Supporting Information

Harnessing J-Aggregation for Dual-Color Cellular Imaging with Chromenoquinoline-Benzimidazole Dyes

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1. General methods

Computational Methods: DFT and TD-DFT calculations were performed with *Gaussian 16.* The ground state (S_0) geometries were optimized at the B3LYP/6-311G+(d,p) level in vacuum. The molecular properties were also investigated using Multiwfn 3.6. the planes defined by the chromenoquinoline scaffold and the benzimidazole moiety were visualized using VMD (Visual Molecular Dynamics) program.

The fluorescent quantum yields in solution were measured on a Hitachi F-7000 spectrophotometer using a standard reference and calculated from the following equation:

$$\Phi_u = \Phi_S \frac{F_u}{F_s} \frac{A_s}{A_u} \frac{n_u^2}{n_s^2}$$

Φ denotes the fluorescent quantum yield; F means the integral intensity of fluorescence, A refers to the absorbance at the excitation wavelength and n is the refraction index of solvents. *u* and *s* represent the testing and the standard samples, respectively.

Cell Culture: Human cervical carcinoma cells (HeLa) and HEK-293T cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, China). The cells were cultured in a Dulbecco's modified Eagle's medium (DMEM, glucose 4.5 g/L, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), streptomycin at 100 mg/mL, penicillin at 100 U/mL, 4 mM L-glutamine at 37 °C in 5% CO_2.

Cell Viability Assay: Cell viability was assessed using the MTT assay. Briefly, cells (5, 000 cells/well) were seeded in 96-well plates. The plates were maintained at 37 °C in a 5% CO_2 incubator for 24 h, followed by exposure to 200 μL of medium containing various concentrations of **5a-Me** with various concentrations (0, 1, 2, 5, 10 and 20 μM), respectively. After 12 h incubation, the culture media were removed, and an MTT solution (20 μL, 5 mg/mL) and 180 μL of fresh medium were added to each well. After 4 h, the medium was removed, and 150 μL DMSO was added to dissolve the formazan crystals (10 min incubation in the dark). The absorbance at 570 nm for each well was measured on a microplate reader, and the cell viability was calculated.

2. Synthetic methods

Synthesis of 2a/2b

Compound **1a**/**1b** (10 mmol), 3-bromopropyne 2.38g (20 mmol), potassium carbonate 4.15g (30 mmol) were dissolved in 30 mL acetone. The mixture was refluxed in the dark at 55 ℃ for 12 h, after which the solvent was evaporated under reduced pressure. The residues were purified by chromatography on silica gel with DCM/EA = 100:10 as eluent to give pure products **2a** and **2b**.

Synthesis of 3a/3b

Compound **2a**/**2b** (4 mmol), 4-aminobenzyl alcohol 0.738g (6 mmol), lanthanum trifluoromethanesulfonate 0.234g (0.4mmol) and cupric iodide 0.762g (4 mmol) were dissolved in 15 mL acetonitrile. The mixture was refluxed at 70 ℃ for 5 h, after which the solvent was evaporated under reduced pressure. The residues were purified by chromatography on silica gel with DCM/EA = 100:10 as eluent to give pure products **3a** and **3b**.

3b: Yield 32%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.99 (s, 1H), 7.87 (d, J = 8.6 Hz, 1H), 7.77 – 7.67 (m, 2H), 7.60 (dd, J = 8.7, 2.0 Hz, 1H), 5.26 (s, 2H), 4.66 (d, J = 5.3 Hz, 2H), 3.20 (dt, J = 8.7, 5.5 Hz, 4H), 2.82 – 2.72 (m, 2H), 2.63 (t, J = 6.5 Hz, 2H), 2.01 (q, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.73, 154.08, 149.70, 147.59, 146.06, 139.64, 130.72, 130.12, 128.95, 128.29, 126.66, 124.79, 122.82, 115.84, 110.51, 107.51, 68.15, 63.21, 49.83, 49.26, 27.39, 22.56, 22.06, 21.20. HRMS (ESI) m/z for $[C_{23}H_{22}N_2O_2+H]^+$: calculated, 359.1681; found, 359.1756.

Synthesis 4a/4b

Compound **3a**/**3b** (1.2 mmol) and manganese dioxide 1.46g (16.8 mmol) were dissolved in 20 mL DCM. The mixture was stirred at room temperature for 5 h. After filtering the manganese dioxide with diatomite, the solvent was evaporated under reduced pressure. The residues were purified by chromatography on silica gel with DCM/EA = 100:5 as eluent to give pure products **4a** and **4b**.

4b: Yield: 78%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.12 (s, 1H), 8.46 (d, J = 1.8 Hz, 1H), 8.20 (s, 1H), 8.04 (dd, J = 8.7, 1.8 Hz, 1H), 7.98 (d, J = 8.7 Hz, 1H), 7.75 (s, 1H), 5.31 (d, J = 1.1 Hz, 2H), 3.23 (dt, J = 8.5, 5.4 Hz, 4H), 2.76 (t, J = 6.7 Hz, 2H), 2.64 (t, J = 6.5 Hz, 2H), 1.89 (q, J = 6.8, 5.9 Hz, 5H).^{13}C NMR (100 MHz, DMSO-d_6) δ 192.70, 154.74, 151.27, 148.06, 146.96, 134.10, 132.23, 131.33, 129.37, 127.64, 126.88, 126.11, 123.95, 116.21, 112.16, 107.18, 67.97, 49.84, 49.27, 22.56, 21.91, 21.01, 20.92. HRMS (ESI) m/z for $[C_{23}H_{20}N_2O_2+H]^+$: calculated, 356.1525; found, 356.1519.

Synthesis of 5a/5b

Compound **4a**/**4b** (0.9 mmol) was dissolved in 6 mL DMF, followed the addition of *o*-phenylenediamine 0.103g (0.95 mmol) and sodium metabisulfite 0.171g (0.9 mmol). The mixture was stirred at 110 ℃ for 4 h. Upon cooling to room temperature, distilled water was added to quench the reaction and then extracted with DCM (3x50 mL). The organic phase was combined and dried over anhydrous Na_2SO_4. After removal of DCM under reduced pressure, the residue was purified by chromatography on silica gel with DCM as eluent to give pure products **5a** and **5b**.

5a: Yield 57%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (d, J = 2.1 Hz, 1H), 8.46 (dd, J = 8.8, 2.0 Hz, 1H), 8.17 – 8.11 (m, 2H), 8.05 (d, J = 8.8 Hz, 1H), 7.67 – 7.60 (m, 2H), 7.27 – 7.20 (m, 2H), 6.53 (dd, J = 9.0, 2.5 Hz, 1H), 5.75 (s, 1H), 5.35 (d, J = 1.1 Hz, 2H), 3.40 (q, J = 7.1 Hz, 4H), 1.14 (t, J = 7.0 Hz, 7H).^{13}C NMR (100 MHz, DMSO-d_6) δ 159.59, 151.46, 150.53, 148.82, 131.52, 129.15, 127.84, 127.02, 126.19, 125.77, 122.69, 110.43, 107.13, 98.24, 79.76, 79.43, 79.10, 68.13,

60.20, 55.32, 44.39, 13.00. HRMS (ESI) m/z for $[C_{27}H_{24}N_4O+H]^+$: calculated, 421.1950; found, 421.2022.

5b: Yield 49%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.63 (d, J = 2.0 Hz, 1H), 8.44 (dd, J = 8.8, 2.0 Hz, 1H), 8.11 (s, 1H), 8.03 (d, J = 8.8 Hz, 1H), 7.75 (s, 1H), 7.64 (t, J = 4.6 Hz, 2H), 7.30 – 7.19 (m, 2H), 5.36 – 5.27 (m, 2H), 3.26 – 3.16 (m, 5H), 2.77 (t, J = 6.4 Hz, 2H), 2.65 (t, J = 6.5 Hz, 2H), 1.93 – 1.85 (m, 4H).^{13}C NMR (100 MHz, DMSO-d_6) δ 170.80, 154.40, 153.82, 151.45, 150.95, 148.89, 146.47, 140.72, 134.60, 131.27, 130.10, 129.06, 127.77, 126.71, 126.14, 125.93, 123.62, 123.06, 116.05, 116.02, 110.16, 107.37, 68.08, 49.84, 49.27, 27.42, 22.56, 22.00, 21.22. HRMS (ESI) m/z for $[C_{29}H_{24}N_4O+H]^+$: calculated, 445.1950; found, 445.2034.

Synthesis of 5a-Me/5b-Me

Compound **5a**/**5b** (0.4 mmol) and iodomethane 0.114g (0.8 mmol) was dissolved in 10 mL DCM in a thick-walled reaction flask. The mixture was stirred at 50 ℃ for 12 h under dark. At the end of the reaction, petroleum ether was added to the solution and the products were precipitated by recrystallization, then filtered and the solids were washed with petroleum ether to give the red solid products **5a-Me** and **5b-Me**.

5a-Me: Yield 43%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.74 (s, 1H), 8.54 – 8.39 (m, 4H), 8.27 (d, J = 8.9 Hz, 2H), 8.15 (s, 1H), 7.88 (dd, J = 6.1, 3.1 Hz, 3H), 7.65 – 7.51 (m, 3H), 6.81 – 6.68 (m, 2H), 6.31 (s, 2H), 5.45 (d, J = 1.2 Hz, 3H), 3.48 (p, J = 6.9 Hz, 7H), 3.40 (s, 5H), 1.17 1.10 (m, 12H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.36, 148.45, 141.12, 135.81, 135.78, 135.76, 132.91, 132.16, 130.10, 129.11, 128.70, 127.40, 126.90, 126.52, 125.76, 121.38, 115.19, 114.95, 114.73, 113.88, 109.17, 67.36, 61.70, 53.33, 45.09, 33.58, 15.60, 13.00. HRMS (ESI) m/z for $[C_{28}H_{26}N_4O+H]^+$: calculated, 435.2107; found, 435.2190.

5b-Me: Yield 45%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (d, J = 2.1 Hz, 1H), 8.44 (dd, J = 8.9, 2.1 Hz, 1H), 8.37 (dd, J = 7.7, 1.5 Hz, 2H), 8.32 (d, J = 8.9 Hz, 1H), 7.87 (dd, J = 6.1, 3.1 Hz, 2H), 7.58 (dd, J = 6.1, 3.1 Hz, 2H), 5.52 (s, 2H), 3.76 (s, 3H), 3.25 – 3.18 (m, 4H),2.77 (t, J = 6.4 Hz, 1H), 2.65 (t, J = 6.5 Hz, 1H).^{13}C NMR (100 MHz, DMSO-d_6) δ 158.05, 151.09, 149.23, 149.16, 144.42, 133.49, 133.41, 133.06, 130.67, 129.12, 127.89, 127.48, 127.44, 126.24, 125.85, 122.97, 122.60, 122.01, 117.99, 114.73, 67.91, 49.84, 49.27, 32.00, 27.84, 25.39, 23.66, 22.33. HRMS (ESI) m/z for $[C_{30}H_{26}N_4O+H]^+$: calculated, 459.2107; found, 459.2180.

3. Photophysical properties of 5a, 5a-Me, 5b and 5b-Me

Table S1. The photophysical properties of **5a**, **5a-Me**, **5b**, **5b-Me** in toluene, ethanol, THF and DMF.

Dye	Solvent	λ_{abs}/ nm	λ_{em}/ nm	Δ_{ss}/ nm
5a	Toluene	420	508	88
	EtOH	420	498	78
	THF	418	476	58
	DMF	418	500	82
5a-Me	Toluene	504	539	35
	EtOH	424	534	110
	THF	418	483	65
	DMF	422	504	82
5b	Toluene	434	532	98
	EtOH	434	531	97
	THF	428	505	77
	DMF	432	534	102
5b-Me	Toluene	530	404	-126
	EtOH	442	532	90
	THF	434	511	77
	DMF	434	535	101

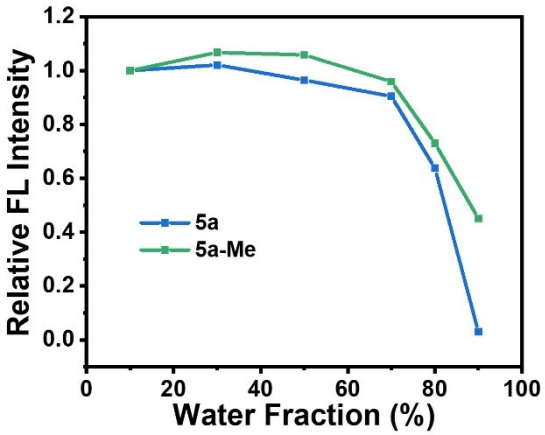

Fig. S1. Relative fluorescence intensities of **5a** and **5a-Me** in DMF solutions containing different proportions of water.

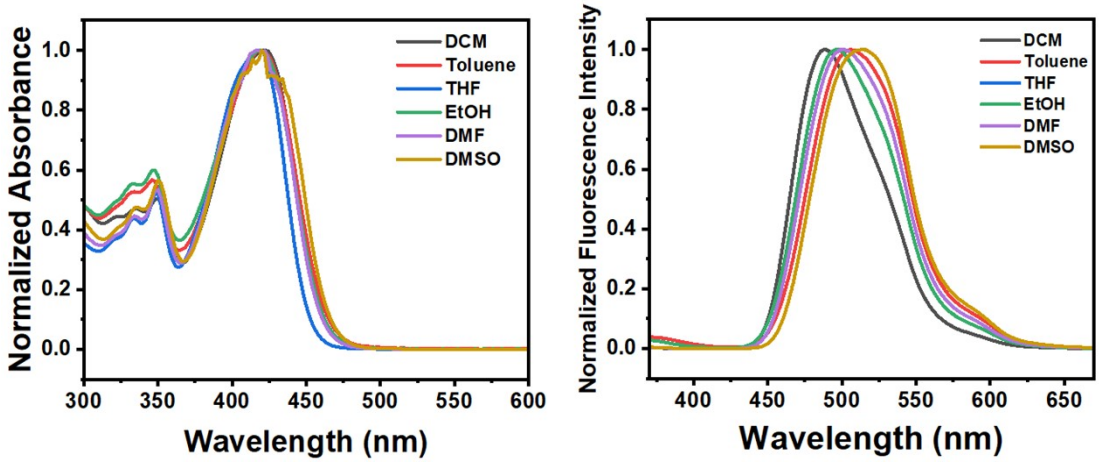

Fig. S2. Normalized absorption and emission spectra of dye **5a** in different solvents.

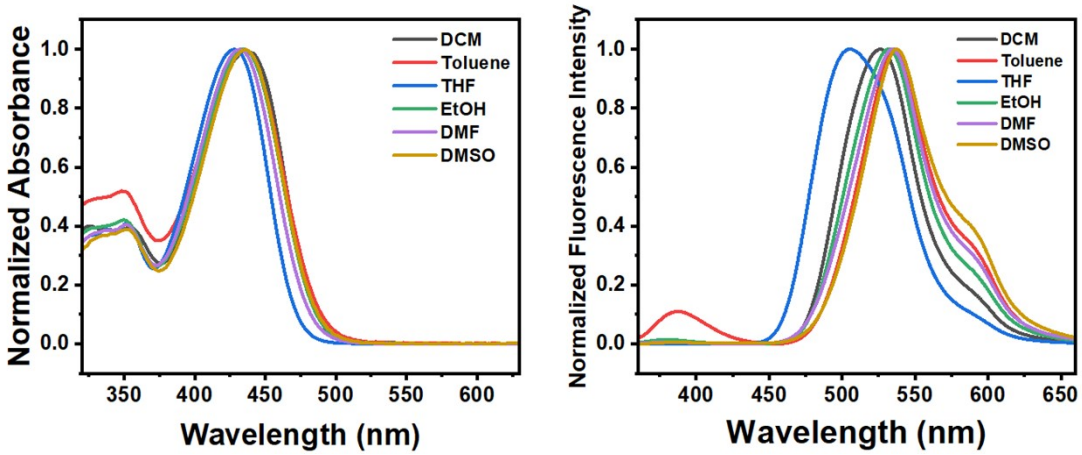

Fig. S3. Normalized absorption and emission spectra of dye **5b** in different solvents.

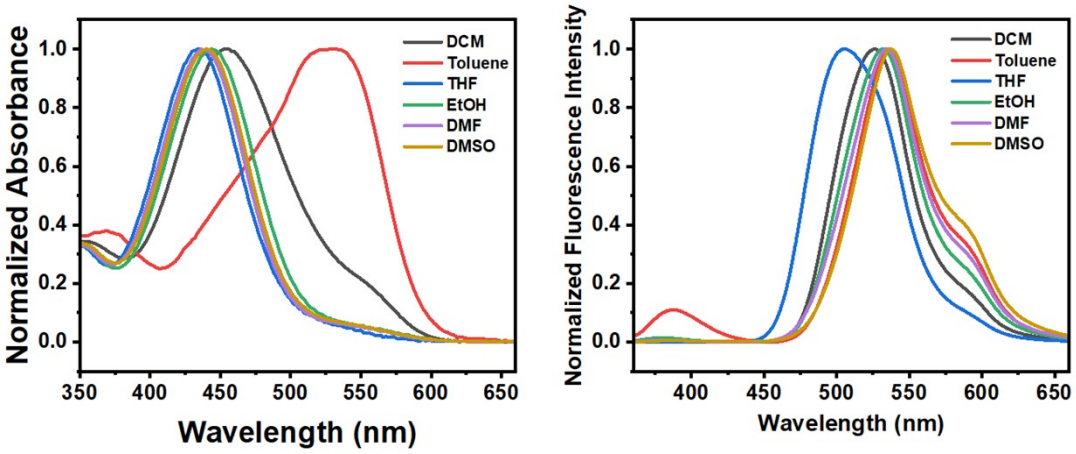

Fig. S4. Normalized absorption and emission spectra of dye **5b-Me** in different solvents.

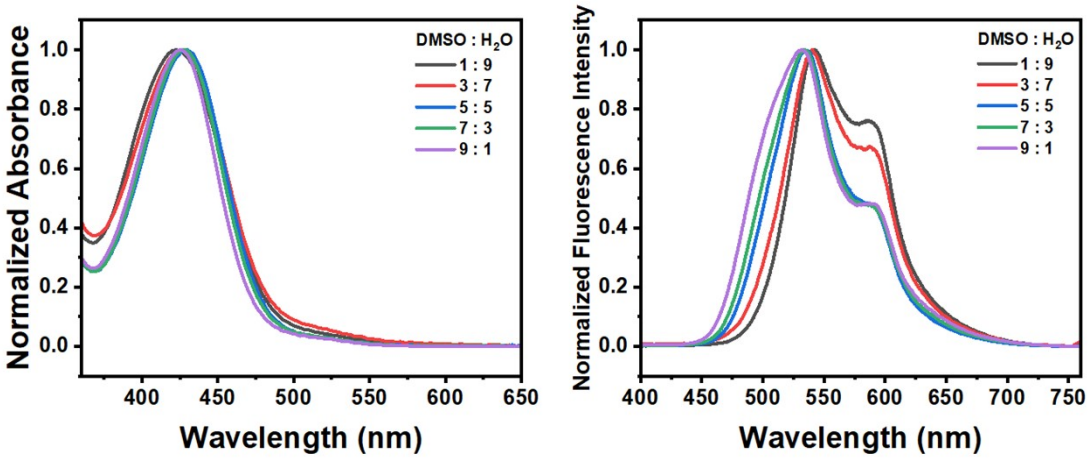

Fig. S5. Normalized absorption and emission spectra of **5a-Me** in different ratios of DMSO-H_2O system.

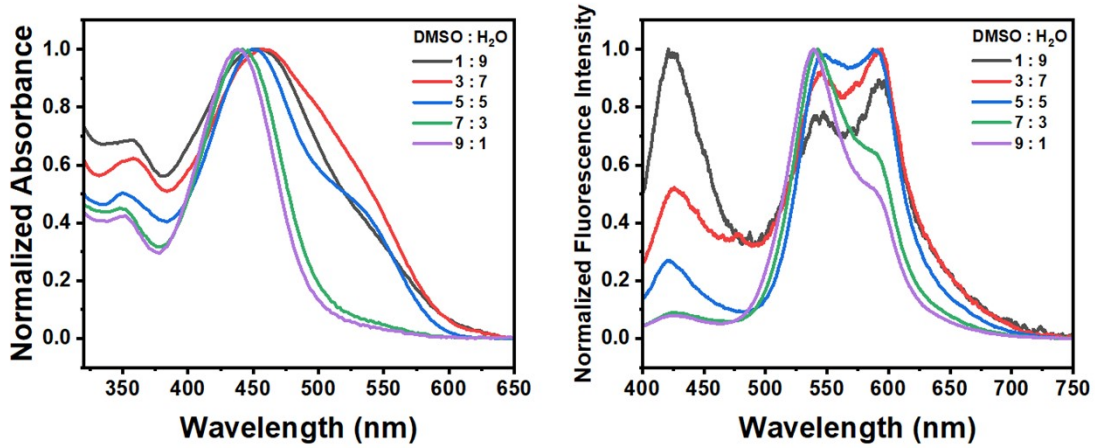

Fig. S6. Normalized absorption and emission spectra of **5b-Me** in different ratios of DMSO-H_2O system.

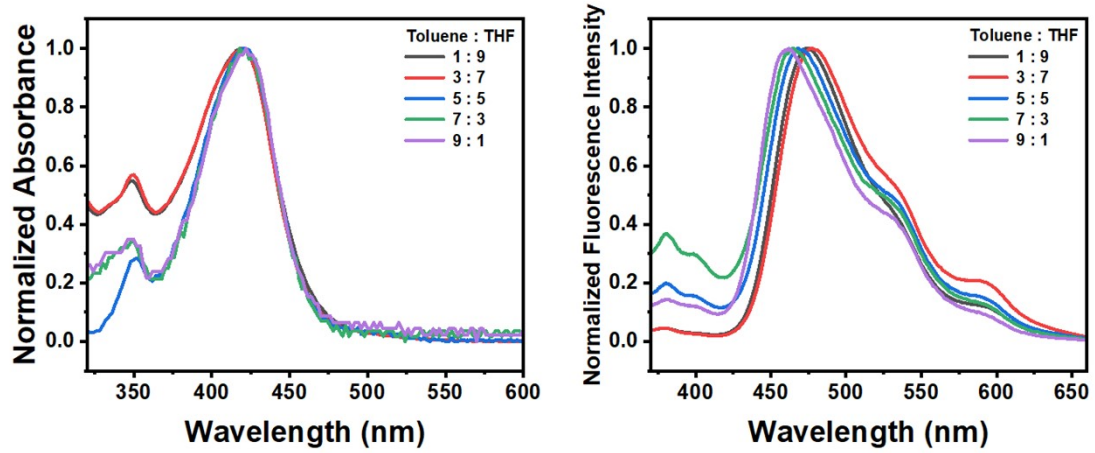

Fig. S7. Normalized absorption and emission spectra of **5a-Me** in different ratios of toluene-THF system.

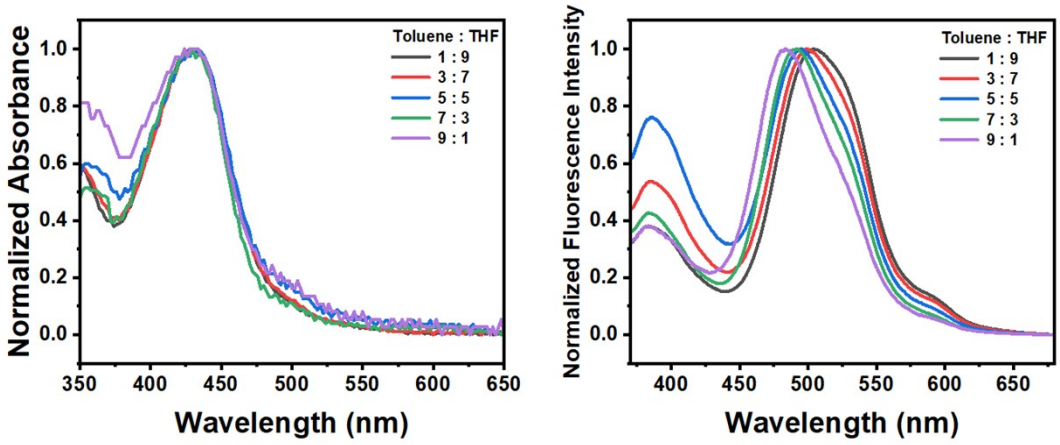

Fig. S8. Normalized absorption and emission spectra of **5b-Me** in different ratios of toluene-THF system.

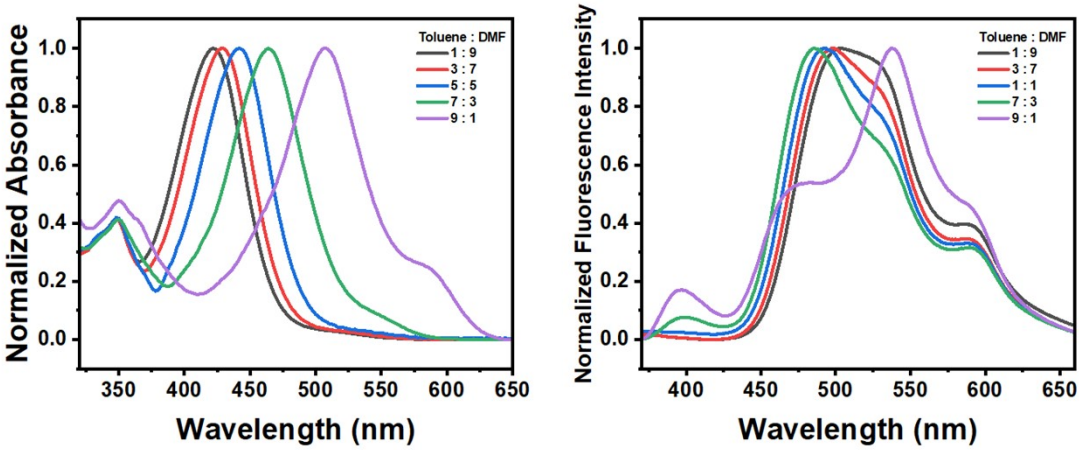

Fig. S9. Normalized absorption and emission spectra of **5a-Me** in different ratios of toluene-DMF system.

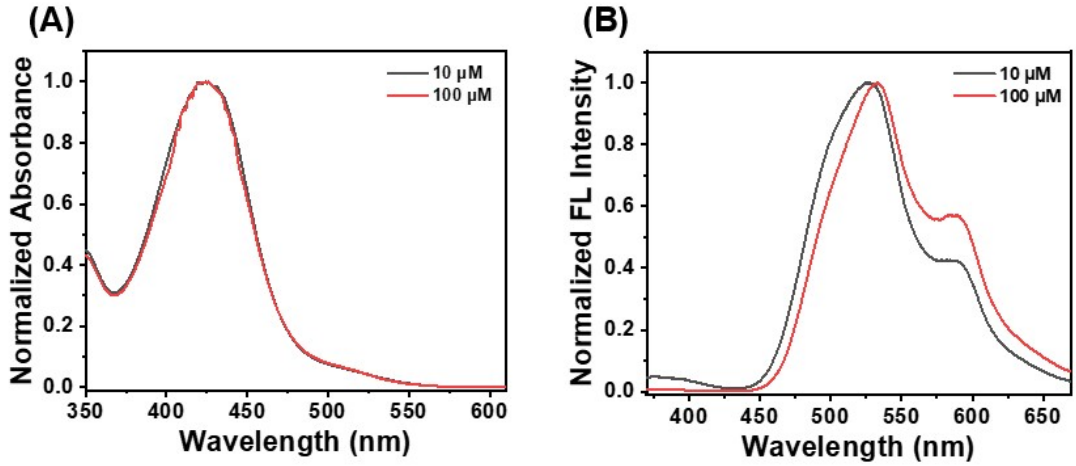

Fig. S10. Normalized absorption (A) and emission spectra (B) of 5a-Me dye in DMSO at different concentrations.

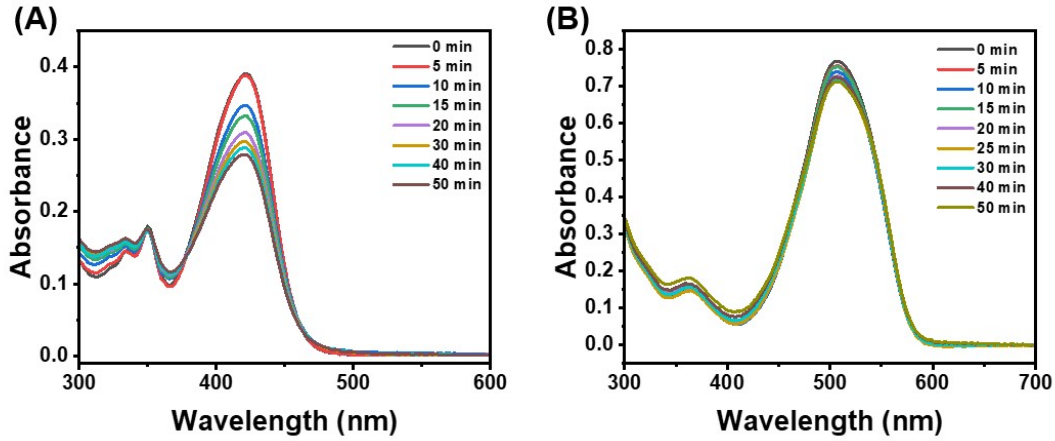

Fig. S11. Photostability test plots of **5a-Me** dye in DMF (A) and DCM (B).

4. Cell culture and fluorescence imaging

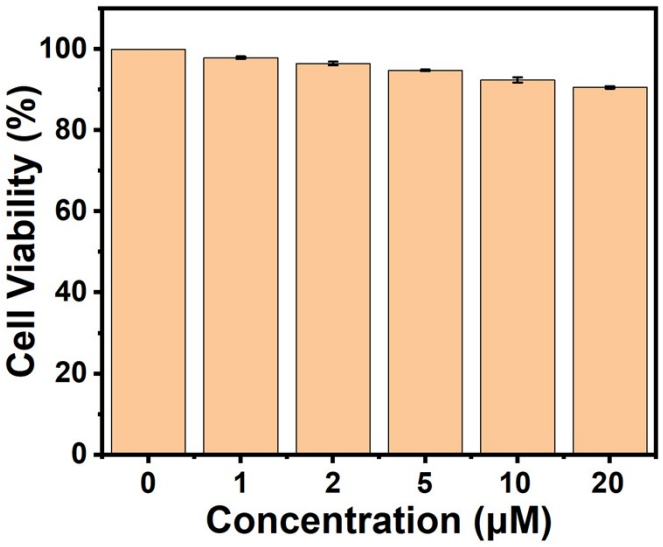

Fig. S12. The survival rate of cells was measured by MTT method at different concentrations of **5a-Me**.

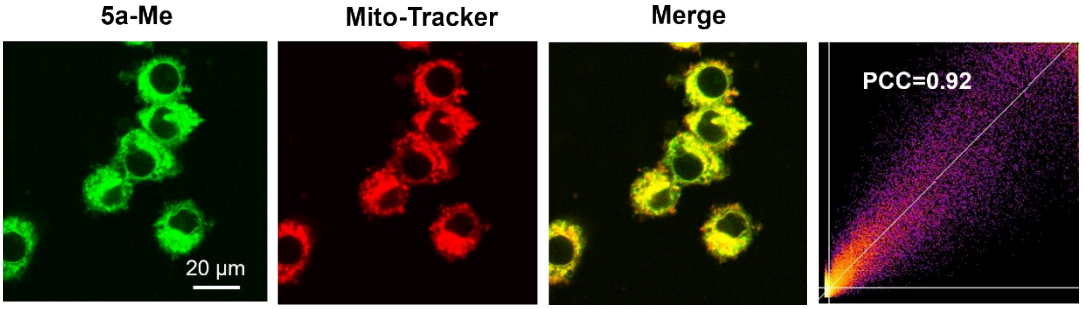

Fig. S13. Mitochondrial colocalization images in HeLa cells. Cells were incubated with **5a-Me** for 20 min followed by incubation with Mito-Tracker for another 20 min. **5a-Me** channel: Ex = 453 nm, Em = 500-550 nm; Mito-Tracker channel: Ex = 632 nm, Em = 650-680 nm.

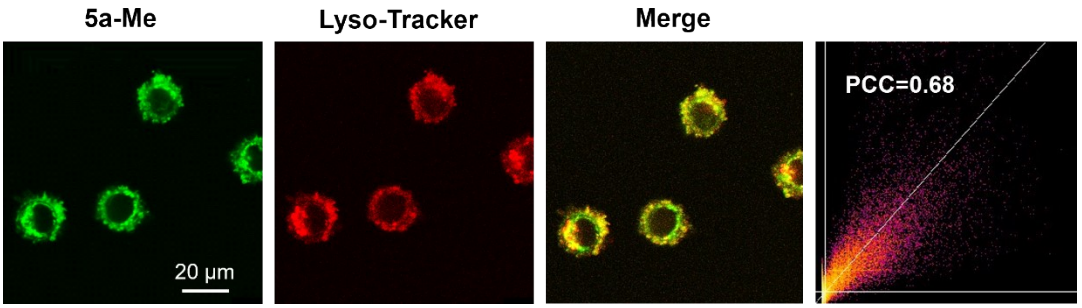

Fig. S14. Lysosome colocalization images in HeLa cells. Cells were incubated with **5a-Me** for 20 min followed by incubation with Lyso-Tracker for another 20 min. **5a-Me** channel: Ex = 453 nm, Em = 500-550 nm; Lyso-Tracker channel: Ex = 632 nm, Em = 660-700 nm.

5. NMR and HRMS spectra

Fig. S15. ^{1}H NMR spectrum of **3b** in DMSO.

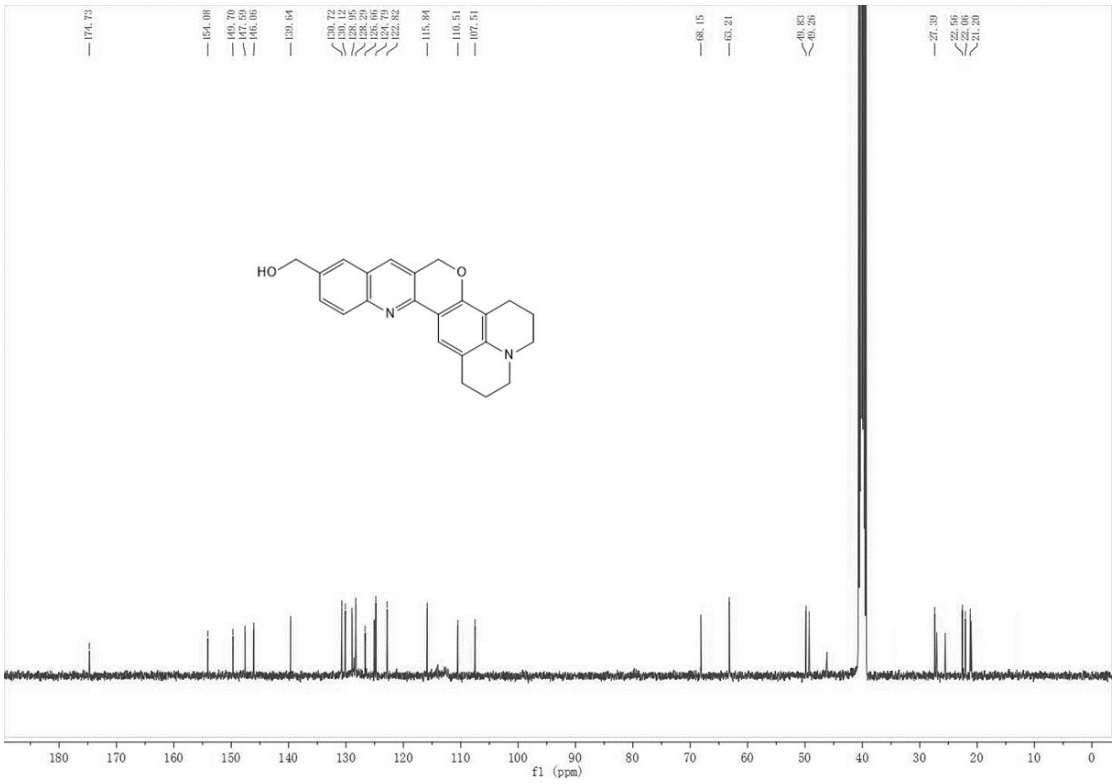

Fig. S16. ^{13}C NMR spectrum of **3b** in DMSO.

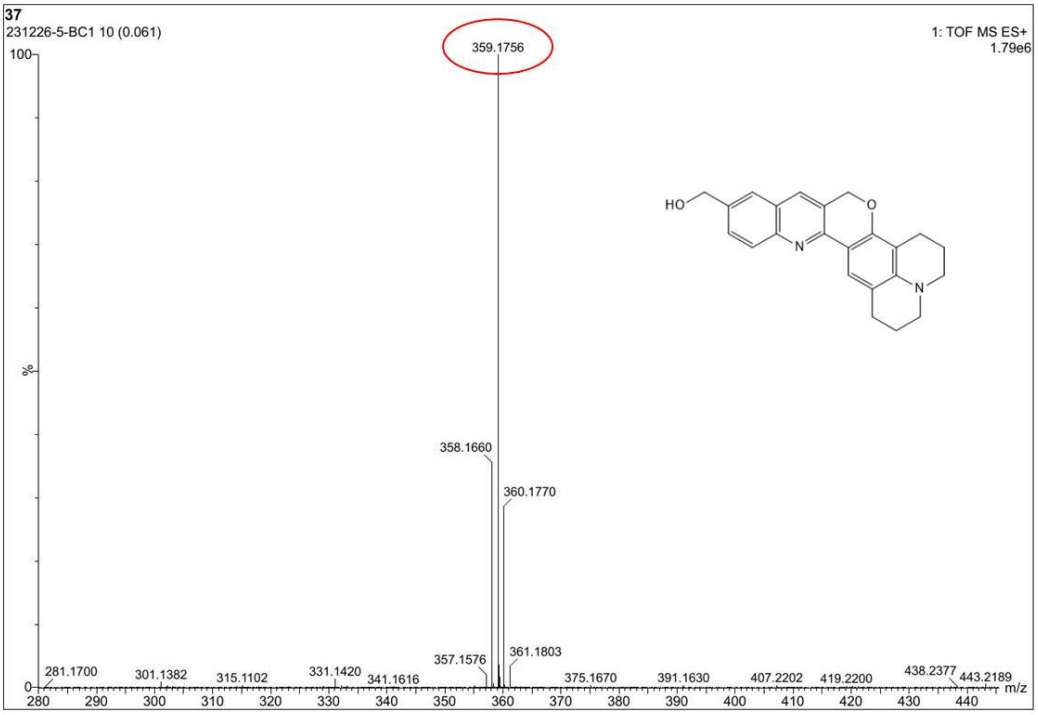

Fig. S17. HRMS spectrum of **3b**.

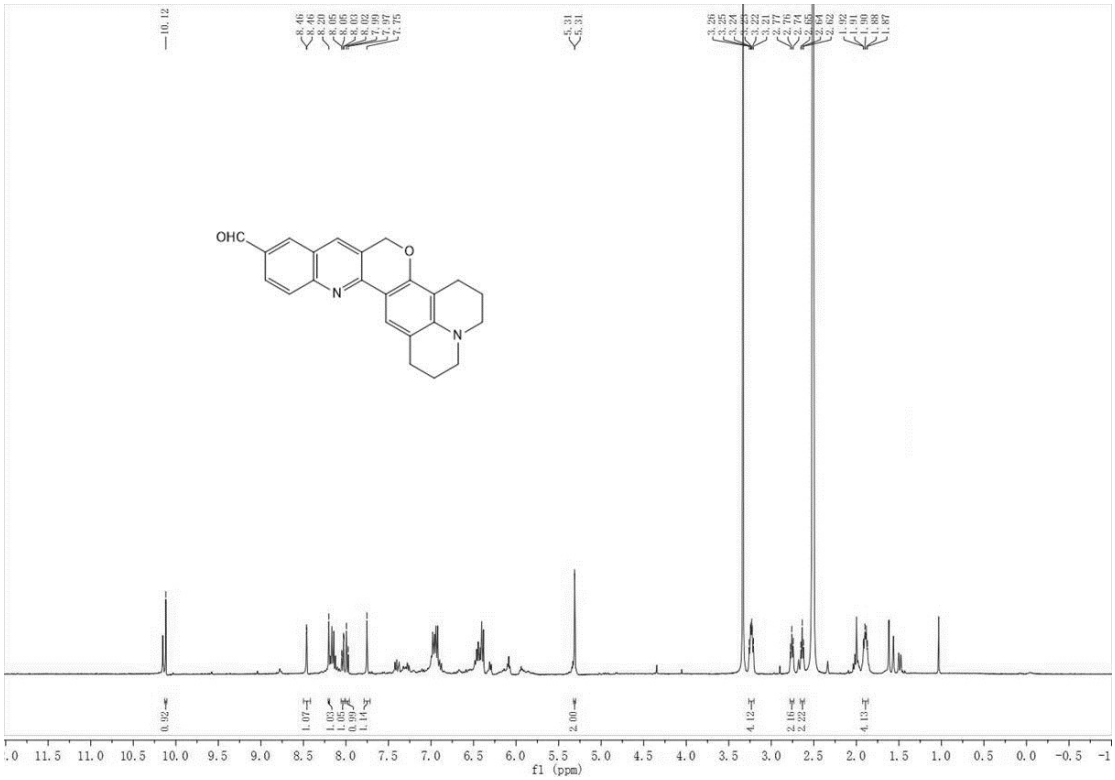

Fig. S18. ^{1}H NMR spectrum of **4b** in DMSO.

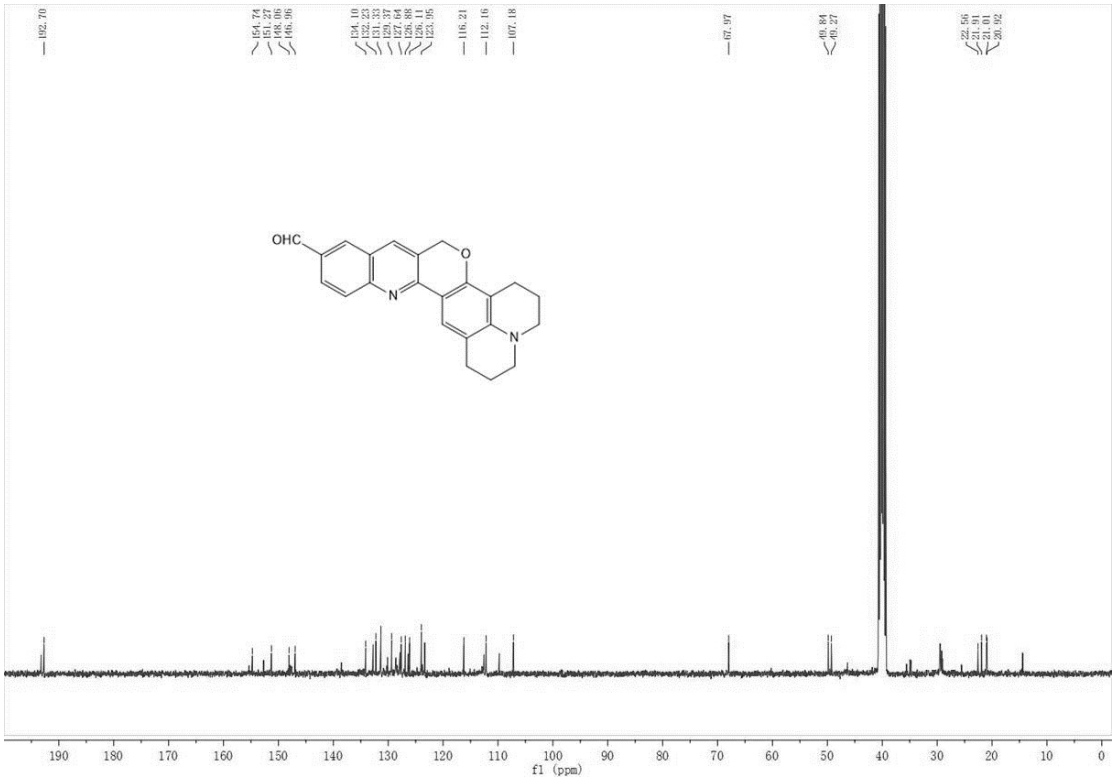

Fig. S19. ^{13}C NMR spectrum of **4b** in DMSO.

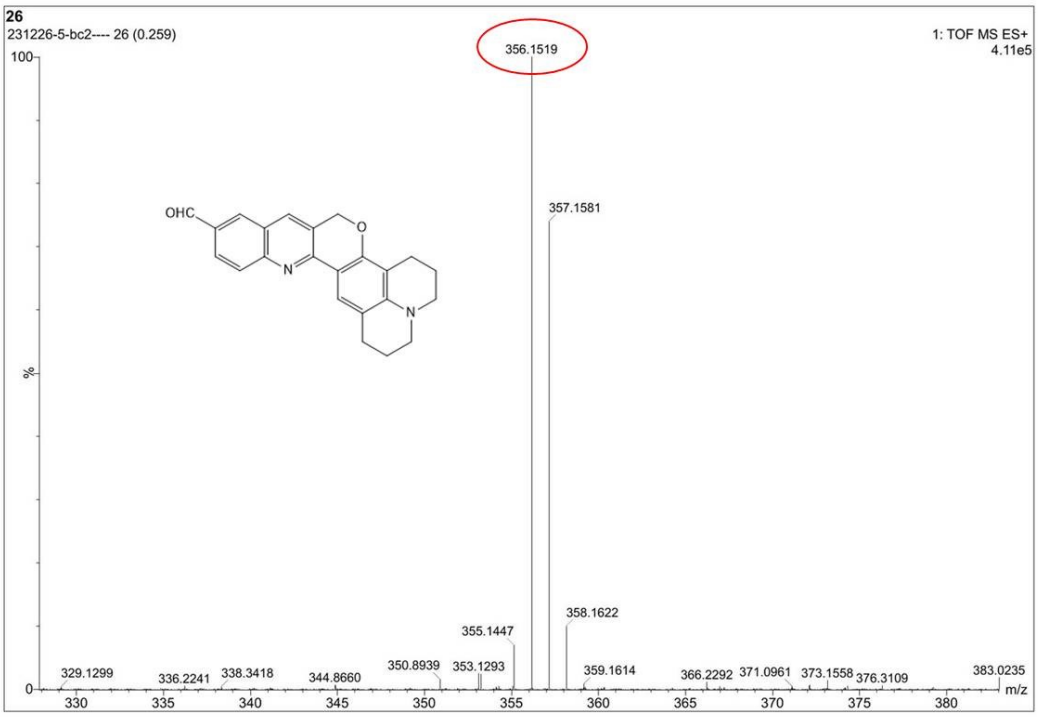

Fig. S20. HRMS spectrum of **4b**.

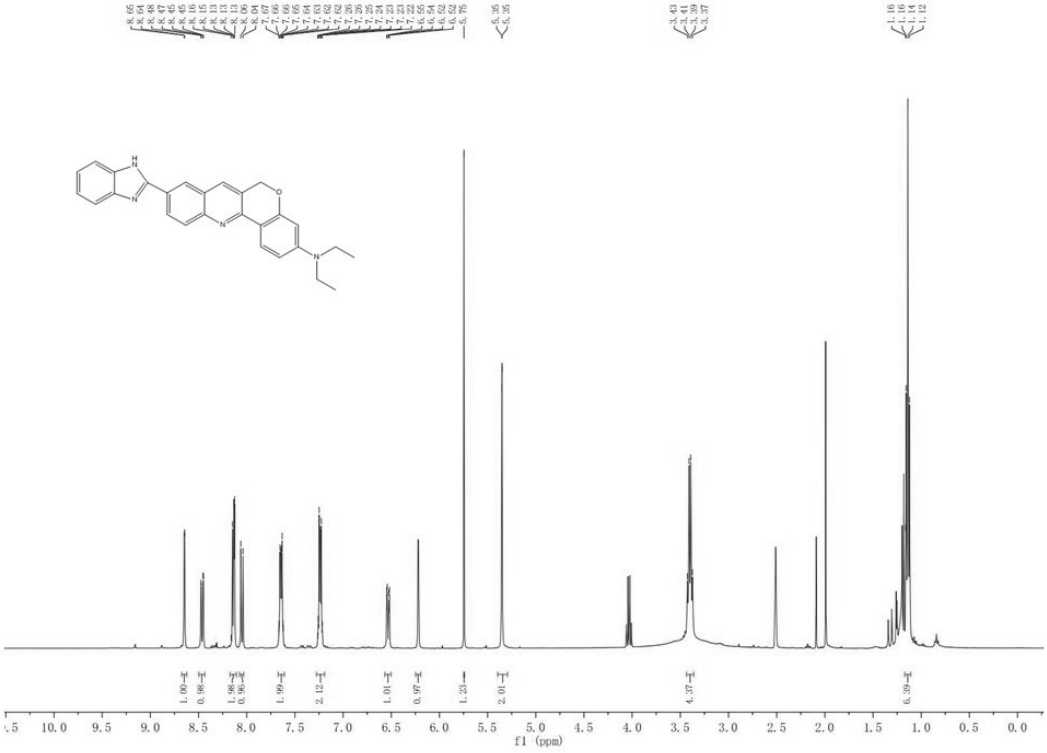

Fig. S21. ^{1}H NMR spectrum of **5a** in DMSO.

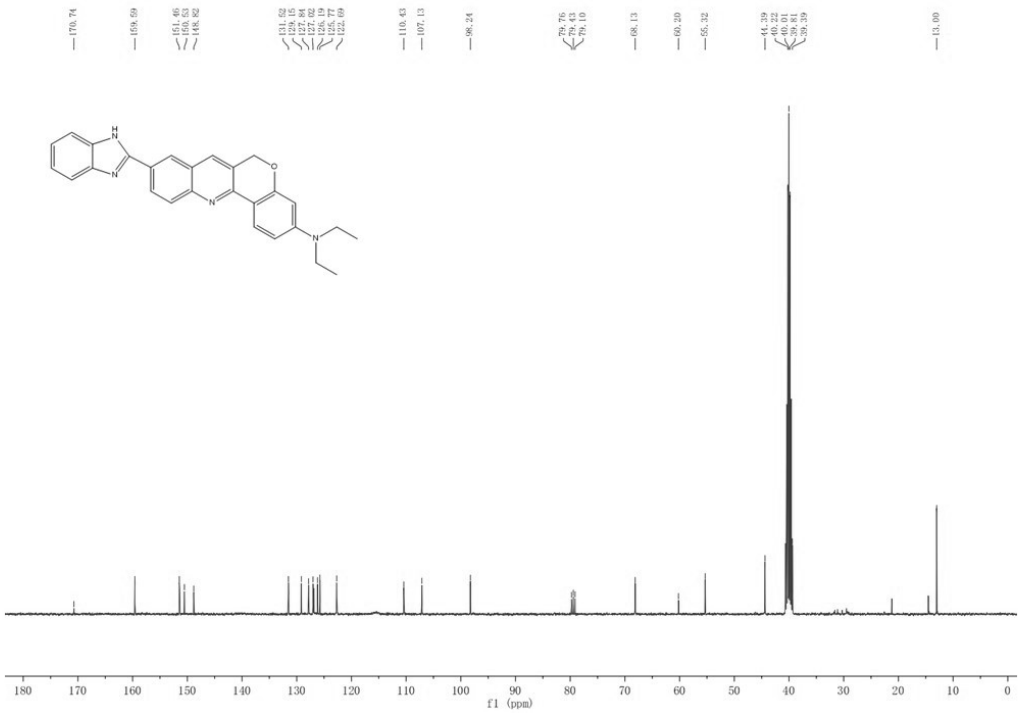

Fig. S22. ^{13}C NMR spectrum of **5a** in DMSO.

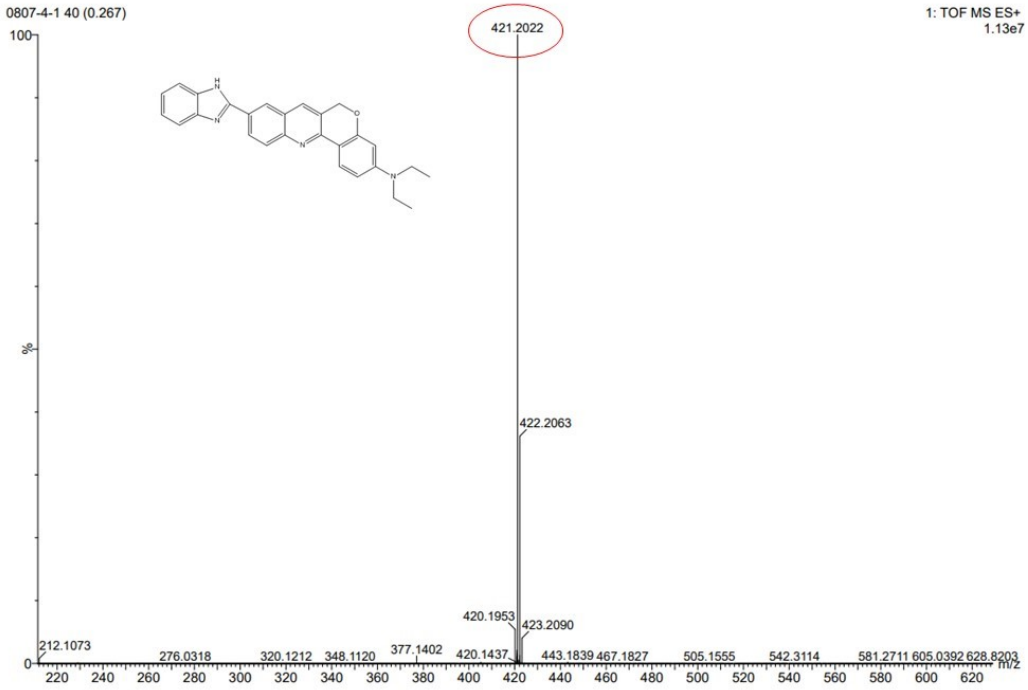

Fig. S23. HRMS spectrum of **5a**.

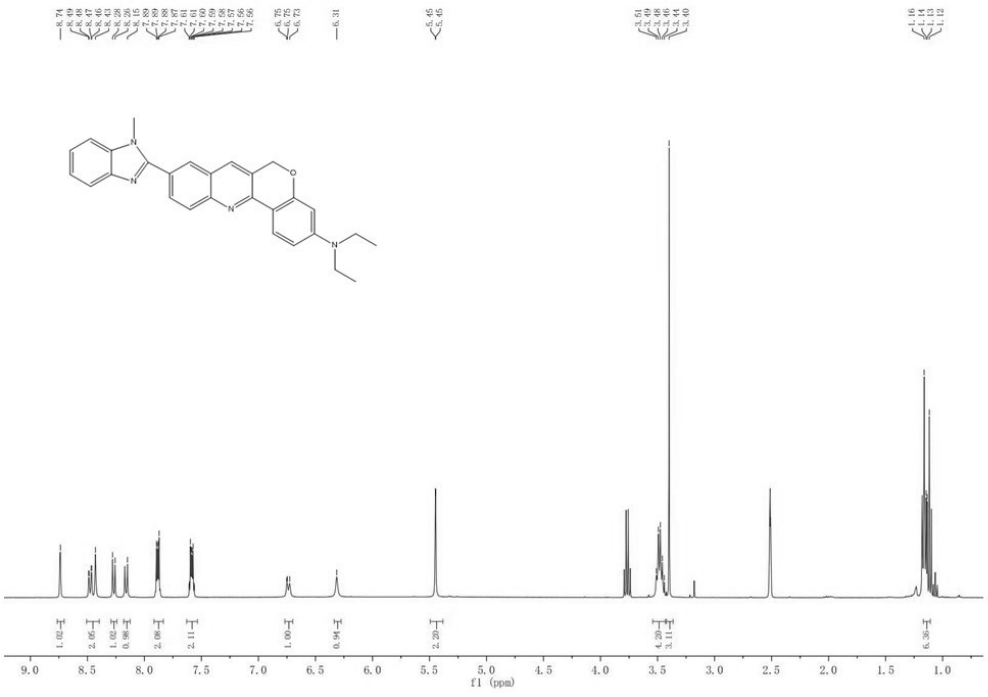

Fig. S24. ^{1}H NMR spectrum of **5a-Me** in DMSO.

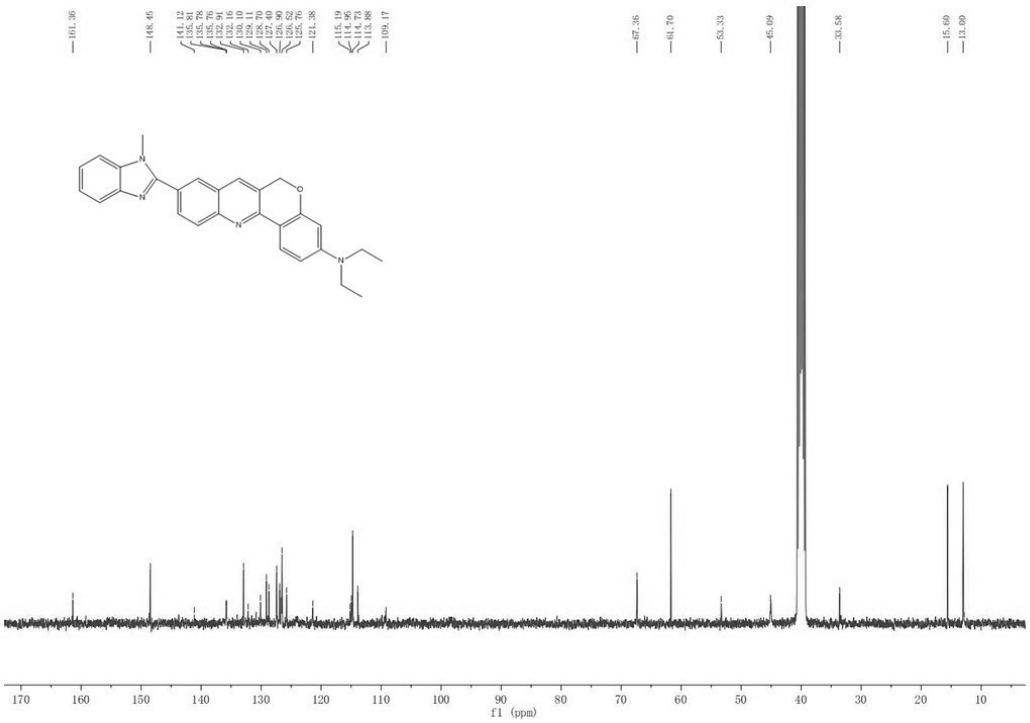

Fig. S25. ^{13}C NMR spectrum of **5a-Me** in DMSO.

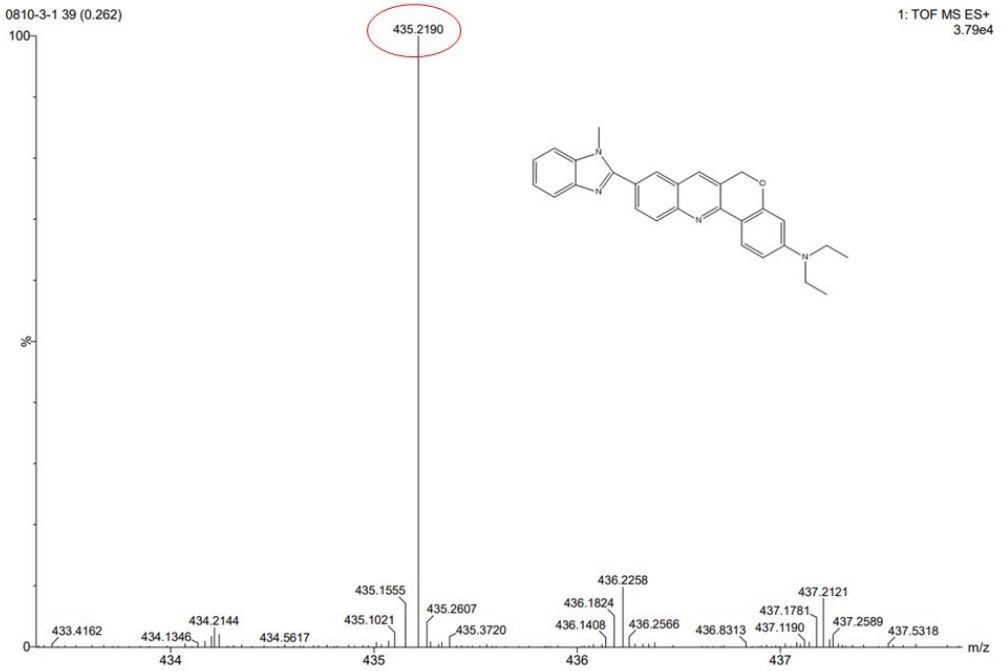

Fig. S26. HRMS spectrum of **5a-Me**.

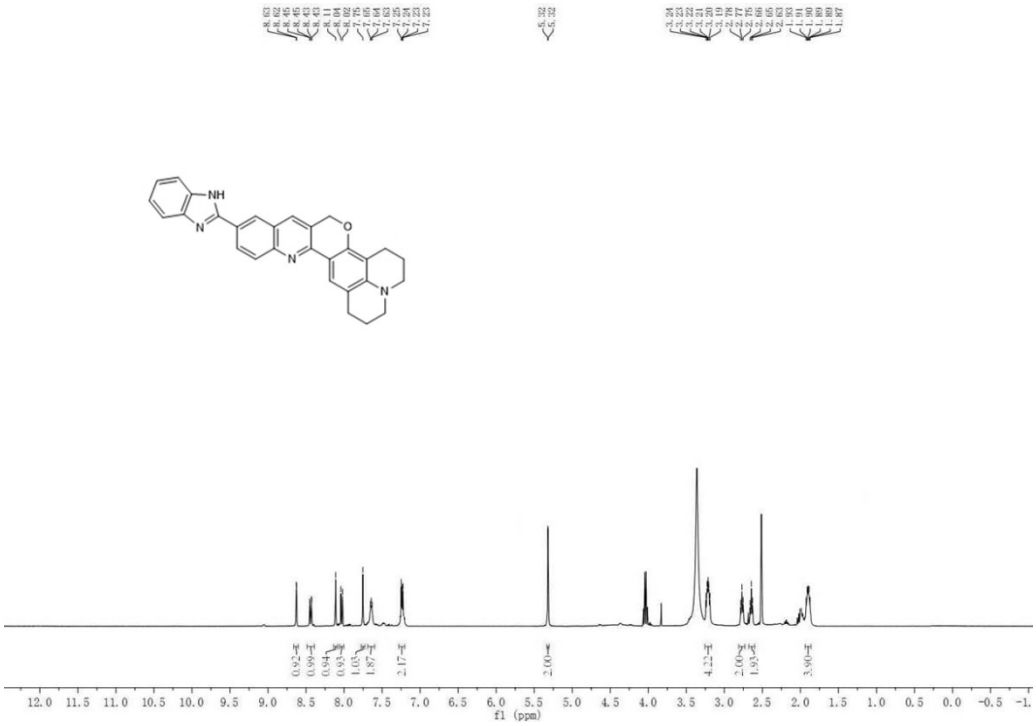

Fig. S27. [1]H NMR spectrum of **5b** in DMSO.

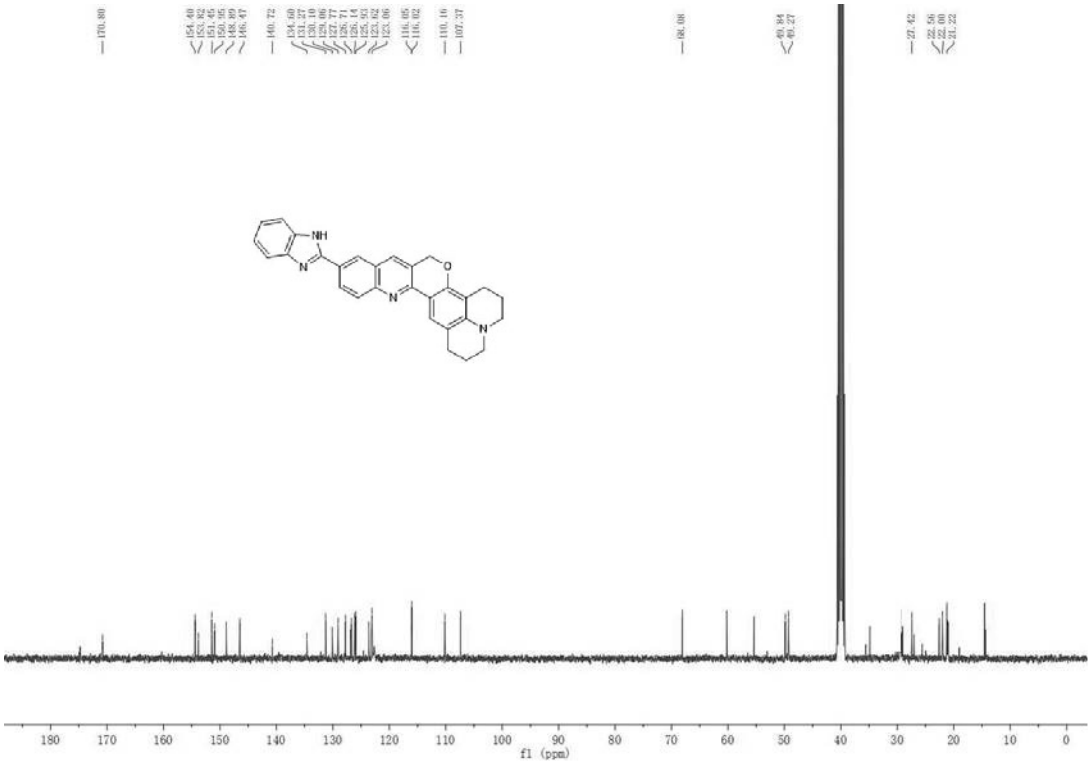

Fig. S28. [13]C NMR spectrum of **5b** in DMSO.

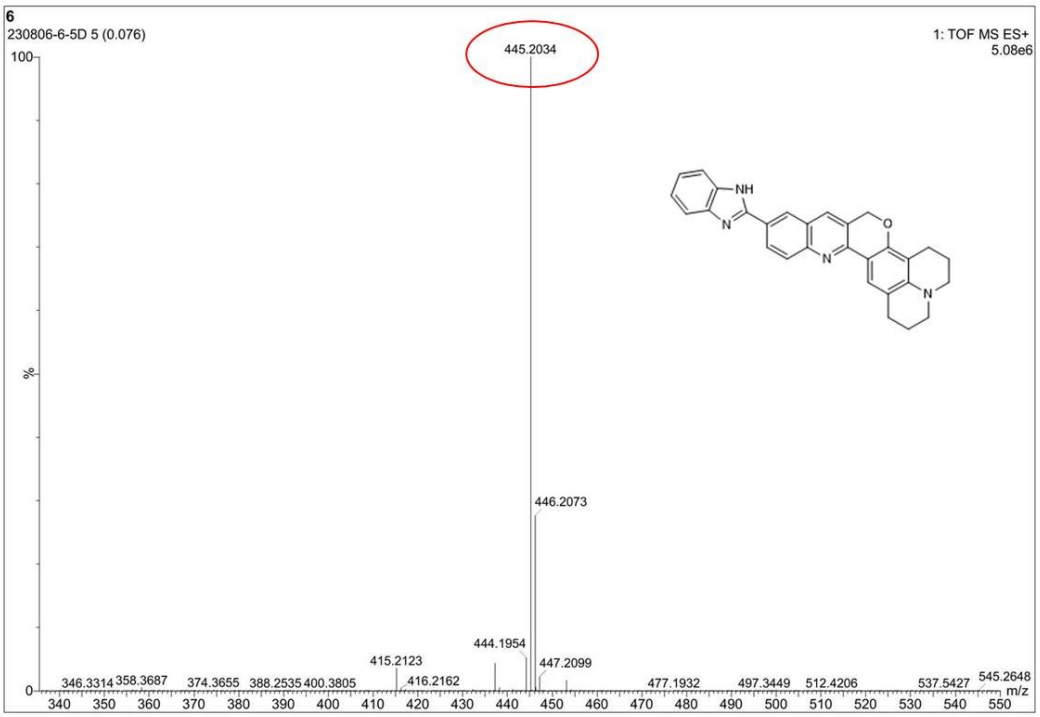

Fig. S29. HRMS spectrum of **5b**.

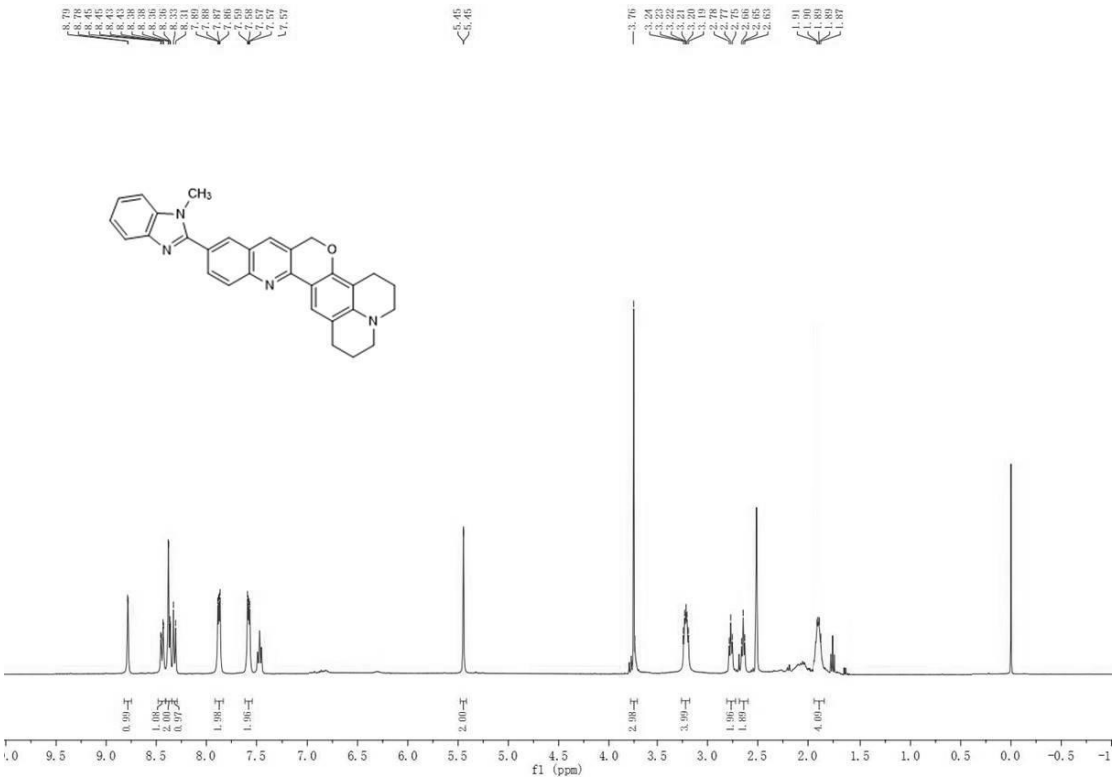

Fig. S30. ^{1}H NMR spectrum of **5b-Me** in DMSO.

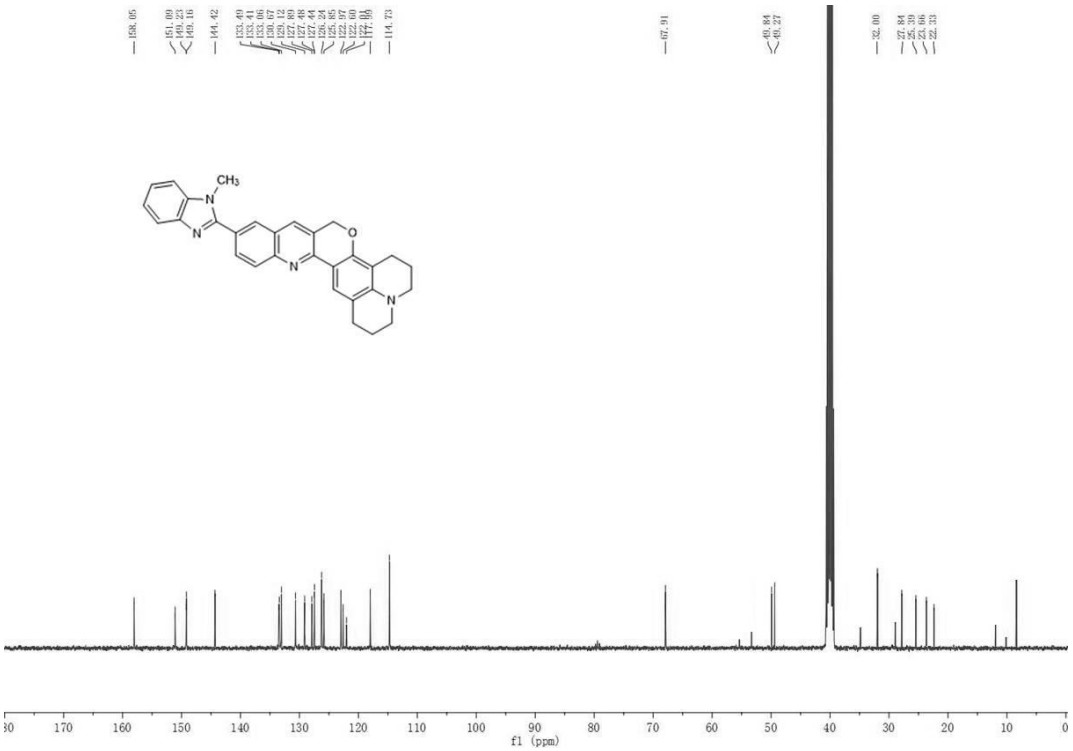

Fig. S31. ^{13}C NMR spectrum of **5b-Me** in DMSO.

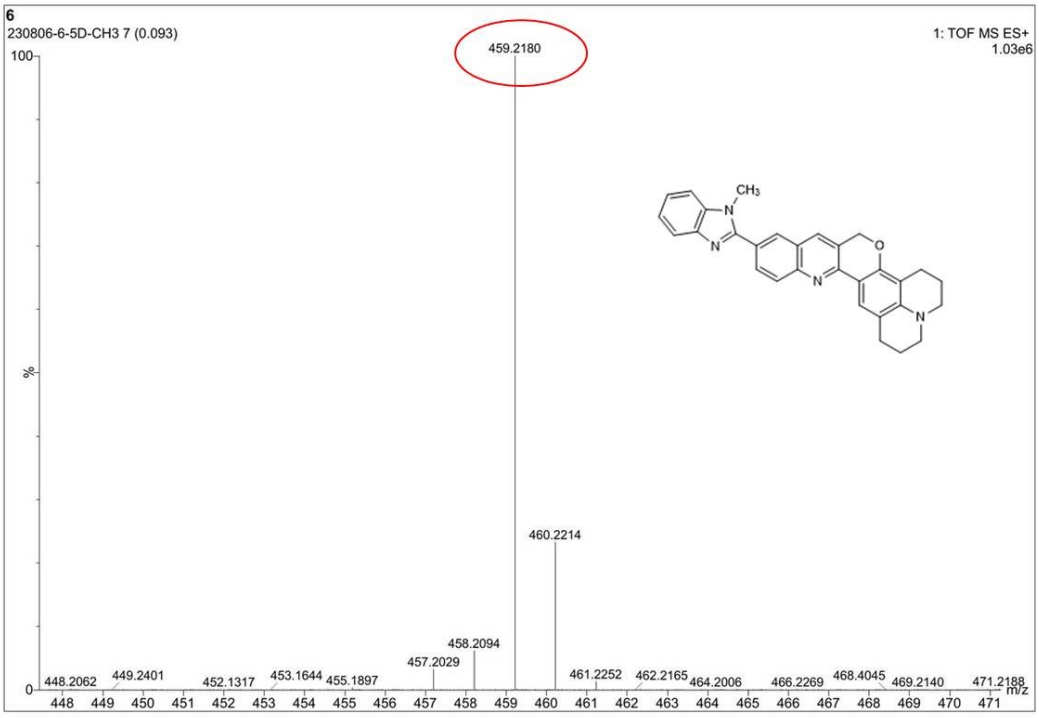

Fig. S32. HRMS spectrum of **5b-Me**.