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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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D-boroAla was previously characterized as an inhibitor of bacterial alanine racemase and D-Ala-D-Ala ligase enzymes (Biochemistry, 28, 1989, 3541). In this study, D-boroAla was identified and characterized as an antibacterial agent. D-boroAla has activity against both Gram-positive and Gram-negative organisms, with minimal inhibitory concentrations down to 8 μg/mL. A structure–function study on the alkyl side chain (NH_2-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. D-boroAla is bactericidal at 1× minimal inhibitory concentration against *Staphylococcus aureus* and *Bacillus subtilis*, which each encode one copy of D-Ala-D-Ala ligase, and at 4× minimal inhibitory concentration against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, which each encode two copies of D-Ala-D-Ala ligase. D-boroAla demonstrated a frequency of resistance of 8×10^{-8} at 4× minimal inhibitory concentration 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 D-Ala-D-Ala ligase targeted antibacterial agents. This study also demonstrates D-boroAla as a possible probe for D-Ala-D-Ala ligase function.

Key words: alanine branch, antibacterial, broad spectrum, cell wall, D-Ala-D-Ala ligase

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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. Methicillin-resistant *Staphylococcus aureus* (MRSA) and several Gram-negative pathogens such as *Pseudomonas aeruginosa*, *Burkholderia* sp., *Acinetobacter baumannii*, and *Klebsiella pneumoniae* are of 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 that 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 (DDL) (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 DDL in the alanine branch of bacterial cell wall biosynthesis (Figure 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.

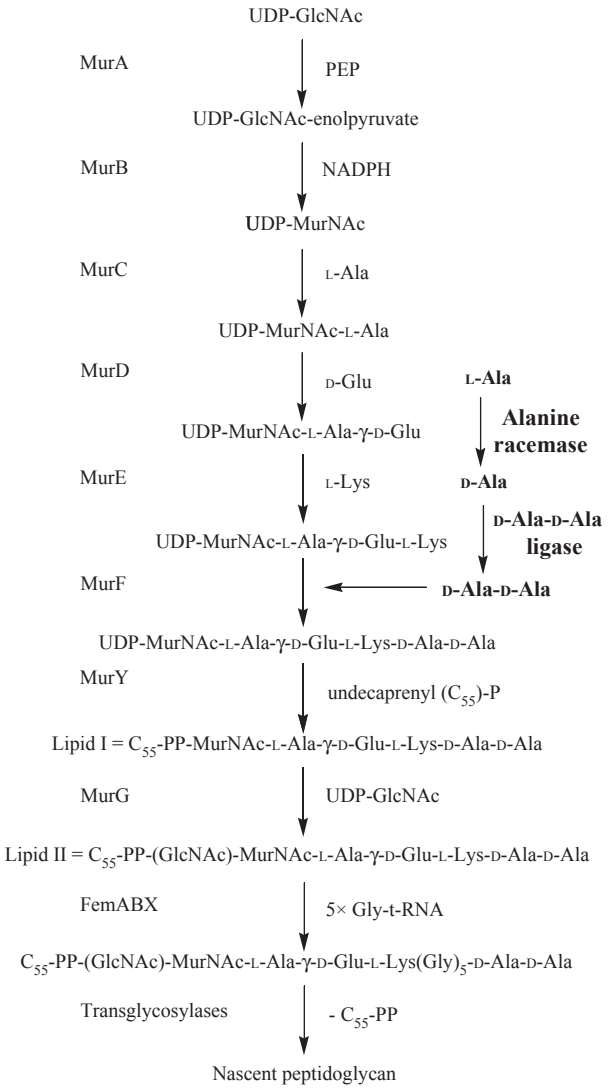
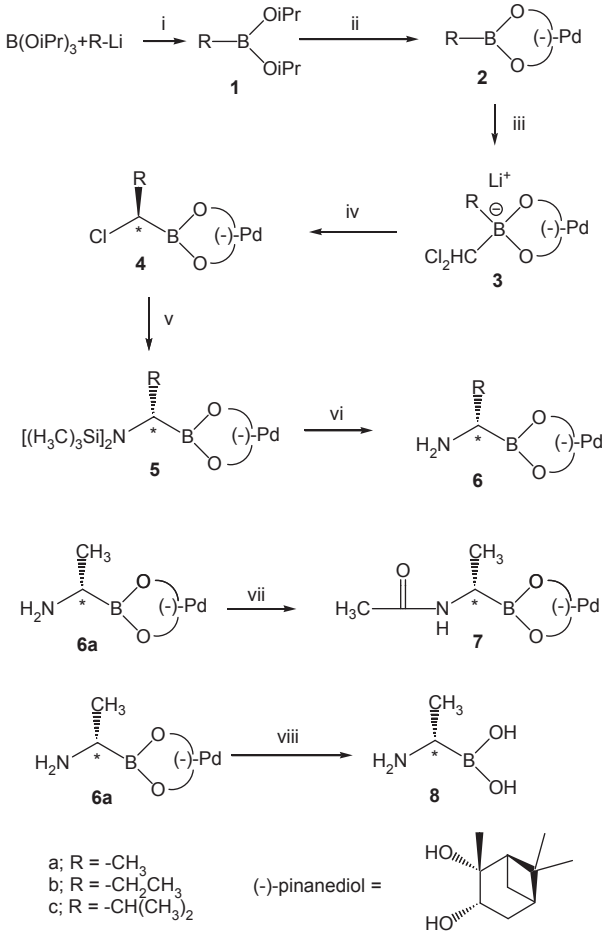


Figure 1: Bacterial cell wall biosynthesis pathway in *Staphylococcus aureus*.



Scheme 1: Reagents and conditions: (i) a-ether, −72 °C, 16 h. b-1 M HCl in ether, 0 °C. (ii) (−)-(Pd). (iii) CH_2Cl_2, BuLi, THF, −100 °C, 30 min. (iv) $ZnCl_2$, 16 h. (v) hexamethyldisilazane, BuLi, THF, −78 °C, 16 h. (vi) 1 M HCl in ether, 0 °C, 1 h. (vii) Triethylamine, acetyl chloride. (viii) phenylboronic acid, H_2O/ether (1:1) +0.2 eq 1 M HCl, 1 h.

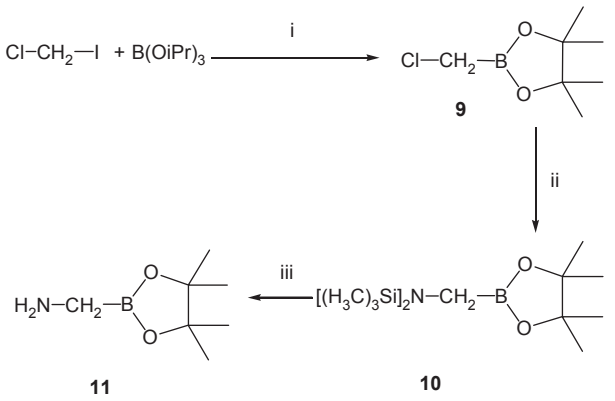
Materials and Methods

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 et al. (14–16) and described previously (11) (Scheme 1). The synthesis strategy used for boroGly pinacol is outlined in Scheme 2.

(−)-Pinanediol (1S)-(1-aminoethyl)-1-boronate (D-boroAla-(−)-pinanediol) (R = CH_3) (**6a**) was synthesized and characterized as described previously (11).

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



Scheme 2: Reagents and conditions: (i) a-ether, BuLi, −78 °C, 1 h, 10 °C, 30 min. b-pinacol, r.t., 16 h. (ii) hexamethyldisilazane, BuLi, THF, −78 °C, 16 h. (iii) 1 M HCl in ether, 0 °C.

(−)-Pinanediol (1S)-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 (1S)-1-amino-2-methylpropane-1-boronate (D-boroValine-(−)-pinanediol) (R = iPr) (**6c**) was synthesized following the procedure described by Kettner and Shenvi (16) for the L-isomer 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).

(1S)-(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 phenylboronic 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 described above for the 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 Clinical and Laboratory Standards Institute guidelines [CLSI, formerly National Committee for Clinical Laboratory Standards (21)]. Twofold serial dilutions of test agents were prepared in 100 μL of Mueller Hinton broth (Difco, Sparks, MD, USA) 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). Minimal inhibitory concentrations were determined against several bacterial pathogens (Table 1), including both Gram-positive and Gram-negative organisms, to determine spectrum of activity. All MIC determinations were performed in triplicate.

Minimal bactericidal concentrations

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 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 $2500 \times g$ 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 were 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 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 antibiotic (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, four samples of 10 mL were moved from each flask to ice-cold 15-mL centrifuge tubes, and the cells were pelleted by centrifuge at $2500 \times g$ 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 $^{13}C_3$-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

Table 1: Spectrum of activity: MICs for D-boroAla-(−)-pinanediol, D-boroAla (no pinanediol), and controls

		MIC (μg/mL)						
				Control antibiotics[a]				
Strain		D-boroAla-(−)-Pd (**6a**)	D-boroAla (**8**)	D-Cycloser	Vanc	Tet	Amp	Cefoxitin
Enterococcus faecium (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
Salmonella typhi (clinical)	G−	8	32	64	>512	0.5	8	2
Escherichia coli K12	G−	32	256	8	>256	0.5	8	2
Shigella sonnei (clinical)	G−	64	>128	32	>512	16	2	2
Pseudomonas aeruginosa (ATCC 27853)	G−	128	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
Burkholderia pseudomallei (strain 1026b)	G−	64	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]

[a]D-Cycloser, D-cycloserine; Vanc, vancomycin; Tet, tetracycline; Amp, ampicillin.
[b]ND, not determined.
MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*; MIC, minimal inhibitory concentrations.

Table 2: Structure–activity correlation

	MIC: μM and (μg/mL)		
Compound	MSSA[a]	MRSA[a]	*Escherichia coli* K12
6a	50 (10)	50 (10)	100 (20)
L-boroAla-(+)-Pd	1000 (250)	ND[a]	400 (100)
6b	200 (50)	1000 (250)	1000 (250)
6c	5000 (1250)	5000 (1250)	5000 (1250)
7	NA[b]	NA[b]	NA[b]
8	200 (25)	200 (25)	400 (50)
11	Weak activity	ND[b]	NA[b]

[a]MSSA and MRSA were both clinical strains as described in the text.
[b]ND, not determined; NA, no activity, minimal inhibitory concentrations (MIC) >5000 μM.
MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus.*

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 (μg/mL)	MBC (μg/mL)	MBC/MIC	DDLs in genome[a]
Staphylococcus aureus (ATCC 29213)	G+	16	16	1	1
Salmonella enterica serovar Typhimurium (ATCC 14028)	G−	32	128	4	2
Bacillus subtilis (ATCC 6633)	G−	16	16	1	1
Escherichia coli K12	G−	32	128	4	2

[a]Based on searches of annotated full genomes for *S. aureus* and *E. coli* at the Welcome 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.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi).
DDL, D-Ala-D-Ala ligase; MBC, minimal bactericidal concentrations; MIC, minimal inhibitory concentrations.

E. coli K12 is given in Table 2. 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 because of 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 is summarized in Tables 1 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*

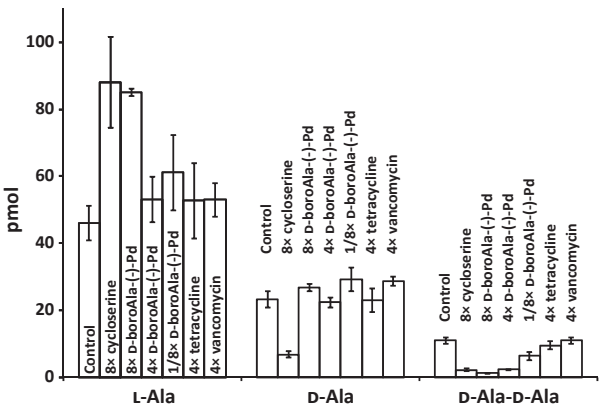


Figure 2: Levels of L-Ala, D-Ala, and D-Ala-D-Ala in *Escherichia coli* as a function of added agents.

enterica serovar Typhimurium at 4× MIC (Table 3). A frequency of resistance determination was performed for D-boroAla-(−)-pinanediol (**6a**) against MSSA (clinical, Table 1) 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 (Figure 2).

A similar result was observed in MRSA (Figure S1). 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) and DDL (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 disk 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 (i) the length of the side chain alkyl group, (ii) the effect of N-acylation, and (iii) 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 boropinanediol 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 8–32 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 1). 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 of *S. aureus* 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}–4×10^{-7}), and just above the normomutable range (8×10^{-9}–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 (Figure 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 DDL (13) – the two enzymes catalyzing the reactions in the alanine branch (Figure 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 whether 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* (Figure 2) and *S. aureus* (Figure S1). This effect is centered on the MIC for D-boroAla (Figure 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 DDL. 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 (one in *S. aureus* and *B. subtilis* and two 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 that

inhibits grown, 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 DDL. There has recently been considerable interest in DDL as a potential target for antibacterial agent development [recently reviewed in (30)], and the observations reported here further support DDL as a viable target for the development of novel antibacterial agents. Future studies directed toward 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.

Acknowledgments

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References

1. Appelbaum P.C. (2007) Reduced glycopeptide susceptibility in methicillin-resistant *Staphylococcus aureus* (MRSA). Int J Antimicrob Agents;30:398–408.
2. Dijkshoorn L., Nemec A., Seifert H. (2007) An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. Nat Rev Microbiol;5:939–951.
3. Klevens R.M., Morrison M.A., Nadle J., Petit S., Gershman K., Ray S., Harrison L.H. *et al.* (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA;298:1763–1771.
4. O'Neill A.J. (2008) New antibacterial agents for treating infections caused by multi-drug resistant Gram-negative bacteria. Expert Opin Investig Drugs;17:297–302.
5. Taubes G. (2008) The bacteria fight back. Science;321:356–361.
6. Fischbach M.A., Walsh C.T. (2009) Antibiotics for emerging pathogens. Science;325:1089–1093.
7. Kluytmans J., Struelens M. (2009) Meticillin resistant *Staphylococcus aureus* in the hospital. BMJ;338:b364.
8. Barrett C.T., Barrett J.F. (2003) Antibacterials: are the new entries enough to deal with the emerging resistance problems? Curr Opin Biotechnol;14:621–626.
9. Abbanat D., Macielag M., Bush K. (2003) Novel antibacterial agents for the treatment of serious Gram-positive infections. Expert Opin Investig Drugs;12:379–399.
10. Baltz R.H. (2009) Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. Curr Opin Chem Biol;13:144–151.
11. Pechenov A., Stefanova M.E., Nicholas R.A., Peddi S., Gutheil W.G. (2003) Potential transition state analogue inhibitors for the penicillin-binding proteins. Biochemistry;42:579–588.
12. Nicola G., Peddi S., Stefanova M., Nicholas R.A., Gutheil W.G., Davies C. (2005) Crystal structure of *Escherichia coli* penicillin-binding protein 5 bound to a tripeptide boronic acid inhibitor: a role for Ser-110 in deacylation. Biochemistry;44:8207–8217.
13. Duncan K., Faraci W.S., Matteson D.S., Walsh C.T. (1989) (1-Aminoethyl)boronic acid: a novel inhibitor for Bacillus stearothermophilus alanine racemase and *Salmonella typhimurium* D-alanine:D-alanine ligase (ADP-forming). Biochemistry;28:3541–3549.
14. Matteson D.S., Sadhu K.M. (1984) Synthesis of 1-amino-2-phenylethane-1-boronic acid derivatives. Organometallics;3:614–618.
15. Matteson D.S., Jesthi P.K., Sadhu K.M. (1984) Synthesis and properties of pinanediol α-amido boronic esters. Organometallics;3:1284–1288.
16. Kettner C.A., Shenvi A.B. (1984) Inhibition of the serine proteases leukocyte elastase, pancreatic elastase, cathepsin G, and chymotrypsin by peptide boronic acids. J Biol Chem;259:15106–15114.
17. Venkatraman S., Wu W., Prongay A., Girijavallabhan V., George Njoroge F. (2009) Potent inhibitors of HCV-NS3 protease derived from boronic acids. Bioorg Med Chem Lett;19:180–183.
18. Coutts S.J., Adams J., Krolikowski D., Snow R.J. (1994) Two efficient methods for the cleavage of pinanediol boronate esters yielding the free boronic acids. Tetrahedron Lett;35:5109–5112.
19. Sadhu K.M., Matteson D.S. (1985) (Chloromethyl)lithium: efficient generation and capture by boronic esters and a simple preparation of diisopropyl (chloromethyl)boronate. Organometallics;4:1687–1689.
20. Martichonok V., Jones J.B. (1997) Cysteine proteases such as papain are not inhibited by substrate analogue peptidyl boronic acids. Bioorg Med Chem;5:679–684.
21. Clinical and Laboratory Standards Institute (2009) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. CLSI document M07-A8, Vol 29, No 2. Eighth edition. Wayne, PA: Clinical and Laboratory Standards Institute.
22. Jamindar D., Gutheil W.G. (2010) A liquid chromatography-tandem mass spectrometry assay for Marfey's derivatives of L-Ala, D-Ala, and D-Ala-D-Ala: application to the in vivo confirmation of alanine racemase as the target of cycloserine in *Escherichia coli*. Anal Biochem;396:1–7.
23. Matteson D.S., Sadhu K.M. (1983) Boronic ester homologation with 99% chiral selectivity and its use in syntheses of the insect pheromones (3S,4S)-4-methyl-3-heptanol and exo-brevicomin. J Am Chem Soc;105:2077–2078.
24. Bjorkholm B., Sjolund M., Falk P.G., Berg O.G., Engstrand L., Andersson D.I. (2001) Mutation frequency and biological cost of

antibiotic resistance in *Helicobacter pylori*. Proc Natl Acad Sci USA;98:14607–14612.
25. Pachon-Ibanez M.E., Fernandez-Cuenca F., Docobo-Perez F., Pachon J., Pascual A. (2006) Prevention of rifampicin resistance in *Acinetobacter baumannii* in an experimental pneumonia murine model, using rifampicin associated with imipenem or sulbactam. J Antimicrob Chemother;58:689–692.
26. Baquero M.R., Nilsson A.I., Turrientes Mdel C., Sandvang D., Galán J.C., Martínez J.L., Frimodt-Møller N., Baquero F., Andersson D.I. (2004) Polymorphic mutation frequencies in *Escherichia coli*: emergence of weak mutators in clinical isolates. J Bacteriol;186:5538–5542.
27. Hanninen M.L., Hannula M. (2007) Spontaneous mutation frequency and emergence of ciprofloxacin resistance in *Campylobacter jejuni* and *Campylobacter coli*. J Antimicrob Chemother;60:1251–1257.
28. Bondi A., Kornblum J., Forte C. (1957) Inhibition of antibacterial activity of cycloserine by alpha-alanine. Proc Soc Exp Biol Med;96:270–272.
29. Zygmunt W.A. (1962) Reversal of D-cycloserine inhibition of bacterial growth by alanine. J Bacteriol;84:154–156.
30. Tytgat I., Colacino E., Tulkens P.M., Poupaert J.H., Prevost M., Van Bambeke F. (2009) DD-ligases as a potential target for antibiotics: past, present and future. Curr Med Chem;16:2566–2580.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Levels of L-Ala, D-Ala, and D-Ala-D-Ala in *Staphylococcus aureus* as a function of no added D-boroAla-(−)-Pd (control) and 4× minimal inhibitory concentrations of added D-boroAla-(−)-Pd.

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