Electronic Supplementary Material (ESI) for Organic and Biomolecular Chemistry

Supplementary Information

Target-selective and fluorescent "switch-on" protein labeling by 6π-azaelectrocyclization

Katsunori Tanaka,* Masataka Kitadani, and Koichi Fukase*

Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho

Toyonaka-shi, Osaka 560-0043, Japan

e-mail: ktzenori@chem.sci.osaka-u.ac.jp and
koichi@chem.sci.osaka-u.ac.jp

General Procedures. All commercially available reagents were used without further purification. Dichloromethane were refluxed over and distilled from CaH_2. Anhydrous DMF was purchased from Aldrich, and anhydrous THF was purchased from Kanto Chemicals, Tokyo. 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 NMR spectra was recorded on a JEOL JNM-LA 500 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). UV-vis spectra were recorded on a JASCO V-530 spectrophotometer and reported as λ_{max} [nm] (ε_{max}[Lmol^{-1}cm^{-1}]). Fluorescence emission spectra were measured either on a JASCO FP-6500 spectrofluorometer.

2-{2-{4-[4-(Dimethylamino)phenylazo]benzoyl}aminoethoxy}ethyl (*E*,*E*)-4-*tert*Butyldiphenylsilanylhydroxy-2-(6-aminostyryl)but-2-enoate (3). To a solution of **2** (30 mg, 40 μmol) in CH_2Cl_2 (3.0 mL) was added TFA (750 μL) at 0 °C and the mixture was stirred at this temperature for 20 min. The solution was neutralized with 1 N aqueous NaOH (3.0 mL) and extracted with CH_2Cl_2. The organic layers were combined, washed with brine, dried over Na_2SO_4, filtrated, and concentrated *in vacuo* to give the crude diamine, which was subjected to the acylation without further purification.

To a solution of the diamine (35% out of the crude products obtained above (14 μmol)) in dry CH_2Cl_2 (1.0 mL) was added 4-[4-(dimethylamino)phenylazo]benzoic acid succinimidyl ester (5.2 mg, 14 μmol) at room temperature and stirred overnight at this temperature. After the solution was concentrated *in vacuo*, the residue was purified by preparative TLC on silica gel (60% ethyl acetate in hexane) to give **3** as a bright red solid (7.2 mg, 62 % for 2 steps); MALDI-TOF-MS *m/z* calcd for $C_{47}H_{53}N_5O_5Si$ $(M+Na)^+$ 818.4, found 818.3; HRESI-MS *m/z* calcd for $C_{47}H_{53}N_5O_5$ Si $(M+Na)^+$ 818.3705, found 818.3687; IR (neat, cm^{-1}) 3355, 2927, 2855, 1713, 1601, 1517; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (d, 2H, *J* = 9.2 Hz), 7.85 (d, 2H, *J* = 8.6 Hz), 7.81 (d, 2H, *J* = 8.7 Hz), 7.66 (m, 4H), 7.41-7.30 (m, 6H), 7.08 (d, 2H, *J* = 8.5 Hz), 6.79 (t, 1H, *J* = 5.8 Hz), 6.75 (d, 2H, *J* = 9.2 Hz), 6.60 (d, 1H, *J* = 16.4 Hz), 6.54 (d, 2H, *J* = 8.5 Hz), 6.46 (d, 1H, *J* = 16.5 Hz), 4.52 (d, 2H, *J* = 5.9 Hz), 4.40 (t, 2H, *J* = 4.7 Hz), 3.80 (t, 2H, *J* = 4.7 Hz), 3.70 (t, 2H, *J* = 4.4 Hz), 3.69 (t, 2H, *J* = 4.4 Hz), 3.10 (s, 6H), 1.05 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.2, 167.0, 155.0, 152.8, 146.6, 143.8, 140.2, 135.6, 135.5, 133.3, 130.3, 129.8, 129.6, 128.0, 127.9, 127.8, 127.7, 122.2, 115.0, 111.5, 69.9, 69.2, 63.7, 61.3, 40.3, 39.9, 26.8, 26.8.

2-{2-{4-[4-(Dimethylamino)phenylazo]benzoyl}aminoethoxy}ethyl (*E*,*E*)-4-*tert*Butyldiphenylsilanylhydroxy-2-{6-[7-(diethylamino)coumarin-3-carboxamide]styryl}but-2-enoate. To a solution of **3** (1.5 mg, 1.9 μmol) in dry DMF (500 μL) were added HATU (1.1 mg, 2.8 μmol) and 7-(dimethylamino)coumarin-3-carboxylic acid (590 μg, 2.3 μmol) at room temperature. After the mixture was stirred at this temperature for 10 min, triethylamine (530 nL, 3.8 μmol) was added to this solution and stirred at room temperature overnight. The resulting mixture was concentrated *in vacuo* to give the crude product, which was directly purified by preparative TLC on silica gel (9 % MeOH in $CHCl_3$) to give the coupling product as a bright red solid (1.5 mg, 76 %): MALDI-TOF-MS *m/z* calcd for $C_{61}H_{66}N_6O_8Si$ $(M+Na)^+$ 1061.5, found 1061.6; HRESI-MS *m/z* calcd for $C_{61}H_{66}N_6O_8$ Si $(M+Na)^+$ 1061.4603, found 1061.4576; IR (neat, cm^{-1}) 3269, 2955, 2917, 2849, 1699, 1617, 1600, 1509; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.9 (s, 1H), 8.76 (s, 1H), 7.86 (d, 2H, *J* = 8.5 Hz), 7.85 (d, 2H, *J* = 9.2 Hz), 7.80 (d, 2H, *J* = 8.6 Hz), 7.68-7.65 (m, 4H), 7.64 (d, 2H, *J* = 8.6 Hz), 7.46 (d, 1H, *J* = 9.0 Hz), 7.42-7.30 (m, 6H), 7.24 (d, 2H, *J* = 8.6 Hz), 6.86 (t, 1H, *J* = 5.9 Hz), 6.73 (d, 2H, *J* = 9.0 Hz), 6.69 (d, 1H, *J* = 16.3 Hz), 6.67 (dd, 1H, *J* = 2.5, 8.9 Hz), 6.58 (d, 1H, *J* = 16.3 Hz), 6.53 (d, 1H, *J* = 2.1 Hz), 4.53 (d, 2H, *J* = 6.0 Hz), 4.42 (t, 2H, *J* = 4.7 Hz), 3.81 (t, 2H, *J* = 4.7 Hz), 3.72 (t, 2H, *J* = 4.6 Hz), 3.70 (t, 2H, *J* = 4.6 Hz), 3.46 (q, 4H, *J* = 7.1 Hz), 3.07 (s, 6H), 1.26 (t, 6H, *J* = 7.2Hz), 1.05 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): Due to

the multiple signals of rotamers, those of representative were shown here: δ 167.0, 166.9, 163.0, 160.9, 157.8, 155.0, 152.8, 152.7, 148.4, 143.8, 141.3, 138.3, 140.2, 135.6, 135.5, 134.4, 134.3, 133.2, 129.9, 128.8, 127.9, 127.8, 127.7, 127.4, 122.2, 120.3, 119.5,111.5, 110.3, 110.2, 108.7, 96.7, 69.9, 69.2, 63.7, 61.3, 45.2, 40.2, 39.9, 26.8, 26.8, 12.5.

2-{2-{4-[4-(Dimethylamino)phenylazo]benzoyl}aminoethoxy}ethyl (*E*,*E*)-2-{6-[7-(Diethylamino)coumarin-3-carboxamide]styryl}-4-hydroxybut-2-enoate (4). To a solution of the coupling product obtained above (1.5 mg, 1.4 μmol) in THF (500 μL) were added acetic acid (1.0 M in THF, 1.4 μL, 1.4 μmol) and TBAF (1.0 M in THF, 1.4 μL, 1.4 μmol) at 0 ℃. After the resulting mixture was warmed to room temperature and stirred for 2 h, the mixture was extracted with $CHCl_3$. The organic layers were combined, washed with brine, dried over Na_2SO_4, filtrated, and concentrated *in vacuo* to give the crude product, which was rapidly purified by preparative TLC on silica gel (9 % MeOH in $CHCl_3$) to give the corresponding allyl alcohol **4** as a bright red solid (1.0 mg, 87 %): MALDI-TOF-MS *m/z* calcd for $C_{45}H_{48}N_6O_8$ $(M+Na)^+$ 823.4, found 823.7; HRESI-MS *m/z* calcd for $C_{45}H_{48}N_6O_8$ $(M+Na)^+$ 823.3425, found 823.3428; IR (neat, cm^{-1}) 2956, 2924, 2853, 1720, 1690, 1602, 1540; ^1H NMR (500 MHz, $CDCl_3$) δ 10.8 (s, 1H), 8.76 (s, 1H), 7.85 (d, 2H, *J* = 8.5 Hz), 7.84 (d, 2H, *J* = 9.2 Hz), 7.80 (d, 2H, *J* = 8.3 Hz), 7.64 (d, 2H, *J* = 8.6 Hz), 7.46 (d, 1H, *J* = 9.1 Hz), 7.32 (2H, *J* = 8.6 Hz), 6.76 (t, 1H, *J* = 6.0 Hz), 6.73 (d, 2H, *J* = 9.0 Hz), 6.70 (d, 1H, *J* = 16.3 Hz), 6.66 (dd, 1H, *J* = 2.3, 8.5 Hz), 6.61 (d, 1H, *J* = 16.4 Hz), 6.54 (d, 1H, *J* = 2.5 Hz), 4.44 (d, 2H, *J* = 7.1 Hz), 4.41 (t, 2H, *J* = 4.5 Hz), 3.82 (t, 2H, *J* = 4.4 Hz), 3.76 (t, 2H, *J* = 5.0 Hz), 3.68 (t, 2H, *J* = 5.2 Hz), 3.48 (q, 4H, *J* = 7.2 Hz), 3.04 (s, 6H), 1.26 (t, 6H, *J* = 7.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): Due to the multiple signals of rotamers, those of representative were shown here: δ 167.8, 167.3, 166.8, 163.0, 160.9, 157.8, 155.1, 152.8, 152.8, 148.3, 143.7, 141.3, 138.4, 134.6, 130.99, 130.14, 128.8, 127.9, 127.4, 125.5, 124.4, 122.28, 120.32, 119.3, 111.5, 110.2, 108.7, 96.7, 69.9, 69.0, 64.0, 59.8, 45.2, 40.2, 38.2, 12.5.

2-{2-{4-[4-(Dimethylamino)phenylazo]benzoyl}aminoethoxy}ethyl (*E*,*E*)-4-Oxo-2-{6-[7-(diethylamino)coumarin-3-carboxamide]styryl}but-2-enoate (1). To a solution of the allyl alcohol **4** obtained above (1.0 mg, 1.25 μmol) in dry CH_2Cl_2 (1.0 mL) was added IBX-polystyrene (38 mg, 38 μmol) and the mixture was stirred at room temperature for 20 min. The solution was filtered and the filtrate was concentrated *in vacuo* to give **1** as a bright red solid (1.0 mg, quant), being pure enough used for subsequent labeling studies after the rapid MS analysis: MALDI-TOF-MS *m/z* calcd for $C_{45}H_{46}N_6O_8$ $(M+H)^+$ 799.3, found 799.8; HRESI-MS *m/z* calcd for $C_{45}H_{46}N_6O_8$ $(M+Na)^+$ 821.3274, found 821.3310; ^1H NMR (500 MHz, $CDCl_3$) δ 10.98 (s, 1H), 10.14 (d, 1H, *J* = 7.1 Hz), 8.77 (s, 1H), 7.87 (m, 2H), 7.85 (d, 2H, *J* = 8.0 Hz), 7.82 (d, 2H, *J* = 8.4 Hz), 7.75 (d, 2H, *J* = 8.5 Hz), 7.47 (d, 1H, *J* = 8.5 Hz), 7.33 (1H, *J* = 5.6 Hz), 7.30 (d, 2H, *J* = 8.6 Hz), 7.08 (d, 1H, *J* = 15.4 Hz), 6.78 (d, 1H, *J* = 16.1 Hz), 6.74 (d, 2H, *J* = 9.0 Hz), 6.57 (dd, 1H, *J* = 2.3, 8.5 Hz), 6.54 (s, 1H), 4.49 (t, 2H, *J* = 4.4 Hz), 3.82 (t, 2H, *J* = 4.3 Hz), 3.76 (t, 2H, *J* = 5.0 Hz), 3.68 (t, 2H, *J* = 5.2 Hz), 3.48 (q, 4H, *J* = 7.0 Hz), 3.07 (s, 6H), 1.26 (t, 6H, *J* = 7.2 Hz).

"Switch-On" HSA labeling by the probe 1: To a mixture of HSA, phospholipase A_2, TGF-β3, orosomucoid, anti-Tau antibody, somatostatin, neurotensin, and ACTH dissolved in 1.35 mL of 0.1 M phosphate buffer (each of 1.0×10^{-8} M, pH 7.4) was added a 1.0×10^{-6} M solution of **1** in 0.1 M phosphate buffer (150 μL), and the mixture was incubated at 25 °C. The final concentrations of each proteins/peptides and **1** were 1.0×10^{-8} M and 1.0×10^{-7} M, respectively. The solution was then directly analyzed by fluorescence spectrometer (excitation of 7-diethylaminocoumarin excitation at 420 nm) and/or HPLC.

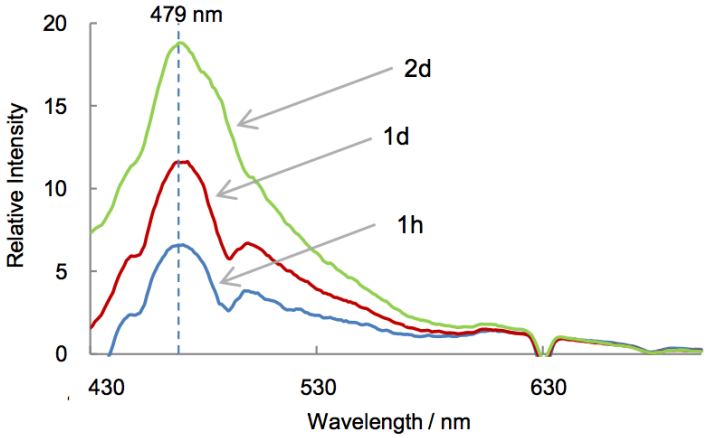

Fig. S-1 Fluorescence spectrum of HSA after treatment with probe **1**. Both incubation and fluorescence analysis were performed at 1.0×10^{-9} M for HSA and 1.0×10^{-8} M for probe **1** in PBS at room temperature (excitation at 420 nm).

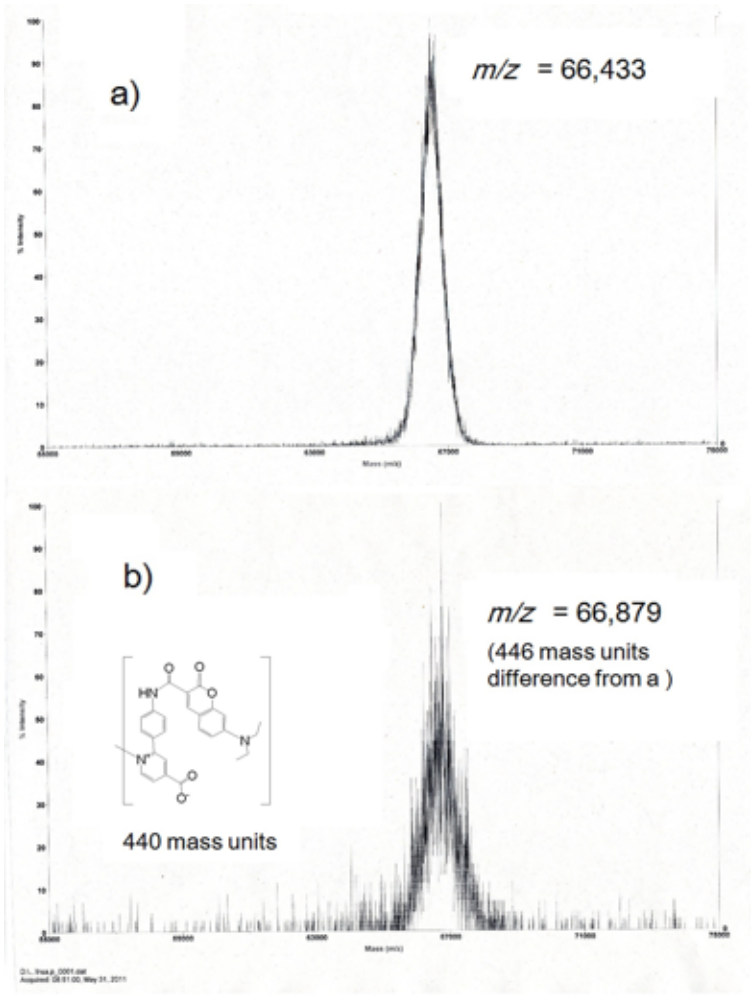

Fig. S-2 MALDI-TOF-MS of (a) HSA (*m/z* calcd for $(M+H)^+$ 66,473) and (b) HSA labeled by **1** (twitter ion form).

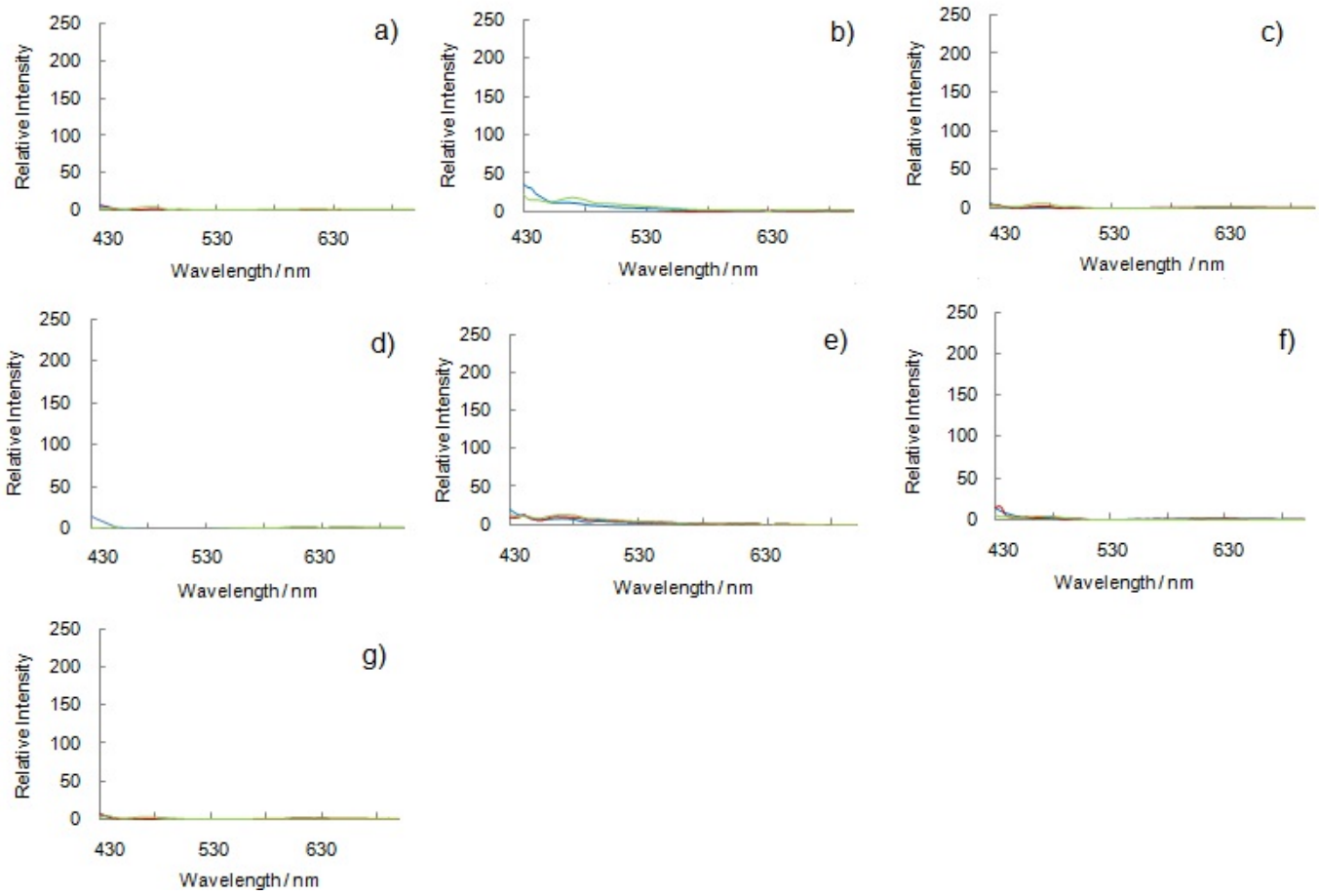

Fig. S-3 Fluorescence spectra of various protein and peptide solutions after treatment with probe **1**. Both incubation and fluorescence analysis were performed at 1.0 x 10^{-8} M for proteins and/or peptides, and at 1.0 x 10^{-7} M for probe **1** in PBS at room temperature (excitation at 420 nm). (a) bovine pancreatic phospholipase A_2, (b) TGF-b3, (c) orosomucoid, (d) anti-Tau antibody, (e) somatostatin, (f) neurotensin, (g) ACTH. Blue: 1 h; red: 1 d; green; 2 d after incubation.

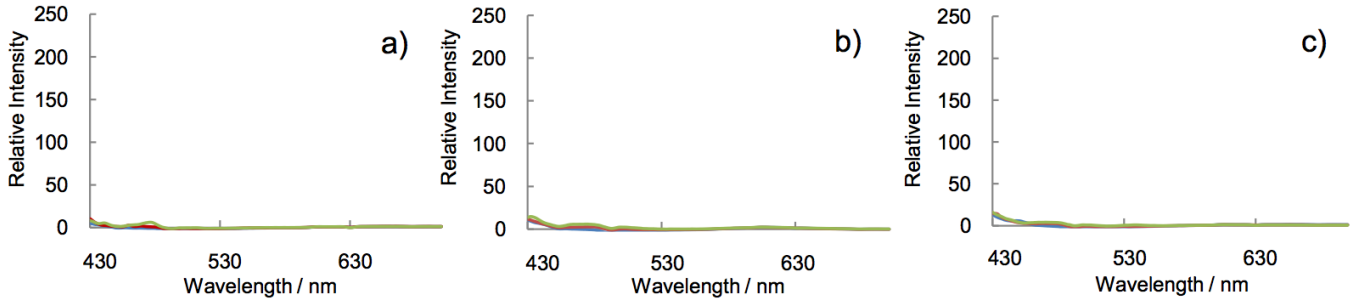

Fig. S-4 Fluorescence spectrua of (a) somatostatin, (b) neurotensin, and (c) ACTH after treatment with probe **1**. Incubation was performed at 1.0 x 10^{-6} M for peptides and 1.0 x 10^{-7} M for **1** in PBS at room temperature. Fluorescence spectra were recorded after 1h (blue), 1d (red) and 2d (green) incubation (excitation at 420 nm).

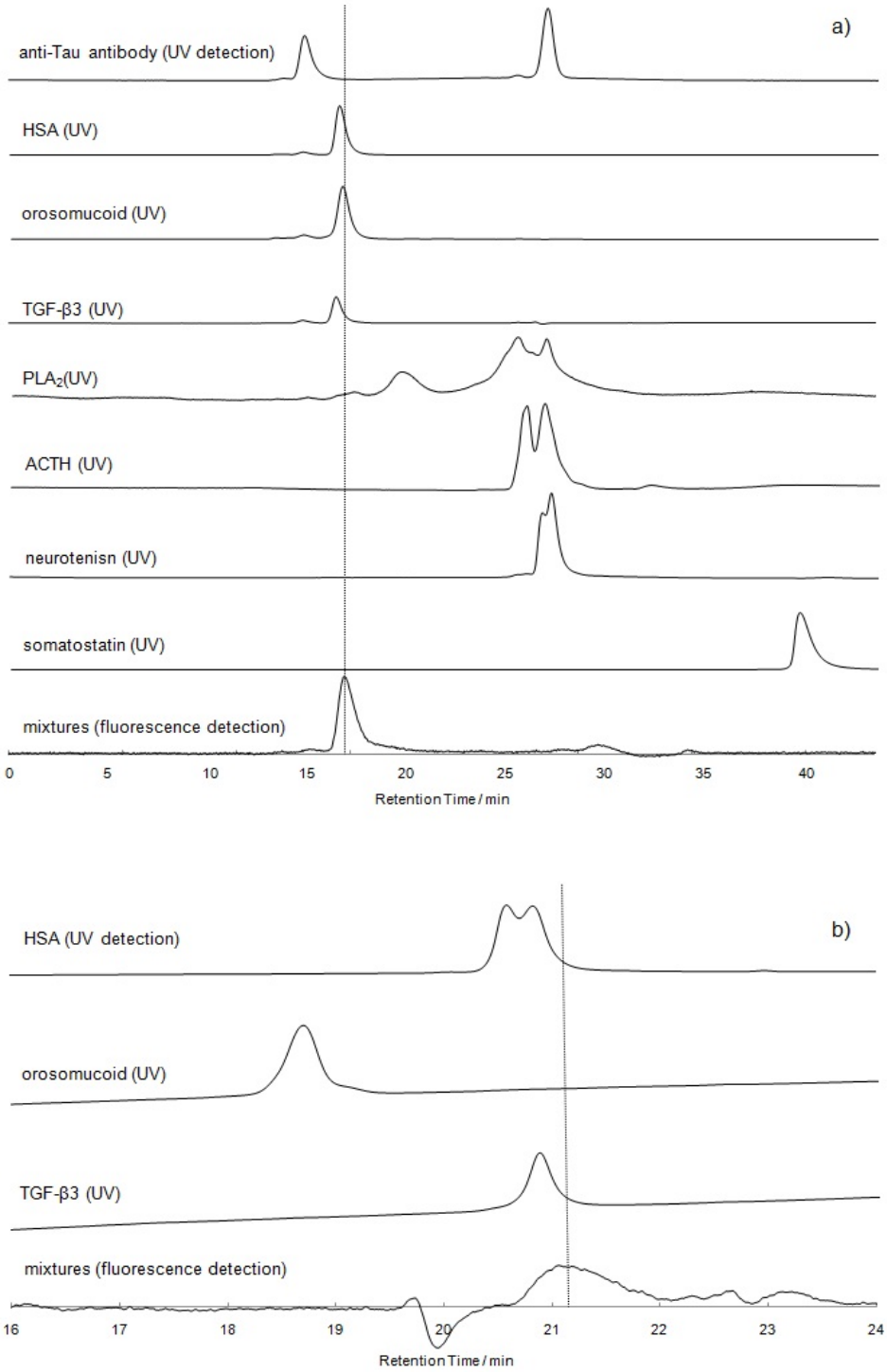

Fig. S-5 HPLC profiles of the individual proteins and peptides as well as their mixture after treatment with probe **1** for 2 days (reaction concentrations: 1.0 x 10^{-8} M for peptides and 1.0 x 10^{-7} M for **1** in PBS, detection: UV at 215 nm, excitation at 420 nm and emission at 480 nm). (a) Size-partitioning gel-filtration on HPLC (column: TSK-Gel G2000SW_{XL}, 7.8 x 300 mm; eluent: 0.02 M PBS containing 0.3 M NaCl, pH 7.0 at rt; flow rate: 0.5 mL/min). Since the labeling of HSA, orosomucoid, or TGF-β3 could not be concluded, these three proteins were further analyzed by reverse phase HPLC. (b) Reverse phase HPLC (column: Nacalai Tesque Protein-R, 2.0 x 150 mm; MeCN in H_2O containing 0.05% TFA (20-60% gradient over 20 min, 0.5 mL/min). Out of HSA and TGF-β3, TGF-β3 being treated with the probe **1** did not show any fluorescence (see Fig. S-3), hence the preferential labeling of HSA was concluded; the conclusion was also supported by the MALDI-TOF-MS of HSA and TGF-β3 treated by **1** (see Figs S-2 and S-6).

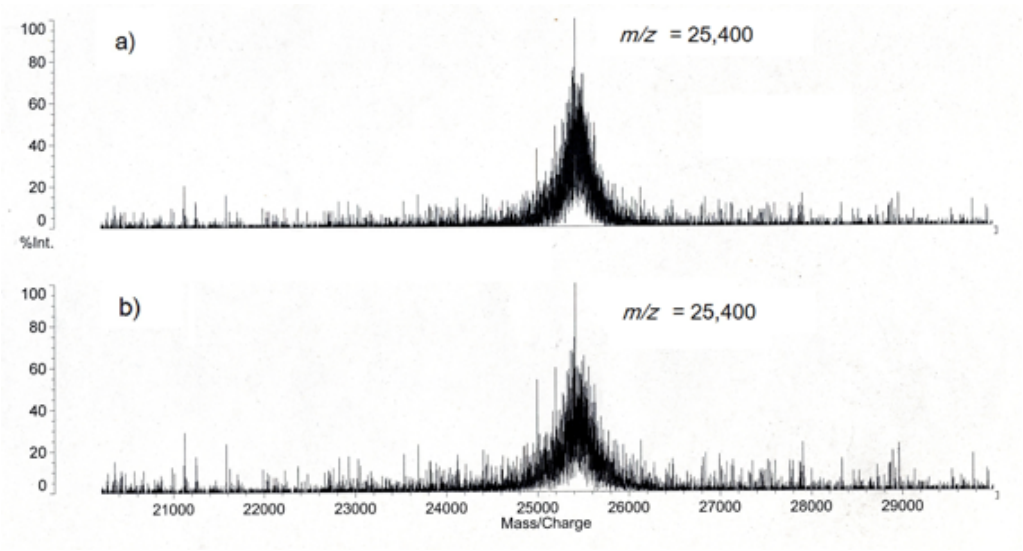

Fig. S-6 MALDI-TOF-MS of (a) TGF-β3 (*m/z* calcd for $(M+H)^+$ 25,429) and (b) TGF-β3 treated by **1**.

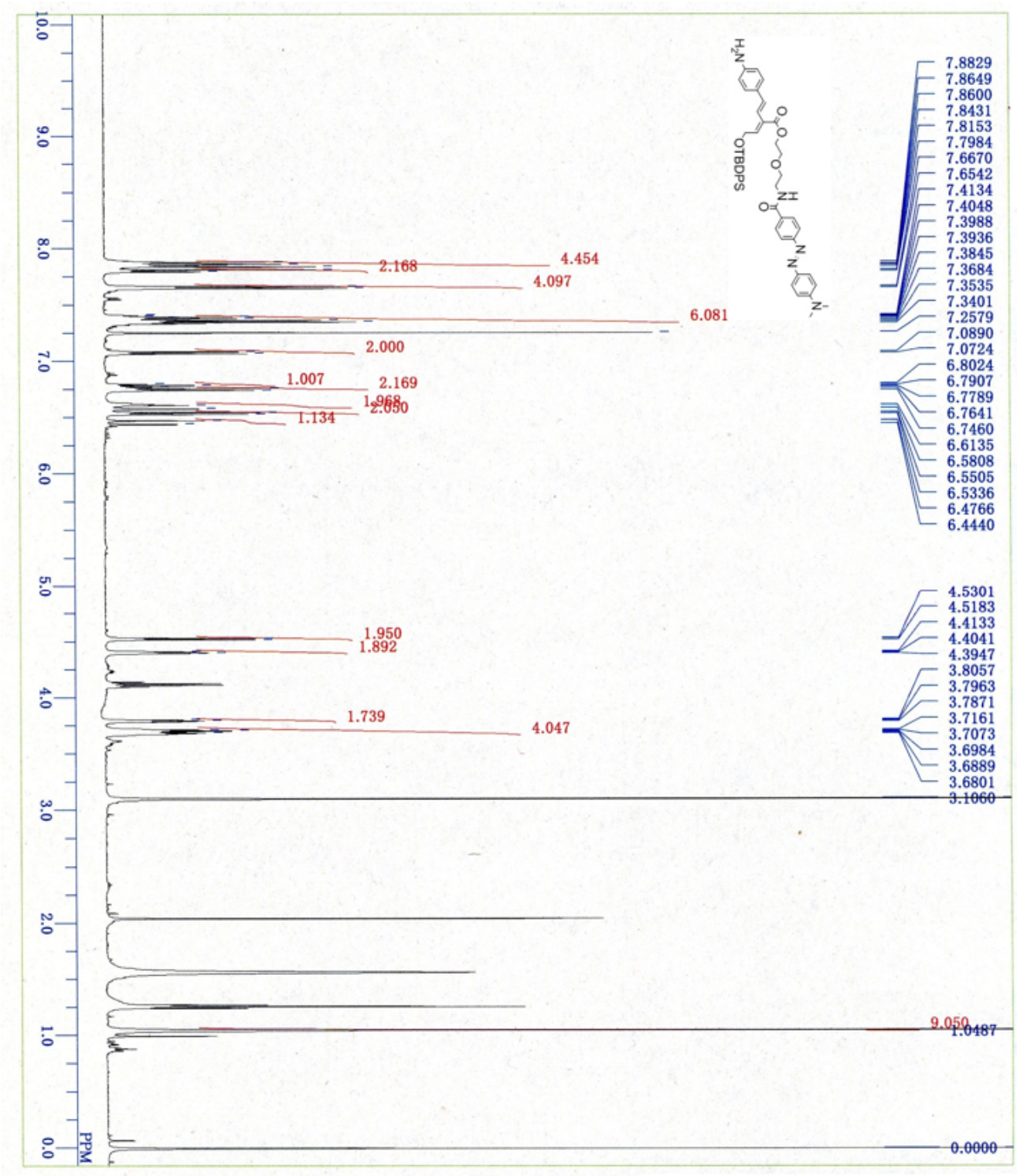

Fig. S-7 ^{1}H NMR of **3** (500 MHz, $CDCl_3$).

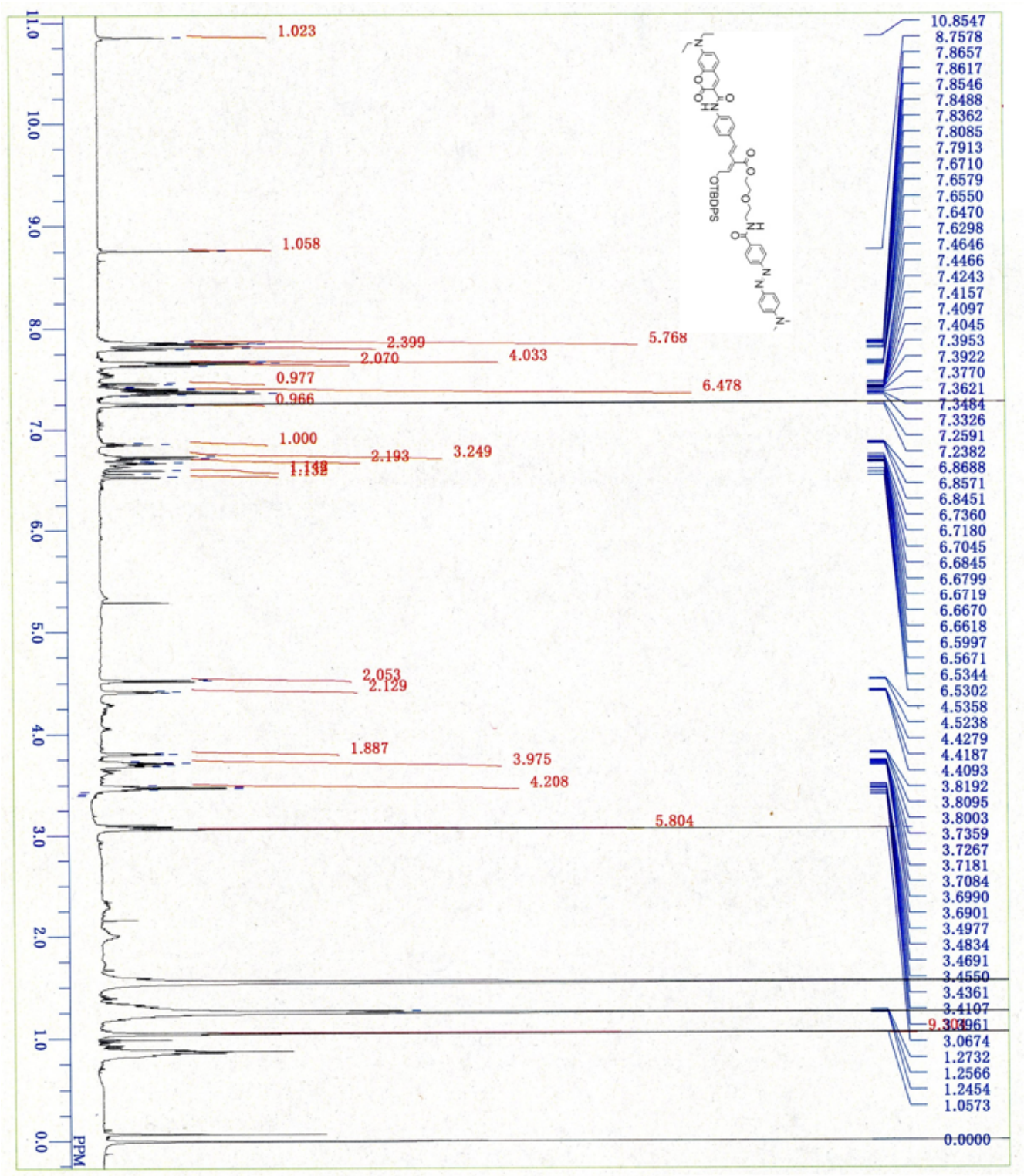

Fig. S-8 ^{1}H NMR of TBDPS-protected **4** (500 MHz, $CDCl_3$).

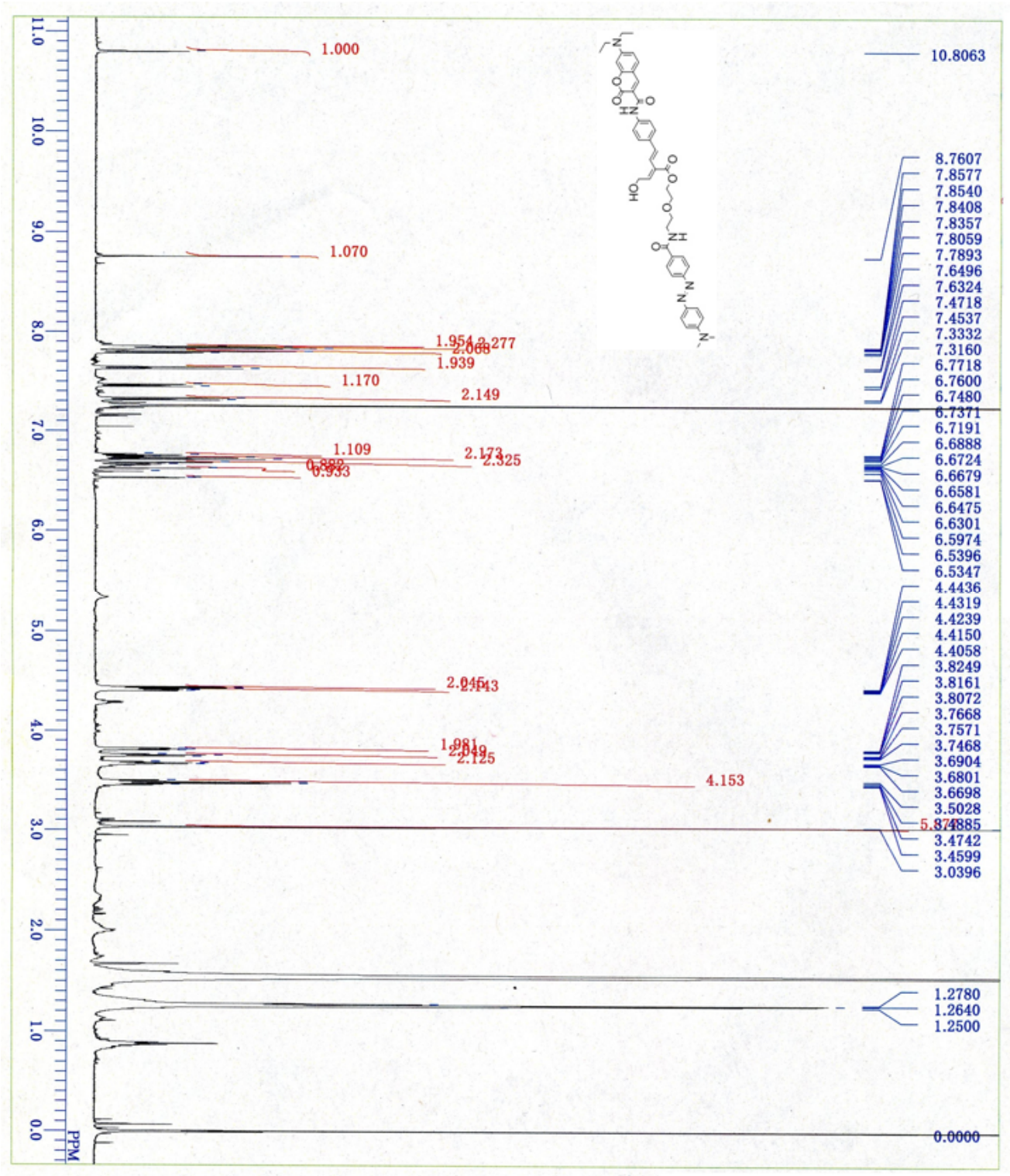

Fig. S-9 ^{1}H NMR of **4** (500 MHz, $CDCl_3$).