Supporting Information for

A Fluorescent Probe for Ratiometric Imaging Exogenous and Intracellular Formed Hypochlorous Acid in Lysosomes

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Table of contents

Page

Materials and instruments……..…….….. S3

Method of spectral measurements…….…….….. S3

Culture and preparation of HeLa and RAW 264.7 cells…….....S4

Cytotoxicity assay…….…….…….....S4

Imaging of HOCl in living cells …….…….....S4

Synthesis…… …….…….….S6

Figure S1…… …….…….S9

Figure S2…… ……..…….S9

Figure S3…….…..…….S10

Figure S4…….…..…….S10

Spectral characterization…….……...…S11-S14

Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments; Mass spectrometric analyses were measured on a Finnigan MAT 95 XP spectrometer; High resolution mass spectrometric (HRMS) analyses were measured on an Agilent 1100 HPLC/MSD spectrometer; NMR spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a Shimadzu UV-2700 power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer with a 1 cm standard quartz cell; The fluorescence imaging of cells was performed with a Nikon A1MP confocal microscope; The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

Method of spectral measurements

Unless otherwise noted, all the measurements were made according to the following procedure. A stock solution (1.0 mM) of **FL-HA** was prepared by dissolving the requisite amount of it in DMF. In a 10 mL tube the test solution of compounds **FL-HA** was prepared by placing 0.1 mL of stock solution, 4.9 mL of DMF, 5 mL of 0.1 M PBS buffer (different pH). After adjusting the final volume to 10 mL with distilled-deionized water, standing at room temperature 3 min, 3 mL portion of it was transferred to a 1 cm quartz cell to measure absorbance or fluorescence. The stock solutions of metal ions for selectivity experiments were prepared respectively by dissolving TBHP (tert -Butyl hydroperoxide), H_2O_2, NO, DTBP (Di-t-butyl peroxide), NaOH, $NaNO_2$, $NaNO_3$, NaI, Na_2S, Cys, GSH, Hcy, $CuSO_4$, $CoCl_2$, $CaCl_2$, $MgSO_4$, KCl and NaClO in twice-distilled water. The slight pH variations of the solutions were achieved by adding the minimum volumes of NaOH (0.1 M) or HCl (0.2 M).

Culture and preparation of HeLa and RAW 264.7 cells

HeLa cells and RAW 264.7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. Before the experiments, seed the HeLa or RAW 264.7 cells in 35-mm glass-bottomed dishes at a density of 2×10^5 cells per dish in 2 mL of culture medium and incubate them inside an incubator containing 5% CO_2 and 95% air at 37 °C. Incubate the cells for 24 h. Cells will attach to the glass surface during this time.

Cytotoxicity assay

In vitro cytotoxicity was measured using the colorimetric methyl thiazolyl tetrazolium (MTT) assay on HeLa cells. Cells were seeded into the 24-well tissue culture plate in the presence of 500 μL Dulbecco's modifed eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO_2 atmosphere for overnight and then incubated for 24 h in the presence of **FL-HA** at different concentrations (0, 5, 10, 20, 30, 50 μM). Then cells were washed with PBS buffer and 500 μL supplemented DMEM medium was added. Subsequently, 50 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. Violet formazan was dissolved in 500 μL sodium dodecyl sulfate solution in the water-DMF mixture. Absorbance of the solution was measured at 570 nm using a microplate reader. The cell viability was determined by assuming 100% cell viability for cells without **FL-HA**.

Imaging of HOCl in living cells

1. Ratiometric imaging of exogenous HOCl in HeLa cells

Before the experiments, the HeLa cells were seeded on two 35-mm glass-bottomed dishes and allowed to adhere for 24 h. the cells were washed with PBS (pH=7.4) buffer three times. Subsequently, the first group was incubating with probe **FL-HA** (10 μM) (containing 0.1 % DMSO as a cosolvent) for 30 min at 37 ˚C, the HeLa cells were rinsed with PBS three times.

The second group was incubating with probe **FL-HA** (10 μM) (containing 0.1 % DMSO as a cosolvent) for 30 min at 37 ˚C, the HeLa cells were rinsed with PBS three times and the cells were incubated with NaOCl (30 μM) for 30 min at 37 ˚C, and then washed with PBS three times, and the fluorescence images were acquired through a Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera

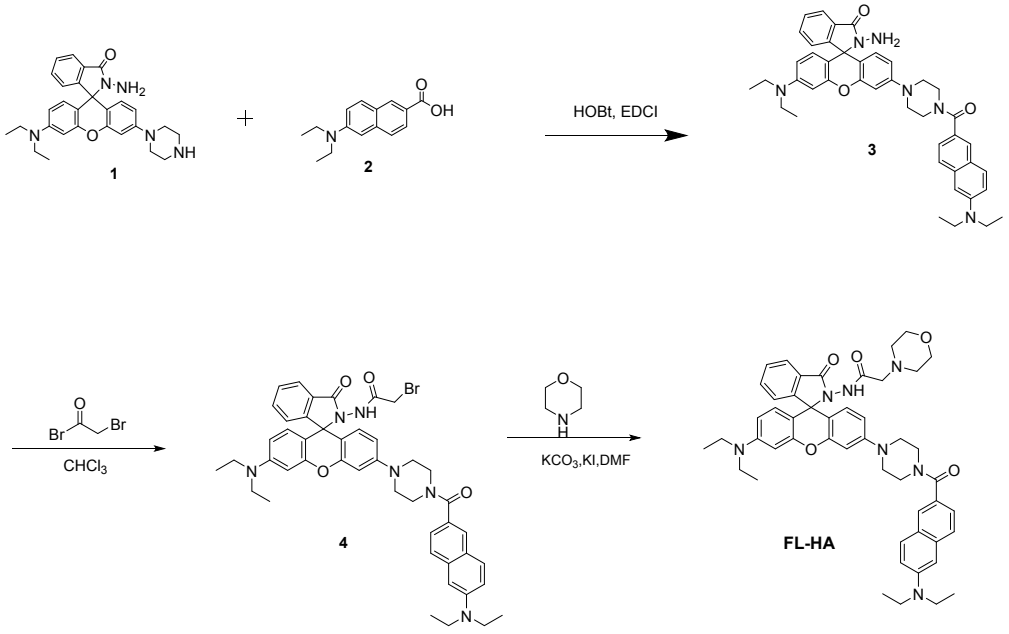
2. Imaging of endogenous HOCl in RAW 264.7 cells

Before the experiments, the RAW 264.7 cells were plated on 6-well plates and allowed to adhere for 24 h and then incubated with probe **FL-HA** (10 μM) for 30 min at 37 ˚C, washed by PBS buffer and subsequently incubated with 2 μg/mL PMA (phorbol 12-myristate13-acetate) and 2 μg/mL LPS (lipopolysaccharides) for 2 h. For the control experiments, the cells without treated with PMA/LPS were incubated with probe **FL-HA** (10 μM) for 2 hours under the same conditions. For negative control group, the RAW 264.7 cells incubated with probe **FL-HA** (10 μM) for 30 min at 37 ˚C, washed by PBS buffer and subsequently incubated with 2 μg/mL PMA, 2 μg/mL LPS and 4-aminobenzoic acid hydrazide (ABH, 200 μM) for 2 h prior to imaging. The cells were washed with PBS (pH=7.4) buffer. The fluorescence images were acquired through a Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera.

Colocation experiment in HeLa cells

HeLa cells were seeded on two 35-mm glass-bottomed dishes and allowed to adhere for 24 h. the cells were washed with PBS (pH=7.4) buffer three times. Subsequently, the cells incubating with probe **FL-HA** (10 μM) (containing 0.1 % DMSO as a cosolvent) and 25 nM LysoTracker Green for 30 min at 37 ˚C, the cells were rinsed with PBS three times and the cells were incubated with NaOCl (30 μM) for 20 min at 37 ˚C, and then washed with PBS three times, and the fluorescence images were acquired through a Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera.

Synthesis



Scheme 1 Synthesis of the probe ***FL-HA***

Compound **1** was synthesized according to the reported method. [1] Yield: 90%

Compound **2** was synthesized according to the reported method. [2] Yield: 80%

Synthesis of compound 3

Compound **2** (181mg, 0.75 mmol, 1.0 eq) was dissolved in DMF (5 mL), HOBt (101 mg, 0.75 mmol, 1.0 eq) and EDCI (144 mg, 0.75 mmol, 1.0 eq) were added to the solution and reacted at 45°C for 30 min, and then added the compound **1** (350 mg, 0.75mmol, 1.0 eq) to the mixture. The reaction mixture was reacted at the room temperature for 3-4 h, and the poured into 20 mL water. The aqueous suspension was extracted with dichloromethane. The combined organic phase was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by column chromatography on silica gel (MeOH / CH_2Cl_2 = 1: 20, v/v) to afford the compound compound **3** as a white powder (360 mg, yield: 70%). ^{1}H NMR (400 MHz, DMSO) δ 7.83 – 7.75 (m, 2H), 7.66 (d, *J* = 8.6 Hz, 1H), 7.49 (dd, *J* = 8.8, 5.2 Hz, 2H), 7.36 (dd, *J* = 8.5, 1.5 Hz, 1H), 7.20 (dd, *J* = 9.2, 2.4 Hz, 1H), 7.01 – 6.97 (m, 1H), 6.92 (d, *J* = 2.1 Hz, 1H), 6.74 (d, *J* = 2.2 Hz, 1H), 6.67 (dd, *J* =

8.8, 2.3 Hz, 1H), 6.43 (d, *J* = 8.7 Hz, 2H), 6.36 (d, *J* = 4.5 Hz, 2H), 4.36 (s, 2H), 4.13 (q, *J* = 5.2 Hz, 1H), 3.68 (s, 3H), 3.50 – 3.43 (m, 4H), 3.38 – 3.30 (m, 4H), 3.25 (s, 3H), 3.17 (d, *J* = 5.2 Hz, 2H), 1.16 (t, *J* = 7.0 Hz, 6H), 1.08 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 170.11, 165.88, 153.34, 153.13, 152.12, 151.80, 148.68, 146.91, 136.07, 132.93, 130.10, 129.96, 128.72, 128.13, 127.97, 127.30, 126.01, 125.36, 124.85, 123.92, 122.72, 116.74, 112.13, 110.27, 108.51, 105.74, 104.61, 102.53, 97.87, 65.12, 63.50, 48.36, 44.27, 44.15, 41.97, 30.26, 29.11, 23.50, 23.08, 14.46, 12.98, 12.90, 11.49. HRMS (EI) *m/z* calculated for $C_{43}H_{48}N_6O_3$: 696.3788. Found 696.3746 (M^+)

Synthesis of compound 4

Compound **3** (100 mg, 0.16 mmol, 1.0 eq) was dissolved in $CHCl_3$ (15 mL), under nitrogen atmosphere, triethylamine (45 μl, 0.32 mmol, 2.0 eq) and bromoacetyl bromide (30 μL, 0.32 mmol, 2.0 eq) were added to the solution and reacted at 0°C for 1h. Then, the mixture was warmed to the room temperature and reacted for 3 h. The reaction mixture was poured into water and was extracted with dichloromethane (3×30 mL). The combined organic phase was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by column chromatography on silica gel (MeOH / CH_2Cl_2 = 1: 20, v/v) to afford the compound compound **4** as a white powder (75 mg, yield: 70%).^{1}H NMR (400 MHz, DMSO) δ 10.00 (s, 1H), 7.85 (dd, *J* = 6.4, 1.8 Hz, 1H), 7.79 (d, *J* = 9.0 Hz, 2H), 7.66 (d, *J* = 8.6 Hz, 1H), 7.60 – 7.55 (m, 2H), 7.36 (dd, *J* = 8.5, 1.6 Hz, 1H), 7.20 (dd, *J* = 9.2, 2.4 Hz, 1H), 7.04 (d, *J* = 6.5 Hz, 1H), 6.92 (d, *J* = 2.2 Hz, 1H), 6.68 – 6.63 (m, 2H), 6.58 (d, *J* = 8.6 Hz, 1H), 6.49 (d, *J* = 8.8 Hz, 1H), 6.39 – 6.31 (m, 2H), 3.76 (s, 2H), 3.47 (q, *J* = 6.9 Hz, 4H), 3.36 – 3.24 (m, 12H), 1.16 (t, *J* = 7.0 Hz, 6H), 1.08 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO) δ 170.11, 165.25, 163.83, 153.37, 153.16, 151.92, 148.91, 146.88, 136.07, 133.93, 129.97, 129.72, 129.13, 128.81, 128.06, 127.32, 126.01, 125.37, 124.81, 124.34, 123.22, 116.72, 111.69, 108.42, 104.55, 104.21, 101.90, 97.45, 65.37, 48.15, 44.27, 44.11, 27.29, 26.15, 23.48, 12.97, 12.90. HRMS (EI) *m/z* calculated for $C_{45}H_{47}BrN_6O_4$: 815.2920. Found 815.2905 (M+H).

Synthesis of compound FL-HA

To a mixture of compound 4 (82.0 mg, 0.10 mmol, 1.0 eq) in dry DMF (3 mL) was added K_2CO_3 (41.4 mg, 0.3 mmol, 3.0 eq) and KI (16.6 mg, 0.10 mmol, 1.0 eq), the reaction solution was stirred for 10 min. Morpholine (44 mg, 0.50 mmol ,5.0 eq) was added into the solution. The reaction was stirred for 6 h at the room temperature. Then, the reaction was poured into water and extracted with ethyl acetate (3×20 mL). The combined extracts were washed with water three times, dried over hydrous Na_2SO_4, and concentrated under reduced pressure. The oil residue was purified by column chromatography on silica gel using ethanol/dichloromethane (v/v 1 : 10) to afford a white solid as compound **FL-HA** (50 mg, yield: 62%) ^{1}H NMR (400 MHz, DMSO) δ 9.41 (s, 1H), 7.85 (d, *J* = 6.8 Hz, 1H), 7.78 (d, *J* = 8.5 Hz, 2H), 7.66 (d, *J* = 8.6 Hz, 1H), 7.58 (dd, *J* = 13.1, 6.7 Hz, 2H), 7.36 (d, *J* = 8.4 Hz, 1H), 7.20 (d, *J* = 9.1 Hz, 1H), 7.06 (d, *J* = 7.1 Hz, 1H), 6.92 (s, 1H), 6.66 (s, 2H), 6.55 (d, *J* = 9.3 Hz, 1H), 6.46 (d, *J* = 8.8 Hz, 1H), 6.40 – 6.29 (m, 2H), 3.68 (s, 4H), 3.50 – 3.42 (m,8H), 3.34 – 3.21 (m, 8H), 2.84 (q, *J* = 14.3 Hz, 2H), 2.12 (s, 4H), 1.16 (t, *J* = 6.9 Hz, 6H), 1.08 (t, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, DMSO) δ 170.12, 167.23, 163.89, 153.47, 153.23, 151.94, 151.63, 148.92, 146.91, 136.08, 133.80, 129.94, 129.45, 129.15, 128.11, 127.30, 126.01, 125.35, 124.85, 124.43, 123.16, 116.75, 111.71, 108.63, 104.61, 104.29, 101.68, 97.29, 66.53, 65.42, 63.50, 59.86, 52.87, 48.14, 44.27, 44.10, 41.97, 30.26, 29.11, 23.50, 23.08, 14.46, 12.98, 12.86, 11.49. HRMS (EI) *m/z* calculated for $C_{49}H_{55}N_7O_5$: 822.4343. Found 822.4319 (M+H).

References:

[1] Yan-Ru Zhang, Xin-Peng Chen, Jing-Shao, Jia-Yi Zhang, Qiong Yuan, Jun-Ying Miao, and Bao-Xiang Zhao, Chem. Commun., 2014, 50, 14241–14244.

[2] Peng-Zhong Chen, Yu-Xiang Weng, Li-Ya Niu, Yu-Zhe Chen, Li-Zhu Wu, Chen-Ho Tung, and Qing-Zheng Yang, Angew. Chem. Int. Ed., 2016, 55, 2759–2763.

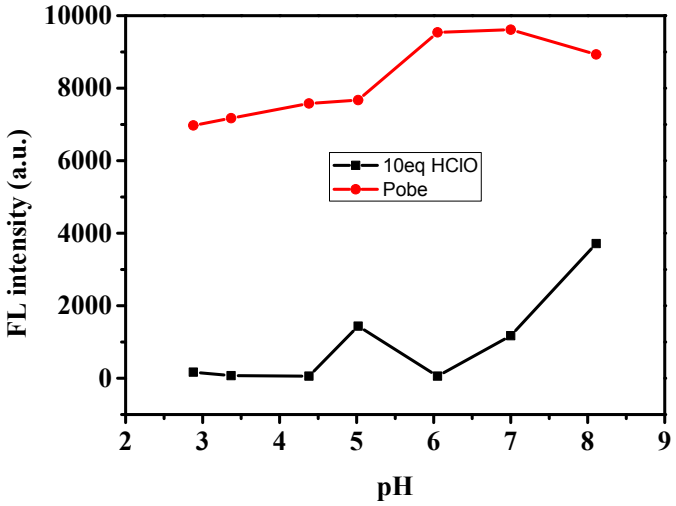


Figure S1 The emission intensity changes (at 455 nm) of the probe **FL-HA** (10μM) before and after addition of NaOCl (10 eq) in PBS buffer with different pH values, containing 50 % DMF as a cosolvent (λ_{ex} = 380 nm).

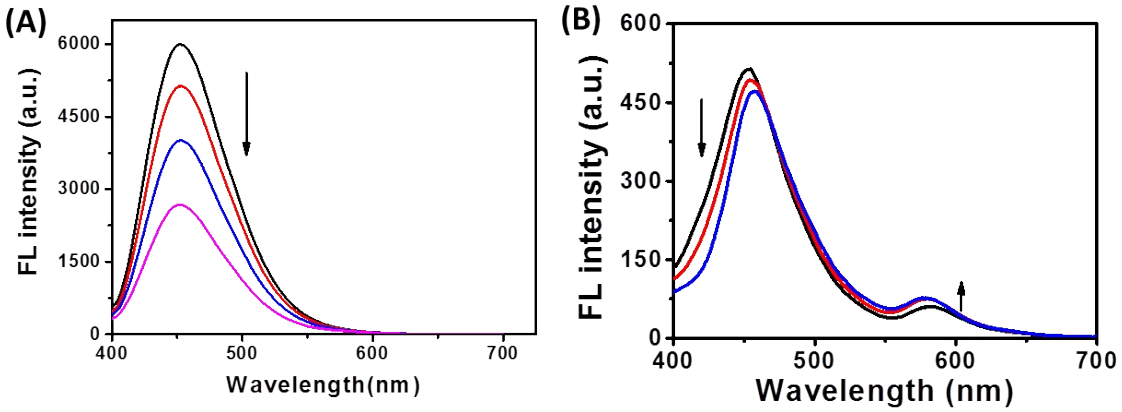


Figure S2 The Fluorescence intensity titration profiles of **FL-HA** (10 μM) in the presence of NaOCl in PBS buffe containing 50 % DMF as a cosolvent excitation at 380 nm. (A) NaOCl 0- 10 eq, (B) NaOCl 20- 30 eq.

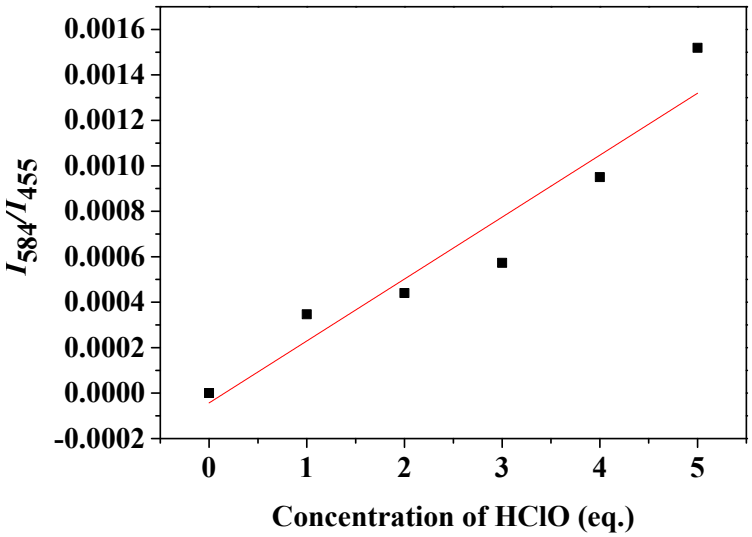


Figure S3 The linear relationship between the fluorescence intensity ratio (I_{584}/I_{455}) and the concentration of HOCl.

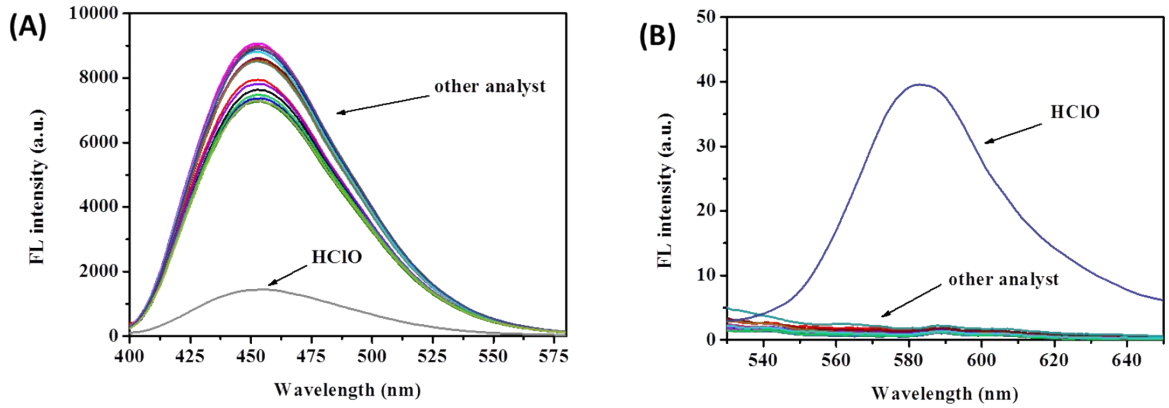


Figure S4 The fluorescence spectra changes of probe **FL-HA** (10 μM) in the presence of various analytes (30 μM) in PBS buffer containing 50% DMF as a cosolvent. (A) Excitation at 380 nm for naphthalene part; (B) Excitation at 530 nm for rhodol fluorophore part.

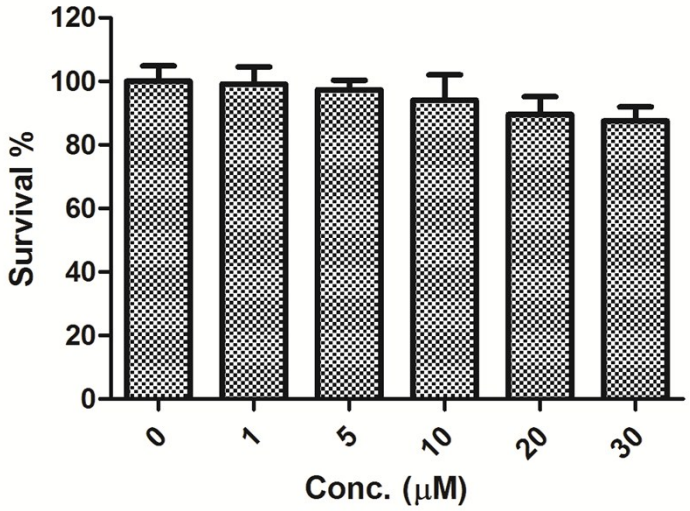


Figure S5 Cytotoxicity assays of **FL-HA** at different concentrations for HeLa cells

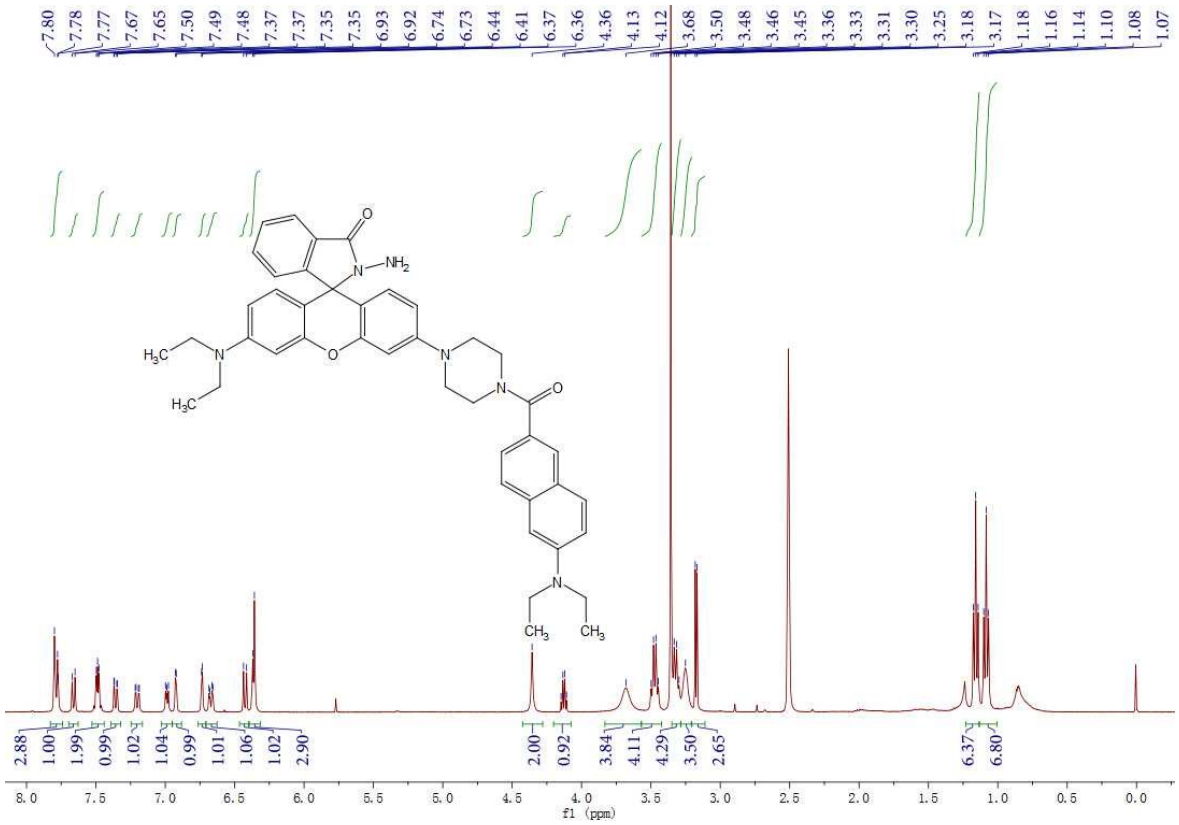


Figure S6 ^{1}H-NMR (DMSO-d_6) spectrum of compound **3**.

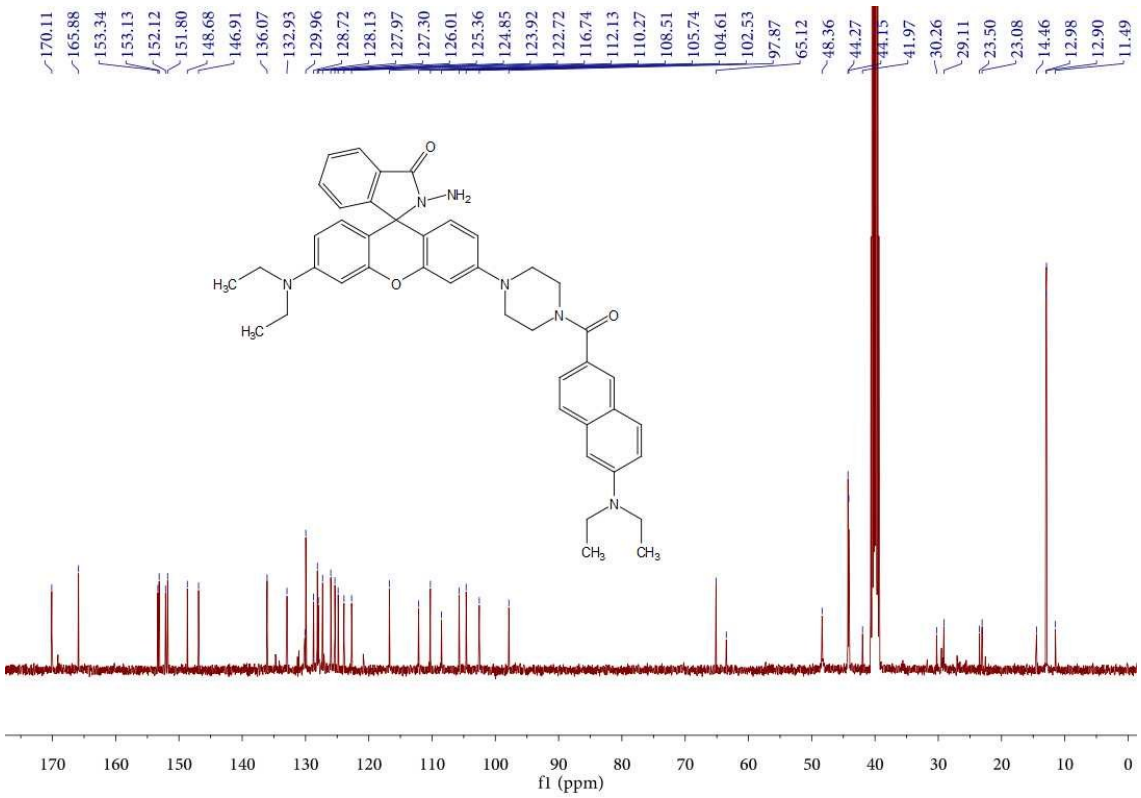


Figure S7 ^{1}C-NMR (DMSO-d_6) spectrum of compound **3**.

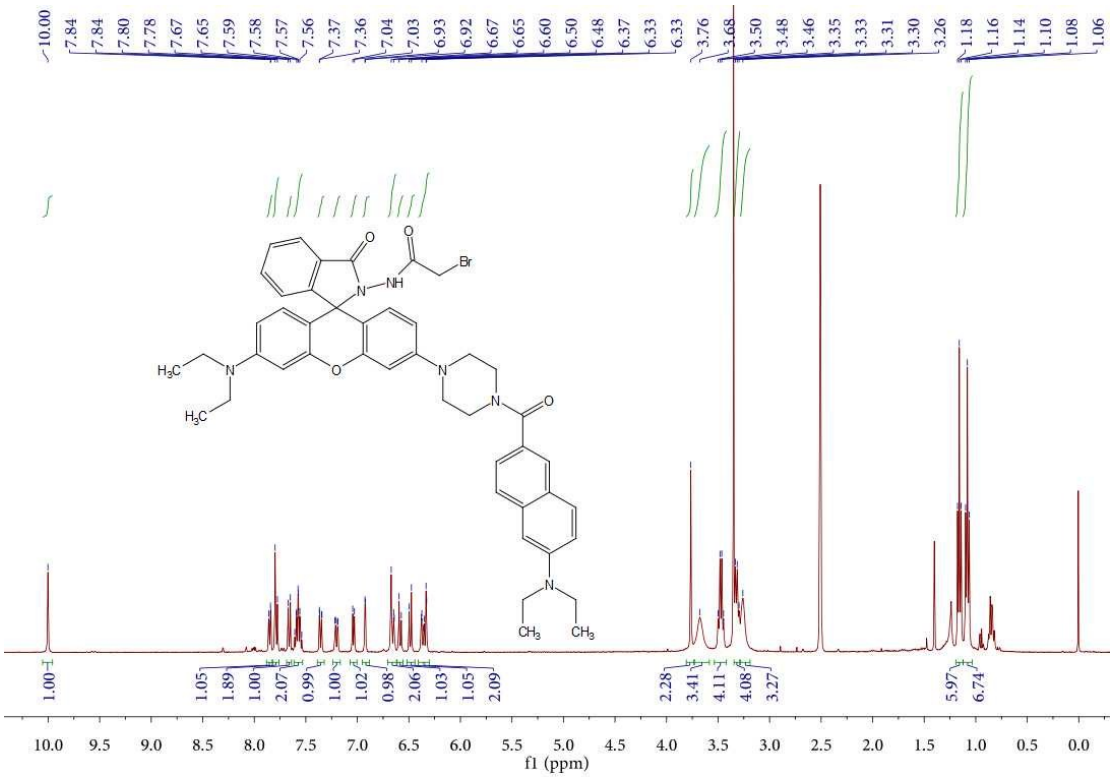


Figure S8 ^{1}H-NMR (DMSO-d_6) spectrum of compound **4**.

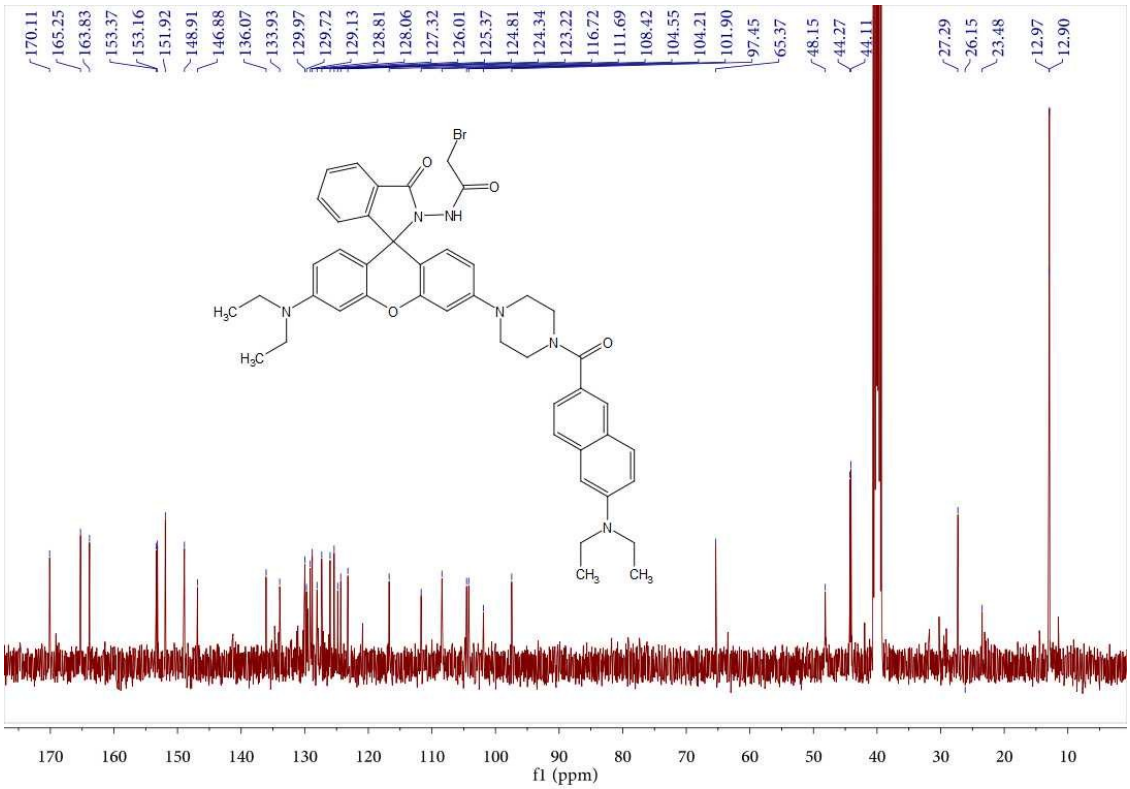


Figure S9 ^{13}C-NMR (DMSO-d_6) spectrum of compound **4**.

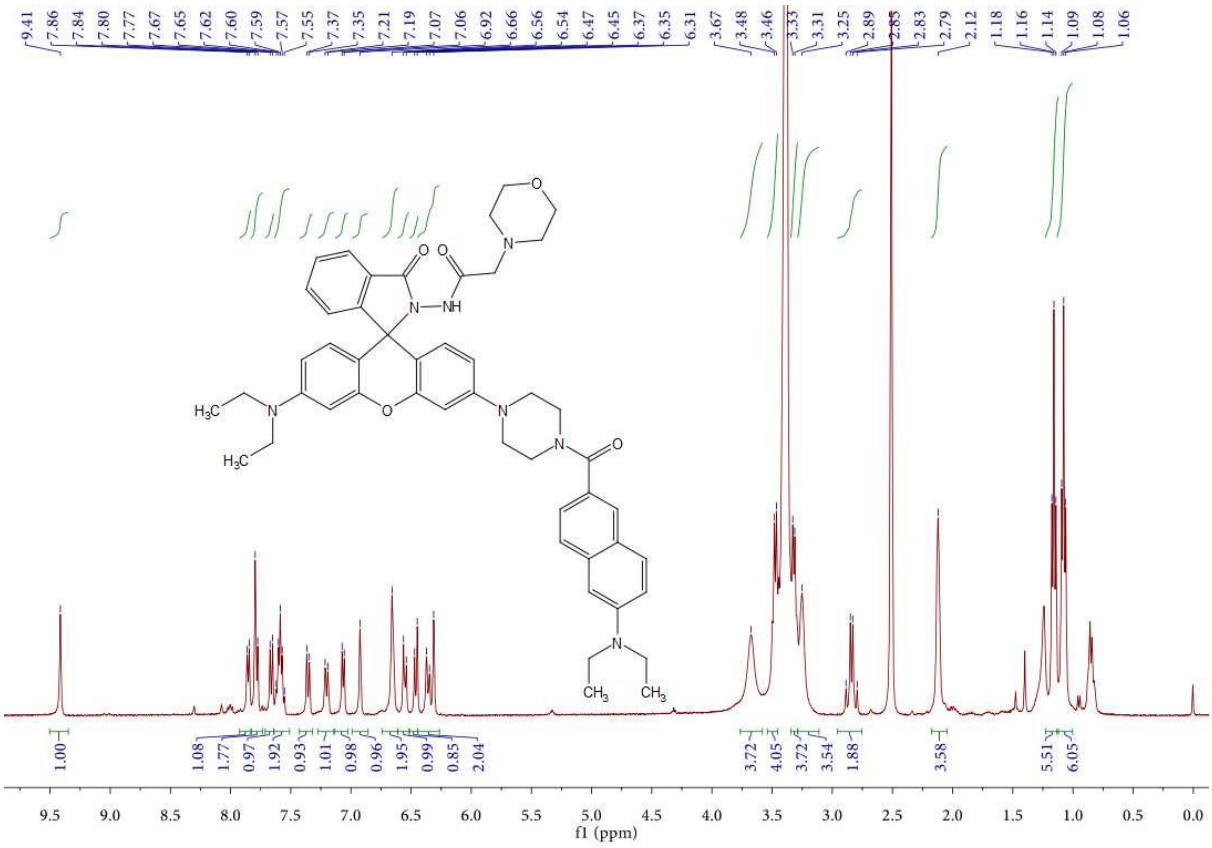


Figure S10 ^{1}H-NMR (DMSO-d_6) spectrum of compound **FL-HA**.

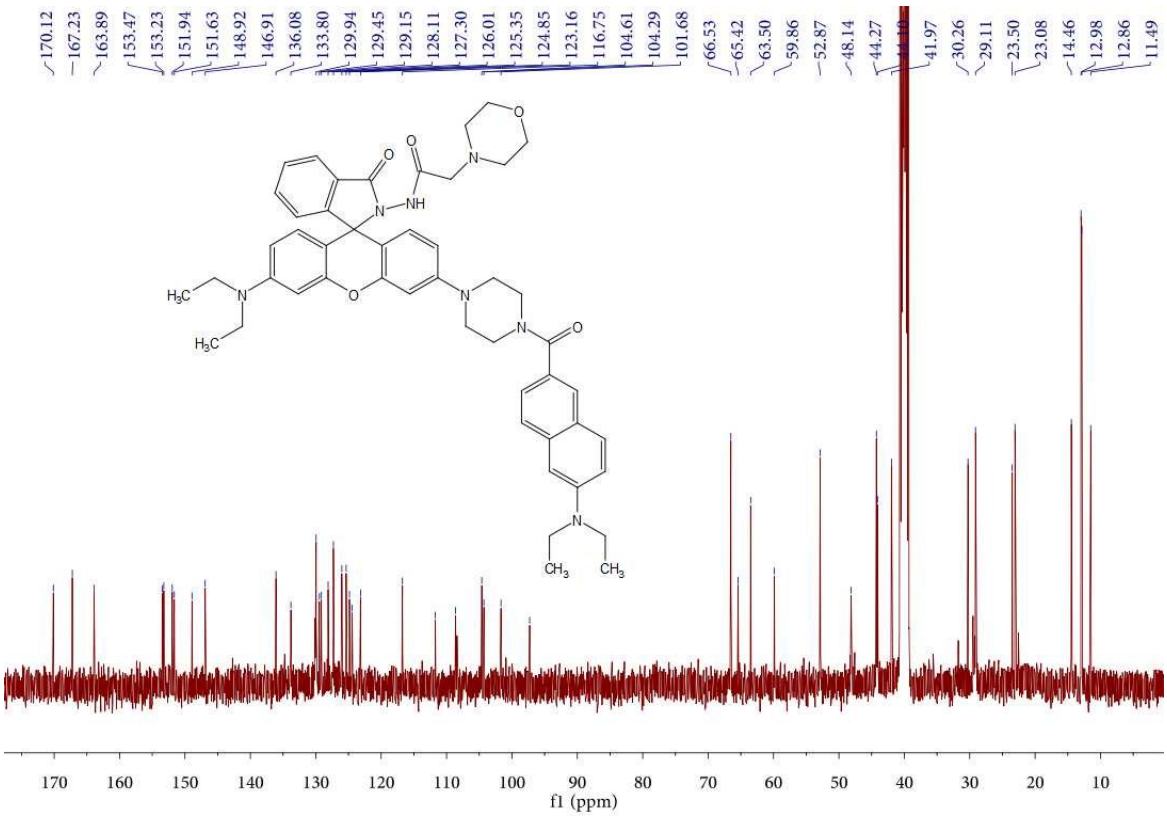


Figure S11 ^{13}C-NMR (DMSO-d_6) spectrum of compound **FL-HA**.

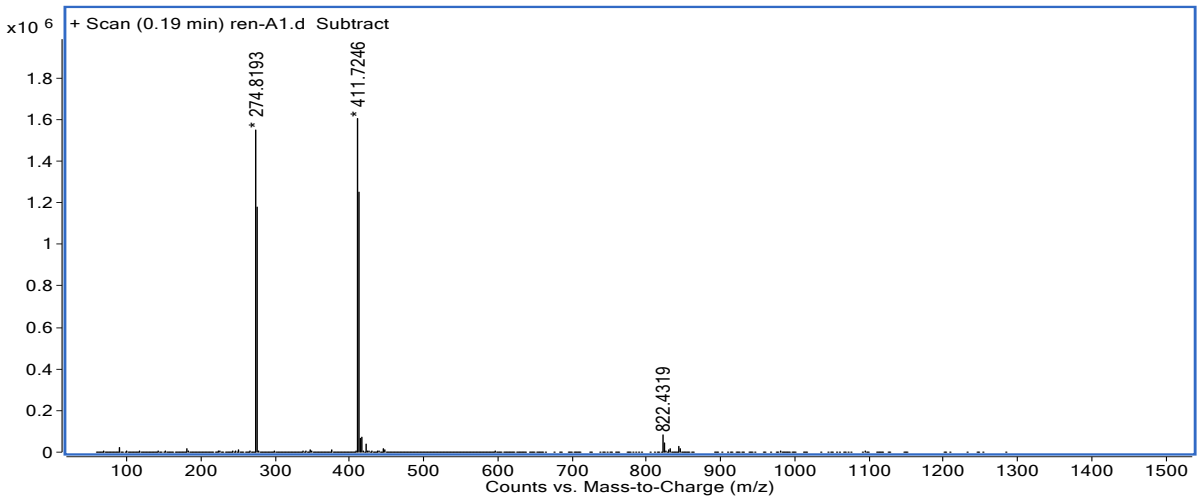


Figure S12 HRMS (ESI) spectrum of **FL-HA.**

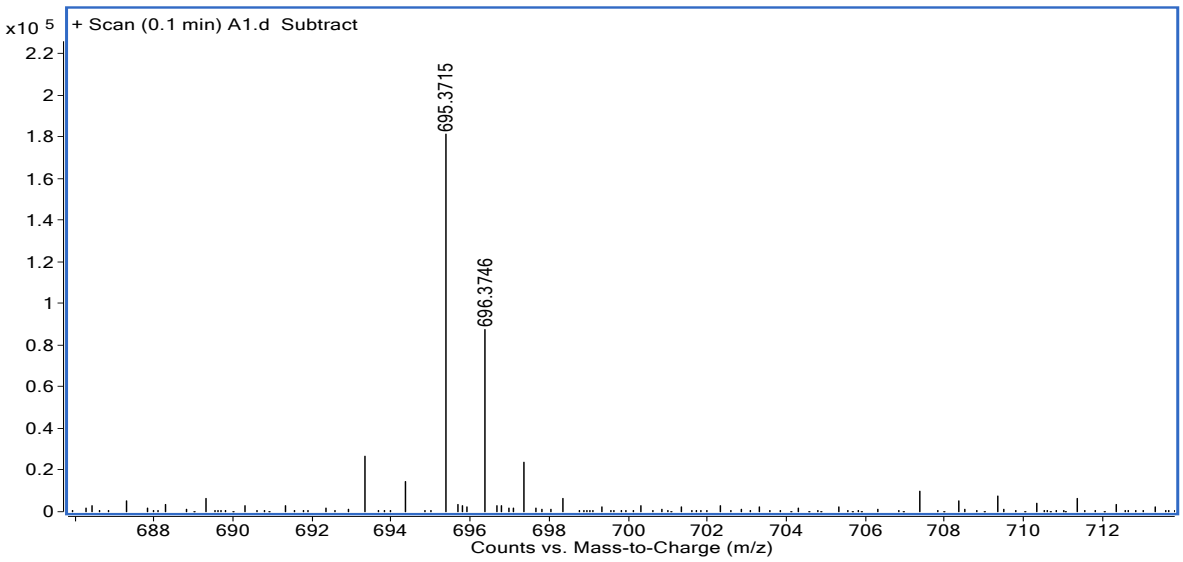


Figure S13 HRMS (ESI) spectrum of compound **3.**

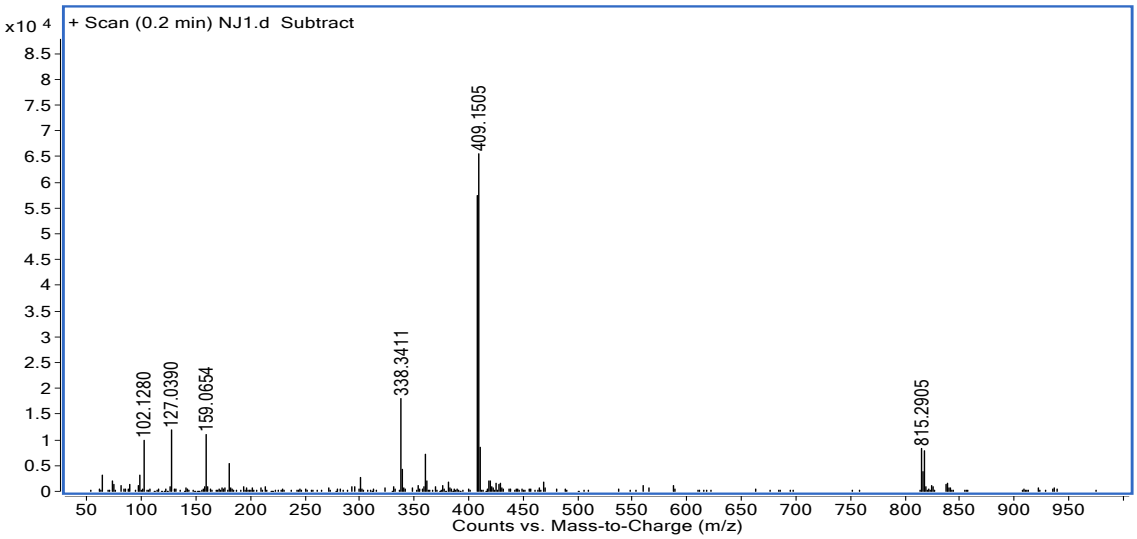


Figure S14 HRMS (ESI) spectrum of compound **4.**

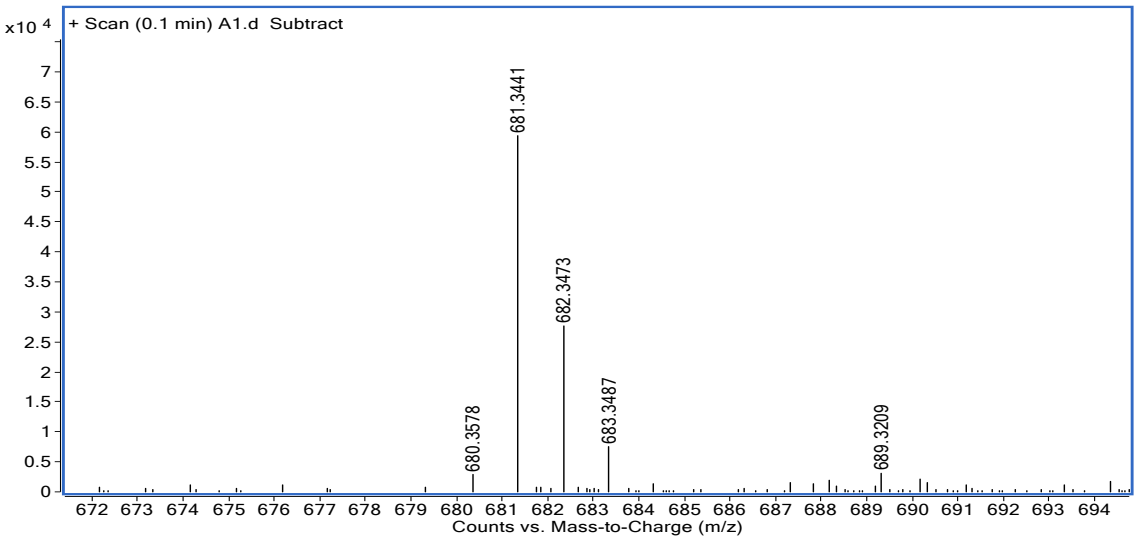


Figure S15 HRMS (ESI) spectrum of product of **FL-HA** reacted with HOCl.