Supplementary Information

A Rapid Response Near-infrared Ratiometric Fluorescent Probe Enabled In Real-Time Peroxynitrite Tracking for Pathological Diagnosing and Therapeutic Assessment in Rheumatoid Arthritis Model

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Materials

All the regents and materials in our exprimenrs , including 2- (4- Diethylamino -2-hydroxybenzoyl) benzoicacid, cyclohexanone, 4-Methoxycinnamaldehyde, 4-(Dimethylamino) cinnamaldehyde, 3-morpholinosydnoniminehydrochloride(SIN-1), 3, 3-bis (aminoethyl) -1-hydroxy -2-oxo-1-triazene (NOC-18), menadione sodium bisulfite (MSB), FeTMPyP, Lipopolysaccharides (LPS), interferon-gamma (IFN-γ), phorbol-12-myristate-13-acetate (PMA), aminoguanidine (AG), apocynin, 4 ,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich (USA). Concentrated H_2SO_4, perchloric acid and acetic acid were purchased from Energy Chemical Co., Ltd (Shanghai, China). Bovine type II collagen (BCol II) (2.0 mg/mL), complete Freund's Adjuvant (CFA) (2.0 mg/mL), and incomplete Freund's Adjuvant (IFA) were purchased from Chondrex (Washington, USA). Dimethyl sulfoxide (DMSO), CH_3OH, Ethanol were analytical grade without further purification. The stock solution of probes **Ratio-A** and **Ratio-B** were prepared in dimethyl sulfoxide (DMSO) at the concentration of 1 mM and were reserved in refrigerator, analytical condition is the phosphate buffered saline (PBS) solution (20 mM, pH=7.4,with 0.1% DMSO).

Apparatus

NMR spectra were measured on a Bruker AVANCEIIIHD 500 MHz (^{1}H NMR 500 MHz, ^{13}C NMR 125 MHz) spectrometers at 37 ± 1 °C with TMS as the internal standard. Mass spectrometry data were obtained on Agilent 1290/6545 UHPLC-Q-TOF mass spectrometer. Absorption spectra were obtained on the UV-vis spectrophotometer (Shimadzu, UV-3600 plus). Fluorescence spectra were determined on a fluorescence spectrometer (Edinburgh, FLS 1000). Fluorescence imaging were acquired using a laser scanning confocal microscope (Olympus FV3000) with an objective lens (× 60). Intracellular fluorescence analysis was carried out on flow cytometry (FACSAria, BD). Kunming mice fluorescence images were collected by PerkinElmer IVIS® Lumina XR Series Ⅲ System. Mice pathological splices were imaged by Nikon Model Eclipse Ci-L microscope. Ultrapure water was prepared using a Milli-Q A10 system. All pH measurements were made with a JENCO 6230 M pH meter.

Determination of the in vitro detection limit

The limit of detection was calculated based on the fluorescence titration curve of **Ratio-A** and **Ratio-B**. The fluorescence intensity of **Ratio-A** and **Ratio-B** were measured by twelve times and the standard deviation of blank measurement was obtained. The detection limit was calculated with the following equation: LOD= 3σ/k. Where σ is the standard deviation of blank measurement, k is the slop between the fluorescence intensity versus $ONOO^-$ concentrations.

Determination of Absolute quantum yields

Ratio-A and **Ratio-B** were diluted to 10 μM for absolute quantum yield (QY), and measurement using fluorometer (Edinburgh，FLS 1000). For **Ratio-A**, λ_{ex} = 480 nm and **Ratio-B**, λ_{ex} = 500 nm.

Generation of ROS/RNS

The sources of different ROS/RNS are described as follows. Specifically, H_2O_2 solution was purchased from Sigma-Aldrich and added into the probe solution directly. KO_2 was dissolved in DMSO to generate superoxide ($O_2^{\bullet-}$).[1] Potassium Nitroprusside Dihydrate was used to generate •NO. *tert*-butyl hydroperoxide (TBHP) was diluted from 70 % stock solution.[2] The source of NaOCl was from NaClO solution that contains 5% available chlorine. Hydroxyl radical (•OH) was generated by Fenton reaction. Briefly, ferrous chloride ($FeCl_2$) was added in the presence of 10 equiv. of H_2O_2, the concentration of •OH was equal to the Fe (II) concentration.[3] Peroxynitrite ($ONOO^-$) solution was synthesized according to literature report. Simply, hydrogen peroxide (0.7 M) was firstly acidified with hydrochloric acid (0.6 M), and then mixed with sodium nitrite (0.6 M), finally, sodium hydroxide (1.5 M) was added within 1-2 seconds to make the solution alkaline. We use a short column of manganese dioxide to remove excess hydrogen peroxide. The concentration of $ONOO^-$ was determined by measuring the absorption of the solution at 302 nm. The $ONOO^-$ concentration was estimated by using an extinction coefficient of 1670 ± 50 $cm^{-1}M^{-1}$ at 302 nm.[4]

Information of confocal imaging

Fluorescent images were acquired on an Olympus FV3000 confocal laser-scanning microscope. Fluorescence collection windows for red channel: 670−750 nm (λ_{ex}=594 nm), green channel: 520−600 nm (λ_{ex} = 488 nm), and blue channel: 430−480 nm (λ_{ex}=405 nm). Cells were plated in the culture dish and allowed to adhere for 24 h before imaging. Cell nucleus dye 4, 6-diamidino-2-phenylindole (DAPI, 100 ng/mL, 1 μL) and our probe **Ratio-A** (10 μL, 1.0 mM) were simultaneously added to the culture plates which were filled with 1 mL fresh complete medium. The cell imaging was carried out after washing cells with corresponding medium for three times after the different treatment.

Flow cytometry analysis of $ONOO^-$ by Ratio-A

Flow Cytometry assay was performed for the detection of $ONOO^-$ with probe **Ratio-A**. The tested cells (2.0×10^5 cells/well) were resuspended by staining buffer, and then added 5 μL of Annexin V-FITC and 10 μL PI staining solution. After mixing, the samples were incubated in ice bath for 30 min and then analyzed by flow cytometry.

Histopathological Analyses

The knee joints were excised and fixed in 4 % (W/V) PBS-buffered paraformaldehyde overnight at room temperature, and then decalcified using 10 % (W/V) EDTA solution. Then, the joints were embedded in paraffin and cut into slices of 5 mm thickness for hematoxylin and eosin (H&E) staining.

Imaging in vivo

All animal experiments were performed accordingto the guidelines issued by The Ethical Committee of Qufu Normal University. Mice were anesthetized prior to injection and during imaging via inhalation of isoflurane. PerkinElmer IVIS® Lumina XR Series Ⅲ System was used for the bio-imaging of animal models. The excitation and emission wavelength were chosen as described in the paper.

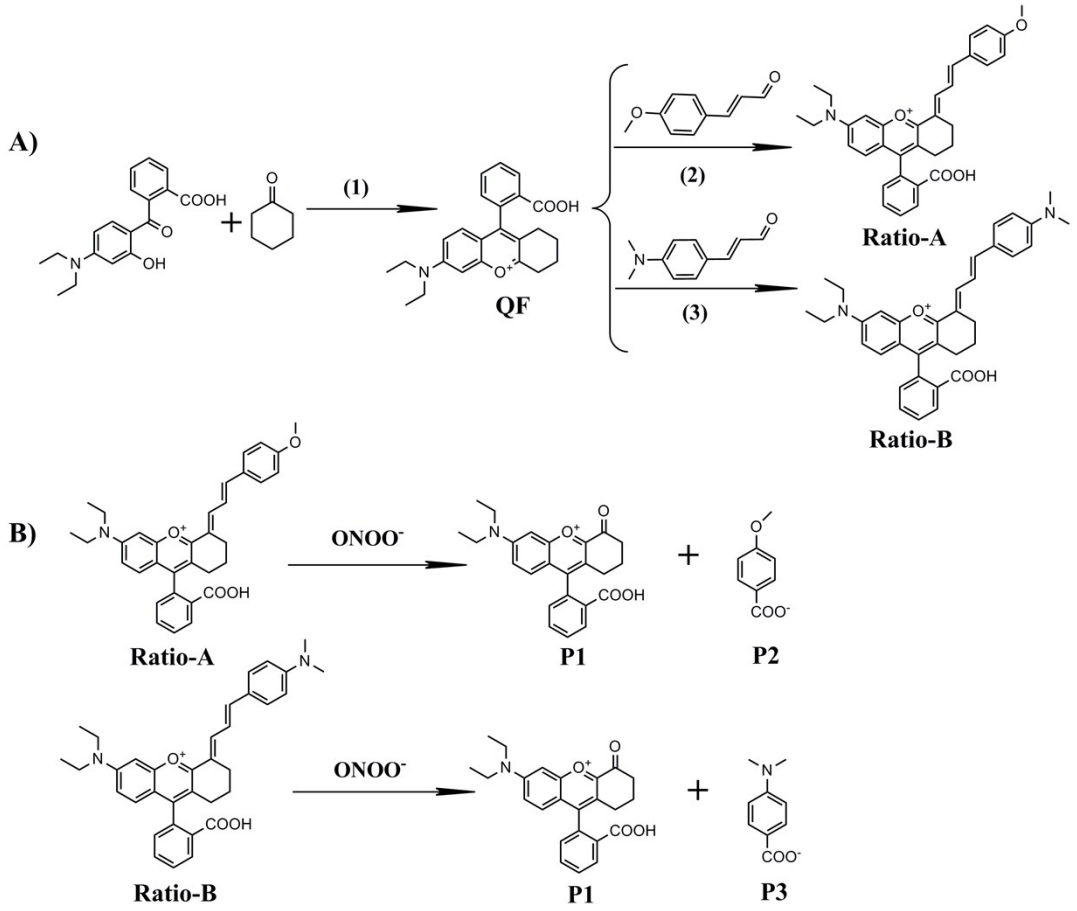

Figure S1. (A). The general synthetic routes for the probes **Ratio-A** and **Ratio-B**. (1). Condensed H_2SO_4, 95 °C, 3 h. (2). Ethanol, 80 °C, 8 h. (3). Ethanol, 80 °C, 8 h. (B). Proposed responding mechanisms toward $ONOO^-$ after 30 minutes at 37 °C in PBS solution (pH=7.4, 20 mM, with 0.1% DMSO).

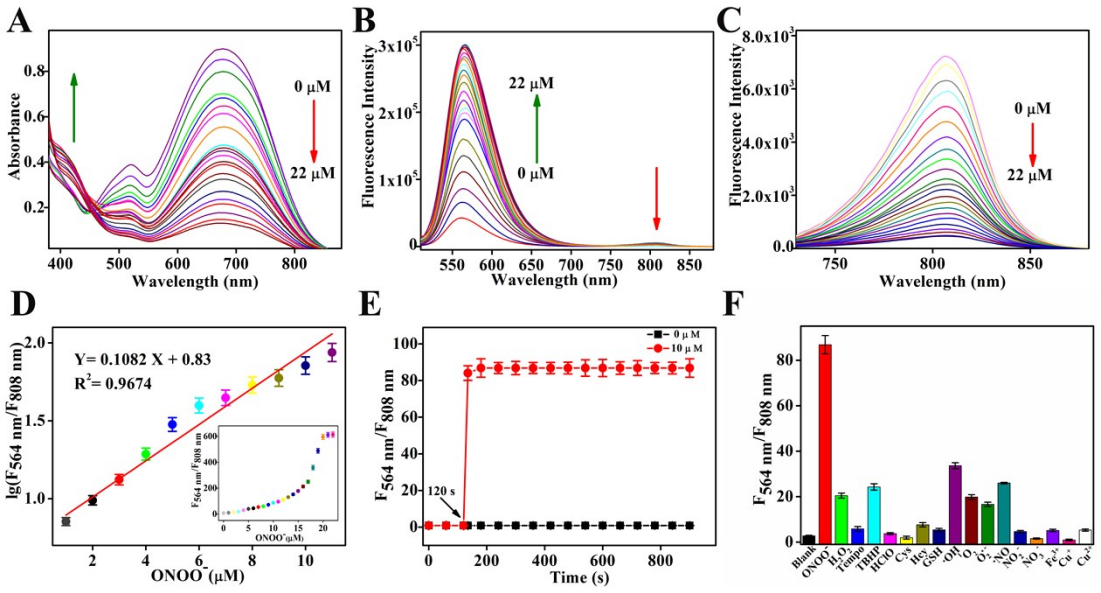

Figure S2. Spectral properties of **Ratio-B** (10 μM) in PBS solution (pH=7.4, 20 mM, with 0.1% DMSO). Data were recorded ten min after the addition of $ONOO^-$. (A) UV absorption spectra of **Ratio-B** in the presence of $ONOO^-$ (0−10 μM). (B) Emission spectra of **Ratio-B** with concentrations of $ONOO^-$ (0–10 μM), λ_{ex} = 480 nm. (C) Emission spectra of **Ratio-B** at 808 nm (λ_{ex} = 680 nm). (D) The linear relationship between $lg(F_{564\ nm}/F_{808\ nm})$ and $ONOO^-$. Insert: ratiometric intensity changes with different concentrations of $ONOO^-$. (E) Time-dependent intensity ratios ($F_{564\ nm}/F_{808\ nm}$) of **Ratio-B** towards $ONOO^-$ (0 μM, 10 μM) during 0 − 900 s, probe was added at 120 s. (F) Ratiometric fluorescence responses ($F_{564\ nm}/F_{808\ nm}$) of **Ratio-B** toward $ONOO^-$ (10 μM) and other ROS/RNS/RSS (20 equivalents), metal ions. The error bars represent ± S.D. (n=3).

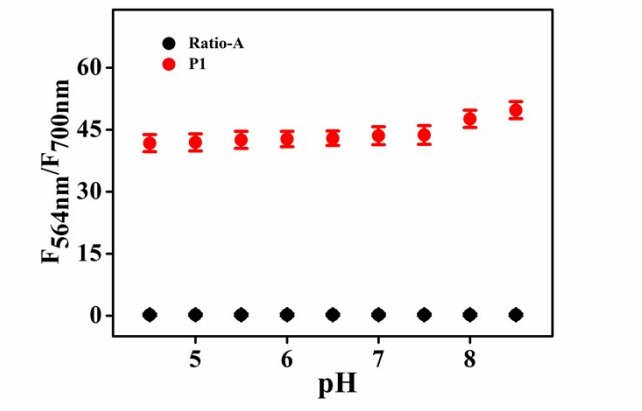

Figure S3: Investigation on the stability of **Ratio-A** (10 μM) for detecting $ONOO^-$ at different pH.

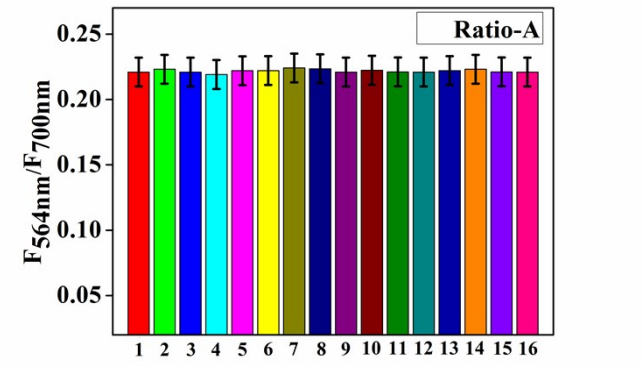

Figure S4. The ratio values of **Ratio-A** (10 μM) in different solvents: 1. Blank; 2. Ethanol; 3. Ethylene Glycol; 4. Methanol; 5. Dichloromethane; 6. Isobutanol; 7. Water; 8. Dimethyl Sulfoxide; 9. N, N-Dimethyl formamide; 10. Acetonitrile; 11.

Chloroform; 12. Acetone; 13. Toluene; 14. Ethylacetate; 15. Dioxane; 16. Diethyl Ether.

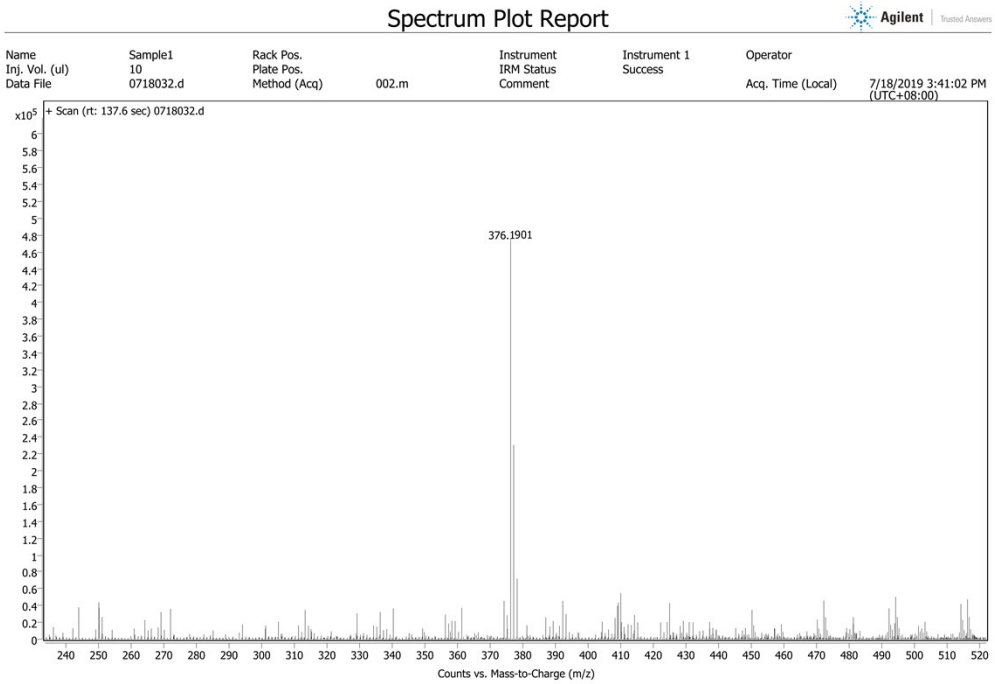

Figure S5. ESI-MS of **QF**

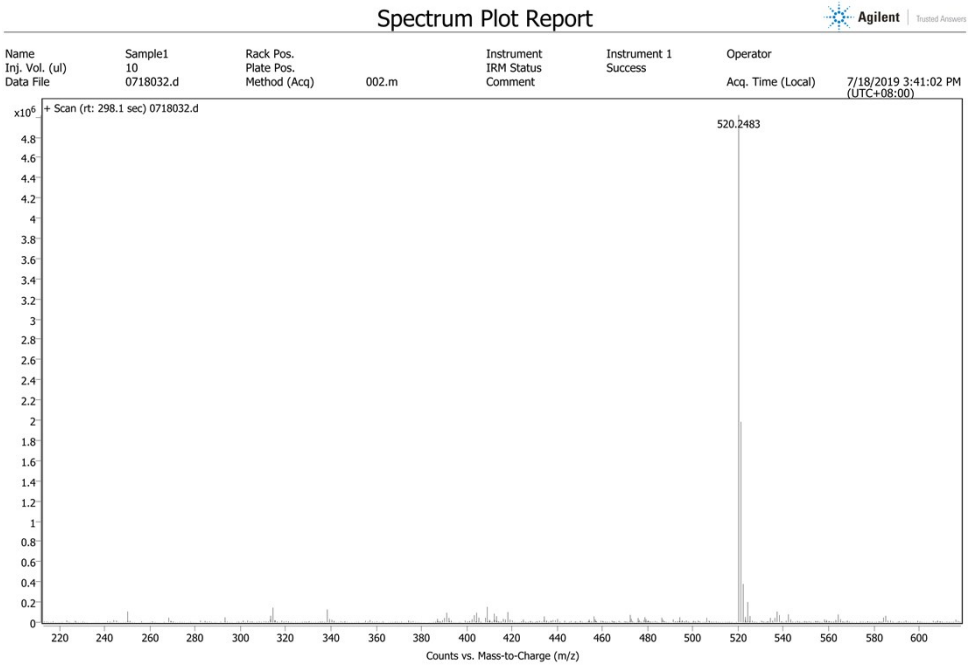

Figure S6. ESI-MS of **Ratio-A**

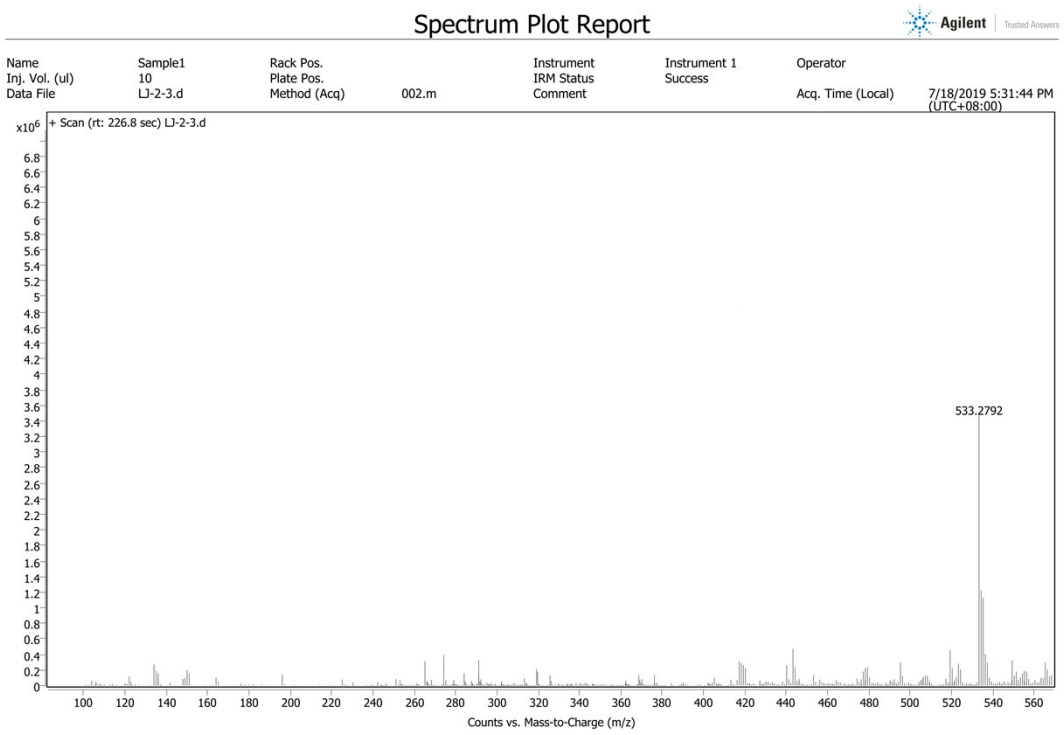

Figure S7. ESI-MS of **Ratio-B**

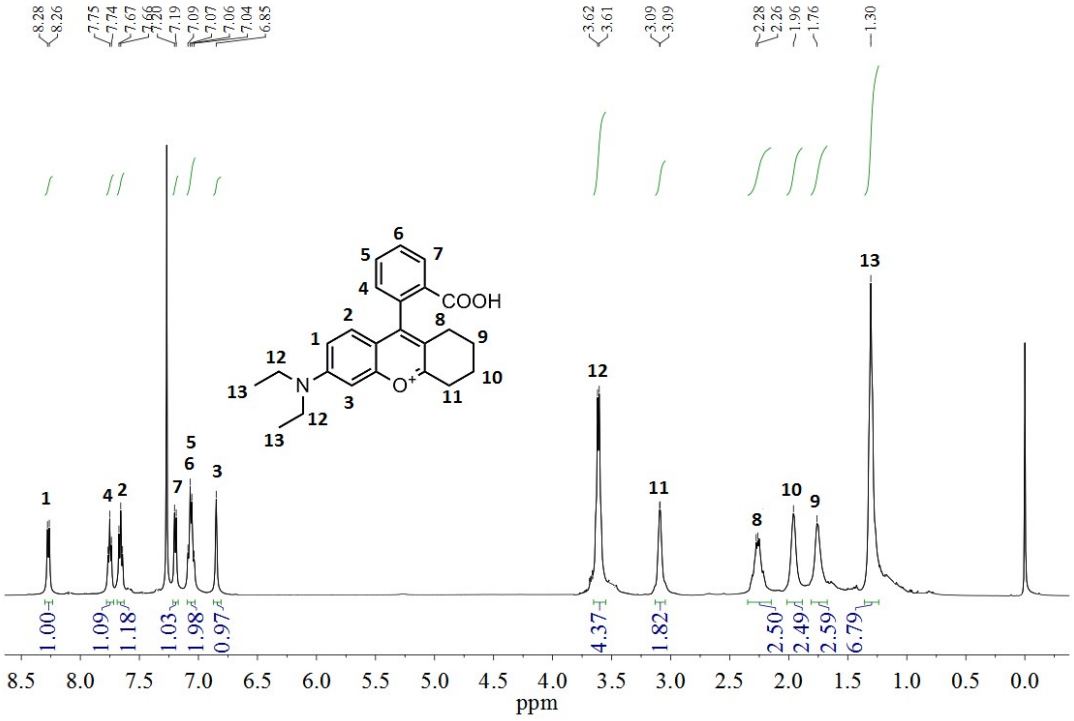

Figure S8. ^{1}H NMR of **QF** in $CDCl_3$

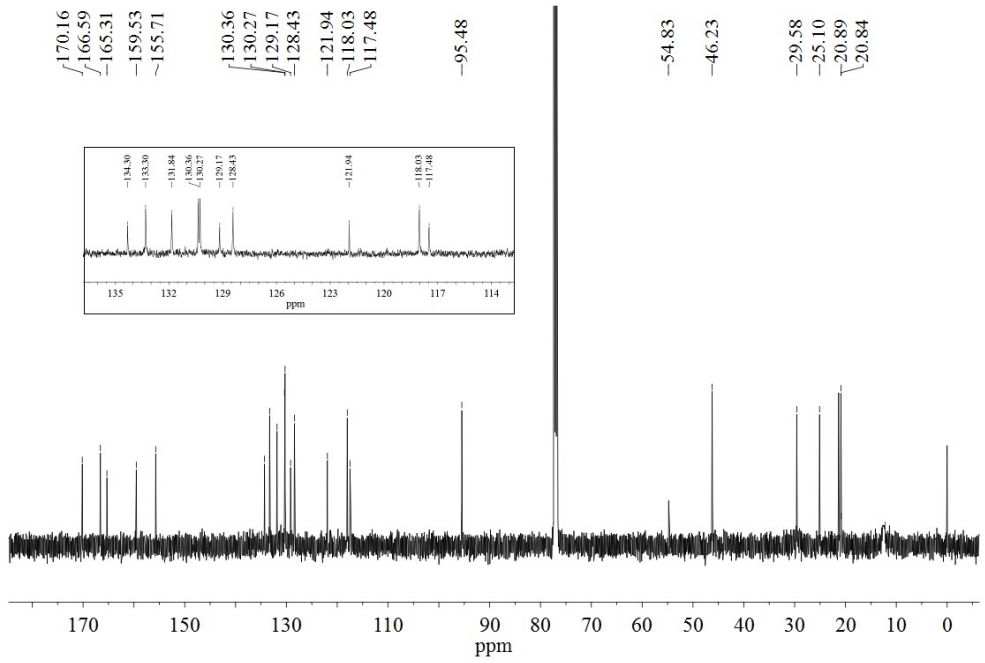

Figure S9. ^{13}C NMR of **QF** in $CDCl_3$

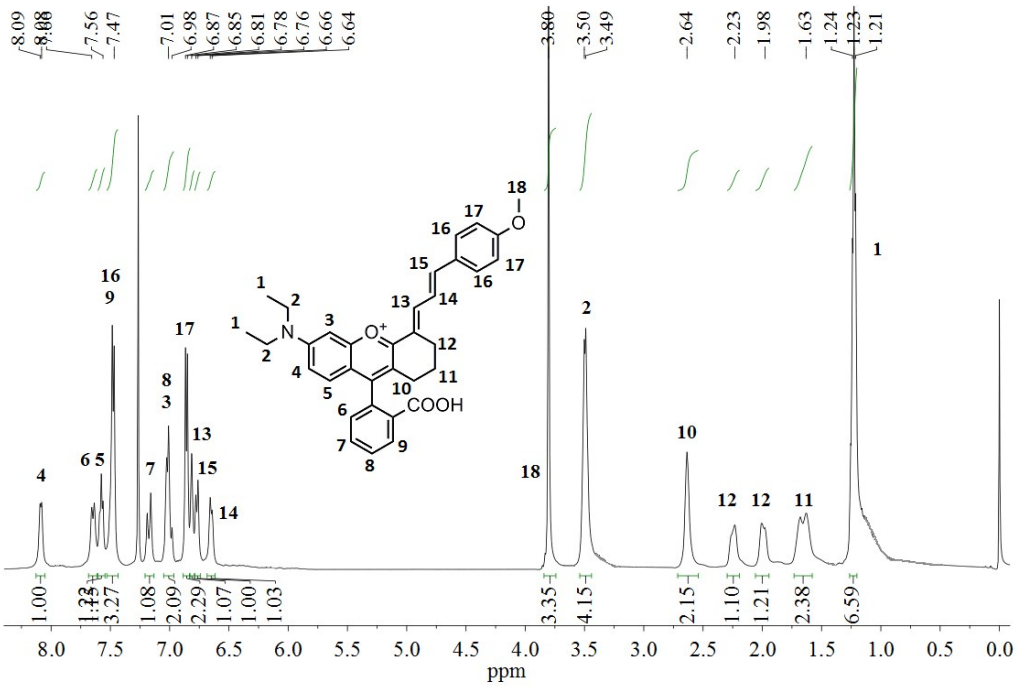

Figure S10. ^{1}H NMR of **Ratio-A** in $CDCl_3$

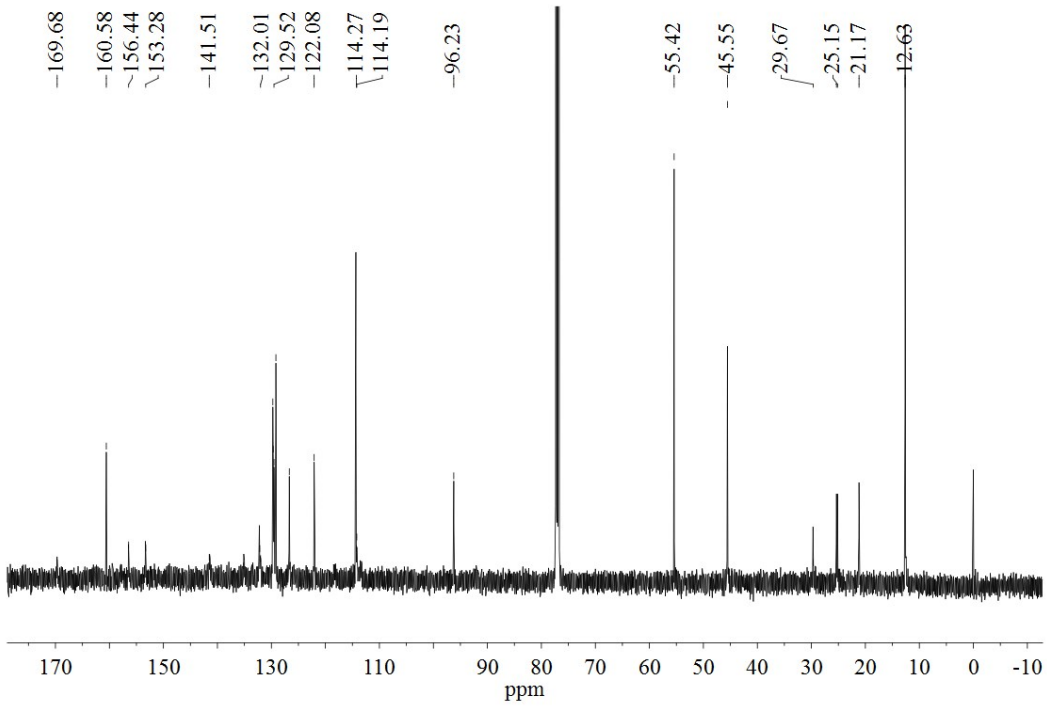

Figure S11. ^{13}C NMR of **Ratio-A** in $CDCl_3$

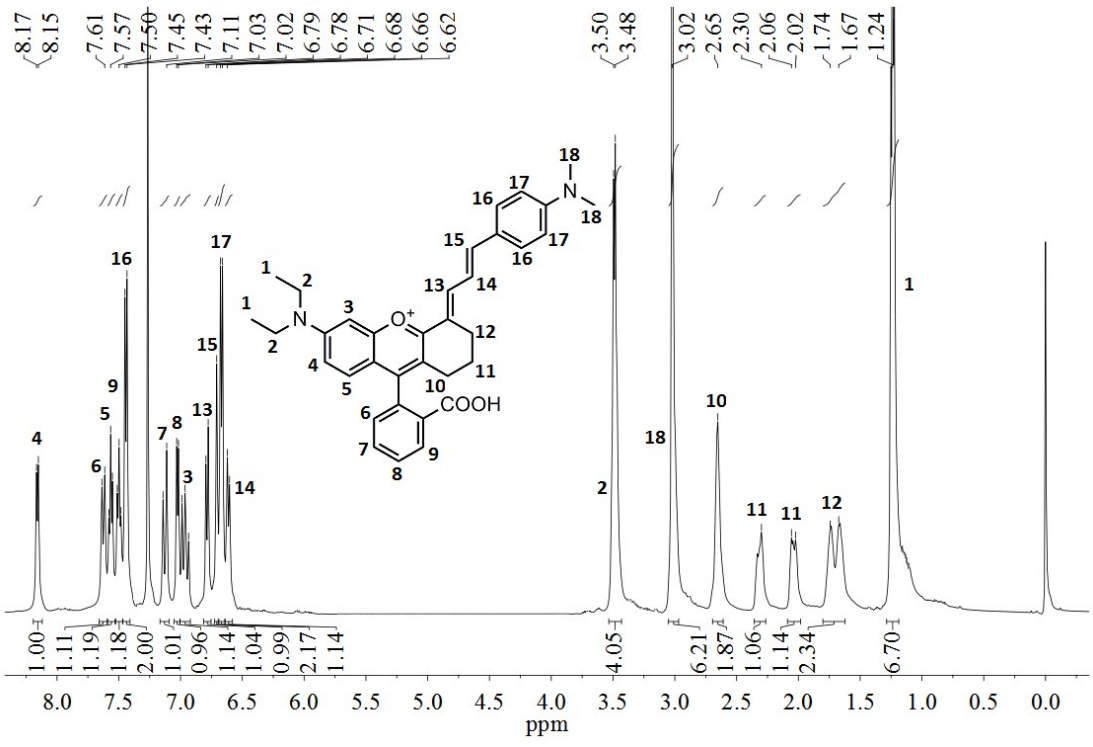

Figure S12. ^{1}H NMR of **Ratio-B** in $CDCl_3$

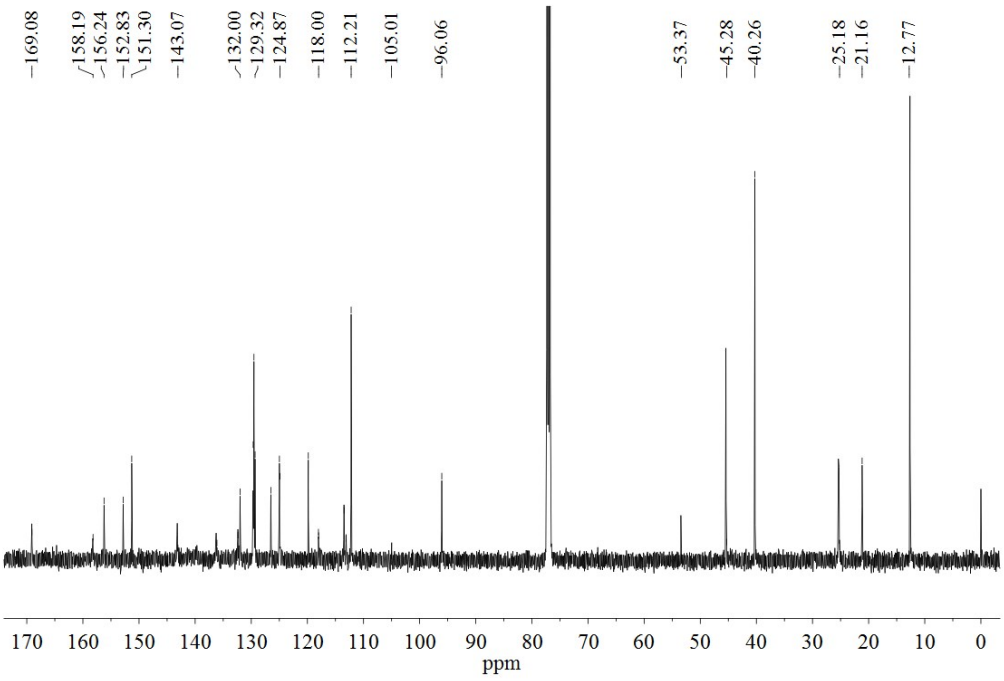

Figure S13. ^{13}C NMR of **Ratio-B** in $CDCl_3$

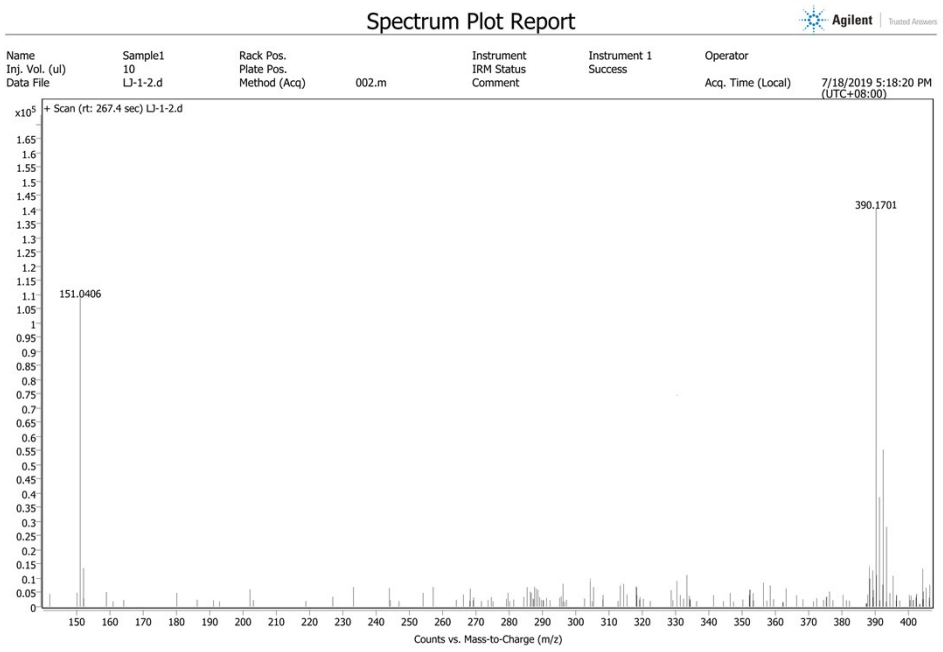

Figure S14. ESI-MS for **Ratio-A** and $ONOO^-$ after 30 minutes at 37°C in PBS solution (pH=7.4, 20 mM, with 0.1% DMSO).

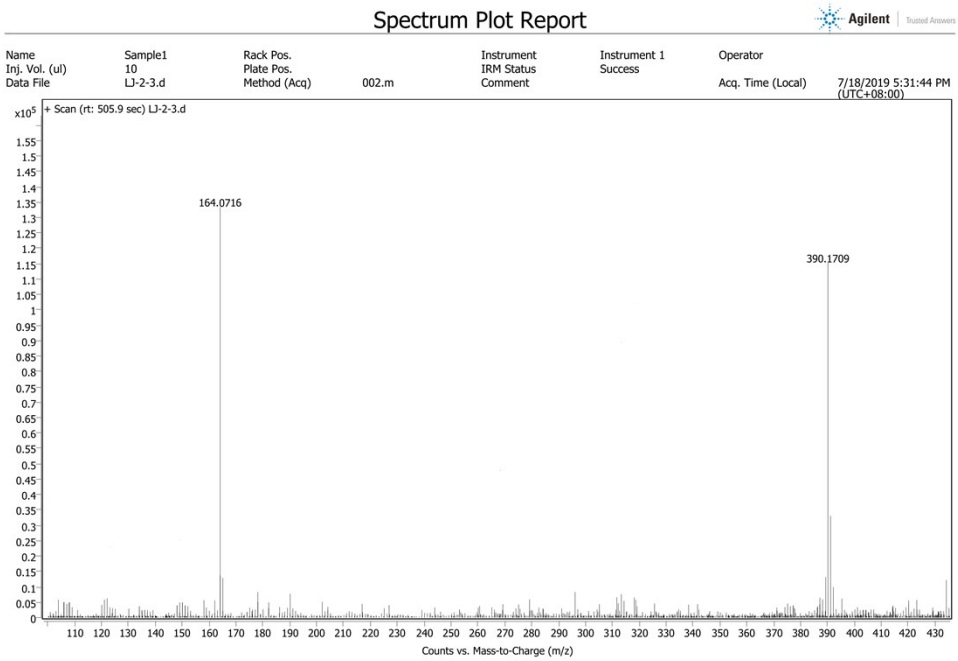

Figure S15. ESI-MS for **Ratio-B** and $ONOO^-$ after 30 minutes at 37 °C in PBS solution (pH=7.4, 20 mM, with 0.1% DMSO).

5. HPLC assay

To further verify the response mechanism during detection process of **Ratio-A** towards $ONOO^-$. High-performance liquid phase (HPLC) experiments were performed. Eluent A was H_2O solution containing 5% methanol, and eluent B was pure methanol. The linear gradient condition was as follows: 0 min = 20% B, 10 min = 100% B, 15 min = 100% B using Hypersil BDS C18, 5μm, 4.6×250 mm column in Agilent 1260 infinity II. The flow rate was set at 1.0 mL. min^{-1} and the monitor wavelength was set as 254 nm. As shown in Figure S16, These results could verify the response mechanism during detection process of **Ratio-A** towards $ONOO^-$.

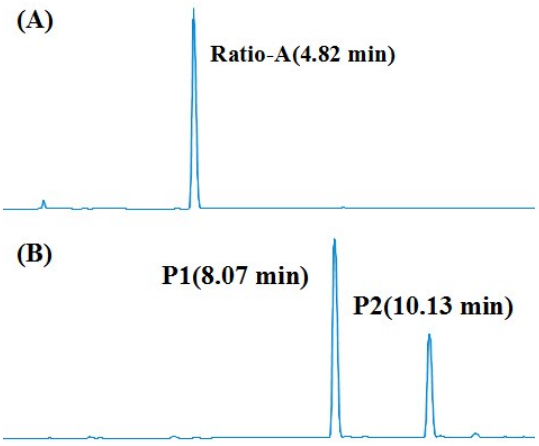

Figure S16. HPLC analysis of **Ratio-A** and **Ratio-A** + $ONOO^-$ (25 μM). Data were recorded ten min after the addition of $ONOO^-$.

The cytotoxicity of probe **Ratio-A** against RAW264.7 cells was assessed by the CCK-8 assay. The cells were seeded into a 96-well cell culture plate at a final density of 5×10^3 cells/well. After the cells were incubated for 24 h at 37 °C under 5% CO_2, different concentrations of **Ratio-A** (0−100 μM) were added to the wells. Subsequently, the cells were incubated for 24 h at 37 °C under 5% CO_2. Then, 10 μL CCK-8 solution was added to each well of the plate and incubated for 4 h at 37 °C under 5% CO_2. The optical density (OD) was measured at 450 nm using a microplate reader (Tecan, Austria). When the amount of the probe **Ratio-A** added was 0, the cell viability value was set to 100%. Accordingly, the IC_{50} value of RAW264.7 cells was calculated to be 232 μM. As shown in Figure S17, the high cells viability indicated that our probe displayed low cytotoxicity in living cells.

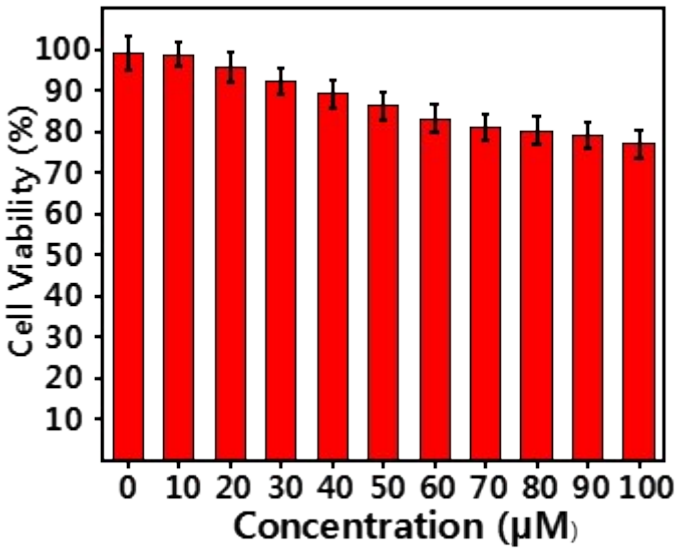

Figure S17. The cell viability of RAW264.7 cells with **Ratio-A**.

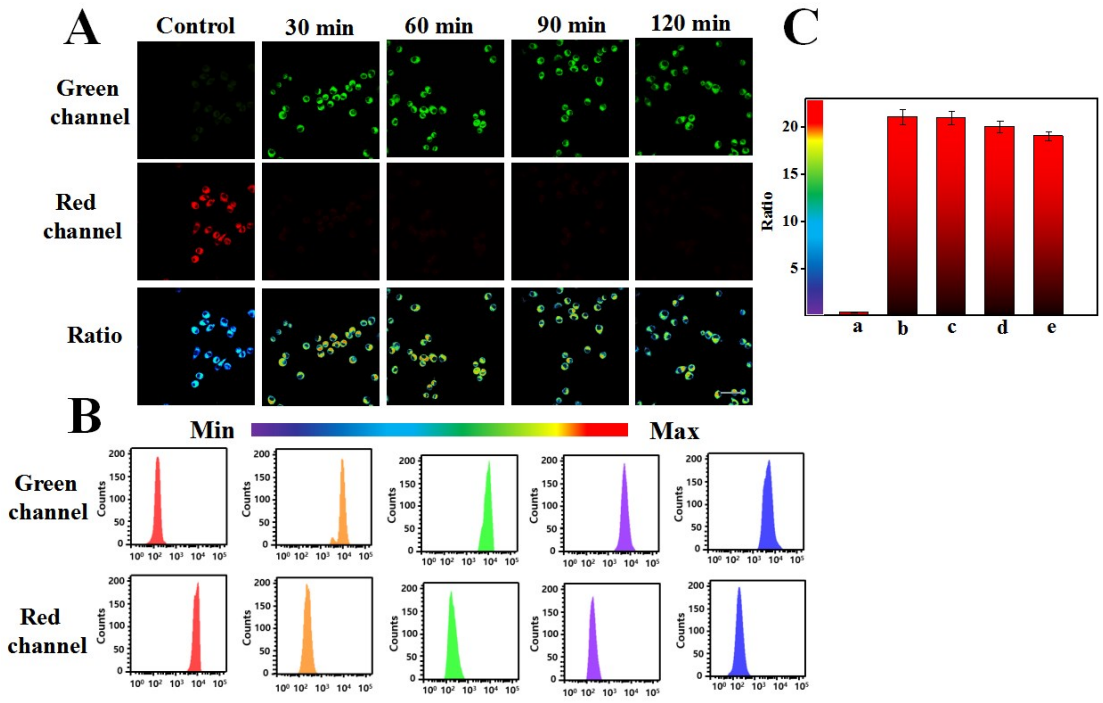

Figure S18. Intracellular retention of **Ratio-A** (10 μM). (A). RAW264.7 cells were stained with **Ratio-A** and then treated with 50 μM SIN-1 for 30 min. Confocal images were acquired at indicated time points. The control cells were incubated in the absence of SIN-1. (B). Flow cytometry analysis of the cells in (A). (C) The fluorescence ratio values (F_{green}/F_{red}) of (A). Scale bar represents 20 μm. The experiments were repeated three times and the data were shown as mean (± S.D.).

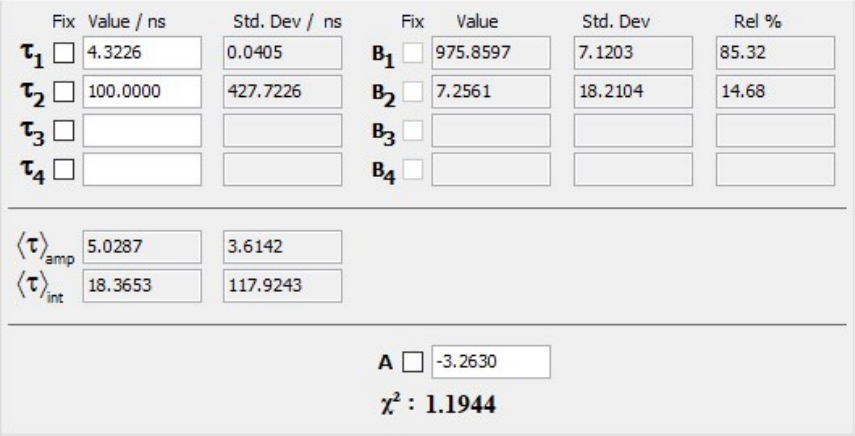

Figure S19. The fluorescence lifetime of **Ratio-A**.

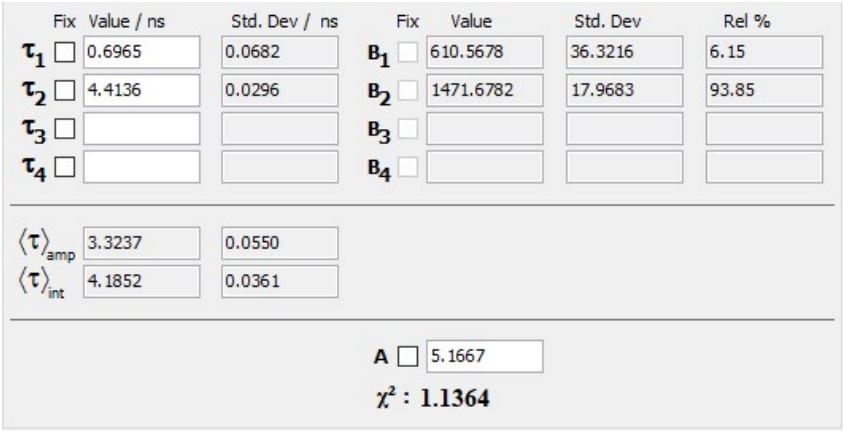

Figure S20. The fluorescence lifetime of **Ratio-B**

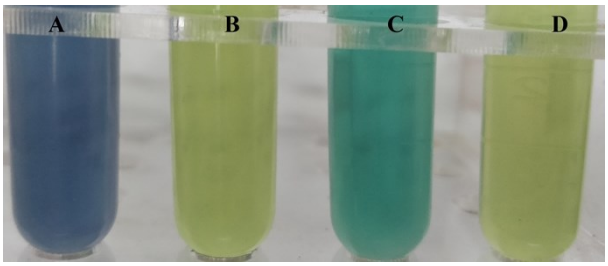

Figure S21. Image of **Ratio-A** (10 μM) and **Ratio-B** (10 μM) in PBS solution (pH 7.4) before and after adding 10.0 equivalents of $ONOO^-$ in 1 minute. A: the color of **Ratio-A**; B: the color of **Ratio-A** after adding $ONOO^-$; C: the color of **Ratio-B**; D: the color of **Ratio-B** after adding $ONOO^-$.

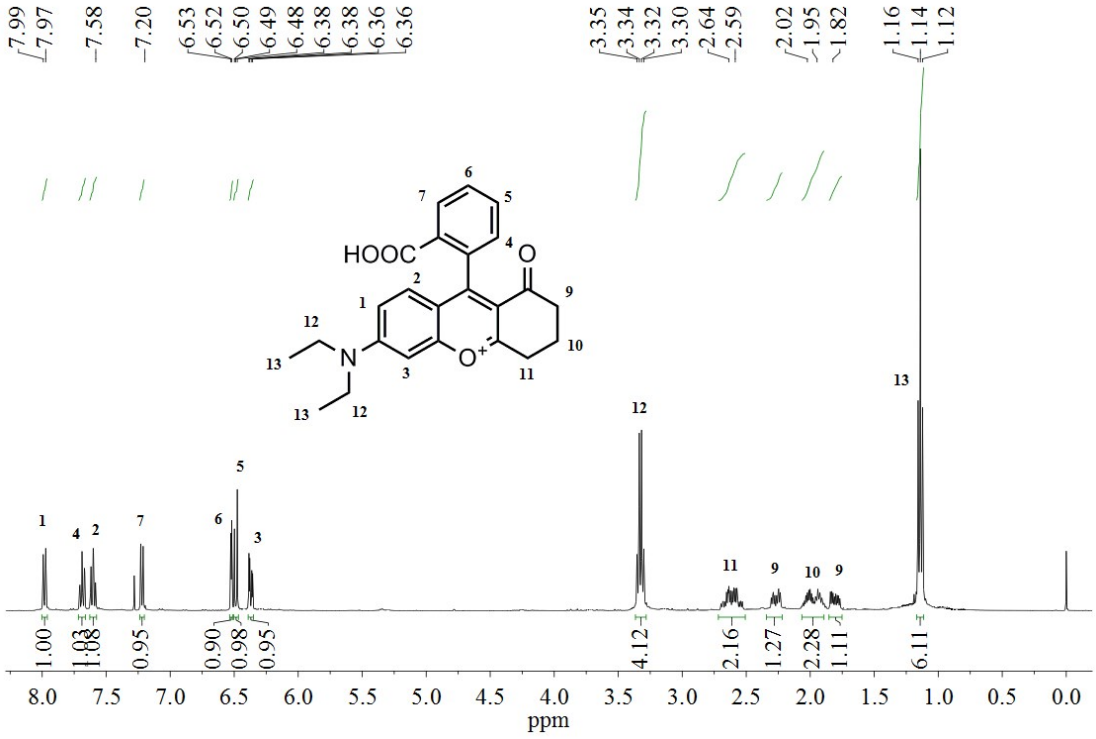

Figure S22. ^{1}H NMR of **P1** in $CDCl_3$

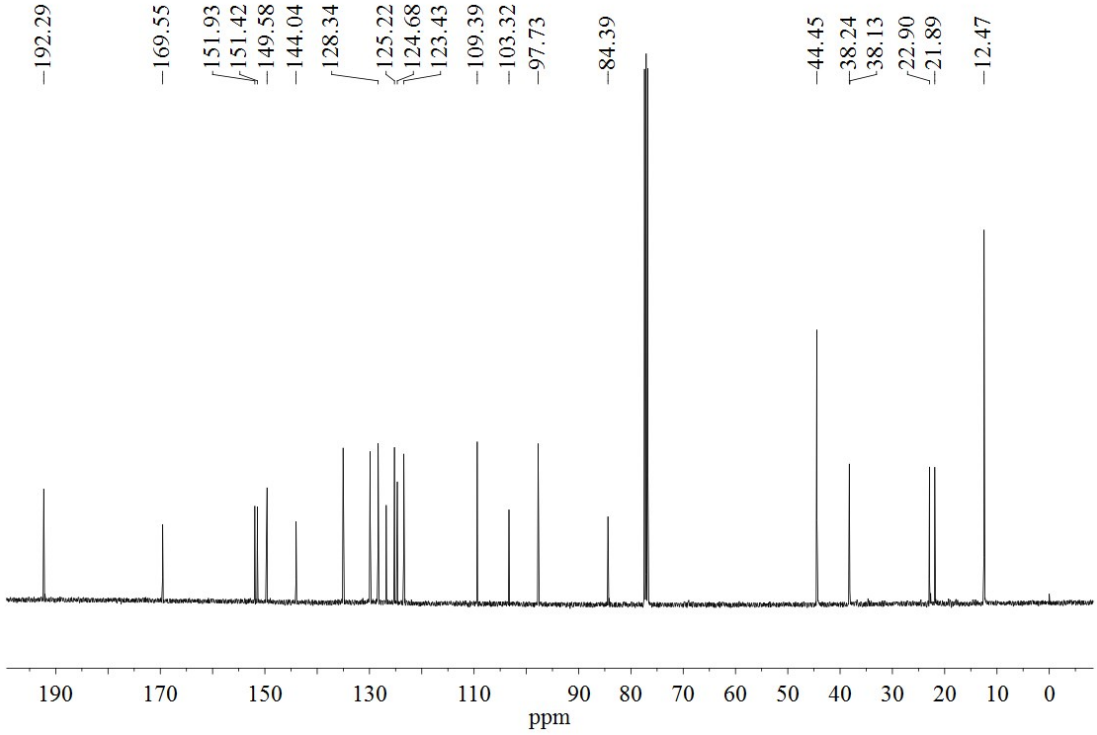

Figure S23. ^{13}C NMR of **P1** in $CDCl_3$

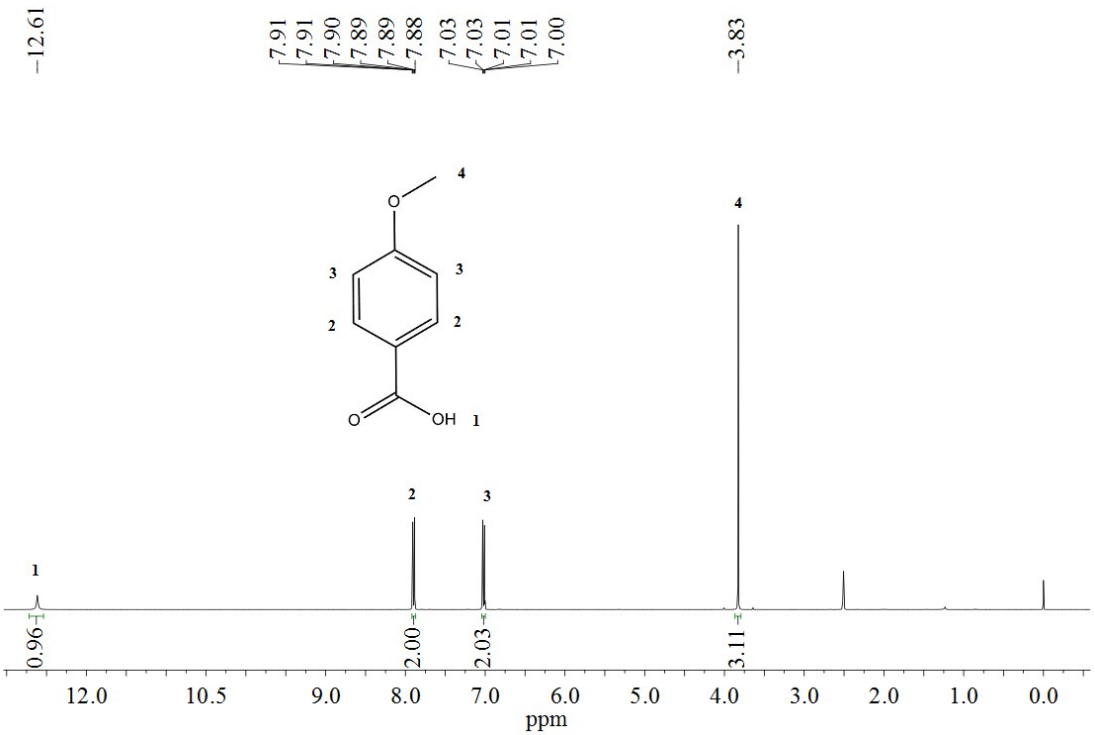

Figure S24. ^{1}H NMR of **P2** in d_6-DMSO.

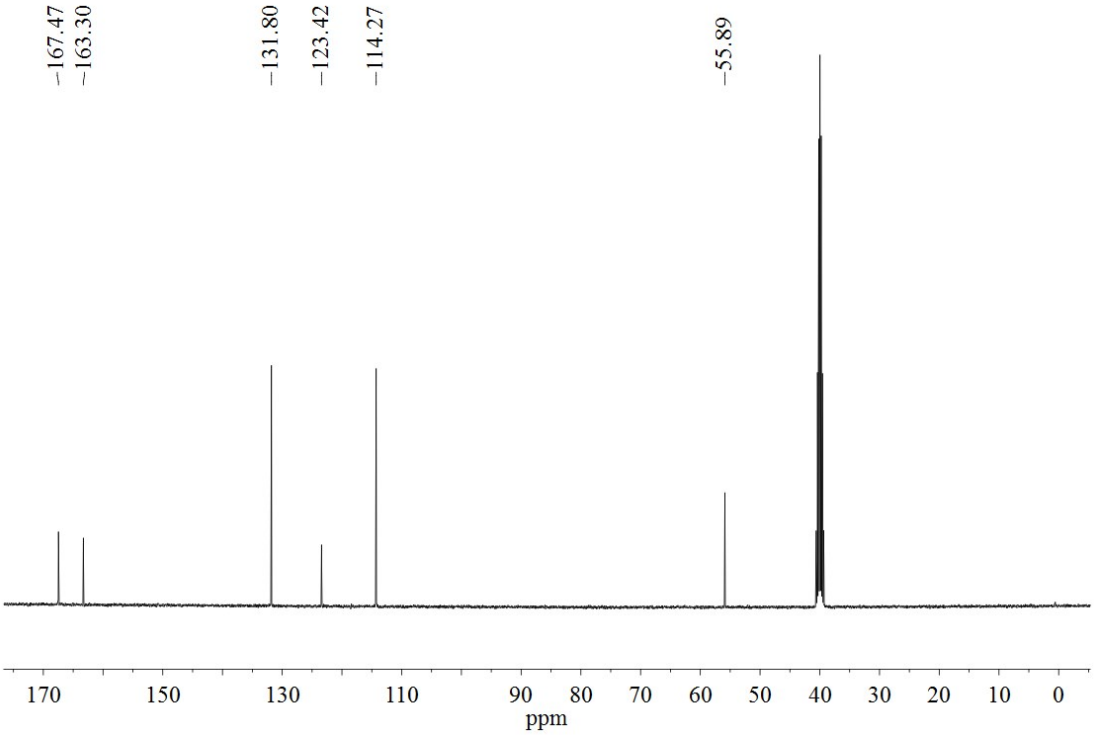

Figure S25. ^{13}C NMR of **P2** in d_6-DMSO.

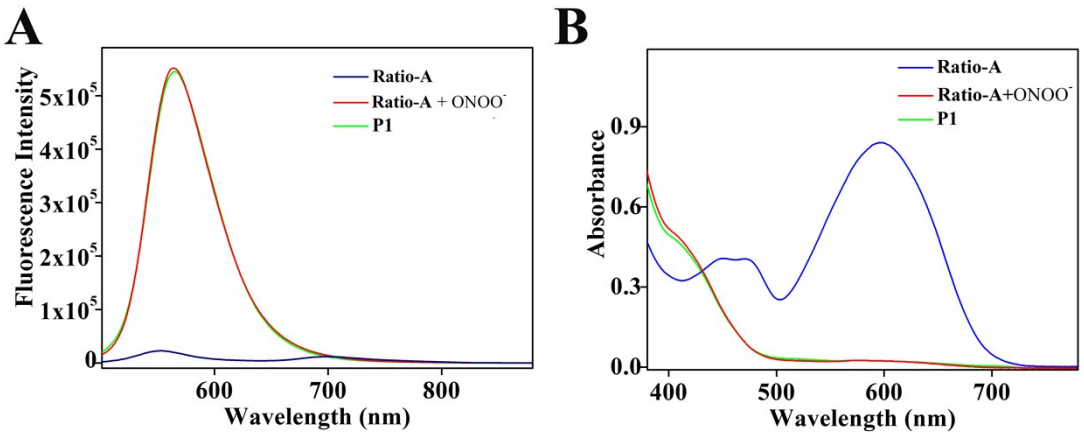

Figure S26. (A) UV-vis absorption spectra and (B) fluorescence spectra of **Ratio-A** (10 μM), **Ratio-A** (10 μM) + $ONOO^-$ (25 μM) and **P1** (10 μM) in PBS solution (pH=7.4, 20 mM, with 0.1% DMSO).

Reference

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