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Characterization of D -boroAla as a Novel Broad Spectrum Antibacterial Agent Targeting D -Ala- D -Ala Ligase

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Abstract

D -boroAla was previously characterized as an inhibitor of bacterial alanine racemase and D -Ala- D -Ala ligase enzymes [Duncan, K., et al *Biochemistry* 1989, 28:3541–9]. In the present study, D -boroAla was identified and characterized as an antibacterial agent. D -boroAla has activity against both Gram-positive and Gram-negative organisms, with MICs down to 8 $\mu\text{g/mL}$. A structure-function study on the alkyl side chain ($\text{NH}_2\text{-CHR-B(OR')}_2$) revealed that D -boroAla is the most effective agent in a series including boroGly, D -boroHomoAla, and D -boroVal. L -boroAla was much less active, and *N*-acetylation completely abolished activity. An LC-MS/MS assay was used to demonstrate that D -boroAla exerts its antibacterial activity by inhibition of D -Ala- D -Ala ligase (DDL). D -boroAla is bactericidal at 1 $\times$ MIC against *Staphylococcus aureus* and *Bacillus subtilis* – which each encode one copy of DDL, and at 4 $\times$ MIC against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium – which each encode two copies of DDL. D -boroAla demonstrated a frequency of resistance of 8×10^{-8} at 4 $\times$ MIC in *S. aureus*. These results demonstrate that D -boroAla has promising antibacterial activity, and could serve as the lead agent in a new class of DDL targeted antibacterial agents. This study also demonstrates D -boroAla as a possible probe for DDL function.

Keywords

antibacterial; cell wall; alanine branch; broad spectrum; D -Ala- D -Ala ligase

INTRODUCTION

Bacterial infections were the major cause of death and morbidity prior to the development of modern antibiotics, and the increasing resistance of pathogenic bacteria to commonly used antibacterial agents is of major public health concern. MRSA (methicillin-resistant *Staphylococcus aureus*) and several Gram-negative pathogens such as *Pseudomonas aeruginosa*, *Burkholderia sp.*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* are of

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particular concern (1–7). Further heightening concern about existing and emerging bacterial drug resistance is the fact that, although a number of new antibacterial agents from known antibacterial classes are under development, only two new class of antibacterial agents have been introduced into clinical practice in the last 40 years – the oxazolidinones as represented by linezolid (8), and the lipopeptides as represented by daptomycin (9, 10). There is therefore an urgent need to identify new classes of antibacterial agents, especially agents which act through novel mechanisms and for which mechanisms of resistance are not yet known (6).

During our efforts to develop transition-state analog inhibitors for bacterial cell wall synthesizing enzymes (11, 12), we observed that *D*-boroAlanine (*D*-Ala with the -COOH group replaced with a -B(OH)₂ group) had effective antibacterial activity. While *D*-boroAla has previously been described as an inhibitor of alanine racemase and *D*-Ala-*D*-Ala ligase (13), it has not previously – to our knowledge – been reported as an antibacterial agent. In this report we describe the antibacterial properties of *D*-boroAla, structure-activity correlation among several *D*-boroAla homologs, and determination of the biochemical mechanism for *D*-boroAla's antibacterial activity. This study demonstrates that *D*-boroAla has broad spectrum antibacterial activity, and targets *D*-Ala-*D*-Ala ligase (DDL) in the alanine branch of bacterial cell wall biosynthesis (Fig. 1).

This study suggests that suitably designed derivatives and analogs of *D*-boroAla could provide unique new antibacterial agents for use in countering drug resistant pathogenic bacteria.

MATERIALS and METHODS

Synthesis of boro-amino acid analogs

The synthesis strategy for alkyl side chain *D*-boro-amino acid analogs was adapted from general procedures for *L*-boro analogs developed by Matteson, Kettner, and coworkers (14–16) and described previously (11) (Scheme 1). The synthesis strategy used for boroGly pinacol is outlined in Scheme 2.

(–)-Pinanediol (1*S*)-(1-aminoethyl)-1-boronate (*D*-boroAla-(–)-pinanediol) (*R* = CH₃) (**6a**) was synthesized and characterized as described previously (11).

(+)-Pinanediol (1*R*)-(1-aminoethyl)-1-boronate (*L*-boroAla-(+)-pinanediol), the opposite enantiomer to **6a**, was synthesized as described previously (11).

(–)-Pinanediol (1*S*)-1-amino-propane-1-boronate (*D*-boroHomoAlanine-(–)-pinanediol) (*R* = Et) (**6b**) was synthesized following the procedure described by Vankatraman *et. al.* for the *L*-isomer (17), substituting (–)-pinanediol for (+)-pinanediol as the stereochemical directing group, and using EtLi in place of EtMgBr as the alkyl anion.

(–)-Pinanediol (1*S*)-1-amino-2-methylpropane-1-boronate (*D*-boroValine-(–)-pinanediol) (*R* = *i*Pr) (**6c**) was synthesized following the procedure described by Kettner and Shenvi for the *L*-isomer (16) and substituting (–)-pinanediol for (+)-pinanediol as the stereochemical directing group.

Acetyl-*D*-boroAlanine-(–)-pinanediol (**7**) was synthesized from *D*-boroAla-(–)-pinanediol as described previously (11).

(1*S*)-(1-aminoethyl)-1-boronate (*D*-boroAlanine) (**8**), the free acid of *D*-boroAla, was prepared from the HCl salt of (**6a**) by treatment with excess benzene boronic acid in a 1:1 water/ether mixture plus 0.2 equivalents of 1 M HCl, followed by extraction of the aqueous

phase 3× with an equal volume of ether (18) (Scheme 1). The pure HCl salt of D-boroAla was obtained from the separated aqueous phase after lyophilization.

Pinacol (chloromethyl)boronate (9) was synthesized following the procedure of Sadhu and Matteson (19), and converted *in situ* to the pinacol ester, pinacol (chloromethyl)boronate (**9**).

Pinacol aminomethylboronate (boroGlycine-pinacol) (11) was synthesized following the procedure as described above for conversion of intermediates **4a–c** to products **6a–c** in Scheme 1, as described by Martichonok and Jones (20).

Antibacterial Properties Characterization

MICs and spectrum of activity—Minimal inhibitory concentrations (MICs) were determined by broth microdilution following CLSI guidelines (Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards (21)). Two-fold serial dilutions of test agents were prepared in 100 µL of Mueller Hinton Broth (Difco) in the wells of microtiter plates. Wells were inoculated with $\sim 1 \times 10^4$ colony forming units (cfu) of the test bacteria, and plates incubated for 16–20 h at 35 °C. The plates were read for turbidity either visually or at 600 nm in a Tecan SpectroFluor Plus microtiter plate reader. The MIC was read as the lowest concentration of test compound for which no turbidity is apparent (Transmittance >90% of a media control well). MICs were determined against several bacterial pathogens (Table 2), including both Gram-positive and Gram-negative organisms, to determine spectrum of activity. All MIC determinations were performed in triplicate.

MBCs—Minimal bactericidal concentrations (MBCs) were performed by plating serially diluted samples from wells of microtiter plates from MIC determination assays onto agar media. After overnight (24 h) incubation at 35 °C, colonies were counted and used to calculate cfu's of the samples. The MBC was defined as the lowest concentration of drug which killed 99.9% (≥ 3 log reduction) of the original inoculum.

Frequency of resistance—A clinical isolate of methicillin-sensitive *S. aureus* (MSSA) was grown for 16 h at 35 °C in 100 mL Mueller Hinton Broth, with shaking at 250 rpm. Bacterial cells were concentrated from 50 mL of this saturated overnight culture by centrifugation at 3000 rpm for 15 min and reconstituted into 5 mL media. Samples of 0.4 mL ($\sim 1 \times 10^9$ cfu) were plated onto 150-mm agar plates containing 75 mL of media with (**6a**) at concentrations of 2× and 4× MIC. As a reference, the cfu of the reconstituted culture was also determined. At each concentration, 3–4 plates were used. Inoculated plates were incubated for 48 h at 35 °C and each plate visually screened for growth of single colonies. Representative subsets of observed colonies were selected using a sterile inoculating loop and re-streaked onto fresh media containing **6a** (at selection concentration) to confirm resistance. The frequency of resistance was calculated by dividing the number of resistant colonies by the total number of cfu's plated.

In vivo Biochemical Mechanism Determination

Determination of in vivo intracellular L-Ala, D-Ala, and D-Ala-D-Ala levels in response to D-boroAla-(–)-pinanediol—Levels of L-Ala, D-Ala, and D-Ala-D-Ala were determined as described in detail previously (22). Bacteria (*Escherichia coli* K12 or MRSA (clinical isolate)) were grown to an OD at 600 nm of 0.6 in either minimal media (*E. coli*) or Mueller Hinton broth (MRSA). To a test culture was added D-boroAla-(–)-pinanediol (**6a**) to 4× MIC, and to control cultures were added no antibiotic, or a control antibiotics (cycloserine, tetracycline, or vancomycin) at 4–8× their respective MICs. Growth inhibition was observed within 15 min. Once growth inhibition was apparent cultures were rapidly

cooled in an ice/water bath, 4 samples of 10 mL were moved from each flask to ice-cold 15-mL centrifuge tubes, and the cells were pelleted by centrifuge at 3000 rpm for 10 min at 2 °C. Cell pellets (~50 µL) were resuspended in 100 µL of ice-cold M9 minimal medium and treated with 200 µL of ice-cold 80% acetone spiked with 20 µM $^{13}\text{C}_3$ -D-Ala as an internal standard. Tubes were kept on ice with occasional vortexing for 5 min. These tubes were again centrifuged, and supernatants were collected into fresh ice-cold microcentrifuge tubes. Samples (15 µL) were derivatized with Marfey's reagent, and analyzed by LC-MS/MS.

RESULTS

Structure-activity correlation

A summary of antibacterial activities of the newly synthesized compounds against MSSA (clinical isolate), MRSA (clinical isolate), and *E. coli* K12 is given in Table 1. D-boroAla-(–)-pinanediol (**6a**) has the most potent activity in this series.

D-boroHomoAla-(–)-pinanediol (**6b**), D-boroVal-(–)-pinanediol (**6c**), and boroGly-(–)-pinacol (**11**) were found to have worse antibacterial activity (higher MIC values) than D-boroAla-(–)-pinanediol (**6a**). Removal of the pinanediol group also reduced antibacterial effectiveness, which is presumably due to the lipophilic pinanediol group facilitating transport across the bacterial membrane. Acetyl-D-boroAla-(–)-pinanediol (**7**) was completely inactive, indicating that a positively charged amino group is required for antibacterial activity. Finally, L-boroAla-(+)-pinanediol (**11**) (the opposite enantiomer of **6a**), had very weak activity.

Antibacterial activity

The spectrum of activity results are summarized in Tables 2 and 3. D-boroAla demonstrated activity against several strains of both Gram-positive and Gram-negative organisms.

D-boroAla-(–)-pinanediol (**6a**) was bactericidal against *S. aureus* and *Bacillus subtilis* at 1× MIC, and against *E. coli* and *Salmonella enterica* serovar Typhimurium at 4× MIC (Table 3). A frequency of resistance determination was performed for D-boroAla-(–)-pinanediol (**6a**) against MSSA (clinical, Table 2) at 2× and 4× MIC (16 and 32 µg/mL respectively). At 2× MIC, a frequency of resistance of 1×10^{-6} was observed whereas at 4× MIC, a frequency of resistance of 8×10^{-8} was observed.

Determination of Biochemical Mechanism

Treating *E. coli* with D-boroAla-(–)-pinanediol (**6a**) at 4× MIC had a profound effect on the intracellular levels of D-Ala-D-Ala (Fig. 2).

A similar result was observed in MRSA (supplemental material). Treating cells with sub-MIC levels of **6a** resulted in only a modest decrease in D-Ala-D-Ala levels (data also not shown). This experiment verifies that D-boroAla exerts its antibacterial activity through inhibition of DDL.

DISCUSSION

Previous biochemical studies (13) have identified D-boroAla as an effective inhibitor of both alanine racemase (saturable time dependent inhibition with $K_I = 20$ mM and $k_{inact} = 0.35$ min⁻¹) and D-Ala-D-Ala ligase (K_I under intracellular conditions against the *S. enterica* serovar Typhimurium enzyme of 18 µM). There have however been no previous reports on the antibacterial activity of D-boroAla. During the course of our investigations on peptide-D-boroAla derivatives as inhibitors of the penicillin-binding proteins (11), we observed

antibacterial activity in some crude peptide-D-boroAla preparations which was lost on purification of the peptide-D-boroAla derivative. A filter disc test of D-boroAla for antibacterial activity revealed surprisingly good activity for D-boroAla(-)-Pd against both *E. coli* and *S. aureus* indicating possible broad spectrum activity, and it seemed worthwhile to further characterize the antibacterial activity of D-boroAla and its homologs.

A structure-activity study was first performed by synthesizing a series of D-boroAla homologs. Three features of D-boroAla were examined including; 1) the length of the side chain alkyl group, 2) the effect of N-acylation, and 3) the presence or absence of the pinanediol protecting group. Pinanediol protecting groups are used in amino boronic acid syntheses to control the stereochemical outcome of the product (15, 16, 23). In aqueous solutions the boropinane diol ester is in equilibrium with the free boronic acid and pinanediol. A control test of racemic pinanediol revealed no antibacterial activity (data not shown). Among the compounds tested in this study, D-boroAla(-)-pinanediol was the most active, with MICs against *E. coli* and *S. aureus* in the 8 to 32 µg/mL range (Tables 1 and 2). Removal of the pinanediol group resulted in higher MICs, likely due to the lipophilic pinanediol group facilitating membrane permeability. L-boroAla showed much lower antibacterial activity, demonstrating stereospecificity of antibacterial activity. This observation indicates that D-boroAla likely acts on a specific macromolecular target, and not simply as a non-specific membrane disrupting agent. Longer and shorter side chain homologs of D-boroAla (e.g. boroGly, D-boroHomoAla, and D-boroVal) demonstrated greatly reduced antibacterial activity. Acetylation of D-boroAla to give acetyl-D-boroAla abolished activity, demonstrating that a positively charged amine group is required for antibacterial activity.

Activity of D-boroAla was then tested against several Gram-negative and Gram-positive pathogenic bacteria to determine spectrum of activity (Table 2). Broad spectrum activity against both Gram-positive and Gram-negative bacteria was observed, with MICs ranging from 8 to 128 µg/mL. Bactericidal activity was apparent at 1× MIC against *S. aureus* and *B. subtilis*, and at 4× MIC against *S. enterica* serovar Typhimurium and *E. coli* (Table 3).

The frequency of resistance at 4× MIC was 8×10^{-8} . This is comparable to or lower than rifampicin resistance frequency in several bacterial strains (24, 25), and falls at the lower end of the weakly hypermutable range (4×10^{-8} to 4×10^{-7}), and just above the normomutable range (8×10^{-9} to 4×10^{-8}) (26, 27).

Given these observations, an obvious question was: what is the molecular target of D-boroAla? Several lines of evidence suggested that D-boroAla would act in the alanine branch of the bacterial cell wall biosynthesis pathway (Fig. 1), including that bacterial cell wall biosynthesis is unique in its requirement for D-Alanine, that the antibacterial activity in this series of compounds is correspondingly specific to D-boroAla (Table 2), and that D-boroAla has previously been described as an inhibitor of both alanine racemase and D-Ala-D-Ala ligase (13) – the two enzymes catalyzing the reactions in the alanine branch (Fig. 1). We have recently developed an assay for the intermediates (L-Ala, D-Ala, and D-Ala-D-Ala) in the alanine branch of bacterial cell wall biosynthesis (22). This assay was used to determine if D-boroAla had a significant impact on the early cell wall intermediates in both *E. coli* and *S. aureus*, and demonstrate that D-boroAla has a substantial effect on the level of D-Ala-D-Ala above its MIC in both *E. coli* (Fig. 2) and *S. aureus* (data not shown). This effect is centered on the MIC for D-boroAla (Fig. 2) – D-boroAla exhibits little effect on D-Ala-D-Ala levels below its MIC, but a pronounced effect above its MIC. It is also notable that the control antibiotic cycloserine exerts its effect on both D-Ala and D-Ala-D-Ala levels, consistent with its known mechanism of action as an alanine racemase inhibitor. From these observations we conclude that D-boroAla exerts its antibacterial activity through inhibition of D-Ala-D-Ala

ligase. As a further test of biochemical mechanism, it is known that in *S. aureus* the addition of D-Ala can antagonize the antibacterial action of cycloserine (28, 29). We have also observed that D-Ala at 2.5 mM antagonizes the antibacterial activity of cycloserine at 2× and 4× MIC, but does not antagonize the antibacterial activity of D-boroAla at 2× and 4× MIC, which is an observation also consistent with DDL as the molecular target of D-boroAla.

The identification of DDL as the molecular target of D-boroAla's antibacterial activity can be used to rationalize the MBC/MIC ratios observed against several bacterial strains (Table 3). The difference in MBC/MIC ratio between bacterial strains appears correlated with the number of copies of DDL in the genomes of these organisms (1 in *S. aureus* and *B. subtilis*, and 2 in *E. coli* and *S. typhimurium*). Our working hypothesis is that at 1× MIC D-boroAla inhibits the one DDL in *S. aureus* and *B. subtilis*, and is bactericidal. However, against *E. coli* and *S. typhimurium* which have two copies of DDL, 1× MIC D-boroAla inhibits only one of the two DDLs which inhibits growth, while the other DDL appears sufficiently active to ensure viability of these organisms. At higher D-boroAla concentrations (4× MIC) the second DDL in *E. coli* and *S. enterica* serovar Typhimurium is also inhibited by D-boroAla, which then causes cell death.

CONCLUSIONS AND FUTURE DIRECTIONS

This study demonstrates that D-boroAla has broad-spectrum antibacterial activity, is bactericidal, and acts on D-Ala-D-Ala ligase. There has recently been considerable interest in D-Ala-D-Ala ligase as a potential target for antibacterial agent development (recently reviewed in (30)), and the observations reported here further support D-Ala-D-Ala ligase as a viable target for the development of novel antibacterial agents. Future studies directed towards characterizing D-boroAla against the two DDL variants found in *E. coli* and *S. enterica* serovar Typhimurium will be of interest to determine the relative affinities of these DDLs for D-boroAla, and correlation with D-boroAla's *in vivo* activity. This study also raises the question of why some bacteria have two copies of DDL. One explanation is that two different DDL enzymes with different kinetic properties may be required to allow efficient bacterial cell growth or survival under different growth conditions. D-boroAla and gene knockout experiments could be used to address this question.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Bacterial cell wall biosynthesis pathway in *S. aureus*.

Figure 2.
Levels of L-Ala, D-Ala, and D-Ala-D-Ala in *E. coli* as a function of added agents.

Scheme 1.

Reagents and conditions: i) a-ether, -72 °C, 16 h. b-1M HCl in ether, 0 °C. ii) (-)-(Pd). iii) CH₂Cl₂, BuLi, THF, -100 °C, 30 min. iv) ZnCl₂, 16h. v) hexamethyldisilazane, BuLi, THF, -78 °C, 16h. vi) 1M HCl in ether, 0 °C, 1h. vii) Triethylamine, acetyl chloride. viii) phenylboronic acid, H₂O/ether (1:1) +0.2 eq 1M HCl, 1h.

Scheme 2.

Reagents and conditions: i) a-ether, BuLi, -78°C , 1h, 10°C , 30 min. b-pinacol, r.t., 16 h. ii) hexamethyldisilazane, BuLi, THF, -78°C , 16 h. iii) 1M HCl in ether, 0°C .

Table 1

Structure-activity correlation.

Cmpd	MIC: μM and ($\mu\text{g/mL}$)		
	MSSA ¹	MRSA ¹	<i>E. coli</i> K12
6a	50 (10)	50 (10)	100 (20)
L-boroAla-(+)-Pd	1000 (250)	ND ¹	400 (100)
6b	200 (50)	1000 (250)	1000 (250)
6c	5000 (1250)	5000 (1250)	5000 (1250)
7	NA ²	NA ²	NA ²
8	200 (25)	200 (25)	400 (50)
11	Weak Activity	ND ²	NA ²

¹ MSSA and MRSA were both clinical strains as described in the text.² ND – not determined, NA – no activity, MIC >5000 μM .

Table 2
Spectrum of activity: MICs for D-boroAla-(−)-pinanediol, D-boroAla (no pinanediol), and controls.

Strain	MIC (μg/mL)							
	D-boroAla- (-)-Pd (6a)	D-boroAla (8)	Control Antibiotics ¹					Cefoxitin
			D-Cycloser	Vanc	Tet	Amp		
<i>Enterococcus faecium</i> (VRE, clinical)	G+	16	>128	32	>512	8	128	>256
MRSA (clinical)	G+	16	64	16	1	0.125	128	>256
MSSA (clinical)	G+	8	64	8	2	0.0625	32	4
<i>Salmonella typhi</i> (clinical)	G-	8	32	64	>512	0.5	8	2
<i>Escherichia coli</i> K12	G-	32	256	8	>256	0.5	8	2
<i>Shigella sonnei</i> (clinical)	G-	64	>128	32	>512	16	2	2
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	G-	128	ND ²	ND ²	ND ²	ND ²	ND ²	ND ²
<i>Burkholderia pseudomallei</i> (strain 1026b)	G-	64	ND ²	ND ²	ND ²	ND ²	ND ²	ND ²

¹ D-Cycloser, D-cycloserine; Vanc, vancomycin; Tet, tetracycline; Amp, ampicillin.

² ND – not determined.

Table 3

MICs and MBCs for D-boroAla-(-)-pinanediol against several bacterial strains, and correlation of MBC/MIC ratio with genomic copies of DDL.

Strain	MIC	MBC	MBC/MIC	DDLs in Genome ¹
(μg/mL)				
<i>Staphylococcus aureus</i> (ATCC 29213)	G+	16	1	1
<i>Salmonella enterica</i> serovar Typhimurium (ATCC 14028)	G-	32	4	2
<i>Bacillus subtilis</i> (ATCC 6633)	G-	16	1	1
<i>Escherichia coli</i> K12	G-	32	4	2

¹ Based on searches of annotated full genomes for *S. aureus* and *E. coli* at the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/>), and the annotated full genomes of *B. subtilis* and *S. enterica* serovar Typhimurium at the Comprehensive Microbial Resource (<http://cmr.jevti.org/cgi-bin/CMR/CmrHomePage.cgi>).