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Capturing a Sulfenic Acid with Arylboronic Acids and Benzoxaborole

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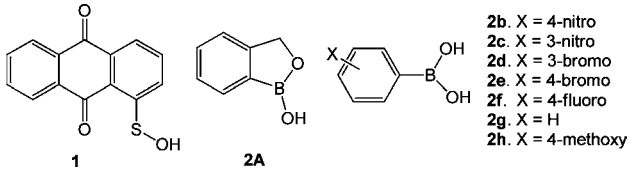
Supporting Information

ABSTRACT: Post-translational redox generation of cysteine-sulfenic acids (Cys-SOH) functions as an important reversible regulatory mechanism for many biological functions, such as signal transduction, balancing cellular redox states, catalysis, and gene transcription. Herein we show that arylboronic acids and cyclic benzoxaboroles can form adducts with sulfenic acids in aqueous medium and that these boron-based compounds can potentially be used to trap biologically significant sulfenic acids. As proof of principle we demonstrate that a benzoxaborole can inhibit the enzyme activity of an iron-containing nitrile hydratase, which requires a catalytic αCys114-SOH in the active site. The nature of the adduct and the effect of the boronic acid's pK_a^B on the stability constant of the adduct are discussed within.

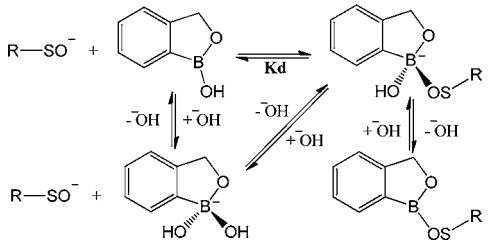
Redox signaling mediated by endogenous reactive oxygen species has emerged as a prevalent signaling pathway that is essential for cell proliferation and survival.[1−3] Specifically, reversible oxidation of reactive cysteine residues in the presence of reactive oxygen species has been shown to participate in celluar processes, such as signal transduction, managing the intracellular redox state, modulating gene transcription, and catalysis.[1,4−12] In the presence of reactive oxygen species, two electron oxidation of a cysteine residue generates sulfenic acid (Cys-SOH) as the initial product, which is often the intermediate to further modifications, such as disulfide bond formation, S-glutathiolation, S-nitrosation, and sulfenyl amide formation.[1,4,13] The reversible nature of cysteine oxidation is analogous to phosphorylation, making them both well suited for regulatory post-transloational modifications. This also means that biological sulfenic acids can be a new class of potential pharmaceutical targets, especially given the growing implications of cysteine-derived sulfenic acids' role in health issues such as cancer,[14−16] heart disease,[17] and scurvy.[18]

Biological cysteine sulfenic acids can be relatively stable when stabilized by the microenvironment within the protein that excludes other sulfur groups and solvent molecules.[1,6,19] In contrast, stable small molecule sulfenic acid compounds are scarce due to their highly reactive nature. Due to the rarity of small stable sulfenic acids, possible interactions between sulfenic acids and boronic acids have not been investigated. Here we used Fries acid **1**,[20] whose sulfenic acid moiety is stabilized by strong resonance interaction with the 9-carbonyl group,[21] to demonstrate that boronic acids (**2A−h**) can form reversible complexes with sulfenic acids in addition to providing the first example of a RSO-B bond. This study thus expands upon the current strategies for trapping biological sulfenic acids, which have been limited mostly to dimedone-based[1,4] or analogous (e.g., β-ketoesters)[22] probes that irreversibly conjugate to the sulfenic acids moiety. This also adds to the growing applications of boronic acids.[23]



First, we identified complexation between boronic acids **2A−h** and sulfenic acid **1** via UV/vis spectrophotometric titrations. The characteristic peak (676 nm; ε 3100 M^{-1} cm^{-1})[24] for the sodium salt of sulfenic acid **1** decreases with sequential additions of boronic acids **2A−h**. This is accompanied by an increase of absorbance centered at 468 nm, which corresponds to the formation of the **1**:**2** adduct (Scheme 1). The **1**:**2** adduct has a λ_{max} that is similar to the free acid of **1** (462 nm) even though under the experimental conditions the observed spectral changes cannot be due to simply the protonation of anionic **1**. This is because all of the boronic acids used, except **2b**, have a higher pK_a value than sulfenic acid **1**, which was found to be 7.61 ± 0.02 (previously reported as 7.51)[24] in 15% v/v acetonitrile/water mixture[25,26] (Table 1) by the half-neutralization method where $[\mathbf{1}] = [\mathbf{1} - H^+]^-$. In other words, mixing the deprotonated **1** with a more basic boronic acid should not result in the generation of the free acid form of **1**. While the substituents on the boronic acids (or benzoxaborole vs arylboronic acids) do not shift the λ_{max} (468 nm) of the **1**:**2** complex, the associated extinction coefficient increases with decreasing pK_a^B value of **2**.

Scheme 1. Various Equilibria for the Adduct Formation between a Sulfenic Acid and 2A[a]



[a]Only one of the stereoisomers is shown for simplicity.

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Table 1. Dissociation Constants (K_d) for Complexes of 1:2A–h from Duplicate UV/vis and ITC Titrations in 15% (v/v) Acetonitrile/Water Mixture at 22 °C

	pK_a^B at 15% v/v $CH_3CN:H_2O$	averaged (K_d; M) from UV/vis titrations	averaged (K_d; M) from ITC
2A	7.8 ± 0.1	$10^{-(3.8\pm0.1)}$	$10^{-(3.9\pm0.2)}$
2b	7.39 ± 0.05	$10^{-(3.6\pm0.1)}$	ND
2c	8.13 ± 0.01	$10^{-(3.53\pm0.01)}$	ND
2d	8.55 ± 0.09	$10^{-(3.8\pm0.1)}$	$10^{-(4.3\pm0.1)}$
2e	9.1 ± 0.1	$10^{-(3.90\pm0.04)}$	ND
2f	9.4 ± 0.02	$10^{-(3.80\pm0.01)}$	ND
2g	9.5 ± 0.06	$10^{-(3.7\pm0.2)}$	$10^{-(3.96\pm0.04)}$
2h	10.13 ± 0.02	$10^{-(3.81\pm0.01)}$	ND

$$ArB(OH)_2 \overset{K_a^B}{\rightleftharpoons} ArB(OH)_3^- + H^+$$

Figure 1 shows a representative titration curve where the corrected absorbance (corrected for dilutions due to sequential

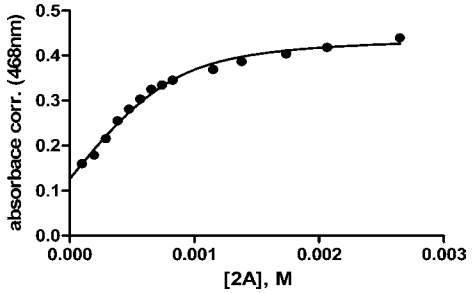

Figure 1. Plot of the corrected absorbance (468 nm) vs [**2A**] for the spectrophotometric titration of the sodium salt of **1** (0.8 mM) with sequential addition of **2A** in 15% (v/v) acetonitrile/water mixture at 22 °C. The data points are fitted into eq S1 to yield $K_d = 10^{-3.90\pm0.01}$ M and $r^2 = 0.9904$.

additions of **2A** stock solution) at 468 nm was plotted against the [**2A**] in the system. The dissociation constant (K_d in unit of M) for complex **1**:**2** in 15% v/v acetonitrile/water at 22 °C can be derived from fitting the titration data to a universal binding expression[27] that describes the top pathway in Scheme 1 while considering mass balance on all the participating species (eq S1). This adduct is likely to be of an SO-B nature, since the sulfur atom is electrophilic and the oxygen atom is nucleophilic in sulfenic acids.[28] While the reactive nature of Fries acid in solution prevented convenient access to a crystal structure, several experimental observations support the SO-B linkage (see Supporting Information, SI). It should be noted that Fries acid also reacted with dimedone and other nucleophiles that attack sulfenic acids, such as cysteinesulfenic acid[28] (SI).

The averaged K_d values from duplicate titrations are listed in Table 1, along with the pK_a values of the boron compounds in 15% v/v acetonitrile/water mixture[25] at 22 °C. The pK_a^B values of the boron compounds **1A–h** in our acetonitrile/water mixtures were also determined in duplicates by the half neutralization methods, and when comparisons can be made the values found here agree well with the published numbers.[29,30] Table 1 shows that the binding affinity between sulfenic acid **1** and **2A–h** is unaffected by the acidity of the boron species. Over a pK_a^B range of 2.7 units from **2b** (pK_a^B = 7.39 ± 0.05) to **2h** (pK_a^B = 10.13 ± 0.02), the dissociation constants for the **1**:**2** adduct are essentially the same within experimental uncertainty. The strength of the adduct formation was also reconfirmed by isothermal calorimetry (ITC) method, where the ITC data determined at 25 °C agree with the K_d values determined from spectrophotometric titrations at 22 °C (Table 1). The good agreement between the two titration methods strengthens confidence in the measured stability constant of the complexes. ITC data also found that, as expected, the formation of the sulfenic acid-boron adduct yielded negative entropy for a two-to-one species complexation process (Table S1). Overall, the dissociation constants obtained for **2A–h** are $\sim10^{-3.8}$ M in 15% v/v acetonitrile/water mixture at 22–25 °C. The insensitivity of the adduct dissociation constant with respect to the acidity of the boron compound observed here can potentially be exploited to enhance boronic acids' selectivity for sulfenic acid over other biological ligands (e.g. catechol,[30] glucose,[30] adenosine monophosphate;[29] see SI).

Decreasing the polarity of the solvent system also minimally perturbed the K_d values of the **1**:**2** adduct. The K_d values for complexes **1**:**2A** and **1**:**2d** in 70% v/v acetonitrile/water mixture[25,26] at 22 °C were found to be $10^{-3.90\pm0.02}$ M and $10^{-4.0\pm0.1}$ M, respectively (Figures S17–20). This is not surprising considering the equilibria that describe the **1**:**2** adduct formation have the same amount of charged species on both sides of the equilibria. Specifically, anioinic **1** and neutral **2** can either form an anionic tetrahedral boronate adduct or a neutral trigonal ester plus a hydroxide. Thus, stabilization of charged species by solvation does not greatly favor a particular side of the equilibria.

Nonetheless, experimental data do support the formation of an anionic tetrahedral **1**:**2** boronate complex over the neutral trigonal species under the conditions employed here. First, the measured pH of the solutions was found to be ~7.5–8.0 both before and after the spectrophotometric titrations. The relatively neutral pH of the reaction mixture at the end of the titration is suggestive of an anionic tetrahedral boronate complex instead of a neutral trigonal adduct. This is because the titrations were done under mM concentration of **1** and **2** meaning that the generation of a neutral **1**:**2** adduct would require the release of ~mM of hydroxide anion and would raise the mixture to pH ~ 11, which was not the case.

Further evidence of a **1**:**2** adduct in solution was provided by the observation of the anionic boronate **1**:**2** complexes using electrospray time-of-flight mass spectroscopy for the boron compounds tested (**2A–f**; Figures S28–34). Upon lowering the ionization temperature or the capillary voltage was found to increase the amount of detectable adduct. While it is true that the neutral trigonal **1**:**2** adduct would not be detected in the MS experiments, the MS data provide further validation to the presence of a **1**:**2** boronate complex in solution. In addition, we observed a small cluster (smaller than that for the **1**:**2** adduct) of MS peaks that correspond to the mass of the boronic acid plus the oxidized form **1**, the sulfinic acid. This suggests that the boronic acids might also form adducts with sulfinic acids. It is possible that the harsh MS ionization conditions promoted the oxidation of **1**. MS data collected at different time intervals after combining **1** and **2** together suggest that the formation of **1**:**2** complex did not enhance the oxidation of the sulfenic acid moiety to its sulfinic acid form.

We also examined the complex formation between **1** and **2** using ^{11}B NMR, which is useful in distinguishing between a neutral trigonal boron ester and an anionic tetrahedral boronate. The insert in Figure 2 shows peaks for the two ionization states of **2A**: 32.9 ppm for the neutral trigonal **2A**

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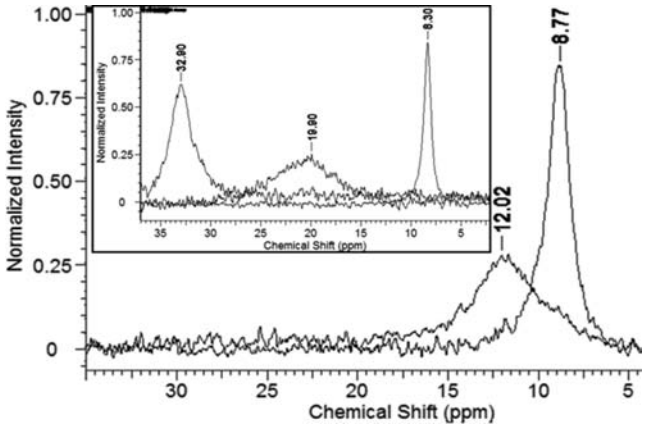

Figure 2. ^{11}B NMR spectra of solutions containing **2A**:**1** complex showing a sharp peak at 8.77 ppm (with 6 mM **2A**, 6 mM sodium salt of **1**) and a broader peak at 12.02 ppm (10 mM **2A**, 5 mM sodium salt of **1**). Insert shows spectra of the neutral trigonal **2A** at 32.9 ppm (10 mM **2A**, 10 mM HCl); anionic boronate **2A**:$^{-}$OH at 8.3 ppm (10 mM **2A**, 20 mM NaOH); and half-neutralized **2A** at 19.9 ppm (10 mM **2A**, 5 mM NaOH).

and 8.3 ppm for the anionic tetrahedral boronate.[30−32] When **2A** was mixed with half equivalence of NaOH, a broader intermediate peak at 19.9 ppm was found, corresponding to equal amount of [**2A**] and [**2A**:OH]$^{-}$ in rapid exchange. When 10 mM of boronic acid **2A** was mixed with half equivalence of the deprotonated sulfenic acid **1**, a broad ^{11}B peak was found centered at ∼12 ppm.[31] The broadness of this peak is most likely due to the **1**:**2A** boronate adduct exchanging with the free **2A** and the **2A**:$^{-}$OH boronate species in solution at pH of 7.5. However, when equal amounts of the sulfenic acid and **2A** were mixed, a sharp peak (at 8.77 ppm) that closely resembles the [**2A**:OH]$^{-}$ boronate species was found (Figure 2), confirming that the **1**:**2** adduct formed under the experimental condition is indeed in the tetrahedral boronate form. Similar ^{11}B NMR results were for the **1**:**2d** adduct (Table S2).

Next, we tested the reversibility of the adduct formation between sulfenic acid **1** and the boron compounds (Figure 3).

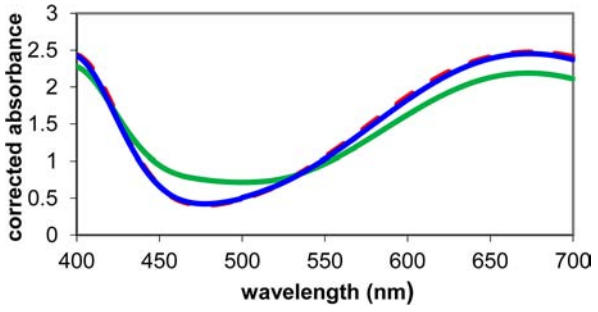

Figure 3. Corrected absorbance vs wavelength of 0.8 mM of deprotonated **1** (red); 0.8 mM of deprotonated **1** plus 4.5 mM of **2d** (green); and adding 4.5 mM of NaOH to the previous mixture (blue) in 15% (v/v) acetonitrile/water at 22 °C.

Boronic acid **2d** possesses no significant absorbance between 400 and 700 nm. Figure 3 shows that when saturating concentration of **2d** was added to the sodium salt of **1** (red dashed curve), a new trace that represents the **1**:**2d** complex (green curve; absorbance increases at 468 nm and decreases at 676 nm) was observed. This follows the top pathway in Scheme 1. The addition of NaOH (4.5 mM in the final mixture) competes off the bound anionic **1** to regenerate the original spectrum (blue curve), which corresponds to the unbound deprotonated **1** in solution (diagonal pathway in Scheme 1). This reversible nature of complex **1**:**2** holds true with all boronic acids **2A**−**h**. It is not surprising that the more nucleophilic (more basic) hydroxide anion can replace a bound sulfenic acid on boron. The boronate formation constant (K ∼ 2.8 × 10^{5} M) between hydroxide and **2d** can be calculated from dividing the K_a^B value of **2d** by the water self-ionization constant (K_w = 10^{-14}). This means that boronic acid **2d** should preferentially bind to the hydroxide anion over the anionic form of sulfenic acid **1** by ∼40 times. Thus, while the stability constant of the **1**:**2** adduct is insensitive to the pK_a^B of the boron compound, it might be affected by the acidity of the sulfenic acid.

Finally, as proof of principle to demonstrate the potential use of benzoxaborole/boronic acids to inhibit enzymes with functionally important sulfenic acids, we used the iron-based nitrile hydratase (NHase; EC 4.2.1.84) from *Rhodococcus erythropolis* as the model system. Nitrile hydratase is one of the most successful biocatalysts: it is responsible for the industrial production of acrylamide and nicotinamide,[33] and NHase's catalytic functionality depends on the presence of a sulfenic acid at the αCys114 (αCys114-SOH) position in the active site.[34,35] Literature data show that the sulfenate oxygen actives a water molecule that can subsequently undergo nucleophilic attack on the nitrile substrate. Oxidation of αCys114-SOH into αCys114-SO2 deactivates the enzyme.

We first photoactivated the iron-containing NHase (Prozomix) derived from *Rhodococcus erythropolis* as per published protocols[34,36] and then employed an enzyme activity assay that converted 2-nitro-5-thiocyanatobenzoic acid (NTBA) into formamide and 5-mercapto-2-nitrobenzoic acid (ε_{412} = 13 600 M^{-1} cm^{-1}), whose appearance can be followed spectrophotometrically.[37] We confirmed the necessity of the catalytic sulfenic acid in the active site by adding the commonly used specific sulfenic acid trap, dimedone. At 1 mM of dimedone, only ∼40% of the original enzyme activity was observed; in the absence of dimedone or boron compounds, the rate constant for thiolate product formation is ∼0.033 min^{-1} under the experimental conditions (Table S3). When the [dimedone] was increased to 2−2.5 mM, no detectable reaction was observed for 10−20 min. Next, we challenged the activated NHase with boron compounds. Parallel experiments were conducted using various concentrations of **2A** or **2g**. The results (Figure 4; Table S3) show that while **2g** has a small influence on the

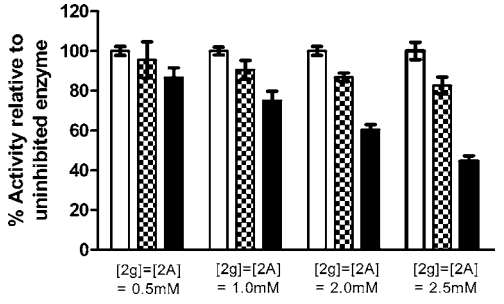

Figure 4. Percent enzyme activity for the NHase-promoted conversion of NTBA into formamide and 5-mercapto-2-nitrobenzoic acid in the absence of boronic acids (unfilled bars; set as 100% activity) and in the presence of **2g** (checkered bars) or **2A** (black solid bars).

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NHase activity, **2A** is much more effective in inhibiting the enzyme activity of NHase. The inhibition is probably due to **2A** complexing to the catalytic αCys114-SOH. The effectiveness of **2A**, which has a benzoxaborole scaffold, over a simple phenylboronic acid **2g** and also **2d** (Table S3), is reminiscent of the superior antifungal properties of benzoxaboroles over acyclic arylboronic acids.[38]

Here we demonstrated that boronic acids/benzoxaborole can form reversible complexes with sulfenic acid. The complex formation constant appears to be insensitive to the acidity of the boron compounds. However, since cysteine-sulfenic acids would have higher pK_a than **1**, it possible that the biological cysteine-SOH would form tighter adducts with benzoxaboroles/boronic acids than the values reported herein. Together with NHase inhibition data, the data indicate that the rational designs of biological sulfenic acid trapping agents should focus on the structural element (e.g., designs based on the active site architect) rather than the acidity of the boron warhead. Nonetheless, compounds of RSO-B nature or complexes of boronic and sulfenic acids can potentially be a valuable new class of chemical agents for trapping, inhibiting, and detecting biological sulfenic acids as well as for synthetic applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, Figures S1−S34, and Table S1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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