Salivary Hydrogen Sulfide Measured with a New Highly Sensitive Self-Immolative Coumarin-Based Fluorescent Probe

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Abstract: Ample evidence suggests that H$_2$S is an important biological mediator, produced by endogenous enzymes and microbiota. So far, several techniques including colorimetric methods, electrochemical analysis and sulfide precipitation have been developed for H$_2$S detection. These methods provide sensitive detection, however, they are destructive for tissues and require tedious sequences of preparation steps for the analyzed samples. Here, we report synthesis of a new fluorescent probe for H$_2$S detection, 4-methyl-2-oxo-2H-chromen-7-yl 5-azidopentanoate (I). The design of 1 is based on combination of two strategies for H$_2$S detection, i.e., reduction of an azido group to an amine in the presence of H$_2$S and intramolecular lactamization. Finally, we measured salivary H$_2$S concentration in healthy, 18–40-year-old volunteers immediately after obtaining specimens. The newly developed self-immolative coumarin-based fluorescence probe (C$_{15}$H$_{15}$N$_3$O$_4$) showed high sensitivity to H$_2$S detection in both sodium phosphate buffer at physiological pH and in saliva. Salivary H$_2$S concentration in healthy volunteers was within a range of 1.641–7.124 µM.

Keywords: fluorescent probe; hydrogen sulfide; azide; assay; Ellman’s Reagent; biological systems; saliva; halitosis

1. Introduction

Ample evidence shows that H$_2$S plays a role of a mediator in many biological systems. For example, H$_2$S has been found to contribute to the regulation of the circulatory system [1–3] nervous system [4,5], reproductive system [6–8] and energy balance [9,10]. In mammalian tissues, H$_2$S is generated endogenously from cysteine and homocysteine. There are at least three enzymes that are responsible for converting sulfur-containing molecules into H$_2$S: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptoppyruvate sulfurtransferase (MPST) [11,12]. Furthermore, H$_2$S is generated in large amounts by microbiota, which is present in the intestines and in the oral cavity. On the one hand, microbiota-produced H$_2$S seems to play a significant physiological role in cardiovascular and gastrointestinal systems [13–19]. On the other hand, the excessive bacterial production of H$_2$S may cause medical complaints such as halitosis, a chronic bad breath condition [20,21].

Fast catabolism and low stability of H$_2$S results in difficulties in the accurate analysis of H$_2$S concentrations. Several methods have been traditionally employed for H$_2$S detection, including colorimetric and electrochemical assays [22], gas chromatography and sulfide precipitation [23,24].
Most of these techniques require lengthy storing and/or complicated processing of analyzed sample. Therefore, new methods that will be useful for rapid and selective evaluation of H$_2$S concentration in biological systems are highly desired. These requirements may be met by techniques employing fluorescent probes, which do not involve sophisticated sample processing and chemical treatment [25].

The goal of the study was to synthesize the probe that: (i) is facile to synthesize with an easy purification procedure (ii) acts fast (within seconds, considering real-time imaging of H$_2$S-related biological processes), (iii) is chemically stable for long-term storage, (iv) shows a linear concentration–signal relationship within physiologically relevant H$_2$S concentrations (v) is stable in aqueous solutions, especially in physiological pH of a body fluids. Finally, in order to confirm the usability of the designed 4-methyl-2-oxo-2H-chromen-7-yl 5-azidopentanoate (1) for the detection of H$_2$S in biological samples, we aimed to establish salivary H$_2$S concentration in healthy volunteers.

2. Results and Discussion

Here, we have designed and synthesized a new fluorescent probe (C$_{13}$H$_{11}$N$_3$O$_4$, compound 1) based on a coumarin scaffold. The operation of compound 1 is based on the azide group to amine group reduction mediated by H$_2$S in combination with spontaneous intramolecular lactamization. The developed probe was characterized by $^1$H-NMR, $^{13}$C-NMR (The Supplementary Materials) Compound 1 showed the desired characteristics for a highly sensitive fluorescent probe for H$_2$S detection. Compound 1 showed a good aqueous solubility and worked at an optimal pH ≈ 7.0, the pH of most of mammalian body fluids. Data analysis revealed a linear relationship between the fluorescence signal and the concentration of aqueous solutions of NaHS, a commonly used H$_2$S donor. Finally, the compound 1 was synthesized from commercially available reagents in a straightforward procedure.

Currently, several methods for H$_2$S detection are used, i.e., colorimetric and electrochemical assays and metal-induced sulfide precipitation. Despite the many advantages of the abovementioned methods their widespread use in biological systems is limited. This is due to the complex, multistep mechanisms of H$_2$S detection, a slow response time, poor water solubility and poor stability in aqueous solutions of the reagents, and non-physiological pH of the reaction environment. Moreover, some of those methods require complicated sample processing steps and the destruction of cells or tissues. During the last years fluorescence-based probes have been attracting increasing interest as a method for H$_2$S detection. The fluorescence-based assays for H$_2$S detection offer high selectivity, sensitivity and biocompatibility, less invasiveness and enable real-time imaging [26–28]. Various fluorescence methods for H$_2$S detection have been elegantly reviewed by Guo et al. [29]. The synthesis and design strategies of fluorescent probes are based on the use of specific chemical reactions and the use of several characteristic properties of H$_2$S. The most commonly used strategy for designing fluorescent probes is the reduction of azide or nitro groups to amine groups [30–34]. Self-immolative probes based on coumarin were designed and synthesized by Han and co-workers [35]. Based on a similar strategy, Zhao and Song demonstrated a series of probes with para-azidobenzyl group attached to the 1,8-naphthalimide [36,37]. Other methods are based on unique dual nucleophilic reactions [25,38,39], high binding affinity towards copper ions [40–42] and a specific addition reaction to unsaturated double bond [43–45].

The evaluation of H$_2$S concentration, or more precisely, free sulphhydril group concentration may also be performed using Ellman’s reagent, i.e., 5,5'-dithiobis(2-nitrobenzoic acid), often referred to as DTNB [46–48]. However, the latter method requires alkaline conditions (pH 8.0) and the test absorbance response is obtained no sooner than after 15 min. Therefore, despite the progress in the field of detection methods, further development of highly sensitive and selective fluorescent probes for H$_2$S detection is still needed to provide valuable information on the functions of H$_2$S in physiological and pathological processes.
Saliva is a promising and increasingly used biological material for clinical investigations \[49,50\]. H$_2$S in saliva may originate from its endogenous synthesis in tissues and from oral microbiota activity. The excessive concentration of H$_2$S in the saliva is associated with halitosis \[20,51,52\].

In our study, using the newly synthesized probe we showed that the concentration of H$_2$S in saliva of healthy 20–40-year-old humans is in the range between 1.641 and 7.124 µM (Table 1).

| Samples of Saliva (n = 15) |
|--------------------------|
| H$_2$S concentration [µM] | Range | Mean | SE  |
| 1.641–7.124              | 3.424 | ±0.547 |

Our results are comparable to previously reported ones, however slightly higher \[53–55\]. Generally, the analysis of H$_2$S is a tricky procedure because of the instability of H$_2$S, its high volatility and rapid oxidation. This can lead to falsely elevated or decreased H$_2$S concentrations. The most used methods for H$_2$S detection are colorimetric assays (mainly the methylene blue method), high-performance liquid chromatography and gas chromatography \[52\]. However, there is much doubt about the reliability of abovementioned methods. Differences between the above methods and the fluorescence method using our probe include different duration of sample preparation and processing, and different measurement conditions. In our study, the collected saliva samples were tested instantly, whereas in other studies the samples were subjected to lengthy processing or storage. For example, in studies by Kaneshiro et al. and Ritz et al. saliva was collected by holding a cotton swab in the mouth for a few minutes \[53,54\]. Other methods require a chemical treatment with strong acid or base before analysis of H$_2$S \[36\]. Some of those treatments can lead to falsely elevated or decreased H$_2$S levels and/or cause irreversible destruction of the analyzed sample. For example, the methylene blue method uses acidic conditions (pH = 2) in which so-called acid labile sulfides (ALS) are formed. This contributes to falsely high H$_2$S level readings. As pointed out by Siegel and Kanehira the methylene blue method may be disturbed by interference with other colored substances that interfere with the measurements, lowering the sensitivity of this method \[55\]. In contrast to the methylene blue method our measurements do not require any chemical pretreatment of the sample. Moreover, compound 1 works in an aqueous medium at pH = 7.4. Finally, the methylene blue method is a single point assay and does not monitor the H$_2$S concentration in real time. There are also doubts about the repeatability of this method \[56\]. Another disadvantage of currently used methods is their long incubation periods, which are needed to achieve detection \[57\]. Ritz et al. analyzed H$_2$S concentrations with the fluorescent probe SF4 which required up to 45 min of incubation with a chemosensor. Considering that H$_2$S is a very volatile compound H$_2$S concentrations may decrease significantly during such a long sample processing time.

3. Materials and Methods

3.1. Materials and Instruments

Unless noted otherwise, reagents and solvents for synthesis were obtained from commercial suppliers and employed without further purification. Commercial reagents for quantitating sulphydryl groups were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Buffer reagents were purchased from Sigma Aldrich (Saint Louis, MO, USA) and were used without purification. All spectroscopic measurements were performed in 0.1 mM sodium phosphate buffer (pH 7.4) or 0.1 M sodium phosphate buffer (pH 8.0). Silica gel P60 (SiliCycle, Québec, QC, Canada) was used for column chromatography and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography and visualized by fluorescence quenching under UV light. UV/Vis spectra were recorded at ambient temperature using a U-1900 spectrophotometer.
(Hitachi, Chiyoda, Tokyo, Japan) and quartz cuvettes. Fluorescence spectra were recorded at ambient temperature in quartz cuvettes using a F7000 spectrofluorometer (Hitachi).

3.2. Synthesis and Sensing Mechanisms

In the design of the probe for H$_2$S we used 7-hydroxy-4-methylcoumarin as a fluorophore due to its good stability and desirable spectroscopic properties, such as large absorption extinction coefficients, sharp fluorescence emissions and excitation and emission in visible region [58,59]. The fluorescence of 7-hydroxy-4-methylcoumarin can be easily controlled by modification of hydroxyl group causing changes of physical and chemical properties and fluorescence quenching. Our probe operates by H$_2$S-mediated reduction of azide group, which generates a primary amine, that can subsequently undergo spontaneous intramolecular lactamization to release 7-hydroxy-4-methylcoumarin and piperidin-2-one (Figure 1). Our scientific concept is analogous to studies reported by Zadlo-Dobrowolska et al. for self-immolative carbonate-based probes [60]. Moreover, in comparison to other fluorogenic assays, self-immolative probes provide a more stable signal with higher signal to noise ratio.

![Mechanism of H$_2$S detection for the self-immolative probe.](image1)

Figure 1. Mechanism of H$_2$S detection for the self-immolative probe.

To confirm the proposed mechanism, the reaction solution was analyzed by high resolution mass spectrometry (HRMS) and NMR analysis. MS and NMR spectra confirmed formation of piperidin-2-one as a product of intramolecular lactamization. A major peak located at 122.2 corresponding to piperidin-2-one (C$_5$H$_9$NO, [M + Na]$^+$: 122.07) was observed (Figure 2, the supplementary materials).

![HRMS confirmed formation of piperidin-2-one in the reaction of the compound 1 with NaHS.](image2)

Figure 2. HRMS confirmed formation of piperidin-2-one in the reaction of the compound 1 with NaHS.
In order to check the validity of the proposed mechanism we synthesized 4-methyl-2-oxo-2H-chromen-7-yl 3-azidopropionate (2) and compared the results of fluorometric measurements for the 4-methyl-2-oxo-2H-chromen-7-yl 5-azidopentanoate (1) with the results obtained for compound 2 under the same reaction conditions. For compound 2 a much lower fluorescence response was recorded, which could be due to progressive autohydrolysis of compound 2 (Figure 3A). The hydrolytic decomposition of the compound 1 is further enhanced by close location of an electron-acceptor azide group in relation to the ester bond in the 7-position of 4-methylcoumarin. In addition, NMR and MS analysis of the assay solution was performed. In contrast to compound 1, the 4-membered product of intramolecular lactamization was absent in the assay solution of compound 2 due to hydrolysis of the ester bond.

To confirm the proposed mechanism of compound 1 in sensing H$_2$S, 4-methyl-2-oxo-2H-chromen-7-yl propionate (3) was synthesized and tested in parallel under the same conditions. The analysis of reaction solution of compound 3 after addition of NaHS by fluorometry showed a minimal fluorescence enhancement (Figure 3B). In this case minimum fluorescence was caused the hydrolysis of the ester bond. The observed lower fluorescence enhancement of compound 3 in comparison to 2, could result from the absence of the azide group in the structure of compound 3. The different responses of 4-methyl-2-oxo-2H-chromen-7-yl propionate (3) and compound 1 highlighted a key role of the azide moiety for the H$_2$S detection mechanism. NMR and MS analysis confirmed the absence of the lactamization product in the reaction mixture.

![Figure 3](image-url)  
*Figure 3.* Time course experiment of compound 2 (0.1 mM, A) and 3 (0.1 mM, B) reacting with NaHS in sodium phosphate buffer (pH = 7.4) at room temperature. Time points represent time range from 1 to 10 min after addition of NaHS (0.1 mM). Fluorescence spectra of compound 1 (C), 2 (A) and 3 (B) were recorded for 10 min after addition of NaHS (0.1 mM).

To sum up, the compound 1 can be used for the determination of H$_2$S levels. Furthermore, it is characterized by a high stability and the lack of susceptibility to autohydrolysis. We showed that the distance of the reaction site and thus the azide group as the electron-acceptor group from the fluorophore reduces the susceptibility to autohydrolysis (Figure 4). The obtained results confirm proposed mechanism of H$_2$S detection for the compound 1. Detection of H$_2$S was achieved by the reduction of azide group mediated by H$_2$S to amine group, then intramolecular lactamization with simultaneous release of highly fluorescent 7-hydroxy-4-methylcoumarin.

Firstly, intermediates 3-azidopropionic acid and 5-azidopentanoic acid were obtained by reacting suitable ester 3-chloropropionate (a) or ester 5-bromopentanoate (b) with sodium azide in H$_2$O (Figure 5). The compound 1 and the compound 2 were synthesized from the corresponding commercially available fluorescent 7-hydroxy-4-methylcoumarin (Figure 6).
Figure 4. Comparison of fluorescence responses for the compound 1, 2 and 3 (0.1 mM). Data were acquired at room temperature after addition of NaHS (0.1 mM) in sodium phosphate buffer (pH = 7.4, 20% CH3CN) with excitation at 365 nm. Means ± SE from three measurements of the fluorescence responses are presented.

![Fluorescence response comparison](image)

**Figure 4.** Comparison of fluorescence responses for the compound 1, 2 and 3 (0.1 mM). Data were acquired at room temperature after addition of NaHS (0.1 mM) in sodium phosphate buffer (pH = 7.4, 20% CH3CN) with excitation at 365 nm. Means ± SE from three measurements of the fluorescence responses are presented.

\[
\text{Cl-OR} \xrightarrow{\text{NaN}_3 \cdot \text{H}_2\text{O}} \text{N}_3\text{-OH} \quad 7 \text{ days} \quad >99\%
\]

3-azidopropanoic acid

\[
\text{Br-OR} \xrightarrow{\text{NaN}_3 \cdot \text{H}_2\text{O}} \text{N}_3\text{-OH} \quad 7 \text{ days} \quad >99\%
\]

5-azidopentanoic acid

**Figure 5.** The synthesis of 3-azidopropanonic (a) acid and 5-azidopentanonic acid (b).

### 3.3. Synthesis of 3-Azidopropanoic Acid and 5-Azidopentanoic Acid

A solution of sodium azide (4 equiv.) in 10 mL water was added dropwise into the ester 3-chloropropionate or ester 5-bromopentanoate (1 equiv.). The reaction mixture was stirred at room temperature for 7 days. After this time the resulting reaction mixture was acidified with solution of HCl (1 M). Then, the mixture was extracted with ethyl acetate for several times. The combined organic layers were dried over anhydrous MgSO\(_4\) followed by filtration and concentrated under reduced pressure. The obtained product was used for the next reaction without purification. Then, the azide-probe (1) and compound 2 was readily synthesized by esterification of 7-hydroxy-4-methylcoumarin with suitable azido acid in CH\(_2\)Cl\(_2\), under a room temperature as shown in Figure 6.

![Synthesis of compounds](image)

**Figure 6.** The synthesis of compounds 1 (a) and 2 (b).
In turn 4-methyl-2-oxo-2\textsubscript{H}-chromen-7-yl propionate (3) was synthesized from the corresponding commercially available 7-hydroxy-4-methylcoumarin with butyric acid in CH\textsubscript{2}Cl\textsubscript{2}, under room temperature as shown in Figure 7.

![Figure 7. The synthesis of 4-methyl-2-oxo-2\textsubscript{H}-chromen-7-yl propionate (3).](image)

### 3.4. Synthesis of 4-Methyl-2-oxo-2\textsubscript{H}-chromen-7-yl 5-Azidopentanoate (1)

5-Azidopentanoic acid (1.2 equiv.), 7-hydroxy-4-methylcoumarin (1 equiv.), and a catalytic amount of N,N-dimethylpyridin-4-amine (DMAP) were dissolved in dry CH\textsubscript{2}Cl\textsubscript{2} (15 mL). Then DCC (2 equiv.) was added. The reaction mixture was stirred at room temperature for 12 h. The reaction was monitored by thin layer chromatography (TLC). After the reaction was completed, the precipitate was filtered and washed several times with CH\textsubscript{2}Cl\textsubscript{2}. The filtrate was concentrated under reduced pressure. The crude product was purified by silica gel chromatography (ethyl acetate/n-hexane 3:7) to obtain the pure product as white solid (72% yield).\textsuperscript{1}H-NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 7.57 (\(d, J = 8.6\) Hz, 1H), 7.13–6.93 (m, 2H), 6.22 (s, 1H), 3.33 (t, \(J = 6.6\) Hz, 2H), 2.62 (t, \(J = 7.3\) Hz, 2H), 2.39 (d, \(J = 1.1\) Hz, 3H), 1.82 (dt, \(J = 12.0, 7.1\) Hz, 1H), 1.76–1.64 (m, 1H); \(^{13}\text{C}-\text{NMR} (100 MHz, CDCl\textsubscript{3}) \(\delta\) 170.94, 160.38, 154.14, 153.01, 153.10, 151.93, 125.41, 117.99, 117.80, 110.45, 110.31, 77.43, 77.11, 76.79, 50.98, 33.65, 28.17, 21.93, 18.64 ppm. Element Anal. calcd for C\textsubscript{15}H\textsubscript{15}N\textsubscript{3}O\textsubscript{4}: C 59.80, H 5.02, N 13.95; found C 59.78, H 4.95, N 13.84.

### 3.5. Synthesis of 4-Methyl-2-oxo-2\textsubscript{H}-chromen-7-yl 3-Azidopropanoate (2)

3-azidopropanoic acid (1.2 equiv.), 7-hydroxy-4-methylcoumarin (1 equiv.) and DMAP (a catalytic amount) were dissolved in dry CH\textsubscript{2}Cl\textsubscript{2} (15 mL). Then DCC (2 equiv.) was added. The reaction mixture was stirred at room temperature for 12 h. The reaction was monitored by thin layer chromatography (TLC). After the reaction was completed, the precipitate was filtered and washed several times with CH\textsubscript{2}Cl\textsubscript{2}. The filtrate was concentrated under reduced pressure. The crude product was purified by silica gel chromatography (ethyl acetate/n-hexane 3:7) to obtain the pure product as white solid (72% yield). \(^{1}\text{H}-\text{NMR} (400 MHz, CDCl\textsubscript{3}) \(\delta\) 7.59 (\(d, J = 8.6\) Hz, 1H), 7.15–7.02 (m, 2H), 6.24 (d, \(J = 0.8\) Hz, 1H), 3.69 (t, \(J = 6.4\) Hz, 2H), 2.86 (t, \(J = 6.4\) Hz, 2H), 2.41 (d, \(J = 0.9\) Hz, 3H); \(^{13}\text{C}-\text{NMR} (100 MHz, CDCl\textsubscript{3}) \(\delta\) 168.88, 160.32, 154.15, 152.69, 151.86, 125.49, 118.03, 117.89, 114.62, 110.30, 46.55, 34.17, 18.66 ppm. Element Anal. calcd for C\textsubscript{13}H\textsubscript{12}N\textsubscript{3}O\textsubscript{4}: C 57.14, H 4.06, N 15.38; found C 57.08, H 4.02, N 15.29.

### 3.6. Synthesis of 4-Methyl-2-oxo-2\textsubscript{H}-chromen-7-yl Propionate (3)

Butyric acid (1.2 equiv.), 7-hydroxy-4-methylcoumarin (1 equiv.), and DMAP (a catalytic amount) were dissolved in dry CH\textsubscript{2}Cl\textsubscript{2} (15 mL). Then DCC (2 equiv.) was added. The reaction mixture was stirred at room temperature for 12 h. The reaction was monitored by thin layer chromatography (TLC). After the reaction was completed, the precipitate was filtered and washed several times with CH\textsubscript{2}Cl\textsubscript{2}. The filtrate was concentrated under reduced pressure. The crude product was purified by silica gel chromatography (ethyl acetate/n-hexane 2:8) to obtain the pure product as a white solid (92% yield). \(^{1}\text{H}-\text{NMR} (400 MHz, CDCl\textsubscript{3}) \(\delta\) 7.58 (\(d, J = 8.6\) Hz, 1H), 7.31–6.75 (m, 2H), 6.22 (s, 1H), 3.33 (t, \(J = 6.6\) Hz, 2H), 2.62 (t, \(J = 7.5\) Hz, 2H), 2.41 (d, \(J = 0.9\) Hz, 3H); \(^{13}\text{C}-\text{NMR} (100 MHz, CDCl\textsubscript{3}) \(\delta\) 172.19, 160.40, 154.11, 153.20, 151.96, 125.37, 118.04, 117.67, 114.34, 110.30, 77.45, 77.13, 76.81, 27.69, 18.63, 8.88 ppm. Element Anal. calcd for C\textsubscript{13}H\textsubscript{12}O\textsubscript{4}: C 67.23, H 5.21; found C 67.19, H 5.17.
3.7. Characterization of the Fluorescence of Compound 1

First, we examined the optical properties of the probe/compound 1. Compound 1 was non-fluorescent in sodium phosphate buffer containing 20% CH$_3$CN at physiological pH 7.4. The sensing ability of H$_2$S for the compound 1 was investigated using aqueous solutions of NaHS, a H$_2$S donor. The solution was analyzed by fluorometry and spectra were recorded in selected time-points after the addition of NaHS. Upon addition of 0.1 mM NaHS, the solution of the compound 1 showed a strong fluorescence enhancement, as expected. A strong emission peak at 445 nm was detected when the reaction mixture was excited at 365 nm. The fluorescence intensity was dramatically increased due to the reduction of azide group to amine by H$_2$S, intramolecular lactamization and the release of highly fluorescent 7-hydroxy-4-methylcoumarin. Within 10 min of reaction with NaHS (100 µM) the compound 1 generated an over 1000-fold fluorescence enhancement (Figure 8).

![Fluorescence response of the self-immolative probe/compound 1 (0.1 mM) to 0.1 mM NaHS. Data were acquired at room temperature in sodium phosphate buffer (pH = 7.4) with excitation at 365 nm. Emission was collected in the time range 1–10 min after the addition of 0.1 mM NaHS. The spectrum at t = 0 min was acquired from a 0.1 mM solution of the compound 1 without the addition of NaHS.](image)

We examined the time courses of the fluorescence intensities of compound 1 in the presence of 100 µM NaHS. The time courses of the fluorescence intensities of the compound 1 (0.1 mM) in the presence of NaHS (0.1 mM) in sodium phosphate (pH = 7.4) buffered acetonitrile (20%, v/v) is displayed in Figure 9. The fluorescence signal increased rapidly at the beginning and reached steady state at around 10 min. When we extended reaction time to 60 min, the fluorescent intensity increased insignificantly, thus we chose 10 min as a test time.
7.927[H₂S] + 666.62 with R² = 0.9852

Figure 9. Time course experiment of the compound 1 (0.1 mM) reacting with NaHS (0.1 mM) in sodium phosphate buffer (pH = 7.4) at room temperature. Time points represent time range from 1 to 60 min after addition of NaHS (0.1 mM). Means ± SE from three measurements of the fluorescence responses are presented.

To evaluate the compound 1 for feasibility of quantitative determination of H₂S concentration we examined the reactivity of the compound 1 in different concentrations of NaHS in sodium phosphate buffered acetonitrile (20% v/v CH₃CN, pH = 7.4) at room temperature. NaHS (NaHS concentration from 20 μM up to 100 μM) was added to the test solution of the compound 1 (0.1 mM). As shown in Figure 10, we observed almost the linear relationship of fluorescence intensity of the compound 1 against varying concentrations of NaHS. The regression analyses was: \( \frac{F_{\text{Ex}}}{F_{\text{Em}}} \) (365/445 nm) = 7.927[H₂S] + 666.62 with R² = 0.985 in 10 min of incubation with NaHS.

Figure 10. The correlation between fluorescence intensity and NaHS concentration determined using a fluorometer: the compound 1 (0.1 mM) with NaHS (20–100 μM) in sodium phosphate buffer (λex = 365 nm) at room temperature. The points represent the mean fluorescence responses at 10 min after the addition of NaHS.
3.8. Quantification of H$_2$S Concentration Using Ellman’s Reagent (5,5-Dithiobis(2-Nitrobenzoic Acid) and the Developed Probe

We determined H$_2$S levels in 20 μM up to 100 μM NaHS solution using DTNB method and our probe. DTNB assay was performed according to the protocol provided by the manufacturer (catalog number: 22,582, Thermo Fisher Scientific, Waltham, MA, USA).

Figure 11 shows time-dependent UV-vis absorption spectra of SH-free DTNB solution (green line-background) and its mixture with NaHS (0.1 mM, orange line). After 15 min incubation, the effect of DTNB with NaHS on absorption spectra was observed as gain of TNB$^{2-}$ (2-nitro-5-thiobenzoate anion) and a loss of DTNB, respectively. We chose 15 min as a test time, because after this time we did not observe any increase in the intensity of absorbance, 15 min is also the incubation time which is required for the measurement procedure recommended by the manufacturer.

![Figure 11](image1.png)

**Figure 11.** Time-dependent UV-vis absorption spectra of SH-free DTNB solution (green line-background) and its mixture with NaHS (0.1 mM, orange line) in the reaction buffer (pH 8.0) at room temperature. The absorbance at 412 nm was recorded 15 min after addition of NaHS.

To determine relationship between changes of absorbance and concentration of NaHS, we recorded the absorbance in different concentrations of NaHS aqueous solution. Concentration of NaHS from 20 μM up to 100 μM, were used. As shown in Figure 12, we observed an increase in the absorbance along with increasing NaHS concentration.

![Figure 12](image2.png)

**Figure 12.** The correlation between absorbance and NaHS concentration determined by the DTNB assay in sodium phosphate buffer (pH 8.0) at room temperature. The absorbance at 412 nm was recorded for 15 min after addition of various concentrations of NaHS from 20 to 100 μM. Values of absorbance are given as means obtained from 3 measurements. Background values were subtracted from the sample values.
Table 2 summarizes the results of H₂S detection (aqueous solution of NaHS, a H₂S donor) which have been obtained by Ellman’s test and by fluorescence method using our probe. In Table 2 are shown the results of H₂S detection (aqueous solution of NaHS, a H₂S donor) which have been obtained by Ellman’s test and by fluorescence method using our probe.

### Table 2. Comparison of detection methods for H₂S.

| Concentration of NaHS [μM] | Ellman’s Test (μM) a | Fluorescence Method Using the Compound 1 (μM) b |
|---------------------------|---------------------|---------------------------------------------|
|                           | C H₂S[μM] | V [μM s⁻¹] | C H₂S[μM] | V [μM s⁻¹] |
|---------------------------|-----------|-----------|-----------|-----------|
| 20                        | 15.82     | 0.018     | 17.00     | 0.028     |
| 40                        | 36.91     | 0.041     | 38.15     | 0.064     |
| 60                        | 57.63     | 0.064     | 58.10     | 0.097     |
| 80                        | 78.25     | 0.087     | 78.60     | 0.13      |
| 100                       | 94.52     | 0.105     | 97.23     | 0.16      |

a Conditions: 10 mM Ellman’s Reagent Solution in sodium phosphate buffer (pH 8.0), room temperature, the absorbance at 412 nm was recorded 15 min after addition of NaHS. b Conditions: 0.1 mM the fluorogenic probe in sodium phosphate buffer (pH 7.4), room temperature, the fluorescence intensity was recorded 10 min after the addition of NaHS.

#### 3.9. H₂S Detection in Saliva

To determine whether the novel compound 1 can be used for the determination of H₂S concentrations in a biological sample we performed competition experiments in 15 samples of saliva. Additionally, we plotted the calibration curve for the compound 1 in range concentrations of NaHS from 1 μM up to 10 μM. As shown in Figure 13, we observed almost the linear relationship of fluorescence intensity of the compound 1 in this range concentration of NaHS. The regression analyses were: \( F_{Ex/Em} (365/445 \text{ nm}) = 60.405[H₂S] + 240.7 \) with \( R² = 0.9987 \). The obtained fluorescence data for saliva samples were converted into H₂S concentrations by means of a calibration curve. The obtained results measured by fluorescence method with the compound 1 for 15 samples of saliva are presented in Table 1.

![Figure 13](image-url)  
**Figure 13.** The correlation between fluorescence intensity and NaHS concentration determined using a fluorometer: the compound 1 (0.1 mM) with NaHS (1–10 μM) in sodium phosphate buffer (\( \lambda_{ex} = 365 \text{ nm} \)) at room temperature. The points represent the mean fluorescence responses at 10 min after the addition of NaHS.
3.10. General Procedure for H$_2$S Detection by the Fluorescence Method

NaHS solutions with appropriate concentrations were prepared using sodium phosphate buffer as a solvent. For the assay, the various volumes of NaHS solution were added respectively to solution of 1, 2 and 3 in sodium phosphate buffer containing 20% CH$_3$CN (pH = 7.4). The total volume of the solution being measured was 2000 µL. The final concentration of the compound 1 was 0.1 mM, while various concentration of NaHS were added (in range from 1 to 10 µM and in range from 20 to 100 µM). The fluorescence response was monitored over time. Emission spectra were collected between 350 nm and 550 nm with $\lambda_{ex} = 365$ nm. Time points represent time range from 1 to 10 min after addition of NaHS. The spectrum at t = 0 min was acquired from a 0.1 mM solution of the compound 1 without NaHS. Fluorescence data and obtained linear calibration curves were used to calculate the reaction rate of NaHS with the compound 1 and concentrations of H$_2$S.

3.11. General Procedure for H$_2$S Detection Using the Ellman’s Test

Procedure was carried out quantitating sulfhydryl groups according to the manual attached by Thermo Fisher Scientific (Catalog number: 22,582). The procedure for the quantification of sulfhydryl groups was performed according to the manual attached by Thermo Fisher Scientific (Catalog number: 22,582).

3.12. General Procedure for H$_2$S Detection in Saliva Using Compound 1

In these experiments an appropriate sample of saliva (0.4 mL) were added to solution of the compound 1 in the in sodium phosphate buffer containing 20% CH$_3$CN (pH = 7.4). The final concentration of the compound 1 was 0.1 mM. The total volume of the solution being measured was 2000 µL. The fluorescence response of the compound 1 was monitored over time with $\lambda_{ex} = 365$ nm and $\lambda_{em} = 445$ nm. The spectrum at t = 0 min was acquired from a solution of the compound 1 without saliva. Fluorescence data were converted into H$_2$S concentrations in sample of saliva by means of a calibration curve.

3.13. Collection of Saliva Samples

The study was performed in compliance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Bioethics Committee of the Medical University of Warsaw (approval no. KB/138/2018). Informed consent was obtained in every case. Samples were obtained from 15 adult volunteers. Demographics and clinical data of the study subjects are listed in Table 3. Inclusion criteria were as follows: healthy, 18–40 years-old, male and female. Exclusion criteria were as follows: chronic general diseases, acute general diseases, current dental problems, halitosis, treatment with any drugs or dietary supplement including probiotics during the last month before the study. Subjects brushed teeth and did not drink and eat for 90 min before saliva collection. Samples were collected directly to Eppendorf tubes after short exposition of subjects to the smell of lemon.

**Table 3.** Demographics and clinical data of the study subjects and H$_2$S levels in the saliva samples obtained using fluorescence method with the compound 1.

| Participant Demographics and Physical Characteristics. | 15 | Range |
|--------------------------------------------------------|----|-------|
| Healthy volunteers (n)                                 |    |       |
| Age (years)                                            | 28 | 18–40 |
| Sex (m/f)                                              | 1.6|       |
| Ethnicity:                                             | 7/8|       |
| Caucasian                                              | 100%|     |
| Other                                                  | -  |       |
4. Conclusions

We synthesized a new fluorescent, self-immolative probe for \( \text{H}_2\text{S} \) detection in biological fluids. The design of compound 1 was based on the combination of two strategies for \( \text{H}_2\text{S} \) detection, i.e., reduction of an azido group to an amine in the presence of \( \text{H}_2\text{S} \) and spontaneous intramolecular lactamization. The compound 1 showed several characteristics that are desirable for evaluation of \( \text{H}_2\text{S} \) concentration in biological systems and human body fluids, including straightforward synthesis, stability, reactivity in aqueous media at physiological pH and fast response time. Finally, we measured salivary \( \text{H}_2\text{S} \) concentration in healthy, 18–40-year-old volunteers immediately after obtaining specimens. Salivary \( \text{H}_2\text{S} \) concentration in healthy humans was within a range of 1.641–7.124 µM.

Supplementary Materials: The Supplementary Materials are available online.

Author Contributions: Conception or design of the work: E.Z., R.O., D.K., M.U. Performing analyzes or interpretation of data for the work: E.Z., M.K., D.K., M.U. Drafting the work: E.Z., D.K., M.U. All authors have approved the final version of the manuscript and agreed to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. The authors declare no conflict of interest.

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Abbreviations

| Abbreviation | Definition                                      |
|--------------|------------------------------------------------|
| ALS          | Acid Labile Sulfide                            |
| CDCl\(_3\)   | Deuterated chloroform                          |
| CH\(_2\)Cl\(_2\) | Dichloromethane                                 |
| CH\(_3\)CN   | Acetonitrile                                    |
| CLSS         | ChemiLuminescent Sulfide Sensors               |
| \(^{13}\)C-NMR | Carbon-13 nuclear magnetic resonance          |
| CuS          | Copper sulfide                                 |
| CTAB         | Cetyltrimethylammonium bromide                 |
| DCC          | \(N,N'\)-Dicyclohexylcarbodiimide              |
| DMAP         | \(N,N\)-Dimethylpyridin-4-amine                 |
| DTNB         | 5,5'-Dithiobis(2-nitrobenzoic acid)            |
| HCl          | Hydrochloric acid                              |
| \(^1\)H-NMR | Proton nuclear magnetic resonance              |
| HRMS         | High Resolution Mass Spectrometry              |
| H\(_2\)S     | Hydrogen sulfide                               |
| MgSO\(_4\)   | Magnesium sulfate                              |
| mM           | Millimolar concentration                       |
| MS           | Mass Spectrometry                              |
| NaHS         | Sodium hydrosulfide                            |
| R\(^2\)      | Coefficient of determination                   |
| SE           | Standard Error                                 |
| SF           | Sulfur fluide                                  |
| TLC          | Thin layer chromatography                      |
| TNB\(^2\)\(^-\) | 2-Nitro-5-thiobenzoate anion                   |
| UV-vis       | Ultraviolet–visible spectroscopy               |
| \(\lambda_{ex}\) | Excitation wavelength                          |
| \(\lambda_{em}\) | Emission wavelength                           |
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Sample Availability: Samples of the compounds 3-azidopropanonic acid, 5-azidopentanonic acid, 4-Methyl-2-oxo-2H-chromen-7-yl 5-Azidopentanoate (1), 4-Methyl-2-oxo-2H-chromen-7-yl 3-Azidopropanoate (2) and 4-Methyl-2-oxo-2H-chromen-7-yl Propionate (3) are available from the authors.