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BORIC ACID EFFECT ON THE HYDROLYSIS OF 4-NITROPHENYL 2,3-DIHYDROXYBENZOATE: MIMIC OF BORATE INHIBITION OF SERINE PROTEASES

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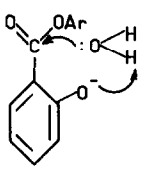
Summary: Boric acid inhibits the hydrolysis of deprotonated 4-nitrophenyl 2,3-dihydroxybenzoate which proceeds with intramolecular general base assistance. This effect is opposite to previously reported borate catalysis of the hydrolysis of salycilic acid esters and mimics borate inhibition of serine proteases.

Boric and boronic acids are widely used as enzyme models owing to their ability to catalyze reactions of hydroxylic compounds via reversible binding to a hydroxyl group and the subsequent intramolecular attack at the given function (ester, imine, nitrile etc.).[1-5] In these systems trivalent boron acts as a Lewis acid like metal ions, which catalyze numerous related reactions[6] and activate many enzymes.[7] In contrast to metals, however, the only function of boric and boronic acids in enzymic systems is the inhibition of serine proteases by the binding to serine hydroxyl.[8-13] This effect was never observed, to our knowledge, in chemical systems.

A suitable model reaction would be the hydrolysis of salycilate aryl esters, which proceeds with the general base intramolecular assistance of deprotonated *ortho*-hydroxy group, as shown in **1**, in a manner closely resembling the mechanism of action of serine hydrolases.[14] The binding of



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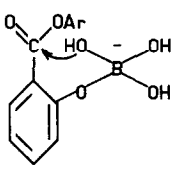

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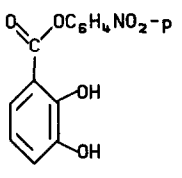


3

boric acid to salycilate *ortho*-hydroxy group leads, however, to acceleration

rather than inhibition of the hydrolysis.[1-3] The reason of this effect is the ability of bound borate to attack ester function,[3] as shown in 2. Evidently, the desired model system must have structural features allowing the binding of borate in a conformation, which excludes this interaction. We have found such behavior in the hydrolysis of ester 3.

The hydrolysis of 3 (prepared as described in Ref.15) was followed by monitoring the absorption of 4-nitrophenolate anion at 25°C using a Hitachi 150-20 UV-VIS spectrophotometer equipped with a thermostatted cell holder. Pseudo-first-order rate constants (k_{obs}) were calculated with the integral first-order rate equation.

The pH-dependence of k_{obs} shown in Fig. 1 is similar to that found for salycilate esters.[1,14] Two plateau regions observed at pH<7 and pH>9 refer to the reactions of neutral (k_1) and deprotonated (k_2) forms of 3, respectively, with water, Scheme 1.

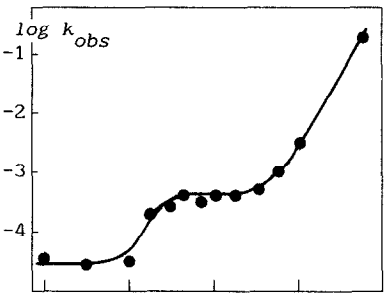
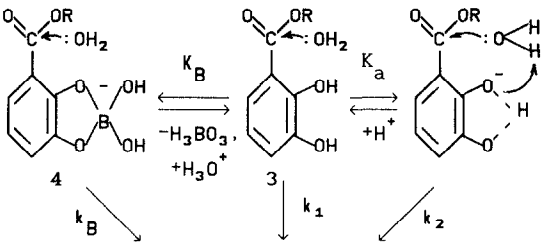


Figure 1. pH profile for the hydrolysis of 3



Scheme 1

The increase in reaction rate at pH>11 can be attributed to the alkaline hydrolysis of deprotonated 3 or, alternatively, to the reaction of doubly deprotonated 3 with water.

Boric acid effects at three pH values are shown in Fig. 2. Evidently, boric acid strongly inhibits the k_2 path, which dominates at higher pH values (Fig.2A), and only slightly effects the k_1 path (Fig.2B). The dependencies in Fig.2A obey eq. (1), which follows from Scheme 1 at fixed pH under conditions when H_3BO_3 is in a great excess over 3 and the k_1 path can be neglected.

$$k_{obs} = \frac{k_{2,app} + k_B[H_3BO_3]_t/K_I}{1 + [H_3BO_3]_t/K_I} \qquad (1)$$

In this equation $k_{2,app} = k_2K_a^S/([H^+]+K_a^S)$ and K_I is an apparent inhibition

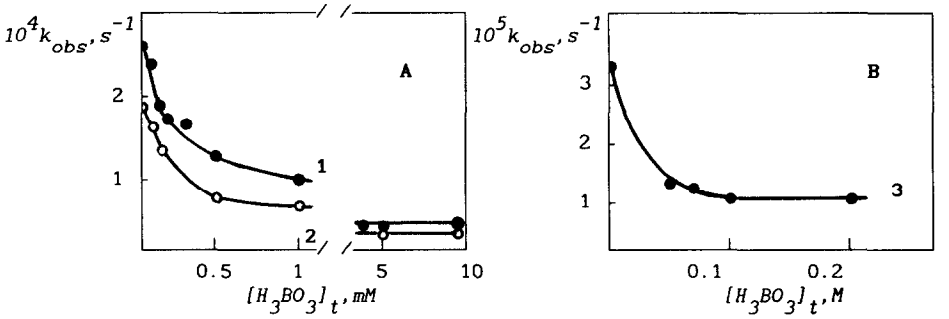


Figure 2. Dependencies of the observed rate constants for the hydrolysis of **3** on total boric acid concentration at pH 9.5 (1), 8.5 (2) and 7.0 (3).

constant defined by the following equation

$$K_I = [H^+](1 + K_a^S/[H^+])(1 + K_a^B/[H^+])/K_B \qquad (2)$$

where K_a^S and K_a^B are the acidity dissociation constants of **3** and boric acid, respectively, and K_B is the equilibrium constant (Sheme 1) defined as

$$K_B = [\mathbf{4}][H_3O^+]\backslash[\mathbf{3}][H_3BO_3] \qquad (3)$$

The rate and inhibition constants found by the fitting of the data in Fig.2A to eq. (1) are collected in Table 1 together with other relevant parameters.

Table 1. Rate and equilibrium parameters for the hydrolysis of **3** in the presence of boric and phenylboronic acids at 25°C.

k_1, s^{-1}	k_2, s^{-1}	pK_a^S	Inhibitor	K_I, M	k_B, s^{-1}	
3.3×10^{-5}	2.6×10^{-4}	8.8	H_3BO_3	2.3×10^{-4}	2.2×10^{-5}	(pH 9.5)
				3.8×10^{-4}	1.8×10^{-5}	(pH 8.5)
inhibition of α-chymotrypsin[8a]:				4.0×10^{-2}		
			$PhB(OH)_2$	2.5×10^{-4}	1.7×10^{-5}	(pH 9.5)
inhibition of α-chymotrypsin[9]:				2.0×10^{-4}		

Rate constants k_B are close to k_1 in accordance with the small boric acid effect on the neutral hydrolysis of non-protonated **3**.

Much stronger effect of boric acid on the k_2 path, as compared to that on k_1 path, shows that the inhibition results from the suppression of the general base assistance to the hydrolysis from the deprotonated *ortho*-hydroxy group. Binding of boric acid to various 1,2-dixydroxybenzenes is well documented.[16] In the case of **3** this binding affords complex **4** (Scheme 1). The respective equilibrium constant K_B can be calculated from the experimental inhibition

constants K_I with equation (2). The data in Table 1 give $K_B=(2\pm1)\times10^{-5}$.

Binding of boric acid to **3** was studied independently by UV spectrophotometry at pH<8 where the hydrolysis was sufficiently slow and $K_B=(5\pm2)\times10^{-5}$ was obtained in a reasonable agreement with the value given above.

Boric acid is a much more powerful inhibitor of the hydrolysis of **3** than of α-chymotrypsin, Table 1. The latter is inhibited more effectively by alkyl- and arylboronic acids, which can interact with the hydrophobic site of the enzyme.[8b,9-11] Therefore we have studied the effect of phenylboronic acid on the hydrolysis of **3**. As one could expect, in this case phenylboronic acid showed the same K_I as boric acid, Table 1.

Comparing the structures of complexes **2** and **4**, one can see that the second hydroxy group provides both the tighter binding of boric acid and the conformation, which excludes interaction of borate anion with the ester function. The binding of boric acid with active sites of serine proteases is also bifunctional, since boron bound to an active-site serine residue gains additional stabilization from the neighboring imidazole group.[8-13] Another common feature of model and enzyme systems is a similar pH-dependence of K_I. In enzyme systems K_I passes through a minimum, the pH-dependence being controlled by dissociation of two groups with pK_a values 6.4 and 8.9.[10] In the case of inhibition of the hydrolysis of **3**, eq. (2) predicts the same type of the pH-dependence, which is shifted to alkaline media and is controlled by pK_a^S and pK_a^B. Thus, the model system proposed in this communication mimics several essential features of boric acid effects on hydrolytic enzymes.

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