Supporting Information for

The Visualization of Mitochondrial Viscosity in Inflammation, Fatty Liver, and Cancer Living Mice by a Robust Fluorescent Probe

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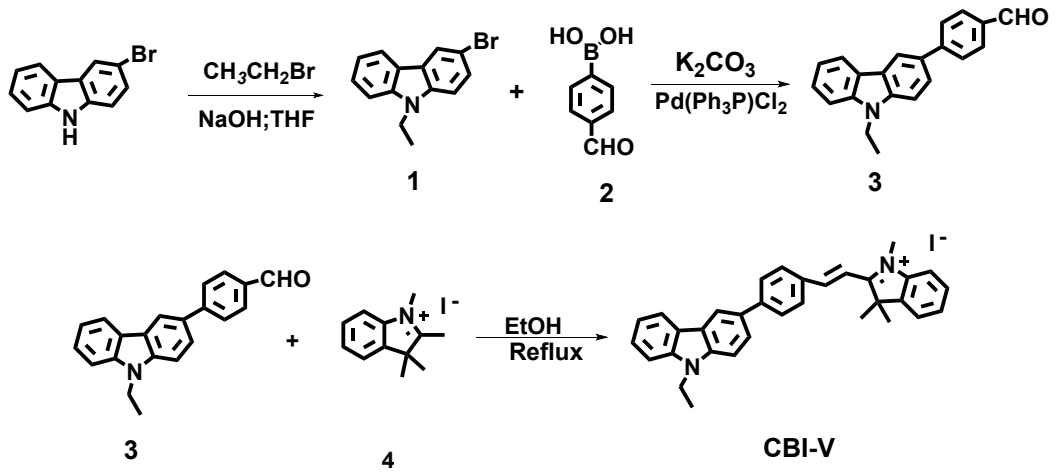
Materials and instruments.

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. UV-vis absorption spectra were obtained on a Shimadzu UV-2700 spectrophotometer and fluorescence spectra were measured on a HITACHI F4600 fluorescence spectrophotometer. The pH measurements were performed with a Mettler-Toledo Delta 320 pH meter. MTT was purchased from J&K Scientific Ltd. TLC analysis carried out on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of them were purchased from the Qingdao Ocean Chemicals. ^{1}H and ^{13}C NMR spectra were measured on an AVANCE III digital NMR spectrometer, using tetramethylsilane (TMS) as internal reference. High resolution mass spectrometric (HRMS) analyses were measured on an Agilent 1100 HPLC/MSD spectrometer. Cell imaging experiment was performed on Nikon A1 Fluorescence Microscopy equipped with a cooled CCD camera. PerkinElmer IVIS spectrum imaging system equipped with COM 8 X-ray controller and -90 °C CCD camera. The animals were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

Synthesis routine of CBI-V.



Scheme S1. The synthetic routine of **CBI-V**.

Synthesis of compound 1 and compound 3.

Firstly, 3-Bromo-9H-carbazole (2.46 g, 10 mmol) and NaOH (0.8 g, 10 mmol) were dissolved in 15 mL mixtures (V_{THF}:V_{H_2O}=3:1) and stirred at room temperature for 1.5 h. Then bromoethane (6.54 g, 60 mmol) were added and heated at 55 °C for 12 h. The resulting sample was purified by silica gel column chromatography (petroleum ether/ethyl acetate, 30:1) to afford compound **1** (white product; 70%). A mixture of compound **1** (274.1 mg, 1 mmol), 4-formylphenylboronic acid (compound **2**, 180 mg, 1.2 mmol), K_2CO_3 (447 mg, 0.03 mmol), and tetrakis(tripheylphosphine)platinum in THF (10 mL) was heated at 60 ℃ for 12 h and the reaction was flushed with nitrogen. After that, the solvent was removed under reduced vacuum. The resulting sample was purified by silica gel column chromatography (petroleum ether/ethyl acetate, 30:1) to afford 4-(9-ethyl-9H-carbazol-3-yl)benzaldehyde (compound **3**) (74%). ^{1}H NMR (400 MHz, DMSO-d6) δ 10.05 (s, 1H), 8.65 (d, J = 1.6 Hz, 1H), 8.28 (d, J = 7.6 Hz, 1H), 8.03 (q, J = 8.8 Hz, 4H), 7.90 (dd, J1 = 8.4 Hz, J2 = 2, 1H), 7.73 (d, J = 8.8 Hz, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.49 (m, 1H), 7.24 (t, J = 7.4 Hz, 1H), 4.48 (q, J = 7.0 Hz, 2H), 1.33 (t, J = 7.0 Hz, 3H)； ^{13}C NMR (101 MHz, DMSO-d6) δ 192.34, 146.71, 139.85, 139.52, 134.00, 129.96, 129.18, 126.79, 125.91, 124.73, 122.72, 122.14, 120.50, 118.97, 118.88, 109.47, 109.14, 36.88, 13.49. HRMS (ESI): calcd. for $C_{21}H_{17}NO$, $[M+H]^+$, m/z, 299.1305, found: 300.1325.

Calculation of fluorescence quantum yield of CBI-V.

The fluorescence quantum yields (Φ_f) were determined using Rhodamine B as the reference according to the literature method. Quantum yields were corrected as follows:

$$\emptyset_f = \emptyset_r\left(\frac{A_r{\eta_s}^2 D_s}{A_s{\eta_s}^2 D_r}\right)$$

Where the s and r indicate the designated and reference samples, respectively. A, η, and D are the absorbance at λ_{ex}, average refractive index of the appropriate solution, and the integrated area under the corrected emission spectrum, respectively

Calculation of two-photon absorption cross section of CBI-V.

$$\delta = \delta_{ref} \frac{F \Phi_{ref} C_{ref} n_{ref}}{F_{ref} \Phi C n}$$

where "ref" subscript stands for reference while the ones without any are for the sample. δ: Two photon absorption cross section; F: Integrated area of Two photon Induced fluorescence spectra; Φ: Fluorescence quantum yield; C: Concentration in moles/Litre, n: Refractive Index of the solvents used.

Viscosity determination and fluorescence spectral measurement.

The stock solution of **CBI-V** (1 mM) was dissolved in DMSO. 10 μM of the probe was used in spectral test by addition of 30 μL stock solution to 3.0 mL different viscosity solutions, which were obtained by mixing methanol-glycerol systems in different proportions. The solutions of the various interfering substance were prepared with the ultrapure water. The resulting solution was shaken well before recording the spectra. For all the measurements, the excitation wavelength, excitation slit widths, and emission slit widths are 520 nm, 5 nm, and 5 nm, respectively.

Cell culture and cell cytotoxicity assays.

Cell culture.

Hela cell, A549 and HepG2 cell were utilized in this work. These cells were cultured in Dulbecco's Modified Eagle Medium media (DMEM, Hyclone) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Sijiqing) and 1 % antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Hyclone) at 37 °C and 5% CO_2.

Cytotoxicity assays.

The cytotoxicity of the probe **CBI-V** to Hela, A549, HepG2 cells were studied by standard MTT assays. 2×10^4 cells/mL cells were seeded in 96-well plates and then incubated with various concentrations of **CBI-V** (0-50 μM) for 24 h. After that, 10 μL of MTT (5 mg/mL) was added to each well and incubated for another 4 h. Finally, the media was discharged, and 100 μL of DMSO was added to dissolve the formazan crystals. The plate was shaken for about 10 min, and each well was analyzed by the microplate reader and detected at the absorbance of 490 nm. The cell viability (%) =

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

OD_{sample}, $OD_{control}$, and OD_{blank} denote the cells incubated with various of concentrations of the probe, the cells without the probe, and the wells containing only the culture media, respectively.

Cell imaging and co-localization experiments.

Before imaging, 1 mL of cells was seeded in the glass bottom culture dishes (Nest) with the density of 1×10^5/mL. The cells were placed on glass cover slips and allowed to adhere for 24 h. When the cells reached about 70 % confluence, they were then subjected to the imaging experiments. For the cell imaging, the cells were incubated with Monensin, nystatin or LPS (the final concentration is 10 μM) for 30 min and **CBI-V** (10 μM) for another 30 min, and then the medium was removed. The residual probe was removed by washing three times using PBS before imaging. Finally, confocal fluorescence imaging was carried out using Nikon fluorescence microscope equipped with the excitations of 561 nm (one-photon mode) and 740 nm (Two-photon mode), and the collection is 570-620 nm.

The co-localization experiments were carried out with commercially available dye MitoTracker Green and DAPI. Firstly, Hela cells were incubated with DAPI (2.0 μM), MitoTracker Green (1.0 μM) and probe **CBI-V** (8.0 μM) simultaneously for 30 min. Then, Hela cells were washed by PBS for three times and imaged at the appropriate test conditions above. The blue channel was collected at 450-500 nm with the excitation of 404 nm. The green channel was collected at 500-550 nm with the excitation of 488 nm. The red channel at 570-620 nm was collected with the excitation of 561 nm.

Preparation of living organs and tumor for imaging experiments.

Four-week old female balb/c mice were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

The mice were inoculated with 4T1 cell and after 14 days a tumor was obtained.

Then the organs (heart, liver, spleen, lung and kidney) and tumor were isolated from the mice. After washing by PBS (pH 7.4) for three times, these isolated organs and tumor were loaded with **CBI-V** (20 μM), respectively, and finally subjected to imaging using an IVIS Lumina XR *in vivo* imaging system with an excitation filter of 560 nm and an emission filter of 620 nm.

Fluorescence imaging of viscosity in zebrafish.

Wild type zebrafish were obtained from the Nanjing Eze-Rinka Biotechnology Co., Ltd. For the fluorescence imaging experiments, 3-day-old zebra fishes were transferred into a 30 mm glass culture dishes using a disposable sterilized dropper. 10 μM of Monensin, nystatin or LPS were added into three dishes respectively for 30 min. Then 10 μM probe **CBI-V** was added for incubated for another 30 min, followed by washing away gently. After that, the zebrafish were transferred into the new glass bottom dishes for imaging. Prior to the imaging, we adopted 1% agarose gel for immobilization of zebrafish, and put zebrafish onto agarose with a little media to ready imaging. Fluorescence images were acquired using Nikon A1R confocal microscope with a 4 × objective lens. The imaging experiments were recorded through a Nikon AIP confocal microscopy. The fluorescence emission was collected at TRICT channel (570-620 nm) upon 561 nm excitation at one-photon mode and 740 nm at two-photon mode.

Construction of inflammatory mice models for imaging experiment.

The animals were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

Four-week old female balb/c mice were purchased from School of Pharmaceutical Sciences, Shandong University and the mice were kindly kept during the experiments. These mice were injected by monensin, nystatin or LPS in abdomen respectively to produce viscosity increasing models. After two days, the triggered mice and normal mice were simultaneously utilized for *in vivo* imaging. Before *in vivo* imaging, the

abdominal fur was removed by an electric shaver, and then the mice were anesthetized by 4% chloral hydrate aqueous solution (100 μL). The probe **CBI-V** (80 μL; 1 mM) dissolved in DMSO was then injected into the abdominal position of the normal mice and triggered mice, respectively. The mice were then imaged by using an *in vivo* imaging system with an excitation filter of 560 nm and an emission filter of 620 nm.

Construction of tumor-bearing mice models for imaging experiment.

The animals were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

4T1 cells were grafted into the mice to produce tumor models and on day 14 post-injection, a tumor was obtained. Then the tumor-bearing mice and normal mice were simultaneously utilized for *in vivo* imaging. Before *in vivo* imaging, the abdominal fur was removed by an electric shaver, then the mice were anesthetized by 4% chloral hydrate aqueous solution (100 μL). **CBI-V** dissolved in DMSO (80 μL; 1 mM) was then injected into the abdominal position of the normal mice and tumor-grafted mice by hypodermic injection, respectively. The mice were then imaged by using an i*n vivo* imaging system with an excitation filter of 560 nm and an emission filter of 620 nm.

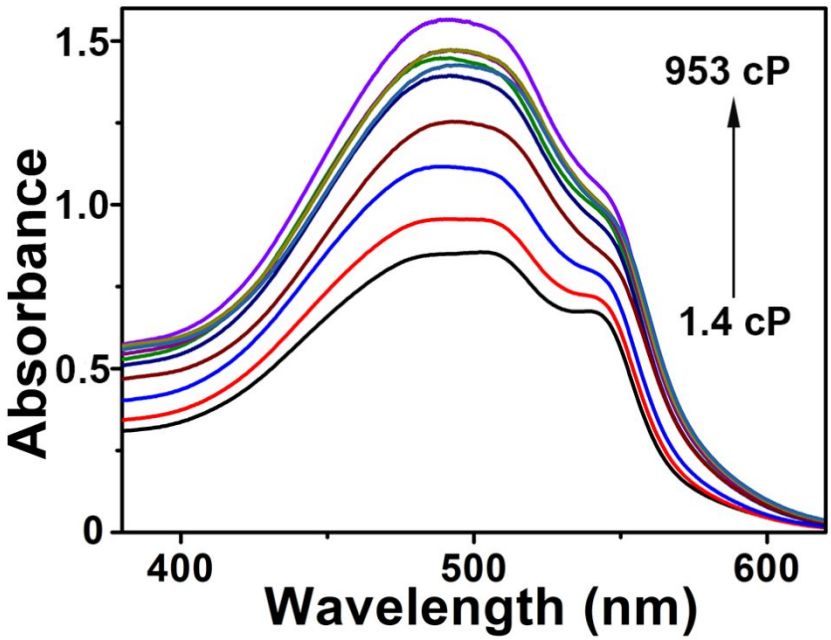


Figure S1. The absorption spectra of **CBI-V** in different viscosity solvents.

Table S1. Test viscosity in the varied of the methyl methanol/glycerol (v/v) mixtures and fluorescence quantum yield (Φ_f) of **CBI-V**.

glycerol / methanol (v: v)	η/cp	Φ_f/%
0:10	1.40	0.02
1:9	3.47	0.34
2:8	11.6	1.07
3:7	16.1	3.78
4:6	25.5	9.04
5:5	91.4	13.21
6:4	148	17.2
7:3	327	20.1
8:2	611	24.4
9:1	795	28.6
10:0	953	29.3

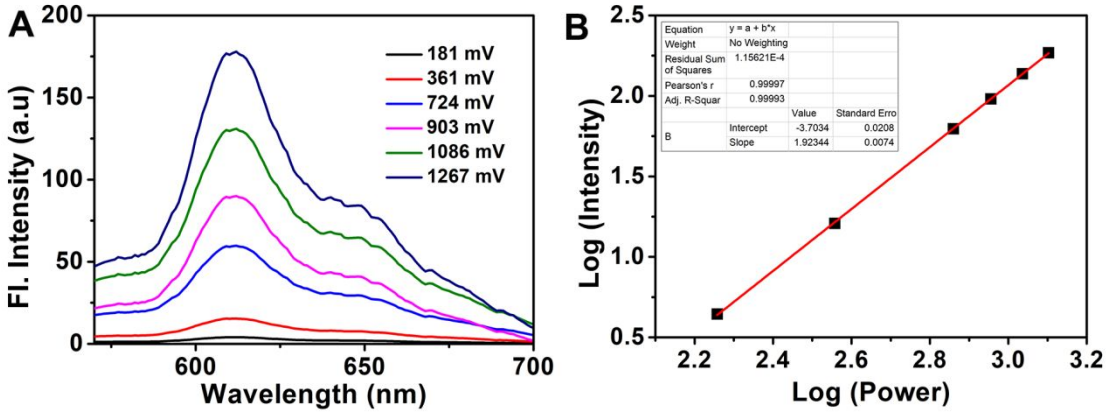


Figure S2. (A) Laser power-dependent two-photon emission spectra of **CBI-V**. (B) The logarithmic plots between the power dependence and relative two-photon induced luminescence intensity.

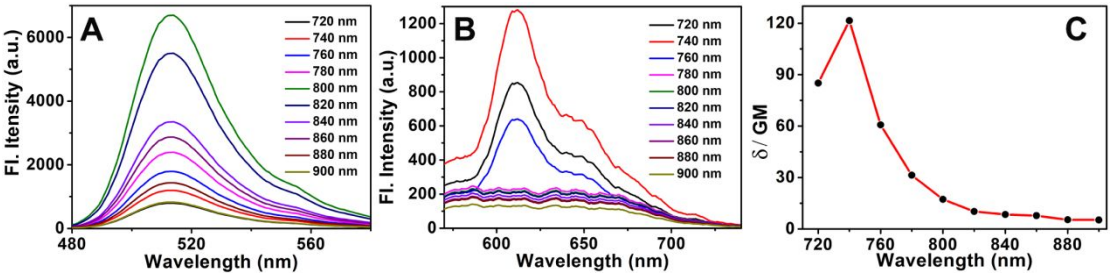


Figure S3. Two-photon fluorescence intensity of 1.0 μM fluorescein (A) and 5.0 μM **CBI-V** (B) at 720-900 nm excitation. (C) Two-photon absorption cross sections (δ) of the compounds **CBI-V** at 720-900 nm in glycerol solution.

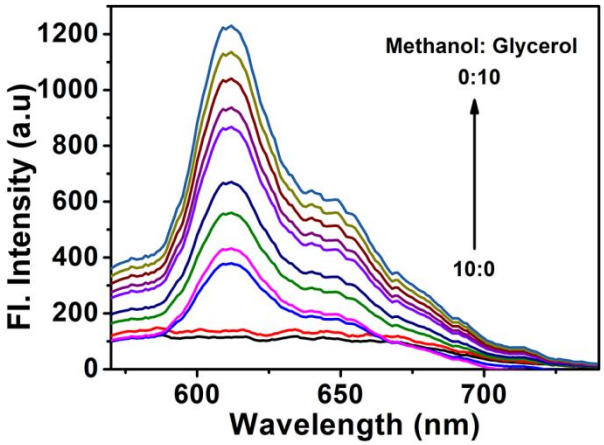


Figure S4. The fluorescence spectra of 5.0 μM **CBI-V** in methanol-glycerol systems at various ratios. λ_{ex}=740 nm.

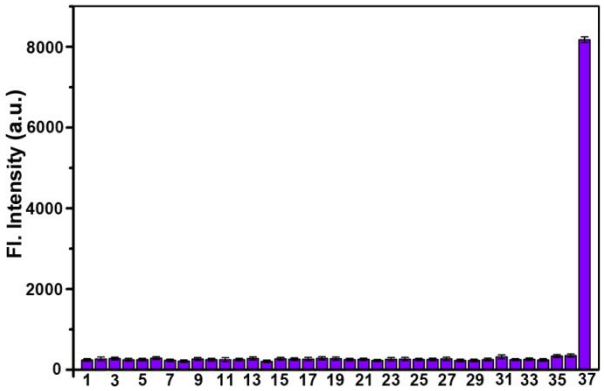


Figure S5. The Relative fluorescence intensities of **CBI-V** (10 μM) to various relevant analytes in phosphate buffer (pH 7.4, 10 mM, 5% DMSO). 1. Only the phosphate buffer; 2, $ZnCl_2$; 3, $MgCl_2$; 4, $CaCl_2$; 5, $CoCl_2$; 6, $SnCl_2$; 7, $CuSO_4$; 8, $MgCl_2$; 9, $HgSO_4$; 10, KNO_3; 11, $NiSO_4.6H_2O$; 12, $(Fe)_2(SO_4)_3$; 13, $FeSO_4$; 14, KI; 15, $Na_2S_2O_3$; 16, NaHS; 17, $NaHSO_3$; 18, Na_2SO_3; 19, NaOAc; 20, $NaNO_2$; 21, $ONOO^-$, 22, NaClO; 23, H_2O_2; 24, OH^-; 25, GSH; 26, glucose; 27, Cys; 28, Thr; 29, Ser; 30, Gln; 31, Asn; 32, Ala; 33, Gly; 34, Trp; 35, FBS; 36, BSA; 37, Glycerol. The spectra were recorded at 25 ℃, the fluorescence intensity was measured at λ_{ex} = 520 nm with both excitation and emission slit widths of 5 nm, and a 700 V PMT voltage.

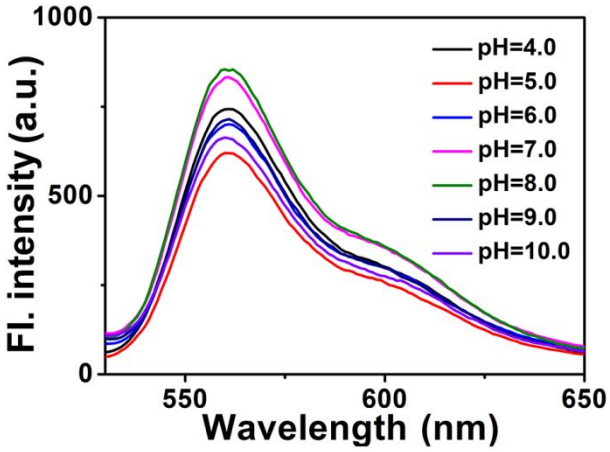


Figure S6. The fluorescence spectra of **CBI-V** in different pH values. The spectra were recorded at 25 ℃, the fluorescence intensity was measured at λ_{ex} = 520 nm with both excitation and emission slit widths of 5 nm, and a 700 V PMT voltage.

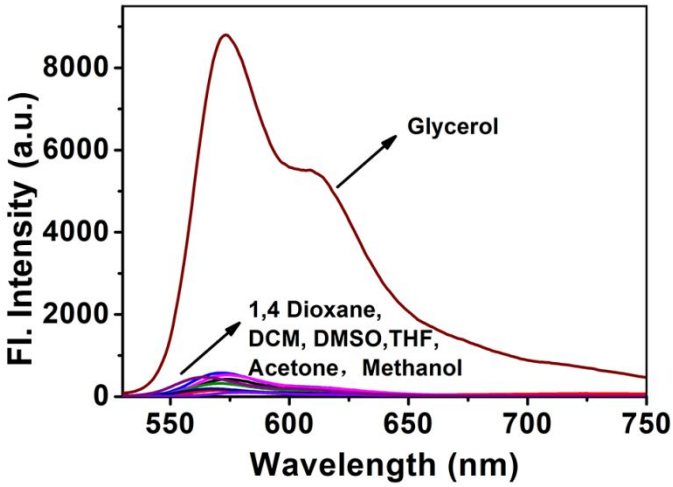


Figure S7. The fluorescence spectra of **CBI-V** in different polarity values. The spectra were recorded at 25 ℃, the fluorescence intensity was measured at λ_{ex} = 520 nm with both excitation and emission slit widths of 5 nm, and a 700 V PMT voltage. nm.

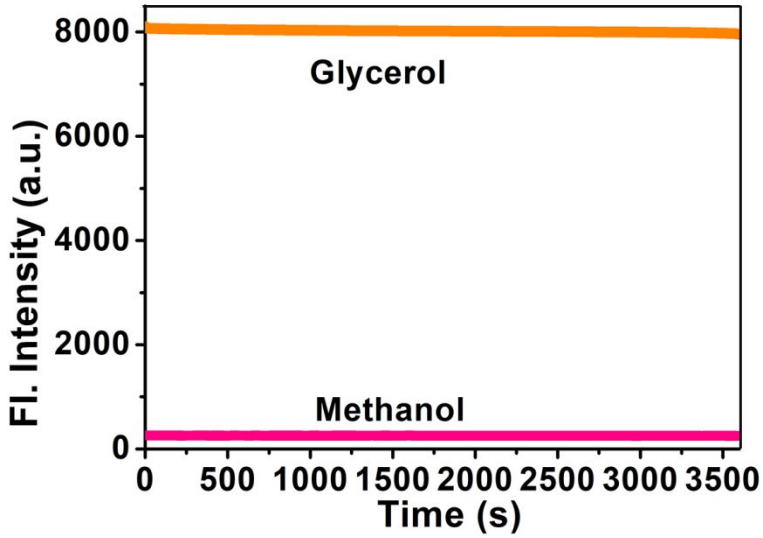


Figure S8. The photo-stability experiments of **CBI-V** under different viscosity condition with continuous irradiation by laser light for 60 min.

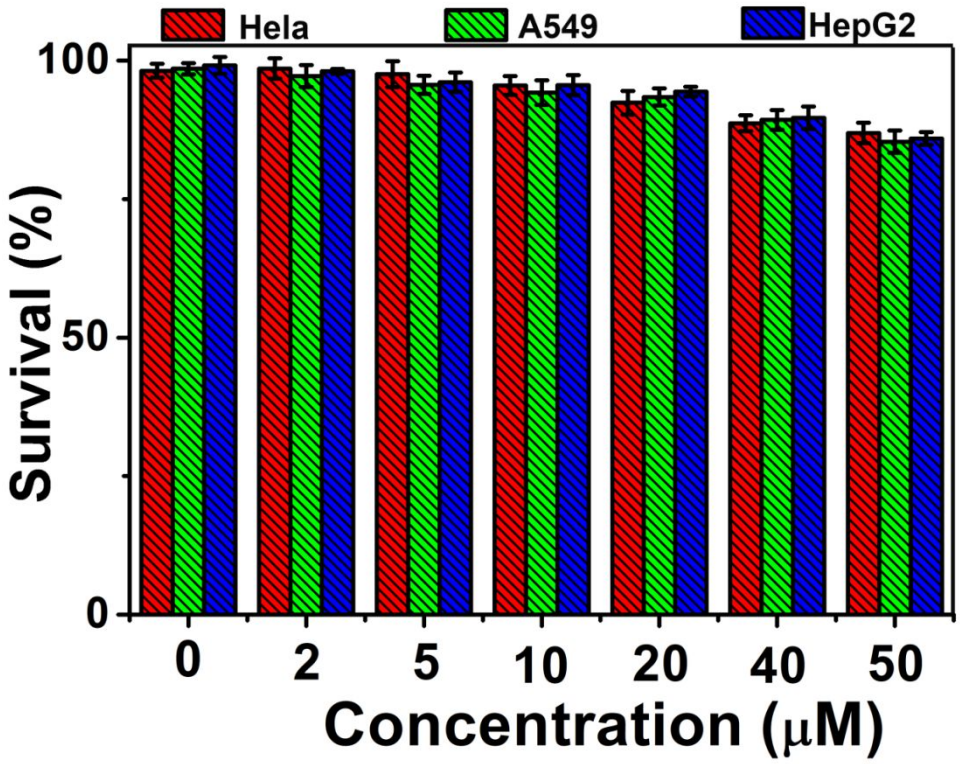


Figure S9. The MTT experiments of **CBI-V** with different concentrations for Hela cells, A549 cells, and HepG2 cells.

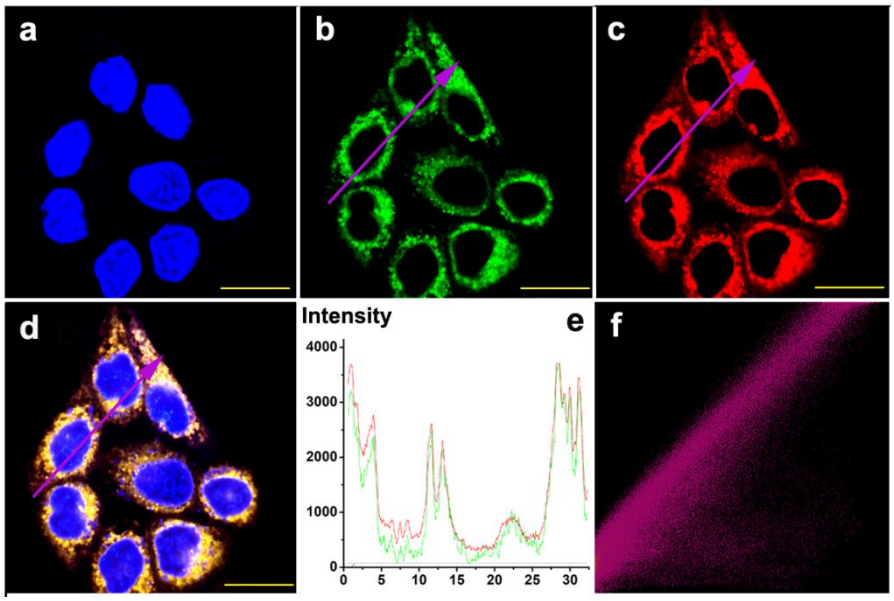


Figure S10. Images of the living HeLa cells costained with (a) 2.0 μM DAPI (λ_{ex}=404 nm, λ_{em}=450-500 nm), (b) 1.0 μM Mito-Tracker Green (λ_{ex}= 488 nm, λ_{em}= 500-550 nm). (c) 8.0 μM probe CBI-V (λ_{ex}= 561 nm, λ_{em}= 570-620 nm). (d) The merged pattern of (a), (b) and (c). (e) Intensity profile of ROI across the cells in the blue, red and green channels. (f) The intensity scatter plot of two channels. Scale bar: 20 μm.

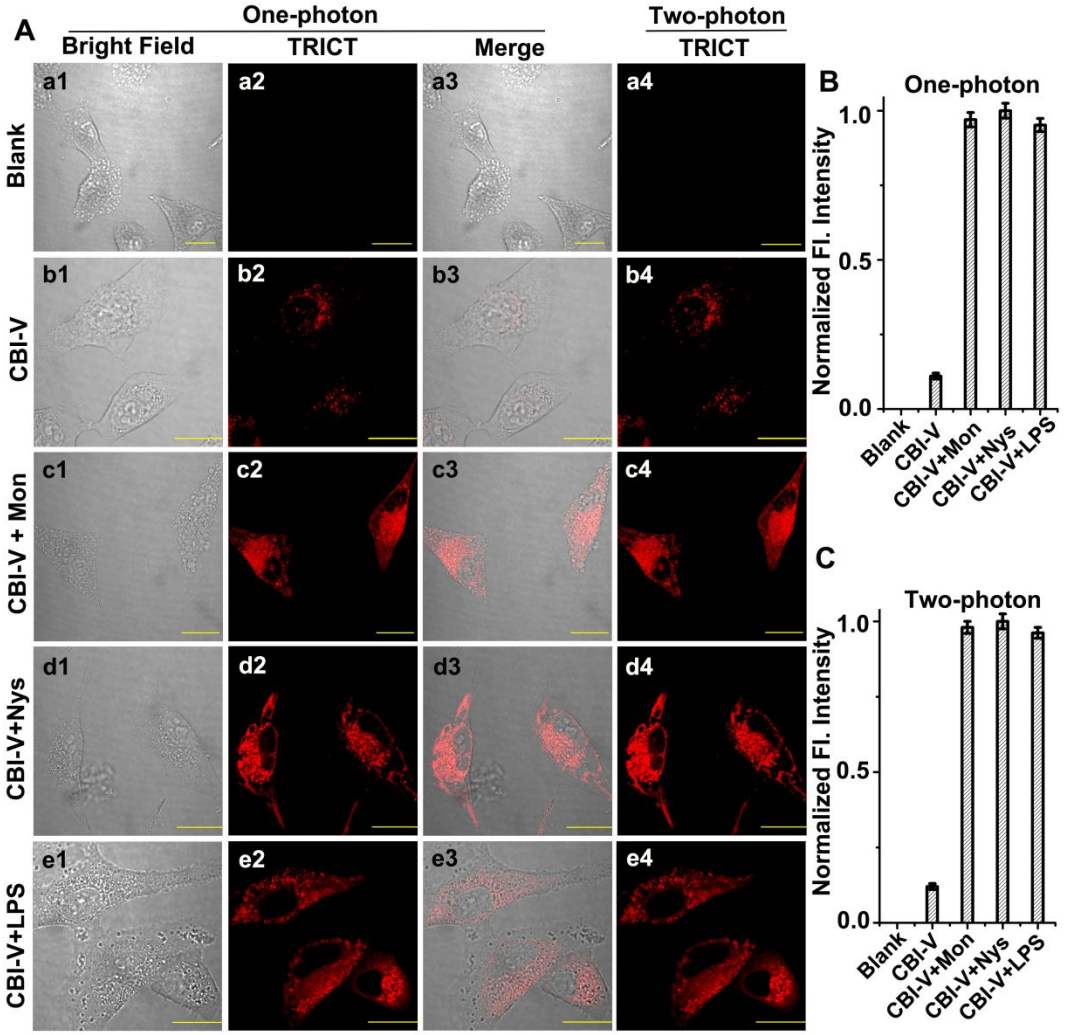


Figure S11. (A). Fluorescence imaging of A549 cells: (a1-a4) Confocal fluorescence images of the A549 cells. (b1-b4) Confocal fluorescence images of the A549 cells incubated with 10 μM probe **CBI-V** for 30 min. (c1-c4) Confocal fluorescence images of the A549 cells incubated with 10 μM Monensin for 30 min and 10 μM probe **CBI-V** for another 30 min. (d1-d4) Confocal fluorescence images of A549 cells incubated with 10 μM nystatin for 30 min and 10 μM probe **CBI-V** for another 30 min. (e1-e4) A549 cells stained with 10 μM LPS for 30 min and **CBI-V** for another 30 min. (B) Normalized fluorescence intensity at One-photon mode. (C) Normalized fluorescence intensity at Two-photon mode. The images were collected at 570-620 nm, one photon excited at 561 nm; Two-photon excited at 740 nm; scale bar: 20 μm, data are mean SD (bars) (n=3).

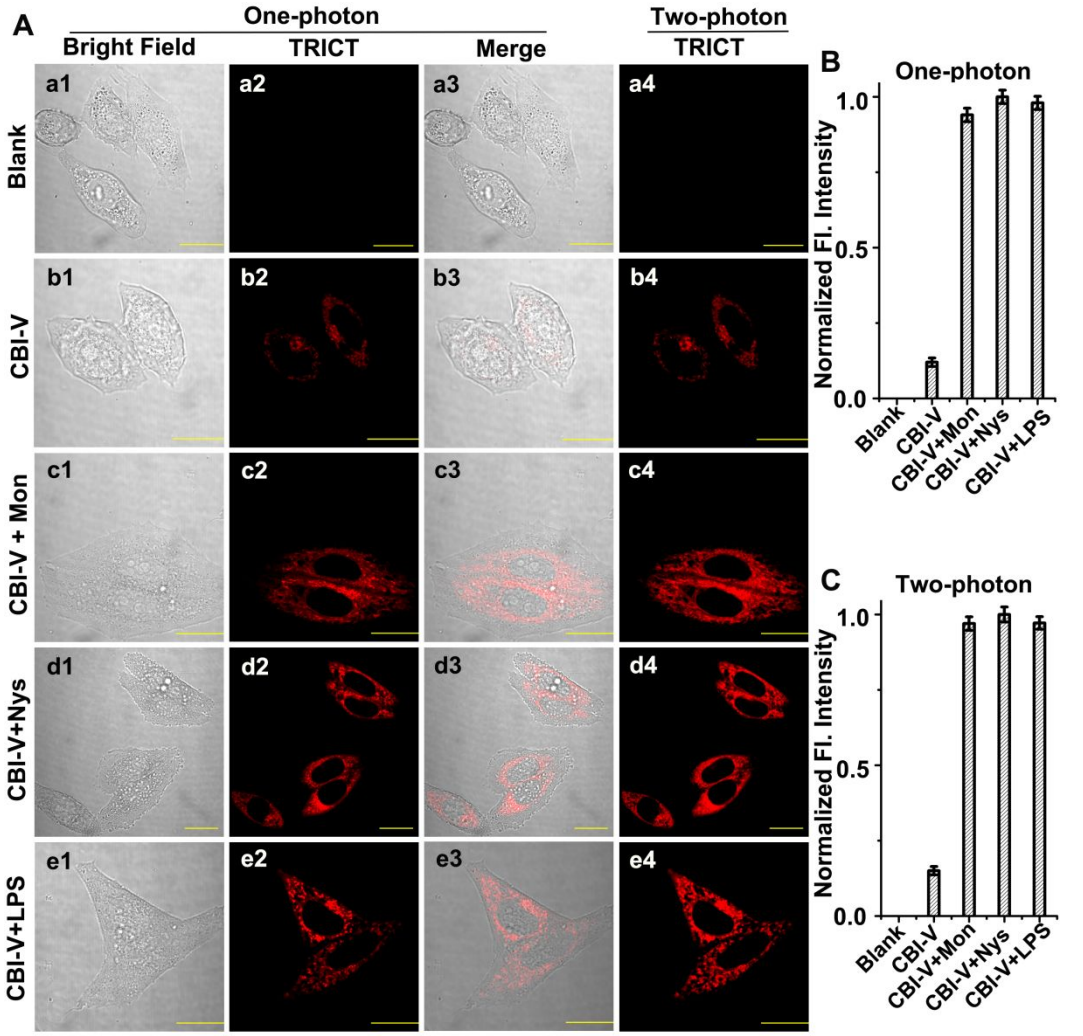


Figure S12. (A) Fluorescence imaging of HepG2 cells: (a1-a4) Confocal fluorescence images of the HepG2 cells. (b1-b4) Confocal fluorescence images of the HepG2 cells incubated with 10 μM free probe **CBI-V** for 30 min. (c1-c4) Confocal fluorescence images of the HepG2 cells incubated with 10 μM Monensin for 30 min and 10 μM probe **CBI-V** for another 30 min. (d1-d4) Confocal fluorescence images of HepG2 cells incubated with 10 μM nystatin for 30 min and 10 μM probe **CBI-V** for another 30 min. (e1-e4) HepG2 cells stained with 10 μM LPS for 30 min and **CBI-V** for another 30 min. (B) Normalized fluorescence intensity at One-photon mode. (C) Normalized fluorescence intensity at Two-photon mode. The images were collected at 570-620 nm, one photon excited at 561 nm. Two-photon excited at 740 nm. scale bar: 20 μm, data are mean SD (bars) (n=3). Scale bar: 20 μm.

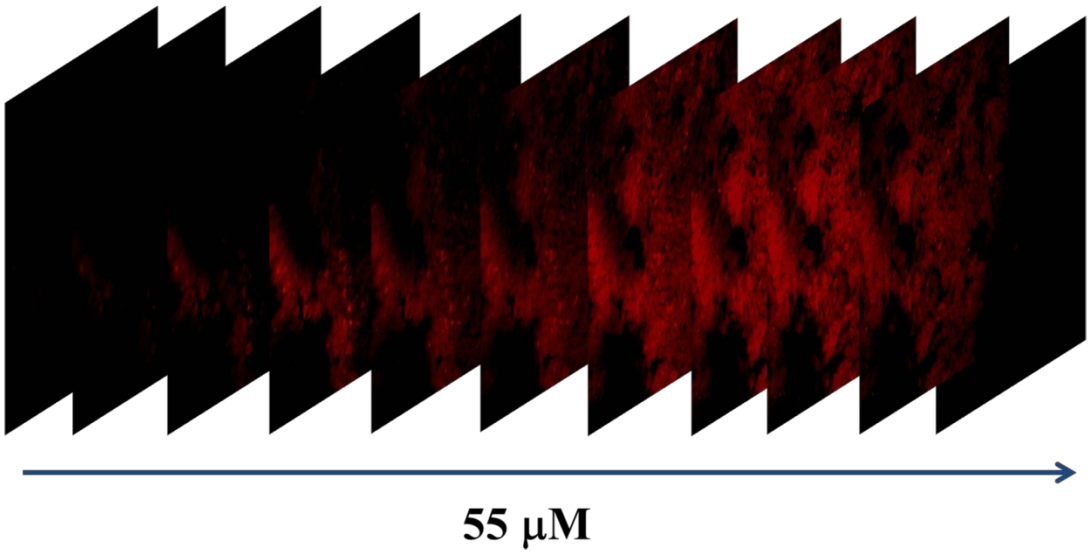


Figure S13. Two-photon fluorescence images of the mormal liver slice pretreated with **CBI-V** (15 μM). The images were collected at 570-620 nm with TP excitation of 740 nm.

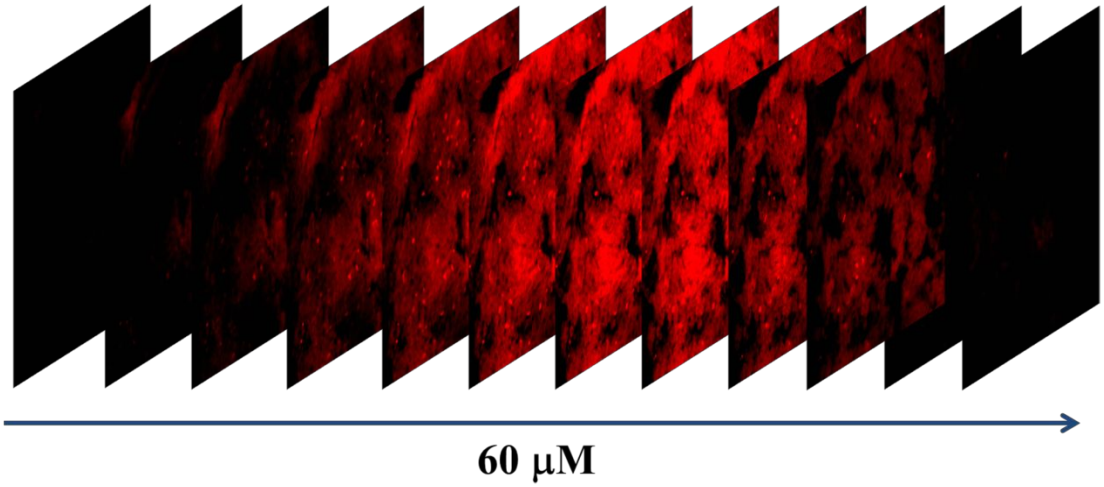


Figure S14. Two-photon fluorescence images of the fatty liver slice pretreated with **CBI-V** (15 μM). The images were collected at 570-620 nm with TP excitation of 740 nm.

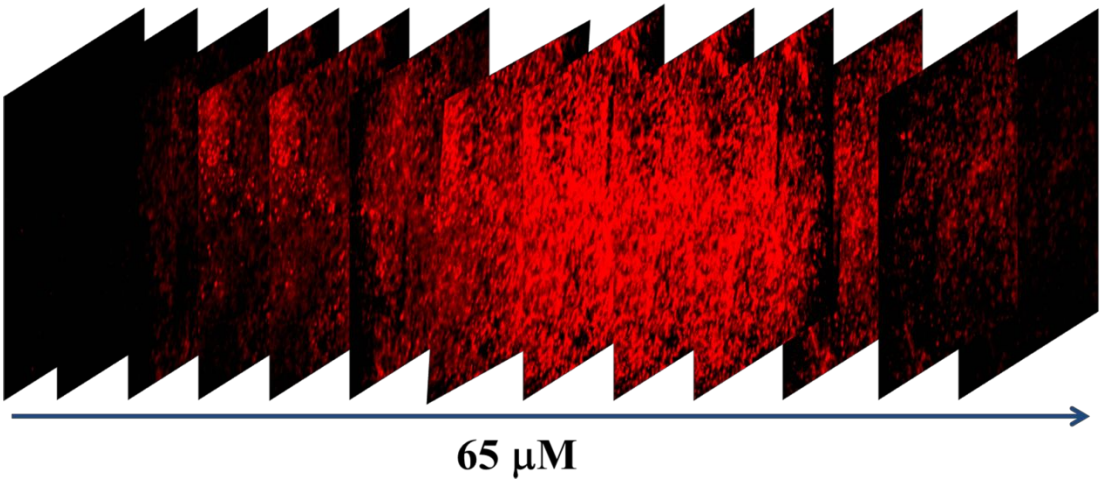


Figure S15. Two-photon fluorescence images of the tumor slice pretreated with **CBI-V** (15 μM). The images were collected at 570-620 nm with TP excitation of 740 nm.

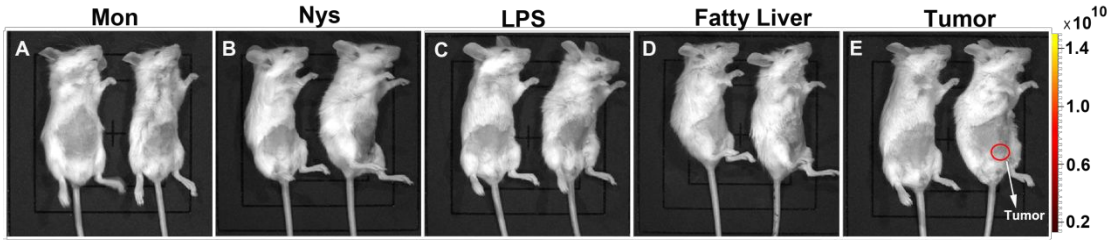


Figure S16. Fluorescence imaging of living mice: (A) Normal living mouse (left) and mouse triggered with Monensin (right). (B) Normal living mouse (left) and mouse induced with nystatin (right). (C) Normal living mouse (left) and mouse induced with LPS (right); (D) Normal living mouse (left) and fatty liver mouse (right); (E) Normal living mouse (left) and tumor-bearing mouse (right); λ_{ex}=560 nm, λ_{em}=620 nm.

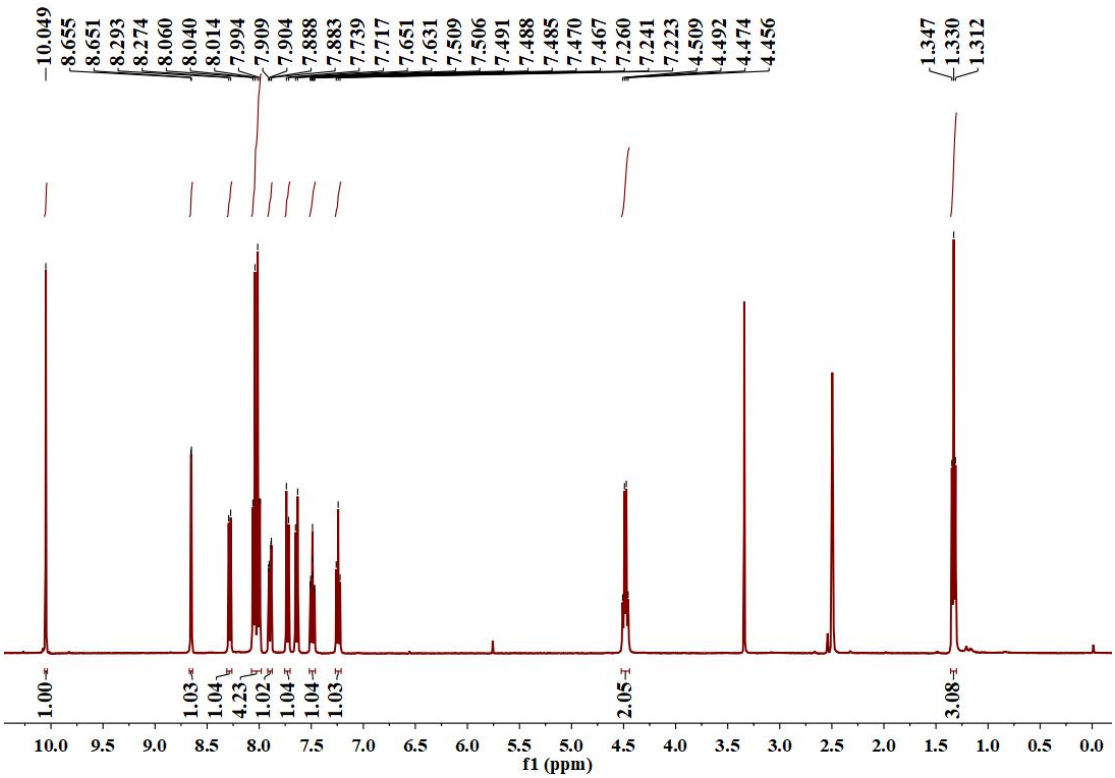


Figure S17. The ^{1}H NMR spectrum of compound **3** in DMSO-*d6*.

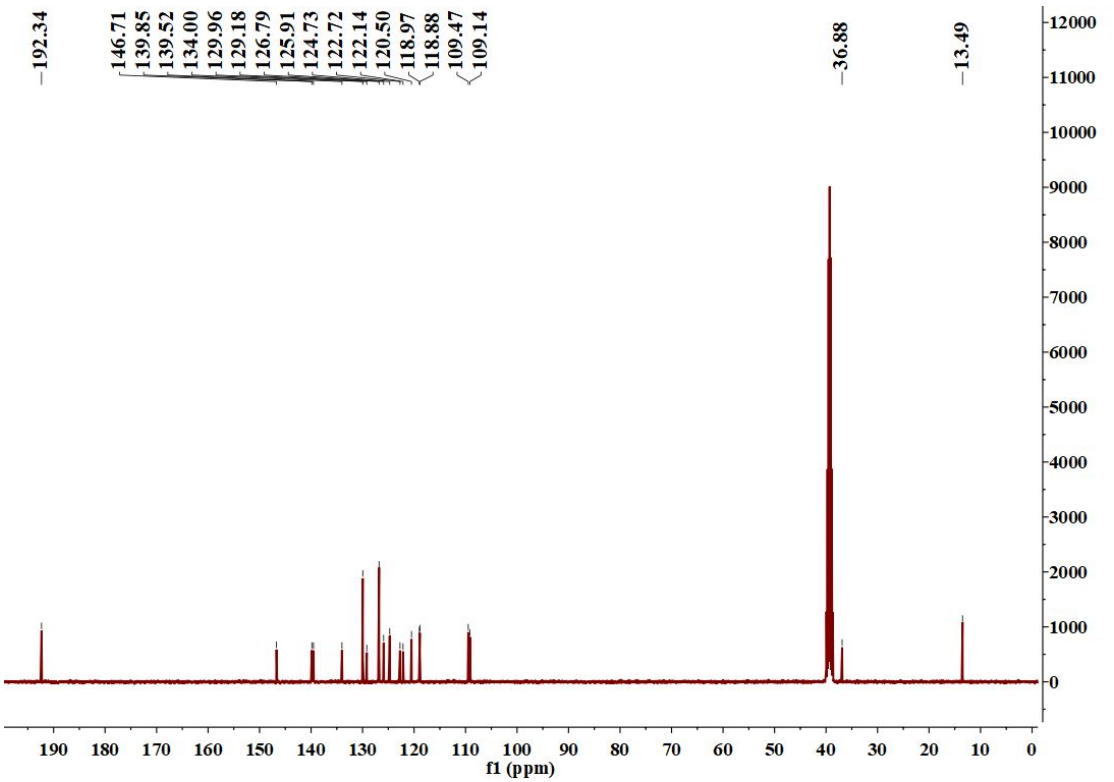


Figure S18. The ^{13}C NMR spectrum of compound **3** in DMSO-*d6*.

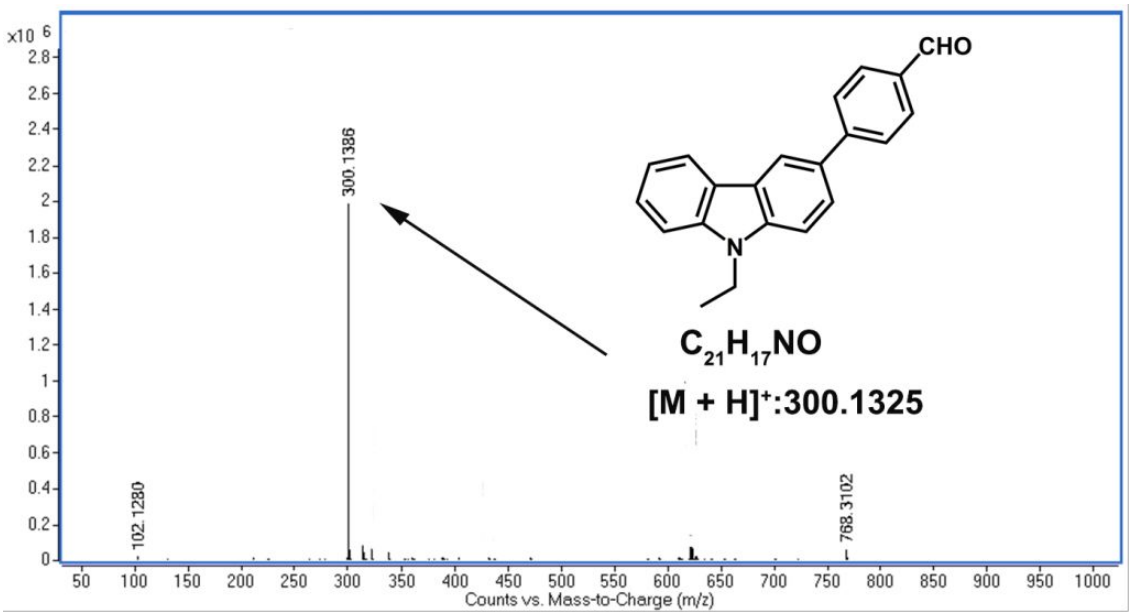


Figure S19. The HRMS spectrum of compound **3**.

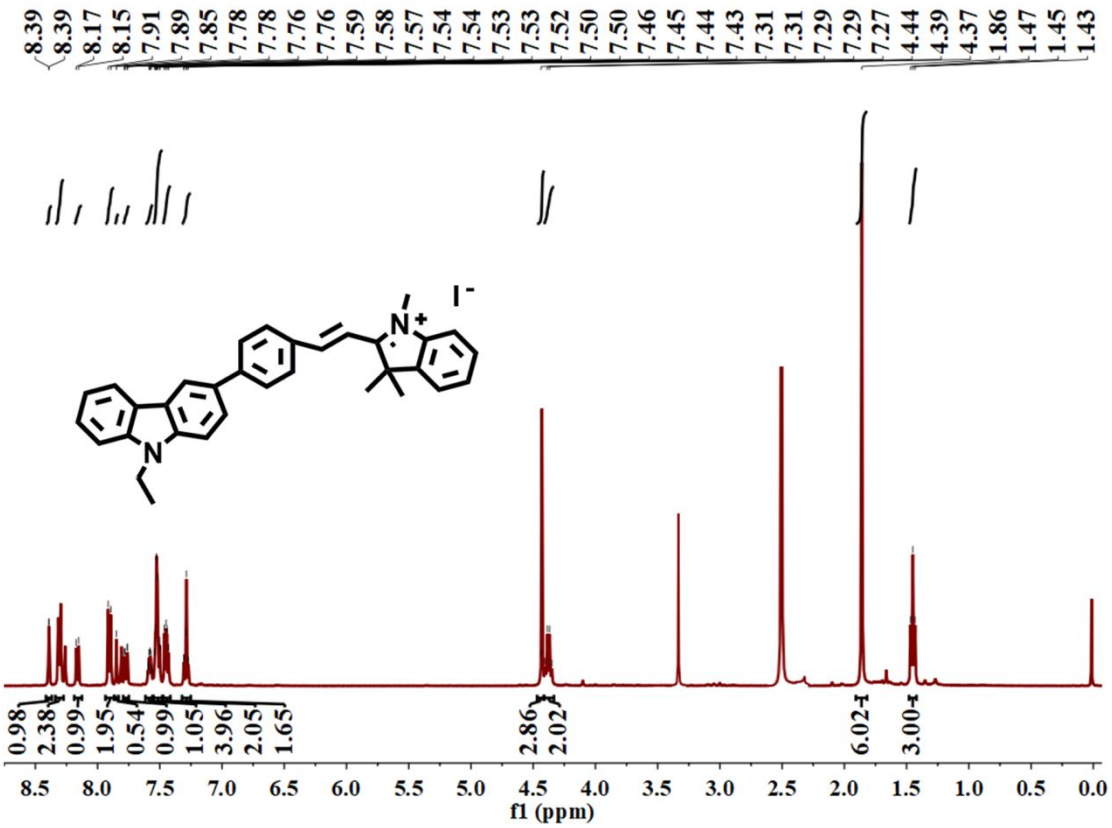


Figure S20. The ^{1}H NMR spectrum of **CBI-V** in DMSO-*d6*.

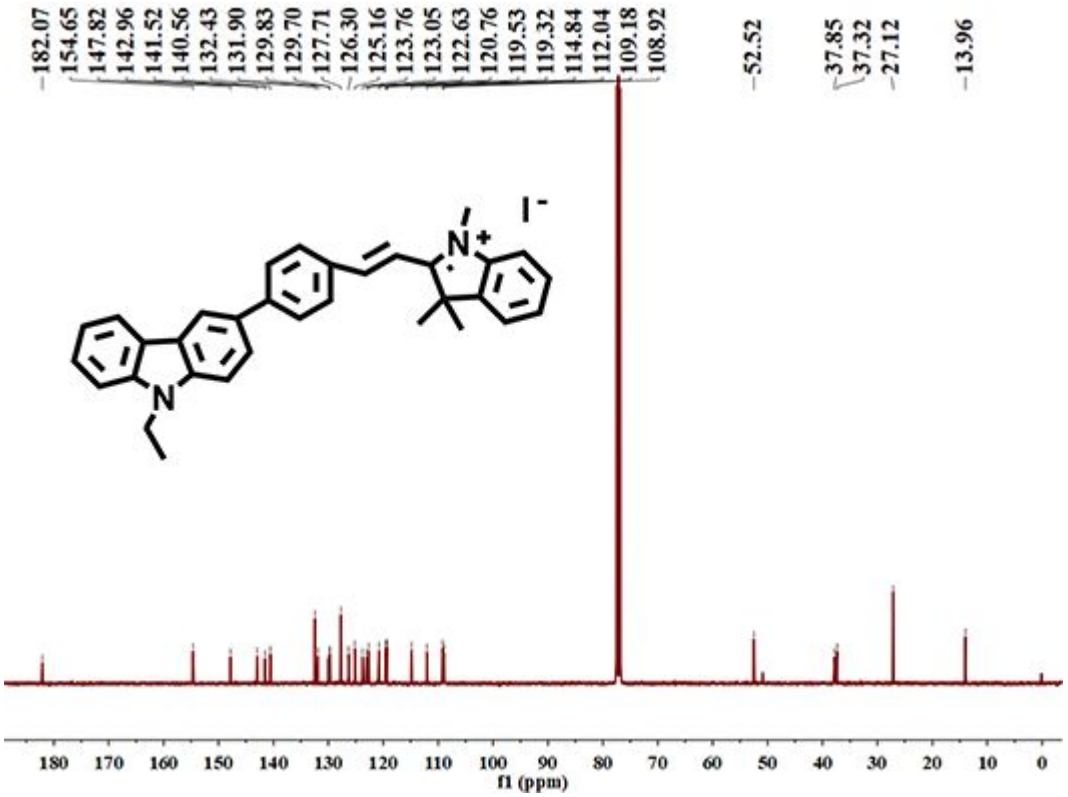


Figure S21. The ^{13}C NMR spectrum of **CBI-V** in chloroform-*d6*.

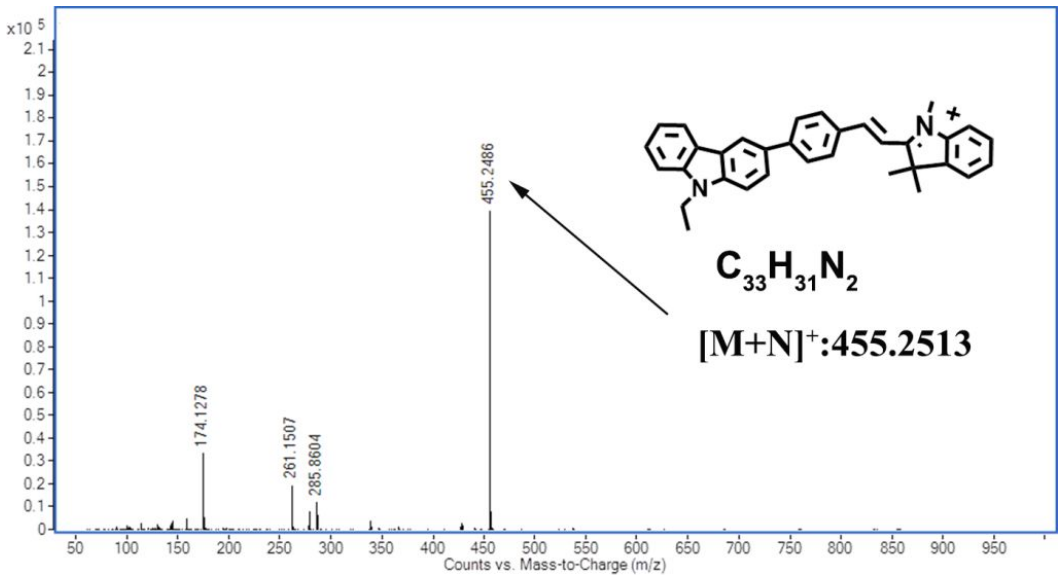


Figure S22. The HRMS spectrum of **CBI-V**.