ACES Asian Chemical Editorial Society

DOI: 10.1002/asia.201500940

CHEMISTRY AN ASIAN JOURNAL Communication

Fluorescent Probes

Multi-Fluorinated Azido Coumarins for Rapid and Selective Detection of Biological H_2S in Living Cells

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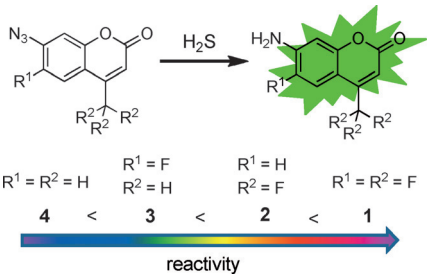
Abstract: Hydrogen sulfide (H_2S) is an endogenously produced gaseous signaling molecule with multiple biological functions. In order to visualize the endogenous in situ production of H_2S in living cells in real time, here we developed multi-fluorinated azido coumarins as fluorescent probes for the rapid and selective detection of biological H_2S. Kinetic studies indicated that an increase in fluorine substitution leads to an increased rate of H_2S-mediated reduction reaction, which is also supported by our theoretical calculations. To our delight, tetra-fluorinated coumarin **1** could react with H_2S fast ($t_{1/2} \approx 1$ min) and selectively, which could be further used for continuous enzymatic assays and for visualization of intracellular H_2S. Bioimaging results obtained with **1** revealed that D-Cys could induce a higher level of endogenous H_2S production than L-Cys in a time-dependent manner in living cell.

Hydrogen sulfide (H_2S) is an important endogenous signaling molecule with multiple functions.[1–3] H_2S can be enzymatically produced by three distinctive pathways, namely cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MPST)/cysteine aminotransferase (CAT), in different organs and tissues.[2] 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.[4] Although 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. Therefore, it is of significant research value to develop efficient methods for the detection of H_2S in living biological systems.

Compared with traditional methods,[5] fluorescent probes should be excellent tools for in-situ and real-time monitoring of H_2S in biological samples because of their non-destructive sensing of biological targets with readily available detection.[6–12] H_2S probes functioning through the reduction of azido groups to amines developed by Chang[6a] and Wang[6b] are the most available in the literature;[6–8, 11, 12] however, most of them do not respond fast enough for real-time and selective detection of H_2S.[7] To achieve real-time detection, we proposed that a multi-fluorinated aromatic azide could react with H_2S efficiently.

Herein, we rationally designed and synthesized a series of fluorinated azido-coumarins as fluorescent probes for H_2S detection (Scheme 1). These fluorinated probes (**1**–**3**) showed a faster H_2S-mediated reduction rate than the control probe **4**, which has been reported by Tang et al.[8j] To our delight, the tetra-fluorinated coumarin **1** showed a very fast response ($t_{1/2} \approx 1$ min) and good selectivity toward H_2S in buffer and in living cells. To the best of our knowledge, probe **1** is the fastest aromatic azido-based H_2S probe.



Scheme 1. Chemical structures of fluorescent probes **1**–**4** and their reactivity toward H_2S.

All fluorinated coumarins were formed via a Pechmann reaction,[13] and the resulting free amines were transformed to azido-based coumarins using a Sandmeyer reaction.[11] All fluorinated azido-coumarins were fully characterized by ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectroscopy and by high-resolution mass spectrometry (HRMS) (see the Supporting Information).

With these probes in hand, we examined their absorption and fluorescence spectra in PBS buffer (pH 7.4). As shown in Figure 1, probes exhibit absorbance peaks at 332 or 344 nm

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/asia.201500940.

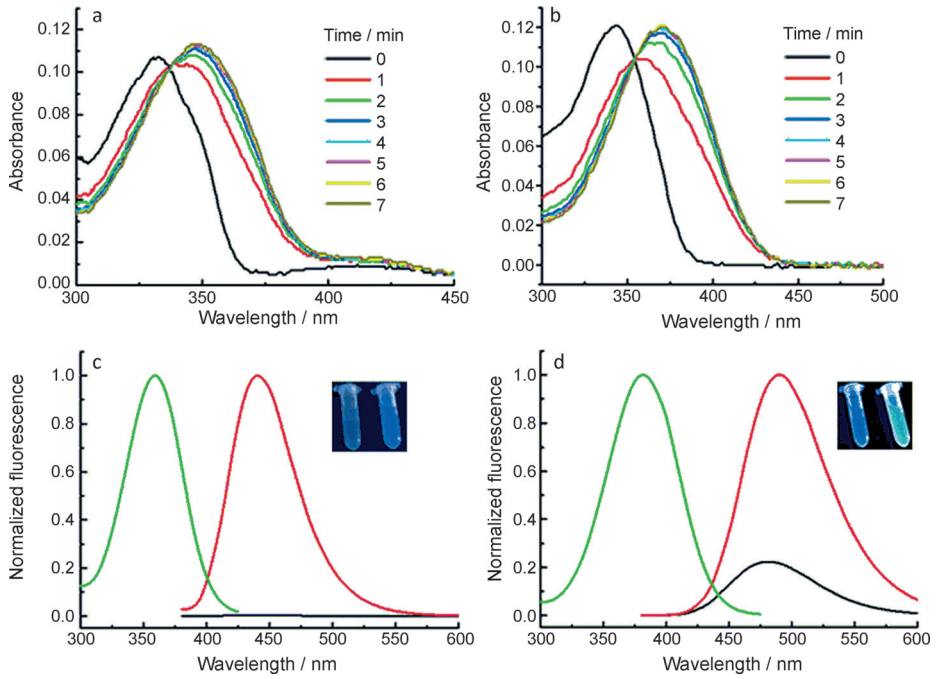

Figure 1. Spectral changes of probe **1** or **3** toward H_2S. (a, b) Absorption spectra of **3** (a) and **1** (b) (10 μM each) upon addition of Na_2S (1 mM) in PBS buffer. (c, d) Normalized excitation and emission spectra of probe **3** (c) or **1** (d) (1 μM each) in the presence of Na_2S (100 μM) in PBS buffer. Black line, probe only; red line, probe and Na_2S; green line, excitation. Inset: photographs of the probe before and after reacting with Na_2S under illumination with a UV lamp (365 nm). Blue and cyan fluorescence were observed by the naked eye for **3** and **1**, respectively.

for **3** and **1**, respectively. After reacting with H_2S, the maximum absorbance shifted to 347 nm or 370 nm for **3** or **1**, respectively. In this process, a well-defined isobestic point was noted at 336 nm or 354 nm for **3** or **1**, respectively, suggesting a clean chemical transformation.[10l] H_2S-mediated reduction of the electron-withdrawing azido group to the electron-donating amino group led to an increased push–pull character of the dye and resulted in large bathochromic shifts in the absorption, a typical characterization for the intramolecular charge transfer (ICT) effect. Probes **3** and **1** showed weak background fluorescence in PBS buffer due to ICT, while a significant fluorescent turn-on response was observed in the presence of H_2S. Compared with **3**, the emission of probe **1** after H_2S activation showed a significant red-shift. These fluorescence changes can be observed directly under a UV lamp (Figure 1c and 1d). Probes **2** and **4** displayed similar spectra (Figure S1, Supporting Information) as those of probes **1** and **3**, respectively.

All probes had good solubility in PBS buffer (Figure S2). The H_2S-mediated reaction could further be probed by NMR and mass spectral measurements. The in situ ^1H NMR spectra of probe **1** or **2** with 1.5 equivalents of H_2S revealed the transformation of azido-coumarin to amino-coumarin (Figure S3), which was further confirmed by HRMS (see the Supporting Information).

Encouraged by these preliminary results, we moved forward to study the reaction kinetics of probes **1**–**4**. To obtain the reaction kinetics, the time-dependent fluorescence signals were acquired for data analysis (Figure 2 and Figure S4). The pseudo-first-order rate, k_{obs}, was obtained by fitting the fluorescence intensity data with a single-exponential function, and the reaction rate (k_2) was calculated as shown in the inset of Figure 2. The addition of a fluorine group on the *o*-position of the aromatic azide in coumarin (probe **3**) improved the reaction rate by about 2-fold compared with **4**, which is consistent with our previous work,[11] while the addition of a trifluoromethyl group in probe **2** led to a more than 10-fold rate improvement. Both *o*-fluorination and trifluoromethylation in coumarin resulted in a further increase in the reaction rate, with $t_{1/2}$ of about 1.09 min and k_2 up to 107 $M^{-1}s^{-1}$ for probe **1**. In a previous work, Tang et al.[12] have developed fluorescent probes with an improved reaction rate by addition of an electronic-withdrawing methoxy group on the *o*-position of the aromatic azide. Guo[8n] and Chen et al.[8m] have

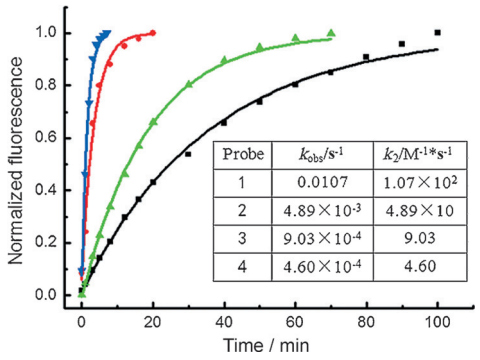

Figure 2. Time-course experiments of probes **1**–**4** (1 μM each) upon addition of Na_2S (100 μM) in PBS buffer (pH 7.4). **1**, blue; **2**, red; **3**, green; **4**, black. Solid lines represent the best fitting. The kinetic data are shown in the table.

developed H_2S fluorescent probes with a good response time of 3 min. In this work, we developed the fastest aromatic azido-based H_2S probe (**1**) up to now.

Next, calculations were performed at the B3LYP/6-31G* level in an attempt to gain insight into the reactivity of **1**–**4**. The results show that the energy of the LUMO decreases from **4** to **1** (Table S2, Supporting Information), implying that the H_2S-mediated reduction becomes more efficient from **4** to **1** along with the lower LUMO energy.[14] On the other hand, the difference between HOMO and LUMO is smaller for **5** than for **6** (see the for Supporting Information and Table S2), implying that the emission for **5** is red-shifted. These calculations are consistent with our experimental results.

To examine the stability of the probes, a control experiment was performed in which each probe was incubated in the absence of any analyte in PBS buffer (Figure S5). The results indicated that the fluorescence change of these probes within 1 h was much smaller than that in the presence of H_2S. In order to evaluate the specificity of these fluorinated coumarins for H_2S, they were incubated with various biologically related species in PBS buffer, and the fluorescence change was measured accordingly (Figure 3, Figure S6, and Figure S7). Among all the

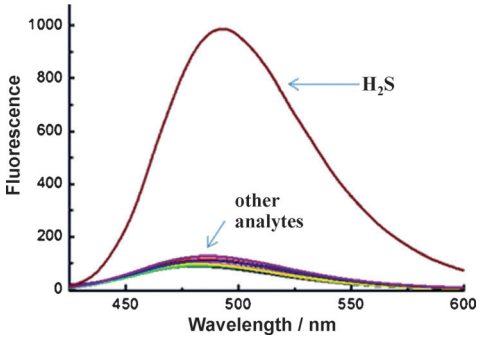

Figure 3. Fluorescent response of probe **1** (1 μM) to various biologically relevant species (100 μM) or H_2S (100 μM) or biothiols (Cys or Hcy, 1 mM; GSH, 5 mM) in PBS (50 mM, pH 7.4). Other species comprise SO_3^{2-}, $S_2O_3^{2-}$, NO^{2-}, H_2O_2, ClO^-, Zn^{2+}, and Fe^{3+}. Excitation, 370 nm.

tested molecules, only H_2S showed a noticeable fluorescence response. The fluorescence increase by biothiols (Cys and GSH) at millimolar concentrations, however, is far below that induced by H_2S. These results suggest that multi-fluorinated azido coumarins are selective to H_2S over other biological species.

Considering that the H_2S-mediated reduction reaction for probe **1** was the fastest, we predominantly used this probe for further tests and biological applications. To gain detailed information about the sensitivity of **1**, the fluorescence intensity change was closely monitored by addition of various concentrations of H_2S to the probe (Figure S8). Data analysis revealed an excellent linear relationship ($r = 0.9944$) between the fluorescence signal at 490 nm and the concentration of H_2S (10–50 μM). The detection limit was determined to be 0.67 μM by using the $3\sigma/k$ method.[11] These results demonstrated that probe **1** could react with H_2S with good sensitivity and quantitatively. In addition, we also investigated the fluorescence response of probe **1** to H_2S at different pH values (Figure S9). The results indicated that the probe can function over a wide range of pH from 6.0 to 8.5.

To further demonstrate the biological applicability of the multi-fluorinated azido coumarins, we used the probe **1** to monitor the CBS activity in vitro. The enzymatic reaction can be visualized by the specific fluorescence response of probe **1** to the enzymatically produced H_2S. Preliminary studies indicated that significant fluorescence appeared at 480 nm during the enzymatic reaction after addition of increased concentrations of CBS and as the concentration of CBS increased (Figure 4). Particularly noteworthy is the continuous nature of the assay. The CBS activity can be inhibited by an analogue of

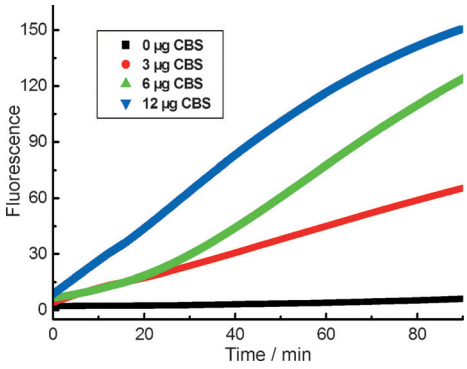

Figure 4. In vitro continuous CBS activity assay based on probe **1**. The time-dependent emission intensity of probe **1** (20 μM) at 480 nm in the presence of 5 mM L-Cys upon addition of 0–12 μg CBS enzyme in 1 mL Tris-HCl (pH 8.0) buffer; excitation, 360 nm.

Cys, L-propargylglycine (PPG),[11] which was also observed with probe **1** by the continuous fluorophotometric assay (Figure S10).

To further demonstrate the biological applicability of probes **1** and **2**, we examined whether they could be used to detect H_2S in living cells in real time. To this end, HEK293A cells were first treated with probe **1** or **2** (2 μM) for 30 min and then with Na_2S (200 μM) for different times. The significant fluorescence increase in the cyan channel (Figures S11 and S12) indicated that probes **1** and **2** can be used for imaging of H_2S in living cells. Bright-field images show that the cells retained good morphology after incubation with **1**, suggesting the probe has low cytoxicity at the experimental concentration. Consequently, we determined the average fluorescence intensity in cells at different time points. The increase in the intensity saturated after 5 min for **1** (Figure S12), implying that this probe can be used for imaging exogenous H_2S in living cells at a rapid speed.

To test whether **1** could detect endogenous production of H_2S, cells were treated with D-Cys or L-Cys and then with **1**. As shown in Figure 5, HEK293A cells incubated with **1** displayed a fairly weak fluorescence under the confocal fluorescence microscope (Figure 5a). In the presence of Cys to induce endogenous H_2S production,[10f] a significant fluorescent enhancement can be observed (Figure 5b, 5c), implying that **1** can be used to visualize cysteine-dependent H_2S production in situ in living cells. The measured average fluorescence indicated that D-Cys induced a higher H_2S production than L-Cys in a time-dependent manner in living cells (Figure 5d and Figures S13–S15). The above results suggested that **1** was cell-permeable and could react with intracellular H_2S efficiently.

In summary, we rationally designed and synthesized a series of multi-fluorinated azido coumarins for rapid sensing of H_2S in buffer and in living cells. The kinetic studies indicated that an increase in fluorine substituents leads to an increased rate of H_2S-mediated reduction reaction, which is also supported by calculations. The tetra-fluorinated coumarin **1** can respond to H_2S rapidly ($t_{1/2} \approx 1$ min) and selectively. In preliminary studies probe **1** was applied for continuous enzymatic assay and for bioimaging in living cells. We believe that this design strategy

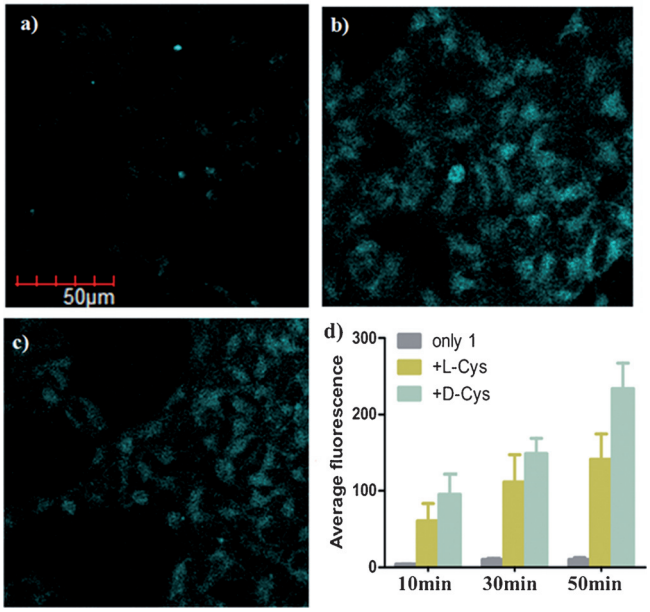

Figure 5. Confocal microscopy images of intracellular H_2S in living cells using **1**. HEK293 cells were incubated with (a) **1** (1 μM) for 30 min; (b) D-cysteine (100 μM) or (c) L-cysteine (100 μM) for 30 min and then **1** (1 μM) for 50 min. Emission was collected at the blue channel (440–540 nm) with excitation at 405 nm. Scale bar, 50 μm. (d) The average fluorescence of the cells shown in panels a–c for 10 min, 30 min and 50 min.

could be employed for the preparation of other H_2S probes that are capable to respond rapidly in future.

Acknowledgements

We thank Professor Gu Yuan and Wei Tan of Peking University for the MS analysis. This work was supported by the MOST (2010CB126102), NSFC (21332004, 21402007, 21572019), 111 project (B14004) and the Fundamental Research Funds for the Central Universities (YS1401).

Keywords: biosensors · fluorescent imaging · fluorescent probes · fluorinated azido coumarin · hydrogen sulfide

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Manuscript received: September 5, 2015
Accepted Article published: September 18, 2015
Final Article published: September 30, 2015