HHS Public Access

Author manuscript

Angew Chem Int Ed Engl. Author manuscript; available in PMC 2019 May 22.

Published in final edited form as:

Angew Chem Int Ed Engl. 2018 May 22; 57(21): 6324–6328. doi:10.1002/anie.201803087.

A Reactive Oxygen Species (ROS)-Responsive Persulfide Donor: Insights into Reactivity and Therapeutic Potential

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Abstract

Persulfides (R–SSH) have been hypothesized as critical components in sulfur-mediated redox cycles and as potential signaling compounds, similar to hydrogen sulfide (H_2S). Hindering the study of persulfides is a lack of persulfide donor compounds with selective triggers that release discrete persulfide species. Herein we report the synthesis and characterization of an ROS-responsive, self-immolative persulfide donor. The donor, termed **BDP-NAC,** showed selectivity towards H_2O_2 over other potential oxidative or nucleophilic triggers, resulting in the sustained release of the persulfide of *N*-acetyl cysteine (NAC) over the course of 2 h, as measured by LCMS. Exposure of H9C2 cardiomyocytes to H_2O_2 revealed that **BDP-NAC** mitigated the effects of a highly oxidative environment in a dose-dependent manner over relevant controls and to a greater degree than common H_2S donors sodium sulfide (Na_2S) and GYY4137. **BDP-NAC** also rescued cells more effectively than a non-persulfide releasing control compound with a Bpin moiety in concert with common H_2S donors and thiols.

Graphical Abstract

Persulfides (R–SSH) have been hypothesized as critical components in sulfur-mediated redox cycles and as potential signaling compounds, similar to hydrogen sulfide (H_2S). Hindering the study of persulfides is a lack of persulfide donor compounds with selective triggers that release discrete persulfide species. Herein we report the synthesis and characterization of a redox-responsive, self-immolative persulfide donor. The donor, termed **BDP-NAC**, shows selectivity towards H_2O_2.

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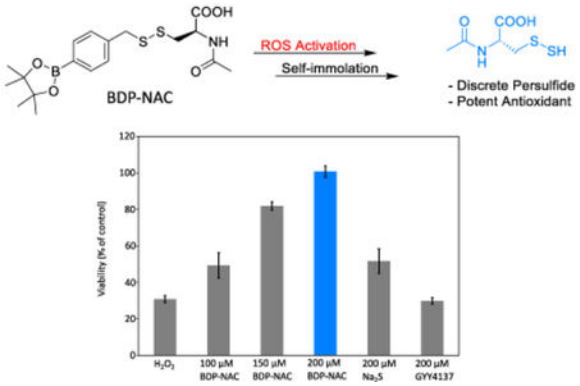

Keywords

Hydrogen sulfide; Gasotransmitter; Self-Immolative; Prodrug; Peroxide

Hydrogen sulfide (H_2S) plays a key signaling role in mammalian biology and has been under investigation as a potential therapeutic via exogenous delivery.[1] To help elucidate its biological roles, chemists have synthesized several types of H_2S releasing compounds (termed H_2S donors) with a variety of biologically relevant triggers, including water,[2] nucleophiles (e.g., thiols, amines),[3] enzymes,[4] and light[5]. Additionally, compounds that release carbonyl sulfide (COS),[6] and sulfur dioxide (SO_2),[7] have recently been reported, allowing for the study of other small molecule sulfur species as potential signaling compounds. These donors aid in our understanding of the physiological roles of H_2S and related compounds, and hold potential therapeutic value via exogenous H_2S delivery.[8] Interestingly, recent studies into the redox chemistry of sulfur species in the body indicate that persulfides (R–SSH) may have physiological roles similar to H_2S, insinuating that some of the physiological effects ascribed to delivery of H_2S may actually be derived from persulfides.[9] Further study of persulfides is needed to differentiate between the roles of H_2S itself and its biological products. Moreover, a clear description of sulfur redox chemistry in a biological context will allow further development of therapeutics that exploit pathways involved in H_2S signaling.

Dean and coworkers first identified persulfides in a biological context in their 1994 report on a protein persulfide intermediate of the cysteine desulfurase NifS.[10] Persulfides are prevalent in mammalian biology, generated via reaction of an oxidized thiol (e.g., a sulfenic acid, R–SOH) with H_2S in a process called S-persulfidation.[11] More nucleophilic than thiols, persulfides have pK_a values a few units lower than their corresponding thiols,[12] as well as greater reduction potentials,[13] making them highly reactive, transiently stable species. In a biological context, persulfides protect thiols from irreversible oxidation, serve as reactive intermediates in sulfur shuttling,[14] and alter enzymatic activity.[15] Some examples of protein persulfidation and the resulting changes in protein activity include: an increased parkin activity upon S-persulfidation resulting in a decrease in Parkinson's symptoms,[16] an increase in activity of GAPDH, protecting cells from apoptosis,[17] and H-Ras activation in cardiac tissue, regulating cellular redox signaling.[18] More recently, studies have confirmed the presence of endogenously produced small molecule persulfides

(e.g., cysteine persulfide and glutathione persulfide) with reported concentrations as high as 150 μM in human and mouse tissue.[9a] Small molecule persulfides likely play a role in regulating cellular redox balance and mediating cellular signaling.[9c] A major barrier in the study of the biological roles of persulfides is a lack of chemical tools capable of generating well-defined persulfide species in response to specific, biologically relevant triggers. Our understanding of H_2S biology has been aided immensely by the synthesis of organic H_2S donors; analogous to H_2S, persulfide donors will be vital tools for understanding how persulfides fit into the overall web of redox signaling.

Polysulfides ($RS–(S)_n–SR$), such as naturally occurring diallyl trisulfide (DATS), are perhaps the best known type of persulfide donor, but their reactivity in biological systems is complex, leading to generation of other redox-active species, including H_2S.[19] As a result, polysulfides are not ideal persulfide donors for use in studying persulfide biology, and the complex product mixture may limit their therapeutic potential. Free persulfides (i.e., R–SSH) have been isolated, but they suffer from poor stability under storage conditions and poor water solubility, and thus have relatively low utility in a practical sense.[19c] Persulfides are also proposed intermediates in several types of H_2S donors,[3] but these compounds all require conditions that cause rapid conversion of the persulfide into H_2S. To date there exist only two families of compounds capable of generating discrete persulfides: Wang and coworkers developed esterase-triggered persulfide prodrugs capable of releasing either a persulfide or hydrogen persulfide (HSSH) and Galardon and coworkers developed a pH-triggered persulfide analog of the nitrosothiol SNAP.[20] These donors generate persulfides without concomitant generation of H_2S and can be viewed as spontaneous persulfide donors due to the ubiquity of esterases *in vitro* and *in vivo*.

We sought to synthesize a discrete persulfide donor scaffold, inert under normal physiological conditions but capable of self-immolation in response to a specific trigger, revealing a discrete persulfide species (Figure 1). As the triggering moiety and persulfide could be readily tuned, this system would enable persulfide generation in response to many types of triggers, providing a valuable set of laboratory tools similar to the self-immolative COS donors recently reported by Pluth and coworkers.[21] In addition to their use as biological tools to study persulfide reactivity, persulfide prodrugs are exciting from a therapeutic standpoint because the reduction potential of persulfides is higher than that of thiols or H_2S, making them prime candidates for scavenging and reducing the harmful effects resulting from high levels of reactive oxygen species (ROS). Therefore, we aimed to synthesize an ROS-responsive persulfide prodrug as a proof of concept. This would allow for a two-stage quenching of ROS: the initial reaction of the ROS with the prodrug to trigger release, followed by the release of the persulfide. We envisioned that such an ROS-triggered persulfide prodrug would be ideal for cytoprotection against harmful levels of ROS.

Aryl boronic esters are relatively easy to synthesize, generally biocompatible, and react selectively with ROS in a B–C bond cleavage reaction to reveal the corresponding phenolate. Therefore, we set out to synthesize a self-immolative persulfide donor containing an aryl boronic ester as an ROS-sensitive trigger. The desired persulfide donor (termed **BDP-NAC** for Bpin-disulfide prodrug-*N*-acetyl cysteine) was synthesized from commercially available 4-tolylboronic acid in four steps (Scheme 1A). Theoretically, any thiol may be installed on

the distal end of the disulfide bond from the trigger/self-immolation moiety. Our choice of *N*-acetyl cysteine (NAC) was motivated by its biocompatibility as well as its ability to protect cells in vitro in highly oxidative environments.[22]

The ability of **BDP-NAC** to mediate the release of the desired NAC persulfide (**NAC-SSH**) in response to ROS was analyzed by LCMS (Figure 2). Aliquots of the reaction mixture of **BDP-NAC** with H_2O_2 were injected at various time points until the peak attributed to **BDP-NAC** (2.9 min) had subsided, revealing near complete decomposition of **BDP-NAC** within 2 h. A peak corresponding to **NAC–SSH** (3.4 min) increased in intensity over the course of the reaction, consistent with our proposed mechanism of persulfide generation. Mass spectrometry evidence also confirmed the presence of the other byproduct of the reaction, 4-hydroxybenzyl alcohol (a result of addition of water to the quinone methide), but the chromatogram peak was weak, likely due to low absorbance at the monitoring wavelength.

In addition to LCMS, we also investigated the reaction of **BDP-NAC** with H_2O_2 utilizing ^{1}H NMR spectroscopy. Experiments were conducted in DMSO-d_6:D_2O (9:1 v/v) due to the hydrophobic nature of **BDP-NAC** and the increased concentration required in NMR spectroscopy compared with LCMS. Shortly after the addition of H_2O_2 to the **BDP-NAC** solution, two new sets of peaks in the aryl region of the ^{1}H NMR spectrum appeared. One was consistent with 4-hydroxybenzyl alcohol, and the other was attributed to the slow hydrolysis of the Bpin moiety of **BDP-NAC,** yielding a boronic acid; boronic acids react with H_2O_2 in a similar fashion as pinacol boronic esters.[23] The reaction was considerably slower under these conditions than in the LCMS experiments. This retardation in reaction rate is likely a result of the high organic solvent content in the reaction.[24] Further insights into the stability of **BDP-NAC** as a persulfide prodrug as well as pertinent controls without the Bpin triggering moiety can be found in the SI (Figure S25 and S26).

To further evaluate the reactivity and trigger selectivity of **BDP-NAC**, a profluorophore (**BDP-Fluor**, Scheme 1B) was synthesized. Drawing inspiration from Xian and coworkers' turnon fluorescence probe (compound **7**) used for detection of sulfane sulfur species (HSSH, RS–$(S)_n$–SR, or S_8), the self-immolative **BDP-Fluor** has the same general structure as **BDP-NAC,** but with a coumarin-based fluorophore as the distal thiol species.[25] As shown in Figure 3A, we expected self-immolation to trigger release of a discrete persulfide, which would then cyclize to form a 5-membered benzodithiolone species, resulting in the release of 7-hydroxycoumarin. Because **BDP-Fluor** itself is not fluorescent, an increase in fluorescence at the characteristic emission wavelength of 7-hydroxycoumarin should only result from persulfide release and subsequent intramolecular cyclization, providing secondary confirmation of persulfide release from these self-immolative prodrug systems.

We tested this design by exposing **BDP-Fluor** to a variety of potential triggers. **BDP-Fluor** showed no evidence of self-immolative behavior (i.e., no fluorescence signal) in the absence of a trigger, but addition of H_2O_2 (100-fold excess) led to a 90-fold increase in fluorescence intensity at the characteristic wavelength of 7-hydroxycoumarin after incubation for 5 h in PBS buffer (Figure 3B and C). When **BDP-Fluor** was treated with other potential triggers, including sodium hypochlorite (NaOCl), cysteine (Cys), glutathione (GSH), lysine (Lys), and potassium superoxide (KO_2), the response was significantly lower, with H_2O_2 showing a

greater than 20-fold response over all of these potential triggers, and a greater than 90-fold response over Lys and KO_2.

As the increase in fluorescence response to thiols was unexpected, further investigation indicated that the fluorescence increase may be attributed to nucleophilic attack by cysteine at the aryl ester position, resulting in the release of 7-hydroxycoumarin. For more discussion on the probe response to thiols and relevant controls, see the SI (Figure S22). Taken together, these results confirm release of the desired persulfide species and demonstrate the selectivity of H_2O_2 as a trigger.

We next aimed to analyze **BDP-NAC** in a biological context. In vitro cytotoxicity studies on H9C2 cardiomyocytes showed that **BDP-NAC** is non-toxic up to 200 μM (Figure S27). As mentioned previously, persulfides have greater reducing potential than their corresponding thiols as well as H_2S. Thus, we envisioned that **BDP-NAC** might be effective in rescuing cells under oxidative stress, either via direct reduction of H_2O_2 or via upregulation of antioxidant pathways mediated by persulfide signaling.[9a, 15a] To this end, we evaluated the protective effects of **BDP-NAC** on H9C2 cells in culture via exogenous delivery of H_2O_2, which stresses the cells and promotes apoptosis (Figure 4). In the absence of **BDP-NAC,** cell viability drastically decreased after exposure to H_2O_2 (100 μM) for 1 h. However, simultaneous application of **BDP-NAC** (100–200 μM) with H_2O_2 showed a dose-dependent increase in cell viability, with no cytotoxicity observed after treatment with 200 μM **BDP-NAC.** At longer treatment times (2 h), **BDP-NAC** rescued a similar percentage of cells compared to H_2O_2-only controls (Figure S29). These results indicate that **BDP-NAC** can successfully mitigate the deleterious effects of a hyperoxidative environment in culture.

To further ensure that persulfide release imparts protection to the cardiomyocytes in the presence of H_2O_2, several control studies were carried out. Exposure of the cells to H_2O_2 with added 4-(hydroxymethyl)benzeneboronic acid pinacol ester (Bpin-OH), a non-persulfide releasing compound with a Bpin moiety, showed an increase in viability compared to H_2O_2 alone but did not rescue cells to the same extent as **BDP-NAC.** We also compared **BDP-NAC** to sodium sulfide (Na_2S), a fast-releasing H_2S donor, and GYY4137, a slow-releasing H_2S donor, under the same experimental conditions. Na_2S had a limited ability to rescue cells while GYY4137 had no effect on viability. Interestingly, **BDP-NAC** was more effective at rescuing cells than Na_2S, even while Na_2S enhanced H9C2 proliferation in the absence of H_2O_2 (Figure S28). This provides further evidence that persulfides may serve to maintain redox homeostasis in cells to a greater extent than H_2S. NAC, a potential thiol byproduct after reaction of **BDP-NAC,** also had no effect on viability. To confirm that **BDP-NAC** derives its activity from ROS-triggered persulfide release, the cardiomyocytes were treated with **BDP-Control,** which has an identical structure to **BDP-NAC,** but without the Bpin triggering moiety. **BDP-Control** also did not rescue cells exposed to H_2O_2 under identical conditions to the previous experiments.

Finally, to recreate the synergistic effects of the Bpin moiety of **BDP-NAC** and the resultant persulfide release, cells were treated with Bpin-OH simultaneously with either NAC, GYY4137, or Na_2S. Each of these combinations was able to mitigate the effects of H_2O_2 on cell viability, but not to the same degree as **BDP-NAC,** with the exception of Bpin-OH +

Na_2S. We suspect that simultaneous treatment of the cardiomyocytes with Bpin-OH and Na_2S gives a greater instantaneous concentration of potential antioxidants than **BDP-NAC,** considering its sustained release. In a system with continuous generation of ROS, delivery of Bpin-OH and Na_2S would likely have a diminished ability to rescue cells compared to sustained release from **BDP-NAC**.

In summary, we have synthesized a self-immolative prodrug that releases a discrete persulfide species (**BDP-NAC**) in the presence of H_2O_2. Persulfide release and trigger specificity were characterized by LCMS, NMR, and fluorescence spectroscopy, demonstrating that sustained release of the persulfide is selectively triggered by H_2O_2. In vitro studies using H9C2 cardiomyocytes under oxidative stress showed that **BDP-NAC** mitigates the harmful effects of highly oxidative environments with greater potency than commonly used H_2S donors Na_2S and GYY4137 as well as relevant controls. **BDP-NAC** not only shows promise therapeutically, but it also provides a modular system for persulfide donors that may be triggered under a variety of conditions. We envision that a library of persulfide donors based on the **BDP-NAC** template will enable the study of persulfide biology in greater depth than is currently possible, providing insight into sulfur redox cycles and sulfur-mediated cell signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Science Foundation (DMR-1454754) and the National Institutes of Health (R01GM123508). We also thank 3M for support of this work through a Non-Tenured Faculty Award to JBM. We thank Dr. Tijana Grove and Dr. Webster Santos and their students for experimental assistance as well as Dr. Mehdi Ashraf-Khorassani for help with the LCMS

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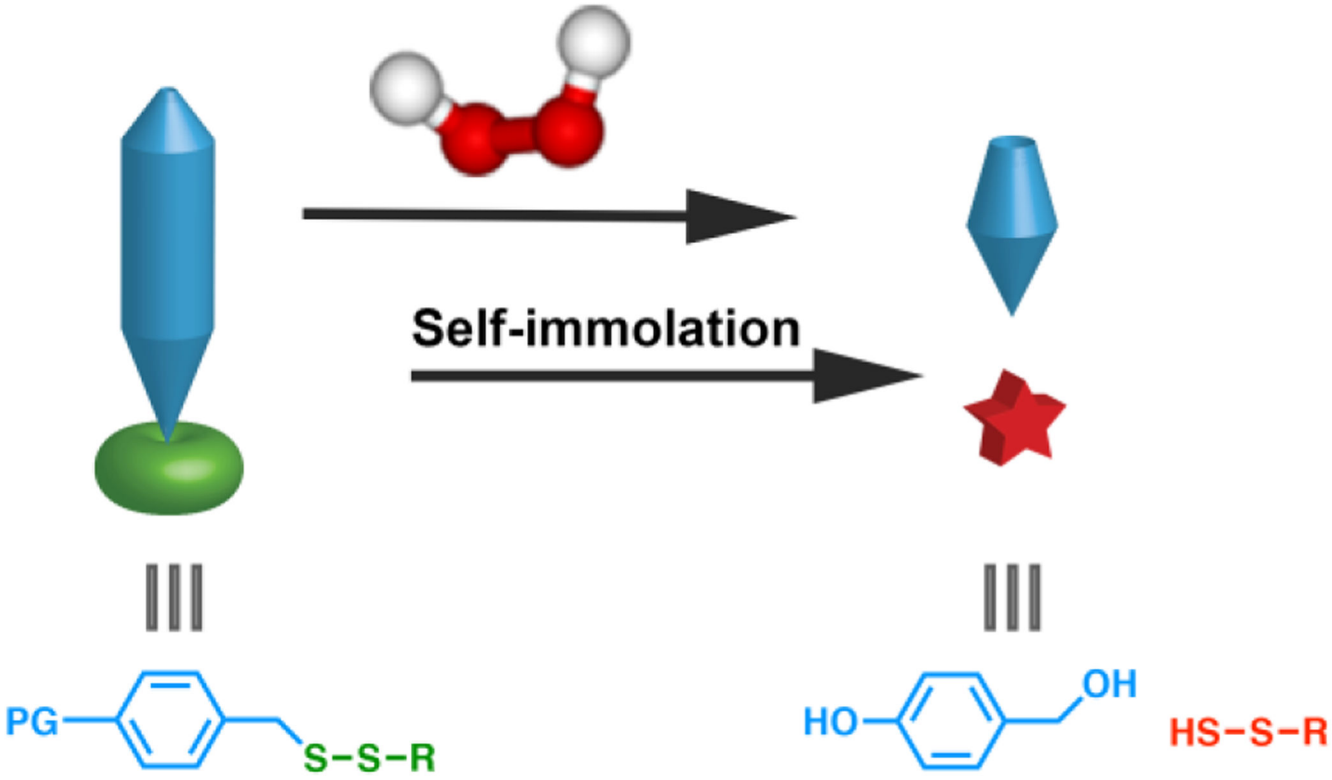


Figure 1.
Cartoon schematic representing the proposed release of a discrete persulfide species from a generalized self-immolative prodrug (PG = protecting group) in the presence of a trigger (H_2O_2 shown here).

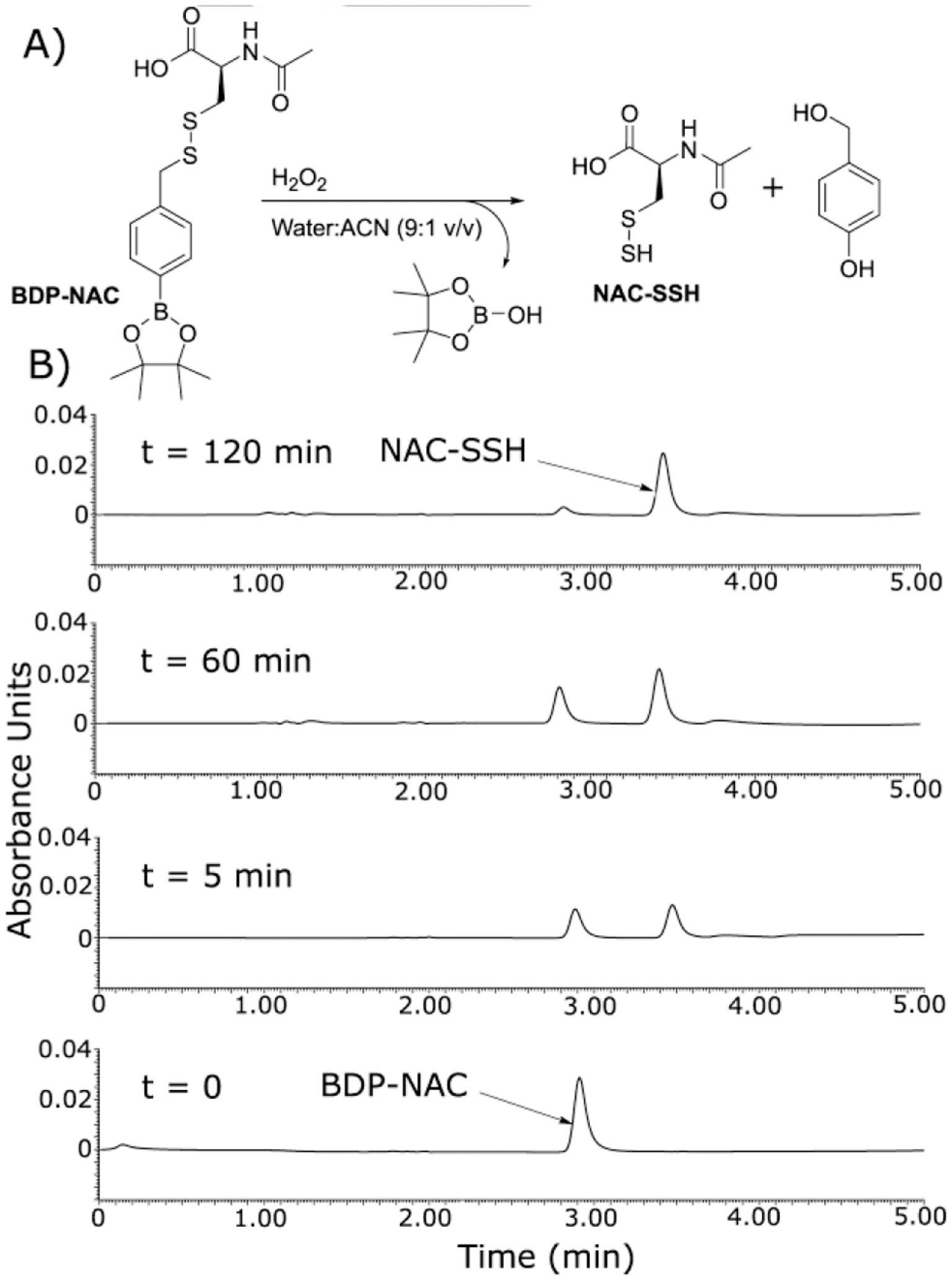


Figure 2.

A) Proposed reaction of **BDP-NAC** in the presence of H_2O_2 leading to the release of **NAC–SSH**. B) LC chromatograms highlighting the conversion of **BDP-NAC** into NAC persulfide (**NAC-SSH**) in the presence of H_2O_2. Timepoints are noted above each LC chromatogram. The peak eluting at 2.9 min corresponds to **BDP-NAC**, and the peak at 3.4 min corresponds to **NAC-SSH** (see Figure S20 for corresponding mass spectrometry data).

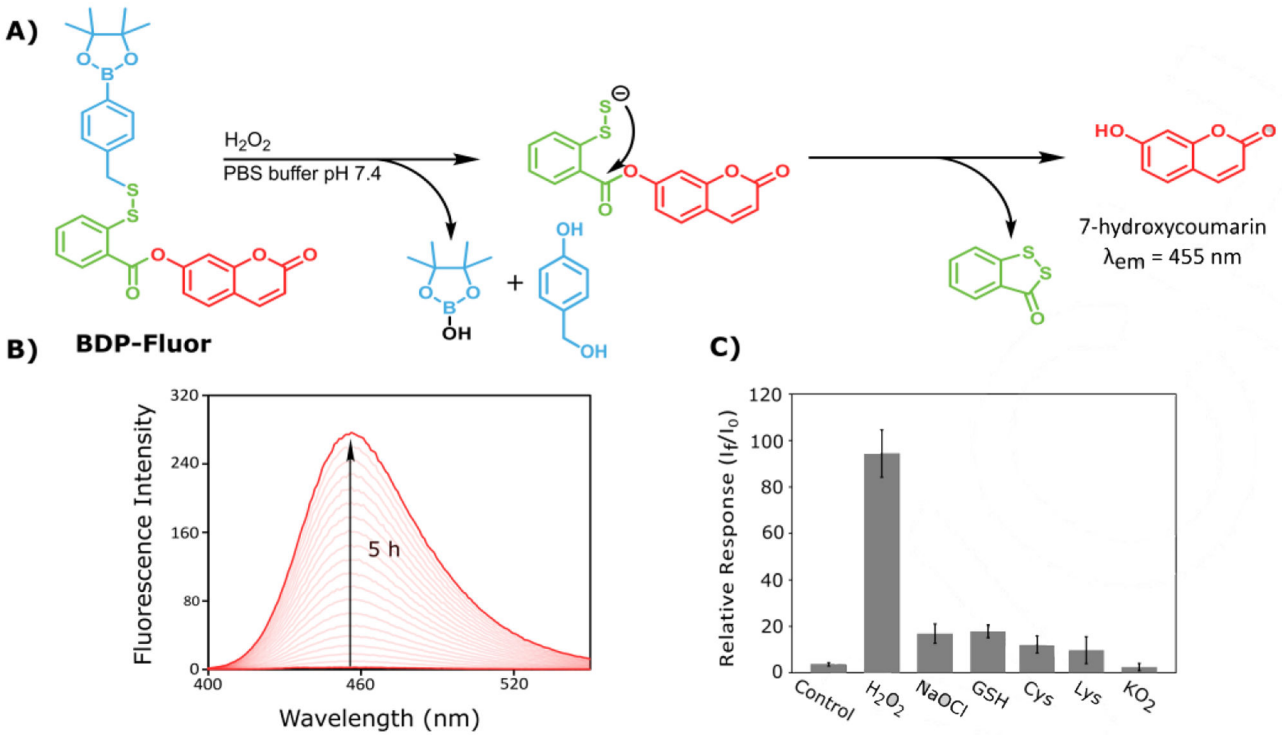


Figure 3.

A) Proposed reaction mechanism for the release of 7-hydroxycoumarin from **BDP-Fluor** in the presence of H_2O_2. B) Representative overlay of the fluorescence spectra of **BDP-Fluor** in the presence of 100-fold excess H_2O_2 resulting from the release of 7-hydroxycoumarin over the course of 5 h. C) Relative response of **BDP-Fluor** (3.3 μM) to each potential trigger (330 μM) or control (no trigger added) represented as the ratio of the final fluorescence (I_f) intensity after 5 h to the initial fluorescence intensity (I_0), showing an increased selectivity for H_2O_2 over other potential triggers.

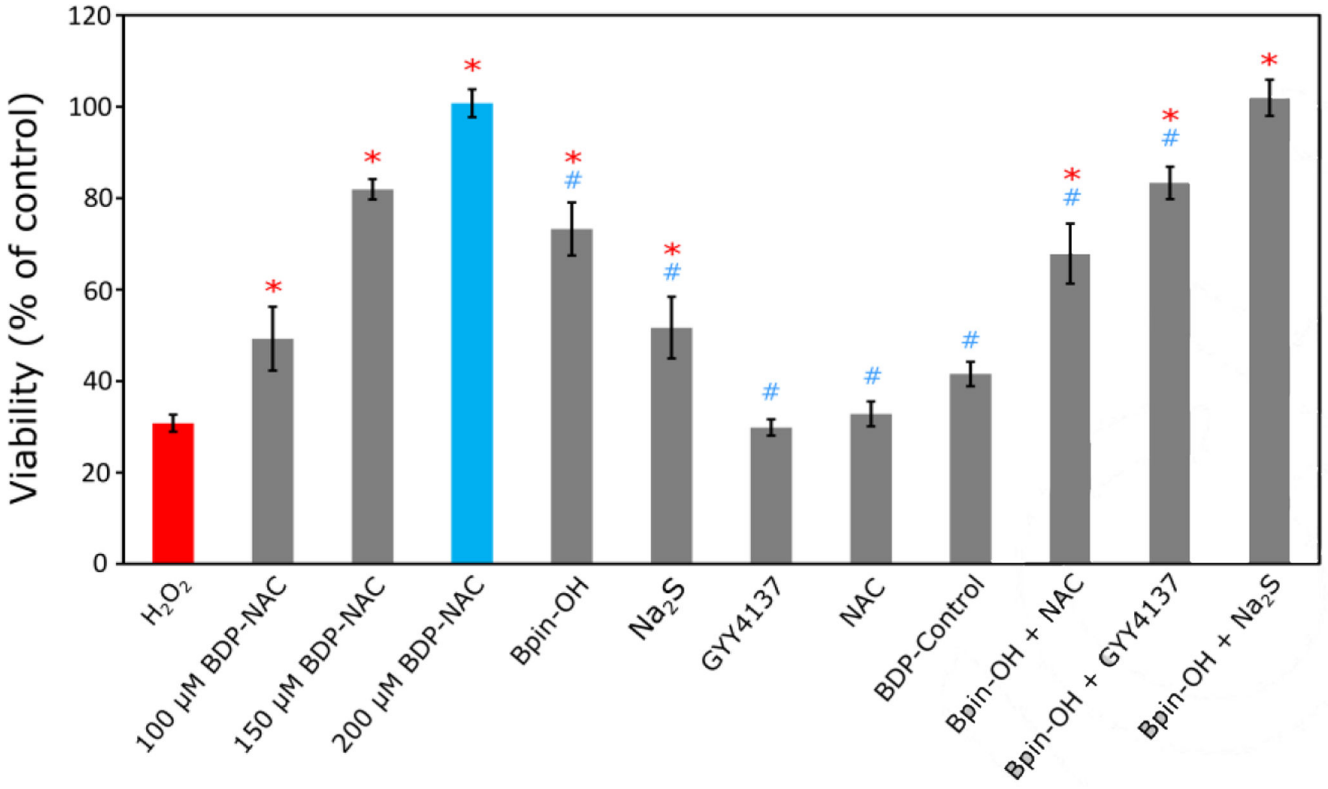
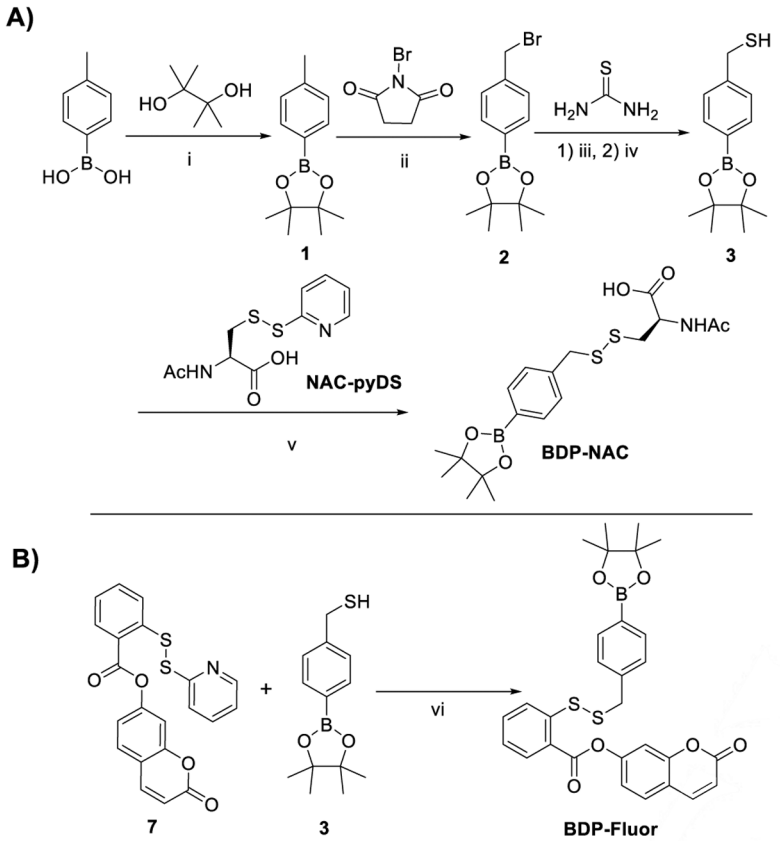


Figure 4.

Viability of H9C2 cardiomyoctyes treated with **BDP-NAC** or various controls and related compounds concurrent with exposure to H_2O_2 (100 μM) for 1 h. Each control compound was applied at a concentration of 200 μM (except for Na_2S (100 μM) in the Bpin-OH + Na_2S treatment group). Quantification of viability was carried out using 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. *P<0.01 for comparisons with the H_2O_2 treatment group and #P<0.01 for comparisons with the **BDP-NAC** (200 μM) treatment group. Group comparisons are indicated as determined by a one-way analysis of variance (ANOVA) with a Student-Newman-Keuls comparisons post-hoc test.



Scheme 1.

A) Synthetic route to **BDP-NAC**. Conditions: i) $MgSO_4$, Et_2O, rt, 16 h; ii) AIBN, C_6H_{12}, reflux, 16 h; iii) 1) EtOH, rt, 4 h; iv) 1 N NaOH, reflux, 45 min; v) NEt_3, $CHCl_3$, rt, 3 h; B) Synthetic route to **BDP-Fluor**. Conditions: vi) $CHCl_3$:MeOH (1:1 v/v), rt, 40 h.