Diazaborines Resist H_2O_2-Mediated Oxidation but React Rapidly with Peroxynitrite in Aqueous Buffer

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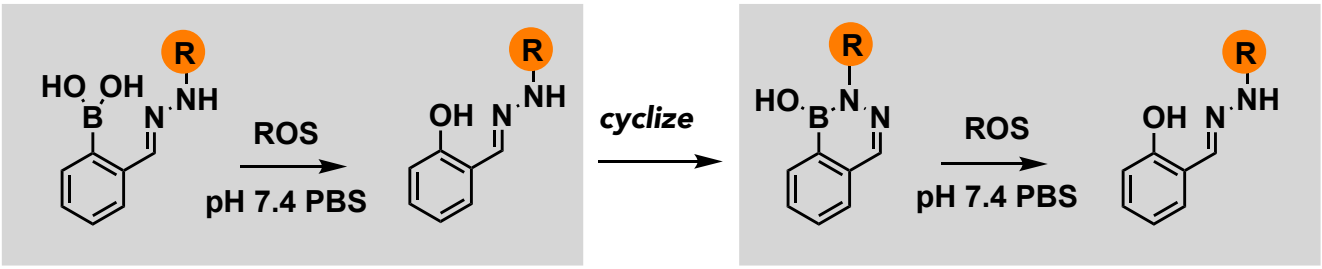
ABSTRACT: Arylboronic acids oxidize with H_2O_2 and peroxynitrite ($ONOO^-$) to yield their corresponding phenols. However, simple arylboronic acids struggle to discriminate between these two biological reactive oxygen species (ROS). Here, we show that diazaborines (DABs) react slowly with H_2O_2 but rapidly with peroxynitrite in aqueous buffer. Moreover, oxidation of DABs with H_2O_2 and $ONOO^-$ first yields a long-lived intermediate that ultimately converts to the phenol. Taken together, our work shows that diazaborines exhibit enhanced kinetic discrimination between H_2O_2 and $ONOO^-$, opening up new opportunities for diazaborine-based tools in chemical biology.



*Reacts rapidly with H_2O_2 **and** $ONOO^-$*

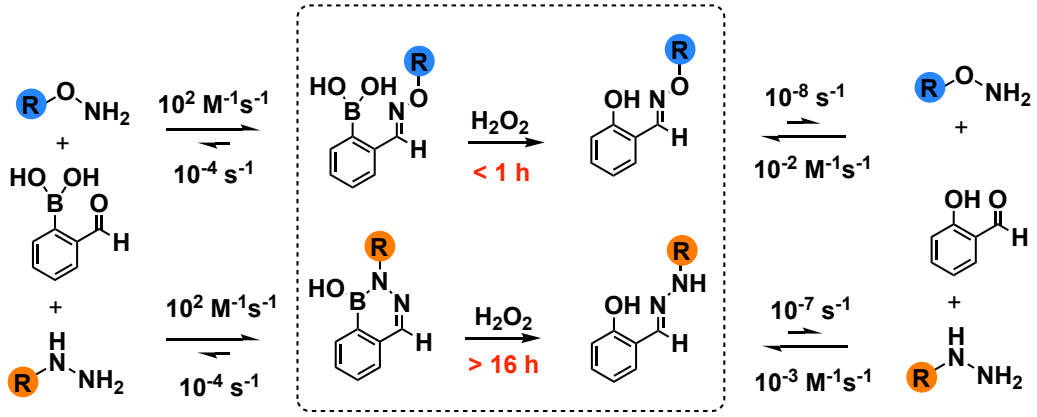
enhanced kinetic discrimination between H_2O_2 and $ONOO^-$

Diazaborines are boron-containing hetereocycles that are emerging as powerful motifs to expand the pharmacophore space and as valuable linkages for bioconjugation chemistry.[1–10] However, in contrast to arylboronic acid oxidation, which has been thoroughly studied and explored for biological applications,[11–13] an analogous evaluation of diazaborine reactivity with common reactive oxygen species (ROS) has not yet been reported. Given the increased use of diazaborines as drug molecules and bioconjugation linkages in biological settings, it is important to establish the reactivity profile diazaborines to common cellular ROS.

As we and others have previously shown, 2-formylphenylboronic acid (2-fPBA) reacts rapidly with alkoxyamines and hydrazines/hydrazides (k_f ~ 10^2 – 10^3 M^{-1} s^{-1}) to yield oximes and diazaborines,[14–16] respectively, in aqueous buffer at physiological pH (Figure 1). We have further shown that the hydrolytic half-lives of boronic acid oximes and acyl-diazaborines are relatively short ($t_{1/2}$ ~ hours at pH 7.4) but increase more than 30,000-fold ($t_{1/2}$ ~ months at pH 7.4) after H_2O_2-mediated oxidation.[15] We attribute this decrease in the exchange dynamics to the loss of boronic acid, a proximal Lewis acid that catalyzes the exchange processes. However, we noted significantly different rates of oxidation for boronic acid

oximes compared to diazaborines, suggesting that diazaborines may be resistant to ROS-mediated oxidation. We reasoned that molecular architectures that facilitate complexation between heteroatom-containing proximal groups with the vacant p-orbital in boron may slow the rate of ROS-mediated oxidation. We further hypothesized that judicious choice of neighboring groups may enhance kinetic discrimination between H_2O_2 and $ONOO^-$-mediated oxidation in a manner analogous to peroxynitrite-specific fluorescent protein sensors,[17] small-molecule probes,[18] and iminoboronate linkages.[5]

Here, we show that *o*-substituted arylboronic acid and diazaborines react with drastically different rates with common biological ROS. In contrast to arylboronic acids, diazaborines more than 1000-fold more slowly with H_2O_2 yet undergo rapid oxidation with $ONOO^-$. Unlike arylboronic acid oxidation, the reaction of diazaborines with H_2O_2 and $ONOO^-$ does not immediately yield a phenol, but rather, prompts the formation of a long-lived intermediate that ultimately converts to the final phenol over the course of hours



Are diazaborines resistant to oxidation?

Figure 1. Alkoxyamines and hydrazines/hydrazides react rapidly with 2-formylphenylboronic acid (2-fPBA) to form boronic acid oximes and diazaborines, respectively. Reaction with H_2O_2 abolishes the rapid dynamics. However, diazaborines react significantly slower to form the phenol, suggesting they may be resistant to ROS-mediated oxidation.

to days in aqueous buffer. Taken together, our results provide the initial groundwork to guide the design of oxidatively-resistant diazaborine-based drugs and bioconjugates and further develop diazaborine-based chemical tools with $ONOO^-$-specific reactivity.

We began by surveying the impact of a variety of neighboring groups on arylboronic acid oxidation. We examined arylboronic acids with neighboring oxime, ether, and bulky hydrazone functionalities and measured their reaction profiles with a library of ROS, including H_2O_2, NaOCl, *tert*-butyl hydroperoxide (t-BuOOH), and peroxynitrite ($ONOO^-$). We initially monitored the ROS-triggered oxidation of each arylboronic acid by ^1H NMR (SI Fig. S1-S25). Time-course UV-visible spectroscopy and/or time-course NMR was then used to measure the second-order rate constant with ROS. Data were fitted to a second-order kinetic model with a non-linear least squares fitting.[19]

Phenylboronic acid (PBA) oxidation by H_2O_2 or $ONOO^-$ is well-established and was used to benchmark the library of *ortho*-substituted compounds. PBA reacts quickly with H_2O_2 (k_2 =4.9±0.9 $M^{-1}s^{-1}$) in

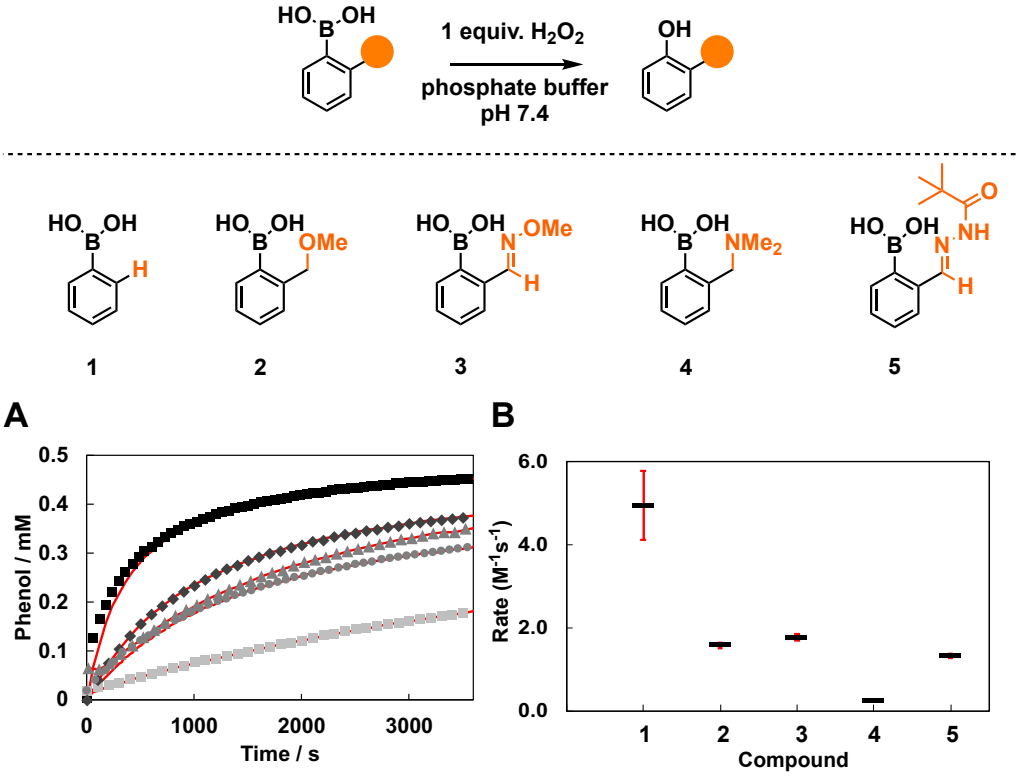


Figure 2. H_2O_2-mediated oxidation of arylboronic acids **1-5**. (A) Arylboronic acids (500 μM) in phosphate buffer (200 mM, pH 7.4) were treated with H_2O_2 (500 μM, 1 equivalent) at 24°C. Datapoints refer to the concentration of phenol product as a function of time as monitored by time-course ultraviolet-visible spectroscopy. Solid red lines represent a fit to a second-order kinetic model. Shown are data for **1** (black squares), **2** (XX), **3** (XX), **4** (light gray squares), and **5** (gray circles)(B) Rate constants for the reactions shown in (A). Each measurement represents the average ± SD of three runs with independent fitting to the kinetic model.

phosphate buffer, pH 7.4, a value in line with recent measurements (Fig. 2A).[20] No significant reaction was seen with the remaining ROS, except with $ONOO^-$. Previous stopped-flow kinetics studies report a rate constant on the order of 10^6 M^{-1} s^{-1} for the reaction between PBA and $ONOO^-$, which is 10^6-times faster than with H_2O_2.{Citation} Consistent with this observation, the reaction between PBA with $ONOO^-$

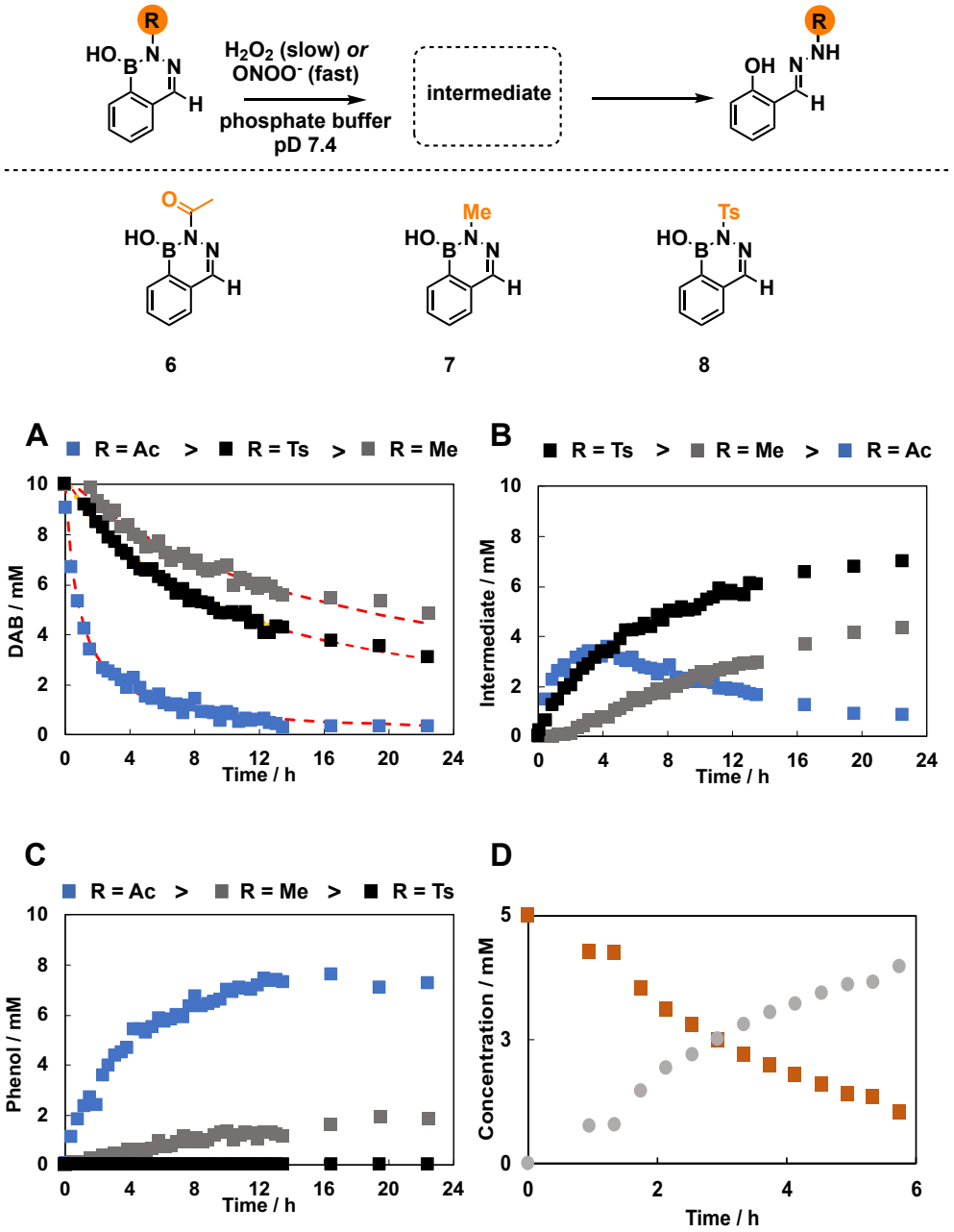


Figure 3. Reaction of DABs **6-8** (10 mM) with H_2O_2 (10 mM) in phosphate buffered (200 mM, pD 7.4) D_2O at room temperature. (A) Representative time-course ^1H NMR plot for reaction of **6** as a function of time showing starting material consumption, intermediate formation and intermediate disappearance with concomitant phenol formation. (B) DAB consumption as a function of time. Dashed line represents a fit to a second-order kinetic model. (C) Intermediate formation as a function of time. (D) Time-course conversion of the intermediate from **6** (orange) to phenol (grey) as a function of time. DAB **6** (5 mM) in phosphate buffer (200 mM, pD 7.4) D_2O was treated with $ONOO^-$ (4 × 5 mM) to generate the intermediate. Data points refer to the concentration of molecular species at the indicated timepoint. Concentrations were determined by ^1H NMR with respect to a dimethylsulfone internal standard.

was too fast to measure by UV-visible spectroscopy. ^1H NMR revealed clean conversion of $ONOO^-$-mediated oxidation of PBA to phenol; however, 4 equivalents of $ONOO^-$ was necessary for complete conversion. We attribute this to the short half-life of $ONOO^-$ in pH 7.4 PBS, which leads to competing decomposition of $ONOO^-$.[17]

The rate of H_2O_2-mediated oxidation of *o*-arylboronic acids bearing a neighboring ether (**2**, k = 1.6±0.1 $M^{-1}s^{-1}$) or oxime (**3**, k = 1.8±0.1 $M^{-1}s^{-1}$) track closely with each other and below that of the oxidation of PBA. *N, N*-dimethylaminomethyl arylboronic acid (**4**), the canonical Wulff-type arylboronic acid, reacts 20-fold more slowly with H_2O_2 than does PBA (k = 0.25±0.02 $M^{-1}s^{-1}$). Importantly, a hydrazone derived from the bulky pivaloyl hydrazide (**5**) reacts with a rate constant in line with the other examined arylboronic acids (k = 1.3±0.1 $M^{-1}s^{-1}$) (Fig. 2B). Compared to arylboronic acids, diazaborines react with both H_2O_2 and $ONOO^-$ (Fig S26-S40b) but show dramatically slower rates of H_2O_2-mediated oxidation. Time-course ^1H NMR of diazaborines (10 mM) in phosphate buffer (PB, 200 mM, pD 7.4 in D_2O) with H_2O_2 (10 mM) revealed slow consumption of the starting material compared to arylboronic acid oxidations (Figure 3A). Acyl diazaborine (**6**) was consumed the fastest (k = 3.1 × 10^{-2} $M^{-1}s^{-1}$), and methyl diazaborine (**7**, k = 1.7 × 10^{-3} M^{-1} s^{-1}) was consumed the slowest. DAB **8**, an anti-bacterial drug that inhibits bacterial fatty acid synthase,[21,1] also reacts slowly with H_2O_2 (k = 3.1 × 10^{-3} $M^{-1}s^{-1}$). DABs thus react approximately 150-1500-times slower with H_2O_2 than does PBA with H_2O_2. We attribute the faster reaction of DAB **6** with H_2O_2 to its non-planar B-N heterocycle structure[14] while **7** and **8** adopt planar heterocycle structure. Strikingly, the expected phenolic products do not grow in concomitantly with starting material consumption. Instead, for all DABs, phenol formation was preceded by the appearance of an intermediate(s) that persists for hours (**6**) or days (**7**, **8**). The oxidation of **6** in the presence of H_2O_2 results in a rapid formation of the intermediate that peaks ~4 h after reaction initiation (Fig 3B). Diazaborines **7** and **8** show a steady rise in intermediate formation with ~20% phenol production from **7** after 24 h and only trace phenol from **8** after 24 h (Fig. 3C; SI Figs S41-S42a). Notably, $ONOO^-$ (~ 4 equivalents) prompts immediate consumption of the starting material. While DAB **7** yields a complex mixture, DAB **6** and **8** are cleanly converted to the intermediate, which, upon standing, ultimately converts to the phenolic product (Fig 3D, SI Figs S43-S44b). No intermediate formation is seen during the H_2O_2- or $ONOO^-$-mediated oxidation of arylboronic acids **1-5**.

Next, we characterized the pH dependence of H_2O_2-mediated oxidation of arylboronic acids and diazaborines. To esablish a comparative baseline, we first measured the rate of H_2O_2-mediated PBA oxidation at different pH conditions in phosphate buffer (SI Figs S42-S45). The rate of H_2O_2-mediated oxidation of PBA is faster at more basic conditions than at acidic conditions. The slowest rate was observed at pH 5.5 (k = 0.013 ± 0.002 $M^{-1}s^{-1}$), followed by pH 6.5 (k = 0.49 ± 0.08 $M^{-1}s^{-1}$), then pH 7.4 (4.94 ± 0.82 $M^{-1}s^{1}$), with the fastest observed at pH 8.5 (k = 39.0±1.0 $M^{-1}s^{-1}$) (SI Figs S45-S47).

We then measured the rate of H_2O_2-mediated oxidation of DAB **6** as a function of pH (Figure 4). Additional acetohydrazide (4 equivalents) was added to prevent hydrolysis to 2-fPBA. In contrast to the oxidation of PBA, the oxidation of DAB **6** yielded its corresponding phenol faster at more acidic pH conditions. However, careful examination of the time-course ^{1}H NMR reveals that the rate of starting material consumption is largely unchanged between pH 6.5 to pH 8.5. Instead, more acidic pH conditions accelerate the conversion of the intermediate to the phenol. At pH 6.5, no detectable intermediate forms, and phenol production is the fastest (Figure 4A). At pD 7.4, a moderate amount of intermediate is formed but is rapidly converted to phenol (Figure 4B), while at pD 8.5, the most intermediate is formed, and it converts the slowest to the phenol (Figure 4C). Repeating the experiment with DAB **8** reveals no significant phenol production between pH 6.5-8.5 over 12 hours. Similar to DAB **6**, however, the rate of starting material consumption is not significantly affected by pH, and the rate of intermediate formation is also largely unaffected by pH from pH 6.5-8.5 (SI Fig 48). Forming the intermediate of **8** at pD 7.4 followed by adjusting the pD to ~4 immediately prompts conversion of the intermediate to the phenol (SI Fig 49). In total, these data indicate that the rate of starting material consumption is pH-independent from

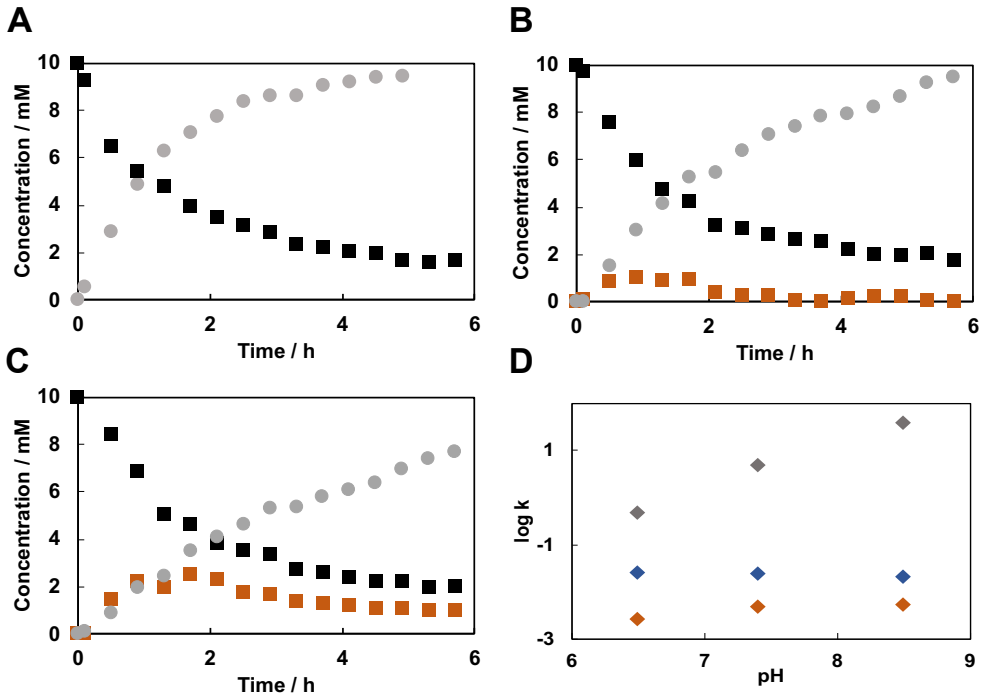
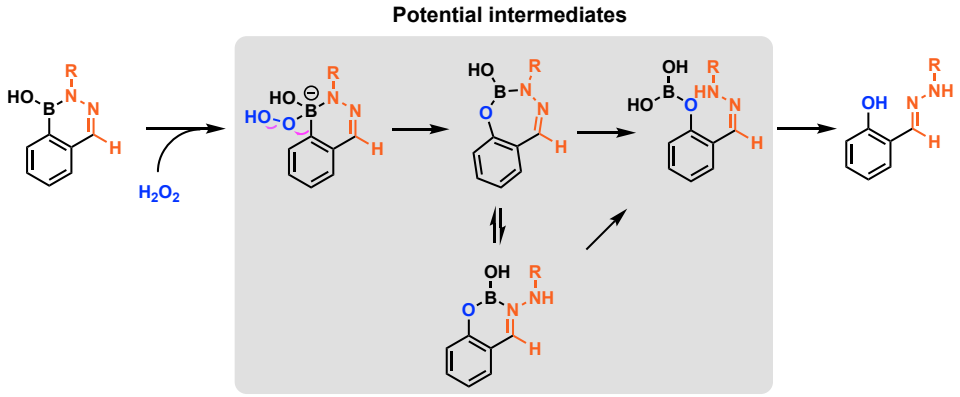


Figure 2. Effect of pH on H_2O_2-mediated oxidation of DABs. (A) Reaction of DAB **6** (10 mM, black squares) with H_2O_2 (10 mM) in buffer (200 mM phosphate buffer, 200 mM, pD 6.5) as a function of time. (B) Same conditions as (A) but at pD 7.4. (C) Same conditions as (A) but at pD 8.5. Datapoints are the concentration of **6** (black sqaures), intermediate (orange squares), and phenol (gray circles) as measured by ^{1}H NMR against a dimethylsulfone internal standard (10 mM). (D) Rate of starting material consumption for PBA (gray), **6** (blue), and **8** (orange) at each pH condition.

6.5-8.5, and higher pH conditions stabilize the intermediate and slow its conversion to the phenol.



Scheme 1. Proposed mechanism of H_2O_2-mediated oxidation of diazaborines and potential intermediates.

A proposed mechanism, based on the related arylboronic acid oxidation mechanism and a recent DFT-computed mechanism of H_2O_2-mediation oxidation of a diazaborine[9] is shown in Scheme 1. Initial adduct formation between hydrogen peroxide and diazaborine is followed by a 1,2-shift to form the C-O bond, followed by hydrolysis of the borate species to give free boric acid and the corresponding phenol. While the precise structure of the intermediate has not been fully elucidated, the foregoing experiments show that the intermediate converts to the phenol in an acid-promoted process, suggesting a potential slow hydrolysis step. Moreover, the intermediate is not a reversible complex formed between H_2O_2 and the diazaborine, as treatment of the intermediate with catalase neither regenerates the starting diazaborine nor affects the rate at which it converts to the phenol (SI Fig 50). A recent report from Raines *et. al.* attributed the resistance of 2-carboxyphenylboronic acid to H_2O_2-mediated oxidation to an impeded C→O shift resulting from borolactone formation.[20] To test this possibility for diazaborines, we formed the intermediate from diazaborine **8** with H_2O_2, decomposed excess H_2O_2 with catalase, and treated the intermediate with tris-carboxyethylphosphine (TCEP) (SI Fig 51). The intermediate is unable to oxidize TCEP, which is in contrast to the reactivity profiles of previously reported isolable boron hydroperoxides.[22] Additionally, ^{13}C NMR analysis of the intermediate shows the emergence of a new ^{13}C shift *ca.* 157 ppm, which is in line with a ^{13}C shift of the carbon bearing a phenolic oxygen and consistent with the ^{13}C spectrum of the tosylhydrazone phenol product independently synthesized from salicylaldehyde and tosylhydrazine (^{13}C ~ 159 ppm) (SI Fig S51). Finally, time-course ^{11}B NMR spectroscopy during H_2O_2 oxidation shows disappearance of starting material (*ca.* 0 ppm) and the growth of a new signal at *ca.* 18 ppm, which is consistent with a reference spectrum of boric acid (SI Figs S53-S55). Notably, there is no distinct ^{11}B signal seen for the intermediate, suggesting that the environment in the intermediate is similar to that of boric acid. Taken together, the intermediate formed upon reaction of diazaborines and H_2O_2 or $ONOO^-$ appears to have undergone the 1,2-shift to form the new C-O bond and is susceptible to acid-promoted but not base-promoted hydrolysis to form the phenol. Efforts are ongoing to crystallographically characterize the intermediate(s) and will be reported in due course.

To close, we have shown that diazaborines react ~2-3 orders of magnitude more slowly with H_2O_2 than do a library of *o*-arylboronic acids. A long-lived intermediate forms in the reaction between H_2O_2 or $ONOO^-$ with diazaborines **6-8**. Peroxynitrite prompts immediate consumption of diazaborine starting material,

leading to the formation of the intermediate, which slowly converts in aqueous buffer to its corresponding phenol. Notably, peroxynitrite is unable to oxidize 2-carboxyphenylboronic acid (2-CPBA), a recently reported arylboronic acid that is resistant to H_2O_2-mediated oxidation (SI Fig S56).[20] Thus, diazaborines may serve as privileged molecular motifs that enhance kinetic discrimination between $ONOO^-$ and H_2O_2. Direct interrogation of the intermediate structure and the exploration of diazaborine-based chemical biology tools are currently underway.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Research was conceived by DWD. Experiments were carried out by JGH, GSH, and ARM. Data analysis was performed by JGH, GSH, and DWD. ‡These authors contributed equally.

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