Potent Inhibition of Mandelate Racemase by Boronic Acids: Boron as a Mimic of a Carbon Acid Center

Amar Nath Sharma
*Dalhousie University*

Lia Grandinetti
*Marquette University*

Erin R. Johnson
*Dalhousie University*

Martin St. Maurice
*Marquette University*, martin.stmaurice@marquette.edu

Stephen L. Bearne
*Dalhousie University*

Follow this and additional works at: [https://epublications.marquette.edu/bio_fac](https://epublications.marquette.edu/bio_fac)

Part of the Biology Commons

**Recommended Citation**
Sharma, Amar Nath; Grandinetti, Lia; Johnson, Erin R.; St. Maurice, Martin; and Bearne, Stephen L., "Potent Inhibition of Mandelate Racemase by Boronic Acids: Boron as a Mimic of a Carbon Acid Center" (2020). *Biological Sciences Faculty Research and Publications*. 824.
[https://epublications.marquette.edu/bio_fac/824](https://epublications.marquette.edu/bio_fac/824)
Potent Inhibition of Mandelate Racemase by Boronic Acids: Boron as a Mimic of a Carbon Acid Center

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

Lia Grandinetti  
Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin

Erin R. Johnson  
Department of Chemistry, 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
Boronic acids have been successfully employed as inhibitors of hydrolytic enzymes. Typically, an enzymatic nucleophile catalyzing hydrolysis adds to the electrophilic boron atom forming a tetrahedral species that mimics the intermediate(s)/transition state(s) for the hydrolysis reaction. We show that para-substituted phenylboronic acids (PBAs) are potent competitive inhibitors of mandelate racemase (MR), an enzyme that catalyzes a 1,1-proton transfer rather than a hydrolysis reaction. The \( K_i \) value for PBA was 1.8 ± 0.1 \( \mu \)M, and \( p\)-Cl-PBA exhibited the most potent inhibition (\( K_i = 81 ± 4 \) nM), exceeding the binding affinity of the substrate by \( \sim 4 \) orders of magnitude. Isothermal titration calorimetric studies with the wild-type, K166M, and H297N MR variants indicated that, of the two Brønsted acid–base catalysts Lys 166 and His 297, the former made the greater contribution to inhibitor binding. The X-ray crystal structure of the MR-PBA complex revealed the presence of multiple H-bonds between the boronic acid hydroxyl groups and the side chains of active site residues, as well as formation of a His 297 N\( \varepsilon_2 \)–B dative bond. The dramatic upfield change in chemical shift of 27.2 ppm in the solution-phase \( ^{11} \)B nuclear magnetic resonance spectrum accompanying binding of PBA by MR was consistent with an sp\(^3\)-hybridized boron, which was also supported by density-functional theory calculations. These unprecedented findings suggest that, beyond substituting boron at carbon centers participating in hydrolysis reactions, substitution of boron at the acidic carbon center of a substrate furnishes a new approach for generating inhibitors of enzymes catalyzing the deprotonation of carbon acid substrates.

Over the past decade, interest in employing boron in the design of enzyme inhibitors and potential therapeutic agents has grown.(1−9) This interest arises because boron-containing biomolecules exhibit low toxicity(6) and are not abundant in nature, although natural boron-containing antibiotics and cell surface signaling molecules do exist.(10) Moreover, the boron atom acts as a Lewis acid(11) with its vacant p orbital readily accepting electrons from donor atoms to form a coordinate covalent (dative) bond with concomitant conversion from a neutral sp\(^2\) center to an anionic sp\(^3\) center. The electron donors are typically the side chains of Ser,(12−15) Thr,(16) His,(17,18) or Lys,(19) which can interact directly with the boron atom or via an intervening water molecule(20) (Scheme 1). Furthermore, the boron atom is often covalently linked to one or two hydroxyl groups; hence, it can act as an acceptor or donor of H-bonds for additional molecular recognition.(21) Consequently, enzymes often bind boron-based inhibitors with high affinity such that the inhibition is effectively irreversible.(6) Traditionally, the boronic acid group has been employed for the design of inhibitors of hydrolytic enzymes, where either water(22) or the hydroxyl group of a Ser or Thr residue of the enzyme(5,6,9,23) reacts with the trigonal planar boronic acid group to yield a tetrahedral adduct that mimics the geometric and electronic features of the tetrahedral intermediate(s) and/or transition states (TSs) formed during hydrolysis (Scheme 1). We hypothesized that the Lewis acidity of the boronic acid functional group might also permit strong interactions with the Brønsted base catalyst(s) present at the active sites of enzymes that
catalyze heterolytic cleavage of a C–H bond. Indeed, many different enzymes catalyze such proton abstraction reactions, often to initiate 1,1-, 1,2-, or 1,3-migrations of protons, aldol or Claisen condensations, or β-elimination reactions.(24–26) Racemases and epimerases that utilize a two-base mechanism(27) offer particularly attractive targets because the Brønsted acid–base catalysts are located on opposite sides of the carbon atom where deprotonation occurs. This active site architecture should afford the potential for interactions between the enzyme and the boron atom of an inhibitor.

As a model system, we explored the inhibition of mandelate racemase (MR) by various boronic acids. MR has served as a powerful paradigm for developing our understanding of how enzymes overcome the thermodynamic and kinetic barriers accompanying the abstraction of a proton from a carbon acid substrate, as well as our general understanding of enzyme catalysis.(24,28−31) The enzyme utilizes a two-base mechanism with either Lys 166 or His 297 acting as the enantiospecific Brønsted base to abstract the α-proton from (S)- or (R)-mandelate, respectively (Scheme 2).(32–34) The resulting aci-carboxylate intermediate is subsequently reprotonated by the conjugate acid of the Brønsted base, located antipodal to the Brønsted base that effected deprotonation, to afford the enantiomeric product. Considering the arrangement of the Lys and His side chains around the stereogenic α-carbon, we envisioned that potent inhibition might arise if a bound boron-bearing inhibitor positioned the boron atom at the location normally occupied by the α-carbon of the substrate, thereby allowing for formation of N–B interactions.(35–42) Herein, we report that derivatives of phenylboronic acid (PBA) are the most potent inhibitors of MR yet described, exceeding the binding affinity of TS analogue inhibitors by ~1–2 orders of magnitude.(43,44) X-ray crystallographic studies reveal that the boronic acid moiety of the inhibitor is “gripped” by key catalytic residues at the active site, capitalizing on the ability of the boronic acid group to form multiple H-bonds. Moreover, crystallographic and 11B nuclear magnetic resonance (NMR) evidence supports the formation of an N–B dative bond between PBA and the Brønsted acid–base catalyst His 297. Our results suggest that replacement of the acidic carbon of a carbon acid substrate with an electrophilic boron atom expands the scope of boronic acids beyond their use as carboxylate isosteres and as inhibitors targeting hydrolytic enzymes.
Materials and Methods

General
All reagents, unless mentioned otherwise, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). 4-Methylphenylboronic acid and methylboronic acid were purchased from TCI America (Portland, OR). The (R)- and (S)-enantiomers of 4-chloromandelic acid were purchased from DSM Pharma Chemicals Regensburg GmbH (Regensburg, Germany). Circular dichroism (CD) spectral measurements were conducted using a JASCO J-810 spectropolarimeter (Jasco Inc., Easton, MI). Binding studies were conducted using a VP-ITC microcalorimeter (MicroCal, Inc., Northampton, MA). $^{11}$B NMR spectra were recorded using a Bruker AVANCE 500 NMR spectrometer at the Dalhousie Nuclear Magnetic Resonance Research Resource (NMR-3) Centre.

Enzyme Purification
StrepII-tagged recombinant variants of MR (wild-type MR, K166M-MR, and H297N-MR) were overexpressed in and purified from *Escherichia coli* BL21(DE3) cells transformed with the appropriate plasmid as described previously. These constructs encode the wild-type or mutant MR gene products as fusion proteins with an N-terminal StrepII tag (MASWSPQFEKGALEVLFQGPGYHM-MR, where M denotes the initial Met of either the wild-type or variant MRs and the StrepII tag is underlined). The purity of the wild-type and variant MRs (≥99%) was assessed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% acrylamide) with staining by Coomassie blue R-250. Protein concentrations were determined from the intrinsic enzyme absorbance at 280 nm using an extinction coefficient ε of 53400 M$^{-1}$ cm$^{-1}$ for all MR variants estimated using the ProtParam program from ExPasy (http://web.expasy.org/protparam/). The StrepII tag was not removed from the enzymes.

Inhibition Studies
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. All inhibition experiments were conducted at 25 °C in Na$^+$-HEPES buffer (0.1 M, pH 7.5) containing MgCl$_2$ (3.3 mM) and bovine serum albumin (BSA, 0.005%). Wild-type MR (150 ng/mL) was assayed with (R)-mandelate (0.5–15.0 mM) as the substrate and varying concentrations of PBA (1.00, 2.00, and 5.00 μM), 4-Br-PBA (0.08, 0.16, and 0.24 μM), 4-Cl-PBA (0.08, 0.16, and 0.24 μM), 4-CH$_3$-PBA (0.50, 1.00, and 1.50 μM), 4-CF$_3$-PBA (0.25, 0.50, and 0.75 μM), 4-CN-PBA (2.50, 5.00, and 7.50 μM), 4-F-PBA (0.20, 0.40, and 0.60 μM), 4-NO$_2$-PBA (0.50, 1.00, and 1.50 μM), 4-OCH$_3$-PBA (4.00, 8.00, and 12.00 μM), cyclohexylboronic acid (1.50, 3.00, and 4.20 mM), and methylboronic acid (0.125, 0.250, and 0.375 M). The apparent kinetic constants $V_{max}$ and $K_m$ were determined by fitting eq 1 to the initial velocity data using nonlinear regression analysis and KaleidaGraph ver. 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.
\[v_i = \frac{V_{\text{max}}[S]}{K_m + [S]}\]

(1)

Competitive inhibition constants \((K_i)\) for the inhibition of MR by the 4-substituted PBAs were determined from plots of the apparent \(K_m/V_{\text{max}}\) values versus inhibitor concentration in accord with eq 2.(50)

\[v_i = \frac{V_{\text{max}}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]}\]

(2)

The IC\(_{50}\) values for the inhibition of wild-type MR by 4-Cl-PBA (40–640 nM) at MR concentrations of 150, 300, and 450 ng/mL were determined, relative to \((R)\)-mandelate (1.0 mM), by fitting eq 3 to the relative velocities \((v_i/v_0)\) obtained at the indicated concentrations. The IC\(_{50}\) value is the concentration of the inhibitor that yields a \(v_i/v_0\) of 0.5, and \(n\) is the Hill number.(50)

\[\frac{v_i}{v_0} = \frac{IC_{50}^N}{IC_{50}^N + [I]^N}\]

(3)

Reversibility and Effect of Enzyme Concentration

The reversibility of inhibition by PBA were evaluated by measuring the recovery of enzyme activity after dialysis. Wild-type MR (1500 ng/mL) was incubated with PBA (0 μM, as a control, and 30 μM each in 3.0 mL) at 25 °C for 20 min, yielding 10% of the enzymatic activity. After dialysis against assay buffer (3 × 250 mL over 24 h), both the control and PBA-containing samples were assayed for enzymatic activity.

Assay with 4-Chloromandelate

The ability of MR to catalyze the racemization of \((R)\) - and \((S)\)-4-chloromandelate was examined using a CD-based assay. The CD spectra of \((R)\) - and \((S)\)-4-chloromandelate (10 mM) in assay buffer were recorded, and a suitable wavelength at which 4-chloromandelate exhibited a pronounced signal was chosen (276 nm). The molar ellipticity was estimated to be 5189 ± 12 deg cm\(^2\) mol\(^{-1}\) from the slope of a plot of the observed ellipticity at 276 nm against five concentrations of \((R)\)-4-chloromandelate (2.0, 4.0, 6.0, 8.0, and 10.0 mM). The values of \(k_{\text{cat}}\) \((V_{\text{max}}/[E]_T)\) and \(K_m\) were then determined in accord with eq 1 by measuring the initial rates for the racemization of \((R)\) - and \((S)\)-4-chloromandelate (0–10 mM) catalyzed by wild-type MR (150 ng/mL).

Isothermal Titration Calorimetry

Ligand solutions containing PBA were prepared in the final dialysis buffer to eliminate heat signals that could arise from buffer mismatch. MR variants and ligand solutions were degassed (Microcal Thermovac) for 15 min prior to being loaded into the sample cell (1.46 mL) and the injection syringe (297 μL), respectively. The stirred cell contained either wild-type MR (45 μM), H297N-MR (50 μM), or K166M-MR (60 μM), and the injection syringe contained various concentrations of PBA (1.0 mM, 6.0 μL/injection with wild-type MR; 2.0 mM, 6.0 μL/injection with H297N-MR; and 26.22 mM, 6.0 μL/injection with K166M-MR). Titrations were conducted at 20 °C. The heat released due to the initial injection (typically 2–5 μL) was excluded from data analyses to minimize the effect of diffusion of the titrant from the syringe tip during the equilibration process. To correct for the heats of dilution and mixing, ligand titrations were also conducted with the sample cell containing only buffer. The
dilution isotherm for each ligand was subtracted from the appropriate binding isotherm prior to curve fitting. Binding affinities and the $\Delta H$ and $\Delta S$ values for binding of PBA to the MR variants were obtained by fitting the calorimetric data with a single-site model using the Origin 7.0 software (OriginLab, Northampton, MA). For K166M-MR, the binding of PBA was weak and the experiment was limited by our inability to utilize higher concentrations of PBA due to precipitation of the protein. Curve fitting was therefore conducted by fixing the molar ratio ($n$) at 1.

$^{11}$B NMR Spectroscopy
All NMR spectra were recorded at 25 °C in Na$^+$-HEPES buffer (0.1 M, pH 7.5) containing MgCl$_2$ (3.3 mM) and D$_2$O (10%). Chemical shifts (δ) of the signals arising from $^{11}$B are reported relative to an external standard of BF$_3$·OEt$_2$ (δ = 0.00 ppm). Samples were in 5 mm quartz tubes (Sigma-Aldrich Canada Ltd.) to reduce the background signal arising from the boron in borosilicate glass. For the spectra of PBA in the presence of MR, the concentration of PBA was fixed at either 300 or 400 μM, and the spectra were recorded with varying amounts of wild-type MR added to the solution (0–300 μM). The background signal arising from borosilicate glass in the spectrometer probe was reduced in the spectra using Whittaker smoothing.(51)

Protein Crystallization
Crystals of wild-type MR were grown in the presence of PBA by the sitting-drop vapor diffusion method against a reservoir volume of 500 μL. The protein solution and reservoir solution were mixed in a 1:1 ratio to give a final volume of 10 μL. Crystals grew spontaneously at 21 °C. The reservoir solution consisted of PEG 4000 [10% (w/v)] and Bis-Tris Propane (BTP; 50 mM, pH 7.0). The protein solution consisted of MR (6 mg/mL) purified as described above, PBA (1.0 mM), MgCl$_2$ (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 4000 [8% (w/v)], BTP (80 mM, pH 7.0), ethylene glycol [5% (w/v)], PBA (0.77 mM), MgCl$_2$ (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 4000 [10% (w/v)], BTP (80 mM), ethylene glycol [20% (w/v)], PBA (0.8 mM), MgCl$_2$ (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-G at the Advanced Photon Source, Argonne National Laboratory, on a Rayonix MarMosaic 300 CCD detector with an X-ray wavelength of 0.979 Å. Diffraction images were autoprocessed using the autoPROC software workflow,(52) which merged and scaled the isotropic data in AIMLESS(53) with the programs TRUNCATE(54) and UNIQUE(55) to automatically 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,(56) with the program Phaser.(57) The molecular replacement models were extended by several rounds of manual model building with COOT(58) and refinement with Phenix.Refine(59) with an X-ray/stereochemistry weight of 2.0 applied 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 PBA were optimized for structure refinement using eLBOW (electronic ligand building and optimization workbench).(60) For each dimer, one active site (chains A, C, E, and G) was modeled with single occupancy by PBA with a C–B–O$^\alpha$–O$^\beta$ dihedral angle of ~145° (intermediate between sp$^2$- and sp$^3$-hydridized), while the other active site in the adjacent subunit (chains B, D, F, and H) was modeled at 50% occupancy by PBA with a C–B–O$^\alpha$–O$^\beta$ dihedral angle of ~165° (~sp$^2$-hydridized) and at 50%
occupancy by PBA with a C–B–Oα–Oβ dihedral angle of \(\sim 120^\circ\) (\(\sim sp^3\)-hybridized). The side chain of His 297 was also modeled in two conformations for chains B, D, F, and H. The model was refined using a ligand definition file for PBA with a C–B–Oα–Oβ dihedral angle of 145°, with loose restraints. For chains B, D, and F, the refinement supported two distinct conformations for PBA and His 297, each at 50% occupancy. However, for chain H, the refinement resulted in both conformations converging to a single conformation for His 297 and a single conformation for PBA, with a C–B–Oα–Oβ dihedral angle of 166°. Consequently, subsequent refinements modeled only a single conformation for PBA and His 297 in chain H. Data collection and processing statistics are summarized in Table S1.

Density-Functional Theory (DFT) Calculations
All calculations were performed using the Gaussian 09(61) and postg(62) programs, with the LC-ωPBE density functional.(63) The exchange-hole dipole moment (XDM) method(62,64) was used to include dispersion interactions in all geometry optimizations, with the following damping-function parameters: \(a_1 = 0.6889\) and \(a_2 = 1.9452\) Å. The geometry optimizations used a mixed basis set, with the 6-31G* basis assigned to boron, carbon, and hydrogen, and the larger 6-31+G* basis assigned to nitrogen and oxygen. The initial geometry was obtained from the coordinates of chain F from the X-ray crystal structure of MR with bound PBA (PDB entry 6VIM) refined with a single PBA at 100% occupancy (i.e., C–B–Oα–Oβ dihedral angle of 167.9°; His 297 Nε2–B and Lys 166 Nζ–B distances of 2.50 and 2.99 Å, respectively) and a single conformation of His 297, utilizing the first shell of residues interacting with PBA (Asn 197, Lys 164, Lys 166, and His 297), the Mg2+ ion and its ligands (H2O, Asp 195, Glu 221, and Glu 247), and Asp 270, which forms a catalytic dyad with His 297.(65) The amino acids were truncated (by hydrogen capping) at the sp3 or α-carbon atoms, the positions of which were held fixed during geometry optimizations, while all other atomic positions were allowed to relax. Several combinations of H atom positions and protonation states were considered, and the most stable retained. The \(^{11}B\) NMR chemical shifts were calculated with the Gauge-Independent Atomic Orbital (GIAO) method,(66) using the 6-31+G* basis set for all atoms. Analogous calculations were also carried out for an isolated PBA molecule. The boron chemical shifts are expressed relative to that for free PBA in assay buffer (i.e., \(\delta = 28.2\) ppm).

Results and Discussion

Inhibition of MR by PBAs
In accord with our hypothesis, PBA was a potent competitive inhibitor of MR with a \(K_i\) value of 1.8 \(\mu\)M (Table 1), binding with an affinity \(\sim 556\)-fold greater than that observed for the substrate (\(K_m = K_S \approx 1\) mM).(67) The onset of inhibition was rapid, and dialysis of the enzyme–inhibitor solution resulted in full recovery of enzyme activity, indicating that the inhibition was reversible. To investigate the dependence of the binding affinity on the identity of substituents on the phenyl ring, we determined the \(K_i\) values for a series of 4-substituted PBAs (Table 1 and Figures S1–S9). Plotting the observed \(\log(K_i^X/K_i^H)\) values against the Hammett substituent constants (\(\sigma_{para}\)) gave a concave-up pattern (Figure 1).(68) The decreased binding affinity of PBA derivatives with bulky electron-withdrawing substituents at the \(para\) position of the phenyl ring likely arose from unfavorable steric and polar interactions as observed previously for the weak binding of 4-nitromandelate,(67) rather than electronic effects. However, for the free energies accompanying binding of PBA derivatives bearing smaller \(para\) substituents (H, CH3, F, Br, Cl, and, surprisingly, OCH3), there was a roughly linear correlation (\(\rho = -2.9 \pm 0.8\)) indicating that electron-withdrawing character favored binding. A similar observation was reported for the inhibition of subtilisin by a limited number of phenylboronic acids (i.e., \(\rho = -0.895\)).(69) [Note that, as shown in Figure S10, using the \(pK_a\) values (Table S2) to adjust the \(K_i\) values assuming either the neutral trigonal or anionic tetrahedral boronic acid was the inhibitor did not significantly alter the shape of the plot, nor did plotting the data as a function of \(\sigma^-_{para}\).] The most potent inhibitor was 4-Cl-PBA, binding with a \(K_i\) value approximately \(1.23 \times 10^5\)-fold lower than that of the substrate.
Table 1. Inhibition of MR by Boronic Acids

<table>
<thead>
<tr>
<th>compound</th>
<th>$K_i$ (μM)$^a$</th>
<th>inhibition mode$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBA</td>
<td>1.8 ± 0.1</td>
<td>C</td>
</tr>
<tr>
<td>4-Br-PBA</td>
<td>0.123 ± 0.007</td>
<td>C</td>
</tr>
<tr>
<td>4-Cl-PBA</td>
<td>0.081 ± 0.004</td>
<td>C</td>
</tr>
<tr>
<td>4-CH₃-PBA</td>
<td>0.67 ± 0.07</td>
<td>C</td>
</tr>
<tr>
<td>4-CF₃-PBA</td>
<td>0.21 ± 0.01</td>
<td>C</td>
</tr>
<tr>
<td>4-CN-PBA</td>
<td>2.93 ± 0.02</td>
<td>C</td>
</tr>
<tr>
<td>4-F-PBA</td>
<td>0.23 ± 0.01</td>
<td>C</td>
</tr>
<tr>
<td>4-NO₂-PBA</td>
<td>0.66 ± 0.05</td>
<td>C</td>
</tr>
<tr>
<td>4-OCH₃-PBA</td>
<td>4.1 ± 0.2</td>
<td>C</td>
</tr>
<tr>
<td>cyclohexylboronic acid</td>
<td>(1.56 ± 0.05) $\times 10^3$</td>
<td>C</td>
</tr>
<tr>
<td>methylboronic acid</td>
<td>$K_i = (1.3 ± 0.1) \times 10^5$; $\alpha K_i = (7.1 ± 0.1) \times 10^5$</td>
<td>LM</td>
</tr>
</tbody>
</table>

$^aK_m = K_S \approx 1000$ μM.(67)

$^b$Competitive (C) and linear mixed-type (LM) inhibition.

Figure 1. Hammett plot for the inhibition of MR by para-substituted phenylboronic acids. The log($K_i^X/K_i^H$) values, where $K_i^X$ is the observed $K_i$ value for a given para-substituted PBA, are plotted against the para-substituent constant ($\sigma_{para}$). The line shown is the linear regression line [slope ($\rho$) = −2.9 ± 0.8] for the PBA derivatives for which $X = H, Br, Cl, CH_3, F,$ and OCH$_3$ (○). The bulkier electron-withdrawing groups (i.e., $X = NO_2, CN$, and CF$_3$) likely deviate from the correlation due to unfavorable steric and polar effects on binding (●).(67)

To assess whether this enhanced binding arose from electronic effects or some fortuitous interaction(s) with the chloro substituent, we determined the molar ellipticity of (R)- and (S)-4-chloromandelate (Figure S11) and investigated the kinetic parameters for MR acting on 4-chloromandelate as an alternative substrate (Figure S12).(70) Although the turnover numbers ($k_{cat}$) for (R)-4-chloromandelate (1343 ± 43 s$^{-1}$) and (S)-4-chloromandelate (1329 ± 33 s$^{-1}$) were greater than the corresponding values for (R)-mandelate (1029 s$^{-1}$) and
(S)-mandelate (775 s⁻¹),(71) the $K_m$ values of (R)-4-chloromandelate (1.09 ± 0.06 mM) and (S)-4-chloromandelate (0.90 ± 0.07 mM) were similar to those of (R)-mandelate (1.03 ± 0.05 mM) and (S)-mandelate (0.76 ± 0.03 mM), respectively.(71) Hence, the enhanced binding of 4-Cl-PBA likely arose from the electron-withdrawing effect of the chloro substituent. Because 4-Cl-PBA exhibited the highest binding affinity of the PBA derivatives examined, we determined the IC₅₀ values for inhibition by 4-Cl-PBA at three different enzyme concentrations (Figure S13) and found that they were independent of enzyme concentration (Table 1). This observation indicated that the PBA derivatives were not behaving as tight-binding inhibitors and fell into “zone A” as defined by Straus and Goldstein (i.e., $K_{i, app}/[E]r > 10$, and IC₅₀ $\sim K_{i, app}$).(72)

To explore the role of the phenyl ring in binding, we examined the ability of MR to bind cyclohexylboronic acid and methylboronic acid (Table 1 and Figure 2). Cyclohexylboronic acid retains the hydrophobic nature of the ligand but removes the planar and aromatic character. The 867-fold loss of binding affinity for cyclohexylboronic acid (Figure S14) relative to PBA suggested that the aromaticity of the phenyl ring contributed $\sim$4.0 kcal/mol to the overall change in free energy accompanying PBA binding. Strikingly, the additional loss of van der Waals interactions resulting from replacement of the phenyl ring with a methyl group led to a massive 72000-fold loss of binding affinity relative to PBA (Table 1). Interestingly, methylboronic acid exhibited linear mixed-type inhibition kinetics (Figure S15), likely arising from concomitant weak binding of the compound at an allosteric site ($\alpha K_i = 710$ mM).

MR binds PBA with an affinity that is $\sim$7-fold greater than that exhibited for the TS analogue inhibitor benzo hydroxamate (BzH; $K_i = 11.7$ μM).(73) Unlike PBA, replacement of the phenyl ring of BzH with a cyclohexyl ring (i.e., cyclohexylhydroxamate; $K_i = 0.25$ mM)(73) revealed that aromaticity of the phenyl ring contributed only $\sim$1.8 kcal/mol to the overall free energy change accompanying BzH binding (Figure 2). Thus, the aromatic character of the inhibitor makes a more substantial contribution to the binding of PBA than to the binding of BzH.

11B NMR Spectroscopy
To obtain information about the hybridization state of the boron of PBA when bound at the active site of MR in the solution state, we utilized 11B NMR spectroscopy. This approach has been employed to study the interaction
of boronic acid-bearing inhibitors with β-lactamase, (74) γ-glutamyl transpeptidase, (75) and a variety of proteases. (18, 76−83) In the absence of MR, the boron of PBA exhibited a signal with a chemical shift of 28.2 ppm in the assay buffer (Figure 3A), which is in accord with previous studies (20, 84) and arises from the neutral trigonal boronic acid species [Ph−B(OH)₂]. Upon addition of MR, a new peak was observed at 0.97 ppm (Figure 3B−D), which was also the sole peak present when the enzyme and PBA were at equal concentrations (Figure 3E), and suggested that there was slow exchange between the MR-bound PBA and free PBA. (79) The ¹¹B NMR signal from PBA bound to MR was significantly sharper than that of the free inhibitor, which has also been observed for aryl boronic acid inhibitors bound to chymotrypsin. (18, 78) The marked upfield shift of the ¹¹B signal of PBA in the presence of MR is consistent with additional electron density on the boron. Interestingly, the upfield chemical shift to 0.97 ppm is greater than the chemical shift values of 2.6 and 5.7 ppm observed for Ph-B(OH)₃⁻ and for the imidazole complex with PBA, respectively. (20) Thus, the ¹¹B NMR spectroscopic studies suggested that the boron of bound PBA likely participated in an N−B interaction (vide infra) with Lys 166 and/or His 297 and may exist either partially or fully in its anionic, sp³-hybridized state.

Contribution of the Brønsted Acid–Base Catalysts to Binding of PBA

Because the ¹¹B NMR spectroscopic studies suggested the possibility of an N−B interaction, we examined the effect of removing the side chains of Lys 166 (K166M) and His 297 (H297N) on the binding of PBA. Neither K166M-MR nor H297N-MR is catalytically active; (32, 45) however, the binding affinity of these variants for PBA
could be assessed using ITC. Although the binding event was only weakly exothermic, the thermograms for the titration of wild-type-MR, K166M-MR, and H297N-MR with PBA (Figure 4) permitted clear differentiation among the abilities of the three MR variants to bind PBA. As shown in Table 2, the $K_d$ value (1.7 μM) for the binding of PBA to wild-type MR was in excellent agreement with the $K_i$ value (1.8 μM) determined from the inhibition experiments. Most interestingly, K166M-MR bound PBA approximately $3 \times 10^3$-fold less tightly than wild-type MR ($\Delta \Delta G_{binding} = 4.6$ kcal/mol), while H297N-MR bound PBA only $\sim 30$-fold less tightly than the wild-type enzyme ($\Delta \Delta G_{binding} = 2.0$ kcal/mol). A similar trend was reported for the binding of BzH with the wild-type, K166M ($\Delta \Delta G_{binding} = 3.5$ kcal/mol), and H297N ($\Delta \Delta G_{binding} = 1.2$ kcal/mol) MR variants.(45) Thus, of the two Brønsted acid–base catalysts, Lys 166 plays a dominant role in the recognition of PBA.

### Table 2. Analysis of Binding Interactions with MR Variants at 20 °C Using ITC

<table>
<thead>
<tr>
<th>MR variant</th>
<th>$K_d$ (μM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (cal mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>1.7 ± 0.3</td>
<td>−0.96 ± 0.07</td>
<td>23.1 ± 0.5</td>
<td>−7.7 ± 0.1</td>
</tr>
<tr>
<td>K166Ma</td>
<td>4700 ± 400</td>
<td>−5.1 ± 0.5</td>
<td>−6.7 ± 1.8</td>
<td>−3.13 ± 0.05</td>
</tr>
<tr>
<td>H297N</td>
<td>52 ± 7</td>
<td>−1.08 ± 0.04</td>
<td>15.9 ± 0.4</td>
<td>−5.74 ± 0.08</td>
</tr>
</tbody>
</table>

*Values estimated by fixing the molar ratio ($n$) at 1.

Figure 4. Representative thermograms (top) and binding isotherms (bottom) for MR variants binding PBA at 20 °C. Titrations of (A) wild-type MR (45 μM) with PBA (1 mM, 6 μL per injection), (B) K166M-MR (60 μM) with PBA (26.22 mM, 6 μL per injection), and (C) H297N-MR (50 μM) with PBA (2 mM, 6 μL per injection). The first injection in each case (top) was 3 μL and was omitted when calculating the thermodynamic parameters (see Table 2). The molar ratio is [PBA]/[MR variant].

### Structure of the MR-PBA Complex

To explore the possibility of formation of an N–B interaction with the side chains of Lys 166 and/or His 297, we determined the X-ray crystal structure of the MR-PBA complex at 2.00-Å resolution. The complex crystallized as a homooctamer in space group $C_{12}1$ due to subtle variations in the structures of the eight subunits and active sites (Table S1). The individual subunits were very similar, with root-mean-square deviations (rmsds) for structural alignments between the α-carbon atoms of the individual subunits ranging from 0.077 to 0.104 Å. In a comparison of individual structural overlays with subunit A, subunits C, E, and G, representing the same face of the octamer as subunit A, had smaller rmsds (0.082 ± 0.006 Å), while subunits B, D, F, and H, representing the opposite face of the octamer from subunit A, had statistically larger rmsds (0.102 ± 0.001 Å). The electron density for the PBA ligands was well-defined over the entire molecule, and all eight active sites of the octamer were occupied by the ligand. PBA interacted with the Mg$^{2+}$ ion and formed multiple H-bonds with the side chains of active site residues (Figure 5A,B and Figure S16). The rmsds for structural alignments of the subunit A α-carbon atoms of the MR-PBA complex and apo-MR (PDB entry 2MNR)(87) and MR complexes with (S)-atrolactate (PDB entry 1MDR),(33) BzH (PDB entry 3UXK),(56) and Cupferron (Cfn, PDB entry 3UXL)(56) were 0.254, 0.284, 0.175, and 0.198 Å, respectively. Hence, there was no crystallographic evidence of large structural
changes accompanying the enzyme’s binding of PBA relative to when substrate or TS analogue inhibitors are bound. Furthermore, there were no gross differences in the conformation of the 20s or 50s loops.

Figure 5. Active site architecture of MR with bound PBA. (A) Representative stereoview showing PBA interacting with active site residues (stick representations) and the Mg$^{2+}$ ion (sphere) in chain A of the octamer. (B) Representative stereoview showing PBA (two conformations, each with 50% occupancy) interacting with active site residues (stick representations) and the Mg$^{2+}$ ion (sphere) in chain B of the octamer. (C) Stereoview showing the bound orientations of PBA (solid stick representations) from chain A (gray) and chain B (green), the intermediate/TS analogue inhibitors BzH (PDB entry 3UXK, blue)\(^{56}\) and Cfn (PDB entry 3UXL, yellow),\(^{56}\) and the substrate analogue (S)-atrolactate (PDB entry 1MDR, pink).\(^{33}\) The Mg$^{2+}$ ions (spheres) in the corresponding MR complexes are shown in the same color as the ligands.

As anticipated, the boron atom of PBA was located between the N$^\text{ζ}$ and N$^{\varepsilon_2}$ atoms of the Brønsted acid–base catalysts Lys 166 and His 297, respectively (Figure 6). Initial refinements revealed that the electron density could accommodate PBA with either a trigonal geometry or a tetrahedral geometry, with only slight differences in the position of the individual atoms. Formation of an N–B bond with the N$^\text{ζ}$ atom of Lys 166 was not supported by the electron density. Indeed, the N$^\text{ζ}$ atom of Lys 166 was directed toward the boronic acid hydroxyl group located $\sim$3.2 Å from the Mg$^{2+}$ ion, suggesting that Lys 166 forms an H-bond with the hydroxyl group, rather than a dative bond with the boron atom. The average (i.e., over the octamer) Lys 166 N$^\text{ζ}$–B distance was $\sim$2.9 Å, which is much longer than the N$^\text{ζ}$–B distance of 1.67 Å observed for covalent tetrahedral adducts with the $\varepsilon$-amino group of Lys in other structures\(^{19}\) and not sufficient to form a dative bond.\(^{88,89}\) When PBA was modeled as a single ligand with either a trigonal planar geometry or a tetrahedral geometry, the average His 297 N$^{\varepsilon_2}$–B distance was $\sim$2.5 or $\sim$2.2 Å, respectively (Table S3), which also did not agree with the expected bond distances of a dative N–B bond. Typically, N$^{\varepsilon_2}$–B distances of $\sim$1.6 Å have been observed in covalent, tetrahedral adducts of boronic acid inhibitors with hydrolytic enzymes\(^{83,90,91}\) and found in calculations.\(^{20}\) When we conducted DFT calculations on our system (\textit{vide infra}), it was clear that the His 297 N$^{\varepsilon_2}$–B distances required for formation of a tetrahedral boronic acid, as suggested from the NMR experiments, were not compatible with the
structure when either sp²- or sp³-hybridized PBA was modeled as a single ligand at the active sites. However, we noted that the electron density in chains B, D, F, and possibly H could accommodate both sp²- and sp³-hybridized PBA, as well as two conformations of the imidazole ring of His 297, at 50% occupancy each (Figure 6). Modeling the PBA ligands and the conformations of His 297 in this manner clearly revealed the presence of a His 297 N²–B dative bond with a length of 1.5 Å in chains B, D, and F. Furthermore, the C–B–Oα–Oβ dihedral angles for the modeled ligands were in reasonable agreement with those calculated using DFT calculations (Figure S17).

![Figure 6](image)

Figure 6. MR with bound PBA at 2.0-Å resolution for all eight subunits (chains A–H) of the homooctamer. The Mg²⁺ ion (sphere) and Brønsted acid–base catalysts Lys 166 and His 297 (stick representations) are shown. The 2Fo – Fc map is represented by a gray mesh contoured at 1.0σ. The simulated annealing omit map (Fo – Fc) is represented by a green mesh centered around PBA, contoured at 2.5σ. The distances are given in angstroms.

In addition to the His 297 N²–B interactions, the two boronic acid hydroxyl groups can form multiple H-bonds and/or electrostatic interactions with the nearby (≤3.6 Å) side chains of Lys 164, Lys 166, His 297, and Glu 317 (Figure 5A,B) that further contribute to the high binding affinity of PBA. Disruption of this H-bonding network upon substitution of Lys 166 with a Met residue may account for the greater contribution to the binding free energy observed for Lys 166, relative to His 297, in the ITC studies.

Superposition of (S)-atrolactate, BzH, and Cfn with PBA at the active site (Figure 5C) revealed that PBA assumes a binding orientation similar to those of the intermediate/TS analogue inhibitors. In chain A, the boron atom was displaced by 0.8 and 0.6 Å from the corresponding benzylic carbon of BzH and the equivalent nitrogen of Cfn, respectively, while in chain B, the corresponding displacements were 0.8 and 0.7 Å for the sp²-hybridized PBA.
and 1.4 and 1.2 Å for the sp³-hybridized PBA, respectively. Consequently, one of the hydroxyl groups of PBA was able to maintain H-bonding interactions with Glu 317 and Lys 164, similar to the interactions observed for (S)-atrolactate, BzH, and Cfn. Interestingly, this hydroxyl group appeared to occupy the sixth coordination site of the Mg²⁺ ion with a Mg²⁺–O distance of ∼3.2 Å, which resulted in a distorted octahedral coordination geometry about the divalent cation (Figure S18). The other boronic acid O atom was strongly coordinated to the active site Mg²⁺ ion with a Mg²⁺–O distance of ∼1.9 Å, which is reminiscent of the complex formed between PBA and the Co³⁺ ion at the active site of the nitrile hydratase from *Pseudonocardia thermophilia*.(92) Finally, comparison of the MR-PBA complex with the complexes of MR with (S)-atrolactate, BzH, and Cfn revealed that, in all cases, the distance between the Nε² atom of His 297 and the boron atom of PBA was shorter than the distances between the Nε² atom and the corresponding atoms of (S)-atrolactate, BzH, and Cfn, regardless of the hybridization state of the boron atom (Figure S19).

**DFT Calculations**

To obtain insights into the effect of varying the His 297 Nε²–B distance on the geometry of the boronic acid group, we employed LC-ωPBE-XDM/6-31+G* calculations(62) using PBA and 4-methylimidazole. The resulting correlation (Figure S17) revealed that the average dihedral angle (C–B–Oα–Oβ) of ∼165° observed for ∼sp²-hybridized PBA in all chains of the X-ray crystal structure was in reasonable agreement with the average His 297 Nε²–B distance of 2.8 Å, also observed in the structure (Table S3). Similarly, for the ∼sp³-hybridized PBA present (at 50% occupancy) in chains B, D, and F, the dihedral angles of ∼143° and the N–B distance of 1.5 Å (Table S3) were also in reasonable agreement with the calculations (Figure S17).

Our observation of a His 297 Nε²–B interaction in the X-ray crystal structure is in accord with the large upfield change in chemical shift of 27.2 ppm observed in the ¹¹B NMR experiments (Figure 3) and is consistent with formation of a tetrahedral boronic acid adduct. Typically, the ¹¹B chemical shift of boronic acids changes to a higher value when the boron atom changes hybridization from sp² to sp³,(93) and observation of such chemical shift changes in the ¹¹B NMR spectra of enzyme–boronic acid complexes has often been taken as support for the formation of sp³-hybridized boronate O–B or N–B adducts.(18,74,79,81–83) In addition to the electron density of chains B, D, and F supporting occupancy by an sp³- and an sp²-hybridized PBA each at 50%, chains A, C, E, G, and H supported the presence of an sp²-hybridized PBA at 100% occupancy. (However, even in those subunits where only a single conformation was modeled, we cannot rule out the possibility that a range of conformations is possible within the individual active sites.) While significant steric constraints present at the enzyme active site may lead to a boron-containing inhibitor assuming an unusual binding mode wherein the boron remains sp²-hybridized as a planar ester in some hydrolytic enzymes,(9,94) this does not seem to be the case for the MR-PBA interaction because the active site certainly tolerates the sp³-hybridized α-carbon of the substrate. Although development of partial negative charge on the boron atom would mimic the negative charge on the α-carbon of the altered substrate in the TS, the development of a full negative charge, mimicking the fully deprotonated intermediate, would likely not favor enhanced binding because it would be accompanied by a (substrate-like) tetrahedral geometry at the α-carbon. It should also be noted that benzoic acid, with an sp²-hybridized carbon at the same position as boron in PBA, is an exceptionally weak inhibitor of MR.(70)

To further elaborate on the ability of PBA to form N–B interactions with the side chains of His 297 and Lys 166, we conducted DFT calculations using an active site model that included PBA and amino acids truncated at the sp³ or α-carbon atoms with different combinations of protonation states (Table S4). Chain F was used as the model, refined with a single PBA molecule and a single conformation for the side chain of His 297, as the initial state. Because of the apparent H-bond between the side chain of Lys 166 and the Oβ atom of PBA (Figure 5A,B), the ε-amino group of Lys 166 was assumed to be protonated. Upon relaxation, the formation of a tetrahedral adduct with the Nε² atom of His 297 was observed (Figure S20). The calculated value of the ¹¹B chemical shift for the adduct was ∼1 ppm (Table S4), which is in excellent agreement with the observed value of 0.97 ppm.
Interestingly, the DFT calculations revealed that the protonation states yielding the lowest-energy configuration were Lys 164-NH$_3^+$, Lys 166-NH$_3^+$, and Glu 317-CO$_2$–. This configuration of protonation states corresponds with that expected at the intermediate, where the Glu 317-CO$_2$$^2$–···HO–B interaction mimics the Glu 317-CO$_2$H···–O–enolate interaction. Thus, it appears that PBA is being recognized as a TS analogue, consistent with its high binding affinity. DFT calculations also revealed that for the protonation state Lys 166-NH$_2$/His 297–Im, a His 297 N$^{\varepsilon 2}$–B bond formed, and only for the protonation state Lys 166-NH$_2$/His 297–ImH$^+$ did a Lys 166 N$^{\varepsilon}$–B bond form (Figure S21). The fact that the X-ray crystal structure furnishes no support for formation of such a Lys 166 N$^{\varepsilon}$–B bond supports the notion that the $\varepsilon$-amino group of Lys 166 is protonated in the MR·PBA complex.

Conclusions

Boronic acids have long been known as potent inhibitors of hydrolytic enzymes, forming metastable tetrahedral adducts with water or the hydroxyl groups of Ser or Thr residues that resemble the intermediate(s) and/or TS(s) formed during hydrolysis. To the best of our knowledge, this is the first report of a boronic acid acting as a potent inhibitor of an enzyme that catalyzes the abstraction of an $\alpha$-proton from a carbon acid substrate. MR binds PBAs with affinities that exceed those observed for the intermediate/TS analogue inhibitors BzH and Cfn by $\sim$1–2 orders of magnitude. This unprecedented and remarkable binding affinity of PBA arises from the interaction of the boronic acid function with multiple catalytic residues at the active site (notably, Lys 164, Lys 166, His 297, and Glu 317), as well as electrostatic interactions with the Mg$^{2+}$ ion and formation of a N$^{\varepsilon 2}$–B interaction with His 297. The observed interactions at the active site highlight the versatility of boronic acid–heteroatom interactions in inhibitor design. Moreover, our observations suggest that boronic acids may serve as inhibitors of other enzymes that share similar active site architectures with MR (i.e., Brønsted acid–base catalysts located on either side of a carbon atom where heterolytic C–H bond cleavage occurs), such as racemases and epimerases, many of which are therapeutic targets.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00478.

- X-ray diffraction data, kinetic and biophysical data, structural figures, and results from DFT calculations (PDF)

Accession Codes

The structure of MR with bound PBA has been deposited in the Protein Data Bank as entry 6VIM. Mandelate racemase, UniProt ID P11444.

Funding

This work was supported by Discovery Grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada to S.L.B. (Grant RGPIN-2016-05083) and E.R.J. (Grant RGPIN-2016-05795).

Notes

The authors declare no competing financial interest.

Acknowledgments

The authors thank Dr. Mike Lumsden (NMR-3) for assistance with the NMR experiments and Oliver Kuehm for assistance with analysis of the ITC data. 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).

References


