Synthesis and Evaluation of Diphenyl Phosphonate Esters as Inhibitors of the Trypsin-like Granzymes A and K and Mast Cell Tryptase

Delwin S. Jackson,[†] Stephanie A. Fraser,[‖] Li-Ming Ni,[†] Chih-Min Kam,[†] Ulrike Winkler,[‖] David A. Johnson,[§] Christopher J. Froelich,[‡] Dorothy Hudig,[‖] and James C. Powers*[,†]

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

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Thirty-six new amino acid and peptidyl diphenyl phosphonate esters were synthesized and evaluated to identify potent and selective inhibitors for four trypsin-like proteases: lymphocyte granzymes A and K, human mast cell tryptase, and pancreatic trypsin. Among five Cbz derivatives of Lys and Arg homologues, Z-(4-AmPhe)^{P}(OPh)$_2$ is the most potent inhibitor for granzyme A, and Z-Lys^{P}(OPh)$_2$ is the best inhibitor for granzyme K, mast tryptase, and trypsin. The amidino P1 residue D,L-(4-AmPhGly)^{P}(OPh)$_2$ was utilized in a series of compounds with several different N-protecting groups and systematic substitutions at P2 in Cbz-AA derivatives and at P3 in Cbz-AA-Ala derivatives. Generally, these phosphonates inhibit granzyme A and trypsin more potently than granzyme K and tryptase. The P2 Thr and Ala dipeptide phosphonates, Cbz-AA-(4-AmPhGly)^{P}(OPh)$_2$, are the most potent inhibitors for granzyme A, and Cbz-Thr-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 2220 M^{-1} s^{-1}) was quite specific with much lower inhibition rates for granzyme K and trypsin (k_{obs}/[I] = 3 and 97 M^{-1} s^{-1}, respectively) and no inhibition with tryptase. The most effective inhibitor of granzyme A was Ph-SO$_2$-Gly-Pro-(4-AmPhGly)^{P}(OPh)$_2$ with a second-order rate constant of 3650 M^{-1} s^{-1}. The most potent inhibitor for granzyme K was 3,3-diphenylpropanoyl-Pro-(4-AmPhGly)^{P}(OPh)$_2$ with a k_{obs}/[I] = 1830 M^{-1} s^{-1}; all other phosphonates inhibited granzyme K weakly (k_{obs}/[I] < 60 M^{-1} s^{-1}). Human mast cell tryptase was inhibited slowly by these phosphonates with Cbz-Lys^{P}(OPh)$_2$ as the best inhibitor (k_{obs}/[I] = 89 M^{-1} s^{-1}). The overall results suggest that scaffolds of Phe-Thr-(4-AmPhe) and Phe-Pro-Lys will be useful to create selective phosphonate inhibitors for granzymes A and K, respectively, and that P4 substituents offer opportunities to further enhance selectivity and reactivity.

Introduction

Cytolytic T lymphocytes (CTL)[1] and natural killer (NK) cells play important roles in the cellular immune system. These immune effector cells recognize and lyse target cells, such as virus-infected cells, tumor cells, or the cells of allografted tissues.[2,3] The mechanism of CTL and NK cell-mediated lysis is an issue that has received a great deal of attention in the past 2 decades. In the process of target cell killing, proteins present in cytoplasmic granules of activated T lymphocytes are released in the proximity of the target cell. The cytoplasmic granules[4,5] contain the pore-forming protein perforin[6,7] and a family of serine proteases termed granzymes. During the killing process, perforin is released and creates pores in the target cell membrane, allowing entry of the granzymes into the cytoplasm of the target cell. The exact role of the granzymes in this process still needs definition. These proteases have been proposed to be involved in killing and/or postkilling processes.[8−10] Granzymes A, B, and K are associated with the characteristic DNA fragmentation which occurs with apoptosis after CTL-mediated killing.[11−13] Granzyme A can activate a cellular thrombin receptor[14] and will also degrade collagen,[15] indicating that it may have several effects that extend beyond the cytolytic reaction.

Studies with peptide substrates have revealed the presence of at least seven distinct lymphocyte granule serine proteases based on their abilities to differentially hydrolyze synthetic substrates. The hydrolytic activities are attributable to granzymes (three chymases, two tryptases, an Asp-ase, and a Met-ase). Murine lymphocyte granules contain at least eight serine proteases (granzymes A to G and M), some of which are identified only by their gene sequence and have unknown substrate specificity. Related serine proteases have been discovered in human lymphocyte granules (which include tryptase, Asp-ase, and chymase activities).[9,16−19] Human granzymes A and K cleave after basic residues, granzyme B cleaves after acidic residues, granzyme M cleaves after methionine, and an undesignated chymase cleaves after large aromatic residues.

While suitable substrates have been discovered, very few papers describe inhibitors for granzymes. Reactive and specific irreversible inhibitors are needed for the granzymes. The three types of granzyme inhibitors reported in the literature are chloromethyl ketones, 4-guanidinobenzoates, and 3-alkoxy-4-chloroisocoumarins.[11,17,20,21] In general, chloromethyl ketones are

* To whom correspondence should be addressed. Tel: (404) 894-4038. Fax: (404) 894-7452. E-mail: james.powers@chemistry.gatech.edu.
[†] Georgia Institute of Technology.
[‖] School of Medicine and The College of Agriculture, Howard Building MC320, University of Nevada, Reno, NV 89557-0046.
[‡] Evanston Hospital, Northwestern University Medical School, Evanston, IL 60201.
[§] Department of Biochemistry, College of Medicine, East Tennessee State University, Johnson City, TN 37614-0581.

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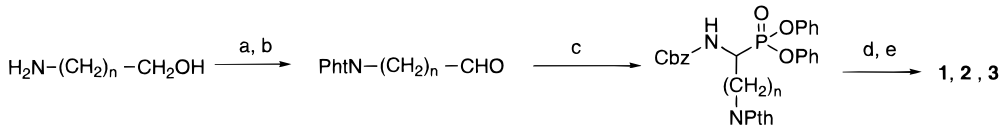
Scheme 1[a]



6a n = 3 **6b** n = 4 **6c** n = 5 | **7a** n = 3 **7b** n = 4 **7c** n = 5 | **8a** n = 3 **8b** n = 4 **8c** n = 5

[a] (a) 145 °C, N_2, phthalic anhydride; (b) DMSO, $(COCl)_2$, DIEA, CH_2Cl_2; (c) $P(OPh)_3$, benzyl carbamate, HOAc, 85−90 °C; (d) NH_2-NH_2, *i*-PrOH; (e) HCl, $CHCl_3$.

potent irreversible inhibitors of serine proteases that alkylate the active site histidine,[22] but a recent report shows that they only weakly inhibit cytotoxic lymphocyte granule serine proteases (k_{obs}/[I] = 3−77 M^{-1} s^{-1} for granzymes A, K, and B).[11] The 4-guanidinobenzoate inhibitors, which are similar to "inverse substrates", are potent inhibitors of granzyme A (k_{obs}/[I] = 540 000 M^{-1} s^{-1}) but are not specific and inhibit other trypsin-like enzymes.[23,24] 3-Alkoxy-4-chloroisocoumarins are potent acylating agents that react with the active-site serine. However, the stability of these acyl-enzymes varied, some of them have short half-lives[20,25] and deacylate to reactivate the granzymes. The limitations of each of these classes of inhibitors illustrate the need for a suitable class of granzyme inhibitors.

Peptidyl (α-aminoalkyl)phosphonate esters are a class of inhibitors that have been used to study serine proteases and shown to be very stable and specific irreversible inhibitors of elastase and various chymotrypsin-like enzymes.[26−30] Serine proteases such as trypsin and thrombin are potently inhibited by peptidyl (α-aminoalkyl)phosphonate diphenyl esters with ornithine-, Lys-, and amidino-containing residues at the P1 site.[31−34] This paper discusses the synthesis and evaluation of Cbz derivatives of Orn, Lys, HomoLys, AmPhGly, and AmPhe diphenyl phosphonate esters (Figure 1). Additionally, a variety of peptidyl (4-AmPhGly)^P$(OPh)_2$ derivatives have been developed to study the structural preferences at P2 and P3 for both granzymes A and K and mast cell tryptase. Inhibitors for granzymes A and K have potential in the treatment of organ transplant rejection[35] and rheumatoid arthritis,[36] and inhibitors for the mast cell tryptase could be used in the treatment of asthma.[37]

Chemistry

The general synthetic pathway for the preparation of the Cbz derivatives of Orn^P, Lys^P, and HomoLys^P is described in Scheme 1. The amino alcohols **6a**−**c** were treated with phthalic anhydride followed by Swern oxidation[38] to give the aldehydes **7a**−**c**. The diphenyl phosphonate esters **8a**−**c** were obtained by treating the aldehydes (**7a**−**c**) with triphenyl phosphite and benzyl carbamate in the presence of acetic acid at 85−90 °C using the Oleksyszyn reaction.[39] Removal of the protecting group with hydrazine in *i*-PrOH solution produced the side-chain unblocked derivatives **1** (Cbz-Orn^P$(OPh)_2$), **2** (Cbz-Lys^P$(OPh)_2$), and **3** (Cbz-HomoLys^P$(OPh)_2$).

Derivatives of (4-AmPhGly)^P$(OPh)_2$ (**14**) were synthesized using the reactions outlined in Scheme 2. The 1-naphthylmethyl carbamate (**10**) was prepared by reacting 1-naphthylmethanol with sodium cyanate in the presence of trifluoroacetic acid.[40] This carbamate and benzyl carbamate were used in the Oleksyszyn reaction with 4-cyanobenzaldehyde and triphenyl phosphite to prepare the diphenyl phosphonate esters **11** and **12.**[34] Subsequent treatment of the nitrile with HCl in $CHCl_3$/EtOH produced the imino ester, which was treated with NH_3/MeOH to give compound **13** and the parent compound Cbz-(4-AmPhGly)^P$(OPh)_2$ (**4**). Deblocking of the Cbz group from compound **4** was accomplished by hydrogenolysis in the presence of HCl to yield compound **14**. The intermediate (4-AmPhGly)^P-$(OPh)_2$ (**14**) was then coupled with the carboxylic acid derivatives, N-blocked amino acid derivatives, or blocked peptide acids using the DCC/HOBt coupling method to give compounds **16**−**36**, **38**, and **40**−**44**. Removal of the side-chain blocking groups from compounds **38** and **40** with TFA yielded compounds **37** and **39**, respectively. Hydrogenolysis of the N-blocking group from compound **34**, followed by treatment with succinic anhydride and 1 equiv of TEA, generated compound **35**. In a similar fashion, **15** was prepared by reaction of succinic anhydride and TEA with compound **14**. The naphthylsulfonyl derivative **22** was made by reacting 1-naphthylsulfonyl chloride with (4-AmPhGly)^P$(OPh)_2$ in the presence of 1 equiv of TEA at room temperature.

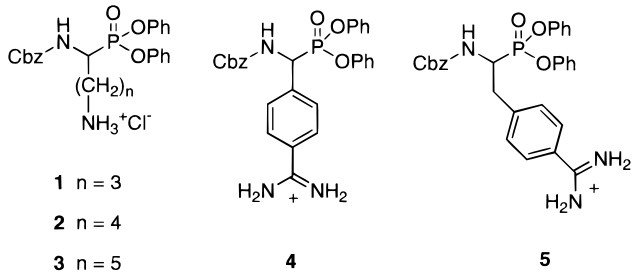
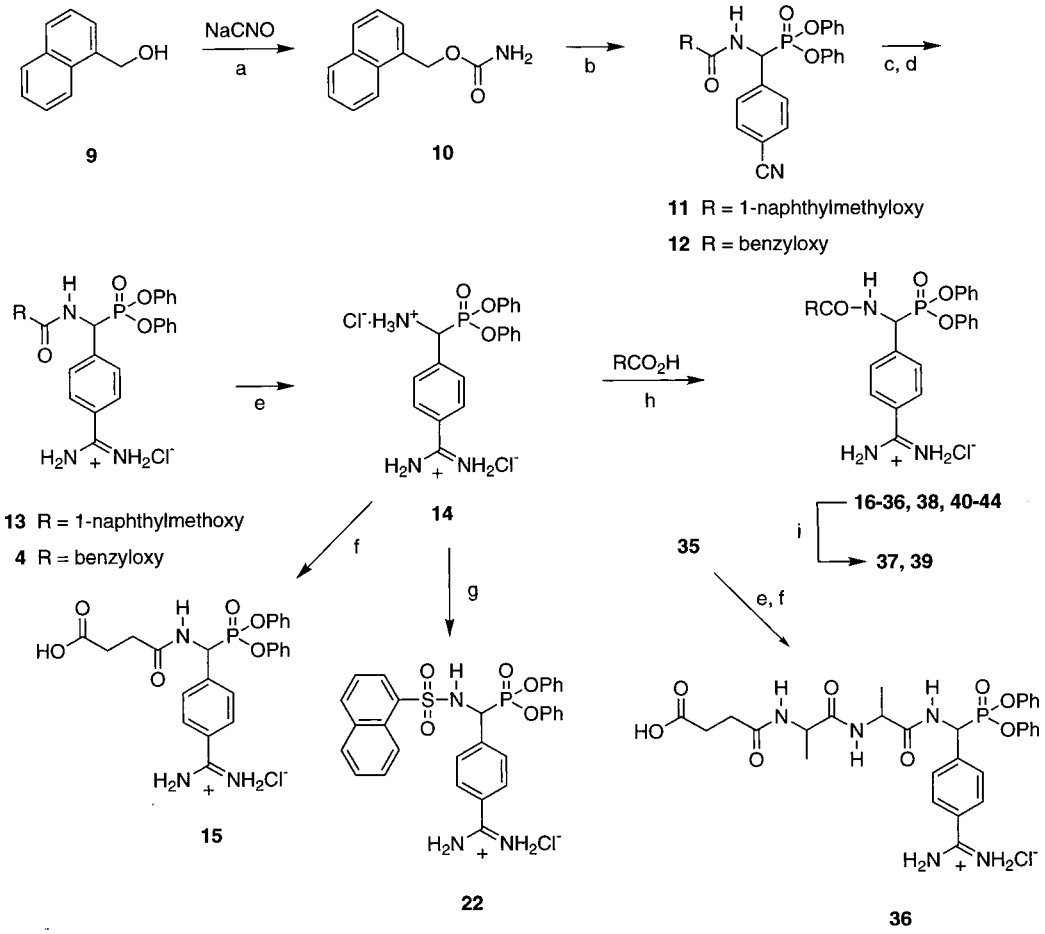


Figure 1. Structures of the amino acid diphenyl phosphonate ester derivatives used to study the primary binding site (S1) of trypsin-like enzymes. Cbz-Orn^P$(OPh)_2$ (**1**) is the phosphonate analogue of ornithine, while Cbz-Lys^P$(OPh)_2$ (**2**) and Homo-Lys^P$(OPh)_2$ (**3**) are related to Lys and homolysine, respectively. Cbz-(4-AmPhGly)^P$(OPh)_2$ (**4**) and Cbz-(AmPhe)^P$(OPh)_2$ (**5**) are phosphonate derivatives of the arginine analogues 4-amidinophenylglycine and 4-amidinophenylalanine, respectively.

Results and Discussion

Evaluation of Compounds. The inhibitory potency of a series of (α-aminoalkyl)phosphonates with basic side chains has been measured with four trypsin-like enzymes: lymphocyte granzymes A and K, mast cell tryptase, and pancreatic trypsin. The results with five Cbz derivatives differing in P1 are reported in Table 1, and the results with various blocking groups or peptide derivatives containing a P1 4-AmPhGly^P$(OPh)_2$ residue are shown in Table 2. The phosphonates used for

Scheme 2[a]



[a] (a) TFA; (b) 4-cyanobenzaldehyde, $P(OPh)_3$; (c) HCl(g), $CHCl_3$/EtOH; (d) NH_3/MeOH, NH_4Cl; (e) H_2, Pd/C, HCl; (f) TEA, succinic anhydride; (g) 1-naphthylsulfonyl chloride, TEA, DMF; (h) RCO_2H = all carboxylic acid derivatives, N-blocked amino acid derivatives, and blocked peptide acids, DCC or EDC, HOBt, TEA; (i) TFA.

Table 1. Inhibition of Trypsin-like Serine Proteases with Amino Acid Diphenyl Phosphonate Derivatives[a]

	$k_{obs}/[I]$ (M^{-1} s^{-1})			
compound	granzyme A	granzyme K	tryptase	trypsin
1, Cbz-(Orn)^P$(OPh)_2$	0.6	NI[c]	NI[c]	NI[c]
2, Cbz-(Lys)^P$(OPh)_2$	1210 ± 59	23	89 ± 1	6240
3, Cbz-(HomoLys)^P$(OPh)_2$	23	1.1 ± 0.1	0.7	21 ± 6
4, Cbz-(4-AmPhGly)^P$(OPh)_2$	1180	3.8 ± 0.7	0.6	2000[b]
5, Cbz-(4-AmPhe)^P$(OPh)_2$	2750 ± 92	2.1 ± 0.2	17 ± 1.4	24[b]

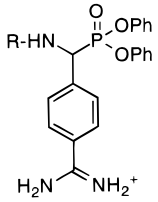
[a] Inhibition constants were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, for trypsin and granzymes A and K or in 0.1 M Hepes, 10% glycerol, 10 mM heparin, pH 7.5, for mast cell tryptase and at 25 °C. The reaction mixtures contained 2% DMSO for trypsin, 7.6% for granzyme A, and 7.1% for granzymes A and K and tryptase. The substrate was Z-Arg-SBzl for granzymes and trypsin and Z-Arg-SBzl or Z-Lys-SBzl for tryptase in the presence of DTNB. The inhibitor concentration ranged from 1.0 to 450 μM. [b] Previously reported data.[34] [c] NI, no inhibition after 25 min of incubation with enzyme.

inhibition studies are racemic mixtures at the phosphonate α-carbon, but only one diastereomer appears to be effective[29] so that true potency will be ~2-fold greater than the numbers reported.

Mechanism of Inhibition. Inhibition of serine proteases by peptidyl phosphonates involves nucleophilic substitution on the phosphorus atom by the oxygen atom of the catalytic Ser195 of the protease (Figure 2). The substitution proceeds through a pentacoordinate phosphorus transition state to give a stable irreversible tetrahedral monophenoxyphosphonyl derivative.[34] The recent X-ray structure of Cbz-(4-AmPhGly)^P$(OPh)_2$ bound to bovine trypsin is consistent with this mechanism and also shows that the second phenoxy group is hydrolyzed during an aging process.[41] The interaction of Cbz-(4-AmPhGly)^P$(OPh)_2$ with trypsin in the crystal structure is stabilized by hydrogen bonds between the amidino nitrogen atoms and the carboxylate oxygens of Asp189 and the oxygens of Gly219, Ser190 ($O\gamma$), and a "buried" water molecule. The inhibited derivatives are extremely stable, and trypsin inhibited by Cbz-(4-AmPhGly)^P$(OPh)_2$ recovered no activity after incubation in a neutral pH buffer for 1 month.

Structural Differences among the Granule Tryptases. Granzymes A and K have several structural features similar to all serine proteases described to date.[42−46] Both granzymes A and K have the characteristic amino-terminal sequence Ile-Ile-Gly-Gly to allow the amino terminus to form an internal salt bridge

Table 2. Inhibition of Trypsin-like Serine Proteases by 4-Amidinophenylglycine Phosphonate Diphenyl Ester Derivatives[a]



		$k_{obsd}/[I]$ (M^{-1} s^{-1})				
compd	R	human granzyme A	rat granzyme A	rat granzyme K	mast cell tryptase	bovine trypsin
4	Cbz	1180		3.8 ± 0.7	0.6	2000[b]
15	Suc	857		0.3 ± 0.07	0.2	45 ± 3
16	*trans*-cinnamoyl	400 ± 10	340 ± 18	3	0.3	110 ± 1
17	3-(2-furyl)acryloyl	1740 ± 41	1740 ± 237	32	NI[c]	360 ± 28
18	3-(2-thienyl)acryloyl	740 ± 18	430 ± 31	4	1	300 ± 22
19	*trans*-3-(3-pyridyl)acryloyl	5	1	3	NI	NI
20	3-phenoxybenzoyl	340 ± 53	470 ± 14	1	NI	50 ± 2
21	2-phenoxybenzoyl	540 ± 3	340 ± 1	1	0.4	670 ± 53
22	1-$NpSO_2$	330 ± 26	30 ± 1	NI	10	170 ± 34
13	1-naphthylmethoxycarbonyl	160 ± 2	90 ± 9	12	NI	30 ± 1
23	Cbz-Ala	1670 ± 11	1270 ± 85	NI	0.8	1850 ± 85
24	Cbz-Val	6.7 ± 0.1		0.7 ± 0.04	21.2 ± 0.2	41 ± 3
25	Cbz-Leu	6.3 ± 0.7		NI	0.3	50 ± 1
26	Cbz-Pro	790 ± 17		34 ± 4	1.2	910 ± 81
27	Cbz-Thr	2220 ± 40		3	NI	97
28	Cbz-Lys	30 ± 1	15 ± 1	1	NI	30 ± 1
29	Cbz-Phe	14 ± 1	8	13	5% inhib	17 ± 1
30	2-phenoxybenzoyl-Pro	610 ± 88		60 ± 5	0.9	7760 ± 310
31	3-phenoxybenzoyl-Pro	330 ± 58		NI	7% inhib	4080 ± 32
32	3,3-diphenylpropanoyl-Pro	250 ± 8		1830 ± 140	NI	2660 ± 40
33	3-phenylpropanoyl-Pro	1510 ± 217		50 ± 2	0.4	1520 ± 27
34	Cbz-Ala-Ala	820 ± 29	480 ± 27	5	2	3620 ± 588
35	Suc-Ala-Ala	260 ± 15		0.3 ± 0.01	0.7	640 ± 36
36	Cbz-Pro-Ala	380 ± 18		5	1	240 ± 10
37	Cbz-Asp-Ala	240 ± 5		6	0.7	2550 ± 145
38	Cbz-Asp(*t*-Bu)-Ala	280 ± 5		NI	3	2000 ± 24
39	Cbz-Lys-Ala	90 ± 9		NI	2	3140 ± 262
40	Cbz-Lys(Boc)-Ala	110 ± 5		NI	8.5 ± 0.4	3100 ± 177
41	Cbz-Phe-Ala	1770 ± 13		4.2 ± 0.3	4	3010 ± 318
42	Boc-D-Phe-Pro	170		56 ± 2	0.4	130[b]
43	Ph-CH_2-SO_2-Gly-Pro	3650 ± 180		87	2	37060 ± 986
44	Cbz-Ala-Ala-Ala	730 ± 15	590 ± 17	7	2	1780 ± 33

[a] Inhibition constants were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, for trypsin and granzymes A and K and in 0.1 M Hepes, 10% glycerol, 10 mM heparin, pH 7.5, for mast cell tryptase and at 25 °C. The reaction mixtures contained 2% DMSO for trypsin, 7.6% for granzyme A, and 7.1% for granzyme K and tryptase. The substrate was Z-Arg-SBzl for granzymes and trypsin and Z-Arg-SBzl or Z-Lys-SBzl for tryptase in the presence of DTNB. The inhibitor concentration ranged from 1.0 to 450 μM. [b] Previously reported data.[34] [c] NI, no inhibition after 25 min of incubation with enzyme.

with Asp194 and the active-site charge relay system, Asp102, His57, and Ser195, at homologous positions flanked by well-conserved peptide segments. Alignment of amino acid sequences shows that granzymes A[43,45] and K[44] and mast cell tryptase[42,46] are similar to bovine trypsin in that each of the enzymes has an Asp189 in the S1 specificity pocket. The molecular model of granzyme A,[43] based on the crystal structure of bovine trypsin,[47] shows a two-amino-acid insertion after Ser217 that extends the entrance to the S1 pocket. Granzyme K and mast cell tryptase do not have this insertion.[42,44] The S2 and S3 subsites derived for granzymes A and K and mast cell tryptase also have some substitutions (Table 3). On the basis of these differences, we expected to observe some differences in the S1, S2, and S3 specificity of the various enzymes. The apparent contributions these substitutions make on the specificity at three subsites of these enzymes are discussed below.

Primary Specificity Pocket. To look at the P1 specificity, we examined a variety of P1 phosphonates. Cbz-blocked derivatives of Lys or AmPhe (an Arg analogue) phosphonates that differed in length by addition or deletion of a CH_2 group were tested (Table 1). The potency of the inhibitors correlates directly with structural differences in the S1 subsites. For instance in the case of trypsin, extension of Cbz-(AmPhGly)^P-$(OPh)_2$ with one CH_2 group to Cbz-(4-AmPhe)$^P(OPh)_2$ decreased the second-order rate constant about 80-fold, from 2000 to 24 M^{-1} s^{-1}. The trypsin structure with Cbz-(4-AmPhGly)$^P(OPh)_2$ shows that the distance between the amidino group and Asp189 is about 3.1 Å,[41] which is similar to the 2.8−3.2-Å distance when Arg is in the S1 pocket (trypsin complexed with Arg15 analogue of the bovine pancreatic trypsin inhibitor).[48] The additional methylene group, as in Cbz-(4-AmPhe)^P-$(OPh)_2$, may create some unfavorable contacts. This trend is more pronounced when examining Cbz-(Lys)^P-$(OPh)_2$ (k_{obs} = 6240 M^{-1} s^{-1}). One-methylene-longer Cbz-(HomoLys)$^P(OPh)_2$ ($k_{obs/[I]}$ = 21 M^{-1} s^{-1}) or one-methylene-shorter Cbz-(Orn)$^P(OPh)_2$ (no inhibition) were both unfavorable. Considering the above data, clearly the proper extension and size at P1 for trypsin is Lys.

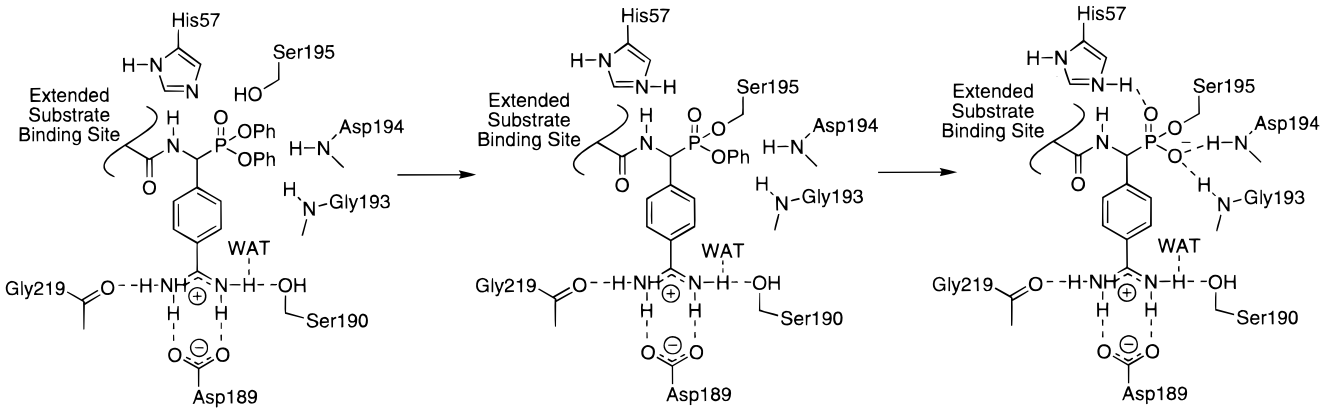


Figure 2. Reaction of a 4-amidinophenylglycine phosphonate diphenyl ester derivative (acyl-AmPhGly^{P}(OPh)$_2$) with a trypsin-like serine protease. The E·I complex (left) reacts to form the monophenoxyserine195 ester (center) which then undergoes slow aging to the tetrahedral monoester (right) in which one phosphonate oxygen is interacting with the oxyanion hole of the serine protease.

Table 3. Influence of Amino Acid Sequence Differences in the S1 Specificity Pockets and at the Extended Substrate Binding Regions of Trypsin-like Serine Proteases on Substrate and Inhibitor Specificity

	substrate recognition subsites[a]									
protease	S1			S1					S2	S3
residue no.[b]	189	215	216	217	218	218a	219	220	99	192
bovine trypsin	Asp	Trp	Gly	Ser	*c*		Gly	Cys	Leu	Gln
granzyme A[d]	Asp	Phe	Gly	Leu	Glu	Asn[e]	Lys	Cys	Arg	Asn
granzyme K[f]	Asp	Gly[g]	Gly	Tyr			Lys	Cys	Gly[h]	Lys
mast cell tryp[i]	Asp	Trp	Asp	Glu			Gly	Cys	Thr	Lys

[a] The subsites refer to those regions of the enzyme which interact with individual residues of a peptide substrate. Thus, the residues in S1 interact with the P1 residue in a substrate. This P1 residue would be a Lys or Arg in the case of trypsin-like enzymes. The primary specificity determinate in S1 is Asp189. The S2 residue often is in a position to interact with the P2 residue of a substrate or inhibitor. Residue 192 is near the S1 pocket and can often interact with the P3 residue of a substrate. [b] Chymotrysinogen numbering. There is no residue 218a in chymotrypsinogen. [c] There is no residue 218 in trypsin. [d] Human granzyme A.[43,45] [e] This insertion extends the entrance to the S1 pocket making a larger opening compared to trypsin. [f] Rat granzyme K (RNK-Tryp-2).[44] [g] This region contains significant changes from trypsin. [h] This region also contains a deletion. [i] Human lung mast cell tryptase α.[42,46]

Also consistent with the predictions based on the modeled structures, granzyme A prefers larger extensions than trypsin at P1. For instance, Cbz-(4-AmPhe)^{P}-(OPh)$_2$ (**5**) with the extra CH_2 group (k_{obs}/[I] = 2750 M^{-1} s^{-1}) is 2-fold more potent than Cbz-4-(AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 1180 M^{-1} s^{-1}). Moreover, compound **5** is a much better inhibitor than any of the Cbz-Lys analogues. However, just as with trypsin, the addition of the CH_2 group when going from Cbz-(Lys)^{P}(OPh)$_2$ to Cbz-(HomoLys)^{P}(OPh)$_2$ results in a significant decrease (52-fold) in the k_{obs}/[I] value and almost a complete loss of inhibition is observed with one less CH_2 group in Cbz-(Orn)^{P}(OPh)$_2$ for granzyme A. Collectively, these observations suggest that the two-amino-acid insertion in the S1 subsite of granzyme A results in an open and accommodating S1 pocket, allowing the enzyme to tolerate bulky extended hydrophobic moieties well.

Distinct structural restrictions also apply to the S1 pocket of granzyme K and human mast cell tryptase. Like trypsin, granzyme K and tryptase do not have the two-amino-acid insertion as in the granzyme A in the S1 pocket. Both granzyme K and tryptase were inhibited best by the P1 Lys derivative **2** (Table 1) and inhibited weakly by 4-AmPhGly (**4**) and HomoLys (**3**) derivatives. Both enzymes were not inhibited by the Orn compound **1**. Tryptase was also inhibited moderately by the 4-AmPhe derivative **5**.

P2 Pocket Specificity. We used two approaches to assess P2 specificity: (1) the N-blocking group of (4-AmPhGly)^{P}(OPh)$_2$ compounds was systematically varied, and (2) a series of Cbz-L-AA-D,L-(4-AmPhGly)^{P}-(OPh)$_2$ analogues were examined. All the *trans*-acryloyl derivatives, compounds **16**−**19**, are similar except for the size of the aromatic ring.

N-Blocking Group. Notably, P2 substitutions result in 100-fold differences in the rate constants for these compounds with Gr A of both species, with (2-furyl)-acryloyl-(4-AmPhGly)^{P}(OPh)$_2$ being the most effective (1740 M^{-1} s^{-1}). Perhaps the potency of this compound is related to the Arg99 side chain hydrogen bonding with the furyl group oxygen, or there may be a small hydrophobic pocket which allows granzyme A to accommodate the furyl ring better. The 2-fold decrease in the second-order rate constants of compounds **18** and **16** versus compounds **17** and **4**, respectively, indicates that the furyl group oxygen and the carbamate oxygen do contribute to the inhibitors' binding to the enzyme. The small 3-arylalkenoyl-containing groups are tolerated better than the bulky N-blocking groups by granzyme A. For rat granzyme A and human granzyme A, the majority of the inhibition constants are the same. The exception is 1-NpSO_2-(4-AmPhGly)^{P}(OPh)$_2$, which is 11-fold less effective with rat granzyme A.

Using the same group of N-blocked derivatives of (4-AmPhGly)^{P}(OPh)$_2$ (see Table 2, compounds **4**, **13**, **15**−**22**), granzyme K and trypsin were not inhibited as well as granzyme A. The best granzyme K inhibitor of the set is (2-furyl)acryloyl-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 32 M^{-1} s^{-1}) which is not surprising since this enzyme has the residue Asn99 in the S2 subsite. For trypsin,

generally, the 3-arylalkenoyl derivatives did not work as well as the parent compound **4**, and compounds with bulky N-blocking groups were not effective inhibitors. All the N-blocked derivatives of (4-AmPhGly)^{P}(OPh)$_2$ inhibited mast cell tryptase poorly.

P2 Amino Acid Derivatives. To further characterize the P2 structural preferences of the tryptase granzymes, one succinyl and a range of Cbz-L-AA-(4-AmPhGly)^{P}(OPh)$_2$ were prepared. The P2 pocket for granzyme A has a distinct preference for small amino acids (Table 2). The inactivation rate constant of Cbz-Ala-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 1670 M^{-1} s^{-1}) is greater than that of the more restricted analogue Cbz-Pro-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 790 M^{-1} s^{-1}), but for this dipeptide series, optimal inhibition is achieved in Cbz-Thr-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 2220 M^{-1} s^{-1}), when the Arg99 in the S2 subsite has the opportunity for H-bonding with the side chain of this compound. Furthermore, Cbz-Thr-(4-AmPhGly)^{P}(OPh)$_2$ had very little effect against granzyme K and trypsin (k_{obs}/[I] = 3 and 97 M^{-1} s^{-1}, respectively) and no inhibition against tryptase, indicating Thr at P2 is selective for granzyme A.

For trypsin, the smaller hydrophobic P2 amino acids provided superior inhibition as they did with granzyme A. For trypsin the smaller less restricted Cbz-Ala-(4-AmPhGly)^{P}(OPh)$_2$ was the most effective with a k_{obs}/[I] = 1850 M^{-1} s^{-1}. The derivatives with large P2 hydrophobic and charged side-chain derivatives behaved as expected, since these side chains would create spatial overlap and unfavorable contacts with the side chain of Leu99. For granzyme K and tryptase, most of these phosphonates were ineffective. Granzyme K and tryptase differed in P2 preferences from the others: Cbz-Pro-(4-AmPhGly)^{P}(OPh)$_2$ was the most effective inhibitor for granzyme K with a k_{obs}/[I] = 34 M^{-1} s^{-1}; Z-Val-(4-AmPhGly)^{P}(OPh)$_2$ was the best dipeptide inhibitor for tryptase with a k_{obs}/[I] = 21 M^{-1} s^{-1}.

P3 Specificity. To examine their P3 structural preferences, granzymes A and K, mast cell tryptase, and trypsin were investigated with analogues of RCO-AA-Ala-D,L-(4-AmPhGly)^{P}(OPh)$_2$. Several N-blocked-Pro-(4-AmPhGly)^{P}(OPh)$_2$ analogues that were developed as thrombin inhibitors based on current literature[49−51] were found to be excellent inhibitors for the enzymes in this study. The derivative Ph-CH$_2$-SO$_2$-Gly-Pro-(4-AmPhGly)^{P}(OPh)$_2$ (**43**) was the most potent of this series against granzyme A (k_{obs}/[I] = 3650 M^{-1} s^{-1}). The Gly makes the P3 extension flexible. Another derivative with a flexible P3 extension, 3-phenylpropanoyl-Pro-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 1510 M^{-1} s^{-1}) (**33**), was not as potent against granzyme A. In the case of the two rigid inhibitors, 2-phenoxybenzoyl-Pro-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 610 M^{-1} s^{-1}) was comparable with Cbz-Pro-(4-AmPhGly)^{P}(OPh)$_2$ (k_{obs}/[I] = 790 M^{-1} s^{-1}), while 3-phenoxybenzoyl-Pro-(4-AmPhGly)^{P}(OPh)$_2$ was 2-fold less effective (k_{obs}/[I] = 330 M^{-1} s^{-1}) against granzyme A.

The trend for flexible vs rigid P3 was also followed, but to a lesser extent, with trypsin. Moderate inhibition was also observed with the flexible P3 compounds **32** and **33** (k_{obs}/[I] = 1520 and 2660 M^{-1} s^{-1}, respectively). Optimal inhibition for trypsin was achieved with another flexible P3 compound, Ph-CH$_2$-SO$_2$-Gly-Pro-(4-AmPhGly)^{P}(OPh)$_2$ (**43**) (k_{obs}/[I] = 37 060 M^{-1} s^{-1}).

The more potent inhibitors for granzyme K contain bulky P3 hydrophobics and P2 Pro. Examining the second-order rate constants for the two phenoxybenzoyl-Pro derivatives, compounds **30** and **31**, illustrates the importance of having the phenyl correctly oriented. Interestingly, the inhibitor with flexibility (**33**) inhibits granzyme K within the same order of magnitude as the rigid compound **30**. Overall, the most potent inactivator of granzyme K is the bulkiest P3 Pro analogue, 3,3-diphenylpropanoyl-Pro-(4-AmPhGly)^{P}(OPh)$_2$, with k_{obs}/[I] = 1830 M^{-1} s^{-1} (**32**).

P4 Specificity. Additionally, the P4 and P5 extended subsite binding of the granzymes was evaluated by comparing Cbz-Ala-Ala-(4-AmPhGly)^{P}(OPh)$_2$ (**34**) to Suc-Ala-Ala-(4-AmPhGly)^{P}(OPh)$_2$ (**35**) and Cbz-Ala-Ala-Ala-(4-AmPhGly)^{P}(OPh)$_2$ (**44**) (Table 2). Examining the second-order rate constant of inhibitor **34** versus inhibitor **35** for granzyme A, a 3-fold decrease in k_{obs}/[I] is observed with P4 Suc, while the tetrapeptide derivative, compound **44** (k_{obs}/[I] = 730 M^{-1} s^{-1}), is more comparable with the tripeptide derivative, compound **34** (k_{obs}/[I] = 820 M^{-1} s^{-1}). Among these tripeptide derivatives with Cbz P4, Cbz-Phe-Ala-(4-AmPhGly)^{P}(OPh)$_2$ was the most effective against granzyme A with a k_{obs}/[I] of 1770 M^{-1} s^{-1}.

For trypsin, changing the N-blocking group from Cbz to Suc in compounds **34** and **35** also resulted in a 6-fold decrease in inhibitory potency. Extending the peptide chain to the tetrapeptide, compound **44**, produced about a 2-fold decrease in k_{obs}/[I] for trypsin, while marginally improving the kinetics for granzyme A. These changes are understandable since this area of the enzymes has the amino acid side chain oriented in a direction going away from the binding pocket. In the tripeptide Cbz series, all of the analogues were found to be potent inhibitors of trypsin, except Cbz-Pro-Ala-(4-AmPhGly)^{P}-(OPh)$_2$ (the one analogue which could possibly be forced to orient unfavorably). For granzyme K and mast cell tryptase, the tripeptides were ineffective.

Comparative Specificity. Each of these enzymes was potently inhibited with some degree of specificity. Specificity is best illustrated with Cbz-Thr-(4-AmPhGly)^{P}(OPh)$_2$ inhibition of granzyme A. This compound potently inactivates granzyme A (k_{obs} = 2220 M^{-1} s^{-1}) while weakly inhibiting granzyme K (k_{obs}/[I] = 3 M^{-1} s^{-1}) and trypsin (k_{obs}/[I] = 97 M^{-1} s^{-1}) and not inhibiting tryptase. The tripeptide derivative Ph-CH$_2$-SO$_2$-Gly-Pro-(4-AmPhGly)^{P}(OPh)$_2$ is the best inhibitor for granzyme A (k_{obs} = 3650 M^{-1} s^{-1}) and also has little activity against granzyme K and tryptase (k_{obs} = 87 and 2 M^{-1} s^{-1}, respectively). However, this compound, Ph-CH$_2$-SO$_2$-Gly-Pro-(4-AmPhGly)^{P}(OPh)$_2$, is also very reactive with trypsin (k_{obs} = 37 060 M^{-1} s^{-1}), making it not absolutely specific. However, it may be possible to obtain selectivity for the granzymes vs trypsin in biological situations since the granzymes are active in granules and susceptible to inhibition. In contrast, trypsin is stored in the pancreas as the inactive zymogen, trypsinogen, which would not be inhibited by phosphonates. When active trypsin is formed in the gut, it is likely that the concentration of the inhibitors would be much reduced due to exhange with blood and gut

fluids, reducing its ability to inhibit trypsin. The best inhibitor of granzyme K is 3,3-diphenylpropanoyl-Pro-(4-AmPhGly)^P(OPh)$_2$, and relative to the best inhibitors for the other trypsin-like enzymes examined, this compound is specific for granzyme K.

Conclusions

Amino acid and peptidyl diphenyl phosphonate esters have been prepared for the study of the relationship of structure to the activities of the granzymes A and K and tryptase. Like trypsin, granzyme K and tryptase show a preference for P1 Lys inhibitor residues consistent with the structure of their narrow hydrophobic S1 pocket. On the other hand, granzyme A is inhibited better by the larger P1 hydrophobic residues such as the 4-amidinophenylalanine derivative. This may be due to a two-amino-acid insertion in the sequence which may enlarge the S1 pocket. Granzyme A is inhibited well by small amino acids at the P2 site; the Thr derivative which has H-bonding character best inhibits granzyme A. For most cases, granzyme A is better inhibited by derivatives containing hydrophobic groups at P3. However, the best overall inhibitor of granzyme A was Ph-CH_2-SO_2-Gly-Pro-(4-AmPhGly)^P(OPh)$_2$, with glycine at the P3 position and Ph-CH_2-SO_2 as an N-blocking group. The conformationally restricted and hydrophobic proline at the P2 site best inhibits granzyme K. The P3 pocket of granzyme K accommodates a large hydrophobic moiety well, similar to the "aryl binding pocket" of thrombin. For tryptase, most of these phosphonates were ineffective except Cbz-(Lys)^P(OPh)$_2$. In summary, this research provides potent inhibitors for both granzymes A and K and suggests scaffolds of Phe-Thr-(4-AmPhGly) and Phe-Pro-Lys for developing better phosphonate inhibitors for granzymes A and K, respectively. Peptide phosphonates have the advantage of being stable in water and gave no apparent side effects when tested in rabbits for 15 min for their anticoagulant effects (C.-M. Kam and J. C. Powers, unpublished results).

Experimental Section

Materials. Benzyl carbamate, triphenyl phosphite, 4-cyanobenzaldehyde, 1-hydroxybenzotriazole (HOBt), 1,3-dicyclohexylcarbodiimide (DCC), and common reagents and solvents were purchased from Aldrich Chemical Co., Milwaukee, WI. The protected amino acid derivatives Z-Ala-OH, Z-Phe-OH, and Z-Lys(Boc)-OH were purchased from Calbiochem-Novabiochem Corp., San Diego, CA. Preparative thin-layer chromatography was performed with plates precoated with 2 mm of silica gel 60 F which were obtained from EM Separations, Gibbstown, NJ. Hepes was purchased from Research Organics Inc., Cleveland, OH; heparin was purchased from Sigma Chemical Co., St. Louis, MO; and 4,4′-dithiodipyridine was purchased from Aldrich Chemical Co., Milwaukee, WI. The substrate Cbz-Arg-SBzl was synthesized as previously described.[52] The substrate Cbz-Lys-SBzl was purchased from Sigma Chemical Co., St. Louis, MO. Melting points were determined on a Mel-Temp II apparatus and are uncorrected. All NMR were recorded on a Varian GEMINI 300 instrument. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Analytical HPLC was performed on a Hewlett-Packard 1090 with a Zorbax Rx-C18 column (4.6 mm × 25 cm). The flow rate was 0.8 mL/min with CH_3CN/H_2O containing 0.1% TFA as eluent.

Synthesis: General Coupling Procedure. The intermediate (4-AmPhGly)^P(OPh)$_2$·HCl was prepared as previously described.[34] All carboxylic acid derivatives, N-blocked amino acid derivatives, and blocked peptide acids were coupled to (4-AmPhGly)^P(OPh)$_2$·HCl using the DCC/HOBt coupling method. Generally, a DMF solution containing 1 mmol of both the amino acid derivative and (4-AmPhGly)^P(OPh)$_2$·HCl was cooled to 0 °C. To this DMF solution were added 1.1 mmol of TEA and 1.2 mmol of HOBt. After the mixture stirred for 15 min at 0 °C, 1.2 mmol of a coupling reagent, DCC or EDC, was added, and the solution was allowed to stir for an additional hour at 0 °C and then overnight at 4 °C. Any solid formed was removed by filtration, and the remaining solution was evaporated to dryness. The residual oil was purified by silica gel column chromatography with $CHCl_3$/MeOH/AcOH (8:2:0.2) as the eluent to purify the final products.

All final products were purified by recrystallization from MeOH/Et_2O or column chromatography and characterized by NMR, mass spectroscopy (MS), and elemental analysis or high-resolution MS, unless otherwise indicated. The ^{1}H NMR spectra indicated that all the derivatives were mixtures of diastereomers because of the pattern observed for the peak at δ 5.9−6.2. A doublet of doublets is usually observed which integrates for 1H, signifying that one doublet comes from one diastereomer while the other originates from a second diastereomer. No attempts were made to resolve the various D,L-(4-AmPhGly)^P(OPh)$_2$ derivatives. The 4-amidino functional group is mostly characterized by two multiplets with chemical shifts in the range of 9.0−9.58 and 9.6−10.5 ppm. Integration shows the presence of two protons in each multiplet.

5-Phthalimidopentanol. A mixture of phthalic anhydride (6.46 g, 43.6 mmol) and 5-aminopentanol (4.5 g, 43.6 mmol) was heated to 145 °C in an open flask for 30 min. A stream of N_2 was applied to expel water vapor. After cooling to room temperature, the reaction residue was dried on vacuum pump. The product was obtained as a colorless oil: yield 100%; ^{1}H NMR ($CDCl_3$) δ 1.4−1.5 (m, 2H), 1.6−1.8 (m, 4H), 3.6 (t, 2H, J = 6.5 Hz), 3.7 (t, J = 7.2 Hz), 7.7 (m, 2H), 7.8−7.9 (m, 2H).

5-Phthalimidopentanal (7b). The preparation of this compound begins by adding oxalyl chloride (4.6 mL, 53.2 mmol) to 60 mL of freshly distilled CH_2Cl_2 containing DMSO (6.9 mL, 96.0 mmol) at −45 °C and stirring for 5 min. The dropwise addition (over 20 min) of phthalic alcohol (10.2 g, 43.6 mmol) in 40 mL of CH_2Cl_2 was followed by an additional 15 min of stirring, and then DIEA (22.8 mL, 130.9 mmol) was introduced. At this time the reaction temperature was warmed to −30 °C, and stirring continued for 30 min. The solvent was removed, and the residue was taken up in 100 mL of EtOAc, filtered to remove any DIEA salt, washed with 6% $NaHCO_3$ and water (3 × 40 mL), and dried over Na_2SO_4. After filtering the solvent was removed yielding the aldehyde, a yellow oil: yield 88%; ^{1}H NMR ($CDCl_3$) δ 1.6−1.9 (m, 4H), 2.5 (t, J = 6.3 Hz, 2H), 3.7 (t, J = 7.8 Hz, 2H), 7.6−7.9 (m, 4H), 9.7 (s, 1H); MS (EI$^+$) m/z 231.1.

Diphenyl *N*-(Benzyloxycarbonyl)amino(4-phthalimidobutyl)methanephosphonate [Cbz-Lys(Pht)^P(OPh)$_2$, 8b]. To a mixture of 5-phthalimidopentanal (8.9 g, 38.5 mmol), triphenyl phosphite (10.1 mL, 38.5 mmol), and benzyl carbamate (5.8 g, 38.5 mmol) was added 100 mL of acetic acid. The solution was stirred at 80−90 °C for 1 h.[39] Acetic acid was removed in vacuo. Crystallizing from methanol yielded the phosphonate product as a white solid: mp 100−102 °C; yield 29.1%; ^{1}H NMR ($CDCl_3$) δ 1.5−2.2 (m, 6H), 3.7 (t, 2H, J = 6.3 Hz), 4.4−4.6 (m, 1H), 5.0−5.3 (m, 2H), 7.0−7.4 (m, 15H), 7.7 (m, 2H), 7.9 (m, 2H). Anal. ($C_{33}H_{31}O_7N_2P$) C, H, N. The analytical data are consistent with reported data.[32]

Diphenyl *N*-(Benzyloxycarbonyl)amino(4-aminobutyl)methanephosphonate Hydrochloride [Cbz-Lys^P(OPh)$_2$, 2]. Cbz-Lys(Pht)^P(OPh)$_2$ (0.6 g, 1.0 mmol) was dissolved in hot *i*-PrOH (60 °C) followed by addition of hydrazine (0.1 mL, 3.0 mmol) via a syringe. The reaction mixture was allowed to stir for 3 h at 60 °C. The solid formed was filtered, and the solvent was removed. The remaining residue was taken up in 100 mL of $CHCl_3$, washed with saturated NaCl (4

× 60 mL), and then dried over Na_2SO_4. After the $CHCl_3$ solution was cooled to 0 °C, the solution was saturated with gaseous HCl. Finally, the solvent was removed, and the residue was triturated in ether to give the product as a white solid: mp 123−126 °C; yield 75%; ^{1}H NMR (DMSO) δ 1.4−2.0 (m, 6H), 2.7−2.8 (m, 2H), 4.2−4.4 (m, 1H), 5.0−5.2 (m, 2H), 7.1−7.4 (m, 15H), 7.8−8.1 (m, 3H); MS (FAB$^+$) *m/z* calcd for $C_{25}H_{29}O_5N_2P$ (M + 1) 469.3. Anal. ($C_{25}H_{29}O_5N_2P$·HCl·0.7H_2O) C, H, N.

4-Phthalimidobutanol. This compound, a colorless oil, was prepared in the same manner as 5-phthalimidopentanol: yield 100%; ^{1}H NMR ($CDCl_3$) δ 1.6−1.7 (m, 2H), 1.7−1.9 (m, 2H), 3.7 (t, J = 7.7 Hz, 2H), 4.35 (t, J = 6.4 Hz, 1H), 7.7−7.8 (m, 2H), 7.8−7.9 (m, 2H), 7.8−7.9 (m, 2H).

4-Phthalimidobutanal (7a). This compound, a colorless oil, was prepared in the same manner as 5-phthalimidopentanal: yield 38%; ^{1}H NMR ($CDCl_3$) δ 2.0−2.1 (m, 2H), 2.5−2.6 (td, J = 6.0, 1.1 Hz, 2H), 3.7−3.8 (t, J = 6.8 Hz, 2H), 7.7−7.8 (m, 2H), 7.8−7.9 (m, 2H), 9.8 (s, 1H); MS (EI$^+$) *m/z* 217.1.

Diphenyl *N*-(Benzyloxycarbonyl)amino(3-phthalimidopropyl)methanephosphonate [Cbz-Orn(Pht)^P(OPh)$_2$, 8a]. This compound was prepared in the same manner as Cbz-Lys(Pht)^P(OPh)$_2$: mp 72−74 °C; yield 47%; ^{1}H NMR ($CDCl_3$) δ 1.5−2.2 (m, 4H), 3.8 (t, J = 6.8 Hz, 2H), 4.5−4.7 (m, 1H), 5.0−5.2 (m, 2H), 7.0−7.4 (m, 15H), 7.7 (m, 2H), 7.9 (m, 2H); MS (FAB$^+$) *m/z* 585.0.

Diphenyl *N*-(Benzyloxycarbonyl)amino(3-aminopropyl)methanephosphonate [Cbz-Orn^P(OPh)$_2$, 1]. This compound was prepared in the same manner as Cbz-Lys^P(OPh)$_2$ with the exception that the product was obtained as an acetate salt: 111−113 °C; yield 66%; ^{1}H NMR (DMSO) δ 1.8 (s, 3H), 1.5−2.1 (m, 4H), 2.6−2.7 (m, 2H), 4.2−4.4 (m, 1H), 5.0−5.2 (m, 2H), 7.1−7.5 (m, 15H), 8.1 (br s, 1H); MS (FAB$^+$) *m/z* 455.0. Anal. ($C_{24}H_{27}O_5N_2P$·$C_2H_4O_2$) C, H, N.

6-Phthalimidohexanol. This compound was prepared in the same manner as 5-phthalimidohexanol from 6-aminohexanol (3.51 g, 30.0 mmol) and phthalic anhydride (4.44 g, 30.0 mmol) to give the product as a brown oil: yield 91%; ^{1}H NMR ($CDCl_3$) δ 1.3−1.8 (m, 8H), 3.6−3.7 (m, 4H), 4.3 (t, J = 6.5 Hz, 1H), 7.7 (m, 2H), 7.8 (m, 2H).

6-Phthalimidohexanal (7c). This compound was prepared in the same manner as 5-phthalimidopentanal from 6-phthalimidohexanol (6.73 g, 27.2 mmol), DMSO (4.3 mL, 59.8 mmol), oxalyl chloride (2.9 mL, 32.7 mmol), and DIEA (14.2 mL, 81.7 mmol) to give the product as a light-brown oil: yield 81%; ^{1}H NMR ($CDCl_3$) δ 1.4 (m, 2H), 1.7 (m, 4H), 2.5 (td, J = 7.1, 1.2 Hz, 2H), 3.7 (t, J = 6.3 Hz, 2H), 7.7−7.9 (m, 4H), 9.8 (s, 1H).

Diphenyl *N*-(Benzyloxycarbonyl)amino(6-phthalimidohexyl)methanephosphonate [Cbz-HomoLys(Pht)^P-(OPh)$_2$, 8c]. This compound was prepared in the same manner as Cbz-Lys(Pht)^P(OPh)$_2$ from 6-phthalimidohexanal (5.38 g, 22.0 mmol), triphenyl phosphite (5.75 g, 22.0 mmol), and benzyl carbamate (3.33 g, 22.0 mmol) to give the product as a light-brown solid: mp 95−96 °C; yield 40%; ^{1}H NMR ($CDCl_3$) δ 1.3−2.1 (m, 8H), 3.7 (t, J = 6.6 Hz, 2H), 4.4−4.5 (m, 1H), 5.1−5.2 (m, 2H), 7.0−7.4 (m, 15H), 7.7 (m, 2H), 7.9 (m, 2H). Anal. ($C_{34}H_{33}O_7N_2P$) C, H, N.

Diphenyl *N*-(Benzyloxycarbonyl)amino(6-aminohexyl)methanephosphonate [Cbz-HomoLys^P(OPh)$_2$, 3]. This compound was prepared in the same manner as Cbz-Lys^P(OPh)$_2$: mp 69−71 °C; yield 59%; ^{1}H NMR ($CDCl_3$) δ 1.2−2.0 (m, 8H), 2.7−2.9 (br s, 2H), 4.4 (m, 1H), 5.0 (m, 2H), 6.0 (d, J = 9.6 Hz, 1H), 7.0−7.3 (m, 15H), 8.0−8.2 (br s, 3H); MS (FAB$^+$) *m/z* 483.1.

1-Naphthylmethyl Carbamate (10). This compound was prepared using methods previously described.[40] Briefly, trifluoroacetic acid (2.22 g, 19.47 mmol) was slowly added to a cooled (0 °C) benzene solution containing 1-naphthylmethanol (1.54 g, 9.73 mmol) and sodium cyanate (1.27 g, 19.47 mmol). The reaction was allowed to go overnight at room temperature. A 20-mL portion of H_2O was added to the mixture, and the organic layer was separated, dried over $MgSO_4$, filtered, and concentrated to give a fluffy white solid. Recrystallization of the crude material from H_2O resulted in the product: mp 126−129 °C; yield 60%; ^{1}H NMR (DMSO) δ 5.4−5.5 (s, 2H), 6.4−6.8 (br s, 2H), 7.4−7.6 (m, 4H), 7.9−8.1 (m, 3H); HRMS (EI$^+$) *m/z* calcd for $C_{12}H_{11}O_2N$ (M + 1) 201.078 97, found 201.079 62.

Diphenyl *N*-(1-Naphthylmethyloxycarbonyl)amino(4-cyanophenyl)methanephosphonate (11). Naphthylmethyl carbamate was used in the Oleksyszyn reaction[39] to prepare 1-naphthylmethyloxycarbonyl-(4-CNPhGly)^P(OPh)$_2$. In this procedure, 4-cyanobenzaldehyde (0.88 g, 6.7 mmol) is reacted with 1-naphthylmethyl carbamate (0.9 g, 4.45 mmol) and triphenyl phosphite (1.38 g, 4.45 mmol) in acetic acid at 85−90 °C for 1.5 h. Removal of the solvent, followed by addition of MeOH and 24 h of cooling at −20 °C gave the product as a white precipitate: mp 146−149 °C; yield 80%; ^{1}H NMR (DMSO) δ 5.5−5.66 (dd, 2H, J = 12.5 Hz), 5.77−5.88 (dd, 1H, J = 10.2 Hz), 6.98−7.02 (d, 4H, J = 8.2 Hz), 7.15−7.25 (m, 2H), 7.25−7.39 (m, 4H), 7.44−7.62 (m, 4H), 7.81−8.08 (m, 7H), 9.0−9.04 (d, 1H, J = 10.2 Hz); HRMS (FAB$^+$) *m/z* calcd for $C_{32}H_{25}O_5N_2P$ (M + 1) 549.1579, found 549.1603. Anal. ($C_{32}H_{25}O_5N_2P$·0.5H_2O) C, H, N.

Diphenyl *N*-(1-Naphthylmethyloxycarbonyl)amino(4-amidinophenyl)methanephosphonate Hydrochloride [Naphthylmethyloxycarbonyl-(4-AmPhGly)^P(OPh)$_2$, 13]. The preparation of this compound proceeds through the imino ester derivative of compound **11**.[34] The synthesis begins by adding compound **11** (1 g, 1.8 mmol) to $CHCl_3$/MeOH (3:2) and cooling to 0 °C. Gaseous HCl was allowed to pass through the solution until saturation was reached, and then the reaction mixture allowed to stir overnight at 4 °C. Removal of the solvent and treatment with Et_2O gave the imino ester. After drying for 24 h, the imino ester and 7 equiv of NH_4Cl were taken up in EtOH and cooled to 0 °C. Addition of 1.5 equiv of NH_3/MeOH (1.8 mL, 1.48 M) was followed by warming to room temperature and stirring for 48 h. The solid material formed was filtered, and the solvent was removed. The residue was resuspended in $CHCl_3$, gaseous HCl was allowed to pass through the solution for about 1 min, and the solvent was concentrated. The final compound was isolated using column chromatography with $CHCl_3$/MeOH/AcOH (8:2:0.2) as the eluent: mp 140−145 °C; yield 10%; ^{1}H NMR (DMSO) δ 5.4−5.7 (dd, 2H), 5.7−5.9 (m, 1H), 6.9−7.7 (m, 15H), 7.7−8.2 (m, 7H), 9.0−9.6 (m, 2H), 9.6−10.4 (s, 2H); HRMS (FAB$^+$) *m/z* calcd for $C_{32}H_{28}O_5N_3P$ (M + 1) 566.1845, found 566.1900. Anal. ($C_{32}H_{28}O_5N_3P$·HCl·3.5H_2O) C, H, N.

Diphenyl *N*-Succinylamino(4-amidinophenyl)methanephosphonate Hydrochloride [Suc-(4-AmPhGly)^P(OPh)$_2$, 15]. Succinic anhydride (33 mg, 0.33 mmol) was added to a stirred DMF solution at 0 °C that contained (4-AmPhGly)^P-(OPh)$_2$·HCl (0.15 g, 0.33 mmol) and was stirred for 15 min. At this time TEA (33.4 mg, 0.33 mmol) was added, and the solution stirred for an additional 30 min at 0 °C and then overnight at room temperature. Any solid that formed was removed by filtration, and the remaining solution was evaporated to dryness. The residual oil was purified by silica gel column chromatography with $CHCl_3$/MeOH/AcOH (7:3:0.2) as the eluent. The crude material was treated with xylene and then toluene after column purification to remove the remaining DMF. The final product was recrystallized from MeOH/Et_2O: mp 90−95 °C; yield 66%; ^{1}H NMR (DMSO) δ 2.38−2.44 (m, 4H), 5.95−6.1 (m, 1H), 7.0−7.1 (m, 4H), 7.15−7.25 (m, 2H), 7.3−7.4 (m, 4H), 7.8−7.9 (m, 4H), 9.2−9.55 (m, 4H); HRMS (FAB$^+$) *m/z* calcd for $C_{24}H_{25}O_6N_3P$ (M + 1) 482.1481, found 482.1541; reversed-phase HPLC (90% MeCN−H_2O) t_R = 2.79 min.

Diphenyl *N*-(*trans*-cinnamoyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [*trans*-cinnamoyl-(4-AmPhGly)^P(OPh)$_2$, 16]: mp 111−118 °C; yield 18%; ^{1}H NMR (DMSO) δ 5.2−5.4 (m, 1H), 6.1−6.4 (m, 1H), 6.6−6.8 (m, 2H), 6.9−7.65 (m, 16H), 7.65−8.0 (m, 3H), 8.4−8.6 (m, 1H), 8.7−8.9 (m, 1H), 9.1−9.7 (m, 3H); MS (FAB$^+$) *m/z* 512.2. Anal. ($C_{29}H_{26}O_4N_3P$·HCl·H_2O) C, H, N.

Diphenyl *N*-(3-(2-furyl)acryloyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [3-(2-furyl)-acryloyl-(4-AmPhGly)^P(OPh)$_2$, 17]: mp 97−105 °C; yield

10%; ^{1}H NMR (DMSO) δ 6.1−6.3 (dd, 1H, J = 9.8 Hz), 6.55−6.63 (m, 1H), 6.7−6.8 (m, 2H), 6.83−6.84 (d, 1H, J = 3.3 Hz), 7.0−7.1 (m, 4H), 7.1−7.25 (m, 3H), 7.3−7.4 (m, 4H), 7.45−7.65 (m, 1H), 7.8−7.95 (m, 4H), 9.2−9.4 (m, 3H), 9.6−9.65 (m, 1H); MS (FAB$^+$) *m/z* 502.4. Anal. ($C_{27}H_{24}O_5N_3P\cdot$HCl) C, H, N.

Diphenyl *N*-(3-(2-thienyl)acryloyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [3-(2-thienyl)acryloyl-(4-AmPhGly)^P(OPh)$_2$, 18]: mp 114−119 °C; yield 17%; ^{1}H NMR (DMSO) δ 6.15−6.25 (dd, 1H, J = 9.0 Hz), 6.65−6.85 (m, 2H), 7.0−7.15 (m, 5H), 7.15−7.25 (m, 2H), 7.3−7.45 (m, 5H), 7.6−7.71 (m, 2H), 7.8−7.9 (m, 4H), 9.5−9.6 (m, 1H), 9.65−10.1 (br s, 3H); MS (FAB$^+$) *m/z* 518.0. Anal. ($C_{27}H_{24}O_4N_3SP\cdot0.5HCl\cdot H_2O$) C, H, N.

Diphenyl *N*-(*trans*-3-(3-pyridyl)acryloyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [*trans*-3-(3-pyridyl)acryloyl-(4-AmPhGly)^P(OPh)$_2$, 19]: mp 103−109 °C; yield 17%; ^{1}H NMR (DMSO) δ 6.15−6.3 (m, 1H), 6.7−6.8 (d, 1H), 6.9−7.6 (m, 14H), 7.7−8.1 (m, 5H), 8.5−8.6 (m, 1H), 8.7−8.9 (m, 1H), 9.1−9.7 (br s, 2H), 9.9−10.0 (m, 1H); HRMS (FAB$^+$) *m/z* calcd for $C_{28}H_{25}O_4N_4P$ (M + 1) 513.1692, found 513.1730; reversed-phase HPLC (90% MeCN−H_2O) t_R = 2.51 min.

Diphenyl *N*-(3-phenoxybenzoyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [3-phenoxybenzoyl-(4-AmPhGly)^P(OPh)$_2$, 20]: mp 85−93 °C; yield 32%; ^{1}H NMR (DMSO) δ 6.2−6.4 (m, 1H), 7.0−7.3 (m, 10H), 7.3−7.5 (m, 8H), 7.6−7.7 (m, 2H), 7.8−8.0 (m, 4H), 9.2−10 (m, 4H); HRMS (FAB$^+$) *m/z* (M + Cl) calcd for $C_{33}H_{28}O_5N_3P$ 578.1845, found 578.1828. Anal. ($C_{33}H_{28}O_5N_3P\cdot$HCl·0.75DMF) C, H, N.

Diphenyl *N*-(2-phenoxybenzoyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [2-phenoxybenzoyl-(4-AmPhGly)^P(OPh)$_2$, 21]: mp 85−93 °C; yield 28%; ^{1}H NMR (DMSO) δ 6.1−6.3 (m, 1H), 6.9−7.1 (m, 5H), 7.1−7.26 (m, 3H), 7.26−7.4 (m, 7H), 7.45−7.6 (m, 3H), 7.75−7.9 (m, 6H), 9.5−10.5 (m, 4H); MS (FAB$^+$) *m/z* 578.0. Anal. ($C_{33}H_{28}O_5N_3P\cdot$HCl·1.5H_2O) C, H, N.

Diphenyl *N*-(1-Naphthylsulfonyl)amino(4-amidinophenyl)methanephosphonate Hydrochloride [1-NpSO$_2$-(4-AmPhGly)^P(OPh)$_2$, 22]. To prepare this compound 1-naphthylsulfonyl chloride (0.15 g, 0.66 mmol) was added to a stirring DMF solution containing (4-AmPhGly)^P(OPh)$_2\cdot$HCl (0.3 g, 0.66 mmol) at 0 °C. After the mixture stirred for 15 min, TEA (0.083 g, 0.82 mmol) was added, and the solution stirred for an additional 30 min at 0 °C and then at 4 °C for 24 h. Any solid that formed was removed by filtration, and the remaining solution was evaporated to dryness. The residual oil was purified by silica gel column chromatography with $CHCl_3$/MeOH/AcOH (9:1:0.1) as the eluent give the product: mp 85−90 °C; yield 16%; ^{1}H NMR (DMSO) δ 6.6−6.8 (m, 1H), 6.9−7.5 (m, 10H), 7.6−7.8 (m, 7H), 7.9−8.3 (m, 5H), 8.5−9.4 (m, 4H); HRMS (FAB$^+$) *m/z* calcd $C_{30}H_{26}O_5N_3PS$ (M + Cl) 572.1409, found 572.1398. Anal. ($C_{30}H_{26}O_5N_3PS\cdot$HCl·0.5$H_2O$) C, H.

Diphenyl *N*-(*N*-benzyloxycarbonylalanyl)amino(4-amidinophenyl)methanephosphonate [Cbz-Ala-(4-AmPhGly)^P(OPh)$_2$, 23]: mp 146−147 °C; yield 14%; ^{1}H NMR (DMSO) δ 1.1−1.3 (m, 3H), 4.25−4.45 (m, 1H), 4.9−5.2 (m, 2H), 5.7−6.1 (m, 1H), 6.9−7.1 (m, 4H), 7.15−7.25 (m, 2H), 7.25−7.4 (m, 9H), 7.5−7.65 (m, 1H), 7.75−7.9 (m, 4H), 9.3−9.5 (m, 1H), 9.9−10.5 (br s, 3H); MS (FAB$^+$) *m/z* 587.2. Anal. ($C_{31}H_{31}N_4O_6P\cdot CH_3CO_2H\cdot H_2O$) C, H, N.

Diphenyl *N*-(*N*-benzyloxycarbonylvalyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [Cbz-Val-(4-AmPhGly)^P(OPh)$_2$, 24]: mp 135−138 °C; yield 30%; ^{1}H NMR (DMSO) δ 0.7−1.0 (m, 6H), 1.9−2.1 (m, 1H), 4.1−4.3 (m, 1H), 5.0−5.1 (m, 2H), 6.0−6.1 (m, 1H), 6.7−6.8 (m, 1H), 7.0−7.6 (m, 16H), 7.8 (d, J = 8.5 Hz, 4H), 9.0−10.5 (br d, 4H); HRMS (FAB$^+$) *m/z* calcd for $C_{33}H_{35}O_6N_4P$ (M + 1) 615.2372, found 615.2434.

Diphenyl *N*-(*N*-benzyloxycarbonylleucyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [Cbz-Leu-(4-AmPhGly)^P(OPh)$_2$, 25]: mp 106−108 °C; yield 20%; ^{1}H NMR (DMSO) δ 0.8−0.9 (m, 6H), 1.5−1.8 (m, 3H), 4.3−4.5 (br s, 1H), 5.6−5.8 (m, 1H), 7.0−7.5 (m, 17H), 7.8−8.1 (m, 4H), 9.1−10.0 (br s, 4H); HRMS (FAB$^+$) *m/z* calcd for $C_{34}H_{37}O_6N_4P$ (M + 1) 629.2529, found 629.2545. Anal. ($C_{34}H_{37}O_6N_4P\cdot$HCl·2.5H_2O) C, H, N.

Diphenyl *N*-(*N*-benzyloxycarbonylprolyl)amino(4-amidinophenyl)methanephosphonate hydrochloride [Cbz-Pro-(4-AmPhGly)^P(OPh)$_2$, 26]: mp 87−95 °C; yield 30%; ^{1}H NMR (DMSO) δ 1.6−1.85 (m, 3H), 2.1−2.3 (m, 1H), 3.25−3.35 (m, 2H), 4.48−4.6 (m, 1H), 4.9−5.1 (m, 2H), 5.9−6.15 (m, 1H), 6.9−7.45 (m, 14H), 7.6−7.64 (d, 1H, J = 8.4 Hz), 7.75−7.95 (m, 4H), 9.1−9.2 (br s, 2H), 9.3−9.4 (br s, 2H), 9.48−9.65 (m, 1H); HRMS (FAB$^+$) *m/z* calcd for $C_{33}H_{33}O_6N_4P$ (M + Cl) 613.2216, found 613.2204. Anal. ($C_{33}H_{33}O_6N_4P\cdot$HCl·DMF) C, H, N.

Diphenyl *N*-(*N*-benzyloxycarbonylthreonyl)amino(4-amidinophenyl)methanephosphonate [Cbz-Thr-(4-AmPhGly)^P(OPh)$_2$, 27]: mp 80−105 °C; yield 17%; ^{1}H NMR (DMSO) δ 1.0−1.2 (2d, 3H), 1.8 (s, 3H), 3.8−3.9 (m, 1H), 4.2−4.3 (m, 1H), 5.0−5.1 (m, 1H), 5.1 (s, 2H), 6.0−6.1 (m, 1H), 7.0−7.4 (m, 15H), 7.8−7.9 (s, 4H), 9.3−10.5 (m, 4H); HRMS (FAB$^+$) *m/z* calcd for $C_{32}H_{33}N_4O_7P$ 617.2165, found 617.2171. Anal. ($C_{32}H_{33}N_4O_7P\cdot0.5CH_3COOH\cdot2H_2O$) C, H, N.

Diphenyl *N*-(*N*-Benzyloxycarbonyllysyl)amino(4-amidinophenyl)methanephosphonate Hydrochloride [Cbz-Lys-(4-AmPhGly)^P(OPh)$_2$, 28]. Blocked Cbz-Lys(Boc)-(4-AmPhGly)$_2$(OPh)$_2$ was prepared and purified using the general procedure from Cbz-Lys(Boc)-OH (0.84 g, 2 mmol) and (4-AmPhGly)^P(OPh)$_2\cdot$HCl (1 g, 2 mmol). Removal of the side-chain protecting group was accomplished by treatment with TFA for 15−20 min at room temperature. The TFA was concentrated, and the remaining material was worked up with Et_2O and purified by preparative TLC using $CHCl_3$/MeOH/AcOH (8:2:0.2) as the solvent: brown oil; yield 20%; ^{1}H NMR (DMSO) δ 1.2−1.7 (m, 8H), 4.2−4.4 (m, 1H), 4.9−5.1 (m, 2H), 6.0−6.15 (m, 1H), 7.0−7.15 (m, 5H), 7.15−7.4 (m, 12H), 7.5−7.55 (d, 1H, J = 8.5 Hz), 7.55−7.75 (m, 1H), 7.8−7.9 (d, 4H, J = 8.2 Hz), 9.4−9.5 (m, 2H), 10.0−11.0 (br s, 4H); HRMS (FAB$^+$) *m/z* calcd for $C_{34}H_{38}O_6N_5P$ (M + 1) 644.2635, found 644.2607; reversed-phase HPLC (90% MeCN−H_2O) t_R = 2.45 min.

Diphenyl *N*-(*N*-benzyloxycarbonylphenylalanyl)amino-(4-amidinophenyl)methanephosphonate hydrochloride [Cbz-Phe-(4-AmPhGly)^P(OPh)$_2$, 29]: mp 89−95 °C; yield 18%; ^{1}H NMR (DMSO) δ 2.6−2.8 (m, 1H), 2.8−2.95 (m, 1H), 4.48−4.7 (m, 1H), 4.85−5.0 (m, 2H), 5.9−6.2 (m, 1H), 6.8−7.5 (m, 20H), 7.5−7.95 (m, 4H), 9.2−9.9 (m, 4H); MS (FAB$^+$) *m/z* 662.7. Anal. ($C_{37}H_{34}O_6N_4P\cdot$HCl·0.25H_2O) C, H, N.

Diphenyl *N*-(*N*-(2-Phenoxybenzoylprolyl))amino(4-amidinophenyl)methanephosphonate Hydrochloride [2-Phenoxybenzoyl-Pro-(4-AmPhGly)^P(OPh)$_2$, 30]. This compound was purified by the same method used with compound **15**: mp 90−95 °C; yield 43%; ^{1}H NMR (DMSO) δ 1.55−1.9 (m, 3H), 2.05−2.3 (m, 1H), 3.2−3.6 (m, 2H), 4.5−4.7 (m, 1H), 5.8−6.15 (m, 1H), 6.7−7.6 (m, 19H), 7.7−7.9 (m, 4H), 9.15−9.5 (m, 4H); HRMS (FAB$^+$) *m/z* calcd for $C_{38}H_{35}O_6N_4P$ (M + 1) 675.2372, found 675.2338. Anal. ($C_{38}H_{35}O_6N_4P\cdot$HCl·0.5DMF) C, H, N.

Diphenyl *N*-(*N*-(3-Phenoxybenzoylprolyl))amino(4-amidinophenyl)methanephosphonate Hydrochloride [3-Phenoxybenzoyl-Pro-(4-AmPhGly)^P(OPh)$_2$, 31]. Purification of this compound was similar to that of compound **15**: mp 104−110 °C; yield 33%; ^{1}H NMR (DMSO) δ 1.5−1.9 (m, 3H), 2.1−2.3 (m, 1H), 3.2−3.6 (m, 2H), 4.5−4.8 (m, 1H), 5.9−6.2 (m, 1H), 6.7−6.9 (m, 1H), 6.9−7.3 (m, 11H), 7.3−7.5 (m, 7H), 7.7−7.9 (m, 4H), 9.1−9.4 (m, 1H), 9.4−9.6 (m, 1H), 9.6−10.0 (br s, 2H); MS (FAB$^+$) *m/z* 675.1. Anal. ($C_{38}H_{35}O_6N_4P\cdot$HCl·$^1/_3H_2O$) C, H, N.

Diphenyl *N*-(*N*-(3,3-Diphenylpropanoylprolyl))amino-(4-amidinophenyl)methanephosphonate Hydrochloride [3,3-Diphenylpropanoyl-Pro-(4-AmPhGly)^P(OPh)$_2$, 32]. Purification of this compound was similar to that of compound **15**: mp 95−98 °C; yield 15%; ^{1}H NMR (DMSO) δ 1.6−1.9 (m, 3H), 2.2−2.3 (m, 1H), 3.15−3.3 (m, 2H), 3.4−3.6 (m, 2H), 4.33−4.55 (m, 2H), 5.9−6.1 (m, 1H), 6.7−6.8 (m, 1H), 6.9−7.5 (m,

18H), 7.6−7.7 (m, 4H), 7.71−7.9 (m, 2H), 8.95−9.05 (m, 2H), 9.25−9.45 (m, 2H); HRMS (FAB$^+$) *m/z* calcd for $C_{40}H_{39}O_5N_4P$ (M + 1) 687.2736, found 687.2770; reversed-phase HPLC (90% MeCN−H_2O) t_R = 2.77 min.

Diphenyl *N*-(*N*-(3-Phenylpropanoylprolyl))amino(4-amidinophenyl)methanephosphonate Hydrochloride [3-Phenylpropanoyl-Pro-(4-AmPhGly)^P(OPh)$_2$, 33]. Purification of this compound was similar to that of compound **15**: mp 61−65 °C; yield 58%; ^{1}H NMR (DMSO) δ 1.6−1.9 (m, 3H), 2.0−2.1 (m, 1H), 2.2−2.3 (m, 1H), 2.7−2.9 (m, 2H), 3.3−3.55 (m, 3H), 4.5−4.7 (m, 1H), 5.85−6.1 (m, 1H), 6.7−6.9 (m, 1H), 6.9−7.45 (m, 14H), 7.5−7.58 (d, 1H, J = 8.2 Hz), 7.77−7.95 (m, 4H), 9.05−9.15 (m, 2H), 9.25−9.5 (m, 2H); HRMS (FAB$^+$) *m/z* calcd for $C_{34}H_{35}O_5N_4P$ (M + 1) 611.2423, found 611.2416; reversed-phase HPLC (90% MeCN−H_2O) t_R = 3.14 min.

Diphenyl *N*-(*N*-benzyloxycarbonylalanylalanyl)amino-(4-amidinophenyl)methanephosphonate hydrochloride [Cbz-Ala-Ala-(4-AmPhGly)^P(OPh)$_2$, 34]: mp 90−95 °C; yield 68%; ^{1}H NMR (DMSO) δ 1.1−1.32 (m, 6H), 4.0−4.2 (m, 1H), 4.4−4.7 (m, 1H), 4.9−5.1 (m, 2H), 5.9−6.1 (m, 1H), 6.9−7.15 (m, 5H), 7.15−7.65 (m, 12H), 7.65−7.7 (m, 1H), 7.8−7.95 (m, 3H), 8.0−8.2 (m, 1H), 9.1−9.6 (m, 4H); MS (FAB$^+$) *m/z* 658.1. Anal. ($C_{34}H_{36}O_7N_5P$·0.5HCl) C, H, N.

Diphenyl *N*-(*N*-Succinylalanylalanyl)amino(4-amidinophenyl)methanephosphonate Hydrochloride [Suc-Ala-Ala-(4-AmPhGly)^P(OPh)$_2$, 35]. The tripeptide Cbz-Ala-Ala-(4-AmPhGly)^P(OPh)$_2$ (1.0 g, 0.14 mmol) was deblocked with 5% Pd/C, H_2, and 1 equiv of HCl. The amine hydrochloride was added to a stirring DMF solution containing succinic anhydride (14 mg, 0.14 mmol) and cooled to 0 °C. After the mixture stirred for 15 min, 1 equiv of TEA (14.1 mg, 0.14 mmol) was added, and the solution stirred an additional 30 min at 0 °C and then overnight at room temperature. Any solid formed was removed by filtration, and the remaining solution was evaporated to dryness. The residual oil was purified by silica gel column chromatography with $CHCl_3$/MeOH/AcOH (7:3:0.2) as the eluent. The crude material was treated with xylene and then toluene to remove the remaining DMF. The final product was recrystallized from MeOH/Et_2O: mp 100−105 °C; yield 50%; ^{1}H NMR (DMSO) δ 1.1−1.3 (m, 6H), 2.3−2.44 (m, 4H), 4.2−4.35 (m, 1H), 4.45−4.57 (m, 1H), 5.95−6.1 (m, 1H), 7.0−7.13 (m, 5H), 7.15−7.25 (m, 2H), 7.3−7.4 (m, 1H), 7.8−7.9 (m, 4H), 8.05−8.17 (m, 2H), 9.2−9.55 (m, 4H); HRMS (FAB$^+$) *m/z* calcd for $C_{30}H_{35}O_8N_5P$ 624.2223 (M + 1), found 624.2255; reversed-phase HPLC (90% MeCN−H_2O) t_R = 2.52 min.

Diphenyl *N*-(*N*-benzyloxycarbonylprolylalanyl)amino-(4-amidinophenyl)methanephosphonate hydrochloride [Cbz-Pro-Ala-(4-AmPhGly)^P(OPh)$_2$, 36]: mp 80−85 °C; yield 10%; ^{1}H NMR (DMSO) δ 1.1−1.4 (m, 4H), 1.7−1.95 (m, 3H), 3.2−3.4 (m, 2H), 4.1−4.4 (m, 2H), 4.95−5.1 (m, 2H), 5.9−6.1 (m, 1H), 6.9−7.12 (m, 1H), 7.17−7.47 (m, 8H), 7.47−7.6 (m, 3H), 7.66−7.7 (d, 4H, J = 8.6 Hz), 7.85−7.9 (m, 1H), 7.93−7.96 (d, 4H, J = 8.2 Hz), 8.15−8.3 (m, 1H), 9.0−9.1 (m, 2H), 9.25−9.4 (m, 2H); HRMS (FAB$^+$) *m/z* calcd for $C_{36}H_{38}O_7N_5P$ (M + 1) 684.2587, found 684.2655; reversed-phase HPLC (90% MeCN−H_2O) t_R = 3.17 min.

Diphenyl *N*-(*N*-Benzyloxycarbonyl(*N*-(*β*-*tert*-butylaspartyl))alanyl)amino(4-amidinophenyl)methanephosphonate Hydrochloride [Cbz-Asp(*t*-Bu)-Ala-(4-AmPhGly)^P(OPh)$_2$, 38]. Purification of this compound was similar to that of compound **15**: mp 105−111 °C; yield 25%; ^{1}H NMR (DMSO) δ 1.1−1.3 (m, 3H), 1.3−1.45 (m, 9H), 4.3−4.4 (m, 1H), 4.4−4.55 (m, 1H), 4.95−5.1 (m, 2H), 5.95−6.1 (m, 1H), 7.0−7.1 (m, 2H), 7.17−7.25 (m, 1H), 7.25−7.45 (m, 7H), 7.45−7.55 (m, 4H), 7.65−7.68 (d, 4H, J = 8.5 Hz), 7.8−7.9 (m, 1H), 7.93−7.96 (d, 3H, J = 8.2 Hz), 9.0 (s, 2H), 9.3−9.45 (m, 2H); HRMS (FAB$^+$) *m/z* calcd for $C_{39}H_{44}O_9N_5P$ (M + 1) 758.2955, found 758.2988; reversed-phase HPLC (90% MeCN−H_2O) t_R = 3.14 min.

Diphenyl *N*-(*N*-Benzyloxycarbonylaspartylalanyl)-amino(4-amidinophenyl)methanephosphonate Hydrochloride [Cbz-Asp-Ala-(4-AmPhGly)^P(OPh)$_2$, 37]. Purification of this compound was similar to that of compound **15**: mp 65−70 °C; yield 60%; ^{1}H NMR (DMSO) δ 1.1−1.3 (m, 3H), 4.3−4.4 (m, 1H), 4.4−4.55 (m, 1H), 4.9−5.1 (m, 2H), 6.0−6.15 (m, 1H), 7.0−7.15 (m, 2H), 7.18−7.5 (m, 10H), 7.5−7.6 (m, 3H), 7.7−7.73 (d, 2H, J = 8.7 Hz), 7.8−7.9 (m, 2H), 7.96−7.99 (d, 2H, J = 7.1 Hz), 8.05−8.1 (m, 1H), 9.1 (s, 2H), 9.3−9.45 (m, 2H); HRMS (FAB$^+$) *m/z* calcd for $C_{35}H_{36}O_9N_5P$ (M + 1) 702.2329, found 702.2332; reversed-phase HPLC (90% MeCN−H_2O) t_R = 3.09 min.

Diphenyl *N*-(*N*-Benzyloxycarbonyl(N-(*ϵ*-*tert*-butyloxycarbonyllysyl))alanyl)amino(4-amidinophenyl)methanephosphonate Hydrochloride [Cbz-Lys(Boc)-Ala-(4-AmPhGly)^P(OPh)$_2$, 40]. Purification of this compound was similar to that of compound **15**: mp 80−86 °C; yield 34%; ^{1}H NMR (DMSO) δ 1.1−1.4 (m, 5H), 1.4−1.7 (m, 15H), 3.88−3.98 (m, 1H), 4.45−4.54 (m, 1H), 4.98−5.02 (m, 2H), 6.0−6.12 (dd, 1H, J = 9.8 Hz), 6.7−6.78 (m, 1H), 6.99−7.02 (d, 1H, J = 8.0 Hz), 7.07−7.10 (d, 1H, J = 8.2 Hz), 7.16−7.25 (m, 1H), 7.25−7.49 (m, 8H), 7.45−7.55 (t, 3H, J = 7.2 Hz), 7.65−7.72 (d, 3H, J = 8.6 Hz), 7.81−7.91 (m, 1H), 7.92−8.0 (d, 3H, J = 8.4 Hz), 8.04−8.1 (m, 1H), 9.0 (s, 2H), 9.3−9.41 (m, 2H); HRMS (FAB$^+$) *m/z* calcd for $C_{42}H_{51}O_9N_6P$ (M + 1) 815.3533, found 815.3586; reversed-phase HPLC (90% MeCN−H_2O) t_R = 3.09 min.

Diphenyl *N*-(*N*-Benzyloxycarbonyllysylalanyl)amino-(4-amidinophenyl)methanephosphonate Hydrochloride [Cbz-Lys-Ala-(4-AmPhGly)^P(OPh)$_2$, 39]. Purification of this compound was similar to that of compound **15**: mp 106−111 °C; yield 50%; ^{1}H NMR (DMSO) δ 1.1−1.4 (m, 6H), 1.4−1.7 (m, 5H), 3.9−4.0 (m, 1H), 4.45−4.55 (m, 1H), 4.98−5.02 (m, 2H), 6.0−6.12 (dd, 1H, J = 9.8 Hz), 6.99−7.02 (d, 1H, J = 8.0 Hz), 7.07−7.10 (d, 1H, J = 8.4 Hz), 7.16−7.25 (m, 1H), 7.25−7.49 (m, 8H), 7.5−7.58 (t, 3H, J = 7.5 Hz), 7.62−7.78 (m, 4H), 7.82−7.92 (m, 2H), 7.96−8.0 (d, 3H, J = 8.6 Hz), 8.04−8.13 (m, 1H), 9.14−9.45 (m, 4H); HRMS (FAB$^+$) *m/z* calcd for $C_{37}H_{43}O_7N_6P$ (M + 1) 715.3009, found 715.3051; reversed-phase HPLC (90% MeCN−H_2O) t_R = 3.13 min.

Diphenyl *N*-(*N*-benzyloxycarbonylphenylalanyl)amino-(4-amidinophenyl)methanephosphonate hydrochloride [Cbz-Phe-Ala-(4-AmPhGly)^P(OPh)$_2$, 41]: mp 120−125 °C; yield 20%; ^{1}H NMR (DMSO) δ 1.1−1.3 (m, 3H), 2.58−2.75 (m, 1H), 2.85−3.0 (m, 1H), 4.15−4.4 (m, 1H), 4.45−4.65 (m, 1H), 4.9−5.0 (m, 2H), 5.9−6.1 (m, 1H), 7.0−7.15 (m, 4H), 7.15−7.4 (m, 15H), 7.4−7.6 (m, 1H), 7.75−7.9 (m, 4H), 8.25−8.4 (m, 2H), 9.35−9.6 (m, 2H), 9.75−10.6 (br s, 2H); HRMS (FAB$^+$) *m/z* calcd $C_{40}H_{40}O_7N_5P$ (M + 1) 734.2744, found 734.2771. Anal. ($C_{40}H_{40}O_7N_5P$·$^1/_4$HCl·3H_2O) C, H, N.

Diphenyl *N*-(Phenylmethylsulfonylglycylprolyl)amino-(4-amidinophenyl)methanephosphonate Hydrochloride [Ph-CH_2-SO_2-Gly-Pro-(4-AmPhGly)^P(OPh)$_2$, 43]. Purification of this compound was similar to that of compound **15**: mp 75−77 °C; yield 25%; ^{1}H NMR (DMSO) δ 1.6−1.8 (m, 2H), 2.0−2.2 (m, 1H), 3.1−3.2 (m, 1H), 3.7−3.8 (m, 2H), 3.85−3.91 (m, 1H), 4.0−4.15 (m, 1H), 4.25−4.4 (m, 2H), 4.55−4.8 (m, 1H), 5.88−6.15 (m, 1H), 6.9−7.4 (m, 15H), 7.74−7.95 (m, 4H), 9.25−9.55 (m, 2H), 9.8−10.4 (m, 2H); HRMS (FAB$^+$) *m/z* calcd for $C_{34}H_{36}O_7N_5PS$ (M + 1) 690.2151, found 690.2466. Anal. ($C_{34}H_{36}O_7N_5PS$·HCl·2H_2O) C, H, N.

Diphenyl *N*-(*N*-Benzyloxycarbonylalanylalanylalanyl)-amino(4-amidinophenyl)methanephosphonate Hydrochloride [Cbz-Ala-Ala-Ala-(4-AmPhGly)^P(OPh)$_2$, 44]. Purification of this compound was similar to that of compound **15**: mp 115−123 °C; yield 30%; ^{1}H NMR (DMSO) δ 1.1−1.32 (m, 9H), 4.0−4.1 (m, 1H), 4.2−4.4 (m, 1H), 4.4−4.6 (m, 1H), 4.95−5.15 (m, 2H), 5.9−6.1 (m, 1H), 6.9−7.15 (m, 5H), 7.2−7.4 (m, 11H), 7.4−7.6 (m, 2H), 7.8−8.0 (m, 5H), 8.0−8.15 (m, 1H), 9.1−9.5 (m, 4H); HRMS (FAB$^+$) *m/z* calcd $C_{37}H_{41}O_8N_6P$ (M + 1) 729.2802, found 729.2852. Anal. ($C_{37}H_{41}O_8N_6P$·0.66HCl·1.33DMF) C, H, N.

Enzyme Inhibition Kinetics. 1. Enzymes. Human granzyme A was purified as previously described.[53] Bovine trypsin was obtained from Sigma Chemical Co., St. Louis, MO. Rat granzymes A and K were prepared from the granules of RNK-16 leukemia cells.[54] Granules were prepared[30] and extracted by freeze−thawing with 1 M NaCl followed by

removal of debris by centrifugation at 10000*g*. Three milliliters of the extract (1−3 mg of protein) was separated by size-exclusion chromatography using a 2.5- × 100-cm column of SuperDex200 (Pharmacia, Piscaway, NJ) in a buffer of 20 mM HEPES (Sigma Chemical Co., St. Louis, MO), pH 7.5, with 1 M NaCl, 0.05% (w/v) NaN_3, 10% betaine (w/v; Sigma), and 0.1 mM EGTA. This initial step with high salt was required to dissociate the granzymes from proteoglycan. Then fractions containing each of the ∼45-kDa Gr A and ∼30-kDa Gr K peaks were pooled from several SD200 runs, diluted with a MonoS buffer of 50 mM MES (Sigma), pH 6, containing 0.05% NaN_3, 10% betaine (w/v; Sigma), and 20% ethylene glycol (enzyme grade; Fisher Biotech, Fair Lawn, NJ) to 0.5 M NaCl, and separated by cation-exchange chromatography with a Pharmacia MonoS 0.5- × 5-cm column using a gradient of 0.5−1.0 M NaCl in the MonoS buffer. Enzyme purity was determined by SDS−PAGE and the identity confirmed by amino acid sequencing.

Mast cell tryptase was purified from human lung tissue. Human lung tissue was obtained from the National Disease Research Institute (Philadelphia). Only the "high-molecular-weight form" of human lung tryptase as described previously[55] was used in these studies because it represents the majority of the tryptase in lung mast cells and adequate quantities of the other form were not available. This material is the same as previously characterized[56] and probably the same as purified by Schwartz.[57] The enzyme was stored in 2 M NaCl in 10 mM MES, pH 6.1, 10% glycerol, 0.01% NaN_3 at 4 °C. Mast cell tryptase protein concentrations were based on $E^{1\%}_{280} = 28$,[56] and enzyme active-site concentrations were determined by MUGB titration.[58] Enzyme concentrations and molar ratios refer to enzyme active sites (monomers). Only enzyme preparations with greater than 90% active sites were used in this study. The enzyme stock solutions were determined to be heparin-free by assessing the presence of heparin with toluidine-blue.[59]

2. Incubation Method. Inhibition of trypsin was initiated by adding 10 μL of inhibitor (0.055−5.0 mM in DMSO) to 0.49 mL of buffer (0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5) containing 10 μL of a trypsin stock solution at 25 °C. At different times, aliquots of 10 μL were withdrawn and added to the assay mixture, and the residual enzymatic activity was measured at 410 nm with a Beckman DU-650 spectrometer. The trypsin stock solution was 5 μM in 1 mM HCl (pH 3) and was stored at −20 °C prior to use. The assay mixture contained 720 μL of buffer, 35 μL of 8 mM Cbz-Arg-SBzl in DMSO,[52] and 35 μL of 16 mM DTNB in DMSO.[60]

The tryptase inhibition reactions were initiated by adding 25 μL of inhibitor (0.5 mM) to 300 μL of buffer (0.1 M Hepes, 10% glycerol, 10 mM heparin, pH 7.5) containing 25 μL of a stock enzyme solution at 25 °C. Aliquots of 50 μL were withdrawn at various time intervals and added to the assay mixture, and the residual enzymatic activity was measured at 405 nm. The concentration of tryptase stock solution was 7 μM. The enzyme stock solution was prepared in 10 mM MES, 2 M NaCl, 10% glycerol, and 0.01% NaN_3, pH 6.1. The assay solution contained 1 mL of buffer, 10 μL of 10 mM Cbz-Lys-SBzl (or Cbz-Arg-SBzl) in DMSO, and 10 μL of 16 mM DTNB in DMSO.

Human recombinant and rat granzyme A and native rat granzyme K inhibition reactions were initiated by adding 10 μL of inhibitor (0.014−5.0 mM in DMSO) to 100 μL of buffer (0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5), to which 10−30 μL of a stock enzyme solution had been previously added at 25 °C. The enzyme stock solutions were 0.3, 0.41, and 0.33 μM in 0.15 M NaCl for human and rat granzymes A and granzyme K, respectively. All the enzyme stock solutions were stored at −20 °C prior to use. Aliquots of 20 (granzyme A) or 30 (granzyme K) μL were withdrawn at various time intervals from the inhibition mixture, and the residual enzymatic activity was measured spectrophotometrically at 405 nm with a Molecular Devices microplate reader. The assay solution contained 200 μL of buffer, 10 (granzyme A) or 20 (granzyme K) μL of 8 mM Cbz-Arg-SBzl in DMSO, and 10 μL of 16 mM DTNB in DMSO.

Pseudo-first-order inactivation rate constants (k_{obs}) were obtained from plots of ln v_t/v_o vs time. Each k_{obs} value was calculated from 5−10 activity determinations which extended to 2−3 half-lives for that particular inhibition reaction, except for tryptase. Very slow inhibition against tryptase was observed so the activity determination was only extended to 1 half-life (more than 1 h). Control experiments were carried out in the same manner as described above, except DMSO was added in place of the inhibitor solution in DMSO. For trypsin three separate k_{obs} values per inhibitor were determined, while for granzymes A two and for granzyme K one or two k_{obs} values were determined.

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(1) Abbreviations: (4-AmPhGly)^P$(OPh)_2$, diphenyl amino(4-amidinophenyl)methanephosphonate or diphenyl 4-amidinophenylglycinephosphonate; (4-AmPhe)^P$(OPh)_2$, diphenyl 1-amino-2-(4-amidinophenyl)ethanephosphonate or diphenyl 4-amidinophenylalaninephosphonate); Boc, *tert*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; (CNPhGly)^P$(OPh)_2$, diphenyl amino(4-cyanophenyl)methanephosphonate or diphenyl 4-cyanophenylglycinephosphonate; CTL, cytotoxic T lymphocyte; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIEA, diisopropyl ethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; FAB, fast atom bombardment; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HRMS, high-resolution mass spectroscopy; HOBt, 1-hydroxybenzotriazole; HomoLys^P$(OPh)_2$, diphenyl 1,6-diaminohexanephosphonate; HPLC, high-performance liquid chromatography; Lys^P$(OPh)_2$, diphenyl 1,5-diaminopentanephosphonate; MES, 2-(4-morpholino)ethanesulfonic acid; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; NK, natural killer; Orn^P$(OPh)_2$, diphenyl 1,4-diaminobutanephosphonate; SBzl, thiobenzyl ester; Suc, succinyl; TEA, triethylamine; TFA, trifluoroacetic acid; 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]-2-(4-amidinophenyl)ethanephosphonate, which is related to 4-amidinophenylalanine, is abbreviated as Cbz-(AmPhe)^P$(OPh)_2$.

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