**Marquette University**

**e-Publications@Marquette**

***Biological Sciences Faculty Research and Publications/College of Arts and Sciences***

***This paper is NOT THE PUBLISHED VERSION*.**

Access the published version via the link in the citation below.

*Biochemistry*, Vol. 60, No. 32 (August 2021): 2508-2518. [DOI](https://doi.org/10.1021/acs.biochem.1c00374). This article is © American Chemical Society and permission has been granted for this version to appear in [e-Publications@Marquette](http://epublications.marquette.edu/). American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

Slow-Onset, Potent Inhibition of Mandelate Racemase by 2-Formylphenylboronic Acid. An Unexpected Adduct Clasps the Catalytic Machinery

Colin D. Douglas

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada

Lia Grandinetti

Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin

Nicole M. Easton

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada

Oliver P. Kuehm

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada

Joshua A. Hayden

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada

Meghan C. Hamilton

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada

Martin St. Maurice

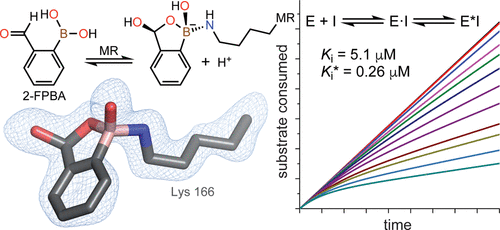
Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin

Stephen L. Bearne

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada

Department of Chemistry, Dalhousie University, Halifax, NS B3H 4R2, Canada

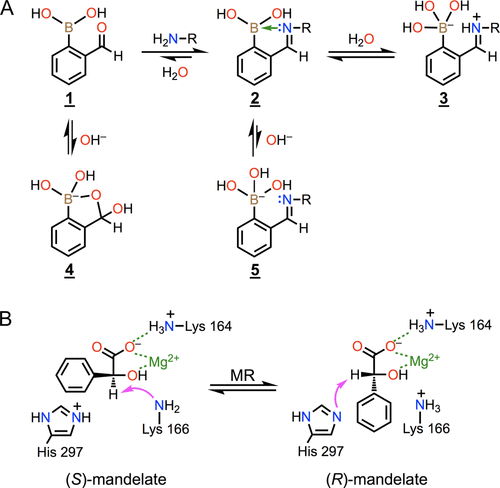
# Abstract



*o*-Carbonyl arylboronic acids such as 2-formylphenylboronic acid (2-FPBA) are employed in biocompatible conjugation reactions with the resulting iminoboronate adduct stabilized by an intramolecular N–B interaction. However, few studies have utilized these reagents as active site-directed enzyme inhibitors. We show that 2-FPBA is a potent reversible, slow-onset inhibitor of mandelate racemase (MR), an enzyme that has served as a valuable paradigm for understanding enzyme-catalyzed abstraction of an -proton from a carbon acid substrate with a high . Kinetic analysis of the progress curves for the slow onset of inhibition of wild-type MR using a two-step kinetic mechanism gave  and  values of and μM, respectively. Hence, wild-type MR binds 2-FPBA with an affinity that exceeds that for the substrate by ∼3000-fold. K164R MR was inhibited by 2-FPBA, while K166R MR was not inhibited, indicating that Lys 166 was essential for inhibition. Unexpectedly, mass spectrometric analysis of the NaCNBH3-treated enzyme–inhibitor complex did not yield evidence of an iminoboronate adduct. 11B nuclear magnetic resonance spectroscopy of the MR·2-FPBA complex indicated that the boron atom was sp3-hybridized , consistent with dative bond formation. Surprisingly, X-ray crystallography revealed the formation of an dative bond between Lys 166 and 2-FPBA with intramolecular cyclization to form a benzoxaborole, rather than the expected iminoboronate. Thus, when *o*-carbonyl arylboronic acid reagents are employed to modify proteins, the structure of the resulting product depends on the protein architecture at the site of modification.

The reaction between aldehydes and the amino group of a Lys residue or the amino terminus of a protein to form an imine (Schiff base) has been widely employed to modify proteins; (1) however, the use of an aldehyde that forms an imine that is stabilized through intramolecular interactions has been less widely employed. Early examples include the use of salicylaldehyde to modify amino groups of proteins in which the imine is stabilized via an intramolecular H-bond (2−5) or a metal chelate. (6−8) More recently, *o*-carbonyl arylboronic acids such as 2-formylphenylboronic acid [2-FPBA, **1** (Scheme 1A)] and 2-acetylphenylboronic acid (2-APBA) have been used to modify the N-terminus of the protein (especially an N-terminal Cys) (9,10) or the  group of Lys residues in proteins, (11−13) serving as the basis for several biocompatible conjugation reactions used in chemical biology. (14−17) Because boron has a vacant p orbital, it is a Lewis acid and a strong electrophile. Hence, either direct coordination of the lone pair of electrons on the imine nitrogen atom to the boron atom (**2**, i.e., an N–B interaction) (11) or a solvent-inserted zwitterionic species (**3**, observed in protic solvents) (18,19) stabilizes the enzyme–iminoboronate adduct by approximately 3–7 kcal/mol (Scheme 1A). (11,14) Furthermore, initial nucleophilic attack by the amine is facilitated by an O–B interaction in the *o*-carbonyl arylboronic acid reagent. (15) More recent strategies have focused on stabilizing iminoboronate-based adducts by employing -nucleophiles such as hydrazines, hydrazides, and hydroxylamines. (14−16,20)

**Scheme 1**



Considering the ability of 2-FPBA to interact with amino groups, we sought to explore 2-FPBA as an inhibitor of mandelate racemase (MR). Indeed, there have been few reports of *o*-carbonyl arylboronic acids as purely active site-directed enzyme inhibitors. (21) MR is the archetype of the enolase superfamily and has served as a useful paradigm for understanding how enzymes deprotonate carbon acid substrates with relatively high  values. (22−24) MR possesses a  (KXK) motif at its active site, which is a characteristic feature of enzymes in the MR and d-glucarate dehydratase subgroups of the enolase superfamily. (25)  serves as a Brønsted base to catalyze the deprotonation of the substrate (*S*)-mandelate (Scheme 1B), and  is believed to modulate the  of . (26,27) Because 3-hydroxypyruvate was shown previously to form an imine with Lys 166 of MR, (28) which irreversibly inactivated the enzyme through formation of an aldehyde/enol(ate) adduct, we anticipated that the enzyme might be susceptible to inhibition by 2-FPBA due to the propensity of this reagent to modify Lys residues through imine formation. Consequently, we rationalized that 2-FPBA should be bound with a high affinity as we recently demonstrated for phenylboronic acid (PBA) (29) and form a stabilized iminoboronate through Schiff base formation with one of the Lys residues present in the KXK motif. Herein, we show that 2-FPBA is the first reversible slow-onset inhibitor of MR, binding with an affinity that exceeds that of PBA. Most surprisingly, X-ray crystallography revealed that, unlike the MR·PBA complex that formed a weak dative bond with His 297 at the active site, MR formed an N–B dative bond between Lys 166 and the boronic acid group accompanied by intramolecular cyclization to form a benzoxaborole derivative, rather than the anticipated iminoboronate. These findings furnish a caveat that alternative products other than iminoboronates may arise when *o*-carbonyl arylboronic acid reagents are employed to modify proteins depending on the protein architecture at the site of modification.

# Materials and Methods

## General

Benzohydroxamate (BzH) and all other reagents, unless mentioned otherwise, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). 2-Formylphenylboronic acid was purchased from TCI America (Portland, OR), and 2-acetylphenylboronic acid, 4-chloro-2-formyphenylboronic acid (4-Cl-2-FPBA), and 2-carboxyphenylboronic acid (2-CPBA) were purchased from Combi-Blocks (San Diego, CA). DNA oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Circular dichroism (CD) spectral measurements were taken using a JASCO J-810 spectropolarimeter (Jasco Inc., Easton, MI). 11B nuclear magnetic resonance (NMR, 160.5 MHz) spectra were recorded using a Brüker AVANCE 500 NMR spectrometer at the Dalhousie Nuclear Magnetic Resonance Research Resource (NMR-3) Centre. Mass spectrometry was conducted at the AIMS Mass Spectrometry Laboratory (Department of Chemistry, University of Toronto, Toronto, ON).

## Site-Directed Mutagenesis

The MR variants K164R and K166R were created using site-directed mutagenesis. For these MR variants, the pET-52b(+)-wtMR plasmid (30) was used as the template for site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and following the protocols described by the manufacturer. Reactions were conducted using *Pfu*Turbo DNA polymerase (Bio Basic Inc., Markham, ON). The forward (F) and reverse (R) synthetic deoxyoligonucleotide primers used to incorporate the desired mutation into the open reading frames encoding the K164R and K166R MR variants were as follows: 5′-GGATTCCGGGCGGTTA**G**GACCAAGATCGGC-3′ (F, K164R), 5′-GCCGATCTTGGTC**C**TAACCGCCCGGAATCC-3′ (R, K164R), 5′-CCGGGCGGTTAAGACC**CG**GATCGGCTATCCG-3′ (F, K166R), and 5′-CGGATAGCCGATC**CG**GGTCTTAACCGCCGG-3′ (R, K166R). The modified codons are underlined, and the altered bases are shown in boldface. After site-directed mutagenesis, mutant plasmids were used to transform competent *Escherichia coli* DH5α cells for plasmid maintenance. Each mutant open reading frame was sequenced using commercial automated DNA sequencing (Robarts Research Institute, London, ON) to ensure that no other alterations in the nucleotide sequence had been introduced.

## Enzyme Purification

StrepII-tagged recombinant variants of MR (wild-type MR, K164R MR, and K166R MR) were overexpressed in and purified from *E. coli* BL21(DE3) cells transformed with the appropriate plasmid as described previously. (30) These constructs encode the wild-type or mutant MR gene products as fusion proteins with an N-terminal StrepII tag (MASWSHPQFEKGALEVLFQGPGYHM1-MR, where M1 denotes the initial Met of either the wild-type or variant MRs and the StrepII tag is underlined). Briefly, two starter cultures containing lysogeny broth (LB, 5 mL) and ampicillin (50 μg/mL) were inoculated with a glycerol stock solution (10 μL each) and incubated overnight at 37 °C while being continuously shaken at 225 rpm. Both starter cultures were used to inoculate LB medium (1.0 L) containing ampicillin (50 μg/mL) in a 2-L Erlenmeyer flask. Cultures producing wild-type MR were incubated for ∼8 h at 37 °C, and cultures producing either K164R or K166R MR were incubated for 2 h at 37 °C followed by 24 h at 25 °C, each with continuous shaking at 225 rpm (no induction by IPTG was used for expression of any enzyme variants). (30) The cells were then harvested by centrifugation (3000*g*, 10 min, 4 °C), and the cell pellet was stored at −20 °C until further processing. Two frozen pellets were thawed and resuspended in ice-cold wash buffer [Tris-HCl buffer (100 mM, pH 8.0) containing NaCl (150 mM) and EDTA (1 mM)]. The cells were lysed using sonication with 6 × 30 s bursts with 1 min cooling intervals, at a constant power setting of 5.5 using a Branson Sonifier 250 instrument. The soluble cell extract was clarified by centrifugation (110000*g*, 30 min, 4 °C), and the supernatant (30–40 mL) was loaded onto a column containing Strep-Tactin Superflow affinity resin (10 mL) (IBA GmbH, Göttingen, Germany) connected to an ÄKTA FPLC instrument. After the column had been washed with wash buffer (5 × the column volume), the enzyme was eluted with wash buffer containing desthiobiotin (2.5 mM). Upon elution, the enzyme was dialyzed against assay buffer [Na+-HEPES buffer (100 mM, pH 7.5) containing MgCl2 (3.3 mM)] and stored at −20 °C. The purity of the enzyme (≥99%) was assessed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% acrylamide) with staining by Coomassie blue R-250. (31) Protein concentrations were determined from the intrinsic enzyme absorbance at 280 nm using extinction coefficients (ε) of 53400 M–1 cm–1 for all MR variants, which were estimated using the ProtParam program from ExPasy (http://web.expasy.org/protparam/). (32) The StrepII tag was not removed from the enzymes.

## MR Assays

MR activity was assayed using a CD-based assay by following the change in ellipticity of mandelate at 262 nm with a 1-cm light path (unless otherwise indicated) as described by Sharp et al. (33) All kinetic assays, including inhibition experiments, were conducted at 25 °C in Na+-HEPES buffer (0.1 M, pH 7.5) containing MgCl2 (3.3 mM). The concentration of (*R*)- or (*S*)-mandelate for assays ranged from 0.25 to 15.0 mM, and the concentrations of wild-type MR, K164R MR, and K166R MR were 3.0, 400, and 3000 nM, respectively. Reactions (total volume of 2.0 mL) were initiated by the addition of the enzyme solution (100 μL) containing 0.1% (w/v) bovine serum albumin. The apparent kinetic constants  and  were determined by fitting eq 1 to the initial velocity data using nonlinear regression analysis and KaleidaGraph version 4.02 from Synergy Software (Reading, PA). All kinetic parameters were determined in triplicate, and average values are reported. The reported errors are the standard deviations.

(1)

## Inhibition Studies

The ability of a variety of aldehydes to inhibit wild-type MR was assessed by measuring the relative activity (i.e., ) of wild-type MR (3.0 nM) with (*S*)-mandelate (2.0 mM) as the substrate and varying concentrations of benzaldehyde (1.0 μM to 5.0 mM), 2-carboxybenzaldehyde (1.0 μM to 2.0 mM), salicylaldehyde (1.0 μM to 2.0 mM), and 2-formylbenzenesulfonate (1.0 μM to 5.0 mM). The  values for inhibition of wild-type MR (3.0 nM) at pH 7.0, 7.5, and 8.0; K164R MR (300 nM); and K166R MR (2300 nM) by 2-FPBA (0.2–20.0, 1.5–10.5, and 1.0–5000.0 μM, respectively) were determined by measuring the relative velocities (i.e., ) with (*S*)-mandelate (10.0 mM) as the substrate at the indicated concentrations of 2-FPBA. The velocities used were those attained at steady state (i.e.,  obtained after full onset of inhibition) while following the reaction for 10 min. The relative velocities for inhibition of wild-type MR (3.0 nM) were also determined for 4-Cl-2-FPBA (0.5–4.0 μM), 2-APBA (50.0–750.0 μM), and 2-CPBA (0.1–3.8 mM) in a similar manner using (*R*)-mandelate (10.0 mM) as the substrate. At higher inhibitor concentrations, the light path length was decreased from 1.0 to 0.5 or 0.2 cm when appropriate.  values were determined by fitting the observed relative velocities ( or  when slow-onset inhibition was observed) to eq 2.

(2)

The reversibility of inhibition by 2-FPBA was evaluated by measuring the recovery of enzyme activity after dilution. Wild-type MR (300 nM) was incubated with 2-FPBA (6.0 μM) at 25 °C, and at various times (2, 7, 11, 16, 20, 24, 29, 44, and 60 min), an aliquot was removed and diluted 100-fold into assay buffer containing (*S*)-mandelate (final concentration of 2.0 mM) and the steady state velocity was then measured using the standard MR assay.

The slow onset of inhibition by 2-FPBA was measured by following the change in the ellipticity at 262 nm over 15 min using a quartz cuvette with a 1.0-cm light path length. The reaction was initiated by addition of either wild-type MR (3.0 nM) or K164R MR (300.0 nM) to a solution of (*S*)-mandelate (5.0 mM) containing varying concentrations of 2-FPBA (0.1–20.0 μM) in assay buffer. Equation 3 was used to fit all of the resulting progress curves simultaneously using nonlinear regression analysis and OriginPro version 9.0 (OriginLab Inc., Northampton, MA), where , , and  are defined by eqs 4–6, respectively, in accord with the two-step, competitive inhibition kinetic mechanism shown in Scheme 2A. (34,35)

(3)

(4)

(5)

(6)

For the inhibition of K164R MR by 2-FPBA, the initial rate (*v*i) was found to be independent of the concentration of 2-FPBA and the progress curves were fit to a single-step, slow-onset inhibition model (Scheme 2B) where eqs 7–9 replaced eqs 4–6, respectively. (34,35)

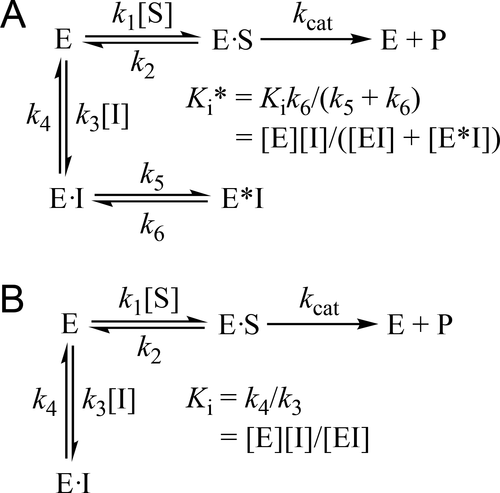
(7)

(8)

(9)

The two- and one-step models were chosen to fit the slow-onset inhibition for wild-type MR and K164R MR, respectively, because these models afforded the fits with better correlation coefficients.

**Scheme 2**



Inhibition experiments with benzohydroxamate (BzH) were conducted at 25 °C in Na+-HEPES buffer (0.1 M, pH 7.5) containing MgCl2 (3.3 mM). The concentration of (*S*)-mandelate in these assays ranged from 0.5 to 10.0 mM. The concentrations of BzH used were 10, 20, and 30 μM with wild-type MR (3.0 nM); 50, 75, and 100 μM with K164R MR (200 nM); and 50, 100, and 200 μM with K166R MR (3000 nM). Competitive inhibition constants () for BzH were determined from plots of the apparent  values versus inhibitor concentration in accord with eq 4. (36)

## Mass Spectrometry (MS)

Samples of MR were prepared for MS by exchanging assay buffer with ammonium acetate (10 mM, pH 7.5). The final concentrations of both the enzyme and the inhibitor were 10 μM. Mass spectra were recorded on an AB/Sciex QStarXL mass spectrometer equipped with an ion spray source operated in positive ion mode. Ions were scanned in the range of *m*/*z* 800–4000 with accumulation times of 1 s per spectrum with no interscan time delay. Experiments were conducted to trap the imine adduct using NaCNBH3. Wild-type MR (10 μM) in assay buffer was incubated with 2-FPBA (10 or 100 μM) for 30 min and then reacted with NaCNBH3 (2.5 mM) overnight at 4 °C.

## 11B NMR Spectroscopy

All NMR spectra were recorded at 25 °C in Na+-HEPES buffer (0.1 M, pH 7.5) containing MgCl2 (3.3 mM) and D2O (10%). Chemical shifts (δ) of the signals arising from 11B are reported relative to an external standard of BF3·OEt2 (δ 0.00). Samples were measured in 5-mm quartz tubes (Sigma-Aldrich Canada Ltd.) to reduce the background signal arising from boron in borosilicate glass. For the spectra of 2-FPBA in the presence of MR, the concentration of 2-FPBA was fixed at 400 μM and the spectra were recorded with varying amounts of wild-type MR added to the solution (0–425 μM). To verify that 2-FPBA was bound at the active site of MR, the competitive inhibitor BzH was added to the sample in excess (5 mM) to displace the 2-FPBA from the active site. As a model reference reaction for possible imine formation, the 11B NMR spectra were also recorded for 2-FPBA (5.0 mM) in the absence and presence of 5, 15, 500, and 3000 mM ethylamine hydrochloride, all in Na+-HEPES buffer (0.1 M, pH 7.5) containing MgCl2 (3.3 mM) and D2O (10%). In addition, the effect of mandelate on the 11B NMR spectrum of 2-FPBA was examined. The 11B NMR spectra were recorded for 2-FPBA (5.0 mM) in the presence of 5.0 and 50.0 mM (*S*)-mandelate in Na+-HEPES buffer (0.1 M, pH 7.5) containing MgCl2 (3.3 mM) and D2O (10%). The background boron signal arising from borosilicate glass in the spectrometer probe was reduced in the spectra using Whittaker smoothing. (37)

## Protein Crystallization

Crystals of wild-type MR were grown in the presence of 2-FPBA by the sitting-drop vapor diffusion method against a 500 μL reservoir volume. The protein solution and reservoir solution were mixed in a 1:1 ratio to a final volume of 10 μL. Crystals grew spontaneously at 21 °C. The reservoir solution consisted of PEG 3350 [4% (w/v)] and Bis-Tris Propane (BTP; 50 mM, pH 7.0). The protein solution consisted of wild-type MR (6 mg/mL) purified as described above, 2-FPBA (1.0 mM), MgCl2 (3.3 mM), and Na+-HEPES buffer (50 mM, pH 7.5). The resulting cubelike crystals (∼50 μm × 40 μm × 40 μm) grew to full size within 15–20 days. Crystals were harvested and transferred to a synthetic stabilizing solution consisting of PEG 4K [8% (w/v)], BTP (80 mM, pH 7.0), ethylene glycol [5% (w/v)], 2-FPBA (0.77 mM), MgCl2 (1.65 mM), and Na+-HEPES buffer (25 mM, pH 7.5). These stabilized crystals were equilibrated in the synthetic stabilizing solution for 5 min and then transferred directly to a cryoprotectant solution consisting of PEG 4K [10% (w/v)], BTP (80 mM), ethylene glycol [20% (w/v)], 2-FPBA (0.8 mM), MgCl2 (1.58 mM), and Na+-HEPES buffer (24 mM, pH 7.5). The cryoprotected crystals were flash-frozen in a stream of nitrogen gas at 100 K.

## Data Collection, Structure Determination, and Refinement

X-ray diffraction data were collected at the Life Sciences Collaborative Access Team beamline 21-ID-D at the Advanced Photon Source, Argonne National Laboratory, on a Dectris Eiger 9M detector with an X-ray wavelength of 1.1271 Å. A total of 3600 diffraction images were collected at an oscillation angle of 0.1° (360° total) with an exposure time of 0.010 s. These images were processed using the autoPROC software workflow, (38) which merged and scaled the isotropic data in AIMLESS (39) with the programs TRUNCATE (40) and UNIQUE (41) to determine the resolution limit. The phases were determined by the molecular replacement method, using the wild-type MR enzyme with bound BzH [Protein Data Bank (PDB) entry 3UXK] as the search model, (42) with the program Phaser. (43) The molecular replacement models were extended by several rounds of manual model building with COOT (44) and refinement with Phenix.Refine. (45) The X-ray/stereochemistry weight was automatically optimized to 3.0 in the final rounds of refinement. Noncrystallographic restraints between each monomer were applied for the first round of refinement but were relieved for subsequent rounds. Water molecules were added to the model in COOT and Phenix.Refine with subsequent manual verification. The final refined ligand coordinates for the 2-FPBA/benzoxaborole adduct were optimized for structural refinement using eLBOW (electronic ligand building and optimization workbench), (46) and a linkage restraint was applied between the ε-nitrogen of Lys 166 and the boron atom of the 2-FPBA/benzoxaborole adduct during the final stages of refinement. The 2-FPBA/benzoxaborole adduct was modeled with full occupancy at each of the eight active sites. Data collection and processing statistics are listed in Table S1.

# Results and Discussion

## Inhibition Kinetics

Surprisingly, the reaction progress curves obtained upon initiation of the racemization reaction by addition of wild-type MR to solutions containing (*S*)-mandelate (5.0 mM) and various concentrations of 2-FPBA revealed an initial “burst” phase followed by a time-dependent decrease in reaction rate until a steady state velocity () was obtained that varied as a function of the inhibitor concentration (Figure 1A). Similarly, when assays were initiated with a substrate after incubation of the enzyme with 2-FPBA for 5 min, an initial lag phase was observed. Our initial inhibition studies with 2-FPBA in the presence of (*S*)-mandelate (10.0 mM) revealed that 2-FPBA was a potent inhibitor of wild-type MR, exhibiting an apparent  value of 3.1 ± 0.3 μM based on the final steady state velocities () (Table 1 and Figure S1), although not irreversible because full activity could be readily regained upon dilution. This degree of inhibition is similar to that observed for the intermediate/transition state analogue inhibitors BzH ( μM), (47) Cupferron ( μM), (48) and *N*-hydroxyformanilide ( μM), (48) as well as PBA ( μM). (29) Two possible kinetic mechanisms were considered to describe the observed slow-onset inhibition: (1) initial reversible binding of the inhibitor to the enzyme to yield an EI complex, which then undergoes reversible isomerization to form a new complex E\*I (Scheme 2A), and (2) reversible binding of the inhibitor to the enzyme to form an EI complex in which the magnitudes of *k*3 and *k*4 are such that the equilibrium is established slowly relative to the rate of enzyme turnover (Scheme 2B). (34,35,49) In both cases, a competitive inhibition model was employed on the basis of BzH displacing the inhibitor in 11B NMR spectroscopy studies and the results obtained from X-ray crystallography experiments (*vide infra*). The kinetic data for the inhibition of wild-type MR by 2-FPBA were analyzed in accord with Scheme 2A and gave an apparent  value of 5.1 ± 1.8 μM,  μM, and  at pH 7.5 (Table 2). Equation 10 was used to calculate an apparent value of .

(10)

Despite the large error in  and  that arises from the lack of sensitivity of the CD-based assay, it is clear that wild-type MR binds 2-FPBA with an affinity that exceeds that for the substrate [for (*S*)-mandelate,  mM (Table 2)] (50) by ∼3000-fold (i.e., /). Interestingly, we found that structurally similar *o*-carbonyl phenylboronic acids were not as potent as inhibitors of MR (Table 1 and Figure S1). For example, 2-APBA was a modest inhibitor ( μM) and 2-carboxyphenylboronic acid (2-CPBA) (51) was an extremely weak inhibitor of wild-type MR ( mM). Clearly, the additional steric bulk arising from the methyl group and oxygen impairs binding. However, 4-chloro-2-FPBA was a slightly better inhibitor ( μM) than 2-FPBA ( μM), which is in accord with the enhanced inhibition previously reported for 4-chloro-PBA relative to PBA. (29) Aromatic aldehydes, including benzaldehyde (5.0 mM), 2-carboxybenzaldehyde (2.0 mM), salicylaldehyde (2.0 mM), and 2-formylbenzenesulfonate (5.0 mM), did not significantly inhibit wild-type MR up to the concentrations indicated (data not shown). Because these aromatic aldehydes are not hydrated to a significant extent in water, (52,53) their inability to inhibit MR suggests that the boronic acid group plays an important role in the binding affinity, which is not surprising considering the potent inhibition of MR exhibited by PBA. (29) Interactions with the boronic acid group could arise through formation of a weak N–B dative bond with His 297 as observed for PBA (29) or perhaps through formation of an intramolecular N–B interaction with an imine [**2** (Scheme 1A)] or an intermolecular N–B interaction with a Lys at the active site.

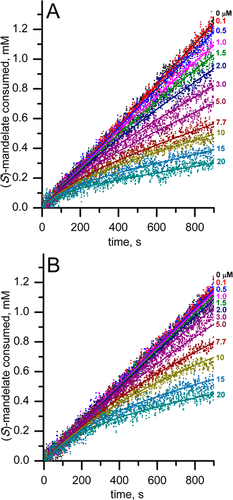


Figure 1. Progress curves for the onset of inhibition of (A) wild-type MR and (B) K164R MR by 2-FPBA. The curves shown for wild-type MR (3.0 nM) and K164R MR (300 nM) are fits of eq 3 to the amount of (*S*)-mandelate (initial concentration of 5.0 mM) consumed over time as described in Materials and Methods. The corresponding concentrations (micromolar) of 2-FPBA are shown to the right of each line, and the values determined for  and  are listed in Table 2.

**Table 1.  Values for Various *o*-Carbonyl Phenylboronic Acids**

|  |  |  |
| --- | --- | --- |
| **MR variant** |  | **(μM)a** |
|  | Inhibition by 2-FPBA |  |
| wild-type |  |  |
| pH 7.0 |  | 7.51 ± 0.59 |
| pH 7.5 |  | 3.12 ± 0.30 |
| pH 8.0 |  | 2.30 ± 0.33 |
| K164R |  | 7.21 ± 0.54 |
| K166R |  | no inhibition observed |
|  | Inhibition by 4-Cl-2-FPBA |  |
| wild-type |  | 1.65 ± 0.08 |
|  | Inhibition by 2-APBA |  |
| wild-type |  | 392 ± 30 |
|  | Inhibition by 2-CPBA |  |
| wild-type |  | ∼19000b |

a values determined in triplicate using the steady state initial velocities. Average values are reported, and the error is the standard deviation.

b value approximated on the basis of 17% inhibition observed at 3.8 mM 2-CPBA, 10 mM (*R*)-mandelate, a  of 0.9 mM, and assuming competitive inhibition.

**Table 2. Kinetic Parameters for Wild-Type MR, K164R MR, and K166R MR with (*R*)- and (*S*)-Mandelate**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **kinetic parametera** | **wild-type MR** |  | **K164R MR** | **K166R MR** |
|  |  | *R* → *S* |  |  |
| (mM) | 0.9 ± 0.2 |  | 1.4 ± 0.2 | 1.0 ± 0.2 |
|  | 670 ± 40 |  | 4.6 ± 0.2 | 0.42 ± 0.05 |
| / (M–1 s–1) | (7.4 ± 0.7) × 105 |  | (3.3 ± 0.4) × 103 | (4.2 ± 1.0) × 102 |
|  |  | *S* → *R* |  |  |
| (mM) | 0.7 ± 0.1 |  | 1.2 ± 0.2 | 1.4 ± 0.2 |
| (s–1) | 590 ± 30 |  | 4.2 ± 0.4 | 0.55 ± 0.09 |
| / (M–1 s–1) | (8.42 ± 1.27) × 105 |  | (3.5 ± 0.6) × 103 | (3.9 ± 0.9) × 102 |
| b | 1.1 ± 0.2 |  | 1.1 ± 0.2 | 0.9 ± 0.3 |
|  |  | Inhibition Studies |  |  |
| (BzH, μM) | 12 ± 2 |  | 54 ± 9 | 99 ± 9 |
| (2-FPBA, μM) | 5.1 ± 1.8 |  | 0.8 ± 0.2 | no inhibition |
| (2-FPBA, μM) | 0.26 ± 0.08 |  | – | no inhibition |

aKinetic parameters determined in triplicate with average values reported. The error is the standard deviation.

bCalculated using the Haldane relationship in which .

## Role of the Active Site Lysine Residues

To assess the possibility of formation of an imine adduct or an intermolecular N–B interaction with 2-FPBA, we investigated the role of Lys 164 and Lys 166 located at the active site. Consequently, we purified and characterized the K164R and K166R MR variants (Table 2). The K164R and K166R MR variants exhibited marked reductions in catalytic efficiency () of 224- and 1762-fold in the *R* → *S* reaction direction and 241- and 2159-fold in the *S* → *R* reaction direction, respectively. Indeed, the K166R variant had been characterized previously by Gerlt and co-workers (54) and our kinetic constants are in excellent agreement with their results. Both K164R and K166R MRs were competitively inhibited by BzH, with  values of 54 ± 9 and 99 ± 9 μM, respectively. The loss of binding affinity corresponded to a decrease in catalytic efficiency as demonstrated previously for this intermediate/transition state analogue inhibitor. (42,47)

As shown in Figure S1, K166R MR was not inhibited by 2-FPBA while K164R MR was strongly inhibited ( μM), although not to the same extent as the wild-type enzyme ( μM). This observation supported the notion that 2-FPBA reacted with Lys 166 at the active site. As observed for wild-type MR, the onset of inhibition of the K164R variant was time-dependent (Figure 1B). However, unlike inhibition of wild-type MR, the initial velocities (*v*i) were not significantly different for K164R MR in the presence of 2-FPBA. Consequently, the inhibition kinetics were fit using the kinetic mechanism shown in Scheme 2B. Interestingly, nearly all reported examples of slow-onset inhibition tend to follow the two-step kinetic model shown in Scheme 2A, with few examples of a single-step mechanism. (55) The values of , , and  were 0.8 ± 0.2 μM, 188 ± 89 M–1 s–1, and (1.5 ± 0.6) × 10–4 s–1, respectively. Hence, K164R MR bound 2-FPBA with an affinity that was reduced by ∼3-fold relative to that observed for wild-type MR (i.e., /).

## Mass Spectrometry and 11B NMR Spectroscopy

Direct evidence of the formation of an MR·2-FPBA adduct using mass spectrometry was inconclusive (Figure S2) likely because of the reversibility of the inhibition leading to rapid dissociation during the aerosolization process. (13) Interestingly, attempts to trap a more stable adduct by reducing the putative imine with NaCNBH3 proved to be unsuccessful, suggesting that the adduct might not be an imine. Consequently, we utilized 11B NMR spectroscopy to investigate the interaction between MR and 2-FPBA. This approach has been employed to study the interaction of boronic acid-bearing inhibitors with a variety of proteases, (56−64) β-lactamase, (65) and γ-glutamyl transpeptidase. (66) In the absence of enzyme, the boron in 2-FPBA exhibited signals with chemical shifts of 29.8 and 8.5 ppm in assay buffer (Figure 2). This observation is in agreement with those of Gutiérrez-Moreno et al., (67) who reported chemical shifts of 29.3 and 8.6 ppm for 2-FPBA in neutral solutions corresponding to the trigonal neutral R-B(OH)2 group [**1** (Scheme 1A)] and the tetrahedral anionic R-B(OR)(OH)2– group formed from the reversible cyclization to the benzoxaborole (**4**), respectively. The observation of the presence of both signals suggests that the ionization of 2-FPBA is a slow process on the NMR time scale, possibly due to the accompanying cyclization. (67) The signal at 29.8 ppm was weak relative to the signal at 8.5 ppm because only 39% of 2-FPBA () (67) exists in the neutral form at pH 7.5. The low signal-to-noise ratio arose because of the requirement to use concentrations of the inhibitor approximately stoichiometric with the enzyme concentration. Upon addition of the enzyme, a new peak was observed at 6.0 ppm, which was also the sole peak present when the enzyme was in slight excess. Surprisingly, this chemical shift differed markedly from that observed for the MR·PBA complex, which exhibited an 11B NMR signal at 0.97 ppm. Furthermore, this chemical shift was slightly more upfield relative to the 11B chemical shift of ∼7.4 ppm observed when we examined the effect of a large excess of ethylamine hydrochloride on the chemical shift of the 11B NMR signal of 2-FPBA under our assay conditions (Figure S3). These observations indicate that the bound species has considerable negative charge associated with the boron atom, which is characteristic of an anionic tetrahedral boronate anion (68) but is often interpreted as evidence for iminoboronate formation. (69) Consequently, the electronic configuration around the boron in the MR·2-FPBA complex differs from that of the MR·PBA complex, suggesting that the two boronic acid species are bound differently at the active site.

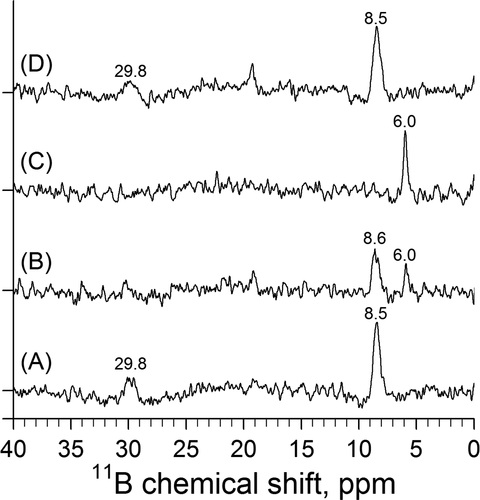


Figure 2. 11B NMR spectra of free 2-FPBA and 2-FPBA bound to MR. The 11B NMR spectra of (A) 2-FPBA (400 μM) in the absence of enzyme, (B) 2-FPBA (400 μM) in the presence of wild-type MR (200 μM), (C) 2-FPBA (400 μM) in the presence of approximately equimolar wild-type MR (425 μM), and (D) 2-FPBA (400 μM) in the presence of wild-type MR (425 μM) and excess BzH (5.0 mM) are shown. All solutions contained Na+-HEPES buffer (0.1 M, pH 7.5), MgCl2 (3.3 mM), and D2O (10%). The pH of these solutions was adjusted to 7.5 using NaOH (6 M). The indicated 11B chemical shifts are relative to that of the external standard BF3·OEt2.

Addition of a large excess of the competitive, intermediate/transition state analogue inhibitor BzH ([BzH] = 417) resulted in total displacement of the 2-FPBA from the active site, regenerating the 11B NMR signals associated with free 2-FPBA (Figure 2). These observations further demonstrated that the interaction of 2-FPBA with MR occurred at the active site and was freely reversible. Finally, as shown in Figure S3, we used 11B NMR spectroscopy to rule out the possibility that inhibition arose from formation of an adduct with mandelate. (70,71)

## Structure of the MR·2-FPBA Complex

To obtain a detailed understanding of how 2-FPBA inhibits MR, we determined the X-ray crystal structure of the MR·2-FPBA complex at 1.91-Å resolution. The complex crystallized as a homooctomer in space group *C*121 (Table S1). The individual subunits were similar, with root-mean-square deviations (rmsds) for structural alignments between the -carbon atoms of the individual subunits ranging from 0.092 to 0.157 Å. The electron density for the 2-FPBA ligands was very well-defined over the entire molecule in all eight active sites. The electron density between the  group of Lys 166 and the bound 2-FPBA was continuous, consistent with formation of a covalent adduct and the requirement for Lys 166 for potent inhibition (Figure 3A). Initially, we modeled the imine adduct (**2**) or the aminol adduct [**S9** (Scheme S1)] at the active site, assuming that Lys 166 had added to the aldehyde group. However, these models failed to fit the electron density well even when partial occupancies were considered, nor did modeling the Lys 166-NH2-boronic acid complex [**6** (Scheme 3)] conform to the observed electron density. Recognizing that 2-FPBA can undergo reversible cyclization to form a benzoxaborole, (67) we modeled the ligand as the product of the nucleophilic addition of the  group of Lys 166 to the boronic acid group with intramolecular addition of a hydroxyl group of the boronic acid to the aldehyde. This cyclic benzoxaborole [**7**, i.e., 1,3-dihydro-1,3-dihydroxy[*c*][2,1]oxaborole (Scheme 3)] (72) afforded an excellent fit to the observed electron density. To the best of our knowledge, this is the first report of this type of adduct for 2-FPBA interacting with a protein.

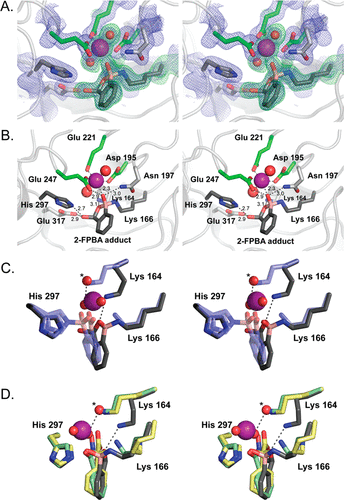
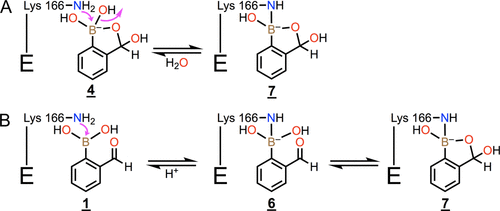


Figure 3. X-ray structure of the 2-FPBA/benzoxaborole adduct at the active site of MR. (A) Stereoview of the representative electron density overlaid on the final refined model for the active site of chain A. The electron density maps represent the final calculated  map (blue, contoured at ) and the simulated annealing omit  map (green, contoured at 3σ) with all atoms of 2-FPBA, Mg2+, Lys 166, and both waters coordinating the Mg2+ ion omitted from the model. (B) Stereoview of the molecular interactions contributing to binding the 2-FPBA/benzoxaborole adduct at the active site of MR (chain A). The carbon atoms are colored green for the metal-chelating residues, gray for the Brønsted acid–base catalysts, and white for additional interacting residues. Interactions are represented with dashed lines. Distances to the ligand are indicated in angstroms. (C) Stereoview of a structural overlay of the active site of MR with the 2-FPBA/benzoxaborole adduct (CPK colors, gray carbon atoms) and the two conformations of PBA (semi-transparent CPK colors, light blue carbon atoms; PDB entry 6VIM, chain B). (D) Stereoview of a structural overlay of the active site of MR with the 2-FPBA/benzoxaborole adduct (CPK colors, gray carbon atoms), BzH (CPK colors, green carbon atoms; PDB entry 3UXK, chain A), and (*S*)-atrolactate (CPK colors, yellow carbon atoms; PDB entry 1MRA). In panels C and D, the water molecule marked with an asterisk is present only in the 2-FPBA/benzoxaborole adduct structure, filling the space normally occupied by the  group of Lys 164 after this residue shifts its position to directly interact with the 2-FPBA/benzoxaborole adduct. In all panels, Mg2+ is represented as a purple sphere and water molecules are represented as red spheres.

**Scheme 3**



Surprisingly, 2-FPBA did not form an iminoboronate at the active site of MR (Figure 3A and Figure S4), consistent with our inability to trap the ligand using NaCNBH3, nor did 2-FPBA form a complex similar to PBA (Figure 3C). (29) Indeed, a clear Nζ–B interaction (1.5 Å) with Lys 166 was present, while the Nε2 atom of His 297 was 4.5 Å from the tetrahedral, sp3-hybridized boron atom. Unlike PBA (PDB entry 6VIM), (29) which coordinated the Mg2+ ion in a bidentate fashion yielding a distorted octahedral coordination geometry, only the single hydroxyl group of the cyclic benzoxaborole was strongly coordinated to the Mg2+ ion with a Mg2+–O distance of 2.3 Å. The side chains of Asp 195, Glu 221, and Glu 247 and two water molecules completed the octahedral coordination of the divalent metal ion. The active site residues Asp 197, Lys 164, His 297, and Glu 317, as well as the Mg2+-chelating residue Glu 247, all formed H-bonds with the adduct with protein–ligand distances of ≤3.0 Å (Figure 3B). Finally, dispersion interactions between the phenyl ring and the hydrophobic pocket at the active site of MR also contributed to the binding affinity of the 2-FPBA/benzoxaborole adduct. Interestingly, the plane of the phenyl ring of the 2-FPBA/benzoxaborole adduct was angled approximately 31° and 24° away from His 297 and toward Lys 166 relative to the plane of the phenyl ring of bound, sp3-hybridized PBA participating in a Nε2–B dative bond with His 297 and the plane of the ring of sp2-hybridized PBA, respectively (Figure 3C). Comparison of the structure of the MR-bound 2-FPBA/benzoxaborole adduct with those structures of MR with bound (*S*)-atrolactate (PDB entry 1MDR) (73) and BzH (PDB entry 3UXK) (42) revealed that one B–OH bond of the 2-FPBA/benzoxaborole adduct aligned with the C-OH group of (*S*)-atrolactate and BzH to coordinate the active site Mg2+ ion. While both (*S*)-atrolactate and BzH interacted with the Mg2+ ion in a bidentate fashion, the ring oxygen of the 2-FPBA/benzoxaborole adduct moved away from the metal ion to form an H-bond with the  group of Lys 164 (3.1 Å) (Figure 3D).

Typically, in the structures of the MR complexes with BzH or (*S*)-atrolactate, the  group of Lys 164 is located such that it is H-bonded to the *N*-hydroxy or carboxyl oxygen of the ligand, respectively. However, in the complex with the 2-FPBA/benzoxaborole adduct, the side chain of Lys 164 occupies an unusual position, moving to form an H-bond (3.1 Å) with the cyclic oxygen of the 2-FPBA/benzoxaborole adduct at all active sites except for that of chain G. In chain G, the electron density was modeled for the side chain of Lys164 in two conformations, each at 50% occupancy: one with the side chain of Lys 164 forming an H-bond with the cyclic oxygen of the 2-FPBA/benzoxaborole adduct [with a water molecule located at the position typically occupied by the  group of Lys 164 in structures with bound (*S*)-atrolactate or BzH] and a second conformation with the side chain of Lys 164 in its “typical” location, forming an H-bond with the oxygen of the water molecule coordinated to the Mg2+ ion (Figure 3C,D and Figure S4).

## Mechanism of Inhibition

In solution, the anionic, tetrahedral species **4** (see Scheme 1A, *vide infra*) comprises ∼61% of the 2-FPBA present at the assay pH of 7.5 ( for 2-FPBA = 7.30 ± 0.05; (67) cf.  (74)). Because 2-FPBA exhibited better inhibition with a change in pH from 7.0 to 8.0 (Table 1 and Figure S1), where MR exhibits little change in the values of and , (27,75) it appears that the anionic, tetrahedral benzoxaborole species **4** is preferentially bound by the enzyme as opposed to the neutral, trigonal boronic acid species **1**. Binding of **4** is not unexpected because MR is competitively inhibited by benzoxaborole with a  value of 3.0 ± 0.4 μM, which is similar to the  value for inhibition by 2-FPBA (J. A. Hayden and S. L. Bearne, unpublished results).

The observed slow-onset inhibition kinetics suggests that inhibition by 2-FPBA may occur via a two-step process (Scheme 2A). Early studies of the reversible binding of diols with arylboronic acids showed that boronic acid ester formation was favored at high pH where the boronate anion was the major species, leading to the notion that adduct formation primarily occurred through the boronate anion. (76,77) However, subsequent studies indicated that the neutral trigonal planar boronic acid species is the reactive species in solution (78−81) and that oxaboroles can form dative bonds more readily than their corresponding phenylboronic acids. (77) Consequently, we propose that the first step (i.e., formation of EI) is binding of the anionic, tetrahedral benzoxaborole species **4** followed by displacement of hydroxide by the  group of Lys 166 to form the Nζ–B bond and yield the E\*I complex (Scheme 3A). Considering the formation of the Nζ–B bond and the multiple H-bonds between the 2-FPBA/benzoxaborole adduct and the active site, the reverse reaction (i.e., *k*6) is likely slow. However, additional studies are required to delineate the potential role of Lys 164 in the onset of inhibition and to rule out alternative mechanisms such as initial binding of the inhibitor as neutral trigonal species **1** with subsequent formation of an Nζ–B bond with the  group of Lys 166, followed by, or concerted with, cyclization to form the cyclic 2-FPBA/benzoxaborole adduct (Scheme 3B). Indeed, because the cyclization is slow in solution on the NMR time scale (*vide supra*), this process could comprise a slow step. Importantly, although formation of an imine is favorable under our assay conditions [i.e.,  for imine formation between ethylamine and 2-FPBA (Figure S5)], our inability to trap an imine by reduction suggests that it is unlikely that the slow step arises from initial imine formation followed by a rearrangement of the orientation of 2-FPBA at the active site. We posit that the binding orientation of 2-FPBA at the active site disfavors imine formation.

Although the CD-based assay is not a sensitive assay, fitting the kinetic data for the slow-onset inhibition of K164R MR by 2-FPBA (Figure 1B) indicated that the bimolecular rate constant for inhibition ( = 188 M–1 s–1) was several orders of magnitude smaller than typical bimolecular rate constants for ES formation (105–107 M–1 s–1). (82) This result is consistent with more than one step being required for formation of the first detectable EI complex, although the specific nature of those steps is not known. (34) Our observation that the binding affinity of 2-FPBA is 3-fold weaker with the K164R variant relative to wild-type MR suggests that the Arg 164 variant causes subtle structural perturbations at the active site due to the added steric bulk or causes alterations in H-bonding that slightly disfavor binding.

# Conclusions

2-FPBA is the first slow-onset inhibitor identified for MR, exhibiting  and  values of 5.1 ± 1.8 and 0.26 ± 0.08 μM, respectively. It is among the most potent inhibitors of MR identified to date (), (29) with a binding affinity that rivals those of transition state analogue inhibitors of the enzyme. (47,48) Most surprisingly, rather than forming the expected iminoboronate, the  group of Lys 166 formed an Nζ–B dative bond with 2-FPBA at the active site, leading to formation of a benzoxaborole adduct. Although benzoxaboroles can be formed from reactions of amines, especially secondary amines, with the aldehyde of 2-FPBA, (83) it appears that the formation of the observed Nζ–B-based 2-FPBA/benzoxaborole adduct is favored and facilitated by the orientation of the bound 2-FPBA at the active site and, possibly, the reduced dielectric environment. (18) Overall, our results indicate that when *o*-carbonyl arylboronic acid reagents are employed to modify amino groups in proteins, the structure of the resulting product depends on the protein architecture at the site of modification.

# Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.1c00374.

* Kinetic data, mass spectrometry data, 11B NMR spectra, table of X-ray crystallographic results, and determination of the imine formation constant (PDF)

# Accession Codes

The structure of MR with the bound 2-FPBA/benzoxaborole adduct has been deposited in the Protein Data Bank as entry 7MQX. Mandelate racemase, UniProt entry P11444.

# Terms & Conditions

Most electronic Supporting Information files are available without a subscription to ACS Web Editions. Such files may be downloaded by article for research use (if there is a public use license linked to the relevant article, that license may permit other uses). Permission may be obtained from ACS for other uses through requests via the RightsLink permission system: http://pubs.acs.org/page/copyright/permissions.html.

# Author Information

* **Corresponding Author**
  + **Stephen L. Bearne** - *Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada*;  *Department of Chemistry, Dalhousie University, Halifax, NS B3H 4R2, Canada*;  https://orcid.org/0000-0003-4433-6174; Email: sbearne@dal.ca
* **Authors**
  + **Colin D. Douglas** - *Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada*
  + **Lia Grandinetti** - *Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin 53201-1881, United States*
  + **Nicole M. Easton** - *Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada*
  + **Oliver P. Kuehm** - *Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada*
  + **Joshua A. Hayden** - *Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada*
  + **Meghan C. Hamilton** - *Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada*
  + **Martin St. Maurice** - *Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin 53201-1881, United States*

# Funding

This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to S.L.B. (Grant RGPIN-2016-05083), a Postdoctoral Fellowship from the Killam Foundation (C.D.D.), a Predoctoral Fellowship from the Killam Foundation (O.P.K.), a Faye Sobey Undergraduate Summer Research Award (N.M.E.), and an NSERC Undergraduate Summer Research Award (J.A.H.).

# Notes

The authors declare no competing financial interest.

# Acknowledgments

The authors thank Dr. Mike Lumsden (NMR-3) for assistance with the NMR experiments and Dr. Matthew Forbes (AIMS Mass Spectrometry Laboratory, University of Toronto) for assistance with the mass spectrometry experiments. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract DE-AC02-06CH11357. Use of LS-CAT Sector 21 was supported by the Michigan Economic Development Corp. and the Michigan Technology Tri-Corridor (Grant 085P1000817).

# Note Added After ASAP Publication

This paper was published ASAP on August 2, 2021 with an error in the title. The corrected version was reposted on August 2, 2021.

# Abbreviations

|  |  |
| --- | --- |
| 2-APBA | 2-acetylphenylboronic acid |
| BPT | Bis-Tris Propane |
| BzH | benzohydroxamate |
| CD | circular dichroism |
| 2-CPBA | 2-carboxyphenylboronic acid |
| 2-FPBA | 2-formylphenylboronic acid |
| HEPES | 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid |
| MR | mandelate racemase |
| MS | mass spectrometry. |

# References

**1** Means, G. E. and Feeney, R. E. (**1968**) Reductive alkylation of amino groups in proteins. *Biochemistry* *7*, 2192– 2201,  DOI: 10.1021/bi00846a023

**2** Williams, J. N. and Jacobs, R. M. (**1966**) A reversible reaction between the ϵ-amino groups of cytochrome *c* and salicylaldehyde. *Biochem. Biophys. Res. Commun.* *22*, 695– 699,  DOI: 10.1016/0006-291X(66)90203-8

**3** Williams, J. N. and Jacobs, R. M. (**1968**) Reversible reaction of ε-amino groups cytochrome *c* with saliclaldehyde to produce cytochrome *c* polymers. *Biochim. Biophys. Acta, Protein Struct.* *154*, 323– 331,  DOI: 10.1016/0005-2795(68)90046-9

**4** Mühlrad, A., Ajtai, K., and Fábián, F. (**1970**) Reaction of myosin with salicylaldehyde. I. The effect of salicylaldehyde on the physicochemical properties of myosin. *Biochim. Biophys. Acta, Bioenerg.* *205*, 342– 354,  DOI: 10.1016/0005-2728(70)90100-3

**5** Mühlrad, A., Ajtai, K., and Fábián, F. (**1970**) Reaction of myosin with salicylaldehyde. II. Effect of salicylalation on the ATPase activity of myosin. *Biochim. Biophys. Acta, Bioenerg.* *205*, 355– 360,  DOI: 10.1016/0005-2728(70)90101-5

**6** Moriya, K., Tanizawa, K., and Kanaoka, Y. (**1989**) Schiff base copper(II) chelate as a tool for intermolecular cross-linking and immobilization of protein. *Biochem. Biophys. Res. Commun.* *161*, 52– 58,  DOI: 10.1016/0006-291X(89)91558-1

**7** Moriya, K., Tanizawa, K., and Kanaoka, Y. (**1989**) Schiff base copper(II) chelate as a tool for immobilization of protein. *Chem. Pharm. Bull.* *37*, 2849– 2851,  DOI: 10.1248/cpb.37.2849

**8** Tanizawa, K., Miyaura, T., and Kanaoka, Y. (**1990**) Introduction of γ-glutamyl residue into chymotrypsin and agarose gel: Application to cross-linking and immobilization of protein. *Chem. Pharm. Bull.* *38*, 2868– 2870,  DOI: 10.1248/cpb.38.2868

**9** Bandyopadhyay, A., Cambray, S., and Gao, J. (**2016**) Fast and selective labeling of N-terminal cysteines at neutral pH via thiazolidino boronate formation. *Chem. Sci.* *7*, 4589– 4593,  DOI: 10.1039/C6SC00172F

**10** Faustino, H., Silva, M. J. S. A., Veiros, L. F., Bernardes, G. J. L., and Gois, P. M. P. (**2016**) Iminoboronates are efficient intermediates for selective, rapid and reversible N-terminal cysteine functionalisation. *Chem. Sci.* *7*, 5052– 5058,  DOI: 10.1039/C6SC01520D

**11** Cal, P. M., Vicente, J. B., Pires, E., Coelho, A. V., Veiros, L. F., Cordeiro, C., and Gois, P. M. (**2012**) Iminoboronates: a new strategy for reversible protein modification. *J. Am. Chem. Soc.* *134*, 10299– 10305,  DOI: 10.1021/ja303436y

**12** Cal, P. M., Frade, R. F., Cordeiro, C., and Gois, P. M. (**2015**) Reversible lysine modification on proteins by using functionalized boronic acids. *Chem. Eur. J.* *21*, 8182– 8187,  DOI: 10.1002/chem.201500127

**13** Akçay, G., Belmonte, M. A., Aquila, B., Chuaqui, C., Hird, A. W., Lamb, M. L., Rawlins, P. B., Su, N., Tentarelli, S., Grimster, N. P., and Su, Q. (**2016**) Inhibition of Mcl-1 through covalent modification of a noncatalytic lysine side chain. *Nat. Chem. Biol.* *12*, 931– 936,  DOI: 10.1038/nchembio.2174

**14** Cambray, S. and Gao, J. (**2018**) Versatile bioconjugation chemistries of *ortho*-boronyl aryl ketones and aldehydes. *Acc. Chem. Res.* *51*, 2198– 2206,  DOI: 10.1021/acs.accounts.8b00154

**15** Akgun, B. and Hall, D. G. (**2018**) Boronic acids as bioorthogonal probes for site-selective labeling of proteins. *Angew. Chem., Int. Ed.* *57*, 13028– 13044,  DOI: 10.1002/anie.201712611

**16** António, J. P. M., Russo, R., Carvalho, C. P., Cal, P. M. S. D., and Gois, P. M. P. (**2019**) Boronic acids as building blocks for the construction of therapeutically useful bioconjugates. *Chem. Soc. Rev.* *48*, 3513– 3536,  DOI: 10.1039/C9CS00184K

**17** van der Zouwen, A. J., Jeucken, A., Steneker, R., Hohmann, K. F., Lohse, J., Slotboom, D. J., and Witte, M. D. (**2021**) Iminoboronates as dual-purpose linkers in chemical probe development. *Chem. Eur. J.* *27*, 3292,  DOI: 10.1002/chem.202005115

**18** Zhu, L., Shabbir, S. H., Gray, M., Lynch, V. M., Sorey, S., and Anslyn, E. V. (**2006**) A structural investigation of the N-B interaction in an *o*-(*N*,*N*-dialkylaminomethyl)arylboronate system. *J. Am. Chem. Soc.* *128*, 1222– 1232,  DOI: 10.1021/ja055817c

**19** Chapin, B. M., Metola, P., Lynch, V. M., Stanton, J. F., James, T. D., and Anslyn, E. V. (**2016**) Structural and thermodynamic analysis of a three-component assembly forming *ortho*-iminophenylboronate esters. *J. Org. Chem.* *81*, 8319– 8330,  DOI: 10.1021/acs.joc.6b01495

**20** Gu, H., Ghosh, S., Staples, R. J., and Bane, S. L. (**2019**) β-Hydroxy-stabilized boron-nitrogen heterocycles enable rapid and efficient C-terminal protein modification. *Bioconjugate Chem.* *30*, 2604– 2613,  DOI: 10.1021/acs.bioconjchem.9b00534

**21** Adamczyk-Woźniak, A., Gozdalik, J. T., Wieczorek, D., Madura, I. D., Kaczorowska, E., Brzezińska, E., Sporzyński, A., and Lipok, J. (**2020**) 5-Trifluoromethyl-2-formylphenylboronic acid. *Molecules* *25*, 799,  DOI: 10.3390/molecules25040799

**22** Kenyon, G. L., Gerlt, J. A., Petsko, G. A., and Kozarich, J. W. (**1995**) Mandelate racemase: structure-function studies of a pseudosymmetric enzyme. *Acc. Chem. Res.* *28*, 178– 186,  DOI: 10.1021/ar00052a003

**23** Gerlt, J. A. (1998) Enzyme-catalyzed proton transfer reactions to and from carbon. In *Bioorganic Chemistry: Peptides and Proteins* (Hecht, S. M., Ed.) pp 279– 311, Oxford University Press, New York.

**24** Bearne, S. L. and St. Maurice, M. (**2017**) A paradigm for CH bond cleavage: structural and functional aspects of transition state stabilization by mandelate racemase. *Adv. Protein Chem. Struct. Biol.* *109*, 113– 160,  DOI: 10.1016/bs.apcsb.2017.04.007

**25** Gerlt, J. A., Babbitt, P. C., and Rayment, I. (**2005**) Divergent evolution in the enolase superfamily: the interplay of mechanism and specificity. *Arch. Biochem. Biophys.* *433*, 59– 70,  DOI: 10.1016/j.abb.2004.07.034

**26** Neidhart, D. J., Howell, P. L., Petsko, G. A., Powers, V. M., Li, R. S., Kenyon, G. L., and Gerlt, J. A. (**1991**) Mechanism of the reaction catalyzed by mandelate racemase. 2. Crystal structure of mandelate racemase at 2.5-Å resolution: identification of the active site and possible catalytic residues. *Biochemistry* *30*, 9264– 9273,  DOI: 10.1021/bi00102a019

**27** Fetter, C. M., Morrison, Z. A., Nagar, M., Douglas, C. D., and Bearne, S. L. (**2019**) Altering the Y137-K164-K166 triad of mandelate racemase and its effect on the observed  of the Brønsted base catalysts. *Arch. Biochem. Biophys.* *666*, 116– 126,  DOI: 10.1016/j.abb.2019.03.011

**28** Nagar, M., Wyatt, B. N., St. Maurice, M., and Bearne, S. L. (**2015**) Inactivation of mandelate racemase by 3-hydroxypyruvate reveals a potential mechanistic link between enzyme superfamilies. *Biochemistry* *54*, 2747– 2757,  DOI: 10.1021/acs.biochem.5b00221

**29** Sharma, A. N., Grandinetti, L., Johnson, E. R., St. Maurice, M., and Bearne, S. L. (**2020**) Potent inhibition of mandelate racemase by boronic acids: boron as a mimic of a carbon acid center. *Biochemistry* *59*, 3026– 3037,  DOI: 10.1021/acs.biochem.0c00478

**30** Narmandakh, A. and Bearne, S. L. (**2010**) Purification of recombinant mandelate racemase: improved catalytic activity. *Protein Expression Purif.* *69*, 39– 46,  DOI: 10.1016/j.pep.2009.06.022

**31** Garfin, D. E. (**1990**) One-dimensional gel electrophoresis. *Methods Enzymol.* *182*, 425– 441,  DOI: 10.1016/0076-6879(90)82035-Z

**32** Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A. (**2003**) ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* *31*, 3784– 3788,  DOI: 10.1093/nar/gkg563

**33** Sharp, T. R., Hegeman, G. D., and Kenyon, G. L. (**1979**) A direct kinetic assay for mandelate racemase using circular dichroic measurements. *Anal. Biochem.* *94*, 329– 334,  DOI: 10.1016/0003-2697(79)90368-3

**34** Morrison, J. F. and Walsh, C. T. (**2006**) The behavior and significance of slow-binding enzyme inhibitors. *Adv. Enzymol. Relat. Areas Mol. Biol.* *61*, 201– 301,  DOI: 10.1002/9780470123072.ch5

**35** Copeland, R. A. (2000) *Enzymes: A Practical Introduction to Structure, Mechanism, and Data analysis*, 2nd ed., pp 318– 333, Wiley-VHC, Inc., New York.

**36** Segel, I. H. (1975) *Enzyme Kinetics*, pp 100– 111, John Wiley and Sons, Inc., New York.

**37** Carlos Cobas, J., Bernstein, M. A., Martín-Pastor, M., and Tahoces, P. G. (**2006**) A new general-purpose fully automatic baseline-correction procedure for 1D and 2D NMR data. *J. Magn. Reson.* *183*, 145– 151,  DOI: 10.1016/j.jmr.2006.07.013

**38** Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Smart, O., Paciorek, W., Womack, T., and Bricogne, G. (**2011**) Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* *67*, 293– 302,  DOI: 10.1107/S0907444911007773

**39** Evans, P. R. and Murshudov, G. N. (**2013**) How good are my data and what is the resolution?. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* *69*, 1204– 1214,  DOI: 10.1107/S0907444913000061

**40** French, G. S. and Wilson, K. S. (**1978**) On the treatment of negative intensity observations. *Acta Crystallogr., Sect. A: Cryst. Phys., Diffr., Theor. Gen. Crystallogr.* *A34*, 517– 525,  DOI: 10.1107/S0567739478001114

**41** Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A., and Wilson, K. S. (**2011**) Overview of the CCP4 suite and current developments. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* *67*, 235– 242,  DOI: 10.1107/S0907444910045749

**42** Lietzan, A. D., Nagar, M., Pellmann, E. A., Bourque, J. R., Bearne, S. L., and St. Maurice, M. (**2012**) Structure of mandelate racemase with bound intermediate analogues benzohydroxamate and Cupferron. *Biochemistry* *51*, 1160– 1170,  DOI: 10.1021/bi2018514

**43** McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (**2007**) Phaser crystallographic software. *J. Appl. Crystallogr.* *40*, 658– 674,  DOI: 10.1107/S0021889807021206

**44** Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (**2010**) Features and development of *Coot*. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* *66*, 486– 501,  DOI: 10.1107/S0907444910007493

**45** Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H., and Adams, P. D. (**2012**) Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* *68*, 352– 367,  DOI: 10.1107/S0907444912001308

**46** Moriarty, N. W., Grosse-Kunstleve, R. W., and Adams, P. D. (**2009**) Electronic Ligand Builder and Optimization Workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* *65*, 1074– 1080,  DOI: 10.1107/S0907444909029436

**47** St. Maurice, M. and Bearne, S. L. (**2000**) Reaction intermediate analogues for mandelate racemase: interaction between Asn 197 and the -hydroxyl of the substrate promotes catalysis. *Biochemistry* *39*, 13324– 13335,  DOI: 10.1021/bi001144t

**48** Bourque, J. R., Burley, R. K., and Bearne, S. L. (**2007**) Intermediate analogue inhibitors of mandelate racemase: *N*-hydroxyformanilide and Cupferron. *Bioorg. Med. Chem. Lett.* *17*, 105– 108,  DOI: 10.1016/j.bmcl.2006.09.079

**49** Morrison, J. F. (**1982**) The slow-binding and slow, tight-binding inhibition of enzyme-catalysed reactions. *Trends Biochem. Sci.* *7*, 102– 105,  DOI: 10.1016/0968-0004(82)90157-8

**50** St. Maurice, M. and Bearne, S. L. (**2002**) Kinetics and thermodynamics of mandelate racemase catalysis. *Biochemistry* *41*, 4048– 4058,  DOI: 10.1021/bi016044h

**51** Graham, B. J., Windsor, I. W., Gold, B., and Raines, R. T. (**2021**) Boronic acid with high oxidative stability and utility in biological contexts. *Proc. Natl. Acad. Sci. U. S. A.* *118*, e2013691118  DOI: 10.1073/pnas.2013691118

**52** McClelland, R. A. and Coe, M. (**1983**) Structure-reactivity effects in the hydration of benzaldehydes. *J. Am. Chem. Soc.* *105*, 2718– 2725,  DOI: 10.1021/ja00347a033

**53** Godoy-Alcántar, C., Yatsimirsky, A. K., and Lehn, J.-M. (**2005**) Structure-stability correlations for imine formation in aqueous solution. *J. Phys. Org. Chem.* *18*, 979– 985,  DOI: 10.1002/poc.941

**54** Kallarakal, A. T., Mitra, B., Kozarich, J. W., Gerlt, J. A., Clifton, J. G., Petsko, G. A., and Kenyon, G. L. (**1995**) Mechanism of the reaction catalyzed by mandelate racemase: structure and mechanistic properties of the K166R mutant. *Biochemistry* *34*, 2788– 2797,  DOI: 10.1021/bi00009a007

**55** Clausen, T., Huber, R., Messerschmidt, A., Pohlenz, H. D., and Laber, B. (**1997**) Slow-binding inhibition of *Escherichia coli* cystathionine β-lyase by l-aminoethoxyvinylglycine: a kinetic and X-ray study. *Biochemistry* *36*, 12633– 12643,  DOI: 10.1021/bi970630m

**56** Adebodun, F. and Jordan, F. (**1988**) 11B nuclear magnetic resonance studies of the structure of the transition-state analog phenylboronic acid bound to chymotrypsin. *J. Am. Chem. Soc.* *110*, 309– 310,  DOI: 10.1021/ja00209a060

**57** Adebodun, F. and Jordan, F. (**1989**) Multinuclear magnetic resonance studies on serine protease transition state analogues. *J. Cell. Biochem.* *40*, 249– 260,  DOI: 10.1002/jcb.240400213

**58** Baldwin, J. E., Claridge, T. D. W., Derome, A. E., Schofield, C. J., and Smith, B. D. (**1991**) 11B NMR studies of an aryl boronic acid bound to chymotrypsin and subtilisin. *Bioorg. Med. Chem. Lett.* *1*, 9– 12,  DOI: 10.1016/S0960-894X(01)81080-5

**59** Zhong, S., Jordan, F., Kettner, C., and Polgar, L. (**1991**) Observation of tightly bound 11B nuclear magnetic resonance signals on serine proteases. Direct solution evidence for tetrahedral geometry around the boron in the putative transition-state analogs. *J. Am. Chem. Soc.* *113*, 9429– 9435,  DOI: 10.1021/ja00025a001

**60** Tsilikounas, E., Kettner, C. A., and Bachovchin, W. W. (**1993**) 11B NMR spectroscopy of peptide boronic acid inhibitor complexes of -lytic protease. Direct evidence for tetrahedral boron in both boron-histidine and boron-serine adduct complexes. *Biochemistry* *32*, 12651– 12655,  DOI: 10.1021/bi00210a013

**61** Sudmeier, J. L., Günther, U. L., Gutheil, W. G., Coutts, S. J., Snow, R. J., Barton, R. W., and Bachovchin, W. W. (**1994**) Solution structures of active and inactive forms of the DP IV (CD26) inhibitor Pro-boroPro determined by NMR spectroscopy. *Biochemistry* *33*, 12427– 12438,  DOI: 10.1021/bi00207a009

**62** Deadman, J. J., Elgendy, S., Goodwin, C. A., Green, D., Baban, J. A., Patel, G., Skordalakes, E., Chino, N., Claeson, G., Kakkar, V. V., and Scully, M. F. (**1995**) Characterization of a class of peptide boronates with neutral P1 side chains as highly selective inhibitors of thrombin. *J. Med. Chem.* *38*, 1511– 1522,  DOI: 10.1021/jm00009a012

**63** London, R. E. and Gabel, S. A. (**2002**) Formation of a trypsin-borate-4-aminobutanol ternary complex. *Biochemistry* *41*, 5963– 5967,  DOI: 10.1021/bi025583z

**64** Transue, T. R., Gabel, S. A., and London, R. E. (**2006**) NMR and crystallographic characterization of adventitious borate binding by trypsin. *Bioconjugate Chem.* *17*, 300– 308,  DOI: 10.1021/bc0502210

**65** Baldwin, J. E., Claridge, T. D. W., Derome, A. E., Smith, B. D., Twyman, M., and Waley, S. G. (**1991**) Direct observation of a tetrahedral boronic acid-β-lactamase complex using 11B NMR spectroscopy. *J. Chem. Soc., Chem. Commun.* 573– 574,  DOI: 10.1039/C39910000573

**66** London, R. E. and Gabel, S. A. (**2001**) Development and evaluation of a boronate inhibitor of γ-glutamyl transpeptidase. *Arch. Biochem. Biophys.* *385*, 250– 258,  DOI: 10.1006/abbi.2000.2169

**67** Gutiérrez-Moreno, N. J., Medrano, F., and Yatsimirsky, A. K. (**2012**) Schiff base formation and recognition of amino sugars, aminoglycosides and biological polyamines by 2-formyl phenylboronic acid in aqueous solution. *Org. Biomol. Chem.* *10*, 6960– 6972,  DOI: 10.1039/c2ob26290h

**68** Stolowitz, M. L., Ahlem, C., Hughes, K. A., Kaiser, R. J., Kesicki, E. A., Li, G., Lund, K. P., Torkelson, S. M., and Wiley, J. P. (**2001**) Phenylboronic acid-salicylhydroxamic acid bioconjugates. 1. A novel boronic acid complex for protein immobilization. *Bioconjugate Chem.* *12*, 229– 239,  DOI: 10.1021/bc0000942

**69** Bandyopadhyay, A. and Gao, J. (**2015**) Iminoboronate formation leads to fast and reversible conjugation chemistry of -nucleophiles at neutral pH. *Chem. Eur. J.* *21*, 14748– 14752,  DOI: 10.1002/chem.201502077

**70** Babcock, L. and Pizer, R. (**1980**) Dynamics of boron complexation reactions. Formation of 1:1 boron acid-ligand complexes. *Inorg. Chem.* *19*, 56– 61,  DOI: 10.1021/ic50203a013

**71** Mishra, S. K. and Suryaprakash, N. (**2015**) A simple and rapid approach for testing enantiopurity of hydroxy acids and their derivatives using 1H NMR spectroscopy. *RSC Adv.* *5*, 67277– 67283,  DOI: 10.1039/C5RA11919G

**72** Luliński, S., Madura, I., Serwatowski, J., Szatyłowicz, H., and Zachara, J. (**2007**) A tautomeric equilibrium between functionalized 2-formylphenylboronic acids and corresponding 1,3-dihydro-1,3-dihydroxybenzo[*c*][2,1]oxaboroles. *New J. Chem.* *31*, 144– 154,  DOI: 10.1039/B611195E

**73** Landro, J. A., Gerlt, J. A., Kozarich, J. W., Koo, C. W., Shah, V. J., Kenyon, G. L., Neidhart, D. J., Fujita, S., and Petsko, G. A. (**1994**) The role of lysine 166 in the mechanism of mandelate racemase from *Pseudomonas putida*: mechanistic and crystallographic evidence for stereospecific alkylation by (*R*)- -phenylglycidate. *Biochemistry* *33*, 635– 643,  DOI: 10.1021/bi00169a003

**74** Brooks, W. L. A., Deng, C. C., and Sumerlin, B. S. (**2018**) Structure-reactivity relationships in boronic acid-diol complexation. *ACS Omega* *3*, 17863– 17870,  DOI: 10.1021/acsomega.8b02999

**75** Landro, J. A., Kallarakal, A. T., Ransom, S. C., Gerlt, J. A., Kozarich, J. W., Neidhart, D. J., and Kenyon, G. L. (**1991**) Mechanism of the reaction catalyzed by mandelate racemase. 3. Asymmetry in reactions catalyzed by the H297N mutant. *Biochemistry* *30*, 9274– 9281,  DOI: 10.1021/bi00102a020

**76** Yan, J., Springsteen, G., Deeter, S., and Wang, B. (**2004**) The relationship among , pH, and binding constants in the interactions between boronic acids and diols—it is not as simple as it appears. *Tetrahedron* *60*, 11205– 11209,  DOI: 10.1016/j.tet.2004.08.051

**77** Baker, S. J., Tomsho, J. W., and Benkovic, S. J. (**2011**) Boron-containing inhibitors of synthetases. *Chem. Soc. Rev.* *40*, 4279– 4285,  DOI: 10.1039/c0cs00131g

**78** Bishop, M., Shahid, N., Yang, J. Z., and Barron, A. R. (**2004**) Determination of the mode of efficacy of the cross-linking of guar by borate using MAS 11B NMR of borate cross-linked guar in combination with solution 11B NMR of model systems. *Dalton Trans.* *2004*, 2621– 2634,  DOI: 10.1039/B406952H

**79** Rietjens, M. and Steenbergen, P. A. (**2005**) Crosslinking mechanism of boric acid with diols revisited. *Eur. J. Inorg. Chem.* *2005*, 1162– 1174,  DOI: 10.1002/ejic.200400674

**80** Iwatsuki, S., Nakajima, S., Inamo, M., Takagi, H. D., and Ishihara, K. (**2007**) Which is reactive in alkaline solution, boronate ion or boronic acid? Kinetic evidence for reactive trigonal boronic acid in an alkaline solution. *Inorg. Chem.* *46*, 354– 356,  DOI: 10.1021/ic0615372

**81** Miyamoto, C., Suzuki, K., Iwatsuki, S., Inamo, M., Takagi, H. D., and Ishihara, K. (**2008**) Kinetic evidence for high reactivity of 3-nitrophenylboronic acid compared to its conjugate boronate ion in reactions with ethylene and propylene glycols. *Inorg. Chem.* *47*, 1417– 1419,  DOI: 10.1021/ic702317n

**82** Fersht, A. (1999) *Structure and Mechanism in Protein Science*, pp 153, 164, 165, W. H. Freeman and Co., New York.

**83** Adamczyk-Woźniak, A., Cyrański, M. K., Fra̧czak, B. T., Lewandowska, A., Madura, I. D., and Sporzyński, A. (**2012**) Imino- and aminomethylphenylboronic acids: stabilizing effect of hydrogen bonds. *Tetrahedron* *68*, 3761– 3767,  DOI: 10.1016/j.tet.2012.02.072