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A study of the effect on nucleophilic hydrolytic activity of pancreatic elastase, trypsin, chymotrypsin, and leucine aminopeptidase by boronic acids in the presence of arabinogalactan: a subsequent study on the hydrolytic activity of chymotrypsin by boronic acids in the presence of mono-, di-, and trisaccharides

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Abstract

The hydrolytic activity of trypsin, chymotrypsin, elastase, and leucine aminopeptidase, is inhibited by different boronic acids. However, all the enzymes are inhibited by the compound *Cbz*Ala(boro)Gly(OH)$_2$. Therefore, these additives can control the nucleophilic hydrolytic activity of these enzymes.

Keywords: Serine proteases; Boronic acids; Inhibition; Hydrolytic activity; Saccharides

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1. Introduction

Ring formation between boronates and hydroxylated compounds such as saccharides [1], amino acids [2], RNA nucleosides [3], and other polar compounds renders these hydroxylated compounds more lipophilic and facilitates their transport across cell membranes and lipid bilayers [4,5]. The association constants for such complexes, in the range of 10–10^4 M^{-1}, would suggest that significant amounts of free boronic acids are available on both sides of the membrane. These two attributes of boronic acids, namely, facile ring formation with suitable functional groups of polar molecules, and relatively small association constants, would seem to make boron- polysaccharide complexes good candidates for the delivery of peptides or peptidomimetics. Examples of polysaccharide drug delivery platforms include the natural polysaccharides guar gum and pectin. Our group has modified and utilized these platforms for colon specific drug delivery because of their unique characteristic to be specifically degraded by colonic bacteria [6,7]. The colon has been discussed as a potential site of peptidic drug delivery due to apparently lower levels of proteases in this region [8]. While the development of colonic delivery systems for peptides and proteins is our long term goal, in the short term, we are interested in studying the inhibitory activity of some boronic acids on various serine proteases found in the GI, such as trypsin, chymotrypsin, elastase, and leucine aminopeptidase, and the effect of added saccharides on the inhibition ability of these boronic acids. Boronic acids are exceptionally potent inhibitors of serine proteases. This characteristic is widely believed to derive from the boronyl group's ability to mimic the transition state of the enzyme-catalyzed reaction [9]. Suenaga et al. [10] proved that the inhibitory effect of phenylboronic acid on chymotrypsin was intensified by added diols but weakened by tripodal additives. Therefore the aim of our study was to assess the effects of saccharides on the inhibition of GI tract serine proteases by boronic acids. In addition, given the recent approval of bortezomib [11] and a number of other boronic acid-based compounds in various stages of research and development [12], our study has implications for other boronic acid therapeutics that might be given orally.

2. Materials and methods

2.1. Enzymatic analysis

α-*Chymotrypsin, Type II*: From bovine pancreas, was purchased from Sigma (MW 25,100). The hydrolytic reaction was carried out according to Kouzoma's method (37 °C, standard pH 8.0, with 50 mM phosphate buffer, 0.2 M NaCl, 0.3 vol% methanol plus 0.03 vol% DMSO) and the progress of the reaction was followed by monitoring the appearance of the absorption band of *p*-nitroaniline at 410 nm (Sub = benzoyl-tyrosine-*p*-nitroanilide).

Trypsin, Type II-S: From porcine pancreas was purchased from Sigma. The hydrolytic reaction was carried out at 37 °C, standard pH 7.5, with 50 mM Tris buffer, 0.02 M $CaCl_2$, 10 vol% DMF, and the progress of the reaction was followed by

monitoring the appearance of the absorption band of *p*-nitroaniline at 410 nm (Sub = benzoyl-arginine-*p*-nitroanilide).

Elastase, Type II-A: From porcine pancreas was purchased from Sigma. The hydrolytic reaction was carried out at 37 °C, standard pH 7.5, with 50 mM phosphate buffer, 0.2 M NaCl, and the progress of the reaction was followed by monitoring the appearance of the absorption band of *p*-nitroaniline at 410 nm (Sub = *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide).

Leucine aminopeptidase, Type VI: From porcine kidney microsomes, was purchased from Sigma. The hydrolytic reaction was carried out at 37 °C, standard pH 7.5, with 50 mM phosphate buffer, 0.02 M $CaCl_2$, 10 vol% DMF, and the progress of the reaction was followed by monitoring the appearance of the absorption band of *p*-nitroaniline at 410 nm (Sub = L-leucine-*p*-nitroanilide).

The five kinds of boronic acids used were phenylboronic acid, 3- aminophenylboronic acid, 4-fluorophenylboronic acid, (Cbz-alanyl)aminomethyl boronic acid, and (Cbz-phenylalanyl)aminomethyl boronic acid. They were all tested for their ability to inhibit trypsin, chymotrypsin, elastase, and leucine aminopeptidase.

2.2. Synthesis and identification

NMR experiments. ^{1}H NMR spectra was recorded at 300 MHz (Varian Unity) instrument. ^{13}C NMR ^{11}B NMR spectras were recorded on a Varian 300 MHz instrument at a frequency of 75.9 MHz and 96.29 MHz, respectively. The chemical shifts were reported with respect to CD_3OD for the peptidyl boronic acids.

2.3. Synthesis of the peptidyl boronic acids

A mixture of triisopropylborate (60 ml, 0.25 mol) and dry pinacol (30 g, 0.25 mol) was heated for 3 h and stirred at 115 °C. The isopropanol was distilled, the residue was cooled, and distilled under vacuo. ^{1}H NMR ($CDCl_3$): δ 1.01 (dd, 6H), 1.10 (s, 12H), 4.20 (sept., 1H). $^{13}C\{^{1}H\}$ NMR ($CDCl_3$): δ 24.39, 24.63, 67.35, 82.48.

A solution of diiodomethane (4.05 ml, 50.2 mmol) and isopropylpinacolboronate (10 ml, 46.5 mmol) in THF (200 ml) was cooled to −78 °C and *n*-BuLi (20 ml of 1.6 M solution) was dropped during 30 min at −78 °C with stirring. The reaction mixture was stirred overnight at room temperature. Then 6 N solution of HCl in ether was added (10 ml) and the solvent was removed. The residue was distilled at 0.1 mmHg.

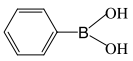
To a cooled solution of hexamethyldisilazane in THF was added *n*-BuLi. The cooling bath was then removed and the mixture was allowed to warm to 0 °C within 0.5 h and then stirred at 0 °C for an additional 0.5 h. The resulting solution of lithiohexamethyldisilizane was cooled again, and the iodomethanedioxaborolane in THF was added within 10 min. The reaction was then allowed to warm to 20 °C and stirred overnight, after which hexane was added and the precipitated LiI was filtered off. The filtrate was concentrated in vacuo and the residue Kugelrohr distilled to give 2-[*bis*(trimethylsilyl)aminomethyl]-4,4,5,5-tetramethyl-1,3,2- dioxaborolane as a colorless liquid. ^{1}H NMR ($CDCl_3$): δ 0.06 (s, 18H), 1.23 (s, 12H), and 2.41 (s, 2H). $^{13}C\{^{1}H\}$ NMR ($CDCl_3$): δ 1.37, 24.65, 29.08, 83.22.

Cesium carbonate (1.21 g, 3.72 mmol) was added at 20 °C to a solution of Cbz-alanine/Cbz-phenylalanine (7.4 mmol) in MeOH. The mixture was stirred overnight, the methanol was then evaporated in vacuo and the resulting cesium salt was dried over P_2O_5 at 0.2 mm for 24 h. It was then dissolved in dry DMF (5 ml) under N_2, the solution cooled to −15 °C, and isobutylchloroformate added in one portion and the mixture stirred at −15 °C for 0.5 h. 2-[*Bis*(trimethylsilyl)aminomethyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (7.43 mmol) was added with stirring followed by water (0.5 ml, 27.6 mmol). After stirring for 3 h at −15 °C the reaction mixture was quenched with 1 M aqueous HCl (40 ml), extracted with EtOAc (60 ml), and washed with water (10 ml). The boronic acid was extracted from EtOAc into 1 M NaOH (3 × 6 ml), washed with EtOAc (2 × 20 ml), and dried on sodium sulfate. After filtering and concentrating in vacuo, the residue was chromatographed (10–25% methanol in chloroform). The resulting boronic dipeptide was obtained as a white solid. ^{1}H NMR (CD_3OD) for *Cbz*Phe(boro)Gly(OH)$_2$: δ 2.32 (AB, $J = 2.64$, 2H), 2.91–3.21 (m, 2H), 4.56 (m, 1H), 5.02 (s, 2H), 7.20–7.34 (m, 10H). ^{13}C NMR (CD_3OD): δ 31.36 (broad), 38.47, 55.23, 67.65, 127.95, 128.67, 128.94, 129.39, 129.52, 130.23, 137.67, 137.87, 158.00, 178.39. ^{11}B NMR (CD_3OD): δ 17.46. Anal. Calcd. for $C_{18}H_{21}BN_2O_5$: C, 60.70; H, 5.94; N, 7.86. Found: C, 60.76; H, 5.85; N, 7.92%.

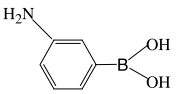
^{1}H NMR (CD_3OD) for *Cbz*Ala(boro)Gly(OH)$_2$: δ 2.81(d, 3H), 2.34 (s, 2H), 4.35 (m, 1H), 5.09 (s, 2H), 7.23–7.35 (m, 5H).^{13}C NMR (CD_3OD): δ 16.70, 30.85 (broad), 47.83, 66.65, 127.80, 138.84, 156.97, 178.30. ^{11}B NMR (CD_3OD): δ 14.43. Anal. Calcd. for $C_{12}H_{17}BN_2O_5$: C, 51.46; H, 6.12; N, 10.00. Found: C, 50.80; H, 5.90; N, 9.71%.

3. Results and discussion

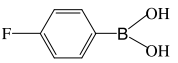
Phenylboronic acid, 3-aminophenylboronic, 4-fluorophenylboronic, *Cbz*Ala(boro)-Gly(OH)$_2$ and *Cbz*Phe(boro)Gly(OH)$_2$(**1–5**) were all tested for their ability to inhibit trypsin, chymotrypsin, elastase, and leucine aminopeptidase. The progress of the reaction was followed by monitoring the appearance of the absorption band at 410 nm (*p*-nitroaniline).



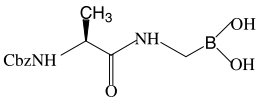
(1) Phenylboronic acid



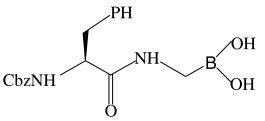
(2) 3-aminophenylboronic acid



(3) 4-Fluorophenylboronic acid



(4) (*Cbz*Ala(boro)Gly(OH)$_2$)



(5) (*Cbz*Phe(boro)Gly(OH)$_2$)

The dissociation constants, K_i, for the inhibitors with all the four enzymes are shown in Table 1. In addition Lineweaver–Burk plots for a representative grouping of inhibitors with each enzyme are illustrated in Figs. 1–4. As can be seen in the plots, the inhibition was competitive and reversible for all the compounds that

Table 1
Inhibition of α-chymotrypsin, trypsin, elastase, and leucine aminopeptidase by boronic acids **1–5**

Boronic acid	Chymotrypsin, K_i (mM)	Trypsin, K_i (mM)	Elastase, K_i (mM)	Aminopeptidase, K_i (mM)
1. Phenylboronic acid	9.70	>0	1.83	>0
2. 3-Aminophenylboronic acid.	>0	>0	3.61	>0
3. 4-Fluorophenylboronic acid	31.01	>0	1.02	>0
4. *Cbz*Ala(boro)Gly(OH)$_2$	1.08	35.9	0.27	0.35
5. *Cbz*Phe(boro)Gly(OH)$_2$	1.96	>0	0.60	1.02

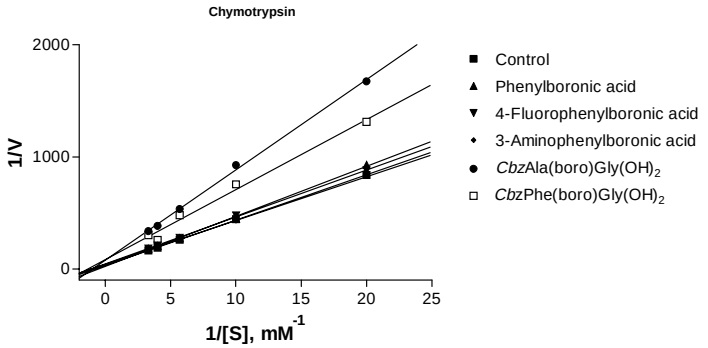


Fig. 1. Lineweaver plot for the inhibition of chymotrypsin without and with inhibitors. Reactions were initiated by adding the substrate benzoyl-tyrosine-*p*-nitroanilide to a solution of chymotrypsin and inhibitor. The final concentration of the inhibitor was 1.071 mM.

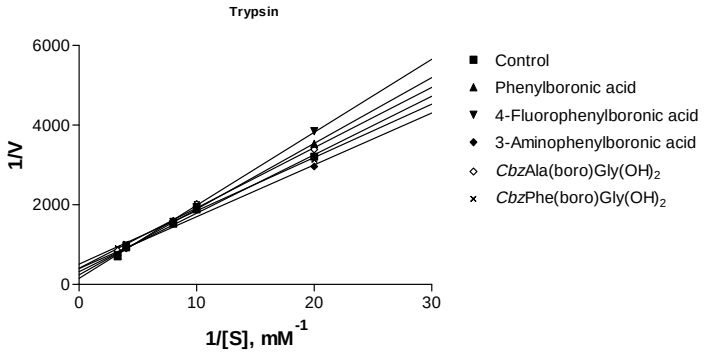


Fig. 2. Lineweaver plot for the inhibition of trypsin without and with inhibitors. Reactions were initiated by adding the substrate benzoyl-arginine-*p*-nitroanilide to a solution of trypsin and inhibitor. The final concentration of the inhibitor was 1.071 mM.

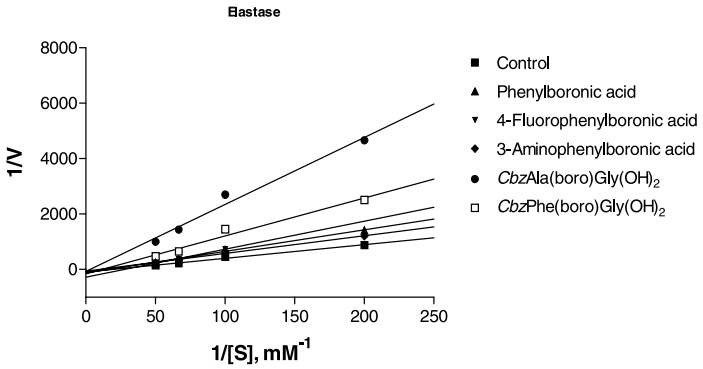


Fig. 3. Lineweaver plot for the inhibition of elastase without and with inhibitors. Reactions were initiated by adding the substrate *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide to a solution of elastase and inhibitor. The final concentration of the inhibitor was 1.071 mM.

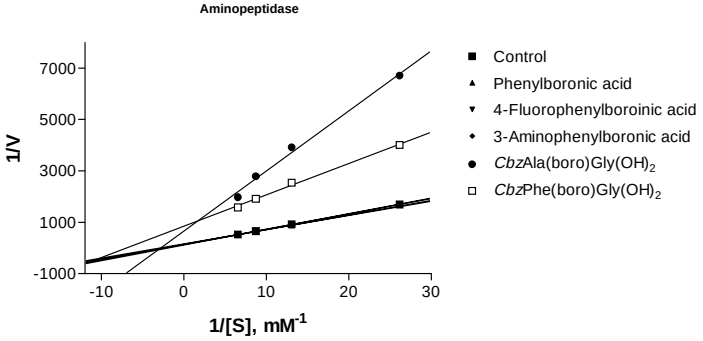


Fig. 4. Lineweaver plot for the inhibition of leucine aminopeptidase without and with inhibitors. Reactions were initiated by adding the substrate leucine-*p*-nitroanilide to a solution of leucine aminopeptidase and inhibitor. The final concentration of the inhibitor was 1.071 mM.

inhibited the enzymes except for *Cbz*Phe(boro)Gly(OH)$_2$ that inhibited leucine aminopeptidase non-competitively and irreversibly.

As can be seen in Table 1, phenylboronic acid and 4-fluorophenylboronic acid revealed moderate inhibition properties for chymotrypsin but 3-aminophenylboronic acid inhibited the enzyme very weakly. This can be explained by the ability of the electron donating group (NH_2) to weaken the acidity of the boron atom, which, in turn, prevents further attack on the nucleophilic center. Another reason for the difference in activity with and without the 3-amino substituent may be due to direct interactions of this group with chymotrypsin. The best inhibition activity was demonstrated by *Cbz*Ala(boro)Gly(OH)$_2$ and *Cbz*Phe(boro)Gly(OH)$_2$ compounds. Dixon plots [13] (Fig. 1) showed that both compounds are competitive inhibitors and that the dissociation constants (K_is) of the enzyme–inhibitor complexes are 1.08 and 1.96 mM for *Cbz*Ala(boro)Gly(OH)$_2$ and *Cbz*Phe(boro)Gly(OH)$_2$, respectively

at 25 °C and pH 7.5. The competitive nature of the inhibition indicates that both compounds bind at the active site of the α-chymoytrypsin.

As for trypsin, none of the boronic acids significantly affected the hydrolytic activity of the enzyme, except for *Cbz*Ala(boro)Gly$(OH)_2$,which inhibited trypsin competitively and reversibly with a K_i value of 35.90 mM (Fig. 2).

All the boronates inhibited elastase, but *Cbz*Ala(boro)Gly$(OH)_2$ and *Cbz*Phe(boro)Gly$(OH)_2$ had the best inhibition abilities with K_i values of 0.27 and 0.60 mM, respectively. These two compounds inhibited the enzyme in a competitive and reversible manner (Fig. 3).

Leucine aminopeptidase was only inhibited by *Cbz*Ala(boro)Gly$(OH)_2$ and *Cbz*Phe(boro)Gly$(OH)_2$ with K_i values of 0.35 and 1.02 mM, respectively. Whereas *Cbz*Ala(boro)Gly$(OH)_2$ inhibited the enzyme competitively and reversibly, *Cbz*Phe(boro)Gly$(OH)_2$ inhibited the enzyme noncompetitively and irreversibly (Fig. 4).

Bachovchin and co-workers [14,15] have demonstrated that the binding mode used by a particular boronic acid inhibitor appears to depend on how well the inhibitor matches the structure of the physiological or natural substrate of the serine protease. In cases in which a boronic acid is a good substrate mimic, formation of a tetrahedral complex with the active site serine is favored. On the other hand, when boronic acids have structures that are not well related to that of the substrate, they tend to coordinate with histidine and serine. The serine proteases chymotrypsin and subtilisin have been shown to be inhibited by aromatic boronic acids with chymotrypsin favoring large aromatic hydrophobic side chains (Phe, Tyr, and Trp) while trypsin favors positively charged groups (Arg and Lys). Consistently, in this study, all the boronic acids that had aromatic groups were able to inhibit chymotrypsin to different extents depending on the substituents incorporated. *Cbz*Phe(boro)Gly$(OH)_2$ and *Cbz*Ala(boro)Gly$(OH)_2$ were expected [16] to act as cysteine proteases inhibitors, but they were proved ineffective up to a concentration of 10 mM. Despite the fact that inhibitors of serine proteases very often inhibit cysteine proteases [17], these two compounds were good inhibitors of chymotrypsin since they are peptidyl boronic acids and it is known that peptidyl boronic acids that are close structural analogues of good substrates for the enzymes can act as potential transition state analogue inhibitors of the representative serine proteases.

Only one boronic acid was able to inhibit trypsin slightly, *Cbz*Ala(boro)Gly$(OH)_2$, although it does not contain charged groups like Arg or Lys. One possible explanation for this behavior is that this compound may be able to fit into the active site and interact with histidine or serine.

It is known that chymotrypsin prefers an aromatic residue in the P1 site whereas pancreatic elastase and leukocyte elastase prefer Ala and Val, respectively. Therefore the peptidyl boronic acids *Cbz*Ala(boro)Gly$(OH)_2$ and *Cbz*Phe(boro)Gly$(OH)_2$ were the most effective inhibitors for elastase with K_i values of 0.27 and 0.60 mM, respectively, while the other boronic acids inhibited the enzyme to a lesser extent.

Aminopeptidases are a family of exopeptidases responsible for cleavage at the N-termini of proteins and peptides. Their activities have been compared in the homogenates of duodenum, jejunum, and ileum of albino rabbits with L-leucine,

L-alanine, and L-arginine-4-methoxy-β-naphthylamide as the substrates [18]. All the compounds showed very weak inhibitory activity toward leucine aminopeptidase except for the compounds *Cbz*Ala(boro)Gly(OH)$_2$ and *Cbz*Phe(boro)Gly(OH)$_2$ which inhibited the enzyme effectively.

3.1. Sugars can increase/decrease the inhibitory effect of boronic acids on the hydrolytic activity of α-chymotrypsin

There are several possible reasons why saccharides might increase or decrease the observed inhibition of serine nucleophilic enzymes by boronic acids. These include: (a) Competition for the boronic acids by the saccharide (reservoir/sink effect), which should decrease the apparent inhibition. (b) Complexation with the boronic acid and binding to the enzyme as the complex, which might increase or decrease the apparent inhibition. (c) Direct effects (allosteric or other) by the saccharide on the enzyme.

Therefore the effect of added mono-, di-, and trisaccharides on the inhibition ability of some boronic acids in the α-chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide (as a substrate) was studied. As expected, the hydrolytic activity was affected to different extents by the addition of saccharides to the boronic-acid-inhibited system depending on the boronic acid and on the kind of saccharide that is used.

Table 2 shows the effects of added saccharides on the activity of chymotrypsin both in the absence and in the presence of certain inhibitors. The results showed that saccharide-only (no inhibitor) additives had no effect on the activity of the enzyme, therefore, the saccharide effects on the inhibition were due to interactions with

Table 2
The effect of the addition of saccharides on the activity of chymotrypsin both in the absence and in the presence of the inhibitors: phenylboronic acid, *Cbz*Phe(boro)Gly(OH)$_2$, and *Cbz*Ala(boro)Gly(OH)$_2$

Compound	% activity no inhibitor	% activity phenylboronic acid	% activity *Cbz*Phe(boro) Gly(OH)$_2$	% activity *Cbz*Ala(boro) Gly(OH)$_2$
No sugar (Inh.)	100.00	66.50	26.04	18.38
+ Lactulose	104.72	66.50	15.48	100.00
+ Lactose	104.72	80.20	23.85	22.02
+ Sucrose	102.90	99.25	23.48	22.36
+ Raffinose	96.97	95.82	34.58	30.64
+ Cellobiose	104.29	89.92	28.55	30.04
+ Glucose	101.20	32.96	44.96	48.86
+ Mannose	100.91	48.67	72.54	41.29
+ Galactose	101.02	59.38	61.30	71.56
+ Methylglucose	111.07	86.16	33.02	31.25
+ Methylgalactose	104.33	84.69	35.58	39.36
+ Methylmannose	106.15	73.33	-	18.95

50 mM phosphate buffer and 0.2 M NaCl, [chymotrypsin] = 7.9×10^{-6} mol dm^{-3}, [Sub] = 9.87×10^{-5} mol dm^{-3}, [boronic acid] = 1.12×10^{-3} mol dm^{-3}, [monosaccharide] = 4.00×10^{-3} mol dm^{-3}, [methylmonosaccharide] = 3.71×10^{-3} mol dm^{-3}, [disaccharide] = 2.1×10^{-3} mol dm^{-3}, [trisaccharide] = 1.21×10^{-3} mol dm^{-3}.

boronic acid and not due to direct effect with the enzyme, and possibility (c) that was mentioned earlier can be ruled out.

For phenylboronic acid, it was found that when D(+)-glucose, D(+)-mannose, and D-galactose were added, the rate of the hydrolytic reaction was further suppressed. The inhibition efficiency for these three saccharides was in the order of D(+)-glucose > D(+)-mannose > D(+)-galactose and this agrees well with the data published by Suenaga et al. [10] On the other hand, with the addition of other monosaccharides such as methyl-α-D-glucopyranoside, methyl-β-D-galactopyranoside, and methyl-α-D-mannopyranoside, the inhibition ability was weakened and some of the enzymatic activity was regenerated completely. The presence of disaccharides or trisaccharides also decreased the inhibitory effect of phenylboronic acid except for lactulose that did not affect the inhibition at all. It is known that for α-D-glucose mono and bisboronates, the sugar ring has a furanose structure with a conformation between T^3_2 and E^3. In addition, methyl-α-D-glucopyranoside reacts with phenylboronic acid (1 mol) to give a crystalline 4,6-cyclic ester which, in turn, forms a 2,3-(diphenylpyroboronate) (containing a seven-membered ring) with an excess of the reagent while phenylboronic acid condenses smoothly with methyl-α-D-mannopyranoside, to give the 2,3:4,6-diester. Therefore, this difference in the structure of the final product may have the effect of either increasing or decreasing the inhibitory activity, i.e., the furanose structure of the ring of phenylboronic acid with glucose and mannose fits the active site of chymotrypsin.

The effect of saccharides on the *Cbz*Phe(boro)Gly$(OH)_2$ inhibition of chymotrypsin shows that the inhibitory effect is weakened by monosacchaarides. The effect of decreasing the inhibition varies for different monosaccharides, and their ability to decrease the inhibition increases in the order D(+)-mannose > D(+)-galactose > D(+)-glucose > methyl-β-D-galactopyranoside > methyl-α-D-glucopyranoside. On the other hand, the addition of disaccharides or a trisaccharide did not affect the inhibition significantly except for the lactulose that removed the inhibition completely (i.e., the hydrolytic activity of the enzyme was regenerated completely). One possible explanation for this is that the association constant of the boronate with lactulose is so high (we could not detect it by NMR due to instantaneous formation of a complex) and therefore it was able to form a complex with the boronic acid and bind to the enzyme as a complex which lead to a decrease in the apparent inhibition. Another possibility is the competition for the boronic acid by lactulose, which decreased the inhibition significantly. This possibility depends on the concentration of the enzyme, boronic acid, and the saccharide.

For *Cbz*Ala(boro)Gly$(OH)_2$, the hydrolytic activity of the enzyme was affected by almost a similar manner as *Cbz*Phe(boro)Gly$(OH)_2$ by the addition of mono-, di-, and trisaccharide.

One possible reason for these effects of sugars is the competition for boronic acids by the saccharides as mentioned earlier, however, this depends upon the relative concentrations of the enzyme, boronic acid, and saccharide component. On the other hand, boronic acids form cyclic esters with saccharides and the reaction occurs reversibly and rapidly at ambient temperature, therefore, the complexation with the boronic acid and binding to the enzyme as the complex must be the reason for our results.

Table 3
The increase/decrease in inhibitory activity of boronic acids with chymotrypsin, trypsin, elastase, and leucine aminopeptidase as a result of the addition of arabinogalactan (1.12 mM)

Compound	Chymotrypsin	Trypsin	Elastase	Leucine aminopeptidase
1. Phenylboronic acid	−4.03	+6.39	+6.50	+5.13
2. 3-Aminophenylboronic acid	−3.85	+3.25	−15.53	+2.67
3. 4-Fluorophenylboronic acid	−2.10	−2.60	−1.43	+0.75
4. *Cbz*Ala(boro)Gly(OH)$_2$	−26.65	+0.89	− 30.67	−50.60
5. *Cbz*Phe(boro)Gly(OH)$_2$	−28.47	+8.15	−21.25	−40.25
Arabinogalactan only—no inhibitor	+6.50	−6.70	–	−3.60

3.2. Effect of a polysaccharide on the inhibition of α-chymotrypsin, trypsin, elastase, and leucine aminopeptidase by the five boronic acids

Since our interest was with the effect of polysaccharides on the inhibitory activity of boronic acids, we examined the effect of arabinogalactan on the inhibitory properties of all boronic acid with the four enzymes as shown in Table 3. With arabinogalactan only (no inhibitor), the activity of chymotrypsin was increased slightly by 6.80%, while the activity of both trypsin and leucine aminopeptidase was decreased by 6.70 and 3.60%, respectively, and elastase was not affected at all. These data show that there is no direct effect by arabinogalactan on the enzymes. However, in the presence of the inhibitors, arabinogalactan decreased the inhibitory activity of both *Cbz*Ala(boro)Gly(OH)$_2$ and *Cbz*Phe(boro)Gly(OH)$_2$ with chymotrypsin, elastase, and leucine aminopeptidase. The most significant effect was with leucine aminopeptidase, where the inhibition activity was decreased by 50.60 and 40.25%, respectively, i.e., the K_i values were decreased from 1.02 to 3.95 mM for *Cbz*Phe(boro)Gly(OH)$_2$ and from 0.35 to 1.26 mM for *Cbz*Ala(boro)Gly(OH)$_2$. Therefore, it seems that the boronic acids form complexes with arabinogalactan and binds to the enzyme as the complex, and this probably decreased the apparent inhibition.

These findings support the view that the enzyme active site 'recognizes' the molecular structure of the boronic acid–saccharide complexes, and that not all saccharides act as 'co-inhibitors' in the boronic acid inhibition system.

4. Conclusion

These preliminary results shows that both compounds, *Cbz*Ala(boro)Gly(OH)$_2$ and *Cbz*Phe(boro)Gly(OH)$_2$ are good inhibitors for chymotrypsin, elastase, and leucine aminopeptidase. For chymotrypsin, this inhibitory activity can be either unaffected or decreased by the addition of monosaccharides, disaccharides, and a trisaccharide. The most important result was obtained with the polysaccharide arabinogalactan that decreased the inhibitory activity of the two inhibitors CbzAla(boro)Gly(OH)$_2$ and CbzPhA(boro)Gly(OH)$_2$ with all the enzymes.

This finding is important since it means that the activity of the inhibitor will be reduced by possible interactions with the polysaccharide. Our study has the potential applicability for boronic acid-based drug delivery systems and the potential effects of saccharides in the body or diet on new boronic acid-based therapeutic agents such as bortezomib.

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