Electronic Supplementary Information

Enzyme-triggered Turn-on Fluorescent Probe Based on Carboxylate-Inducing Detachment of Fluorescence Quencher

Masahiro Oe, Koji Miki* and Kouichi Ohe*

Department of Energy and Hydrocarbon Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

Index

1. Experimental Section

1.1. Materials and Methods

Potassium carbonate (K_2CO_3), *N,N*-dimethylformamide (DMF), acetone, methanol (MeOH), hexane, ethyl acetate (AcOEt) and sodium hydroxide (NaOH) were purchased from nacalai tesque (Japan). Pyridine, dimethylsulfoxide (DMSO), chloroform ($CHCl_3$), acetonitrile (CH_3CN) and acetic acid (AcOH) were purchased from FUJIFILM Wako Pure Chemicals Inc. (Japan). Dichloromethane (CH_2Cl_2) and diethyl ether (Et_2O) was purchased from Kishida Chemical Co., Ltd. (Japan). Silica gel (SiO_2, 230–400 mesh) for column chromatography was purchased from Silicycle (Canada). CH_3CN, pyridine and DMF was distilled over CaH_2 under N_2 before use. MeOH was distilled over Mg under N_2 before use. All other materials were purchased and used without further purification.

Buffered aqueous solutions (pH 2.0-2.2) were prepared by dissolving hydrochloric acid (HCl) and potassium chloride (KCl) in water (MilliQ). Buffered aqueous solutions (pH 3.1-6.9) were prepared by dissolving citric acid and sodium dihydrogenphosphate (NaH_2PO_4) in water (MilliQ). Buffered aqueous solutions (pH 7.5-8.7) were prepared by dissolving NaH_2PO_4 and disodium hydrogenphosphate (Na_2HPO_4) in water (MilliQ). Buffered aqueous solutions (pH 9.0~11.0) 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.

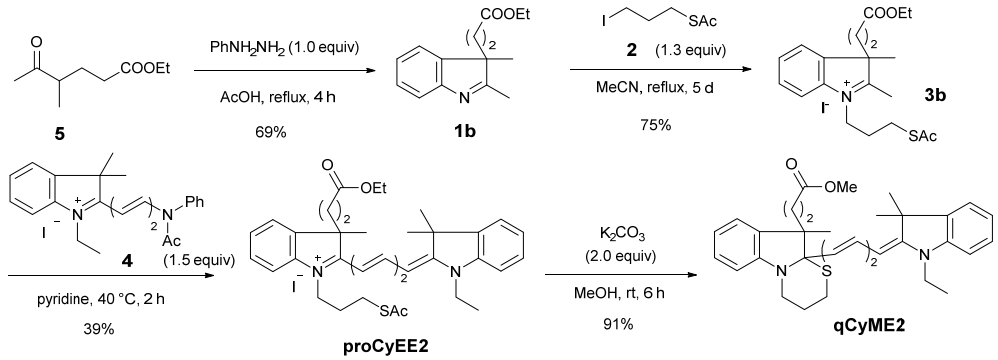
High-resolution mass spectra were measured by Exactive Plus Orbitrap (ESI, Thermo Fisher Scientific Inc., USA). NMR spectra were recorded on JEOL JNM-ECZ 400 (400 MHz) or JEOL JNM-ECX 400 spectrometer (400 MHz).

1.2. Synthesis

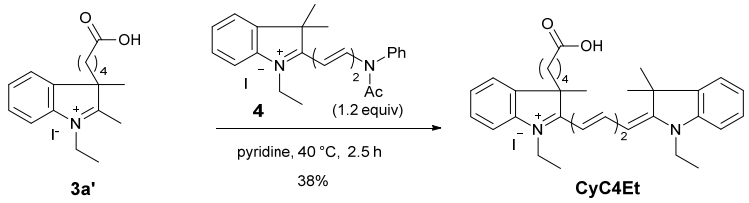
Synthesis of qCyME2 and CyC4Et

Fischer indole synthesis form ethyl 4-methyl-5-oxohexanoate (**5**)[1] in AcOH afforded **1b**. **proCyEE2** having an acethylthio group was obtained in a similar way for **proCyC4**. After the deprotection of an acetyl group of **proCyEE2** under basic conditions, **qCyME2** was obtained. **CyC4Et** was obtained in similar way for **proCyC4**.

Scheme S1 Synthesis of **qCyME2**



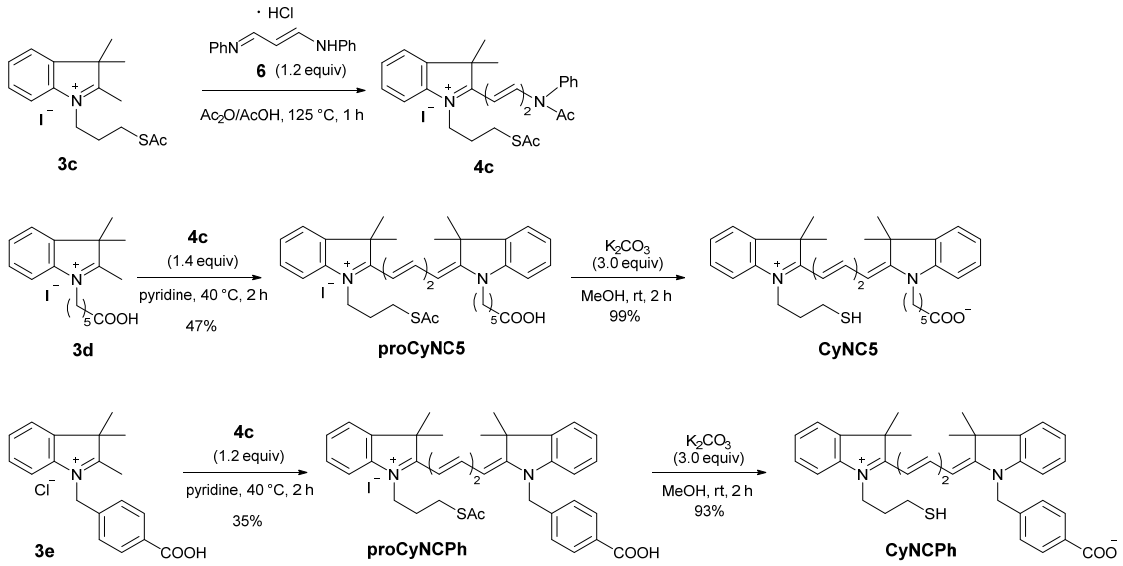
Scheme S2 Synthesis of **CyC4Et**



Synthesis of CyNC5 and CyNCPh

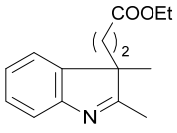
Cy5 derivative **proCyNC5** was prepared from benzoindole **3c**[2], **3d**[3] and malonaldehyde dianilide hydrochloride (**6**)[4]. **CyNC5** was synthesized by removing the acetyl group of **proCyNC5**. **CyNCPh** was obtained in a similar manner to **CyNC5** using benzoindole **3e**[5] instead of **3d**.

Scheme S3 Synthesis of **CyNCPh** and **CyNC5**



Synthesis of 1b

To a solution of **5** (0.51 g, 3.0 mmol) in AcOH (4 mL) was added phenylhydrazine (0.49 g, 4.5 mmol) at room temperature. After refluxing for 3.5 h, the reaction mixture was diluted with CH_2Cl_2. The organic solution was washed with H_2O (20 mL) and saturated $NaHCO_3$ aqueous solution (20 mL). The organic solvent was removed under reduced pressure and the residue was subjected to column chromatography on SiO_2 (eluent: hexane-AcOEt, v:v = 3:1). The organic solvent was removed under reduced pressure to afford benzoindole **1b** (0.51 g, 2.1 mmol, 69%) as a reddish brown oil.



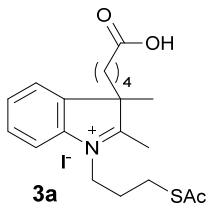
1b

1b: IR(ATR) 2970, 2932, 2872, 1731, 1578, 1449, 1372, 1303, 1242, 1165, 1096, 1016, 939, 863, 775, 754, 654, 577, 530 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 1.15 (t, J = 7.2 Hz, 3H), 1.32 (s, 3H), 1.54-1.62 (m, 1H), 1.66-1.74 (m, 1H), 2.01-2.16 (m, 1H), 2.23-2.30 (m, 4H), 3.95-4.03 (m, 2H), 7.18-7.26 (m, 2H), 7.31 (t, J = 7.6 Hz, 1H), 7.54 (d, J = 7.6 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 14.0, 15.5, 22.4, 29.0, 31.5, 57.0, 60.3, 120.0, 121.6, 125.2, 128.0, 142.4, 154.3, 172.7, 186.1. HRMS (ESI) calcd for $C_{15}H_{20}NO_2$ ([M^+]) 246.1494, found 246.1489.

Synthesis of 3a-b

To a solution of 5-(2,3-dimethyl-3*H*-indol-3-yl)pentanoic acid (**1a**)[6] (0.73 g, 3.0 mmol) in CH_3CN (3 mL) was added *S*-3-iodopropyl thioacetate (**2**)[7] (0.89 g, 3.6 mmol) at room temperature. After refluxing for 2 days, the organic solvent was removed under reduced pressure. The residue was dissolved in a small amount of CH_2Cl_2. After the solution was added to Et_2O, the oily product was gradually separated from etheral solution. The oily product was

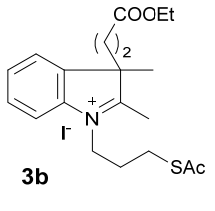
washed with Et_2O. The volatiles were removed under reduced pressure to afford benzoindole **3a** (1.1 g, 2.3 mol, 78%) as a brown oil.



3a

3a: IR(ATR) 2934, 2865, 1724, 1686, 1625, 1605, 1461, 1417, 1388, 1355, 1134, 1021, 954, 919, 764, 730, 698, 626, 566 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 0.65-0.78 (m, 1H), 0.81-0.93 (m, 1H), 1.42-1.56 (m, 2H), 1.67 (s, 3H), 2.13-2.29 (m, 6H), 2.33 (s, 3H), 3.07 (t, J = 7.2 Hz, 2H), 3.12 (s, 3H), 4.72-4.85 (m, 2H), 7.59-7.66 (m, 3H), 7.87-7.90 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 16.7, 22.3, 23.2, 24.1, 25.9, 28.3, 30.7, 33.2, 37.0, 48.3, 58.9, 115.4, 123.6, 129.5, 130.1, 139.7, 141.3, 176.3, 195.5, 196.3. HRMS (ESI) calcd for $C_{20}H_{28}NO_3S$ ([M^+]) 362.1784, found 362.1789.

To a flame-dried Schlenk flask, ethyl 3-(2,3-dimethyl-3*H*-indol-3-yl)propanoate (**1b**) (0.25 g, 1.0 mmol), *S*-3-iodopropyl thioacetate (**2**) (0.34 g, 0.14 mmol) and CH_3CN (1.5 mL) were successively added at room temperature under nitrogen atmosphere. After refluxing for 5 days, the organic solvent was removed under reduced pressure. The residue was dissolved in a small amount of CH_2Cl_2. After the solution was added to Et_2O, the oily product was gradually separated from etheral solution. The oily product was washed with Et_2O. The volatiles were removed under reduced pressure to afford benzoindole **3b** (0.37 g, 0.76 mmol, 75%) as a reddish brown oil.

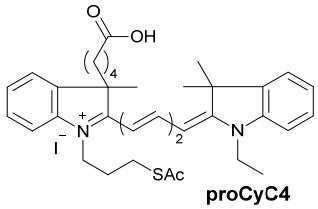


3b

3b: IR(ATR) 3431, 2977, 2934, 1726, 1684, 1623, 1587, 1463, 1374, 1354, 1305, 1252, 1183, 1134, 1020, 954, 858, 765, 731, 697, 626 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 1.11 (t, J = 7.2 Hz, 3H), 1.75 (s, 3H), 1.85-1.92 (m, 1H), 2.01-2.07 (m, 1H), 2.28 (t, J = 7.2 Hz, 2H), 2.36 (s, 3H), 2.51-2.68 (m, 2H), 3.11-3.32 (m, 5H), 3.85-3.98 (m, 2H), 4.68-4.76 (m, 1H), 4.81-4.89 (m, 1H), 7.63-7.71 (m, 3H), 7.93-7.98 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 13.6, 17.0, 23.4, 25.7, 27.7, 29.0, 30.4, 31.2, 48.1, 57.8, 60.5, 115.3, 123.8, 129.6, 129.8, 138.0, 141.2, 171.7, 195.0, 195.6. HRMS (ESI) calcd for $C_{20}H_{28}NO_3S$ ([M^+]) 362.1784, found 362.1796.

Synthesis of proCyC4, proCyEE2 and CyC4Et

In a flame-dried Schlenk flask, benzoindole **3a** (1.3 g, 2.7 mmol), **4**[8] (1.4 g, 3.5 mmol) and pyridine (11 mL) were successively added at room temperature under nitrogen atmosphere. After stirring at 40 °C for 6 h, the organic solvent was removed under reduced pressure and the residue was subjected to column chromatography on SiO_2 (eluent: CH_2Cl_2-MeOH, v:v = 30:1 to 15:1) to afford a crude product. The organic solvents were removed under reduced pressure and the residue was dissolved in a small amount of CH_2Cl_2. After adding ether slowly, the product was gradually precipitated. The precipitate was washed with ether. The volatiles were removed under reduced pressure to afford Cy5 derivative **proCyC4** (1.1 g, 1.5 mmol, 56%) as a red solid.

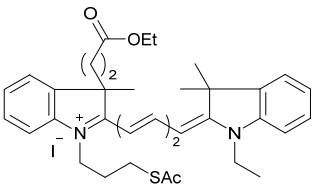


proCyC4

proCyC4: mp 112-114 °C; IR(ATR) 2938, 2877, 1689, 1479, 1446, 1375, 1329, 1145, 1100, 1039, 995, 921, 801, 771, 747, 707, 554 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 0.57-0.67 (m, 1H), 0.90-0.99 (m, 1H), 1.41-1.49 (m, 4H), 1.57-1.64 (m, 1H), 1.72 (s, 3H), 1.78 (s, 3H), 1.79 (s, 3H), 2.02-2.11 (m, 3H), 2.24-2.34 (m, 1H), 2.39 (s, 3H), 2.50-2.57 (m, 1H), 2.65-2.72 (m, 1H), 3.04 (t, J = 7.2 Hz, 2H), 4.06-4.15 (m, 4H), 6.23 (d, J = 13.6 Hz, 1H), 6.29 (d, J = 13.2 Hz, 1H), 6.75 (dd, J = 12.4, 12.8 Hz, 1H), 7.05 (d, J = 8.4 Hz, 1H), 7.10 (d, J = 7.6 Hz, 1H), 7.18-7.33 (m, 3H), 7.35-7.39 (m, 3H), 8.24 (dd, J = 13.2, 12.8 Hz, 1H), 8.34 (dd, J = 13.2, 12.8

Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 12.7, 23.6, 24.7, 26.5, 27.7, 28.0, 28.2, 30.9, 39.7, 39.9, 41.2, 43.3, 49.8, 53.8, 94.7, 104.0, 104.1, 110.1, 110.6, 122.4, 122.6, 125.1, 125.5, 126.9, 128.7, 128.8, 139.4, 141.7, 141.8, 142.9, 153.1, 154.5, 170.9, 173.3, 195.8. HRMS (ESI) calcd for $C_{36}H_{45}N_2O_3S$ ([M^+]) 585.3145, found 585.3157.

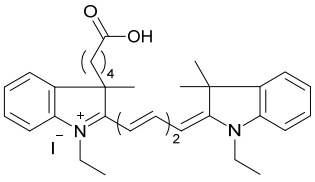
To a flame-dried Schlenk flask, benzoindole **3b** (0.18 g, 0.37 mmol), **4** (0.22 g, 0.57 mmol) and pyridine (7 mL) were successively added at room temperature under nitrogen atmosphere. After stirring at 40 °C for 2 h, the organic solvent was removed under reduced pressure and the residue was subjected to column chromatography on SiO_2 (eluent: CH_2Cl_2-MeOH, v:v = 60:1 to 30:1) to afford a crude product. The organic solvent was removed under reduced pressure and the residue was dissolved in a small amount of CH_2Cl_2. After adding a solution (hexane-ether, v:v = 1:1) slowly, the product was gradually precipitated. The precipitate was washed with hexane. The volatiles were removed under reduced pressure to afford Cy5 derivative **proCyEE2** (0.10 g, 0.14 mmol, 39%) as a red solid.



proCyEE2

proCyEE2: mp 112-113 °C; IR(ATR) 2976, 2926, 1730, 1688, 1478, 1444, 1375, 1329, 1177, 1145, 1099, 1061, 996, 922, 802, 771, 747, 706, 626 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 1.15 (t, J = 7.2 Hz, 3H), 1.45 (t, J = 7.2 Hz, 3H), 1.57-1.65 (m, 1H), 1.74 (s, 3H), 1.74 (s, 3H), 1.76 (s, 3H), 1.91-1.99 (m, 1H), 2.06-2.18 (m, 2H), 2.38 (s, 3H), 2.45-2.53 (m, 1H), 2.57-2.65 (m, 1H), 3.13 (t, J = 6.8 Hz, 2H), 3.90-4.02 (m, 2H), 4.20-4.27 (m, 4H), 6.57 (d, J = 13.6 Hz, 1H), 6.64 (d, J = 12.8 Hz, 1H), 7.08-7.22 (m, 4H), 7.25-7.32 (m, 2H), 7.36-7.41 (m, 3H), 7.91 (dd, J = 13.2, 13.2 Hz, 1H), 7.98 (dd, J = 13.6, 12.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 13.2, 14.5, 26.9, 28.1, 28.4, 28.5, 28.5, 29.9, 31.3, 36.6, 40.4, 43.8, 50.0, 52.9, 61.1, 104.5, 105.4, 110.6, 111.2, 122.7, 122.8, 125.2, 126.0, 127.5, 129.2, 129.5, 138.2, 141.9, 141.9, 143.5, 152.4, 154.4, 169.2, 172.9, 173.8, 196.2. HRMS (ESI) calcd for $C_{36}H_{45}N_2O_3S$ ([M^+]) 585.3145, found 585.3157.

Cy5 derivative **CyC4Et** was obtained in the similar manner to **proCyC4** using benzoindole **3a'**[9] instead of **3a**.



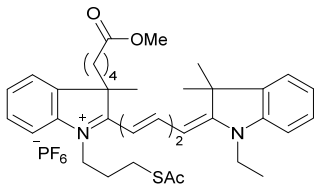
CyC4Et

CyC4Et: a red solid (38%): mp 109-111 °C; IR(ATR) 3021, 2971, 2942, 1739, 1478, 1444, 1366, 1229, 1217, 1153, 1100, 1067, 920, 817, 771, 707, 604, 577, 539, 515 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 0.57-0.65 (m, 1H), 0.88-0.97 (m, 1H), 1.37-1.44 (m, 6H), 1.46-1.54 (m, 2H), 1.73 (s, 3H), 1.77 (s, 3H), 1.78 (s, 3H), 2.13 (ddd, J = 13.7, 13.7, 2.3 Hz, 1H), 2.21-2.28 (m, 1H), 2.32-2.39 (m, 1H), 2.13 (ddd, J = 13.7, 13.2, 3.2 Hz, 1H), 4.08-4.18 (m, 4H), 6.30 (d, J = 13.8 Hz, 2H), 6.78 (dd, J = 12.4, 12.3 Hz, 1H), 7.07-7.13 (m, 2H), 7.19-7.24 (m, 2H), 7.31-7.39 (m, 4H), 8.14 (dd, J = 13.2, 12.8 Hz, 1H), 8.19 (dd, J = 12.8, 12.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 12.5, 23.4, 24.4, 27.8, 27.9, 28.1, 33.9, 39.3, 39.5, 41.0, 49.5, 53.7, 103.5, 103.6, 110.0, 110.3, 122.3, 122.3, 125.0, 125.1, 126.3, 128.5, 128.6, 139.4, 141.5, 141.5, 142.3, 152.9, 153.9, 170.7, 172.7, 177.4. HRMS (ESI) calcd for $C_{33}H_{41}N_2O_3$ ([M^+]) 497.3163, found 497.3166.

Synthesis of proCyME4

To a flame-dried Schlenk flask, Cy5 derivative **proCyC4** (48 mg, 68 μmol) and DMF (1.3 mL) were successively added at room temperature under nitrogen atmosphere. To this solution were added EtN(*i*-Pr)$_2$ (24 μL, 0.14 mmol), HATU (52 mg, 0.14 mmol), HOBt (9.3 mg, 69 μmol) and MeOH (8.4 μL, 0.27 mmol) in order at 0 °C. After stirring at room temperature for 7 h, the reaction mixture was diluted with CH_2Cl_2. The organic solutions was washed with saturated $NaHCO_3$ aqueous solution (10 mL×3) and dried over Na_2SO_4. The organic solvents were removed under

reduced pressure and the residue was subjected to column chromatography on SiO_2 (eluent: CH_2Cl_2-MeOH, v:v = 45:1) to afford a crude product. The organic solvents were removed under reduced pressure and the residue was dissolved in a small amount of CH_2Cl_2. After adding a solution (hexane-ether, v:v = 1:1) slowly, the product was gradually precipitated. The precipitate was washed with hexane. The volatiles were removed under reduced pressure to afford Cy5 derivative **proCyME4** (45 mg, 60 μmol, 89%) as a red solid.



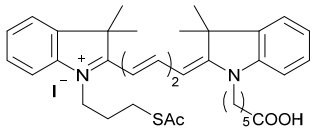
proCyME4

proCyME4: mp 80-82 °C; IR(ATR) 2926, 2856, 1732, 1691, 1477, 1444, 1372, 1330, 1147, 1042, 992, 925, 831, 751, 708, 552 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 0.53-0.65 (m, 1H), 0.85-0.98 (m, 1H), 1.44 (t, J = 7.2 Hz, 3H), 1.47-1.52 (m, 2H), 1.69 (s, 3H), 1.72 (s, 3H), 1.74 (s, 3H), 2.02-2.10 (m, 2H), 2.12-2.18 (m, 3H), 2.29 (ddd, J = 12.3, 12.3, 4.6 Hz, 1H), 2.38 (s, 3H), 2.99 (t, J = 7.2 Hz, 2H), 3.56 (s, 3H), 4.05 (t, J = 7.6 Hz, 2H), 4.11 (q, J = 7.2 Hz, 2H), 6.21 (d, J = 13.2 Hz, 1H), 6.26 (d, J = 14.0 Hz, 1H), 6.71 (dd, J = 12.8, 12.4 Hz, 1H), 7.07 (d, J = 7.6 Hz, 1H), 7.12 (d, J = 8.8 Hz, 1H), 7.20-7.31 (m, 3H), 7.35-7.41 (m, 3H), 7.85 (dd, J = 13.2, 12.8 Hz, 1H), 7.93 (dd, J = 13.2, 12.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 25.4, 34.6, 35.2, 36.4, 37.5, 37.7, 37.9, 40.1, 42.3, 47.1, 48.4, 49.8, 55.2, 56.7, 58.4, 98.2, 98.7, 103.6, 104.0, 113.4, 113.4, 115.5, 116.0, 116.6, 118.5, 118.5, 126.8, 128.7, 128.8, 129.8, 137.4, 138.7, 152.1, 154.2, 154.6, 172.0. HRMS (ESI) calcd for $C_{37}H_{47}N_2O_3S$ ([M^+]) 599.3302, found 599.3315.

Synthesis of proCyNC5 and proCyNCPh

To a solution of benzoindole **3c** (0.77 g, 1.9 mmol) in AcOH/Ac_2O (3.5 mL/8.0 mL) was added malonaldehyde dianilide hydrochloride (**6**) (0.60 g, 2.3 mmol) at room temperature. After stirring at 125 °C for 1 h, and cooled to room temperature. After the solution was added to cold water, and kept it at 0 °C for 12 h. The oily product was dissolved in a small amount of CH_2Cl_2, and washed with water (30 mL) and brine (30 mL) and dried over $MgSO_4$. The organic solvent was removed under reduced pressure. The crude oil **4c** (0.59 g) was used for the next step without further purification.

To a flame-dried Schlenk flask, benzoindole **3d** (0.29 g, 0.74 mmol), **4c** (0.59 g) and pyridine (7 mL) were successively added at room temperature under nitrogen atmosphere. After stirring at 40 °C for 2 h, the organic solvent was removed under reduced pressure and the residue was subjected to column chromatography on SiO_2 (eluent: CH_2Cl_2-MeOH, v:v = 15:1) to afford a crude product. The organic solvent was removed under reduced pressure and the residue was dissolved in a small amount of CH_2Cl_2. After adding ether slowly, the product was gradually precipitated. The precipitate was washed with ether. The volatiles were removed under reduced pressure to afford Cy5 derivative **proCyNC5** (0.25 g, 0.35 mmol, 47%) as a red solid.

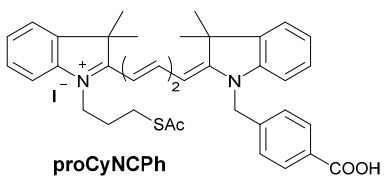


proCyNC5

proCyNC5: mp 99.5-97 °C; IR(ATR) 2970, 2926, 2862, 1726, 1685, 1578, 1477, 1444, 1373, 1330, 1214, 1172, 1128, 1083, 1040, 991, 917, 788, 745, 705, 621, 572, 551 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 1.53-1.60 (m, 2H), 1.74-1.85 (m, 16H), 2.07-2.14 (m, 2H), 2.34 (s, 3H), 2.46 (t, J = 7.4 Hz, 2H), 3.08 (t, J = 6.8 Hz, 2H), 4.07 (t, J = 8.2 Hz, 2H), 4.23 (t, J = 7.3 Hz, 2H), 6.35 (d, J = 13.7 Hz, 1H), 6.57 (d, J = 13.8 Hz, 1H), 6.98 (dd, J = 12.8, 12.4 Hz, 1H), 7.10-7.14 (m, 2H), 7.19-7.24 (m, 2H), 7.35-7.39 (m, 4H), 8.00-8.07 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 24.2, 26.1, 26.2, 26.8, 27.6, 28.0, 28.0, 30.7, 34.0, 43.2, 44.2, 49.2, 49.3,

103.8, 103.8, 110.4, 110.6, 122.2, 122.2, 125.0, 125.1, 126.4, 128.5, 128.6, 141.0, 141.1, 141.8, 141.9, 153.4, 153.5, 172.5, 172.9, 177.0, 195.6. HRMS (ESI) calcd for $C_{36}H_{45}N_2O_3S$ ([M^+]) 585.3145, found 585.3151.

Cy5 derivative **proCyNCPh** was obtained in the similar manner to **proCyNC5** using benzoindole **3e** instead of **3d**.

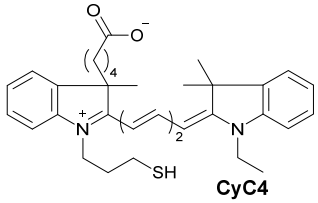


proCyNCPh

proCyNCPh: a red solid (35%): mp 188.5-189.5 °C; IR(ATR) 3465, 2971, 1739, 1577, 1476, 1441, 1336, 1229, 1217, 1127, 1093, 1035, 988, 914, 789, 743, 706, 539, 527 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.73 (s, 6H), 1.80 (s, 6H), 2.02-2.09 (m, 2H), 2.31 (s, 3H), 2.96 (t, J = 7.8 Hz, 2H), 4.18 (t, J = 6.8 Hz, 2H), 5.40 (s, 2H), 6.22 (d, J = 13.2 Hz, 1H), 6.32 (d, J = 13.7 Hz, 1H), 6.52 (dd, J = 12.4, 12.4 Hz, 1H), 7.20-7.28 (m, 2H), 7.30-7.38 (m, 5H), 7.43 (dd, J = 7.8, 7.3 Hz, 2H), 7.51-7.54 (m, 2H), 7.99 (d, J = 8.2 Hz, 2H), 8.25 (dd, J = 13.3, 12.8 Hz, 1H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 26.9, 27.7, 28.1, 28.9, 30.5, 44.0, 47.9, 50.5, 50.9, 104.3, 105.4, 111.8, 112.3, 123.5, 123.5, 126.1, 126.7, 127.2, 127.2, 127.4, 129.8, 129.8, 131.4, 140.6, 140.6, 142.2, 142.8, 143.3, 143.9, 155.4, 156.4, 174.4, 176.0, 196.9. HRMS (ESI) calcd for $C_{38}H_{41}N_2O_3$ ([M^+]) 605.2832, found 605.2838.

Synthesis of CyC4, CyNC5 and CyNCPh

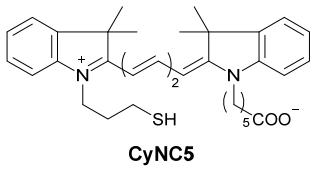
To a solution of Cy5 derivative **proCyC4** (9.9 mg, 16 μmol) in MeOH (1 mL) was added K_2CO_3 (6.6 mg, 48 μmol) at room temperature. After stirring at room temperature for 2 h, the reaction mixture was diluted with CH_2Cl_2. The organic solution was washed with saturated $NaHCO_3$ aqueous solution (10 mL×2) and dried over Na_2SO_4. The organic solvent was removed under reduced pressure to afford Cy5 derivative **CyC4** (7.2 mg, 13 μmol, 83%) as a blue solid.



CyC4

CyC4: mp 160-162 °C; IR(ATR) 2926, 2860, 1738, 1570, 1479, 1443, 1373, 1329, 1217, 1178, 1145, 1100, 1069, 1038, 988, 920, 801, 746, 706, 591, 546 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4, 25 °C) δ 0.49-0.64 (m, 1H), 0.83-0.98 (m, 1H), 1.32-1.43 (m, 5H), 1.66 (s, 3H), 1.69 (s, 3H), 1.69 (s, 3H), 1.99-2.19 (m, 5H), 2.42 (ddd, J = 12.8, 12.4, 3.7 Hz, 1H), 2.62 (t, J = 6.9 Hz, 2H), 4.12-4.20 (m, 4H), 6.32 (d, J = 13.7 Hz, 1H), 6.33 (d, J = 13.8 Hz, 1H), 6.61 (dd, J = 12.4, 12.4 Hz, 1H), 7.20-7.30 (m, 4H), 7.35-7.42 (m, 3H), 7.47 (d, J = 7.4 Hz, 1H), 8.20 (dd, J = 13.7, 12.8 Hz, 1H), 8.25 (dd, J = 13.3, 12.8 Hz, 1H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 12.6, 22.3, 25.2, 25.7, 27.8, 28.0, 32.5, 34.4, 40.1, 41.8, 43.3, 50.7, 54.8, 104.4, 104.6, 111.6, 112.0, 123.4, 123.5, 126.1, 126.5, 126.9, 129.8, 129.8, 140.6, 142.9, 143.0, 144.5, 154.6, 156.0, 172.5, 174.9, 177.0. HRMS (ESI) calcd for $C_{34}H_{43}N_2O_2S$ ([M^+]) 543.3040, found 543.3049.

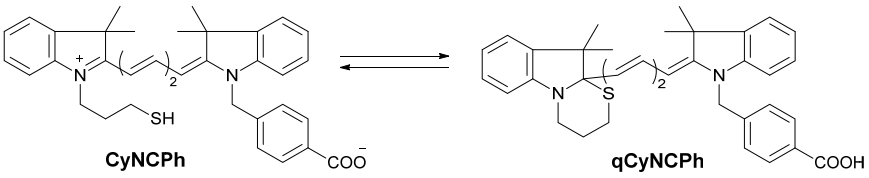
Cy5 derivatives **CyNC5** and **CyNCPh** were synthesized in a similar manner to **CyC4** using Cy5 derivatives **proCyNC5** and **proCyNCPh** instead of **proCyC4**, respectively.



CyNC5

CyNC5: a blue solid (99%): mp 149.5-151 °C; IR(ATR) 3347, 3249, 2966, 1650, 1487, 1451, 1387, 1335, 1255, 1172, 1070, 1017, 925, 799, 698, 609, 566, 538 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4, 25 °C) δ 1.46-1.54 (m, 2H), 1.65-1.73 (m, 14H), 1.80-1.88 (m, 2H), 2.05-2.12 (m, 2H), 2.32 (t, J = 7.3 Hz, 2H), 2.67 (t, J = 6.8 Hz, 2H), 4.13 (t, J = 7.3 Hz, 2H), 4.22 (t, J = 7.3 Hz, 2H), 6.31-6.36 (m, 2H), 6.64 (dd, J = 12.3, 12.4 Hz,

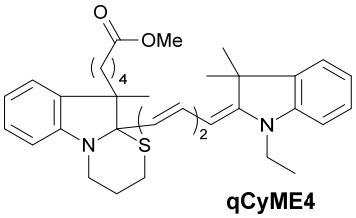
1H), 7.23-7.34 (m, 4H), 7.39-7.44 (m, 2H), 7.50 (dd, J = 6.4, 6.0 Hz, 2H), 8.23-8.30 (m, 2H). ^{13}C NMR (100 MHz, MeOH-d_4, 25 °C) δ 22.3, 25.8, 27.3, 27.9, 28.2, 32.4, 34.8, 35.7, 43.4, 44.9, 50.5, 50.7, 104.1, 104.7, 111.8, 112.2, 123.4, 123.4, 126.2, 126.4, 126.8, 129.7, 129.8, 142.5, 142.7, 143.5, 143.5, 155.4, 155.8, 174.4, 175.1, 177.7. HRMS (ESI) calcd for $C_{34}H_{43}N_2O_2S$ ([M^+]) 543.3040, found 543.3046.



Because pure **qCyNCPh** was obtained after the purification, spectral data of **qCyNCPh** were shown. In MeOH-d_4, a mixture of **CyNCPh** and **qCyNCPh** was obserbed. **qCyNCPh**: a greenish blue solid (93%): mp 185-186 °C; IR(ATR) 2970, 2926, 1702, 1596, 1479, 1453, 1376, 1332, 1255, 1212, 1133, 1105, 1042, 1016, 988, 920, 798, 740, 707, 554 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 0.91 (s, 3H), 1.25-1.39 (m, 4H), 1.62-1.78 (m, 7H), 2.37 (d, J = 12.3 Hz, 1H), 2.92 (dd, J = 12.8, 12.4 Hz, 1H), 3.39 (dd, J = 13.7, 12.8 Hz, 1H), 3.60 (d, J = 13.7 Hz, 1H), 4.69 (s, 2H), 5.28 (d, J = 11.9 Hz, 1H), 5.62 (d, J = 15.1 Hz, 1H), 6.08 (dd, J = 13.7, 11.4 Hz, 1H), 6.41 (d, J = 7.3 Hz, 1H), 6.52 (d, J = 7.3 Hz, 1H), 6.78-6.84 (m, 3H), 7.01 (d, J = 5.5 Hz, 2H), 7.10-7.20 (m, 5H), 7.92 (d, J = 6.4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 19.2, 19.9, 26.1, 26.7, 28.5, 41.1, 45.5, 46.0, 50.0, 86.8, 96.7, 106.0, 108.4, 118.7, 119.7, 121.6, 121.7, 121.7, 124.9, 125.7, 125.7, 127.3, 127.7, 129.6, 130.4, 130.4, 136.7, 138.5, 139.1, 144.8, 147.7, 155.6, 171.8. HRMS (ESI) calcd for $C_{36}H_{39}N_2O_2S$ ([M^+]) 563.2727, found 563.2735.

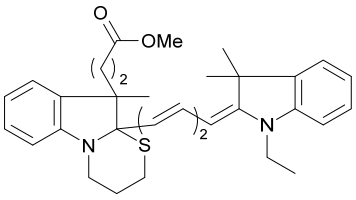
Synthesis of qCyME4 and qCyME2

To a flame-dried Schlenk flask, Cy5 derivative **proCyME4** (8.4 mg, 11 μmol), K_2CO_3 (3.5 mg, 25 μmol) and MeOH (1.1 mL) were successively added at room temperature under nitrogen atmosphere. After stirring at room temperature for 2 h, the reaction mixture was diluted with CH_2Cl_2. The organic solution was washed with saturated $NaHCO_3$ aqueous solution (10 mL×2) and dried over Na_2SO_4. The organic solvents were removed under reduced pressure to afford Cy5 derivative **qCyME4** (6.0 mg, 11 μmol, 95%) as a green solid. **qCyME4** obtained were a mixture of diastereoisomers (dr = ca. 2:1)



qCyME4: mp 79-80.5 °C; IR(ATR) 2964, 2907, 1733, 1593, 1479, 1455, 1357, 1337, 1261, 1103, 926, 802, 773, 738, 551 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 0.88-1.43 (m, 10H), 1.48-1.93 (m, 10H), 2.18 (t, J = 7.6 Hz, 2H), 2.34-2.46 (m, 1H), 2.97-3.07 (m, 1H), 3.44-3.51 (m, 1H), 3.57-3.67 (m, 6H), 5.38 (d, J =12.0 Hz, 1H), 5.72 (d, J = 15.2 Hz, 1H), 6.20-6.28 (m, 1H), 6.57 (dd, J = 7.6, 7.4 Hz, 2H), 6.76-6.84 (m, 2H), 6.89-6.95 (m, 2H), 6.99-7.08 (m, 1H), 7.12-7.17 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 11.3, 16.3, 19.9, 24.0, 25.6, 26.9, 28.4, 34.2, 35.9, 36.7, 41.3, 45.6, 51.6, 53.5, 88.4, 95.4, 105.7, 108.8, 118.3, 119.2, 121.7, 123.8, 124.1, 126.7, 127.5, 127.8, 130.4, 136.7, 137.3, 139.0, 144.5, 148.4, 155.5, 174.3. HRMS (ESI) calcd for $C_{35}H_{45}N_2O_2S$ ([M^+]) 557.3196, found 557.3199.

Cy5 derivative **qCyME2** was synthesized in a similar manner to **qCyME4** using Cy5 derivative **proCyEE2** instead of **proCyME4**. **qCyME2** obtained were a mixture of diastereoisomers (dr = ca. 7:2)



qCyME2

qCyME2: a green solid (91%): mp 83-85 °C; IR(ATR) 2964, 2926, 1735, 1593, 1479, 1456, 1357, 1262, 1086, 1022, 927, 801, 743, 520 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 0.73-1.88 (m, 16H), 2.10 (t, J = 7.2 Hz, 2H), 2.33-2.43 (m, 1H), 2.94 (dd, J = 13.1, 11.8 Hz, 1H), 3.38-3.61 (m, 7H), 5.30 (d, J =11.8 Hz, 1H), 5.68 (d, J = 14.9 Hz, 1H), 6.18 (dd, J = 11.3, 10.9 Hz, 1H), 6.50 (dd, J = 7.2, 6.8 Hz, 2H), 6.70-6.77 (m, 2H), 6.81-6.93 (m, 2H), 7.00-7.10 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) δ 11.3, 16.5, 19.9, 27.0, 28.4, 29.8, 31.6, 36.7, 41.3, 45.6, 51.6, 53.0, 88.1, 95.4, 105.7, 108.9, 118.5, 119.3, 121.7, 123.5, 124.0, 126.0, 127.8, 127.9, 130.6, 135.6, 137.8, 139.1, 144.5, 148.5, 155.7, 174.4. HRMS (ESI) calcd for $C_{33}H_{41}N_2O_2S$ ($[M^+]$) 529.2883, found 529.2893.

1.3. UV-vis and Fluorescence Spectra

UV-vis absorption spectra of dyes were measured by UV-vis-NIR spectrophotometer (UH5300, Hitachi High-Technologies Co., Japan). Emission spectra of dyes were measured by fluorescence spectrophotometer (RF-6000, Shimadzu Co., Japan). The sample solutions (5.0×10^{-6} M) were prepared by mixing a solution of probes in DMSO (30 μL, 5.0×10^{-4} M) with deoxygenated buffered aqueous solution (2970 μL). To estimate pK_a values, the absorbance or the fluorescence intensity were fitted to sigmoid curve (Fig. 2a, 4b and S4). The maximum absorbance or fluorescence intensity of each probe solution are defined as 1. The estimated pK_a values are shown in Table S1. Fluorescence spectra of **qCyME4**, **CyC4**, **CyNC5**, and **CyNCPh** are summarized in Figs. S1, S2, and S3. When pH value of a buffered solution of **qCyC4** was changed from 10.6 to 7.2, **qCyC4** was observed to be converted to the activated form **CyC4** within 10 seconds by UV-vis absorption measurement.

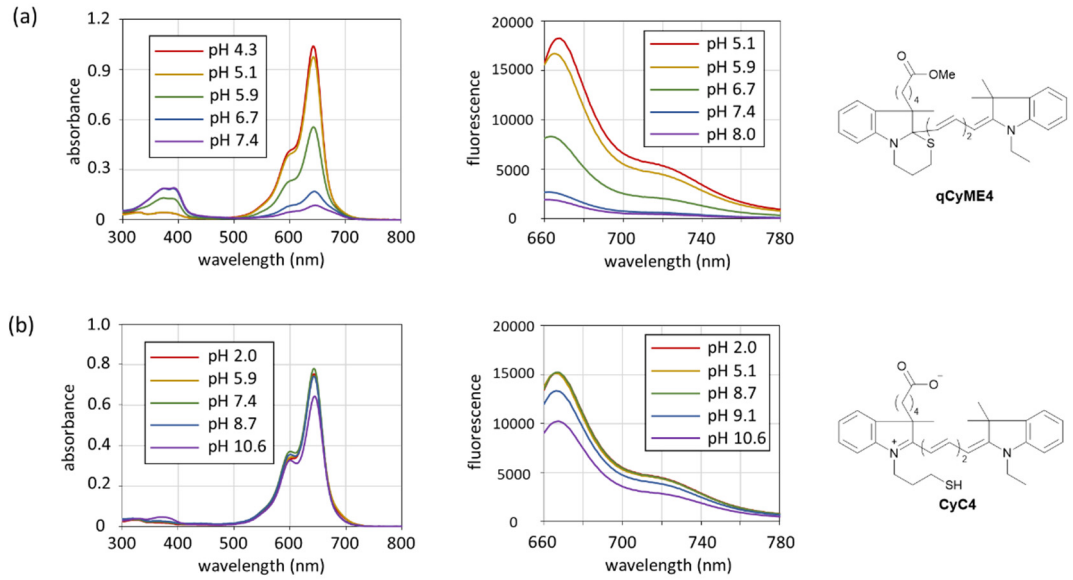


Fig. S1 UV-vis absorption spectra and fluorescence spectra (λ_{ex} = 650 nm) of (a) **qCyME4** and (b) **CyC4** (5.0×10^{-6} M) in various pH buffered solutions.

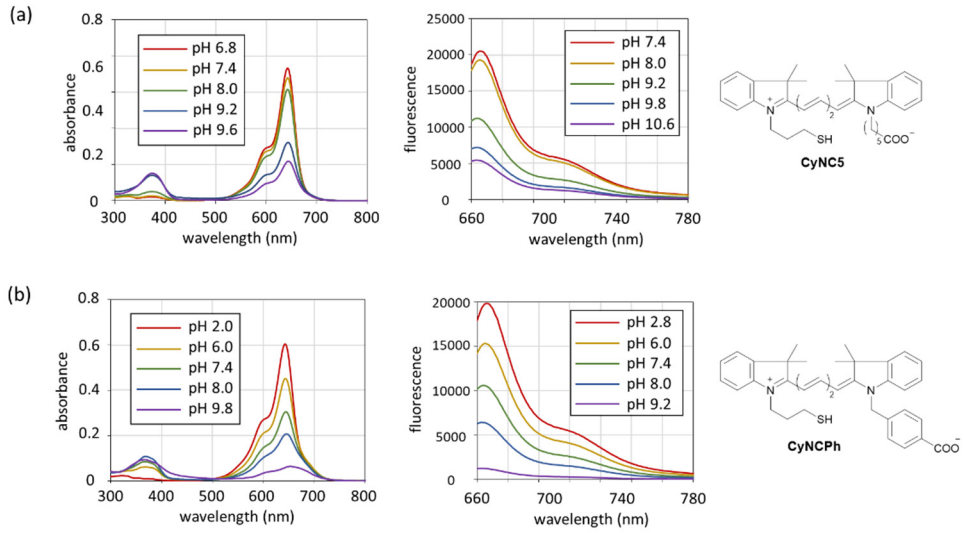


Fig. S2 UV-vis absorption spectra and fluorescence spectra (λ_{ex} = 650 nm) of (a) **CyNC5** and (b) **CyNCPh** (5.0×10^{-6} M) in various pH buffered solutions.

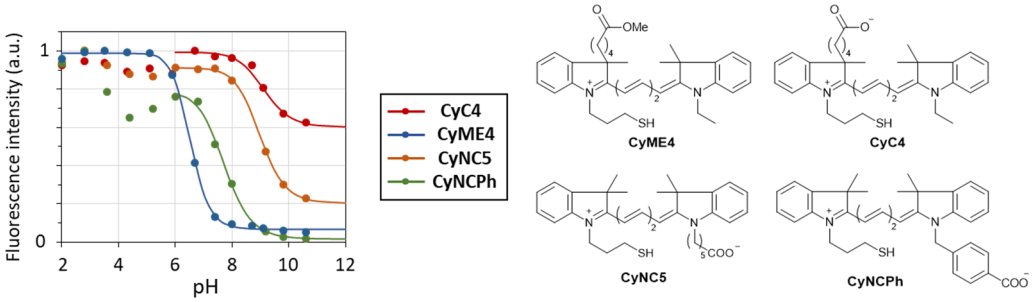


Fig. S3 Normalized fluorescence intensity at 670 nm (λ_{ex} = 650 nm) of **CyME4** (blue), **CyC4** (red), **CyNC5** (orange) and **CyNCPh** (green) (5.0×10^{-6} M) in various pH buffer solutions.

Table S1. pK_a values of **CyME4**, **CyC4**, **CyNC5** and **CyNCPh**

	pK_a (absorbance)	pK_a (fluorescence)
CyME4	5.8	6.6
CyC4	8.6	9.1
CyNC5	8.7	9.0
CyNCPh	7.8	7.7

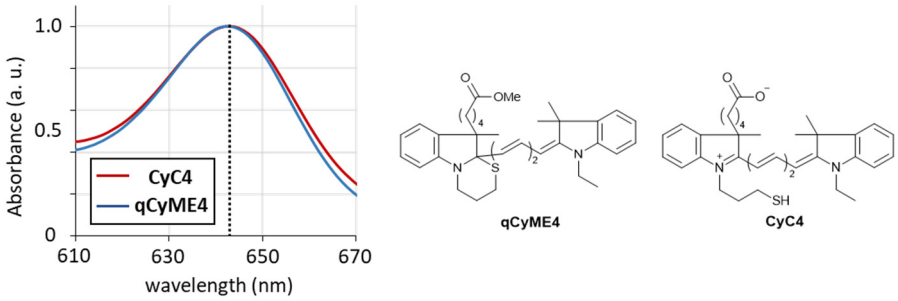


Fig. S4 Normalized absorbance spectra of **qCyME4** (blue) and **CyC4** (red) in the buffer solution (pH 2.0).

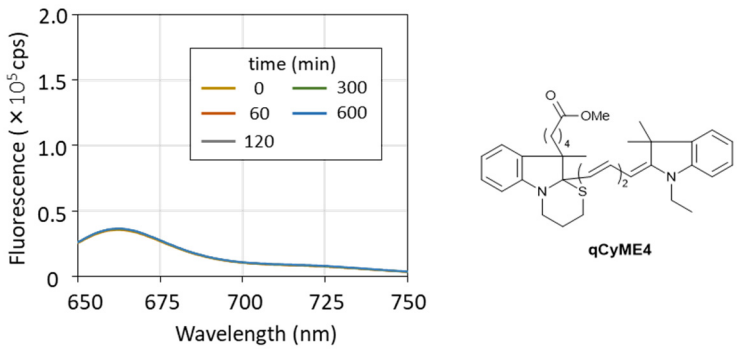


Fig. S5 Fluorescence spectra (λ_{ex} = 640 nm) of **qCyME4** (5.0×10^{-6} M) incubated for 600 min at 37 °C. All spectra without esterase are overlapped.

1.4. Enzyme Assay

Enzyme assay with esterase

Esterase extracted from porcine liver (Roche, Switzerland) was diluted with 0.1 M phosphate buffer solution (pH 7.5) to make different esterase concentrations. Esterase solution (200 μL) was mixed with the probe (20 μL, dissolved in DMSO) solution in the phosphate buffer solution (1780 μL) at 37 °C. Final concentration of each probe in the solution was 5.0 μM. The esterase concentration was 0–1.0 U/mL, 1.0 U/mL and 0–1.0 U/mL for assay with probe **qCyME4**, **qCyN**[2] and **qCyME2**, respectively. Fluorescence intensity excited at 650 nm was recorded every 5 minutes for 60 min at 37 ˚C. The fluorescence intensity at 0 min is defined as 1.

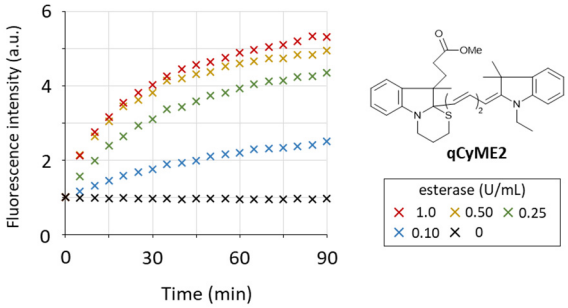


Fig. S6 Time dependent fluorescence intensity change at 670 nm (λ_{ex} = 650 nm) of **qCyME2** solution with 0–1.0 unit/mL esterase at 37 °C.

Inhibition Assay by using qCyME4

For inhibition assay of enzyme activity, final concentration of probe **qCyME4** was fixed to 5.0 μM. Final concentration of esterase was fixed to 1.0 U/mL for the assay of probe **qCyME4**. Esterase inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) was dissolved in 0.1 M phosphate buffer solution (pH 7.5, 100 μL) at different concentrations, and then mixed with the phosphate buffer solution (100 μL) of esterase. The mixed solution was incubated at 37 °C for 15 min to inhibit enzyme activity. To the inhibitor-treated PLE solution was then added a probe (20 μL, dissolved in DMSO) solution in the phosphate buffer solution (1780 μL). Enzymatic reaction was performed for 60 min at 37 °C and monitored by fluorescence spectrophotometer. Excitation wavelength for measurement was 640 nm.

1.5. *In Vitro* Cell Experiments

Cell culture

Human cervical epithelial adenocarcinoma cell line (HeLa) was purchased from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and cultured well-humidified incubator with 5% CO_2 and 95% air at 37 °C.

MTT cytotoxicity Assay

HeLa cells were seeded into a 96-well microtiter plate at a density of 4×10^5 cells. The cells were incubated for 24 hours for cell attachment. HeLa cells were incubated for an additional 6 h with different concentrations of **qCyME4** (0, 0.01, 0.1, 1, 10 and 100 μM). Then the cells were washed with PBS two times. Subsequently, MTT (200 μL, 0.5 mg/mL) was added to each well and the cells were incubated at 37 °C. After 4 h, the remaining MTT solution was removed, and the formazan crystals were dissolved in 200 μL of DMSO with gentle agitation for 5 min. The absorbance at 550 nm was measured using 800TS^{TM} Absorbance Microplate Reader (BioTek Instruments, USA).

Confocal fluorescence imaging

LSM 710 (Zeiss, Germany) was used for laser scanning confocal microscopy of HeLa cells. HeLa cells were seeded in a 35 mm dish (μ-Dish$^{35\ mm,\ high}$, ibidi, Germany) at a density of 4×10^5 cells. The cells were incubated for 24 hours for cell attachment. **qCyME4** dissolved in DMSO (0.2 mM) was diluted in the cell culture media to have a final concentration of 1 μM. The existing culture medium was replaced with fresh medium containing the probe and incubated. Fluorescence images of the cells were acquired by confocal laser scanning microscope (63× objective lens). Excitation lasers of 633 nm (emission: 647–759 nm) was used for detecting fluorescence signals. For inhibition study, before treatment with the probe, HeLa cells were pretreated with AEBSF (1 mM) for 30 min.

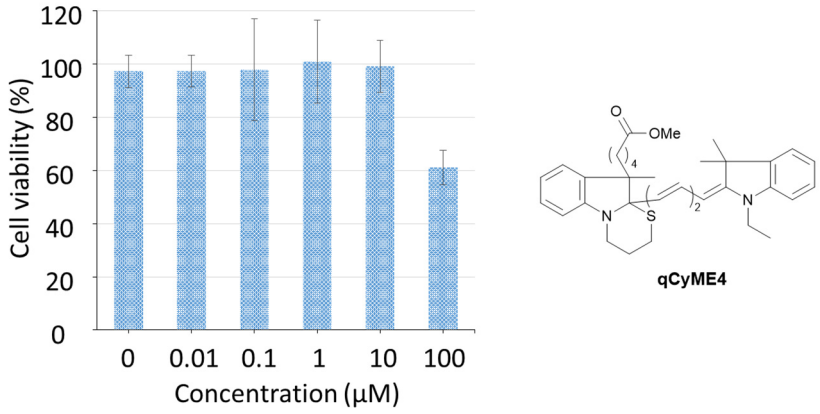


Fig. S7 Cytotoxicity experiment of **qCyME4** in living HeLa cells by MTT assay for 6 h.

1.6. Analysis by NMR

NMR spectra of **proCyC4** were measured in various conditions. Blue α, green β, red γ and purple δ express α-, β-, γ- and δ-methylene protons of **proCyC4**, respectively. ^{1}H NMR spectrum of **proCyC4** having carboxylate is shown in Fig. S8a. These methylene protons of **proCyC4** were assigned by referring H-H COSY NMR spectrum of **proCyC4** (Fig. S9). All of α-, β-, γ- and δ-methylene protons of **proCyC4** were observed to be a set of two non-equivalent signals. On the other hand, only γ- and δ-methylene protons of **proCyME4** having methyl ester were observed to be non-equivalent (Fig. S8b). These results indicate that the separation of the signals of γ- and δ-methylene protons is caused by a chiral carbon atom, and α- and β-methylene protons could be separated by limitation of mobility of alkyl chain through intramolecular electrostatic interaction. The cyclic structure might be formed through the interaction between carboxylate and iminium cation. This signal separation is not caused by the nucleophilic attack of a carboxylate on an iminium carbon because **CyC4Et**, having a carboxylic acid group, retains the iminium structure even under basic conditions (Fig. S10). The formation of this cyclic structure was also supported by the analyses of ^{1}H NMR spectra of **proCyC4** in different concentration (Fig. S11). In a saturated solution of **proCyC4** in $CDCl_3$, the difference of chemical shifts of two α- and δ-methylene protons were 0.096 ppm and 0.437 ppm, respectively (Fig. S11a). As the concentration become lower, the difference of chemical shifts became larger (Fig. S11b, c). The concentration effect on signal separation could be explained by intramolecular electrostatic interaction, not intermolecular interaction. We next examined temperature-dependence of signal separation (Fig. S12). Although the peak separation become slightly smaller at -30 °C, no obvious difference was observed in ^{1}H NMR spectra of **proCyC4** measured at -30 °C, 0 °C, 20 °C and 40 °C. These results also indicate that the intermolecular interaction is negligible.

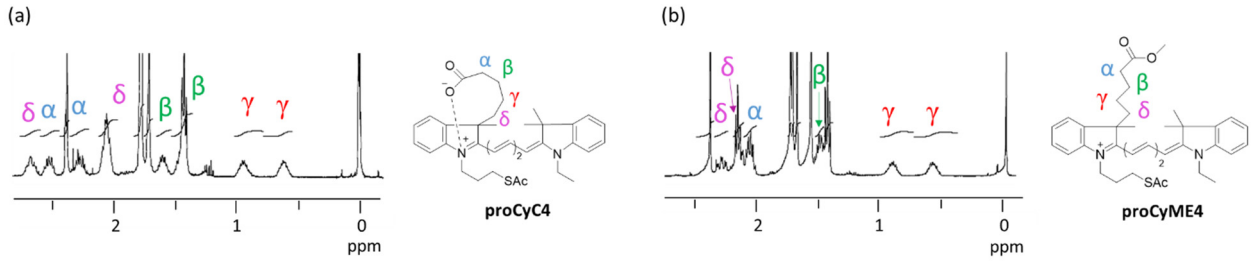


Fig. S8 ^{1}H NMR spectra ($CDCl_3$, 400 MHz, 24 °C) of (a) **proCyC4** and (b) **proCyME4**.

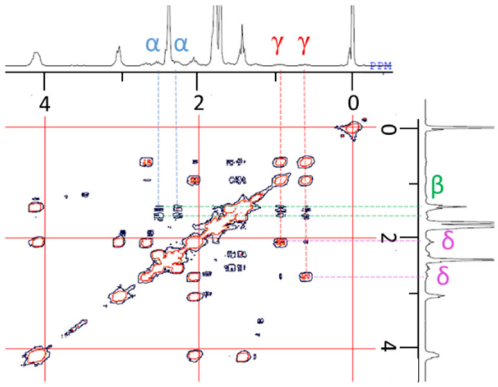


Fig. S9 H-H COSY NMR spectrum ($CDCl_3$, 400 MHz, 24 °C) of **proCyC4**.

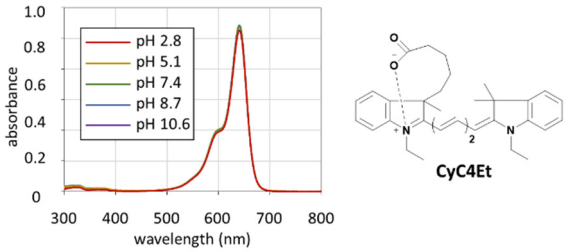


Fig. S10 UV-vis absorption spectra of **CyC4Et** (5.0×10^{-6} M) in various pH buffered solutions.

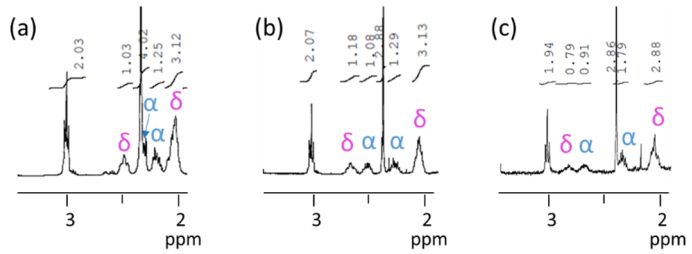


Fig. S11 ^{1}H NMR spectra ($CDCl_3$, 400 MHz, 24 °C) of **proCyC4** in $CDCl_3$ with (d) 1/10 times and (e) 1/100 times diluted concentrations of (c) solution A.

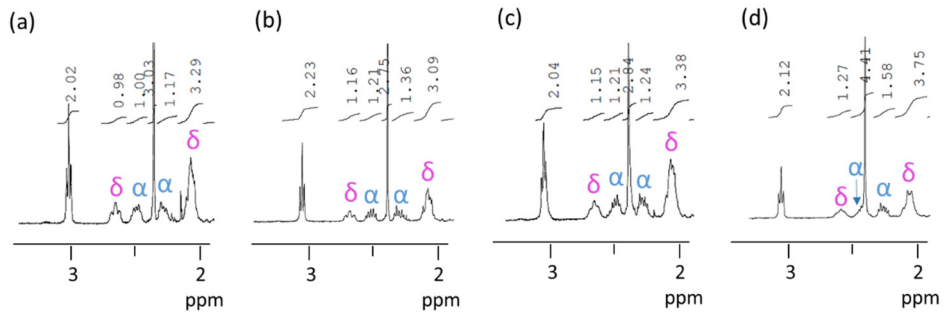


Fig. S12 ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of **proCyC4** measured at (a) 40 °C, (b) 20 °C, (c) 0 °C and (d) -30 °C.

1.7. Theoretical calculation.

The density functional theory (DFT) calculation of **CyC4** and **CyME4** was conducted at M06-2X/6-31G(d) level[10,11] by using Gaussian 16 package.[12]

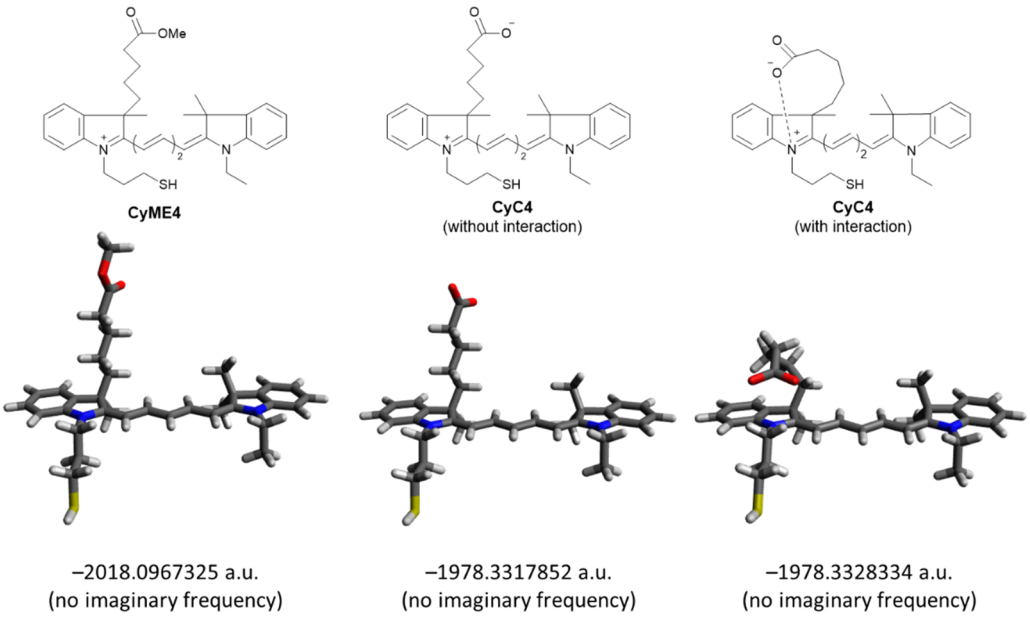
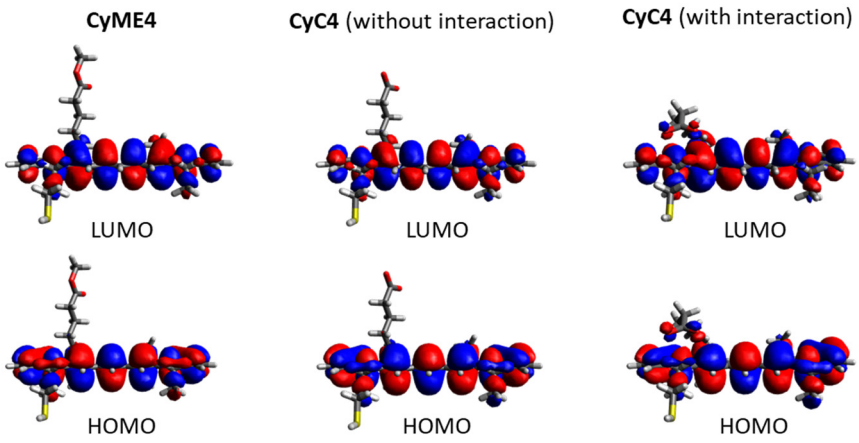


Fig. S13 The energy-minimized structures of **CyME4**, **CyC4** (without interaction) **CyC4** (with interaction).

Table S2. Energy gap of **CyME4** and **CyC4**.



	dye	**CyME4**	**CyC4** (without interaction)	**CyC4** (with interaction)
HOMO → LUMO	energy gap	2.4221 eV (511.89 nm)	2.4219 eV (511.92 nm)	2.4912 eV (497.68 nm)
	oscillator strength	2.1247	2.1274	2.0698

At M06-2X/6-31G(d) level (solvent=water) for TD-DFT calculation.

<Cartesian coordinates for optimized geometry of **CyME4**)>

C,0,6.5829861473,0.9910141201,2.4364247064
C,0,7.1350991319,0.1100229046,1.5069814775
C,0,6.3294769858,-0.605366839,0.6173426247
C,0,4.9603368721,-0.3951068424,0.7026023603
C,0,4.390182358,0.4709854515,1.6315903717
C,0,5.1978940468,1.1769294561,2.5060952558
N,0,3.9307255304,-0.9689954885,-0.0714755852
C,0,2.7108744169,-0.5589400708,0.3371614379
C,0,2.8835950231,0.4481219033,1.4799728496
C,0,1.5314951155,-1.0291632836,-0.2290444746
C,0,0.2501630457,-0.5961897576,0.1217120044
C,0,-0.9199388824,-1.0702147416,-0.4581406248
C,0,-2.1687428399,-0.5936784755,-0.0606374141
C,0,-3.3764883735,-1.0205505321,-0.6053216584
C,0,-4.6349246055,-0.5596993203,-0.2179876886
C,0,4.1925806139,-1.943345306,-1.1212898917
C,0,4.1816345738,-3.371982592,-0.5749157147
N,0,-5.7717697527,-0.9734472511,-0.8069037645
C,0,-6.9111264273,-0.4151941313,-0.1897242073
C,0,-6.4942421981,0.4402715959,0.8262133886
C,0,-4.9821430627,0.4331792241,0.8955704913
C,0,-8.250182803,-0.6317859188,-0.4835519155
C,0,-9.1858136623,0.0637695938,0.2859638216
C,0,-8.786642013,0.9325569574,1.3015784884
C,0,-7.4296881074,1.1269309362,1.5806361524
C,0,-5.8672339474,-1.942521455,-1.8962236461
C,0,-5.9252425611,-3.3721335496,-1.3690958968
C,0,-4.4355249209,1.8410279139,0.5942602122
C,0,-4.5231010405,-0.0756470044,2.2738219933
C,0,2.2422567562,-0.0674136568,2.781567494
C,0,2.3554235202,1.85535853,1.1149247526
C,0,2.8512036593,2.3876397755,-0.2276585012
C,0,2.3636012201,3.810665928,-0.4893085798
C,0,2.8484978685,4.3396881144,-1.8326243377
C,0,2.363623059,5.7411596576,-2.1128237389
O,0,2.8096702741,6.1861948575,-3.2956167903
O,0,1.6605486287,6.4004211673,-1.3811128001
C,0,4.473652108,-4.3743962342,-1.685464451
S,0,4.4428521689,-6.0621288198,-0.9789537354
H,0,7.2329360139,1.5336590527,3.1144519277
H,0,8.2106640483,-0.0278823447,1.4707096213
H,0,6.7669013779,-1.2940985456,-0.0974366194
H,0,4.7689890594,1.8600108141,3.2343829215
H,0,1.5980477432,-1.78824102,-1.0028820979
H,0,0.1382446259,0.1622359353,0.8923576257
H,0,-0.8621208737,-1.8261505247,-1.2387431484
H,0,-2.1720934303,0.1597971272,0.7229176746
H,0,-3.3255658657,-1.7722744916,-1.3871469361
H,0,3.4480873154,-1.8117176985,-1.9099873904
H,0,5.1641478496,-1.7006495912,-1.5592458707
H,0,4.9328684808,-3.457940623,0.2186268979
H,0,3.2037390137,-3.578844254,-0.1247896375
H,0,-8.5699903276,-1.31098448,-1.2662296948
H,0,-10.2426083449,-0.0799610716,0.0867653013
H,0,-9.535613817,1.4595277038,1.8829539223
H,0,-7.1207876777,1.8007821258,2.375120057
H,0,-6.7632837673,-1.6939103422,-2.4683332781
H,0,-5.0162845834,-1.7900711199,-2.5622557717
H,0,-6.0075300316,-4.0718560006,-2.2038486679
H,0,-5.0222354338,-3.6113649648,-0.8006790528
H,0,-6.7919886283,-3.5058168586,-0.7160139004
H,0,-3.3447236017,1.8725605325,0.6342540906
H,0,-4.8237165782,2.5398266958,1.3410719739
H,0,-4.7583185054,2.1777607933,-0.3945245699
H,0,-4.9110587698,0.5931695534,3.0476113212
H,0,-4.9076480491,-1.0816952341,2.4620688932
H,0,-3.4347030252,-0.0979003664,2.3585288587
H,0,1.1544181866,-0.1252757366,2.7031972053
H,0,2.6260651081,-1.0594191222,3.0347065586
H,0,2.490716392,0.6177503894,3.5974827426
H,0,1.2598544077,1.8556000464,1.1281237886
H,0,2.6716112617,2.5308339209,1.9203837096

H,0,3.9489277794,2.3652224212,-0.2513603778
H,0,2.5021233698,1.7300249892,-1.0351993139
H,0,1.2679672688,3.8394587759,-0.4631137866
H,0,2.7110649044,4.4762101866,0.309455319
H,0,3.9437497735,4.3474428912,-1.8844814945
H,0,2.5116057738,3.6989581369,-2.6563969795
H,0,3.7209484082,-4.3006334561,-2.4740356913
H,0,5.4577703298,-4.1849721246,-2.1206397314
H,0,4.742079925,-6.7208873297,-2.1089536226
C,0,2.3975089156,7.5094142195,-3.6493180039
H,0,2.8413291999,7.7087808679,-4.6226626054
H,0,2.7566401018,8.2286624064,-2.9106884277
H,0,1.3086967497,7.5648208988,-3.7067482062

<Cartesian coordinates for optimized geometry of **CyC4** (without interaction)>

C,0,-9.9102218096,-2.977749158,-0.4957538968
C,0,-9.561656609,-4.3273611416,-0.5404007159
C,0,-8.2256015833,-4.7335317726,-0.4947724396
C,0,-7.2686271952,-3.7328992683,-0.4011687199
C,0,-7.5963968487,-2.3803377263,-0.3660368207
C,0,-8.9234095918,-1.989523683,-0.4090228569
N,0,-5.8657366623,-3.8593138249,-0.3286180186
C,0,-5.2574228632,-2.6550964396,-0.2955239349
C,0,-6.3273016477,-1.5580912505,-0.2882863627
C,0,-3.8735234831,-2.5141511496,-0.2856044128
C,0,-3.1962681424,-1.2967461671,-0.1958397202
C,0,-1.8113599056,-1.1740306143,-0.186169752
C,0,-1.1954172087,0.0723995694,-0.0957139914
C,0,0.1846159661,0.2616096563,-0.0802498628
C,0,0.820036552,1.4994620813,0.0025687807
C,0,-5.1941314959,-5.1508164256,-0.3676255589
C,0,-4.8598291908,-5.5664782562,-1.8013805602
N,0,2.1595329443,1.6261768856,0.0524464362
C,0,2.5543929117,2.9802372226,0.0626665254
C,0,1.4135829728,3.7781464041,0.0642722562
C,0,0.1854551591,2.8935668524,0.0343167516
C,0,3.8374846945,3.5094281308,0.0678128837
C,0,3.9461440351,4.902047331,0.0876975492
C,0,2.8144198061,5.7172513188,0.0998805257
C,0,1.5326443451,5.1568530563,0.0868613102
C,0,3.1230648532,0.5287435217,0.0187493543
C,0,3.4662635592,0.1281076301,-1.4118248851
C,0,-0.6500161855,3.109849622,1.3097827525
C,0,-0.6317091714,3.1765096459,-1.2390802267
C,0,-6.2166251012,-0.6602464572,-1.5344234339
C,0,-6.3057977106,-0.7190875663,1.0117886782
C,0,-6.2809089149,-1.5377547087,2.3006178174
C,0,-6.354678475,-0.655820682,3.5458005613
C,0,-6.310366502,-1.4500547739,4.8434260992
C,0,-6.3710280202,-0.5987557479,6.1373156951
O,0,-6.3502900989,-1.2482162037,7.2123843457
O,0,-6.4324307974,0.6472902137,6.0030390489
C,0,-4.1690561754,-6.9251624032,-1.8185879091
S,0,-3.7944928076,-7.3767134112,-3.5518135627
H,0,-10.9558406242,-2.6913873257,-0.5325612514
H,0,-10.3388680975,-5.0808476615,-0.6138333311
H,0,-7.963110394,-5.7851743572,-0.5371258582
H,0,-9.1965956771,-0.9381957983,-0.3786252124
H,0,-3.2642711318,-3.4107358682,-0.3511935296
H,0,-3.7672041758,-0.3744346757,-0.1247754459
H,0,-1.195614868,-2.0689857083,-0.2506422109
H,0,-1.8537760379,0.9350672395,-0.0326303915
H,0,0.8032958361,-0.6284582885,-0.1436758717
H,0,-4.2954696115,-5.0929077962,0.2511102603
H,0,-5.8577106779,-5.8794934,0.104888763
H,0,-5.784668606,-5.6049003646,-2.3885178046
H,0,-4.2135391045,-4.8073130508,-2.256993026
H,0,4.7230786971,2.883593362,0.0500644676
H,0,4.9328911318,5.3531642886,0.0915870794

H,0,2.9293837383,6.7957857046,0.1155848661
H,0,0.6517980844,5.7930980777,0.091454644
H,0,4.0100413941,0.864405905,0.559665048
H,0,2.7065165131,-0.3063112941,0.5847285366
H,0,4.1950674906,-0.6855614292,-1.40408735
H,0,2.5727047832,-0.2089988298,-1.9444010405
H,0,3.895335818,0.9745651909,-1.9549576933
H,0,-1.5444500581,2.4833756106,1.3235231681
H,0,-0.9666969727,4.1560536782,1.3536650354
H,0,-0.0573819553,2.890789554,2.2020247121
H,0,-0.9467940191,4.2240788848,-1.2341679098
H,0,-0.0270981826,3.0019763242,-2.1332813578
H,0,-1.5263041779,2.553045294,-1.29700578
H,0,-5.2992585085,-0.0675210707,-1.5271730931
H,0,-6.2361901272,-1.2607569539,-2.4480102503
H,0,-7.0671337568,0.0275444683,-1.5546839505
H,0,-5.4499200511,-0.0347641544,0.9953758304
H,0,-7.2029173967,-0.0861578573,0.9929880066
H,0,-7.1196579692,-2.2477336257,2.3020890512
H,0,-5.361080653,-2.1383293948,2.3342753312
H,0,-5.5282382969,0.0654292075,3.537110257
H,0,-7.2735001129,-0.0570340574,3.5196933804
H,0,-7.1389897692,-2.1706757614,4.8763654743
H,0,-5.395324663,-2.057072173,4.8850566177
H,0,-3.2378153974,-6.8865560082,-1.2484324567
H,0,-4.8174542449,-7.6878374318,-1.3807465135
H,0,-3.2102417693,-8.5557847635,-3.2915809306

<Cartesian coordinates for optimized geometry of **CyC4** (with interaction)>

C	6.23993487	-2.33297436	-1.87693423	C	-4.74471039	-1.21338514	-1.77591797
C	6.84515299	-1.20873213	-1.31103525	C	1.90010487	-1.50700984	-2.35424782
C	6.0853523	-0.21357967	-0.69588984	C	1.98129063	-2.43440703	0.04441737
C	4.71308709	-0.40753139	-0.65582621	C	3.97968415	-3.38670158	1.42278134
C	4.09061612	-1.53169664	-1.18798474	C	3.5562934	-2.62424877	2.68954046
C	4.8532212	-2.49821241	-1.82696917	C	4.47464889	4.13757408	0.47845114
N	3.72223874	0.45953083	-0.14827893	S	4.56772706	5.56961822	-0.65731208
C	2.49944641	-0.01328625	-0.37227895	C	2.83184556	-3.64756936	0.43645487
C	2.5954025	-1.40887514	-0.98428124	C	3.68193888	-1.08917632	2.52607045
C	1.33166364	0.71362824	-0.06611266	O	4.84705773	-0.66391574	2.32221063
C	0.05832055	0.20262911	-0.17035116	O	2.63011107	-0.40280038	2.59764017
C	-1.11886487	0.91959865	0.12718863	H	6.85240965	-3.08308296	-2.36616521
C	-2.36103342	0.33842819	0.00825034	H	7.9233201	-1.09678249	-1.36142269
C	-3.57854837	0.99519331	0.28426415	H	6.5530317	0.66870392	-0.27223234
C	-4.83193964	0.43136386	0.1654534	H	4.38927352	-3.36869977	-2.28129844
C	4.03984681	1.67360038	0.5931028	H	1.44693719	1.7303767	0.29696553
C	4.12641468	2.89056132	-0.32622224	H	-0.07464783	-0.83072775	-0.48394407
N	-5.97562555	1.09720597	0.48052952	H	-1.03611403	1.95408649	0.45549995
C	-7.11670622	0.32762173	0.20019	H	-2.38464611	-0.69782233	-0.32005313
C	-6.71647312	-0.92199154	-0.2708377	H	-3.50756285	2.02833936	0.61238267
C	-5.20305007	-0.97416447	-0.32687233	H	3.28029908	1.77490063	1.37159258
C	-8.45455484	0.67414565	0.33766694	H	4.97743051	1.47420224	1.11482189
C	-9.40188996	-0.29482479	-0.00513822	H	4.88877406	2.71499956	-1.09450129
C	-9.0183941	-1.55275872	-0.46727692	H	3.17076579	3.03274246	-0.84476093
C	-7.66274316	-1.87412058	-0.60492311	H	-8.76451718	1.6530459	0.68733531
C	-6.04722117	2.47207971	0.95663912	H	-10.45630013	-0.05616594	0.08991971
C	-6.05566874	3.47161468	-0.19561132	H	-9.77496251	-2.28567406	-0.72655001
C	-4.67871198	-2.0666306	0.6220346	H	-7.36303077	-2.85274448	-0.97083337

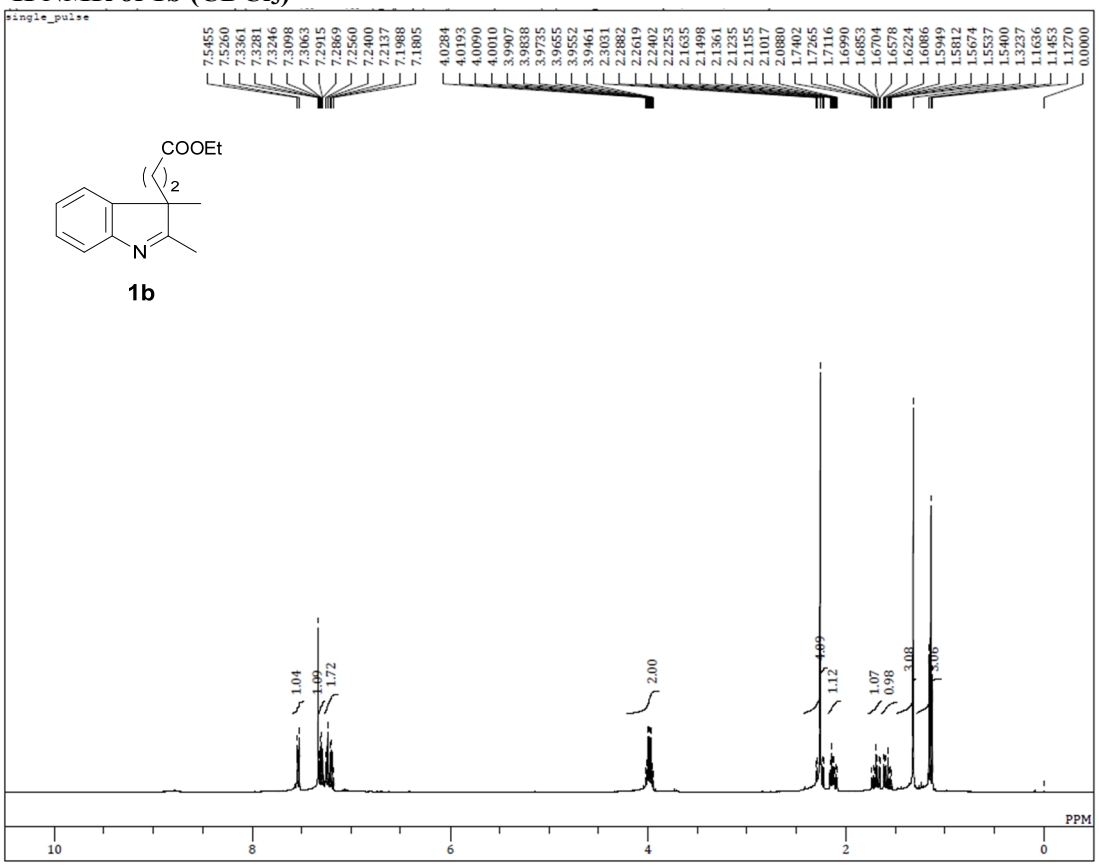
H	-6.95373397	2.55987895	1.55977924
H	-5.20451677	2.64276214	1.63029864
H	-6.11031624	4.4915496	0.192437
H	-5.1469883	3.37526092	-0.79642271
H	-6.91910874	3.29926192	-0.84435206
H	-3.58779229	-2.12200953	0.61572776
H	-5.07088236	-3.03740154	0.30387721
H	-5.01085349	-1.87939986	1.64687256
H	-5.15007059	-2.16699065	-2.12770541
H	-5.11189516	-0.4196466	-2.43236346
H	-3.65657809	-1.25636665	-1.85904904
H	0.82357381	-1.34107593	-2.28018707
H	2.31767355	-0.780256	-3.05664008
H	2.06161566	-2.51086528	-2.7595163
H	1.04642133	-2.79890111	-0.3973992
H	1.71495636	-1.89317226	0.95816089
H	4.39547909	-4.36455354	1.69491548
H	4.79121571	-2.82421644	0.95002182
H	4.22384684	-2.91186316	3.51043877
H	2.53481611	-2.89428615	2.98280577
H	3.71007475	4.32775776	1.23546656
H	5.43839369	4.01132067	0.97737034
H	4.89561506	6.49186468	0.26061193
H	2.14272458	-4.35872602	0.91081921
H	3.21606067	-4.15391676	-0.45705833

2. References

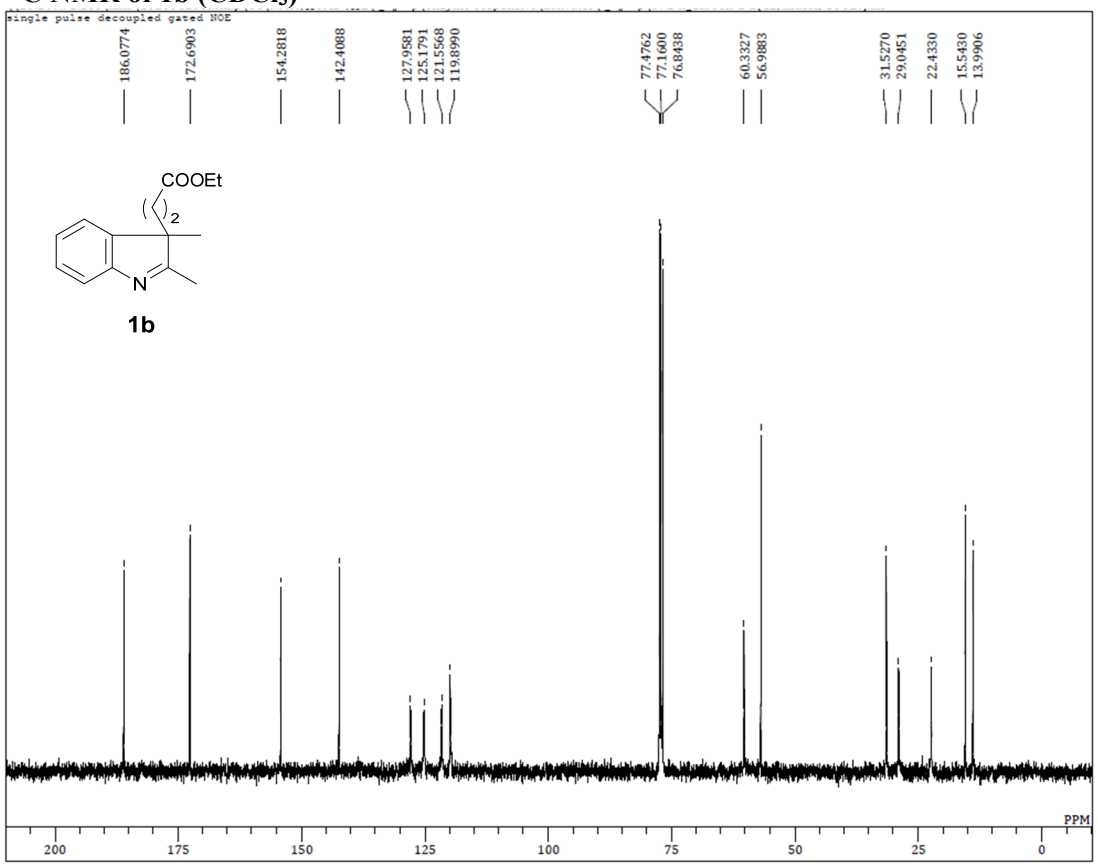
1. V. S. Arutyunyan, M. G. Zalinyan and M. T. Dangyan, *Armyanskii Khimicheskii Zhurnal,* 1978, **31**, 421.
2. M. Oe, K. Miki, H. Mu, H. Harada, A. Morinibu and K. Ohe, *Tetrahedron Lett.,* 2018, **59**, 3317.
3. R. L. Simmons, R. T. Yu and A. G. Myerws, *J. Am. Chem. Soc.,* 2011, **133**, 15870.
4. S. Xu, Q. Wang, Q. Zhang, L. Zhang, L. Zuo, J.-D. Jiang and H.-Y. Hu, *Chem. Commun.*, 2017, **53**, 11177.
5. X. Chen, X. Zhao, M. Li, *Guangdong Huagong,* 2008, **35**, 25.
6. V. E. Kuznetsova and A. V. Chudinov, *Russ. Chem. Bull. Int. Ed.* 2008, **57**, 608.
7. E. Block, E. V. Dikarev, R. S. Glass, J. Jin, B. Li, X. Li and S. Zhang, *J. Am. Chem. Soc.,* 2006, **128**, 14949.
8. L. Bi, Patent WO2014/058535A1, April 17, 2014.
9. V. E. Kuznetsova, V. A. Vasiliskov, A. S. Zasedatelev and A. V. Chudinov, *Mendeleev Commun.,* 2008, **18**, 138.
10. Y. Zhao and D. G. Truhlar, *Theor. Chem. Acc.,* 2008, **120**, 215.
11. (a) W. J. Hehre, R. Ditchfield and J. A. Pople, *J. Chem. Phys*., 1972, **56**, 2257. (b) P. C. Hariharan and J. A. Pople, *Theor. Chim. Acta,* 1973, **28**, 213.
12. Gaussian 16, Revision B.01, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, G. A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A. V. Marenich, J. Bloino, B. G. Janesko, R. Gomperts, B. Mennucci, H. P. Hratchian, J. V. Ortiz, A. F. Izmaylov, J. L. Sonnenberg, D. Williams-Young, F. Ding, F. Lipparini, F. Egidi, J. Goings, B. Peng, A. Petrone, T. Henderson, D. Ranasinghe, V. G. Zakrzewski, J. Gao, N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H.; Nakai, T. Vreven, K. Throssell, J. A., Jr. Montgomery, J. E. Peralta, F. Ogliaro, M. J. Bearpark, J. J. Heyd, E. N. Brothers, K. N. Kudin, V. N. Staroverov, T. A. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. P. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, J. M. Millam, M. Klene, C. Adamo, R. Cammi, J. W. Ochterski, R. L. Martin, K. Morokuma, O. Farkas, J. B. Foresman and D. J. Fox, Gaussian, Inc., Wallingford CT, 2016.

3. NMR spectra

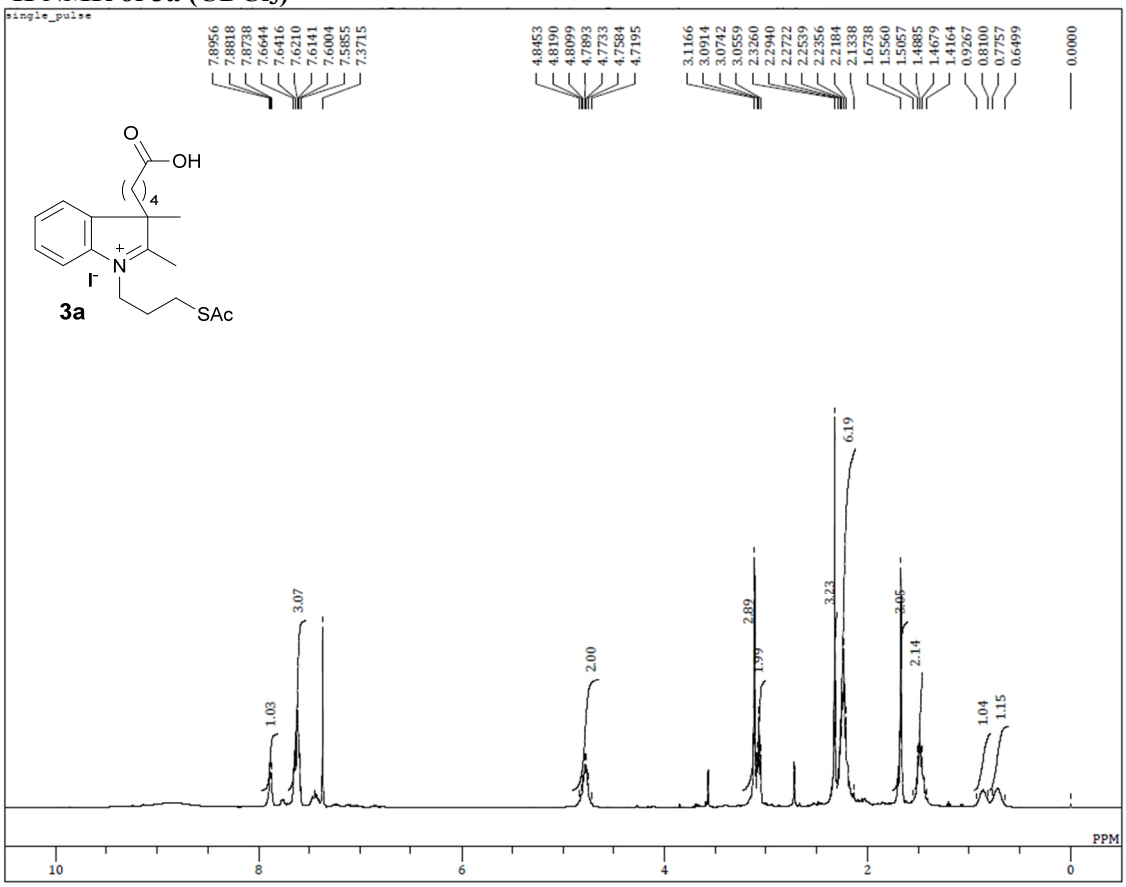
^{1}H NMR of 1b ($CDCl_3$)



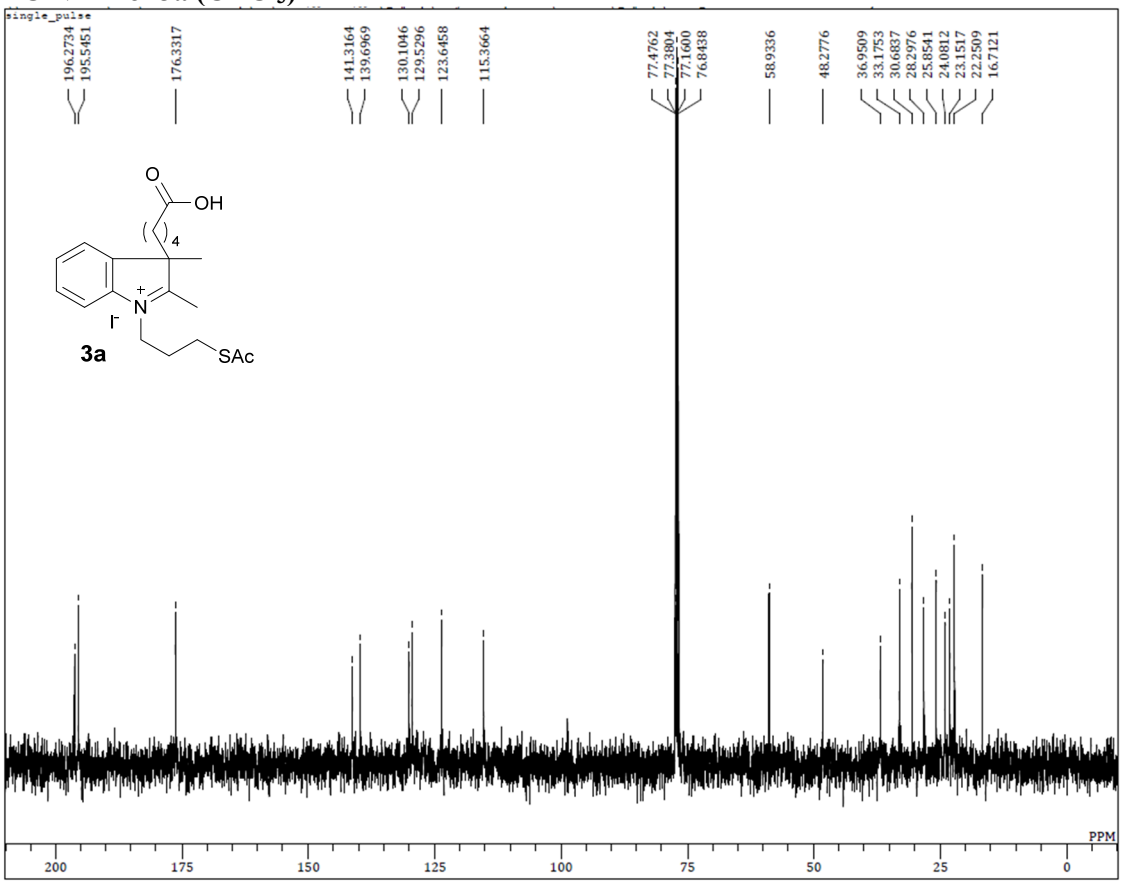
^{13}C NMR of 1b ($CDCl_3$)



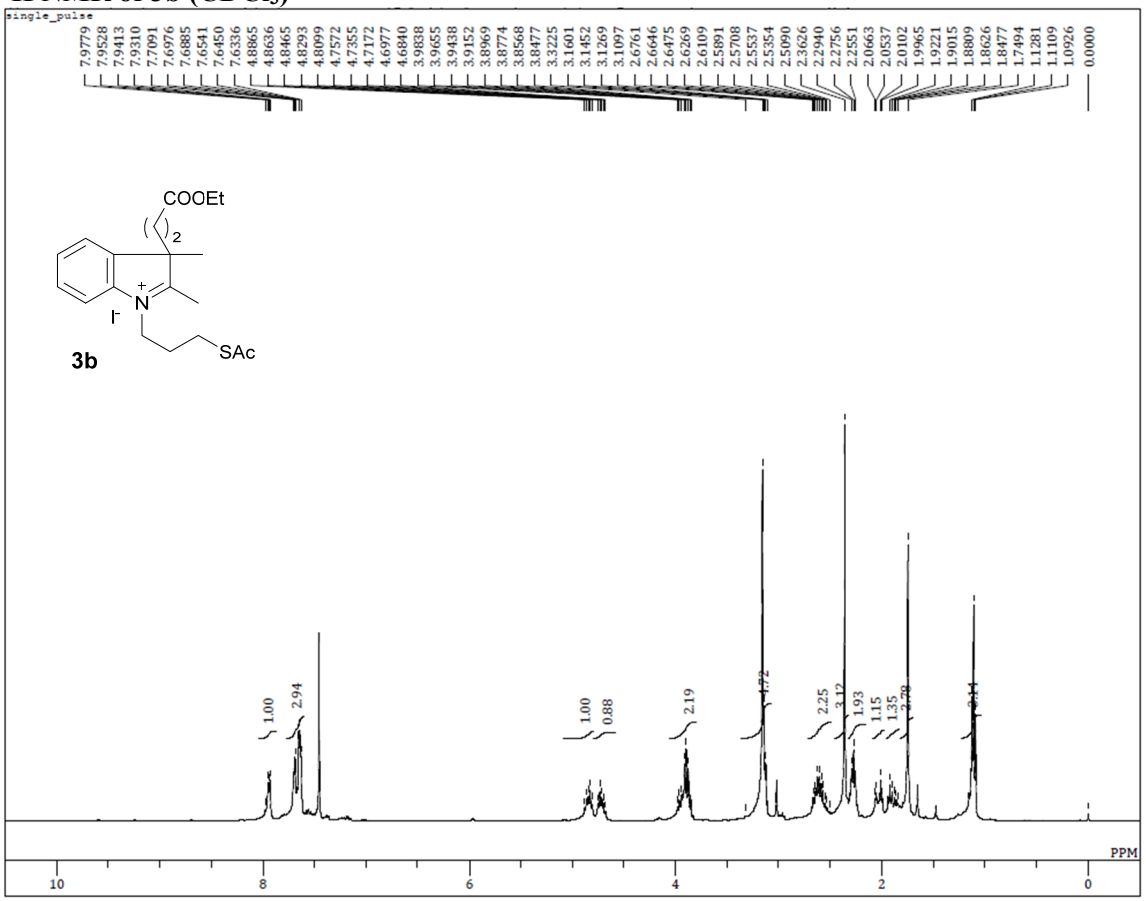
^{1}H NMR of 3a ($CDCl_3$)



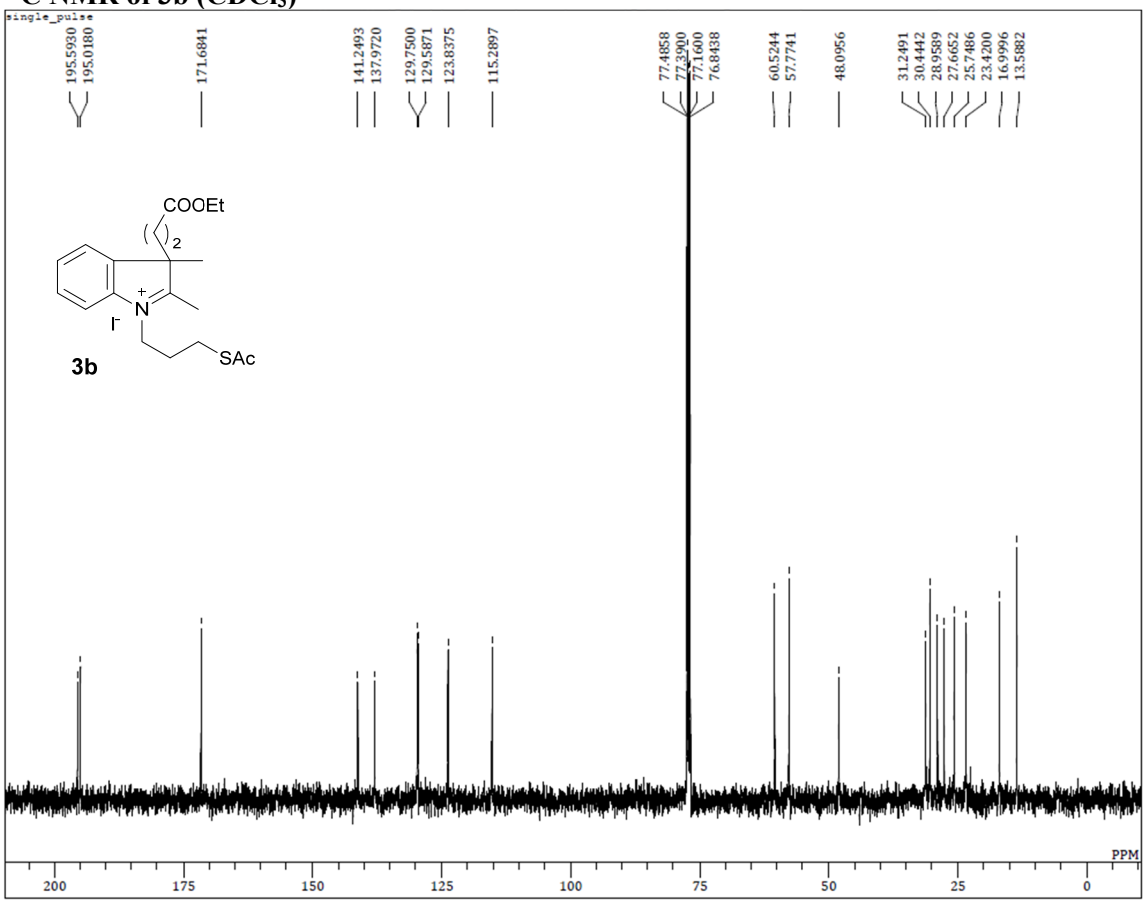
^{13}C NMR of 3a ($CDCl_3$)



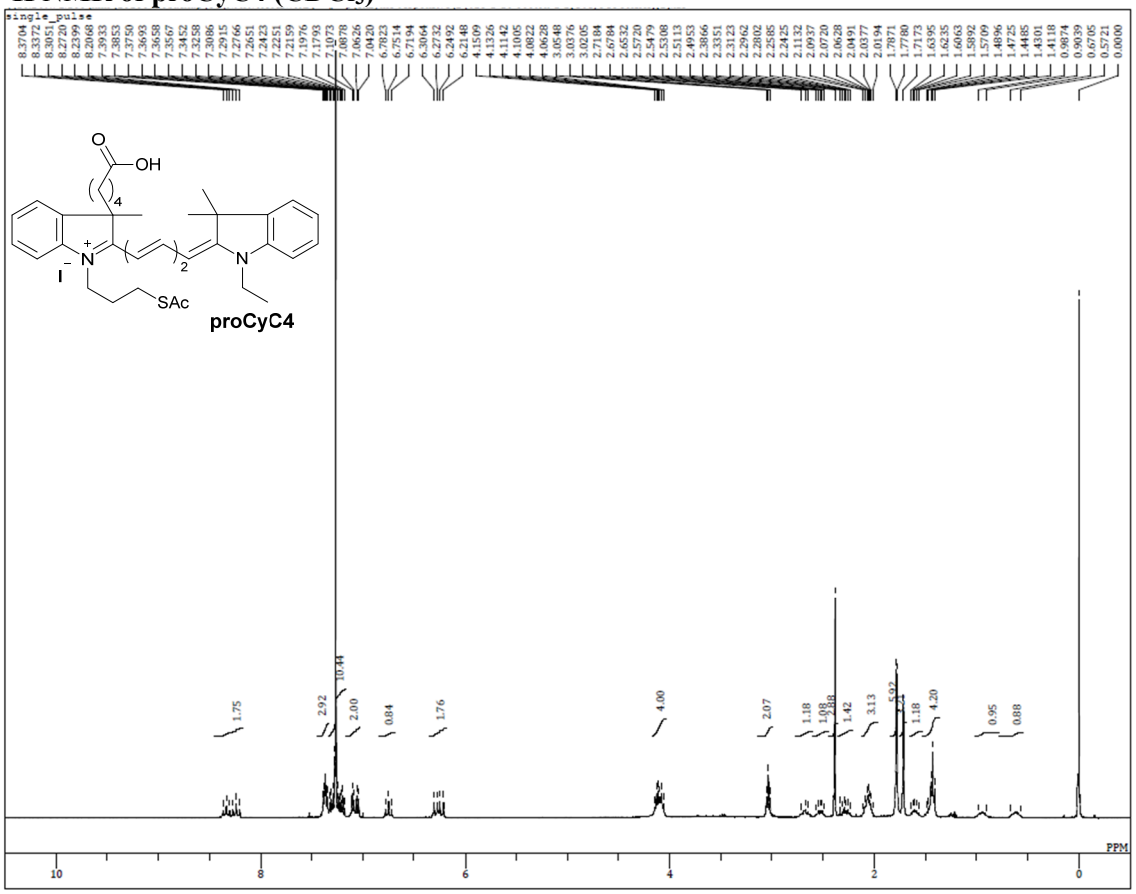
^{1}H NMR of 3b ($CDCl_3$)



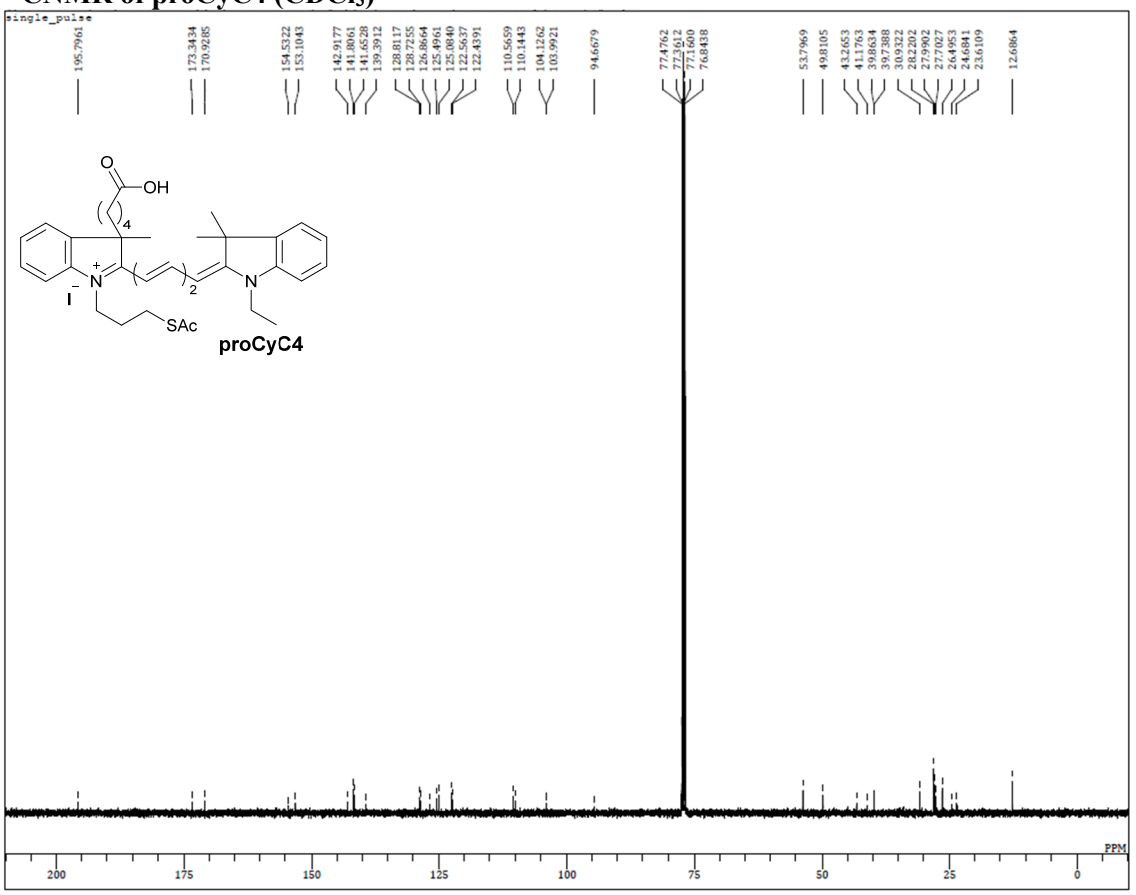
^{13}C NMR of 3b ($CDCl_3$)



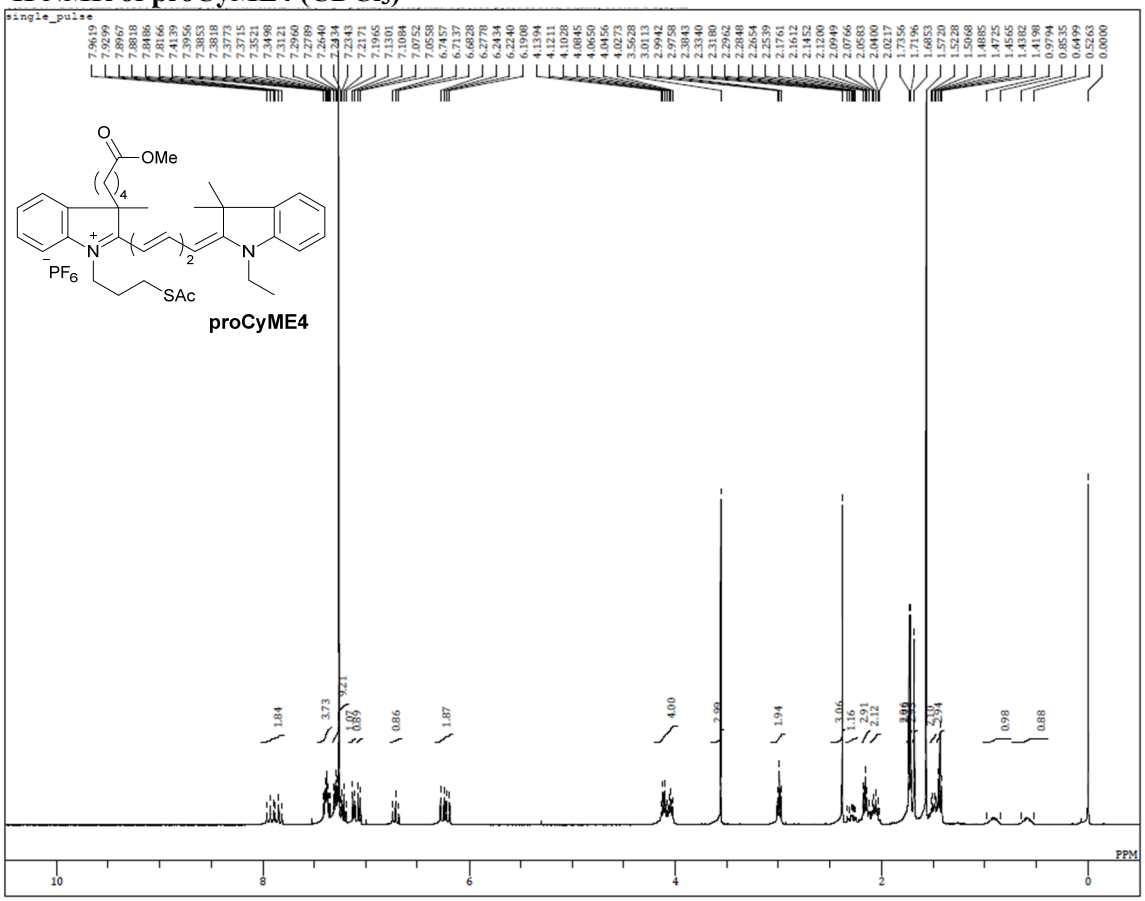
^{1}H NMR of proCyC4 ($CDCl_3$)



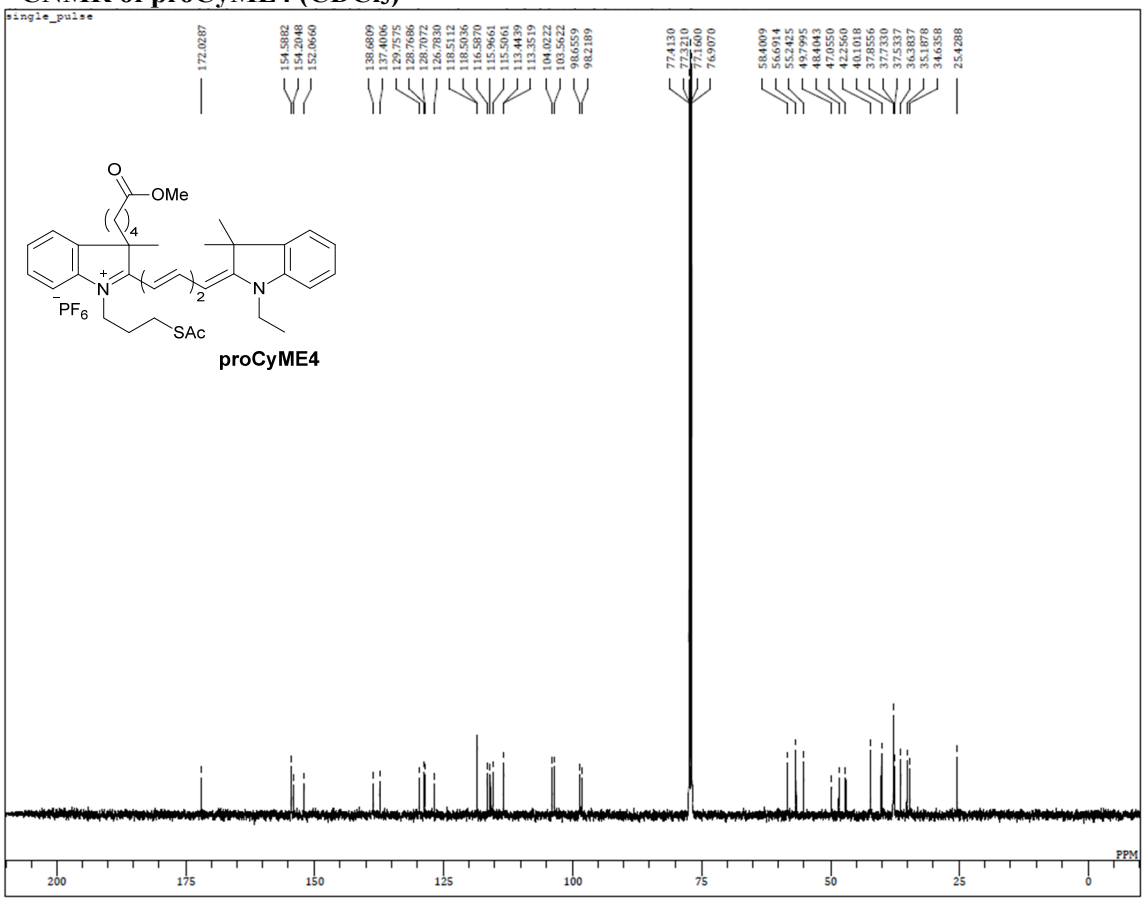
^{13}CNMR of proCyC4 ($CDCl_3$)



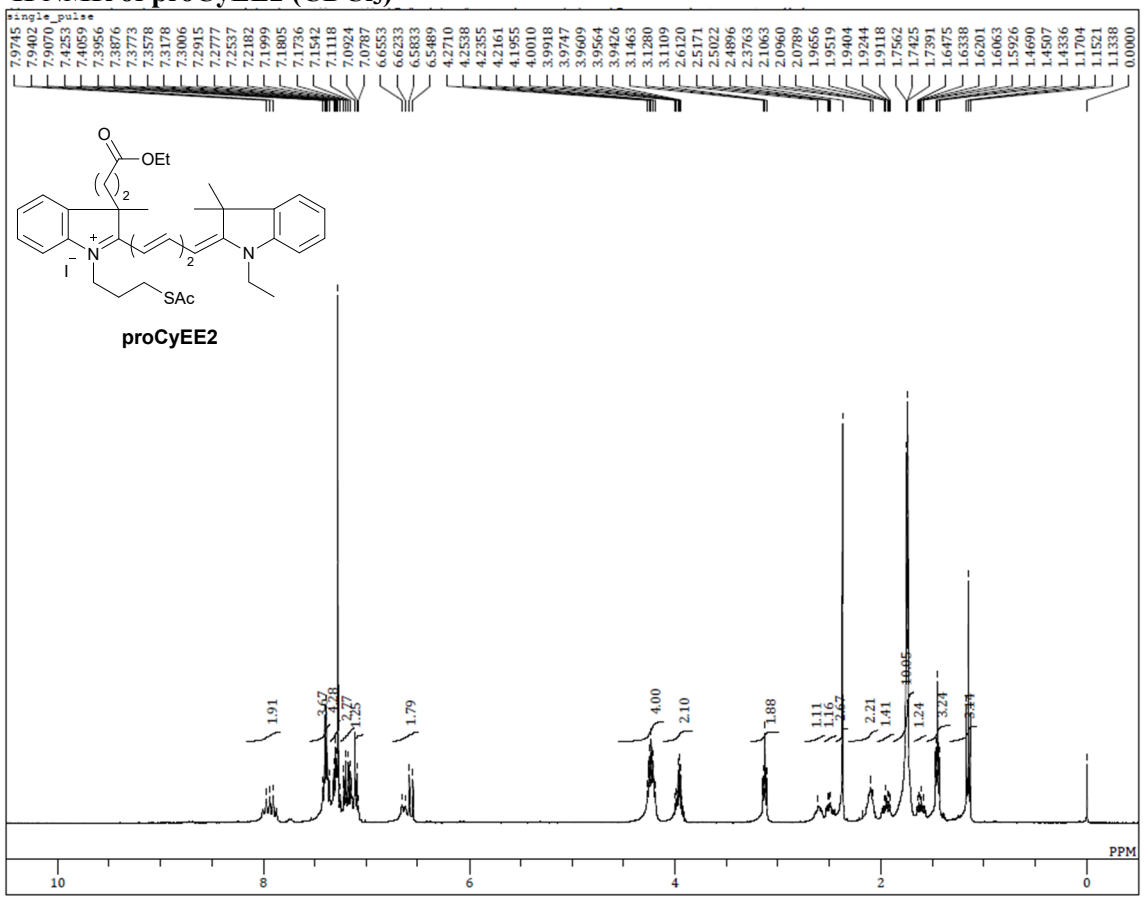
^{1}H NMR of proCyME4 ($CDCl_3$)



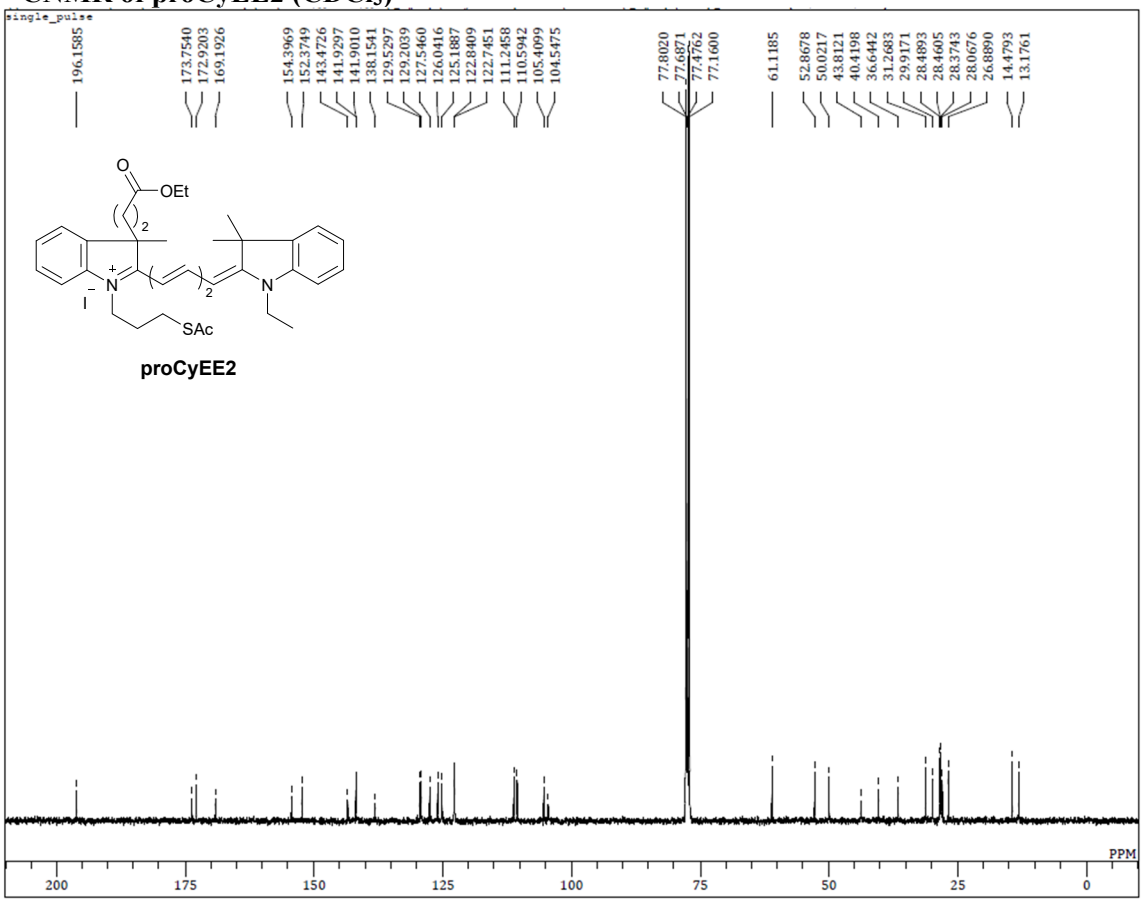
^{13}CNMR of proCyME4 ($CDCl_3$)



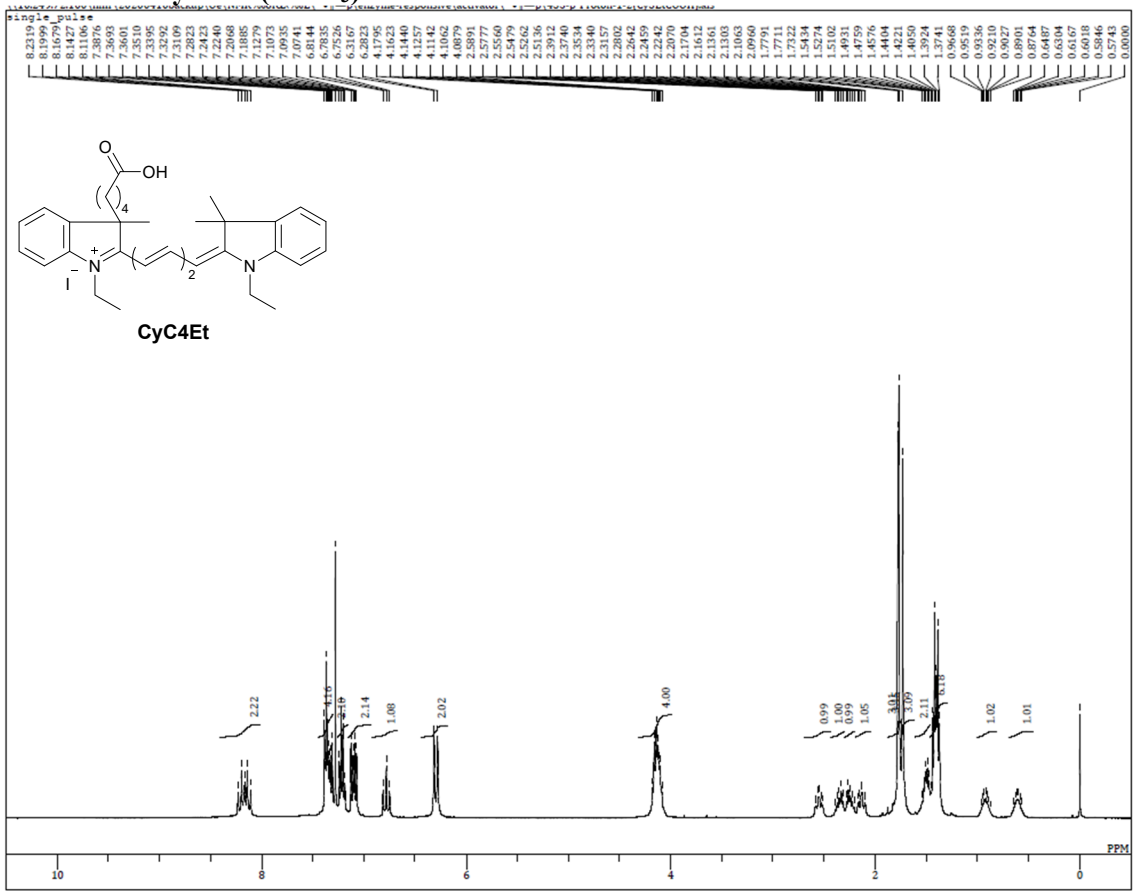
^{1}H NMR of proCyEE2 ($CDCl_3$)



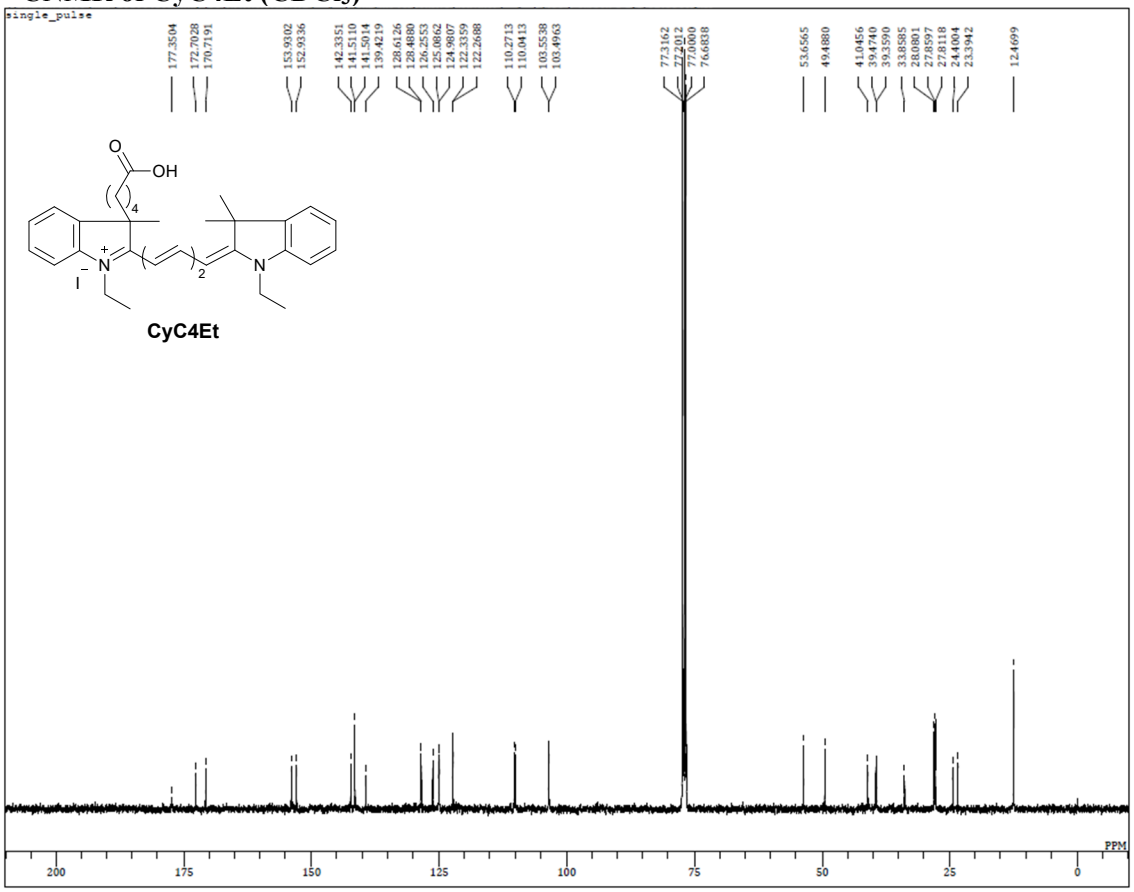
^{13}CNMR of proCyEE2 ($CDCl_3$)



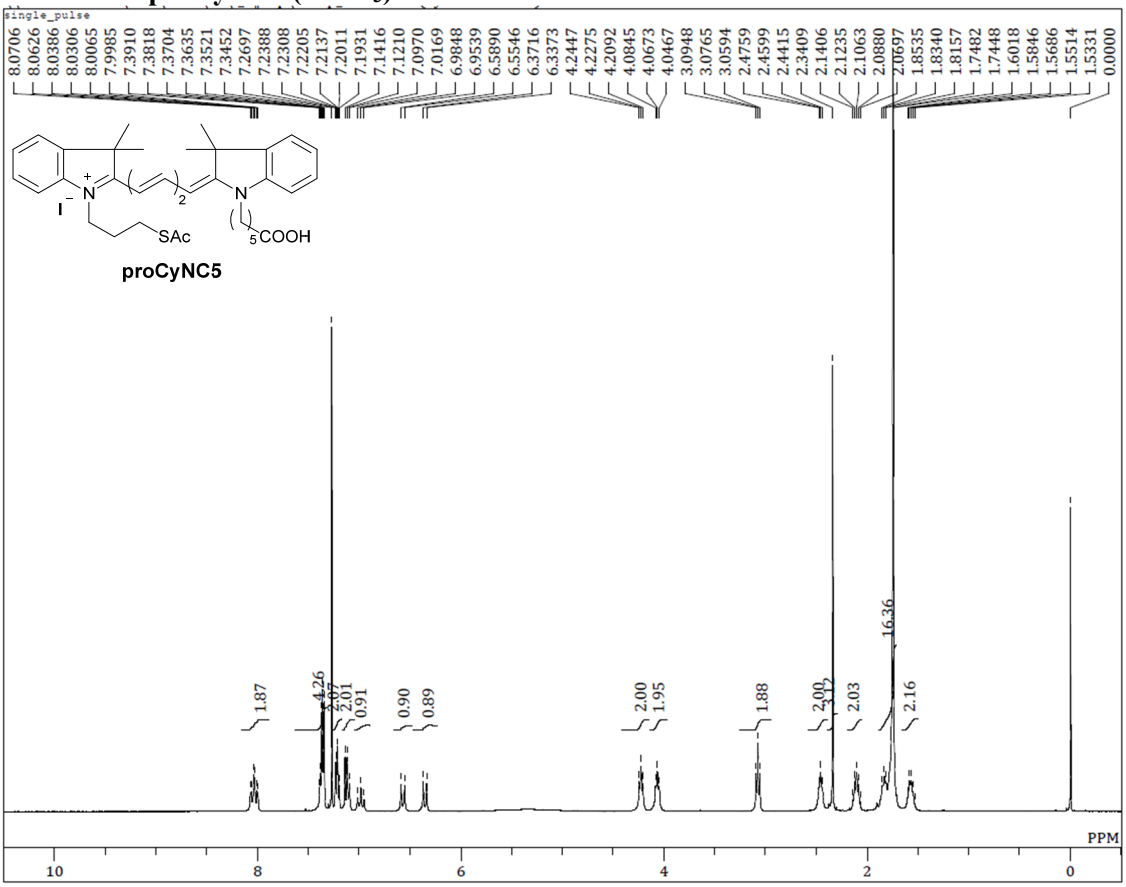
^{1}H NMR of CyC4Et ($CDCl_3$)



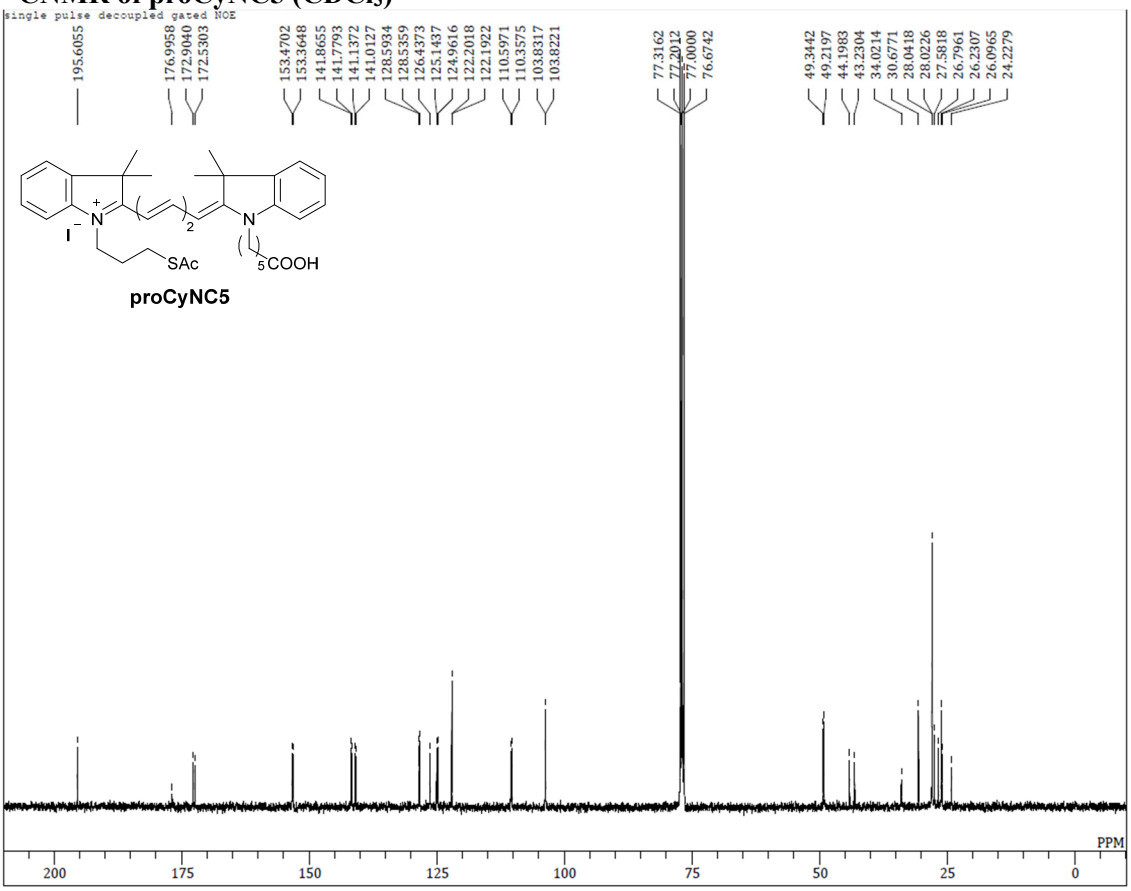
^{13}CNMR of CyC4Et ($CDCl_3$)



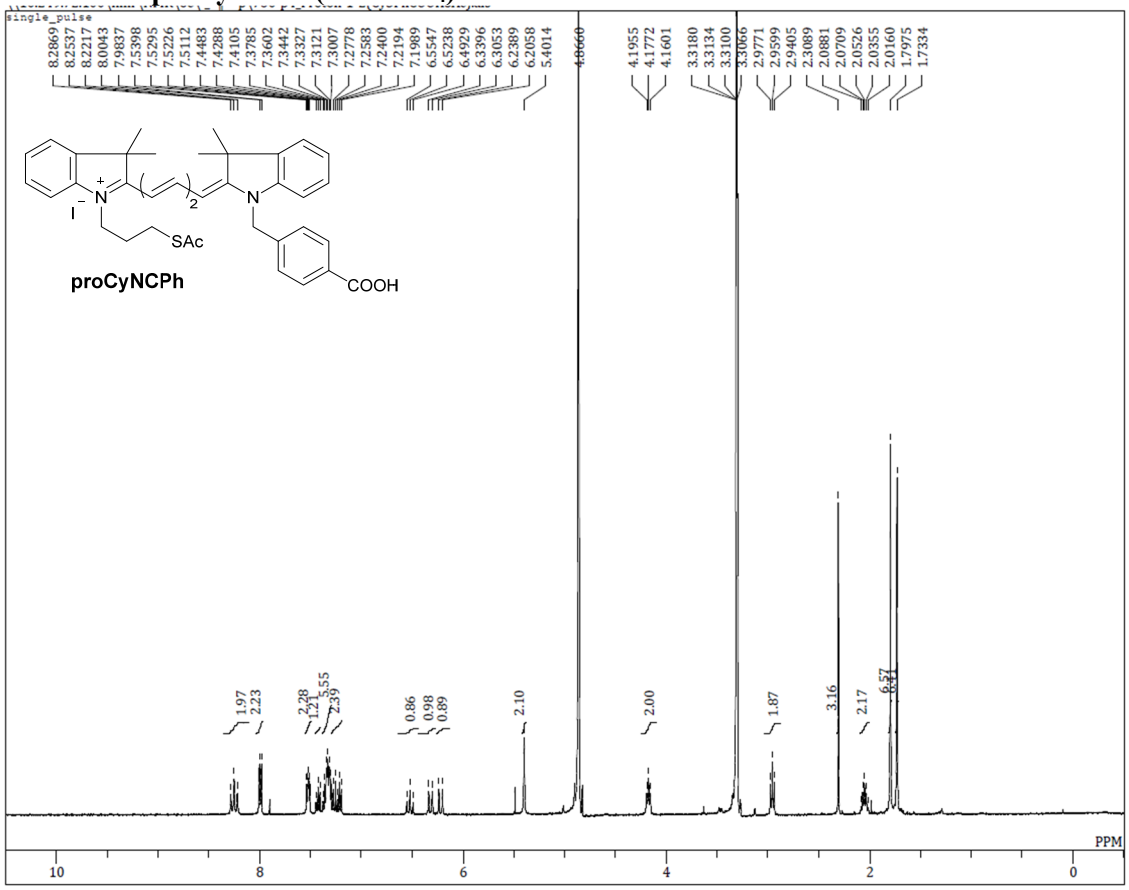
^{1}H NMR of proCyNC5 ($CDCl_3$)



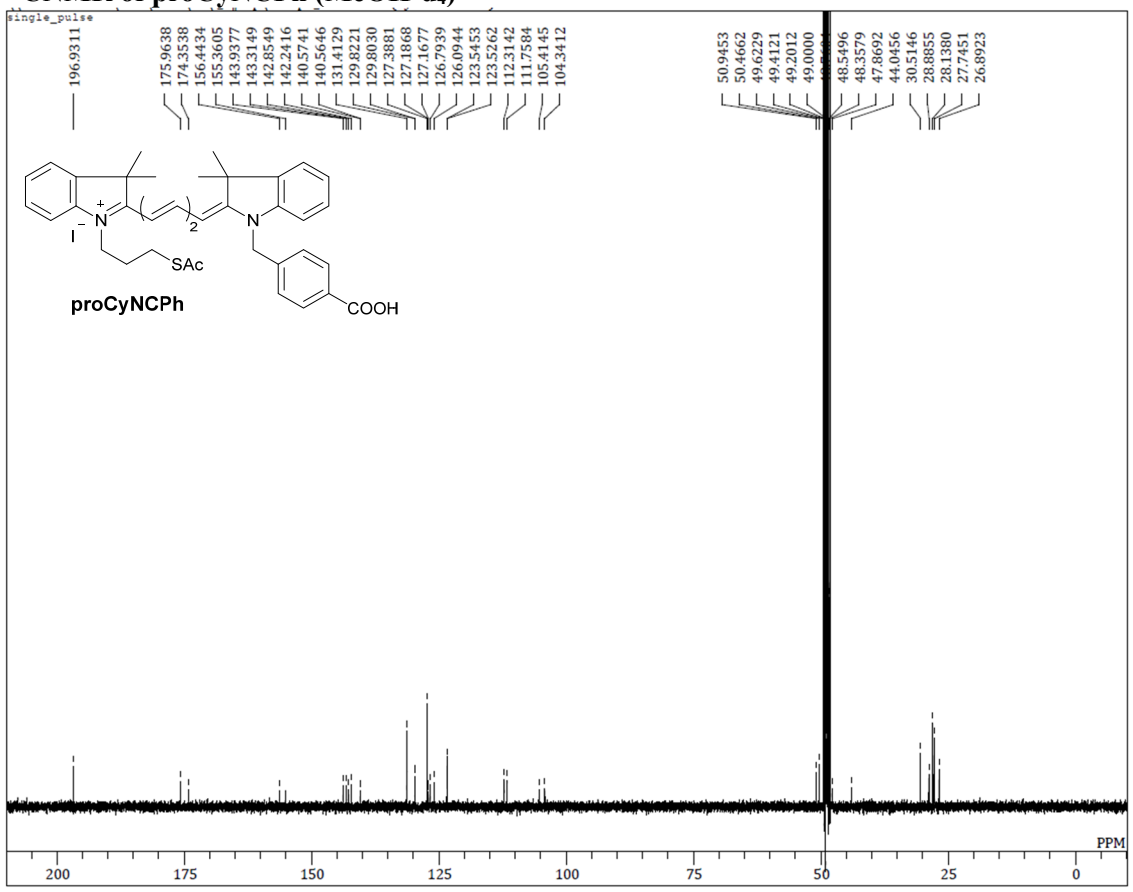
^{13}CNMR of proCyNC5 ($CDCl_3$)



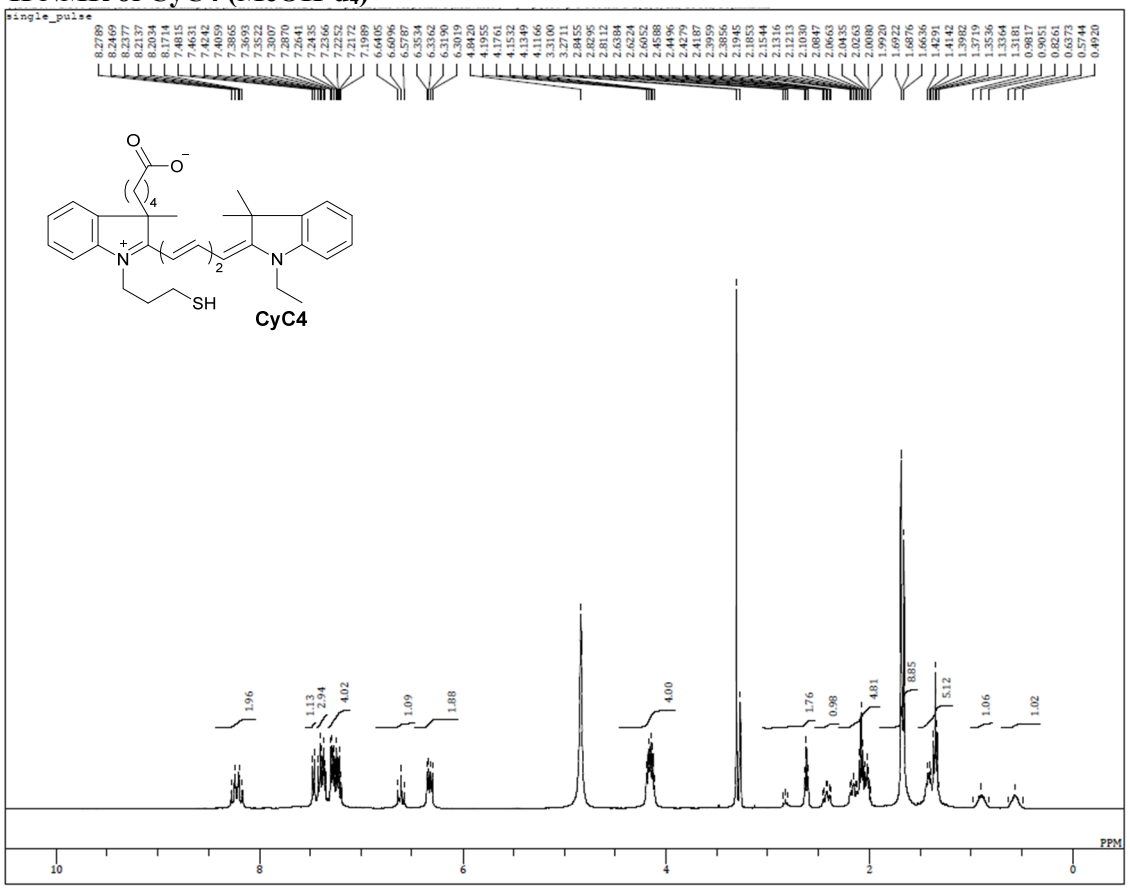
^{1}H NMR of proCyNCPh (MeOH-d$_4$)



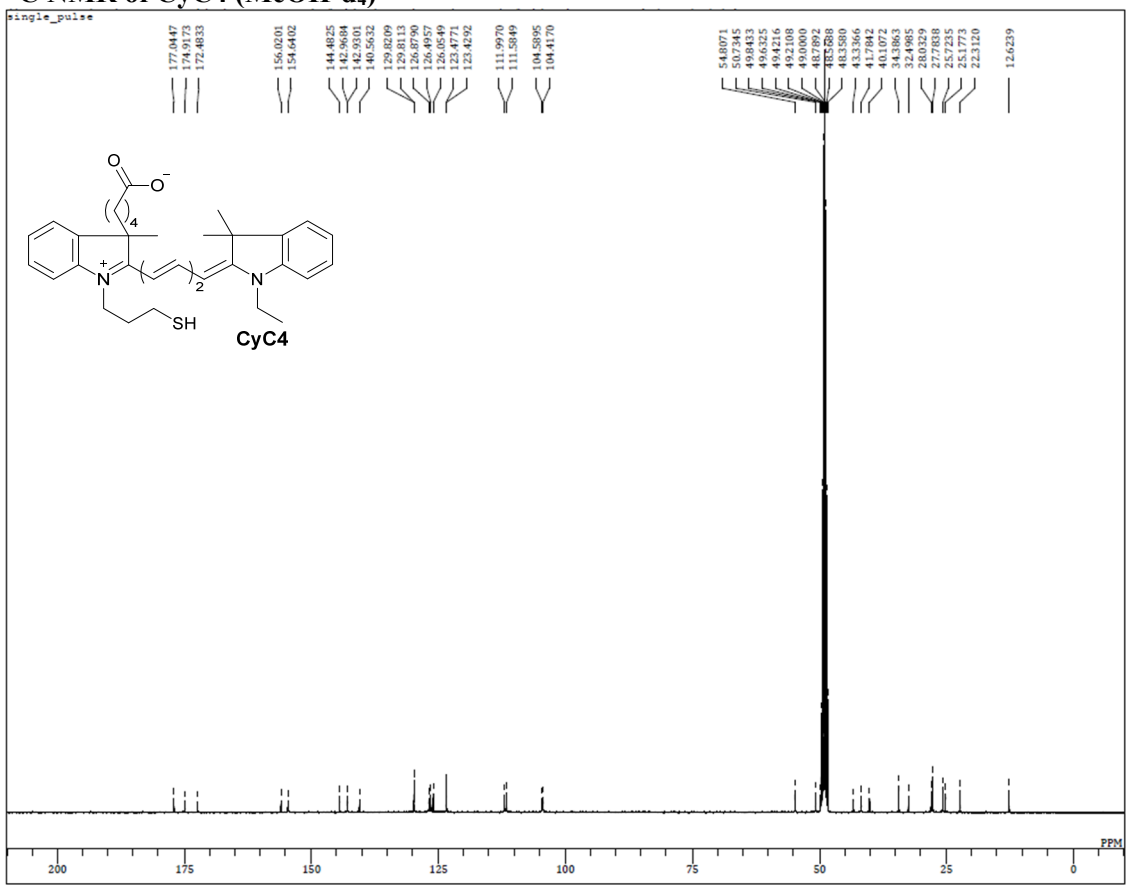
^{13}CNMR of proCyNCPh (MeOH-d$_4$)



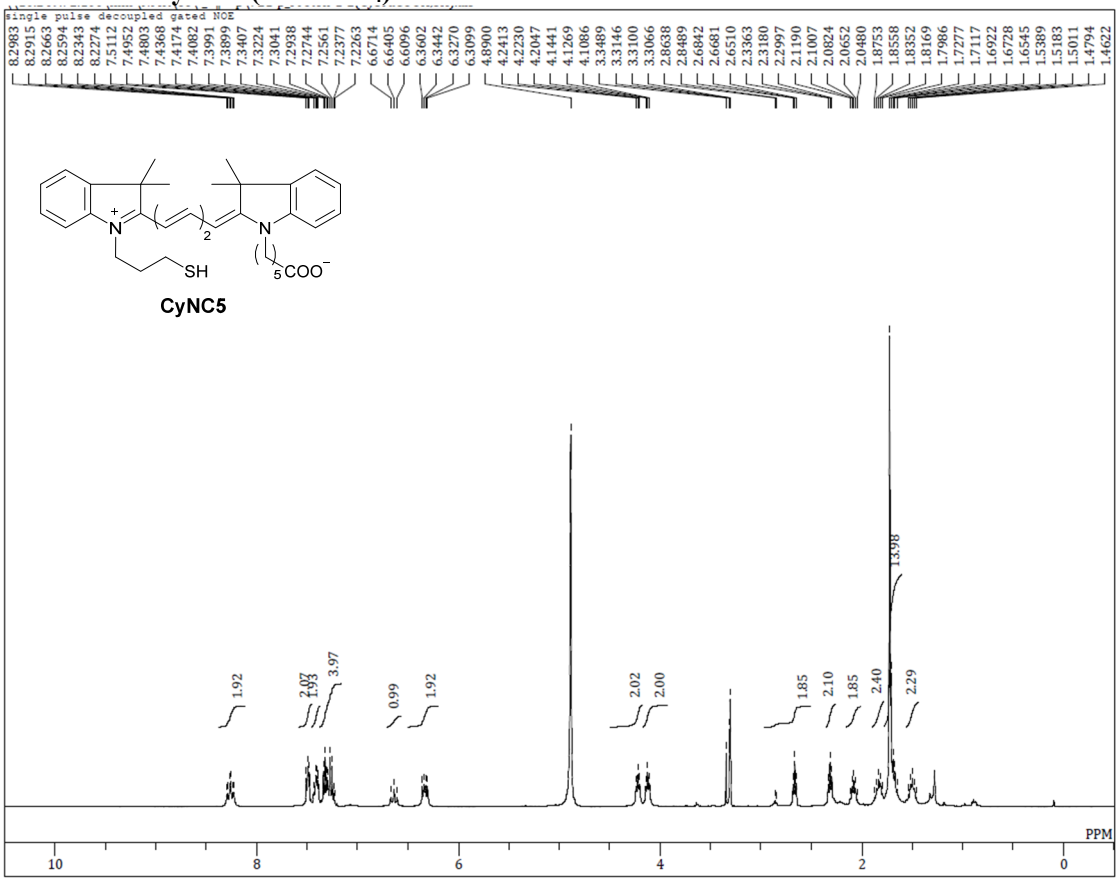
^{1}H NMR of CyC4 (MeOH-d$_4$)



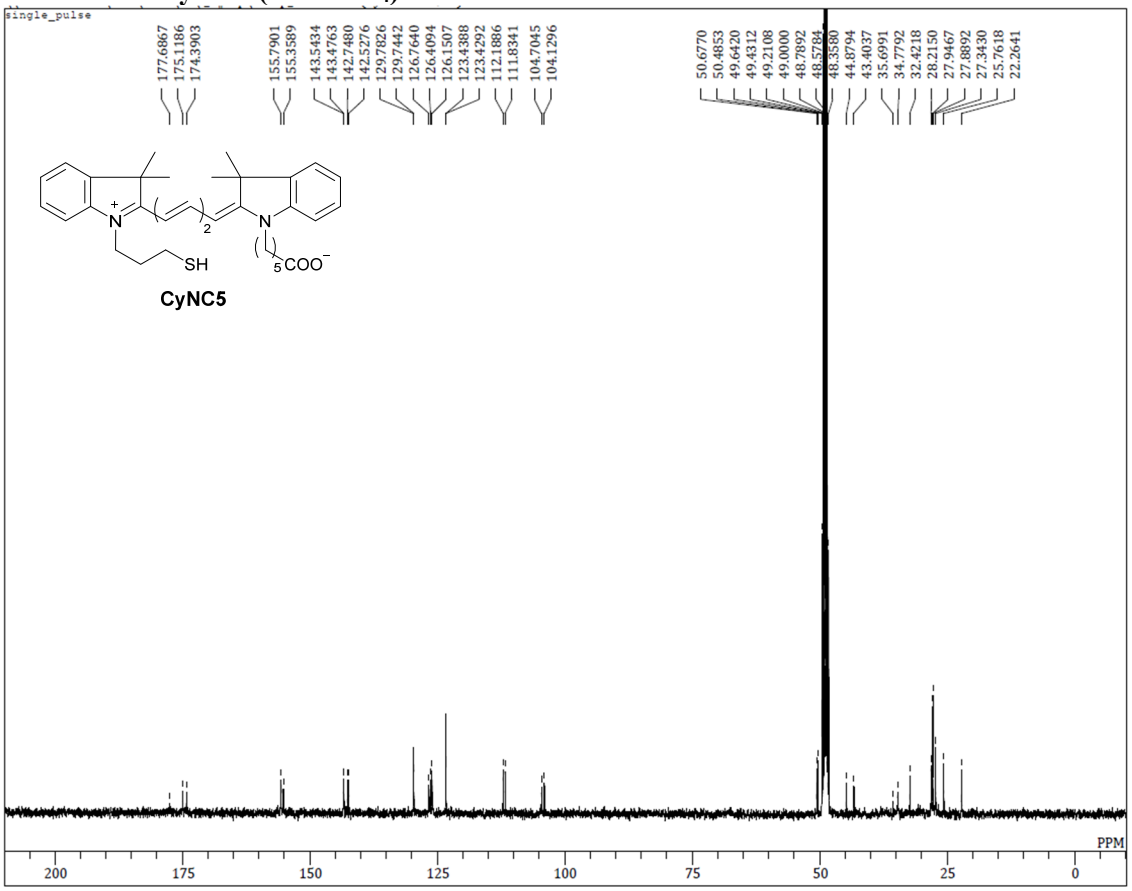
^{13}C NMR of CyC4 (MeOH-d$_4$)



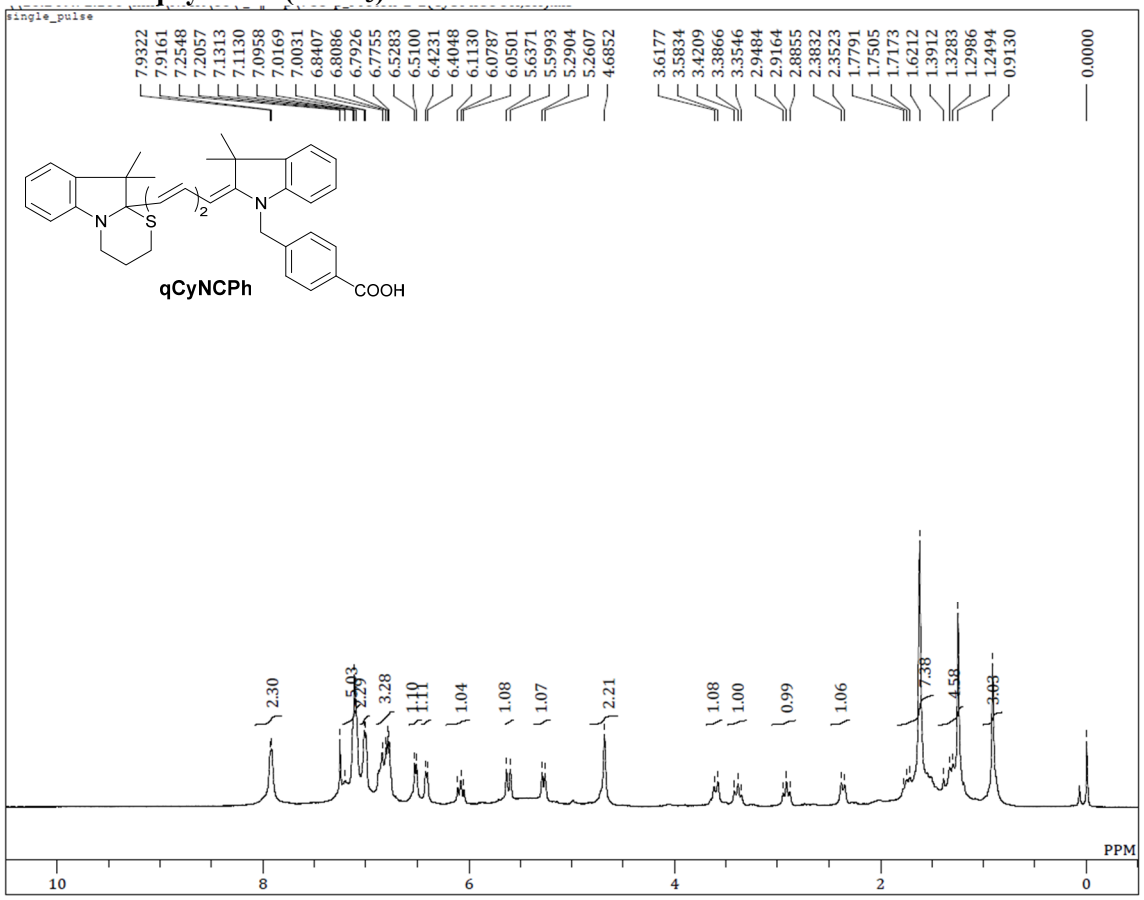
^{1}H NMR of CyNC5 (MeOH-d$_4$)



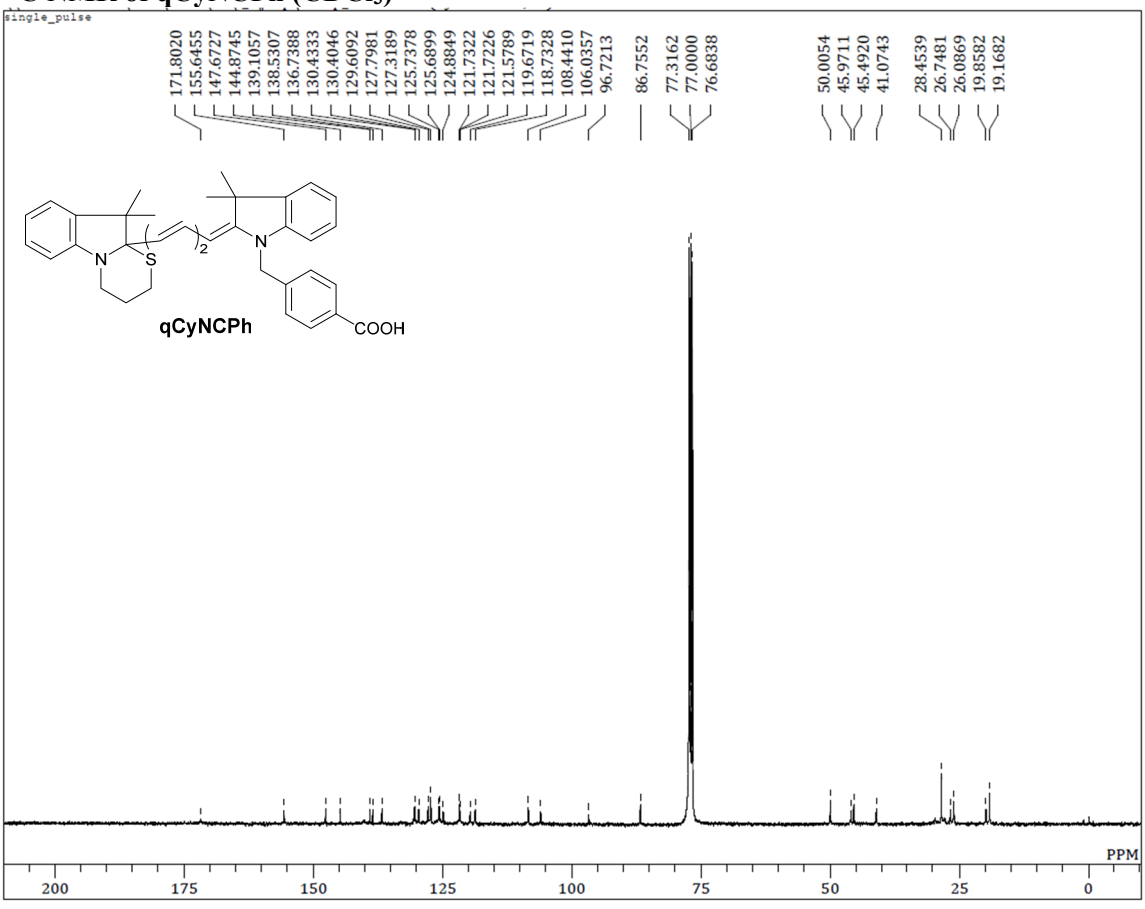
^{13}C NMR of CyNC5 (MeOH-d$_4$)



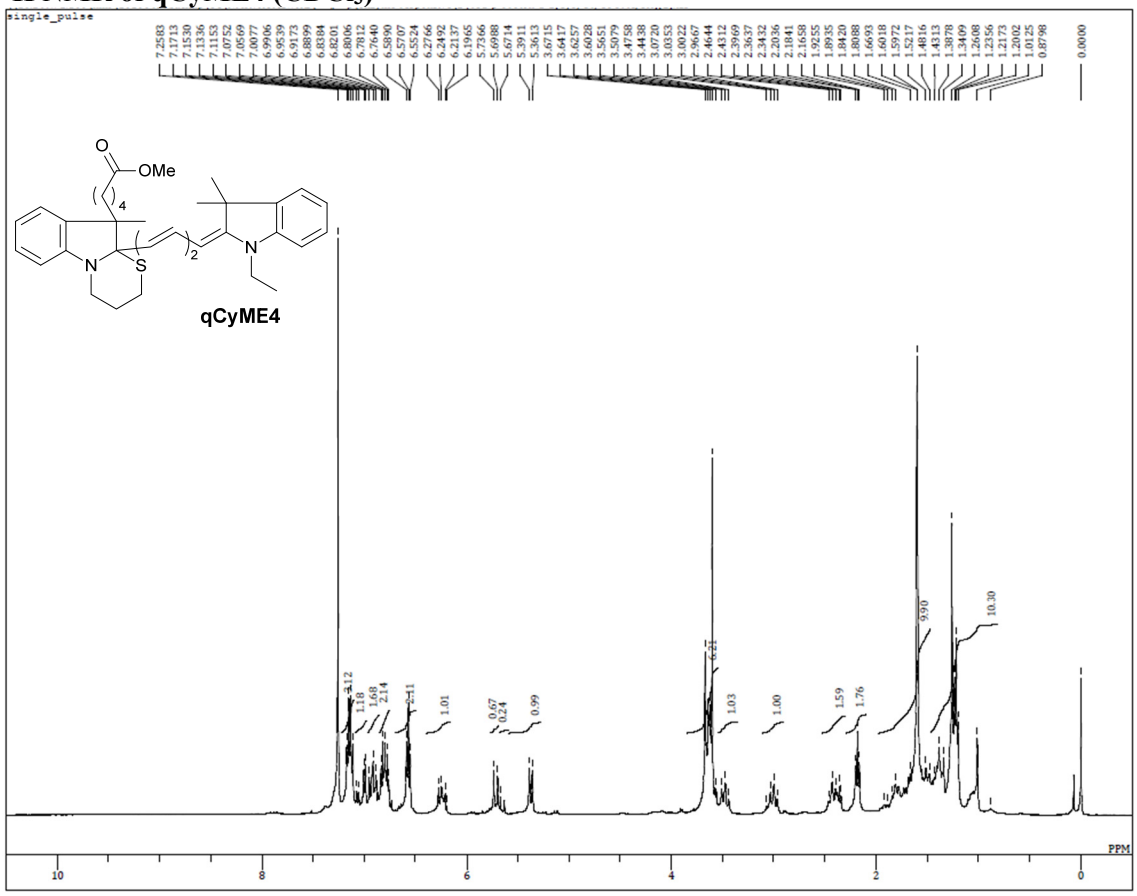
^{1}H NMR of qCyNCPh ($CDCl_3$)



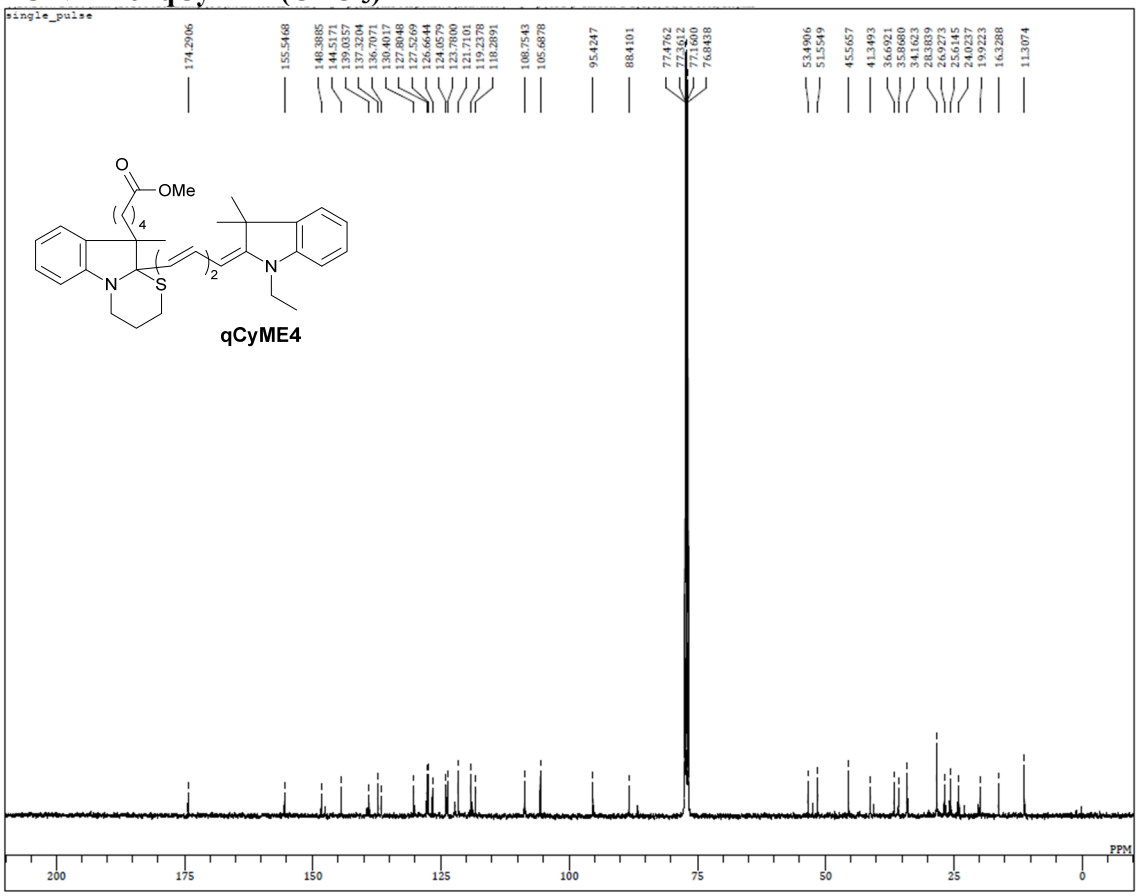
^{13}C NMR of qCyNCPh ($CDCl_3$)



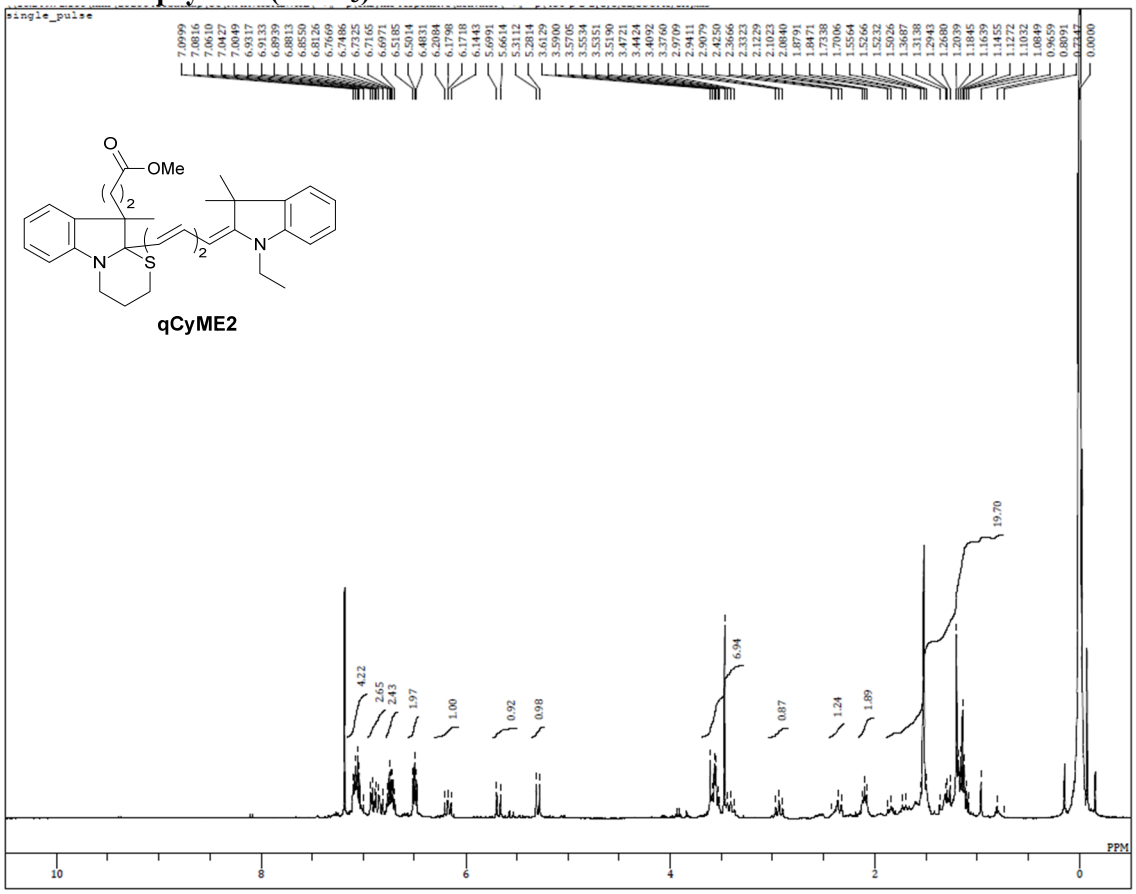
^{1}H NMR of qCyME4 ($CDCl_3$)



^{13}C NMR of qCyME4 ($CDCl_3$)



^{1}H NMR of qCyME2 ($CDCl_3$)



^{13}C NMR of qCyME2 ($CDCl_3$)