Supporting Information for

A ratiometric two-photon fluorescent probe for rapid detection of HClO in living systems

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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. The imaging experiments used 40 × objective lens. Two-photon imaging were obtained by Nikon A1MP confocal microscopy. The Mai Tai DeepSee laser offers 2.5 W of average power and 350 nm (690-1040 nm) in useable tuning range. The pulse width is 100fs (800nm) and the pulse frequency of two-photon illumination is 80 AMHz. Scan resolution $\geqslant$ 4096×4096, gray level $\geqslant$ 12bit. Scanning speed: 20 s/scan. Living cells fresh were cultivated using fresh medium. All procedures for this study were approved by the Animal Ethical Experimentation Committee of Shandong University according to the requirements of the National Act on the use of experimental animals (China).

Determination of the detection limit

The detection limit was determined from the fluorescence titration data based on a reported method. CR-HClO (10.0 μM) was titrated with different concentrations of HClO, the linear relationship between the fluorescence intensity ratio (I_{580}/I_{471}) and the concentration of HClO (λ_{ex} = 400 nm) was fitted based on the fluorescence titration

Detection limit =3σ/k

Where “σ” is the standard deviation of the blank sample and “k” is the slope of the linear regression equation.

M).

Culture and preparation of HeLa cells

HeLa 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 A549 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. Incubating 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 CR-HA at different concentrations (0 μM, 5 μM, 10 μM, 20 μM, 30 μM, 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 **CR-HA**.

Imaging of HClO in living cells

1. Ratiometric imaging of exogenous HClO 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 CR-HA (10 uM) (containing

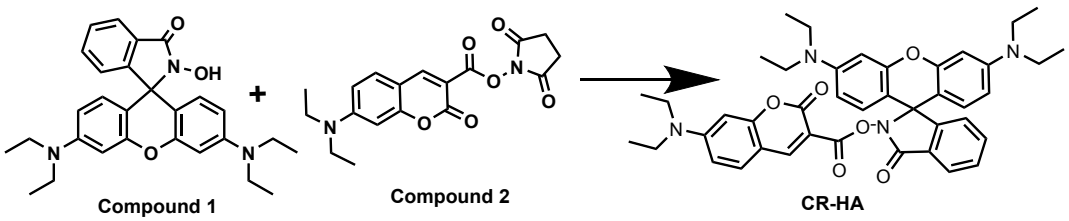
0.1 % DMSO as a cosolvent) for 30 min at 37 ℃. The HeLa cells were rinsed with PBS three times.

The second group was incubating with probe CR-HA (10 M) (containing 0.1 % DMSO as a cosolvent) for 30 min at 37 ℃. the HeLa cells were rinsed with PBS three times and the cells were incubated with HClO (30 uM) 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 HClO 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 CR-HA (10 M) for 30 min at 37 ℃, washed by PBS buffer and subsequently incubated with 2 μg/mL PMA (phorbol 12-myristate13 acetate) and 2 ug/mL LPS (lipopolysaccharides) for 2 h. For the control experiments, the cells without treated with PMA/LPS were incubated with probe CR-HA (10 uM) for 2 hours under the same conditions. For negative control group, the RAW 264.7 cells incubated with probe CR-HA (10 uM) for 30 min at 37 C, washed by PBS buffer and subsequently incubated with 2 ug/mL PMA, 2 μg/mL LPS and 4-aminobenzoic acid hydrazide (ABH, 200 uM) 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

Synthesis



Scheme S1 Synthesis of the probe **CR-HA**

Compounds 1 and Compounds 2 were synthesized according to the reported method [1,2]

***Synthesis of compound* CR-HA**

Compound **1** (51.2 mg, 0.112 mmol, 1 eq) and Compound **2** (40 mg, 0.112 mmol, 1 eq) were dissolved in 3 mL of dichloromethane, add 3 drops of triethylamine and react at room temperature for 4 hours at atmosphere of N2 .Then concentrated under reduced pressure. The resulting residue was purified by column chromatography on silica gel (MeOH / CH_2Cl_2 = 1:50, v/v) to afford compound CR-HA (31 mg, yield: 39%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H), 7.99 (d, *J* = 6.8 Hz, 1H), 7.56 – 7.47 (m, 2H), 7.16 (t, *J* = 7.4 Hz, 2H), 6.82 (d, *J* = 9.1 Hz, 2H), 6.54 (dd, *J* = 9.0, 2.1 Hz, 1H), 6.38 (d, *J* = 7.7 Hz, 5H), 3.49 – 3.31 (m, 12H), 1.26 – 1.15 (m, 18H).

^{13}C NMR (101 MHz, DMSO) δ 164.97, 162.70, 158.37, 153.60, 153.37, 150.75, 149.90, 148.74, 132.92, 132.31, 129.65, 128.78, 128.57, 123.88, 122.56, 110.52, 108.25, 107.86, 107.65, 105.35, 97.81, 96.39, 64.85, 44.87, 44.18, 12.90

HRMS (EI) *m/z* calculated for $C_{42}H_{46}N_4O_6$: 700.3338 Found: 701.3338 (M+H).

References:

[1]Akira Nakamura, Hodaka Kanou, Junki Tanaka, Akira Imamiya, Tomohiro Maegawa and Yasuyoshi Miki* Org. Biomol. Chem., 2018, **16**, 541–544.

[2]Tokiko Murase, Toshitada Yoshihara , Keiichi Yamada , Seiji Tobita, Bull. Chem. Soc. Jpn., 2013,**86**, 510-519.

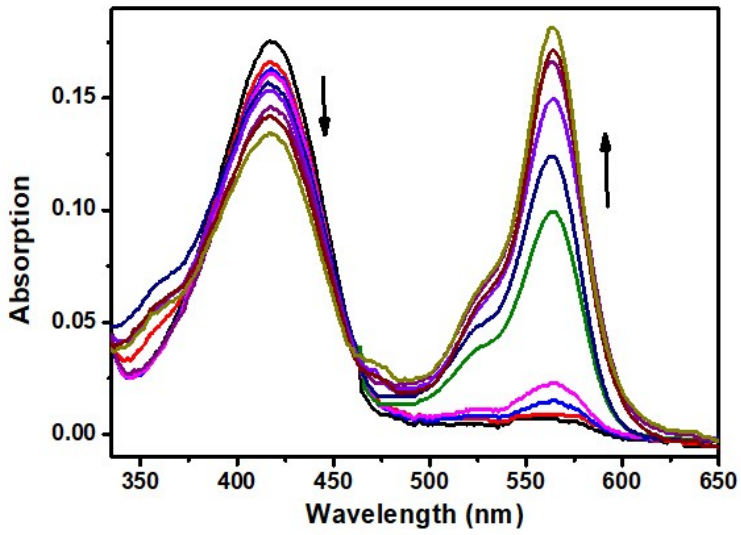


Figure S1 The absorption spectral changes of probe CR-HA (10 μM) in the absence or presence of HClO (1-500 μM).

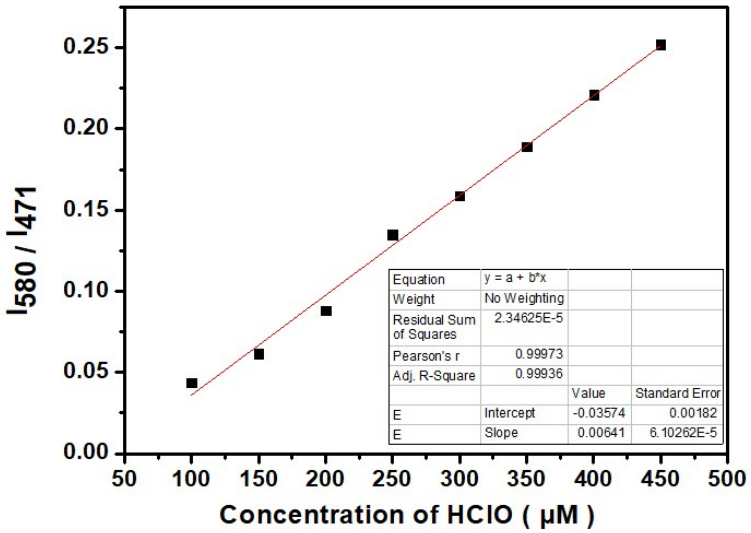


Figure S2 The linear relationship between the fluorescence intensity ratio (I_{580}/I_{471}) and the concentration of HClO (λ_{ex}=400 nm).

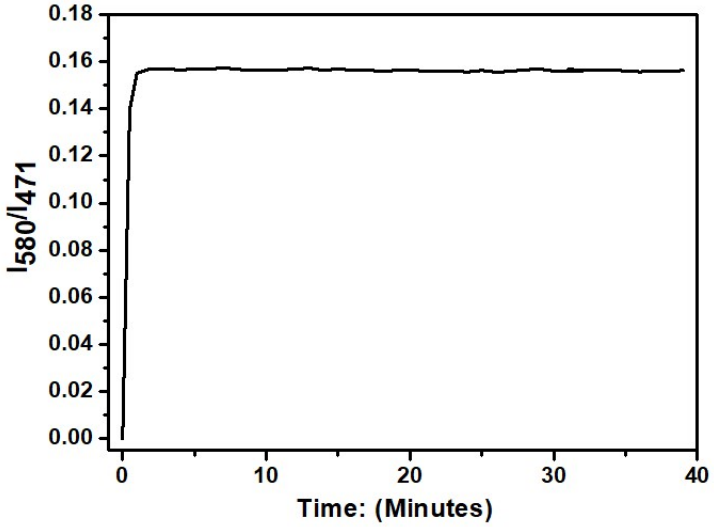


Figure S3.Time-dependent the fluorescence intensity ratio (I_{580}/I_{471}) spectra of CR-HA (10 μM) in the presence of HClO (300 μM) in PBS buffer containing 40% DMF as a cosolvent.

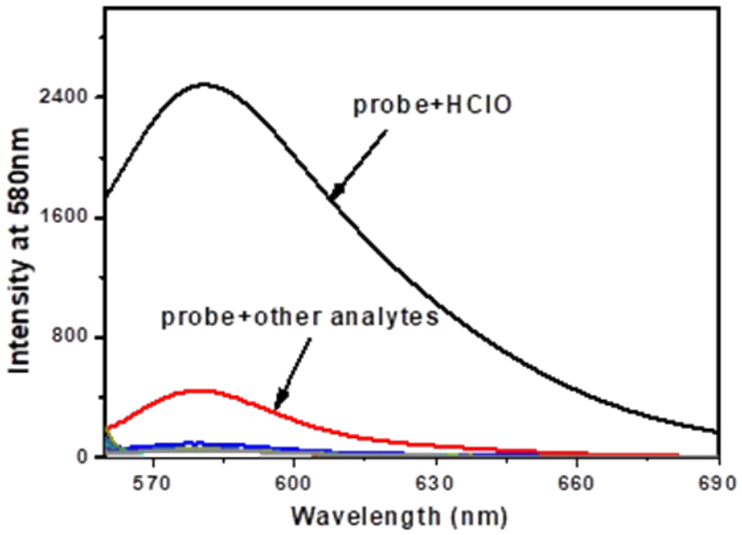


Figure S4 The fluorescence spectra changes of probe **CR-HA** (10 μM) in the presence of various analytes (100 μM) in PBS buffer containing 40% DMFas a cosolvent (λ_{ex}=550 nm)

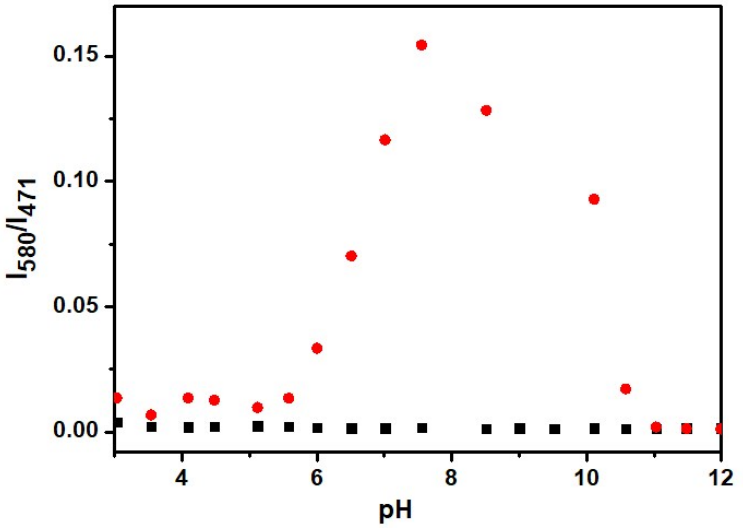


Figure S5 The fluorescence intensity ratio (I_{580}/I_{471}) of probe **CR-HA** (10 μM) before and after addition of HClO (100 μM) in PBS buffer with different pH values, containing 40 % DMF as a cosolvent (λ_{ex} = 550 nm)

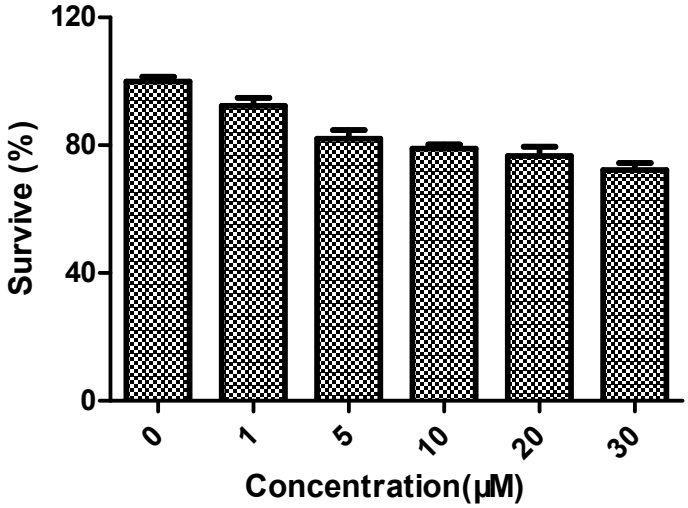


Figure S6 Cytotoxicity assays of **CR-HA** at different concentrations for HeLa cells.

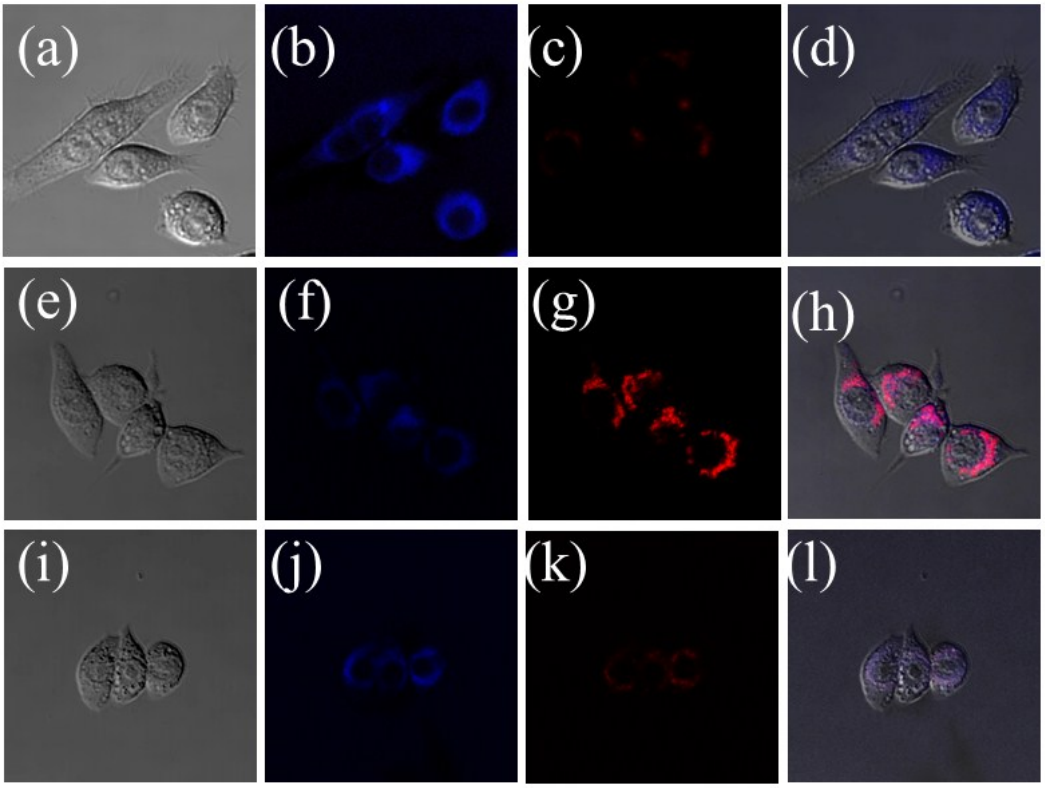


Figure S7. Imaging of endogenous HClO in RAW 264.7 cells stained with the probe CR-HA. (a) Brightfield image of RAW 264.7 macrophages cells costained only with CR-HA; (b) Fluorescence images of (a) from blue channel; (c) Fluorescence images of (a) from red channel; (d) Merged pictures; (e) Brightfield image of RAW 264.7 macrophages cells stimulated with PMA and LPS costained with CR-HA; (f)Fluorescence images of (e) from

blue channel; (g)Fluorescence images of (e) from red channel; (h) Merged pictures; (i) Brightfield image of RAW 264.7 macrophages cells stimulated with PMA and LPS and ABH costained with CR-HA; (j)Fluorescence images of (i) from blue channel; (k)Fluorescence images of (i) from red channel; (l) Merged pictures.

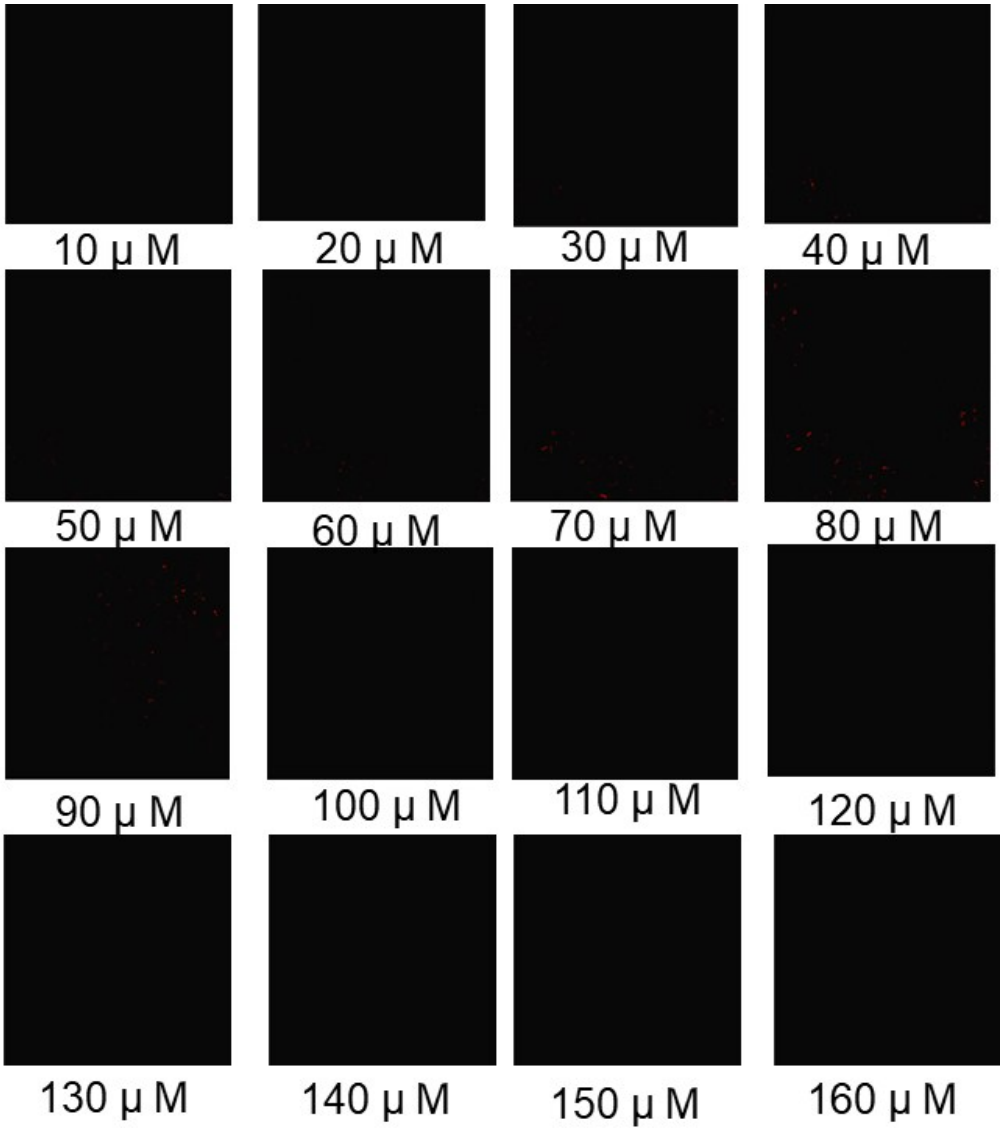


Figure S8 Two-photon fluorescence images of a fresh rat liver slice incubated with 30 μM CR-HA in the absence of HClO at the depths of approximately 0~160 μm. (λ_{ex} = 800 nm)

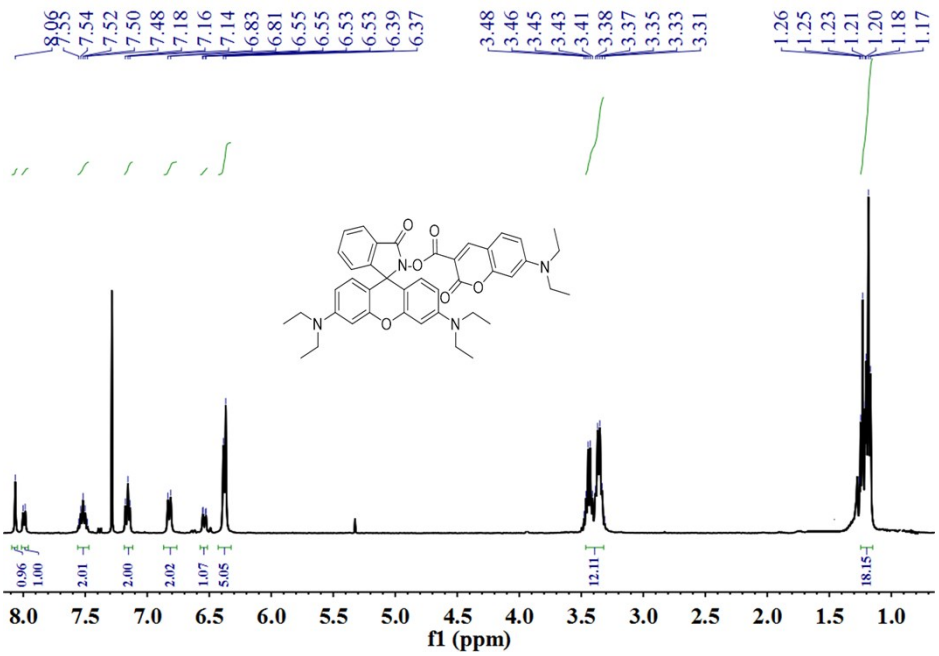


Figure S9 ^{1}H-NMR($CDCl_3$-*d*) spectrum of compound **CR-HA.**

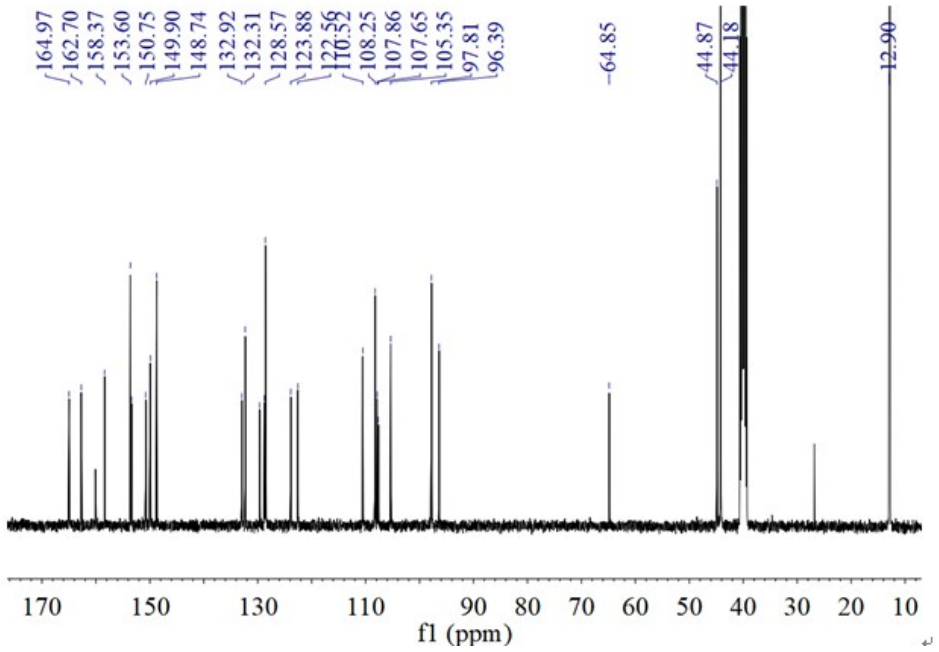


Figure S10 ^{13}C-NMR (DMSO-d_6) spectrum of compound **CR-HA**.

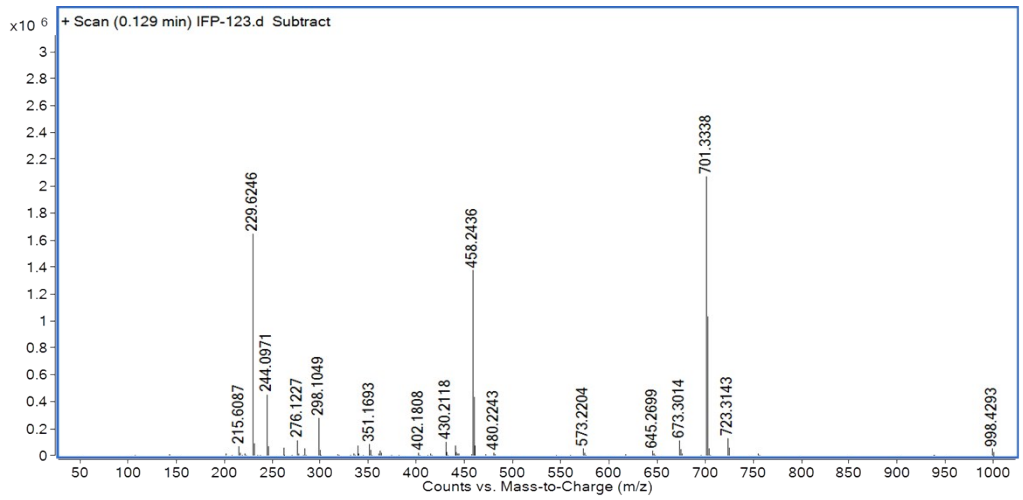


Figure S11 HRMS (ESI) spectrum of **CR-HA.**

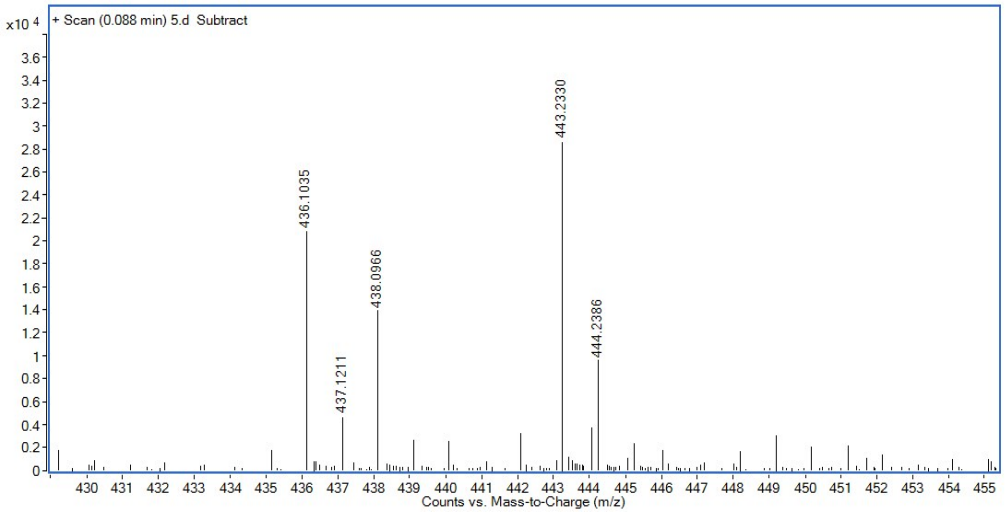


Figure S12 HRMS (ESI) spectrum of product of **CR-HA** reacted with HClO.