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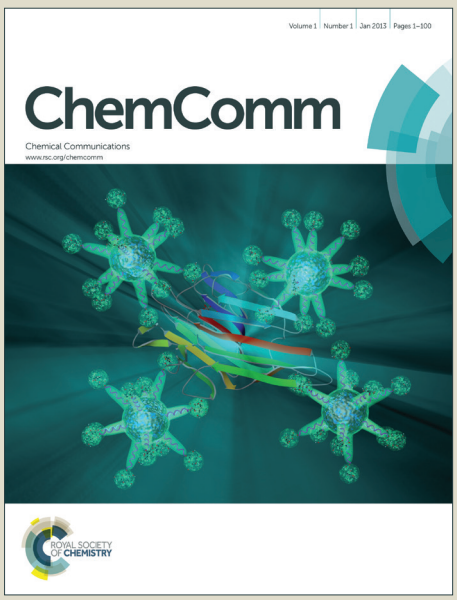

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A Highly Selective Sulfinate Ester Probe for Thiol Bioimaging

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Satish R. Malwal, Ajay Labade, Abhijeet S. Andhalkar, Kundan Sengupta, and Harinath Chakrapani*

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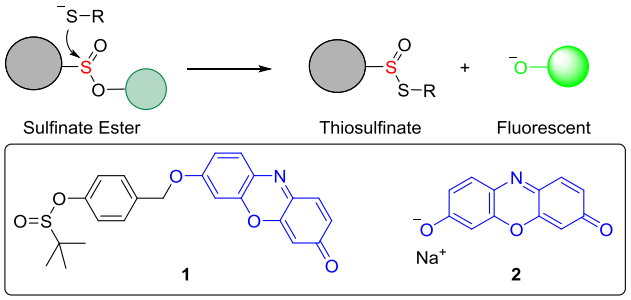
Abstract We describe here hitherto unexplored chemistry of the sulfinate ester functional group as being highly selective towards nucleophilic substitution by thiols in physiological pH. Using this cleavable trigger, an optical thiol probe that is suitable for thiol bioimaging has been developed.

Thiols are present in nearly all cells and mediate numerous cellular processes[1-3] including cellular signaling, protection from cytotoxic agents, and maintenance of homeostasis.[4] Levels of thiols are thus tightly regulated in cells and variations are associated with disease states including diabetes, arteriosclerosis, neurodegenerative disorders certain cancers and acquired immunodeficiency syndrome (AIDS).[5] Hence, reliable tools to detect and image thiols inside cells become essential for diagnostics.[6] Among available options, optical probes, which upon entry into cells undergo an attack or cleavage by sulfhydryl group to produce a highly fluorescent molecule, are desirable due to their simplicity as well as convenience.[7, 8] The aforementioned sensor or probe consists of two portions, first, the thiol cleavable or reactive group and second, the latent fluorophore. While numerous classes of latent fluorophores are reported, the number of distinct thiol-selective activation strategies remains few.[7, 9, 10] Here, we report a novel sulfinate ester probe that is selectively cleaved by thiols in physiological pH and this functional group is suitable for use as a thiol selective cleavable trigger for sensing biological thiols in physiological media as well as within cells.

Esters of sulfinic acids have long been used in asymmetric synthesis[11, 12] and previous investigations have revealed that these groups can be cleaved by nucleophiles to produce an alcohol and a thiosulfinate.[13, 14] The thiophillicity of sulfur-based functional groups is well established[15] and we therefore anticipated that such sulfinate esters might be reactive with biological thiols as well (Scheme 1). Upon reaction with a thiol and cleavage, an alcohol is liberated; the choice of an aryl alcohol which can fluoresce or develop color upon release might allow us to develop novel thiol probes. To our knowledge, such sulfinate ester-based sensors are not reported.

We considered **1** which contains a *tert*-butyl sulfinyl functional group as a possible thiol probe (Scheme 2).[16] Resorufin (**2**), a highly fluorescent molecule, is routinely used as a fluorescent as well as a colorimetric probe (Scheme 2).[17] When alkylated at the phenolic position, the resulting ether is weakly fluorescent and upon cleavage to release the free phenolate **2**. When **1** reacts with thiols, the liberated phenol would undergo self-immolation to generate **2**, which is highly fluorescent in physiological pH (Scheme 1).



Scheme 1. Design of a sulfinate ester as a thiol probe: the sulfinate ester attached with a latent fluorophore is expected be cleaved by a thiol to produce a thiosulfinate and a fluorescent alcohol. Inset: compounds **1** and **2**.

The sulfinate ester **1** was prepared from commercially available 4-hydroxybenzaldehyde in 4 steps (ESI, Scheme S1). The sulfinate ester **1** was found to be stable in buffer and weakly fluorescent (Figure 1a). Upon addition of cysteine (10 equiv), we find a significant increase in fluorescence after 30 min (Figure 1a). When the UV-visible spectrum was recorded, similarly we found that **1** did not show significant absorbance in the visible region (Figure 1b) and upon addition of cysteine, a significant color change is recorded (Figure 1b). This probe is hence suitable for visual detection of thiols as well. This probe was found to fluoresce only when exposed to free thiol containing biomolecules such as glutathione; none of the other biological nucleophiles including amino acids and the disulfide cystine were capable of eliciting a significant increase in fluorescence (Figure 1c).

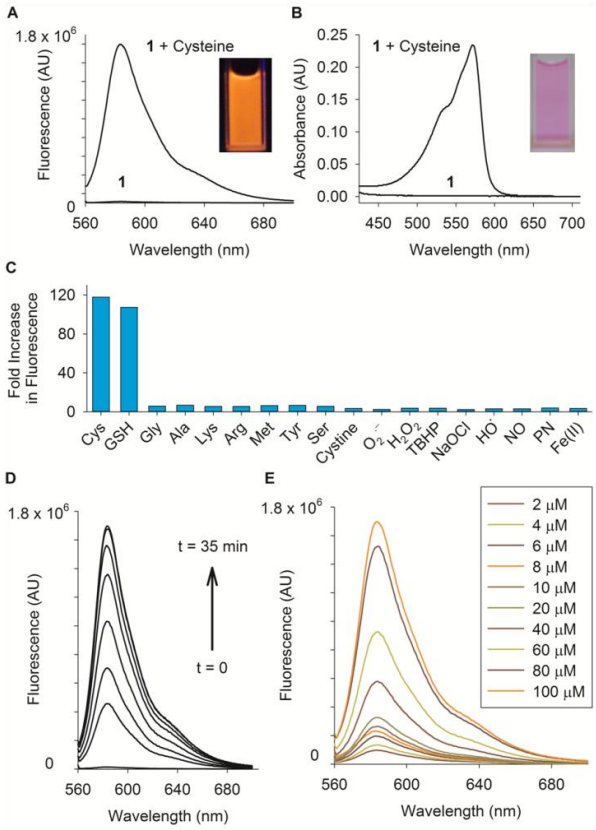


Figure 1. (a) Fluorescence spectra recorded with **1** (10 μM) alone in pH 7.4 buffer and upon reaction with cysteine (100 μM) after 30 min; inset: a photograph of the cuvette after additon of cysteine. (b) Absorbance spectra recorded with **1** (50 μM) alone in pH 7.4 buffer and upon reaction with cysteine (500 μM) after 30 min; inset: a photograph of the cuvette after additon of cysteine. (c) Fold-increase in fluorescence after exposure of **1** (10 μM) to 10 eq. of various glutathione (GSH), amino acids, ROS, RNS and Fe(II) in pH 7.4 buffer after 30 min (d) Fluorescence spectra recorded when **1** (10 μM) was reacted with cysteine (100 μM) in pH 7.4 buffer. (e) Thiol probe **1** (10 μM) was independently exposed to various equivalents of cysteine and fluorescence spectra were recorded after 30 min. Inset: A plot of fluorescence intensity and concentration. Linear regression analysis (R^2 = 0.9949) gave a slope of 21380.35. Detection limit was estimated to be 2.77×10^{-8} M at signal to noise ratio of 3:1.

Biological thiols are first responders during oxidative stress caused by elevated levels of reactive oxygen species (ROS) in cells and hence, detecting thiols during elevated levels of ROS assumes importance. When **1** was reacted with various biologically relevant ROS including superoxide, hydrogen peroxide, the organic peroxides (*tert*-butyl hydroperoxide, TBHP), sodium hypochlorite and hydroxyl radical, we found no significant increase in fluorescence (Figure 1c) suggesting that this probe was not cleaved by common ROS and would hence be suitable for study of cellular events related to thiol response during elevated ROS as well. Another important reactive species that is generated in cells is nitric oxide (NO) and associated reactive nitrogen species (RNS) such as peroxynitrite (PN). When **1** was exposed to these RNS, we found no evidence for increase in fluorescence, again, suggesting that the sulfinate ester was stable when exposed to these RNS. Finally, we found no increase in fluorescence when **1** was reacted with Fe(II), a metal ion that mediates a number of biological processes. Together our data suggests that the probe **1** is inert to conditions frequently encountered during cellular stress.

Next, cysteine (10 eq.) was added to a solution of **1** and fluorescence spectra were periodically recorded (Figure 1d). Curve fitting gave a pseudo first order rate constant of 0.0414 min^{-1}. During the time course of the thiol reaction, the sulfinate ester **1** in the absence of a thiol was found to be stable in pH ranging from 5-9 suggesting stability in the pH range encountered in physiological conditions (see SI).[13, 14] Next, **1** was reacted with increasing equivalents of cysteine and fluorescence emission was recorded after 30 min. A concentration-dependent increase in fluorescence response was recorded (Figure 1e). Based on this data, we estimated a detection limit for cysteine of 28 nM (3 × standard deviation/slope; for GSH, the detection limit was estimated to be 33 nM) supporting the potential use of this probe for detecting thiols at low concentrations (see ESI).

Having established that **1** was an excellent fluorogenic probe for thiols the capability of **1** to permeate cells to detect thiols was evaluated. Human adenocarcinoma DLD-1 cells were first treated with **1** and viable cells were estimated after 24 h using a standard cell viability assay. We found that DLD-1 cells tolerated **1** well (>95% viable cells) at 20 μM (ESI, Figure S3). Live cell imaging of DLD-1 cells treated with **1** using a confocal microscope revealed that this probe permeated cells and fluoresced upon reaction with thiols (Figure 2a). Next, DLD-1 cells were incubated with 1 mM *N*-phenylmaleimide (NPM) following which these cells were treatment with **1**; NPM is reactive with thiols and has been previously used to block free thiols inside cells.[18] During this experiment, we found no fluorescence signal attributable to the formation of **2**. Taken together, these results suggest that **1** is highly selective to activation by thiols and when free thiols within live cells are unavailable no signal is recorded and support the use of **1** for thiol bioimaging.

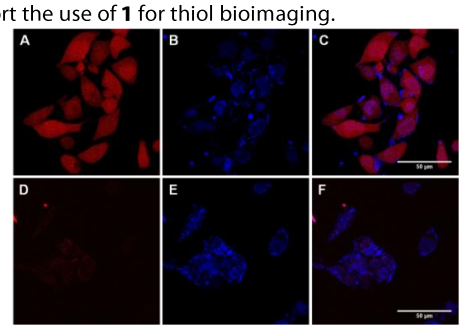


Figure 2. Live cell imaging carried out with DLD-1 cells: (a) **1**, red channel; (b) **1**, Hoechst33258; (c) overlay; (d) **1** co-treated with *N*-phenylmaleimide (NPM), a competitive thiol-reactive electrophile; (e) **1** + NPM, Hoechst33258; and (f) overlay. Probe: excitation 561 nm and emission 568 -797 nm; Hoechst33258: excitation 360 nm and emission 426-797 nm.

Next, in order to study the mechanism of cleavage of the sulfinate ester in the presence of thiols, the model sulfinate ester **3** (Figure 3) was synthesized by reacting para cresol **4** with *t*-butylsulfinyl chloride (see ESI). A model thiosulfinate **5** (Scheme 2) was similarly synthesized by reacting thiophenol with *t*-butylsulfinyl chloride (See ESI). The sulfinate ester **3** was treated with sub-stoichiometric amounts of thiophenol (0.2 equiv) and HPLC analysis of the reaction mixture (Figure 3) revealed the generation of **5** and para-cresol **4** along with unreacted **3**. This observation suggested

that a thiol-mediated displacement of the phenol occurred. When equimolar amounts of **3** and thiophenol were reacted, we found a significant decrease in **3** with concomitant formation of **5** and **4** suggesting that the thiosulfinate ester was an intermediate during reaction of sulfinate esters with thiols.

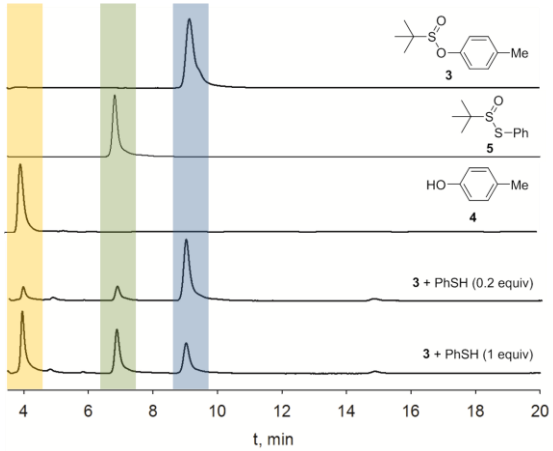
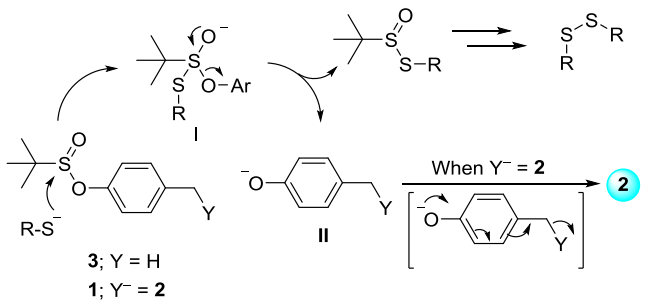


Figure 3. HPLC traces for authentic **3**, **4**, **5** and reaction mixtures of **3** and PhSH (0.2 equiv) and **3** and PhSH (1 equiv) recorded after 1 h.

Based on the aforementioned results, a mechanism for reaction of sulfinate esters was proposed (Scheme 2). Reaction of a thiol with the sulfinate ester produces intermediate **I**.[13, 14] Previous studies have shown that such a hypervalent sulfur intermediate was likely when sulfinate esters were reacted with nucleophiles.[6, 14, 19] Decomposition of **I** gives a thiosulfinate and the phenolate **II**. When the thiol probe **1** was used, the phenolate **II** could rearrange to produce the flurophore **2**. Previous reports[4, 20] indicate that the thiosulfinate ester might further react with a thiol to produce a disulfide.[4] When **5** was treated with excess of thiophenol, indeed, we find the production of PhS-SPh (see ESI, Figure S1). Similarly, when *N*-(4-nitrobenzoyl)-cysteine methyl ester (10 equiv) was reacted with **3**, we found complete decomposition within 30 min with concomitant formation of the corresponding disulfide (see ESI, Figure S2).[21]



Scheme 2. Proposed mechanism for thiol-mediated cleavage of sulfinate esters

Taken together, we report a novel sulfinate ester based "turn on" fluorescence as well as colorimetric probe for selective detection and estimation of thiols. This probe can be synthesized with ease from commercial starting material and was inert to a diverse array of potential interfering agents including ROS,[22] RNS and metal ions. To our knowledge, this is the first report of a thiol probe that uses a sulfinate ester as the reactive functional group. This probe was found to be cell permeable and useful for bioimaging biological thiols in live cells. Sulfinate esters have been viewed thus far as interesting intermediates for assymmetric synthesis and their reactions with amine nucleophiles have received considerable attention.[11, 12] We report new chemistry of this functional group that might find applications in thiol-based bioimaging and can, in principle, be extended to covalent modification of biomacromolecules and thiol-induced drug delivery.[23,24] The authors thank IISER Pune, the Department of Biotechnology, India and Council for Scientific and Industrial Research (CSIR) for financial support.

Notes and references

[a]Indian Institute of Science Education and Research Pune, Dr. Homi Bhabha Road, Pashan Pune 411 008, Maharashtra, India.

† Electronic Supplementary Information (ESI) available: Preparative procedures, characterization data, assay protocols and spectral data. See DOI: 10.1039/c000000x/

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