Synthesis and properties of a lysosome-targeted fluorescent ionophore based on coumarins and squaramides

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Supporting Information

Generals. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance AV 400 spectrometer and the deuterium solvents as standards. LR and HR ESI mass spectra were measured on Waters UPLC/Quattro Premier XE and Bruker maXis 4G ESI-Q-TOF mass spectrometers, respectively. Analytical thin-layer chromatography (TLC) was performed on silica gel plates 60 GF254. Detection on TLC was made by use of iodine, UV (254 or 365 nm) and 2% ninhydrin ethanol solution. Liposomes were prepared by extrusion through nuclepore track-etched polycarbonate membranes (100 nm, Whatman, Florham Park, New Jersey, USA) using an Avanti's Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA). Fluorescence spectra were measured on a Perkin Elmer LS55 spectrofluorimeter. MTT-based cytotoxicity was measured on a Tecan Infinite M1000 PRO microplate reader. LysoTracker™ Deep Red co-staining was carried out on an OLYMPUS FLUOVIEW FV1000 laser scanning confocal microscopy, and acridine orange staining was carried out on a Carl Zeiss Axio Observer A1 microscope.

EYPC and pyranine were purchased from Sigma Chemical Co. (St Louis, USA). AO was purchased from Aladdin Chemical Co. (Shanghai, China). LysoTracker™ Deep Red was

purchased from Beyotime Institute of Biotechnology (Shanghai, China). Anti-fluorescence quenching reagent was purchased from Beijing Solarbio Science & Technology Co (Beijing, China). All the other chemicals and reagents were obtained from commercial sources and used without further purification.

Chemistry

*Compound **3***. To a solution of 7-amino-4-methylcoumarin (100 mg, 0.57 mmol) in ethanol (3 mL) was added diethyl squarate (845 μL, 5.71 mmol) and zinc trifluoromethylsulfonate (59 mg, 0.16 mmol). The resulting solution was refluxed for 10 h. The formed precipitates were collected through filtration, washed subsequently with ethanol and distilled water and dried under vacuum to give compound **3** (90 mg, 80%). ^{1}H NMR (DMSO-d_6, 400 MHz, Fig. S1) δ 11.05 (s, 1H), 7.74 (d, J = 8.8 Hz, 1H), 7.44 (s, 1H), 7.40 (dd, J = 8.8 Hz, 2.0 Hz, 1H), 6.28 (s, 1H), 4.80 (q, J = 7.2 Hz, 2H); 2.40 (s, 3H), 1.45 (t, J = 7.2 Hz, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz, Fig. S2) δ 187.1, 184.1, 179.3, 169.3, 159.7, 153.7, 153.0, 141.4, 126.3, 115.2, 115.1, 112.3, 105.8, 69.9, 17.9, 15.5; negative LR ESI MS: 298.72 ([M–H]$^-$) (Fig. S3); positive HR ESI MS for $C_{16}H_{14}O_5N$ ([M+H]$^+$) calcd: 300.0866, found: 300.0884 and negative HR ESI MS for $C_{16}H_{12}O_5N$ ([M−H]$^-$) calcd: 298.0721, found: 298.0722 (Fig. S4).

*Compound **1***. To a solution of compound **3** (52 mg, 0.17 mmol) in DMSO (2 mL) was added 4-morpholin-4-ylaniline (35 mg, 0.18 mmol) and zinc trifluoromethylsulfonate (19 mg, 0.05 mmol). The resulting solution was heated at 60 ^{o}C for 20 h. The formed precipitates were collected through filtration, washed subsequently with methanol and distilled water and dried under vacuum to give compound **1** (17 mg, 24%). ^{1}H NMR (DMSO-d_6, 400 MHz, Fig. S5) δ 10.12 (s, 1H), 9.88 (s, 1H), 7.75 (d, J = 8.4 Hz, 1H), 7.62 (s, 1H), 7.37(s, 1H), 7.33 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H),6.26 (s, 1H), 3.74 (t, J = 4.4 Hz, 4H), 3.07 (t, J = 4.4 Hz, 4H), 2.41 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz, Fig. S6) δ 166.2, 163.9, 159.8, 154.1, 153.0, 147.7, 142.1, 130.2, 126.5, 120.0, 115.7, 114.4, 111.9, 104.9, 66.0, 48.6, 17.9; negative LR ESI MS: 430.73 ([M–H]$^-$) (Fig. S7); positive HR ESI MS for $C_{24}H_{22}O_5N_3$ ([M+H]$^+$) calcd: 432.1554, found: 432.1553 and negative HR ESI MS for $C_{24}H_{20}O_5N_3$ ([M−H]$^-$) calcd: 430.1408, found: 430.1413 (Fig. S8).

Compound 2. To a solution of compound **3** (50 mg, 0.17 mmol) in DMSO (2 mL) was added benzenamine (37 mg, 0.40 mmol) and zinc trifluoromethylsulfonate (50 mg, 0.13 mmol). The resulting solution was heated at 60 °C for 2 d. The formed precipitates were collected through filtration, washed subsequently with methanol and distilled water and dried under vacuum to give compound **2** (13 mg, 23%). ^{1}H NMR (DMSO-d_6, 400 MHz, Fig. S9) δ 10.21 (s, 1H), 10.00 (s, 1H), 7.79 (d, J = 8.4 Hz, 1H), 7.63 (d, J = 2.0 Hz, 1H), 7.48 (d, J = 8.0 Hz, 2H), 7.42(s, 1H), 7.38 (d, J = 8.4 Hz, 2H), 7.14-7.01 (m, 1H), 6.28 (s, 1H), 2.42 (s, 3H); negative LR ESI MS: 345.74 ([M–H]$^-$) (Fig. S10); positive HR ESI MS for $C_{24}H_{22}O_5N_3$ ([M+H]$^+$) calcd: 347.1026, found: 347.1024 and negative HR ESI MS for $C_{24}H_{20}O_5N_3$ ([M−H]$^-$) calcd: 345.0881, found: 345.0883 (Fig. S11).

Procedures for the measurement of lysosome targeting [1]

Literature procedures were adopted to carry out the lysosome-targeting experiments. Specifically, HeLa cells were incubated at glass slide for 24 h and PBS was added to wash for three times. Compound **1** or **2** was added and incubated at 37 °C for 6h. Then the glass slide was washed with PBS and the LysoTracker™ Deep Red (1 μM) was added for an additional 2 h. The supernatant was removed, and cells growing on the glass slide were washed with PBS. Paraformaldehyde (4%, 1 mL) was added to fix cells for 0.5 h. Then the anti-fluorescence quenching reagent was added and the cells growing on the glass slide were examined by laser scanning confocal microscopy (Ex/Em 405/425-470 nm for blue fluorescence; Ex/Em 561/575-640 nm for red fluorescence).

Procedures for the ion transport test [2,3]

Literature procedures were adopted to prepare the EYPC liposomes and measure the pH discharge activity. Specifically, EYPC (20 mg) was dispersed in 25 mM HEPES buffer (0.1 mM HPTS, 50 mM NaCl, pH 7.0). The mixture was vortexed for 1 min. The dispersion was then incubated at room temperature for 5 min, followed by another 1 min of vortexing and 20 min of incubation at ambient temperature. The sample was subjected to seven freeze/thaw cycles (77 K/325 K), followed by extrusion through a 100 nm Nuclepore membrane (15 times). After extrusion, the dispersion was incubated at room temperature for 1 h, and subject

to gel filtration (Sephadex G-25, eluted with 25 mM HEPES buffer (50 mM NaCl, pH 7.0)) to get rid of the non-entrapped pyranine.

To 1.92 mL of 25 mM HEPES buffer (50 mM NaCl, pH 8.0) was added the vesicle dispersion (0.06 mL), followed by the addition of compound **1** or **2** in DMSO (20 μL). The appearance of fluorescence intensity was monitored as a function of time (ex 460 nm, em 510 nm, ex/em 3.0 nm/3.0 nm). After 300 s, 5 wt% aqueous Triton X-100 (50 μL) was added. The ion-transporting ability was estimated by the relative FI that was calculated using the following equation: relative FI (%) = $(I - I_0)/(I_{total} - I_0) \times 100$, where I_0, I and I_{total} represent the fluorescence intensities of the dispersion at the initial time, after a period of time and after the addition of 5 wt% aqueous Triton X-100, respectively.

Procedures for acridine orange staining

Literature procedures were adopted to carry out acridine orange staining experiments. [4,5] Specifically, HeLa cells were incubated for 24 h and the Petri dish was washed with PBS. Compound **1** or **2** was added and incubated at 37 ^oC for 4 h. Then the Petri dish was washed with PBS and Acridine Orange solution (5 μg/mL) was added. Incubation was conducted for an additional 0.5 h. The supernatant was removed, and the Petri dish was washed with PBS. Standard DMEM culture media (2.0 mL) was added and the cells were examined by fluorescent inverted microscope (Ex BP 470/40 nm and Em BP 525/50 nm for green fluorescence; Ex BP 546/12 nm and Em BP 575-640 nm for red fluorescence).

MTT assay [4]

HeLa cells and A549 cell (3×10^3 cells/well) were incubated for 24 h in 96-well plate. Compound **1** or **2** was added and incubated at 37 ^oC for 48 h. Then MTT (5 mg/mL, 10 μL) was added and incubated for 4 h. The supernatant was removed, and DMSO (100 μL) was added. The absorbance values were measured on a Tecan Infinite M1000 PRO microplate reader.

References:

1. L. Li, M. Wu, H. Zhang, K. Li and X. Yu, *Sci. China Chem.*, 2017, **47**, 1029–1034.

2. Y.-M. Lu, L.-Q. Deng, X. Huang, J.-X. Chen, B. Wang, Z.-Z. Zhou, G.-S. Hu and W.-H. Chen, *Org. Biomol. Chem.*, 2013, **11**, 8221–8227.
3. Z. Li, L.-Q. Deng, J.-X. Chen, C.-Q. Zhou and W.-H. Chen, *Org. Biomol. Chem.*, 2015, **13**, 11761–11769.
4. X.-H. Yu, C.-C. Peng, X.-X. Sun and W.-H. Chen, *Eur. J. Med. Chem.*, 2018, **152**, 115–125.
5. A. M. Rodilla, L. Korrodi-Gregório, E. Hernando, P. Manuel-Manresa, R. Quesada, R. Pérez-Tomás and V. Soto-Cerrato, *Biochem. Pharm.*, 2017, **126**, 23–33.

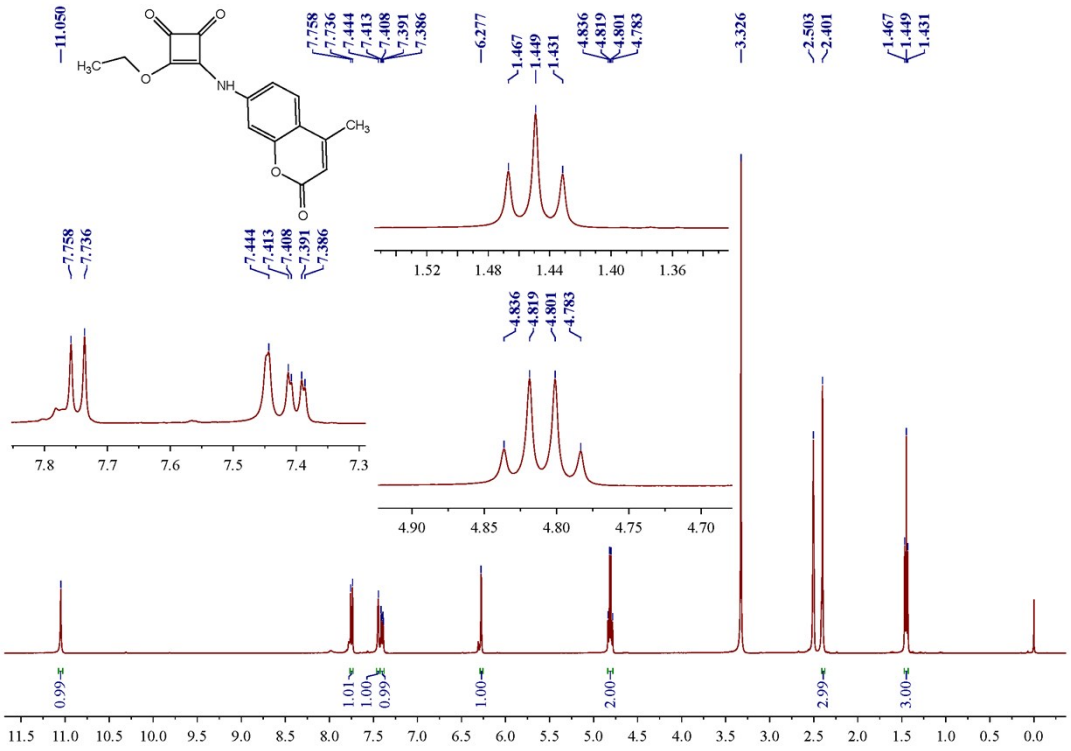

Fig. S1. ^{1}H NMR (DMSO-d_6, 400 MHz) of compound **3**.

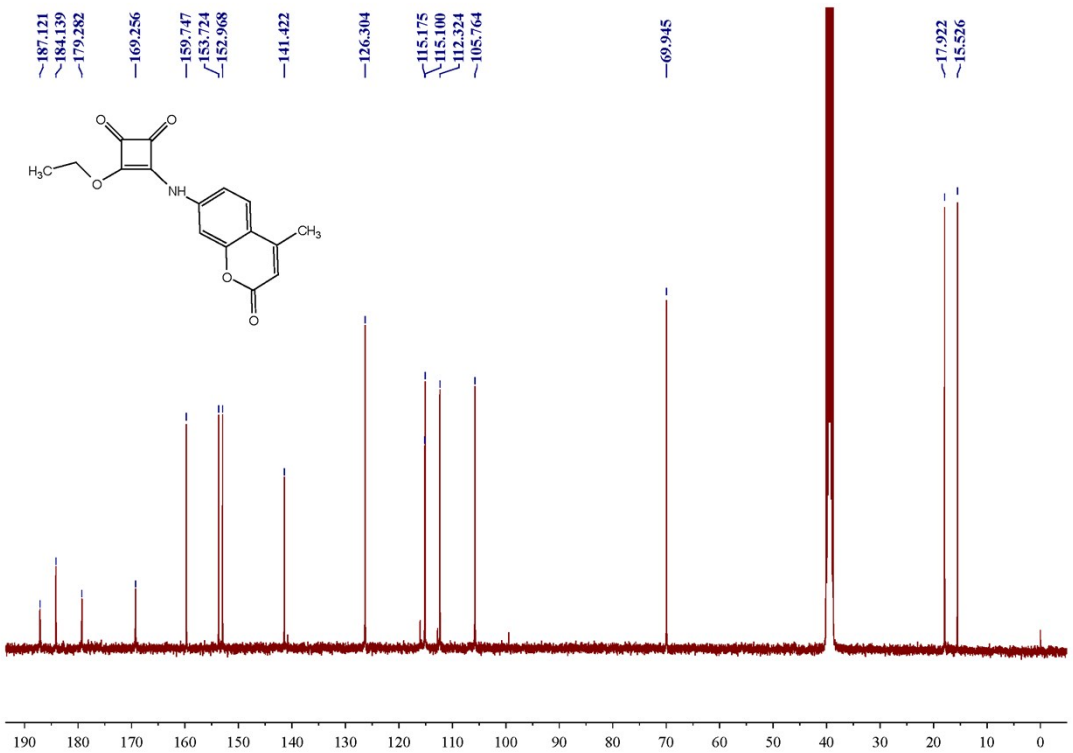

Fig. S2. ^{13}C NMR (DMSO-d_6, 100 MHz) of compound **3**.

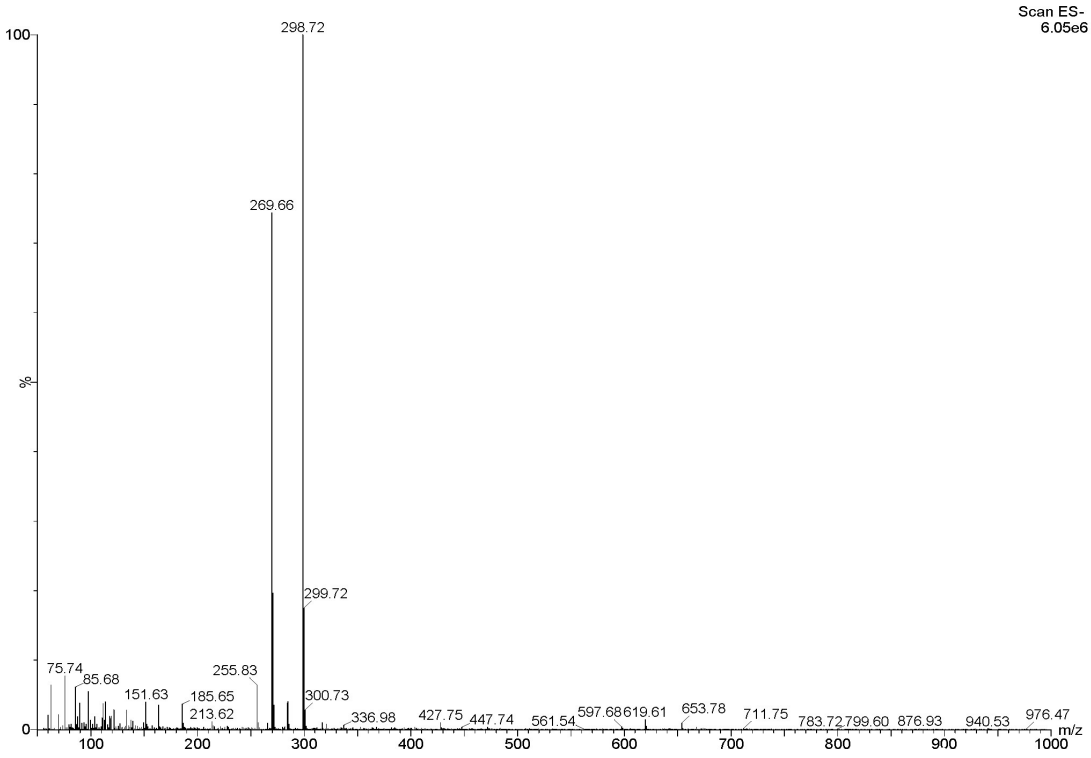

Fig. S3. Negative LR ESI MS of compound **3**.

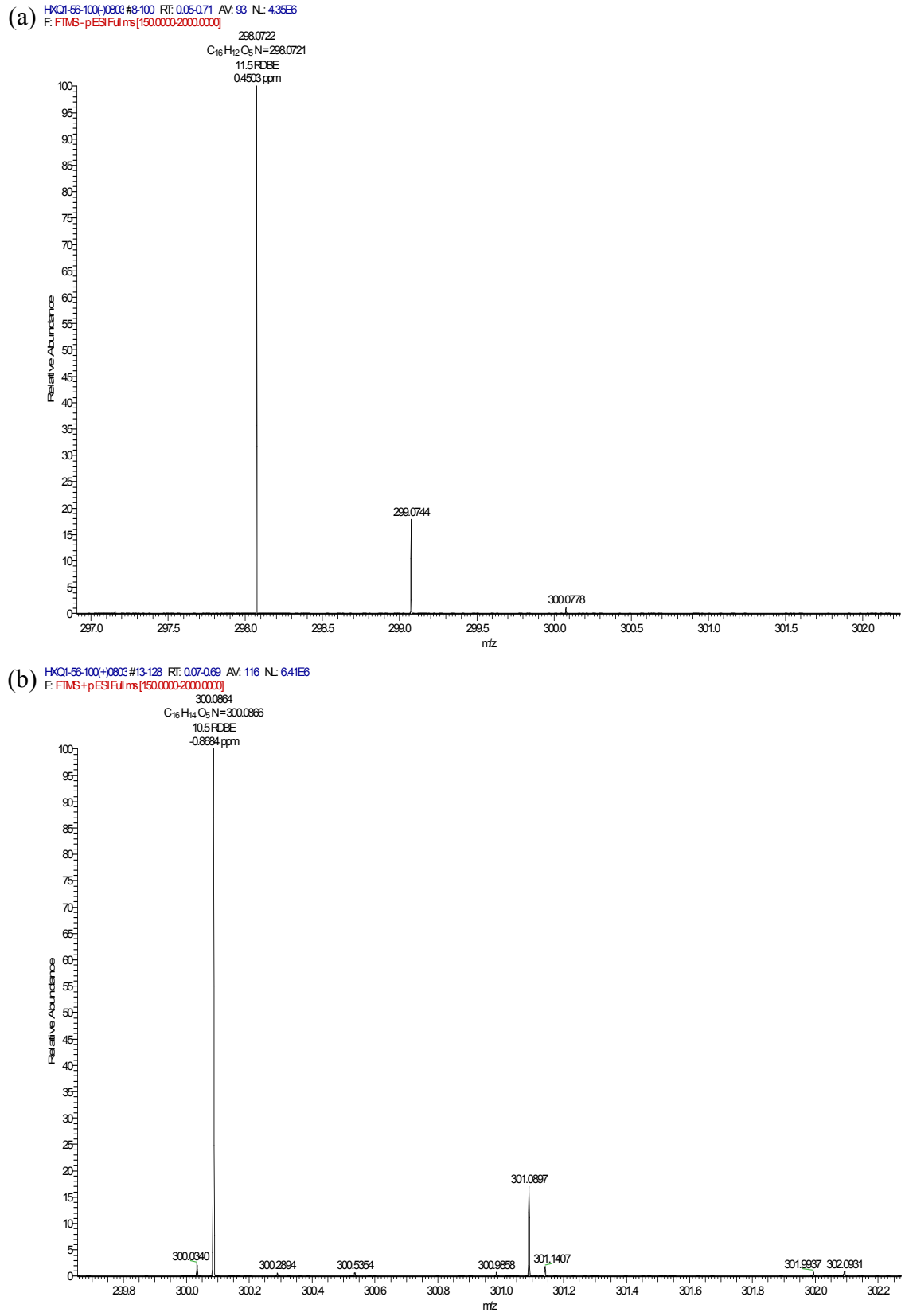

Fig. S4. (a) Negative and (b) positive HR ESI MS of compound **3**.

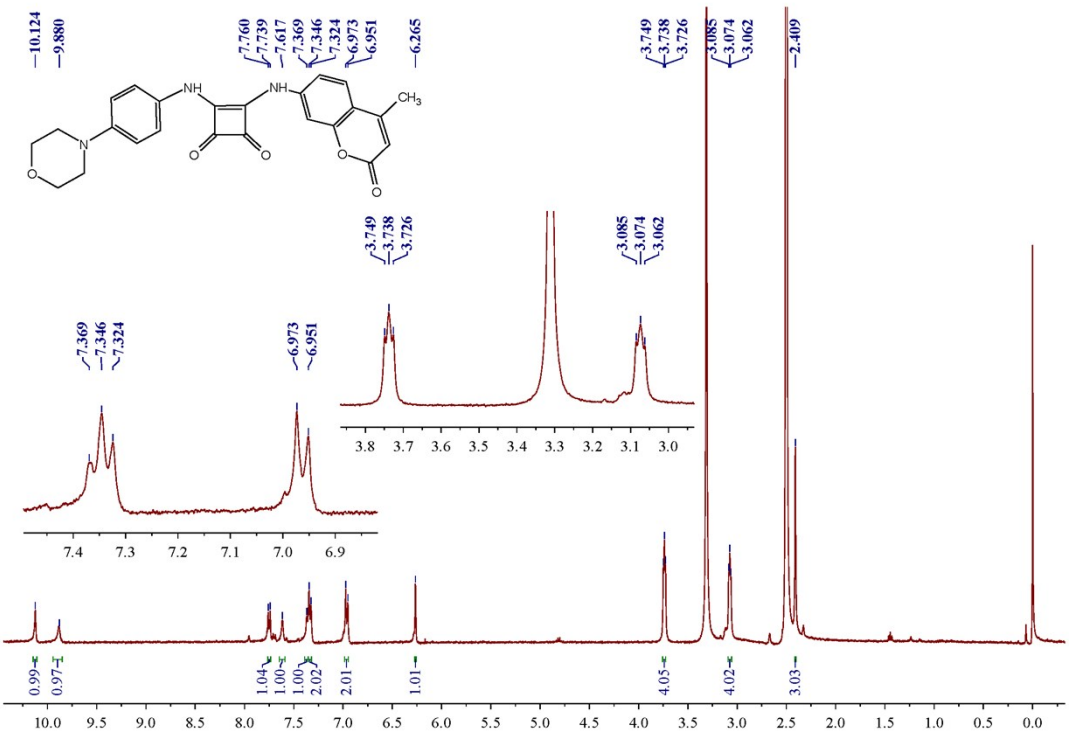

Fig. S5. ^{1}H NMR (DMSO-d_6, 400 MHz) of compound **1**.

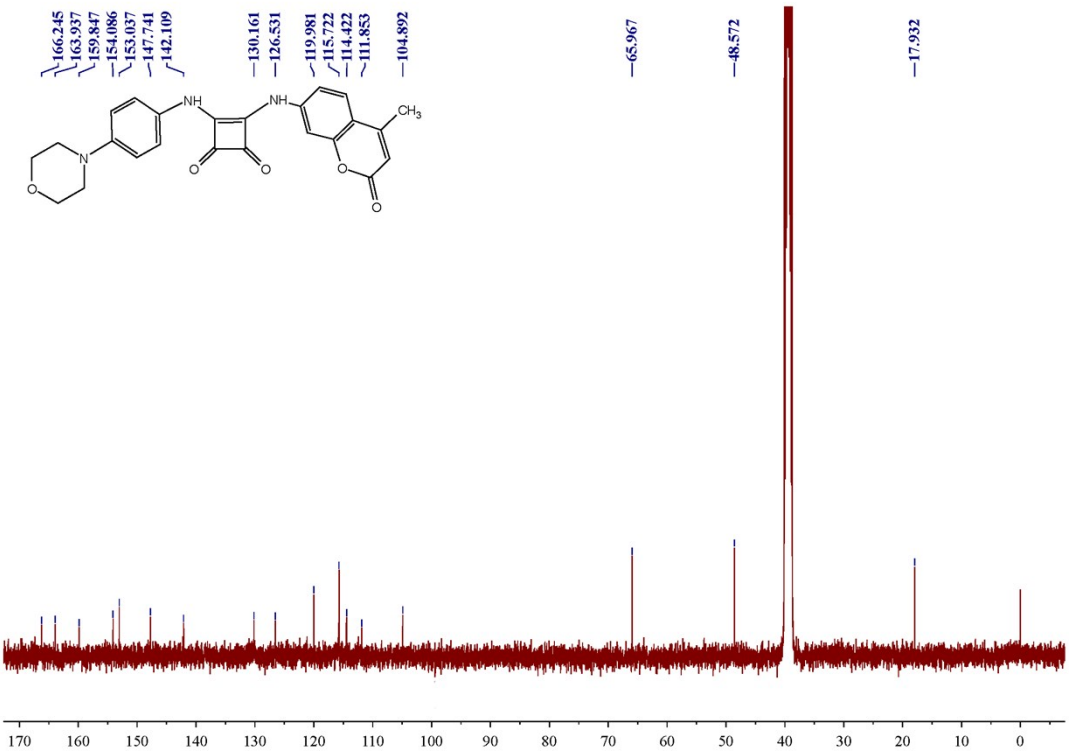

Fig. S6. ^{13}C NMR (DMSO-d_6, 100 MHz) of compound **1**.

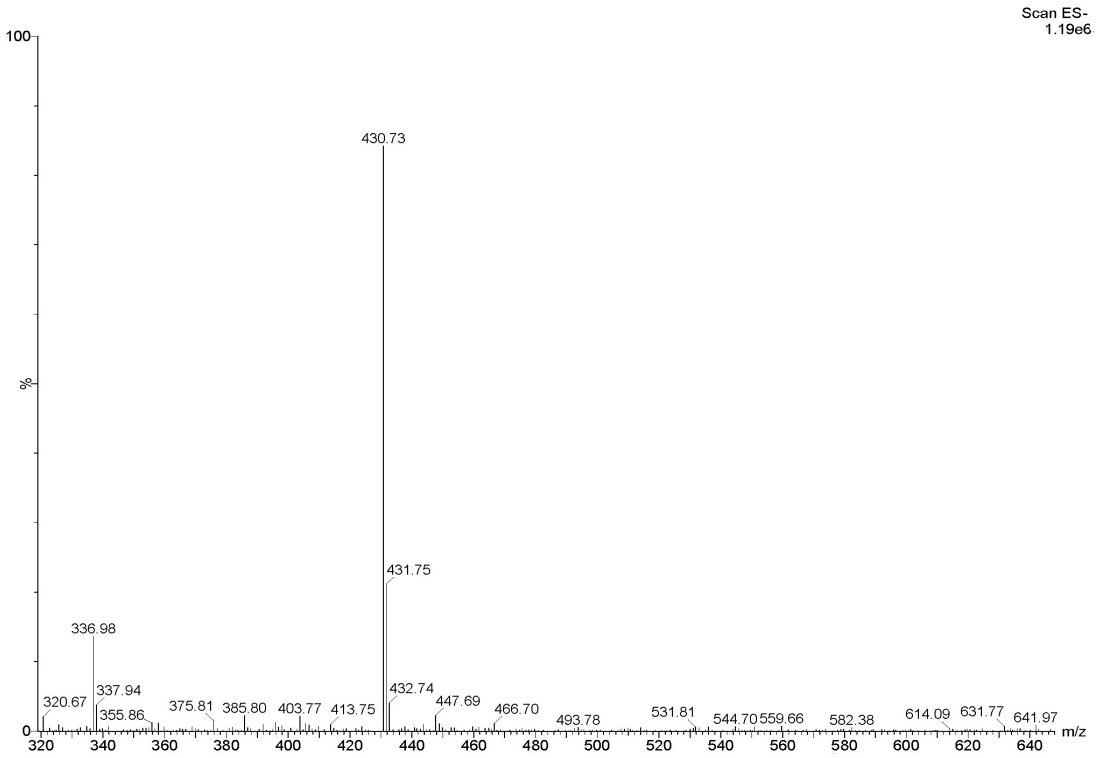

Fig. S7. Negative LR ESI MS of compound **1**.

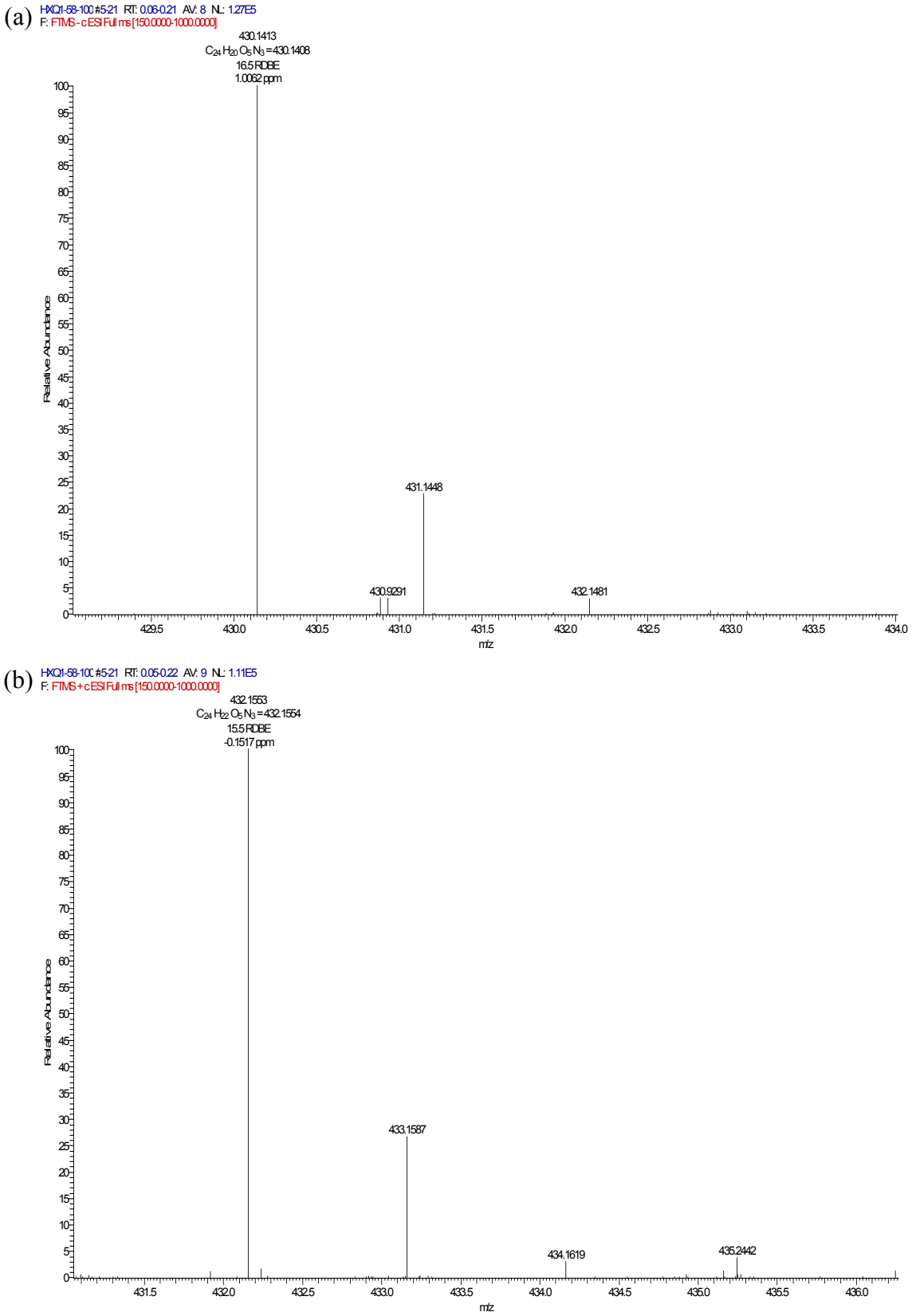

Fig. S8. (a) Negative and (b) positive HR ESI MS of compound **1**.

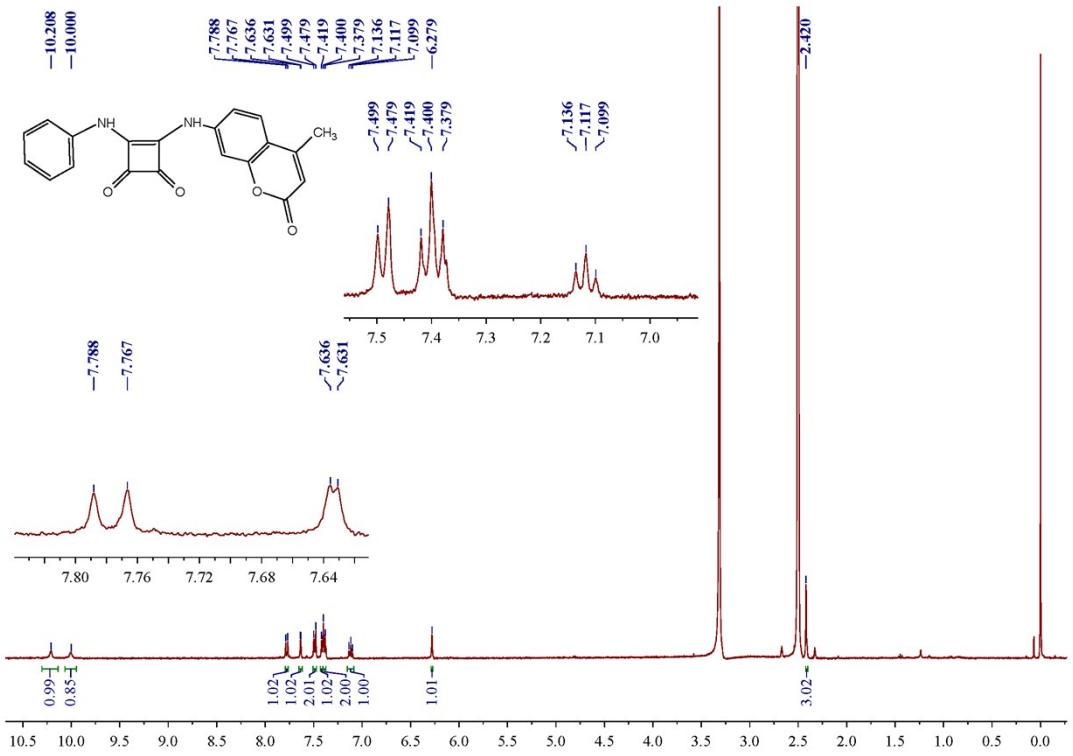

Fig. S9. ^{1}H NMR (DMSO-d_6, 400 MHz) of compound **2**.

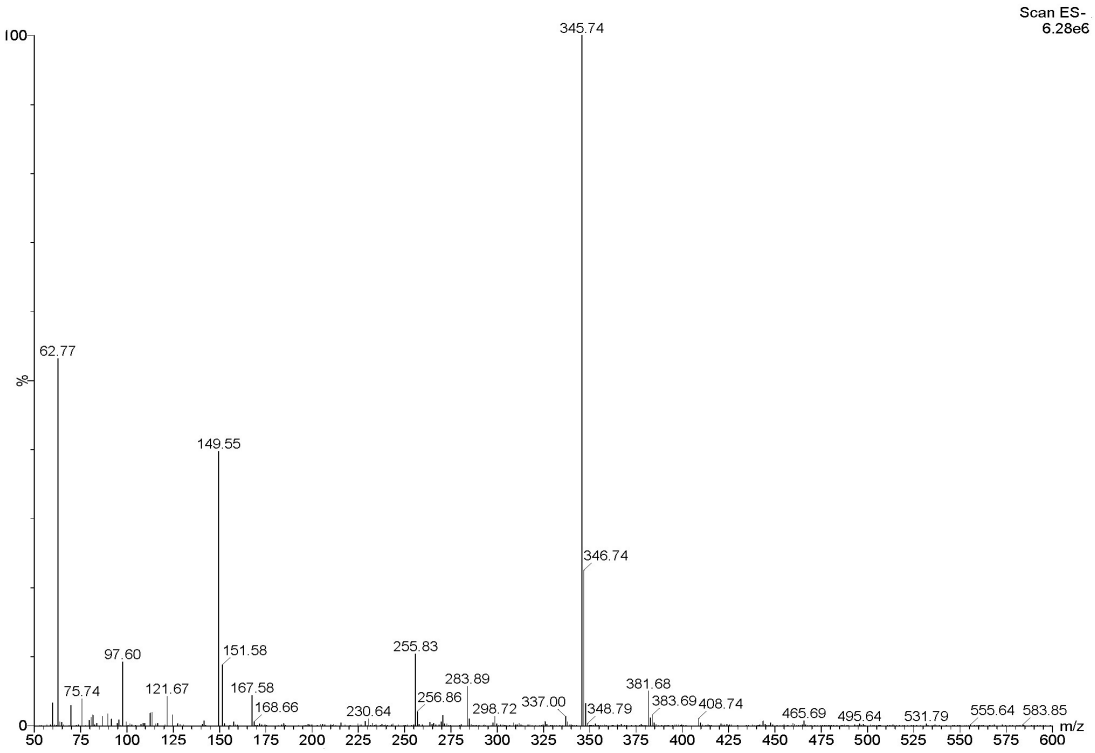

Fig. S10. Negative LR ESI MS of compound **2**.

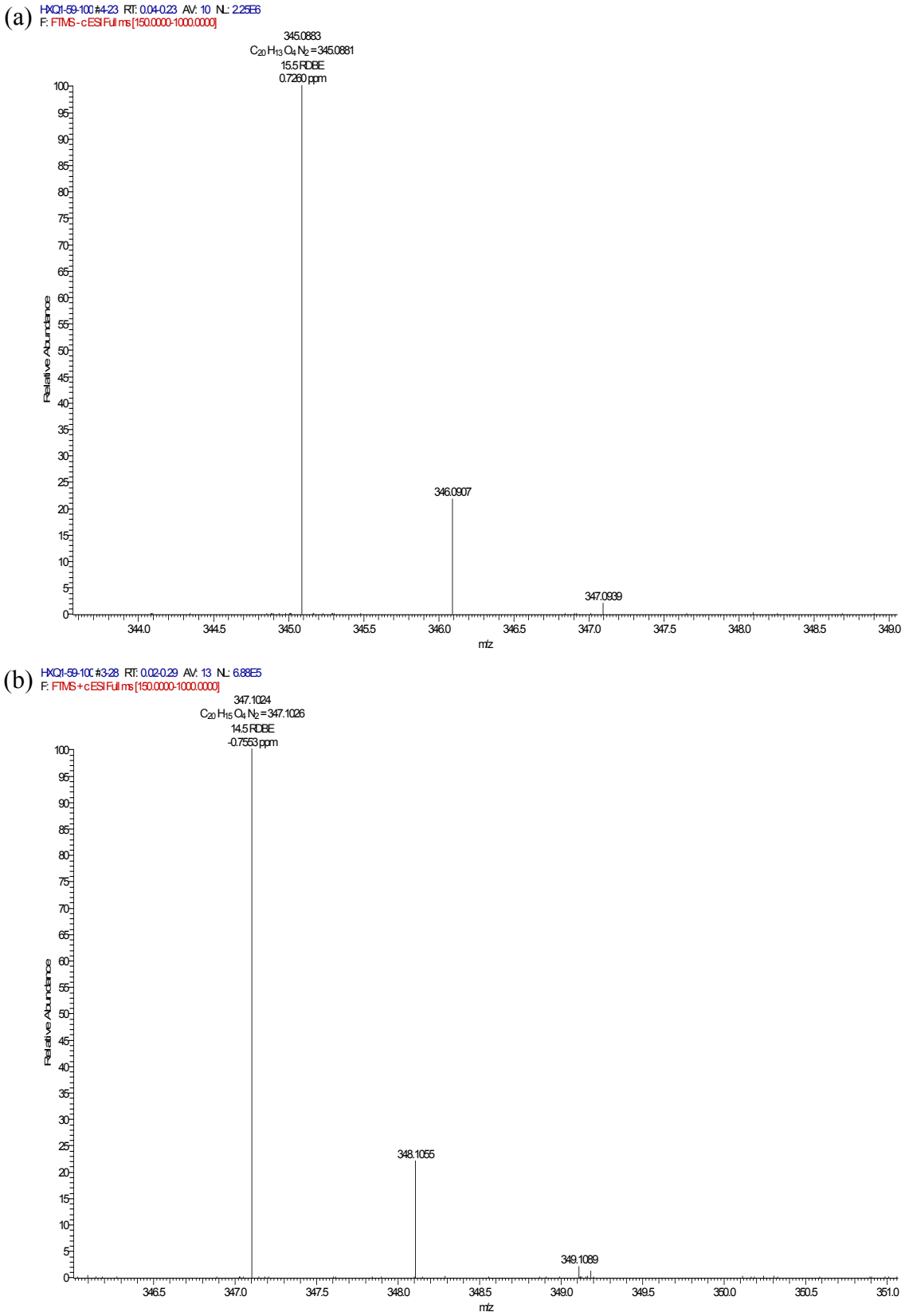

Fig. S11. (a) Negative and (b) positive HR ESI MS of compound **2**.

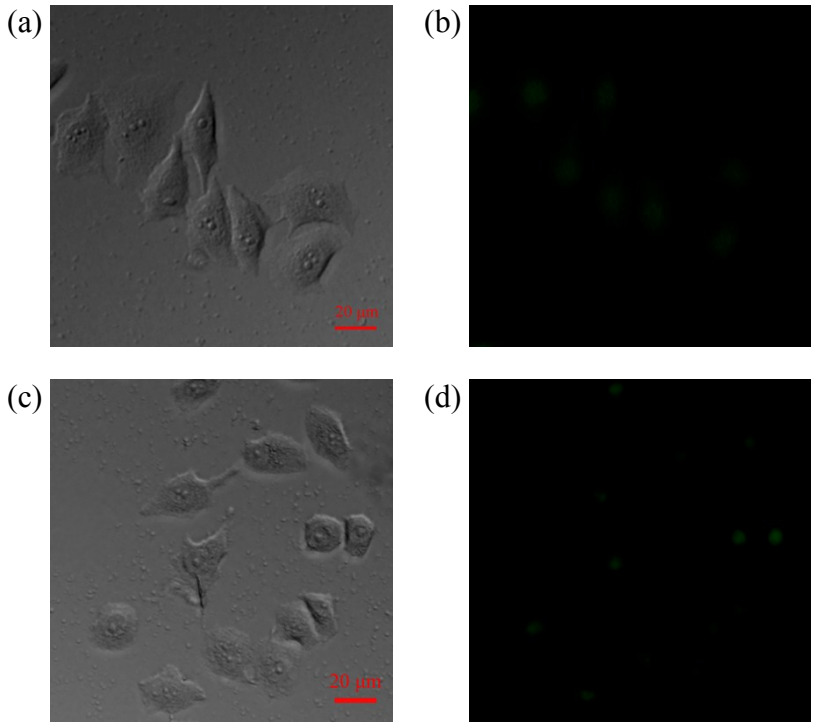

Fig. S12. Imaging of HeLa cells by compound **1**. (a) Bright field (10 μM); (b) Green fluorescence (10 μM); (c) Bright field (20 μM) and (d) Green fluorescence (20 μM). Green fluorescence: Ex BP 470/40 nm, Em BP 525/50 nm.

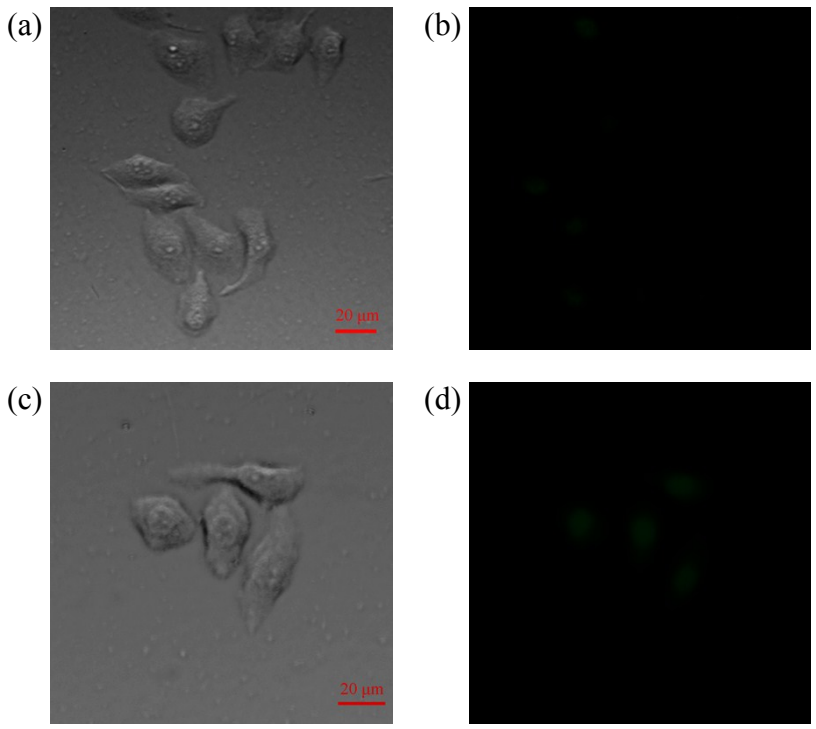

Fig. S13. Imaging of HeLa cells by compound **2**. (a) Bright field (10 μM); (b) Green fluorescence (10 μM); (c) Bright field (20 μM) and (d) Green fluorescence (20 μM). Green fluorescence: Ex BP 470/40 nm, Em BP 525/50 nm.

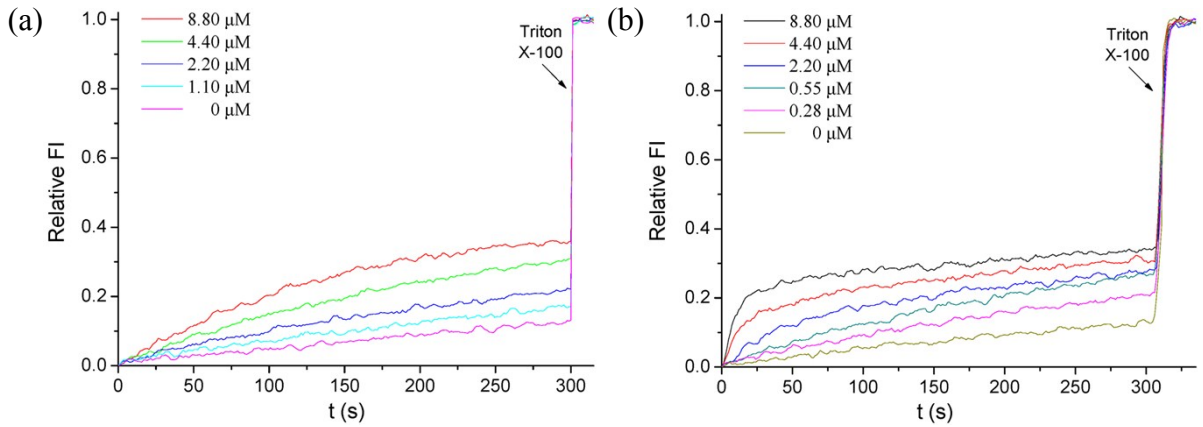

Fig. S14. Plots of the relative fluorescent intensity (FI) of HPTS against time in the presence of compound **1** (a) or **2** (b) of varying concentrations in EYPC-based liposomes. Intravesicular conditions: 0.1 mM HPTS in 25 mM HEPES (50 mM NaCl, pH 7.0) and extravesicular conditions: 25 mM HEPES (50 mM NaCl, pH 8.0).

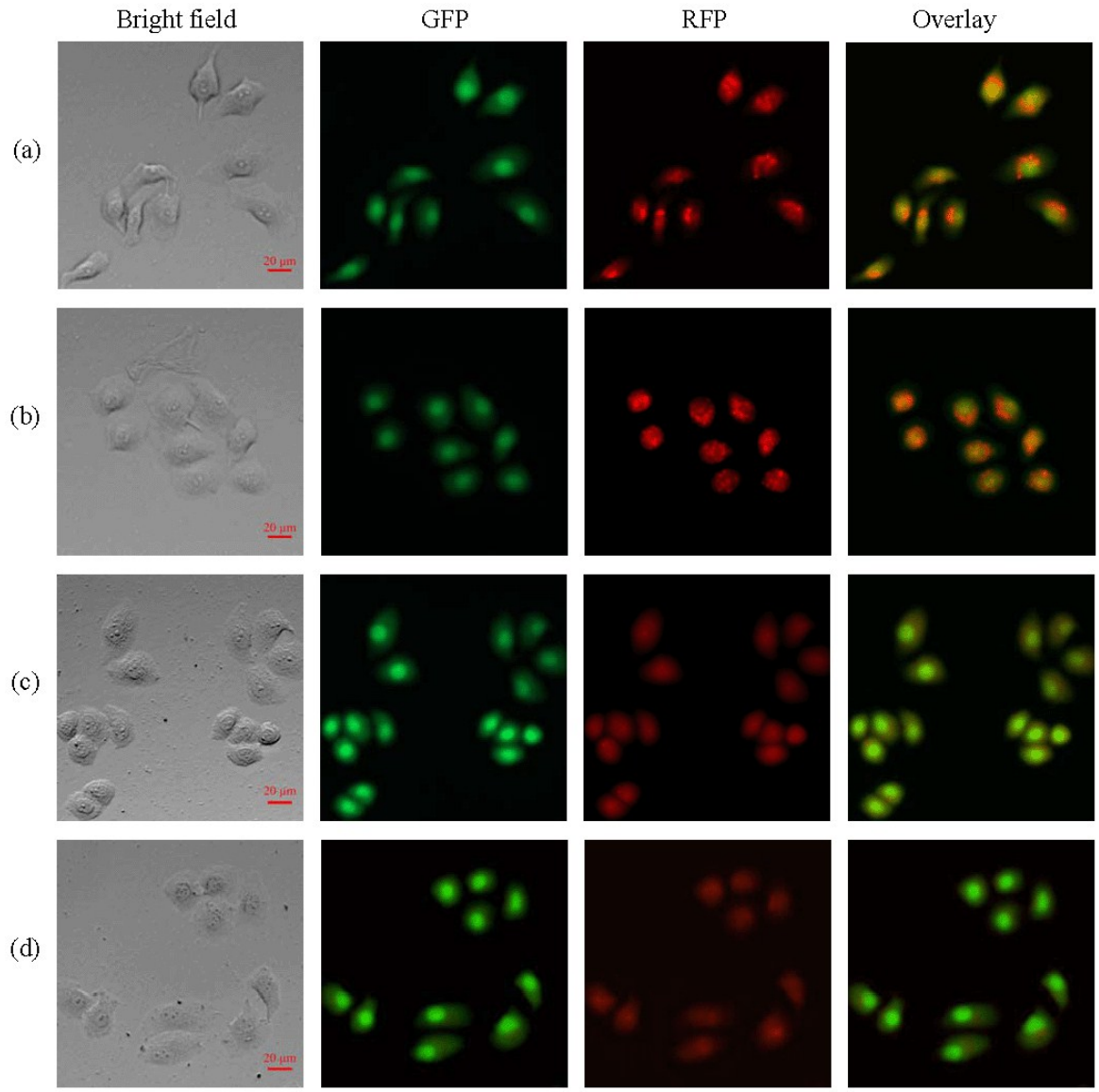

Fig. S15. Acridine orange staining of HeLa cells. (a) Untreated cells (control); (b, c, d) Cells treated with compound **1** of 5 μM, 10 μM and 20 μM for 4 h, respectively. Green fluorescence: Ex BP 470/40 nm, Em BP 525/50 nm; Red fluorescence: Ex BP 546/12 nm, Em BP 575-640 nm.

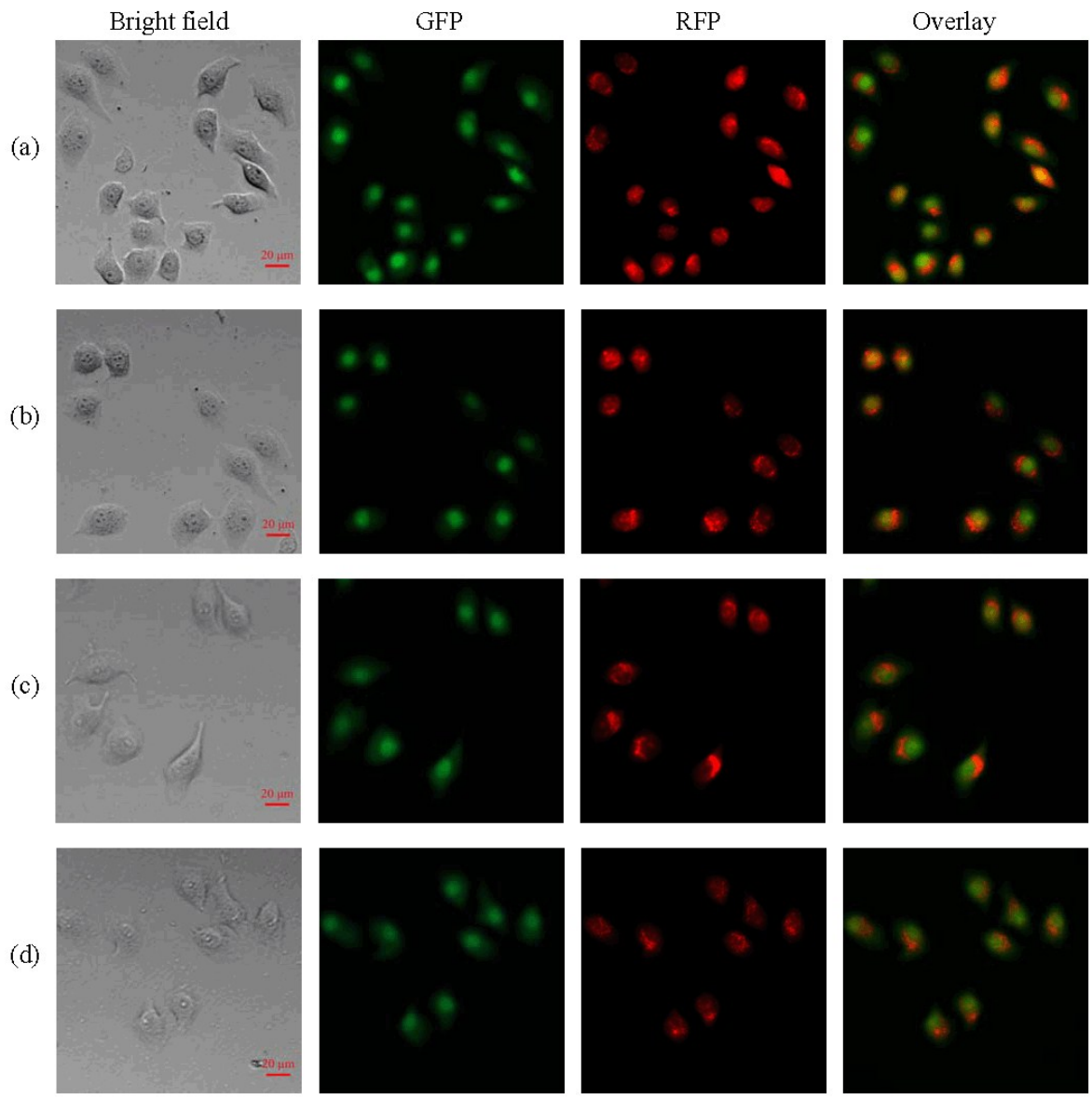

Fig. S16. Acridine orange staining of HeLa cells. (a) Untreated cells (control); (b, c, d) Cells treated with compound **2** of 5 μM, 10 μM and 20 μM for 4 h, respectively. Green fluorescence: Ex BP 470/40 nm, Em BP 525/50 nm; Red fluorescence: Ex BP 546/12 nm, Em BP 575-640 nm.

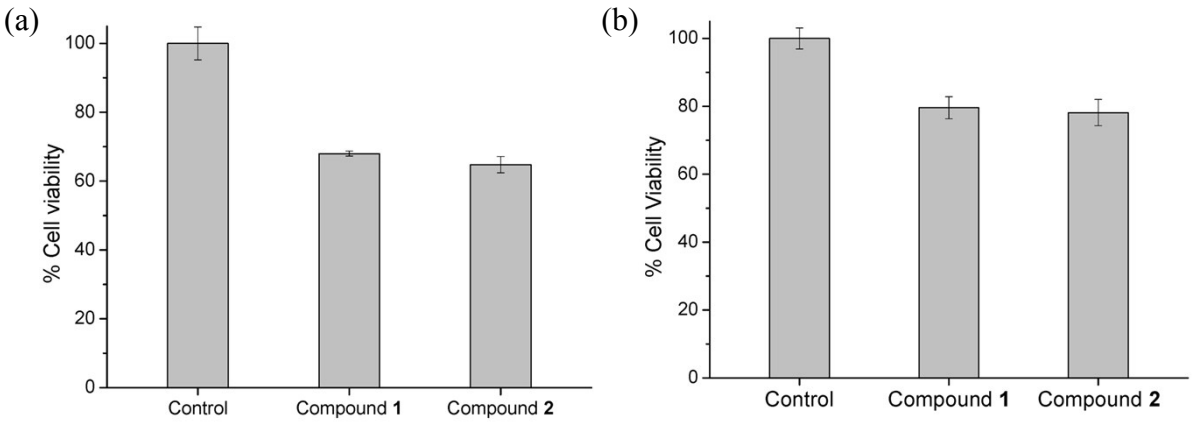

Fig. S17. MTT-based cell viability after the HeLa (a) and A549 (b) cancer cells were treated with compounds **1** and **2** (25 μM) for 48 h separately.