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Supplementary Information

A reversible ratiometric two-photon lysosome-targeted probe for real-time monitoring pH changes in living cells

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Contents

1. Materials and general methods

The solution of $MgCl_2$, $CaCl_2$, KCl, $ZnCl_2$, NaF, $NaNO_3$, KI, $Fe(ClO_4)_3$, Na_2SO_3, NaClO, $FeSO_4$, GSH, Cys, Hcys, VC, Na_2S and H_2O_2 were prepared in distilled water with a concentration of 10 mM, respectively.

The pK_a of probe was calculated using the Henderson-Hasselbach-type mass action equation ($pH = pK_a + c*\log[(R - R_{min}) /(R_{max} - R)] + \log (I_a/I_b)$) through analyzing fluorescence intensity ratio changes as a function of pH. Where R_{max} (or R_{min}) is the maximum (or minimum) ratio value, c is the slope and I_a/I_b is the ratio of absorption intensity in acid to the absorption intensity in base at the wavelength chosen for the denominator of R. The pK_a value for **1** was 5.62.

Two-photon absorption cross sections were measured using the two-photon-induced fluorescence measurement technique. The two-photon absorption cross sections (δ) were determined by comparing their two-photon excitation fluorescence (TPEF) to that of fluorescein in different solvents, according to the following equation:

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

In the equation, the subscript ref stands for the reference molecule. δ is the two-photon absorption cross-section value, n is the refractive index of the solution, Φ is the fluorescence quantum yield, c is the concentration of solution, F is the TPEF integral intensities of the solution emitted at the exciting wavelength.

2. Synthesis

Compound **3** and **6** were synthesized according to the literature procedure.

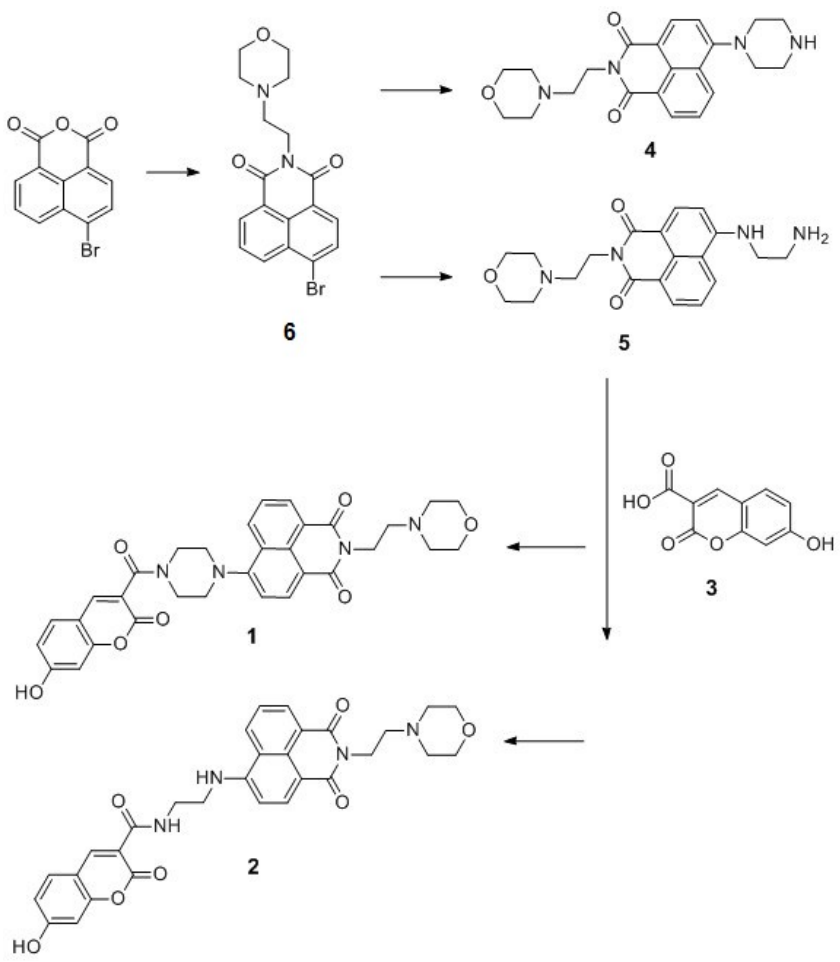
Synthesis of compound **4**. A mixture of compound **6** (2 g, 5.15 mmol) and piperazine (664 mg, 7.7 mmol) in 20 mL ethylene glycol monomethyl ether was refluxed for 10 h under the protection of Ar. After cooled to room temperature, the reaction mixture was concentrated under vacuum, and the crude product was purified by silica column chromatography to afford compound **4** (1.75g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, J = 7.3 Hz, 1H), 8.51 (d, J = 8.0 Hz, 1H), 8.36 (d, J = 8.4 Hz, 1H), 7.75 – 7.68 (m, 1H), 7.26 (d, J = 8.2 Hz, 1H), 4.31 (t, J = 6.9 Hz, 2H), 3.69 – 3.63 (m, 4H), 3.42 (d, J = 2.9 Hz, 6H), 3.17 (q, J = 7.3 Hz, 2H), 2.68 (t, J = 6.9 Hz, 2H), 2.58 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.64, 165.16, 156.45, 133.71, 132.58, 131.17, 127.50, 124.61, 118.85, 116.88, 68.37, 57.50, 55.14, 53.34, 47.66, 46.37, 38.43. ESI-MS m/z [$(M + H)^+$]: 395.20.

Synthesis of compound **5**. Compound **6** (331 mg, 1 mmol) was dissolved in 1,4-dioxane (20 mL) at room temperature. Then 1,2-diaminoethane (ethylenediamine) (10 mmol, 0.7 mL) and triethylamine (5 mmol, 0.7 mL) were added in order. And the solution was refluxed for 12 h, the reaction was completed. The solvent was evaporated under reduced pressure. Purification by neutral alumina column afford compound **5** (147 mg, 40%). ^{1}H NMR (400 MHz, DMSO-D6) δ 8.70 – 8.63 (m, 1H), 8.40 – 8.35 (m, 2H), 8.20 (d, J = 8.7 Hz, 2H), 7.63 (t, J = 8.0 Hz, 1H), 6.76 (d, J = 8.9 Hz, 1H), 4.10 (t, J = 7.6 Hz, 5H), 3.36 (d, J = 7.1 Hz, 8H), 2.87 (t, J = 6.7 Hz, 3H), 2.50 (d, J = 6.8 Hz, 8H). ^{13}C NMR (100 MHz, DMSO-D6) δ 165.53, 164.65, 152.54, 152.33, 135.95, 132.40, 131.14, 123.50, 121.92, 109.45, 105.60, 68.07, 57.60, 55.27, 47.15, 39.23, 38.25. ESI-MS m/z [$(M + H)^+$]: 369.19.

Synthesis of probe **2**. Compound **3** (380 mg, 1 mmol) and HBTU (569 mg, 1.5 mmol) in DMF (50 mL), then compound **5** (316 mg, 0.86 mmol) and DIPA (0.21 mL, 1.5 mmol) were added. After the reaction mixture was stirred for 7 h at room

temperature, then evaporated under reduced pressure. Purification by neutral alumina column afford probe **2** (162 mg, 34%). ^{1}H NMR (400 MHz, DMSO-D6) δ 8.92 (s, 1H), 8.64 (d, J = 8.1 Hz, 1H), 8.41 (d, J = 6.8 Hz, 1H), 8.27 – 8.18 (m, 1H), 7.97 (s, 1H), 7.71 – 7.61 (m, 1H), 7.12 (d, J = 9.1 Hz, 1H), 6.88 (d, J = 8.6 Hz, 1H), 6.03 (dd, J = 9.0, 1.9 Hz, 1H), 5.72 (s, 1H), 4.21 – 4.05 (m, 2H), 3.55 – 3.46 (m, 4H), 3.13 (d, J = 11.9 Hz,4H), 2.57 – 2.47 (m, 2H), 2.43 (d, J = 4.7 Hz, 4H). ESI-MS m/z [$(M + H)^{+}$]: 557.22.

3. Scheme caption and spectra



Scheme S1. Synthesis routes of **1**, **2** and **4**, **5**.

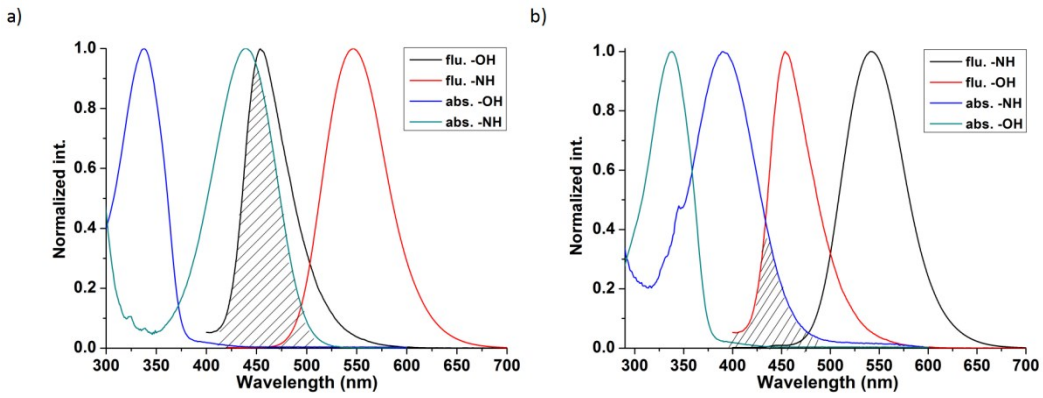


Figure S1. Normalized absorption spectra (abs) and fluorescence spectra (flu) of 5 μM **3** and 5 μM a) **4** or b) **5** at pH 5.0 in B−F buffers. The gray area represents the overlap between the fluorescence spectrum of **3** and the absorption spectrum of a) **4** or

b) **5**.

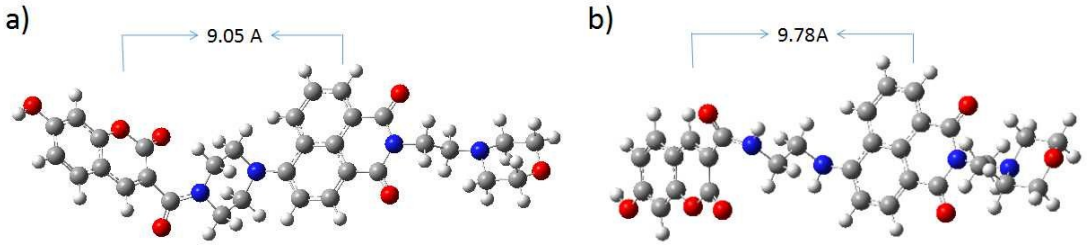


Figure S2. The distance of a) **1**, b) **2** between the two part fluorophores coumarin and naphthalimide.

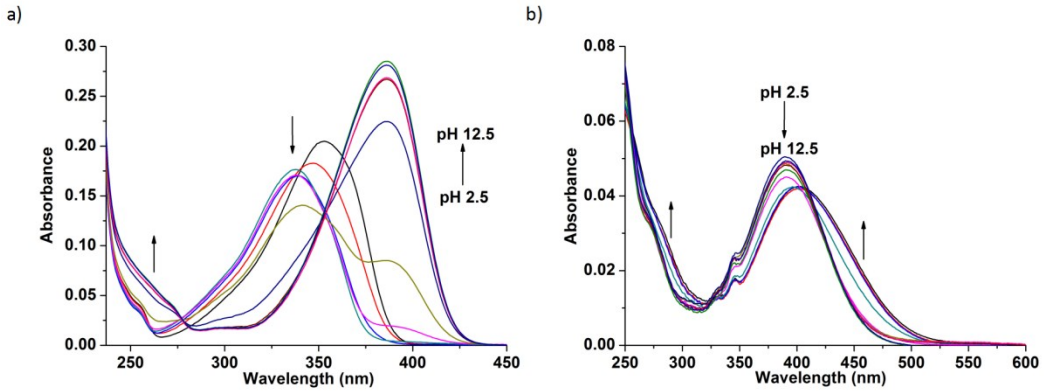


Figure S3. Absorption spectra of 5 μM a) **3**, b) **4** under various pH (2.5-12.5) in B-F buffer solution.

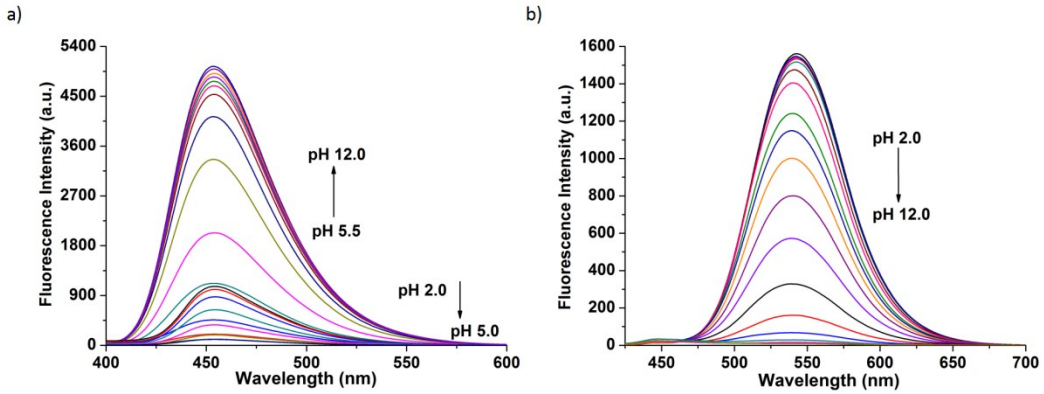


Figure S4. Fluorescence spectra of 5 μM a) **3** (λ_{ex} = 380 nm, slit: 1.0 nm/2.5 nm), b) **4** (λ_{ex} =380 nm, slit: 2.5 nm/5.0 nm) at various pH (2.0-12.0) in B-F buffer solution.

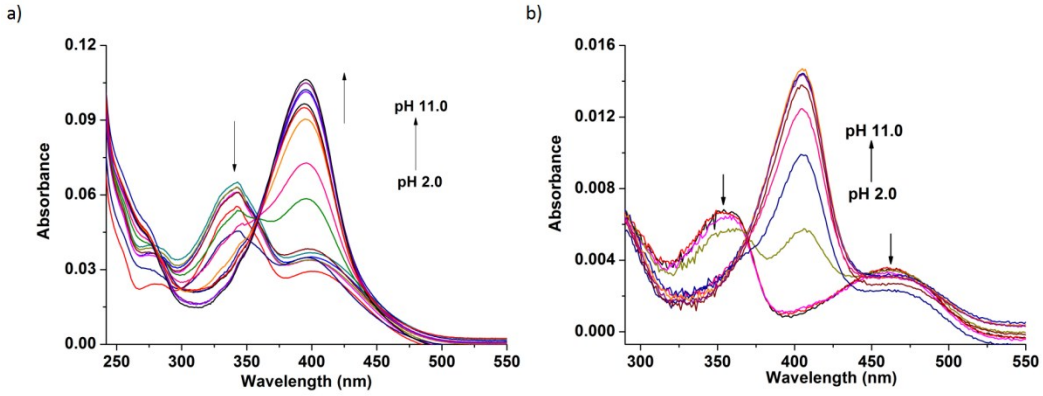


Figure S5. Absorption spectra of 5 μM a) **1**, b) **2** under various pH (2.0-11.0) in B-F buffer solution.

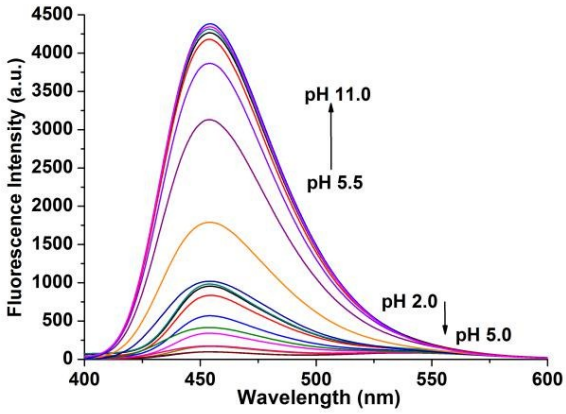


Figure S6. Fluorescence spectra of the mixture of 5 μM **3** and 5 μM **4** at various pH in B-F buffers (λ_{ex} = 380 nm, slit: 1.0 nm/2.5 nm).

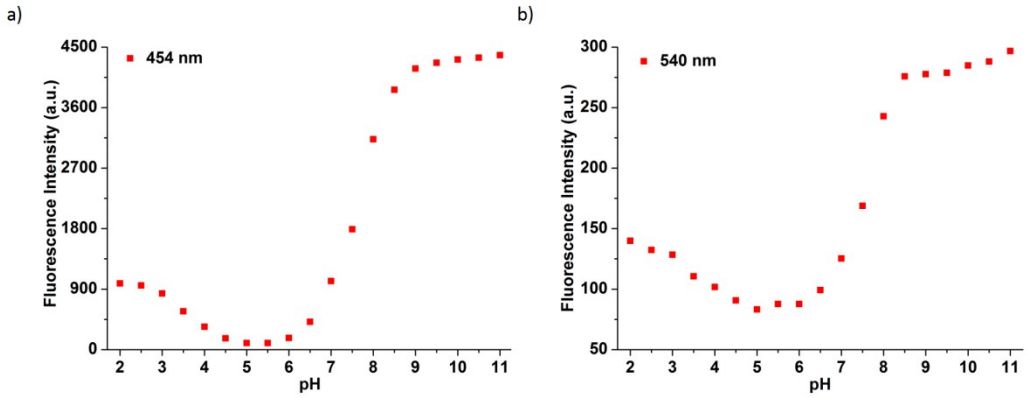


Figure S7. Fluorescence intensity at a) 454 nm and b) 540 nm of the mixture of 5 μM

3 and 5 μM **4** at various pH in B-F buffers (λ_{ex} = 380 nm, slit: 1.0 nm/2.5 nm).

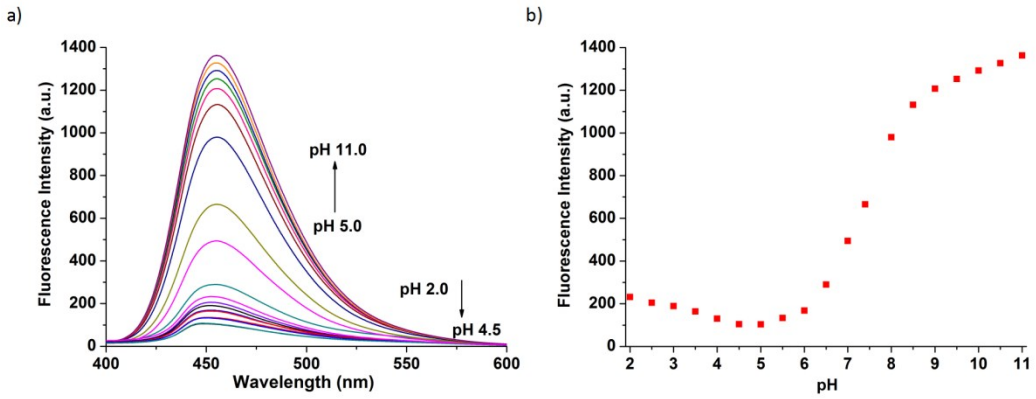


Figure S8. a) Fluorescence spectra of 5 μM **2** in B−F buffers at various pH values (λ_{ex} = 380 nm, slit: 5.0 nm/ 5.0 nm). b) The plot of the fluorescence intensity with changing pH values.

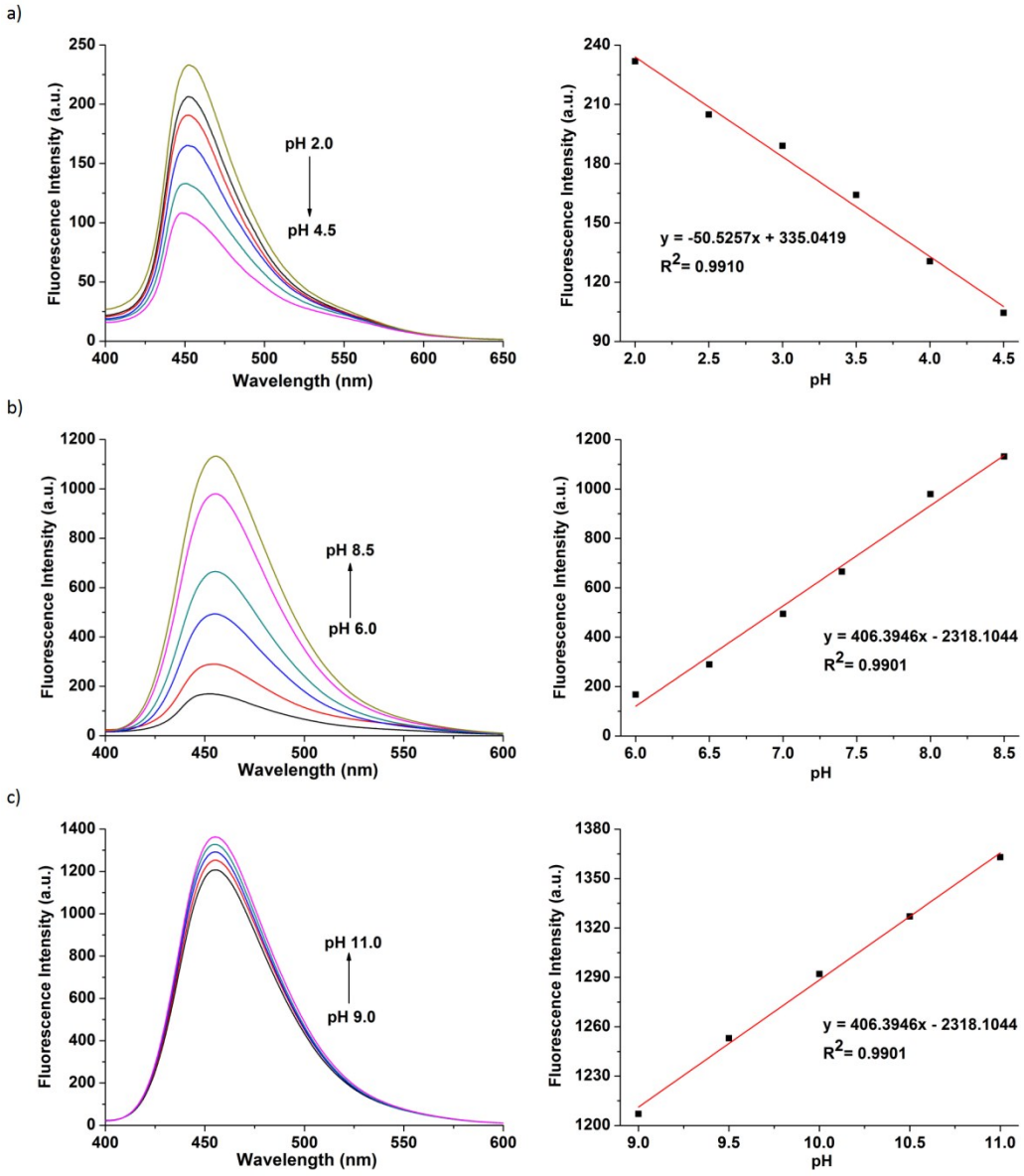


Figure S9. Fluorescence spectra of 5 μM **2** and the linear plots of emission intensity ratio to pH a) 2.0-4.5, b) 6.0-8.5, c) 9.0-11.0.

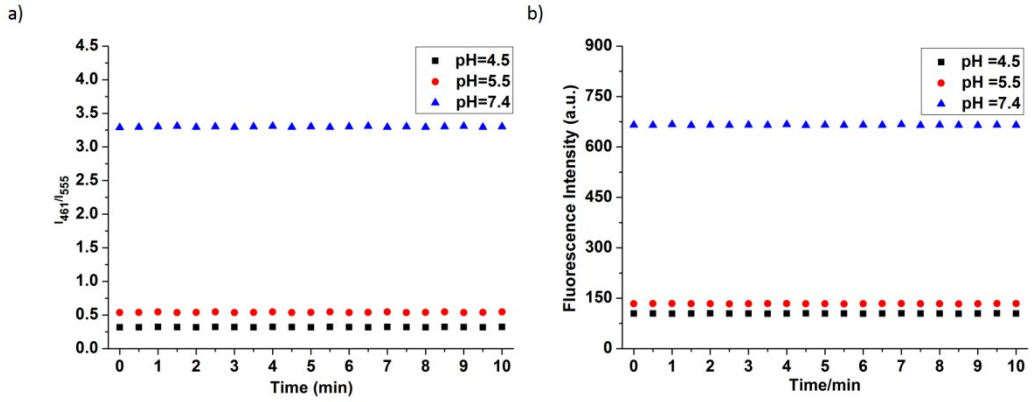


Figure S10. Time courses of a) the fluorescence emission ratios I_{461}/I_{555} of 5 μM **1** and b) intensity of 5 μM **2** in B−F buffers at pH 4.5, 5.5 and 7.4.

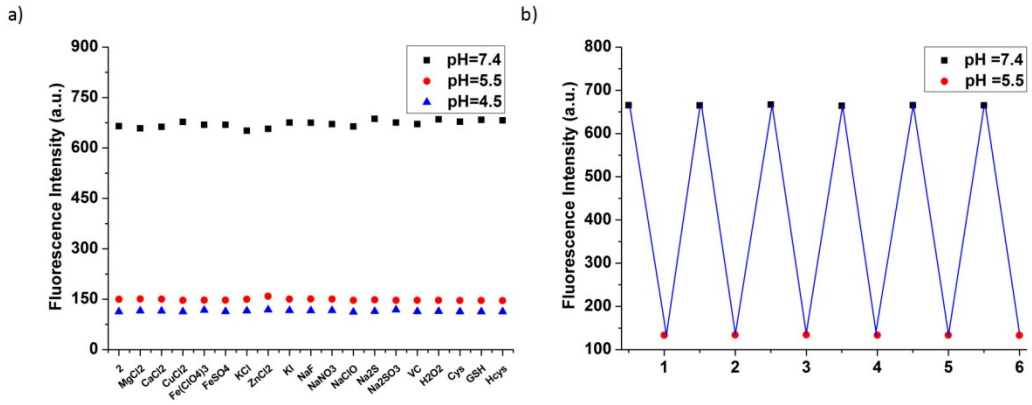


Figure S11. a) The fluorescence intensity of 5 μM **2** by adding various species (25 μM) at pH 4.5, 5.5 and 7.4. b) pH reversibility study of 5 μM **2** between pH 5.5 and 7.4.

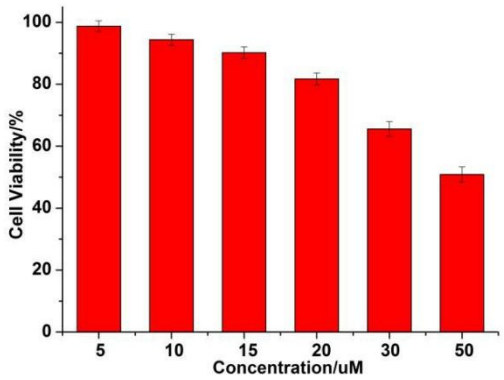


Figure S12. MTT assay to determine **1** on HeLa cells. Statistically significant values derived by ANOVA are indicated by bar marks.

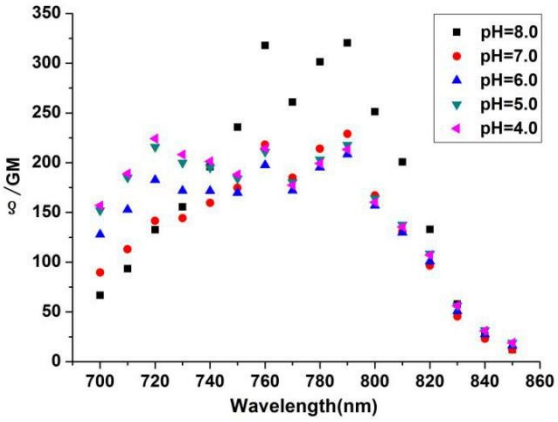


Figure S13. Two-photon cross sections of **1** at various pH (4.0-8.0) under different excitation wavelength.

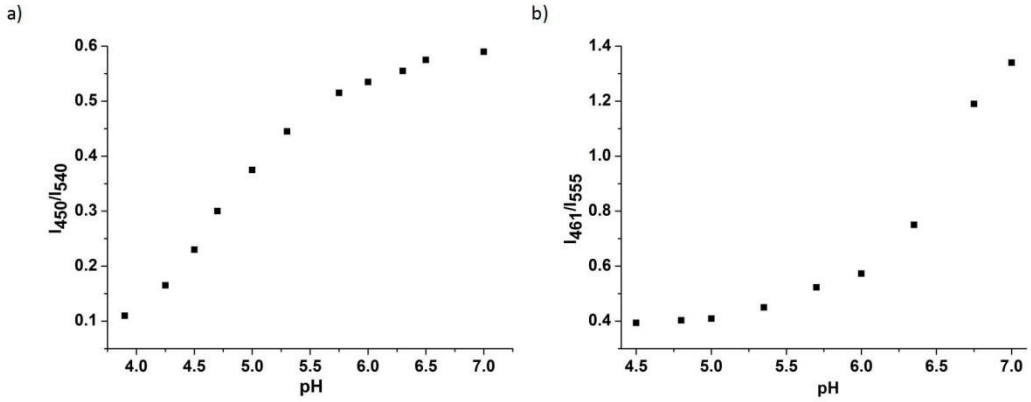


Figure S14. Calibration of the pH response of the a) LysoSensor dye, LSBY TBD160,

b) **1** in a cell lysate background, showing the fit of the ratio of emission bands with pH.

4. TP Bioimaging

4.1. Cell cytotoxicity

The cytotoxic effect of **1** was determined by an MTT assay following the manufacturer instruction (Sigma-Aldrich, MO). HeLa cells were initially propagated in a 25 cm^2 tissue culture flask in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 μg/mL), and streptomycin (100 μg/mL) in a CO_2 incubator. For cytotoxicity assay, cells were seeded into 96-well plates (approximately 104 cells per well), and various concentrations of compound **1** (5, 10, 20, 30, and 50 μM) made in DMEM were added to the cells and incubated for 24 h. Solvent control samples (cells treated with DMSO). Following incubation, the growth media was removed, and fresh DMEM containing MTT solution was added. The plate was incubated for 2−3 h at 37 °C. Subsequently, the supernatant was removed, the insoluble colored formazan product was solubilized in DMSO, and its absorbance was measured in a microtiter plate reader (Infinite M200, TECAN, Switzerland) with an excitation wavelength at 550 nm.

4.2. Cell culture and colocalization experiment

HeLa cells were procured from the biomedical engineering center of Lanzhou University (Lanzhou, China). The cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 μg/mL), and streptomycin (100 μg/mL). Cells were maintained under a humidified atmosphere of 5% CO_2 and at 37 °C incubator as mentioned before. For cell imaging studies, cells were seeded into a confocal dish and incubated at 37 °C in a CO_2 incubator for one day. Then the cells were washed three times with PBS buffer (pH 7.4) and divided into several groups. For group one cells were washed three times with phosphate buffered saline (pH 7.4) and incubated with 5 μM probe in DMEM at 37 °C for 60 minutes in a CO_2 incubator and observed under Olympus FV1000 laser confocal microscope IX81.

To confirm the probe's subcellular localization, acidic organelle specific LysoTracker Red (100 nM) were used to co-stain cells with **1**. Cells internalized with 5 μM **1** for 30 min were washed with fresh medium. LysoTracker Red was then added. Cells were continuously incubated for an additional 30 min for observation of colocalization of **1** with LyoTracker Red. LysoTracker Red was excited at 570 nm and its red emission was collected using a 580/600 nm filter set.

4.3. Fluorescence imaging for cells

Two-photon fluorescence images of dye labeled cells and tissues were obtained by exciting the probes with a modelocked titanium-sapphire laser source (Mai Tai DeepSee, 80 MHz, 90 fs) set at wavelength 760 nm with Olympus FV1000 laser confocal microscope IX81 with 60 objective, numerical aperture (NA)=0.7, and ZOOM *2. The images signals at 425-500 and 525-600 nm range were collected by internal PMTs in 12 bit unsigned 800*800 pixels at 40 Hz scan speed.

4.4. The experiment of pK_a calibration

In order to correlate the observed time-dependent ratiometric changes with pH following nigericin treatment, a cell lysate background was used (0.7 mL). To this suspension was added the probe **1** or the LysoSensor probe, as above, and 2 μM of nigericin was also added. The pH was adjusted to 3.9 and increased incrementally up to 7.0 (pH meter) by addition of NaOH aqueous solution. Then the intensity ratio variation with pH was plotted. Using the LysoSensor probe, an apparent pK_a of 4.74 was determined, by an iterative least squares fitting algorithm operating in Origin. Parallel experiments with probe **1** in the cell lysate gave an apparent pK_a of 5.96.

5. NMR and ESI-mass Data

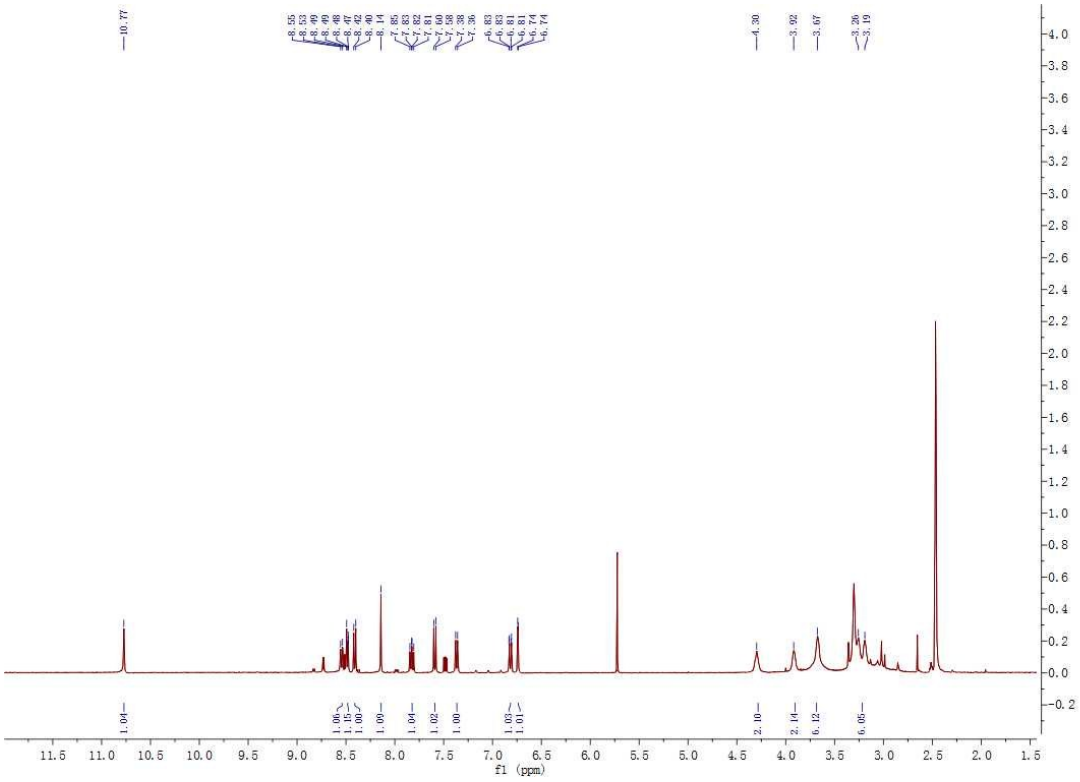


Figure S15. ^{1}H NMR spectrum of **1** (DMSO-D6).

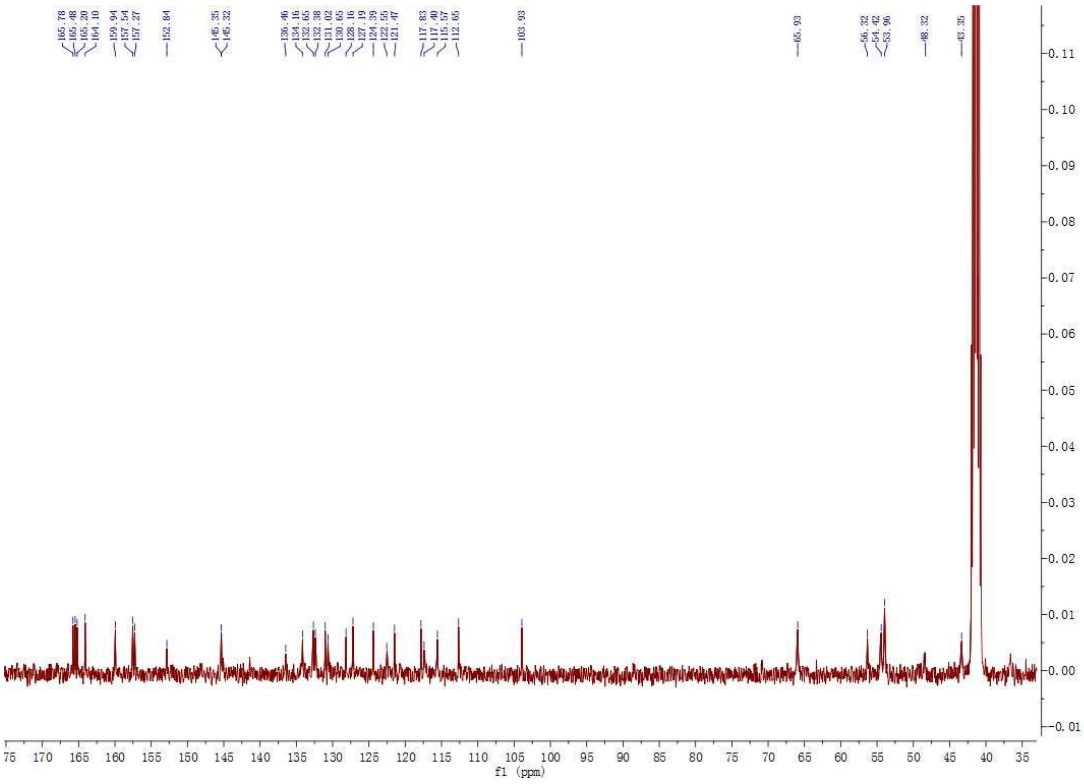


Figure S16. ^{13}C NMR spectrum of **1** (DMSO-D6).

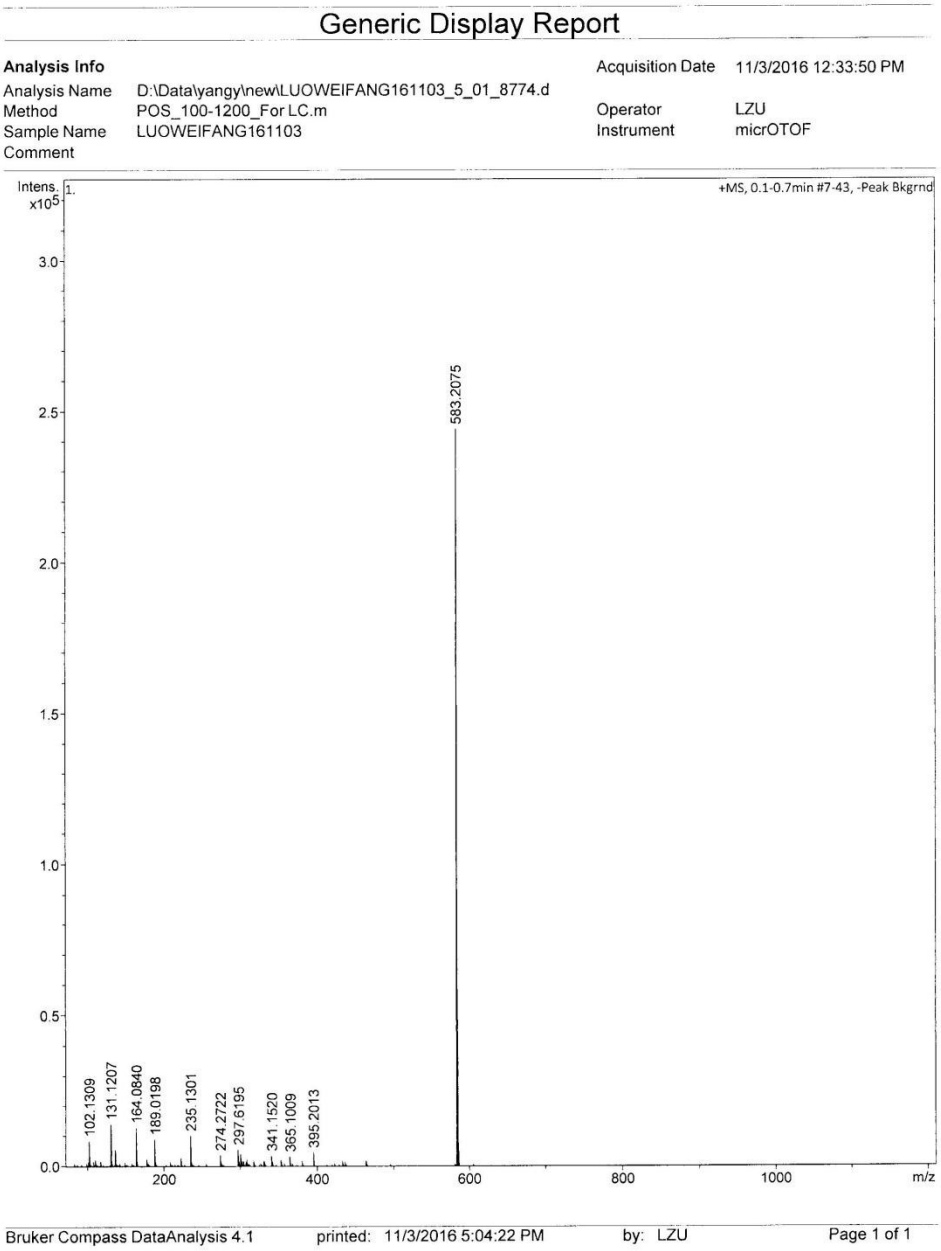


Figure S17. ESI mass spectrum of **1**.

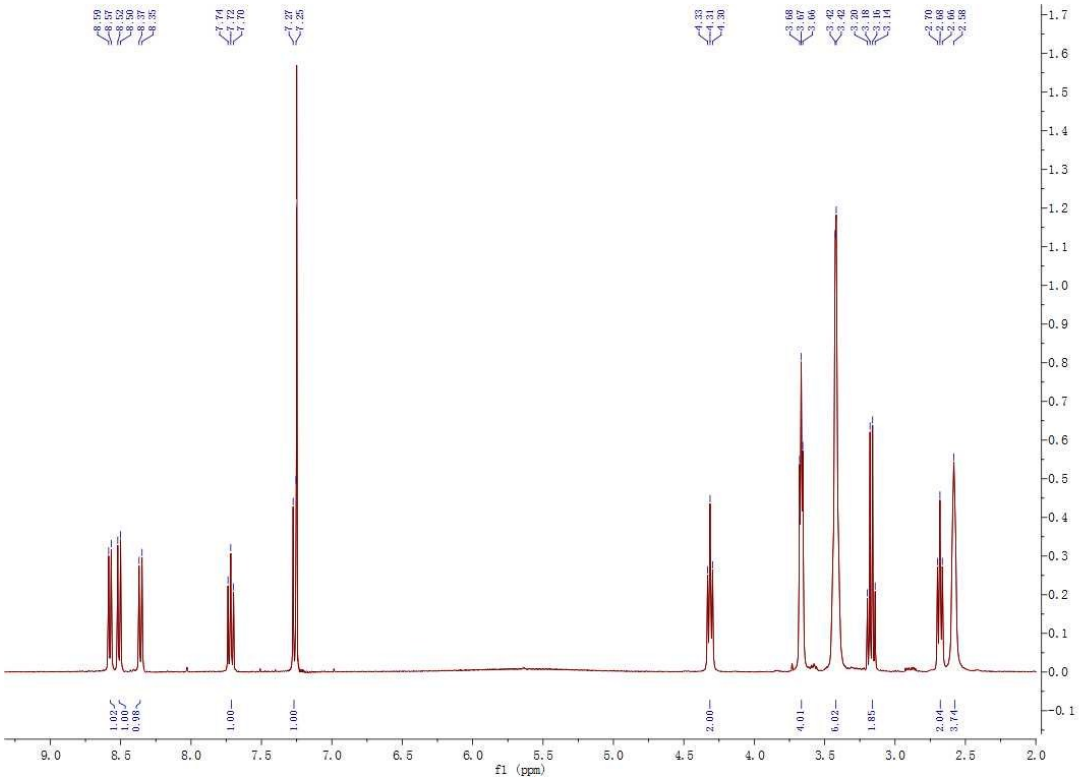


Figure S18. ^{1}H NMR spectrum of **4** ($CDCl_3$).

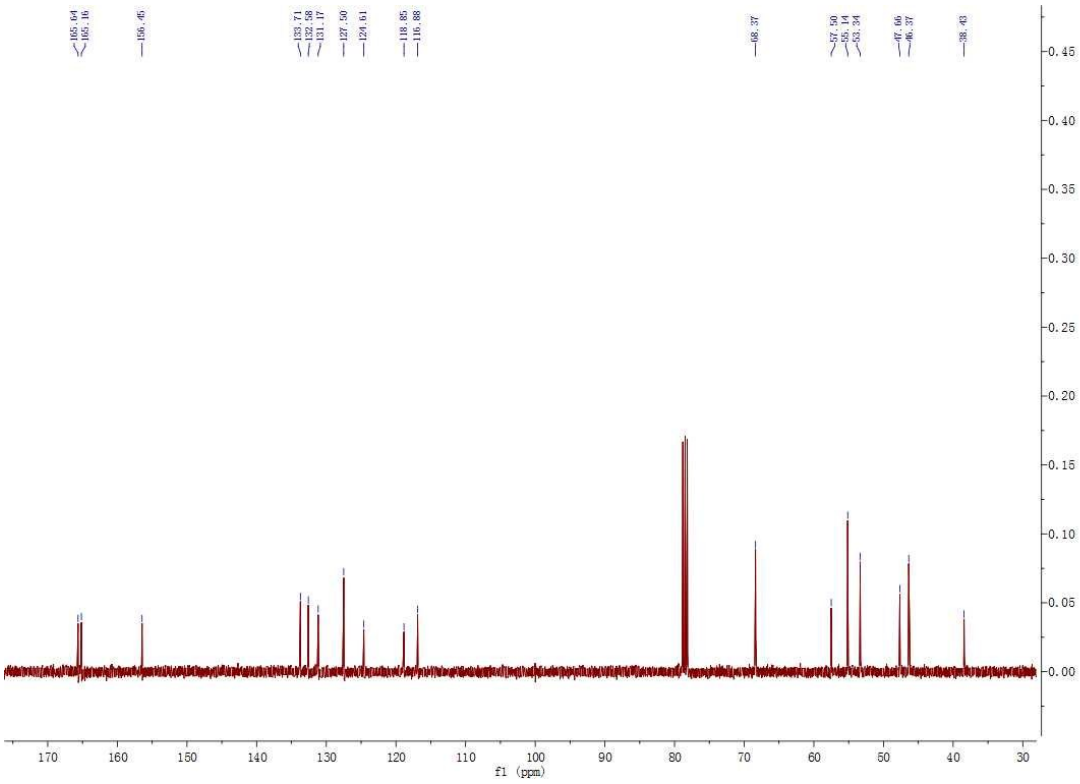


Figure S19. ^{13}C NMR spectrum of **4** ($CDCl_3$).

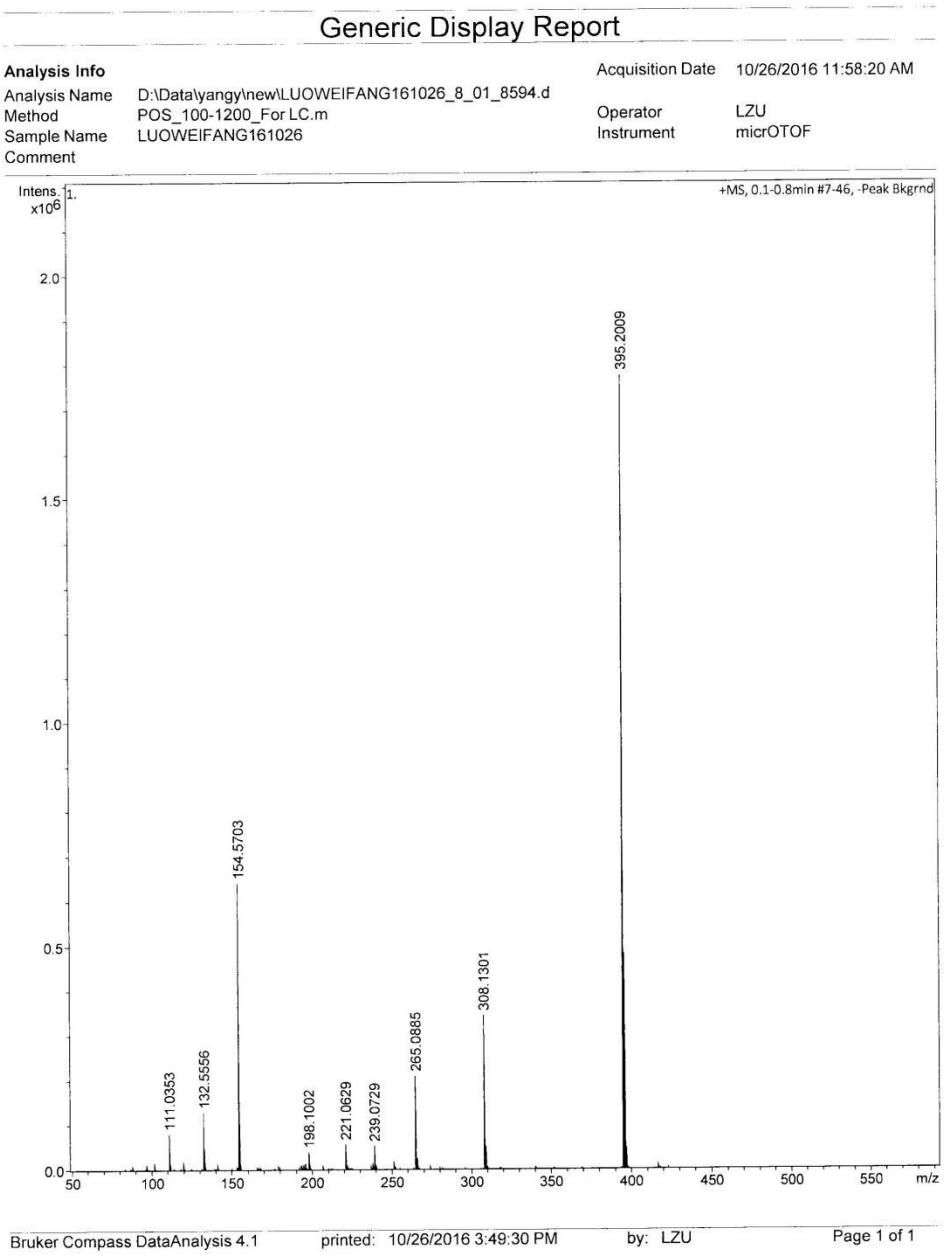


Figure S20. ESI mass spectrum of **4**.

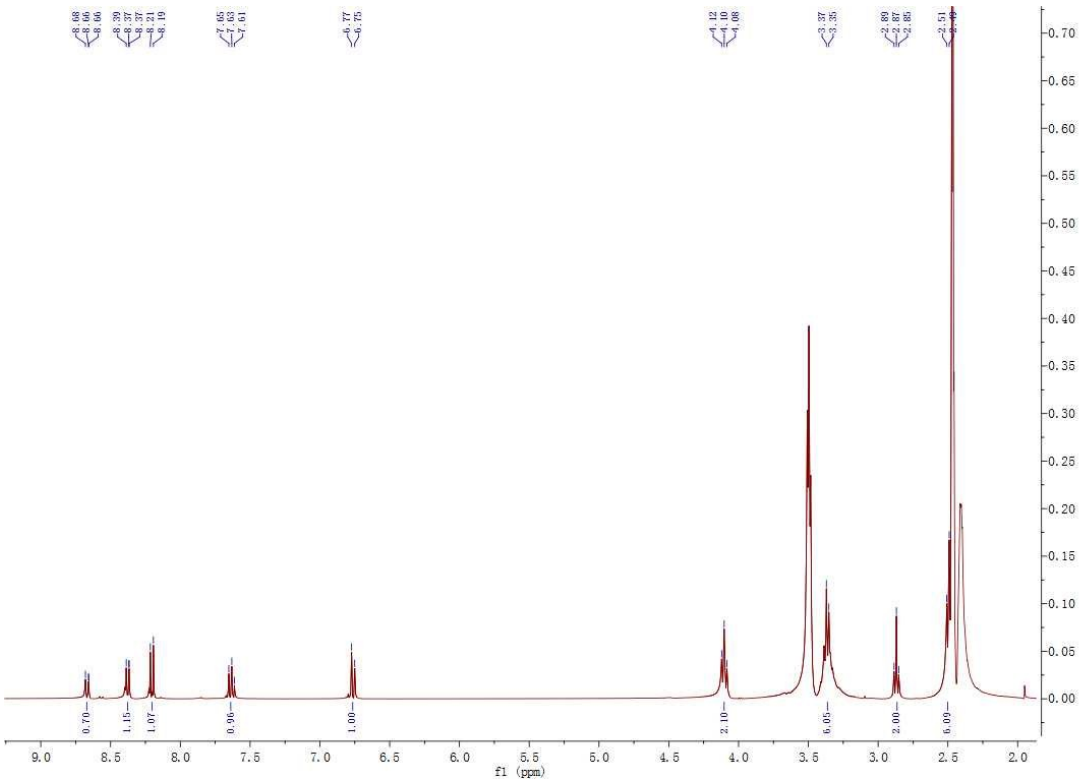


Figure S21. ^{1}H NMR spectrum of **5** (DMSO-D6).

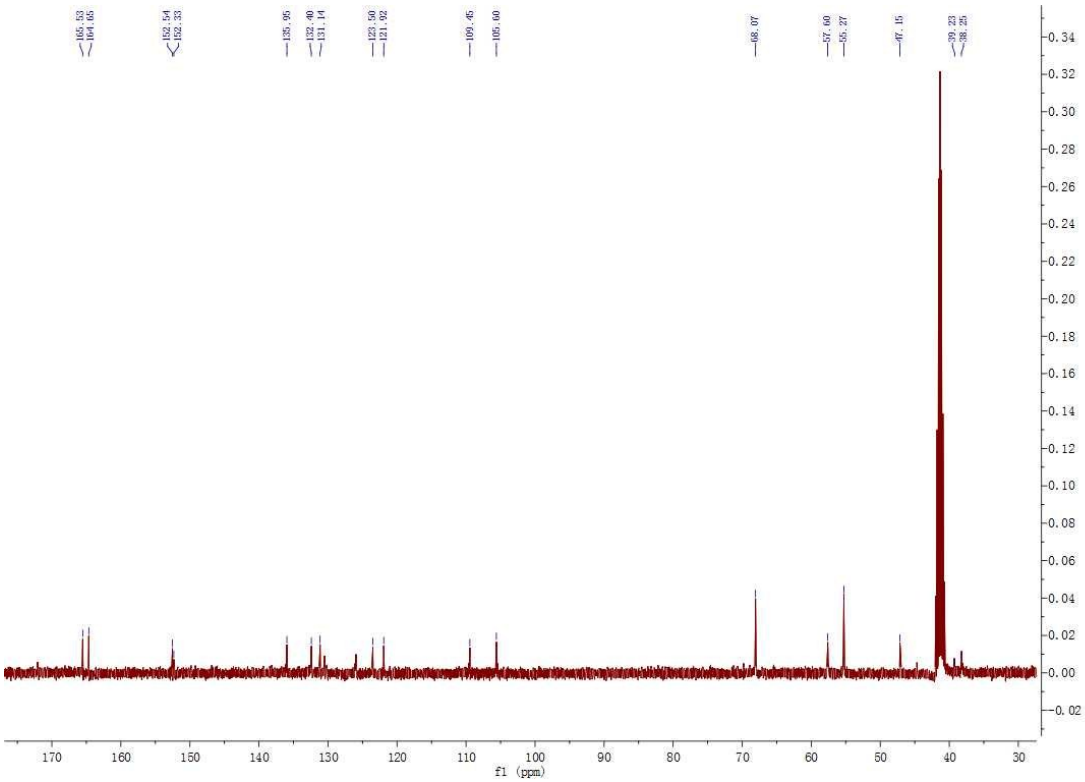


Figure S22. ^{13}C NMR spectrum of **5** (DMSO-D6).

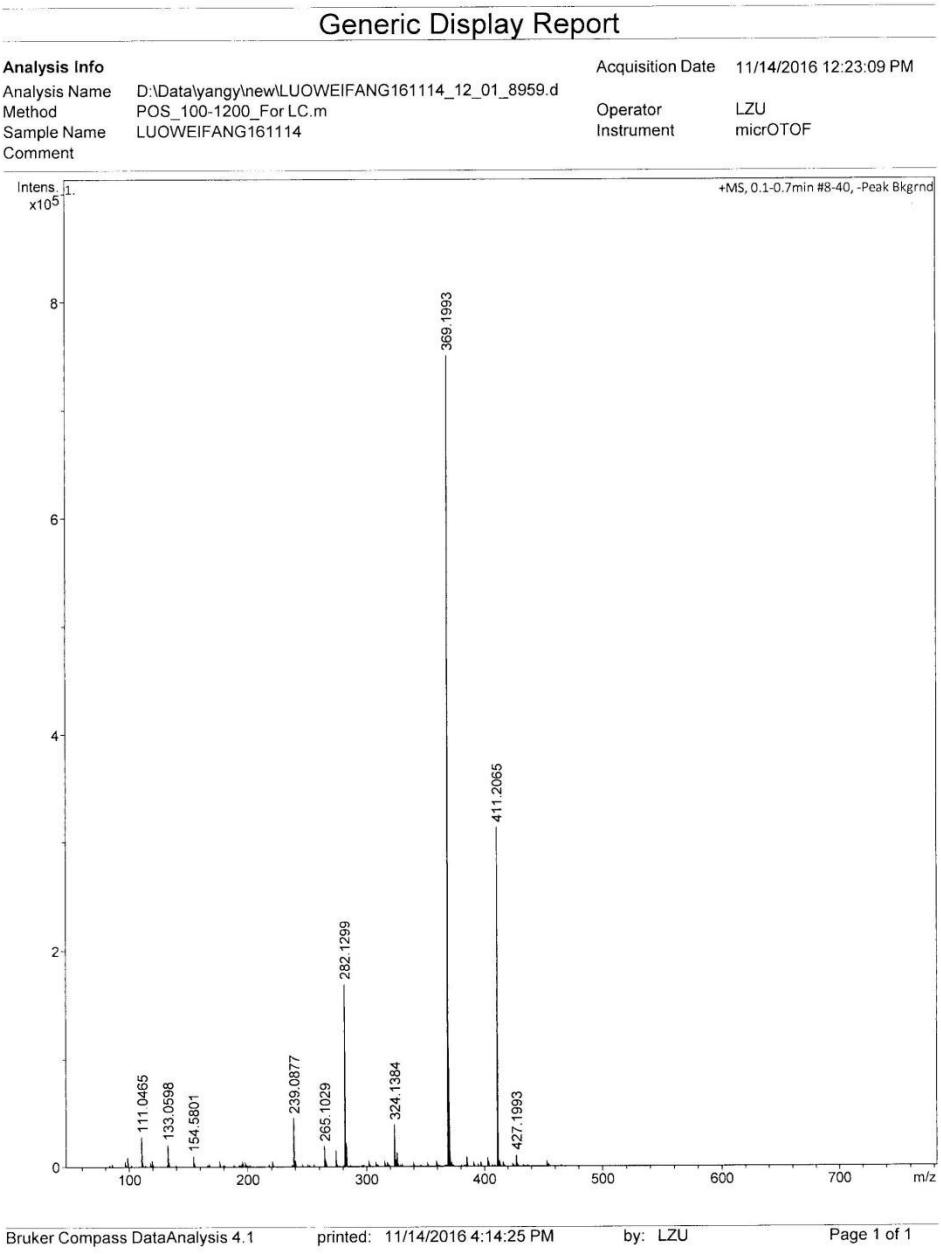


Figure S23. ESI mass spectrum of **5**.

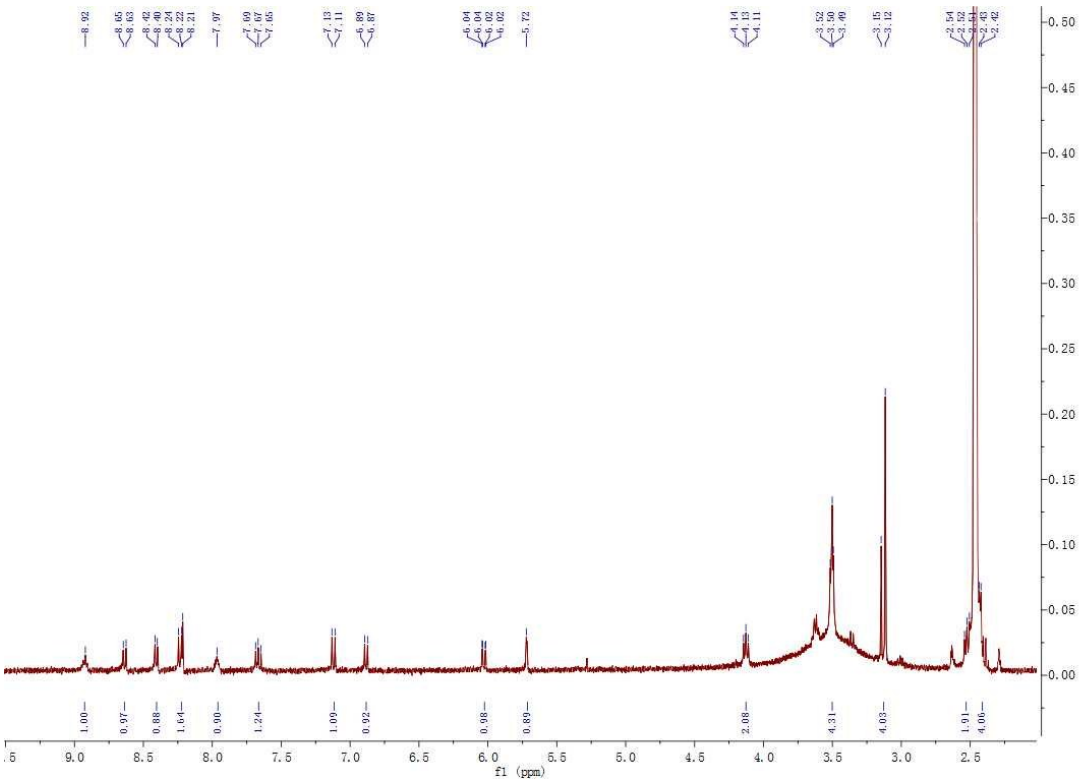


Figure S24. ^{1}H NMR spectrum of **2** (DMSO-D6).

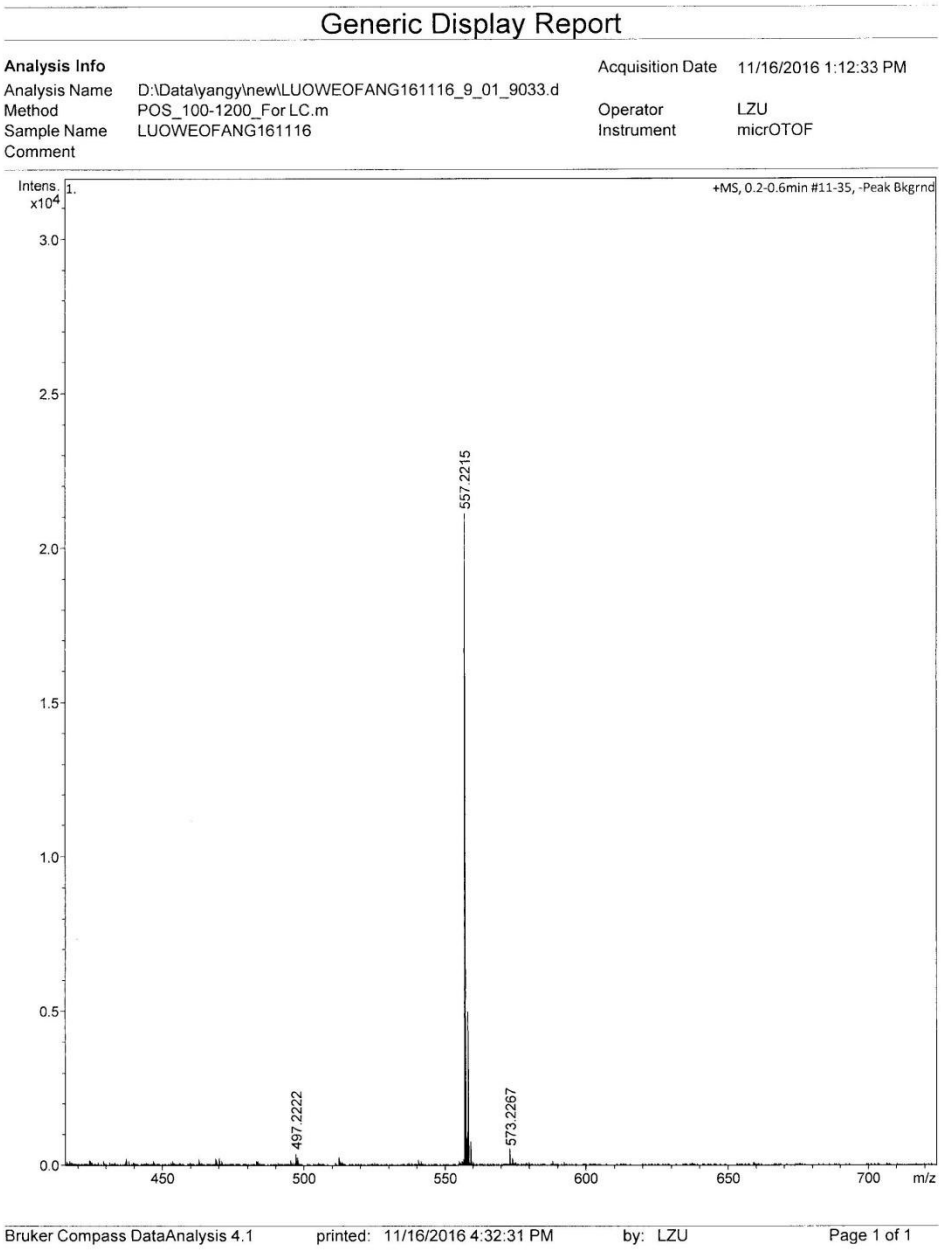


Figure S25. ESI mass spectrum of **2**.