Electronic Supplementary Material (ESI) for RSC Advances.

Development of a novel hypochlorite ratio probe based on coumarin and its application in living cells

1. Materials and Instruments

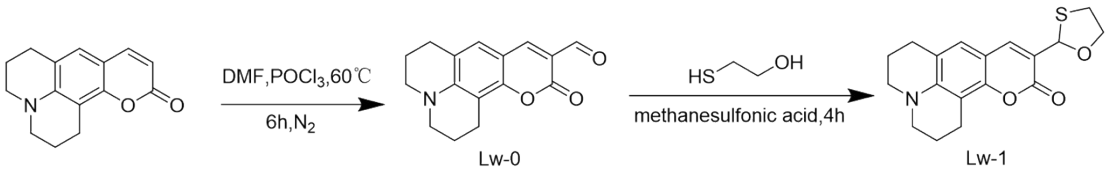
All chemicals and solvents used in this study were analytical-grade reagents purchased from reputable reagent companies unless otherwise stated. Sodium hypochlorite was procured from Aladdin and stored at 4°C, while $POCl_3$ and coumarin 6H were purchased from Anneji and Aladdin, respectively. β- Mercaptoethanol, methanesulfonic acid, and DMF were purchased from McLean, and dichloromethane was purchased from Xilong Scientific. The absorption and emission spectra were measured using the Ravleigh UV-2100 ultraviolet-visible spectrophotometer and ShimadzuRF 5301PC fluorescence spectrophotometer, respectively. NMR spectra were recorded at 500 MHz and 126 MHz using Bruker Avance III HD 500 MHz with DMSO-d6 as the solvent and TMS as the internal standard. HT-29 human colon cancer cells and LO2 human normal liver cells were obtained from the cell bank of Guangxi Key Laboratory of Traditional Chinese Medicine Pharmacodynamics. McCoy's 5A in complete medium, 1640 incomplete medium, and phosphate buffer PBS were purchased from Jiangsu Kaiji Biotechnology Co., Ltd. Fetal bovine serum FBS and 0.25% EDTA Trypsin (1 ×) were procured from Gibco Company in the United States, while dimethyl sulfoxide (cell culture grade) was obtained from Beijing Solebao Technology Co., Ltd. CellTier 96® The Aquarius One Solution Cell Promotion Assay (MTS) was purchased from Promega Corporation in the United States. The high-content cell imaging analysis system and high-throughput ELISA screening system models used for cell imaging were the Operetta CLS and EnVisionXcite, respectively, both from PerkinElmer in the United States.

2. Synthesis and Characterization of Lw-1

Add 2.0 ml of anhydrous DMF, which has been pretreated with a molecular sieve under N2 protection, to 2.0 ml of $POCl_3$ at room temperature and stir for about 30 min until the solution gradually turns pale yellow. Then, 0.5 mmol of 2,3,5,6-1H, 4H-tetrahydroquinoline, and [9,9a, 1-gh] coumarin are fully dissolved in 10 ml of DMF as another solution, mixed with the previous solution, and stirred at room temperature for 30 min to ensure complete mixing of the two solutions. The resulting mixture is then heated and subjected to a reaction at 60 °C for approximately 12 hours. After completion of the reaction, quench $POCl_3$ with ice water by slowly adding 100 ml of ice water to the reaction mixture, followed by adjusting the pH of the solution to about 7.0 with a 20% mass concentration NaOH solution. This will lead to the formation of a large number of reddish-brown precipitates in the solution. The precipitates are collected through filtration, and the filtrate is removed to obtain the filter residue. The product is then purified through column chromatography separation technology, using a developing agent consisting of a petroleum ether and ethyl acetate system, with a petroleum ether to ethyl acetate ratio of 1:3. Finally, a reddish-brown product, LW-0, can be obtained with a yield of approximately

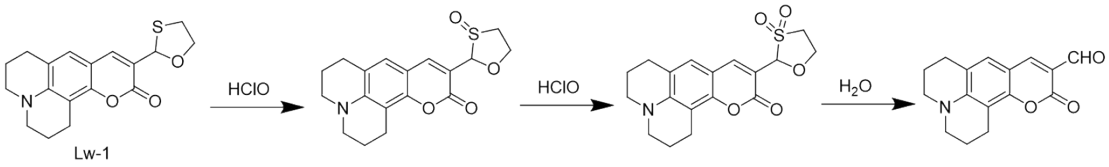
83.2%.^{1}H NMR (500 MHz, $CDCl_3$) δ 10.08 (s, 1H), 8.11 (s, 1H), 6.96 (s, 1H), 3.36 (q, *J* = 6.1 Hz, 4H), 2.87 (t, *J* = 6.4 Hz, 2H), 2.75 (t, *J* = 6.3 Hz, 2H), 1.97 (p, *J* = 6.1 Hz, 4H).^{13}C NMR (126 MHz, $CDCl_3$) δ 187.98, 162.25, 153.74, 149.30, 144.95, 128.26, 119.80, 112.84, 108.02, 106.07, 77.11, 50.34, 49.93, 27.22, 20.87, 19.92, 19.89.

Then, 180mg of Lw-0 was accurately weighed and mixed with 10 mL of CH_2Cl_2 under N_2 protection. 1.2 mL of 2-mercaptoethanol was added to the mixture and thoroughly stirred. Subsequently, 0.2mL of methanesulfonic acid was added to the mixture and heated at 60°C for reflux condensation. The reaction proceeded for approximately 3 hours. The resulting product was purified by column chromatography using a solvent system of petroleum ether and ethyl acetate in a ratio of 2:1, affording an orange-yellow solid, Lw-1, with a yield of approximately 37.1%. The yield of the yellow solid was approximately 31.3%.^{1}H NMR (500 MHz, CDCl3) δ 7.73 (d, J = 0.9 Hz, 1H), 6.89 (s, 1H), 6.16 (s, 1H), 4.51 (ddd, J = 9.2, 5.8, 3.5 Hz, 1H), 4.02 (td, J = 8.8, 6.0 Hz, 1H), 3.28 (dt, J = 8.2, 5.6 Hz, 4H), 3.16 (ddd, J = 9.9, 6.6, 2.3 Hz, 2H), 2.90 (t, J = 6.5 Hz, 2H), 2.77 (t, J = 6.4 Hz, 2H), 2.02 – 1.93 (m, 4H).9, 112.90, 112.60, 40.17. ^{13}C NMR (151 MHz, CDCl3) δ 161.75, 151.20, 146.09, 138.81, 125.49, 119.24, 118.67, 108.27, 106.57, 81.72, 72.13, 50.14, 49.76, 33.11, 27.63, 21.60, 20.69, 20.45. HRMS (ESI): calcd for C18H19NO3S ([M + H]+): 330.1086, found: 330.1137.



Scheme S1 Synthesis procedures of Lw-1

3. Proposed sensing mechanism of QClO in response to HClO.



Scheme S2 Synthesis procedures of Lw-1

4. Cell Culture and high-content imaging

(1) During the logarithmic growth phase of HT-29 human colon cancer cell cultivation, the following steps were conducted: firstly, the old culture medium was discarded using a pipette, and cells were rinsed with DPBS. Secondly, 0.25% EDTA Trypsin was added, and the culture bottle was placed back in the incubator for about 5 minutes to allow for digestion. Thirdly, the culture bottle was taken out, and a majority of cells were gently detached from the bottle by tapping and spraying alcohol. Fourthly, a complete culture medium containing

McCoy's 5A was quickly added to terminate digestion. The medium was collected using a Babbittian tube, transferred to a 15 mL centrifuge tube, and centrifuged at 1000 rpm for 3 minutes. Finally, the supernatant was discarded, and the cells were resuspended in 5 mL of McCoy's 5A complete culture medium and adjusted to a density of 1 × 105 cells/mL. A total of 1 mL of cell suspension and 1 mL of complete culture medium were added to each well of a 6-well plate, containing 10 w cells per well, and were placed in a 37 ℃, 5% CO2 constant temperature incubator for routine cultivation.

(2) Following 24 hours of cell adhesion, the supernatant was aspirated and discarded using a vacuum pump. The experimental groups were treated with hypochlorite at different concentrations (10, 40, 80 μM) in a culture medium, and were incubated in a 5% CO2 constant temperature incubator at 37 ℃ in the dark for 15 minutes. After incubation with hypochlorous acid for 15 minutes, the culture medium containing the hypochlorous acid solution was discarded, and cells were washed with DPBS buffer 1-2 times. Cells were then incubated with a 5 μM concentration of the fluorescent probe in the dark for 20 minutes. After 20 minutes, the fluorescent probe was discarded, cells were cleaned with DPBS, and a fresh and complete culture medium was added. The cells were then ready for imaging.

(3) The high-content imaging instrument used in this study was equipped with three channels: Brightfield, DAPI, and GFP channels. Nine fields of view were selected for photography in each well, and the same parameters were set for experimental result processing.

5. Cell toxicity assay of Lw-1

The fluorescent probe solution was prepared as follows: 1.4 mg of the fluorescent probe was accurately weighed using an analytical balance, dissolved in DMSO, and a stock solution with a final concentration of 5 mmol/L was prepared and stored in the refrigerator at -20 ℃ for further use. Experimental groups were organized, including a blank solvent control group (complete medium group) and a fluorescence probe gradient group (2.5, 5, 10, 20, 40, 80, 160 μM), with each group consisting of 5 compound wells.

The cytotoxicity experiment was carried out in the following steps:

(1) LO2 human normal liver cells were cultured in the logarithmic growth phase, and the old culture medium was discarded using a pipette. DPBS was added to clean the cells, and 0.25% EDTA Trypsin was added. The culture bottle was placed back in the incubator, and the time for digestion was recorded for approximately 5 minutes. The culture bottle was then taken out, and most of the cells were gently detached from the culture bottle by tapping it. Alcohol was sprayed, and the culture medium containing 1640 was quickly added to terminate digestion. The culture medium was taken from the wall of the culture bottle and blown down using a Babbittian

tube. It was then transferred to a 15 mL centrifuge tube and placed in a centrifuge at 1000 rpm. After centrifugation for 3 minutes, the supernatant was discarded using a Babbittian tube, and 5 mL of 1640 complete culture medium was added for resuspension. The cells were counted, and the cell density was adjusted to 4 × 104 cells/mL. A 100 μL cell suspension was added to each well in a 96-well plate, consisting of 4000 cells per well, with 5 replicate wells in each group and a total of 7 gradient groups (2.5, 5, 10, 20, 40, 80, 160 μM), as well as the culture medium control group. The plate was placed in a constant temperature incubator at 37 °C and 5% CO2 for routine cultivation.

(2) After 24 hours of cell adhesion, the supernatant was aspirated and discarded using a vacuum pump. A blank solvent control group and a medication group were set up according to the experimental group, with 5 wells in each group.

(3) After the administration was completed, the plate was incubated for 24 and 48 hours. The culture medium was removed from the 96 well plates using a vacuum pump, and MTS solution (fresh medium: Cell Tier 96 Aqueous One Solution Reagent=9:1) was added to each well using a row gun. The plate was then placed back in the incubator and incubated in the dark for 2 hours. The absorbance value (OD value) at a wavelength of 490 nm was read using a high-flux ELISA screening system.

(4) Cell growth and survival rate were analyzed using GraphPad Prism 8 software. The cell survival rate was calculated using the formula: Cell survival rate = (A administration group/Control) × 100%.

To investigate the toxic effect of fluorescent probes on normal cells, LO2 human normal liver cells were selected, and the toxic effect of fluorescent probes was observed at 7 dosing concentrations. The cell survival rate was calculated using MTS experiments at the corresponding time points after 24 and 48 hours of administration. The results showed that when the fluorescent probe intervened with LO2 cells for 24 and 48 hours, the dosing concentration was between 2.5-40 μM, and the cell survival rate was above 90%. This indicates that within this concentration range, the fluorescent probe showed no toxicity to normal cells. The subsequent experimental concentration was set at 40 μM, and there was no obvious toxicity below this concentration. The results are presented in Figure S1 and Table S1.

Table S1 Survival rates of LO2 cells at 24 and 48 hours after intervention with different concentrations of fluorescent probes

Dosage concentration（μM）	24-hour survival rate（%）	48-hour survival rate（%）
2.5	122.58	104.91
5	129.22	113.17
10	117.88	110.40
20	112.16	104.84
40	91.51	97.80
80	74.96	78.12
160	56.96	26.43
IC50（μM）	174.1	117.6

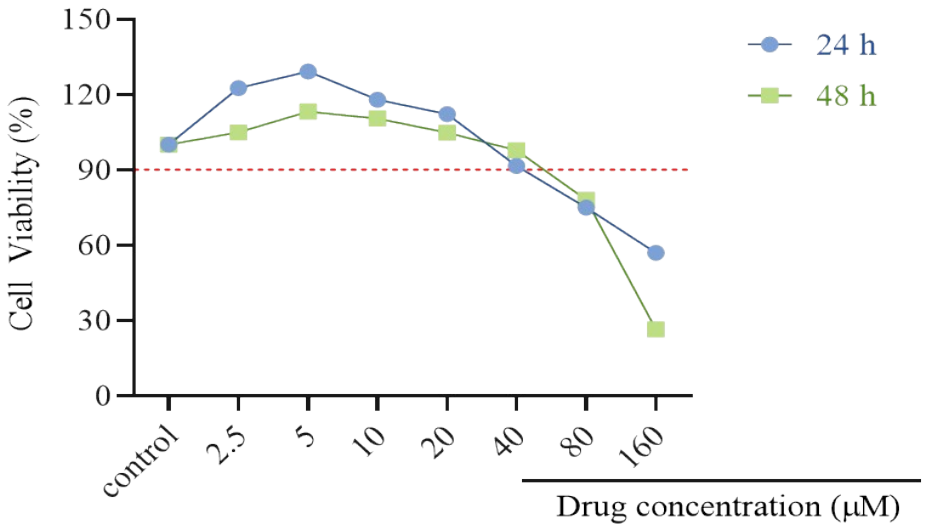


Figure S1 Survival rates of LO2 cells after 24 and 48 hours of intervention with different concentrations of fluorescent probes

6. ^{1}H NMR, ^{13}C NMR and HRMS of Lw-1 and Lw-0

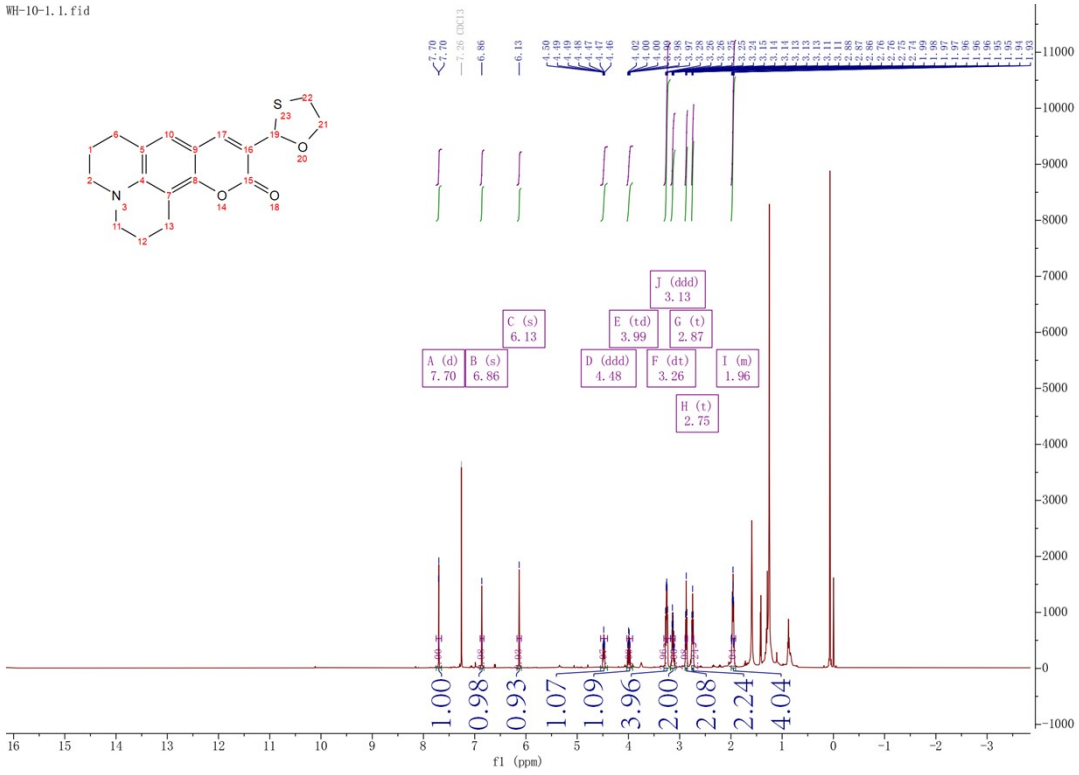


Figure S3 ^{1}H NMR Spectra of Lw-1

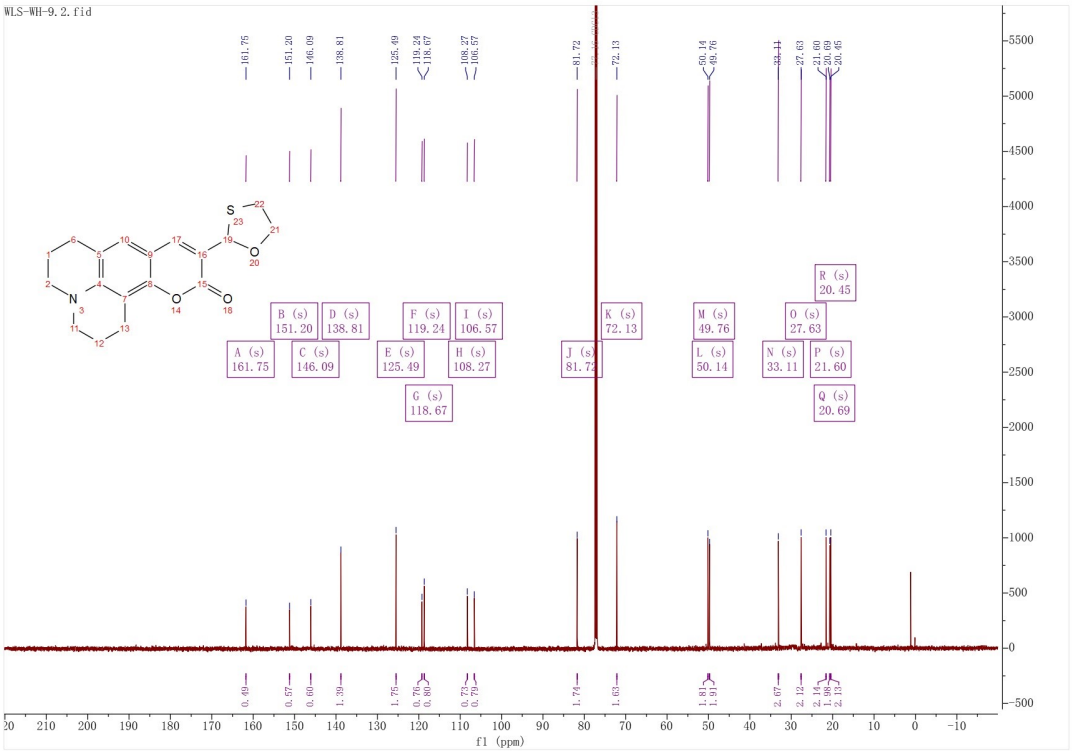


Figure S4 ^{13}C NMR Spectra of Lw-1

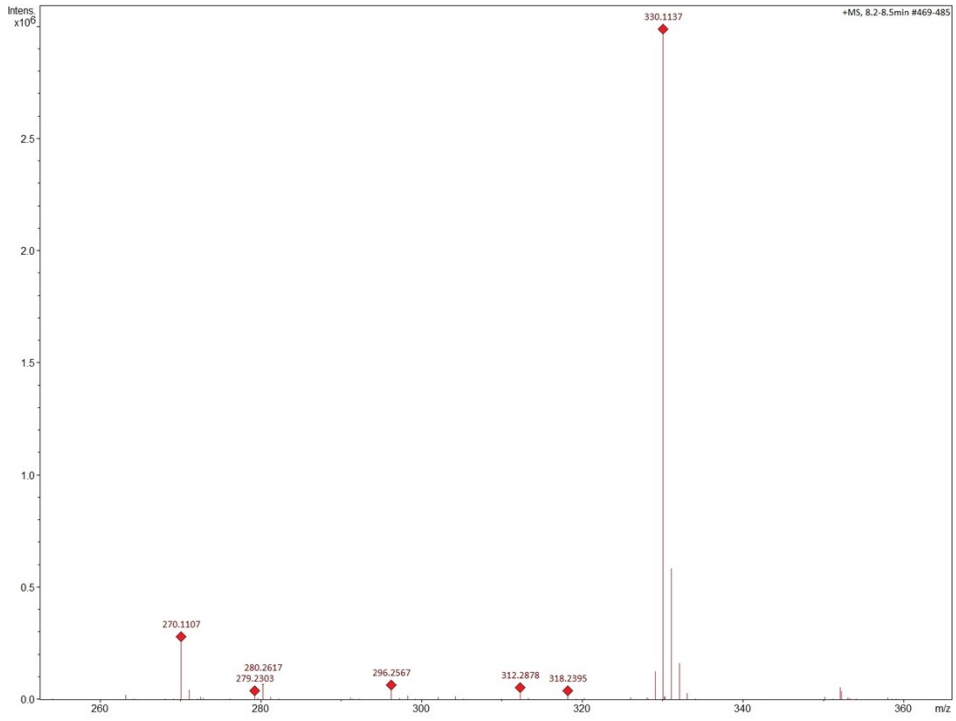


Figure S5 Mass spectrum of Lw-1

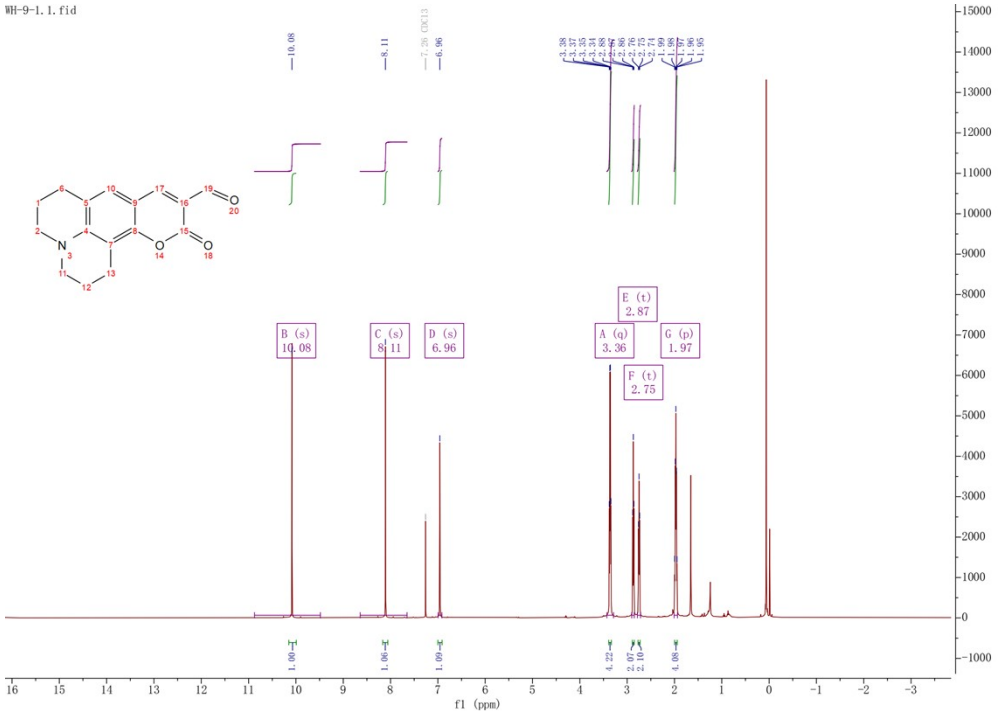


Figure S6 ^{1}H NMR Spectra of Lw-0

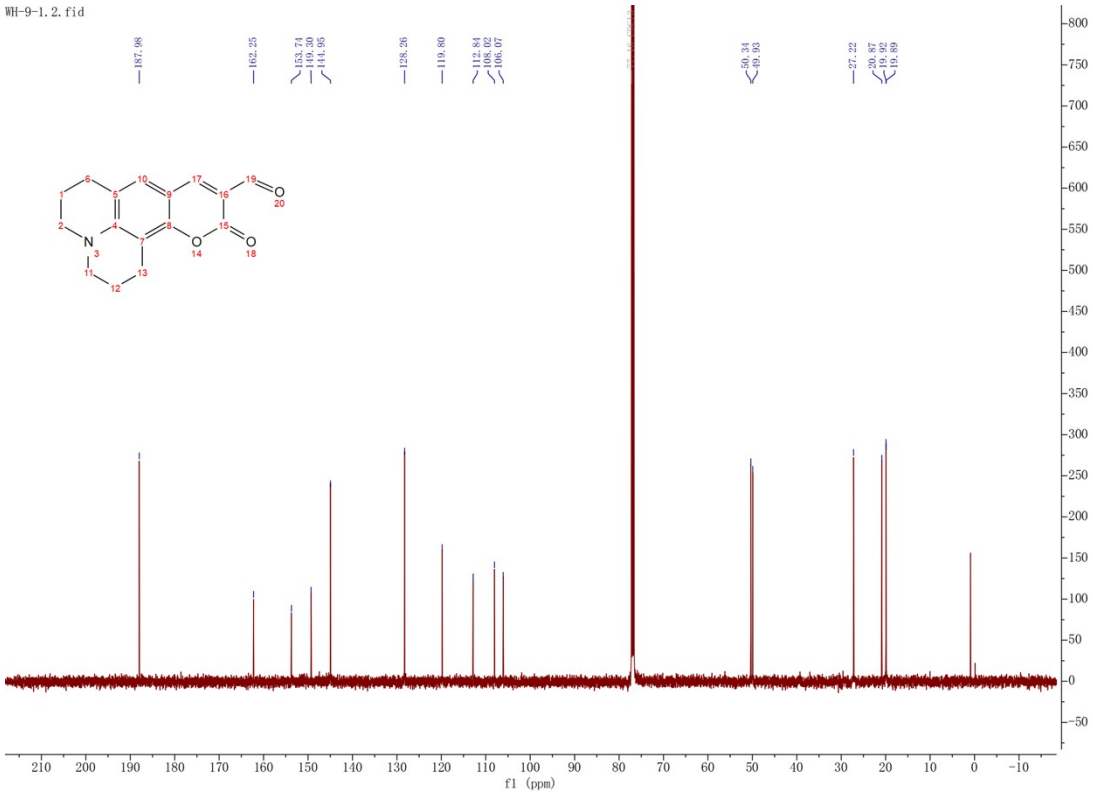


Figure S7 ^{13}C NMR Spectra of Lw-0

7.Fluorescence map of Lw-1 before and after adding HClO

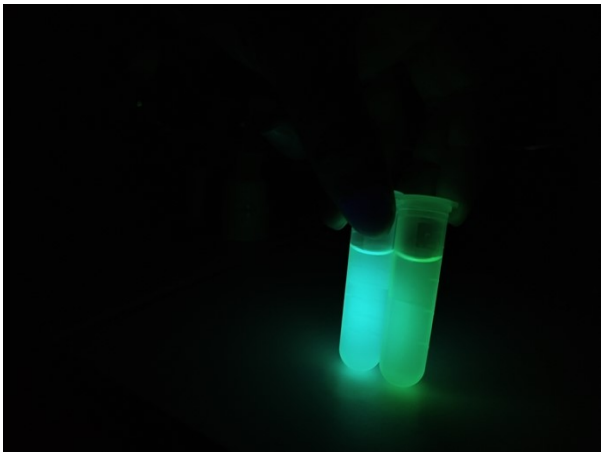


Figure S8 Fluorescence diagram of solution under 365nm ultraviolet lamp, with Lw-1 on the right and NaClO added on the left

8.Error bar of Lw-1's linear response to HClO

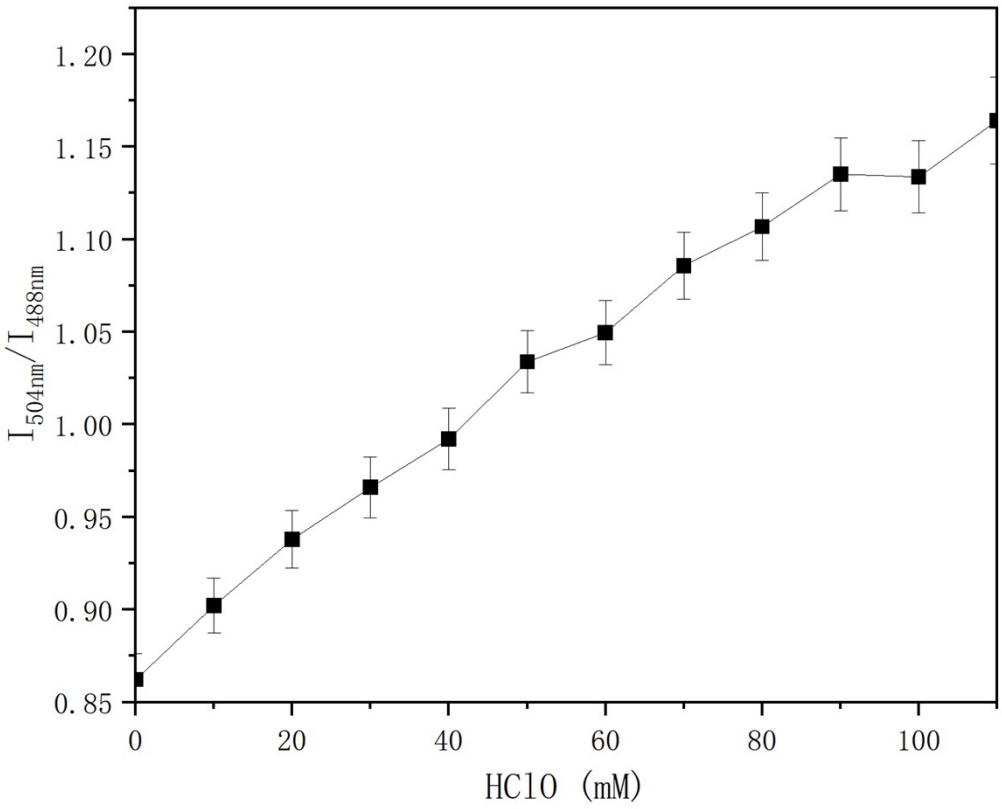


Figure S9 Error bar of Lw-1's linear response to HClO