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A near-infrared fluorescent probe for hydrogen sulfide in living cells

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ABSTRACT

Fluorescent probes for hydrogen sulfide have received considerable attention because of the biological significance of H_2S recognized recently. However, near-infrared fluorescent probes for H_2S are still rare. In this work, a new near-infrared fluorescent probe for H_2S was developed based on the reduction reaction of azide with H_2S to amine based on the fluorophore of dicyanomethylene-4*H*-chromene because of its long excitation and emission wavelength. The probe has a high selectivity for H_2S over competitive anions and sulfide-containing analytes. Finally, the probe was applied to sense H_2S in living cells.

1. Introduction

Hydrogen sulfide (H_2S) has emerged as an important biological messenger and much attention has been paid to the design of fluorescent probes for H_2S to meet the requirement of accurate measurement of H_2S particularly in living systems [1–3]. H_2S is recognized as the third most important gasotransmitter for regulating cardiovascular, neuronal, immune, endocrine, and gastrointestinal systems after nitric oxide and carbon monoxide [4]. H_2S is produced endogenously by enzymes of cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfur transferase (3MST) in the cytosols and mitochondria of mammalian cells from sulfur-containing molecules [4]. The endogenous levels of H_2S are believed to be related with some diseases like Alzheimer's disease [5], Down's syndrome [6], diabetes [7] and liver cirrhosis [8]. Thus, visualization of the distribution and concentration of H_2S in living systems would be very important and helpful to elucidate the biological roles of H_2S. Compared with reported methods such as colorimetric [9], electrochemical analysis [10] and gas chromatography [11], small molecule fluorescent probes offer high sensitivity, real-time imaging, and high spatiotemporal resolution, and have more potential to be a suitable tool.

The design of fluorescent probes for H_2S is mainly based on specific chemical reactions by taking advantage of the nucleophilic or reducing properties of H_2S [1–3]. Xian et al. reported a fluorescein-derived probe for selective detection of H_2S through a nucleophilic substitution reaction between H_2S and the disulfide moiety [12]. Qian et al. reported a ratiometric fluorescent probe using a similar method [13]. He e used the nucleophilic attack of H_2S on the aldehyde functionality to design an elegant fluorescent probe [14]. Very recently, Guo et al. reported a new fluorescent probe based on the selective nucleophilic addition of H_2S to a specific merocyanine derivative [15]. Nagano et al. [16] and Zeng et al. [17] used the displacement strategy to design off-on fluorescent probes with improved selectivity. In addition, the most applied reaction to sense H_2S is the reduction of azide with H_2S to amine pioneered by Chang [18] and Wang [19]. The azide compound is usually not fluorescent. After reduction to the amine counterpart which is highly fluorescent, an off–on fluorescence response is then observed. This approach has been expanded to design azide-containing fluorescent probes by altering fluorophores to naphthalimide [20], resorufamine [21], NBD [22], BMF [23], coumarin [24,25], cresyl violet [26], genetically encoded fluorescent protein [27], pyrene [28], and phenanthroimidazole [29]. However, near-infrared fluorescent probes using this strategy to sense H_2S are still rare [30]. To penetrate sufficiently into tissues and avoid autofluorescence interference from cellular, near-infrared fluorescent probes are required.

In this paper, we reported a new near-infrared fluorescent probe **1** for H_2S. Dicyanomethylene-4*H*-chromene (DCMC) fluorophore is selected because of its long excitation and emission wavelength. The azide-derived compound **1** was expected to be reduced with H_2S to give the amine-derived compound which would highly

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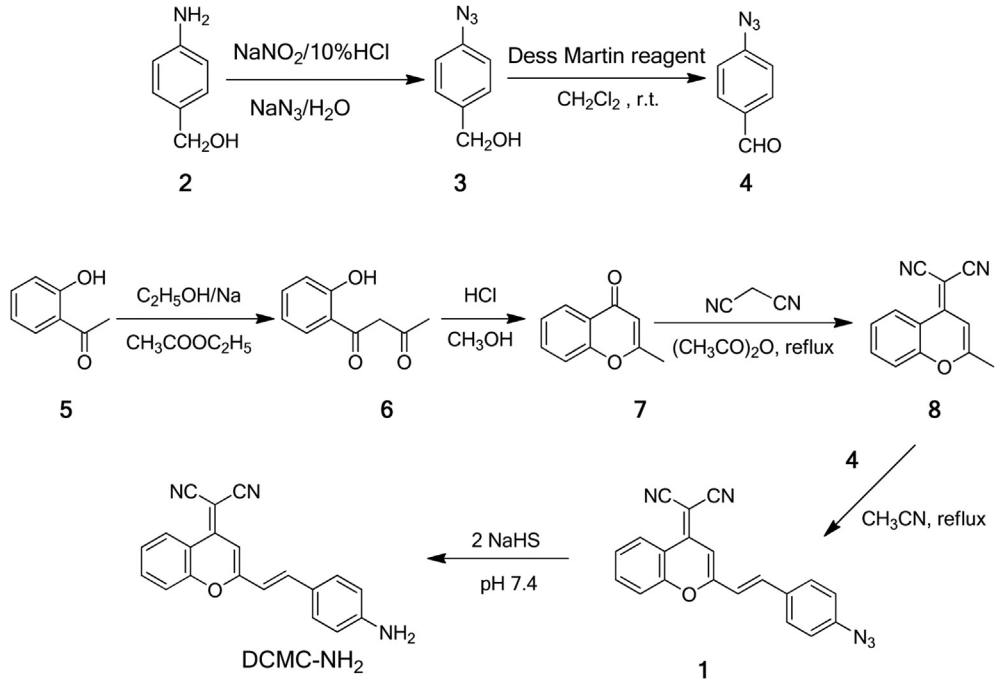

Scheme 1. The synthesis of compound **1** and DCMC−NH_2.

fluoresce (NaHS was used as a hydrogen sulfide source). The synthesis of **1** and DCMC−NH_2 were shown in Scheme 1.

2. Results and discussion

2.1. The optical behaviors of *1* with H_2S in CH_3CN

The emission spectra and fluorescence titration experiments of **1** with H_2S were recorded in CH_3CN firstly (Fig. 1). The free **1** is nonfluorescent. When 10 equiv of NaHS was added to the solution of **1**, a new emission band centered at 635 nm appeared immediately even after 1 s and increased in intensity significantly after 2 min almost to complete the reaction (354-fold fluorescence increase). In an amplification reaction with the same concentration of **1** (10 μM) and H_2S (100 μM), the product was separated and purified after 1 h, and proved to be the compound **DCMC−NH_2** characterized with ^{1}H NMR, ^{13}C NMR and HRMS (see Supporting information). The job plot indicates 2 equiv of HS^- react with 1 equiv of compound **1** to convert the azide to amine group (Scheme 1, Fig. S1). We then checked the fluorescence emission of **1** with 2 equiv of NaHS, and found after 30 min the reaction was completed. By means of kinetic experiments, it was found that if the concentration of NaHS was over 10 equiv, the reaction can be finished in minutes (Fig. 2). Therefore, we used 10 equiv of H_2S to examine the performance of **1** in all the following experiments.

The absorption titration of **1** with NaHS was then performed and also reflected the reduction process of **1** to the amine derivative. Compound **1** exhibits maximum absorption at 420 nm. On addition of 10 equiv of H_2S to the solution of **1**, the absorbance at 420 nm decreased sharply to its limiting value, while an absorption band at 470 nm developed which induced the colour change from yellow to orange (Fig. 3) with an isosbestic point at 448 nm. This new absorption band is in line with the one of DCMC−NH_2.

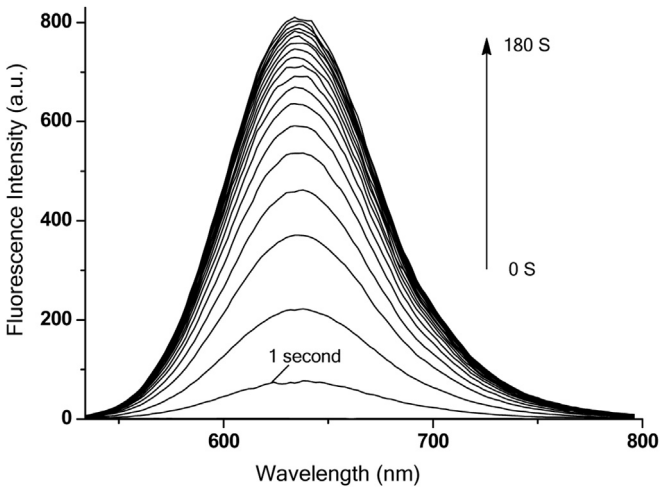

Fig. 1. Fluorescent emission spectra of 10 μM compound **1** in the presence of 10 equiv of NaHS in CH_3CN solution (NaHS was dissolved in water in the concentration of 10 mM). Data were collected every 10 S. Excitation at 491 nm.

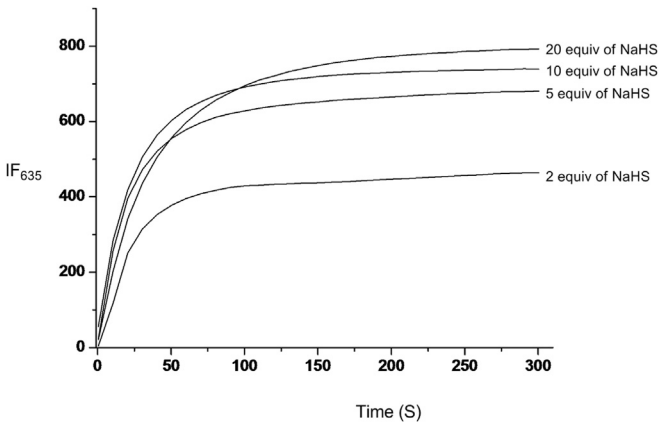

Fig. 2. Time dependence of fluorescence intensity of **1** (10 μM) at 635 nm with various concentration of NaHS.

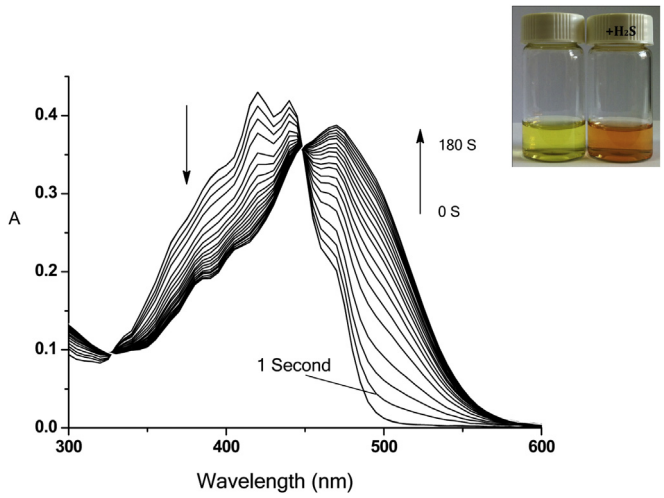

Fig. 3. UV–Vis absorption spectra of 10 μM **1** in the presence of 10 equiv H_2S in CH_3CN. Inset: colour change of the solution of **1** with the addition of H_2S.

2.2. The optical behaviors of *1* with H_2S in aqueous solutions

More importantly, the fluorescence responses of **1** to NaHS in aqueous solution (CH_3CN:phosphate buffer = 1:1, pH 7.4, 50 mM) were detected. As shown in Fig. 4, the addition of NaHS induced an appearance of emission centered at 655 nm. The time-dependent fluorescence responses were detected and the results showed that the reaction was completed within 60 min (96-fold fluorescence increase). However, the background fluorescence of **1** is so weak, and only in 10 min a high fluorescence increase is observed which responses the reaction of **1** with NaHS, then the timescale allows **1** to sense NaHS in real-time intracellular imaging. The absorption titration of **1** with NaHS displayed a same change with that one in CH_3CN (Fig. S2). And the job plot indicates in aqueous solution **1** reacts with HS^- also with a 1:2 stoichiometry (Fig. S3). We reason that the longer reaction time of **1** with NaHS in aqueous solution compared with that in CH_3CN is due to the not so good water-solubility of **1**. Therefore, to induce some hydrophilic groups to compound **1** is now in progress in our group.

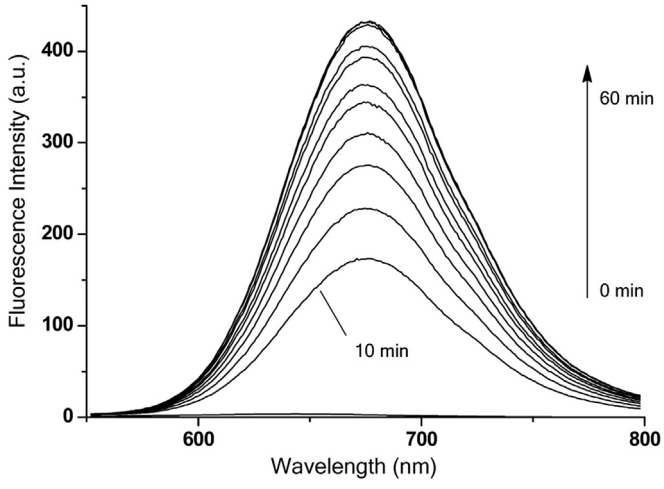

Fig. 4. Fluorescent emission spectra of 10 μM compound **1** in the presence of 10 equiv of NaHS in aqueous solution (CH_3CN: phosphate buffer = 1:1, pH 7.4, 50 mM). Data were collected every 10 min. Excitation at 491 nm.

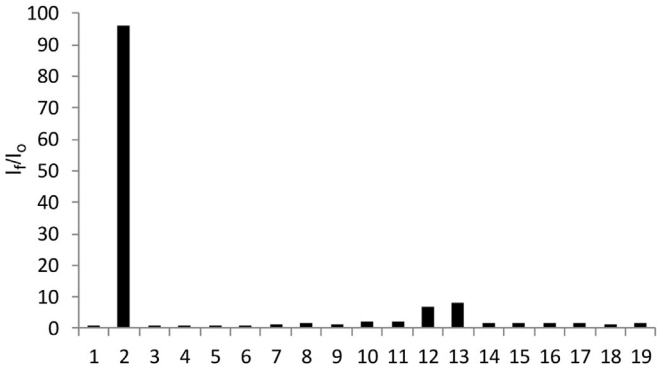

Fig. 5. Fluorescence responses of 10 μM **1** to various analytes in aqueous solution (CH_3CN:50 mM phosphate buffer = 1:1, pH = 7.4). Excitation at 491 nm. Bars represent the final fluorescence intensity of **1** with 1 mM analytes over the original emission of free **1**. (1) free **1**; (2) NaHS; (3) F^-; (4) Cl^-; (5) Br^-; (6) I^-; (7) N_3^-; (8) NO_2^-; (9) HCO_3^-; (10) HSO_3^-; (11) SO_4^-; (12) $S_2O_3^-$; (13) $S_2O_4^-$; (14) $S_2O_5^-$; (15) CN^-; (16) HPO_4^-; (17) PO_4^-; (18) cysteine; (19) glutathione.

2.3. The selectivity of *1* for H_2S

The fluorescence titration of **1** with various analytes was conducted to examine the selectivity. As shown in Fig. 5, the addition of 100 equiv of F^-, Cl^-, Br^-, I^-, N_3^-, NO_2^-, HCO_3^-, HSO_3^-, SO_4^-, $S_2O_5^-$, CN^-, HPO_4^-, PO_4^-, cysteine and glutathione produced a nominal change in the fluorescence spectra of **1**. Other tested analytes including 100 equiv of $S_2O_3^-$ and $S_2O_4^-$ induced fluorescence but with much smaller enhancement (5- and 6-fold, respectively). More importantly, the presence of $S_2O_3^-$ and $S_2O_4^-$ do not interfere with the fluorescence response of **1** for H_2S. Then probe **1** has a very high selectivity for H_2S.

2.4. Cell imaging of *1* with H_2S

We then sought to examine whether **1** can sense H_2S in living cells. The human umbilical vein endothelial cells (HUVEC) were incubated with 10 μM **1** for 30 min first alone and exhibited almost no background fluorescence (Fig. 6a). Then the cells were incubated with 50 μM NaHS next and after 5 min displayed enhanced red fluorescence (Fig. 6b). After 30 min incubation with NaHS, a higher turn-on fluorescence response can be observed (Fig. 6c). These experiments indicate **1** can be used to detect H_2S in living cells.

3. Conclusion

In conclusion, we reported a dicyanomethylene-4*H*-chromene derived fluorescent probe **1** for H_2S based on the reduction of azide with H_2S to amine compound. **1** is nonfluorescent. With the conversion to the fluorescent amine compound by H_2S, a large fluorescence increase is obtained with emission centered at 655 nm in aqueous solution. Concomitantly, the solution colour changes from yellow to orange with the convenience and aesthetic appeal of a colorimetric assay. The probe has a high selectivity for H_2S over competitive anions and sulfide-containing analytes. Finally, the probe was applied to sense H_2S in living cells. In order to shorten the reaction time, we are currently increasing the water-solubility of the compound by introducing some hydrophilic groups.

4. Experimental

4.1. Material and instruments

Unless otherwise noted, materials were obtained from Aldrich and were used without further purification. ^1H NMR and ^{13}C NMR

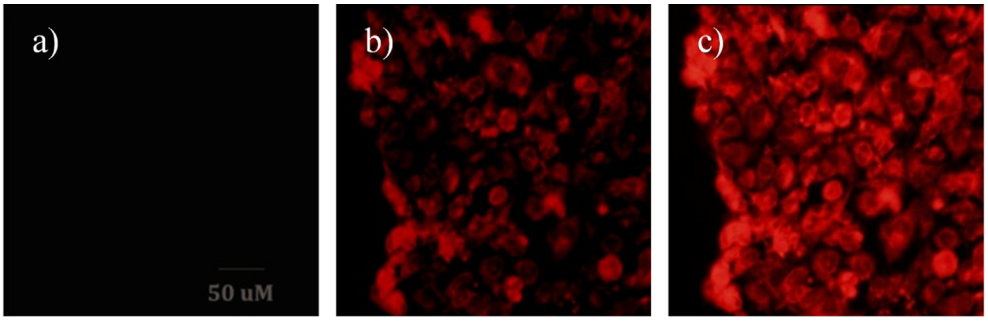

Fig. 6. Fluorescence images of HUVEC incubated with 10 μM **1** and H_2S. Cells treated with **1** in the (a) absence and (b) presence of 50 μM of H_2S incubated for 5 min, and (c) presence of 50 μM of H_2S incubated for 30 min.

spectra were recorded using Bruker 400 MHz. Chemical shifts were given in ppm and coupling constants (*J*) in Hz. UV absorption spectra were obtained on Agilent Cary 60 UV/VIS Spectrometer. Fluorescence emission spectra were obtained using Cary Eclipse fluorescence spectrophotometer (Agilent).

4.2. Synthesis

4.2.1. Synthesis of (4-azidophenyl)methanol (*3*)

To a solution of 4-aminobenzyl alcohol (300 mg, 2.44 mmol) in 5 mL of 10% HCl aqueous solution was added $NaNO_2$ (201 mg, 2.92 mmol) in 3 mL aqueous solution at 0 °C and stirred for 30 min. Then NaN_3 (190 mg, 2.92 mmol) in 3 mL aqueous solution was added at 0 °C and stirred for another hour. The reaction mixture was warmed to 25 °C, diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4, concentrated in vacuo and subjected to silica gel chromatography. A yellow oil **3** (315 mg, 86% yield) was obtained by silica gel column chromatography using Petroleum ether/EtOAc (5:1, v:v) as eluent. ^1H NMR (500 MHz, $CDCl_3$) δ 7.23 (d, $J = 8$ Hz, 2H), 6.93 (d, $J = 8.5$ Hz, 2H), 4.51 (s, 2H), 2.04 (s, br, 1H).

4.2.2. Synthesis of 4-azido-benzaldehyde (*4*)

Compound **3** (306 mg, 2.06 mmol) was dissolved in 15 mL dry CH_2Cl_2. Dess-Martin reagent (1.3 g, 3.08 mmol) was added and the mixture was stirred for 2 h at room temperature, at which point oxidation was completed. The mixture was diluted with EtOAc (60 mL), washed with saturated $Na_2S_2O_3$ (10 M), saturated aqueous $NaHCO_3$ (10 mL), and brine. Then organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using Petroleum ether/EtOAc (50:1, v:v) as eluent to afford **4** as a yellow oil. Yield: 283 mg (93.5%). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.85 (s, 1H), 7.78 (d, $J = 8.5$ Hz, 2H), 7.06 (d, $J = 8.5$ Hz, 2H).

4.2.3. Synthesis of 2-methyl-4H-chromen-4-one (*7*)

A solution of 2-hydroxyacetophenone (5 g, 36.75 mmol) in dry ethyl acetate (60 mL) was added sodium (4 g, 0.17 mmol), and the reaction mixture was stirred at room temperature for 18 h. Cold 0.5 N HCl (50 mL) was added and the aqueous layer was separated. The remained organic layer was dried and evaporated in vacuo to obtain the crude diketone **5**. A solution of the crude diketone with several drops of concentrated HCl in methanol (40 mL) was stirred at room temperature for 4 h. The methanol was removed in vacuo to get the residue, followed by the addition of ethyl acetate (50 mL) and washed with brine (50 mL). The organic layer was dried, evaporated in vacuo and purified with silica gel chromatography using Petroleum ether/EtOAc (9:1, v:v) as eluent to obtain **7** as a yellow solid. Yield: 3.23 g (54.9%). ^1H NMR ($CDCl_3$, 500 MHz) δ 8.93 (d, $J = 8.5$ Hz, 1H), 7.72 (t, $J = 7.5$ Hz, 1H), 7.46 (d, $J = 8$ Hz, 2H), 6.73 (s, 1H), 2.44 (s, 3H).

4.2.4. Synthesis of 2-(2-methyl-4H-chromen-4-ylidene) malononitrile (*8*)

A single round-bottomed flask equipped with a magnetic stirring bar, containing 2-methyl-4*H*-chromen-4-one **7** (2 g, 12.5 mmol), acetic anhydride (40 mL) was charged with malononitrile (1 g, 15.15 mmol). The reaction mixture was stirred vigorously and heated at reflux temperature for 14 h. After that, the mixture was concentrated in vacuo to remove acetic anhydride. Then water (30 mL) was added to the mixture and refluxed for another 0.5 h. The mixture was filtered and the product was recrystallized in ethanol to give a yellow solid. Yield: 350 mg (13.5%). ^1H NMR ($CDCl_3$, 500 MHz) δ 8.92 (d, $J = 9$ Hz 1H), 7.72 (t, $J = 7.5$ Hz, 1H), 7.46 (d, $J = 8$ Hz, 2H), 6.72 (s, 1H), 2.44 (s, 3H).

4.2.5. Synthesis of (E)-2-(2-(4-azidostyryl)-4H-chromen-4-ylidene) malononitrile (*1*)

A mixture of 4-azido-benzaldehyde (155.6 mg, 0.75 mmol), 2-(2-methyl-4*H*-chromen-4-ylidene)malononitrile **7** (110 mg, 0.75 mmol), piperidine (5 drops), and freshly distilled acetonitrile (6 mL) were refluxed under argon for 24 h. The yellow precipitate was filtered and washed with 50 mL of acetonitrile. The crude product was purified by recrystallization from methanol to afford compoud as a yellow solid. Yield: 110 mg (43.5%). ^1H NMR ($CDCl_3$, 500 MHz) δ 8.92 (d, $J = 8$ Hz, 1H), 7.75 (t, $J = 8$ Hz, 1H), 7.60–7.55 (m, 4H, Ar–H), 7.46 (t, $J = 7.5$ Hz, 1H), 7.10 (d, $J = 8.5$ Hz, 2H), 6.87 (s, 1H), 6.77 (d, $J = 16$ Hz, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz) δ 157.3, 152.7, 152.3, 142.2, 137.6, 134.7, 131.4, 129.5, 126.0, 125.9, 119.8, 118.6, 118.3, 117.8, 116.7, 115.6, 106.9, 63.0. HRMS (API-ES) calcd for $C_{20}H_{11}N_5O$ [M^+] 337.0964, found 337.0960.

4.2.6. Synthesis of ***DCMC–NH₂***

A solution of **1** (30 mg, 0.089 mmol) in dry CH_3CN (50 mL) was added NaHS (996.8 mg, 17.8 mmol) which was dissolved in 5 mL water. The reaction mixture was stirred at temperature for 1 h. Then the mixture was filtered to get a brownish solid without further purification. Yield: 17 mg (61.4%). ^1H NMR ($CDCl_3$, 500 MHz) δ 8.92 (d, $J = 8.5$ Hz, 1H), 7.72 (t, $J = 8$ Hz, 1H), 7.57–7.53 (m, 2H), 7.47–7.42 (m, 3H), 6.81 (s, 1H), 6.70 (d, $J = 8.5$ Hz, 2H), 6.62 (d, $J = 16$ Hz, 1H), 4.05 (s, 2H), ^{13}C NMR ($CDCl_3$, 500 MHz) δ 160.2, 153.0, 152.6, 152.5, 141.1, 135.5, 131.2, 126.4, 125.0, 122.8, 119.4, 118.3, 117.7, 116.9, 114.3, 112.9, 105.2, 57.8. HRMS (ESI) calcd for $C_{20}H_{14}N_3O$ [MH^+] 312.1137, found 312.1135.

4.3. Imaging of HUVEC

HUVEC were cultured in DMEM/F12 medium (Hyclone, China) under 95% humidified atmosphere with 5% CO_2 at 37 °C. Besides DMEM/F12, HUVEC culture medium contained 1% penicillin-streptomycin and 10% fetal bovine serum which were purchase

from Invitrogen, USA. Cells passage was conducted every two days with 0.025% trypsin solution digest.

The staining experiment was conduct when HUVEC were confluence around 70–80%. 50 μM **1** in the culture media was added to the cells and the cells were incubated for 1 h at 37 °C. After washing twice to remove the remaining sensor, the cells were treated with 50 μM NaHS for 60 min. The cell sample was observed by IX 73 Olympus microscope.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dyepig.2013.03.014.

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