Toward a Selective, Sensitive, Fast-Responsive, and Biocompatible Two-Photon Probe for Hydrogen Sulfide in Live Cells

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Supporting Information

ABSTRACT: Hydrogen sulfide has emerged as an exciting endogenous gasotransmitter in addition to nitric oxide and carbon dioxide. Noninvasive detection methods for hydrogen sulfide thus become indispensable tools for studying its diverse roles in biological systems. Accordingly, fluorescent probes for hydrogen sulfide have received great attention in recent years. A practically useful fluorescent probe for bioimaging of hydrogen sulfide should be selective, sensitive, fast-responsive, biocompatible, observable in the biological optical window, and capable of deep-tissue imaging. These sensing properties, however, are extremely difficult to achieve at the same time. Disclosed here is the two-photon fluorescent probe that meets all of these criteria. The probe belongs to a Michael acceptor system, which raised a serious selectivity issue over the competing biothiols such as cysteine and glutathione. We have addressed the selectivity issue by optimizing the electronic and steric interactions between biothiols and the probe, in addition to achieving very high sensitivity, fast-response, and biocompatibility. Also, the sensing mechanism suggested in the literature was revised. The probe thus enables us to image the endogenously produced hydrogen sulfide with negligible interference from other biothiols in live cells. The excellent sensing properties of the probe combined with its capability of bioimaging thus make it a practically useful tool for further studying biological roles of hydrogen sulfide.

Hydrogen sulfide (H₂S), existing with its hydrosulfide anion (HS⁻) under physiological conditions, has emerged as an intriguing endogenous gasotransmitter in addition to nitric oxide and carbon monoxide.¹ Recent studies indicate that H₂S is associated with diverse physiological processes; it is known to modulate neuronal activity, relax smooth muscle, regulate insulin release, induce angiogenesis, suppress inflammation and protect cells against oxidative stress.²⁻⁵ The diverse biological roles displayed by H₂S require efficient assay methods for measuring and imaging of its fluctuation in biological systems. A colorimetric assay based on the conversion of N,N-dimethyl-4-phenylenediamine to methylene blue by H₂S in the presence of the oxidizing agent Fe³⁺ in hydrochloric acid, so-called the methylene blue method, has been widely used.⁶ For continuous measuring of H₂S, electrochemical assays including potentiometric methods based on silver/sulfide ion-selective electrode membranes and polarographic methods are available.⁷,⁸ These methods typically require sample preparation steps that are not amenable to intracellular detection. For in vivo analysis, fluorescent probes that enable sensitive and noninvasive detection of H₂S become indispensable tools.

Accordingly, a number of fluorescent probes for H₂S have been developed in a recent few years,⁹⁻¹⁶ mostly through the reaction-based approach that utilizes the unique reaction characteristics of H₂S: the strong nucleophilicity and reduction capability. Several challenging issues in the development of fluorescent probes for H₂S are how to secure (i) selectivity over competing biothiols such as glutathione (GSH), cysteine (Cys), and homocysteine (Hcy), (ii) high sensitivity to detect the endogenously produced H₂S, (iii) fast response within a few minutes, (iv) biocompatibility such as cell permeability, intracellular stability, and low toxicity, and (v) signaling in the biological optical window. Unfortunately, the existing probes hardly satisfy all of these criteria, hampering further application of them to tackle biological issues. We wish to
Table 1. Pros and Cons of Known Fluorescent Probes for Hydrogen Sulfide (H2S)

<table>
<thead>
<tr>
<th>types of probe</th>
<th>reaction modes</th>
<th>sensing properties</th>
<th>limitations</th>
<th>key references</th>
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<tbody>
<tr>
<td>aryl azide/arylulfonyl azide</td>
<td>reduction of azide</td>
<td>sensitive; biocompatible</td>
<td>interference from Cys; GSH; slow response (&gt;1 h in the case of aryl azide); photochemically labile</td>
<td>17–26</td>
</tr>
<tr>
<td>disulfide–ester/enone–ester</td>
<td>disulfide exchange/Michael addition, followed by ester hydrolysis</td>
<td>selective; chemically labile and produce reactive nitrene species, which would limit their use to monitor rapidly fluctuating endogenous H2S levels in biological processes</td>
<td>low sensitivity”, slow response (&gt;0.5–1 h); potential interference from esterase</td>
<td>28–30</td>
</tr>
<tr>
<td>Cu(II) complex</td>
<td>demetalation</td>
<td>selective; fast response (&lt;1 min)</td>
<td>low sensitivity“</td>
<td>31, 32</td>
</tr>
<tr>
<td>aldehyde–enoate/aldoxime</td>
<td>tandem carbonyl and Michael addition</td>
<td>sensitive; biocompatible</td>
<td>interference from Cys and GSH and slow response (&gt;1 h) in the case of simple Michael acceptors</td>
<td>33, 49</td>
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“An exogenous source of H2S was used to obtain fluorescent cellular images.

disclose the new probing system that addresses all of the issues to a practically useful level.

Since the initial report by Chang and co-workers, several fluorescent probes based on H2S-mediated reduction of different aryl azides have been developed. These azide type probes, however, generally show slow response to H2S (>1 h for signal saturation), which would limit their use to monitor the rapid fluctuation of endogenous H2S levels in biological processes. This slow reactivity can be overcome by using more electrophilic azides such as arylsulfonyl azides (within a few minutes for signal saturation); however, in this case significant interference from other biothiols stands out, especially from glutathione (GSH) that is the most abundant biothiol. This “GSH-interference” in the azide-based sensing systems is a critical issue that needs to be overcome without sacrificing the reactivity. Moreover, aryl azides are photochemically labile and produce reactive nitrene species, which would generate undesired fluorescent products; the nitrene species generated from aryl azides indeed have been used for bioconjugation. Those probes based on the disulfide exchange or conjugate addition followed by intramolecular ester hydrolysis reactions developed by Xian and Qian, respectively, seem to be promising; however, for bioimaging application, their performance need to be secured from the undesired but ubiquitous cellular enzymatic ester hydrolysis. Also, their low sensitivity that requires an exogenous source of H2S for its cellular imaging needs to be improved. The probe based on the copper-sulfide precipitation process developed by Nagano and co-workers, following the original demetalation approach by Chang and co-workers, shows high selectivity and fast response; however, they also show low sensitivity to H2S, plausibly owing to the coordination-based sensing mechanism, and thus require an exogenous source of H2S to obtain its cellular images. He and co-workers recently disclosed a novel approach to H2S, which utilizes the conjugate addition capability of H2S to α,β-unsaturated carbonyl moiety. Their original probes, methyl o-formyl cinnamates conjugated to a pyrazole or a BODIPY dye, however, sense H2S with a marginal selectivity and low reactivity (1–8 h for signal saturation).

During the preparation of this manuscript, new probes for H2S with distinct optical properties including ratiometric behavior have been appeared, however, none of these probes also satisfied all the above-mentioned criteria together (high selectivity, high sensitivity, fast response, and bioimaging capability). For tissue imaging applications, additional issues such as photobleaching of the probe and autofluorescence from tissues become serious concerns. In this context, most of the existing probes also pose limitations as they are one-photon excitable at short wavelengths. On the contrary, the two-photon probes based on the nonlinearly intensity-dependent absorption process can provide higher spatial resolution in bioimaging compared with one-photon probes. Furthermore, the low-energy near-infrared excitation light enables deeper tissue penetration and also alleviates the photobleaching and autofluorescence issues. Cho and co-workers thus reported an aryl azide type two-photon probe for H2S, with which they were able to image the endogenously produced H2S in cells and in a sliced tissue for the first time. Since the probe is aryl azide-based one, it exhibits a rather slow response as well as a quite sizable interference from GSH at the cellular concentration level; this GSH-interference would degrade the reliability of the imaging data (see our own data in the following). A related system was further applied to reveal reduction in mitochondrial H2S production in Parkinson’s disease gene knockout astrocytes.

As noted above, the existing fluorescent probes still pose serious limitations with respect to the selectivity, sensitivity, response time, or cellular stability (Table 1). Herein, we wish to disclose a rational approach to address those challenging issues pertinent to the development of the practically useful fluorescent probe for H2S. Our interest in two-photon probes for biological analytes prompted us to exploit the tandem addition approach to develop a two-photon fluorescent probe for H2S, with particular missions of securing selectivity, sensitivity, and reactivity to a practically useful level.

RESULTS AND DISCUSSION

Design and Synthesis of Two-Photon Probe P1. Our initially designed probe P1 has a feature of (3-aryl-3-oxoprop-1-enyl)benzaldehyde derived from acedan, 1-(6-dimethylaminonaphthalen-2-yl)ethanone. Acedan is a well-known two-photon excitable dye with small molecular size, from which various bioimaging probes have been derived in recent years. The enone and aldehyde functionalities in P1 are expected to react with hydrogen sulfide either in its neutral H2S form or in anionic SH− form, possibly in the tandem carbonyl addition and the Michael addition fashion. We expected that P1 would be nonfluorescent because it is a conjugated aromatic aldehyde of which the nonradiative n→π* transition would be the lowest energy transition. In contrast, tandem addition of H2S to the formyl group and the enone β-carbon would generate the acedan moiety that is two-photon excitable and highly fluorescent (Scheme 1a).

P1 can be synthesized from an acedan derivative 6 by the conventional aldol condensation with 2-([1,3]dioxolan-2-yl)benzaldehyde 8, followed by deprotection of the acetal group. The acedan analogue 6 was synthesized from 6-bromo-2-naphthol 5 according to the improved synthetic route developed by us (Scheme 1b). DOI: 10.1021/acs.analchem.5b00366w Anal. Chem. 2015, 87, 1188–1195
As expected, P1 showed little fluorescence when excited at 378 nm but gave strong fluorescence with $\lambda_{\text{max}}$ at 524 nm upon treatment with H$_2$S (in the form of Na$_2$S) in pH 7.4 HEPES buffer at room temperature (Figure 1a). The signal enhancement was rather fast even in the HEPES buffer, showing 97%-signal saturation after 15 min when 10 $\mu$M of the probe and 100 $\mu$M of Na$_2$S were used.

**Figure 1.** (a) Fluorescence titration of P1 (10 $\mu$M) with Na$_2$S (100 $\mu$M), (b) Time-dependent fluorescence intensity changes of P1 (10 $\mu$M) upon addition of biothiols (Na$_2$S, 100 $\mu$M; GSH, 10 $\mu$M; Cys, 200 $\mu$M; Hcy, 50 $\mu$M). The spectra were recorded in pH 7.4 HEPES buffer containing 1% CH$_3$CN at 25 $^\circ$C under excitation at 378 nm.

On the basis of the encouraging results, we evaluated the response of P1 toward major biothiols, GSH and Cys at their physiologically relevant concentrations of 10 mM and 200 $\mu$M respectively. 47,48 Disappointingly, P1 showed a poor selectivity toward the biothiols under the conditions (Figure 1b), particularly toward Cys whose cellular concentration is lower than that of GSH. Thus, in the case of the Michael acceptor probes, this “Cys-interference” becomes a critical issue. It is evident that those probes based on simple Michael acceptors are not suitable for the selective imaging of H$_2$S in living systems. During our study, although a more reactive enone derivatives based probe was reported, 49 which showed an improved selectivity over the competing biothiols but only in unusually high content of organic solvent (PBS buffer containing 40% acetonitrile). We have found that competition from other biothiols becomes significantly suppressed as the content of organic solvent increases (Figure S1 in the Supporting Information); obviously such a buffer media with high content of organic solvent is not compatible with living systems.

**Evolution of P1 into P2 and Further into P3 that Show Excellent Selectivity over Other Biothiols.** Although P1 shows outstanding sensing properties, significant interference from biothiols is a serious drawback. To address the low selectivity drawback met with P1 and the (3-aryl-3-oxoprop-1-enyl)benzaldehyde type probes, in general, in biocompatible aqueous media, we focused on the steric and electronic effects on the thiol conjugate addition. Any steric strain increased near the enone $\beta$-carbon, the electrophilic site for thiol nucleophiles, would hinder the conjugate addition by sterically demanding thiols such as GSH. In contrast, increasing the steric crowding around the enone $\beta$-carbon would hinder little the conjugate addition of H$_2$S that is the least sterically demanding biothiol. To increase the steric hindrance around the enone $\beta$-carbon, we introduced 3-methoxy as an additional ortho-substituent at the $\beta$-phenyl ring of P1. In this case, the resulting electronic perturbation in the $\beta$-phenyl moiety would also influence the electrophilicity of enone $\beta$-carbon toward the sulphydryl nucleophile.

To identify substituent effects on the electrophilicity at the $\beta$-carbon, we carried out computational calculations for simplified probe systems P1’–P3’ where the 2-hydroxyethylamino group in the probe was replaced with $N$-methylamino group (Table S1 in the Supporting Information). To our delight, the introduction of the methoxy group also increased the positive charge at the enone $\beta$-carbon ($0.013$ eV in the case of P1’ versus $0.008$ eV in the case of P2’), which would make it more electrophilic. On the basis of these results, we introduced an additional methoxy group at the para-position of phenyl ring, as in the model compound P3’, gratifyingly, in this case the enone $\beta$-carbon becomes more electropositive ($+0.013$ eV) than that of P2’, that is, even more electrophilic than that of P2’. On the basis of the calculation results, we synthesized P2 and P3 (Figure 2), 3-methoxy and 3,5-dimethoxy derivatives of...
P1, by adopting the similar aldol strategy used in the synthesis of P1, starting from the same acetal analogue and the corresponding benzaldehyde counterparts synthesized separately (see the Supporting Information).

Also, probe P4 was prepared as a reference of aryl azide type probes (Figure 2), and its selectivity toward biothiols was evaluated for a comparison purpose.

With P2 and P3 at hand, we evaluated their selectivity and sensitivity toward the biothiols. When nonfluorescent P2 (10 μM) was treated with H$_2$S (100 μM) in pH 7.4 HEPES buffer, the solution emitted strong fluorescence. As expected from the calculation results, P2 showed a faster response to H$_2$S than P1: After 5 min, P2 gained 92%-signal saturation, whereas P1 gained only 78%-saturation (Figure 3a). To our delight, the interference from Cys is also significantly reduced at their biologically relevant concentrations, in addition to a large improvement from the case of P1 in reducing the interference from GSH probably due to increased steric hindrance at the enone β-carbon of P2. The reduced Cys-interference suggests that an electronic factor is also influencing on the conjugate addition of Cys to P2, in addition to the steric factor. Although further scrutinization is necessary, it seems that the carboxyl group of Cys, in its anionic form, can have repulsive interaction against the electron-rich methoxy-substituted β-phenyl moiety in P2.$^{50}$ This finding is crucial to minimize the problematic Cys-interference in sensing H$_2$S through the Michael addition reaction. Gratifyingly, an even better selectivity was achieved with the dimethoxy-substituted P3 without losing its reactivity (Figure 3b); thus, the interference from Cys is dramatically reduced, showing only 7%-enhancement with respect to that of H$_2$S at their physiologically relevant concentrations after 10 min. This minor interference from Cys can be further suppressed when the media was changed to pH 7.4 HEPES buffer containing 10% FBS (fetal bovine serum), a common cell and tissue culture media (Figure 3c).$^{51}$ The negligible interference from the most interfering Cys in the biological culture media promises further application of P3 to imaging of H$_2$S in cells and tissues. As mentioned above, the interference from biothiols significantly decreases as the polarity of media decreases; therefore, an even better selectivity is expected to be obtained inside cells or tissues, whose media are known to be less polar than the neat aqueous buffer solution. The above results demonstrate that in sensing H$_2$S we can suppress the severe interference from biothiols such as Cys and GSH to a minimum level, by tuning steric and electronic effects of the Michael acceptor in the aldehyde–enone type probes. With the optimized probe P3, we can also realize fast response toward H$_2$S.

At this point, it should be noted that an aryl azide probe, P4, shows much slower response (signal saturation requires more than 2 h; Figure S5 in the Supporting Information) as well as poor selectivity toward biothiols under otherwise the same conditions (Figure 3d). The selectivity of such aryl azide type probes can be improved, by reducing their sensitivity toward GSH, in particular, using electron-rich aryl azides; however, in this case, their reactivity toward hydrogen sulfide should be sacrificed.$^{53}$ Such drawbacks should impede use of aryl azide-type probes for imaging of hydrogen sulfide in living systems. In contrary, the optimized aldehyde–enone type probe P3 shows excellent selectivity toward H$_2$S over the competing biothiols and various other substrates such as amino acids (Ala, Glu, Lys, Met), lipoic acid (a disulfide), NO$_2^-$, SO$_4^{2-}$, S$_2$O$_3^{2-}$, SCN$^-$, H$_2$O$_2$, Γ, and citrate (Figure 3e). P3 also shows a good linear response to [H$_2$S] down to 0.1 μM (Figure 3f), offering a very high sensitivity toward H$_2$S. The limit of detection was determined to be 50 nM on the basis of signal-to-noise ratio of three (Figure S8 in the Supporting Information). This limit of detection value is much lower than those observed by most of the existing small-molecule fluorescent probes for H$_2$S.$^{52}$ The very high sensitivity of P3 to H$_2$S indeed allowed us to detect the endogenously produced H$_2$S, a difficult task by most of the known probes.

P3 shows the maximum fluorescence intensity at neutral pH, but moderate intensity at lower or higher pH values (Figure S9 in the Supporting Information). The decreased fluorescence in either acidic or basic conditions are owing to (i) an equilibrium shift from the more nucleophilic HS$^-$ to the less nucleophilic H$_2$S under acidic conditions and (ii) a competition from HO$^−$ that can also add to the formyl group under basic conditions.

**Sensing Mechanism Revisited.** At this point, we tried to identify the presumed reaction product of the probe (P3) with

![Figure 3. Time-dependent fluorescence intensity changes of (a) P2, (b,c) P3, and (d) P4 (each at 10 μM), upon addition of biothiols at biologically relevant concentrations (Na$_2$S, 100 μM; GSH, 10 mM; Cys, 200 μM; Hcy, 50 μM; Na$_2$S was used as a source of H$_2$S/HS$^-$). The spectra were recorded in pH 7.4 HEPES buffer containing 1% CH$_3$CN (10% FBS in the case of c) at 25 °C under excitation at the maximum absorbance wavelength, $\lambda_{max}$ (P2, 370 nm; P3, 375 nm in the case of b and 360 nm in case of c because of spectral change in different media; P4, 339 nm). (e) Selective fluorescence response of P3 (10 μM) toward Na$_2$S (100 μM) over other common bioanalytes (Ala, Glu, Lys, Met), lipoic acid, NO$_2^-$, SO$_4^{2-}$, S$_2$O$_3^{2-}$, SCN$^-$, H$_2$O$_2$, Γ, and sodium citrate; each at 100 μM concentration), obtained after 30 min of incubation at 25 °C. (f) Fluorescent titration of P3 (10 μM) with Na$_2$S (0–50 μM), recorded after 5 min of each addition; the inset shows the fluorescence intensity depending on [Na$_2$S]. The spectra in panels e and f were recorded in pH 7.4 HEPES buffer containing 1% CH$_3$CN at 25 °C under excitation at 375 nm.](image-url)
hydrogen sulfide, the cyclic sulfide compound A in Scheme 2, but failed. We have found that a major product that can be identified on silica gel plate is not stable for a prolonged period of time; it can be isolated by fast column chromatography on silica gel and purified by recrystallization from a mixture of chloroform and hexane (Scheme S5 in the Supporting Information). Surprisingly, the hydrogen sulfide adduct was not the cyclic sulfide A as suggested in literature but the carbocyclic compound (B) that was identified by extensive NMR analyses (1H, 13C, DEPT-135, COSY, HETCOR, and HMBC).

Again, from the emission spectra of P3 with Na2S and the isolated product B (Figure S12 in the Supporting Information), it was observed that both of them have same emission wavelength, supporting that carbocyclic compound B was the reaction product of P3 with H2S in pH 7.4 HEPES buffer also.

Another important finding is that the acetal 17 which lacks aldehyde functionality does not respond to hydrogen sulfide in pH 7.4 HEPES buffer (Figure S13 in the Supporting Information); in other words, the direct Michael addition of hydrogen sulfide at the enone moiety is not effective to give a fast fluorescence response similar to that with P3. Therefore, it should be concluded that hydrogen sulfide makes an initial adduct formation with the aldehyde group in P3 to accelerate the intramolecular Michael addition to the enone moiety, of which enolate is stabilized by the intramolecular aldol condensation to the aldehyde group, producing the cyclic compound B as the final fluorescent product (Scheme 2).

Therefore, the sensing mechanism of hydrogen sulfide with (3-aryl-3-oxoprop-1-enyl)benzaldehyde type probes should be revised to be the Michael addition promoted by initial formation of sulfide—aldehyde adduct followed by subsequent aldol condensation that stabilize the anionic conjugate adduct. This mechanism is reasonable in a view of the well-established aldol reaction between an enolate and an aldehyde.

**Fluorescence Imaging of Endogenous H2S in Cells.**

Given that P3 is highly selective, fast-responsive, very sensitive, and two-photon excitable, we set out to demonstrate its potential in bioimaging of H2S. Prior to the bioimaging studies, a good level of cell viability in the presence of P3 was confirmed by MTT assay (Figure S15 in the Supporting Information).

We evaluated the sensing capability of H2S in live cells by two-photon fluorescence microscopy. Our major concern was...
whether P3 could detect the endogenously produced H$_2$S because most of the reported systems required an exogenous source of H$_2$S to obtain its cellular images. So far, only a few probes are shown to detect the endogenous H$_2$S in cells,\textsuperscript{20,21,33,35,39,49} but, in these cases, fluorescence from interfering biothiols (GSH and Cys) seems to be also included, as inferred from the poor selectivity observed by the aryl azide-based probe P4. To see how such interference from other biothiols results in the cell imaging data, we have also evaluated P1 that shows low selectivity among the biothiols under their biologically relevant concentrations. The cellular imaging of H$_2$S with P3 and P1 was carried out by two-photon microscopy (TPM) in the absence and presence of exogenous sources of H$_2$S or inhibitors that suppress the intracellular production of H$_2$S.

HeLa cells incubated with P3 (10 \mu M, 30 min) show strong fluorescence, apparently from the endogenous H$_2$S (Figure 4-a(ii, upper row), whereas the probe-untreated cells show dark image (Figure 4a-i, upper row). As P3 shows negligible interference from other biothiols in HEPES buffer containing 10% FBS (Figure 3d), we may assume that the probe-treated cell image is predominantly caused by the endogenous H$_2$S. When the cells were incubated with an exogenous H$_2$S sources such as Na$_2$S, GSH or Cys prior to the probe incubation, there was a substantial increase in the fluorescence intensity in all the cases (Figure 4a-iii, upper row; Figure S14 in the Supporting Information). It is known that GSH is converted to Cys by \gamma-glutamyl transpeptidase,\textsuperscript{53} and Cys is converted to H$_2$S by both cystathionine \beta-synthase (CBS) and cystathionine \gamma-lyase (CSE).\textsuperscript{54} On the contrary, the fluorescence signal decreased significantly when the cells were preincubated with P3, which is a selective probe,\textsuperscript{55} PAG acts as an inhibitor of CSE, a major endogenous source of H$_2$S.\textsuperscript{56,57} From our imaging results with P3, it is also evident that P3 is more effective (1.4-times) than PMA in suppressing the endogenous H$_2$S level in HeLa cells (Figure 4b). The p-values for the intensity bar of both the PMA/P3 and PAG/P3 compared to the control are less than 0.05, strongly supporting against null hypothesis.

The imaging data obtained with the less selective probe P1 (Figure 4, lower row) are brighter than those obtained with P3 (Figure 4, upper row). In particular, a comparison of the image data obtained with P3 and P1 in the presence of PAG that strongly suppresses H$_2$S generation (Figure 4v) clearly show significant interference from other biothiols in the case of less selective probe, P1. Now, it is evident that the cell imaging data obtained with less selective probes do not properly represent the H$_2$S levels in biological systems.

**EXPERIMENTAL SECTION**

Details of experimental procedures and methods are given in the Supporting Information.

**Synthesis of the Probes P1–P4.** Only the procedures for the aldol condensation between the acedan derivative 6 and aldehyde 16, followed by deprotection in the synthesis of P3 (Scheme S3 in the Supporting Information) are described here, and the all other synthetic procedures are given in the Supporting Information.

A solution of acedan derivative 6 (230 mg, 1.003 mmol) and aldehyde 16 (477 mg, 2.002 mmol) in absolute ethanol (5 mL) at room temperature was treated with a catalytic amount of sodium hydroxide (23 mg), and the resulting mixture was heated to reflux for 3 h. After being cooled to room temperature, the reaction mixture was condensed by rotary evaporation, and the residue was treated with water (10 mL) and then extracted with dichloromethane (10 mL \times 3). The combined organic phase was dried over anhydrous MgSO$_4$ and concentrated, and the crude residue was purified by column chromatography (ethyl acetate/hexane = 1/1) to give compound 17 (383 mg, 85%).\textsuperscript{1}H NMR (CDCl$_3$, 300 MHz, 298 K): $\delta$ 8.34 (s, 1H), 8.09 (d, $J$ = 15.9 Hz, 1H), 7.96 (d, $J$ = 8.7, 18 Hz, 1H), 7.85 (d, $J$ = 15.9 Hz, 1H), 7.64 (d, $J$ = 9.0 Hz, 1H), 7.56 (d, $J$ = 8.7 Hz, 1H), 6.92 (d, $J$ = 2.4 Hz, 1H), 6.86 (dd, $J$ = 8.7 Hz, 2.1 Hz, 1H), 6.75 (d, $J$ = 1.8 Hz, 1H), 6.52 (d, $J$ = 2.4 Hz, 1H), 6.04 (s, 1H), 4.22–4.16 (m, 2H), 4.14–4.04 (m, 2H), 3.94–3.89 (m, 5H), 3.87 (s, 3H), 3.37 (t, $J$ = 5.1 Hz, 2H).\textsuperscript{13}C NMR (CDCl$_3$, 75 MHz, 298 K): $\delta$ 190.9, 161.7, 160.9, 148.3, 139.5, 138.0, 136.8, 132.3, 131.0, 130.6, 126.4, 126.3, 125.9, 125.6, 118.8, 117.2, 104.2, 103.1, 101.4, 99.6, 65.6, 61.2, 56.0, 55.7, 45.9.

A solution of acetal 17 (383 mg, 0.852 mmol) in CH$_3$CN (7.5 mL) at 0 °C was treated with concentrated HCl (0.5 mL) dropwise. After it was stirred for 5 min at 0 °C, the reaction mixture was quenched by a saturated NaHCO$_3$ solution (10 mL) and then extracted with dichloromethane (10 mL \times 3). The combined organic phase was dried over anhydrous MgSO$_4$ and concentrated to give yellow residue, which was purified by column chromatography (ethyl acetate/hexane = 1/1) to afford P3 (300 mg, 87%) as an orange solid. Further purification by recrystallization (chloroform/hexane = 1/3, 4 mL) afforded pure P3 (270 mg, 78%), which was used for all the sensing experiments.\textsuperscript{1}H NMR (CDCl$_3$, 300 MHz, 298 K): $\delta$ 10.33 (s, 1H), 8.32 (s, 1H), 8.23 (d, $J$ = 15.3 Hz, 1H), 7.97 (d, $J$ = 8.4 Hz, 1H), 7.69 (d, $J$ = 8.7 Hz, 1H), 7.60 (d, $J$ = 8.4 Hz, 1H), 7.35 (d, $J$ = 15.6 Hz, 1H), 7.07 (d, $J$ = 1.5 Hz, 1H), 6.91 (d, $J$ = 8.1 Hz, 1H), 6.80 (s, 1H), 6.72 (s, 1H), 3.95–3.90 (m, 8H), 3.42 (t, $J$ = 4.8 Hz, 2H).\textsuperscript{13}C NMR (CDCl$_3$, 75 MHz, 298 K): $\delta$ 191.7, 189.1, 161.6, 160.4, 148.5, 138.2, 137.4, 135.1, 131.8, 131.2, 130.7, 130.0, 126.5, 126.4, 125.5, 122.2, 118.9, 104.3, 104.2, 103.5, 61.3, 56.3, 56.0, 45.8. HRMS (m/z): [M$^+$] calcd. for C$_{16}$H$_{23}$NO$_5$, 405.1576; found, 405.1574 [M$^+$].
argin) were prepared every time before experiments and kept under a closed vial. For spectrometric measurements, a required amount of the thiol stock solution was added to a probe solution (10 μM) taken in a cuvette (3 mL) in such a way that the final volume increment remains within less than 1% of total volume. After addition of thiols, the cap of the cuvette was closed immediately and shook gently to mix thoroughly, and then incubated for the given time prior to spectrometric measurements.

Imaging Experiments. Cell samples were prepared from HeLa human cervical carcinoma cells (for details of sample preparation, see the Supporting Information).

A Ti:sapphire laser (Chameleon Ultra II, Coherent) with 140 fs pulse width and 80 MHz pulse repetition rate was used as an excitation light source for TPM imaging. Output from the source was first passed through a half wave plate and a polarizer for power control. For advanced performance, a TPM instrument was equipped with upright microscope (BX51, Olympus) that has 20x and 40x objective lens (XLUMPLFLN, NA 1.0, Olympus) with 1.0 numerical aperture (NA). The objective lens focused the excitation beam into cell and tissue samples. The excitation focus was scanned in the x–y plane of the sample by resonant (GSI Lumonics, 8 kHz resonant frequency) and galvanometric (6215H, Cambridge Technology) scanners and in the z-axis by an objective translator (P-725.4CL, PI) through the two dichroic mirrors (102SDCSP, 680DCSP, Hamamatsu). Emission light from the sample was collected back by the objective lens and was reflected on a dichroic mirror (680DCLP, Chroma) toward photomultiplier tubes (PMTs, H7421-40, Hamamatsu). Signals from the PMTs were collected by a frame grabber (Alta, Bitflow), and images were displayed in real time. Images were quantified by performing a software function of “maximum intensity projection” in the Matlab and Amira tools, which counts the mean pixel intensity after excluding an upper and a lower common threshold values for all images. We took images from several regions of the same sample for each experiment. Finally, the average intensity of those images was reported as the image intensity of that particular sample (as shown in Figure S14 in the Supporting Information). Two-photon excitation wavelength was 880 nm. Laser powers were 3 mW at the focal surface.

CONCLUSION

Hydrogen sulfide has emerged as an exciting endogenous gasotransmitter together with nitric oxide and carbon dioxide, which necessitate efficient detection tools such as fluorescent probes. Recent efforts disclosed several unique fluorescent probes for hydrogen sulfide, mostly by exploring its strong nucleophilicity and reduction capability. A practically useful probe of hydrogen sulfide for bioimaging application should be selective (against competing biothiols), sensitive (enough to detect endogenous hydrogen sulfide, fast-responsive, biocompatible (stable to hydrolytic enzymes and nontoxic), observable in the biological optical window. All of these properties, however, are extremely difficult to secure at the same time, limiting further applications of the known probes. Through a rational approach we have developed a two-photon fluorescent probe that meets all of the criteria. The probe belongs to a Michael acceptor system, which raised a serious selectivity issue, in particular toward cysteine and glutathione. We have addressed the critical selectivity issue by optimizing the steric and electronic interactions between the biothiols and probe.

The probe that has an electron-rich and sterically demanding Michael acceptor shows near-complete selectivity toward hydrogen sulfide over the competing biothiols, along with excellent other sensing properties including very high sensitivity, fast-response, and bioimaging capability. Also, the literature sensing mechanism suggested for such (3-aryl-3-oxoprop-1-enyl)benzaldehyde type probes has been revised to be tandem conjugate addition promoted by initial formation of the sulfide–aldehyde adduct and an intramolecular aldol reaction. The probe enabled us to image the endogenously produced hydrogen sulfide with negligible interference from other biothiols in live cells by two-photon microscopy. The excellent sensing properties of the probe combined with its bioimaging capability thus make it a powerful tool for studying the biological roles of hydrogen sulfide. Also, the rational approach shown here can be applied to develop fluorescent probes with additional features such as ratiometric or fluorescence resonance energy transfer properties, which are necessary for further biological investigations.

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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