Supporting Information

Superior Fluorogen-Activating Protein Probes Based on 3-Indole-Malachite Green

Qingyang Zhang[†, ‡, ǁ], Qinghua Wang[†, ‡, ǁ], Ying Sun[‡], Limin Zuo[§], Verena Fetz[⊥] and Hai-Yu Hu[*, †, ‡]

[†]State Key Laboratory of Bioactive Substances and Function of Natural Medicine, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, 100050, China. haiyu.hu@imm.ac.cn.

[‡]Beijing Key Laboratory of Active Substances Discovery and Drugability Evaluation, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100050, China.

[§]Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100050, China.

[⊥]Department of Chemical Biology, Helmholtz-Zentrum für Infektionsforschung (HZI), Inhoffenstrasse 7, 38124 Braunschweig, Germany

[ǁ]Q. Zhang and Q. Wang contributed equally.

Contents

Abbreviations

BSA= Bovine serum albumin

DCM = Dichloromethane

DMF = Dimethylformamide

DMEM = Dulbecco's modified Eagle's medium

DMSO = Dimethyl Sulfoxide

EA = Ethyl Acetate

ESI-MS = Electrospray Ionisation Mass Spectrometry

equiv = equivalents

FAP = fluoregen activating protein

FBS = Fetal Bovine Serum

GFP = Green Fluorescent Protein

HOMO = Highest Occupied Molecular Orbitals

HPLC = High Performance Liquid Chromatography

HRMS = High Resolution Mass Spectrometry

LB = Luria-Bertani

LC-MS = Liquid Chromatography Mass Spectrometry

LUMO = Lowest Unoccupied Molecular Orbital

MG = Malachite Green

MS = Mass Spectrometry

MTS = 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NMR = Nuclear Magnetic Resonance

NHS = *N*-hydroxysuccinimide

OD = Optical Density

PBS = Phosphate Buffered Saline

PE = Petroleum Ether

rt = Room Temperature

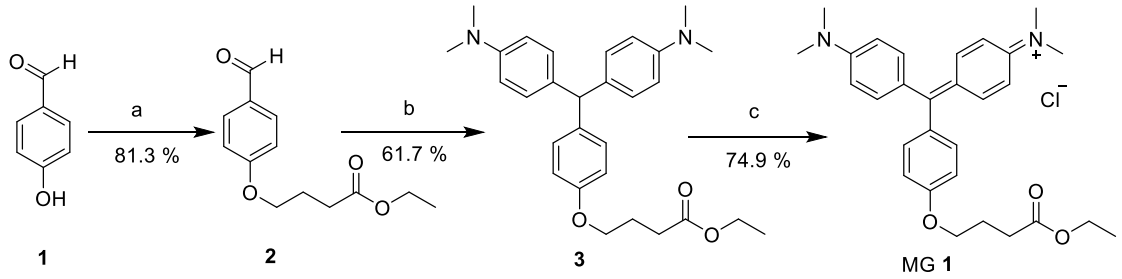
TD-DFT = Time-Dependent Density Functional Theory

TSB = Tryptic Soy Broth

General methods

All the chemicals were purchased from J&K. Commercially available reagents were used without further purification. The synthesis of MGs was according to the previous literature.[1] Fluorescence emission spectra and full wavelength absorption spectra were performed on 2300 EnSpire multimode plate reader. OD values in MTS assays were also measured by 2300 EnSpire multimode plate reader. The confocal laser scanning microscopic imaging studies were conducted with ZEISS LSM 800 Confocal Microscope. Fluorescence quantum yield was measured with Edinburgh photonics FLS980. All ^{1}H NMR spectra were recorded at 400 MHz or 500 MHz, respectively. ^{13}C NMR spectra were recorded at 125 MHz or 150 MHz, respectively. MS was measured with Thermo LCQ Deca XP Max mass spectrometer for ESI.

Synthetic procedures and characterized data



Scheme S1. Synthesis of MG **1**. Reagents and conditions: (a) ethyl 4-bromobutanoate, K_2CO_3, DMF, 80 °C, 2 h; (b) *N*,*N*-dimethylaniline, $ZnCl_2$, 100 °C, overnight; (c) tetrachloro-*p*-benzoquinone, ethyl acetate, 80 °C, 8 h.

Compound **2**

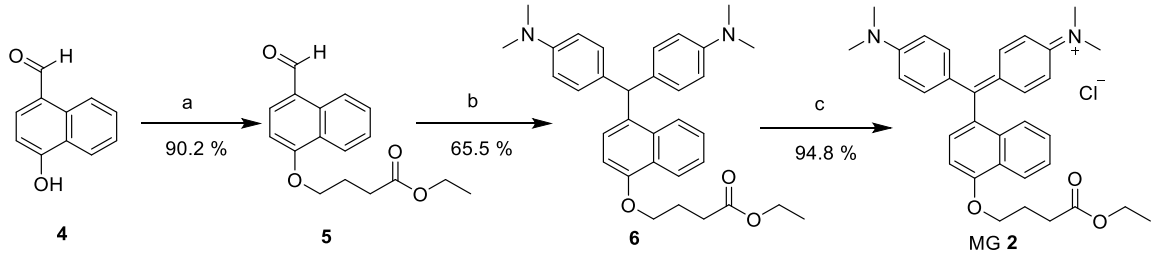
A mixture of compound **1** (200 mg) and K_2CO_3 (453 mg) was stirred in anhydrous DMF (10 mL). Ethyl-4-bromobutyrate (383 mg) was dropwise added and the mixture was stirred at 80 ℃ for 2h. After cooling, the salts were filtered and DMF was evaporated to dryness. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 6 : 1) to give yellow oil 314 mg, yield 81.3 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.82 (s, 1H, -C*H*O), 7.77 (d, J = 8.8 Hz, 2H, -Ar), 6.94 (d, J = 8.8 Hz, 2H, -Ar), 4.13–4.04 (m, 4H, -CH_2-), 2.48 (t, J = 7.2 Hz, 2H, -CH_2-), 2.13–2.06 (m, 2H, -CH_2-), 1.21 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 190.90, 173.07, 163.95, 132.33, 132.06, 130.00, 114.81, 114.22, 67.20, 60.62, 30.67, 24.48, 14.29. HRMS (*m*/*z*) (M+H): calcd. for $C_{13}H_{17}O_4$ 237.1121, found 237.1115.

Compound **3**

A mixture of compound **2** (244 mg), anhydrous $ZnCl_2$ (281 mg), *N*, *N*-dimethylaniline (375 mg) and anhydrous EtOH (10 mL) was stirred at 100 °C overnight. After cooling to rt, the mixture was concentrated to remove remainder *N*, *N*-dimethylaniline. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 10 : 1) to give yellow oil 293 mg, yield 61.7 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.03 (d, J = 8.4 Hz, 2H, -Ar), 6.99–6.97 (m, 4H, -Ar), 6.79 (d, J = 8.8 Hz, 2H, -Ar), 6.69–6.67 (m, 4H, -Ar), 5.33 (s, 1H, -C*H*-), 4.15 (dd, J_1 = 14.4 Hz, J_2 = 7.2 Hz, 2H, -CH_2-), 3.98 (t, J = 6.0 Hz, 2H, -CH_2-), 2.92 (s, 12H, -CH_3), 2.51 (t, J = 7.2 Hz, 2H, -CH_2-), 2.11 – 2.08 (m, 2H, -CH_2-), 1.26 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 173.43, 157.13, 130.35, 130.07, 116.15, 115.71, 114.88, 114.19, 112.97, 66.79, 60.56, 54.33, 41.15, 31.01, 24.86, 14.38. HRMS (*m*/*z*) (M+H): calcd. for $C_{29}H_{36}O_3N_2$ 461.2799, found 461.2794.

MG **1**

Compound **3** (193 mg) was dissolved in EA (25 mL). Tetrachloro-*p*-benzoquinone (123 mg) was added and the mixture was refluxed for 5 h. After cooling to rt, the solid was filtered from the reaction mixture and washed with EA (3 × 25 mL). The solid was purified by silica gel column chromatography (DCM : MeOH= 10 : 1) to give solid 155 mg, yield 74.9 %. m.p.: 151-153 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.36 (d, J = 8.8 Hz, 4H, -Ar), 7.30 (d, J = 8.8 Hz, 2H, -Ar), 7.06 (d, J = 8.8 Hz, 2H, -Ar), 6.95 (d, J = 9.2 Hz, 4H, -Ar), 4.19–4.14 (m, 4H, -CH_2-), 3.56 (s, 12H, -CH_3), 2.56 (t, J = 7.2 Hz, 2H, -CH_2-), 2.22–2.17 (m, 2H, -CH_2-), 1.28 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (125 MHz, Chloroform-*d*) δ 178.03, 173.24, 164.23, 156.81, 143.70, 140.92, 137.93, 131.93, 127.44, 119.57, 115.11, 113.82, 67.70, 60.83, 41.36, 30.80, 24.58, 14.47. HRMS (*m*/*z*) (M^+): calcd. for $C_{29}H_{35}O_3N_2^+$ 459.2642, found 459.2631.



Scheme S2. Synthesis of MG **2**. Reagents and conditions: (a) ethyl 4-bromobutanoate, K_2CO_3, DMF, 80 °C, 2 h; (b) *N*, *N*-dimethylaniline, $ZnCl_2$, 100 °C, overnight; (c) tetrachloro-*p*-benzoquinone, ethyl acetate, 80 °C, 8 h.

Compound **5**

A mixture of compound **4** (400 mg) and K_2CO_3 (643 mg) was stirred in anhydrous DMF (20 mL). Ethyl-4-bromobutyrate (907 mg) was dropwise added and the mixture was stirred at 80 °C for 2h. After cooling, the salts were filtered and DMF was evaporated to dryness. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 6 : 1) to give white solid 600 mg, yield 90.2 %. m.p.: 72-74 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.20 (s, 1H, -C*H*O), 9.30 (d, *J* = 8.4 Hz, 1H, -Ar), 8.33 (d, *J* = 8.4 Hz, 1H, -Ar), 7.91 (d, *J* = 8.8 Hz, 1H, -Ar), 7.70 (t, *J* = 7.6 Hz, 1H, -Ar), 7.58 (t, *J* = 7.6 Hz, 1H, -Ar), 6.92 (d, *J* = 8.0 Hz, 1H, -Ar), 4.31 (t, *J* = 6.0 Hz, 2H, -CH_2-), 4.19–4.14 (m, 2H, -CH_2-), 2.64 (t, *J* = 8.0 Hz, 2H, -CH_2-), 2.34–2.28 (m, 2H, -CH_2-), 1.26 (t, *J* = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 192.23, 172.94, 159.91, 139.60, 131.89, 129.52, 126.35, 125.47, 124.97, 124.86, 122.30, 103.56, 67.55, 60.59, 30.82, 24.40, 14.19. HRMS (*m*/*z*) (M+H): calcd. for $C_{17}H_{19}O_4$ 287.1278, found 287.1278.

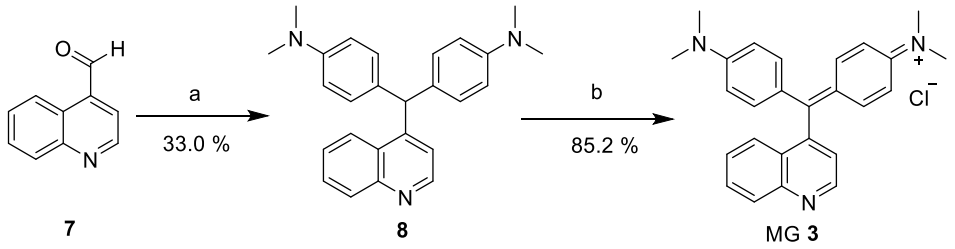
Compound **6**

A mixture of compound **5** (200 mg), anhydrous $ZnCl_2$ (200 mg), *N*, *N*-dimethylaniline (254 mg) and anhydrous EtOH (10 mL) was stirred at 100 °C overnight. After cooling to rt, the mixture was concentrated to remove remainder *N*, *N*-dimethylaniline. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 10 : 1) to give yellow oil 234 mg, yield 65.5 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.28 (d, *J* = 9.2 Hz, 1H, -Ar), 7.96 (d, *J* = 8.4 Hz, 1H, -Ar), 7.43–7.37 (m, 2H, -Ar), 6.97 (d, *J* = 8.4 Hz, 4H, -Ar), 6.84 (d, *J* = 8.0 Hz, 2H, -Ar), 6.66 (d, *J* = 8.4 Hz, 4H, -Ar), 6.00 (s, 1H, -C*H*-), 4.18–4.13 (m, 4H, -CH_2-), 2.91 (s, 12H, -CH_3), 2.62 (t, *J* = 7.2 Hz, 2H, -CH_2-), 2.28–2.21 (m, 2H, -CH_2-), 1.26 (t, *J* = 7.2 Hz, 3H, -CH_3). HRMS (*m*/*z*) (M+H): calcd. for $C_{33}H_{39}O_3N_2$ 511.2961, found 511.2955.

MG **2**

Compound **6** (127 mg) was dissolved in EA (25 mL). Tetrachloro-*p*-benzoquinone (123 mg) was added and the mixture was refluxed for 5 h. After cooling to rt, the solid was filtered from the reaction mixture and washed with EA (3 × 25 mL). The solid was purified by silica gel column chromatography (DCM : MeOH= 10 : 1) to give solid 120 mg, yield 94.8 %. m.p.: 157-159 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.35 (d, *J* = 8.4 Hz, 1H, -Ar), 7.47 (d, *J* = 8.0 Hz, 1H, -Ar), 7.38–7.34 (m, 4H, -Ar), 7.33–7.29 (m, 2H, -Ar), 6.97–6.95 (m, 2H, -Ar), 6.91 (d, *J* = 8.8 Hz, 4H, -Ar), 4.35

(t, J = 6.0Hz, 2H, -CH_2-), 4.21–4.16 (m, 2H, -CH_2-), 3.35 (s, 12H, -CH_3), 2.66 (t, J = 7.2 Hz, 2H, -CH_2-), 2.37–2.31 (m, 2H, -CH_2-), 1.28 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 176.0, 173.0, 158.9, 156.8, 143.5, 140.2, 136.0, 134.9, 129.9, 128.5, 128.1, 126.1, 125.9, 125.6, 122.6, 119.3, 113.8, 104.2, 67.7, 60.6, 41.1, 30.9, 24.5, 14.2. HRMS (*m*/*z*) (M^+): calcd. for $C_{33}H_{37}O_3N_2^+$ 509.2799, found 509.2796.



Scheme S3. Synthesis of MG **3**. Reagents and conditions: (a) *N*, *N*-dimethylaniline, $ZnCl_2$, 100 °C, overnight; (b) tetrachloro-*p*-benzoquinone, ethyl acetate, 80 °C, 8 h.

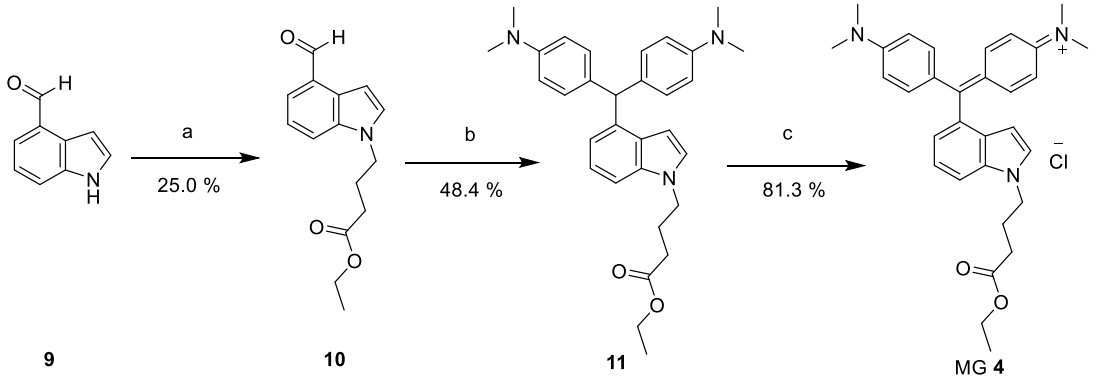
Compound **8**

A mixture of compound **7** (200 mg), anhydrous $ZnCl_2$ (346 mg), *N*, *N*-dimethylaniline (462 mg) and anhydrous EtOH (10 mL) was stirred at 100 °C overnight. After cooling to rt, the mixture was concentrated to remove remainder *N*, *N*-dimethylaniline. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 10 : 1) to give solid 160 mg, yield 33.0 %. m.p.: 174-176 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.79 (d, J = 4.8 Hz, 1H, -Ar), 8.14–8.12 (m, 1H, -Ar), 8.06–8.03 (m, 1H, -Ar), 7.67–7.63 (m, 1H, -Ar), 7.47–7.42 (m, 1H, -Ar), 6.98–6.94 (m, 5H, -Ar), 6.68–6.65 (m, 4H, -Ar), 6.08 (s, 1H, -C*H*-), 2.93 (s, 12H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 149.63, 149.16, 147.61, 130.64, 129.98, 129.47, 129.05, 127.55, 126.61, 124.53, 121.94, 112.57, 50.93, 40.58. HRMS (*m*/*z*) (M+H): calcd. for $C_{26}H_{28}N_3$ 382.2283, found 382.2266.

MG **3**

Compound **8** (81 mg) was dissolved in EA (25 mL). Tetrachloro-*p*-benzoquinone (62 mg) was added and the mixture was refluxed for 5 h. After cooling to rt, the solid was filtered from the reaction mixture and washed with EA (3 × 25 mL). The solid was purified by silica gel column chromatography (DCM : MeOH= 10 : 1) to give solid 75 mg, yield 85.2 %. m.p.: 128-130 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.06–9.05 (m, 1H, -Ar), 8.25 (d, J = 8.5 Hz, 1H, -Ar), 7.78–7.75 (m, 1H, -Ar), 7.48– 7.46 (m, 1H, -Ar), 7.45–7.38 (m, 4H, -Ar), 7.31–7.30 (m, 2H, -Ar), 6.99–6.97 (m,

4H, -Ar), 3.40 (s, 12H, -CH_3). ^{13}C NMR (125 MHz, Chloroform-*d*) δ 169.12, 157.22, 149.55, 148.45, 145.83, 143.86, 139.92, 137.60, 130.53, 130.20, 128.33, 127.68, 127.39, 125.75, 124.13, 119.84, 114.94, 112.89, 40.61. HRMS (*m*/*z*) (M^+): calcd. for $C_{26}H_{26}N_3^+$ 380.2121, found 380.2121.



Scheme S4. Synthesis of MG **4**. Reagents and conditions: (a) ethyl 4-bromobutanoate, K_2CO_3, DMF, 80 °C, 2 h; (b) *N*, *N*-dimethylaniline, $ZnCl_2$, 100 °C,overnight; (c) tetrachloro-*p*-benzoquinone, ethyl acetate, 80 °C, 8 h.

Compound **10**

A mixture of compound **9** (200 mg) and K_2CO_3 (383 mg) was stirred in anhydrous DMF (20 mL). Ethyl-4-bromobutyrate (325 mg) was dropwie added and the mixtrure was stirred at 80 °C for 2h. After cooling, the salts were filtered and DMF was evaporated to dryness. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 6 : 1) to give yellow oil 90 mg, yield 25.0 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.25 (s, 1H, -C*H*O), 7.66–7.62 (m, 2H, -Ar), 7.37–7.33 (m, 1H, -Ar), 7.30 (s, 2H, -Ar), 4.28 (t, *J* = 6.8 Hz, 2H, -CH_2-), 4.15–4.10 (m, 2H, -CH_2-), 2.28 (t, *J* = 6.8 Hz, 2H, -CH_2-), 2.20–2.13 (m, 2H, -CH_2-), 1.24 (t, *J* = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 193.04, 172.59, 136.66, 131.19, 128.52, 127.20, 126.32, 120.91, 115.61, 101.89, 60.64, 45.45, 30.87, 25.54, 14.17. HRMS (*m*/*z*) (M+H): calcd. for $C_{15}H_{18}O_3N$ 260.1281, found 260.1277.

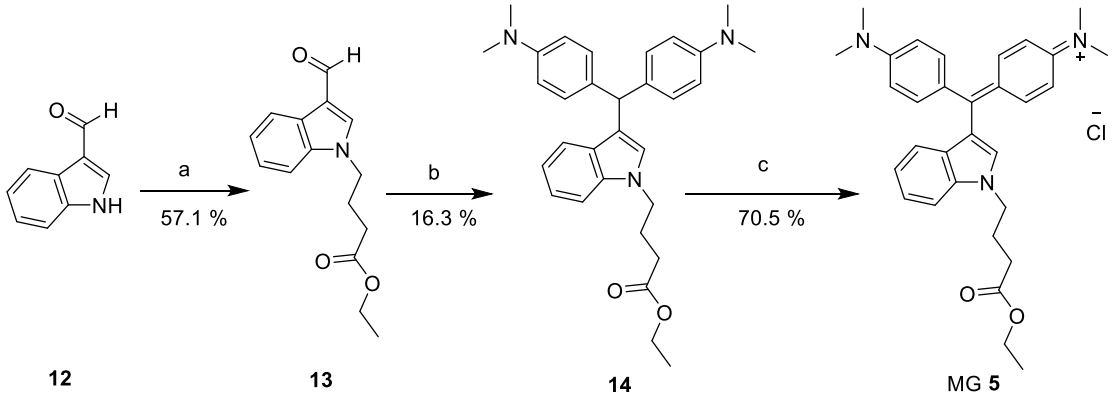
Compound **11**

A mixture of compound **10** (50 mg), anhydrous $ZnCl_2$ (53 mg), *N*, *N*-dimethylaniline (70 mg) and anhydrous EtOH (10 mL) was stirred at 100 °C overnight. After cooling to rt, the mixture was concentrated to remove remainder *N*, *N*-dimethylaniline. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 10 : 1) to give yellow oil 45 mg, yield 48.4 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.21 (d, *J* = 8.0 Hz, 1H, -Ar), 7.13–7.09 (m, 1H, -

Ar), 7.06–7.04 (m, 4H, -Ar), 6.97 (d, J = 4.8 Hz, 1H, -Ar), 6.69–6.67 (m, 5H, -Ar), 6.34–6.33 (m, 1H, -Ar), 5.77 (s, 1H, -C*H*-), 4.18–4.10 (m, 4H, -CH_2-), 2.91 (s, 12H, -CH_3), 2.32–2.28 (m, 2H, -CH_2-), 2.17–2.14 (m, 2H, -CH_2-), 1.25 (t, J = 7.2 Hz, 3H, -CH_3). HRMS (*m*/*z*) (M+H): calcd. for $C_{31}H_{38}O_2N_3$ 484.2964, found 484.2942.

MG **4**

Compound **11** (30 mg) was dissolved in EA (15 mL). Tetrachloro-*p*-benzoquinone (18 mg) was added and the mixture was refluxed for 5 h. After cooling to rt, the solid was filtered from the reaction mixture and washed with EA (3 × 25 mL). The solid was purified by silica gel column chromatography (DCM : MeOH= 10 : 1) to give solid 26 mg, yield 81.3 %. m.p.: 72-74 ℃. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.68 (d, J = 8.0 Hz, 1H, -Ar), 7.40 (d, J = 8.8 Hz, 4H, -Ar), 7.35–7.31 (m, 1H, -Ar), 7.14–7.13 (m, 1H, -Ar), 7.06–7.04 (m, 1H, -Ar), 6.90 (d, J = 8.8 Hz, 4H, -Ar), 5.77 (d, J = 2.8 Hz, 1H, -Ar), 4.27 (t, J = 7.2 Hz, 2H, -CH_2-), 4.18–4.11 (m, 2H, -CH_2-), 3.35 (s, 12H, -CH_3), 2.38–2.34 (m, 2H, -CH_2-), 2.22–2.18 (m, 2H, -CH_2-), 1.27 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (125 MHz, Chloroform-*d*) δ 177.89, 172.95, 156.89, 140.92, 136.38, 131.84, 131.78, 130.60, 128.71, 127.86, 121.38, 115.05, 113.67, 101.85, 60.95, 45.89, 41.36, 31.28, 25.71, 14.46. HRMS (*m*/*z*) (M^+): calcd. for $C_{31}H_{36}O_2N_3^+$ 482.2802, found 482.2788.



Scheme S5. Synthesis of MG **5**. Reagents and conditions: (a) ethyl 4-bromobutanoate, K_2CO_3, DMF, 80 °C, 2 h; (b) *N*, *N*-dimethylaniline, $ZnCl_2$, 100 °C,overnight; (c) tetrachloro-*p*-benzoquinone, ethyl acetate, 80 °C, 8 h.

Compound **13**

A mixture of compound **12** (200 mg) and K_2CO_3 (380 mg) was stirred in anhydrous DMF (20 mL). Ethyl-4-bromobutyrate (322 mg) was dropwise added and the mixture was stirred at 80 ℃ for 2h. After cooling, the salts were filtered and DMF was evaporated to dryness. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous

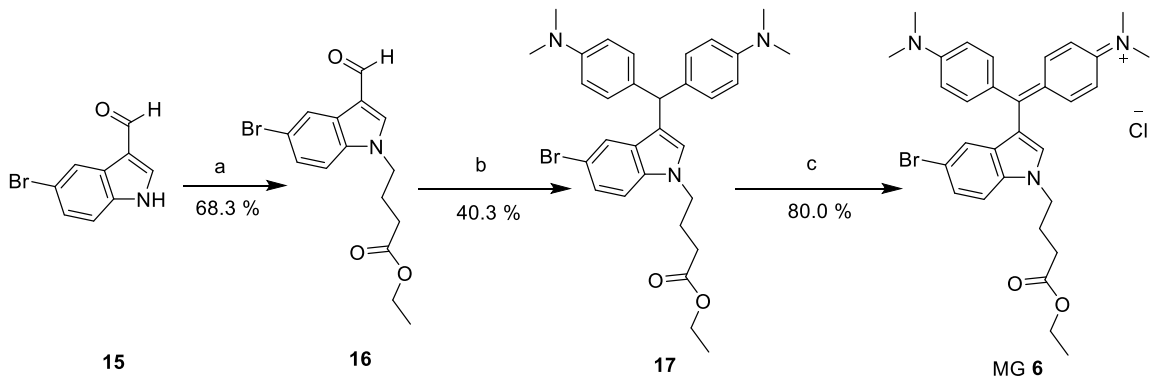
Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 6 : 1) to give yellow oil 204 mg, yield 57.1 %. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.98 (s, 1H, -C*H*O), 8.31–8.29 (m, 1H, -Ar), 7.71 (s, 1H, -Ar),7.42–7.35 (m, 1H, -Ar), 7.35–7.29 (m, 2H, -Ar), 4.26 (t, J = 7.0 Hz, 2H, -CH_2-), 4.13 (dd, J_1 = 14.5 Hz, J_2 = 7.0 Hz,2H, -CH_2-), 2.32 (t, J = 7.0 Hz, 2H, -CH_2-), 2.23–2.17 (m, 2H, -CH_2-), 1.24 (t, J = 7.0 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 184.49, 172.35, 138.31, 137.11, 125.39, 124.06, 122.94, 122.14, 118.24, 110.04, 60.75, 46.14, 30.75, 24.94, 14.17. HRMS (*m*/*z*) (M+H): calcd. for $C_{15}H_{18}O_3N$ 260.1281, found 260.1276.

Compound **14**

A mixture of compound **13** (168 mg), anhydrous $ZnCl_2$ (177 mg), *N*, *N*-dimethylaniline (235 mg) and anhydrous EtOH (20 mL) was stirred at 100 °C overnight. After cooling to rt, the mixture was concentrated to remove remainder *N*, *N*-dimethylaniline. The residue was extracted with water (40 mL) and ethyl acetate (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 10 : 1) to give yellow oil 51 mg, yield 16.3 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.31–7.24 (m, 1H, -Ar), 7.18–7.14 (m, 1H, -Ar), 7.10–7.08 (m, 3H, -Ar), 7.00–6.93 (m, 2H, -Ar), 6.71–6.68 (m, 4H, -Ar), 6.55–6.53 (m, 1H, -Ar), 6.46 (s, 1H, -Ar), 5.50 (s, 1H, -C*H*-), 4.13–4.08 (m, 4H, -CH_2-), 2.92 (s, 12H, -CH_3), 2.27–2.26 (m, 2H, -CH_2-), 2.12–2.08 (m, 2H, -CH_2-), 1.24 (t, J = 7.2 Hz, 3H, -CH_3). HRMS (*m*/*z*) (M+H): calcd. for $C_{31}H_{38}O_2N_3$ 484.2964, found 484.2946.

MG-**5**

Compound **14** (41 mg) was dissolved in EA (15 mL). Tetrachloro-*p*-benzoquinone (25 mg) was added and the mixture was refluxed for 5 h. After cooling to rt, the solid was filtered from the reaction mixture and washed with EA (3 × 25 mL). The solid was purified by silica gel column chromatography (DCM : MeOH= 10 : 1) to give solid 31 mg, yield 70.5 %. m.p.: 102-104 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.14 (s, 1H, -Ar), 7.62 (d, J = 9.2 Hz, 1H, -Ar), 7.47–7.45 (m, 4H, -Ar), 7.38–7.34 (m, 1H, -Ar), 7.18–7.12 (m, 2H, -Ar), 6.86–6.83 (m, 4H, -Ar), 4.66 (t, J = 7.2 Hz, 2H, -CH_2-), 4.12– 4.06 (m, 2H, -CH_2-), 3.25 (s, 12H, -CH_3), 2.54–2.50 (m, 2H, -CH_2-), 2.33–2.30 (m, 2H, -CH_2-), 1.21 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 172.80, 172.31, 155.65, 144.61, 139.20, 138.65, 128.58, 125.10, 123.72, 121.27, 119.18, 112.58, 111.81, 60.67, 47.22, 40.60, 30.99, 25.03, 14.16. HRMS (*m*/*z*) (M^+): calcd. for $C_{31}H_{36}O_2N_3^+$ 482.2802, found 482.2793.



Scheme S6. Synthesis of MG **6**. Reagents and conditions: (a) ethyl 4-bromobutanoate, K_2CO_3, DMF, 80 °C, 2 h; (b) *N*, *N*-dimethylaniline, $ZnCl_2$, 100 °C,overnight; (c) tetrachloro-*p*-benzoquinone, ethyl acetate, 80 °C, 8 h.

Compound **16**

A mixture of compound **15** (200 mg) and K_2CO_3 (380 mg) was stirred in anhydrous DMF (20 mL). Ethyl-4-bromobutyrate (322 mg) was dropwise added and the mixture was stirred at 80 °C for 2h. After cooling, the salts were filtered and DMF was evaporated to dryness. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 6 : 1) to give white solid 244 mg, yield 68.3 %. m.p.: 82-84 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.96 (s, 1H, -C*H*O), 8.46 (s, 1H, -Ar), 7.71 (s, 1H, -Ar), 7.44–7.41 (m, 1H, -Ar), 7.30–7.27 (m, 1H, -Ar), 4.26 (t, *J* = 7.2 Hz, 2H, -CH_2-), 4.17–4.11 (m, 2H, -CH_2-), 2.33 (t, *J* = 7.2 Hz, 2H, -CH_2-), 2.23–2.16 (m, 2H, -CH_2-), 1.26 (t, *J* = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 184.18, 172.25, 138.64, 135.77, 127.08, 126.84, 124.82, 117.64, 116.61, 111.47, 60.84, 46.32, 30.61, 24.93, 14.17. HRMS (*m*/*z*) (M+H): calcd. for $C_{15}H_{17}O_3NBr$ 338.0386, found 338.0399.

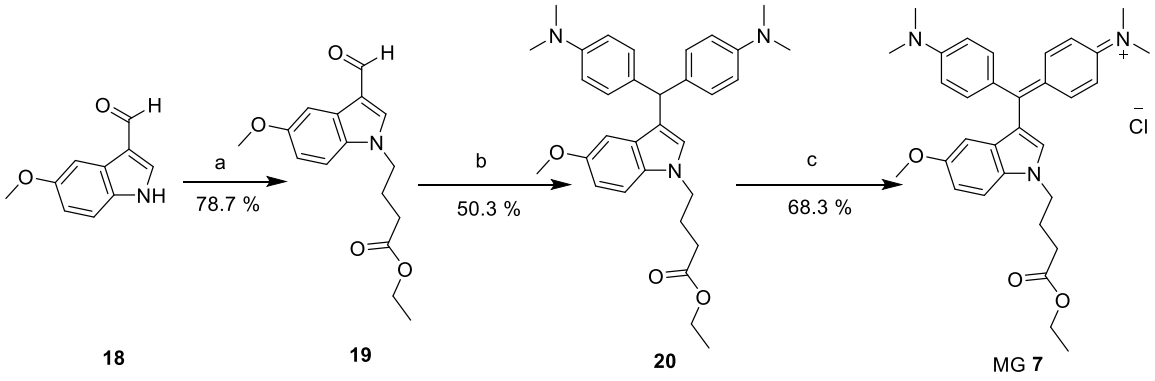
Compound **17**

A mixture of compound **16** (200 mg), anhydrous $ZnCl_2$ (210 mg), *N*, *N*-dimethylaniline (613 mg) and anhydrous EtOH (20 mL) was stirred at 100 °C overnight. After cooling to rt, the mixture was concentrated to remove remainder *N*, *N*-dimethylaniline. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 10 : 1) to give yellow oil 150 mg, yield 40.3 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.40 (s, 1H, -Ar), 7.25 (d, *J* = 8.0 Hz, 1H, -Ar), 7.17–7.15 (m, 1H, -Ar), 7.06–7.04 (m, 4H, -Ar), 6.68–6.66 (m, 4H, -Ar), 6.48 (s, 1H, -Ar), 5.43 (s, 1H, -C*H*-), 4.14–4.04 (m, 4H, -CH_2-), 2.92 (s, 12H, -CH_3), 2.25–2.22 (m, 2H, -CH_2-), 2.11–2.04 (m, 2H, -CH_2-), 1.24 (t, *J* = 7.2 Hz, 3H, -CH_3). HRMS (*m*/*z*) (M+H): calcd. for $C_{31}H_{37}O_2N_3Br$

562.2064, found 562.2043.

MG **6**

Compound **17** (70 mg) was dissolved in EA (15 mL). Tetrachloro-*p*-benzoquinone (25 mg) was added and the mixture was refluxed for 5 h. After cooling to rt, the solid was filtered from the reaction mixture and washed with EA (3 × 25 mL). The solid was purified by silica gel column chromatography (DCM : MeOH= 10 : 1) to give solid 60 mg, yield 80.0 %. m.p.: 128-130 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.21 (s, 1H, -Ar), 7.54 (d, J = 8.4 Hz, 1H, -Ar), 7.44–7.42 (m, 5H, -Ar), 7.22 (s, 1H, -Ar), 6.86–6.83 (m, 4H, -Ar), 4.67 (t, J = 7.6 Hz, 2H, -CH_2-), 4.10–4.05 (m, 2H, -CH_2-), 3.26 (s, 12H, -CH_3), 2.54–2.50 (m, 2H, -CH_2-), 2.30–2.26 (m, 2H, -CH_2-), 1.21 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 172.86, 171.70, 155.93, 144.89, 139.32, 137.30, 130.08, 127.79, 123.71, 119.71, 117.88, 117.12, 113.32, 112.74, 60.65, 47.28, 40.64, 30.90, 25.05, 14.16. HRMS (m/z) (M^+): calcd. for $C_{31}H_{35}O_2N_3Br^+$ 560.1907, found 560.1902.



Scheme S6. Synthesis of MG **6**. Reagents and conditions: (a) ethyl 4-bromobutanoate, K_2CO_3, DMF, 80 °C, 2 h; (b) *N*, *N*-dimethylaniline, $ZnCl_2$, 100 °C,overnight; (c) tetrachloro-*p*-benzoquinone, ethyl acetate, 80 °C, 8 h.

Compound **19**

A mixture of compound **18** (300 mg) and K_2CO_3 (473 mg) was stirred in anhydrous DMF (20 mL). Ethyl-4-bromobutyrate (401 mg) was dropwise added and the mixture was stirred at 80 °C for 2h. After cooling, the salts were filtered and DMF was evaporated to dryness. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 6 : 1) to give yellow oil 389 mg, yield 78.7 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.95 (s, 1H, -C*H*O), 7.79 (d, , J = 2.4 Hz, 1H, -Ar), 7.66 (s, 1H, -Ar), 7.30–7.27 (m, 1H, -Ar), 6.98–6.96 (m, 1H, -Ar), 4.23 (t, J = 6.8 Hz, 2H, -CH_2-), 4.16–4.11 (m, 2H, -CH_2-), 3.13 (s, 3H, -OCH_3), 2.32 (t, J = 7.6 Hz, 2H, -CH_2-),

2.23–2.16 (m, 2H, -CH_2-), 1.25 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 184.59, 172.51, 156.83, 138.50, 132.13, 126.31, 118.16, 114.75, 111.02, 103.54, 60.91, 55.95, 46.48, 30.85, 25.13, 14.31. HRMS (*m*/*z*) (M+H): calcd. for $C_{16}H_{20}O_4N$ 290.1387, found 290.1382.

Compound **20**

A mixture of compound **19** (286 mg), anhydrous $ZnCl_2$ (269 mg), *N*, *N*-dimethylaniline (263 mg) and anhydrous EtOH (20 mL) was stirred at 100 °C overnight. After cooling to rt, the mixture was concentrated to remove remainder *N*, *N*-dimethylaniline. The residue was extracted with water (40 mL) and EA (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the residue was purified by silica gel column chromatography (PE : EA = 10 : 1) to give yellow oil 255 mg, yield 50.3 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.23–7.16 (m, 1H, -Ar), 7.85 (d, J = 8.4 Hz, 4H, -Ar), 6.82–6.79 (m, 1H, -Ar), 6.69–6.67 (m, 5H, -Ar), 6.45 (s, 1H, -Ar), 5.43 (s, 1H, -C*H*-), 4.13–4.07 (m, 2H, -CH_2-), 4.05–4.01 (m, 2H, -CH_2-), 3.67 (s, 3H, -OCH_3), 2.90 (s, 12H, -CH_3), 2.22 (t, J = 6.8 Hz, 2H, -CH_2-), 2.10–2.05 (m, 2H, -CH_2-), 1.22 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 172.99, 153.74, 153.49, 148.96, 133.44, 132.18, 129.58, 128.37, 128.18, 128.16, 126.67, 119.54, 113.93, 112.92, 111.86, 111.53, 110.15, 109.92, 102.55, 101.27, 60.59, 55.98, 46.92, 45.41, 41.02, 31.23, 25.61, 14.33. HRMS (*m*/*z*) (M+H): calcd. for $C_{32}H_{40}O_3N_3$ 514.3064, found 514.3052.

MG **7**

Compound **20** (41 mg) was dissolved in EA (15 mL). Tetrachloro-*p*-benzoquinone (25 mg) was added and the mixture was refluxed for 5 h. After cooling to rt, the solid was filtered from the reaction mixture and washed with EA (3 × 25 mL). The solid was purified by silica gel column chromatography (DCM : MeOH= 10 : 1) to give solid 28 mg, yield 68.3 %. m.p.: 140-142 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.05 (s, 1H, -Ar), 7.55–7.46 (m, 5H, -Ar), 7.00–6.98 (m, 2H, -Ar), 6.97–6.84 (m, 4H, -Ar), 6.62 (s, 1H, -Ar),4.62 (t, J = 6.4 Hz, 2H, -CH_2-), 4.12–4.07 (m, 2H, -CH_2-), 3.64 (s, 3H, -OCH_3), 3.25 (s, 12H, -CH_3), 2.53–2.50 (m, 2H, -CH_2-), 2.32–2.28 (m, 2H, -CH_2-), 1.22 (t, J = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 172.93, 172.47, 157.23, 155.84, 144.93, 139.23, 133.73, 129.76, 119.34, 114.05, 112.84, 112.69, 112.57, 104.63, 60.82, 55.86, 47.59, 40.73, 31.08, 25.17, 14.32. HRMS (*m*/*z*) (M^+): calcd. for $C_{32}H_{38}O_3N_3^+$ 512.2908, found 512.2900.

Crystallography of FAP/MG complex

According to the literatures, Crystallography of FAP/MG complex reveals that MG binds FAP as left-handed propeller conformers with somewhat variable rotation angles along *C*2 axis (Figure S1).[2]

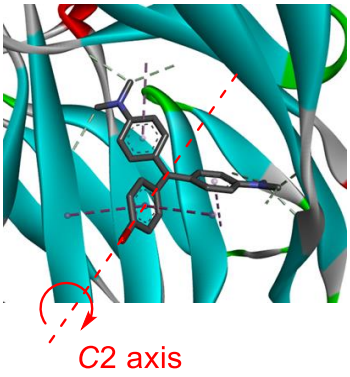


Figure S1. MG binding FAP. Red dotted line marks *C*2 symmetry axis that passes through ring C, and the central carbon. Ring C of MG as left-handed propeller conformers with somewhat variable rotation angles front view along *C*2 axis of MGs via the triarylmethane plane.

Spatial structures of MGs 1-7 calculated by TD-DFT

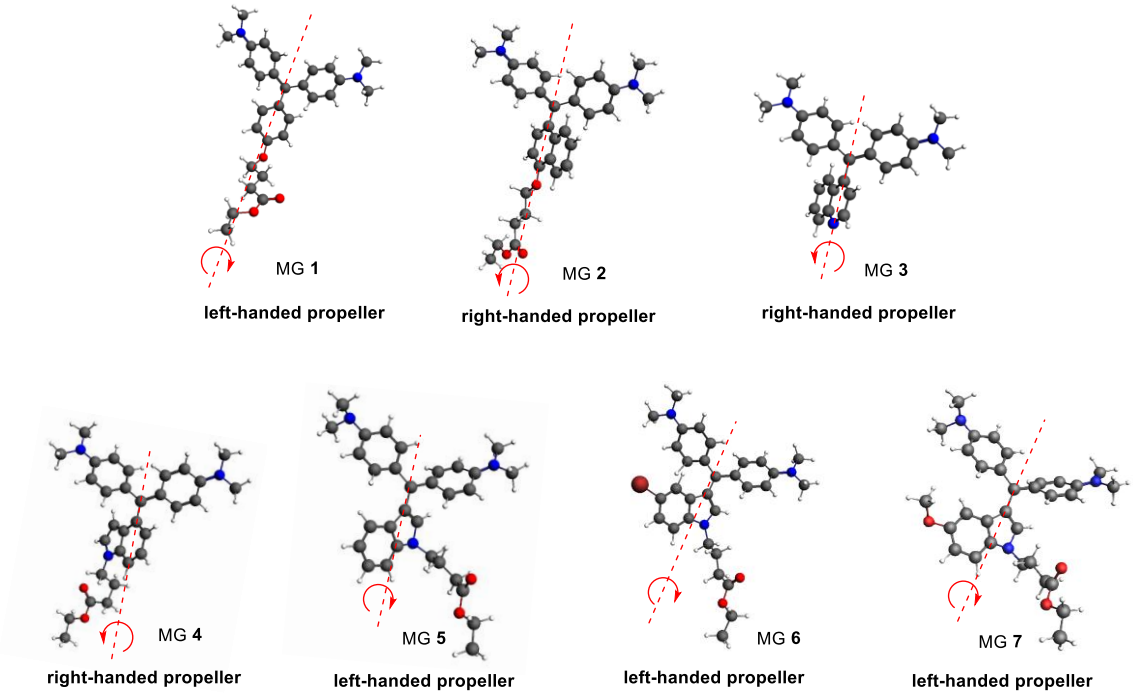


Figure S2. TD-DFT calculated that the ring C of MG **1**, MG **5**, MG **6**, and MG **7** were left-handed propeller, while MG **2**, MG **3**, MG **4** were right-handed propeller.

UV-vis absorption and fluorescence spectra

MGs **1-7** were each dissolved in DMSO to generate stock solutions of 10 mM, respectively. Immediately before UV-Vis measurement, aliquots of fluorogen stocks were diluted in PBS to obtain a final concentration of 10 μM. Wavelength interval: 5.0 nm.

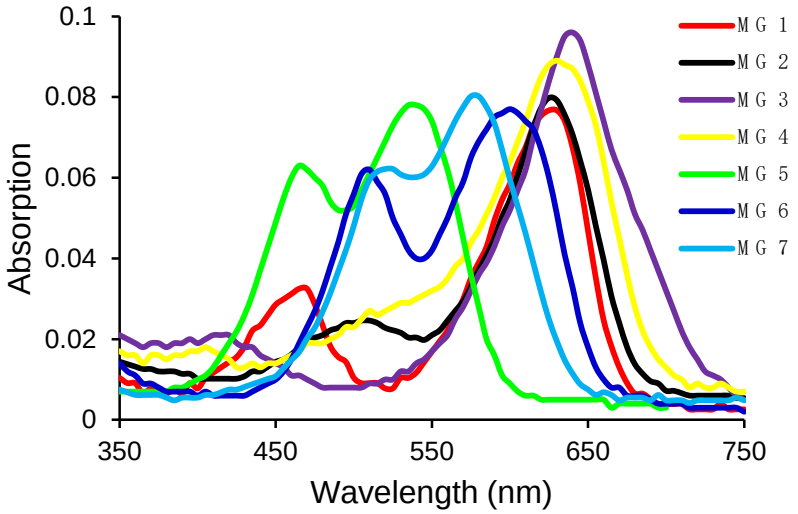


Figure S3. Absorption spectra of MGs **1-7**. The UV-Visible spectra of MGs **1-7** are shown in red, black, violet, yellow, green, blue and cerulean lines, respectively.

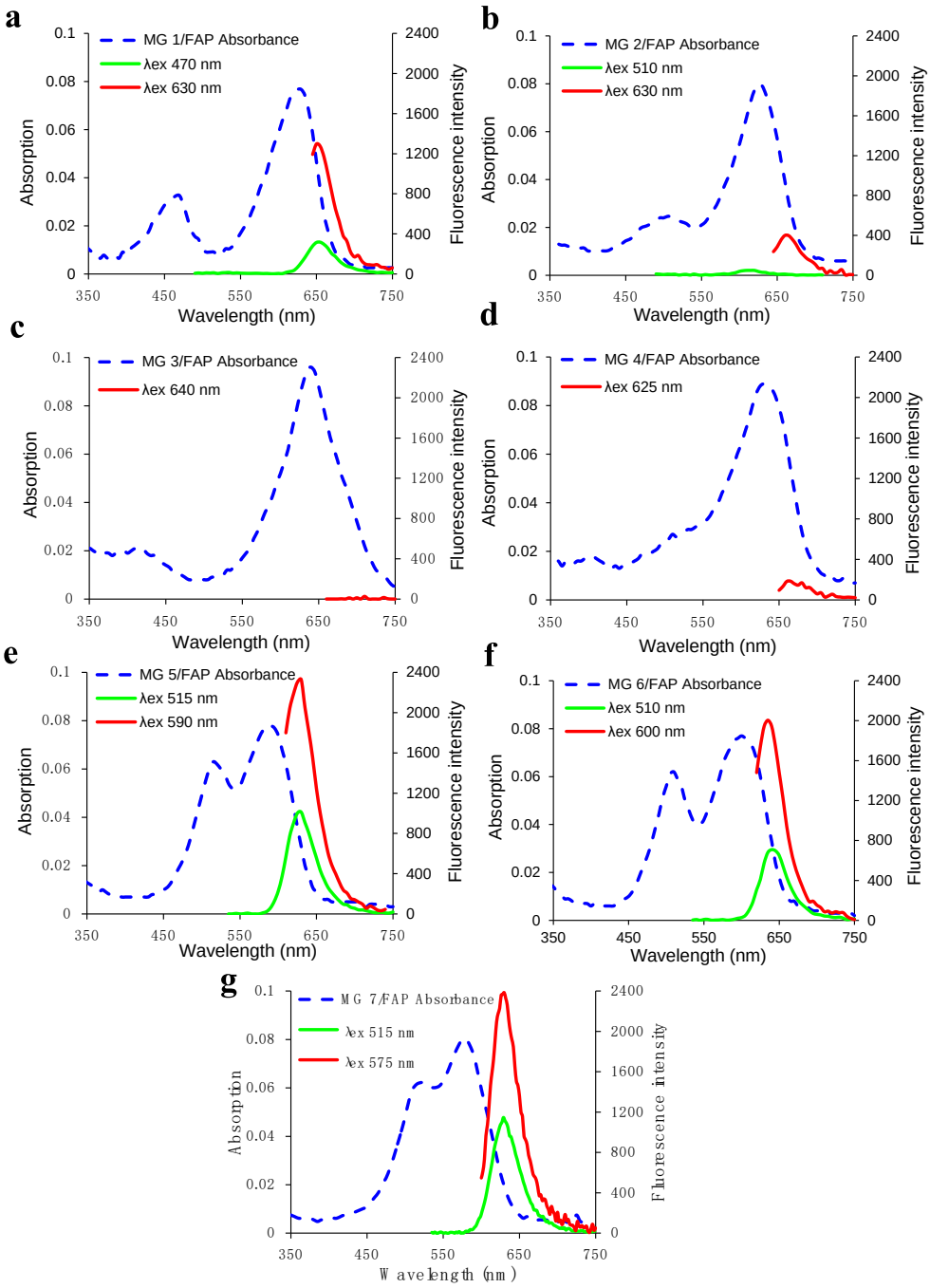


Figure S4. Absorption (dashed lines) and fluorescent emission (solid lines) spectra of MGs **1**-**7**/FAP_dH6.2. (a) MG **1**/FAP_dH6.2 (Y band excitation: λ_{ex} = 470 nm, X band excitation: λ_{ex} = 630 nm) of; (b) MG **2**/FAP_dH6.2 (Y band excitation: λ_{ex} = 510 nm, X band excitation: λ_{ex} = 630 nm); (c) MG **3**/FAP_dH6.2 (X band excitation: λ_{ex} = 640 nm); (d) MG **4**/FAP_dH6.2 (X band excitation: λ_{ex} = 625 nm); (e) MG **5**/FAP_dH6.2 (Y band excitation: λ_{ex} = 515 nm, X band excitation: λ_{ex} = 590 nm); (f) MG **6**/FAP_dH6.2 (Y band excitation: λ_{ex} = 510 nm, X band excitation: λ_{ex} = 600 nm). (g) MG **7**/FAP_dH6.2 (Y band excitation: λ_{ex} = 515 nm, X band excitation: λ_{ex} = 575 nm).

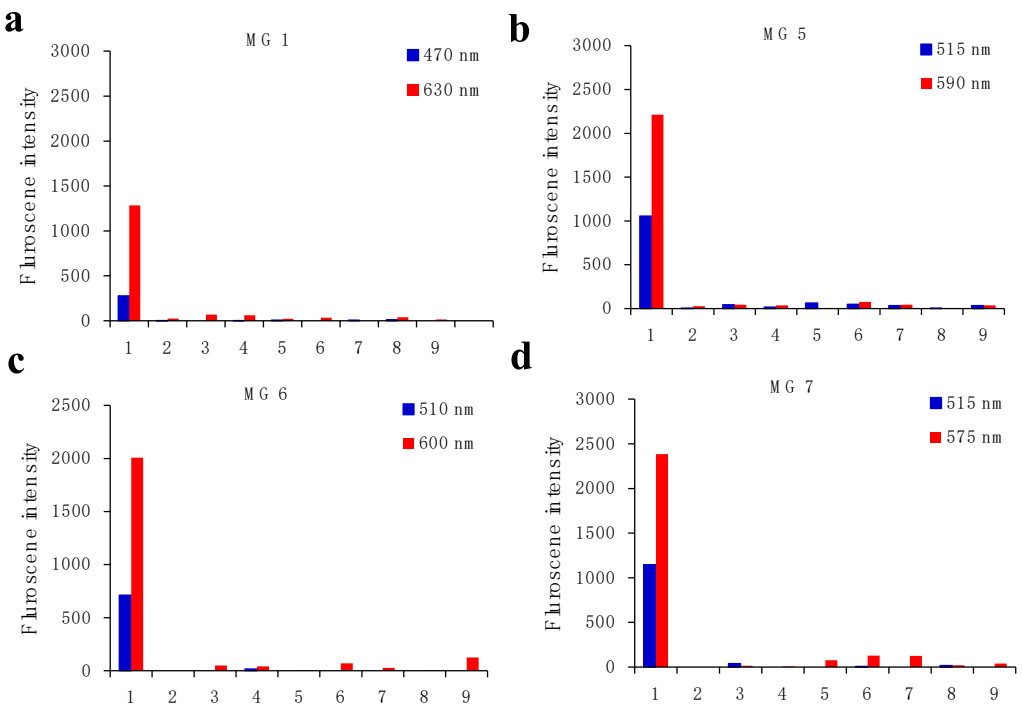


Figure S5. Fluorescence responses of MG **1**, MG **5**, MG **6** and MG **7** to various species: 1: FAP_dH6.2 (2 μM), 2: PBS, 3: BSA (5 μM), 4: Aprotinin (5 μM), 5: Endoproteinase Glu-C (5 μM), 6: Human IgG (H+L) (5 μM), 7: Mouse IgG (H+L) (5 μM), 8: Rabbit IgG (H+L) (5 μM), 9: Goat IgG (H+L) (5 μM). All spectra were acquired in PBS (pH 7.4) at 37 °C, (a) MG **1** (Y band excitation: λ_{ex} = 470 nm, X band excitation: λ_{ex} = 630 nm); (b) MG **5** (Y band excitation: λ_{ex} = 515 nm, X band excitation: λ_{ex} = 590 nm); (c) MG **6** (Y band excitation: λ_{ex} = 510 nm, X band excitation: λ_{ex} = 600 nm); (d) MG **7** (Y band excitation: λ_{ex} = 515 nm, X band excitation: λ_{ex} = 575 nm).

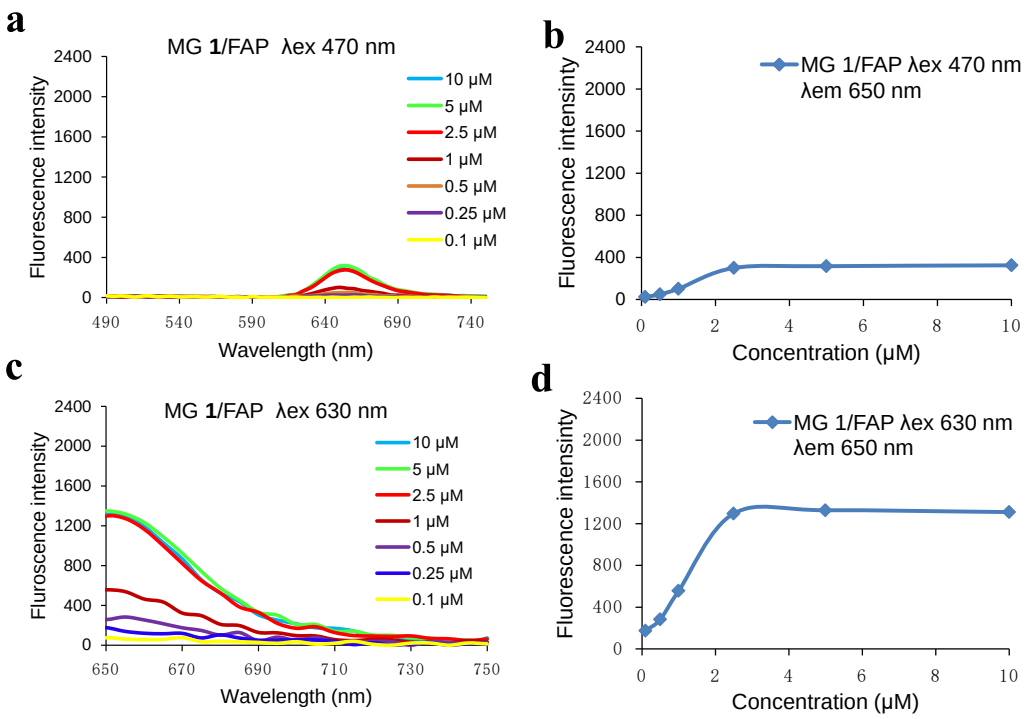


Figure S6. Fluorescent emission spectra and a plot of fluorescence intensity at 650 nm in the presence of different amounts of MG **1** bound to 2 μM FAP_dH6.2. (a) Fluorescent emission spectra of the MG **1**/FAP_dH6.2 complexes when excited at 470 nm; (b) A plot of fluorescence intensity of the MG **1**/FAP_dH6.2 complexes using ex/em of 470 nm/650 nm; (c) Fluorescent emission spectra of the MG **1**/FAP_dH6.2 complexes when excited at 630 nm; (d) A plot of fluorescence intensity of the MG **1**/FAP_dH6.2 complexes using ex/em of 630 nm/650 nm.

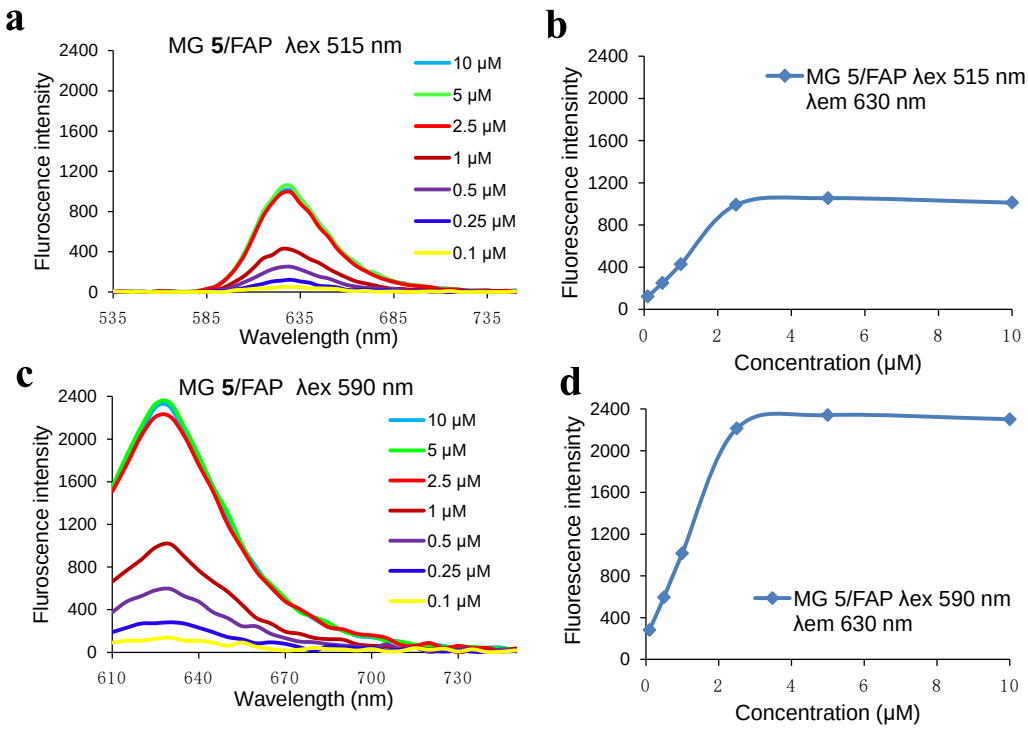


Figure S7. Fluorescent emission spectra and a plot of fluorescence intensity at 630 nm in the presence of different amounts of MG **5** bound to 2 μM FAP_dH6.2. (a) Fluorescent emission spectra of the MG **5**/FAP_dH6.2 complexes when excited at 515 nm; (b) A plot of fluorescence intensity of the MG **5**/FAP_dH6.2 complexes using ex/em of 515 nm/630 nm; (c) Fluorescent emission spectra of the MG **5** /FAP_dH6.2 complexes when excited at 590 nm; (d) A plot of fluorescence intensity of the MG **5** /FAP_dH6.2 complexes using ex/em of 590 nm/630 nm.

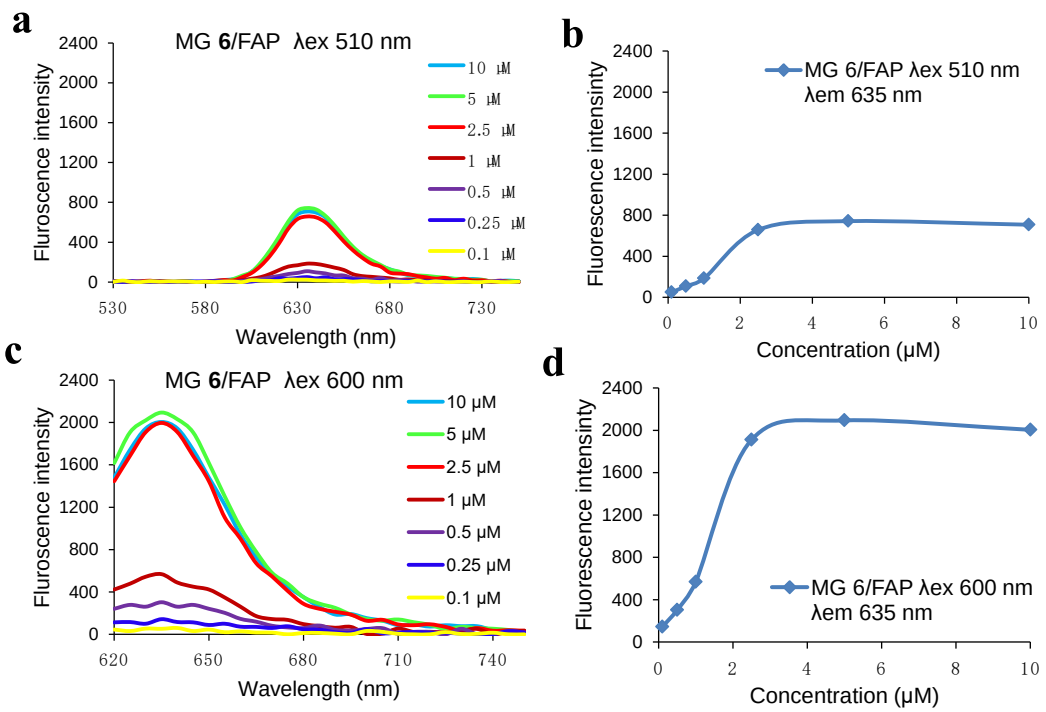


Figure S8. Fluorescent emission spectra and a plot of fluorescence intensity at 635 nm in the presence of different amounts of MG **6** bound to 2 μM FAP_dH6.2. (a) Fluorescent emission spectra of the MG **6**/FAP_dH6.2 complexes when excited at 510 nm; (b) A plot of fluorescence intensity of the MG **6**/FAP_dH6.2 complexes using ex/em of 510 nm/635 nm; (c) Fluorescent emission spectra of the MG **5** /FAP_dH6.2 complexes when excited at 600 nm; (d) A plot of fluorescence intensity of the MG **6** /FAP_dH6.2 complexes using ex/em of 600 nm/635 nm.

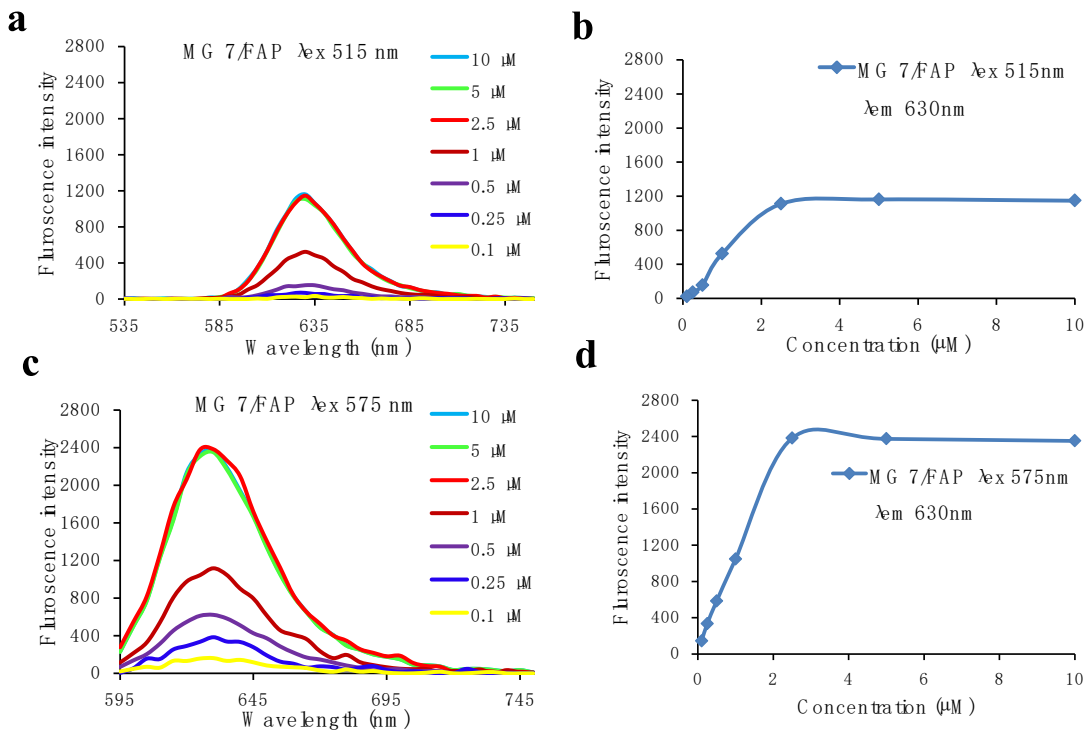


Figure S9. Fluorescent emission spectra and a plot of fluorescence intensity at 630 nm in the presence of different amounts of MG **7** bound to 2 μM FAP_dH6.2. (a) Fluorescent emission spectra of the MG **7**/FAP_dH6.2 complexes when excited at 515 nm; (b) A plot of fluorescence intensity of the MG **7**/FAP_dH6.2 complexes using ex/em of 515 nm/630 nm; (c) Fluorescent emission spectra of the MG **5** /FAP_dH6.2 complexes when excited at 575 nm; (d) A plot of fluorescence intensity of the MG **7** /FAP_dH6.2 complexes using ex/em of 575 nm/630 nm.

Fluorescent OFF-ON-OFF studies

Aliquots of fluorogen stocks of MG **5** and MG **6** were diluted in PBS to obtain a final concentration of 2.5 μM, respectively. MG **5** or MG **6** is fluorescence-off in solution, but intensely fluorescent upon binding to FAP_dH6.2 (2 μM). Figure S8 shows the fluorescence spectral changes of MG **5** and MG **6** during the titration of MG **1**. Wavelength interval: 5.0 nm.

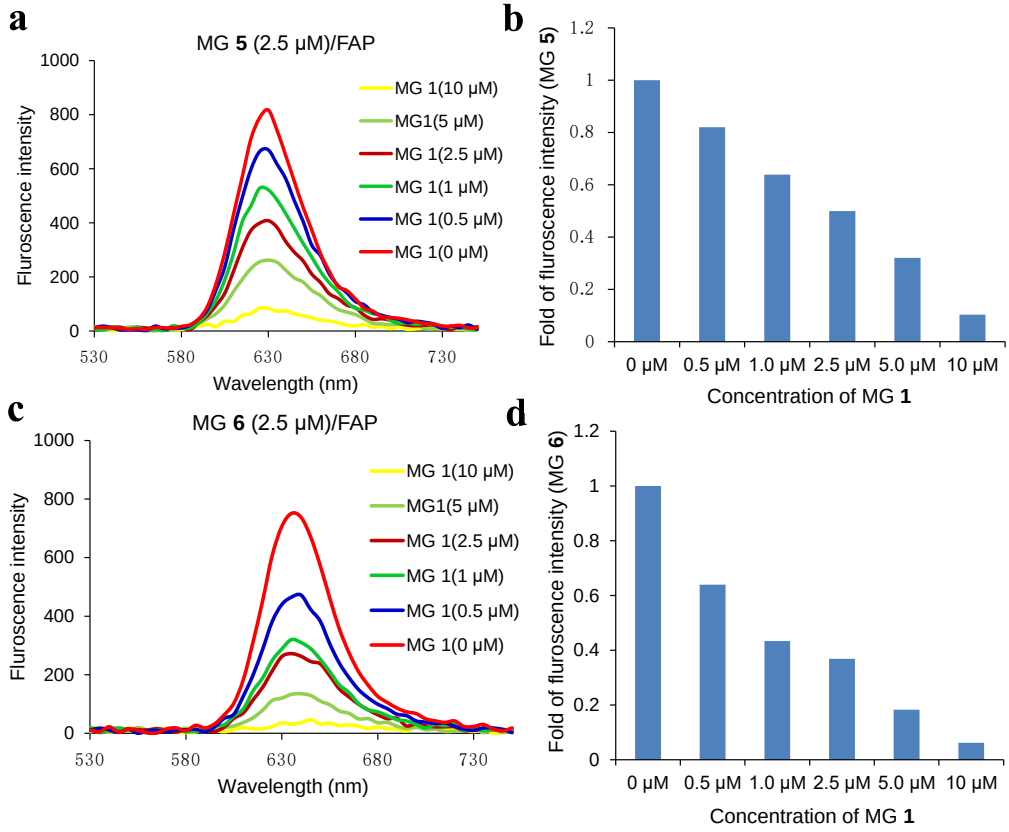


Figure S10. Comparison of the fluorescent emission intensity level in the MG **5**/FAP_dH6.2 (a,b) and MG **6**/ FAP_dH6.2 (c,d) complexed with the different concentrations of MG **1**. (a) Fluorescent emission spectra of the MG **5**/ FAP_dH6.2 complexed with the different concentrations of MG **1** when excited at 510 nm; (b) The relative emission intensity level of the MG **5**/ FAP_dH6.2 bound to the different concentrations of MG **1** using ex/em of 510 nm/630 nm; (c) Fluorescent emission spectra of the MG **6**/ FAP_dH6.2 complexed with the different concentrations of MG **1** when excited at 510 nm; (d) The relative emission intensity level of the MG **6**/ FAP_dH6.2 bound to the different concentrations of MG **1** using ex/em of 515 nm/635 nm.

FAP_dH6.2 expression in *E. coli* and purification

The MG-binding clone (FAP_dH6.2) was derived from the parent MG-binding FAPs through random and targeted mutagenesis efforts, producing clone that retained functional fluorogen activation in the reducing environment of the cells[3] The pET23_FAP6.2_His plasmid was obtained from Prof. Mark Brönstrup' Lab (Helmholtz Centre for Infection Research). Transetta (DE3) Chemically Competent Cells (TransGen Biotech, Beijing, China) were transformed with pET23_FAP_dH6.2 and selected for ampicillin resistance yielding *E. coli*_dH6.2 The expression of FAP_dH6.2 was under 1μM final concentration of IPTG. Purification of HIS-tagged dH6.2 was performed using Ni-NTA-agarose (CWbiotech, Beijing, China). 500 mL LB containing 100 μM ampicillin were inoculated from a 5 mL overnight culture of *E. coli*_dH6.2. At OD_{600} of 0.8 the expression of FAP_dH6.2 was induced by the addition of 1 mM IPTG and the culture was kept under continuous agitation overnight. For protein purification the cells were centrifuged (9000 ×g, 2 min, 4°C) and resuspended in 20 mM Tris, 500 mM NaCl pH7.9. The cells were disrupted by sonication at 4°C. The cell lysate was centrifuged at 12000 ×g for 1 h at 4°C and imidazole was added to the supernatant to a final concentration of 30 mM. The supernatant was then incubated with 1mL prewashed Ni-NTA-agarose (washed with 1 mL H_2O followed by 5 × 1 mL 20 mM Tris, 500 mM NaCl, pH7.9) for 2h at 4°C. Using a poly-prep chromatopgraphy column (BioRad, Hercules, US) the Ni-NTA-agarose was washed with 20 mL of 20 mM Tris, 500 mM NaCl, 30 mM imidazole, pH7.9 FAP_dH6.2-His was eluted with 2 mL 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 300 mM imidazole, pH 7.5 and dialyzed for 2 h against 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5. Glycerol was added to a final concentration of 10% for storage of FAP_dH6.2 at -20°C. Protein concentration was determined using a BCA protein assay kit (Biomiga, San Diego, US).

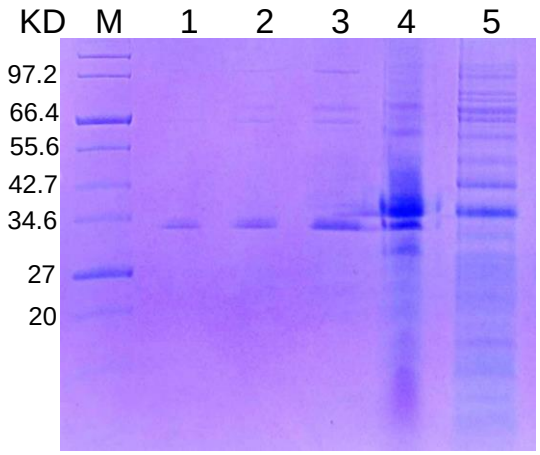


Figure S11. Tricine-SDS-PAGE analysis of the purified FAP_dH6.2 protein. M: protein marker; 1: 2 μl purified FAP_dH6.2 protein; 2: 4 μl purified FAP_dH6.2 protein; 3: 10 μl purified FAP_dH6.2 protein; 4: induced cells ; 5: uninduced cells.

The cytotoxicity assays

The human hepatoma HepG2 cells, HEK293A cells and rat fibroblast L6 cells were seeded on a 96-wells containing 7500 cells per well in 100 μL DMEM with 10 % FBS and incubated for overnight before adding MG **1**, MG **5**, MG **6 and** MG **7**. Upon incubation with different concentration of MG **1**, MG **5**, MG **6** or MG **7** at 37 °C for 48 h, 20 μL MTS was added into each well. After 4 h incubation at 37 °C, the absorbance was measured at 490 nm using a 2300 EnSpire multimode plate reader. Each experiment was repeated three times.

Table S1. The cytotoxicity assays of MG **1**, MG **5**, MG **6** and MG **7** in the human hepatoma HepG2 cells, HEK293A cells and rat fibroblast L6 cells

IC_{50}	MG **1** (μM)	MG **5** (μM)	MG **6** (μM)	MG **7** (μM)
HepG2 cells	19.60	28.23	16.50	13.03
HEK293A cells	2.64	26.95	5.30	0.90
L6 cells	7.413	27.98	18.78	3.79

Confocal imaging of *E. coli*_dH6.2 treated with MG 1, MG 5 or MG 1/MG 5

50 mL LB containing 100 μM ampicillin were inoculated from a 0.5 mL overnight culture of *E. coli*_dH6.2. At OD_{600} of 0.8 the expression of dH6.2 was induced by the addition of 1mM IPTG and the culture was kept under continuous agitation overnight. *E. coli*_dH6.2 strain overnight culture was harvested and washed twice with PBS (pH 7.4). The washed cells were resuspended in PBS with an OD_{600} of 0.5-0.7. Then 200 μL aliquots *E. coli*_dH6.2 strain was incubated with 2 μM of MG **1** or MG **5**. After incubation at 37 °C for 15 min, the cells were treated with 20 μg/mL of Hoechst33258 at 37 °C for 30 min. For MG **5**/MG **1** group, 200 μL aliquots *E. coli*_dH6.2 strain was incubated with 2 μM of MG **5** at 37 °C for 15min, then 2 μM MG **1** was added and incubated for another 15min at 37 °C. The cells were treated with 20 μg/mL of Hoechst33258 at 37 °C for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads.[4] Fluorescence images were acquired with ZEISS LSM 710 Confocal Microscope (Nikon Eclipse TE2000-E, CFI Plan-Apochromat VC 63 × oil immersedoptics), using a high pressure He-Ne lamp and diode laser for

excitation (λ_{ex} = 405 ± 20 nm and λ_{em} = 460 ± 25 nm; λ_{ex} =514 ± 20 nm and λ_{em} = 630 ± 25 nm, Scale bar = 10 μm).

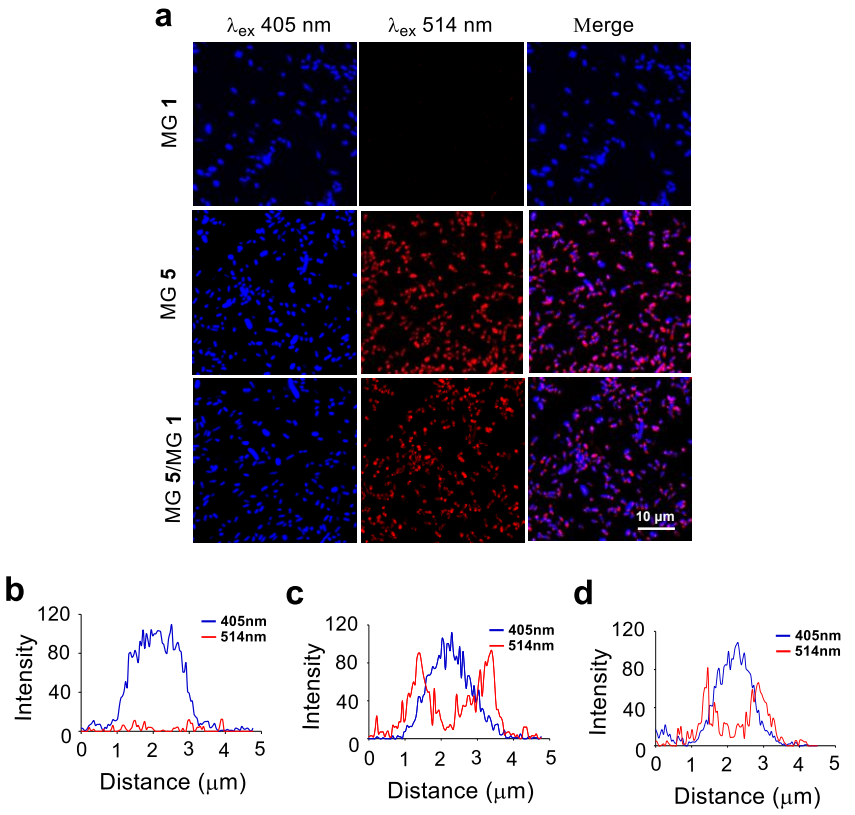


Figure S12. (a) Confocal microscopic images and (b-d) quantification of fluorescence intensity of *E. coli*_dH6.2 cells incubated with MG 1, MG 5, and MG 5 for 15min then 1.0 equiv MG 1 for 15 min. respectively. Blue fluorescence signals from Hoechst 33258 stain; red fluorescence signals from MG/FAP (λ_{ex} =514 ± 20 nm, λ_{em} = 630 ± 25 nm), Scale bar = 50 μm.

FAP_dH6.2 and FAP_dH6.2_GFP expression in HEK293A cells

Plasmids expressing surface displayed scFv's were generated as follows. A 885 bp PCR-amplicon was amplified from the pET23_dH6.2_His plasmid using as primers: 5'-CGGGATCC ATGCAAGTCCAGTTGCAAG-3'(forward),5'-GCGAATTCTCACTGCTGCTGCTGCTGCTCGAG TGAACCG-3'(reverse). This molecule was cut with *BamH* I and *EcoR* I sites and ligated into pCDNA 3.1+ (Invitrogen) between the *BamH* I and *EcoR* I sites to produce vector pCDNA 3.1. Then they were transformed into DH5α *E. coli* to ampicillin resistance, and Lac- colonies picked for DNA sequencing. DNA was prepared from selected transformants using TIANprep Mini Plasmid kit (TIAMGEN, Beijing, China) and transiently transfected into HEK293A cells (~ 1 μg DNA per 10^5 cells) in an 8-

well chamber using Lipofectamine 2000 (Invitrogen) following the protocols supplied by the manufacturer. [5]

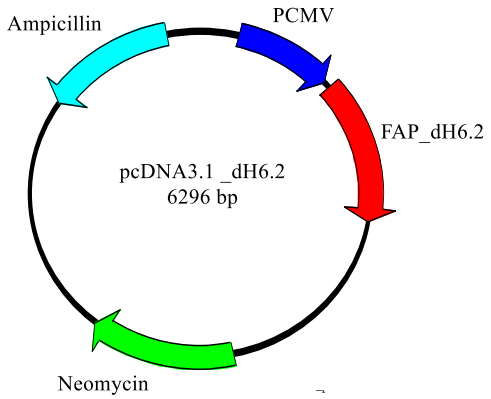


Figure S13. Graphical map of FAP_dH6.2 in pcDNA3.1

FAP_dH6.2 sequence (red):

TTAACTTAGCTTGGTACCGAGCTCGGATCCATGCAAGTCCAGTTGCAAGAATCTGGACCTGGT
TTGGTTAAACCATCTGAGACACTTTCCCTAACCTGCACAGTCAGTGGCGCATCAATTTCCTCCT
CGCACTATTATTGGGGTTGGATTAGACAACCACCTGGAAAGGGCCCAGAGTGGATAGGGTCT
ATGTACTACTCGGGTAGAACTTACTATAATCCTGCCTTGAAGTCACGTGTTACCATATCCTCTG
ATAAGTCAAAAAACCAATTCTTTTTAAAACTCACTAGCGTTACCGCTGCTGATACAGCTGTCT
ATTATGCTGCCCGTGAGGGCCCAACGCATTATTATGATAATTCAGGCCCAATACCTAGCGATG
AGTATTTTCAACATTGGGGCCAGGGCACTTTAGTTACAGTTTCCGGTGGTGGCGGCTCTGGTG
GCGGTGGCAGCGGCGGTGGTGGTTCCGGAGGCGGCGGTTCTCAGGTGCAGCTGCAAGAGTCG
GGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGCCTCC
ATCAGCAGTAGTCATTACTACTGGGGCTGGATTCGCCAGCCCCCAGGGAAGGGGCCTGAGTG
GATTGGGAGTATGTATTATAGTGGGAGAACGTACTACAACCCGGCCCTCAAGAGTCGAGTCA
CCATATCATCAGACAAGTCGAAGAACCAGTTCTTCTTGAAGTTGACCTCTGTAACCGCCGCGG
ACACGGCCGTGTATTACGCTGCGAGGGAGGGACCCACACATTACTATGATAATAGTGGTCCA
ATACCTTCGGATGAGTATTTCCAGCACTGGGGCCAGGGTACACTGGTCACTGTCTCCGGAGGT
GGCGGTTCACTCGAGCACCACCACCACCACCACTGAGAATTCTGCAGATATCCAGCACAGTGG
CGGCCGCTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGT
TGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCA
CTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAATAGGTGTCATTCTATTCT
GGGGGGTTGGGGGTGGGGGCAGGACAACCAAGGGGG

The plasmid pET23_dH6.2_His plasmid using was constructed by PCR amplification of dH6.2 as primers: 5'-CGGGATCCATGCAAGTCCAGTTGCAAG-3'(forward), 5'-GCGAATTCTCACTGCTGCTGCTGCTCGAGTGAACCG-3'(reverse), referred to as dH6.2 in this work) monomer and ligation into the eukaryotic expression vector pcDNA3.1_dH6.2. An annealed DNA insert encoding the amino acid SG4S was ligated upstream from the GFP subunit, followed by the ligation of PCR amplified DNA encoding an additional GFP subunit. The plasmid

pcDNA3.1_dH6.2_GFP was constructed by ligation of double stranded DNA encoding of SG4S followed by the amino acids upstream of the GFP sequence of pEGFP-N1 using as primers: 5'-TCACTGTCTCCGGAGGTGGCGGTTCAGGTGGTGGCGGCTCTATGGTGAGCAAGGGCGAGGA-3'(forward), 5'-TCAGTGGTGGTGGTGGTGGTGCTTGTACAGCTCGTCCATG-3'(reverse). Plasmid sequences were verified by DNA sequencing. Plasmid sequences were verified by DNA sequencing. Then they were transformed into DH5α *E. coli* to ampicillin resistance, and Lac- colonies picked for DNA sequencing. DNA was prepared from selected transformants using TIANprep Mini Plasmid kit (TIAMGEN, Beijing, China) and transiently transfected into HEK293A cells (~ 1 μg DNA per 10^5 cells) in an 8-well chamber using Lipofectamine 2000 (Invitrogen) following the protocols supplied by the manufacturer.

FAP_dH6.2_GFP sequence (FAP: red and GFP: green):

ATAGGGCGATTGAGCTGCCCTTCGGGATCCATGCAAGTCCAGTTGCAAGAATCTGGACCTGGT
TTGGTTAAACCATCTGAGACACTTTCCCTAACCTGCACAGTCAGTGGCGCATCAATTTCCTCCT
CGCACTATTATTGGGGTTGGATTAGACAACCACCTGGAAAGGGCCCAGAGTGGATAGGGTCT
ATGTACTACTCGGGTAGAACTTACTATAATCCTGCCTTGAAGTCACGTGTTACCATATCCTCTG
ATAAGTCAAAAAACCAATTCTTTTTAAAACTCACTAGCGTTACCGCTGCTGATACAGCTGTCT
ATTATGCTGCCCGTGAGGGCCCAACGCATTATTATGATAATTCAGGCCCAATACCTAGCGATG
AGTATTTTCAACATTGGGGCCAGGGCACTTTAGTTACAGTTTCCGGTGGTGGCGGCTCTGGTG
GCGGTGGCAGCGGCGGTGGTGGTTCCGGAGGCGGCGGTTCTCAGGTGCAGCTGCAAGAGTCG
GGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGCCTCC
ATCAGCAGTAGTCATTACTACTGGGGCTGGATTCGCCAGCCCCCAGGGAAGGGGCCTGAGTG
GATTGGGAGTATGTATTATAGTGGGAGAACGTACTACAACCCGGCCCTCAAGAGTCGAGTCA
CCATATCATCAGACAAGTCGAAGAACCAGTTCTTCTTGAAGTTGACCTCTGTAACCGCCGCGG
ACACGGCCGTGTATTACGCTGCGAGGGAGGGACCCACACATTACTATGATAATAGTGGTCCA
ATACCTTCGGATGAGTATTTCCAGCACTGGGGCCAGGGTACACTGGTCACTGTCTCCGGAGGT
GGCGGTTCAGGTGGTGGCGGCTCTATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGT
GCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGG
GCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTG
CCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTAC
CCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGA
GCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGG
GCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATC
CTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCA
GAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGC
TCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACC
ACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTC
CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGCACCAC
CACCACCACCACTGAGAATCG

Confocal imaging of HEK293A _dH6.2 and HEK293A _dH6.2_GFP treated with MG 1, MG 5 or MG 1/MG 5[6]

After transient transfection for 36 h, the HEK293A _dH6.2 cells and HEK293A _dH6.2_GFP cells (0.3 mL, 1 × 10^5 cells/mL) were incubated with 2 μM of MG **5** at 37 °C for 15min, then 2 μM MG **1** was

added and incubated for another 15min at 37 °C and then treated with 2 μg/mL of Hoechst 33258 at 37 °C for 5 min. After incubation, cells attached on 8-well chamber were observed with ZEISS LSM 710 Confocal Microscope equipped with a 40 × immersion lens. The bright field image (Fig. S13 and S14) shows that, as expected, the FAP or GFP-positive cells represent a subset of the whole population about 10-20 % of the total.

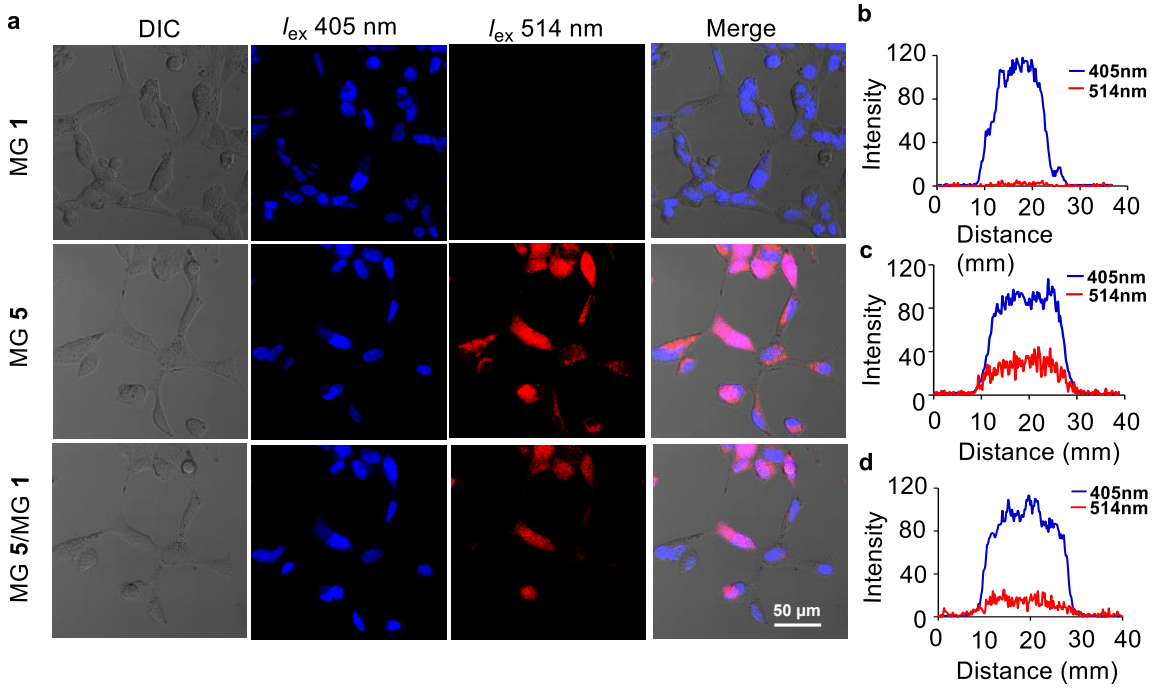


Figure S14. (a) Confocal microscopic images and (b-d) quantification of fluorescence intensity of HEK293A _dH6.2 cells incubated with MG **1**, MG **5**, and MG **5** for 15min then 1.0 equiv MG **1** for 15 min. respectively. Blue fluorescence signals from Hoechst 33258 stain; red fluorescence signals from MG/FAP (λex =514 ± 20 nm, λem = 630 ± 25 nm), Scale bar = 50 μm.

Z-stacks of confocal images using a 60× objective were collected for two channels (GFP channel and MG **5** channel) from the entire volume of the cell using a step size of 0.2 μm, which allows the generation of three dimensional reconstructions that provide much more information.

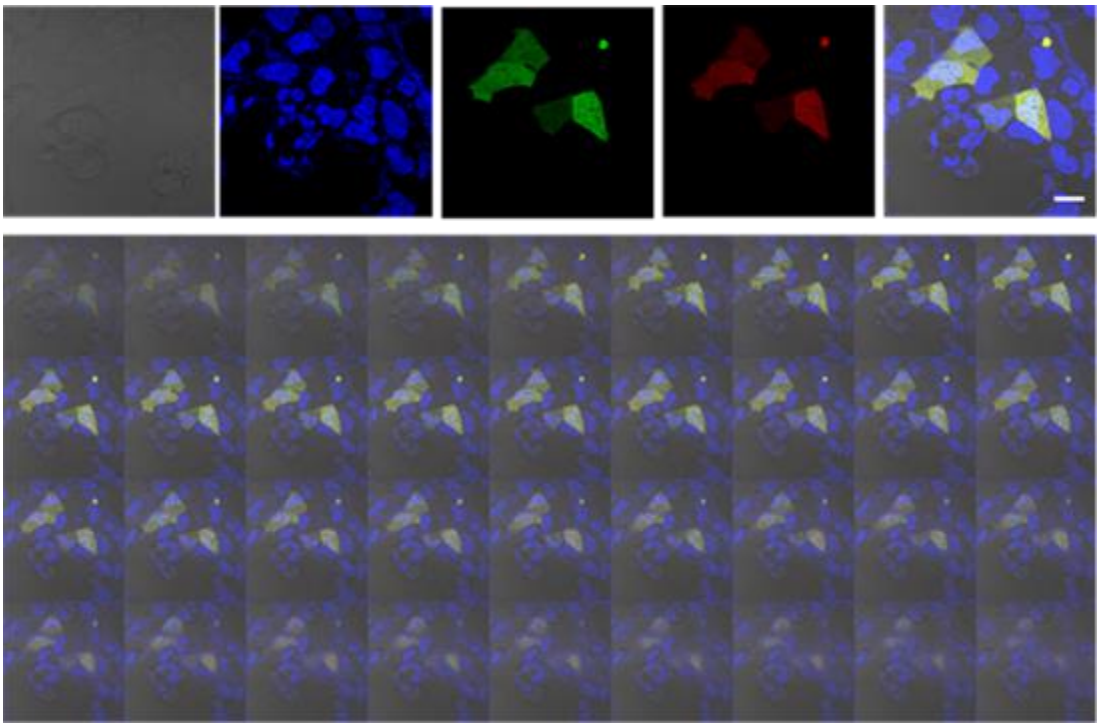


Figure S15. Z-stacks of confocal microscopic images of HEK293A_GFP_ dH6.2 cells incubated with MG **5**. Blue fluorescence signals from Hoechst 33258 stain; green from GFP (λex =488 ± 20 nm, λem = 500 ± 25 nm); red from MG/FAP (λex =514 ± 20 nm, λem = 630 ± 25 nm); merge from bright-field, and blue, green, red fluorescence signals. Scale bar = 20 µm

The confocal microscopy analysis reveals that only GFP fluorescence-positive cells were labeled with MG **5** besides high level co-localization of GFP signal with MG **5**/FAP signal, which indicates the remarkable selectivity of MG **5** to FAP. Moreover, Z-stacks confocal imaging studies showed a clear intracellular distribution of both of the GFP and MG **5**/FAP signals, including the cell nuclei in living cells. In the dead HEK293A_GFP_dH6.2 cells, DNA diffused in the whole cell, in which Hoechst, GFP and FAP/MG **5** signals were merged together.

References

(1) Szent-Gyorgyi, C.; Schmidt, B. F.; Creeger, Y.; Fisher, G. W.; Zakel, K. L.; Adler, S.; Fitzpatrick, J. A.; Woolford, C. A.; Yan, Q.; Vasilev, K. V.; Berget, P. B.; Bruchez, M. P.; Jarvik, J. W.; Waggoner, A., *Nat Biotechnol* **2008**, *26*, 235-240.

(2) (a) Szent-Gyorgyi, C.; Stanfield, R. L.; Andreko, S.; Dempsey, A.; Ahmed, M.; Capek, S.; Waggoner, A. S.; Wilson, I. A.; Bruchez, M. P., *J Mol Biol* **2013**, *425*, 4595-4613; (b) Duxbury, D. F., *Chemical Reviews* **1993**, *93*, 381-433.

(3) Yan, Q.; Schwartz, S.L.; Maji, S.; Huang, F.; Szent-Gyorgyi, C.; Lidke, D. S.; Lidke, K. A.; Bruchez , M. P., *Chemphyschem* **2014**, *15*, 687–695.

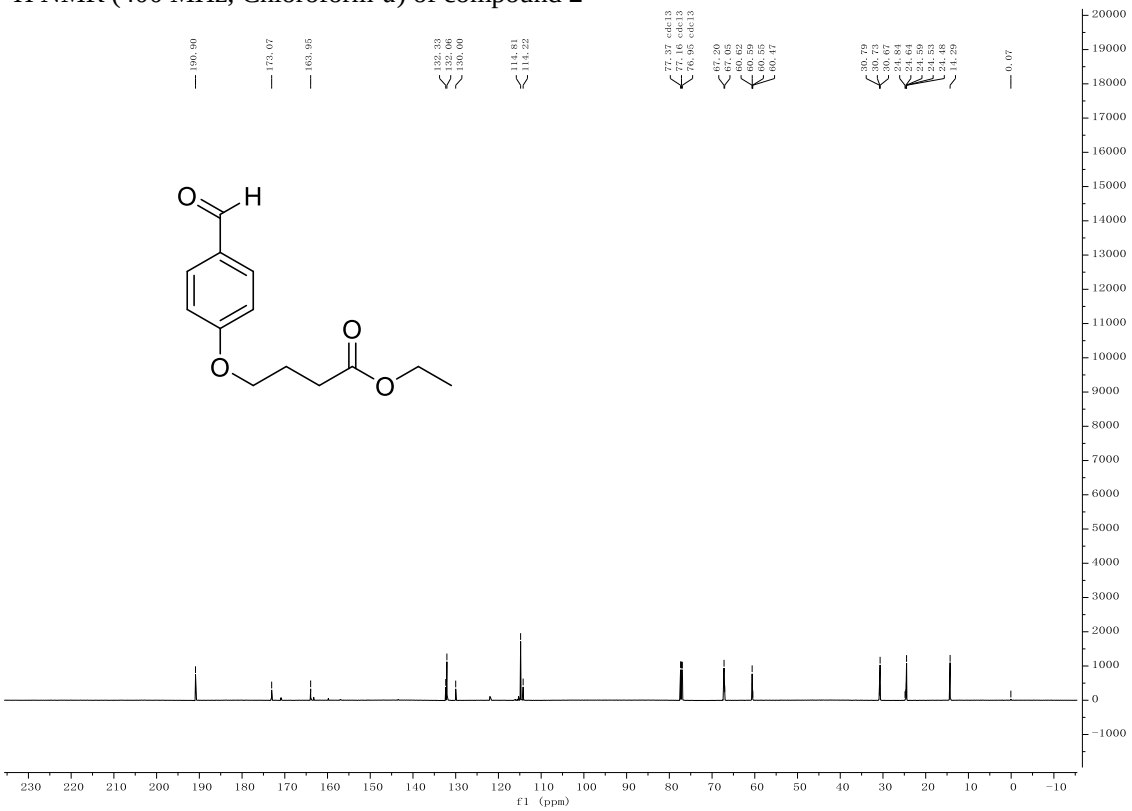
(4) Skinner, S. O.; Sepulveda, L. A.; Xu, H.; Golding, I., *Nat Protoc* **2013**, *8*, 1100-13.

(5) Rizzuto, R.; Brini, M.; Pizzo P.; Murgia, M.; Pozza, T.; *Curr Biol* **1995**, *5*, 635-642.

(6) Zhou, Z.; Zhang, C. C.; Zheng, Y.; Wang, Q., *J Med Chem* **2016**, *59*, 410-8.

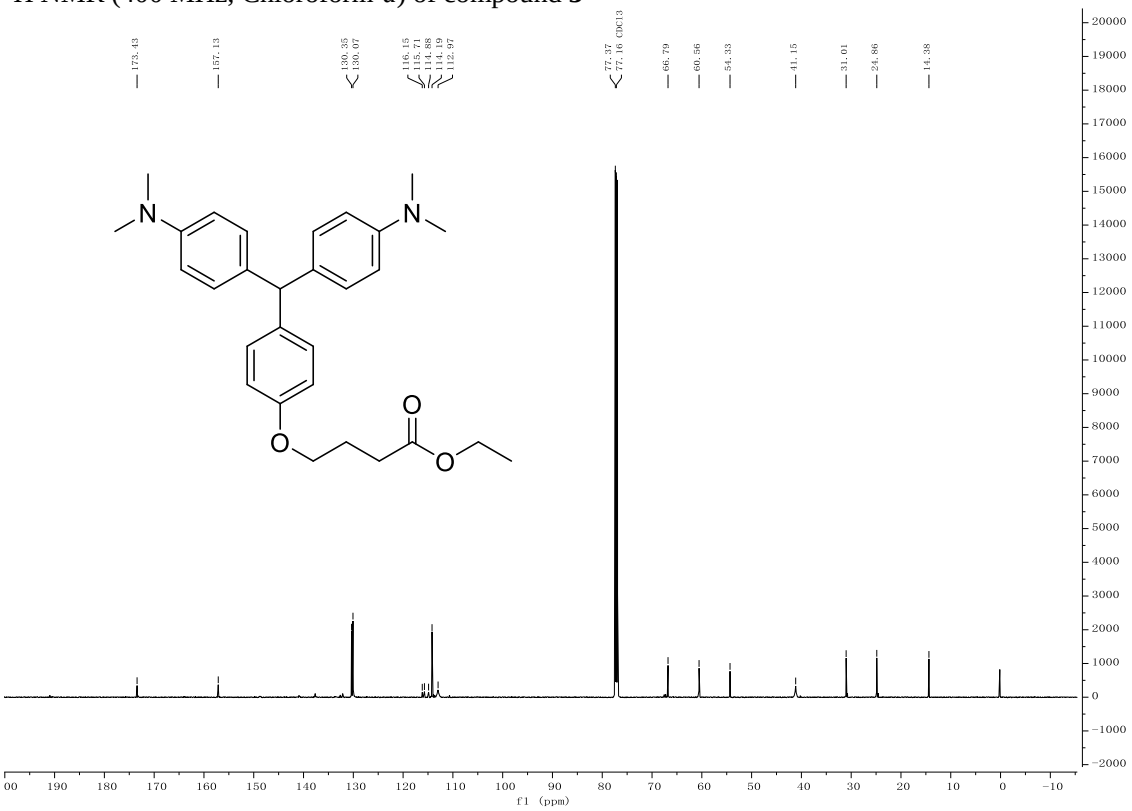
Copies of ^{1}H NMR and ^{13}C NMR spectrum of compounds

^{1}H NMR (400 MHz, Chloroform-*d*) of compound **2**

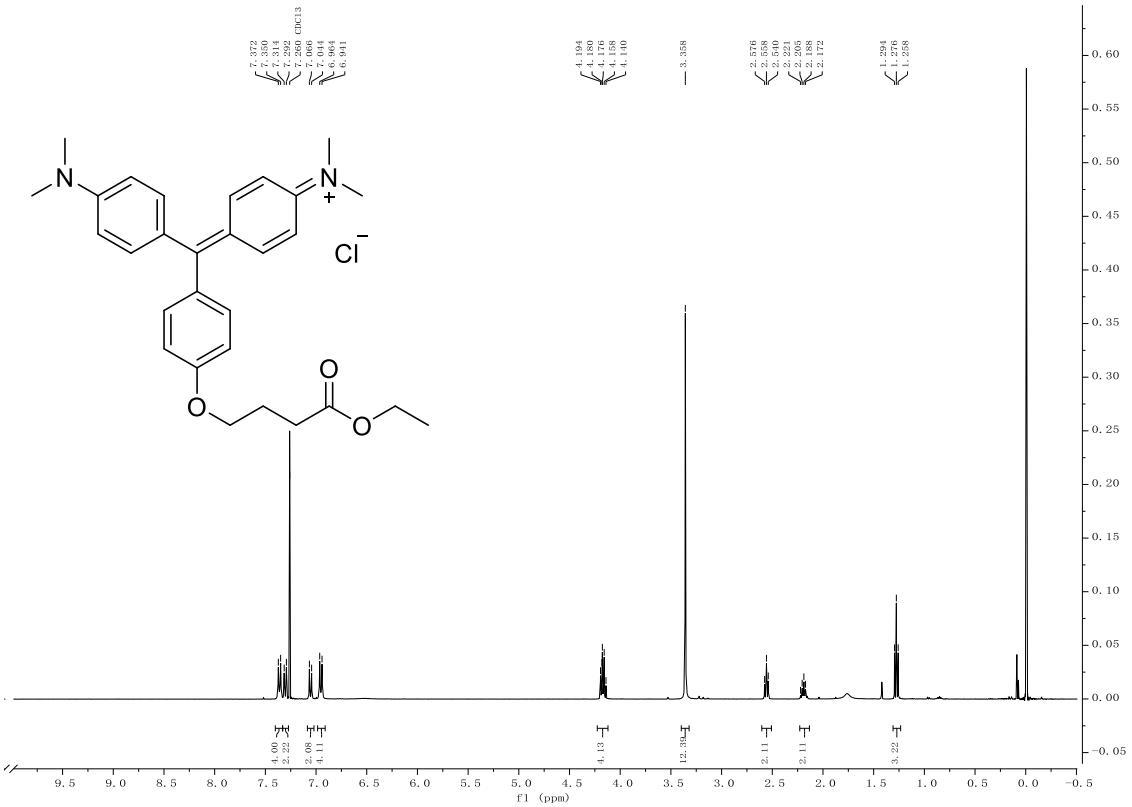


^{13}C NMR (150 MHz, Chloroform-*d*) of compound **2**

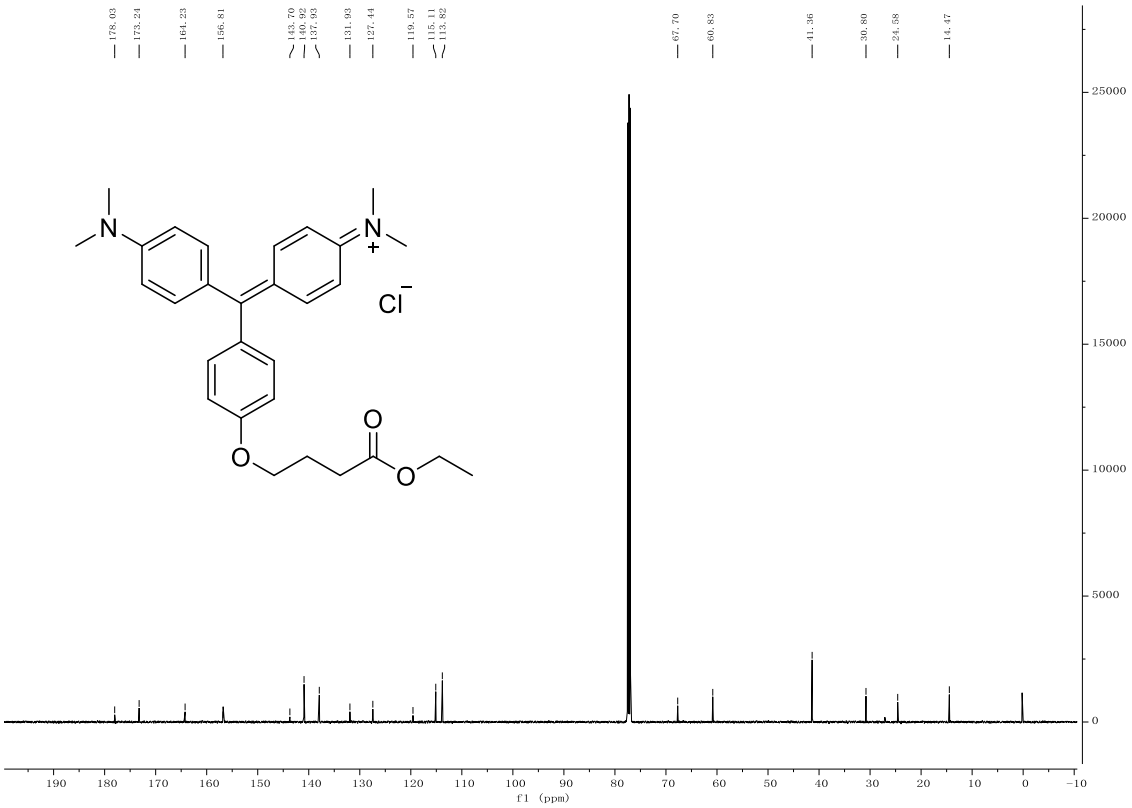
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **3**



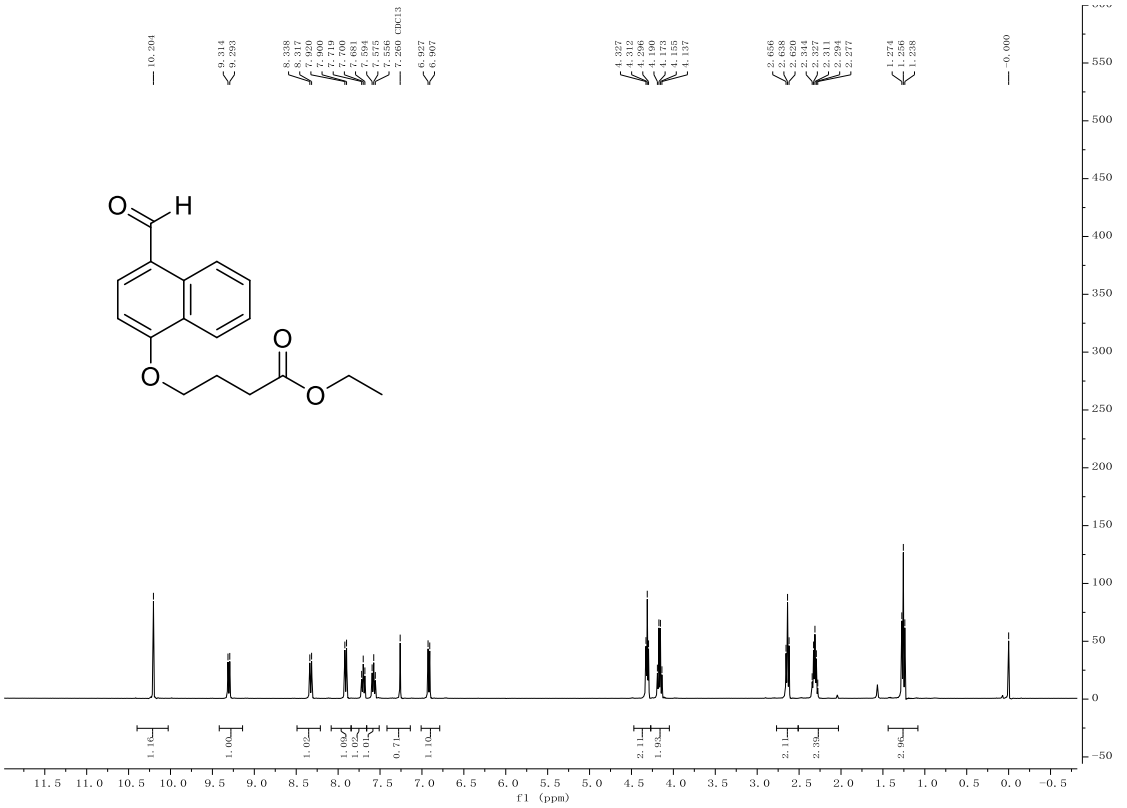
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **3**



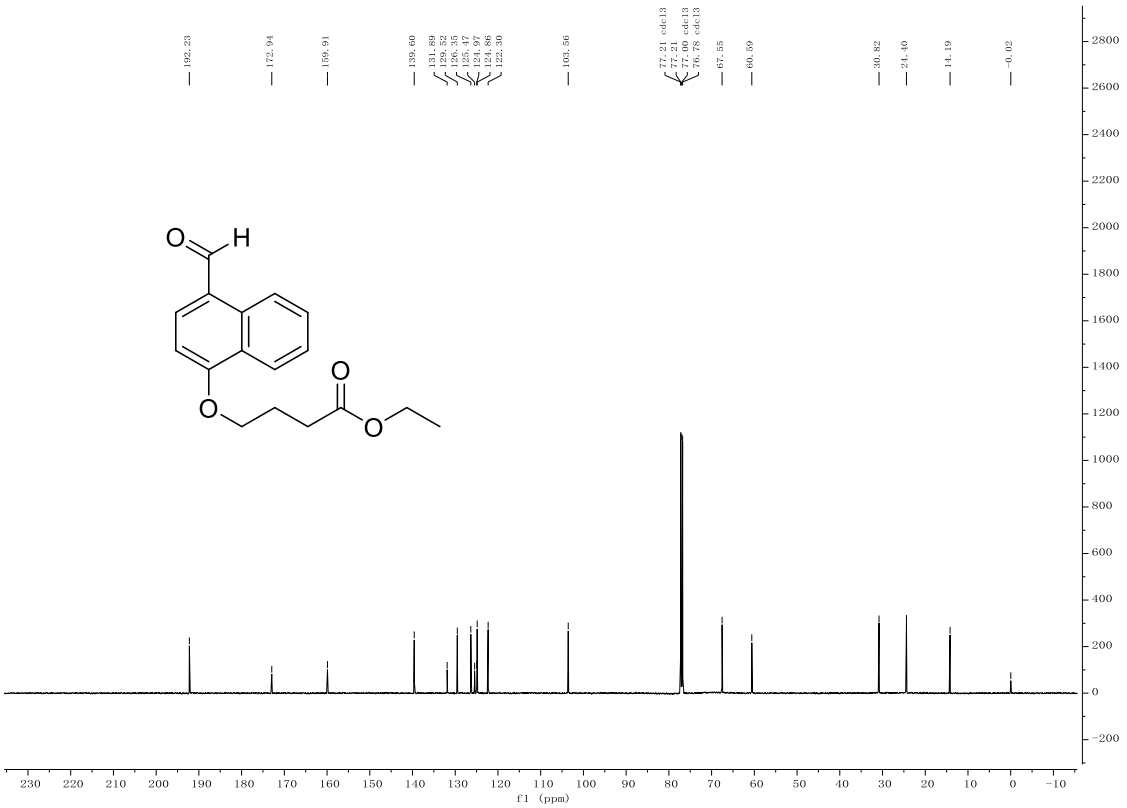
^{1}H NMR (400 MHz, Chloroform-*d*) of MG **1**



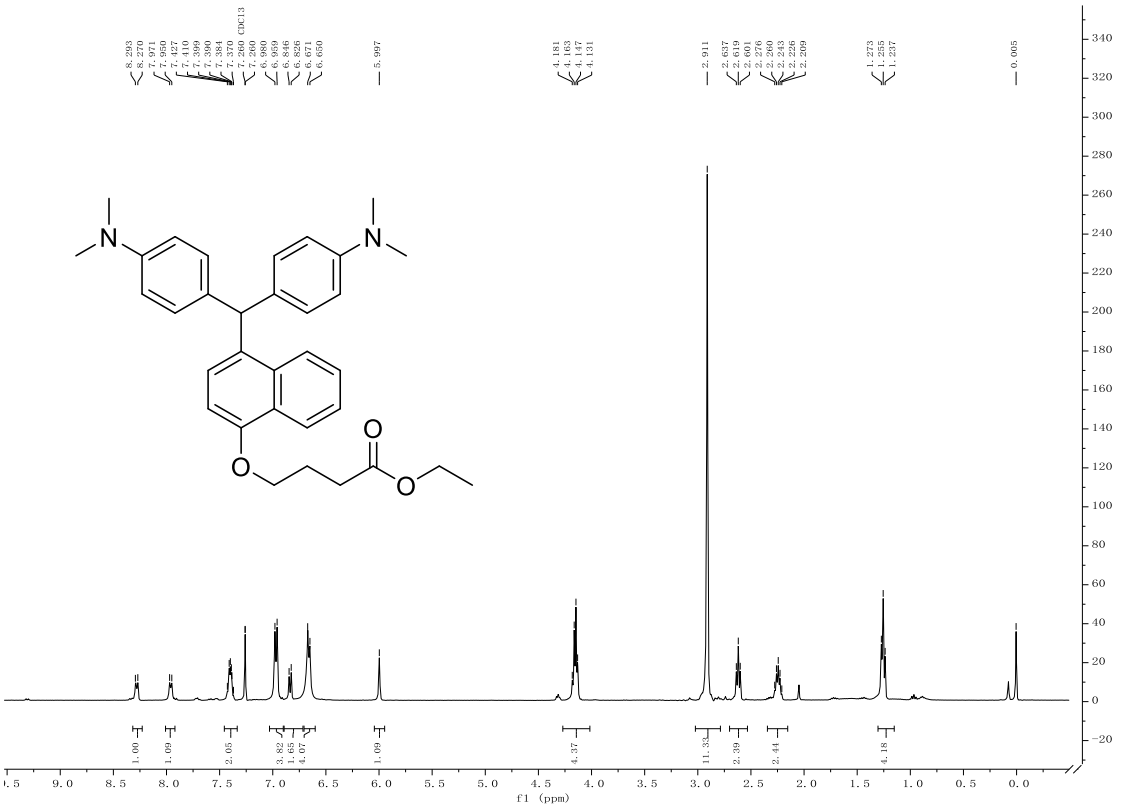
^{13}C NMR (125 MHz, Chloroform-*d*) of MG **1**



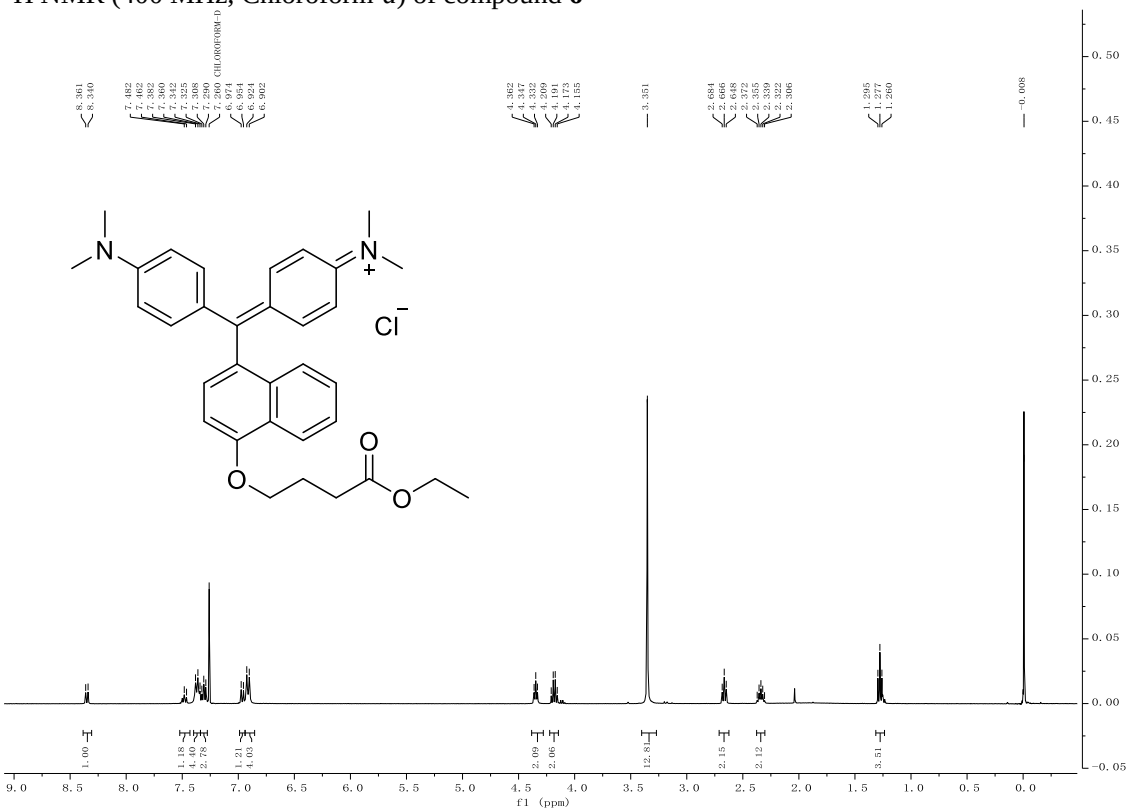
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **5**



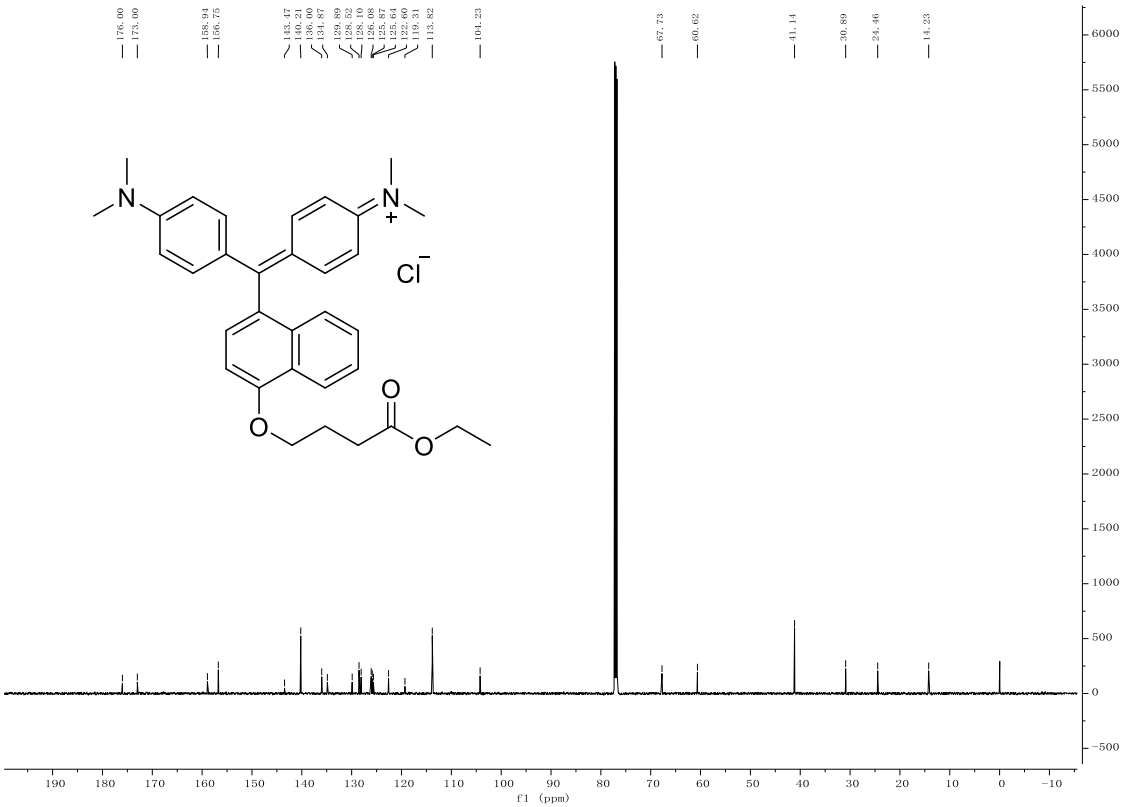
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **5**



^{1}H NMR (400 MHz, Chloroform-*d*) of compound **6**

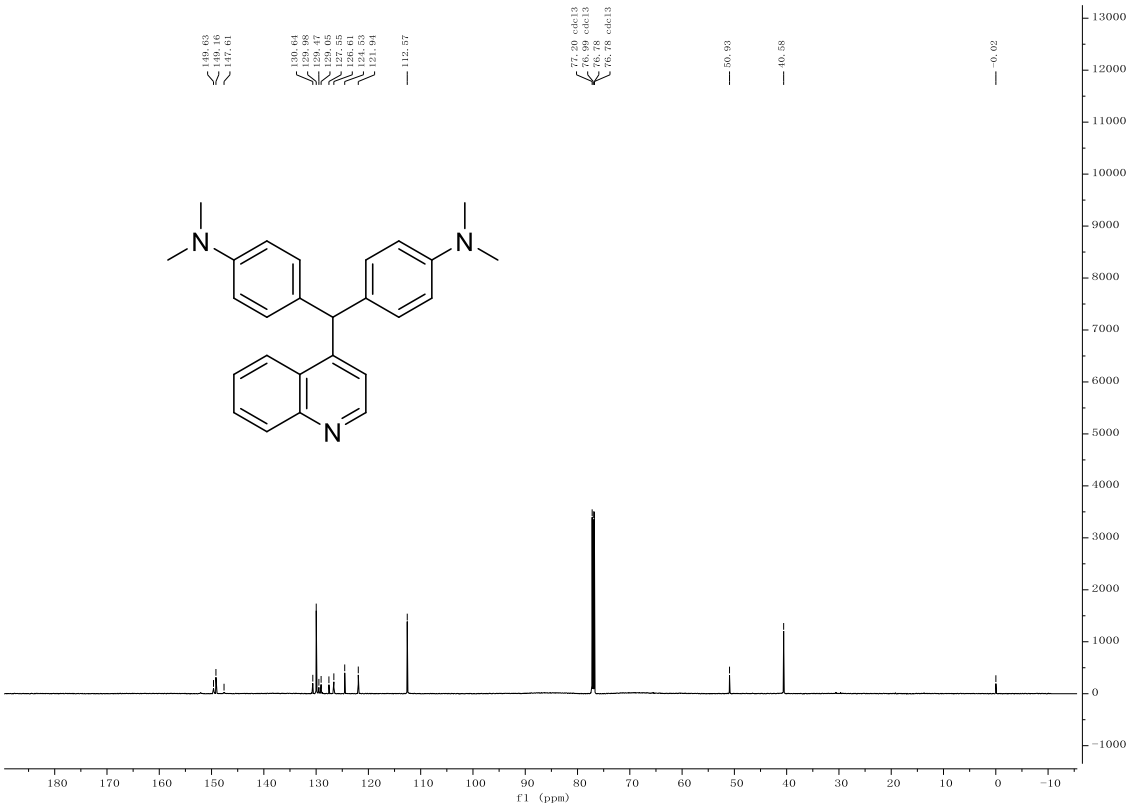


^{1}H NMR (400 MHz, Chloroform-*d*) of **MG-2**

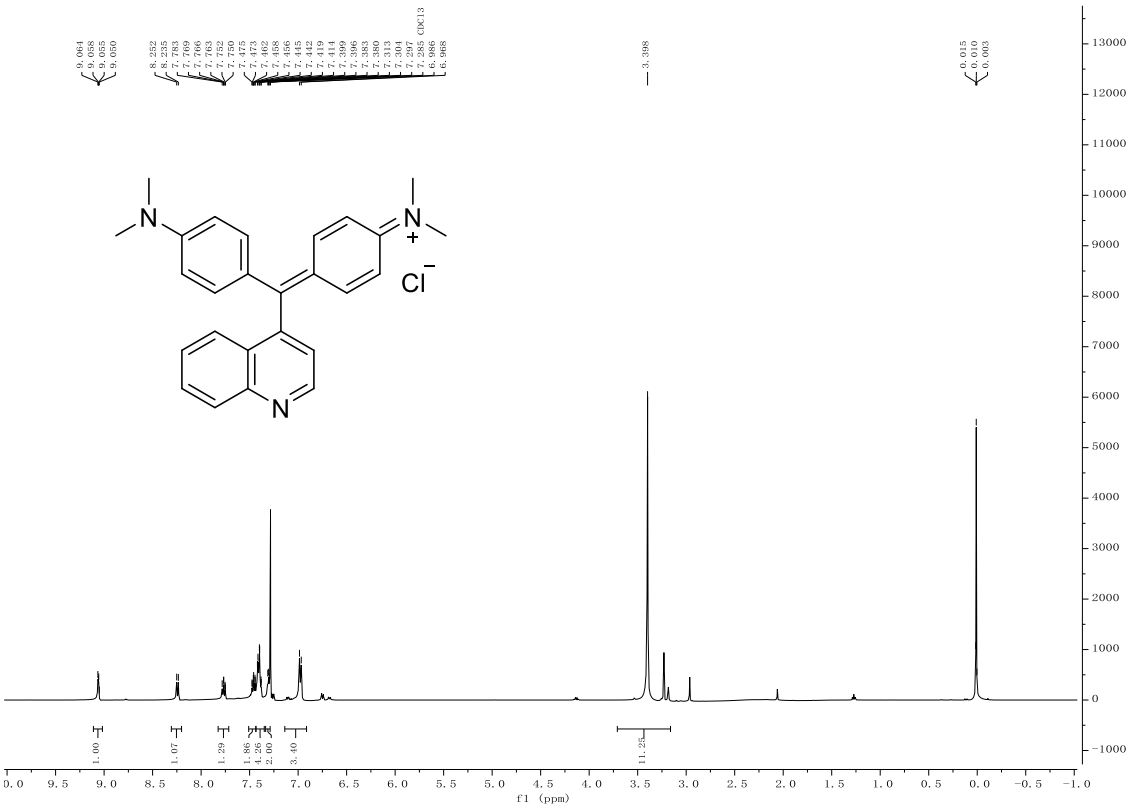


^{13}C NMR (150 MHz, Chloroform-*d*) of **MG-2**

^{1}H NMR (400 MHz, Chloroform-*d*) of compound **8**

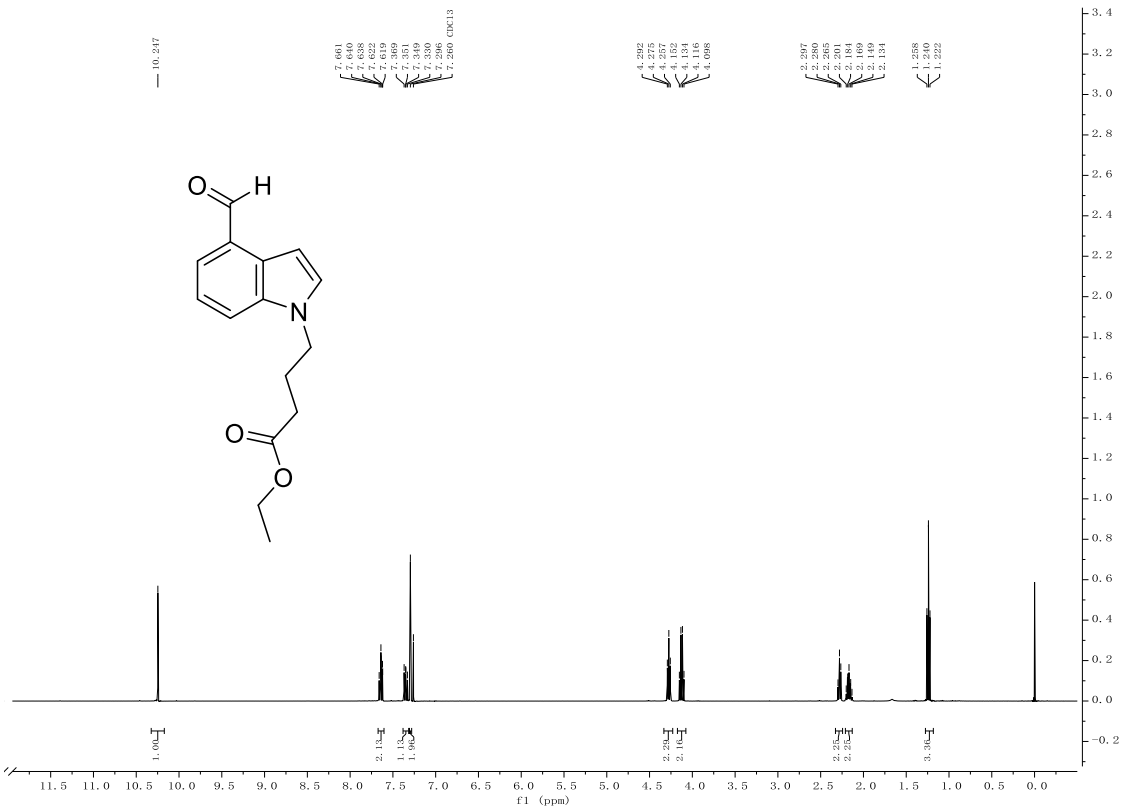


^{13}C NMR (150 MHz, Chloroform-*d*) of compound **8**

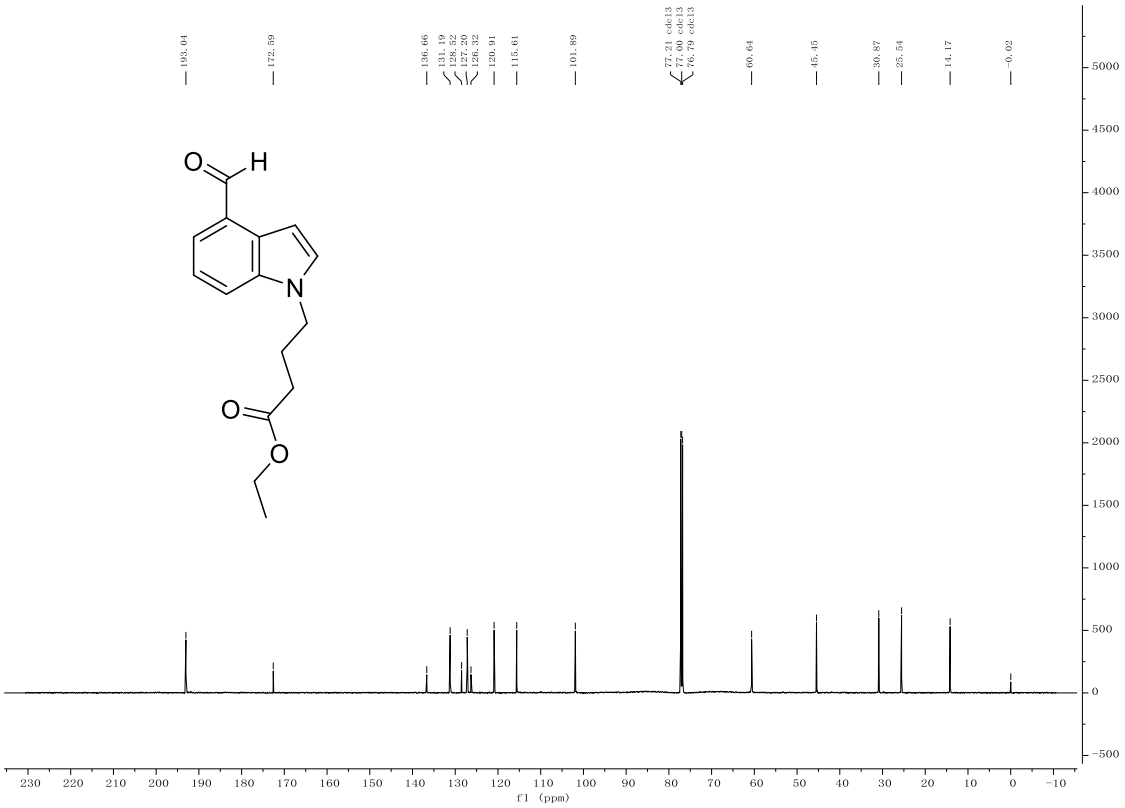


^{1}H NMR (500 MHz, Chloroform-*d*) of MG **3**

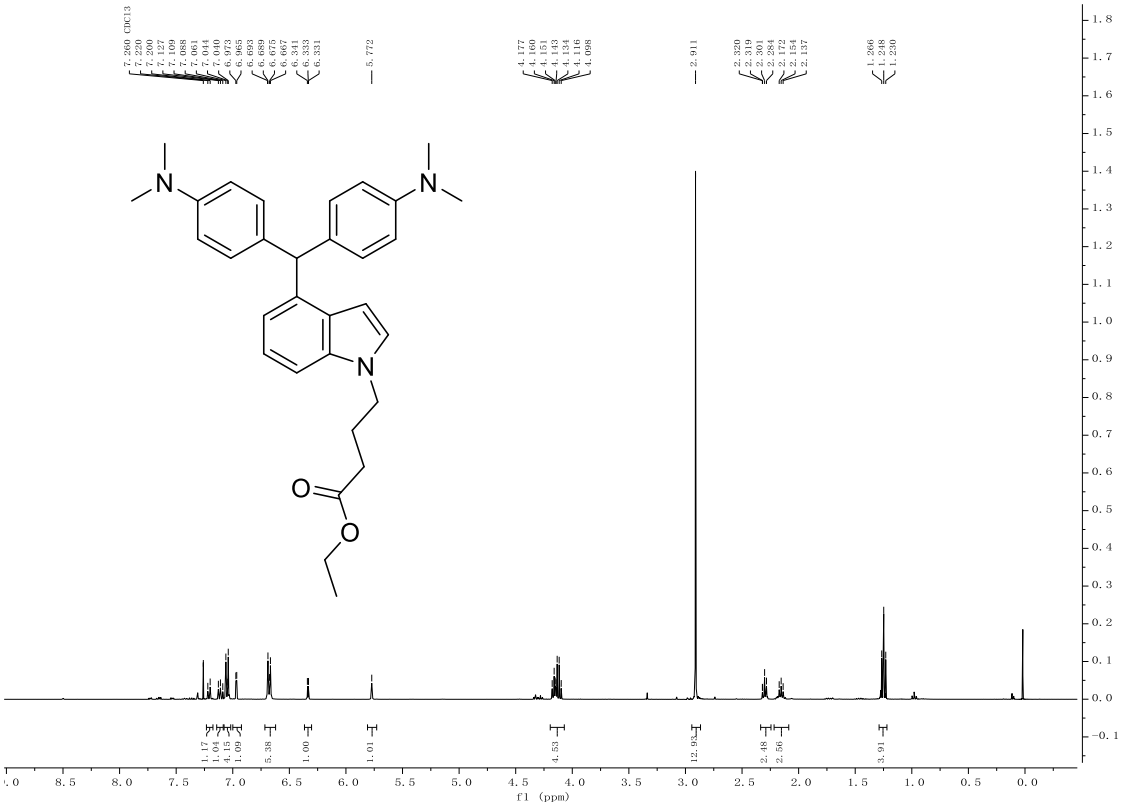
^{13}C NMR (125 MHz, Chloroform-*d*) of MG **3**



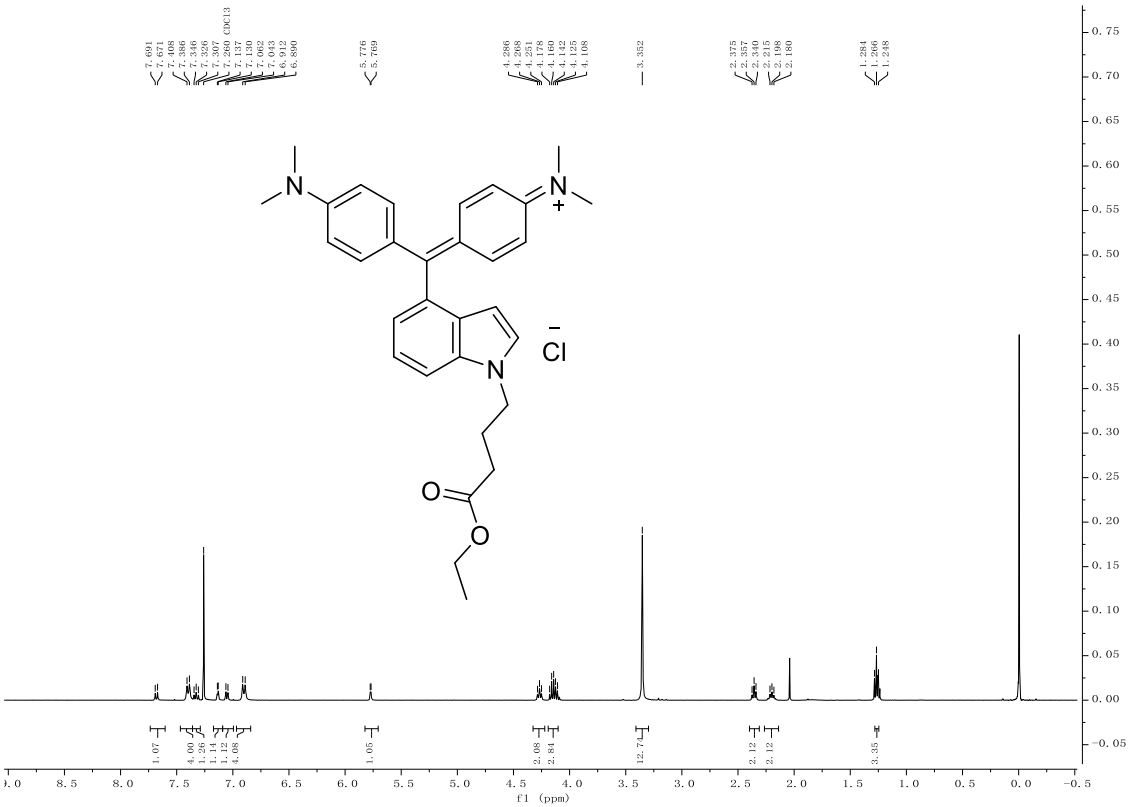
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **10**



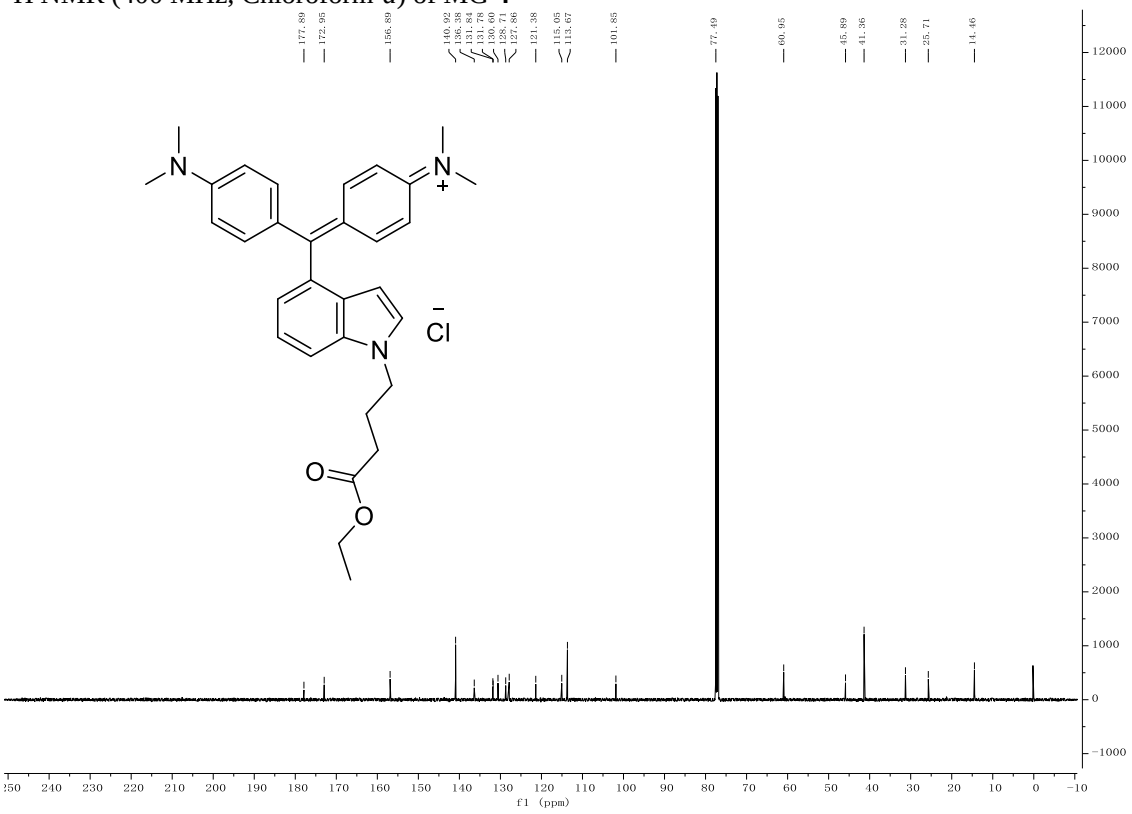
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **10**



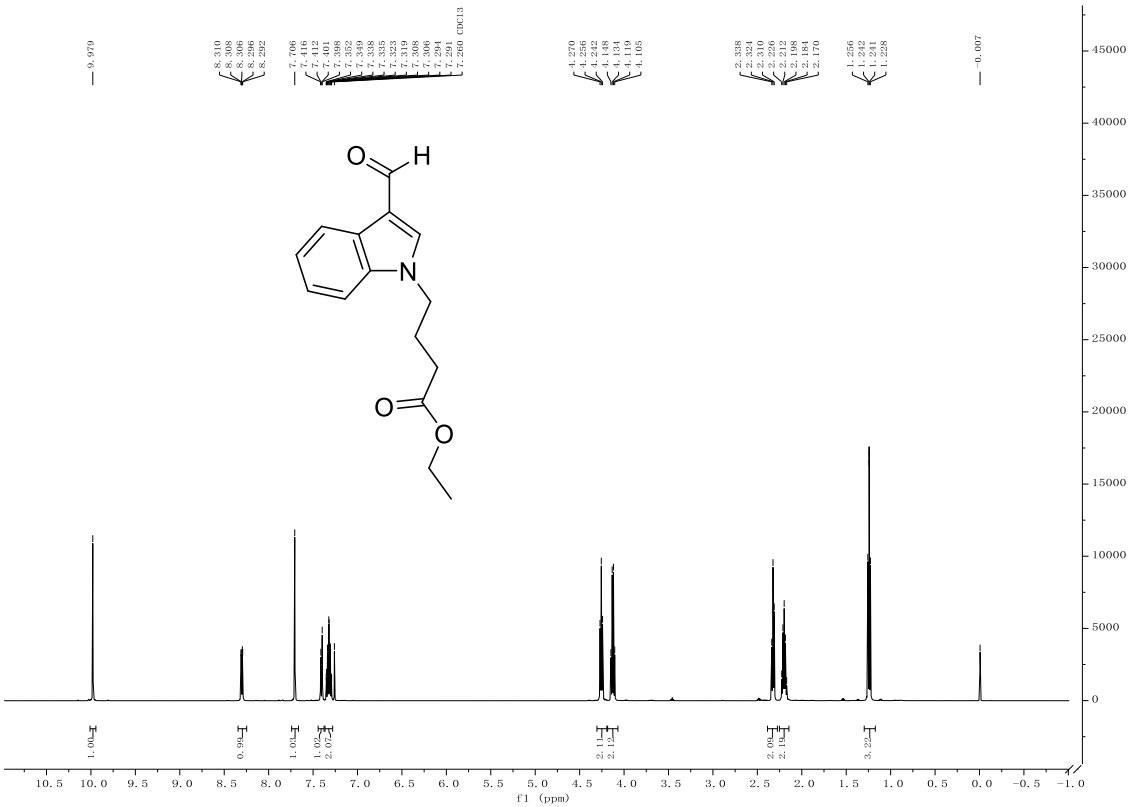
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **11**



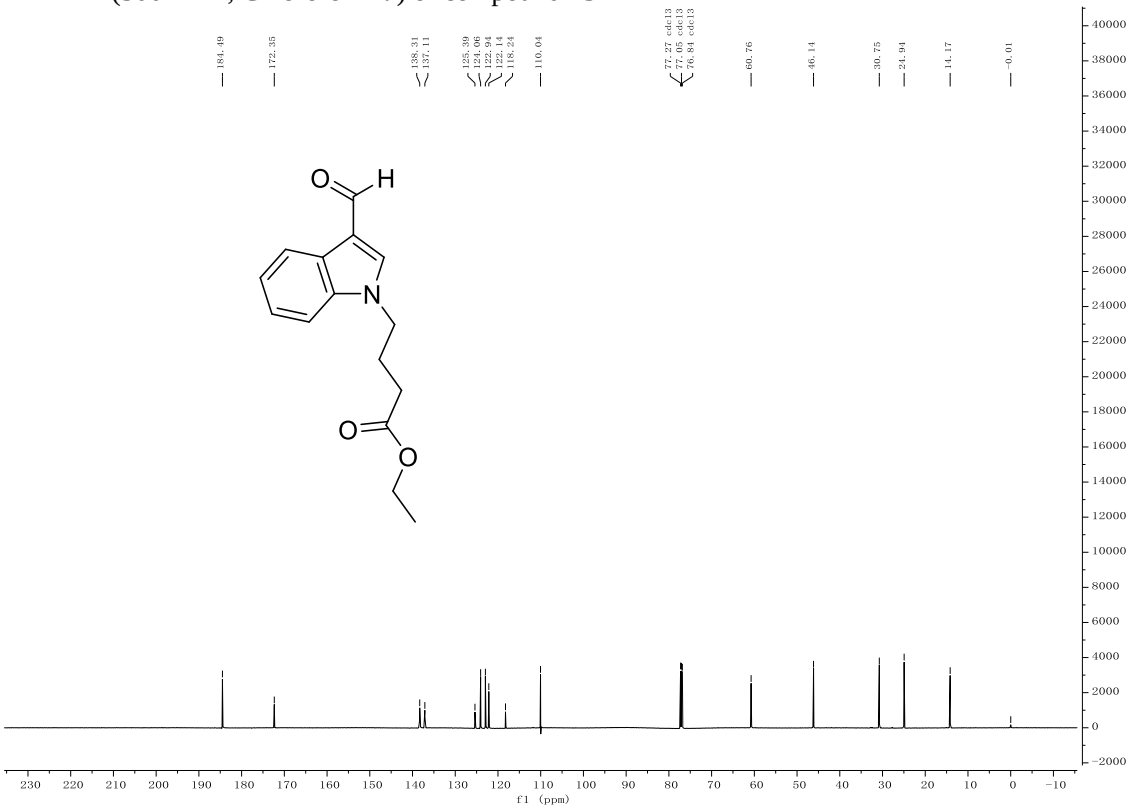
^{1}H NMR (400 MHz, Chloroform-*d*) of MG **4**



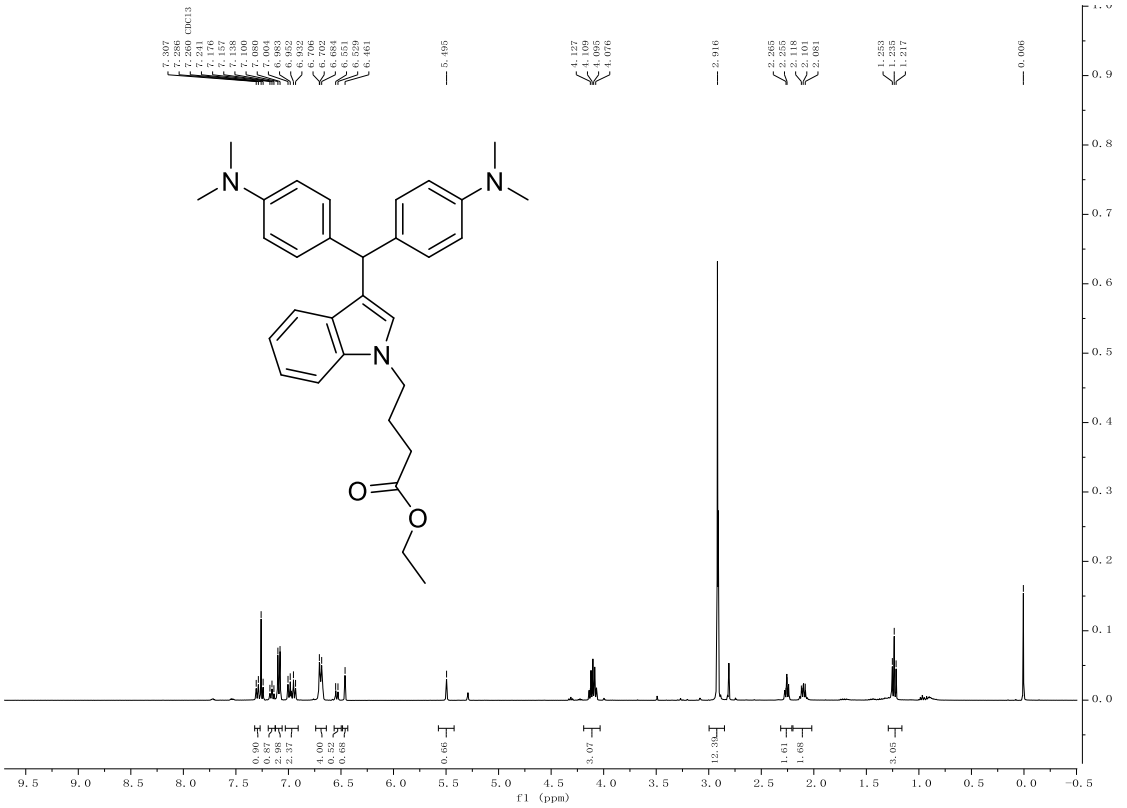
^{13}C NMR (125 MHz, Chloroform-*d*) of MG **4**



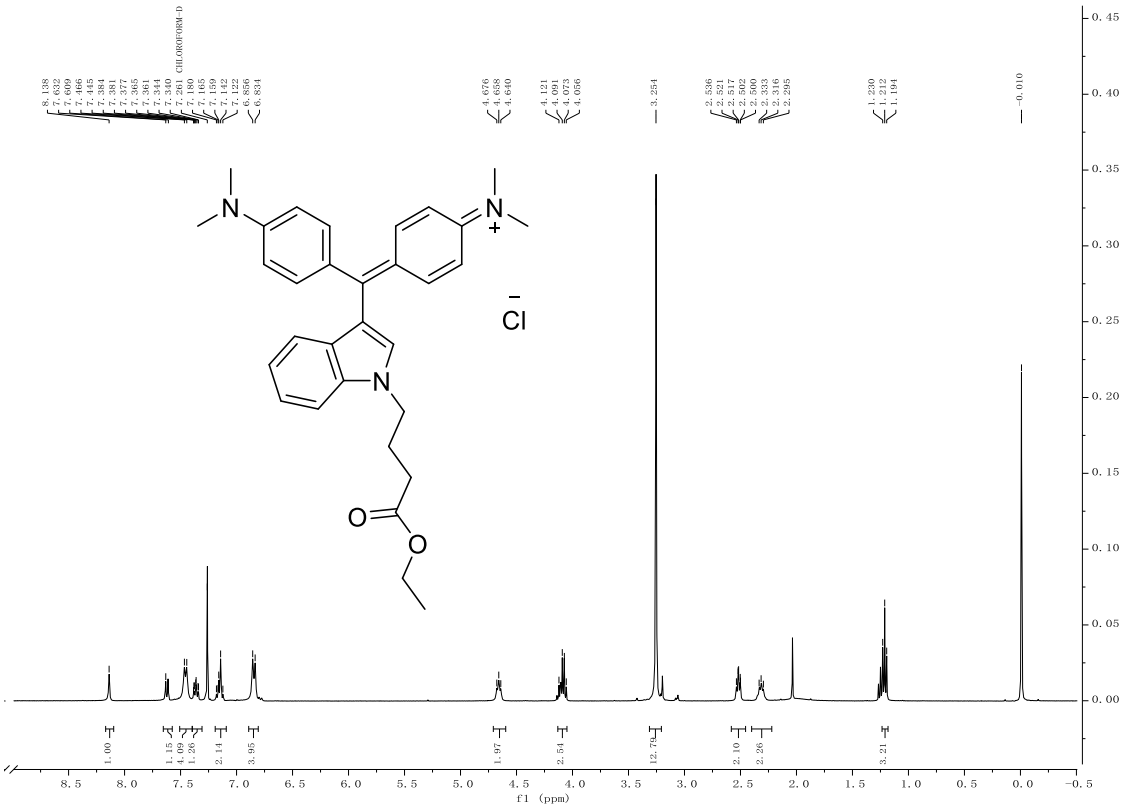
^{1}H NMR (500 MHz, Chloroform-*d*) of compound **13**



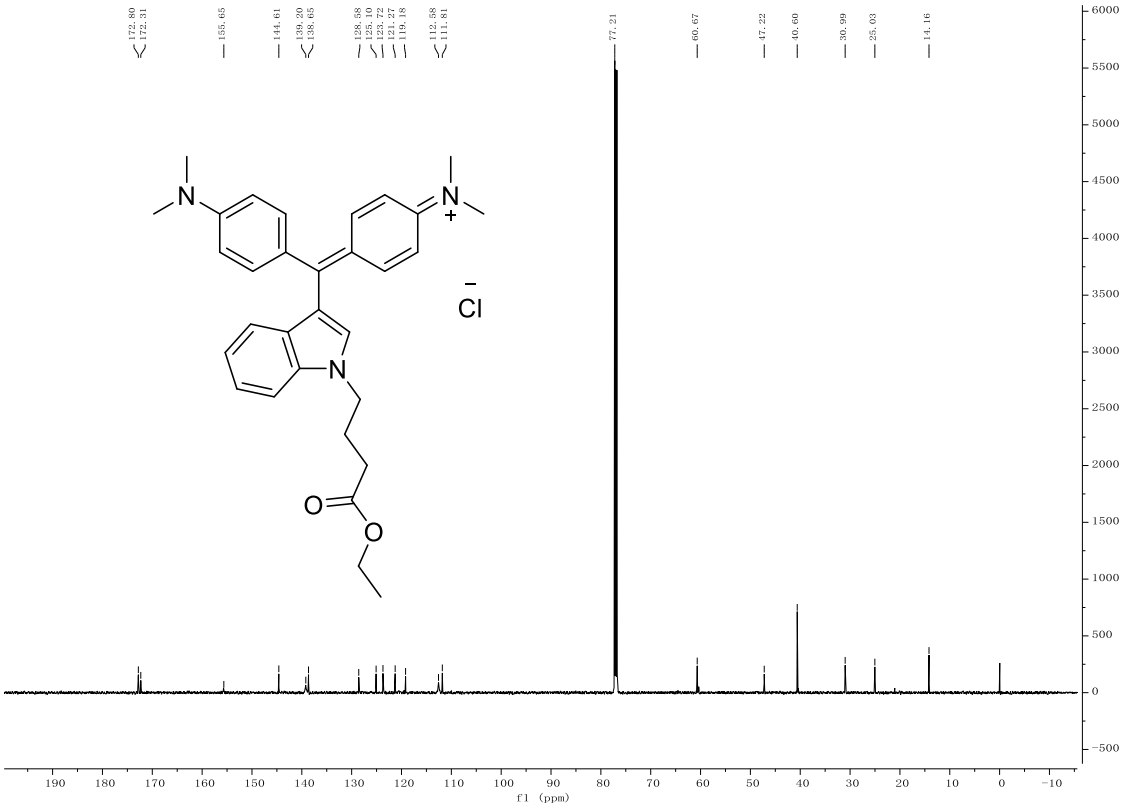
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **13**



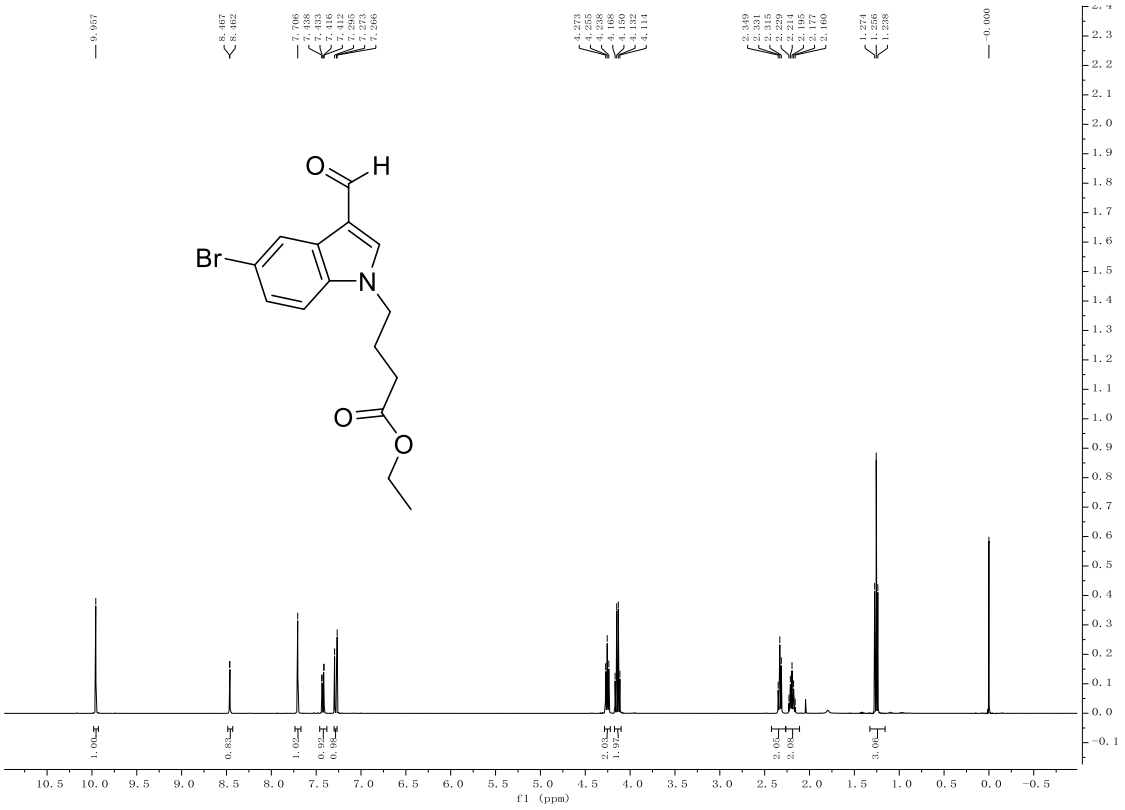
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **14**



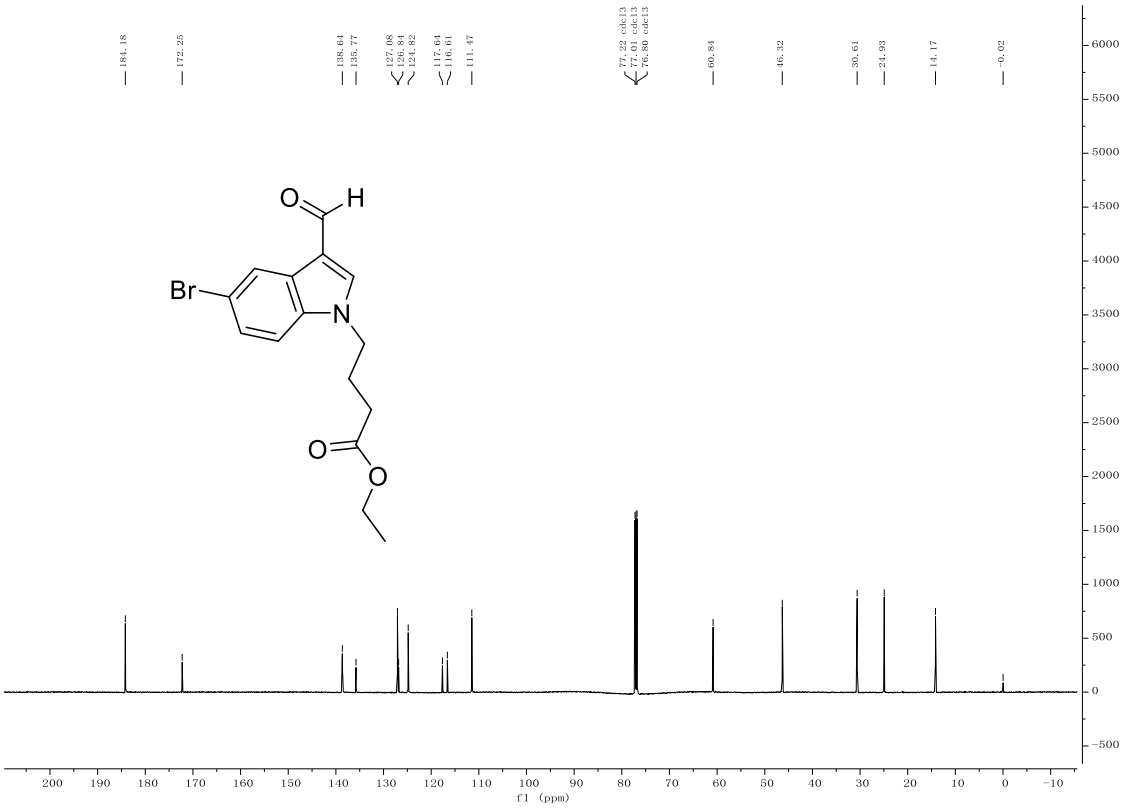
^{1}H NMR (400 MHz, Chloroform-*d*) of MG **5**



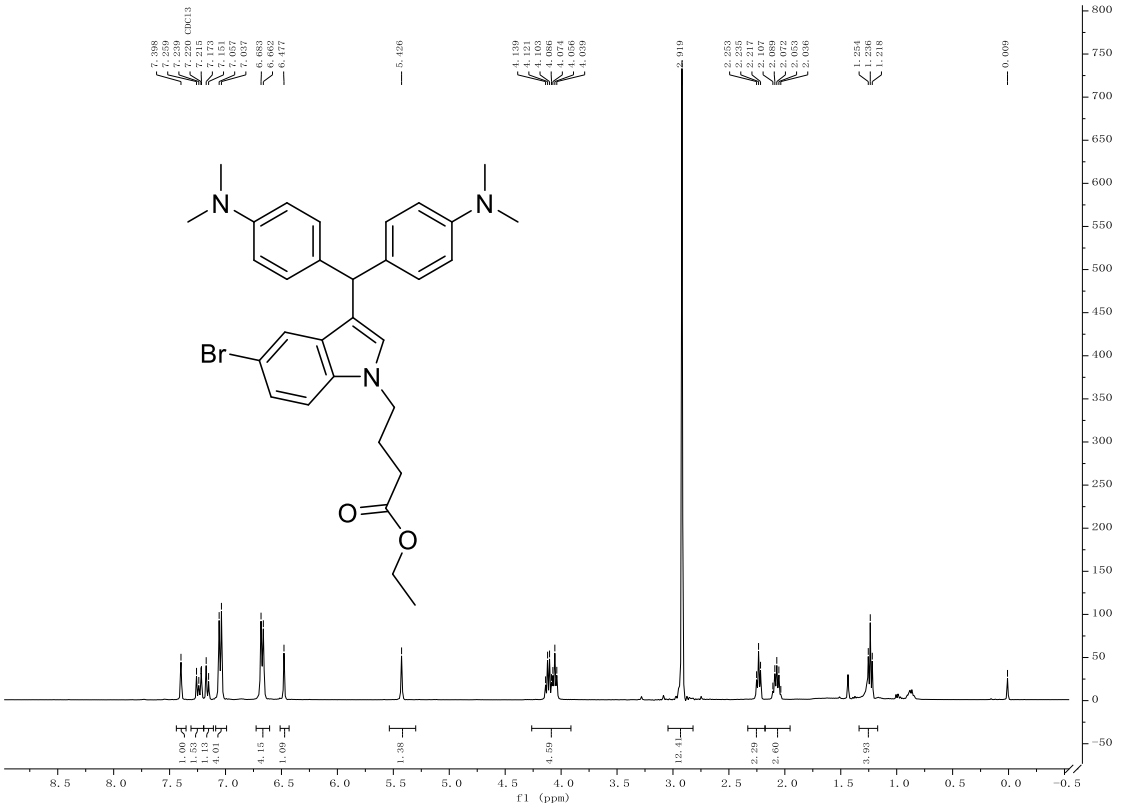
^{13}C NMR (150 MHz, Chloroform-*d*) of MG **5**



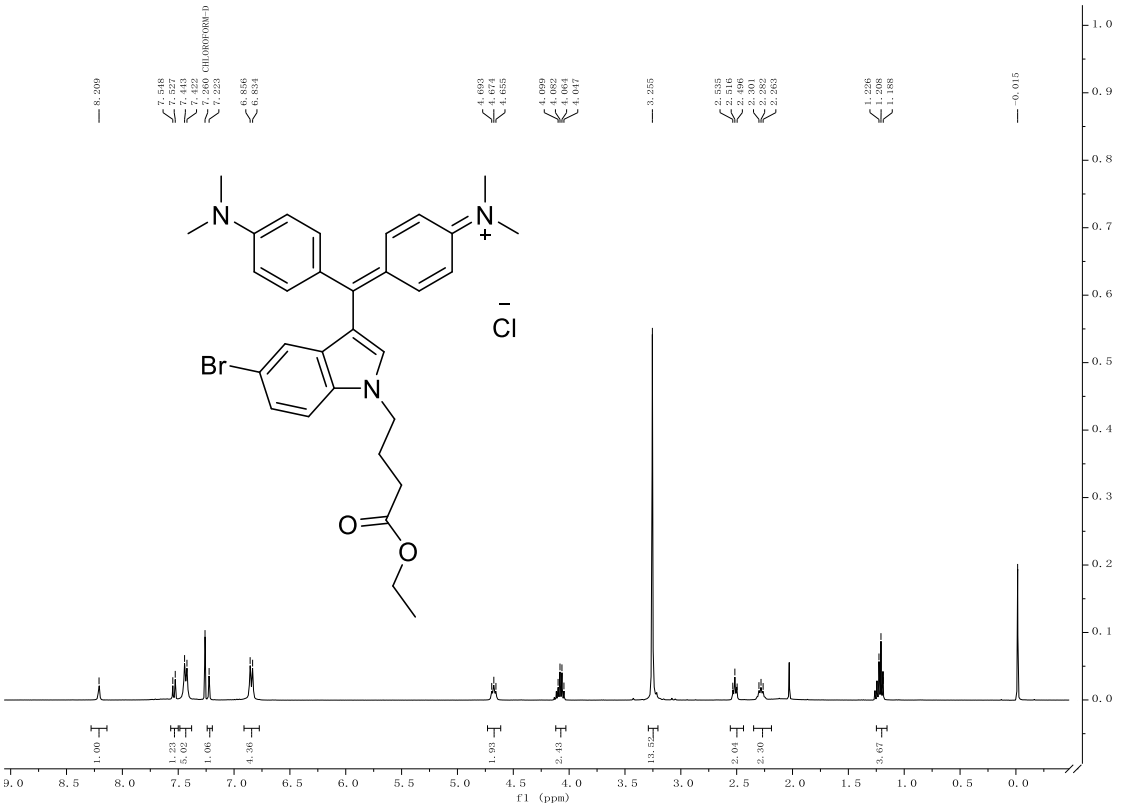
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **16**



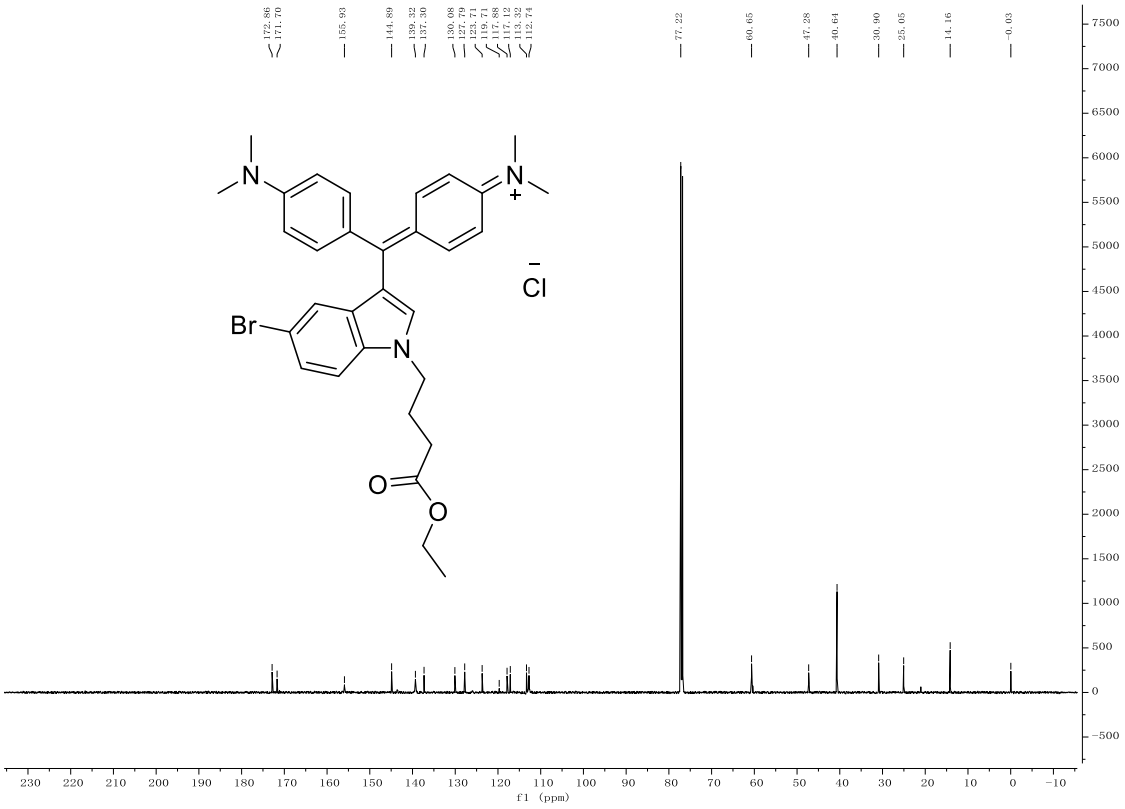
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **16**



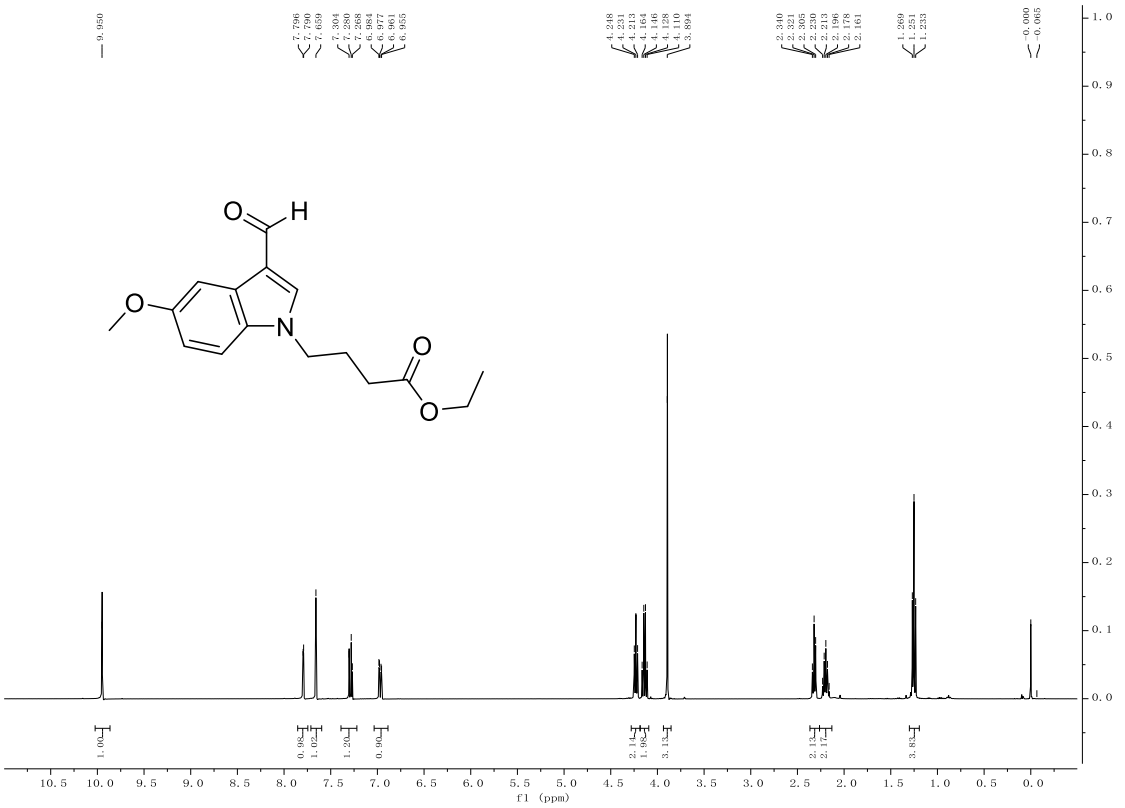
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **17**



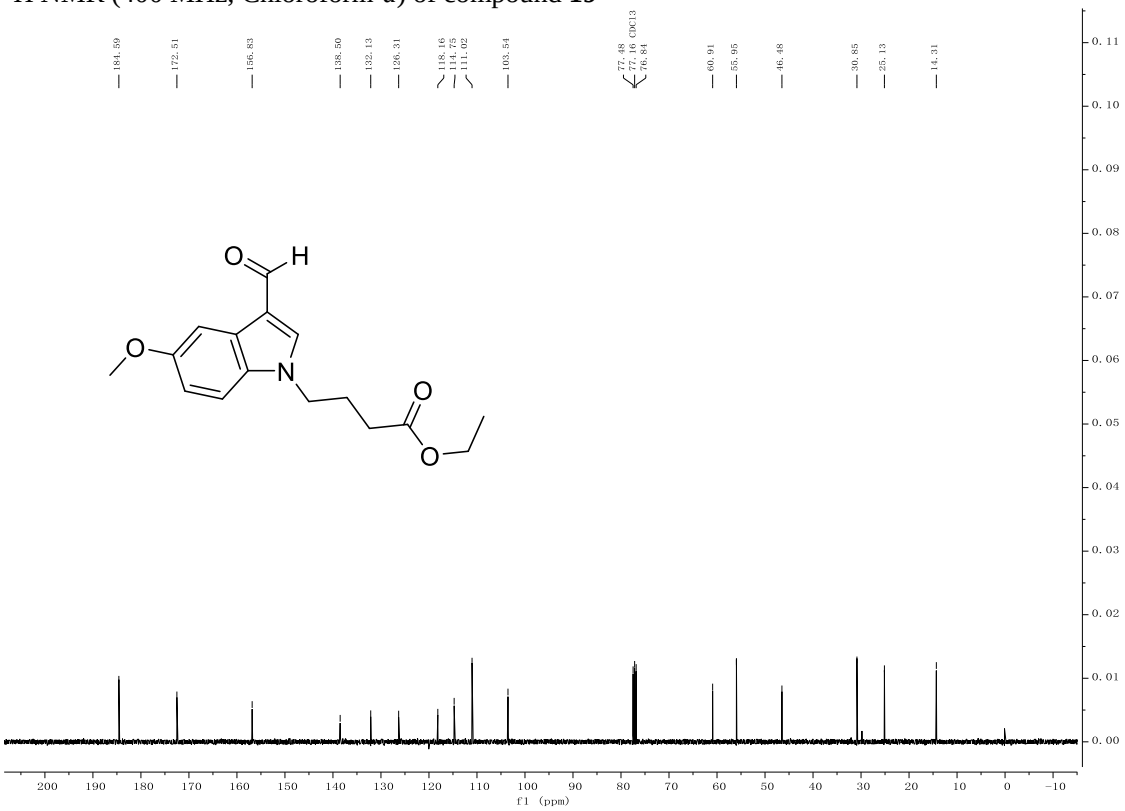
^{1}H NMR (400 MHz, Chloroform-*d*) of MG **6**



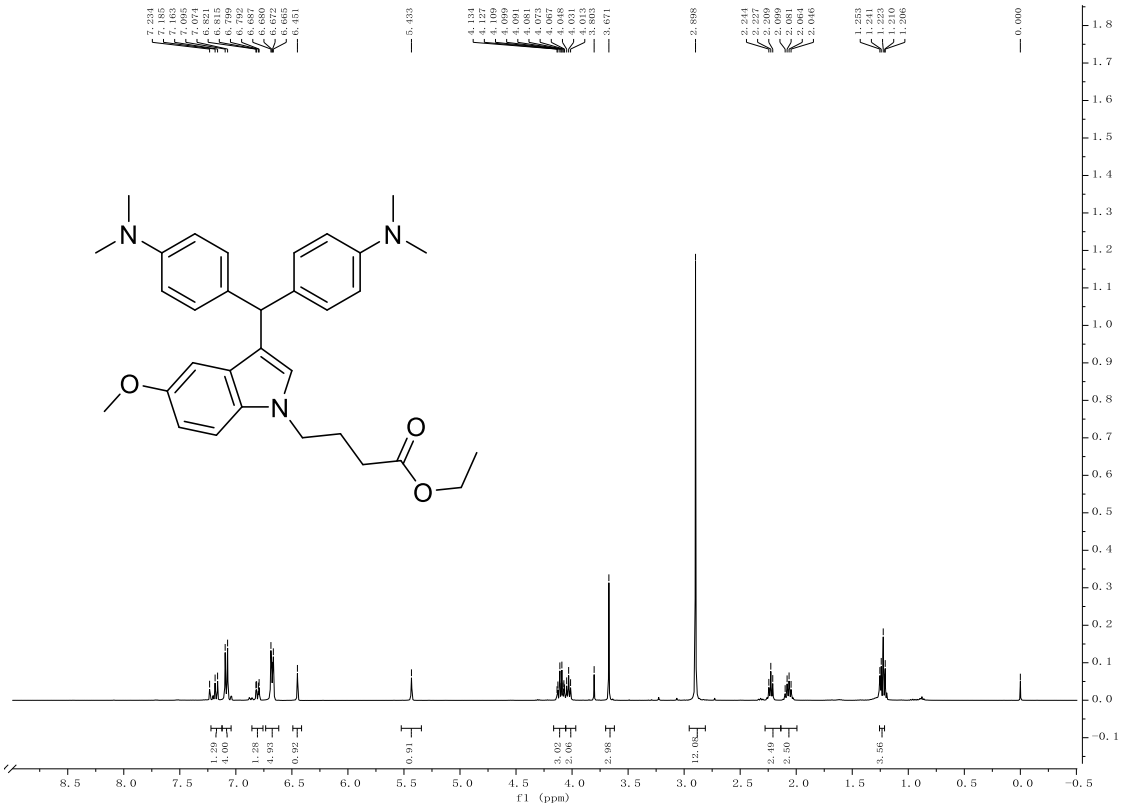
^{13}C NMR (150 MHz, Chloroform-*d*) of MG **6**



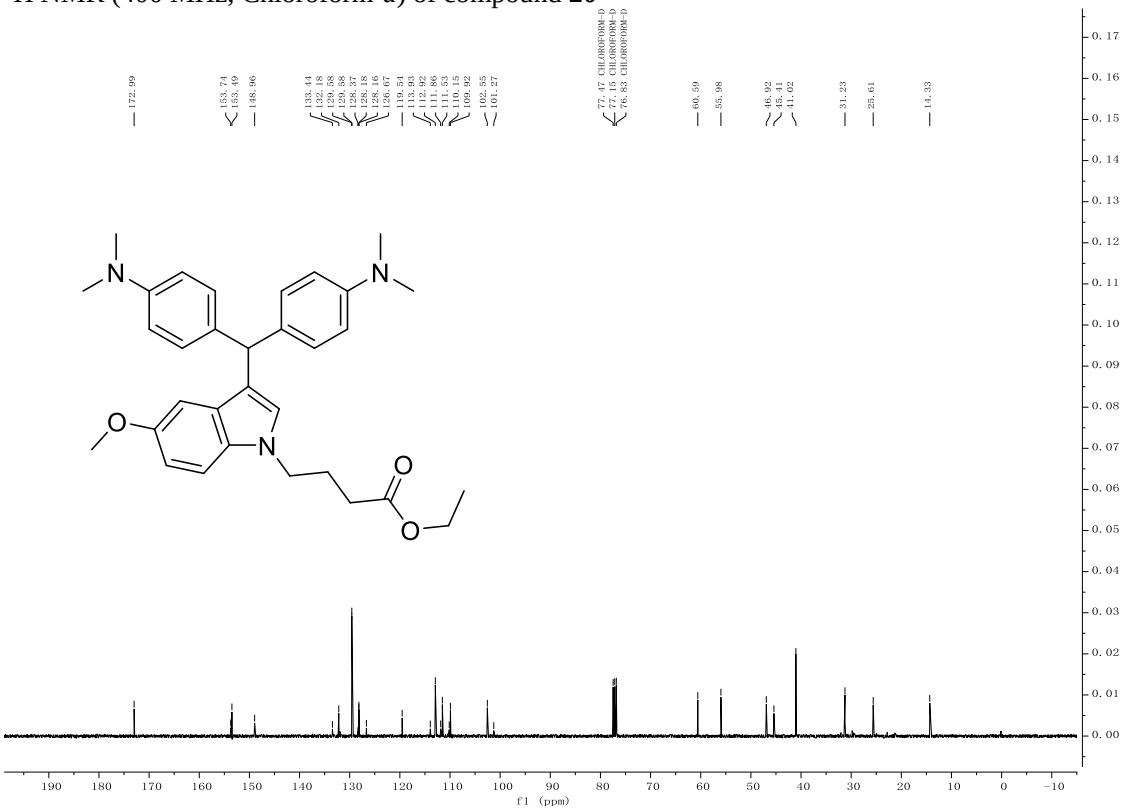
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **19**



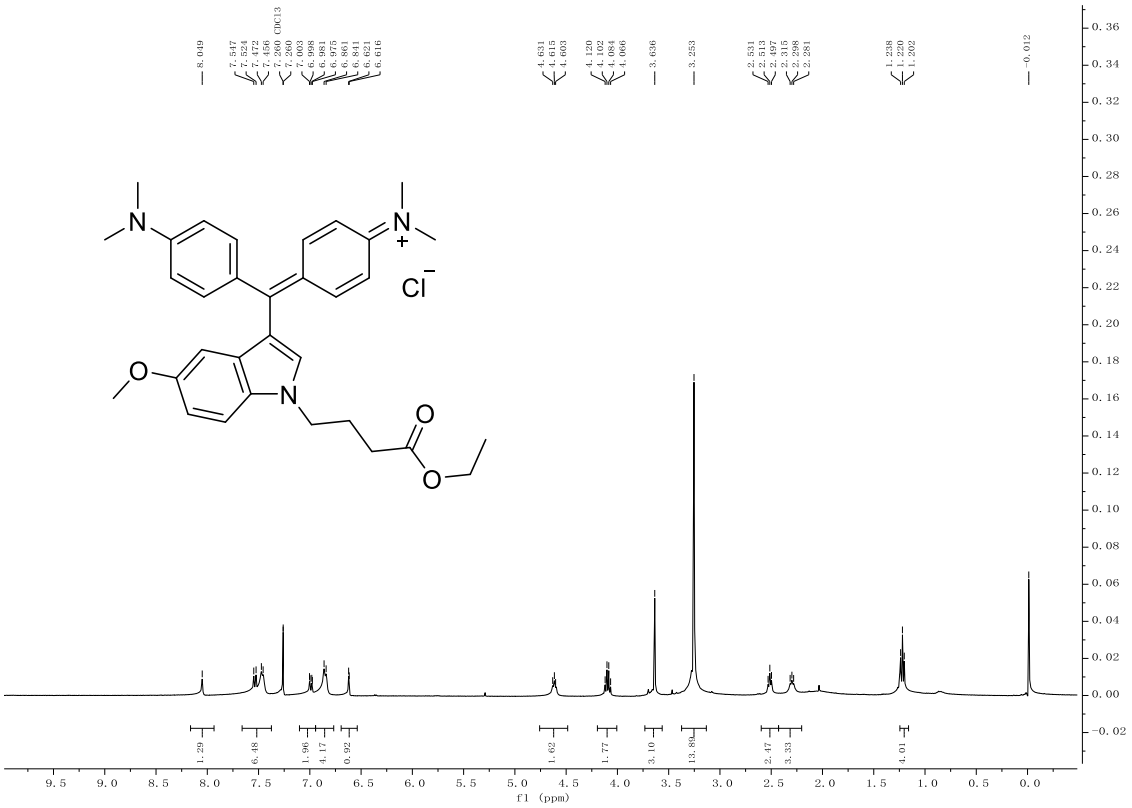
^{13}C NMR (100 MHz, Chloroform-*d*) of compound **19**



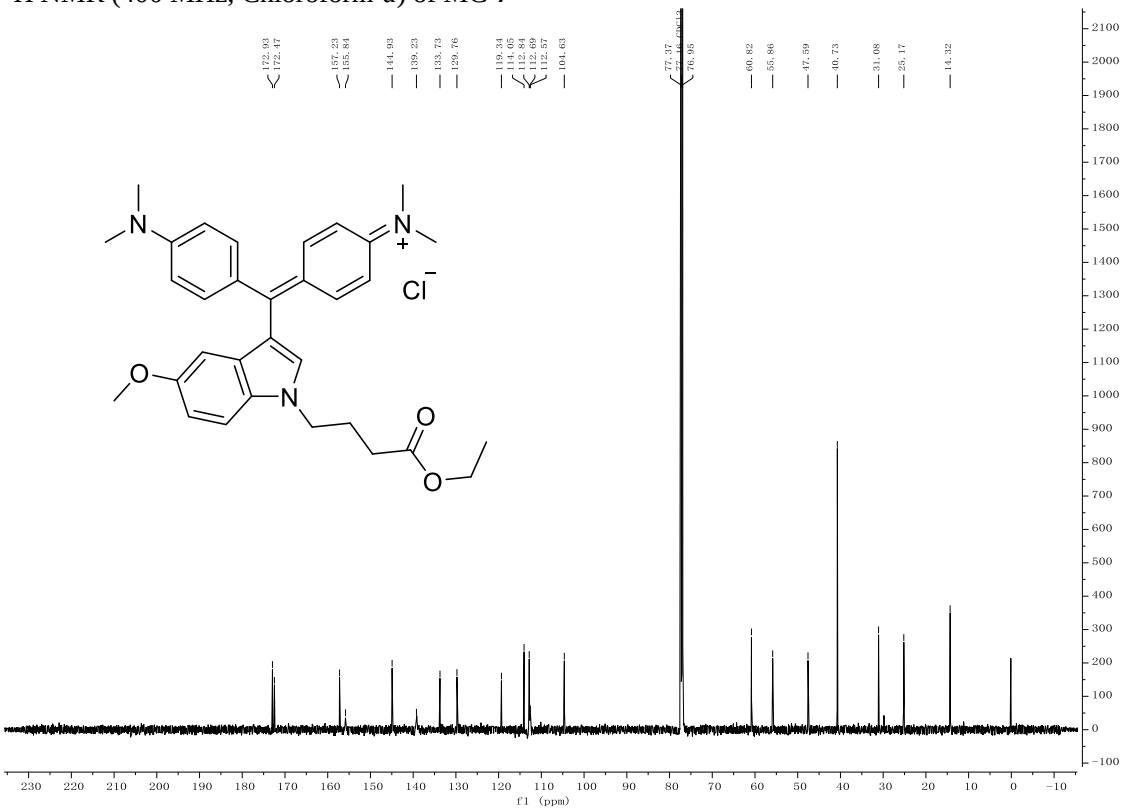
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **20**



^{13}C NMR (100 MHz, Chloroform-*d*) of compound **20**



^{1}H NMR (400 MHz, Chloroform-*d*) of MG **7**



^{13}C NMR (150 MHz, Chloroform-*d*) of MG **7**