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Screening of boronic acids for strong inhibition of the hydrolytic activity of α-chymotrypsin and for sugar sensing associated with a large fluorescence change

Hikaru Suenaga, Hiromasa Yamamoto and Seiji Shinkai*

CHEMIRECOGNICS Project, ERATO, Research Development Corporation of Japan, 2432-3 Aikawa-cho, Kurume, Fukuoka 830, Japan

Abstract: The inhibitory effect of arylboronic acids which act as a transition state analogue for certain peptidases is efficiently intensified by added saccharides: this finding enables us to control the enzyme activity by a combination of arylboronic acids and polyols including sugars. In particular, the combined system of 3-biphenylboronic acid and D-glucose strongly inhibited the hydrolysis reaction mediated by α–chymotrypsin, the inhibitory effect ($K_i = 1.1\times10^{-7}$ mol dm^{-3}) being stronger than that of a specific inhibitor, chymostatin ($K_i = 4.8\times10^{-7}$ mol dm^{-3}). Hence, saccharides act as a *'co-inhibitor'* in the boronic acid inhibition system. To search for such fluorescent boronic acids that are useful for sensitive detection of sugars in water, 8 aromatic boronic acids have been screened. Among them biphenyl-3-boronic acid and naphthalene-2-boronic acid could fully satisfy three prerequisites for sugar sensing: strong fluorescence intensity, large pH-dependent change in I_{max}, and shift of the pH-I_{max} profile to lower pH region in the presence of sugars.

INTRODUCTION

Trigonal boron compounds contain a vacant 2p orbital which easily reacts as a Lewis acid with nucleophiles such as hydroxide, alkoxide, or imidazole to give a tetrahedral boron adduct. 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 (1-4). Apparently, phenylboronic acid acts as a novel *'transition state analogue'* for these enzymes and the K_i values for phenylboronic acid are estimated to be 2.3×10^{-4}-8.0×10^{-4} mol dm^{-3} for subtilisin (1 and 2) and 1.9×10^{-4} mol dm^{-3} for α–chymotrypsin (1 and 2).

Meanwhile, it is known that boronic acids form cyclic esters with saccharides and the reaction occurs reversibly and rapidly at ambient temperature. It was recently demonstrated by a few groups that boronic acids serve as a useful interface to selectively recognize saccharides in water (5-14). For example, saccharides in water can be spectrophotometrically detected by boronic-acid-appended porphyrins (7) or boronic-acid-appended fluorophores (8 and 12). Saccharide detection 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 the complexation of saccharides with the boronic acid moieties changes this equilibrium to deaggregation because of the enhanced hydrophilicity of the complexed porphyrin. 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 phenylboronic acid in α–chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-L-tyrosine-

p-nitroanilide (Sub). Surprisingly, added saccharides efficiently intensified the inhibitory effect and in certain saccharides the distinct D/L discrimination in the inhibitory effect was observed (15 and 16).

Sugars Can Intensify the Inhibitory Effect of Phenylboronic Acid on the Hydrolytic Activity of α-Chymotrypsin

α-Chymotrypsin was purchased from Sigma (Type II: MW 25100). The hydrolytic reaction was carried out according to Kouzuma's method (17) (37 °C, standard pH 8.0 with 5.0×10^{-2} mol dm^{-3} phosphate buffer, 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 at 410 nm (*p*-nitroaniline: P).

Plots of [P] *vs.* reaction time are illustrated in Fig. 1. The hydrolytic activity of α-chymotrypsin (6.08×10^{-7} mol dm^{-3}) was not affected by the addition of saccharides (7.69×10^{-2} mol dm^{-3}) whereas it was moderately inhibited by the addition of phenylboronic acid (6.31×10^{-3} mol dm^{-3}). When saccharides were added to the boronic-acid-inhibited system, the rate of the hydrolytic reaction was further suppressed. The inhibition efficiency for monosaccharides is in the order of D-talose > D-fructose > D-glucose > D-mannose > D-galactose. This order is not necessarily consistent with the order of the association constant with phenylboronic acid (D-fructose > D-galactose > D-mannose > D-glucose) (8,11-12). Interestingly, the distinct D/L enantioselectivity in the inhibitory effect was observed for fructose and glucose: in both saccharides the inhibitory effect for D-isomers is larger than that for L-isomers (Fig. 1). The particularly large difference was observed for fructose: the inhibitory effect of L-fructose was even weaker than that of phenylboronic acid itself. The findings support the view that the enzyme active site '*recognizes*' the molecular structure of boronic acid-saccharide complexes. We here determined the K_i for phenylboronic acid in the absence and the presence of D-fructose. Dixon plots (18) showed that both systems feature a competitive inhibition but the K_i for phenylboronic acid plus D-fructose (6.3×10^{-4} mol dm^{-3}) is much smaller than that for phenylboronic acid (1.9×10^{-3} mol dm^{-3}).

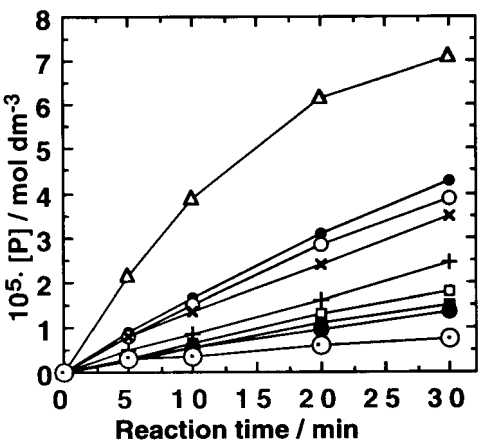


Fig. 1 Plots of [*p*-nitroaniline (P)] vs. reaction time: 37 °C, [α-chymotrypsin]=6.08×10^{-7} mol dm^{-3}, [Sub]=7.56×10^{-5} mol dm^{-3}, [phenylboronic acid]=6.31×10^{-3} mol dm^{-3}, [saccharide]=7.69×10^{-2} mol dm^{-3}: (Δ) control (in the absence of phenylboronic acid and in the presence of saccharides (7.69×10^{-2} mol dm^{-3})); (O) L-fructose, (▲) no saccharide, (×) D-galactose, (+) D-mannose, (□) L-glucose, (■) D-glucose, (●) D-fructose, and (⊙) D-talose in the presence of phenylboronic acid.

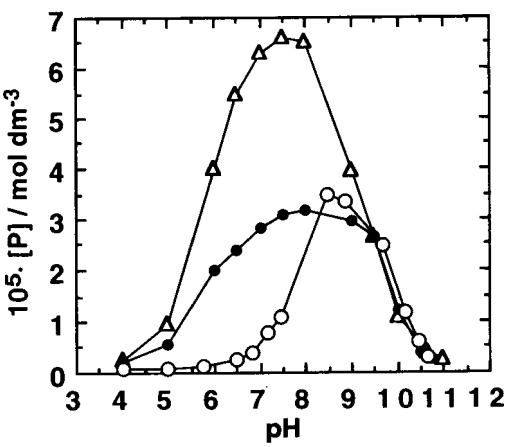
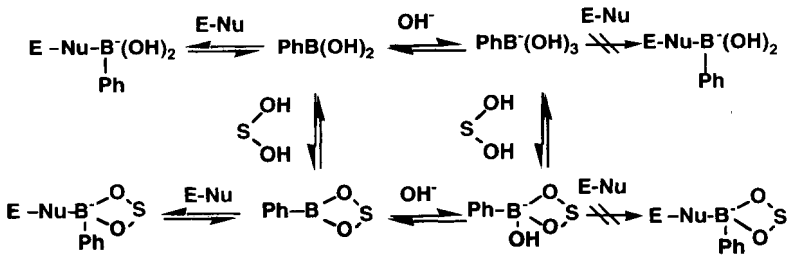


Fig. 2 Plots of [*p*-nitroaniline (P)] vs. reaction time: 37 °C, [α-chymotrypsin]=6.08×10^{-7} mol dm^{-3}, [Sub]=7.56×10^{-5} mol dm^{-3}, [phenylboronic acid]=6.31×10^{-3} mol dm^{-3}, [saccharide]=7.69×10^{-2} mol dm^{-3}: (Δ) control (in the absence of phenylboronic acid and in the presence of D-fructose (7.69×10^{-2} mol dm^{-3})), (●) in the presence of phenylboronic acid, and (O) in the presence of phenylboronic acid and D-fructose

The pH dependence is shown in Fig. 2. In the absence of phenylboronic acid the maximum enzyme activity was observed at pH 7.5. In the presence of phenylboronic acid, on the other hand, the enzyme activity is suppressed and the maximum appeared at around pH 8.0. In the presence of both phenylboronic acid and D-fructose the enzyme activity is strongly suppressed at pH 4~9 and the maximum activity shifts to pH 8.5. How can we rationalize this strange pH dependence? The reaction processes involved in the present system are expressed as in Scheme 1. As shown in Fig. 2, the inhibitory effect is scarcely seen above pH 9.5. This implies that $PhB^{-}(OH)_3$ and $[Ph(HO)B^{-}]O_2S$ 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 $PhBO_2S$ to give the boron adducts. It is already known that the pK_a for $PhBO_2S$ is lower by *ca.* 2.5 pK units than that for $PhB(OH)_2$: that is, $PhBO_2S$ is more acidic as a Lewis acid than $PhB(OH)_2$ (8 and 11). Hence, the nucleophilic reaction between E-Nu and $PhBO_2S$ 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, $PhBO_2S$ is totally converted to $[Ph(HO)B^{-}]O_2S$ and cannot react with the nucleophile in the enzyme active site. This kinetic situation gives the maximum activity at pH 8.5.



Scheme 1 E-Nu and S<OH denote α-chymotrypsin and saccharide, respectively.

Control of α-Chymotrypsin Hydrolytic Activity by a Combination of Phenylboronic Acids and Polyols

The hydrolytic activity of α-chymotrypsin is inhibited by phenylboronic acids. It was shown that the inhibitory effect is intensified by added diols but rather weakened (*i.e.*, the hydrolytic activity is regenerated) by tripodal additives. Hence, the activity can be controlled in a range of 0-100%. This is a novel method to control the activity of the nucleophilic hydrolytic enzymes.

As shown in Fig. 3A, the catalytic activity decreased with increasing **17** concentrations. The order of the inhibitory effect was **17c** > **17b** > **17a**: *i.e.*, the stronger the electron-withdrawing ability, the higher the inhibitory effect. In **17c** the reaction was totally suppressed at [**17c**] = 1.23×10^{-2} mol dm^{-3}. In the presence of 6.31×10^{-3} mol dm^{-3} **17a** the enzyme activity was reduced to 40%.

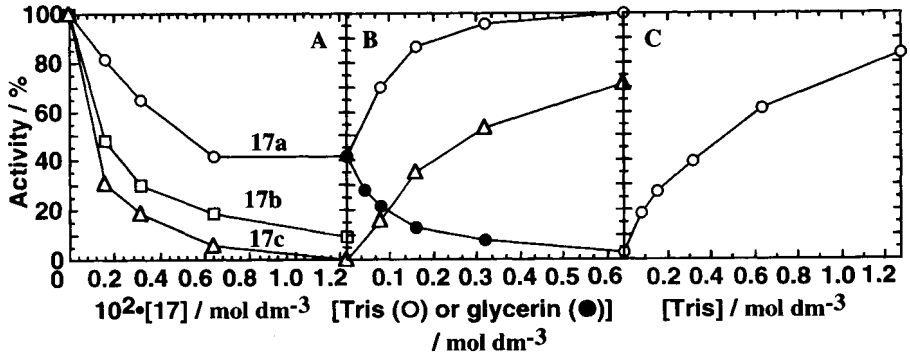
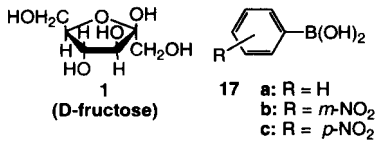
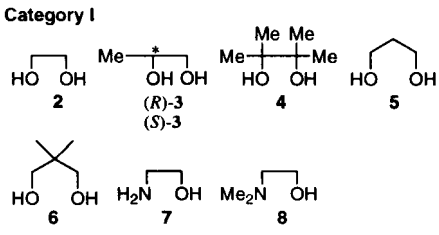
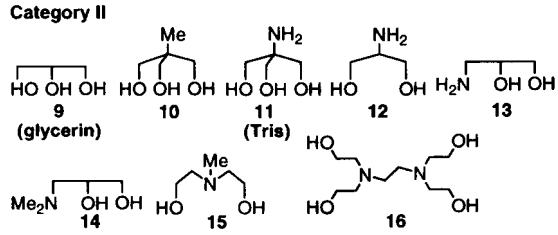


Fig. 3 Control of the hydrolytic activity by the combination of **17** with Tris or glycerin; [α-chymotrypsin] = 6.08×10^{-7} mol dm^{-3}.

The additive effect was investigated at these reaction conditions. Figure 4 summarizes the effect of additives in category I. D-Fructose **1** used as a representative of saccharides showed a strong inhibitory effect. Compound **2** showed a weak inhibitory effect but **5** is almost ineffective. (*R*)-**3** showed the inhibitory effect greater than (*S*)-**3**. Compounds **4** and **6** with additional methyl groups provided turbid solutions (13) and the rate measurement was not reproducible. Compounds **7** and **8** weakly recovered the enzyme activity. Conceivably, the electron-donating ability of amines weakens the acidity of the boron atom and it no longer undergoes the attack of the nucleophilic center. Figure 5 summarizes the effect of additives in category II. As expected, **11**, **12**, **15** and **16** could regenerate the 100% enzyme activity and **10**, **13** and **14** showed the moderate regeneration effect. The sole exception in category II is glycerin **9**. The molecular model predicts that three oxygens in **9** cannot covalently interact with the same boron atom: that is, although **9** is a triol, it actually acts as a diol in the complexation with **17a**.







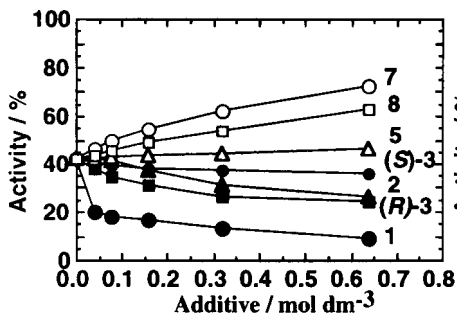


Fig. 4 Effect of additives in category I

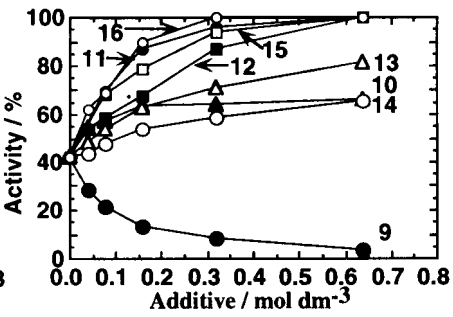


Fig. 5 Effect of additives in category II

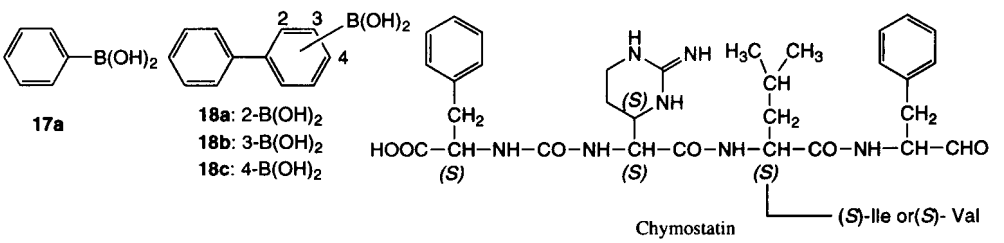
Now, all additives have successfully been classified. We here demonstrate the control of the hydrolytic activity by the combination of **17** as an inhibitor with several additives (Fig. 3B, 3C). At [**17a**] = 6.31×10^{-3} mol dm^{-3} glycerin is added: as seen in Fig. 3B the activity can be suppressed to 3% of the regular activity in the absence of **17**. When Tris **11** is added to this solution, the activity can be recovered to 83% activity (Fig. 3C). At [**17c**] = 1.23×10^{-2} mol dm^{-3} where the enzymatic reaction is totally inhibited, Tris **11** is added: as seen in Fig. 3B the activity can be recovered up to 72%. These results clearly indicate that the activity of α-chymotrypsin can be controlled in a range of 0% -100% by the present method.

In conclusion, the present study shows that phenylboronic acids are unique inhibitors, the inhibitory effect of which can be either intensified or weakened by additives. The finding is useful not only to prove the

hybridization of the boron atom in the enzyme active site but also to control the enzyme activity. We believe that this method is more generally applicable to the control of nucleophilic enzyme activity.

Strong Inhibitory Effect of Sugar•Biphenylboronic Acid Complexes on the Hydrolytic Activity of α–Chymotrypsin

Boronic acids act as transition state analogues for certain peptidases. The inhibitory effect of 2-, 3-, and 4-biphenylboronic acids (**18a**, **18b** and **18c**) on the hydrolytic activity of α–chymotrypsin has been investigated. These inhibitors were employed to monitor by fluorescence the binding event (covalent-bond formation with either serine residue (195) or histidine residue (57)) occurring in the active site. It was shown that the decrease in the fluorescence intensity, which is induced by covalent-bond formation with the boronic acid moiety, is well correlated with the inhibitory effect estimated by kinetic measurements. The inhibitory effect appeared in the order of **18a** < **18c** <<**18b** (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 **18b** 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}) (19).* 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).

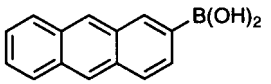


Scheme 2 Inhibitors

Screening of Fluorescent Boronic Acids Which Show a Large Fluorescence Change for Sugar Sensing

Boronic acids can form cyclic esters with diols in basic aqueous solution. It has been shown that this interaction is superior to the hydrogen-bonding interaction for sugar sensing in an aqueous system because the boronic acid-diol interaction is very useful as a potential method to 'touch' polyhydroxylic saccharides and selectively recognize them (refs. 5-14). Aromatic boronic acids (ArB(OH)$_2$) are particularly suitable to this purpose because of the chemical stability of the B-C linkage. Czarnik *et al.*(12) demonstrated that in 2-anthrylboronic acid **19** anionic boron formed through saccharide complexation quenches the anthracene fluorescence, by which one can easily detect the saccharide-binding event. Although this is a novel and very useful system for sugar sensing, the decrease in the fluorescence intensity is relatively small (I in the presence of saccharide /I_0 in the absence of saccharide = *ca.* 0.7) (12). In the course of our studies on the inhibitory effect of ArB(OH)$_2$ on the hydrolytic activity of α-chymotrypsin (15,16,20), we noticed that the saccharide-binding can change biphenylboronic acids (**18**) into nonfluorescent molecules (*i.e.*, $I/I_0 \approx 0$). This unexpected finding suggests that the efficiency of photoinduced electron transfer in ArB(OH)$_2$ is governed by a subtle balance in the HOMO level between the aryl moiety and the boronic acid moiety. It is undoubted, therefore, that in order to search for more efficient sugar sensing systems screening of fluorescent ArB(OH)$_2$ warrants further investigation. We here tested 7 fluorescent ArB(OH)$_2$ in addition to **19** and found an interesting relationship between the aromatic ring structure and I/I_0 (21).

Compounds **18**, **20-22** were synthesized from corresponding bromides via lithiation followed by the reaction with $B(OR)_3$. The products were identified by IR and ^1H NMR spectral evidence and elemental analysis. The fluorescence spectra for 7 aromatic boronic acids were measured at 25 °C in water : DMSO = 99:1 v/v using isosbestic points in the absorption spectra as excitation wavelengths. As a measure of quantum yields we used I_{max}/A_{ex} where I_{max} and A_{ex} denote the fluorescence intensity at the emission maximum and the absorbance at the excitation wavelength. As a sugar D-fructose which possesses the greatest association constants with boronic acids (8-14,22) was used: in basic pH region at [D-fructose] = 3.30×10^{-2} mol dm^{-3} one can regard that boronic acids are totally converted to their D-fructose complexes. The medium pH was adjusted with 1.0 mol dm^{-3} HCl and 1.0 mol dm^{-3} NaOH. The results are summarized in Table 1. Among them biphenyl-3-boronic acid and naphthalene-2-boronic acid could fully satisfy three prerequisites for sugar sensing: strong fluorescence intensity, large pH-dependent change in I_{max}, and shift of the pH-Imax profile to lower pH region in the presence of sugars.



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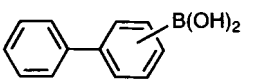
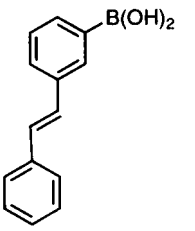


18a: 2-$B(OH)_2$
18b: 3-$B(OH)_2$
18c: 4-$B(OH)_2$



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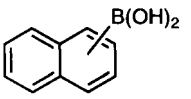

21a: 1-$B(OH)_2$
21b: 2-$B(OH)_2$

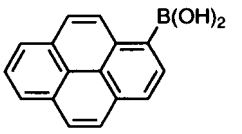

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TABLE 1. Fluorescence properties of aromatic boronic acids at 25°C[a]

Boronic acid	Excitation	Emission	*I*max / *A*ex				I_{max} at pH 11 / I_{max} at pH 3	
(conc. mol dm^{-3})	/nm	/nm	Without sugar		With sugar		Without sugar	With sugar
			pH 3	pH11	pH 3	pH11		
19[b](7.50×10^{-7})	348	416	-	-	-	-	0.61	0.61
18a(3.35×10^{-4})	230	340	0.30	0	0.30	0	0	0
18b(3.35×10^{-6})	246	324	1043	0	1043	310	0	0.30
18c(3.35×10^{-6})	246	324	1081	98	1081	836	0.09	0.77
20(3.35×10^{-5})	296	360	39	8	39	13	0.21	0.33
21a(3.35×10^{-6})	268	344	777	60	777	694	0.08	0.89
21b(3.35×10^{-6})	268	344	3310	0	3310	605	0	0.18
22(3.35×10^{-6})	338	376	3700	2729	3700	2547	0.74	0.69

[a][Sugar (D-fructose)] = 3.30×10^{-2} mol dm^{-3}.

[b] Cited from reference 12.

CONCLUSION

The original aim of this study was to synthesize diphenylboronic acids and to monitor the inhibition event occurring in the active site of α-chymotrypsin by fluorescence spectroscopy. Through this study we found that biphenyl-3-boronic acid and naphthalene-2-boronic acid can act as excellent fluorescent probes for sugar sensing and we unexpectedly found that the inhibitory effect of **18b** plus D-glucose is comparable with that of a specific inhibitor, chymostatin. Since **18b** itself only moderately inhibits the enzyme 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 **18b**•D-glucose complex is not yet clarified, the present findings suggest that the development of new boronic acid derivatives leads to exploitation of super inhibitors for nucleophilic hydrolytic enzymes. The present studies show that phenylboronic acids are unique inhibitors, the inhibitory effect of which can be either intensified or weakened by the additives. The finding is useful not only to prove the hybridization of the boron atom in the enzyme active site but also to control the enzyme activity. We believe that this method is more generally applicable to the control of nucleophilic enzyme activity.

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