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o-Fluorination of Aromatic Azides Yields Improved Azido-Based Fluorescent Probes for Hydrogen Sulfide: Synthesis, Spectra, and Bioimaging

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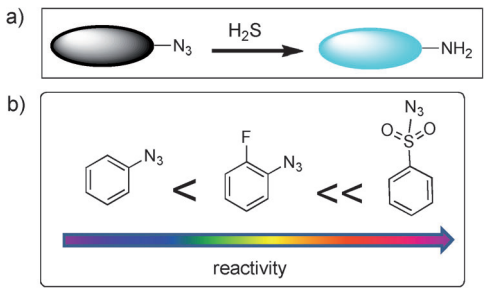
Abstract: Hydrogen sulfide (H_2S) is an endogenously produced gaseous signaling molecule with multiple biological functions. To visualize the endogenous in situ production of H_2S in real time, new coumarin- and boron-dipyrromethene-based fluorescent turn-on probes were developed for fast sensing of H_2S in aqueous buffer and in living cells. Introduction of a fluoro group in the *ortho* position of the aromatic azide can lead to a greater than twofold increase in the rate of reaction with H_2S. On the basis of *o*-fluorinated aromatic azides, fluorescent probes with high sensitivity and selectivity toward H_2S over other biologically relevant species were designed and synthesized. The probes can be used to in situ to visualize exogenous H_2S and D-cysteine-dependent endogenously produced H_2S in living cells, which makes them promising tools for potential applications in H_2S biology.

Keywords: bioimaging · dyes/pigments · fluorescent probes · FRET · hydrogen sulfide

Introduction

Hydrogen sulfide (H_2S) is an important endogenous signaling molecule (gasotransmitter) along with nitric oxide (NO) and carbon monoxide (CO).[1] In vivo, H_2S can be enzymatically generated in many organs (heart, liver, kidney, brain, ileum, uterus, etc.) and tissues (connective tissues, adipose tissues, etc.).[2] The production of endogenous H_2S and exogenous administration of H_2S have been demonstrated to exert protective effects in many pathologies, including preservation of mitochondrial function, relaxation of vascular smooth muscles, inhibition of apoptosis, intervention of neuronal transmission, regulation of inflammation, and stimulation of angiogenesis.[3] Studies have shown that the endogenous concentration of H_2S is correlated with numerous diseases, including the symptoms of Alzheimer's disease, Down's syndrome, diabetes, and liver cirrhosis.[4] Although H_2S has been recognized to be linked to numerous physiological and pathological processes, many of its underlying molecular events remain unknown. Therefore, research to develop efficient methods for fast detection of H_2S in living biological systems is of significant value.

Traditionally, the major methods for H_2S detection are colorimetry, electrochemical assay, gas chromatography, and sulfide precipitation.[5] However, these methods are destructive and require tedious preparation sequences. Fluorescence-based methods have recently emerged as a highly desirable and sensitive approach for in situ and real-time visualization of H_2S in living biological systems.[6–11] These probes, which are largely based on specific H_2S-induced reactions, include reduction-based probes,[6–8] probes based on precipitation of metal sulfides,[9] and nucleophile-based probes.[10] Previously, we developed several H_2S probes that can be used to in situ visualize the chiral-sensitive cysteine-dependent H_2S production in living cells.[11] Despite the great success of these fluorescent probes for H_2S biology,[6–11] most of them showed a delayed response (typically



Scheme 1. Schematic drawing showing a) the fluorescent turn-on mechanism based on the reduction of azide to amine with H_2S and b) the reactivity of azido-based compounds toward H_2S.

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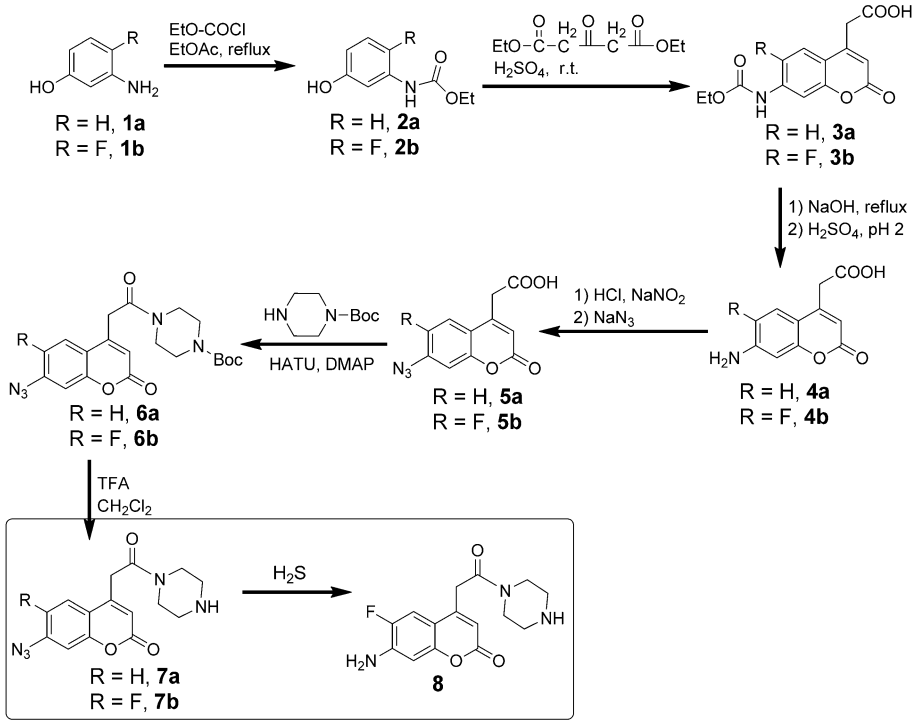
>20 min) toward H_2S. Herein, we intended to develop fast-response H_2S probes based on the reduction of aromatic azides by H_2S. To this end, we rationally designed azido-based H_2S fluorescent probes with improved reaction rate by introduction of a fluoro group in the *ortho* position of aromatic azides (Scheme 1). The developed coumarin- and boron-dipyrromethene (BODIPY)-based fluorescent probes are highly selective and show fast response to H_2S in buffer and in living cells.

Results and Discussion

Rational Design and Synthesis of Coumarin-Based Probes

H_2S probes functioning through the reduction of azido groups to amines are known in the literature;[6,7] however, most of them, based on aromatic azides,[6a] do not respond fast enough for real-time H_2S detection.[7] Sulfonyl azide can react with H_2S very fast but suffers from relatively low selectivity in living biological systems.[6b] Therefore, we proposed that electron-withdrawing groups in the *ortho* position of the aromatic azide could improve its reaction rate with H_2S (Scheme 1).

Firstly, we chose coumarin as model fluorophore because of its good quantum yield, water solubility, and easy preparation. As shown in Scheme 2, treatment of commercially available 3-aminophenol (**1a**) and 3-amino-4-fluorophenol (**1b**) with ethyl chloroformate led to the formation of carbamate **2**. Coumarin **3** was formed by a Pechmann reaction of carbamate **2** with diethyl-1,3-acetone dicarboxylate in 70% H_2SO_4.[12] Compound **3** was hydrolyzed in refluxing 0.1 M NaOH, and the resulting free amine **4** was transformed into azido-based coumarin **5** by means of a Sandmeyer reaction. To improve the cell permeability of the probe,[6a] a piperazidine unit was coupled with **5** to provide **7**, which can be further modified through coupling reactions with the piperazidine linker. The structures of probes **7a** and **7b** were confirmed by ^1H, ^{19}F, and ^{13}C NMR spectroscopy and high-resolution mass spectrometry (HRMS).



Scheme 2. Synthesis of fluorescent probes **7a** and **7b** for H_2S detection.

Spectroscopic Characterization of Coumarin-Based Probes

The fluorescence spectra of probes **7a**, **7b** and their reaction with H_2S (Na_2S was used as an equivalent) were investigated. As shown in Figure 1, both **7a** and **7b** did not display noticeable fluorescence in phosphate-buffered saline (PBS, 20 mM, pH 7.4). On treatment of the probes with H_2S, more than 100-fold fluorescence enhancement was obtained for both **7a** and **7b**, which implies that azide moieties were reduced by H_2S to produce strongly fluorescent compound **8** (Scheme 2). The mass spectrum also indicated that the reaction produced **8** (ESI-MS $[M+H]^+$: found 306.19; calcd 306.12).

To obtain the kinetic parameters, the fluorescence signal at 450 nm was plotted as a function of time (Figure 1c). This revealed that the fluorescence intensity reached the steady state after about 40 and 100 min at 25 °C for **7b** and **7a**, respectively (**7a**: $\Phi=0.002$, $\Phi=0.72$ after reaction with H_2S; **7b**: $\Phi=0.012$, $\Phi=0.75$ after reaction with H_2S). The pseudo-first-order rate constant k_{obs} was found to be 10.4×10^{-4} and $4.82\times10^{-4}\,s^{-1}$ for **7b** and **7a**, respectively, by fitting the fluorescence intensity data with a single-exponential function. The reaction rates k_2 under the test conditions were calculated as 4.16 and $1.93\,M^{-1}s^{-1}$ for **7b** and **7a**, respectively. Thus, introduction of a fluoro group in the *ortho* position of the aromatic azide led to an approximately 2.2-fold improvement of the reaction rate with H_2S. Since the pH in mitochondria normally lies in the weakly alkaline range (ca. 8.0),[13] we investigated the reaction kinetics of **7b** with H_2S in a pH 8.0 buffer. The reaction half-time of 8.35 min (Figure 1d) implies that the probe could be used for fast H_2S detection in mitochondria in living cells.[11c] To the best of our knowledge, this is the first investigation of the reactivity of *o*-fluorinated aromatic azides toward H_2S.

In solution, probe **7b** exhibited a noticeable absorbance at

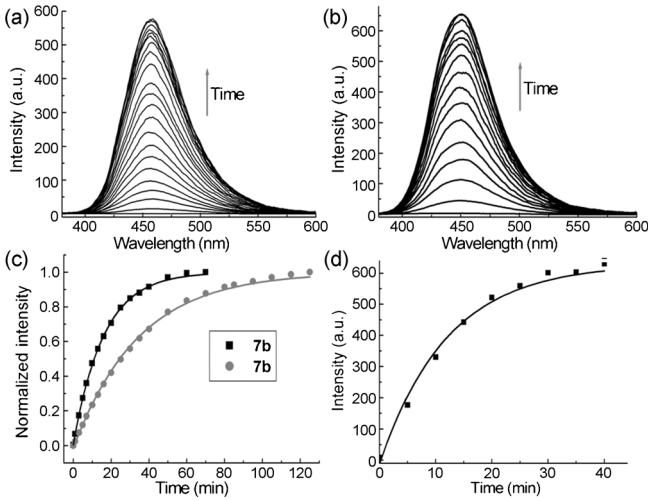


Figure 1. Time-dependent fluorescence spectra of a) **7a** (2.5 μM) and b) **7b** (2.5 μM) on reaction with H_2S (250 μM) at 25 °C. Excitation at 350 nm. c) The relationship between fluorescence intensity at 450 nm and reaction time of **7** (2.5 μM) on treatment with H_2S (250 μM) at 25 °C. d) The relationship between fluorescence intensity at 450 nm and reaction time of probe **7b** (2.5 μM) on treatment with H_2S (250 μM) at 37 °C and pH 8.0. The solid line represents the best fit to a single-exponential function.

335 nm, which shifted to 350 nm after treatment with H_2S (Supporting Information, Figure S1). A well-defined isosbestic point at 341 nm was observed, indicative of a clean chemical reaction between **7b** and H_2S.

We investigated the dependence of the fluorescence signal of probe **7b** on H_2S concentration (Figure 2). As expected, a strong emission peak at 450 nm could be detected when the reaction mixture was excited at 350 nm. Further data analysis revealed a good linear relationship ($r = 0.99$) between the fluorescence signal at 450 nm and the concentration of H_2S (0–150 μM). The detection limit was determined to be 0.61 μM by setting the signal-to-noise ratio to 3:1.[11] The results demonstrated that probe **7b** could react with H_2S both qualitatively and quantitatively.

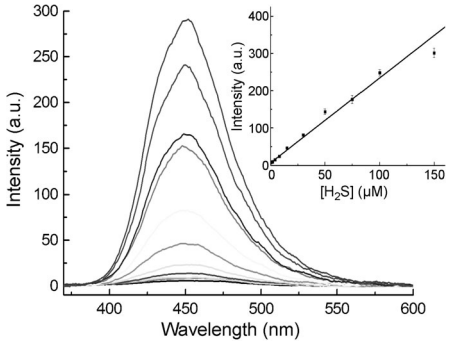


Figure 2. Fluorescence spectra of **7b** (2.5 μM) on reaction with different concentrations of H_2S (0–150 μM). The linear relationship between fluorescence intensity at 450 nm and H_2S concentration is shown in the inset. Error bars are plus/minus standard deviation. The solid linear line represents the best fit. The fluorescence intensity was acquired at 37 °C after incubation of the probe with H_2S for 30 min (excitation at 350 nm).

A major challenge of H_2S detection in biological systems is to develop highly selective probes that exhibit distinct responses to H_2S over other cellular molecules. To investigate the selectivity of probe **7b**, various biologically relevant species were incubated with probe **7b** in PBS and their fluorescence responses measured (Figure 3). These biologically relevant species included reactive sulfur species (glutathione (GSH), cysteine (Cys), HSO_3^{2-}), reactive oxygen species (H_2O_2), reactive nitrogen species (NO_2^-), other anions (HCO_3^-, I^-, etc.), and cations (Zn^{2+}, Ca^{2+}, Mg^{2+}). The fluorescence increases of the test molecules, however, were far lower than that of H_2S. Small-molecules thiols such as GSH and Cys in concentrations of 5 and 1 mM, respectively, did not trigger significant fluorescence enhancement. Thus, according to fluorescence spectra, probe **7b** is highly selective toward H_2S over other biologically relevant thiols and anions.

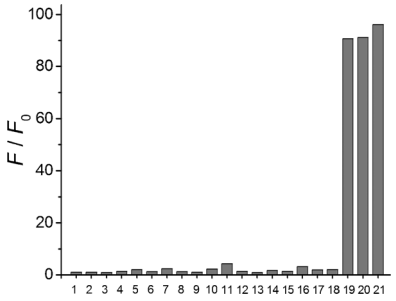


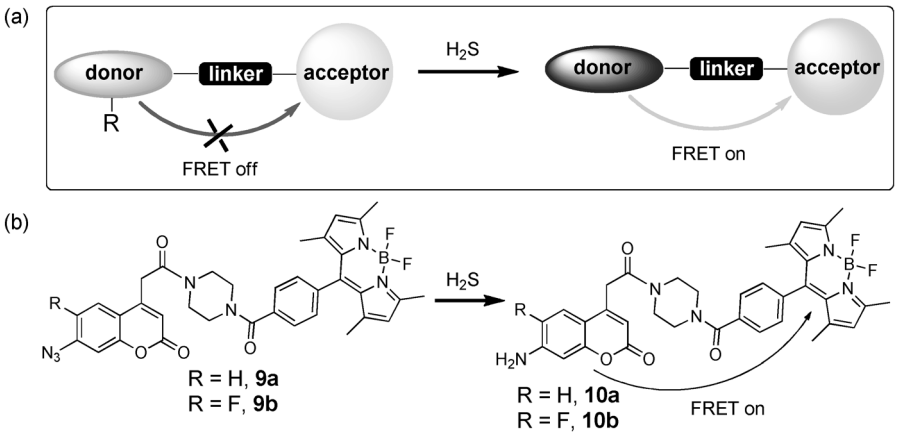
Figure 3. Relative fluorescent response of probe **7b** (2.5 μM) to various biologically relevant species (1 mM) and GSH (5 mM) in PBS (pH 7.4) for 30 min. 1, probe **7b** alone; 2, $MgSO_4$; 3, $CaCl_2$; 4, NaF; 5, NaI; 6, NaOAc; 7, NaN_3; 8, $NaHCO_3$; 9, $Na_2C_2O_4$; 10, Na_2SO_3; 11, $NaHSO_3$; 12, $Zn(OTf)_2$; 13, $NaNO_2$; 14, ascorbic acid; 15, H_2O_2; 16, 2-mercaptoethanol; 17, GSH; 18, Cys; 19, GSH + Na_2S; 20, Cys + Na_2S; 21, Na_2S. F/F_0 is the emission intensity ratio of the test sample to probe **7b** alone (excitation at 350 nm, emission at 450 nm).

Bioimaging Based on 7b

To demonstrate the biological applicability of probe **7b** in vivo, we investigated whether it can be used to image H_2S in living cells. HEK293 cells were treated with probe **7b** (5 μM) for 30 min and then washed with PBS to remove excess **7b**. The **7b**-loaded cells were incubated with different concentrations of Na_2S (50–250 μM) for another 30 min. The cells were subsequently imaged by confocal fluorescence microscopy (Supporting Information Figure S2). The addition of both **7b** and Na_2S resulted in brighter fluorescence than the addition of higher concentration of Na_2S. These results clearly indicated that probe **7b** can be used for imaging H_2S in living cells in a concentration-dependent fashion.

Synthesis and Characterization of BODIPY-Based Probes

The emission of the coumarin fluorophore in probe **7b** is centered in the blue region. We aimed to use fluorescence



Scheme 3. a) Schematic showing a FRET-based turn-on mechanism based on the reduction of azide (R) to amine by H_2S and b) the chemical response of FRET-based molecules toward H_2S.

resonance energy transfer (FRET) to achieve longer-wavelength emission of our H_2S probes. As shown in Scheme 3a, a general strategy for H_2S probes could be based on a two-fluorophore cassette comprised of a reaction-site-containing FRET donor (here azido-containing coumarin) and a FRET acceptor linked by a rigid spacer. FRET occurs after removing the reaction site in the FRET donor, which results in an increase in fluorescence of the FRET acceptor. We employed BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) as FRET acceptor (Supporting Information, Figure S4), because BODIPY dyes are photostable and have high quantum yields.[14]

Probes **9a** and **9b** (Scheme 3b) were synthesized by coupling reaction of **7** and BODIPY and characterized by ^1H, ^{13}C, and ^{19}F NMR spectroscopy and HRMS. The ^{19}F NMR spectrum of **9b** shows two peaks at −129.5 and −146.8 ppm (see Supporting Information), which are consistent with two kinds of F atoms in **9b**. The fluorescence spectra of probes **9a**, **9b** and their reaction with H_2S were investigated. As shown in Figure 4, both **9a** and **9b** show weak fluorescence of the BODIPY fluorophore in PBS buffer (pH 7.4, containing 10% DMF as cosolvent). On treatment of the probes with H_2S, significant fluorescence enhancement of both coumarin and BODIPY emission was observed for both **9a** and **9b** (**9a**: $\Phi = 0.040$, $\Phi = 0.21$ after reaction with H_2S; **9b**: $\Phi = 0.025$, $\Phi = 0.20$ after reaction with H_2S). The increase in BODIPY fluorescence should be due to FRET from the coumarin donor. The kinetics of the reaction of H_2S with **9** is similar to that of **7** (Figure 4c), that is, *o*-fluorination of aromatic azides can improve the reaction rate of azide reduction by H_2S approximately twofold. The reaction half-time of **9b** and H_2S at 37 °C at pH 8.0 can reach 7.5 min (Figure 4d), which implies that the probe has potential applications in fast sensing of H_2S in biological systems.

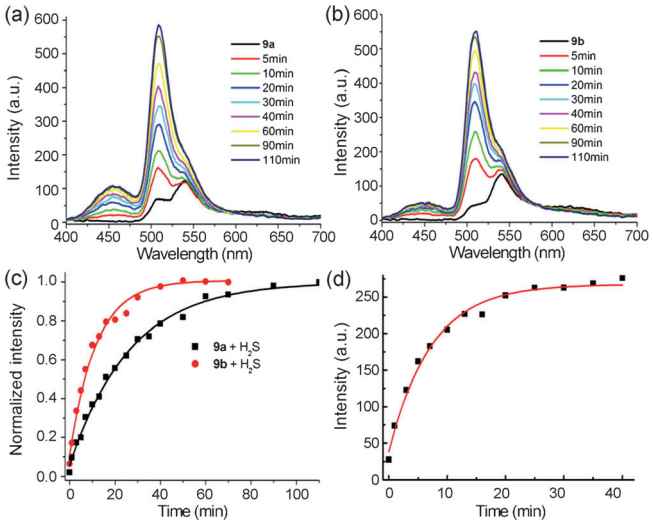


Figure 4. Time-dependent fluorescence spectra of a) **9a** (5 μM) and b) **9b** (5 μM) on reaction with H_2S (1 mM) at 25 °C. Excitation at 360 nm. c) The relationship between fluorescence intensity at 457 nm and reaction time of probes **9** (5 μM) on treatment with H_2S (1 mM) at 25 °C. d) The relationship between fluorescence intensity at 508 nm and reaction time of probe **9b** (5 μM) on treatment with H_2S (1 mM) at 37 °C and pH 8.0. The solid line represents the best fit to a single-exponential function.

To investigate the selectivity of probes **9**, various biologically relevant species were incubated with **9** in PBS buffer (pH 7.4, containing 10% DMF) and their fluorescence responses measured (Figure 5). The test compounds including biological thiols did not trigger significant fluorescence enhancement of the BODIPY fluorophore for both **9a** and **9b**. Thus, the FRET-based probes **9** are highly selective toward H_2S according to fluorescence spectra and thus suitable for biological applications.

We also investigated the fluorescence response of probe **9b** to H_2S at different pH values (Supporting Information Figure S6). This indicated that the probe can function over a wide range of pH from 6.5 to 8.5.

Bioimaging with a BODIPY-Based Probe

Probe **9b** was employed to stain living cells for bioimaging. The probe-treated cells were incubated with Na_2S (0–250 μM) for 30 min. Compared with cells treated only with the probe, the addition of both probe and Na_2S resulted in significant fluorescence increase of the green channel (Figure 6). Furthermore, the good morphologies of cells in bright-field transmission images suggested satisfactory biocompatibility of the probe. Therefore, the new FRET-based probe **9b** can be used to detect intracellular H_2S.

Our further goal was to use BODIPY-based probe **9b** to detect endogenously produced H_2S from living cells. Our previous work indicated that D-Cys can serve as a precursor

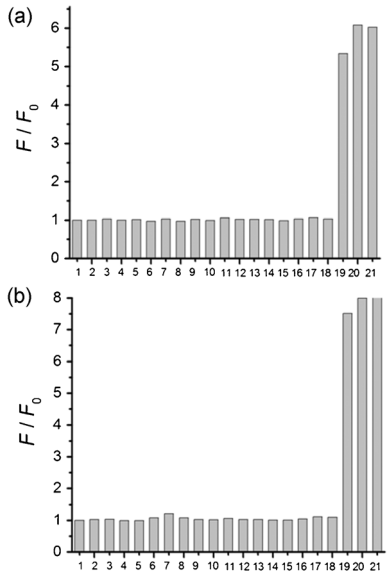


Figure 5. Relative fluorescent responses of probes **9** (5 μM) to various biologically relevant species (1 mM) and GSH (5 mM) in PBS (pH 7.4, containing 10% DMF) for 30 min. a) Test with **9a**. b) Test with **9b**. 1, probe **9** alone; 2, $MgSO_4$; 3, $CaCl_2$; 4, NaF; 5, NaI; 6, NaOAc; 7, NaN_3; 8, $NaHCO_3$; 9, $Na_2C_2O_4$; 10, Na_2SO_3; 11, $NaHSO_3$; 12, $Zn(OTf)_2$; 13, $NaNO_2$; 14, ascorbic acid; 15, H_2O_2; 16, 2-mercaptoethanol; 17, GSH; 18, Cys; 19, GSH + Na_2S; 20, Cys + Na_2S; 21, Na_2S. Excitation at 360 nm, emission at 508 nm.

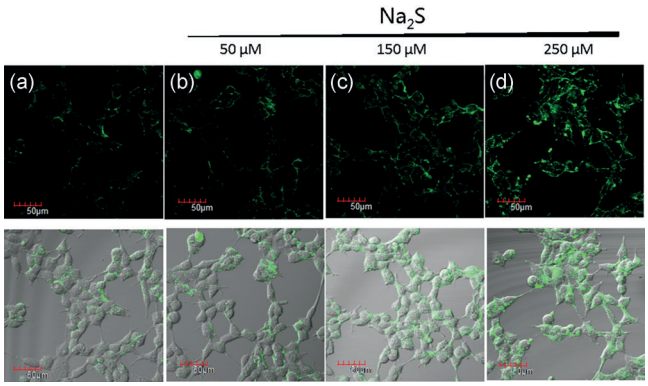


Figure 6. Confocal microscopy images of exogenous H_2S in living cells with probe **9b**. HEK293 cells were incubated with **9b** (2 μM) for 30 min (a) and with **9b** (2 μM) for 30 min and then Na_2S (50–250 μM) for 30 min (b–d). The bright-field images are shown below. Scale bar: 50 μm.

that can be converted to H_2S through enzymatic H_2S biosynthesis in living human cells.[11c] In this work, cells were co-incubated with 50 μM D-Cys and probe **9b** for 30 min. The cells showed clearly enhanced fluorescence responses compared to those without the addition of Cys (Figure 7 a,b). The fluorescence triggered by **9b** could be attributed to enzymatically generated endogenous H_2S from D-Cys in living cells. Inhibition experiments were performed by adding DL-propargylglycine (DL-PPG), an analogue of cysteine, as a known inhibitor for H_2S biogenesis.[11c] The fluorescence signal change implied the suppression of endogenous H_2S production (Figure 7 b,c), which should be due to enzyme inactivation by the inhibitor.

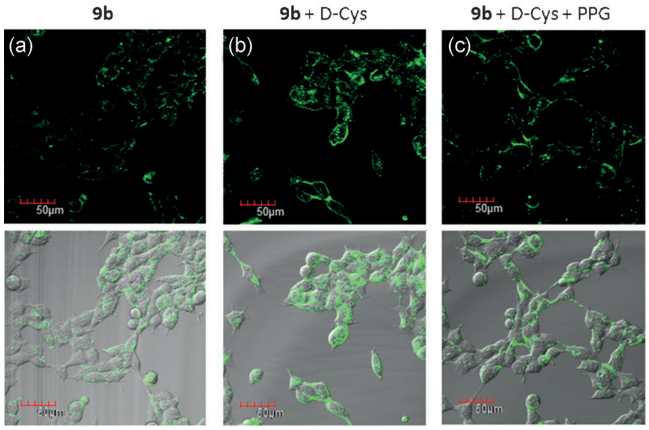


Figure 7. Confocal microscopy images of D-Cys-dependent endogenous H_2S production in living cells with probe **9b**. HEK293 cells were incubated with a) **9b** (2 μM) for 30 min, b) **9b** (2 μM) and D-Cys (50 μM) for 30 min, and c) DL-PPG (50 $\mu g\,mL^{-1}$) for 20 min and then **9b** (2 μM) and D-Cys (50 μM) for 30 min. The bright-field images are shown below. Scale bar: 50 μm.

Conclusions

To visualize biological H_2S in living cells, we have developed new coumarin- and BODIPY-based fluorescent probes for H_2S. Introduction of a fluoro group in the *ortho* position of the aromatic azide improved the reaction rate with H_2S more than twofold. This strategy should enable relatively facile tuning of the H_2S reaction rate by means of probe design. For example, one can add two or more fluoro substituents to the aromatic azide to achieve faster sensing of H_2S than with the single fluoro group in this work. We believe that extension of this work by such a strategy could produce fast azido-based fluorescent probes for real-time H_2S bioimaging in living cells. On the other hand, the probes developed in this work are highly sensitive and selective toward H_2S over other biologically relevant species. Confocal imaging indicated that the probes could monitor changes in intracellular H_2S levels. We further employed a BODIPY-based probe for bioimaging of D-Cys-dependent endogenous H_2S production in living human cells, which suggests potential applications of our probes in H_2S biology.

Experimental Section

Materials and Methods

All chemicals and solvents used for synthesis were purchased from commercial suppliers and used without further purification. The progress of reactions was monitored by TLC on precoated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized with UV light. Merck silica gel 60 (70–200 mesh) was used for purification by column chromatography. ^1H and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million rela-

tive to TMS as internal standard or residual solvent peaks ($CDCl_3$: 7.26 ppm, DMSO: 2.50 ppm). High-resolution mass spectra (HRMS) were obtained on a Varian 7.0 T FTICR-MS. Spectroscopic measurements were performed in PBS (20 mM, pH 7.4) buffer. Probes were dissolved in DMSO to prepare stock solutions with a concentration of 1–10 mM. The UV/Vis spectra were recorded on a CARY 100 Bio (Varian, USA). Fluorescence studies were carried out with a Varian Cary Eclipse spectrophotometer at 25 °C.

Synthesis

*Ethyl 3-hydroxy-6-fluorophenylcarbamate (**2b**)*

4-Amino-3-fluorophenol (**1b**, 5.0 g, 39.3 mmol) in EtOAc (50 mL) was heated to reflux for 30 min. Ethyl chloroformate (3 mL, 31.5 mmol) was added to the refluxing mixture in drops over 30 min. The reaction mixture was further heated to reflux for 1 h. The mixture was filtered while hot and the filtrate was evaporated to dryness. Propan-2-ol was added to the off-white residue, and the dissolved mixture was kept at 4 °C overnight to obtain **2b** as precipitate in a yield of 75% (4.71 g). TLC (CH_2Cl_2:MeOH 40:1): $R_f = 0.47$; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta =$ 9.35 (s, 1H), 8.99 (s, 1H), 7.28 (d, $J = 3.9$ Hz, 1H), 6.94 (t, $J = 9.7$ Hz, 1H), 6.61–6.33 (m, 1H), 4.11 (q, $J = 7.0$ Hz, 2H), 1.20 ppm (t, $J = 7.1$ Hz, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 154.1$, 153.7, 148.8, 146.4, 126.9, 115.8, 110.78, 110.2, 60.8, 14.6 ppm; HRMS calcd for $[M+H]^+$: 200.0723; found: 200.0715.

*Ethyl 2-(6-fluoro-7-(ethoxycarbonylamino)-2-oxo-2H-chromen-4-yl)acetic acid (**3k**)*

70% H_2SO_4 (20 mL) was added dropwise to a mixture of **2b** (1.99 g, 10.0 mmol) and diethyl-1,3-acetone dicarboxylate (1.81 mL, 10.0 mmol). After stirring overnight, TLC showed no starting material, and then the solution was poured into 200 mL of ice/water. The precipitate was filtered to give the desired produce as a light pink solid. The product **3b** was purified by column chromatography on silica to give a yellow powder (2.62 g, yield 85%). TLC (CH_2Cl_2:MeOH 40:1): $R_f = 0.30$; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 9.88$ (s, 1H), 7.86 (d, $J = 6.8$ Hz, 1H), 7.60 (d, $J = 11.5$ Hz, 1H), 6.43 (s, 1H), 4.19 (q, $J = 7.1$ Hz, 2H), 3.88 (s, 2H), 1.26 ppm (t, $J = 7.1$ Hz, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 170.9$, 160.1, 153.9, 150.7, 150.1, 149.8, 148.2, 130.9, 130.8, 115.5, 114.7, 111.9, 111.7, 109.2, 61.6, 37.4, 14.8 ppm; ^{19}F NMR (376.5 MHz, [D$_6$]DMSO): $\delta = -129.62$ ppm; HRMS calcd for $[M+H]^+$: 310.0727; found: 310.0727.

*2-(6-Fluoro-7-amino-2-oxo-2H-chromen-4-yl)acetic acid (**4b**)*

Compound **3b** (3.09 g, 10.0 mmol) was added to 20 mL of sodium hydroxide solution (4.00 g, 100 mmol). The mixture was stirred under reflux for 3 h and then cooled to 0 °C. The pH was adjusted to 2.0 by dropwise addition of conc. H_2SO_4. A light yellow precipitate was observed. The reaction mixture was cooled to 0 °C and the precipitate was filtered off to give the desired produce as a light yellow solid **4b** (1.66 g, yield 70%). **4b** was pure enough for use in the next reaction without further purification. TLC (CH_2Cl_2:MeOH 5:1 with one drop AcOH): $R_f = 0.30$; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.35$ (d, $J = 12.1$ Hz, 1H), 6.63 (d, $J = 7.6$ Hz, 1H), 6.25 (s, 2H), 6.09 (s, 1H), 3.76 ppm (s, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 170.6$, 160.4, 151.5, 150.1, 148.3, 145.9, 141.2, 111.4, 107.3, 101.7, 37.7 ppm; ^{19}F NMR (376.5 MHz, [D$_6$]DMSO): $\delta = -138.29$ ppm; HRMS calcd for $[M+H]^+$: 238.0515; found: 238. 0508.

*2-(6-Fluoro-7-azido-2-oxo-2H-chromen-4-yl)acetic acid (**5b**)*

A solution of $NaNO_2$ (2.07 g, 30.0 mmol) in water (7 mL) was added dropwise to a cooled solution of amino acid **4b** (2.37 g, 10.0 mmol) in 20% H_2SO_4 (100 mL). The reaction temperature was maintained at 0–5 °C. After stirring for 30 min, a solution of NaN_3 (2.60 mg, 40.0 mmol) in water (10 mL) was added dropwise into the mixture at 0 °C. The resulting reaction mixture was stirred overnight, after which it was extracted with EtOAc (3 × 100 mL). The organic layer was washed with brine and then dried with anhydrous sodium sulfate. After filtration, the organic phase was concentrated under vacuum. The product **5a** was purified by column chromatography on silica to give a light yellow solid (947 mg, yield 36%). TLC (CH_2Cl_2:MeOH 40:1): $R_f = 0.50$; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.69$ (d, $J = 11.7$ Hz, 1H), 7.39 (d, $J = 7.2$ Hz, 1H), 6.49 (s, 1H), 3.90 ppm (s, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 170.8$, 159.7, 151.7, 150.3, 149.6, 149.3, 131.8, 116.8, 116.3, 113.2, 113.0, 109.6, 37.4 ppm; ^{19}F NMR (376.5 MHz, [D$_6$]DMSO): $\delta = -131.13$ ppm; HRMS calcd for $[M+H]^+$: 264.0421; found: 264. 0419.

*Fluoro-Containing Coumarin Boc-Amine Derivative **6b***

HATU (932 mg, 2.45 mmol) and DIPEA (437 μL, 2.50 mmol) were added to a CH_2Cl_2 solution (20 mL) of coumarin acid **5b** (526 mg, 2.00 mmol) and mono-*t*Boc-piperazine (456 mg, 2.45 mmol) at room temperature. The solution was stirred at room temperature overnight and then poured into 200 mL of ice/water. The precipitate was filtered off to give the desired produce as a light pink solid. The product **6b** was purified by column chromatography on silica to give a yellow powder (733 mg, yield 85%). TLC (CH_2Cl_2:MeOH 40:1): $R_f = 0.4$; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.33$–7.13 (m, 1H), 6.95 (d, $J = 7.0$ Hz, 1H), 6.21 (s, 1H), 3.75 (s, 2H), 3.65–3.53 (m, 2H), 3.43 (m, 6H), 1.42 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 166.0$, 159.5, 154.3, 152.3, 150.3, 149.9, 148.5, 132.2, 132.1, 116.3, 112.0, 111.8, 109.0, 80.5, 45.9, 41.8, 37.1, 28.3 ppm; ^{19}F NMR (376.5 MHz, $CDCl_3$): $\delta = -129.67$ ppm; HRMS calcd for $[M+H]^+$: 432.1683; found: 432.1679.

*Fluoro-Containing Coumarin Amine Derivative **7b***

Compound **7b** was obtained by treatment of **6b** (431 mg, 1.00 mmol) with TFA:CH_2Cl_2 (1:1) solution. The product **7b** was purified by column chromatography on silica to give a faintly yellow powder (315 mg, yield 95%). TLC (CH_2Cl_2:MeOH 20:1): $R_f = 0.20$; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.64$ (d, $J = 10.8$ Hz, 1H, Ar), 7.41 (d, $J = 6.0$ Hz, 1H, Ar), 6.37 (s, 1H, Ar), 3.97 (s, 2H), 3.41 (br s, 5H), 2.76 (s, 2H), 2.66 ppm (s, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 166.2$, 159.2, 151.0, 149.7, 148.7, 131.1, 116.8, 115.4, 112.9, 112.7, 108.9, 46.3, 45.5, 45.1, 42.2, 35.9 ppm; ^{19}F NMR (376.5 MHz, [D$_6$]DMSO): $\delta = -131.42$ ppm; HRMS calcd for $[M+H]^+$: 332.1153; found: 332.1155.

*Coumarin-BODIPY Probe **9a***

HATU (932 mg, 2.45 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 437 μL, 2.50 mmol) were added to a CH_2Cl_2 solution (20 mL) of coumarin acid **7a** (626 mg, 2.00 mmol) and BODIPY-COOH (737 mg, 2.00 mmol), at room temperature. The solution was stirred at room temperature overnight. TLC showed no starting material, and then the solution was poured into 200 mL of ice/water. The precipitate was filtered off to give the desired produce as a light pink solid. The product **9a** was purified by column chromatography on silica to give a yellow powder (1.12 g, yield 85%). TLC (CH_2Cl_2:MeOH = 100:1): $R_f = 0.2$; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.57$ (d, $J = 7.6$ Hz, 2H), 7.51 (d, $J = 6.8$ Hz, 1H), 7.38 (d, $J = 7.6$ Hz, 2H), 6.96–6.92 (m, 2H), 6.21 (s, 1H), 5.98 (s, 2H), 3.84 (s, 2H), 3.70–3.63 (m, 8H), 2.53 (s, 6H), 1.36 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 169.8$, 166.5, 159.8, 156.0, 154.8, 148.7, 144.3, 142.6, 140.0, 137.2, 135.5, 131.1, 128.6, 128.1, 126.1, 121.5, 116.0, 115.5, 115.1, 107.3, 46.0, 42.0, 38.6, 37.1, 29.6, 14.6 ppm; ^{19}F NMR (376.5 MHz, $CDCl_3$): $\delta = -146.1$ ppm (m); HRMS calcd for $[M-H]^-$: 662.2499; found: 662.2476,.

*Fluoro-Containing Coumarin-BODIPY Probe **9b***

HATU (932 mg, 2.45 mmol) and DIPEA (437 μL, 2.50 mmol) were added to a CH_2Cl_2 solution (20 mL) of coumarin acid **7b** (662 mg, 2.00 mmol) and BODIPY-COOH (737 mg, 2.00 mmol) at room temperature. The solution was stirred at room temperature overnight. TLC showed no starting material, and then the solution was poured into 200 mL of ice/water. The precipitate was filtered off to give the desired product as a light pink solid. The product **9b** was purified by column chromatography on silica to give a yellow powder (1.17 g, yield 85%). TLC (CH_2Cl_2:MeOH 100:1): $R_f = 0.2$; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 7.59 (d, $J = 8.0$ Hz, 2H), 7.40 (d, $J = 7.6$ Hz, 2H), 7.27 (d, $J = 9.6$ Hz, 1H), 7.03 (d, $J = 6.8$ Hz, 1H), 6.28 (s, 1H), 5.99 (s, 2H), 3.81 (br s, 2H), 3.71–3.66 (m, 8H), 2.55 (s, 6H), 1.38 ppm (s, 6H); ^{13}C NMR (100 MHz,

$CDCl_3$): δ = 169.8, 166.1, 159.5, 156.0, 152.4, 150.4, 150.0, 148.1, 142.7, 139.9, 137.3, 135.5, 132.5, 131.1, 128.6, 128.1, 121.5, 116.3, 111.7, 109.2, 46.0, 42.1, 38.6, 37.2, 29.7, 14.6 ppm; ^{19}F NMR (376.5 MHz, $CDCl_3$): δ = −129.5, −146.1 ppm (m). HRMS calcd for $[M-H]^-$: 680.2404; found: 680.2391.

Cell Culture and Bioimaging

HEK-293 cells were cultured at 37 °C and 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (GIBICO) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 4 mM L-glutamine. The cells were maintained in exponential growth and then seeded in a glass-bottom 35 mm plate at a density of about 2×10^4 per well. Cells were passaged every 2–3 d 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 in the blue or green channel with 405 nm excitation.

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FULL PAPER

Fluorescent Probes

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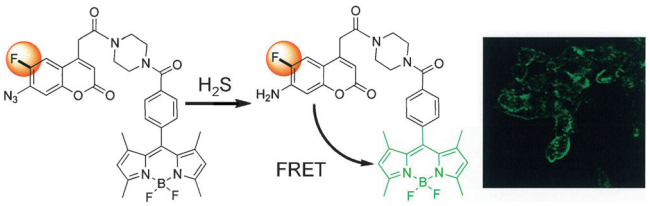
***o*-Fluorination of Aromatic Azides Yields Improved Azido-Based Fluorescent Probes for Hydrogen Sulfide: Synthesis, Spectra, and Bioimaging**



Just add fluorine! On the basis of introducing fluoro substituents into the *ortho* position of aromatic azides, which more than doubles their rate of reaction with H_2S, new BODIPY-based fluorescent probes were developed for real-time bioimaging of H_2S. These probes can be used for in situ visualization of D-cysteine-dependent endogenously produced H_2S in living cells (see figure).