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Synthesis and antimicrobial activity of α-aminoboronic-containing peptidomimetics

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A library of 175 dipeptidomimetics and tripeptidomimetics containing an α-amino boronic acid or boronate has been synthesized, and the activity toward *Mycobacterium tuberculosis*, *Candida albicans*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* has been screened. Although there is no clear structure–activity relationship, several compounds exhibit promising activity against different pathogens. Copyright © 2013 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: α-amino boronic acid; α-amino boronate; antimicrobials; Matteson homologation

Introduction

Infections have been accompanying humans throughout our history, and no general treatment was available until the discovery of sulfa [1]. However, the main breakthrough came with the discovery of penicillin, and over the following years, several new compounds were developed [2]. However, antibiotic resistance was encountered shortly after this new class of compounds had been introduced [3–5]. Whenever new effective drugs have been discovered, pathogens have, using different mechanisms, developed defense systems, and because of the sometimes uncontrolled use of antibiotics, more and more microbes develop resistance to an increasing number of the available drugs.

In our laboratories, we have been concentrating much of our efforts on developing drugs based on peptides. This approach is, of course, known from nature where peptides that protect the host organism from infections are found in most species [6]. Our interest stems from the discovery of lactoferricin/lactoferrin, found in mother's milk, acting as an antibiotic for the newborn before they have had the chance of developing their own immune system by encountering different infectious agents [7–9]. Starting with the secondary structure of lactoferricin, the minimum requirements for antimicrobial activity were identified as being a pentadecapeptide containing tryptophan as an important constituent [10–12]. Further studies led to the identification of shorter and shorter peptides still exhibiting antibiotic activity [9]. By incorporating amino acid residues not encoded for in nature, it was possible to synthesize tripeptides with high activity if the amino acids contained two side chains with a size equal to or larger than a phenyl ring and two positive charges [10–12].

This work was then extended to include β-boronic acids and esters as non-natural moieties in the peptides. By using the Matteson homologation [13] for introducing substituents stereoselectively, a number of compounds were synthesized, subsequently coupled with amino acids and tested for antimicrobial activity. With the exception of one compound being active against *Candida albicans*, all of the other compounds synthesized turned out to be highly selective against *Mycobacterium tuberculosis* (MTB) [14,15]. It was therefore decided to expand the range of the syntheses and testing to include α-amino boronic peptidomimetics in order to gain insight into the influence of boron in antimicrobial compounds.

Material 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 Aldrich Chemical Co. Inc (Milwaukee, WI, USA). NMR spectra were recorded in $CDCl_3$ on a Varian Mercury 400 plus (399.65/100.54 MHz; Varian Inc., Palo Alto, CA, USA), and the residual signal from $CHCl_3$ in $CDCl_3$ was used as internal standard and set to 7.26 ppm for ^1H 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, UK). Mass spectra were measured on a Thermo electron LTQ Orbitrap XL + electrospray ion source [Ion Max (Thermo Fisher Scientific, Waltham, MA, USA)]. Samples were dissolved in pure methanol and infused by syringe pump at a flow rate of 5 μl/min. The molecular ion was not stable enough for detection in compounds containing boronic acid.

Compounds were synthesized according to the route shown in Scheme 1. All synthetic and spectroscopic details of compounds **1–175** are described in the supporting information. It should be

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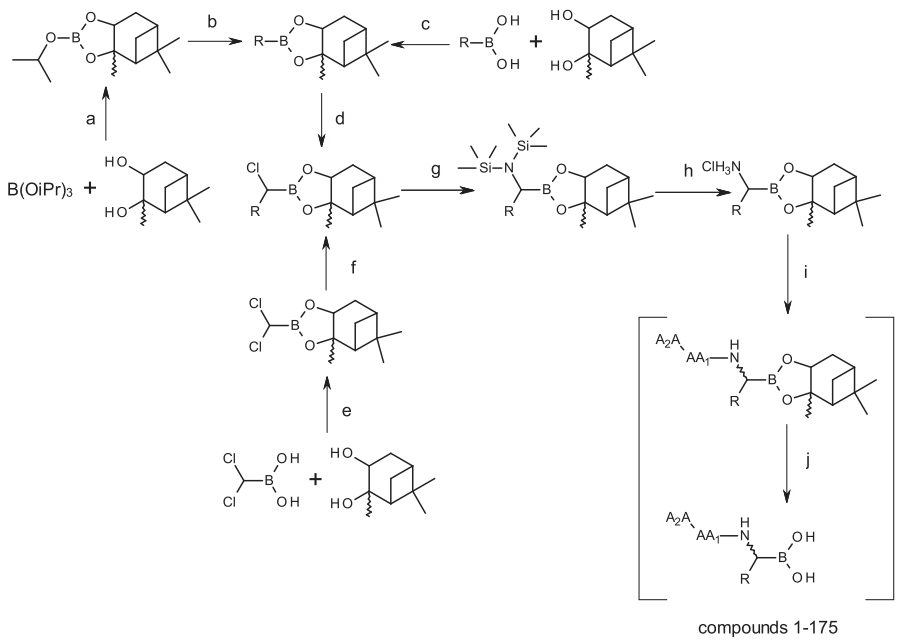

Scheme 1. General outline for synthesis of α-aminoboronic acids and boronates. (a) PhMe, reflux; (b) 1. RMgCl, Ar, Et_2O, −70 °C, 2. HCl/dioxane 4 M; (c) Et_2O, $MgSO_4$, r.t.; (d) 1. CH_2Cl_2, *n*-BuLi, Ar, THF, −100 °C, 2. $ZnCl_2$, −78 °C; (e) THF, $MgSO_4$, r.t.; (f). 1. RMgCl, Ar, Et_2O, −78 °C, 2. $ZnCl_2$, −78 °C; (g) $[(CH_3)_3Si]_2NLi$, Ar, THF, −78 °C; (h) HCl/dioxane 4 M, pentane, 0 °C; (i) amino acid, 1-HOBt, EDC, *N*-methylmorpholine, CH_2Cl_2, 0 °C; (j) $PhB(OH)_2$, Et_2O/H_2O, r.t.

noted that the route via f is not as reliable as the route via d regarding the control of stereochemistry, but compounds should be of sufficient purity for producing valid biological results [16].

Biological Testing

In the screening experiments, MTB (H37Rv, from the laboratory collection of Dr L. Heifets, National Jewish Medical and Research Center, Denver, CO 80206, USA), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Streptococcus pyogenes* (ATCC 19615), *C. albicans* (ATCC90028) and *Pseudomonas aeruginosa* (ATCC 27853) were included.

The liquid media used for growth were Luria-Bertani (Becton, Dickinson and Company, Sparks, MD, USA) and Mueller-Hinton (bioMerieux, 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 were examined for visible growth. The compounds were tested at concentrations of 500, 50 or 5 mg/l. This assay was performed in triplicate, and the consensus value was reported.

MTB was cultivated in Middlebrook 7H9 medium (Difco) or in Middlebrook 7H10 agar plates at 37 °C. For testing, MTB was cultivated in 4 ml of broth until the culture reached a concentration of approximately 1×10^8 colon-forming unit (CFU)/ml and then diluted ten times in phosphate buffered saline to yield a suspension with minimal viscosity and a concentration of bacteria of approximately 1×10^7 CFU/ml. From this suspension, 100 µl was added to a tube with media with the tested agent in concentrations 500, 50 or 5 mg/l. After cultivation at 37 °C in the tube where growth was determined, 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 aforementioned broth activity assays were tested was plated on agar to determine the presence of bacterial or fungal growth by CFU count.

All results from the testing are included in the supporting information, while only compounds active at 5 or 50 mg/l are included in the tables presented in the paper.

Results and Discussion

In our laboratories, we have been studying different types of peptidomimetics as antimicrobials. From these studies, it is clear that compounds not containing boron have to contain at least three amino acids for activity [10–12] and that no activity against MTB has been detected. On the other hand, β-amino boronic

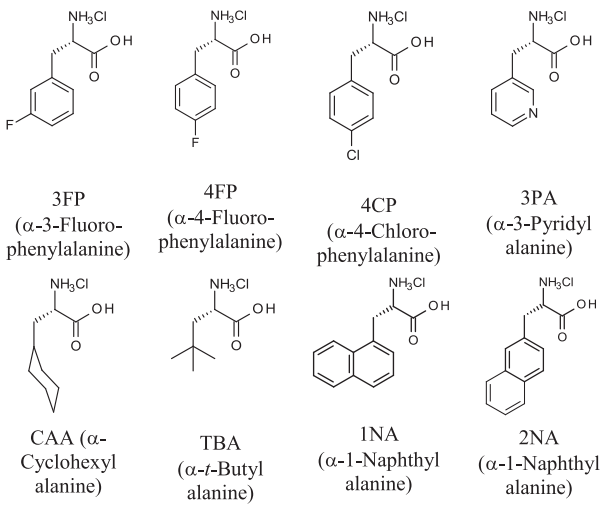

Figure 1. Abbreviation of the non-natural amino acids used.

acid/ester-containing dipeptides are highly active and selective toward MTB with the exception of one single compound, which is also active against *C. albicans* [14,15]. This prompted us to synthesize a number of α-amino boronic acids/boronates and to couple them with a selection of amino acids, including compounds not naturally coded for (Figure 1). In order to verify that it is not in fact the amino boronic moiety that causes the effect, all starting compounds were also tested, and none of them exhibited activity below 500 mg/l.

Activity against mycobacteria and yeasts

Mycobacteria and yeasts are often neglected when designing test panels of pathogens, but they may be important targets for

Table 1. Activity of α-amino boronic peptidomimetics toward *Mycobacterium tuberculosis* and *Candida albicans* for compounds with activity <500 mg/l

N	R	Amino acid 1	Amino acid 2	Pinanediol/acid	*Mycobacterium tuberculosis*		*Candida albicans*	
					Liquid	Solid	Liquid	Solid
3	Me	Ala	—	(−)-acid*	50	50	—	—
4	Me	Lys	—	(−)-acid*	50	50	500	500
5	Me	Phe	—	(−)-pinanediol	50	—	—	—
6	Me	Phe	—	(−)-acid*	50	50	—	—
30	*i*-Pr	Ala	Lys	(+)-acid**	500	—	50	50
32	*i*-Pr	Ala	Phe	(+)-acid**	500	500	50	500
39	*i*-Pr	Ile	1NA	(+)-pinanediol	50	50	—	—
40	*i*-Pr	Ile	1NA	(+)-acid**	50	500	—	—
41	*i*-Pr	Ile	2NA	(+)-pinanediol	500	50	500	500
43	*i*-Pr	Phe	Lys	(−)-pinanediol	50	50	500	500
46	*i*-Pr	Phe	Trp	(−)-pinanediol	-	500	500	50
57	Phe	Lys	—	(−)-pinanediol	5	500	—	—
63	Phe	Ala	Lys	(+)-acid**	50	50	500	—
65	Phe	Ala	CHA	(−)-acid*	500	—	—	5
68	Phe	Ala	2NA	(−)-pinanediol	500	50	500	5
73	Phe	Phe	Ala	(+)-pinanediol	50	500	—	—
74	Phe	Phe	Ala	(+)-acid**	500	500	500	5
77	Phe	Phe	Lys	(+)-pinanediol	50	500	500	500
82	Phe	3FP	Ala	(+)-acid**	50	50	—	—
84	Phe	3FP	Ile	(+)-acid**	500	50	—	—
87	Phe	4FP	Leu	(+)-pinanediol	50	500	—	—
92	4FP	Ala	Lys	(−)-acid*	50	50	—	—
110	Bn	Ala	Phe	(−)-acid*	5	5	—	—
115	Bn	Ala	4FP	(−)-pinanediol	500	50	5	500
119	Bn	Val	Leu	(+)-acid**	500	500	—	50
124	Bn	Leu	CHA	(+)-pinanediol	50	50	—	500
125	Bn	Leu	CHA	(+)-acid**	50	50	500	50
127	Bn	Leu	Phe	(+)-acid**	500	50	—	—
128	Bn	Leu	3PA	(+)-pinanediol	500	500	500	50
130	Bn	Leu	2NA	(+)-pinanediol	—	50	—	50
132	Bn	Ile	Lys	(−)-pinanediol	50	500	500	500
133	Bn	Ile	Lys	(−)-acid*	—	—	500	—
136	Bn	Ile	CHA	(−)-pinanediol	50	50	500	—
140	Bn	Ile	3FP	(−)-pinanediol	50	50	—	—
141	Bn	Ile	3FP	(−)-acid*	50	50	—	—
142	Bn	Ile	4FP	(−)-pinanediol	50	50	500	500
143	Bn	Ile	4FP	(−)-acid*	500	500	—	—
144	Bn	Ile	4CP	(−)-pinanediol	50	50	500	500
151	Bn	Phe	Phe	(−)-acid*	50	500	500	500
158	Pe	Ala	CHA	(+)-pinanediol	50	500	—	50
160	Pe	Ala	3PA	(+)-pinanediol	500	500	500	50
164	Pe	Ala	2NA	(+)-pinanediol	50	50	—	500
170	Pe	Leu	CHA	(+)-pinanediol	50	50	—	500
172	Pe	Leu	3PA	(+)-pinanediol	500	50	500	—
175	Pe	Leu	2NA	(+)-acid**	50	50	500	—

* (−)-acid derived from (−)-pinanediol boronate.
** (+)-acid derived from (+)-pinanediol boronate.

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Table 2. Activity of α-amino boronic peptidomimetics toward *Staphylococcus aureus* and *Streptococcus pyogenes* for compounds with activity <500 mg/l

N	R	Amino acid 1	Amino acid 2	Pinanediol/acid	*Staphylococcus aureus*		*Streptococcus pyogenes*	
					Liquid	Solid	Liquid	Solid
4	Me	Lys	—	(−)-acid*	50	50	n.t.	n.t.
18	Me	Phe	Trp	(+)-pinanediol	500	500	50	500
21	*i*-Pr	Ala	—	(+)-pinanediol	50	—	—	—
34	*i*-Pr	Ile	CHA	(+)-acid**	50	500	500	500
41	*i*-Pr	Ile	2NA	(+)-pinanediol	50	500	—	500
74	Phe	Phe	Ala	(+)-acid**	—	5	—	—
93	Bn	Ala	—	(−)-pinanediol	50	—	—	—
94	Bn	Ala	—	(−)-acid*	5	5	5	5
95	Bn	Ala	—	(+)-pinanediol	—	—	—	—
97	Bn	Lys	—	(−)-pinanediol	—	5	500	—
105	Bn	Ala	Lys	(−)-pinanediol	50	—	—	—
107	Bn	Ala	Lys	(+)-pinanediol	—	—	5	500
114	Bn	Ala	3FP	(−)-acid*	5	—	n.t.	n.t.
150	Bn	Phe	Phe	(−)-pinanediol	—	50	—	500
170	Pe	Leu	CHA	(+)-pinanediol	50	50	—	500
174	Pe	Leu	2NA	(+)-pinanediol	50	50	—	—

n.t., Compounds have not been tested against this bacterial strain.
* (−)-acid derived from (−)-pinanediol boronate.
** (+)-acid derived from (+)-pinanediol boronate.

Table 3. Activity of α-amino boronic peptidomimetics toward *Escherichia coli* and *Pseudomonas aeruginosa* for compounds with activity ≤500 mg/l

N	R	Amino acid 1	Amino acid 2	Pinanediol/acid	*Escherichia coli*		*Pseudomonas aeruginosa*	
					Liquid	Solid	Liquid	Solid
26	*i*-Pr	3PA	—	(−)-pinanediol	—	—	—	5
147	Bn	Phe	Ala	(−)-acid*	—	—	50	—
160	Pe	Ala	3PA	(+)-pinanediol	—	5	500	—
163	Pe	Ala	1NA	(+)-acid**	50	—	—	—

* (−)-acid derived from (−)-pinanediol boronate.
** (+)-acid derived from (+)-pinanediol boronate.

future therapy, a fact that is accentuated by the emergence of resistant and extensively resistant strains [17,18], and even strains of MTB that do not respond to any of the drugs presently available in clinics have been reported [18,19]. Thus, the activity of the 175 synthesized α-amino boronic peptidomimetics against MTB and *C. albicans* was tested, revealing 158 active compounds with 45 of them having an activity of <500 mg/l (Table 1). This is interesting in view of our previous studies of non-boron-containing peptides and peptidomimetics, which have never exhibited any activity against MTB while several are active against *Candida*. It should also be noted that the active compounds include both dipeptides and tripeptides and that there is no need for high lipophilicity/bulk or charge in the compounds for being active, as was the case for non-boron-containing compounds. However, it is clear that the activity in general is not as high as for the β-amino boronic compounds previously investigated [14,15].

Structure–activity relations are not obvious when looking at the results, but there are some points that can be made. The only compounds active against MTB at the lowest concentration tested are **57**, a dipeptide with a phenyl substituent at the boron moiety and lysine as amino acid, and **110**, a tripeptide with benzyl boronic acid. β-Boronates/acids containing a phenyl group do not usually exhibit antitubercular activity while lysine is often found in highly active compounds, and a benzyl substituent usually leads to high antitubercular activity if the amino acid moiety is not alanine. On the other hand, a methyl substituent is tolerated in both moieties of dipeptides of α-compounds, which is in contrast to the β-compounds where larger groups have to be incorporated in order to achieve high activity. The necessity for the boronic moiety was also confirmed by synthesizing the amino acid analogs of compounds **6** and **151** (Phe-Ala and Phe-Phe-Phe). Neither of these compounds had any activity against MTB, while the boronic compounds were active at 50 mg/l.

As for activity against yeast, compounds **65**, **68**, **74** and **115** are active at the lowest level tested for. These compounds are all tripeptides with a phenyl or benzyl substituent α to the boron and an alanine as either the middle or end amino acid. In view of earlier results, this is difficult to explain, but some compounds are more active as boronic acids and others as esters has been noticed before [14,15]. It should also be pointed out that the stereochemistry of the boron moiety can be switched without this affecting the activity, as judged from the data for compounds **68** and **74**, which is also in agreement with what is observed for the β-compounds.

Activity against Gram-positive bacteria

The constant need for new antibiotics for fighting resistant bacteria made it interesting to test for activity against *S. aureus* and *S. pyogenes*. Fewer compounds exhibited activity against Gram-positive bacteria (97 compounds in total), while only a few of the compounds were active at concentrations $<$500 mg/l (Table 2).

Fewer compounds exhibited activity against Gram-positive bacteria (97 compounds in total), and furthermore, only a small minority of those compounds that did exhibit activity were active at concentrations $<$500 mg/l (Table 2).

As was the case for activity against MTB and *C. albicans*, it is difficult to see any clear trends for structure–activity (e.g. boronic acids are as likely as boronates to be active, and both dipeptides and tripeptides exhibit the same level of activity), but there are some interesting results. As for the compounds in the β-series, a benzyl substituent at the amino boronate/boronic acid is beneficial for activity. Another important point is that compounds seem to exhibit high selectivity, affecting only one pathogen, an exception being **94**, which is active against both *S. aureus* and *S. pyogenes* with inhibition at 5 mg/l. Interestingly, another compound, **74**, is not only active toward *S. aureus* but has the same level of activity toward MTB. Alanine is also present in all but one of the active compounds, a fact that has been pointed out as being in contrast to the activity of other boron-containing compounds we investigated.

Activity against Gram-negative bacteria

Of the 175 compounds tested, only 38 exhibited any effect against the Gram-negative pathogens included in our study, and of these, only four had an activity of ≤50 mg/l (Table 3).

As for the other pathogens, the results are difficult to interpret in terms of structure–activity as there are both boronic acids and boronates that are active and the same is true for dipeptides and tripeptides. The selectivity pattern is also the same as described earlier as the activity exhibited is for only one of the bacterial strains. Alanine is present in all three active tripeptides, and there seems to be a preference for more lipophilic compounds because a pentyl substituent is present at the boronic moiety in two of the active compounds.

Cytotoxicity

As part of our investigations into antitubercular drugs [14,15], it was an interesting observation that compound **110** was the only α-amino boronic compound exhibiting high activity and selectivity against MTB. This compound was therefore included in the set of compounds tested for cytotoxicity as this would be an issue in developing boron-containing compounds into potential drugs. Employing HepG2 (Cyprotex Discovery Ltd, Macclesfield, UK) did not reveal any cytotoxicity in the concentration range 0.04–100 μM, suggesting low toxicity for α-amino boronic acids.

Conclusions

A library of dipeptides and tripeptides containing α-amino boronate and α-amino boronic acid has been synthesized and tested against a selection of different pathogens. A large portion of these compounds exhibit biological activity, in particular against MTB and *C. albicans*, while fewer are highly active against Gram-negative/Gram-positive bacteria, which is interesting in view of the more complex membrane structure of mycobacteria and fungi. The observation that changes in lipophilicity and overall charge of the peptides tested show no obvious correlation with activity may be an indication that the mode of action is more complex than just interaction with the cell membrane. Another interesting fact is that, of the 175 compounds tested, only six compounds exhibit activity below 500 mg/l against two different pathogens. In conclusion, the high activity of certain compounds tested makes them ideal for further research regarding structure–activity studies as well as mode of action studies, especially when taking into account the low toxicity revealed in the preliminary testing.

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