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Letter

Esterase-Activated Hydrogen Sulfide Donors with Self-Reporting Fluorescence Properties and Highly Tunable Rates of Delivery

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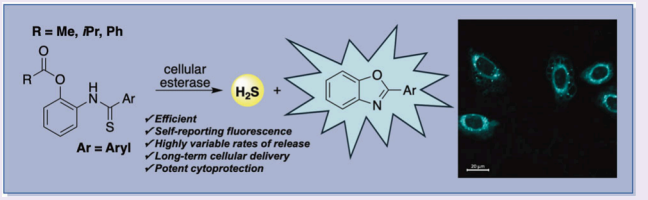
ABSTRACT: Hydrogen sulfide (H_2S) has emerged as a significant biomolecule with diverse activities, akin to other gaseous signaling molecules such as nitric oxide (NO) and carbon monoxide (CO). In the present study, we report on the development of esterase-activated donors that track their direct cellular donation of H_2S by enlisting a cyclization reaction onto a thioamide that forms a fluorogenic byproduct. This simple donor design provides a noninvasive method for monitoring the biological delivery and activity of H_2S, along with access to a library of compounds with highly variable rates of H_2S delivery. These studies culminated with the identification of a slow-release, yet highly efficient, donor (**ZL-DMA-Ph**) that was shown to self-report its gradual and continuous cellular donation of H_2S for up to 24 h which, in addition to better mimicking the natural biosynthesis of H_2S, provided impressive cytoprotection in a cellular cardiotoxicity model, even at submicromolar concentrations. In total, these findings indicate that the esterase-triggered fluorogenic donors identified in this study will offer new opportunities for exploring the chemical biology and therapeutic potential of exogenous H_2S supplementation.



Hydrogen sulfide (H_2S) is a small molecule that has garnered significant interest over the past several decades due to its immense biological activity.[1−4] Endogenously and enzymatically generated in mammals,[5−7] H_2S functions as a gaseous signaling molecule (i.e., gasotransmitter), similar to nitric oxide (NO) and carbon monoxide (CO), regulating key physiological and pathophysiological processes within the human body.[8−12] To date, H_2S has been implicated in vasodilation,[13−15] neurotransmission,[16−18] inflammation,[19−21] and cytoprotection.[22−24] Not surprisingly, diminished H_2S levels are linked to several illnesses, including neurodegenerative disorders,[25,26] diabetes,[27−29] inflammatory-related conditions,[30] and cardiovascular disease.[31−35] These strong associations underscore the significance and potential therapeutic utility of donor compounds, which can offset the downregulation of H_2S by delivering it in a controlled fashion that mimics its natural enzymatic production.

Among the previously reported H_2S donors, aryl thioamides are of particular interest due to their promising pharmacological effects,[36−39] especially in cardiovascular-related disease models. However, this important donor class has several drawbacks, including extremely low H_2S-releasing efficiencies and an undefined mechanism of release, making it difficult to attribute the activity of these compounds to their production of H_2S rather than the donor itself or unidentified byproducts.

Recently, our group was successful in implementing a general design strategy that augments both the efficiency and selectivity of H_2S release from thioamide-based donors by enlisting a cyclization reaction, initiated by a specific biological stimulus, to afford a heterocyclic byproduct (i.e., a key control compound) in conjunction with the release of H_2S (Scheme 1A).[40] In addition to providing a wide array of stimulus-responsive donors by simply changing the identity of the nucleophile and/or protecting group (Scheme 1B and 1C), we also recognized a unique opportunity with this template to generate donors that simultaneously supply compounds with useful biological and/or chemical properties alongside the liberated H_2S given the privileged nature of heterocyclic structures.[41−44]

As a proof of concept, we developed a reactive oxygen species (ROS)-activated donor (**QH642**) that self-reports its H_2S donation by forming a benzoxazole-based fluorophore (Scheme 1C).[45] The real-time tracking and monitoring of H_2S release from synthetic donors remains a challenge, especially in complex biological systems, which highlights the significance and benefits of donors, such as **QH642**, with a built-in, turn-on fluorescence response to H_2S delivery. Presently, several stimulus-responsive (i.e., ROS-activated,[45−48] thiol-activated,[49,50] and light-activated[51,52]) donors with self-reporting fluorescence properties have been reported and represent one of the greatest advancements in the field of H_2S chemical

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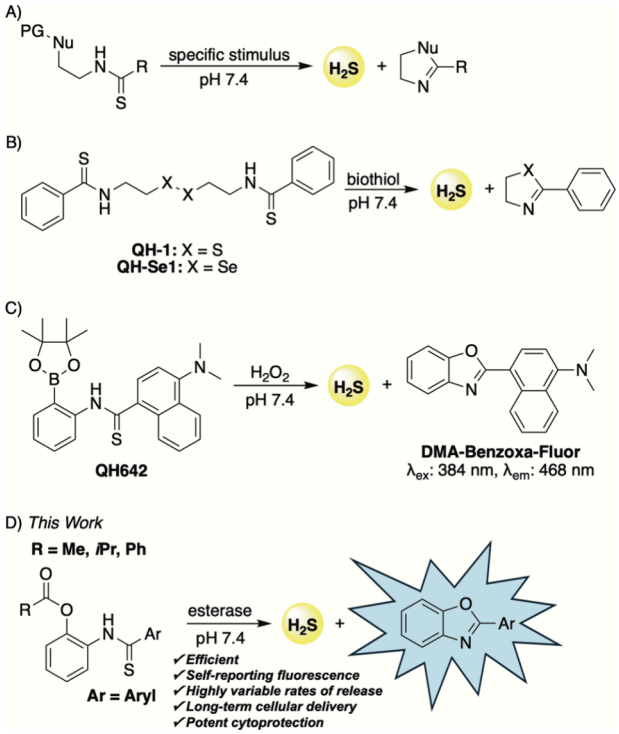
Scheme 1. Stimuli-Responsive H_2S Donation from Aryl Thioamides



[A]A general design strategy for increasing the efficiency and selectivity of H_2S release from thioamide-based donors. PG = protecting group and Nu = nucleophile. [B]Thiol-activated H_2S donors with high efficiency due to intramolecular thiol or selenol assistance.[40] [C]An ROS-activated donor that self-reports its H_2S release by forming a fluorescent benzoxazole byproduct.[45] [D]The first example of an esterase-activated donor with self-reporting fluorescence properties.

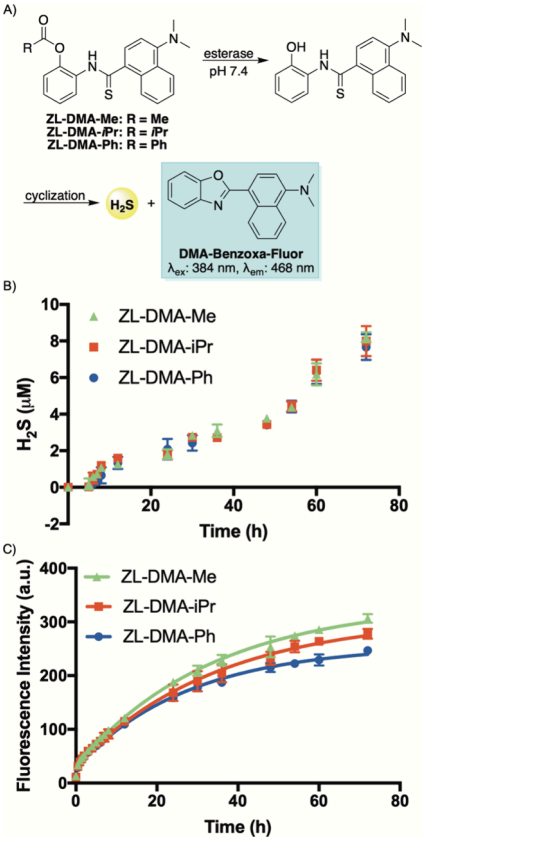


Figure 1. A) Proposed mechanism for H_2S release from donors **ZL-DMA-Me**, **ZL-DMA-*i*Pr**, and **ZL-DMA-Ph**. **B)** Time-course for H_2S release from **DMA** donors (100 μM) in PBS (pH 7.4, 37 °C) and in the presence of esterase (1 U/mL). Released H_2S was quantified spectrophotometrically by using a methylene blue assay (670 nm). Plotted as the mean ± STDEV from three independent experiments. **C)** Time-dependent fluorescence emission (λ_{ex}: 384 nm, λ_{em}: 468 nm) from **DMA** donors (20 μM) in PBS (pH 7.4, 37 °C) and in the presence of esterase (1 U/mL). Data were fit to obtain pseudo first-order rate constants (k_{obs}): **ZL-DMA-Me**: 0.00026 min^{-1}, **ZL-DMA-*i*Pr**: 0.00022 min^{-1}, **ZL-DMA-Ph**: 0.00018 min^{-1}). Plotted as the mean ± STDEV from three independent experiments.

biology, as donors with these characteristics have the potential to correlate H_2S concentration with biological activity.

Looking to expand upon this valuable donor class, we aimed to use our thioamide-based scaffold to introduce an enzyme-activated donor with self-reporting capabilities (Scheme 1D). Enzymes exhibit exquisite specificity and fast reaction kinetics. Moreover, given their catalytic nature, their use as a trigger can provide the prolonged liberation of H_2S to better compensate for situations in which its production is attenuated and can do so without the consumption of biologically important analytes, preventing further perturbation of cellular homeostasis.

To this end, esterases, which are ubiquitous enzymes that catalyze the hydrolysis of esters,[53] have been targeted in the past for the development of highly efficient H_2S donors with tunable kinetics.[54−57] However, to the best of our knowledge, these previous donors all lack the ability to self-monitor their direct release of H_2S.

Using our previous ROS-activated, self-reporting system as a model,[45] we reasoned that the implementation of an ester functionality would afford an esterase-sensitive system that, once activated, would furnish a nucleophilic phenol that would then cyclize onto the adjacent thioamide, forming **DMA-Benzoxa-Fluor** alongside its release of H_2S (Figure 1A).

Accordingly, we initiated our studies by synthesizing a small library of esters (methyl, isopropyl, and phenyl) from 2-aminophenol (see Supporting Information) to generate donors **ZL-DMA-Me**, **ZL-DMA-*i*Pr**, and **ZL-DMA-Ph** (Figure 1A). In our previous studies, we established the extraordinary hydrolytic stability of aryl thioamides.[40,45] Thus, we initially sought to evaluate the reactivity of these donors toward esterase (1 U/mL) in PBS (pH 7.4).

Using a methylene blue assay to quantify the amount of released sulfide at various time points,[58] we observed that 20 μM of donor only yielded negligible amounts of H_2S, even within a 24-h period. However, LCMS studies confirmed that the esters were, in fact, hydrolyzed quite rapidly, suggesting that the ensuing cyclization onto the electron-rich thioamide was sluggish. This is in agreement with our previous finding that Lewis-acid activation greatly accelerates the ring-closure of the same phenolic intermediate.[45]

Based on this result, we repeated this same experiment using 100 μM **DMA** donors. At this concentration, while in the presence of esterase (1 U/mL), H_2S release was established with approximately 8 μM detected at 72 h (Figure 1B). Using this gas trapping method (i.e., the methylene blue assay), which is not the most accurate way of measuring H_2S, especially after long incubation periods given its instability and transient nature, little distinction between methyl (**ZL-DMA-**

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Me), isopropyl (**ZL-DMA-*i*Pr**), and phenyl (**ZL-DMA-Ph**) esters were observed in terms of both rate and yield of H_2S.

Therefore, after confirmation that ester hydrolysis of **ZL-DMA-Ph** led to clean formation of **DMA-Benzoxa-Fluor** (see Supporting Information), we also monitored the reaction using fluorescence spectroscopy to gain more insight (λ_{ex}: 384 nm, λ_{ex}: 468 nm, Figure 1C). From a calibration curve (Figure S2), it was determined that **DMA-Benzoxa-Fluor** (and thus H_2S) was formed in a yield greater than 75% after a 3-day exposure of **DMA** donors (20 μM) to esterase (1 U/mL) in PBS (pH 7.4, 37 °C). Moreover, this method also inferred slight differences in rates of release among methyl, isopropyl, and phenyl esters with **ZL-DMA-Me** being the fastest and **ZL-DMA-Ph** being the slowest, which aligns well with suspected ester stability based on both electronic and steric effects.

To further evaluate ester stability, hydrolysis selectivity studies were conducted with **ZL-DMA-Ph** in PBS (pH 7.4, 37 °C) and in the presence of glutathione, cysteine, serine, and lysine (Figure S4). While small amounts of fluorescence were observed after 20 h due to hydrolysis and the formation of **DMA-Benzoxa-Fluor**, it paled in comparison to the fluorescence obtained when esterase (1 U/mL) was introduced, confirming that hydrolysis and the ensuing release of H_2S are greatly facilitated by the enzyme.

While structural modifications of the ester appeared to have a subtle effect on the rate of H_2S delivery, we speculated that alterations of the aryl thioamide itself would have a much greater impact. With the goal of keeping things simple while still forming a functional fluorescent byproduct that coincides with H_2S donation, we elected to exchange the electron-rich *N*,*N*-dimethylnaphthylamine from the **DMA** donor series for an electron-deficient isoquinoline moiety (**ISO** donors) with the expectation of augmenting H_2S liberation (Figure 2A).

With a new series of donors in hand (**ZL-ISO-Me**, **ZL-ISO-*i*Pr**, and **ZL-ISO-Ph**), H_2S release was again assessed using the spectrophotometric methylene blue assay. In the presence of esterase (1 U/mL), sulfide donation from the **ISO** series of donors (20 μM) was clearly visible in just a few hours, indicating a substantial boost in activity that greatly exceeded our expectations (Figure 2B).

To further substantiate this reactivity enhancement, the reaction between **ISO** H_2S donors and esterase was also examined by using fluorescence spectroscopy. First, we confirmed the hydrolytic stability and selectivity of phenyl ester **ZL-ISO-Ph** (Figure S4) and that its hydrolysis cleanly produced **ISO-Benzoxa-Fluor** (see Supporting Information). Then, after establishing its photophysical properties (λ_{ex}: 340 nm, λ_{em}: 400 nm), the reaction between the donor (20 μM) and esterase (1 U/mL) in PBS (pH 7.4, 37 °C) was monitored by recording the resultant fluorescence intensity at the λ_{max} of **ISO-Benzoxa-Fluor** (Figure 2C). In stark contrast to the **DMA** donor series, the reaction between **ISO** H_2S donors (20 μM) and esterase (1 U/mL) appeared to plateau in just a few hours, supplying **ISO-Benzoxa-Fluor** (and thus H_2S) in a yield greater than 80% (Figure S3) and at an observed first-order rate nearly 2 orders of magnitude faster. Like the **DMA** donor group, more subtle variations in reactivity were observed when comparing methyl (**ZL-ISO-Me**), isopropyl (**ZL-ISO-*i*Pr**), and phenyl (**ZL-ISO-Ph**) esters within the same **ISO** series.

Given that our two experimental methods (i.e., methylene blue and fluorescence spectroscopy) for tracking **ISO** donor progress could be used under identical conditions (i.e., donor and enzyme concentration), we anticipated similar donor

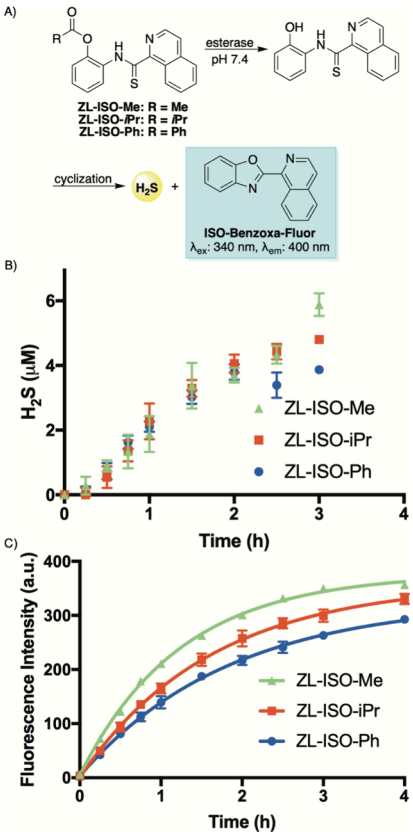

Figure 2. A) Proposed mechanism for H_2S release from donors **ZL-ISO-Me**, **ZL-ISO-*i*Pr**, and **ZL-ISO-Ph**. **B)** Time-course for H_2S release from **ISO** donors (20 μM) in PBS (pH 7.4, 37 °C) and in the presence of esterase (1 U/mL). Released H_2S was quantified spectrophotometrically using a methylene blue assay (670 nm). Plotted as the mean ± STDEV from three independent experiments. **C)** Time-dependent fluorescence emission (λ_{ex}: 340 nm, λ_{em}: 400 nm) from **ISO** donors (20 μM) in PBS (pH 7.4, 37 °C) and in the presence of esterase (1 U/mL). Data was fit to obtain pseudo first-order rate constants (k_{obs}): **ZL-ISO-Me**: 0.013 min^{-1}, **ZL-ISO-*i*Pr**: 0.010 min^{-1}, **ZL-ISO-Ph**: 0.009 min^{-1}). Plotted as the mean ± STDEV from three independent experiments.

peaking times being achieved with both methods given the expected correlation between the fluorescence and H_2S concentration. To evaluate this further, we elected to react our most efficient donor (**ZL-ISO-Me**) with esterase for an extended 8 h period using both the methylene blue assay and fluorescence spectroscopy to monitor the reaction progress. As can been seen in Figure S8, when both sets of data are plotted on the same *x*-axis, peaking times from both experiments are in unison, confirming that there is a strong association between fluorescence and H_2S concentration, as predicted.

To summarize the results of our small structure−activity relationship (SAR) study, it appears that H_2S release rates can be modified from this framework in two distinct ways, producing vastly different results. If small changes in H_2S release rates are desired, it can be achieved by altering the structure of the ester, with bulkier groups affecting the rate of hydrolysis, producing a slight deceleration in H_2S donation. Conversely, if a more marked adjustment is required, this can be realized by tailoring the electronics of the thioamide with more electron-rich thioamides, releasing H_2S much more slowly by impeding the requisite cyclization.

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We next sought to assess whether similar differences in activity were observed in the cellular milieu. Having already established that **DMA-Benzoxa-Fluor** could be imaged and used to track H_2S delivery in live cells,[45] we elected to first examine our **ISO** donor series and, in particular, **ZL-ISO-Ph** given the heightened control over its H_2S release (Figure 3).

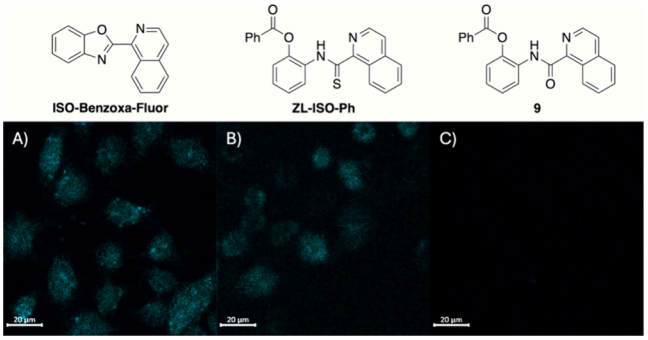

Figure 3. Live HeLa cells stained with **A) ISO-Benzoxa-Fluor** (500 μM), **B) ZL-ISO-Ph** (500 μM), or **C) 9** (500 μM) for 1 h prior to imaging. Scale bar was set to 20 μm.

Cultured HeLa (human cervical cancer) cells were treated with 500 μM **ISO-Benzoxa-Fluor**, **ZL-ISO-Ph**, or **9** (amide control). After incubating for 1 h, cells were washed and then imaged using a confocal microscope. As highlighted in Figure 3, cells exposed to **ISO-Benzoxa-Fluor** (Figure 3A) exhibited a blue fluorescence, confirming its photophysical properties (λ_{ex}: 340 nm, λ_{em}: 400 nm), while restricted, were still suitable for cellular imaging experiments at higher concentrations. Likewise, cells treated with **ZL-ISO-Ph** displayed a blue hue (Figure 3B), indicating the release of H_2S through the formation of **ISO-Benzoxa-Fluor**. Compound **9**, however, which lacks the ability to cyclize upon hydrolysis,[45] did not elicit a discernible fluorescent signal (Figure 3C).

To further validate the ability of **ZL-ISO-Ph** to modulate cellular H_2S levels, confocal imaging experiments were repeated with the addition of **SS2**, a previously reported sensor with high selectivity and sensitivity toward H_2S.[59,60] First, we confirmed that **SS2** could be used to monitor esterase-triggered H_2S donation from **ZL-ISO-Ph** in PBS as it was found to report a reactivity and selectivity profile similar to that of our previous findings (Figures S5 and S6). Cells were then treated with **SS2** alone (10 μM), which resulted in weak green fluorescence, presumably due to low levels of endogenous H_2S (Figure 4A). However, with the addition of just 40 μM **ZL-ISO-Ph**, a noticeable enhancement in green fluorescence was observed (Figure 4B), confirming elevated levels of cellular H_2S, imparted by the donor.

Having established the capacity for **ZL-ISO-Ph** to deliver H_2S to live human cells, we sought to further exploit its self-reporting fluorescence properties by tracking its reactivity and cellular H_2S delivery in real-time (Figure 5A). To accomplish this, cultured HeLa cells were treated with **ZL-ISO-Ph** and imaged at different time points (Figure 5B). The resulting cellular fluorescence intensity at each time point was then quantified and plotted for comparison. As highlighted in Figures 5B and 5C, time-dependent fluorescence was observed with a greater than 50% increase in relative fluorescence intensity occurring between the first 10 min time point and the final 6 h time point. Moreover, the fluorescence intensity, while gradually increasing over the first several time points, appeared

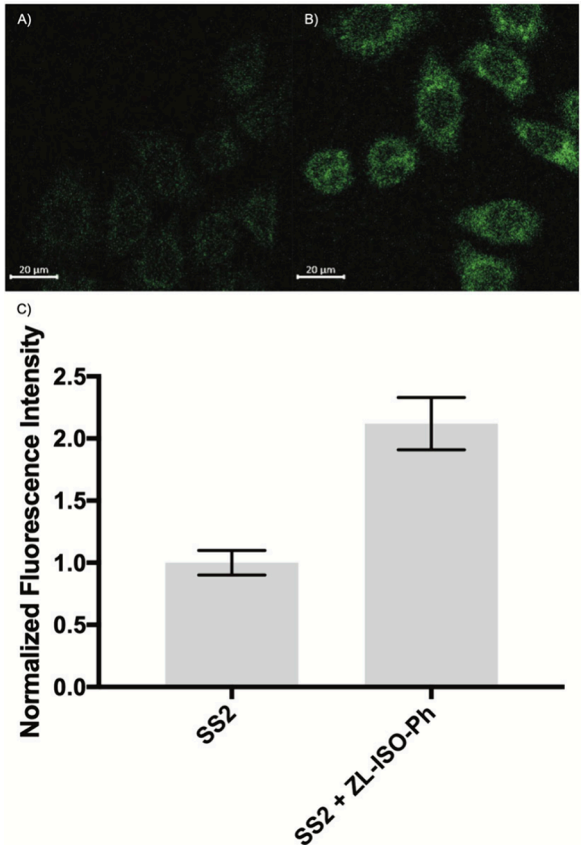

Figure 4. Visualization of H_2S release from **ZL-ISO-Ph** in live HeLa cells in a culture. Cells were treated with either **A) SS2** (10 μM) alone or **B) SS2** (10 μM) and **ZL-ISO-Ph** (40 μM). Scale bar was set to 20 μm. **C)** Quantified fluorescence intensity of the corresponding cellular images. Fluorescence was normalized and plotted as the mean ± STDEV from three independent imaging experiments.

to reach a maximum after just a few hours, which is on par with the reaction kinetics from our earlier fluorimeter studies in buffer.

Intrigued by these results, we also assessed the ability of **ZL-DMA-Ph** to track its own real-time cellular H_2S delivery by monitoring its formation of **DMA-Benzoxa-Fluor** (Figure 6A). Given the diminished reactivity of **DMA** donors in buffer, we surmised that the slow and sustained cellular delivery of H_2S throughout a 24 h period might be achieved with **ZL-DMA-Ph** and confirmed from this simple confocal imaging experiment.

To test our hypothesis, HeLa cells were treated with **ZL-DMA-Ph** and confocal images were obtained at various time points, starting from 10 min and up to 24 h after the administration of donor (Figure 6B). The relative cellular fluorescence intensity at each time point was then quantified and plotted for comparison (Figure 6C). As expected, significant time-dependent fluorescence was again observed. However, distinct from **ZL-ISO-Ph**, which appeared to plateau in terms of its H_2S release after only a couple of hours (Figure 5), the relative fluorescence intensity appeared to rise significantly between 9 and 24 h after the administration of **ZL-DMA-Ph**, signifying a prolonged cellular delivery of H_2S.

In total, the results of these experiments not only confirm esterase-triggered H_2S release from this novel donor platform, but that the rates of release can vary significantly through simple structural changes to the aryl thioamide, giving rise to donors that self-report their continuous cellular delivery of H_2S

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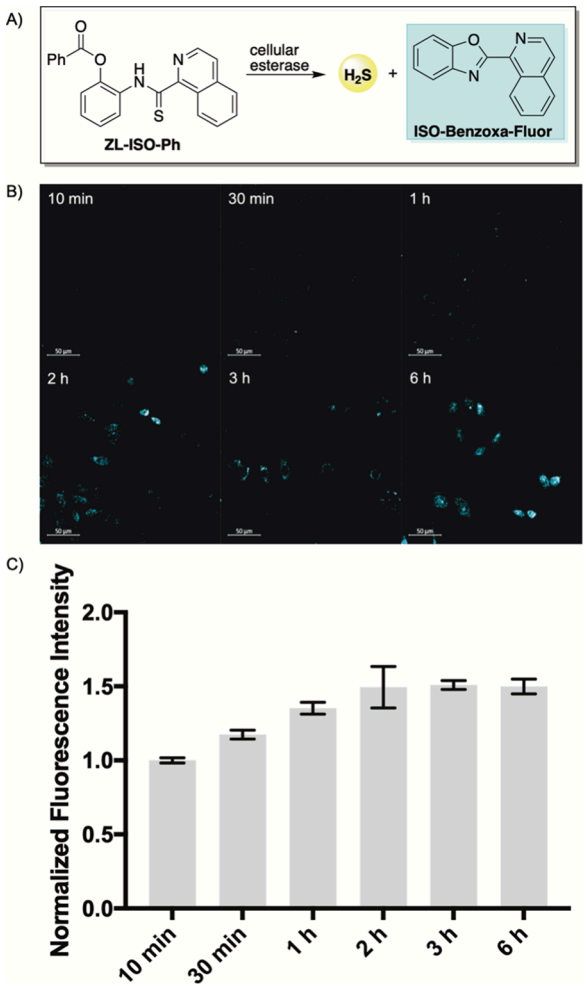

Figure 5. **A**) Time-dependent cellular fluorescence studies with **ZL-ISO-Ph** (500 μM) in live HeLa cells. **B**) Resulting confocal images after incubation periods of 10 min, 30 min, 1, 2, 3, and 6 h. Scale bar was set to 50 μm. **C**) Quantified fluorescence intensity of corresponding cellular images. Fluorescence was normalized and plotted as the mean ± STDEV from three independent imaging experiments.

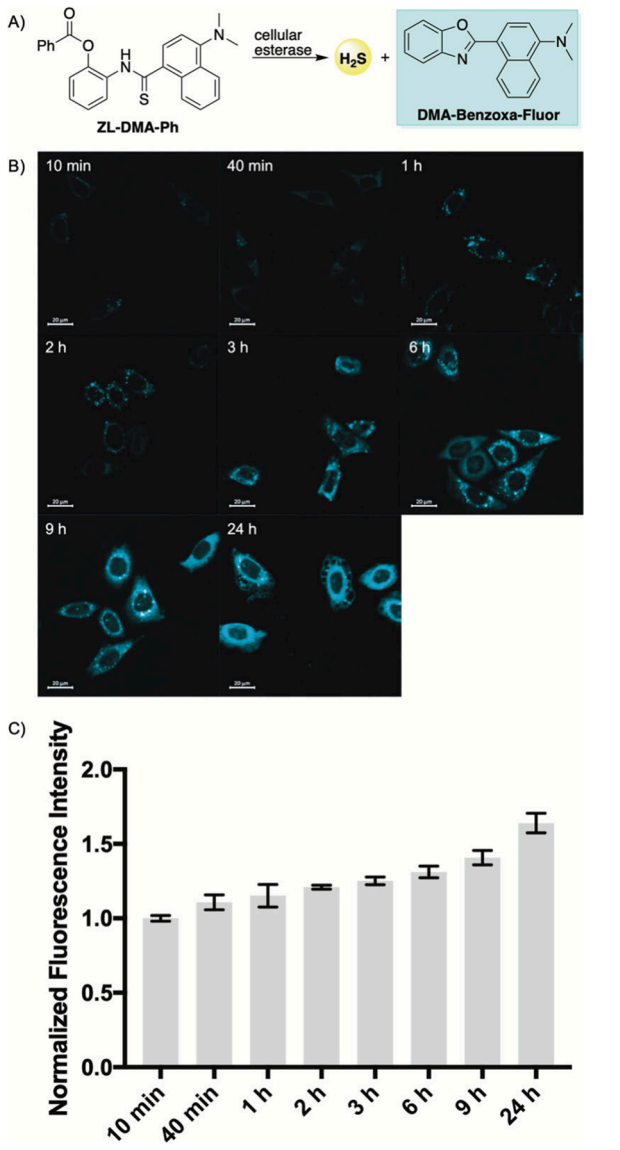

Figure 6. **A**) Time-dependent cellular fluorescence studies with **ZL-DMA-Ph** (40 μM) in live HeLa cells. **B**) Resulting confocal images after incubation periods of 10 min, 40 min, 1, 2, 3, 6, 9, and 24 h. Scale bar was set to 20 μm. **C**) Quantified fluorescence intensity of corresponding cellular images. Fluorescence was normalized and plotted as the mean ± STDEV from three independent imaging experiments.

throughout the course of an entire day, not only mimicking the endogenous production of H_2S, but doing so without the consumption of essential analytes both during the initiation and imaging process, which underscores their usefulness as a tool for exploring H_2S biology and medicine.

Ergo, we were also interested in providing some initial insight into the cytoprotective effects of these donors against H_2O_2-induced oxidative stress. Having previously established the low cytotoxicity of **DMA-Benzoxa-Fluor** in cultured H9c2 (rat cardiomyocyte) cells,[45] we elected to assess the antioxidative properties of **ZL-DMA-Ph** in this cell line. In our hands, a brief 1 h exposure of H9c2 cells to H_2O_2 (1 mM) reduced viability to approximately 60% (Figure 7). However, when cardiomyocytes were pretreated with small amounts of **ZL-DMA-Ph** to provide enriched levels of H_2S in a continuous and controlled manner for 48 h prior to the addition of peroxide, concentration-dependent cellular protection was observed (Figure 7). Moreover, significant cell rescue from H_2O_2-induced oxidative stress occurred at a donor concentration as low as 200 nM, which is on par with the cytoprotective effects of some previously reported esterase-sensitive donors.[57] This data also affirms that the antioxidative effects of H_2S are not due to its direct scavenging of H_2O_2, but rather its activation of antioxidative proteins through its involvement in cellular signaling.[61−64] To further validate that cellular protection imparted by **ZL-DMA-Ph** was due to its release of H_2S, additional control compounds were tested under identical experimental conditions with neither amide (**5**) nor the cyclized byproduct (**DMA-Benzoxa-Fluor**) providing any defense against H_2O_2-induced oxidative damage (Figure 7).

In summary, we successfully prepared a library of esterase-activated donors that are the first to track and self-report their direct donation of H_2S by forming a fluorogenic byproduct. From a small SAR study, two distinct self-reporting donor systems with highly variable rates of H_2S delivery were uncovered, underscoring the flexibility and ease with which

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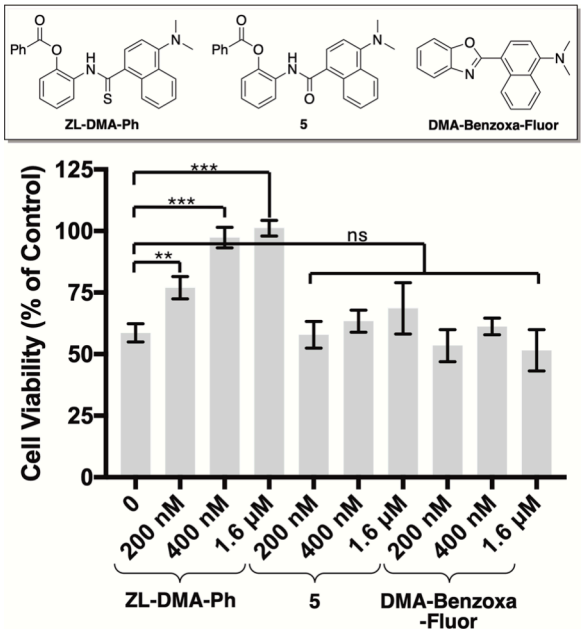

Figure 7. Viability of H9c2 cells upon pretreatment with DMSO (delivery vehicle), **ZL-DMA-Ph**, **5**, or **DMA-Benzoxa-Fluor** for 48 h prior to exposure to H_2O_2 (1 mM) for 1 h. Results are expressed as the mean ± STDEV from three independent experiments. Two-tailed unpaired *t* test: ***$p < 0.0005$, **$p < 0.005$, ns = not significant ($p > 0.05$).

H_2S release can be regulated from this scaffold. These studies led to the identification of a particular compound, **ZL-DMA-Ph**, that was shown to self-report its gradual and continuous cellular donation for up to 24 h, which better mimics the natural biosynthesis of H_2S and confers impressive therapeutic benefits, even at submicromolar concentrations. In total, these findings indicate that the donors identified in this study will offer new opportunities for exploring H_2S biology and medicine.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.4c00396.

Reagents, chemical synthesis, ^{1}H NMR data, absorbance and emission spectra, fluorescence assays, and additional supplementary data (PDF)

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Notes

The authors declare no competing financial interest.

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