Strong inhibitory effect of sugar·biphenylylboronic acid complexes on the hydrolytic activity of α-chymotrypsin

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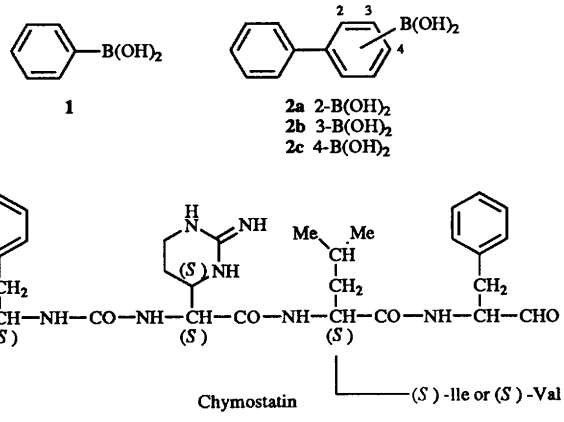
Boronic acids act as transition-state analogues for certain peptidases. The inhibitory effect of 2-, 3- and 4-biphenylylboronic acids (**2a**, **2b** and **2c**) on the hydrolytic activity of α-chymotrypsin has been investigated. These inhibitors were employed to monitor the binding event [formation of covalent bond with either serine residue (195) or histidine residue (57)] occurring in the active site by a fluorescence method. It was shown that the decrease in the fluorescence intensity, which is induced by the formation of a covalent bond with the boronic acid moiety, is well correlated with the inhibitory effect estimated by kinetic measurements. The inhibitory effect appeared in the order **2a** < **2c** ≪ **2b** (K_i = 1.6 × 10^{-6} mol dm^{-3}). Interestingly, the inhibitory effect was further intensified by added saccharides. In particular, the combined system of **2b** and D-glucose strongly inhibited the enzyme reaction, *the inhibitory effect* (K_i = 1.1 × 10^{-7} mol dm^{-3}) *being stronger than that of a specific inhibitor, chymostatin* (K_i = 4.8 × 10^{-7} mol dm^{-3}). Hence, saccharides act as a '*co-inhibitor*' in the boronic acid inhibition system. This is a novel and efficient inhibition system for α-chymotrypsin (and probably more generally for other peptidases).

Introduction

Boronic acids adopt an sp^2-hybridized boron atom which easily reacts as a Lewis acid with nucleophiles such as hydroxide, alkoxide or imidazole to give a tetrahedral boron adduct.[1] This adduct formation also occurs in the active site of certain hydrolytic enzymes such as subtilisin and α-chymotrypsin and the active site serine (or histidine) is usually the fourth ligand of the tetrahedral structure.[2] In fact, phenylboronic acid **1** acts as a novel '*transition-state analogue*' for these enzymes and the K_i-values are estimated to be 0.23–0.80 mmol dm^{-3} for subtilisin and 0.19 mmol dm^{-3} for α-chymotrypsin.[1,2] We previously found that the inhibitory effect of compound **1** is efficiently intensified by added saccharides.[3,4] The synergistic effect is attributed to the generation of a more acidic boron atom through self-complexation with saccharides; this efficiently reacts with the active site serine (or histidine) in α-chymotrypsin.[3,4] To obtain further insight into the inhibition mechanism we investigated several fluorescent boronic acids which act as photo-induced electron-transfer probes in the active site of α-chymotrypsin. We unexpectedly found that biphenylylboronic acids **2** show a remarkably strong inhibitory effect in the presence of certain saccharides, which is comparable with that of chymostatin, a specific inhibitor for α-chymotrypsin. This system is useful as an expeditious method to inhibit totally or control conveniently the activity of α-chymotrypsin.

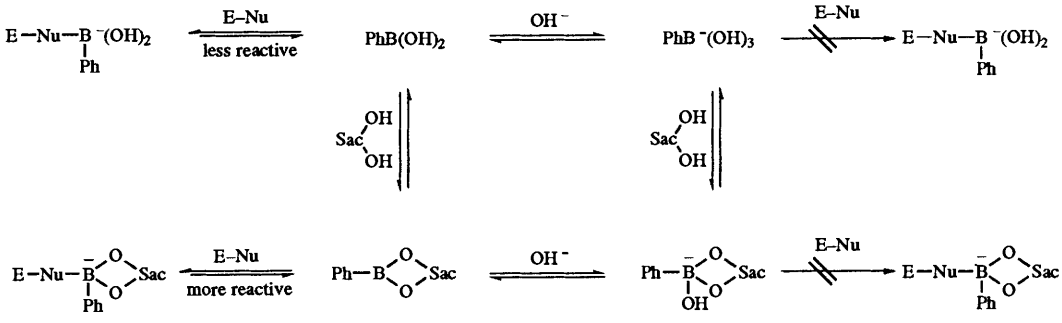


Results and discussion

pH Dependence of the inhibitory effect

It was recently demonstrated by several groups that boronic acids serve as a useful means of detecting saccharides in water.[5-12] For example, saccharides in water can be spectrophotometrically detected by boronic acid-appended porphyrins or boronic acid-appended fluorophores.[7,8,12] Detection of saccharides with boronic acid-appended porphyrins is based on the idea that absorption and fluorescence spectra of porphyrins change sensitively in response to a shift in the aggregation–deaggregation equilibrium and that the complexation of saccharides with the boronic acid moieties changes this equilibrium to deaggregation because of the enhanced hydrophilicity of the complexed porphyrin.[7] Here, it occurred to us that phenylboronic acid working as an inhibitor in the enzyme active site should be withdrawn upon complexation with saccharides because of the enhanced hydrophilicity of the saccharide-complexed inhibitor. As a result the enzyme activity should be regenerated. To test this intriguing hypothesis we investigated the influence of added saccharides on the inhibition ability of compound **1** in α-chymotrypsin-catalysed hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide (Sub).[3] However, to our surprise, added saccharides efficiently intensified the inhibitory effect and in certain saccharides a distinct enantiomeric (D/L) discrimination in the inhibitory effect was observed.[3]

The pH dependence for compound **1** is shown in Fig. 1. In the absence of compound **1** the maximum enzyme activity is observed at pH 7.5. In the presence of compound **1**, on the other hand, the enzyme activity is suppressed over a wide pH range and the maximum appeared at pH ~ 8.0. In the presence of both compound **1** and D-fructose the enzyme activity is strongly suppressed at pH 4–7: above pH 7 the activity gradually increases and at maximum activity (pH 9.0) it becomes higher than that in the presence of only compound **1**. In the presence of both compound **1** and D-glucose the inhibition at pH 4–7 is not



Scheme 1 E-Nu and Sac$<^{OH}_{OH}$ denote α-chymotrypsin and saccharide, respectively

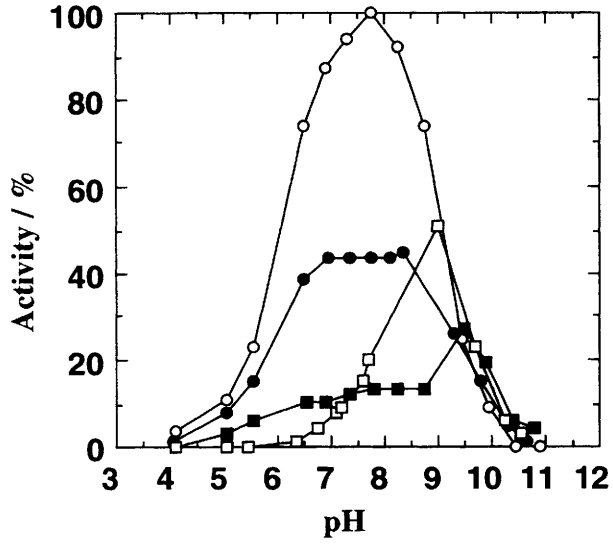


Fig. 1 Plots of activity *vs.* pH for compound **1**: 37 °C, [α-chymotrypsin] = 6.08 × 10^{-7} mol dm^{-3}, [Sub] = 7.56 × 10^{-5} mol dm^{-3}, [**1**] = 6.31 × 10^{-3} mol dm^{-3}, [saccharide] = 7.69 × 10^{-2} mol dm^{-3}: control (○) [in the absence of compound **1** and in the presence of D-fructose or D-glucose (7.69 × 10^{-2} mol dm^{-3})], in the presence of compound **1** (●) and in the presence of both compound **1** and either D-fructose (□) or D-glucose (■)

so conspicuous as that in the presence of both compound **1** and D-fructose but the activity does not increase even at pH 7–9. In any case the pH–activity profile in the presence of both boronic acid **1** and the saccharide is more or less the same irrespective of the saccharide used. How can we rationalize these pH dependences? The reaction processes involved in the present system are expressed as in Scheme 1.

As shown in Fig. 1, the inhibitory effect is scarcely seen above pH 9.5. This implies that anionic species generated at high pH {*i.e.*, $PhB^-(OH)_3$ and $[Ph(HO)B^-]O_2$–Sugar} do not react with the active-site serine (or histidine) in α-chymotrypsin (E–Nu:Nu denotes either serine or histidine acting as a nucleophile in the active site). On the other hand, it can react with $PhB(OH)_2$ and $PhBO_2$–Sugar to give the corresponding boron adducts. It is already known that the pK_a for $PhBO_2$–Sugar is lower by ~2.5 pK-units than that for $PhB(OH)_2$: that is, $PhBO_2$–Sugar is more acidic as a Lewis acid than is $PhB(OH)_2$.[8,11,12] Hence, the nucleophilic reaction between E–Nu and $PhBO_2$–Sugar occurs in preference to that between E–Nu and $PhB(OH)_2$. This difference causes the large inhibitory effect at pH 4–9. Above pH 9.5, on the other hand, $PhBO_2$–Sugar is totally converted into $[Ph(HO)B^-]O_2$–Sugar and cannot react with the nucleophile in the enzyme active site. This kinetic situation gives the maximum activity at around pH 9. It is known that, in complexation with compound **1**, D-fructose has an association constant larger than that for D-glucose:[5,7,8,11,12] hence, D-fructose forms the inhibitory species $PhBO_2$–Sugar more efficiently than does D-glucose at low pH, but the major complex species is converted into the non-inhibitory species $[Ph(HO)B^-]O_2$–Sugar at high pH. Therefore, the distribution of pH-dependent species can well explain the pH–activity profiles in Fig. 1.

Fig. 2 shows the pH–activity profiles for three biphenylylboronic acid isomers. Added 2-isomer **2a** suppresses the enzyme activity to some extent but added saccharides scarcely intensify the inhibitory effect. The steric crowding around the boronic acid moiety probably hampers the efficient binding of saccharides. The pH–activity profiles for 4-isomer **2c** are more or less similar to those for the phenylboronic acid **1**: in the presence of compound **2c** the enzyme activity is suppressed at all pH ranges and the co-existence of D-fructose gives rise to both the strong inhibitory effect at pH 4–7 and the recovery of the activity at pH 8–10. The results suggest that complexes **2c**·saccharide are bound to the enzyme active site in a manner similar to **1**·saccharide complexes. On the other hand, 3-isomer **2b** shows unique pH–activity profiles. Addition of fructose moderately intensifies the inhibitory effect of **2b** at pH 4–7 but lessens the inhibition at pH 7–9, and the L-isomer is more effective than the D-isomer. Addition of glucose strongly intensifies the inhibitory effect and at pH 4–7 α-chymotrypsin totally loses its hydrolytic activity. Both L- and D-isomer are effective but at pH 8–10 the D-isomer is more effective than the L-isomer.

Fluorescence change in compound 2b bound to the active site

To obtain further insight into the complexation event occurring at the active site of α-chymotrypsin we measured the fluorescence spectra of compound **2b**, which showed the strongest inhibitory effect. The excitation wavelength is 246 nm, which is an isosbestic point in the pH-dependent absorption spectrum. Typical fluorescence spectra are shown in Fig. 3. In Fig. 4 the fluorescence intensity (I_{324}) at the emission maximum (324 nm) is plotted against pH. In the absence of α-chymotrypsin the fluorescence intensity decreases with increasing medium pH (Fig. 4A), which occurs in response to a change from fluorescence Ph–Ph–$B(OH)_2$ to non-fluorescent Ph–Ph–$B^-(OH)_3$.† Hence, the apparent pK_a is estimated to be 8.6. When D-glucose was added, the pH–I_{324} profile shifted slightly to lower pH: the apparent pK_a was then 8.2. In contrast, when D-fructose was added, a large pH–I_{324} profile shift was induced at pH 6–9 and the pH–I_{324} did not descend to zero even

† Previously, Mohler and Czarnik proposed 2-anthrylboronic acid as a fluorescent receptor for saccharide sensing: ref. 12(*b*). The fluorescence change induced by addition of saccharide (I in the presence of saccharide/I_0 in the absence of saccharide) is ~0.7. As shown in Fig. 4, compound **2b** shows I/I_0 = 0–0.3, indicating that compound **2b** is much superior as a fluorescent receptor to 2-anthrylboronic acid.

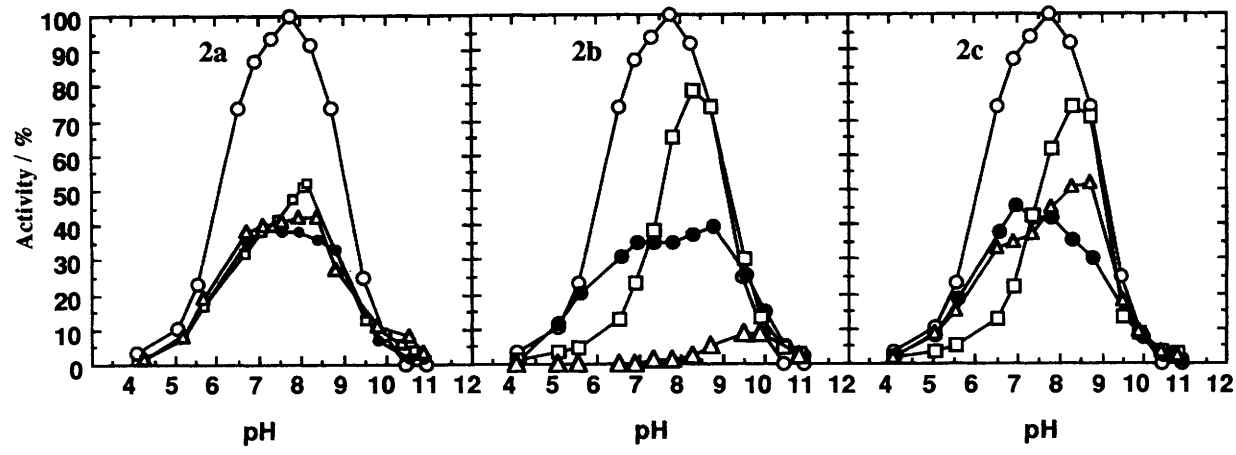


Fig. 2 Plots of activity *vs.* pH for compounds **2**: 37 °C, [α-chymotrypsin] = 6.08×10^{-7} mol dm^{-3}, [Sub] = 7.56×10^{-5} mol dm^{-3}, [**2**] = 6.31×10^{-3} mol dm^{-3}, [saccharide] = 7.69×10^{-2} mol dm^{-3}: control (○) [in the absence of compounds **2** and in the presence of D-fructose or D-glucose (7.69×10^{-2} mol dm^{-3})], in the presence of compounds **2** (●) and in the presence of both compounds **2** and D-fructose (□) or D-glucose (△)

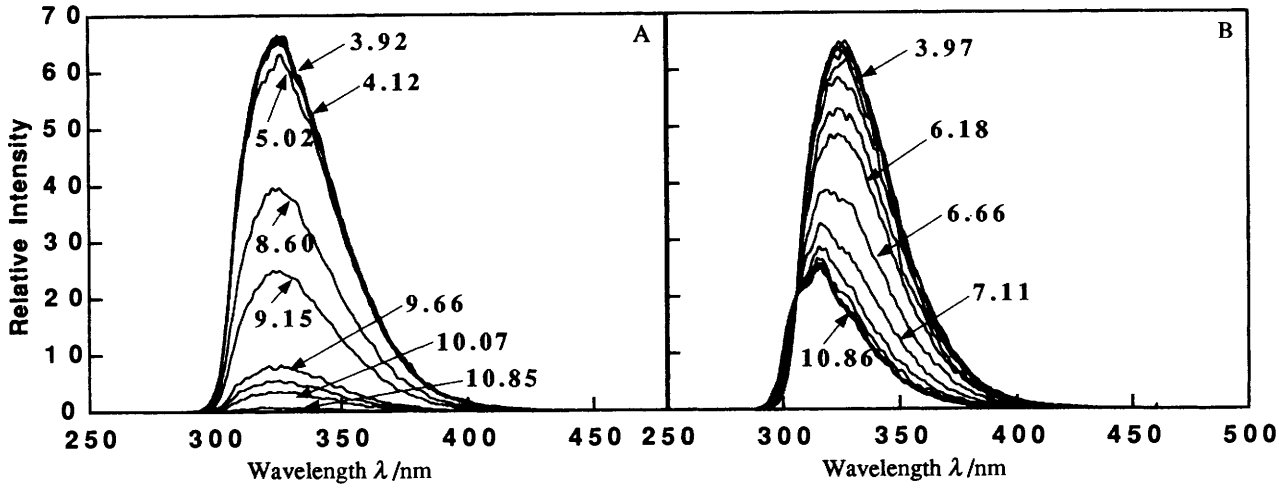


Fig. 3 Fluorescence spectra of compound **2b** (A) and compound **2b** plus D-fructose (B): 37 °C, [**2b**] = 3.35×10^{-6} mol dm^{-3}, [D-fructose] = 3.30×10^{-2} mol dm^{-3}, excitation 246 nm. Numbers in figures denote medium pH

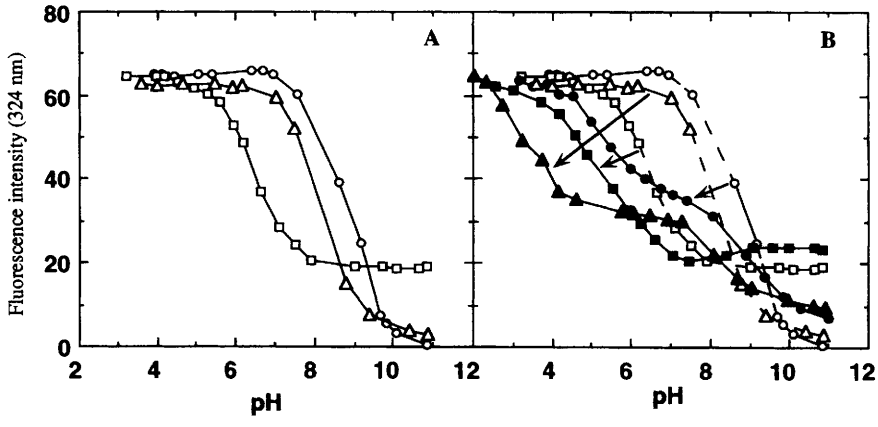


Fig. 4 Plots of the fluorescence intensity of compound **2b** in the absence (A) and the presence (B) of α-chymotrypsin *vs.* pH: 37 °C, [α-chymotrypsin] = [**2b**] = 3.35×10^{-6} mol dm^{-3}, [saccharide] = 3.30×10^{-2} mol dm^{-3} (when it is added), excitation 246 nm, emission 324 nm: no saccharide (○), with D-fructose (□) and with D-glucose (△). For comparison we show in B the fluorescence intensity without α-chymotrypsin by dotted lines, and changes induced by α-chymotrypsin addition are indicated by arrows

at high pH. The apparent pK_a was then 6.8. The difference in the profile shift is accounted for by the difference in the affinity with boronic acids: the association constant for D-fructose (4370 mol^{-1} dm^3 for phenylboronic acid)[13] is much greater than that for D-glucose (110 mol^{-1} dm^3 for phenylboronic acid).[13] On the other hand, it is not so easy to offer a rationale for the difference in the quenching efficiency at high pH. Two possible explanations come to mind. The first rationale is related to the steric

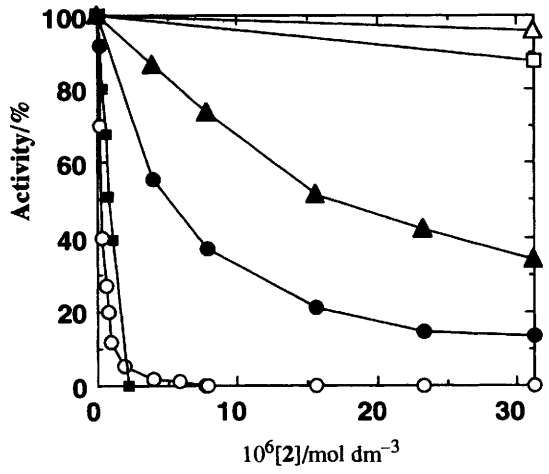


Fig. 5 Plots of [2] *vs.* activity: **2a** (△), **2b** (●), **2c** (□), **2b** + D-fructose (▲), **2b** + D-glucose (○) and chymostatin (■). The measurement conditions are similar to those recorded in the caption to Fig. 2 (37 °C; standard pH 8.0 with 50 mmol dm^{-3} phosphate buffer).

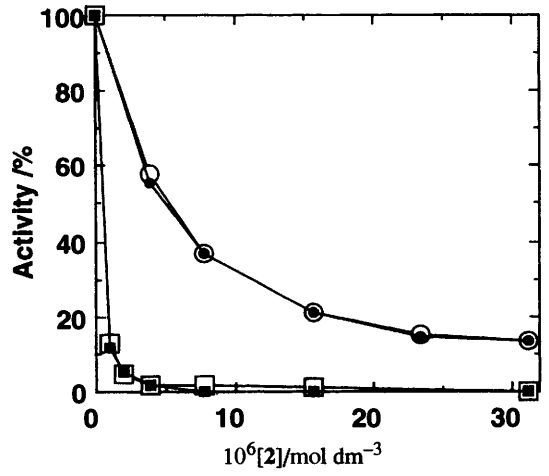
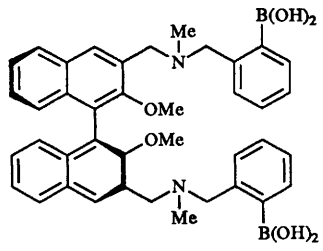


Fig. 6 Plots of [**2b**] *vs.* activity: **2b** (●), **2b** + methyl α-D-glucopyranoside (○), **2b** + D-glucose (■) and **2b** + 6-deoxy-D-glucose (□). The measurement conditions are similar to those recorded in the caption to Fig. 2.

effect. We recently found that in a photo-induced electron-transfer system of compound **3** the quenching efficiency is sensitively affected by a dihedral angle between the naphthalene (Ar) plane and the lone-pair orbital in the nitrogen atom.[14] This suggests that in an Ar(fluorescent)–B^- system the quenching efficiency is affected by the angle of the sp^3-hybridized orbital in the B^- atom. Conceivably, D-fructose and D-glucose give different dihedral angles, which leads to the different quenching efficiencies. The second rationale is related to the electronic effect. Since $ArBO_2$–Sugar is more acidic than $ArB(OH)_2$,[8,11,12] the electron density of the boron atom in $[Ar(HO)B^-]O_2$–Sugar should be lower than that in $ArB^-(OH)_3$. In compound **2b**, therefore, the quenching efficiency in $[Ph–Ph(HO)B^-]O_2$–Sugar is inferior to that in $Ph–Ph–B^-(OH)_3$.

The pH–I_{324} profiles in the presence of α-chymotrypsin are more or less similar to those in the absence of α-chymotrypsin. This indicates that compound **2b** is converted into a non-fluorescent or less fluorescent molecule through the nucleophilic attack of a functional group (serine or histidine) in the active site of α-chymotrypsin. Careful examination of a plot for compound **2b** in the presence of α-chymotrypsin reveals that there are two independent pK_a-values, 5.0 and 8.6. These two



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values are attributed to the enzyme-bound acid **2b** and free acid **2b**, respectively. Although both D-fructose and D-glucose induce a shift of the pH–I_{324} profile to lower pH, the magnitude induced by addition of D-glucose is much greater than that induced by addition of D-fructose, and with D-glucose two independent pK_a-values (3.2 and 8.2) are observable. With D-fructose compound **2b** is predominantly converted into a less fluorescent **2b**•D-fructose complex because of the high affinity with D-fructose. Hence, the formation of a complex with α-chymotrypsin scarcely changes the fluorescence intensity. With D-glucose, in contrast, compound **2b** predominantly exists as the fluorescent free species because of its low affinity with D-glucose, and the binding of the **2b**•D-glucose complex to the active site causes the remarkable shift of the pH–I_{324} profile to lower pH. The apparent pK_a-values are 3.2 and 8.2. Since the higher pK_a (8.2) is nearly consistent with that of compound **2b** in the absence of α-chymotrypsin (8.6), it is assigned to the pK_a-value of unbound acid **2b**. The lower pK_a (3.2) is attributed to a new species which is different both from **2b**•D-glucose and from **2b**•α-chymotrypsin (pK_a 5.0). Hence, this should be a ternary complex **2b**•D-glucose•α-chymotrypsin. The pK_a-value is lower by 5.4 p*K*-units than that of free boronic acid **2b** and by 1.8 p*K*-units than that for enzyme-bound acid **2b**. The remarkably large pK_a shift implies that complex **2b**•D-glucose is strongly bound to the active site of α-chymotrypsin.

Strong inhibitory effect of compound 2b plus glucose which is stronger than that of chymostatin

The foregoing fluorescence studies suggest that compound **2b** would show a very strong inhibitory effect in the presence of D-glucose. We therefore compared the inhibitory effect with that of chymostatin, a specific inhibitor for α-chymotrypsin.[15] As shown in Fig. 5, *the combined inhibitor of compound* **2b** *and* D-*glucose shows a remarkably strong inhibitory effect at* (0–20) × 10^{-7} mol dm^{-3} *and which is even stronger than that of chymostatin.* In chymostatin the activity is entirely suppressed at 2.2 × 10^{-6} mol dm^{-3}. In the combined inhibitor, on the other hand, the activity gradually decreases to zero and it is totally suppressed at 7.8 × 10^{-6} mol dm^{-3}. The combined inhibitor system can be expressed in the form of three equations (1)–(3) in addition to the conventional enzyme-catalysed reaction system. Although the **2b**•D-glucose complex strongly binds to the enzyme active site and inhibits the enzyme activity, equations (1) and (2), which have not so large binding constants, retard the abrupt decrease in the activity.[13] On the other hand, the acid **2b** itself shows a moderate inhibitory effect, which is much weaker than that of chymostatin (Fig. 5). It is also seen from Fig. 5 that compounds **2a** and **2c** show a further, weak inhibitory effect.

$$\textbf{2b} + \text{D-glucose} \rightleftharpoons \textbf{2b}\text{•D-glucose} \quad (1)$$

$$\text{Enzyme} + \textbf{2b} \rightleftharpoons \text{Enzyme•}\textbf{2b} \quad (2)$$

$$\text{Enzyme} + \textbf{2b}\text{•D-glucose} \rightleftharpoons \text{Enzyme•}\textbf{2b}\text{•D-glucose} \quad (3)$$

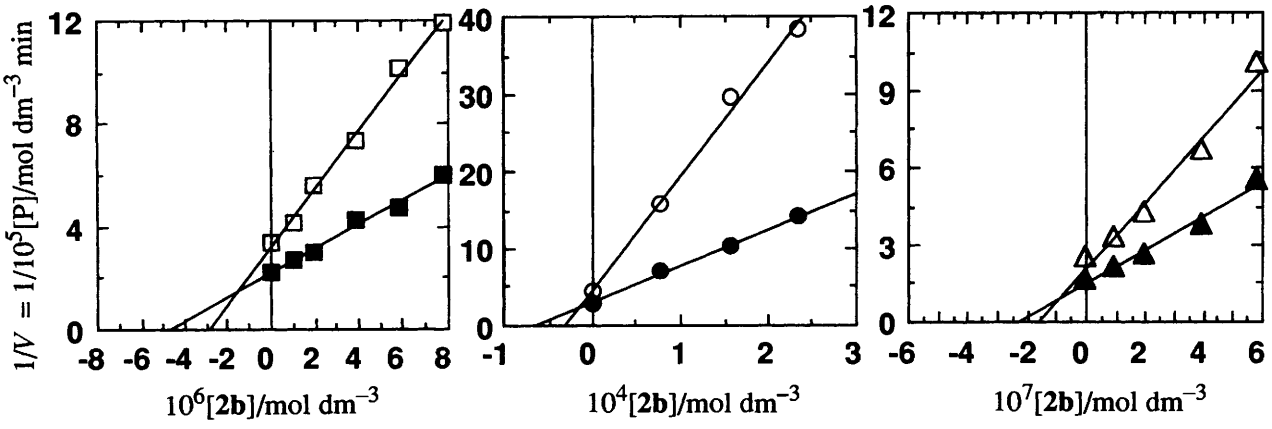
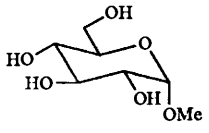


Fig. 7 Typical Dixon plots: 37 °C, [α-chymotrypsin] = 6.08×10^{-7} mol dm^{-3}, [Sub] = 7.56×10^{-5} mol dm^{-3} (open symbols) and 1.51×10^{-4} mol dm^{-3} (filled symbols), [saccharide] = 7.69×10^{-2} mol dm^{-3}: (a) (□, ■) **2b** only, (b) (○, ●) **2b** + D-fructose and (△, ▲) **2b** + D-glucose

Table 1 K_i-values for inhibitors (37 °C; pH 8.0)

Inhibitor	10^7 K_i/mol dm^{-3}
2b	16
2b + D-Fructose	130
2b + L-Fructose	11
2b + D-Glucose	1.1
Chymostatin	4.8

To obtain insight into the complexation site of D-glucose with acid **2b**, we compared the activity of methyl α-D-glucopyranoside **4** with that of 6-deoxy-D-glucose **5** as a 'co-inhibitor'. In methyl α-D-glucopyranoside **4** the sole complexation site is the 4,6-diol moiety, whereas in 6-deoxy-D-glucose **5** the sole complexation site is the 1,2-diol moiety. As shown in Fig. 6, the inhibitory effect in the presence of compound **2b** and methyl α-D-glucopyranoside **4** is consistent with that in the presence of only compound **2b**. On the other hand, the inhibitory effect in the presence of the acid **2b** and 6-deoxy-D-glucose **5** is comparable with that in the presence of both the acid **2b** and D-glucose. The results clearly establish that the complexation site in D-glucose is the 1,2-diol functionality.



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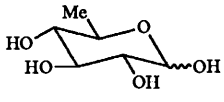

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We have estimated K_i-values for acid **2b**, **2b** + D-fructose, **2b** + D-glucose and chymostatin by use of a Dixon plot.[16] Although the combined inhibitor systems may not necessarily result in a straight line in the Dixon plot, because of the presence of additional equilibria, the plots at [**2b**] = 0.95×10^{-6} to 7.81×10^{-6} mol dm^{-3} gave satisfactory straight lines (Fig. 7). We therefore determined the apparent K_i-value from the intercept with the $1/V = 0$ line. Also, as commercially available chymostatin is a mixture of (*S*)-Ile and (*S*)-Val species, one must consider that this also gives rise to the apparent K_i. The results are summarized in Table 1. Several interesting points can be made about the data in Table 1. First, system **2b** + L-fructose has a K_i-value smaller than that for free acid **2b** whereas system **2b** + D-fructose has a K_i-value larger than that for acid **2b**. The difference is due to the medium pH (8.0) employed for the kinetic measurements: although both D-fructose and L-fructose similarly intensify the inhibitory effect at low pH and rather weaken it at high pH (Fig. 2), the inhibitory effect appears more strikingly in L-fructose. At pH 8.0 two isomers operate in opposite direction on the enzyme activity. Anyhow, it is interesting that the inhibitory effect of acid **2b** reflects the absolute configuration of covalently bound saccharides. Secondly, D-glucose is more effective as a 'co-inhibitor' by two orders of magnitude than is D-fructose. As described previously, the affinity of D-fructose with boronic acids is much greater than that of D-glucose.[5,7,8,11,12] This trend means that D-fructose·boronic acid complexes are relatively stable and therefore less reactive with α-chymotrypsin. In contrast, D-glucose·boronic acid complexes are relatively unstable and therefore active for nucleophilic attack of α-chymotrypsin. Of course, one has to take the shape selectivity of α-chymotrypsin for substrates into account but we believe that the stability of saccharide·boronic acid complexes is also operative for the determination of the inhibitory effect. Thirdly, and most importantly, *the K_i-value for system* **2b** + D-*glucose is smaller by more than* 4-*fold than that for chymostatin*. It is very interesting that in spite of its simple structure the inhibitory effect of the **2b**·D-glucose complex is stronger than that of the specific α-chymotrypsin inhibitor. At present the binding mode is not yet clear except for the fact that the boronic acid moiety forms a covalent linkage with either serine or histidine.[17] We consider, however, that because of the superinhibitory effect the binding mechanism merits further investigation. We are now trying to understand the binding mode in the active site by computational methods and ^{11}B NMR spectroscopy.[18]

Conclusions

The original aim of this study was to synthesize biphenylylboronic acids and to monitor the inhibition event occurring at the active site of α-chymotrypsin by fluorescence spectroscopy. Through this study we unexpectedly found that the inhibitory effect of the system **2b** + D-glucose is comparable to that of a specific inhibitor, chymostatin. Since acid **2b** itself only moderately inhibits the enzyme's activity, it follows that the effect is remarkably enhanced by complexation with D-glucose. Therefore, one may call D-glucose a 'co-inhibitor'. Although the detailed binding mode of the **2b**·D-glucose complex is not yet clarified, the present findings suggest that the development of new boronic acid derivatives leads to exploitation of superinhibitors for nucleophilic hydrolytic enzymes. We are currently studying different boronic acids bearing different acidity functions, different saccharides possessing different stereostructures and different enzymes having similar mechanisms, expecting that the large inhibitory effect will be reproduced by a simple, combined system of boronic acids and sugars.

Experimental

General procedures

All experiments were carried out under a nitrogen atmosphere. Tetrahydrofuran (THF) was distilled from sodium–benzophenone immediately prior to use. ^{1}H NMR spectra were recorded on a Bruker ARX-300 spectrometer. Mass spectrometry was performed on a Hitachi M-2500 instrument. IR spectra were obtained as KBr disks using a Shimadzu FT-IR 8100 spectrometer. UV spectra were measured on a Shimadzu UV-2200 spectrometer. Melting points were determined on a Yanaco (MP-500D) micro melting point apparatus and are uncorrected.

Materials

Compounds **1** and **2a** were purchased from Aldrich. Compounds **2b** and **2c** were synthesized from the corresponding biphenylyl bromides (*vide post*). Chymostatin was purchased from Sigma.

Biphenyl-4-ylboronic acid 2c. To a tetrahydrofuran (THF) solution (30 cm^3) of 4-bromobiphenyl (2.33 g, 10.0 mmol) at −78 °C was added a hexane solution (10 cm^3) of butyllithium (16 mmol) and the reaction mixture was stirred for 1 h under nitrogen. The obtained lithium reagent was added to another flask which contained a THF (50 cm^3) solution of trimethyl borate (6.41 g, 61.6 mmol) *via* a cannula by nitrogen pressure at −78 °C. The reaction mixture was stirred for 1 h, and then was gradually warmed to room temp. After addition of 2 mol dm^{-3} HCl (10 cm^3), the mixture was stirred at room temp. for 15 h. After removal of the solvent under reduced pressure, diethyl ether (50 cm^3) was added and the organic phase was washed twice with water. The organic layer was separated, and dried over anhydrous magnesium sulfate. Concentration of the solution resulted in an oily product, which was triturated with hexane. Finally, the solid was reprecipitated from methanol–water to give *title compound* **2c** (40%), mp 263–266 °C; ν_{max}(KBr disk)/cm^{-1} 3393 (OH); δ_H(300 MHz; CD_3OD), 7.24 (1 H, d, ArH), 7.33 (2 H, t, ArH), 7.46–7.53 (4 H, m, ArH), 7.59 (1 H, d, ArH) and 7.73 (1 H, d, ArH) (Found: C, 72.5; H, 5.6. $C_{12}H_{11}BO_2$ requires C, 72.78; H, 5.60%); *m/z* (SIMS$^+$, glycerol) 254 ([M + glycerol − 2H_2O]$^+$).

Biphenyl-3-ylboronic acid 2b. This *compound* was synthesized in a similar manner to its 4-isomer **2c**: yield 43%, mp 203–205 °C; ν_{max}(KBr disk)/cm^{-1} 3274 (OH); δ_H(300 MHz; CD_3OD), 7.24 (1 H, d, ArH), 7.33 (3 H, t, ArH), 7.50–7.55 (4 H, m, ArH) and 7.73 (1 H, s, ArH) (Found: C, 72.9; H, 5.6%); *m/z* (SIMS$^+$, glycerol) 254 ([M$^+$ + glycerol − 2H_2O]$^+$).

Estimation of the α-chymotrypsin activity

α-Chymotrypsin was purchased from Sigma (Type II: MW 25100). The hydrolytic reaction was carried out according to Kouzuma's method[19] [37 °C; standard pH 8.0 with 50 mmol dm^{-3} phosphate buffer, 0.3 vol% methanol plus 0.8 vol% dimethyl sulfoxide (DMSO)] and the progress of the reaction was followed by monitoring the appearance of the absorption band at 410 nm (*p*-nitroaniline: P) (see Fig. 7). The activity was estimated from the liner A_{410} *vs.* time plots for the initial 10 min.

Fluorescence measurements

A DMSO solution (3.3 cm^3) containing compound **2b** (1.01 × 10^{-4} mol dm^{-3}), an aqueous solution (3.3 cm^3) containing saccharide (1.0 mol dm^{-3}) and an aqueous solution (4.22 cm^3) containing α-chymotrypsin (7.97 × 10^{-5} mol dm^{-3}) were added to a buffered aqueous solution (100 cm^3) adjusted with 1.0 mol dm^{-3} HCl and 1.0 mol dm^{-3} NaOH. The final concentrations were 3.35 × 10^{-6} mol dm^{-3} for compound **2b**, 3.30 × 10^{-2} mol dm^{-3} for saccharide and 3.35 × 10^{-6} mol dm^{-3} for α-chymotrypsin. After the solution was stored for 5 min at 25 °C, the fluorescence spectra were measured in a 1 cm cell with a Perkin-Elmer Model LS50B fluorescence spectrophotometer.

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