Supplementary Information

Visualization of Lung Inflammation to Pulmonary Fibrosis via Peroxynitrite Fluctuation

Zixuan Zhan[†], Rui Liu[†], Li Chai, [§] Yongcheng Dai[†] and Yi Lv[† ‡*]

[†]Key Laboratory of Green Chemistry & Technology, Ministry of Education, College of Chemistry, Sichuan University, Chengdu, Sichuan 610064, China

[‡]Analytical & Testing Center, Sichuan University, Chengdu, Sichuan 610064, China

[§]Core Facility of West China Hospital, Sichuan University, Chengdu, Sichuan, 610041, China

E-mail: lvy@scu.edu.cn; Tel & Fax: +86 28 85412798

Content

1.General Experimental Section

Materials and Instrumentations

^{1}H NMR, ^{13}C NMR spectra were measured on a Bruker AM400 NMR spectrometer. Proton Chemical shifts of NMR spectra were given in ppm relative to internals reference TMS (1H, 0.00 ppm). ESI-MS and HRMS spectral data were recorded on a Finnigan LCQ^{DECA} and a Bruke Daltonics Bio TOF mass spectrometer, respectively. Photoluminescence spectra were performed on a Hitachi F-7000 fluorescence spectrophotometer. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Cells and tissue imaging was performed with an Nikon Ni-E multiphoton laser scanning confocal microscope and flow cytometry experiment was performed in BD FACSAria SORP.

Preparation of Solutions of Cations and Anions

1 mmol of inorganic salt (NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $ZnCl_2$, $MgCl_2 \cdot 6H_2O$, $FeCl_2$, $FeCl_3 \cdot 6H_2O$, $AlCl_3 \cdot 6H_2O$, glutathione (GSH), cysteine (Cys) were dissolved in distilled water (100 mL) to afford 1×10^{-2} mol/L aqueous solution. The stock solutions were diluted to desired concentrations with water when needed.

Preparation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

Hypochlorite was prepared by dilution of commercial NaClO solution in deionized water and assayed using a spectrophotometer using $\varepsilon_{292nm} = 350\ M^{-1}cm^{-1}$.

Tert-butyl hydroperoxide (TBHP) was prepared by dilution of commercial TBHP solution.

Hydrogen peroxide was prepared by dilution of commercial H_2O_2 solution directly.

Nitric oxide (NO) was prepared by using $Na_2Fe(CN)_5NO \cdot 2H_2O$

Peroxynitrite stock ($ONOO^-$) was prepared by following literature procedure[1] and the concentration of peroxynitrite was estimated by using a spectrophotometer $\varepsilon_{302nm} = 1670\ M^{-1}cm^{-1}$.

Hydroxy radical was prepared by addition of Fe^{2+} solution into a solution containing excess H_2O_2 through Fenton chemistry.

Superoxide was generated from KO_2 with a saturated solution of KO_2 in DMSO.

Fluorescence Analysis

Probe was dissolved in dimethylformamide (DMF) for a stock solution (500 μM). The final test solution of **rTPONOO-1 and rTPONOO-2** (5×10^{-6} mol/L) was prepared in PBS solution (containing 1%DMF, 50 mM PBS, pH 7.4). The resulting solutions were shaken well at room temperature before recording spectra.

Cell Cytotoxicity Assay

The cytotoxicity was evaluated by MTT assay. A549 cells and RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37℃ in 96-well microplates at 37°C under 5% CO_2 for 24 h. The medium was next replaced by fresh medium containing various concentrations of **rTOPNOO-1** (0-20 μM) for 12 h. Each concentration was tested in six replicates. Afterwards, cells were washed twice with PBS and incubated with 0.5 mg/mL MTT reagent for 2 h at 37°C and then 150 μL of DMSO was added to dissolve formazan. Finally, the absorbance at 490 nm was measured by multidetection microplate reader.

Cell Culture and Imaging.

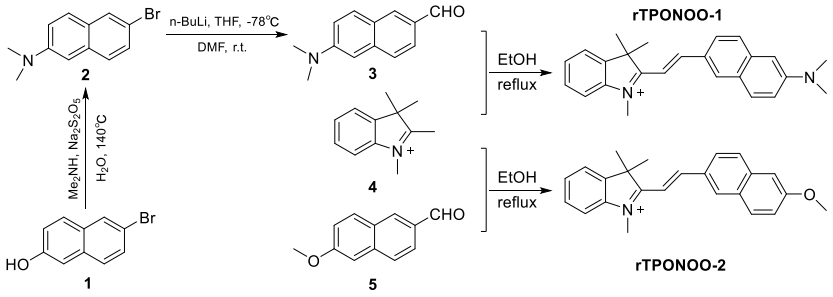
A549 cells and RAW 264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM)

containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37℃ in a 5% CO2/95% air incubator. For detection of exogenous ONOO-, the cultured cells in a 6-well plate were washed three times with PBS and then incubated with 10 μM of **rTPONOO-1** for 30 min at 37 °C in a 5% CO2/95% air incubator. After washing twice with PBS, the cells were cultured for another 30 min with different concentrations of ONOO-. The cells were washed twice carefully with PBS and imaged. For detection of endogenous ONOO-, RAW 264.7 cells in a 6-well plate were stimulated by LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 12 h and then treated with **rTPONOO-1** for another 0.5 h in PBS. The cells were washed three times carefully with PBS and imaged.

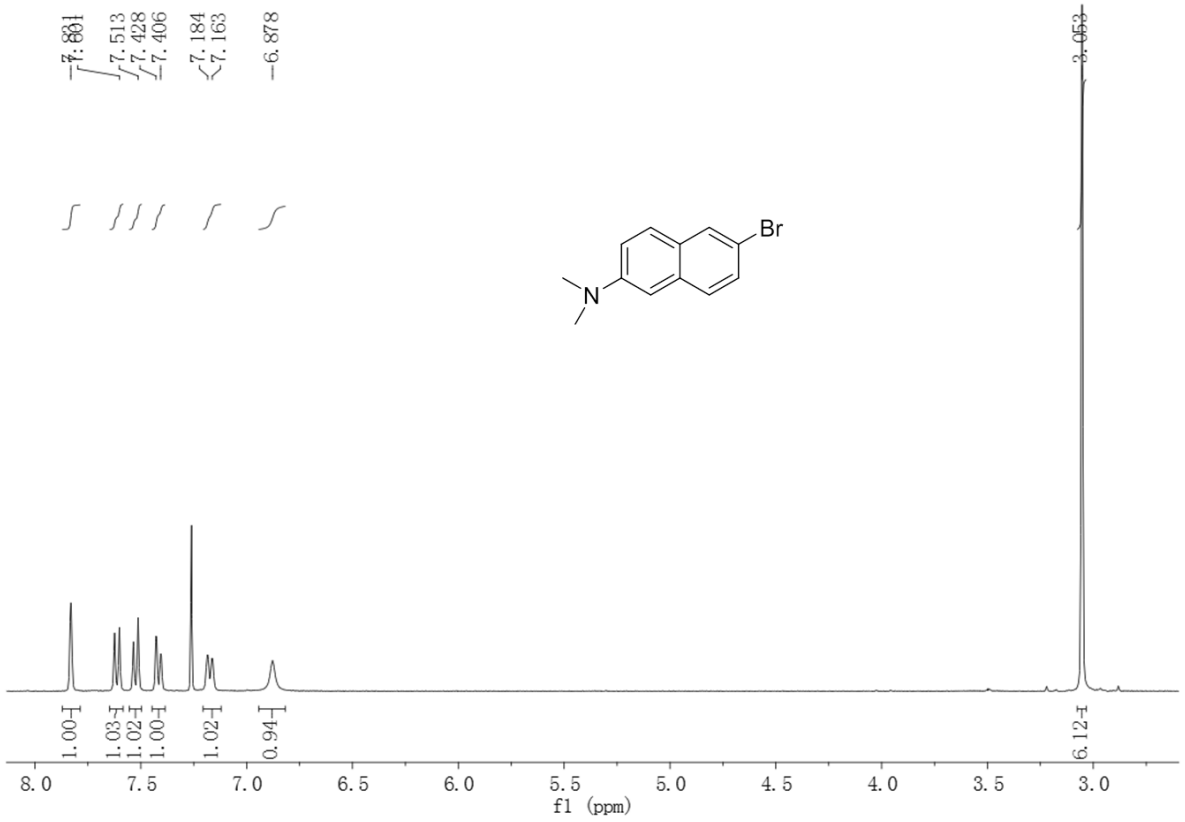
Flow Cytometry Experiments

Cells were treated as described in Cell culture and imaging part. Before measurement by flow cytometry, cells were scraped off gently and collected into a clean 2 mL centrifuge tube. Then, cells were spun down (500 rpm, room temperature, 3 min). After discarding the supernatant, 1 mL of warm PBS was added gently to re-suspend the cell pellet. Finally, cells were analyzed on a BD flow cytometer equipped with 405 nm and 488 nm Ar laser and fluorescence was collected by 525/50 and 695/40 channel.

2.Synthesis and Characterization of Compounds



Scheme S1. Synthesis of rTPONOO-1 and rTPONOO-2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1.02
1.00
1.02
0.94
6.12
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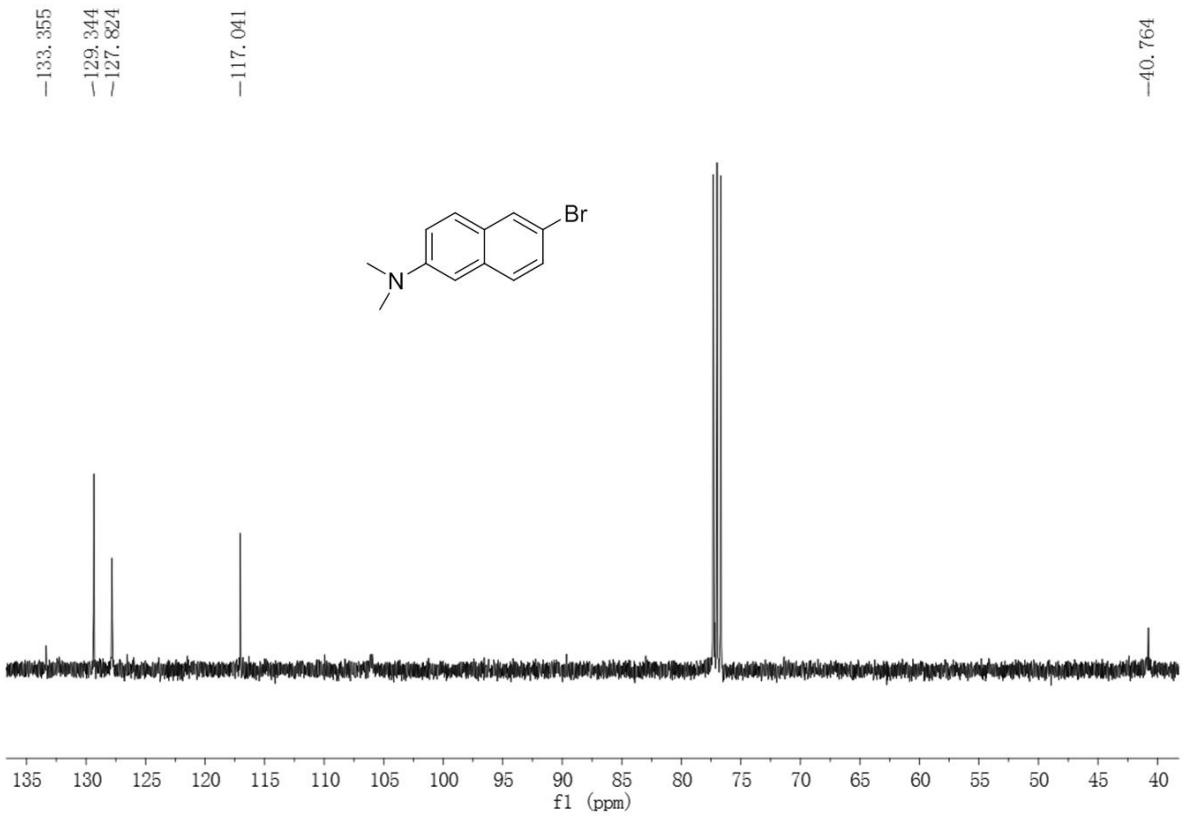
3.0
f1 (ppm)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65
60
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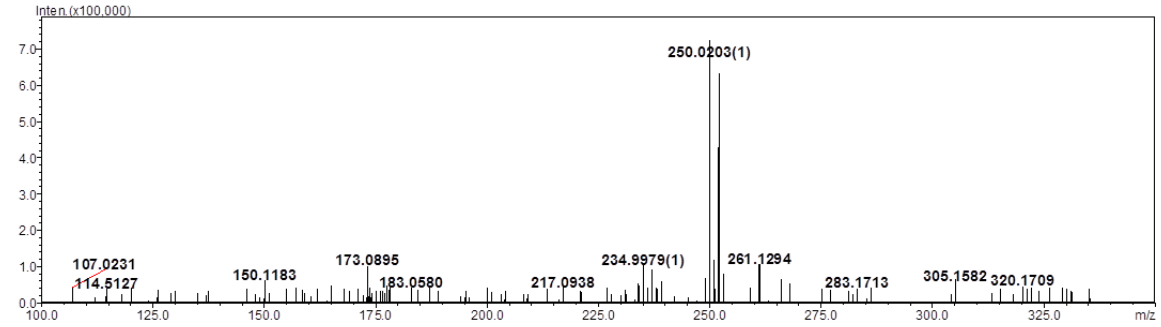
50
45
40
f1 (ppm)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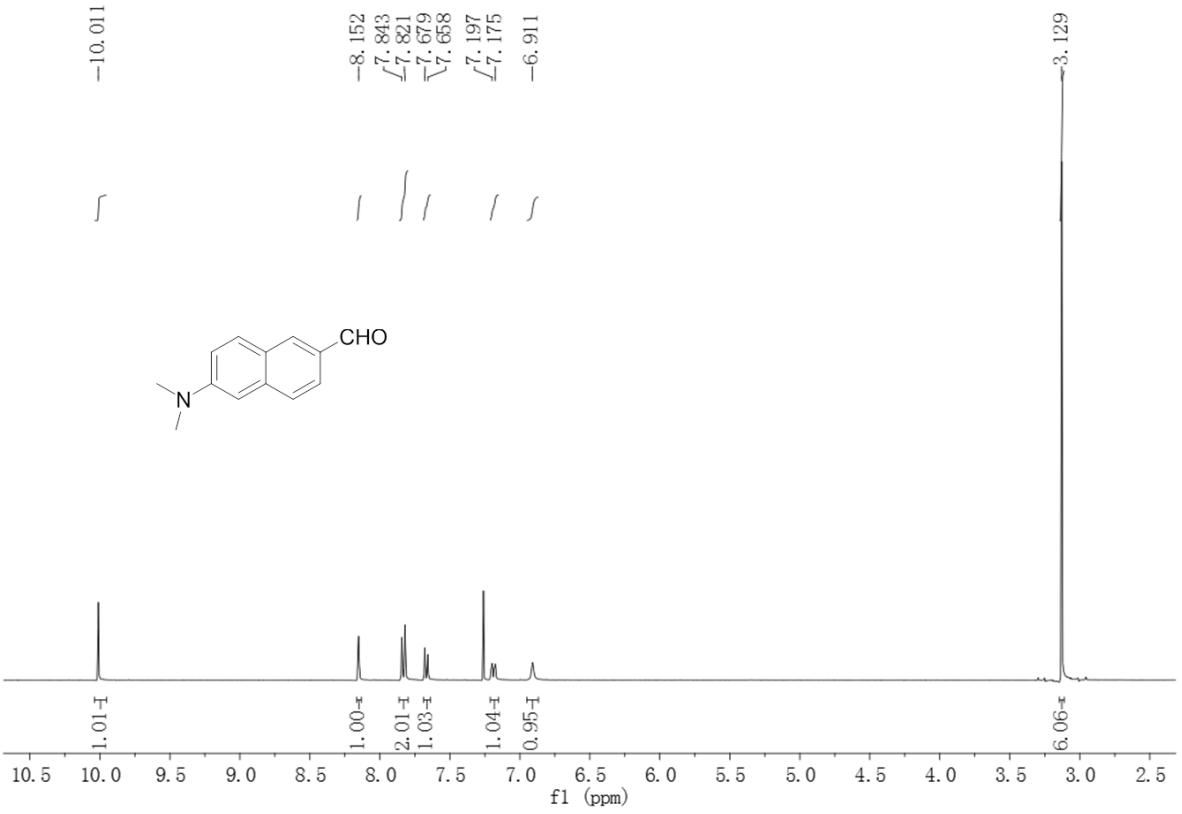
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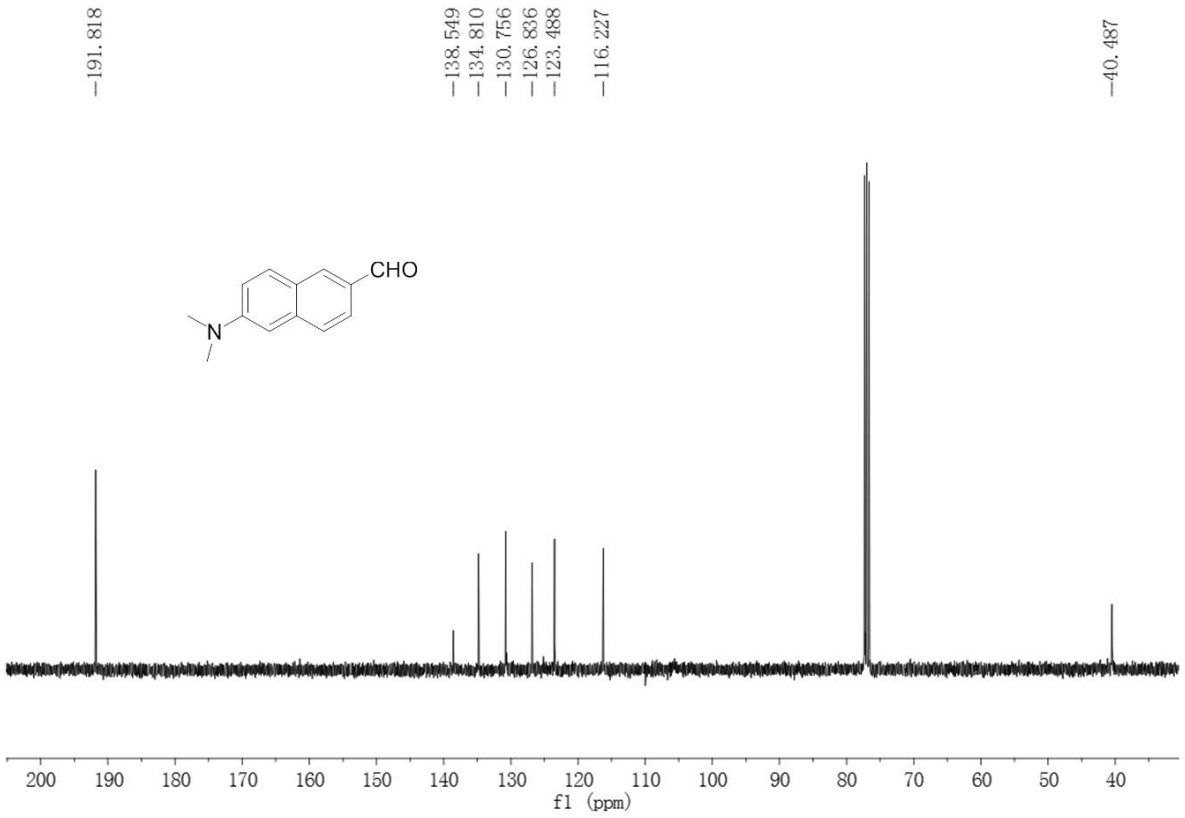
f1 (ppm)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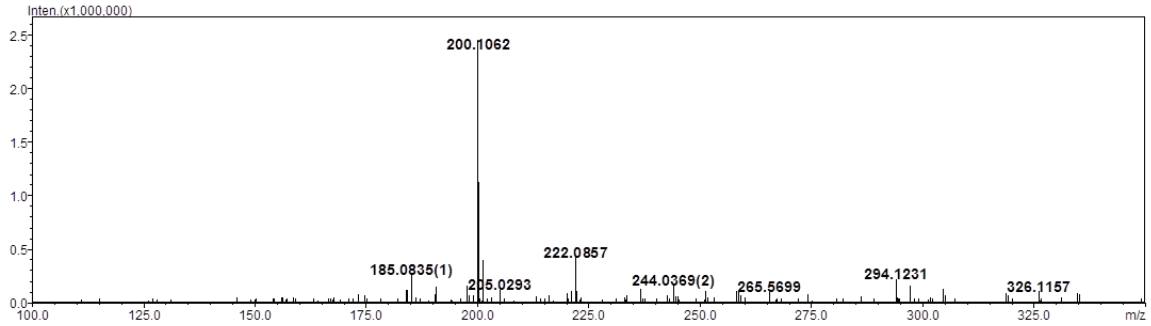
f1 (ppm)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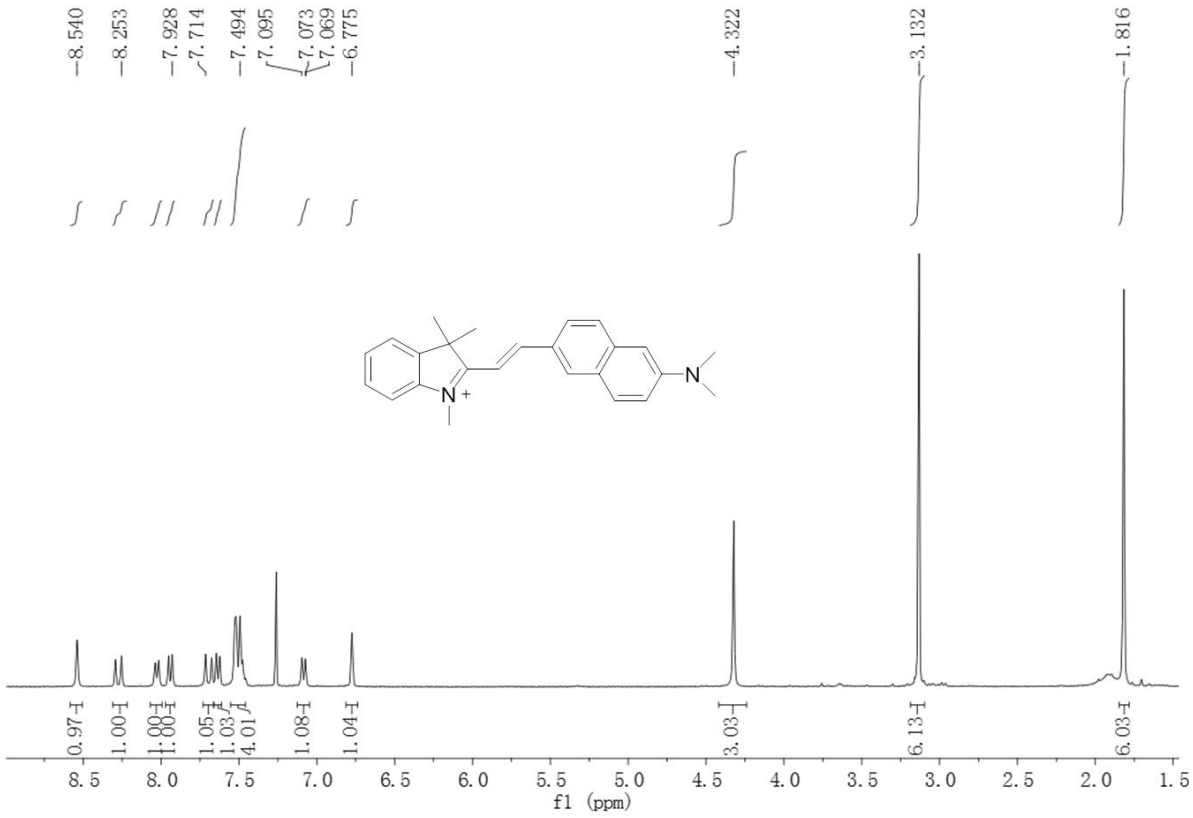
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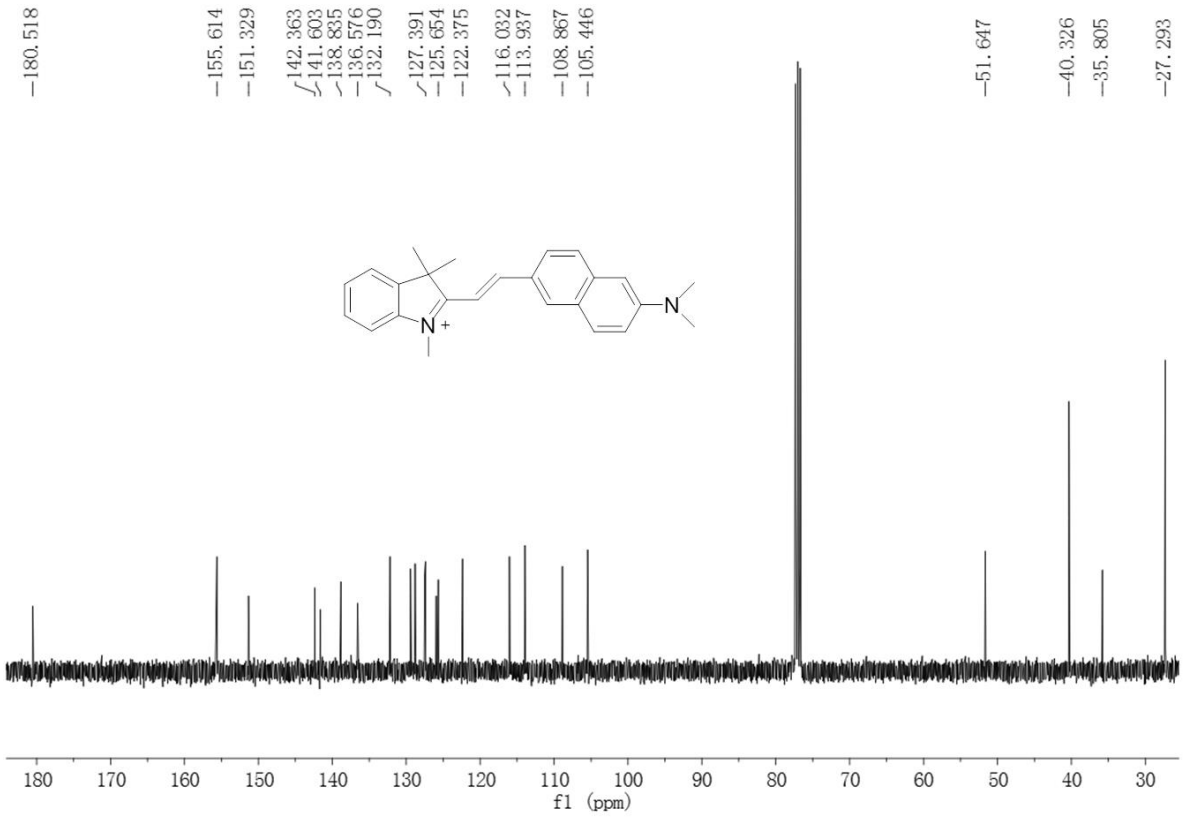
6.03
f1 (ppm)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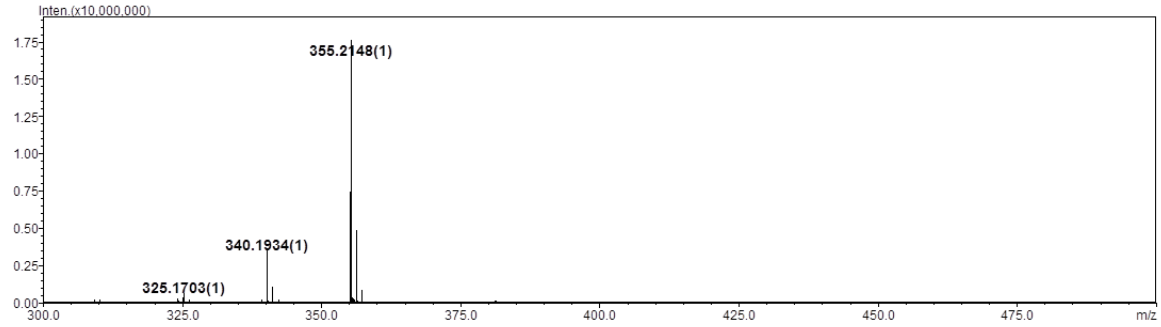
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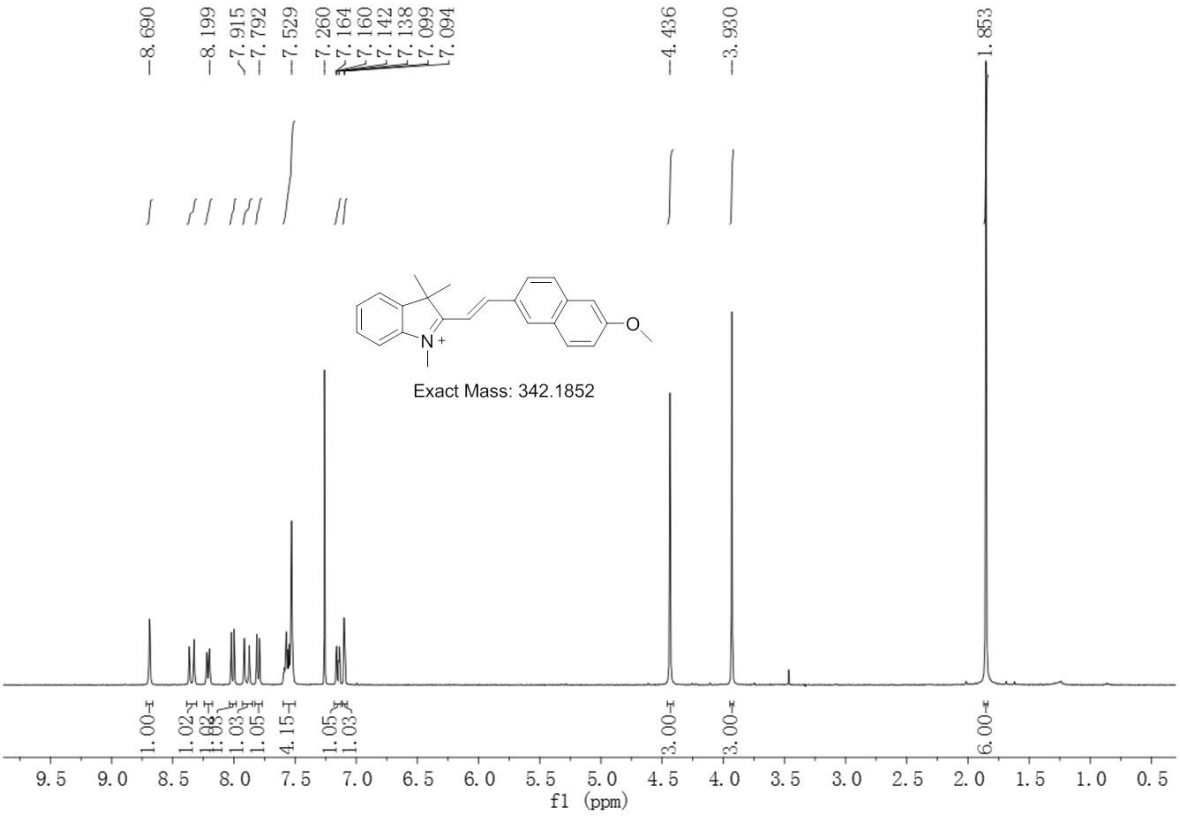
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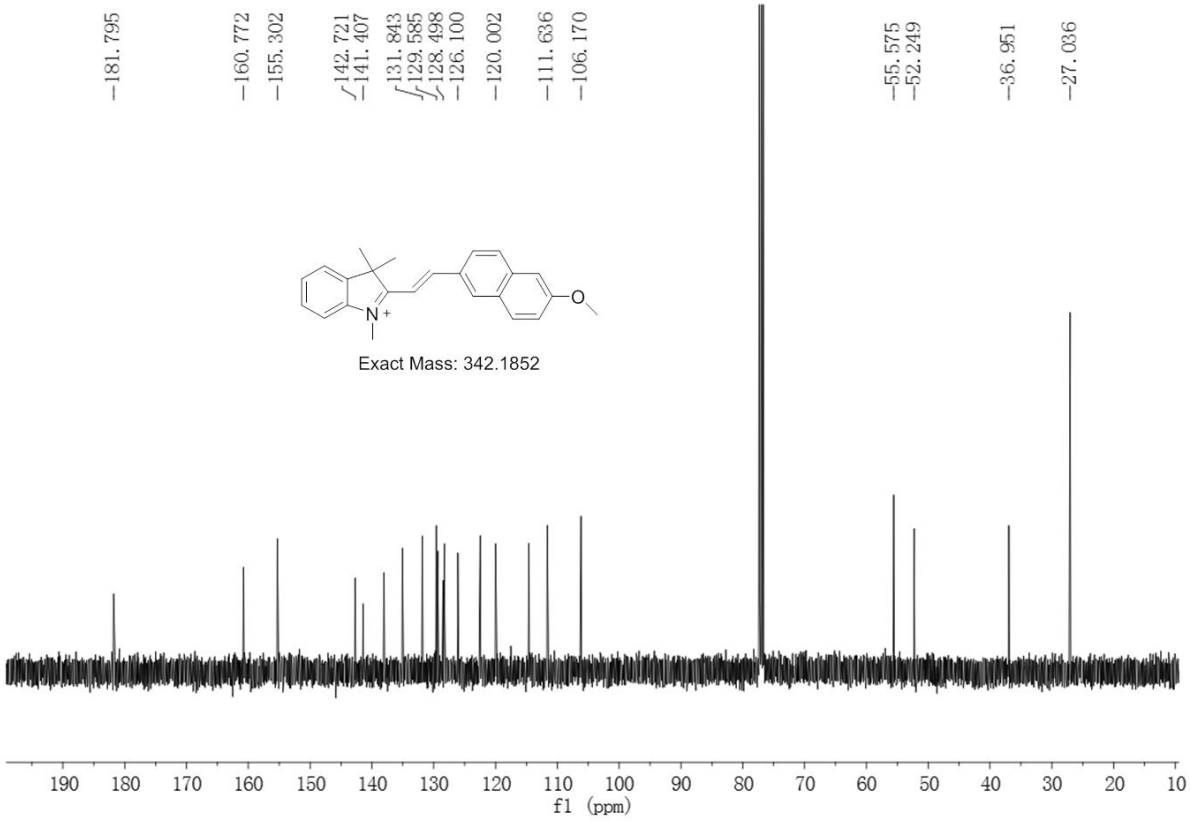
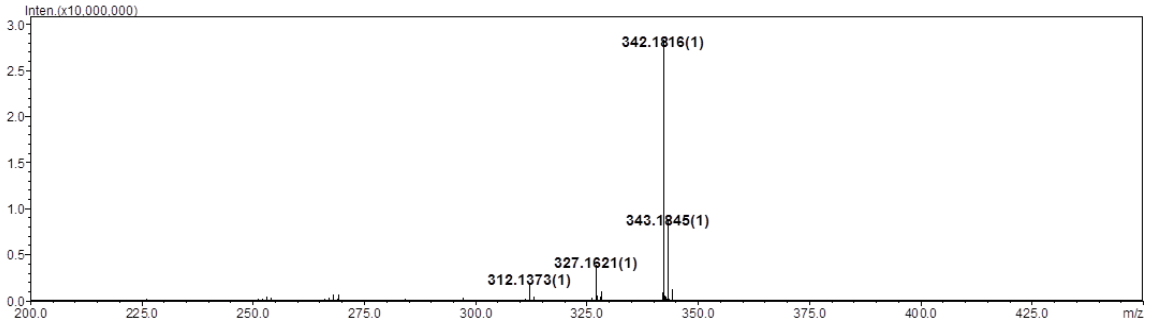
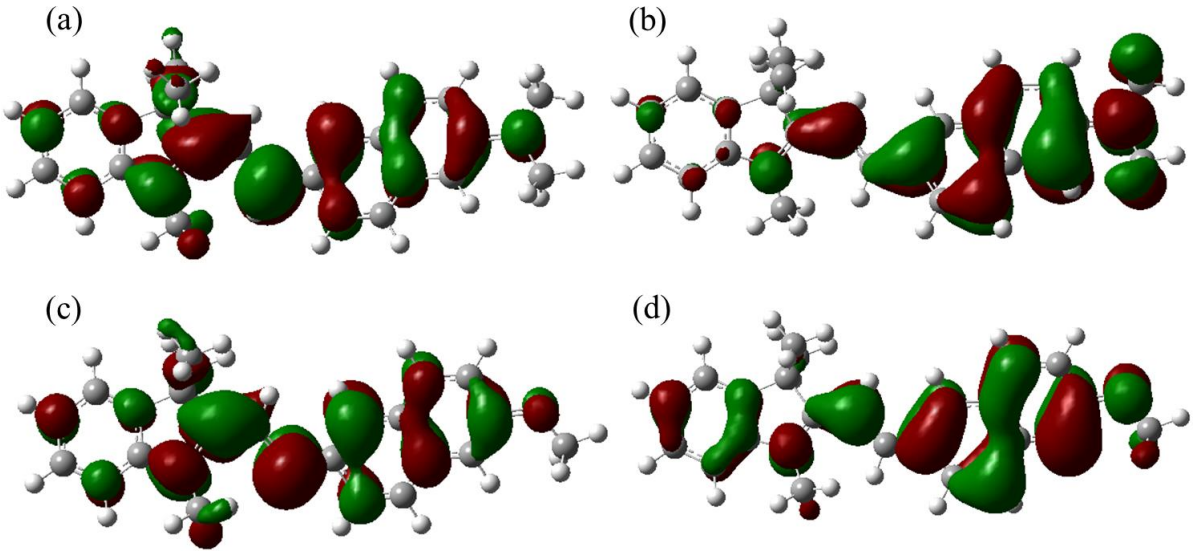
f1 (ppm)

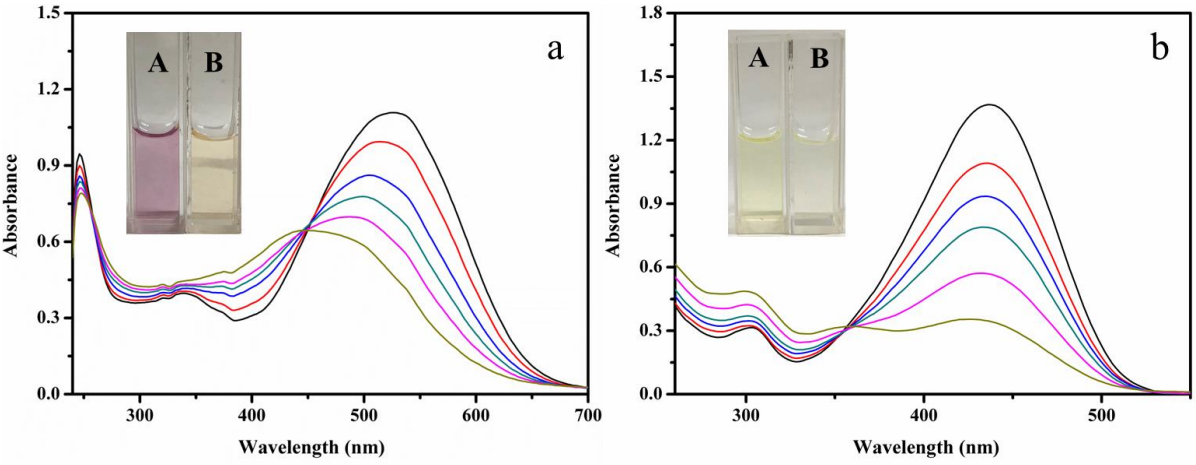




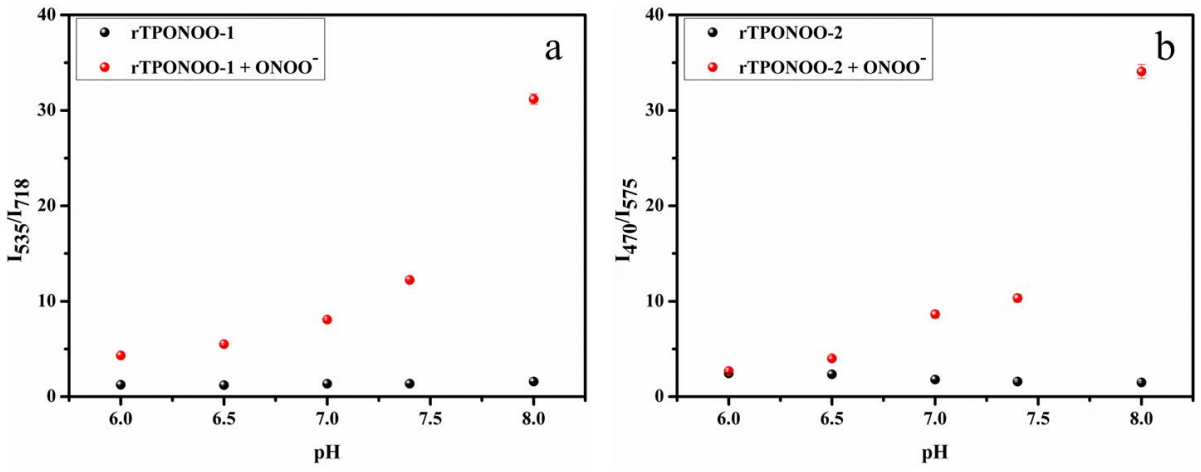
3. Figure S1 ^{1}HNMR, ^{13}CNMR and HRMS of compounds



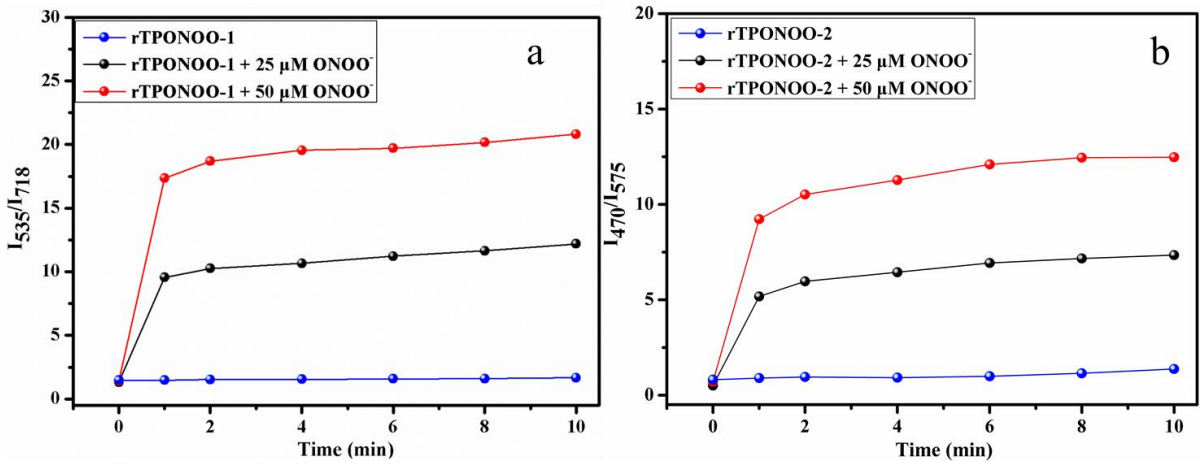
4. Figure S2 HOMO and LUMO energy level of **rTPONOO-1** and **rTPONOO-2** （a）LUMO orbital of **rTPONOO-1** （b）HOMO orbital of **rTPONOO-1** （c）LUMO orbital of **rTPONOO-2** （d）HOMO orbital of **rTPONOO-2**



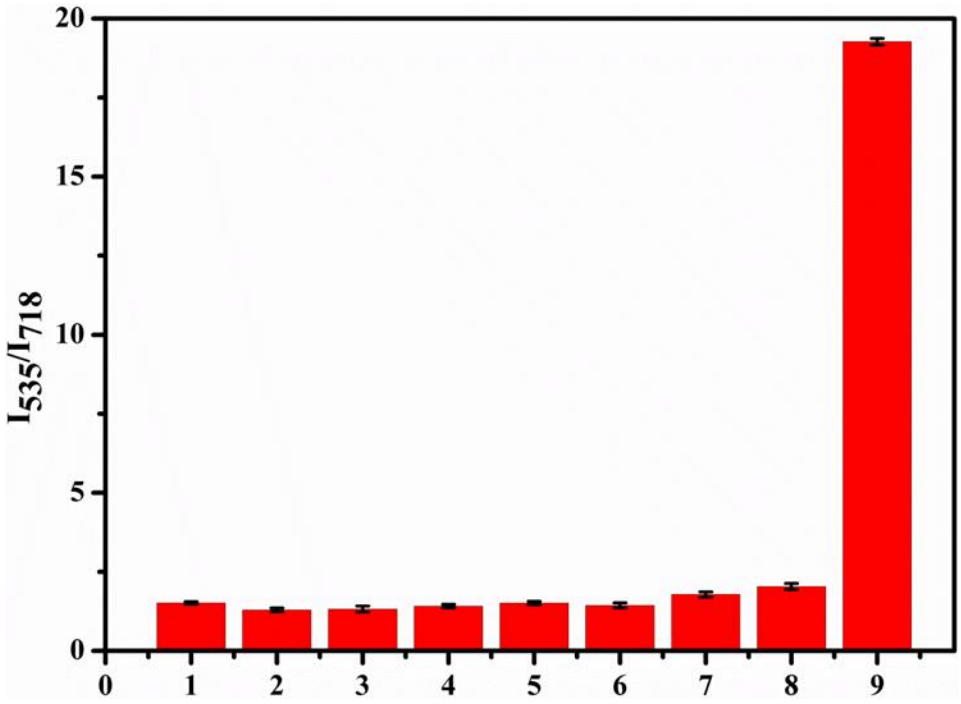
5. Figure S3 The absorption spectra of **rTPONOO-1** (10 μM) and **rTPONOO-2** (10 μM) with addition of different concentrations of $ONOO^-$（0, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM）in PBS buffer solution containing 1% DMF（50 mM, pH = 7.4 ）（a）**rTPONOO-1** （b）**rTPONOO-2**. Inset: images of **rTPONOO-1** (10 μM) or **rTPONOO-2** (10 μM) in the absence (A) and presence (B) of $ONOO^-$.



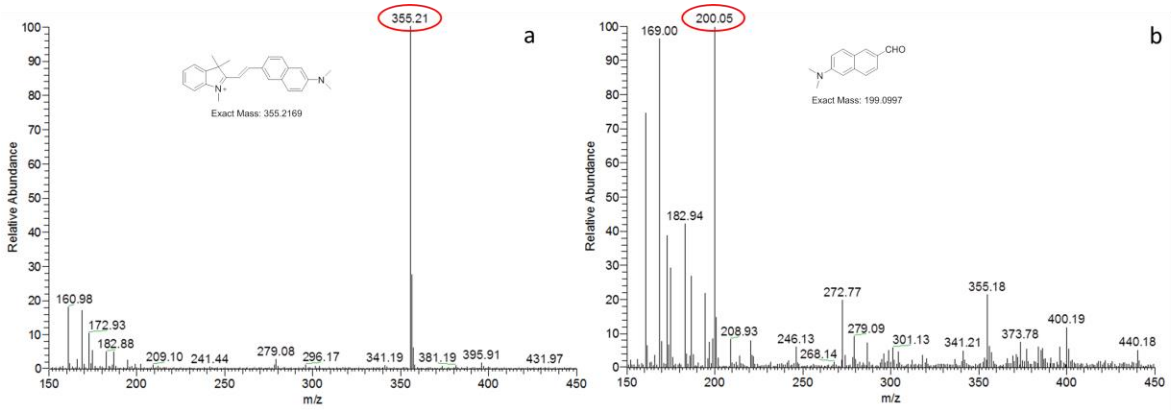
6. Figure S4 . The effects of pH on the fluorescence of **rTPONOO-1** (5 μM) and **rTPONOO-2** (5 μM) before and after addition of $ONOO^-$ (50 μM) in PBS buffer solution containing 1% DMF（50 mM, pH = 7.4 ）.（a）**rTPONOO-1** （b）**rTPONOO-2**.



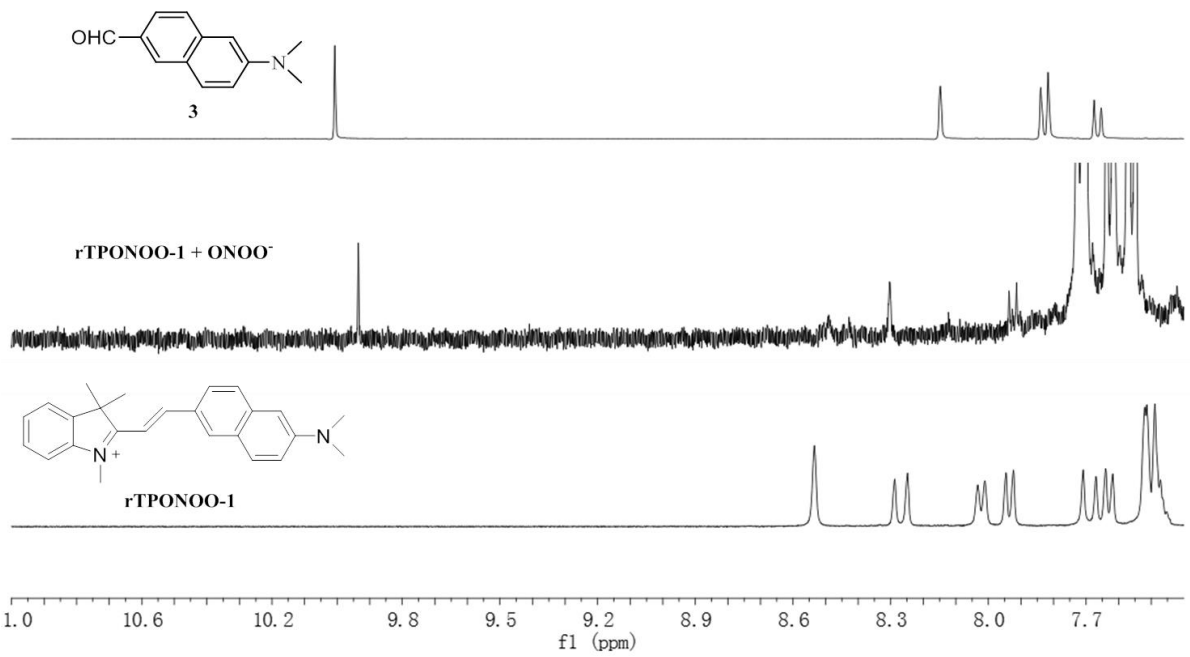
7. Figure S5 . The reaction time of **rTPONOO-1** (5 μM) and **rTPONOO-2** (5 μM) toward different concentrations of $ONOO^-$ in PBS buffer solution containing 1% DMF（50 mM, pH = 7.4 ）.（a）**rTPONOO-1** （b）**rTPONOO-2**.



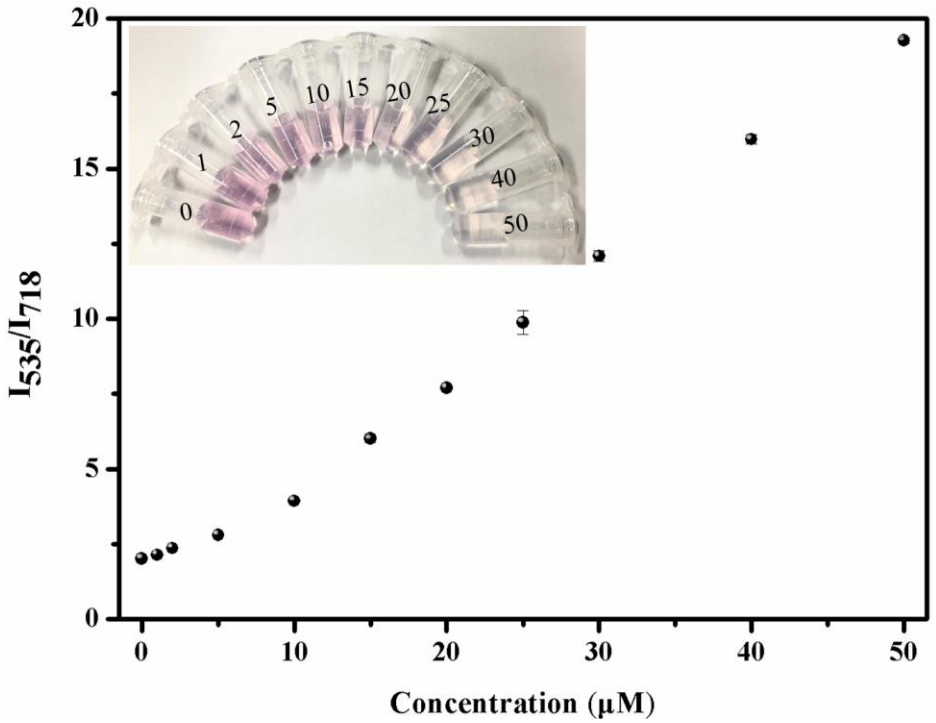
8. Figure S6 . The fluorescence ratio (I_{535}/I_{718}) of **rTPONOO-1** (5 μM) in the presence of $ONOO^-$ (50 μM) and biologically relevant analytes (100 μM) [1, **rTPONOO-1**; 2, Na^+; 3, K^+; 4, Ca^{2+}; 5, Mg^{2+}; 6, Zn^{2+}; 7, Al^{3+}; 8, Fe^{3+}; 9, $ONOO^-$] in PBS buffer solution containing 1% DMF (50 mM, pH = 7.4). (λ_{ex} = 375 nm and λ_{ex} = 525 nm).



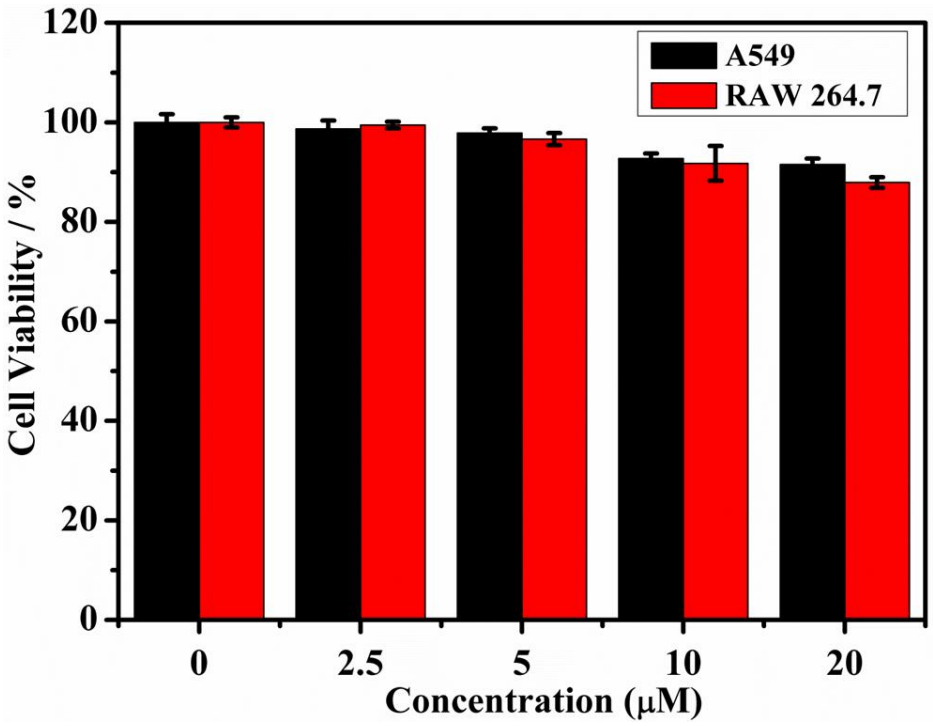
9. Figure S7 . ESI spectra of （a） **rTPONOO-1** （b） **rTPONOO-1** upon addition of $ONOO^-$.



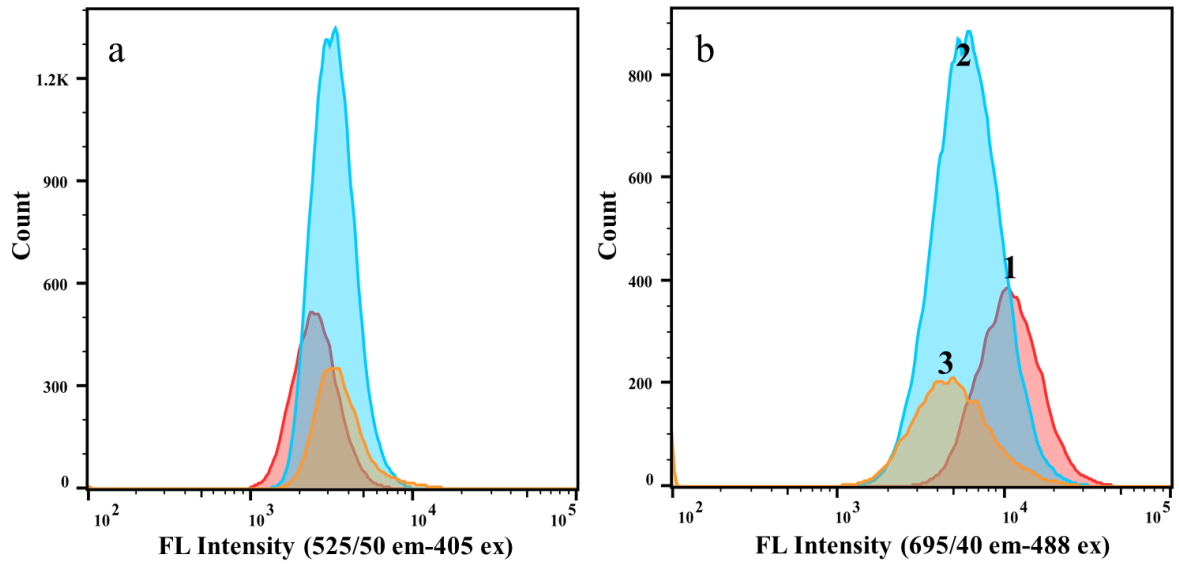
10. Figure S8 . The NMR titration analysis of the mixture of **rTPONOO-1** and $ONOO^-$ in DMSO-d_6.



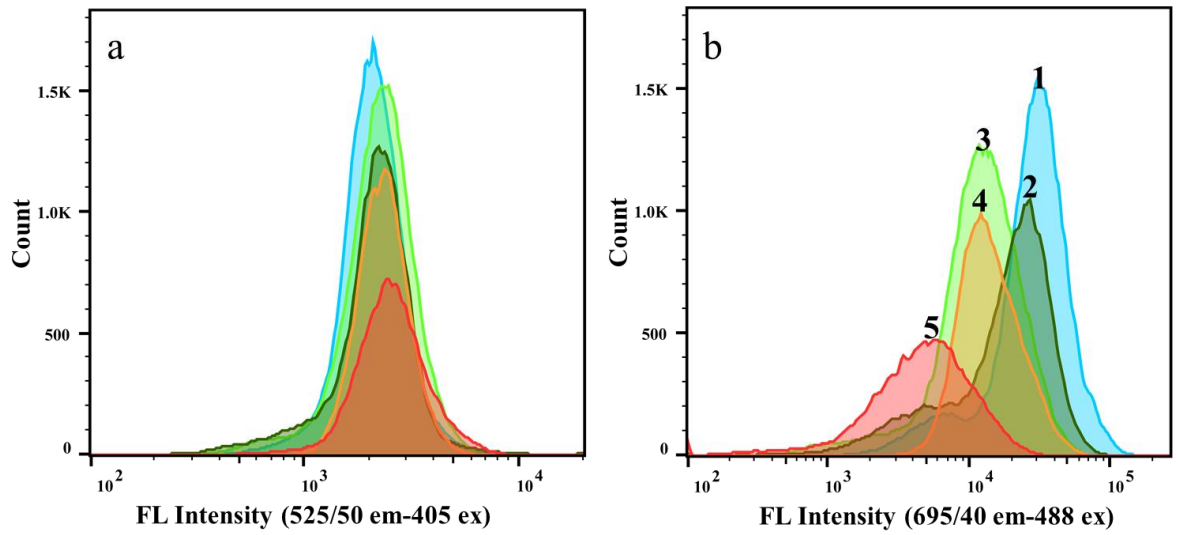
11. Figure S9 . The fluorescence ratio (I_{535}/I_{718}) of **rTPONOO-1** (5 μM) in the different concentrations of $ONOO^-$ (0 – 50 μM). Inset: images of **rTPONOO-1** (5 μM) in the different concentrations of $ONOO^-$ (0 – 50 μM).



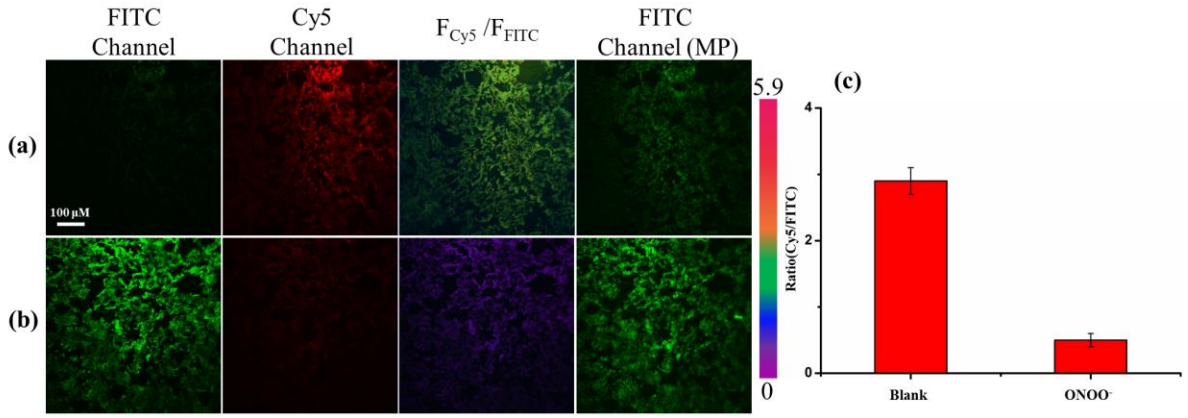
12. Figure S10 . Cell viability of A549 and RAW 264.7 treated with different concentrations of **rTPONOO-1**.



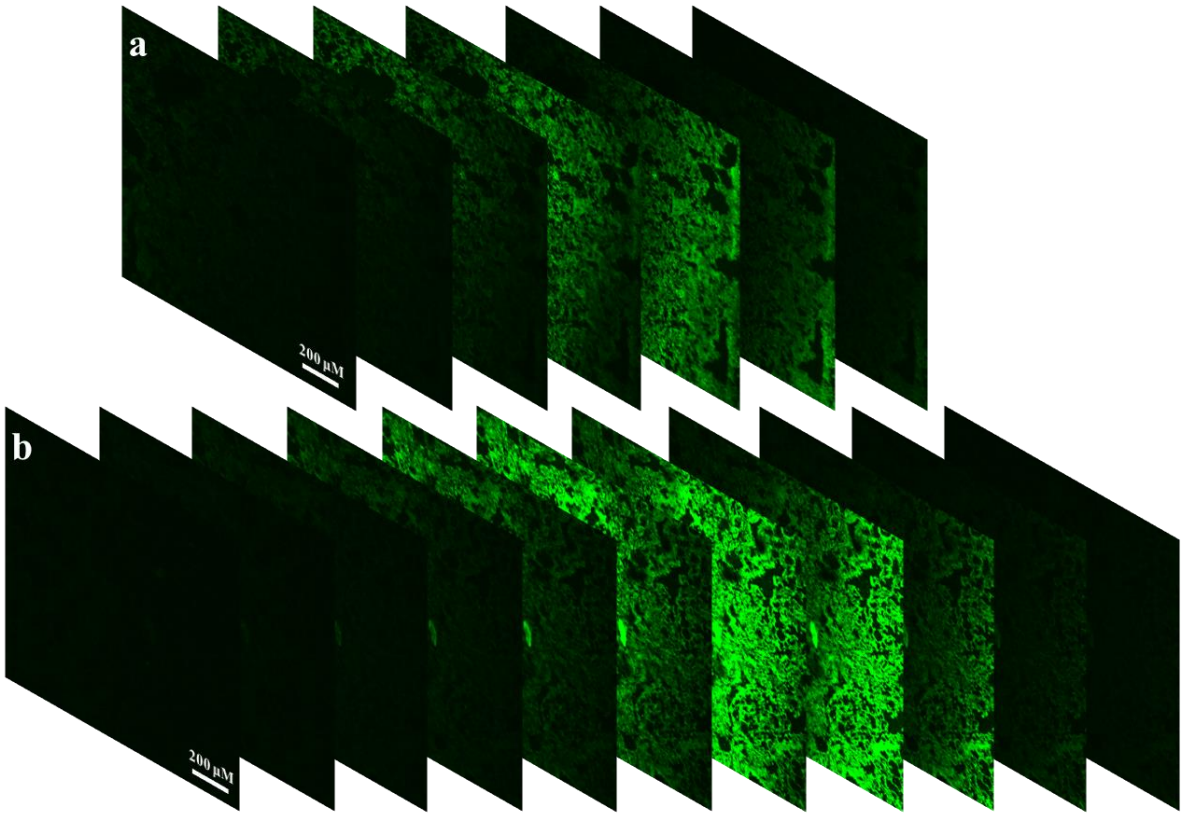
13. Figure S11 . Detecting exogenous $ONOO^-$ in A549 cells treated with (red line) **rTPONOO-1** (10 μM) for 30 min; (blue line) **rTPONOO-1** (10 μM) for 30 min and then 0.6 mM SIN-1 for another 30 min; (orange line) **rTPONOO**-1 (10 μM) for 30 min and then 1.2 mM SIN-1 for another 30 min. (a) The FL intensity record in 525/50 nm, λ_{ex} = 405 nm; (b) The FL intensity record in 695/40 nm, λ_{ex} = 488 nm.



14. Figure S12 . Detecting exogenous and endogenous $ONOO^-$ in RAW 264.7 cells treated with (blue line) **rTPONOO-1** (10 μM) for 30 min; (deep green line) 0.6 mM SIN-1 for 30 min and then **rTPONOO-1** (10 μM) for another 30 min; (green line) 1.2 mM SIN-1 for 30 min and then **rTPONOO-1** (10 μM) for another 30 min; (orange line) LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 12 h, then **rTPONOO-1** (10 μM) for 30 min; (orange line) LPS (1 μg/mL) and IFN-γ (50 ng/mL) and NAC (1 mM) for 12 h, then **rTPONOO-1** (10 μM) for 30 min. (a) The FL intensity record in 525/50 nm, λ_{ex} = 405 nm; (b) The FL intensity record in 695/40 nm, λ_{ex} = 488 nm.



15. Figure S13 . Fluorescence imaging of $ONOO^-$ in frozen lung slice (a)The slice was treated with **rTPONOO-1** (20 μM) for 1 h; (b) The slice was treated with **rTPONOO-1** (20 μM) for 1 h and then 200 μM $ONOO^-$ for another 1 h; (c) Average intensity ratios from ratio images of (a) and (b). Cy5 channel: λ_{ex} = 488 nm; FITC channel: λ_{ex} = 405 nm; FITC channel (MP): λ_{ex} = 800 nm.



16. Figure S14 . Images of lung slice at different depths (a) The frozen lung slice was treated with **rTPONOO-1** (20 μM) for 1 h, the images were captured every 10 μm from 0 to 70 μm (b) The frozen lung slice was treated with **rTPONOO-1** (20 μM) for 1 h and then 200 μM $ONOO^-$ for another 1 h the images were captured every 10 μm from 0 to 110 μm.