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Additional Information

# 2,4-dinitrophenyl ether-containing chemodosimeters for the selective and sensitive “*in vitro*” and “*in vivo*” detection of hydrogen sulfide

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## Abstract

Four probes (i.e. **D1-D4**) for the selective and sensitive fluorogenic detection of HS<sup>-</sup> have been prepared and characterized. HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solutions of **D1-D4** are essentially non-fluorescent. Changes in the emission using **D1-D4** in the presence of anions (F<sup>-</sup>, Cl<sup>-</sup>, Br<sup>-</sup>, I<sup>-</sup>, N<sub>3</sub><sup>-</sup>, CN<sup>-</sup>, SCN<sup>-</sup>, AcO<sup>-</sup>, CO<sub>3</sub><sup>2-</sup>, PO<sub>4</sub><sup>2-</sup>, SO<sub>4</sub><sup>2-</sup>, HS<sup>-</sup> and OH<sup>-</sup>), biothiols (GSH, Cys, Hcy, Me-Cys and lipoic acid), reducing agents (SO<sub>3</sub><sup>2-</sup> and S<sub>2</sub>O<sub>3</sub><sup>2-</sup>) and oxidants (H<sub>2</sub>O<sub>2</sub>) demonstrated that only HS<sup>-</sup> is able to induce the appearance of intense emission bands in the 400-520 nm range in the four probes. The selectivity observed was ascribed to a unique hydrogen sulfide-induced hydrolysis of the 2,4-dinitrophenyl ether moiety that yielded the corresponding free highly fluorescent alcohols. The potential detection of intracellular HS<sup>-</sup> was also studied.

## **Keywords**

Hydrogen sulfide, fluorescence, dinitrophenyl ether, in vivo detection, chemodosimeter

## **Introduction**

Hydrogen sulfide (H<sub>2</sub>S), a colourless and flammable gas with high solubility in water and in organic solvents, is well-known for its unpleasant “rotten eggs” smell. Hydrogen sulfide has been traditionally studied for its toxicity,(1) its presence as a corrosive industrial refuse and emission in the energy industry,(2) as parameter for geological activity,(3) as quality control of foods and as an important compound related with the presence of microbial activity in anaerobic environments.(4) Moreover, this simple molecule has been recently recognized to play a relevant role in living systems. For instance hydrogen sulfide has been documented to be the third gaseous transmitter (the other two being nitric oxide and carbon monoxide).(5-7) The production of H<sub>2</sub>S in mammalian systems has been attributed to at least three endogenous enzymes, i.e. cystathionine b-synthase (CBS), cystathionine g-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MPST).(8-11) These enzymes use cysteine or cysteine derivatives as substrates and convert them into hydrogen sulfide in different organs and tissues. In addition to these enzymatic pathways, there are also a range of simple chemical events that may liberate hydrogen sulfide from the intracellular pool of “labile” sulfur such as the so-called “sulfane pool” (compounds containing sulfur atoms bound only to other sulfur atoms).(12) The production of endogenous hydrogen sulfide and exogenous administration of hydrogen sulfide has been demonstrated to exert protective effects in many pathologies. For example, hydrogen sulfide has been shown to relax vascular smooth muscle, induce vasodilation of isolated blood vessels, and reduce blood pressure.(13) Hydrogen sulfide has proved also to inhibit leukocyte adherence in mesenteric microcirculation in vascular inflammation in rats, suggesting that hydrogen sulfide is a potent anti-inflammatory molecule.(14) Additionally, it has become evident that hydrogen sulfide is a potent antioxidant and, under chronic conditions, can up-regulate antioxidant defense.(15)

However hydrogen sulfide exact mechanism of action and production in living systems is still under active investigation. For example it is known that hydrogen sulfide can react readily with methemoglobin to form sulfhemoglobin, which act as the metabolic sink for H<sub>2</sub>S. As a potential reductant, hydrogen sulfide is likely to be consumed by endogenous oxidant species such as hydrogen peroxide, superoxide,

peroxynitrite, etc. This process is significant and it provides a mechanism by which the concentration of hydrogen sulfide changes. Besides, abnormal levels of hydrogen sulfide have been associated with various diseases such as chronic kidney disease, liver cirrhosis,(16) Alzheimer,(17) and Down syndrome.(18)

Due to the important roles that hydrogen sulfide plays in environmental and in biological processes the development of selective detection systems for this compound has recently become a focus of importance. In particular it was a major challenge to clarify the complex contributions of H<sub>2</sub>S in certain diseases by finding methods for selective tracking of this small molecule within living systems. Current techniques for hydrogen sulfide detection, such as colorimetry,(19-21) electrochemical assays,(22) gas chromatography(23) and metal-induced sulfide precipitation(24) show some shortcomings typically involving destruction of the sample and/or poor selectivity.

In addition to the above mentioned classical methodologies, fluorescence is an attractive highly sensitive technique for studying biomolecule distribution in living cells. For instance, very recently, fluorescent probes for monitoring redox cycles *in vivo* have been described.(25) In this area, several fluorescent probes, designed taking into account supramolecular chemistry concepts, for the selective recognition of H<sub>2</sub>S or HS<sup>-</sup> have been described.(26) Most of the fluorescent probes reported are based on the hydrogen sulfide-induced reduction of azides or nitro moieties, electronically connected with a fluorophore, to amines.(27-36) This reduction induced perturbations in the electronic structure of the signaling unit that is reflected in emission changes. Another important group of probes are constructed under a displacement paradigm and are based on non-fluorescent Cu(II) complexes.(37-46) In these systems the hydrogen sulfide-induced demetallation of the probes induced marked emission enhancements. Other sulfide-selective fluorescent probes used Michael type addition reactions coupled with emission changes.(47-50) Also, cyclization processes that yielded fluorescent compounds have recently been used for the design of fluorescent probes for sulfide anion.(51-53) In other examples, hydrolysis reactions coupled with emission changes have been also used for the preparation of other hydrogen sulfide sensitive probes.(54-56) Finally, selenium containing BOIPY dyes have also been used for the reversible detection of sulfide in solution and in living cells.(57,58) Among probes that used hydrolysis reactions, as far as we know only three probes containing 2,4-dinitrophenyl ethers have been described and used for the selective and sensitive recognition of HS<sup>-</sup>

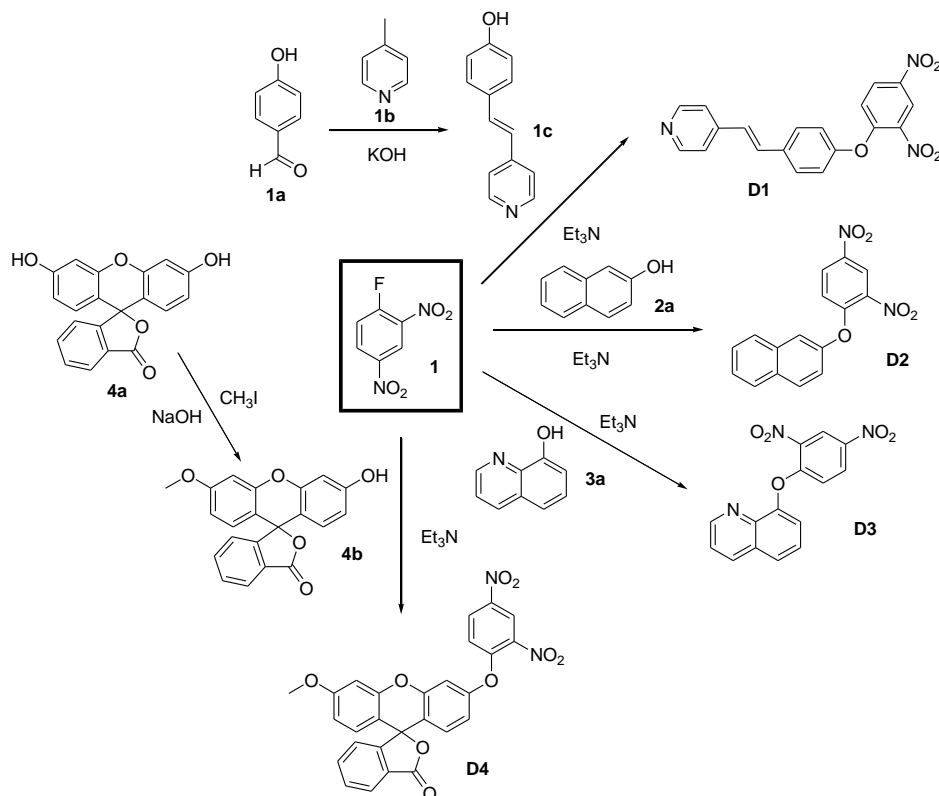
anion. In particular, Lin and co-workers prepared a NIR dye functionalized with a 2,4-dinitrophenyl ether moiety.(59) PBS (pH 7.0)-ethanol 9:1 v/v solutions containing CTAB and this probe were non-fluorescent whereas addition of increasing quantities of HS<sup>-</sup> anion induced a progressive appearance of an emission band. This emission enhancement was ascribed to a thiolysis of the 2,4-dinitrophenyl ether moiety. The same sensing mechanism was used by Spring and co-workers that prepared a 1,8-naphthalimide fluorophore equipped with a 2,4-dinitrophenyl ether group.(60) Finally, Liu and Feng prepared an ESIPT (excited state intramolecular proton transfer) probe functionalized with a 2,4-dinitrophenyl ether moiety for the rapid chromo-fluorogenic recognition of hydrogen sulfide.(61) The above described facts demonstrate that the design of new fluorogenic probes for hydrogen sulfide is a timely field of interest. Such simple systems may be used to control H<sub>2</sub>S concentrations in target industrial processes, for in situ environmental, geological monitoring and detection of hydrogen sulfide levels in living systems. Nevertheless, in spite of these possible applications, there are still relatively few examples of optical probes for the selective and sensitive detection of hydrogen sulfide (*vide ante*).

Bearing in mind the above mentioned concepts and following our interest in the development of new approaches for optical sensing,(62) we show herein a family of chemodosimeters (**D1-D4**) composed by selected fluorophores functionalized with 2,4-dinitrophenyl ether moieties for the detection of hydrogen sulfide. The synthesis and sensing features of probe **D3** were recently published by us in a short communication.(63) These dosimeters were easily synthesized through nucleophilic aromatic substitution reactions with 1-fluoro-2,4-dinitrobenzene with different fluorophores. The four probes displayed sensing features in buffered aqueous solution containing 1% of DMSO and gave marked emission enhancements only in the presence of HS<sup>-</sup> anion, whereas other bio-thiols were unable to induce emission changes. The probes showed excellent sensitivities, with limits of the detection in the 0.05-0.30 μM range, and some of the chemosensors were used for the fluorescence detection of both exogenous and endogenous HS<sup>-</sup> in living cells.

## Results and discussion

**Synthesis and characterizations of the probes:** The path of synthesis and the structures of probes **D1-D4** are shown in Scheme 1. **D1-D4** are 2,4-dinitrophenyl ether derivatives which are easily obtained in good yield (65 to 88%) through an aromatic

nucleophilic substitution reaction between 1-fluoro-2,4-dinitrobenzene (**1**) and alcohols **1c**, **2a**, **3a** and **4b**. Compounds **2a** and **3a** are commercially available. Alcohol **1c** was prepared through the base-catalysed condensation between 4-hydroxybenzaldehyde (**1a**) and 4-picoline (**1b**), whereas alcohol **4b** was obtained through methylation of fluorescein (**4a**) with methyl iodide.

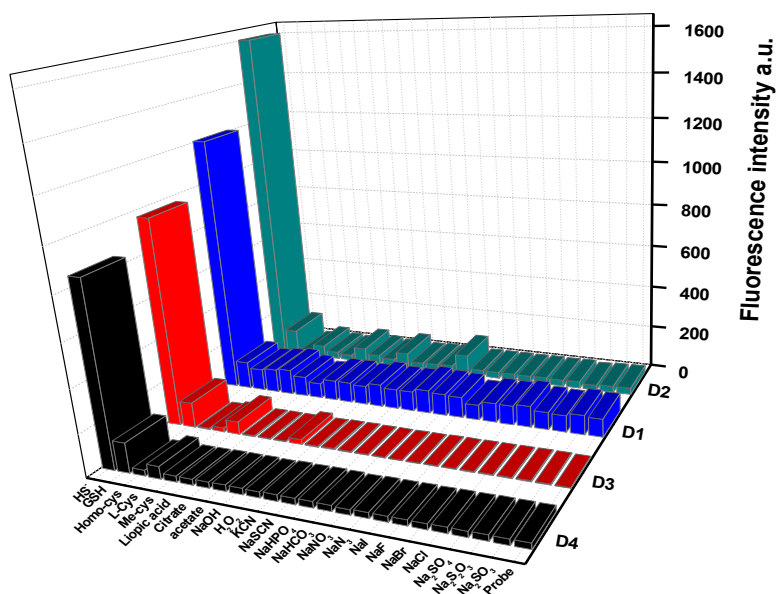


**Scheme 1.** Synthesis and chemical structure of chemodosimeters **D1-D4**.

All new probes were characterized by  $^1\text{H}$ ,  $^{13}\text{C}$ -NMR and HRMS. At this respect, the more indicative signals in the  $^1\text{H}$ -NMR spectra of dosimeter **D1** in  $\text{DMSO-}d_6$  were the doublets centred at 7.81 and 7.32 ppm attributed to the protons of the *trans* double bond. Moreover the protons of the 2,4-dinitrophenyl ether moiety appeared at 8.91 (1H, d), 8.47 (1H, dd) and 7.57 (1H, d) ppm. On changing to chemodosimeter **D2**, the most characteristic signals in the  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ ) spectrum were also those of the 2,4-dinitrophenyl ether moiety centred at 8.97, 8.41 and 7.39 ppm. Nearly the same pattern was observed for **D3** in which the dinitrophenyl ether protons were located at 8.93 (1H, d), 8.18 (1H, dd) and 7.83 (1H, d) ppm. Dealing with fluorescein derivative **D4**, the signals ascribed to the dinitrophenyl ether appeared at nearly the same chemical shift

than those observed for **D1-D3**, whereas the methoxy moiety appeared as singlet at 3.76 ppm (in DMSO- $D_6$ ).

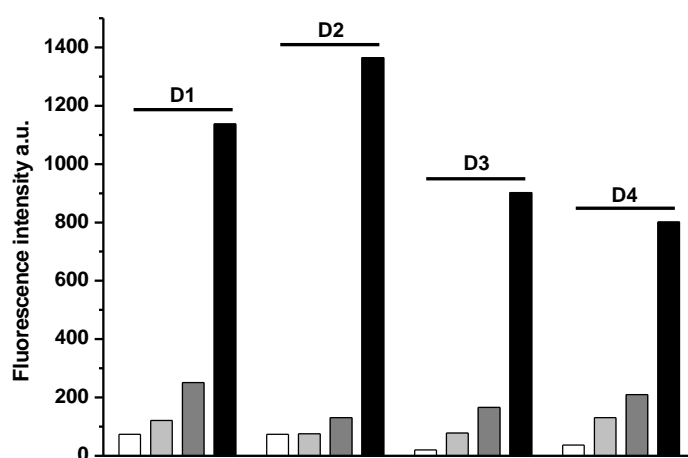
**Sensing features of the probes:** The four synthesized probes contain a chromo-fluorogenic subunit functionalized with a sulfide-sensitive 2,4-dinitrophenyl ether moiety. It is well known that the dinitrophenyl group has been often used for tyrosine protection in peptide synthesis.(64) The removal of this protective group is carried out using thiols under basic conditions. In our prepared probes the dinitrophenyl ether was electronically coupled with the fluorophore and the rupture of the ether bond was expected to induce changes in the emission spectra of the fluorogenic subunit. This simple sensing mechanism was used, very recently, in the preparation of selective and sensitive chemodosimeters for  $HS^-$  anion in water and in cellular media (*vide ante*). (59-61) The high selectivity obtained for  $HS^-$  versus thiols (*vide infra*) relies on the fact that  $H_2S$  is a small molecule with a  $pK_a$  of 6.9, while typical cellular free thiols (i.e. GSH and Cys) have  $pK_a$  values of ca. 8.5.(65) Taking into account these differences, at physiological pH, the thiolysis of 2,4-dinitrophenyl ethers is chemoselective for  $HS^-$  over GSH, Cys and other biologically-relevant thiols.



**Figure 1.** Emission intensity of **D1** (at 515 nm upon excitation at 390 nm), **D2** (at 415 nm upon excitation at 350 nm), **D3** (at 516 nm upon excitation at 450 nm) and **D4** (at 515 nm upon excitation at 450 nm) dosimeters (5  $\mu M$ ) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solutions upon addition of 10 equivalents of anions, bio-thiols, reducing agents and oxidants (after 60, 70,

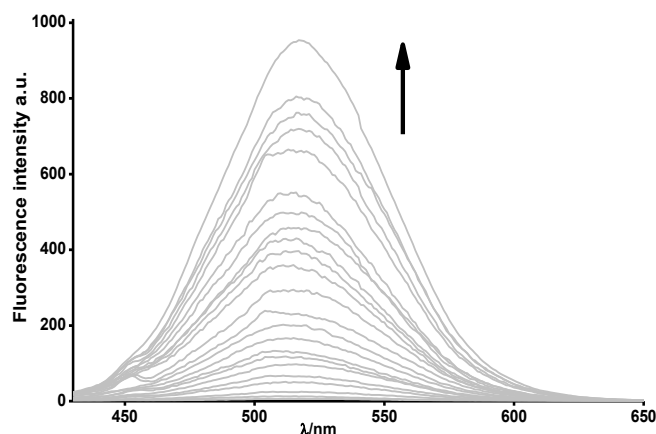
30 and 50 minutes of the addition for **D1**, **D2**, **D3** and **D4** respectively). The temperature was set at 25°C.

In a first step we tested the fluorescent response of the probes **D1-D4** (5  $\mu\text{M}$ ) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of an excess (10 equivalents) of selected anions ( $\text{F}^-$ ,  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ ,  $\text{N}_3^-$ ,  $\text{CN}^-$ ,  $\text{SCN}^-$ ,  $\text{AcO}^-$ ,  $\text{CO}_3^{2-}$ ,  $\text{PO}_4^{2-}$ ,  $\text{SO}_4^{2-}$ ,  $\text{HS}^-$  and  $\text{OH}^-$ ), biothiols (GSH, Cys, Hcy, Me-Cys and lipoic acid), reducing agents ( $\text{SO}_3^{2-}$  and  $\text{S}_2\text{O}_3^{2-}$ ) and oxidants ( $\text{H}_2\text{O}_2$ ). As it can be seen in Figure 1 the four dosimeters were practically non-fluorescent, however addition of  $\text{HS}^-$  induced a very remarkable fluorescent “turn-on” response, whereas addition of the other chemicals tested induced negligible changes in the emission behaviour. Besides, the fluorescent behavior of the four probes in the presence of GSH 6 mM (GSH concentrations in cells are in the 1-10 mM range) was also tested. As could be seen in Figure 2 GSH was unable to induce a remarkable fluorescent enhancement of the four probes even at mM concentrations.

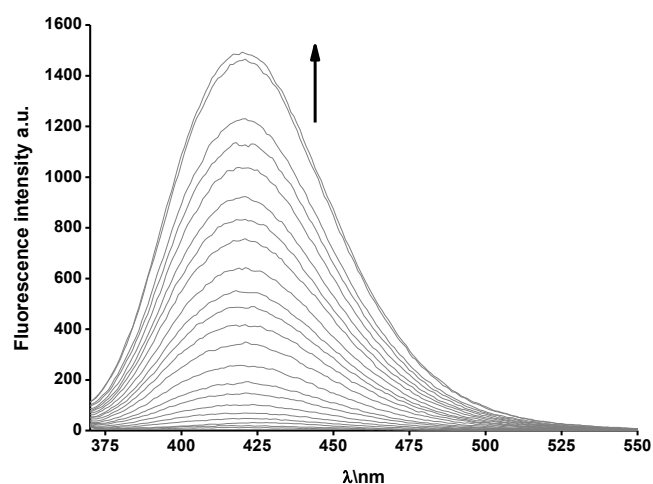


**Figure 2.** Emission intensity of **D1** (at 515 nm upon excitation at 390 nm), **D2** (at 415 nm upon excitation at 350 nm), **D3** (at 516 nm upon excitation at 450 nm) and **D4** (at 515 nm upon excitation at 450 nm) dosimeters (5  $\mu\text{M}$ ) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solutions alone (white bars) and upon addition of 50  $\mu\text{M}$  GSH (light grey bars), 6 mM GSH (dark grey bars) and 50 mM  $\text{HS}^-$  (black bars). The temperature was set at 25°C.



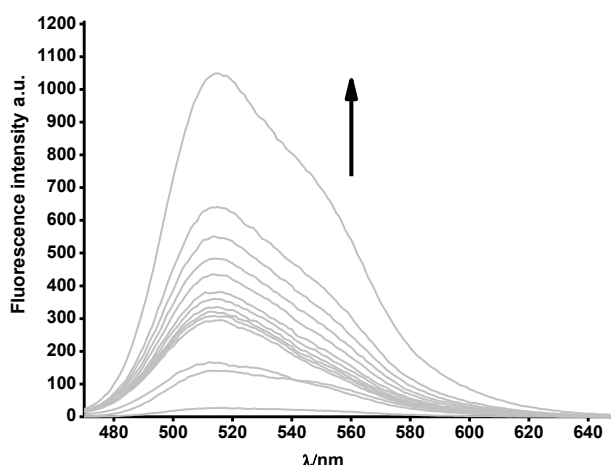


**Figure 3.** Fluorescence enhancement of dosimeter **D1** (5  $\mu\text{M}$ ) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of increasing quantities of  $\text{HS}^-$  anion (from 0 to 10 equivalents) after 60 minutes of the addition ( $\lambda_{\text{ex}} = 390 \text{ nm}$ ). The temperature was set at 25°C.

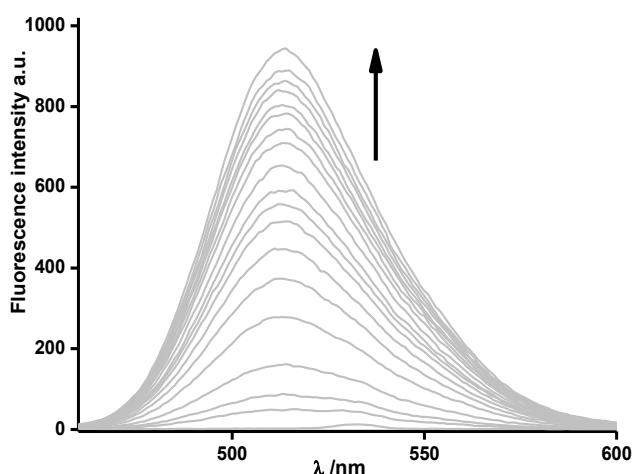


**Figure 4.** Fluorescence enhancement of dosimeter **D2** (5  $\mu\text{M}$ ) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of increasing quantities of  $\text{HS}^-$  anion (from 0 to 10 equivalents) after 70 minutes of the addition ( $\lambda_{\text{ex}} = 350 \text{ nm}$ ). The temperature was set at 25°C.

More in detail, addition of 10 equivalents of  $\text{HS}^-$  anion to HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution of dosimeter **D1** induced the appearance of an emission band at 515 nm ( $\lambda_{\text{ex}} = 390 \text{ nm}$ ) that reached its maximum intensity after 60 minutes (15-fold enhancement) (see Figure 3). For **D2**, addition of  $\text{HS}^-$  induced a 46-fold enhancement of the emission intensity at 415 nm ( $\lambda_{\text{ex}} = 350 \text{ nm}$ ) after 70 minutes (Figure 4).



**Figure 5.** Fluorescence enhancement of dosimeter **D4** (5  $\mu\text{M}$ ) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of increasing quantities of  $\text{HS}^-$  anion (from 0 to 10 equivalents) after 30 minutes of the addition ( $\lambda_{\text{ex}} = 450 \text{ nm}$ ). The temperature was set at  $25^\circ\text{C}$ .



**Figure 6.** Fluorescence enhancement of dosimeter **D3** (5  $\mu\text{M}$ ) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of increasing quantities of  $\text{HS}^-$  anion (from 0 to 10 equivalents) after 50 minutes of the addition ( $\lambda_{\text{ex}} = 450 \text{ nm}$ ). The temperature was set at  $25^\circ\text{C}$ .

Nearly the same enhancement (32-fold) in the intensity at 515 nm ( $\lambda_{\text{ex}} = 450 \text{ nm}$ ) was obtained upon addition of  $\text{HS}^-$  anion to probe **D4** (Figure 5). In this case the response was faster (when compared with **D1** and **D2**) as the maximum enhancement was observed after 30 minutes upon  $\text{HS}^-$  addition. Besides, dosimeter **D3** gives the larger response, in this case the addition of  $\text{HS}^-$  anion induced a remarkable 344-fold enhancement in the emission band at 516 nm ( $\lambda_{\text{ex}} = 450 \text{ nm}$ ) after 50 minutes (see

Figure 6). The subtle differences in response time and in emission enhancement upon addition of HS<sup>-</sup> anion to the four dosimeters could be ascribed to the different electronic properties of the probes although in all cases a clear highly selective *turn-on* response was observed.

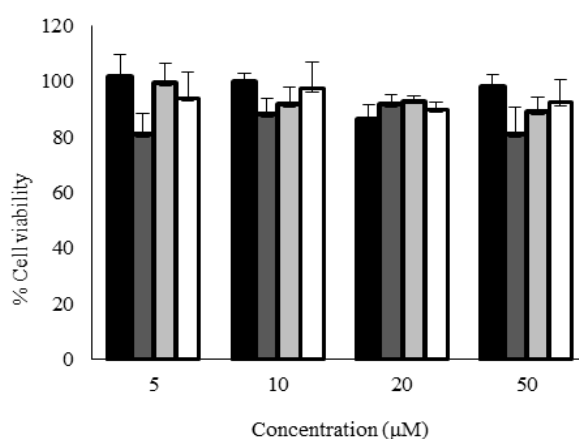
After assessing the highly selective turn-on response of **D1-D4** to hydrogen sulfide, the sensitivity of the probes was studied from titration profiles with HS<sup>-</sup> obtained in HEPES-DMSO 99:1 v/v solutions. From these studies a limit of detection (LOD) of 0.20 μM for HS<sup>-</sup> was determined for **D1**. Nearly the same response and sensitivity was obtained with probe **D2** (LOD = 0.29 μM). A more sensitive response was found on changing to dosimeters **D3** and **D4**. In these cases the titration profiles obtained allowed to calculate LOD of 0.06 and 0.05 μM for **D3** and **D4**, respectively. In all cases the probes displayed LODs below the hydrogen sulfide concentration required to elicit physiological responses (10-1000 μM).(66)

The selective emission enhancements observed for **D1-D4** in the presence of HS<sup>-</sup> is ascribed in all cases to an HS<sup>-</sup>-induced hydrolysis of the 2,4-dinitrophenyl ether groups that results in the release of the highly fluorescent alcohols **1c**, **2a**, **3a** and **4b**. In order to corroborate this hypothesis probes **D1-D4** were dissolved in ethanol and an excess of Na<sub>2</sub>S was added. The reactions were stirred at room temperature for 3 h and then the solvent was eliminated in a rotary evaporator. The crudes obtained were purified by silica column chromatography and <sup>1</sup>H and <sup>13</sup>C confirmed the presence of the alcohols **1c**, **2a**, **3a** and **4b** (for **D1**, **D2**, **D3** and **D4** respectively) as main products of the reaction.

The sensing features of **D1-D4** probes were quite similar to those reported for analogous fluorophores functionalized with 2,4-dinitrophenylethers (see Table 1). In particular, reaction times when using hydrolysis of 2,4-dinitrophenylether as sensing mechanism are in the range of minutes whereas the detection limits are in the 0.05-0.29 μM interval. Table 1 also shows the typical sensing parameters for probes for hydrogen sulfide when using different sensing mechanisms. From the data in the table it is apparent that the best response, in terms of reaction time, are obtained with probes in which the sensing mechanism is related with demetallation of Cu(II) complexes (from few seconds to minutes). As seen also in Table 1, lower limits of detection for hydrogen sulfide are obtained when demetallation processes and reduction reactions are used as transduction mechanisms.

**Table 1.** Mechanism used in the development of fluorogenic probes for hydrogen sulfide.

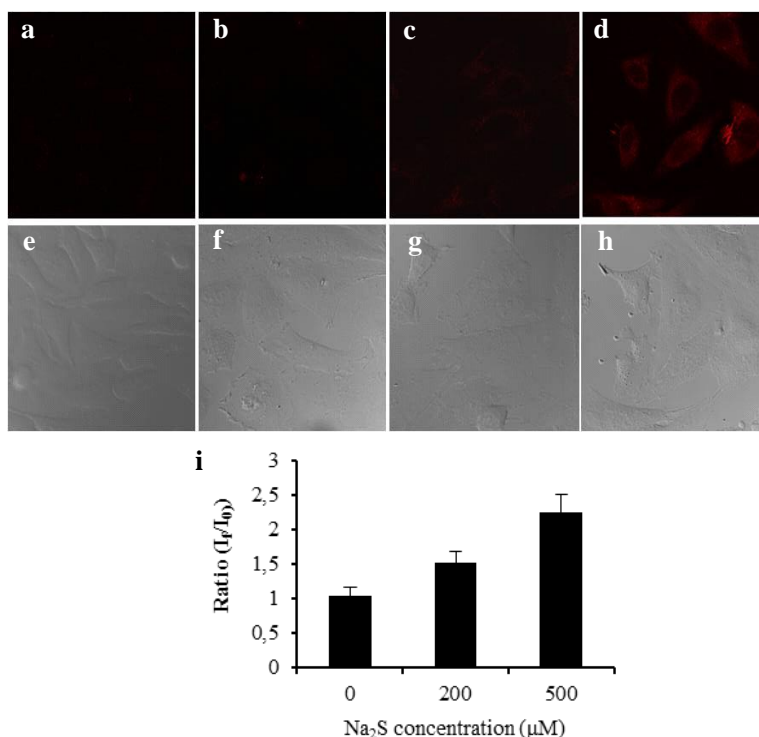
Mechanism	Time	Limit of detection	References
hydrolysis of 2,4-dinitrophenylether ( <b>D1-D4</b> )	30-70 minutes	0.05-0.29 $\mu\text{M}$	this paper
hydrolysis of 2,4-dinitrophenylether	5-20 minutes	0.05-0.48 $\mu\text{M}$	59-61
demetallation of Cu(II) complexes	few seconds-5 minutes	0.01-16 $\mu\text{M}$	37-46
Michael addition reactions	2-60 minutes	0.12-1 $\mu\text{M}$	47-50
other hydrolysis reactions	30-60 minutes	0.05-9 $\mu\text{M}$	54-56
reduction reactions	3-120 minutes	0.01-5 $\mu\text{M}$	27-36
oxidation of Se-containing fluorophores	5 minutes	not reported	57,58
cyclization reactions	2-50 minutes	0.1-100 $\mu\text{M}$	51-53



**Figure 7.** Cell viability assays. HeLa cells were treated with dosimeters **D1** (black), **D2** (dark grey), **D3** (grey) and **D4** (white) at concentrations of 5,10,20,50  $\mu\text{M}$  during 24h.

**Cell viability assays:** The selective emission enhancement of **D1-D4** in the presence of  $\text{HS}^-$  and the poor response observed upon the addition of bio-thiols (GSH, Cys and Hcy) strongly suggest that these probes could be used for  $\text{HS}^-$  imaging in living cells. Based on these observations, the cytotoxicity of **D1-D4** was first evaluated. For this purpose HeLa cells were treated with probes **D1-D4** at different concentrations (5, 10, 20 and 50  $\mu\text{M}$ ) over a 24-hour period and cell viability was determined by using a WST-1 assay. The obtained results are shown in Figure 7. As seen, the four dosimeters were essentially non-toxic in the range of concentrations tested.

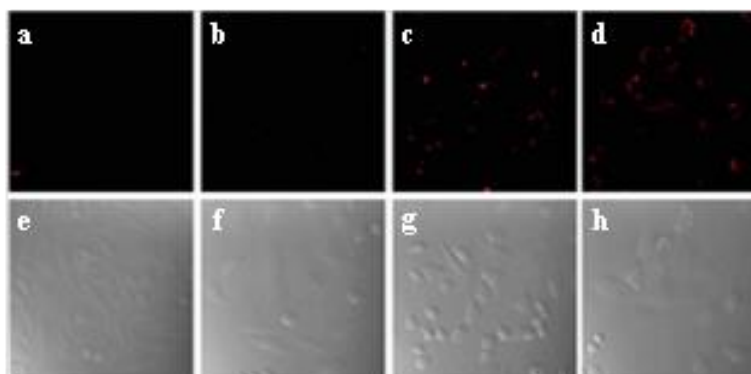
**Detection of HS<sup>-</sup> in HeLa cells:** Once assessed the biocompatibility of the prepared probes, and with the aim to test the dosimeters in highly competitive environments, we prospectively used probes **D1-D4** for the fluorescence imaging of sulfide in living cells. Of the four probes tested, only **D1** and **D3** gave remarkable results due to the low solubility of **D2** and **D4** at the concentrations required in the confocal microscopy studies.



**Figure 8.** Detection of HS<sup>-</sup> levels in living cells using dosimeter **D1**. (a, e) Transmitted light and fluorescence images of untreated HeLa cells. (b, f) HeLa cells incubated with **D1** for 90 minutes. (c, g) HeLa cells incubated with **D1** for 30 minutes at 37 °C and then with 200  $\mu\text{M}$   $\text{Na}_2\text{S}$  for 60 minutes. (d, h) HeLa cells incubated during 30 min with **D1** and 500  $\mu\text{M}$   $\text{Na}_2\text{S}$  for other 60 minutes. (i) Average  $I_f/I_0$  intensity ratios after addition of 200 and 500  $\mu\text{M}$  of  $\text{Na}_2\text{S}$  in PBS buffer. The excitation and emission wavelength were 450-470 nm and 510-530 nm. Representative fluorescence images from replicate experiments ( $n = 3$ ) are shown. Error bars are (SD).

In a typical experiment, HeLa cells were incubated in DMEM supplemented with 10% fetal bovine serum. To conduct fluorescence microscopy studies, HeLa cells were seeded in 24 mm glass coverslips in 6-well plates and were allowed to settle for 24 h. Cells were treated with the probes in PBS-DMSO 99:1 v/v at final concentrations of

10 and 30  $\mu\text{M}$ , for **D1** and **D3** respectively. After 30 minutes, the medium was removed and solutions of different concentrations of NaHS in PBS were added (0, 100, 200 and 500  $\mu\text{M}$ ) and cells were incubated for another 10-minute period.



**Figure 9.** Detection of  $\text{HS}^-$  levels in living cells using probe **D3**. HeLa cells were incubated with **D3** (30  $\mu\text{M}$ ) for 30 min at 37°C in DMEM. Transmitted light and fluorescence images were captured for (a) HeLa cells, (b) HeLa cells incubated with **D3**, and HeLa cells incubated with **D3** in the presence of  $\text{HS}^-$  at concentrations of (c) 200  $\mu\text{M}$  and (d) 500  $\mu\text{M}$ . The range of excitation was 330-380 nm and emission was monitored for wavelengths exceeding 420 nm.

The results are shown in Figures 8 (for **D1**) and 9 (for **D3**). The control experiment (HeLa cells without dosimeters) and the cells incubated with **D1** and **D3** showed no fluorescence, whereas a clear and marked enhancement in intracellular emission was found in the  $\text{HS}^-$ -treated cells. The emission enhancement observed was clearly dependent of the amount of NaHS added. The enhancement in the intracellular fluorescence intensity with **D1** was quantified by a standard image analysis and the results are shown in Figure 8i. A similar response (not shown) was observed for **D3**.

## Conclusions

In summary, here we reported the synthesis and sensing features of four compounds (**D1-D4**) as fluorescent turn-on probes for hydrogen sulfide. These chemodosimeters were based in a different fluorophores (i.e. styryl pyridine, naphthalene, quinoline and fluoresceine for **D1**, **D2**, **D3** and **D4**, respectively) functionalized with a 2,4-dinitrophenyl ether moiety. This chemical modification made the final probes poorly emissive. Probes **D1-D4** were easy to prepare and were able to selectively detect the  $\text{HS}^-$  anion in HEPES(10 mM, pH 7.4)-DMSO 99:1 v/v solutions via a remarkable *turn-*

on emission. The emission enhancements observed were due to a selective sulfide-induced hydrolysis of the 2,4-dinitrophenyl ether moiety that released the free fluorophores. **D1-D4** can selectively and sensitively detect HS<sup>-</sup> anion in water over other anions, biothiols and common oxidants. The titration profiles obtained upon addition of increasing quantities of HS<sup>-</sup> anion to aqueous solutions of the four dosimeters allowed us to determine LODs of 0.20, 0.29, 0.06 and 0.05 μM for **D1**, **D2**, **D3** and **D4** respectively. Besides the four dosimeters showed remarkably low sensitivities below the hydrogen sulfide concentration required to elicit physiological responses. From cell viability assays it was found that the four probes were essentially non-toxic. Moreover, real-time fluorescence imaging measurements confirmed that probes **D1** and **D3** can be used to detect intracellular HS<sup>-</sup> at micromolar concentrations.

## Experimental section

**Materials and methods:** UV-visible spectra were recorded with a JASCO V-650 Spectrophotometer. Fluorescence measurements were carried out in a JASCO FP-8500 Spectrophotometer. <sup>1</sup>H and <sup>13</sup>C-NMR spectra were acquired in a BRUKER ADVANCE III (400 MHz), where mass spectra were carried out in a TRIPLETOF T5600 (ABSciex, USA) spectrometer. The chemicals 4-picoline, 2-hydroxynaphthalene, 8-hydroxyquinoline, fluorescein, iodomethane, 1-fluoro-2,4-dinitrobenzene, 4-hydroxybenzaldehyde (98%), acetic anhydride (98%), hexadecyltrimethylammonium bromide (CTABr) and sodium bicarbonate were purchased from Sigma-Aldrich. Triethylamine (98%) was purchased from J. T. Baker. Analytical-grade solvents, potassium hydroxide, sodium sulfate and hydrochloric acid (37%) were purchased from Scharlau (Barcelona, Spain).

**Synthesis of 4-((E)-2-(pyridin-4-yl)vinyl)phenol (1c):** 4-hydroxybenzaldehyde (**1a**, 500 mg, 4.1 mmol) and 4-picoline (**1b**, 0.4 mL, 4.1 mmol) were refluxed in acetic anhydride (5 mL) for 24 h. After cooling to room temperature a precipitate appeared. This was collected and recrystallized from ethanol. Then, the solid was dissolved in ethanol-water (20 mL, 1:1 v/v) containing potassium hydroxide (400 mg, 7.1 mmol) and refluxed for 6 h. Afterwards, the crude reaction was neutralized with diluted hydrochloric acid. The product **1c** precipitated and was isolated as a yellow solid after filtration (749 mg, 3.8 mmol, 85% yield).

$^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  12.10 (s, 1H), 10.79 (d,  $J$  = 5.6 Hz, 2H), 9.75 (dd,  $J$  = 21.4, 11.6 Hz, 5H), 9.29 (d,  $J$  = 16.4 Hz, 1H), 9.10 (d,  $J$  = 8.5 Hz, 2H).

**Synthesis of 4-(4-(2,4-dinitrophenoxy)styryl)pyridine (D1):** **1c** (200 mg, 1.17 mmol) was dissolved in acetone (5 mL) and then triethyl amine (0.5 ml, 3.6 mmol) was added. After that, 1-fluoro-2,4-dinitrobenzene (217.7 mg, 1.17 mmol) dissolved in acetone (5 mL) was added to the crude reaction and the mixture refluxed for 30 min. Then, the acetone was evaporated and the residue taken up in 5% hydrochloric acid (10 mL). A precipitate appeared that was filtered, washed with water and then suspended in 5% sodium hydroxide (15 mL) and stirred for 15 min. After that, the solid was filtered, washed with water and purified by silica column chromatography using hexane-acetone 1:1 v/v as eluent. Dosimeter **D1** was isolated as yellow solid (310 mg, 0.85 mmol, 74% yield).

$^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  8.91 (d,  $J$  = 2.8 Hz, 1H), 8.56 (d,  $J$  = 6.0 Hz, 2H), 8.47 (dd,  $J$  = 9.3, 2.8 Hz, 1H), 7.81 (d,  $J$  = 8.7 Hz, 2H), 7.57 (dd,  $J$  = 4.9, 3.4 Hz, 3H), 7.34 – 7.25 (m, 4H).

$^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  154.7, 153.9, 150.1, 144.2, 141.6, 139.6, 134.3, 131.8, 129.7, 129.4, 126.6, 122.0, 121.0, 120.7, 119.7.

HRMS-EI  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{13}\text{N}_3\text{O}_5$  363.0855; found: 364.0944 ( $\text{M}+\text{H}^+$ ).

**Synthesis of 2-(2,4-dinitrophenoxy)naphthalene (D2):** Dosimeter **D2** was prepared by the same experimental procedure used for **D1**. The final product was isolated as pale yellow solid (725 mg, 2.33 mmol, 88 % yield).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.97 (d,  $J$  = 2.7 Hz, 1H), 8.41 (dd,  $J$  = 9.3, 2.7 Hz, 1H), 8.09 (d,  $J$  = 8.9 Hz, 1H), 8.04 – 8.00 (m, 1H), 7.96 – 7.92 (m, 1H), 7.67 (ddd,  $J$  = 9.4, 4.2, 2.3 Hz, 3H), 7.40 – 7.37 (m, 1H), 7.18 (s, 1H).

$^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  156.3, 151.2, 141.6, 139.6, 134.2, 131.7, 131.2, 128.9, 128.1, 127.6, 126.5, 122.2, 119.7, 118.8, 117.6.

HRMS-EI  $m/z$ : calcd for  $\text{C}_{16}\text{H}_{10}\text{N}_2\text{O}_5$  310.0590; found: 310.0817 ( $\text{M}^+$ ).

**Synthesis of 8-(2,4-dinitrohydroxy)quinoline (D3):** Dosimeter **D3** was prepared by the same experimental procedure used for **D1**. The final product was isolated as pale yellow solid (703 mg, 2.25 mmol, 85 % yield).



$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.93 (d,  $J = 2.8$  Hz, 1H), 8.79 (dd,  $J = 4.2, 1.7$  Hz, 1H), 8.25 (dd,  $J = 8.4, 1.7$  Hz, 1H), 8.18 (dd,  $J = 9.3, 2.8$  Hz, 1H), 7.83 (dd,  $J = 7.9, 1.7$  Hz, 1H), 7.65 – 7.56 (m, 2H), 7.47 (dd,  $J = 8.4, 4.2$  Hz, 1H), 6.79 (d,  $J = 9.3$  Hz, 1H).

$^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  157.4, 151.0, 149.4, 141.4, 140.6, 138.9, 136.4, 130.3, 128.7, 126.8, 122.4, 120.9.

HRMS-EI  $m/z$ : calcd for  $\text{C}_{15}\text{H}_{10}\text{N}_3\text{O}_5$  311.0542; found: 312.0628 ( $\text{M}+\text{H}^+$ ).

**Synthesis of 3-hydroxy-6-methoxy-3H-spiro[isobenzofuran-1,9-xanthen]-3-one (4b):** Fluorescein (**4a**, 2 g, 6.18 mmol) was suspended in methanol (50 mL) and NaOH (160 mg, 4 mmol) was then added. The final mixture was stirred until complete dissolution of fluorescein. After evaporation of methanol, the disodium salt (2.0 g, 5.32 mmol) was suspended in DMF (50 mL) containing methyl iodide (332  $\mu\text{L}$ , 5.32 mmol) and the reaction was then stirred at room temperature for 48 h. After this time, the crude was diluted with 5%  $\text{NaHCO}_3$  (50 mL) and extracted with diethyl ether (2 x 25 mL). The organic phase was washed with brine (20 mL), dried over  $\text{Na}_2\text{SO}_4$ , and evaporated to give an orange precipitate. This solid was dissolved in methanol (30 mL), 2M NaOH (10 mL) was added and the mixture stirred at room temperature for 2 h. After evaporation of methanol, the crude was diluted with water (20 mL) and impurities extracted with diethyl ether (2 x 10 mL). The aqueous phase was acidified (pH 2.0) by the addition of concentrated HCl and then extracted with diethyl ether (3 x 15 mL), washed with brine (25 mL) and dried over  $\text{Na}_2\text{SO}_4$ . After evaporation of the diethyl ether a brown solid was obtained. The final product was purified by silica column chromatography using diethyl ether-hexane 2:1 v/v as eluent. Compound **4b** was isolated as brown solid (831.2 mg, 2.4 mmol, 45% yield).

**Synthesis of 3-(2,4-dinitrophenoxy)-6-methoxy-3H-spiro[isobenzofuran-1,9-xanthen]-3-one (D4):** Dosimeter **D4** was prepared by the same experimental procedure used for **D1**. The final product was isolated as brown solid (162 mg, 0.315 mmol, 65 % yield).

$^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  8.84 (d,  $J = 2.8$  Hz, 1H), 8.58 (d,  $J = 3.1$  Hz, 1H), 8.45 (dd,  $J = 9.2, 2.6$  Hz, 1H), 8.01 (d,  $J = 7.6$  Hz, 1H), 7.90 – 7.77 (m, 1H), 7.73 (t,  $J = 7.3$  Hz, 1H), 7.37 (d,  $J = 9.2$  Hz, 1H), 7.22 (dd,  $J = 9.8, 5.1$  Hz, 1H), 6.92 (ddd,  $J = 20.1, 13.2, 5.6$  Hz, 1H), 6.74 (dd,  $J = 8.9, 2.5$  Hz, 1H), 6.67 (d,  $J = 8.8$  Hz, 1H), 6.45 (d,  $J = 9.7$  Hz, 1H), 3.76 (s, 1H).

$^{13}\text{C}$  NMR (101 MHz, DMSO)  $\delta$  158.3, 155.0, 144.7, 141.2, 140.9, 139.1, 138.7, 138.5, 136.6, 129.7, 127.9, 126.6, 126.0, 125.8, 125.6, 124.8, 123.0, 121.7, 120.3, 117.8, 114.8, 115.8, 113.0, 110.1, 107.8, 56.1, 18.6.

HRMS-EI m/z: calcd for  $\text{C}_{27}\text{H}_{16}\text{N}_2\text{O}_9$  512.0856; found: 513.0935 ( $\text{M}+\text{H}^+$ ).

**Cell culture conditions:** The HeLa human cervix adenocarcinoma cells were purchased from the German Resource Centre for Biological Materials (DSMZ) and were grown in DEM supplemented with 10% FBS. Cells were maintained at 37 °C in an atmosphere of 5%  $\text{CO}_2$  and 95% air and underwent passage twice in a week.

**WST-1 cell viability assays:** Cells were cultured in sterile 96 –well plates at a seeding density of  $2.5 \times 10^3$  cells/well for HeLa and were allowed to settle for 24 h. Dosimeters **D1**, **D2**, **D3** and **D4** were added to the cells at final concentrations of 10, 20, 30 and 50  $\mu\text{M}$ . After 23 h, WST-1 (7  $\mu\text{L}$  of a 50 mg/ml solution) was added to each well. Cells were further incubated for 1 h (a total of 24 h of incubation was therefore studied). Then shaken thoroughly for 1 min. on a shaker and the absorbance was measured at 450 nm against a background control as blank using a microplate ELISA reader. The reference wavelength is 690 nm.

**Live confocal microscopy:** HeLa cells were seeded in 24 mm glass coverslips in 6-well plates at a seeding density of  $10^5$  cells/well. After 24 hours, cells were treated with **D1** and **D3** at a final concentration of 10 and 30  $\mu\text{M}$ , respectively. After 30 minutes, the medium was removed to eliminate compounds and washed with PBS. Then a solution of  $\text{Na}_2\text{S}$  in PBS was added at a final concentrations of 0, 100, 200, 500  $\mu\text{M}$  and cells were incubated during 1h at 37 °C. After that, slides were washed twice with PBS to remove traces of the dosimeters. Then slides were visualized under a confocal. Confocal microscopy studies were performed by Confocal Microscopy Service (CIPF). The images were acquired with a Leica TCS SP2 AOBS (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) inverted laser scanning confocal microscope using oil objectives: 63X Plan-Apochromat-Lambda Blue 1.4 N.A. The excitation wavelengths were 450 nm (argon laser) for both dosimeters. Two-dimensional pseudo colour images (255 colour levels) were gathered with a size of 1024 x 1024 pixels and Airy 1 pinhole diameter. All confocal images were acquired using the same settings and the distribution of fluorescence was analyzed using the Image J Software. Identical experiments were done three times to obtain reproducible results.

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