Supplementary Information

Development of bis-unsaturated ester aldehydes as amino-glue probes: Sequential double azaelectrocyclization as promising strategy for bioconjugation

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General Procedures. All commercially available reagents were used without further purification. Preparative separation was usually performed by column chromatography on silica gel (FUJI silysia LTD, BW-200 and BW-300) and by thin layer chromatography on silica gel (Merck, 20 x 20 cm, Silica gel 60 F_{254}, 1 mm). ^{1}H and ^{13}C NMR spectra were recorded on both JEOL Lambda 500 NMR spectrometer and Delta ECA 500 NMR spectrometer, and chemical shifts were represented as δ-values relative to the internal standard TMS. MALDI-TOF-mass spectra were measured on PerSeptive Biosystems, Voyager RP-DE/H and SHIMADZU AXIMA-CFR mass spectrometers equipped with a nitrogen laser (λ = 337 nm). High resolution mass spectra (HRMS) were measured on a Bruker micro-TOF mass spectrometer under positive electrospray ionization (ESI) conditions. IR spectra were recorded on a *JASCO* FT/IR-8000 Fourier Transform Infrared Spectrometer. Fluorescence images of the labeled and/or bioconjugated cells were captured on OLYMPUS fluorescence microscope, IX71-23FL/DIC.

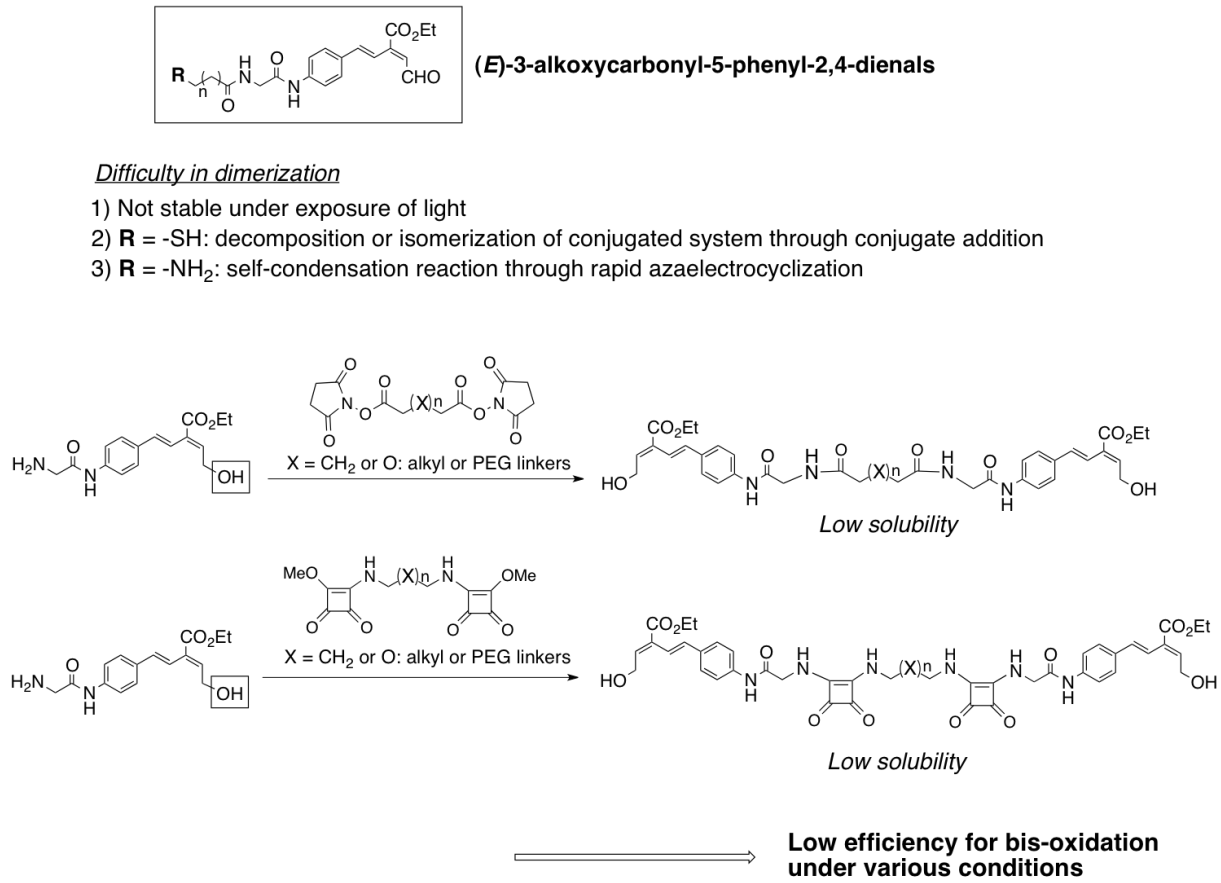
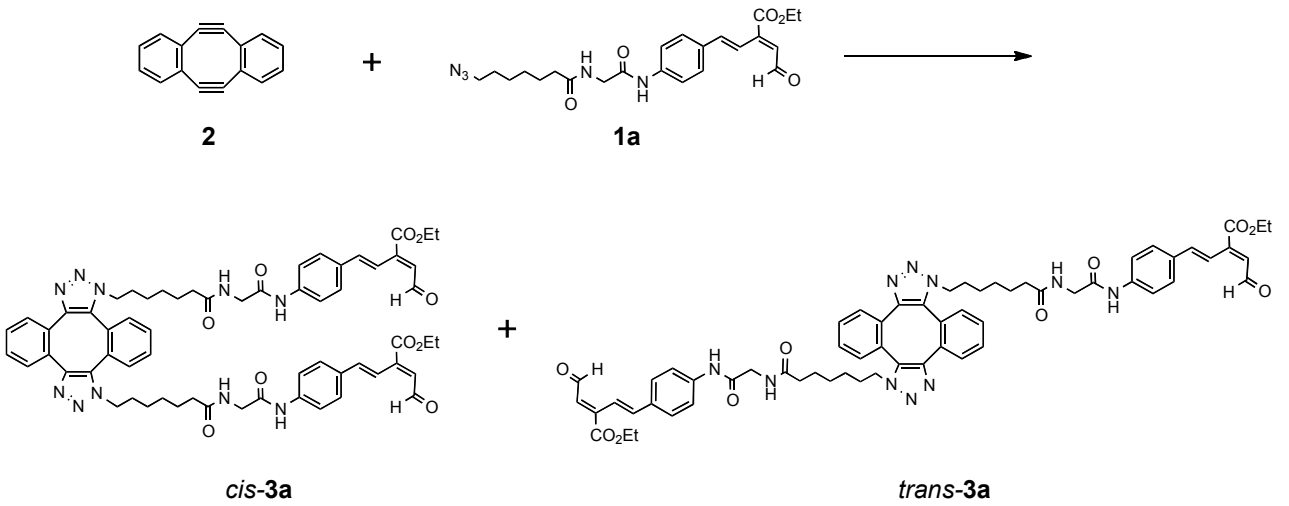


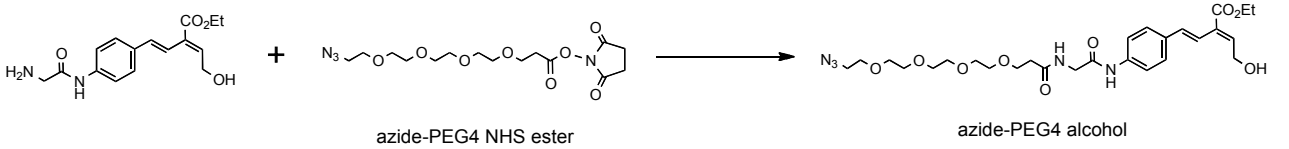
Fig. SI-1 Dimerization trials of (*E*)-3-alkoxycarbonyl-5-phenyl-2,4-dienals.

Preparation of *cis*- and *trans*-3a. To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**2**) (8.0 mg, 40 ⌈mol) in dry $CHCl_3$ (3.0 mL) was added azide aldehyde (**1a**) (36.4 mg, 80 μmol) in $CHCl_3$ (0.2 mL) at room temperature. After stirring for 2 h at this temperature, the mixture was concentrated *in vacuo* to yield the mixture of *cis*- and *trans*-**3a** in quantitative yield as a yellow solid (44.4 mg). The two isomers were separated by repeating the preparative thin layer chromatography on silica gel ($CHCl_3$: MeOH = 25 : 1) to give one isomer of **3a** (isomer-1, 24 mg, 53%) and another isomer of **3a** (isomer-2, 11 mg, 25%). Data for **3a** (isomer-1): ^{1}H NMR (500 MHz, $CDCl_3$) δ = 10.07 (d, 2H, *J* = 7.2 Hz), 9.23 (brs, NH x 2), 7.89 (brt, NH x 2), 7.63 (dd, 2H, *J* = 7.4, 1.4 Hz), 7.51 (d, 4H, *J* = 8.6 Hz), 7.42-7.49 (m, 6H), 7.38 (d, 4H, *J* = 8.6 Hz), 7.26 (d, 2H, *J* = 16.0 Hz), 6.98 (d, 2H, *J* = 16.0 Hz), 6.56 (d, 2H, *J* = 7.2 Hz), 4.37-4.41 (m, 2H), 4.27 (q, 4H, *J* = 7.2 Hz), 4.12-4.18 (m, 2H), 4.07 (t, 4H, *J* = 5.2 Hz), 2.00-2.14 (m, 4H), 1.56-1.61 (m, 2H), 1.33-1.43 (m, 6H), 1.30 (t, 6H, *J* = 7.2 Hz), 0.94-1.07 (m, 4H), 0.63-0.70 (m, 2H), 0.50-0.60 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ =191.3 (2C), 175.0 (2C), 168.2 (2C), 166.5 (2C), 146.0 (2C), 144.9 (2C), 141.0 (2C), 139.4 (2C), 135.0 (2C), 132.3 (2C), 131.5 (2C), 131.2 (2C), 130.7 (2C), 130.4 (2C), 129.5 (2C), 129.3 (2C), 128.4 (4C), 126.5 (2C), 119.9 (4C), 118.2 (2C), 62.0 (2C), 48.5 (2C), 45.1 (2C), 35.8 (2C), 29.4 (2C), 28.0 (2C), 25.4 (2C), 25.1 (2C), 14.1 (2C); IR (KBr, cm^{-1}) 3276, 2931, 1663, 1530, 1246, 755; ESI-MS *m/z* calcd for $C_{62}H_{66}N_{10}O_{10}Na$ $(M+Na)^+$ 1133.4856, found 1133.4857.

Data for **3a** (isomer-2): ^{1}H NMR (500 MHz, $CDCl_3$) δ = 10.06 (d, 2H, *J* = 7.2 Hz), 9.37 (brs, NH x 2), 7.55-7.56 (m, 2H), 7.49-7.51 (m, 6H), 7.36-7.41 (m, 6H), 7.25-7.28 (m, 4H), 6.97 (d, 2H, *J* = 16.0 Hz), 6.56 (d, 2H, *J* = 7.2 Hz), 4.31-4.39 (m, 2H) 4.27 (q, 4H, *J* = 7.2 Hz), 4.09-4.15 (m, 2H), 3.92-4.02 (m, 4H), 2.11-2.15 (m, 4H), 1.64-1.70 (m, 4H), 1.45-1.50 (m, 4H), 1.29 (t, 6H, *J* = 7.2 Hz), 1.11-1.18 (m, 8H); ^{13}C NMR (125 MHz, $CDCl_3$) δ =191.4 (2C), 174.6 (2C), 168.0 (2C), 166.4 (2C), 146.1 (2C), 145.9 (2C), 140.9 (2C), 139.1 (2C), 133.3 (2C), 132.3 (2C), 131.7 (2C), 130.8 (2C), 130.7 (2C), 130.4 (2C), 130.2 (2C), 129.2 (2C), 128.43 (4C), 128.35 (2C), 120.0 (4C), 118.4 (2C), 62.0 (2C), 48.5 (2C), 44.6 (2C), 35.6 (2C), 29.9 (2C), 28.4 (2C), 26.1 (2C), 25.1 (2C), 14.1 (2C); IR (KBr, cm^{-1}) 3280, 2931, 1663, 1520, 1247, 756; ESI-MS *m/z* calcd for $C_{62}H_{66}N_{10}O_{10}Na$ $(M+Na)^+$ 1133.4856, found 1133.4857.

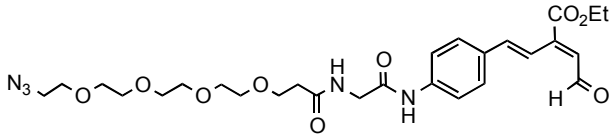


Preparation of *cis*- and *trans*-3b. To a solution of aminoalcohol (structure shown below, 46 mg, 150 μmol) in DMF (1.2 mL) was added azide-PEG4 NHS ester (58 mg, 150 μmol) at room temperature. After stirring for overnight at this temperature, the mixture was concentrated *in vacuo*. The residue was purified by preparative thin layer chromatography on silica gel ($CHCl_3$: MeOH = 8 : 1) to give the corresponding azide-PEG4 alcohol (54 mg, 62%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ = 8.84 (brs, NH), 7.60 (brs, NH), 7.54 (d, 2H, J = 8.6 Hz), 7.35 (d, 2H, J = 8.6 Hz), 6.82 (t, 1H, J = 6.0 Hz), 6.75 (d, 2H, J = 12.0 Hz), 4.54 (d, 2H, J = 6.0 Hz), 4.27 (q, 2H, J = 7.2 Hz), 4.08 (d, 2H, J = 6.0 Hz), 3.83 (t, 2H, J = 5.5 Hz), 3.70-3.72 (m, 2H), 3.60-3.64 (m, 12H), 3.37 (t, 2H, J = 5.2 Hz), 2.56 (t, 2H, J = 5.5 Hz), 1.34 (t, 3H, J = 7.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 172.9, 167.8, 166.9, 140.5 (2C), 138.0, 134.3, 132.8, 130.8, 127.3 (2C), 119.9 (2C), 70.6, 70.55 (2C), 70.5, 70.3, 70.2, 70.0, 67.5, 61.1, 59.8, 50.6, 44.5, 36.7, 14.3 ; IR (KBr, cm^{-1}) 3272, 2875, 2108, 1653, 1535, 1252, 1109; ESI-MS *m/z* calcd for $C_{27}H_{39}N_5O_9Na$ $(M+Na)^+$ 600.2640, found 600.2640.



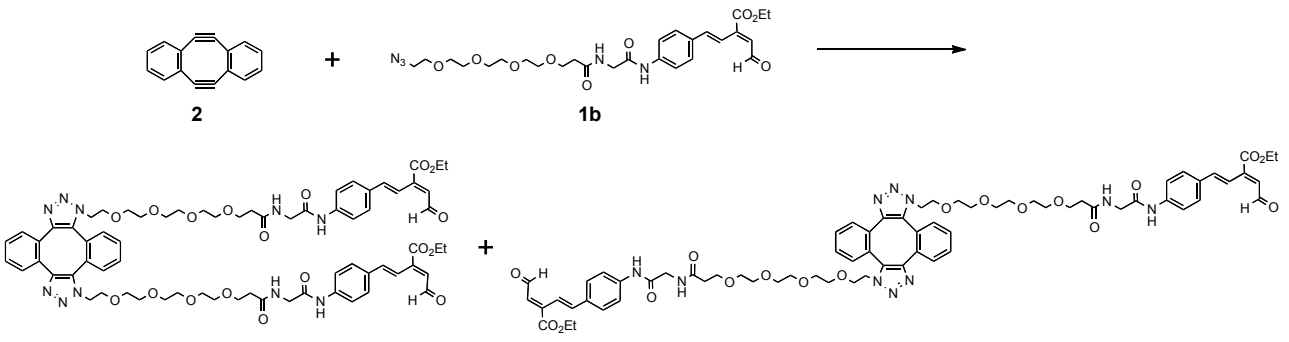
To a solution of the azide-PEG4 alcohol obtained above (22 mg, 38 μmol) in CH_2Cl_2 (2.0 mL) was added Dess-Martin periodinane (16 mg, 38 μmol) at room temperature. After stirring for 15 min at this temperature, the mixture was concentrated under reduced pressure. The residue was purified by preparative thin layer chromatography on silica gel ($CHCl_3$: MeOH = 8 : 1) to give the corresponding aldehyde **1b** (16.5 mg, 76%) as a yellow solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ = 10.14 (d, 1H, J = 7.2 Hz), 8.93 (brs, NH), 7.63 (d, 2H, NH, J = 8.6 Hz), 7.47 (d, 2H, J = 8.6 Hz), 7.32 (d, 1H, J = 16.0 Hz), 7.05 (d, 1H, J = 16.0 Hz), 6.64 (d, 1H, J = 7.2 Hz), 4.35 (q, 2H, J = 7.2

Hz), 4.10 (d, 2H, J = 6.0 Hz), 3.85 (t, 2H, J = 5.4 Hz), 3.72-3.74 (m, 2H), 3.60-3.65 (m, 12H), 3.38 (t, 2H, J = 5.0 Hz), 2.57 (t, 2H, J = 5.4 Hz), 1.38 (t, 3H, J = 7.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 191.3, 172.9, 168.0, 166.5, 146.1, 141.1, 139.5, 131.4, 130.8, 128.4 (2C), 119.8 (2C), 118.1, 70.6, 70.5 (2C), 70.3 (2C), 70.2, 69.9, 67.6, 62.0, 50.6, 44.7, 36.7, 14.1 ; IR (KBr, cm^{-1}) 3317, 2872, 2104, 1668, 1532, 1248, 1114; ESI-MS *m/z* calcd for $C_{27}H_{37}N_5O_9Na$ $(M+Na)^+$ 598.2484, found 598.2484.



1b

To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**2**) (2.6 mg, 13 μmol) in dry $CHCl_3$ (0.5 mL) was added aldehyde (**1b**) (15.1 mg, 26 μmol) in $CHCl_3$ (1.0 mL) at room temperature. After stirring for 2 h at this temperature, the mixture was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$: MeOH = 15 : 1) to give a mixture of *cis*- and *trans*-**3b** (17.7 mg, 100%) as a yellow solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ = 10.14 (d, 2H, J = 7.2 Hz), 9.15 (brs, NH x 2), 7.39-7.72 (m, 18H), 7.32 (d, 2H, J = 15.8 Hz), 7.04 (d, 2H, J = 15.8 Hz), 6.63 (d, 2H, J = 6.9 Hz), 4.44-4.49 (m, 2H), 4.27-4.37 (m, 6H), 4.05-4.09 (m, 4H), 3.77-3.81 (m, 6H), 3.34-3.67 (m, 26H), 2.51-2.55 (m, 4H), 1.37 (t, 6H, J = 7.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 191.3 (2C), 172.8 (2C), 168.0 (2C), 166.5 (2C), 146.1 (2C), 145.6, 144.9, 141.2 (2C), 139.6 (2C), 135.4, 134.6, 132.5, 131.4 (2C), 131.31, 131.27, 130.9, 130.7 (2C), 130.4, 130.2, 130.1, 129.2, 129.1, 128.5, 128.4 (4C), 128.1, 126.4, 119.9 (4C), 118.0 (2C), 70.5 (2C), 70.5 (2C), 70.4 (2C), 70.34, 70.30, 70.21 (2C), 70.15 (2C), 69.2, 69.1, 67.4 (2C), 62.2, 62.0, 48.3, 48.2, 44.5 (2C), 36.7 (2C), 14.1 (2C); IR (KBr, cm^{-1}) 3324, 2924, 1668, 1532, 1250, 1113, 755; ESI-MS *m/z* calcd for $C_{70}H_{82}N_{10}O_{18}Na$ $(M+Na)^+$ 1373.5701 , found 1373.5703.

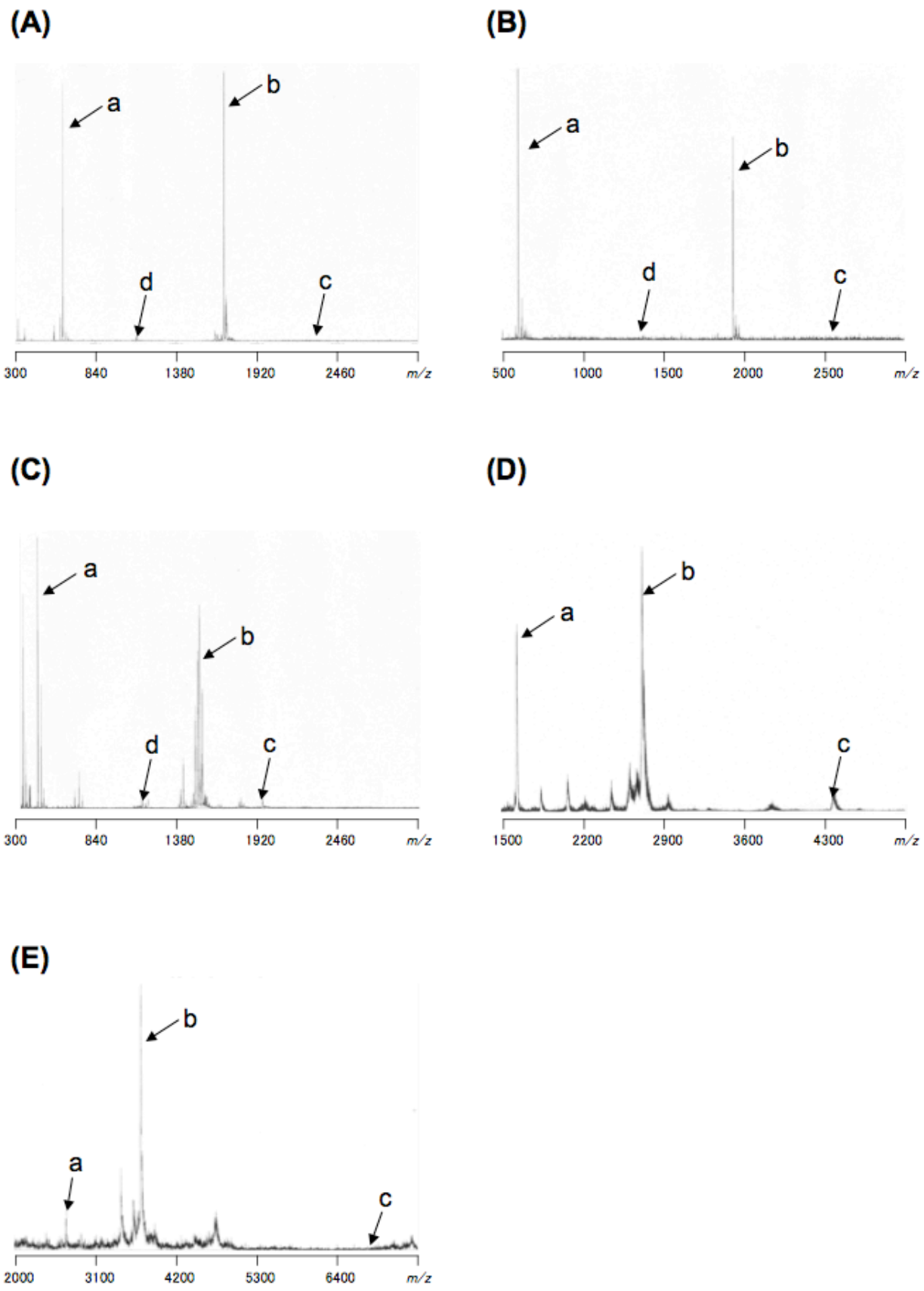


2 + **1b**

mixture of *cis*- and *trans*-**3b**

General protocol for protein conjugation. To a solution of cyclic RGDyK peptide (620 μg , 1.0

μmol) in PBS (25 μL) was added a solution of *cis*- and/or *trans*-**3a** (550 μg, 500 nmol) in DMSO (25 μL) (concentration of **3a**: 10 mM). After the resulting solution was kept at 25 ℃ for 10 min, 10 μL of the reaction mixture was added to a solution of HSA in PBS (66 μg, 1.0 nmol, 90 μL) (final reaction concentrations: 1.0×10^{-3} M for cyclic RGDyK+**3a** and 1.0×10^{-5} M for HSA, containing 5% DMSO). After the resulting solution was kept at 37 ℃ for 30 min, the mixture was directly analyzed by MALDI-TOF-MS.



Figs. SI-2 MALDI-TOF-MS spectra of mono-azaelectrocyclization products by the reaction of **3** with various peptides (peptide: 20 mM, **3**: 10 mM, 25 °C, 10 min in PBS buffer containing 50% DMSO).

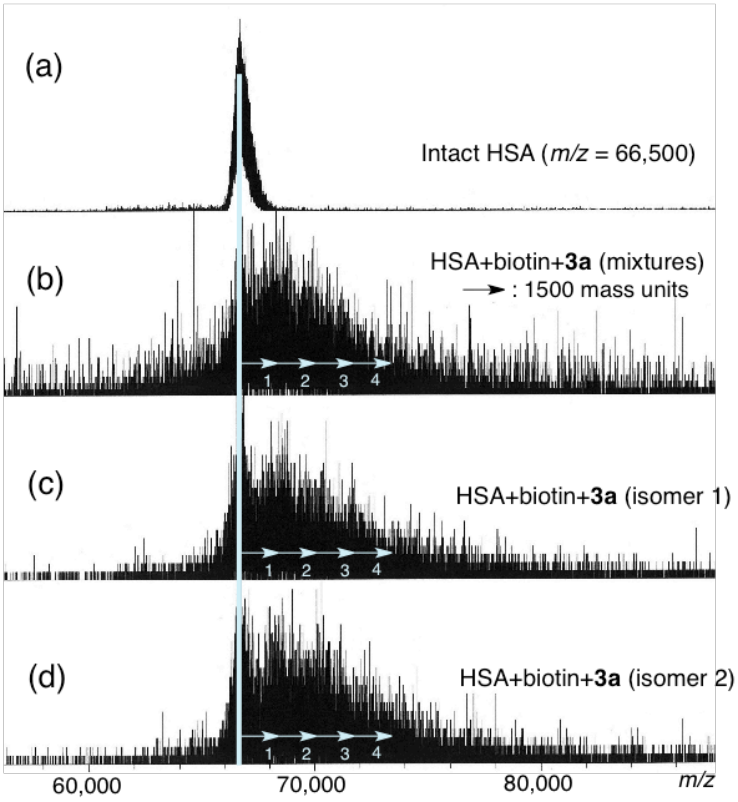
(A) Cyclic RGDyK + **3a** (mixtures). Peak **a**: Cyclic RGDyK (*m/z* calcd for $C_{27}H_{42}N_9O_8$ $(M+H)^+$ 620.3, found 620.5). Peak **b**: cyclic RGDyK＋**3a** (mono-azaelectrocyclization product, *m/z* calcd for $C_{89}H_{106}N_{19}O_{17}$ $(M-O+H)^+$ 1696.8, found 1696.9). Peak **c**: 2 molecules of RGDyK＋**3a** (homo-coupling product, not detected). Peak **d**: **3a** (not detected).

(B) Cyclic RGDyK + **3b** (mixtures). Peak **a**: Cyclic RGDyK. Peak **b**: Cyclic RGDyK＋**3b** (mono-azaelectrocyclization product, *m/z* calcd for $C_{97}H_{122}N_{19}O_{25}$ $(M-O)^+$ 1936.9, found 1936.6). Peak **c**: 2 molecules of RGDyK＋**3b** (homo-coupling product, not detected). Peak **d**: **3b** (not detected).

(C) Biotin-PEG-NH_2 + **3a** (mixtures). Peak **a**: Biotin-PEG-NH_2 (*m/z* calcd for $C_{18}H_{35}N_4O_5S$ $(M+H)^+$ 419.2, found 419.8). Peak **b**: Biotin-PEG-NH_2＋**3a** (mono-azaelectrocyclization product, *m/z* calcd for $C_{80}H_{99}N_{14}O_{14}S$ $(M-O)^+$ 1494.7, found 1494.8). Peak **c**: 2 molecules of biotin＋**3a** (homo-coupling product, almost negligible). Peak **d**: **3a** (almost negligible).

(D) Somatostatin + **3a** (mixtures). Peak **a**: Somatostatin (*m/z* calcd for $C_{76}H_{104}N_{17}O_{20}S_2$ $(M+H)^+$ 1638.7 found 1638.7). Peak **b**: Somatostatin＋**3a** (mono-azaelectrocyclization product, *m/z* calcd for $C_{138}H_{168}N_{27}O_{29}$ $(M+H-H_2O)^+$ 2714.2, found 2714.3). Peak **c**: 2 molecules of somatostatin＋**3a** (homo-coupling product, very small peak).

(E) Disialoglycopeptide **4a** + **3a** (mixtures). Peak **a**: Disialoglycopeptide **4a** (*m/z* calcd for $C_{112}H_{190}N_{15}O_{70}$ $(M+H)^+$ 2866.2 found 2866.1). Peak **b**: **4a**＋**3a** (mono-azaelectrocyclization product, *m/z* calcd for $C_{174}H_{254}N_{25}O_{79}$ $(M-O+H)^+$ 3942.7, found 3942.4). Peak **c**: 2 molecules of **4a**＋**3a** (not detected).



Figs. SI-3 MALDI-TOF-MS of HSA (10^{-5} M) conjugated to the biotin-PEG-NH_2 pretreated with **3a** (**3a**: 10^{-3} M, 37 °C, 30 min in PBS buffer containing 5% DMSO). (a) Intact HSA. (b) Conjugation in the presence of the *cis*- and *trans*-mixture of **3a** (theoretical mass unit difference for each conjugation product: *m/z* = 1493.8). (c) Conjugation of one isomer of **3a** (isomer-1). (d) Conjugation of another isomer of **3a** (isomer-2).

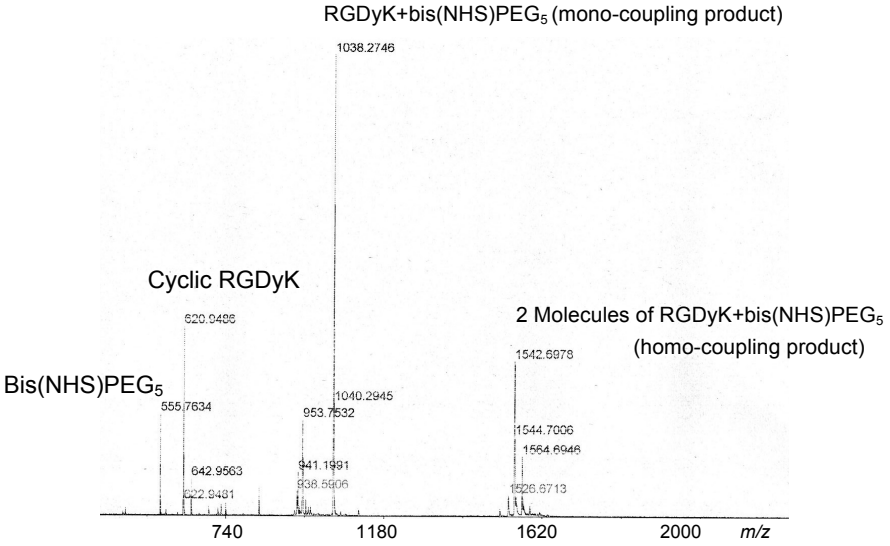


Fig. SI-4 MALDI-TOF-MS spectrum of the reaction between bis(NHS)PEG_5 and cyclic RGDyK under the identical conditions applied to Figs. SI-2. Structure of bis(NHS)PEG_5 is shown in Fig. SI-1 (X = -CH_2OCH_2-, n = 5). ***Note that the significant amount of the homo-coupling product is produced***. RGDyK+bis(NHS)PEG_5 (mono-coupling product, *m/z* calcd for $C_{45}H_{69}N_{10}O_{18}$ $(M+H)^+$ 1037.5, found 1038.3). 2 Molecules of RGDyK+bis(NHS)PEG_5 (homo-coupling product, *m/z* calcd for $C_{68}H_{105}N_{18}O_{23}$ $(M+H)^+$ 1541.8, found 1542.7).

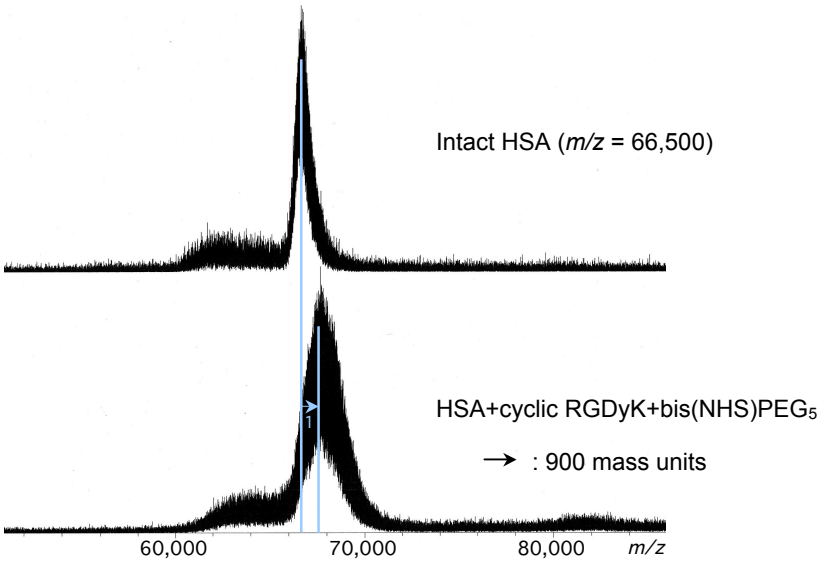


Fig. SI-5 MALDI-TOF-MS of HSA (10^{-5} M) conjugated to the mono-coupling product of RGDyK peptide with bis(NHS)PEG_5 (10^{-3} M), which was obtained in **Fig. SI-4** and isolated by HPLC. Bioconjugation was performed under the identical conditions in Fig. 3. Theoretical mass unit difference for each conjugation product: *m/z* = 923.5.

FITC-labeling of disialoglycopeptide 4a. Disialoglycopeptide **4a** (3.0 mg, 1.0 μmol) was added to a solution of 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester (470 μg, 1.0 μmol) in DMF (200 μL) and H_2O (200 μL) at room temperature. After the mixture was stirred overnight at room temperature, the solvent was removed *in vacuo* and the residue was purified by HPLC [column: Nacalai Tesque 5C_{18}-AR300, 4.6×250 mm; MeCN in H_2O (containing 0.1% TFA); 10-100% gradient over 40 min; 1 mL/min; UV detection at 245 nm]. The fractions **a** (retention time at 11.9 min), **b** (retention time at 12.5 min), and **c** (retention time at 12.8 min) contain the desired mono-labeled products, which were lyophilized to give the FITC-labeled **4b**, 930 μg, 770 μg, and 780 μg, respectively. Fraction **a**, which was expected to be the *N*-terminus labeling product, was used for cell surface bioconjugation trials: MALDI-TOF-MS detected the three peaks characteristic for disialoglycans (see ref. 8c and 14 in the text): *m/z* calcd for $C_{133}H_{200}N_{15}O_{76}$ (disialoside-form, $M+H)^+$ 3225.1, found 3225.6, for $C_{122}H_{181}N_{14}O_{68}Na$ (monosialoside-form, $M-H+Na)^+$ 2954.8, found 2954.3, and for $C_{111}H_{164}N_{13}O_{60}Na_2$ (asialo-form, $M-H+Na+Na)^+$ 2686.5, found 2686.5.

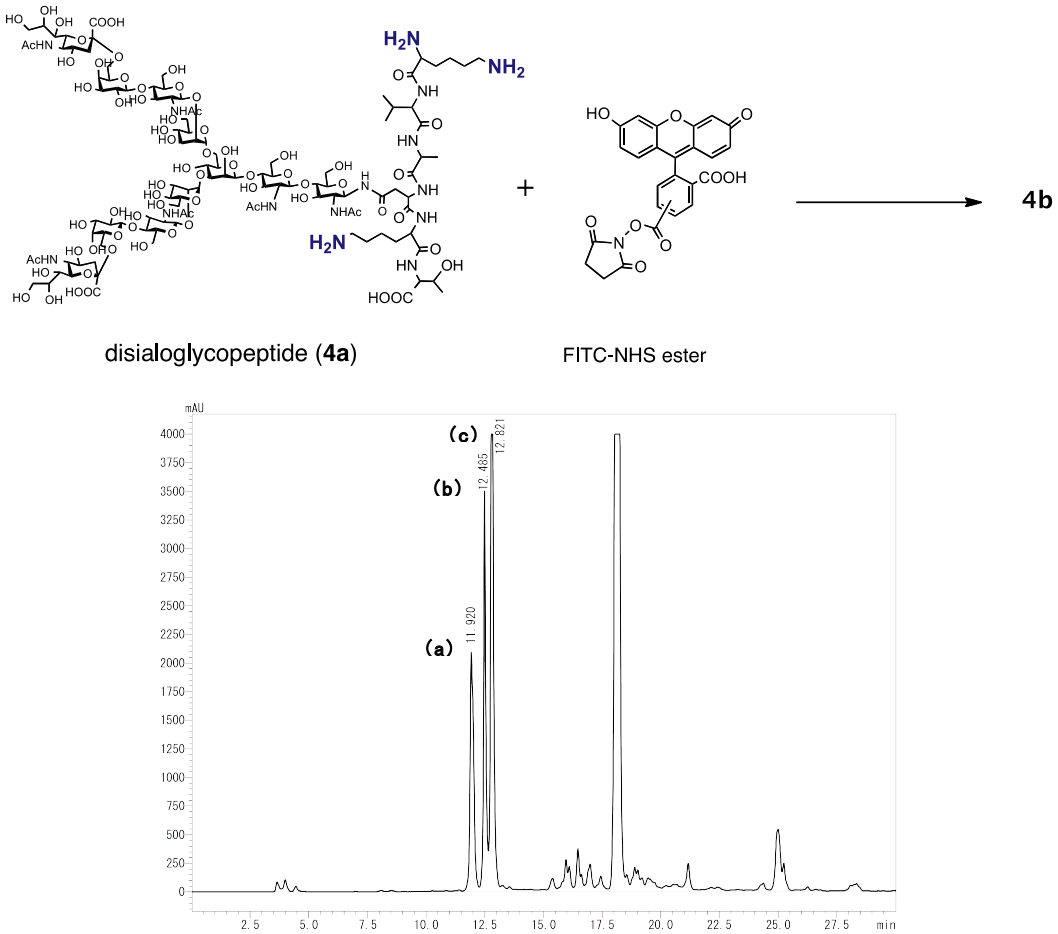


Fig. SI-6 Reaction of disialoglycopeptide with FITC-NHS ester and HPLC profile.

Cell culture and cell surface conjugation. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 5% antibyomyco. Cells of 2 x 10^5 were seeded on 13 mm diameter glass cover slips and grown overnight at 37 °C, 5% CO_2. Cells were washed twice with PBS (pH 7.2-7.4) to exclude residual conjugation inhibitors. Washing buffer was replaced with either TAMRA-NH_2+**3a** or the disialoglycopeptide **4b**+**3a** in PBS at 1 x 10^{-6} M concentration containing 0.005% DMSO, which were prepared according the procedure described above. After incubation for 5 min at 37° C, 5% CO_2, cells were washed twice with cultured medium to stop the conjugation reaction. In order to check the cell viability, cells were treated with the PI (propidium iodide) (Invitrogen, 3.5 μg/700 μL in medium) for 30 min at 37° C, 5% CO_2. Cells were then fixed with 2% paraformaldehyde and 4% sucrose in PBS for 15 min at room temperature. After nuclei and chromosomes were labeled with 2 μg/mL DAPI (4',6-diamino-2-phenylindole, Invitrogen), they were mounted with Prolong Gold (Invitrogen) to analyze by microscopy.

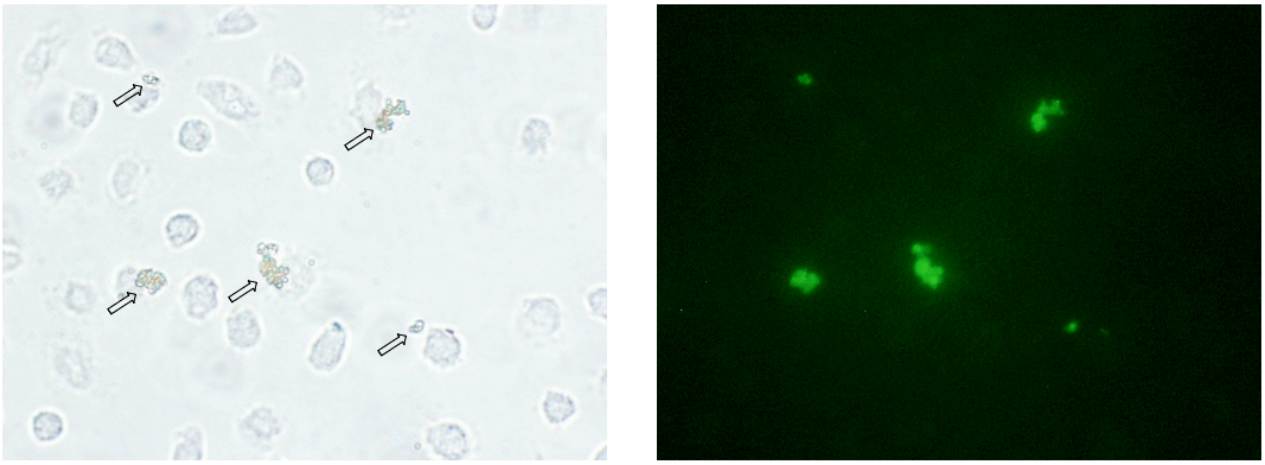


Fig. SI-7 Microscopy images of HeLa cells treated with disialoside **4b**+**3a** at a concentration of 10^{-4} M in PBS for 5 min at 37 °C, 5% CO_2. Left panel: phase contrast, right panel: FITC.

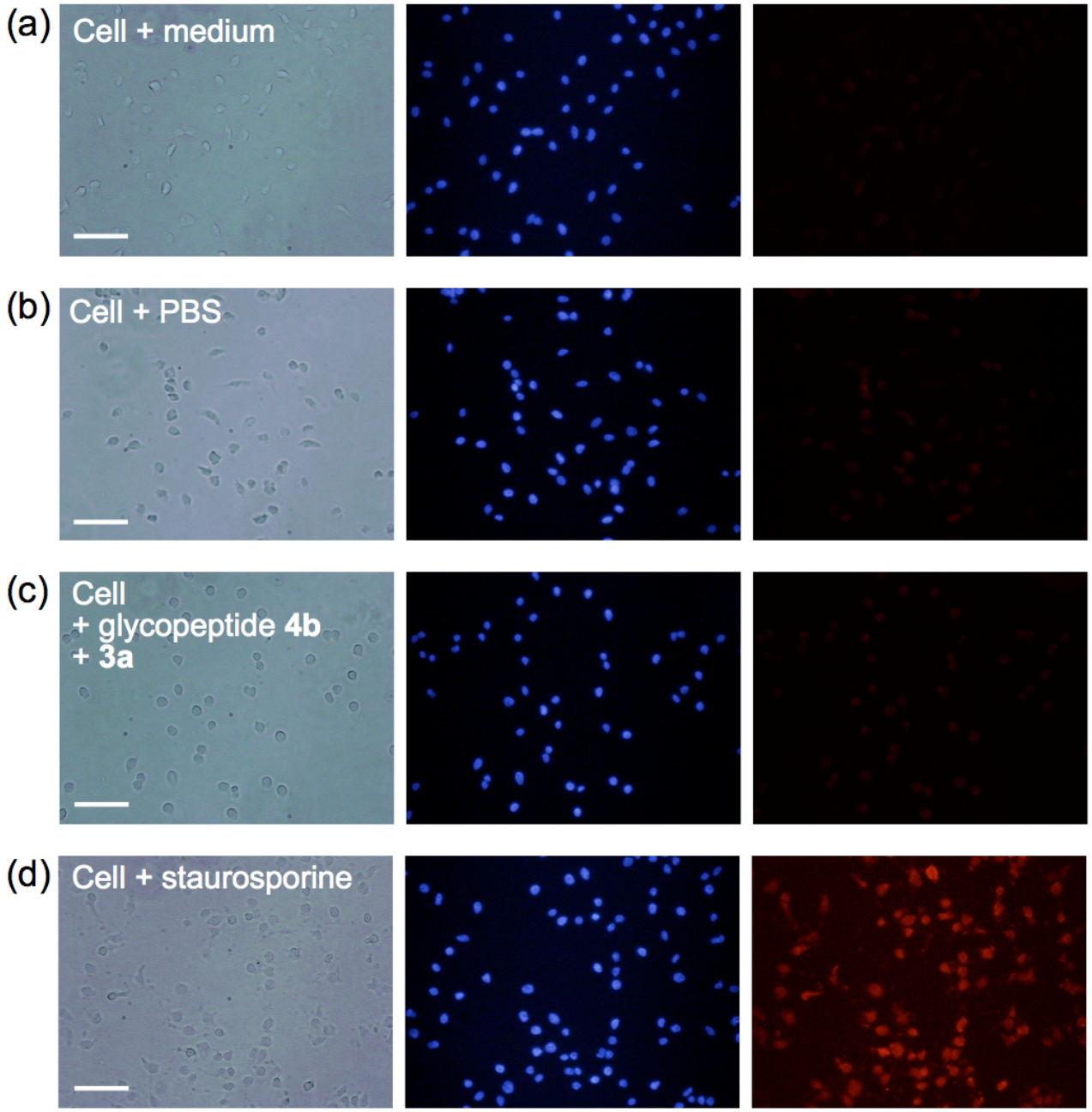
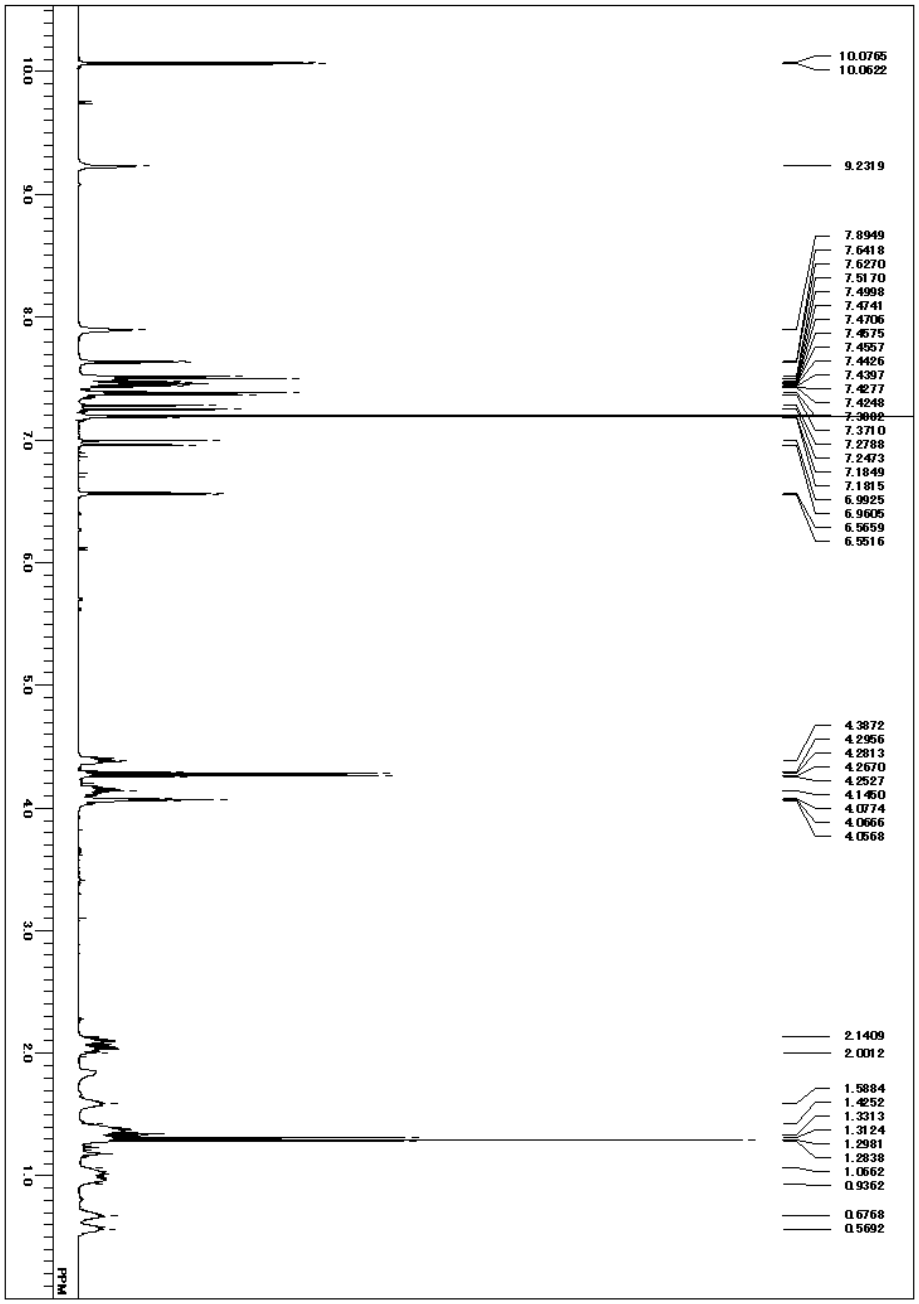


Fig. SI-8 Microscopy images of HeLa cells treated with medium, PBS, **4b**+**3a**, and staurosporine for 5 min at 37 °C, 5% CO_2; bars indicate 100 μm. Cells treated with (a) medium, (b) PBS, (c) 1 x 10^{-6} M of **4b**+**3a** (a mixture of the *cis*- and *trans*-isomers), and (d) 2 μM of staurosporine to induce the apoptosis. Cells were treated with PI (red) to check viability. Nuclei and chromosomes were also labeled with DAPI (blue). From left to right: phase contrast, DAPI, and PI.



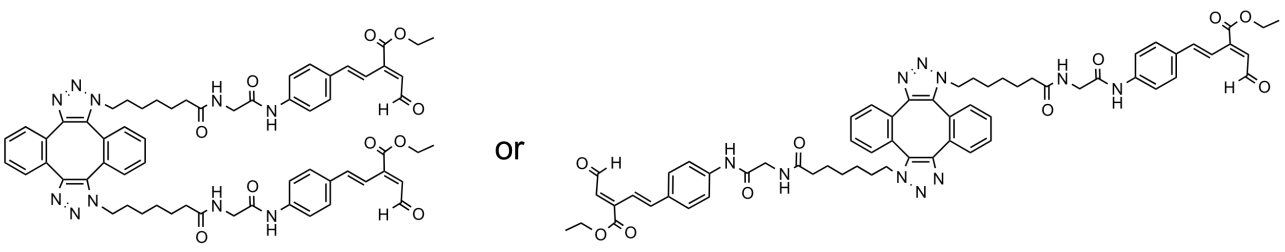
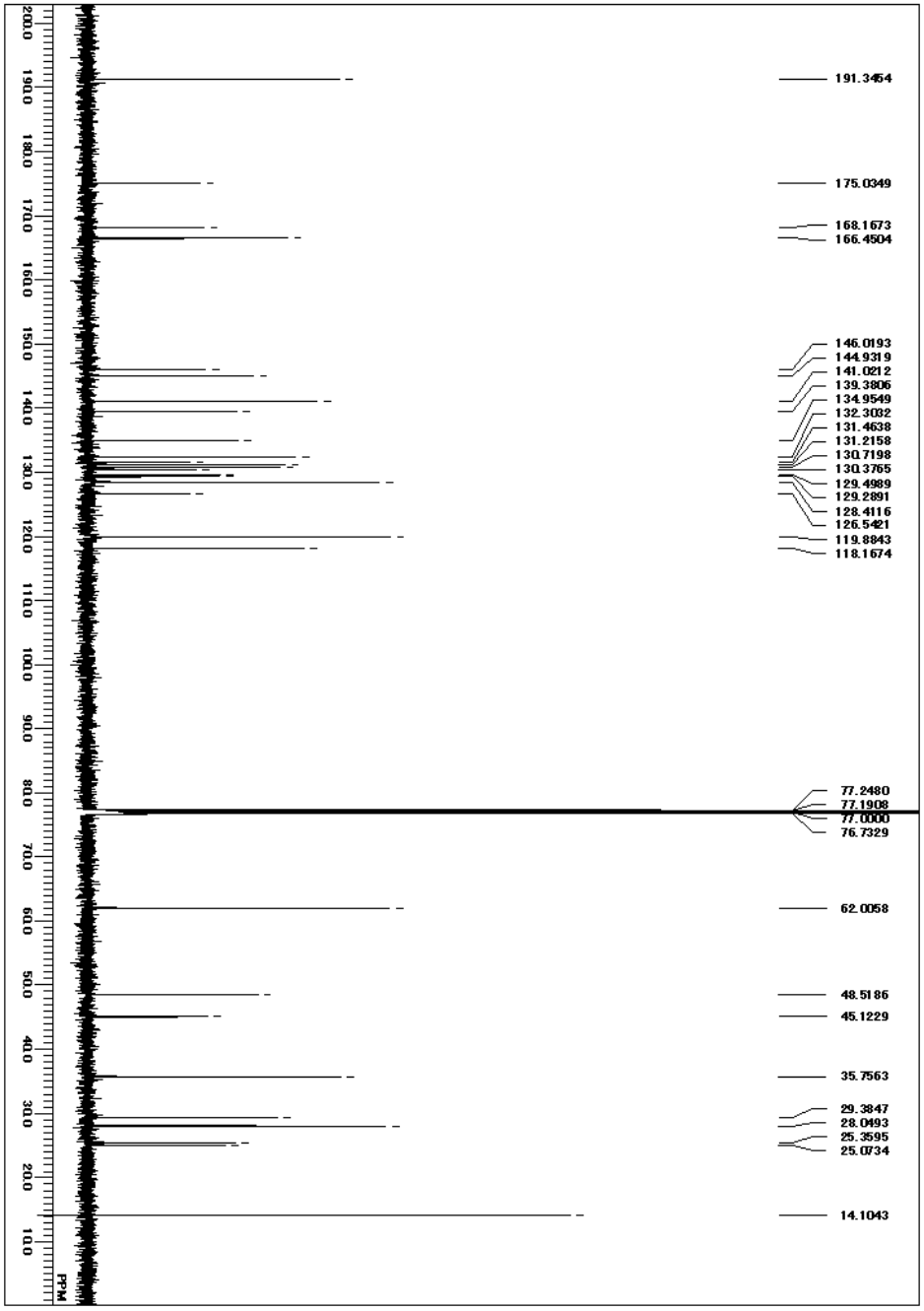


Fig. SI-9 ^{1}H NMR spectrum of one isomer of **3a** (isomer-1).



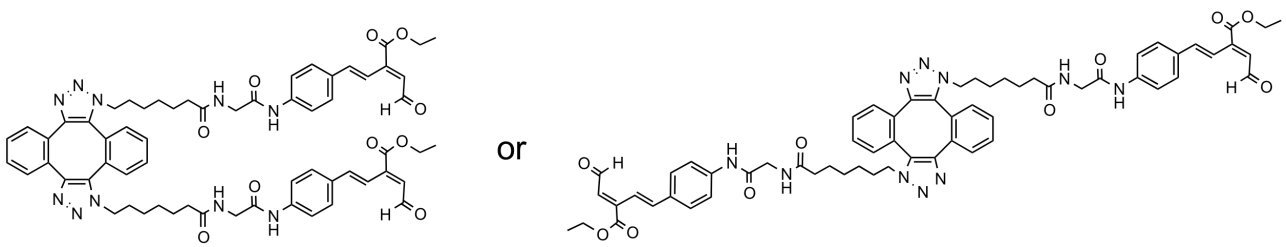
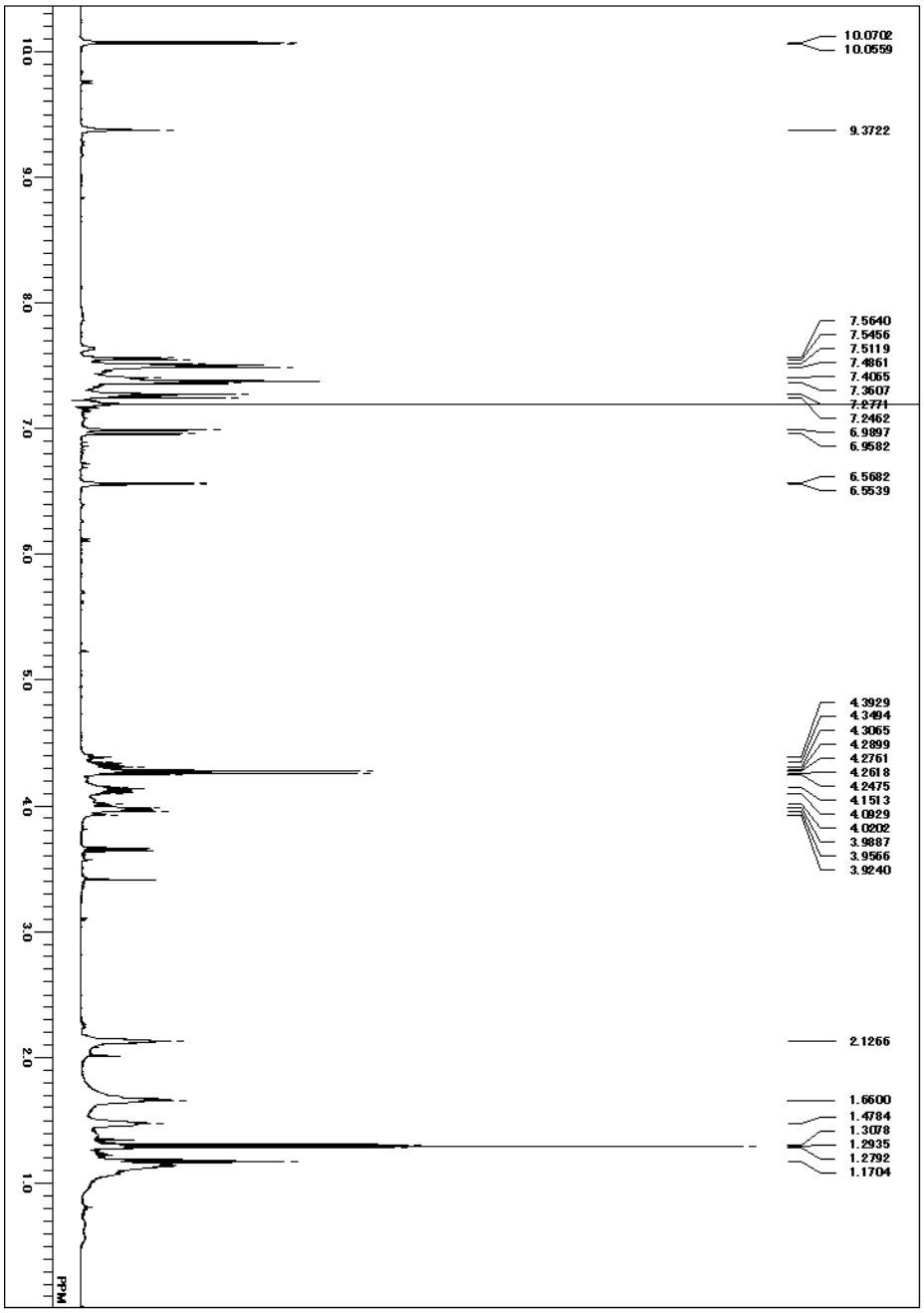


Fig. SI-10 ^{13}C NMR spectrum of one isomer of **3a** (isomer-1).



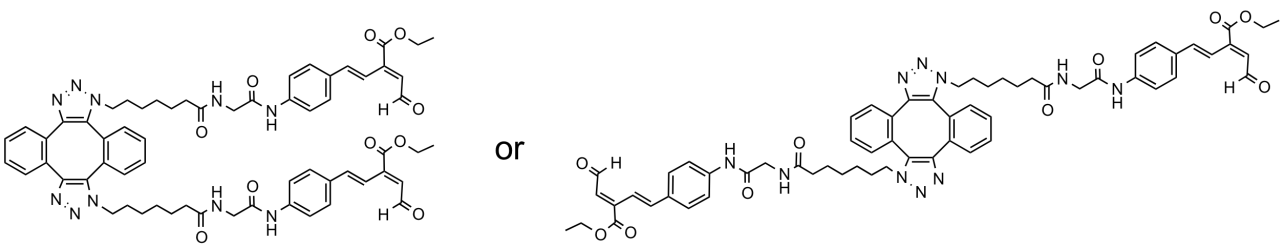
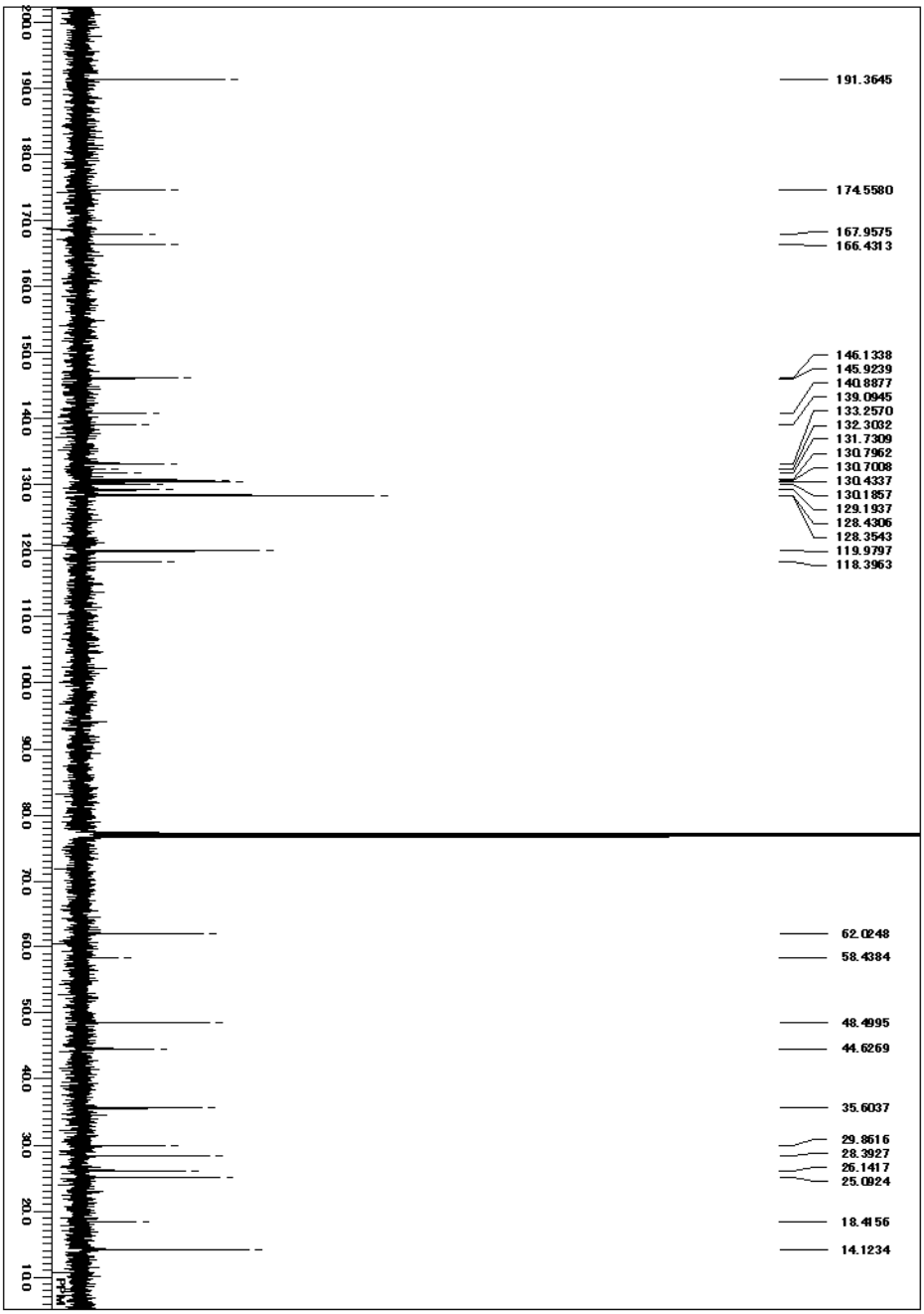


Fig. SI-11 ^{1}H NMR spectrum of another isomer of **3a** (isomer-2).



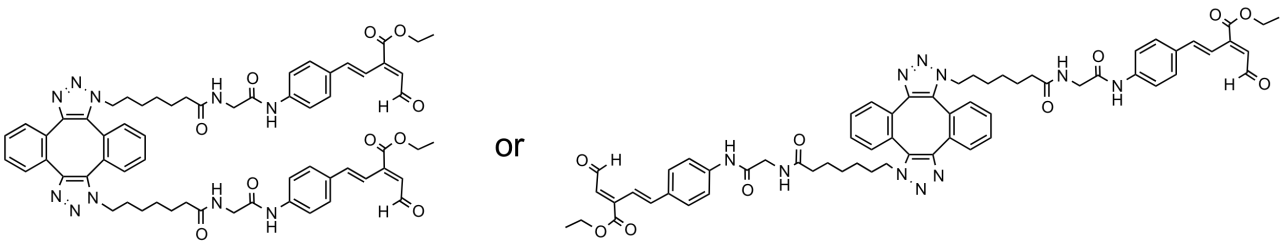
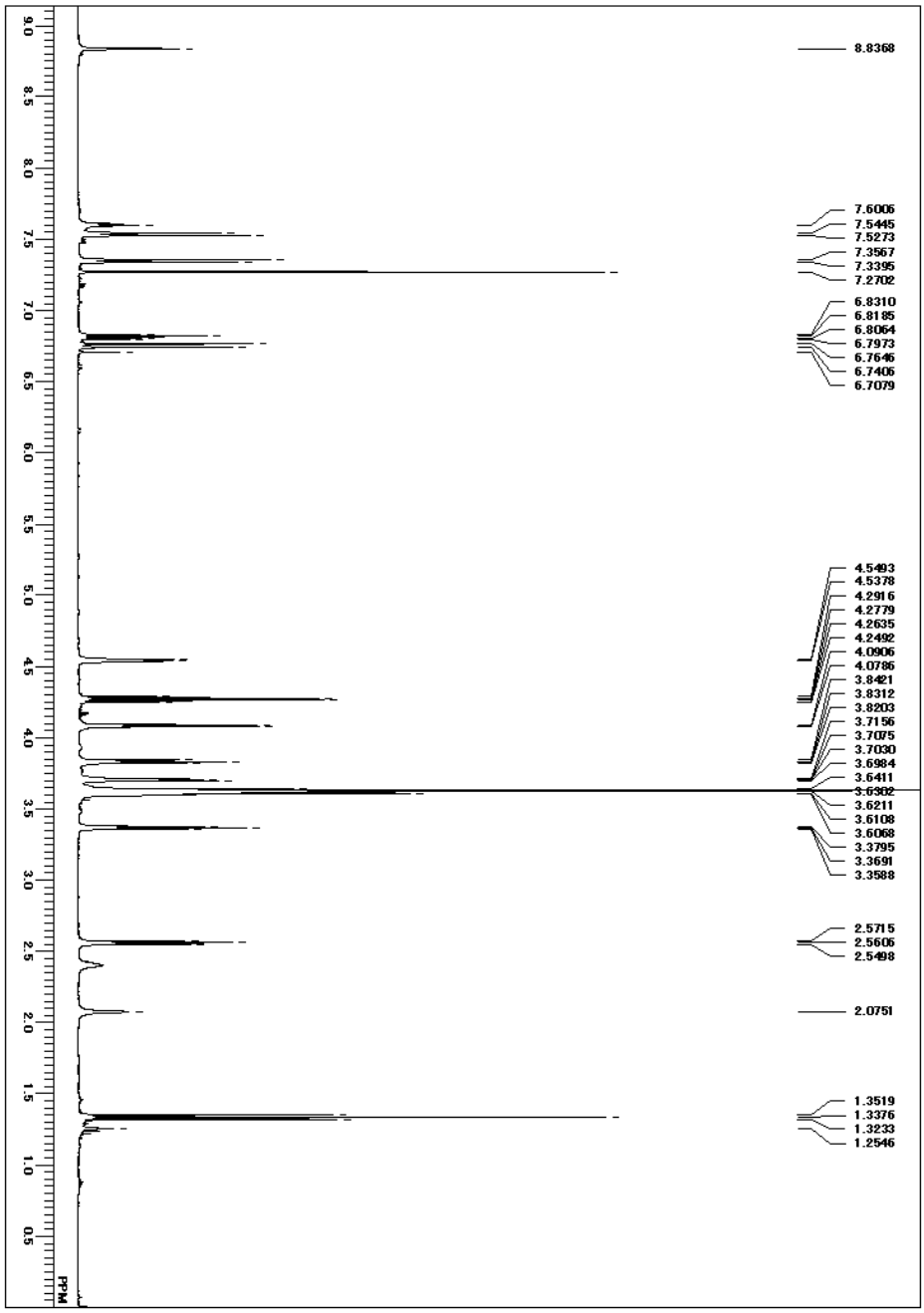


Fig. SI-12 ^{13}C NMR spectrum of another isomer of **3a** (isomer-2).



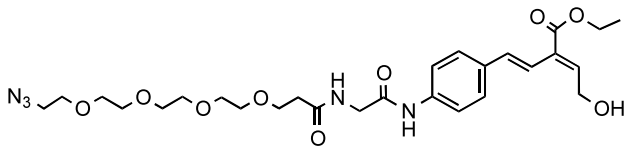
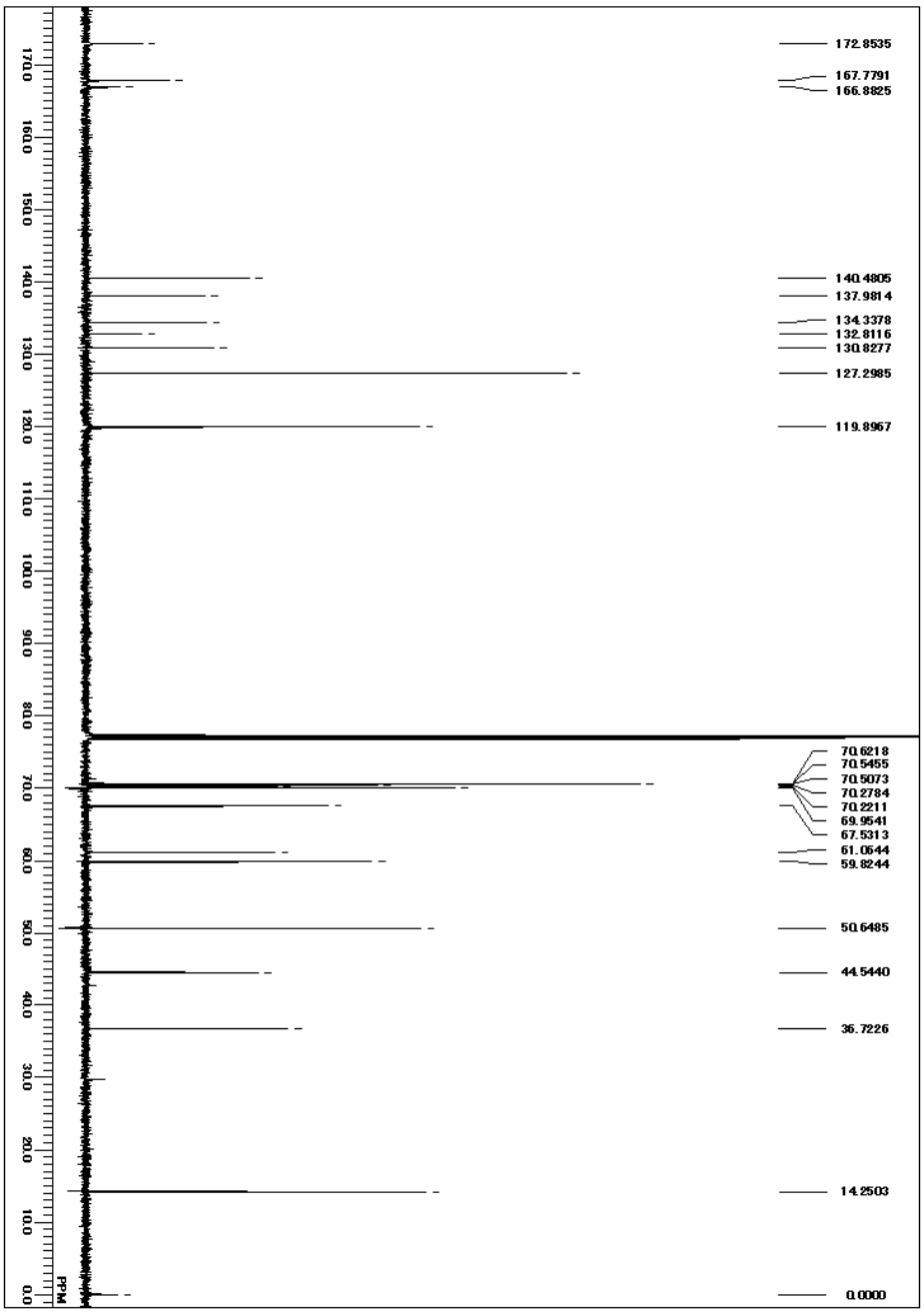


Fig. SI-13 ^{1}H NMR spectrum of azide-PEG4 alcohol.



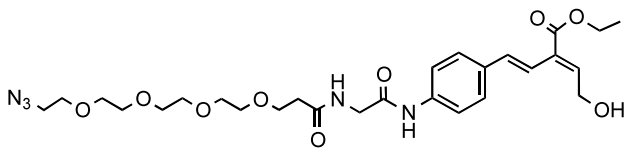
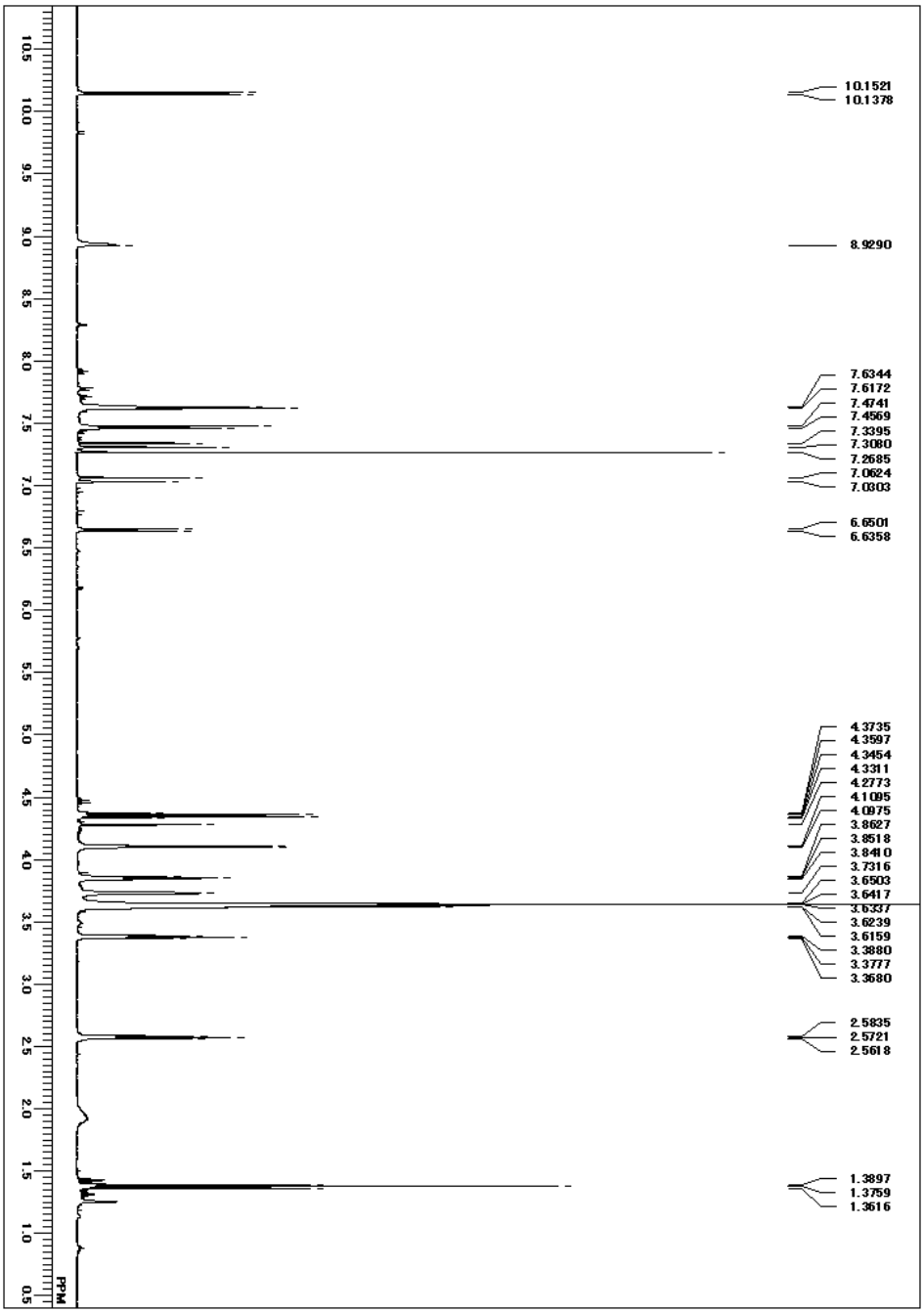


Fig. SI-14 ^{13}C NMR spectrum of azide-PEG4 alcohol.



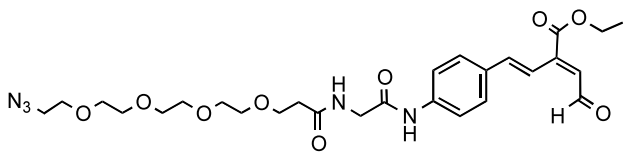
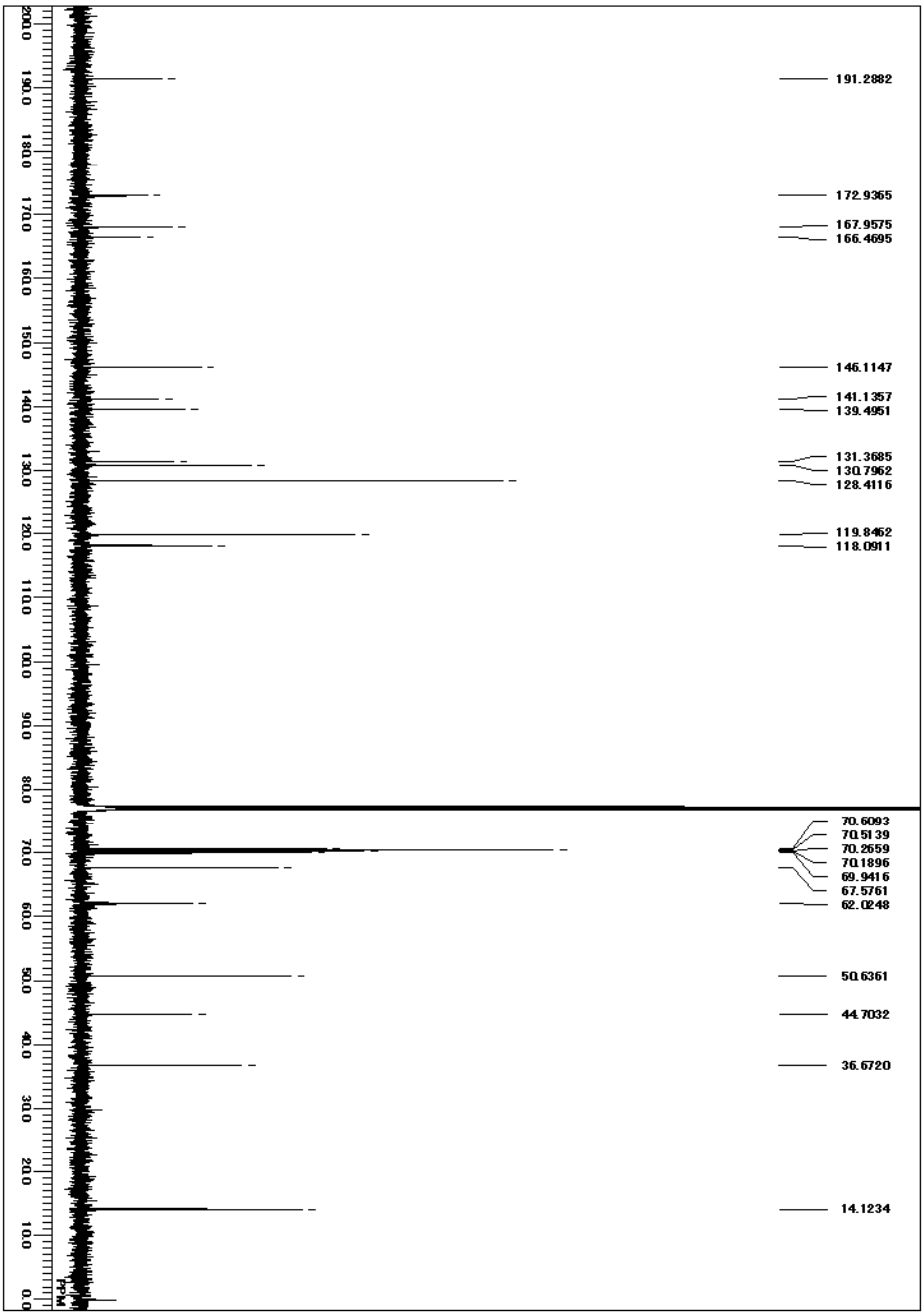


Fig. SI-15 ^{1}H NMR spectrum of **1b**.



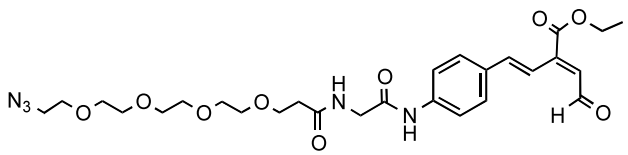
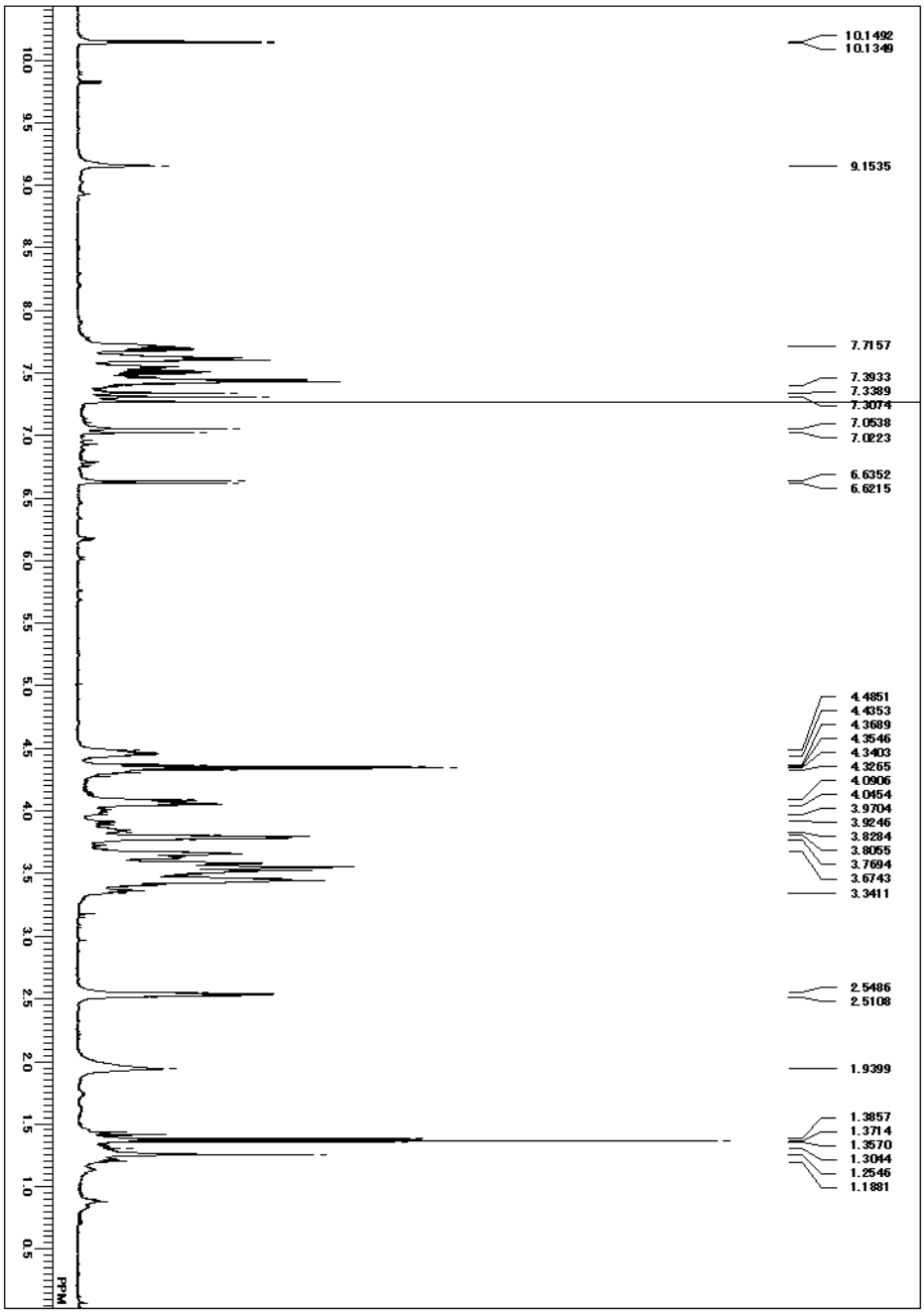


Fig. SI-16 ^{13}C NMR spectrum of **1b**.



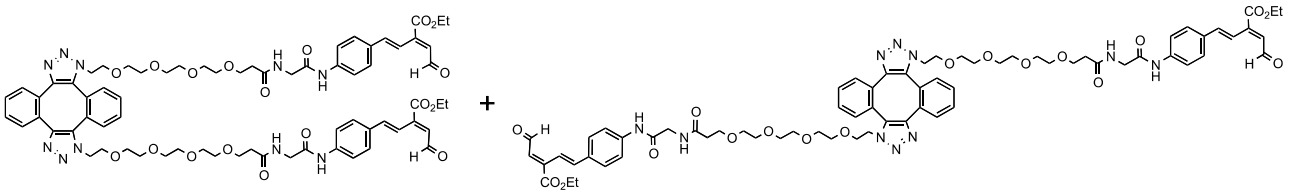
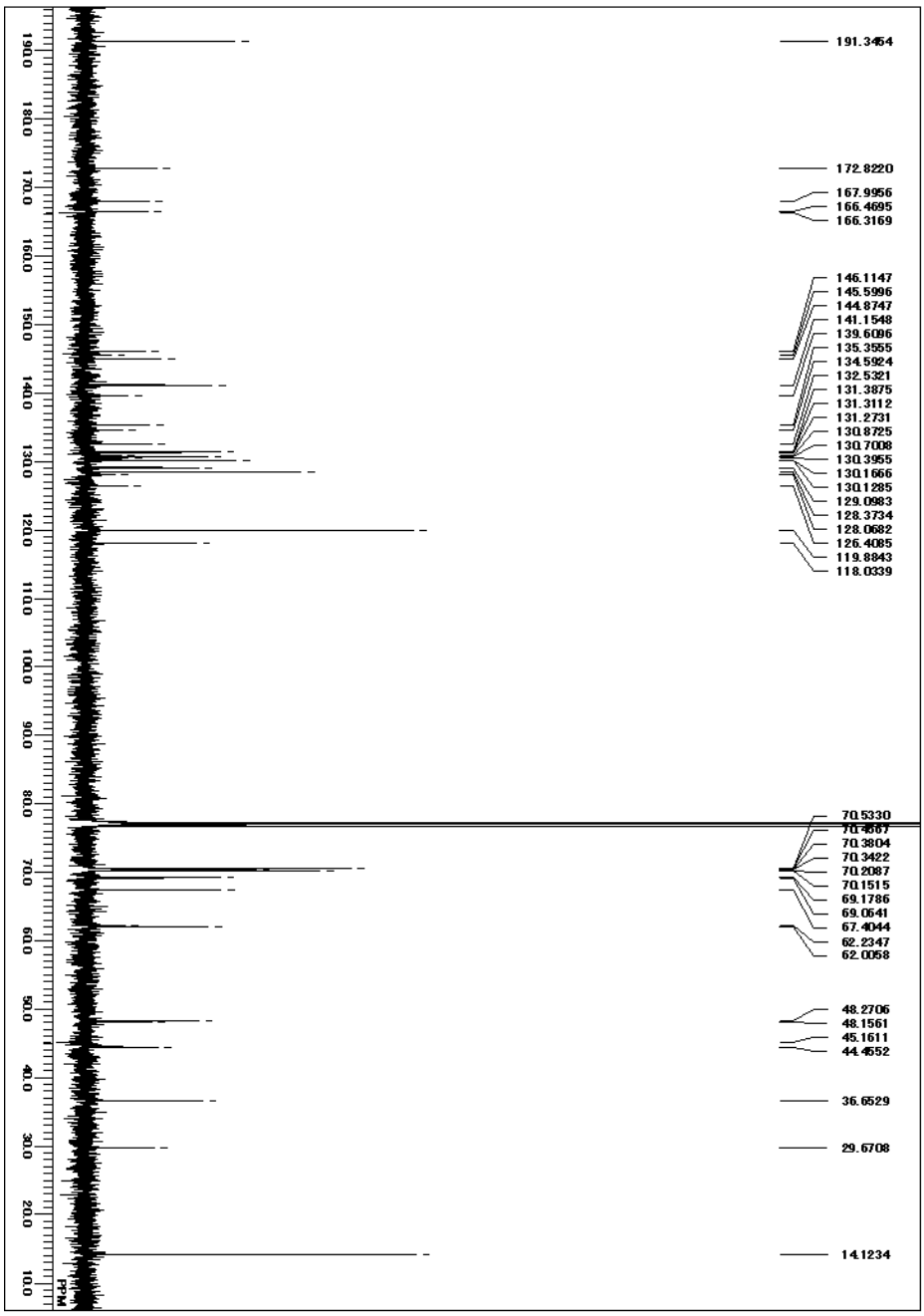


Fig. SI-17 ^{1}H NMR spectrum of *cis*- and *trans*-**3b** as mixtures.



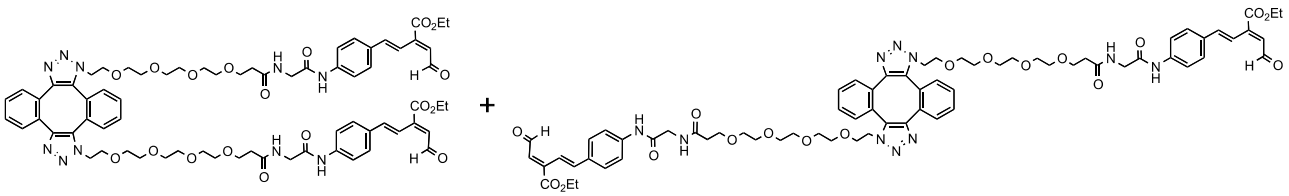


Fig. SI-18 ^{13}C NMR spectrum of *cis*- and *trans*-**3b** as mixtures.