A Fluorescently Ratiometric Natural Probe for Selective Detection of Sulfur Dioxide Derivative and Host-Guest Supramolecular Regulation

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Abstract

The natural sanguinarine (SG) was first used as a fluorescent probe to develop a novel ratiometric sensor for the selective HSO$_3^-$ detection. The nucleophilic addition reaction of HSO$_3^-$ occurs at the C=N$^+$ group of SG and the subsequent breakage of the conjugated π cycle leads to a decrease in the SG iminium fluorescence that is accompanied by an increase in the alkanolamine fluorescence. Therefore, a ratiometric fluorescence method with a large wavelength shift can be established for the HSO$_3^-$ detection. Furthermore, cucurbit[8]uril was used as an efficient host to encapsulate SG for an improved selectivity for the HSO$_3^-$ detection over H$_2$S. Our method benefits little interference from other common anions and cations for the HSO$_3^-$ detection, suggesting a promising application in real sample analysis. Besides the sensor development, the interaction of the natural SG with HSO$_3^-$ was first demonstrated in this work to further get an insight into the SG’s pharmacology.

Keywords: Sanguinarine, ratiometric fluorescence, HSO$_3^-$, detection, cucurbit[8]uril, selectivity.
Introduction

As major environmental pollutants, SO₂/H₂S derivatives (including sulfurous acid (H₂SO₃), sulfite (SO₃²⁻), bisulfite (HSO₃⁻), hydrogen sulfide (H₂S), sulphion (S²⁻), etc) can cause heavy damages to the ecological environment and human health especially with a long-term exposure.¹-⁶ To prevent oxidation, some foods and beverages are stored also using sodium sulfite as an efficient preservative. However, asthma and other allergic symptoms can arise with an excess of sulfite involving in foods and beverages.⁷ Furthermore, it is accepted that endogenous SO₂ and its derivatives in mammals can modulate a wide range of physiological processes.⁸ Therefore, there is a great demand to develop a straightforward method for reliable detection of SO₂ derivatives.

Variant Fluorescent probes have been utilized for the detection of SO₂ derivatives by nucleophilic addition to double bonds (C=O, C=C, C=N, and N=N), reduction of azide, nitryl, and selenoxide groups, and the Michael addition reaction.³-⁵,⁹,¹⁰ The probes with fluorescent ratiometric responses have received much attention due to their ability to eliminate fluctuations of the measuring environments by self-calibration. Special efforts must be made to synthesize the ratiometric probes. For example, fluorophores with the levulinate moiety can be selectively deprotected by SO₃²⁻/HSO₃⁻ to allow occurrence of excited-state intramolecular proton transfer (ESIPT) or internal charge transfer (ICT) with a subsequent red-shifted emission.¹¹-¹³ The nucleophilic addition of SO₃²⁻/HSO₃⁻ to double bonds interrupts the intramolecular charge transfer (ICT) process or fluorescence resonance energy transfer (FRET) process and leads to a ratiometric change of the fluorescence signal.¹⁴-¹⁹ However, developing ratiometric probes for the selective SO₃²⁻/HSO₃⁻ detection without elaborate synthesis procedure but with a large emission wavelength shift is still under the way.

We have being continued to search natural products that have selective molecular
recognition capacities$^{20-23}$ along with promising theranostic potentials. Sanguinarine (SG), a natural benzophenanthridine alkaloid, is well known for its antitumor property and cancer treatment.$^{24-28}$ It has been reported that the C6=N5$^+$ double bond in SG can be attacked by a nucleophilic reagent (for example, OH$^-$) towards converting the positively charged iminium form to the alkanolamine form (Scheme 1).$^{20,29,30}$ These two forms emit at two bands with a wavelength separation of at least 100 nm. Therefore, SG can serve as a natural ratiometric probe for the straightforward SO$_2$ derivative detection without the elaborate probe synthesis procedure.

In this work, we first investigated the possibility of SG interacting with HSO$_3^-$ for the HSO$_3^-$ detection by converting the iminium form to the alkanolamine form. Interestingly, the supramolecular encapsulation of SG with cucurbit[8]uril (CB[8])$^{31}$ can improve the selectivity for the HSO$_3^-$ analysis.

**Experimental**

**Reagents and chemicals**

Cucurbit[n]urils (CB[n], n=5, 6, 7, 8) were purchased from Strem Chemicals, Inc. (Massachusetts, USA). SG and metal ions were purchased from Aladdin Reagent Co. (Shanghai, China). Na$_2$SO$_3$ and Na$_2$S (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) were used as the sulfur-containing species. At the optimized pH 6.2, they exist mainly at HSO$_3^-$ and H$_2$S ($p$K$_{a1}$ and $p$K$_{a2}$ for H$_2$SO$_3$ are about 1.9 and 7.2, while those for H$_2$S are about 6.9 and 14.1, respectively). In this work, for convenience, we nominate them as HSO$_3^-$ and H$_2$S in all the text, although they shouldn’t exist at these states at pH other than 6.2. Other reagents were of analytical grade (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) and used without any purification. Milli-Q water (18.2 MΩ; Millipore Co, Billerica, USA) was used in all of the experiments.
**Fluorescence measurements**

Fluorescence spectra were acquired with a F-2700 spectrofluorometer (Hitachi High-Technology Corp, Japan). Fluorescence was measured in a quartz cell with a path length of 1 cm under excitation at 327 nm. SG at the specified concentration was added into the solution buffered with 50 mM PBS (0.1 M K+) at the specified pH, and the resulting solutions were allowed incubation for 30 min before addition of CB[n]. Fluorescence measurements were measured at 20 °C after incubating the resultant solution with HSO₃⁻ or other species for 30 min.

**Absorption spectra measurements**

Absorption spectra were determined with a UV2550 spectrophotometer (Shimadzu Corp., Japan), equipped with a temperature-controlled circulator (Julabo Laborteknik GmbH, Seelbach, Germany) at 20 °C and a quartz cell with a path length of 1 cm.

**¹H NMR experiments**

¹H NMR experiments were acquired with a Bruker Avance 600 MHz NMR spectrometer. Samples were prepared by dissolving SG in 0.5 mL D₂O (4 mM). After NMR scanning, 20 mM HSO₃⁻ was then added to allow incubation for 30 min before the NMR measurements.

**Sample detection**

Tap water and rainwater samples were determined using the standard addition method. The final SG and CB[8] concentrations were maintained at 1 μM and 5 μM, respectively. Into the water samples were added PBS buffer (50 mM, 0.1 M K⁺, pH 6.2) containing SG and CB[8] before fluorescence measurement. Then an appropriate Na₂SO₃ standard was added to allow incubation for 30 min before recording fluorescence. The calibration curve was used to get the recovery and the sample results.

**Results and Discussion**
In aqueous solution, SG exists in the iminium and alkanolamine forms with their populations dependent on pH.\textsuperscript{20,29,30} As shown in Fig. 1A, increasing the solution pH (from 4.5 to 9.2) cause an increase in the alkanolamine emission band ($F_a$) and a decrease in the iminium emission band ($F_i$) of 1 $\mu$M SG because of the OH$^-$ attack with the C6=N5$^+$ bond (Scheme 1).\textsuperscript{18,29,30} The $F_a$ band at 409 nm and the $F_i$ band at 565 nm can be obtained with excitation at 327 nm.\textsuperscript{20} The fluorescence intensity at 409 nm as a function of pH predicts a pK$_a$ of about 7.6 (Figure 1D), which is in good agreement with the previous reports.\textsuperscript{20,29,30} Such large emission wavelength shift (156 nm) due to the breakage of the conjugated $\pi$ cycle (Scheme 1) is advantageous in developing novel sensors with an ideal contrast in signal response, although up to now, only the nucleophilic addition reaction of OH$^-$ has been investigated. In this work, we tried to use this property to develop a fluorescent sensor for selective detection of HSO$_3^-$.

As shown in Fig. 1B, at pH 6.2, increasing the CB[8] concentration up to 6 $\mu$M shifts the emission band of SG (1 $\mu$M) from 565 nm to 544 nm, accompanying a final 12-fold fluorescence increase. This host-guest interaction can be confirmed by the solution color change under UV illumination (inset of Fig. 1B). This indicates that the CB[8] cavity provides a hydrophobic microenvironment to protect SG from attacking by water molecules and other species since SG at pH 6.2 is positively charged, as confirmed by other SG analogues with a solvent polarity-sensitive fluorescence behavior.\textsuperscript{34} The pH-dependent experiments with CB [8] (Fig. 1C) show that the iminium form still remains even increasing the solution pH to 9.2, suggesting a strong binding of the SG iminium form with CB[8] and inhibition of the
nucleophilic interaction of OH\(^{-}\) to produce the alkanolamine form. For example, at pH 9.2, the fluorescence of the SG alkanolamine form in the presence of CB[8] is 2.4-fold lower than that obtained in the absence of CB[8]. Since CB[n] has a weak binding with the SG alkanolamine form\(^{32}\) and the fluorescence of the alkanolamine form is not strongly affected by CB[8], we can estimate that at pH 9.2, only 42% SG (totally in 1 μM) is converted to the alkanolamine form in the presence of 5 μM CB[8]. Because the used PBS buffer can’t be extended to higher pH, we extrapolated the pK\(_a\) value of the CB[8]-bound SG to be about 9.4, which is about 1.8 pK\(_a\) unit higher than that obtained without CB[8]. However, a pK\(_a\) shift of 3.7 has been observed in a previous report for the SG encapsulation by CB[7] in water solution without substantial amount of cation ions.\(^{32}\) This is caused by the high ion strength of 0.1 M K\(^+\) used in this work thus to weaken the encapsulation binding with CB[n] due to the competitive electrostatic binding of cation ions with the electronegative portals of CB[n].\(^{35}\) On the other hand, this host-guest encapsulation will decrease the background fluorescence in developing SG-based ratiometric sensor when operating in weakly acidic solution at which SO\(_2\) can exist dominantly as HSO\(_3^−\) (pK\(_{a1}\) and pK\(_{a2}\) for H\(_2\)SO\(_3\) are about 1.9 and 7.2). For example, at pH 6.2, the presence of CB[8] leads to a 6.8-fold decrease in fluorescence of the alkanolamine form (Fig. 1D). We thus used pH 6.2 as the optimal buffer condition to prevent the OH\(^{-}\) reaction with SG.

We then measured the fluorescence response of SG (1 μM) to HSO\(_3^−\) and H\(_2\)S at pH 6.2 with their concentrations up to 300 μM. As shown in Fig. 2A, addition of HSO\(_3^−\) cause a decrease in the iminium band at 565 nm and an increase in the alkanolamine band but appearing at 435 nm. This suggests a specific interaction of SG with HSO\(_3^−\) to produce a product different from the reaction occurring with OH\(^{-}\) (Scheme 1). Note that this 130 nm emission wavelength shift provides an ideal contrast in developing a ratiometric method for the HSO\(_3^−\) detection, as confirmed by the clear color change under UV illumination (inset of Fig. 2A). The fluorescence ratio at 435 nm and 565 nm (F\(_a\)/F\(_i\)) is linear to the HSO\(_3^−\) concentration between 0 and 30 μM
(Fig. 2E). The detection limit is estimated to be about 1.2 μM, assuming a signal-to-noise ratio of 3. However, addition of H2S within the same concentration range similarly leads to the alkanolamine band emitting but at 430 nm and brings smaller F/Fi values (Fig. 2B). Within the linear response range, the response slope (Δ(Fa/Fi) vs. ΔC) for HSO3− is only 11-fold higher than that for H2S (Fig. 2E). These facts suggest that HSO3− has a stronger nucleophilic addition reaction with SG in comparison to H2S (Scheme 1) most likely because of the higher electron density of the sulfur atom in HSO3−.

To improve the response specificity of SG to HSO3−, we then checked the fluorescence response of SG (1 μM) to HSO3− and H2S in the presence of 5 μM CB[8]. As shown in Fig. 2C, the CB[8] addition causes a more noticeable increase and decrease in the fluorescence of SG at 435 nm and 544 nm in response to HSO3− at pH 6.2, although this host-guest encapsulation blunts the response with the linear range for the HSO3− response increasing up to about 200 μM (Fig. 2F). This suggests that this encapsulation can expand the HSO3− response range. The change in the solution color under UV illumination upon the HSO3− addition becomes much more distinct (inset of Fig. 2C). The detection limit in this case is estimated to be about 16 μM, assuming a signal-to-noise ratio of 3. However, H2S is much less efficient in decreasing the iminium band and increasing the alkanolamine band (Fig. 2D). Within the linear response range, the response slope (Δ(Fa/Fi) vs. ΔC) for HSO3− in the presence of CB[8] is 45-fold higher than that for H2S (Fig. 2F). Furthermore, coexistence of H2S (up to 8 mM) with 180 μM HSO3− only slightly increases the F/Fi value (Fig. 3). These facts demonstrate that the CB[8] encapsulation can improve the selectivity of the SG response to HSO3− against H2S because of the stronger inhibition of the nucleophilic addition32 by CB[8] with the sulfur atom in H2S that has a lower electron density than that in HSO3−. Thus, less interference from H2S is expected for the HSO3− detection. Note that upon addition of HSO3− or H2S in the absence and presence of CB[8], the absorption band of SG almost exists at about 327 nm (Fig. S2), suggesting that the ratiometric
sensor for the HSO$_3^-$ detection can be operated at a single excitation wavelength.

$^1$H NMR was then used to further confirm the interaction of SG with HSO$_3^-$.

As shown in Fig. 4, the $^1$H NMR chemical shifts of SG alone in D$_2$O can be assigned to 4.45 (N5-CH$_3$), 6.13 (C2-O-CH$_2$-O-C3), 6.35 (C7-O-CH$_2$-O-C8), 6.96 (H-1), 7.45-7.81 (H-4, H-9, H-10, H-11, H-12), and 9.30 (H-6). After addition of 5 equivalent HSO$_3^-$, these chemical shifts changed to 2.69 (N5-CH$_3$), 5.53 (H-6), 5.94-6.04 (C2-O-CH$_2$-O-C3 and C7-O-CH$_2$-O-C8), 6.94 (H-1), and 7.10-7.68 (H-4, H-9, H-10, H-11, H-12), respectively. These data suggest that the two types of protons in N5-CH$_3$ and H-6 experience the largest chemical environment alteration by shifting to the high field in the $^1$H NMR upon interaction with HSO$_3^-$, demonstrating that the electron cloud densities around these protons are increased. Therefore, we can conclude that the C6=N5$^+$ group undergo a nucleophilic addition by HSO$_3^-$, as depicted in Scheme 1.

The effect of common anions and cations on the HSO$_3^-$ response specificity was then examined. As shown in Fig. 5A, in comparison with the significant response for 200 μM HSO$_3^-$, representative anions of F$, SO_4^{2-}$, Cl$, I$, Br$, NO_3^-$, Ac$^-$, and H$_2$PO$_4^-$ alone in solution at 1 mM still keep the F$_0$/F$_i$ value at the background level. Coexistence of these anions with HSO$_3^-$ has no obvious effect on the HSO$_3^-$ response. Note that the reductive I$^-$ is inefficient in interacting with SG. The HSO$_3^-$ specificity over other anions can be discriminated by the naked eye under UV illumination (Fig. 5C). Furthermore, typical cations such as Cu$^{2+}$, Pb$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, Al$^{3+}$, Ca$^{2+}$, Fe$^{3+}$, Mg$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, and Hg$^{2+}$ alone and coexisted with HSO$_3^-$ (each cation was at 200 μM and EDTA was added to avoid the direct interaction of cations with HSO$_3^-$) don’t induce significant changes in the F$_0$/F$_i$ value (Fig. 5B). The solution colors under UV illumination (Fig. 5D) also confirm the specificity for the HSO$_3^-$ response.

Finally, we explored application of our method in detecting HSO$_3^-$ using tap water and rainwater as the typical samples. Standard addition method was used to confirm feasibility of our method. The recoveries of six samples were close to 100% (Table S1), demonstrating that
our method can be used to the real sample analysis. Therefore, our method has potential applications for the selective detection of HSO$_3^-$ in water samples.

Conclusions

In summary, we developed a novel fluorescent method for the selective HSO$_3^-$ assay based on its nucleophilic addition reaction with SG. The reaction occurs at the C=N$^+$ group to bring a decrease in the SG iminium fluorescence and an increase in the alkanolamine fluorescence. Thus, a ratiometric fluorescence method can be established for the HSO$_3^-$ detection. Furthermore, the host-guest encapsulation with CB[8] is confirmed to be an efficient way to improve the detection selectivity of HSO$_3^-$ against other species including, for example, H$_2$S. Other common anions and cations have no interference with the HSO$_3^-$ response, suggesting a promising application of our method in real sample analysis. In this work, we first demonstrated occurrence of interaction of the natural benzophenanthridine alkaloid SG with HSO$_3^-$ and a ratiometric fluorescence method was developed with a large emission wavelength shift. Since SO$_2$ exists in cells and SG is usually used as an anti-cancer drug, our work also demonstrates the potential SG pharmacology by interaction with SO$_2$ derivatives.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 21675142) and the National and Zhejiang Undergraduate Training Program for Innovation and Entrepreneurship (Grant No. 201810345017).

Supporting Information
Fluorescence spectra of the SG-CB[n] system in the absence and presence of HSO$_3^-$, and the sample analysis results. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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**Figure Captions**

Scheme 1. The structure conversion of SG from the iminium form to the alkanolamine form upon its interaction with HSO$_3^-$ following the emission wavelength shifts.

Fig. 1 Effect of solution pH on the emission spectra of 1 μM SG in 50 mM PBS (0.1 M K$^+$) in the (A) absence and (C) presence of 5 μM CB[8]. (B) Fluorescence spectra of SG (1 μM) in 50 mM PBS (0.1 M K$^+$, pH 6.2) upon increasing the CB[8] concentration. Inset: Fluorescence intensity at 544 nm upon increasing the CB[8] concentration and photographs under UV illumination in the (left) absence and (right) presence of 5 μM CB[8]. (D) Dependence of fluorescence intensity at 409 nm on solution pH in the absence and presence of CB[8].

Fig. 2 Fluorescence spectra of SG (1 μM) in 50 mM PBS (0.1 M K$^+$, pH 6.2) upon increasing the concentration of (A, C) HSO$_3^-$ or (B, D) H2S in the (A, B) absence and (C, D) presence of 5 μM CB[8], respectively. Photographs under UV illumination in the absence (left) and presence...
(right) of HSO$_3^-$ without and with CB[8] are shown in Inset of (A, 50 μM HSO$_3^-$) and (C, 200 μM HSO$_3^-$), respectively. Also shown in inset of (B) and (D) are the F$_0$/F$_1$ changes upon increasing the HSO$_3^-$ or H$_2$S concentration without and with CB[8], respectively. (E) Fluorescence spectra of SG (1 μM) and CB[8] (5 μM) with HSO$_3^-$ alone (180 μM) and coexisted with H$_2$S.

Fig. 3  Fluorescence spectra of SG (1 μM) and CB[8] (5 μM) with HSO$_3^-$ alone (180 μM) and coexisted with H$_2$S.

Fig. 4  $^1$H NMR spectra of SG (4 mM) and its interaction with HSO$_3^-$ (20 mM) in D$_2$O.

Fig. 5  Fluorescence ratio changes of SG (1 μM) in the presence of (A) anions (HSO$_3^-$ at 200 μM and others at 1 mM) and (B) cations (200 μM, EDTA 3 mM) in 50 mM PBS (pH 6.2, 0.1 M K$^+$) containing 5 μM CB[8]. Also shown are photographs of these solutions under UV illumination in the presence of  (C) anions and (D) cations, respectively.
Scheme 1. The structure conversion of SG from the iminium form to the alkanolamine form upon its interaction with OH⁻ (at alkalic solution) and HSO₃⁻ (at acidic solution) following the emission wavelength shifts. The CB[8] binding with the iminium form of SG highly favors the nucleophilic addition of HSO₃⁻ over H₂S.
Fig. 1  Effect of solution pH on the emission spectra of 1 μM SG in 50 mM PBS (0.1 M K⁺) in the (A) absence and (C) presence of 5 μM CB[8]. (B) Fluorescence spectra of SG (1 μM) in 50 mM PBS (0.1 M K⁺, pH 6.2) upon increasing the CB[8] concentration. Inset: Fluorescence intensity at 544 nm upon increasing the CB[8] concentration and photographs under UV illumination in the (left) absence and (right) presence of 5 μM CB[8]. (D) Dependence of fluorescence intensity at 409 nm (Fₐ) on solution pH in the absence and presence of CB[8].
Fig. 2  Fluorescence spectra of SG (1 μM) in 50 mM PBS (0.1 M K+, pH 6.2) upon increasing the concentration of (A, C) HSO$_3^-$ or (B, D) H$_2$S in the (A, B) absence and (C, D) presence of 5 μM CB[8], respectively. Photographs under UV illumination in the absence (left) and presence (right) of HSO$_3^-$ without and with CB[8] are shown in Inset of (A, 50 μM HSO$_3^-$) and (C, 200 μM HSO$_3^-$), respectively. Also shown are the F/F$_i$ change upon increasing the HSO$_3^-$ or H$_2$S concentration (E) without and (F) with CB[8], respectively.
Fig. 3  Fluorescence spectra of SG (1 μM) and CB[8] (5 μM) with HSO₃⁻ alone (180 μM) and coexisted with H₂S.
Fig. 4  $^1$H NMR spectra of SG (4 mM) and its interaction with HSO$_3^-$ (20 mM) in D$_2$O.
Fig. 5  Fluorescence ratio changes of SG (1 μM) in the presence of (A) anions (HSO$_3^-$ at 200 μM and others at 1 mM) and (B) cations (200 μM, EDTA 3 mM) in 50 mM PBS (pH 6.2, 0.1 M K$^+$) containing 5 μM CB[8]. Also shown are photographs of these solutions under UV illumination in the presence of (C) anions and (D) cations, respectively.