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Supporting information

# A dual-site controlled ratiometric probe revealing the simultaneous down-regulation of pH in lysosomes and cytoplasm during autophagy

Minggang Tian, Chuang Liu, Baoli Dong, Yujing Zuo, Weiying Lin*

Institute of Fluorescent Probes for Biological Imaging, School of Chemistry and Chemical Engineering, School of Materials Science and Engineering, University of Jinan, Jinan, Shandong 250022, P. R. China.

Email: weiyinglin2013@163.com

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## Materials

All chemicals used are of analytical grade, Rhodamine b and 2,4-dihydroxybenzaldehyde were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). 2,2-Dimethyl-1,3-dioxane-4,6-dione etc. was purchased from J&K Chemical (Beijing, China). The solvents used in the spectral measurement are of chromatographic grade.

## Spectroscopic measurements

The UV-visible-near-IR absorption spectra of dilute solutions were recorded on a U2910 spectrophotometer using a quartz cuvette having 1 cm path length. One-photon fluorescence spectra of dilute solutions were obtained on a HITACH F-2700 spectrofluorimeter equipped with a 450-W Xe lamp. PBS buffer solution: 10 mM, NaCl, $NaHPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, pH = 7.40.

## Cell culture and staining methods

HepG2 and A549 cells were grown in H-DMEM (Dulbecco's Modified Eagle's Medium, High Glucose) supplemented with 10% FBS (Fetal Bovine Serum) in a 5% $CO_2$ incubator at 37 °C. For living cells imaging experiment of the probes, the culture medium surrounding the cells were firstly removed, and the cells were washed with PBS twice. Then the cells were incubated in 1 mL of culture medium. On the other hand, 1 mM stock solutions of the probe in DMSO were prepared. After that, 5 μL of stock solutions were mixed evenly with 1 mL culture medium (pH 7.4) in a tube. The cells were incubated with the above mixed solutions at 37 °C.

## Fluorescent imaging methods

Confocal fluorescence images were obtained with a Nikon A1R confocal laser scanning microscope. The differential interference contrast (DIC) images were taken

with 561 nm sapphire laser. To acquire the fluorescence images, $\lambda_{ex}$ = 405 nm, $\lambda_{em}$ = 425-475 nm for blue channel; $\lambda_{ex}$ = 561 nm, $\lambda_{em}$ = 570-620 nm for red channel.

**Starvation induced autophagy.** For autophagy experiments, the live HepG2 cells were firstly incubated with 5 μM of **Cyto-Lyso** for 30 min. Then the cells were washed with PBS twice, and incubated in PBS for 1 h and 2 h to trigger the starvation-induced autophagy. For control experiments, the cells were incubated with fresh culture medium for 2 h. These cells were then imaged under Nikon A1R microscopes.

Inhibition of autophagy with $NH_4Cl$ and CQ. Live HepG2 cells were initially incubated with 5 μM of **Cyto-Lyso** for 30 min. Then the cells were washed with PBS twice, and incubated in PBS with different amount of $NH_4Cl$ or CQ for 2 h. These cells were then imaged under Nikon A1R microscopes.

**Western Blotting (WB) experiments**

Hepg2 cells with a density of $10^5$ cells/mL were initially cultured in cell culture plate for 24 h to afford adherence. Afterwards, three groups of cells were further cultured in culture medium, PBS buffer solution, and PBS buffer containing 100 μM of $NH_4Cl$ for 2 h, respectively. The three group of cells were then lysed with cell 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 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 Atg-7 proteins in TBS + Tween 20 (TBST) at a dilution of 1:1000, using Tublin (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.

**Cytotoxicity test**

The cytotoxicity of the **Mito-Lyso** has been evaluated with HeLa cells using the reagent MTT. A 96-well plate was used to perform the cell viability experiment. Suspension of HeLa cells with cell concentration of 10000 cells/mL was firstly prepared, and the suspension was added to the plate (200 μL per well). At the same time, culture medium without cells was also introduced into the wells (200 μL per well) as blank. The cells were incubated with different concentration of **Mito-Lyso** for different time. Change the culture medium of all the wells, and after that 10 μL of MTT (5mg/mL) was added to each well. The well plate was incubated for 4 h, and then the culture medium in each well was removed. 200 μL of DMSO was added to dissolve the formazan, and finally the absorbance was measured with a microplate reader at 620 nm. The cell survival rates were finally calculated following the equation below:

$$\text{Survival rate (\%)} = \frac{A_{sample} - A_b}{A_c - A_b} \times 100$$

Where $A_{sample}$ is the absorbance of the wells with cells treated with the probes, $A_b$ is the absorbance of wells pretreated with only culture medium, and $A_c$ is the absorbance of wells with cells treated with no reagents.

**Figures used in the manuscript**

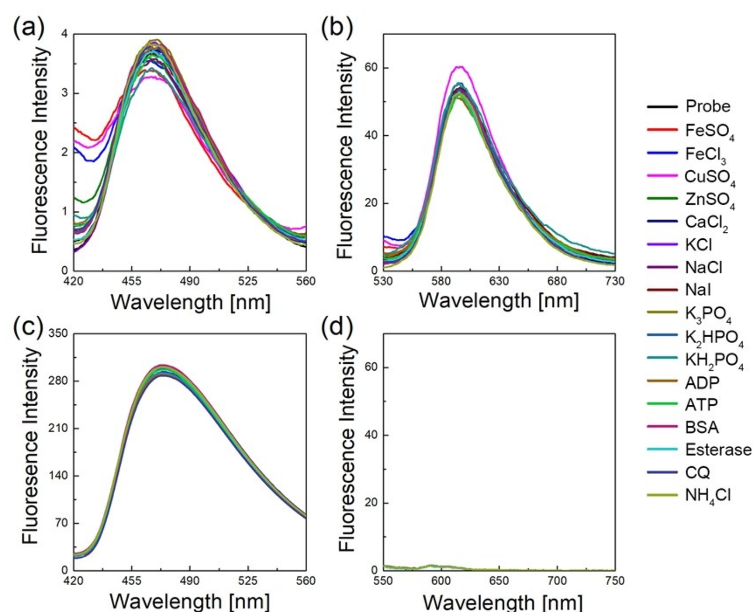


**Figure S1.** The fluorescence spectra of the probe **Cyto-Lyso** in the absence and presence of various reagents in PBS buffer with pH of 4.0 (a,b) and 8.0 (c,d). (a,c) $\lambda_{ex}$ = 405 nm; (b,d) $\lambda_{ex}$ = 500 nm; concentration of $CaCl_2$, KCl, NaCl, NaI: 20 mM; concentration of CQ and $NH_4Cl$: 500 μM; concentration of other reagents: 100 μM.

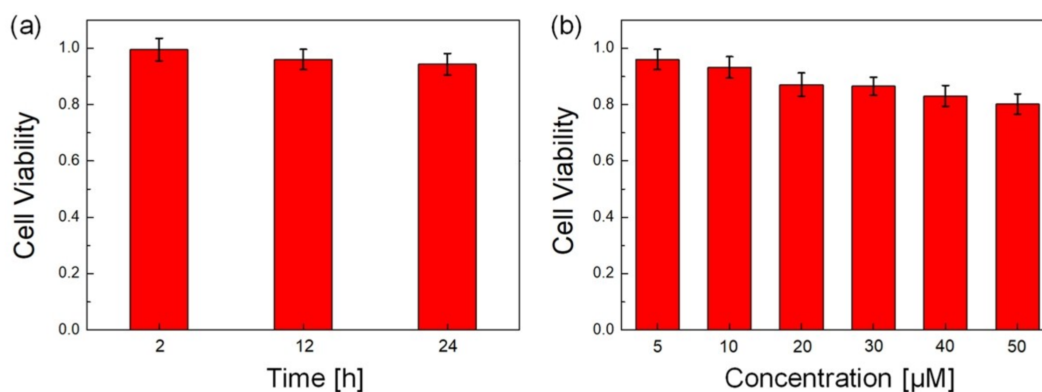


**Figure S2.** The cell viability of HepG2 cells after the incubation with 5 μM **Cyto-Lyso** for 2 h, 12 h, and 24 h (a), and incubation with 5-50 μM **Cyto-Lyso** for 12 h (b).

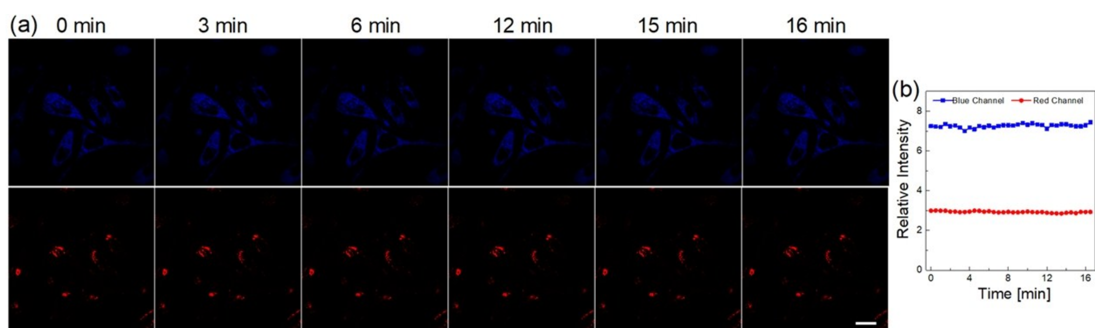


**Figure S3.** The fluorescence images of HepG2 cells pre-incubated in PBS buffer for 1 h, stained with Cyto-Lyso for 30 min, and then ceaselessly scanned with 405 nm and 561 nm laser for different time (a); the time-dependent intensity in blue and red channels. Bar = 20 μm.

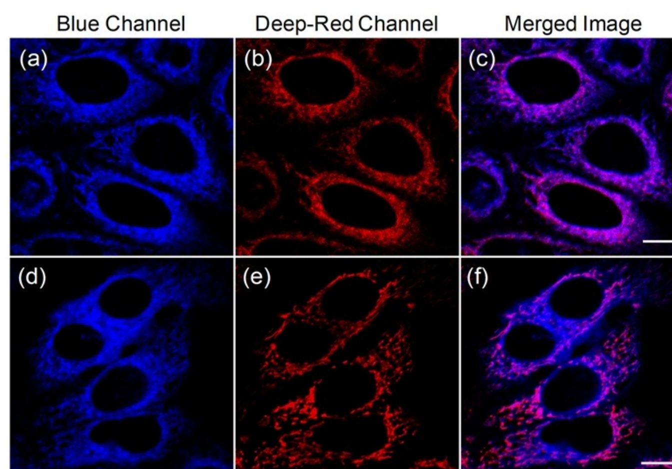


**Figure S4.** The fluorescence images of HepG2 cells co-stained with **Cyto-Lyso**/ERTR (a-c) and **Cyto-Lyso**/MTDR (d-f) for 30 min. (a,d) blue channel; (b,e) deep-red channel, $\lambda_{ex}$ = 561 nm for (b), $\lambda_{ex}$ = 647 nm for (e); (c,f) merged images. Bar = 10 μm.

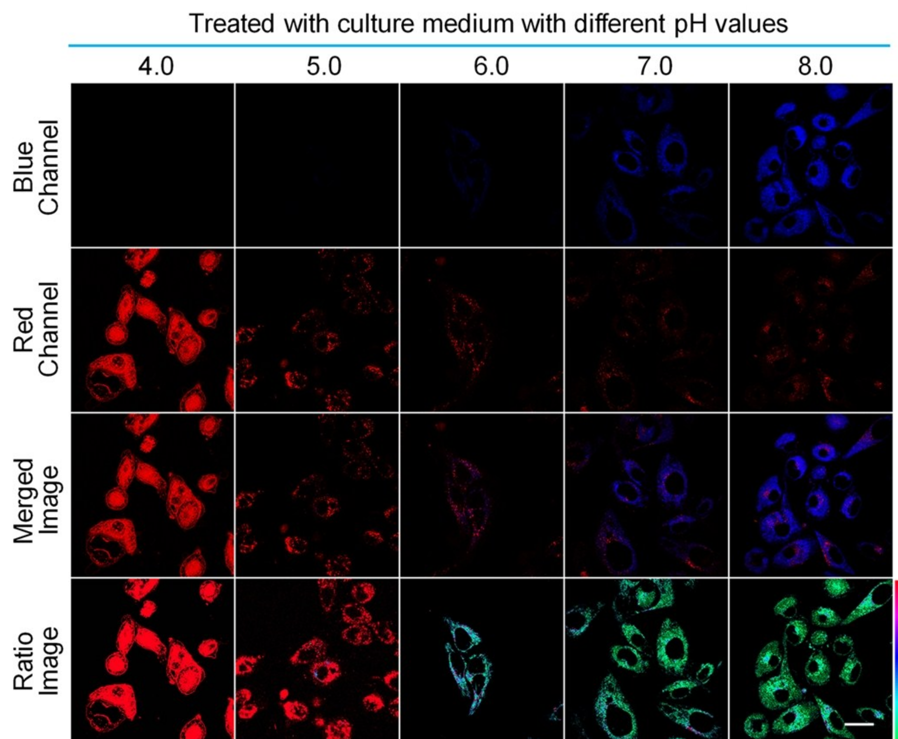


**Figure S5**. The fluorescent and ratiometric images of HepG2 cells pre-treated with cultural medium with different pH values for 60 min then stained with 5 μM **Cyto-Lyso** for 30 min. Bar = 20 μm.

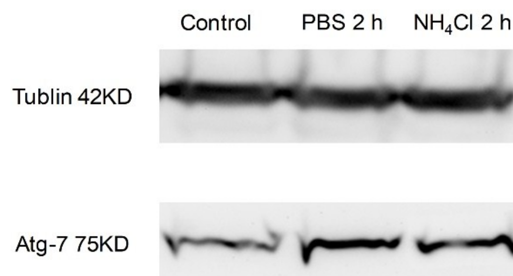


**Figure S6**. The Western blot showing the expression of Tublin and Atg-7 in Hepg2 cells. Control group of cell was cultured in culturing medium, and the other two groups of cells were cultured in PBS and PBS containing 100 μM of $NH_4Cl$, respectively.

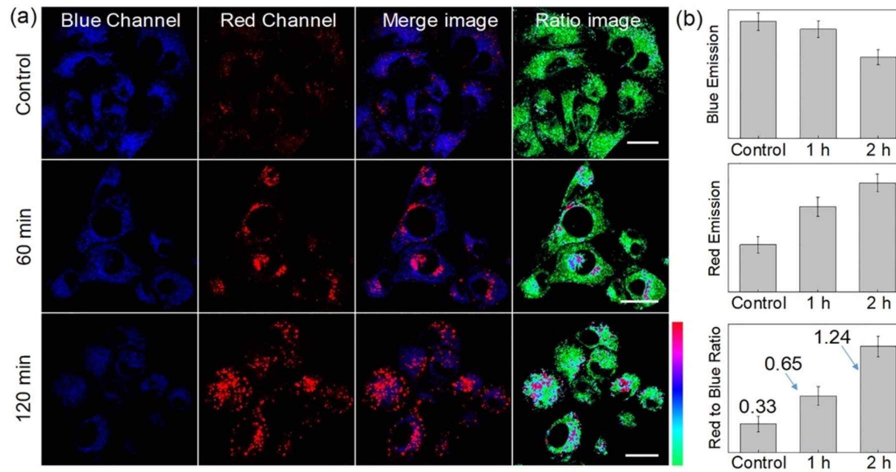


**Figure S7.** The fluorescent images of 4T1 cells pre-stained with 5 μM **Cyto-Lyso** for 30 min (a) then incubated with culture medium for 120 min (Control) and PBS buffer for 60 min and 120 min. The mean intensity in blue channel, red channel, and the intensity ratio of red to blue channel of the three groups of cells. Bar = 20 μm.

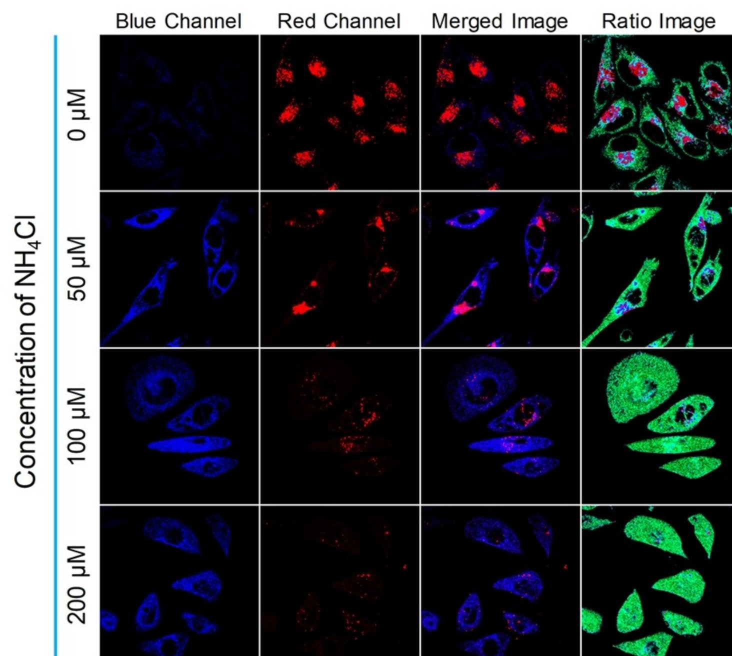


**Figure S8.** The fluorescence images of HepG2 cells pre-stained with 5 μM **Cyto-Lyso** for 30 min (a) then incubated with PBS buffer containing different amount of $NH_4Cl$ for 2 h. Bar = 20 μm.

**The synthesis of the probe Cyo-Lyso.**

The probe **Cyto-Lyso** is synthesized following the synthetic route shown in Scheme 1b.

Synthesis of 2-(2-aminoethyl)-3',6'-bis(diethylamino)spiro[isoindoline-1,9'-xanthen]-3-one (**1**). Rhodamine b (10 mmol) was initially added in 50 mL EtOH in a round-bottom flask. Ethylenediamine (100 mmol) was then added into the flask, which were vigorously stirred. The mixture was heated to 90 $^{o}C$ for 16 hours to accomplish the reaction, which was cooled down to 25 $^{o}C$ and poured into ice water. The crude product could be obtained by filtration, and purified by column chromatography with $CH_2Cl_2/CH_3OH$ (v/v, 100:1) as eluent, to afford white-powder product with a yield of 68 %. $^{1}H$ NMR (400 MHz, Chloroform-*d*) δ 7.95 – 7.86 (m, 1H), 7.52 – 7.41 (m, 2H), 7.15 – 7.06 (m, 1H), 6.45 (d, *J* = 8.9 Hz, 2H), 6.39 (d, *J* = 2.6 Hz, 2H), 6.29 (dd, *J* = 8.9, 2.6 Hz, 2H), 3.35 (q, *J* = 7.2 Hz, 8H), 1.18 (t, *J* = 7.0 Hz, 12H).

Synthesis of 7-hydroxy-2-oxo-2H-chromene-3-carboxylic acid (**2**). Into a round-bottom flask with 50 mL EtOH, 2,4-dihydroxybenzaldehyde (10 mmol) and 2,2-dimethyl-1,3-dioxane-4,6 -dione (10 mmol) were added. 400 μL of pyrrolidine was added and the mixture was stirred vigorously. The system was heated to reflux for 12 hours to accomplish the reaction. Then the mixture was cooled down to 25 $^{o}C$ and poured into ice water. The crude product could be obtained by filtration, and purified by recrystallization in ethanol to afford white powder with a yield of 82 %. $^{1}H$ NMR (400 MHz, DMSO-$d_6$) δ 8.46 (s, 1H), 7.64 (d, *J* = 8.6 Hz, 1H), 6.80 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.70 (d, *J* = 2.1 Hz, 1H).

Synthesis of N-(2-(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-ethyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxamide (**Cyto-Lyso**). Compound **1** (1 mmol) and compound **2** (1 mmol) were added into a flask. Afterwards, EDCI (2 mmol), HOBT (2 mmol), and DCC (2 mmol) were added. 10 mL DMF was then added to dissolve the reagents, and the mixture was stirred for 24 at room temperature to finish the reaction. The system was poured into water and extracted with dichloromethane. The organic layer was dried with $MgSO_4$ and distilled. The pure product could be

obtained by column chromatography with $CH_2Cl_2/CH_3OH$ (v/v, 80:1) as eluent, to afford yellow powder with a yield of 45 %. $^1H$ NMR (400 MHz, DMSO-$d_6$) δ 11.07 (s, 1H), 8.66 (s, 1H), 8.41 (t, $J$ = 5.5 Hz, 1H), 7.89 – 7.68 (m, 2H), 7.57 – 7.42 (m, 2H), 6.99 (dd, $J$ = 5.9, 2.7 Hz, 1H), 6.88 (dd, $J$ = 8.6, 2.3 Hz, 1H), 6.79 (d, $J$ = 2.2 Hz, 1H), 6.47 – 6.32 (m, 4H), 6.27 (dd, $J$ = 8.9, 2.6 Hz, 2H), 3.19 (dq, $J$ = 16.3, 6.5, 5.6 Hz, 12H), 1.02 (t, $J$ = 7.0 Hz, 12H). $^{13}C$ NMR (100 MHz, DMSO-$d_6$) δ 168.07, 167.42, 164.12, 161.88, 161.79, 161.18, 161.16, 156.71, 154.09, 153.01, 148.73, 148.11, 133.19, 132.37, 132.18, 132.03, 130.71, 130.09, 129.11, 128.71, 114.79, 113.94, 113.92, 111.51, 108.54, 105.33, 102.20, 97.72, 67.87, 64.62, 44.05, 38.56, 37.92, 30.27, 28.83, 23.72, 22.86, 14.35, 12.83, 11.26. HRMS (ESI): m/z, for $C_{40}H_{41}N_4O_6^+$, Calc., 673.3021, found, 673.3148; for $C_{40}H_{42}N_4O_6^{2+}$, Calc., 337.1550, found, 337.1608.

## Characterizations of the probes

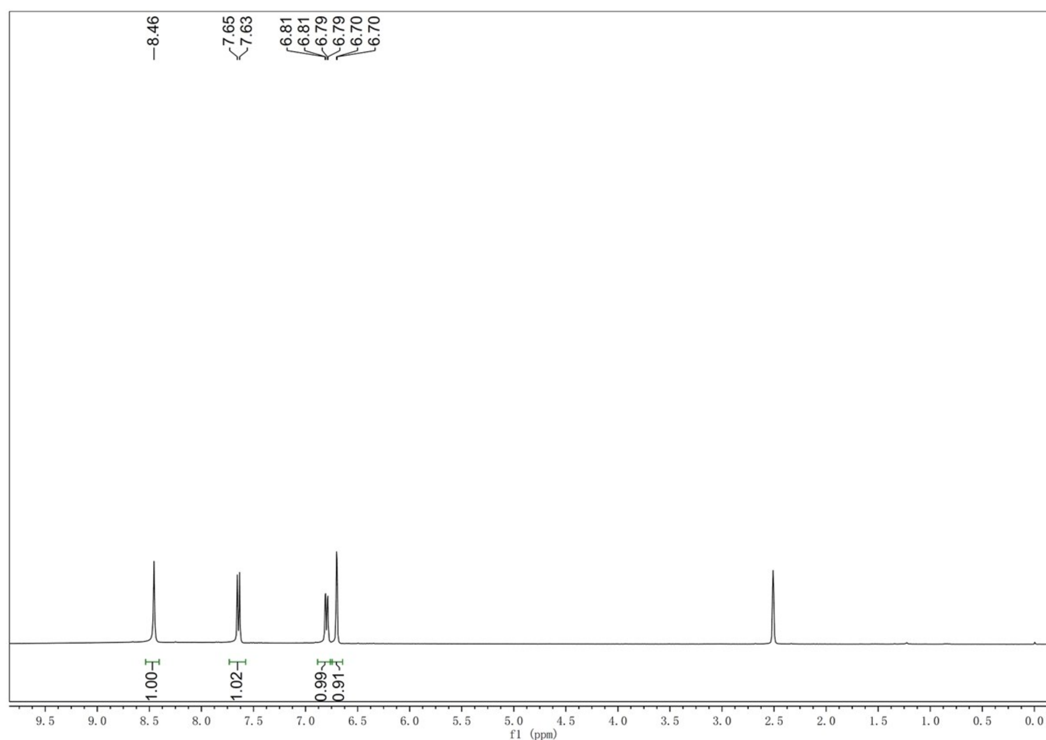


**Figure S9.** The $^{1}H$ NMR spectra of **1** in $CDCl_3$.

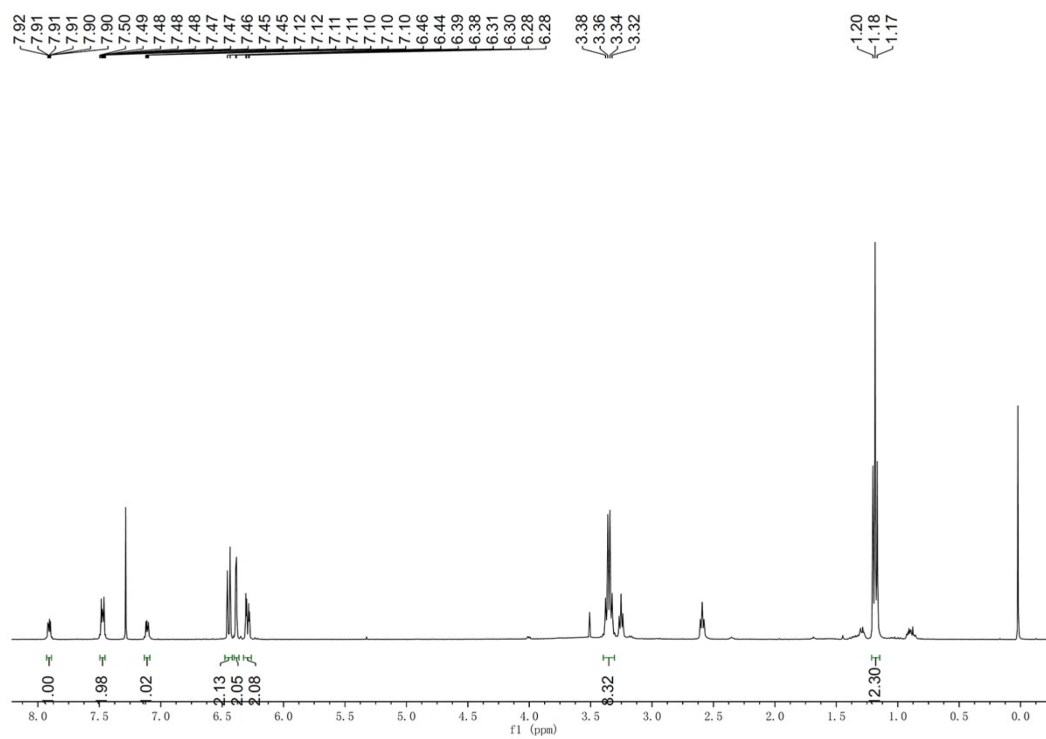


**Figure S10.** The $^{1}H$ NMR spectra of **2** in $CDCl_3$.

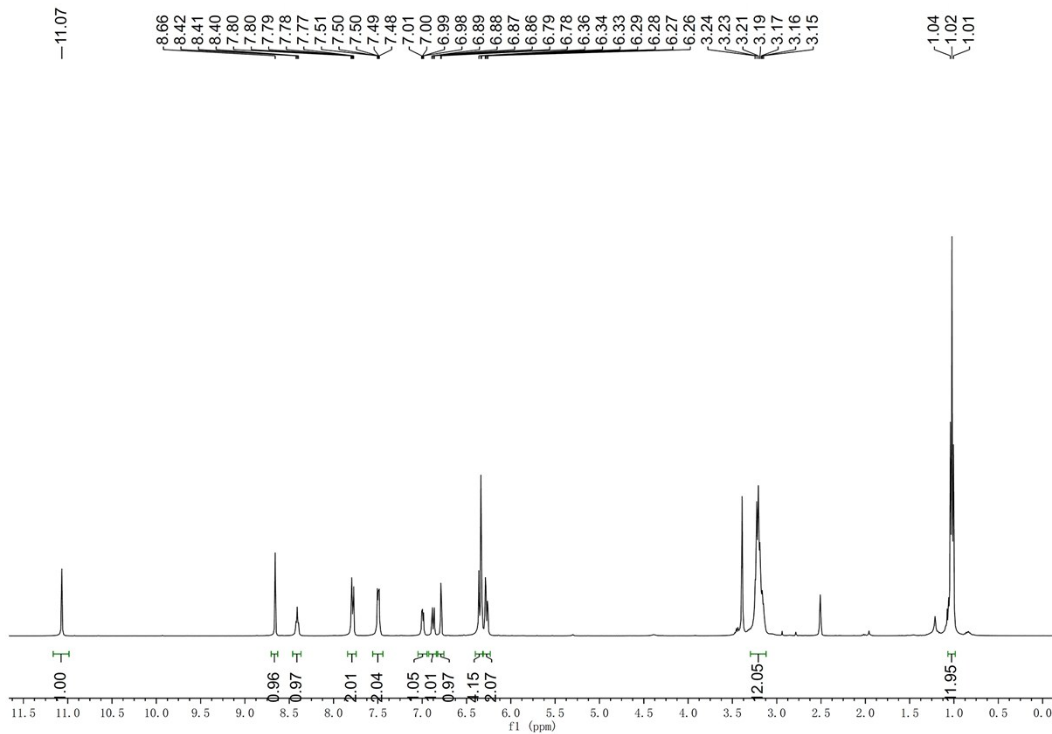

**Figure S11.** The $^{1}$H NMR spectra of **Cyto-Lyso** in DMSO-$d_6$.

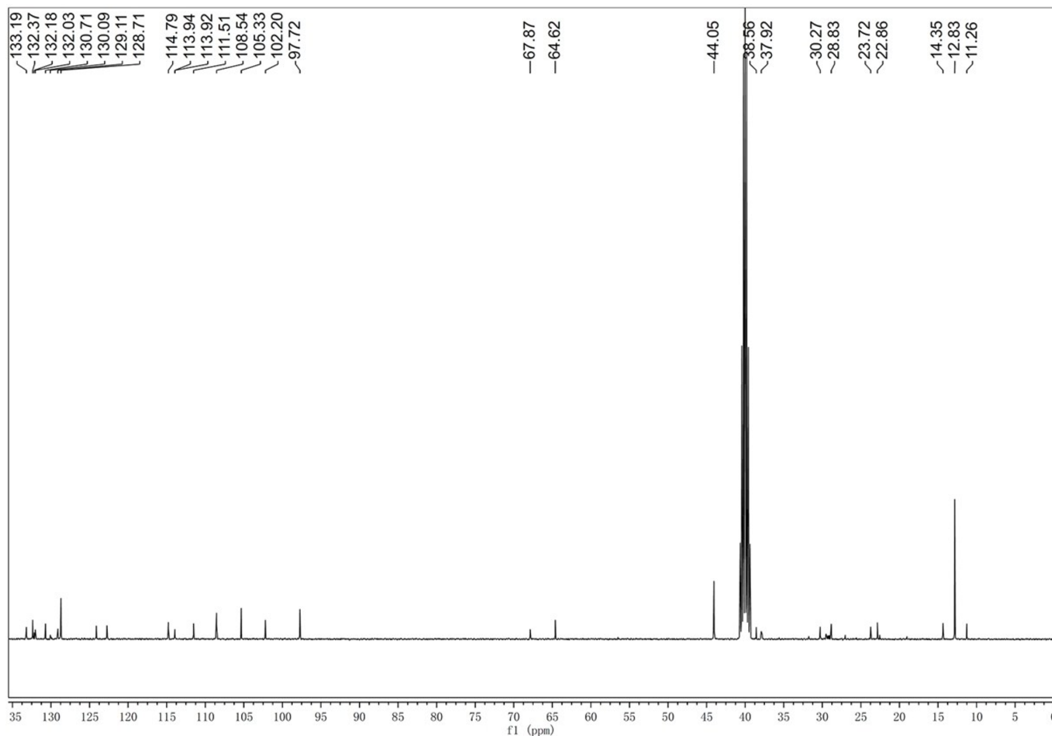

**Figure S12.** The $^{13}$C NMR spectra of **Cyto-Lyso** in DMSO-$d_6$.

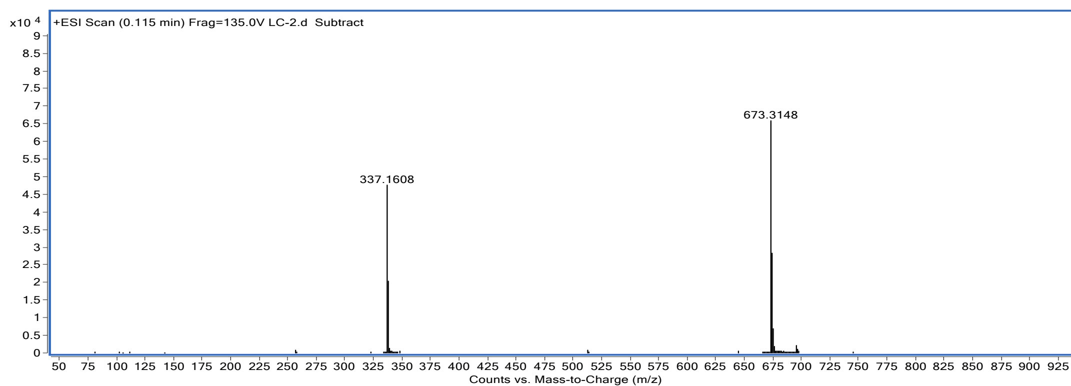


**Figure S13.** The HRMS spectra of **Cyto-Lyso**.