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# DIPHENYLBORINIC ACID IS A STRONG INHIBITOR OF SERINE PROTEASES≠

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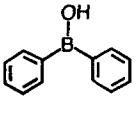
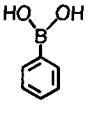
**Abstract.** Diphenylborinic acid, a commercially available and reasonably air stable compound, was found to be a strong competitive inhibitor of three serine proteases. Compared to phenylboronic acid, it was a thirty-fold better inhibitor of α-chymotrypsin, a fifteen-fold better inhibitor of subtilisin BPN', and a sixty-fold better inhibitor of bovine trypsin. The $pK_a$ and inhibitory ability of methylphenylborinic acid was also determined.

Boronic acids have been studied as competitive inhibitors of serine proteases for more than twenty-five years.[1] Nonetheless, interest in these compounds remains high due to their potential clinical uses,[2] and their ability to act as structural probes of enzyme binding sites.[3] Despite numerous X-ray and NMR studies, some of the details concerning the structures of the enzyme/inhibitor complexes remain controversial, particularly when the inhibitors are simple, "non substrate-like" boronic acids.[4] In some cases there is clear evidence for a covalent tetrahedral adduct with the active-site serine hydroxyl.[4,5] In other cases there is no doubt that the boron is coordinated to the active-site histidine.[6]

Our interest in this area stems from our recent efforts to develop molecular transport devices using boron acids.[7] While conducting experiments with diphenylborinic acid, **1**, we became curious about its ability to inhibit serine proteases. Inhibition with asymmetric borinic acids has been reported before,[8] the most recent study by the Jones research group.[9] In general, borinic acids are better inhibitors than boronic acids. The major detraction with borinic acids is their susceptibility to air oxidation. Diarylborinic acids, however, are reasonably air stable compounds. For example, a solution of **1** in phosphate buffer, at pH 7.4, was found to be > 90 % pure after standing on the bench top for 24 hours. Compound **1** has a $pK_a$ of 6.2.[10] At neutral pH it readily combines with vicinal diols to form anionic, tetrahedral "ate" complexes.[7] The expected inhibitory ability of **1** was hard to predict, *a priori,* since it was difficult to estimate the relative importance of various opposing factors such as increased acidity, enzyme binding site specificity, inhibitor hydrophobicity, loss of a potential active-site hydrogen, etc. We felt that if **1** were a good protease binder then it may have utility in clarifying some of the structural and mechanistic ambiguities concerning this class of transition-state-analogue inhibitors.




**1**

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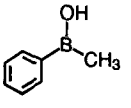


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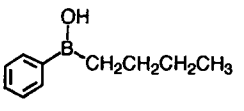



**4**

Inhibition studies were carried out at 22 °C in sodium phosphate solution buffered at pH 7.4. Due to their instability, borinic acids are usually synthesized and stored as their aminoethanol esters. Previous kinetic studies with the esters of alkylborinic acids used the esters directly, since they were found to be rapidly hydrolyzed to the acids.[8,9] The aminoethanol ester of **1**, however, is reasonably stable at neutral pH.[11] To avoid any ambiguity due to slow ester hydrolysis, the aminoethanol group was removed beforehand by acid extraction,[11] and the free acid used in the experiments. Enzyme activity was monitored spectrophotometrically with the following standard substrates, N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide, 0.015 - 0.075 mM, (α-chymotrypsin, 56 nM); 4-nitrophenylbutyrate, 0.015 - 0.12 mM (subtilisin BPN', 250 nM); and N-benzoyl-DL-arginine-4-nitroanilide, 0.30 - 0.60 mM, (bovine trypsin, 1.23 μM). Inhibition constants were determined from Lineweaver-Burk plots which were consistent with competitive inhibition.

**Table 1.** Inhibition Constants (dissociation) and $pK_a$'s.[a]

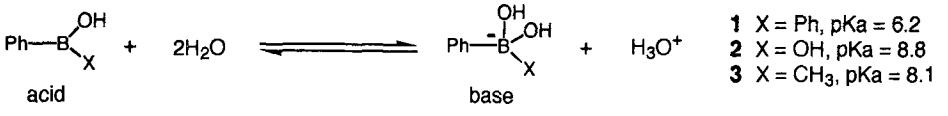
| | | $K_i$ /μM ± 10% (lit.) | | |
|---|---|---|---|---|
| Inhibitor | $pK_a$ ± 0.1 (lit.) | Chymotrypsin | Subtilisin | Trypsin |
| **1** | 6.1 (6.2)[b] | 20 | 30 | 170 |
| **2** | 8.8 (8.85)[c] | 640 (200)[d] | 470 (230)[d] | 10200 |
| **3** | 8.1 | 30 | - | - |
| **4** | - | (8)[e] | (1)[e] | |

[a]All experimental measurements are the average of at least two independent determinations. [b]Reference 10. [c]Reference 7. [d]Reference 12. [e]Reference 9.

In the event, **1** was found to be a strong competitive inhibitor of the three serine proteases examined. Compared to phenylboronic acid, **2**, it was a thirty-fold better inhibitor of chymotrypsin, a fifteen-fold better inhibitor of subtilisin, and a sixty-fold better inhibitor of trypsin (Table 1). As a way of calibrating our results with those of Jones, we also examined methylphenylborinic acid, **3**. This compound was synthesized according to the method of Brown.[13] It was found to be moderately air sensitive which made inhibition studies problematic. Our pragmatic procedure involved synthesizing **3** as its aminoethanol ester, and deprotecting a fresh sample just before use.[11] Evaluation of its inhibition of chymotrypsin produced a $K_i$ of 30 μM which correlates reasonably well with the $K_i$ of 8 μM reported for the structurally related butylphenylborinic acid **4** (Table 1). The sub-millimolar inhibition of trypsin by **1** is a notable result as the enzyme has a strong preference for an arginyl residue at its $S_1$ specificty site, and therefore is poorly inhibited by arylboronic acids. There is much interest in trypsin-like enzymes as they play important roles in regulatory systems such as blood coagulation and fibrinolysis.[2] Compound **1** should be a useful, and readily available, transition-state probe for NMR and X-ray studies of these enzymes.[6] With regard to NMR studies, a salient point is that outside the extreme-narrowing range, $^{11}B$ NMR signals have the unusual feature of becoming narrower as the molecular correlation time and magnetic field strength increase.[14]

The increased inhibition exhibited by borinic acids has been attributed, in part, to the increased electrophilicity of the boron center,[9] although to our knowledge this has never been strictly proved. To confirm this reasoning we determined the $pK_a$'s for **1**, **2**, and **3**, to be 6.1, 8.8 and 8.1, respectively (Table 1).[15] Thus, the $pK_a$'s reflected, qualitatively, the order of $K_i$'s. There are, of course, other factors that influence enzyme-inhibitor binding such as solvation changes, electrostatics, hydrogen bonding, and steric effects. For the subtilisin-boronic acid system, in particular, these factors have been examined in detail and will not be discussed

here.[3] The lower $pK_a$ of **3** compared to **2** is somewhat counter-intuitive if stability of the conjugate base is the only criterion considered; a methyl group is not expected to stabilize an adjacent negative charge better than a hydroxyl group. A reasonable explanation is provided by examining both sides of the acid-base equilibrium for **3** and **2**. It appears that replacing the methyl group in **3** with a hydroxyl results in stabilization of the acid more than the conjugate base.




The subtilisin inhibition ability of **1** was examined as a function of pH. Previous studies on subtilisin inhibition by boronic acids produced bell-shaped curves with an invariant $pK_1$ of about 7, and a $pK_2$ that depended on the boronic acid (usually greater than 8).[12,16] Since $pK_1$ corresponded to the $pK_a$ of the active-site imidazole and $pK_2$ matched the boronic acid $pK_a$, the profile was rationalized, mechanistically, in terms of a neutral boronic acid binding with an alkaline enzyme active-site. The plot of $1/K_i$ versus pH for subtilisin inhibition by **1** is shown in Figure 1. Although the curve is transposed to lower pH values, with $K_{i(opt)}$ at pH 6.8, the profile is still consistent with the above binding mechanism.[17,18] In this case, the pKa of inhibitor **1** is lower than the pKa of the enzyme active-site, *i.e.*, $pK_2 < pK_1$.[19] The Alberty-Massey equation was used to calculate pK values of 6.2 and 7.4.[18]

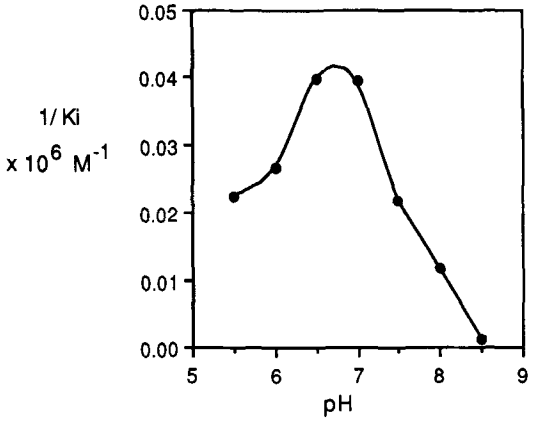



**Figure 1.** pH profile for subtilisin inhibition by **1**.

In conclusion, diphenylborinic acid, **1**, proved to be a strong competitive inhibitor of the three serine proteases examined. The commercial availability and increased stability of this compound makes it an attractive probe for X-ray and NMR studies of enzyme/inhibitor complexes.

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≠Molecular recognition with boron acids, part 8. For part 7, see: Paugam, M. -F.; Valencia, L. S.; Smith, B. D. *J. Am. Chem. Soc.*, submitted.

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