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Pentapeptide Boronic Acid Inhibitors of *Mycobacterium tuberculosis* $MycP_1$ Protease

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Abstract

Mycosin protease-1 ($MycP_1$) cleaves ESX secretion-associated protein B (EspB) that is a virulence factor of *Mycobacterium tuberculosis*, and accommodates an octapeptide, AVKAASLG, as a short peptide substrate. Because peptidoboronic acids are known inhibitors of serine proteases, the synthesis and binding of a boronic acid analog of the pentapeptide cleavage product, AVKAA, was studied using $MycP_1$ variants from *M. thermoresistible* ($MycP_{1mth}$), *M. smegmatis* ($MycP_{1msm}$) and *M. tuberculosis* ($MycP_{1mtu}$). We synthesized the boropentapeptide, $HAlaValLysAlaAlaB(OH)_2$ (**1**) and the analogous pinanediol PD-protected $HAlaValLysAlaAlaBO_2(PD)$ (**2**) using an Fmoc/Boc peptide strategy. The pinanediol boropentapeptide **2** displayed IC_{50} values 121.6±25.3 μM for $MycP_{1mth}$, 93.2±37.3 μM for $MycP_{1msm}$ and 37.9±5.2 μM for $MycP_{1mtu}$. Such relatively strong binding creates a chance for crystalizing the complex with **2** and finding the structure of the unknown $MycP_1$ catalytic site that would potentially facilitate the development of new anti-tuberculosis drugs.

Mycobacterium tuberculosis exerts a staggering human and economic toll: in 2012, an estimated 8.6 million people developed tuberculosis and 1.3 million died from the disease[1]. *M. tuberculosis* secretes several highly immunogenic proteins across the cell wall using the ESX-1 transport system[2], and these virulence factors cause lung tissue inflammation and necrosis[3]. *In vivo* inhibition of $MycP_1$ protease, in a mouse model of infection[4], led to a lower mortality rate than in untreated animals. In addition, a *M. tuberculosis* strain with a mutation affecting the catalytic activity of $MycP_1$ was less virulent than a wild type strain[4].

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Inhibition of $MycP_1$ protease, which is one of the components of the ESX-1 transport system, is an attractive target for drug development[5-11]

It was recently shown that *M. smegmatis* $MycP_1$[11] and *M. thermoresistibile* $MycP_1$[12] process *M. tuberculosis* EspB at positions Ala^{358} and Ala^{386}. We confirmed that the octapeptide, (H)AVKAASLG(OH), mimicked the natural substrate in a fluorescent resonance energy transfer (FRET) experiment using an internally quenched peptide, (Abz)AVKAASLG(DNP) with an N-terminal, *ortho*-aminobenzoic acid (Abz) fluorescent group and a C-terminal, 2,4-dinitrophenyl (DNP) quencher[12]. Cleavage of this octapeptide substrate by $MycP_1$ liberated the readily measured, fluorescent pentapeptide (Abz)AVKAA(OH), and this FRET system also provided a convenient means for screening potential $MycP_1$ inhibitors[13]. Peptidyl boronic acids and their cyclic boronic esters with 1,2-diols are known inhibitors of various serine proteases[14-17] in the nanomolar range, including peptidoboronic acids that show selectivity towards *M. tuberculosis*.[18] The mechanism of action of peptidoboronate inhibitors involves the formation of tetrahedral complexes with active-site serines.[19-21] Variability in the activity of structurally related peptidoboronic acids and peptidoboronates in the literature[22] prompted us to determine if either the boronic acid analog **1** or the boronate **2** (Fig. 1) of the pentapeptide (H)AVKAA(OH) would inhibit the MycP1 protease and provide lead structures for the development of still other, clinically useful inhibitors.

Solution-phase synthesis of $HAlaValLysAlaAlaB(OH)_2$ (**1**) and the pinanediol PD-protected $HAlaValLysAlaAlaBO_2(PD)$ (**2**) involved the initial coupling of FmocLys(Cbz)OH (**3**) to HAlaOtBu to afford the dipeptide, FmocLys(Cbz)AlaOtBu, Fmoc-deprotection, and an additional coupling to (Cbz)AlaVal(OH) to provide the tetrapeptide intermediate, (Cbz)AlaValLys(Cbz)AlaOtBu (**4**) (Fig. 2). Acid-catalyzed removal of the tert-butoxy group furnished the Cbz-protected tetrapeptide, and HATU-promoted condensation[23] with ((R)-boroalanine-(1S,2S,3R,5S)-(+)-2,3-pinanediol ester provided the protected pentapeptide **5**, purified by a combination of flash silica gel and preparative layer silica gel chromatography. Hydrogenolysis[24] afforded **2** and acid-catalyzed hydrolysis of **2** provided $(H)AlaValLysAlaAlaB(OH)_2$ (**1**), albeit in low yield.

We have previously characterized the activity of $MycP_1$ variants from *M. thermoresistible* ($MycP_{1mth}$) and *M. smegmatis* ($MycP_{1msm}$) using a quenched fluorescent peptide assay[13]. In addition to these variants, we also expressed and purified $MycP_1$ from *M. tuberculosis* ($MycP_{1mtu}$). We characterized the activity $MycP_{1mtu}$ and found significant differences in enzyme activity relative to other $MycP_1$ homologs. In particular, the specific activity of $MycP_{1mtu}$ was 28.2±2.0 nmol/min/mg, which was four times higher than that of $MycP_{1mth}$ homolog (Table 1). This difference in enzyme activity was not surprising because the peptide substrate, (Abz)AVKAASLGK(DNP)OH was based on the cognate *M. tuberculosis* substrate $EspB_{mtu}$ residues 354-362 (AVKAASLG). This recognition region displayed sequence variations in *M. thermoresistible* $EspB_{mth}$ (*i.e.*, SVKPAAGG) and in *M. smegmatis* $EspB_{msm}$ (*i.e.*, SLKPASAG), and consequently, the affinity determinants in the $MycP_{1mth}$ and $MycP_{1msm}$ binding sites differed from those of $MycP_{1mtu}$. Nevertheless, all three species variants had measureable activity using the quenched fluorescent octapeptide as a substrate.

The potency of the synthetic boronic acid analogs **1** and **2** was tested using the three $MycP_1$ variants. Because compound **1** exhibited poorer inhibition than compound **2** in preliminary testing, we focused on the characterization of compound **2**. As expected, all were inhibited to some extent, but the inhibitor showed relatively tighter binding to the cognate $MycP_{1mtu}$ enzyme (Fig. 3) than their binding to the two others. The concentrations necessary to achieve 50% inhibition of $MycP_1$ (IC_{50} values) were as follows: $MycP_{1mtu}$ = 37.9±5.2 μM, $MycP_{1mth}$ = 121.6±25.3 μM, and $MycP_{1msm}$ = 93.2±33.7 μM. It has been reported that the inhibition of boronic acid peptides could vary over several orders of magnitude depending on the chemical structure of amino acid at critical positions of peptide[18]. While the inhibition of $MycP_1$ variants by the boronic acid analog **2** is relatively moderate, it could be improved by using a combinatorial chemistry approach to analyze a library of peptides with variable sequence. For example, $MycP_1$ displays a higher activity against a substrate peptide with Met in P1 position.[12] therefore, boronic acid analogs with aliphatic side groups in this position could be explored in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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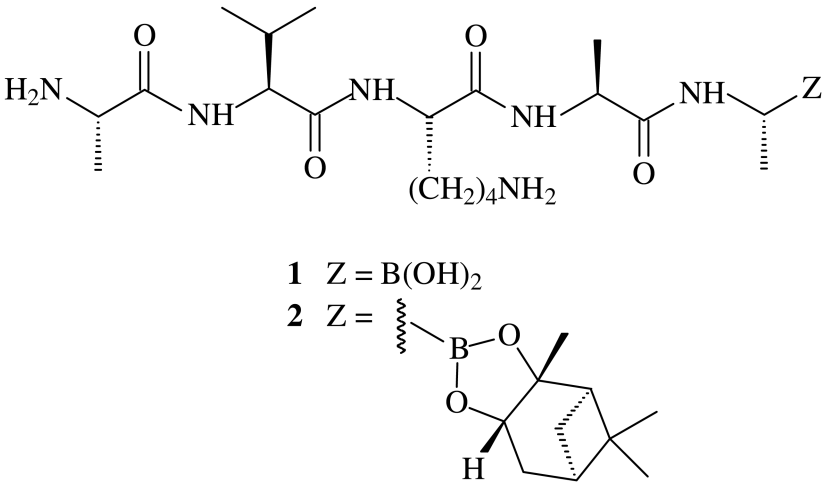

Figure 1. Boronic acid analogs, HAlaValLysAlaAlaB(OH)$_2$ (1) and the pinanediol PD-protected HAlaValLysAlaAlaBO$_2$(PD) (2)

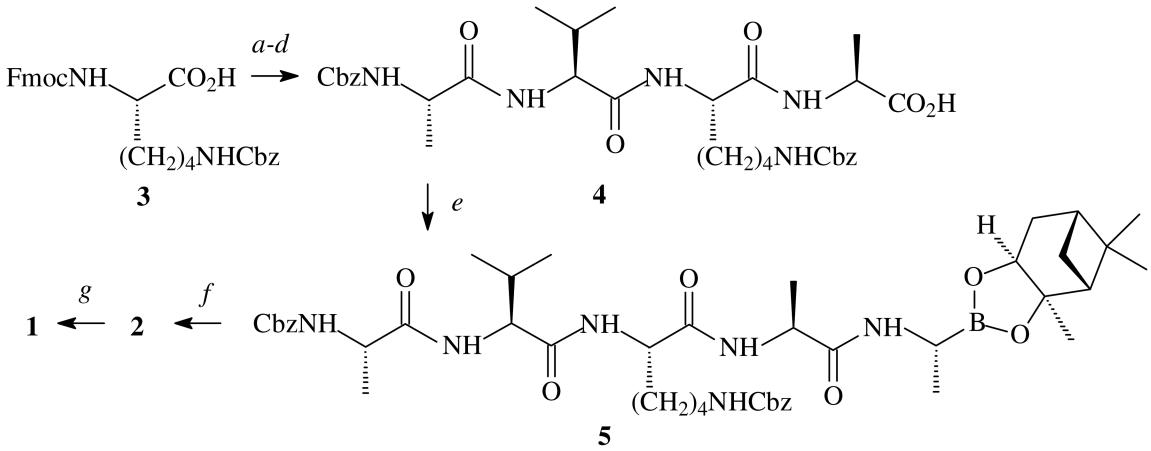

Figure 2. Synthesis of HAlaValLysAlaAlaB(OH)$_2$ (1) and HAlaValLysAlaAlaBO$_2$(PD) (2)

Legend : *a*, HAlaOtBu, hydroxybenzotriazole (HOBt), iPr$_2$NEt, EDC-HCl, N,N-dimethylformamide (DMF), 0°C to 25°C, 24 h; *b*, piperidine, DMF, 25°C, 1 h; *c*, CbzAlaValOH, HOBt, iPr$_2$NEt, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMF, 0° to 25°C, 24 h; *d*, CF$_3$CO$_2$H, CH$_2$Cl2, 25°C, 24 h; *e*, ((R)-boroalanine-(1S,2S,3R,5S)-(+)-2,3-pinanediol ester hydrochloride, i-Pr$_2$NEt, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), DMF, 0°C, 0.5 h; *f*, H$_2$, 10% Pd/C, 25°C, 17 h; *g*, 2-methylpropylboronic acid, 3M HCl, 25°C, 17 h.

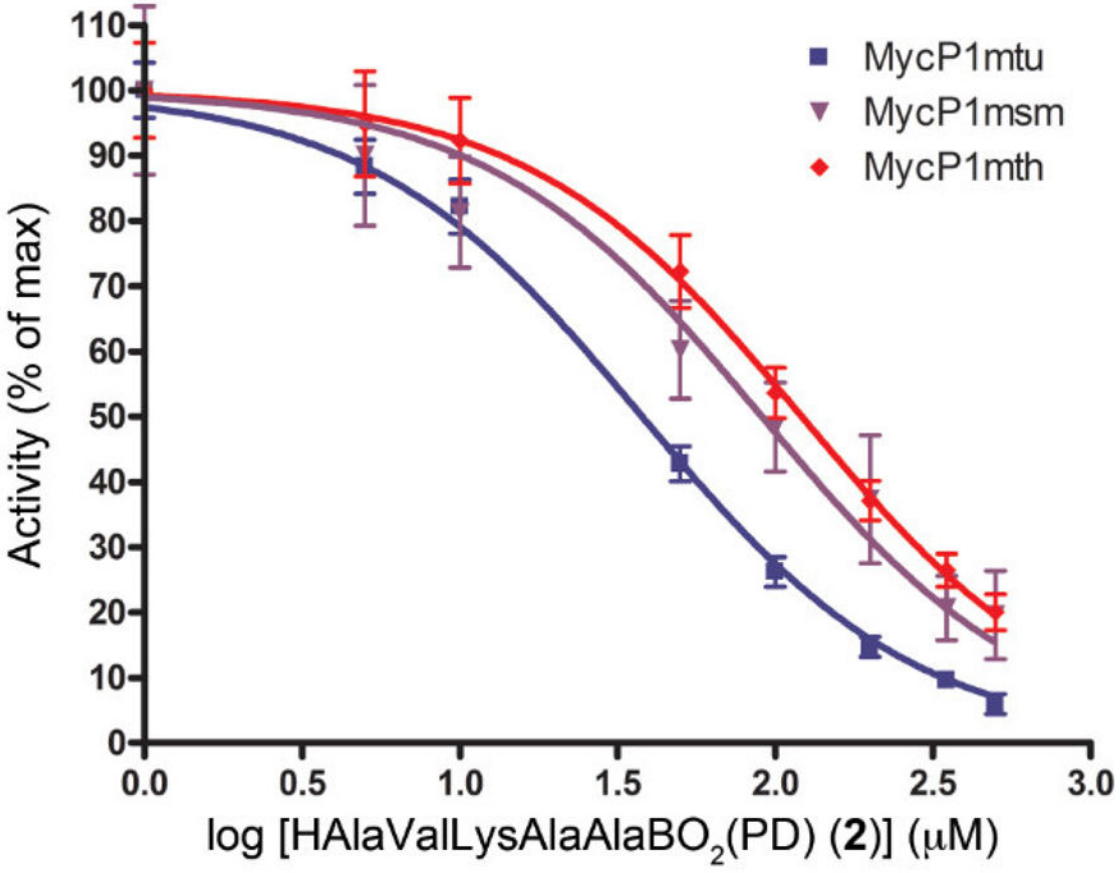

Figure 3. Determination of IC_{50} of HAlaValLysAlaAlaBO$_2$(PD) (2) for MycP$_1$ variants

The IC_{50} of **2** was measured for MycP$_1$ variants from *M. tuberculosis* (MycP$_{1mtu}$), *M. thermoresistible* (MycP$_{1mth}$), and *M. smegmatis* (MycP$_{1msm}$), using a quenched fluorescent peptide (Abz)AVKAASLGK(DNP)OH). Activity of MycP$_1$ is plotted as a function of the logarithm of the concentration of **2**. Calculated IC_{50} values were: MycP$_{1mtu}$ = 37.9±5.2 μM, MycP$_{1mth}$ = 121.6±25.3 μM, and MycP$_{1msm}$ = 93.2±33.7 μM.

Table 1

Michaelis-Menten parameters for the three $MycP_1$ variants

$MycP_1$ variant	K_m (μM)	V_{max} (nmol/min/mg)
M. tuberculosis	79±11	28.2±2.0
M. thermoresistible	60±12	6±0.6
M. smegmatis	86±42	1.8±0.4