Supporting Information

Imaging of lysosomal pH changes with a fluorescent sensor containing a novel lysosome-locating group

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Materials and methods

All of the solvents used were of analytic grade. Analyte solutions of NaCl, KCl, $CaCl_2$, $MgCl_2\ 6H_2O$, $CoCl_2\ 6H_2O$, $PbCl_2$, $CuCl_2\ 2H_2O$, $BaCl_2\ 2H_2O$, $NiCl_2\ 6H_2O$, $HgCl_2$, $CrCl_3$, $ZnCl_2$, $CdCl_2$, $FeCl_3\ 6H_2O$, $AgNO_3$, $NaNO_3$, NaBr, $NaH_2PO_4\ 2H_2O$, NaAc, $NaClO_4$, KI, K_2CO_3, $Na_2S\ 9H_2O$, Na_2HPO_4, and NaClO were prepared by dissolving the salts in distilled water to final concentrations of 5.0 mM for $HgCl_2$ and 25 mM for the other ions. **Rlyso** and **R** were dissolved in dimethyl sulphoxide (DMSO) to produce 3 mM stock solutions. Aliquots were then diluted to 10 μM with a Britton-Robinson buffer solution containing 40 mM acetic acid, phosphoric acid, and boric acid. Slight variations in the pH of the solutions were achieved by adding minimal volumes of NaOH or HCl. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a VARIAN INOVA-400 spectrometer. Chemical shifts (δ) were reported as ppm (in $CDCl_3$ or CD_3SOCD_3, with TMS as the internal standard). Mass spectrometric (MS) data were obtained with HP1100LC/MSD MS and an LC/Q-TOF-MS instruments. Fluorescence measurements were performed on a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018). Excitation and emission slit widths were modified to adjust the fluorescence intensity to a suitable range. Absorption spectra were measured on a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. All pH measurements were performed using a Model PHS-3C meter.

Synthesis

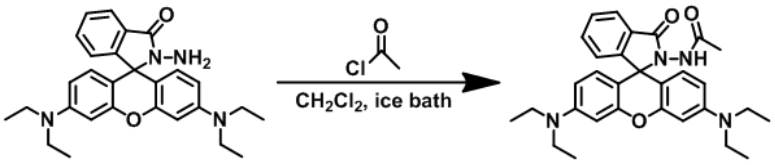
Synthesis of **1**:

1 was synthesized from rhodamine B by the procedure published in literature[1].

Synthesis of **2**:

2 was prepared as previously described.[2]

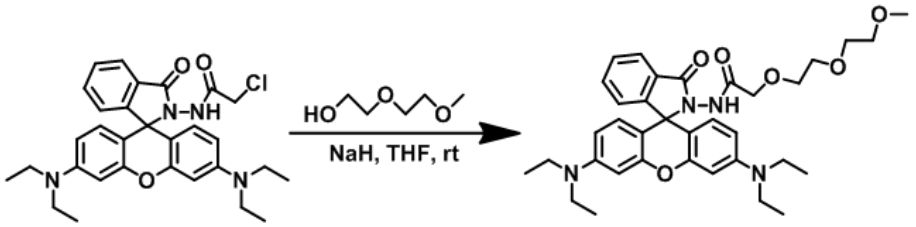
Synthesis of **R**:



1 **R**

To a 100 ml flask, **1** (0.91 g, 2.00 mmol) was dissolved in 30 mL dichloromethane, and then cooled with ice bath. Then 0.14 mL (2.00 mmol) acetyl chloride was dissolved in 10 mL dichloromethane and added dropwise to the above 100 mL flask with vigorous stirring. All reagents and glass apparatuses should be dried before use. After addition, the stirred mixture was allowed to stand in ice bath for 2 h, then the solvent was removed under reduced pressure. The crude compound was purified by flash column chromatography on silica gel (1:1 hexane-ethyl acetate) to afford a white solid (780 mg, 78 %). ^{1}H NMR (400 MHz, CD_3SOCD_3), δ: 1.08 (t, *J* = 8 Hz, 12H), 1.70 (s, 3H), 3.32 (q, *J* = 8Hz, 8H), 6.31 (m, 4H), 6.51 (d, *J* = 8Hz, 2H), 6.98 (d, *J* = 4Hz, 1H), 7.53 (m, 2H), 7.80 (d, *J* = 4Hz, 1H), 9.46 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$), δ: 12.62, 18.91, 20.92, 44.42, 65.91, 66.74, 97.57, 98.18, 107.92, 123.48, 123.59, 124.03, 124.62, 128.21, 128.80, 129.31, 129.47, 133.15, 133.80, 148.94, 149.20, 150.30, 151.76, 153.67, 154.20, 165.30, 166.31, 167.87, 175.09 ppm; TOF MS: m/z calcd for $C_{30}H_{34}N_4O_3Na^+$ $[M + Na]^+$: 521.2529, found: 521.2526.

Synthesis of **Rlyso**:



2 **Rlyso**

Sodium hydride (120 mg of 60% suspension in mineral oil, 3.00 mmol) was dispersed in 10 mL freshly dry tetrahydrofuran. To this slurry was added dropwise methylcarbitol (90 mg, 0.75 mmol, dissolved in 5 mL tetrahydrofuran) at 0 ^{o}C over a period of 60 min. **2** (200 mg, 0.37 mmol, dissolved in 5 mL tetrahydrofuran) was added dropwise at 0 ^{o}C over 30 min. The reaction mixture was stirred at room temperature for 24 h. After elimination of the solvent in a rotary evaporator under reduced pressure, the residue was purified by flash column chromatography on silica gel (1:2 dichloromethane-ethyl acetate) to afford a white solid (99 mg, 43 %).^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.15 (t, *J* = 8 Hz, 12H), 3.30 (q, *J* = 12 Hz, 11H), 3.38 (t, *J* = 4 Hz, 2H), 3.46 (t, *J* = 4 Hz, 2H), 3.53 (d, *J* = 2 Hz, 4H), 3.97 (s, 2H), 6.29 (d, *J* = 8 Hz, 2H), 6.34 (s, 2H), 6.66 (d, *J* = 8 Hz, 2H), 7.12 (d, *J* = 8 Hz, 1H), 7.48 (m, 2H), 7.95 (d, *J* = 4 Hz, 1H), 8.01 (s, 1H);^{13}C NMR (100 MHz, $CDCl_3$), δ: 12.63, 44.31, 58.95, 65.99, 70.10, 70.21, 70.40, 70.85, 71.92, 97.49, 104.40, 107.92, 123.45, 124.07, 128.23, 129.20, 129.45, 133.06, 148.95, 151.61, 153.74, 164.72, 167.69 ppm; TOF MS: m/z calcd for $C_{35}H_{44}N_4O_6Na^+$ $[M + Na]^+$: 639.3159, found: 639.3151.

Cell incubation

The mammalian cells MCF-7, Hela, and Raw 264.7 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). The cells were seeded in 24-well flat-bottomed plates and then incubated for 24 h at 37 ^{o}C under 5% CO_2. **Rlyso**/**R** (10 μM) was then added to the cells and incubation for another 30 min followed. The cells were washed three times with phosphate-buffered saline (PBS). Fluorescence imaging was performed using an OLYMPUS FV-1000 inverted fluorescence microscope with a 100×objective lens.

Lysosome staining in live cells: LysoSensorTM Green DND-189 (1 μM, Invitrogen) was used to co-stain the cells.

Chloroquine-induced lysosomal pH change: Raw 264.7 cells internalised with **Rlyso** (10 μM) were washed with PBS. Chloroquine (100 μM) was then added.

Lysosomal pH change during apoptosis: Hela cells precultured with **Rlyso** were washed with PBS, then treated with 2 μM dexamethasone, and the fluorescence images were recorded every 2 min for 1 h. The fluorescence intensity value was extracted from six areas of each image, and the values in each case were averaged.

Cytotoxicity experiments

Cell viability measurements were performed by reducing 3-(4,5-dimethylthiahiazol-2-y1)-2,5-diphenytetrazolium bromide (MTT) to formazan crystals using mitochondrial dehydrogenases (Mosmann, 1983). Briefly, Hela and Raw 264.7 cells were seeded in 96-well microplates (Nunc, Denmark) at a density of 1×10^5 cells/mL in 100 μL of medium containing 10% FBS. After 24 h of cell attachment, the plates were washed with 100 μL/well PBS. The cells were then cultured in a medium with 10 μM **Rlyso**/**R** for 12 h. Cells in a culture medium without fluorescent dyes were used as the control. Six replicate wells were used for each control and test concentration. MTT (10 μL, 5 mg/mL) in PBS was subsequently added to each well. The plates were then incubated at 37 ℃ for 4 h in a 5% CO_2 humidified incubator. The medium was carefully removed, and the purple products were lysed in 200 μL DMSO. The plate was shaken for 10 min, and the absorbance was measured at 405 and 488 nm using a microplate reader (Thermo Fisher Scientific). Cell viability was expressed as a percentage of the control culture value.

Photodegradation Experiments

In vitro: The photodegradation experiments were performed by placing the sample solutions in square quartz cells (l × 1 cm^2) and then irradiating the samples with a 500 W I–W lamp at room temperature. To eliminate heat and the absorbance of short-wavelength light, a cold trap [2 L solution of 50 g/L $NaNO_2$ in 22 cm (height) × 7 cm (width) × 22 cm (length)] was set up between the cells and the lamp. The distance between the cells and the lamp was 30 cm (Fig. S1) The irreversible bleaching of the dyes at the emission peak was monitored as a function of time. All samples were tightly sealed and not deoxygenated with nitrogen prior to the test to simulate the actual conditions during practical applications (unless specified otherwise).

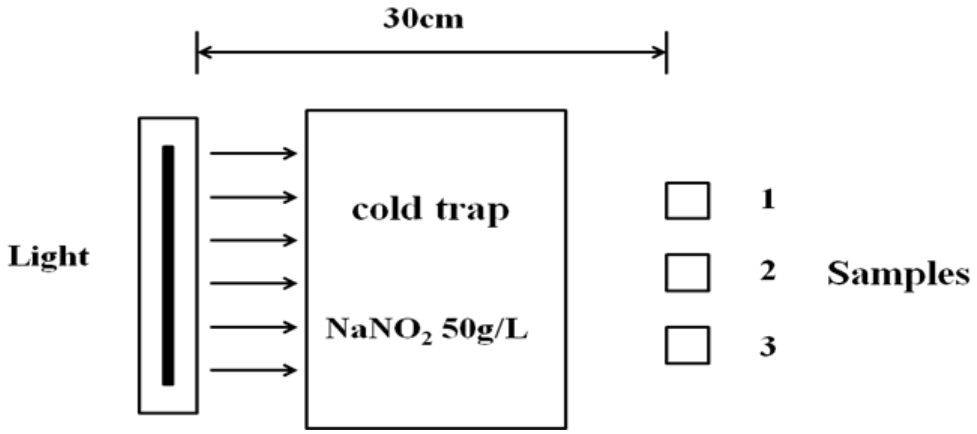


Fig. S1 Photodegradation experiments in vitro

In vivo: Hela cells were incubated with **Rlyso** (10 μM) via the procedure described in the preceding section. The cells were then washed three times with PBS, exposed to laser illumination (λ_{ex} = 559 nm), and then imaged for 657 sec. Data were obtained from eight real-time image areas; the values observed in each case were averaged.

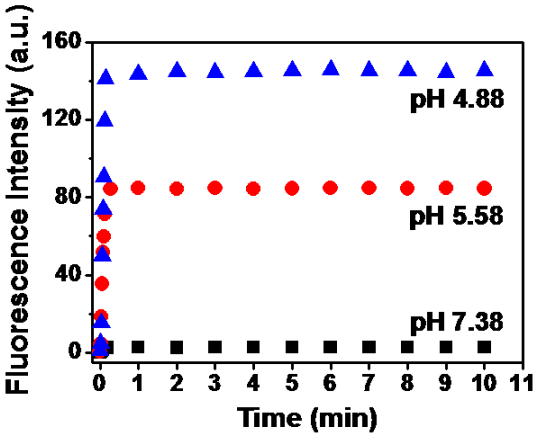


Fig. S2 The time courses of fluorescence intensity of **Rlyso** in Britton-Robinson buffer solution at different pH values (4.88, 5.58 and 7.38, respectively). Conditons: excitation wavelength is 559 nm, emission wavelength is 578 nm.

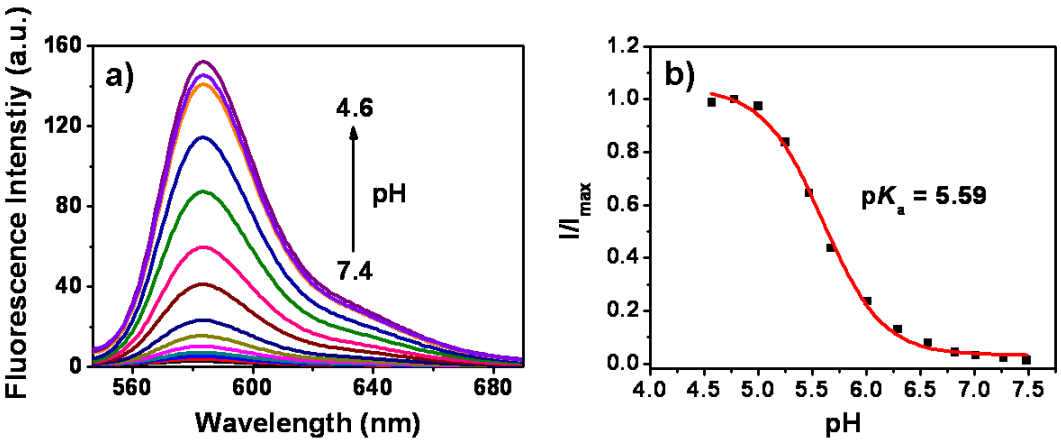


Fig. S3 a) Fluorescence spectra of **R** at various pH in Britton-Robinson buffer solution, λ_{ex} = 525 nm. b) pH titration curve of **R**, λ_{ex} = 559 nm, λ_{em} = 582 nm.

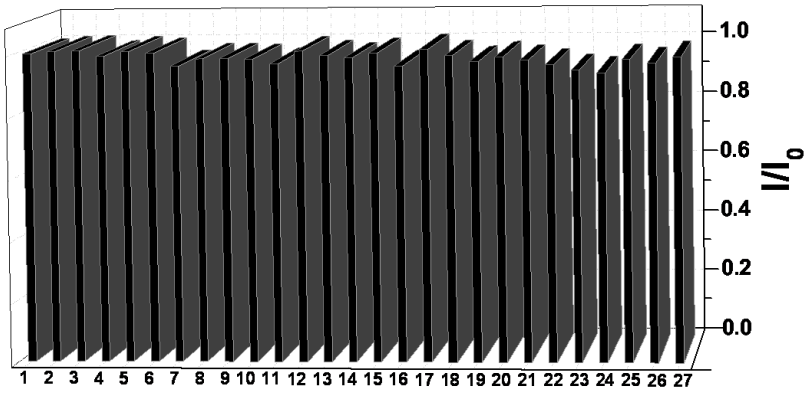


Fig. S4 Fluorescence intensity of **Rlyso** in the absence or prescence of miscellaneous ions in Britton-Robinson buffer solution at pH 4.87. From left to right: H^+, Na^+, K^+, Ca^{2+}, Mg^{2+}, Co^{2+}, Pb^{2+}, Cu^{2+}, Ba^{2+}, Ni^{2+}, Hg^{2+}, Cr^{3+}, Zn^{2+}, Cd^{2+}, Fe^{3+}, Ag^+, Cl^-, NO_3^-, Br^-, $H_2PO_4^-$, Ac^-, ClO_4^-, I^-, CO_3^{2-}, S^{2-}, HPO_4^-, ClO^-. Condition: excitation wavelength is 559 nm, emission wavelength is 578 nm. 200 μM for all the ions.

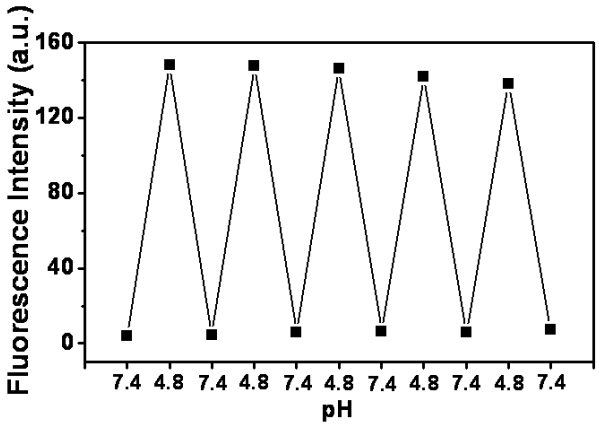


Fig. S5 Fluorescence intensity of **Rlyso** in Britton-Robinson buffer solution. pH was changed repeatedly between 4.8 and 7.4. Conditons: excitation wavelength is 559 nm, emission wavelength is 578nm.

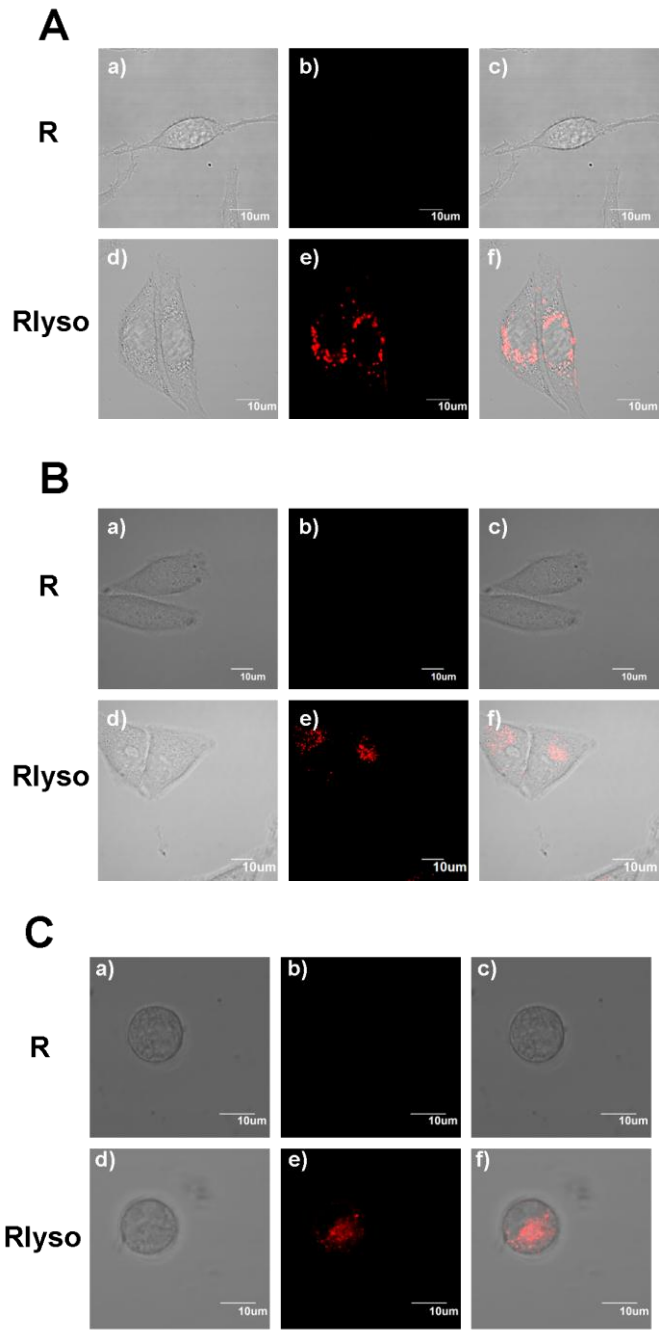


Fig. S6 Staining of Hela (A), MCF-7 (B) and Raw 264.7 (C) cells with **R** and **Rlyso**. a) and d) Bright field images; b) and e) confocal fluorescence images; c) and f) merge of a) and b), d) and e), respectively. The probes were excited at 559 nm and the fluorescence was collected at 575-620 nm.

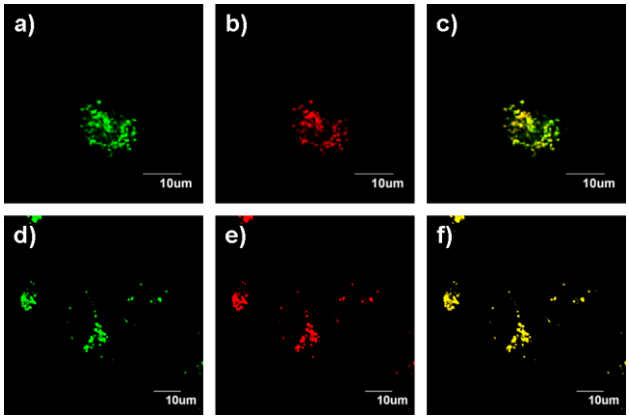


Fig. S7 Rlyso colocalizes to lysosomes in live Raw 264.7 (a-c) and Hela (d-f) cells. Cells were stained with: a) and d) 1 μM LysoSensor Green, b) and e) 10 μM **Rlyso**. c) and f) areas of colocalization appear in yellow. **Rlyso** and LysoSensor Green were excited at 559 nm and 488 nm, respectively. The fluorescence images were recorded at 575-620 nm (**Rlyso**) and 495-515 nm (LysoSensor Green), respectively.

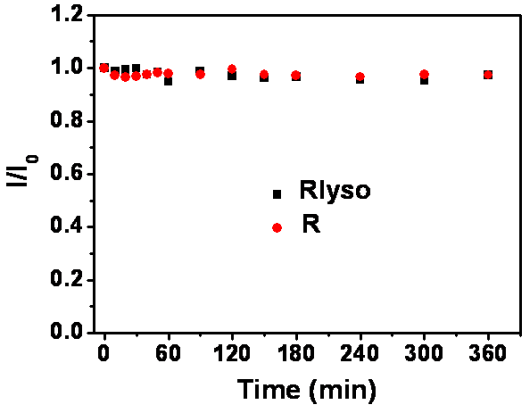


Fig. S8 Photostability of **Rlyso** and **R** at various time points in vitro.

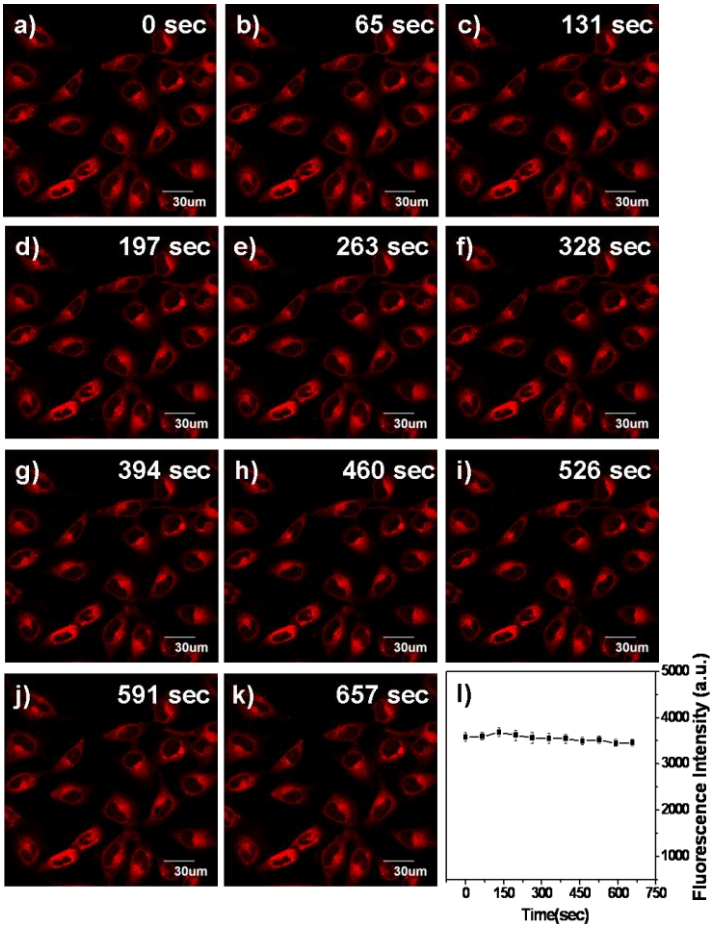


Fig. S9 Photostability of **Rlyso** in cells illuminated with a laser. a)-k) Confocal fluorescence images of **Rlyso** at various time points. l) Plot of the fluorescence intensity as a function of time.

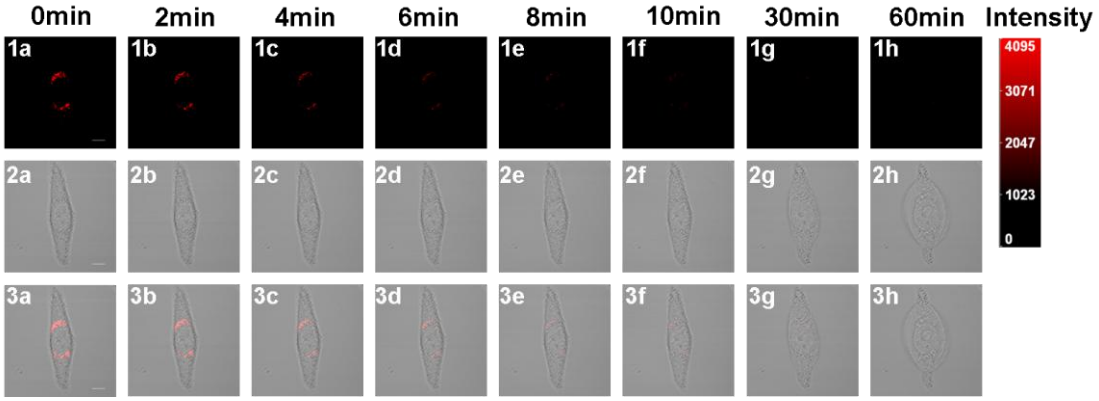


Fig. S10 Time dependent fluorescence changes of **Rlyso** in Hela cells undergoing apoptotic death. a, b, c, d, e, f, g and h indicated times of 0, 2, 4, 6, 8, 10, 30 and 60 min, respectively. 1, 2 and 3 represent red channel image (Ex@559nm, Em@575-620nm), bright field and overlap of 1 and 2, respectively.

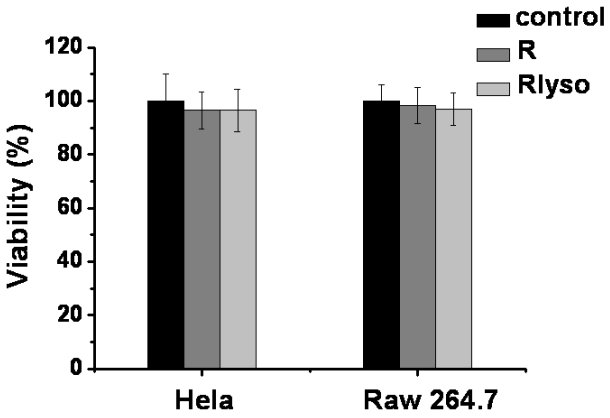


Fig. S11 Cytotoxicity of **Rlyso** and **R** on Hela and Raw 264.7 cells. Cells were incubated with 10 μM **Rlyso**/**R** in FBS buffer for 12 h, cell viabilities were examined using Thermo Fisher Scientific.

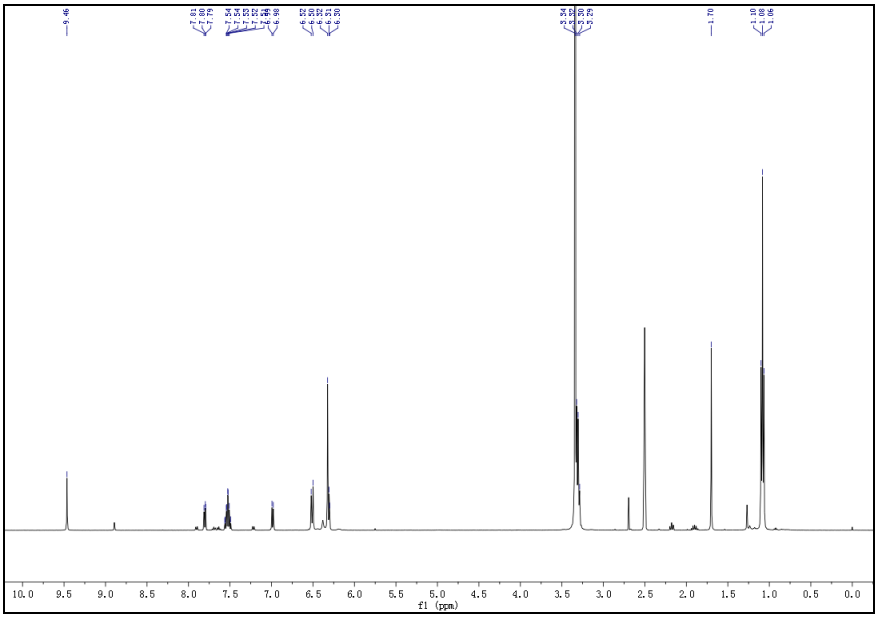


Fig. S12 ^{1}H-NMR of **R**.

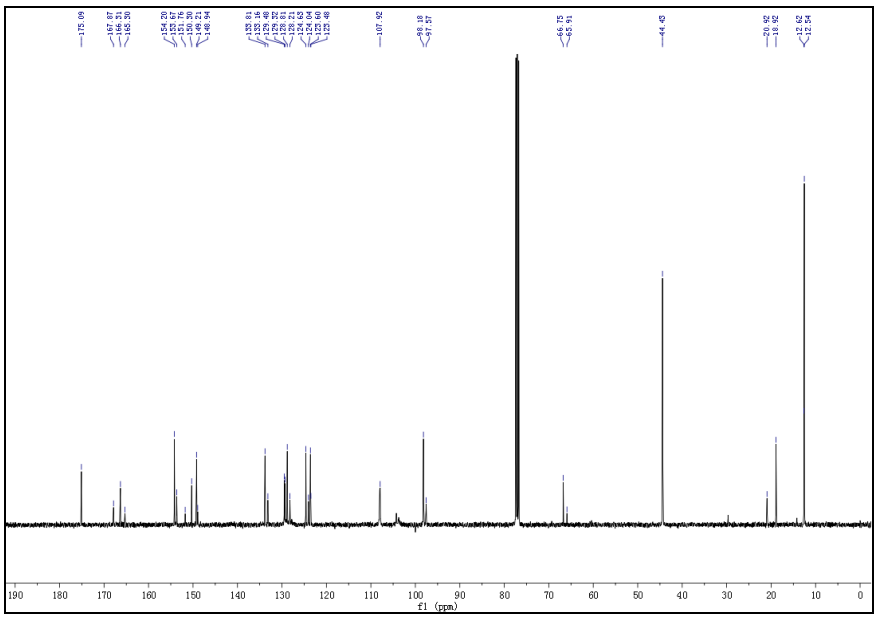


Fig. S13 ^{13}C-NMR of **R**.

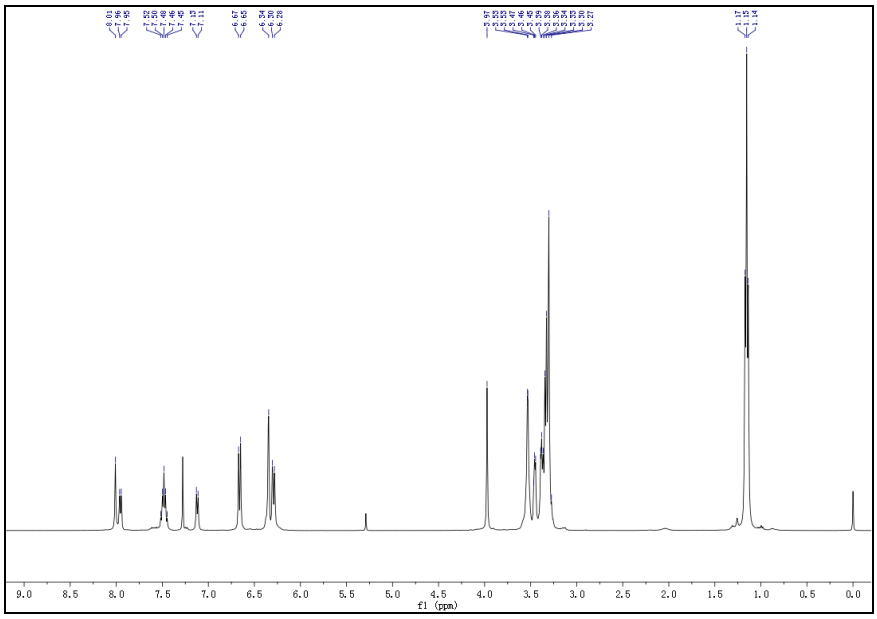


Fig. S14 ^{1}H-NMR of **Rlyso**.

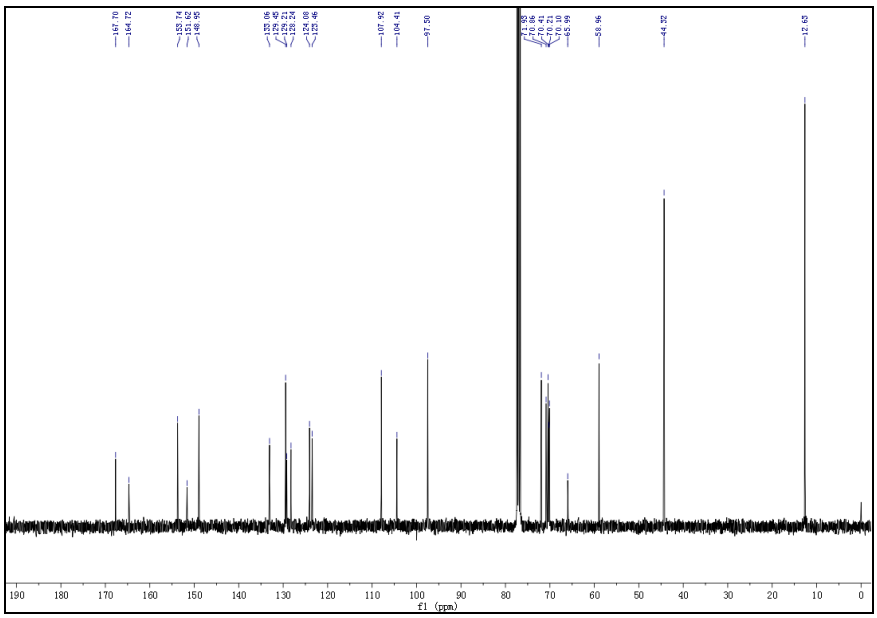


Fig. S15 ^{13}C-NMR of **Rlyso**.

Reference

1 V. Dujols, F. Ford and A. W. Czarnik, *J. Am. Chem. Soc*., 1997, **119**, 7386-7387.

2 H. Zhu, J. Fan, J. Lu, M. Hu, J. Cao, J. Wang, H. Li, X. Liu and X. Peng, *Talanta*, 2012, **93**, 55-61.