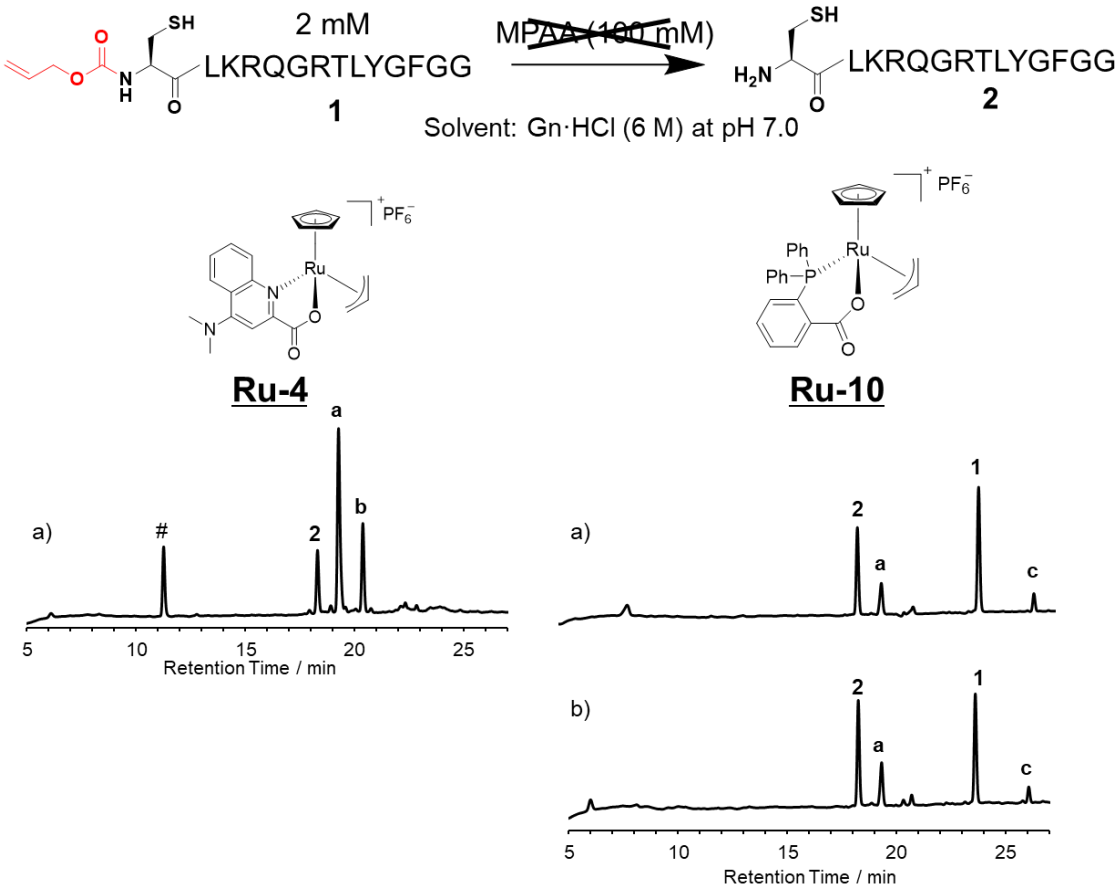


Figure S6. Reaction between peptide **1** and Ru complexes in the absence of MPAA. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10–35% for 25 min with a 5C_{18}-AR-II column. Peptide **1** was reacted with **Ru-4** or **Ru-10** (10 mol%). Reaction time: a) 30 min, b) 1 h. # = ligand dissociated from Ru

Calculated mass of [Peptide **2**+allyl+H]$^+$: 1595.8; Mass Found [Peak **a**+H]$^+$: 1595.7.

Calculated mass of [Peptide **2**+2allyl+H]$^+$: 1635.9; Mass Found [Peak **b**+H]$^+$: 1635.8.

Calculated mass of [Peptide **1**+allyl+H]$^+$: 1679.9; Mass Found [Peak **c**+H]$^+$: 1679.9.

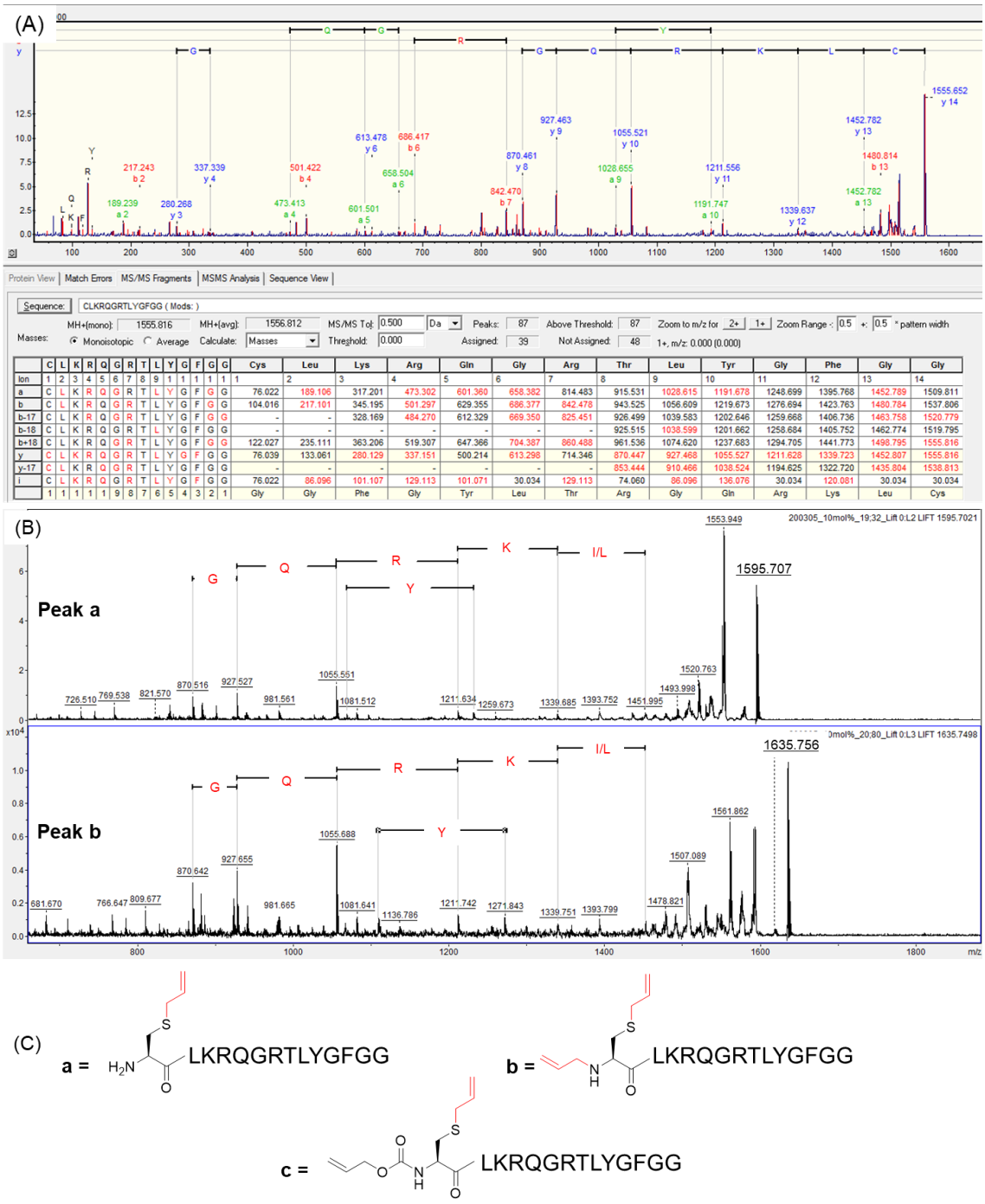


Figure S7. MS/MS analysis with MALDI LIFT-TOF/TOF of peptide **2**, peak **a** and **b** shown in Figure S5. (A) Analysis of MS/MS spectrum with lift mode of peptide **2** by biotools (Bruker). (B) MS/MS spectra of peak **a** and peak **b**. The detected y ion pattern was consistent with peptide **2**, which suggested that the allyl groups were transferred via Ru catalysts to N-terminal Cys of peptide **2**. (C) Plausible chemical structures of peak **a**, peak **b**, peak **c**.

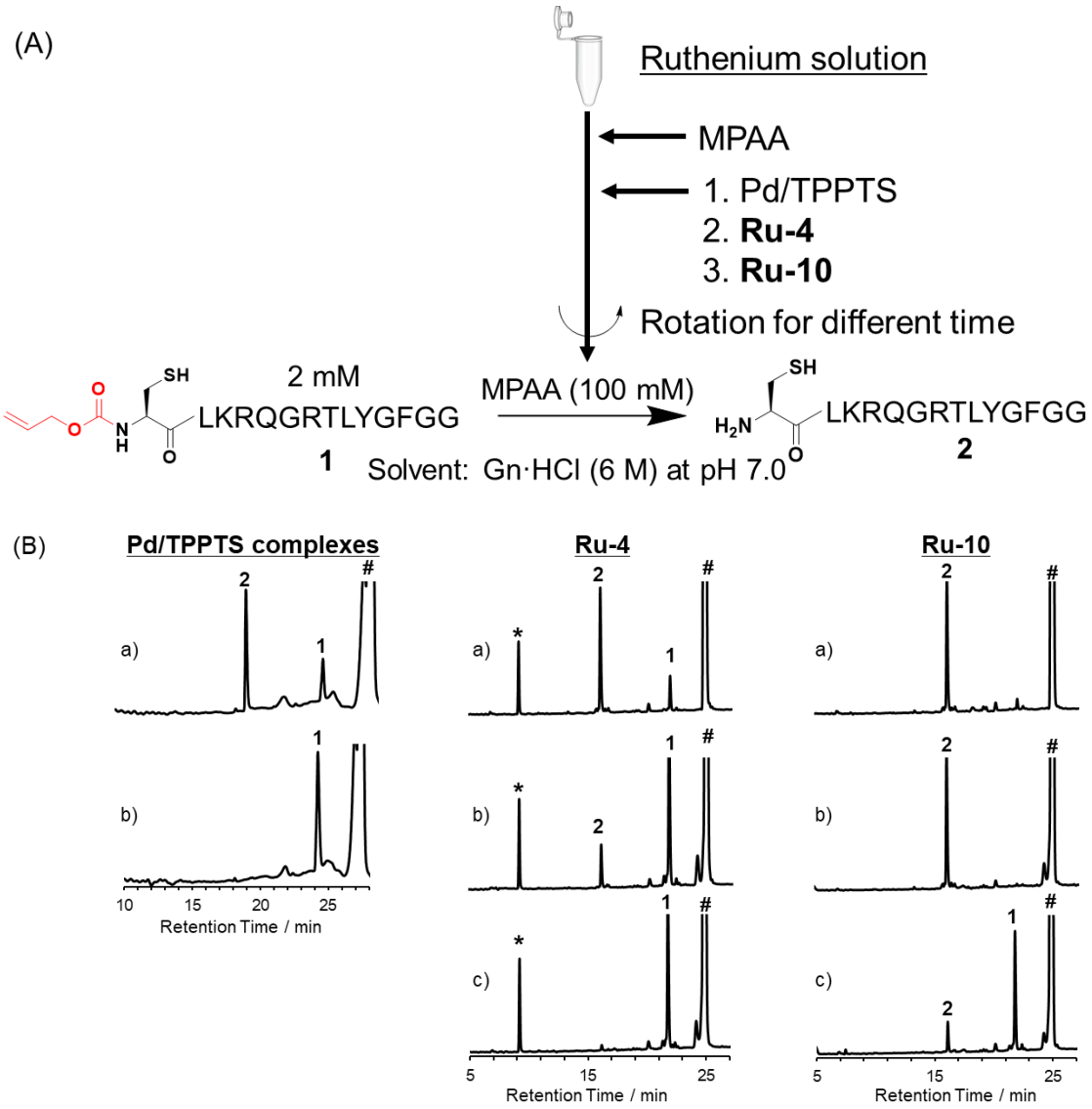


Figure S8. Deactivation test to examine the stability of Ru complexes toward MPAA. (A) General procedure of this experiment. (B) HPLC analysis of deactivation of Pd/TPPTS complexes and Ru complexes toward MPAA. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10–35% for 25 min. Deactivation time: a) 3 min, b) 10 min, c) 30 min. # = MPAA, * = ligand dissociated from Ru.

Table S2. Inactivation of metal complexes with MPAA.

			Conversion yields [%][a] at each deactivation time		
Entry	Metal complexes	(mol%)	3 min	10 min	30 min
1	**Pd/TPPTS**	200	69	<5	–
2	**Ru-4**	10	>95	18	<5
3	**Ru-10**	10	>95	>95	20

[a]The conversion yields were calculated from peak areas analyzed by HPLC (shown in Figure S7).

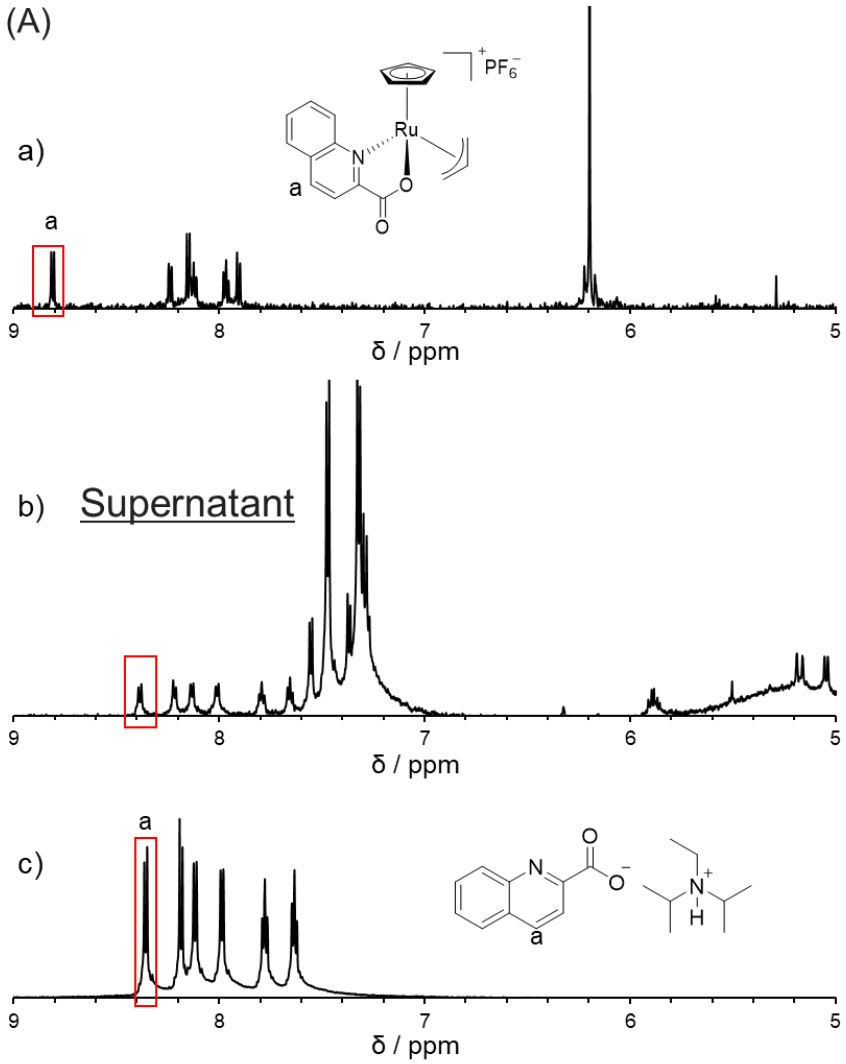


Figure S9. Elucidation of deactivation mechanism of Ru complexes by MPAA. (A) ^{1}H NMR spectra of a) **Ru-3** in CD_3CN, b) the supernatant after mixing **Ru-3**, MPAA, and DIEA in acetone-d_6 and c) quinoline carboxylic acid bearing DIEA salt. The chemical shift of **a** position of **Ru-3** was shifted to a higher magnetic field (8.80 ppm → 8.38 ppm), indicating that compound **S4** was dissociated from Ru by ligand exchange with the thiolate ion of MPAA.

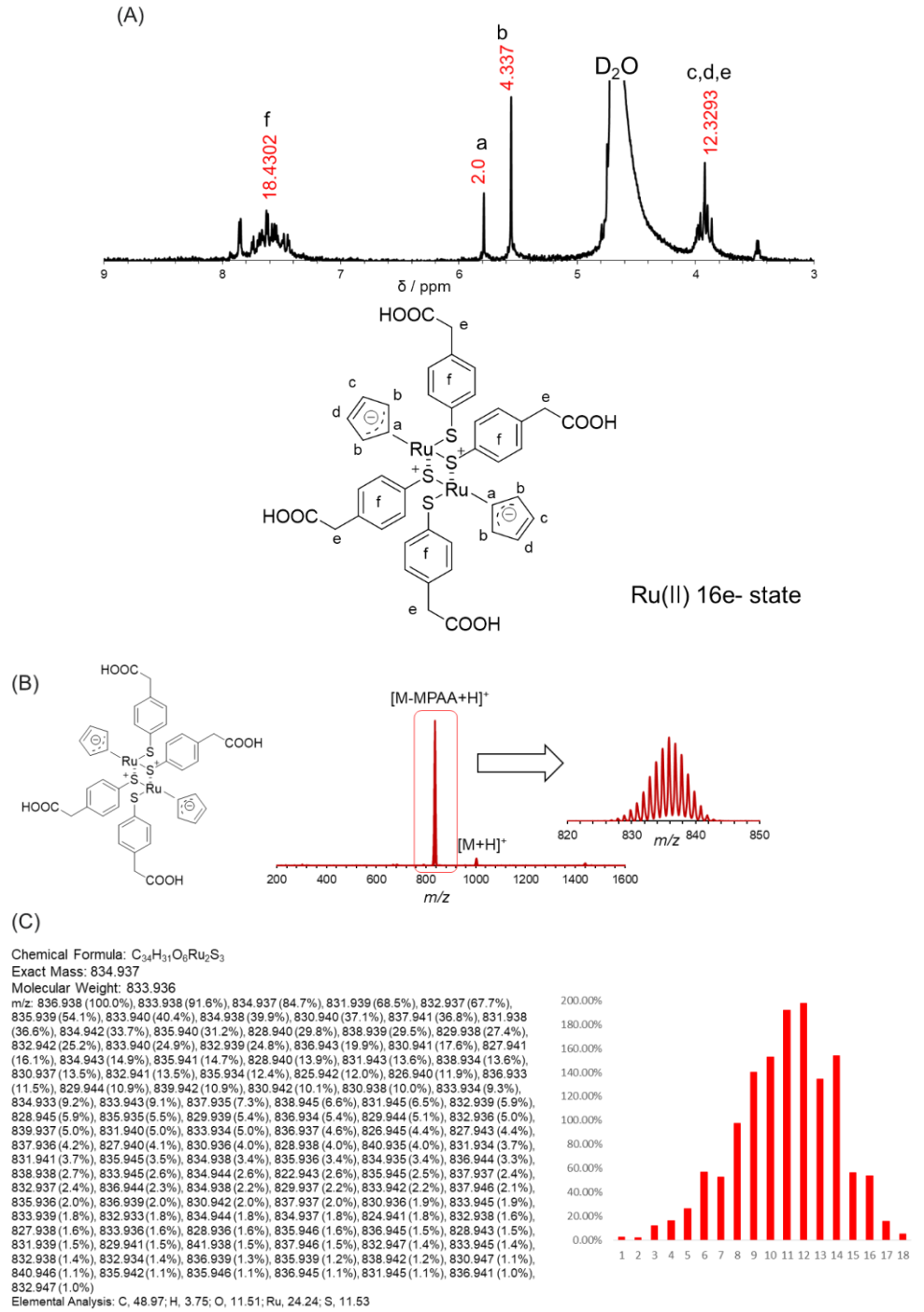


Figure S10. (A) ^{1}H NMR spectrum of the precipitation (binuclear Ru complexes) in a mixture of CD_3CN and D_2O. (B) MALDI-TOF mass spectrum of a binuclear Ru complex after ligand exchange between **Ru-3** and MPAA. $[M\text{-}MPAA+H]^+$: 835.9; Mass Found: 835.8, $[M+H]^+$: 1002.9; Mass Found: 1002.8. (C) Expected mass distribution of the binuclear Ru complex. It was suggested that major 15 kinds of isotopes should be observed, which was consistent with the observed mass spectra.

Part 2. Chemical synthesis of linker histone H1.2

Peptide synthesis. All peptides were synthesized using Intavis ResPep SL (Intavis). Amino acids protected by 9-fluorenylmethoxycarbonyl (Fmoc) group were coupled with HBTU as activator and N, N-diisopropylethylamine (DIEA) as base. For the coupling of Fmoc-His(Trt)-OH, Fmoc-Cys(Trt)-OH, or Alloc-Cys(Trt)-OH, DIC (6.0 equiv.) and HOAt (6.0 equiv.) were employed to avoid racemization during coupling reactions.

Synthesis of peptides 4, 6, 8, 8a, 10. To prepare the C-terminal hydrazide peptide, 2-Trt(2-Cl) resin was used. Briefly, the resin was swelled in 50% DCM/DMF for 30 min. After removing the solvent, 5% hydrazine in DMF was added to the resin and the mixture was agitated for 10 min at r.t. then the solvent was drained and washed by DMF. Next, 5% MeOH/DMF 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 each amino acid (4.0 equiv.), HBTU (3.8 equiv.) and DIEA (8.0 equiv.). The mixture was stirred for 60 min, and then washed by DMF, DCM and DMF three times, respectively. After automated SPPS, the resin was cleavage with TFA cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole) and the mixture was gently stirred at room temperature for 2 h. Then 10-fold amount of cold ether was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. Each peptide precipitation was dissolved in 6 M Gn·HCl and 0.2 M NaH_2PO_4 at pH 3.0 (peptide concentration: 2–3 mM). The solution was cooled to -15 °C and 1.0 M $NaNO_2$ aq was added (10 equiv. against peptide). The mixture was stirred at -15 °C for 15 min, and then 1 M MESNa aq (50 equiv. against each peptide) was added to the reaction mixture. The pH was adjusted to 6.5–7.0 with 6 N NaOH aq and the solution was stirred at room temperature for 30 min. The peptide solution was diluted by mixture of water/acetonitrile containing 0.1 % TFA and purified by HPLC and identified by MALDI-TOF mass spectrometry.
Peptide **4** [27.2 mg, 19% yield (scale: 20 μmol)]. Peptide **6** [10.2 mg, 24% yield (scale: 10 μmol)]. Peptide **8** [11.8 mg, 46% yield (scale: 10 μmol)]. Peptide **8a** [5.0 mg, 21% yield (scale: 10 μmol)]. Peptide **10** [12.2 mg, 21% yield (scale: 10 μmol)].

Synthesis of peptides 3, 3a. Fmoc-Lys(Boc)-Alko resin (0.25 mmol/g) was used for C-terminal carboxyl peptides. After automated SPPS, the peptide was cleaved with TFA cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole). The mixture was rotated at room temperature for 2 h and filtered to remove the resin. Then cold ether was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. The crude

peptide was dissolved in mixture of water/acetonitrile containing 0.1 % TFA and purified by RP-HPLC and identified by MALDI-TOF mass spectrometry.
Peptide **3** [46.5 mg, 24% yield (scale: 20 μmol)]. Peptide **3a** [8.1 mg, 8% yield (scale: 10 μmol)].

One-pot five-segment ligation to afford full-length histone H1.2. Peptide **3** (374 nmol, 3.65 mg) was dissolved in 187 μL NCL buffer (6 M Gn.HCl, 0.2 M NaH_2PO_4, 100 mM MPAA at pH 7.0) (the concentration of peptide was 2.1 mM). Then, 1.05 equiv. of a powdered peptide **4** (392 nmol, 2.74 mg) was added to the reaction mixture and it was stirred at 37 °C for 2 h under argon atmosphere (first NCL). Then, 2.5 μL **Ru-4** solution in acetonitrile (30 mM) (20 mol%) was added to the reaction solution and the mixture was stirred for 30 min at 37 °C (first deprotection). Then, 3.0 μL TCEP solution (500 mM) was added. After stirring 10 min, a powdered peptide **6** (406 nmol, 1.74 mg) and the reaction solution was stirred at 37 °C for 1.5 h (second NCL). 2.5 μL **Ru-4** solution in acetonitrile (30 mM) (20 mol%) was added to the reaction solution and the mixture was stirred for 30 min at 37 °C (second deprotection). Then, 2.0 μL TCEP solution (500 mM) was added. After stirring 10 min, a powdered peptide **8** (409 nmol, 1.05 mg) and the reaction solution was stirred at 37 °C for 2 h (third NCL). Then, 2.5 μL **Ru-4** solution in acetonitrile (30 mM) (20 mol%) was added to the reaction solution and the mixture was stirred for 30 min at 37 °C (third deprotection). Finally, powdered peptide **10** (516 nmol, 3.00 mg) and 15.0 μL TCEP (500 mM) were added and the pH was adjusted to around 7.0. The mixture was stirred at 37 °C for 2 h (fourth NCL). For analysis of each reaction, 1.0 μL aliquot from each reaction mixture was treated with a 15.0 μL MESNa aq (1.0 M) and 2.0 μL TCEP solution (500 mM), followed by stirring for 10 s and injection into analytical HPLC. The peptide solution was diluted by a mixture of water/acetonitrile containing 0.1% TFA and purified by HPLC to afford the desired product **11** (2.52 mg, 88 nmol) in 23% isolated yield. Peptide **11a** with citrullination and peptide **11b** with phosphorylation were prepared in a similar way on a 260 nmol scale. **11a** was isolated in 22% yield (1.63 mg, 57 nmol), and **11b** was isolated in 13% yield (0.98 mg, 35 nmol).

Free-radical desulfurization to obtain histone H1.2. To peptide 11 (1.32 mg, 46 nmol) were added 9.0 μL denaturing buffer, 36.0 μL TCEP solution (500 mM dissolved in denaturing buffer), and 9.0 μL glutathione solution (1 M). To the mixture were 6.0 μL VA-044 solution (200 mM). Final concentration of the mixture was peptide 11 (0.8 mM), TCEP (300 mM), glutathione (150 mM) and VA-044 (20 mM). The reaction mixture was stirred at 37 °C under argon atmosphere for 5 h. The peptide solution was diluted by mixture of water/acetonitrile containing 0.1 % TFA and purified by HPLC to afford desired full-length linker histone H1.2 (12) (1.34 mg) quantitatively. Histone H1.2 bearing R53Cit (12a) or S172ph (12b) was prepared in a similar way. 12a was isolated in 66% yield (0.75 mg, 26 nmol) from 40 nmol scale, and 12b was isolated in

85% yield (0.85 mg, 30 nmol) from 35 nmol scale.

Measurement of Ru content by inductively coupled plasma mass spectrometry (ICP-MS). The protein solution was analyzed by XSERIES 2 ICP-MS (Thermo Scientific). Ruthenium ICP standard (Merck Millipore), Hydrochloric acid 30% (Merck Millipore), and Ultrapure Water (Wako Pure Chemical Industries) were used for the preparation of 0.3 μg/L (ppb), 1.0, 5.0, 7.5, 10.0 ppb Ru solution in 0.1 N HCl aq. 0.17 mg of chemically synthesized histone H1.2 (**12**) was dissolved in 10 ml of 0.1 M HCl aq. The concentration of Ru in H1.2 solution was measured as 6.29 ppb. The palladium content attached on synthesized H1.2 was calculated as 0.000037% (w/w).

Reconstitution of histone octamer. The same amount of histone H2A, H2B H3, and H4 were dissolved to a concentration of about 0.8 mg mL^{-1} in denaturing buffer [20 mM Tris·HCl (pH 7.5), 7 M Gn·HCl, and 20 mM 2-mercaptoethanol]. After the denaturation by incubation for 2 h at 4 ºC, the histone solution was transferred to an Oscillatory Cup molecular weight cutoff 3500 instrument (Cosmo Bio, BTE-212949). The mixture was dialyzed against 1 L of dialysis buffer [10 mM Tris·HCl (pH 7.5), 2 M NaCl, and 2 mM 2-mercaptoethanol] at 4 °C at least three times. The solution was concentrated with an Amicon Ultra instrument (3 K, 0.5 mL) and filtered using Centrifugal filter units 0.22 μm GV Durepore (Merck Millipore). Then, the solution was purified by size-exclusion chromatography with a Superdex 200 Increase 5/150 GL column.

Reconstitution of nucleosomes. The purified octamer solution (1.0 equiv.) was added to 193 bp 601 DNA solution [0.7 equiv, containing 2 M NaCl and 10 mM Tris·HCl (pH 7.5)]. The final concentration of octamer was adjusted to 0.3 mg/mL. The mixture was dialyzed against 1 L of dialysis buffer [10 mM Tris-HCl (pH 7.5), 2 M NaCl, and 1 mM 2-mercaptoethanol] for 2 h at 4 ºC. Then, the concentration of NaCl was changed to 1, 0.85, 0.65, and 0.25 M to form nucleosomes.

Reconstitution of chromatosomes. Each amount of H1.2 (**12**, **12a**, and **12b**) were mixed with the nucleosomes (0.2 μM), which was formed with the 193 bp 601 DNA in the presence of Nap1 (0.3 μM), in 10 μL of the reaction buffer, containing 35 mM Tris-HCl (pH 8.0), 70 mM NaCl, 0.01 mM PMSF, 0.05 mM EDTA, 5% glycerol, 1.2 mM dithiothreitol, 1.1 mM 2-mercaptoethanol, and 5 μg/ml bovine serum albumin. After incubation at 37 °C for 30 min, 2 μL of 30% sucrose was added and the samples were loaded onto 5% native polyacrylamide gels in 1×Trisborate-EDTA (TBE) buffer (90 mM Tris base, 90 mM boric acid and 2 mM EDTA). The gel was stained with ethidium bromide. The band intensities of the chromatosomes and nucleosomes were

quantitated with a LAS-4000 image analyzer (GE Healthcare) using MultiGauge ver. 3.2 (Fujifilm) and the efficiency of the formation of the chromatosomes was calculated.

(A)

3

150

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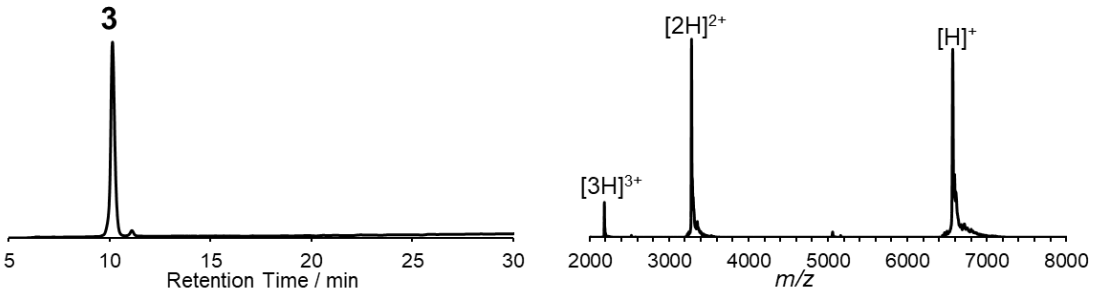
KKAAKSAAKAVKPKAAKPKVVKPKKAAPKKK

212



3a

150

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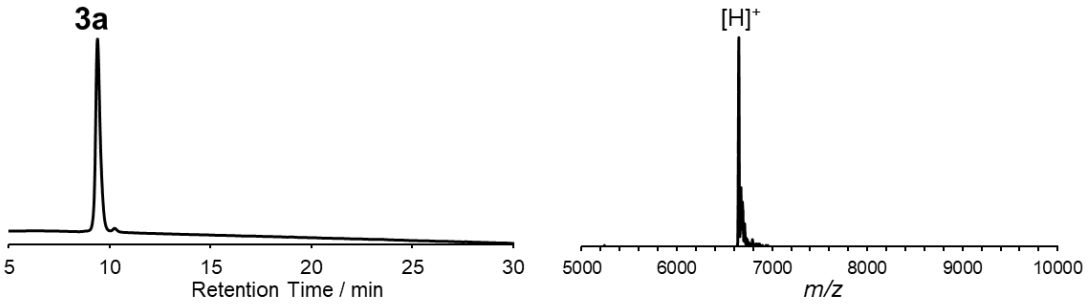
PKKAAKSAAKAVKPKAAKPKVVKPKKAAPKKK

212



(B)

4

100

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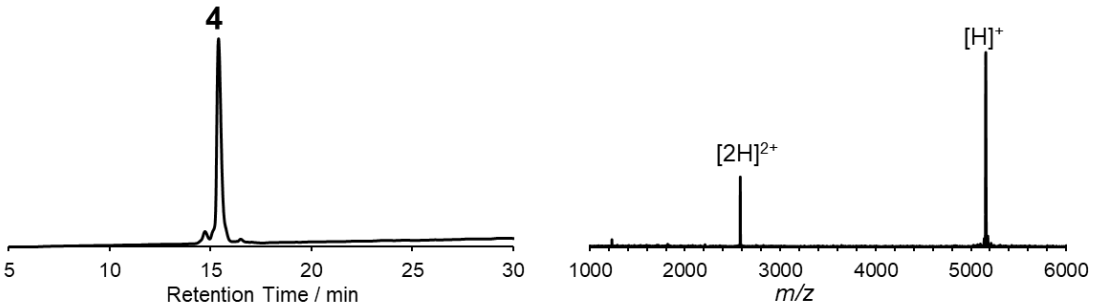
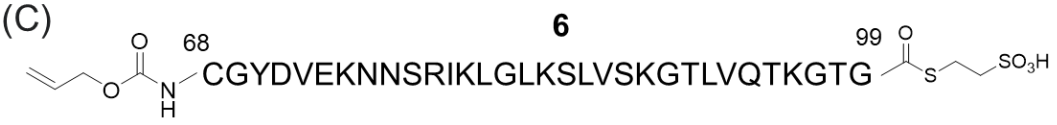
149



(C)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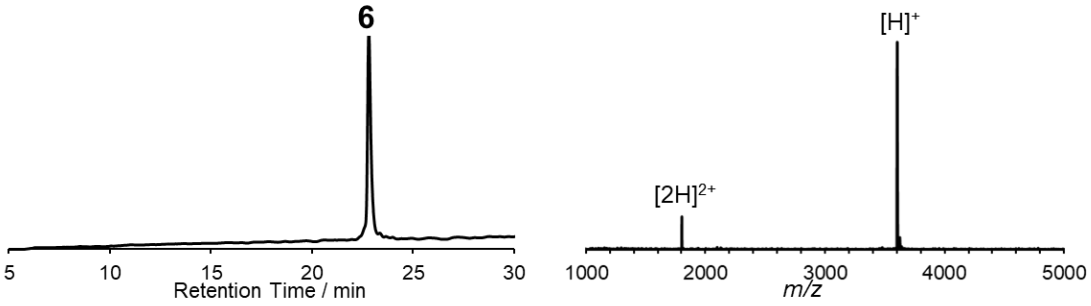
99
SO3H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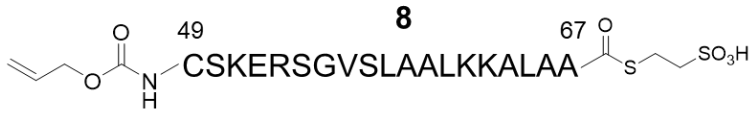
[H]+
[2H]2+
5
10
15
20
25
30
Retention Time / min
1000
2000
3000
4000
5000
m/z

(D)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49
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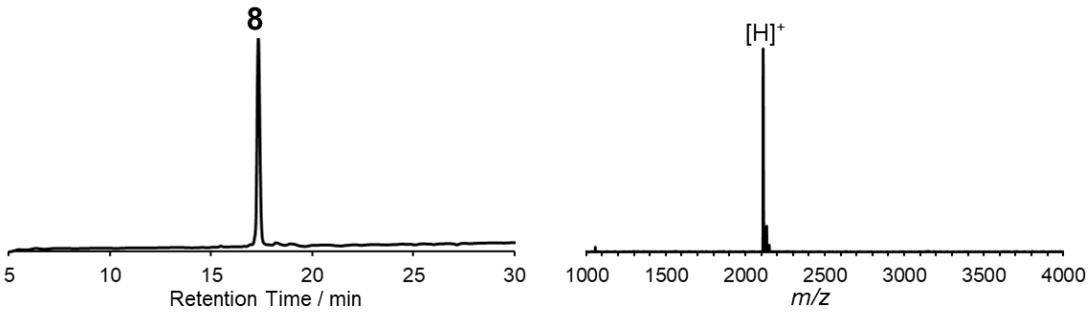
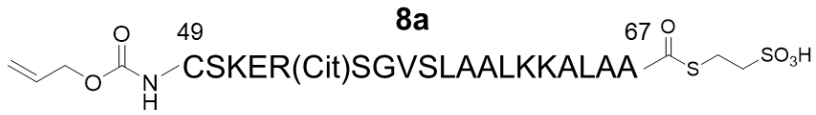
67
SO3H


8
[H]+
5
10
15
20
25
30
Retention Time / min
1000
1500
2000
2500
3000
3500
4000
m/z


8a
49
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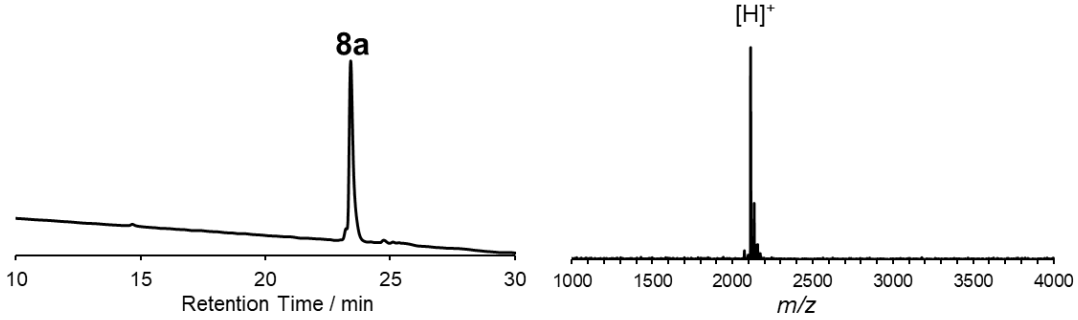
67
SO3H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[H]+
10
15
20
25
30
Retention Time / min
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1500
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m/z

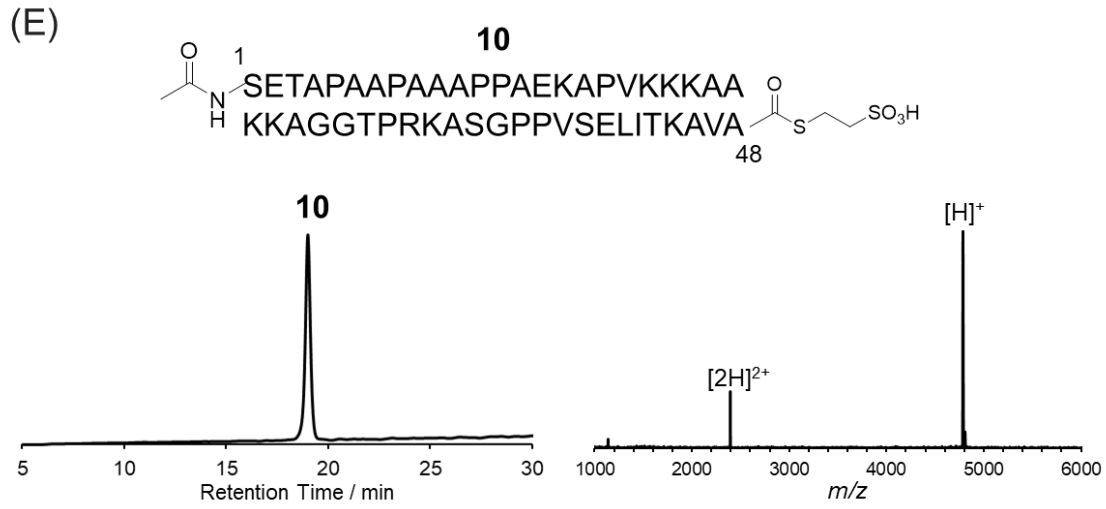


Figure S11. Synthesis of peptides **3**, **3a**, **4**, **6**, **8**, **8a**, **10**. HPLC charts of purified peptides, and MALDI-TOF mass spectra (A) of peptide **3** and **3a**, (B) of peptide **4**, (C) of peptide **6**, (D) of peptide **8** and **8a**, and (E) of peptide **10**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 10–46% for 30 min with Protein-R.

Calculated mass of **3** $[M+H]^+$: 6569.3; Mass Found $[M+H]^+$: 6570.4.

Calculated mass of **3a** $[M+H]^+$: 6649.3; Mass Found $[M+H]^+$: 6650.7.

Calculated mass of **4** $[M+H]^+$: 5154.9; Mass Found $[M+H]^+$: 5154.9.

Calculated mass of **6** $[M+H]^+$: 3602.8; Mass Found $[M+H]^+$: 3601.9.

Calculated mass of **8** $[M+H]^+$: 2111.1; Mass Found $[M+H]^+$: 2111.4.

Calculated mass of **8a** $[M+H]^+$: 2112.0; Mass Found $[M+H]^+$: 2112.9.

Calculated mass of **10** $[M+H]^+$: 4785.6; Mass Found $[M+H]^+$: 4786.5.

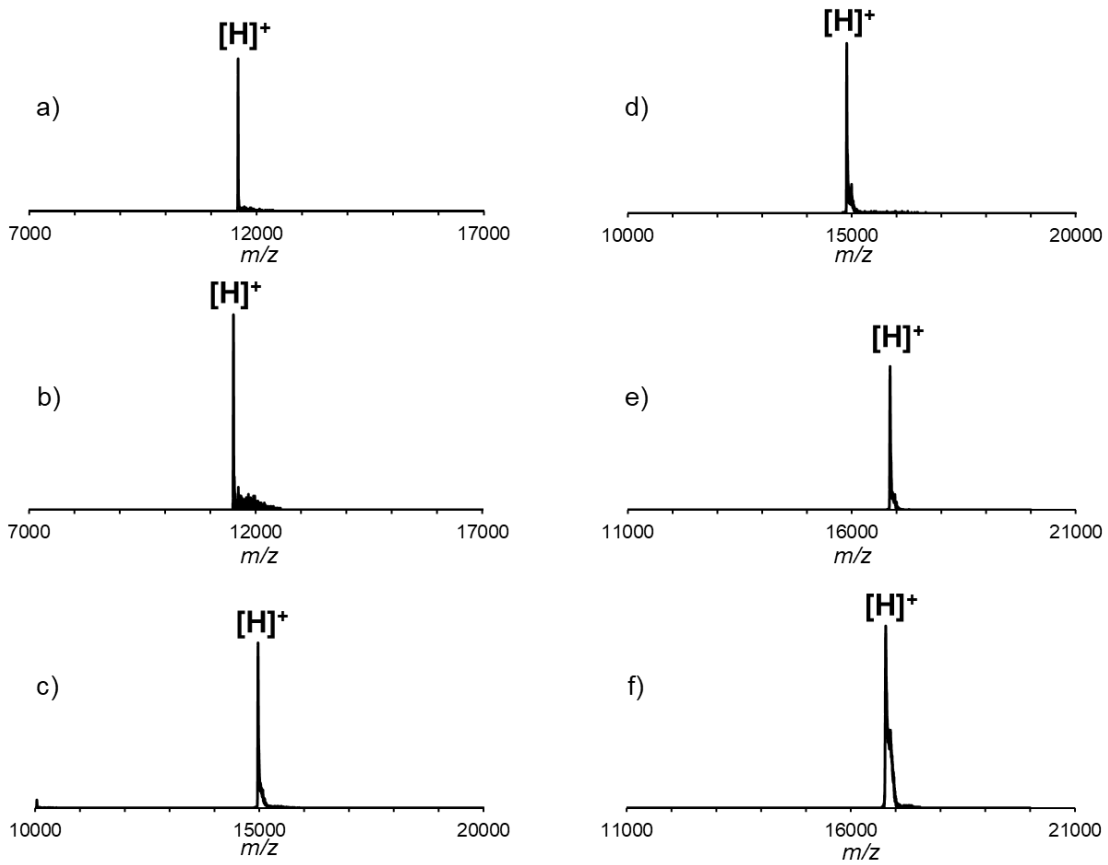


Figure S12. Identification of ligation intermediates **5'**, **5**, **7'**, **7**, **9'**, and **9**. MALDI-TOF mass spectra a) of peptide **5'**, b) of peptide **5**, c) of peptide **7'**, d) of peptide **7**, e) of peptide **9'**, and f) peptide **9**.

Calculated mass of **5'** $[M+H]^+$: 11584.3; Mass Found $[M+H]^+$: 11585.0.

Calculated mass of **5** $[M+H]^+$: 11500.3; Mass Found $[M+H]^+$: 11502.3.

Calculated mass of **7'** $[M+H]^+$: 14961.3; Mass Found $[M+H]^+$: 14963.3.

Calculated mass of **7** $[M+H]^+$: 14877.2; Mass Found $[M+H]^+$: 14879.7.

Calculated mass of **9)** $[M+H]^+$: 16846.6; Mass Found $[M+H]^+$: 16847.8.

Calculated mass of **9** $[M+H]^+$: 16762.5; Mass Found $[M+H]^+$: 16763.7.

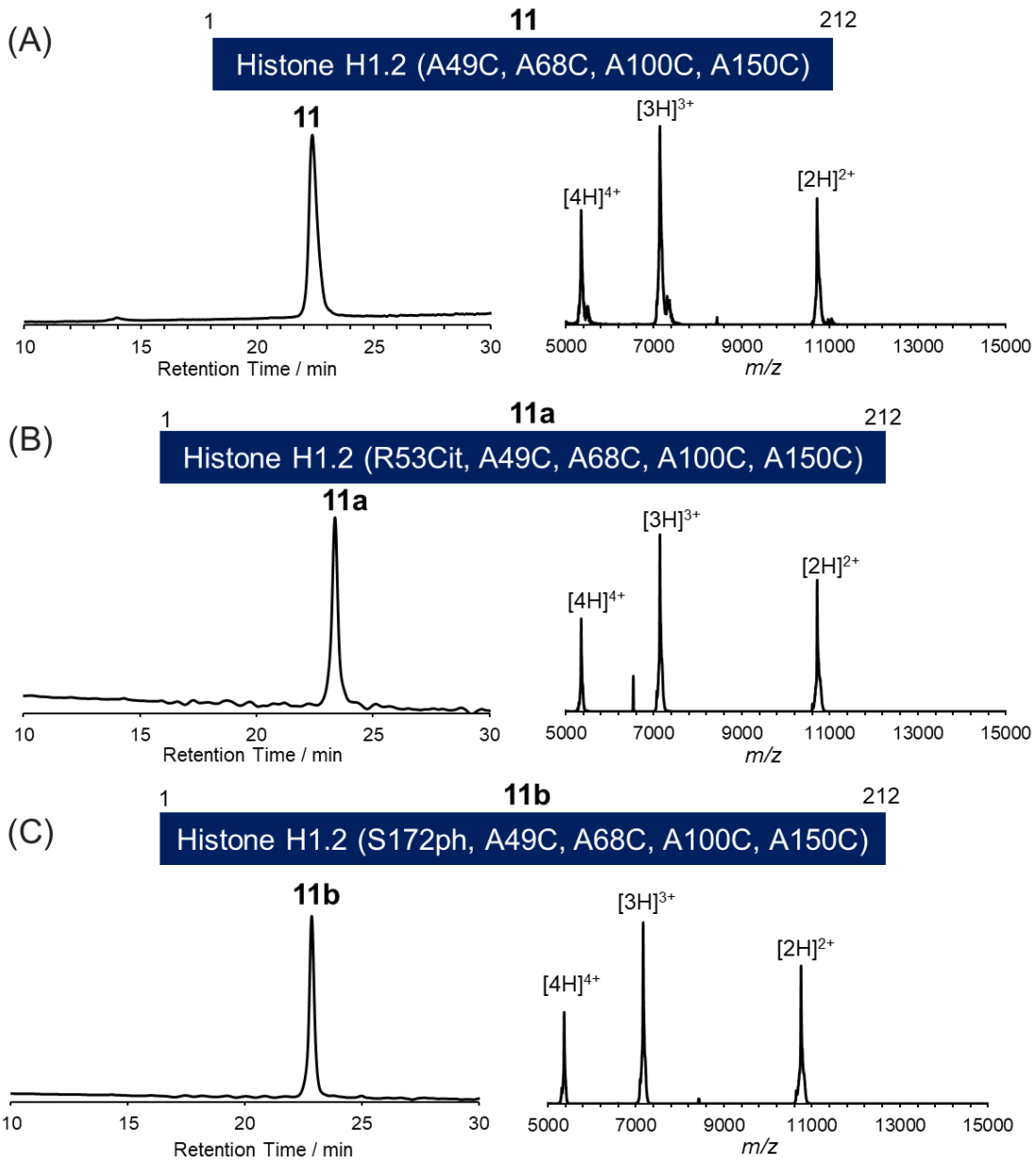


Figure S13. Identification of peptide **11**, **11a**, and **11b** after one-pot five-segment ligation using Ru catalyst. HPLC charts and MALDI TOF mass spectra of purified (A) peptide **11**, (B) **11a**, and (C) **11b**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.

Calculated mass of **11** $[M+2H]^{2+}$: 10702.4; Mass Found $[M+2H]^{2+}$: 10703.5.

Calculated mass of **11a** $[M+2H]^{2+}$: 10702.9; Mass Found $[M+2H]^{2+}$: 10703.8.

Calculated mass of **11b** $[M+2H]^{2+}$: 10742.4; Mass Found $[M+2H]^{2+}$: 10743.1.

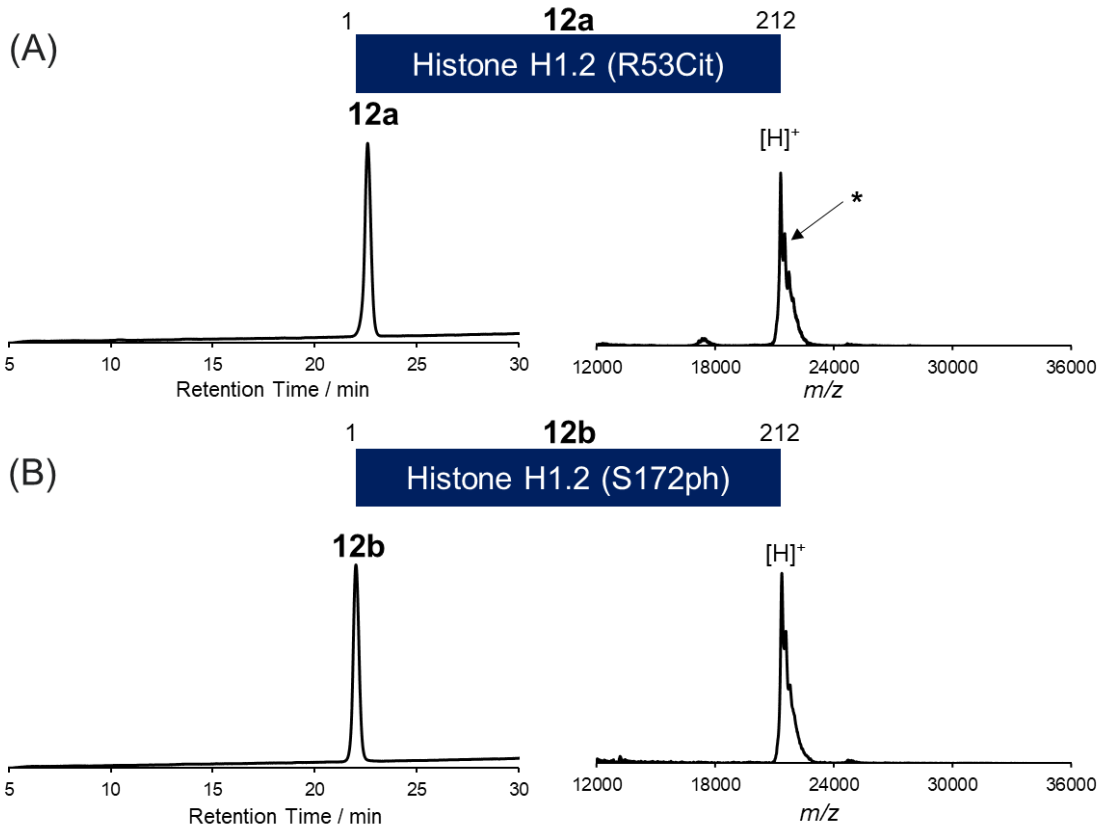


Figure S14. Identification of peptide **12a** and **12b** after desulfurization. HPLC charts and MALDI TOF mass spectra of purified (A) peptide **12a**, (B) **12b**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R. * corresponds to a sinapinic adduct.

Calculated mass of **12a** $[M+H]^+$: 21277.6; Mass Found $[M+H]^+$: 21279.1.

Calculated mass of **12b** $[M+H]^+$: 21370.6; Mass Found $[M+H]^+$: 21371.5.

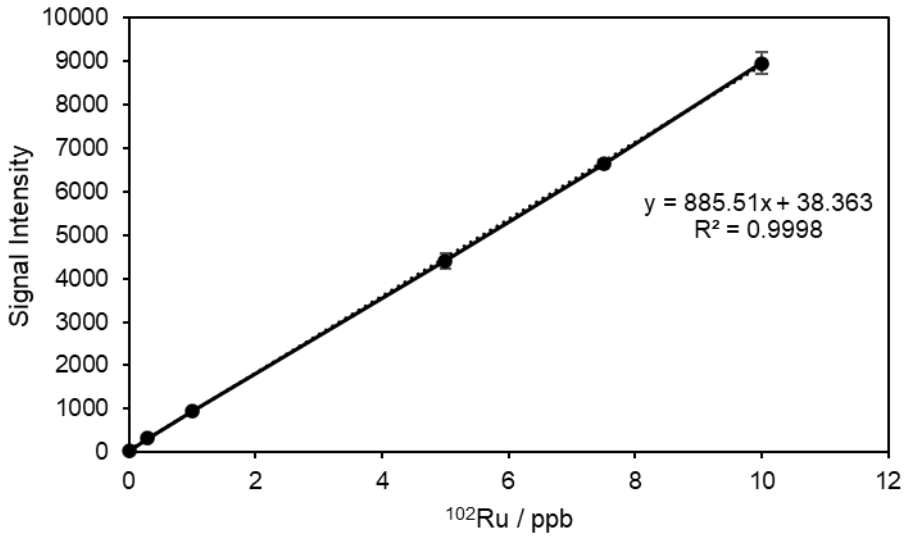


Figure S15. Calibration curve using Ruthenium ICP standard (Merck Millipore) to determine Ru contents on synthetic proteins.

Table S3. Determination of Ru concentration of H1.2 or HP1α solution.

Entry	Protein		Signal Intensity	Concentration (ppb)
1	Histone H1.2 (**12**)	1st	5617.735	6.301
		2nd	5660.762	6.349
		3rd	5767.829	6.47
		Average	5682.109	6.373
2	HP1α (**21**)	1st	1439.114	1.582
		2nd	1397.107	1.534
		3rd	1432.113	1.574
		Average	1422.778	1.563

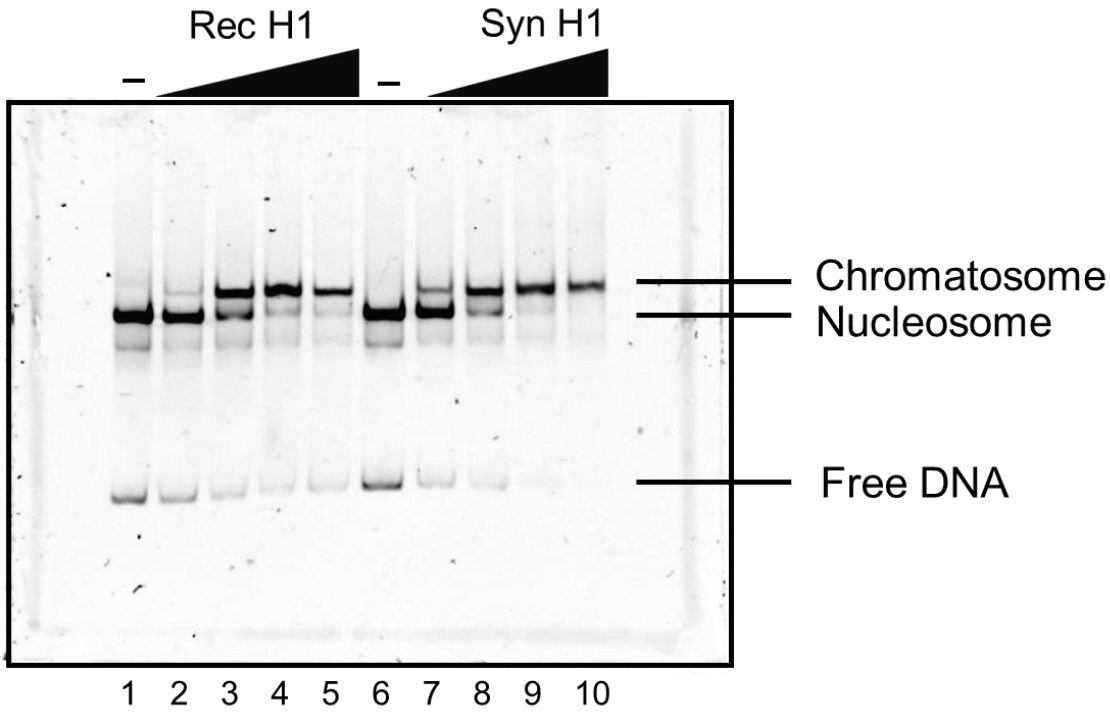


Figure S16. Representative gel image of the H1 binding assay. Increasing amounts of recombinant or synthetic H1.2 (0 μM: Lanes 1 and 6; 0.4 μM: lanes 2 and 7; 0.6 μM: lanes 3 and 8; 0.75 μM: lanes 4 and 9; 0.9 μM: lanes 5 and 10) were mixed with nucleosomes (0.1 μM) in presence of Nap1 (0.3 μM). After an incubation at 37 ºC, the complexes were detected by non-denaturing 5% PAGE with ethidium bromide staining.

Part 3. Chemical synthesis of HP1α

Peptide synthesis. All peptides were synthesized using Intavis ResPep CF (Intavis) or Initiator + Alstra (Biotage). Amino acids protected by 9-fluorenylmethoxycarbonyl (Fmoc) group (4.0 equiv.) were coupled with HBTU (3.8 equiv.) as activator and DIEA (8.0 equiv.) as base. For the coupling of Fmoc-His(Trt)-OH, Fmoc-Cys(Trt)-OH, and Fmoc-Cys(Acm)-OH, HBTU (3.8 equiv.), HOBt (4.0 equiv.), and DIEA (4.0 equiv.) were employed to avoid racemization during coupling reactions. For the coupling of Alloc-Cys(Trt)-OH,[1] Alloc-Cys(Phacm-Fmoc)-OH, Fmoc-Asp(tBu)-(Dmb)Gly-OH (Novabiochem) Fmoc-Ser(tBu)-Thr(psiMe,Mepro)-OH (Novabiochem), DIC and HOAt were employed.

Synthesis of peptides 13. Fmoc-Ser(tBu)-Alko resin (0.23 mmol/g) was used for C-terminal carboxyl peptides. After automated SPPS using ResPep CF (Intavis) of parallel synthesis, the peptide was cleaved with TFA cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole). The mixture was rotated at room temperature for 2 h and filtered to remove the resin. Then cold ether was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. The crude peptide was dissolved in mixture of water/acetonitrile containing 0.1 % TFA and purified by RP-HPLC and identified by MALDI-TOF mass spectrometry. Peptide **13** [17.4 mg, 16% yield (scale: 20 μmol)].

Synthesis of peptides 14. To prepare the C-terminal hydrazide peptide, 2-Trt(2-Cl)-resin (1.60 mmol/g, Watanabe Chemical Industries) was used. The resin [167 mg for 50 μmol scale synthesis (final concentration: 0.30 mmol/g)] was swelled in 50% DCM/DMF for 30 min. After removing the solvent, 9-fluorenylmethyl carbazate (Fmoc-hydrazine) (50 μmol, 1.0 equiv.) and DIEA (100 μmol, 2.0 equiv.) in 800 μL DMF was added to the resin and the mixture was stirred overnight at room temperature then the solvent was drained and washed by DMF three times. Next, 5% MeOH/DMF (800 μL) was added and stirred for 10 min. After removing the solvent, the resin was washed by DMF four times. Each amino acid was coupled with automated SPPS using Initiator + Alstra (Biotage), and after the coupling of Gly 134, Alloc-Cys(Phacm-Fmoc)-OH was introduced. After the Fmoc deprotection with 20% piperidine in DMF, two Fmoc-Arg(Pbf)-OHs and Boc-Arg(Pbf)-PH were coupled. To remove the alloc group on the resin were added $Pd(PPh_3)_4$ (0.20 equiv.) and $PhSiH_3$ (20 equiv.) in degassed DCM and the mixture was stirred for 10 min under argon stmosphere. Then, the remaining amino acids were coupled using Initiator + Alstra (Biotage). The resin was cleavage with TFA cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole) and the mixture was gently stirred at room temperature for 2 h. To reduce oxidized

Met and prevent removals of the acm groups, premixed 1% (w/v) of tetrabutylammonium iodide (TBAI) and 10% (v/v) of dimethyl sulfide dissolved in the cleavage cocktail was added to the peptide solution and the whole mixture was stirred for 5 min. Then 10-fold amount of cold ether was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. The crude peptide was dissolved in mixture of water and acetonitrile (70:30), and lyophilized. The powdered crude peptide was dissolved in 6 M Gn·HCl and 0.2 M NaH_2PO_4 at pH 3.0 (peptide concentration: 1 mM), and acetylacetone (2.0 equiv.) and 4-mercaptophenol (100 equiv.) were added.[4] The mixture was rotated overnight at 37 °C, and after completion of the conversion of hydrazine into thioester, the excess thiol moieties was removed by ether extraction. After the removal of ether under reduced pressure, the peptide solution was neutralized with 6 N NaOH aq, reduced with TCEP solution and diluted with mixture of water and acetonitrile to induce self-cyclization. The peptide solution was purified by HPLC and identified by MALDI-TOF mass spectrometry. Peptide **14** [14.0 mg, 6.7% yield (scale: 50 μmol)].

Synthesis of peptides 15, **15a**, **16**, **16a**, **16b**, **16c**, **20**, **20a**, **20b**, **23**, **24.** To prepare the C-terminal hydrazide peptide, 2-Trt(2-Cl)-resin (1.60 mmol/g, Watanabe Chemical Industries) was used. The resin [67 mg for 20 μmol scale synthesis (final concentration: 0.30 mmol/g)] was swelled in 50% DCM/DMF for 30 min. After removing the solvent, 9-fluorenylmethyl carbazate (Fmoc-hydrazine) (20 μmol, 1.0 equiv.) and DIEA (40 μmol, 2.0 equiv.) in 800 μL DMF was added to the resin and the mixture was agitated overnight at room temperature then the solvent was drained and washed by DMF three times. Next, 5% MeOH/DMF (800 μL) was added and stirred for 10 min. After removing the solvent, the resin was washed by DMF four times. After automated SPPS using ResPep CF (Intavis) of parallel synthesis (for peptides **15**, **15a**, **16**, **16a**, **16b**, **16c**, **23**, **24**) or Initiator + Alstra (Biotage) (for peptides **20**, **20a**, **20b**), the resin was cleavage with TFA cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole) and the mixture was gently stirred at room temperature for 2 h. To reduce oxidized Met and prevent acm removals during TFA cleavage for peptides **16**, **16a**, **16b**, **16c**, **20**, **20a**, **20b**, premixed 1% (w/v) of TBAI and 10% (v/v) of dimethyl sulfide dissolved in the cleavage cocktail was added to the peptide solution and the whole solution was stirred for another 10 min. Then 10-fold amount of cold ether was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. Each peptide precipitates except peptide **24** was dissolved in 6 M Gn·HCl and 0.2 M NaH_2PO_4 at pH 3.0 (peptide concentration: 2–3 mM). The solution was cooled to -15 °C and 1.0 M $NaNO_2$ aq was added (10 equiv. against peptide). The mixture was stirred at -15 °C for 15 min, and then 1 M MESNa aq (100 equiv. against peptide) was added to the reaction mixture. The pH was adjusted to 6.5–7.0 with 6 N NaOH aq and the solution was stirred at room temperature for 30 min. After the reduction of disulfide bonds with TCEP solution, the peptide

solution was diluted by mixture of water/acetonitrile containing 0.1 % TFA and purified by HPLC and identified by MALDI-TOF mass spectrometry. Peptide **15** [27.2 mg, 19% yield (20 μmol scale)]. **15a** [8.2 mg, 7% yield (25 μmol scale)]. **16** [10.2 mg, 24% yield (10 μmol scale)]. **16a** [15.3 mg, 10% yield (25 μmol scale)]. **16b** [17.8 mg, 11% yield (25 μmol scale)]. **16c** [7.7 mg, 5% yield (25 μmol scale)]. **20** [7.1 mg, 7% yield (12.5 μmol scale)]. **20a** [3.5 mg, 3% yield (12.5 μmol scale)]. **20b** [4.6 mg, 4% yield (12.5 μmol scale)]. **23** [15.6 mg, 10% yield (25 μmol scale)]. **24** [18.0 mg, 16% yield (25 μmol scale)].

Synthesis of peptide 22 with C-terminal Ub. Fmoc-Ser(tBu)-Alko resin (0.23 mmol/g, 25 μmol) was used for C-terminal carboxyl peptides. Each amino acid was coupled with automated SPPS using ResPep CF (Intavis). After the coupling of Glu 155, Alloc-Lys(Fmoc)-OH (Watanabe Chemical Industries) was coupled, and the peptide sequence of C-terminal ubiquitin was extended. To increase the coupling efficiency and avoid aspartimide formation, Fmoc-Ser(tBu)-Thr(psiMe,Mepro)-OH and Fmoc-Asp(tBu)-(Dmb)Gly-OH were employed, respectively. After the coupling of Cys46, $Pd(PPh)_4$ (0.20 equiv.) and $PhSiH_3$ (20 equiv.) in degassed DCM were added to the resin and the mixture was stirred for 10 min under argon stmosphere. After the completion of the removal of the alloc group, Alloc-Cys(Trt)-OH was coupled. The resin was cleaved with TFA cocktail (90% TFA, 5% thioanisole, 3% EDT and 2% anisole). The mixture was rotated at room temperature for 2 h and filtered to remove the resin. Then cold ether was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. The crude peptide was dissolved in mixture of water/acetonitrile containing 0.1 % TFA and purified by RP-HPLC and identified by MALDI-TOF mass spectrometry. Peptide **22** [9.3 mg, 4% yield (scale: 25 μmol)].

One-pot four-segment ligation to afford ligated intermediate 17. Peptide **13** (440 nmol, 2.44 mg) was dissolved in 200 μL NCL buffer (6 M Gn.HCl, 0.2 M NaH_2PO_4, 100 mM MPAA, 3 mM TCEP at pH 7.0). A powdered peptide **14** (400 nmol, 1.66 mg) was added to the reaction mixture and it was stirred at 37 °C for 4 h under argon atmosphere (first NCL). Then, 4.0 μL **Ru-4** solution in acetonitrile (20 mM) (20 mol% toward peptide **14**) was added to the reaction solution and the mixture was stirred for 20 min at 37 °C (first deprotection). Then, 1.6 μL TCEP solution (500 mM) was added. After stirring 10 min, a powdered peptide **15** (470 nmol, 2.20 mg) and the reaction solution was stirred at 37 °C for 2 h (second NCL). 4.4 μL **Ru-4** solution in acetonitrile (20 mM) (20 mol% toward peptide **15**) was added to the reaction solution and the mixture was stirred for 20 min at 37 °C (second deprotection). Then, 15.0 μL TCEP solution (500 mM) was added. After stirring 5 min, a powdered peptide **16** (625 nmol, 3.75 mg) and the reaction solution was stirred overnight at 37 °C (third NCL). For analysis of each reaction, 1.0 μL aliquot from

each reaction mixture was treated with a 15.0 μL MESNa aq (1.0 M) and 2.0 μL TCEP solution (500 mM), followed by stirring for 10 s and the solution was injected into analytical HPLC. To reduce disulfide bonds before purification, 30 μL TCEP solution (500 mM) was added to the peptide solution, and the mixture was stirred for 5 min at room temperature. The solution was diluted by mixture of water/acetonitrile containing 0.1% TFA and purified by HPLC to afford the desired product **17** (2.83 mg, 141 nmol) in 35% isolated yield. Peptides **17c**, **17d**, **17e** were prepared in similar ways. **17c** was isolated in 24% yield (1.92 mg, 96 nmol) from 400 nmol scale, **17d** was isolated in 33% yield (2.62 mg, 131 nmol) from 400 nmol scale, and **17e** was isolated in 29% yield (4.53 mg, 226 nmol) from 780 nmol scale.

One-pot four-segment ligation for HP1α with Ub (from N-terminus to C-terminus, scheme S2). For the ligation from N-terminus to C-terminus direction, peptide **16** (4.60 mg, 740 nmol) and peptide **24** (2.82 mg, 614 nmol) were dissolved in 154 μL NCL buffer (6 M Gn.HCl, 0.2 M NaH_2PO_4, 150 mM MPAA, 30 mM TCEP at pH 7.0). After stirring for 4 h at 37 °C, the solution was diluted by mixture of water/acetonitrile containing 0.1% TFA and purified by HPLC to afford the desired product **16**–**24** (4.15 mg, 393 nmol) in 64% isolated yield.

The powdered peptide **16-24** (5.07 mg, 480 nmol) was dissolved in 120 μL NCL buffer (6 M Gn.HCl, 0.2 M NaH_2PO_4, 150 mM MPAA at pH 3.0). Acetylacetone (1.20 μmol, 2.5 equiv.) was added to the peptide solution, and the mixture stirred for 4 h at 37 °C. For analysis of each reaction, 1.0 μL aliquot from each reaction mixture was treated with a 15.0 μL MESNa aq (1.0 M) and 2.0 μL TCEP solution (500 mM), followed by stirring for 10 s and injection into analytical HPLC. Peptide **16**–**24** bearing MPAA thioester at its C-terminus was employed for the next reaction directly.

One-pot four-segment ligation for HP1α with Ub (from C-terminus to N-terminus). For the ligation from N-terminus to C-terminus direction, peptide **22** (4.47 mg, 472 nmol) and peptide **23** (2.85 mg, 472 nmol) were dissolved in 189 μL NCL buffer (6 M Gn.HCl, 0.2 M NaH_2PO_4, 100 mM MPAA, 20 mM TCEP at pH 7.4) (first NCL). After stirring overnight 37 °C and being confirmed that thioesters of peptide **23** were hydrolyzed, 8.0 μL MPAA solution (500 mM) was added. After stirring 1 min, 4.7 μL **Ru-4** solution in acetonitrile (20 mM) (20 mol% toward peptide **22**) was added to the reaction solution and the mixture was stirred for 20 min at 37 °C (first deprotection). Then, 12.5 μL TCEP solution (1.0 M) was added. After stirring 5 min, a powdered peptide **14** (480 nmol, 2.00 mg) and the reaction solution was stirred overnight at 37 °C (second NCL). 8.0 μL MPAA solution (500 mM) was added, and the reaction solution was stirred for 1 min. 4.4 μL **Ru-4** solution in acetonitrile (20 mM) (20 mol% toward peptide **14**) was added to the reaction solution and the mixture was stirred for 20 min at 37 °C (second deprotection). Then,

23.0 μL TCEP solution (1.0 M) was added. After stirring 5 min, 120 μL peptide **16-24** bearing MPAA thioester solution (4.0 mM) and the pH was adjusted to 7.0 (third NCL). For analysis of each reaction, 1.0 μL aliquot from each reaction mixture was treated with a 15.0 μL MESNa aq (1.0 M) and 2.0 μL TCEP solution (500 mM), followed by stirring for 10 s and injection into analytical HPLC. After stirring overnight 37 °C, the solution was diluted by mixture of water/acetonitrile containing 0.1% TFA and purified by HPLC to afford the desired product **25** (2.79 mg, 94 nmol) in 20% isolated yield.

Free-radical desulfurization to peptide 18. To peptide **17** (2.83 mg, 141 nmol) were added 35.2 μL denaturing buffer, 88.0 μL TCEP solution (500 mM dissolved in denaturing buffer), and 35.2 μL glutathione solution (500 mM). To the mixture was added 17.6 μL VA-044 solution (300 mM). Final concentration of the mixture was peptide **17** (0.8 mM), TCEP (250 mM), glutathione (100 mM) and VA-044 (30 mM). The reaction mixture was stirred overnight at 37 °C under argon atmosphere. The peptide solution was diluted with mixture of water/acetonitrile containing 0.1 % TFA and purified by HPLC to afford desired peptide **18** (1.81 mg, 90 nmol) in 64% isolated yield. Peptides **18c**, **18d**, **18e**, **26** were prepared in similar ways. **18c** was isolated in 60% yield (1.15 mg, 58 nmol) from 96 nmol scale, **18d** was isolated in 77% yield (2.02 mg, 101 nmol) from 131 nmol scale, **18e** was isolated in 49% yield (2.21 mg, 111 nmol) from 226 nmol scale, and **26** was isolated in 70% yield (1.86 mg, 63 nmol) from 90 nmol scale.

Removal of the acm groups and a solubilizing tag with silver acetate. Peptide **18** (1.81 mg, 90 nmol) was dissolved in 181 μL degassed mixture of water and acetic acid (1:1). Then, silver acetate (0.90 mg, 5.43 μmol) was added. Final concentration of the mixture was peptide **18** (0.5 mM), AgOAc (30 mM). The reaction mixture was stirred overnight at 37 °C under argon atmosphere. After reaction completion, 181 μL DTT solution (1 M) dissolved in denaturing buffer was added to the peptide solution, and the mixture was stirred for 20 min at room temperature. After centrifugation, the supernatant was diluted with mixture of water and purified by HPLC to afford desired peptide **19** (1.05 mg, 56 nmol) in 62% isolated yield. Peptides **19c**, **19d**, **19e** were prepared in similar ways. **19c** was isolated in 74% yield (0.80 mg, 43 nmol) from 58 nmol scale, **19d** was isolated in 67% yield (1.27 mg, 68 nmol) from 101 nmol scale, **19e** was isolated in 81% yield (1.69 mg, 90 nmol) from 111 nmol scale, and **27** was isolated in 58% yield (1.02 mg, 36 nmol) from 62 nmol scale.

NCL and following the allyl removal with Ru-4 to afford 21. Peptide **20** (0.50 mg, 26 nmol) was dissolved in 17.5 μL NCL buffer (6 M Gn.HCl, 0.2 M NaH_2PO_4, 100 mM MPAA, 2 mM TCEP at pH 7.0). A powdered peptide **20** (0.27 mg, 32 nmol) was added to the reaction mixture

and it was stirred at 37 °C for 2 h under argon atmosphere. Then, 0.6 μL **Ru-4** solution in acetonitrile (10 mM) was added to the reaction solution and the mixture was stirred for 20 min at 37 °C. To the reaction mixture was added 5.0 μL TCEP solution (500 mM) to reduce disulfide bonds. After stirring for 5 min, the reaction solution was diluted with a mixture of water/acetonitrile containing 0.1 % TFA and purified by HPLC to afford the desired full-length HP1α **21** (0.37 mg, 14 nmol) in 53% isolated yield. Proteins **21a**, **21b**, **21c**, **21d**, and **21e** were prepared in similar ways. **21a** was isolated in 53% yield (0.74 mg, 28 nmol) from a 53 nmol scale, **21b** was isolated in 46% yield (0.32 mg, 12 nmol) from a 26 nmol scale, **21c** was isolated in 60% yield (0.80 mg, 27 nmol) from a 45 nmol scale, **21d** was isolated in 60% yield (1.06 mg, 40 nmol) from a 67 nmol scale, **21e** was isolated in 70% yield (0.93 mg, 31 nmol) from a 44 nmol scale, and **21f** was isolated in 31% yield (0.41 mg, 11 nmol) from a 36 nmol scale.

Refolding of chemically synthesized HP1α. Chemically synthesized HP1α were dissolved to a concentration of approximately 4.0 mg mL^{-1} in unfolding buffer [20 mM Tris·HCl (pH 7.5), 7 M Gn·HCl, and 20 mM 2-mercaptoethanol]. After the denaturation by incubation for 1 h at room temperature, the mixture was transferred to an Oscillatory Cup molecular weight cutoff 3500 instrument (Cosmo Bio) or Slide-A-Lyzer™ MINI Dialysis Device (7 K MWCO) (Thermo Fisher). The solution was dialyzed against 500 mL of dialysis buffer [20 mM Tris·HCl (pH 7.5), 100 mM NaCl, and 2.0 mM 2-mercaptoethanol] at 4 °C at least three times. Then, the mixture was transferred to Protein Lobind tube (Eppendorf) and the concentration was determined from the absorption at 280 nm wavelength.

Measurement of Ru content on synthesized HP1α by ICP-MS. 5.0 μL of 128 μM synthetic HP1α (**21**) was mixed with 10 ml of 0.1 M HCl aq, and the Ru concentration was measure in the same way as synthesized histone H1.2 (**12**). The concentration of Ru in HP1α solution was measured as 1.55 ppb. The palladium content attached on synthesized HP1α was calculated as 0.00011% (w/w).

Investigation of aggregation of HP1α by DLS. Solution of recombinant HP1α protein (NOVUS Biologicals) in dialysis buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM 2-mercaptoethanol] was filtered using Centrifugal filter units 0.22 μm GV Durepore (Merck Millipore) to remove large particles. To 12 μL HP1α solution (70 μM) was added 0.4 μL Na_2PdCl_4 (10 mM) in milliQ (Pd: 5 equiv.), 0.4 μL **Ru-4** (10 mM) in acetonitrile (Ru: 5 equiv.), or 0.32 μL **Ru-4** (0.5 mM) (Ru: 0.2 equiv.), respectively. Each mixture was incubated at room temperature for 3 min. The whole solution was transferred to quartz cell (ZEN2112, Malvern), and the particle size was measured by Zetasizer Nano ZSP (Malvern).

Measurement of CD spectrum. After refolding of chemically synthesized HP1α by salt dialysis, 300 μL HP1α solution (12 μM) was transferred to a quartz cell, and the CD spectrum was recorded with J-1500 (JASCO).

Synthesis of fluorescent-labelled H3K9me3 peptide and Sgo1 peptide. Fmoc-NH-SAL Resin (0.40 mmol/g) was used for the preparation of H3K9me3 peptide and Sgo1 peptide. For the incorporation of K9me3, Fmoc-Lys(me^3)-OH·HCl was employed, which was synthesized in our previous research.[5] After automated SPPS using ResPep CF (Intavis) of parallel synthesis, N-Fmoc-β-Ala-OH (Tokyo Chemical Industry) was coupled to inhibit a side reaction which occurred during next reaction.[6] Then, 1.50 equiv. Fluorescein isothiocyanate isomer-I (FITC) (Dojindo Molecular Technologies, Inc.) dissolved in DMF and DIEA (3.0 equiv.) were added to the resin, and the mixture was stirred overnight at room temperature. The peptide was cleaved with TFA cocktail (92.5% TFA, 5% TIPS, 2.5% H_2O). The mixture was rotated at room temperature for 2 h and filtered to remove the resin. Then cold ether was added, vortexed and centrifuged 5,000 × g at room temperature for 1 min. Ether was decanted and washed with ether three times. The crude peptide was dissolved in mixture of water/acetonitrile containing 0.1 % TFA and purified by RP-HPLC and identified by MALDI-TOF mass spectrometry.

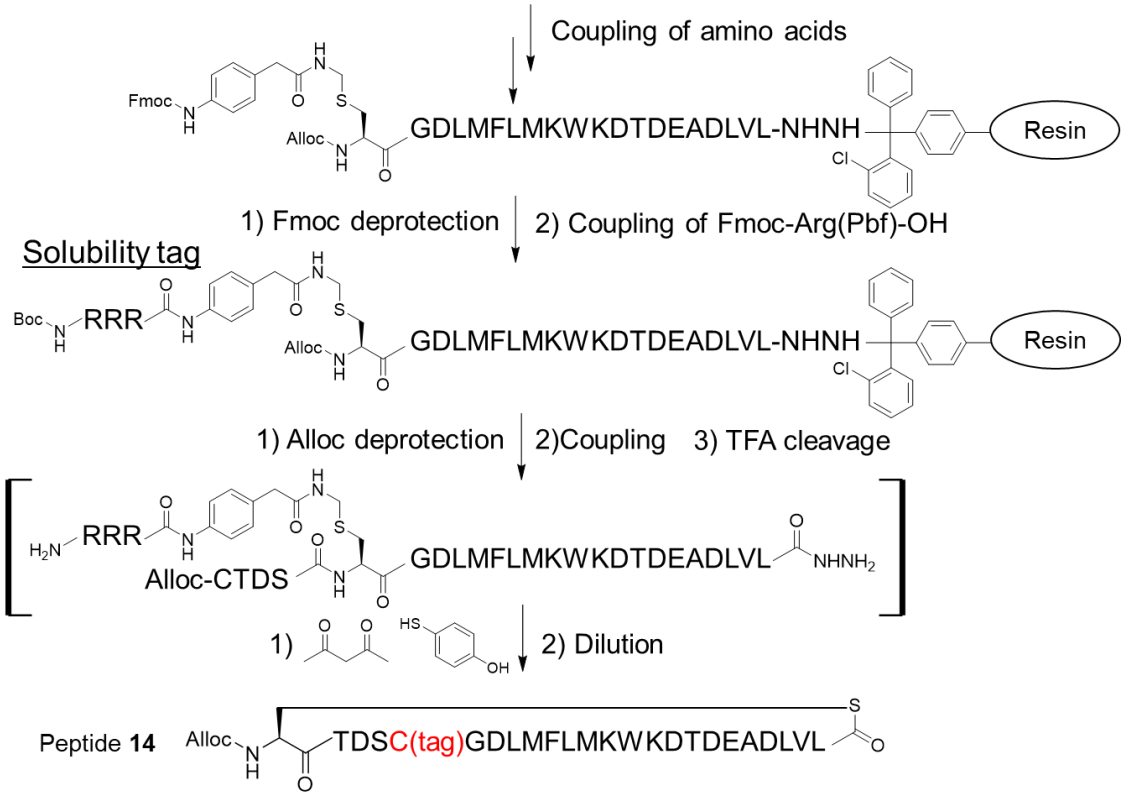
Measurement of fluorescence anisotropy. To analyze the interaction between H3K9me3 peptide and CD of HP1α, to 4.5 μL HP1α solution at different concentrations in binding buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM 2-mercaptoethanol] was added 0.5 μL fluorescein-conjugated H3K9me3 peptide solution (1 μM). The final concentrations of the mixtures of HP1α were 0.5, 1, 5, 10, 20, 50, 100, and 120 μM. The mixtures were incubated at 25 °C for 30 min. The samples were transferred to a Non-binding 384 well Black Plate (Greiner Bio-one) and the polarization was measured by SpectraMax M5 (Molecular Device) (excitation: 485 nm, detection: 525 nm). To analyze the interaction between Sgo1 peptide and CSD of HP1α, to 4.5 μL HP1α solution at different concentrations in the binding buffer was added 0.5 μL fluorescein-conjugated Sgo1 peptide solution (200 nM). The final concentrations of the mixtures of HP1α were 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 3 μM and 10, and 15 μM only for **21f**. After incubation at 25 °C for 30 min, the polarization was measured by SpectraMax M5 (Molecular Device) (excitation: 485 nm, detection: 525 nm). Curve-fitting was performed with Graphpad Prism 8 software.

Electrophoretic mobility shift assays (EMSAs). Each amount of HP1α was incubated with 0.125 pmol of 193 bp 601 DNA in 5 μL of binding buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM 2-mercaptoethanol for 15 min at 37 °C. Then, 1 μL of 30% sucrose was

added, and the samples were loaded onto 8% native polyacrylamide gels in 0.5×TBE buffer. Gels were run at room temperature at 150 V for 1 h. Gels were stained with SYBR Gold (Invitrogen), visualized using a Gel Doc EZ Imager (BIO RAD) and quantified using ImageJ. Curve-fitting was performed with Graphpad Prism 8 software.

Measurement of complexes of DNA and HP1α by DLS. Solution of HP1α proteins in binding buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM 2-mercaptoethanol] was filtered using Centrifugal filter units 0.22 μm GV Durepore (Merck Millipore) to remove large particles. To 11 μL HP1α solution (11 μM) was added 1 μL 193 bp 601 DNA solution (600 nM), and the mixture was incubated 37 °C for 10 min. The whole solution was transferred to quartz cell (ZEN2112, Malvern), and the particle size was measured by Zetasizer Nano ZSP (Malvern) three times.

Scheme S1. Synthetic strategy of peptide **14** bearing a solubilizing tag.



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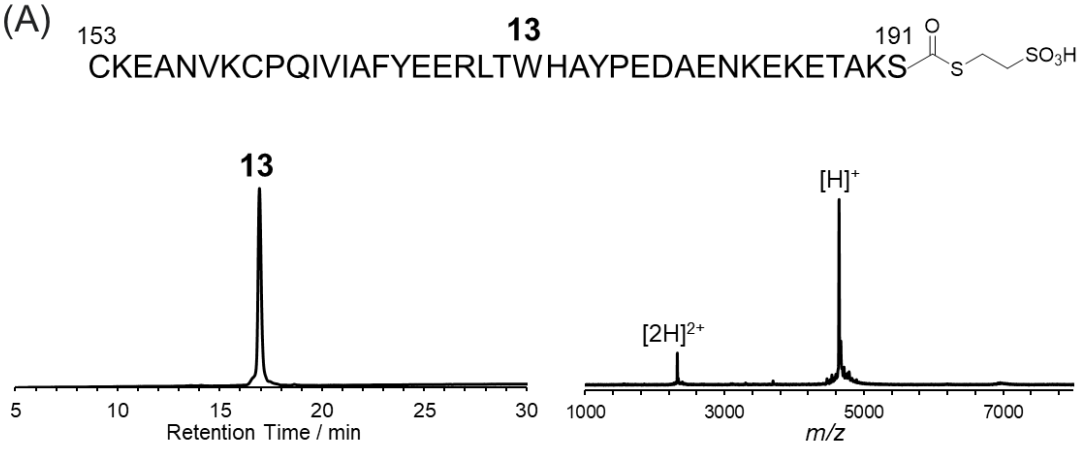
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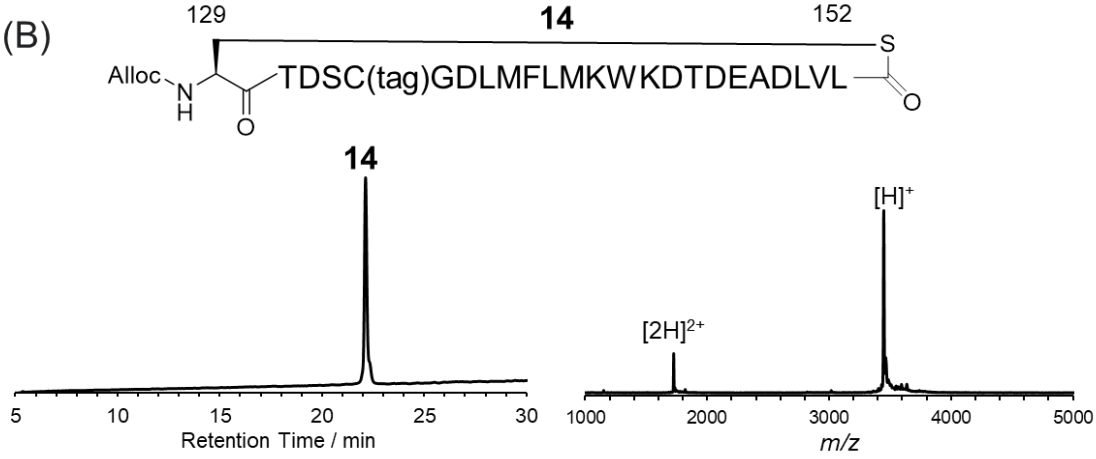
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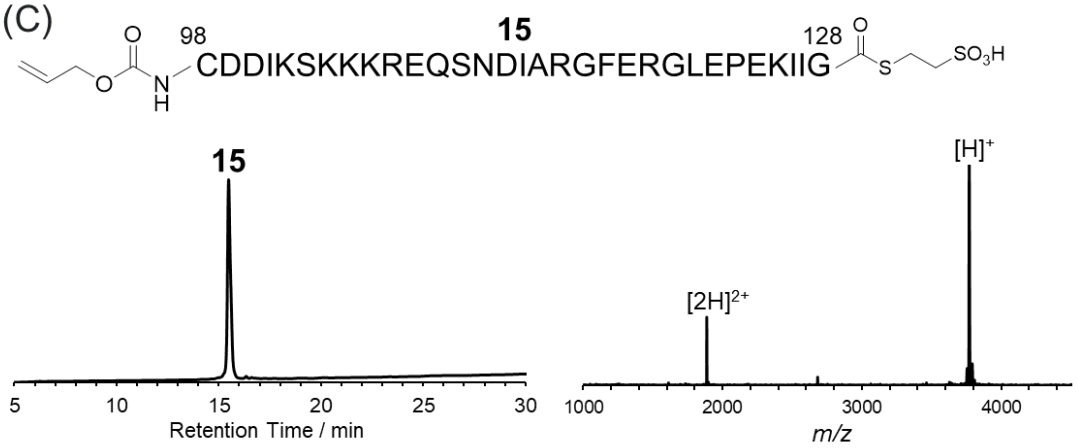
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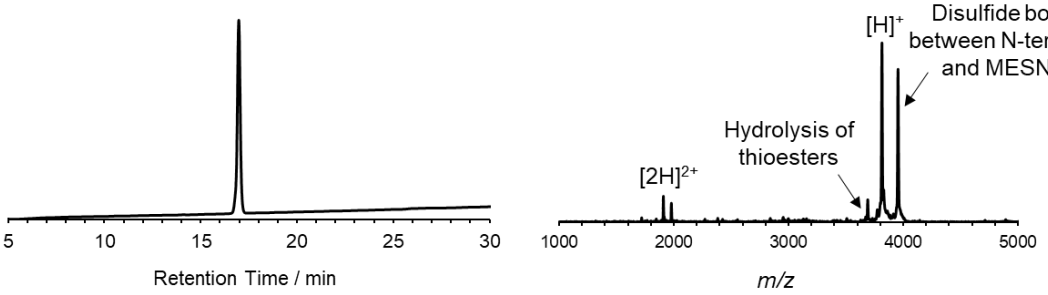

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16

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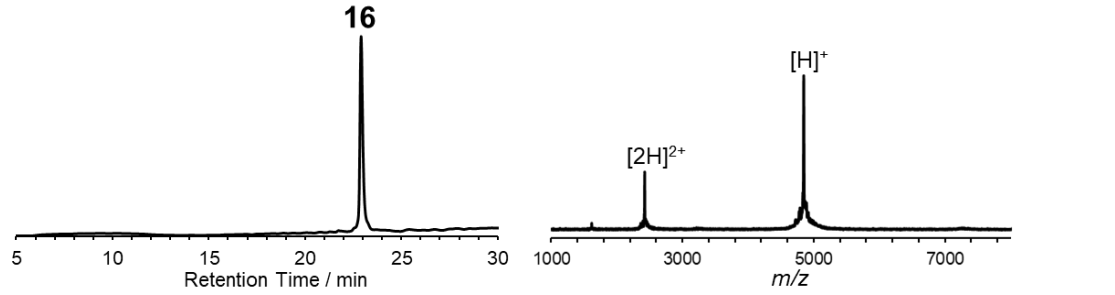

16a

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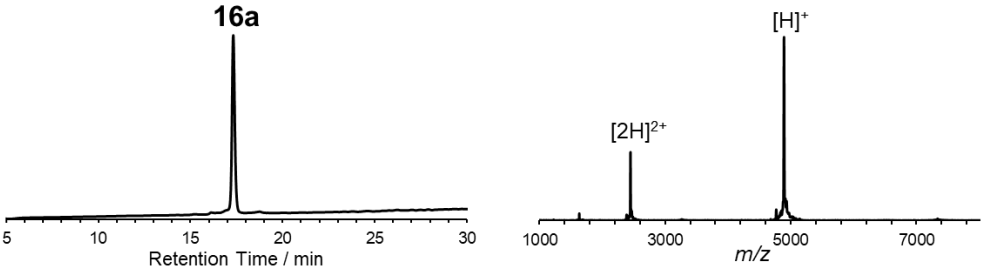
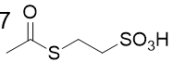
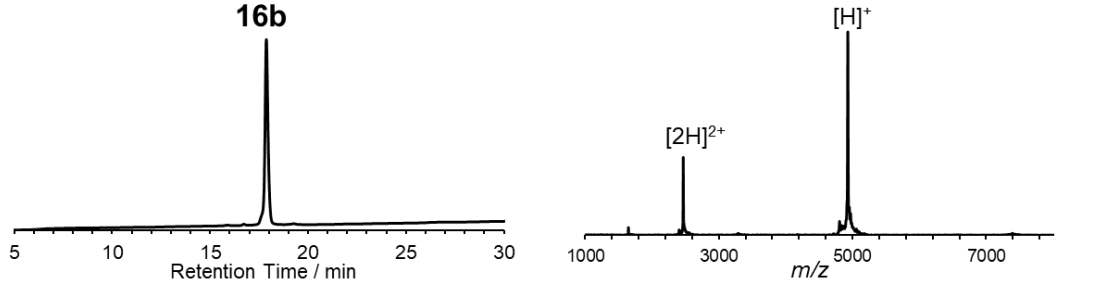
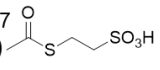


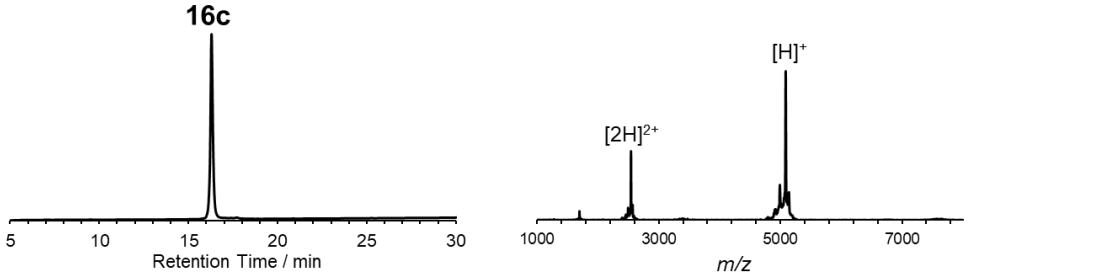
59 **16b**

C(Acm)PELISEFMKKYKKMKEGENNKPREKSESNKRKSNFS(ph)NS 97 

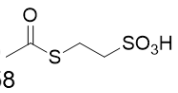


59 **16c**

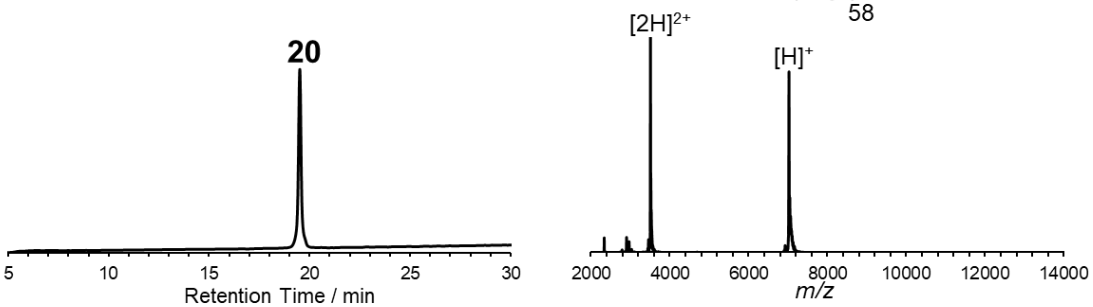
C(Acm)PELISEFMKKYKKMKEGENNKPREKSESNKRKS(ph)NFS(ph)NS(ph) 97 



(E) **20**

1 MGKKTKRTADSSSSEDEEEYVVEKVLDRRVV
KGQVEYLLKWKGFSEEHNTWEPEKNLD(allyl) 

58



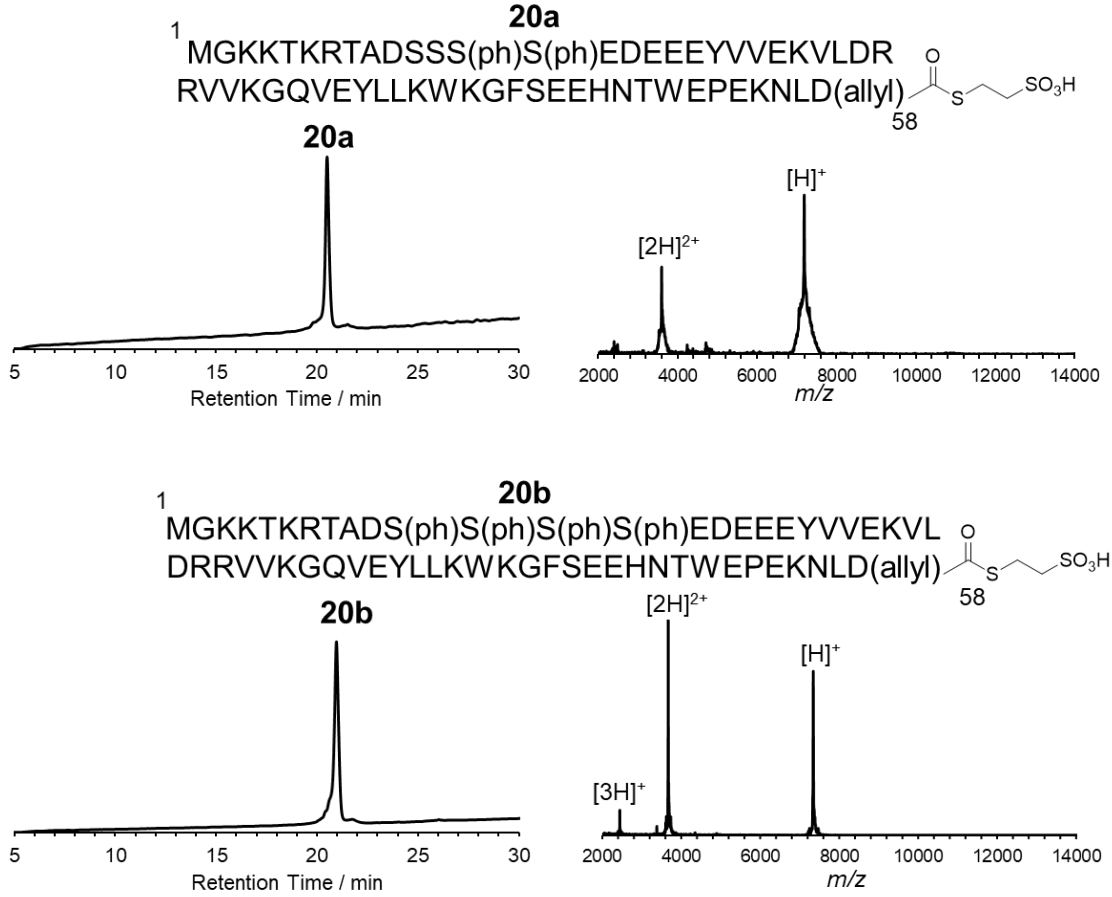


Figure S17. Synthesis of peptides **13**, **14**, **15**, **15a**, **16**, **16a**, **16b**, **16c**, **20**, **20a**, **20b**. HPLC charts of purified peptides, and MALDI-TOF mass spectra (A) of peptide **13**, (B) of peptide **14**, (C) of peptides **15**, **15a**, (D) of peptides 1**6**, **16a**, **16b**, **16c**, (E) of peptide **20**, **20a**, **20b**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.

Calculated mass of **13** $[M+H]^+$: 4642.2; Mass Found $[M+H]^+$: 4642.0.

Calculated mass of **14** $[M+H]^+$: 3449.0; Mass Found $[M+H]^+$: 3449.3.

Calculated mass of **15** $[M+H]^+$: 3770.3; Mass Found $[M+H]^+$: 3770.9.

Calculated mass of **15a** $[M+H]^+$: 3812.3; Mass Found $[M+H]^+$: 3813.2.

Calculated mass of **16** $[M+H]^+$: 4847.6; Mass Found $[M+H]^+$: 4848.3.

Calculated mass of **16a** $[M+H]^+$: 4890.6; Mass Found $[M+H]^+$: 4891.0.

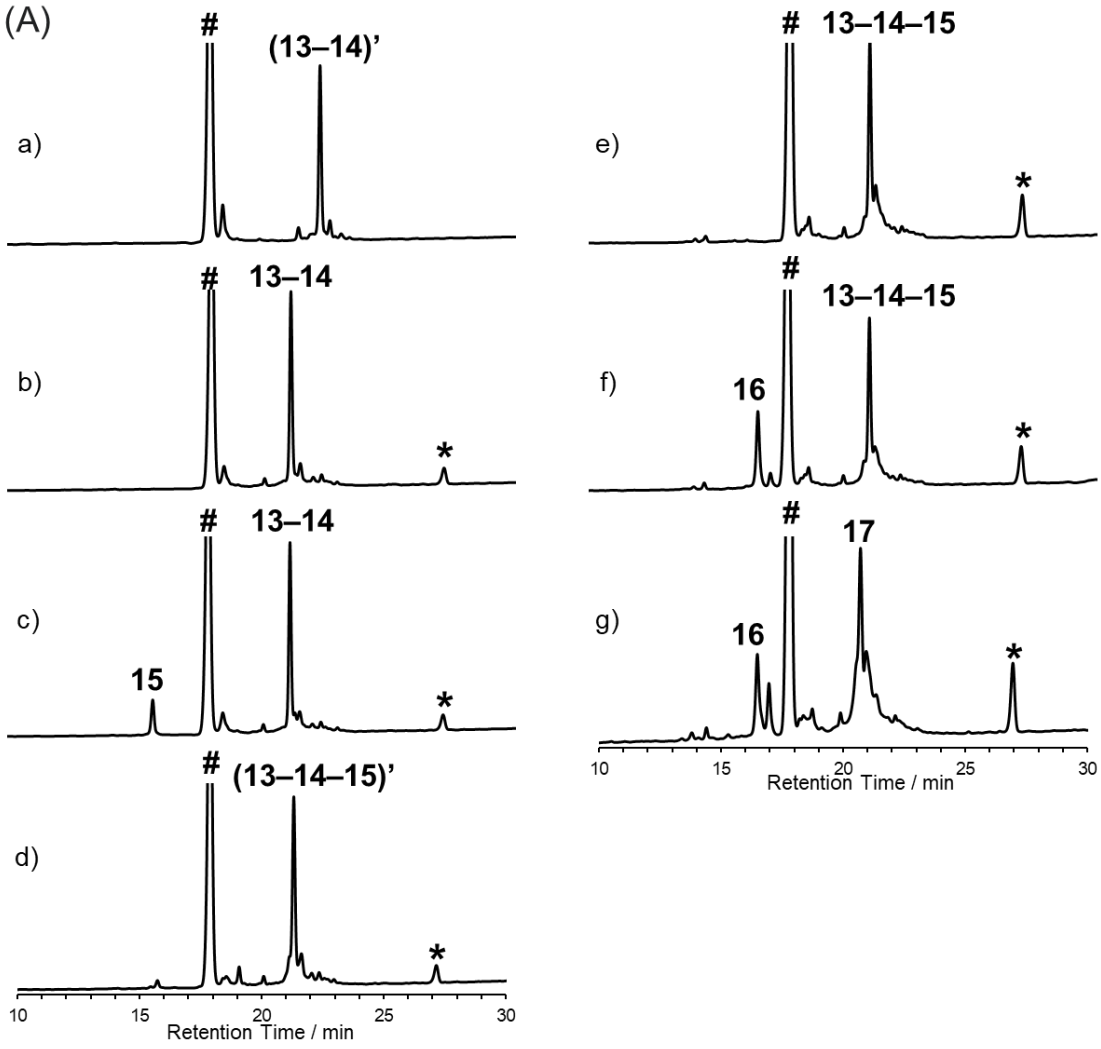
Calculated mass of **16b** $[M+H]^+$: 4928.6; Mass Found $[M+H]^+$: 4929.4.

Calculated mass of **16c** $[M+H]^+$: 5088.5; Mass Found $[M+H]^+$: 5088.3.

Calculated mass of **20** $[M+H]^+$: 7024.2; Mass Found $[M+H]^+$: 7026.0.

Calculated mass of **20a** $[M+H]^+$: 7184.2; Mass Found $[M+H]^+$: 7185.4.

Calculated mass of **20b** $[M+H]^+$: 7344.2; Mass Found $[M+H]^+$: 7343.5.


(A)
a)
#
(13–14)'
b)
#
13–14
*
c)
#
13–14
15
*
d)
#
(13–14–15)'
*
10
15
20
25
30
Retention Time / min
e)
#
13–14–15
*
f)
#
13–14–15
16
*
g)
#
17
16
*
10
15
20
25
30
Retention Time / min

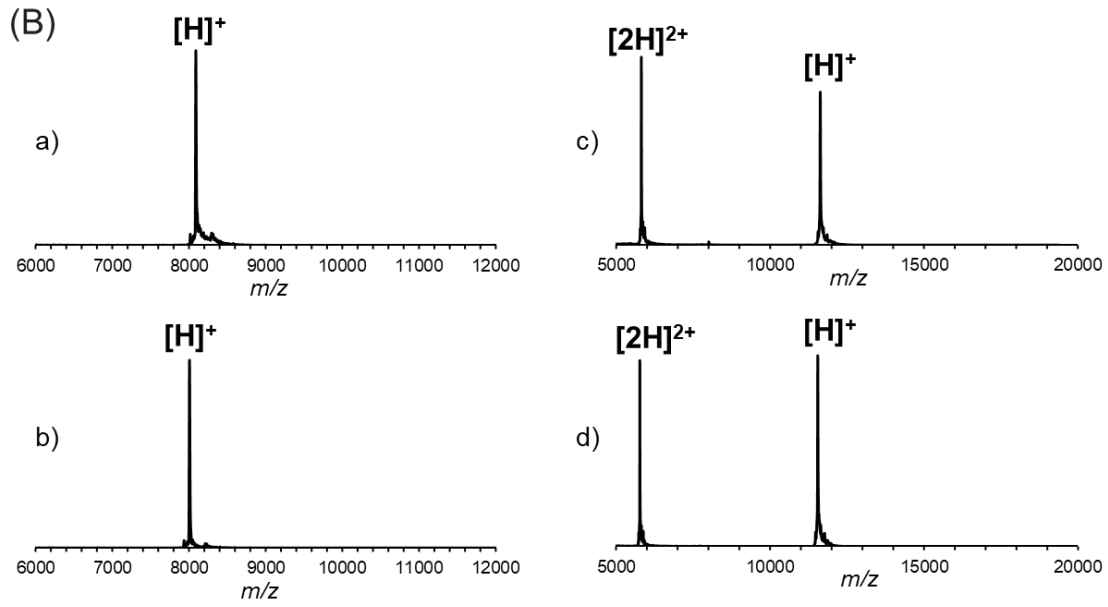


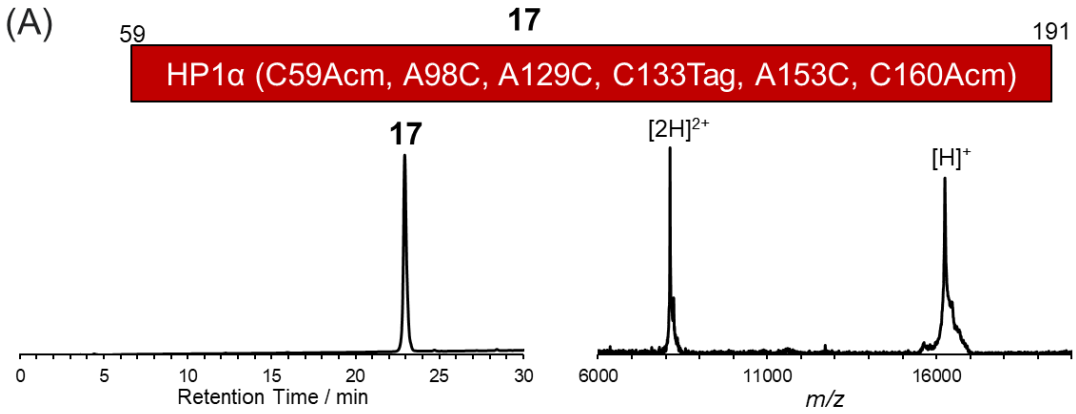
Figure S18. Reaction tracking of one-pot four-segment ligation to afford peptide **17**. (A) Reaction tracking by HPLC. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with $5C_{18}$-AR-300. **(13–14)'**= Alloc-protected **13**–**14**, **(13**–**14**–**15)'**=Alloc-protected **13**–**14**–**15**. # = MPAA, * = allyl-MPAA. a) 1st NCL (t = 4 h). b) 1st deprotection with **Ru-4** (20 mol%). c) 2nd NCL (before). d) 2nd NCL (t = 2 h). e) 2nd deprotection. f) 3rd NCL (before). g) 3rd NCL after overnight. (B) Identification of ligation intermediates **(13**–**14)'**, **13**–**14**, **(13**–**14**–**15)'**, and **13**–**14**–**15**. MALDI-TOF mass spectra a) of peptide **(13**–**14)'**, b) of peptide **13**–**14**, c) of peptide **(13**–**14**–**15)'**, d) of peptide **13**–**14**–**15**.

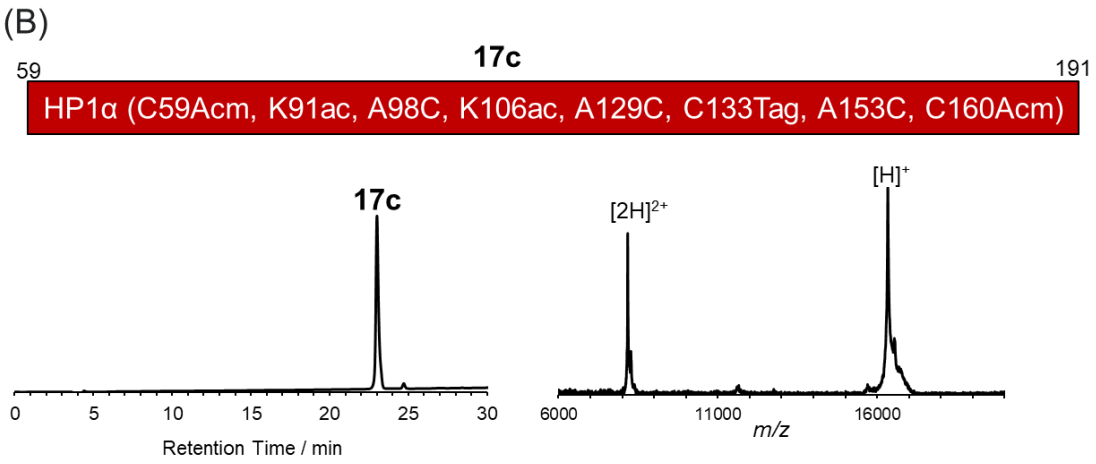
Calculated mass of **(13**–**14)'** $[M+H]^+$: 8090.2; Mass Found $[M+H]^+$: 8090.5.

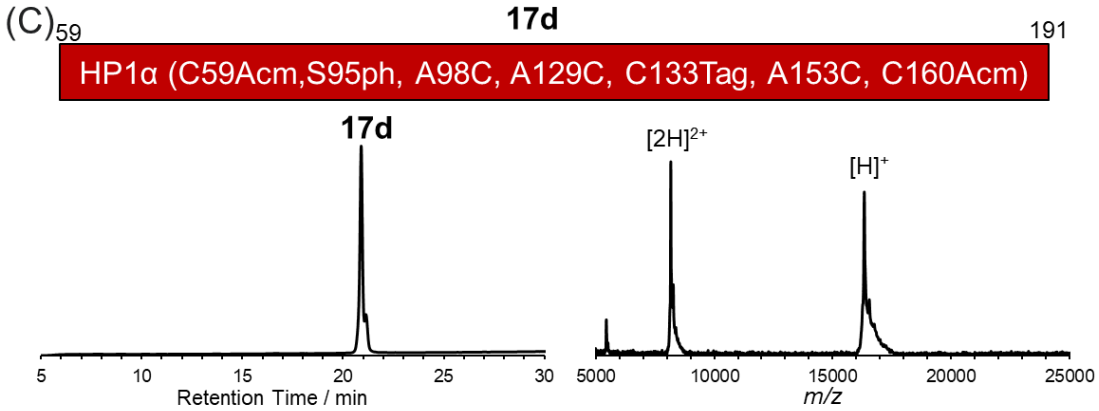
Calculated mass of **13**–**14** $[M+H]^+$: 8006.2; Mass Found $[M+H]^+$: 8005.8.

Calculated mass of **(13**–**14**–**15)'** $[M+H]^+$: 11630.9; Mass Found $[M+H]^+$: 11632.2.

Calculated mass of **13**–**14**–**15** $[M+H]^+$: 11546.8; Mass Found $[M+H]^+$: 11547.9.


(A)
17
59
191
HP1α (C59Acm, A98C, A129C, C133Tag, A153C, C160Acm)
17
[2H]2+
[H]+
0
5
10
15
20
25
30
Retention Time / min
6000
11000
16000
m/z


(B)
17c
59
191
HP1α (C59Acm, K91ac, A98C, K106ac, A129C, C133Tag, A153C, C160Acm)
17c
[2H]2+
[H]+
0
5
10
15
20
25
30
Retention Time / min
6000
11000
16000
m/z


(C)
17d
59
191
HP1α (C59Acm,S95ph, A98C, A129C, C133Tag, A153C, C160Acm)
17d
[2H]2+
[H]+
5
10
15
20
25
30
Retention Time / min
5000
10000
15000
20000
25000
m/z

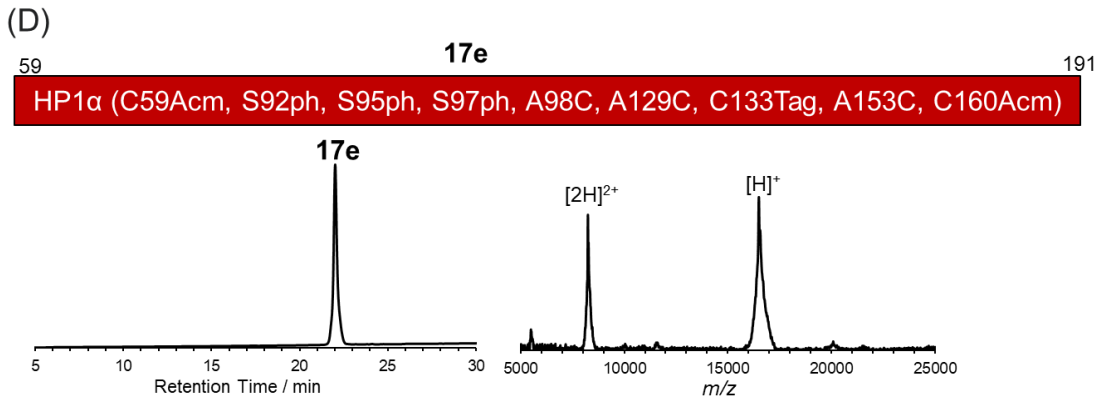


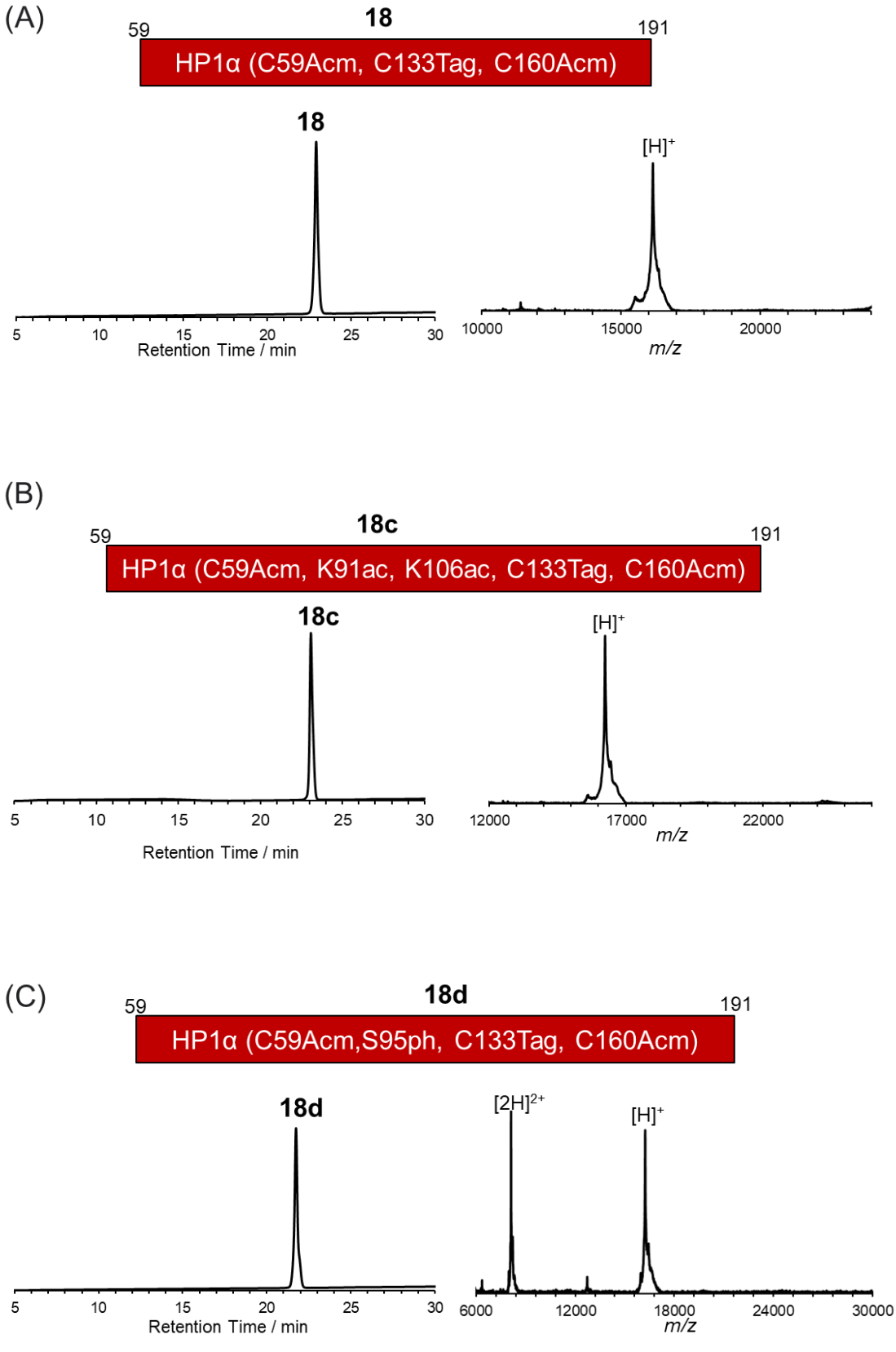
Figure S19. Identification of peptide **17** after one-pot multiple peptide ligation. HPLC charts and MALDI TOF mass spectra of purified peptides (A) **17**, (B) **17c**, (C) **17d**, (D) **17e**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.

Calculated mass of **17** $[M+H]^+$: 16254.6; Mass Found $[M+H]^+$: 16255.0.

Calculated mass of **17c** $[M+H]^+$: 16338.6; Mass Found $[M+H]^+$: 16339.2.

Calculated mass of **17d** $[M+H]^+$: 16334.8; Mass Found $[M+H]^+$: 16334.5.

Calculated mass of **17e** $[M+H]^+$: 16493.7; Mass Found $[M+H]^+$: 16493.1.


(A)
18
59
191
HP1α (C59Acm, C133Tag, C160Acm)
18
[H]+
5
10
15
20
25
30
Retention Time / min
10000
15000
20000
m/z
(B)
18c
59
191
HP1α (C59Acm, K91ac, K106ac, C133Tag, C160Acm)
18c
[H]+
5
10
15
20
25
30
Retention Time / min
12000
17000
22000
m/z
(C)
18d
59
191
HP1α (C59Acm,S95ph, C133Tag, C160Acm)
18d
[2H]2+
[H]+
5
10
15
20
25
30
Retention Time / min
6000
12000
18000
24000
30000
m/z

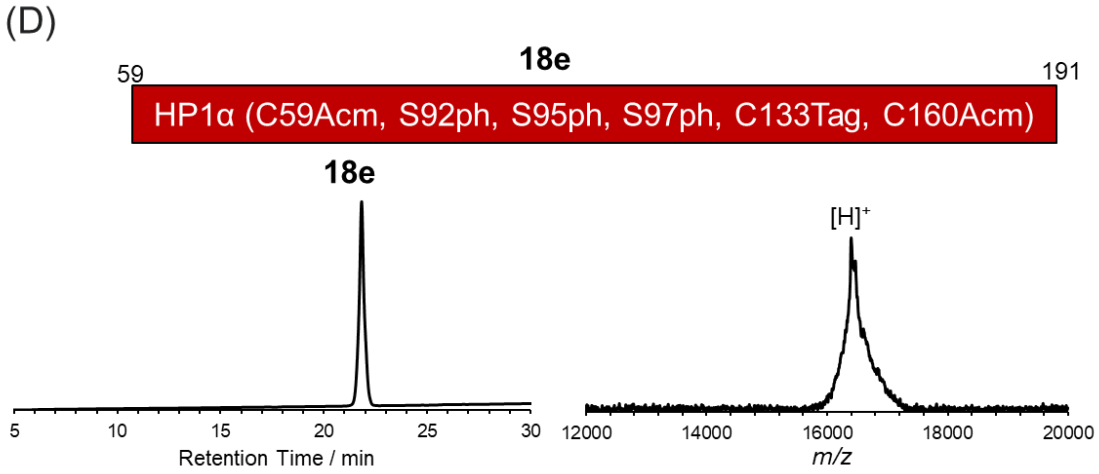


Figure S20. Identification of peptide **18** after desulfurization. HPLC charts and MALDI TOF mass spectra of purified peptides (A) **18**, (B) **18c**, (C) **18d**, (D) **18e**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.

Calculated mass of **18** $[M+H]^+$: 16157.6; Mass Found $[M+H]^+$: 16158.2.

Calculated mass of **18c** $[M+H]^+$: 16241.7; Mass Found $[M+H]^+$: 16242.6.

Calculated mass of **18d** $[M+H]^+$: 16236.6; Mass Found $[M+H]^+$: 16236.7.

Calculated mass of **18e** $[M+H]^+$: 16396.5; Mass Found $[M+H]^+$: 16397.1.

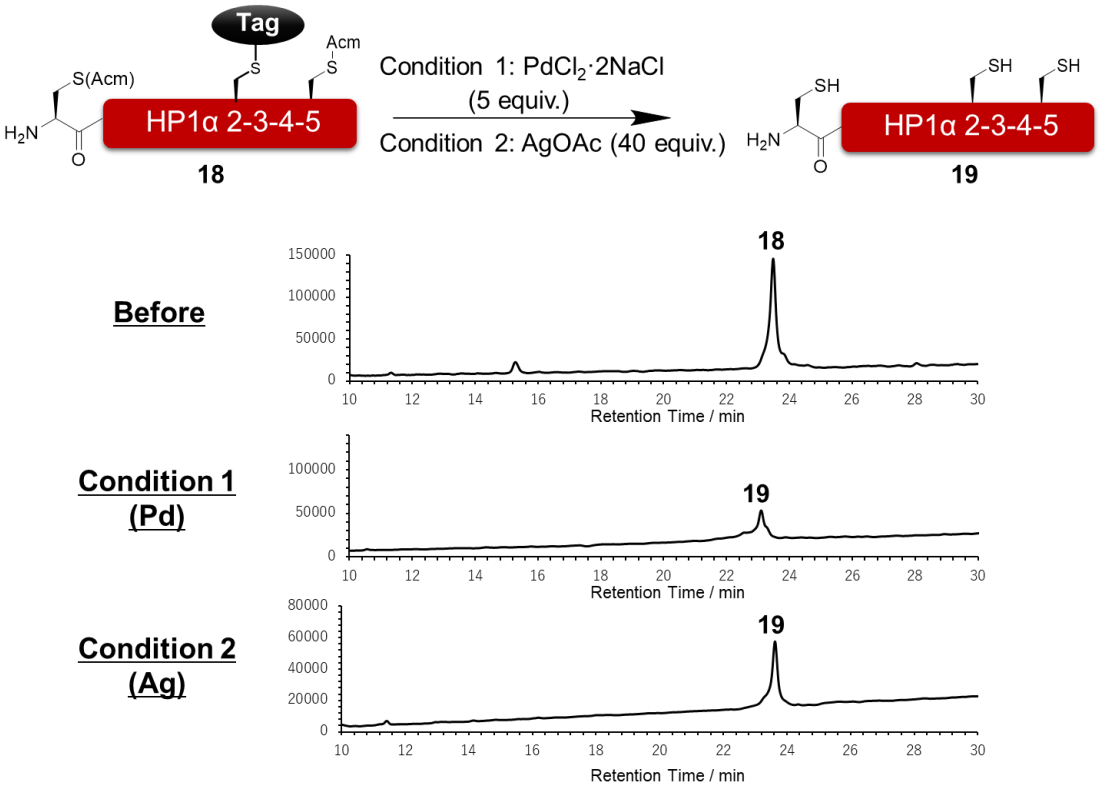
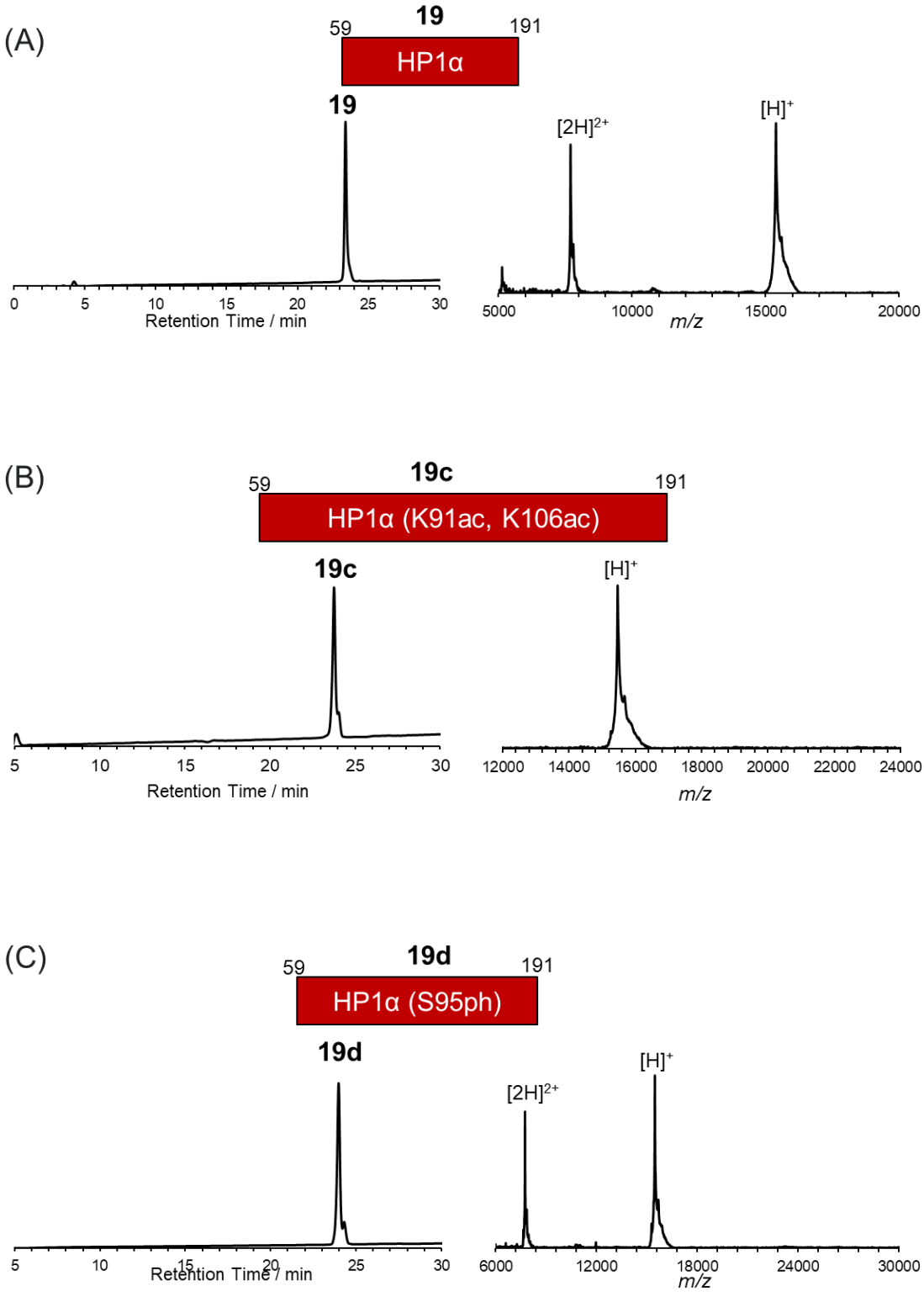


Figure S21. Removal of the acm groups and the solubilizing tag of peptide **18** with the treatment of the Pd complex or the Ag complex. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.

Notes: The recovered and lyophilized **19** after deprotection with $PdCl_2$ was not dissolved under the denaturing conditions and not properly refolded.


(A)
19
59
191
HP1α
19
[2H]2+
[H]+
0
5
10
15
20
25
30
Retention Time / min
5000
10000
15000
20000
m/z
(B)
19c
59
191
HP1α (K91ac, K106ac)
19c
[H]+
5
10
15
20
25
30
Retention Time / min
12000
14000
16000
18000
20000
22000
24000
m/z
(C)
19d
59
191
HP1α (S95ph)
19d
[H]+
[2H]2+
5
10
15
20
25
30
Retention Time / min
6000
12000
18000
24000
30000
m/z

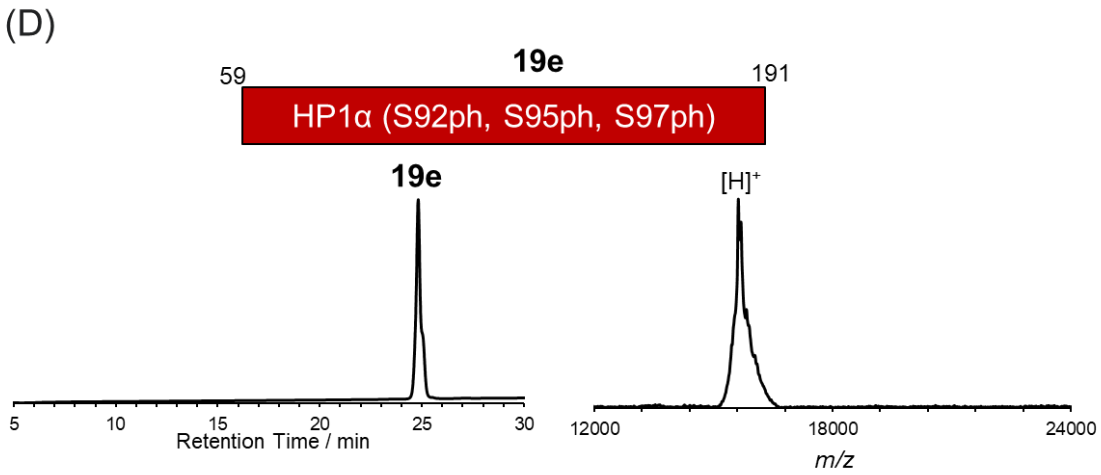


Figure S22. Identification of peptide **19** after the removal of the acm groups and the solubilizing tag. HPLC charts and MALDI TOF mass spectra of purified (A) peptides **19**, (B) **19c**, (C) **19d**, (D) **19e**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.

Calculated mass of **19** $[M+H]^+$: 15384.5; Mass Found $[M+H]^+$: 15384.8.

Calculated mass of **19c** $[M+H]^+$: 15467.5; Mass Found $[M+H]^+$: 15468.0.

Calculated mass of **19d** $[M+H]^+$: 15463.4; Mass Found $[M+H]^+$: 15463.9.

Calculated mass of **19e** $[M+H]^+$: 15623.4; Mass Found $[M+H]^+$: 15624.8.

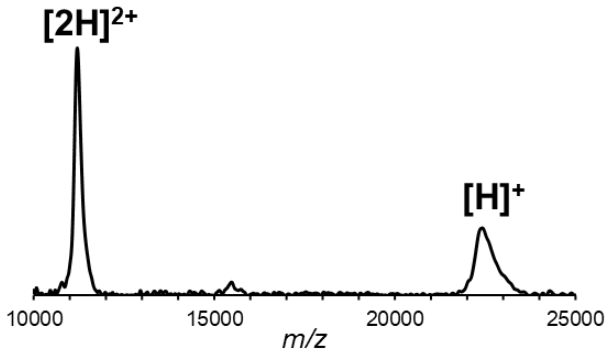


Figure S23. MALDI TOF mass spectrum of ligation intermediate **21"**. Calculated mass of **21"** $[M+H]^+$: 22265.80; Mass Found $[M+H]^+$: 22266.98

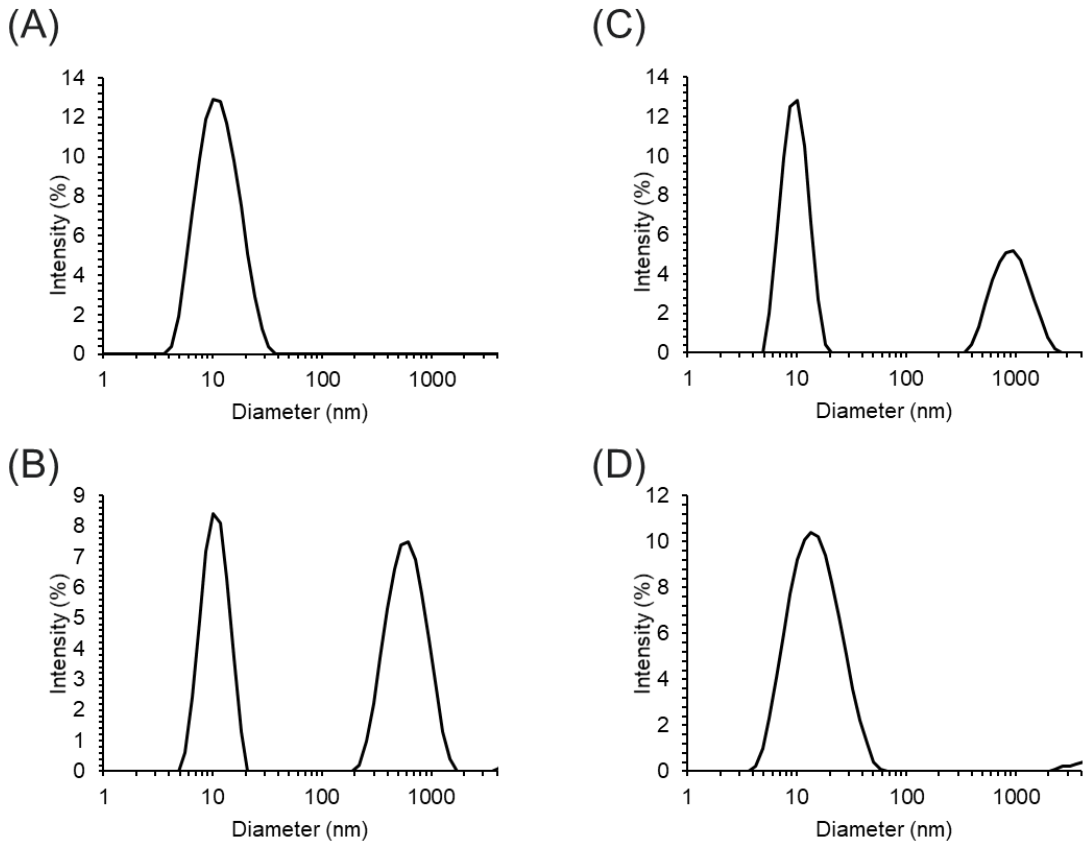
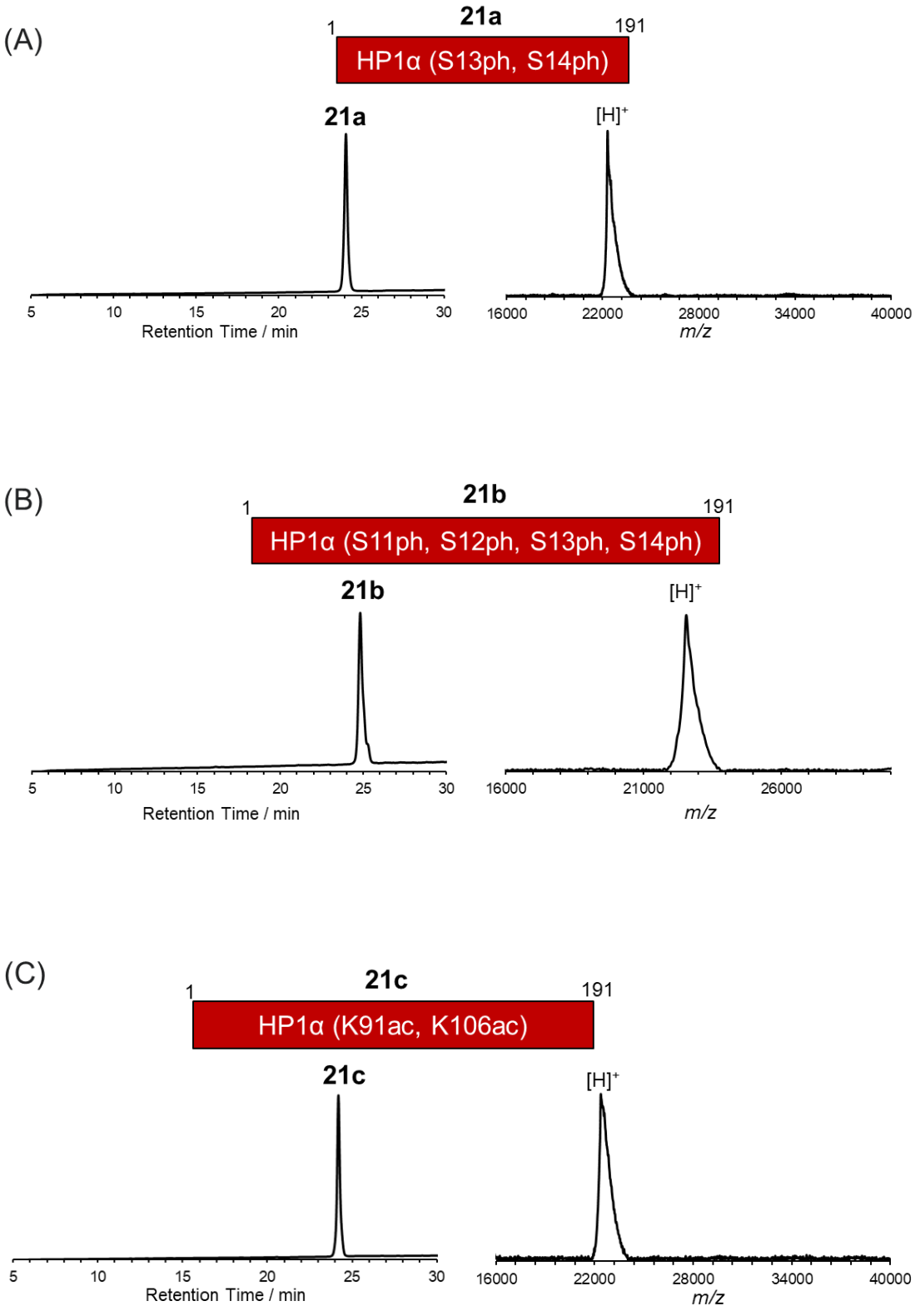


Figure S24. DLS measurement of the aggregation of HP1α (70 μM) (A) in the absence of metal complexes and in the presence of (B) Pd (5 equiv.), (C) Ru (5 equiv.), and (D) Ru (0.2 equiv.).

Table S4. Z-average particle diameter of HP1α under different conditions.

	Z-Average Particle Diameter [nm]
(A) No metal	10.16
(B) Pd 5 equiv.	33.88
(C) Ru 5 equiv.	16.26
(D) Ru 0.2 equiv.	12.89


(A)
21a
1
191
HP1α (S13ph, S14ph)
21a
[H]+
5
10
15
20
25
30
Retention Time / min
16000
22000
28000
34000
40000
m/z
(B)
21b
1
191
HP1α (S11ph, S12ph, S13ph, S14ph)
21b
[H]+
5
10
15
20
25
30
Retention Time / min
16000
21000
26000
m/z
(C)
21c
1
191
HP1α (K91ac, K106ac)
21c
[H]+
5
10
15
20
25
30
Retention Time / min
16000
22000
28000
34000
40000
m/z

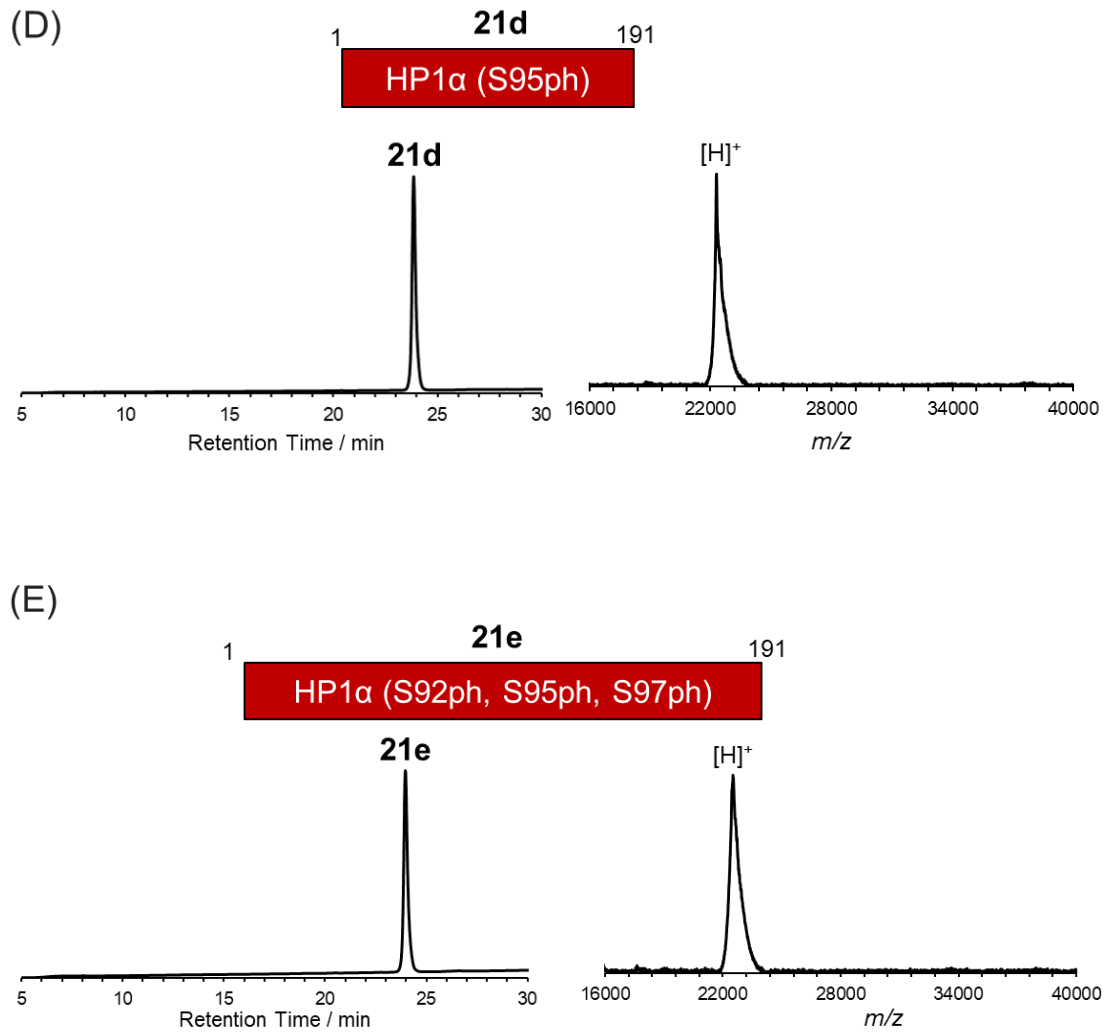


Figure S25. Identification of peptide **21** after NCL and following the removal of the allyl group with **Ru-4**. HPLC charts and MALDI TOF mass spectra of purified proteins (A) **21a**, (B) **21b**, (C) **21c**, (D) **21d**, and (E) **21e**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.

Calculated mass of **21a** $[M+H]^+$: 22386.7; Mass Found $[M+H]^+$: 22384.8.

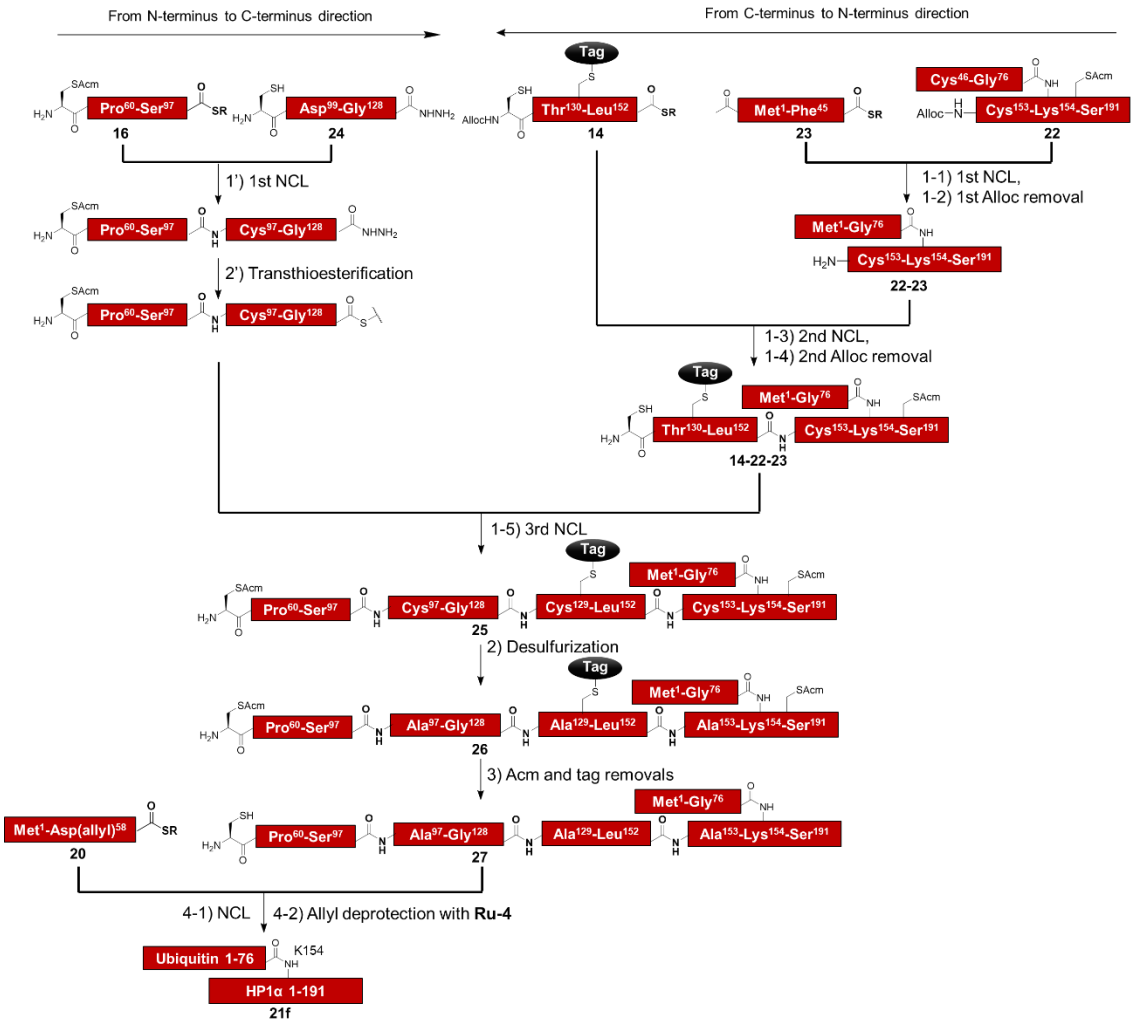
Calculated mass of **21b** $[M+H]^+$: 22546.7; Mass Found $[M+H]^+$: 22547.2.

Calculated mass of **21c** $[M+H]^+$: 22310.8; Mass Found $[M+H]^+$: 22310.2.

Calculated mass of **21d** $[M+H]^+$: 22306.7; Mass Found $[M+H]^+$: 22304.7.

Calculated mass of **21e** $[M+H]^+$: 22466.7; Mass Found $[M+H]^+$: 22468.5.

Scheme S2. Synthetic strategy of **21f** bearing a branched C-terminal ubiquitin fragment.



From N-terminus to C-terminus direction. 1') Peptides (4 mM), MPAA (100 mM), TCEP (20 mM) in denaturing buffer at pH 7.0, 37 ºC, overnight. 2') peptides (4 mM), MPAA (150 mM), acetyl acetone (10 mM) in denaturing buffer at pH 3.0, 37 ºC, 3 h.

From C-terminus to N-terminus direction. NCL condition: peptides (2 mM), MPAA (100 mM) in denaturing buffer at pH 7.0, 37 ºC. Removal condition: **Ru-4** (20 mol%), 37 ºC, 20 min.

2) Peptide (0.8 mM), TCEP (300 mM), GSH (150 mM), VA-044 (20 mM) in denaturing buffer at pH 7.0. 3) Peptide (0.5 mM), AgOAc (30 mM), H_2O/AcOH (1:1), 37 ºC, overnight. 4) NCL condition: peptides (1.0 mM), MPAA (100 mM) in denaturing buffer at pH 7.0, 37 ºC. Removal condition: **Ru-4** (40 mol%), 37 ºC, 20 min.

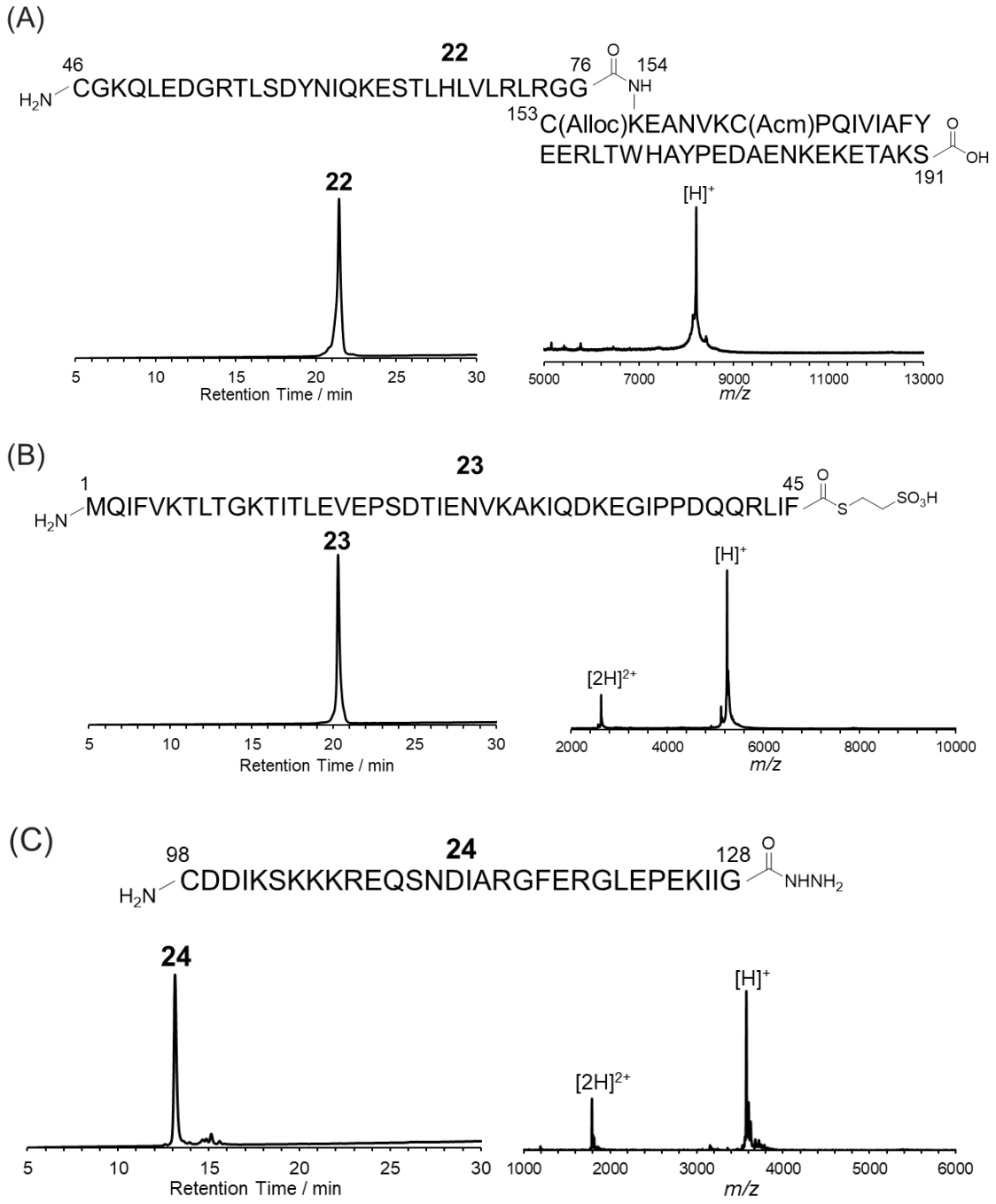


Figure S26. Synthesis of peptides **22**, **23**, **24**. HPLC charts of purified peptides, and MALDI-TOF mass spectra of peptides (A) **22**, (B) **23**, and (C) **24**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.

Calculated mass of **22** $[M+H]^+$: 8209.2; Mass Found $[M+H]^+$: 8209.4

Calculated mass of **23** $[M+H]^+$: 5239.1; Mass Found $[M+H]^+$: 5238.5.

Calculated mass of **24** $[M+H]^+$: 3576.0; Mass Found $[M+H]^+$: 3576.8.

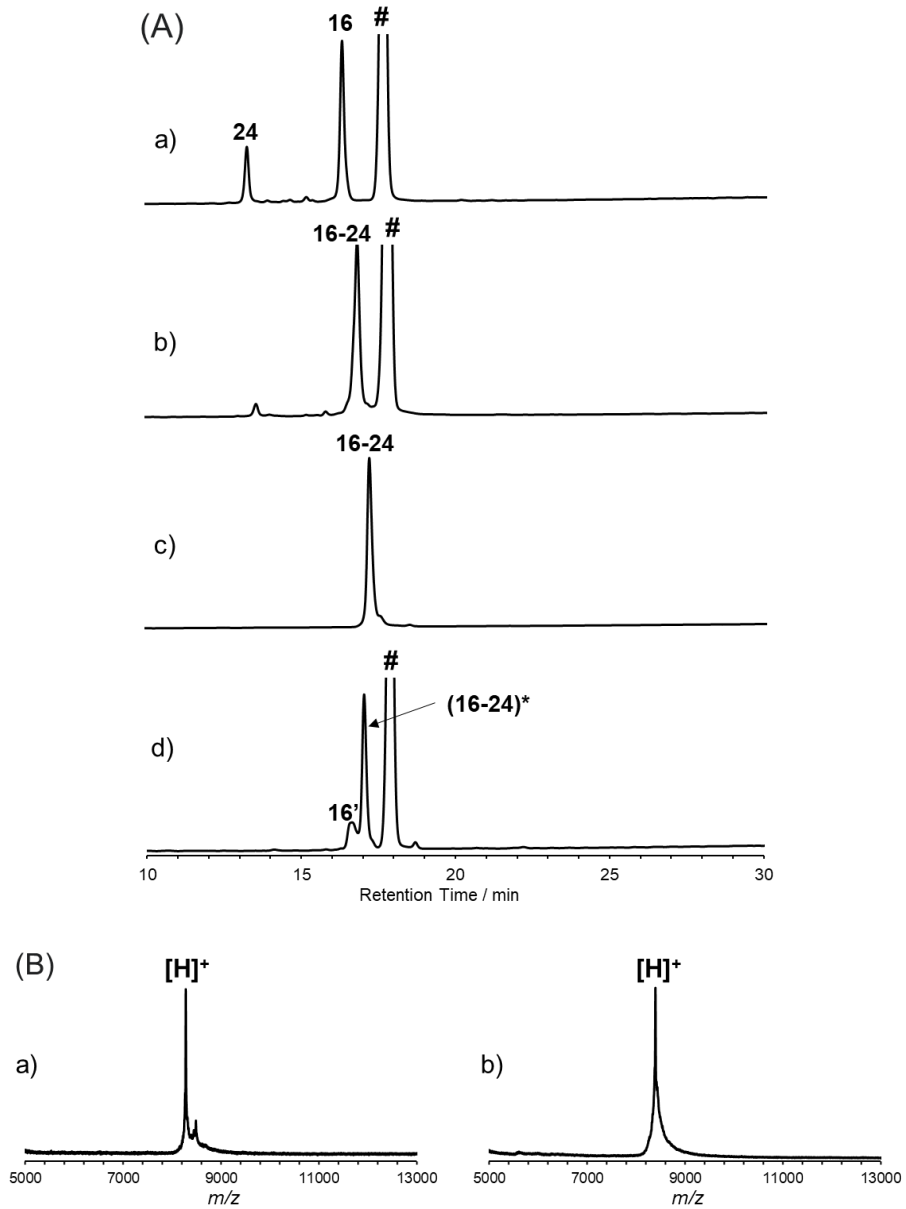
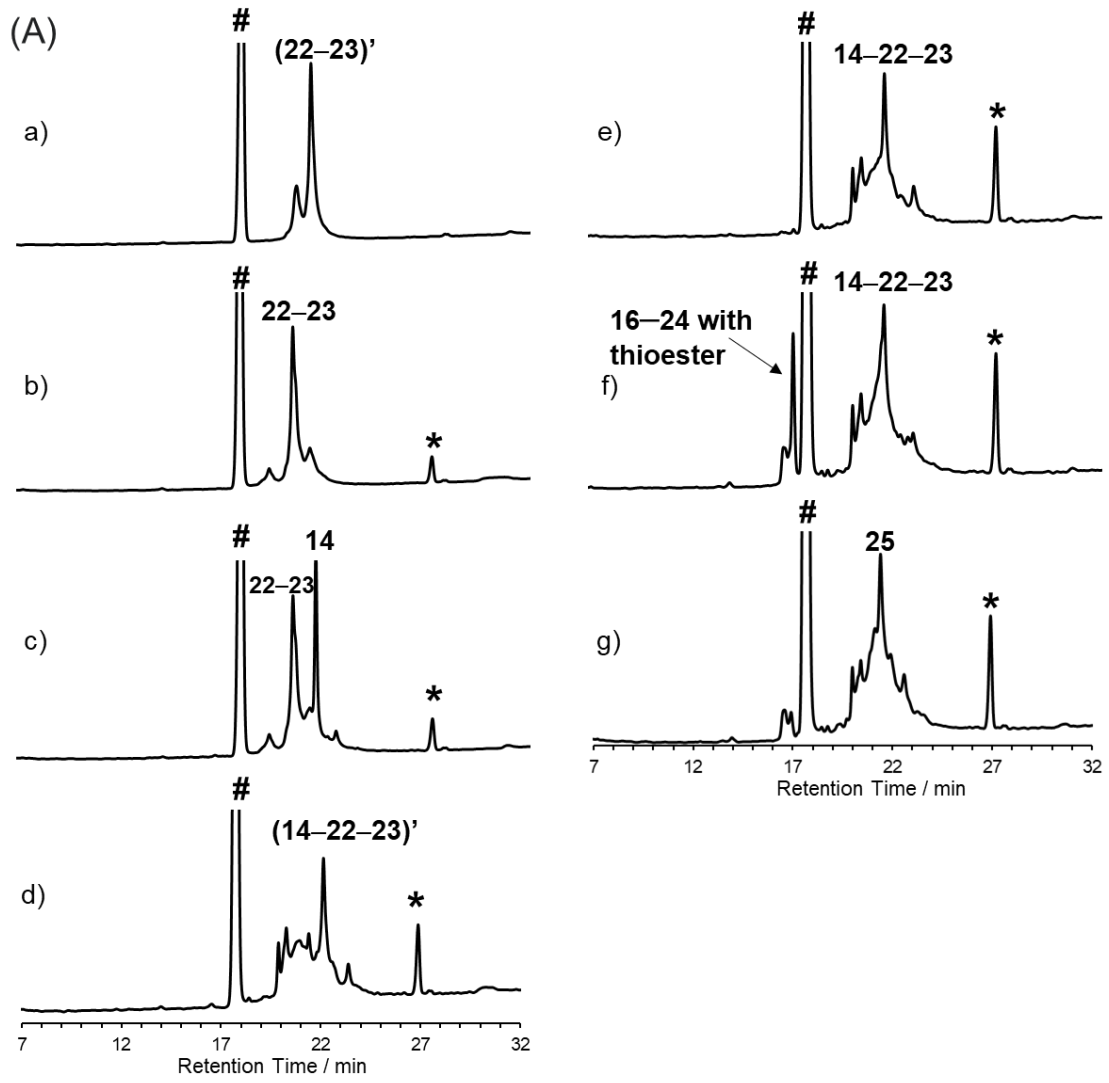


Figure S27. Reaction tracking of NCL and subsequent transthioesterification. (A) Reaction tracking by HPLC. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with $5C_{18}$-AR-300. # = MPAA. a) NCL between peptide **24** and **16** (t = 2 min); b) after overnight reaction. c) Purified peptide **16**–**24**. d) Transthioesterification from hydrazine using acetylacetone. **16'** = hydrolyzed peptide **16**. (**16**–**24**)* = **16**–**24** with a thioester. (B) MALDI-TOF mass spectra of ligation intermediates peptide **16**–**24** and (**16–24)***.

Calculated mass of **16**–**24** $[M+H]^+$: 8280.6; Mass Found $[M+H]^+$: 8280.4.

Calculated mass of (**16**–**24)*** $[M+H]^+$: 8390.8; Mass Found $[M+H]^+$: 8391.8.


(A)
a)
#
(22–23)'
b)
#
22–23
*
c)
#
14
22–23
*
d)
#
(14–22–23)'
*
7
12
17
22
27
32
Retention Time / min
e)
#
14–22–23
*
f)
#
14–22–23
16–24 with
thioester
*
g)
#
25
*
7
12
17
22
27
32
Retention Time / min
(B)
a)
[H]+
8000
10000
12000
14000
16000
18000
20000
m/z
b)
[H]+
8000
10000
12000
14000
16000
18000
20000
m/z
c)
[2H]2+
[H]+
6000
13000
20000
m/z
d)
[2H]2+
[H]+
7000
14000
21000
m/z

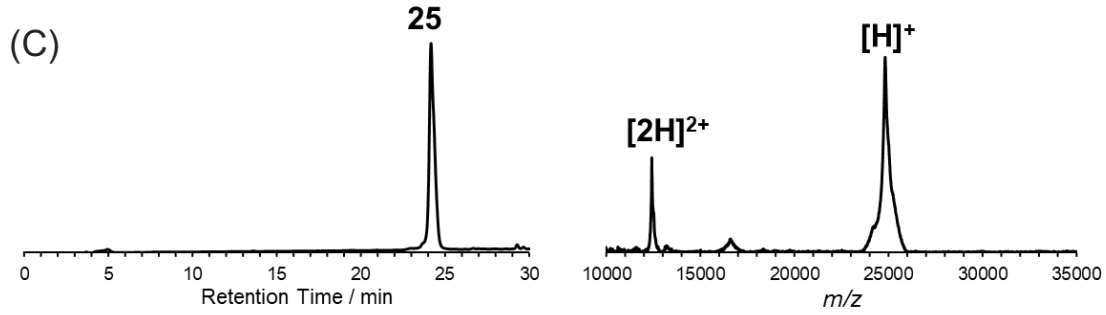


Figure S28. Reaction tracking of one-pot four-segment ligation to afford peptide **25**. (A) Reaction tracking by HPLC. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with 5C_{18}-AR-300. **(22–23)'**= Alloc-protected **22–23**, **(14–22–23)'**=Alloc-protected **14–22–23**. # = MPAA, * = allyl-MPAA. a) 1st NCL (overnight). b) 1st deprotection with **Ru-4** (20 mol%). c) 2nd NCL (before). d) 2nd NCL (overnight). e) 2nd deprotection. f) 3rd NCL (before). g) 3rd NCL (overnight). (B) Identification of ligation intermediates. MALDI-TOF mass spectra of peptide (**22**–**23)'**, **22–23**, (**14–22–23)'**, **14–22–23**. Calculated mass of (**22**–**23)'** $[M+H]^+$: 13306.3; Mass Found $[M+H]^+$: 13308.4. Calculated mass of **22–23** $[M+H]^+$: 13222.2; Mass Found $[M+H]^+$: 13226.7. Calculated mass of (**14–22–23)'** $[M+H]^+$: 16670.2; Mass Found $[M+H]^+$: 16672.1. Calculated mass of **14–22–23** $[M+H]^+$: 16586.2; Mass Found $[M+H]^+$: 16588.3. (C) Identification of peptide **25** after one-pot multiple peptide ligation. HPLC charts and MALDI TOF mass spectra of purified peptide **25**. Calculated mass of **25** $[M+H]^+$: 24832.6; Mass Found $[M+H]^+$: 24833.7.

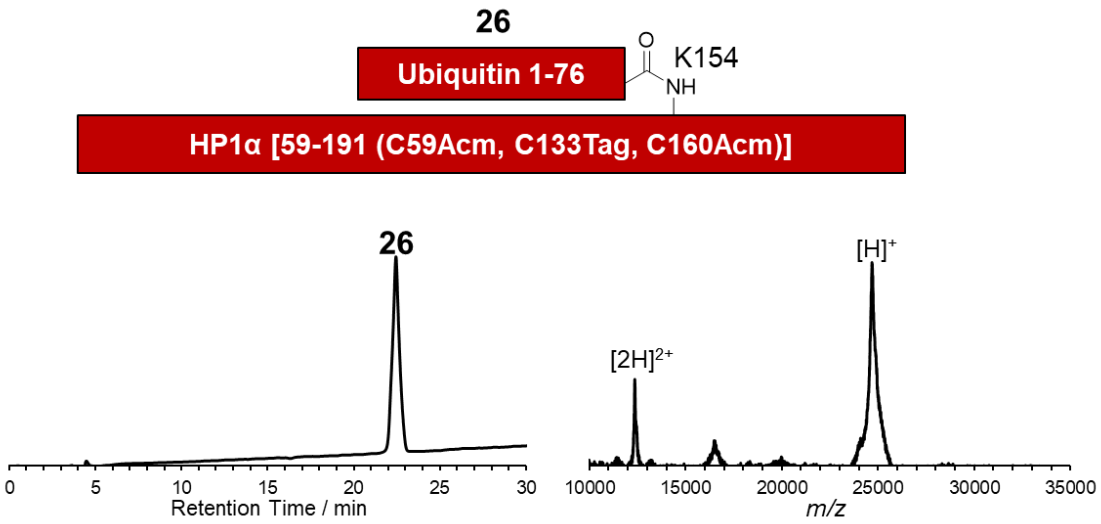


Figure S29. Identification of peptide **26** after desulfurization. HPLC charts and MALDI TOF mass spectra of purified peptide **26**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.
Calculated mass of **26** $[M+H]^+$: 24704.4; Mass Found $[M+H]^+$: 24705.3.

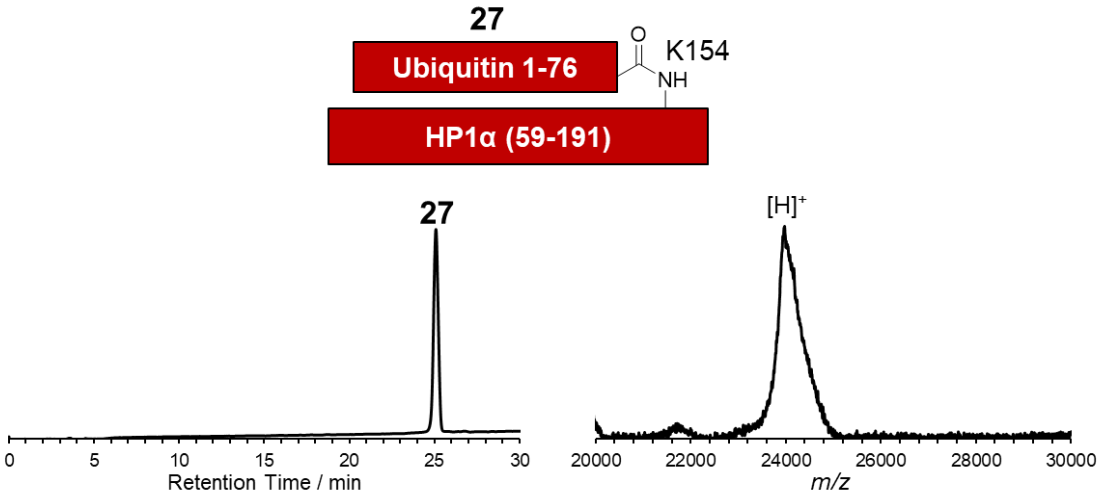


Figure S30. Identification of peptide **27** after the removal of the acm groups and the solubilizing tag. HPLC charts and MALDI TOF mass spectra of purified peptide **27**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R.
Calculated mass of **27** $[M+H]^+$: 23931.21; Mass Found $[M+H]^+$: 23933.93.

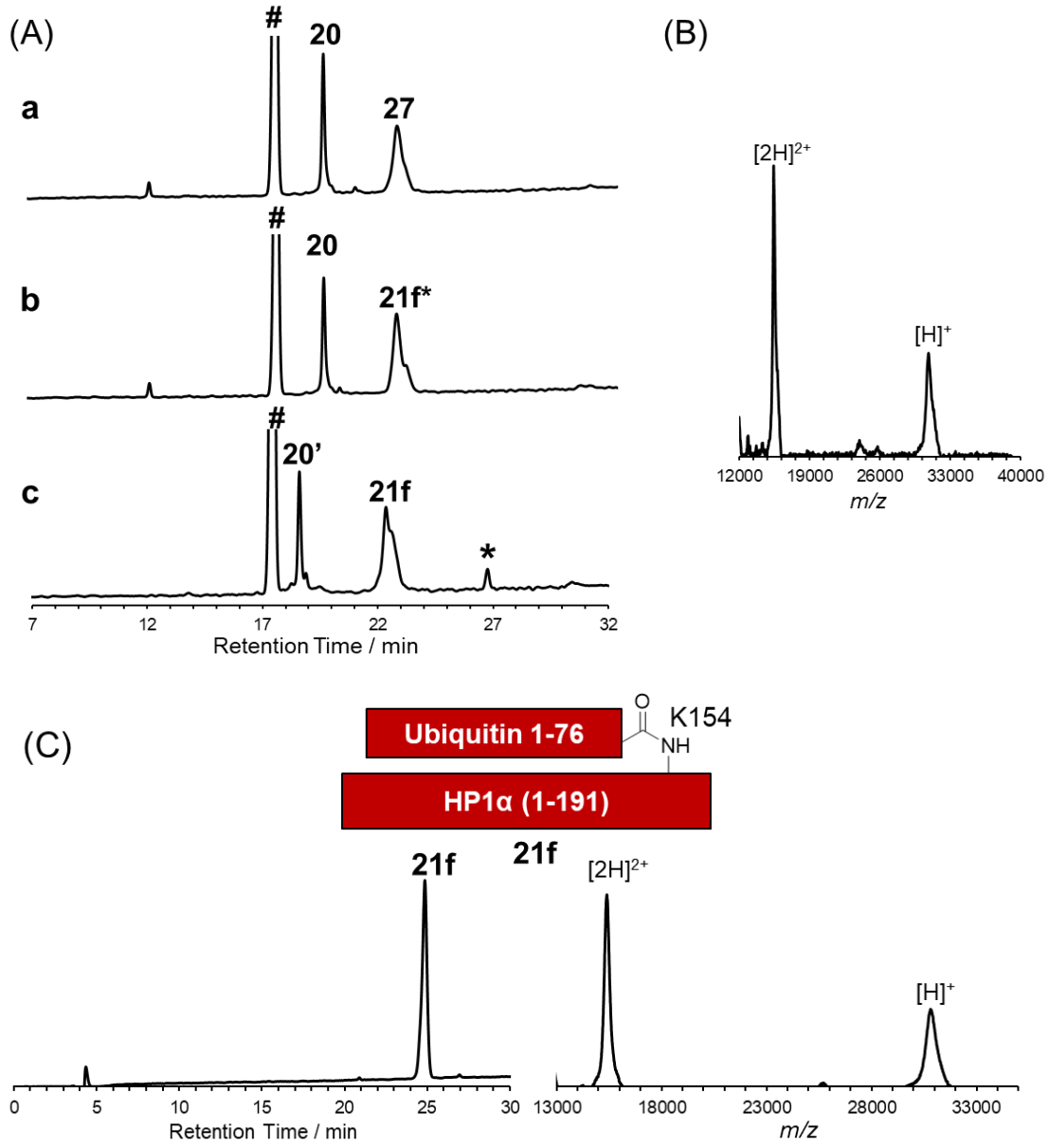


Figure S31. Reaction tracking of NCL between peptide **20** and **27**, followed by the allyl deprotection with **Ru-4**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with 5C_{18}-AR-300. (A) **20'** = Allyl deprotected **20**, **27*** = **27** bearing allyl-protected Asp58. # = MPAA, * = allyl-MPAA. a) NCL (t = 3 min). b) . b) NCL (t = 3 h). c) Allyl deprotection with **Ru-4** (40 mol%). (B) MALDI-TOF mass spectrum of ligation intermediate peptide **21f***. Calculated mass of **21f*** $[M+H]^+$: 30812.5; Mass Found $[M+H]^+$: 305815.3. (C) Identification of peptide **21f**. HPLC chart and MALDI TOF mass spectra of purified peptide **21f**. HPLC peaks were monitored at 220 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. Gradient: 15–51% for 30 min with Protein-R. Calculated mass of **21f** $[M+H]^+$: 30772.5; Mass Found $[M+H]^+$: 30773.0.

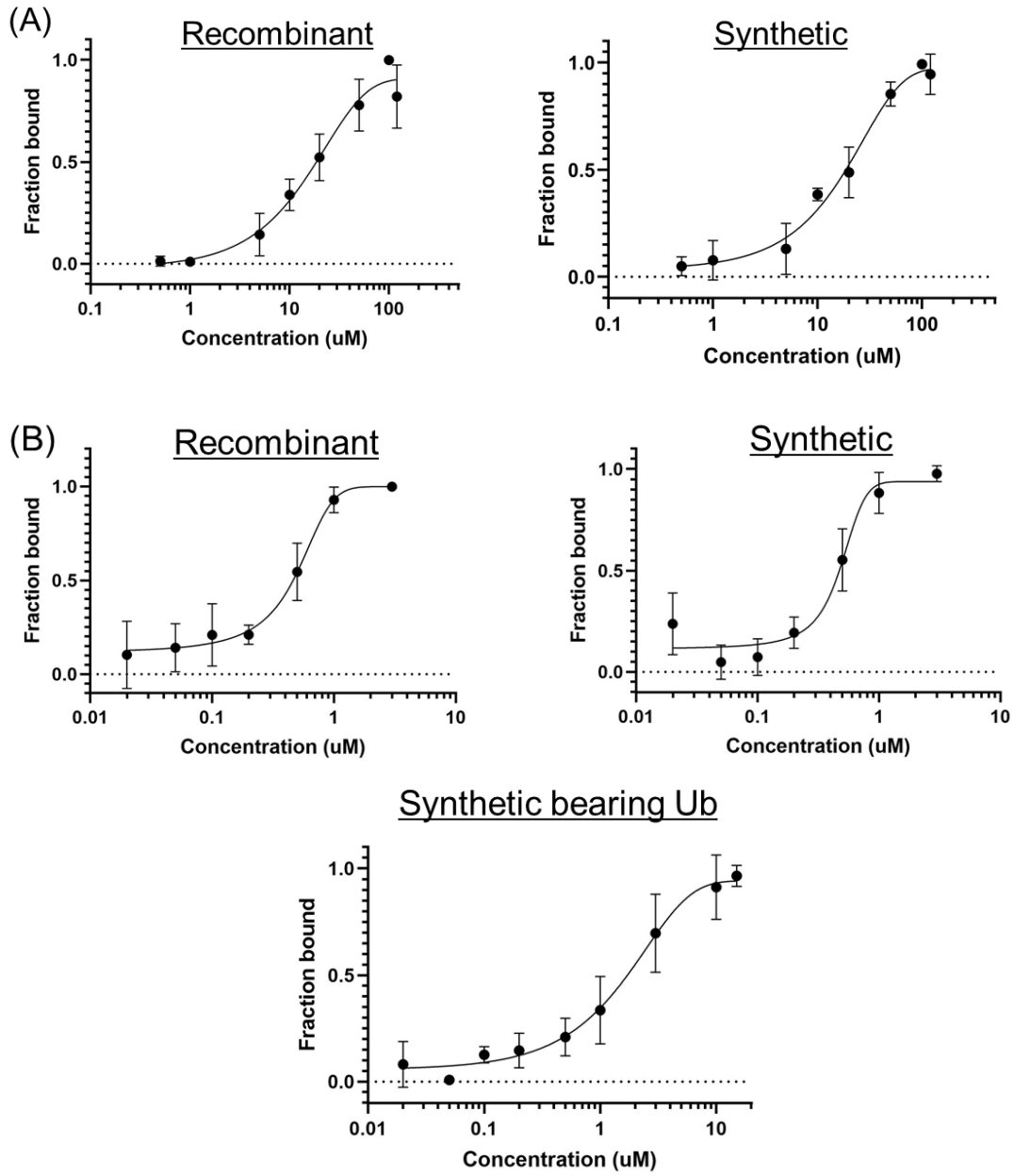
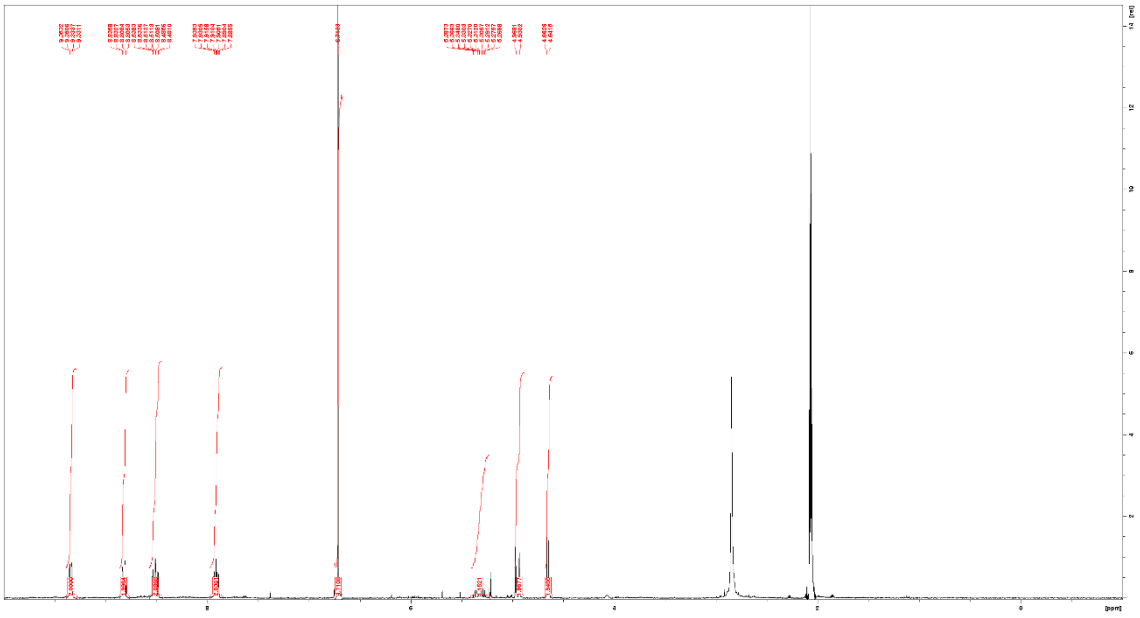
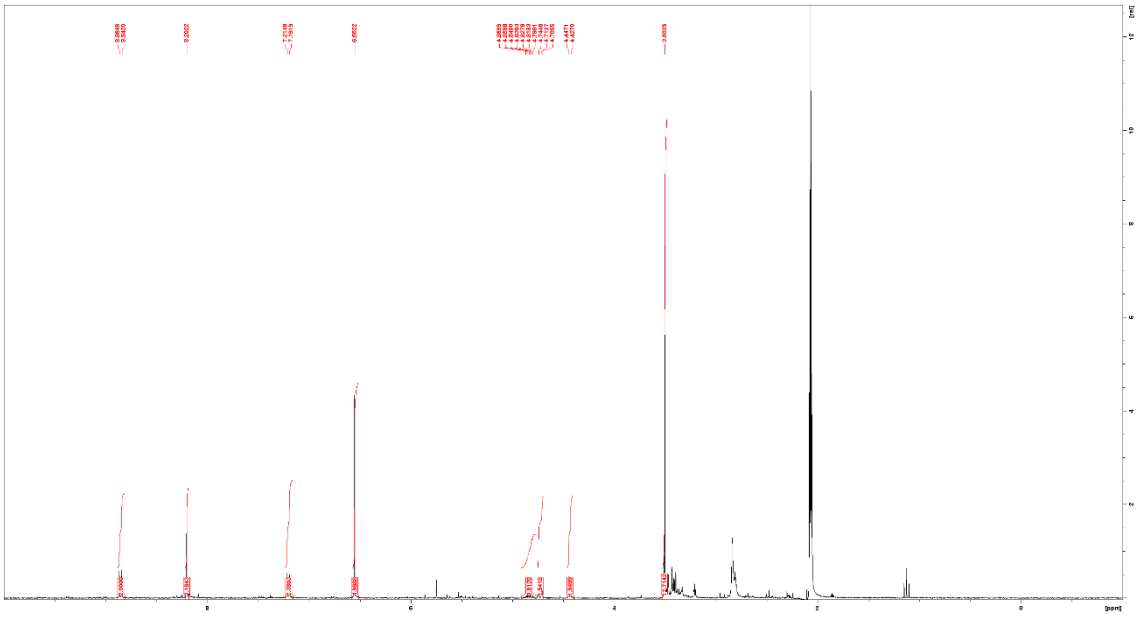


Figure S32. Measurement of fluorescence anisotropy. (A) Interaction between HP1α (**21**) and H3K9me3 peptide. (B) Interaction between HP1α or HP1α bearing Ub at K154 with Sgo1 peptide.

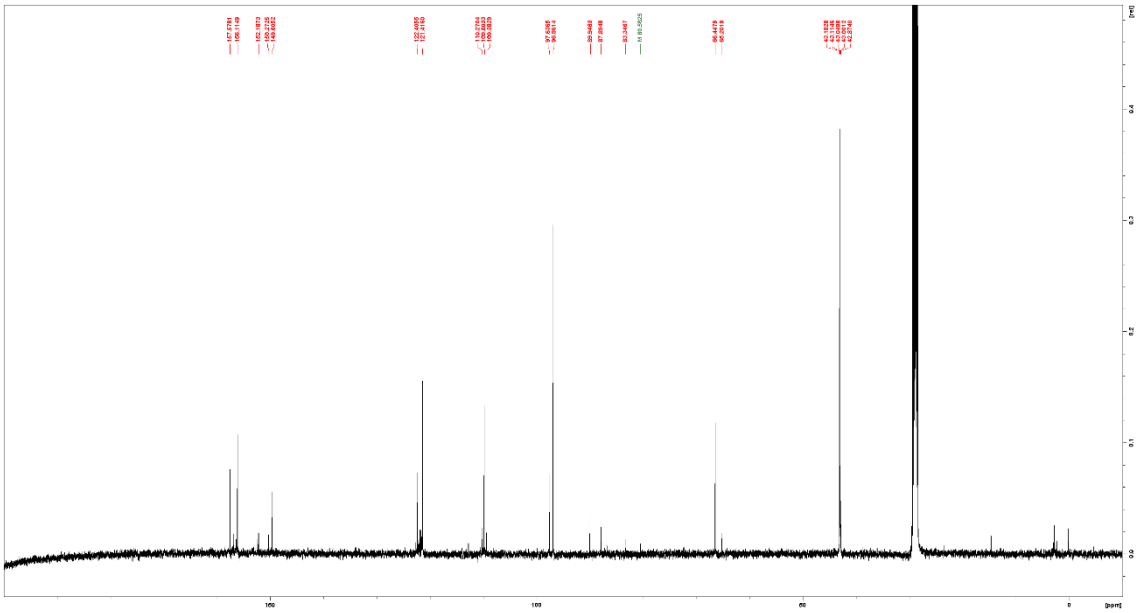
Ru-5 (^{1}H NMR, acetone-d_6, 300 MHz)



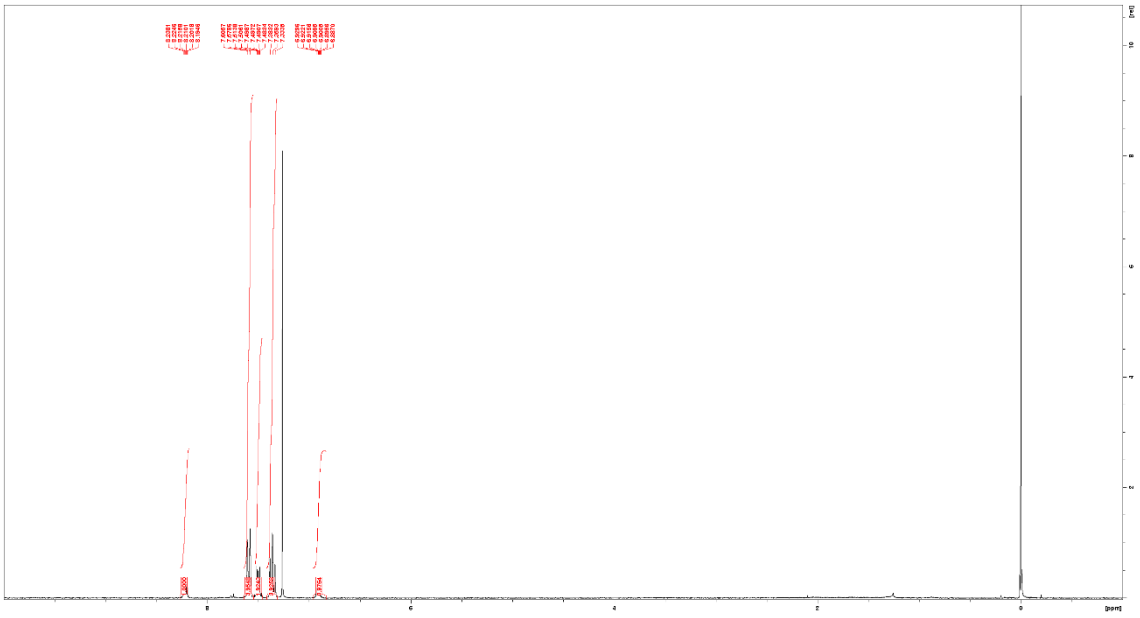
Ru-6 (^{1}H NMR, acetone-d_6, 300 MHz)



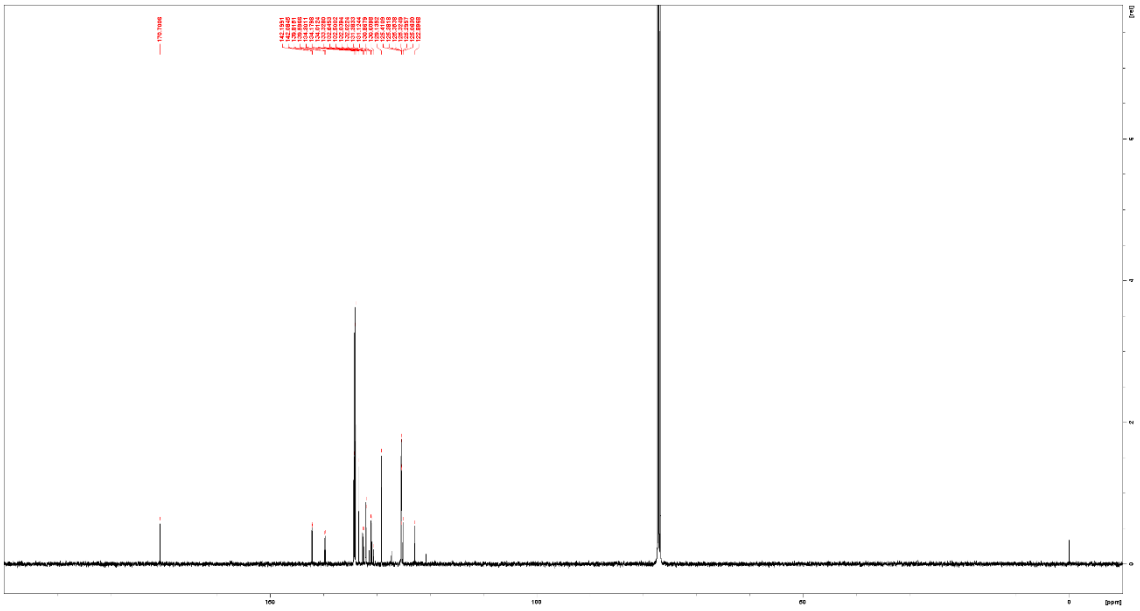
Ru-6 (^{13}C NMR, acetone-d_6, 126 MHz)



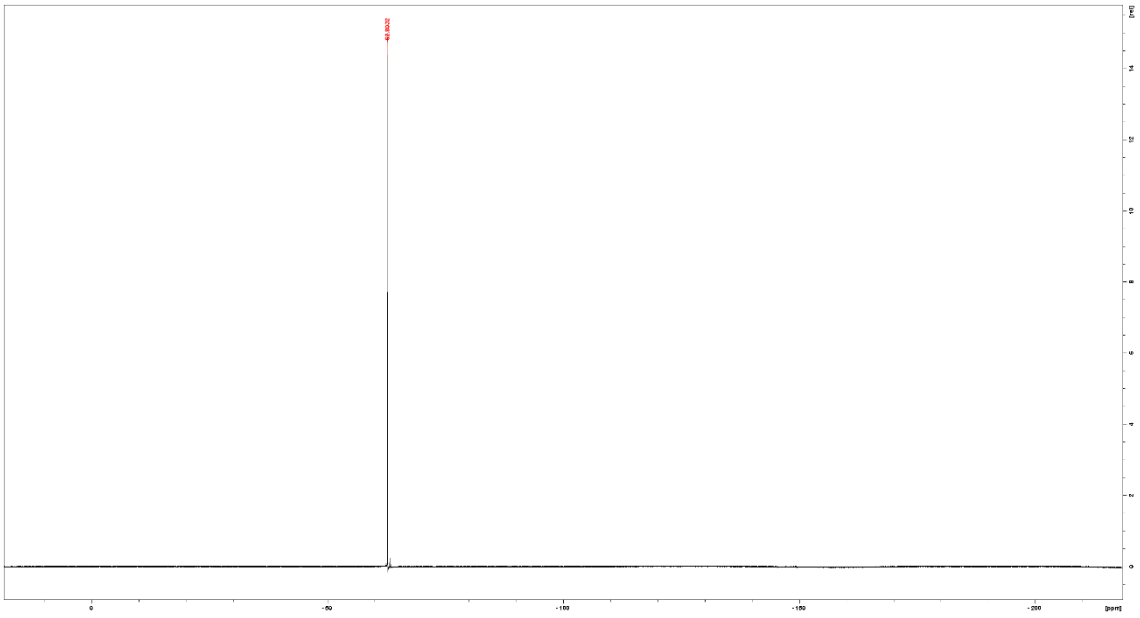
Compound S17 (^{1}H NMR, $CDCl_3$, 300 MHz)



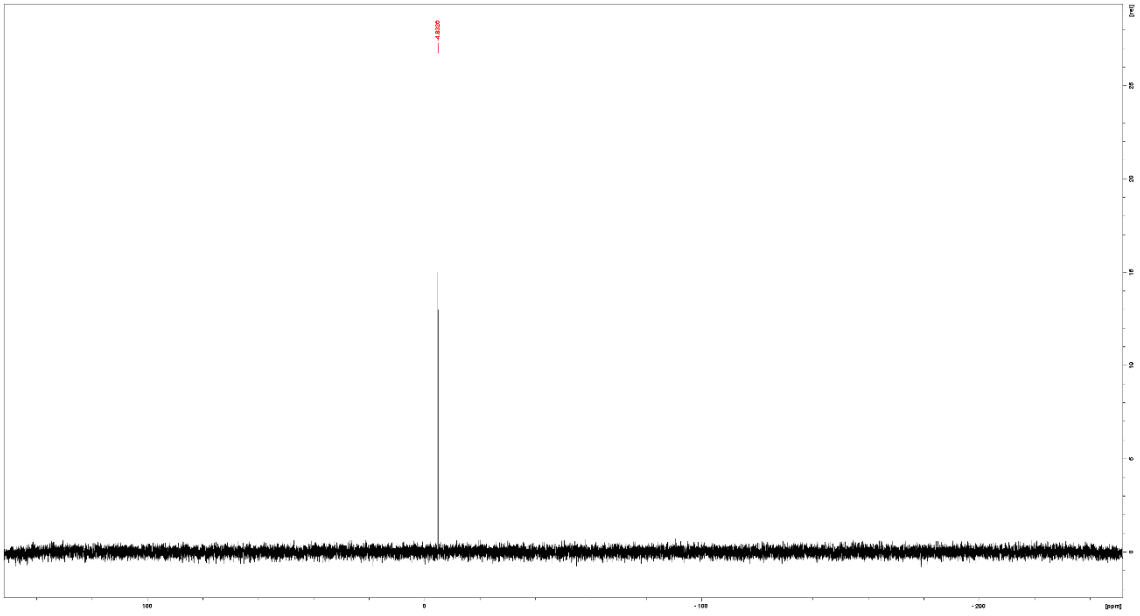
Compound S17 (^{13}C NMR, $CDCl_3$, 126 MHz)



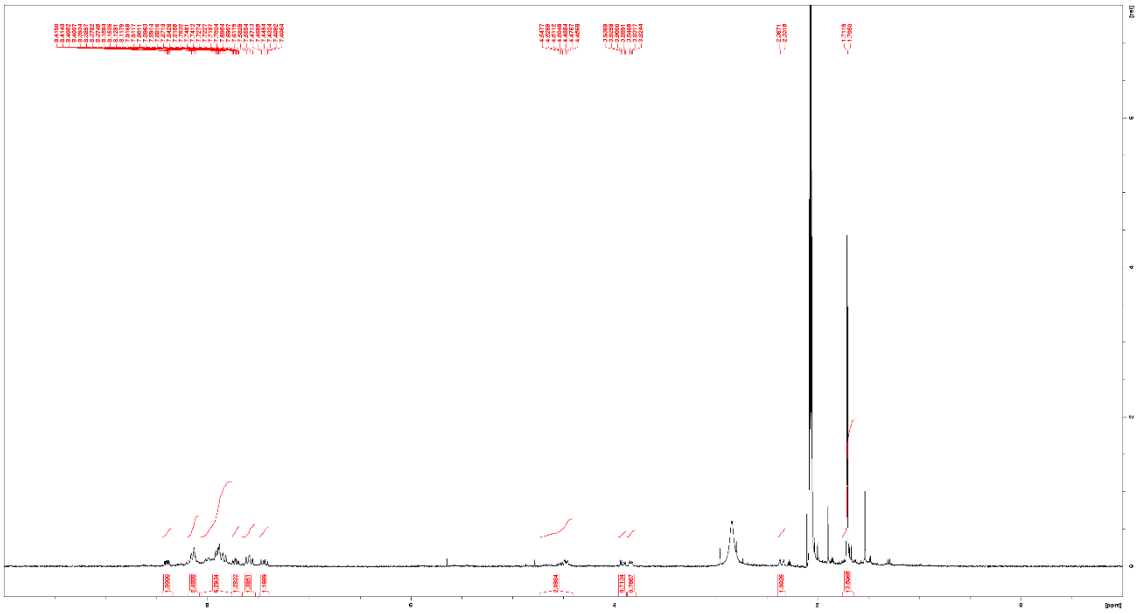
Compound S17 (^{19}F NMR, $CDCl_3$, 282 MHz)



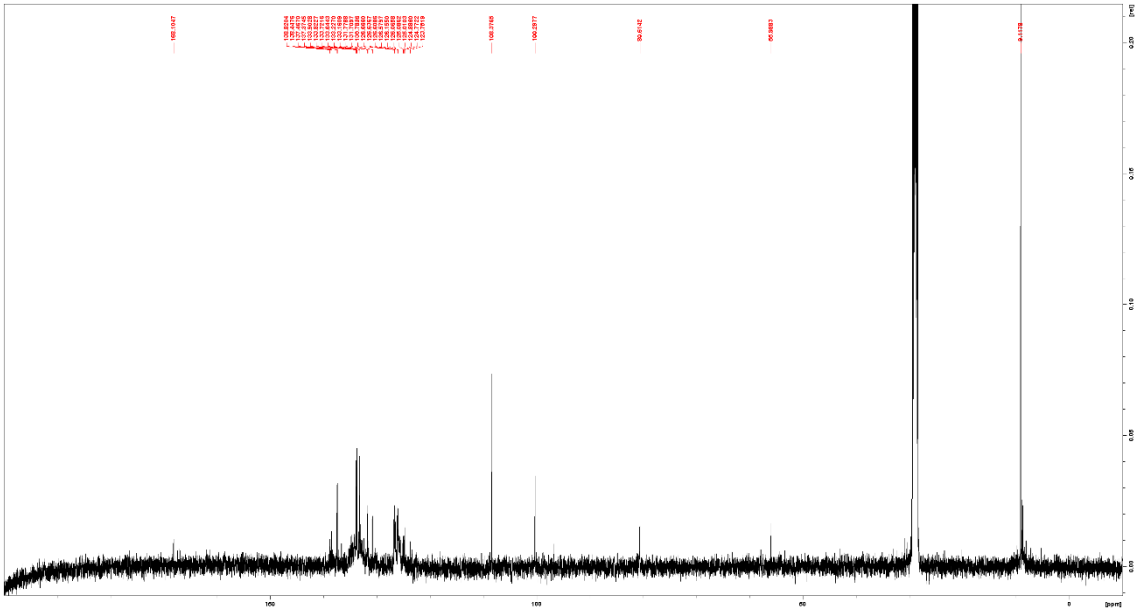
Compound S17 (^{31}P NMR, $CDCl_3$, 122 MHz)



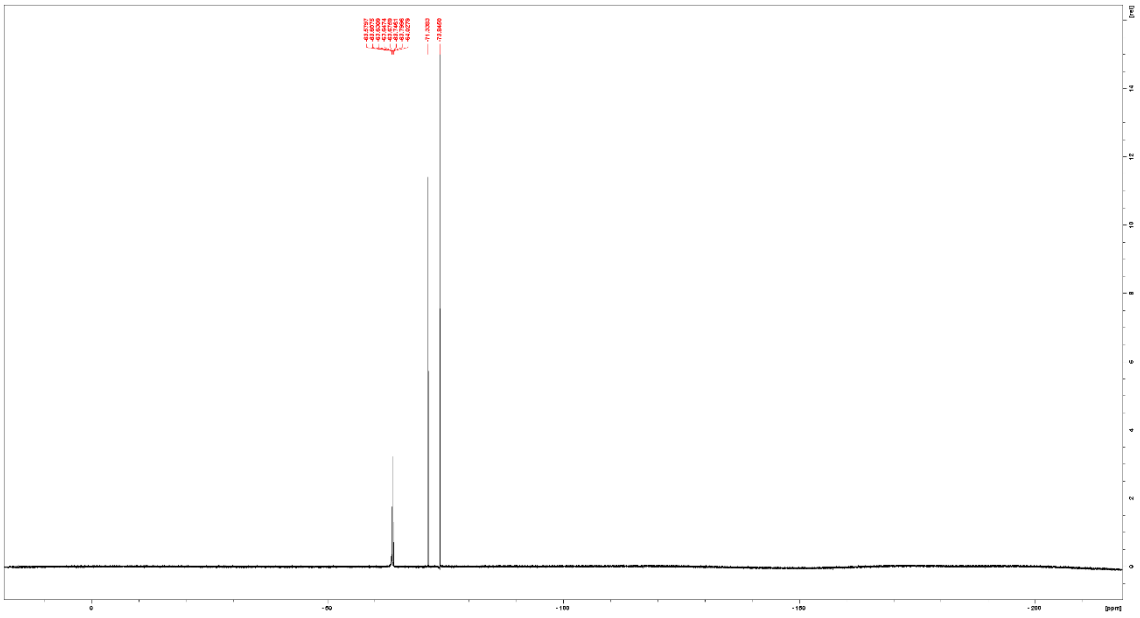
Ru-9 (^{1}H NMR, acetone-d_6, 300 MHz)



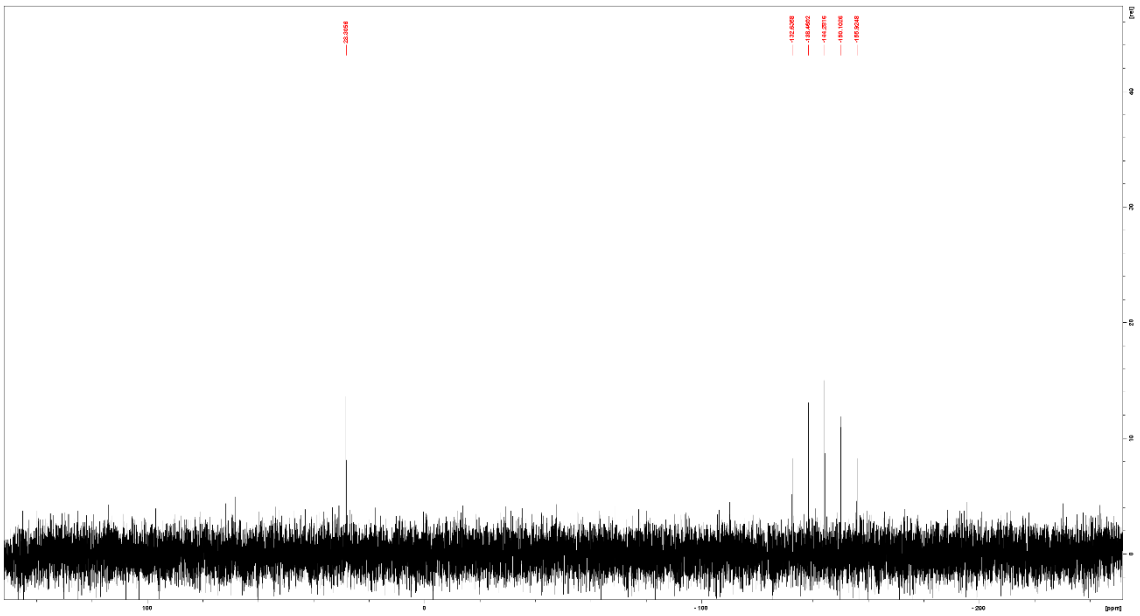
Ru-9 (^{13}C NMR, acetone-d_6, 126 MHz)



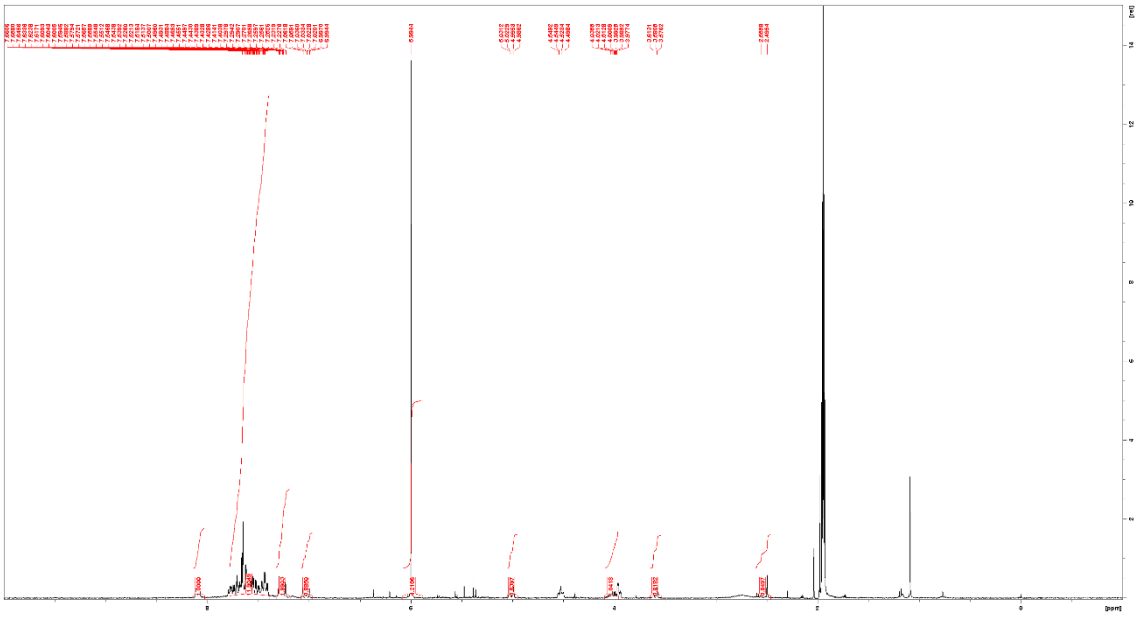
Ru-9 (^{19}F NMR, acetone-d_6, 282 MHz)



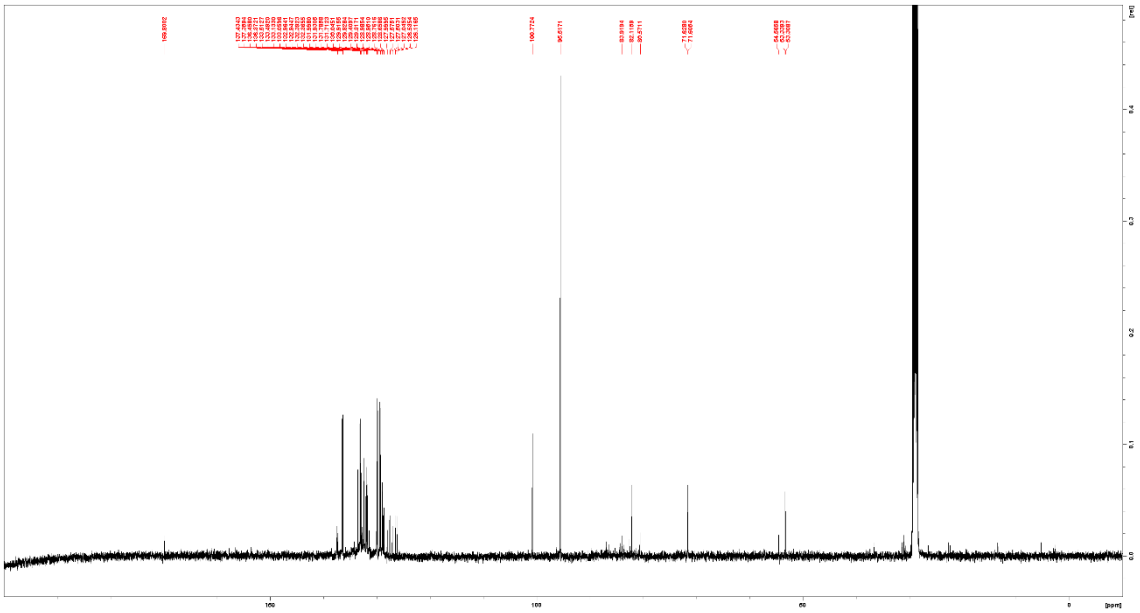
Ru-9 (^{31}P NMR, acetone-d_6, 122 MHz)



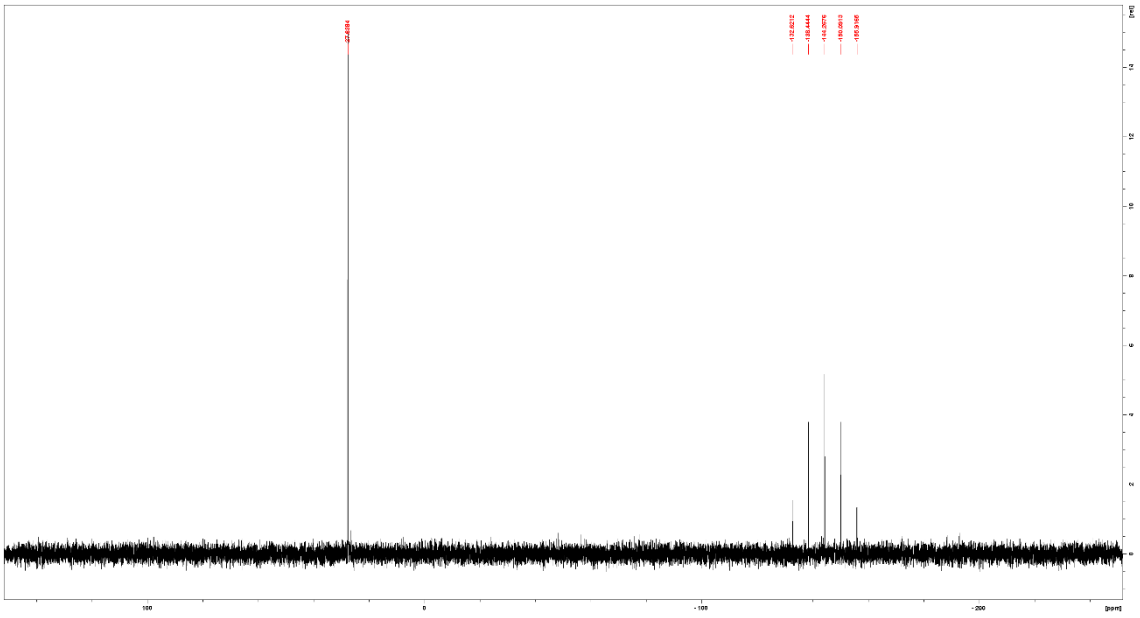
Ru-10 (^{1}H NMR, acetone-d_6, 300 MHz)



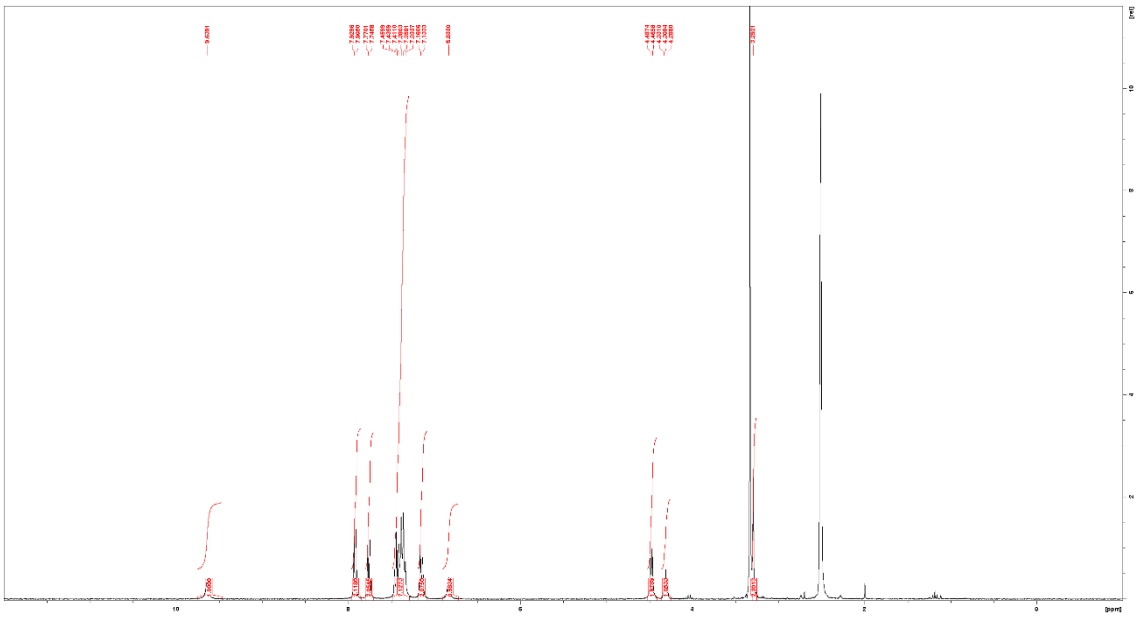
Ru-10 (^{13}C NMR, acetone-d_6, 126 MHz)



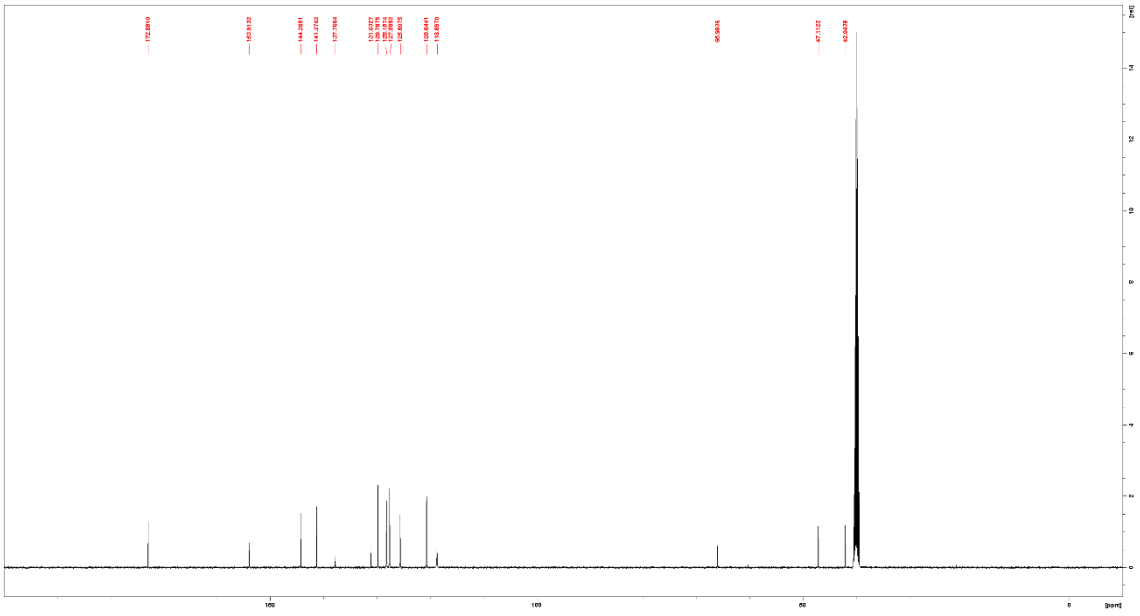
Ru-10 (^{31}P NMR, acetone-d_6, 122 MHz)



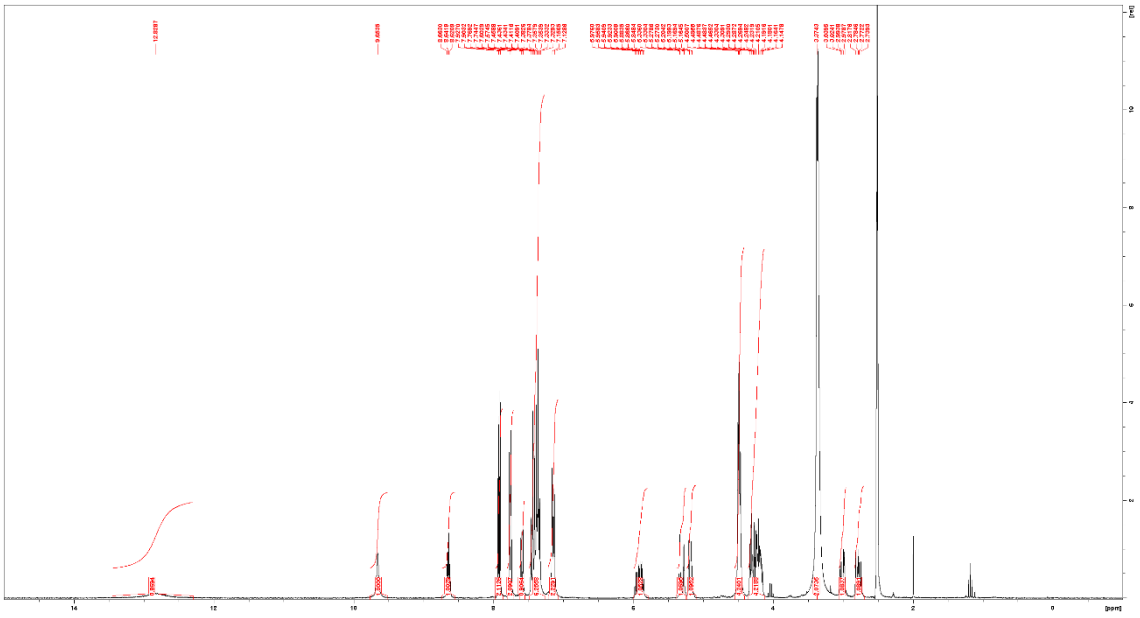
Compound S22 (^{1}H NMR, DMSO-d_6, 300 MHz)



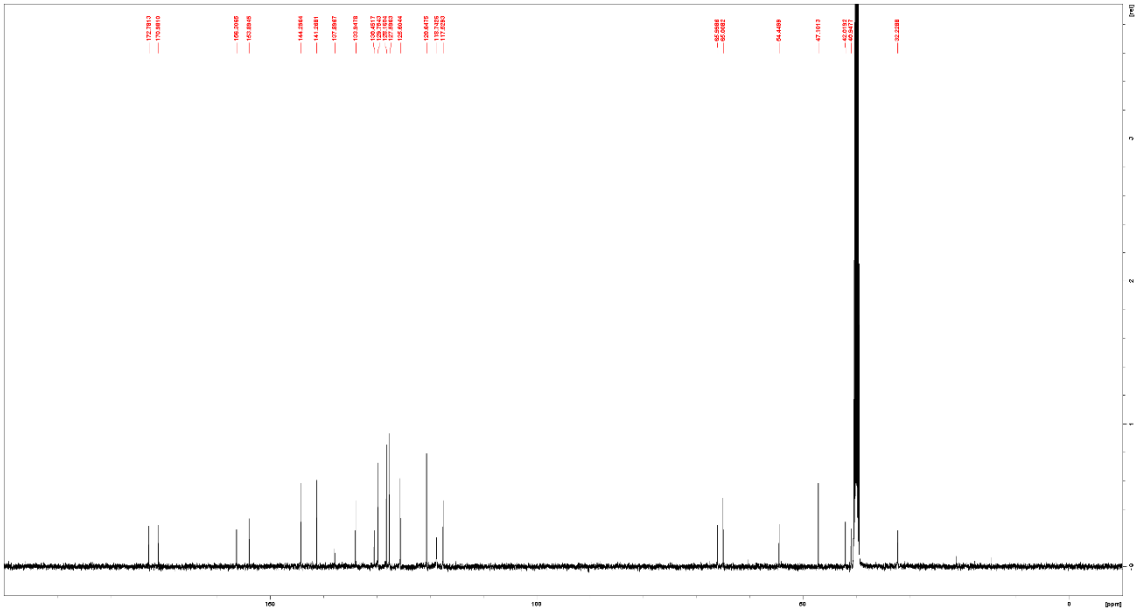
Compound S22 (^{13}C NMR, DMSO-d_6, 126 MHz)



Compound S25 (^{1}H NMR, DMSO-d_6, 300 MHz)



Compound S25 (^{13}C NMR, DMSO-d_6, 126 MHz)



DFT calculation of QA

Job type: Geometry optimization.

Method: RB3LYP

Basis set: 6-31G**

Number of shells: 73

Number of basis functions: 230

Multiplicity: 1

SCF model:

A restricted hybrid HF-DFT SCF calculation will be performed using Pulay DIIS + Geometric Direct Minimization

Solvation: water [SM8]

Optimization:

Step	Energy	Max Grad.	Max Dist.
1	-590.525813	0.029698	0.106594
2	-590.532134	0.008043	0.024179
3	-590.532893	0.003875	0.011996
4	-590.532952	0.001898	0.004050
5	-590.532970	0.000247	0.001016

DFT calculation of QA-NMe_2

Job type: Geometry optimization.

Method: RB3LYP

Basis set: 6-31G**

Number of shells: 100

Number of basis functions: 300

Multiplicity: 1

SCF model:

A restricted hybrid HF-DFT SCF calculation will be performed using Pulay DIIS + Geometric Direct Minimization

Solvation: water [SM8]

Optimization:

Step	Energy	Max Grad.	Max Dist.
1	-724.292394	0.137472	0.105205
2	-724.335718	0.106866	0.100282
3	-724.366354	0.088211	0.111854
4	-724.393167	0.072441	0.113475

5	-724.410548	0.060326	0.113555
6	-724.428910	0.046442	0.093769
7	-724.444415	0.036352	0.121898
8	-724.456513	0.026977	0.111594
9	-724.465407	0.019927	0.133102
10	-724.472134	0.011391	0.102434
11	-724.474919	0.008344	0.090615
12	-724.477477	0.006518	0.123210
13	-724.478939	0.006221	0.093506
14	-724.480363	0.005286	0.101958
15	-724.482063	0.005133	0.093561
16	-724.469583	0.048715	0.085786
17	-724.483881	0.007963	0.109682
18	-724.486474	0.006039	0.099217
19	-724.474139	0.031203	0.130148
20	-724.487199	0.011138	0.134416
21	-724.490917	0.007251	0.137198
22	-724.494362	0.005812	0.140644
23	-724.497402	0.004002	0.121377
24	-724.499927	0.004579	0.124291
25	-724.501782	0.003669	0.134096
26	-724.503240	0.005027	0.132749
27	-724.504084	0.005295	0.098214
28	-724.504888	0.004592	0.168707
29	-724.505452	0.007152	0.143357
30	-724.505929	0.005150	0.158925
31	-724.506439	0.003438	0.132142
32	-724.506856	0.003235	0.112476
33	-724.507212	0.004083	0.074020
34	-724.507444	0.005215	0.034402
35	-724.507608	0.001595	0.037918
36	-724.507643	0.001277	0.025358
37	-724.507655	0.001161	0.013170
38	-724.507664	0.001201	0.008144
39	-724.507674	0.000965	0.010364
40	-724.507687	0.001057	0.021074

41	-724.507717	0.001317	0.017961
42	-724.507744	0.001492	0.024247
43	-724.507792	0.001554	0.051033
44	-724.507908	0.002644	0.082056
45	-724.508080	0.003775	0.094440
46	-724.508290	0.004129	0.087910
47	-724.508503	0.002948	0.090551
48	-724.508653	0.002957	0.066883
49	-724.508745	0.001353	0.097099
50	-724.508802	0.003856	0.022917
51	-724.508870	0.001090	0.065423
52	-724.508927	0.001723	0.081625
53	-724.508963	0.001173	0.068229
54	-724.508981	0.003320	0.026116
55	-724.508984	0.000673	0.014943
56	-724.509004	0.000428	0.019824
57	-724.509010	0.001421	0.012098
58	-724.509014	0.001069	0.032300
59	-724.509021	0.000144	0.013948
60	-724.509023	0.000080	0.004083
61	-724.509023	0.000086	0.002028

DFT calculation of o-DPPBz

Job type: Geometry optimization.

Method: RB3LYP

Basis set: 6-31G**

Number of shells: 134

Number of basis functions: 409

Multiplicity: 1

SCF model:

A restricted hybrid HF-DFT SCF calculation will be performed using Pulay DIIS + Geometric Direct Minimization

Solvation: water [SM8]

Optimization:

Step	Energy	Max Grad.	Max Dist.
1	-1224.888197	0.017331	0.111093

2	-1224.892350	0.005579	0.148984
3	-1224.893344	0.003917	0.137561
4	-1224.894332	0.003012	0.123546
5	-1224.895474	0.003847	0.113284
6	-1224.896506	0.007044	0.119651
7	-1224.897151	0.009414	0.116471
8	-1224.897009	0.013440	0.131747
9	-1224.897879	0.009453	0.086321
10	-1224.898342	0.007791	0.131784
11	-1224.898874	0.007508	0.053191
12	-1224.899056	0.005084	0.036523
13	-1224.899198	0.003581	0.053694
14	-1224.899335	0.001795	0.043748
15	-1224.899436	0.001996	0.067468
16	-1224.899525	0.001113	0.027425
17	-1224.899556	0.000815	0.013623
18	-1224.899562	0.000217	0.001941
19	-1224.899562	0.000093	0.001037

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