

Supporting Information

Chemical synthesis of dual labeled protein via differently protected alkynes enables intramolecular FRET analysis

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Table of contents

Experimental section

Scheme S1. Synthetic route of carboxylic acid compounds containing silyl-protected alkynes (**S2a-c**).

¹H and ¹³C NMR spectra of **S2a-c**.

Figure S1. Synthesis of peptide **1a**, **1b**, and **1c**.

Figure S2. Desulfurization of peptide **1a**, **1b**, and **1c**.

Figure S3. MALDI-TOF mass spectra of prolonged desulfurization product of peptide **1a**.

Figure S4. HPLC spectra of selective deprotection of peptide **2a**, **2b**, and **2c**.

Figure S5. Synthesis of peptide **4a** and **4b**.

Figure S6. Identification of peptide **5a**, **6**, and **7** by MALDI-TOF mass spectrometry.

Figure S7. One-pot dual labeling of peptide **4b**.

Figure S8. Identification of peptide **8** and **9**.

Figure S9. Identification of peptide **10**.

Figure S10. Desulfurization of peptide **10** yields **11**.

Figure S11. Identification and Cy3 conjugation of peptide **12**.

Figure S12. UV-vis spectrum of dual dye-labeled TP1 (**13**).

Experimental Section

General methods and materials

MALDI-TOF mass spectra were recorded with microflex (BRUKER), using Protein Calibration Standard 2 as an external standard. Reversed-phase HPLC was performed on a 5C₁₈-AR-II, 5C₁₈-AR-300 and Protein-R column (Nacalai tesque, 4.6ID and 10ID × 250 mm for analysis and purification, respectively) with a PU-2080 plus Intelligent HPLC Pump (JASCO) and MD-2018 plus Photodiode Array Detector (JASCO) at 195 to 650 nm. All solvents and reagents were commercially available and used without further purification. All peptides were synthesized using Intavis ResPep SL (Intavis). Amino acids protected by 9-fluorenylmethoxycarbonyl (Fmoc) group were coupled with *O*-(1*H*-Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) as activator and 4-methylmorpholine (NMM) as base. The isolated yields of each peptide were estimated by using the molecular weights of TFA salt at Arg and Lys positions.

Synthesis of compound S1a

Transient TMS-protection of propargyl amine has been done according to the previous report (P. Wipf, Y. Aoyama, T. E. Benedum, *Org. Lett.*, 2004, 6, 3593-3595). Briefly, a mixture of propargylamine (1.65 g, 30 mmol), chlorotrimethylsilane (7.58 mL, 60 mmol), and Et₃N (12.5 mL, 90 mmol) in 50 mL of CH₂Cl₂ was stirred at rt for 21 h, quenched with water (60 mL), and extracted with CHCl₃ (30 mL, twice). The combined organic layers were washed with brine (90 mL), dried by MgSO₄, and concentrated under reduced pressure to give 1,1,1,3,3,3-hexamethyl-2-prop-2-ynyldisilazane (3.25 g, 54%). ¹H NMR (600 MHz, CDCl₃) δ 3.56 (d, 2H), 2.17 (t, 1H), 0.18 (s, 18H).

The compound **S1a** was synthesized by following previous protocols (I. E. Valverde, A. F. Delmas, V. Aucagne, *Tetrahedron*, 2009, 65, 7597-7602). A solution of TMS-protected propargyl amine (1,1,1,3,3,3-hexamethyl-2-prop-2-ynyldisilazane, 0.78 g, 3.91 mmol) in anhydrous THF (7 mL) was cooled to -78°C, and *n*-BuLi was added dropwise (2.44 mL, 1.6 M in hexanes, 3.91 mmol, 1.0 equiv). The solution was allowed to stir at -78°C for 15 min, then was warmed to 0 °C and a solution of DMES-Cl (7.83 mmol, 2 equiv) in anhydrous THF (1.1 mL) was added dropwise. The reaction mixture was stirred for 2 h at rt then quenched with a 1 M aqueous HCl solution (20 mL). Its pH

was adjusted to 9–10 with an aqueous 6 M NaOH aq. The aqueous layer was extracted with EtOAc (3×10 mL), the combined organic fractions were dried over Na₂SO₄, filtered and concentrated in reduced pressure and the residue was purified by column chromatography to give **S1a** as an orange oil (0.45 g, 57%). ¹H NMR (600 MHz, CDCl₃) δ 3.46 (s, 2H), 7.75 (s, 1H), 1.01 (t, 3H), 0.61 (q, 2H), 0.15 (s, 6H).

Synthesis of compound **S1b**

The compound **S1b** was synthesized by following previous protocols (I. E. Valverde, A. F. Delmas, V. Aucagne, Tetrahedron, 2009, 65, 7597-7602). Briefly, a solution of propargylamine (283 mg, 5.1 mmol) in anhydrous THF (8 mL) was cooled to -78°C. *n*-BuLi (3.1 mL, 1.6 M in hexanes, 5.1 mmol, 1.0 equiv) was added dropwise. The solution was allowed to stir for 15 min at 78°C, then was warmed to 0°C and TES-Cl (1.0 mL, 6.0 mmol, 1.2 equiv) was added dropwise. The reaction mixture was stirred for 4 h at rt then quenched with an aqueous NH₄Cl solution (12 mL). After removal of THF by evaporation under vacuum, the aqueous slurry was diluted with 12 mL of a 1 M HCl solution. The aqueous phase was extracted with EtOAc (3 × 10 mL). The combined organic fractions were dried over Na₂SO₄, filtered and concentrated in vacuo to give **S1b** as an orange oil (873 mg, quant.). ¹H NMR (600 MHz, CDCl₃): δ 3.84 (s, 2H), 1.01 (m, 9H), 0.63 (m, 6H). ESI-MS: *m/z*=339.26(calcd): 339.3395[2M+H]⁺ (found), *m/z*=361.25 (calcd) , 361.3360[2M+K]⁺ (found).

Synthesis of compound **S1c**

The synthesis of TMS-protected propargyl amine (1,1,1,3,3,3-hexamethyl-2-prop-2-ynyldisilazane) was done as described above. A solution of 1,1,1,3,3,3-Hexamethyl-2-prop-2-ynyldisilazane (1.00 g, 5.01 mmol) in anhydrous THF (10 mL) was cooled to -78 °C, *n*-BuLi was added dropwise (3.13 mL, 1.6 M in hexanes, 5.01 mmol, 1.0 equiv). The solution was allowed to stir at -78 °C for 15 min, then was warmed to 0 °C and a solution of TBS-Cl (10.0 mmol, 2 equiv) in anhydrous THF (2.0 mL) was added dropwise. The reaction mixture was stirred for 4 h at rt then quenched with a 1 M aqueous HCl solution (30 mL). Then the water layer was extracted by EtOAc (3×10 mL). Its pH was adjusted to 9–10 with an aqueous 6 M NaOH aq. The aqueous layer was extracted with EtOAc (3×30 mL), the combined organic fractions were dried over Na₂SO₄, filtered and concentrated in vacuum to give **S1c** as an orange oil (0.38 g, 45%).

¹H NMR (600 MHz, CDCl₃) δ 3.46 (s, 2H), 0.95 (s, 9H), 0.12 (s, 6H).

Synthesis of 4-oxo-4-(3-(dimethylethylsilyl)prop-2-yn-1-ylamino)butanoic acid (S2a)

The procedure was followed as described in the previous report (S. Loison, et al., J. Med. Chem., 2012, 55, 8588-8602). A solution of succinic anhydride (73.7 mg, 0.74 mmol), compound **S1a** (86.7 mg, 0.61 mmol) and 4-DMAP (75.0 mg, 0.61 mmol) in CH₂Cl₂ (3 mL) was stirred for 1 h at r.t.. The aqueous layer was acidified with a 1 N HCl solution (10ml) and was extracted with EtOAc (3 × 10mL), dried under Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography to give **S2a** as a white powder (78.0 mg, 53%). ¹H NMR (600 MHz, CDCl₃) δ 5.84 (bs, 1H), 4.12 (d, 2H), 2.76 (t, 2H), 2.56 (t, 2H), 1.01 (t, 3H), 0.61 (q, 2H), 0.16 (s, 6H); ESI-MS: *m/z*=505.21 (calcd): 505.3195 [2M+Na]⁺ (found), *m/z*=521.18 (calcd) : 521.2949 [2M+K]⁺ (found).

Synthesis of 4-oxo-4-(3-(triethylsilyl)prop-2-yn-1-ylamino)butanoic acid (S2b)

The procedure was followed as described above. A solution of succinic anhydride (252 mg, 2.52 mmol), compound **S1b** (326 mg, 1.93 mmol) and 4-DMAP (200 mg, 2 mmol) in CH₂Cl₂ (5 mL) was stirred for 2h at room temperature. Then, the mixture was washed with a solution of 5% Na₂CO₃. The aqueous layer was acidified with a 1 N HCl solution and was extracted with AcOEt(3 × 5 mL), dried under Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography to give **S2b** as a white powder (193 mg, 38%). ¹H NMR (600 MHz, CDCl₃): δ 5.85 (bs, 1H), 4.12 (d, 2H), 2.76-2.55 (m, 4H), 2.12 (s, 1H) 1.01 (t, 9H) 0.63 (q, 6H); ESI-MS: *m/z*=270.42(calcd): 270.2193 [M+H]⁺ (found), *m/z*=292.42 (calcd) : 292.2058[M+Na]⁺ (found), *m/z*=308.42 (calcd) , 308.1820[M+K]⁺ (found).

Synthesis of 4-oxo-4-(3-(t-butyl-dimethylsilyl)prop-2-yn-1-ylamino)butanoic acid (S2c)

The procedure was followed as described above. A solution of succinic anhydride (247 mg, 2.46 mmol), compound **S1c** (348 mg, 2.05 mmol) and DMAP (250 mg, 2.05 mmol) in CH₂Cl₂ (8 mL) was stirred for 2 h at r.t.. The aqueous layer was acidified with a 1 N HCl solution (3×10 ml) and was extracted with EtOAc (3 × 10 mL), dried under Na₂SO₄

and concentrated *in vacuo*. The residue was purified by column chromatography to give compound **S2c** as a white powder (462 mg, 84%). ¹H NMR (600 MHz, CDCl₃) δ 5.84 (bs, 1H), 4.12 (d, 2H), 2.75 (t, 2H), 2.57 (t, 2H), 0.95 (s, 9H), 0.13 (s, 6H); ESI-MS: *m/z*=292.13 (calcd): 292.1865 [M+Na]⁺ (found), *m/z*=308.10 (calcd) : 308.1565 [M+K]⁺ (found), *m/z*=561.27 (calcd) : 561.3665 [2M+Na]⁺ (found), *m/z*=577.24 (calcd) : 577.3375 [2M+K]⁺ (found).

Synthesis of peptide 1a-1c

TentaGel Resin (0.23 mmol/g, 15 μmol scale, HiPep Lab.) was used for C-terminal amide peptides. After automated SPPS, coupling of alkyne derivatives to the N-terminal of the peptide was conducted with compounds **S2a-c** (4 eq), HBTU (3.8 eq) and DIEA (8 eq). The mixture was rotated at room temperature for 1h. Then the resin was washed by DMF and CH₂Cl₂. The peptide was cleaved by cleavage cocktail (95% TFA, 2.5% H₂O, 2.5% TIPS). The mixture was rotated at room temperature for 2 h, and filtered to remove the resin. Then ether (8 mL) was added, vortexed and centrifuged 5,000 g at room temperature for 1min. Ether was decanted and washed with ether three times. Obtained crude peptides were purified by RP-HPLC and identified by MALDI-TOF mass spectrometry. The isolated yields of peptide **1a**, **1b**, and **1c** were 19, 25, 22%, respectively.

Desulfurization of peptide 1a-1c

The reaction condition was referred to the previous study (Q. Wan, S. J. Danishefsky, *Angew. Chem. Int. Ed.*, 2007, 46 9248-9252). To the peptide solution (0.37 μmol in 200 μL of degassed water), TCEP-HCl solution (0.5 M, 200 μL) adjusted to pH7, *t*-BuSH (*v/v* = 5%, 22 μL) and VA-044 (0.1 M, 10 μL) were added. The reaction mixture was stirred at 37°C, and the reaction was monitored by HPLC. The products were identified by MALDI-TOF mass spectrometry.

Selective deprotection of silyl-protected alkynes on peptide 2a, 2b and 2c

A solution of 0.1 M KF aq and/or 0.1 M AgNO₃ aq were added to peptide (0.10–0.25 μmol) solution in water and this solution was adjusted so that the concentration of peptide was 1 mM. Then, this mixture was agitated at the respective temperature. The reaction was monitored by HPLC and the products were identified by MALDI-TOF

mass spectrometry.

Synthesis of peptide 4a and 4b

TentaGel Resin (0.23 mmol/g, 10 μ mol scale) was used for amide-terminal peptides. After automated SPPS, coupling of silyl-protected alkyne to the N-terminal of the peptide **S3** was conducted with compound **S2b** or **S2c** (4 eq), HBTU (3.8 eq) and DIEA (8 eq). The mixture was rotated at room temperature for 1h. Then the resin was washed by DMF and CH_2Cl_2 . To deprotect Dde-protected lysine of peptide **S4a** and **S4b**, hydrazine monohydrate (3% in 500 μ L DMF) was added to the resin. Then, the mixture was agitated at r.t. for 10 min and DMF was drained. This operation was repeated three times. Then, the resin was washed by DMF and CH_2Cl_2 . DMES-alkyne carboxylic acid **S2a** (4 eq) was coupled to the side-chain amino group with HBTU (3.8 eq) and DIEA (8 eq). Peptide was cleaved by cleavage cocktail (95% TFA, 2.5% H_2O , 2.5% TIPS). The mixture was rotated at room temperature for 2 h, and filtered to remove the resin. Then ether (8 mL) was added, vortexed and centrifuged 5,000 g at r.t. for 1min. Ether was decanted and this operations was repeated three times. Obtained crude peptide **4a** and **4b** were purified by RP-HPLC and identified by MALDI-TOF mass spectrometry.

One-pot DMES removal and CuAAC

To a solution of peptide **4a** or **4b** in $\text{H}_2\text{O}/\text{tBuOH}$ (8:2) was added azido-(PEG)₃-NH₂ (150 mM), CuSO_4 (20 mM), THPTA (50 mM). Then, the solution was degassed by Argon bubbling. To the mixture solution were added amino guanidine chloride (500 mM), sodium ascorbic acid (200 mM) and potassium fluoride (100 mM). The final concentration of the reaction mixture was peptide (1 mM), azido-(PEG)₃-NH₂ (4 mM for **4a** and 1 mM for **4b**), CuSO_4 (1 mM), THPTA (5 mM), sodium ascorbic acid (10 mM) and KF (2 mM for **4a** and 1.5 mM for **4b**). The reaction was conducted at 37°C and the product was confirmed by RP-HPLC and MALDI-TOF mass spectrometry.

Deprotection of TBS and click reaction in one-pot

0.1 M KF aq or 0.1 M AgNO_3 aq were added to peptide **5a** solution in water and the concentration was adjusted to peptide (1 mM), AgNO_3 (10 mM) and KF (10 mM). Then, the mixture was agitated at 37°C and the product was monitored and identified by RP-HPLC and MALDI-TOF mass spectrometry, respectively. After adding NaCl to make

precipitation of AgCl, the solution was diluted by milliQ. To the solution were added 5-azidovaleric acid (150 mM), CuSO₄ (50 mM), THPTA (50 mM). Then, the solution was degassed by Argon bubbling. To the mixture solution were added amino guanidine (500 mM), sodium ascorbic acid (200 mM). The final concentration of the reaction mixture was peptide (0.5 mM), 5-azidovaleric acid (5 mM), CuSO₄ (2 mM), THPTA (5 mM), sodium ascorbic acid (15 mM) and amino guanidine (15 mM). The reaction was conducted at 37°C and the product was confirmed by RP-HPLC and MALDI-TOF mass spectrometry.

Synthesis of doubly dye-labeled TP1 protein

1) Synthesis of peptide fragment 8

To prepare the C-terminal hydrazide peptide, 2-Cl-(Trt)-Cl resin (50 μmol scale) was used. The preparation procedure was followed as described in previous report (J. Zheng, S. Tang, Y. Qi, Z. Wang, L. Liu, Nat. Prot., 2014, 8, 2483-2495). Briefly, the resin was swelled in 50% DCM/DMF for 50 min. After removing the solvent, 5% hydrazine in DMF (400 μL) was added to the resin and the mixture was agitated for 1 h at RT then the solvent was drained and washed by DMF. This operation was conducted again, and the resin washed by DMF, DCM and DMF. Next, 5% MeOH/DMF (400 μL) was added and stirred for 10 min. After removing the solvent, the resin was washed by DMF, DCM and DMF. Immediately, to the resin were added Fmoc-Arg(Pbf)-OH (4 eq), HBTU (3.8 eq) and DIEA (8 eq). The mixture was stirred for 60 min, and then washed by DMF, DCM and DMF three times respectively. After manual SPPS, coupling of DMES-protected alkyne to the N-terminal of the peptide was conducted with compound **S2a** (4 eq), HBTU (3.8 eq) and DIEA (8 eq). The mixture was rotated at r.t. for 1h. Then the resin washed by DMF and CH₂Cl₂.

2) Synthesis of peptide fragment 9

Tenta Gel Resin (0.23 mmol/g, 10 μmol scale) was used for the synthesis of peptide **9**. After coupling of K(Dde) to the C-terminal, to the resin added the solution of NH₂OH · HCl (4.5 eq) and imidazole (3.4 eq) in NMP/DMF=5/1. The mixture were agitated for 1 h at RT and then drained, washed by DCM. This operation was conducted again and the deprotection of Dde group was confirmed by Kaiser Test. TBS-alkyne carboxylic acid **S2c** was coupled to the side-chain amino group with HBTU (3.8 eq) and

DIEA (8 eq), and then automated SPPS was continued. After SPPS, peptide was cleaved by cleavage cocktail (95% TFA, 2.5% H₂O, 2.5% TIPS). The mixture was rotated at room temperature for 2 h, and filtered to remove the resin. Then ether (8 mL) was added, vortexed and centrifuged 5,000 g at room temperature for 1 min. Ether was decanted and washed with ether three times. Obtained crude peptides were purified by RP-HPLC and identified by MALDI-TOF mass spectrometry.

3) Native chemical ligation

To the solution of peptide **8** in 6 M Gn-HCl and 0.2 M NaH₂PO₄ (pH 3.0) was added the solution of NaNO₂ (10 eq) in water under -15°C, and the mixture was stirred for 15 min. To the mixture were added the solution of peptide **9** (1.2 eq) and MPAA (40 eq) in 6 M Gn-HCl and 0.2 M NaH₂PO₄ (pH 6.5) and the value of pH of the mixture was adjusted to pH 6.9-7.0 and the reaction mixture was stirred for 4 h at r.t.. The reaction was monitored by RP-HPLC, and the ligation product (peptide **10**) was identified by MALDI-TOF mass spectrometry.

4) Desulfurization

Peptide **10** was dissolved in 500 mM TCEP in denaturing solution (6 M Gn-HCl, 0.2 M NaH₂PO₄ pH 6–7). To the solution were added tBuSH and 100 mM VA-044aq. Final concentration of the mixture was peptide **10** (1 mM), TCEP (200 mM), tBuSH (5% v/v) and VA-044 (2 mM). The reaction mixture was placed at 37°C. The product (peptide **11**) was confirmed by RP-HPLC and MALDI-TOF mass spectrometry.

5) DMES deprotection and Cy5 conjugation in one-pot

To a solution of peptide **11** in H₂O/*t*-BuOH (6:4) were added Cy5-azide (50 mM, Sigma), CuSO₄ (50 mM), THPTA (100 mM). Then, the solution was degassed by argon bubbling. To the mixture solution were added amino guanidine chloride (1 M), sodium ascorbic acid (200 mM) and KF (100 mM) sequentially. The final concentration of the reaction mixture is shown as follows; peptide **11** (1 mM), Cy5-azide (1.5 mM), CuSO₄ (1.5 mM), THPTA (5 mM), sodium ascorbic acid (20 mM), KF (2 mM) and amino guanidine chloride (20 mM). The reaction was conducted at 30°C and the product peptide **12** was confirmed by RP-HPLC and MALDI-TOF mass spectrometry.

6) TBS deprotection and Cy3 conjugation in one-pot

0.1 M KF aq or 0.1 M AgNO₃ aq were added to peptide **12** (0.10–0.25 μmol) solution in water and the concentration was adjusted; peptide (1 mM), AgNO₃ (10 mM) and KF (10 mM). Then, the mixture was stirred at 37°C for 1 h. The reaction was quenched by addition of NaCl, which can trap silver cation as AgCl salt.

To the supernatant were added Cy3-azide (50 mM, Sigma), CuSO₄ (50 mM), THPTA (100 mM). Then, the solution were degassed by Argon bubbling. To the mixture solution were added amino guanidine (1 M), sodium ascorbic acid (200 mM). The final concentration of the reaction mixture is as follows; peptide (0.5 mM), Cy3-azide (5 mM), CuSO₄ (1 mM), THPTA (5 mM), sodium ascorbic acid (20 mM) and amino guanidine (20 mM). The reaction was conducted at 37°C and the product peptide **13** was confirmed by RP-HPLC and MALDI-TOF mass spectrometry.

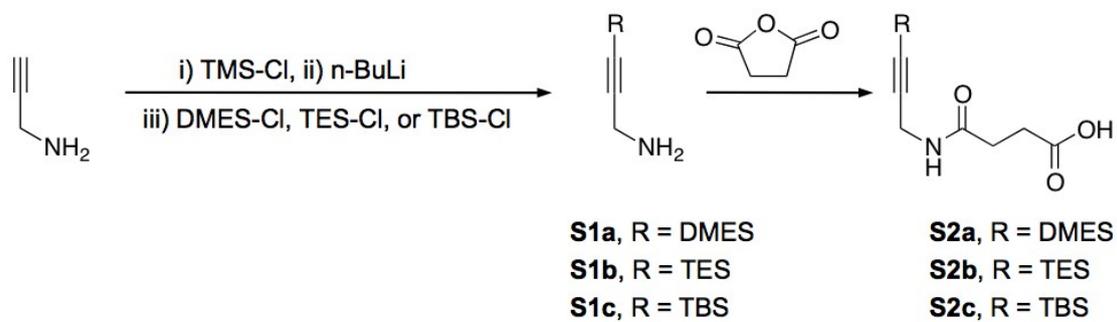
UV-Vis measurements of Cy3-Cy5-labeled TP1

UV-Vis spectrum was recorded on Shimadzu UV-2550 UV-Visible spectrophotometer. The spectrum was measured in 1xPBS (gibco, 1 mM KH₂PO₄, 155 mM NaCl, 3 mM Na₂HPO₄, pH 7.4) using a cell with a 1 cm path length. The sample contains 5 μM labeled TP1 (**13**) and 6 M Gn-HCl.

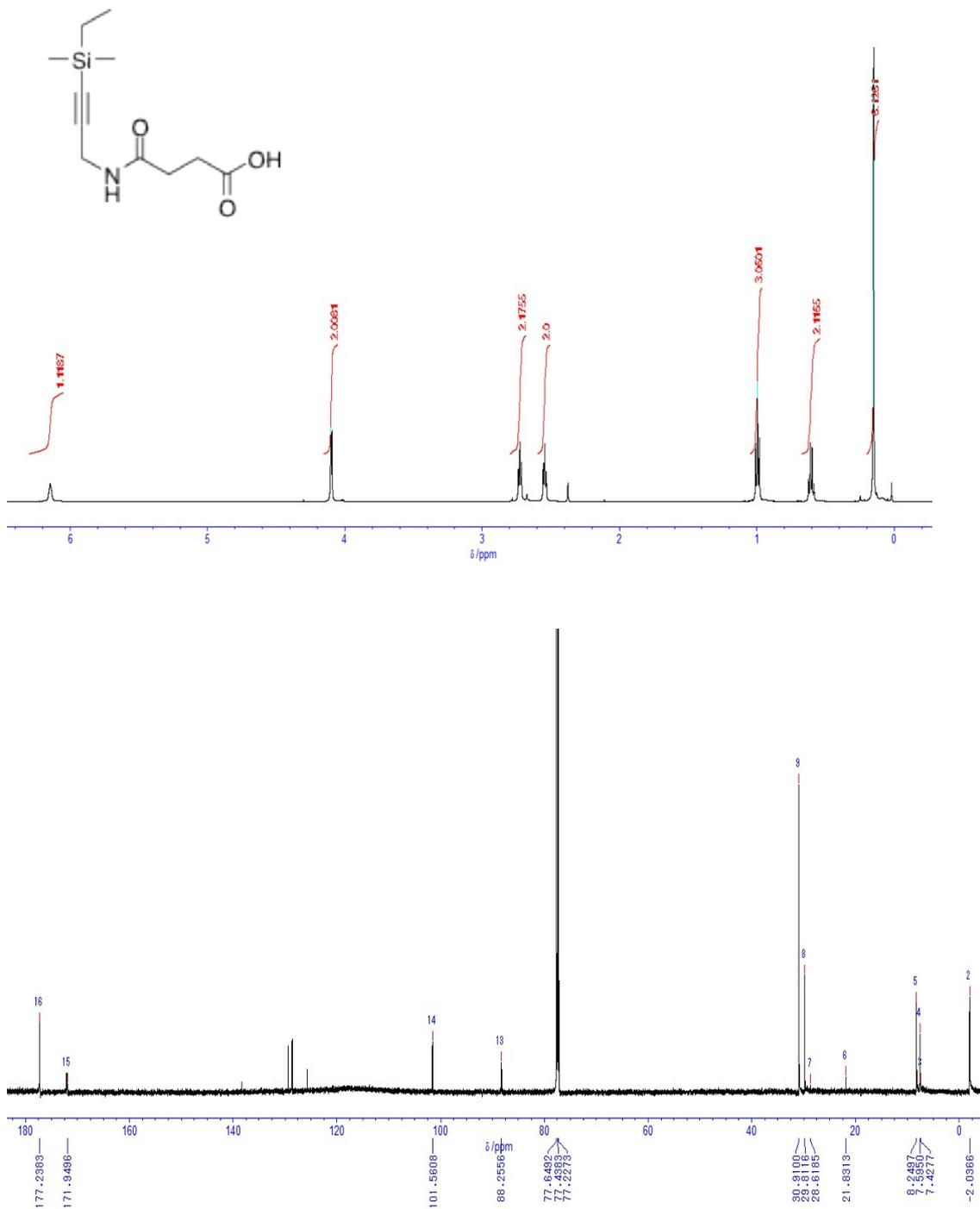
Fluorescence measurements of Cy3-Cy5-labeled TP1

Fluorescence spectrum was recorded on Shimadzu RF-5300PC spectrofluorophotometer. The spectrum was measured in 1xPBS (gibco, 1 mM KH₂PO₄, 155 mM NaCl, 3 mM Na₂HPO₄, pH 7.4) using a cell with a 1 cm path length. The samples contain 2 μM labeled TP1 (**13**) and 0–9 M urea in the denaturation experiment, or 0.5, 1.0, or 2.0 μM of 150-mer ssDNA (5'-CCTTTGTCTCACAGGTGGGAGGCTGGCGTGCGACCTTCATCGGCTCCCACGTCTCTGCCTCCGTGCTCTGGCAGCTGGAGTGGGTGCA GCATGCAGCAAGGATACGGAGATGCCATGGTGGGCCACCGTGACTGCCGACCCTTCTAGGAC-3') in the DNA binding experiment. All of the obtained spectra were normalized by the total area below the spectra curve.

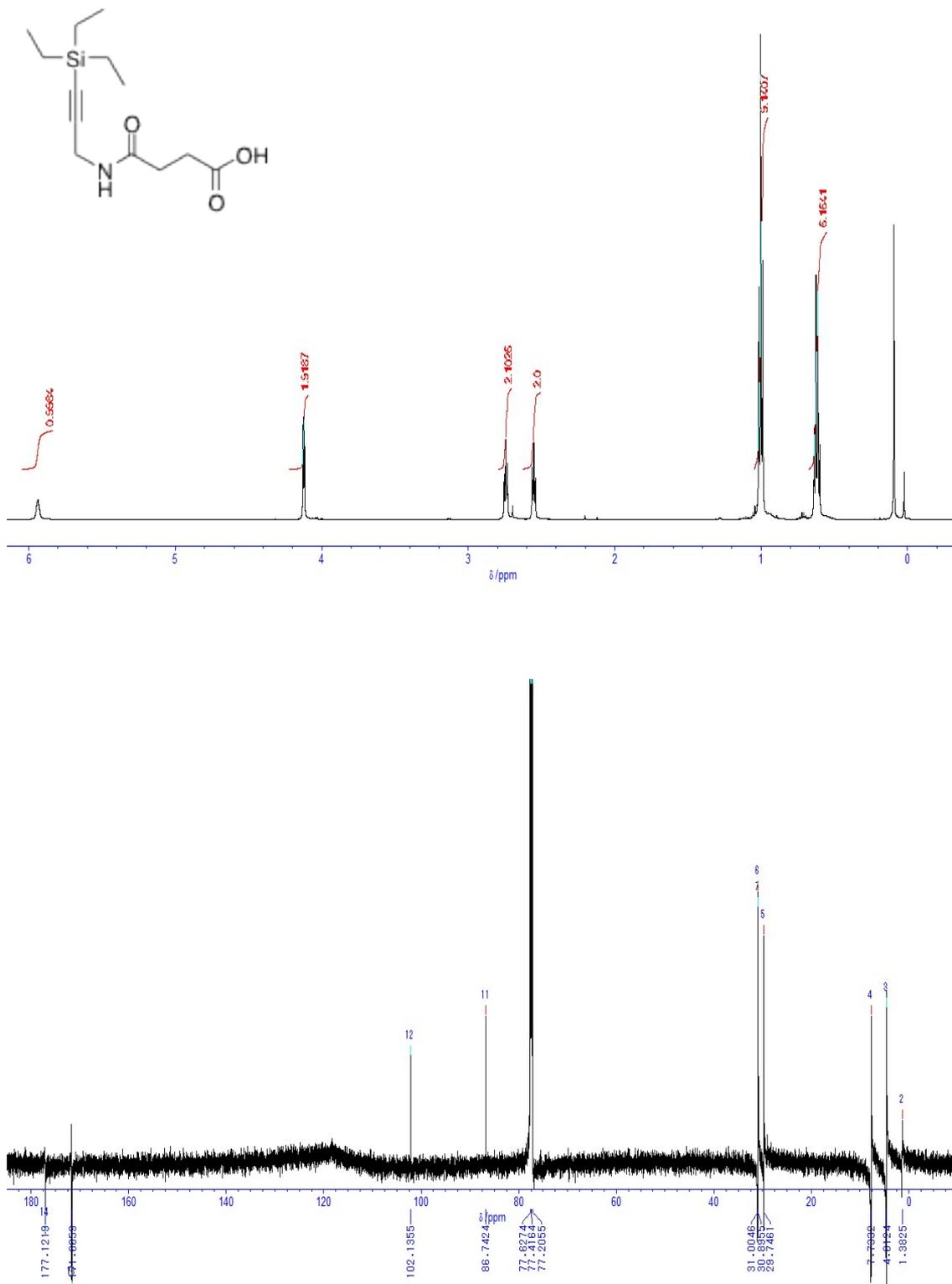
Scheme S1. Synthetic route of carboxylic acid compounds containing silyl-protected alkynes (**S2a-c**).



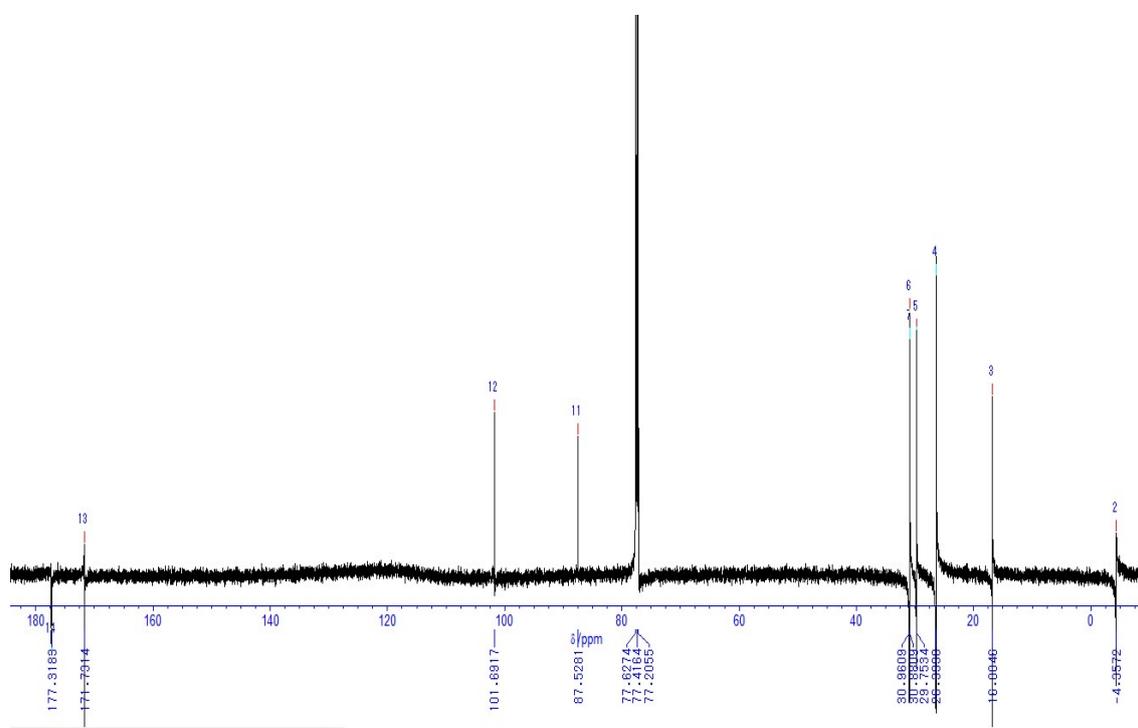
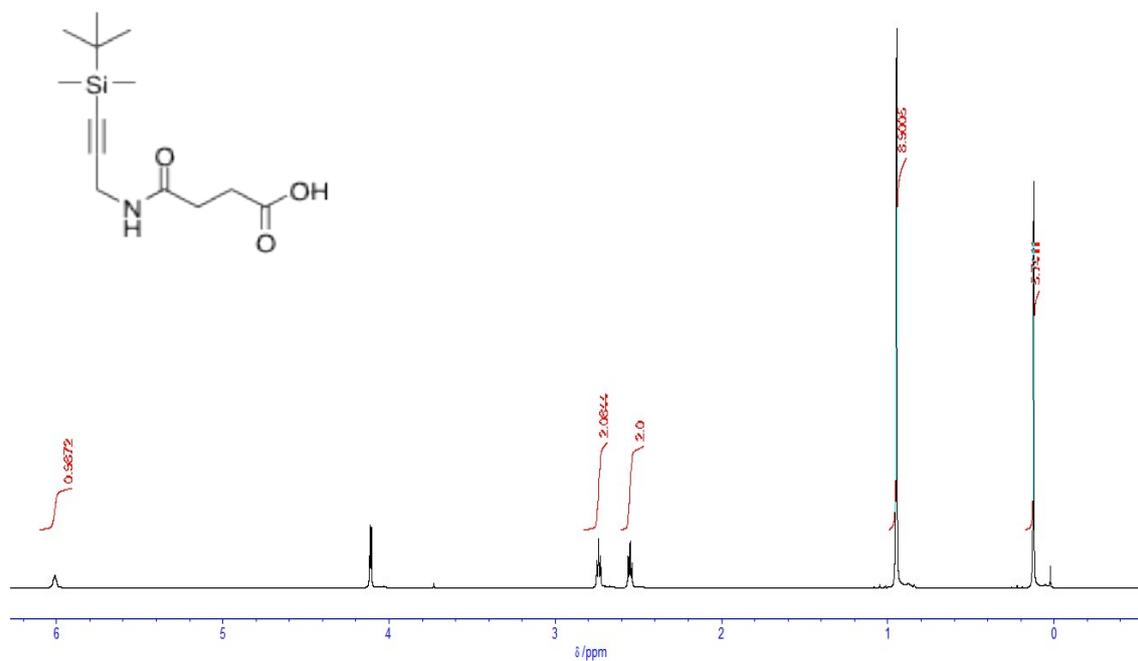
^1H and ^{13}C NMR spectra of **S2a**.



^1H and ^{13}C NMR spectra of **S2b**.



^1H and ^{13}C NMR spectra of **S2c**.



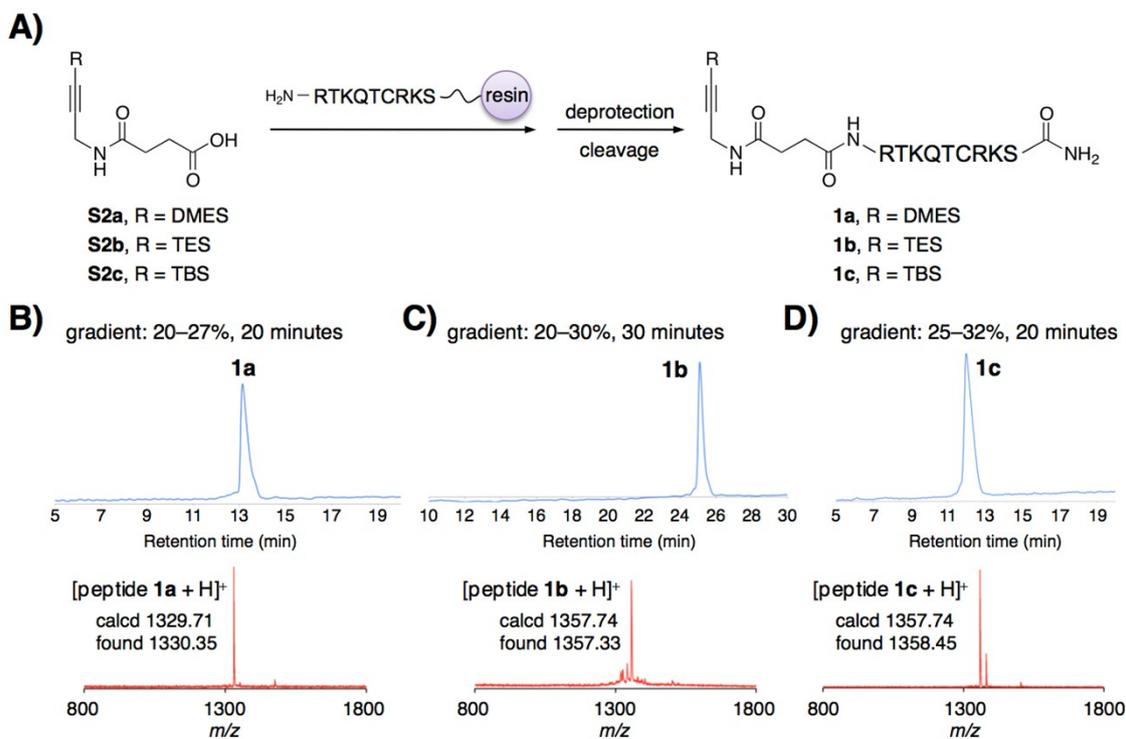


Figure S1. Synthesis of peptide **1a**, **1b**, and **1c**. (A) Synthetic scheme of peptide **1a-1c** by coupling of carboxylic acid compounds to the N-terminus of 9-aa peptide. Identification of purified peptide **1a** (B), **1b** (C), and **1c** (D). HPLC charts of purified peptides were shown in blue. HPLC peaks were monitored at 214 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. MALDI-TOF mass spectra of each peptide were shown in red.

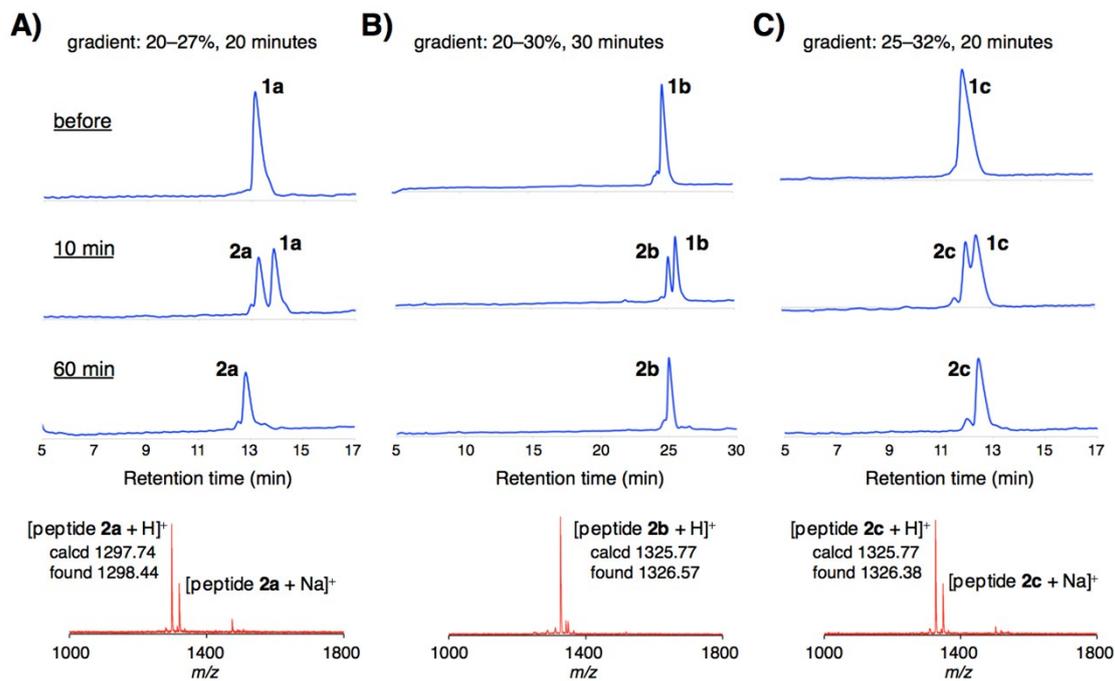


Figure S2. Desulfurization of peptide **1a** (A), **1b** (B), and **1c** (C). HPLC charts of each reaction mixture were shown in blue. HPLC peaks were monitored at 214 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. MALDI-TOF mass spectra of desulfurized peptide **2a**, **2b**, and **2c** were shown in red.

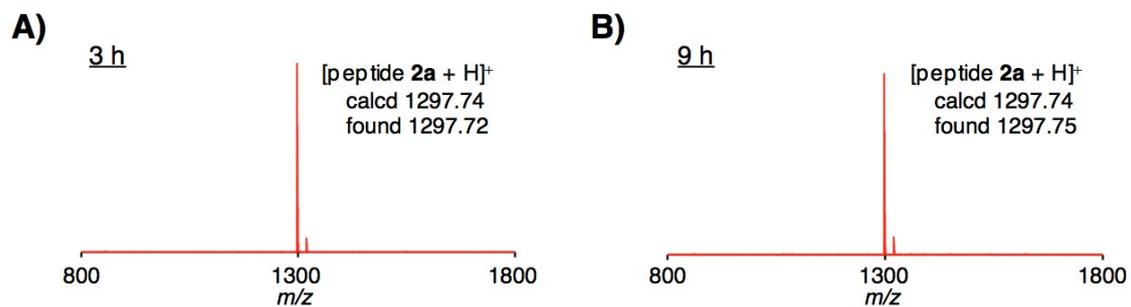


Figure S3. MALDI-TOF mass spectra of prolonged desulfurization product of peptide **1a**. The measurements were done at 3 (A) and 9 h (B) reaction time after purification by ZipTip (Merk).

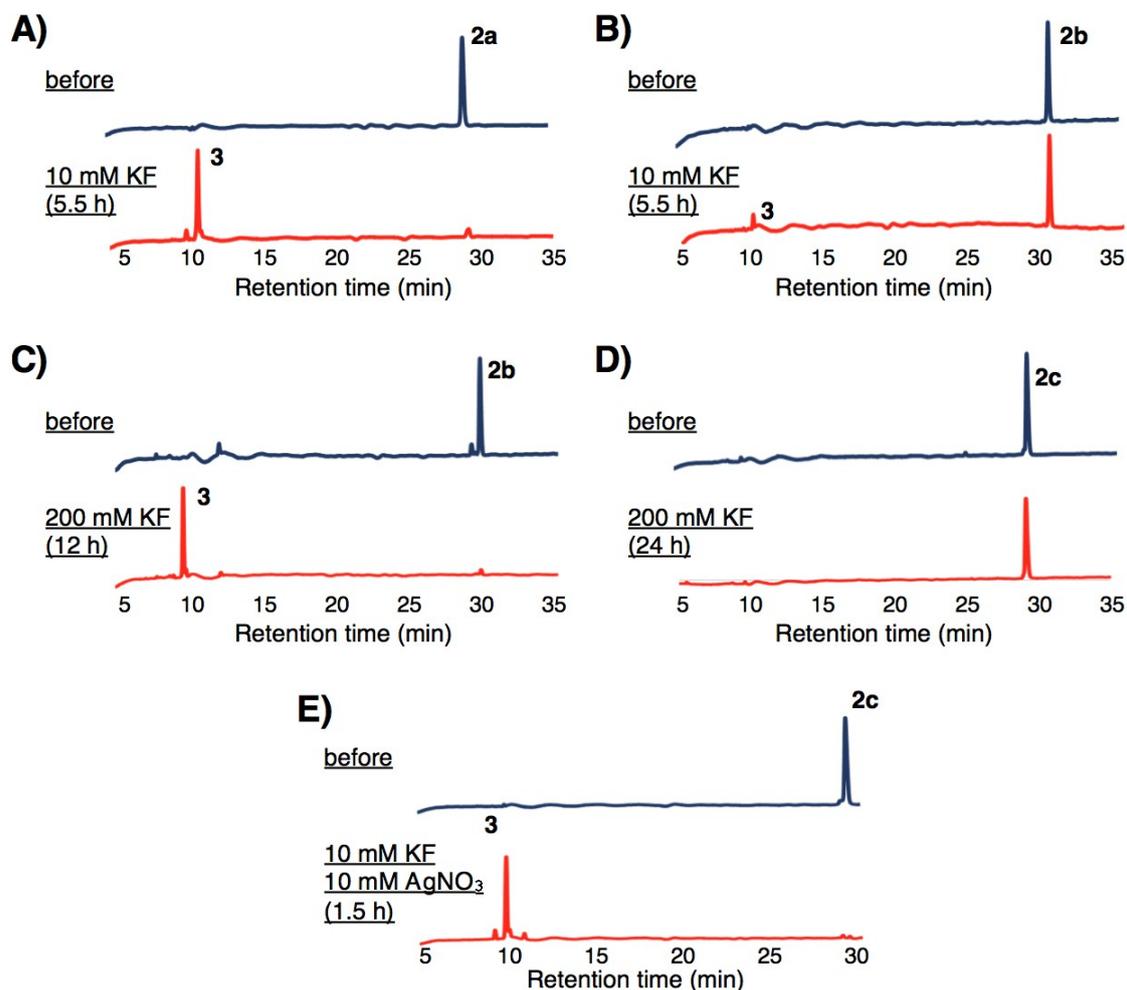


Figure S4. HPLC spectra of selective deprotection of peptide **2a**, **2b**, and **2c**. The deprotection conditions are as follows: peptide **2a** in 10 mM KF at 37°C, 5.5 h (A), **2b** in 10 mM KF at 37°C, 5.5 h (B), **2b** in 200 mM KF at 37°C, 12 h (C), **2c** in 200 mM KF at 37°C, 24 h (D), and **2c** in 10 mM KF and 10 mM AgNO₃ at r.t., 1.5 h (E). HPLC charts of before and after reaction were shown in dark blue and red, respectively. HPLC peaks were monitored at 214 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. The deprotected peptide **3** was identified by MALDI-TOF mass spectrometry (calculated mass: [peptide **3** + H]⁺ = 1211.68).

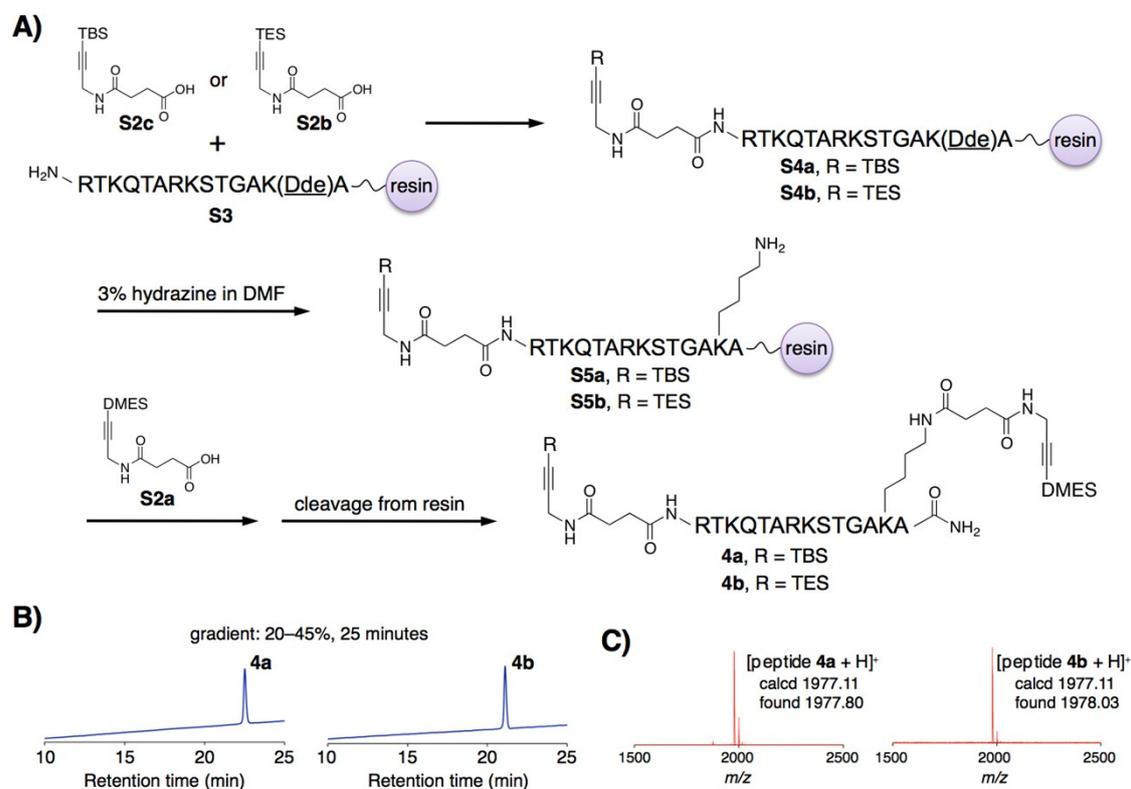


Figure S5. Synthesis of peptide **4a** and **4b**. (A) Synthetic scheme of peptide **4a** and **4b**. TBS- and TES-protected alkynes were introduced at N-terminal of the peptide. DMES-protected alkyne moiety was introduced by using selective deprotection of Dde protecting group at C-terminal Lys. (B) HPLC spectra of purified peptide **4a** and **4b**. HPLC peaks were monitored at 214 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. (C) MALDI-TOF mass spectra of peptide **4a** and **4b**.

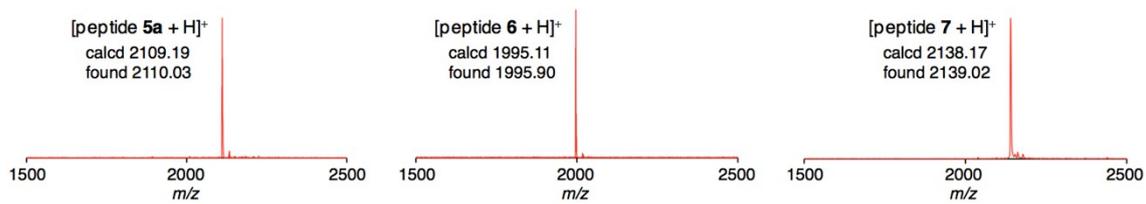


Figure S6. Identification of peptide **5a**, **6**, and **7** by MALDI-TOF mass spectrometry.

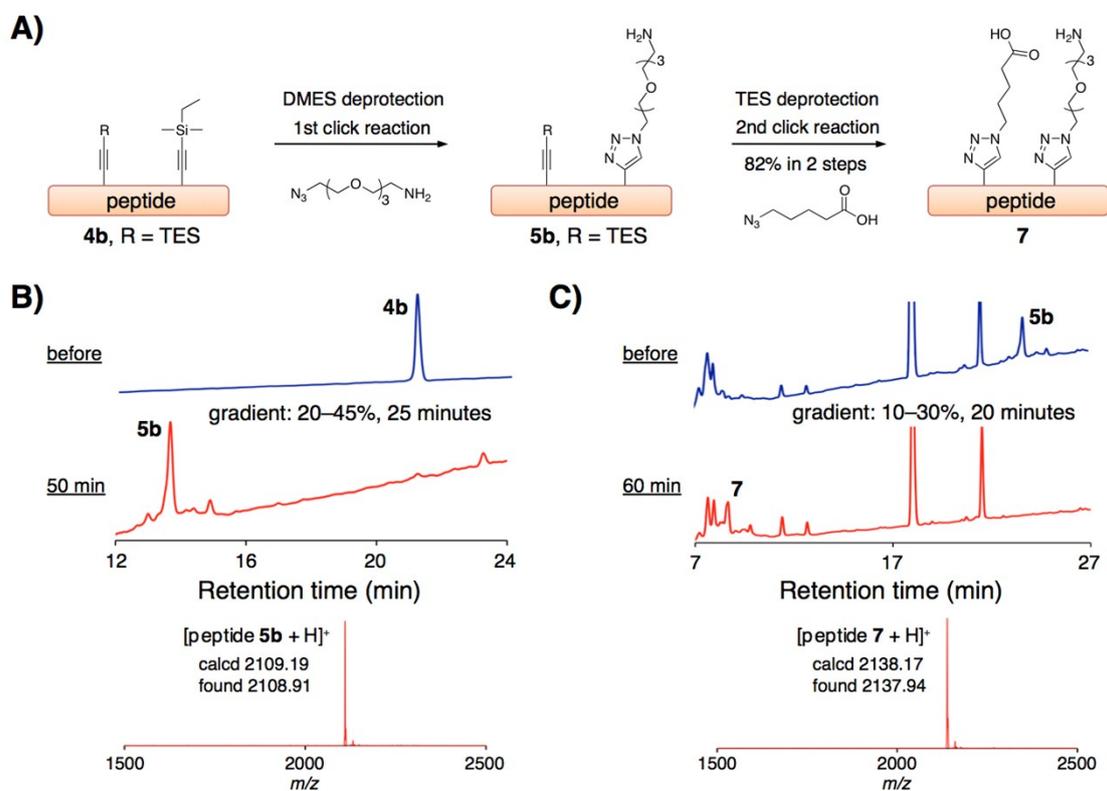


Figure S7. One-pot dual labeling of peptide **4b**. (A) Reaction scheme of the dual labeling of peptide **4a**. The reaction from **4b** to **7** was executed in one-pot (no purification at peptide **5b**). HPLC profiles and MALDI-TOF mass spectra of first conjugation (B), second click reaction (C) are shown. HPLC charts of before and after reaction were shown in blue and red, respectively. HPLC peaks were monitored at 214 nm in the linear gradient with water/acetonitrile containing 0.1% TFA.

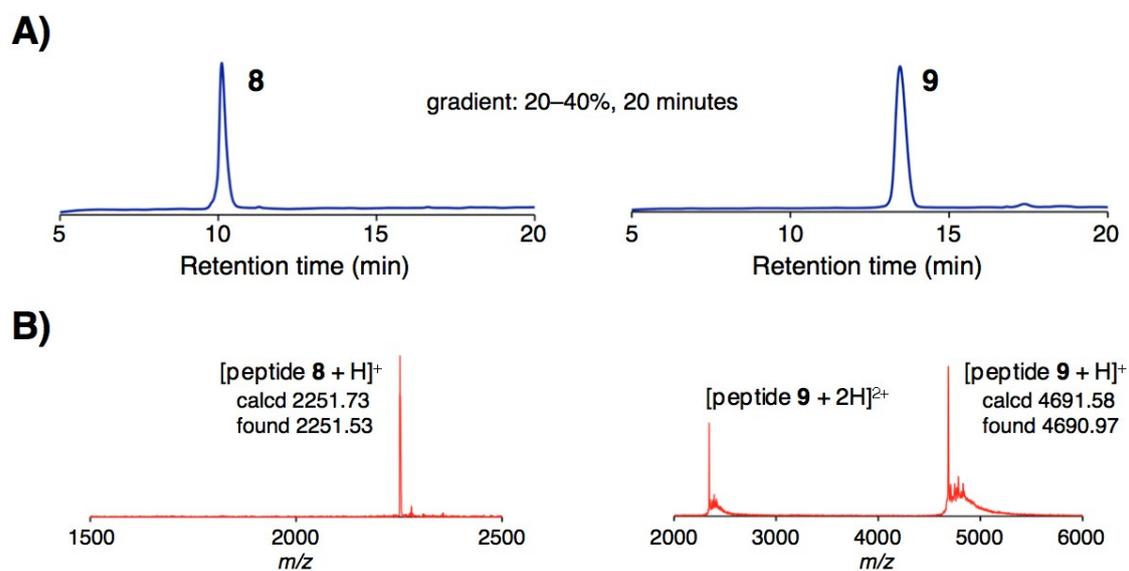


Figure S8. Identification of peptide **8** and **9**. (A) HPLC charts of purified peptide **8** and **9**. HPLC peaks were monitored at 214 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. (B) MALDI-TOF mass spectra of peptide **8** and **9**.

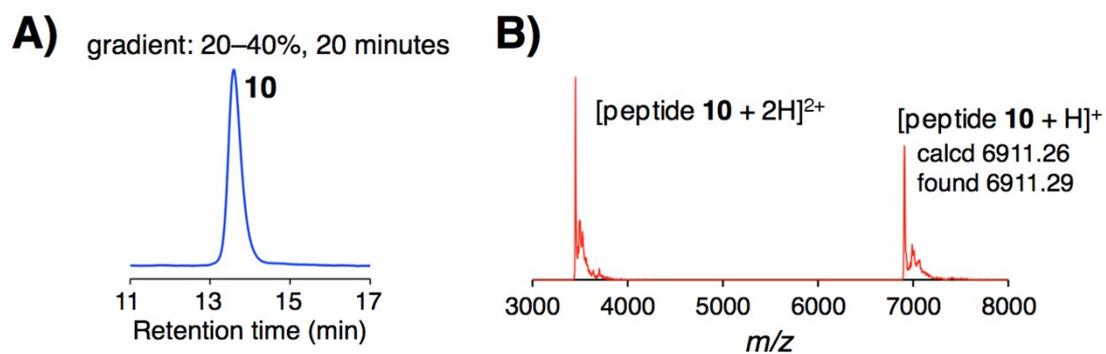


Figure S9. Identification of peptide **10**. (A) HPLC chart of purified peptide **10**. HPLC peaks were monitored at 214 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. (B) MALDI-TOF mass spectrum of peptide **10**.

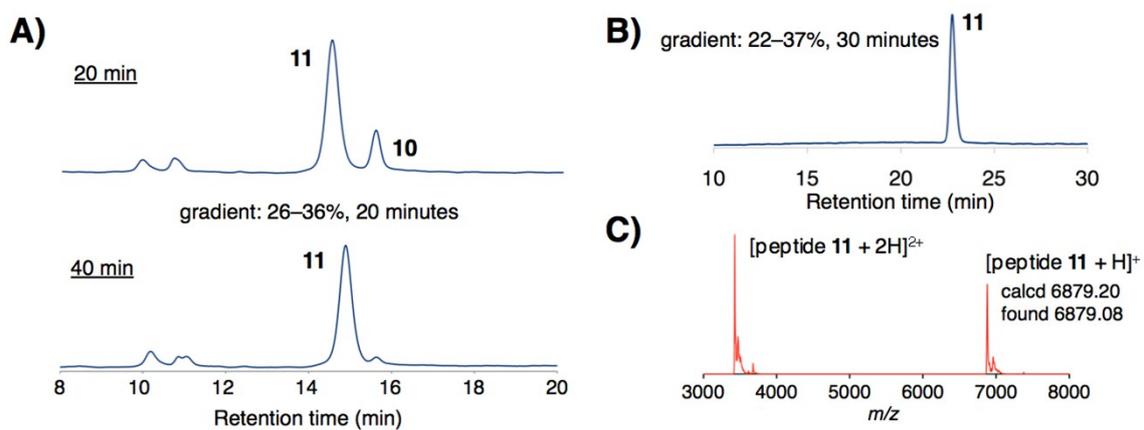


Figure S10. Desulfurization of peptide **10** yields **11**. (A) Reaction monitoring by HPLC. (B) HPLC chart of purified peptide **11**. HPLC peaks were monitored at 214 nm in the linear gradient with water/acetonitrile containing 0.1% TFA. (C) MALDI-TOF mass spectrum of peptide **11**.

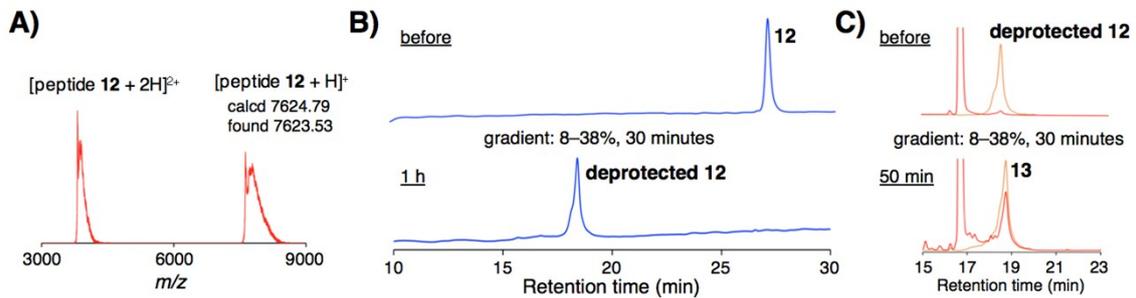


Figure S11. Identification and Cy3 conjugation of peptide **12**. (A) MALDI-TOF mass spectrum of peptide **12**. (B) HPLC monitoring of TBS deprotection of peptide **12**. (C) HPLC monitoring of Cy3-conjugating CuAAC reaction. HPLC peaks were monitored at 214 nm (blue), 647 nm for Cy5 (yellow) and 550 nm for Cy3 (red) in the linear gradient with water/acetonitrile containing 0.1% TFA.

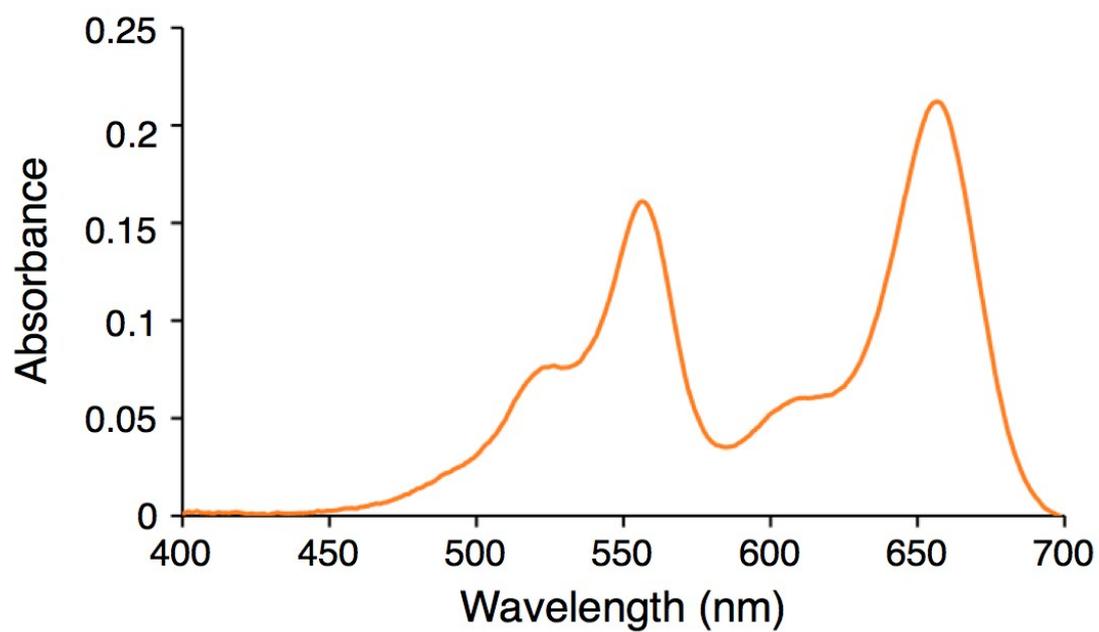


Figure S12. UV-vis spectrum of dual dye-labeled TP1 (**13**). Two significant peaks derived from Cy3 (~560 nm) and Cy5 (~650 nm) were observed.