# Electronic Supplementary Information for:

# Reactive oxygen species-triggered off-on fluorescence donor for imaging hydrogen sulfide delivery in living cells

Yiming Hu,<sup>ab</sup> Xiaoyi Li,<sup>a</sup> Yu Fang,<sup>a</sup> Wen Shi,<sup>ab</sup> Xiaohua Li,<sup>a</sup> Wei Chen,<sup>c</sup> Ming Xian<sup>c</sup> and Huimin Ma\*ab

<sup>a</sup> Beijing National Laboratory for Molecular Sciences, Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China.

E-mail: mahm@iccas.ac.cn

<sup>b</sup> University of Chinese Academy of Sciences, Beijing 100049, China.

<sup>c</sup> Department of Chemistry, Washington State University, Pullman, Washington 99164, United States.

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### 1. Apparatus and Reagents

High resolution electrospray ionization mass spectra (HR-ESI-MS) were performed on Bruker SolariX 9.4T FT-MS instrument. Electrospray ionization mass spectra (ESI-MS) were recorded on Shimadzu LC-MS 2010 instrument. High resolution electron impact ionization mass spectra (HR-EI-MS) were collected on Waters GCT instrument. <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained through Bruker Fourier 300 or Bruker Avance III 400 HD spectrometers, and the data were reported as ppm. UV-Vis absorption spectra were recorded on Shimadzu UV-2600 spectrophotometer in 1-cm quartz cells. Fluorescence spectra were measured on Hitachi F-4600 spectrophotometer in 1×1 cm quartz cells with a PMT voltage at 700 V; the excitation and emission slit widths were set at 10 nm for collection. MTT analysis were made on Molecular Devices SpectraMax i3 microplate reader. Fluorescence imaging was performed on an FV 1200-IX83 confocal laser scanning microscope and processed by the Olympus software FV10-ASW. The pH measurements were determined by Mettler Toledo FE28 pH meter.

Dimethyl sulfoxide, N-Acetyl-L-cysteine (NAC), phorbol-12-myristate-13-acetate (PMA), CA from bovine erythrocytes, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and tert-butyl hydroperoxide (TBHP) were purchased from Sigma-Aldrich. Sodium hydride, triethylamine, tin chloride and lithium chloride were obtained from Acros. 3-Nitro-1,8-naphthalic anhydride was purchased from Aladdin. 4-Hydroxymethylphenylboronic acid pinacol ester, N,N-dimethyl-*p*-phenylenediamine dihydrochloride, 1,1'-thiocarbonyldiimidazole (TCDI), and n-butylamine were obtained from Alfa Aesar. CellROX Deep Red Reagent (CRDR, ROS probe) and fetal bovine serum (FBS) was purchased from Thermo Fisher. Cell lines (HeLa and RAW264.7), Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute-1640 medium (RPMI-1640) were obtained from KeyGEN BioTECH Co., LTD, Nanjing, China. New born calf serum was purchased from Zhejiang Tianhang Biological Technology Co., Ltd. WSP-1 (H<sub>2</sub>S probe) was obtained from Shanghai Maokang Biotechology Co., Ltd. The stock solution (0.5 mM) of NAB was prepared in DMSO and stored in small aliquots at -20 °C to avoid frequently freezing and thawing. Ultrapure water with resistivity over 18 M $\Omega$ ·cm (Millipore) was used throughout the experiments.

#### 2. Synthesis of Fluorescent H<sub>2</sub>S Donor (NAB)

NAB was prepared according to the procedures illustrated in Scheme S1 in the Electronic Supplementary Information, which is briefly described below.



Scheme S1. Synthetic procedure of fluorescent H<sub>2</sub>S donor NAB.

Compound **1** was synthesized according to the previously reported route with some modifications (Ao et al, *Org. Biomol. Chem.* **2017**, *15*, 6104). 3-Nitro-1,8-naphthalic anhydride (1.2 g, 5.0 mmol) and n-butylamine (1 ml, 10 mmol) were dissolved in EtOH (80 mL). The mixture was heated to reflux overnight, and then cooled to room temperature. The solvent was removed under reduced pressure and the obtained residue was further subjected to silica gel column chromatography eluting with CH<sub>2</sub>Cl<sub>2</sub>, affording compound **1** as a white solid (1.4 g, 94% yield). Next, SnCl<sub>2</sub> (5.4 g, 28 mmol) was dissolved in the concentrated HCl (11 mL) and added dropwise to a solution of compound **1** (1.4 g, 4.7 mmol) in CH<sub>3</sub>OH (50 mL). The resulting mixture was stirred at room temperature for 2 h. After the mixture was basified with NaOH to pH 8-9 and the product was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic phase was collected, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and subjected to evaporation. The crude product was purified by silica gel chromatography with CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (v/v, 250:1) as eluent, affording NAH as a yellow solid (0.90 g, yield 71%).

NAH (0.80 g, 3 mmol) and triethylamine (2.5 mL, 18 mmol) were dissolved in anhydrous DMF (10.5 mL) under vigorous stirring, and then TCDI (1.1 g, 6 mmol) in anhydrous DMF (20 mL) was added dropwise to the aforementioned solution under nitrogen atmosphere. The reaction was allowed to stir for 24 h at room temperature and finally quenched with water. The mixture was extracted by CH<sub>2</sub>Cl<sub>2</sub> and washed with LiCl (5%, 35 mL) for three times. The organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated under reduced pressure. The residue was recrystallized from CH<sub>2</sub>Cl<sub>2</sub> and **NANCS** was obtained as a yellow crystal (0.62 g, yield 67%). <sup>1</sup>H and <sup>13</sup>C NMR spectra are shown in Figures S1 and S2. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.56 (d, *J* = 7.2 Hz, 1H), 8.35 (d, *J* = 5.4 Hz, 1H), 8.12 (d, *J* = 8.3 Hz, 1H), 7.96 (s, 1H), 7.78 (t, *J* = 7.8 Hz, 1H), 4.15 (t, *J* = 7.5 Hz, 2H), 1.74 – 1.64 (m, 2H), 1.50 – 1.37 (m, 2H), 0.97 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C

NMR (75 MHz, 298 K, CDCl<sub>3</sub>)  $\delta$  163.55, 162.93, 139.07, 133.06, 132.09, 131.48, 130.86, 128.58, 128.44, 128.28, 126.34, 124.64, 122.91, 40.45, 30.15, 20.34, 13.82. HR-EI-MS: m/z calcd. for [C<sub>17</sub>H<sub>14</sub>N<sub>2</sub>O<sub>2</sub>S]<sup>+</sup>, 310.0776; found, 310.0780 (Figure S3).



Figure S1. <sup>1</sup>H NMR spectrum of NANCS (300 MHz, CDCl<sub>3</sub>, 298 K).



Figure S2. <sup>13</sup>C NMR spectrum of NANCS (75 MHz, CDCl<sub>3</sub>, 298 K).



Figure S3. HR-EI-TOF MS of NANCS.

**NANCS** (0.62 g, 2 mmol) and 4-hydroxymethylphenylboronic acid pinacol ester (0.47 g, 2 mmol) were dissolved in ice-cold anhydrous THF (30 mL), followed by the addition of sodium hydride (60% in mineral oil, 100 mg, 2.5 mmol). The mixture was stirred at 0 °C for 20 min, and then removed from ice bath, followed by stirring for another 4 h at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by preparatory TLC (PE/EtOAc, v/v, 20:1), affording **NAB** as pale yellow solid (0.12 g, 11%). <sup>1</sup>H and <sup>13</sup>C NMR spectra are shown in Figures S4 and S5. <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>) δ 11.80 (s, 1H), 8.74 (s, 1H), 8.56 (d, *J* = 10.2 Hz, 1H), 8.40 (d, *J* = 7.2 Hz, 1H), 8.32 (d, *J* = 7.2 Hz, 1H), 7.83 (t, *J* = 7.6 Hz, 1H), 7.72 (d, *J* = 6.9 Hz, 2H), 7.51 (d, *J* = 7.8 Hz, 2H), 5.65 (s, 2H), 4.04 (t, *J* = 7.2 Hz, 2H), 1.64 – 1.59 (m, 2H), 1.39-1.34 (m, 2H), 1.30 (s, 12H), 0.93 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, 298 K, CDCl<sub>3</sub>) δ 188.19, 163.97, 163.53, 140.64, 137.75, 135.16, 133.54, 132.13, 130.60, 129.70, 128.26, 127.75, 127.57, 127.46, 126.08, 123.75, 122.63, 83.97, 67.41, 40.39, 30.20, 24.88, 20.37, 13.85. HR-ESI-MS: m/z calcd. for (C<sub>30</sub>H<sub>32</sub>BN<sub>2</sub>O<sub>5</sub>S<sup>-</sup>, [M-H]<sup>-</sup>), 543.2130; found, 543.2134 (Figure S6).



Figure S4. <sup>1</sup>H NMR spectrum of NAB (300 MHz, DMSO-d<sub>6</sub>, 298 K).



Figure S5. <sup>13</sup>C NMR spectrum of NAB (100 MHz, CDCl<sub>3</sub>, 298 K).



Figure S6. HR-ESI-MS of NAB.

### 3. Preparation of Reactive Oxygen Species

ClO<sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, NO, ONOO<sup>-</sup>, <sup>1</sup>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, TBHP and TBO• were generated according to the previous report (Li et al, *Anal. Chem.* **2017**, *89*, 5519). •OH was generated from the reaction between Cu<sup>2+</sup> and ascorbate (Manevich et al, *Radiat. Res.* **1997**, *148*, 580).

# 4. General Procedure for Spectral Measurement

Unless otherwise noted, all the spectral measurements were carried out in 20 mM phosphate buffer (pH 7.4) according to the following procedure in triplicate. In a 2-mL test tube, 100  $\mu$ L of the phosphate buffer (200 mM, pH 7.4) was added, followed by mixing 20  $\mu$ L of 0.5 mM NAB stock solution and appropriate volume of H<sub>2</sub>O<sub>2</sub> solution. For fluorescence measurements, the final volume was adjusted to 1 mL by ultrapure water and incubated at 37 °C. At predetermined time points, the solution was transferred to the quartz cell for spectra scan with the excitation wavelength of 405 nm. For absorbance measurements, 3 mL of the reaction solution was prepared as above. The blank solution without H<sub>2</sub>O<sub>2</sub> was used for comparison.

## 5. Methylene Blue (MB) Colorimetric Assay of H<sub>2</sub>S

To a solution of the phosphate buffer (20 mM, pH 7.4) containing CA (100  $\mu$ g/mL), appropriate H<sub>2</sub>O<sub>2</sub> and NAB were added. The mixture were diluted with water to 2 mL for desirable

concentration. The vials were placed in thermostat at 37 °C for 7 h, and 1.5 mL of the reaction aliquots were taken out to mix with 1.5 mL of a freshly prepared solution (referred to the MB solution), which contained 300  $\mu$ L of Zn(OAc)<sub>2</sub> (1%, w/v), 600  $\mu$ L of FeCl<sub>3</sub> (30 mM in 1.2 M HCl) and 600  $\mu$ L of N,N-dimethyl-*p*-phenylenediamine dihydrochloride (20 mM in 7.2 M HCl). After reaction for 30 min, the absorbance of the mixture was determined at 670 nm against a blank: 1.5 mL of the MB solution and 1.5 mL of the phosphate buffer (20 mM, pH 7.4) including NAB (10  $\mu$ M) and CA (100  $\mu$ g/mL). H<sub>2</sub>S concentration in each sample was calculated against the H<sub>2</sub>S calibration curve made by measuring a series of Na<sub>2</sub>S solutions under the same condition against a blank: 1.5 mL MB solution and 1.5 mL phosphate buffer (20 mM, pH 7.4).

## 6. Cytotoxicity Assay

The cytotoxicity of NAB to HeLa and RAW264.7 cells was investigated by standard MTT assay according to the previous report (Li et al, *Anal. Chem.* **2017**, *89*, 5519). The cytotoxicity of PMA to RAW264.7 cells was also examined. In brief, RAW264.7 cells were seeded in 96-well plates at a density of 7000 cells/well and incubated with PMA in serum-free growth media at varied concentrations. After that, the plate was incubated at 37 °C for 1 h and the culture media were removed subsequently. Then, 100  $\mu$ L of the MTT solution (0.5 mg/mL in RPMI-1640) was added to each well and the cells were grown for another 4 h in a humidified 5% CO<sub>2</sub> incubator. After discarding the supernatant, 100  $\mu$ L of DMSO was added to each well for dissolving the formed formazan. The absorbance at 490 nm was determined within 10 min.

# 7. Cell Culture and Fluorescence Imaging

HeLa and RAW264.7 cells were cultured as described before (Li et al, *Anal. Chem.* **2017**, *89*, 5519). For fluorescence imaging, HeLa cells were incubated with NAB (10  $\mu$ M) and exogenous H<sub>2</sub>O<sub>2</sub> in DMEM at 37 °C, and then washed by DMEM. RAW264.7 cells were first treated with PMA (1  $\mu$ g/mL) for 1 h to induce phagocytosis-associated endogenous H<sub>2</sub>O<sub>2</sub> generation (Srikun et al, *J. Am. Chem. Soc.* **2008**, *130*, 4596). Then the cells were washed with RPMI-1640, followed by incubation with NAB (10  $\mu$ M). The cell imaging was conducted with the excitation wavelength of 405 nm and the fluorescence emission was collected between 550–650 nm through a 100× 1.4 NA objective.

To explore the H<sub>2</sub>S release capacity of NAB, fluorescent probe WSP-1 was utilized to image H<sub>2</sub>S in HeLa cells (Li et al, *PLoS One* **2014**, *9*, e90340). After incubation with NAB and H<sub>2</sub>O<sub>2</sub>, the cells were washed with DMEM and loaded with WSP-1 (15  $\mu$ M) for 45 min. The cell imaging was carried out with the excitation wavelength of 488 nm and the fluorescence emission was collected between 510–540 nm through a 100× 1.4 NA objective. The pixel intensity of living cells in each fluorescence image was counted for at least 5 cells.

To investigate the ROS change after the treatments of PMA and NAB, CRDR was utilized to image ROS in RAW264.7 cells. After incubation with PMA and/or NAB, the cells were washed with RPMI-1640 and loaded with CRDR (5  $\mu$ M) for 30 min. The cell imaging was carried out with the excitation wavelength of 635 nm, and the fluorescence emission was collected between 660–760 nm through a 100× 1.4 NA objective. The pixel intensity of living cells in each fluorescence image was counted for at least 5 cells.

#### 8. Statistical Tests

The t analysis was made according to our previous method (Gong et al, Chem. Sci. 2016, 7, 788).



#### 9. Other Supplementary Figures

**Figure S7.** (A) Absorption and (B) fluorescence spectra of 10  $\mu$ M NAB reacting with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> at 37 °C for (a) 0 h and (b) 7 h; (c) the corresponding spectra of 10  $\mu$ M NAH in the same media of 20 mM phosphate buffer (pH 7.4).  $\lambda_{ex} = 405$  nm.



Figure S8. ESI-MS of the reaction solution of NAB with H<sub>2</sub>O<sub>2</sub>.



**Figure S9.** Effects of (A) pH at 37 °C and (B) temperature at pH 7.4 on the fluorescence of 10  $\mu$ M NAH (**■**) and 10  $\mu$ M NAB reacting with (**●**) 0 and (**▲**) 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 2 h.  $\lambda_{ex/em} = 405/577$  nm.



**Figure S10.** Fluorescence changes of NAB (10  $\mu$ M) in the presence of different species: (1) blank; (2) KCl (150 mM); (3) MgCl<sub>2</sub> (2.5 mM); (4) CaCl<sub>2</sub> (500  $\mu$ M); (5) ZnCl<sub>2</sub> (100  $\mu$ M); (6) glucose (10 mM); (7) vitamin C (1 mM); (8) glutamic acid (1 mM); (9) glutathione (5 mM); (10) cysteine (5 mM); (11) glycine (1 mM); (12) alanine (1 mM); (13) lysine (1 mM); (14) H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M).  $\lambda_{ex/em} = 405/577$  nm.



**Figure S11.** (A) Absorption spectra of the MB solution upon introducing CA (100  $\mu$ g/mL) with (a) 10  $\mu$ M NAB, (b) 10  $\mu$ M NAB + 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, (c) 20  $\mu$ M NAB + 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and (d) 30  $\mu$ M NAB + 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>; (e) CA (100  $\mu$ g/mL) was pre-treated with acetazolamide (50  $\mu$ M) for 20 min, followed by incubating with 10  $\mu$ M NAB + 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and finally the reaction solution was introduced into the MB solution. (B) H<sub>2</sub>S calibration curve obtained with Na<sub>2</sub>S.



**Figure S12.** Effect of  $H_2O_2$  on the production of  $H_2S$  from NAB. Absorbance at 670 nm of methylene blue formed by NAB (10  $\mu$ M) in the presence of (a) 0, (b) 100, (c) 200 and (d) 300  $\mu$ M  $H_2O_2$ .



**Figure S13.** Absorbance change of 10  $\mu$ M methylene blue in the presence of (a) 0, (b) 100, (c) 200 and (d) 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>.



**Figure S14.** The absorbance of the formed methylene blue at 670 nm: Na<sub>2</sub>S (10  $\mu$ M) is mixed with (a) 0, (b) 100, (c) 200 and (d) 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 0.5 or 7 h. The results are the mean  $\pm$  standard deviation of three separate measurements.



**Figure S15.** Cell viability of (A) HeLa and (B) RAW264.7 cells treated with NAB at varied concentrations for 24 h. The results are the mean  $\pm$  standard deviation of five separate measurements.



**Figure S16.** Confocal fluorescence images of HeLa cells with NAB (10  $\mu$ M) in the presence of (A) 0 and (C) 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for different periods of time. Scale bar, 10  $\mu$ m. The normalized intensity of the images in panels A and C is shown in panels B and D, respectively [the pixel intensity is obtained by subtracting that (control) from the 0-h image in panel A; the fluorescence intensity from the 8-h image in panel C is defined as 1.0]. The results are presented as mean ± standard deviation of five cells.



Figure S17. (A) Confocal fluorescence images of HeLa cells under different conditions. (a) Cells

incubated with WSP-1 (15  $\mu$ M) for 45 min; (b) cells pre-treated with NAB (10  $\mu$ M) for 4 h and then incubated with WSP-1 (15  $\mu$ M) for 45 min; (c-e) cells pre-treated with varied NAB (10, 20 and 30  $\mu$ M, respectively) in the presence of H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) for 4 h and then incubated with WSP-1 (15  $\mu$ M) for 45 min. Scale bar, 10  $\mu$ m. (B,C) The normalized intensity of the images from (B) the WSP-1 channel ( $\lambda_{ex} = 488$  nm,  $\lambda_{em} = 510-540$  nm) and (C) the NAB channel ( $\lambda_{ex} = 405$  nm,  $\lambda_{em} = 550-650$ nm) in panel A [the pixel intensity is obtained by subtracting that (the corresponding control) from image a; the fluorescence intensity from the corresponding image e is defined as 1.0]. The results are presented as mean ± standard deviation of at least five cells.



**Figure S18.** (A) Confocal fluorescence images of HeLa cells. Cells pre-treated with NAB (10  $\mu$ M) in the presence of (a) 100, (b) 200 and (c) 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 4 h and then incubated with WSP-1 (15  $\mu$ M) for 45 min. Scale bar, 10  $\mu$ m. (B) The normalized intensity of the images in panel A (the fluorescence intensity from image a is defined as 1.0). The results are presented as mean  $\pm$  standard deviation of five cells.



**Figure S19.** Viability of RAW264.7 cells treated with PMA (1  $\mu$ g/mL) for 1 h and then incubated with NAH at varied concentrations for 4 h. Control is the untreated cells. The results are the mean  $\pm$  standard deviation of five separate measurements. It is seen that NAH cannot recover the viability of the PMA-stimulated cells.



**Figure S20.** (A) Confocal fluorescence images of RAW264.7 cells under different conditions. (a) Cells incubated with CRDR (5  $\mu$ M) for 30 min; (b) cells pre-stimulated with PMA (1 $\mu$ g/mL) for 1 h were incubated with CRDR (5  $\mu$ M) for 30 min; (c) cells pre-stimulated with PMA (1 $\mu$ g/mL) for 1 h were treated with NAB (10  $\mu$ M) for 4 h and then incubated with CRDR (5  $\mu$ M) for 30 min. The red channel ( $\lambda_{ex} = 635$  nm,  $\lambda_{em} = 660-760$  nm) for CRDR; the green channel ( $\lambda_{ex} = 405$  nm,  $\lambda_{em} = 550-650$  nm) for NAB. (B,C) The pixel intensity of the images from (B) the red and (C) green channels in panel A. The pixel intensity is obtained by subtracting the intensity (the corresponding control) from image a; the fluorescence intensity from the image b of red channel and image c of green channel is defined as 1.0, respectively. The results are presented as mean  $\pm$  standard deviation of at least five cells.