Supporting Imformation

A two-photon fluorescent probe for real-time monitoring autophagy by ultrasensitive detection of the change in lysosomal polarity

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Materials and methods

All reagents and solvents were commercially purchased. ^{1}H NMR spectra were recorded on Bruker-400 MHz spectrometers and ^{13}C NMR spectra were recorded on 100 MHz spectrometers. The one-photon excited fluorescence (OPEF) spectra measurements were performed using a Hitachi F-7000 fluorescence spectrophotometer. The one-photon absorption (OPA) spectra were recorded on a Tech-comp UV 1000 spectrophotometer. The fluorescence quantum yields were detected by HORIB FluoroMax-4P. The two-photon cross section was tested in THF with 500 μM probe **Lyso-OC**. The fluorescence lifetime were detected by Edinburgh Instruments FLSP920 Steady State and Transient State Fluorescence Spectrometer. The test solution of probe **Lyso-OC** (10 μM) in various solvents, including 1,4-dioxane, ethyl acetate, THF, benzonitrile , n-Hexanol, MIPK, DMSO, ethylene glycol, acetonitrile methanol, water, or mixed solvent was prepared. All pH measurements were performed using a Model PHS-3C meter calibrated at room temperature (23 ± 2 ° C) with standard buffers of pH 9.21 and 4.01.

Synthesis

As shown in Scheme S1, **Lyso-OC** can be easily synthesized in a six steps process from cheap materials. The structures of **Lyso-OC** and the intermediates were all confirmed by ^{1}H NMR and ^{13}C NMR. OC7 could be synthesized from 3-iodophenol according to the literature method[1].

Compound OC7 (1.0 g, 3 mmol), NHydroxybenzotrizole (0.60 g, 4.5 mmpl), N, N-Diisopropylethylamine (1.2 g, 9.3 mmol), 4-(2-Aminoethyl) morpholine (1.2 g, 8.9 mmol) and EDC·HCl (0.65 g, 3.4 mmol) were added to anhydrous DMF (30 mL) was stirred at room temperature for a night under N_2 atmosphere. Then, the mixture was poured into water and extracted with CH_2Cl_2 (20 mL, three times). The organic layer was dried over Na_2SO_4 and concentrated to yield a crude product. The crude material was purified through column chromatography (silica gel, EtOAc:PE=1:1) to give 0.3 g of **Lyso-OC**. ^{1}H NMR (400 MHz, CDCl3): δ 9.13 (s, 1H), 8.86 (s, 1H), 7.63 (d, J = 8.0 Hz, 1H), 7.53 (s, 1H), 7.50 (s, 1H), 7.49 (s, 1H), 7.46 (s, 1H), 6.91 (d, J = 8.9 Hz, 2H), 3.85 (s, 3H), 3.78 (s, 4H), 3.61 (s, 2H), 2.61 (d, J = 31.8 Hz, 6H). ^{13}C NMR (101 MHz, CDCl3) δ 161.65, 161.09, 160.49, 154.31, 147.46, 133.52, 133.05, 129.87, 129.53, 128.24, 118.81, 118.13, 118.00, 114.24, 114.14, 95.16, 87.03, 66.49, 56.74, 55.32, 53.26, 36.30.

Calculations

Calculations were run by Guassian09 software. All geometry optimizations and molecular orbitals calculations were performed at the MPWB95/6-311+g* level, excitation calculations of the first excited states were run using TD method at the MPWB95/6-311+g* level.

Measurement of two-photon absorption cross-section (δ)

Two-photon excitation fluorescence (TPEF) spectra were measured using femtosecond laser pulse and Ti: sapphire system (680~1080 nm, 80 MHz, 140 fs, Chameleon II) as the light source. All measurements were carried out in air at room temperature. Two-photon absorption cross-sections were measured using two-photon-induced fluorescence measurement technique. The TPA cross sections (δ) are determined by comparing their TPEF to that of fluorescein in different solvents, according to the following equation:

$$\delta = \delta_{ref} \frac{\Phi_{ref}}{\Phi} \frac{c_{ref}}{c} \frac{n_{ref}}{n} \frac{F}{F_{ref}}$$

Here, the subscripts ref stands for the reference molecule. δ is the TPA cross-section value, c is the concentration of solution, n is the refractive index of the solution, F is the TPEF integral intensities of the solution emitted at the exciting wavelength, and Φ is the fluorescence quantum yield. The δ_{ref} value of reference was taken from the literature.

Absorption and fluorescence quantum yields measurements

The relative fluorescence quantum yields were determined with Rhodamine B (ΦF = 0.97) in ethanol as a standard and calculated using the following equation:

$$\Phi_x = \Phi_s (F_x / F_s) (A_s / A_x) (\lambda_{exs}/\lambda_{exx}) (n_x/n_s)^2$$

Where Φ represents quantum yield; F stands for integrated area under the corrected emission spectrum; A is absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; n is the refractive index of the solution (because of the low concentrations of the solutions (10^{-7}~10^{-8} mol/L), the refractive indices of the solutions were replaced with those of the solvents); and the subscripts x and s refer to the unknown and the standard, respectively.

Measurement of Lifetime

The fluorescence lifetime were detected by Edinburgh Instruments FLSP920 Steady State and Transient State Fluorescence Spectrometer. The sample is illuminated with a short pulse of light and the intensity of the emission versus time is recorded. Originally, these short light pulses were generated using flash lamps that had widths on the order of several nanoseconds. Modern laser sources can now routinely generate pulses with widths on the order of picoseconds or shorter. At first, the fluorescence lifetimes of Lyso-OC (10 μM) in different solvents, including methanol, acetonitrile, ethylene glycol, dimethyl sulfoxide (DMSO), methyl isopropenyl ketone (MIPK), n-hexanol, benzonitrile, tetrahydrofuran (THF), ethyl acetate and 1, 4-dioxane, were tested. And then, the fluorescence lifetimes of Lyso-OC (10 μM) in water / THF solvent mixtures were tested by same method.

Cytotoxicity assays

MTT (5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide) assay was performed as previously reported to test the cytotoxic effect of the probe in cells. MCF-7 cells were passed and plated to ca. 70% confluence in 96-well plates 24 h before treatment. Prior to **Lyso-OC** treatment, DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS was removed and replaced with fresh DMEM, and aliquots of **Lyso-OC** stock solutions (1 mM MeOH) were added to obtain final concentrations of 5, 10, and 15 μM respectively. The treated cells were incubated for 24 h at 37 °C

under 5% CO_2. Subsequently, cells were treated with 5 mg/mL MTT (40 mL/ well) and incubated for an additional 4 h (37 °C, 5% CO_2). Then the cells were dissolved in DMSO (150 mL/well), and the absorbance at 570 nm was recorded. The cell viability (%) was calculated according to the following equation: Cell viability % = OD570 (sample) / OD570 (control) × 100, where OD570 (sample) represents the optical density of the wells treated with various concentration of **Lyso-OC** and OD570 (control) represents that of the wells treated with DMEM containing 10% FBS. The percent of cell survivlvalues is relative to untreated control cells.

TEM protocol

Cell specimens were received pelleted in Eppendorf tubes. Fresh 3% glutaradehyde in 0.1 M phosphate buffer was added to re-suspend the pellet to ensure optimal fixation, and left overnight at 4 °C. The specimens were then washed in 0.1 M phosphate buffer at 4 °C, twice at 30 min intervals. Secondary fixation was carried out in 2% aqueous osmium tetroxide for 2 hours at room temperature, followed by washing in buffer as above. Continuing at room temperature, this was followed by dehydration through a graded series of ethanol: 75% (15 min), 95% (15 min), 100% (15 min) and 100% (15 min). 100% ethanol was prepared by drying over anhydrous copper sulphate for 15min. The specimens were then placed in an intermediate solvent, propylene oxide, for two changes of 15 mins duration. Resin infiltration was accomplished by placing the specimens in a 50/50 mixture of propylene oxide/Araldite resin. The specimens were left in this mixture overnight at room temperature. The specimens were left in full strength Araldite resin for 6-8 hrs at room temperature (with change of resin after 3-4 hrs) after which they were embedded in fresh Araldite resin for 48-72 hrs at 60 °C. Semi-thin sections approximately 0.5 μm thick were cut on a Leica ultramicrotome and stained with 1% Toluidine blue in Borax. Ultra-thin sections, approx. 70-90 nm thick, were cut on a Leica ultramicrotome and stained for 25 mins with saturated aqueous uranyl acetate followed by staining with Reynold’s lead citrate for 5mins. The sections were examined using a FEI Tecnai Transmission Electron Microscope at an accelerating voltage of 80 kVv. Electron micrographs were taken using a Gatan digital camera.

Western Blotting

MCF-7 cells were seeded at a density of 1.0×10^5 per well in cell culture plate. After 24 h, some cells were incubated for different times (20 min, 40 min, 1 h, 2 h, 3 h, 4 h) with Hanks’s Balanced Salt Solution (HBSS) and another cells were incubuted for different times (20 min, 40 min, 1 h, 2 h, 3 h, 4 h) with Hanks’s Balanced Salt

Solution (HBSS) in the presence of 100 μM 3-Methyladenine (3-MA). Both adherent and floating cells were collected and lysed with protein lysis buffer containing 0.5% Triton X 100, 100 mM Tris – HCl, 150 mM NaCl, 0.1 U/mL aprotinin for 10 s on ice and centrifuged at 3000 g for 5 min. Protein concentration was determined by Bradford assay and equal protein resolved on 15 % SDS – PAGE. Protein was electroblotted on a polyvinylidene-difluoride (PVDF) membrane at 200 mA for 90 min at 4 °C. The membrane was blocked using 5 % skimmed milk for 1.5 h at room temperature, incubated overnight at 4 °C with primary antibody against the LC3 proteins in TBS + Tween 20 (TBST) at a dilution of 1:1000, using GAPDH (primary antibody dilution, 1:1000) as loading control. After washing three times, 10 min each time, with TBST, the membrane was incubated in the appropriate HRP-conjugated secondary antibody (diluted 1:3000 in skimmed milk) at room temperature for 1.0 h. The membrane was then incubated with enhanced chemiluminescence reagent (ECL) solution for certain time. The membrane was washed three times with TBST and signal visualized by using EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel) by exposing to X-ray film.

Cell image

Cells (including MCF-7 cells, HeLa cells, HELF cells and CHO cells) were seeded in 24 well plates at a density of 1×10^4 cells per well and grown for 96 hours. For live cell imaging cell cultures were incubated with the **Lyso-OC** (10% water: 90% cell media) at concentrations 10 μM and maintained at 37 °C in an atmosphere of 5 % CO_2 and 95 % air for incubation times ranging for 45 min. The cells were then washed with PBS (3 × 1 mL per well) and 1 mL of HBBS was added to each well. The cells were imaged using confocal laser scanning microscopy and oil immersion lenses at 37 °C in an atmosphere of 5 % CO_2 in an ZEISS image chamber for different times. Excitation energy of 760 nm was used and the fluorescence emission measured at 490 - 550 nm

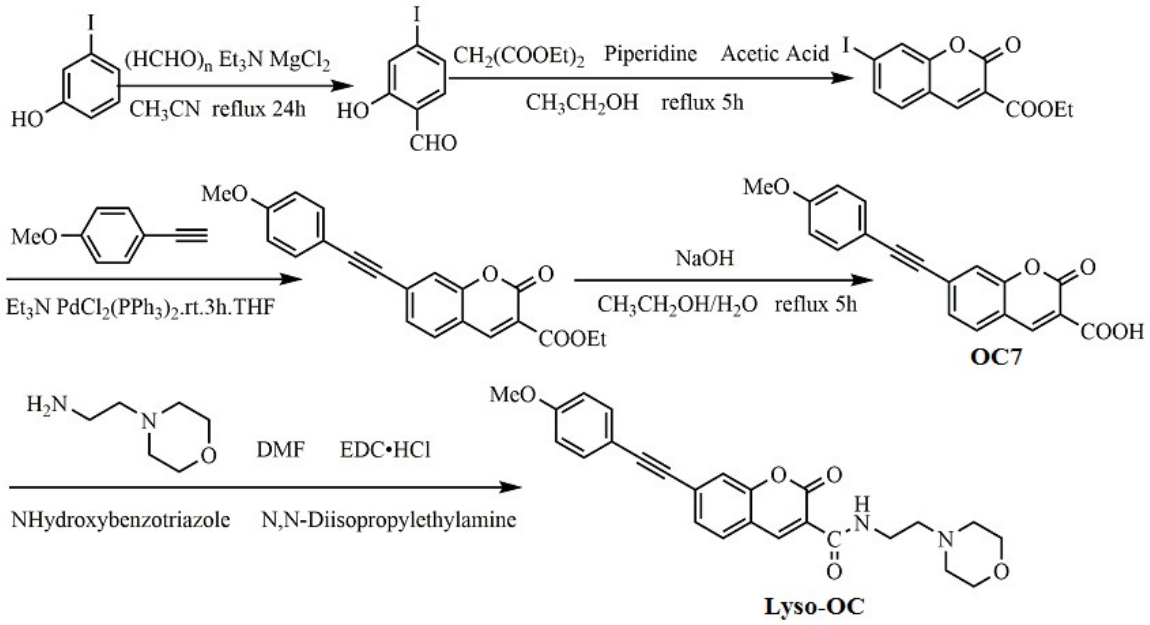
For confocal microscopy, cells were luminescently imaged on a Zeiss LSM 710 META upright confocal laser-scanning microscope using magnification 40x, 63x and 100x oil immersion lenses for monolayer cultures and tissue sections. Image data acquisition and processing was performed using Zeiss LSM Image Browser, Zeiss LSM Image Expert and Image J.

Two-photon fluorescence imaging in living tissues

The liver of rat is extracted and then snap frozen in liquid nitrogen cooled isopentane. Fresh tissues from PBS-perfused animals were sectioned at 20 μm in the sagittal plane using a cryostat (Leica 1950). Sections were mounted on glass slides and cover-slipped using an aqueous mountant (Vectashield, Vector Labs). Image data acquisition and processing was performed using Zeiss LSM Image Browser, Zeiss LSM Image Expert and Image J.

Animal Method

All procedures involving animals were approved by and conformed to the guidelines of the Anhui University Animal Care Committee, School of life science. We have taken great efforts to reduce the number of animal used in these studies and also taken effort to reduce animal suffering from pain and discomfort.



Scheme S1 Synthesis route of **Lyso-OC**.

Table S1 Spectral properties of **Lyso-OC** in various solvents.

Solvents	Dielectric Constant(ε)	Refractive Index(*n*)	Δf	λ_{abs}(nm)[a]	λ_{em}(nm)[b]	$\Delta\lambda$(nm)[c]
1,4-dioxane	2.21	1.4224	0.0205	375	461	86
Ethyl acetate	6.1	1.372	0.2012	370	470	100
THF	7.5	1.407	0.2087	375	471	94
Benzonitrile	25.9	1.53	0.2356	375	500	125
n-Hexanol	13.03	1.418	0.2433	375	496	121
MIPK	13.1	1.39	0.2532	375	495	120
DMSO	47.2	1.474	0.2649	374	519	145
Ethylene glycol	37.7	1.432	0.2744	375	521	146
Acetonitrile	37.5	1.344	0.3055	375	519	144
Methanol	33.6	1.326	0.3101	375	523	148
Water	80.1	1.34	0.3212	376	525	149

The dielectric constant data of the solvents were measured at 25 °C. Solvent-dependent spectra are often interpreted in terms of the Lippert-Mataga equation.

$$f_{(\varepsilon)} = (\varepsilon-1) / (2\varepsilon+1) \text{ and } f(n^2) = (n^2-1) / (2n^2+1)$$

$$\Delta f = f(\varepsilon) - f(n^2)$$

Where ε and n are the dielectric constant and the refractive indices of the solvent, respectively

Table S2 Fluorescence lifetime of **Lyso-OC** in various solvents.

Solvents	T(ns)
1,4-dioxane	2.01
Ethyl acetate	1.39
THF	1.50
Benzonitrile	2.09
n-Hexanol	1.03
MIPK	1.32
DMSO	0.77
Ethylene glycol	0.29
Acetonitrile	0.95
Methanol	< 0.1
Water	< 0.1

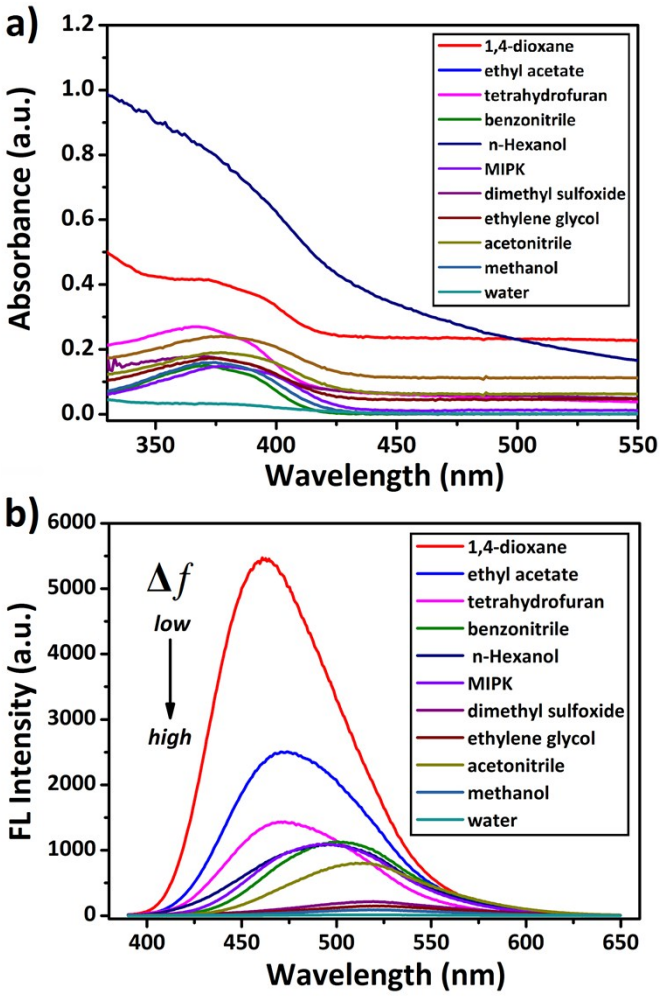


Figure S1. (a) Absorption spectra of **Lyso-OC** (10 μM) in different solvents. (b) Fluorescence emission spectra of **Lyso-OC** (10 μM) in different solvents. λ_{ex} = 375 nm.

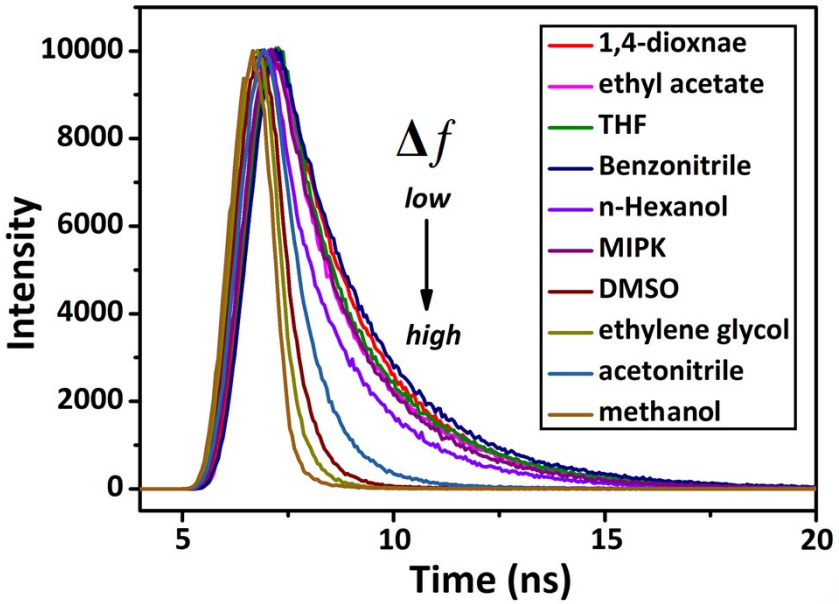


Figure S2. Fluorescence lifetimes of **Lyso-OC** (10 μM) in different solvents. λ_{ex} = 375 nm.

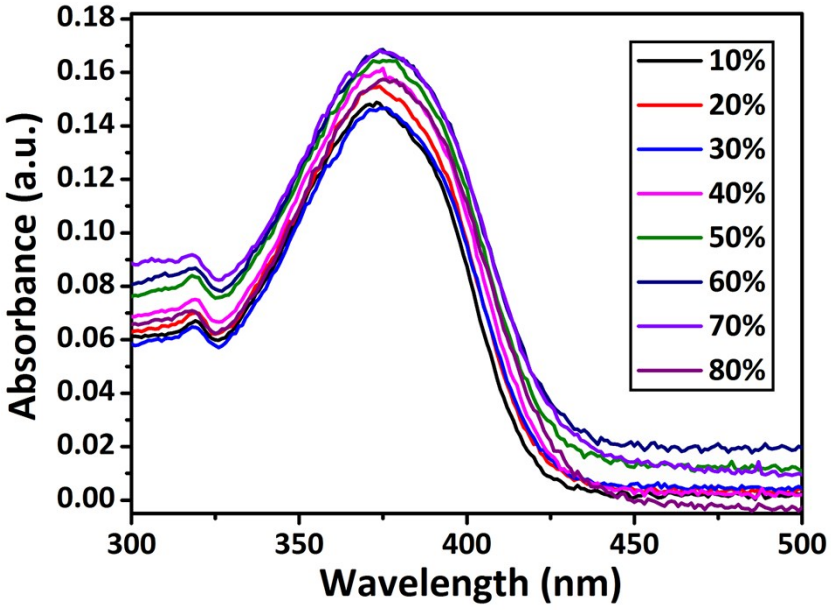


Figure S3. Absorption spectra of **Lyso-OC** (10 μM) in water/THF solvent mixtures (the percentage in the box indicates the water content).

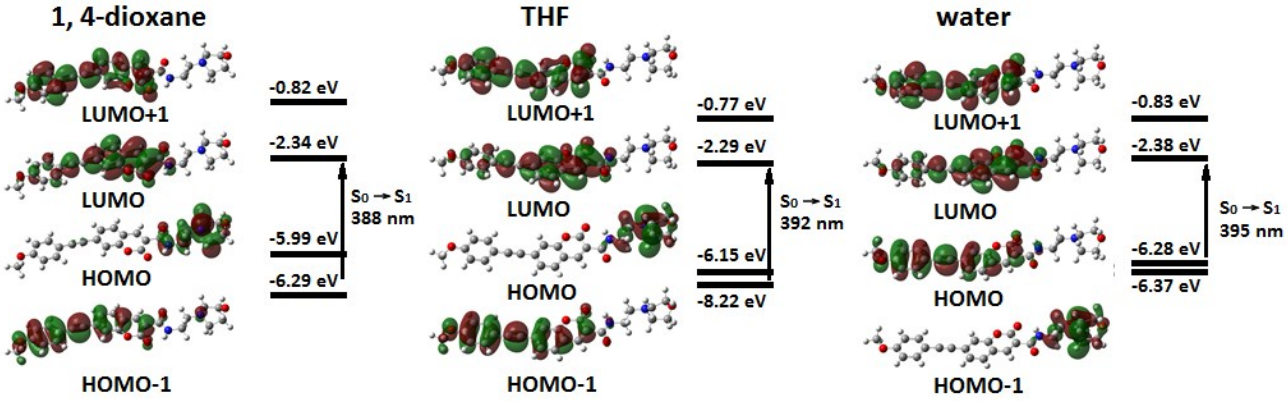


Figure S4. Optimized structures, HOMOs-1, HOMOs, LUMOs and LUMOS+1 of **Lyso-OC** in the ground state for 1, 4-dioxane, THF and water.

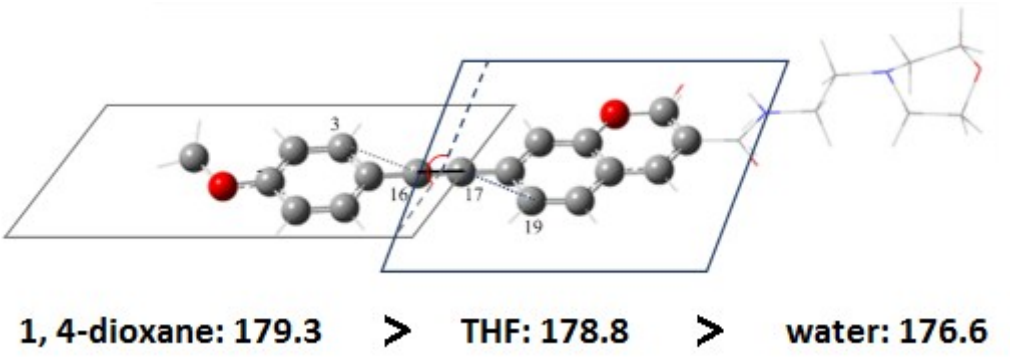


Figure S5. The dihedral angel between 16 and 17 of **Lyso-OC** in 1, 4-dioxane, THF and water.

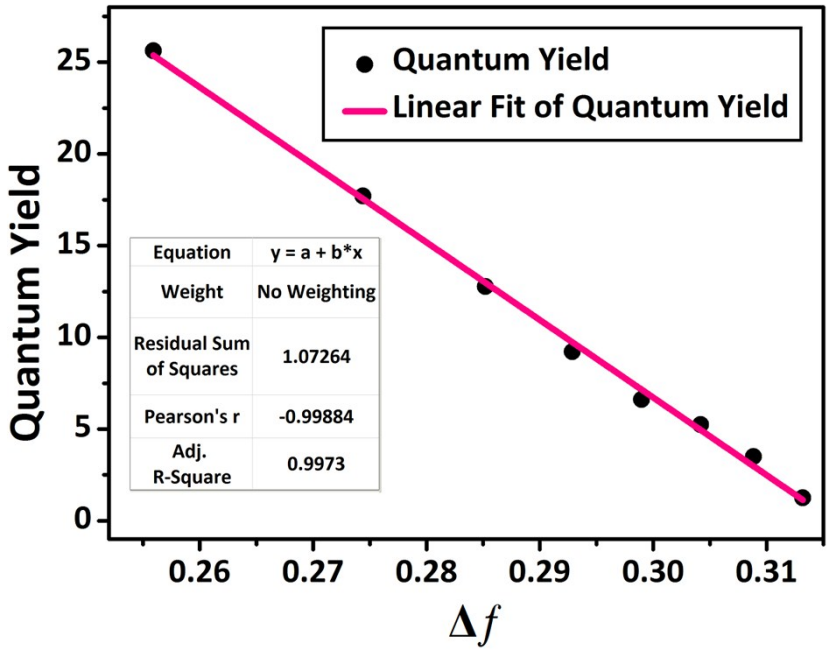


Figure S6. Linearity of quantum yield of **Lyso-OC** (10 μM) versus the solvent parameter Δf.

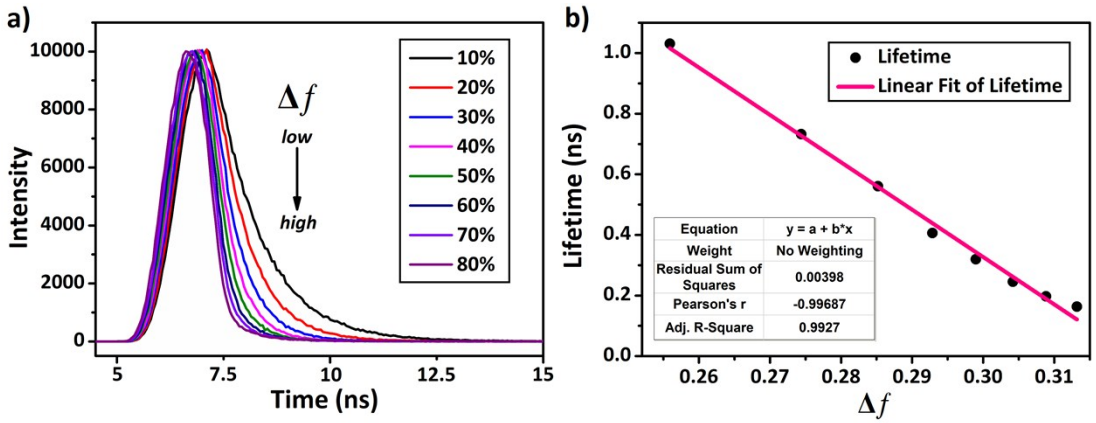


Figure S7. a) Fluorescence lifetimes of **Lyso-OC** (10 μM) in water / THF solvent mixtures (the percentage in the box indicates the water content). b) Linearity of lifetimes versus the solvent parameter Δf. λ_{ex} = 375 nm.

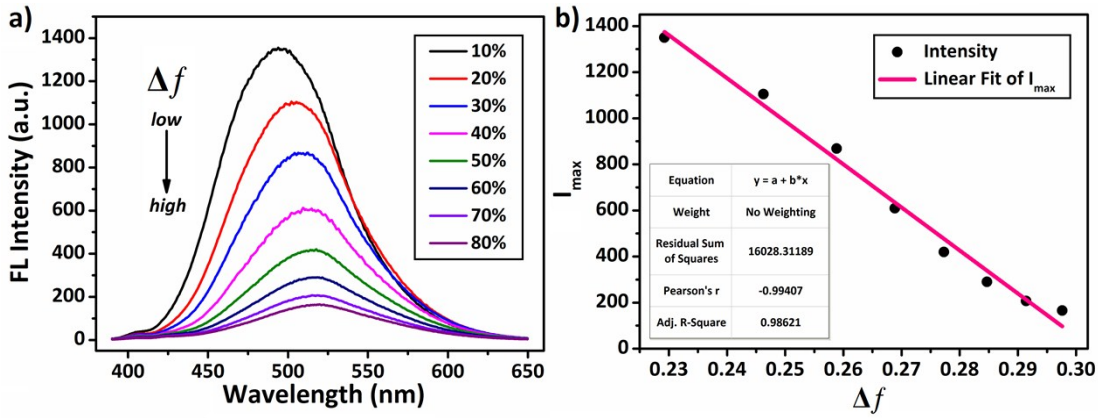


Figure S8. a) Fluorescence emission spectra of **Lyso-OC** (10 μM) in methanol/THF solvent mixtures (the percentage in the box indicates the methanol content). b) Linearity of I_{max} versus the solvent parameter Δf. λ_{ex} = 375 nm.

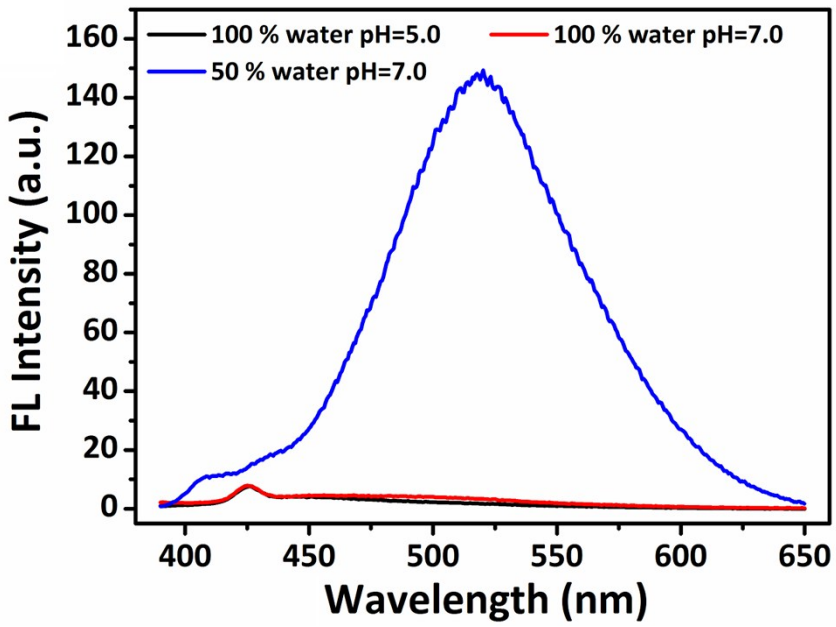


Figure S9. The fluorescence spectra of **Lyso-OC** (10 μM) under different water contents (50% and 100%) and different pH values (5.0 and 7.0). λ_{ex} = 375 nm.

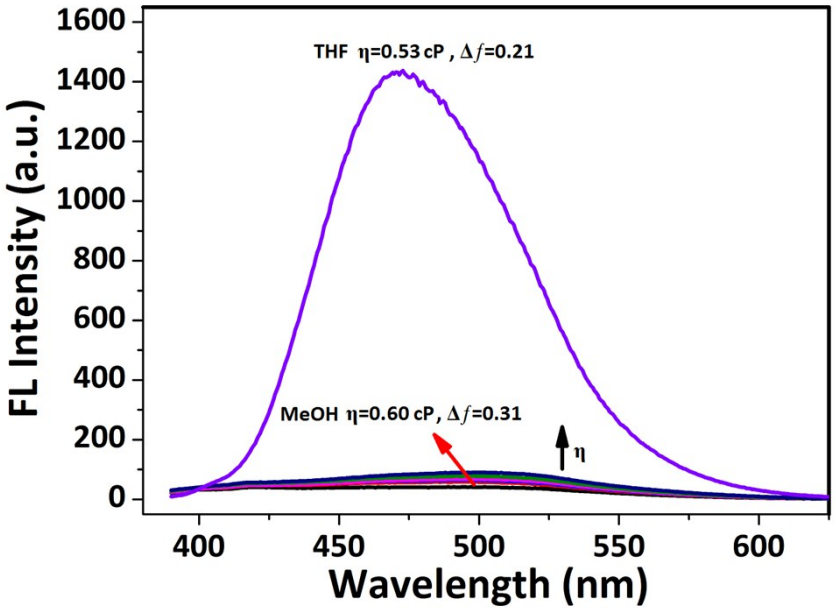


Figure S10. The fluorescence spectra of **Lyso-OC** (10 μM) in methanol-glycerol system under different viscosity. THF and methanol have almost the same viscosity (0.53 cP vs 0.60 cP) but different polarity (0.21 vs 0.31). The fluorescence intensity of **Lyso-OC** displayed huge difference in them. The fluorescence intensity changed little with increasing viscosity from 0.60 cP to about 100 cP.

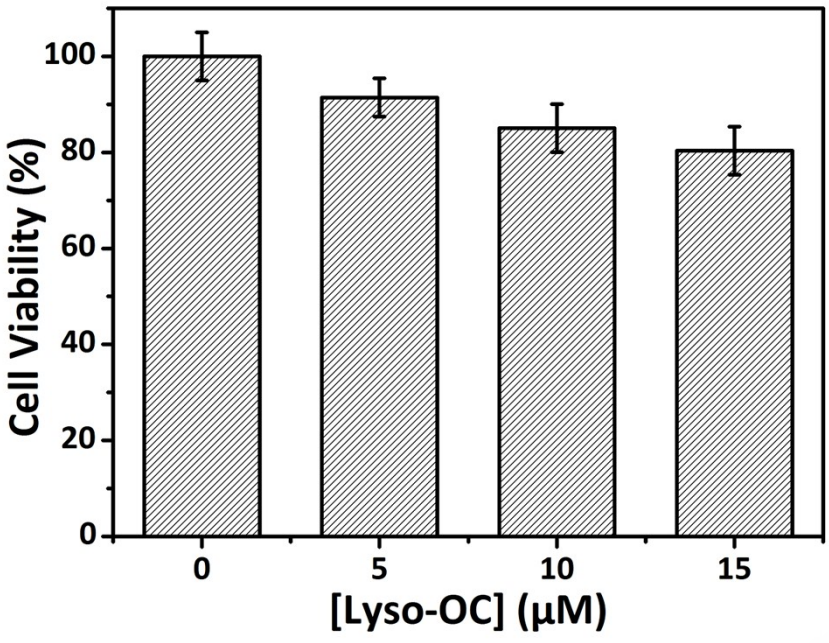


Figure S11. Cytotoxicity of **Lyso-OC** at various concentrations (0 μM, 5.0 μM, 10.0 μM, 15.0 μM) in living MCF-7 cells.

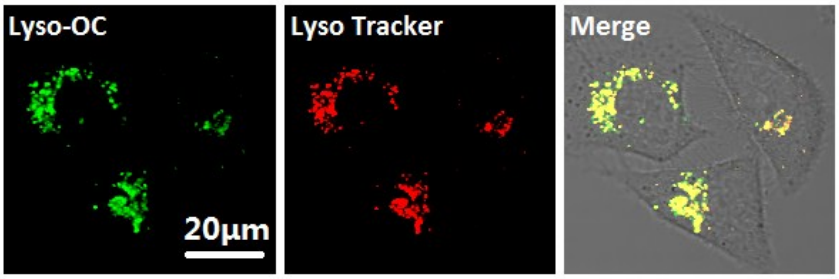


Figure S12. Fluorescence imaging of HeLa cells co-stained with Lyso-OC (10.0 μM) and Lyso Tracker Red (0.5 μM) for 45 min. The scale bar represents 20 μm.

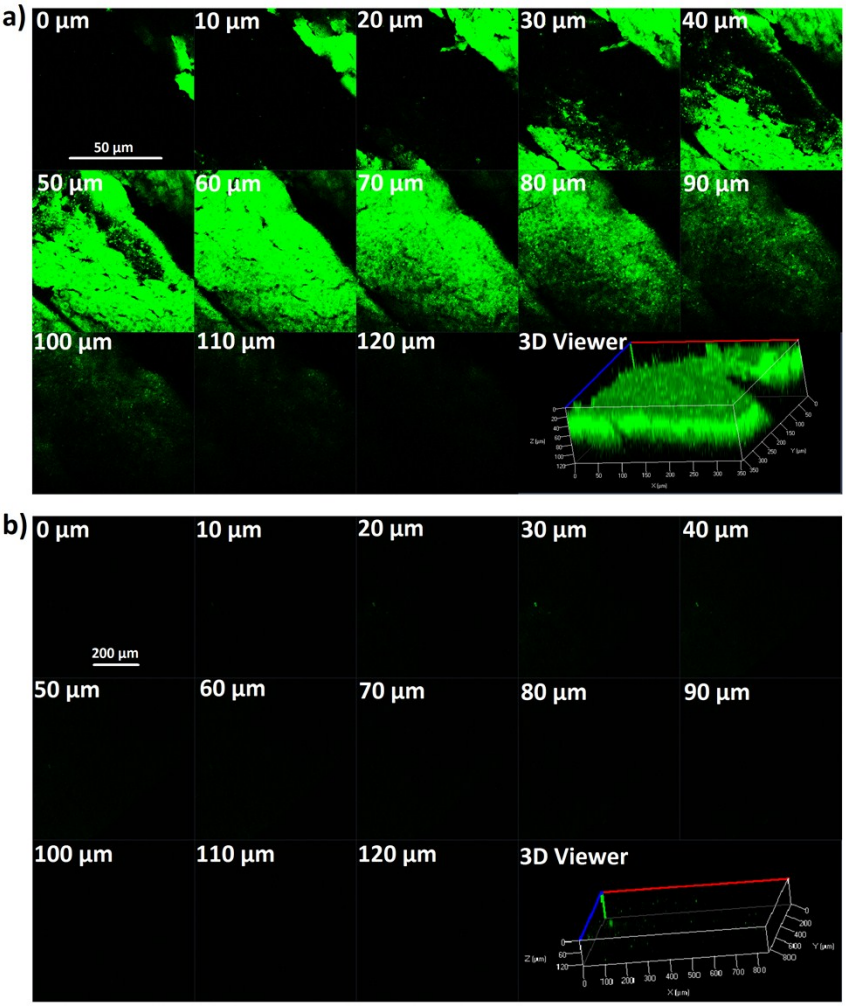


Figure S13. a) Two-photon fluorescence imaging and the 3D viewer of the liver slide staining with **Lyso-OC** (10.0 µM). b) Two-photon fluorescence imaging and the 3D viewer of the liver slide staining without **Lyso-OC**. λ_{ex} = 760 nm, λ_{em} = 490-550 nm.

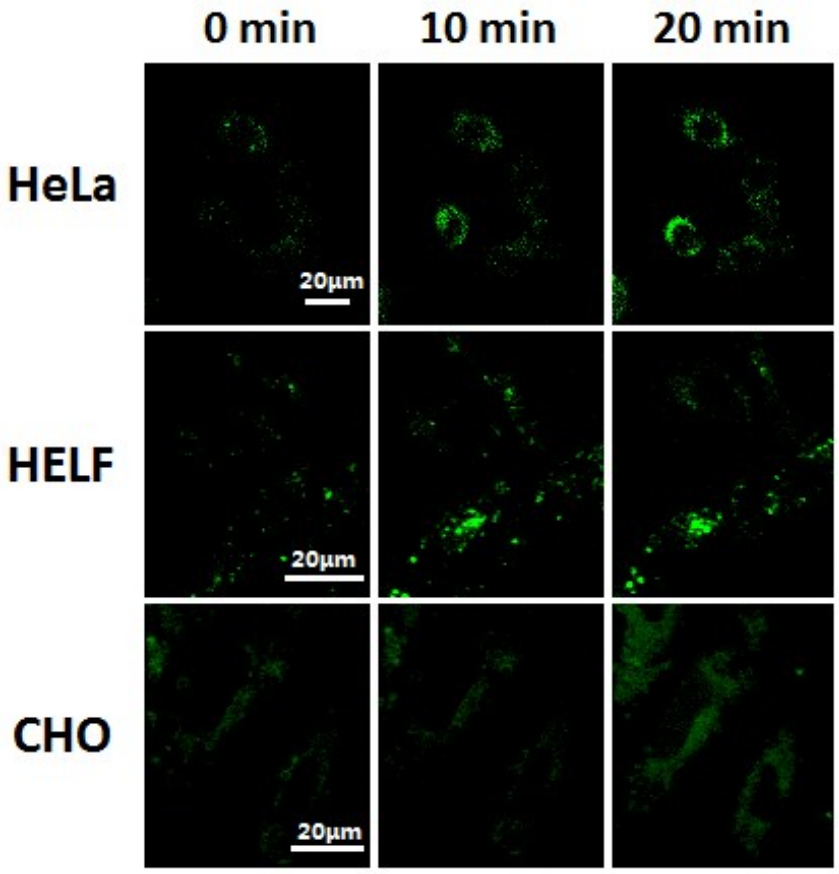


Figure S14. Confocal fluorescence images of HeLa cells, HELF cells and CHO cells. Cells were cultured in DMEM (containing 10% FBS) and stained with **Lyso-OC** (10 μM) for 45 min (λ_{ex} = 760 nm, λ_{em} = 490-550 nm). The stained cells were treated with DMSO (10 μL) for different times. The confocal fluorescence images were recorded. The scale bar represents 20 μm.

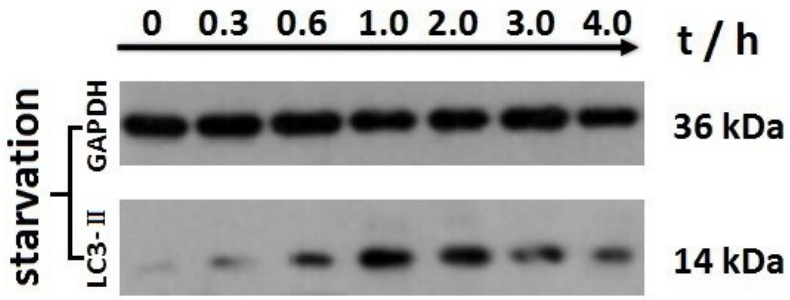


Figure S15. Western blot showing the expression of GAPDH and LC3-II in MCF-7 cells. Cells were incubated for different times in Hank's Balanced Salt Solution (HBSS).

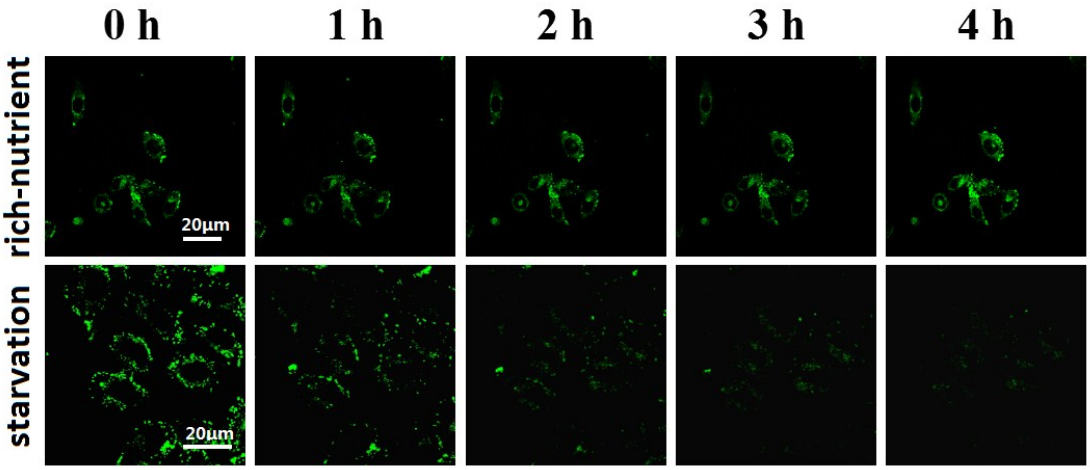


Figure S16. Real-time fluorescence imaging of HeLa cells stained with **Lyso-OC** (10 μM) in rich-nutrient (in control) and starvation (in autophagy), respectively. λ_{ex} = 760 nm, λ_{em} = 490-550 nm. The scale bar represents 20 μm.

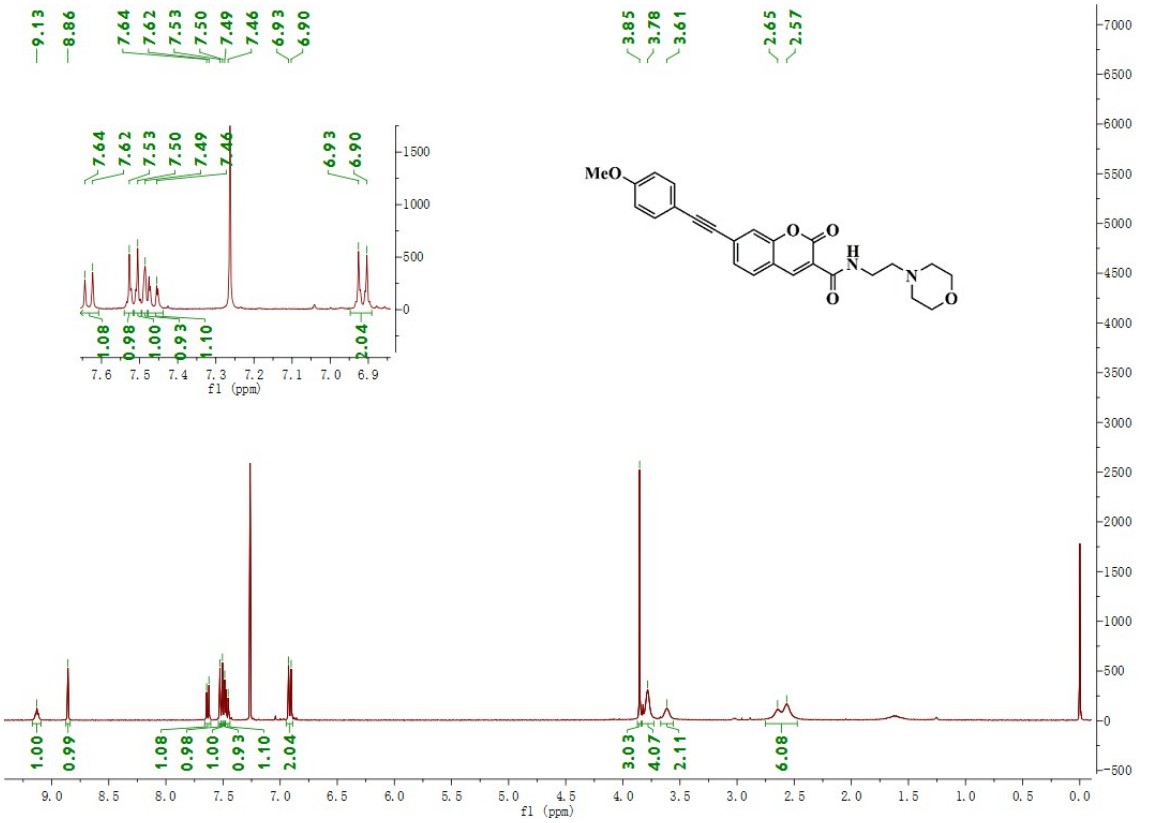


Figure S17. ^{1}H-NMR spectrum of **Lyso-OC** recorded in $CDCl_3$.

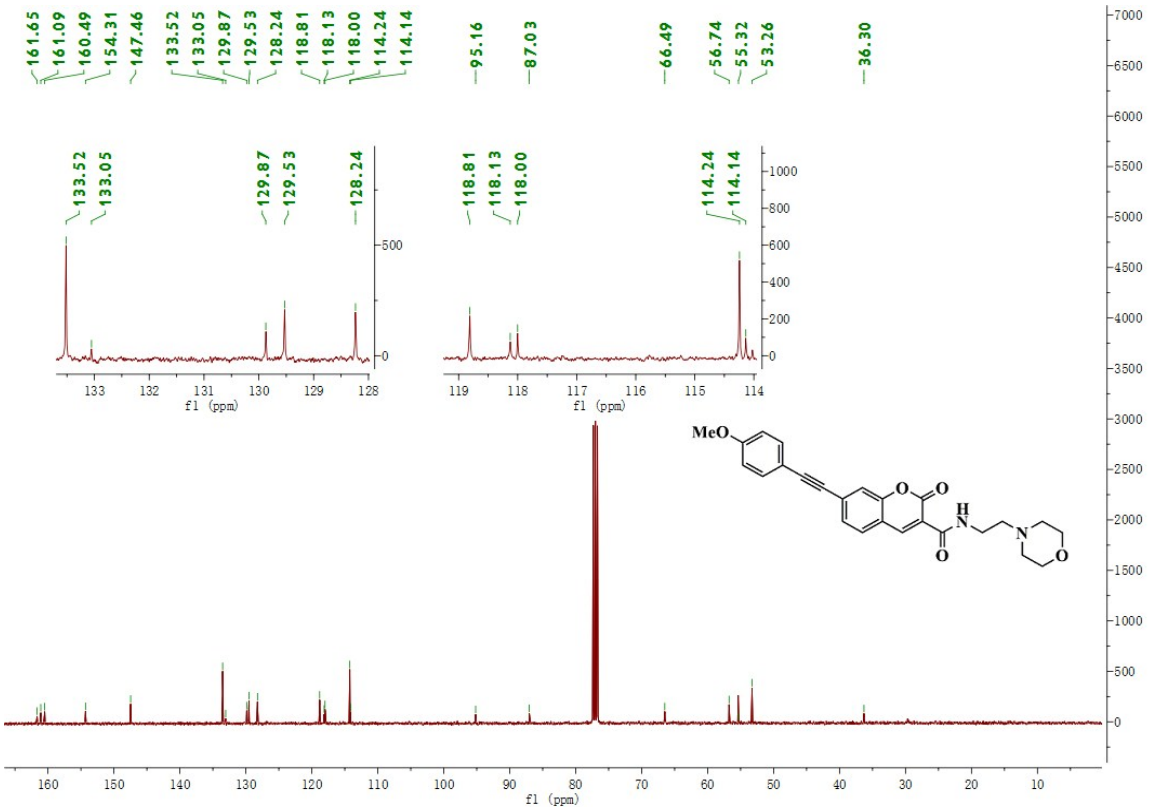


Figure S18. ^{13}C-NMR spectrum of **Lyso-OC** recorded in $CDCl_3$.

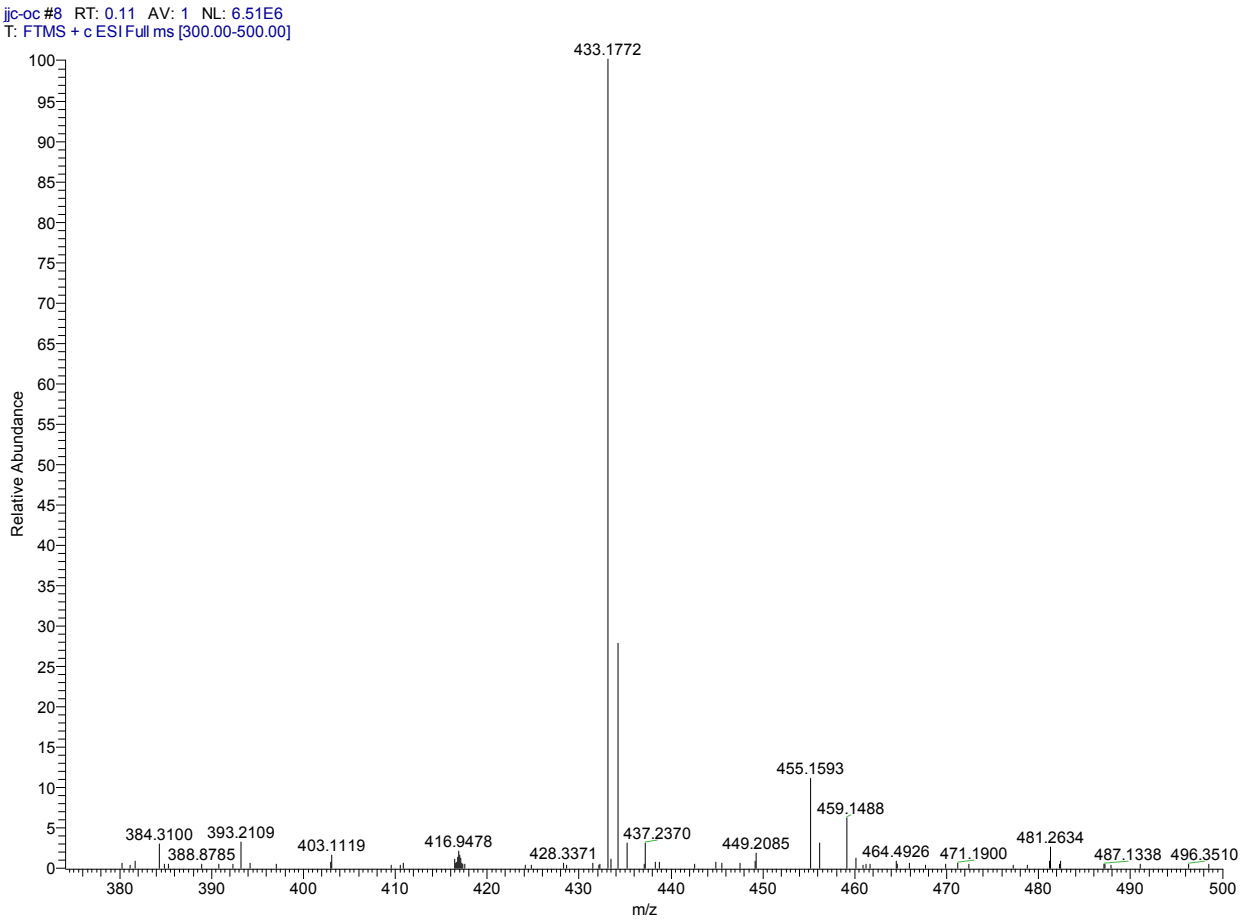


Figure S19. ESI-MS spectrum of **Lyso-OC**.

References

(1) Yin, H.; Zhang, B.; Yu, H.; Feng, Y.; Zhu, M.; Guo, Q.; Meng, X. *J. Org. Chem.* **2015**, *80*, 4306.