Supplementary Information for

Construction of a mitochondria-targeted fluorescent probe for the detection of viscosity in living cells and zebrafish

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Experimental

1.Materials and Instrumentations

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. All experiments used ultra-pure water. Solvents were purified by standard methods prior. Ultra-pure water (18.2 MΩ cm) is used by ULPURE. TLC analysis was carried out on silica gel plates, and column chromatography was conducted over silica gel (mesh 200-300); both of them were purchased from Qingdao Ocean Chemicals. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance III HD 600 MHz NMR spectrometer (United States of America). High-resolution mass spectrometric (HRMS) analyses were measured on Brooke Solan X 70 FT-MS, Agilent 6540T. UV-vis absorption spectra were obtained on a Shimadzu UV-2700 spectrophotometer, and fluorescence spectra were measured on a HITACHI F4700 fluorescence spectrophotometer. The fluorescence imaging of cells was performed with a Leica TCS SP8 CARS confocal microscope.

2. Steps of synthesis

2.1 Synthesis routine of the probe PMI-MT

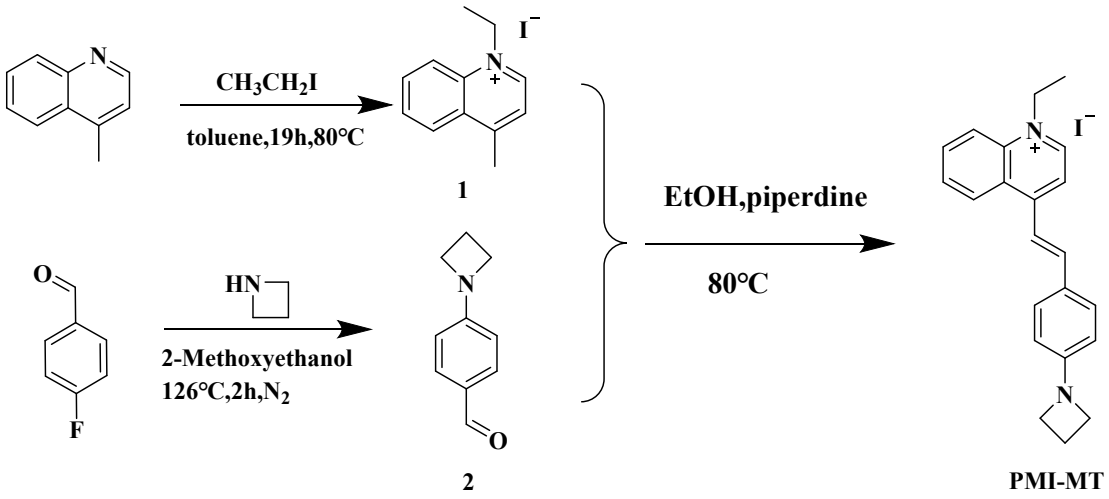


Fig. S1 Steps in the synthesis of the compound **PMI-MT**

2.2 Synthesis of the compound 1 and compound 2

Compound l and Compound 2 were synthesized by the reported method.[1, 2]

2.3 Structural formulae of compounds DB-1

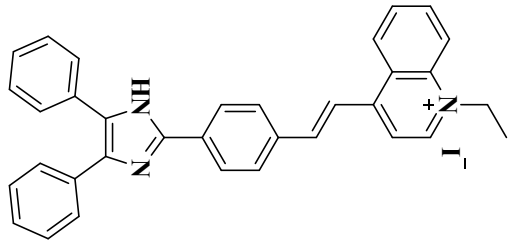


Fig. S2 Structural formulae of compounds **DB-1**.

2.4 Synthesis of the probe PMI-MT

Compound 1(1 mmol) and compound 2(1 mmol) were dissolved in 10 ml of anhydrous ethanol, then an appropriate amount of piperidine was added as a catalyst, and the reaction was carried out under nitrogen protection for 8 h. The crude product was obtained by decompression and rotary distillation to remove the solvent, and the black-red solid was purified by chromatography silica gel column (DCM: MeOH=10:1) to obtain 121 mg of red solid product with a yield of 27.38 %. H NMR (600 MHz, DMSO-d6) δ 9.12 (d, J = 6.7 Hz, 1H), 9.02 (dd, J = 8.7, 1.4 Hz, 1H), 8.37-8.31 (m, 2H), 8.21 (ddd, J = 8.6, 6.9, 1.3 Hz, 1H), 8.16 (d, J = 15.6 Hz, 1H), 8.03-7.95 (m, 2H), 7.87-7.82 (m, 2H), 6.52-6.45 (m, 2H), 4.45 (s, 2H), 4.00 (t, J = 7.4 Hz, 4H), 3.32 (s, 3H), 2.39 (tt, J = 8.0, 6.8 Hz, 2H). δ ^{13}C NMR (151 MHz, Chloroform-*d*) 155.03, 147.50, 141.65, 139.61, 138.13, 137.22, 135.59, 131.33, 130.88, 128.03, 126.81, 123.83, 122.79, 118.64, 117.81, 117.67, 115.79, 113.91, 112.02, 55.15, 45.52, 28.87.ESI: m/z: calcd for [$C_{24}H_{24}N_4$] 368.2001, found: 368.2003.

3. Optical studies and analysis

A stock solution (1 mM) of the probe **PMI-MT** was initially prepared in dimethyl sulfoxide (DMSO). All spectrometric probes were used at a concentration of 10 μM. The adjunction of 20 μL of stock solution was added to 2.0 mL of different solvent systems to obtain the probe **PMI-MT** diluent. The solutions of various interfering substances (cations, anions, amino acids and active small molecules) were prepared with twice-distilled water. The providing solutions were mixed well before texting the spectra. Unless otherwise specified, the required fluorescence spectral measurement is generally an excitation wavelength of 550 nm, an excitation slit width of 5.0 nm, and an emission slit width of 5.0 nm. The analytes 1–33:(1) Glu; (2) $ZnCl_2$; (3) NO_2^-; (4) $NiCl_2$; (5) Hg^+; (6) $CaCl_2$; (7) GSH; (8) Hcy; (9) Cys; (10) Asp; (11) ALA; (12) lle; (13) Leu; (14) His; (15) $ONOO^-$; (16) H_2O_2; (17) O_2^-; (18) •OH; (19) NaClO; (20) NaF; (21) NaHS; (22) Na_2SO_3; (23)

$NaHSO_3$;(24)Na_2SO_4 (25) NaSCN; (26) NaBr; (27) Na_3PO_4 ; (28) $NaHPO_4$; (29)Na_2CO_3 ; (30) $FeCl_3$; (31) $FeSO_4$; (32) PBS;(33) Glycerol.

4. Calculation of fluorescence quantum yield

The fluorescence quantum yield of probe **DB-1** and probe **PMI-MT** was measured using Rhodamine B as a reference. The calculation formula is as follows:

$$\Phi_h = \Phi_l \frac{F_h}{F_l} \frac{A_l}{A_h} \left(\frac{n_h}{n_l}\right)^2$$

The quantum yield of rhodamine B (Φ_l = 0.69) is Φ_l in Eq. Φ_l; and F, A, and n denote the integrated area, absorbance, and refractive index of the fluorescence spectrum, respectively. The subscript “h” denotes the sample, and “l” denotes rhodamine B.

5. Calculation of molar extinction coefficient

The molar extinction coefficient ϵ expresses the ability of a substance to absorb light at a specific wavelength and is expressed in units of $L \cdot mol^{-1} \cdot cm^{-1}$:

$$A = \epsilon c L$$

Where A is the absorbance, c is the concentration of the solution (mol/L), and L is the optical range (cm), the absorbance of the solution at each concentration was measured. The A - c curve was plotted to the slope k = ϵ L, and combined with the optical range L (1 cm) to calculate: ϵ = k / L.

6. Calculation of fluorescence brightness of fluorescent probes

The brightness of a fluorescent probe is determined by a combination of the molar extinction coefficient (ϵ) and the fluorescence quantum yield (Φ), calculated as:

$$\text{Brightness} = \epsilon \times \Phi$$

7. Culture and preparation of HepG2 cells

HepG2 cells were cultured in DMEM (Dulbecco’s modified Eagle's medium) supplemented with

10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 at 37 °C. Before the experiments, the HepG2 cells in 35-mm glass-bottomed dishes were cultured to a density of 2×10^5 cells per dish. Incubate the cells for 24 h. Cells will attach to the glass surface during this time.

8. Cytotoxicity assay

HepG2 cells were seeded into 96-well plates, and 0, 1, 2, 5, 10, 20, 30, 40 and 50 μM (final concentration) of the probe **PMI-MT** were added respectively. Subsequently, the cells were cultured at 37 °C in an atmosphere of CO_2 (5%) and air (95%) for 24 h. Next, CCK8 (10 μL, 5 mg/mL) was injected into every well and incubated for 4 h. The absorbance of the solution was measured at 450 nm by way of a microplate reader. The cell viability was determined by assuming 100% cell viability for cells without the probe **PMI-MT**.

Cell viability (%) = $(OD_{sample} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$.

9. Co-location experimental imaging

The cells were incubated with **PMI-MT** (10 μM) and Mito-Tracker Green (500 nM, MTG, a commercial dye targeting mitochondrial) for 15 min in an incubator of 95% air and 5% CO_2 at 37 °C. Afterward, wash thrice with PBS (10 mM, pH 7.4) before co-staining fluorescence imaging. The Pearson coefficient analyzed the mitochondrial localization ability of **PMI-MT**. laser confocal imaging. **PMI-MT**: λ_{ex} = 550 nm, λ_{em} = 650-750 nm.Mito-Tracker Green: λ_{ex}= 488 nm, λ_{em}= 500-550 nm

10. Imaging of mitochondrial fluorescence in cells

HepG2 cells were incubated with oleic acid (0 μM, 100 μM) for 30 min, and then incubated with the probe **PMI-MT** for 20 min. Afterward, the medium was removed and the cells were rinsed three times with PBS (10 mM, pH 7.4) for confocal imaging. λ_{ex} = 550 nm, λ_{em} = 650-750 nm.

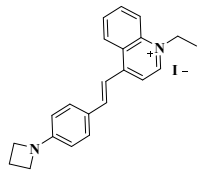
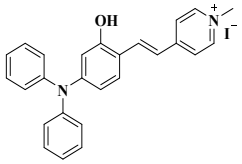
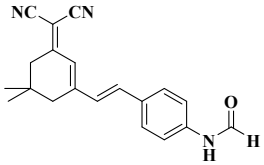
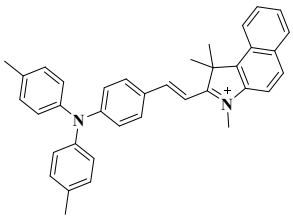
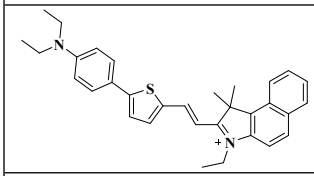
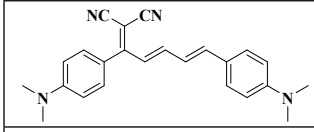
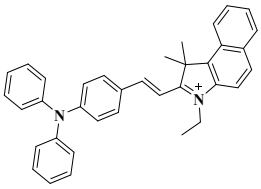
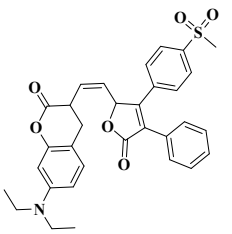
11. Confocal imaging of intracellular viscosity

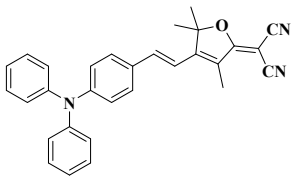
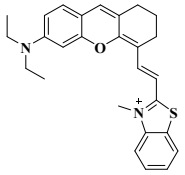
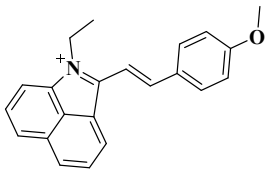
For cellular viscosity change, the HepG2 cells were respectively incubated with dexamethasone (10 μM), monensin (10 μM), nystatin (10 μM) for 30 min and then incubated with the probe **PMI-MT** for 20 min. Afterward, the medium was removed and the cells were rinsed three times with PBS (10 mM, pH 7.4) for confocal imaging. λ_{ex} = 550 nm, λ_{em} = 650-750 nm.

12. Confocal imaging of zebrafish viscosity

3-day-old zebra fish were transferred into a 30 mm glass culture dishes using a disposable sterilized dropper. Zebrafish were divided into multiple groups and incubated with CCCP (10 μM), monensin (10 μM), nystatin (10 μM), LPS (10 μM), dexamethasone (10 μM) for 30 min and then incubated with the probe **PMI-MT** for 20 min. Afterward, the medium was removed and the zebrafish were rinsed three times with PBS (10 mM, pH 7.4) for confocal imaging. λ_{ex} = 550 nm, λ_{em} = 650-750 nm.

Table S1. The latest research progress of viscosity probes

Probe structure	$\lambda_{ex}/\lambda_{em}$ (nm)	Targeted organelles	Imaging	References
	550/681	mitochondrial	Living cells and zebrafish	This work
	475/610	mitochondrial	Living cells	J. Mol. Struct, 2024, 1308
	425/650	mitochondrial	Living cells	Sens. Actuators B-Chem., 2025, 424
	567/697	mitochondrial	Living cells and zebrafish	Spectrochim. Acta, Part A , 2025, 332, 125831
	610/783	mitochondrial	Living cells and mice	Talanta, 2025, 286, 127470
	490/640	mitochondrial	Living cells and zebrafish	New J. Chem. 2024, 38
	545/650	mitochondrial	Living cells and zebrafish	Anal. Methods. 2024, 293, 16
	500/650	mitochondrial	Living cells and mice	Spectrochim. Acta. A Mol. Biomol. Spectrosc. 2024, 307, 123637

	550/670	mitochondrial	Living cells	Chem. Commun. 2023, 59, 12735
	720/780	mitochondrial	Living cells and mice	Heliyon. 2023, 9, e18704
	410/477	mitochondrial	Living cells	Analyst. 2023, 148, 4174

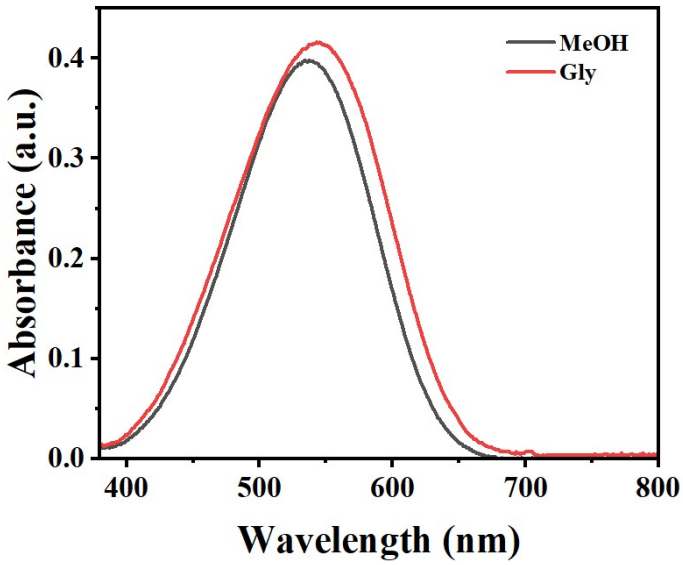


Fig. S3. Absorption spectra of the probes **PMI-MT** in various solvents (MeOH and Gly).

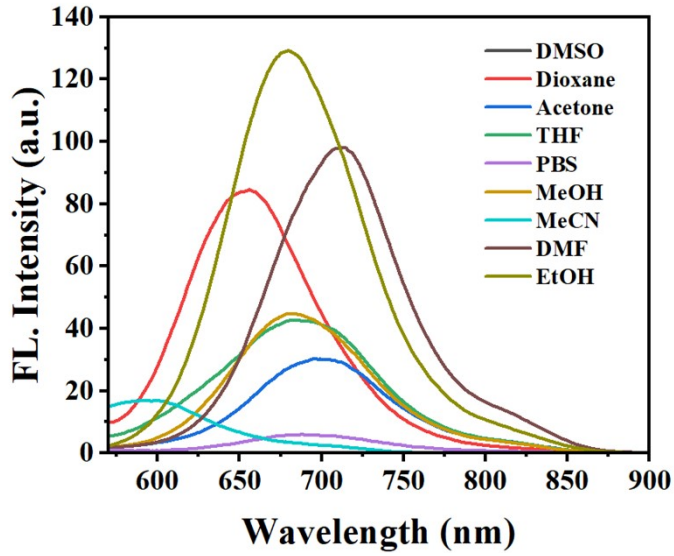


Fig. S4. Spectral properties of the probes **PMI-MT** in various solvents. (DMSO, Dioxane, Acetone, THF, PBS, MeOH, MeOH, MeCN, DMF and EtOH).

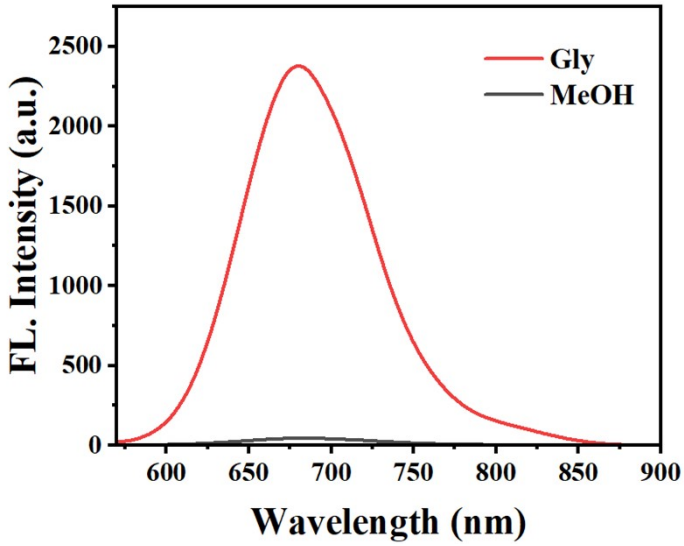


Fig. S5. Spectral properties of the probes **PMI-MT** in various solvents. (MeOH and Gly).

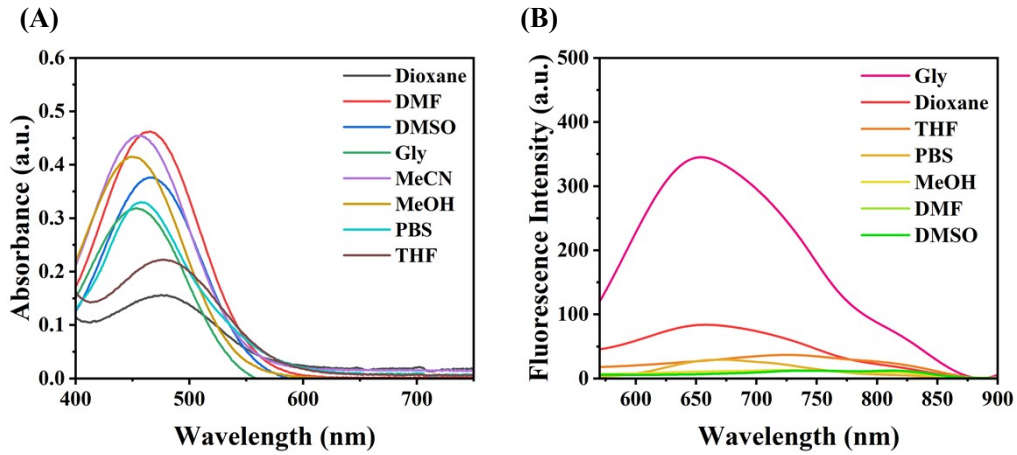


Fig. S6. (A) Absorption spectra of the probe **DB-1** in different solvents. (B) Fluorescence intensity spectra of the probe **DB-1** in different solvents. λ_{ex} = 470 nm, slit width: d_{ex} = d_{em} = 5 nm.

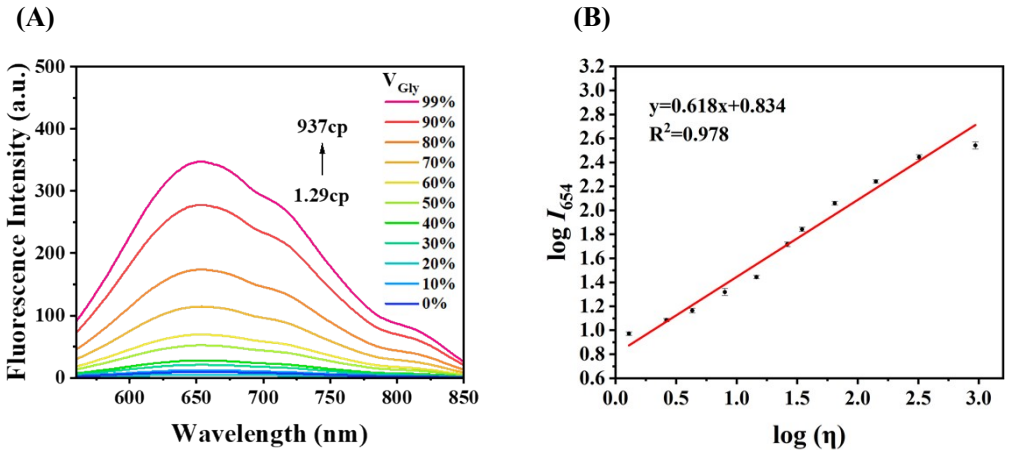


Fig. S7. (A) Fluorescence spectra of the probe **DB-1** (10 μM) in mixtures of different glycerol/MeOH ratios. (B) Linear response in mixtures of different glycerol/MeOH.

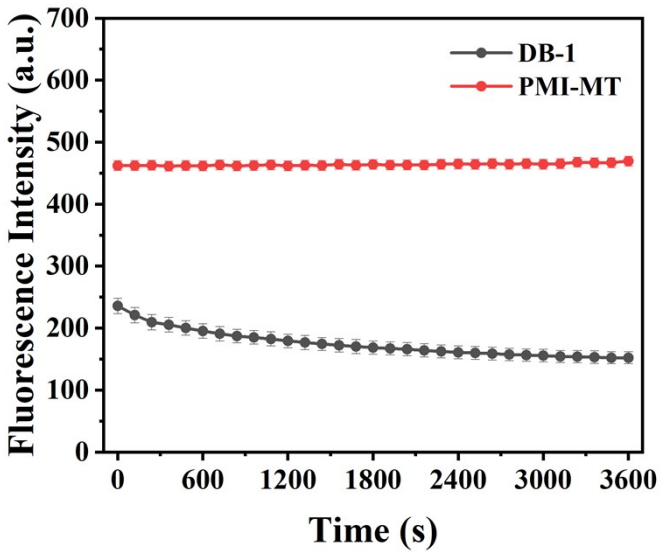


Fig. S8. The photostability of the probe **PMI-MT** (10 μM) and **DB-1** (10 μM) with continuous laser

scanning during 60 min in 80% glycerol solutions.

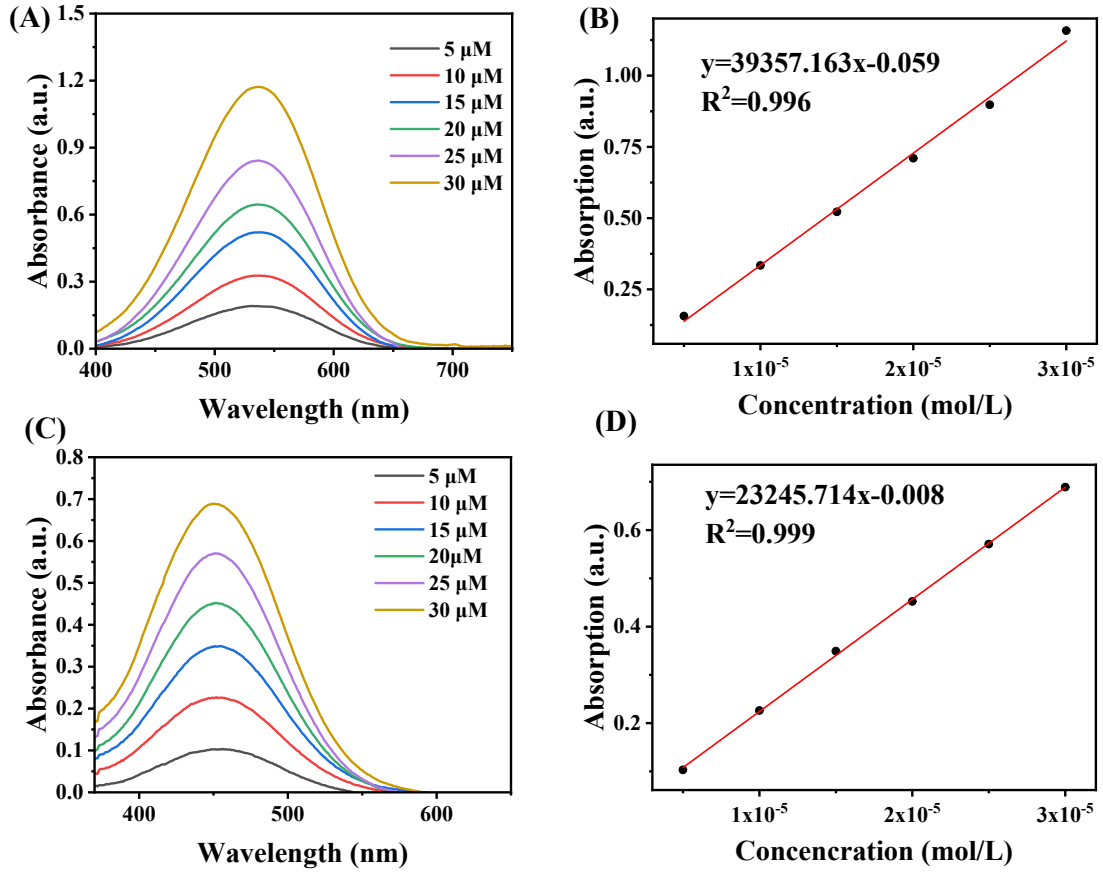


Fig. S9. (A) Absorption spectra of different concentrations of probe **PMI-MT** in methanol. (B) Linearity of different concentrations of probe **PMI-MT** with their absorbance in methanol. (C) Absorption spectra of different concentrations of probe **DB-1** in methanol. (D) Linearity of different concentrations of probe **DB-1** with their absorbance in methanol.

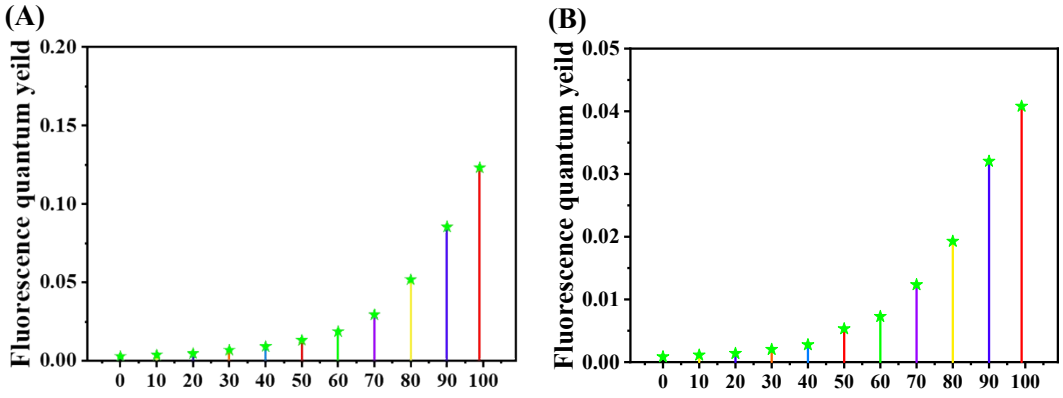


Fig. S10. (A) Fluorescence quantum yield (Φ_f) of **PMI-MT** in the Glycerol /MeOH (v/v) mixture. (B) Fluorescence quantum yield (Φ_f) of **DB-1** in the Glycerol /MeOH (v/v) mixture.

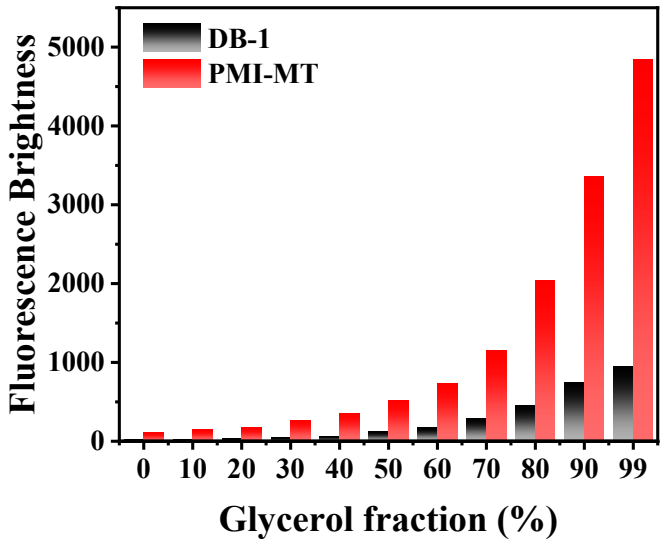


Fig. S11. Fluorescence brightness of probe **DB-1** and probe **PMI-MT** in methanol-glycerol systems of different viscosities.

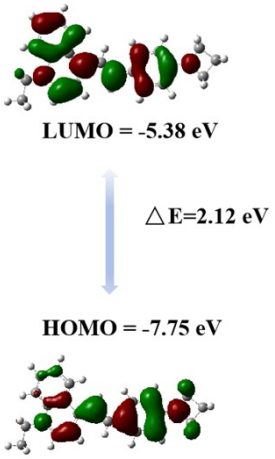


Fig. S12. Frontier molecular orbital maps of LUMO and HOMO of **PIM-MT.**

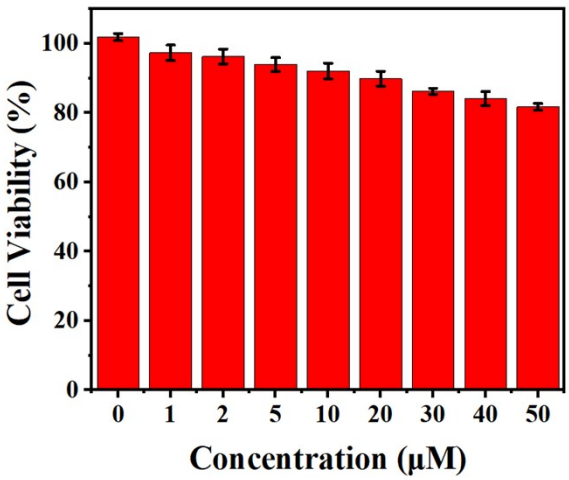


Fig. S13. Cytotoxicity assays of the probe **PMI-MT** at concentrations for HepG2 with CCK8.

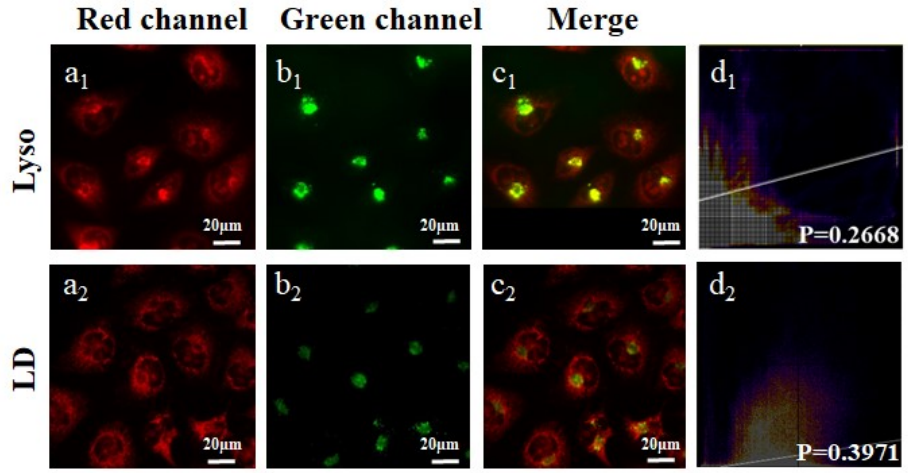


Fig. S14. The colocalization imaging of HepG2 cells staining with **PMI-MT** and different Tracker. (a_1 – a_5) The cells were stained with **PMI-MT** (10 μM) for 15 min and Lyso-Tracker Green for 10 min, λ_{ex} = 504 nm, λ_{em} = 501-521 nm. Scale bar: 20 μm; (b_1- b_5) The cells were stained with **PMI-MT** (10 μM) for 15 min and BODIPY 493/503 for10 min, λ_{ex} = 493 nm, λ_{em} =500-513nm.

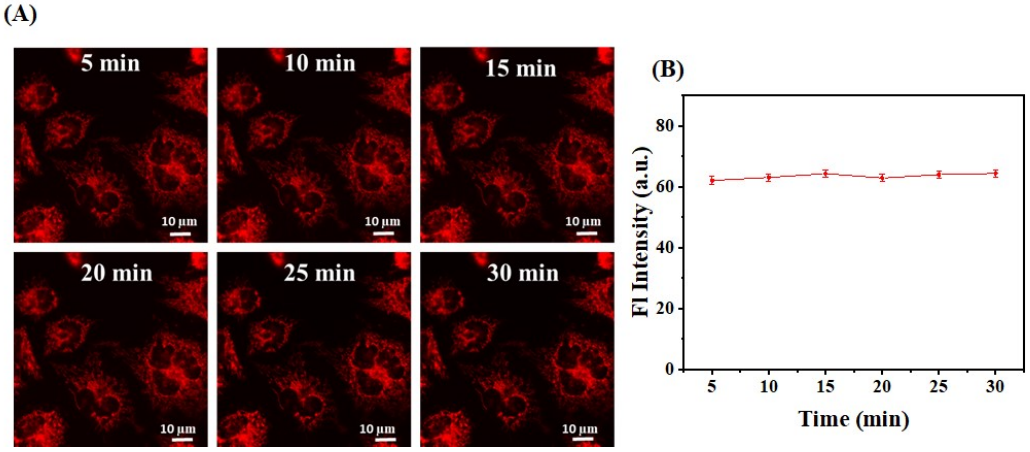


Fig. S15. The photostability map of the probe in HepG2 cells with constant laser irradiation was obtained by taking a measurement every five minutes.

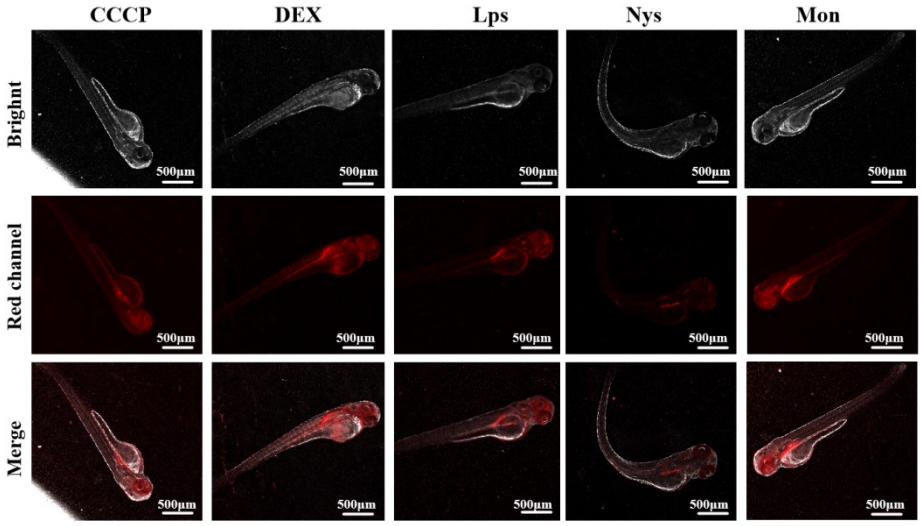


Fig. S16. Fluorescence images of zebrafish induced with 10 μM of different drugs (CCCP, Dexamethasone, Lipopolysaccharide, Nystatin, and Monensin) for 30 min and treated with probe **PMI-MT** for 10 min. Scale bars: 500 μm.

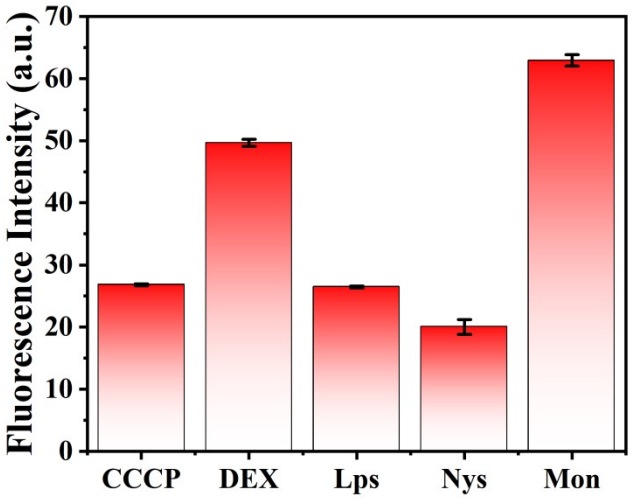


Fig. S17. Fluorescence intensity of zebrafish induced with 10 μM of different drugs (CCCP, Dexamethasone, Lipopolysaccharide, Nystatin,and Monensin) for 30 min and treated with probe **PMI-MT** for 10 min.

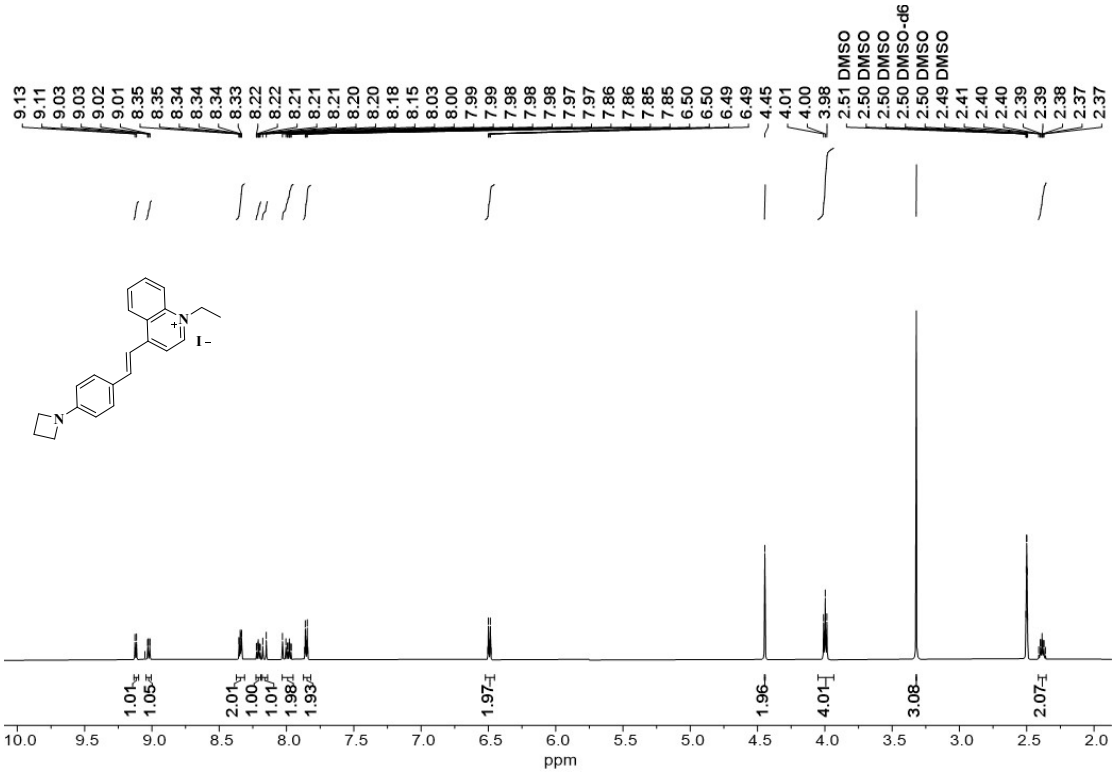


Fig. S18. The ^{1}H NMR spectrum of the compound **PMI-MT** in DMSO.

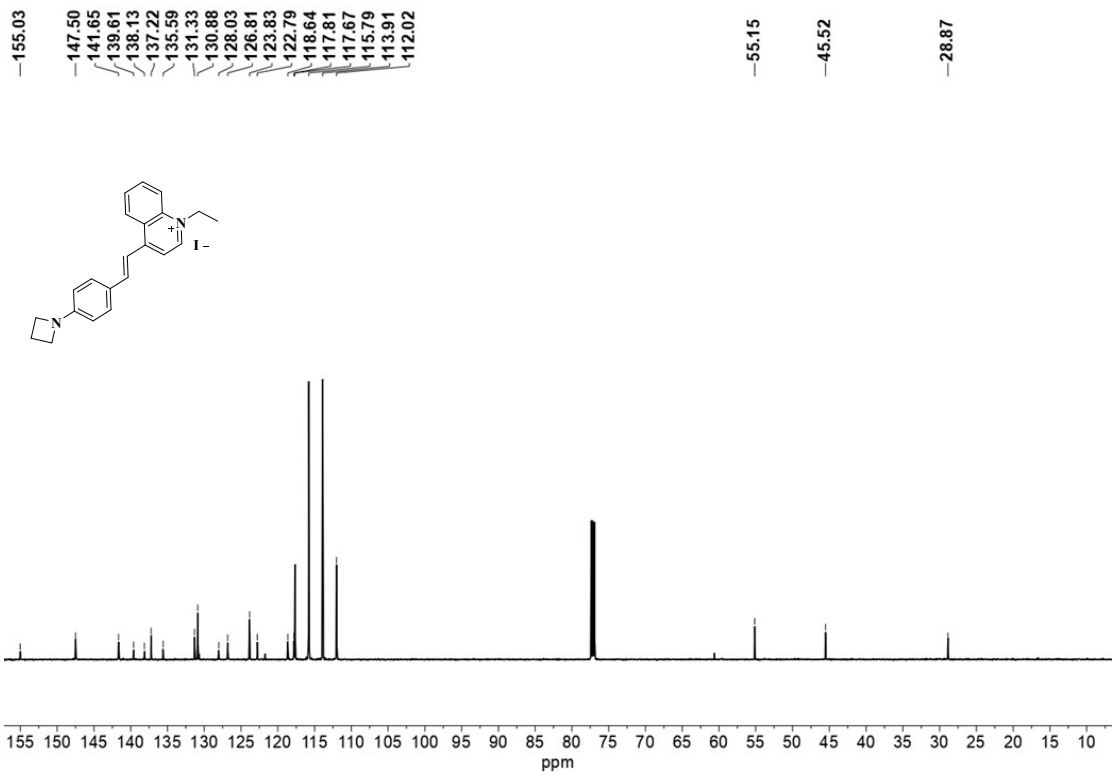


Fig. S19. The ^{13}C NMR spectrum of the probe **PMI-MT** in $CDCl_3$.

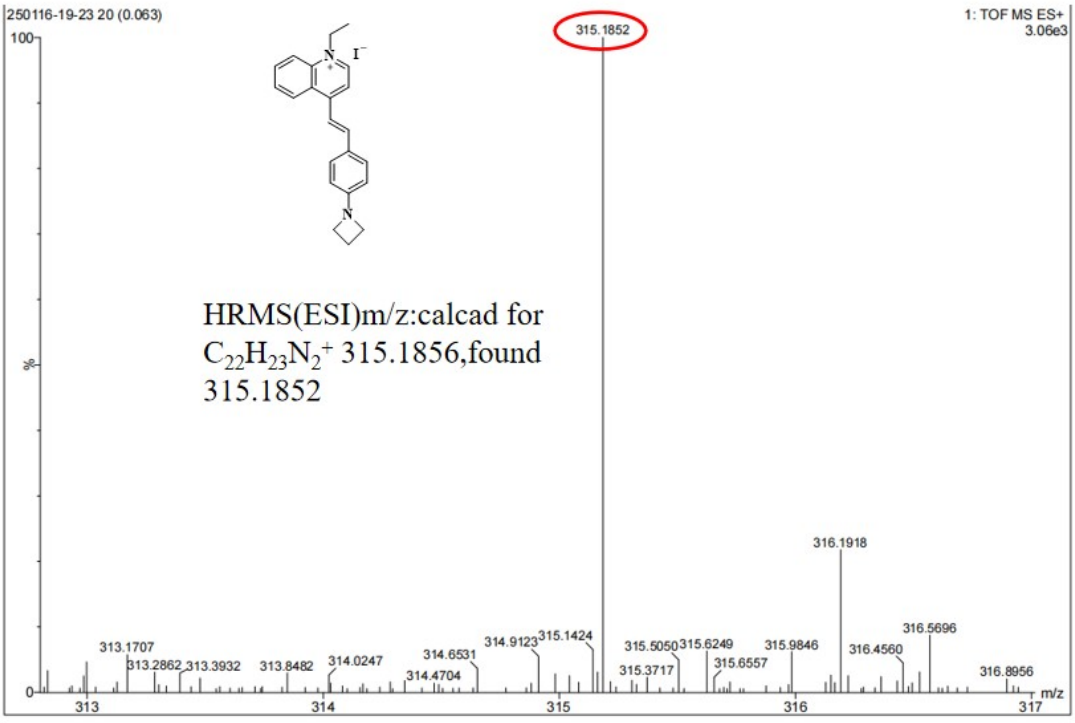


Fig. S20. The HRMS spectrum of the probe **PMI-MT**.

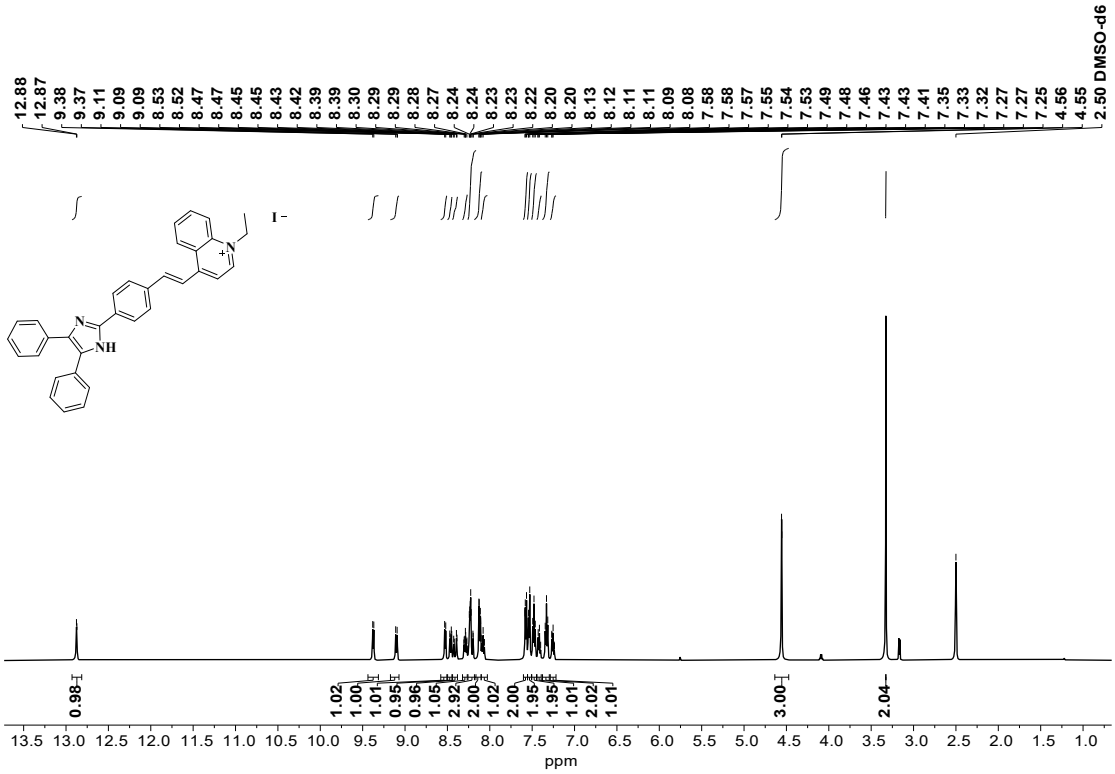


Fig. S21. The ^{1}H NMR spectrum of the compound **DB-1** in DMSO.

References

1. M. Mečiarová, Š. Toma and P. Magdolen, *Ultrason. Sonochem.*, 2003, **10**, 265-270.
2. N. Wang, J. Su, Q. Deng, A. Liu, F. Qi, J. Zhang, L. Long, S. Kong, C. Liu and R. Tang, *Dyes Pigm.*, 2020, **177**.