Supporting Information

A NIR molecular rotor photosensitizer for efficient PDT and synchronous mitochondrial viscosity imaging

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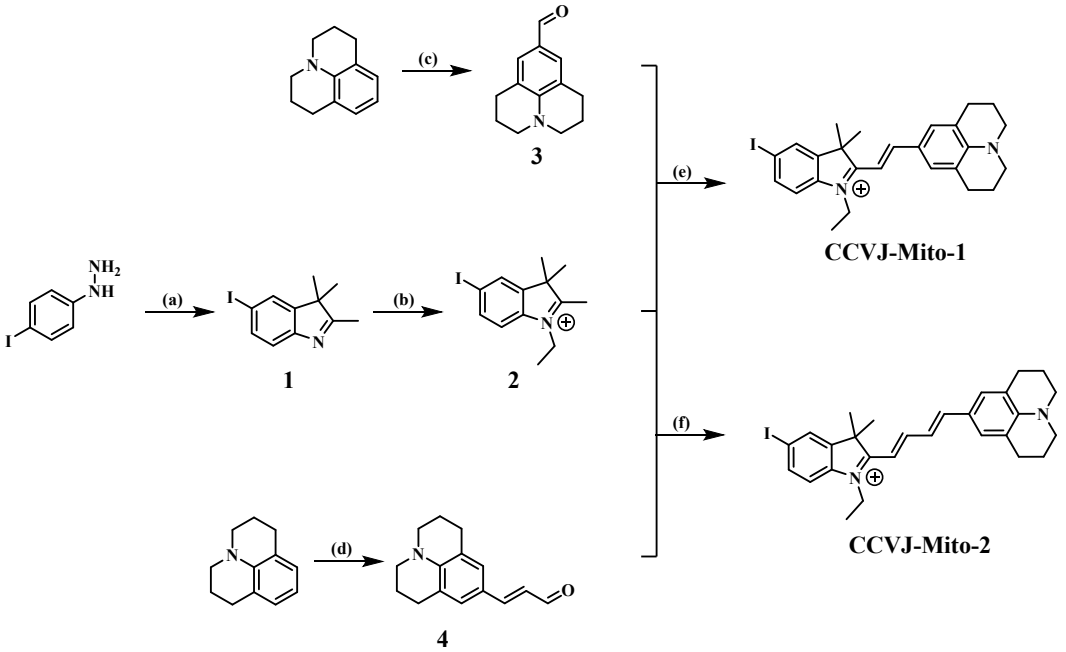
1. Experiment

1.1 Materials and methods

All the reagents and solvents were commercially available and used without further purification. Cell culture related items, including fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, PBS, and penicillin/streptomycin, were purchased from Invitrogen. DCFH-DA, calcein AM and propidium iodide (PI) were purchased from Abcam. 96-well of ibidi® culture plates were purchased from ibidi GmbH. Mass spectra were measured with a PC Sciex API 150 EX ESI-MS system. NMR spectra were acquired by a Bruker 400 MHz NMR spectrometer. A FiveEasy TM Fe20 pH meter was employed to measure pH values. Absorption and emission spectra were recorded on Molecular Devices SpectraMax ID5 Microplate Reader. Fluorescence imaging was conducted with a Leica TCS SP5 confocal scanning microscope.

1.2 Synthesis

CCVJ-Mito-1 and **CCVJ-Mito-2** were synthesized according to the protocol depicted in **Scheme S1**.[1, 2]



Scheme S1 Synthetic route of the compound **CCVJ-Mito-1** and **CCVJ-Mito-2**. (a) 3-methyl-2-butanone, acetic acid, yield 76.5%; (b) iodoethane, acetonitrile, yield 79.1%; (c) $POCl_3$, DMF, yield 87.6%; (d) $POCl_3$, 3-dimethylaminoacrolein, yield 17.0%; (e) EtOH, reflux, yield 52.8%; (f) EtOH, reflux, yield 61.5%.

Synthesis of Compound **1**

Compound (4-iodophenyl)hydrazine (2.34 g, 10.0 mmol) and 3-methyl-2-butanone (1.72 g, 20.0 mmol) were dissolved in 50 mL of acetic acid and then heated to reflux for 4 h under N_2. The mixture was cooled to room temperature and acetic acid was evaporated under reduced pressure. The crude product was purified with silica gel chromatography to obtain compound **1** as light yellow oil (2.18 g, 76.5%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.81 (d, J = 1.8 Hz, 1H), 7.60 (dd, J = 8.0, 1.8 Hz, 1H), 7.24 (d, J = 8.0 Hz, 1H), 2.18 (s, 3H), 1.22 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 188.87, 153.75, 149.20, 136.60, 131.10, 122.00, 90.73, 54.17, 22.72, 15.52.

Synthesis of Compound **2**

Compound **1** (1.43 g, 5.0 mmol) were mixed with iodoethane (1.56 g, 10.0 mmol) in 50 mL of acetonitrile. The solution was refluxed for 16 h under N_2. Then, the solution was cooled to room temperature and the precipitate was filtered and washed with 50 mL of ethyl ether for three times to give compound **2** as colorless solid (1.74 g, 79.1%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.30 (d, J = 1.6 Hz, 1H), 7.99 (dd, J = 8.4, 1.6 Hz, 1H), 7.78 (d, J = 8.4 Hz, 1H), 4.45 (q, J = 7.3 Hz, 2H), 2.80 (s, 3H), 1.52 (s, 6H), 1.40 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 196.66, 144.64, 141.09, 138.07, 132.95, 117.78, 96.81, 54.72, 43.66, 22.14, 14.37, 13.10.

Synthesis of Compound **3**

$POCl_3$ (2.0 g, 13.0 mmol) was added dropwise to a solution of DMF (1.83 g, 25.0 mmol) in dichloromethane (50 mL) at 0℃ under N_2. After stirred for 30 min, julolidine (1.73 g, 10.0 mmol) was added to the mixture and further heated at reflux for 4 h. Then, the mixture was cooled to room temperature, quenched with cold water (100 mL) and neutralized with sodium hydroxide. The organic layer was separated and dried with Na_2SO_4. Solvent was evaporated under vacuum and compound **3** was obtained as light green solid (1.76 g, 87.6%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.50 (s, 1H), 7.21 (s, 2H), 3.33-3.20 (m, 4H), 2.68 (t, J = 6.3 Hz, 4H), 1.84 (dt, J = 11.4, 5.8 Hz, 4H). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 189.75, 147.98, 129.34, 123.80, 120.39, 49.72, 27.51, 21.16.

Synthesis of Compound **4**

$POCl_3$ (2.0 g, 13.0 mmol) was added dropwise to a solution of 3-dimethylaminoacrolein (1.98 g, 20.0 mmol) in dichloromethane (30 mL) at 0℃ under N_2. After stirred for 30 min, julolidine (1.73 g, 10.0 mmol) was added to the mixture and further stirred for 12 h. Then, the mixture was quenched with cold water (100 mL) and neutralized with sodium hydroxide. The organic layer was separated

and dried with Na_2SO_4. Solvent was evaporated under vacuum and the residue was purified by silica gel chromatography with dichloromethane to afford compound **4** as yellow solid (387 mg, 17.0%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.52 (d, *J* = 7.9 Hz, 1H), 7.25 (d, *J* = 7.9 Hz, 1H), 7.00 (s, 2H), 6.45 (dd, *J* = 15.5, 8.0 Hz, 1H), 3.27-3.23 (m, 4H), 2.73 (t, *J* = 6.4 Hz, 4H), 1.94 (q, *J* = 6.1 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 193.66, 154.52, 145.78, 128.26, 122.68, 120.98, 120.70, 49.95, 27.68, 21.44.

Synthesis of Compound **CCVJ-Mito-1**

Compound **2** (88 mg, 0.2 mmol) was mixed with compound **3** (40 mg, 0.2 mmol) in 20 mL of ethanol, then the mixture was refluxed overnight under N_2. Solvent was evaporated under vacuum and the residue was purified by silica gel chromatography with dichloromethane : methanol (20:1, v/v) to afford **CCVJ-Mito-1** as deep purple solid (66 mg, 52.8%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.25-8.10 (m, 2H), 7.96-7.50 (m, 3H), 7.42 (d, *J* = 8.4 Hz, 1H), 7.02 (d, *J* = 15.1 Hz, 1H), 4.40 (q, *J* = 7.0 Hz, 2H), 3.45 (t, *J* = 5.7 Hz, 4H), 2.74 (t, *J* = 6.1 Hz, 4H), 1.91 (q, *J* = 5.8 Hz, 4H), 1.70 (s, 6H), 1.31 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.61, 154.47, 150.30, 145.03, 141.33, 137.72, 131.98, 122.37, 115.06, 102.86, 102.31, 94.01, 92.04, 50.65, 50.57, 28.24, 27.26, 26.98, 20.88, 13.31.

Synthesis of Compound **CCVJ-Mito-2**

Compound **2** (66 mg, 0.15 mmol) was mixed with compound **4** (34 mg, 0.15 mmol) in 20 mL of ethanol, then the mixture was refluxed overnight under N_2. Solvent was evaporated under vacuum and the residue was purified by silica gel chromatography with dichloromethane : methanol (20:1, v/v) to afford **CCVJ-Mito-2** as green solid (60 mg, 61.5%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (dd, *J* = 14.3, 11.6 Hz, 1H), 8.15 (d, *J* = 1.6 Hz, 1H), 7.85 (dd, *J* = 8.4, 1.7 Hz, 1H), 7.68 (d, *J* = 14.3 Hz, 1H), 7.46 (d, *J* = 8.4 Hz, 1H), 7.28-7.19 (m, 3H), 6.75 (d, *J* = 14.4 Hz, 1H), 4.26 (q, *J* = 7.2 Hz, 2H), 3.40-3.37 (m, 4H), 2.72 (t, *J* = 6.3 Hz, 4H), 1.89 (p, *J* = 5.8 Hz, 4H), 1.68 (s, 6H), 1.32 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.25, 156.82, 154.42, 148.48, 145.07, 141.36, 137.77, 132.06, 130.27, 123.60, 122.93, 122.32, 115.20, 108.41, 92.28, 50.69, 50.32, 27.39, 26.81, 21.03, 18.00, 13.05.

1.3 General procedure for viscosity detection in solution

Stock solution was prepared by dissolving **CCVJ-Mito-1** or **CCVJ-Mito-2** in DMSO with concentration of 5 mM. Then, the probe was diluted to 5 μM with various solvents. 530 nm and 630 nm were set as excitation wavelength for **CCVJ-Mito-1** and **CCVJ-Mito-2**, respectively.

1.4 Cell culture and fluorescence imaging

Cells were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS at

37 °C under 5% CO_2.

Colocalization imaging: Cells were seeded in a confocal dish (35 mm) for 24 h with density of about 10^4 cells, and then co-incubated with probes (1 μM) and **Mito-tracker Green** (100 nM) for 30 min. The cells were washed with PBS for three times and confocal imaging was conducted. Excitation wavelength was set to 543 nm, 633 nm and 488 nm for **CCVJ-Mito-1**, **CCVJ-Mito-2** and **Mito-tracker Green**, respectively. Corresponding emission was collected at 600-650 nm, 700-800 nm and 500-540 nm.

Viscosity dynamics imaging: Cells were seeded in a confocal dish (35 mm) for 24 h with density of about 10^4 cells, and then treated with Nystatin (10 μM and 20 μM) or10 μM Cu^{2+} for 30 min.[3,4] Cells treated with DMEM alone was employed as control. Cells were washed with PBS for three times and further incubated with 1 μM **CCVJ-Mito-1** or **CCVJ-Mito-2** for another 30 min. After washed with PBS for three times, confocal imaging was conducted. The detailed settings of fluorescence imaging were shown in **Figure S18**.

1.5 Procedure for PDT test in solution and in living cells

Determination of $^1O_2$ production efficiency: With **DPBF** as $^1O_2$ indicator,[5] the concentration of DPBF in 1 mL DMF was adjusted to make the absorbance at 415 nm close to 1.0. Then, 5 μM of **CCVJ-Mito-1** or **CCVJ-Thio-Mito-2** was added, respectively. After that, the mixture solutions were exposed to light irradiation and the absorption spectrum was recorded with a UV-vis spectrophotometer (590 nm, 50 mW/cm^2 for **CCVJ-Mito-1** and 660 nm, 60 mW/cm^2 for **CCVJ-Mito-2**). With **SOSG** as $^1O_2$ indicator,[6] 5 μM of SOSG and **CCVJ-Mito-1** or **CCVJ-Mito-2** were mixed in deionized water. Then, the mixture solutions were exposed to light irradiation and the fluorescence spectrum was recorded with a Molecular Devices SpectraMax ID5 Microplate Reader (590 nm, 50 mW/cm^2 for **CCVJ-Mito-1** and 660 nm, 60 mW/cm^2 for **CCVJ-Mito-2**) with excitation at 450 nm.

Phototoxicity experiment in cells: Cells (5×10^3 /well) were incubated on a 96-well plate for 24 h. Then, the cells were treated with different concentrations of probes (0, 0.25, 0.5, 0.75, 1 μM) for 1 h. After that, the old medium was replaced with fresh medium. For light irradiation, cells were irradiated with LED light for 5 min. After an additional 12 h incubation, the cell viability was measured using CCK8 assay.

Live/Dead cell co-staining:[7] HeLa cells were seeded in a confocal dish (35 mm) for 24 h with

density of about 10^5 cells. Then, the cells were treated with 1 μM of probes for 1 h and irradiated with light for 5 min. HeLa cells were further cultured for 6 h and incubated with 500 nM of calcein AM and 10 μM of PI under dark for another 30 min. After washing with PBS, confocal imaging was conducted with Leica TCS SP5. 488 nm and 543 nm were set as excitation wavelength for calcein AM and PI, respectively. Corresponding emission at 500-550 nm for calcein AM, 580-610 nm for PI in **CCVJ-Mito-1** group and and 580-650 nm for PI in **CCVJ-Mito-2** group were collected.

Determination of cellular ROS generation in cells: Cells were seeded in a confocal dish (35 mm) for 24 h with density of about 10^4 cells. Then, the cells were treated with 1 μM of probes for 1 h. Afterwards, 10 μM DCFH-DA was added and incubated for another 30 min. The cells were then washed with PBS and irradiated with LED light for 5 min. Confocal imaging was conducted with Leica TCS SP5 at 488 nm excitation and the fluorescence channel at 500-550 nm was collected.

1.6 Mitochondrial viscosity imaging during PDT

Cells were seeded in a confocal dish (35 mm) for 24 h with density of about 10^4 cells. Then, the cells were treated with 1 μM of probes for 1 h and irradiated for 5 min. Afterwards, cells were incubated under dark for another 1 h. The cells were then washed with PBS and confocal imaging was conducted with Leica TCS SP5.

2. Supplementary Figures

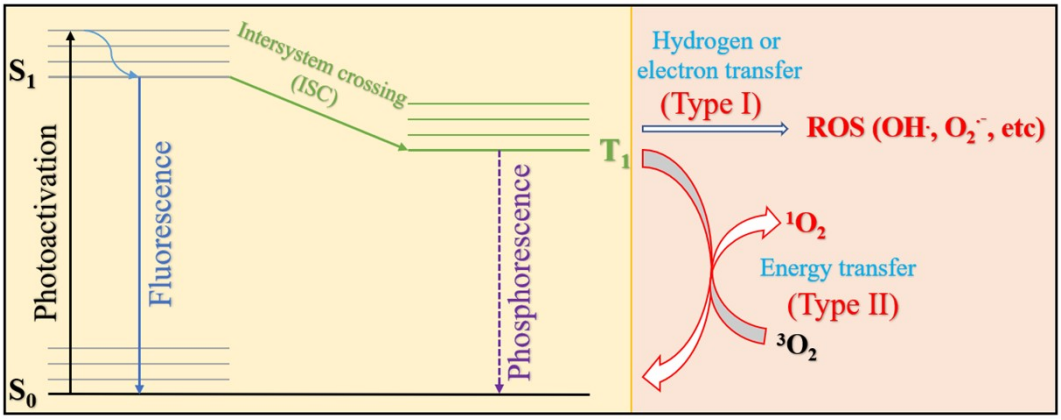


Fig. S1. Schematic illustration of Type I and type II PDT.

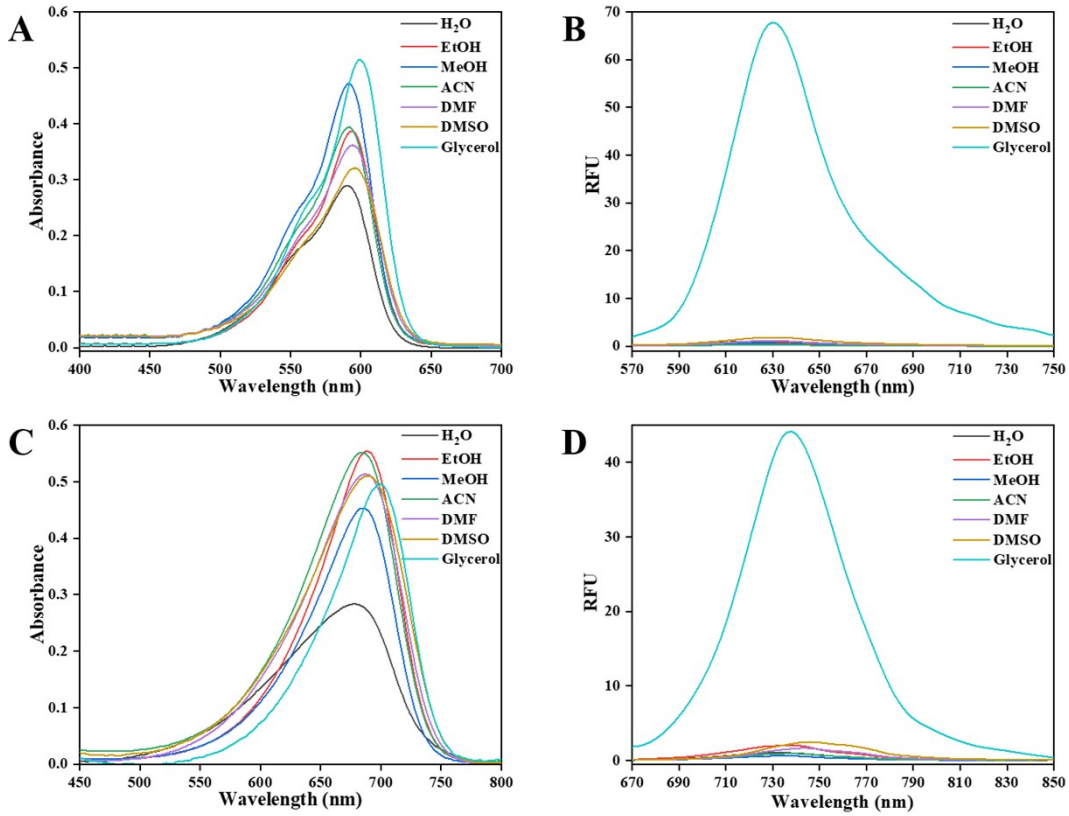


Fig. S2. Absorbance and fluorescence of **CCVJ-Mito-1** (A and B) and **CCVJ-Mito-2** (C and D) in different solvents.

Table S1 Photophysical properties of **CCVJ-Mito-1** and **CCVJ-Mito-2.**

Compounds	**Solvents**	**λ_{abs} (nm)**	**ε (M^{-1} cm^{-1})**	**λ_{em} (nm)**	**Stokes shift (nm)**	**Φ_F**
CCVJ-Mito-1	H_2O	590	57962	625	35	0.028
	EtOH	593	77423	623	30	0.046
	MeOH	592	94443	624	32	0.042
	ACN	592	78802	625	33	0.016
	DMF	594	72435	628	34	0.058
	DMSO	595	64286	631	36	0.096
	Glycerol	600	102881	631	31	0.53
CCVJ-Mito-2	H_2O	678	56813	733	55	0.029
	EtOH	688	110824	736	48	0.055
	MeOH	685	90605	733	48	0.035
	ACN	684	110435	740	56	0.021
	DMF	687	102653	744	57	0.045
	DMSO	689	102817	746	57	0.087
	Glycerol	700	992478	739	39	0.49

Note: For **CCVJ-Mito-1**, Nile Blue was used as reference with Φ_F=0.30 in DMSO;[8] For **CCVJ-Mito-2**, methylene blue (**MB**) was used as reference with Φ_F=0.52 in water.[9]

Compounds	$S^0 \rightarrow S^1$ (eV)	$f_{S^0 \rightarrow S^1}$	$S^0 \rightarrow T^1$ (eV)	$S^1 \rightarrow T^1$ (eV)
CCVJ-Mito-1	2.0962	1.9364	1.3115	0.7847
CCVJ-Mito-2	1.8207	2.3685	1.0997	0.721

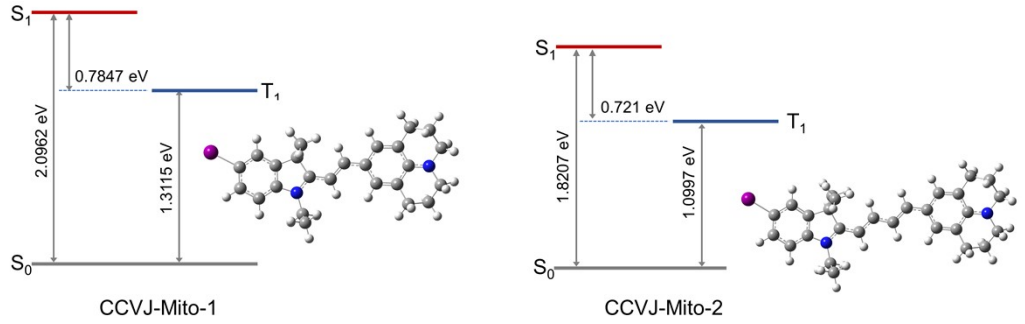


Fig. S3. DFT calculation results for **CCVJ-Mito** compounds[10]. The geometries of **CCVJ-Mito-1** and **CCVJ-Mito-2** were optimized based on the density functional theory (DFT)//B3LYP/6-31G(d) or DFT//B3LYP/6-31G(d)/LANL2DZ method. The excitation energies for the singlet and triplet excited states of **CCVJ-Mito-1** and **CCVJ-Mito-2** were predicted with time-dependent density functional theory (TDDFT) based on the H_2O-TDDFT//B3LYP/6-31G(d) or H_2O-TDDFT//B3LYP/6-31G(d)/LANL2DZ method.

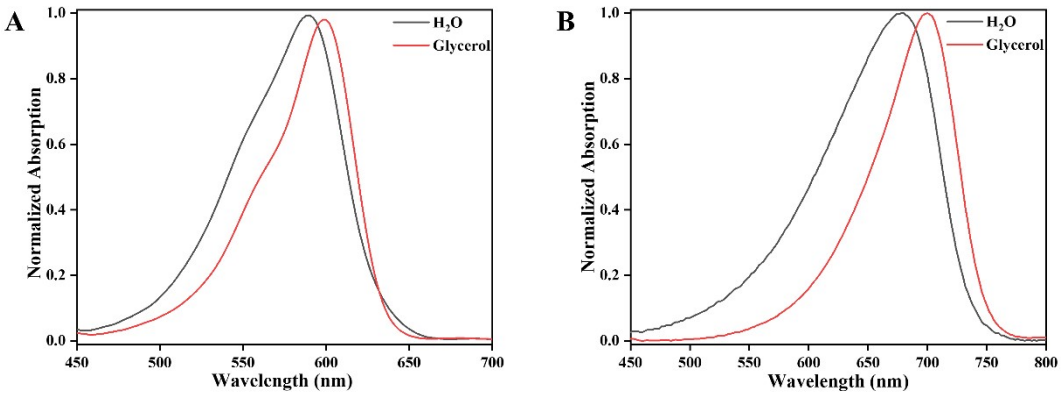


Fig. S4. UV-Vis absorbance of (A) **CCVJ-Mito-1** and (B) **CCVJ-Mito-2** in H_2O (black line) and glycerol (red line).

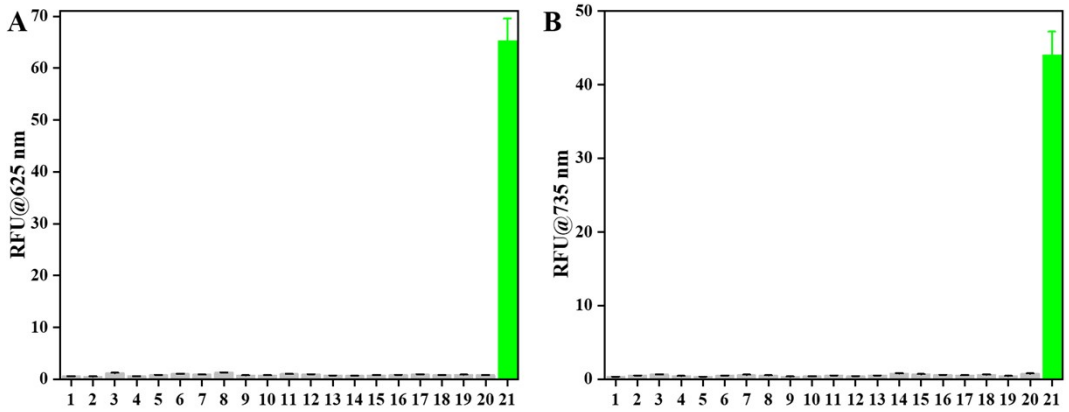


Fig. S5. Fluorescence response of (A) **CCVJ-Mito-1** and (B) **CCVJ-Mito-2** towards various common biomolecules. (1) Blank; (2) Na^{+}; (3) Ca^{2+}; (4) Mg^{2+}; (5) PO_4^{3-}; (6) NO_3^{-}; (7) SO_3^{2-}; (8) H_2O_2; (9) HClO; (10) $O_2^{\cdot-}$; (11) $HO\cdot$; (12) $ONOO^{-}$; (13) Cys; (14) HCy; (15) GSH; (16) ATP; (17) ADP; (18) AMP; (19) PPi; (20) BSA; (21) Glycerol.

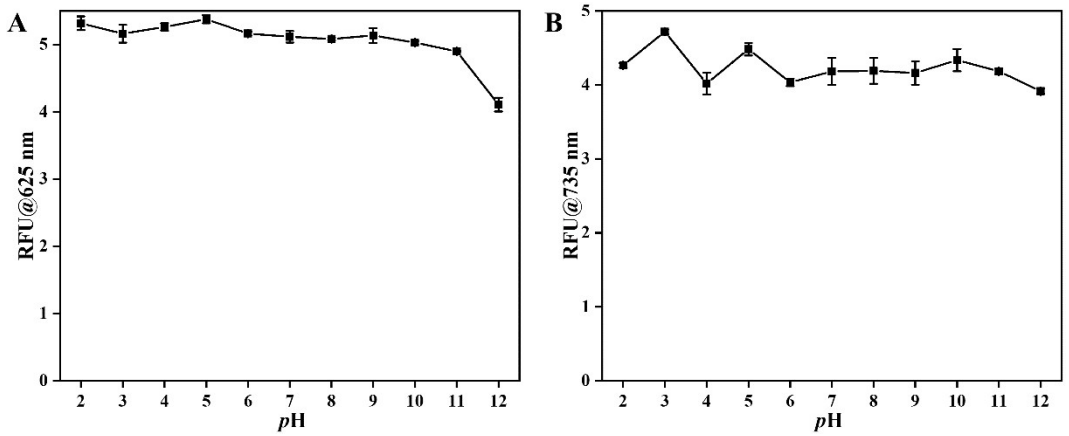


Fig. S6. Fluorescence of (A) **CCVJ-Mito-1** and (B) **CCVJ-Mito-2** in solutions (water/glycerol=50/50) with different *p*H.

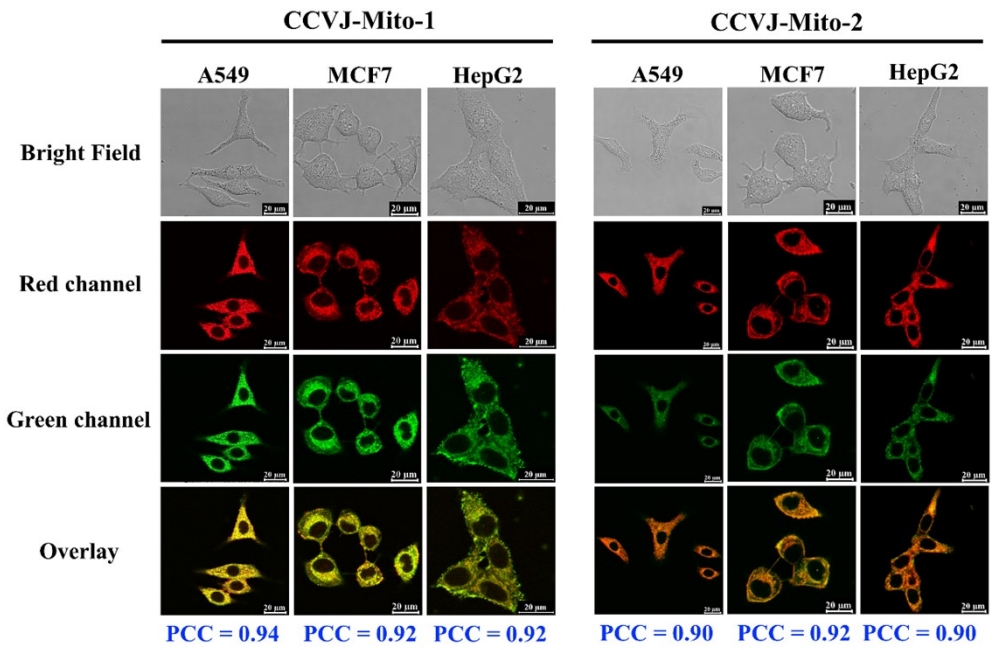


Fig. S7. Colocalization images of various cells incubated with **CCVJ-Mito-1** (1 μM) and **CCVJ-Mito-2** (1 μM) as well as **Mito-tracker Green** (100 nM). Emission: 600-650 nm for **CCVJ-Mito-1** (red channel), 700-800 nm for **CCVJ-Mito-2** (red channel) and 500-540 nm for **Mito-tracker Green** (green channel). Excitation: 543 nm for **CCVJ-Mito-1**, 633 nm for **CCVJ-Mito-2** and 488 nm for **Mito-tracker Green**. Scale bar=20 μm.

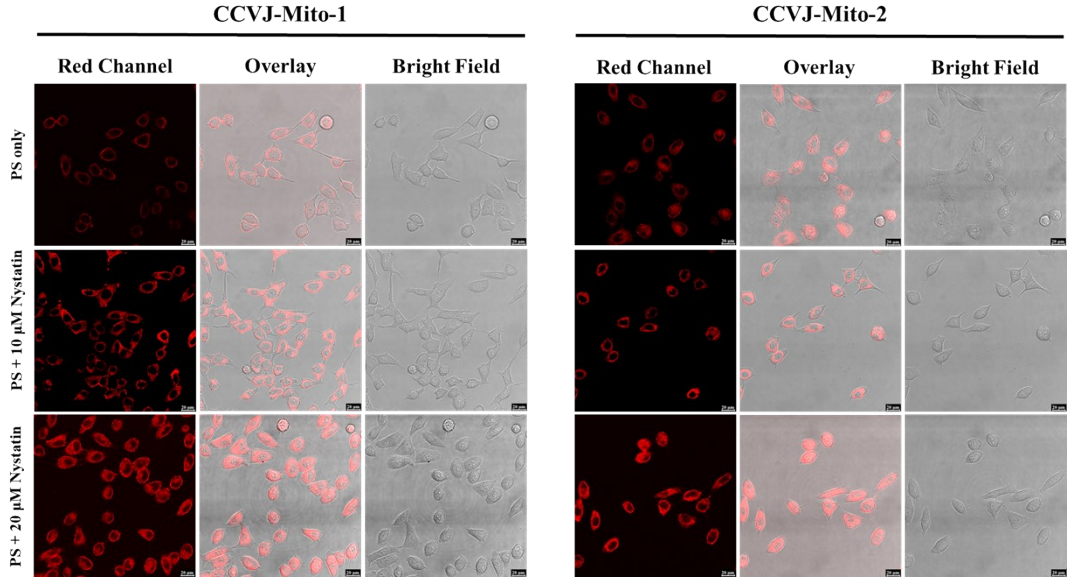


Fig. S8. CLSM images of HeLa cells treated with different concentrations of Nystatin. For **CCVJ-Mito-1**, Ex=543 nm, Em=600-650 nm; For **CCVJ-Mito-2**, Ex=633 nm, Em=700-800 nm. Scale bar = 20 μm.

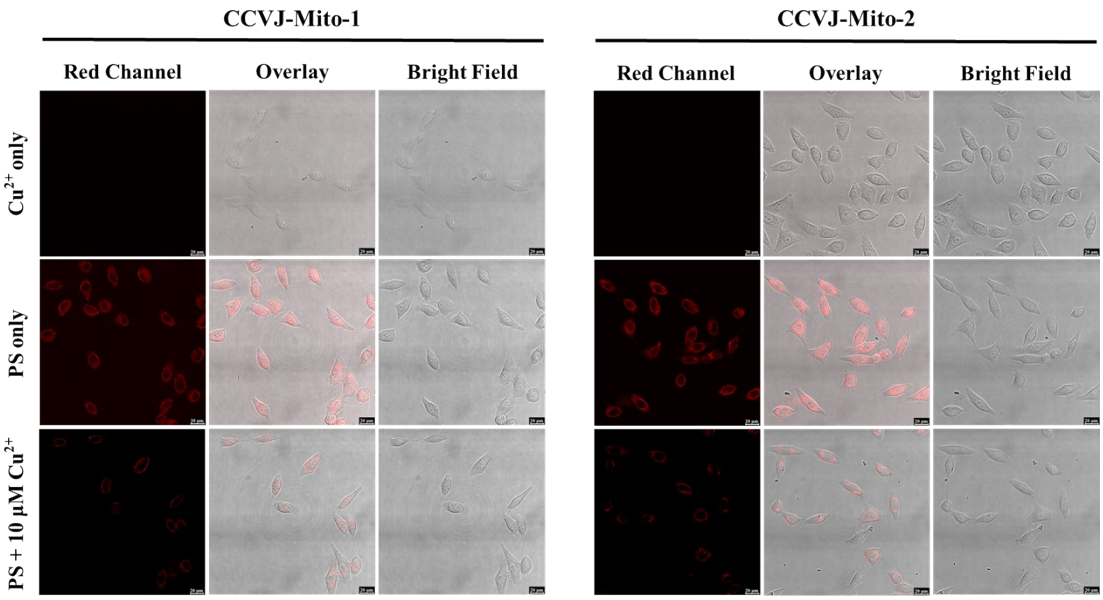


Fig. S9. CLSM images of HeLa cells treated w/o Cu^{2+}. For **CCVJ-Mito-1**, Ex=543 nm, Em=600-650 nm; For **CCVJ-Mito-2**, Ex=633 nm, Em=700-800 nm. Scale bar = 20 μm.

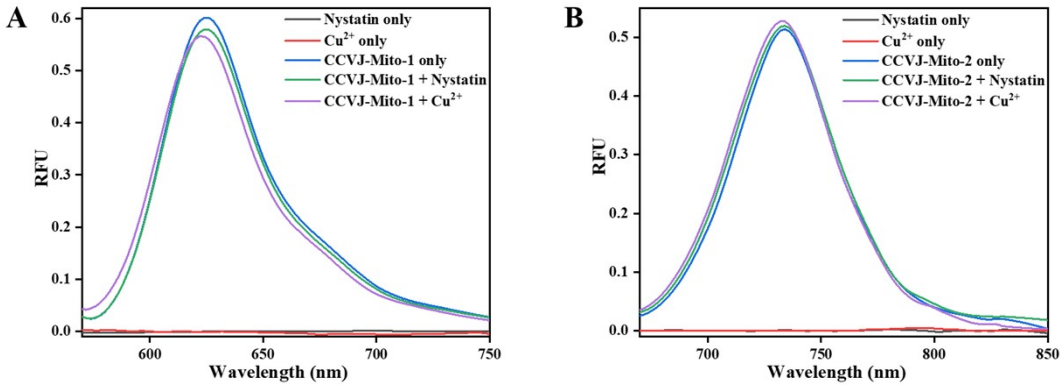


Fig. S10. Fluorescence variation of (A) **CCVJ-Mito-1** (5 μM) and (B) **CCVJ-Mito-2** (5 μM) after incubation with Nystatin (10 μM) or Cu^{2+} (10 μM) at 37℃ for 1 h. 530 nm and 630 nm were set as excitation wavelength for **CCVJ-Mito-1** and **CCVJ-Mito-2**, respectively.

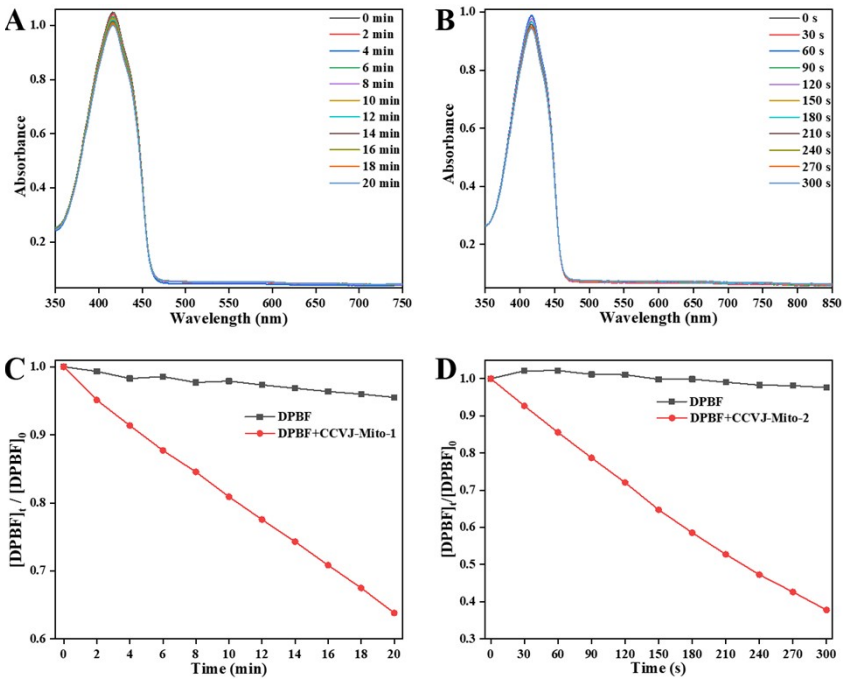


Fig. S11. Photodegradation curves of **DPBF** (A and B) under light irradiation (A: 590 nm, 50 mW/cm^2; B: 660 nm, 60 mW/cm^2). Quantification results of photodegradation plots of **DPBF** with/without (C) **CCVJ-Mito-1**; (D) **CCVJ-Mito-2**.

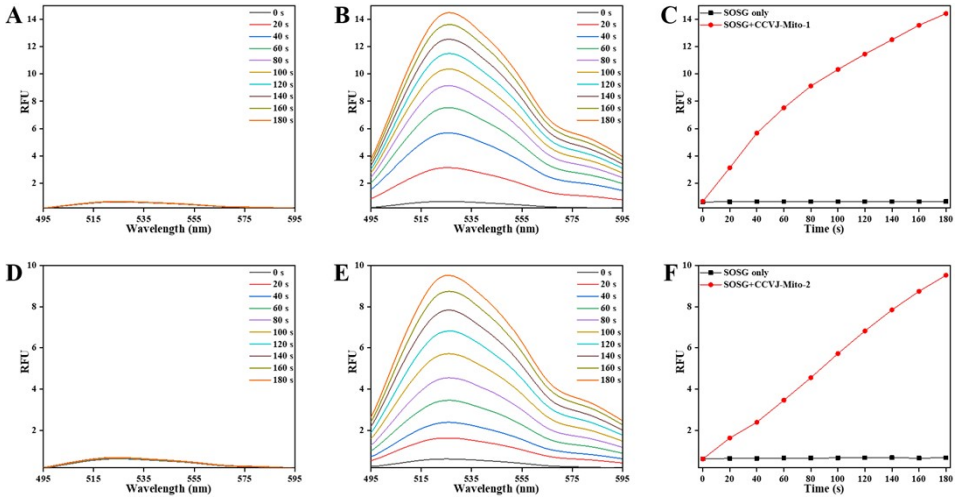


Fig. S12. PDT efficiency of **CCVJ-Mito-1** (A-C) and **CCVJ-Mito-2** (D-F) in water with **SOSG** (5 μM) as $^1O_2$ indicator. (A) **SOSG** alone in water; (B) **SOSG** incubated with **CCVJ-Mito-1** (5 μM) in water; (C) Plot of fluorescence at 525 nm as function of $^1O_2$ production; (D) **SOSG** alone in water; (E) **SOSG** incubated with **CCVJ-Mito-2** (5 μM) in water; (F) Plot of fluorescence at 525 nm as function of $^1O_2$ production. A-C was conducted under 590 nm light irradiation with power of 50 mW/cm^2, while D-F was conducted under 660 nm light irradiation with power of 60 mW/cm^2.

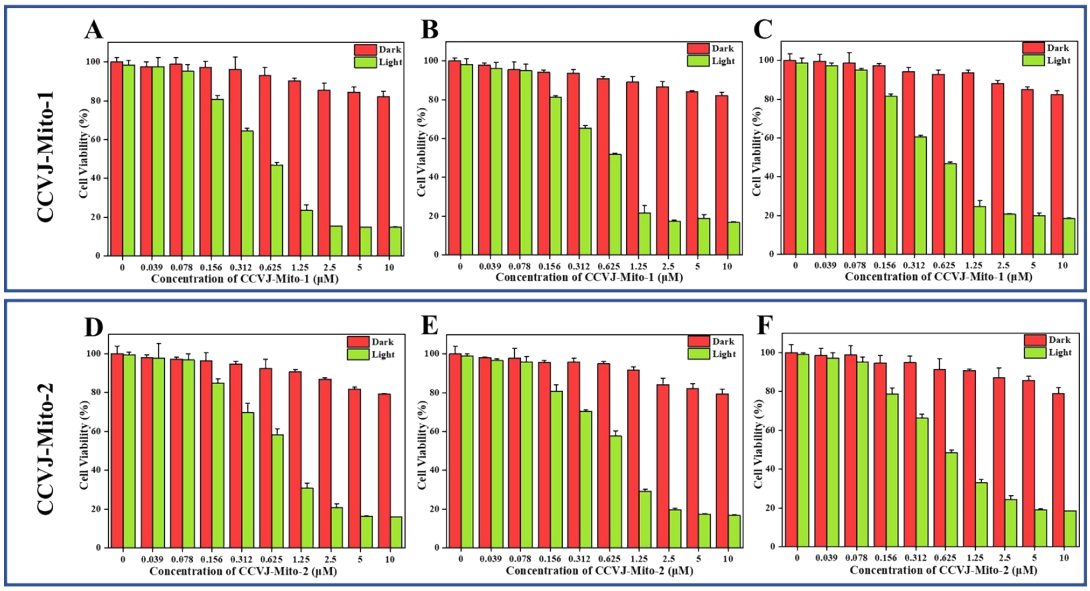


Fig. S13. Phototoxicity of **CCVJ-Mito-1** (A-C) and **CCVJ-Mito-2** (D-F) towards A549 (A, D), MCF7 (B, E) and HepG2 (C, F) cells.

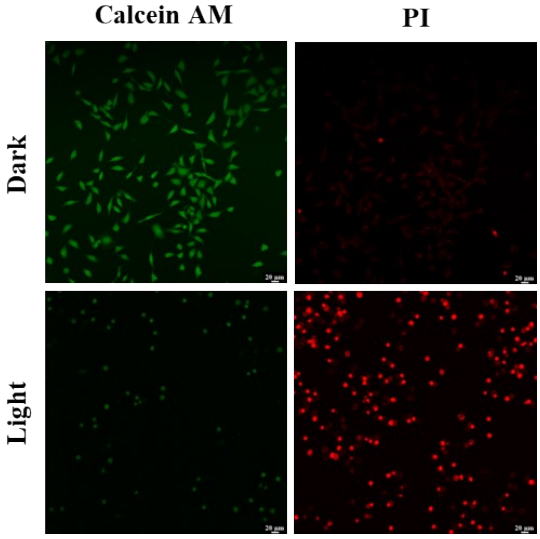


Fig. S14. Live/dead cell staining in **CCVJ-Mito-1** treated HeLa cells with/without light irradiation. Emission: 500-550 nm for calcein AM and 580-610 nm for PI. Excitation: 488 nm for calcein and 543 nm for PI. Scale bar=20 μm.

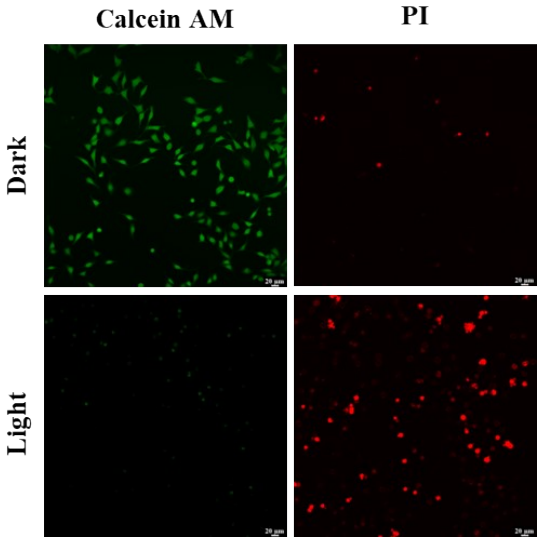


Fig. S15. Live/dead cell staining in **CCVJ-Mito-2** treated HeLa cells with/without light irradiation. Emission: 500-550 nm for calcein AM and 580-610 nm for PI. Excitation: 488 nm for calcein AM and 543 nm for PI. Scale bar=20 μm.

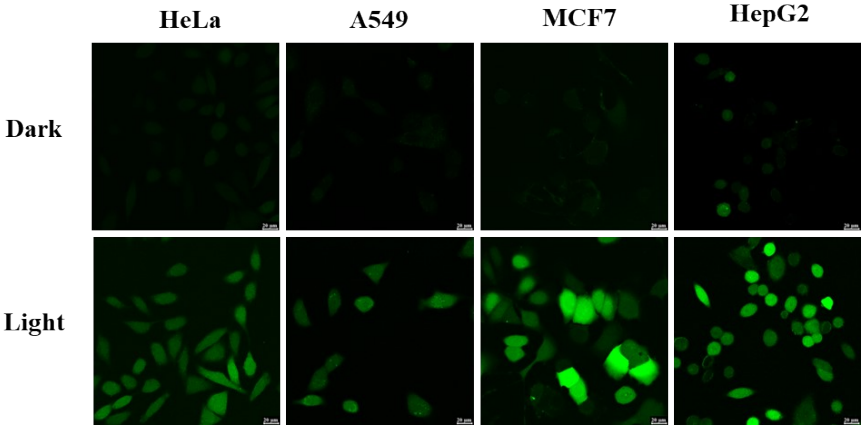


Fig. S16. Determination of cellular ROS generation in different cell lines by **CCVJ-Mito-1**.

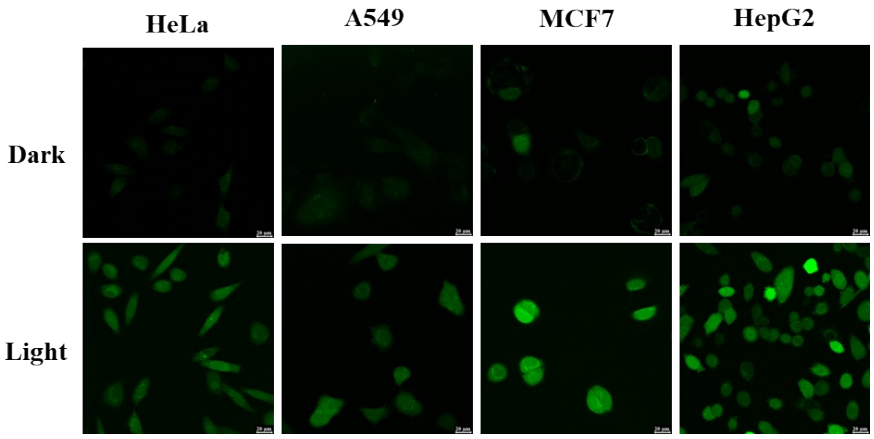


Fig. S17. Determination of cellular ROS generation in different cell lines by **CCVJ-Mito-2**.

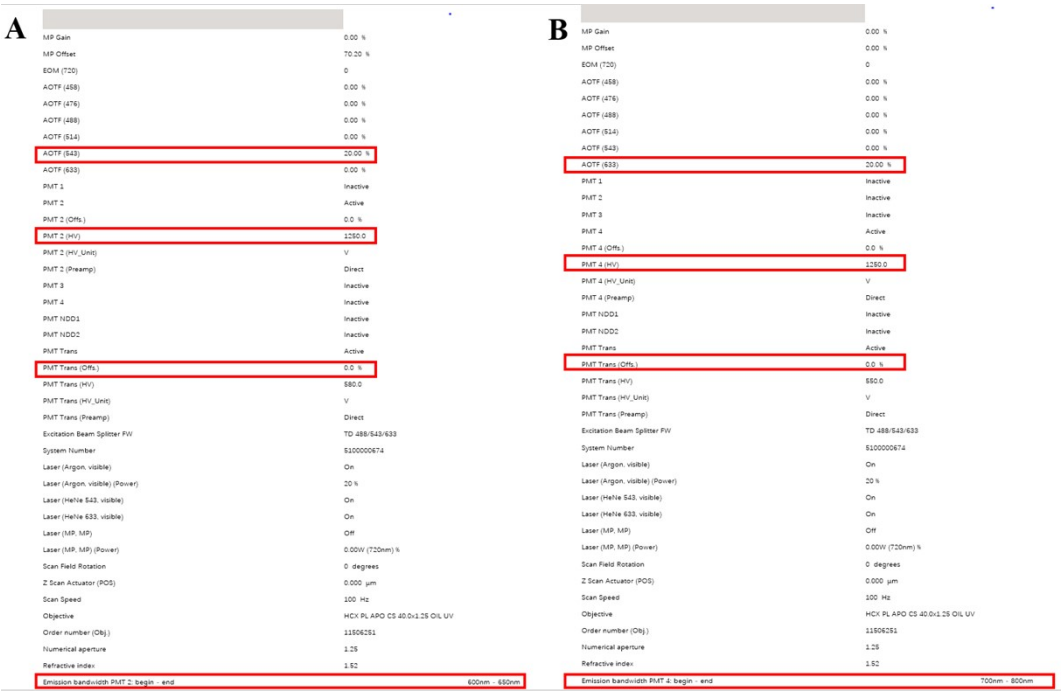
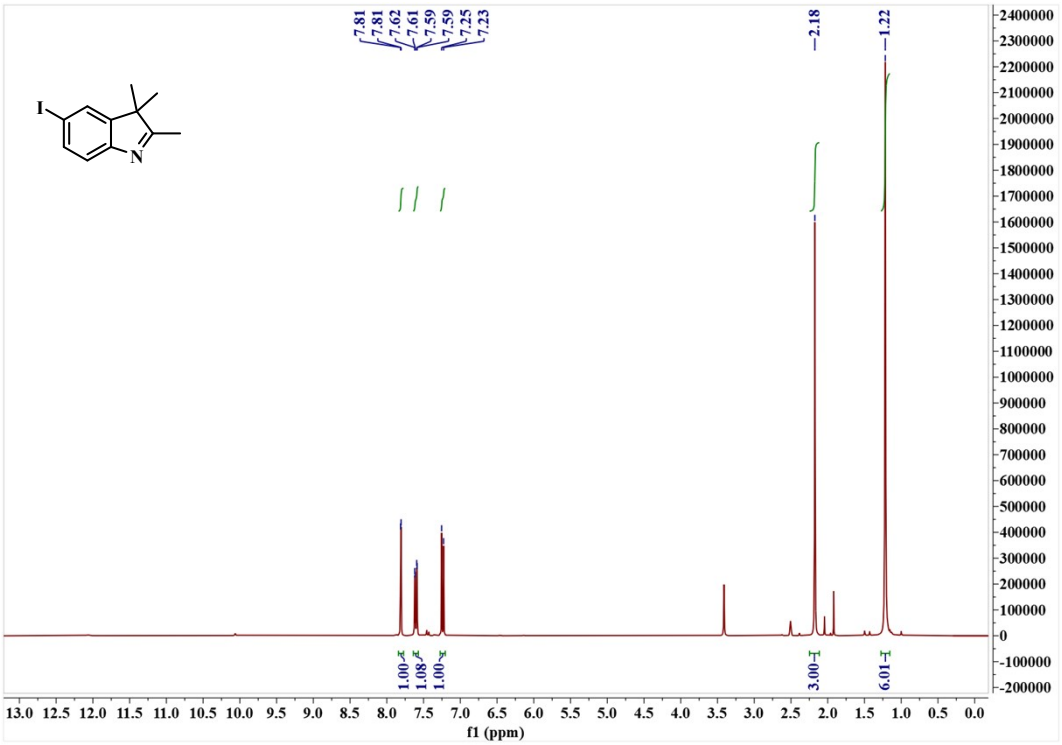
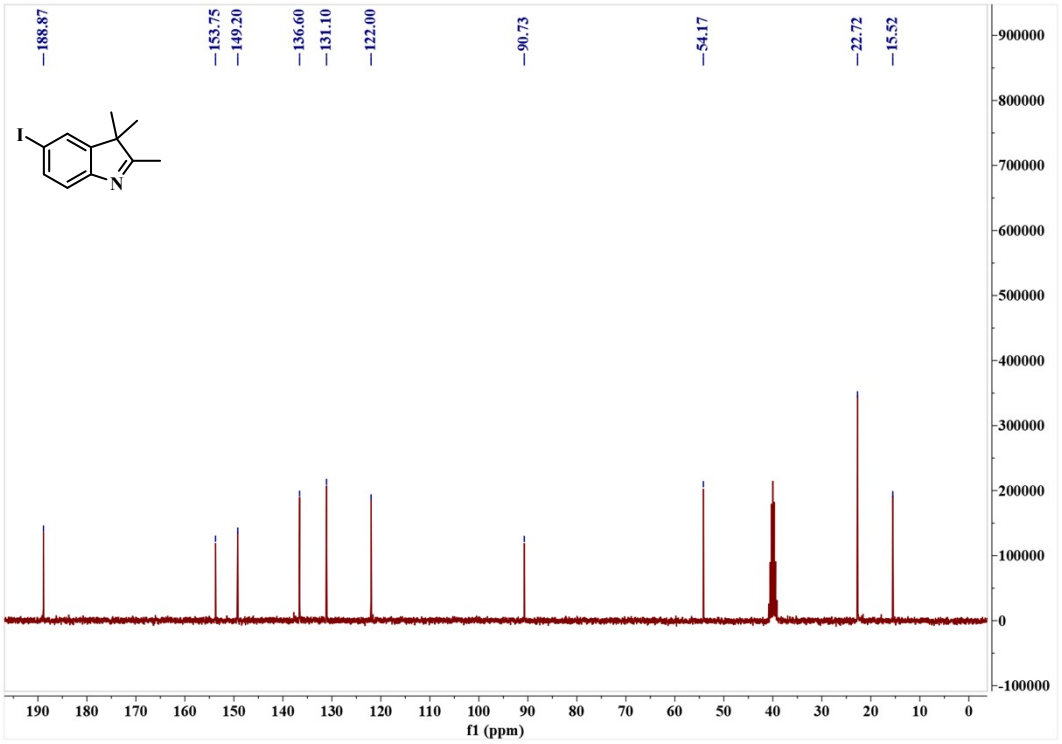


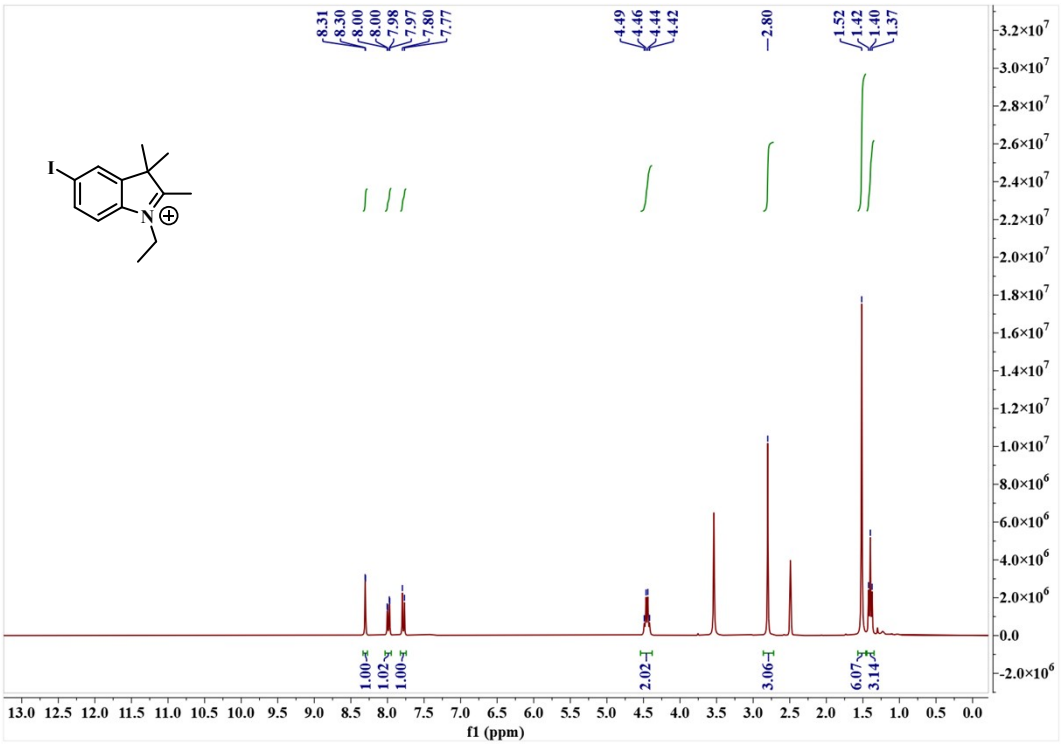
Fig. S18. Detail settings of fluorescence imaging for (A) **CCVJ-Mito-1** and (B) **CCVJ-Mito-2**.



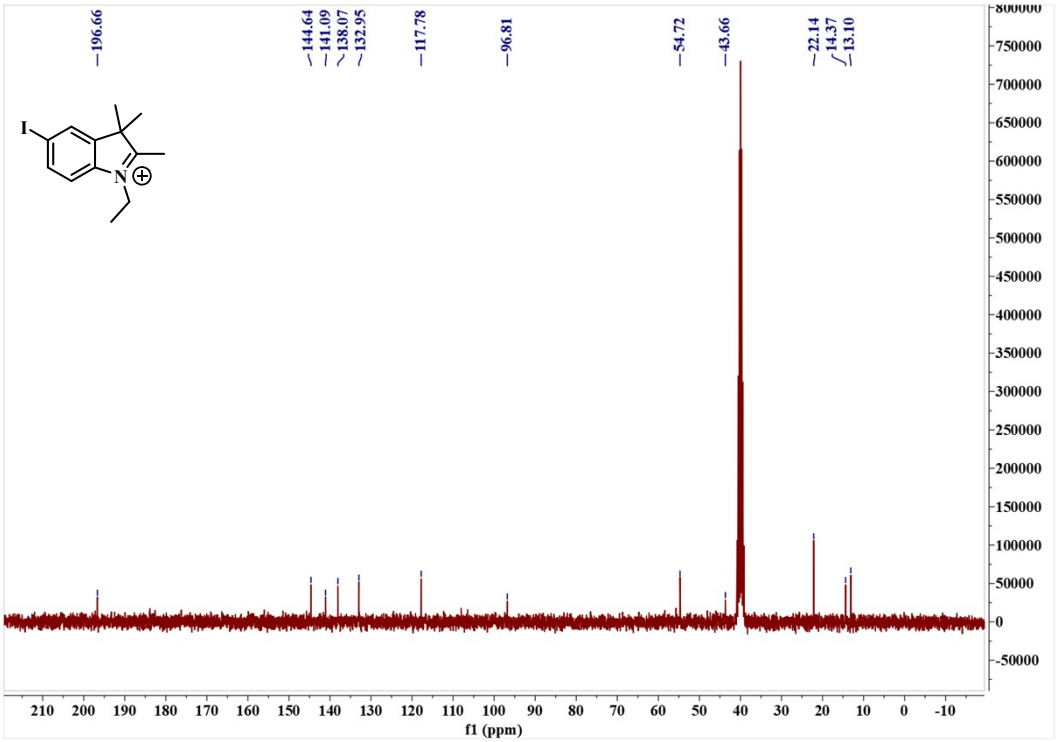
^{1}H NMR spectrum of compound **1** in DMSO-d_6



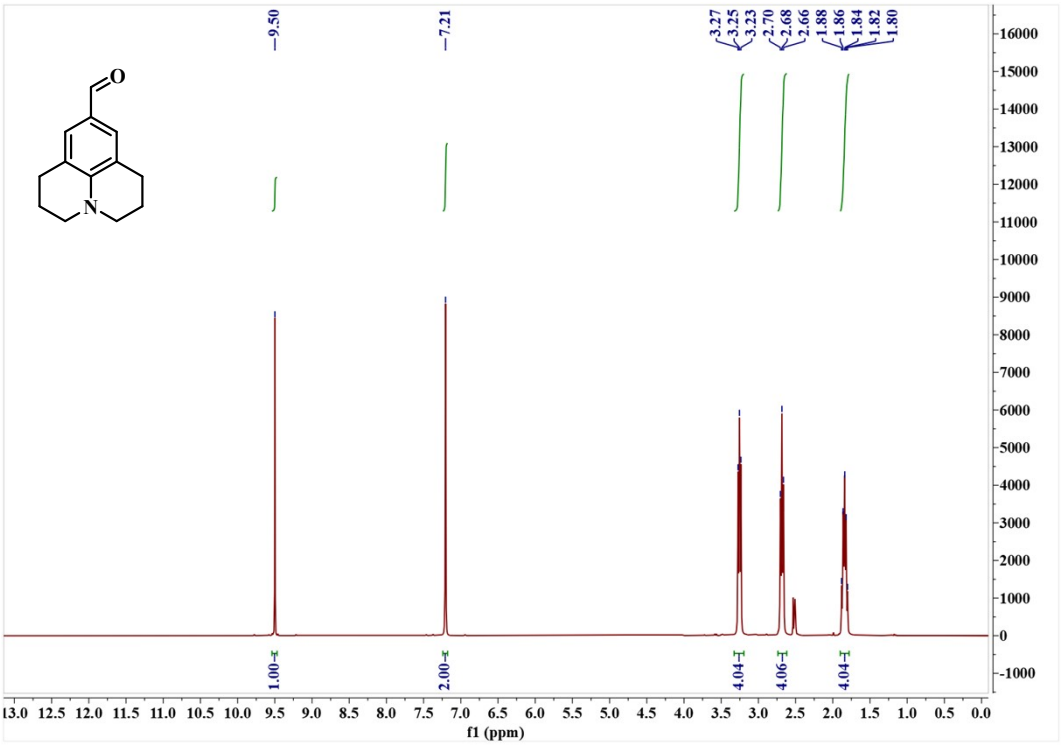
^{13}C NMR spectrum of compound **1** in DMSO-d_6



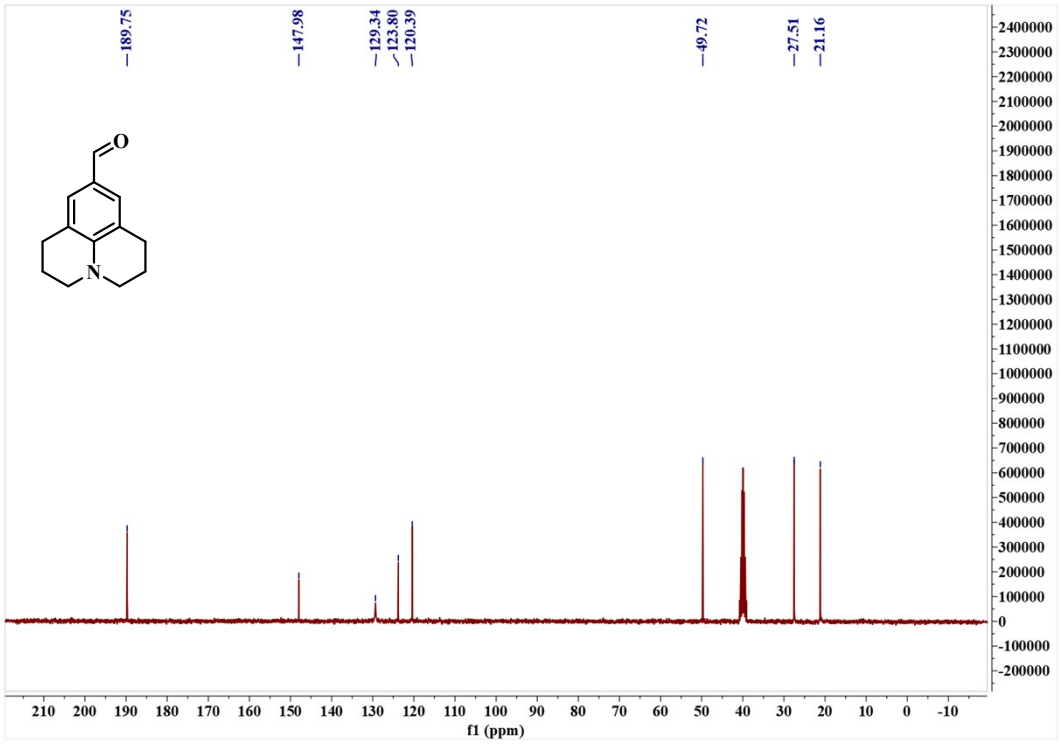
^{1}H NMR spectrum of compound **2** in DMSO-d_6



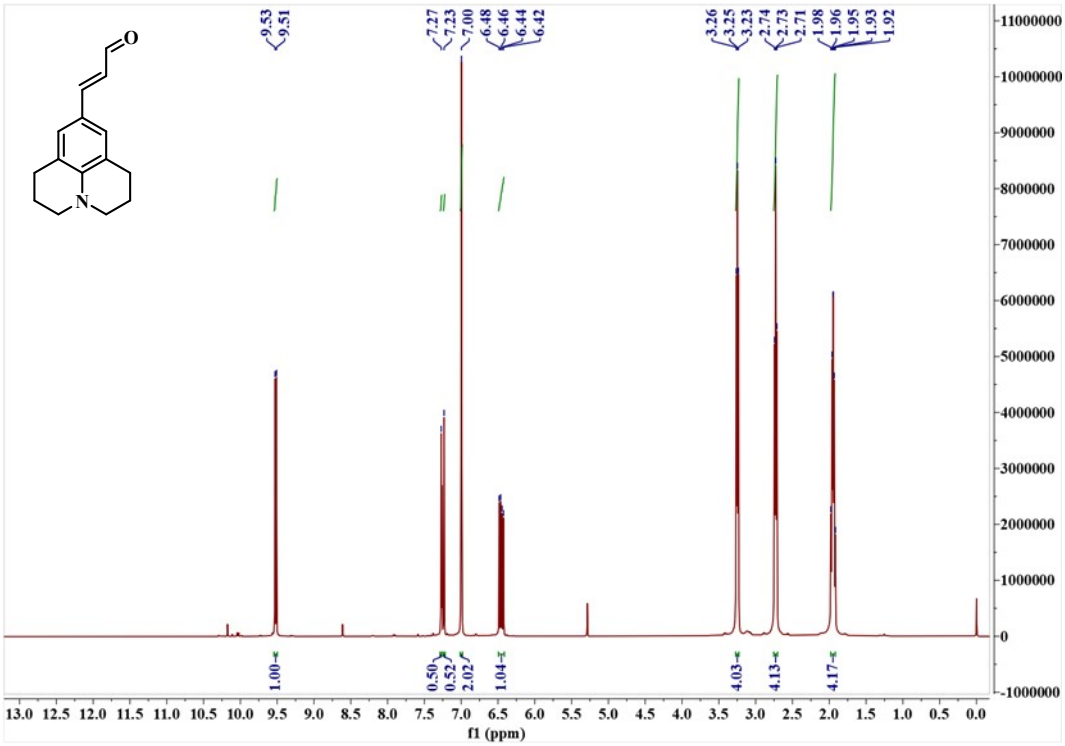
^{13}C NMR spectrum of compound **2** in DMSO-d_6



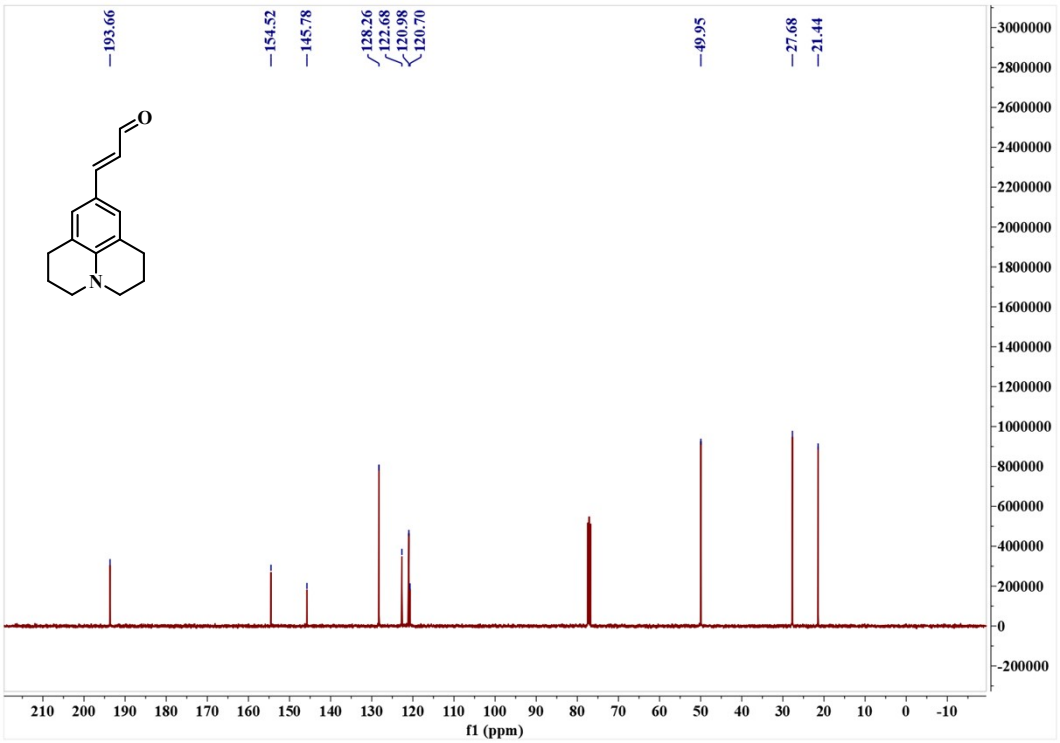
^{1}H NMR spectrum of compound **3** in DMSO-d_6



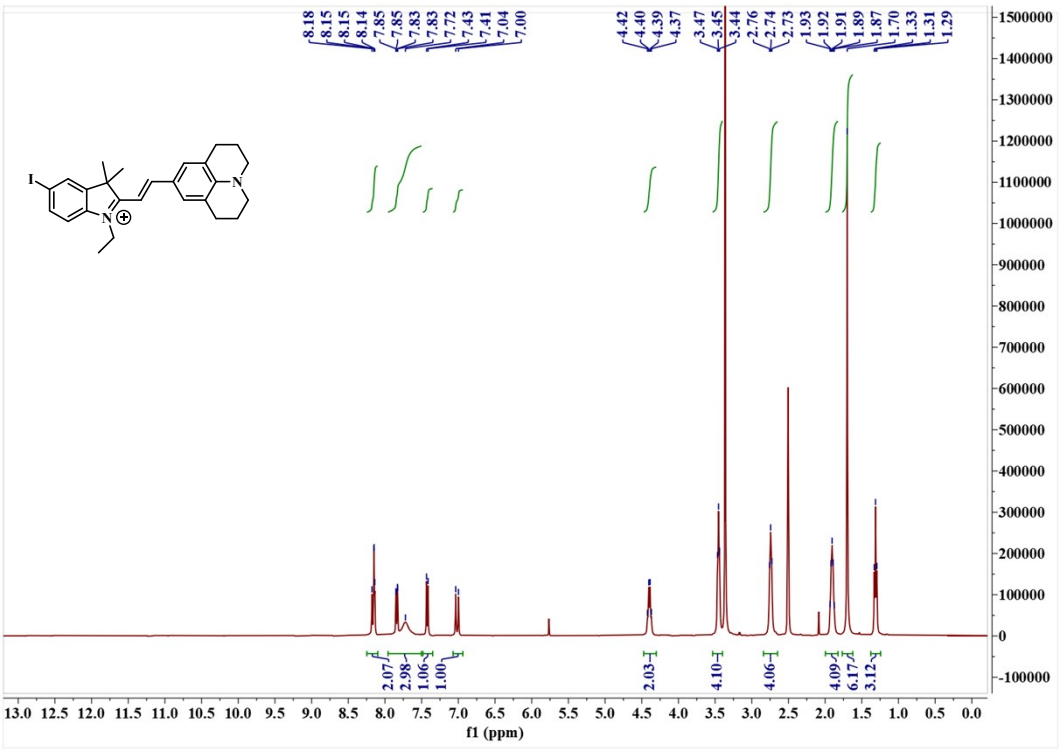
^{13}C NMR spectrum of compound **3** in DMSO-d_6



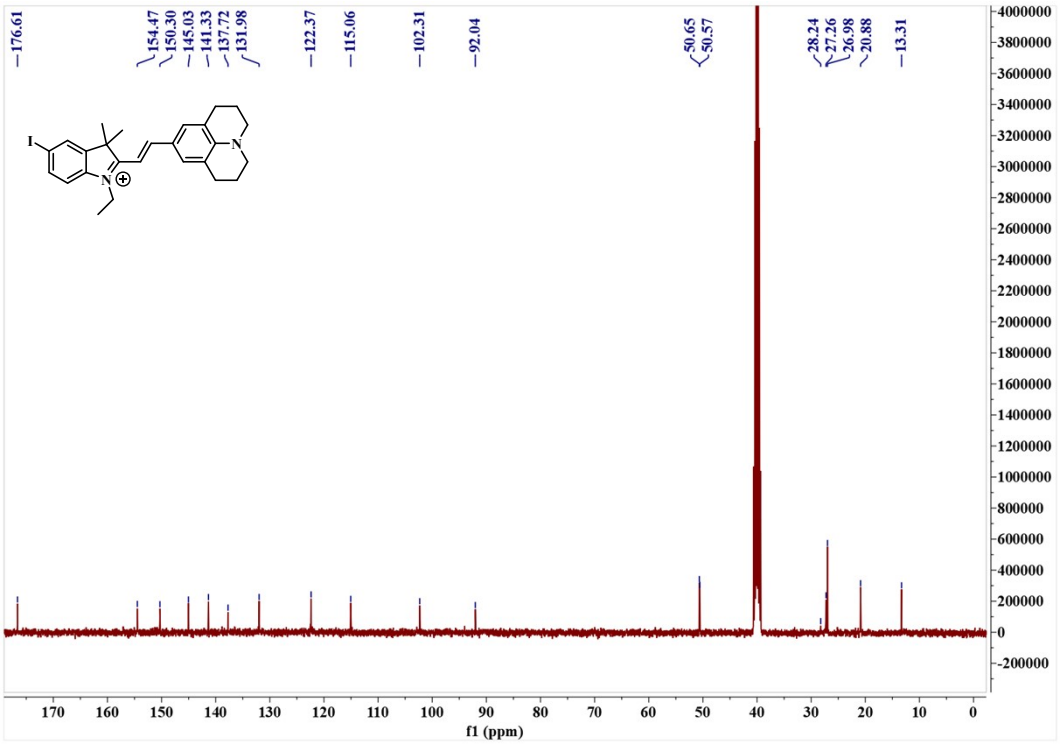
^{1}H NMR spectrum of compound **4** in $CDCl_3$



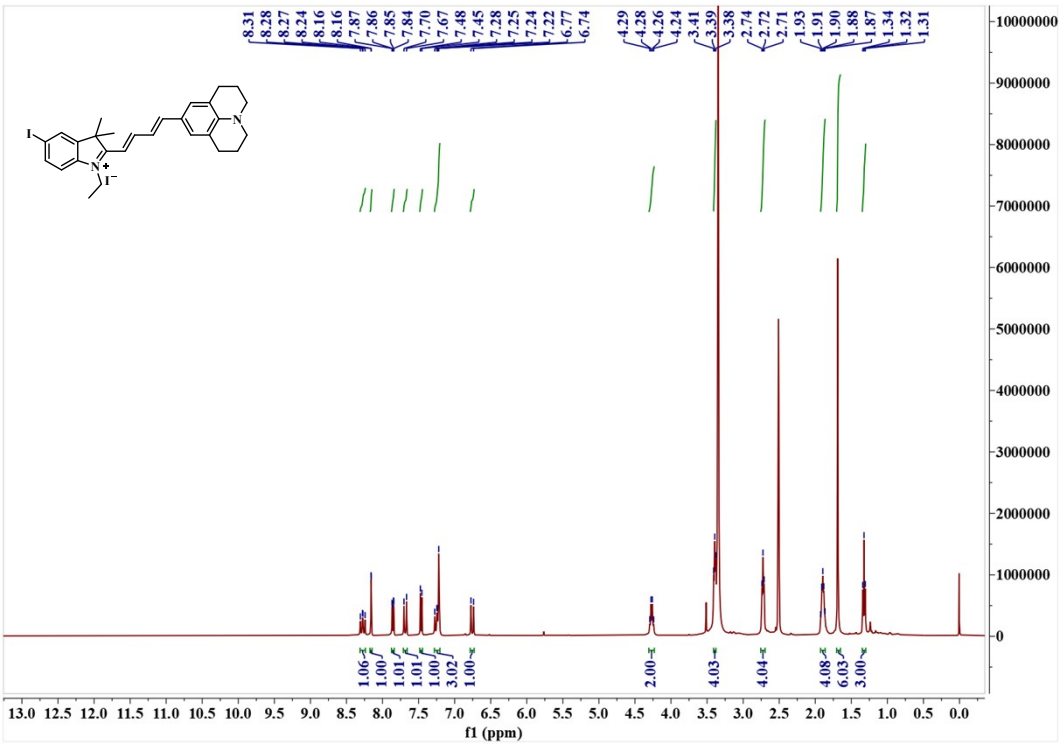
^{13}C NMR spectrum of compound **4** in $CDCl_3$



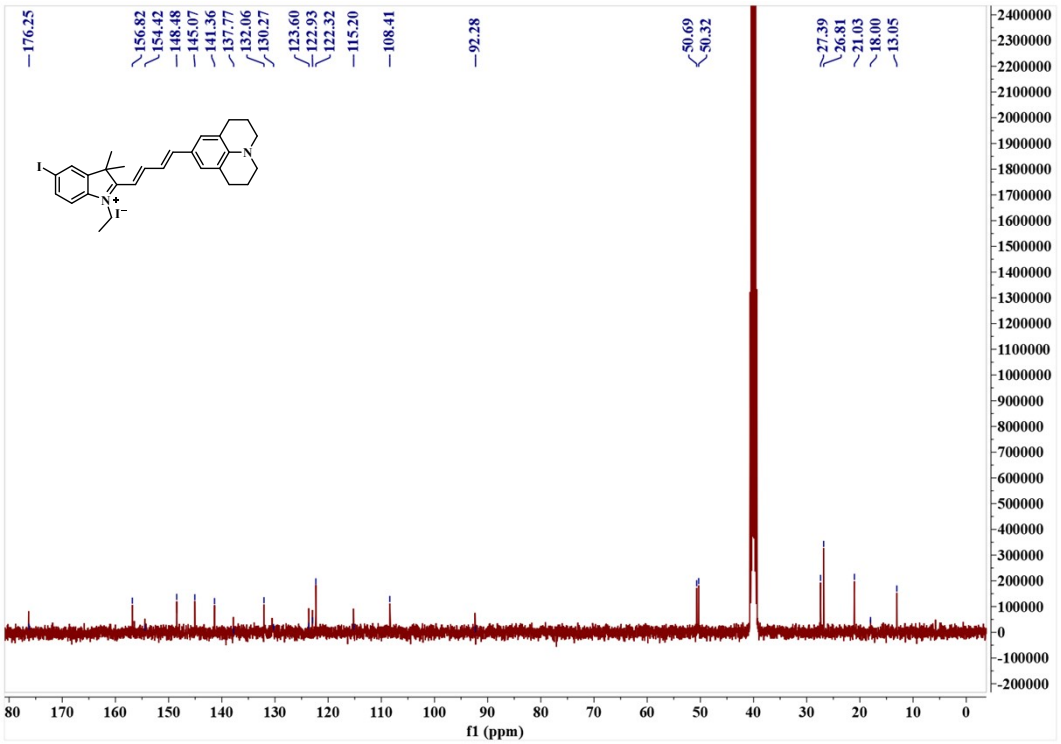
^{1}H NMR spectrum of compound **CCVJ-Mito-1** in DMSO-d_6



^{13}C NMR spectrum of compound **CCVJ-Mito-1** in DMSO-d_6



^{1}H NMR spectrum of compound **CCVJ-Mito-2** in DMSO-d_6



^{13}C NMR spectrum of compound **CCVJ-Mito-2** in DMSO-d_6

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