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BORINIC ACID INHIBITORS AS PROBES OF THE FACTORS INVOLVED IN BINDING AT THE ACTIVE SITES OF SUBTILISIN CARLSBERG AND α-CHYMOTRYPSIN

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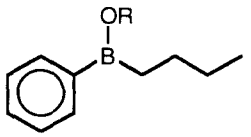
Abstract: Unsymmetrical borinic acids, with butyl, phenyl and 2-phenylethyl substituents, have been prepared and evaluated as inhibitors of the serine proteases subtilisin Carlsberg and α-chymotrypsin. These borinic acids are powerful inhibitors of each enzyme and the results provide additional information on the factors controlling binding to the active sites of such serine proteases.

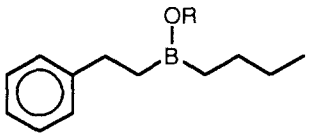
Hydrolytic enzymes are one of the most important classes of enzymes used for asymmetric synthetic applications in organic chemistry.[1] They are highly stereoselective, accept a broad variety of unnatural substrates and are easily to use synthetically since no cofactors are needed. However, before their utility in asymmetric synthesis can be optimized, it will become necessary to develop a detailed understanding of the factors determining their binding specificity, including the contributions of forces such as hydrophobic and hydrogen bonding, and electrostatic interactions.[2] One of the most direct methods for evaluating active site binding is to use reversible inhibitors, and it is this approach to probing binding specificity that has been adopted in this study. The present work is part of a program to systematically investigate the binding of selected substrates and inhibitors to synthetically useful proteases.[3] It builds on the well documented competitive inhibition of serine proteases by boronic acids, which block the active site by formation of a tetrahedral transition-state analog adduct with the catalytically active serine residue.[4,5] Our previous work on boronic acid inhibitors had provided some additional insights into the binding properties of the S_1 pocket of the serine protease subtilisin Carlsberg.[3] However, for probing the binding site regions outside the S_1 pocket, simple boronic acids are less suitable and more highly substituted boron derivatives are needed. An increase of the structural complexity of inhibitors containing boron is achieved by replacing one of the boronic acid hydroxyl groups by alkyl or aryl substituents, thus leading to borinic acids. At present, there are very few examples of borinic acid inhibitors of serine proteases,[6,7] with the inhibition of lipoprotein lipase and cholesterol esterase by n-butyl-phenylborinic acid **1** being the best documented.[7]

In this communication, the inhibition of two representative serine proteases by borinic acids **1a - 3a** are described. The enzymes selected were α-chymotrypsin, which has been intensively studied [8] and has been routinely used in amino acid resolutions,[8,9] and subtilisin Carlsberg, another well-known serine protease that has been applied synthetically.[10] Other major factors in the choice of these enzymes were the complementary data available on the competitive inhibition by structurally related boronic acids [3,11-13], and the high resolution X-ray crystal structures [14] available as bases for graphical analyses of the kinetic results.

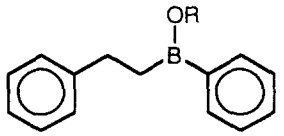
The three borinic acids selected for the initial studies were n-butyl-phenylborinic acid (**1a**),[7,15] n-butyl-(2-phenylethyl)borinic acid (**2a**) and phenyl-(2-phenylethyl)borinic acid (**3a**). Acids **1a** and **2a** were prepared from dibutyl phenylboronate and dibutyl (2-phenylethyl)boronate, respectively, with n-butyllithium in ether at -70 °C under an argon atmosphere. They were not isolated but were converted directly into the corresponding 2-aminoethyl esters **1b** and **2b** by subsequent treatment of the crude product with ethanolamine. Compound **3b**, the 2-aminoethyl ester of **3a**, was obtained by first preparing dibutyl (2-phenylethyl)boronate by hydroboration of styrene with $HBBr_2 \cdot SMe_2$ and quenching of the reaction mixture with n-butanol,[16] followed by treatment of the product with phenyllithium, and finally addition of ethanolamine as outlined above. The esters **1b - 3b** were purified by recrystallization from toluene/ether/hexane. The melting point of **1b** (104 - 105 °C) was in agreement with the literature data (104.5 - 105.5 °C [8], 108 °C [15]), as were the spectral data. Esters **2b** and **3b** showed the expected NMR, mass spectra, and elemental analyses. Free borinic acids are known to be air-sensitive and often do not crystallize.[15] Accordingly, their stable and easily purified 2-aminoethyl ester derivatives **1b - 3b** were used directly in the kinetic evaluations. The esters **1b - 3b** hydrolyze rapidly after injection into the aqueous medium [7] to give the free acid inhibitors **1a - 3a** needed.



1a (R = H)
1b (R = $CH_2CH_2NH_2$)



2a (R = H)
2b (R = $CH_2CH_2NH_2$)



3a (R = H)
3b (R = $CH_2CH_2NH_2$)

The enzyme kinetics were performed under steady-state conditions at 25°C using a pH-stat. In order to prevent any oxidation of the free borinic acids, all measurements were done under argon using solvents degassed with argon. For subtilisin Carlsberg, the substrate was N-p-tosyl-L-arginine methyl ester (TAME) and the following basic procedure was employed. After adjusting the pH to 7.0 with 0.2 M NaOH, 10 μL of the inhibitor solution (4-10 mM in CH_3CN) was added to the reaction mixture containing 0.3M TAME solution (0.4 - 8.94 mL), 1 M KCl solution (1 mL), and water to give a final volume of 10 mL. After equilibration for 2 min, the pH was adjusted to 7.8, the reaction was initiated by addition of 50 μL of enzyme solution (4.0 μM in 0.01 M phosphate buffer pH 7.8) and the uptake of 0.2 M NaOH was recorded directly into a PC. The kinetic data for α-chymotrypsin-catalyses were determined similarly, using the following stock solutions: 3.8 mM N-acetyl-L-tyrosine ethyl ester (ATEE) as substrate (0.4 - 8.94 mL), 1M KCl, inhibitor solution (0.5 -25 μM in CH_3CN). The results for α-chymotrypsin were consistent with competitive inhibition and the K_I values, calculated using the GRAFIT program, are recorded in Table 1. For subtilisin Carlsberg the curves for substrate hydrolysis in the presence of borinic acids showed a change of slope in the Lineweaver-Burk plot, a phenomenon that we do not yet understand. The K_I values of Table 1 for subtilisin

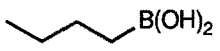
Table 1: Inhibition constants of some borinic and boronic acids

inhibitor	$K_I/\mu M$ (subtilisin Carlsberg)	$K_I/\mu M$ (α-chymotrypsin)
1a	1.1 [a]	8.1 [d]
2a	2.6 [b]	1.8 [d]
3a	1.6 [c]	0.31 [d]
4	1300 [3]	n.a.
5	100 [3]	0.2 [11]
6	270 [3]	0.04 [12]

[a] [TAME] = 0.12 - 0.27 M, [b] [TAME] = 0.09 - 0.27 M, [c] [TAME] = 0.12 - 0.27 M , [d] [ATEE] = 0.15 - 3.39 mM, n.a. = not available. All determinations at least in duplicate with standard deviations of 4 - 6%.

Carlsberg were calculated from the higher ranges of substrate concentrations, whose values are given in the Table, within which curves characteristic for competitive inhibition were observed.

The Table 1 data show that all the borinic acids are excellent inhibitors of both enzymes. For α-chymotrypsin, a comparison of the K_I values of **1a** and **2a** demonstrates that the presence of a 2-phenylethyl residue is more favorable to good binding than that of a smaller phenyl group. This is consistent with the results for the corresponding boronic acid inhibitors **5** and **6** and is rationalized by the preference of the S_1 binding pocket for the longer aromatic sidechains. In addition, replacement of the butyl group of **2a** by phenyl to give **3a** improves the borinic acid binding significantly, presumably as a result of the increased electrophilicity of the boron due to the replacement of an aliphatic substituent by an aromatic group. This facilitates the attack of the boron by the hydroxyl group of the active site serine 195.



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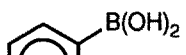

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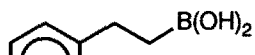

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However, all borinic acids are worse inhibitors of α-chymotrypsin than the corresponding boronic acids by an order of magnitude, showing that the geometry and dimensions of the key binding regions of the active site of α-chymotrypsin are such that introductions of additional groups at the boron atom are poorly tolerated. In contrast, for subtilisin Carlsberg, which has a more open S_1 pocket than α-chymotrypsin, all the borinic acids are approximately two orders of magnitude more effective as inhibitors than the corresponding boronic acids. A further difference between the two enzymes is that **1a** is a slightly better inhibitor for subtilisin Carlsberg than **2a**. This leads to the conclusion that the increased electrophilicity of the boron in the alkyl-arylborinic acid **1a** over that in

the dialkylborinic acid **2a** is a factor that helps to override any preference of the S_1 binding pocket of subtilisin Carlsberg for the larger 2-phenylethyl group of **2a**. However, the ratio of K_I values for **1a** and **2a** is almost identical to that observed for phenylboronic acid (**5**) and (2-phenylethyl)boronic acid (**6**), indicating that similar effects determine the relative binding affinities of both borinic and boronic acids. The better inhibition of subtilisin Carlsberg by **3a** compared to **2a** is also attributable to the higher electrophilicity of the boron atom in **3a**.

The present data do not permit unequivocal conclusions to be drawn regarding the orientations of the borinic acids in their EI-complexes, and further investigations on this aspect are in progress.

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