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Research Article

Boron-Containing Peptidomimetics – A Novel Class of Selective Anti-tubercular Drugs

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Medical treatment for tuberculosis is complicated nowadays by the appearance of new multiresistant strains, and therefore, new antibiotics are in great need. Here, we report the synthesis and *in vitro* testing of a new class of highly selective antimicrobial boron-containing peptidomimetics with compounds exhibiting activity against *Mycobacterium tuberculosis* at ≤5 μg/mL. The new approach developed makes it possible to synthesize variously substituted β-aminoboronic acids and their derivatives with a high level of diastereoselectivity.

Key words: antibacterial peptides, beta-aminoboronates, beta-aminoboronic acids, boronic acids, Matteson homologation, peptidomimetics, tuberculosis

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Present Situation

Today, the World Health Organization estimates that one-third of the world's population is infected by *Mycobacterium tuberculosis* with 9.2 million new cases diagnosed and 1.7 million deaths recorded in 2006. Infected persons who develop symptoms are usually suffering from other trauma such as malnutrition or HIV infection, a clear indication of TB being a problem mainly in economically deprived areas, but more and more new cases are also being diagnosed in Europe. In addition, drug-resistant and extremely drug-resistant strains of *M. tuberculosis* are more frequently found. In response to this, the Global Alliance for TB Drug Development has been formed.

History

Tuberculosis (TB) in animals has been dated as far back as 8000 BC as evidenced from fossil bones (1), and in humans, it has been detected in Egyptian mummies from 2400 BC as well as in an Incan mummy from 700 BC (1,2). This indicates that mycobacteria have been plaguing mankind for millennia and that the bacteria have not been confined geographically.

Written record of a chronic lung disease, which may be tuberculosis, appears almost two millennia before BC in legal texts from Hammurabi's reign, and from ancient Greek sources, it seems clear that Hippocrates (460–370 BC) had identified tuberculosis as the disease that was called 'phthisis' and that was the most common disease of the time. From Roman times to the present day, reference has been made to tuberculosis, both in romantic literature as well as in medical texts. Throughout history, it appears that while there have not been any pandemics of TB, it has existed in populations and that it was only in times and areas of high population density that it was detected.

At various times, it was believed that TB was hereditary, but some physicians recognized the contagious nature of the disease, and in the 19th century, Robert Koch finally identified the source of the disease to be a microbe that was named *M. tuberculosis*.

Therapy

Early treatment for TB was based on the assumption that the body would heal itself if the patient was brought to an environment where there were no affected people. This was the beginning of sanatorium treatment. However, results were ambiguous, and many patients suffered relapses, so there was a constant search for new treatment regimes. A method that had been suggested as early as 1771 by Bourru (1) was to induce lung collapse, and similar methods were developed over time exhibiting good results. Collapse therapy remained the treatment of choice until the Second World War when antibiotics were introduced.

With the introduction of streptomycin (1), it was, for the first time, possible to strike directly at the bacillus. Due to the development of resistance, new compounds were soon needed, and over the next two decades, p-aminosalicylic

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acid, isoniazid, pyrazanamide, cycloserine, ethambutol, and rifampicin (3) were found to be active in combination therapy. To this day, the first-line treatment remains a combination of isoniazid, pyrazanamide, ethambutol, and rifampicin with aminoglycosides and (fluoro)quinolones being added to the repertoire of possible drugs. All of the first-line drugs were developed in the 1960s, while the broad-spectrum fluoroquinolones used in TB treatment were patented in the early 1980s (4). There are some experimental drugs in Phase II testing, but it is rare that completely new structural classes are developed.

There are few examples of naturally occurring boron-containing compounds exhibiting biological activity, but boromycin and aplasmomycin are examples of macrocycles that exhibit antibiotic activity (5). In addition, synthetic boron-containing compounds have been shown to act as enzyme inhibitors (6), and bortezomib, an amino boronic acid-containing compound, is used in cancer therapy (7), but to the best of our knowledge, peptidomimetics with amino boronic moieties have not earlier been used as antibiotics.

Materials and Methods

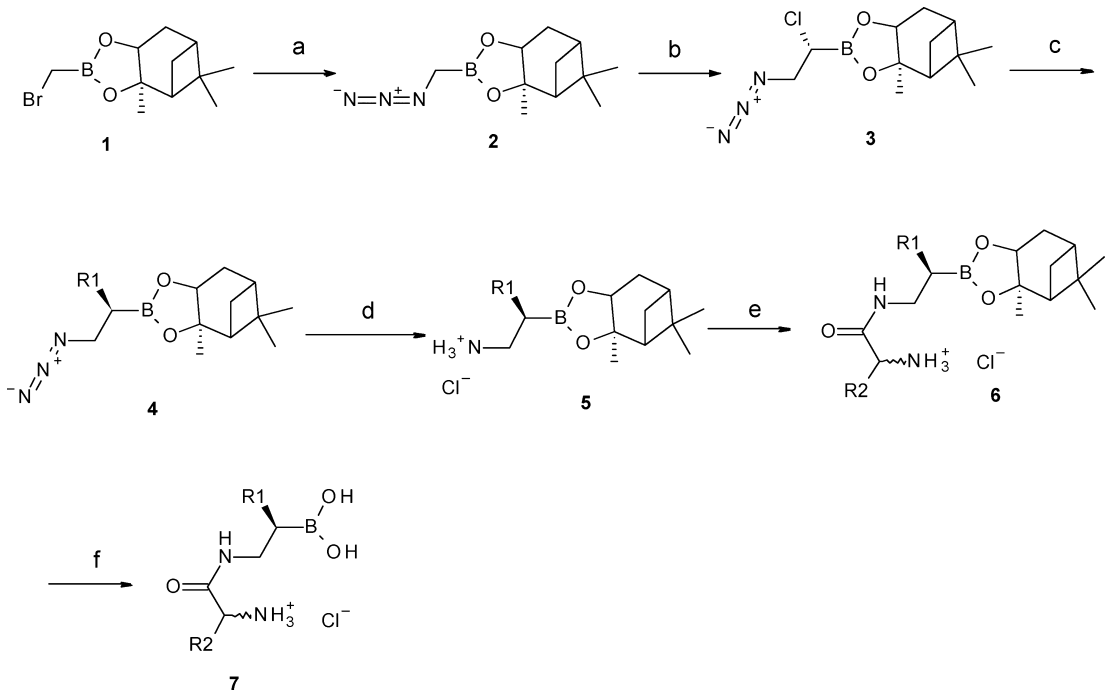
Chemistry

THF was freshly distilled from sodium benzophenone ketyl. All reactions were performed under an atmosphere of argon in oven-dried glassware. n-Butyllithium 2.7 M in heptane, $ZnCl_2$ 1 M in diethyl ether, and all Grignard reagents were purchased from Sigma-Aldrich Norway AS, Oslo, Norway. NMR spectra were recorded in $CDCl_3$ on a Varian Mercury 400 plus (399.65/100.54 MHz), and the residual signal from $CHCl_3$ in $CDCl_3$ was used as internal standard and set to 7.26 ppm for ^{1}H and 77 ppm for ^{13}C. ^{13}C NMR spectra were obtained with broadband proton decoupling. Signals from carbons α to boron were not detected. IR spectra were recorded on a Varian 7000e FT-IR spectrometer. Optical rotation was measured on an AA-10R polarimeter (Optical Activity Ltd. Ramsey, Cambridgeshire, United Kingdom). Mass spectra were measured on a Thermo electron LTQ Orbitrap XL+ Electrospray ion source (ION-MAX). Samples were dissolved in pure methanol and infused by syringe pump at a flow rate of 5 μL/min. No molecular ion was detected for compounds containing boronic acid.

Compounds were synthesized according to the route shown in Scheme 1. All synthetic and spectroscopic details of compounds **1**–**7** are described in Supporting information (Appendix S1).

Biological testing

In the screening experiments, *M. tuberculosis* (H37Rv, from the laboratory collection of Dr. L. Heifets; National Jewish Medical and Research Center, Denver, CO, USA),



Scheme 1: General outline for synthesis of α-substituted β-aminoboronic acids and boronates exemplified using (–)-pinanediol. (a) NaN_3, $(Bu)_4$ N^+Br^-, CH_2Cl_2/H_2O; (b) 1. CH_2Cl_2, n-BuLi, Ar, THF, −100 °C, 2. $ZnCl_2$, −78 °C; (c) R1-MgCl, $ZnCl_2$, −78 °C, Ar, THF. For (4a) super-hydride, −78 °C, Ar, THF; (d) 1. $LiAlH_4$/THF, 2. HCl/MeOH; (e) 1. N-boc-amino acid, 1-HOBt, EDC, N-Methylmorpholine, CH_2Cl_2, 2. HCl/MeOH; (f) A: 3 M HCl/H_2O, 90 °C B: phenylboronic acid, Et_2O/H_2O r.t.

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922), *Streptococcus pyogenes* (ATCC 19615), and *Pseudomonas aeruginosa* (ATCC 27853) were included. The liquid media used for growth were Luria-Bertani (Becton Dickinson, Sparks, MD, USA) and Mueller-Hinton (bio-Merieux, Paris, France) broths and Middlebrook 7H9 medium (Difco) for mycobacteria.

The strains were grown at 37 °C, and after suitable cell concentrations had been reached, 100 μL of each cell suspension was added to a tube with growth media and test compound. Cultivation of the bacteria was then carried out in the presence of each test compound at 37 °C, and the tubes examined for visible growth. The compounds were tested at concentrations of 500, 50, or 5 mg/L. This assay was repeated twice.

Mycobacterium tuberculosis (strain H37Rv) was cultivated in Middlebrook 7H9 medium (Difco) or on Middlebrook 7H10 agar plates at 37 °C. For testing, *M. tuberculosis* was cultivated in 4 mL of broth until the culture reached a concentration of approximately 1×10^8 cfu/mL and then diluted 10 times in PBS to yield a suspension with minimal viscosity and a concentration of bacteria of approximately 1×10^7 cfu/mL, and 100 μL of this suspension was added to a tube with media with tested agent in concentrations 500.0, 50.0, or 5.0 mg/L. After cultivation at 37 °C from the tube where growth was determined, the aliquots were plated on Middlebrook 7H12 agar to determine the presence of bacterial growth and identification of bactericidal or bacteriostatic effect of tested peptide.

In a further experiment, a sample of cells from the test tubes, in which the above broth activity was assayed, were plated on agar to determine the presence of bacterial growth. The CFUs were counted.

Results and Discussions

Our interest in antimicrobial peptides stems from the discoveries regarding lactoferrin/lactoferricin and their role as anti-infective agents in breast-feeding children (8). Further studies led to the identification of the active region of the peptides, which was further developed into results about minimum requirements for peptide structure to preserve antibiotic activity (9). Synthetic peptides were then investigated to improve activity as well as a means to improve uptake and to avoid side effects, for example enzymatic degradation (10,11). From these results, it was clear that the smallest peptidomimetics still exhibiting desired effect were tripeptides with certain features, that is, two bulky side-groups and two charged side chains (12).

It was therefore decided to modify the C-terminal end of the peptides: first, by employing β-amino acids as this introduces more flexibility (13) and enzymatic stability (14) into the molecule and second, by making use of boronic acids as isosters for the carboxylic acid (Figure 1) (15).

In parallel to earlier observations on (naturally occurring) amino acids, the first dipetides synthesized did not exhibit any, or only minor, effect against *S. aureus*, *E. coli*, *S. pyogenes*, or *P. aeruginosa* while being active against *M. tuberculosis* with MIC values ranging from 5 to 500 μg/mL (Table 1).

It was therefore decided to explore this new class of compounds and investigate the influence on tuberculosis activity by extended synthesis. Parameters to screen included what amino acid was incorporated, the effect of the substituent α to the boron, and whether there was a difference in activity between the boronic acids and esters. In addition, the effect of the stereochemistry on either side of the amide bond was also included as a parameter.

Matteson homologation is an efficient procedure for the synthesis of enantiomerically pure α-chloroalkylboronates, and stereochemistry is controlled by the use of a diol as chiral director (16). Subsequent nucleophilic substitution of the chlorine atom by nucleophiles proceeds with stereocontrol (17), and there are a number of publications devoted to applications of chiral α-chloroalkylboronates in asymmetric synthesis. Combining Matteson homologation and nucleophilic substitution thus appeared interesting as a method for the synthesis of substituted derivatives of β-amino boronic acids (Scheme 1). In our studies, both (+)- and (–)-pinanediol were used as chiral directors and were obtained from the corresponding pinene according to a well-known procedure (18).

The starting material, 1,3-propandiol-bromomethyl boronate, was obtained by a procedure analogous to a published procedure (19) with a yield of 57% and reesterification by pinanediol gave **1** in 93% yield. (This approach is one step longer than direct esterification with pinanediol, but loss of precious pinanediol is avoided.) In the next step, bromine was substituted by azide, and the

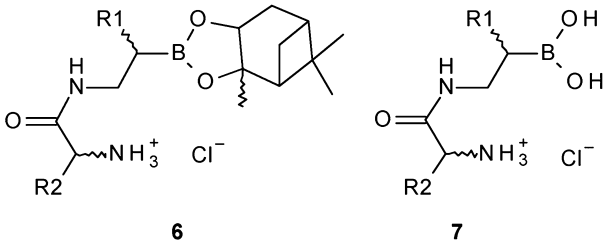


Figure 1: General scaffold for the target compounds library, where R1= H, methyl, phenethyl, benzyl, 4-(F)-benzyl, 4-(CF_3O)-benzyl, 2-naphthylmethyl, phenyl. R= remaining portion of (l)- or (d)-lysine, (l)-phenylalanine, (l)-alanine, (l)-arginine.

Table 1: Antimicrobial activity of (L)-lysine pinanediol boronates 6 (R2= remaining portion of lysine)

				MIC values (μg/mL)							
				Mycobacterium tuberculosis		*Escherichia coli*		*Staphylococcus aureus*		*Pseudomonas aeruginosa*		*Streptococcus pyogenes*	
N	R1	R2 for	Acid/Ester	Liq.	Solid	Liq.	Solid	Liq.	Solid	Liq.	Solid	Liq.	Solid
6a	H	L-Lys	(−)pinanediol	≤500	≤500	≤500	–	–	–	–	–	≤500	–
6b	Me	L-Lys	(−)pinanediol	≤50	≤500	–	–	–	–	–	–	–	–
6h	Benzyl	L-Lys	(−)pinanediol	≤5	≤5	–	–	≤500	≤500	≤500	≤500	≤500	≤500
6v	2-naphthylmethyl	L-Lys	(−)pinanediol	≤5	≤50	–	≤500	≤50	≤500	≤500	≤500	≤500	≤50
6w	Phenyl	L-Lys	(−)pinanediol	≤500	≤500	–	–	–	–	–	–	–	–

resulting azidomethyl boronate **2** was homologated to the corresponding 1-chloro -2-azidoethyl boronate **3**, with the stereochemistry governed by the chiral director (20). Compound **3** was then reacted with a Grignard reagent to obtain the substituted 2-azidoethyl boronates **4** with opposite stereochemistry, in high yield. (If full conversion was not obtained in this step, subsequent reactions were performed on the mixtures, and impurities were removed in the purification at this stage.) Reduction to the amine hydrochorides **5** was high-yielding reactions using standard techniques. Coupling reactions were performed as in the literature (21) to obtain compound **6,** and in the case where the boronic acid **7** was wanted, deprotection was performed using either transesterification with phenyl boronic acid (22) or simple hydrolysis in water with hydrochloric acid.

As is evident from Tables 1 and 2, all compounds synthesized exhibit antitubercular activity to some extent. As for the boronic acids/esters, there is no clear trend in activity as there are compounds for which the acid is more active while the opposite is true for other compounds. Also, the larger groups incorporated into the α-position are more active than the smaller groups as seen from the low activity for unsubstituted boronate and methyl boronate. This suggests that, as for our earlier results, a certain bulk is necessary for activity. Whether this is purely an effect of size or whether it is related to lipophilicity is not clear at this stage. Interestingly, the activity of the phenyl substituted compounds is also low. That there is no discrimination between compounds of opposite stereochemistry at the α-position is also an interesting observation, and it may be argued that the mechanism does not involve enzymes.

At the N-terminal end, arginine and lysine exhibit high activity; this is also in accordance with our earlier observations on antibacterial peptides in which charge is essential for activity. On the other hand, charge is not necessary for antitubercular activity, as seen from compounds with phenylalanine that are also highly active.

Conclusions

By employing a newly developed method for synthesizing α-substituted-β-boronic acids and esters, a novel class of antitubercular compounds has been synthesized. Initial studies aiming at clarifying the mode of action have been initialized, but more work will be needed to develop these compounds into much needed antitubercular drugs.

Acknowledgments

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Table 2: Antitubercular activity of α-substituted-β-amino boronic acid derivatives 6 and 7 (R2= remaining portion of amino acid)

				MIC values (μg/mL)	
				Mycobacterium tuberculosis	
N	R1	R2 for	Acid/Ester	Liq.	Solid
6a	H	L-Lys	(–)pinanediol	≤500	≤500
6b	Me	L-Lys	(–)pinanediol	≤50	≤500
6c	Me	L-Phe	(–)pinanediol	≤500	≤500
6d	Phenethyl	D-Lys	(–)pinanediol	≤50	≤50
6e	Phenethyl	L-Phe	(–)pinanediol	≤50	≤50
7e	Phenethyl	L-Phe	Acid	≤500	≤500
6f	Phenethyl	L-Lys	(–)pinanediol	≤50	≤50
7f	Phenethyl	L-Lys	Acid	≤500	≤500
6g	Benzyl	L-Arg	(–)pinanediol	≤5	≤50
7h	Benzyl	L-Lys	Acid	≤5	≤5
6h	Benzyl	L-Lys	(–)pinanediol	≤5	≤5
6i	Benzyl	D-Lys	(–)pinanediol	≤50	≤500
7i	Benzyl	D-Lys	Acid	≤5	≤500
6j	Benzyl	L-Phe	(–)pinanediol	≤5	≤50
6k	Benzyl	L-Phe	(+)pinanediol	≤50	≤50
6l	Benzyl	L-Lys	(+)pinanediol	≤50	≤50
6m	Benzyl	L-Ala	(+)pinanediol	≤50	≤500
6n	4-(F)-benzyl	L-Lys	(–)pinanediol	≤50	≤50
7n	4-(F)-benzyl	L-Lys	Acid	≤5	≤50
6o	4-(F)-benzyl	L-Phe	(–)pinanediol	≤50	≤50
7o	4-(F)-benzyl	L-Phe	Acid	≤50	≤50
6p	4-(CF_3O)-benzyl	D-Lys	(–)pinanediol	≤50	≤50
7p	4-(CF_3O)-benzyl	D-Lys	Acid	≤500	≤500
6q	4-(CF_3O)-benzyl	L-Lys	(–)pinanediol	≤50	≤500
6r	4-(CF_3O)-benzyl	L-Ala	(–)pinanediol	≤50	≤50
6s	4-(CF_3O)-benzyl	L-Phe	(–)pinanediol	≤5	≤50
7s	4-(CF_3O)-benzyl	L-Phe	Acid	≤50	≤500
6t	2-naphthylmethyl	L-Phe	(–)pinanediol	≤5	≤5
6u	2-naphthylmethyl	L-Lys	(–)pinanediol	≤5	≤50
6v	Phenyl	L-Phe	(–)pinanediol	≤500	≤500
6w	Phenyl	L-Lys	(–)pinanediol	≤500	≤500

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Supporting Information

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

Appendix S1. Supplementary material.