Supporting Information for

A near-infrared ratiometric fluorescent probe based on C=N double bond for monitoring SO_2 and its application in biological imaging

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1. Materials and Instruments:

Common reagents or materials stated in this work were all obtained from commercial suppliers without further purification except as otherwise noted. The solvents were purified before using by standard methods. Ultrapure water was used throughout the analytical experiments. All reactions were performed under nitrogen protection and monitored by thin-layer chromatography (TLC). The products were purified by silica gel (200-300 mesh) column chromatography. TLC plates and silica gels were purchased form Qingdao Ocean chemicals. All the intermediates were analyzed by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectrometry (HRMS). The ^{1}H NMR and ^{13}C NMR were carried out on AVANCE III 400 MHz Digital NMR Spectrometers (Bruker Daltonics Corp, USA), using tetramethylsilane (TMS) as an internal reference. HRMS spectra were recorded on Bruker apex-Ultra mass spectrometer in electrospray ionization (ESI) mode (Bruker Daltonics Corp, USA). The absorption spectra were taken on a Shimadzu UV-2700 spectrophotometer (Shimadzu Suzhou instruments Mfg. Co, Ltd). The fluorescence spectra were taken on a Hitachi F4600 spectrofluorimeter (Hitachi High-Tech Science) with a 10 mm quartz cuvette. $NaHSO_3$ was used as the source of SO_2. The fluorescence imaging of cells was performed with Nikon A1MP confocal microscopy (Nikon instruments Inc.), and live-animal imaging experiments were performed with PerkinElmer IVIS Lumina Series III Pre-clinical (PerkinElmer Inc.) *in vivo* imaging system. The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter.

2. Synthesis of compounds

The compound 1-3 were synthesized according to the previously reported method [1].

Synthesis of compound 1:

2-methoxyphenothiazine (1.145 g, 5 mmol), 1-bromobutane (1.36 g, 10 mmol), NaOH (0.4 g, 10 mmol) and KI (12 mg, 0.072 mmol) were dissolved in anhydrous DMSO (10 mL).The resulting mixture was stirred at 95 ℃ for 6 h under an argon atmosphere. After cooling to room temperature, the reaction mixture was poured into

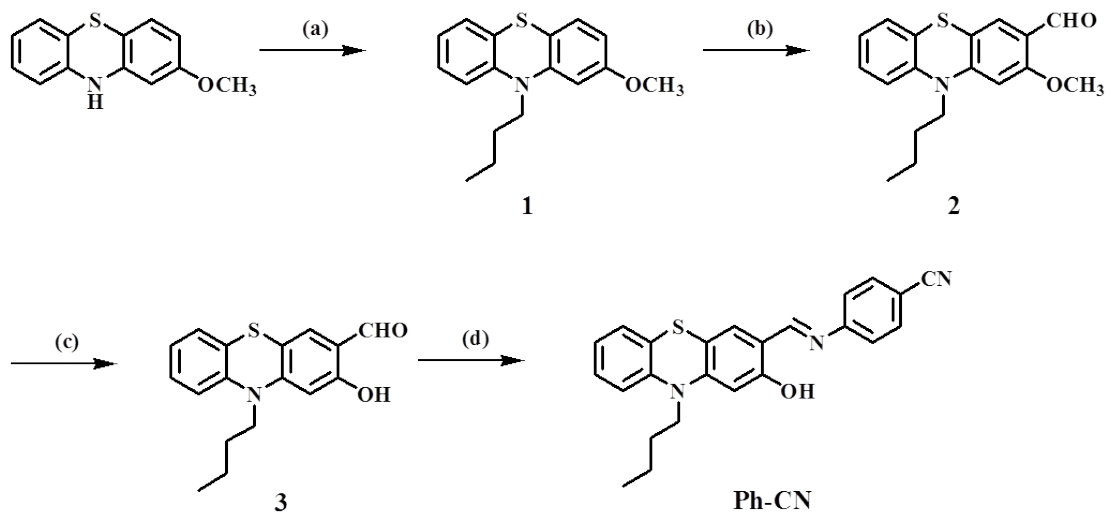
100 mL water and extracted with dichloromethane (3×100 mL). The organic layer was separated and washed successively with brine and water, dried over anhydrous Na_2SO_4 then vacuum evaporated, the crude product was further purified by silica gel flash chromatography (petroleum $ether_2$/CH_2Cl_2, 30:1) to afford colorless oil compound **1** (71 % yield).

Synthesis of compound 2:

Dimethylformamide (464 μL, 6 mmol) was added to phosphorous oxychloride (559 μL, 6 mmol) at 0 ℃ under argon atmosphere. The resulting mixture was allowed to stir at this temperature for 15 min. Then a portion of compound **1** (1.4 g, 2 mmol) (dissolved in 2 mL anhydrous DMF) was added to the cooled reagent with stirring. The mixture warmed to 60 ℃and stirred for 4 h, then poured into ice water (100 mL). The clear solution obtained was neutralized by $NaHCO_3$ solution (10 %). The resulting sticky mass was extracted with dichloromethane (3×100 mL). The organic layers were separated, combined and washed successively with brine and water, dried over anhydrous Na_2SO_4 and vacuum evaporated. The crude product was further purified by silica gel flash chromatography (petroleum $ether_2$/ethyl acetate, 15:1) to afford yellow powder (65 % yield).

Synthesis of compound 3:

Compound **2** (313 mg, 1 mmol), Aluminium chloride anhydrous (62.6 mg, 2 mmol) was added to anhydrous dichloromethane (5 mL) at 0 ℃ under argon atmosphere. The resulting mixture was allowed to stir at this temperature for 15 min then stirred at room temperature 6 h, poured into ice water (80 mL). The mixture was extracted with CH_2Cl_2 (3×60 mL). Combined CH_2Cl_2 extracts were washed with water, dried over anhydrous Na_2SO_4 and vacuum evaporated. The crude product was further purified by silica gel flash chromatography (petroleum $ether_2$/CH_2Cl_2, 5:1) to afford yellow oil (61 % yield).



Scheme S1. Synthesis of probe **Ph-CN**

3. Measurements

Sample preparation and spectrum measurement:

The stock solution of **Ph-CN** (1.0 mM) was prepared in DMSO. $NaHSO_3$ (10 mM) were dissolved in ultrapure water to prepare stock solution respectively. The multiple species stock solutions Hcy, GSH, NaCNS, Na_2S, glyoxal, Cys, KCl, $MgCl_2$, H_2O_2, NaF, $NaNO_2$, CH_3COONH_4, $Na_2S_2O_3$, KNO_3, $CaCl_2$, Na_2SO_3, benzaldehyde, Na_2SO_3, TBHB, KI, NaBr, NH_4PO_4, $NaHSO_3$ were prepared in distilled water at 10 mM. The test solution was obtained by placing the requisite amount of the probe **Ph-CN** solution (10 μM) in PBS buffer (10 mM, pH 7.4, 30 % CH_3CN) as final concentration. Unless otherwise stated, for all the records, the excitation wavelength is 375 nm.

Cytotoxic assay:

To evaluated the cytotoxicity of **Ph-CN**, the cell viability of **Ph-CN** was measured by standard MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The HeLa cells were seeded into a 96-well plate and cultured for 12 h. Then the cells were cultured with different concentrations (0, 1, 2, 5, 10, 20, 40 μM) of **Ph-CN** for another 24 h. 10 μL of MTT were added to each well and further incubated for 4 h. After aspirating the supernatant, 100 μL of DMSO were added to

each well. The plate was shaken for 10 min and the absorption at 375 nm was measured by a microplate reader.

The cell viability (%) = $(OD_s - OD_b) / (OD_c - OD_b) \times 100$ %. (1)

As it shown in the formula above, s, b and c represent the sample group, the blank group and the control group respectively.

Detection limit:

The detection limit was calculated based on a reported method [2]. According to the result of titrating experiment, the study suggested a plot of fluorescence intensity at ($I_{460\ nm}/I_{660\ nm}$) versus the concentrations of SO_2 exhibited good linearity (R^2 = 0.9949) in the range of 0-150 eq. Limit of detection is calculated from the mean of the blank, the standard deviation of the blank, and the corresponding linear regression equation. The theoretical detection limit was calculated to be 0.16 μM based on the following formula:

$$LOD=3\sigma/s$$

$$\sigma = \sqrt{\frac{\sum(\bar{x} - x_i)^2}{n-1}}$$

$\bar{x}$ is the mean of the blank measures, x_i is the values of blank measures; n is the rested number of blank measure; and s is the slope of the linear regression equation.

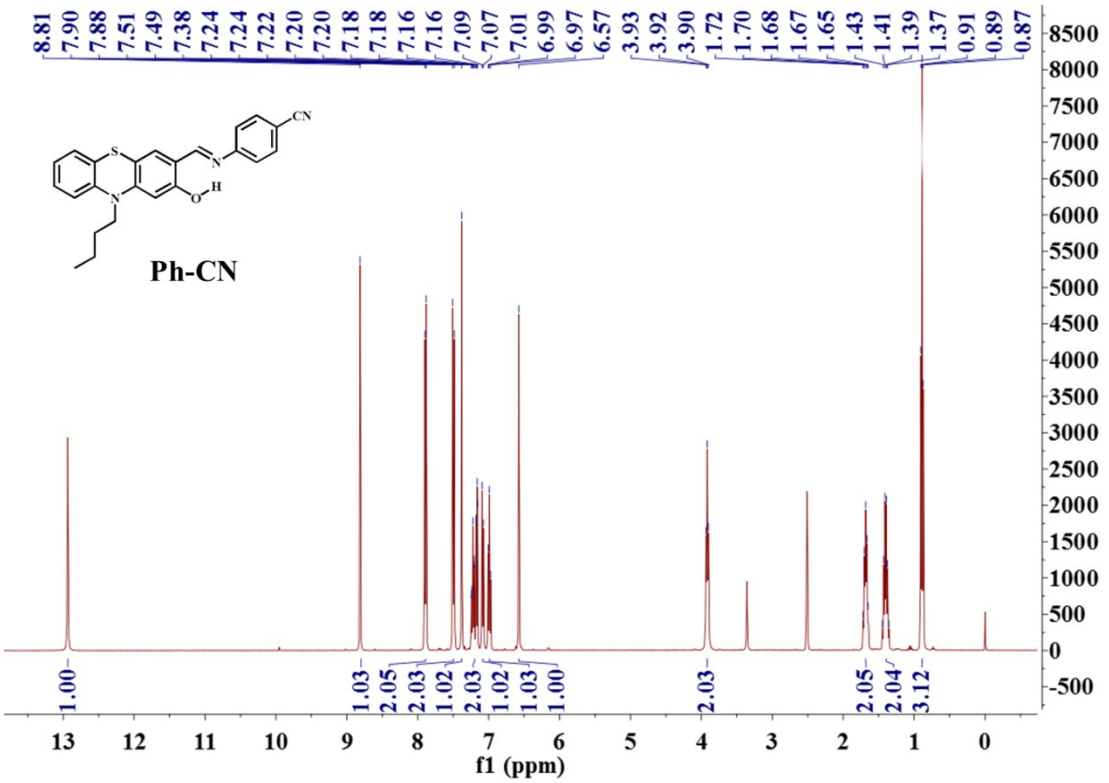


Fig. S1. ^{1}H NMR spectrum of the probe **Ph-CN** in d_6-DMSO.

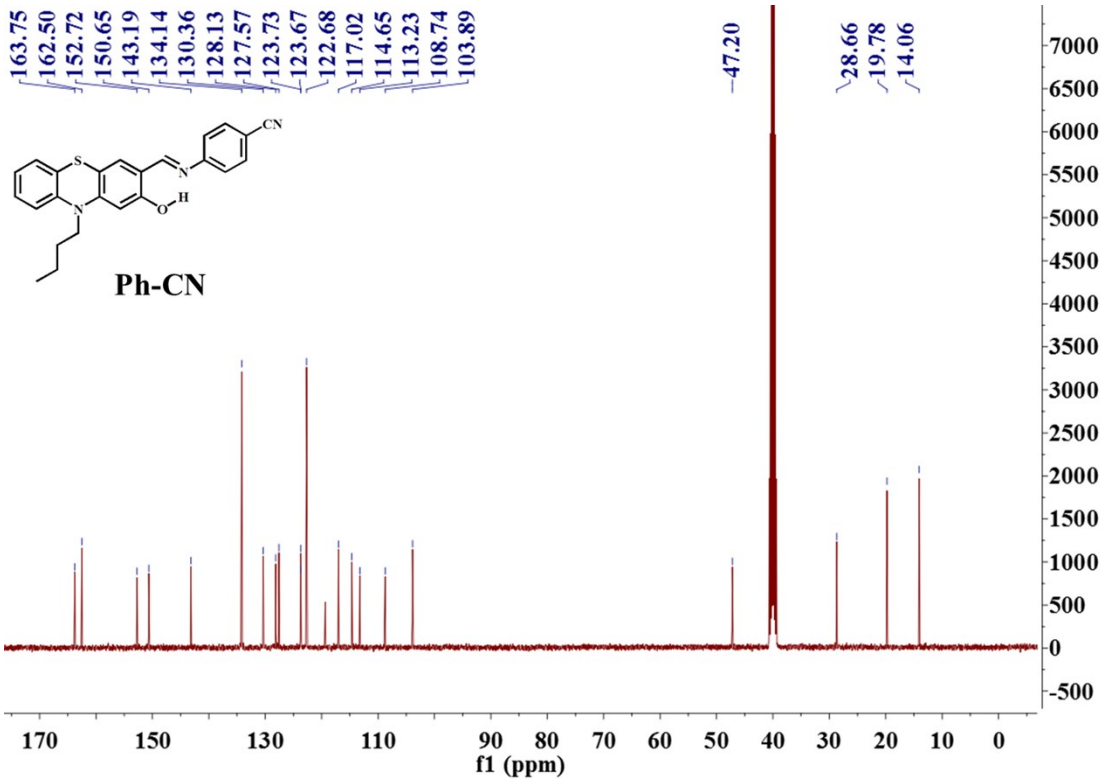


Fig. S2. ^{13}C NMR spectrum of the probe **Ph-CN** in d_6-DMSO**.**

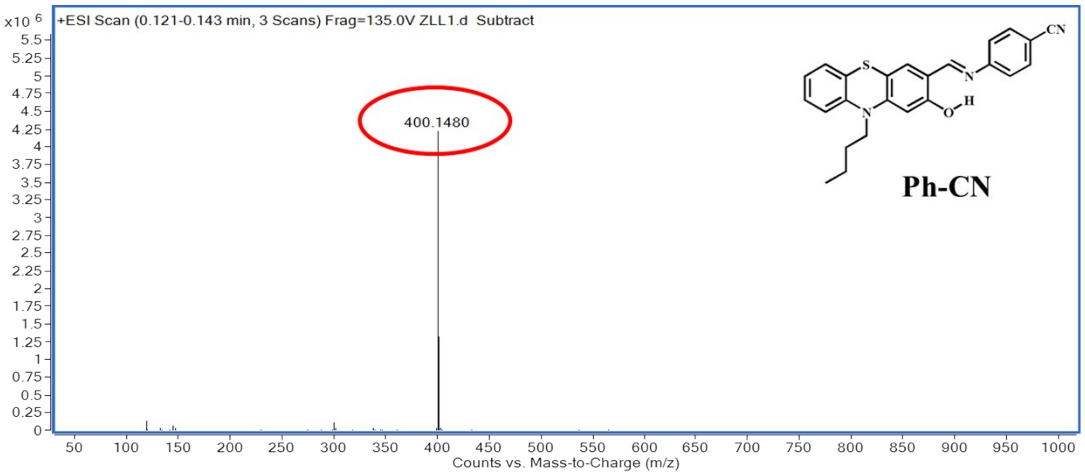


Fig.S3. HR-MS spectrum of the probe **Ph-CN**. HR-MS m/z: Calcd for $[C_{24}H_{20}N_3OS]^+$: 400.1484; Found 400.1480.

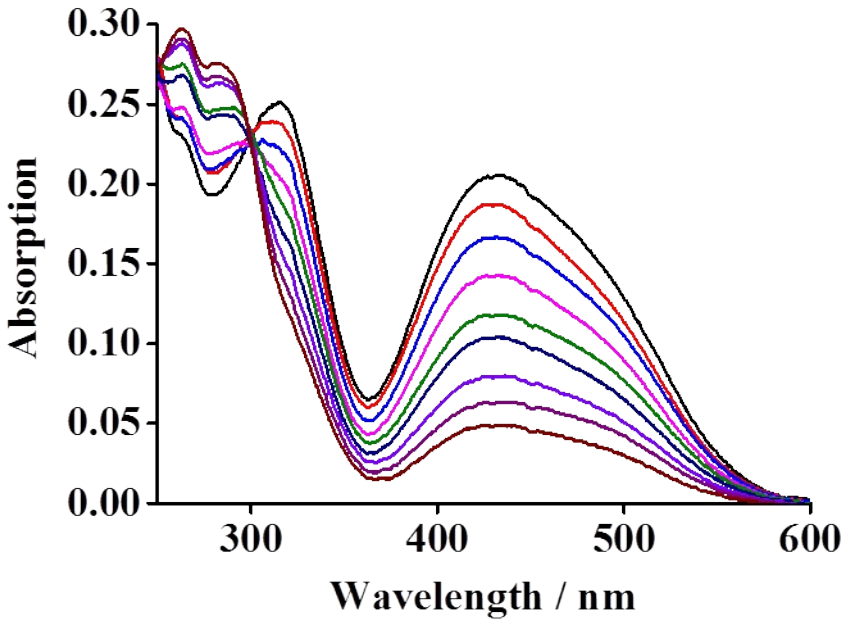


Fig. S4. Absorption spectra of probe **Ph-CN** (10 μM) treated with $NaHSO_3$ (0-150 eq) in PBS solutions (pH 7.4, 10 mM, 30 % CH_3CN).

Table S1. Comparing properties of probe **Ph-CN** with previous reported HSO_3^- probes.

	LOD	**C=N reaction site**	**Emission wavelength**	**Bioimaging application**

Org. Lett.12 (2010) 5624–5627	4.9×10^{-5} M	\	584 nm	\
Agric. Food Chem. 59 (2011) 11935–11939	5.8×10^{-5} M	\	553 nm	\
RSC Adv. 2 (2012) 10869–10873	5×10^{-6} M	\	373 nm	\
Sens. Actuators B 188 (2013) 1196–1200	1×10^{-5} M	\	520 nm	\
Anal. Sci. 30 (2014) 589–593	1.2×10^{-5} M	\	441 nm	\
Sens. Actuators B 231 (2016) 752–758	1.74×10^{-6} M	\	695 nm	Detecting SO_2 in cells
Chin. J. Chem. 28 (2010) 55–60	3.7×10^{-7} M	\	458 nm	\
Sens. Actuators B 184 (2013) 274–280	2×10^{-6} M	\	515 nm	Detecting SO_2 in cells
Polym. Chem. 4 (2013) 5416–5424	3.6×10^{-6} M	\	466 nm	\
J. Fluoresc. 27 (2017) 799–804	1.9×10^{-7} M	\	388 nm	\
J. Organomet. Chem. 781 (2015) 59–64	2.73×10^{-6} M	\	530 nm	\
Sens. Actuators B 243 (2017) 971–976	3.5×10^{-7} M	\	600 nm	Detecting SO_2 in cells and zebrafish
Org. Biomol. Chem. 13 (2015) 8663–8668	3.5×10^{-7} M	\	563 nm	Monitoring SO_2 in cells
Spectrochim. Acta A 149 (2015) 208–215	1.97×10^{-6} M	\	407 nm and 524 nm	\
J. Mater. Chem. B 4 (2016) 7888–7894	1.86×10^{-6} M	\	600 nm	Observing SO_2 in cells and tissues
Sens. Actuators B 152 (2011) 8–13	8.9×10^{-6} M	\	580 nm	\
Analyst 138 (2013) 3018–3025	5.8×10^{-6} M	\	580 nm	\
Anal. Chim. Acta 788 (2013)165–170	2×10^{-7} M	\	592 nm	\
New J. Chem. 42 (2018) 3063–3068	2.92×10^{-7} M	\	631 nm	Detecting SO_2 in cells
Talanta 162 (2017)	2.3×10^{-7} M	\	630 nm	Detecting SO_2 in

107–113				cells
Sens. Actuators B 272 (2018)195–202	7.7×10^{-7} M	\	530 nm	Detecting SO_2 in cells and zebrafish
Sens. Actuators B 247 (2017) 421–427	3.5×10^{-7} M	\	695 nm	Detecting SO_2 in cells
Sens. Actuators B 254 (2018) 709–718	1.61×10^{-7} M	\	600nm	Monitoring SO_2 in cells
RSC Adv. 6 (2016) 18662–18666,	3×10^{-5} M	\	628 nm	Sensing SO_2 in cells
Sens. Actuators B 268 (2018) 157–163	4.12×10^{-6} M	\	545 nm	Detecting SO_2 in cells and zebrafish
Sens. Actuators B 190 (2014) 792–799	0.56×10^{-6} M	Yes	530 nm	Visualizing SO_2 in cells
This work	1.6×10^{-7} M	Yes	660 nm	Monitoring SO_2 *in vivo*

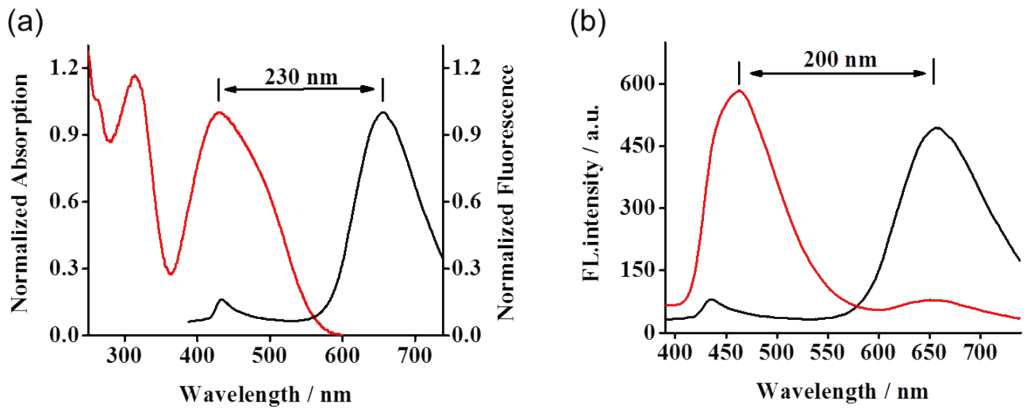


Fig. S5. The stoke shift and emission peak spacing of probe **Ph-CN.**

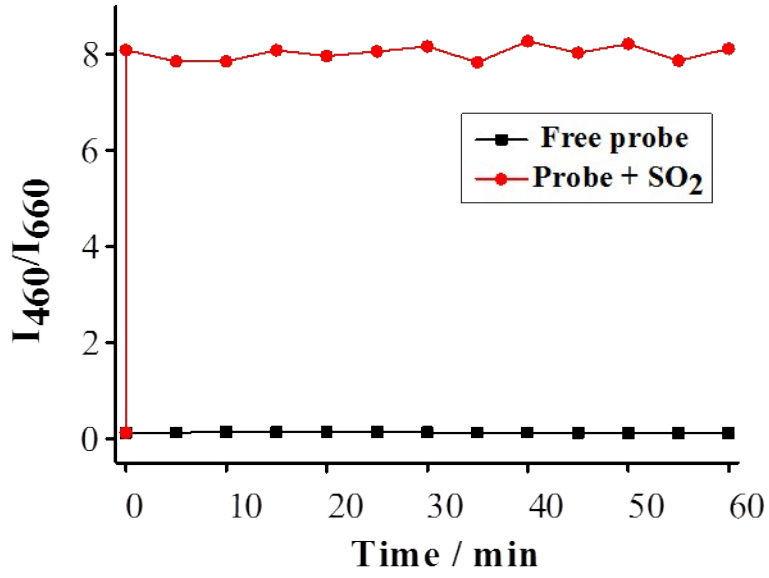


Fig. S6. Photostability profiles of fluorescent ratios (I_{460}/I_{660}) of the probe **Ph-CN** in the absence or presence of $NaHSO_3$ under continues irradiation (375 nm).

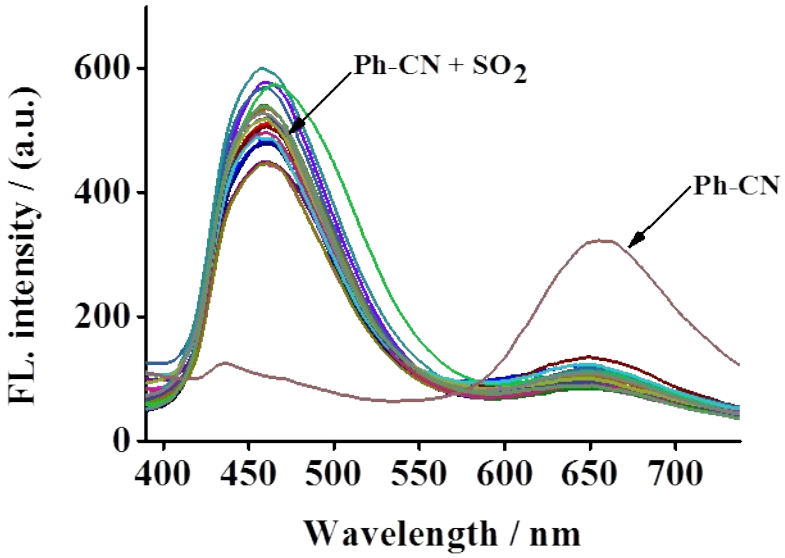


Fig. S7. The interference study of **Ph-CN** towards $NaHSO_3$ and other analytes (2 mM). The reactions of **Ph-CN** (10 μM) with : 1, Hcy; 2, GSH; 3, SCN^-; 4, S^{2-}; 5, glyoxal; 6, Cys; 7, K^+; 8, Mg^{2+}; 9, H_2O_2; 10, F^-; 11, NO_2^-; 12, AcO^-; 13, $S_2O_3^{2-}$; 14, NO_3^-; 15, Ca^{2+}; 16, benzaldehyde; 17, SO_4^{2-}; 18, TBHB; 19, I^-; 20, Br^-; 21, PO_4^{3-}; 22, blank; 23, HSO_3^- in PBS solutions (pH 7.4, 10 mM, 30 % CH_3CN). λ_{ex} = 375 nm.

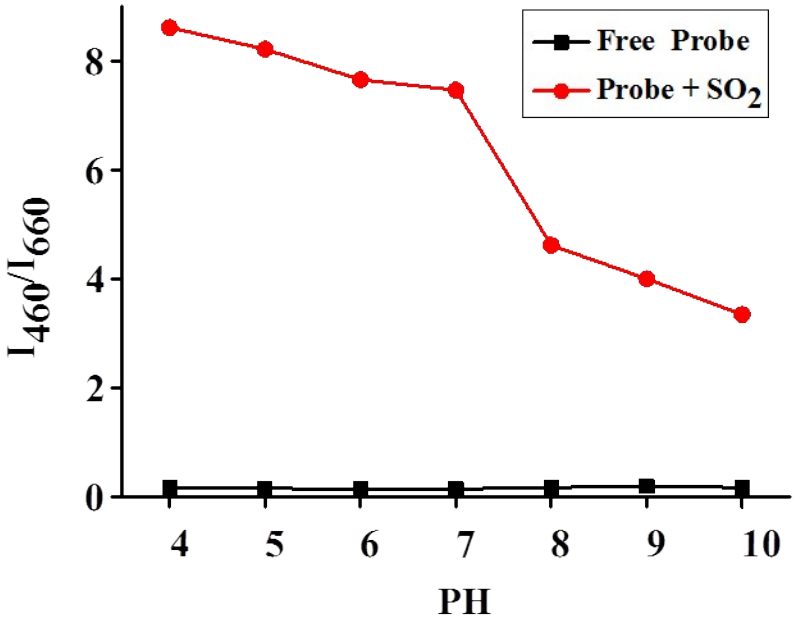


Fig. S8. pH dependence of probe **Ph-CN** (10 μM) in the absence or presence of $NaHSO_3$ (150 eq) in different pH (range from 4.0 to 10.0). λ_{ex} = 375 nm.

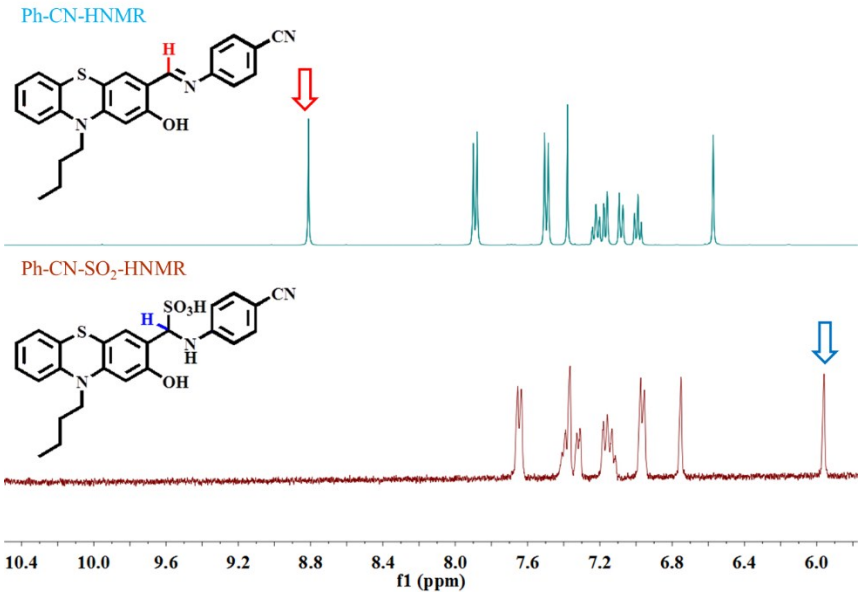


Fig. S9. The mechanism study by ^{1}H NMR at the range of 5 to 10 ppm. The ^{1}H NMR of probe **Ph-CN** and **Ph-CN-SO$_2$** is measured in DMSO-d_6 and CD_3CN 30% (CD_3CN/ D_2O= V_1 / V_2=3/ 7) respectively.

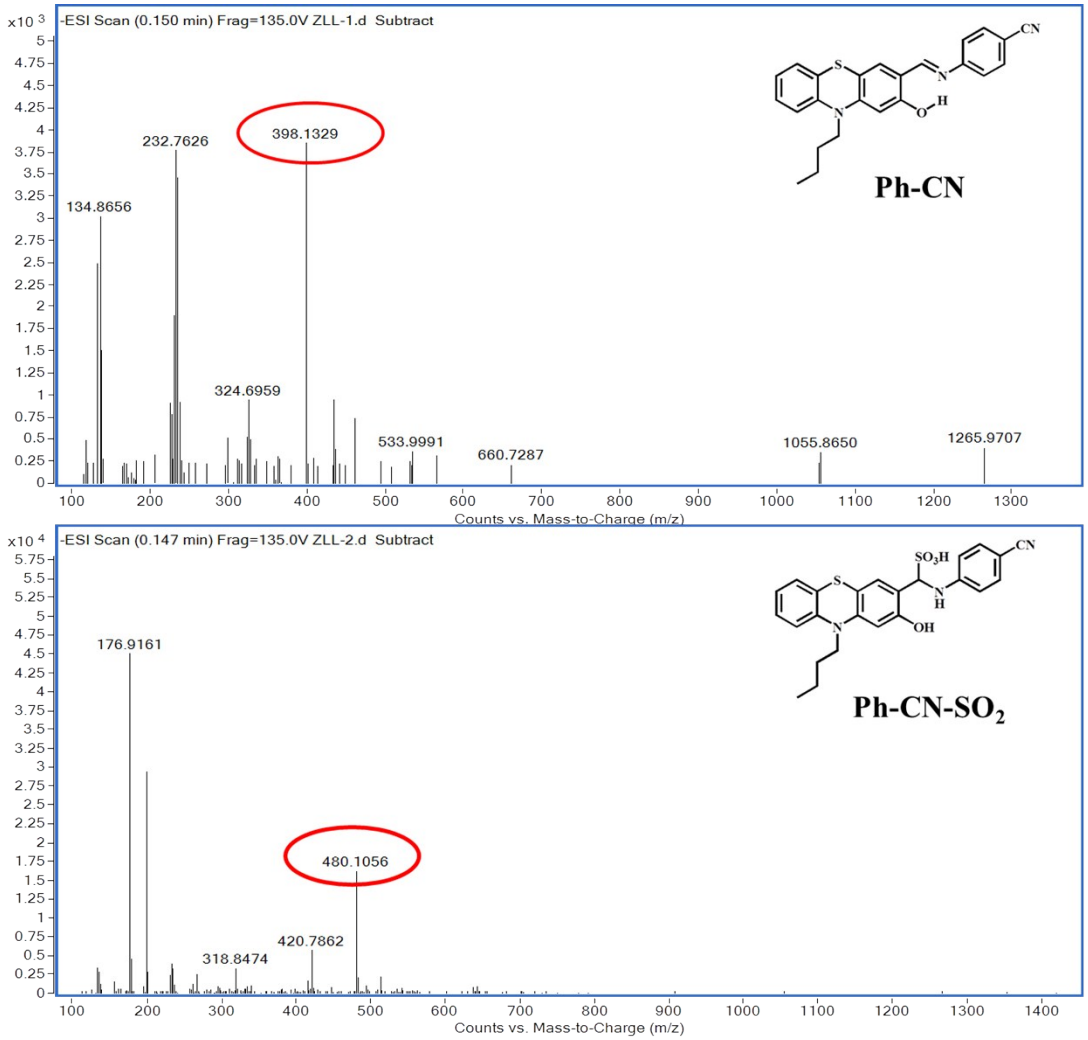


Fig. S10. HR-MS spectrum of reaction products of **Ph-CN** and SO_2 in CH_3CN (HR-MS m/z of **Ph-CN**: Calcd for $[C_{24}H_{20}N_3OS]^-$: 398.1327; Found 398.1329. m/z of **Ph-CN-SO$_2$**: Calcd for $[C_{24}H_{22}N_3O_4S_2]^-$: 480.1057; Found 480.1056).

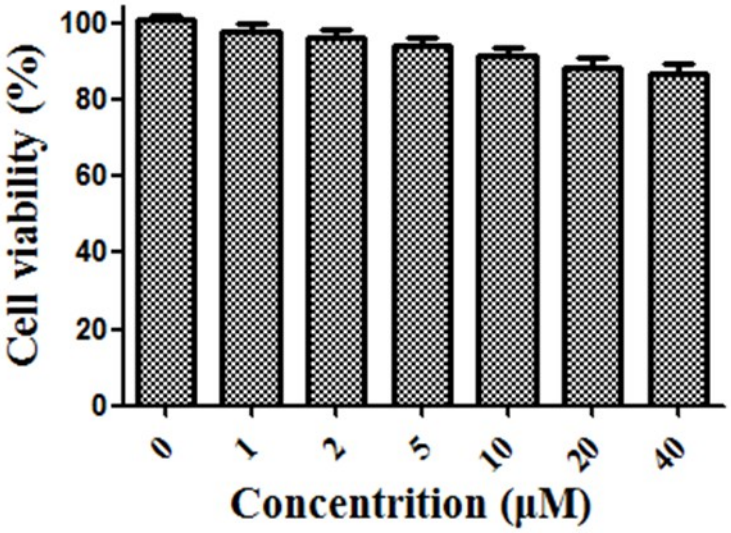


Fig. S11. Viability of HeLa cells treated with various concentrations (0-40 μM) of **Ph-CN** for 24 h. Error bars represent mean values ± SD (n = 3).

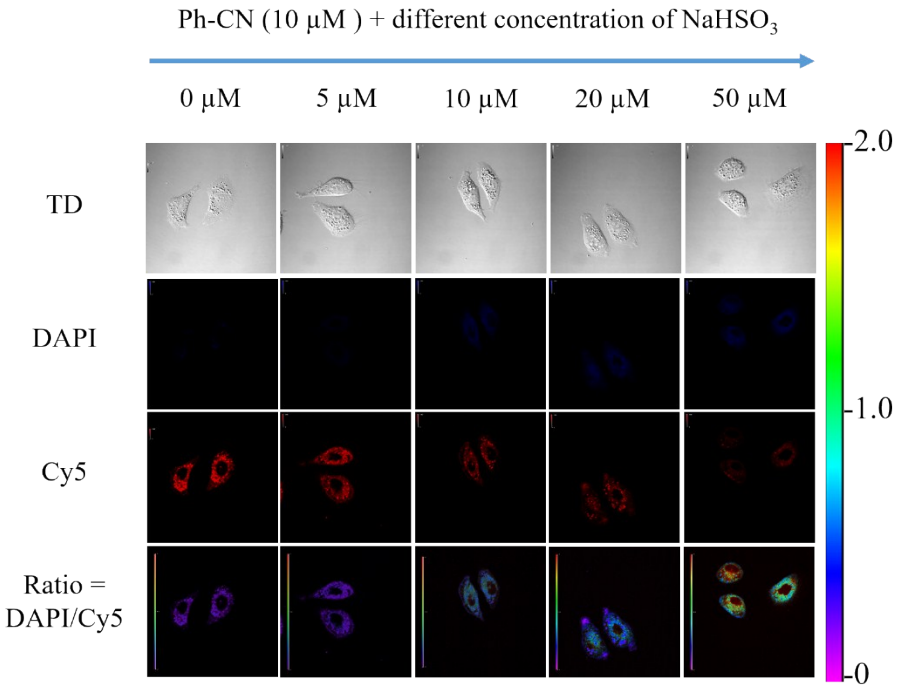


Fig. S12. Confocal images of HeLa cells stained with 10 μM **Ph-CN** and the increasing concertation of $NaHSO_3$ (5 μM, 10 μM, 20 μM and 50 μM). Channel of DAPI, λ_{ex} = 405 nm, λ_{em} = 425−475 nm. Channel of Cy5, λ_{ex} = 405 nm, λ_{em} = 663−738 nm. The fluorescence ratio is the ratio of DAPI and Cy5 channel.

Supplementary Reference

[1] W. Chen, L. Zhu, Y. Hao, X. Yue, J. Gai, Q. Xiao, et al., Detection of thiophenol in buffer, in serum, on filter paper strip, and in living cells using a red-emitting amino phenothiazine boranil based fluorescent probe with a large Stokes shift, Tetrahedron, 73 (2017) 4529-4537.

[2] M. Shortreed, R. Kopelman, M. Kuhn, B. Hoyland, Fluorescent Fiber-Optic Calcium Sensor for Physiological Measurements, Analytical Chemistry, 68 (1996) 1414-1418.