A 4-Methylumbelliferone-based Fluorescent Probe for Sodium New Houttuyfonate

Yang, Xiaofeng*(杨小峰) Wang, Liping(王丽萍) Zhao, Minglei(赵明雷)
Qi, Haiping(齐海萍) Wu, Yao(毋尧)

Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education, Institute of Analytical Science, Department of Chemistry, Northwest University, Xi'an, Shaanxi 710069, China

A new fluorogenic probe for sodium new houttuyfonate (SNH) was proposed. 4-Methylumbelliferyl-2,4-dinitrobenzenesulfonate (4-MUDNBS) was a nonfluorescent compound and was synthesized via the one-step reaction of 4-methylumbelliferone (4-MU) with 2,4-dinitrobenzenesulfonyl chloride. In basic media, SNH was decomposed to produce sodium sulfite, which then reacted with 4-MUDNBS to yield highly fluorescent 4-MU, hence leading to the fluorescence increase of the reaction solution. A linear correlation existed between the emission intensity and the concentration of SNH within the range from 0.5 to 15 μg•mL^{-1} with a detection limit of 0.15 μg•mL^{-1} (3σ). The effect of substituents on the benzenesulfonyl moiety of the probe is discussed, and the presence of electronegative groups is favorable for the proposed cleavage reaction.

Keywords sodium new houttuyfonate, 4-methylumbelliferone, fluorescent probes, fluorescence spectroscopy

Introduction

Sodium new houttuyfonate (SNH, sodium lauroyl-α-hydroxyethyl sulfonate) is a novel antimicrobial medicine for clinical practice in recent years (Scheme 1). Previous studies showed that houttuyfonate homologues (HOU-C_n) had immunoregulatory activities and typical adjuvanticity, and could improve the immune ability of mice and inhibit the growth of *Staphylococcus aureus*, *Bacillus subtilis* and *pneumococci*.[1-3] And the immunoregulation and antibacterial activity of SNH were higher and stronger than that of sodium houttuyfonate.[4,5] HPLC method has been successfully applied to the determination of SNH in injections.[6,7] Though HPCL method is accurate, specific, it suffered from the disadvantages of low sensitivity, narrow linear range and time-consuming.

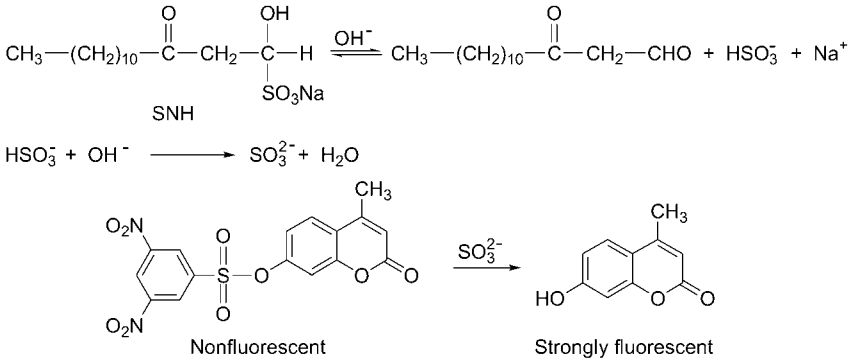
Fluorimetric analysis is widely used for its high sensitivity, but no work has been reported for the fluorimetric determination of SNH. Herein, we developed a simple and sensitive fluorogenic probe for determining SNH with a new interaction mechanism.

It was reported that strongly electron-withdrawing nitro group is the most effective in labilizing the carbon-sulfur bond in benzenesulfonamide.[8] The resulting benzenesulfonamide can be readily cleaved by thiolate anion, derived from thiol under basic conditions through nucleophilic aromatic substitution.[9,10] Based on this mechanism, several fluorescent probes for thiol-containing compounds have been developed.[11-14] In our recent studies, we observed that other nucleophiles, such as sulfide anion and bisulfite can also lead to the cleavage reaction. Encouraged by this observation, we envisioned that this cleavage reaction might be utilized for the determination of SNH, and such an idea has been attempted in this work by designing a new fluorogenic probe, 4-methylumbelliferyl-2,4-dinitrobenzenesulfonate (4-MUDNBS), for the selective determination of SNH in basic solution.

In the present study, protection of the hydroxyl group in 4-MU with 2,4-dinitrobenzenesulfonyl chloride gives a nonfluorescent 4-MUDNBS. Upon incubation with SNH in basic solution at room temperature, the equilibrium reaction between SNH and sodium bisulfite in aqueous solution moves toward right, and thus sulfite is formed, which can then react with 4-MUDNBS to yield the highly fluorescent 4-MU and hence leading to a dramatic increase in fluorescence intensity (Scheme 1). Based on this mechanism, a new fluorescent probe for SNH was developed. The fluorescence increase is linearly with SNH concentration in the range 0.5—15 μg•mL^{-1} with a detection limit of 0.15 μg•mL^{-1}. The effect of substituents on the benzenesulfonyl unit of the probe is discussed, and the presence of electronegative groups is favorable for the cleavage reaction.

* E-mail: xfyang@nwu.edu.cn; Tel.: 0086-029-88302604; Fax: 0086-029-88303798
Received December 28, 2009; revised February 27, 2010; accepted March 23, 2010.
Projected supported by the Science & Technology Department (No. SJ08B05) and the Education Department (No. 09JK787) of Shaanxi Province of China, and NWU Graduate Experimental Research Funds (No. 09YSY19).

Scheme 1 Proposed mechanism for the detection of SNH with 4-MUDNBS



Experimental

Apparatus

The fluorescence spectra and relative fluorescence intensity were measured with a Sanco CRT-970 spectrofluorimeter (Shanghai, China) with a 10 mm quartz cuvette. The excitation and emission wavelength bandpasses were both set at 10 nm. The absorption spectra were measured with a Shimadzu UV-1700 spectrophometer. The pH was measured with a Model pHs-3B meter (Shanghai, China). IR spectra were taken in KBr pellets on a Bruker Tensor 27 IR spectrometer. Mass spectra were obtained with AXIMA-CFR plus MALDI-TOF Mass Spectrometer. ^{1}H NMR spectra were measured with Varian unity INOVA-400 spectrometer (400 MHz) with tetramethylsilane (TMS) as internal standard. Elemental analysis were obtained with a vario EL III Elemental Analyzer.

Reagents

A stock solution of 0.2 mg•mL^{-1} SNH was prepared by dissolving appropriate amount of SNH in 50 mL water. This stock solution was stored at 4 ℃ in the refrigerator. Standard test solutions were prepared daily by appropriate dilution of the stock solution. 4-MUDNBS solution (1.0 mmol•L^{-1}) was prepared by dissolving 20.3 mg of the probe in 50 mL ethyl acetate. 4-MUTS solution (1.0 mmol•L^{-1}) was prepared by dissolving 8.25 mg of 4-MUTS in 25 mL ethyl acetate. 4-MUNBS solution (2.0 mmol•L^{-1}) was prepared by dissolving 18.1 mg of 4-MUNBS in 25 mL ethyl acetate. A 0.1 mol•L^{-1} NH_3NH_4Cl buffer solution (pH 8.9) was employed. The source of reagents were as follows: SNH was obtained from Qinghai Xiadu Pharmaceutical Co., LTD; 4-MU was obtained from Beijing Hengye Zhongyuan Chemical Reagent Co., 2-nitrobenzenesulfonyl chloride and 2,4-dinitrobenzenesulfonyl chloride were obtained from Alfa Aesar. *p*-Toluenesulfonyl chloride was obtained from Tianjin Chemical Reagent Plant.

All the reagents were of analytical-reagent grade, and doubly distilled water was used throughout.

Synthesis of 4-MUDNBS

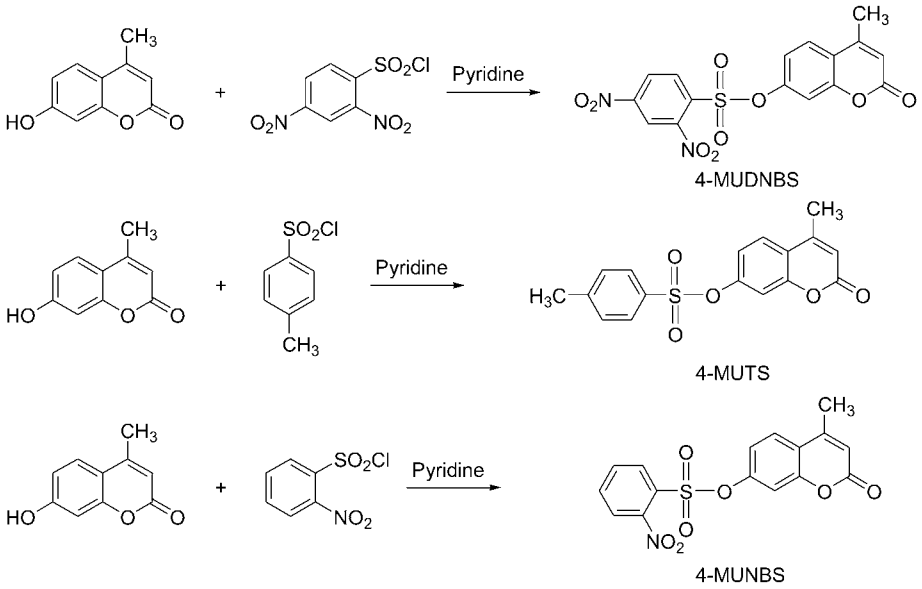
A mixture of 4-MU (0.1 g, 0.57 mmol) and 2,4-dinitrobenzenesulfonyl chloride (1.2 equiv.) in 5 mL dried pyridine was stirred overnight at room temperature (Scheme 2). The resulting reaction solution was poured into saturated brine and yellow precipitate was observed. The precipitate was filtered off and dried to give a light yellow solid. This solid was subjected to silica gel (200—300 mesh) chromatography eluted with chloroform-ethyl acetate (7∶1, *V*∶*V*) to afford the desired product (0.16 g, 69% yield) as a white solid. The product was shown to consist of only one substance by thin layer chromatography (TLC), and its R_f value was 0.68 (chloroform/ethyl acetate, *V*∶*V*=4∶1). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.12 (d, *J*=2.0 Hz, 1H), 8.60 (dd, *J*=2.0, 8.8 Hz, 1H), 8.29 (d, *J*=8.4 Hz, 1H), 7.85 (d, *J*=8.4 Hz, 1H), 7.35 (d, *J*=2.4 Hz, 1H), 7.22 (dd, *J*=2.4, 8.8 Hz, 1H), 6.47 (d, *J*=0.8 Hz, 1H), 2.41 (d, *J*=1.2 Hz, 3H); IR (KBr) *v*: 1732.4, 1557.7, 1537.7 cm^{-1}; MS (MALDI-TOF) *m*/*z*: 406.1 (M^+). Anal. calcd for $C_{16}H_{10}H_2O_9S$: C 47.30, H 2.48, N 6.89; found C 47.65, H 2.45, N 6.38.

Synthesis of 4-methylumbelliferyl-*p*-toluenesulfonate (4-MUTS)

A mixture of 4-MU (0.2 g, 1.14 mmol) and *p*-toluenesulfonyl chloride (1.5 equiv.) in 5 mL dried pyridine was stirred overnight at room temperature (Scheme 2). The resulting reaction solution was poured into saturated brine and white precipitate was observed. The precipitate was filtered off and dried to give a white solid. This solid was dissolved by 60 mL of CH_2Cl_2, washed with 0.1 mol•L^{-1} Na_2CO_3 (10 mL×2) and water (10 mL×2), and dried over anhydrous Na_2SO_4. The solvent was removed by evaporation to dryness, giving the desired product as white solid (0.23 g, 61.3% yield). The product was shown to consist of only one substance by thin layer chromatography (TLC), and its R_f value was 0.72 (ethyl acetate/petroleum ether, *V*∶*V*=1∶1). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.73 (dd, *J*=1.6, 6.8 Hz, 2H), 7.57 (d, *J*=8.4 Hz, 1H), 7.34 (dd, *J*=0.8, 8.0 Hz, 2H), 7.11 (dd, *J*=2.4, 8.8 Hz, 1H), 6.83 (d, *J*=1.2 Hz, 1H), 6.28 (d, *J*=1.2 Hz, 1H), 2.48 (s, 3H), 2.42 (d, *J*=1.6 Hz, 3H); MS (MALDI-TOF) *m*/*z*: 330.0 (M^+). Anal. calcd for $C_{17}H_{14}O_5S$: C 61.81, H 4.27; found C 61.47, H 4.29.

CHINESE JOURNAL OF CHEMISTRY

Scheme 2 Synthesis of 4-MUDNBS, 4-MUTS and 4-MUNBS



Synthesis of 4-methylumbelliferyl-2-nitrobenzenesulfonate (4-MUNBS)

Following the procedure for the preparation of 4-MUTS, 4-MUNBS was prepared as a white solid (66.7% yield) from 4-Mu and 2-dinitrobenzenesulfonyl chloride. R_f = 0.57 (chloroform/ethyl acetate, V : V = 3 : 1). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.02 (d, J = 7.6 Hz, 1H), 7.89—7.87 (m, 2H), 7.75—7.71 (m, 1H), 7.62 (d, J = 9.2 Hz, 1H), 7.26 (dd, J = 2.4, 8.8 Hz, 2H), 6.30 (dd, J = 1.2, 2.4 Hz, 1H), 2.43 (d, J = 1.2 Hz, 3H); MS (MALDI-TOF) m/z: 362.0 (M^+). Anal. calcd for $C_{16}H_{11}NO_7S$: C 53.19, H 3.07, N 3.88; found C 53.16, H 3.25, N 3.83.

Procedure

The fluorogenic reaction was performed in a 10 mL volumetric tube. Typically, to a test tube containing 0.4 mL of 0.1 mmol•L^{-1} 4-MUDNBS, different concentration of SNH was added, and then 2.0 mL of 0.1 mol•L^{-1} NH_3-NH_4Cl buffer solution (pH 8.9) was added and the solution was diluted to 10 mL with water. The reaction solution was kept at room temperature for 35 min, and then the fluorescence intensity of the above solution was recorded at an emission wavelength of 445 nm with excitation wavelength set at 362 nm. In the meantime, a blank solution containing no SNH was prepared and measured under identical conditions for comparison.

Results and discussion

Spectral characteristics

The fluorescence spectra of 4-MUDNBS in the absence and presence of SNH are shown in Figure 1. It can be observed that 4-MUDNBS itself was nonfluorescent prior to its mixing with SNH. However, a significant enhancement of fluorescence with an emission maximum at 446 nm was observed when SNH was introduced. This may result from the fact that sodium sulfite is generated for the decomposition of SNH in basic media, which then reacted with 4-MUDNBS to generate the strongly fluorescent 4-MU through nucleophilic reaction, and thus leading to the fluorescence increase of the reaction mixture. Furthermore, the fluorescence spectra of 4-MUDNBS solution containing different concentrations of SNH were measured, and a characteristic fluorescence emission maximum centered at about 446 nm was recorded (Figure 1), which were consistent with the fluorescence emission maximum of 4-MU at the present conditions. In addition, it can be observed that the fluorescence emission increases dramatically upon increasing the SNH concentration, which forms the basis of SNH determination.

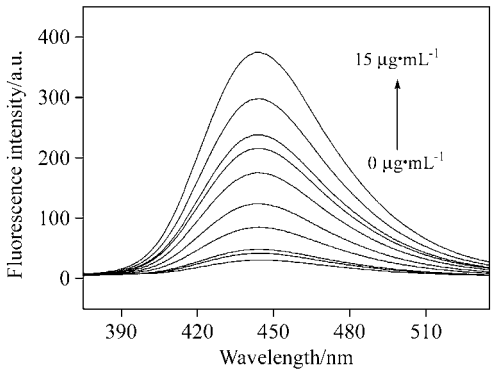


Figure 1 Fluorescence emission spectra (excitation at 350 nm) of 4-MUDNBS (4.0 μmol•L^{-1}) toward different concentrations of SNH (final concentrations: 0, 0.5, 1.0, 3.0, 5.0, 7.0, 9.0, 10, 12, 15 μg•mL^{-1}) after incubation at room temperature for 35 min in pH 8.9 NH_3-NH_4Cl buffer.

Effect of substituents on the cleavage reaction

To investigate the effect of substituents on the benzenesulfonyl moiety of the probe on the cleavage reaction, the fluorescent responses of 4-MUDNBS, 4-MUNBS and 4-MUTS toward SNH were compared and the results are shown in Figure 2. It can be observed that the fluorescence signal of 4-MUDNBS increased dramatically with the reaction time prolonged, while the fluorescence signal of 4-MUNBS and 4-MUTS remained almost unchanged. The significant difference between them lies in the substituents on the benzenesulfonyl moiety of the probe. For 4-MUDNBS, the nitro group on the *ortho* and *para* position of benzenesulfonyl unit can effectively labilize the C—S bond,[8] and hence the cleavage reaction can proceed readily. While for 4-MUTS, because of the lack of electronegative groups, the cleavage of the sulfonate is generally very unfavorable. As for 4-MUNBS, though the presence of the nitro group in its *ortho* position, the cleavage reaction is very slowly compared with that of 4-MUDNBS. Based on the above results, it can be concluded that the electrowithdrawing groups on the *ortho* and *para* position of benzenesulfonyl moiety of the probe can markedly facilitate the nucleophilic reaction.

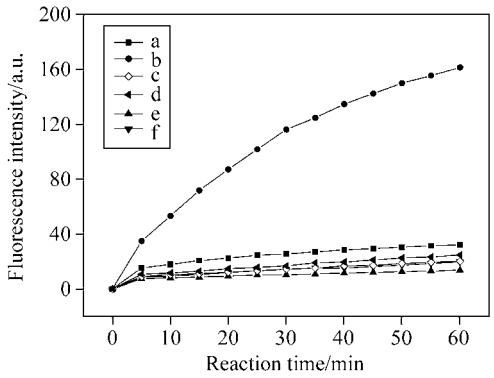


Figure 2 Time dependent fluorescence intensity changes observed during reaction of 4-MUDNBS (or 4-MUNBS, 4-MUTS) with SNH (5.0 μg•mL^{-1}) in pH 8.9 NH_3-NH_4Cl buffer. (a) 4-MUDNBS; (b) 4-MUDNBS + SNH; (c) 4-MUNBS; (d) 4-MUNBS+SNH; (e) 4-MUTS; (f) 4-MUTS+SNH. The concentrations of 4-MUDNBS, 4-MUNBS and 4-MUTS are 4.0 μmol•L^{-1}.

Effect of reaction time

The effect of reaction time on the fluorescence response of 4-MUDNBS with SNH is demonstrated in Figure 3. It can be seen that the fluorescence intensity of the reaction system increased rapidly with the reaction time prolonged. Meanwhile the blank signal also increased slowly at the same conditions. The effect of the reaction time on the F/F_0 value was also investigated and the results are shown in Figure 3. It can be seen that F/F_0 value of the reaction system increased rapidly at the beginning, while the F/F_0 value increased very slightly after 30 min. Therefore, a 35 min reaction time was selected in the following experiment.

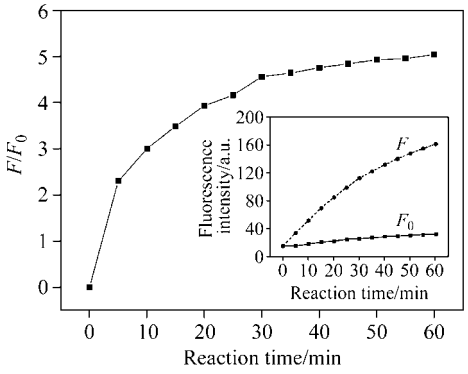


Figure 3 Effect of reaction time on fluorescence intensity of 4-MUDNBS (4.0 μmol•L^{-1}) cleavage system by SNH (5.0 μg•mL^{-1}). F and F_0 are the fluorescence intensity of 4-MUDNBS solution in the presence and absence of SNH, respectively. Other conditions were the same as those described in the experimental section.

Effect of pH

The pH had a great effect on the sensitivity of the proposed method and was selected considering the following three aspects. (i) It was reported that SNH can decompose to produce lauroyl acetaldehyde and sodium sulfite in basic media,[15] and the latter is an essential compound responsible for the proposed fluorogenic reaction. Therefore, to ensure the decompose of SNH to yield sulfite, the reaction solution should be kept at basic conditions. (ii) As the pK_a of the 4-MU phenolic proton is 7.8,[16] it requires relatively high pH medium (pH *ca.* 9) to achieve maximum fluorescence intensity. (iii) Because the nucleophilic attack by hydroxide can take place and thus leading to the cleavage of the sulfonate,[8] a high background signal may be recorded under high pH media.

In order to select the optimum conditions for the determination of SNH with 4-MUDNBS, the effect of pH was studied in the range 8.3—9.8, and the results are shown in Figure 4. It can be observed that both the fluorescence signal of reaction system and blank solution increased with increasing pH. It can also be observed from Figure 4 (insert curve) that F/F_0 increased with increasing pH, obtained the maximum value at pH 8.9, and thereafter decreased. Hence, to obtain a high F/F_0 value, pH 8.9 of NH_3-NH_4Cl buffer was selected for the fluorogenic reaction in the subsequent experiments.

Study of interferences

In order to investigate the possibility of practical application in determination of pharmaceutical preparation, the interference from some metal ions and excipients often contained in tablets, such as lactose, starch, and mannitol, were tested in the chosen condition. The effects of foreign substances were tested by analyzing a standard solution of SNH (5.0 μg•mL^{-1}) to which

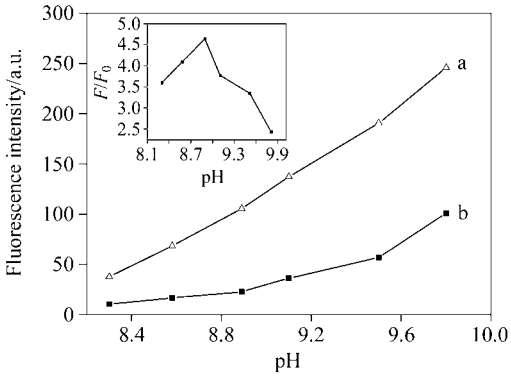


Figure 4 Effect of pH on the fluorescence intensity of 4-MUDNBS (4.0 μmol•L^{-1}) in the presence (a) and absence (b) of SNH (5.0 μg•mL^{-1}) in pH NH_3-NH_4Cl buffer solution. Inset: fluorescence enhancement factor F/F_0 as a function pH value.

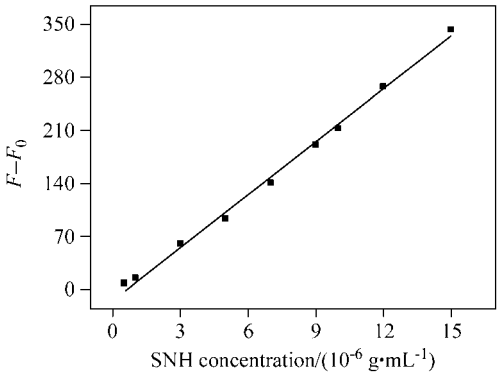


Figure 5 Linear plots of ($F-F_0$) vs. SNH concentration over the entire dynamic concentration range. Other conditions were the same as those described in the experimental section.

increasing amounts of interfering substances was added. The tolerable concentration ratios with respect to 5.0 μg•mL^{-1} SNH for interference at 5% level are reported in Table 1. From the result it was observed that most of the foreign substances studied did not interfere in the determination of SNH, except for Cu^{2+}, citrate and ascorbic acid.

Analytical characteristics of 4-MUDNBS for SNH

The calibration graphs for the determination of SNH with 4-MUDNBS were constructed under the optimum experimental conditions. A very good linear relationship (R = 0.998) was observed up to SNH concentration ranging from 0.5 to 15 μg•mL^{-1} (Figure 5) and the detection limit, calculated following the IUPAC criteria, was 0.15 μg•mL^{-1} (3σ). The standard deviation for seven replicate measurements of a solution containing 5.0 μg•mL^{-1} SNH was 1.7%.

Application

Three synthetic samples of SNH with ions and excipients were determined under the optimal condition. The recovery of the method was acquired using the standard addition method by adding a known amount of standard to the synthetic samples. As presented in Table 2, the determination results were satisfactory. From the results presented it can be concluded that the proposed method is a reliable method for the determination of SNH.

Mechanism for the fluorescence enhancement

As is well known, there is a reversible equilibrium reaction between SNH and sodium bisulfite in aqueous solution (Scheme 1), SNH, lauroyl acetaldehyde and sodium bisulfite are all present in the solution, and the equilibrium lies toward lauroyl acetaldehyde and sodium bisulfite in basic media. To investigate whether the dramatic increase in fluorescence intensity results from

Table 1 Effects of several cations and excipients on the fluorescent determination of SNH (5.0 μg•mL^{-1}) with 4-MUDNBS

Metal ion	Coexisting concentration/(μmol•L^{-1})	Change of F/%	Excipient	Coexisting concentration/(μg•mL^{-1})	Change of F/%
K^+	1500	3.21	Starch	500	−0.40
Mg^{2+}	1500	−2.09	Mannitol	500	−3.27
Ca^{2+}	1500	−0.56	Glucose	500	−1.12
Cd^{2+}	150	−0.91	Sucrose	500	−4.96
Zn^{2+}	150	−3.78	Citrate	5	−0.44
Al^{3+}	15	−2.40	Ascorbic acid	5	1.10
Cu^{2+}	0.15	−0.21			

Table 2 Results for the determination of SNH in synthetic samples (n=3)[a]

Number	Amount/(μg•mL^{-1})	Main interferent	Amount found/(μg•mL^{-1})	RSD/%
1	5.00	K^+, Al^{3+}, starch, sucrose	4.78±0.03	0.6
2	5.00	Ca^{2+}, Zn^{2+}, mannitol, ascorbic acid	5.04±0.19	3.9
3	5.00	Cd^{2+}, Mg^{2+}, glucose, citrate	4.97±0.03	0.7

[a] The concentration of all cations was 15 μmol•L^{-1}. The concentration of starch, sucrose, mannitol, ascorbic acid, glucose and citrate was 5.0 μg•mL^{-1}.

the deprotection of an benzenesulfonate by sulfite, the same concentration of SNH, lauroyl acetaldehyde and sodium bisulfite were added to the 4-MUDNBS solution and the fluorescence signals were recorded under identical conditions, respectively. It was found that no fluorescence increase was observed for lauroyl acetaldehyde, while a 1,2-fold higher fluorescence signal was obtained by sodium bisulfite than that of SNH. Based on the above results, we thus believe that the fluorescence increase of the system is ascribed to SNH's decomposing product, sodium sulfite. Based on the above experiment, a possible detection mechanism was given as shown in Scheme 1. In basic media, SNH was decomposed to produce sodium sulfite, which can then react with 4-MUDNBS to yield the highly fluorescent 4-MU and hence leading to a dramatic increase in fluorescence intensity.

Conclusion

In summary, a 4-MU-based fluorogenic probe for SNH was prepared and its fluorogenic behavior was investigated. The method is based on the decomposition of SNH to produce sodium sulfite, which then reacted with 4-MUDNBS to yield the strongly fluorescent 4-MU, hence leading to the fluorescence increase of the reaction solution. The method is proved to be simple, selective and highly sensitive for SNH assay.

References

1 Ye, X.-L.; Li, X.-G.; Yuan, L.-J.; Zhang, B.-S. *Colloids Surf., A*: *Physicochem. Eng. Aspects* **2006**, *279*, 218.
2 Wang, D.-Y.; Yu, Q.-H.; Eikstadt, P.; Hammond, D.; Feng, Y.; Chen, N. *Int. Immunopharmacol*. **2002**, *2*, 1411.
3 Wang, D.-Y.; Noda, Y.; Zhou, Y.; Nitta, A.; Nabeshima, T.; Yu, Q. H. *Int. Immunopharmacol*. **2004**, *4*, 1083.
4 Yuan, L.-J.; Li, X.-G.; He, H.-M. *Acta Chin. Med. Pharmacol*. **2004**, *32*, 7.
5 Ye, X.-L.; Li, X.-G.; Yuan, L.-J.; He, H.-M. *Colloids Surf., A*: *Physicochem. Eng. Aspects* **2005**, *268*, 85.
6 Zhang, L.-H.; Jing, N. *Chin. Hosp. Pharm. J.* **2005**, *25*, 9845.
7 Ji, B.; Wang, D.; Wang, D. K.; Xue, M.-Y.; Liu, S.-P.; Xu, S.; Zhuang, R. *Chin. J. Pharm. Anal*. **2006**, *26*, 86.
8 Searles, S.; Nukima, S. *Chem. Rev*. **1959**, *59*, 1077.
9 Fukuyama, T.; Cheung, M.; Jow, C.-K.; Hidai, Y.; Kant, T. *Tetrahedron Lett*. **1997**, *38*, 5831.
10 Nihei, K-I.; Kato, M.-J.; Yamane, T.; Palma, M.-S.; Konno, K. *Synlett* **2001**, *7*, 1167.
11 Maeda, H.; Matsuno, H.; Ushida, M.; Katayama, K.; Saeki, K.; Itoh, N. *Angew. Chem., Int. Ed*. **2005**, *44*, 2922.
12 Jiang, W.; Fu, Q.; Fan, H.; Ho, J.; Wang, W. *Angew. Chem., Int. Ed*. **2007**, *46*, 8445.
13 Shibata, A.; Furukawa, K.; Abe, H.; Tsuneda, Y.; Ito, S. *Bioorg. Med. Chem. Lett*. **2008**, *18*, 2246.
14 Bouffard, J.; Kim, Y.; Swager, T. M.; Weissleder, R.; Hilderbrand, S. A. *Org. Lett*. **2008**, *10*, 37.
15 Yang, X.-F.; Yao, H.-C.; Zhai, J.-B.; Li, H. *J. Fluoresc*. **2007**, *17*, 15.
16 Adamczyk, M.; Cornwell, M.; Huff, J.; Rege, S.; Rao, T. V. S. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1985.

(E0912283 Zhao, C.; Zheng, G.)

