Supporting Information

Observation of the generation of peroxynitrite in mouse liver after acetaminophen overdose with a boronate-based ratiometric fluorescence probe

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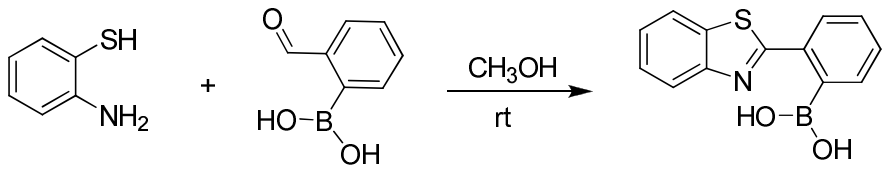
1. General methods

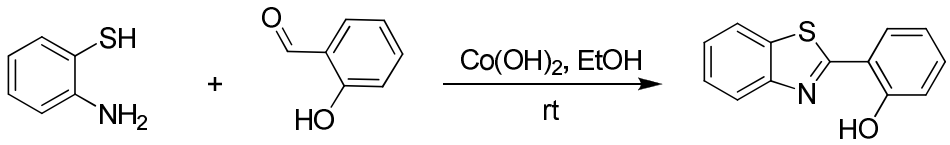
All reagents and solvents were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard procedures. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Flash chromatography was performed using silica gel 60 (200–300 mesh). Absorption spectra were taken on a Shimadzu UV-2450 spectrophotometer. Fluorescence spectra were taken on Shimadzu RF-5301 fluorescence spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 100 MHz on Bruker AVANCF-400 instrument, respectively. High resolution mass spectra were obtained on a Varian QFT-ESI mass spectrometer.

2. Synthesis



Probe BTPB: A mixture of 2-formylphenylboronic acid (0.24 g, 1.60 mmol) and 2-aminobenzothiol (0.2 g, 1.60 mmol) was dissolved in 4 mL methanol and the reaction was stirred at room temperature. Full consumption of the starting materials was achieved within 30 min of stirring (confirmed by TLC, DCM: EtOAc = 9:1). The organic solvent was then evaporated and the residue was purified by column chromatography on silica gel (eluent DCM: MeOH = 200:1 to 50:1) to afford 0.21 g off-white solid (51.5% isolated yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.16

(dd, J = 8.0, 0.6 Hz, 1H), 8.04 (s, 2H), 8.00-7.95 (m, 1H), 7.89-7.83 (m, 1H), 7.58-7.45 (m, 5H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.71, 153.58, 135.55, 135.31, 133.18, 130.42, 129.05, 128.72, 126.95, 125.76, 123.10, 122.77 (CH×2). HRMS (ESI) m/z [M-H]$^-$ calcd. for $C_{13}H_{10}BNO_2S$ 254.0598, found 254.0631. m.p.: 221.1-223.7℃.



Compound BTP: A mixture of 2-hydroxybenzaldehyde (0.59 g, 4.80mmol) and 2-aminobenzothiol (0.50 g, 4.00 mmol) was dissolved in 15 mL ethanol. Cobalt hydroxide (II) (0.04 g, 0.40 mmol) was added and the reaction was stirred at room temperature. Full consumption of the starting materials was achieved within 4.5 h of stirring (confirmed by TLC, PE: EtOAc = 2:1). The organic solvent was then evaporated and the residue was purified via silica gel column chromatography (eluent PE: EtOAc = 3:1) to afford 0.34 g off-white solid (37.2% isolated yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.60 (s, 1H), 8.17 (dd, J = 13.9 Hz, 7.8 Hz, 2H), 8.07 (d, J = 8.0 Hz, 1H), 7.55 (t, J = 7.4 Hz, 1H), 7.51-7.39 (m, 2H), 7.09 (d, J = 8.2 Hz, 1H), 7.03 (t, J = 7.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.70, 156.74, 151.90, 134.71, 132.96, 129.00, 126.95, 125.57, 122.59, 122.48, 120.24, 118.80, 117.44. HRMS (ESI) m/z [M-H]$^-$ calcd. for $C_{13}H_9NOS$ 226.0405, found 226.0336. m.p.: 128.5-130.3℃.

3. Preparation of the test solution

Deionized water and medical grade CTAB were used for spectroscopic studies. Superoxide solution ($O_2^{\bullet-}$) was prepared by adding KO_2 (1 mg) to dry dimethyl sulfoxide (1 mL) and stirring vigorously for 10 min. Hydroxyl radical ($^{\bullet}OH$) was generated *in situ* by the Fenton reaction (to generate $^{\bullet}OH$, Fe^{2+} was added in the presence of 10 eq of H_2O_2). Hypochlorite and hydrogen peroxide solution were prepared by dilution of commercial NaClO solution and H_2O_2 solution in deionized water. Nitric oxide (·NO) was generated from Sodium Nitroferricyanide(III) Dihydrate (SNP), which was added into degassed deionized water under N_2 then stirred for 30 min at 25 ℃. The aqueous solution of $NaNO_2$ was freshly prepared and used as nitrite (NO^{2-}). Peroxynitrite solution was synthesized as literature reported.[1] The peroxynitrite concentration was estimated by using an extinction coefficient of 1670 $M^{-1}cm^{-1}$ at 302 nm. $\mathbf{C}_{ONOO^-} = Abs_{302}$ nm/1.67 (mM). $^1O_2$ was generated from the online reaction of NaClO solution (100 μM) with H_2O_2 (100 μM). And *tert*-butoxy radical (·O^{t}Bu) were generated by Fenton reaction of 25 mM Fe^{2+} with 5 mM *t*-BuOOH.

4. Monitoring *in situ* the formation of $ONOO^-$ in liver from the mice treated with APAP overdose

Male C57BL/6 mice with an average weight of 18 to 20 g were purchased from laboratory animal center of Xi'an Jiaotong University. Mice were housed in an environmentally controlled room with 12-h light/dark cycle

and allowed free access to food and water. The experimental protocols were performed in accordance with the 'Principles of Laboratory Animal Care' and guidelines of the laboratory animal care committee of Xi'an Jiaotong University. Mice were fasted overnight before the experiments. All mice were divided into two groups. One group of mice was injected intraperitoneally with APAP at 400 mg/kg, and the other group was injected intraperitoneally with saline as the control. At 6 h After APAP injection, 10% chloral hydrate (0.1 mL/10 g) was injected as an anesthetic during sacrifice. Mouse abdomen and chest were surgically opened. The tip of the sternum was clamped with the hemostat, and then the hemostat was placed over the head of mouse to expose the heart. The same method was applied to mouse abdomen to expose the liver. An infusion needle was passed into the left ventricle, and then a small incision was made to the right atrium. Initial perfusion (5 min) was performed with saline to remove blood. At this point, the perfusate is clear and the liver is white and bloodless. BTPB (1.0 μM) was next added to the saline (supplemented with 0.6 mM L-arginine) for tissue staining during perfusion (3.0 min). The liver was then excised and washed with PBS. The liver tissues were quickly embedded in OCT compound and stored at -20 °C. The tissues were then cryosectioned at an interval of 10 μm and mounted onto a glass slide. One part of the liver sections were quickly observed using Nikon C2 confocal microscope over the wavelength range of 417 - 477 nm with an excitation wavelength at 408

nm, and another part were stained with hematoxylin and eosin (HE).

5. HPLC analysis

BTPB and BTP were separated on a Shimadzu LC-30AD UFHPLC system equipped with UV-vis absorption detectors. 50 μL of sample was injected into the UFHPLC system equipped with a C_{18} column (Shim-pack, GIST-HP, 100×2.1 mmL.D., 3 μm) equilibrated with CH_3CN. The compounds were separated by gradient elution with CH_3CN : tri-distilled water (containing 0.05% formic acid) from 60% to 90% using a flow rate of 1 mL/min. The column temperature was 40 °C. The detection wavelength was set at 290 nm.

6. MTT assay

HepG2 cells were seeded in each well of 96-well plates at a density of 3,000 cells/well and incubated for 24 hours for cell attachment. The stock solution of probe BTPB dissolved in DMSO (1.2 mM) was diluted with a cell culture medium containing 10% FBS to have a final concentration of 0, 5, 10, 20, and 40 μM. Existing culture medium was replaced with 200 μL of the fresh one containing probe BTPB, and the cells were incubated for 24 hours. The culture media was then removed. Portions of 100 μL MTT solution (0.5 mg in 1 mL of Hanks' balanced salt solution) were added to the wells and the cells were further incubated at 37 °C for 2 h. The absorption of each well was measured at a wavelength of 570 nm with a multiwell scanning spectrophotometer (ELISA reader). Cell viability was

calculated as a percentage compared to untreated control cells (Fig. S10). Data are expressed as a mean ± standard deviation. Statistical significance compared to the untreated control cells was calculated by determining *p* values by using the *t*-test.

7. Supplementary data

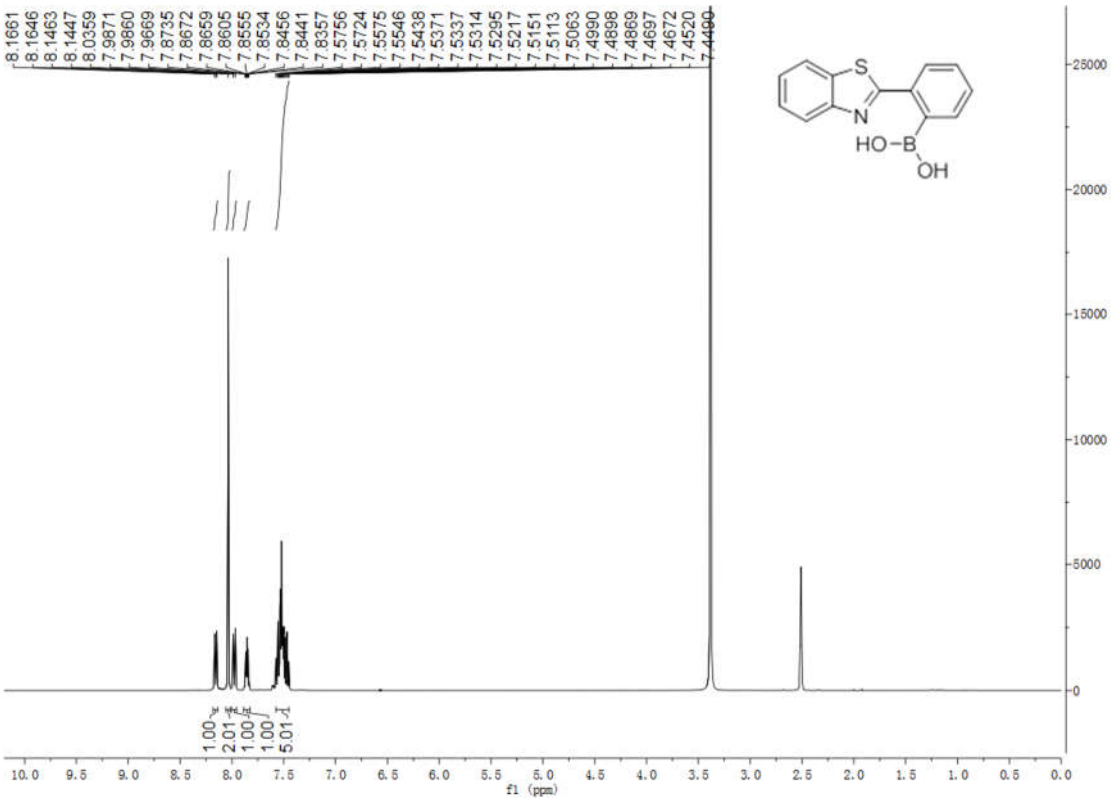


Fig. S1 ^{1}H NMR chart of Probe BTPB

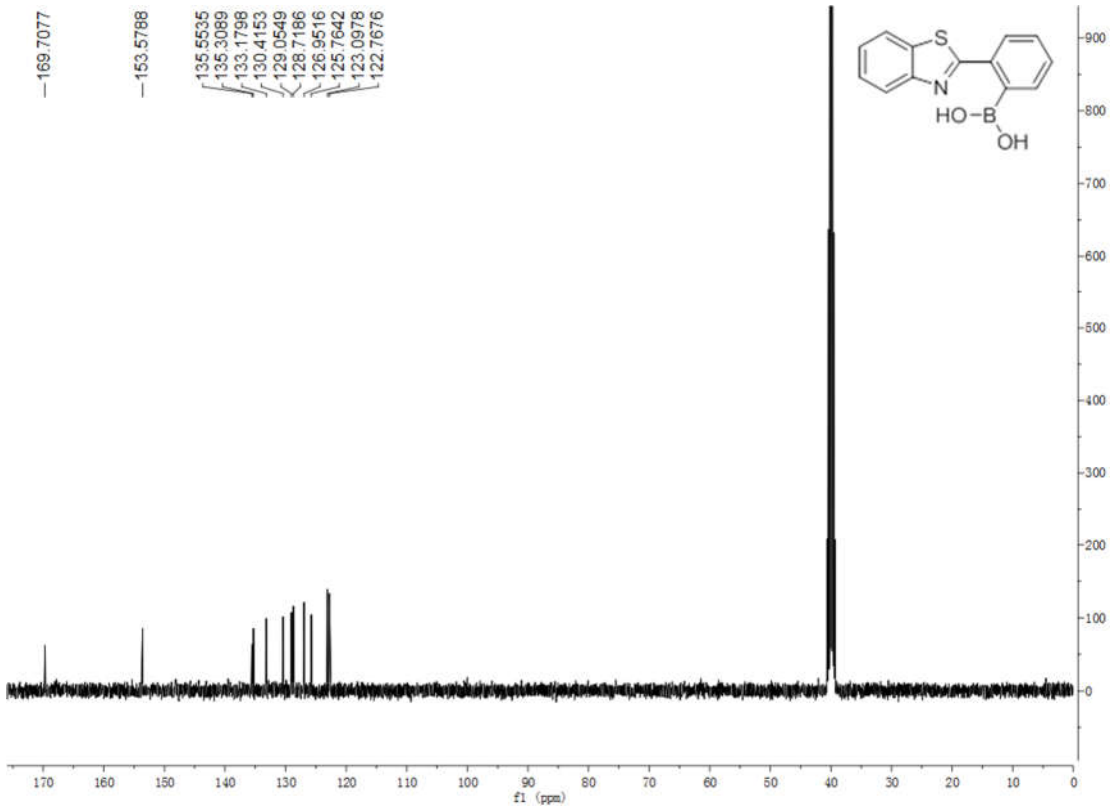


Fig. S2 ^{13}C NMR chart of Probe BTPB

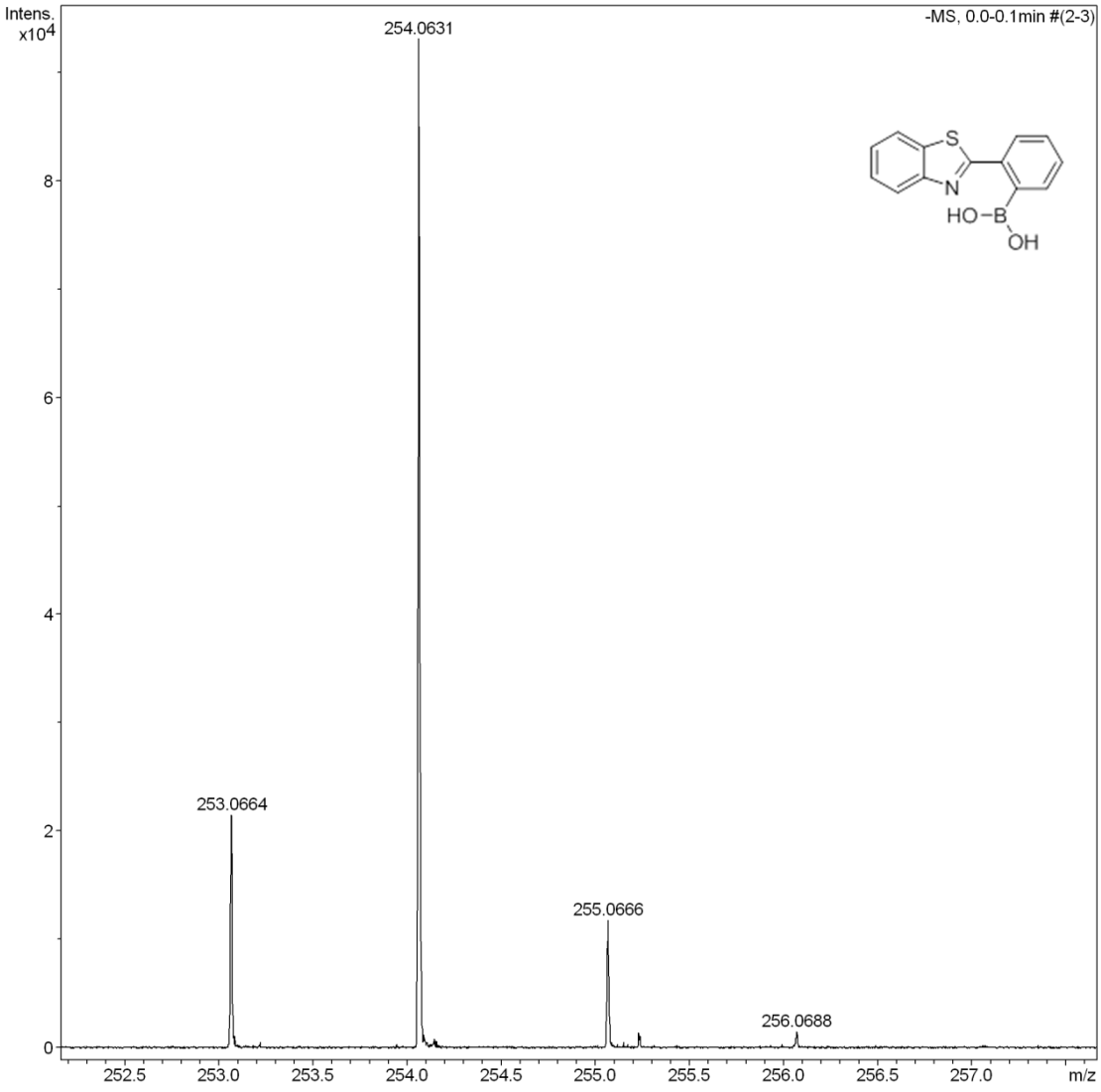


Fig. S3 HRMS chart of Probe BTPB

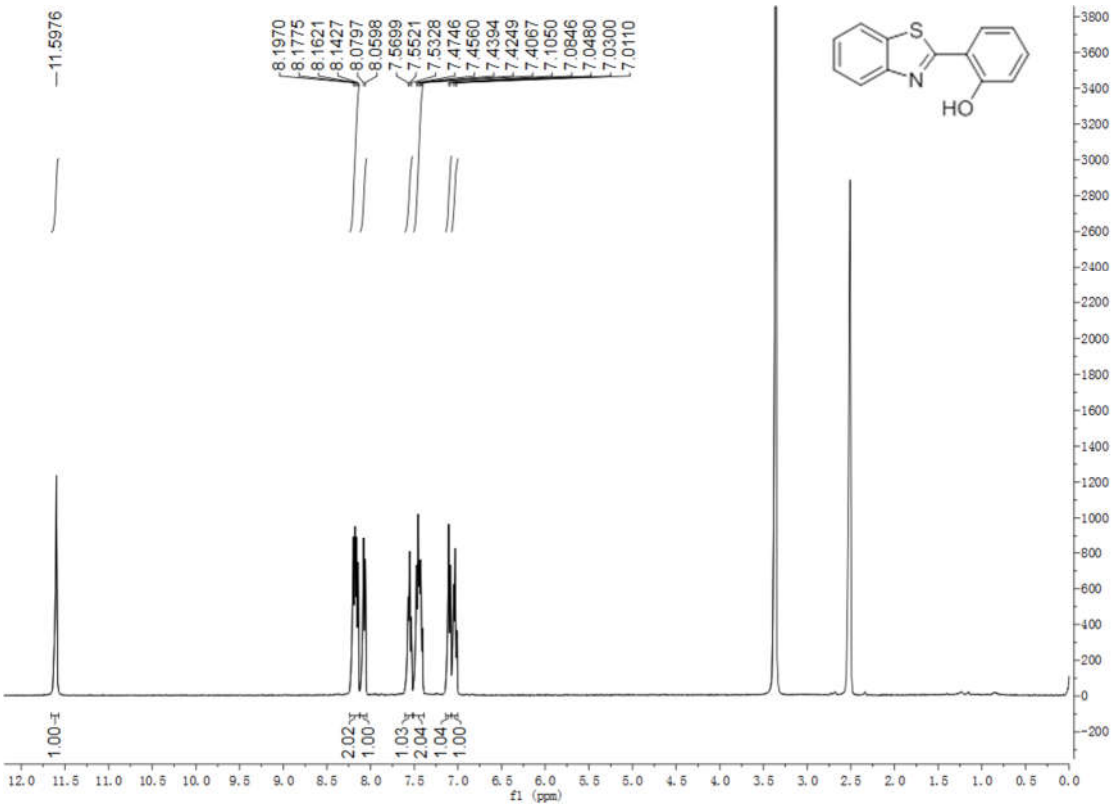


Fig. S4 ^{1}H NMR chart of compound BTP

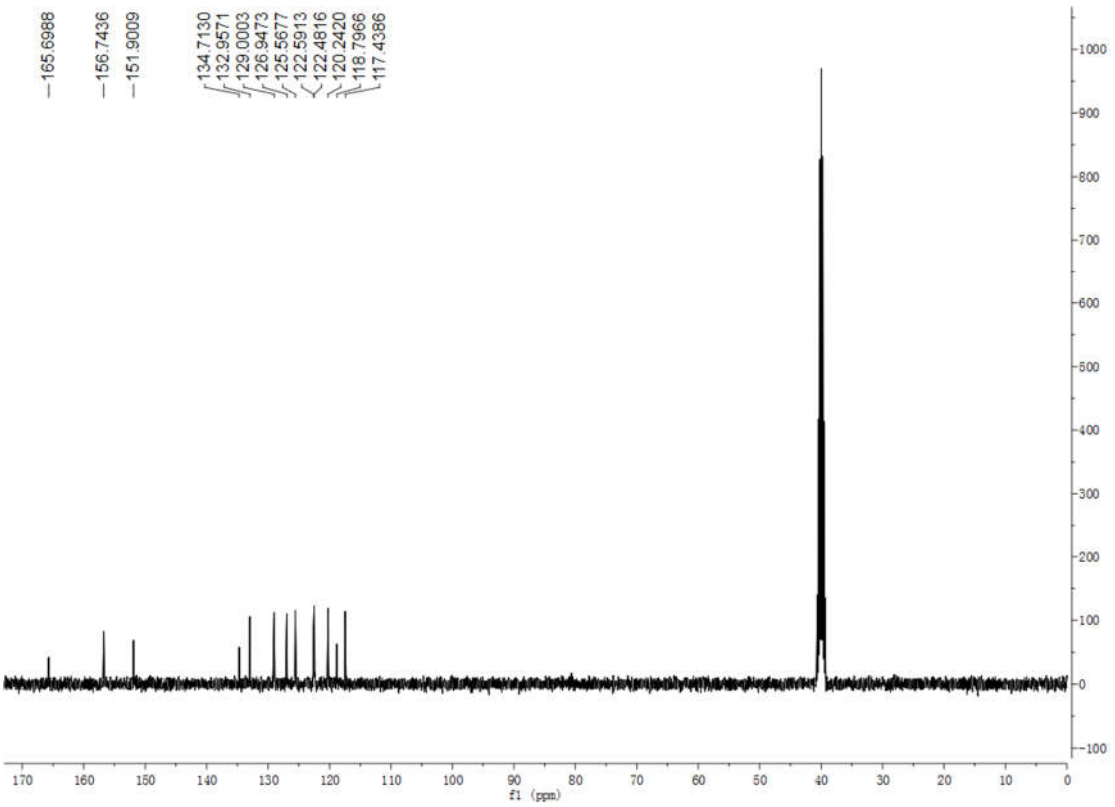


Fig. S5 ^{13}C NMR chart of compound BTP

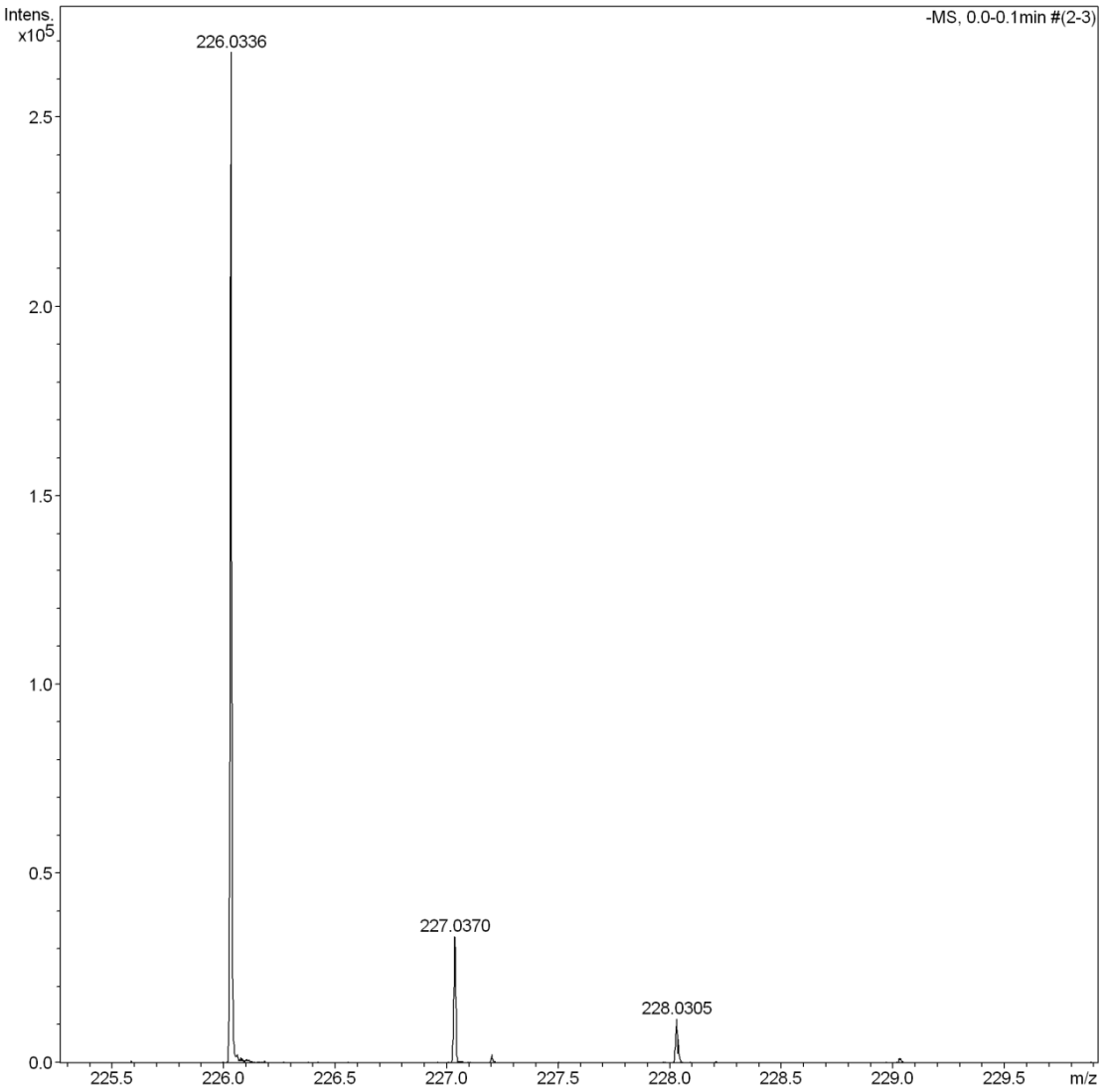


Fig. S6 HRMS chart of compound BTP

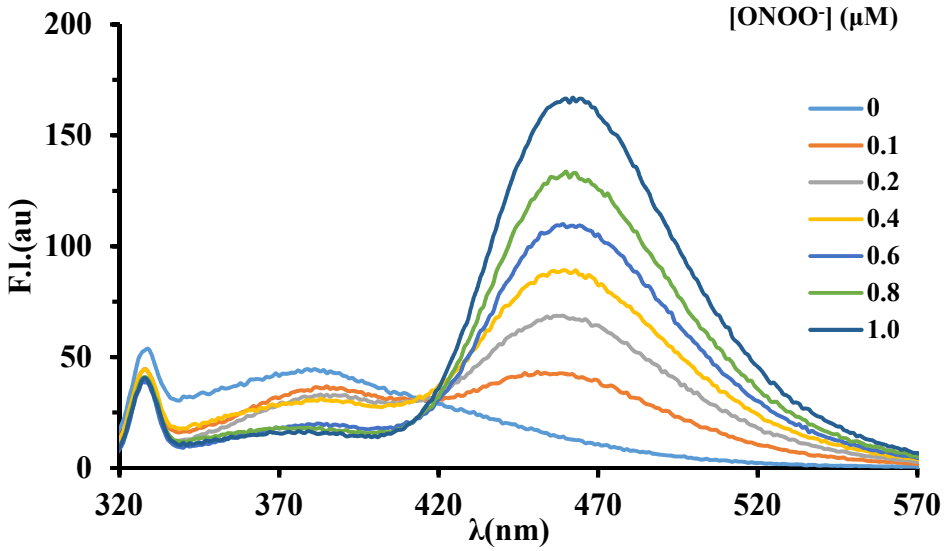


Fig. S7 Fluorescence emission spectra of BTPB (1.0 μM) to OONO- (0 - 1 equiv), λ_{ex} = 296 nm. All spectra were obtained in 100.0 mM phosphate buffer (pH 7.4) under vigorous stirring for 1 min after incubation of the probe with $ONOO^-$ for 30 seconds at 25.0 °C.

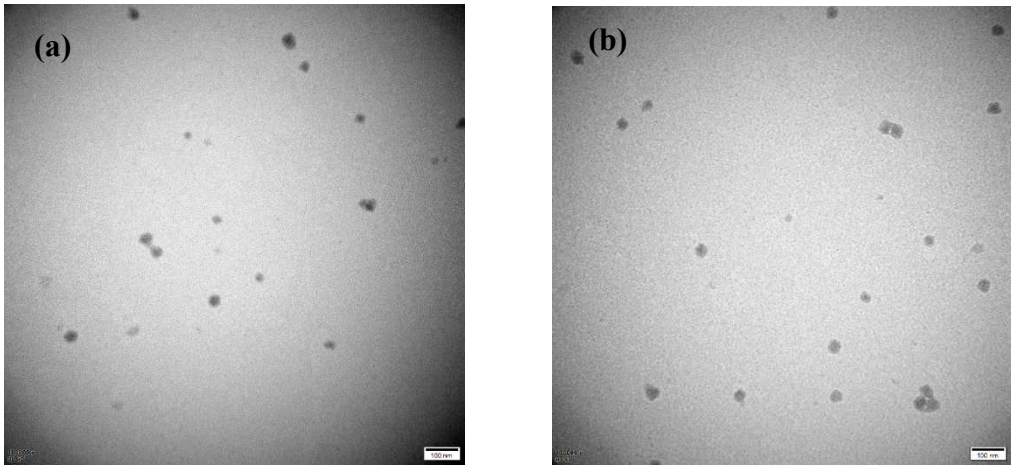


Fig. S8 The formation of micelles of BTPB (1.0 μM) before (a) and after (b) reaction with $ONOO^-$ (1.0 μM) in 100.0 mM phosphate buffer (pH 7.4) with 1.0 mM cetyltrimethylammonium bromide (CTAB).

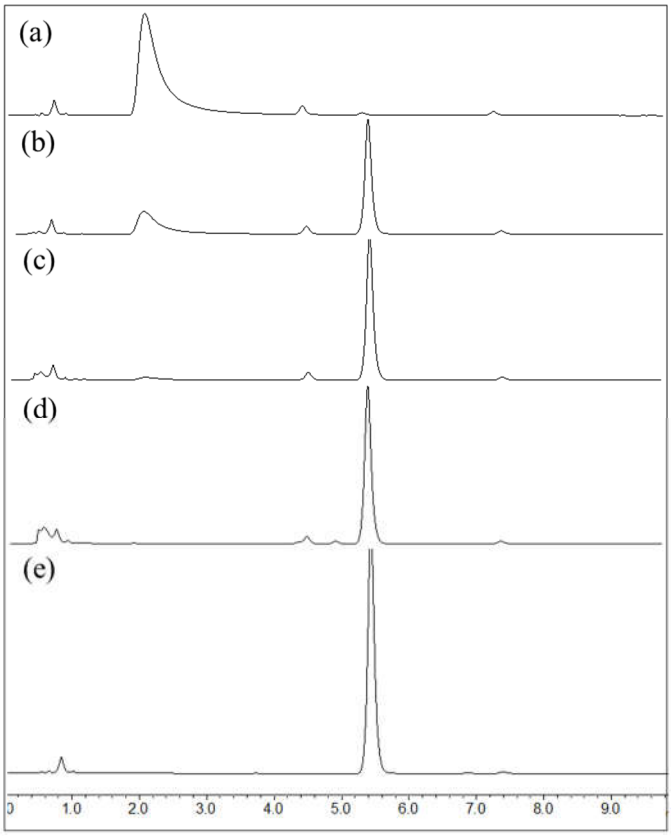


Fig. S9 The reaction between BTPB and $ONOO^-$ by HPLC analyses. (a) 10 μM BTPB. (b) 10 μM BTPB and 0.3 equiv $ONOO^-$. (c) 10 μM BTPB and 0.7 equiv $ONOO^-$. (d) 10 μM BTPB and 1.0 equiv $ONOO^-$. (e) 10 μM BTP. Samples were prepared in 100.0 mM phosphate buffer (pH 7.4) after incubation of BTPB with $ONOO^-$ (0 – 1.0 equiv) for 1 min at 25.0 °C. BTPB eluted at 2.0 min, BTP eluted at 5.5 min by gradient elution with CH_3CN : tri-distilled water (containing 0.05% formic acid) from 60% to 90% using a flow rate of 1 mL/min. The column temperature was 40 °C. The detection wavelength was set at 290 nm.

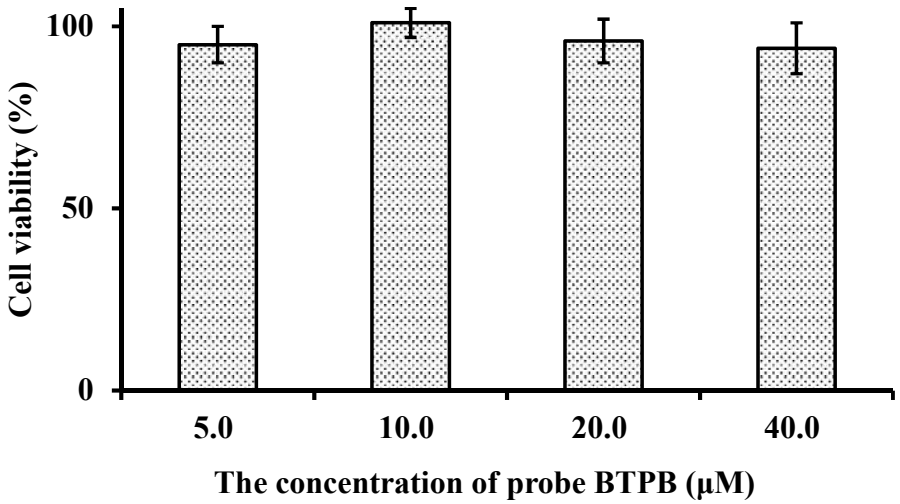


Fig. S10 Cytotoxicity of probe BTPB in cultured HepG2 cells. Cells were incubated with the probes at corresponding concentrations for 24 h. Cell viability was measured by MTT assay and the results are reported as percentage relative to untreated cells (mean ± SD) (n=5).

8. Reference

1. R. M. Uppu, W. A. Pryor, *Anal. Biochem.*, 1996, **236**, 242-249.