A Novel Near-Infrared Fluorescent Probe for Peroxynitrite Imaging in Cellular and Organ Injury

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Experimental section

1. Materials and instruments

All other chemical solvents were purchased from commercial suppliers and were pretreated according to the standard method before use. ^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance) spectra were determined using a Bruker 400-MHz spectrometer. ESI-HRMS (high-resolution mass spectroscopy) was measured using a Mariner instrument. UV–vis titrations were tested using a Shimadzu UV2600. Fluorescence spectra were measured using an Eclipse fluorescence spectrophotometer (Agilent, USA) at room temperature. The fluorescence images of cells were obtained using an inverted fluorescence microscope (Nikon, Ts2-FL). The pH was recorded by pH meter (Mettler Toledo pHS-210).

2. Preparation of DCI-ONOO

Compound DCI-OH (290 mg, 1 mmol) and N, N-diisopropylethylamine (0.10 mL, 1.2 mmol) were dissolved in anhydrous dichloromethane (10 mL). Then diphenylphosphinyl chloride (400 mg, 1.5 mmol) in anhydrous dichloromethane (10 mL) was added to the above mixture dropwise at 0 °C. After the reaction for 0.5 h, the reaction mixture was treated under vacuum to remove the solvent. The obtained residue was purified by column chromatography to obtain yellow solid **DCI-ONOO**. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.82 (ddd, J = 12.6, 8.3, 1.4 Hz, 4H), 7.52 – 7.45 (m, 2H), 7.40 (tdd, J = 6.7, 3.7, 1.4 Hz, 4H), 7.32 (d, J = 8.8 Hz, 2H), 7.17 (dd, J = 8.7, 1.2 Hz, 2H), 6.88 (d, J = 16.1 Hz, 1H), 6.78 (d, J = 16.1 Hz, 1H), 6.72 (s, 1H), 2.51 (s, 2H), 2.35 (s, 2H), 0.98 (s, 6H). ^{13}C NMR (101 MHz, Chloroform-d) δ 169.29, 153.74, 135.94, 132.74, 132.71, 132.19, 131.83, 131.73, 131.24, 129.87, 128.98,

128.86, 128.81, 128.67, 123.54, 121.38, 121.33, 113.53, 112.74, 78.66, 42.98, 39.15, 32.05, 28.02. HRMS (ESI): calcd for $C_{31}H_{28}N_2O_2P$ [M + H]+ 491.1883, found 491.1883; $C_{31}H_{28}N_2O_2P$ $[M + Na]^+$ 513.1702, found 513.1705.

3. General preparation for optical measurements

The stock solution of **DCI-ONOO** and various analytes were prepared in DMSO and deionized water respectively. All spectra were measured in PBS-DMSO (10 mM, pH 7.4, v:v = 7:3) solution. For the measurements, excitation wavelength was 462 nm, the excitation and the emission slit width are 5 nm.

4. Cell culture and fluorescence imaging

The HepG-2 cells were grown in DMEM medium (Hyclone, USA) supplemented with 10% FBS, 1% of penicillin-streptomycin at 37 °C under 5% CO2. For the first control experiment, the cells were incubated with **DCI-ONOO** (10 μM) for another 30 min at 37 °C under 5% CO_2. For the second experiment, the cells were pretreated with $ONOO^-$ (20 and 50 μM) for 30 min, then incubated **DCI-ONOO** for 30 min at 37 °C. All the cells in the experiments were washed with PBS buffer (10 mM, pH = 7.4, 1 mL × 2) before imaging. The fluorescence images of cells were obtained using an inverted fluorescence microscope (Nikon, Ts2-FL, 20 × magnification).

5. Cell viability assays

The cytotoxicity of the probe was examined by MTT assay. HepG-2 cells were digested by 0.25% of trypsin,and transferred into 96-well plates with a density of 1×104 cells per hole. The cells were incubated in DMEM supplemented with 10 % (v/v) fetal bovine serum in a cell culture incubator containing 5 % CO_2 at 37 ℃ for 24 h, and then different concentrations of **DCI-ONOO** (0, 1, 2.5, 5, 10, 15, 20, 25 μM) were added to the 96-well plates. After 12 h incubation at the same condition, 20 μL MTT (5 mg/mL, dissolved in DMSO) was added and incubated for another 4 h. The cells were washed three times with PBS (10 mM)，and dissolved with DMSO (150 μL) per hole. The culture medium was removed, and 150 μL DMSO was added into each well. The absorbance (OD value) of each well at 490 nm was recorded by the microplate spectrophotometer system. Cell viability was calculated based on the

equation: Cell viability (%) = [OD490 (Probe) - OD490(Blank)] / [OD490(Control) - OD490(Blank)] × 100%.

6. APAP-induced Organ Injury model and in vivo imaging

The animal experiment protocol was reviewed and approved by Xinxiang Medical University, complying with the Laboratory Animal Care and Use Guidelines issued by Xinxiang Medical University（Ethical Review Approval Number：XYLL— 20240327）. The control group was intravenously injected with 100 μL (100 μM) DCI-ONOO. The experimental group received an intraperitoneal injection of APAP (400 mg/kg), followed by an intravenous injection of 100 μL (100 μM) DCI-ONOO after 24 h. All animals were anesthetized with isoflurane, and fluorescence images were obtained at different time points after administration using a small animal in vivo optical imaging system. After the above experiments, the mice were euthanized under anesthesia. The abdominal cavity was opened, different organs were separated, and fluorescence imaging was performed on the different tissues using a camera.

7. Generation of various ROS/RNS.

The sources of ROS/RNS are described as follows, 30 % H2O2 solution was purchased from Sigma-Aldrich and diluted before using. KO2 was dissolved in DMSO to generate superoxide (O2•−).

1). Potassium Nitroprusside Dihydrate was used to generate•NO. tert-butyl hydroperoxide (TBHP) was diluted from 70 % stock solution.

2). The source of NaClO was from NaClO solution that contains 5% available chlorine.

3). Hydroxyl radical (•OH) was generated by Fenton reaction. Briefly, ferrous chloride (FeCl2) was added in the presence of 10 equiv. of H2O2, the concentration of •OH was equal to the Fe (II) concentration.

4). Peroxynitrite (ONOO−) solution was synthesized according to the reported literature. 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. A short column of manganese dioxide was used 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.

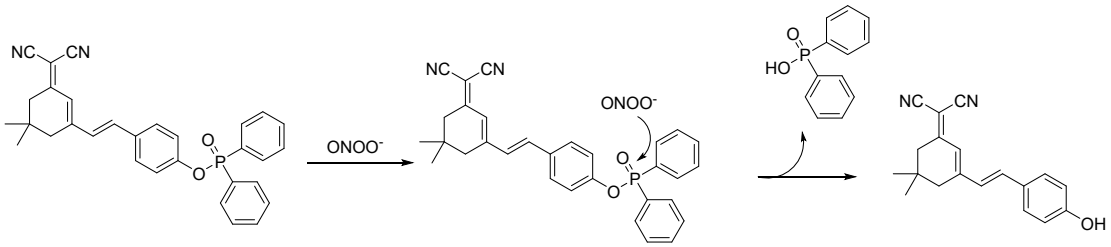

Figure S1. Reaction process of $ONOO^-$ interact with DCI-ONOO.

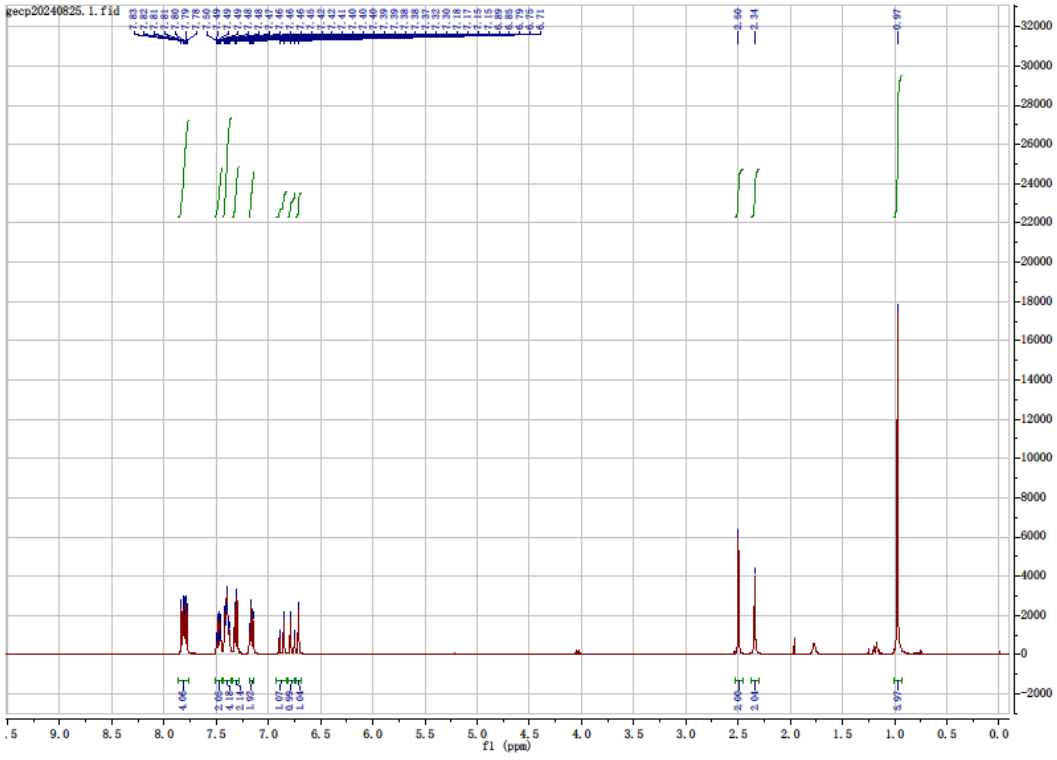

Figure S2. ^1H NMR spectrum of **DCI-ONOO**

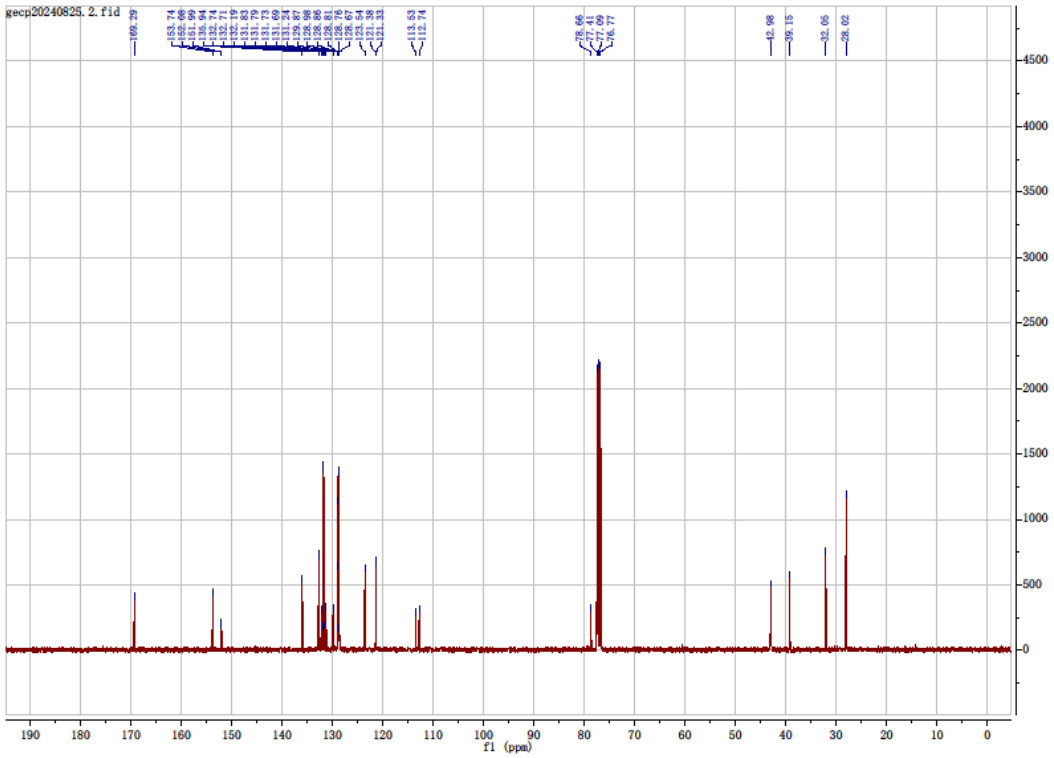

Figure S3. ^{13}C NMR spectrum of **DCI-ONOO**

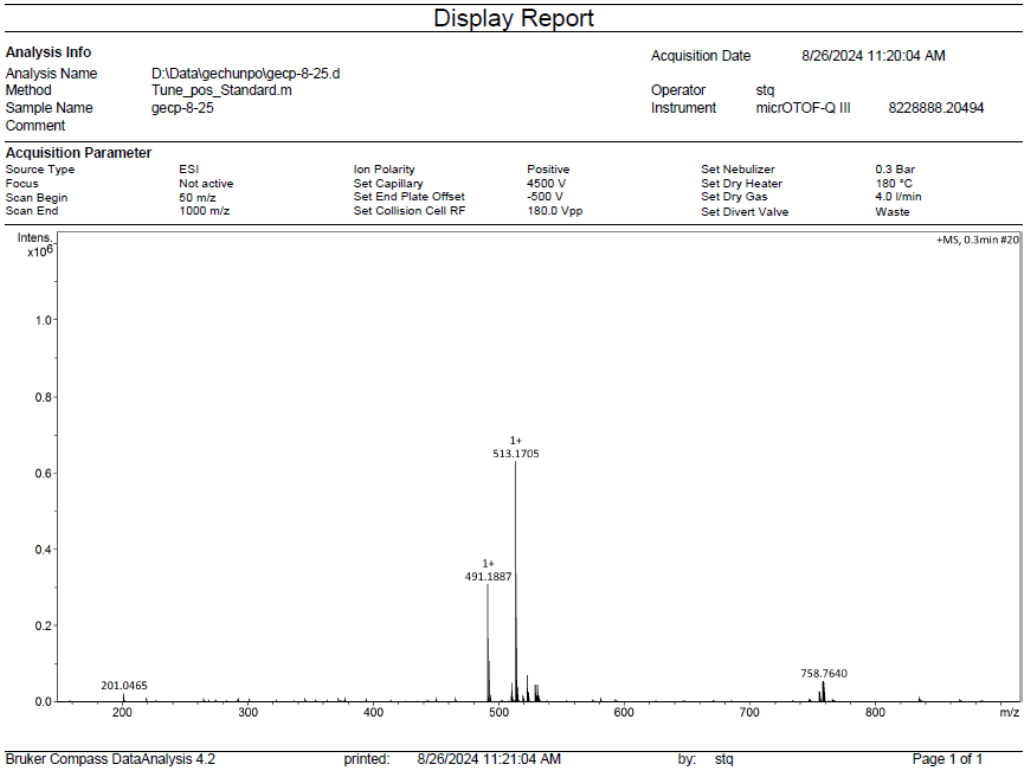

Figure S4. High resolution mass spectrum of **DCI-ONOO**

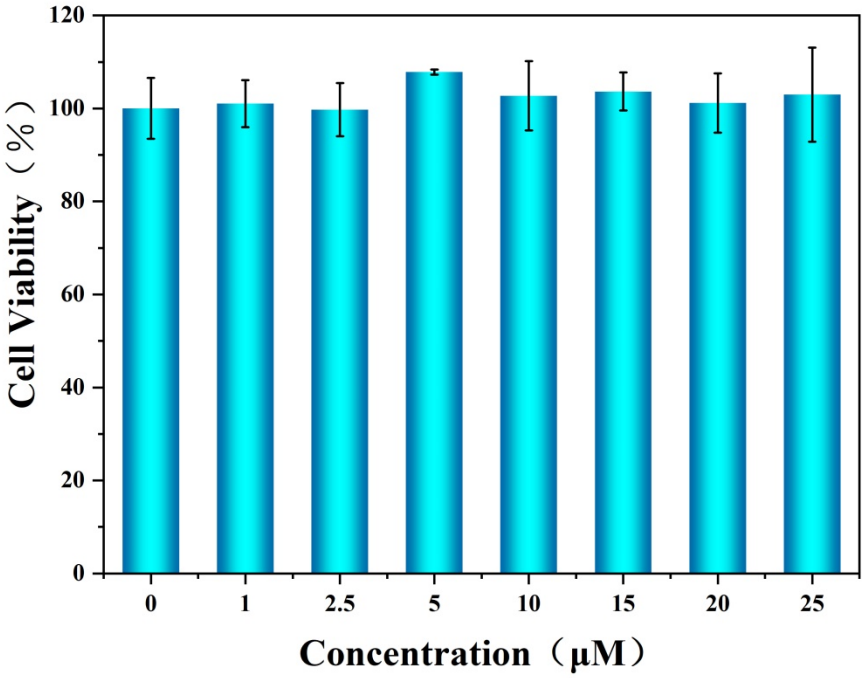

Figure S5. The cytotoxicity of **DCI-ONOO** in living HepG2 cells for 24 h.