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Synthesis and Kinetic Studies of an Amidine-containing Phosphonofluoridate: a Novel Potent Inhibitor of Trypsin-like Enzymes

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Abstract—The amidine-containing α-aminoalkylphosphonofluoridate **3** (Cbz-(4-AmPhGly)^{P}(OPh)(F)) is a very potent inhibitor of trypsin-like enzymes. It was prepared by hydrolyzing the corresponding phosphonate diphenyl ester **4** followed by reaction of fluoride with the phosphonochloridate prepared from the intermediate phosphonic acid monoester **5**. Compound **3** is the most potent amidine-containing organophosphorus inhibitor yet reported for trypsin-like enzymes. It inhibits trypsin and thrombin with second-order rate constants (k_{obs}/[I]) of $2.6\times10^5\,M^{-1}\,s^{-1}$ and $1.0\times10^5\,M^{-1}\,s^{-1}$, respectively, showing a 130-fold and a 1250-fold rate enhancement over the corresponding diphenyl ester (**4**). It also inactivates trypsin 2 orders of magnitude more potently than simple phosphonofluoridates such as DFP,[1] Sarin and Soman. The phosphonofluoridate **3** does not inhibit other serine proteases such as porcine pancreatic elastase (PPE) and the esterase acetylcholinesterase (AChE). The phosphonofluoridate **3** is hydrolyzed rapidly in buffer solution and has a $t_{1/2}$ of 4.5 s at pH 7.5.

Introduction

Organophosphonofluoridates or phosphonyl fluorides have been known to be very potent inhibitors for serine proteases and esterases for over 80 years. Classic phosphonofluoridates including diisopropyl phosphonofluoridate (DFP), isopropylmethyl phosphonofluoridate (Sarin), 1,2,2-trimethylpropylmethyl phosphonofluoridate (Soman) are extremely toxic and are considered to be general serine protease inhibitors with poor selectivity toward individual enzyme.[2] The inhibitors phosphorylate the active site serine hydroxy group of serine proteases and esterases due to the electrophilicity of the phosphorus atom induced by the adjacent fluorine atom. Phosphonofluoridates such as **1** and **2**, which resemble peptide substrates, are more potent inhibitors for chymotrypsin and elastase than DFP.[3,4] Unlike simple organophosphonofluoridates, the amino acid-derived phosphonofluoridates possess substrate recognition features and exhibit very good selectivity toward individual target serine proteases.[3,4]

Diphenyl ester derivatives of α-aminoalkyl phosphonates esters and extended peptide derivatives have also been shown to irreversibly phosphorylate the active site Ser-195 of serine proteases. Phosphonate esters with alanine and valine-like P1 residues are effective inhibitors of porcine pancreatic elastase and human neutrophil elastase;[3,5,6] those with ornithine, lysine, homolysine[7,8] and arginine-like[9] P1 residues are inhibitors of trypsin-like enzymes; those with phenylalanine-like P1 residues are inhibitors of chymotrypsin-like enzymes.[3–6] Other reported α-aminoalkyl phosphonate diphenyl ester derivatives include side chain 4-amidinophenyl,[10] 4-methoxybutyl and the *n*-hexyl groups[11] at the P1 site. The various P1 side chain functional groups enhance the selectivity of the phosphonate esters toward a variety of target serine proteases. Extended peptidyl phosphonates where a tetrahedral phosphorus center replaces the scissile peptide bond of a substrate are transition analogue inhibitors for serine proteases, metalloproteases, aspartic proteases, and D-Ala-D-Ala ligase, and mimic

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the tetrahedral intermediate formed during normal substrate hydrolysis.[12–19]

In a continuing effort to develop inhibitors for blood coagulation enzymes, we reported a series of amidine-containing phosphonate diphenyl and mixed esters specific for trypsin-like enzymes.[10] Some of these compounds were very potent and specific serine protease inhibitors, but were ineffective in clotting assays due to their moderate inhibition rates. This inspired us to synthesize an amidine-containing phosphonofluoridate, which has a better leaving group, with the hope that it would lead to effective anticoagulants. In this paper, we report the synthesis and the kinetic evaluation of the phosphonofluoridate Cbz-(4-AmPhGly)^{P}(OPh)(F) (**3**). This organophosphonofluoridate is a very potent inhibitor of trypsin-like enzymes, but is hydrolytically unstable (Fig. 1).

Results

Chemistry

The diphenyl ester Cbz-(4-AmPhGly)^{P}(OPh)$_2$ (**4**) was prepared using the Oleksyszyn reaction.[10] The monoester **5** was then obtained by hydrolysis with NH_3 in a mixture of water and DMF containing a catalytic amount of 18-crown-6. Other bases including NaOH, KOH and Et_3N resulted in incomplete hydrolysis. The crown ether clearly plays a role in the hydrolysis reaction since the reaction was incomplete without it. The phosphonic acid monoester **5** was converted to a phosphonochloridate intermediate using freshly distilled thionyl chloride. The phosphonochloridate was converted in situ to the phosphonofluoridate **3** by reaction with KF in the presence of crown ether.[4] This transformation is complete since no phosphonochloridate was detected in the final product by mass spectroscopy. The phosphonofluoridate **3** was unstable in the presence of nucleophiles and formed Cbz-(4-AmPhGly)^{P}(OPh)(OEt) when $CHCl_3$ (containing 0.75% EtOH) was used during the work up procedure. The ^{19}F NMR chemical shifts of **3** are −70.07 ppm and −71.53 ppm with a ratio of 2:1 and coupling constants of 1,131 Hz and 1,130 Hz, respectively, indicating the existence of a P-F bond.[3] The ^{1}H and ^{31}P NMR spectra of **3** also indicates the presence of two diastereomers. For example, the two doublets for the α-carbon proton of the phosphonofluoridate were found at δ 5.4–5.6 in the ^{1}H NMR spectrum of **3**. Similar patterns have been observed with other phosphonates where the existence of diastereomers was confirmed.[3] The coupling constants for the α-hydrogen doublet ($J = 9.4$ Hz) are also consistent with the reported P-H coupling constants of phosphonofluoridate **1** and **2**.[3] The ^{31}P NMR spectrum of **3** has two doublets (10.88 and 15.90 ppm) with coupling constants of 1130 Hz and 1135 Hz, respectively, which are almost identical to those found in the ^{19}F NMR. However, the ^{31}P NMR failed to show the 2:1 ratio of the two diastereomers found in the ^{19}F NMR. The discrepancy in the ratio of diastereomers between the ^{31}P and ^{19}F NMR spectra may be a result of the poor resolution of the ^{31}P NMR spectrum due to the low inhibitor concentration used. Recording ^{31}P NMR spectrum requires a much higher sample concentration to obtain the same sensitivity (Scheme 1).

Inhibition kinetics

The phosphonofluoridate **3** was readily hydrolyzed in aqueous solution. A DMSO solution of the inhibitor lost 80% of its inhibitory activity after 1 h at room temperature. In order to prevent its hydrolysis in this solvent, the inhibitor was dissolved in dry DMF and stored at 0 °C. This DMF solution was stable for at least 1 h which was adequate to perform all the necessary kinetic measurements for one enzyme and obtain reproducible data. A fresh inhibitor solution was used with each enzyme.

Incubation of enzyme with phosphonofluoridate **3** did not display a simple logarithmic decrease in residual activity with respect to time. The rapid first-order hydrolysis reaction of the inhibitor must be taken into

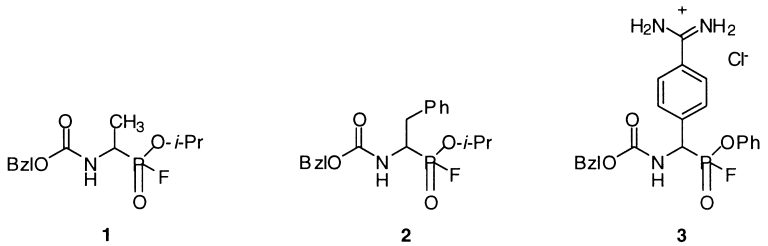
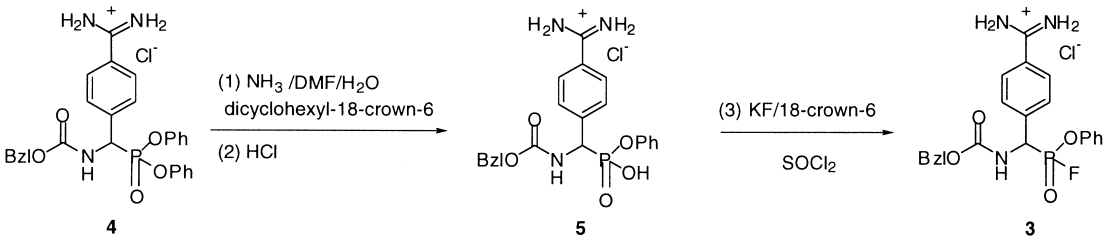


Figure 1. Structures of amino acid related phosphonofluoridates. The alanine derivative **1** is abbreviated Cbz-Ala^{P}(O-*i*-Pr)(F), the phenylalanine derivative **2** Cbz-Phe^{P}(O-*i*-Pr)(F) and the 4-amidinophenylglycine derivative **3** Cbz-(4-AmPhGly)^{P}(OPh)(F).



Scheme 1. Synthesis of phenyl *N*-benzyloxycarbonylamino-(4-amidinophenyl)methane phosphonofluoridate hydrochloride [Cbz-(4-AmPhGly)^{P}(OPh)(F), **3**].

account. The kinetic inhibition mechanism is described by eq 1 where E·I is the reversible Michaelis complex formed between the inhibitor and the enzyme, E-I is the irreversibly inhibited enzyme, and I′ is the hydrolysis product of the inhibitor.

$$\begin{array}{ccccccc} E + I & \underset{K_I}{\rightleftharpoons} & E \cdot I & \xrightarrow{k'_i} & E{-}I + F^- \\ & & \downarrow{\scriptstyle k_h} & & \\ & & I' & & \end{array} \quad (1)$$

In most cases the concentration of inhibitor will be lower than K_I and the second-order inhibition rate constant will be k'_i/K_I. The inhibition rate (k_{obs}) which is equivalent to k'_i/K_I and the hydrolysis rate of inhibitor (k_h) in water can then determined by the method of Ashani et al.[20] Figure 2 illustrates a typical plot of the inhibition of trypsin by **3** from which the k'_i/K_I and k_h values of the inhibitor can be calculated. Due to competing hydrolysis of the inhibitor, the enzyme retained 14% of its original activity after completion of the reaction. Similar results (10–20% residual activity) were obtained with the other enzymes.

Discussion

The phosphonyl fluoride **3** is a very potent inhibitor for trypsin, thrombin, tryptase, plasmin, and chymotrypsin (Table 1) despite its rapid hydrolysis in aqueous solution. It is the most potent amidine-containing organophosphorus inhibitor yet reported for trypsin-like enzymes and is 130-fold more potent for trypsin and 1250-fold better for thrombin than corresponding diphenyl ester **4** (k_{obs}/[I] values are 2200 $M^{-1}s^{-1}$ for trypsin and 88 $M^{-1}s^{-1}$ for thrombin).[10] It is also a 10-fold better inhibitor for thrombin compared to the tripeptide Boc-D-Phe-Pro-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 11,000 $M^{-1}s^{-1}$), the best amidine-containing phosphonate thrombin inhibitor which we have previously reported.[10] The monoester (Cbz-(4-AmPhGly)^{P}(OPh)(OH)) was found not to inhibit thrombin and tryptase and is a very weak irreversible inhibitor for trypsin (k_{obs}/[I] = 0.3 $M^{-1}s^{-1}$).

The amino acid related phosphonofluoridate **3** had enhanced inhibitory potency and selectivity for trypsin-like enzymes compared to simple organophosphonate inhibitors. Phosphonofluoridate **3** inhibited trypsin 257-fold, 139-fold and 267-fold more potently than DFP, Sarin and Soman (k_{obs}/[I] values are 970 $M^{-1}s^{-1}$, 1800 $M^{-1}s^{-1}$ and 680 $M^{-1}s^{-1}$, respectively).[21] The phosphonofluoridate **3** did not demonstrate much greater potency for chymotrypsin (k_{obs}/[I] = 29,000 $M^{-1}s^{-1}$) compared to DFP, Sarin and Soman (k_{obs}/[I] 15,000 $M^{-1}s^{-1}$, 23,000 $M^{-1}s^{-1}$ and 200,000 $M^{-1}s^{-1}$, respectively, for chymotrypsin). In contrast, the phenylalanine analogue **2** displays higher reactivity for

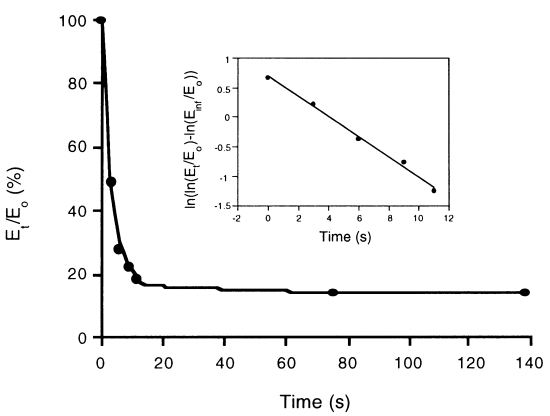


Figure 2. A typical plot for the inhibition of trypsin by **3**. Trypsin was incubated with inhibitor **3** in a reaction mixture which was composed of 0.13 μM trypsin. 1.3 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$ and 8% DMF at 25 °C. Aliquots of 25 μL were withdrawn at various intervals and the residual enzymatic activity was measured with the trypsin substrate Cbz-Arg-SBzl (35 μM) in the presence of DTNB (50 μM) in 2 mL of 0.1 M Hepes, pH 7.5 buffer containing 1% DMSO and 0.01 M $CaCl_2$. The insert is a plot of $\ln(\ln(E_t/E_0 - \ln(E_\infty/E_0))$ versus time. Only the first five points are plotted.

Table 1. Inhibition of serine proteases by Cbz-(4-AmPhGly)^{P}(OPh)(F) (**3**) and the hydrolysis rate of Cbz-(4-AmPhGly)^{P}(OPh)(F) (**3**) in buffer solutions[a]

Enzymes	$k_{obs}/[I]\times10^{-5}$ ($M^{-1}s^{-1}$)	k_h (s^{-1})
Bovine trypsin	2.6 ± 0.2	0.18
Human thrombin	1.0 ± 0.0	0.24
Mast cell tryptase	0.4 ± 0.1	0.17
Plasmin	0.59 ± 0.08	0.17
Kallikrein	0.9 ± 0.3	0.15
α-chymotrypsin	0.29 ± 0.01	0.30
HNE	0.027 ± 0.007	0.26
PPE	NI[b]	
AChE	NI[b]	

[a]Enzyme was incubated with inhibitor in an appropriate buffer to form an inhibition reaction mixture at 25 °C. Specifically, the inhibition reaction mixtures were composed of: 0.13 μM trypsin, 1.3 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$ and 8% DMF; 0.07 μM thrombin, 0.8 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 428 mM NaCl and 8% DMF; 0.07 μM tryptase, 0.8 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 8 mM heparin, 166 mM NaCl, 0.8 mM Mes, 9% glycerol and 8% DMF; 0.5 μM plasmin, 5 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$, 4% glycerol and 8% DMF; 0.12 kallikrein, 1.3 μM inhibitor in an 88 mM Hepes, pH 7.5 buffer containing 1.1 mM $CaCl_2$ and 8% DMF; 1.7 μM chymotrypsin, 16 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$ and 8% DMF; 1.7 μM PPE or HNE, 16 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$ and 8% DMF; 0.3 μM AChE, 3 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 415 mM NaCl and 8% DMF. Aliquots (25–50 μL) were withdrawn at various intervals and the residual enzymatic activity was measured as described in the experimental section. The k_{obs} and k_h values were measured and calculated by the method of Ashani et al.[20]

[b]No inhibition was observed after incubation with inhibitor (16 μM) for 5 min.

chymotrypsin ($k_{obs}/[I]$ 180,000 $M^{-1}s^{-1}$) and has high selectivity (160 $M^{-1}s^{-1}$ for elastase).[34] The positively charged amidine-containing phosphonofluoridate **3** appears to take advantage of its complementarity with the S1 subsite (pocket with Asp-189) of trypsin-like enzymes. It inhibits the structurally homologous trypsin, thrombin, plasmin and mast cell tryptase much faster than chymotrypsin and HNE and does not inhibit PPE and AChE at all.

It is worth noting that hydrolysis rate of **3** is much faster in Hepes buffer (pH 7.5) than that of **1** and **2** in phosphate buffer (pH 7.0). The average half-life time of the inhibitor **3** in aqueous buffer which can be calculated from the values of k_h in Table 1 is only 4.5 s. It is 100 times faster than that of the reported phosphonofluoridates **1** and **2**.[34] The phenoxy group on phosphorus atom in **3** may act as better electron-withdrawing group (compared to isopropoxy in **1** and **2**) which increases the positive charge on phosphorus resulting in facilitated hydrolysis. The positively charged amidino group might also catalyze the hydrolysis reaction.

The phosphonofluoridate **3** contains two chiral centers (α-carbon and P) and is racemic and diasteromeric. It is likely that the configuration at the chiral carbon centers plays a role in the inhibition reaction, as observed in the case of the Cbz-(4-AmPhGly)^{P}(OPh)$_2$ (**4**) inhibition of trypsin[22] and phosphonofluoridate inhibitors for AChE.[23] It is not clear that the chiral phosphorus center will have the same effect on the reactivity of the inhibitor. It has been reported that there is no significant difference in inhibition rates of chymotrypsin by different phosphorus diastereomers of the phosphonate Ac-Ala-Pro-Phe^{P}(OPh)NHCH(CH$_3$)CO$_2$CH$_3$,[3] while with α-lytic protease a twofold rate difference is observed and the two products have the same stereochemistry at phosphorus.[24]

The mechanism of irreversible inactivation of serine proteases by (4-AmPhGly)^{P}(OPh)$_2$ derivatives involves nucleophilic replacement of one of the phenoxy groups on phosphorus atom by the Ser-195 oxygen atom at the active site to generate a stable enzyme–inhibitor complex containing a P–O–Ser–195 bond.[10] A recent report of the X-ray structure of the complex of Cbz-(4-AmPhGly)^{P}(OPh)$_2$ (**4**) with bovine trypsin has confirmed the tetrahedral phosphorus center which resembles the tetrahedral transition state in substrate hydrolysis.[22] The second phenoxy group is lost by hydrolysis in an aging reaction. The reported enzyme–inhibitor is stabilized by a number of hydrogen bonds including those between the amidino nitrogen atoms and the carboxylate oxygens of Asp-189, carbonyl oxygen of Gly-219, Ser-190 Oγ and a 'buried' water molecule 557 in the S1 binding pocket. The inactivation mechanism of phosphonofluoridate **3** with serine proteases should be similar to that of its diphenyl ester analogue **4**.

In derivatives of α-aminoalkyl phosphonates, there is a relationship between the reactivity of the leaving group on phosphorus center and the enzyme inhibitory potency. For example, in the case of phosphonate diphenyl ester, Cbz-(4-AmPhGly)^{P}(OPh)$_2$, we have found that replacing the two phenyl groups with two *para*-chlorophenyl groups dramatically improve its inhibitory potency against trypsin (20-fold) and thrombin (100-fold). The much greater activity of **3** compared to the diphenyl ester phosphonates is probably the result of facilitated nucleophilic substitution on the phosphorus atom by Ser-195, due to the presence of the adjacent electronegative fluorine atom.

In summary, the phosphonofluoridate **3** is a very potent and moderately specific inhibitor for trypsin-like

enzymes. It has low stability toward hydrolysis compared to other amino acid-like phosphonofluoridates. A good phosphonate anticoagulant will require a leaving group which is less reactive than fluoride, but more reactive than phenoxy.

Experimental

Synthesis

Benzyl carbamate, triphenyl phosphite, 4-cyanobenzaldehyde, crown ethers, and all common chemical reagents and solvents were purchased from Aldrich Co. (Milwaukee, WI, USA) and were used without further purification unless otherwise specified. The ^{1}H NMR spectra of all phosphonates were recorded in DMSO-d_6 solutions (chemical shifts are given in ppm with TMS as the internal standard) on a Varian Gemini 300 MHz instrument. The ^{19}F NMR spectrum of phosphonofluoridate **3** was obtained in a freshly prepared DMSO solution at 470.6 MHz on a Varian XL-400 instrument; chemical shifts are reported relative to trifluorotoluene (external standard) at −63.0 ppm with downfield chemical shifts being positive. The ^{31}P NMR chemical shifts of **3** in a freshly prepared DMSO solution are reported relative to external H_3PO_4 at 0.00 ppm with positive values being downfield at 161.2 MHz on a Varian XL-400 instrument. Melting points were measured on a Mel-Temp II apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. All final products were characterized by NMR, mass spectroscopy (MS), high-resolution MS, and elemental analysis.

Phenyl hydrogen *N*-benzyloxycarbonylamino-(4-amidinophenyl)methanephosphonate hydrochloride (Cbz-(4-AmPhGly)^P(OPh)(OH)) (5). The phosphonate Cbz-(4-AmPhGly)^P(OPh)$_2$ (0.55 g, 1.0 mmol) and *cis*-dicyclohexano-18-crown-6 (dicyclohexyl-18-crown-6, 0.037 g, 0.1 mmol) were dissolved in 10 mL of a mixture of DMF/water (80%, v/v) followed by addition of a 30% ammonia solution (0.16 mL, 2.6 mmol). The reaction mixture was allowed to stir for 3 days. The reaction was monitored by TLC (silica gel, $CHCl_3$/MeOH/HOAc, 8/2/0.2). A solution of 2 M HCl (1.4 mL, 2.8 mmol) was added and the solvent was evaporated under reduced pressure. The residue was taken up in 50 mL $CHCl_3$. After removal of the precipitate, $CHCl_3$ was removed in vacuo. The residue was triturated in EtOAc to obtain the product as a white solid (0.38 g): mp 130–139 °C; yield, 80%; ^{1}H NMR (DMSO-d_6) δ 5.0 (m, 3H), 6.7 (m, 1H), 7.0–7.4 (m, 10H), 7.5 (m, 4H), 7.7–7.8 (m, 2H), 8.3 (s, 2H), 9.5 (s, 1H); MS (FAB$^+$) m/z 440.1 $(M+H)^+$. Anal. calcd for $C_{22}H_{22}O_5N_3P{\cdot}HCl{\cdot}H_2O$: C, 53.50; H, 5.10; N, 8.59. Found: C, 53.89; H, 5.47; N, 8.99.

Phenyl *N*-benzyloxycarbonylamino-(4-amidinophenyl)-methyl phosphonofluoridate hydrochloride (Cbz-(4-AmPhGly)^P(OPh)(F)) (3). To a mixture of Cbz-(4-AmPhGly)^P(OPh)(OH) (**5**, 0.25 g, 0.53−mmol), KF (0.061 g, 1.1 mmol) and 18-crown-6 (0.025 g, 0.095 mmol), freshly distilled $SOCl_2$ was added dropwise until all the solid was submerged. The reaction mixture was allowed to stir overnight at room temperature. Excess $SOCl_2$ was evaporated in vacuo. The residue was taken up in 50 mL dry CH_2Cl_2. Insoluble material was removed by filtration and the CH_2Cl_2 was then evaporated. The residue was triturated in EtOAc to obtain the product as a white solid (0.10 g): mp, 77–82 °C; yield, 40%; ^{1}H NMR (DMSO-d_6) δ 5.0 (m, 2H), 5.5 (m, 1H), 7.0–7.4 (m, 10H), 7.8 (m, 4H), 8.8 (br s, 1H), 9.1–9.4 (m, 4H); ^{19}F NMR (DMSO-d_6) δ −71.53 (d, J = 1130 Hz), −70.07 (d, J = 1131 Hz) (ratio of diastereomers = 2:1); ^{31}P NMR (DMSO-d_6) δ 10.88 (d, J = 1135 Hz), 15.90 (d, J = 1130 Hz); HRMS (FAB$^+$) m/z calcd for $C_{22}H_{22}O_4N_3PF$ 442.1432 $(M+H)^+$, found 442.1444. Anal. calcd for $C_{22}H_{21}O_4N_3PF{\cdot}HCl{\cdot}2H_2O$: C, 48.05; H, 5.50; N, 8.64. Found: C, 47.92; H, 5.33; N, 8.39.

Enzyme inactivation

Hepes was purchased from Research Organics Inc. (Cleveland, OH, USA). Bovine trypsin, acetylcholinesterase (AChE), plasmin, and α-chymotrypsin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Porcine pancreatic elastase (PPE) was purchased from Calbiochem Corp. (La Jolla, CA, USA). Human neutrophil elastase (HNE) was purchased from Athens Research and Technology, Inc. (Athens, GA, USA). Human thrombin was a generous gift from Dr S. Krishnaswamy of Emory University and mast cell tryptase was a gift of Dr David A. Johnson of East Tennessee State University.

The substrate Cbz-Arg-SBzl·HBr was synthesized according to reported procedures.[25] The substrates Cbz-Lys-SBzl·HCl, Suc-Phe-Leu-Phe-SBzl, and Suc-Ala-Ala-Ala-NA were purchased from Sigma Chemical Co. DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) and MES (2-(4-morpholino)ethanesulfonic acid) were purchased from Aldrich Co.

Incubation method

The enzyme inhibition reactions were initiated by adding 25 μL of the inhibitor solution (10–190 μM in DMF) to a mixture of 250 μL of an appropriate buffer and 25 μL of a stock enzyme solution (0.8–20 μM) at 25 °C. All inhibitor solutions were freshly prepared using dry DMF for each enzyme and stored at 0 °C prior to use. The inhibitor solutions were used for up to 1 h. Aliquots of 25 μL (50 μL for HNE) from the incubation mixture were withdrawn at various time intervals, added to an

assay solution, and the residual enzymatic activity was measured spectrophotometrically at 405 nm with a Beckman model DU650 at 25 °C. Control experiments were carried out using DMF instead of inhibitor solution. The assay solution was formed by adding 10 μL of a substrate solution (6 mM in DMSO) and 10 μL DTNB solution (16 mM in DMSO) to 2 mL (1 mL for AChE and chymotrypsin) of an appropriate buffer.

The buffers used for the inhibition and assay of enzymatic activity were: buffer A (0.1 M Hepes, 0.5 M NaCl, 0.1% PEG, pH 7.5) for thrombin and AChE; buffer B (0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5) for trypsin, chymotrypsin, plasmin, kallikrein, PPE, and HNE; buffer C (0.1 M Hepes, 10% glycerol, 10 mM heparin, pH 7.5) for mast cell tryptase. The concentrations of stock enzyme solutions were: trypsin, 1.6 μM in 1 mM HCl; thrombin, 0.9 μM in a 50 mM sodium citrate, pH 6,5 buffer containing 150 mM NaCl; mast cell tryptase, 0.8 μM in a 10 mM MES, pH 6.1 buffer containing 2 M NaCl, 10% glycerol and 0.01% NaN_3; plasmin, 6 μM in 50% glycerol/H_2O (v/v); kallikrein, 1.5 μM in buffer B; chymotrypsin, 20 μM in 1 mM HCl; AChE, 3.8 μM in water; PPE and HNE, 20 μM in a 50 mM NaOAc, pH 5.5 buffer containing 150 mM NaCl. All enzyme stock solutions were stored at −20 °C prior to use. The inhibition reaction mixtures (final concentrations) were composed of: 0.13 μM trypsin, 1.3 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$ and 8% DMF; 0.07 μM thrombin, 0.8 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 428 mM NaCl and 8% DMF; 0.07 μM tryptase, 0.8 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 8 mM heparin, 166 mM NaCl, 0.8 mM Mes, 9% glycerol and 8% DMF; 0.5 μM plasmin, 5 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$, 4% glycerol and 8% DMF; 0.12 μM kallikrein, 1.3 μM inhibitor in an 88 mM Hepes, pH 7.5 buffer containing 1.1 mM $CaCl_2$ and 8% DMF; 1.7 μM chymotrypsin, 16 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$ and 8% DMF; 1.7 μM PPE or HNE, 16 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 1 mM $CaCl_2$ and 8% DMF; 0.3 μM AChE, 3 μM inhibitor in an 80 mM Hepes, pH 7.5 buffer containing 415 mM NaCl and 8% DMF. The enzyme assay solutions were: substrate Cbz-Arg-SBzl (35 μM) for thrombin in 2 mL of a 0.1 M Hepes, pH 7.5 buffer containing 78 μM DTNB, 1% DMSO and 0.5 M NaCl; Cbz-Arg-SBzl (35 μM) for trypsin and kallikrein in 2 mL of a 0.1 M Hepes, pH 7.5 buffer containing 78 μM DTNB, 1% DMSO and 0.01 $CaCl_2$; substrate Cbz-Lys-SBzl (30 μM) for mast cell tryptase in 2 mL of a 0.1 M Hepes, pH 7.5 buffer containing 78 μM DTNB, 1% DMSO, 9% glycerol and 10 mM MES; Cbz-Lys-SBzl (30 μM) for plasmin in 2 mL of a 0.1 M Hepes, pH 7.5 buffer containing 78 μM DTNB, 1% DMSO and 0.01 M $CaCl_2$; Suc-Phe-Leu-Phe-SBzl (50 μM) for chymotrypsin in 1 mL of a 0.1 M Hepes, pH 7.5 buffer containing 153 μM DTNB, 2% DMSO and 0.01 M $CaCl_2$; acetylthiocholine (50 μM) for AChE in 1 mL of a 0.1 M Hepes, pH 7.5 buffer containing 153 μM DTNB, 2% DMSO and 0.5 M NaCl; Suc-Ala-Ala-Ala-NA (50 μM) for PPE and HNE in 1 mL of a 0.1 M Hepes, pH 7.5 buffer containing 2% DMSO and 0.01 $CaCl_2$.

The inhibition rate k_{obs} and first order hydrolysis rate k_h of the inhibitor were obtained using the method of Ashani et al.[20] using eqs (2)–(4) where E_0 is the activity of the enzyme at zero time, E_t is the activity of the enzyme at time t, I_0 is the concentration of the inhibitor at zero time, and I_t is the concentration of the inhibitor at time t. E_∞ is the residual enzymatic activity after the inhibition and hydrolysis reactions are complete. This kinetic method enables the second-order rate constant to be measured while the inhibitor undergoes simultaneous hydrolysis.

$$\ln \frac{E_t}{E_0} = I_0 \frac{k_i}{k_h}(e^{-k}h^t - 1) \quad (2)$$

$$\ln \frac{E_\infty}{E_0} = -I_0 \frac{k_i}{k_h} \quad (3)$$

$$\ln\left(\ln \frac{E_t}{E_0} - \ln \frac{E_\infty}{E_0}\right) = \ln \ln \frac{E_0}{E_\infty} - k_h t \quad (4)$$

Each k_{obs} value was determined using at least five data points which were used to plot a single straight line. The residual enzymatic activity was followed for at least twenty half-lives (3 min). The second-order inhibition rate $k_{obs}/[I]$ was obtained from the average of at least three independent measurements of the inhibition rate k_{obs} for each enzymatic reaction. Standard deviations reflect the experimental errors in the determination of the separate inhibition rate constants.

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References and Notes

1. Abbreviations: AChE, acetylcholinesterase; (4-AmPhGly)-$P(OPh)_2$, diphenyl amino(4-amidinophenyl)methanephosphonate or diphenyl 4-amidinophenylglycinephosphonate; Cbz, benzyloxycarbonyl; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid;

FAB, fast atom bombardment; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HNE, human neutrophil elastase; HRMS, high resolution mass spectroscopy; k_{obs}, pseudo first-order rate constant; $k_{obs}/[I]$, apparent second-order inactivation rate constant; MES, 2-(4-morpholino)ethanesulfonic acid; NI, no inhibition; PEG, polyethylene glycol; PPE, porcine pancreatic elastase; SBzl, thiobenzyl ester; TLC, thin layer chromatography. The (α-aminoalkyl)phosphonic acids are analogues of natural α-amino acids and are designated by the generally accepted three-letter abbreviations for the amino acid followed by a superscript P. For example, diphenyl 1-*N*-(benzyloxycarbonyl)amino-(4-amidinophenyl)-methanephosphonate, which is related to phenylglycine, is abbreviated as Cbz-(4-AmPhGly)^{P}$(OPh)_2$.

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