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Authors: S. Israel Suarez, Rynne Ambrose, Madison A. Kalk, and John C. Lukesh III

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Selenosulfides Tethered to *Gem*-Dimethyl Esters: A Robust and Highly Versatile Framework for H_2S Probe Development**

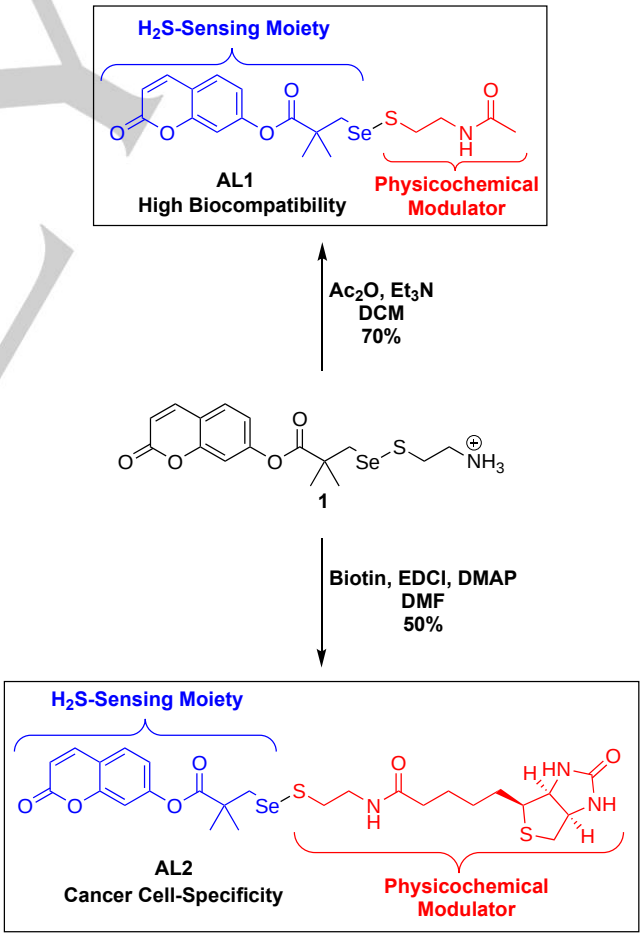
S. Israel Suarez, Rynne Ambrose, Madison A. Kalk, and John C. Lukesh III*

Abstract: Selenosulfides coupled to *gem*-dimethyl esters provide an exceptional platform for H_2S probe development. With the sulfur half being nonessential to its high reactivity and selectivity towards H_2S, we highlight the unique flexibility of our design by improving its biocompatibility and tissue specificity through structural modifications of its sulfide moiety.

Hydrogen sulfide (H_2S), once regarded as merely a toxic and foul-smelling gas, has recently been recognized as an endogenous signaling molecule and important physiological mediator within mammalian systems.[1–3] Although several H_2S-regulated processes have been discovered in recent years, questions surrounding its metabolism, distribution, and prevalence still persist. Therefore, the development of new bioanalytical methods for probing the biological signaling pathways of H_2S is needed to better discern its primary and secondary roles as a signaling molecule. To this end, reaction-based fluorescent probes have proven to be invaluable chemical tools for aiding H_2S research by providing a non-destructive method for the real-time detection and visualization of H_2S within biological samples.[4–10] Developing reaction-based probes that can selectively detect H_2S within a biological setting, however, does pose significant challenges. With other biological thiols being present in appreciably higher concentrations (e.g. glutathione), reaction-based probes must effectively key in on reactivity differences that distinguish H_2S from these other species. With a suppressed pK_a (6.8) relative to that of other biothiols (pK_a: ~8–9), H_2S resides predominately in its reactive HS^- form under physiological conditions, rendering it a vastly superior reductant and nucleophile at neutral pH.[11] Chang and co-workers were the first to develop a turn-on fluorescent probe that capitalized on this increased reactivity with their design of an azide-caged rhodamine dye.[12] With the addition of H_2S, reduction of the aryl azide to the aryl amine afforded a significant increase in fluorescence intensity upon a 30–60 min incubation period. In an alternative approach, Xian and He were the first to develop reaction-based probes that relied on cascade processes to release a fluorescent reporter.[13–18] These probes also displayed good selectivity as they effectively exploited the double nucleophilic character of H_2S—another distinctive feature in terms of its reactivity.

Still, with low water solubility and inefficient intracellular delivery, many H_2S-reactive probes have proven unsuccessful in their ability to function in a biological setting. The extreme hydrophobic nature of some even required the use of an organic co-solvent (such as acetonitrile) for *in vitro* assays or surfactants for cellular assays.[6] Therefore, the continued development of reaction-based fluorescent probes with improved physicochemical properties is highly desirable and would provide a significant contribution to the chemical toolkit that is currently available for studying H_2S physiology.

Taking inspiration from these earlier designs, we set out to develop chemical probes that would not only display remarkable reactivity and selectivity towards H_2S, but unprecedented modularity, such that issues related to insufficient water solubility, cell permeability, and/or tissue specificity could be easily addressed. To achieve this desired versatility without comprising reactivity and selectivity, we conjectured that the employment of a selenosulfide functionality would be key for accessing a probe



Scheme 1. Synthesis of versatile, reaction-based fluorescent probes for H_2S detection.

[*] S. Israel Suarez, Rynne Ambrose, Madison A. Kalk, and Prof. John C. Lukesh III
Department of Chemistry, Wake Forest University
Wake Downtown Campus
Winston-Salem, NC 27101
E-mail: lukeshjc@wfu.edu

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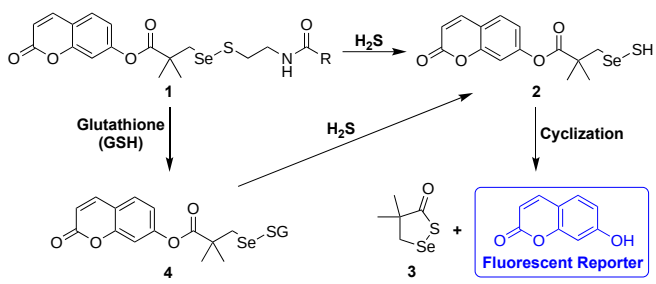

Scheme 2. Proposed mechanism for H_2S detection with **AL1**.

with these desired attributes. Although selenosulfides have been rarely reported within the chemical literature,[19,20] we reasoned that reactivity differences between selenium and sulfur could be exploited such that the selenium half—when tethered to a second electrophilic partner—would effectively scavenge and detect H_2S, while the sulfur half would provide a suitable handle for mediating its physicochemical properties. Therefore, we first synthesized selenosulfide **1** from 3-bromopivalic acid (see supporting information) as it provided an amino group for subsequent conjugation reactions. And in an initial effort to afford high biocompatibility, we then appended an acetyl group to access probe **AL1** (Scheme 1).

Our proposed mechanism for H_2S detection by **AL1** is depicted in Scheme 2. We hypothesized that an initial exchange reaction between H_2S and **AL1** would quickly generate **2** and *N*-acetylcysteamine as an innocuous byproduct (note: cysteamine is a prescribed therapeutic for the treatment of corneal cystinosis given its low toxicity and superb antioxidant activity).[21] With selenium's heightened electrophilic character, nucleophiles will typically attack at selenium much more rapidly than at sulfur.[22] In fact, exchange reactions at selenium have been reported to be orders of magnitude faster than analogous exchange reactions where sulfur serves as the electrophilic partner.[23,24] This feature was thought to ensure that our probe would not be consumed by other biothiols—a serious limitation of earlier probes—as their attack at selenium would simply regenerate a similar selenosulfide poised for nucleophilic attack by H_2S (**4**, Scheme 2).[14,18] We predicted that once intermediate **2** was formed, however, a rapid cyclization event would release the fluorescent reporter and thus confirm the presence of H_2S.

Based on this proposed mechanism, implementation of the *gem*-dimethyl group was predicted to serve several distinct functions. For one, it was expected to operate as a steric shield, blocking access to the ester carbonyl and shutting down hydrolysis and the direct nucleophilic attack of other biothiols and nucleophiles. In turn, this would reduce background fluorescence and increase the overall selectivity of the probe. Indeed, the introduction of *gem*-dimethyl groups is a common technique used in medicinal chemistry to improve plasma and chemical stability of biologically active compounds.[25] Secondly, while intermolecular attack at the ester would be diminished, we theorized that the requisite cyclization event was likely to be both thermodynamically and kinetically enhanced by the presence of those same methyl groups as introduction of alkyl substituents is known to favor intramolecular reactions (i.e. The Thorpe-Ingold Effect).[26]

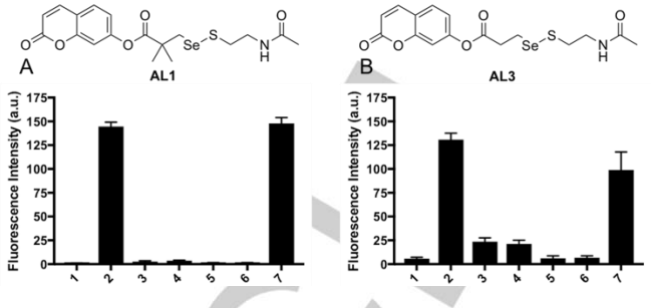

Figure 1. Fluorescence response (λ_{ex}: 330 nm) of probes (20 μM) in PBS buffer (pH 7.4) and in the presence of various biological thiols and nucleophiles during a 10 min incubation period: (1) PBS buffer alone; (2) 50 μM Na_2S; (3) 1 mM glutathione; (4) 100 μM cysteine; (5) 100 μM serine; (6) 100 μM lysine; (7) 1 mM glutathione + 50 μM Na_2S. Plotted as the mean +/− STD from three independent experiments. **(A)**: Probe **AL1** and **(B)**: Probe **AL3**.

To assess our hypothesis, the selectivity of our design towards H_2S was initially gauged by incubating **AL1** (20 μM) with various biological thiols and nucleophiles in PBS buffer (Figure 1A). After reacting for 10 min at room temperature, the resulting turn-on response was measured using fluorescence spectroscopy. The results from this assay were indeed stunning. In the presence of 50 μM Na_2S (an H_2S equivalent at physiological pH) a greater than 100-fold increase in relative fluorescence intensity was observed. Yet, in the presence of other biologically relevant thiols and nucleophiles, no increase in fluorescence was detected, even when significantly higher concentrations were used. Moreover, this assay confirmed that **AL1** is not consumed by the presence of other thiols and that its reactivity towards H_2S is retained in their presence (Figure 1A, Column 7). To confirm that the *gem*-dimethyl group affords enhanced differentiation, we also generated **AL3** which lacks the steric shield alpha to the ester carbonyl. The same selectivity studies with **AL3** unequivocally

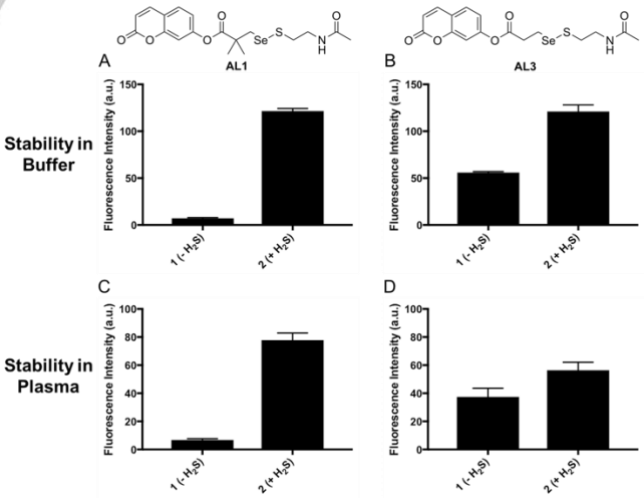

Figure 2. Fluorescence response (λ_{ex}: 330 nm) of probes (20 μM) under varying conditions: (**A** and **B**): (1) 24-hour incubation period in PBS buffer alone; (2) 24 hr incubation period in PBS buffer followed by the addition of Na_2S (50 μM). (**C** and **D**): (1): 10 min incubation period in fetal bovine plasma alone; (2): 10 min incubation period in fetal bovine plasma and in the presence of Na_2S (50 μM). Plotted as the mean +/− STD from three independent experiments. **AL1**: **2A** and **2C** and **AL3**: **2B** and **2D**.

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demonstrate the significance of the *gem*-dimethyl group as a noticeable reduction in selectivity was observed in their absence (Figure 1B).

To further establish the increased durability of **AL1** relative to **AL3**, we tested the stability of both compounds in PBS buffer over a 24 h incubation period (Figures 2A and 2B). While **AL3** was shown to be highly susceptible to hydrolysis in buffer alone (Figure 2B, Column 1), **AL1** exhibited superb stability during the 24 h period (Figure 2A, Column 1). Moreover, the subsequent addition of Na_2S to **AL1** resulted in impressive turn-on fluorescence (Figure 2A, Column 2). These results indicate that **AL1** is not only resistant to hydrolysis, but that its reactivity towards H_2S is preserved, even after prolonged exposure to buffer. The stability of **AL1** and **AL3** was also assessed in fetal bovine plasma (Figures 2C and 2D). Again, **AL1** displayed remarkable stability as little fluorescence was emitted in the absence of Na_2S (Figure 2C, Column 1). Furthermore, even within this complex environment, **AL1** showed a marked turn-on response when Na_2S was present (Figure 2C, column 2). Probe **AL3**, on the other hand, underwent significant decomposition in a very short period of time indicating that its ester moiety is significantly more labile, especially in the presence of plasma proteins (Figure 2D, Column 1).

The efficiency of **AL1** in sensing H_2S in PBS buffer (pH 7.4) and at room temperature was also assessed (Figure 3A). Under these conditions, the resulting fluorescence intensity reached a maximum in less than two minutes, highlighting that our designed probe is not only selective but also highly reactive towards H_2S—a necessary feature for detecting analytes which are short-lived. To determine if **AL1** could also be used to detect H_2S quantitatively in buffer, the resulting fluorescence was measured in response to a series of different concentrations of H_2S (Figure 3B). Indeed, the ensuing fluorescence intensity increased in a linear fashion in the range of 0–20 μM H_2S and with a detection limit of 0.05 μM.

Probe **AL1** was also shown to be useful in imaging H_2S in live cells using confocal microscopy. Cultured HeLa cells were incubated with **AL1** (12.5 μM) for 45 min and then washed to remove any excess probe. Under these conditions we did not observe any fluorescent cells, signifying that **AL1** was indeed stable within a cellular environment (Figure 4A). However, upon treatment with 50 μM Na_2S, a strong blue fluorescence within

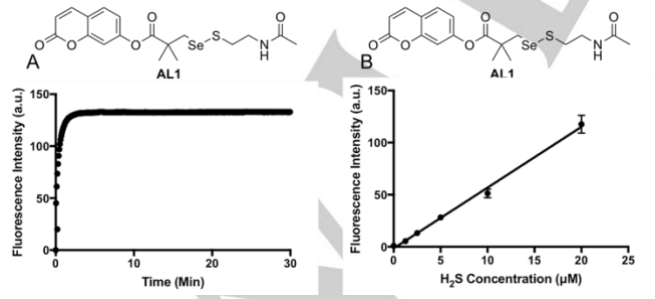

Figure 3. (A) Time-dependent fluorescence emission (λ_{ex}: 330 nm) of **AL1** (20 μM) in the presence of Na_2S (50 μM). **(B)** Fluorescence Intensity (λ_{ex}: 330 nm) of **AL1** (40 μM) in response to increasing Na_2S concentrations. Plotted as the mean +/− STD from three independent experiments.

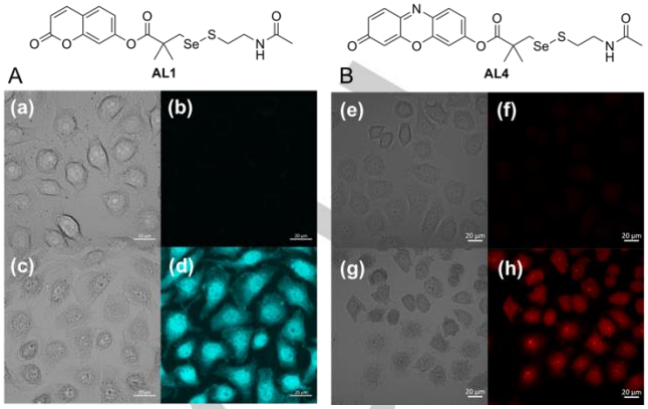

Figure 4. (A) Visualization of H_2S in live HeLa cells with **AL1** (12.5 μM). Imaging: ex: 405 nm, em: 452 nm. **a** and **b**: No Na_2S added (control); **c** and **d**: Addition of Na_2S (50 μM). Scale bar is set to 20 μm. **(B)** Visualization of H_2S in live HeLa cells with **AL4** (5 μM). Imaging: ex: 561 nm, em: 587 nm. **e** and **f**: No Na_2S added (control); **g** and **h**: Addition of Na_2S (25 μM). Scale bar is set to 20 μm.

cells was observed. To show that our framework was highly adaptable, we also generated **AL4.** After determining that **AL4** displayed similar selectivity and reactivity towards H_2S (Figures S1–S3), we also assessed its ability to visualize H_2S in live cells. Probe **AL4** utilizes resorufin as its latent fluorescent reporter which has the added benefit of longer excitation and emission wavelengths, further boosting its potential in tissue imaging applications. Under similar conditions, a strong fluorescence was again observed within HeLa cells upon treatment with **AL4** (5 μM) and Na_2S (25 μM) (Figure 4B).

We next set to out to showcase the high versatility of our design by demonstrating that we could target selenosulfide **1** to specific tissue or cell types by simply modifying its sulfide linker. As a proof of concept, we generated **AL2**, a cancer cell-specific

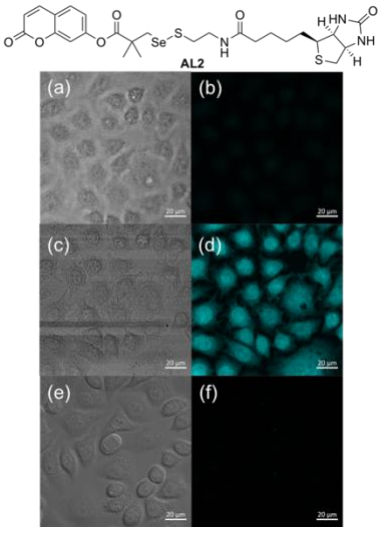

Figure 5. a and **b**: Fluorescence images of live HeLa cells treated with **AL2** (10 μM); **c** and **d**: Fluorescence images of live HeLa cells treated with **AL2** (10 μM) and Na_2S (50 μM); **e** and **f**: Fluorescence images of live HeLa cells pre-treated with biotin (2 mM) followed by treatments with **AL2** (10 μM) and Na_2S (50 μM). Imaging: ex: 405 nm, em: 452 nm. Scale bar is set to 20 μm.

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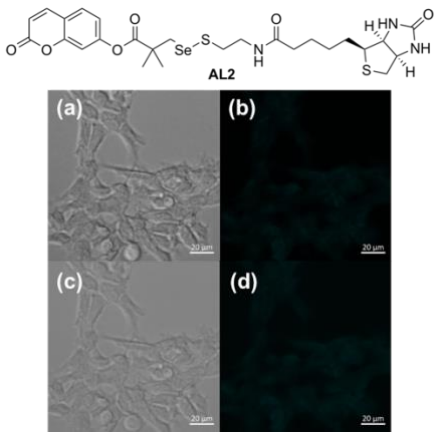

Figure 6. Fluorescence images of Live HEK293 cells. **a** and **b**: Treatment with **AL2** (10 μM) alone; **c** and **d**: Treatment with **AL2** (10 μM) followed by treatment with Na_2S (50 μM). Imaging: ex: 405 nm, em: 452 nm. Scale bar is set to 20 μm.

H_2S probe, by coupling biotin to its amine handle (Scheme 1).[27–29] Fluorescence imaging experiments were then conducted using both a biotin positive (HeLa) and biotin negative (HEK293) cell line to confirm its selective targeting and delivery.[27] As seen in Figure 5, when HeLa cells were treated with **AL2** alone, minimal fluorescence was witnessed within cells. However, upon the addition of Na_2S, a strong blue fluorescent signal was emitted from within, confirming both the intracellular delivery of **AL2** and its high selectivity towards H_2S. To confirm that **AL2** was targeting biotin cell surface receptors, in a separate experiment, HeLa cells were pre-treated with biotin prior to the addition of **AL2**. Under these conditions, the subsequent addition of Na_2S did not produce any fluorescence within cells, denoting that the intracellular delivery of **AL2** was in fact mediated via biotin transporters (Figures 5e and 5f). The selectivity of **AL2** was further established using HEK293 (noncancerous human embryonic kidney) cells. Under identical conditions, negligible fluorescence was observed, even upon the addition of exogenous Na_2S, indicating that the cellular uptake of **AL2** within HEK293 cells (biotin negative) was much less efficient (Figure 6).

With many forms of cancer having increased expression of H_2S-producing enzymes, cancer-cell specific probes such as **AL2** may prove useful in elucidating the roles of H_2S in cancer initiation and progression.[30–35] Additionally, we note that this technology may also find utility as a diagnostic for certain cancers where both a specific cell surface receptor (e.g. biotin, folate, B12, etc.) and H_2S are highly over-expressed as the demonstrated tissue specificity of our H_2S-selective probe provides a convenient dual-biomarker for turn-on fluorescence.[36,37] Finally, given our proposed mechanism for H_2S detection (Scheme 2), and the fact that **3** is cleanly generated as a byproduct (see supporting information), scaffolds similar to **AL2** may be advantageous for the specific capture and removal of H_2S within cancerous cells and tissue. Moreover, with high expression levels of H_2S being linked to several diseases, its precise down-regulation may prove beneficial,[38] highlighting the potential for selenosulfides, and frameworks akin to **AL1** and **AL2**, for serving as useful theranostics.

In closing, we have successfully shown that selenosulfides tethered to *gem*-dimethyl esters are exceptional frameworks for H_2S probe development. In addition to their high reactivity and selectivity towards H_2S, their remarkable versatility allows for their physicochemical properties to be easily tuned through structural modifications of their sulfide handle. Given the distinct chemical differences of both sulfur and selenium, we foresee the use of selenosulfides as a new strategy for tuning the physicochemical and biological properties of molecular cargo.

Experimental Section

Supporting information and the ORCID identification number(s) for the authors of this article can be found under:

Keywords: H_2S • selenosulfides • chemical probes • selenium chemistry

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