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Self-Immolative Prodrugs: Effective Tools for the Controlled Release of Sulfur Signaling Species

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Abstract

H₂S, persulfides (R-SSH), and related sulfur species have recently received attention due to the pronounced physiological effects they elicit. Delivering sulfur signaling molecules in a controlled manner presents many challenges, as many available donors have short half-lives, lack water solubility, and exhibit poor trigger specificity. Self-immolative prodrugs provide a unique ability to impart stability to H₂S precursors and persulfides while allowing for trigger specificity by tuning the functional group installed on the self-immolative linker. This strategy has proven successful in delivering sulfur signaling species under specific conditions and may lead to the further elucidation of persulfide interactions within biological systems, affording effective therapeutics.

Graphical Abstract

Keywords

self-immolation; gasotransmitter; perthiol; redox; reactive oxygen species

Introduction

In the late 1990s and early 2000s hydrogen sulfide (H₂S) was discovered as a key signaling molecule in mammals.^{1–2} Combined with its endogenous production and membrane permeability, the signaling role of H₂S placed this gas in a family of compounds known as

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“gasotransmitters,” along with carbon monoxide (CO) and nitric oxide (NO).³ The recognition of H₂S as a gasotransmitter has led to a flurry of research in the redox and signaling biology communities as the signaling pathways in which H₂S is involved continues to be investigated.^{4–5} Additionally, reductions in endogenous H₂S production are associated with a variety of disease states, including cardiovascular disease,⁶ cancer,^{7–8} inflammation,⁹ and colonic diseases,¹⁰ among others. Many of the aforementioned studies performed to elucidate the physiological role of H₂S were carried out using inhalation of H₂S gas or with “donor salts” such as sodium hydrosulfide (NaSH) and sodium sulfide (Na₂S). While useful in many early studies on H₂S biology, these H₂S sources present delivery challenges due to a lack of methods for their targeted delivery and the short half-life of the gas in vivo.

To aid in our understanding of H₂S physiology, chemists have synthesized stimuli-responsive H₂S prodrugs (referred to as H₂S donors) with specific triggers and extended, tunable release profiles.¹¹ Although not an exhaustive list, nucleophiles,^{12–14} enzymes,¹⁵ and light,^{16–17} have been utilized to trigger H₂S release from small molecule and/or polymeric donor platforms.^{18–21} These H₂S donors may enable the study of the physiological roles of H₂S better than donor salts due to their extended release profiles, which more accurately mimic the measured and steady production of endogenous H₂S. Two critical components to consider when analyzing the utility of H₂S donors are the triggering mechanism(s) and the byproducts released. Triggering mechanisms dictate the kinetics of H₂S release and influence targeting capabilities depending on local concentrations of the trigger. Byproducts of H₂S release are also a key consideration as they may have independent biological activity that could complicate biological studies. Synthetic H₂S donors have also gained attention as potential therapeutics in conditions where exogenous H₂S delivery may be beneficial.²² A select few H₂S donors are in clinical trials for a variety of indications.²³

While there exist several scaffolds from which H₂S donors have been synthesized, one of the most widely used prodrug motifs for delivery of many types of compounds is the self-immolative linker. The self-immolative prodrug platform is based on the 1,4- or 1,6-benzyl elimination reaction, first introduced by Katzenellenbogen et al 1981.²⁴ The general structure of these self-immolative prodrugs involves a trigger unit or an “input” functionality, the self-immolative benzyl spacer, and an “output” compound on the other end of the self-immolative spacer. In the presence of a specific stimulus, the triggering moiety is cleaved to reveal an unstable intermediate that then self-immolates to release the desired output compound. This general prodrug strategy has been employed in signal amplification applications as well as in drug delivery systems for a myriad of drugs from anticancer agents to anesthetics.²⁵ Furthermore, this self-immolative design has been utilized in a variety of polymeric systems to trigger depolymerization under distinct conditions.²⁶ These self-immolative polymers find applications as drug delivery platforms, sensory materials, self-healing materials, and patterned plastics.^{27–30}

In the context of H₂S donors, the self-immolative motif has been implemented by Pluth and Chakrapani in a class of dual carbonyl sulfide (COS) / H₂S donors based on a benzyl thiocarbamate structure (R–OC(S)NH–R), termed “caged thiocarbamates” (Scheme 1). The

first description of caged thiocarbamates in COS/H₂S delivery was for the development of H₂S analyte replacement probes based on the reduction of aryl azides to the corresponding aniline by H₂S.³¹ Upon reduction of the azide, a self-immolation reaction occurs, resulting in the release of COS, which is rapidly converted into H₂S in the presence of the ubiquitous enzyme carbonic anhydrase (CA), along with the release of a fluorescent output. This system was capable of detecting H₂S in the micromolar concentration range in biologically relevant settings. Since this initial analyte replacement probe based on the thiocarbamate COS/H₂S donor motif, an expanded array of triggers has become available. These include self-immolative donors responsive to hydrogen peroxide,³² tetrazines,³³ light,³⁴ and esterases.³⁵ In addition to demonstrating the variety of targeting capabilities that may be installed into a self-immolative system, Pluth and coworkers have employed esterase-responsive caged thiocarbamates to demonstrate that COS may have physiological roles that differ from H₂S.³⁶ In this work, COS reduced cellular respiration and ATP synthesis in BEAS 2B cells, which was attributed to COS inhibition of the enzyme cytochrome c oxidase, revealing a complex interplay between the beneficial roles that H₂S plays and a more harmful role that its precursor COS may enact under certain conditions.

As our understanding of H₂S physiology expands, an appreciation has grown for other endogenously produced sulfur-containing small molecules, particularly those containing one or more sulfur-sulfur bonds where at least one sulfur atom has an oxidation state of 0 (sulfane sulfur).³⁷ These compounds generally have structures with two or more sulfur atoms bound in a chain with organic substituents on one or both ends of the polysulfide moiety (i.e., R-(S)_n-X, where X = H, or carbon-containing functionality and n ≥ 2). Persulfides (R-SSH) are an especially interesting subset of sulfane sulfur species as they are presumed intermediates in H₂S signaling and are produced endogenously by the same enzymes responsible for H₂S production.³⁸ Calculations have shown that persulfides have lower pK_a's and greater reducing potential than the corresponding thiols and H₂S, indicating a potential role in maintaining redox balance in the cytoplasm. Additionally, small molecule persulfides are capable of persulfidating enzymes under conditions where H₂S cannot, further implicating the role persulfides may play as antioxidants. Early research into the role of these small molecule persulfides has been hampered by a lack of donor molecules, as the few persulfide donors that have been reported suffer from limited benchtop stability, poorly understood release mechanisms, and/or ill-defined persulfide species. The aforementioned problems of persulfide stability make synthesizing and directly delivering free persulfides challenging. Therefore, a prodrug approach must be used to protect the persulfide under conditions that would normally degrade it, yet allow for the degradation of the protecting group under desired conditions to release the persulfide payload.

Currently Available Persulfide Donors

The lack of generally stable, water-soluble, and stimuli-specific persulfide donors has limited the study of this important transiently stable species. Currently there are only a few examples of persulfide donors. The first discrete persulfide donor was developed by Galardon and coworkers in the form of a persulfide derivative of S-nitrosoacetyl penicillamine (SNAP, Scheme 2a).³⁹ This SNAP derivative utilizes an S-to-N methoxycarbonyl transfer mechanism to uncage the persulfide on the order of 2–15 minutes

at 25 °C, with more basic environments providing faster persulfide release. The initial report of this prodrug inspired us and others to design novel, varied persulfide donors for specific applications.

Following Galardon's report, Wang and coworkers developed an esterase-sensitive persulfide donor that utilizes an O-to-S relay deprotection mechanism as a means of releasing its persulfide payload (Scheme 2b).⁴⁰ In this work, several ester-methyldisulfide derivatives were synthesized, and strong protective effects from these donors were observed in ischemia/reperfusion (I/R) injury in murine models. Wang then extended this design, developing a prodrug system that releases hydrogen persulfide (H₂S₂).⁴¹ This prodrug utilizes a well-known trimethyl lock template,⁴² exploiting steric hindrances in three precisely placed methyl groups to drive the release of H₂S₂ in response to esterase (e.g., porcine liver esterase, PLE) or alkaline phosphatase (ALP) enzymes. Researchers measured H₂S₂ release using the persulfide-responsive fluorescent probe DSP-3⁴³ and found that peak concentrations of H₂S₂ reached 10–15 μM within 15 min of enzyme addition to 100 μM prodrug.

Finally, Xian and coworkers recently reported persulfide prodrugs that utilized the same O-to-S relay deprotection mechanism as a means of releasing persulfides (Scheme 2c).⁴⁴ The structure of the prodrug is similar to Wang's persulfide donors, but utilizing silyl protecting groups at the 1 position instead of esters. This system is quite modular, enabling the release of nitrosothiol (HSNO) and persulfides in response to specific triggers that remove silyl protecting groups (e.g., dilute acid and F⁻). The reactions used to synthesize these prodrugs were high-yielding and modular, and their clever use of common protecting groups may also lead to bio-orthogonal triggers to release persulfides.

BDP NAC: An ROS-Responsive Persulfide Prodrug

Inspired by these previous designs, we set out to synthesize a modular persulfide prodrug, allowing for the tuning of both the input stimulus and output persulfide. Given the nature of persulfides as strong antioxidants, we aimed first to develop a persulfide donor scaffold that releases persulfides in the presence of reactive oxygen species (ROS).⁴⁵ Aryl boronic esters are cleaved selectively in the presence of ROS⁴⁶, releasing generally biocompatible byproducts, making this an ideal protecting group for ROS-triggered release. The prodrug, termed BDP-NAC for Bpin-disulfide prodrug-N-acetyl cysteine, contains an aryl boronic acid pinacol ester (Bpin) functional group *para* to a benzyl *N*-acetylcysteine disulfide. We envisioned that cleavage of the Bpin moiety by ROS to reveal the corresponding phenolate would result in a 1,6-elimination (i.e., self-immolation) in water to release *p*-hydroxybenzyl alcohol and the corresponding NAC persulfide species (Scheme 1d).

The synthesis of BDP-NAC was accomplished in 5 steps with an overall yield of 30% using an efficient synthetic route (Scheme 3). Firstly, *p*-tolyl boronic acid was protected with pinacol in the presence of MgSO₄. Next, the resulting tolyl-Bpin species (1) was subjected to radical bromination with NBS. The resulting benzyl bromide (2) was treated with thiourea to form the thiuronium salt, which was subsequently hydrolyzed under basic conditions in one pot to yield the corresponding Bpin-thiol (3). Finally, a disulfide exchange reaction was

carried out with the thiol and activated NAC disulfide (NAC-pyDS) to yield the prodrug BDP-NAC.

To determine whether the release of NAC persulfide proceeded under the conditions that were hypothesized, decomposition of BDP-NAC triggered by H₂O₂ was evaluated by LCMS. H₂O₂ was added to a solution of BDP-NAC in H₂O/ACN (9:1 v/v), and aliquots were removed at different reaction times and injected into the LCMS. As shown in Figure 1, BDP-NAC eluted at 2.9 min with no secondary peaks observed before addition of peroxide. Five min after the addition of H₂O₂, a second peak of comparable intensity was seen, and high resolution mass spectral analysis confirmed that this peak corresponded to *N*-acetylcysteine persulfide (NAC-SSH). Two h after H₂O₂ addition, the peak corresponding to NAC-SSH was the predominate peak observed, with minimal BDP-NAC remaining.

Additionally, BDP-NAC was analyzed by ¹H NMR spectroscopy to further elucidate persulfide release kinetics as well as confirm the formation of *p*-hydroxybenzyl alcohol. An initial ¹H NMR spectrum was taken of BDP-NAC in DMSO-d₆/D₂O (9:1 v/v) to mark a t₀ timepoint. H₂O₂ (10-fold excess) was added to the reaction mixture, and reaction progress was monitored by taking ¹H NMR spectra at several timepoints after H₂O₂ addition. As shown in Figure 3, there were only two peaks observed in the aromatic region of the ¹H NMR spectrum (a and a') before the addition of H₂O₂. Upon addition of peroxide, a second set of aromatic peaks (c and c') was immediately observed that was consistent with a sample of pure *p*-hydroxybenzyl alcohol. Over the course of the reaction, a third set of aromatic peaks was observed (b and b'), and these were attributed to the hydrolysis product of BDP-NAC. The half-life of BDP-NAC under these conditions was calculated to be 7.5 ± 0.3 h calculated by tracking the disappearance of the BDP-NAC aryl peaks. The discrepancy between this half-life and the more rapid reaction rate observed from LCMS is commensurate with previous literature reports for self-immolative systems, in which greater degrees of organic solvent content causes a reduction in reaction rate.⁴⁷

To probe the reactivity of the Bpin moiety to various oxidants and nucleophiles, a derivative of BDP-NAC was synthesized with a coumarin-based fluorophore installed in place of the distal thiol species. Drawing inspiration from Xian and coworkers,⁴⁸ this pro-fluorophore was designed to release 7-hydroxycoumarin only under conditions that would first cleave the Bpin moiety, allowing for self-immolation to occur. The pro-fluorophore, termed BDP-Fluor, exhibited a greater than 90-fold response to H₂O₂, while other oxidants and nucleophiles exhibited much lower fluorescence turn-on under the same conditions. These findings further confirmed that BDP-NAC responded specifically to H₂O₂ and not to other oxidants or nucleophiles.

We next directed our efforts to analyze BDP-NAC in a biological setting. BDP-NAC exhibited no cytotoxicity towards H9C2 cardiomyocytes at concentrations exceeding 200 μM. As mentioned, persulfides possess greater reduction potentials than their corresponding thiols and H₂S; thus, we proposed that BDP-NAC could be effective in rescuing cells from the deleterious effects of oxidative stress, either by direct reduction of H₂O₂ or by regulation of antioxidative pathways within cells. For this purpose, we examined the cytoprotective effects of BDP-NAC on H9C2 cells by exogenously delivering H₂O₂, which promotes

apoptosis through oxidative stress pathways. In the presence of only H₂O₂ (100 μM), cell viability drastically decreased after exposure for 1 h (Figure 5). In contrast, synchronous exposure of BDP-NAC (100 – 200 μM) and H₂O₂ (100 μM) revealed a dose-dependent increase in viability, retaining 100% cell viability with the addition of 200 μM BDP-NAC.

To validate our initial conclusion that the persulfide component of BDP-NAC is responsible for rescuing cells under these conditions, several controls were examined for their efficacy in eliminating oxidative stress under these conditions. H9C2 exposure to H₂O₂ concurrently with Bpin-OH, a control compound containing a Bpin moiety, but lacking the distal persulfide, exhibited significantly less capacity to rescue cells from oxidative stress than BDP-NAC at an equal concentration. The controls Na₂S (an instantaneous H₂S donor), GYY4137 (a slow-releasing H₂S donor), NAC, and BDP-Control (a compound that contains all of BDP-NAC's functionalities except for the Bpin moiety) were all unsuccessful in rescuing cells under oxidative conditions. To probe the importance of persulfides in this system even further, groups of controls were used to mimic important functionalities of BDP-NAC without being covalently bound. Bpin-OH + NAC and Bpin-OH + GYY4137 were able to impart some protective effects in the presence of H₂O₂, but significantly less than BDP-NAC at the same concentration. Bpin-OH and Na₂S together mimicked the cytoprotective effects of BDP-NAC, but we attribute this protective effect to the much larger instantaneous concentrations of available antioxidants in this group of controls over 1 h reaction time as compared to BDP-NAC.

Conclusion

The self-immolative prodrug platform has been utilized in numerous prodrugs systems since its conception. The benchtop stability it imparts, coupled with the ease of functionalization to the self-immolative linker, have proven invaluable. In particular, the use of this platform in the field of persulfide donors has opened the doors to a new class of prodrugs capable of releasing virtually any persulfide with high trigger specificity. The field of synthetic persulfide donors is expanding at a rapid pace, and with careful design of these prodrugs, the biological mechanisms by which persulfides enact their physiological effects may be unraveled, also potentially yielding effective, real-world therapeutics.

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Biosketches

John B. Matson received his bachelor's degree in Chemistry from Washington University in St. Louis and his PhD in Chemistry from Caltech, working under the guidance of Professor Robert H. Grubbs. After a postdoctoral fellowship at Northwestern University with Professor Samuel I. Stupp, he began his independent career at Virginia Tech in 2012. His

research focuses on development of new materials for biology, medicine, and sustainability. In particular, he is interested in new methods and materials for delivery of signaling molecules, particularly sulfur-containing compounds. Kearsley M. Dillon (right) and Chad R. Powell (left) are graduate students in the Matson group working on delivery of sulfur-containing signaling molecules.

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Figure 1.
Illustration of payload release from a generalized self-immolative prodrug.

Figure 2.

A) Proposed reaction of BDP-NAC with H_2O_2 to release NAC-SSH. B) LC chromatograms demonstrating conversion of BDP-NAC into NAC-SSH. Powell, C. R.; Dillon, K. M.; Wang, Y.; Carrazzone, R. J.; Matson, J. B., A Persulfide Donor Responsive to Reactive Oxygen Species: Insights into Reactivity and Therapeutic Potential. *Angew. Chem. Int. Ed.* **2018**, 57 (21), 6324–6328. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Figure 3.

¹H NMR spectroscopic kinetics of persulfide release from BDP-NAC. The peaks a and a' correspond to aromatic peaks on BDP-NAC, whereas c and c' correspond to aromatic peaks of *p*-hydroxybenzyl alcohol. Peaks b and b' are attributed to aromatic peaks found in hydrolyzed BDP-NAC. The half-life of BDP-NAC under these conditions was calculated to be 7.5 ± 0.3 h based on the slope of the pseudo-first order kinetics plot calculated by integrating a and a' over time.

Figure 4.

A) Putative mechanism of fluorophore release from BDP-Fluor. B) Representative fluorescence spectra of BDP-Fluor subjected to 100-fold excess H_2O_2 . C) Image depicting 7-hydroxycoumarin release from a reaction mixture after 24 h (visualized under UV irradiation). D) Relative response of BDP-Fluor (3.3 μM) to each oxidant or nucleophile (330 μM) or control (no trigger added), shown as the ratio of fluorescence intensity (I_f) after 5 h to the initial response before addition of the potential trigger compound (I_0).

Figure 5.

Cell viability of H9C2 cardiomyocytes treated with H₂O₂ (100 μM) and BDP-NAC or other controls (100–200 μM for BDP-NAC, 200 μM for controls except Na₂S at 100 μM) for 1 h. Cell quantification was carried out with Cell Counting Kit-8 (CCK-8). Results are expressed as the mean ± SEM (n = 10–15 for each treatment group) with 2–3 independent experiments for each. *P < 0.01 for comparisons with H₂O₂ group and #P < 0.01 for comparison with the most concentrated (200 μM) BDP-NAC treatment group. Group comparisons were determined by ANOVA with a Student-Newman-Keuls comparisons post-hoc test. Powell, C. R.; Dillon, K. M.; Wang, Y.; Carrazzone, R. J.; Matson, J. B., A Persulfide Donor Responsive to Reactive Oxygen Species: Insights into Reactivity and Therapeutic Potential. *Angew. Chem. Int. Ed.* **2018**, 57(21), 6324–6328. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Scheme 1.

Generalized depiction of caged thiocarbamates with self-immolative linkers for release of COS/H₂S.

(a) Direct deprotection persulfide donor

(b) Esterase-triggered persulfide donor

(c) 1,2-O,S Relay persulfide donor

(d) 1,6-Elimination persulfide donor

Scheme 2.

Release mechanisms for currently available persulfide prodrugs.

Scheme 3.

Synthetic route to BDP-NAC. Powell, C. R.; Dillon, K. M.; Wang, Y.; Carrazzone, R. J.; Matson, J. B., A Persulfide Donor Responsive to Reactive Oxygen Species: Insights into Reactivity and Therapeutic Potential. *Angew. Chem. Int. Ed.* **2018**, 57(21), 6324–6328. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.