Inhibition of Mn^{2+}_2-Arginase by Borate Leads to the Design of a Transition State Analogue Inhibitor, 2(*S*)-Amino-6-boronohexanoic Acid

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Given the prominence of arginine catabolism in the regulation of diverse metabolic pathways such as ureagenesis[1] and nitric oxide biosynthesis,[2] the synthesis and evaluation of nonreactive arginine analogues as possible enzyme inhibitors or receptor antagonists is a rapidly-growing focus of medicinal chemistry.[2,3] To date, only one enzyme of mammalian arginine catabolism, rat liver arginase, has yielded an X-ray crystal structure to guide structure-based inhibitor design efforts.[4] This trimeric metalloenzyme contains a binuclear manganese cluster in the active site of each subunit required for maximal catalytic activity.[5] Arginine hydrolysis is achieved by a metal-activated solvent molecule that symmetrically bridges the $Mn^{2+}-Mn^{2+}$ ion pair in the native enzyme. The reaction coordinate of hydrolysis is postulated to proceed through a tetrahedral intermediate resulting from nucleophilic attack of metal-bridging hydroxide ion at the guanidinium carbon of arginine (Figure 1a).[4]

The tetrahedral borate anion is a modest, noncompetitive inhibitor of arginase, with $K_{is} = 1.0$ mM and $K_{ii} = 0.26$ mM; inhibition is even more pronounced in the presence of product ornithine, which is a competitive inhibitor with $K_i = 1.0$ mM.[6,7] In order to understand the mode of inhibition, we now report the X-ray crystal structure of the ternary arginase−ornithine−borate complex. The tetrahedral borate anion mimics binding

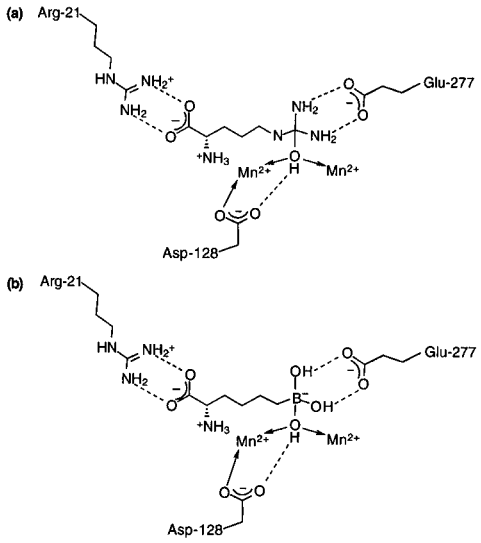

Figure 1. (a) The proposed tetrahedral intermediate of arginase-catalyzed arginine hydrolysis resulting from nucleophilic attack of metal-bridging hydroxide ion at the guanidinium carbon of arginine.[4] (b) Arginine analogue 2(*S*)-amino-6-boronohexanoic acid (**6**), which in the hydrated form illustrated is a structural analogue of the tetrahedral intermediate.

interactions postulated for the tetrahedral transition state(s) in the catalytic reaction (Figure 1a).[4] This result provides an important foundation for the design, synthesis, and evaluation of the first boronic acid analogue of arginine, 2(*S*)-amino-6-boronohexanoic acid (**6**). The high affinity of this inhibitor for arginase is proposed to result from the structural similarity between its hydrated form and the proposed tetrahedral intermediate (and flanking transition states) for arginase-catalyzed arginine hydrolysis (Figure 1b).

Crystal Structure of the Arginase−Ornithine−Borate Complex. Crystals of rat liver arginase were prepared as described[8] and gradually transferred to a buffer solution containing 10 mM ornithine and 10 mM sodium borate. X-ray diffraction data to 3.0 Å resolution were collected and processed as previously described[4] (28 047 total reflections (20−3 Å), 13 114 unique reflections (9−3 Å) used in refinement, 74% complete with $R_{merge} = 0.062$). The atomic coordinates of native rat liver arginase[4] served as the starting model for refinement with X-PLOR.[9] Refinement of the arginase−ornithine−borate complex converged smoothly to a final crystallographic R factor of 0.190 for 9−3 Å data ($R_{free} = 0.301$), with root-mean-square deviations from ideal bond lengths and angles of 0.013 Å and 1.6°, respectively.

The crystal structure of the arginase−ornithine−borate complex reveals the net displacement of the manganese-bridging solvent molecule of the native enzyme by an oxygen of the tetrahedral borate anion. No other structural changes are observed in the manganese coordination polyhedra, and the average metal−metal separation is 3.5 Å as predicted from EPR studies[7] (the average separation is 3.3 Å in the native enzyme[4]). Although the low resolution of this structure determination precludes a definitive conclusion on the binding conformation of ornithine, an interaction between the α-carboxylate of ornithine and the side chain of Arg-21 is evident in two of the three arginase subunits (data not shown).

Synthesis and Evaluation of 2(*S*)-Amino-6-boronohexanoic Acid (6). Boronic acids are effective aminopeptidase and serine protease inhibitors because they presumably bind as tetrahedral

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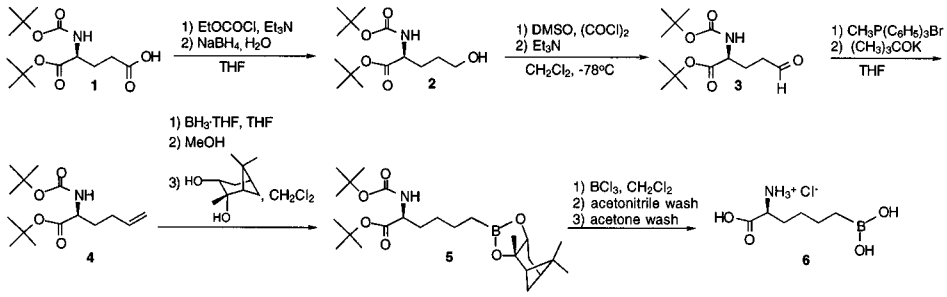
Scheme 1



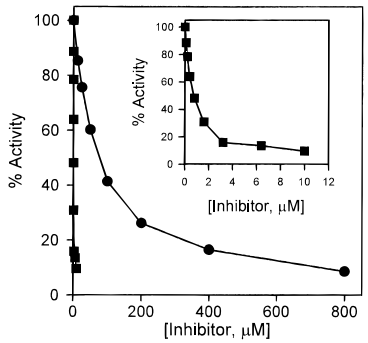


Figure 2. Inhibition of arginase by *N*-hydroxyarginine (●) and **6** (■). Inset: expanded plot showing inhibition by **6**; $IC_{50} = 0.8\ \mu M$.

transition state analogues.[10] The electron-deficient boron atom of a boronic acid invites the addition of a suitable nucleophile (e.g., a protein-bound nucleophile or a solvent molecule) to yield a stable, anionic tetrahedral species. Based on the structure of the ternary arginase−ornithine−borate complex and based on the previously-characterized behavior of boronic acid-based inhibitors of aminopeptidases, we postulated that the boronic acid analogue of arginine, 2(*S*)-amino-6-boronohexanoic acid (**6**), would bind avidly to arginase as the hydrated anion to mimic the tetrahedral intermediate and its flanking transition states (Figure 1b).

The compound 2(*S*)-amino-6-boronohexanoic acid (**6**) is the first example of a boronic acid-based arginine isostere, and the synthesis is outlined in Scheme 1. Sodium borohydride reduction of the ethyl carbonate anhydride of **1** affords the primary alcohol **2** (45% yield).[11] Swern oxidation[12] of **2** yields the desired aldehyde **3** which is used directly without further purification. The subsequent Wittig reaction[13] of crude **3** with triphenylphosphonium methylide yields olefin **4** in 22% yield over the two steps. The one-pot synthesis of **5** is achieved by hydroboration (4-fold excess of borane at −78 °C)[14] followed by treatment with methanol to quench unreacted borane and protection with (1*S*,2*S*,3*R*,5*S*)-(+)-pinanediol (31% yield).[15] Complete deprotection with BCl_3 yields 2(*S*)-amino-6-boronohexanoic acid (**6**) as a white semicrystalline solid (42% yield; 1.3% overall yield).[16] Crystal structure determination of **6** confirms the trigonal planar geometry of the boronic acid moiety. Complete details of the synthesis and characterization of compounds **1**−**6** and the crystal structure determination of **6** are found in the Supporting Information.

The inhibitory activity of **6** against arginase was evaluated using the radioactive [^{14}C-guanidino]arginine assay of Rüegg and Russell[17] as described.[6b] In initial experiments, IC_{50} values for **6** and *N*-hydroxyarginine ($K_i = 42\ \mu M$)[18] were compared. Assays performed in 100 mM CHES (pH = 9.0) containing 1 mM arginine yield an IC_{50} of 80 μM for *N*-hydroxyarginine and an IC_{50} of 0.8 μM for **6** (Figure 2). A more complete kinetic analysis of **6** was complicated by nonlinearity of kinetic replots. The origin of this nonlinearity is not clear, since **6** is a reversible inhibitor that shows no evidence of slow-binding behavior (a detailed analysis of the inhibition kinetics of **6** as well as other boronic acid-based inhibitors, will be published at a future date). Additional evidence for high affinity of **6** was derived from competition binding experiments using **6** and *N*-hydroxyarginine, as monitored by fluorescence spectroscopy (data not shown).[19] These experiments indicate that $K_d \leq 0.1\ \mu M$ for **6** at pH 7.5 and pH 9.0.

The compound 2-amino-6-boronohexanoic acid (**6**) is one of the most potent inhibitors of Mn^{2+}_2-arginase reported to date. Previously reported inhibitors include various free amino acids (millimolar K_i values), *N*-hydroxyarginine ($K_i = 42\ \mu M$), *N*-hydroxyindospicine ($K_i = 20\ \mu M$), *N*-hydroxylysine ($K_i = 4\ \mu M$), and N-hydroxy-nor-arginine ($K_i = 0.5\ \mu M$).[18,20] It is generally agreed that the closer the structural analogy between an inhibitor and the catalytic transition state, the tighter the inhibitor is expected to bind.[21] We propose that the high affinity of **6** toward arginase arises from the fact that in the hydrated form, it is the closest structural analogue of the tetrahedral intermediate (and its flanking transition states) generated to date. Future work will involve the X-ray crystal structure determination of the complex between arginase and **6** in order to verify the proposed binding mode outlined in Figure 1.[22]

Supporting Information Available: Details of synthesis and characterization of intermediates and final product **6** and X-ray crystal structure determination of final product **6** (12 pages). See any current masthead page for ordering and Internet access instructions.

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