Expedited Articles

Novel Amidine-Containing Peptidyl Phosphonates as Irreversible Inhibitors for Blood Coagulation and Related Serine Proteases

Jozef Oleksyszyn,† Bogdan Boduszek,‡ Chih-Min Kam,‡ and James C. Powers*,‡

School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332-0400

Received November 10, 1993•

A series of new peptidyl (α-aminoalkyl)phosphonate diphenyl esters containing the 4-amidinophenyl group were synthesized and tested as irreversible inhibitors for thrombin and other trypsin-like enzymes. These phosphonates irreversibly inhibited several coagulation enzymes and trypsin. Boc-D-Phe-Pro-(4-AmPhGly)^P(OPh)$_2$ is the best human thrombin inhibitor in the series with a k_{obs}/[I] value of 11 000 M^{-1} s^{-1}, and it inhibits thrombin more than 5-fold more effectively than the other enzymes tested. Z-(4-AmPhGly)^P(OPh)$_2$ is the best inhibitor for plasma kallikrein with a k_{obs}/[I] value of 18 000 M^{-1} s^{-1}. Generally, the (4-AmPhGly)^P(OPh)$_2$ derivatives are better inhibitors of thrombin and trypsin than the corresponding (4-AmPhe)^P(OPh)$_2$ derivatives which contain an extra CH_2 separating the amidinophenyl group from the peptide backbone. The amidino phosphonates did not inhibit acetylcholinesterase and were chemically stable in neutral buffers. In addition, the inhibited trypsin derivative did not regain any enzyme activity after removal of excess inhibitor and incubation in a pH 7.5 buffer for 1 day. Boc-D-Phe-Pro-(4-AmPhGly)^P(OPh)$_2$ and D-Phe-Pro-(4-AmPhe)^P(OPh)$_2$ prolonged the prothrombin time ca. 2-fold and prolonged the activated partial thromboplastin time ca. 3–4-fold in human plasma at concentrations of 63 and 125 μM, respectively. The novel amidine-containing peptidyl phosphonates reported here are thus effective anticoagulants *in vitro*, and they may have utility for use *in vivo*.

Introduction

Thrombin plays a pivotal role in blood coagulation since it cleaves fibrinogen to form the fibrin clot and can activate factor V, VIII, XIII, and protein C in processes which are essential for the control of thrombosis and hemostasis. Thrombin also stimulates platelet secretion and aggregation in blood; it is an important mediator of many nonhemostatic cellular events and is thought to play an important role in disease processes including restenosis following vascular surgery or tissue plasminogen activator treatment. Thrombin and other blood coagulation enzymes (factor VIIa, IXa, Xa, XIa, XIIa, and protein C) are members of the trypsin family of serine proteases. Since thrombin is a powerful trigger for thrombus formation in the blood, it has been targeted along with other enzymes in the coagulation pathway in the design of new antithrombotic drugs.[1] Nonpeptidyl inhibitors of thrombin include the amidine NAPAP[2] and the arginine derivative Argatroban which inhibit thrombin reversibly with K_I in the submicromolar range.[3] Peptide inhibitors containing the chloromethyl ketone, aldehyde, boronic acid, or trifluoromethyl ketone functional groups and the thrombin specific D-Phe-Pro-Arg sequence or a related sequence are potent and specific transition-state analog inhibitors for thrombin.[4]

Peptidyl phosphonates where the scissile peptide bond of a peptide substrate is replaced by an (α-aminoalkyl)-phosphonic acid residue have been previously reported as inhibitors of serine proteases,[5] and in particular, peptidyl derivatives of (α-aminoalkyl)phosphonates diphenyl esters have been shown to be specific and potent irreversible inhibitors of elastases and various chymotrypsin-like enzymes.[6] Good interactions of the amino acid side chain of the inhibitor with the S_1 pocket and with the extended substrate binding sites (S_2, S_3)[7] of the serine protease are essential for effective inhibition.[6] Recently, arginine and ornithine analogs and peptidyl phosphonates containing (α-amino-δ-methoxybutyl)phosphonyl or (α-amino-n-hexyl)phosphonyl residues at the P_1 site have been reported to be inhibitors of trypsin and thrombin.[8] Here we report a series of new phosphonate diphenyl esters with 4-amidinophenyl groups as arginine analogs (structures shown in Figure 1) as irreversible inhibitors for thrombin and related enzymes.

Results and Discussion

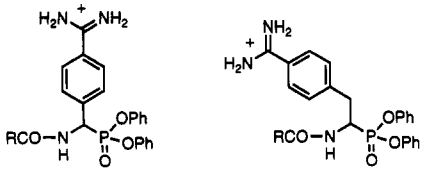
Chemistry. Peptide derivatives of 4-amidinophenylglycine phosphonate diphenyl ester were prepared using the reactions outline in Scheme 1. The parent compound Z-(4-AmPhGly)^P(OPh)$_2$ (**3**) was prepared by α-amidoalkylation[9] of triphenyl phosphite with 4-cyanobenzaldehyde and benzyl carbamate (60–90 °C, AcOH, 2 h) to give Z-(4-CN-PhGly)^P(OPh)$_2$ (70%) which was converted to the imino ester (dry HCl in $CHCl_3$/ethanol) and then to the amidine derivative **3** (dry NH_3 in methanol, 70–80%). Hydrogenolysis of Z-(4-AmPhGly)^P(OPh)$_2$ (**3**) gave H-(4-AmPhGly)^P(OPh)$_2$ (60–80%) which was then coupled respectively with Z-Pro-OH, Boc-D-Phe-Pro-OH, and 2-NpSO_2-Gly-OH using CDI to give Z-Pro-(4-AmPhGly)^P-(OPh)$_2$ (**4**), Boc-D-Phe-Pro-(4-AmPhGly)^P(OPh)$_2$ (29%, **5**), and 2-NpSO_2-Gly-(4-AmPhGly)^P(OPh)$_2$ (30%, **7**). Hydrogenolysis of **5** gave D-Phe-Pro-(4-AmPhGly)^P(OPh)$_2$

* Address for corresponding author: James C. Powers, School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332-0400. (404) 894-4038. Fax (404) 894-7452.

† Current address: OsteoArthritis Sciences, Inc., One Kendall Square, Bldg. 200, Cambridge, MA 02139.

• Abstract published in *Advance ACS Abstracts*, January 1, 1994.

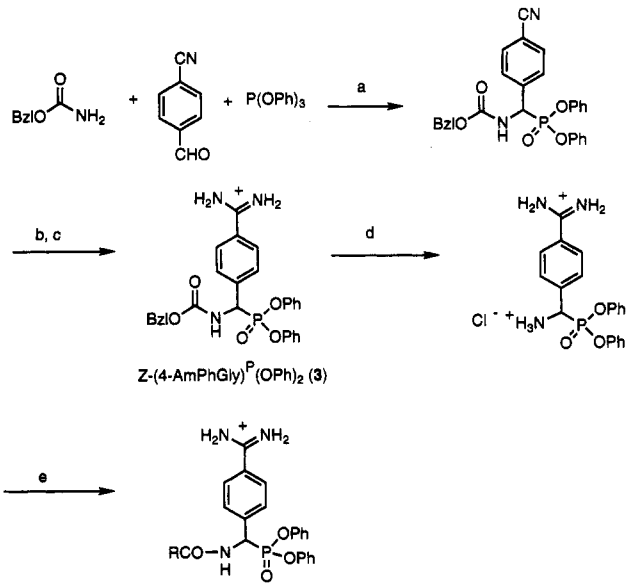
0022-2623/94/1837-0226$04.50/0



RCO-(4-AmPhGly)^P(OPh)$_2$ (1) RCO-(4-AmPhe)^P(OPh)$_2$ (2)

Figure 1. Structures of phosphonate derivatives of 4-amidinophenylglycine [diphenyl amino(4-amidinophenyl)methanephosphonate, RCO-(4-AmPhGly)^P(OPh)$_2$, 1] and 4-amidinophenylalanine [diphenyl 1-amino-2-(4-amidinophenyl)ethanephosphonate, RCO-(4-AmPhe)^P(OPh)$_2$, 2].

Scheme 1[a]



Z-(4-AmPhGly)^P(OPh)$_2$ (3)

[a] Reagents: (a) AcOH, 60–90 °C, 2h; (b) dry HCl in $CHCl_3$/ethanol; (c) dry NH_3 in CH_3OH; (d) 2 N HCl in CH_3OH, Pd/C; (e) RCOOH, CDI.

(24%, 6). The 4-amidinophenylalanine phosphonate diphenyl ester derivative Z-(4-AmPhe)^P(OPh)$_2$ (8) was synthesized from 4-cyanophenylacetaldehyde using the same reaction scheme with slightly modified conditions (α-amidoalkylation, 25–35%; amidination sequence, 50–70%). Hydrogenolysis produced H-(4-AmPhe)^P(OPh)$_2$ (85–90%, 9) which was coupled with Boc-D-Phe-Pro-OH to give Boc-D-Phe-Pro-(4-AmPhe)^P(OPh)$_2$ (59%, 11). Z-Pro-(4-AmPhe)^P(OEt)$_2$ (10) was obtained as a transesterification product during the amidination of Z-Pro-(4-CN-Phe)^P(OPh)$_2$ (50%). Deblocking of 11 with dry HCl/$CHCl_3$ produced D-Phe-Pro-(4-AmPhe)^P(OPh)$_2$ (80%, 12).

Inhibition Kinetics. Acyl and peptidyl derivatives of the diphenyl esters of 4-amidinophenylglycine phosphonate and 4-amidinophenylalanine phosphonate are effective irreversible inhibitors of human thrombin and human plasma kallikrein (Table 1). The best human thrombin inhibitor is Boc-D-Phe-Pro-(4-AmPhGly)^P(OPh)$_2$ (5) with a second-order inhibition rate constant (k_{obs}/[I]) of 11 000 M^{-1} s^{-1} which is more than 1 order of magnitude better than any of the other compounds tested with human thrombin. This inhibitor is also quite specific since only trypsin is effectively inhibited by 5 (at $^1/_5$ the rate). The Boc group is clearly making significant interactions with human thrombin's extended substrate binding site since the deblocked derivative 6 and the parent Z-derivative 3 are substantially poorer inhibitors than 5 by 16- and 140-fold, respectively. The 2-$NpSO_2$-Gly-(4-AmPhGly)^P-(OPh)$_2$ derivative 7, which has the same $NpSO_2$-Gly moiety as NAPAP, also inhibits bovine thrombin poorly. Unexpectedly, the 4-amidinophenylglycine derivatives are better inhibitors of both trypsin and thrombin than the corresponding 4-amidinophenylalanine derivatives [RCO-(4-AmPhe)^P(OPh)$_2$] which contain an extra methylene group in the side chain, and they also appear to be more effective than the recently reported arginine derivative Ac-D-Phe-Pro-Arg^P(OPh)$_2$.[8] The higher thrombin inhibitory potency of the Boc derivative 5 (relative to 6) and of the AmPhGly derivatives (relative to AmPhe derivatives) may indicate that there are substantial differences in the binding mode of these phosphonates to thrombin relative to other peptide-based inhibitors.[10] As expected, the diethoxy derivative 10 is not an inhibitor of thrombin or trypsin. Both human plasma kallikrein and bovine trypsin are effectively inhibited by the Z-derivative 3, which indicates that even more potent inhibitors could be prepared by the introduction of more appropriate peptide sequences for these two enzymes. It is also likely that more potent inhibitors for human factor XIIa could be produced by changes in the tripeptide sequence of 5.

Inhibition Mechanism. The inhibition mechanism of serine proteases by peptidyl phosophonates involves phosphonylation of the active-site serine via a pentacovalent intermediate with loss of one phenoxy group to form a stable phosphonylated derivative which may undergo slow aging to form a serine phosphonomonoester upon loss of the second phenoxy group. The serine phosphonate derivative has a tetrahedral geometry similar to the tetrahedral intermediate formed in peptide bond hydrolysis. The ^{31}P NMR of a freshly prepared complex of trypsin inhibited by 3 showed a broad signal at 25.55 ppm (downfield relative to H_3PO_4), while 3 alone gave a signal at 14.87 ppm. Similar results are also observed in the complex of chymotrypsin inhibited by Suc-Val-Pro-Phe^P(OPh)$_2$, which has a chemical shift of 25.98 ppm.[6] The more than 10 ppm difference in chemical shift is consistent with a tetrahedral geometry at the phosphorus atom[11] and is similar to the chemical shift observed in simple (α-aminoalkyl)phosphonate diethyl esters.[12] The two bulky phenoxy group in the inhibitor molecule result in a change in the O–P–O angle to a value larger than 109° with a resulting upfield chemical shift.

The phosphonates and phosphonylated enzyme derivatives are remarkably stable. The UV spectrum of 3 showed maximum absorbance at 246 nm at neutral pH in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer and no change was observed after incubation for 3 days at room temperature. Trypsin inactivated by 3, 5, 6, and 7 did not regain any activity after incubation for 24 h in the pH 7.5 buffer. None of the derivatives (3, 4, 5, 6, and 7) inhibited acetylcholinesterase.[13]

Anticoagulant Activity. Compounds 3, 5, 6, and 12 were tested in human plasma with the prothrombin (PT) and activated partial thromboplastin time (APTT) coagulant assays, and the PT was prolonged from 14.6 s to 17.0, 19.8, 29.3, and 28.0 s in the presence of 125 μM of 3, 6, and 12 and 63 μM of 5, respectively. At the same inhibitor concentrations, the APTT was prolonged from 34.0 s to 61.2, 73.0, 106, and >140 s, respectively for 3, 6, 12, and 5. Both compounds 5 and 12 prolonged the PT ca. 2-fold and prolonged the APTT ca. 3–4-fold although their thrombin inhibition rates differed by over 200-fold.

Table 1. Rates of Inhibition of Thrombin and Other Trypsin-like Serine Proteases by Peptide Phosphonate Derivatives of 4-Amidininophenylglycine and 4-Amidinophenylalanine[a]

		$k_{obsd}/[I]$ (M^{-1} s^{-1})				
no.	inhibitor	human thrombin	bovine thrombin	human factor XIIa	human plasma kallikrein	bovine trypsin
3	Z-(4-AmPhGly)^P(OPh)$_2$	80	170	20	18000	2000
4	Z-Pro-(4-AmPhGly)^P(OPh)$_2$	20	70	2.6	60	100
5	Boc-D-Phe-Pro-(4-AmPhGly)^P(OPh)$_2$	11000	12000	52	160	2200
6	D-Phe-Pro-(4-AmPhGly)^P(OPh)$_2$	700	730	2.8	250	110
7	2-NpSO$_2$-Gly-(4-AmPhGly)^P(OPh)$_2$		170	25	960	470
8	Z-(4-AmPhe)^P(OPh)$_2$	0.2				24
9	H-(4-AmPhe)^P(OPh)$_2$	2.2				32
10	Z-Pro-(4-AmPhe)^P(OEt)^2	NI				NI
11	Boc-D-Phe-Pro-(4-AmPhe)^P(OPh)$_2$	0.7				130
12	D-Phe-Pro-(4-AmPhe)^P(OPh)$_2$	54				50
	Ac-D-Phe-Pro-Arg^P(OPh)$_2$[b]	1200				

[a] Enzyme (0.03–2.3 μM) was incubated with the inhibitor (1.7–420 μM) in 0.3–0.6 mL of 0.1 M Hepes, 0.01 M $CaCl_2$ (0.5 M NaCl for human thrombin), pH 7.5, 8–12% Me_2SO at 25 °C. Aliquots (25–150 μL) were withdrawn at various intervals and the residual enzymatic activity were measured. Residual activity of trypsin and other coagulation enzymes were assayed with Z-Phe-Gly-Arg-NA (100–120 μM) and Z-Arg-SBzl (80–90 μM), respectively. The k_{obs} values were calculated from pseudo-first-order plots. [b] $k_{obs}/[I]$ value was calculated from the data obtained from ref 8c.

Conclusion

The 4-amidinophenylglycine and 4-amidinophenylalanine phosphonate derivatives which we have synthesized are stable in neutral pH buffer, are potent inhibitors of thrombin and other coagulation serine proteases, are inactive toward acetylcholinesterase, and form stable enzyme–inhibitor complexes. In addition, they appear to be more easily synthesized and more potent than the recently reported ornithine and arginine phosphonate derivatives. The stability of the inhibitors make them suitable for *in vivo* studies, and preliminary experiments indicate that some of the compounds are active in a rabbit model of coagulation. The stability of the enzyme–inhibitor complex makes these compounds suitable for crystallographic investigations. Molecular modeling studies and experiments aimed at extending these 4-amindinophenyl derivatives to other trypsin-like enzymes are in progress.

Experimental Section

Synthesis of Inhibitors. Benzyl carbamate, triphenyl phosphite, 4-cyanobenzaldehyde, 1,1'-carbonyldiimidazole (CDI), 1,3-dicyclohexylcarbodiimide (DCC), and all common chemicals were obtained from the Aldrich Co., Milwaukee, WI. Blocked amino cid derivatives were obtained from Aldrich or Bachem Bioscience Inc., Philadelphia, PA. The NMR spectra were recorded on a Varian Gemini 300 MHz instrument. ^{31}P NMR spectra were obtained at 161.895 MHz using broad-band ^1H decoupling on a Varian XL-400 instrument; chemical shifts were reported relative to 85% phosphoric acid (sealed capillary) at 0.000 ppm with positive values downfield. Elemental analyses were performed by Atlantic Microlabs of Atlanta, GA.

Diphenyl [*N*-(Benzyloxycarbonyl)amino](4-cyanophenyl)methanephosphonate [Z-(4-CN-PhGly)^P(OPh)$_2$, 3a]. This compound was synthesized from 9.75 g of 4-cyanobenzaldehyde, 7.65 g of benzyl carbamate, and 13.5 mL of triphenyl phosphite in 20 mL of glacial acetic acid using the α-amidoalkylation procedure described previously:[9] yield 70%; mp 135–138 °C. Anal. ($C_{28}H_{23}N_2O_5P$·0.5H_2O) C, H, N.

Diphenyl [*N*-(Benzyloxycarbonyl)amino](4-amidinophenyl)methanephosphonate Hydrochloride [Z-(4-AmPhGly)^P(OPh)$_2$, 3]. A solution of 7 g of the cyano derivative Z-(4-CN-PhGly)^P(OPh)$_2$ in 150 mL of dry chloroform and 15 mL of absolute ethanol was saturated with dry HCl at 0 °C and was kept in the refrigerator until no starting materials were present (checked periodically by TLC, ca. 24 h required). An excess of pentane was added, and the precipitate was removed by filtration and dried *in vacuo*. The solid was then dissolved in 200 mL of dry methanol, and dry ammonia (one equivalent is required) was passed through the solution for ca. 20 min. Methanol and excess ammonia were evaporated as fast as possible. Fresh methanol (100 mL) was added, and the solution was heated at 50 °C for 8 h until TLC shows the absence of the imino ether. The solvent was evaporated, and the resulting oil was dissolved in chloroform. Addition of ether caused the oil to solidify. The solid was again dissolved in chloroform and precipitated with ether to give the product: yield 70–80%; mp 154–158 °C dec; ^{31}P NMR 14.87 ppm. Anal. ($C_{28}H_{27}N_3O_5ClP$·0.3NH_4Cl·H_2O) C, H, N, Cl.

An improved amidination procedure was developed subsequently and should be used in future syntheses. The imino ester is dissolved in a freshly prepared solution of ammonia (1.5 equiv) in methanol, ammonium chloride (1 equiv) is added, and the mixture is then stirred at room temperature for 1 day.

Diphenyl Amino(4-amidinophenyl)methanephosphonate Dihydrochloride [(4-AmPhGly)^P(OPh)$_2$, 3b]. A sample (1.8 g) of compound **3** was dissolved in 150 mL of 2 N HCl in methanol and stirred under 1 atm of hydrogen in the presence of 5% Pd/C catalyst. After hydrogenolysis, the catalyst was removed and the solvent was evaporated. The residue was crystallized from ethanol–ether to give the product: yield 60–80%; mp 213–215 °C. Anal. ($C_{20}H_{22}N_3O_3Cl_2P$·0.5H_2O) C, H, N, Cl.

Diphenyl [*N*-(Benzyloxycarbonyl)-L-prolylamino](4-amidinophenyl)methanephosphonate Hydrochloride [Z-Pro-(4-AmPhGly)^P(OPh)$_2$, 4]. To 0.5 g of Z-proline (2 mmol) in 3 mL of dry DMF at 0 °C was added 0.45 g of CDI (2.77 mmol), and the mixture was kept at 0 °C for 1 h. Then 0.9 g of compound **3b** (2 mmol) was added. After the mixture was stirred for 18 h at 0–5 °C, 10 mL of water was added. The oily residue was washed with water and solidified by washing with cold 0.1 N HCl. One gram of the compound was obtained and used for next step without purification: ^{31}P NMR spectra, 12.31, 12.62 (free base) and 15.11, 15.41 ppm (hydrochloride). [After addition of one drop of concentrated HCl to the NMR tube, 14.22, 14.54 ppm (ratio 1:1), the chemical shift difference is caused by a solvent effect due to the excess concentrated HCl.]

Boc-D-Phe-L-Pro-OH (4a). Boc-D-Phe-OH (2.66 g, 10 mmol) and L-Pro-OBzl (2.42 g) were coupled using DCC, and Boc-D-Phe-Pro-OBzl was obtained in 83% yield: ^1H NMR ($CDCl_3$) δ 7.34 (s, 5H), 7.23 (m, 5H), 5.4 (d, 1H), 5.15 (q, 2H), 4.65 (m, 1H), 4.35 (m, 1H), 3.5 (m, 1H), 3.1–2.9 (m, 2H), 2.6 (m, 1H), 2.0–1.7 (m, 4H), 1.43 (s, 9H).

Hydrogenolysis was performed in methanol with 5% water using 5% Pd/C as a catalyst. The product was obtained as a white solid and recrystallized from aqueous methanol: yield 85%; mp 173–174 °C; ^1H NMR (DMSO) δ 7.3–7.15 (m, 5H), 7.0 (d, 1H), 4.45 (m, 1H), 4.10 (m, 1H), 3.6–2.7 (m, 4H), 2.2–1.6 (m, 4H), 1.30 (s, 9H); MS (FAB$^+$) *m/e* 363 (M + 1); $[\alpha]^{20}_D$ = −91.5° (0.14 g/mL in methanol). Anal. ($C_{19}H_{26}N_2O_5$) C, H, N.

Diphenyl [*N*-[(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-prolyl]amino](4-amidinophenyl)methanephosphonate Hydrochloride [Boc-D-Phe-L-Pro-(4-AmPhGly)^P(OPh)$_2$, 5]. CDI (0.17 g, 1.05 mmol) was added to a solution of 0.36 g (1.0 mmol)

of Boc-D-Phe-Pro-OH in 2 mL in dry DMF at 0 °C. After the mixture was stirred at 0 °C for 1 h, 0.45 g (1.0 mmol) of the dihydrochloride **3b** was added, and the solution was stirred at 0 °C for 48 h. Water (10 mL) was added, and the oil residue was decanted and washed with distilled water. The oil residue was dissolved in chloroform, and the solution was washed with 4% $NaHCO_3$, water, and 0.05 N HCl. After drying with $MgSO_4$ and removal of the solvent, the resulting oil was dried *in vacuo* for a few hours to give 0.22 g of product: yield 29%; mp 185–190 °C; ^{31}P NMR, 12.42, 12.66, 12.79 ppm (stereoisomers and conformers, free base); 15.12, 15.38, 15.58 (hydrochloride); MS (FAB$^+$) *m/e* 726 (M − Cl). Anal. ($C_{39}H_{45}N_5O_7ClP\cdot0.5H_2O$) C, H, N, Cl.

Diphenyl [*N*-(D-Phenylalanyl-L-prolyl)amino](4-amidinophenyl)methanephosphonate Dihydrochloride [D-Phe-Pro-(4-AmPhGly)^P(OPh)$_2$, 6]. The Z group in compound 4 was deblocked by hydrogenolysis in 1 N HCl in methanol with 0.1 g of 5% Pd/C. The catalyst was removed by filtration, the solvent was evaporated, and the oily residue (2HCl·Pro-(4-AmPhGly)^P(OPh)$_2$) was dried *in vacuo*. A solution of 0.43 g of Boc-D-Phe-OH (1.64 mmol) and 0.34 g of CDI (2 mmol) in 2 mL of dry DMF was kept at 0 °C for 1 h, and a sample of the dihydrochloride (0.9 g, 1.64 mmol) was added. After incubation for 24 h, 10 mL of water was added and the oil which formed was decanted. After washing with 0.1 N HCl, the oil was solidified and dried *in vacuo*. The dry solid was dissolved in 1 N HCl/methanol and stirred for 1 h. The solvent was removed by evaporation to give an oil which was dried *in vacuo* to give 0.3 g of product: yield 24%; mp 220–224 °C; ^{31}P NMR 16.95, 17.25, 17.60 ppm (stereoisomers and conformers); MS (FAB$^+$) *m/e* 626 (M + 1 − 2HCl). Anal. ($C_{34}H_{38}N_5O_5Cl_2P\cdot5H_2O$) C, H, N, Cl.

Diphenyl [*N*-[(2-Naphthylsulfonyl)glycyl]amino](4-amidinophenyl)methanephosphonate Hydrochloride [2-Np-SO$_2$-Gly-(4-AmPhGly)^P(OPh)$_2$, 7]. This compound was prepared in the same manner as 5 from 0.3 g (1.13 mmol) of (2-naphthylsulfonyl)glycine, 0.2 g (1.23 mmol) of CDI, and 0.45 g (1.0 mmol) of dihydrochloride **3b** to give the product: yield 30% (0.21 g); mp 205–210 °C dec; ^{31}P NMR 11.76 (free base), 14.74 ppm (hydrochloride). Anal. ($C_{32}H_{30}N_4O_6ClPS\cdot H_2O$) C, H, N, S.

4-Cyanophenylacetaldehyde (8a). This compound was prepared from 4-cyanobenzaldehyde using a modification of a procedure previously used in the multistep synthesis of 2-phenylpropanal.[14]

Ethyl 3-(4-Cyanophenyl)-2,3-epoxypropionate. Ethyl chloroacetate (6.2 g, 50 mmol) and 4-cyanobenzaldehyde (6.6 g, 50 mmol) were dissolved in 100 mL of dry benzene. Freshly prepared sodium ethoxide solution in absolute ethanol (1.3 g sodium in 25 mL ethanol) was added, and the mixture was stirred at room temperature for 20 h. Water (100 mL) was added with stirring, and after several minutes the organic layer was separated, washed with water, dried ($MgSO_4$), filtered, and evaporated to give the desired epoxy product as a yellow oil: yield 70–80%; ^1H NMR ($CDCl_3$) δ 7.68 (d, 2H), 7.43 (d, 2H), 4.30 (m, 2H), 4.16 (d, 1H), 3.48 (d, 1H), 1.34 (t, 3H).

Sodium 3-(4-Cyanophenyl)-2,3-epoxypropionate. Ethyl 3-(4-cyanophenyl)-2,3-epoxypropionate (10.9 g, 50 mmol) was dissolved in 50 mL of absolute ethanol and cooled with an ice bath. A freshly prepared sodium ethoxide solution (1.2 g of sodium in 25 mL of ethanol) was added dropwise during a 10-min period. Dropwise addition of 1 g of water to the stirred mixture caused separation of the sodium salt of the epoxy compound. The mixture was stirred for 3 h, and the salt was collected by filtration, washed with 50 mL ethanol and ether several times, and dried: yield 75–85%; ^1H NMR (D_2O) δ 7.58 (d, 2H), 7.33 (d, 2H), 3.93 (d, 1H), 3.39 (d, 1H).

A 1 N HCl solution (50 mL, 50 mmol) was added to the sodium salt of the epoxy compound (10.6 g, 50 mmol) dissolved in 50 mL of water. This resulted in the separation of an oil which solidified after a short time. This material was refluxed with 100 mL of toluene for 2.5 h to effect decarboxylation. The organic layer was separated, washed with water, dried (Na_2SO_4), filtered, and evaporated to give 4-cyanophenylacetaldehyde as a yellow brownish oil which solidified after several hours, yield 35–45%. This product is unstable and was used immediately for next step; otherwise, polymerization occurs. A sample of the crude product was purified by recrystallization from ether–hexane to give white crystals: mp 63–65 °C; NMR ($CDCl_3$) δ 9.81 (s, 1H), 7.65 (d, 2H), 7.35 (d, 2H), 3.84 (s, 2H); MS *m/e* 146 (M + 1). Anal. ($C_9H_7NO\cdot0.5H_2O$) C, H, N.

Diphenyl 1-[*N*-(Benzyloxycarbonyl)amino]-2-(4-cyanophenyl)ethanephosphonate [Z-(4-CN-Phe)^P(OPh)$_2$, 8b]. This compound was synthesized from crude 4-cyanophenylacetaldehyde, benzyl carbamate, and triphenyl phosphite using a modification of the previously described amidoalkylation procedure.[9] Benzyl carbamate (6.1 g, 40 mmol) and 4-cyanophenylacetaldehyde (4.7 g, 32 mmol) were dissolved in 50 mL of toluene and refluxed for 1 h. The toluene was evaporated, glacial acetic acid (10 mL) and triphenyl phosphite (8.5 mL, 32 mmol) were added to the residue, and the mixture was heated at 80 °C for 2 h. The volatile materials were removed by evaporation *in vacuo*, and the resulting oil was dissolved in 50 mL of methanol. The solution was refrigerated overnight, and the white precipitate was filtered, dried, and recrystallized from 50 mL of hot methanol. The undissolved product was discarded, and the filtrate was cooled down to give white crystals: yield 25–35%; mp 136–137 °C; ^1H NMR (DMSO) δ 8.20 (d, 1H), 7.75 (d, 2H), 7.54 (d, 2H), 7.35 (m, 5H), 7.3–7.1 (m, 10H), 4.95 (q, 2H), 4.60 (m, 1H), 3.4–3.0 (m, 2H); MS (FAB$^+$) *m/e* 513 (M + 1). Anal. ($C_{29}H_{25}N_2O_5P$) C, H, N.

Diphenyl 1-Amino-2-(4-cyanophenyl)ethanephosphonate Hydrobromide [4-CN-Phe^P(OPh)$_2$·HBr, 8c]. Z-(4-CNPhe)^P(OPh)$_2$ (0.57 g, 1.1 mmol) was mixed with 1.0 mL of 30% HBr in acetic acid. The mixture was protected against moisture and kept at room temperature for 1 h. Addition of 50 mL of dry ether and stirring for several hours resulted in formation of a yellow brownish solid which was filtered, washed with ether, and dried to give the product (0.48 g, 95%): mp 197–199 °C; ^1H NMR (DMSO) δ 9.0 (b, 3H), 7.85 (d, 2H), 7.65 (d, 2H), 7.4–7.0 (m, 10H), 4.7 (m, 1H), 3.4 (m, 2H); MS (FAB$^+$) *m/e* 379 (M − Br).

Diphenyl 1-[*N*-(Benzyloxycarbonyl)amino]-2-(4-amidinophenyl)ethanephosphonate [Z-(4-AmPhe)^P(OPh)$_2$, 8]. Z-(4-CNPhe)^P(OPh)$_2$ (1.03 g, 2.0 mmol) was dissolved in a mixture of methanol and chloroform (1:1, 20 mL). The solution was cooled to 0 °C, saturated with dry HCl, and kept at 5 °C for 48 h, and then the solvent was evaporated at room temperature *in vacuo*. The resulting semisolid was treated with 50 mL of dry ether, filtered, washed with dry ether, and dissolved in 10 mL of methanol containing 51 mg (3.0 mmol) ammonia. Ammonium chloride (107 mg, 2.0 mmol) was added, the mixture was stirred at room temperature for 1 day, the solvent was removed at room temperature *in vacuo*, the resulting semisolid was dissolved in 50 mL of chloroform and filtered, and the filtrate was evaporated to give the crude product. The crude product was treated with 50 mL of dry ether and stirred, and the undissolved solid collected, washed with ether, and dried to give the amidine product 8 as a yellow powder: yield 50–70%; mp >140 °C dec; ^1H NMR (DMSO) δ 9.4–9.2 (d, 4H), 8.25 (d, 1H), 7.8 (d, 2H), 7.6 (d, 2H), 7.38 (m, 5H), 7.3–7.1 (m, 10H), 4.95 (q, 2H), 4.6 (m, 1H), 3.4–3.1 (m, 2H); MS (FAB$^+$) *m/e* 530 (M − Cl). Anal. ($C_{29}H_{29}N_3O_5ClP$) C, H, N.

Diphenyl 1-Amino-2-(4-amidinophenyl)ethanephosphonate [(4-AmPhe)^P(OPh)$_2$·2HCl, 9]. The amidine 8 (1.0 g, 1.76 mmol) was dissolved in 150 mL of methanol containing 2 mmol of HCl, a catalyst (0.5 g of 5% Pd/C) was added, and the mixture was hydrogenated. After hydrogenolysis, the catalyst was removed by filtration and the filtrate was evaporated to give a near white crystalline product: yield 85–90%; mp >180 °C dec; ^1H NMR (DMSO) δ 9.5–9.3 (d, 4H), 9.2–9.0 (m, 3H), 7.9–7.1 (m, 14H), 4.55 (m, 1H), 3.2–3.5 (m, 2H); MS (FAB$^+$) *m/e* 396 (M + 1 − 2HCl). Anal. ($C_{21}H_{24}N_3O_3Cl_2P$) C, H, N.

Diphenyl 1-[[*N*-(Benzyloxycarbonyl)-L-prolyl]amino]-2-(4-cyanophenyl)ethanephosphonate [Z-Pro-(4-CN-Phe)^P-(OPh)$_2$, 10a]. To a cooled solution of the phosphonate 8c (0.41 g, 0.9 mmol) and Z-Pro-OH (0.25 g, 1.0 mmol) in 20 mL of CH_2Cl_2 was added 0.14 mL of triethylamine. After the solution was stirred for 10 min, DCC (0.21 g, 1.0 mmol) was added and the mixture was stirred at 0 °C for 2 h and at room temperature for 24 h. During this period, dicyclohexylurea (DCU) formed and was removed by filtration, and the filtrate was evaporated. The residue was dissolved in 50 mL of ethyl acetate and filtered, and the filtrate was washed with 50 mL of 1 N HCl, water, 6% $NaHCO_3$, and water. The organic layer was dried, filtered, and evaporated to give crude product which was then recrystallized from ether to give white crystals: yield 52% (0.29 g); mp 131–132

°C; ^{1}H NMR (DMSO) δ 8.75 (m, 1H), 7.8–7.2 (m, 19H), 5.06 (m, 2H), 4.95 (m, 1H), 4.6 (q, 1H), 4.2 (m, 1H), 3.5–3.1 (m, 3H), 2.0–1.4 (m, 4H); MS (FAB$^+$) *m/e* 610 (M + 1). Anal. ($C_{34}H_{32}N_3O_6P$) C, H, N.

Diethyl 1-[[*N*-(Benzyloxycarbonyl)-L-prolyl]amino]-2-(4-amidinophenyl)ethanephosphonate [Z-Pro-(4-AmPhe)^P(OEt)$_2$, 10]. This compound was obtained during the amidination procedure of dipeptide **10a** when ethanol was used instead of methanol. Transesterification occurred, and the phenyl groups were replaced by ethyl groups: yield 50% (0.11 g); mp >130 °C dec; ^{1}H NMR (DMSO) δ 9.4–9.2 (m, 4H), 8.4 (m, 1H), 7.8–7.1 (m, 9H), 5.0 (s, 2H), 4.5 (m, 1H), 4.2 (m, 1H), 4.0 (m, 4H), 3.5–2.9 (m, 4H), 2.1–1.4 (m, 4H), 1.25 (m, 6H); MS (FAB$^+$) *m/e* 531 (M − Cl). Anal. ($C_{26}H_{36}N_4O_6ClP$) C, H, N.

Diphenyl 1-[[*N*-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-prolyl]amino]-2-(4-cyanophenyl)ethanephosphonate [Boc-D-Phe-Pro-(4-CN-Phe)^P(OPh)$_2$, 11a]. The phosphonate 8c (0.46 g, 1 mmol) was coupled with Boc-D-Phe-Pro-OH (0.36 g, 1.0 mmol) using DCC. The product was obtained as a glass-like solid: yield 86% (0.62 g); mp 85–90 °C; ^{1}H NMR (DMSO) δ 8.25 (2d, 1H), 7.8–7.0 (m, 19H), 4.8 (m, 1H), 4.4 (m, 1H), 4.3 (m, 1H), 4.2 (m, 1H), 3.5–2.8 (m, 4H), 2.0–1.4 (m, 4H), 1.3 (m, 9H); MS (FAB$^+$) *m/e* 723 (M + 1). Anal. ($C_{40}H_{43}N_4O_7P\cdot0.25H_2O$) C, H, N.

Diphenyl 1-[[*N*-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-L-prolyl]amino]-2-(4-amidinophenyl)ethanephosphonate [Boc-D-Phe-Pro-(4-AmPhe)^P(OPh)$_2$, 11]. CDI (0.13 g, 0.8 mmol) was added to Boc-D-Phe-Pro-OH (0.24 g, 0.67 mmol) in 2 mL of DMF. A solution of amidine **9** (0.31 g, 0.67 mmol) in 2 mL of DMF was added to a cooled solution (0 °C) of the peptide, and the mixture was incubated at 0 °C for 2 h and room temperature for 48 h. DMF was removed *in vacuo*, the residue oil was dissolved in 100 mL of $CHCl_3$ and filtered, and the organic layer was washed subsequently with 50 mL of 0.05 M HCl, 10% KCl, 6% $NaHCO_3$, and water. The organic layer was dried, filtered, and evaporated to give a crystalline product which was then dried *in vacuo*: yield 59% (0.31 g); mp 145–155 °C dec; ^{1}H NMR (DMSO) δ 10.1–9.7 (m, 2H), 9.4–9.0 (m, 3H), 8.0–7.0 (m, 19H), 6.75 (m, 1H), 4.9–4.0 (m, 3H), 3.5–2.6 (m, 6H), 2.0–1.4 (m, 4H), 1.3 (s, 9H); MS (FAB$^+$) *m/e* 740 (M − Cl). Anal. ($C_{40}H_{46}N_5O_7P\cdot0.3HCl\cdot1.5H_2O$) C, H, N.

Diphenyl 1-[*N*-(D-Phenylalanyl-L-prolyl)amino]-2-(4-amidinophenyl)ethanephosphonate Dihydrochloride [D-Phe-Pro-(4-AmPhe)^P(OPh)$_2$, 12]. Compound 11 (0.11 g) was dissolved in 2 mL $CHCl_3$ and the solution was saturated with dry HCl. An oily precipitate formed, and the mixture was kept at room temperature for 2 h before evaporating to dryness to obtain the crystalline product: yield 80% (0.08 g); mp >165 °C dec; ^{1}H NMR (DMSO) δ 9.4–9.2 (d, 4H), 8.5 (m, 3H), 8.2 (m, 1H), 7.9–7.0 (m, 19H), 4.8–3.9 (m, 3H), 3.5–2.8 (m, 6H), 2.0–1.1 (m, 4H); MS (FAB$^+$) *m/e* 640 (M + 1 − 2Cl); exact mass calcd for $C_{35}H_{39}N_5O_5P$ (M + 1 − 2HCl) 640.2689, found 640.2786.

Enzyme Inactivation. Hepes and Mes were purchased from Research Organics Inc., Cleveland, OH, and 4,4′-dithiodipyridine was purchased from Aldrich Chemical Co., Milwaukee, WI. Bovine and porcine pancreatic trypsin, bovine thrombin, and human erythrocyte acetylcholinesterase were obtained from Sigma Chemical Co., St. Louis, MO. Human plasma kallikerin and factor XIIa were generous gifts of Dr. Kazuo Fujikawa of the University of Washington. Human thrombin was kindly provided by Dr. S. Krishnaswamy of Emory University. The substrates Z-Arg-SBzl·HCl and Z-Phe-Gly-Arg-NA·HCl were prepared as previously described.[15,16]

Incubation Method. An aliquot of inhibitor (25 or 50 μL) in Me_2SO was added to 0.28–0.55 mL of a buffered enzyme solution (0.03–2.3 μM) to initiate the inactivation. Aliquots (25–150 μL) were withdrawn at various intervals, and the residual activity was measured as described below. A 0.1 M Hepes, 0.01 $CaCl_2$, pH 7.5 buffer was utilized for all enzymes except for human thrombin which was assayed in 0.1 M Hepes, 0.5 NaCl, pH 7.5 buffer. Inhibitors concentrations were 1.7–420 μM. Trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (100–120 μM),[15] and the hydrolysis rates of the 4-nitroanilides were measured at 410 nm (ϵ_{410} = 8800 M^{-1} cm^{-1}).[17] Other enzymes were assayed with Z-Arg-SBzl (80–90 μM), and the hydrolysis rates of thioesters were measured with assay mixtures containing 4,4′-dithiodipyridine (ϵ_{324} = 19 800 M^{-1} cm^{-1}).[18] Acetylcholinesterase was assayed with acetylthiocholine (0.36 mM) in 0.1 M phosphate, pH 8.0 buffer in the presence of 0.47 mM 5,5′-dithiobis(2-nitrobenzoic acid).[19]

Pseudo-first-order inhibition rate constants (k_{obs}) were obtained from plots of ln v_t/v_0 vs time. The apparent second-order inhibition rate constants (k_{obs}/[I]) shown in Table 1 are typically the average of duplicate or triplicate experiments.

Stability of Inhibited Trypsin. Reactivation of inhibited trypsin was measured after the removal of excess inhibitor from the reaction mixture by centrifugation twice at 0 °C for 1 h using Amicon Centricon-10 microconcentrators. The enzymatic activity of the solution was assayed at various times as described above.

^{31}P NMR of the Complex of Trypsin and Z-(4-AmPhGly)^P(OPh)$_2$ (3). A solution of **3** (10% solution in Me_2SO) was added in 25-μL portions to 100 mg of porcine pancreatic trypsin in 75 mL of Hepes buffer, pH 7.5, with stirring at room temperature until the solution shows no enzymatic activity. The solution was adjusted to pH 4 and then concentrated at 4 °C through an Amicon UM membrane (M_r cutoff 10 000) at 80 psi of nitrogen over a period of 0.5 h until 5–7 mL of solution remained. Fresh Mes buffer (75 mL, 0.1 M Mes, 0.1 M $CaCl_2$, pH 5.5) was added and the concentration process repeated three times before the residual solution was transfered to a 10-mm NMR tube. After addition of ca. 15% v/v of D_2O, the spectrum was run at 161.895 MHz using broad ^{1}H decoupling on a Varion XL-400 instrument at room temperature. The solution was freshly prepared and the spectrum run almost immediately. Chemical shift data are reported relative to 85% phosphoric acid (sealed capillary) at 0.000 ppm with positive values downfield. The trypsin–inhibitor complex showed one broad signal at 25.55 ppm, and compound **3** showed a signal at 14.87 ppm under similar conditions.

Acknowledgment. This work was supported by a grant from the National Institutes of Health to the Georgia Institute of Technology (HL 29307). We thank Dr. Kazuo Fujikawa of the University of Washington for supplying human plasma kallikrein, human factor XIIa, Dr. S. Krishnaswamy for providing human thrombin, Dr. Leslie Gelbaum for running the ^{31}P NMR spectra, and Dr. David Shrout for performing preliminary experiments on synthesis of 4-cyanophenylacetaldehyde.

References

(1) For reviews, see: (a) Stürzebecher, J. Inhibitors of Thrombin. In *The Thrombin*; Machovich, R., Ed.; CRC Press: Boca Raton, FL, 1984; Vol. 1, pp 131–160. (b) Powers, J. C.; Kam, C.-M. Synthetic Substrates and Inhibitors of Thrombin. In *Thrombin: Structure and Function*; Berliner, L. J., Ed.; Plenum Press: New York, 1992; pp 117–158. (c) Shebuski, R. J. Emerging Drug Discovery Targets in Thrombosis and Coagulation. *Annu. Rep. Med. Chem.* **1991**, *26*, 93–101.

(2) Abbreviations: Ac, acetyl; (4-AmPhGly)^P(OPh)$_2$, diphenyl amino-(4-amidinophenyl)methanephosphonate (or diphenyl [(4-amidinophenyl)glycyl]phosphonate); (4-AmPhe)^P(OPh)$_2$, diphenyl 1-amino-2-(4-amidinophenyl)ethanephosphonate (or diphenyl [(4-amidinophenyl)alanyl]phosphonate); Argatroban, (2*R*,4*R*)-4-methyl-1-[N^α-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; APTT, activated partial thromboplastin time; Boc, *tert*-butyloxycarbonyl; NAPAP, N^α-[[(2-naphthylsulfonyl)glycyl]-DL-4-amidinophenylalanyl]piperidine; 2-NpSO$_2$-Gly, (2-naphthylsulfonyl)glycyl; PT, prothrombin time; Z, benzyloxycarbonyl.

(3) Argatroban: (a) Kikumoto, R.; Tamao, Y.; Tezuka, T.; Tonomura, S.; Hara, H.; Ninomiya, K.; Hijikata, A.; Okamoto, S. Selective Inhibition of Thrombin by (2R,4R)-4-Methyl-1-[N^α-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)-sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid. *Biochemistry* **1984**, *23*, 85–90. NAPAP: (b) Stürzebecher, J.; Markwardt, F.; Voigt, B.; Wagner, G.; Walsmann, P. Cyclic Amides of N^α-Arylsulfonylaminoacylated 4-amidinophenylalanine-Tight Binding Inhibitors of Thrombin. *Thrombos. Res.* **1983**, *29*, 635–642. (c) Voigt, B.; Stürzebecher, J.; Wagner, G.; Markwardt, F. Syntheses of N^α-(Arylsulfonyl)-4-amidinophenylalanyl-proline and of N^α-(Arylsulfonylglycyl)-4-amidinophenylalanyl-proline and Investigation on Inhibition of Serine Proteinases 33. Synthetic Inhibitors of Serine Proteinases. *Pharmazie* **1988**, *43*, 412–414.

(4) Peptidyl chloromethyl ketones: (a) Kettner, C.; Shaw, E. D-Phe-Pro-Arg-CH_2Cl-A Selective Label for Thrombin. *Thromb. Res.* **1979**, *14*, 969–973. (b) Kettner, C.; Shaw, E. Inactivation of Trypsin-like Enzymes with Peptides of Arginine Chloromethylketones.

Methods Enzymol. **1981**, *80*, 826–842. Peptide aldehydes: (c) Bajusz, S.; Barabas, E.; Tolnay, P.; Szell, E.; Bagdy, D. Inhibition of Thrombin and Trypsin by Tripeptide Aldehydes. *Int. J. Peptide Protein Res.* **1978**, *12*, 217–221. (d) Bajusz, S.; Szell, E.; Bagdy, D.; Barabas, E.; Horvath, G.; Dioszegi, M.; Fittler, Z.; Szabo, G.; Juhasz, A.; Tomori, E.; Szilagyi, G. Highly Active and Selective Anticoagulants: D-Phe-Pro-Arg-H, A Free Tripeptide Aldehyde Prone to Spontaneous Inactivation, and Its Stable N-Methyl Derivative, D-MePhe-Pro-Arg-H. *J. Med. Chem.* **1990**, *33*, 1729–1735. (e) Shuman, R. T.; Rothenberger, R. B.; Campbell, C. S.; Smith, G. F.; Gifford-Moore, D. S.; Gesellchen, P. D. Highly Selective Tripeptide Thrombin Inhibitors. *J. Med. Chem.* **1993**, *36*, 314–319. Boronic acid derivatives: (f) Kettner, C.; Mersinger, L.; Knabb, R. The Selective Inhibition of Thrombin by Peptides of Boroarginine. *J. Biol. Chem.* **1990**, *265*, 18289–18297. (h) Tapparelli, C.; Metternich, R.; Ehrhardt, C.; Zurini, M.; Claeson, G.; Scully, M. F.; Stone, S. R. *In Vitro* and *In Vivo* Characterization of A Neutral Boron-containing Thrombin Inhibitor. *J. Biol. Chem.* **1993**, *268*, 4734–4741. (i) Claeson, G.; Philipp, M.; Agner, E.; Scullu, M. F.; Metternich, R.; Kakkar, V. V.; Desoyza, T.; Niu, L.-H. Benzyloxycarbonyl-D-Phe-Pro-methoxypropylboroglycine: A Novel Inhibitor of Thrombin with High Selectivity Containing A Neutral Side Chain at the P1 Position. *Biochem. J.* **1993**, *290*, 309–312. Trifluoromethyl ketone: (j) Neises, B.; Tarnus, C. Thrombin Inhibition by the Tripeptide Trifluoromethylketone D-Phe-Pro-Arg-CF_3 (MDL 73756). *Thromb. Haemostas.* **1991**, *65*, 1290.

(5) (a) Bartlett, P. A.; Lamden, L. A. Inhibition of Chymotrypsin by Phosphonate and Phosphonamidate Peptide Analogs. *Bioorg. Chem.* **1986**, *14*, 356–377. (b) Lamden, L. A.; Bartlett, P. A. Aminoalkylphosphonofluoridate Derivatives: Rapid and Potentially Selective Inactivators of Serine Peptidases. *Biochem. Biophys. Res. Commun.* **1983**, *112*, 1085–1090. Simple phosphonates are also inhibitors of thrombin, see for example: Kovach, I. M.; McKay, L. Reversible Modulation of Serine Protease Activity by Phosphonate Esters. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1735–1740.

(6) (a) Oleksyszyn, J.; Powers, J. C. Irreversible Inhibition of Serine Proteases by Peptidyl Derivatives of α-Aminoalkylphosphonate Diphenyl Esters. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 143–149. (b) Oleksyszyn, J.; Powers, J. C. Irreversible Inhibition of Serine Proteases by Peptide Derivatives of (α-Aminoalkyl)-phosphonate Diphenyl Esters. *Biochemistry* **1991**, *30*, 485–493.

(7) The nomenclature used for the individual amino acid residues (P_1, P_2, etc.) of a substrate and the subsites (S_1, S_2, etc.) of the enzyme is described in Schecter, I.; Berger, A. On the Size of the Active Site in Protease. 1. Papain. *Biochem. Biophys. Res. Comm.* **1967**, *27*, 157–162.

(8) (a) Fastrez, J.; Jespers, L.; Lison, D.; Renard, M.; Sonveaux, E. Synthesis of New Phosphonate Inhibitors of Serine Proteases. *Tetrahedron Lett.* **1989**, *30*, 6861–6864. (b) Cheng, L.; Goodwin, C. A.; Scully, M. F.; Kakkar, V. V.; Claeson, G. Substrate-related Phosphonopeptides, A New Class of Thrombin Inhibitors. *Tetrahedron Lett.* **1991**, *32*, 7333–7336. (c) Wang, C.-L.; Taylor, T. L.; Mical, A. J.; Spitz, S.; Reilly, T. M. Synthesis of Phosphonopeptides As Thrombin Inhibitors. *Tetrahedron Lett.* **1992**, *33*, 7667–7670. (d) Hamilton, R.; Walker, B. J.; Walker, B. A Convenient Synthesis of N-Protected Diphenyl Phosphonate Ester Analogues of Ornithine, Lysine and Homolysine. *Tetrahedron Lett.* **1993**, *34*, 2847–2850.

(9) Oleksyszyn, J.; Subotkowska, L.; Mastalerz, P. Diphenyl 1-Aminoalkanephosphonates. *Synthesis* **1979**, 985–986.

(10) Kettner et al., ref 4f, report K_I values with thrombin of 3.6 and <3.6 pM for Boc-D-Phe-Pro-boroArg-$C_{10}H_{16}$ and D-Phe-Pro-boroArg-$C_{10}H_{16}$. Whitting, J. I.; Pouliott, C.; Catalfamo, J. L.; Fareed, J.; Fenton, J. W. II. Thrombin Inhibition with Dipeptidyl Argininals. *Thrombosis Res.* **1988**, *50*, 461–468 report K_I values of 7.2 and 6.5 nM with D-Phe-Pro-Arg-H and Boc-D-Phe-Pro-Arg-H with α-thrombin. Markwardt, F.; Wagner, G.; Stürzebecher, J.; Walsmann, P. N^{α}-Arylsulfonyl-ω-(4-amidinophenyl)-α-aminoalkylcarboxylic Acid Amides. Novel Selective Inhibitors of Thrombin. *Thrombosis Res.* **1980**, *17*, 425–431 report that an 4-AmPhGly derivatives has a K_I value of 61 μM compared to 2.3 μM for the corresponding 4-AmPhe derivative.

(11) Vogel, H. J. ^{31}P-NMR Studies of Phosphoproteins. In *Phosphorus-31 NMR, Principles and Application*; Gorenstein, D. G., Ed.; Academic Press: New York, 1984; pp 105–154.

(12) The ^{31}P NMR chemical shift of camphonyl-HN-CH(CH_2Ph)-P(O)-$(OEt)_2$ is ca. 24 ppm. Bartlett, P. A.; Lamden, L. A. Inhibition of Chymotrypsin by Phosphonate and Phosphonamidate Peptide Analogs. *Bioorg. Chem.* **1986**, *14*, 356–377.

(13) Less than 5% inhibition was observed when human erythrocyte acetylcholinesterase was incubated for 1 h with **3**, **4**, **5**, **6**, and **7** at concentrations of 83, 70, 105, 80, and 60 μM, respectively.

(14) Allen, C. F. H.; van Allan, J. α-Phenylpropionaldehyde. *Organic Syntheses*; Wiley: New York, 1955; Collect. Vol. III, pp 733–734.

(15) Tanaka, T.; McRae, B. J.; Cho, K.; Cook, R.; Frake, J. E.; Johnson, D. A.; Powers, J. C. Mammalian Tissue Trypsin-like Enzymes: Comparative Reactivities of Human Skin Tryptase, Human Lung Tryptase, and Bovine Trypsin with Peptide 4-Nitroanilide and Thioester Substrates. *J. Biol. Chem.* **1983**, *258*, 13552–13557.

(16) McRae, B. J.; Kurachi, K.; Heimark, R. L.; Fujikawa, K.; Davie, E. W.; Powers, J. C. Mapping the Active Sites of Bovine Thrombin, Factor IXa, Factor Xa, Factor XIa, Factor XIIa, Plasma Kallikrein, and Trypsin with Amino Acid and Peptide Thioester: Development of New Sensitive Substrates. *Biochemistry* **1981**, *20*, 7196–7206.

(17) Erlanger, B. F.; Kokowsky, N.; Cohen, W. Preparation and Properties of Two Chromogenic Substrates of Trypsin. *Arch. Biochem. Biophys.* **1961**, *95*, 271–278.

(18) Grassetti, D. R.; Murray, J. F., Jr. Determination of Sulfhydryl Groups with 2,2′- or 4,4′-Dithiodipyridine. *Arch. Biochem. Biophys.* **1967**, *119*, 41–49.

(19) Ellman, G. L.; Courtney, D.; Andres, V.; Featherstone, R. M. A New and Rapid Colorimetric Determination of Acetylcholinesterase. *Biochem. Pharmacol.* **1961**, *7*, 88–95.