Supplementary Information

Time-lapse Monitoring of TLR2 Ligand Internalization with Newly Developed Fluorescence Probes

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Contents:

Files of live cell imaging analysis movies

1. (Data for Figure 3) Live-cell imaging of fluorescence styleben-type-labeled Pam2CSK4 (**1e**) endocytosis, either (A) as TLR2/CD14-dependent in HEK-Blue TLR2 or (B) as TLR2/CD14 independent in HEK293. The Fluorescence imaging depicts Styleben-Pam2CSK4 (**1e** at 100 nM) in green.

File 1: 1_HEK293_F-Tlr2 ligand_stilbene_1e.mov
(for Figure 3A: Live-cell imaging of fluorescence(stilbene)-labeled Pam2CSK4 (**1e**) endocytosis in HEK293)

File 2: 2_HEKBlue_F-Tlr2 ligand_stilbene_1e.mov
(for Figure 3B: Live-cell imaging of fluorescence(stilbene)-labeled

Pam2CSK4 (**1e**) endocytosis in HEK-Blue TLR2)

2. (Data for Figure 4) Live-cell imaging of fluorescence-labeled Pam2CSK4 (**1a**) endocytosis, either (A) as TLR2 independent in HEK293 or (B) as TLR2/CD14-dependent in HEK-Blue TLR2. Fluorescence imaging depicts Hoechst 33342 staining in blue and Pam2CSK4-Alexa Fluor® 594 (**1a** at 100 nM) in red.

File 3: 3_HEK293_F-Tlr2 ligand_1a.mov

(for Figure 4A: Live-cell imaging of fluorescence-labeled Pam2CSK4 (**1a**) endocytosis in HEK293)

File 4: 4_HEKBlue-F-Tlr2 ligand_1a.mov

(for Figure 4B: Live-cell imaging of fluorescence-labeled Pam2CSK4 (**1a**) endocytosis in HEK-Blue TLR2)

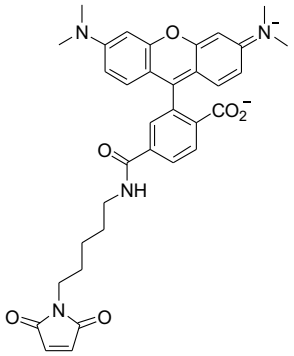
1. Synthesis of Fluorescence-labeled TLR2 ligands

Reagents and general experimental procedures:

Amino acid derivatives and Rink amide resin were purchased from Watanabe Chemical Industries, Ltd. Alexa Fluor® 594 C_5 Maleimide was purchased from Thermo Fisher Scientific, and other fluorescence probes were synthesized as described in the following section. Unlabeled Pam2CSK4 was purchased from InvivoGen and also synthesized as reported for use as a positive control. All other reagents were purchased from commercial supplier (TCI, Nacalai Tesque, and Life Technologies Japan Ltd.) and were used without further purification. Reverse Phase HPLC was performed on a Prominence system (Shimadzu Corporation). RP-HPLC was carried out by using a COSMOSIL 5C_4-AR-300 Packed column (5 μm, 4.6 x 250 mm) at a flow rate of 1 ml/min and a COSMOSIL 5C_{18}-AR-II Packed column (5 μm, 10 x 250 mm) at a flow rate of 3 ml/min. Mass spectra (MS) of synthetic compounds were obtained on an electrospray ionization quadrupole time on flight (ESI-QTOF) mass spectrometer (micrOTOF-QII-HC; BRUKER). Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) spectra were measured at 25 ºC in an indicated solvent with JEOL ECX 400 and analyzed Alice 2 (JEOL). The proton chemical shifts in $CDCl_3$ and CD_3OD are reported

in parts per million (δ) from tetramethylsilane as an internal standard and coupling constants are in Hertz (Hz). The absorption spectra were measured with a UV 3600 Plus UV-VIS-NIR spectrophotometer (Shimadzu Corporation) or V-530 UV/VIS Spectrophotometer (JASCO) in 1 μM concentration. Fluorescence spectra were recorded on a RF-6000 spectro fluorophotometer (Shimadzu Corporation) or FP-6500 spectrofluorometer (JASCO) in 1 μM concentration.

Synthesis of 6-Carboxytetramethylrhodamine C_5 Maleimide (S1)



To a stirred solution of 6-TAMRA (2.93 mg, 6.81 μmol), *N*-(5-aminopentyl)-maleimide trifluoroacetic acid salt[1)] (2.02 mg, 6.81 μmol), WSC·HCl (1.31 mg, 6.81 μmol) and 1-hydroxybenzotriazole (HOBt) (0.92 mg, 6.81 μmol) in DMF (40 μL) was added Et_3N (2.85 μL, 20.4 μmol). The mixture was stirred under dark condition for overnight, and concentrated *in vacuo*. The resulting residue was purified by RP-HPLC [COSMOSIL 5C_{18}-AR-II Packed column, 580 nm linear gradient of 50–80% (v/v) of MeOH (0.1% TFA) (solvent B) in H_2O (0.1% TFA) (solvent A) over 20 min, 1 mL/min] to give 6-TAMRA C_5 maleimide **S1** as a solid (0.99 mg, 24%). **Absorbance and Emission wavelength** (in PBS): λ_{abs} = 552 nm, λ_{em} = 577 nm. **^{1}H NMR** (400 MHz, CD_3OD) δ 8.40 (d, J = 8.4 Hz, 1H), 8.18 (dd, J = 8.4 Hz, 1.6 Hz, 1H), 7.80 (d, J = 1.6 Hz, 1H), 7.16 (d, J = 9.6 Hz, 2H), 7.06 (dd, J = 9.6 Hz, 2.4 Hz, 2H), 6.99 (d, J = 2.8 Hz, 2H), 6.76 (s, 2H), 3.48 (t, J = 7.2 Hz, 2H), 3.37 (t, J = 7.2 Hz, 2H), 3.30 (br, 12H), 1.66-1.56 (m, 4H), 1.36-1.31 (m, 2H); **HRMS** (ESI-Q-TOF): calculated for $C_{34}H_{34}N_4NaO_6^+$ $[M+Na]^+$: 617.2371; found: 617.2374.

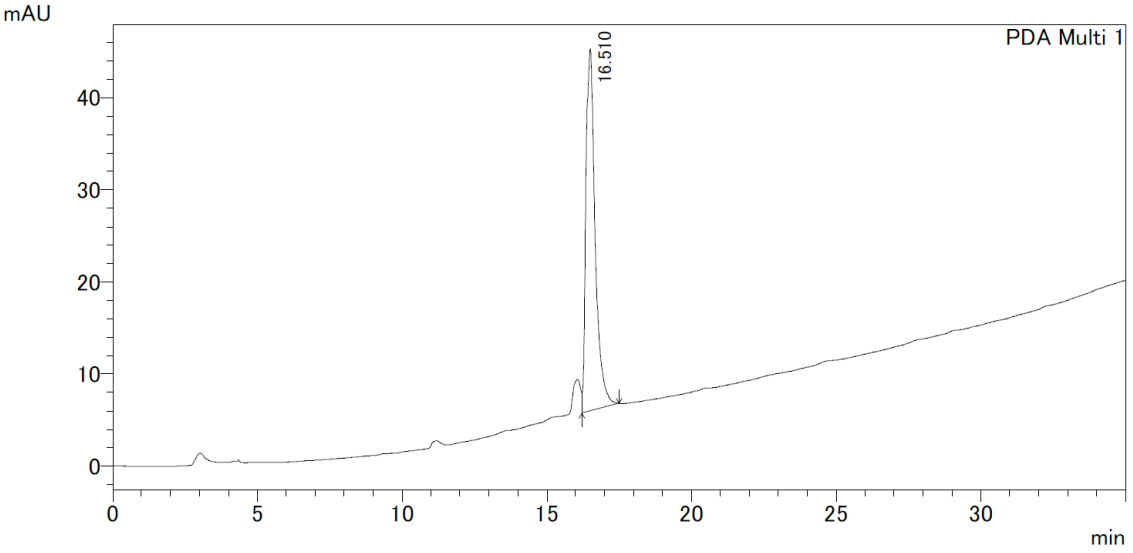
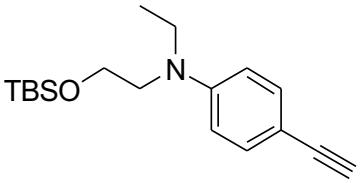


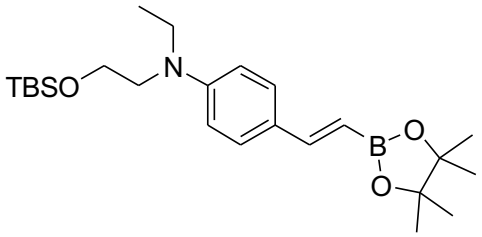
Fig. S1 HPLC elution profile of compound **S1**. COSMOSIL $5C_{18}$-AR-II Packed column, 254 nm linear gradient of 40–100% (v/v) of MeOH (0.1% TFA) in H_2O (0.1% TFA) over 30 min, 1 mL/min.



Synthesis of *N*-{2-[(*tert*-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-ethynylaniline (S2)

A 30 mL round flask equipped with a magnetic stirring bar was charged with 2-[ethyl(4-ethynylphenyl)amino]ethan-1-ol (161 mg, 0.851 mmol), tetrahydrofuran (4.3 mL), imidazole (482 mg, 7.08 mmol) and *tert*-butyldimethylsilylchloride (520 mg, 3.45 mmol). The solution was stirred at room temperature for 15 h. The reaction mixture was diluted with water, and extracted three times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residual oil was purified by silica gel column chromatography (*n*-hexane/EtOAc = 10/1) to provide *N*-{2-[(*tert*-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-ethynylaniline **S2** as a brown oil (256 mg, 99%). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.32 (d, J = 9.2 Hz, 2H), 6.58 (d, J = 9.2 Hz, 2H), 3.75 (t, J = 6.4 Hz, 2H), 3.45-3.38 (m, 4H), 2.96 (s, 1H), 1.15 (t, J = 7.2 Hz,

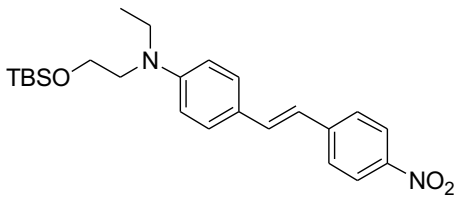
3H), 0.88 (s, 9H), 0.03 (s, 6H); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 147.98, 133.38 (2C), 111.04 (2C), 107.84, 84.94, 74.52, 60.50, 52.31, 45.42, 25.87 (3C), 18.26, 12.03, -5.40 (2C); **HRMS** (ESI-Q-TOF): calculated for $C_{18}H_{29}BNNaOSi^+$ $[M+Na]^+$ 326.1911, found 326.1916.



Synthesis of

(*E*)-*N*-{2-[(tert-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-[2-(4,4,5,5-tetramethyl-1,3,2-dioxaborol an-2-yl)vinyl]aniline (S3)

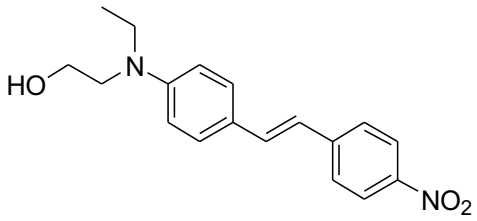
A test tube equipped with a magnetic stirring bar was charged with *N*-{2-[(tert-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-ethynylaniline **S2** (29.0 mg, 95.5 μmol) and carbonylchlorohydridotris(triphenylphosphine)ruthenium(II) (5.6 mg, 5.9 μmol). After argon purge, toluene (0.38 mL) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (69 μL, 0.48 mmol) were added to the solution, and the mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with ether and washed three times with water and once brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residual oil was purified by silica gel column chromatography (*n*-hexane/EtOAc = 10/1) to provide (*E*)-*N*-{2-[(tert-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-[2-(4,4,5,5-tetramethyl-1,3,2-di oxaborolan-2-yl)vinyl]aniline **S3** as a yellow oil (34 mg, 81%). **^{1}H NMR** (400 MHz, CDCl3): δ 7.36 (d, J = 9.2 Hz, 2H), 7.31 (d, J = 18.4 Hz, 1H), 6.62 (d, J = 9.2 Hz, 2H), 5.89 (d, J = 18.4 Hz, 1H), 3.75 (t, J = 6.4 Hz, 2H), 3.46-3.39 (m, 4H), 1.30 (s, 12H), 1.16 (t, J = 7.2 Hz, 3H), 0.89 (s, 9H), 0.03 (s, 6H); **^{13}C NMR** (100 MHz, CDCl3): δ 149.78, 148.49, 128.62 (2C), 125.15 (2C), 111.19 (2C), 82.95 (2C), 60.61, 52.39, 45.52, 25.89 (3C), 24.80 (4C), 18.27, 12.18, -5.38 (2C); **HRMS** (ESI-Q-TOF): calculated for $C_{24}H_{42}BNNaO_3Si^+$ $[M+Na]^+$ 454.2919, found 454.2924.



Synthesis of

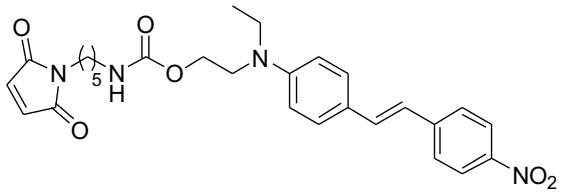
(*E*)-*N*-{2-[(tert-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-(4-nitrostyryl)aniline (S4)

A test tube equipped with a magnetic stirring bar was charged with (*E*)-*N*-{2-[(tert-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-[2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)vinyl]aniline **S3** (58.7 mg, 136 μmol), sodium carbonate (215.1 mg, 2.03 mmol), 1-bromo-4-nitrobenzene (82.5 mg, 408 μmol) and [1,1'-bis(diphenylphosphino)ferrocene]palladium(II) dichloride dichloromethane adduct (5.8 mg, 7.1 μmol). After argon purge, *tert*-amyl alcohol (2.0 mL) and water (0.5 mL) were added to the solution. The mixture was stirred at 80 ºC for 21 h. The reaction mixture was cooled to room temperature, diluted with EtOAc and filtered through Celite®. Filtrate was washed with brine, and organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residual oil was purified by silica gel column chromatography (*n*-hexane/EtOAc = 20/1) to give (*E*)-*N*-{2-[(tert-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-(4-nitrostyryl)aniline **S4** as a red solid (36 mg, 62%). **^{1}H NMR** (400 MHz, CDCl3): δ 8.17 (d, J = 8.8 Hz, 2H), 7.55 (d, J = 8.8 Hz, 2H), 7.41 (d, J = 8.8 Hz, 2H), 7.19 (d, J = 16.4 Hz, 1H), 6.90 (d, J = 16.4 Hz, 1H), 6.68 (d, J = 8.8 Hz, 2H), 3.79 (t, J = 6.4 Hz, 2H), 3.50-3.43 (m, 4H), 1.19 (t, J = 7.2 Hz, 3H), 0.90 (s, 9H), 0.05 (s, 6H); **^{13}C NMR** (100 MHz, CDCl3): δ 148.47, 145.75, 145.13, 133.66, 128.61 (2C), 125.96 (2C), 124.15 (2C), 123.55, 121.03, 111.55 (2C), 60.58, 52.40, 45.55, 25.89 (3C), 18.28, 12.17, -5.38 (2C); **HRMS** (ESI-Q-TOF): calculated for $C_{24}H_{34}N_2NaO_3Si^+$ $[M+Na]^+$ 449.2231, found 449.2238.



Synthesis of (*E*)-2-{ethyl[4-(4-nitrostyryl)phenyl]amino}ethan-1-ol (S5)

A 10 mL round flask equipped with a magnetic stirring bar was charged with (*E*)-*N*-{2-[(tert-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-(4-nitrostyryl)aniline **S4** (33.0 mg, 77.4 μmol), 1,4-dioxane (1.4 mL) and hydrogen chloride solution (ca. 4 M in 1,4-dioxane, 195 μL). The solution was stirred at room temperature for 1 h, and then quenched with saturated $NaHCO_3$ aq. while being cooled in ice bath. The reaction mixture was diluted with EtOAc and washed twice with water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residual oil was purified by silica gel column chromatography (*n*-hexane/EtOAc = 1/1) to give (*E*)-2-{ethyl[4-(4-nitrostyryl)phenyl]amino}ethan-1-ol **S5** as a red solid (21 mg, 87%). **^{1}H NMR** (400 MHz, CDCl3): δ 8.17 (d, J = 9.2 Hz, 2H), 7.56 (d, J = 9.2 Hz, 2H), 7.42 (d, J = 9.2 Hz, 2H), 7.19 (d, J = 16.8 Hz, 1H), 6.92 (d, J = 16.8 Hz, 1H), 6.76 (d, J = 9.2 Hz, 2H), 3.84 (t, J = 6.0 Hz, 2H), 3.55-3.45 (m, 4H), 1.20 (t, J = 7.2 Hz, 3H). All the spectral data were in agreement with those reported by Zhang *et al* [2].

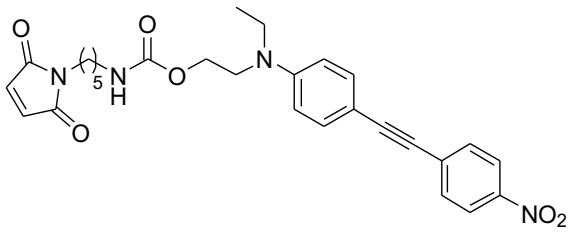


Synthesis of maleimide linker-connected stilbene-type fluorophore (S6)

A test tube equipped with a magnetic stirring bar was charged with (*E*)-2-{ethyl[4-(4-nitrostyryl)phenyl]amino}ethan-1-ol **S5** (17.6 mg, 56.3 μmol) and *N*-(5-aminopentyl)-maleimide trifluoroacetic acid salt [1] (23.7 mg, 112.6 μmol). After argon purge, tetrahydrofuran (0.25 mL), triethylamine (15 μL) and diphenylphosphoryl

azide (25 μL) were added to the solution and the mixture was refluxed for 1 d. The reaction mixture was cooled to room temperature, diluted with EtOAc, and concentrated *in vacuo*. The sticky residue was purified by silica gel column chromatography with (*n*-hexane/EtOAc = 3/2) to give linker-connected tolan-type fluorophore **S6** as a yellow oil (5.4 mg, 18%). **^{1}H NMR** (400 MHz, CDCl3): δ 8.18 (d, J = 9.2 Hz, 2H), 7.56 (d, J = 9.2 Hz, 2H), 7.43 (d, J = 8.8 Hz, 2H), 7.20 (d, J = 16.4 Hz, 1H), 6.91 (d, J = 16.4 Hz, 1H), 6.73 (d, J = 8.8 Hz, 2H), 6.68 (s, 2H), 4.67 (bs, 1H), 4.22 (t, J = 6.4 Hz, 2H), 3.58 (t, J = 6.4 Hz, 2H), 3.51 (t, J = 7.2 Hz, 2H), 3.45 (q, J = 7.2 Hz, 2H), 3.16 (q, J = 6.4 Hz, 2H), 1.62-1.50 (m, 4H), 1.35-1.23 (m, 2H), 1.20 (t, J = 7.2 Hz, 3H). **^{13}C NMR** (100 MHz, CDCl3): δ 170.84 (2C), 148.29, 145.82, 145.01, 134.05 (2C), 133.54, 128.64 (2C), 126.04 (2C), 124.14 (2C), 124.07, 121.40, 111.76 (2C), 99.89, 49.04, 45.18, 40.82, 37.53, 29.69, 29.36, 28.17, 23.77, 12.13. **HRMS** (ESI-Q-TOF): calculated for $C_{28}H_{32}N_4NaO_6^+$ $[M+Na]^+$ 543.2214, found 543.2218.

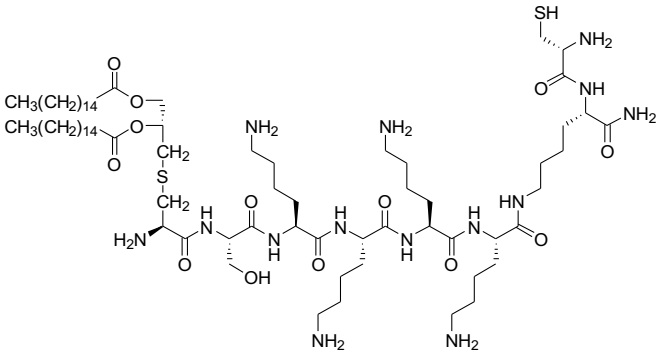
Synthesis of maleimide linker-connected tolan-type fluorophore (S7)



By a procedure identical with that described for synthesis of linker-connected tolan-type fluorophore from (*E*)-2-{ethyl[4-(4-nitrostyryl)phenyl]amino}ethan-1-ol, the 4′- [*N*- Ethyl- *N*- (2-hydroxyethyl)amino]- 4- nitrodiphenylacetylene [3] (37.4 mg, 120 μmol) was converted into linker-connected stilbene-type fluorophore **S7** as an orange oil (5.7 mg, 9%). **^{1}H NMR** (400 MHz, CDCl3): δ 8.18 (d, J = 8.8 Hz, 2H), 7.59 (d, J = 8.8 Hz, 2H), 7.40 (d, J = 9.2 Hz, 2H), 6.71-6.68 (m, 4H), 4.67 (bs, 1H), 4.21 (t, J = 6.4 Hz, 2H), 3.58 (t, J = 6.4 Hz, 2H), 3.52 (t, J = 7.2 Hz, 2H), 3.44 (q, J = 7.2 Hz, 2H), 3.16 (q, J = 6.4 Hz, 2H), 1.63-1.48 (m, 4H), 1.31-1.24 (m, 2H), 1.20 (t, J = 7.2 Hz, 3H). **^{13}C NMR** (100 MHz, CDCl3): δ 170.84 (2C), 156.52, 148.34, 146.19, 134.06 (2C), 133.43 (2C), 131.59, 131.40 (2C), 123.61 (2C), 111.43 (2C), 108.32, 97.01, 86.39, 77.21, 45.13, 37.53, 29.69, 29.35, 28.17, 23.78, 17.37, 12.13. **HRMS** (ESI-Q-TOF): calculated for

$C_{28}H_{30}N_4NaO_6^+$ $[M+Na]^+$ 541.2058, found 541.2063.

Synthesis of Pam_2CSK_4 containing the linker (3)



Pam_2CSK_4 containing a linker (**3**) was synthesized by solid-phase peptide synthesis (SPPS) on Rink Amide resin (100-200 Mesh, 81.0 mg, 0.047 mmol) using Fmoc protected amino acids (0.141 mmol) and *N,N′*-diisopropylcarbodiimide (DIC) (21.8 μL, 0.141 mmol)/HOBt (19.1 mg, 0.141 mmol) in DMF (800 μL). The following protected amino acids were employed: Fmoc-L-Lys(Dde)-OH (75.1 mg, 0.141 mmol), Boc-L-Cys(Trt)-OH (65.4 mg, 0.141 mmol), Fmoc-L-Lys(Boc)-OH (66.1 mg, 0.141 mmol ×4) and Fmoc-L-Ser(Trt)-OH (80.3 mg, 0.141 mmol). Fmoc groups were cleaved by treatment with 20% piperidine in DMF (1 mL, 3 min×1 then 20 min×1). Dde groups were cleaved by treatment with 5% hydrazine in DMF (1 mL, 1 min×1 then 20 min×1). The coupling of Fmoc-Pam_2Cys-OH (**2**) [4] (84.1 mg, 0.094 mmol) was carried out using DIC (14.6 μL, 0.094 mmol) and HOBt (12.7 mg, 0.094 mmol) in DMF (800 μL). Progress of the manual couplings was monitored by standard Kaiser test. After completion of the Pam_2CSK_4 synthesis, the resin was thoroughly washed with DMF (3 mL), CH_2Cl_2 (3 mL), and MeOH (3 mL) and dried *in vacuo* to a constant weight. Cleavage of the peptide from the resin was achieved by stirring the resin with a cleavage cocktail composed of trifluoroacetic acid (TFA), CH_2Cl_2 and triisopropylsilane (TIS) (50:50:2 = in vol. ratio, 1 mL, 3 min×5). The combined solutions were concentrated *in vacuo*. The crude product was purified by HPLC [COSMOSIL $5C_4$-AR-300 Packed column, 220 nm, linear gradient of 75–100% (v/v) of MeOH (0.1% TFA) (solvent B) in H_2O (0.1% TFA) (solvent A) over 20 min, 1 mL/min] to give the desired compound **3** (23% based on Rink amide resin loading capacity). **^{1}H NMR** (400

MHz, CD_3OD): δ5.27-5.21 (m, 1H), 4.54 (t, *J* = 6.0 Hz, 1H), 4.43 (dd, *J* = 12.0 Hz, 2.4 Hz, 1H), 4.38-4.23 (m, 5H), 4.19-4.09 (m, 3H), 3.92 (dd, *J* =11.2 Hz, 5.6 Hz, 1H), 3.78 (dd, *J* =11.2 Hz, 6.4 Hz, 1H), 3.24-3.12 (m, 3H), 3.06-3.04 (m, 2H), 2.94 (t, *J* = 7.2 Hz, 8H), 2.91-2.86 (m, 2H), 2.81 (dd, *J* =14.4 Hz, 8.0 Hz, 1H), 2.34 (t, *J* = 7.6 Hz, 2H), 2.32 (t, *J* =7.6 Hz, 2H), 1.95-1.67 (m, 10H), 1.76-1.64 (m, 10H), 1.64-1.56 (m, 4H), 1.56-1.39 (m, 10H), 1.28 (br, 48H), 0.89 (t, *J* =7.2 Hz, 6H). **HRMS** (ESI-Q-TOF): calculated for $C_{74}H_{145}N_{14}O_{13}S_2^+$ $[M+H]^+$ 1502.0551, found 1502.0554.

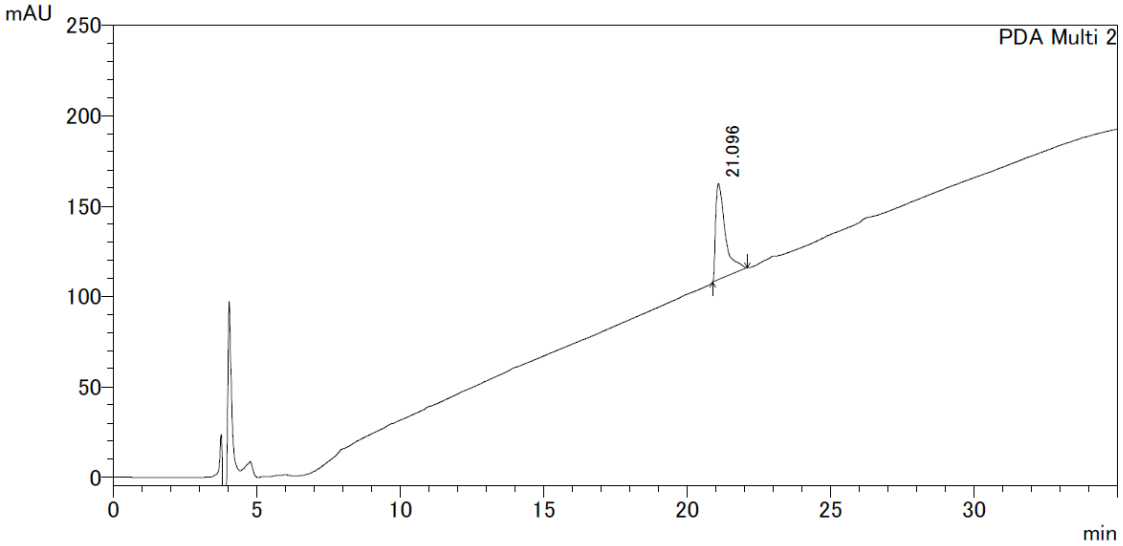
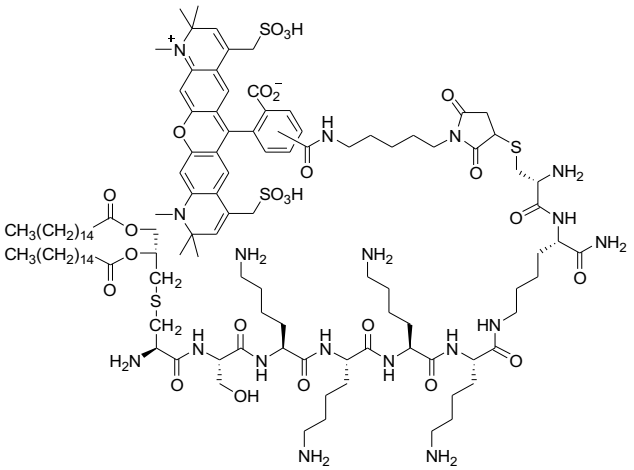


Fig. S2 HPLC elution profile of compound **3**. COSMOSIL $5C_4$-AR-300 Packed column, 220 nm linear gradient of 70–100% (v/v) of MeOH (0.1% TFA) in H_2O (0.1% TFA) over 30 min, 1 mL/min.

Synthesis of Alexa-labeled Pam_2CSK_4 (1a)



To a solution of **3** (1.0 mg, 0.66 μmol) in MeOH (200 μL) was added Alexa Fluor® 594 C_5 Maleimide (0.40 mg, 0.44 μmol). The mixture was stirred under dark condition for overnight, and concentrated *in vacuo*. The resulting mixture was purified by RP-HPLC [COSMOSIL $5C_4$-AR-300 Packed column, 594 nm, linear gradient of 75–100% (v/v) of MeOH (0.1% TFA) (solvent B) in H_2O (0.1% TFA) (solvent A) over 20 min, 1 mL/min] to give Alexa-labeled Pam_2CSK_4 **1a** as a solid (0.90 mg, 86%). **Absorbance and Emission wavelength** (in PBS): λ_{abs} = 590 nm, λ_{em} = 617 nm. **^{1}H NMR** (400 MHz, CD_3OD) 5'-isomer: δ 8.61 (br, 1H), 8.23 (d, J = 8.0 Hz, 1H), 7.40 (d, J= 8.4 Hz, 1H), 7.38-7.25 (m, 2H), 6.82 (s, 2H), 5.88-5.86 (m, 2H), 5.26-5.18 (m, 1H), 4.51-4.47 (m, 1H), 4.43-4.38 (m, 1H), 4.37-4.22 (m, 6H), 4.16-4.06 (m, 3H), 3.92-3.82 (m, 1H), 3.79-3.71 (m, 1H), 3.71-3.61 (m, 2H), 3.59-3.54 (m, 2H), 3.52-3.46 (m, 4H), 3.38-3.11 (m, 24H), 2.95-2.84 (m, 10H), 2.79 (dd, J =14.8 Hz, 7.6 Hz, 1H), 2.54 (dd, J =19.2 Hz, 6.4 Hz, 1H), 2.33 (t, J = 7.6 Hz, 2H), 2.30 (t, J =7.6 Hz, 2H), 1.95-1.18 (m, 88H), 0.89 (t, J =7.2 Hz, 6H); 6'-isomer: δ 8.08-7.99 (m, 2H), 7.57 (d, J= 8.8 Hz, 1H), 7.38-7.25 (m, 2H), 6.82 (s, 2H), 5.88-5.86 (m, 2H), 5.26-5.18 (m, 1H), 4.51-4.47 (m, 1H), 4.43-4.38 (m, 1H), 4.37-4.22 (m, 6H), 4.16-4.06 (m, 3H), 3.92-3.82 (m, 1H), 3.79-3.71 (m, 1H), 3.71-3.61 (m, 2H), 3.59-3.54 (m, 2H), 3.52-3.46 (m, 4H), 3.38-3.11 (m, 24H), 2.95-2.84 (m, 10H), 2.79 (dd, J =14.8 Hz, 7.6 Hz, 1H), 2.54 (dd, J =19.2 Hz, 6.4 Hz, 1H), 2.33 (t, J = 7.6 Hz, 2H), 2.30 (t, J =7.6 Hz, 2H), 1.95-1.18 (m, 88H), 0.89 (t, J =7.2 Hz, 6H). **HRMS** (ESI-Q-TOF): calculated for $C_{118}H_{192}N_{18}O_{25}S_4^{2+}$ $[M+2H]^{2+}$ 1194.6589, found 1194.6589.]

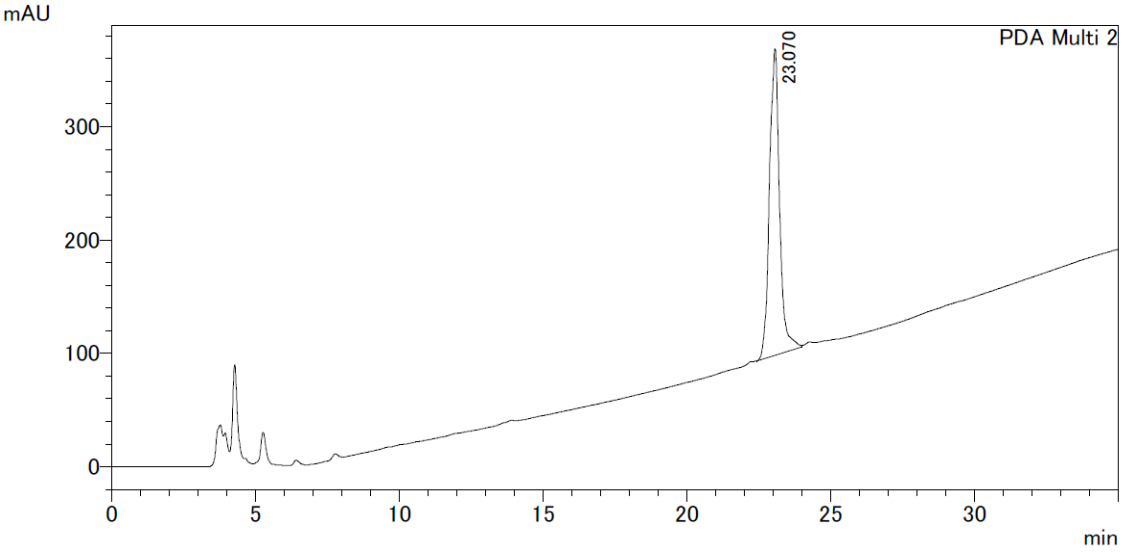
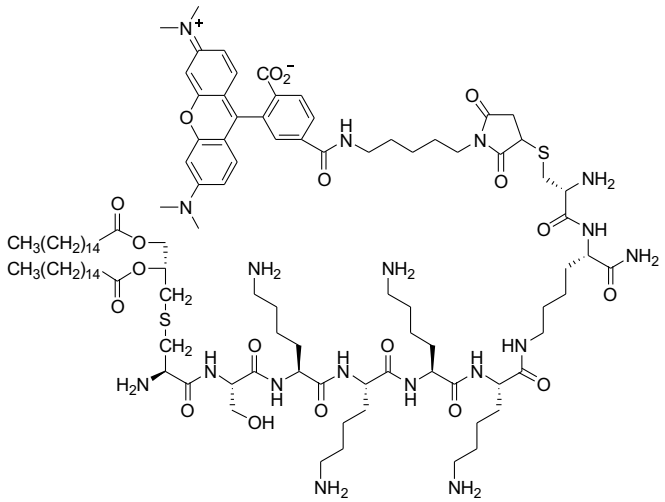


Fig. S3 HPLC elution profile of compound **1a**. COSMOSIL 5C_4-AR-300 Packed column, 220 nm linear gradient of 70–100% (v/v) of MeOH (0.1% TFA) in H_2O (0.1% TFA) over 30 min, 1 mL/min.

Synthesis of 6-TAMRA-labeled Pam$_2$CSK$_4$ (1b)



By a procedure identical with that described for synthesis of **1a** from **3**, Pam$_2$CSK$_4$ derivative **3** (1.0 mg, 0.66 μmol) and 6-TAMRA C_5 maleimide (0.26 mg, 0.44 μmol) were converted into 6-TAMRA-labeled Pam$_2$CSK$_4$ **1b** as a solid (0.88 mg, 95%). **Absorbance and Emission wavelength** (in PBS): λ_{abs} = 518 nm, λ_{em} = 577 nm. **^{1}H NMR** (400 MHz, CD_3OD): δ 8.34 (d, J = 8.4 Hz, 1H), 8.17 (d, J = 8.4 Hz, 1H), 7.78 (s, 1H), 7.18 (d, J = 9.6 Hz, 2H), 7.05 (dd, J = 9.6 Hz, 2.4Hz, 2H), 6.99 (s, 2H), 5.27-5.21

(m, 1H), 4.54 (t, J = 6.0 Hz, 1H), 4.43 (dd, J = 12.0 Hz, 2.4 Hz, 1H), 4.38-4.23 (m, 6H), 4.15-4.09 (m, 3H), 3.92 (dd, J =11.2 Hz, 5.6 Hz, 1H), 3.78 (dd, J =11.2 Hz, 6.4 Hz, 1H), 3.53-3.47 (m, 4H), 3.38-3.11 (m, 18H), 2.94 (br, 8H), 2.91-2.86 (m, 2H), 2.81 (dd, J =14.4 Hz, 8.0 Hz, 1H), 2.49 (dd, J =19.2 Hz, 3.6 Hz, 1H), 2.34 (t, J = 7.6 Hz, 2H), 2.32 (t, J =7.6 Hz, 2H), 1.95-1.18 (m, 88H), 0.89 (t, J =7.2 Hz, 6H); **HRMS** (ESI-Q-TOF): calculated for $C_{108}H_{180}N_{18}O_{19}S_2^{2+}$ $[M+2H]^{2+}$ 1048.6551, found 1048.6559.

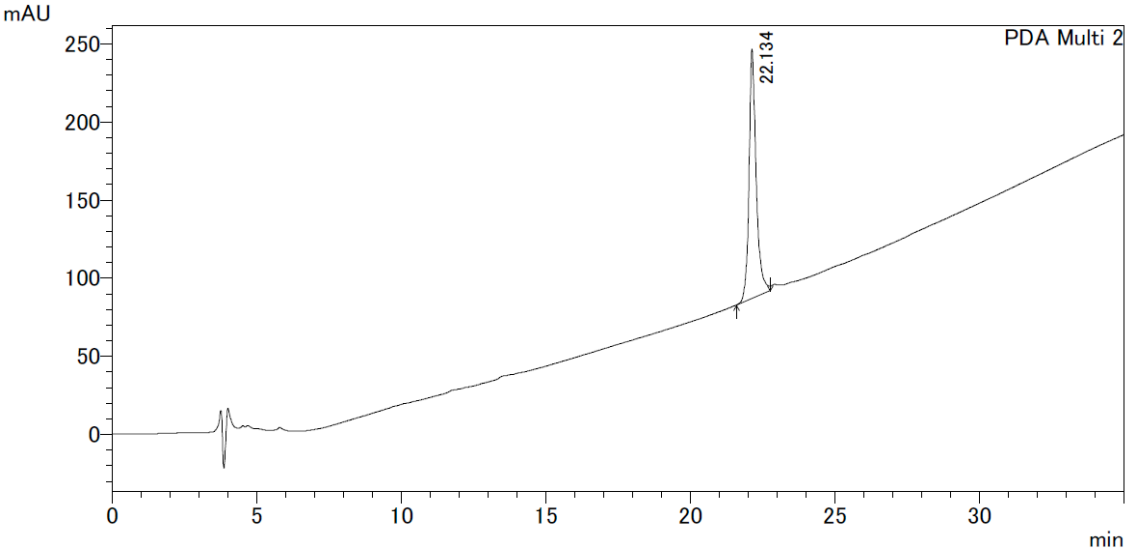
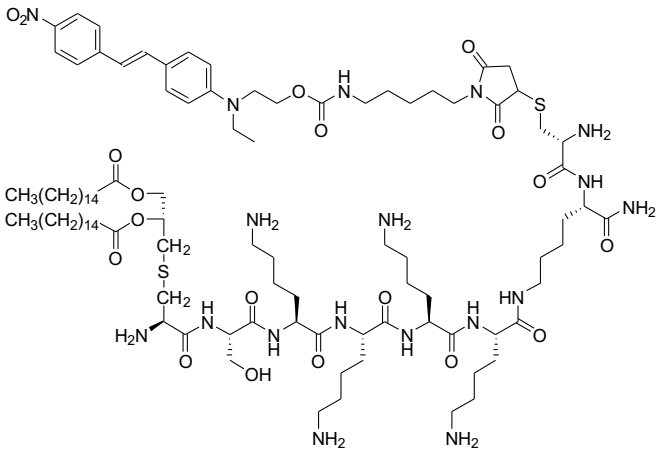


Fig. S4 HPLC elution profile of compound **1b**. COSMOSIL 5C_4-AR-300 Packed column, 220 nm linear gradient of 70–100% (v/v) of MeOH (0.1% TFA) in H_2O (0.1% TFA) over 30 min, 1 mL/min.

Synthesis of stilbene-type-labeled Pam$_2$CSK$_4$ (1c)



By a procedure identical with that described for synthesis of **1a** from **3**, Pam_2CSK_4 derivative **3** (3.0 mg, 2 μmol) and stilbene-type fluorophore (0.50 mg, 1 μmol) were converted into stilbene-type-labeled Pam_2CSK_4 **1c** as an orange solid (0.7 mg, 36%). **Absorbance and Emission wavelength** (in PBS): λ_{abs} = 443 nm, λ_{em} = 477 nm. **^{1}H NMR** (400 MHz, CD_3OD): δ 8.18 (d, J = 9.2 Hz, 2H), 7.68 (d, J = 9.2 Hz, 2H), 7.47 (d, J = 8.8 Hz, 2H), 7.32 (d, J = 16.4 Hz, 1H), 7.02 (d, J = 16.4 Hz, 1H), 6.78 (d, J = 8.8 Hz, 2H), 5.20 (br, 1H), 4.61 (br, 1H), 4.45-4.42 (m, 2H), 4.38-4.19 (m, 9H), 4.15-4.10 (m, 1H), 3.96-3.91 (m, 1H), 3.82-3.77 (m, 1H), 3.64-3.59 (m, 1H), 3.53-3.42 (m, 5H), 3.13-3.11 (m, 1H), 3.10-2.73 (m, 16H), 2.51 (dd, J =20.8 Hz, 2.8 Hz, 1H), 2.35-2.28 (m, 6H), 1.95-1.18 (m, 91H), 0.89 (t, J =7.2 Hz, 6H). **HRMS** (ESI-Q-TOF): calculated for $C_{102}H_{178}N_{18}O_{19}S_2^{2+}$ $[M+2H]^{2+}$ 1011.6473, found 1011.6473.

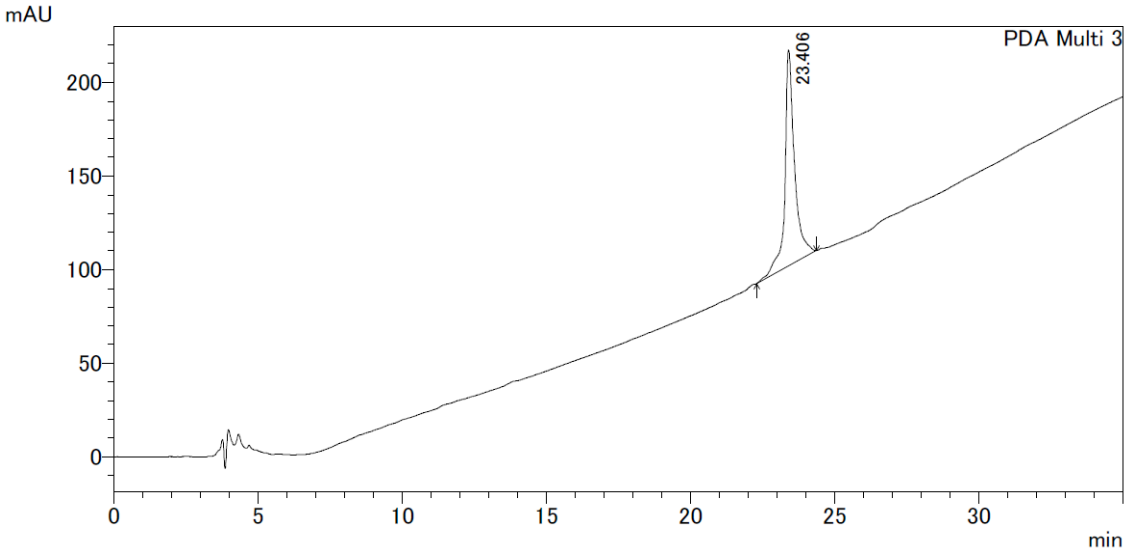
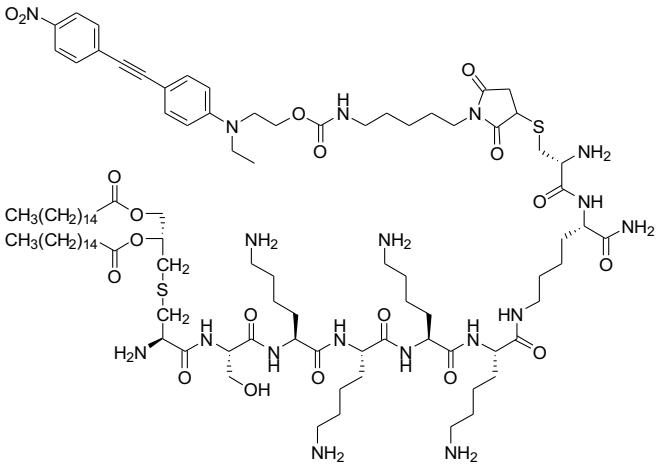


Fig. S5 HPLC elution profile of compound **1c**. COSMOSIL $5C_4$-AR-300 Packed column, 220 nm linear gradient of 70–100% (v/v) of MeOH (0.1% TFA) in H_2O (0.1% TFA) over 30 min, 1 mL/min.

Synthesis of tolan-type-labeled Pam_2CSK_4 (1d)



By a procedure identical with that described for synthesis of **1a** from **3**, Pam_2CSK_4 derivative **3** (3.0 mg, 2 μmol) and tolan-type fluorophore (0.51 mg, 1 μmol) were converted into tolan-type-labeled Pam_2CSK_4 **1d** as an orange solid (1.0 mg, 51%). **Absorbance and Emission wavelength** (in PBS): λ_{abs} = 295, 420 nm, λ_{em} = 543 nm (Ex = 270 nm), 723 nm (Ex = 380 nm). **^{1}H NMR** (400 MHz, CD_3OD) δ 8.19 (d, J = 8.8 Hz, 2H), 7.93 (d, J = 9.1 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H), 6.81 (d, J = 9.1 Hz, 2H), 5.20 (br, 1H), 4.61 (br, 1H), 4.46-4.41 (m, 2H), 4.38-4.20 (m, 9H), 4.15-4.09 (m, 1H), 3.97-3.90 (m, 1H), 3.84-3.79 (m, 1H), 3.58-3.42 (m, 6H), 3.36-3.32 (m, 2H), 3.19-3.16 (m, 1H), 3.13-3.12 (m, 2H), 3.04-2.77 (m, 14H), 2.51 (dd, J =20.8 Hz, 2.8 Hz, 1H), 2.37-2.29 (m, 4H), 1.93-1.25 (m, 91H), 0.90 (t, J = 6.8 Hz, 6H). **HRMS** (ESI-Q-TOF): calculated for $C_{102}H_{174}N_{18}Na_2O_{19}S_2^{2+}$ $[M+2Na]^{2+}$ 1032.6214, found 1032.6222.

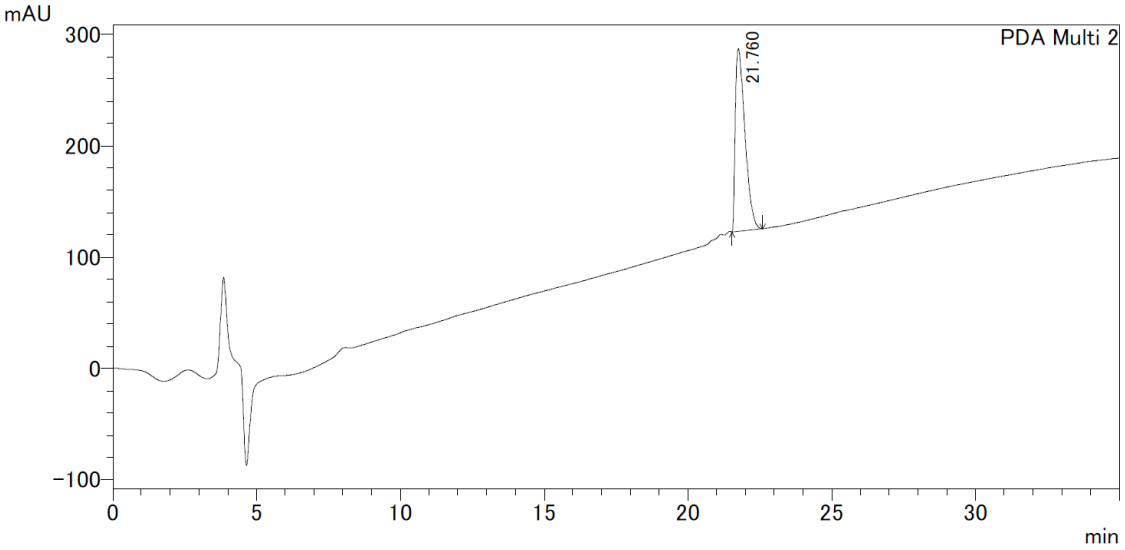
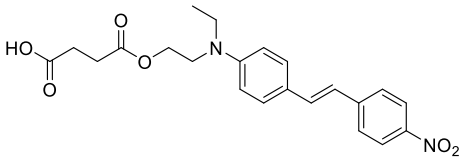


Fig. S6 HPLC elution profile of compound **1d**. COSMOSIL 5C_4-AR-300 Packed column, 220 nm linear gradient of 70–100% (v/v) of MeOH (0.1% TFA) in H_2O (0.1% TFA) over 30 min, 1 mL/min.

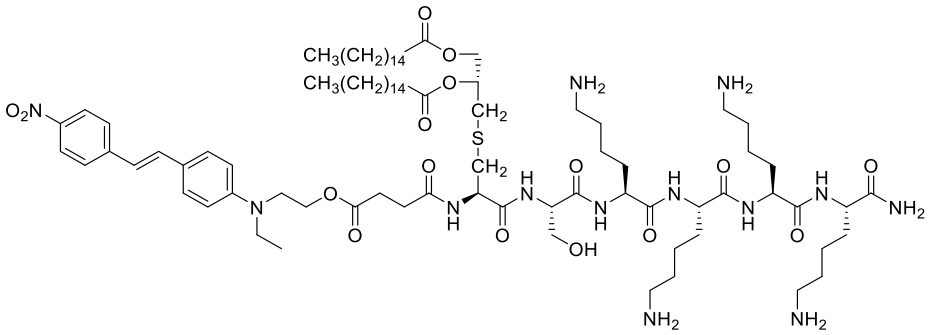
Synthesis of succinic acid mono 2-[ethyl[4-[2-(4-nitrophenyl)ethenyl]phenyl]amino]ethoxy ester (S8)



Succinic anhydride (16 mg, 0.16 mmol) was stirred with dry toluene (1.6 mL), *N*-hydroxy succinimide (5.5 mg, 48 μmol) and DMAP (2.0 mg, 16 μmol) at room temperature for 2 h. For dissolving succinic anhydride, the solution was heated at 60 °C for 5 min. After being cooled, to the solution was added (*E*)-*N*-{2-[(tert-butyldimethylsilyl)oxy]ethyl}-*N*-ethyl-4-(4-nitrostyryl)aniline **S4** (50 mg, 0.16 mmol) and Et_3N (22 μL, 0.16 mmol), and stirred at room temperature. For dissolving **S4**, the solution was added dry DMF (0.16 mL), and stirred overnight. AcOEt (47.5 mL) was added, and the organic layer was washed with 10 % citric acid and brine and dried over Na_2SO_4. The solvent was concentrated in vacuo, and removed in vacuo. The residue was purified by silica-gel column chromatography (AcOEt: CH_2Cl_2= 2: 5) to give **S8** (45 mg, 68 %). **Absorbance and Emission wavelength** (in 1,4-dioxane): λ_{abs} = 427 nm, λ_{em} = 564 nm. **^{1}H NMR** (500MHz, $CDCl_3$): δ = 8.18 (dd, *J* = 1.9 Hz, 8.9 Hz,

2H), 7.56 (dd, *J* = 2.2 Hz, 8.9 Hz, 2H), 7.43 (dd, *J* = 2.6 Hz, 9.1 Hz, 2H), 7.19 (dd, *J* = 3.0 Hz, 16.2 Hz, 1H), 6.92 (dd, *J* = 5.9 Hz, 16.4 Hz, 1H), 6.71-6.78 (br, 2H), 4.29 (t, *J* = 6.5 Hz, 2H), 3.59-3.66 (m, 2H), 3.43-3.51 (m, 2H), 2.67-2.70 (m, 2H), 2.61-2.64 (m, 2H), 1.20 (t, *J* = 6.8 Hz, 3H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ = 170.3, 155.6, 148.0, 145.9, 144.9, 133.4, 128.6, 126.1, 124.1, 121.7, 111.9, 79.9, 62.3, 48.5, 45.3, 42.4, 28.3, 12.2. **HRMS** (ESI-Q-TOF): calculated for $C_{22}H_{23}N_2O_6^+$ $[M+H]^+$ 411.1562, found 411.1568.

Synthesis of
***Nα*-amide-[(*R*)-*N*-([ethyl[4-[2-(4-nitrophenyl)ethenyl]phenyl]amino]ethoxy oxobutanoic**
ester)-*S*-[2,3-di(palmitoyloxy)-(2*R*)-propyl]-cysteinyl-L-seryl-L-lysinyl-L-lysinyl-L-lysinyl-L-lysine (1e)



Compound **1e** was synthesized by solid-phase peptide synthesis (SPPS) on Rink Amide resin (100-200 Mesh, 20.0 mg, 0.0108 mmol) using Fmoc protected amino acids (0.0216 mmol) and *N,N'*-diisopropylcarbodiimide (DIC) (3.34 μL, 0.0216 mmol)/HOBt (2.92 mg, 0.0216 mmol) in DMF (100 μL). The following protected amino acids were employed: Fmoc-L-Lys(Boc)-OH (10.12 mg, 0.0216 mmol ×4), Fmoc-L-Ser(Trt)-OH (12.3 mg, 0.0216 mmol) and Fmoc-Pam_2Cys-OH **(2)** [4] (19.32 mg, 0.0216 mmol). Fmoc groups were cleaved by treatment with 20% piperidine in DMF (1 mL, 3 min×1 then 20 min×1). The coupling of compound **S8** (8.88 mg, 0.0216 mmol) was carried out using DIC (3.34 μL, 0.0216 mmol) and HOBt (2.92 mg, 0.0216 mmol) in DMF (100 μL). Progress of the manual couplings was monitored by standard Kaiser test. After completion of the Pam_2CSK_4 synthesis, the resin was thoroughly washed with DMF (3 mL), CH_2Cl_2 (3 mL), and MeOH (3 mL) and dried *in vacuo* to a constant weight.

Cleavage of the peptide from the resin was achieved by stirring the resin with a cleavage cocktail composed of trifluoroacetic acid (TFA), CH_2Cl_2 and triisopropylsilane (TIS) (50:50:2 = in vol. ratio, 1 mL, 3 min×5). The combined solutions were concentrated *in vacuo*. The crude product was purified by HPLC [COSMOSIL 5C_4-AR-300 Packed column, 220 nm, linear gradient of 75–100% (v/v) of MeOH (0.1% TFA) (solvent B) in H_2O (0.1% TFA) (solvent A) over 20 min, 1 mL/min] to give the desired compound **1e** (25% based on Rink amide resin loading capacity). **Absorbance and Emission wavelength** (in PBS): λ_{abs} = 436 nm, λ_{em} = 477 nm. **^{1}H NMR** (400MHz, CD_3OD): δ: 8.18 (d, *J* = 8.8 Hz, 2H), 7.68 (d, *J* = 8.9 Hz, 2H), 7.47 (d, *J* = 8.8 Hz, 2H), 7.31 (d, *J* = 16.4 Hz, 1H), 7.03 (d, *J* = 16.2 Hz, 1H), 6.78 (d, *J* = 8.8 Hz, 2H), 5.21-5.19 (m, 1H), 4.47 (dd, *J* = 8.4, 5.3 Hz, 1H), 4.41-4.38 (m, 1H), 4.36-4.26 (m, 7H), 4.12 (dd, *J* = 11.9, 6.9 Hz, 1H), 3.92-3.90 (m, 1H), 3.80-3.76 (m, 1H), 3.64 (t, *J* = 5.9 Hz, 2H), 3.50-3.47 (m, 2H), 3.12-3.04 (m, 1H), 2.97-2.70 (m, 11H), 2.65-2.63 (m, 2H), 2.59-2.58 (m, 2H), 2.31-2.28 (m, 4H), 1.86-1.63 (m, 16H), 1.58-1.56 (m, 4H), 1.49-1.46 (m, 8H), 1.28-1.26 (br m, 48H), 1.19 (t, *J* = 7.0 Hz, 3H), 0.90-0.88 (m, 6H). **HRMS** (ESI-Q-TOF): calculated for $C_{87}H_{151}N_{13}O_{16}S^+$ $[M+2H]^{2+}$ 833.0556, found 833.0561.

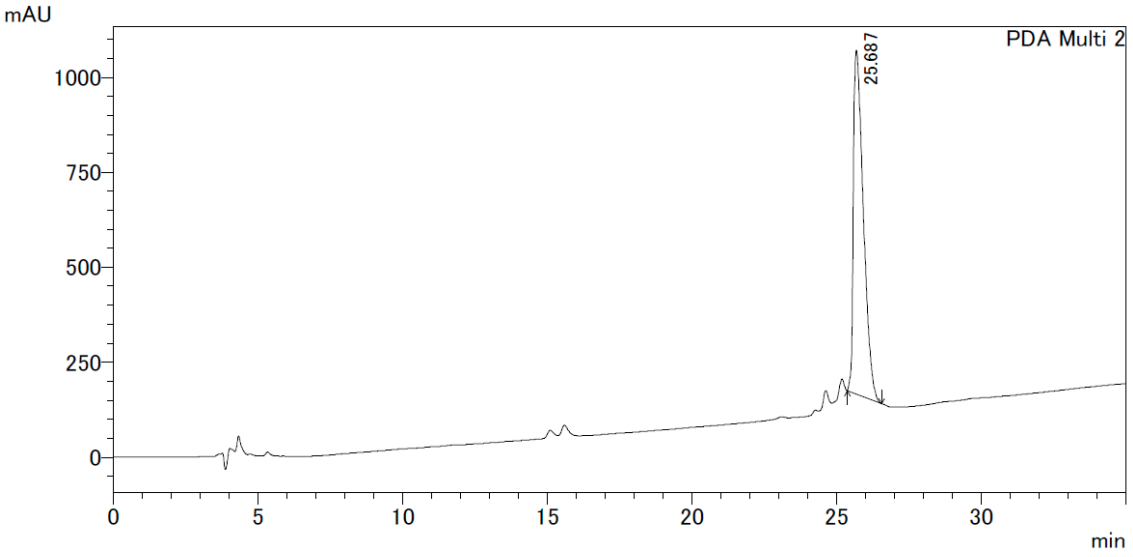
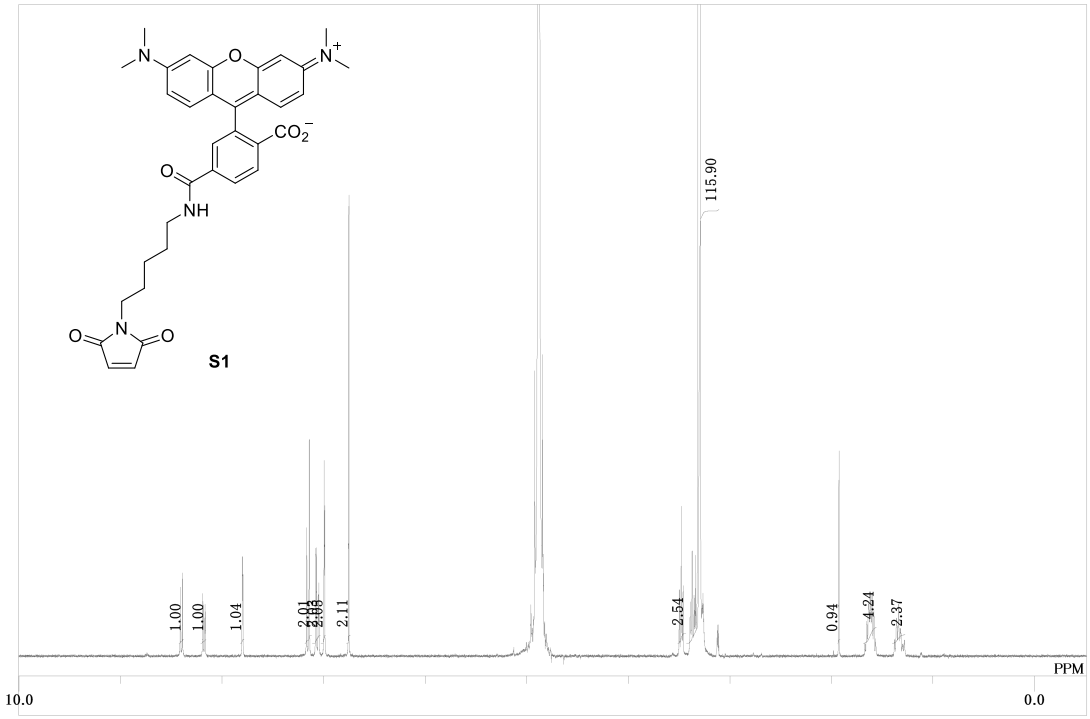


Fig. S7 HPLC elution profile of compound **1e**. COSMOSIL 5C_4-AR-300 Packed column, 220 nm linear gradient of 70–100% (v/v) of MeOH (0.1% TFA) in H_2O (0.1% TFA) over 30 min, 1 mL/min.

References

1) S. R. Banerjee, P. Schaffer, J. W. Babich, J. F. Valliant, J. Zubieta, *Dalton Trams.* 2005, 3886.
2) F. Gao, J. Liu, H. Peng, N. Hu, H. Li, S. Zhang, *J. Fluoresc.* 2010, **20**, 703.
3) T. A. Cross, M. C. Davis, *Synth. Commun.* 2008, **38**, 499.
4) T. Hida, K. Hayashi, K. Yukishige, S. Tanida, N. Kawamura, S. Harada, *J. Antibiot.* 1995, **48**, 589.
5) B. He, J. Du, H. Lin, *J. Am. Chem. Soc.* 2012, **134**, 1922.

2. NMR spectra

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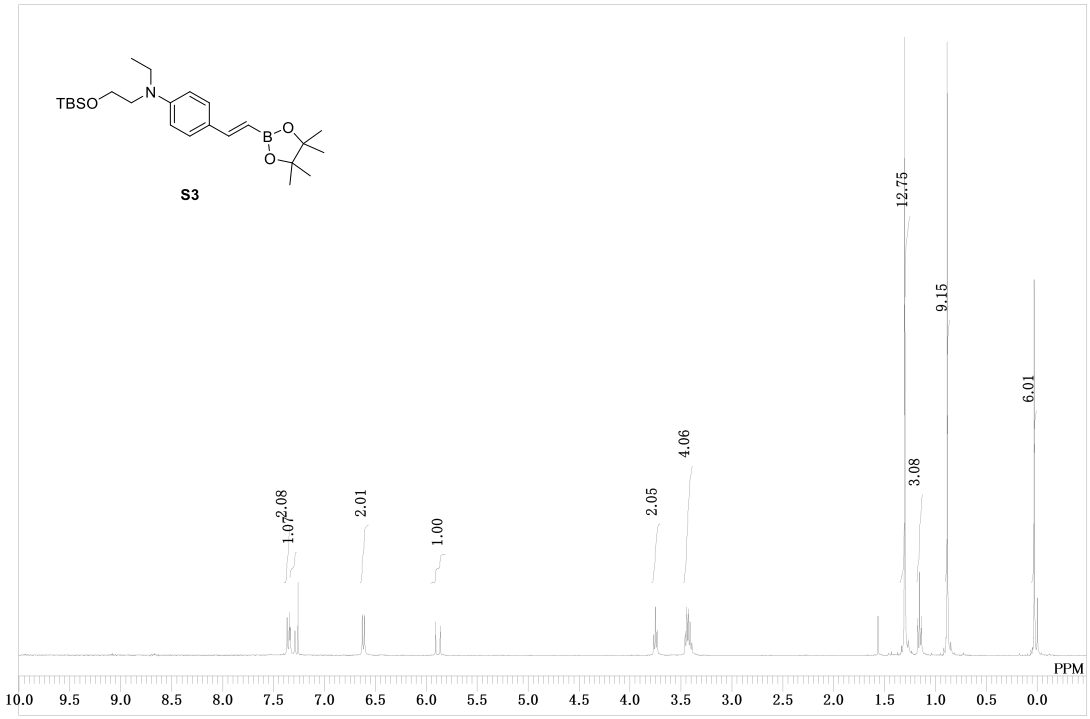
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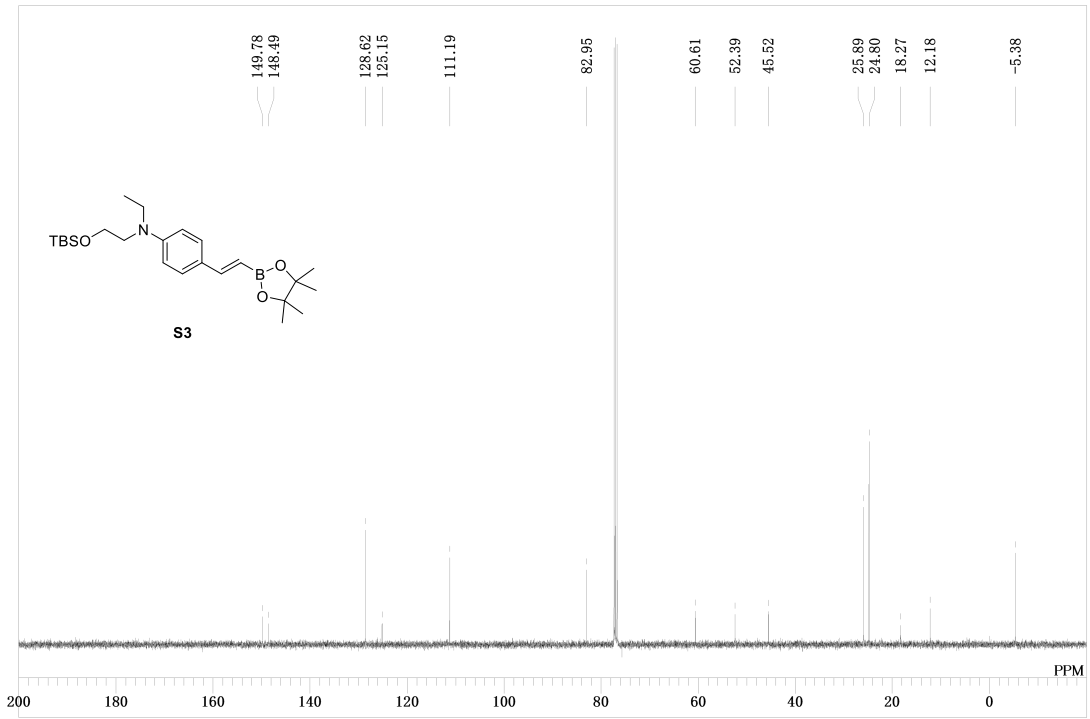
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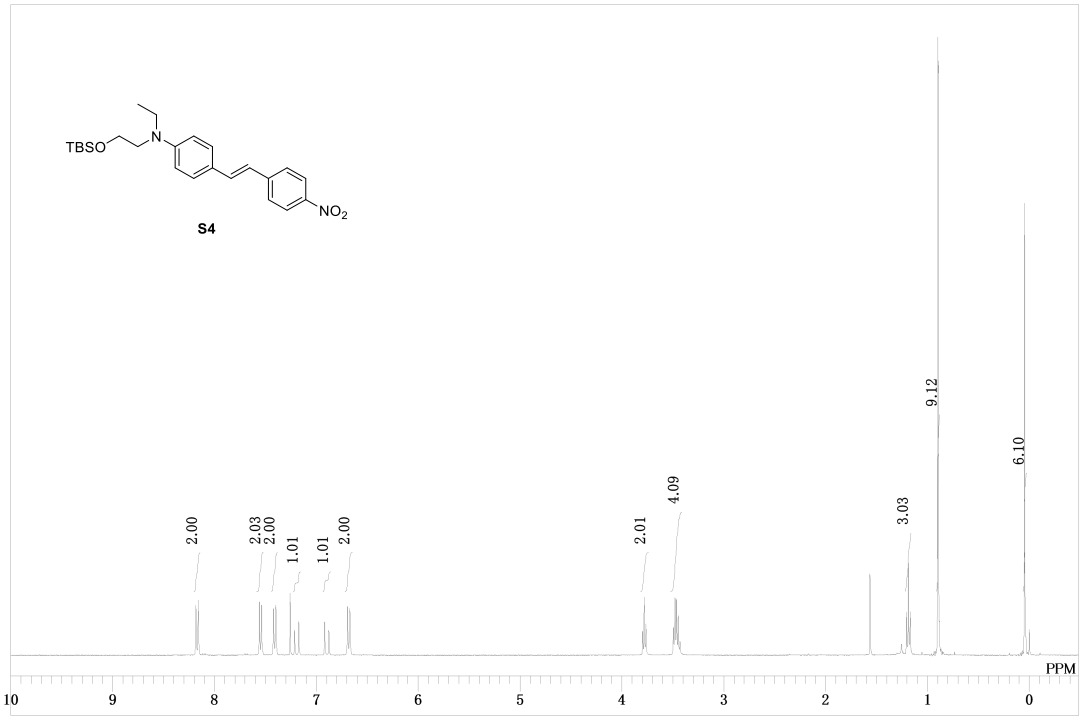
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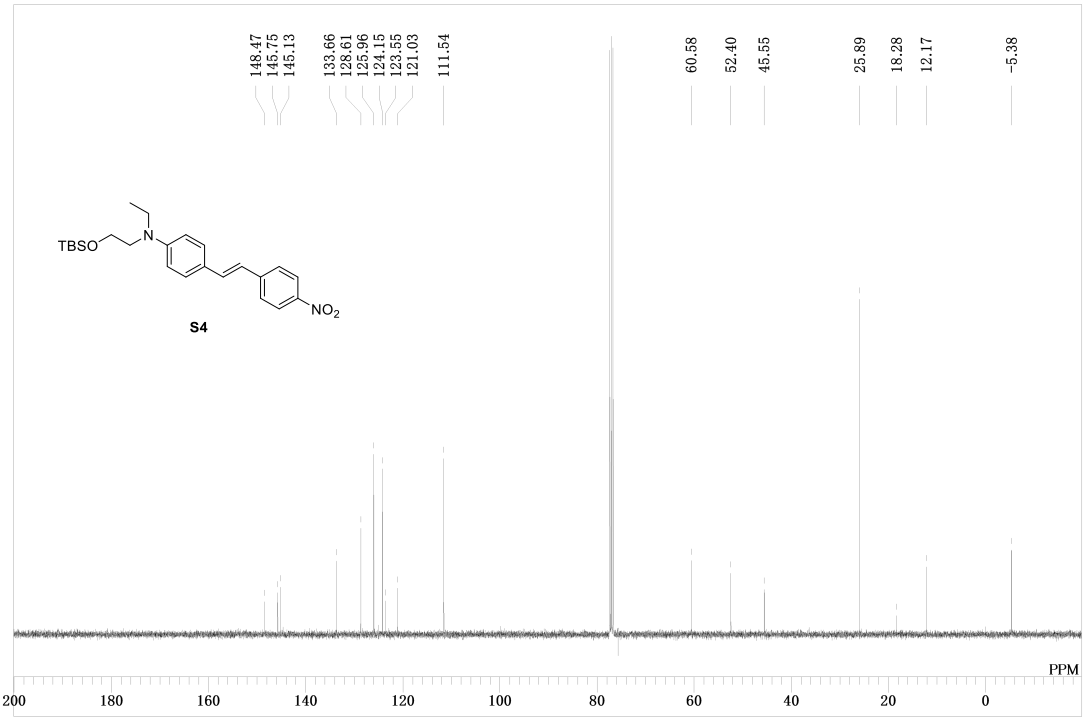
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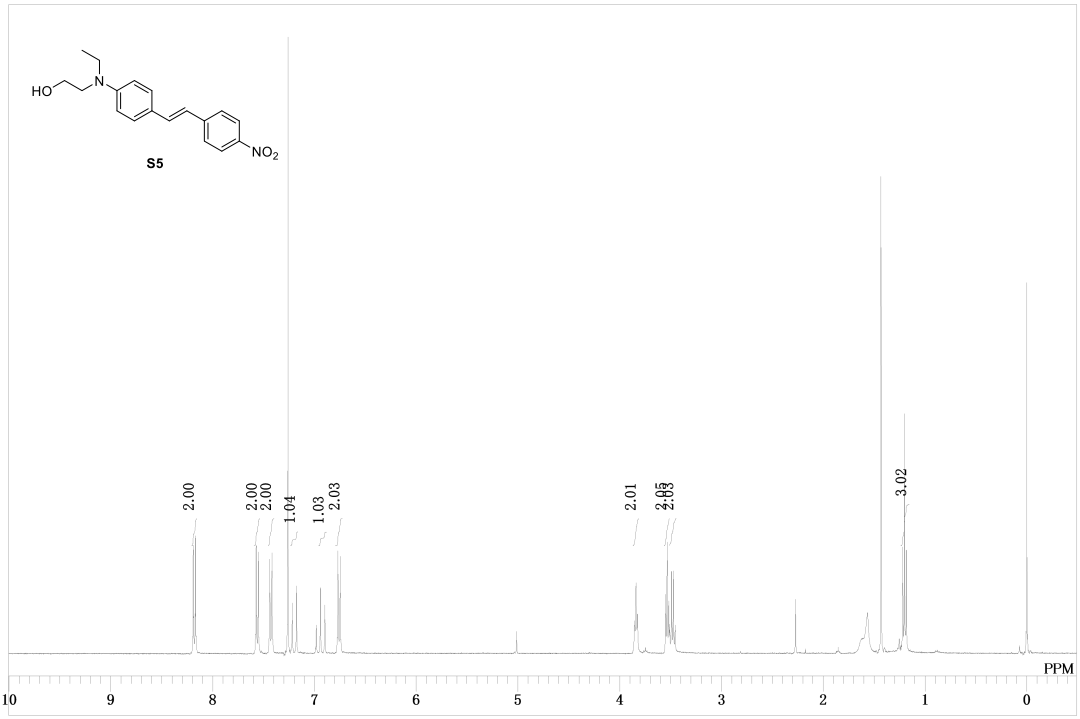
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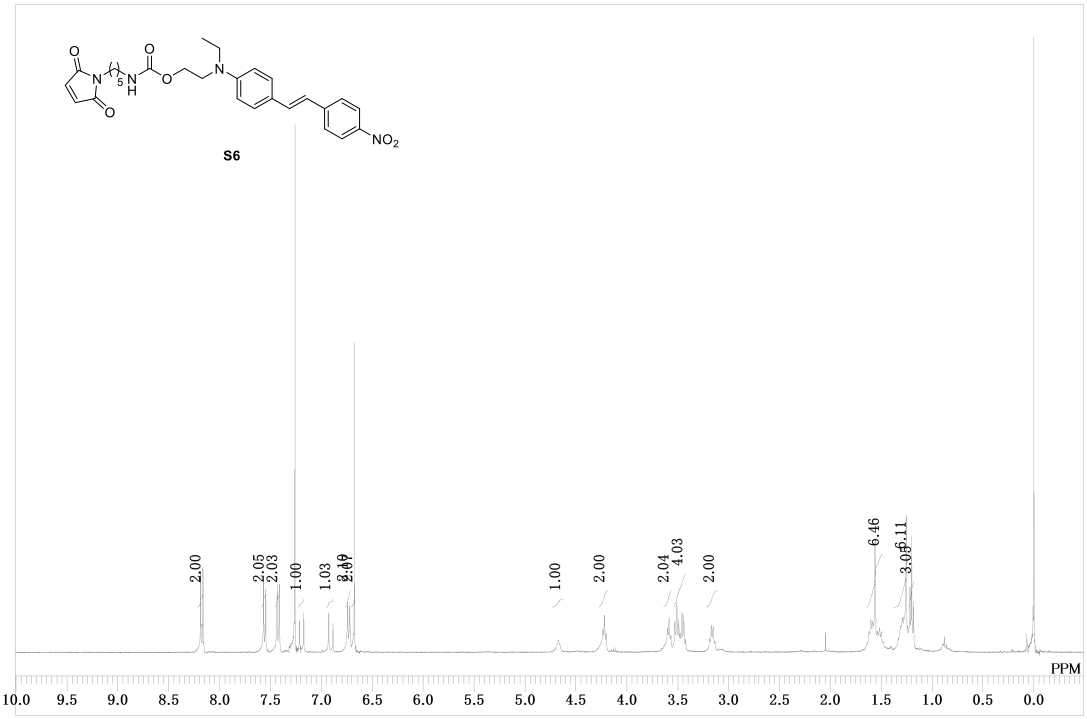
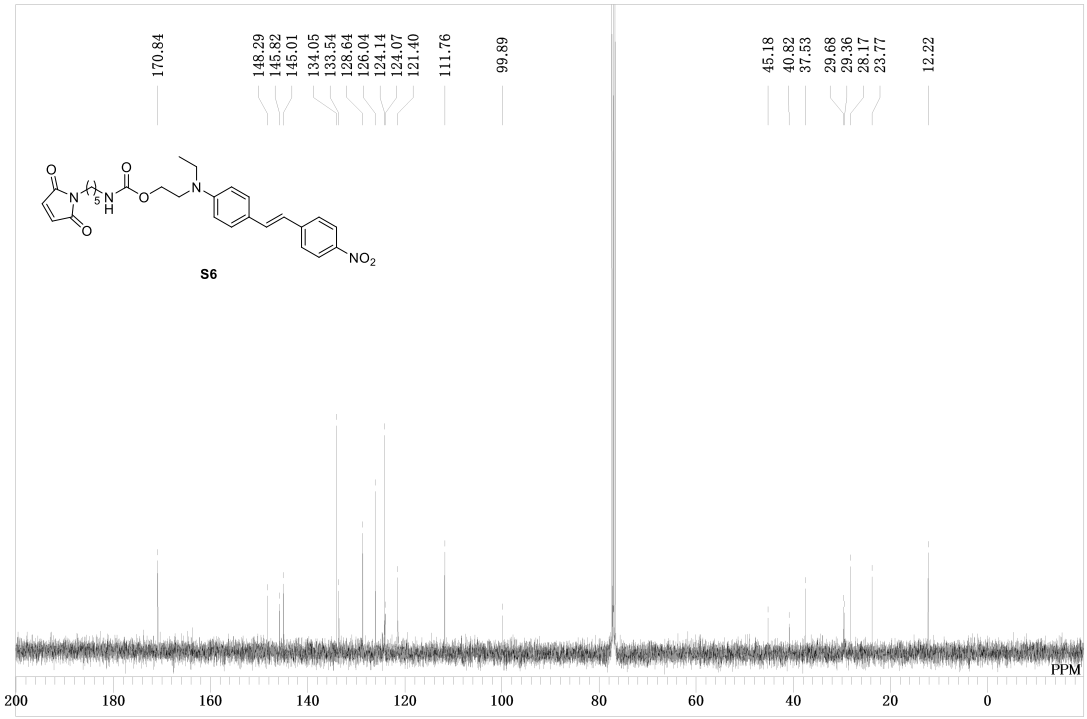
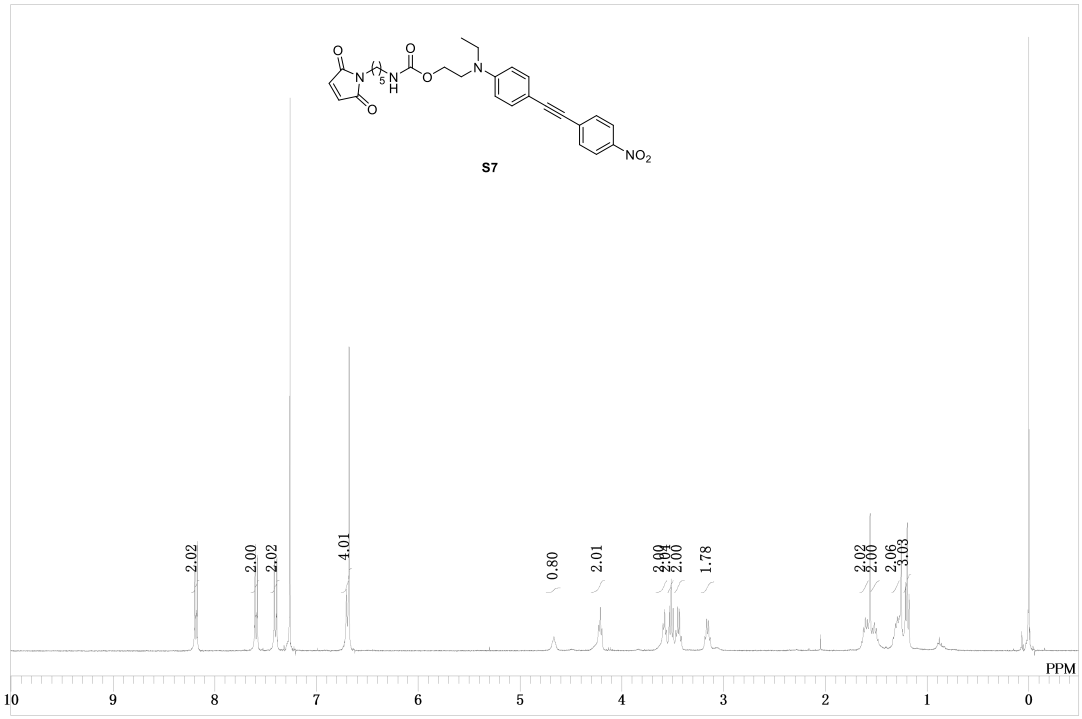
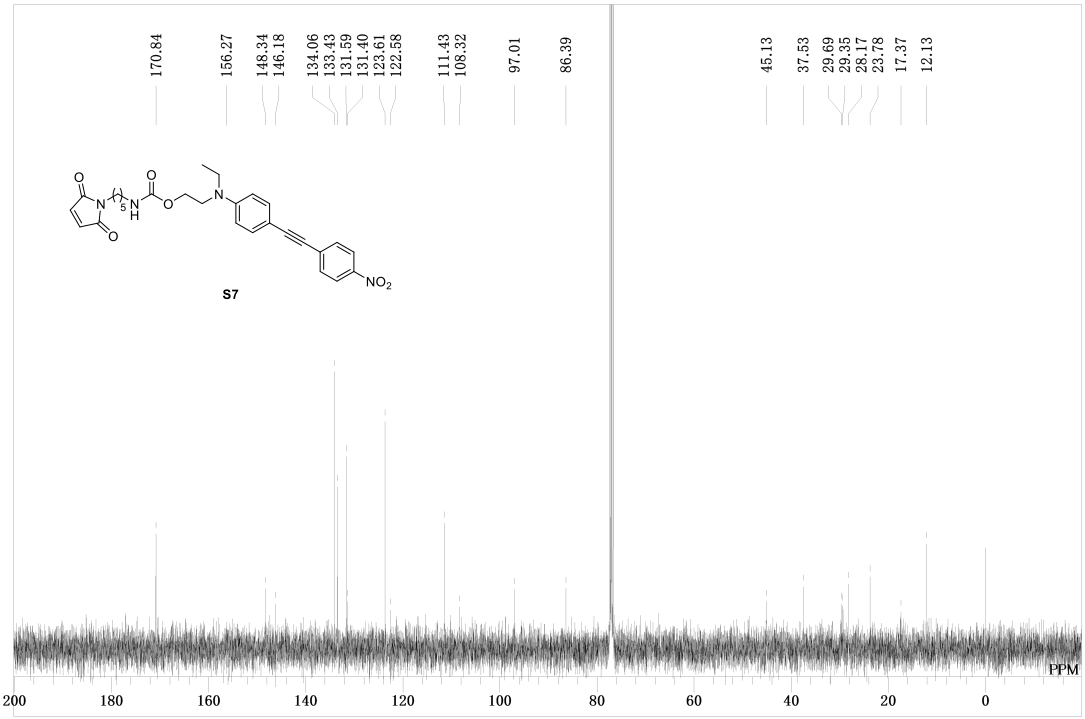
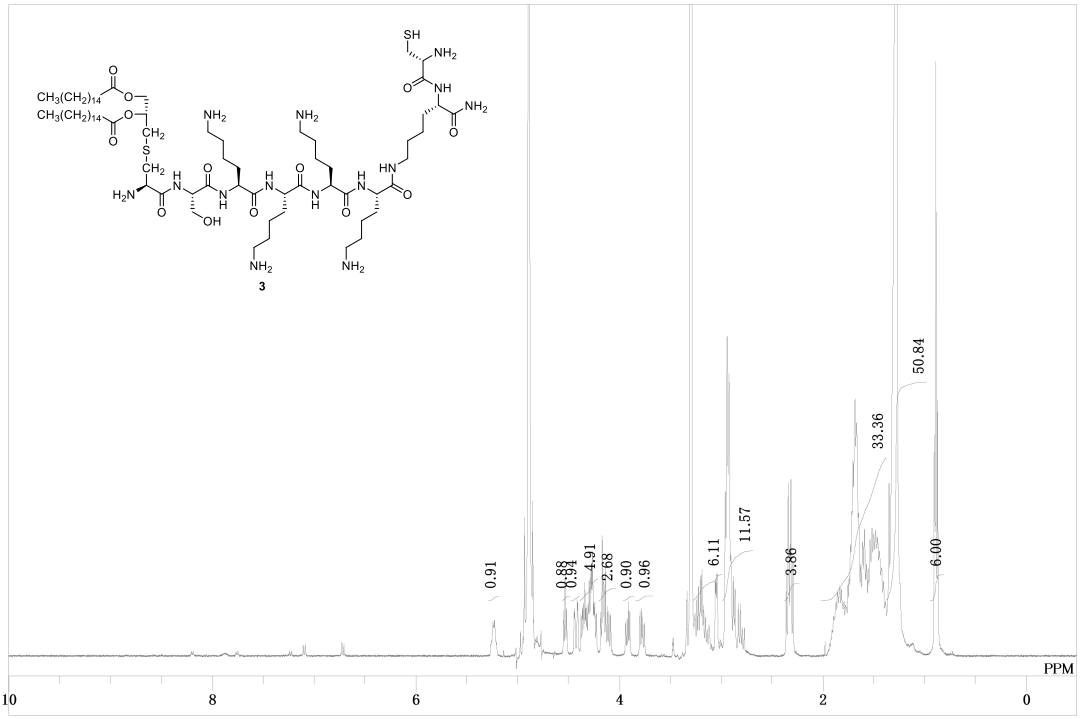
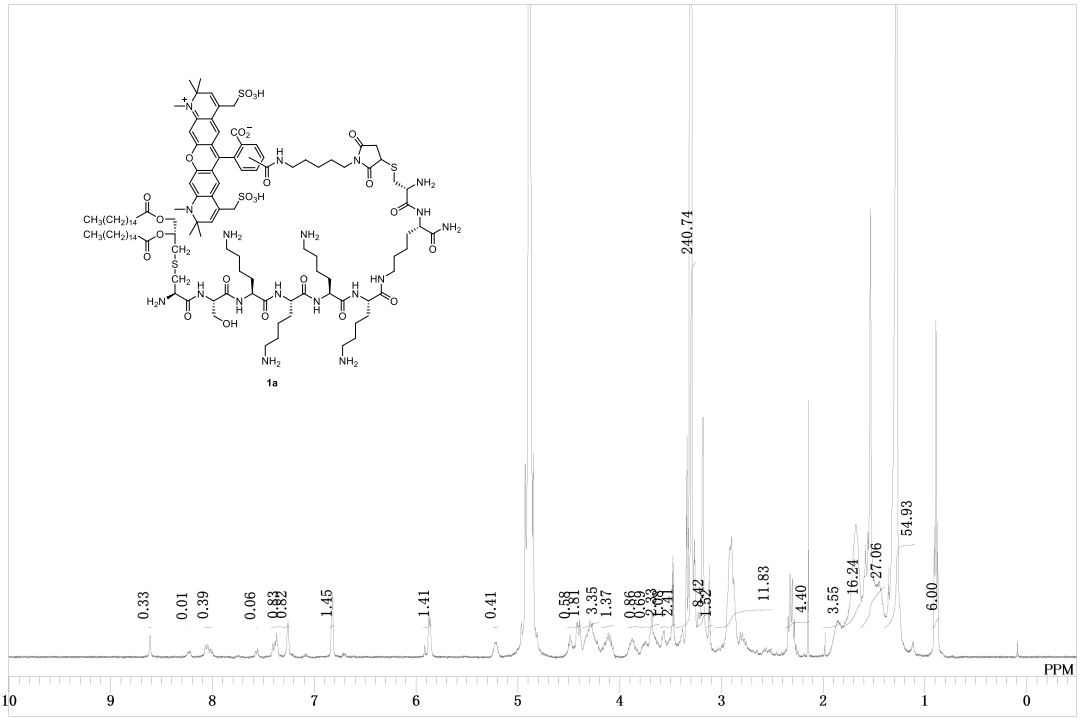

S6
2.00
2.05
2.03
1.00
1.03
1.00
2.00
2.04
4.03
2.00
6.46
6.11
3.05
PPM
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

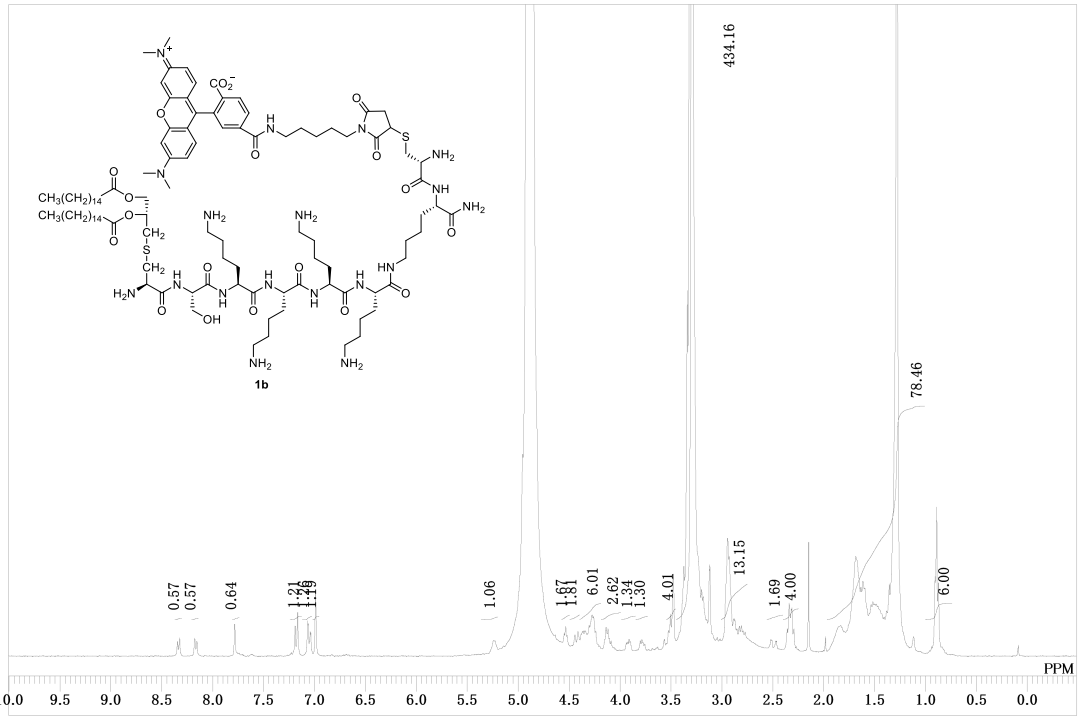

170.84
148.29
145.82
145.01
134.05
133.54
128.64
126.04
124.14
124.07
121.40
111.76
99.89
45.18
40.82
37.53
29.68
29.36
28.17
23.77
12.22
S6
PPM
200 180 160 140 120 100 80 60 40 20 0

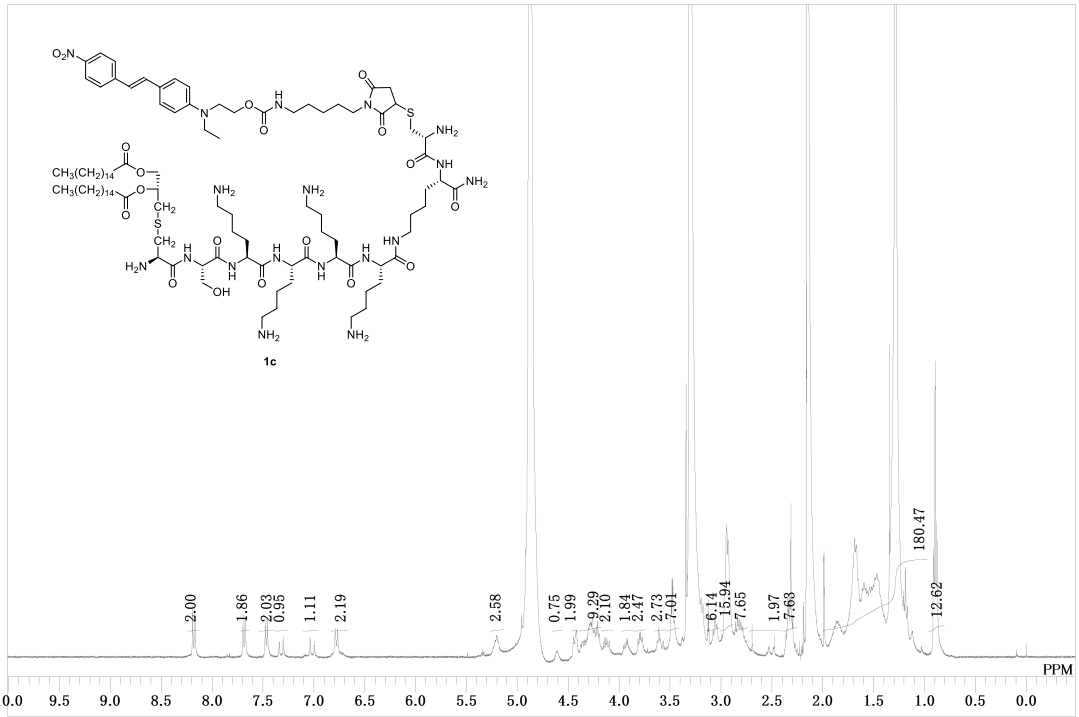

S7
2.02
2.00
2.02
4.01
0.80
2.01
1.78
2.02
2.06
3.03
PPM
10
9
8
7
6
5
4
3
2
1
0

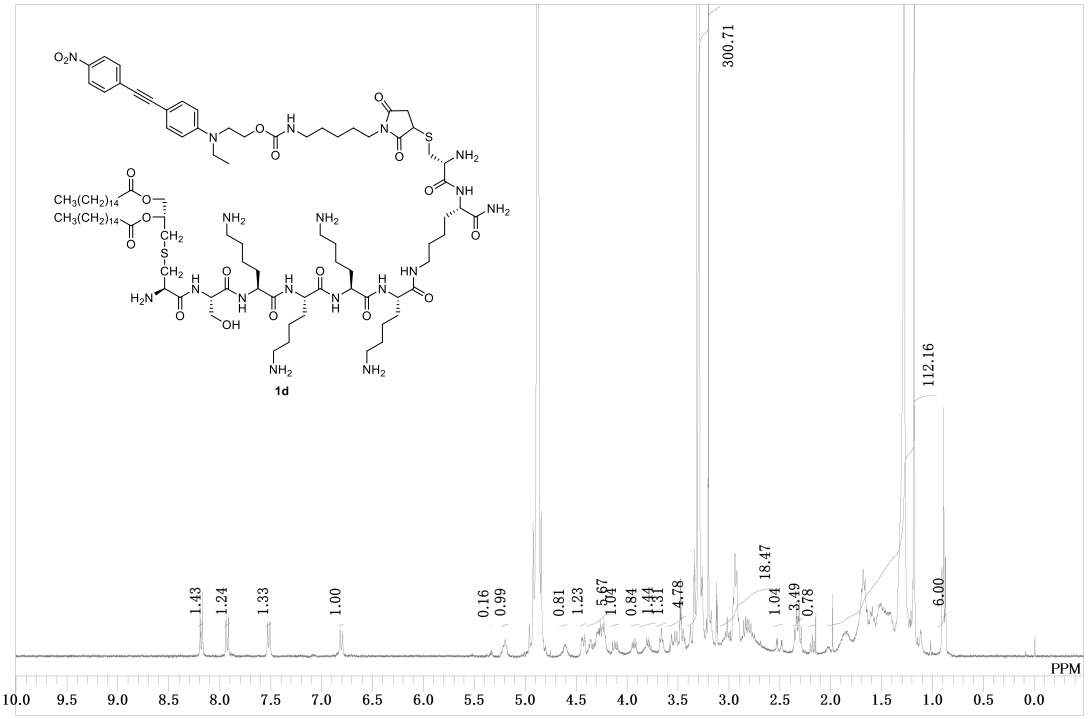

S7
170.84
156.27
148.34
146.18
134.06
133.43
131.59
131.40
123.61
122.58
111.43
108.32
97.01
86.39
45.13
37.53
29.69
29.35
28.17
23.78
17.37
12.13
PPM
200
180
160
140
120
100
80
60
40
20
0

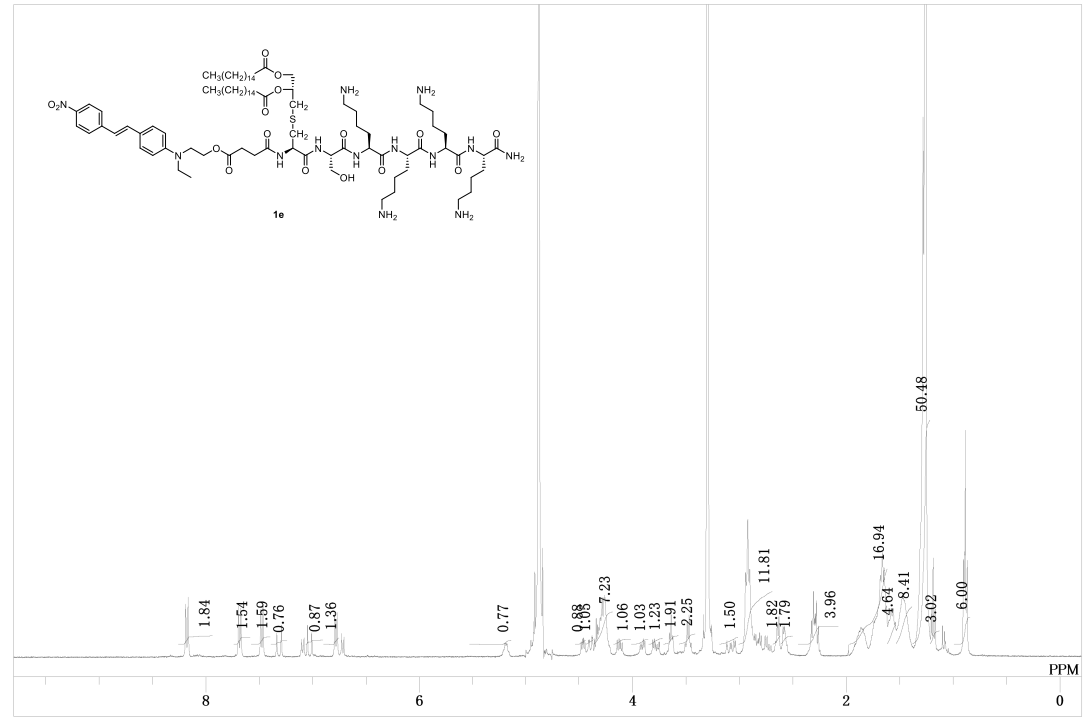

3
0.91
0.88
0.93
4.91
2.68
0.90
0.96
6.11
11.57
3.86
33.36
50.84
6.00
PPM
10
8
6
4
2
0


1a
0.33
0.01
0.39
0.06
1.45
1.41
0.41
3.35
1.37
240.74
1.52
11.83
4.40
3.55
16.24
27.06
54.93
6.00
PPM
10
9
8
7
6
5
4
3
2
1
0


1b
434.16
78.46
13.15
6.01
6.00
4.01
2.62
1.34
1.30
1.69
4.00
1.67
1.06
0.57
0.57
0.64
PPM
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0


1c
180.47
12.62
2.00
1.86
2.03
0.95
1.11
2.19
2.58
0.75
1.99
9.29
2.10
1.84
2.47
2.73
7.01
6.14
15.94
7.65
1.97
7.68
PPM
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0


1d
300.71
112.16
18.47
1.43
1.24
1.33
1.00
0.16
0.99
0.81
1.23
5.67
1.04
0.84
1.44
1.31
4.78
1.04
3.49
0.78
6.00
PPM
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0


1e
50.48
16.94
11.81
1.84
1.54
1.59
0.76
0.87
1.36
0.77
7.23
1.06
1.03
1.23
1.91
2.25
1.50
1.82
1.79
3.96
4.64
8.41
3.02
6.00
PPM
8 6 4 2 0