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## A Bioorthogonal Reaction of N-Oxide and Boron Reagents

Justin Kim and Carolyn R. Bertozzi\*

Abstract: The development of bioorthogonal reactions has classically focused on bond-forming ligation reactions. In this report, we seek to expand the functional repertoire of such transformations by introducing a new bond-cleaving reaction between N-oxide and boron reagents. The reaction features a large dynamic range of reactivity, showcasing second-order rate constants as high as  $2.3 \times 10^3 \,\mathrm{M}^{-1} \,\mathrm{s}^{-1}$  using diboron reaction partners. Diboron reagents display minimal cell toxicity at millimolar concentrations, penetrate cell membranes, and effectively reduce N-oxides inside mammalian cells. This new bioorthogonal process based on miniscule components is thus well-suited for activating molecules within cells under chemical control. Furthermore, we demonstrate that the metabolic diversity of nature enables the use of naturally occurring functional groups that display inherent biocompatibility alongside abiotic components for organism-specific applications.

he field of bioorthogonal chemistry strives to meet the demands for methods that can facilitate the molecular analysis of biological processes. Its foundational applications were in the chemical targeting of biomolecules with probes and affinity reagents, both in cultured cells and living organisms, and in building hybrid biomolecules for therapeutic applications.<sup>[1]</sup> In the context of these pursuits, chemists have compiled a reaction compendium that includes the Staudinger ligation,<sup>[2]</sup> copper-mediated<sup>[3]</sup> and metal-free<sup>[4]</sup> azide–alkyne cycloadditions, and tetrazine ligations.<sup>[5]</sup>

An emerging application of bioorthogonal chemistry is the modulation of the activity of a target molecule under the control of a small-molecule switch.<sup>[6]</sup> Small-molecule-based chemical uncaging strategies are a compelling complement to photochemical activation methods<sup>[7]</sup> in applications demanding tissue penetrance, subcellular targeting at the multi-cell level, or cellular pretargeting. To this end, reactions that impart new functionality on their targets beyond the attachment of probes are particularly useful but remain relatively rare in the compendium—recent advances in transitionmetal-<sup>[6a,8]</sup> and non-metal-mediated<sup>[6b,9]</sup> uncaging strategies notwithstanding.

The tetrazine ligation reaction has recently been adapted by Chen and co-workers for the bioorthogonal uncaging of modified lysine residues on target proteins within cells<sup>[6b]</sup> and by Robillard and co-workers for the activation of prodrugs

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in vitro;<sup>[9d]</sup> notably, however, the performance of the reaction in the context of ligation reactions remains unmatched. In many ways, current bioorthogonal methods reflect an evolutionary history of selection for bond-forming reactions. Perhaps simpler, faster, and more tolerant bond-cleaving reactions could be constructed anew from a bioorthogonal set outside the ligation-centric compendium. Such reactions would fulfill the demand for novel triggers, which, in combination with a generic immolative linker,<sup>[9a-c,e]</sup> would comprise a general scheme for the uncaging of biological effectors. We thus turned to new sources for the development of bioorthogonal reactions.

Organism-specific variations in biological reactivity allow the potential discovery of bioorthogonal reaction partners not just from outside but also within biology. Natural organisms possess considerable metabolic diversity, such that functional groups endogenous to one species (e.g., terminal alkynes)<sup>[10]</sup> can be orthogonal in another. Trimethylamine *N*-oxide (TMAO, **1**; Figure 1) provides another case in point. Elasmobranchs,<sup>[11]</sup> deep-sea teleosts,<sup>[12]</sup> and other osmoconforming



**Figure 1.** Determination of kinetic parameters. A) The reaction between TMAO (1) and *para*-nitrophenylboronic acid (2) features biologically compatible reagents and byproducts. B) The reaction of *N*,*N*-dialkylani-line-derived *N*-oxide **6** and phenylboronic acid is three orders of magnitude faster. C) General scheme for the use of a triggered reaction for the uncaging of biological effectors.

marine invertebrates acquire high concentrations of urea to maintain high internal osmolalities. The chaotropic effects of urea are countered by proportionally high concentrations of the potent kosmotrope TMAO.<sup>[13]</sup> In deep-sea fish, where the kosmotropic effects of TMAO are crucial for countering high

 <sup>[\*]</sup> Dr. J. Kim, Prof. C. R. Bertozzi
 Department of Chemistry and Howard Hughes Medical Institute Stanford University
 380 Roth Way, Stanford, CA 94305 (USA)
 E-mail: bertozzi@stanford.edu
 Supporting information and ORCID(s) from the author(s) for this

article are available on the WWW under http://dx.doi.org/10.1002/ anie.201508861.

hydrostatic pressures, its concentration can reach up to  $400 \text{ mm}^{[12]}$  Even at extreme levels, *N*-oxides are inert to biomolecules. Critically, for most organisms, including humans, TMAO is not a component of osmoregulation. Very low concentrations of this analyte (ca. 1  $\mu$ M) are present in the serum as a metabolic byproduct of gut-microbiota-derived trimethylamine.<sup>[14]</sup> By refining our notion of bioor-thogonality and looking to nature for inspiration, we have identified *N*-oxides as potential reaction partners with inherent biocompatibility.

Similarly, boron-containing compounds fit the description that they are sometimes, yet infrequently, found in biological systems. Indeed, natural-product antibiotics such as boromycin incorporate boron into their structures. Furthermore, boronic acids are widely appreciated for their relatively benign toxicology profile<sup>[15]</sup> and have been incorporated into chemical biology methods, for example, in fluorogenic tetraserine-binding<sup>[16]</sup> and peroxide-responsive probes.<sup>[17]</sup> Given the positive qualities of this functional group, we wished to combine *N*-oxides with boronic acids to generate a new bioorthogonal reaction.

The hydroxydeboration reaction between TMAO and alkyl boranes was discovered by Köster and Morita in 1967.<sup>[18]</sup> Its remarkable functional-group tolerance and quantitative nature continue to render it a mild, reliable alternative to hydrogen peroxide mediated deborylative oxidations under conditions challenging for total synthesis.<sup>[19]</sup> Kabalka and Hedgecock's subsequent report that the dihydrate of TMAO is just as effective as its anhydrous form<sup>[20]</sup> offered the first hint that the reaction would work in aqueous media, a necessary criterion for bioorthogonality.

We first set out to determine the kinetic parameters for the reaction between TMAO (1) and *para*-nitrophenylboronic acid (2) in water. The reaction progress was monitored by measuring the UV/Vis absorption of the *para*-nitrophenol product (3) under pseudo-first-order conditions (Figure 1). We determined a second-order rate constant of  $2.83 \pm 0.05 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$  for the reaction at room temperature, which is several orders of magnitude below the current standards of bioorthogonal reactivity<sup>[1b-e]</sup> but nonetheless a starting point for kinetic optimization.

Conjecturing that the concomitant C-B bond migration and N–O bond cleavage events are rate-limiting (Figure 1A), we expected that the principal determinants of the reaction rate would be the leaving-group ability of the tertiary amine and the migratory aptitude of the boronic acid. Focusing first on the former, the reaction could indeed be accelerated by turning to N,N,N-dialkylaryl N-oxides, which produce superior leaving groups compared to trialkyl amines.<sup>[21]</sup> The kinetic parameters for this reaction were measured by employing the fluorogenic N, N, N-dialkylaryl N-oxide  $6^{[22]}$ which was obtained through mCPBA mediated oxidation of the parent rhodol fluorophore.<sup>[23]</sup> The reaction of N-oxide **6** with phenylboronic acid in phosphate-buffered saline (PBS, pH 7.4) proceeded with a second-order rate constant of  $1.28 \pm$  $0.11 \times 10^{-3} \text{ m}^{-1} \text{ s}^{-1}$  (Figure 1 B). While gratified by the rate acceleration of three orders of magnitude relative to our baseline reaction using TMAO, we sought even faster rates for use in biological systems.

We thus turned to the second factor, namely the migratory aptitude of the boronic acid. We anticipated that significant gains could be achieved through direct weakening of the dissociating bond. Accordingly, we considered exchanging the migrating C–B bond for a B–B bond. The reduction of *N*-oxides by bis(pinacolato)diboron (B<sub>2</sub>pin<sub>2</sub>), first reported by Carter et al. in 2002,<sup>[24]</sup> exploits the cleavage of a weak B–B bond (68 kcal mol<sup>-1</sup>) and the formation of strong B–O bonds (125 kcal mol<sup>-1</sup>) to provide an enthalpic driving force of about 180 kcal mol<sup>-1</sup>. This powerful reaction, which superficially effects nothing more than deoxygenation, has seen scant use.<sup>[25]</sup> We immediately sought to characterize its reaction kinetics and adapt it for biological systems.

We first evaluated the kinetics of the reaction using *N*-oxide **6** (Figure 2 A). Impressively, fluorescence measurements with a stopped-flow fluorometer under pseudo-first-order conditions revealed a second-order rate constant of  $8.05 \pm 0.076 \times 10^2 \,\text{m}^{-1} \,\text{s}^{-1}$  in PBS (pH 7.4). We also synthesized the HaloTag linker-bound profluorophore **8**, designed for use in cell-labeling studies (see below), and found the second-order rate constant for its reaction with B<sub>2</sub>pin<sub>2</sub> to be even higher at  $1.71 \pm 0.043 \times 10^3 \,\text{m}^{-1} \,\text{s}^{-1}$ , which is likely due to steric relaxation (Figure 2 B).

We then explored the reaction on a biomolecule. The 34 kDa HaloTag protein was ligated to compound 8 to produce HaloTag-8, which was purified by size-exclusion chromatography and treated with B<sub>2</sub>pin<sub>2</sub> under pseudo-firstorder conditions. Analysis by stopped-flow fluorometry revealed a second-order rate constant of  $2.30 \pm 0.073 \times$  $10^3 \text{ M}^{-1} \text{s}^{-1}$  (Figure 2 C). It should be noted, however, that pseudo-first-order kinetics obtained under saturating conditions can obscure important information regarding the deactivation of the diboron reagent through sequestration or off-target reactivity. To address this issue, a 64 kDa GFP-HaloTag fusion protein was expressed and ligated to compound 10 to produce GFP-HaloTag-10. Conjugate 11 (500 nm) was then treated with stoichiometric to slightly superstoichiometric quantities of  $B_2 pin_2$  (1–25 µM) and analyzed by in-gel fluorescence imaging. Figure 2D shows that 5-10 equivalents of B<sub>2</sub>pin<sub>2</sub> are necessary to fully reduce the conjugated fluorophore 10 in < 15 min. Considering that 2 equivalents of reductant are theoretically required, this experiment validates the robustness of the reaction and suggests minimal off-target reactivity.

Having confirmed the compatibility of our *N*-oxide/ diboron reaction with proteins, we explored the viability of the reaction in mammalian (Jurkat) cell lysate (Figure 2 E). Lysates were made to a final protein concentration of 1 mgmL<sup>-1</sup>, and variable concentrations of B<sub>2</sub>pin<sub>2</sub> were reacted with a 1  $\mu$ M solution of probe **6**. The fluorescence intensities were then measured after 30 min. Consistent with prior kinetic data, adding just 5 equivalents of B<sub>2</sub>pin<sub>2</sub> was sufficient to drive the reaction to completion in mammalian cell lysate within the allotted time.

An organism-centric approach to bioorthogonal reaction development, a central facet of our thesis, enables the implementation of reactions that would be overlooked under more stringent searches for abiotic reaction partners. As a point of emphasis, we also performed the preceding



**Figure 2.** Evaluating the bioorthogonality of diboron reagents. A)  $B_2pin_2$  rapidly reduces *N*-oxide **6** in PBS (pH 7.4). The second-order rate constant was calculated from fluorescence measurements of fluorophore **7** under pseudo-first-order conditions with saturating concentrations of  $B_2pin_2$ . B) Steric relaxation around the *N*-oxide enhances the reaction rate, and the second-order rate constant exceeds  $10^3 \text{ m}^{-1} \text{ s}^{-1}$ . C) *N*-Oxide **9** conjugated to a 34 kDa HaloTag protein rapidly reacts with  $B_2pin_2$  in PBS. The reaction conversions for the lowest and highest  $B_2pin_2$  concentrations used in the kinetics experiments are displayed. Data represent the mean of triplicate experiments. The vertical dotted lines indicate the half-lives of the reactions at the respective saturating concentrations of  $B_2pin_2$ . D) Bis(*N*-oxide) profluorophore **10** (500 nm) was conjugated to a 64 kDa GFP–HaloTag fusion and treated with stoichiometric to slightly superstoichiometric quantities of  $B_2pin_2$ . The red and green channels represent the TAMRA and GFP signals, respectively. E) *N*-Oxide profluorophore **6** (1 µm) was reacted with 0 (black), 5 (light blue), 50 (middle blue), or 500 µm (dark blue)  $B_2pin_2$  in Jurkat and *E. coli* cell lysates containing 1 mg mL<sup>-1</sup> of protein; the fluorescence was then normalized to the level of a positive control based on fluorophore **7** (violet). Data represent the mean of triplicate experiments. Error bars represent the standard deviation. F) Time-dependent fluorescence measurements reveal the stability of *N*-oxide profluorophore **6** to mammalian cell lysate. Data represent the mean of triplicate experiments. Error bars represent the standard deviation and triplicate experiments. G) The viability of three mammalian cell lines at a range of  $B_2pin_2$  concentrations was evaluated after 24 hours using an MTT assay. Data represent the mean of triplicate experiments. Error bars represent the standard deviation.

experiment in *E. coli* cell lysate. *E. coli*, like several other facultative anaerobes, possess an *N*-oxide reductase<sup>[26]</sup> and were predicted to be incompatible with our method. Indeed, the results confirmed this hypothesis: Our negative control containing no  $B_2pin_2$  displayed high levels of fluorescence (Figure 2 E). Time-dependent fluorescence measurements of *N*-oxide probe **6** in *E. coli* cell lysate confirmed the gradual reduction of the *N*-oxides with a half-life of about 15 min (Figure 2 F). Gratifyingly, no degradation was observed in mammalian cell lysate over 11 hours. Therefore, the *N*-oxide/ diboron reaction is not suitable for all systems, but should still be powerful in many.

To ascertain the toxicity of the diboron reagents to cells, we subjected three mammalian cell lines (HEK293T, HeLa, and MEF) to the MTT cell viability assay (Figure 2 G). Each cell line was treated with twofold serial dilutions of  $B_2pin_2$ starting at 1 mM in 0.5% DMSO/DMEM (DMEM = Dulbecco's modified eagle medium). The maximum concentration of  $B_2pin_2$  was dictated by its solubility limitations in the medium. The MTT assays indicated that the human cell lines are relatively insensitive to the diboron reagents, at least at the concentrations that were tested, and whereas for MEF cells, some toxicity was observed at higher diboron levels, the IC<sub>50</sub> value was > 1 mM. No aberrations in cell morphology were observed for HEK293T cells upon treatment with 1 mm  $B_2pin_2$  for 24 hours (Figure S5). Furthermore, no gross changes in the time to confluency were observed between cells treated with  $B_2pin_2$  and DMSO vehicles.

Next, we demonstrated the mutual orthogonality of our N-oxide/diboron reaction with a representative cohort of bioorthogonal reactions commonly in use today: the aminooxy-aldehyde condensation, the azide-cyclooctyne cycloaddition, and the tetrazine-cyclopropene ligation (Figure 3). To showcase the robustness of our new reaction, we executed these experiments amidst the complexity of a cellular system. Utilizing a combination of chemical, genetic, and metabolic engineering techniques, four populations of HEK293T cells were endowed with distinct cell-surface modifications. Population 1 was modified by sodium periodate to display cellsurface aldehydes derived from sialic acid moieties;<sup>[27]</sup> population 2 was modified with azides through the metabolic incorporation of Ac<sub>4</sub>ManNAz;<sup>[2]</sup> population 3 was modified with a cyclopropene through the metabolic incorporation of Ac<sub>4</sub>ManNCp;<sup>[28]</sup> and population 4 was modified by cell-surface expression of an N-terminal HaloTag-EGFR fusion protein followed by the ligation of HaloTag-linked bis(Noxide) TAMRA profluorophore 10. Each cell population was labeled with a distinct combination of Hoechst 33342 and





**Figure 3.** The N-oxide/diboron reaction is compatible with common bioorthogonal reactions. A) HEK293T cell surfaces were modified with aldehydes, N-oxides, azides, or cyclopropenes, mixed, and then treated with a cocktail of bioorthogonal reagents. Each population was analyzed by flow cytometry for reaction with aminooxy-Alexa Fluor 488 (light blue), B<sub>2</sub>pin<sub>2</sub> (middle blue), DIBAC-Cy5 (dark blue), and tetrazine-Cy7 (violet). The mean normalized fluorescence intensity of each fluorophore from three biological replicates is shown. Error bars represent the standard deviation. B) N-Oxide-modified HEK293T cells were treated with aminooxy, DIBAC, and tetrazine reagents with or without a diboron reagent. N-Oxides are not reactive towards hydroxylamines, cyclooctynes, or tetrazines.

Syto 41 nuclear stains, combined, then treated with a reagent cocktail consisting of 10 mM aniline, 100  $\mu$ M aminooxy-Alexa Fluor 488, 10  $\mu$ M DIBAC-Cy5, 20  $\mu$ M tetrazine-Cy7, and 100  $\mu$ M B<sub>2</sub>pin<sub>2</sub> in pH 6.7 PBS. B<sub>2</sub>pin<sub>2</sub> was applied at a concentration of 100  $\mu$ M to rigorously challenge the aminooxy condensation reaction, the reaction most likely to be perturbed by diboron reagents. The mixture of cells was then analyzed by flow cytometry.

Impressively, each of the cell types was labeled highly selectively by its corresponding bioorthogonal partner, and the labeling efficiency was undiminished in the presence of the complete complement of reactive functional groups (Figure 3). Notably, aminooxy functional groups are fully compatible with diboron reagents, and the *N*-oxide functional group is neither reduced by nor reactive towards tetrazines, which are known to be susceptible to degradation through nucleophilic attack.

Finally, to demonstrate the utility of the N-oxide/diboron reaction in an intracellular uncaging application, we first had to determine the membrane permeability of each of the compounds and evaluate their bioorthogonality in live cells. We transiently transfected HEK293T cells with a cytosolic GFP-HaloTag fusion construct and then treated the cells with  $100 \,\mu\text{M}$  profluorophore **10**. After three washes, the cells were treated with 1 mm, 100 µm, 10 µm, or 0 µm B<sub>2</sub>pin<sub>2</sub> for 45 min (Figures 4; see also the Supporting Information, Figure S1). The TAMRA signal increased upon addition of B2pin2 and also co-localized with the GFP signal. These data indicate that both profluorophore 10 and the diboron reagent are cellpermeable, that the reaction can be performed intracellularly, and, impressively, that fluorophore activation can be observed at a diboron concentration of 10 µM with full activation requiring at most 100 µM B<sub>2</sub>pin<sub>2</sub>, which is well below the toxic levels.



**Figure 4.** B<sub>2</sub>pin<sub>2</sub> activates *N*-oxides on cytosolic proteins in mammalian cells. HEK293T cells transfected with GFP–HaloTag were incubated with 100 μm of profluorophore **10**, washed, treated with 0 or 100 μm B<sub>2</sub>pin<sub>2</sub>, and then imaged by confocal microscopy after 45 min. The merge is a composite of GFP, TAMRA, and Hoechst 33342 fluorescence with a bright-field image.

Fast rates of ligand hydrolysis, even for bulky pinacol derivatives, enable the utilization of diboron species even amidst the diols present in biological milieu.  $B_2(OH)_4$  is as nontoxic and competent at reducing *N*-oxides (Figures S2–S4) as  $B_2pin_2$ . Furthermore, the tolerance of the reaction to sterically demanding ligand structures on the boron reagents indicates that even diboron species transiently ligated to saccharides or other cellular diols may still be competent at reducing *N*-oxides.

In conclusion, we have sought new reactions to expand the functional repertoire of bioorthogonal transformations beyond bond-forming ligation reactions. We initiated our search for new bioorthogonal reactivity by relaxing its definition. Rather than exploring chemical space completely outside the realm of biology, we focused our search for molecular components within organisms that are metabolically orthogonal to models of human disease. As a result, we could readily find novel reaction components with inherent biocompatibility.

We have thus described a powerful new reaction between *N*-oxide and boron reagents. The reaction features fast reaction kinetics that rival the fastest bioorthogonal reactions reported to date while introducing functionality that has yet to gain significant attention. Owing to the benignity of the reaction conditions towards live cells and its intracellular compatibility, this reaction holds much promise as a method for the small-molecule activation of biomolecules in vivo.

We envision that this method will find application in the spatially and temporally controlled chemical uncaging of important naturally occurring trialkyl amines, such as the epigenetically relevant dimethyllysine histone residues.<sup>[29]</sup> Furthermore, this reaction acts as a powerful trigger, which, when interfaced with chemical or biological effectors through immolative linkers<sup>[9]</sup> containing an *N*-oxide, should enable the small-molecule activation of enzymes and chemotherapeutics.



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