Electronic Supplementary Information

A ratiometric fluorescence probe for peroxynitrite prepared by *de novo* synthesis and its application in assessing mitochondrial oxidative stress status in cells and *in vivo*

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1. General experimental procedures.

Reagents: Cyclohexanone, phosphorus tribromide (PBr_3), 4-(diethylamino)salicylaldehyde, cesium carbonate (Cs_2CO_3), 2,3,3-Trimethylindolenine, iodoethane (CH_3CH_2I), 2-hydroxy-4-methoxybenzaldehyde, boron tribromide (BBr_3) were purchased from Aladdin (Shanghai, China). 3-Morpholinosydnonimine hydrochloride (SIN-1), minocycline hydrochloride, lipopolysaccharide (LPS), were purchased from Sigma-Aldrich (St. Louis, USA). Unless noted, all the chemicals were of analytical reagent grade and used as received without further purification.

Instruments: Nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance II NMR spectrometer (Germany). ^1H NMR and ^{13}C NMR were conducted at 400, 100 MHz, respectively. Mass spectra (MS) was recorded on a Bruker Autoflex MALDI-TOF mass spectrometer (Germany). Element analysis was operated on Perkin Elmer 2400 elemental analyzer (USA). The fluorescence spectra were obtained on a Hitachi F-4600 spectrophotometer (Japan). The absorption spectra were collected on an Agilent CARY 60 UV-vis spectrophotometer (USA). HPLC was carried out on an Agilent 1260 LC system with a C18 column (USA). Fluorescence imaging of cells were carried out by a Nikon confocal fluorescence microscope (Japan). Fluorescence imaging of mice was performed on an IVIS Lumina XR small animal optical in vivo imaging system (USA).

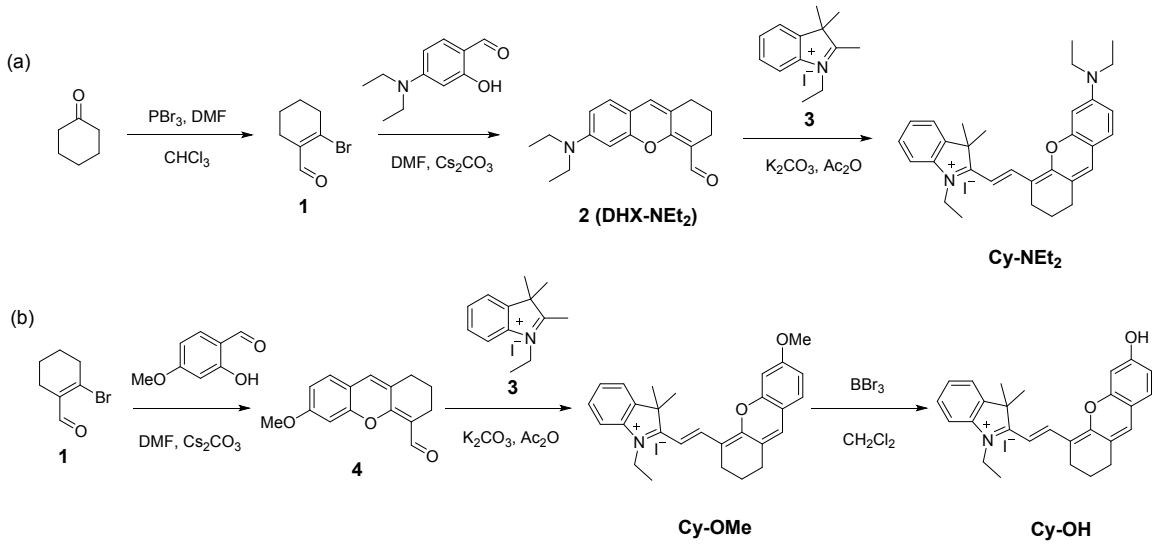
General procedure for fluorescence measurement: The stock solution of Cy-NEt_2 (500 μM) was prepared by dissolving Cy-NEt_2 in DMF. The stock solution of $ONOO^-$ was

prepared by mixing three solutions, including hydrogen peroxide (0.7 M), hydrochloric acid (0.6 M), sodium nitrite (0.6 M) and sodium hydroxide (1.5 M). The concentration of the $ONOO^-$ stock solution was calculated by measuring the absorbance at 302 nm with a molar extinction coefficient of 1670 $M^{-1}cm^{-1}$. The solution of Cy-NEt_2 with $ONOO^-$ was prepared with 50 μL Cy-NEt_2 stock solution and appropriate volume of $ONOO^-$ stock solution, and then were diluted to 5 mL with phosphate buffer solution (PBS, 0.1 M, pH 7.4). The fluorescence spectra were recorded with the excitation at 360 nm and 710 nm, and the emission was collected at 420-600 nm and 720-800 nm. The excitation slit and emission slit were both set at 5.0 nm.

Cell incubation and fluorescence imaging: The HepG2 cells were obtained from the Biomedical Engineering Center of Hunan University (Changsha, China) and cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) at 37 °C in a 5% CO_2 atmosphere. Then, the cells were plated on a 35 mm culture dish and allowed to adhere for 24 h. Fluorescence images for subcellular localization were studied in HepG2 cells by using Mito-Tracker Green and Lyso-Tracker Green. And fluorescence images for monitoring mitochondrial $ONOO^-$ level and accessing mitochondrial oxidative stress were also studied in HepG2 cells. All the cells were incubated at 37 °C and washed three times with PBS buffer solutions (pH 7.4) before fluorescence image. All images were obtained by confocal fluorescence microscope.

Fluorescence imaging in mice: Kunming (KM) mice were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China), used and kindly kept in all the experimental process. All animal operation was carried out according to the regulations issued by the Ethical Committee of Xiangtan University. The abdominal fur of the mice was removed by an electric shaver. All the mice were anesthetized with chloral hydrate (10% in saline) before image and remained anesthetized throughout the image period. And the mice were placed into the imaging chamber and imaged by small animal optical in vivo imaging system.

2. Synthesis of probes.



Scheme S1. Synthetic route for (a) Cy-NEt_2 and (b) Cy-OMe and Cy-OH.

Compound 1. PBr_3 (0.9 mL, 9.5 mmol) was slowly added to the solution of DMF (1.2 mL, 15.5 mmol) and $CHCl_3$ (10 mL) at 0 °C. After the mixture was stirred for 1 h, cyclohexanone (0.4 mL, 3.9 mmol) dissolved in $CHCl_3$ (10 mL) was added and the mixture was continued stirring for 18 h at 25 °C. The mixture was poured into ice and solid $NaHCO_3$ was slowly added until pH = 7. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (100 mL). The organic layer was dried over Na_2SO_4, and the solvent was removed by evaporation under reduced pressure to provide a yellow oil. Yield: 0.62 g (80%). Compound **1** was found to be rather volatile and unstable at 25 °C but could be stored for months under N_2 at -20 °C.

Compound 2 (DHX-NEt_2). Compound **1** (0.75 g, 4.0 mmol), 4-(diethylamino)salicylaldehyde (0.39 g, 2.0 mmol) and Cs_2CO_3 (1.95 g, 6.0 mmol) were

dissolved in anhydrous DMF (20 mL). The mixture was stirred for 48 h at 25 °C. The insoluble substance was then filtered on a pad of silica gel and the filtrate was concentrated. The resulting residue was dissolved in CH_2Cl_2 (100 mL), followed by washing with water for three times and drying by Na_2SO_4. The solvent was removed by evaporation under reduced pressure, and the residue was subjected to silica gel chromatography with CH_2Cl_2/EtOAc (9/1, v/v) as eluent to afford a deep yellow solid. Yield: 0.36 g (64%). ^{1}H NMR (400 MHz, $CDCl_3$, Fig. S1) δ 10.25 (s, 1H), 6.99 (d, J = 8.8 Hz, 1H), 6.62 (s, 1H), 6.42-6.39 (m, 1H), 6.35 (s, 1H), 3.37 (q, J = 7.2 Hz, 4H), 2.52 (t, J = 5.6 Hz,2H), 2.43 (t, J = 6.0 Hz, 2H), 1.68 (t, J = 6.0 Hz, 2H), 1.19 (t, J = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$, Fig. S2): δ 187.2, 162.1, 154.2, 149.6, 128.2, 127.6, 123.1, 111.3, 110.3, 107.8, 97.2, 46.2, 29.8, 21.6, 20.7, 12.6; MS (TOF, Fig. S3): 284.1.

Compound 3. 2,3,3-Trimethylindolenine (0.32 g, 2.0 mmol) and iodoethane (1.56 g, 10.0 mmol) were dissolved in CH_3CN (30 mL). The mixture was refluxed and stirred for 15 h. After the removal of solvent under reduced pressure, the resulting solid was recrystallized in acetone to obtain a white solid product. Yield: 0.51 g (81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (d, J = 4.0 Hz, 1H), 7.60-7.58 (m, 3H), 4.77 (q, J = 8.0 Hz, 2H), 3.16 (s, 3H), 1.66-1.61 (m, 9H). MS (TOF): 188.2.

Compound 4. Compound **1** (0.75 g, 4.0 mmol), 2-hydroxy-4-methoxybenzaldehyde (0.30 g, 2.0 mmol) and Cs_2CO_3 (1.95 g, 6.0 mmol) were dissolved in anhydrous DMF (20 mL). The mixture was stirred for 16 h at 25 °C. The insoluble substance was then filtered on a pad of silica gel and the filtrate was concentrated. The resulting residue was dissolved

in CH_2Cl_2 (100 mL), followed by washing with water for three times and drying by Na_2SO_4. The solvent was removed by evaporation under reduced pressure, and the residue was subjected to silica gel chromatography with CH_2Cl_2/EtOAc (10/1, v/v) as eluent to afford a deep yellow solid. Yield: 0.36 g (75%). ^{1}H NMR (400 MHz, $CDCl_3$, Fig. S4) δ 10.31 (s, 1H), 7.09 (d, *J* = 9.2 Hz, 1H), 6.67-6.66 (m, 3H), 3.85 (s, 3H), 2.57 (t, *J* = 6.0 Hz, 2H), 2.45 (t, *J* = 6.0 Hz, 2H), 1.72 (t, *J* = 6.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, Fig. S5): δ 187.6, 161.4, 160.9, 153.4, 127.5, 127.0, 126.6, 114.7, 112.6, 111.0, 100.5, 55.7, 29.9, 21.5, 20.4; MS (TOF, Fig. S6): 243.1.

Compound Cy-NEt$_2$. Compound **2** (0.28 g, 1.0 mmol), K_2CO_3 (0.28 g, 2.0 mmol) and compound **3** (0.23 g, 1.2 mmol) were dissolved in anhydrous Ac_2O (5 mL). The mixture was stirred for 18 h at 25 °C. The deep green solution obtained was concentrated and the resulting residue was dissolved in CH_2Cl_2, followed by washing with water for three times and drying by Na_2SO_4. The solvent was removed by evaporation under reduced pressure, and the residue was subjected to silica gel chromatography with CH_2Cl_2/CH_3OH (100/3, v/v) as eluent to afford a dark green solid product. Yield: 0.38 g (65%) ^{1}H NMR (400 MHz, $CDCl_3$, Figure S7) δ 8.50 (d, *J* = 14.0 Hz, 1H), 7.45-7.40 (m, 4H), 7.30-7.20 (m, 2H), 6.80-6.78 (m, 1H), 6.49 (s, 1H), 6.12 (d, *J* = 13.6 Hz, 1H), 4.27-4.25 (m, 2H), 3.55 (d, *J* = 6.4 Hz, 4H), 2.79-2.72 (m, 4H), 1.94 (s, 2H), 1.78 (s, 6H) 1.48 (t, *J* = 7.2 Hz, 3H), 1.29 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, Figure S8): δ 172.8, 163.6, 156.4, 152.2, 142.2, 141.7, 140.9, 138.6, 129.8, 129.0, 125.6, 122.5, 122.4, 115.2, 112.5,

112.4, 110.9, 99.5, 95.8, 49.5, 45.4, 40.3, 29.7, 28.8, 28.6, 24.8, 20.7, 12.6; MS (TOF, Figure S9): 453.3. Elem. anal. (%) calcd. for $C_{31}H_{37}IN_2O$: C, 64.14, H, 6.42, N, 4.83. Found: C, 63.65, H, 6.58, N, 4.61.

Compound Cy-OMe. Compound **4** (0.24 g, 1.0 mmol), K_2CO_3 (0.28 g, 2.0 mmol) and compound **3** (0.23 g, 1.2 mmol) was dissolved in anhydrous Ac_2O (5 mL). The reaction mixture was stirred for 16 h at 80 °C, the deep blue solution obtained was concentrated and the resulting residue was dissolved in CH_2Cl_2, followed by washing with water for three times and drying by Na_2SO_4. The solvent was removed by evaporation under reduced pressure, and the residue was subjected to silica gel chromatography with CH_2Cl_2/CH_3OH (100/2, v/v) as eluent to afford a blue solid product. Yield: 0.28 g (52%) ^{1}H NMR (400 MHz, $CDCl_3$, Figure S10) δ 8.65 (d, *J* = 14.0 Hz, 1H), 7.49 (t, *J* = 7.2 Hz, 2H), 7.41-7.38 (m, 3H), 7.20 (t, *J* = 7.2 Hz, 1H), 6.91-6.88 (m, 2H), 6.54 (d, *J* = 14.8 Hz, 1H), 4.55 (d, *J* = 7.2 Hz, 2H), 3.97 (s, 3H), 2.82 (t, *J* = 5.6 Hz, 2H), 2.76 (t, *J* = 5.6 Hz, 2H), 1.97-1.94 (m, 2H), 1.81 (s, 6H), 1.54 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, Figure S11): δ 176.6, 163.1, 161.8, 154.5, 145.8, 141.8, 141.1, 133.9, 129.3, 128.9, 127.4, 127.3, 122.6, 115.8, 115.6, 113.2, 112.5, 103.9, 100.9, 56.4, 50.6, 41.7, 29.3, 28.3, 24.9, 20.3, 13.0; ppm. MS (TOF, Figure S12): 412.2. Elem. anal. (%) calcd. for $C_{28}H_{30}INO_2$: C, 62.34, H, 5.61, N, 2.60. Found: C, 62.39, H, 5.58, N, 2.57.

Compound Cy-OH. BBr_3 (0.95 mL, 10 mmol) was slowly added to the solution of Cy-OMe (0.27 g, 0.5 mmol) and $CHCl_2$ (20 mL) at 0 °C. The mixture was stirred for 16 h

at 25 °C. The mixture was poured into a saturated solution of $NaHCO_3$ at 0 °C. Then the aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with water and dried over Na_2SO_4, The solvent was removed by evaporation under reduced pressure, and the residue was subjected to silica gel chromatography with CH_2Cl_2/CH_3OH (9/1, v/v) as eluent to afford a blue solid product. Yield: 0.24 g (90%) ^1H NMR (400 MHz, $CDCl_3$, Figure S13) δ 8.59 (d, *J* = 14.4 Hz, 1H), 7.49-7.40 (m, 2H), 7.34-7.19 (m, 4H), 7.09 (d, *J* = 8.0 Hz, 2H), 6.20 (d, *J* = 14.4 Hz, 1H), 4.19 (t, *J* = 6.0 Hz 2H), 2.67 (d, *J* = 4.0 Hz, 4H), 1.91 (s, 2H), 1.79 (s, 6H) 1.08 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, Figure S14): δ 176.1, 163.0, 154.6, 144.9, 141.7, 141.6, 136.6, 128.9, 128.7, 126.5, 125.3, 122.8, 114.8, 111.5, 103.2, 50.4, 46.7, 28.7, 24.2, 20.5, 11.7; MS (TOF, Figure S15): 398.2. Elem. anal. (%) calcd. for $C_{27}H_{28}INO_2$: C, 61.72, H, 5.37, N, 2.67. Found: C, 61.57, H, 5.34, N, 2.58.

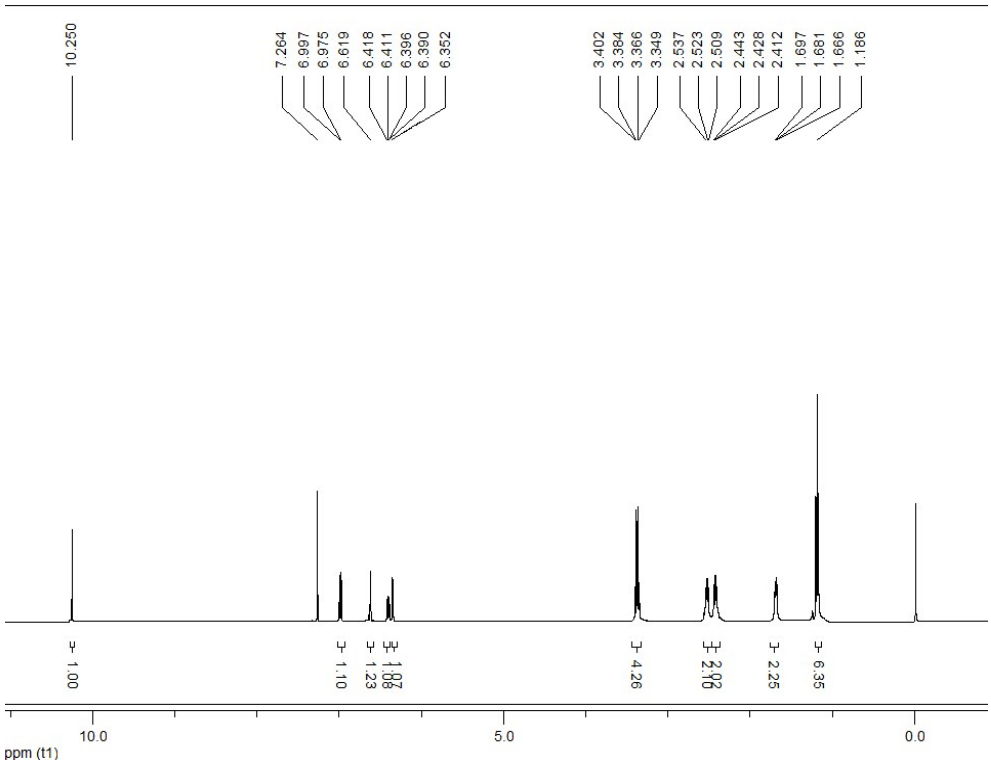


Fig. S1. ^{1}H NMR spectra of Compound 2 (DHX-NEt_2) in $CDCl_3$.

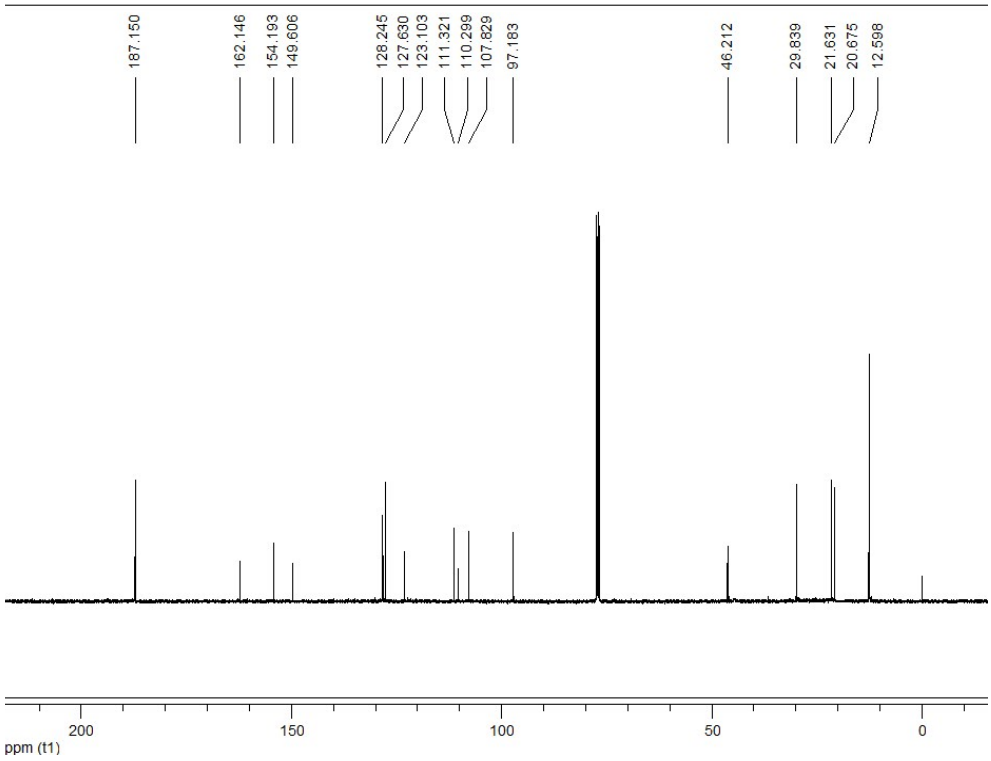


Fig. S2. ^{13}C NMR spectra of Compound 2 (DHX-NEt_2) in $CDCl_3$.

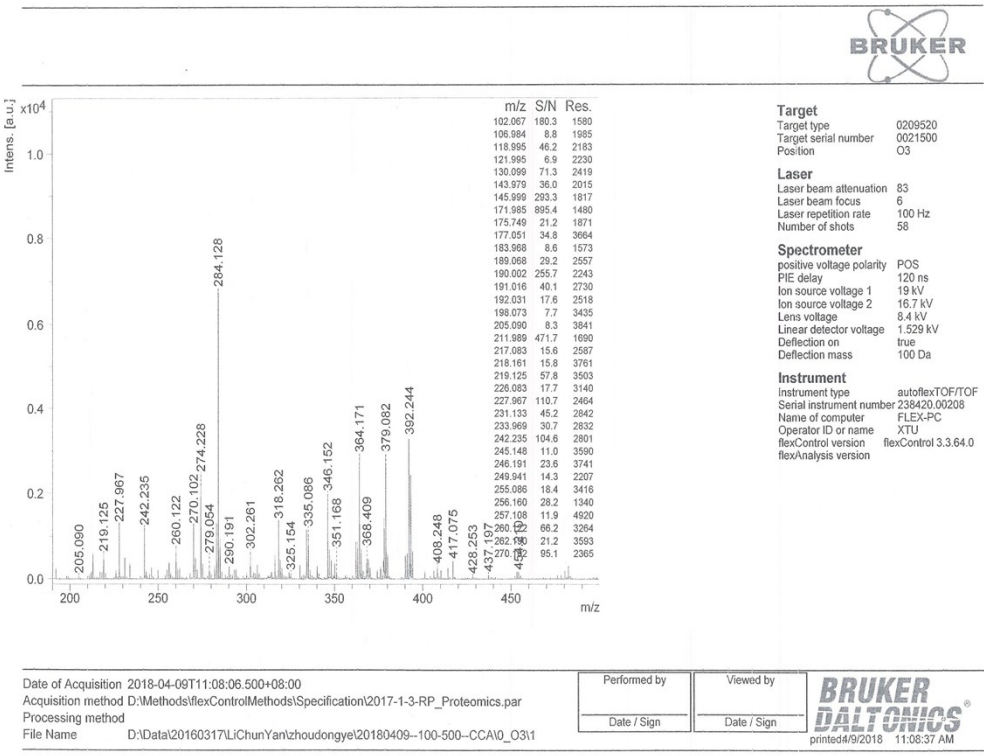


Fig. S3. Mass spectra of Compound 2 (DHX-NEt_2).

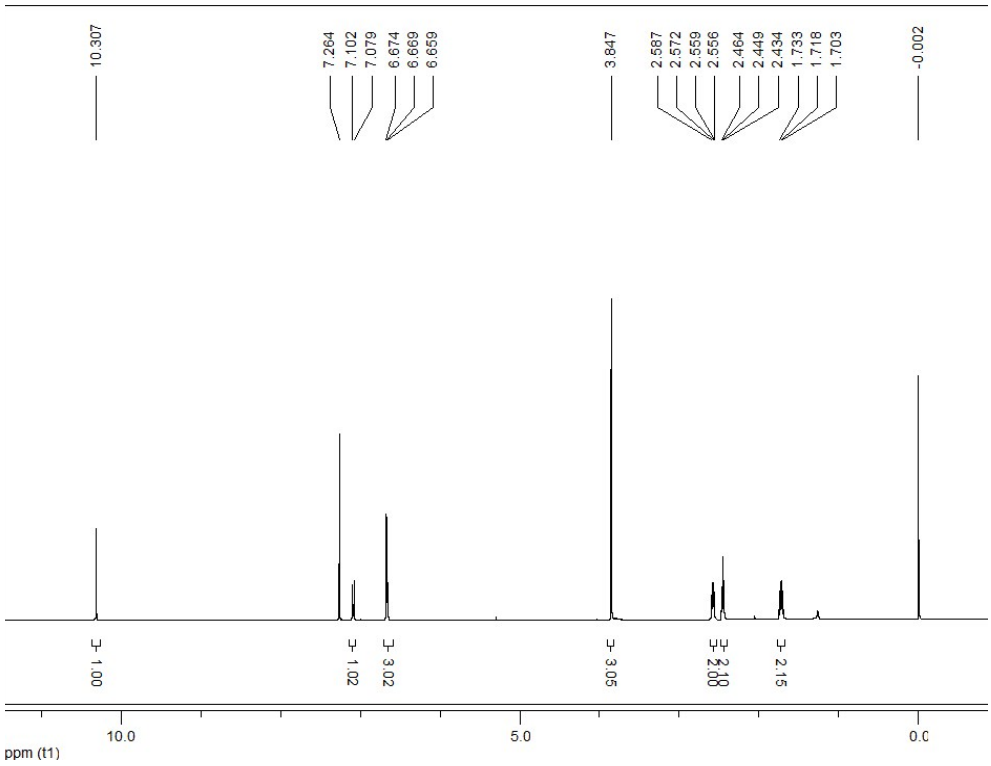


Fig. S4. ^{1}H NMR spectra of Compound 4 in $CDCl_3$.

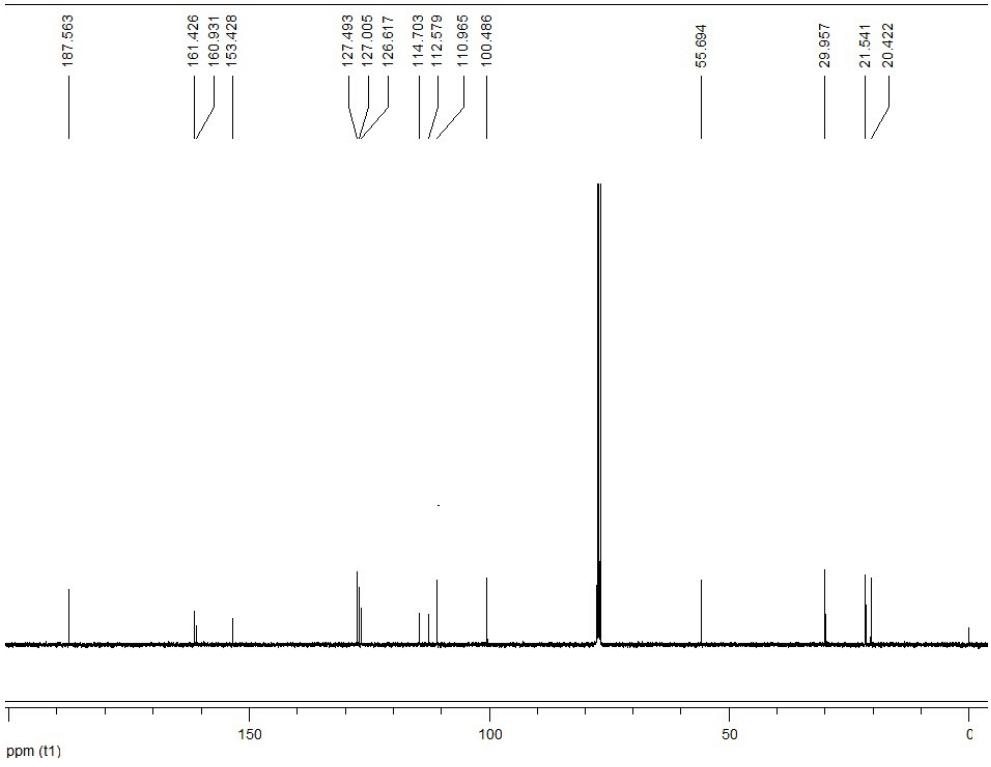


Fig. S5. ^{13}C NMR spectra of Compound 4 in $CDCl_3$.

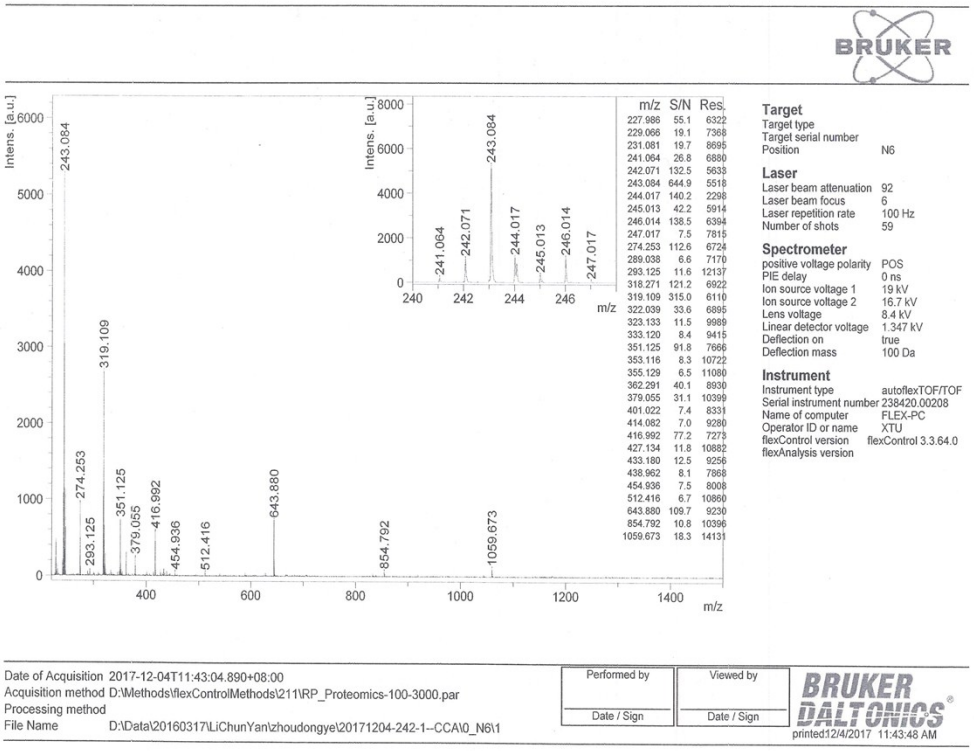


Fig. S6. Mass spectra of Compound 4.

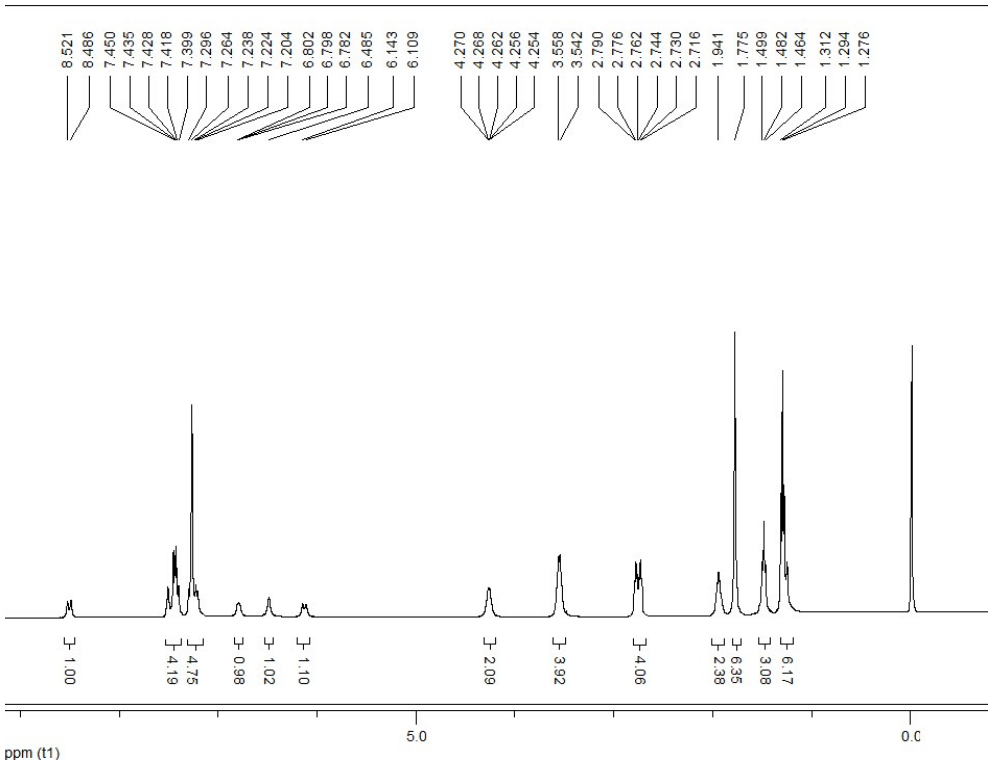


Fig. S7. ^{1}H NMR spectra of Cy-NEt_2 in $CDCl_3$.

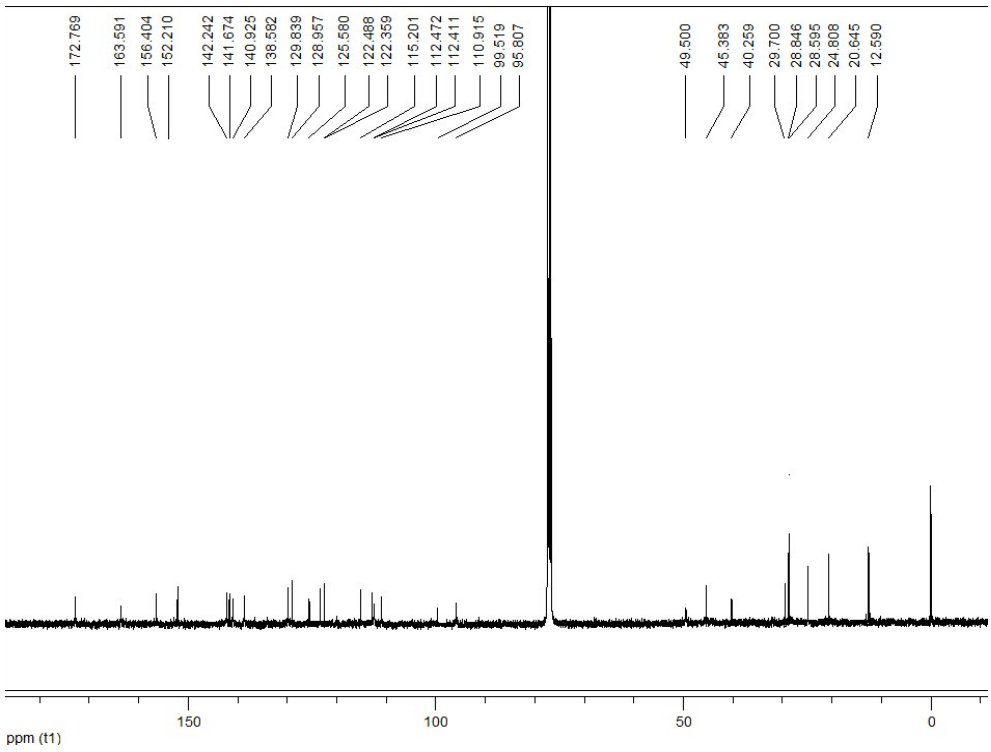


Fig. S8. ^{13}C NMR spectra of Cy-NEt_2 in $CDCl_3$.

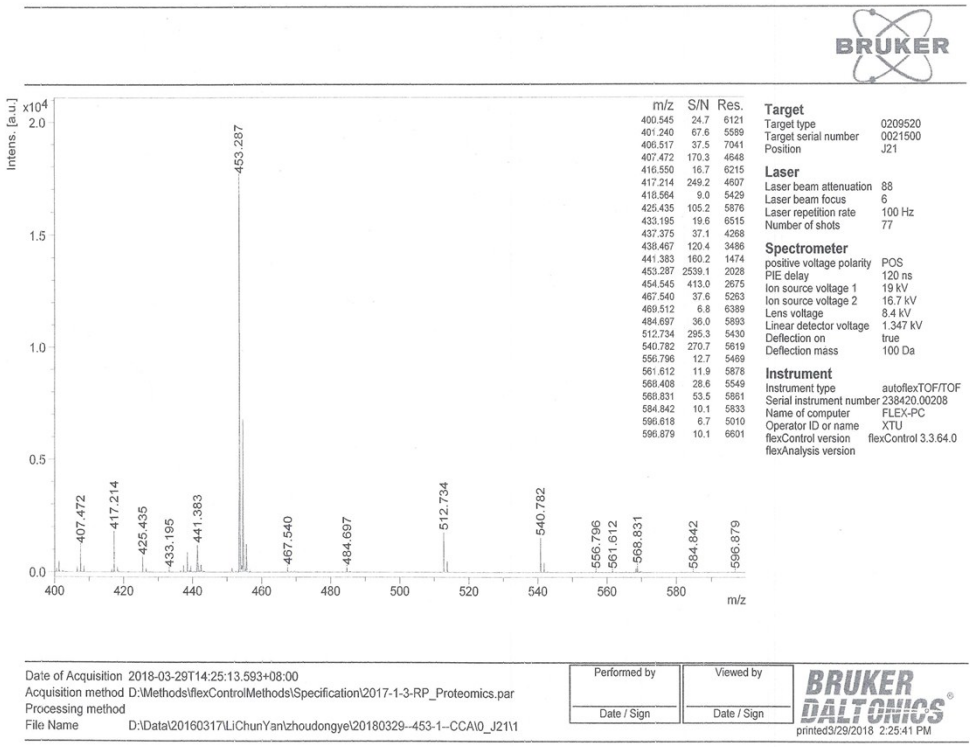


Fig. S9. Mass spectra of Cy-NEt_2.

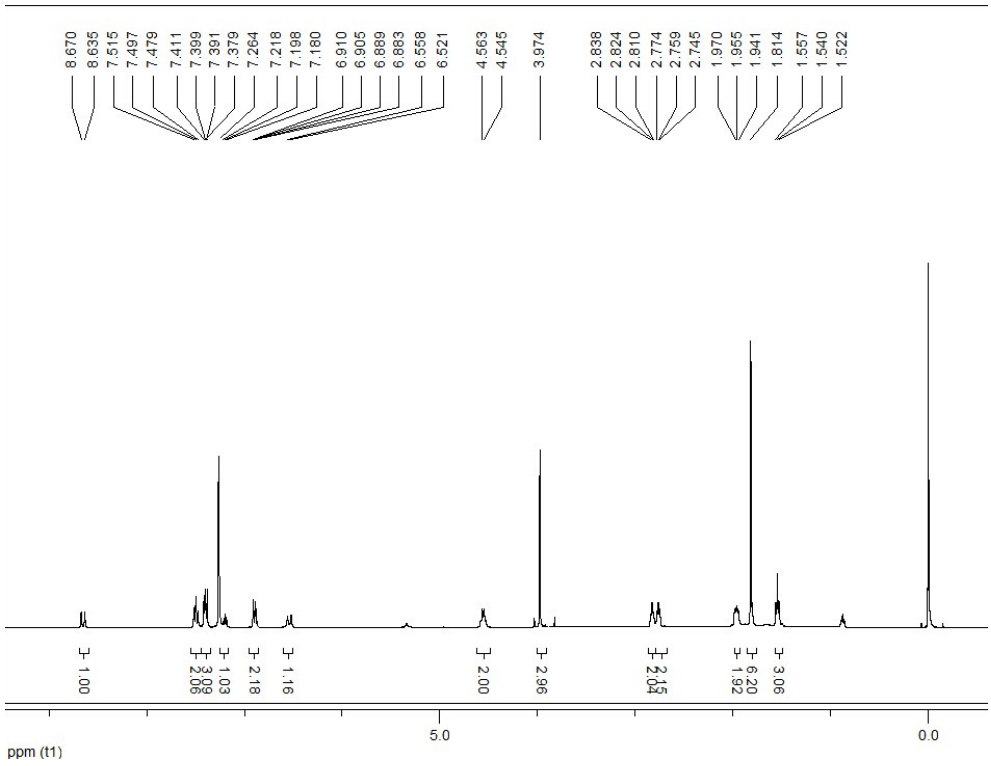


Fig. S10. ^{1}H NMR spectra of Cy-OMe in $CDCl_3$.

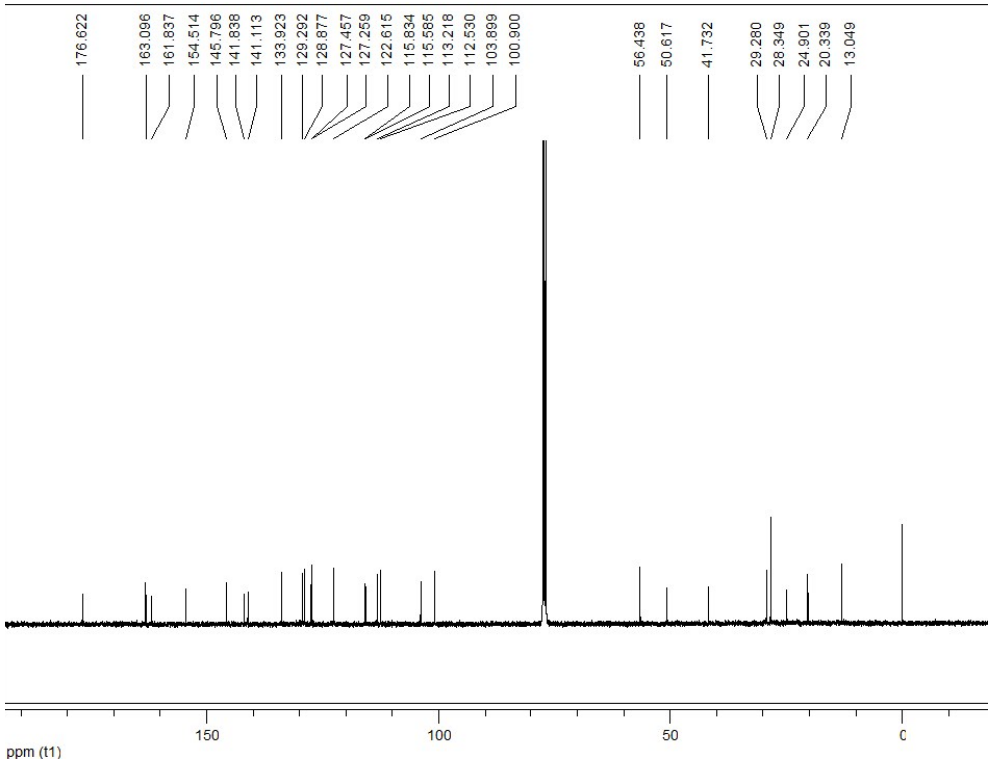


Fig. S11. ^{13}C NMR spectra of Cy-OMe in $CDCl_3$.

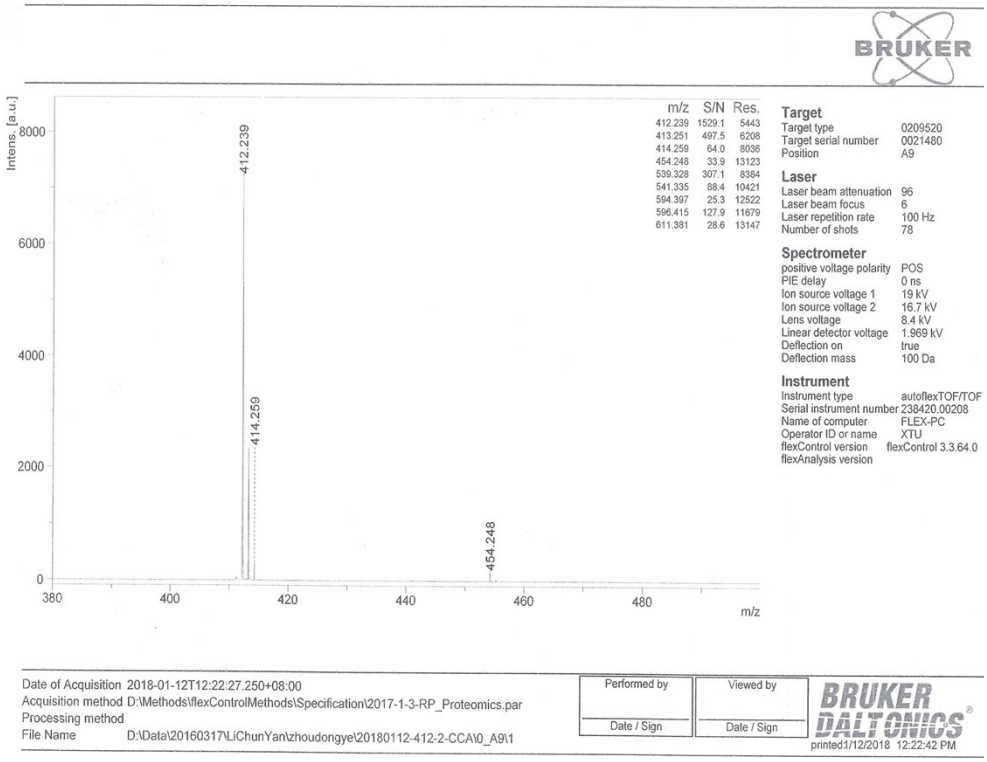


Fig. S12. Mass spectra of Cy-OMe.

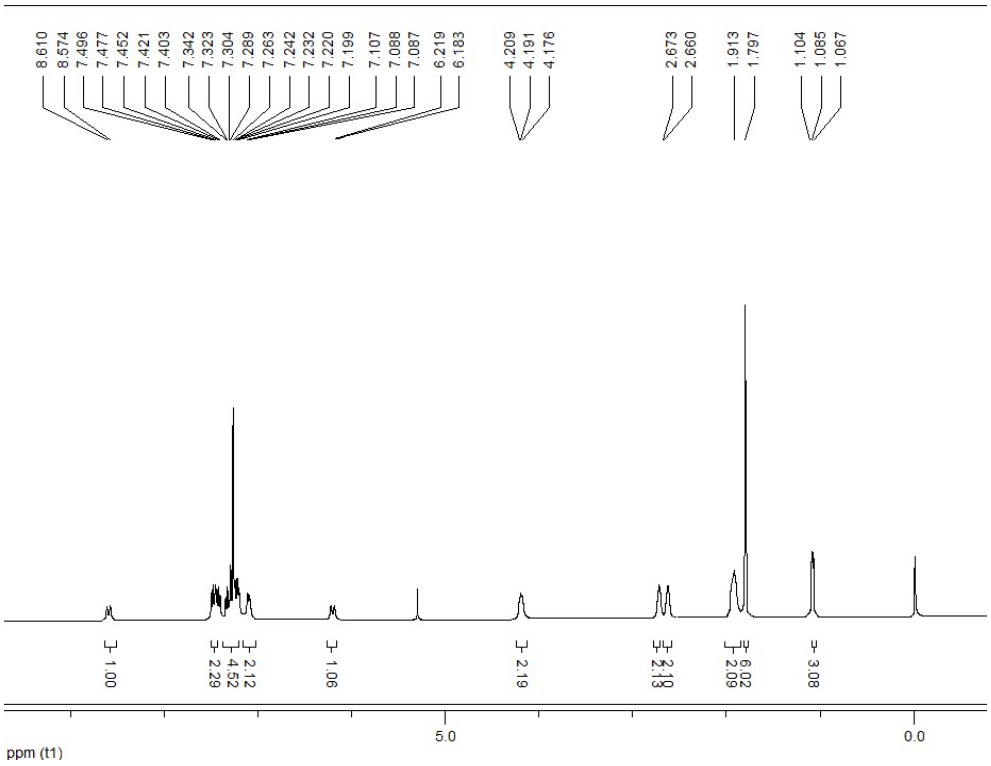


Fig. S13. ^{1}H NMR spectra of Cy-OH in $CDCl_3$.

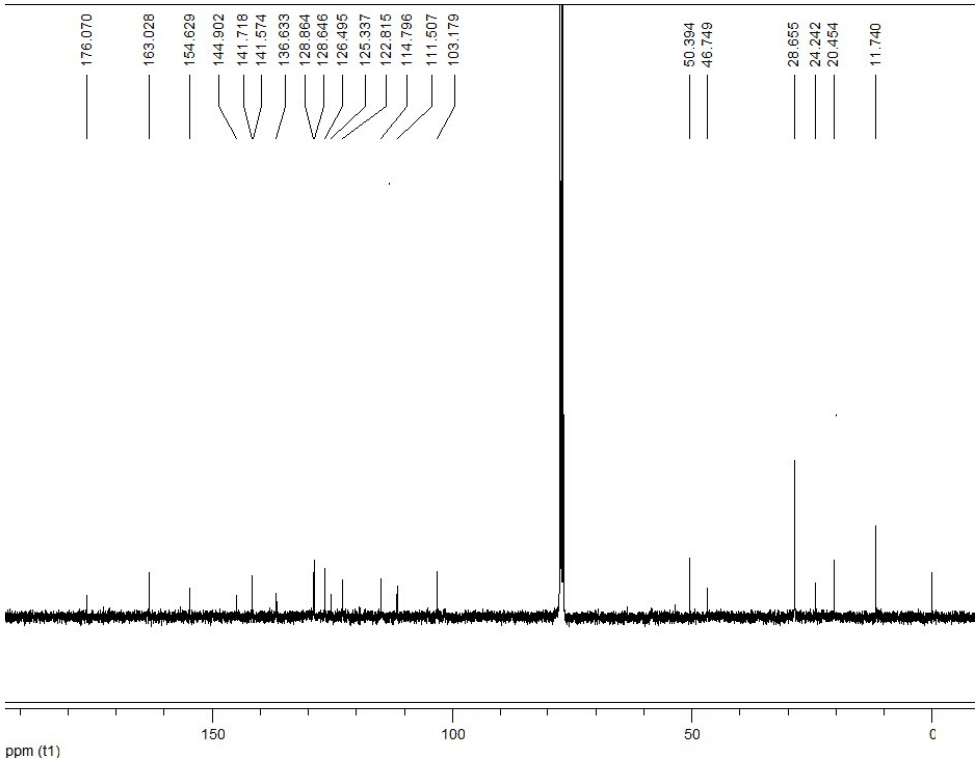


Fig. S14. ^{13}C NMR spectra of Cy-OH in $CDCl_3$.

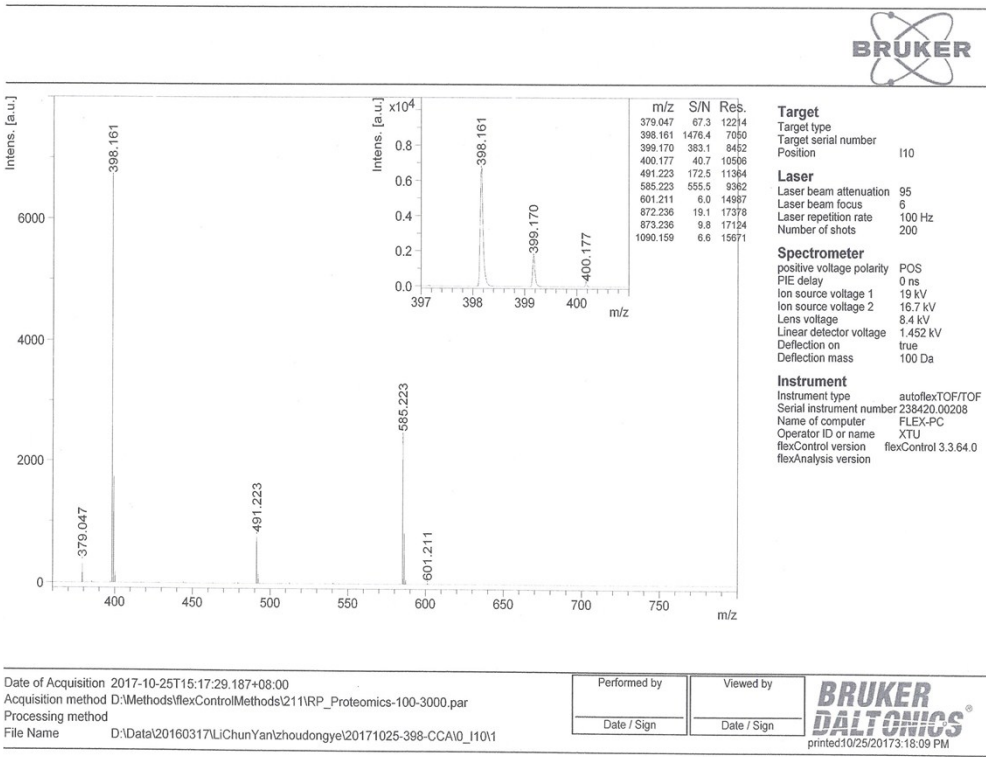


Fig. S15. Mass spectra of Cy-OH.

3. Spectral data.

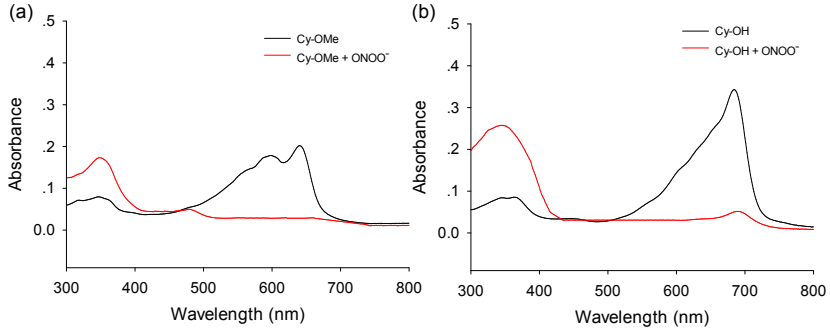


Fig. S16. The absorption spectra of (a) Cy-OMe (5 μM), (b) Cy-OH (5 μM) before and after the addition of $ONOO^-$ (30 μM).

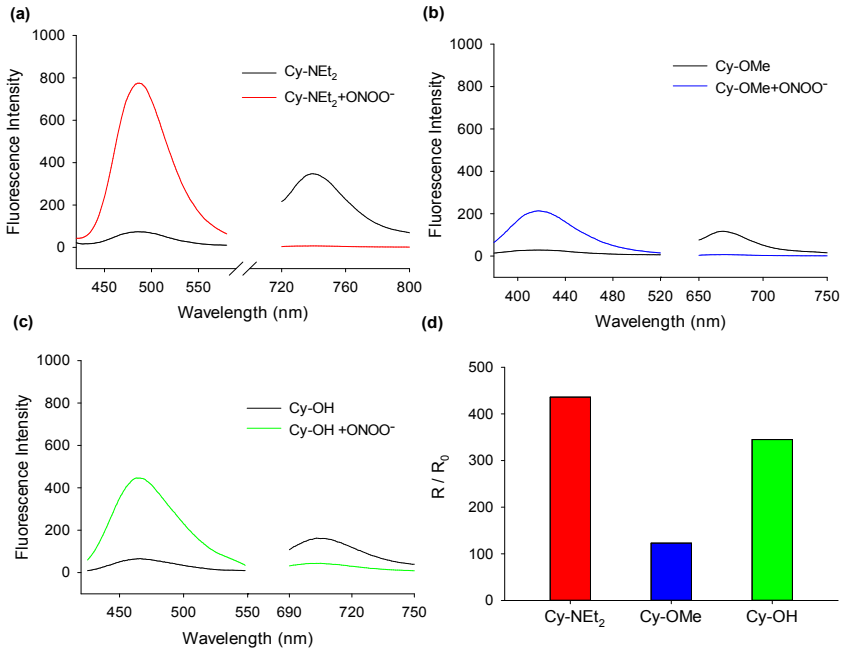


Fig. S17. The fluorescence spectra of (a) Cy-NEt_2 (5 μM), (b) Cy-OMe (5 μM), (c) Cy-OH (5 μM) to $ONOO^-$ (30 μM) in PBS buffer solution (pH 7.4). (d) The change of fluorescence intensity ratio of Cy-R after the addition of $ONOO^-$. R_0 is the ratio of the two emission

peaks before the addition of $ONOO^-$; R is the ratio of the two emission peaks after the addition of $ONOO^-$.

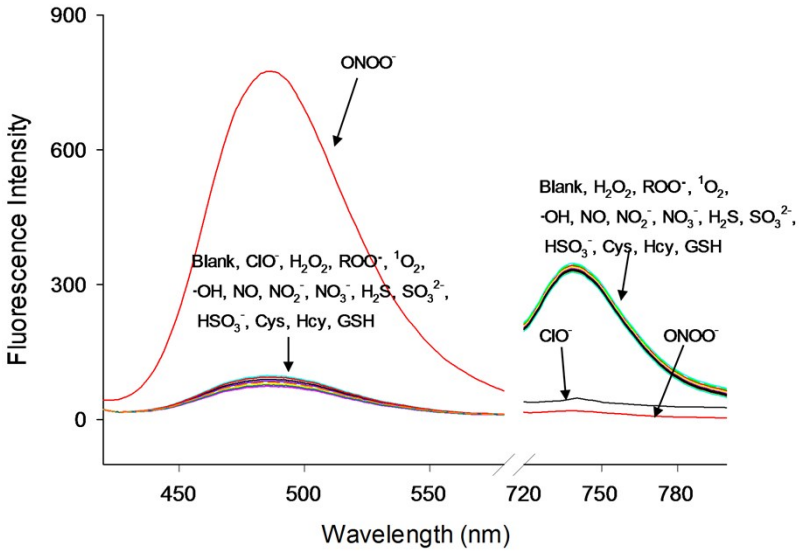


Fig. S18. The fluorescence spectra of Cy-NEt_2 (5 μM) to $ONOO^-$ (30 μM) and other various species (300 μM) in PBS buffer solution (pH 7.4). λ_{ex} = 360 nm and λ_{ex} = 710 nm.

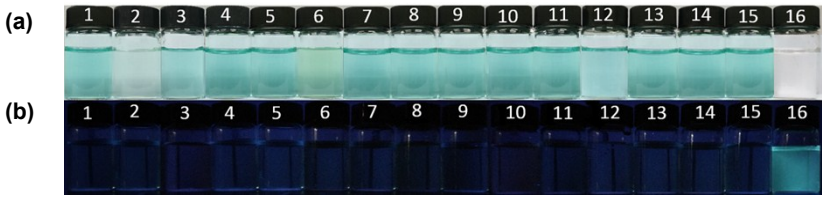


Fig. S19. The color (a) and fluorescence (b) image of Cy-NEt_2 (5 μM) to $ONOO^-$(30 μM) and other various species (300 μM) in PBS buffer solution (pH 7.4). 1: Blank, 2-6: ROS (ClO^-, H_2O_2, $ROO\cdot$, $^1O_2$, $\cdot OH$), 7-9: RNS (NO, NO_2^-, NO_3^-), 10-12: RSS (H_2S, SO_3^{2-}, HSO_3^-), 13-15: Biothiols (Cys, Hcy, GSH), 16: $ONOO^-$.

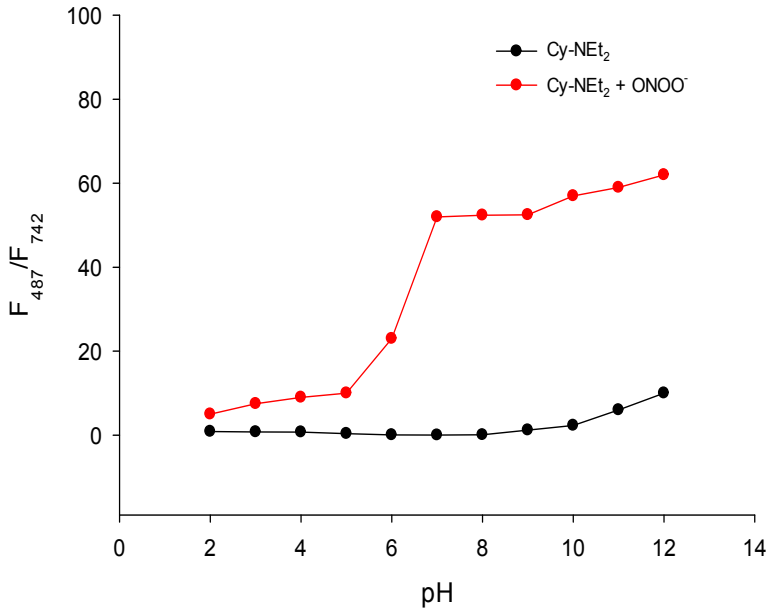


Fig. S20. Effect of pH on the fluorescence intensity ratio (F_{487}/F_{742}) of Cy-NEt_2 (5 μM) before and after the addition of $ONOO^-$ (30 μM). λ_{ex} = 360 nm and λ_{ex} = 710 nm.

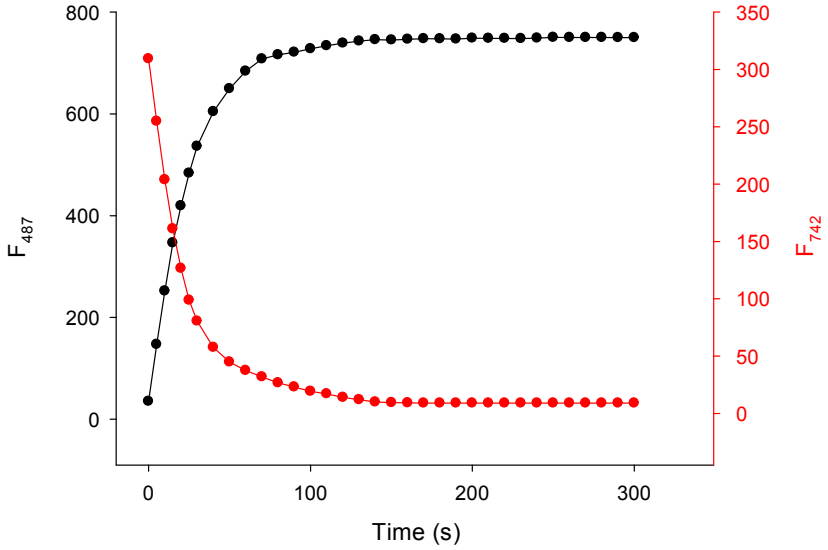


Fig. S21. The time course of fluorescence response of Cy-NEt_2 (5 μM) to $ONOO^-$ (30 μM) in PBS buffer solution (pH 7.4). λ_{ex} = 360 nm and λ_{ex} = 710 nm.

4. Response mechanism.

Based on the evidence from the changes of fluorescence spectra, a possible response mechanism of Cy-NEt_2 to $ONOO^-$ was proposed (Fig. S15a). Cy-NEt_2 displays NIR emission due to the large π-conjugation system. After adding $ONOO^-$, the C=C bond of the probe undergoes nucleophilic addition and then oxidative cleavage to obtain two main cleavage products (Indole-CHO and DHX-NEt_2). DHX-NEt_2 gives a green fluorescence owing to its small π-conjugation system. So, the probe inhibits a ratiometric fluorescence response towards $ONOO^-$ with large emission shift.

In order to prove the response mechanism of Cy-NEt_2 to $ONOO^-$, the mass spectra analysis was carried out (Fig. S15b). For free Cy-NEt_2, the peak is at m/z = 453.3. Upon the addition of $ONOO^-$, the peak at m/z = 453.3 declines and two new peaks at m/z = 204.1 corresponding to Indole-CHO and m/z = 284.1 corresponding to DHX-NEt_2 arise. HPLC experiments were also performed to confirm the proposed mechanism (Fig. S15c). Cy-NEt_2 itself displays a signal peak at 9.78 min. Upon adding $ONOO^-$, the signal peak at 9.78 min disappears and two signal peaks at 3.84 min and 12.18 min appear, which are attributed to two main cleavage products. Interestingly, the signal peak at 12.18 min shows the same retention time with standard sample of DHX-NEt_2 (12.18 min). All the above results are in good agreement with the proposed mechanism.

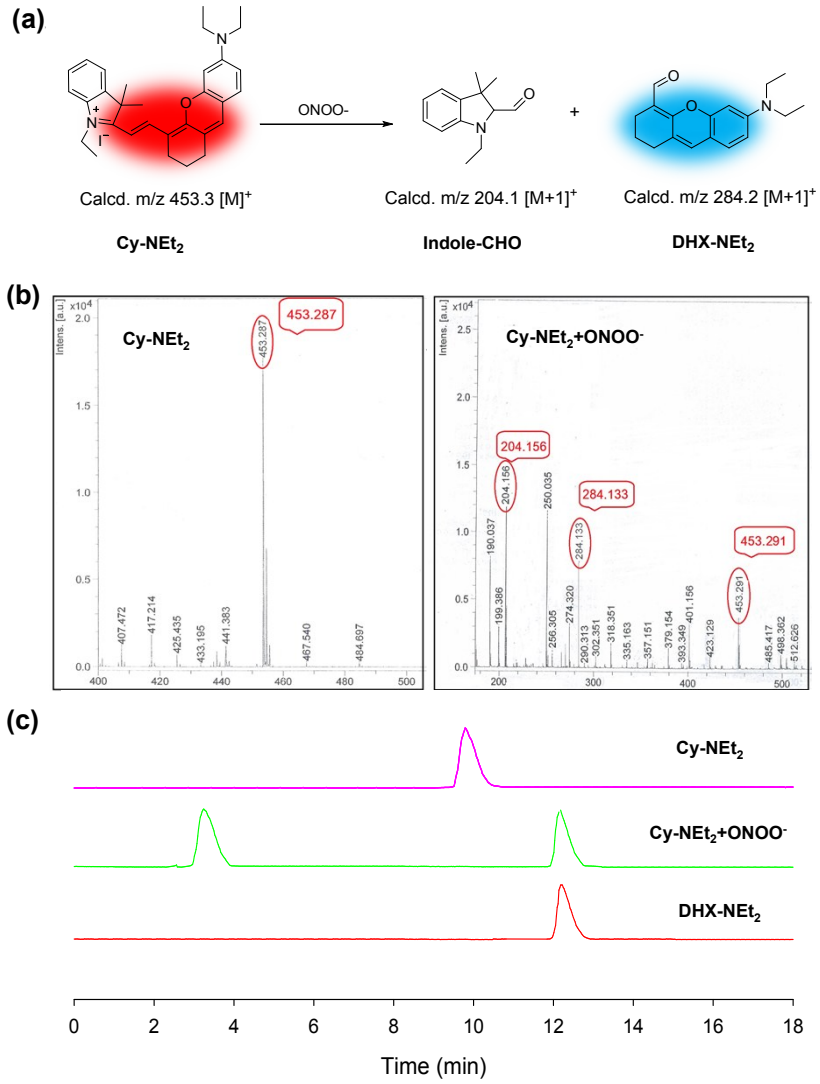


Fig. S22. (a) Proposed response mechanism of Cy-NEt_2 to $ONOO^-$. (b) Mass spectra of Cy-NEt_2 before and after the addition of $ONOO^-$. (c) HPLC chromatograms of Cy-NEt_2 before and after the addition of $ONOO^-$. The HPLC mobile phase: solvent A (CH_3CN), solvent B (0.02 M ammonium acetate buffer), A/B = 4/1 (v/v).

5. Biological assays.

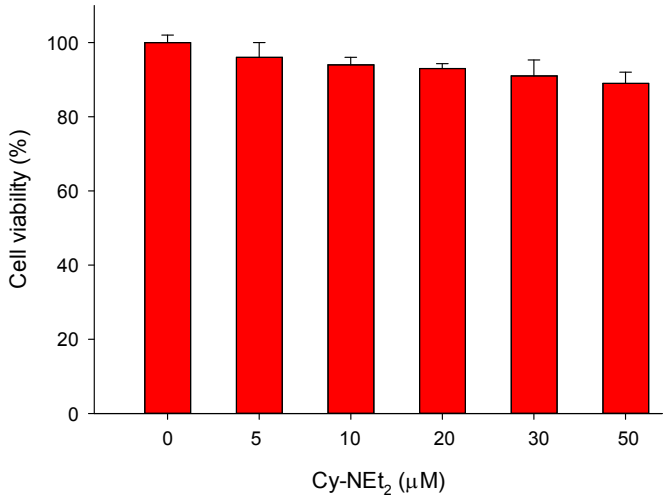


Fig. S23. MTT assay for estimating cell viability (%) of HepG2 cells treated with various concentrations of Cy-NEt_2 (0-50 μM) for 24 h.

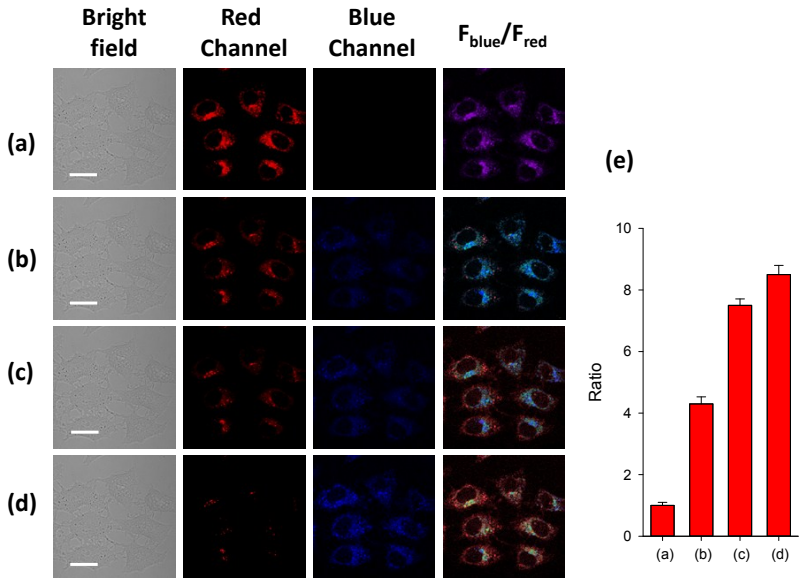


Fig. S24. Fluorescence images of HepG2 cells pretreated with SIN-1 (100 μM) for 30 min, and then incubated with Cy-NEt_2 (5 μM) for (a) 0 min, (b) 10 min, (c) 20 min, (d) 30 min. (e) Fluorescence intensity ratio (F_{blue}/F_{red}) is obtained from the image a-d and the ratio of image a is defined as 1.0. Scale bar: 10 μm. Red channel: λ_{ex} = 640 nm, λ_{em} = 680-780 nm; Blue channel: λ_{ex} = 405 nm, λ_{em} = 425-525 nm.

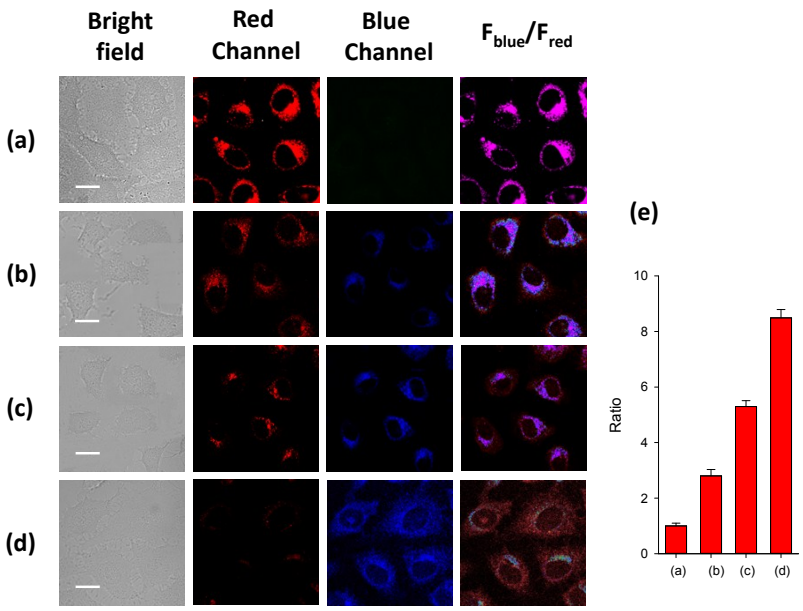


Fig. S25. Fluorescence images of HepG2 cells incubated with different concertrations of LPS (a) 0 mg/mL, (b) 0.25 mg/mL, (c) 0.5 mg/mL, (d) 1.0 mg/mL for 10 h, and then stained with Cy-NEt_2 (5 μM) for 30 min. (e) Fluorescence intensity ratio (F_{blue}/F_{red}) is obtained from image a-d and the ratio of image a is defined as 1.0. Scale bar: 10 μm. Red channel: λ_{ex} = 640 nm, λ_{em} = 680-780 nm; Blue channel: λ_{ex} = 405 nm, λ_{em} = 425-525 nm.