

## ***Supporting information***

### **A two-photon ratiometric fluorescent probe for the synergistic detection of mitochondrial SO<sub>2</sub>/HClO crosstalk in cells and in vivo**

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## 1. General methods

**Materials and reagents:** 6-Hydroxy-2-naphthaldehyde, (4-bromobutyl)triphenyl phosphonium bromide and malononitrile were purchased from Sigma-Aldrich Co. (Steinheim, Germany). Tert-butylhydroperoxide (t-BuOOH, 70%), Fe<sup>II</sup> (EDTA), sodium hypochlorite (NaClO, 10% available chlorine), and hydrogen peroxide solution (30%) were purchased from Aladdin Chemistry Co. (Shanghai, China). Anion (Cl<sup>-</sup>, Br<sup>-</sup>, I<sup>-</sup>, HSO<sub>3</sub><sup>-</sup>, CH<sub>3</sub>COO<sup>-</sup>, ClO<sub>4</sub><sup>-</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, HS<sup>-</sup>, SCN<sup>-</sup>, S<sub>2</sub>O<sub>3</sub><sup>2-</sup>, SO<sub>4</sub><sup>2-</sup>) were prepared by dissolving the appropriate amount of pure salts in deionized water and stored at 4 °C. Various ROS and RNS including HClO, ·OH, H<sub>2</sub>O<sub>2</sub>, TBHP, TBO·, NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, NO, ONOO<sup>-</sup> and O<sub>2</sub><sup>-</sup> were obtained according to reported methods (See details in following information). GSH, Cys, Hcy, Rhodamine123, lipopolysaccharide (LPS), fluorescein, N-ethylmaleimide (NEM), 4-aminobenzoic acid hydrazide (4-ABAH), 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich Co. (Steinheim, Germany). Acetonitrile, pyridine, CH<sub>2</sub>Cl<sub>2</sub>, methanol were analytical grade without further purification. Water used in all experiments was the double-distilled water.

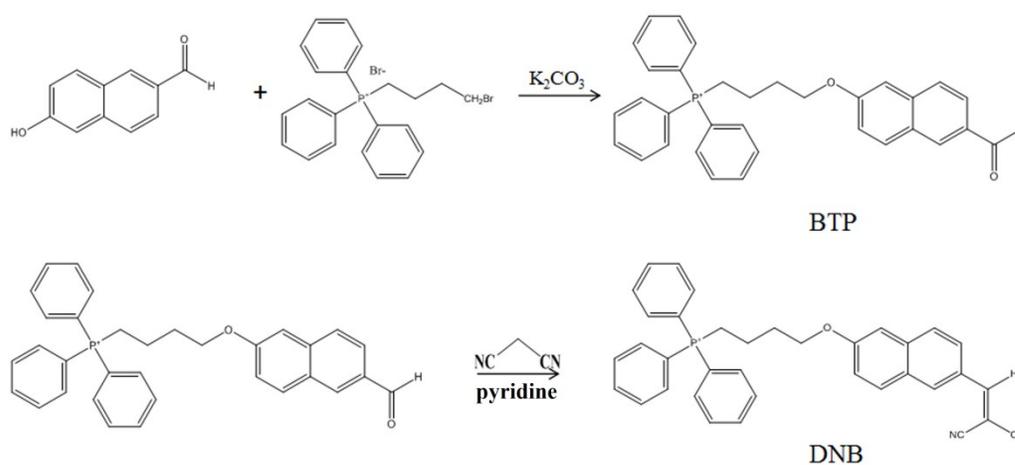
**Instruments:** TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 300-400), both of which were obtained from the JianYou Chemical Company (Yan Tai, China). All pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China). Absorption spectra were obtained on 300 bio UV-vis spectrophotometer (Varian USA). Hitachi F-4600 fluorescence spectrophotometer (Hitachi Japan) with a Xenon lamp and 1.0-cm quartz cells was used to collect the fluorescence record. ESI-MS and MS data were recorded on Agilent 1100 Series LC-MSD Trap-SL (ion trap) mass spectrometer. <sup>1</sup>H NMR, <sup>13</sup>C NMR spectra were measured with Bruker ascend 500 (500.1 MHz, <sup>1</sup>H; 125.8 MHz <sup>13</sup>C) instrument. The fluorescence images of cells were acquired using the Carl Zeiss LSM880 with a multiphoton laser scanning microscope (Jena, Germany) with an objective lens (× 10/40).

**Generation of various ROS, RSS and RNS:** The concentration of the commercially available stock H<sub>2</sub>O<sub>2</sub> solution was estimated by optical absorbance at 240 nm (43.6 M<sup>-1</sup>cm<sup>-1</sup>).<sup>1</sup> The hydroxyl radical (·OH) was generated by Fenton reaction between Fe<sup>II</sup> (EDTA) and H<sub>2</sub>O<sub>2</sub> quantitatively, and the concentrations of Fe<sup>II</sup> (EDTA) represented the concentrations of ·OH.<sup>2</sup> T-BuOOH (TBHP) was obtained from Aladdin and was diluted to the

needed concentration. Tert-butoxy radical (TBO $\cdot$ ) were generated by Fenton reaction of TBHP with Fe<sup>II</sup> (EDTA).<sup>3</sup>The sources of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> were respectively the NaNO<sub>2</sub> and NaNO<sub>3</sub>. Nitric oxide (NO) was generated from diethylamine NONOate.<sup>4</sup> Peroxynitrite (ONOO<sup>-</sup>) was prepared according to the reported methods; the concentration of peroxynitrite was estimated by using an extinction coefficient of 1670 M<sup>-1</sup>cm<sup>-1</sup> (302 nm).<sup>5</sup>Superoxide (O<sub>2</sub><sup>-</sup>) was prepared from KO<sub>2</sub>.

**Absorption and Fluorescence Analysis:** Absorption spectra were obtained with 1.0-cm glass cells. Fluorescence emission spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. Fluorescent spectra of probe to HClO were recorded at the excitation wavelength of 420 nm, with the excitation / emission slit width of 10 nm/10 nm. Fluorescent spectra of probe to SO<sub>2</sub> were recorded at the excitation wavelengths of 350 nm and 460 nm, with the excitation / emission slit width of 10 nm/10 nm. All the optical properties were evaluated under the physiological conditions using 2.0 μM of probe in DMSO/PBS buffer solution (v/v: 10/90; pH = 7.4, 20 mM). The mixture was equilibrated before measurement.

## 2. Synthesis route of probe (DNB)

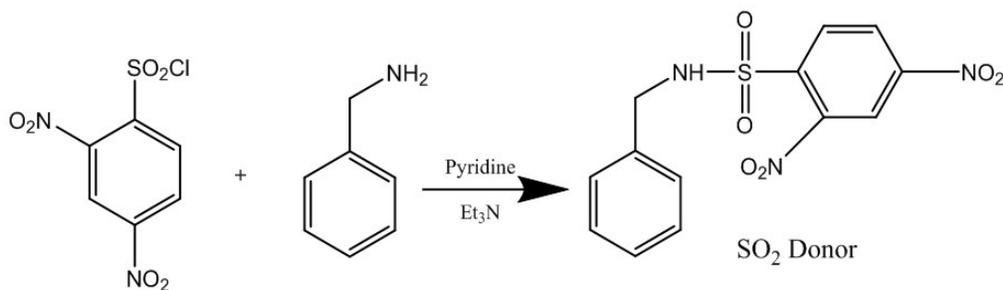


Scheme S1

The synthesis route of probe (DNB)

## 3. Synthesis of SO<sub>2</sub> donor

Benzylamine (0.321 g, 3 mmol) was dissolved in 10ml dry pyridine. To the solution, 0.2 ml Et<sub>3</sub>N was added. The solution was cooled to 0 °C. Then, a solution of 2,4-dinitrobenzenesulfonyl chloride (0.792 g, 3 mmol) in dry pyridine (5 mL) was slowly added. After stirring for 15 min, the mixture was heated to room temperature and stirred for another 3h. The resultant mixture was poured into 20 ml water and filtered. The crude product was recrystallized in CH<sub>3</sub>OH to get yellow solid, N-benzyl-2,4-dinitrophenylsulfonamide (BTSA). ESI-MS m/z calcd for C<sub>13</sub>H<sub>11</sub>N<sub>3</sub>O<sub>6</sub>S [M+H]<sup>+</sup>, 338.04, found 338.10. <sup>1</sup>H NMR (CD<sub>3</sub>Cl, 500MHz), δ (ppm): 8.86 (d, J =2.0 Hz, 1H), 8.364 (s, J, 1H), 8.120 (d, J = 8.5 Hz, H), 7.107 (d, J = 7.5 Hz, 3H), 6.996 (d, J = 6.5 Hz, 2H), 3.709 (s, 2H). <sup>13</sup>C NMR (CD<sub>3</sub>Cl, 125MHz), δ (ppm): (148.6, 146.82, 138.32, 136.13, 131.24, 127.75, 127.69, 126.09, 126.01, 119.70, 44.35).



Scheme S2 The synthesis route of SO<sub>2</sub> donor

#### 4. Quantum yield calculation

The fluorescence quantum yields were determined by comparing the integrated area of the corrected emission spectrum of samples with a reference. In this work, fluorescence quantum yields for DNB, DNB-HSO<sub>3</sub><sup>-</sup> and BTP were determined in the reference of fluorescein ( $\Phi = 0.98$ , 0.1 M NaOH).<sup>6</sup> The quantum yields were calculated using the expression:  $\Phi_{\text{sample}} = \Phi_{\text{standard}} \times (A_{\text{standard}} F_{\text{sample}} / A_{\text{sample}} F_{\text{standard}})$ , where  $\Phi_{\text{sample}}$  and  $\Phi_{\text{standard}}$  are the fluorescence quantum yields of the sample and the standard, respectively;  $F_{\text{sample}}$  and

$F_{\text{standard}}$  are the integrated fluorescence intensities of the sample and the standard spectra, respectively;  $A_{\text{sample}}$  and  $A_{\text{standard}}$  are the optical densities at the excitation wavelength of the sample and the standard, respectively.

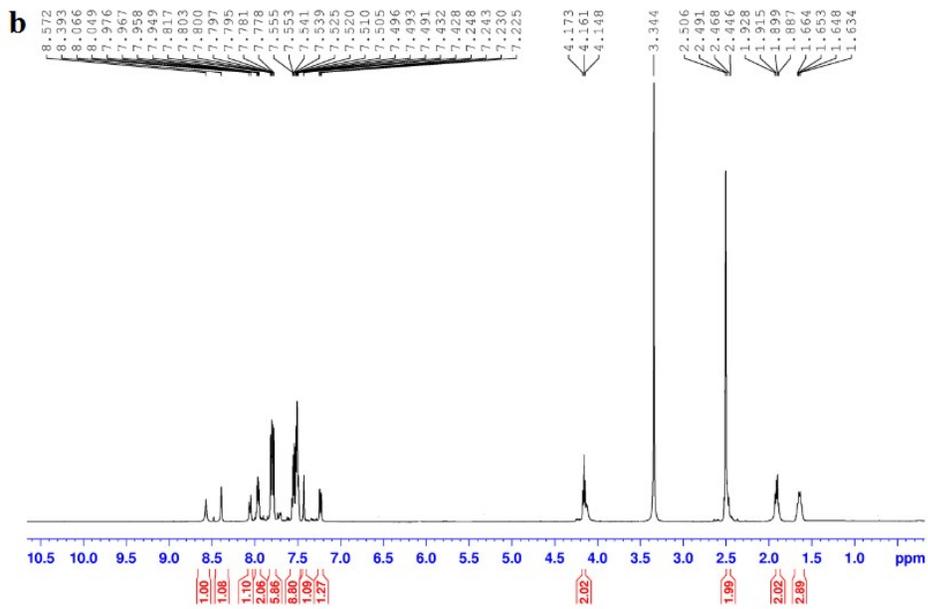
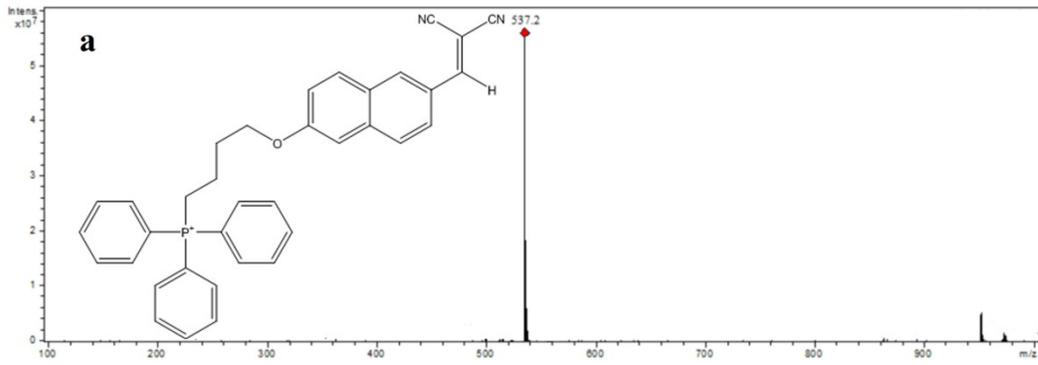
## 5. Determination of limits of detection

To determine the detection limit of probe by fluorescence spectra titration, detection limits for  $\text{HSO}_3^-$  and  $\text{HClO}$  were calculated by the formula: detection limit =  $3 \text{ SD} / k$ , where  $k$  is the slope of the curve equation and  $\text{SD}$  represents the standard deviation for the fluorescence intensity ratio responses of probe to  $\text{HSO}_3^-$  or  $\text{HClO}$ .

## 6. Measurement of two-photon cross section

In this study, Rhodamine B was used to determine the two-photon cross section ( $\delta$ ) by fluorescence measurement technique. The two-photon induced fluorescence intensity was measured at 680–940 nm by using Rhodamine B (5.0  $\mu\text{M}$ ) in methanol as the reference, whose two-photon property has been well characterized in the literature.<sup>7,8</sup> TP absorption cross-section ( $\delta$ ) was calculated by using the following equation:  $\delta = \delta_r (S_s \Phi_r \phi_r C_r) / (S_r \Phi_s \phi_s C_s)$ . In the formula, the subscripts of  $s$  and  $r$  stand for the probe and Rhodamine B, respectively;  $S$  represents the fluorescence intensity;  $\Phi$  symbolizes the fluorescence quantum yield;  $\phi$  is the overall fluorescence collection efficiency of the experimental apparatus and  $C$  is the concentration of the molecules in solution,  $\delta_r$  is the two-photon absorption cross section of the reference molecule.

## 7. $^1\text{H}$ NMR, $^{13}\text{C}$ NMR and MS for probe.



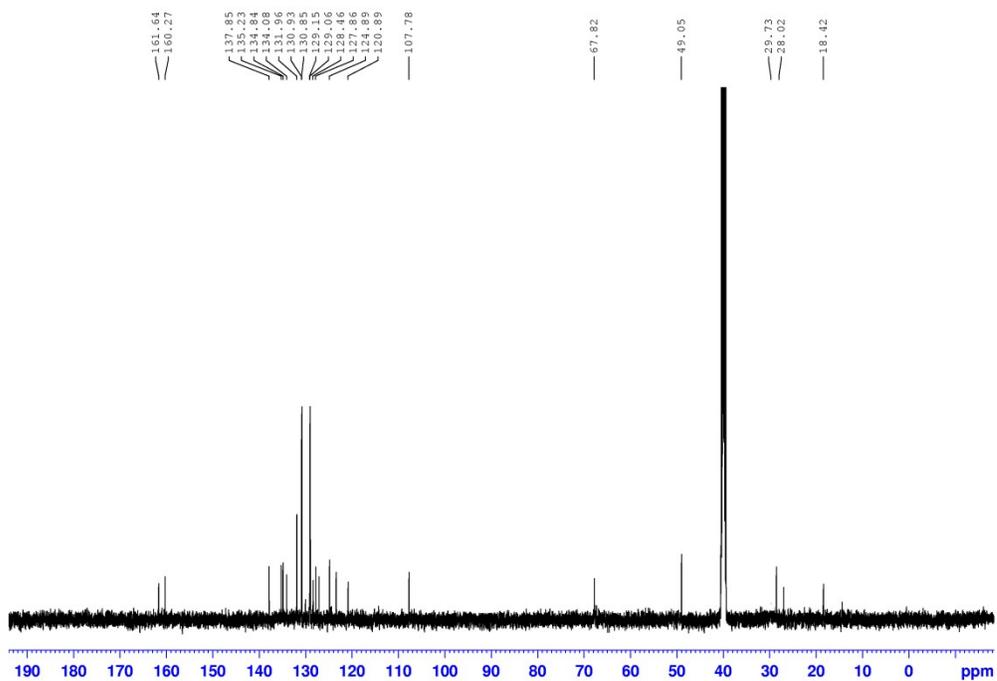
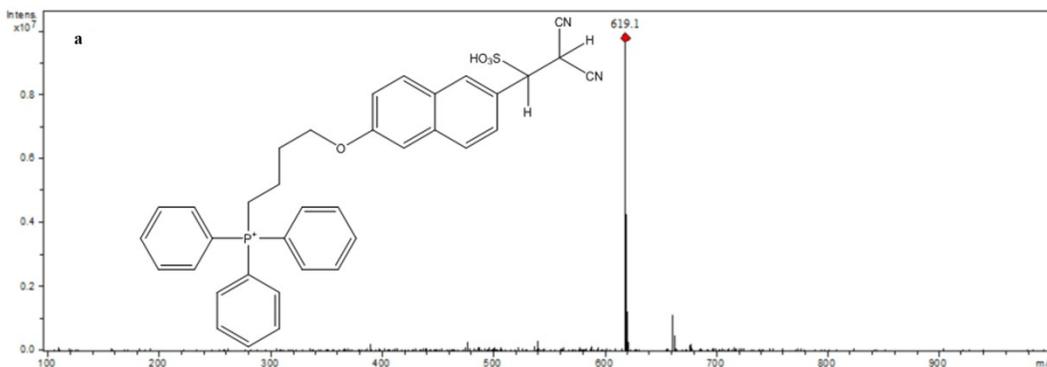
**c**

Fig. S1 Characterization of probe DNB: (a) Mass Spectra:  $m/z$  537.2; (b)  $^1\text{H}$  NMR spectrum ( $\text{DMSO-d}_6$ ) and (c)  $^{13}\text{C}$  NMR spectrum ( $\text{DMSO-d}_6$ ).

### 8. Mass spectra for probe to $\text{HClO}$ , $\text{HSO}_3^-$ and successive reaction to $\text{HClO}$ and $\text{HSO}_3^-$ , respectively.



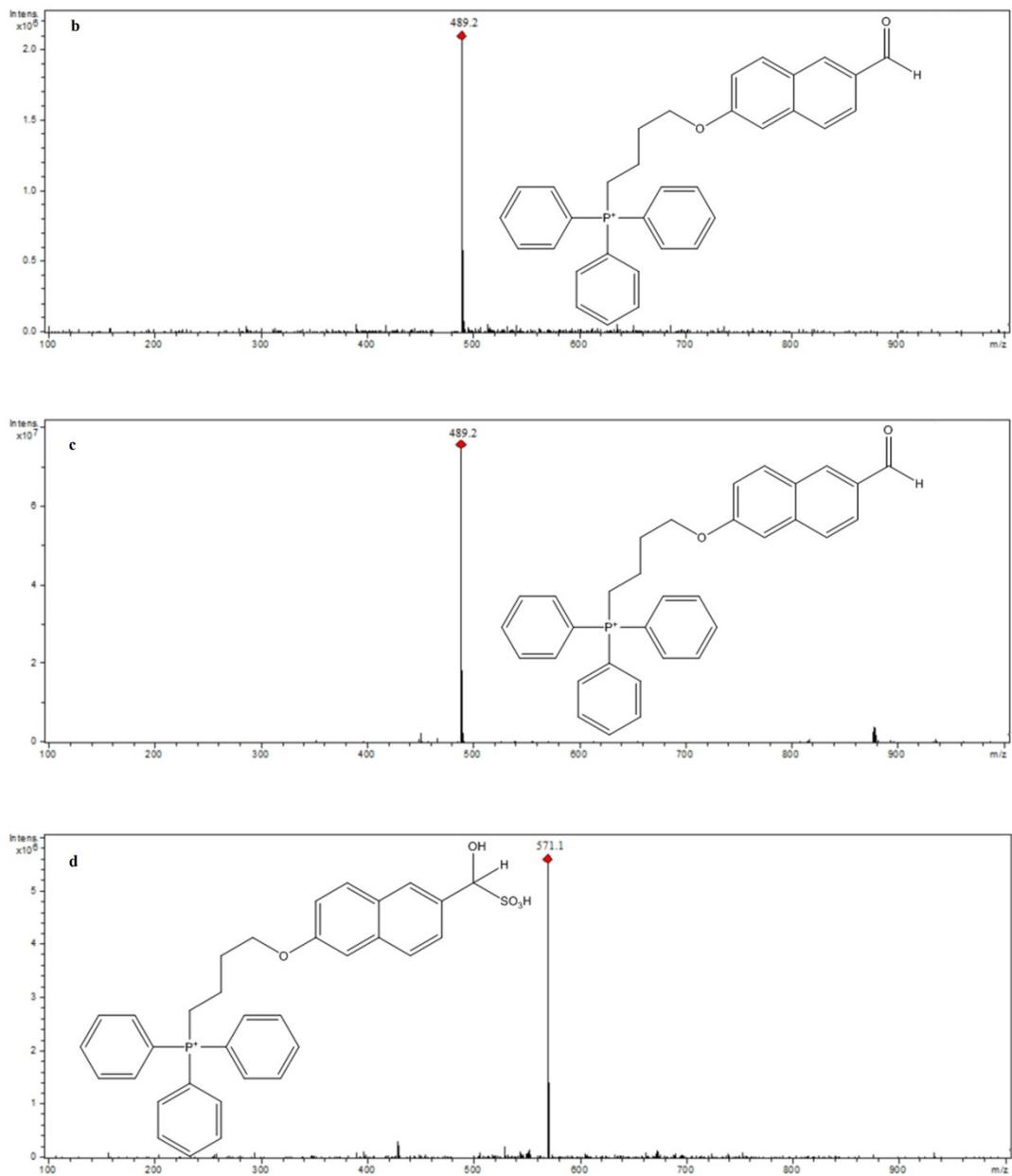
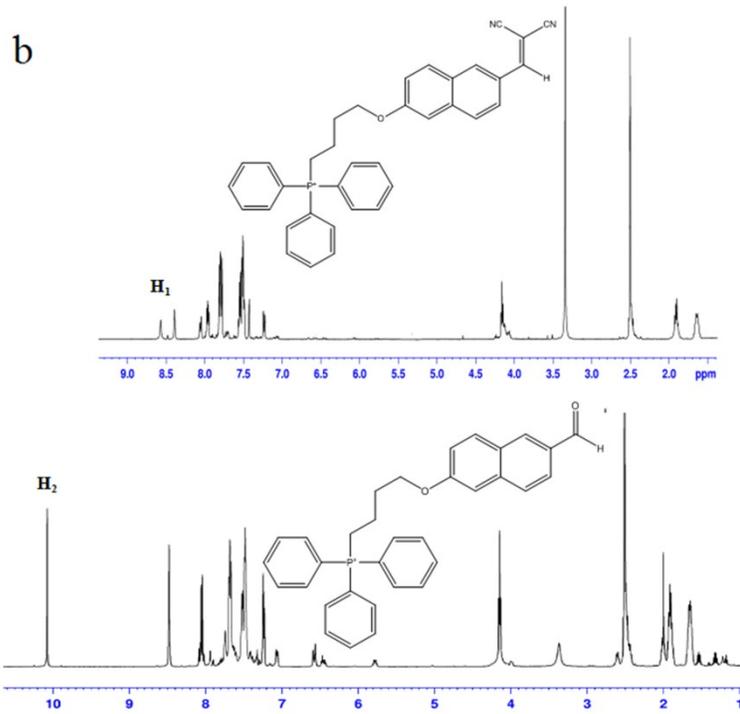
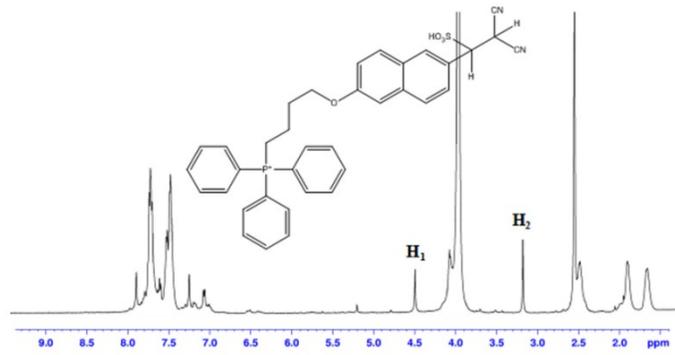


Fig. S2 (a) Mass spectra of DNB-HSO<sub>3</sub><sup>-</sup>; (b) Mass spectra of reaction product of DNB to HClO (BTP); (c) Mass spectra of HClO added to DNB-HSO<sub>3</sub><sup>-</sup>; (d) Mass spectra of HSO<sub>3</sub><sup>-</sup> added to BTP.

### 9. <sup>1</sup>H NMR titration of probe with HSO<sub>3</sub><sup>-</sup> and HClO.



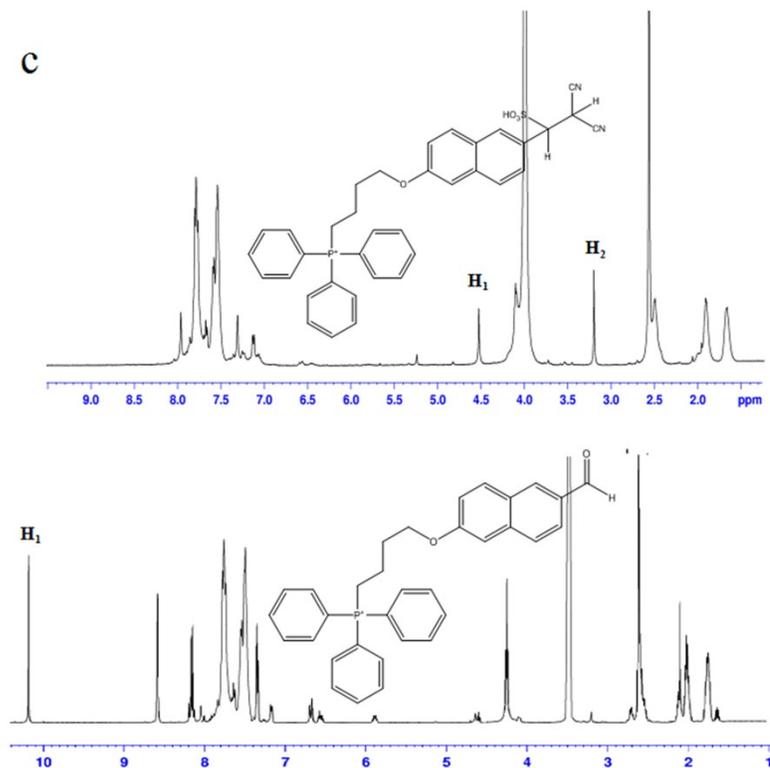


Fig. S3 (a) Partial  $^1\text{H}$  NMR spectra of DNB in DMSO- $d_6$  and  $^1\text{H}$  NMR spectra of DNB in the presence of  $\text{HSO}_3^-$  in DMSO- $d_6/\text{D}_2\text{O}$ ; (b) Partial  $^1\text{H}$  NMR spectra of DNB and in the presence of  $\text{HClO}$  in DMSO- $d_6/\text{D}_2\text{O}$ , and (c) partial  $^1\text{H}$  NMR spectra of DNB- $\text{HSO}_3^-$  in the presence of  $\text{HClO}$  in DMSO- $d_6/\text{D}_2\text{O}$ .

#### 10. Absorption spectra of probe to $\text{HClO}$ and $\text{HSO}_3^-$ , respectively.

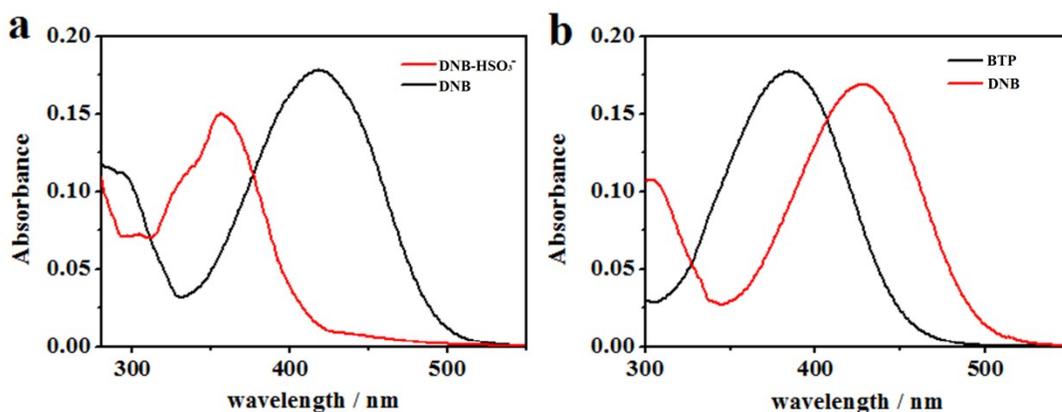


Fig. S4 (a) UV-vis of probe DNB ( $2\ \mu\text{M}$ ) upon addition of  $\text{HSO}_3^-$  (DMSO/PBS buffer solution (v:v=10/90, pH = 7.4, 20 mM)); (b) UV-vis of probe DNB ( $2\ \mu\text{M}$ ) upon addition of  $\text{HClO}$  (DMSO/PBS buffer solution (v:v=10/90, pH = 7.4, 20 mM));

#### 11. Time-dependence change of fluorescence intensity of probe to $\text{HSO}_3^-$ and $\text{HClO}$

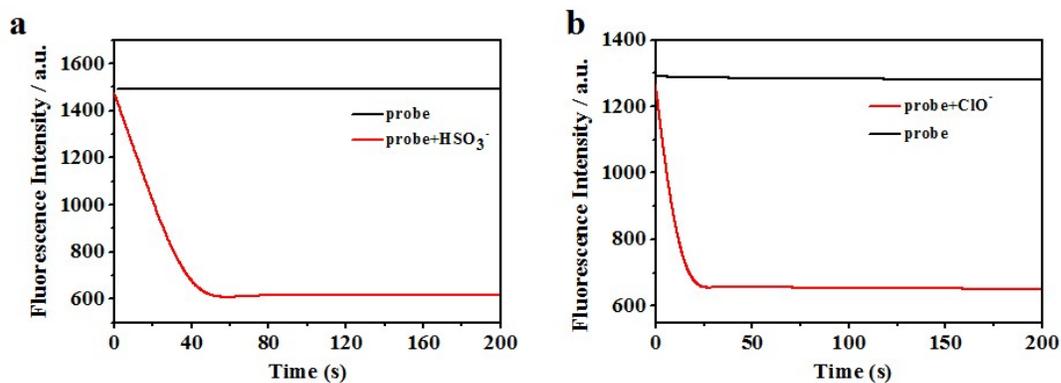


Fig. S5 (a) Time-dependent fluorescence intensity of probe (2 μM) in the presence of HSO<sub>3</sub><sup>-</sup> 15 μM (DMSO/PBS buffer solution (10/90, v: v, pH = 7.4, 20 mM) λ<sub>ex</sub>=460 nm); (b) Time-dependent fluorescence intensity of probe (2 μM) in the presence of HClO (15 μM) (DMSO/PBS buffer solution (10/90, v: v, pH = 7.4, 20 mM), λ<sub>ex</sub>= 420 nm).

## 12. Effect of pH Values to probe and probe derivatization.

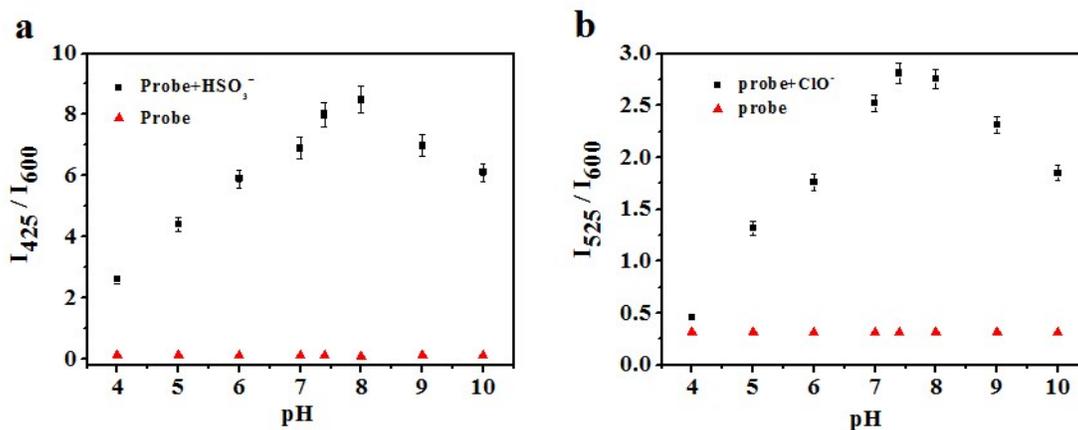


Fig. S6 (a) Fluorescence emission ratio (I<sub>425 nm</sub>/I<sub>600 nm</sub>) of DNB (2 μM) in the absence (▲) and presence (■) of sulfite (15 μM) under different pH conditions (DMSO/PBS buffer solution (10/90, v: v, pH = 7.4, 20 mM), λ<sub>ex</sub>=350 nm, λ<sub>ex</sub>=460 nm); (b) Fluorescence emission ratio (I<sub>525 nm</sub>/I<sub>600 nm</sub>) of DNB (2 μM) in the absence (▲) and presence (■) of sulfite (15 μM) under different pH conditions (DMSO/PBS buffer solution (50/50, v: v, pH =

7.4, 20 mM),  $\lambda_{ex}=420$  nm).

### 13. Temperature-dependence change of fluorescence intensity of probe to $\text{HSO}_3^-$ and $\text{HClO}$

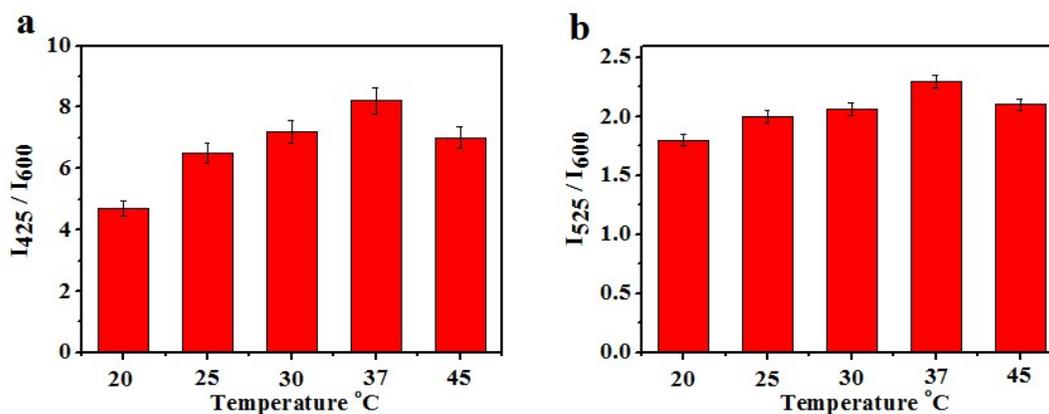


Fig. S7. (a) The effect of temperature on the fluorescence intensity ratio ( $I_{425\text{ nm}}/I_{600\text{ nm}}$ ) of the probe DNB (2  $\mu\text{M}$ ) to  $\text{HSO}_3^-$  (15  $\mu\text{M}$ ), (DMSO/PBS buffer solution (10/90, v/v, pH = 7.4, 20 mM),  $\lambda_{ex}=350$  nm,  $\lambda_{em}=460$  nm); (b) The effect of temperature on the fluorescence intensity ratio ( $I_{525\text{ nm}}/I_{600\text{ nm}}$ ) of the probe DNB (2  $\mu\text{M}$ ) to  $\text{HClO}$  (15  $\mu\text{M}$ ), (DMSO/PBS buffer solution (10/90, v/v, pH = 7.4, 20 mM),  $\lambda_{ex}=420$  nm).

### 14. MTT assay for probe.

To evaluate the cytotoxicity of probe DNB, HeLa cells and Raw 264.7 cells were planted into 96-well microtiter plates in DMEM or RPMI 1640 with 10% fetal bovine serum incubator containing 5%  $\text{CO}_2$  gas at 37°C for 24 h. Then the cells were incubated for 24 h at 37 °C in a 5%  $\text{CO}_2$ /95% air upon different concentrations probe of 0  $\mu\text{M}$  to 50  $\mu\text{M}$  respectively. Following, 25  $\mu\text{L}$  methylthiazolyl tetrazolium (MTT) (5 mg/mL) was added to each well and incubated for 4 h. The cytotoxicity tests were performed using MTT assays in compliance with ISO standard 10993-5. All experiments were performed in eight replicates. The cell viability was evaluated using a MTT assay; the results were expressed as mean values  $\pm$  standard deviation (S.D.).

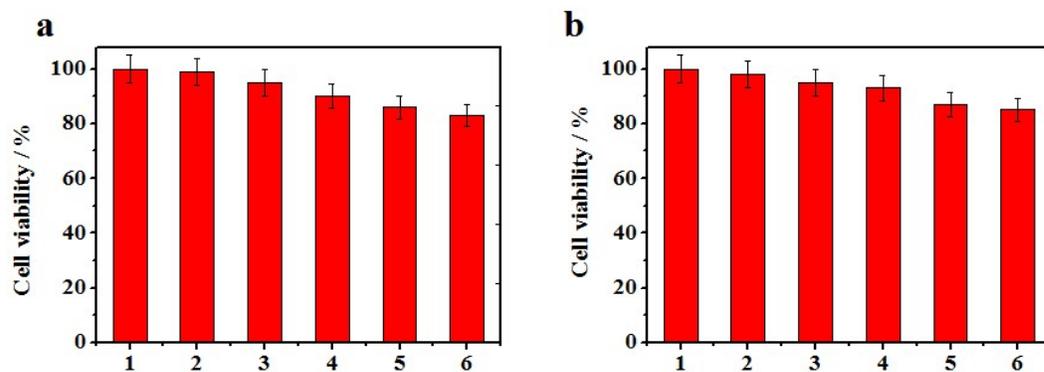


Fig. S8. (a) Cell viability of HeLa cells treated with different concentrations of probe DNB (1~6: 0, 5, 10, 20, 30 and 50  $\mu\text{M}$ ) for 24 h in fresh medium; (b) Cell viability of Raw 264.7 cells treated with different concentrations of probe (1~6: 0, 5, 10, 20, 30 and 50  $\mu\text{M}$ ) for 24 h in fresh medium.

### 15. $\text{SO}_2$ generation by BTSA in the presence of Cys.

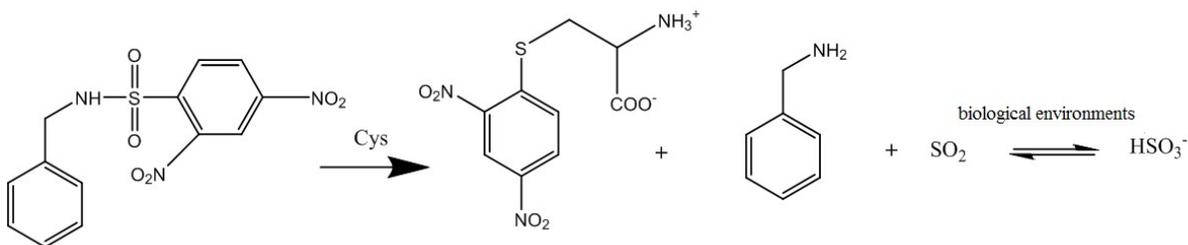
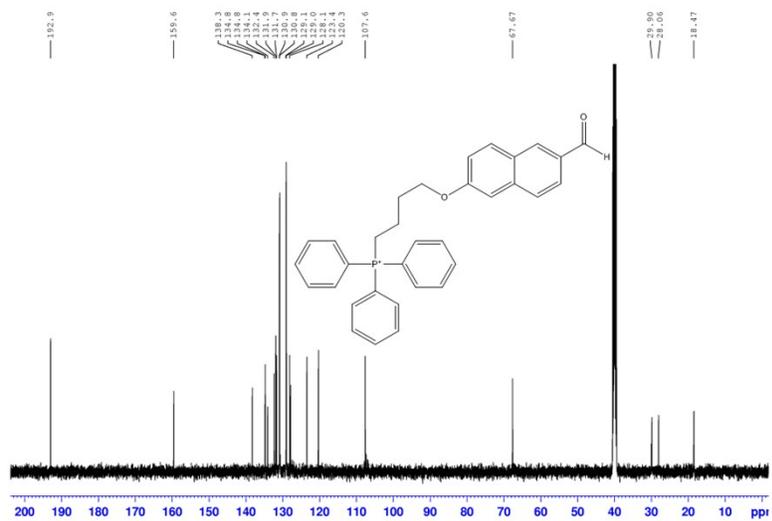
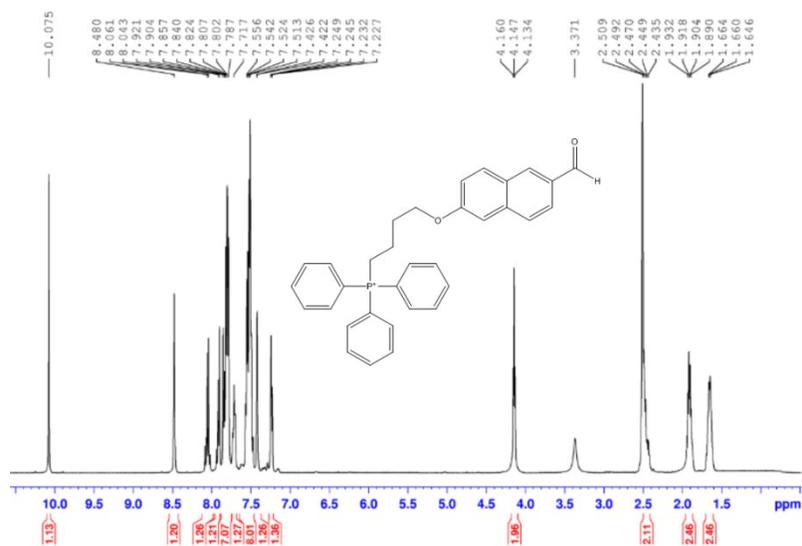


Fig. S9. Reaction process of  $\text{SO}_2$  donor with thiols.

### 16. $^1\text{H}$ NMR, $^{13}\text{C}$ NMR of BTP and $\text{SO}_2$ donor



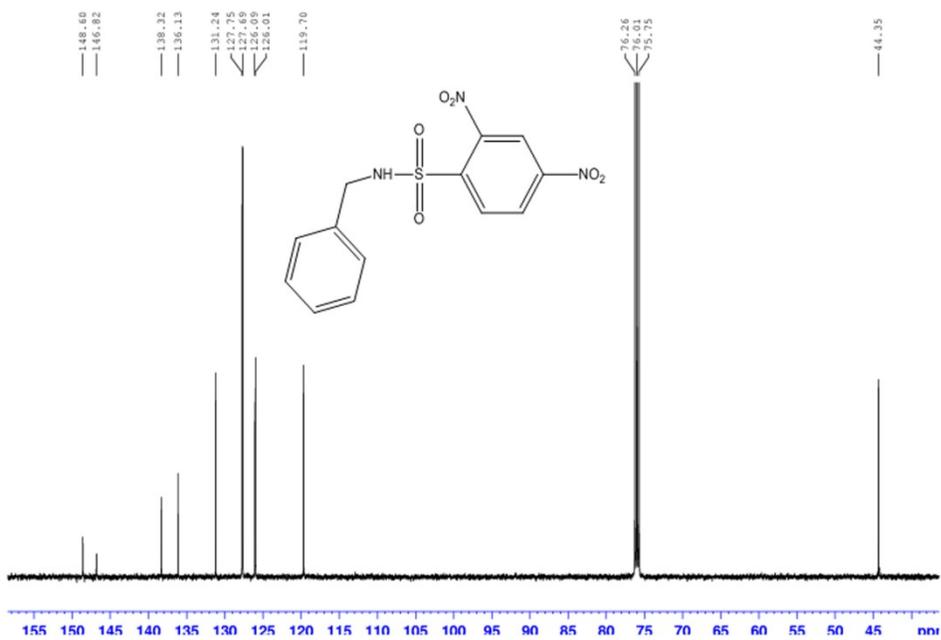
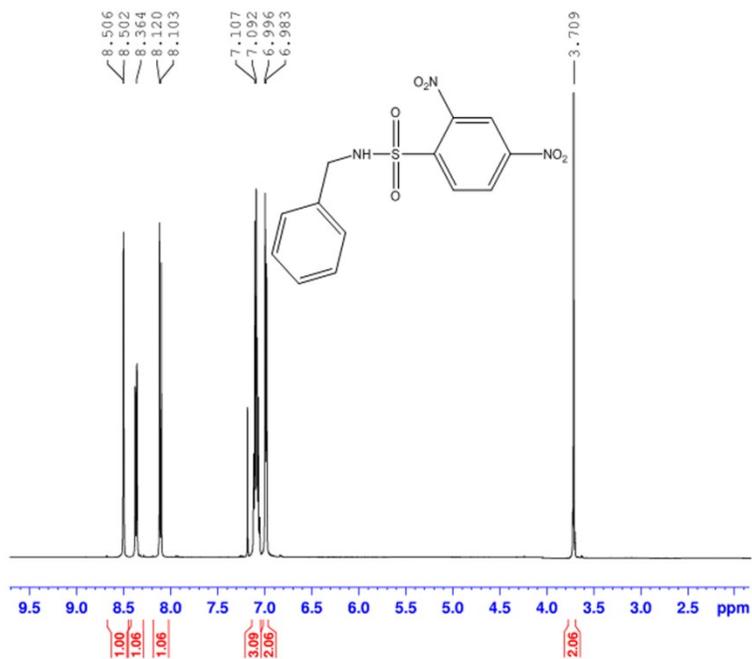


Fig. S10. <sup>1</sup>H NMR, <sup>13</sup>C NMR of BTP and SO<sub>2</sub> donor

### 17. $^1\text{H}$ NMR spectra of BTP in the presence of $\text{HSO}_3^-$

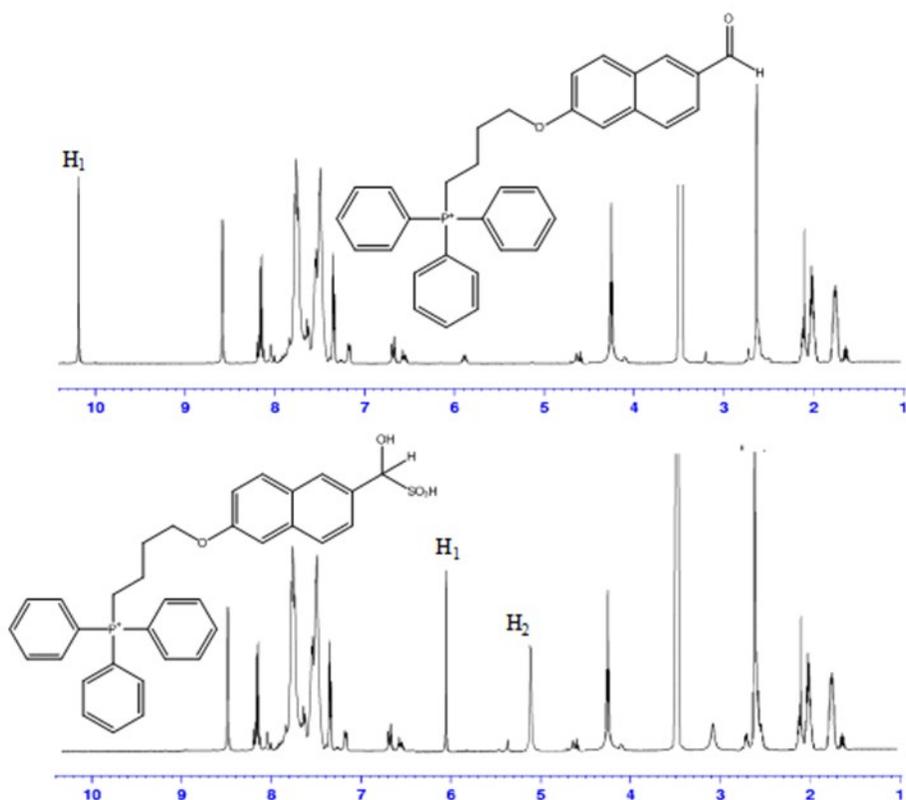


Fig. S11, partial  $^1\text{H}$  NMR spectra of BTP in the presence of  $\text{HSO}_3^-$  in  $\text{DMSO-d}_6/\text{D}_2\text{O}$

### 18. Investigate the transform from $\text{BTP-HSO}_3^-$ to BTP

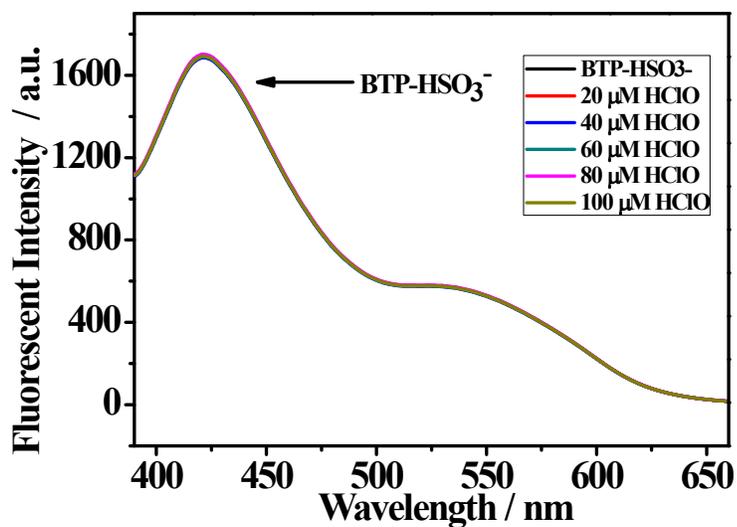


Fig.S12: Investigate the transform from  $\text{BTP-HSO}_3^-$  to BTP. Fluorescence response of  $\text{BTP-HSO}_3^-$  (2  $\mu\text{M}$ ) to various concentration of  $\text{HClO}$  from (0  $\mu\text{M}$  to 100  $\mu\text{M}$ ), ( $\lambda_{\text{exc}}$  = 380 nm).

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