Supporting Information

An Amphiphilic Squarylium Indocyanine Dye for Long-term Tracking of Lysosomes

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Materials and Methods

4-Bromophenylhydrazine hydrochlorid (97%), 3-methyl-2-butanone (98%), o-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) (99%), 3,4-dihydroxycyclobut-3-ene-1,2-dione (98%) and ethyldiisopropylamine (99%), 4-bromomethylbenzoic acid (97%), were purchased from Alfa Aesar and used without further purifications. All solvents were dried prior to use with appropriate drying agents. Anhydrous *N, N*-dimethylformamide (DMF) was obtained from Alfa Aesar and used directly. Analytical thin layer chromatography (TLC) was carried out on Yantai chemical industry silica gel plates and visualized by UV. Tali TMcell viability kit was purchased from Invitrogen. MTT cell viability kit was purchased from R&D Systems. Cell viability assays by TaliTMand MTT kits were performed according to the manufacture's specifications. LysoTracker Red DND99 was purchased from Lifetechologies. DPPC for membrane model was purchased from Sigma. The pH measurements were carried out using a pH meter (Mettler Toledo S40K) which was calibrated with pH 5 and pH 7 buffers before use. ^{1}H and ^{13}C-NMR spectra were recorded on Bruker 400 (400 MHz ^{1}H; 100 MHz ^{13}C) spectrometer at room temperature. Mass spectra (MS) were measured with a XEVO-G2QTOF (ESI) (Waters, USA). UV-visible spectra were obtained on Submitted to a spectrometer

(Cintra 20, GBC, Australia). Fluorescence spectroscopic studies were performed on a fluorescence spectrophotometer (Horiba Jobin Yvon FluoroMax-4 NIR, NJ, USA). Fluorescent images were collected using an AMG EVOS f1 microscope. Confocal images were collected using a Leica TCS SP2 AOBS confocal microscope.

Experimental Section

Isothermal Titration Calorimetry

All ITC experiments were carried out with a Nano ITC (TA Instruments Waters, LLC, UT). 1,2-Dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC) used as membrane model was purchased from Sigma. DPPC were dispersed in different buffers (pH = 5 or 7) to form the micelles (0.01 mM) under ultrasonic. The solutions of **LysoCy** in the same buffer (0.1 mM) were prepared for the binding experiments. The ITC cell was filled with 1.2 mL of DPPC micelle solution and added up to 25 injections (250 μL) of the dye solution at 25 °C for each experiment. The titration gap was 300 s to ensure the heat fully equilibrated. Finally, the raw data were collected and analyzed by TA Instruments NanoAnalyze^{TM} software and the titration curves were yielded using the independent model. The thermodynamic parameters including enthalpy change (ΔH), entropy change (ΔS) and affinity constant (K_A) of were given by above software.

Molecular Calculations and Modeling

Geometry-optimized structure of **LysoCy** was calculated at the Ground state B3LYP/3–21G level with the Gaussian 09W software package (**Fig. S13**).

Cell Cultures

S2 cells were cultured at 25 °C in Schneider's Drosophila Medium (Sigma) with 10% fetal bovine serum (FBS), penicillin (100 unit/mL), and streptomycin (100 μg/mL). COS-7 cells were grown at 25 °C in DMEM medium (Gibco) containing 10% FBS, penicillin (100 unit/mL) and streptomycin (100 μg/mL) in 5% CO_2 conditioned air.

Lysosome Tracking

COS-7 cells were seeded in cell culture dishes at a density of 5×10^4 cells per dish. Subsequently, the cells were incubated with **LysoCy** at 0.1 mM concentration or with a commercial lysosome marker, LysoTracker Red DND99 (Lifetechologies, L7528), at recommended concentration (10 mM) by the manufacturer. For double labeling, cell were incubated with **LysoCy** containing medium, and then replaced with fresh medium containing LysoTracker. The fluorescent images were captured on a fluorescent microscope (AMG-EVOSf1) after washing twice with PBS.

Dynamic Lysosomes Tracking

Dynamic monitoring assays were conducted in a 35 mm^2 cell culture dish, about 2.5×10^5 cells per well. When COS-7 cells adhered to the bottom of the dish after 6 h of cell seeding, **LysoCy (**0.1 mM**)** and LysoTracker (10 mM) were added into individual cell culture dishes, respectively. Real time images were obtained on a fluorescent microscope.

Cytotoxicity Assays

Cytotoxicity assays of lysosome markers were performed using TaliTM viability kit on S2 cells. Dead cells were monitored using a green fluorescent probe Dead Cell Green (Invitrogen, Catalog A10787), which efficiently penetrates the dead cell membranes and labels the dead cell nuclei, but not the healthy cells. The measurement was conducted after 48 h incubation with **LysoCy** or LysoTracker. The culture medium was replaced with 100 μL fresh cell culture medium containing 1 μL Dead Cell Green for dead cells labeling. After incubation for 0.5 h, the percentage of viable cells was measured.

Synthesis and Characterization of 2,3,3-Trimethyl-5-bromo-3h-indole (1):

4-Bromophenylhydrazine hydrochloride (2.23 g, 10 mmol) and 3-methyl-2-butanone (0.86 g, 10 mmol) were dissolved in acetic acid (10 mL) in a three-necked flask equipped with a stirrer. The mixture was refluxed for 12 hours under an argon atmosphere then cooled to room temperature. The solvent was removed under reduced

pressure. The residue was dissolved in dichloromethane and then washed with saturated aqueous solution of sodium bicarbonate. The organic layer was separated, washed with water, dried, and then concentrated with a rotary evaporator to afford 1.88 g (7.9 mmol, 79% yield) product **2** as pale brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (s, 3H), 2.27 (s, 3H), 1.30 (s, 6H). HRMS (ESI-TOF): m/z calcd for $[M+H]^+$, 238.02; found, 238.0232.

Synthesis and Characterization of 5-Bromo-1-(p-carboxybenzyl)-2,3,3-trimethylindolenium Bromide (2)

Compound **1** (1.19 g, 5 mmol) and 4-bromomethylbenzoic acid (1.08 g, 5 mmol) were dissolved in acetonitrile (10 mL) in a three-necked flask under stirring. The mixture was refluxed for 14 hours under an argon atmosphere then cooled to room temperature. The solvent was removed under reduced pressure and the residue was washed with diethyl ether for three times. The diethyl ether was then removed with a rotary evaporator vacuum to afford compound **2** as a dry brown powder (1.72 g, 3.8 mmol, 76% yield). ^{1}H NMR (400 MHz, MeOD) δ 8.10 (s, 3H), 7.76 (s, 1H), 7.67 (s, 1H), 7.49 (s, 2H), 5.93 (s, 2H), 1.72 (s, 6H). HRMS (ESI-TOF): m/z calcd for $[M-2H-Br]^-$, 372.06; found, 372.0614.

Synthesis and Characterization of Compound 3

Compound **2** (1.35 g, 3 mmol) and 3,4-dihydroxycyclobut-3-ene-1,2-dione (171 mg, 1.59 mmol) were dissolved in a mixture of n-butyl alcohol (5 mL), toluene (5 mL) and pyridine (5 mL). The solution was refluxed for 24 hours under an argon atmosphere then cooled to room temperature. The mixture was added into diethyl ether (100 mL) dropwise. The resulting aquamarine blue precipitate was collected as a crude product. Then the crude product was re-dissolved in *N, N*-dimethylformamide (DMF) (1 mL) and precipitated in diethyl ether (100 mL) under stirring. The resulting solid was washed with diethyl ether for three times. The diethyl ether was removed with a rotary evaporator vacuum to afford compound 4a as an aquamarine blue powder (805 mg, 0.98 mmol, yield 65%). HRMS (ESI-TOF): Calcd for $[M+H]^+$,

821.08, Found, 821.0685.

Synthesis and Characterization of Compound 4

Compound **3** (411 mg, 0.5 mmol), ethyldiisopropylamine (1.65 mL, 10 mmol) and o-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) (1.9 g, 5 mmol) were dissolved in 5 mL anhydrous DMF in a three-necked flask under argon atmosphere under stirring. *tert*-Butyl *N*-(2-aminoethyl) carbamate (644 mg, 4 mmol) was added to the solvent subsequently. After stirring for 2 hours at room temperature, the mixture was precipitated in the mixed solvent of diethyl ether and n-hexane (100 mL) for two times. The resulting blue precipitate was washed with diethyl ether for three times then dried under vacuum to afford a blue powder (404 mg, 0.37 mmol, yield 73%). HRMS (ESI-TOF): m/z calcd for $[M+H]^+$, 1105.30; found, 1105.3121; $[M+Na]^+$, 1127.30; found, 1127.2941.

Synthesis and Characterization of LysoCy

Compound **4** (331 mg, 0.3 mmol) was dissolved in 5 mL CH_2Cl_2, followed by the addition of 5 mL CF_3COOH under argon atmosphere. After stirring for 2 hours at room temperature, the mixture was precipitated in diethyl ether (100 mL). The resulting blue precipitate was washed with diethyl ether for three times, and then dried under vacuum to yield the final product **LysoCy** as a blue powder (250 mg, yield 92%). ^{1}H NMR (400 MHz, MeOD) δ 7.89 (s, 2H), 7.70 (s, 1H), 7.48 (s, 1H), 7.38 (s, 2H), 7.15 (s, 1H), 5.98 (s, 1H), 5.45 (s, 2H), 3.66 (s, 3H), 3.16 (s, 2H), 3.04 (s, 3H), 1.80 (s, 6H). ^{13}C NMR (100 MHz, MeOD, δ) 178.85, 172.37, 170.63, 145.27, 143.00, 140.12, 134.68, 132.34, 129.29, 127.85, 127.02, 118.60, 113.16, 105.20, 88.50, 64.92, 50.88, 43.36, 41.06, 40.36, 38.79, 27.33, 18.57, 15.62. HRMS (ESI-TOF): m/z calcd for $[M+H]^+$, 905.2026; found, 905.2025; $[M+Na]^+$, 927.1845; found, 929.1827

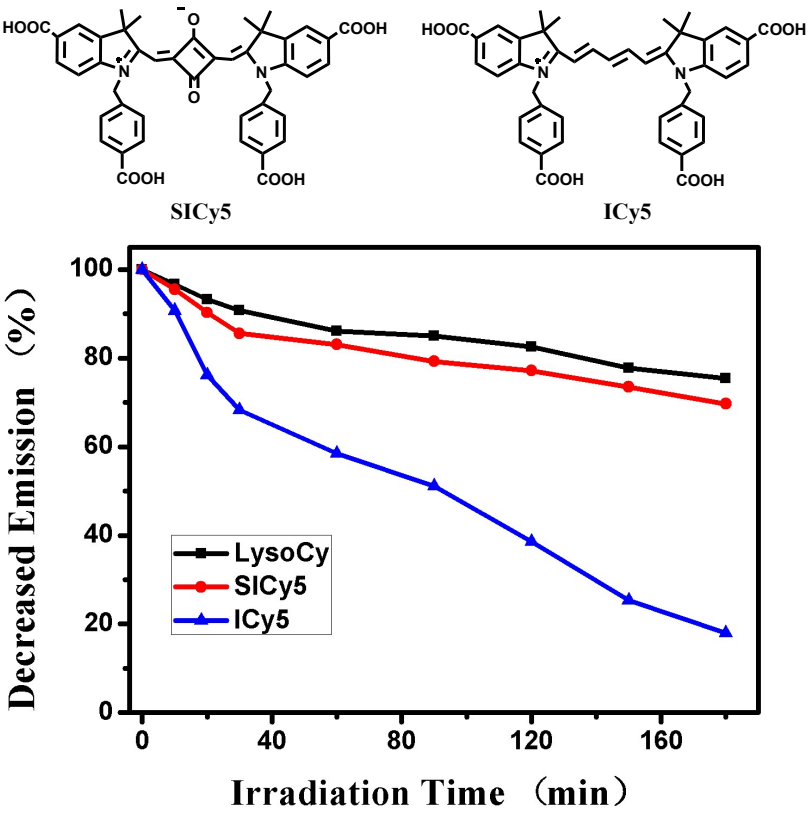

Fig. S1 Time-dependent emission of aqueous LysoCy, SICy5 and ICy5. (Irradiated under a 420 nm xenon lamp with 3 mW/cm^2)

Characterizations of the Products

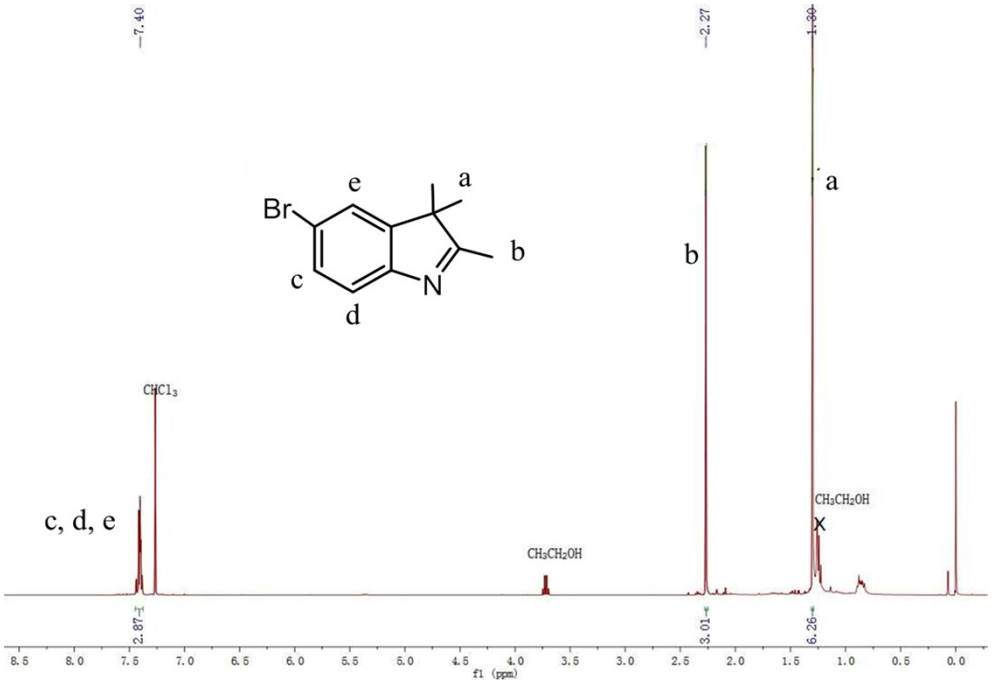

Fig. S2 ^{1}H NMR spectrum of **1** (in $CDCl_3$).

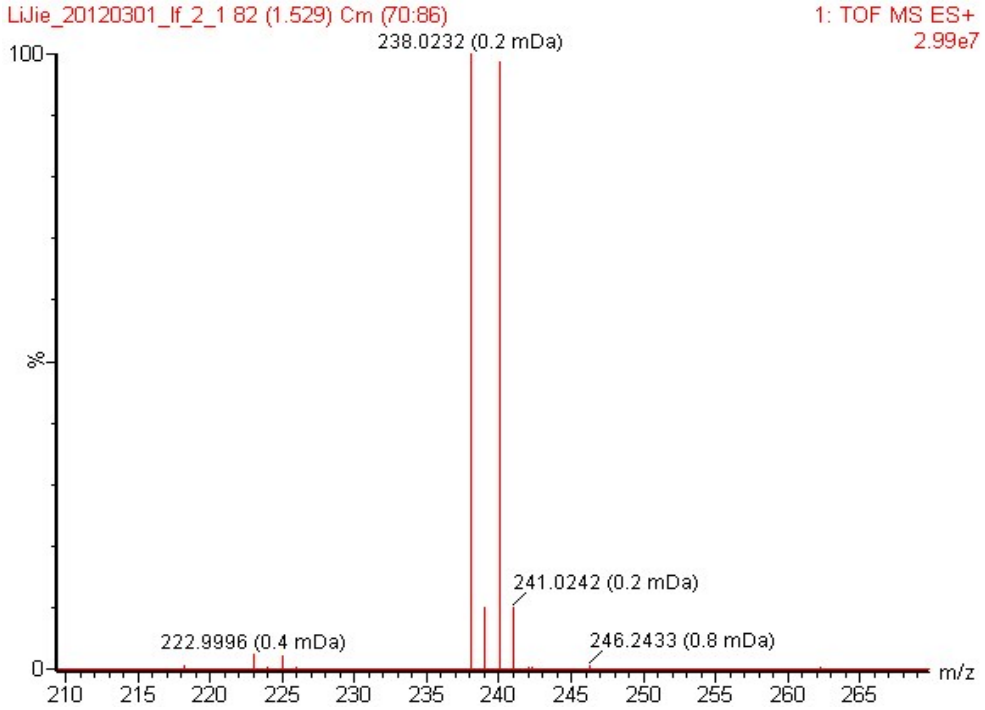

Fig. S3 HRMS (ESI^{-}) of **1.**

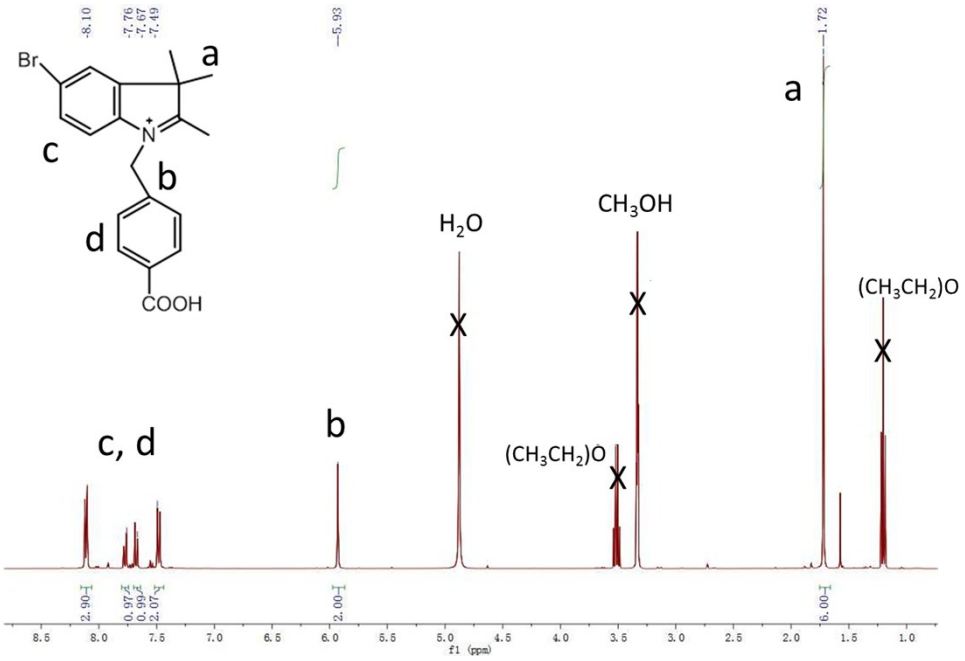

Fig. S4 ^{1}H NMR spectrum of **2** (in MeOD).

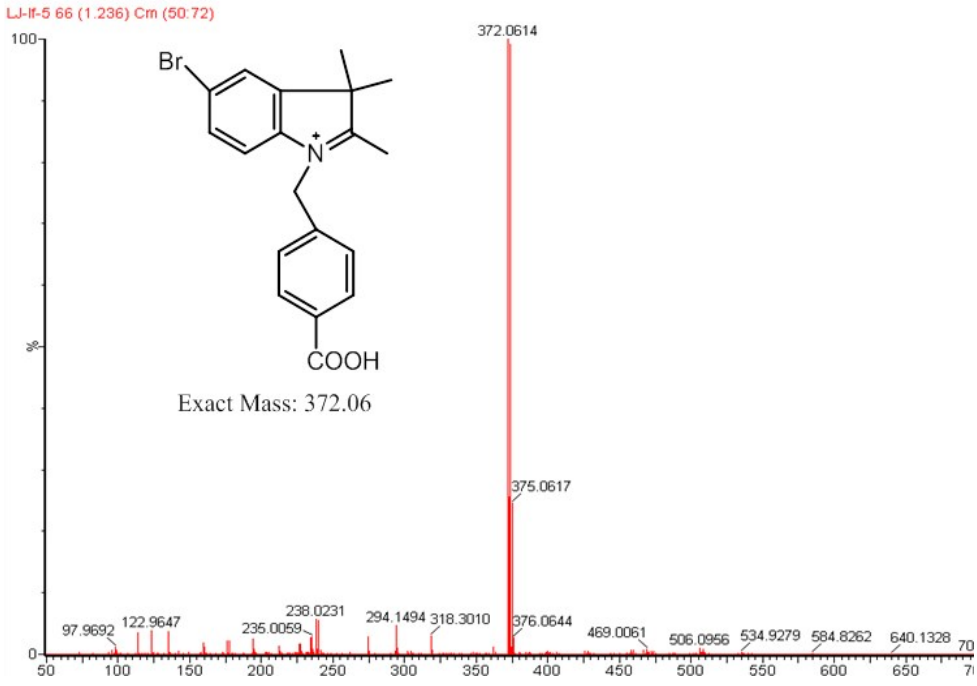

Fig. S5 HRMS (ESI^{-}) of **2.**

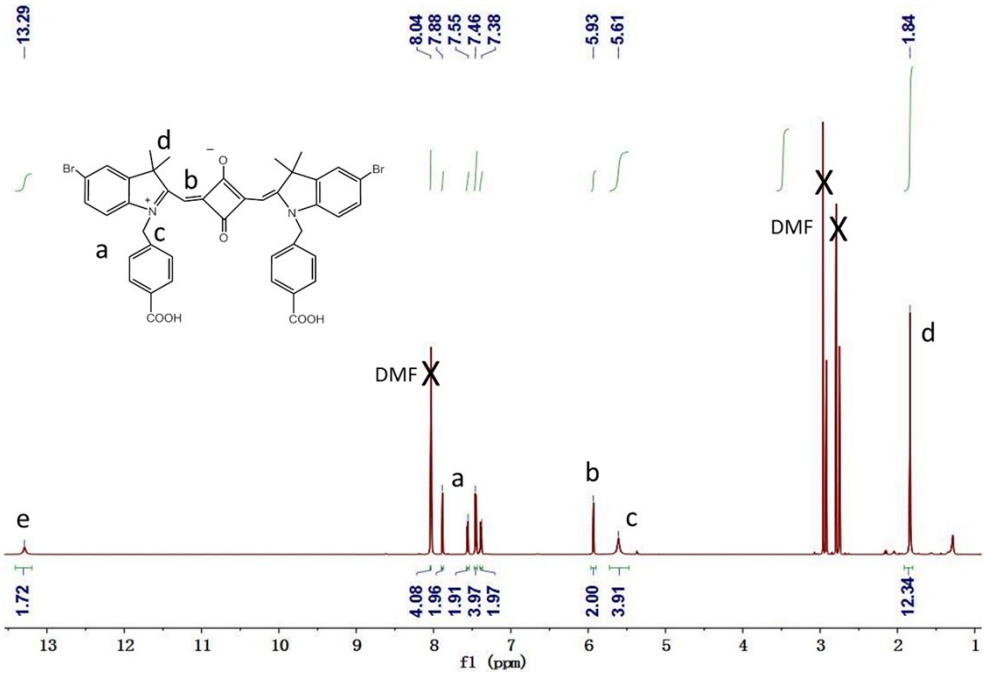

Fig. S6 ^{1}H NMR spectrum of **3** (in DMF).

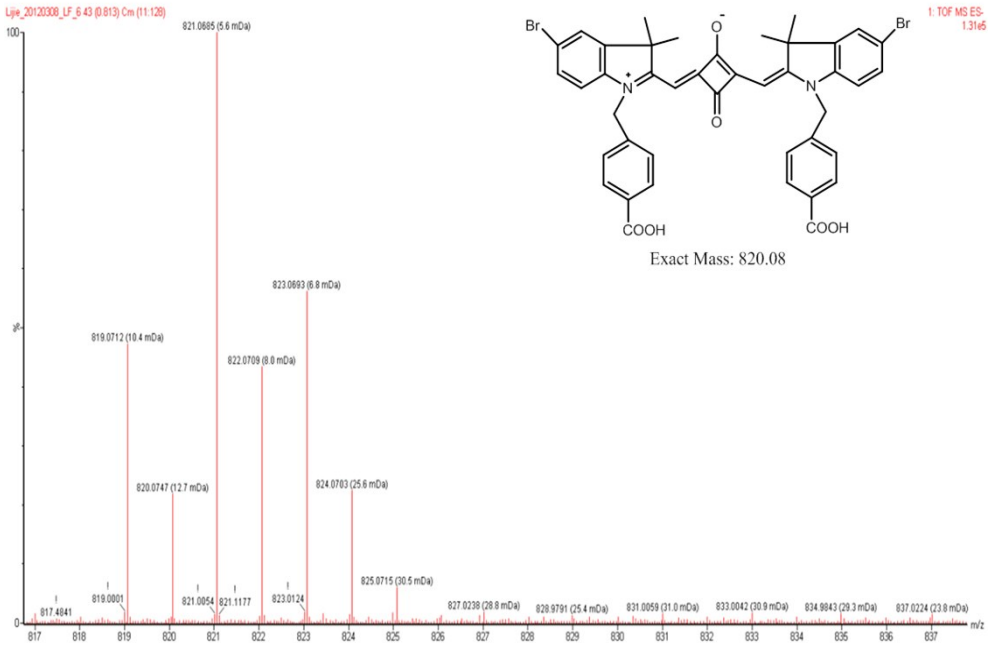

Fig. S7 HRMS (ESI^{-}) of **3.**

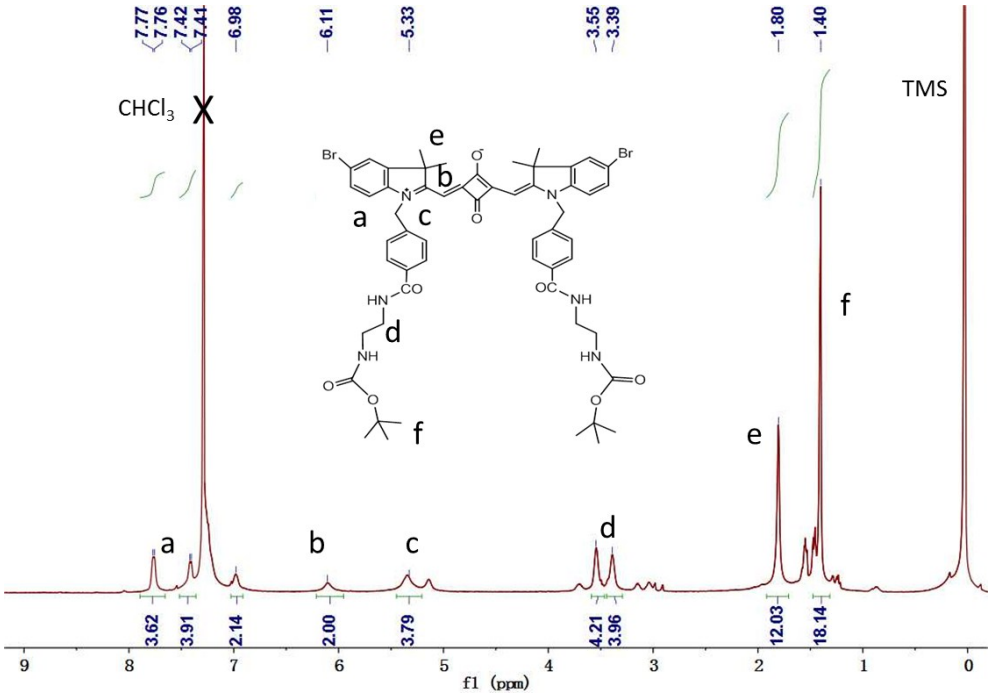

Fig. S8 ^{1}H NMR spectrum of **4** (in $CDCl_3$).

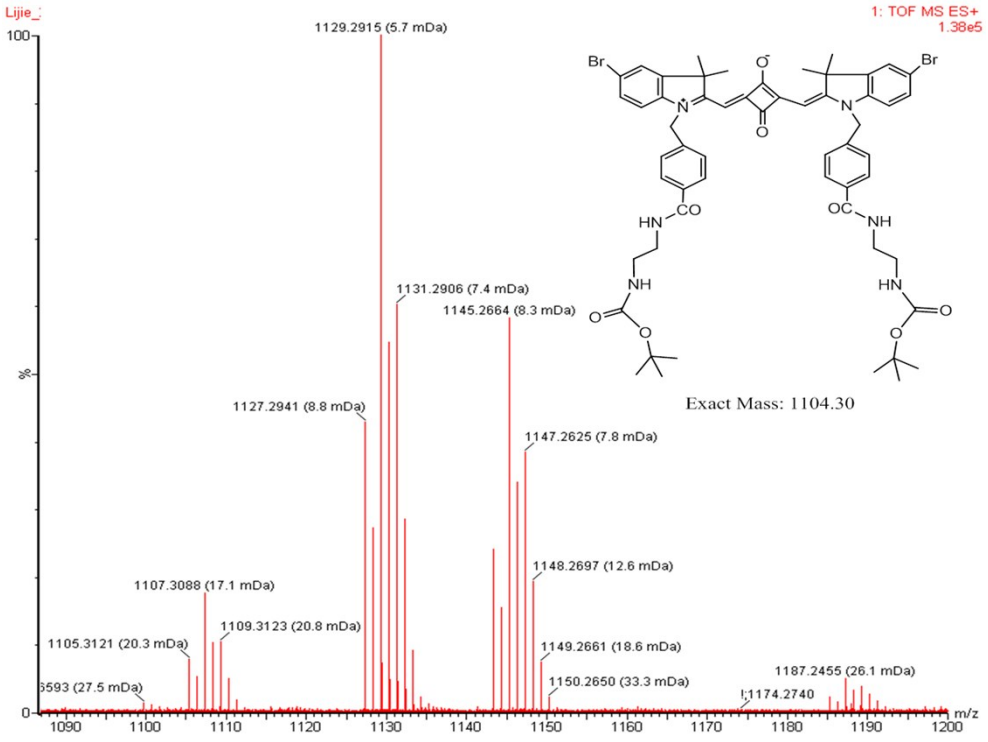

Fig. S9 HRMS (ESI^{-}) of **4.**

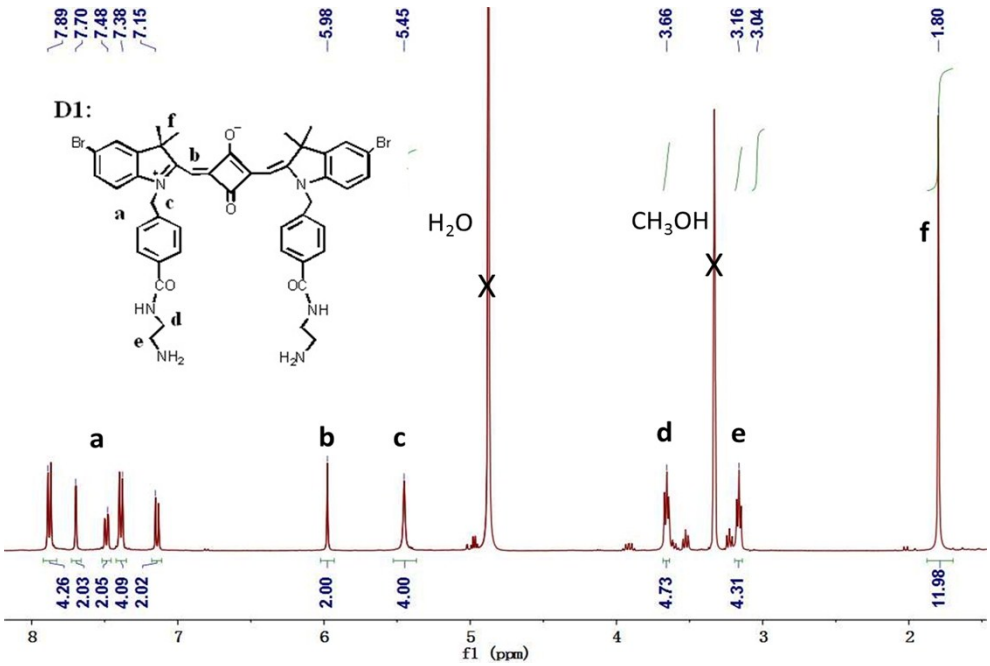

Fig. S10 ^{1}H NMR spectrum of **LysoCy** (in MeOD).

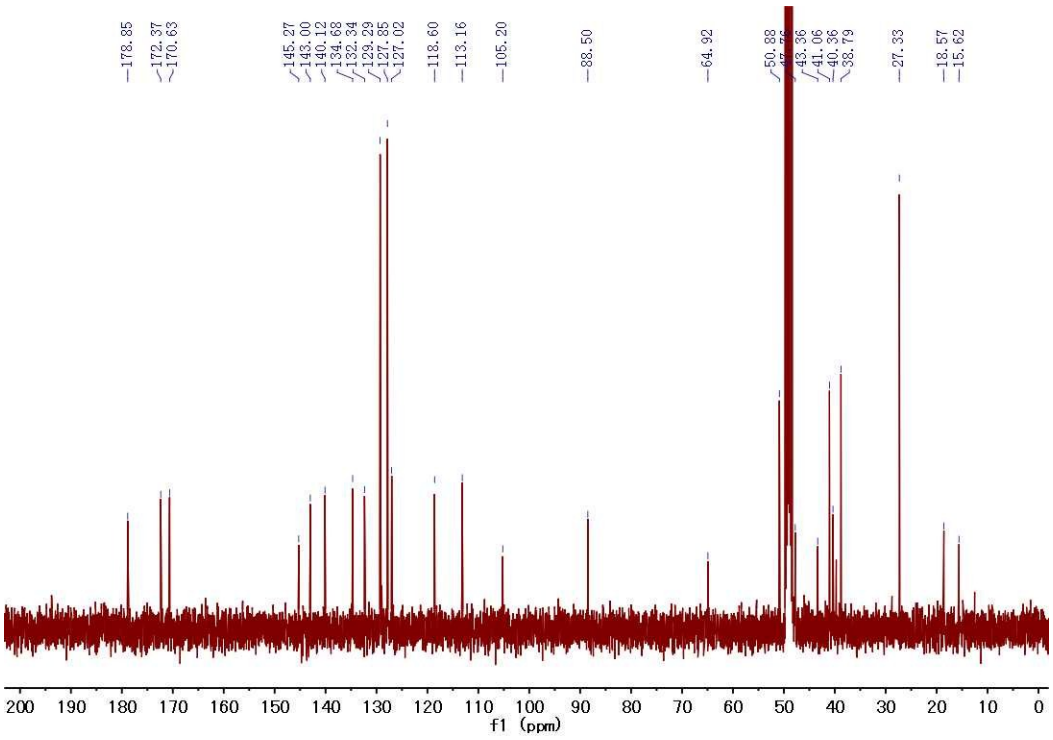

Fig. S11 ^{13}C NMR spectrum of **LysoCy** (in MeOD).

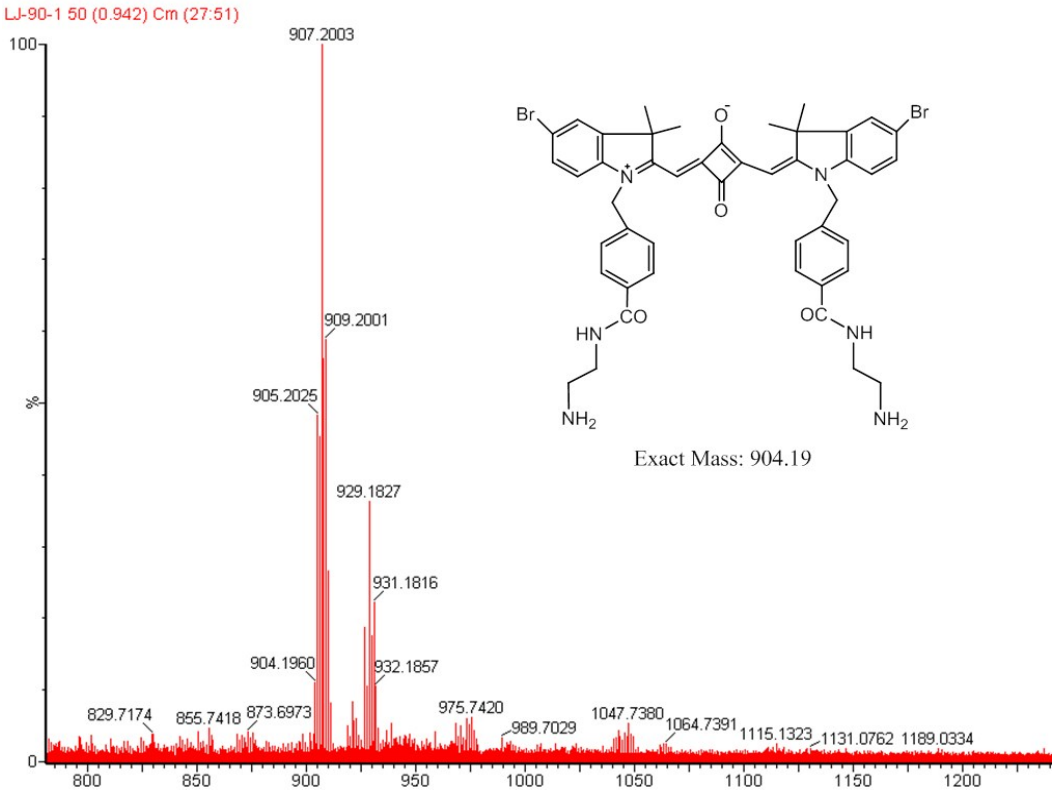

Fig. S12 HRMS (ESI$^-$) of **LysoCy.**

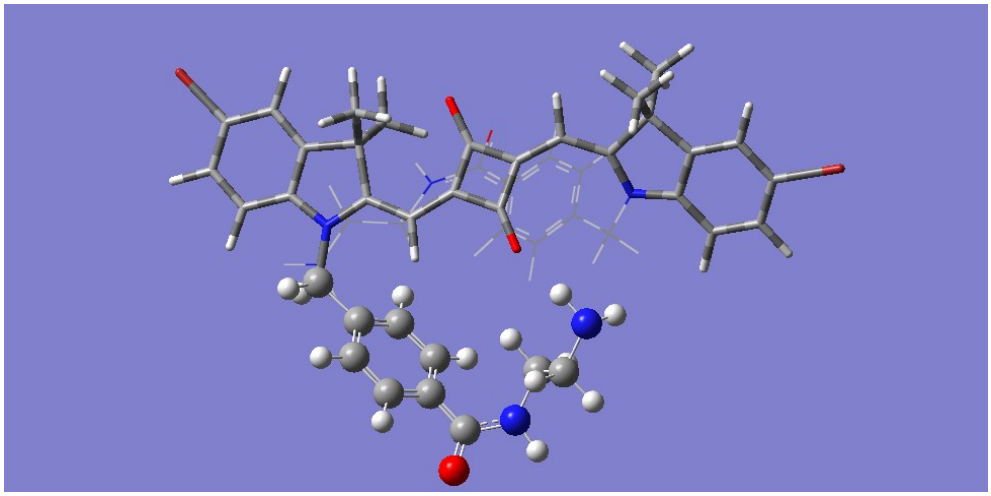

Fig. S13 Geometry-optimized models for **LysoCy**. A ball-stick model with atom colors is displayed (carbon, grey; nitrogen, blue; oxygen, red; bromine dark red; hydrogen, white).

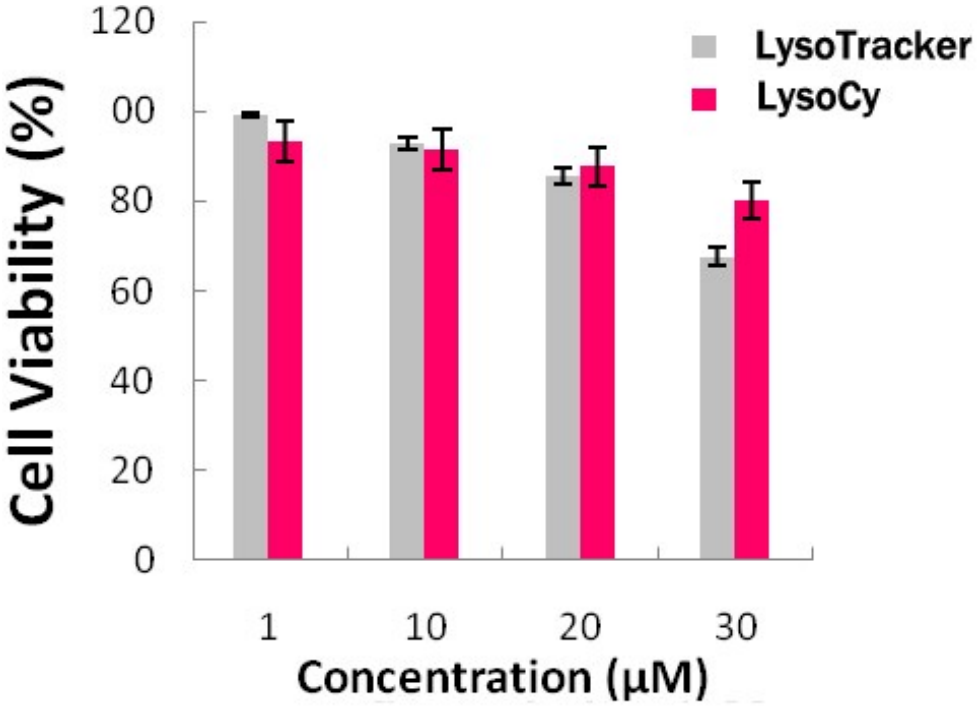

Fig. S14 Cytotoxicity assays of **LysoCy** and LysoTracker.