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ARTICLE

Investigation of Thiolysis of NBD Amines for the Development of H_2S Probes and Evaluating the Stability of NBD dyes

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In order to evaluate the thiolysis of NBD (7-nitro-1,2,3-benzoxadiazole) amines for development of H_2S probes, herein we investigated the reactivity and selectivity of a series of NBD amines for the first time. The piperazinyl- and piperidyl-based NBD probes could react efficiently with micromolar H_2S in buffer (pH 7.4), while such NBD(S) (nitrobenzothiadiazole) derivatives showed much slow thiolysis even in the presence of millimolar H_2S. Low reactivity was also observed for thiolysis of these ethylamino-, ethanolamino- and anilino-based NBD probes. Therefore, almost NBD amines used in bioimaging should be stable, in consideration of the presence of only micromolar endogenous H_2S *in vivo*. Moreover, the piperazinyl-NBD derivatives could be efficient in development of fluorescent H_2S probes and for directly visualizing H_2S by paper-based detection.

Introduction

Hydrogen sulfide (H_2S) is an important endogenous signalling molecule with multiple biological functions.[1-6] H_2S could be enzymatically produced *in vivo* by three distinctive pathways including cystathionine *β*-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MPST)/cysteine aminotransferase (CAT) in different organs and tissues.[7-10] In the central nervous system, the biological concentration of H_2S is in the range of 50-160 μM. The sulfide level in plasma blood is in the range of 10-100 μM. Studies have shown that the H_2S level *in vivo* is correlated with numerous diseases, including the symptoms of Alzheimer's disease, Down syndrome, diabetes and liver cirrhosis.[1,11,12] Despite H_2S has been recognized to be linked to numerous physiological and pathological processes, many of its underlying molecular events *in vivo* remain largely unknown and could be further explored. Therefore, it presents significant research value to develop efficient chemical or advanced methods for selective and sensitive detection of biological H_2S.

H_2S-triggered chemical reactions,[13,14] including nucleophilic addition,[15-19] reduction of azide or nitro to amine,[20-25] copper precipitation,[26] thiolysis of dinitrophenyl ether,[27,28] and others,[29-43] have been successfully employed to develop fluorescent and/or colorimetric probes for detection of biological H_2S. Among these organic reactions, the thiolysis of NBD amine[29] and NBD thioether[30] discovered by our group and Pluth group, respectively, were successful for development of efficient H_2S probes. We[31-36] and others[37-39] further employed the thiolysis of NBD amines to develop efficient fluorescent H_2S probes. The thiolysis of NBD ether was also explored by us and others for biothiols' probes.[40-42] However, our research indicated that the rate for thiolysis of NBD probes varied from 1 to over 100 $M^{-1}s^{-1}$ due to different chemical structures.[31-36] In this study, we aim to investigate the reactivity and selectivity of NBD amines for further understanding such thiolysis reaction.

On the other hand, though the success for thiolysis of NBD probes in H_2S detection,[31-42] the rising of issue whether NBD-based dyes are stable in bioimaging should be addressed, because endogenous H_2S exists *in vivo* and NBD-based dyes were widely used in bioimaging.[44-48] Herein, we prepared a series of NBD amines and studied their thiolysis reactions in buffer solution. The reaction rates of NBD amines towards H_2S varied from 43.2 to 0.05 $M^{-1}s^{-1}$ or smaller values dependent on the chemical structures. A sensitive colorimetric probe was obtained for direct visualization of low micromolar H_2S and for paper-based H_2S detection. Our studies also point out that almost NBD amines should be stable dyes for bioimaging.

Results and discussion

One major challenge in development of H_2S probes is discovery of a chemical reaction to effectively separate the reactivity of biothiols and H_2S. To address this challenge, we discovered such a reaction of thiolysis of NBD amine in 2013.[29]

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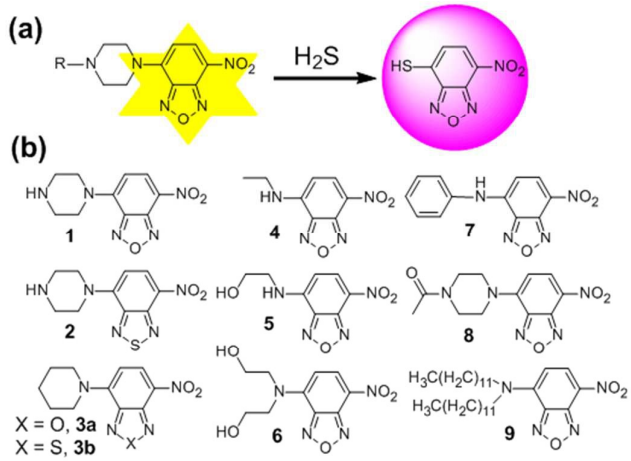
Consequently, this reaction was widely applied for H_2S probes by us and others.[31-39] The NBD amines show yellow fluorescence in buffer, while the thiolysis product 4-thiol-7-nitrobenzofurzan (NBD-SH) is non-fluorescence but shows unique red color.[30] Therefore, NBD-based probes could be used to develop colorimetric and fluorescent dual-channel probes (Scheme 1). However, we observed that different NBD-based probes have various reaction rates toward H_2S,[31-42] and hope to further understand the thiolysis of NBD amines. In this work, we prepared a series of NBD derivatives **1-9** (Scheme 1) for comparative studies of their thiolysis reactions. The coupling reaction of NBD-Cl (or NBD(S)Cl) and commercial amines under basic conditions produced the target probes (see experimental part). Such facile and economic synthesis is important for the wide use of the probes reported herein. The structural characterizations of all probes were confirmed by ^{1}H NMR, ^{13}C NMR, and high resolution mass spectra.



Scheme 1. Fluorescent and colorimetric H_2S probes based on thiolysis of NBD amine (a) and chemical structures of NBD amines in this work (b).

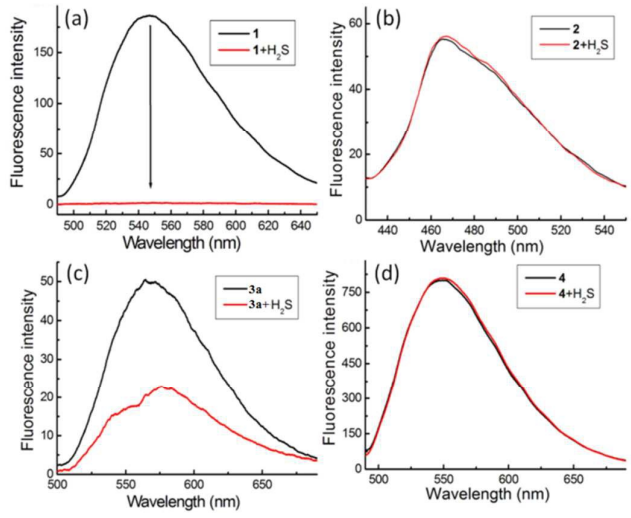


Figure 1. The emission spectra (excitation = 470 nm) of probes **1-4** (1 μM) before (black line) and after (red line) reaction with H_2S (100 μM) for 30 min in PBS buffer (pH 7.4).

Because the biological H_2S level normally stands in the range of nanomolar to micromolar concentrations, we firstly tested the fluorescence spectra of the reaction between NBD probes and 100 μM H_2S (using Na_2S as an equivalent) in PBS buffer (pH 7.4, containing 20% DMSO) for 30 min. As shown in Figures 1 and S1-S2, all NBD probes exhibited a certain emission around 560 nm upon excitation at 470 nm. After reaction with H_2S, large fluorescence change for **1**, **3a** and **8** was observed, while other probes (**2**, **3b**, **4-7**) did not display any obviously fluorescence change. The fluorescence turn-off for probe **1** is largest, and the final decrease factor was about 14-fold. Furthermore, the fluorescent turn-off response of the probes could be observed by naked eye under 365 nm UV lamp (Figure S3), and we found that high concentration H_2S (2 mM) could also trigger fluorescent turn-off of probes including **5**. These preliminary results encouraged us to further check the reactivity and selectivity of these probes.

To obtain the reaction kinetics, the time-dependent fluorescence signal at 560 nm was recorded for data analysis (Figures 2, S4-S7). The pseudo-first-order rate, k_{obs} was determined by fitting the fluorescence intensity data with single exponential function. The linear fitting between k_{obs} and H_2S concentrations gives the reaction rate (k_2). The thiolysis rate (Table 1) for **1**, **3a**, **5** was found to be 43.26, 1.10, 0.05 M^{-1} s^{-1}, respectively, which showed near 1000-fold difference for thiolysis of NBD amines. Probes **2**, **4**, and **6** have similar slow thiolysis rate with that of **5** even in the presence of millimolar H_2S. The thiolysis rate of **7** in millimolar H_2S is too slow to be observed. The k_2 for probe **8** is 21.77 M^{-1} s^{-1}, which is slight slower than **1**. The thiolysis reactions in 1% DMSO-containing buffer gave similar kinetic data as that in 20% DMSO-containing buffer (Figure S2). These results clearly indicated that piperazinyl- and piperidyl-based probes could react efficiently with micromolar H_2S under physiological conditions, while other NBD amines could react with millimolar H_2S slowly. Previous studies indicated that H_2S executes physiological effects at a wide range of concentrations between 10 and 300 μM.[4] Therefore, NBD amines including **2**, **4-7** should be enough stable in the presence of biological H_2S.

Pluth et al. have reported that H_2S and biothiols could deactivate the NBD-based thiol labeling reagents (thiolysis of NBD thioether bond),[49] implying that NBD thioethers could not be used in *in vivo* experiments. In our previous work,[40,50,51] we have indicated that micromolar biothiols could efficiently react with several NBD ethers which contained electron-withdrawing group neighboring the NBD ether bond. Herein we hope to state that since many NBD dyes are analogs of ethylamino-NBD, such dyes could be safety used for biological studies even in the presence of micromolar endogenous H_2S. However, the piperazinyl- and piperidyl-based NBD amines could react efficiently with micromolar H_2S under physiological conditions. The NBD(S) amines should be good alternatives for the piperazinyl- and piperidyl-based amines, because compounds **2** and **3b** are stable toward micromolar H_2S and biothiols. We hope that this work could help scientists to choose a suitable linker for labelling with NBD in biological studies.

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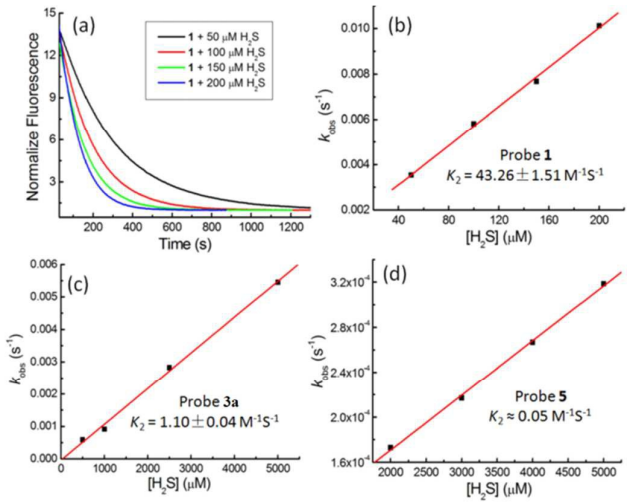

Figure 2. Kinetic studies for thiolysis of NBD amines. (a) Time-dependent fluorescence intensity at 560 nm (excitation = 470 nm) of probe **1** (1 μM) upon reaction with different concentrations of H_2S in PBS buffer (pH 7.4). The linear relationship of k_{obs} versus H_2S concentrations gives reaction kinetics constant k_2 for **1** (b) or **3a** (c) or **5** (d) and H_2S.

	λ_{max} (nm)	Em. (nm)	k_{obs} (s^{-1}) ([H_2S])	k_2 (M^{-1} s^{-1})
1	492	550	$4.4*10^{-3}$ (75 μM)	43.26
2	471	466	$3.8*10^{-4}$ (1 mM)	ND
3a	505	565	$2.6*10^{-4}$ (250 μM)	1.10
3b	498	562	ND	ND
4	480	550	$2.8*10^{-4}$ (7 mM)	ND
5	476	551	$3.2*10^{-4}$ (3 mM)	0.05
6	496	554	$1.3*10^{-4}$ (7 mM)	ND
7	475	558	ND	ND
8	480	550	$2.0*10^{-3}$ (200 μM)	21.77
9	490	552	ND	ND

Table 1. Spectra characterizations and kinetic data of **1**-**9**. ND, not determine.

Considering that NBD probes have been widely used in member biology due to the environmental sensitivity of this fluorophore, we also prepared a lipid-containing NBD dye **9** for thiolysis studies. As shown in Figure 3, the fluorescence change of **9** was much small in the presence of 100 μM H_2S. Time-dependent emission of **9** in millimolar H_2S (5 mM) only triggered slight decrease. Therefore, lipid-containing NBD probes should be stable in the presence of endogenous H_2S *in vivo* and could be used for biological studies *in vivo*.

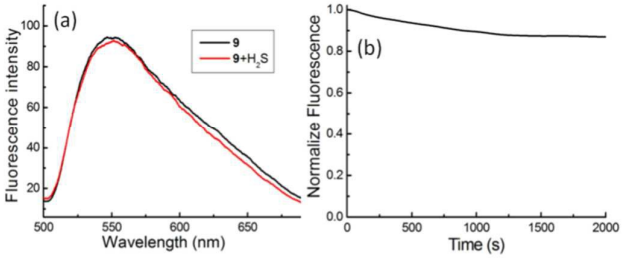

Figure 3. (a) The emission spectra (excitation = 470 nm) of **9** (1 μM) before (black line) and after (red line) reaction with H_2S (100 μM) for 30 min in SDS-containing PBS buffer (pH 7.4, containing 40 mM SDS). (b) Time-dependent emission intensity at 550 nm of **9** (1 μM) upon reaction with H_2S (5 mM) in PBS buffer.

We also checked the UV-Vis absorbance spectrum of NBD probes upon treatment with H_2S. As shown in Figures 4, S6 and S7, all probe solutions exhibited noticeable absorbance at 460-510 nm, due to the NBD absorbance. After treatment with H_2S, all probe solutions except **7** exhibited time-dependent increase absorbance at 530-550 nm with various rates, which could be attributed to the produce of NBD-SH. Pluth group firstly indentified the production of coloric NBD-SH from thiolysis of NBD probes.[30] For probe **1**, the isosbestic point was at 510 nm, indicating a clear transformation. We also observed the concentration-dependent absorbance spectrum changes through treatment of the probe solution with different concentrations H_2S in PBS buffer (data not shown). The reaction kinetics (Figure S7) based on absorbance spectra were consistent with that from fluorescent tests (Table 1), namely, **1**, **3a** and **8** are fast-response toward micromolar H_2S, while **2**, **4**-**6** are slow-response toward even millimolar H_2S. NBD probes are also water-soluble based on absorbance spectra (Figure S8).

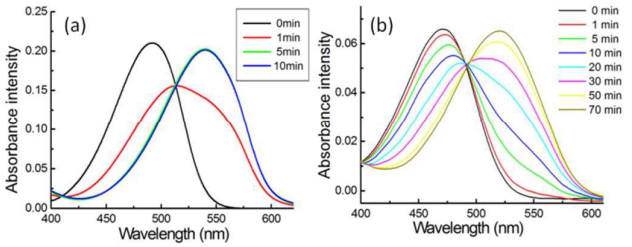

Figure 4. The absorbance spectra of probes **1** or **2** (10 μM) in the presence of 100 μM (for **1**, a) or 2 mM H_2S (for **2**, b) in PBS buffer (50 mM, pH 7.4, containing 20% DMSO).

Inspiring with the above results, we further checked the color change of NBD probes for directly visualizing different concentrations of H_2S. The obviously colorimetric change of probes upon H_2S activation is compound **8**, which turned from light brown to red through reaction with H_2S (Figure 5). We further used **8** for visualization of various concentrations of H_2S (10-100 μM) at room temperature. The results indicated that low concentration of H_2S (10 μM) have triggered obvious color change even for 5 min reaction; and 1 h incubation led to clearer color change for low micromolar H_2S (Figure 5a). Therefore, we can clearly see physiological level of micromolar H_2S based on **8**. Moreover, **8** could be further employed for paper-based detection of H_2S (Figure 5b). To our delight, the **8**-labelling paper could response with H_2S over 10 to 1000 μM range. The unassisted visual detection of low concentration of H_2S based on probe **8** and paper-based method is an appealing method. This no instrumentation requirement provides access

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to simple detection methods for applications in which instrumentation or laboratory costs are unavailable. Considering the facile synthesis of **8** and its colorimetric response with low concentration H_2S, we believe the probe **8** has various future applications.

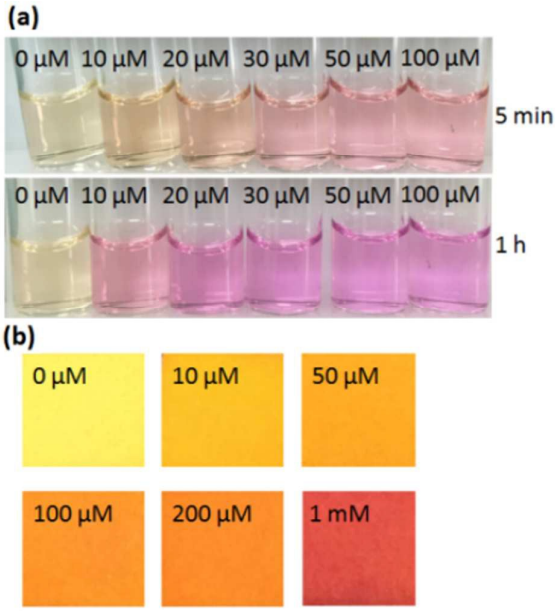

Figure 5. NBD probe **8** for colorimetric and paper-based detection of H_2S. (a) Photographs of probe **8** (10 μM) in the presence of different concentrations of H_2S for 5 min or 1 h. (b) Paper-based tests of probe **8** (1 mM) in the presence of different concentrations of H_2S for 30 min.

Because **1** had the fastest response rate toward H_2S, we further evaluated its usage as H_2S fluorescence probe. We checked the fluorescence signal change of probe **1** with various concentrations of H_2S (Figure 6). As expected, fluorescence decrease at 560 nm could be detected when the reaction mixture was excited at 470 nm. Higher concentration of H_2S induced weaker fluorescence of the reaction solution in the range of H_2S (5-40 μM). The detection limit was determined to be 0.57 μM. These demonstrated that probe **1** could be used to fluorescent detection of low micromolar H_2S.

To investigate the selectivity of NBD probes, various reactive sulfur species were incubated with probe in PBS buffer and their fluorescence responses were tested (Figures 7 and S9). Small-molecules thiols such as glutathione (GSH) and cysteine (Cys) at 1 mM triggered no obvious fluorescence change for probes **1** and **8**. SO_3^{2-} showed a certain fluorescence decrease, which is far below that for H_2S. While for probes **4** and **5**, no obvious fluorescence decrease was observed for the tested species. The pK_a of H_2S is around 6.9, while the typical free thiols have higher pK_a values about 8.5. Thus, it should be possible to selectively detect H_2S over biological thiols based on the thiolysis of NBD-based amines. Our studies further indicated piperazinyl-NBD derivatives are selective toward H_2S over biothiols.

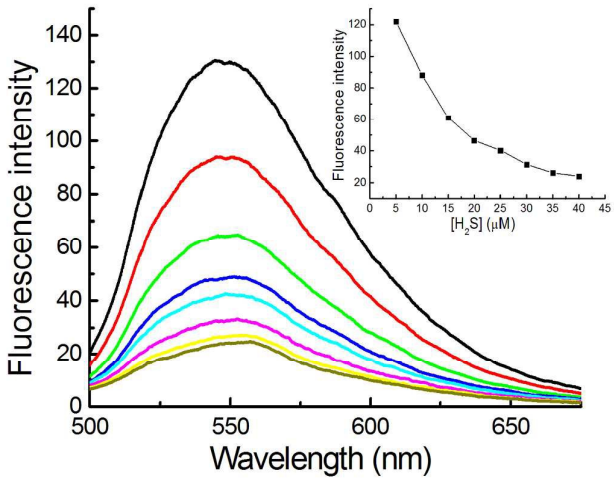

Figure 6. Concentration-dependent fluorescence spectra of probe **1** (1 μM) upon reaction with H_2S (5-40 μM) for 20 min in PBS buffer (pH 7.4). Inset: emission intensity at 560 nm versus H_2S concentrations.

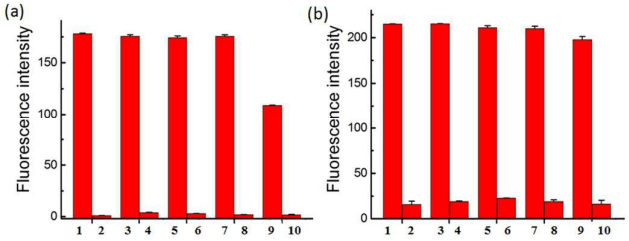

Figure 7. Fluorescence responses at 560 nm of the probe **1** (1 μM, a) or **8** (1 μM, b) to various biologically relevant species in PBS (pH 7.4, containing 1% DMSO) for 30 min. Lanes 1, 3, 5, 7, 9, probe or probe to 1 mM Cys, 1 mM GSH, 1 mM Hcy, or 100 μM SO_3^{2-}, respectively. Lanes 2, 4, 6, 8, 10, probe to 100 μM H_2S or to both H_2S and Cys, GSH, Hcy, or SO_3^{2-}, respectively.

To test the biological applicability of probe **1**, we examined whether it can be used to detect exogenous H_2S in living cells. HeLa cells were treated with probe **1** and then washed with PBS to remove excess **1**. The **1**-loaded cells were incubated with H_2S and subsequently imaged using a confocal fluorescence microscopy (Figure 8). The addition of both probe **1** and H_2S resulted in nearly no fluorescence while the cells treated with only probe **1** showed obvious yellow fluorescence. These preliminary studies suggested that NBD probe **1**could be used for visualization of H_2S in cells selectively.

Conclusions

In summary, ten NBD amines were synthesized and characterized for study of thiolysis reaction in PBS buffer. The NBD-based piperazinyl- and piperidyl-probes could react efficiently with micromolar H_2S in buffer, while the NBD(S)

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(nitrobenzothiadiazole) probes showed much slow thiolysis even in the presence of millimolar H_2S in buffer. Low reactivity was also observed for ethylamino-, ethanolamino- and anilino-based probes. The acetylpiperazinyl NBD was found to be efficient in directly visualizing H_2S by naked eye and paper-based H_2S detection. The piperazinyl-NBD could be used for highly selective detection and bioimaging of H_2S. This study also points out that many NBD amines used in bioimaging should be stable dyes, in consideration of the presence of only micromolar H_2S *in vivo*.

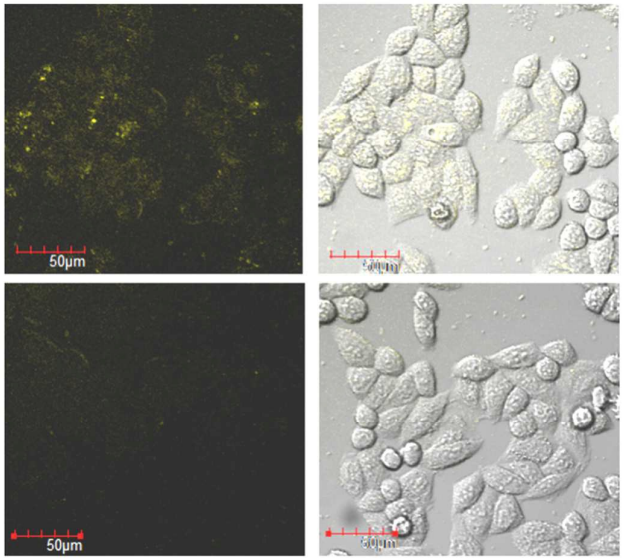

Figure 8. Confocal microscopy images of exogenous H_2S in living cells using probe **1**. HeLa cells were incubated with (a) **1** (5 μM) for 30 min, (b) **1** (5 μM) for 30 min, washed by PBS buffer, and then Na_2S (100 μM) for 30 min. The merge images between fluorescent and bright-field images are right. Scale bar, 50 μm.

Experimental

Materials and Methods

All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiments without further purification. The progress of the reaction was monitored by TLC on pre-coated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized by basic $KMnO_4$, UV light or iodine. Merck silica gel 60 (100-200 mesh) was used for general column chromatography purification. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm; DMSO-d_6 = 2.5 ppm). ^1H NMR coupling constants (*J*) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), m (multiple). High-resolution mass spectra (HRMS) were obtained on a Agilent 6540 UHD Accurate-Mass Q-TOFLC/MS or Varian 7.0 T FTICR-MS. The UV-visible spectra were recorded on a UV-3600 UV-VIS-NIR spectrophotometer (SHIMADZU, Japan). Fluorescence study was carried out using F-280 spectrophotometer (Tianjin Gangdong Sci & Tech., Development. Co., Ltd).

General Procedure for Spectroscopic Studies

All spectroscopic measurements were performed in phosphate-buffered saline buffer (PBS, 50 mM, pH 7.4, containing 20% DMSO or 1% DMSO). Compounds were dissolved into DMSO to prepare the stock solutions with a concentration of 10.0 or 1.0 mM. 1-1000 mM Stock solutions of Na_2S in degassed (by bubbling N_2 for 30 min) PBS buffer were used as H_2S source. Probes were diluted in PBS buffer (pH = 7.4, 50 mM) to afford the final concentration of 1-10 μM. For the selectivity experiment, different biologically relevant molecules (100 mM) were prepared as stock solutions in PBS buffer. Appropriate amount of biologically relevant species were added to separate portions of the probe solution and mixed thoroughly. All measurements were performed in a 3 ml corvette with 2 ml solution. The reaction mixture was shaken uniformly before emission spectra were measured. If it is not stated specially, the excitation wavelength is 470 nm and the emission at 560 nm was recorded. For compound **9**, 40 mM sodium dodecyl sulfate (SDS) was added in the test buffer.

Colorimetric and Paper-Based Assay for H_2S

Probe **8** (10 μM) in 1 mL PBS buffer (50 mM, pH 7.4, containing 20% DMSO) was treated with Na_2S (final concentration 10, 20, 30, 50 or 100 μM). The resulted solution was incubated at 25 ºC for 5 min and 1 h and photos of the solution were recorded. Filter papers (7 mm*7 mm) were immersed in probe **8** solution (1 mM, CH_3CN:DMSO = 9:1) for 10 min and then dried on glass plate for 2 h. The dye-loaded papers were soaked into various concentrations of H_2S solution (10 μM to 1 mM) and took out immediately for reaction at 25 ºC in a sealed container. After 30 min incubation, the photos of papers were recorded.

Cell Culture and Bioimaging

HeLa cells were cultured at 37 ºC, 5% CO_2 in DMEM/HIGH GLUCOSE (GIBICO) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine. The cells were maintained in exponential growth, and then seeded in glass-bottom 35 mm plate at the density about 2 × 104/well. Cells were passaged every 2-3 days and used between passages 3 and 10. Cells were imaged on a confocal microscope (Olympus FV1000 UPLSAPO40X) with a 40 × objective lens. All images were analyzed with Olympus FV1000-ASW. Emission was collected at green channel (500-600 nm) with 488 nm excitation.

Synthesis

4-Nitro-7-(piperazin-1-yl)benzo[c][1,2,5]oxadiazole (1). A mixture of mono-*t*Boc-piperazine (223 mg, 1.2 mmol) and NBD-Cl (200 mg, 1.0 mmol) was dissolved in 20.0 mL dichloromethane. N,N-Diisopropylethylamine (DIPEA, 0.25 mL,

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1.5 mmol) was added to the reaction mixture.[33] After stirring at room temperature for 4 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with methanol/dichloromethane = 1.5/100 to give the protected product. Then the product was dissolved in 10.0 mL dichloromethane, 10.0 mL CF_3COOH was added. After stirring at room temperature for 3 h, the resulting solution was evaporated by distillation under reduced pressure to give a red solid product **1** (188 mg, 75%). R_f = 0.7 (methanol/dichloromethane = 1/10); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.29 (brs, 1 H), 8.56 (d, J = 9.2 Hz, 1 H), 6.75 (d, J = 9.2 Hz, 1 H), 4.30 (t, J = 5.2 Hz, 4 H), 3.39 (t, J = 5.2 Hz, 4 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 144.9, 144.7, 144.5, 136.2, 122.6, 104.5, 46.1, 42.2; HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{10}H_{12}N_5O_3$: 250.0935, found: 250.0936.

4-Nitro-7-(piperazin-1-yl)benzo[c][1,2,5]thiadiazole (2). A mixture of mono-*t*Boc-piperazine (115 mg, 0.62 mmol) and NBD(S)-Cl (110 mg, 0.5 mmol) was dissolved in 20.0 mL dichloromethane. N,N-Diisopropylethylamine (DIPEA, 0.13 mL, 0.75 mmol) was added to the reaction mixture. After stirring at room temperature for 2 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with methanol/dichloromethane = 2/100 to give the protected product. Then the product was dissolved in 15.0 mL dichloromethane, 15.0 mL CF_3COOH was added. After stirring at room temperature for 2 h, the resulting solution was evaporated by distillation under reduced pressure to give a red solid product **2** (106 mg, 80%). R_f = 0.7 (methanol/dichloromethane = 1/10); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.58 (d, J = 8.8 Hz, 1 H), 6.93 (d, J = 8.8 Hz, 1 H), 4.20-4.10 (m, 4 H), 3.23-3.16 (m, 4 H), 1.23 (brs, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.7, 147.6, 146.9, 131.8, 128.6, 106.4, 47.8, 43.7; HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{10}H_{12}N_5O_2S$: 266.0712, found: 266.0703.

4-Nitro-7-(piperidyl)benzo[c][1,2,5]oxadiazole (3a). A mixture of piperidine (43 mg, 0.5 mmol) and NBD-Cl (150 mg, 0.75 mmol) was dissolved in 10.0 mL dry THF. N,N-Diisopropylethylamine (DIPEA, 0.25 mL, 1.5 mmol) was added to the reaction mixture. After stirring at room temperature for 1 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with petroleum ether/dichloromethane = 1/2 to give a red solid product **3a** (110 mg, 88%). R_f = 0.3 (petroleum ether/dichloromethane = 1/2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (d, J = 8.8 Hz, 1 H), 6.27 (d, J = 8.8 Hz, 1 H), 4.14-4.07 (m, 4 H), 1.86-1.80 (m, 6 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 145.1, 144.9, 144.6, 136.3, 120.0, 103.0, 50.9, 25.8, 23.4; HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{11}H_{13}N_4O_3$: 249.0988, found: 249.0979.

4-Nitro-7-(piperidyl)benzo[c][1,2,5]thiadiazole (3b). A mixture of piperidine (27 mg, 0.3 mmol) and NBD(S)-Cl (60 mg, 0.28 mmol) was dissolved in 15.0 mL dry CH_2Cl_2. N,N-Diisopropylethylamine (DIPEA, 50 μL, 0.3 mmol) was added to the reaction mixture. After stirring at room temperature for 4 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with petroleum ether/dichloromethane = 1/1 to give a red solid product **3b** (59.7 mg, 68%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60-8.50 (m, 1 H), 6.61-6.55 (m, 1 H), 4.05 (d, J = 5.6 Hz, 4 H), 1.88-1.76 (m, 6 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.9, 148.7, 147.6, 132.4, 128.8, 105.3, 51.3, 26.2, 24.5; HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{11}H_{13}N_4O_2S$: 265.0754, found: 265.0752.

4-Nitro-7-(ethylamino)benzo[c][1,2,5]oxadiazole (4). A mixture of ethylamine (0.38 mL, 2 M THF solution, 0.75 mmol) and NBD-Cl (100 mg, 0.5 mmol) was dissolved in 10.0 mL dry THF. N,N-Diisopropylethylamine (DIPEA, 0.25 mL, 1.5 mmol) was added to the reaction mixture. After stirring at room temperature for 1 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with petroleum ether/dichloromethane = 1/1 to give a red solid product **4** (70 mg, 67%). R_f = 0.6 (petroleum ether/dichloromethane = 1/2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, J = 8.4 Hz, 1 H), 6.17 (d, J = 8.4 Hz, 1 H), 3.59-3.53 (m, 2 H), 1.47 (t, J = 7.2 Hz, 3 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 144.7 144.2, 143.9, 137.7, 120.4, 98.7, 64.9, 13.1; HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_8H_9N_4O_3$: 209.0669, found: 209.0647.

4-Nitro-7-(2-ethanolamino)benzo[c][1,2,5]oxadiazole (5). A mixture of 2-ethanolamine (61 mg, 1.0 mmol) and NBD-Cl (300 mg, 1.5 mmol) was dissolved in 10.0 mL dry THF. N,N-Diisopropylethylamine (DIPEA, 0.5 mL, 3.0 mmol) was added to the reaction mixture. After stirring at room temperature for 1 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with petroleum ether/ethyl acetate = 1/1 to give a red solid product **5** (110 mg, 49%). R_f = 0.3 (petroleum ether/ethyl acetate = 1/1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.41 (brs, 1 H), 8.51 (d, J = 8.8 Hz, 1 H), 6.46 (d, J = 8.8 Hz, 1 H), 4.94 (brs, 1 H), 3.71-3.67 (dd, J = 5.6 Hz, 11.2 Hz, 2 H), 3.62-3.48 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 145.5, 144.3, 143.9, 137.8, 120.4, 99.3, 58.8, 46.0; HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_8H_9N_4O_4$: 225.0624, found: 225.0620.

4-Nitro-7-(diethanolamino)benzo[c][1,2,5]oxadiazole (6). A mixture of diethanolamine (53 mg, 0.5 mmol) and NBD-Cl (150 mg, 0.75 mmol) was dissolved in 10.0 mL dry DMF. N,N-Diisopropylethylamine (DIPEA, 0.25 mL, 1.5 mmol) was added to the reaction mixture. After stirring at room temperature for 1 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with ethyl acetate to give a red solid product **6** (120 mg, 89%). R_f = 0.4 (ethyl acetate); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45 (d, J = 9.2 Hz, 1 H), 6.55 (d, J = 9.2 Hz, 1 H), 5.00 (brs, 2 H), 4.40-4.00 (m, 4 H), 3.76 (t, J = 5.6 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 146.0, 144.7, 143.4, 136.0,

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119.7, 102.6, 58.2, 56.3; HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{10}H_{13}N_4O_5$: 269.0886, found: 269.0881.

4-Nitro-7-(anilino)benzo[c][1,2,5]oxadiazole (7). A mixture of aniline (93 mg, 1.0 mmol) and NBD-Cl (300 mg, 1.5 mmol) was dissolved in 10.0 mL dry THF. N,N-Diisopropylethylamine (DIPEA, 0.5 mL, 3.0 mmol) was added to the reaction mixture. After stirring at room temperature for 1 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with petroleum ether/ethyl acetate = 6/1 to give a red solid product **7** (150 mg, 58%). R_f = 0.4 (petroleum ether/ethyl acetate = 6/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, *J* = 8.4 Hz, 1 H), 7.74 (brs, 1 H), 7.55-7.51 (m, 2 H), 7.42-7.35 (m, 3 H), 6.73 (d, *J* = 8.4 Hz, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 144.9, 144.1, 142.3, 137.7, 137.6, 129.6, 126.4, 123.8, 123.0, 101.5; HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{12}H_9N_4O_3$: 257.0669, found: 257.0664.

4-Nitro-7-(4-acetylpiperazin-1-yl)benzo[c][1,2,5]oxadiazole (8). A mixture of compound **1** (250 mg, 1.0 mmol) and acetic anhydride (0.22 mL, 1.8 mmol) was dissolved in 20.0 mL dichloromethane. After stirring at room temperature for 3 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with methanol/dichloromethane = 0.8/100 to give a red solid product **8** (151 mg, 52%). R_f = 0.2 (methanol/dichloromethane = 2/100); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.52 (d, *J* = 8.8 Hz, 1 H), 6.62 (d, *J* = 8.8 Hz, 1 H), 4.27-4.10 (m, 4 H), 3.80-3.70 (m, 4 H), 2.06 (s, 3 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.7, 145.4, 144.7, 144.7, 136.2, 121.1, 103.1, 48.8, 44.2, 21.1; HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{12}H_{14}N_5O_4$: 292.1046, found: 292.1047.

4-Nitro-7-(didodecylamino)benzo[c][1,2,5]oxadiazole (9). A mixture of dodecylamine (88 mg, 0.25 mmol) and NBD-Cl (75 mg, 0.375 mmol) was dissolved in 10.0 mL dry THF. N,N-Diisopropylethylamine (DIPEA, 0.13 mL, 0.75 mmol) was added to the reaction mixture. After stirring at room temperature for 1 h, the resulting solution was evaporated by distillation under reduced pressure. The residue was purified by flash column chromatography eluting with petroleum ether/dichloromethane = 3/1 to give a red solid product **9** (96 mg, 74%). R_f = 0.3 (petroleum ether/dichloromethane = 3/1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, *J* = 9.2 Hz, 1 H), 6.07 (d, *J* = 9.2 Hz, 1 H), 4.00-3.70 (m, 4 H), 1.79-1.70 (m, 4 H), 1.38-1.27 (m, 36 H), 0.88 (t, *J* = 6.8 Hz, 6 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 144.6, 144.6, 144.0, 135.6, 119.8, 101.6, 64.7, 31.1, 28.8, 28.8, 28.8, 28.7, 28.5, 21.9, 13.7; HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{30}H_{53}N_4O_3$: 517.4118, found: 517.4104.

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