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A Lysosome-Targetable Fluorescent Probe for Imaging Hydrogen Sulfide in Living Cells

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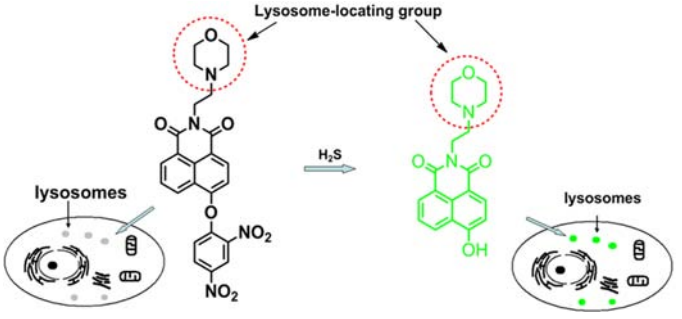
ABSTRACT



In this work, a 1,8-naphthalimide-derived fluorescent probe for H_2S based on the thiolysis of dinitrophenyl ether is reported. This probe exhibits turn-on fluorescence detection of H_2S in bovine serum and lysosome-targetable fluorescent imaging of H_2S with excellent selectivity.

Hydrogen sulfide (H_2S) is well-known as a toxic gas with the characteristic smell of rotten eggs, but is now also considered the third most important gasotransmitter for regulating cardiovascular, neuronal, immune, endocrine, and gastrointestinal systems, along with nitric oxide and carbon monoxide.[1] H_2S is produced endogenously in mammalian systems from L-cysteine in reactions catalyzed mainly by two pyridoxal-5′-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE).[2] As a signal molecule, it modulates neuronal transmission,[2] relaxes smooth muscle,[3] regulates the release of insulin,[4] and is involved in inflammation.[5] The endogenous levels of H_2S are believed to be related with some diseases such as Alzheimer's disease,[6] Down's syndrome,[7] diabetes,[8] and liver cirrhosis.[9] Inhibitors of H_2S, and H_2S donors, in animal disease models have shown potential for therapeutic exploitation of H_2S.[10] Thus, visualization of the distribution and concentration of H_2S in living systems would be very important and helpful to elucidate the biological roles of H_2S.

Small molecule fluorescent probes offer high sensitivity, real-time imaging, and high spatiotemporal resolution and have excellent potential as useful tools.[11,12] A few fluorescent probes for H_2S have been reported.[13] These probes

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(1) Kimura, H. *Amino Acids* **2011**, *41*, 113.
(2) Li, L.; Rose, P.; Moore, P. K. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 169.
(3) Dombkowski, R. A.; Russell, M. J.; Olson, K. R. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *286*, R678.
(4) Kaneko, Y.; Kimura, Y.; Kimura, H.; Niki, I. *Diabetes* **2006**, *55*, 1391.
(5) Zanardo, R. C. O.; Brancaleone, V.; Distrutti, E.; Fiorucci, S.; Cirino, G.; Wallace, J. L. *FASEB J.* **2006**, *20*, 2118.
(6) Eto, K.; Asada, T.; Arima, K.; Makifuchi, T.; Kimura, H. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 1485.
(7) Kamoun, P.; Belardinelli, M.-C.; Chabli, A.; Lallouchi, K.; Chadefaux-Vekemans, B. *Am. J. Med. Genet. A* **2003**, *116A*, 310.
(8) Yang, W.; Yang, G.; Jia, X.; Wu, L.; Wang, R. *J. Physiol.* **2005**, *569*, 519.
(9) Fiorucci, S.; Antonelli, E.; Mencarelli, A.; Orlandi, S.; Renga, B.; Rizzo, G.; Distrutti, E.; Shah, V.; Morelli, A. *Hepatology* **2005**, *42*, 539.
(10) Szabo, C. *Nat. Rev. Drug Discovery* **2007**, *6*, 917.
(11) Xu, Z.; Yoon, J.; Spring, D. R. *Chem. Soc. Rev.* **2010**, *39*, 1996.
(12) Ueno, T.; Nagano, T. *Nat. Methods* **2011**, *8*, 642.
(13) Lin, V. S.; Chang, C. J. *Curr. Opin. Chem. Biol.* **2012**, *16*, 595.

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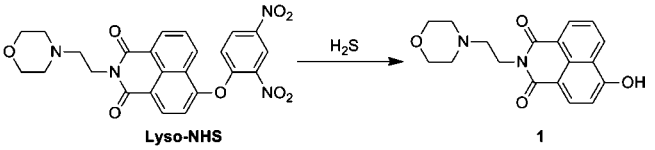
can image H_2S in blood samples and living cells but without location specificity, in particular subcellular localization. The distribution of H_2S producing enzymes in tissues is known to a first approximation.[14] For example, CSE is distributed in smooth muscle cells, liver, and pancreas, whereas CBS is found in the brain, liver, kidney, and pancreas.[14] CBS is also found in the endosomal-lysosomal system.[15] However, the distribution and function of H_2S in different organelles are still unclear. Therefore, organelle-specific fluorescent probes for H_2S are especially required to help understand the detailed network of H_2S biology in cells.

The design of fluorescent probes for H_2S is mainly based on specific chemical reactions by taking advantage of the reducing or nucleophilic properties of H_2S. For example, Chang[16] and Wang[17] et al. pioneered an approach of using the reduction of azide with H_2S to amine to sense H_2S, which has been expanded to design azide-containing fluorescent probes by altering fluorophores.[18−23] A ratiometric fluorescent probe was developed in terms of this strategy.[20] Xian and co-workers constructed a H_2S probe through a nucleophilic substitution reaction between H_2S and the disulfide moiety.[24] He et al. used the nucleophilic attack of H_2S on the aldehyde functionality to design a fluorescent probe to sense H_2S.[25] Lin et al. reported a near-infrared fluorescent probe for H_2S based on thiolysis of dinitrophenyl ether.[26] However, most of these probes require complex synthesis and display a response time of ∼1−2 h. 1,8-Naphthalimide is a cell-permeable fluorophore possessing a visible emission wavelength, high photostability, and facile synthesis of various fluorescent probes by easily introducing different functional groups to the aromatic 'naphthalene' moiety and the '*N*-imide site'.[27−36] In our work, we introduced a dinitrophenyl ether group into the 4-position of 1,8-naphthalimide, which acts as the H_2S reactive site,[26] and a 4-(2-aminoethyl)morpholine, which is a lysosome-targetable group,[37] onto the *N*-imide termus, thereby, efficiently yielding the fluorescent probe **Lyso-NHS** (Scheme 1). To our best knowledge, **Lyso-NHS** is the first fluorescent probe that can image H_2S in lysosomes of living cells in minutes.

Scheme 1. Mechanism of H_2S Sensing by **Lyso-NHS**



The pH value of lysosomes is in the range of 4.0−6.0.[38] To monitor H_2S in lysosomes, the fluorescent probe should first have the ability to survive in this acidic environment and display no fluorescence response. In order to verify the workability of **Lyso-NHS** within this pH range, the influence of pH on the fluorescence of **Lyso-NHS** was first determined by fluorescence titration. The fluorescence at 555 nm of **Lyso-NHS** remains unaffected at pH 8.2−4.2 and then gradually increases from pH 4.2 to 2.03 due to the inhibition of the photoinduced electron transfer (PET) process from the morpholine nitrogen to the fluorophore (Figure 1). The pK_a value of **Lyso-NHS** is 3.12. Therefore, the fluorescence of **Lyso-NHS** will not change in lysosomes, which makes **Lyso-NHS** fit the purpose.

The emission spectra and fluorescence titration experiments of **Lyso-NHS** with H_2S were then recorded in aqueous solution (CH_3CN/PBS = 1:9, pH = 7.4) (Figure 2a). The free **Lyso-NHS** displays quite weak fluorescence. When H_2S was added progressively from 0 to 10 equiv to the solution of **Lyso-NHS** (NaHS was used as a hydrogen sulfide source), the fluorescence intensity of the emission band centered at 555 nm increased in intensity significantly (42-fold) due to the thiolysis of the dinitrophenyl ether by H_2S (Scheme 1).

The formation of compound **1** was confirmed by MS analysis (Figure S1) and the HPLC retention time compared with those of independently synthesized **1**, which is responsible for the fluorescence emission and enhancement at 555 nm (Figure S2). Moreover, the product was purified and characterized with ^1H and ^{13}C NMR, which is

(14) Yamamoto, J.; Sato, W.; Kosugi, T.; Yamamoto, T.; Kimura, T.; Taniguchi, S.; Kojima, H.; Maruyama, S.; Imai, E.; Matsuo, S.; Yuzawa, Y.; Niki, I. *Clin. Exp. Nephrol.* **2013**, *17*, 32.
(15) Leisle, L.; Ludwig, C. F.; Wagner, F. A.; Jentsch, T. J.; Stauber, T. *EMBO J.* **2011**, *30*, 2140.
(16) Lippert, A. R.; New, E. J.; Chang, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 10078.
(17) Peng, H.; Cheng, Y.; Dai, C.; King, A. L.; Predmore, B. L.; Lefer, D. J.; Wang, B. *Angew. Chem., Int. Ed.* **2011**, *50*, 9672.
(18) Das, S. K.; Lim, C. S.; Yang, S. Y.; Han, J. H.; Cho, B. R. *Chem. Commun.* **2012**, *48*, 8395.
(19) Montoya, L. A.; Pluth, M. D. *Chem. Commun.* **2012**, *48*, 4767.
(20) Wan, Q.; Song, Y.; Li, Z.; Gao, X.; Ma, H. *Chem. Commun.* **2013**, *49*, 502.
(21) Wu, Z.; Li, Z.; Yang, L.; Han, J.; Han, S. *Chem. Commun.* **2012**, *48*, 10120.
(22) Yu, F.; Li, P.; Song, P.; Wang, B.; Zhao, J.; Han, K. *Chem. Commun.* **2012**, *48*, 2852.
(23) Chen, S.; Chen, Z.-j.; Ren, W.; Ai, H.-w. *J. Am. Chem. Soc.* **2012**, *134*, 9589.
(24) Liu, C.; Pan, J.; Li, S.; Zhao, Y.; Wu, L. Y.; Berkman, C. E.; Whorton, A. R.; Xian, M. *Angew. Chem., Int. Ed.* **2011**, *50*, 10327.
(25) Qian, Y.; Karpus, J.; Kabil, O.; Zhang, S.-Y.; Zhu, H.-L.; Banerjee, R.; Zhao, J.; He, C. *Nat. Commun.* **2011**, *2*, 495.
(26) Cao, X.; Lin, W.; Zheng, K.; He, L. *Chem. Commun.* **2012**, *48*, 10529.
(27) Duke, R. M.; Veale, E. B.; Pfeffer, F. M.; Kruger, P. E.; Gunnlaugsson, T. *Chem. Soc. Rev.* **2010**, *39*, 3936.
(28) Xu, Z.; Qian, X.; Cui, J. *Org. Lett.* **2005**, *7*, 3029.
(29) Xu, Z.; Xiao, Y.; Qian, X.; Cui, J.; Cui, D. *Org. Lett.* **2005**, *7*, 889.
(30) Xu, Z.; Qian, X.; Cui, J.; Zhang, R. *Tetrahedron* **2006**, *62*, 10117.
(31) Xu, Z.; Baek, K.-H.; Kim, H. N.; Cui, J.; Qian, X.; Spring, D. R.; Shin, I.; Yoon, J. *J. Am. Chem. Soc.* **2009**, *132*, 601.
(32) Xu, Z.; Han, S. J.; Lee, C.; Yoon, J.; Spring, D. R. *Chem. Commun.* **2010**, *46*, 1679.
(33) Xu, Z.; Pan, J.; Spring, D. R.; Cui, J.; Yoon, J. *Tetrahedron* **2010**, *66*, 1678.
(34) Xu, Z.; Yoon, J.; Spring, D. R. *Chem. Commun.* **2010**, *46*, 2563.
(35) Xu, Z.; Zheng, S.; Yoon, J.; Spring, D. R. *Analyst* **2010**, *135*, 2554.
(36) Wang, M.; Xu, Z.; Wang, X.; Cui, J. *Dyes Pigm.* **2013**, *96*, 333.
(37) Yu, H.; Xiao, Y.; Jin, L. *J. Am. Chem. Soc.* **2012**, *134*, 17486.
(38) Christensen, K. A.; Myers, J. T.; Swanson, J. A. *J. Cell Sci.* **2002**, *115*, 599.

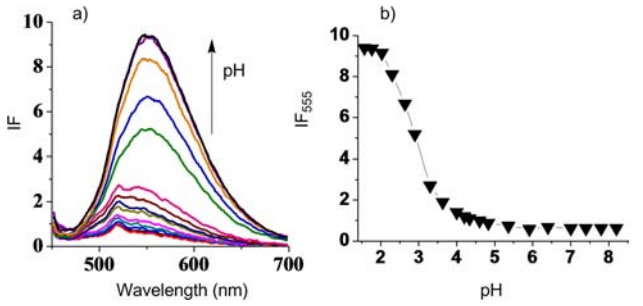


Figure 1. Influence of pH on the fluorescence of **Lyso-NHS** in aqueous solution (CH_3CN/PBS = 1:9). Excitation wavelength is 450 nm. [**Lyso-NHS**] = 10 μM.

identical to compound **1** (see the Supporting Information for spectral data). The pH-dependent fluorescence of **Lyso-NHS** with the addition of H_2S confirmed the applicability of **Lyso-NHS** in lysosomes (Figure S3). The time-dependent fluorescence responses were next detected with the addition of 10 equiv of H_2S, and the results showed that the reaction was completed within 20 min (Figure 2b–c). Notably, the background fluorescence of **Lyso-NHS** is very weak, and within minutes a high fluorescence increase is observed which relays the reaction of **Lyso-NHS** with H_2S (Figure 2c); therefore, the time scale allows **Lyso-NHS** to sense H_2S in real-time intracellular imaging. There was good linearity between the fluorescence intensity and the concentrations of H_2S in the range 0 to 100 μM with a detection limit of 0.48 μM (Figure S4). The absorption titration of **Lyso-NHS** with H_2S was subsequently performed and also reflected the thiolysis of the dinitrophenyl ether. Compound **Lyso-NHS** exhibits maximum absorption at 360 nm. On addition of 0–10 equiv of H_2S to the solution of **Lyso-NHS**, the absorbance at 360 nm decreased sharply to its limiting value, while an absorption band at 440 nm developed which induced the color change from colorless to yellow (Figures 2d, S5).

The fluorescence titration of **Lyso-NHS** with various analytes was conducted to examine the selectivity. As shown in Figure 3, the addition of 100 equiv of Na^+, Ca^{2+}, K^+, Mg^{2+}, HCO_3^-, F^-, Cl^-, Br^-, I^-, NO_3^-, $S_2O_3^{2-}$, $S_2O_4^{2-}$, $S_2O_5^{2-}$, SO_3^-, N_3^-, CO_3^{2-}, CH_3COO^-, OH^-, SO_4^{2-}, H_2O_2, HSO_4^-, homocysteine, and ascorbic acid produced a nominal change in the fluorescence spectra of **Lyso-NHS**. Other tested analytes including 100 equiv of Zn^{2+}, cysteine, and glutathione induced fluorescence but with much smaller enhancement. Therefore the probe **Lyso-NHS** has a very high selectivity for H_2S.

The tests in buffer solutions have shown the potential utility of **Lyso-NHS** in biological samples. We first checked the fluorescence response of **Lyso-NHS** with H_2S in bovine serum. The background fluorescence of bovine serum sample is relatively weak. With the addition of NaHS, the fluorescence intensity of emission of the bovine serum sample with **Lyso-NHS** increases significantly. It should be noted that the fluorescence enhancement is observed

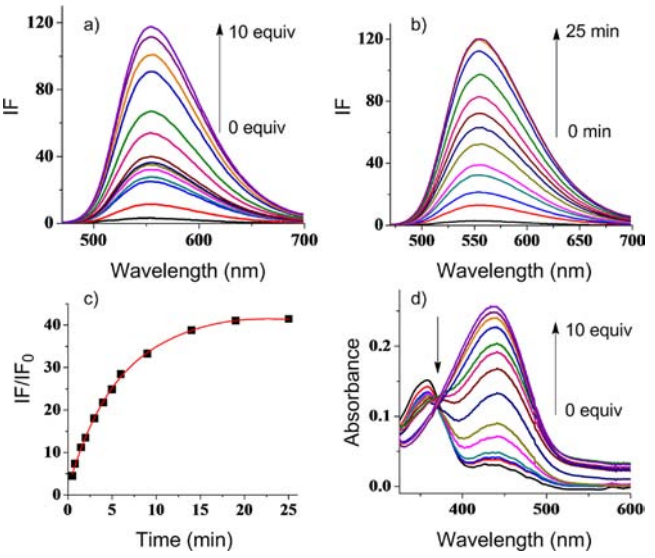


Figure 2. (a) Fluorescent emission spectra of 10 μM compound **Lyso-NHS** in the presence of 0–10 equiv of H_2S in aqueous solution (CH_3CN/PBS = 1:9, pH = 7.4, 37 °C) (NaHS was dissolved in water at a concentration of 10 mM). Excitation at 450 nm. (b) Time dependence of fluorescence profiles of **Lyso-NHS** (10 μM) with 10 equiv of H_2S. (c) Time dependence of fluorescence intensity of **Lyso-NHS** (10 μM) at 555 nm with 10 equiv of H_2S. (d) UV–vis absorption spectra of 10 μM compound **Lyso-NHS** in the presence of 0–10 equiv of H_2S in an aqueous solution (CH_3CN/PBS = 1:9, pH = 7.4, 37 °C).

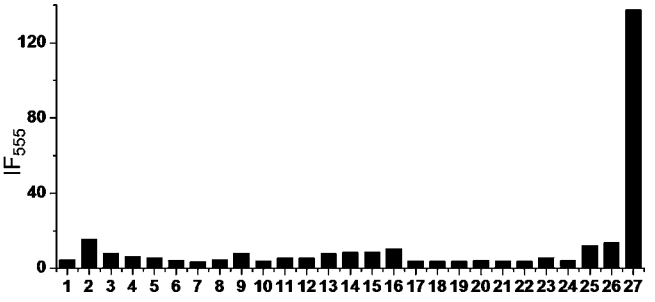


Figure 3. Fluorescence responses of 10 μM **Lyso-NHS** to various analytes in aqueous solution (CH_3CN/PBS = 1:9, pH = 7.4, 37 °C). Excitation at 450 nm. Bars represent the final fluorescence intensity of **Lyso-NHS** with 1 mM analytes over the original emission of free **Lyso-NHS**. (1) Free **Lyso-NHS**; (2) Zn^{2+}; (3) Na^+; (4) Ca^{2+}; (5) K^+; (6) Mg^{2+}; (7) HCO_3^-; (8) F^-; (9) Cl^-; (10) Br^-; (11) I^-; (12) NO_3^-; (13) $S_2O_3^{2-}$; (14) $S_2O_4^{2-}$; (15) $S_2O_5^{2-}$; (16) SO_3^-; (17) N_3^-; (18) CO_3^{2-}; (19) CH_3COO^-; (20) SO_4^{2-}; (21) H_2O_2; (22) HSO_4^-; (23) homocysteine; (24) ascorbic acid; (25) cysteine; (26) glutathione; (27) NaHS.

immediately with the addition of NaHS and reaches the maximum value in minutes (Figure S6a). The concentration-dependent fluorescence responses of **Lyso-NHS** with NaHS were next detected, and a linear relationship for the fluorescence intensity of **Lyso-NHS** versus hydrogen sulphide concentration was exhibited. As seen in Figure S6b,

an excellent linear correlation between the added NaHS concentration and the fluorescence intensity of **Lyso-NHS** at 555 nm was observed. The fast responses and excellent linear relationship provided a real-time quantitative detection method for hydrogen sulfide in biological samples. The fluorescence titration of **Lyso-NHS** in bovine serum with various analytes was also conducted, which showed **Lyso-NHS** to have a high selectivity for H_2S (Figure S7).

We then sought to examine whether **Lyso-NHS** can localize to the lysosome and sense H_2S in living cells. The cell permeability of **Lyso-NHS** was first investigated. MCF-7 cells were incubated with 5 μM **Lyso-NHS** for 30 min and exhibited no fluorescence (Figure 4a). Then the cells were incubated with 20 μM NaHS and after 5 min displayed enhanced green fluorescence (Figure 4b). After 10 min of incubation with NaHS, a higher turn-on fluorescence response can be observed (Figure 4c). These experiments indicate **Lyso-NHS** can be used to detect H_2S in living cells. The cytotoxicity of **Lyso-NHS** was examined toward MCF-7 cells cells by an MTT assay (Figure S8). The results showed that $>90\%$ MCF-7 cells survived after 12 h (5.0 μM **Lyso-NHS** incubation), and after 24 h the cell viability remained at $\sim$80%, demonstrating **Lyso-NHS** to be minimally toxic toward cultured cell lines.

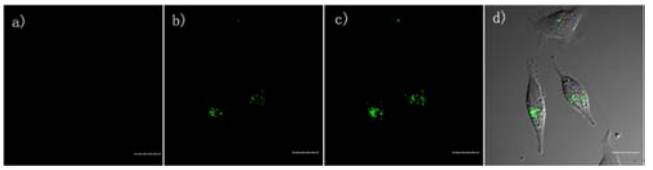


Figure 4. Time-dependent exogenous H_2S released from NaHS (20 μM) in MCF-7 cells stained with **Lyso-NHS** (5.0 μM) at 37 °C: (a) 0 min; (b) 5 min; (c) 10 min; (d) merged images of (c) and bright field. Scale bars = 20 μm.

The fluorescence localization was examined by costaining cells with a commercially available lysosome-specific dye Neutral Red (NR) (5 μM) (Figure 5a–b). As shown in Figure 5c, the fluorescence patterns of **Lyso-NHS** and the **NR** signals overlapped perfectly, indicating that the fluorescence response of **Lyso-NHS** to H_2S was localized at the lysosome. The intensity profiles of the linear regions of interest across MCF-7 cells stained with **Lyso-NHS** and **NR** also display close synchrony (Figure 5e). The high Pearson coefficient and overlap coefficient are 0.885 and 1.419, respectively (Figure 5f). These experiments indicate **Lyso-NHS** can specifically localize in lysosomes and be used to detect H_2S in lysosomes of living cells.

In conclusion, we have reported a 1,8-naphthalimide-derived fluorescent probe for H_2S based on thiolysis of

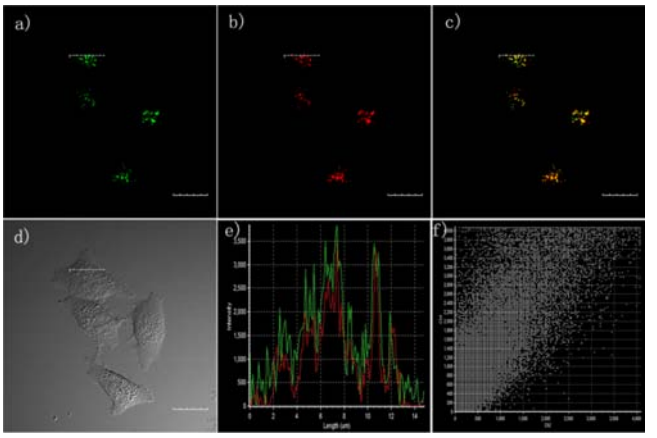


Figure 5. Lyso-NHS colocalizes to lysosomes in MCF-7 cells. (a) 5.0 μM Lyso-NHS with 20 μM of H_2S incubated 10 min at 37 °C (Channel 1: λ_{ex} = 450 nm, λ_{em} = 520–560 nm). (b) 5.0 μM **NR** (Channel 2: λ_{ex} = 559 nm, λ_{em} = 565–610 nm). (c) Merged images of (a) and (b). (d) Bright field image. (e) Intensity profile of regions of interest (ROI) across MCF-7 cells. (f) Intensity correlation plot of dyes **Lyso-NHS** and **NR**. Scale bars = 20 μm.

dinitrophenyl ether, known as **Lyso-NHS**. Due to rapid conversion to the fluorescent compound **5** by H_2S, a large fluorescence increase is obtained with emission centered at 555 nm in aqueous solution. Concomitantly, the solution color changes from colorless to yellow with the convenience and aesthetic appeal of a colorimetric assay. The probe has a high selectivity for H_2S over competitive analytes. This probe is applicable to H_2S detection in bovine serum and live cell imaging and has the ability to detect intracellular H_2S in lysosomes. The successful application of our probe to detect lysosomal H_2S will help to study the biological role of H_2S in lysosomes and encourage the appearance of new H_2S probes suitable for other organelle localizations.

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Supporting Information Available. Synthesis, characteristics and spectroscopic data of **Lyso-NHS**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.