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Reaction-based epoxide fluorescent probe for *in vivo* visualization of hydrogen sulfide

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ABSTRACT

Hydrogen sulfide (H_2S) has emerged as the most important biosynthetic gasotransmitters along with nitric oxide (NO) and carbon monoxide (CO). In this study, we report the design and the synthesis of a new epoxide fluorescent probe 7-glycidyloxy-9-(2-glycidyloxycarbonylphenyl)-2-xanthone (FEPO) for use in *in vivo* visualization of hydrogen sulfide. The probe employs a fluorescein as a fluorophore, and is equipped with an operating epoxide unit. FEPO functions *via* epoxide ring opening upon nucleophilic attack of H_2S. This ring opening strategy may open a new avenue for the development of various H_2S fluorescent sensors. FEPO showed high selectivity and high sensitivity for H_2S. FEPO's cytotoxicity was tested using MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) assay. Furthermore, the use of confocal imaging of H_2S and *in vivo* imaging in live zebra fish demonstrated FEPO's potential biological applications. We anticipate that, owing to their ideal properties, probes of this type will find great uses in exploring the role of H_2S in biology.

1. Introduction

Understanding the pathophysiological and pharmacological roles of gasotransmitters has been challenging and remain widely unexplored. Knowledge in gasotransmitters and their biological importance have significant implication for drug discovery (Moore et al., 2003; Olson and Donald, 2009). Among the various gasotransmitters, hydrogen sulfide (H_2S) is considered as one of the most important species and have shown to exert protective effects in relaxation of vascular smooth muscles (Yang et al., 2008), reduced blood pressure (Kamoun et al., 2003), mediation of neurotransmission (Abe and Kimura, 1996), inhibition of insulin signalling (Li et al., 2005; Peng et al., 2010), and regulation of inflammation (Eto et al., 2002). In addition, alteration in H_2S levels lead to diseases such as Alzheimer's disease, Down's syndrome (Yang et al., 2005), diabetes (Fiorucci et al., 2005), and liver cirrhosis (Chiku et al., 2009). In mammalian systems, the endogenous H_2S is synthesized from cysteine or its derivatives by several enzymes, such as cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), cysteine aminotransferase (CAT), and 3-mercaptopyruvate sulfurtransferase (MST) (Dominy and Stipanuk, 2004; Kabil and Banerjee, 2010; Shibuya et al., 2009; Dorman et al., 2002).

Interest in understanding the physiological and pathological functions of H_2S (Wang, 2002) continues to increase. Methods such as colorimetry (Lei and Dasgupta, 1989; Jiménez et al., 2003), gas chromatography (Radford-Knoery and Cutter, 1993; Bérubé et al., 1999), and electrochemical analysis (Doeller et al., 2005; Searcy and Peterson, 2004) are available for H_2S detection. However, these methods are less ideal for a fast, accurate, and real-time determination due to the high reactivity of H_2S. Therefore, new methods are needed for the efficient detection of sulfide in biological systems. Fluorescence imaging is one of the best techniques for the determination and measurement of intracellular molecules due to its high selectivity and sensitivity. It does not destroy, specimen tissues or cells, providing it an advantage over analytical methods.

Currently, a variety of fluorescent probes for tracking the H_2S in biological samples are available, reflecting the diverse state-of-the-art OFF/ON fluorescent mechanisms. Sasakura et al. (2011) designed a fluorescent probe for cellular bioimaging based on the azamacrocyclic Cu^{2+} complex chemistry. Chen et al. (2013) reported a new fluorescent probe based on the selective nucleophilic addition of H_2S to a specific merocyanine derivative. Qian et al. (2011) developed fluorescent probes based on H_2S-induced tandem chemical reactions. Lippert et al. and Lin et al. developed azide based and cell-trappable fluorescent probes for H_2S

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detection, respectively (Lippert, 2014; Lin et al., 2013). These methods have been expanded to design different fluorescent probes by altering the fluorophores to naphthalimide, rhodamine, porous conjugated polymer, cyanine, 7-benzo[d]thiazol-2-yl-9,9-(2-methoxy ethoxy)ethyl-9H-fluorene, coumarin, cresyl violet, genetically encoded fluorescent protein, pyrene, and phenanthro imidazole (Montoya and Pluth, 2012; Liu et al., 2013, 2012, 2014; Zhou et al., 2013; Cao et al., 2011; Li et al., 2013; Wan et al., 2013; Chen et al., 2012; Zheng et al., 2012). However, these probes are not suitable for real time imaging of quick H_2S related biological processes. As a result, fast reactions that are sensitive to H_2S are continuously investigated to enhance the response rate of H_2S probes. Selectivity of H_2S over biological thiols is also an important factor for choosing a good sensing reaction for H_2S. Electrophiles that have been used in this strategy include various combinations of disulfides, dinitrophenyl ethers, esters, aldehydes, and α,β-unsaturated carbonyl groups. However, to our knowledge, no probes utilizing epoxide group as electrophilic triggers have been found so far in the H_2S database.

In this study, we report the synthesis of an epoxide fluorescent probe 7-glycidyloxy-9-(2-glycidyloxycarbonylphenyl)-2-xanthone (FEPO) and its direct application in *in vivo* imaging of H_2S in HeLa cells and live zebra fish. The results of this work proposed a paradigm in the adoption of epoxide moiety as a recognition receptor for nucleophilic attack to distinguish H_2S from other biothiols and analytes, providing a promising methodology for selective determination of H_2S.

2. Materials and methods

2.1. General information of materials and methods

Unless otherwise noted, all materials used in this study were obtained from commercial sources and were used without further purification. All IR spectra were recorded as KBr pellets on a Nicolet Avatar instrument in the frequency range 400–4000 cm^{-1}. LC mass spectrometric data was determined with a Micromass Quattro mass spectrometer and its corresponding chromatography was performed with a RP C-18 column (4.6 × 250 mm^2 5 μm) at elution flow rate of 0.8 mL/min using acetonitrile/water as a solvent mixture. The ^1H NMR and ^{13}C NMR spectra of the compounds were recorded in chloroform ($CDCl_3$) at room temperature using tetramethylsilane as an external standard on a Unity-300 NMR spectrometer. HPLC analysis was performed on AKTA basic 10 systems equipped with an UV-900 detector, Frac-920 fraction collector and RP-C18 column (5 μm, 4.6 × 250 mm^2), with flow rate of 0.8 mL/min with a solvent system consisting of methanol/water solvent mixture. UV–visible and fluorescence spectra were measured with U-3010 UV–visible spectrophotometer and F-9000 fluorescence spectrophotometer at room temperature, respectively. Mass spectrometric data were determined with a Bruker Autoflex II ESI mass spectrometer.

2.2. Synthesis of FEPO

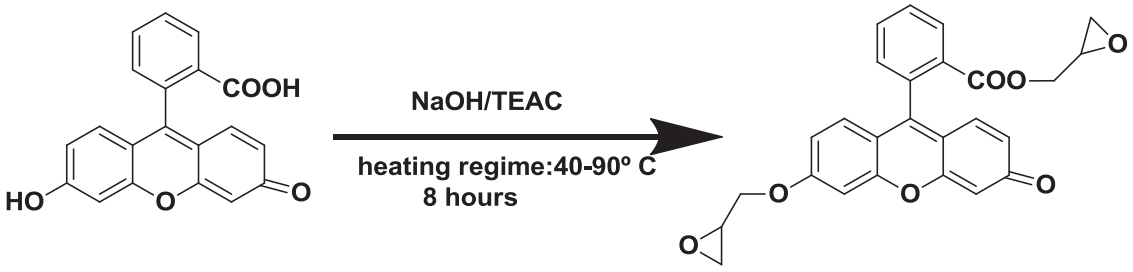
Mixture of fluorescein (1 g, 3 mmol) and granulated NaOH (0.397 g, 9.94 mmol) were treated with epichlorohydrin (5.53 ml, 60.2 mmol) and tetraethyl ammonium chloride (0.01 g, 1 mmol). The mixture was heated with stirring to 40 °C. Stirring was continued with the following heating regime: 40 °C (1 h), 50 °C (1 h), 60 °C (1 h), 70 °C (2 h), 80 °C (2 h), and 90 °C (2 h) (Scheme 1) (Korotkikh et al., 1999). The precipitate was filtered off and the mother liquor was passed through neutral aluminum oxide column, evaporated under vacuum, washed with hexane, and dried under vacuum at 30–40 °C. The viscous orange substance crystallized readily upon addition of water to its acetone solution. It was then purified using high performance liquid chromatography (HPLC) with the yield of 45%. Selected IR bands (ν in cm^{-1}):1727(s) (carboxylic carbonyl (COO^-)), 1643(s) (quinoid carbonyl (C=O)), and 962(w), 904(m), 855(s) (C–O of epoxide ring). ^1H NMR (300 MHz, $CDCl_3$, δ, ppm): 8.30 (1H, dd, J= 7.7, 1.2 Hz), 7.74 (2H, m), 7.34 (1H, dd, J=7.4, 1.2 Hz), 7.00 (1H, t, J=2.1), 6.91 (1H, d, J=13.0), 6.89 (1H, d, J=13.0), 6.81 (1H, t, J=2.4), 6.57 (1H, dd, J=9.6, 1.8), 6.48 (1H, d, J=1.8), 4.42 (1H, dd, J=11.0, 2.7), 4.31 (1H, dd, J=13.1, 3.1), 4.02 (1H, m), 3.98 (1H, m), 3.42 (1H, m), 2.99 (2H, t, J=4.5), 2.80 (1H, m), 2.71 (1H, m), 2.42 (1H, m). ^{13}C NMR (300 MHz, $CDCl_3$, δ, ppm): δ 185.7 (C=O), 165.6 (COO), 162.7, 158.9, 154.1, 149.8, 134.6, 132.7, 131.2, 130.5, 130.4, 130.2, 130.1, 129.7, 128.9, 117.9, 115.2, 113.6, 113.5, 105.9, 101.2, 101.0, 69.5 (O–CH_2), 69.4 (O–CH_2), 52.4 (OCH), 49.7 (OCH_2 epoxy ring), 44.5 (OCH_2 epoxy ring). LC-Mass: Retention time of 20.9 min with mass at 445 *m/z*. ESI (positive mode, *m/z*): Calculated: 445.1, found 445.2 for $[M]^+$.

2.3. General procedure for analysis

The stock solutions of all the test analytes (1) cysteine; (2) homocysteine; (3) glutathione; (4) alanine; (5) histidine; (6) lysine; (7) sodium nitrate (NO_3^-); (8) sodium thiocyanate (SCN^-); (9) sodium sulfate (SO_4^{2-}); (10) sodium sulfite (SO_3^{2-}); (11) sodium carbonate (Na^+); (12) potassium chloride (K^+); (13) magnesium chloride (Mg^+); (14) sodium chloride (Cl^-); (15) calcium chloride (Ca^{2+}); (16) sodium nitrite (NO_2^-); (17) hydrogen peroxide (H_2O_2); (18) hydroxyl radical ($OH^•$); (19) superoxide anion (O_2^-); (20) hypochlorous acid (HClO) and (21) sodium hydrosulfide (HS^-) were prepared in PBS (10 mM, pH=7.4). The sensing behavior of FEPO to H_2S (obtained from a source of NaHS), was measured by UV–visible and fluorescence spectral methods under simulated physiological conditions using phosphate-buffered saline (PBS; 20 mM, pH 7.4) containing 1% methanol at room temperature.

2.4. Cell culture, cytotoxicity and confocal imaging

HeLa cell lines were purchased from Invitrogen and were used to measure the *in vitro* cell cytotoxicity with increasing concentrations of probe. Cells were cultured in DMEM (Dulbecco's



Scheme 1. Synthetic scheme of FEPO.