Electronic Supplementary Information

pH-Responsive Near-Infrared Fluorescent Cyanine Dyes for Molecular Imaging Based on pH Sensing

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1. Experimental Section

1.1. Materials and Methods

Triphenylphosphine, iodine, Triton X-100, chloroform ($CHCl_3$), thiophenol, potassium carbonate (K_2CO_3), *N,N*-dimethylformamide (DMF), acetone, and methanol (MeOH) were purchased from nacalai tesque (Japan). 3-Bromopropionic acid, 1,1,2-trimethyl-1*H*-benzo[e]indole, 2-nitrobenzenesulfonyl chloride (NsCl), and Dulbecco's modified Eagle's medium (DMEM) with/without phenol red were purchased from Tokyo Chemical Industry (TCI) Co., Ltd. (Japan). Pyridine, dimethylsulfoxide (DMSO), and acetonitrile (CH_3CN) were purchased from Wako Pure Chemicals Inc. (Japan). Dichloromethane (CH_2Cl_2) was purchased from Kishida Chemical Co., Ltd. (Japan). Imidazole was purchased from Apollo Scientific, Ltd. Silica gel (SiO_2, 230-400 mesh) for column chromatography was purchased from Silicycle (Canada). Basic alumina (Al_2O_3) for column chromatography was purchased from Merck (Germany). Buffered aqueous solutions (pH 2.5~5.8) were prepared by dissolving citric acid and sodium dihydrogenphosphate (NaH_2PO_4) in water (MilliQ). Buffered aqueous solutions (pH ~7.4) were prepared by dissolving NaH_2PO_4 and disodium hydrogenphosphate (Na_2HPO_4) in water (MilliQ). Buffered aqueous solutions (pH 9.2~11.5) were prepared by dissolving sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) in water (MilliQ). All buffered aqueous solutions were stored in refrigerator and used within one week.

UV-vis absorption spectra of dyes were measured by UV-vis-NIR spectrophotometer (V-570, JASCO Inc., Japan and UH5300, Hitachi High-Technologies Co., Japan). The sample solutions (5.0×10^{-6} M) were prepared by mixing a solution of dyes in DMSO (10 μL, 1.5×10^{-3} M) with deoxygenated buffered aqueous solution (3.0 mL) with/without Triton X-100 (2.0×10^{-3} M).

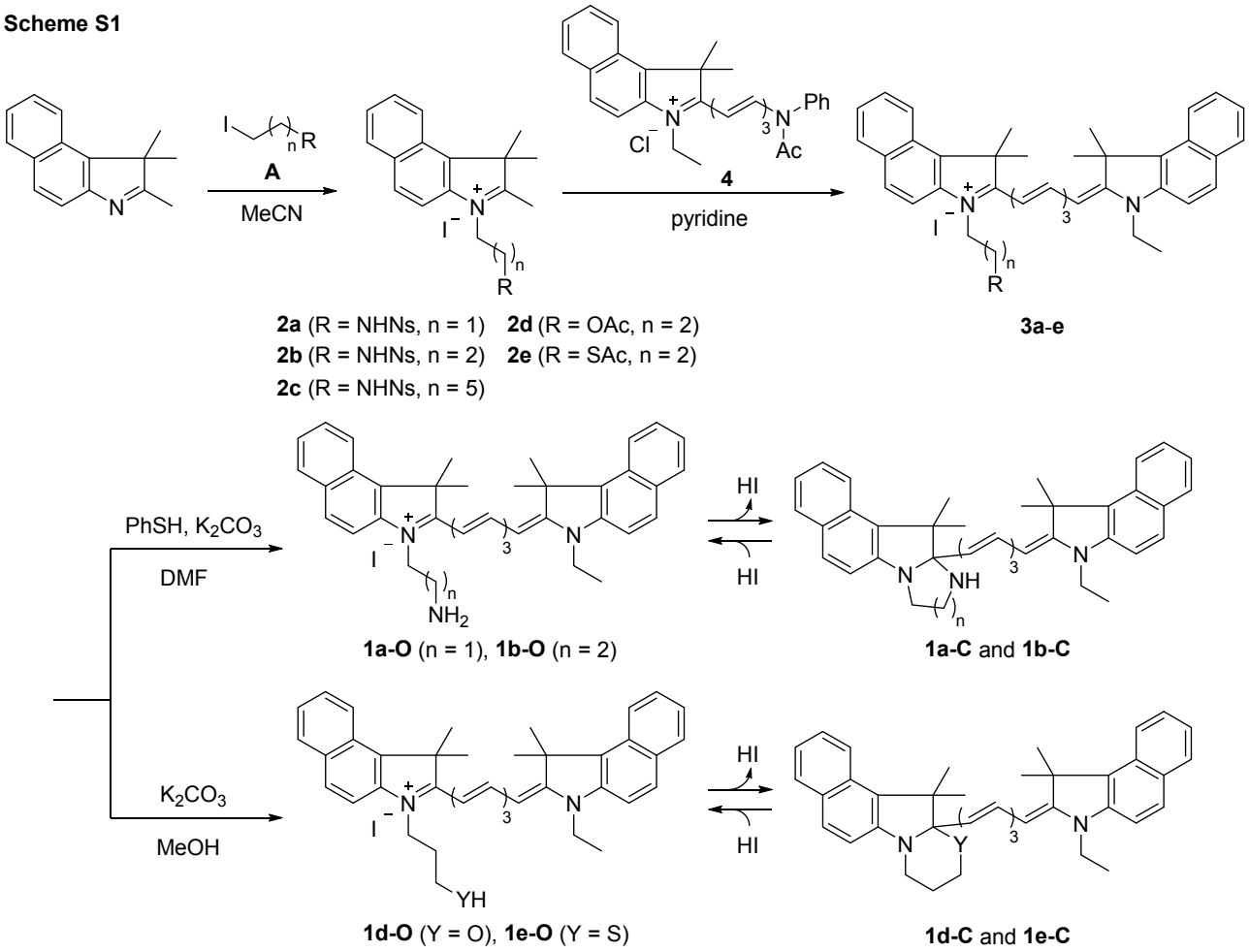
Emission spectra of dyes were measured by fluorescence spectrophotometer (FluoroMax-3 equipped with integrating sphere, HORIBA Jobin Yvon IBH Ltd., UK and RF-6000, Shimadzu Co., Japan). The sample solutions were prepared in the same manner shown in the method of UV-vis absorption measurement.

Low-resolution mass spectra were measured by ultraflex (MALDI-TOF, Bruker Co., USA). High-resolution mass spectra were measured by JMS-HX110A (FAB, JEOL Ltd., Japan) or EXACTIVE (ESI, Thermo Fisher Scientific Inc., USA).

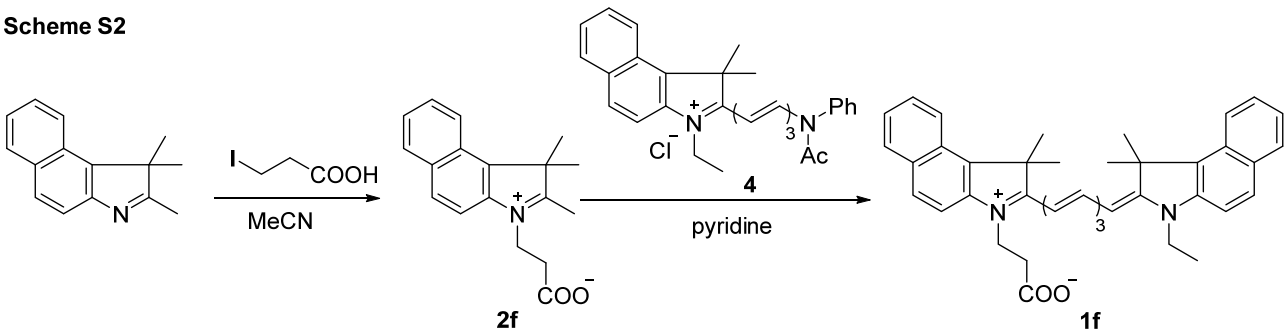
1.2. Synthesis of ICG derivatives 1a, 1b, and 1d-f

ICG derivatives **1a-f** were prepared from 1,1,2-trimethyl-1*H*-benzo[e]indole for 2~3 steps (Schemes S1 and S2). The synthesis of ICG derivative **1c** was reported in our previous work.[1]

Scheme S1



Scheme S2



1.2.1. Synthesis of iodides A

Iodides **A**, *N*-(2-iodoethyl)-2-nitrobenzenesulfoamide and *N*-(3-iodopropyl)-2-nitrobenzenesulfoamide, were prepared according to the synthesis of *N*-(6-iodohexyl)-2-nitrobenzenesulfoamide.[1] The synthetic procedures of these compounds are briefly summarized below. 3-Iodopropyl acetate[2] and *S*-3-iodopropyl thioacetate[3] were prepared according to the reported procedures. 3-Bromopropionic acid was purchased from TCI, Japan.

Synthesis of *N*-(3-iodopropyl)-2-nitrobenzenesulfoamide: To a solution of 3-amino-1-propanol (1.5 g, 20 mmol) and pyridine (1.6 mL, 20 mmol) in CH_2Cl_2 (40 mL) was slowly added 2-nitrobenzenesunfonyl chloride (2.2 g, 10 mmol) at 0 °C. After stirring for 1 h at room temperature, the reaction mixture was washed with 0.5 M HCl aqueous solution (20 mL×5). The organic layer was dried over $MgSO_4$. The organic solvents were removed under reduced pressure to afford *N*-(3-hydroxypropyl)-2-nitrobenzenesulfonamide (2.2 g, 8.3 mmol, 83%) as a pale yellow oil. To a solution of triphenylphosphine (2.1 g, 8.2 mmol) and imidazole (1.1 g, 17 mmol) in CH_2Cl_2 (130 mL) was added iodine (1.0 g, 8.1 mmol) at room temperature. After stirring for 15 min for room temperature, to this mixture was slowly added *N*-(3-hydroxypropyl)-2-nitrobenzenesulfonamide (2.2 g, 8.3 mmol) in CH_2Cl_2 (30 mL). After stirring at room temperature overnight, the organic solvents were removed under reduced pressure. The residue was subjected to column chromatography on SiO_2 (eluent: hexane-AcOEt, v:v = 2:1) to afford *N*-(3-iodoprophyl)-2-nitrobenzenesulfoamide (0.73 g, 2.0 mmol, 24%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C) δ 2.06 (q, J = 6.5, 2H), 3.22 (m, 4H), 7.74-7.79 (m, 2H), 7.88-7.90 (m, 1H), 8.16-8.12 (m, 1H).

Synthesis of *N*-(2-iodoethyl)-2-nitrobenzenesulfoamide: *N*-(2-Hydroxyethyl)-2-nitrobenzenesulfonamide was similarly synthesized from 2-amino-1-ethanol as a pale yellow solid in 77% yield. *N*-(2-iodoethyl)-2-nitrobenzenesulfoamide was similarly synthesized from *N*-(2-hydroxyethyl)-2-nitrobenzenesulfonamide as a white solid in 63% yield.

1.2.2. Synthesis of benzoindoles 2

To a solution of 1,1,2-trimethyl-1*H*-benzo[e]indole (0.68 g, 3.2 mmol) in acetonitrile (40 mL) was added *N*-(2-iodoethyl)-2-nitrobenzenesulfoamide (1.5 g, 4.2 mmol) at room temperature. After stirring the mixture at

100 °C for 3 days, the organic solvent was removed under reduced pressure. The residue was dissolved in a small amount of CH_2Cl_2. After adding ether slowly, the product was gradually precipitated. The resulting mixture was stored in refrigerator overnight. The precipitate was removed by filtration and washed with cold ether. The volatiles were removed under reduced pressure to afford benzoindole **2a** (0.56 g, 0.99 mmol, 30%) as a bluish purple solid.

2a: mp 125-130 °C (decomp.); IR(KBr) 3436, 3047, 2979, 2870, 1633, 1615, 1581, 1543, 1469, 1431, 1339, 1204, 1163, 1107, 1087, 1032, 992, 927, 863, 813, 779, 735, 696, 654, 584, 551, 524 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.80 (s, 6H), 2.99 (s, 3H), 3.58-3.64 (m, 2H), 4.74 (t, J = 5.0 Hz, 2H), 7.75 (t, J = 7.5 Hz, 1H), 7.81 (t, J = 7.2 Hz, 1H), 7.84-7.91 (m, 2H), 7.94 (dd, J = 7.3, 1.8 Hz, 1H), 8.01 (dd, J = 7.6, 1.5 Hz, 1H), 8.11 (d, J = 8.9 Hz, 1H), 8.23 (d, J = 8.3 Hz, 1H), 8.30 (d, J = 8.9 Hz, 1H), 8.40 (d, J = 8.2 Hz, 1H), 8.48 (t, J = 6.4 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, 25 °C) δ 14.4, 21.5, 40.6, 48.4, 55.8, 113.3, 123.4, 124.9, 127.2, 127.3, 128.4, 129.5, 129.7, 130.6, 132.2, 133.0, 133.1, 134.5, 136.7 138.7, 147.3, 198.4. HRMS (FAB) calcd for $C_{23}H_{24}N_3O_4S$ ([M^+]) 438.1488, found 438.1496.

Benzoindole **2b** and **2d-f** were obtained in the similar manner using *N*-(3-iodopropyl)-2-nitrobenzenesulfoamide, 3-iodopropyl acetate,[2] *S*-3-iodopropyl thioacetate,[3] and 3-bromopropionic acid instead of *N*-(3-iodoethyl)-2-nitrobenzenesulfoamide, respectively. Compounds **2d**[4] and **2f**[5] were known.

2b: a bluish white solid (76% yield): mp 201-206 °C (decomp.); IR(KBr) 3436, 3187, 3105, 3056, 3030, 2997, 2969, 2926, 1634, 1583, 1537, 1472, 1443, 1409, 1368, 1343, 1167, 1116, 1085, 853, 828, 784, 745, 730, 657, 589, 560 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.77 (s, 6H), 2.10-2.18 (m, 2H), 2.92 (s, 3H), 3.14-3.22 (m, 2H), 4.60 (t, J = 7.5 Hz, 2H), 7.75 (t, J = 7.6 Hz, 1H), 7.81 (t, J = 7.5 Hz, 1H), 7.87-7.94 (m, 2H), 7.98-8.04 (m, 2H), 8.08 (d, J = 9.2 Hz, 1H), 8.23 (d, J = 8.3 Hz, 1H), 8.27-8.33 (m, 2H), 8.37 (d, J = 8.0 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, 25 °C) δ 14.0, 21.7, 27.2, 40.3, 45.8, 55.7, 113.2, 123.5, 124.7, 127.3, 127.5, 128.6, 129.8, 129.9, 130.8, 132.0, 132.9, 133.1, 134.5, 137.0 138.5, 147.9, 196.9. HRMS (FAB) calcd for $C_{24}H_{26}N_3O_4S$ ([M^+]) 452.1644, found 452.1639.

2e: a brown oil (39%): IR(neat) 3426, 3106, 2972, 2930, 1687, 1634, 1615, 1581, 1523, 1466, 1393, 1354, 1236, 1217, 1132, 1026, 954, 919, 868, 810, 789, 753, 661, 626 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, 25 °C) δ 1.87

(s, 6H), 2.29 (s, 3H), 2.32 (tt, *J* = 7.3, 7.5 Hz, 2H), 3.07 (t, *J* = 7.3 Hz, 2H), 3.22 (s, 3H), 4.93 (t, *J* = 7.5 Hz, 2H), 7.66 (t, *J* = 7.5 Hz, 2H), 7.74 (t, *J* = 7.8 Hz, 2H), 7.96-8.13 (m, 5H). ^{13}C NMR (126 MHz, $CDCl_3$, 25 °C) δ 16.8, 22.6, 25.8, 28.3, 30.5, 49.0, 55.8, 112.4, 122.5, 1237.4, 127.6, 128.4, 129.9, 131.4, 133.5, 136.8, 138.2, 195.3, 195.7. HRMS (FAB) calcd for $C_{20}H_{24}NOS$ ([M^+]) 326.1579, found 326.1586.

1.2.3. Synthesis of ICG derivatives 3

In a flame-dried Schlenk flask, benzoindoles **2a** (0.20 g, 0.35 mmol), **4**[6] (0.20 g, 0.35 mmol), and pyridine (5 mL) were successively added at room temperature under nitrogen atmosphere. After stirring at 40 °C for 1 h, the organic solvent was removed under reduced pressure. The residue was diluted with $CHCl_3$ (20 mL). The organic solution was washed with brine (20 mL) and dried over Na_2SO_4. The organic solvent was removed under reduced pressure and the residue was subjected to column chromatography on SiO_2 (eluent: $CHCl_3$-MeOH, v:v = 10:1) to afford a crude product. The crude product was washed with a mixture of CH_2Cl_2 and ether (v:v = 3:1) and EtOAc by centrifugation. The volatiles were removed under reduced pressure to afford ICG derivative **3a** (0.21 g, 0.24 mmol, 69%) as a green solid.

3a: mp 182-187 °C (decomp.); IR(KBr) 3435, 2926, 1626, 1542, 1508, 1420, 1355, 1147, 1100, 1064, 1011, 925, 879 cm^{-1}; ^{1}H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.46 (t, *J* = 7.1 Hz, 3H), 1.99 (s, 6H), 2.00 (s, 6H), 3.51-3.54 (m, 2H), 4.29-4.34 (m, 4H), 6.33 (d, *J* = 13.7 Hz, 1H), 6.42 (d, *J* = 13.7 Hz, 1H), 6.57-6.66 (m, 2H), 7.27-7.33 (m, 1H), 7.42-8.04 (m, 16H), 8.18-8.25 (m, 2H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 13.0, 27.5, 27.5, 27.7, 39.2, 41.6, 51.9, 52.5, 54.5, 104.1, 111.9, 121.2, 123.2, 123.4, 125.1, 125.6, 126.0, 126.2, 128.5, 128.8, 129.2, 129.4, 129.7, 131.0, 131.1, 131.2, 131.4, 131.9, 133.7, 135.1, 137.9, 140.5, 141.4. Signals in aromatic region were overlapped. HRMS calcd for $C_{45}H_{45}N_4O_4S$ ([M^+]) 737.3162, found 737.3161.

ICG derivatives **3b**, **3d**, and **3e** were obtained in the similar manner using benzoindoles **2b**, **2d**, and **2e** instead of benzoindoles **2a**, respectively.

3b: a dark yellow solid (80%): mp 171-176 °C (decomp.); IR(KBr) 3443, 2928, 1626, 1542, 1508, 1475, 1420, 1355, 1308, 1147, 1099, 1062, 1009, 964, 924, 834, 752, 720, 665, 586, 521 cm^{-1}; ^{1}H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.45 (t, *J* = 7.1 Hz, 3H), 1.94 (s, 12H), 2.00-2.13 (m, 2H), 3.25-3.34 (m, 2H), 4.21-4.32 (m,

4H), 6.26 (d, J = 11.9 Hz, 1H), 6.40 (d, J = 13.7 Hz, 1H), 6.57-6.64 (m, 2H), 7.05-8.19 (m, 19H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 13.0, 23.8, 27.5, 27.7, 29.0, 40.4, 41.7, 51.9, 52.5, 103.6, 103.7, 111.6, 111.9, 121.2, 123.2, 123.3, 123.4, 125.1, 125.9, 126.0, 126.2, 128.6, 128.7, 129.6, 129.7, 131.0, 131.1, 131.6, 131.8, 133.5, 133.6, 134.3, 135.2. Signals in aromatic region were overlapped. HRMS calcd for $C_{46}H_{47}N_4O_4S$ ([M^+]) 751.3318, found 751.3317.

3d: a dark green solid (48%): mp 178-182 °C (decomp.); IR(ATR) 2974, 1735, 1508, 1415, 1351, 1056, 1003, 916, 718, 664 cm^{-1}; ^{1}H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.45 (t, J = 7.1 Hz, 3H), 1.97 (s, 12H), 2.17-2.23 (m, 2H), 3.31 (s, 3H), 4.17 (t, J = 6.0 Hz, 2H), 4.24-4.33 (m, 4H), 6.27 (d, J = 13.2 Hz, 1H), 6.42 (d, J = 13.7 Hz, 1H), 6.58-6.66 (m, 2H), 7.41-7.66 (m, 7H), 7.94-8.24 (m, 8H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 13.0, 20.8, 27.5, 27.7, 27.7, 40.5, 41.9, 51.9, 52.5, 62.8, 111.8, 112.0, 123.4, 125.7, 126.2, 128.7, 128.8, 129.5, 129.6, 131.1, 131.1, 131.6, 131.9, 133.1, 133.5, 134.2, 135.5, 140.5, 141.3, 172.6. Signals in aromatic region were overlapped. HRMS (ESI) calcd for $C_{42}H_{45}N_2O_2$ ([M^+]) 609.3476, found 609.3465.

3e: a dark green solid (52%): mp 179-184 °C (decomp.); IR(KBr) 3435, 2925, 2864, 1626, 1508, 1467, 1421, 1355, 1308, 1147, 1090, 1064, 1008, 924, 879, 772, 720, 665 cm^{-1}; ^{1}H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.45 (t, J = 6.6 Hz, 3H), 1.99 (s, 6H), 2.00 (s, 6H), 2.00-2.16 (m, 2H), 2.34 (s, 3H), 3.02 (t, J = 7.1 Hz, 2H), 4.21-4.30 (m, 4H), 6.28 (d, J = 13.3 Hz, 1H), 6.41 (d, J = 12.6 Hz, 1H), 6.56-6.66 (m, 2H), 7.43-7.66 (m, 7H), 7.96-8.25 (m, 8H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 13.0, 27.0, 27.5, 27.7, 29.0, 30.6, 40.4, 43.8, 52.0, 52.5, 103.8, 103.8, 111.7, 111.9, 123.3, 123.4, 125.8, 126.2, 127.2, 128.7, 128.8, 129.5, 129.6, 131.1, 131.1, 131.7, 131.9, 133.2, 133.5, 134.4, 140.6, 141.2, 197.0. Signals in aromatic region were overlapped. HRMS (FAB) calcd for $C_{42}H_{45}N_2OS$ ([M^+]) 625.3253, found 625.3263.

1.2.4. Synthesis of ICG derivatives 1a and 1b

ICG derivative **3a** (78 mg, 90 μmol) and DMF (5 mL) were added in a flame-dried Schlenk flask at room temperature under nitrogen atmosphere. To this solution were added thiophenol (85 μL, 0.83 mmol) and K_2CO_3 (0.11 g, 0.83 mmol) at room temperature. After stirring at room temperature for 3 h, the reaction mixture was diluted with CH_2Cl_2 (20 mL). The organic solution was successively washed with saturated

$NaHCO_3$ aqueous solution (20 mL×2) and 1 N HCl aqueous solution (20 mL), and dried over Na_2SO_4. The organic solvent was removed under reduced pressure and the resulting residue was dissolved in a small amount of CH_2Cl_2. After slow addition of hexane to this solution, the precipitate was corrected by centrifugation. The volatiles were removed under reduced pressure to afford ICG derivative **1a** (23 mg, 33 μmol, 38%) as a green solid. ICG derivative **1a** was obtained as a mixture of **1a-C** and **1a-O**, which was inseparable. ICG derivative **1b** was synthesized in a similar manner.

1a: IR(KBr) 3435, 2926, 1637, 1561, 1509, 1421, 1356, 1108, 1011, 927 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.50 (t, J = 7.1 Hz, 3H), 1.99 (s, 6H), 2.00 (s, 6H), 3.30-3.35 (m, 2H), 4.25-4.47 (m, 4H), 6.20-6.72 (m, 4H), 7.30-8.30 (m, 15H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 13.3, 27.2, 27.3, 27.4, 27.5, 27.7, 27.8, 31.7, 36.9, 37.7, 41.2, 51.2, 52.6, 112.3, 123.1, 123.6, 126.9, 127.3, 128.5, 129.0, 129.2, 129.8, 131.0, 131.2, 131.6, 132.1, 134.1, 140.1, 141.2. **1a-C** was not observed in MeOH. Signals in aromatic region were overlapped. HRMS (FAB) calcd for $C_{39}H_{42}N_3$ ($[M^+]$) 552.3379, found 552.3381.

1b: a green solid (46%): IR(KBr) 3536, 2969, 1626, 1561, 1509, 1469, 1416, 1357, 1237, 1154, 1109, 1088, 1067, 1010, 964, 925, 879, 834 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.47 (t, J = 6.9 Hz, 3H), 2.00 (s, 12H), 2.05-2.22 (m, 2H), 3.14-3.18 (m, 2H), 4.22-4.28 (m, 4H), 6.28 (d, J = 13.3 Hz, 1H), 6.51 (d, J = 14.2 Hz, 1H), 6.58-6.70 (m, 2H), 7.41-8.27 (m, 15H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 13.1, 26.7, 27.4, 27.8, 38.3, 40.8, 41.7, 51.6, 52.9, 102.9, 111.4, 111.7, 112.1, 123.1, 123.3, 123.5, 125.6, 126.5, 128.6, 128.8, 128.9, 129.4, 129.7, 130.6, 131.1, 131.1, 131.7, 132.0, 133.0, 133.8, 136.0, 140.3, 141.1. **1b-C** was not observed in MeOH. Signals in aromatic region were overlapped. HRMS (FAB) calcd for $C_{40}H_{44}N_3$ 566.3535; found 566.3543.

1.2.5. Synthesis of ICG derivatives 1d and 1e-C

ICG derivative **3e** (12 mg, 16 μmol) and MeOH (1 mL) were added in a flame-dried Schlenk flask at room temperature under nitrogen atmosphere. To this solution was added K_2CO_3 (4.7 mg, 34 μmol) at room temperature. After stirring at room temperature for 3 h, the reaction mixture was diluted with CH_2Cl_2 (10 mL). The organic solution was washed with saturated $NaHCO_3$ aqueous solution (10 mL×2) and dried over Na_2SO_4.

The organic solvent was removed under reduced pressure and the residue was subjected to column chromatography on Al_2O_3 (basic alumina, eluent: CH_2Cl_2) to afford ICG derivative **1e-C** (6.5 mg, 9.1 μmol, 57%) as a brown solid. Under the slightly basic conditions, ICG derivative **1e-C** could be purified without the contamination of **1e**. ICG derivative **1d** was synthesized in a similar manner.

1d: an orange solid (52%): IR(ATR) 2974, 1727, 1506, 1468, 1410, 1351, 1196, 1145, 1081, 1057, 1003, 911, 876, 804, 774, 663 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.44 (t, J = 7.1 Hz, 3H), 1.97 (s, 6H), 1.97 (s, 6H), 2.02-2.09 (m, 2H), 3.73 (t, J = 5.7 Hz, 2H), 4.22-4.31 (m, 4H), 6.32-6.38 (m, 2H), 6.59 (t, J = 12.4 Hz, 2H), 7.47-7.76 (m, 6H), 7.96-8.07 (m, 7H), 8.21 (d, J = 8.2 Hz, 2H). In MeOH-d_4, **1d-C** was observed as a minor component (~5%), due to low solubility of **1d-C** in MeOH. In $CDCl_3$, the signals of **1d-C** were observed as a mixture of diastereoisomers of olefins. ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 12.9, 27.5, 27.6, 31.4, 40.2, 42.2, 52.1, 52.2, 59.6, 104.3, 104.3, 111.8, 112.0, 123.3, 123.3, 125.9, 126.0, 126.0, 127.0, 128.7, 128.7, 128.7, 1129.5, 129.5, 129.5, 131.1, 131.1, 131.7, 131.8, 133.3, 133.4, 134.7, 135.0, 140.7, 140.7, 141.2. Signals in aromatic region were overlapped. HRMS (ESI) calcd for $C_{40}H_{44}N_3$ 566.3535; found 566.3543. Because **1d** is a mixture of **1d-C** and **1d-O**, ^1H NMR spectrum of **1d** is complicated.

1e-C: **1e-C** was obtained as a mixture of diastereoisomers of olefins. The major compounds might be *cis* and *trans* isomers (ratio: ca. 1:1) at double bond next to 1,3-thiazinane ring. Other minor compounds (~10%) were also observed. The following data were measured by using the mixture of isomers. mp 105-110 °C (decomp.); IR(KBr) 3435, 2926, 1623, 1561, 1421, 1356, 1091, 1065, 1011, 925, 760 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 1.13 (s, 3H), 1.24 (t, J = 7.2 Hz, 3H), 1.26 (t, J = 7.2 Hz, 3H), 1.52 (s, 3H), 1.59 (s, 3H), 1.63-1.88 (m, 7H, including signals of methyl proton: 1.67 (s, 3H), 1.91 (s, 6H), 1.91 (s, 6H), 2.40-2.48 (m, 2H), 2.97-3.04 (m, 2H), 3.49-3.57 (m, 2H), 3.72-3.83 (m, 6H), 5.41 (d, J = 11.9 Hz, 1H), 5.86 (d, J = 15.6 Hz, 1H), 6.13 (dd, J = 11.0, 14.2 Hz, 1H), 6.38 (dd, J = 11.0, 14.6 Hz, 1H), 6.57 (dd, J = 11.2, 14.4 Hz, 1H), 6.93-7.28 (m, 17H), 7.36 (dd, J = 7.5, 7.5 Hz, 2H), 7.42 (t, J = 7.5, 7.5 Hz, 2H), 7.70-7.78 (m, 8H), 7.90 (d, J = 8.7 Hz, 2H), 7.97 (d, J = 8.7 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 11.6, 20.8, 21.3, 24.2, 26.7, 27.2, 36.7, 41.4, 47.4, 51.6, 88.3, 95.8, 108.8, 111.1, 121.4, 121.5, 121.6, 121.8, 124.3, 126.1, 126.5, 127.1, 128.3, 128.5, 128.7, 129.2, 129.3, 129.3, 129.4, 129.5, 129.8, 130.8, 135.5, 137.1, 141.8, 145.7, 157.2. Signals in aromatic

region were overlapped. HRMS calcd for $C_{40}H_{43}N_2S$ ([M^+]) 583.3147, found 583.3137.

1.2.6. Synthesis of ICG derivatives 1f

In a flame-dried Schlenk flask, benzoindoles **2f** (0.50 g, 0.14 mmol), **4**[6] (0.66 g, 0.14 mmol), and pyridine (3 mL) were successively added at room temperature under nitrogen atmosphere. After stirring at 40 °C for 1 h, the organic solvent was removed under reduced pressure. The residue was diluted with $CHCl_3$ (20 mL). The organic solution was washed with brine (20 mL) and dried over Na_2SO_4. The organic solvent was removed under reduced pressure and the residue was subjected to column chromatography on SiO_2 (eluent: $CHCl_3$-MeOH, v:v = 10:1) to afford a crude product. The crude product was dissolved in a small amount of CH_2Cl_2. After slow addition of diethyl ether to this solution (v:v = 3:1), the precipitate was corrected by centrifugation. The precipitate was washed with a small amount of EtOAc. The volatiles were removed under reduced pressure to afford ICG derivative **1f** (0.070 g, 0.12 mmol, 88%) as a green solid.

1f: mp 180-185 °C (decomp.); IR(ATR) 2974, 1727, 1506, 1468, 1410, 1351, 1196, 1145, 1081, 1057, 1003, 911, 876, 804, 774, 663 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, 25 °C) δ 1.45 (t, J = 6.9 Hz, 3H), 1.96 (s, 6H), 1.97 (s, 6H), 2.79 (t, J = 6.9 Hz, 2H), 4.28-4.34 (m, 2H), 4.41-4.48 (m, 2H), 6.28 (d, J = 13.8 Hz, 1H), 6.44 (d, J = 13.7 Hz, 1H), 6.53-6.70 (m, 2H), 7.40-7.64 (m, 6H), 7.85-8.24 (m, 7H), 8.52-8.55 (m, 2H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 13.1, 27.5, 27.7, 40.6, 41.3, 51.8, 52.5, 59.8, 112.0, 112.0, 123.2, 123.4, 125.6, 125.7, 12612, 128.5, 128.8, 129.4, 129.5, 131.0, 131.0, 131.1, 131.5, 131.9, 133.1, 133.5, 138.8, 140.5, 141.1, 149.8, 169.3. Signals in aromatic region were overlapped. HRMS (ESI) calcd for $C_{40}H_{40}N_2O_2$ ($M+H^+$): 581.3163; found 581.3154.

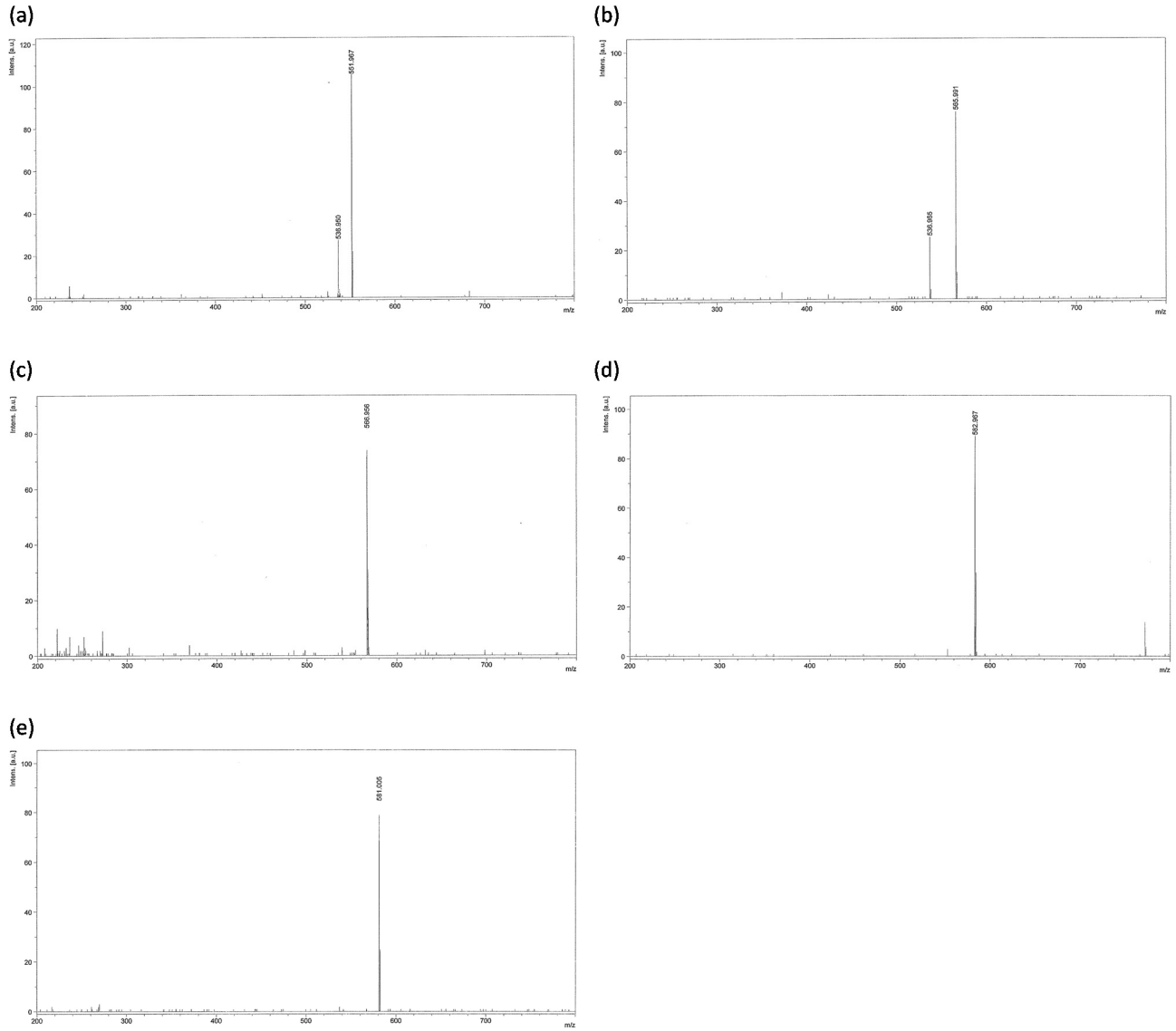


Figure S1. Low-resolution MALDI-TOF mass spectra of (a) **1a**, (b) **1b**, (c) **1d**, (d) **1e**, and (e) **1f**. Matrix: CHCA.

1.3. UV-vis and fluorescence spectra of pH-responsive dyes 1

The UV-vis absorption spectra of **1** in buffered solutions with and without Triton X-100 are summarized in Figures S2 and S3.

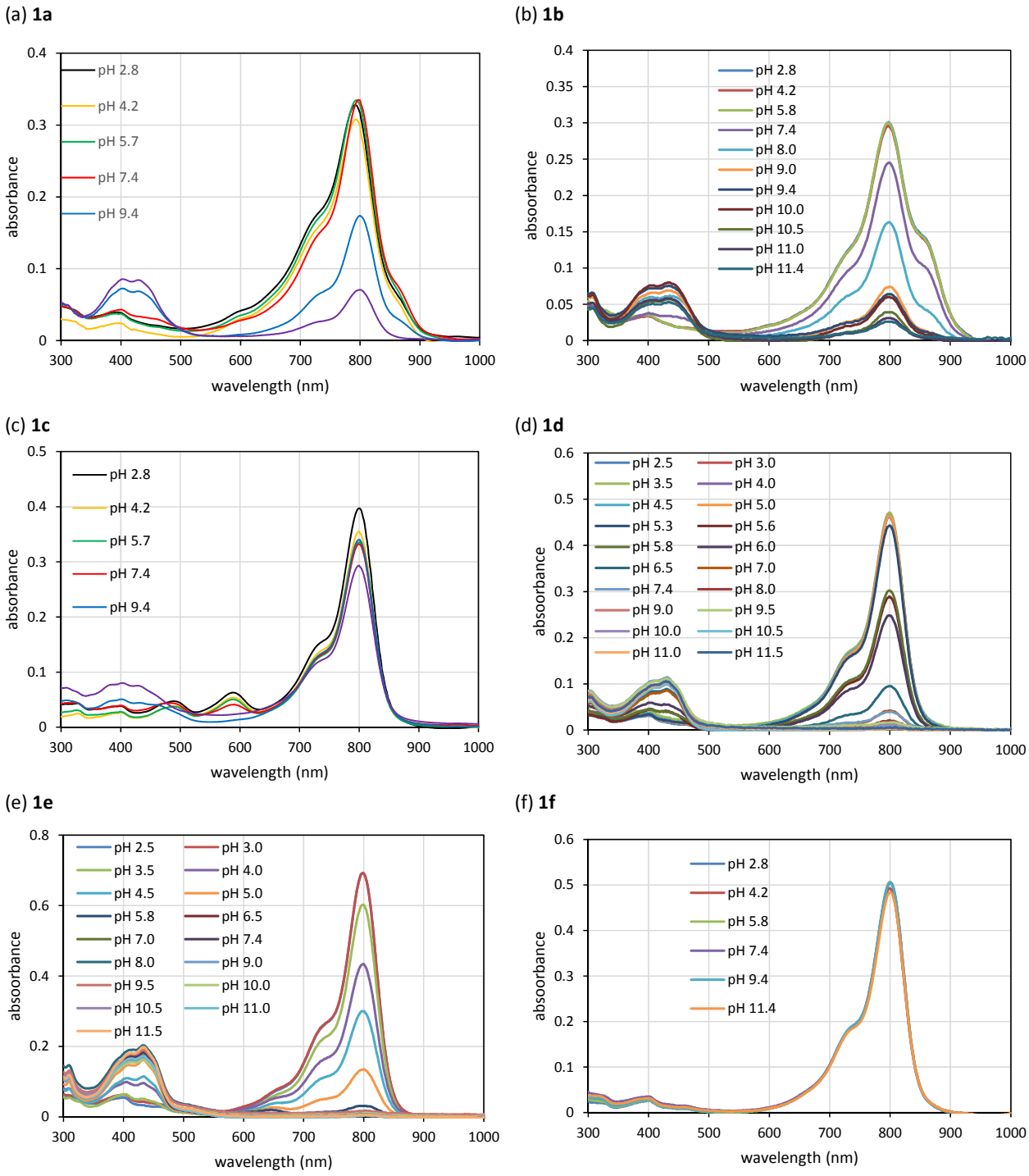


Figure S2. UV-vis absorption spectra of **1a-f** (5×10^{-6} M) in buffered solutions with Triton X-100 (2×10^{-3} M).

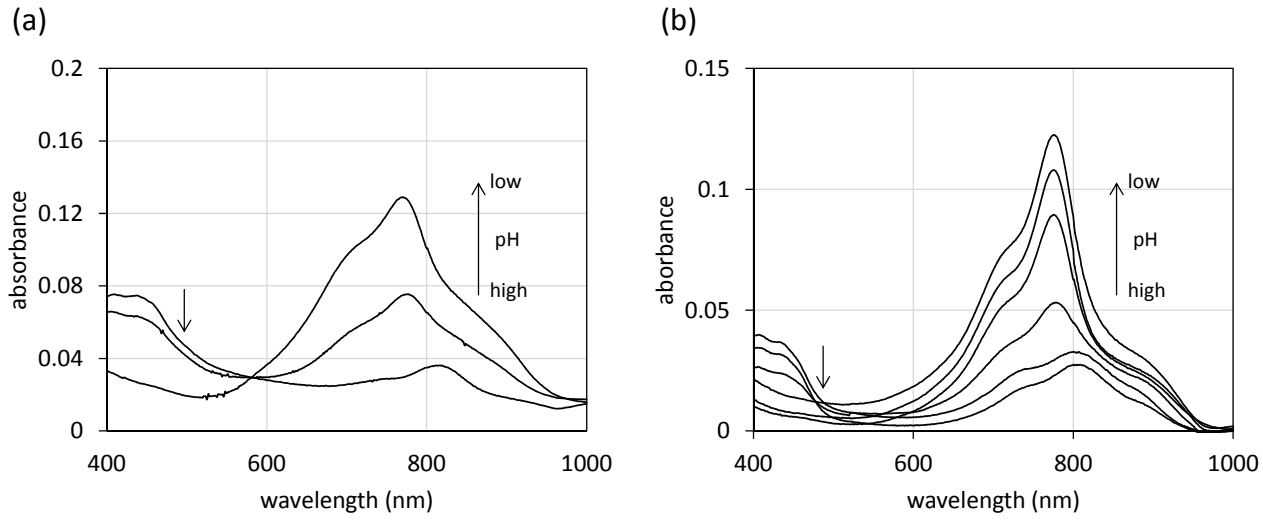


Figure S3. UV-vis absorption spectra of (a) **1a** (pH 11.4, 7.8, 5.7) and (b) **1b** (pH 11.2, 9.2, 7.4, 5.8, 4.7, 2.5) in buffered solutions without Triton X-100.

The fluorescence spectra of **1** in buffered solutions with Triton X-100 are summarized in Figure S4.

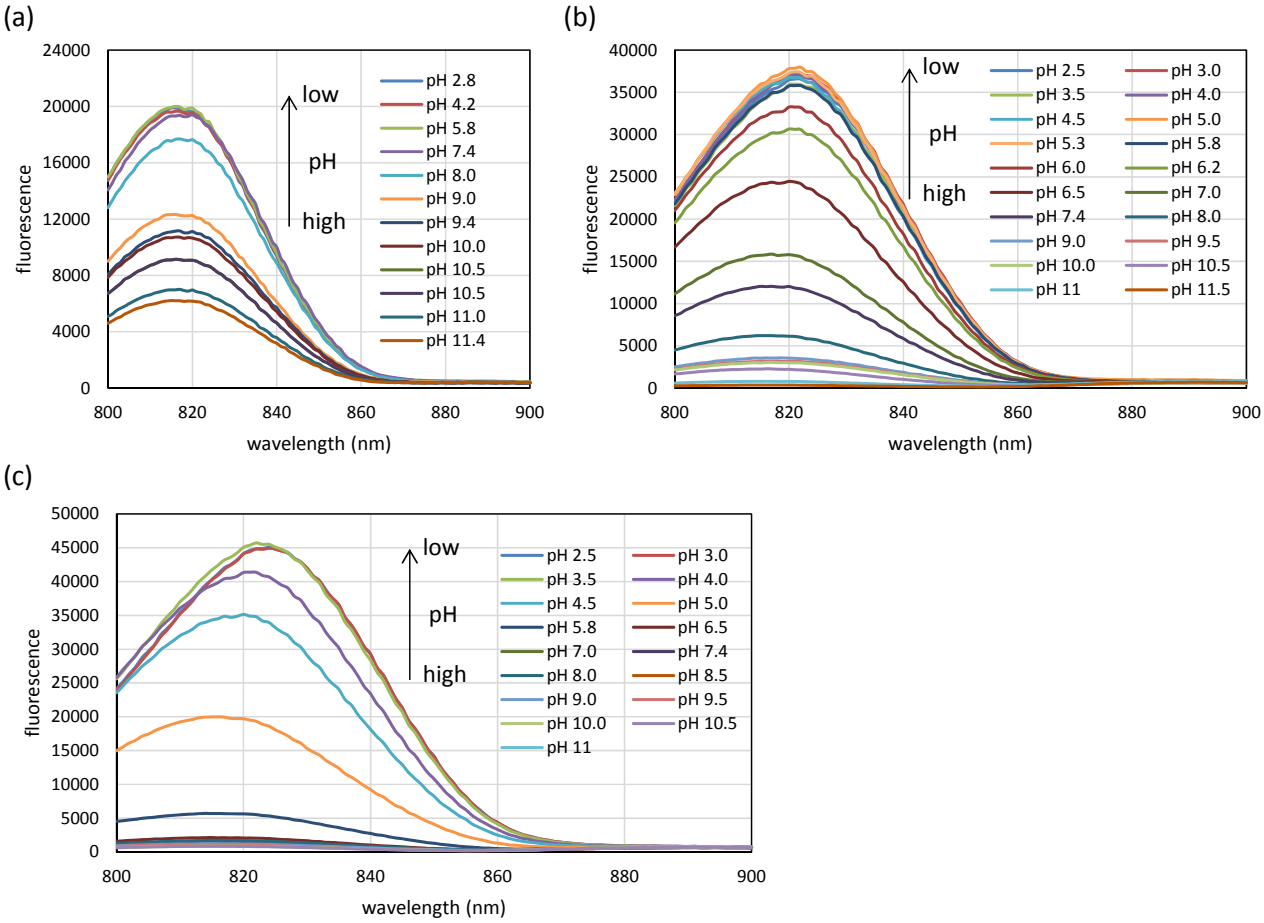


Figure S4. Fluorescence spectra of **1b**, **1d** and **1e** (5×10^{-6} M) in buffered solutions with Triton X-100 (2×10^{-3} M).

The UV-vis absorption spectra of **1** and **3** in organic solvents are summarized in Figures S5. When a solution of **1d-C**/**1d-O** in $CHCl_3$ was treated with sat. $NaHCO_3$ aq., **1d-C** was observed as a major component (Figure S5c, green). The solvent was removed under reduced pressure and the residue was dissolved in EtOH, the ratio of **1d-C** was decreased (Figure 5Sc, blue). When a solution of **1d-C**/**1d-O** in $CHCl_3$ was treated with 1N HCl aq., **1d-O** was observed as a major component (Figure S5c, red). This observation indicates that (1) when dyes were dissolved in EtOH, a part of dyes **1-C** was converted to **1-O**, and (2) when dyes were dissolved in $CHCl_3$, dyes **1-C** were not converted to **1-O**.

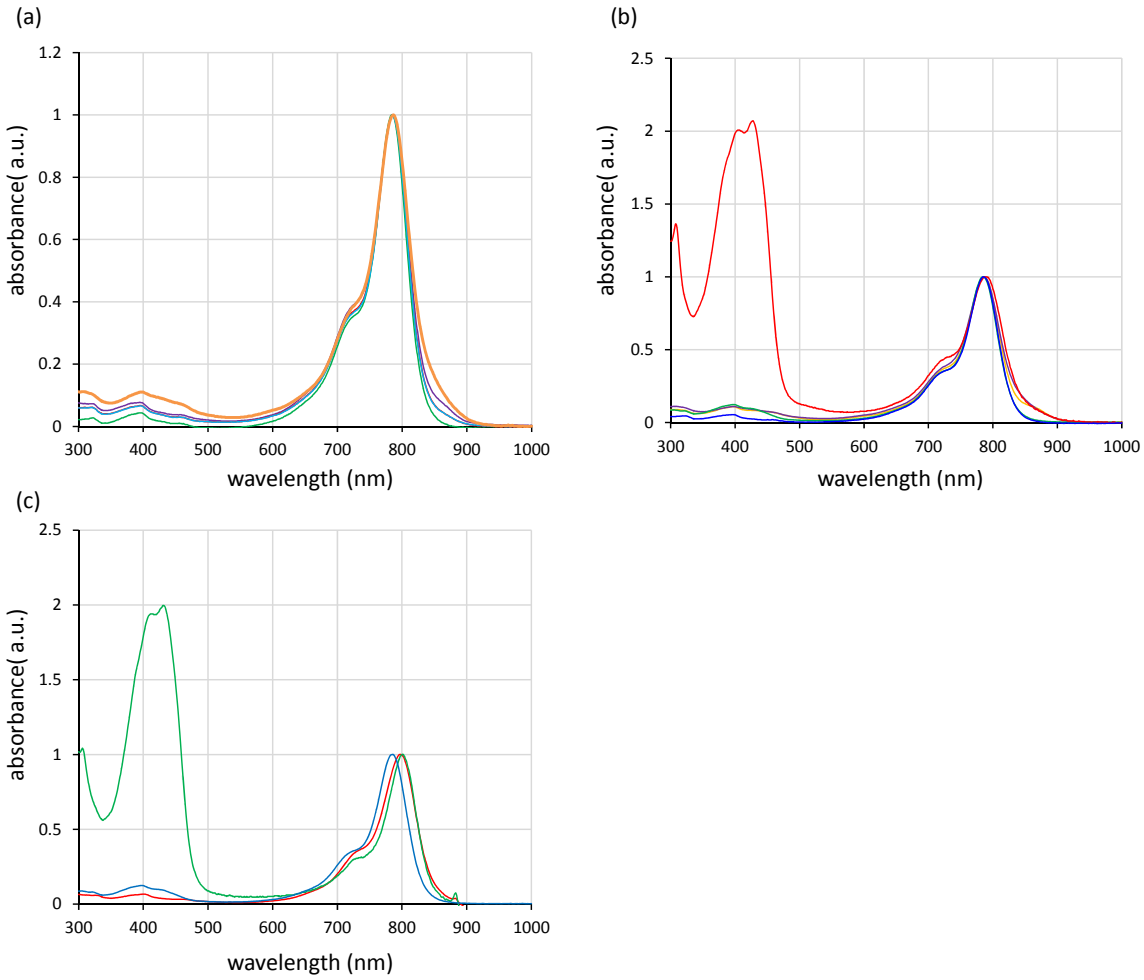


Figure S5. Normalized UV-vis absorption spectra of (a) **3a** (purple), **3b** (red), **3d** (green), and **3e** (blue) in EtOH, (b) **1a** (orange), **1b** (purple), **1d** (green), **1e** (red), and **1f** (blue) in EtOH, and (c) **1d** in EtOH (blue), in $CHCl_3$ which was treated with 1N HCl aq. before measuring (red), and in $CHCl_3$ which was treated with sat. $NaHCO_3$ aq. before measuring (green).

Molar extinction coefficient values and relative quantum yields in EtOH were measured (Table S1). Relative quantum yields were determined by using the reported method.[7] Those of **3** and **1f** were similar to ICG. Due to low solubility of **1-C** in EtOH, molar extinction coefficients of **1-O** are not accurate. Relative quantum yields of **1a-O**, **1b-O**, and **1d-O** were similar to that of ICG. The quantum yield of **1e** might be underestimated, because **1e-C** was a major component in EtOH (see Figure S5b, red).

Table S1. Photophysical properties of **1** and **3** in EtOH.

	ε ($cm^{-1}M^{-1}$) [a)]	relative quantum yield (%) [b)]
3a-O	1.9×10^5	10.2±0.4
3b-O	1.9×10^5	11.5±0.4
3d-O	2.0×10^5	11.6±0.5
3e-O	2.0×10^5	11.0±0.5
1a-O	5.6×10^4 [c)]	11.5±1.0
1b-O	7.8×10^4 [c)]	12.2±0.5
1d-O	7.0×10^4 [c)]	12.6±0.6
1e-O	1.4×10^4 [c)]	7.1±0.4
1f-O	1.7×10^5	12.0±0.7

[a)] Determined at 785 nm. [b)] Indocyanine green (ICG, Φ = 13.2% (EtOH)) was used as a standard. [c)] Due to low solubility of **1-C** in EtOH, the values are not accurate.

The absorption and fluorescence spectra were fitted to sigmoid curves to determine pKa values (Figure S6). The pKa values are summarized in Table S2.

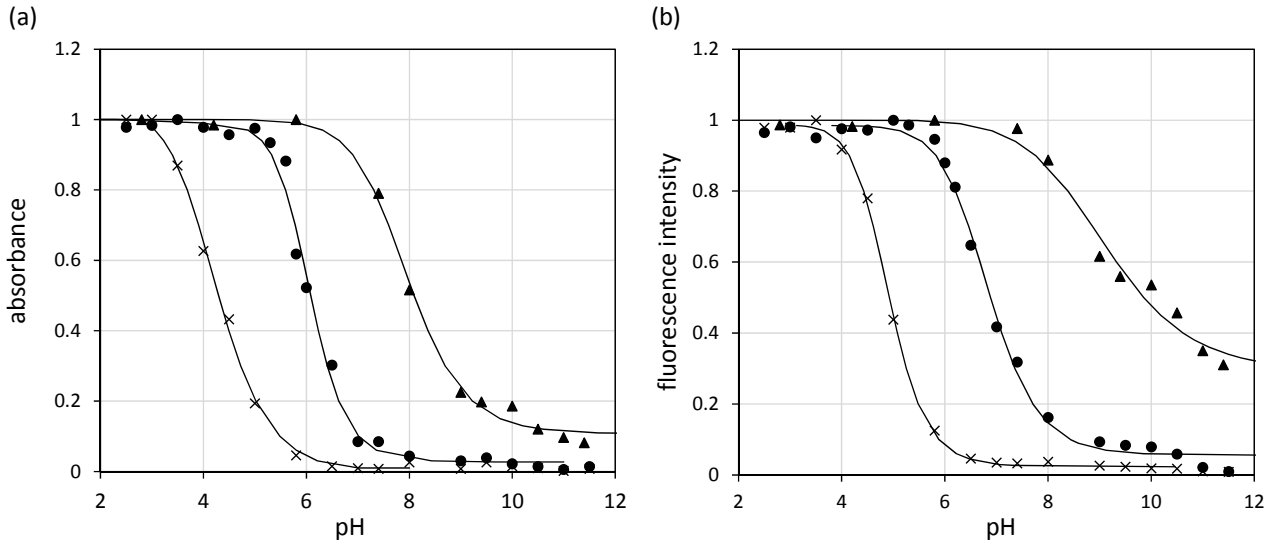


Figure S6. (a) UV–vis absorbance of **1b** (triangle), **1d** (circle) and **1e** (cross) in buffered aqueous solutions normalized at 780 nm, and (b) Fluorescence intensities of them normalized at 820 nm (λ_{ex} = 780 nm).

Table S2. pKa values of selected **1** and **3**.

	pKa (absorbance)	pKa (fluorescence)
1b	8.1	9.9
1d	6.1	6.9
1e	4.3	4.9

1.4. Photobleaching test of dyes.

The photostability of dyes **1a-O**, **1d-O**, **3a-O**, **3d-O**, and ICG (commercial, standard) was checked. Solutions of dyes were dissolved in EtOH (2 mL). To minimize the influence of the background signal, the absorbance of all solutions was adjusted to approximately 1.0–1.7. All solutions were transferred into quartz cells in air and irradiated using a 150 W Xenon light source (MAX-150, Asahi Spectra Co., Ltd., Japan, illuminance: 20 $\mu W \cdot cm^{-2}$ (above 400 nm) at the sample level, wavelength: 400–800 nm) with a visual light module and an optical filter (cutoff < 400 nm). The time-dependent photobleaching was monitored by measuring the absorbance at 785 nm (Figure S7). UV–vis absorption spectra in near-infrared region are summarized in Figure S8. The photostability of **1-O** was worse than that of **3-O** and ICG. In the case of **1d**, the absorbance of **1d-O** increased during the initial 10 min irradiation, probably because of the photo-induced ring-opening of **1d-C**. Similar photo-induced ring-opening was reported by Raymo et al.[8]

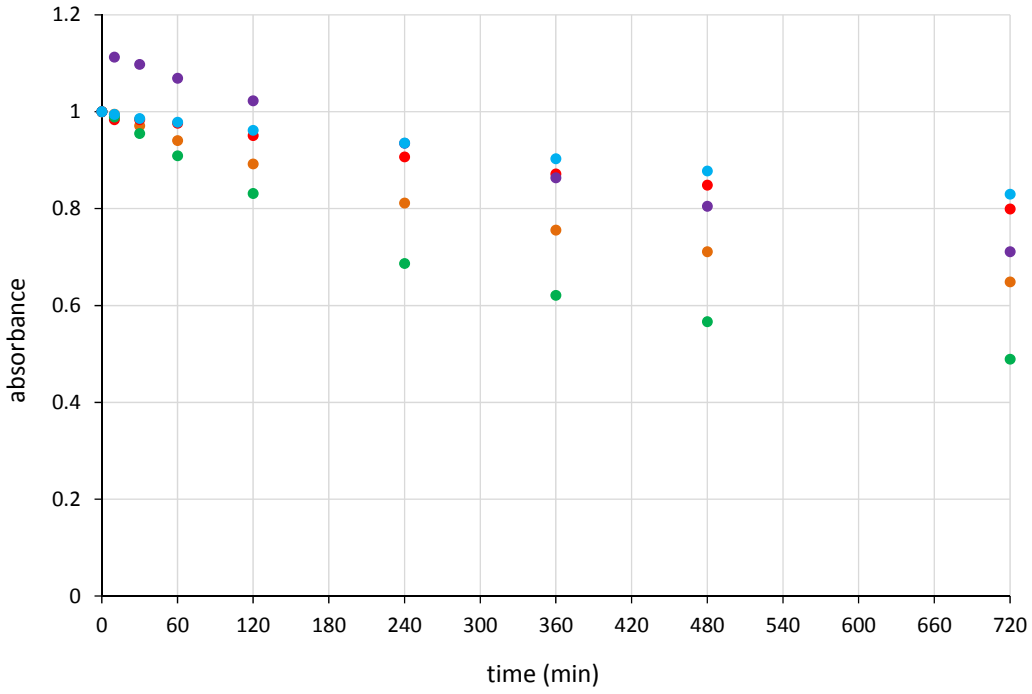


Figure S7. Time-dependent change of absorbance at 785 nm of **1a-O** (green), **1d-O** (purple), **3a-O** (orange), **3d-O** (red), and indocyanine green (ICG, blue) in EtOH after continuous photoirradiation (20 $\mu W \cdot cm^{-2}$, visible light).

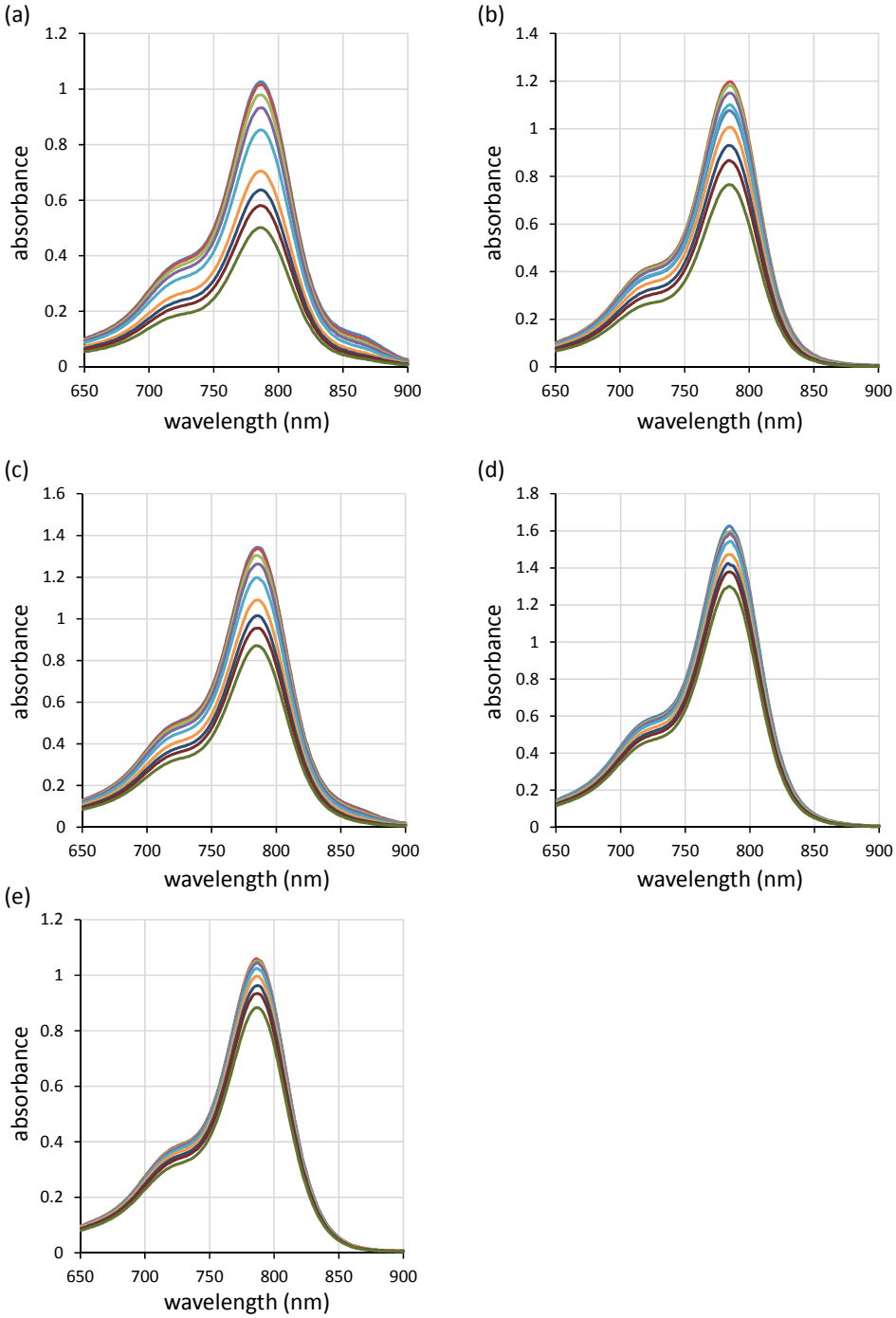


Figure S8. Time-dependent change of absorbance spectra of (a) **1a-O**, (b) **1d-O**, (c) **3a-O**, (d) **3d-O**, and (e) ICG in EtOH. Absorbance spectra were measured before and after 10 min, 30 min, 60 min, 120 min, 240 min, 360 min, 480 min, and 720 min irradiation.

1.5. *In vitro* cell imaging experiment: cell internalization

The human cervical epithelial adenocarcinoma cell line (HeLa) was purchased from American Type Culture Collection. Cells were cultured in 10% FBS-Dulbecco's modified Eagle's medium (DMEM) and cultured well-humidified incubator with 5% CO_2 and 95% air at 37 °C. We prepared two 12-well cell-culture plates for each *in vitro* experiments. HeLa cells ($8x10^4$ cells/well in 12-well plate) were cultured with **1d**, **1e**, or the commercially-available ICG (1.0×10^{-5} M or 1.0×10^{-4} M, 2.0 mL) in DMEM without phenol red for 4 h. During 4 h incubation, one of plate was placed on ice (4 °C), and another was placed on a hot plate (37 °C). Every 1 h, the fluorescence intensities of cells were measured by optical imaging device IVIS-lumina (ICG filter: λ_{ex} = 745 nm, λ_{em} = 820 nm, PerkinElmer Inc.). After 4 h incubation, cells were washed with phosphate buffer saline (1.0 mL × 2) and the wells were refilled by 10% FBS-DMEM (1.0 mL). Fluorescence intensities from cells were measured by IVIS imaging device. Images were analyzed using Living Image 2.50-Igor Pro 4.09 software (PerkinElmer Inc.), according to the manufacturer's instructions. The fluorescence dyes in the same batch was injected to three wells and one set of experiment was repeated twice to confirm reproducibility, and representative images are shown (Figure S10). In order to emphasize the fluorescence intensity from the cells, we set a threshold and represent intense fluorescence above it.

Table S3. Fluorescence intensities from cells incubated with ICG (1.0×10^{-5} M).

	Incubated at 4 °C			Incubated at 37 °C		
	F_4 ($\times10^{10}$ cps)	F_4 / F'_4	s.d.	F_{37} ($\times10^{10}$ cps)	F_{37} / F'_{37}	s.d.
0 h	51.3	1.00	0.16	47.3	1.00	0.14
1 h	46.8	0.91	0.15	52.2	1.10	0.15
2 h	46.4	0.90	0.14	521.	1.10	0.15
3 h	47.3	0.92	0.14	52.6	1.11	0.15
4 h	47.2	0.92	0.15	52.6	1.11	0.15

Table S4. Fluorescence intensities from cells incubated with **1e** (1.0×10^{-5} M).

	Incubated at 4 °C			Incubated at 37 °C		
	F_4 ($\times10^{10}$ cps)	F_4 / F'_4	s.d.	F_{37} ($\times10^{10}$ cps)	F_{37} / F'_{37}	s.d.
0 h	1.43	1.00	0.009	1.27	1.00	0.020
1 h	1.39	1.02	0.008	1.81	1.43	0.039
2 h	1.49	1.06	0.030	1.94	1.53	0.027
3 h	1.52	1.08	0.029	1.98	1.56	0.040
4 h	1.40	1.05	0.048	2.07	1.64	0.034

Table S5. Fluorescence intensities from cells incubated with **1d** (1.0×10^{-5} M).

	Incubated at 4 °C			Incubated at 37 °C		
	F_4 ($\times10^{10}$ cps)	F_4 / F'_4	s.d.	F_{37} ($\times10^{10}$ cps)	F_{37} / F'_{37}	s.d.
0 h	4.06	1.00	0.17	3.69	1.00	0.14
1 h	4.33	0.97	0.18	5.95	1.61	0.22
2 h	4.41	1.05	0.17	6.44	1.74	0.23
3 h	4.49	1.07	0.18	6.59	1.79	0.24
4 h	4.59	0.98	0.18	6.85	1.86	0.24

Table S6. Fluorescence intensities from cells incubated with **1e** (1.0×10^{-4} M).

	Incubated at 4 °C			Incubated at 37 °C		
	F_4 ($\times10^{10}$ cps)	F_4 / F'_4	s.d.	F_{37} ($\times10^{10}$ cps)	F_{37} / F'_{37}	s.d.
0 h	3.44	1.00	0.038	3.03	1.00	0.029
1 h	3.50	1.06	0.038	4.52	1.49	0.097
2 h	3.64	1.09	0.076	4.94	1.63	0.024
3 h	3.73	1.11	0.10	5.11	1.68	0.069
4 h	3.63	1.13	0.082	5.16	1.70	0.039

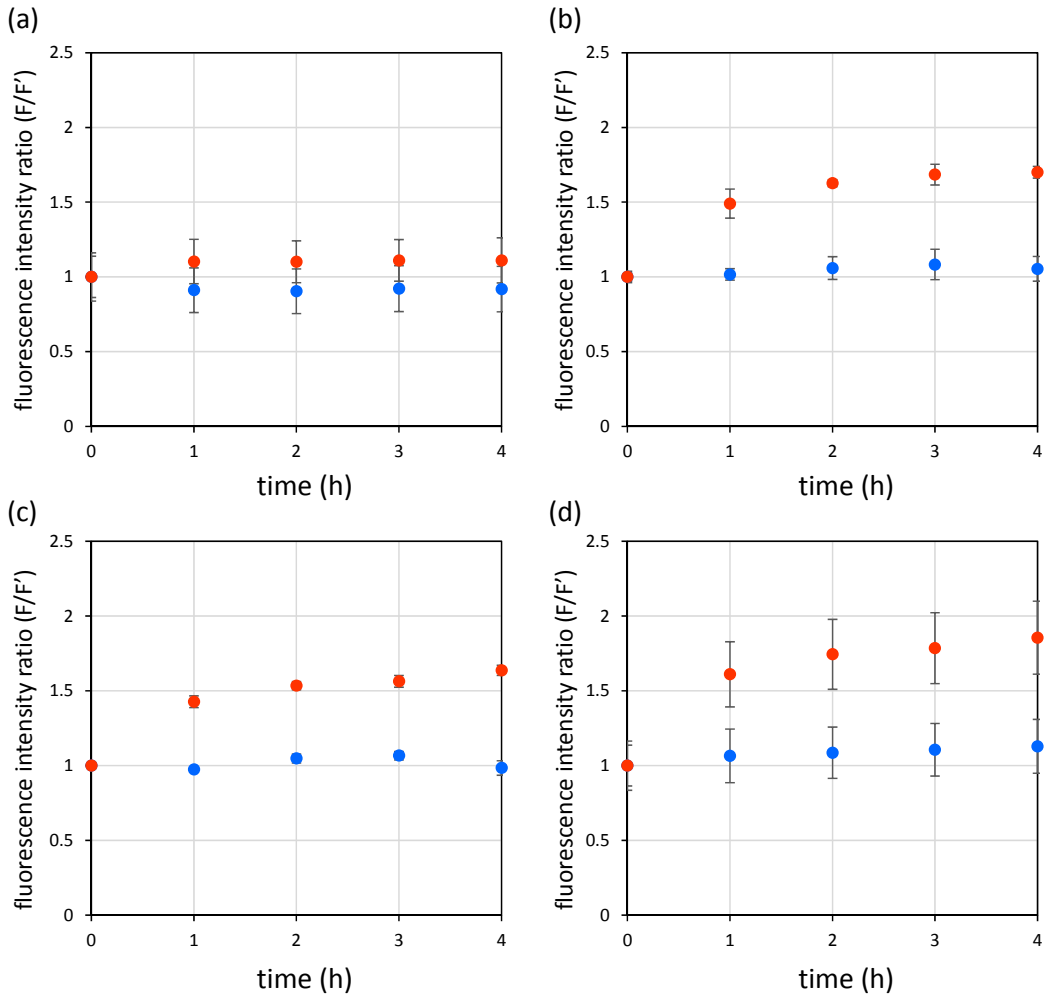


Figure S9. Fluorescence intensity ratios (F/F') of HeLa cells incubated in DMEM solutions (2.0 mL) with (a) ICG (1.0×10^{-5} M), (b) **1e** (1.0×10^{-4} M), (c) **1e** (1.0×10^{-5} M), or (d) **1d** (1.0×10^{-5} M) at 4 °C (blue) and 37 °C (orange). F': fluorescence intensities before injection (0 h).

Table S7. Fluorescence intensities from cells after 4 h incubation.

	concentration	Incubated at 4 °C		Incubated at 37 °C		F_{37}/F_4
	(M)	F (cps)	s.d. (cps)	F (cps)	s.d. (cps)	
ICG	1.0×10^{-5}	4.78E+09	6.29E+08	1.09E+10	1.68E+09	2.3
1e	1.0×10^{-4}	5.10E+08	1.47E+08	7.62E+09	5.77E+08	14.9
1d	1.0×10^{-5}	1.13E+08	5.51E+07	3.16E+09	2.31E+08	4.3
1e	1.0×10^{-5}	4.54E+09	8.25E+08	1.96E+10	2.62E+09	27.9

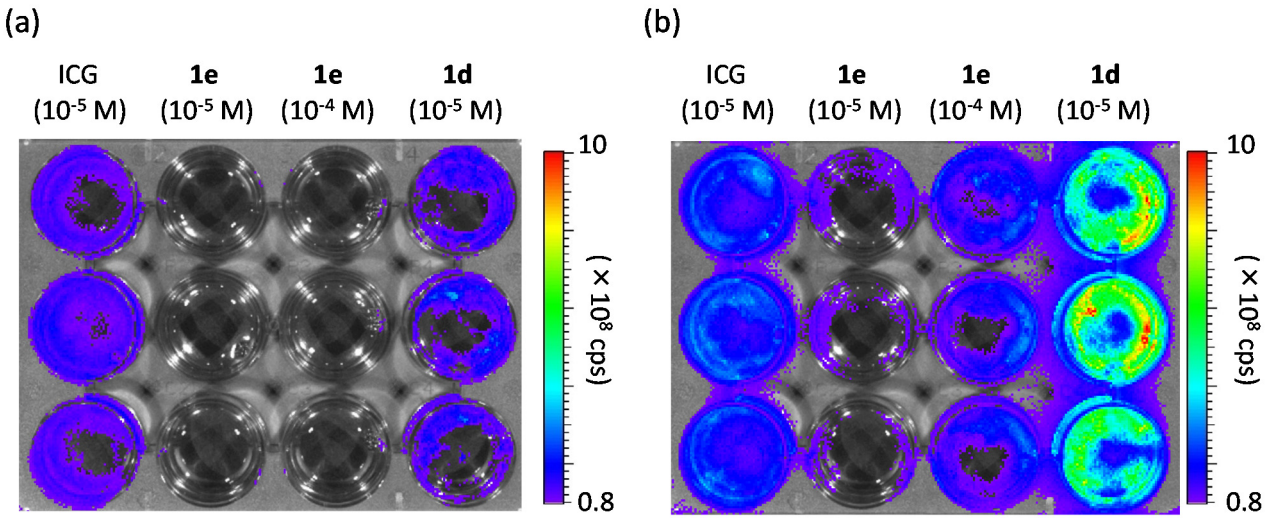


Figure S10. Fluorescence images from HeLa cells incubated for 4 h in DMEM solutions (2.0 mL) with ICG (1.0×10^{-5} M), **1e** (1.0×10^{-4} M), **1e** (1.0×10^{-5} M), or **1d** (1.0×10^{-5} M) at (a) 4 °C and (b) 37 °C.

To eliminate the influence of background fluorescence emitted from dyes in medium, fluorescence intensities (F'') of dyes in HeLa cells were calculated by using the following equation,

$$F'' = F_1 - F_2$$

where F_1 = fluorescence intensity of dye-containing incubation medium with cells (Tables S2-S5) and F_2 = fluorescence intensity of dye-containing incubation medium without cells (control experiments). Time-dependent changes of F'' were summarized in Figure S11. In the case of ICG, similar mean values of fluorescence intensities with quite large standard deviations were detected in all experiments because of strong fluorescence. In the cases of **1d** and **1e**, the fluorescence intensities of cells incubated at 37 °C were clearly stronger than those of cells incubated at 4 °C. Based on these results, by considering the influence of baseline

emission of dyes without cells, we concluded that there are significant differences between fluorescence of cells incubated at 37 °C and 4 °C.

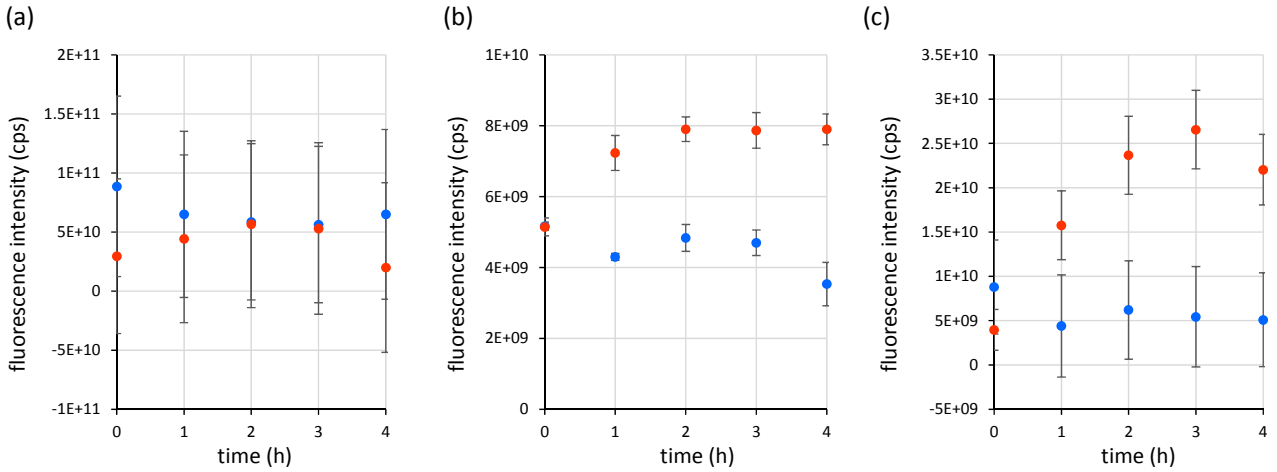


Figure S11. Fluorescence intensity (F'') of dyes in HeLa cells incubated in DMEM solutions (2.0 mL) with (a) ICG (1.0×10^{-5} M), (b) **1e** (1.0×10^{-5} M), or (c) **1d** (1.0×10^{-5} M) at 4 °C (blue) and 37 °C (red).

1.6. Theoretical calculation

The density function theory (DFT) calculations were performed for the geometry optimization of **1e-C** at the B3LYP/6-31G(d) lelvel[9,10] by using Gaussian 09 package.[11] The grand and excited states of **1e-C** were calculated by the time-dependent (TD) DFT calculations at the B3LYP/6-31G(d) level[7,8] by using Gaussian 09 package. From excitation energies and oscillator strengths, the UV-vis spectrum shown in Figure S12 was estimated. This spectrum is quite similar to UV-vis absorption spectrum of **1e-C**.

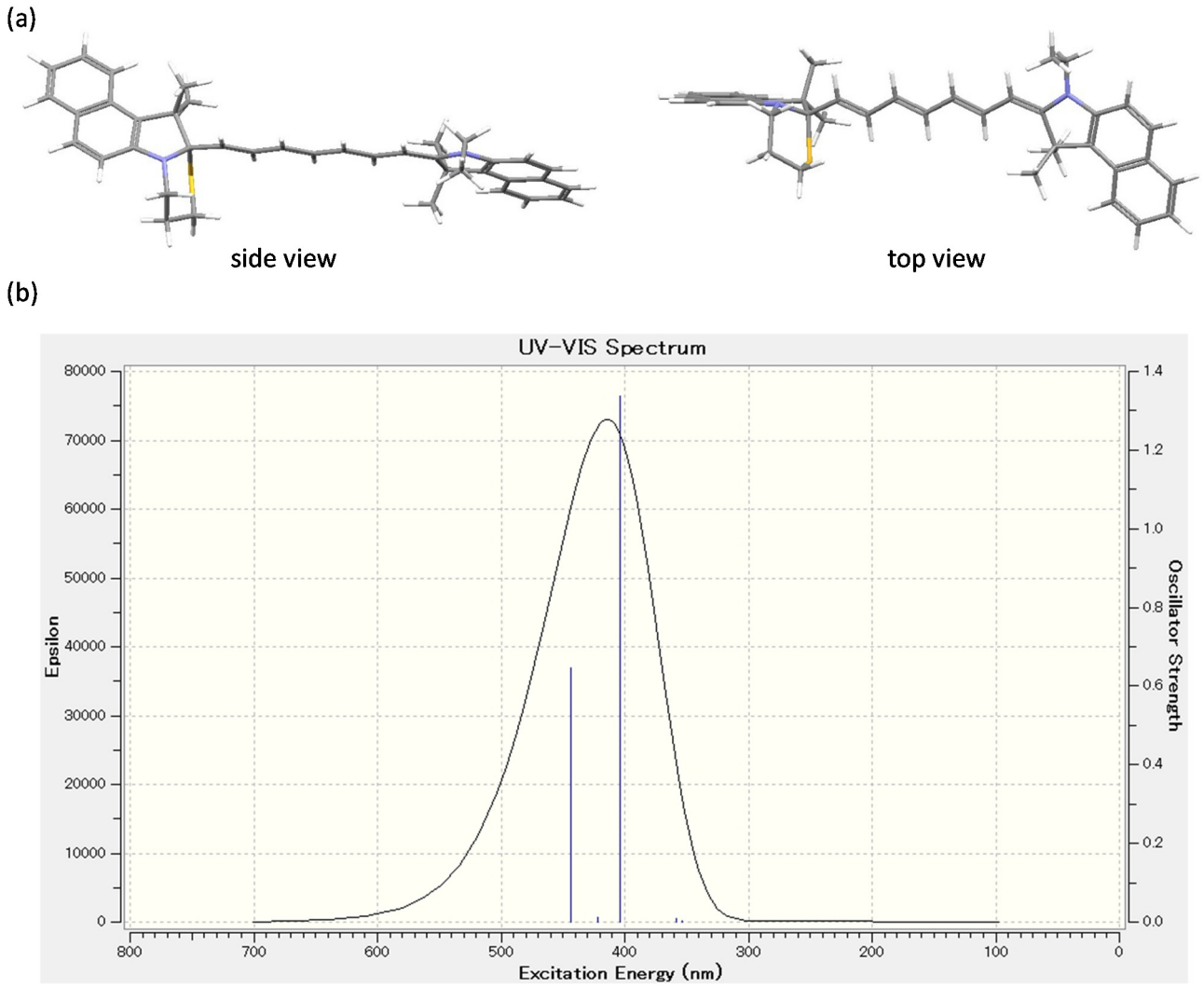


Figure S12. (a) Optimized structure of **1e-C** visualized by Mercury (The Cambridge Crystallographic Data Centre). (b) UV-vis absorption spectrum calculated by Gaussian09.

<Cartesian coordinates for optimized geometry of 1e-C>

C,-4.3099315876,-2.1160135377,-3.0943517366
C,-4.9822423313,-3.1989967881,-3.771256339
C,-4.2944779207,-4.4202093654,-3.9906296644
C,-2.9956971814,-4.6036510059,-3.5730918633
C,-2.3410643223,-3.5374789311,-2.9162626677
C,-2.9628905032,-2.3142546791,-2.6963434399
C,-5.0528690138,-0.9189141797,-2.8714129269
C,-6.3572007555,-0.7946666501,-3.2937807011
C,-7.0063197148,-1.8560915523,-3.9688991906
C,-6.3261455494,-3.0294620768,-4.1979644256
N,-1.0489597191,-3.5552366311,-2.376100791
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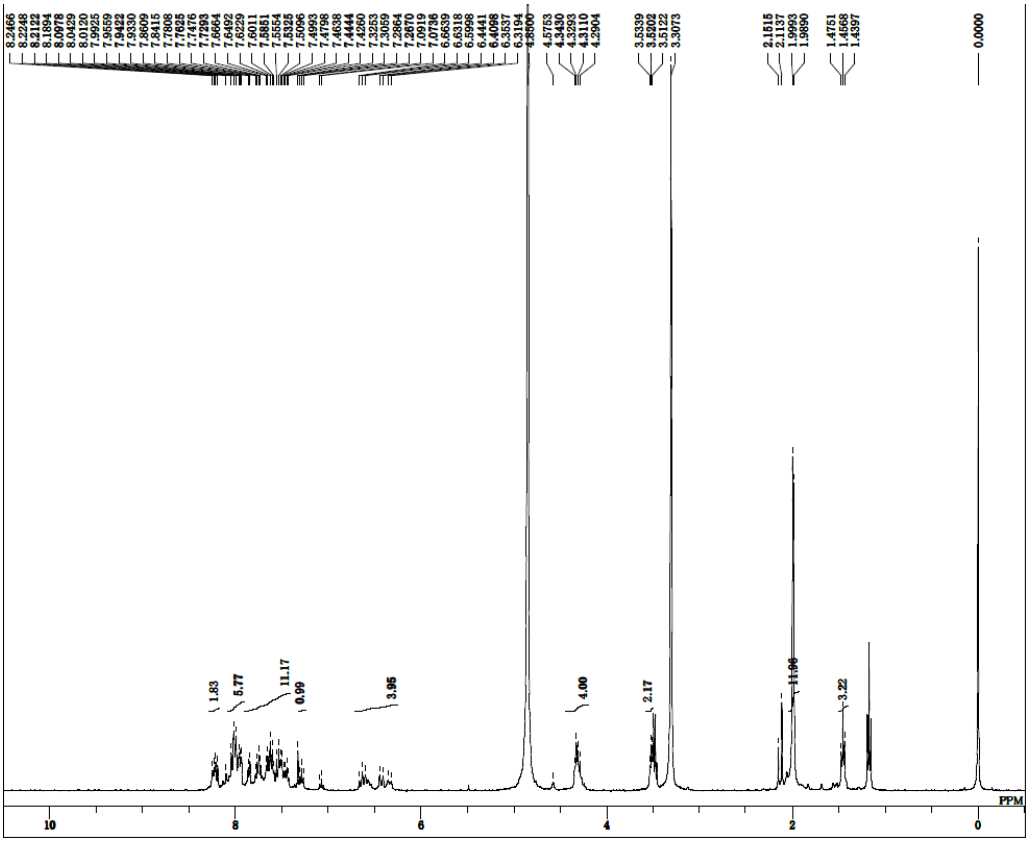
3. NMR spectra

^{1}H NMR of 3a (MeOH-d_4)



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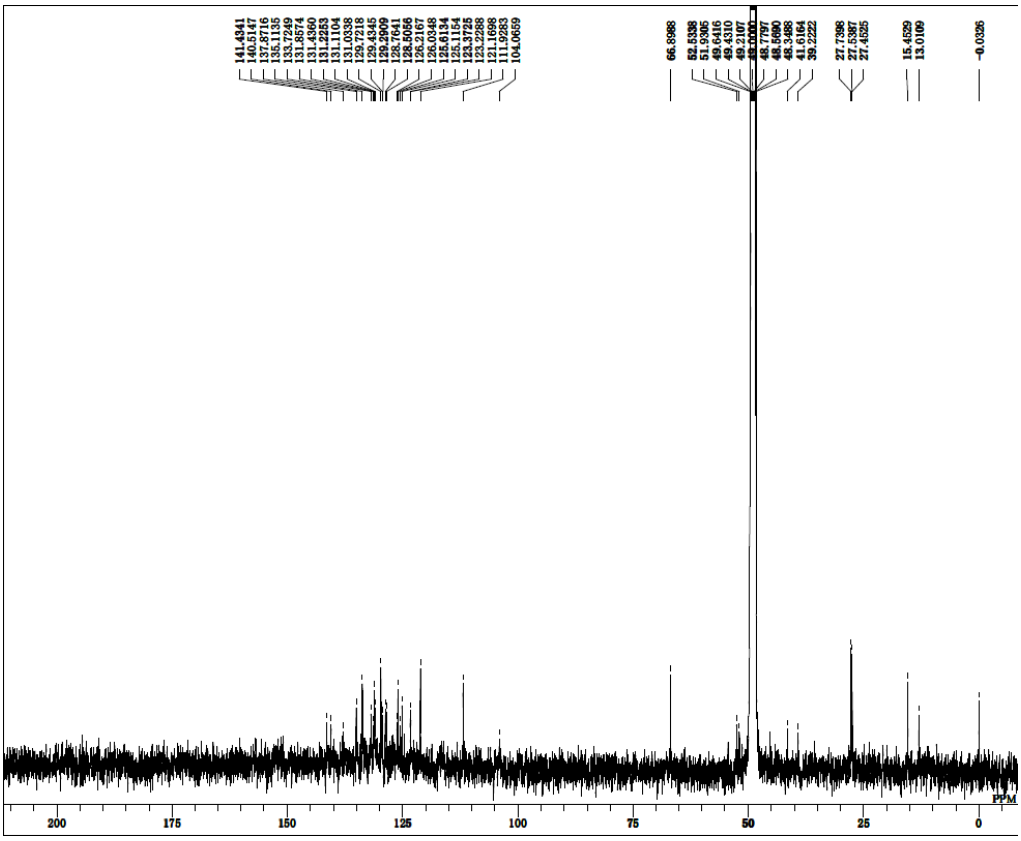

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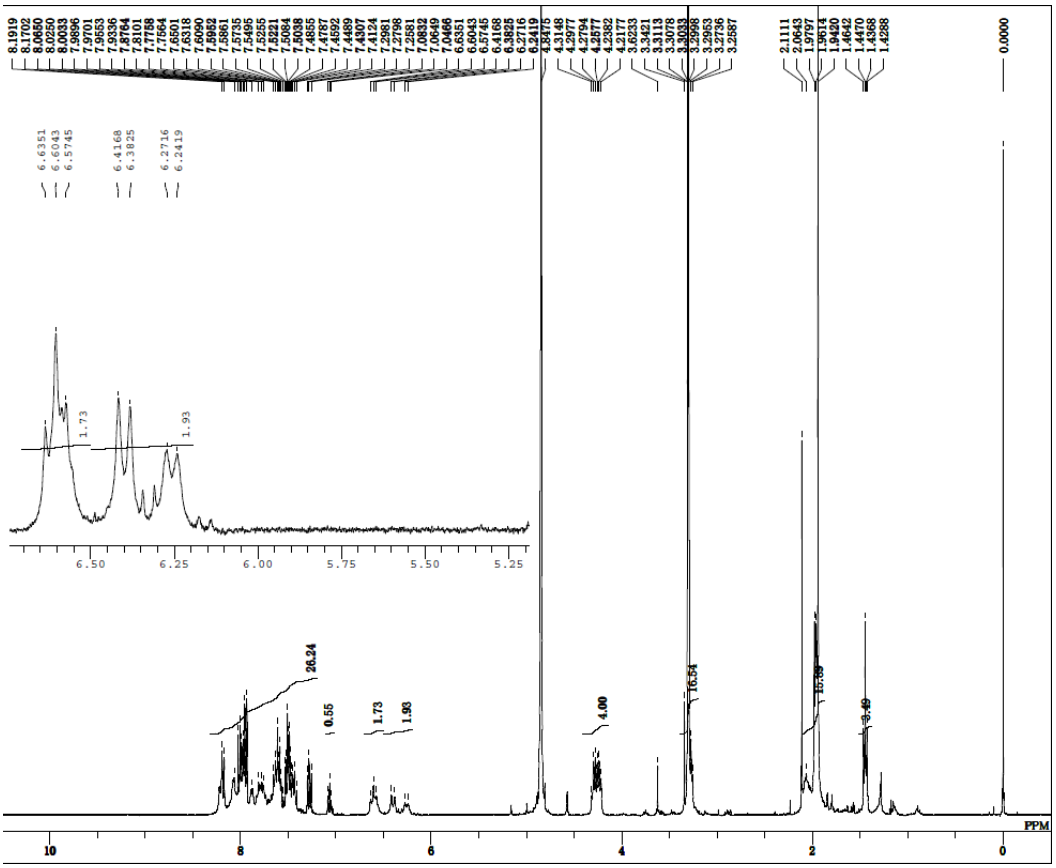

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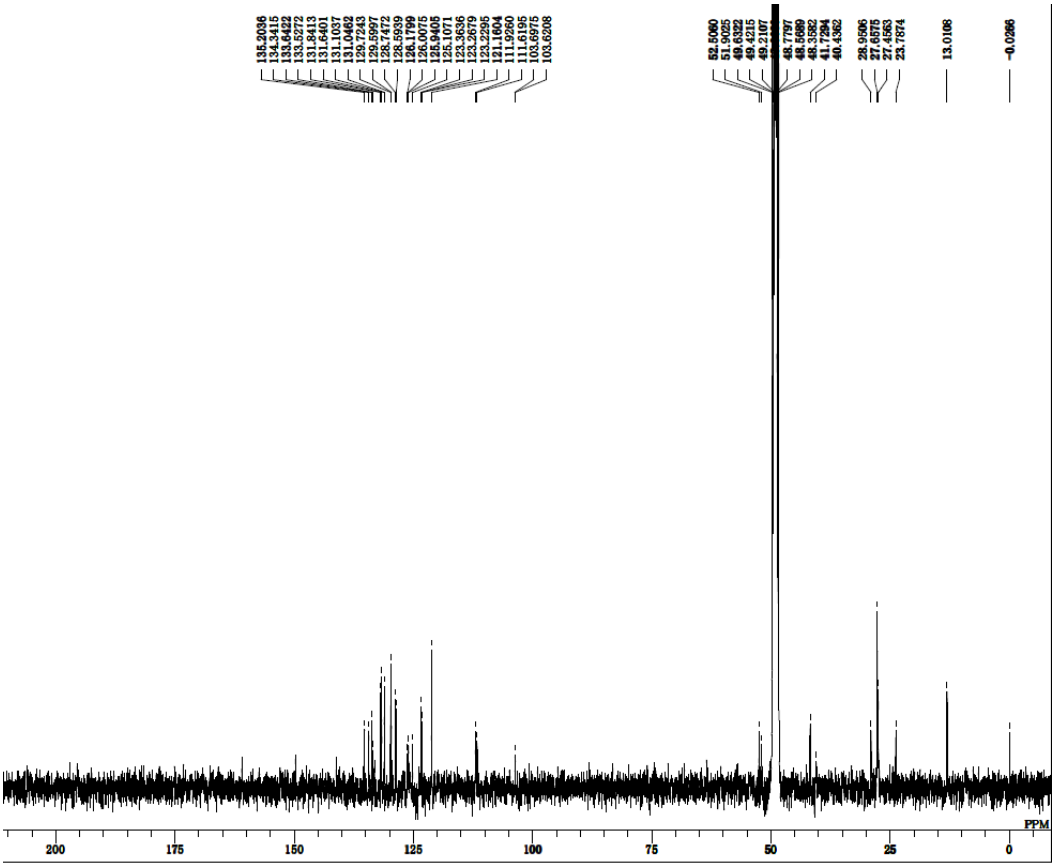

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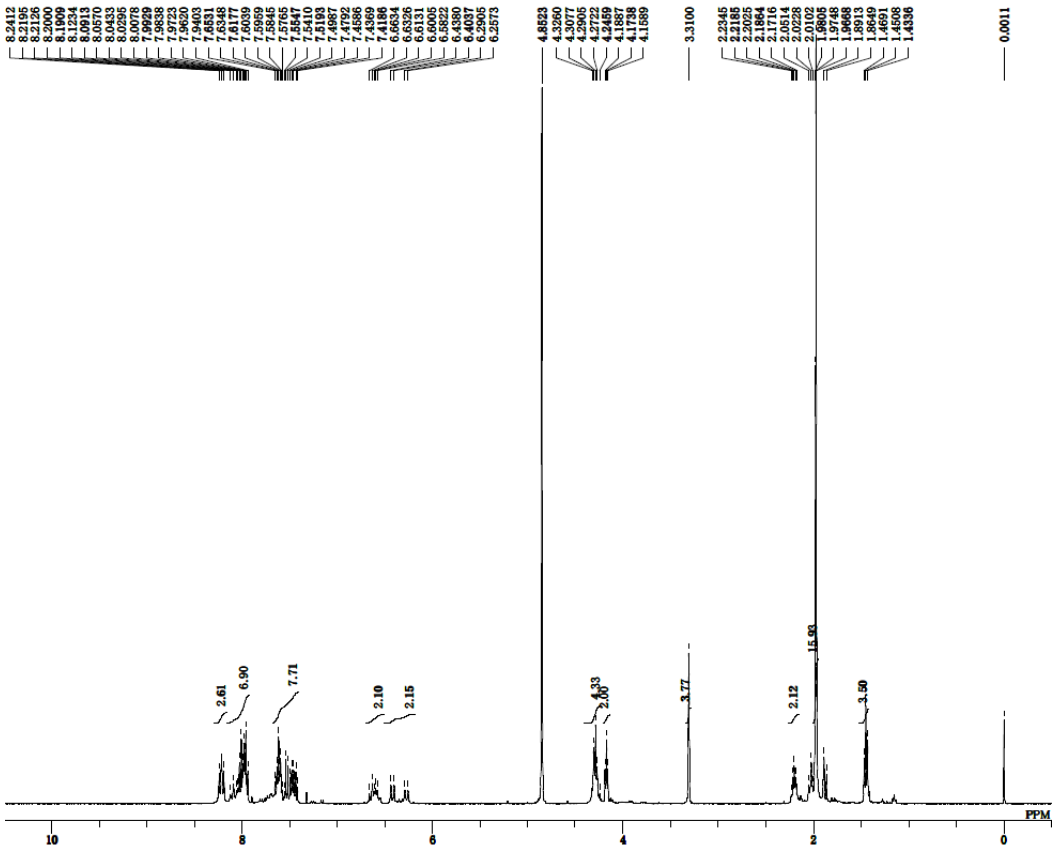

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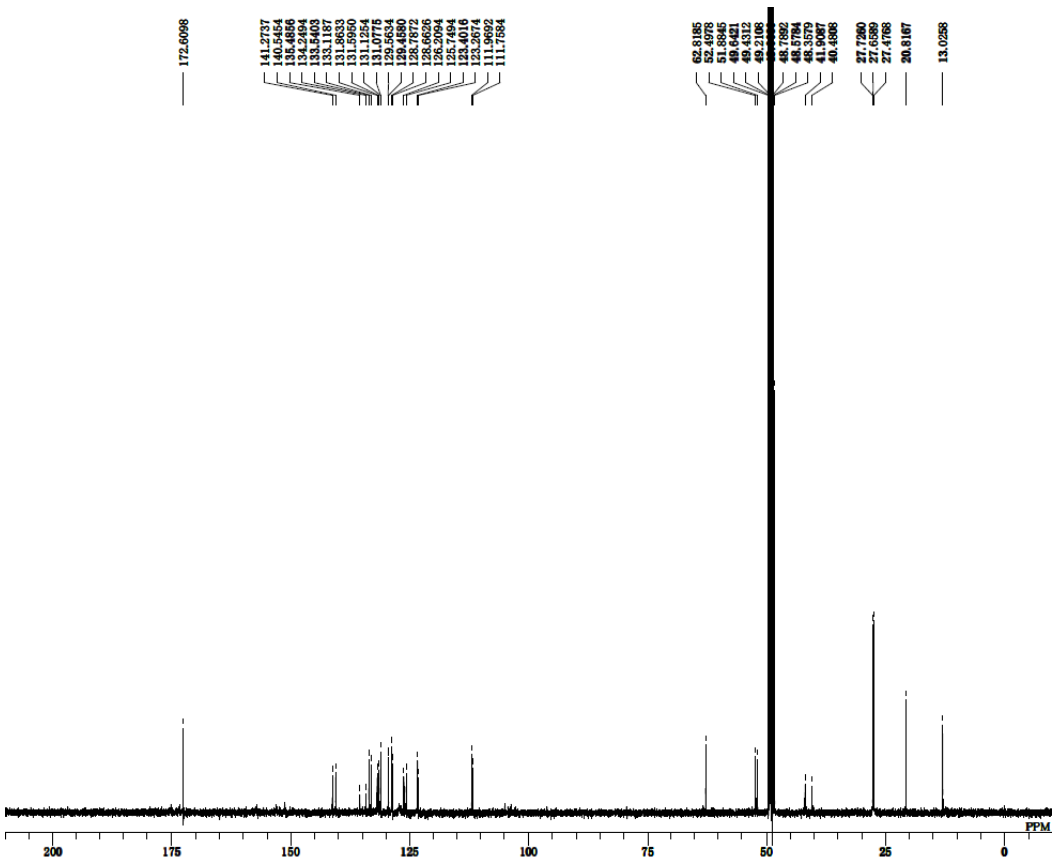

Electronic Supplementary Information

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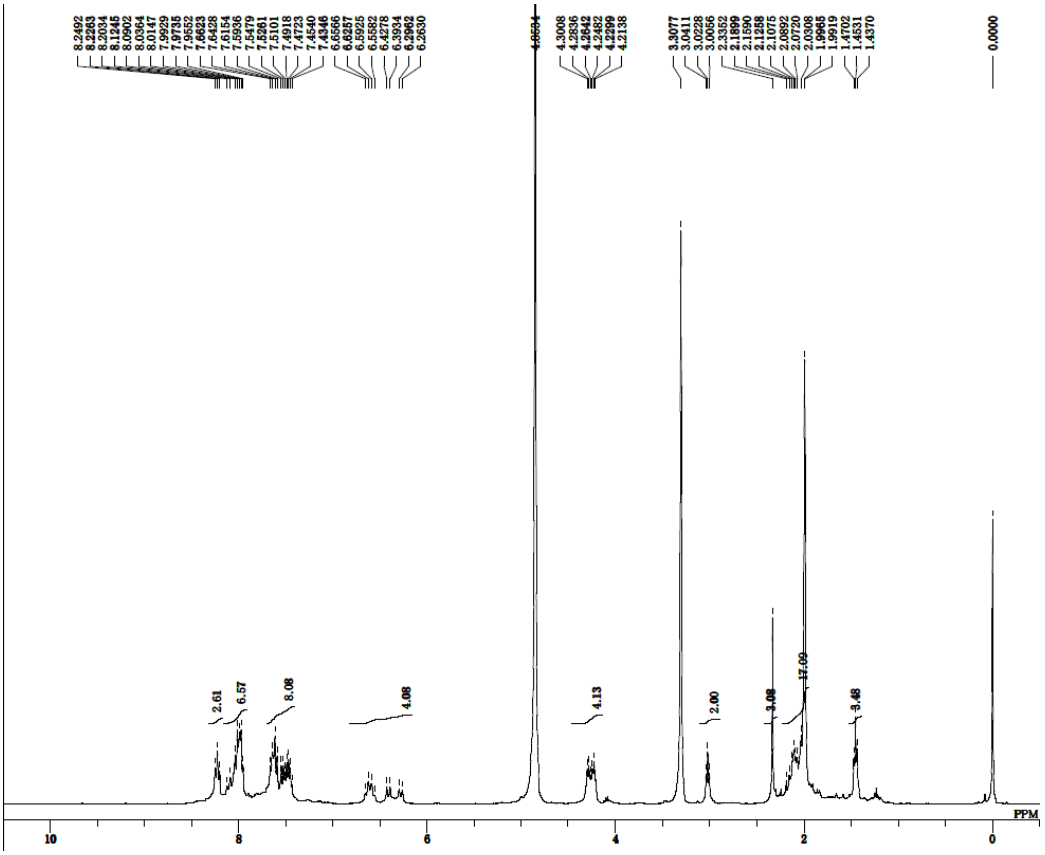

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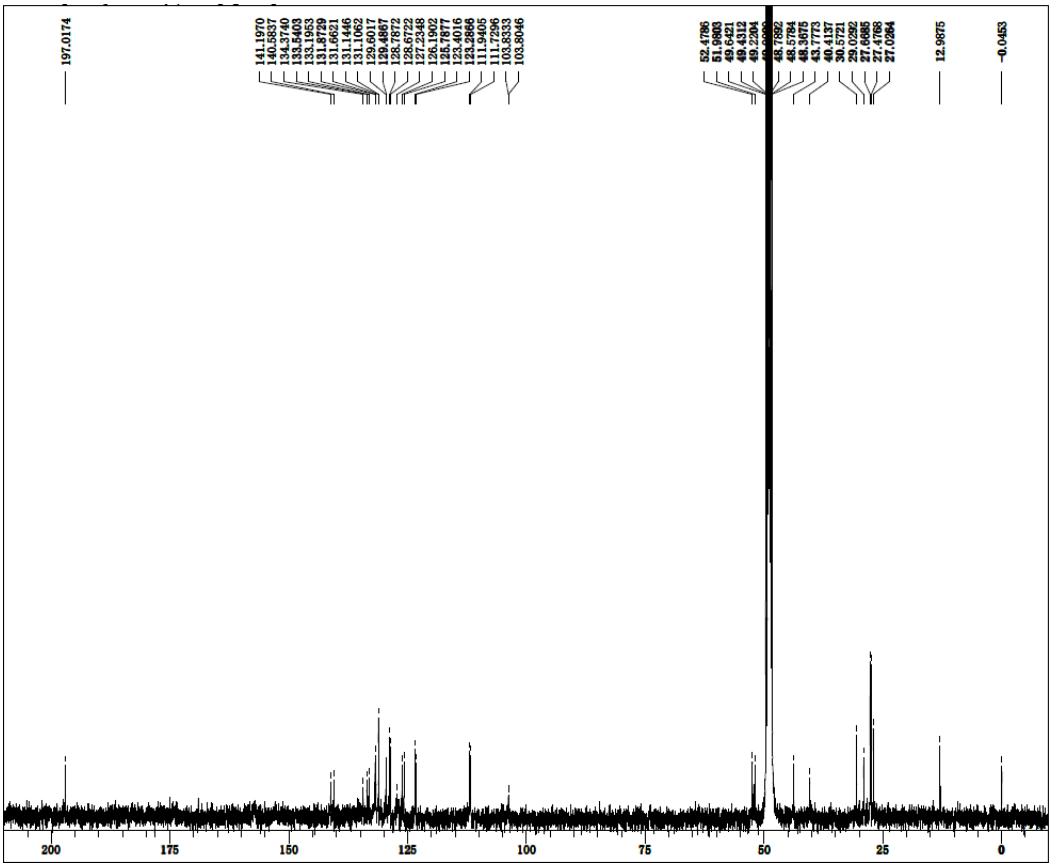

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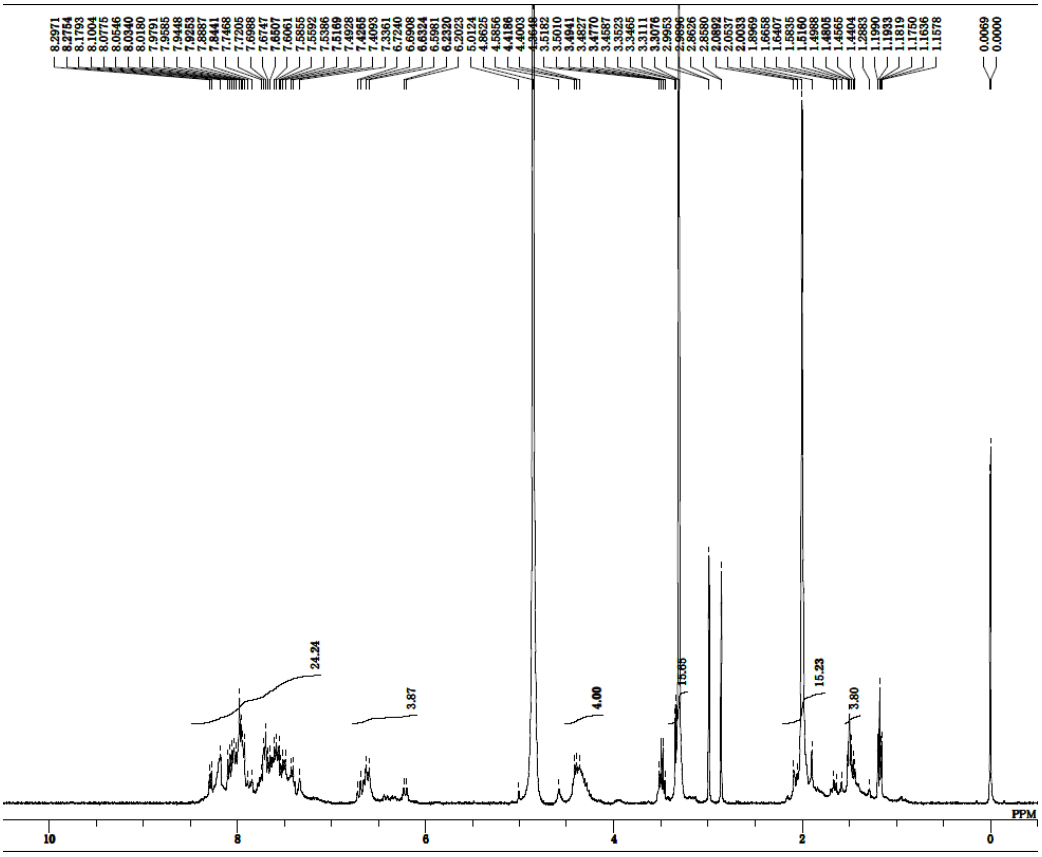

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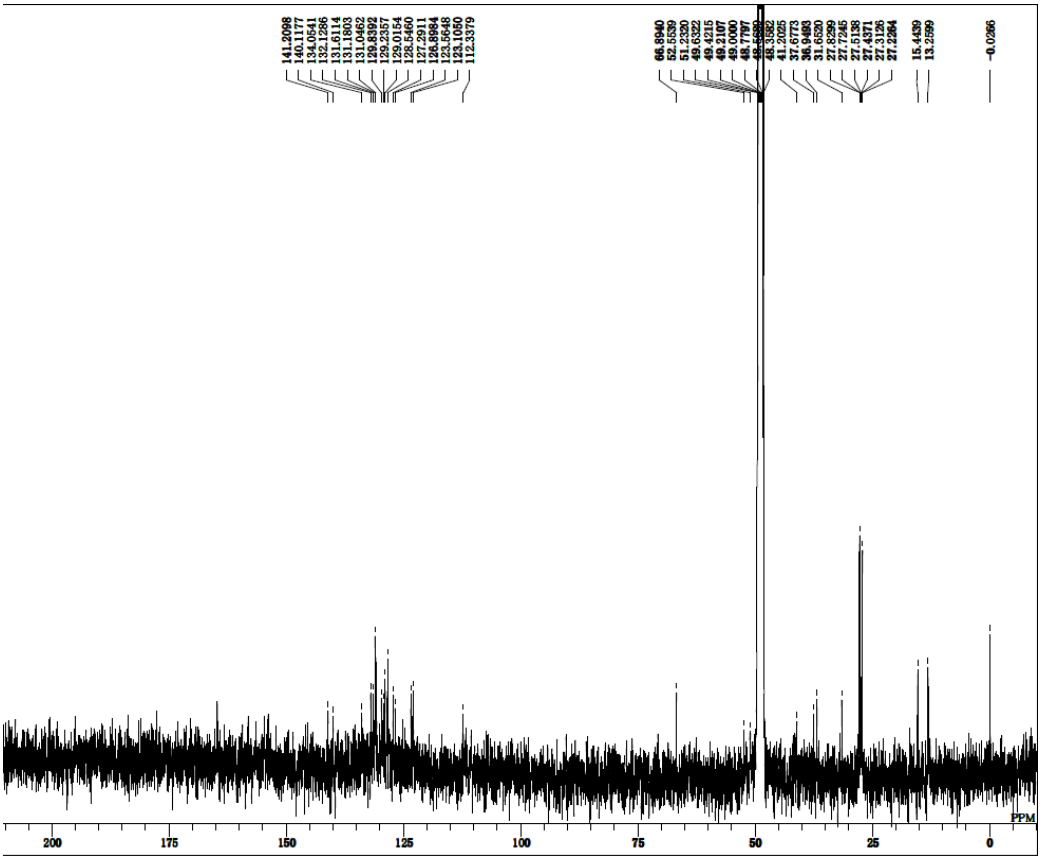

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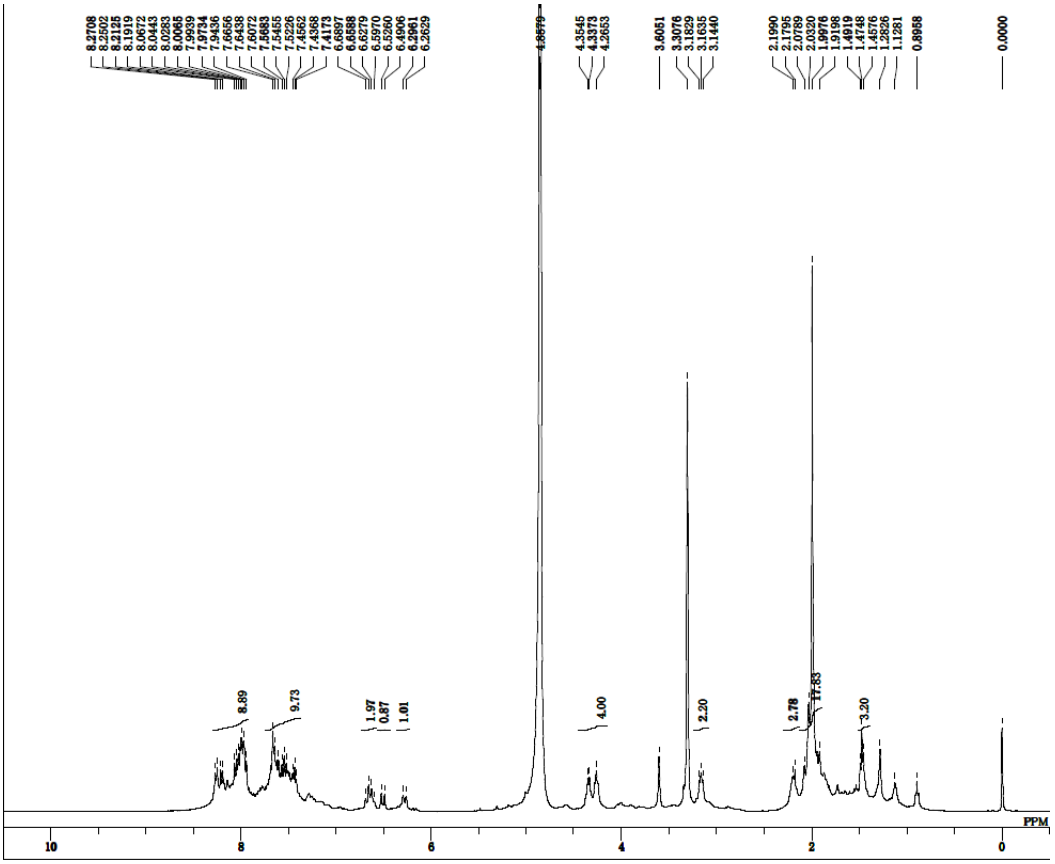

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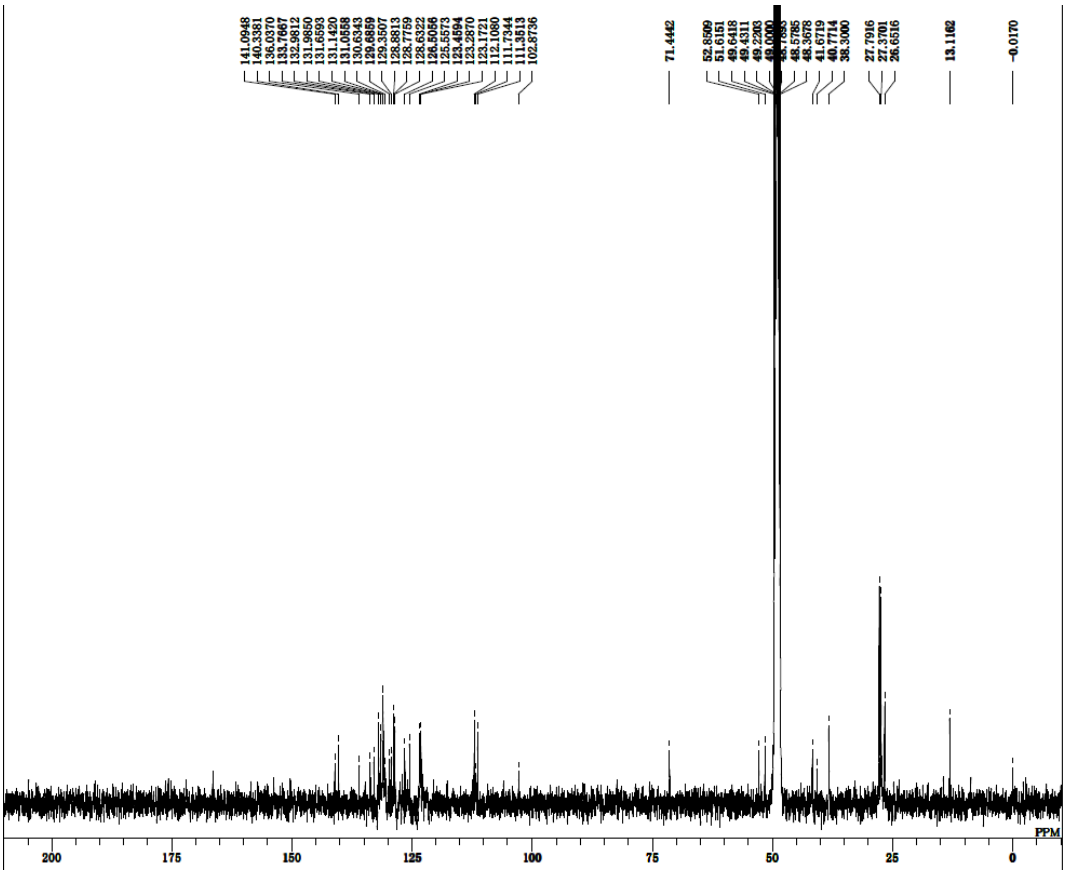

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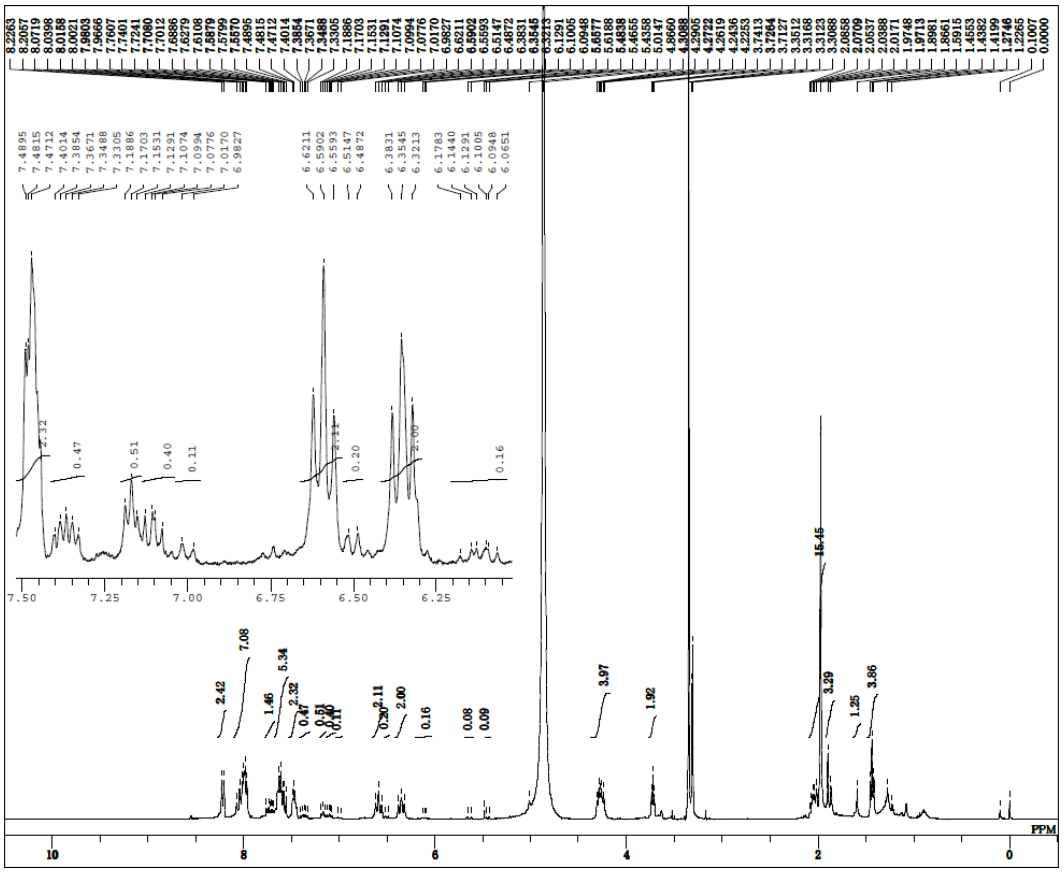

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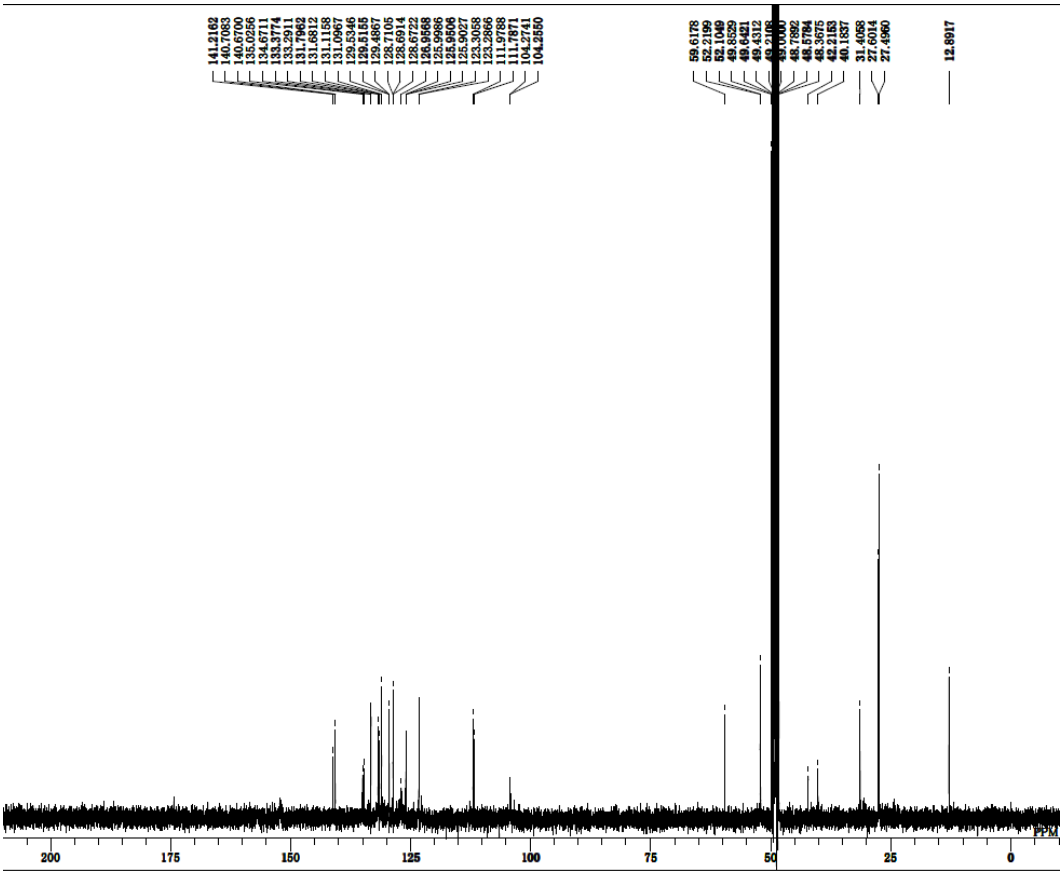

Electronic Supplementary Information

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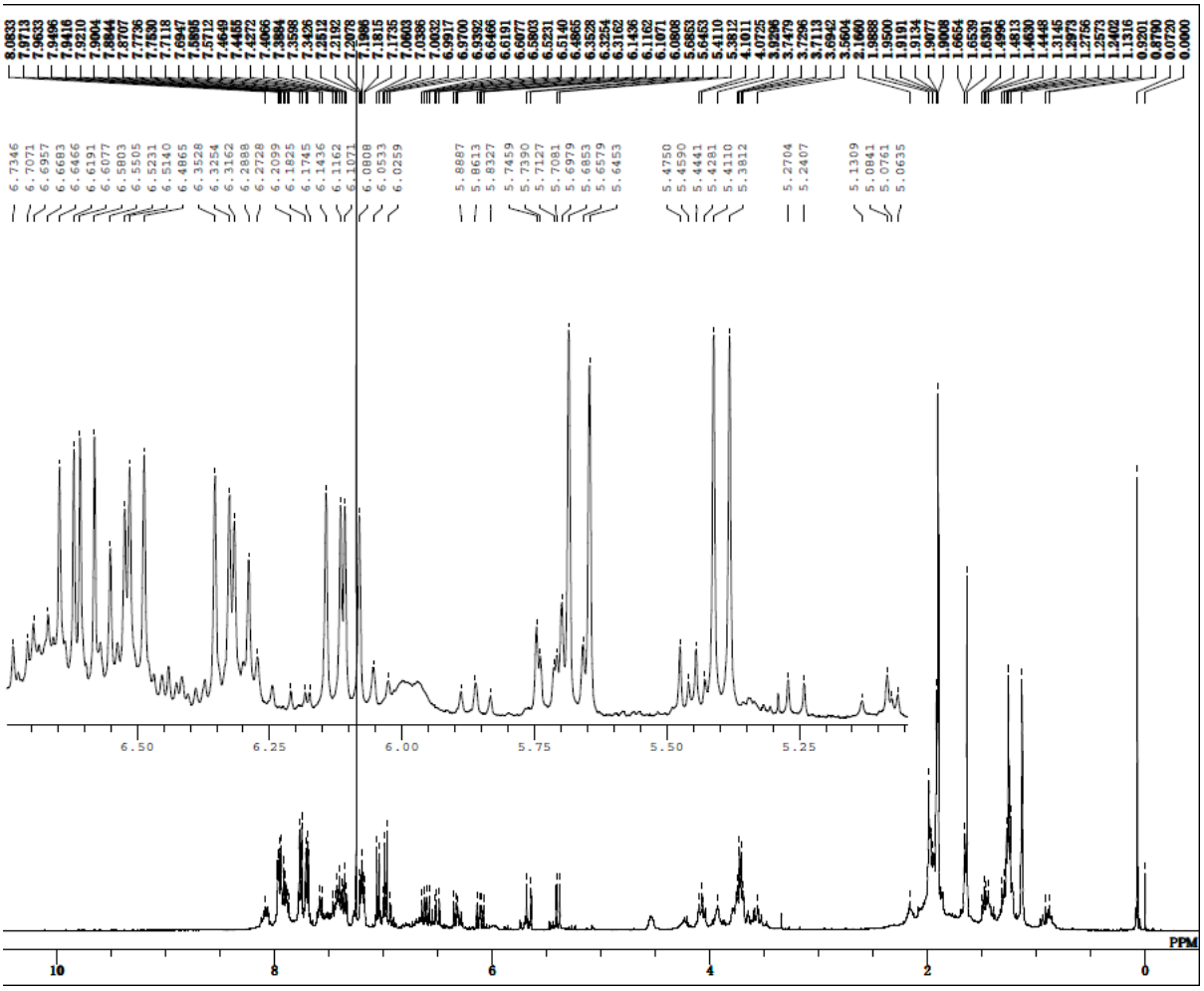

Electronic Supplementary Information

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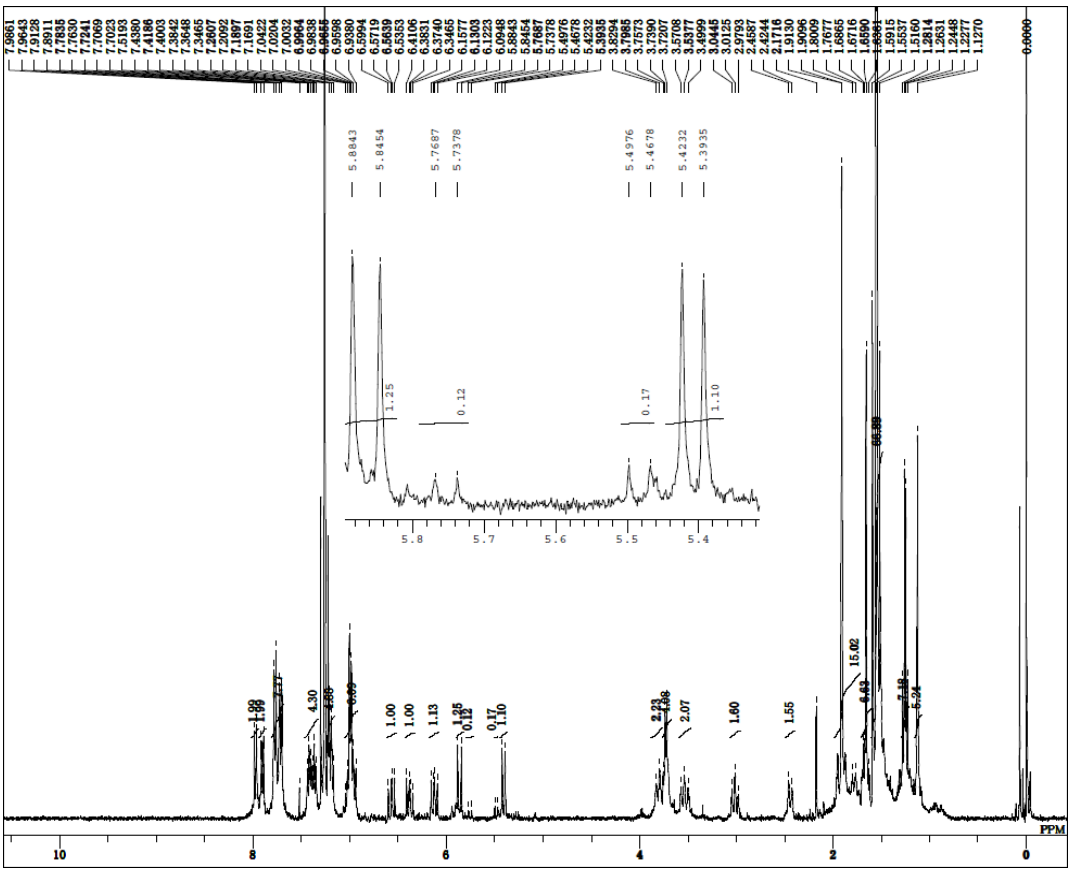

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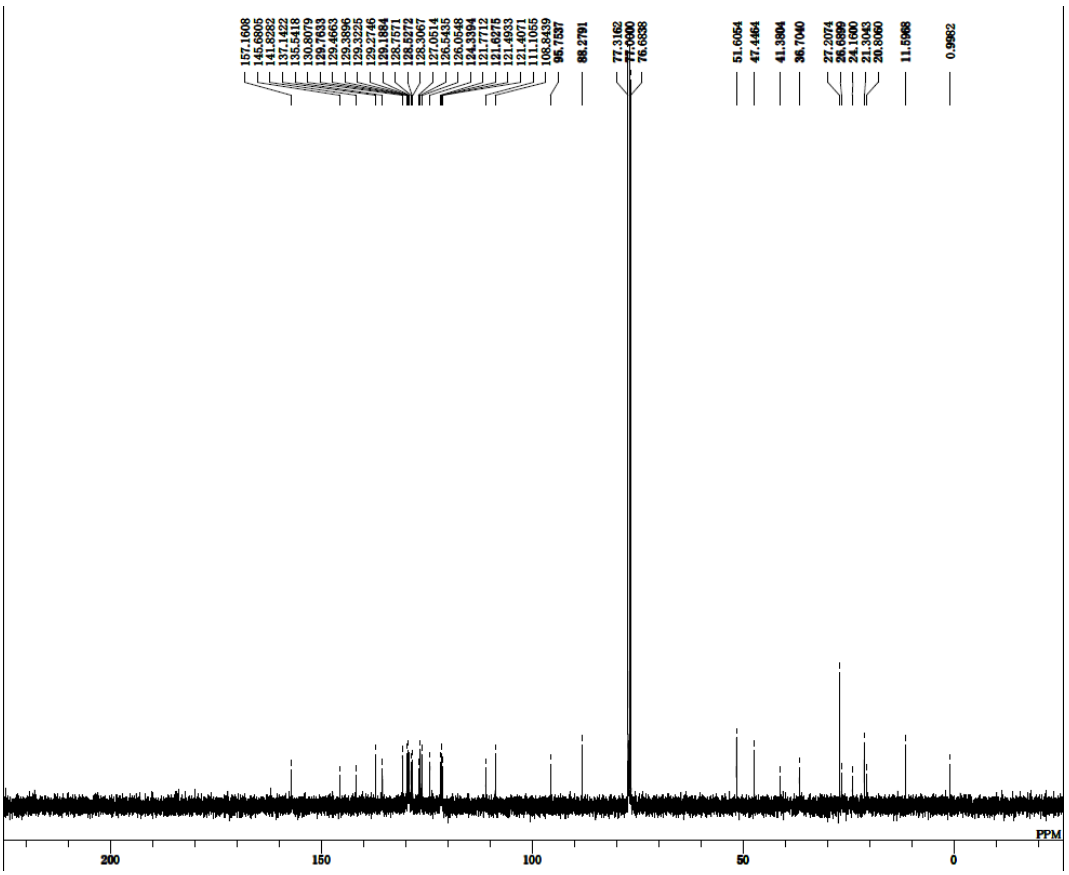

Electronic Supplementary Information

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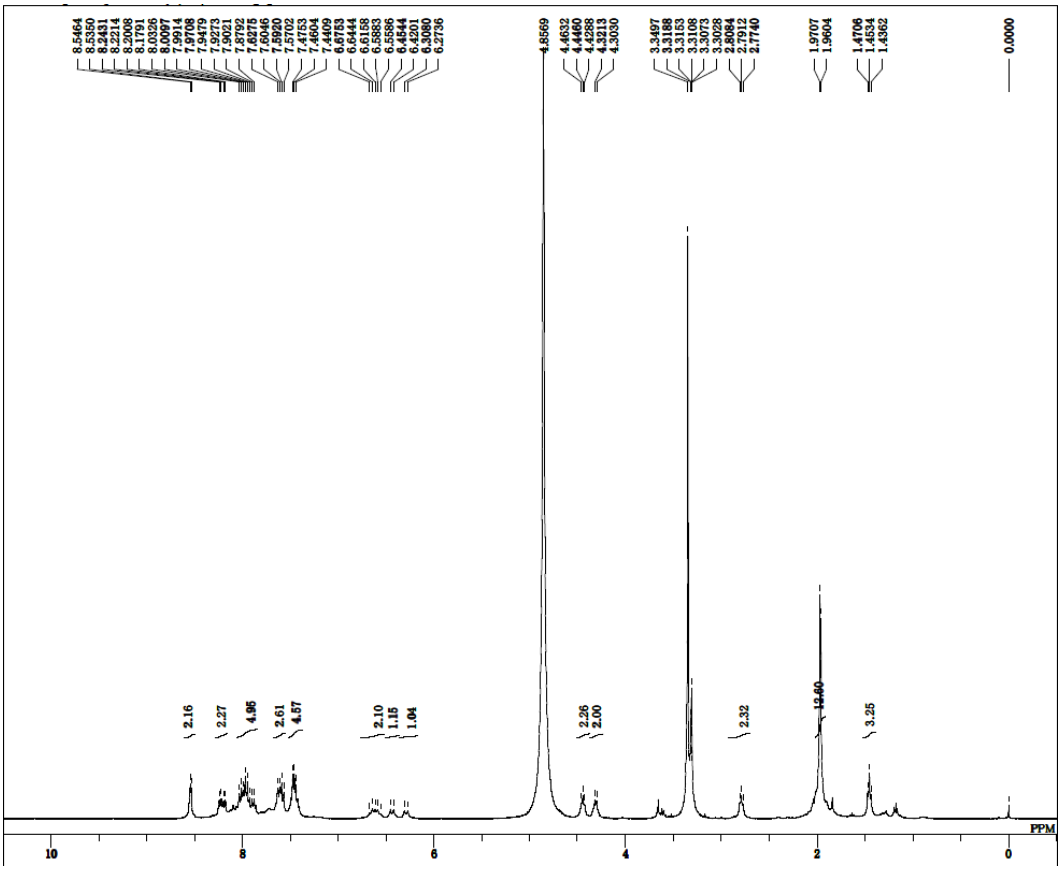

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