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Noncovalent inhibition of the serine proteases, α-chymotrypsin and trypsin by trifluoro(organo)borates

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A series of potassium organotrifluoroborates were synthesized. Their stability to hydrolysis was determined in D_2O, TRIS and phosphate buffer. It was found that in both D_2O and TRIS buffers, these compounds are quite stable, whereas in phosphate buffer rapid hydrolysis occurs. Based on these results, a study was undertaken to determine whether potassium organotrifluoroborates can serve as protease inhibitors. It was found that potassium organotrifluoroborates increased inhibition by at least an order of magnitude over the corresponding boronates. Dixon plots showed that these compounds are reversible competitive inhibitors of α-chymotrypsin and trypsin. Based on ^{19}F NMR, we speculate that they inactivate the enzymes as a result of the formation of hydrogen-bonds between fluorine atoms of the inhibitors and the serine protease.

Introduction

Serine proteases have been shown to play a multifarious role in health and disease. As a result, there has been considerable interest in the design and development of synthetic inhibitors of these enzymes.[1]

Inhibitors of serine proteases divide naturally into two classes: covalent and noncovalent. Covalent inhibitors bind covalently to the active site of the enzyme (typically to the nucleophilic serine hydroxyl of the catalytic triad). The resulting tetrahedral intermediate resembles the transition state during a normal substrate hydrolysis. Such inhibitors usually contain electrophiles such as aldehydes, trifluoromethyl ketones, pentafluoroethyl ketones, α-ketoamides, α-ketoesters, α-diketones, α-ketoheterocycles, organoboronic acids and organophosphonate esters. All of these inhibitors interact with the protease covalently and reversibly.[1–4] Irreversible covalent inhibitors include a variety of acylating and alkylating agents such as β-lactams and mono-halomethyl ketones.[1,3,5] It is very important that covalent inhibitors such as these are highly selective, since nonselectivity may result in toxic side effects.[2,5] For this reason, non-covalent inhibitors are often preferred over covalent inhibitors and are commonly studied as potential protease inhibitors.[6] These may be more selective and both chemically and metabolically less reactive than covalent inhibitors. The binding of non-covalent inhibitors relies on hydrogen bonds, electrostatic interactions, hydrophobic interactions and/or Van der Waal interactions *etc*. Thrombin, factor Xa (fXa) and trypsin are the only serine proteases for which small, potent, selective noncovalent inhibitors have been developed which are ultimately intended as drug development candidates.[7–10] These inhibitors are structural mimics of peptide substrates, maintaining a hydrogen bond array to the enzyme as well as complementing hydrophobic surfaces in the active site. In addition, aryl derivatives of benzamidine form intimate indirect contacts with the active site of trypsin. The inhibitors are linked to the enzyme by a sulfate ion that forms an indirect network of three-centered hydrogen bonds.[11]

Peptide boronic acids are exceptionally potent transition state analogue[12] inhibitors of serine proteases. Bachovchin and coworkers found that when a boronic acid derivative is a good analogue of the substrate, formation of a tetrahedral complex with the active-site serine is favored, while boronic acids with structures not well related to that of the substrate tend to coordinate with both histidine and serine.[13,14]

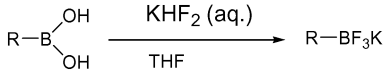
Substitution of H or OH by F in a biologically important molecule can have substantial effects because a new set of chemical interactions are introduced. Several studies have shown that the incorporation of fluorine atoms into serine protease inhibitors enhances their activity as a result of the formation of hydrogen bonds between the fluorine atoms and the serine protease.[15] In addition, difluoroborane analogues of several 1-acylaminoboronic acids are found to be stable and more easily purified than the acids. Since they hydrolyze readily in aqueous buffer, they are convenient precursor forms for boronic acid amino acid analogue type transition-state inhibitors.[16]

In this article, we examine trifluoro(organo)borate salts as inhibitors of trypsin and α-chymotrypsin. Their potential activity could be advantageous since these salts are much more stable than the acids and are much easier to handle and purify.[18] The molecules that were used are simple compounds and, hence, their activity was moderate. In order to observe slow, tight binding characteristics however, interactions between inhibitor and enzyme secondary subsites are necessary.

Results and discussion

Synthesis and stability

Vedejs *et al.* showed that hydroxyl ligands[17] of arylboronic acids could be displaced with potassium hydrogen difluoride (KHF_2), thereby leading to potassium aryltrifluoroborates (Scheme 1). Pure compounds were obtained by extraction of the resultant solid with acetone so that all the excess KHF_2 and the other salts (HBF_4, KF *etc.*) are removed (Fig. 1). The potassium organotrifluoroborates do not show sensitivity towards oxygen or moisture and are therefore far more stable than the corresponding organoboronic acids or esters.[18] Also, unlike trivalent boron substituents, trifluoroborate is an electron-donating substituent.[19] Until now the only utility of



Scheme 1 Formation of potassium organotrifluoroborates.

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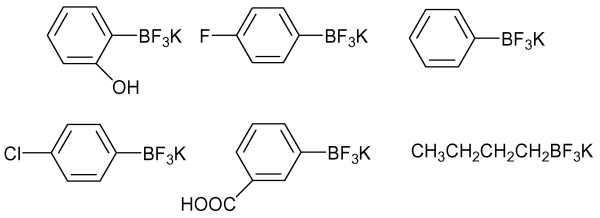

Fig. 1 Potassium organotrifluoroborates.

potassium organotrifluoroborates has been in the field of organic synthesis.[20,21]

Since the enzymatic study was carried out in an aqueous environment, it was necessary to determine the stability of the salts in this medium. Using ^{1}H, ^{11}B and ^{19}F NMR analysis, the potassium organotrifluoroborates in D_2O did not hydrolyze after incubation for 30 minutes. After 24 hours however, partial hydrolysis took place ranging from 10–30%. The aryl derivatives were more stable than the butyl salt, which displayed 30% hydrolysis. In TRIS buffer (in D_2O, pH = 7.00), ^{1}H and ^{11}B NMR indicated the presence of the salts only, except for the butyl salt which showed about 50% hydrolysis. Upon incubation of the salts (6.02 mM) with α-chymotrypsin (0.02 mg ml^{-1}) in 0.05 M TRIS buffer (in D_2O, pH = 7.00) for 30 minutes, ^{11}B NMR showed only a very small degree of hydrolysis for the aryl salts, ranging from 2–10% depending on the functional group on the aromatic ring, and about 50% hydrolysis for the butyl derivative. The ^{11}B NMR peaks observed for the salts were broader than for the salts alone in DMSO. On standing for 48 hours in the buffer and enzyme, the ^{11}B NMR of all the samples indicated a complete hydrolysis of the salts to the acids.

Enzyme-inhibition studies

For this study, α-chymotrypsin and trypsin from bovine pancreas were selected as representative serine proteases. Both these enzymes have a very strong structural similarity and use the same mechanism for cleavage, a mechanism that very much resembles the mechanism for the acid-catalyzed hydrolysis of an amide.

The inhibitory activity of the trifluoro(organo)borates was evaluated by determining the dissociation constant (K_i) using Dixon plots.[22] These plots show that the inhibition is competitive for all the compounds. A Dixon plot of one of the best inhibitors (potassium trifluorophenylborate) with chymotrypsin is shown in Fig. 2. In addition, the values of the inhibition constants (K_i) are listed in Table 1 and Table 2 for α-chymotrypsin and trypsin, respectively. As can be seen from these values, all the trifluoroborate salts are much more potent than the corresponding acids. For trypsin, the difference in activity between the trifluoroborate salts and the boronic acids ranges from a 12–32 fold increase in inhibitory activity, while for

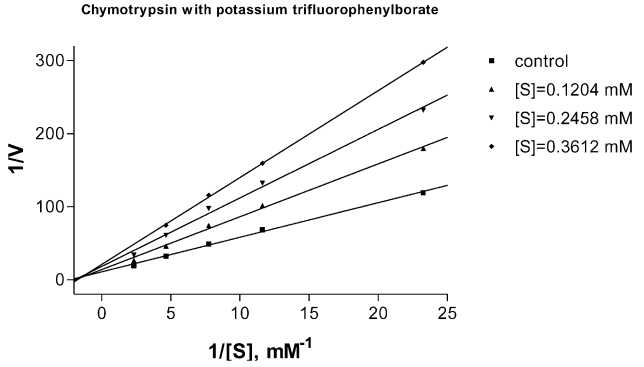

Fig. 2 Lineweaver–Burk plot for the inhibition of chymotrypsin with and without potassium trifluorophenylborate. Reactions were initiated by adding the substrate benzoyl-tyrosine-*p*-nitroanilide to a solution of chymotrypsin and inhibitor.

Table 1 Competitive inhibition of α-chymotrypsin by boronic acids and their salts

Compound	K_i/mM	
	X = $(OH)_2$	X = F_3K
PhBX	9.70	0.20
4-F-PhBX	>10.0	1.84
4-Cl-PhBX	0.65	0.57
2-OH-PHBX	0.61	0.14
3-HOOC-PhBX	1.42	0.69
$CH_3CH_2CH_2CH_2BX$	>10.0	2.84
KF (control)	>10.0	
KBF_4 (control)	>10.0	

Table 2 Competitive inhibition of trypsin by boronic acids and their salts

Compound	K_i/mM	
	X = $(OH)_2$	X = F_3K
PhBX	>10.0	1.11
4-F-PhBX	>10.0	0.29
4-Cl-PhBX	>10.0	1.50
2-OH-PHBX	>10.0	1.06
3-HOOC-PhBX	2.87	0.26
$CH_3CH_2CH_2CH_2BX$	>10.0	0.62
KF (control)	>10.0	
KBF_4 (control)	>10.0	

chymotrypsin there is a 2–47 fold increase. Both, potassium fluoride and potassium tetrafluoroborate were used as controls and showed no activity/very weak activity (Table 1 and Table 2).

When phosphate buffer was used for the enzymatic assay, the trifluoroborate salts showed the same activity as the free boronic acids, whereas in the TRIS buffer, the salts were much more potent than the free acids. In phosphate buffer, there was complete hydrolysis of the salts as evidenced by ^{1}H and ^{19}F NMR, whereas in TRIS buffer, hydrolysis increased with time, *i.e.* it took 48 h for complete hydrolysis. As observed by ^{19}F NMR, a sample containing 6.02 mM of inhibitor (d_6-DMSO) and 0.044 mM of phosphate buffer (D_2O, pH 7.00) was completely hydrolyzed to give the acid and KF immediately.

In TRIS buffer, the fluorine atoms hydrogen bond with the OH and NH of the TRIS, thus forming O–H · · · F and N–H · · · F hydrogen bonds, which may stabilize the salt in the buffer. These bonds are in turn displaced by other hydrogen bonds in the serine protease. In contrast, no hydrogen bonding occurs in the phosphate buffer and the salt is hydrolyzing, therefore possessing an inhibitory property the same as that of the acids.

Phenyl potassium trifluoroborate decomposes to phenylboronic acid in the presence of Li or Mg *via* dissociation into phenyl difluoroborane and LiF or MgF_2, followed by hydrolysis.[17] Since the TRIS buffer contains $CaCl_2$ there was the possibility of the formation of CaF_2 and phenyl difluoroborane, followed by hydrolysis. Therefore, a TRIS buffer was prepared without $CaCl_2$ (pH = 7.00). The inhibitory properties using this buffer were the same as those observed previously, indicating that no formation of phenyl difluoroborane due to the presence of $CaCl_2$ takes place. Therefore, the compound remains as the trifluoroborate salt in the buffer.

Mode of binding

Until now, it was accepted that only boronic acids inhibit serine proteases and trifluoroborates have never been tested biologically for this purpose. Owing to the high electron density, the trifluoroborate group is capable of participating in hydrogen bonding as an electron donor. This property confers additional mechanisms of interaction with enzyme or receptor subsites

for trifluoropotassium borates, which cannot be found for the fluorine free compound. The different proteolytic stabilities of the compounds seem to result from a specific interaction between the substrate and the enzyme induced by the high electron density of the BF_3K group.

^{19}F NMR studies on enzyme–inhibitor complex

Fluorine NMR is a useful tool for investigating enzyme–inhibitor interactions owing to its high sensitivity and lack of background interference from protein.[23,24] The high sensitivity of the fluorine chemical shift to their local environment can be used to probe details of a receptor–ligand complex. In order to investigate the structure of the enzyme–inhibitor complex of a sample compound (trifluoro-4-fluorophenylboronate), we determined the ^{19}F NMR spectra of inhibitor alone and of inhibitor and the enzyme. In the former case, two signals were observed at −136.366 and −114.189 ppm for the $-BF_3K$ group and for the aromatic fluorine, respectively. Upon addition of α-chymotrypsin/trypsin to the sample, two new broad signals appeared at −133.435 and −107.803 ppm for the $-BF_3K$ and the aromatic fluorine respectively (Fig. 3). These two new peaks were downfield shifted from the original peaks. The peak at −133.435 is consistent with the formation of an enzyme-bound complex derived from the hydrogen-bond formation between the fluorine atoms and the serine protease. On the other hand, the −107.803 ppm signal is assigned to other noncovalent enzyme-bound aromatic fluorine atoms with the hydrophobic pocket. As can be seen in Fig. 3, the broad signals for the bound-inhibitor are in relatively rapid equilibrium with the free inhibitor. The increased line widths for the signals of the enzyme-bound species are consistent with other ^{19}F NMR studies on chymotrypsin bound inhibitors and are attributed to the slower tumbling of the macromolecular complex.[25,26] Saika and Slichter[27] have developed a quantitative theory for the ^{19}F chemical shifts and these shifts have been related[28] to changes in ionic character, bond order and degree of hybridization of the bond involving the fluorine atom. In general, an increase in electronegativity of the atom attached to fluorine decreases the ionic character of the fluorine, which thereby increases the paramagnetic contribution to shielding, *i.e.* causes a downfield shift. Hence, the downfield shift seen for the fluorine atoms of the salt arise from the formation of hydrogen bond between two fluorine atoms and the N–H and O–H groups in the active site. The amide N–H groups that constitute the oxyanion hole are believed to contribute to catalysis by stabilizing the tetrahedral substrate oxyanion formed during catalysis. The third fluorine atom is hydrogen bonded to the N–H in the oxyanion hole (Fig. 4).

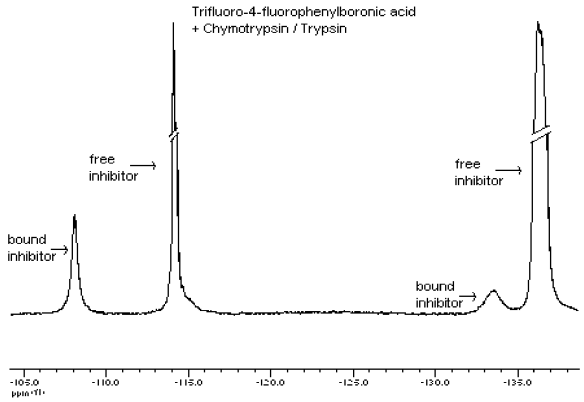

Fig. 3 ^{19}F NMR (282 MHz) spectrum of α-chymotrypsin and trifluoro-4-fluorophenyl-boronate (0.602 M inhibitor, 0.08 mM enzyme in 0.1 M KCl with 10% D_2O).

The downfield shift of the ^{19}F signal of an enzyme-bound species is consistent with other studies. The ^{19}F signals of various *p*-fluorophenylalanine inhibitors are shifted 1–2 ppm downfield upon binding to chymotrypsin.[29,30] Current ^{19}F chemical shift theory attributes these downfield shifts to Van der Waal interactions with protein protons and thus to binding of the *p*-fluorophenyl ring into the aromatic ring pocket. Gorenstein and Shah[31] observed ~4 ppm downfield shift for *N*-acetyl-*DL*-*p*-fluorophenylalinal and this is one of the largest protein-induced shifts observed for a reversibly bound inhibitor to chymotrypsin.

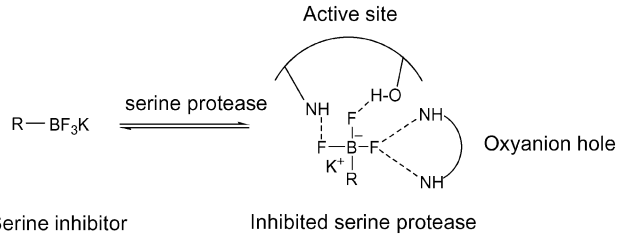

Fig. 4 A trifluoroborate inhibitor forming hydrogen bonding with α-chymotrypsin which is subsequently inhibited from further activity.

Conclusion

The potassium trifluoro(organo)borates show high stability toward air and water, which is not the case for the vast majority of other organoboron compounds. The compounds were found to be simple noncovalent, competitive and reversible inhibitors of the serine proteases α-chymotrypsin and trypsin. If the postulated hydrogen bonding interactions are formed in the active-site of the target enzyme and are responsible for the inactivation, our concept would be useful for further development of selective noncovalent type inhibitors. The potency and/or selectivity can be improved by taking advantage of binding interactions at the S subsites of the protease. This is the first example of protease inhibition by $KRBF_3$ compounds. While more potent inhibitors are known, our intent was to show a concept and not to optimize inhibition at this stage.

Experimental

General procedures

^{1}H, ^{13}C, ^{11}B and ^{19}F NMR were recorded on a Varian 300 MHz instrument at frequencies of 300, 75.9, 96.29 and 282.23 MHz, respectively. Shift values were reported with respect to TMS (^{1}H and ^{13}C), $BF_3{\cdot}OEt_2$ (^{11}B) and CCl_3F (^{19}F). Coupling constants are reported in Hertz and refer to apparent peak multiplicities. Biological assays were performed on a $UVIKON_{xs}$-BIO-TEK instrument. All chemicals and enzymes were purchased from Sigma-Aldrich. Solvents were purchased as analytical reagent grade.

General procedure for preparation of trifluoroborate salts

The boronic acid (1 mmol) dissolved in 4 ml of THF was treated with excess aqueous KHF_2 (6 mmol) and allowed to stir overnight at room temperature. The THF solvent was removed under a reduced pressure using a rotary evaporator and water was removed by lyophilization. The resultant solid was extracted with acetone, the combined acetone fractions concentrated under a reduced pressure and the residue washed with ether to obtain trifluoroborate salt.

Potassium butyltrifluoroborate. Yield 90%. (Found: C, 29.01; H, 5.42%. Calc. for $C_4H_9BF_3K$: C, 29.29; H, 5.53%); δ_H(300 MHz; d_6-DMSO) 0.08 (2 H, t, *J* 7.6, CH_2B), 0.77 (3 H, t, *J* 7.05, CH_3), 1.12 (4 H, m, CH_2CH_2); δ_C(75.9 MHz; d_6-DMSO) 15.07, 26.67, 28.81; δ_F(282.23 MHz; d_6-DMSO) −137.23; δ_B(96.29 MHz; d_6-DMSO) 4.09.

Potassium 4-chlorophenyltrifluoroborate. Yield 86%. (Found: C, 32.60; H, 5.21%. Calc. for $C_6H_4BClF_3K$: C, 32.99; H,

1.85%); δ_H (300 MHz; d_6-DMSO) 7.08 (2 H, d, *J* 8.1, Ph), 7.29 (2 H, d, *J* 8.1, Ph); δ_C(75.9 MHz; d_6-DMSO) 126.79, 133.81; δ_F(282.23 MHz; d_6-DMSO) −139.69; δ_B(96.29 MHz; d_6-DMSO) 2.41.

Potassium 3-carboxyphenyltrifluoroborate. Yield 84%. (Found: C, 36.55; H, 2.06%. Calc. for $C_7H_5BF_3KO_2$: C, 36.87; H, 2.21%); δ_H(300 MHz; d_6-DMSO) 7.17 (1 H, t, *J* 7.35, Ph), 7.50 (1 H, d, *J* 7.2, Ph), 7.61 (1 H, d, *J* 7.8, Ph), 7.94 (1 H, s, Ph); δ_C(75.9 MHz; d_6-DMSO) 126.96, 129.24, 133.26, 136.55, 169.34; δ_F(282.23 MHz; d_6-DMSO) −139.97; δ_B(96.29 MHz; d_6-DMSO) 6.34.

Potassium 4-fluorophenyltrifluoroborate. Yield 96%. (Found: C, 35.33; H, 1.90%. Calc. for $C_6H_4BF_4K$: C, 35.68; H, 2.00%); δ_H(300 MHz; d_6-DMSO) 6.84 (2 H, t, *J* 8.55, Ph), 7.29 (2 H, t, *J* 6.9, Ph); δ_C(75.9 MHz; d_6-DMSO) 113.22, 113.46, 133.43, 133.53; δ_F(282.23 MHz; d_6-DMSO) −139.26, −119.07; δ_B(96.29 MHz; d_6-DMSO) 4.13.

Potassium 2-hydroxyphenyltrifluoroborate. Yield 88%. (Found: C, 35.93; H, 2.49%. Calc. for $C_6H_5BF_3O$: C, 36.03; H, 2.52%); δ_H(300 MHz; d_6-DMSO) 6.48 (1 H, d, *J* 8.1, Ph), 6.60 (1 H, t, *J* 6.6, Ph), 6.91 (1 H, t, *J* 7.65, Ph), 7.11 (Ph, d, *J* 6.6, Ph), 7.32 (1H, bs, OH); δ_C(75.9 MHz; d_6-DMSO) 114.02, 119.03, 127.70, 133.59, 160.01; δ_F(282.23 MHz; d_6-DMSO) −134.38; δ_B(96.29 MHz; d_6-DMSO) 6.38.

Potassium phenyltrifluoroborate. Yield 90%. (Found: C, 39.02; H, 2.62%. Calc. for $C_6H_5BF_3K$: C, 39.16; H, 2.74%). δ_H(300 MHz; d_6-DMSO) 7.01 (3 H, m, Ph), 7.30 (2 H, d, *J* 6.6, Ph); δ_C(75.9 MHz; d_6-DMSO) 125.65, 126.92, 131.95; δ_F(282.23 MHz; d_6-DMSO) −139.53; δ_B(96.29 MHz; d_6-DMSO) 3.08.

Fluorine NMR spectra

NMR samples were prepared by dissolving lyophilized enzyme in 900 µl of 0.1 M KCl with about 10% D_2O added to provide the field frequency lock signal. Enzyme concentration was typically about 0.08 mM. Inhibitor boronic salt was dissolved in DMSO (100 µl) and added directly to the NMR sample. The amount of the inhibitor added was 0.602 M, enough to saturate the enzyme.

Biochemical assays

Determination of inhibition constants (K_i) to chymotrypsin. α-Chymotrypsin Type II from bovine pancreas was purchased from Sigma-Aldrich (MW 25 000). The inhibitors were tested by competitive assay against benzoyl-tyrosine-*p*-nitroanilide as a substrate. The hydrolytic reaction was carried out at 37 °C at pH = 7.00, with 50 mM TRIS buffer and 0.02 M $CaCl_2$. Inhibitor stock solutions in DMSO were prepared with concentrations ranging from 6.02 mM to 0.0602 mM. A stock enzyme solution of 0.2 mg ml^{-1} was made in 1 mM HCl solution. A series of substrate concentrations (dissolved in 36.6% DMSO and 63.4% methanol) ranging from 0.043 mM to 0.43 mM were prepared. The assay was performed by adding 700 µl TRIS buffer to a 1.5 ml Eppendorf, followed by 100 µl inhibitor and 100 µl enzyme. The mixture was incubated for 10 minutes at 37 °C. Then, 100 µl of substrate was added to the reaction followed by incubation for 20 minutes at 37 °C. The reaction was stopped by adding 200 µl of 30% acetic acid solution. The progress of the reaction was followed by monitoring the appearance of the absorption band of *p*-nitroaniline at 410 nm. K_i Values for reversible competitive inhibitors were estimated by the method of Lineweaver and Burk. Data were fitted to the best straight line by the least-squares procedure.

Assay of inhibitors of trypsin. Trypsin Type IIs from bovine pancreas was purchased from Sigma-Aldrich. The inhibition constants were determined exactly as was done for chymotrypsin, except that the substrate was benzoyl-arginine-*p*-nitroanilide dissolved in DMF.

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