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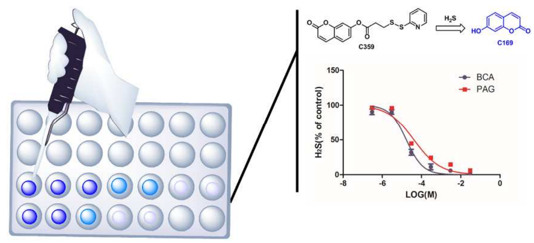
Graphical Abstract



C359, a novel fluorescent probe for highly selective detection of H_2S over biothiols, was applied to evaluate CSE inhibitors.

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ARTICLE

Development of a highly selective H_2S fluorescent probe and its application to evaluate CSE inhibitors

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In this paper, we developed a novel fluorescent probe **C359** for highly selective detection of H_2S over other relevant biothiols. **C359** is designed to contain a thiol-specific cleavable disulfide bond. H_2S-mediated the disulfide cleavage and subsequent intramolecular cyclization released the masked 7-Hydroxyl coumarin, displaying a remarkable fluorescence enhancement. With the promising features in hand, **C359** has been applied to detect the activity of CSE (one of H_2S-producing enzyme) and build up an assay for screening CSE inhibitors. We anticipated that the enzyme assay using **C359** could provide a powerful methodology for screening more potent and selective enzyme inhibitors.

Introduction,

Hydrogen sulfide (H_2S) has been recognized as the third gaseous transmitter of signaling molecules in biological system, succeeding nitric oxide (NO) and carbon monoxide (CO). Therefore, H_2S is a topic of great interest in chemistry and biology. In 1996, the role of H_2S in human neuromodulation was first reported by Abe and Kimura, which had become a research prelude on the biological signaling function of H_2S [1]. Since then, all aspects of the research on the biological functions of H_2S has been unfolded, such as cardioprotective [2-5], neuroprotective [6-8] and gastroprotective effects [9], the regulation of insulin release [10] and anti-inflammatory effects [11], which make it known that H_2S also plays an important, probably even pivotal role in human and other biological systems.

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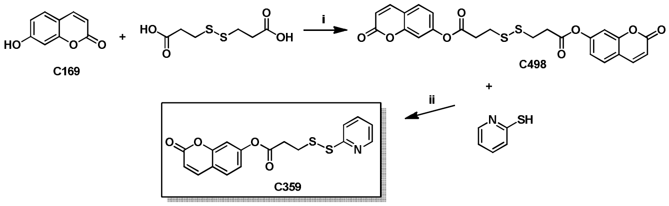
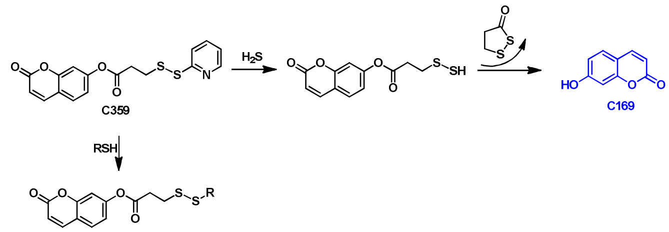
In mammalian systems, the generation of H_2S derives from several enzymatic pathways. For example, both cystathionine γ-lyase (CSE, EC 4.4.1.1) and cysta thionine β-synthase (CBS, EC4.2.1.22), two pyridoxal 5′-phosphate (PLP) dependent enzymes, are able to convert cysteine into H_2S within different organs and tissues[12-17]. Recently, it was reported that 3-mercaptopyruvate sulphurtransferase (3-MST, EC2.8.1.2) and cysteine aminotransferase (CAT cysteine lyase (CL, EC4.4.1.10)) can also catalyzed the production of H_2S[18]. To understanding the biological roles of H_2S, the inhibitors of these enzymes would be valuable tools. Up to now, some inhibitors of these enzymes have been reported, such as PAG, BCA and AOAA, which are the inhibitors of CSE and CBS. Methylene blue assay is a colorimetric method for H_2S detection [19], which has been used as a common enzyme activity assay to evaluate inhibitors of H_2S-producing enzymes. However, by using this method, complicated sample processing is often required, and variable results are often yielded. To obtain more potent and selective inhibitors, new and efficient inhibition assays are needed. In recent years, fluorescence-based assays are fast emerging in the field of biological molecule detection because of their high sensitivity and convenience [20]. As the studies on H_2S biological functions are proceeding, more and more fluorescent probes for H_2S detection have been reported [21]. However, most of these probes are only applied to cell imaging, and rare of them used to the research on enzyme activities, which is of great value in understanding the role of H_2S. Herein, a highly selective H_2S fluorescence probe over other thiols was designed and synthesized. Furthermore, an inhibition assay of CSE by using this fluorescent probe was developed.



Scheme 1. The synthesis of **C359**.



Scheme 2. Proposed mechanism of the reaction of **C359** with H_2S and thiols.

Results and discussion

Design and synthesis of probe C359: CSE, a PLP-dependent enzyme, is able to catalyze the production of H_2S using Cysteine as a substrate. Therefore, interference from cysteine could occur in the assay of CSE activity when using a H_2S fluorescent probe as an assay tool, due to the similar reactivity of thiol-containing compounds [22]. Thus, it is highly desirable to construct a selective fluorescent probe for detecting H_2S over thiols. Recently, Xian's group reported a series of selective probes for H_2S with 2-(2-pyridinyldithio)-benzoic group [21g]. These probes can be triggered by H_2S to undergo a tandem nucleophilic substitution-cyclization reaction, and then to release the fluorophores and render the fluorescence turn-on. Although these probes exhibit highly selectivity between thiols and H_2S, poor water solubility and slow reaction rate limit their biological application. In order to improve water solubility and fluorescence turn-on rate of these probes, they added surfactant CTAB. However, the need for a surfactant may also limit their application in living systems. With this consideration in mind, a selective and fast reactive disulfide containing probe (**C359**) was designed and synthesized. **C359** was readily synthesized in two steps using the procedure shown in scheme 1.

7-Hydroxyl coumarin (**C169**) is a commercial available fluorescent dye with a broad absorption band and an emission band centered around 350 nm ($\varepsilon = 1.7 \times 10^7$ M^{-1} cm^{-1}, in EtOH) and 450 nm (Φ_f = 0.09), respectively. By introducing a 3-(pyridine-2yldisulfanyl)-propionyl group into **C169**, we tend to build up a highly selective fluorescent probe for H_2S over other related thiols. Notably, the introduction of this cage unit further reduced the fluorescent quantum yield to be 0.005, making **C359** a promising probe with low background interference. Moreover, **C359** exhibits fast reactivity with H_2S due to its good solubility in buffer system under experimental conditions and the less steric effect of 3-(pyridine-2yldisulfanyl)-propionyl group. As shown in scheme 2, the probe **C359** could react with H_2S to afford an intermediate with a nucleophile SSH, then a spontaneous intramolecular nucleophilic reaction would be triggered as the electrophile carbonyl is present in a suitable site, to release a five membered cyclic lactone ring and the fluorescent product **C169**. In sharp contrast, the reaction between thiols and **C359** yielded a compound with disulfide bond, and this compound could not undergo an intramolecular nucleophilic attack to release **C169**, which leads to a high discriminative detection of H_2S over thiols.

To validate the proposed mechanism of the reaction between **C359** and H_2S, ^1H NMR and HPLC analysis were further performed to confirm the production of **C169** in the reaction. The partial ^1H NMR spectra of **C359** in the absence and presence of H_2S, and that of **C169** are shown in Fig. 1. Upon addition of H_2S to the solution of **C359**, new peaks at 7.9, 7.5, 6.8, 6.7 and 6.2 ppm assigned to **C169** were observed, suggesting the formation of **C169**. In the HPLC spectra, the retention time of standard **C359** and **C169** are around 5.8 min and 2.3 min respectively. Upon addition of H_2S to the solution of **C359**, the peak around 5.8 min decreased, while a new peak with the retention time around 2.3 min appeared simultaneously (Fig. S1). These observations confirm that treatment of **C359** with H_2S led to formation of **C169**.

The spectroscopic response of C359 to H_2S

Initially, we evaluated the optical properties of **C359** toward H_2S in 200mM Tris HCl buffer (pH=7.4) by monitoring the changes in the absorption and fluorescence spectra. In the absence of H_2S, **C359** displayed a strong absorption at 280 nm with a shoulder band around 310 nm. Introduction of H_2S led to buildup of a new band at 350 nm. Two well-defined isosbestic points at 305 and 275 nm were noted. The absorption band at 350 nm is the characteristic feature of **C169**, indicating the formation of **C169** (Fig. 2a). In the fluorescence spectra, free **C359** exhibited a weak emission feature centered on 450 nm. Upon gradual addition of H_2S to the solution of **C359** in 200mM Tris HCl buffer (pH=7.4), a remarkable enhancement of fluorescence intensity at 450 nm was observed (Fig. 2b). Notably, the fluorescence intensity increased linearly with concentrations of H_2S increased from 0 to 35 μM, indicating that **C359** is a promising probe for detecting H_2S at micromolar concentration level under physiological conditions. Based on the titration experiments, the detection limits were evaluated to be 5.0×10^{-8} M (Fig. S3), which are comparable to previously reported probes.

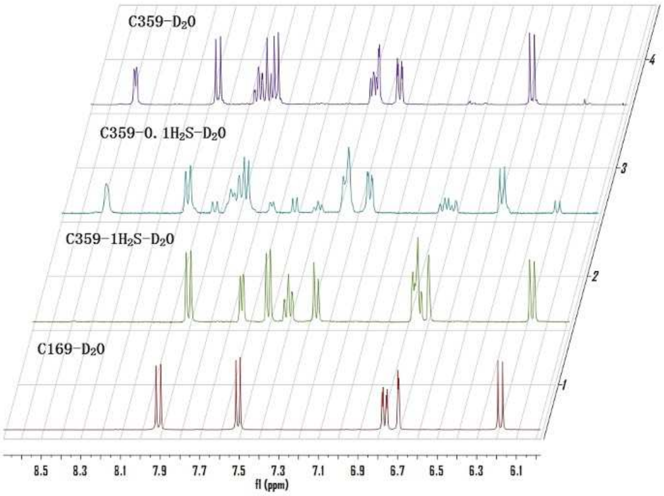


Fig. 1 Partial 1H NMR spectra of **C359**, **C359**+H_2S and **C169**.

Interference from other related analytes was then investigated under the same condition. As shown in Fig. 3, no obvious fluorescence change was observed upon addition of 50 equiv of F^-, Cl^-, Br^-, I^-, SO_4^{2-}, SO_4^{2-}, NO_3^-, CO_3^{2-}, SCN^-, $S_2O_5^{2-}$, N_3^-, and NO_2^-, as well as relevant thiols and cofactor PLP. More interestingly, only addition of H_2S introduced remarkable fluorescent enhancement. These results indicate the excellent capacity of **C359** for selective detection of H_2S over the other competitive anions, thiols and enzyme cofactor.

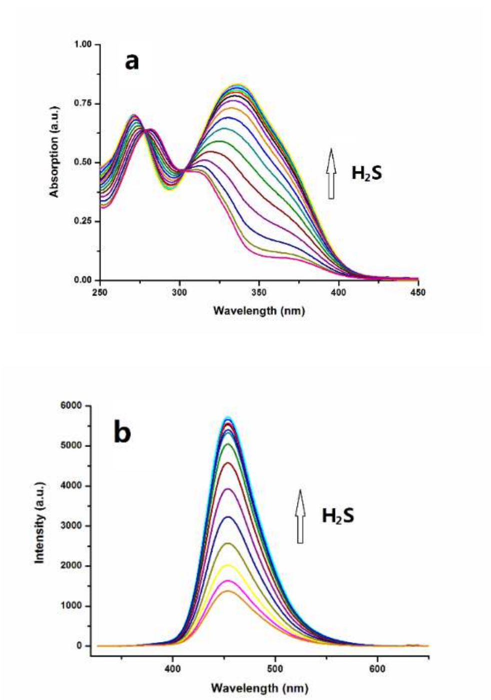


Fig. 2 (a) Absorption spectra of **C359** (5×10-6 M) in the presence of different concentrations of NaHS (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 equiv) in Tris HCl buffer (200mM, pH=7.4). (b) Fluorescence spectra of C359 (λex = 305 nm) in the presence of different concentrations of NaHS (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 equiv).

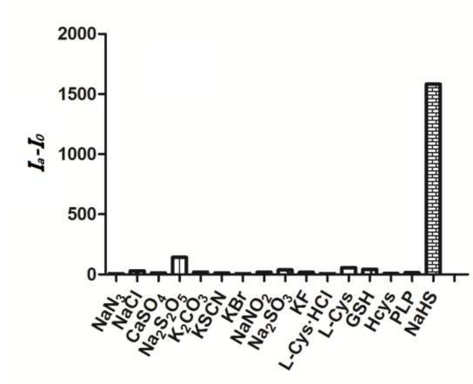


Fig. 3 Fluorescence response of **C359** (450nm) in the absence and presence of 50 equiv of various anions, thiols and PLP in Tris HCl buffer (200mM, pH=7.4). Each Measuring was performed after 2 min of mixing. I_0 represents the fluorescence intensity of **C359** only, and I_a represents the intensity in the presence of various anions, PLP and thiols.

The application of C359 to evaluation of CSE inhibitors

As shown in Fig. 3, no fluorescence response was observed upon the incubation of **C359** with CSE/CBS substrate (Cys) or cofactor (PLP), enabling **C359** a promising probe for testing activity of H_2S-producing enzymes and screening their inhibitors. To test our proposal, CSE was chosen in our enzyme inhibition assay due to the commercial availability of recombinant CSE (GST-CSE). To assess the ability of **C359** for monitoring activity of CSE, treatment of CSE with **C359** was performed firstly. As shown in Fig. 4, significant fluorescent signal over the background was recorded when Cys was used as a substrate. In the absence of Cys, significantly reduced fluorescence intensity was noted. The weak fluorescence response of **C359** toward Cys in the absence of CSE further confirmed the role of CSE in production H_2S. These observations indicated that **C359** can be employed to monitor CSE catalyzing Cys to produce H_2S. With this result in hand, we then assessed the capability of **C359** for the evaluation of CSE inhibitors. Two known inhibitors, BCA and PAG, were used to test the validity of the inhibition assay. As shown in Fig. 5, two inhibition curves of BCA and PAG against CSE were obtained using **C359** as a reporter. Interestingly, IC_{50} of BCA and PAG was determined to be 16.75μM and 40.81μM respectively, which are comparable to the literature data by using methylene blue as a reporter (Table 1). These results indicated that **C359** is an ideal fluorescent probe for monitoring enzyme activity, which further allows the buildup of a useful methodology to screen more potent and selective inhibitors of H_2S-producing enzyme.

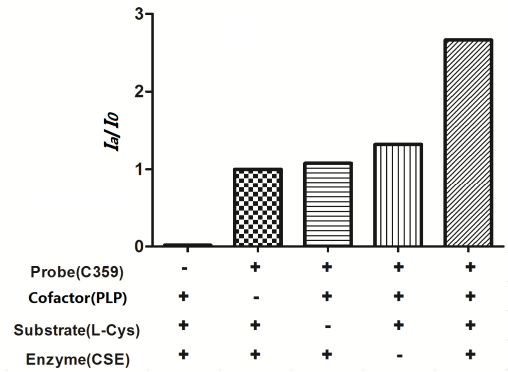


Fig.4 Utility of **C359** as a H_2S probe and assay for CSE activity. I_0 represents the fluorescence intensity of **C359** only, and I_a represents the intensity in the presence of Cys, PLP and enzyme.

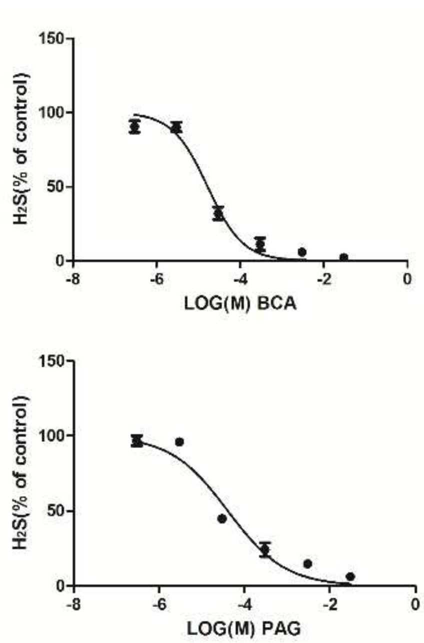


Fig. 5 Inhibition curves for BCA and PAG against CSE. Data are presented as mean±SEM; n=3; *P<0.05 versus control.

Table 1. IC_{50} of CSE inhibitors

Inhibitors	IC_{50} (μM)	IC_{50}[a] (μM)
BCA	16.75	14.0
PAG	40.81	40.0

a Literature data (ref. 19)

Conclusions

In conclusion, a fluorescent probe **C359** was designed and synthesized, which show high selectivity for H_2S. The reaction between **C359** and H_2S triggered the disulfide bond cleavage and subsequent intramolecular cyclization, releasing 7-Hydroxyl coumarin and resulting in a remarkable fluorescence enhancement. Other relevant thiols introduce no observable fluorescent response. More importantly, **C359** is capable of monitoring CSE activity, which further allows the buildup of an inhibition assay of CSE by using this fluorescent probe.

Experimental Section

General Method: All chemical reagents and solvents for synthesis were purchased from commercial suppliers and were used without further purification. Sodium hydrogen sulfide (NaSH) was purchased from Sigma. cystathionine γ-lyase (CSE), BCA and PAG were purchased from Cayman Chemical. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-400 spectrometer with chemical shifts reported in ppm (in DMSO-d_6) at room temperature. The analytical HPLC was performed on Waters 600E HPLC system. Mass spectra were measured on a HP 1100 LC-MS spectrometer. UV-vis absorption spectra were recorded on a Varian Cary 100 spectrophotometer. Fluorescence spectra were measured with a Varian CARY Eclipse Fluorescence spectrophotometer. Spectral-grade solvents were used for measurements of UV-vis absorption and fluorescence.

Synthesis of C359: 3, 3′-dithiodipropionic acid (0.500g，2.38mmol）and triethylamine (431μl ， 3.09mmol) were dissolved in dichloromethane (40mL). After the solution was cooled to 0 ºC, oxalyl chloride (264μl ， 3.09mmol) was added dropwise. The reaction mixture was stirred at room temperature for 1 hour. After removed solvent under vacuum, the resulting mixture added in the stirring solution of 7-hydroxyl coumarin (771mg，4.76mmol）and triethylamine (1ml) in dichloromethane (40mL), the reaction mixture was stirred at room temperature overnight. The solvent was removed under vacuum, and the residual solid was purified by flash chromatography (silica gel) to afford **C498** 2.13g (90%). ^1H NMR (400 MHz, DMSO-d_6, δ ppm): 8.05 (d, 2H), 7.75 (d, 2H), 7.23 (s,1H), 7.14 (m, 2H), 6.46 (d, 2H), 5.74 (s,1H), 3.07 (m, 8H). ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 170.5, 160.3, 154.7, 153.3, 144.5, 130.1, 119.2, 117.4, 116.3, 110.6, 34.3, 32.9. **C498** (666mg，1.34mmol) and 2-thiol pyridine (180mg，1.62mmol) were dissolved in ethyl acetate (20ml). After the addition of 3 drops of BF_3 ether solution, the reaction mixture was stirred at room temperature for 3 days. White solid was appear, and after filtering, filter cake was washed by cold ethyl acetate to afford 390mg of **C359** (81%). ^1H NMR (400 MHz, DMSO-d_6, δ ppm): 8.47 (d, 1H), 8.06 (d, 2H), 7.84-7.75 (m,3H), 7.26 (m, 2H), 7.14 (d, 1H), 6.47 (d, 1H), 3.17 (m,2H), 3.05 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 169.9, 159.6, 158.6, 153.9, 152.5, 149.5, 143.7, 137.9, 129.3, 121.4, 119.5, 118.5, 116.7, 115.6, 109.9, 33.4, 32.6. HR-MS (ESI-TOF) (m/z): $C_{17}H_{14}N_1O_4S_2$ calcd, 360.0359; found, 360.0356 $[M + 1]^+$,

UV and FL Spectroscopic measurements: Stock solutions of probe **C359** (5×10^{-3} M) and NaHS (1.5×10^{-2} M) were prepared in deionized H_2O. 3 mL Tris HCl buffer (200mM, pH=7.4) was firstly added to a 5 mL cuvette, and then 3 μL of probe stock solutions and 0-16 μL of NaHS stock solutions were added. The resulting solution was thoroughly shaken before recording the spectra.

CSE enzyme inhibition assay using C359: The standard reaction was performed in the presence of 2μg of CSE, 6μM PLP, 0.5mM of L-cysteine as substrate, and 10μM of **C359** as the probe in 100μL Tris HCl buffer (200mM, pH=7.4). The concentration of inhibitors were varied from 0-5mM, and the assays were incubated for 75 minutes before fluorescence was measured. Data are presented as mean±SEM; n=3; *P<0.05 versus control.

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