Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Selective Detection of Ozone in Inflamed Mice using a Novel Activatable Chemiluminescent Probe

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Materials and General Experimental Methods

Reagents and Apparatus. All chemicals were purchased from commercial suppliers and used without further purification. phorbol 12-myristate 13-acetate (PMA) was dissolved in dimethyl sulfoxide (DMSO) to form solution and divided into several parts for daily experiments. To keep activity of PMA, all these solutions were stored at -20 ℃ before use. Twice-distilled water was used throughout all experiments. NMR spectra were recorded on a Bruker-400 spectrometer, using TMS as an internal standard. Thin layer chromatography (TLC) was conducted using silica gel 60 F254, and column chromatography was carried out over silica gel (100-200 mesh), both of them were obtained from Qingdao Ocean Chemicals (Qingdao, China). The in vivo imaging was carried out using an IVIS Lumina XR (IS1241N6071) *in vivo* imaging system.

Spectral experiments. The 10 mM **CL-O_3** and **CL-PEG-O_3** stock solutions were prepared by dissolving **CL-O_3** or **CL-PEG-O_3** in dimethyl sulfoxide (DMSO) and stored at -20 ℃. The test solution of **CL-O_3** and **CL-PEG-O_3** was prepared by diluting an appropriate volume of stock solution of **CL-O_3** and **CL-PEG-O_3** into PBS (pH 7.4, 10% DMSO) before each use. The chemiluminescent response of **CL-O_3** and **CL-PEG-O_3** toward O_3 was measured by IVIS Lumina XR and the response mechanism of **CL-O_3** towards **O_3** was further confirmed by absorption and fluorescence spectra, high-performance liquid chromatography (HPLC) analysis and mass spectrometry. The fluorescence spectra were recorded from 500 to 620 nm with an excitation at 420 nm on the F-7000 fluorescence spectrophotometer. In the selectivity experiment, the solutions of various testing species were prepared in twice-distilled water. The selectivity experiment and chemiluminescence imaging of the solution were both acquired by IVIS Lumina XR. The kinetics of reaction between **CL-O_3** and O_3 were measured by Microplate reader for 8 h. Except kinetic experiment, the test solutions were both oscillated at 37 °C for 40 min before the test.

Preparation of O_3 and Concentration of O_3 Calculation. O_3 prepared by ozone generator. Oxygen passed through the ozone generator and then bubble into PBS at 0 °C for 30 min. The UV-vis absorption spectra of O_3 in PBS was detected immediately. The concentration of O_3 is calculated using the Beer-Lambert law:

$$A = Kbc$$

in which A is the absorption of O_3 at 260 nm, K of O_3 is 2900 L mol^{-1} cm^{-1}, b is the absorbent layer thickness that is 1 cm.

Detection Limit Calculation. The detection limit was calculated based on the chemiluminescence and fluorescence titration. The detection limit is calculated using the following equation:

$$\text{Detection limit} = 3\sigma/k$$

where σ is the standard deviation of blank measurements, k is the slope between the chemiluminescence or fluorescence intensity versus sample concentration.

Selectivity Experiment. The superoxide anion radical ($O_2^{\cdot-}$) was freshly prepared by dissolving potassium superoxide (KO_2) in dimethyl sulfoxide (DMSO). Singlet oxygen ($^1O_2$) was freshly prepared by mixing ClO^- with H_2O_2 in a ratio of 1:1. The hydroxyl radical (·OH) was freshly prepared by mixing $FeCl_2$ with H_2O_2 in a ratio of 1:1. The hydrogen sulfide (S^{2-}) was prepared from Sodium sulfide (Na_2S). The Na^+, Mg^{2+} and Zn^{2+} were prepared from NaCl, $MgCl_2$ and $ZnCl_2$, respectively. All analytes involved in the selectivity experiment were incubation with CL-O_3 (10 μM) in PBS (pH 7.4, 10% DMSO) at 37 °C for 40 min.

Detection of O_3 in PBS solutions with Paper-Based O_3 Sensor. The paper-based O_3 sensors was made by cutting qualitative filter paper into rectangular pieces with the same size and then absorbed the solution of **CL-O_3**. Later, the paper-based sensors were dipped into freshly prepared ozone solutions that were diluted to different concentrations. Images were acquired

after 40 min on the IVIS Lumia XR system.

Detection of O_3 in Ambient Air with Paper-Based O_3 Sensor. The paper-based O_3 sensors that with 200 μM **CL-O_3** were prepared. And the sensors were placed for 1 h in a fume hood with closed ventilation system and window, and the O_3 generator was worked at the same time. A negative control was prepared in the same manner, but the sensors were placed in a normal fume hood with closed ventilation system and window for 1 h. Then the images were acquired after 40 min on the IVIS Lumia XR system.

Cytotoxicity Study of CL-O_3 for RAW 264.7 Cells. The cytotoxicity of CL-O_3 was determined by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. RAW 264.7 cells were placed in a 96-well plate in 200 μL of culture medium and incubated in 5% CO_2 at 37 °C for 24 h. Then the culture medium was removed and cells were treated with different concentrations (0-40 μM) of CL-O_3. After 12 h of incubation, CL-O_3 was removed and MTT solution (5.0 mg/mL) was added into each well. Residual MTT solution was removed after 4 h, and then dimethyl sulfoxide (DMSO, 150 μL) was added to each well to dissolve the formazan crystals. After the plates were shaken for 20 min at 37 °C, the absorbance values of the wells were recorded by use of a microplate reader at 490 nm. The cytotoxic effects (VR) of CL-O_3 were assessed by the following equation: $VR = A/A_0 \times 100\%$, where A and A_0 represent absorbance of the experimental group and control group, respectively. The assays were performed in five sets for each concentration.

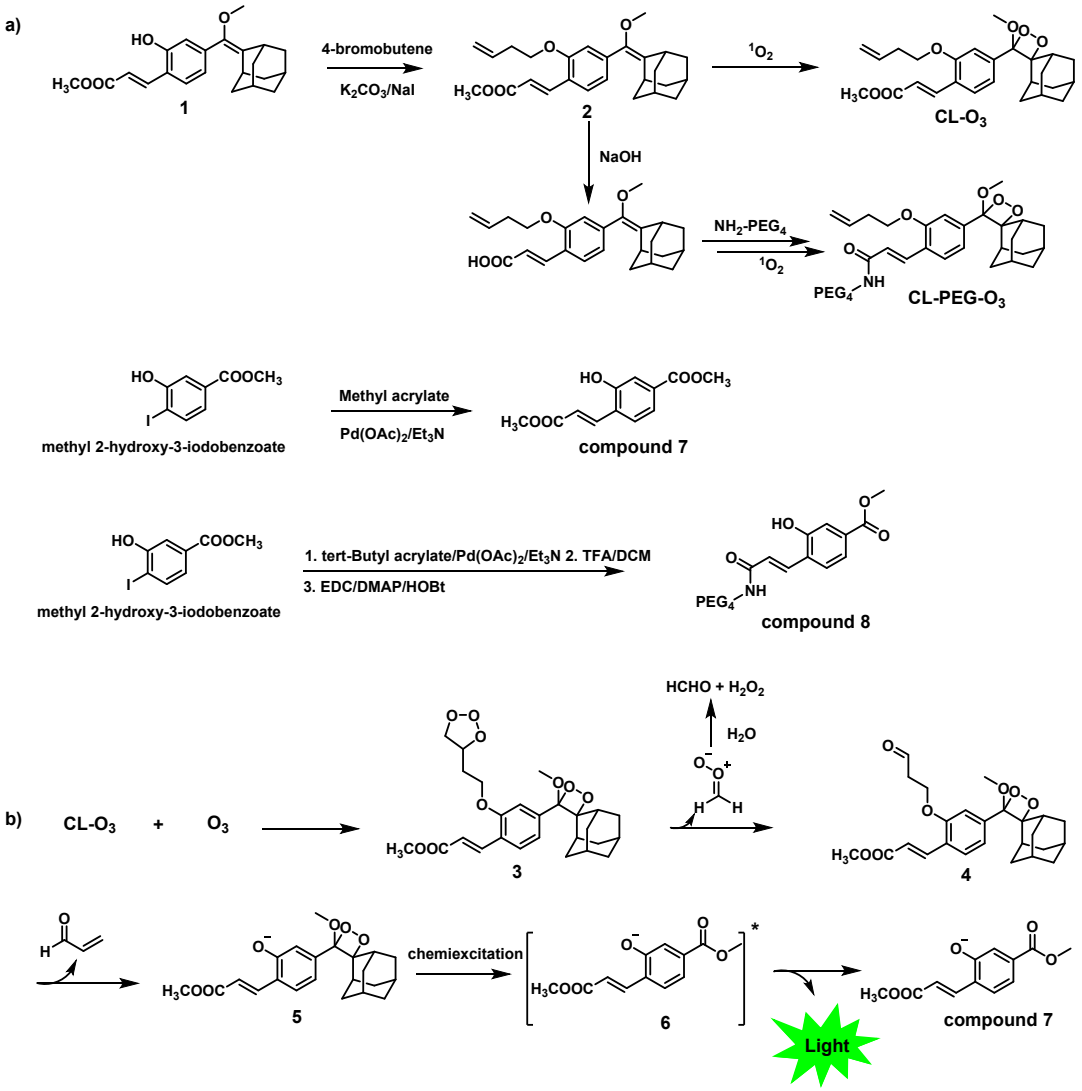
Chemiluminescence Imaging of O_3 *in vivo*. All animal procedures were performed in accordance with protocol No. SYXK (Xiang) 2008-0001 approved by the Laboratory Animal Center of Hunan and experiments were approved by the Animal Ethics Committee of College of Biology (Hunan University). All female Kunming and nude mice (20-25 g) were used in accordance with institutional

ethics committee regulations and guidelines on animal welfare. For the detection of exogenous O_3, the mixture of O_3 and **CL-O_3** or **CL-PEG-O_3** in PBS solutions was subcutaneously injected into Kunming mice. For the detection of endogenous O_3, different concentrations of PMA ware subcutaneously injected into Kunming mice and further incubated for 10 min, and then treated with **CL-O_3** for another 10 min. All of the whole body chemiluminescence images of mice were recorded by the IVIS Lumia XR system (IS1241N6071). The imaging parameters: Bioluminescence modes: open filter; Exposure time: 60 s; Field of view: D.

Table S1 Currently reported O_3 probes.

Name of probe	Type of probe	Excitation /emission (nm)	Detection limit (nM)	Applications	Reference
CL-O_3 and CL-PEG-O_3	Chemiluminescent probe	None/550 and None/530	8.5/517	*In vivo* and paper-based sensor for visualize O_3 in environmental sample	This work
Trp-Cy	Fluorescent probe	630 /770	17	Exogenous and endogenous O_3 in cell	*Chem. Commun.*, **2012**, *48*, 684
Acy7	Fluorescent probe	570 /690	10	Exogenous and endogenous O_3 *in vivo*	*Chem. Sci.*, **2019**, *10*, 2805
DCM-O_3	Fluorescent probe	560 /680	620	Exogenous O_3 in cell	*Spectrochim. Acta. A Mol. Biomol. Spectrosc.*, **2021** *248*, 119192
HBT-OZO	Fluorescent probe	385 /460	34	Paper-based sensor for visualize O_3 in environmental sample	Sensor Actuat B-Chem., **2018** *266*, 717
	Fluorescent probe	497 /523		environmental sample and exogenous O_3 in cell	*Nat chem.*, **2009**, *1,* 316
	Fluorescent probe	550 /580	5.9	Exogenous O_3 in cell	*Sci Rep.*, **2013**, *3*, 2830
	Bioluminescence probe	None/560 or 580	0.01 or 0.11	Environmental sample	*Chem. Commun.*, **2016**, *52*, 1128

Scheme S1 (a) Synthesis procedure of **CL-O$_3$**, **CL-PEG-O$_3$**, compound **7** and compound **8** and (b) the response mechanism of **CL-O$_3$** with O_3.



Synthesis of CL-O$_3$. Compound **2** (50 mg, 0.12 mmol) and methylene blue (10 mg) was dissolved in 50 mL dichloromethane in two-necked round bottom flask, then the reaction mixture was cooled to 0 ℃. Oxygen was bubbled into the solution while irradiating with a yellow light for 30 min. After reaction completion, the reaction mixture was filtered to remove excess methylene blue, and the filtrate was concentrated under reduced pressure. The crude product was purified by silica gel column with PE: EA (14:1), giving a viscous colorless oil (35 mg, 66% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.87 (d, J = 16.3 Hz, 1H), 7.82 (d, J = 8.1 Hz, 1H), 7.18 (s, 2H), 6.74 (d, J = 16.3 Hz, 1H), 5.97 – 5.87 (m, 1H), 5.22 – 5.17 (m, 1H), 5.12 (d, J = 8.8 Hz, 1H), 4.26 – 4.15 (m, 2H), 3.73 (s, 3H), 3.15 (s, 3H), 2.56 (d, J = 6.6 Hz, 2H), 1.81 – 1.42 (m, 10H), 1.22 (d, J = 2.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.80, 157.59, 139.58, 138.14, 134.06, 129.03, 119.53, 117.70, 111.85, 95.61, 68.01, 51.64, 49.97, 39.25, 36.35, 34.79, 33.71, 33.27, 33.20, 32.31, 31.70, 31.53, 29.69, 27.45, 26.02, 25.88. HRMS (ESI): calcd for

$C_{26}H_{33}O_6^+$ [M+Na] $^+$ 463.2091, found 463.2092

Synthesis of CL-PEG-O_3. Compound **2** (100 mg, 0.24 mmol) was dissolved in 20 mL THF: H_2O (3:1) solution, and NaOH (48 mg, 1.2 mmol) was added. The mixture was stirred at 50 ℃ for 2 h. After completion, the pH of the solution was adjusted to 6. The mixture was extracted with EA (50 mL×2) and concentrated under reduced pressure. The crude product was re-dissolved in 10 mL N, N-Dimethylformamide, and HATU was further introduced and stirred at room temperature for 10 min. then NH_2-PEG_4 was added and the mixture was stirred for another 2 h. After completion, the mixture was diluted with dichloromethane and washed with H_2O (50 mL×3). The organic layer was separated, dried and concentrated under reduced pressure. The product and methylene blue (5 mg) was dissolved in 50 mL dichloromethane in two-necked round bottom flask, then the reaction mixture was cooled to 0 ℃. Oxygen was bubbled into the solution while irradiating with a yellow light for 30 min. After reaction completion, the reaction mixture was filtered to remove excess methylene blue, and the filtrate was concentrated under reduced pressure. The crude product was purified by silica gel column with PE: EA (8:1), giving a light pink solid. (31 mg, 20%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.89 (d, *J* = 15.8 Hz, 1H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.36 (dd, *J* = 8.0, 1.5 Hz, 2H), 6.71 (d, *J* = 15.8 Hz, 1H), 6.01 – 5.86 (m, 1H), 5.27 – 5.20 (m, 1H), 5.17 (dd, *J* = 10.3, 1.7 Hz, 1H), 4.16 (t, *J* = 6.6 Hz, 2H), 3.69 (m, 10H), 3.67 (s, 3H), 3.65 (d, *J* = 5.1 Hz, 3H), 3.63 (d, *J* = 7.2 Hz, 1H), 3.56 (dd, *J* = 5.8, 3.3 Hz, 2H), 3.36 (s, 3H), 2.65 (q, *J* = 6.7 Hz, 2H), 2.32 (d, *J* = 3.7 Hz, 2H), 2.05 (d, *J* = 13.1 Hz, 4H), 1.94 (d, *J* = 3.0 Hz, 4H), 1.77 (dd, *J* = 9.1, 2.5 Hz, 2H), 1.60 (d, *J* = 12.7 Hz, 2H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 203.39, 166.25, 157.56, 138.62, 135.02, 134.52, 130.73, 128.72, 128.17, 128.10, 125.68, 124.03, 120.49, 117.40, 111.77, 111.25, 71.88, 70.52, 70.40, 70.16, 70.04, 69.26, 67.85, 58.96, 52.26, 39.47, 38.82, 37.42, 33.63, 32.80, 30.43, 27.94, 27.50, 17.90. MS (ESI): calcd for $C_{34}H_{49}NO_9$ [M] 615.3407, found 615.46

Synthesis of compound 7. Methyl 2-hydroxy-3-iodobenzoate (0.277 g, 1 mmol), methyl acrylate (0.258 g, 3 mmol) and Et_3N (0.45 mL) were dissolved in anhydrous ACN. Then $Pd(OAc)_2$ (34 mg, 0.15 mmol) were added. The flask was sealed and the solution was stirred at 120℃ for 2 hours. Upon completion, the solvent was evaporated under reduced pressure. The residue was purified by column chromatography on silica gel PE: EA (5:1) to afford compound 7 as a white solid (152 mg, 65%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.87 (d, *J* = 16.2 Hz, 1H), 7.74 (d, *J* = 8.1 Hz, 1H), 7.52 (d, *J* = 1.7 Hz, 1H), 7.38 (d, *J* = 8.1, 1.7 Hz, 1H), 6.74 (d, *J* = 16.2 Hz, 1H), 3.84 (s, 3H), 3.73 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.31, 166.20, 157.30, 139.30, 132.34, 129.54, 125.59, 120.11, 119.83, 117.06,

52.70, 51.95. HRMS (ESI): calcd for $C_{12}H_{13}O_5^+$ [M+H] $^+$ 237.0757, found 237.0765

Synthesis of compound 8. Methyl 2-hydroxy-3-iodobenzoate (0.277 g, 1 mmol), tert-Butyl acrylate (0.384 g, 3 mmol) and Et_3N (0.45 mL) were dissolved in anhydrous ACN. Then $Pd(OAc)_2$ (34 mg, 0.15 mmol) were added. The flask was sealed and the solution was stirred at 120℃for 2 hours. Upon completion, the solvent was filtered and concentrated under reduced pressure. The crude product was re-dissolved in 20 mL CH_2Cl_2 and 6 mL TFA was added. The reaction mixture was stirred at r.t for 3 h. Upon completion, the solvent was evaporated under reduced pressure and the residue was washed with Et_2O. The residue was re-dissolved in 20 mL CH_2Cl_2. Then EDCI (0.191 g, 1 mmol), DMAP (0.122 g, 1 mmol), HOBt (0.135 g, 1 mmol) and NH_2-PEG_4 (1.5 eq) were added. Upon completion, the reaction mixture was diluted with CH_2Cl_2 and washed with brine. The solvent was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel CH_2Cl_2 to afford compound 8 as colorless oil. (80 mg, 20%).^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.93 (d, *J* = 15.9 Hz, 1H), 7.60 (s, 1H), 7.49 (d, *J* = 8.8 Hz, 1H), 7.19 (d, *J* = 4.6 Hz, 1H), 6.83 (d, *J* = 15.9 Hz, 1H), 3.90 (s, 3H), 3.68 (t, *J* = 5.4 Hz, 12H), 3.64 – 3.60 (m, 2H), 3.60 – 3.56 (m, 2H), 3.38 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.31, 166.77, 156.02, 135.82, 131.50, 129.03, 126.82, 123.56, 120.91, 117.60, 71.81, 70.38, 70.34, 70.30, 70.21, 70.05, 69.98, 58.93, 52.20, 39.61. MS (ESI): calcd for $C_{20}H_{29}NO_8^+$ [M+H] $^+$ 412.1966, found 412.16

Optical properties

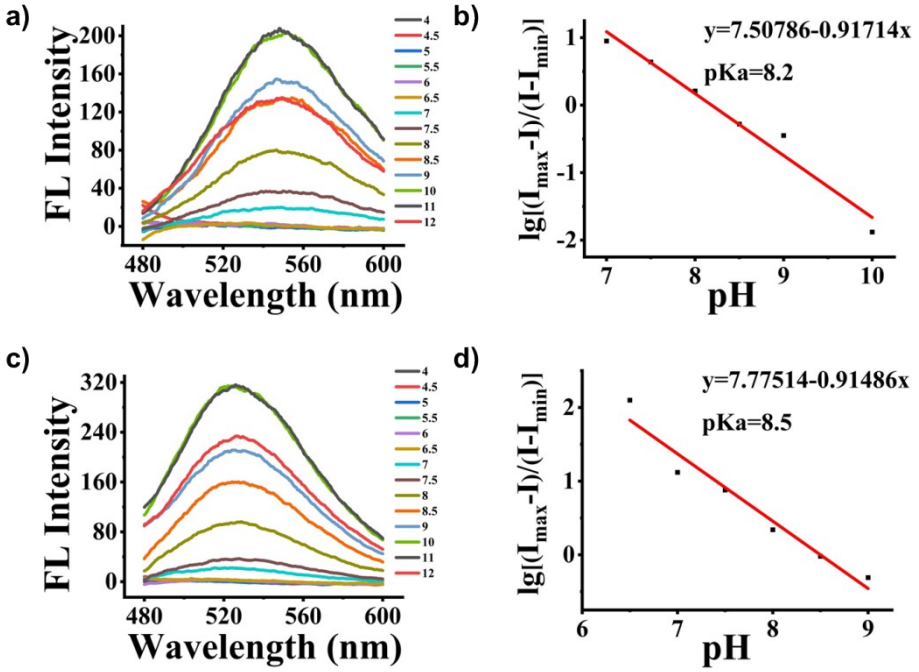


Fig. S1. (a, b) The fluorescence emission spectra of compound **7** (10 μM) and compound **8** (10 μM) in buffer solution with different pH values from pH 4.0 to pH 12. (λ_{ex}=440 nm). (c, d) The linear relationship between lg[(I_{max}-I)/(I-I_{min})] and pH (7.0-10.0 or 6.5-9.0) of compound **7** or compound **8**. The pKa value was calculated using the Henderson–Hasselbalch equation: lg[(I_{max}-I)/(I-I_{min})] = pH – p*Ka.*

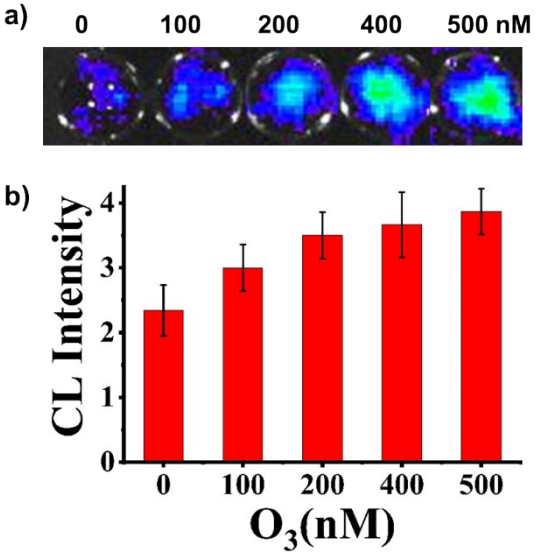


Fig. S2. The response of **CL-O_3** to nanomolar concentrations of O_3. (a) The images of **CL-O_3** reacting with different concentrations of O_3 (0, 100, 200, 400, 500 nM). (b) The chemiluminescence intensity of (a). (mean ±SD, where n = 3)

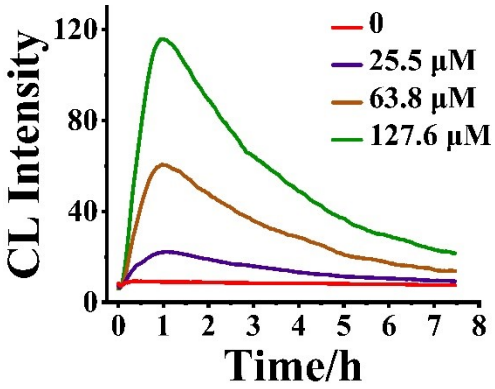


Fig. S3. Chemiluminescence kinetic profile of CL-PEG-O_3 (10 μM) for different O_3 concentrations (0, 25.5, 63.8 and 127.6 μM) at 37 °C for 7.5 h.

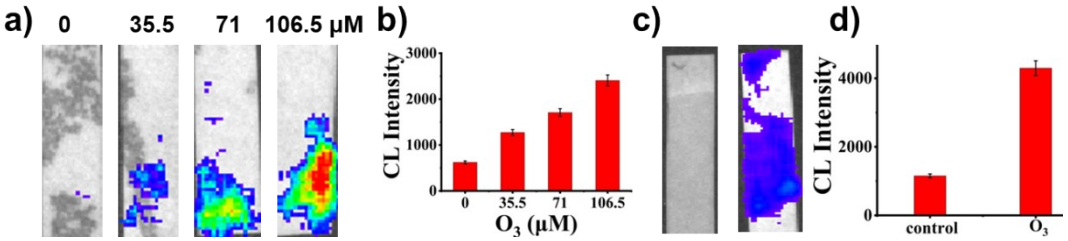


Fig. S4. Paper-based sensor for the detection of O_3 in PBS solutions (a) and in O_3 atmosphere (c). (b, d) The chemiluminescence intensity of (a) and (c). (mean ±SD, where n = 3).

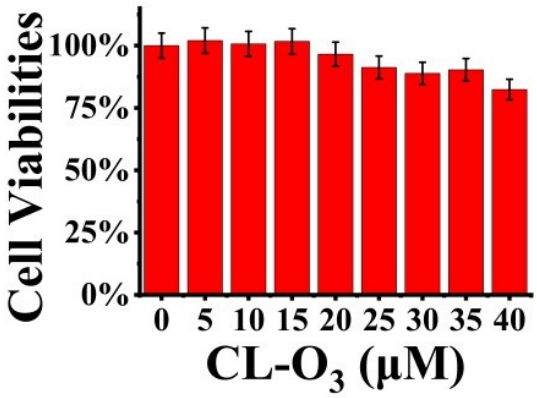


Fig. S5. Cell viabilities (%) estimated by MTT proliferation tests versus incubation concentrations of **CL-O_3**. RAW264.7 cells were incubated with (0, 5, 10, 15, 20, 25, 30, 35, 40 μM) **CL-O_3** at 37 ℃ for 12 h. (mean ±SD, where n = 3)

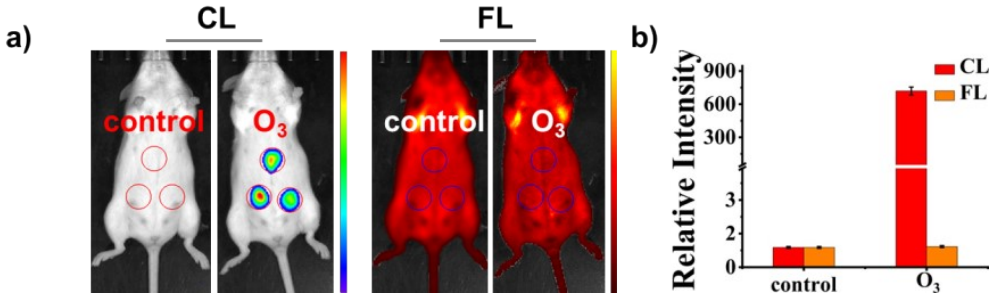


Fig. S6. (a) Chemiluminescence (left) and fluorescence (right) images of Kunming mice. b) Relative chemiluminescence and fluorescence intensity from (a) (mean ±SD, where n = 3)

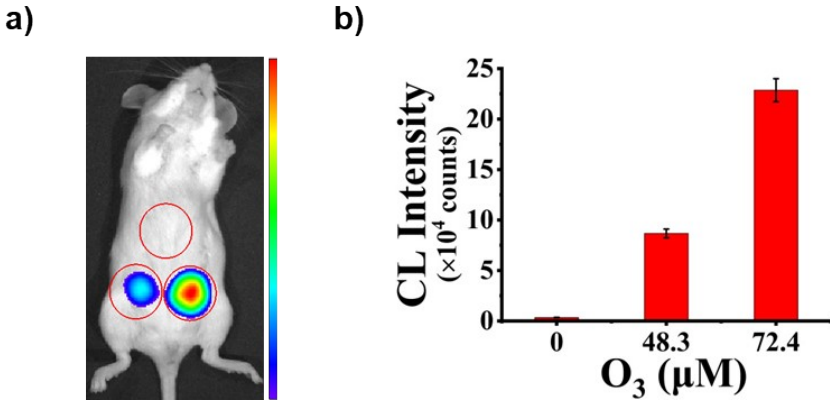


Fig. S7. (a) Chemiluminescence image of different concentrations of exogenous O_3 (0, 48.3, 72.4 μM) in Kunming mice using **CL-O_3** (20 μM). (b) The chemiluminescence intensity from (a). (mean ±SD, where n = 3)

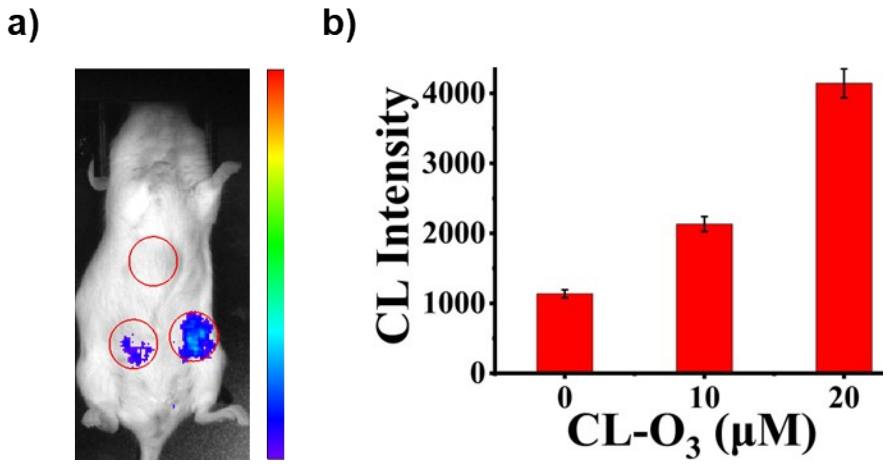


Fig. S8. (a) Chemiluminescence image of exogenous O_3 (112 μM) in Kunming mice using different concentration of **CL-O_3** (0, 10 μM, 20 μM in PBS solution containing 10% DMSO). (b) The chemiluminescence intensity from (a). (mean ±SD, where n = 3)

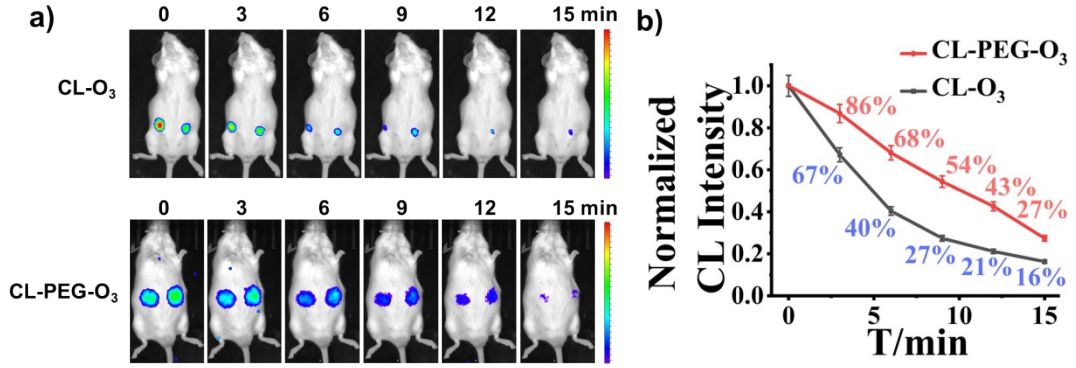


Fig. S9. Real-time chemiluminescence imaging of **CL-O$_3$** (10 μM) + **O$_3$** (98 μM) and **CL-PEG-O$_3$** (10 μM) + **O$_3$** (98 μM)

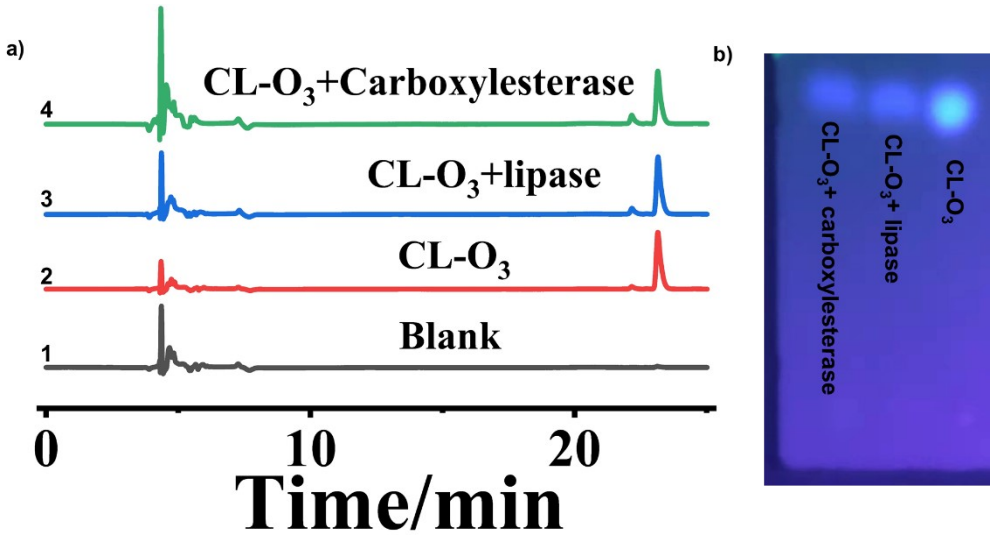


Fig. S10. (a) HPLC analysis of PBS (containing 30% DMSO), **CL-O_3** (100 μM), **CL-O_3** (100 μM) + lipase (0.8 mg/mL) and **CL-O_3** (100 μM) + carboxylesterase (20 μg/mL). (b) TLC monitoring of the reaction mixtures under excitation of 365 nm.

MS and NMR spectra

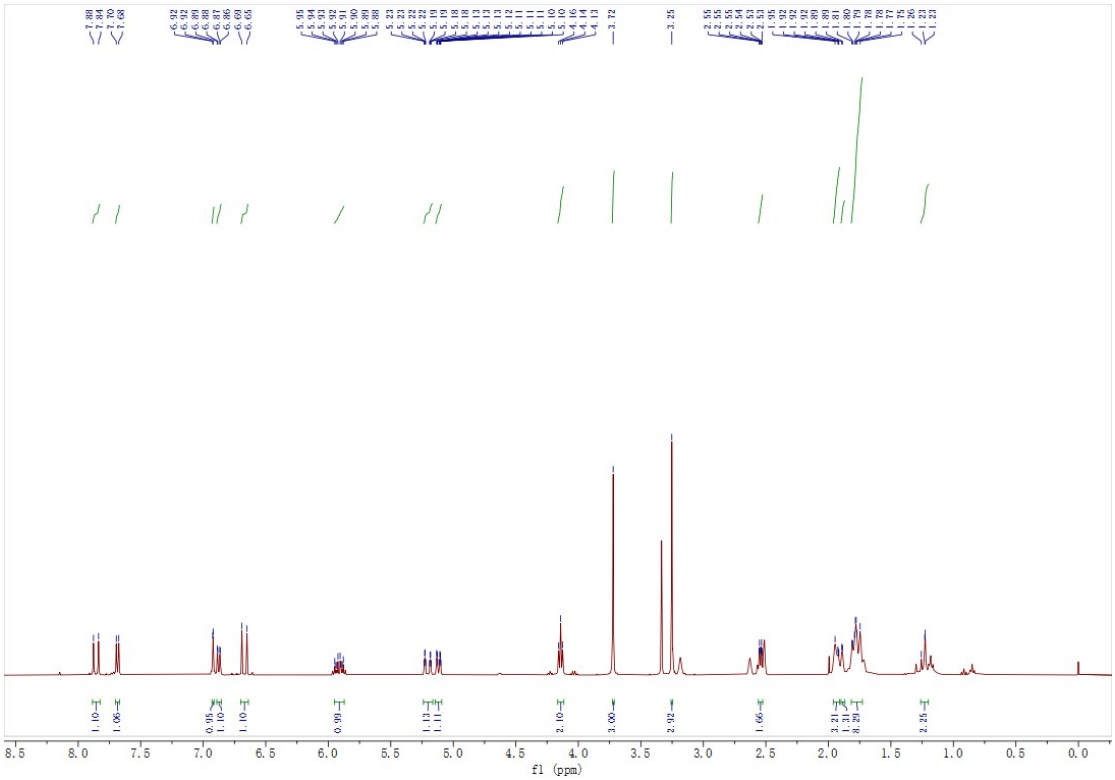


Fig. S11. ^{1}H NMR of compound **2.**

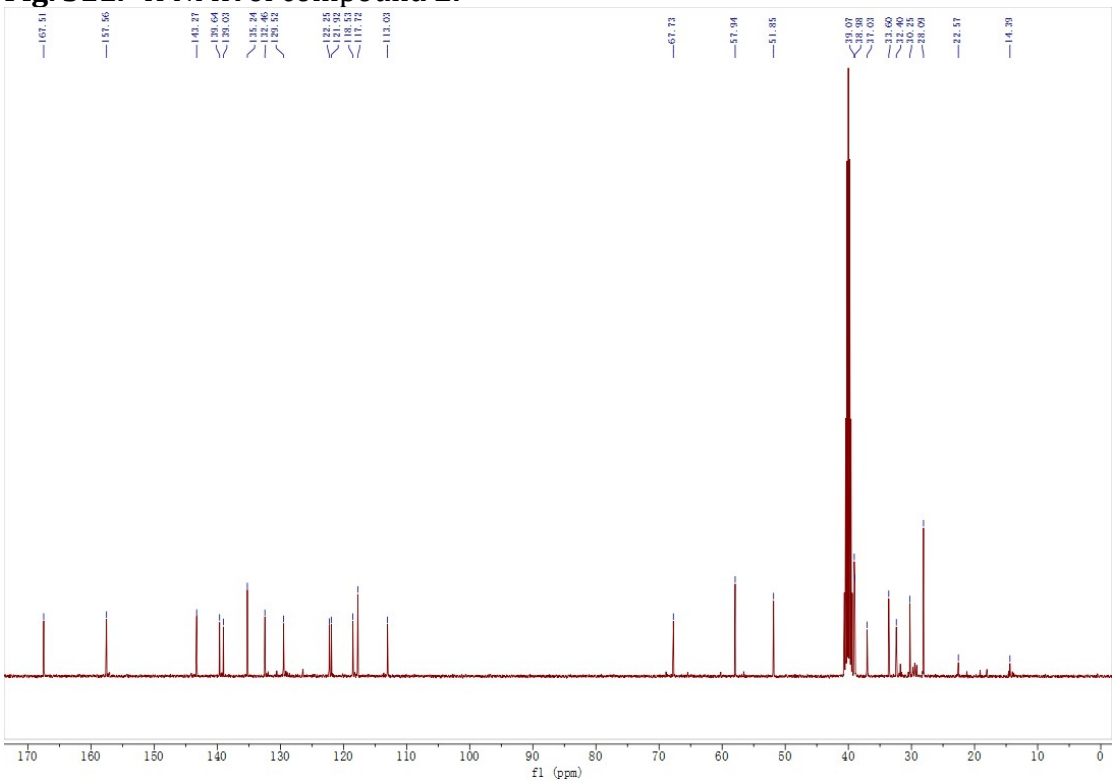


Fig. S12. ^{13}C NMR of compound **2.**

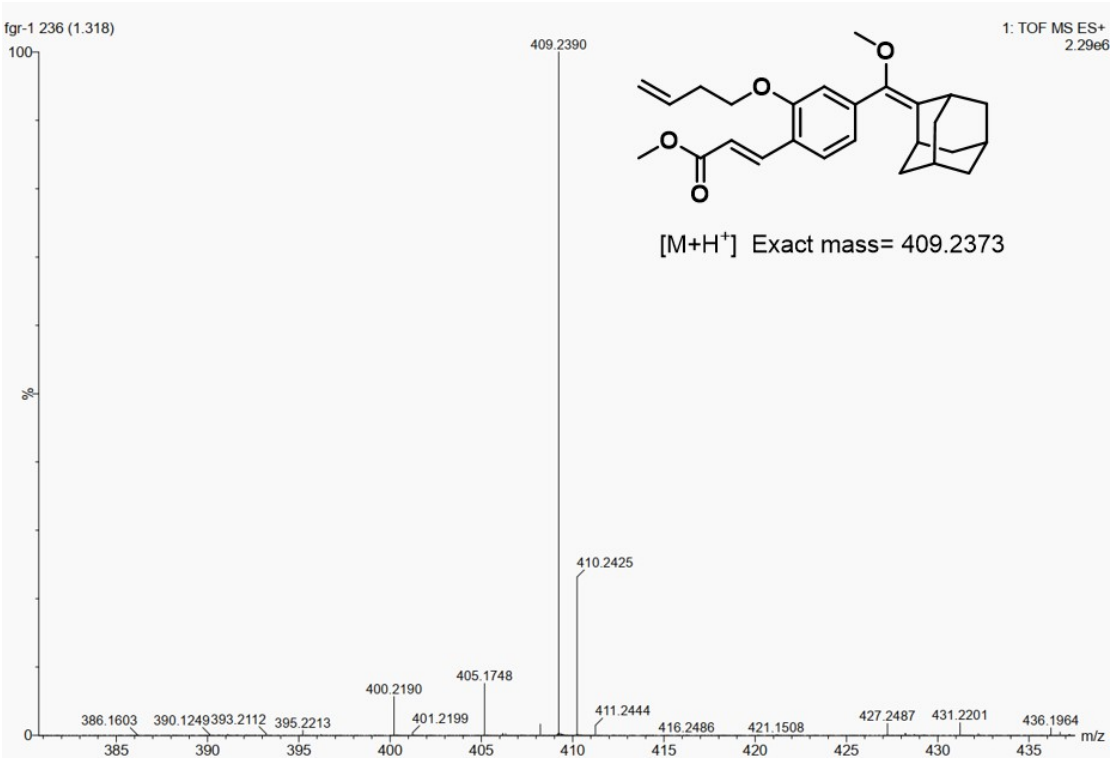


Fig. S13. HRMS spectrum of compound **2.**

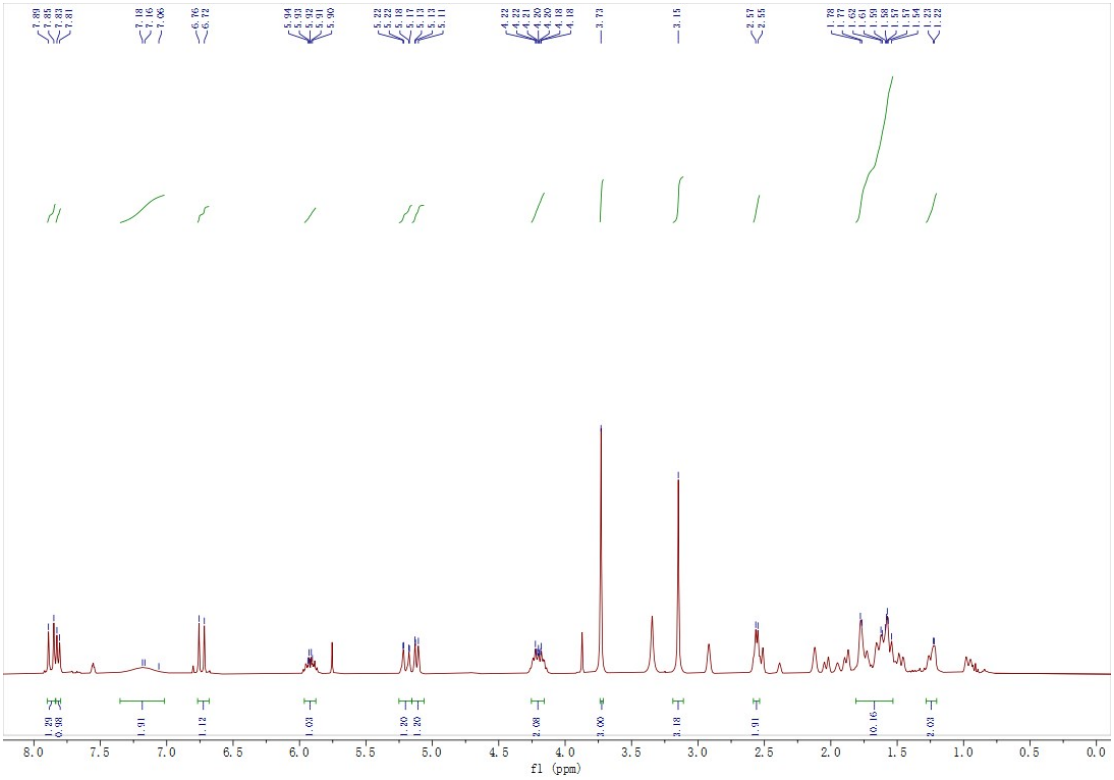


Fig. S14. ^{1}H NMR of **CL-O$_3$**.

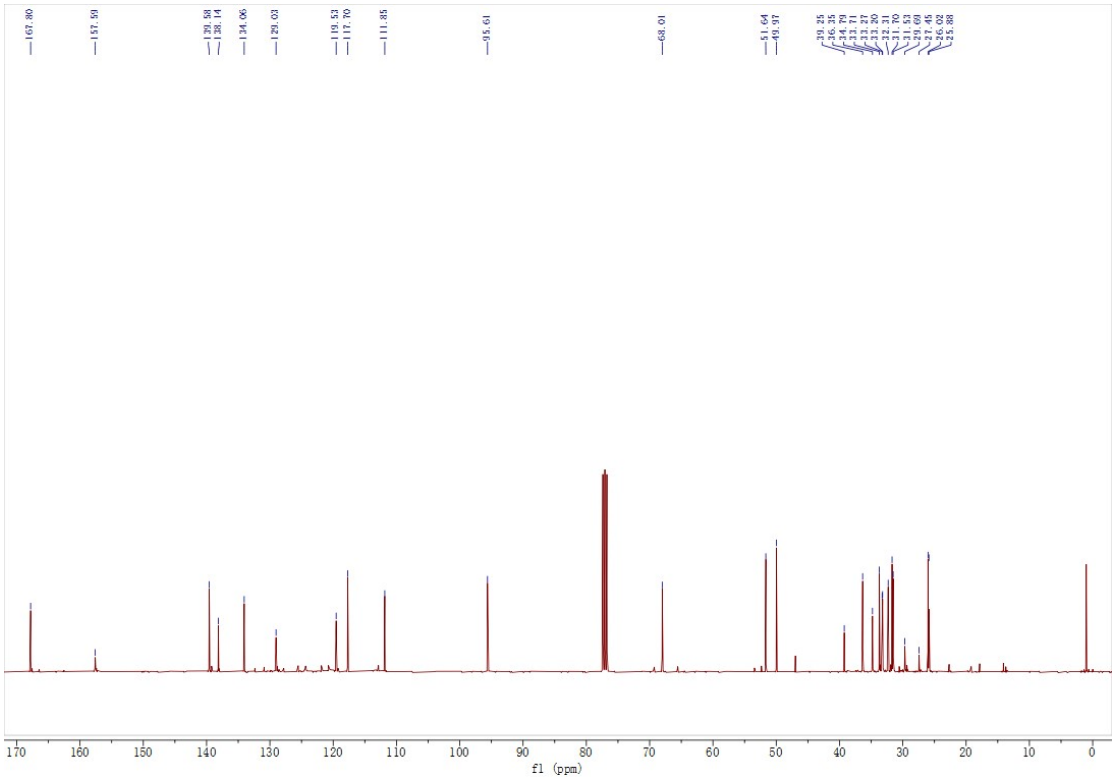


Fig. S15. ^{13}C NMR of **CL-O_3**.

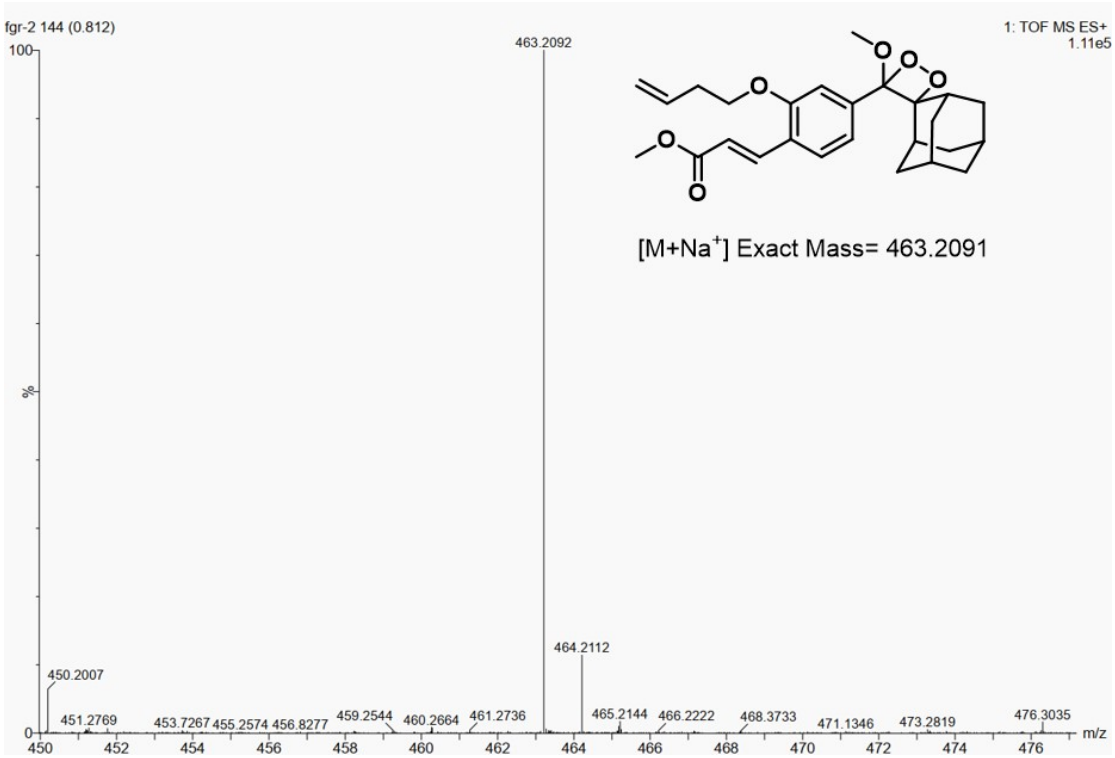


Fig. S16. HRMS spectrum of **CL-O_3.**

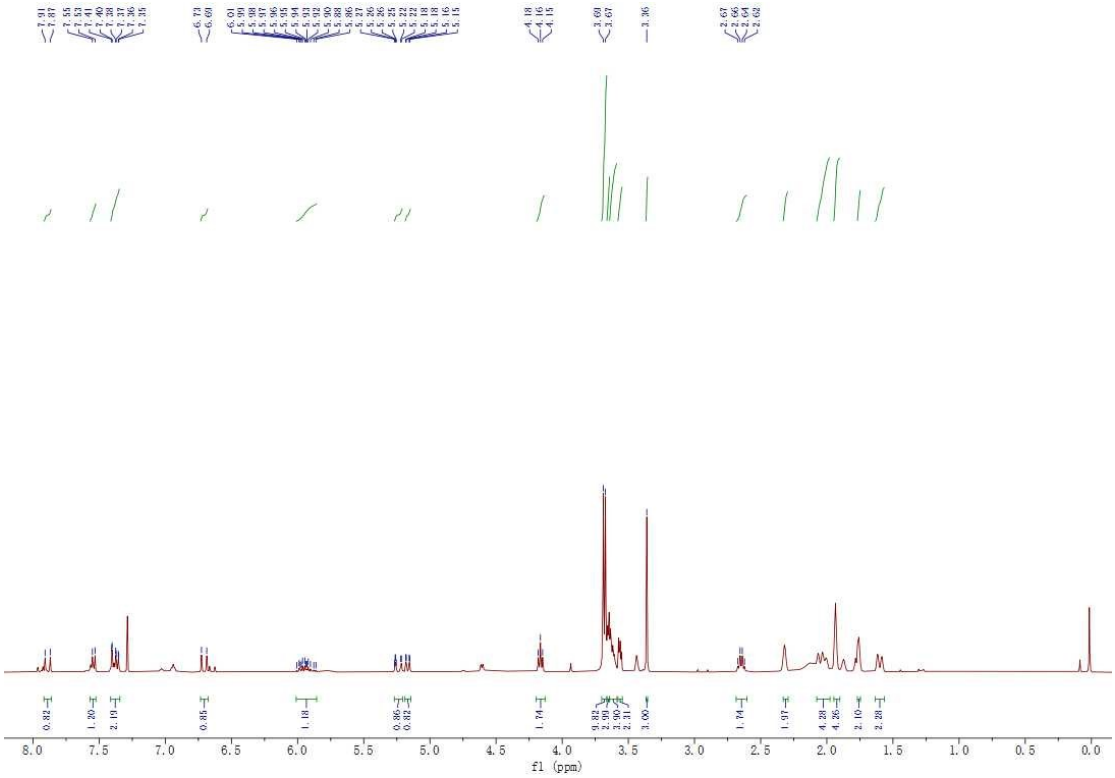


Fig. S17. ^{1}H NMR of **CL-PEG-O$_3$.**

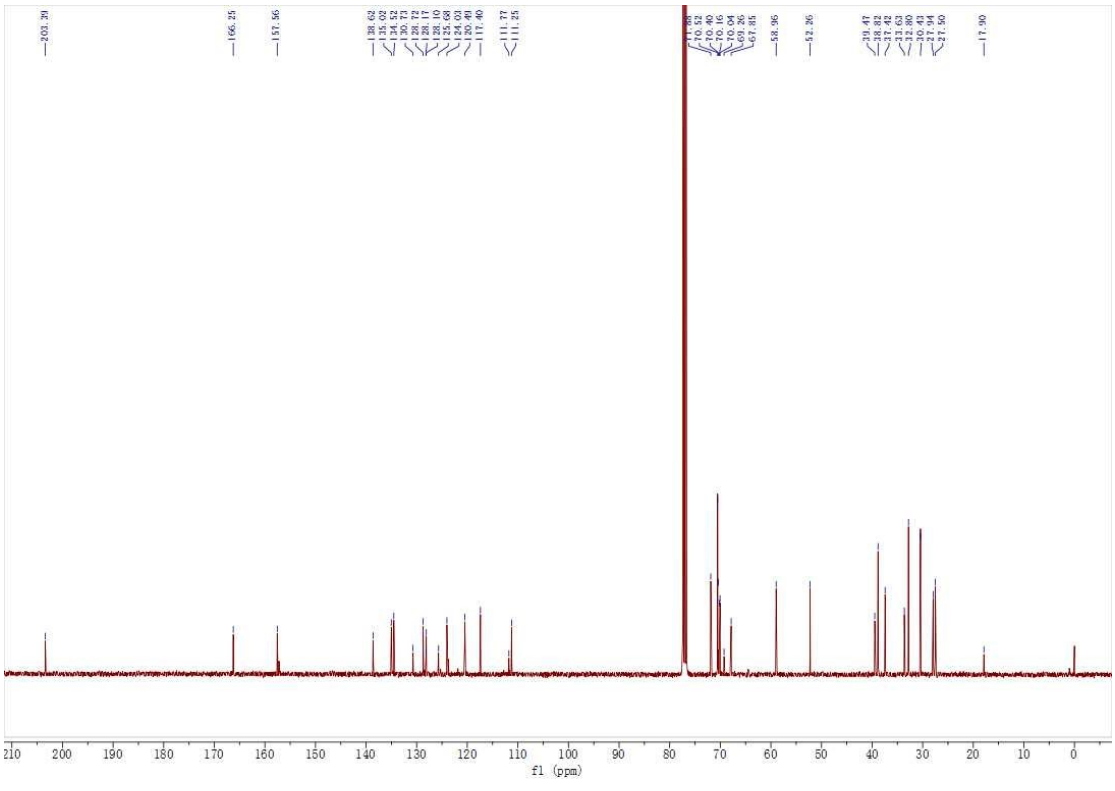


Fig. S18. ^{13}C NMR of **CL-PEG-O$_3$.**

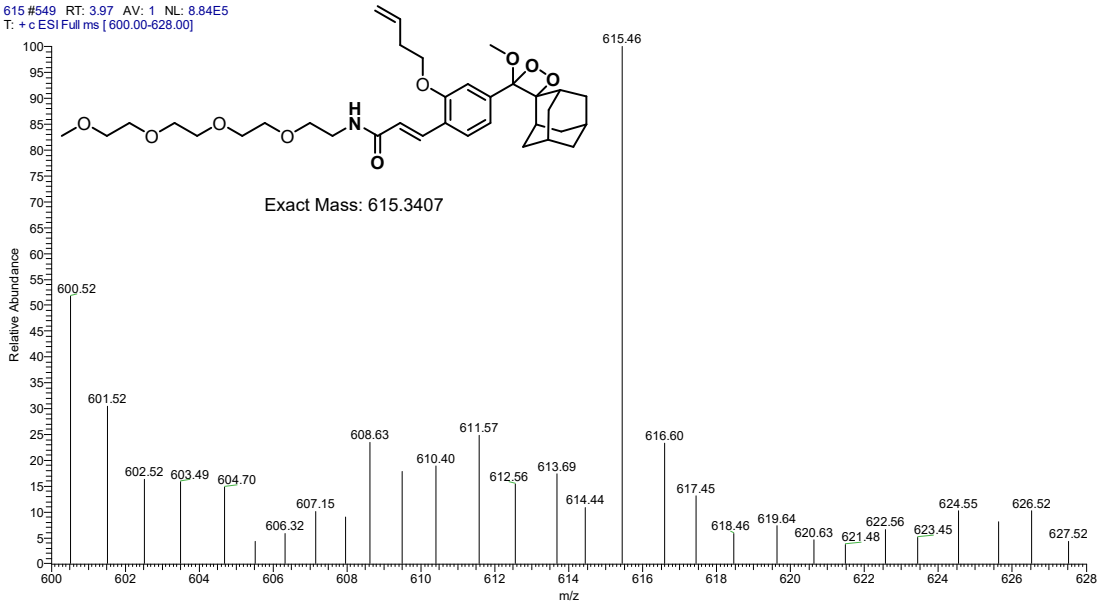


Fig. S19. MS spectrum of **CL-PEG-O_3.**

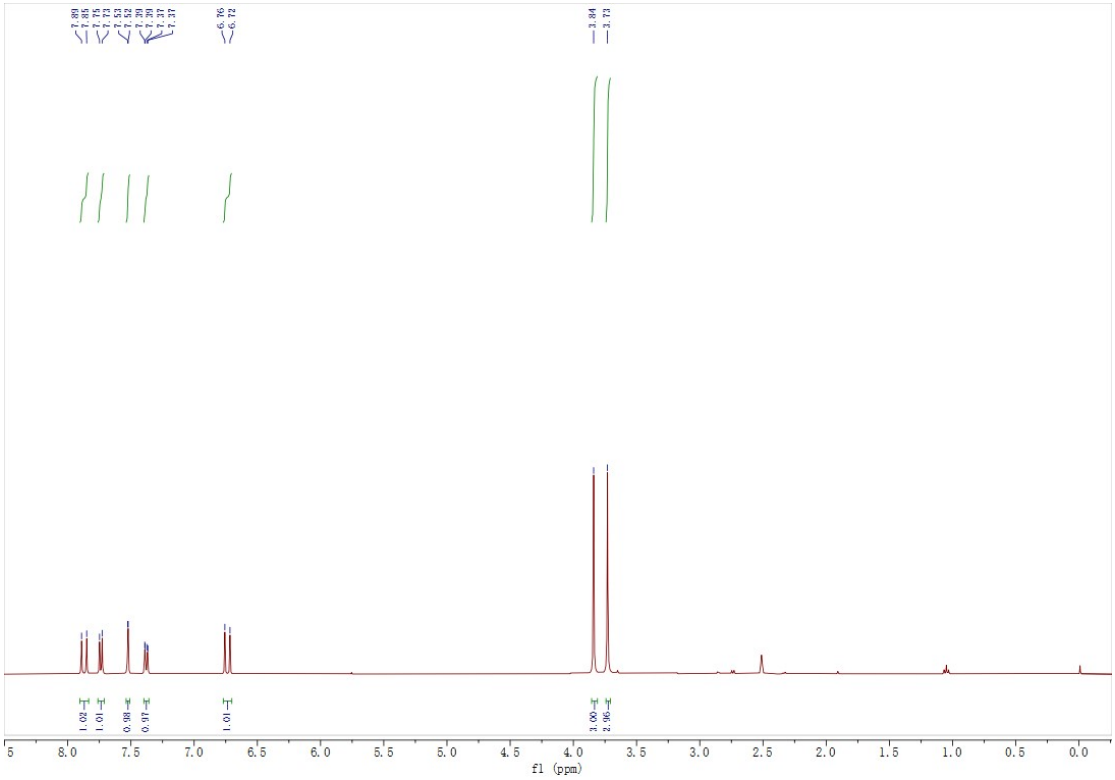


Fig. S20. ^{1}H NMR of compound **7**.

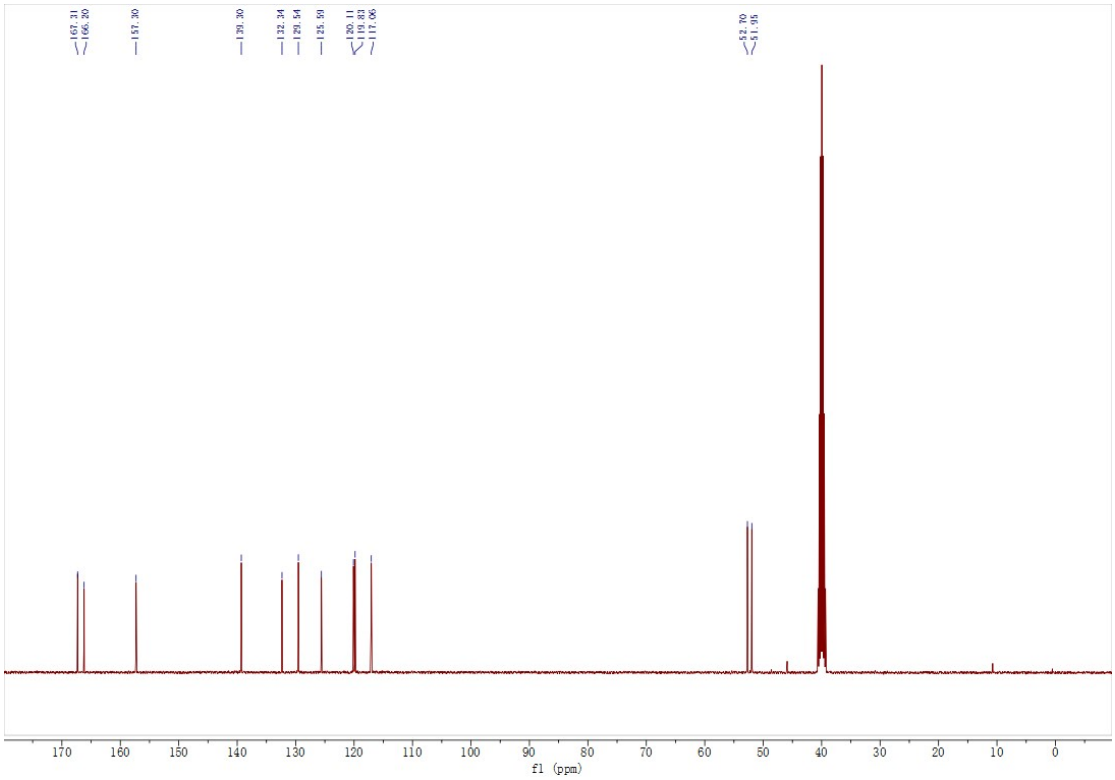


Fig. S21. ^{13}C NMR of compound **7**.

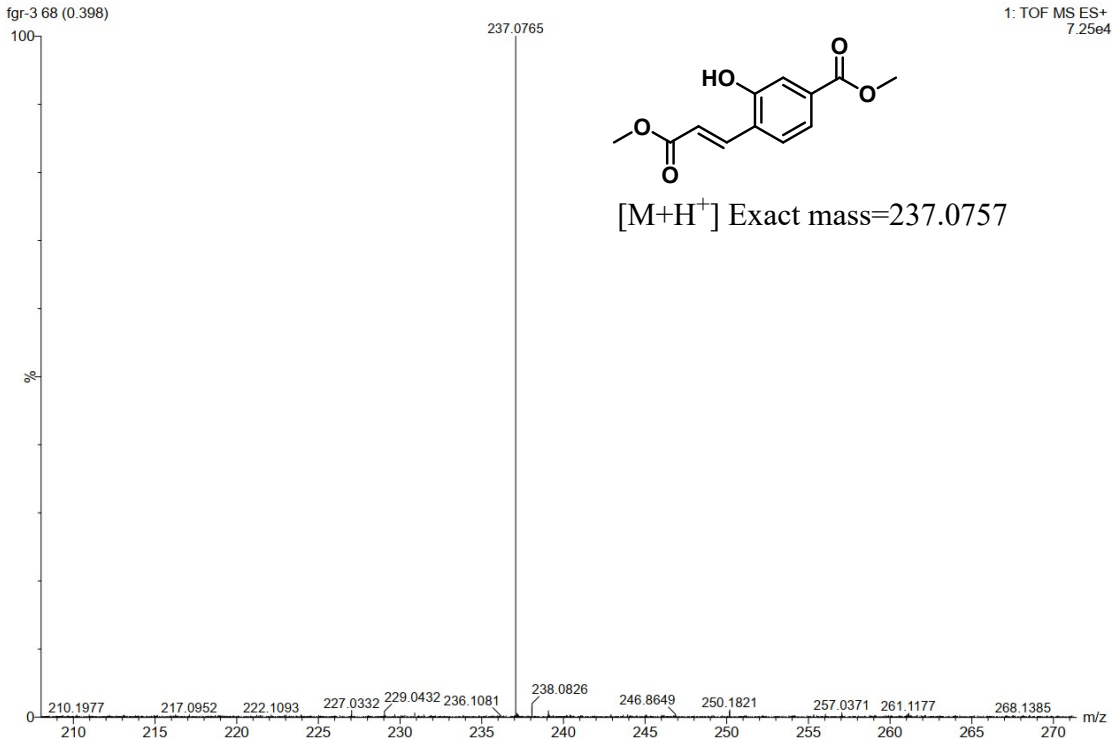


Fig. S22. HRMS spectrum of compound **7**.

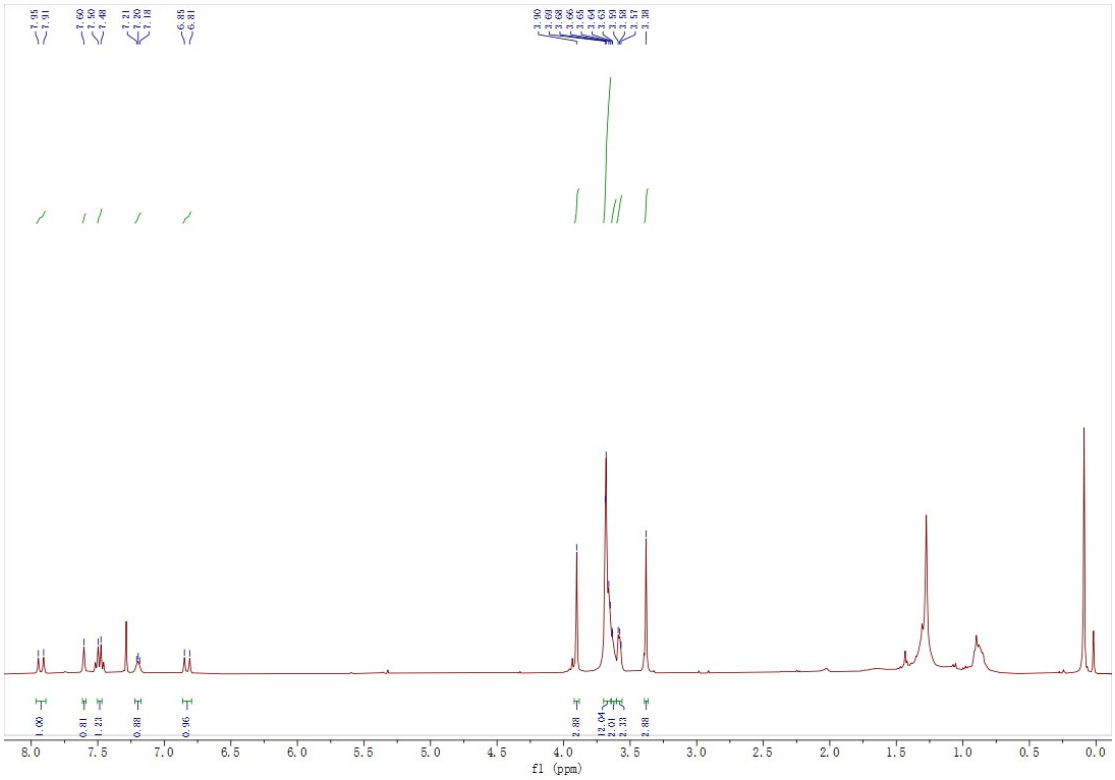


Fig. S23. ^{1}H NMR of compound **8**.

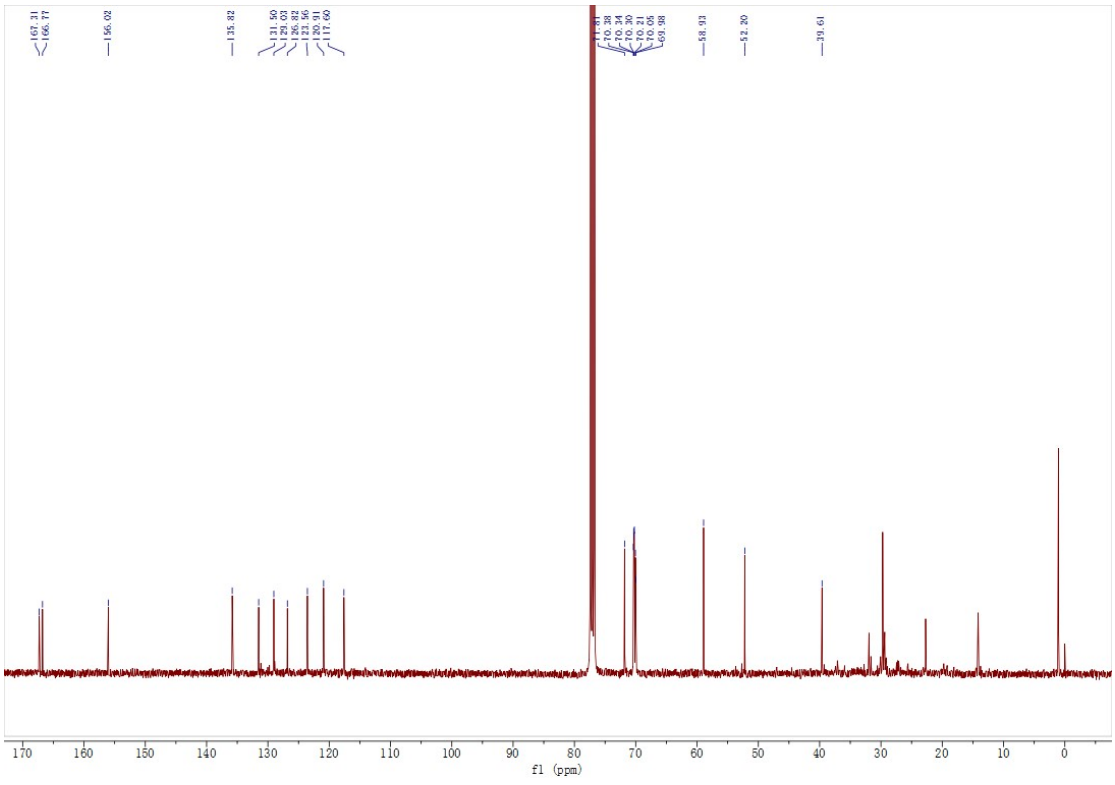


Fig. S24. ^{13}C NMR of compound **8**.

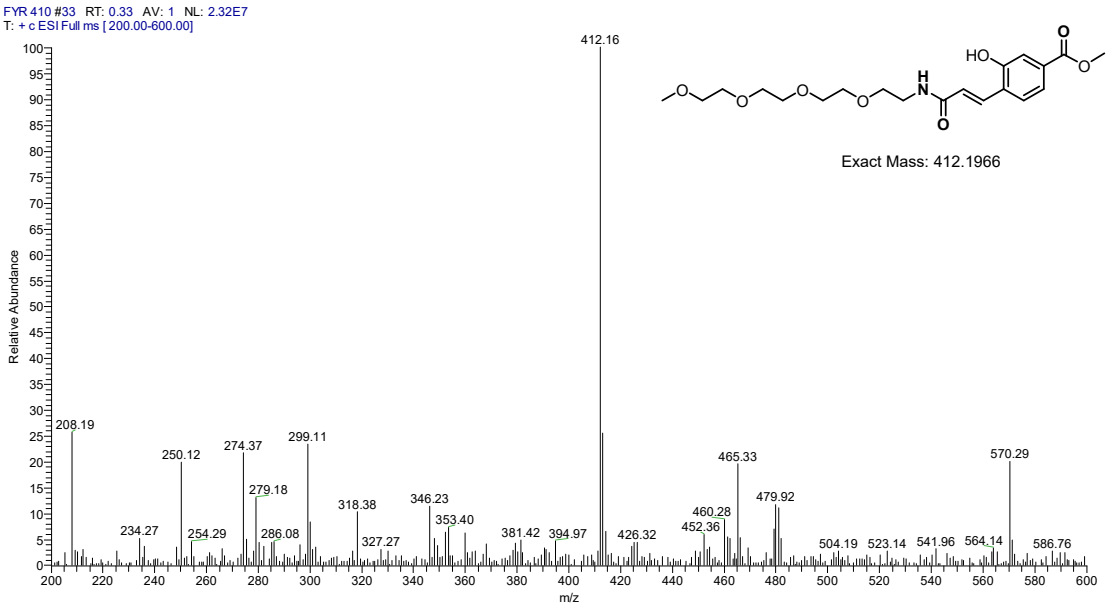


Fig. S25. MS spectrum of compound **8**.