O]H_2O, 400 μM **9**, and 5% DMF. The mixture was maintained at room temperature for 1.5 (21H3) or 21 h (2H6) and quenched to pH ~4 with 23% $HClO_4$. The resulting acid was isolated for MS analysis as described above.

Hydrolysis of Amide 2a by NPN 43C9 in [^{18}O]H_2O and Incubation of Amide 10 with NPN 43C9 in [^{18}O]H_2O. The reaction mixtures contained NPN 43C9 (7.8 μM), *p*-nitro amide **2a** (2 mM), and either 10% (v/v) of 95% [^{18}O]H_2O (4.8 M) or 18% of 95% [^{18}O]H_2O (8.8 M) in ATE, pH 9.0. The mixtures were incubated at 37 °C for 7 days, at which point the 10% [^{18}O]H_2O reaction had proceeded to 38% completion and the 18% [^{18}O]H_2O reaction had proceeded to 36% completion. The reactions were quenched with 23% $HClO_4$ to pH ~4 and acid **9** and amide **2a** were isolated by HPLC for MS as described for the hydrolysis of ester **1a** in [^{18}O]H_2O. The uncatalyzed hydrolysis was performed in 20% [^{18}O]H_2O in a similar manner, with the exceptions that no antibody was present and the concentration of **2a** was 800 μM.

A 2 mM solution of the *m*-nitro amide **10** in 10% (v/v) [^{18}O]H_2O in ATE (pH 9, I = 0.1) was also incubated with NPN 43C9 (7.8 μM) at 37 °C as described above for the reaction of *p*-nitro amide **2a**. The reaction was quenched after 7 days with 23% $HClO_4$. Analysis by HPLC indicated that a negligible amount (<1 μM) of acid **9** was obtained. The *m*-nitro amide was then isolated by HPLC and analyzed for ^{18}O content as described above. The results of these experiments are shown in Table I.

pH-Rate Studies. pH-rate profile assays for antibody 43C9 were conducted in the following manner. Velocities were determined spectrophotometrically by measuring the initial linear absorbance change at 404 nm (release of *p*-nitroaniline). Antibody 43C9 [10 μM; average value determined from a bicinchoninic acid (BCA) assay and absorbance at 280 nm, assuming a molecular weight of 150000 for IgG] was preincubated at 37 °C (ATE buffer, pH 7.9-10.62, I = 0.1) and reactions were initiated by addition of amide **2a** in DMF to give a substrate concentration of 200-2000 μm, (total organic phase was 5%). Matching ATE buffers in 99.7% D_2O were prepared analogously as described above in water. pD was determined by adding +0.4 unit to the meter reading. Assays in D_2O were conducted and monitored in a similar manner as described above for assays conducted in H_2O.

Data Analysis for pH-Rate Profile Assays. The pH and pD variation of the rate constants for antibody 43C9 was fitted to eq 1 by the HA-

$$\log y = \log [C/(1 + H/K)] \quad (1)$$

BELL program of Cleland.[19] This program assumes a drop in kinetic constant only at a low pH, where y data input is either in the form V_{max} or V_{max}/K.

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Differential Binding Energy: A Detailed Evaluation of the Influence of Hydrogen-Bonding and Hydrophobic Groups on the Inhibition of Thermolysin by Phosphorus-Containing Inhibitors

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Abstract: Two series of phosphorus-containing peptide analogues, **3** (Cbz-Gly-ψ($PO_2^-CH_2$)Leu-Xaa) and **4** (Cbz-Gly-ψ-(PO_2^-NH)Leuψ[CO_2]Xaa), have been synthesized and evaluated as inhibitors of the zinc endopeptidase thermolysin. In comparison with the previously reported phosphonamidates **1**, the phosphinates **3** lose only 0.1 kcal/mol in binding affinity, whereas the depsipeptides **4** are bound 2.7 kcal/mol more weakly; these values are contrasted to the 4.0 kcal/mol reduction in binding affinity observed for the phosphonates **2** (Cbz-Gly-ψ(PO_2^-O)Leu-Xaa) in comparison to **1** (Cbz-Gly-ψ(PO_2^-NH)Leu-Xaa). The observed effects are interpreted through consideration of the differences in active-site and solvent interactions. For the comparison between the diamides **1** and the depsipeptides **4**, a full accounting of the balance between these interactions can be approached. The arylphosphonates **5** (Aryl-ψ(PO_2^-O)Leu-Leu) were synthesized and evaluated to investigate the importance of phosphonate basicity on the overall binding affinity of these zinc-coordinating inhibitors; the inhibitor K_i values were found to be independent of phosphonate pK_a, indicating that the basicity of the phosphonate moiety exerts counterbalancing effects on the energies of zinc coordination and solvation. For analysis of the influence of structural variations on observed affinity, the definition of "differential binding energy" is introduced as a practical alternative to the concept of "intrinsic binding energy".

The principles of molecular recognition are important for understanding the selectivity of biological transformations and for attempts to mimic such processes through the design of artificial inhibitors and catalysts. Quantitative information on the energy of interaction between protein binding sites and the molecules that associate with them is indispensible in this regard, since this energy is the currency that governs all such transactions. The contribution that an individual functional group can make to the overall energy of interaction can be determined by comparison of related systems in which the functional group has been removed from either the ligand[1] or the protein,[2] and it is most accurately assessed when structural information on the complex is available.

The bacterially derived endopeptidase thermolysin has proven to be an advantageous system for the detailed evaluation of protein-ligand binding effects. Along with the mammalian digestive enzyme carboxypeptidase A, thermolysin is one of the prototypical members of the class of zinc proteases, and it has played a prominent role in the design of inhibitors for this medicinally important class of enzymes.[3] The selectivity of ther-

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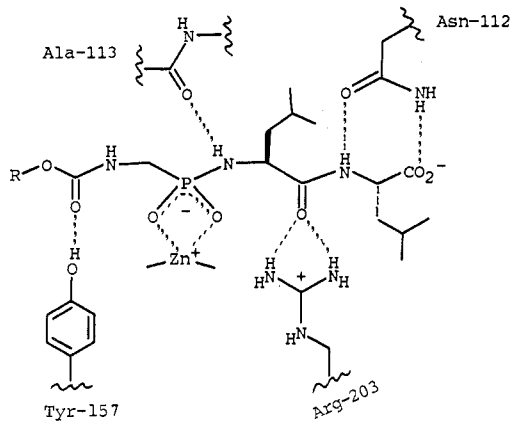
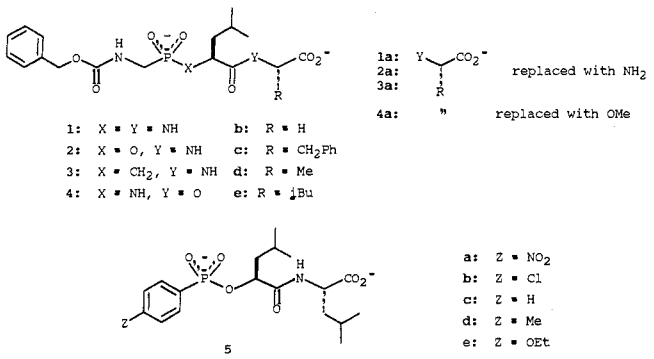


Figure 1. Interactions between the inhibitors and thermolysin in the vicinity of the phosphorus and amide linkages.

molysin for a range of substrates has been determined,[4] and the structural[5] as well as energetic features[6,7] of its interaction with a number of potent inhibitors have also been investigated.

Prominent among these zinc protease inhibitors are the phosphorus derivatives **1**, in which the carbonyl group of an oligopeptide is replaced with a tetrahedral phosphorus oxy anion. These



phosphonamidates have been shown to be "transition-state analogues"[8] by virtue of the correlation between the K_i values of a series of inhibitors and the K_m/k_{cat} values of the corresponding substrates.[7a] The structure of the thermolysin complex of one of these inhibitors, Cbz-Gly-$\psi(PO_2^-NH)$Leu-Leu (**1e**),[9] was determined by Matthews et al.,[10] and the specific, polar interactions depicted in Figure 1 were identified. Subsequently, a related set of phosphonate esters **2** was prepared and evaluated.[7b] While replacement of the phosphorus-linked NH moiety with an oxygen atom results in a uniform decrease of 4 kcal/mol in binding affinity across the series,[11] the structure of the complex with Cbz-Gly-$\psi(PO_2^-O)$Leu-Leu (**2e**) is essentially superimposable with that of **1e**,[10] in spite of the inability of the ester to donate a hydrogen bond to the carbonyl oxygen of Ala-113 (see Figure 1). The loss of this hydrogen-bonding interaction was judged to be the major factor in the reduced binding affinity of the esters, after consideration of the differences in solvation and metal coordination strength between the phosphonamidate and phosphonate moieties.[7b] This interpretation was supported through free energy perturbation calculations by Bash et al.,[11] who were able to reproduce the 4 kcal/mol difference in binding energy for the amides and esters **1** vs **2** by separate calculations of the differences in the energy of their interactions with the enzyme active site and with solvent.

Recently, Kollman and Merz carried out similar calculations in a predictive sense, computing what the effect would be of replacement of the NH group with a methylene unit.[12] Although the phosphinates **3** are also unable to hydrogen bond to Ala-113, it was predicted that they would bind to thermolysin with almost the same affinity as the phosphonamidates **1**, a prediction that was borne out by experiment. Independently, Grobelny et al. reported similar observations and suggested a reevaluation of the roles of hydrogen bonding, zinc coordination, and solvation effects in the binding of these inhibitors.[13] They concluded that the solvation effects are unimportant in accounting for the differences in affinity between the various phosphorus derivatives. They suggested instead that the basicity of the phosphorus moiety, through the influence of this basicity on the strength of the phosphonate–zinc coordination, accounts for most of the difference in binding affinities and that the hydrogen bond plays a minor role.

Although information on the relationship between phosphorus oxy anion basicity and the strength of metal ion coordination is available,[14] detailed analysis of these effects has been complicated by the fact that the energy of solvation of such anions is not accessible experimentally. In the latter respect, a more readily analyzed substitution would be a carboxylic ester for amide replacement, since the relevant difference in solvation energy for these functional groups is known experimentally.[15] In this report, we address three topics: (1) the complete description of the synthesis and evaluation of the phosphinate inhibitors **3**; (2) the design, synthesis, and evaluation of a series of arylphosphonates **5** to determine the effect of phosphonate basicity directly; and (3) the synthesis and evaluation of the depsipeptides **4** to investigate the effect of an ester for amide substitution in the context of a different set of hydrogen-bonding interactions.

Synthesis of Inhibitors

The synthesis of the phosphinate inhibitors, **3**, is illustrated in Scheme I. Conversion of the phosphinic acid **6**[16] to the bis(trimethylsilyl)phosphonite derivative **7**,[17] followed by conjugate addition to methyl α-(isobutyl)acrylate[18] and esterification with diazomethane, affords phosphinate **8** in 87% yield, This material is resolved by hydrolysis with subtilisin Carlsberg (pH 8.1, borate buffer).[19] A mixture of diester **8R** and the acids **9A** and **9E** is isolated from the reaction mixture, which implies that the enzyme hydrolyzes specifically the diastereomers with the *S* configuration at carbon, corresponding to the desired L stereochemistry. Cleavage of the phosphinate ester, as a result of intramolecular catalysis by the carboxylate moiety,[20] cannot be completely

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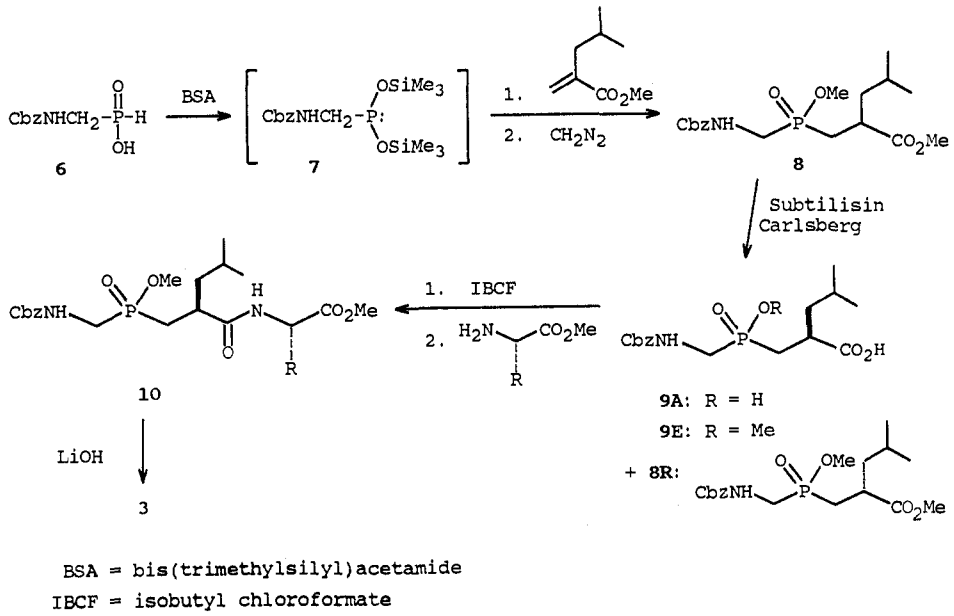
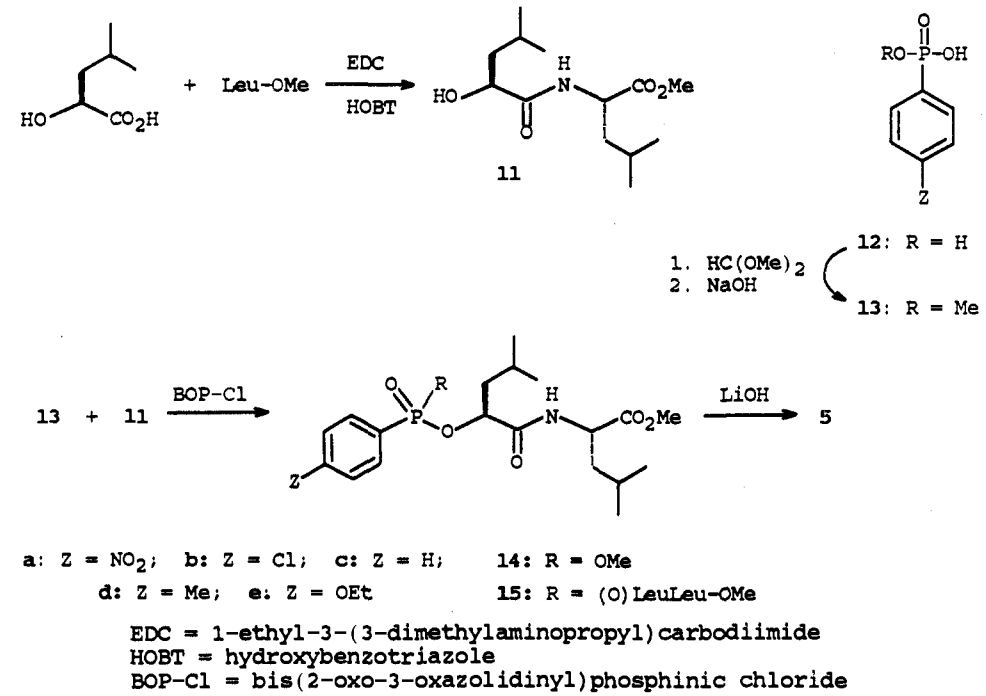
Scheme I



Scheme II



suppressed, although we investigated a variety of buffers for the resolution [Tris, phosphate, and triethylammonium bicarbonate (TBK)]. The monoester **9E** and diacid **9A** can be obtained in a ratio of 3:1 and separated by anion-exchange chromatography; the major fraction is concentrated, acidified, and extracted at 0 °C to give the monoester **9E** in pure form. When the material is isolated directly by lyophilization, only the diacid **9A** is observed, presumably as a result of the acidification that occurs on evaporation of triethylamine from an acidic salt.

A number of procedures were investigated to couple **9E** with the requisite amino acid esters. Condensation of **9E** with alanine methyl ester using a conventional diimide procedure[21] with 1-hydroxybenzenetriazole hydrate (HOBT) gives the peptide **10d** as a mixture of four diastereomers, which suggests either that the phosphinate derivative is epimerized under the conditions or that the enzymatic resolution is not stereospecific. However, activation of **9b** with isobutyl chloroformate at −5 °C, followed by coupling to alanine methyl ester at −30 to −5 °C, affords only two diastereomers of **10d**. Hydrolysis of both of these materials with LiOH yields a single product, **3d**, demonstrating that the subtilisin-catalyzed hydrolysis is indeed stereospecific. The remaining phosphinates **3** were synthesized in this manner with no evidence of racemization.

Synthesis of the arylphosphonates **5** is shown in Scheme II. Although a monomethyl phosphonate can be esterified via the methyl phosphonochloridate,[22] the yield for this process is typically

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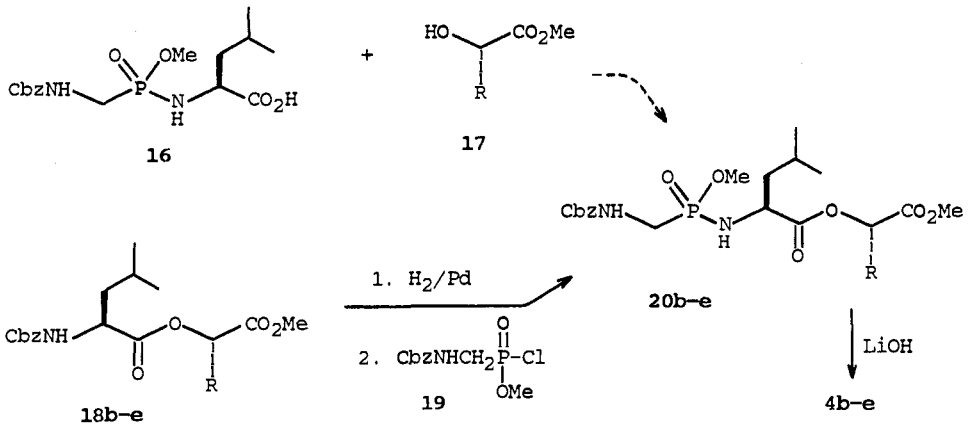
Scheme III



20–40%; hence, we investigated more efficient coupling procedures. When methyl phenylphosphonate (**13c**) and the hydroxy derivative **11** are stirred with bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl), a product mixture is obtained with the symmetrical dialkyl ester **15c** as the major compound and the starting material and the anticipated diester **14c** as minor products. A number of mechanisms can be proposed for this double esterification, including nucleophilic demethylation of the unsymmetrical diester **14** followed by a second BOP-Cl-induced coupling, or intramolecular catalysis of an ester-exchange reaction via a cyclic anhydride similar to those postulated for related phosphonyl peptide derivatives.[20] Because replacement of the triethylamine with less nucleophilic bases such as *N*-methylmorpholine or imidazole gives similar results, we infer that the amine is not responsible for demethylation. The problem of dialkylation was overcome simply by employing 2 equiv of alcohol and BOP-Cl and driving the formation of symmetrical diester **15** to completion by holding the reaction mixture at reflux overnight. These compounds are isolated after chromatography in 30–80% yield, and they are converted into the desired diacids **5** by hydrolysis with excess aqueous LiOH in acetonitrile, followed by ion-exchange chromatography.

The first route investigated for synthesis of the phosphonamidate tripeptide analogues **4b–e** was the more versatile, in that the varied residue was incorporated late in the synthesis by coupling the α-hydroxy esters **17** with the phosphonamidate dipeptide **16** (Scheme III). However, the lability of **16** complicates the esterification reaction sufficiently that the less general route via the depsipeptides **18** proved to be more practical. Conventional diimide coupling affords the esters **18**, which are deprotected by hydrogenolysis immediately prior to addition to the phosphonochloridate **19**.[23]

Deprotection of the dimethyl esters **20** requires carefully controlled conditions, since both acid-sensitive (the P–NH linkage) and base-sensitive moieties (the internal ester) are present in the molecule. While conventional demethylation procedures (excess LiOH, lithium *n*-propyl mercaptide, trimethylsilyl bromide) proved unsatisfactory in this case, the desired product can be obtained in good purity by hydrolysis with a deficiency of LiOH (1.5–1.8 equiv). The byproduct from incomplete hydrolysis is the phosphonamidate methyl ester, which can be separated readily from the desired compound by anion-exchange chromatography. The synthesis of the phosphonamidate Cbz-Gly-ψ(PO_2^-NH)Leu-OMe (**4a**) was straightforward; leucine methyl ester is coupled to the chloridate **19**, and the phosphorus methyl ester of the product is cleaved selectively with trimethylsilyl bromide (TMS-Br).[24]

Results and Discussion

The inhibition constants, K_i, of the phosphorus-containing tripeptide analogues **1–4** are listed in Table I, and in Figure 2 is depicted the correlation between the K_i values of the parent diamides **1** with, separately, the modified derivatives **2–4**.

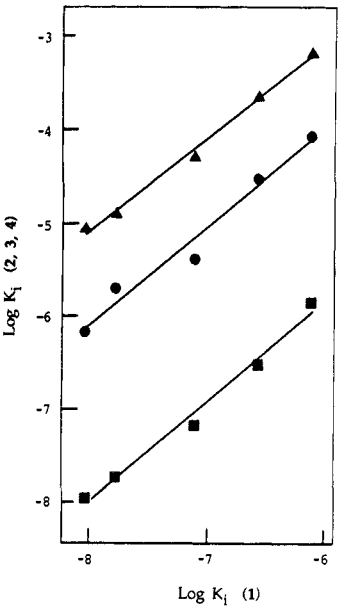
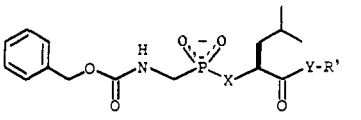


Figure 2. Comparison of K_i values for phosphonate esters **2** (▲), phosphinates **3** (■), and carboxylate esters **4** (●) with K_i values of the phosphonamidate peptides **1**. The lines represent least-squares fits to the data, with slopes of 0.99 (▲), 1.07 (■), and 1.05 (●), respectively.

Table I. Inhibition Constants for Binding of Compounds **1–4**[a] to Thermolysin



	K_i, nM			
series [R'[b]]	1[c]	2[d]	3[e]	4
a [H(Me)[f]]	760	660000	1400	85000
b [Gly]	270	230000	300	29700
c [Phe]	78	53000	66	4100
d [Ala]	16.5	13000	18.4	1990
e [Leu]	9.1	9000	10.6	676

[a] **1**: X = Y = NH; **2**: X = O, Y = NH; **3**: X = CH_2, Y = NH; **4**: X = NH, Y = O. [b] The amino acid abbreviation is used to designate the amino acid or analogue represented by Y–R'. [c] Data from ref 7a. [d] Data from ref 7b. [e] Data first reported in ref 12. [f] Y–R' = OMe in the case of **4a**.

Replacement of the Phosphorus-Linked NH Moiety with CH_2 or O. As demonstrated previously for the phosphonates **2**,[7b] replacement of the NH group of the phosphonamidates **1** with a CH_2 moiety (**3**) alters the affinity of the inhibitors by a relatively

(23) The glycine phosphonic acid derivative of **19** was synthesized by the route of: Oleksyszyn, J.; Subotkowska, L. *Synthesis* **1980**, 906–907.

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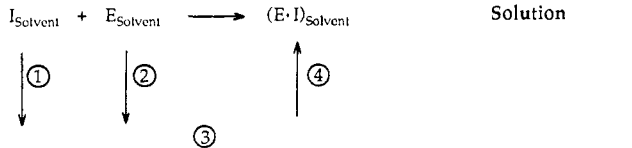
Scheme IV



		Difference in Energy
Step 1	Desolvation of inhibitor	$\Delta G_{Solvent}$
Step 2	Desolvation of enzyme	0
Step 3	Active-site interactions	$\Delta G_{Active\text{-}Site}$
Step 4	Resolvation of E·I complex	= 0

$$\Delta\Delta G = \Delta G_{Solvent} + \Delta G_{Active\text{-}Site}$$

For 1 vs. 2 and 1 vs. 3, $\Delta G_{Active\text{-}Site} = \Delta G_{Ligand} + \Delta G_{H\text{-}bond}$

For 1 vs. 4, $\Delta G_{Active\text{-}Site} = \Delta G_{Lewis\text{-}base} + \Delta G_{H\text{-}bond}$

constant amount across the 2 orders of magnitude in absolute binding energy spanned by each series. The linear relationship (slope ≈1) between the K_i values for the phosphonamidates **1** and those for the phosphonates **2** and phosphinates **3** suggests that all of these inhibitors bind to thermolysin similarly, an interpretation that has been validated by X-ray crystallography.[10,25] However, in contrast to the phosphonates **2**, which bind an average of 4 kcal/mol less tightly than the phosphonamidate inhibitors **1**, the phosphinates **3** are bound with virtually the same affinity: only 0.1 kcal/mol less tightly than the corresponding phosphonamidates.[26]

The observed difference in binding affinities clearly reflects more than the presence or absence of a hydrogen bond between the phosphorus linkage and the carbonyl oxygen of alanine-113 of thermolysin. The difference in inhibitor–active site interaction must be balanced against the difference in inhibitor solvation, as underscored by the thermodynamic cycle illustrated in Scheme IV.[7a] As that analysis reveals, desolvation of the enzyme (step 2) is identical for related inhibitors; hence it is not a factor in the relative affinity of one inhibitor vs another. Resolvation of the E·I complex (step 4) is similarly unimportant for these inhibitors, since the phosphorus linkage is buried inside the active site and variations at this position are not accessible to the solvent. The similarities in dimension and geometry for the three types of inhibitors, as well as the absence of electronic barriers to rotation around the linking atoms, suggest that conformational differences do not play a role in their relative affinities.[28] The difference in binding energies arises from steps 1 and 3, the desolvation of the inhibitors and their active-site interactions, respectively. The most obvious active-site interactions that differ in energy between the inhibitors **1**, **2**, and **3** are the hydrogen bond of the phosphonamidates (vs any steric or electronic repulsion between the alanine-113 carbonyl group and the CH_2 and O moieties) and coordination of the zinc cation by the phosphorus oxy anions. The difference in binding affinities can be summarized by the following equation:[7b]

$$\Delta\Delta G = \Delta G_{solvent} + \Delta G_{ligand} + \Delta G_{H\ bond} \quad (1)$$

Since the binding affinities of the phosphinates **3** and phosphonamidates **1** are approximately equal, the difference in active-site interactions must be balanced by the difference in their solvation. In contrast, for the phosphonates **2**, the loss of active-site interaction relative to the phosphonamidates is not balanced by a comparable decrease in solvation energy, which accounts for the fact that these compounds are bound 4 kcal/mol less tightly than the phosphonamidates. The importance of $\Delta G_{solvent}$ as a factor in the relative binding affinity of these inhibitors has been questioned by Grobelny, Goli, and Galardy as the result of their measurement of the octanol/water partition coefficients for the related compounds *n*-hexyl-$\psi(PO_2^-X)$Leu-Trp-NHMe (where X = NH, O, and CH_2).[13] They found a negligible difference in the three partition coefficients, an observation that, at first glance, is surprising for compounds that differ so significantly in their potential hydrogen-bonding and polar interactions. However, when it is recognized that 1-octanol is itself capable of hydrogen bonding and that under the conditions of the partition experiment it contains 5% water,[29] it is apparent that an octanol/water partition coefficient is an inappropriate measure of the $\Delta G_{solvent}$ in eq 1 and Scheme IV. What would be relevant for this analysis are the vapor phase/water distribution coefficients, values that are inaccessible experimentally due to the ionic nature of these molecules.

Although these solvation differences cannot be determined experimentally, they can be assessed theoretically by the free energy perturbation technique, as shown by Bash et al.[11] and more recently by Merz and Kollman.[12] By mutation of one structure into another computationally, the differences in the energies of interaction with the solvent, as well as with an enzyme active site, can be calculated for compounds that are closely related in structure. Together these differences provide a prediction of the relative binding affinities of the two compounds. This approach was first applied to the phosphonate–phosphonamidate comparison (**2** vs **1**) and was shown to reproduce accurately the observed difference in binding affinity of 4 kcal/mol.[11] More recently, it was used in a predictive sense with the calculation of the binding affinity of the phosphinates **3** in advance of their experimental evaluation.[12] The prediction that the methylene analogues **3** would be weaker inhibitors than the phosphonamidates **1** by 0.3 kcal/mol compares very favorably with the experimental finding of 0.1 kcal/mol. The fact that these calculations predict closely the difference in binding energies lends credence to the individual calculations that lead to this figure, namely, the differences in solvation and in active-site interaction. These calculations support

Table II. Comparison of Arylphosphonic Acid pK_{a1} and K_i Values

compound	pK_{a1}[a]	K_i, μM
5a (NO_2)	1.24	23 ± 2.2
5b (Cl)	1.55	23 ± 2.3
5c (H)	1.83	26 ± 3.2
5d (Me)	1.98	26 ± 2.4
5e (OEt)	2.06	22 ± 2.3

[a] pK_{a1} values for $ArPO_3H_2$ taken from ref 29.

(25) Matthews, B. W., personal communication.

(26) Shortly before the initial report of these results,[12] Grobelny et al.[13] described the synthesis of Cbz-Gly($PO_2^-CH_2$)Leu-Leu (**3e**) as a mixture of diastereomers at the $P_{1'}$ position, and their determination of a K_i value for this compound of 180 nM, an order of magnitude higher than we find. The explanation for this discrepancy lies in the different conditions under which the inhibition constants were determined, specifically the salt concentration. The K_i values reported by Grobelny et al. were determined at a salt concentration of 0.5 M, where the kinetic parameters for substrate hydrolysis are considerably different than in 2.5 M NaBr, the conditions under which the K_i values recorded in Table I and reported previously were measured.[7] The kinetic parameters for the thermolysin-catalyzed hydrolysis of the substrate *N*-[(2-furylacryloyl)glycyl]-L-leucinamide (faGLa) were determined under prescribed conditions, which includes 2.5 M NaBr. The data were fit to a hyperbolic curve by using Cleland's HYPER program to obtain a K_m of 6.5 ± 0.5 mM and a K_{cat} of 423 ± 11 s^{-1}. The reported K_m of 30 mM was obtained under different conditions, most notably in the absence of NaBr.[27] In light of the difference in K_m values determined as a function of NaBr concentration, we attempted to characterize the effects of the salt on the kinetic parameters of faGLa. At 100 mM NaBr, the K_m for faGLa is ca. 53 mM while the k_{cat} (525 ± 44 s^{-1}) is virtually unaffected. These parameters are only estimates, however, since the nature of the UV assay does not allow for faGLa concentrations above 10 mM. What is revealed by this study is that NaBr can have a pronounced effect on the kinetic parameters for the hydrolysis of certain substrates.

(27) Feder, J. *Biochem. Biophys. Res. Commun.* **1968**, *32*, 326–332. Feder, J.; Schuck, J. M. *Biochemistry* **1970**, *9*, 2784.

(28) Calculations of the conformational behavior of these derivatives reveal similar energy profiles for rotation around the P–O–C and P–CH_2–C bonds (MacroModel V2.5, Still, W. C.; Richards, N. G. J.; Guida, W. C.; Lipton, M.; Liskamp, R.; Chang, G.; Hendrickson, T.; Department of Chemistry, Columbia University, New York, NY 10027).

(29) Leo, A.; Hansch, C.; Elkins, D. *Chem. Rev.* **1971**, *71*, 525–555.

the expectation that the methylene compounds are less strongly solvated than the phosphonates and the phosphonamidates and corroborate the view that reduction in the solvation energy is the major factor in counteracting the reduced active-site interactions for the methylene analogues in comparison to the phosphonamidates. In essence, while the phosphonamidates are pulled into the active site by favorable binding interactions, the phosphinates are pushed into the active site by unfavorable solvent interactions.

Influence of Phosphonate Basicity on Inhibitor Affinity. A limited amount of experimental information is available that can lead to an estimation of the magnitude of ΔG_{ligand}.[14] However, this information suggests that coordination of a phosphorus oxy anion with a divalent transition-metal cation in aqueous solution is only moderately sensitive to the basicity of the anion, with Brønsted coefficients significantly less than 0.5.[14] In their reinterpretation of our previous analysis, Grobelny et al. have suggested that basicity of the phosphorus ligand is the major factor in determining the relative affinities of the phosphonamidate, phosphinate, and phosphonate inhibitors, noting that the NH and CH_2 compounds have similar pK_a's, while the O derivatives are ca. 1.5 pK units less basic.[13] For this difference in pK_a to account for a significant part of the 3 orders of magnitude difference in binding affinity would require a greatly enhanced Brønsted coefficient, a possibility that is rationalized on the basis of a reduced dielectric constant in the enzyme active site.

The para-substituted arylphosphonates **5** were designed to examine the influence of phosphonate basicity on binding affinity. The pK_{a1} values for the parent phosphonic acids are known (Table II), and the substituent effects are comparable to those for benzoic acid ionization.[30] The range of acidity of these derivatives (0.82 pH unit) is sufficient to reveal unambiguously an influence on binding affinity comparable to that suggested by Grobelny et al. The arylphosphonates were chosen in preference to the corresponding phosphonamidates to avoid complications due to any influence of the aryl substituent on the strength of a hydrogen bond between the phosphonamide NH and the alanine-113 carbonyl oxygen.

The phenylphosphonate **5c** was modeled onto the structure of the complex between Cbz-Gly-$\psi(PO_2^-O)$Leu-Leu (**2e**) and thermolysin.[10] The phenyl substituent can be accommodated in the S_1 region of the active site without any obvious steric interference from the protein. Moreover, the para position of this ring appears to protrude from the active site without making any contact with the enzyme, suggesting that substituents at this position will not influence binding either sterically or through changes in solvation. Therefore, the only variation in binding affinity of the aryl phosphonates to thermolysin should be due to the differences in metal coordination, balanced, of course, by the differences in solvation of these phosphonate anions.

In Table II are compared the pK_{a1} values of the parent arylphosphonic acids and the inhibition constants of the inhibitors **5**. It is clear that neither the size nor the hydrophobicity of the para substituent has a systematic effect on the binding affinity, in support of the assumption that this group does not interact directly with the enzyme. Figure 3 shows the relationship between pK_a and $-\log K_i$ graphically. The horizontal line indicates that the basicity of the phosphorus oxy anion has no effect on binding affinity. These results demonstrate, for eq 1, that any difference in coordination energy is offset by a similar change in solvation energy. While the energy of interaction between the inhibitors and the thermolysin active site may well increase with the increasing basicity, there is no net effect on the binding affinity since the energy required to desolvate the phosphorus oxy anion increases proportionally, an eventuality neglected in the analysis by Grobelny et al.[13] In the earlier analysis of Bartlett and Marlowe,[7b] ΔG_{ligand}, as an indirect effect of the phosphorus substituent, was discounted as a factor in explaining the higher affinity of the phosphonamidates **1** in comparison to the phosphonates **2**. That analysis was also incomplete, in the sense that the indirect effect of the substituent on the energy of solvation of the anionic oxygens was not considered explicitly. Rather than introduce another term in eq 1 to describe this aspect of solvation, it can be recast as eq 2, in which the two offsetting, indirect effects are subsumed in

$$\Delta\Delta G = \Delta G_{solvent} + \Delta G_{indirect} + \Delta G_{H\ bond} \quad (2)$$

$\Delta G_{indirect}$, defined as the differential influence of the phosphorus substituent on oxy anion coordination and solvation. Figure 3 demonstrates that $\Delta G_{indirect}$ is an insignificant factor in determining the binding affinities of the phosphorus-containing inhibitors and underscores the original interpretation that these affinities can be attributed to the importance and relative magnitudes of the hydrophobic and hydrogen-bonding characteristics of the phosphorus substituents themselves.

Replacement of the P_1'–P_2' NH Moiety with O. The crystal structures of a variety of thermolysin–inhibitor complexes[5,10] indicate that the amide linkage between the P_1'–P_2' residues is also hydrogen bonded to the enzyme. The NH moiety donates a hydrogen bond to the side chain carbonyl of asparagine-112, and the carbonyl oxygen accepts hydrogen bonds from the guanidinium groups of arginine-203 (Figure 1). To probe the importance of these interactions, the series of inhibitors was synthesized in which the P_1'–P_2' amide bond is replaced with an ester linkage. Although this substitution is in principle similar to that described above for the phosphonamidate–phosphonate comparison, in this case the differences in solvation of carboxylic amides and esters are known experimentally.[15] In deciding what compounds to prepare, we chose the phosphonamidates in order to preserve the overall affinity of the inhibitors, and the methyl ester **4a** as the counterpart to amide **1a**, instead of the carboxylic acid, to preserve the neutrality of this group under the assay conditions.

The K_i values for these inhibitors are included in Table I, and their comparison against the diamides is shown in Figure 2 (circles). The linear relationship in Figure 2 (slope $\approx$1) suggests that the phosphonamide esters **4** bind in the same fashion as the peptides **1**, **2**, and **3**. However, these compounds show on average a 92-fold decrease in binding affinity in comparison to the diamides **1**, which corresponds to a loss in binding energy of 2.7 ± 0.1 kcal/mol.

In spite of the obvious differences between the two systems, it is remarkable that the relative binding affinities of the amide and ester derivatives are so similar for the phosphorus and carboxyl linkages. As before, the theoretical thermodynamic cycle depicted in Scheme IV is useful for analyzing the differences in binding affinity between the ester and amide analogues **4** and **1**. The difference in binding energy between ester and amide arises again from steps 1 and 3. In this instance, however, the contribution of step 1 is known: comparison of the vapor/water distribution coefficients of simple esters and amides indicates that the solvation energy of a secondary amide is ca. 6.8 kcal/mol greater than that of the analogous ester.[15] Since the observed difference in binding affinity is 2.7 kcal/mol in favor of the amides **1**, the active-site interactions (step 3) must favor these derivatives by 9.5 kcal/mol, i.e., a factor of 10^7.

As was the case for the phosphorus substituent, two active-site interactions are perturbed by the replacement of the P_1'–P_2' NH moiety with O: hydrogen bond donation to the side-chain oxygen of asparagine-112 ($\Delta G_{H\ bond}$), and the strength of the hydrogen bonds accepted from the guanidinium group of arginine-203 ($\Delta G_{Lewis\ base}$) (see Figure 1). Although the influence of the substituted atom on the latter interaction is an indirect effect (analogous to the influence of phosphorus substituents on the strength of the oxy anion–zinc interaction), in this case it is more significant because of conjugation between the nitrogen and the carbonyl group. In contrast to ΔG_{ligand} above, the indirect effects of carbonyl basicity on hydrogen bonding to the arginine side chain and on solvation energy cannot be absorbed into a $\Delta G_{indirect}$ (see eq 2), since it is an inseparable component of the experimentally determined $\Delta G_{solvent}$.

Although amide carbonyls are more basic than ester carbonyls, the absolute magnitude of this difference is somewhat ambiguous

(30) Jaffe, H. H.; Freedman, L. D.; Doak, G. O. *J. Am. Chem. Soc.* **1953**, *75*, 2209–2211.

(e.g., ca. 10^3–10^7).[31] If we use as a model a simple amide and ester measured under similar conditions, amides are some 4 orders of magnitude more basic than esters [e.g., pK_a(*N*,*N*-dimethylacetamide·H^+) = −0.4, pK_a(ethyl acetate·H^+) = −4.5].[30b] This difference in basicity may be translated directly into hydrogen bond strength, since the Brønsted coefficient for such interactions is close to unity within a series of related bases.[32] Thus, the carbonyl–arginine hydrogen bond(s) may contribute on the order of 5.5 kcal/mol to the difference in active-site interaction. While this accounting cannot be exact, it is a reasonable implication that the hydrogen bond between the NH group and the side-chain carbonyl of asparagine-112 is on the order of 4 kcal/mol more stabilizing than the interaction between the ester oxygen and this side chain.[33] Note that this value is distinct from the strength of the hydrogen bond itself, since it includes any destabilization from oxygen–oxygen repulsion in the ester complexes. For this system, then, we can approach a complete accounting of the energy differences that are responsible for the observation that the amides are bound with an overall affinity of 2.7 kcal/mol more than the esters (eq 3).

$$\Delta\Delta G = \Delta G_{solv} + \Delta G_{H\ bond\ donor} + \Delta G_{Lewis\ base} \qquad (3)$$

$$2.7 = -6.8 + 4 + 5.5\ (kcal/mol)$$

Conclusions

We have determined the influence of selected structural alterations on the binding energies of a series of phosphorus-based thermolysin inhibitors. Although these structural changes have been small, they have dramatic effects on affinity, effects that can be analyzed by considering the separate contributions from active-site and solvent interactions. As an operational measure of the importance of a hydrogen-bonding group to binding, the bottom line is similar in both instances: the amides are bound more strongly than the esters because the NH group increases the active-site interactions to a greater extent than it increases (directly or indirectly) the interactions with solvent. This net advantage, shown for two series of inhibitors to be between 2.7 and 4.0 kcal/mol, incorporates both the favorable hydrogen bond donation of the amide NH and the potential electronic repulsion of the oxygen lone pairs. However, the differences in binding energy determined in this manner clearly depend on the basis for comparison. One can similarly calculate an incremental binding energy due to a hydrophobic methylene group, in comparison to oxygen, to be 3.8 kcal/mol (the advantage of the phosphinates **3** over the phosphonates **2**).[2b]

Jencks' definition of intrinsic binding energy envisages an incremental change in binding affinity in response to an incremental change in structure.[1a,34] It is a conceptual definition, since it relies implicitly on "other things being equal". Previous evaluations of the intrinsic binding energy of a group X have taken as a basis molecules in which X is replaced with a hydrogen[2] or an electron lone pair.[7b] Such replacements are reasonable approximations when X is comparatively large. However, it is impossible to make a structural alteration that does not have a number of ramifications with respect to active-site and solvent interactions. Unfortunately, these may be of a magnitude similar to the intrinsic effects themselves when X is small. The intrinsic binding energy due to hydrogen bonding from the phosphonamidate NH group, for example, should be based on a comparison with a hypothetical compound in which the hydrogen is absent but all of the other characteristics of the molecule are maintained. Although this hypothetical compound is approximated by the phosphonate esters, as described above there are a number of inescapable differences.

Fersht has evaluated the intrinsic binding energy from a hydrogen bond through the site-specific removal of hydroxyl and amino substituents from binding sites in tyrosine tRNA synthetase and phosphofructokinase.[2] In this approach as well, the observed effects reflect a number of influences in addition to hydrogen bonding, including differences in active-site solvation, in entropic contributions from the fixation of conformationally mobile groups, and in van der Waals effects as a result of the structural changes. Acknowledging that it is difficult, indeed perhaps impossible, to determine such an intrinsic binding energy, simply because a true basis molecule does not exist, Fersht has suggested the term "apparent binding energy".[2b]

In hindsight, it may be more pragmatic to define a "differential binding energy", in which the basis for comparison is acknowledged directly. A differential binding energy is in essence the difference between the two intrinsic binding energies of the functional groups involved in the comparison, with the contribution from the hypothetical basis molecule removed by the subtraction. In a practical sense, such differential effects are what one considers intuitively in devising an enzyme inhibitor or altering the structure of a lead compound. Moreover, recognition of the basis for comparison also reconciles contrasting approaches to determining the value of a hydrogen-bonding interaction in a protein–ligand complex. From Fersht's work,[2] the replacement of a hydrogen atom with a hydroxyl group results in an increase in differential binding energy of only 0.5–1.0 kcal/mol. The basis in this comparison is clearly different from that which relates the esters and amides described above, and there is no inconsistency in the finding that the differential binding energies from these substitutions are not the same.

Experimental Section[35]

***N*-[2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]oxyphosphinyl]methyl]-4-methylpentanoyl]-L-alanine Dilithium Salt (3d).** A solution of 26.2 mg (0.057 mmol) of diester **10d** (see below) in 0.12 mL of CH_3CN and 0.12 mL of 1 N LiOH was stirred at room temperature for 48 h. The reaction mixture was concentrated under reduced pressure to remove the CH_3CN and then lyophilized to yield a white solid. Purification of this material by anion-exchange chromatography over Sephadex DEAE with a linear gradient from H_2O to 0.5 M TBK buffer followed by cation-exchange chromatography (Dowex, Li^+ form) and lyophilization provided 23 mg (91% yield) of **3a** as a white solid: IR (KBr) 3345 br, 2950, 1700, 1620, 1550, 1446, 1155 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 0.71 (d, 3, J = 6.3), 0.77 (d, 3, J = 6.2), 1.18 (d, J = 7.2) and 1.20 (m, 5), 1.38 (m, 2), 1.48 (m, 2), 1.70 (m, 1), 2.53 (m, 1), 3.11 (ddd, 2, J = 10.2), 3.99 (q, 1, J = 7.2), 5.01 (s, 2), 7.31 (m, 5); ^{31}P NMR (D_2O) δ 37.13; MS (FAB) m/z 435 $[(M - Li)H]^+$, 442 $(MH)^+$; HRMS (FAB) calcd for $Li_2C_{19}H_{27}N_2O_7P$ 441.1954, found 441.1951. Anal. Calcd for $Li_2C_{19}H_{27}N_2O_7P$: P, 7.02. Found: P, 5.94; 85% of the solid is inhibitor.

The following compounds were prepared in a similar manner.

2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]oxyphosphinyl]methyl]-4-methylpentanamide lithium salt (3a): yield, 94%; ^1H NMR (250 MHz, D_2O) δ 0.724 (d, J = 6.2) and 0.754 (d, 6, J = 6.2), 1.20 (m, 1), 1.35

(31) (a) Arnett, E. M. *Progress in Physical Organic Chemistry*; Wiley-Interscience: New York, 1963; pp 223–403. (b) Arnett, E. M.; Mitchell, E. J.; Murty, T. S. S. R. *J. Am. Chem. Soc.* **1974**, *96*, 3875–3891.

(32) Hine, J.; Hanh, S.; Hwang, J. *J. Org. Chem.* **1988**, *53*, 884–887. Hine, J. *Structural Effects on Equilibria in Organic Chemistry*; Wiley-Interscience: New York, 1975; Sections 6–8.

(33) It is conceivable that the bifurcated hydrogen bond between the carbonyl oxygen and the guanidinium moiety is more sensitive to the Lewis basicity of the oxygen than predicted from a Brønsted coefficient of unity. In this case, the estimated contribution from the amide hydrogen bond to Asn-112 would be less than 4 kcal/mol.

(34) Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4046–4050.

(35) General procedures: Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Subtilisin Carlsberg was obtained from Sigma Chemical Co. (protease type VIII: bacterial, subtilisin Carlsberg, subtilopeptidase A). Acetonitrile was distilled from calcium hydride (under nitrogen) and further deoxygenated by stirring under 0.1 mmHg vacuum for 30 min followed by a nitrogen purge. Methylene chloride was freshly distilled from CaH_2. Dimethylformamide was distilled from calcium oxide at 35 mmHg vacuum and stored over 3-Å molecular sieves. Triethylammonium bicarbonate (TBK) buffer was prepared by bubbling CO_2 gas through an aqueous solution of triethylamine until the desired pH was attained. NMR spectra were determined in $CDCl_3$ on high-field instruments (≥200 MHz), unless otherwise indicated. ^1H chemical shifts are reported in ppm on the δ scale relative to tetramethylsilane as 0 ppm (samples acquired in D_2O are referenced to methanol as 3.30 ppm). ^1H NMR data are presented as follows: chemical shift (multiplicity, number of protons, coupling constants in hertz). ^{13}C chemical shifts are referenced to $CDCl_3$ (77.0 ppm) or CD_3OD (49.0 ppm). ^{31}P chemical shifts are reported downfield from trimethyl phosphate (3.086 ppm). IR spectra were obtained on neat films, unless otherwise indicated. Flash chromatography (silica) was performed by the method of Still, Kahn, and Mitra (*J. Org. Chem.* **1978**, *42*, 2923).

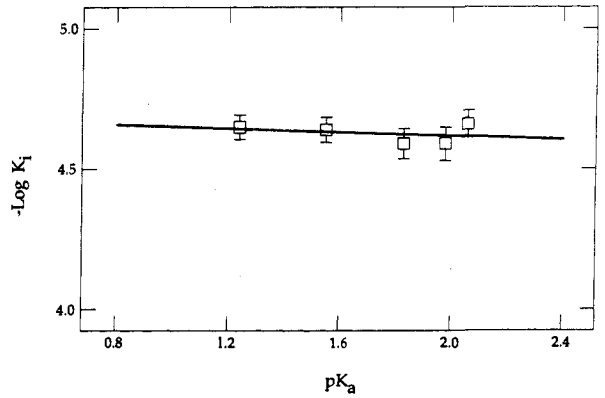


Figure 3. Relationship between K_i values for arylphosphonates **5** and pK_{a1} values of the parent arylphosphonic acids (from ref 30). The line represents a least-squares fit to the data (slope −0.03).

(m, 2), 1.47 (ddd, 1, J = 6.1, 14), 1.67 (ddd, 1, J = 7.4, 14), 2.54 (m, 1), 3.13 (m, 2), 5.00 (s, 2), 7.30 (m, 5); ^{13}C NMR (102 MHz, D_2O) δ 23.5, 24.9, 27.9, 33.9 (d, J = 91), 40.8, 42.7, 45.2 (d, J = 8), 69.5, 130.0, 130.8, 131.2, 171.5, 177.0; ^{31}P NMR (123 MHz, D_2O) δ 37.0; MS (FAB) m/z 363 $(MH)^+$; HRMS (FAB) calcd for $LiC_{16}H_{24}O_5P$ 363.170, found 363.1672. Anal. Calcd for $LiC_{16}H_{24}N_2O_5P$: P, 8.56. Found: P, 5.90; 69% of the solid is inhibitor.

***N*-[2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]oxyphosphinyl]methyl]-4-methylpentanoyl]glycine dilithium salt (3b)**: yield, 81%; ^{1}H NMR (400 MHz, D_2O) δ 0.719 (d, 3, J = 6.2), 0.768 (d, 3, J = 6.2), 1.25 (m, 1), 1.37 (m, 2), 1.49 (m, 2), 1.72 (m, 1), 2.57 (m, 1), 3.13 (m, 2), 3.59 (dd, 2, J = 13.2), 4.70 (s, 2), 7.30 (m, 5); ^{13}C NMR (D_2O) δ 15.04, 16.35, 19.42, 25.30 (d, J = 91.5), 32.80, 35.13, 36.54 (d, J = 7.60), 37.24, 52.80, 60.93, 121.43, 122.17, 122.62, 130.31, 170.21, 171.81; ^{31}P NMR (D_2O) δ 37.15; MS (FAB) m/z 427 $(MH)^+$; HRMS (FAB) calcd for $Li_2C_{18}H_{25}N_2O_7P$ 427.1760, found 427.1779. Anal. Calcd for $Li_2C_{18}H_{25}N_2O_7P$: P, 7.27. Found: P, 6.14; 84% of the solid is inhibitor.

***N*-[2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]oxyphosphinyl]methyl]-4-methylpentanoyl]-L-phenylalanine dilithium salt (3c)**: yield, 78%; IR (KBr) 3340 br, 1700, 1618, 1528, 1415, 1151 cm^{-1}; ^{1}H NMR (400 MHz, D_2O) δ 0.67 (d, 3, J = 5.7), 0.72 (d, 3, J = 5.4), 1.24–1.40 (m, 5), 2.48 (m, 1), 2.73 (dd, 1, J = 14, 9.2), 2.87 (dd, 1, J = 7.6), 2.90–3.02 (m, 2), 4.35 (dd, 1, J = 3), 5.01 (s, 2), 7.06–7.15 (m, 5), 7.27–7.31 (m, 5); ^{31}P NMR (D_2O) δ 36.93; MS (FAB) m/z 511 $[(M = Li)H]^+$; HRMS (FAB) calcd for $Li_2C_{25}H_{31}N_2O_7P$ 517.2267, Found 517.2270. Anal. Calcd for $Li_2C_{25}H_{31}N_2O_7P$: P, 6.00. Found: P, 5.18; 86% of the solid is inhibitor.

***N*-[2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]oxyphosphinyl]methyl]-4-methylpentanoyl]-L-leucine dilithium salt (3e)**. Yield, 99%. This inhibitor was further purified by reverse-phase HPLC over a Whatman Partisil 100DS3 semipreparative column with a concave gradient from 75% 0.05 M triethylammonium acetate buffer (pH 7.5)/CH_3CN to 10% 0.05 M triethylammonium acetate buffer/CH_3CN: IR (KBr) 3320 br, 2955, 1705, 1600, 1548, 1410, 1150 cm^{-1}; ^{1}H NMR (400 MHz, D_2O) δ 0.72 (d, 6, J = 6.3) and 0.74 (d), 0.77 (d, 6, J = 6.2), 1.28–1.55 (m, 6), 1.61–1.78 (m, 1), 2.56–2.61 (m, 1), 3.05–3.18 (m, 2), 4.08 (dd, 1, J = 5.5), 5.00 (s, 2), 7.30 (m, 5); ^{13}C NMR (D_2O) δ 20.9, 21.1, 22.3, 22.6, 24.5, 31.7, (d, J = 90), 39.0, 40.7, 41.0 (d, J = 40), 42.2, 53.7, 67.1, 127.6, 128.3, 128.7, 136, 158, 177.6, 179.5; ^{31}P NMR (D_2O) δ 37.0; MS (FAB) m/z 483 $(MH)^+$; HRMS (FAB) calcd for $Li_2C_{22}H_{33}N_2O_7P$ 483.2428, found 483.2425. Anal. Calcd for $Li_2C_{22}H_{33}N_2O_7P$: P, 6.42. Found: P, 3.07; 48% of the solid is inhibitor.

Methyl *N*-[[(*N*-Carbobenzoxyamino)methyl]hydroxyphosphinyl]-L-leucinate Lithium Salt (4a). Isobutylene gas was bubbled into 10 mL of CH_2Cl_2 at 0 °C for 10 min. Trimethylsilyl bromide (460 μL, 3.44 mmol) was added in one portion and the solution was stirred for 5 min under Ar. A solution of 266 mg of methyl *N*-[[(carbobenzoxyamino)-methyl]methoxyphosphinyl]-L-leucinate (0.69 mmol) in 2 mL of CH_2Cl_2 was added over 10 min. The dimethyl ester flask was rinsed with additional CH_2Cl_2 and the rinses were added to the reaction flask (total CH_2Cl_2, 15 mL). The reaction was allowed to warm to room temperature over 2 h. The volatile materials were removed by a stream of dry N_2, $CHCl_3$ was added, and the evaporation was repeated. The product was purified by anion-exchange chromatography (see below): yield, 42–51%; ^{1}H NMR (D_2O) δ 0.88 (2 d, 6), 1.47 (m, 2), 1.72 (m, 1), 3.30 (2 d, 2), 3.68 (s, 3), 3.71 (m, 1), 5.01 (s, 2), 7.31 (s, 5); ^{13}C NMR (D_2O) δ 21.5, 22.1, 24.0, 38.0, 42.8 (J_{PC} = 216), 48.1 (J_{PC} = 813), 55.2, 66.7, 127.5, 128.1, 128.5, 136.1, 157.8, 174.9; ^{31}P NMR (D_2O) δ 20.6; HRMS (FAB) calcd for $C_{16}H_{24}N_2O_6PLi$ 378.4165, found 378.4159. Anal. Calcd for $C_{16}H_{24}N_2O_6PLi$: P, 8.19. Found: P, 7.07; 86.3% of solid is inhibitor.

General Method for the Preparation of Inhibitors 4b–e. The corresponding dimethyl ester (**20b–e**; see below) (0.36 mmol) was dissolved in 1.5 mL of THF in an NMR tube. Aqueous LiOH (1.6 equiv of 2.0 N) was added and the hydrolysis was followed by ^{31}P NMR spectroscopy. After 18–24 h, the THF was removed by a stream of Ar, and the aqueous phase was purified by anion-exchange chromatography (25 × 150 mm, DEAE Sephadex, 0–0.25 M triethylammonium bicarbonate, pH 7.5 (TBK buffer), 250 mL of total buffer). The dianion fractions containing inhibitor (as monitored by A_{257}) were pooled and most of the excess triethylamine was removed by azeotropic evaporation with MeOH or absolute EtOH. The triethylammonium salt of the inhibitor was converted to the lithium salt by passing it through a small column of Dowex 50W-X cation-exchange resin in the lithium form. Lyophilization provided the dilithium salt as a hygrosopic white powder. After being equilibrated with the atmosphere, the powder was analyzed to determine the percentage of phosphorus in the sample and this was used to determine the relative amount of inhibitor by weight [the balance of the powder was assumed to be lithium (bi)carbonate salts].

***O*-[*N*-[[*N*-Carbobenzoxyamino)methyl]hydroxyphosphinyl]-L-leucyl]-hydroxyethanoate dilithium salt (4b)**: yield, 46–63%; ^{1}H NMR (D_2O) δ 0.71 (2 d, 6), 1.21 (m, 1), 1.26 (m, 2), 3.00 (m, 2), 3.10 (m, 2), 3.25 (dd, 1, J = 5.8, 6.4), 4.90 (s, 2), 7.25 (s, 5); ^{13}C NMR (D_2O) δ 21.0, 21.4, 22.0, 40.6, 52.6 (J_{PC} = 844), 55.1, 66.8, 127.5, 128.0, 128.5, 136.1, 157.8, 179.6, 83.4; ^{31}P NMR (D_2O) δ 20.8; HRMS (FAB) calcd for $C_{17}H_{23}N_2O_8PLi_2$ 428.3487, found 428.3489. Anal. Calcd for $C_{17}H_{23}N_2O_8PLi_2$: P, 7.23. Found: 4.92; 68.1% of solid is inhibitor.

***O*-[*N*-[[(*N*-Carbobenzoxyamino)methyl]hydroxyphosphinyl]-L-leucyl]-(*S*)-2-hydroxy-3-phenylpropanoate dilithium salt (4c)**: yield, 47%; ^{1}H NMR (D_2O) δ 0.83 and 0.85 (d, 6), 1.30 (m, 2), 1.61 (m, 1), 3.04 (ABX, 2, J = 10, 12), 3.22 (m, 2), 3.61 (dt, 1, J = 5, 9), 4.49 (m, 1), 5.10 (m, 2), 7.26 (m, 5), 7.38 (m, 5); ^{13}C NMR (D_2O) δ 21.0, 22.4, 24.2, 38.1, 39.5 (J_{PC} = 142), 44.0, 54.7, 55.4, 67.1, 126.6, 126.9, 127.2, 127.4, 127.5, 128.1, 128.5, 128.7, 136.1, 137.0, 158.1, 172.1, 176.8; ^{31}P NMR (D_2O) δ 20.7; HRMS (FAB) calcd for $C_{24}H_{29}N_2O_8PLi_2$ 487.5007, found 487.5006. Anal. Calcd for $C_{24}H_{29}N_2O_8PLi_2$: P, 6.35. Found: P, 3.42; 53.8% of solid is inhibitor.

***O*-[*N*-[[(Carbobenzoxyamino)methyl]hydroxyphosphinyl]-L-leucyl]-(*S*)-2-hydroxypropanoate dilithium salt (4d)**: yield, 55–59%; ^{1}H NMR (D_2O) δ 0.73 (2 d, 6), 1.23 (m, 1), 1.27 (m, 2), 1.43 (d, 3, J = 5.1), 3.15 (m, 2), 3.25 (dd, 1, J = 5.7, 6.2), 3.81 (m, 1), 4.90 (s, 2), 7.25 (s, 5); ^{13}C NMR (D_2O) δ 17.6, 21.2, 23.4, 23.9, 39.7 (J_{PC} = 142), 41.2, 50.8, 53.7, 67.2, 127.6, 127.8, 127.9, 128.2, 136.2, 158.1, 172.3, 179.6; ^{31}P NMR (D_2O) δ 20.9; HRMS (FAB) Calcd for $C_{18}H_{25}N_2O_8PLi_2$ m/z 442.3758, found m/z 442.3761. Anal. Calcd for $C_{18}H_{25}N_2O_8PLi_2$: P, 7.00. Found: 6.33; 90.4% of solid is inhibitor.

***O*-[*N*-[[(Carbobenzoxyamino)methyl]hydroxyphosphinyl]-L-leucyl]-(*S*)-2-hydroxy-4-methylpentanoate dilithium salt (4e)**: yield, 43–61%; ^{1}H NMR (D_2O) δ 0.83 & 0.84 (d, 12, J = 4.1), 1.27 (m, 2), 1.30 (m, 2), 1.41 (m, 1), 1.43 (m, 1), 3.08 (m, 1), 3.22 (m, 1), 4.28 (m, 1), 4.98 (s, 2), 7.28 (s, 5); ^{13}C NMR (D_2O) δ 17.7, 21.3, 22.4, 24.2, 39.7 (J_{PC} = 130), 41.9 (J_{PC} = 8), 50.8, 54.6, 67.3, 127.7, 128.0, 128.2, 128.4, 136.1, 158.2, 176.9, 180.1; ^{31}P NMR (D_2O) δ 20.6; HRMS (FAB) calcd for $C_{21}H_{31}N_2O_8PLi_2$ m/z 484.4570, found m/z 484.4572. Anal. Calcd for $C_{21}H_{31}N_2O_8PLi_2$: P, 6.39. Found: P, 4.45; 69.7% of solid is inhibitor.

***N*-[2(*S*)-[(Phenylhydroxyphosphinyl)oxy]-4-methylpentanoyl]-L-leucine dilithium salt (5c)**. BOP-Cl (1.1 mmol) was added to a stirring solution of monomethyl phosphonate **13c** (see below) (0.5 mmol), hydroxy analogue **11** (1.1 mmol), and triethylamine (2.2 mmol) in CH_3CN (3 mL) under argon at room temperature; formation of a white precipitate was observed immediately. The reaction mixture was heated to reflux, which resulted in the dissolution of the precipitate, and was kept at this temperature for 16 h. After cooling to room temperature, the solvent was removed in vacuo and the residue was applied directly to a chromatography column, which was eluted with 80% EtOAc/hexane to give 256 mg of the diester **15c** a white solid. This material was dissolved in 5 mL of CH_3CN and 3.2 mL of 1 N LiOH, and the solution was kept at room temperature for approximately 80 h. After concentration of this mixture, the crude product was purified by anion-exchange chromatography over Sephadex DEAE with a linear gradient from H_2O to 0.5 M TBK buffer. Cation-exchange chromatography (Dowex, Li^+ form) and lyophilization afforded the dianion **5c** as a white solid in 50% yield based on **13c**: ^{1}H NMR (400 MHz, D_2O) δ 0.637 (d, 3, J = 6.3), 0.731 (d, 3, J = 6.3), 0.865 (d, 3, J = 6.2), 0.895 (d, 3, J = 6.2), 1.26–1.40 (m, 1), 1.45–1.60 (m, 5), 4.19 (t, 1, J = 7.3), 4.47 (m, 1), 7.40 (m, 3), 7.78 (ddd, 2, J = 7.8, 13, 16.6); ^{13}C NMR (D_2O) δ 22.2, 22.5, 23.6, 23.6, 24.8, 25.9, 42.8, 43.6 (d, J = 3.6), 54.5, 74.8 (d, J = 5.5), 129.0 (d, J = 14), 131.8 (d, J = 2.7), 132.6 (d, J = 9.3), 135.1 (d, J = 179), 174.4 (d, J = 3.2), 179.7; ^{31}P NMR (D_2O) δ 14.8; MS (FAB) m/z 398 $(MH)^+$;

HRMS (FAB) calcd for $Li_2C_{18}H_{26}NO_6P$ 398.1896, found 398.1916; 91% of the solid is inhibitor.

The following compounds were prepared in a similar manner.

***N*-[2(*S*)-[[(*p*-Nitrophenyl)hydroxyphosphinyl]oxy]-4-methylpentanoyl]-L-leucine dilithium salt (5a)**: yield, 65%; ^{1}H NMR (400 MHz, CD_3OD) δ 0.759 (d, 3, J = 6.5), 0.831 (d, 3, J = 6.6), 0.891 (d, J = 5.9) and 0.896 (d, 6, J = 6.0), 1.48–1.71 (m, 6), 4.20 (dd, 1, J = 4.4, 8.8), 4.50 (ddd, 2, J = 4.2, 8.7, 21), 8.04 (dd, 2, J = 8.7, 12), 8.22 (dd, 2, J = 2.3, 8.7); ^{13}C NMR (CD_3OD) δ 22.2, 22.5, 23.7, 23.8, 25.2, 26.0, 26.1, 43.4, 44.2 (d, J = 4.6), 54.5, 75.0 (d, J = 5.8), 123.7 (d, J = 14.2), 134.1 (d, J = 9.8), 143.9 (d, J = 176), 173.9 (d, J = 2.8), 179.0; ^{31}P NMR (CD_3OD) δ 10.1; HRMS (FAB) calcd for $Li_2C_{18}H_{25}N_2O_8P$ 443.1675, found 443.1755; 85% of the solid is inhibitor.

***N*-[2(*S*)-[[(*p*-Chlorophenyl)hydroxyphosphinyl]oxy]-4-methylpentanoyl]-L-leucine dilithium salt (5b)**: yield, 61%; ^{1}H NMR (400 MHz, CD_3OD) δ 0.695 (d, 3, J = 6.5), 0.807 (d, 3, J = 6.6), 0.903 (d, J = 3.4) and 0.918 (d, 6, J = 3.5), 1.47–1.59 (m, 6), 4.25 (dd, 1, J = 3, 9), 4.42 (ddd, 1, J = 4.2, 8.2, 9.5), 7.38 (dd, 2, J = 2.8, 4.7), 7.79 (dd, 2, J = 4.7, 8.3); ^{13}C NMR (CD_3OD) δ 22.2, 22.4, 23.8, 23.8, 25.1, 26.2, 43.4, 44.2 (d, J = 4.7), 54.5, 74.7 (d, J = 5.6), 129.0 (d, J = 5.6), 134.6 (d, J = 9.9), 134.8 (d, J = 182), 137.7 (d, J = 3.8), 174.2 (d, J = 2.5), 179.1; ^{31}P NMR (CD_3OD) 21.8; MS (FAB) m/z 432 $(MH)^+$; HRMS (FAB) calcd for $Li_2C_{18}H_{25}ClNO_6P$ 432.1505, found 432.1491; 78% of the solid is inhibitor.

***N*-[2(*S*)-[[(*p*-Methylphenyl)hydroxyphosphinyl]oxy]-4-methylpentanoyl]-L-leucine dilithium salt (5d)**: yield, 58%; ^{1}H NMR (400 MHz, CD_3OD) δ 0.633 (d, 3, J = 6.5), 0.778 (d, 3, J = 6.6), 0.910 (d, J = 5.8) and 0.922 (d, 6, J = 4.5), 1.40–1.67 (m, 6), 2.34 (s, 3), 4.26 (dd, 1, J = 9.0, 4.2), 4.39 (ddd, 1, J = 4.1, 8.1, 9.5), 7.20 (dd, 2, J = 3.2, 7.8), 7.70 (dd, 2, J = 8.0, 12); ^{13}C NMR (CD_3OD) δ 21.5, 22.2, 22.4, 23.8, 25.0, 26.2, 43.3, 44.1 (d, J = 4.9), 54.5, 74.6 (d, J = 5.6), 129.5 (d, J = 14.4), 132.5 (d, J = 183), 133.0 (d, J = 9.5), 141.8 (d, J = 30), 174.6 (d, J = 2.3), 179.1; ^{31}P NMR (CD_3OD) δ 15.3; MS (FAB) m/z 412 $(MH)^+$; HRMS (FAB) calcd for $Li_2C_{19}H_{28}NO_6P$ 412.2053, found 412.2061; 92% of the solid is inhibitor.

***N*-[2(*S*)-[[(*p*-Ethoxyphenyl)hydroxyphosphinyl]oxy]-4-methylpentanoyl]-L-leucine dilithium salt (5e)**: yield, 31%; ^{1}H NMR (400 MHz, CD_3OD) δ 0.773 (d, 3, J = 6.3), 0.814 (d, 6, J = 6.0), 0.858 (d, 3, J = 6.3), 1.38 (t, 6, J = 7.0), 1.46 (m, 4), 1.57 (m, 2), 4.00 (dd, 1, J = 5.3, 8.6), 4.14 (q, 2, J = 7.0), 4.43 (m, 1), 7.00 (dd, 2, J = 2.8, 8.7), 7.66 (dd, 2, J = 8.7, 12.1); ^{13}C NMR (CD_3OD) δ 14.9, 22.2, 22.5, 23.4, 23.5, 24.6, 25.4, 42.1, 43.4, 54.6, 65.2, 74.5 (d, J = 4.9), 115.2 (d, J = 15), 128.0 (d, J = 200), 134.3 (d, J = 11), 161.4, 174.6, 180.0; ^{31}P NMR (CD_3OD) δ 15.6; MS (FAB) m/z 442 $(MH)^+$; HRMS calcd for $Li_2C_{20}H_{30}NO_7P$ 442.2161, found 442.2159; 81% of the solid is inhibitor.

Methyl 2-[[[(*N*-Carbobenzoxyamino)methyl]methoxyphosphinyl]methyl]-4-methylpentanoate (8). To an oxygen-free solution of 140 mg (0.61 mmol) of phosphinic acid **6**[16] and 430 mg (3.05 mmol) of methyl α-isobutylacrylate[18] (deoxygenated by three freeze–thaw cycles) in 0.4 mL of acetonitrile was added 0.76 mL of bis(trimethylsilyl)acetamide (BSA) and the reaction was stirred under argon at room temperature overnight. The mixture was diluted with 5 mL of methanol and cooled to 0 °C, and ethereal diazomethane was added until a yellow color persisted. Glacial acetic acid was added to consume excess diazomethane, and the solution was evaporated under reduced pressure. The residue was dissolved in EtOAc, and the solution was washed with water and saturated $NaHCO_3$, dried ($MgSO_4$), and concentrated to a yellow oil, which was purified by chromatography (EtOAc) to give 204 mg (87% yield) of phosphinate **8** as a white solid (mixture of diastereomers): mp = 74–83 °C; IR ($CHCl_3$) 3445, 2963, 2245, 1725, 1514, 1203, 1100 cm^{-1}; ^{1}H NMR (250 MHz) δ 0.89 (t, 6, J = 6.6), 0.90 (t, 6, J = 6.6), 1.33 (m, 2), 1.57 (m, 4), 1.81 (q, 2, J = 16), 1.82 (q, 2, J = 16), 2.20 (m, 4), 2.88 (m, 1), 3.35 (m, 1), 3.69 (s, m, 16), 5.12 (s, 4), 7.35 (m, 10); ^{13}C NMR δ 175.9 (d, J = 3.9), 175.5 (d, J = 1.8), 156.2, 156.1, 136.2, 136.1, 128.6, 128.2, 128.0, 67.8, 67.2, 52.0 (d, J = 6.4), 51.7 (d, J = 6.4), 43.2, 43.0, 37.1 (d, J = 6.3), 37.0 (d, J = 8.0), 25.8, 25.7, 22.5 (d, J = 8.0), 22.0 (d, J = 8.0); ^{31}P NMR δ 51.6, 50.4; MS (EI) m/z 385 (1.11), 91 (100). Anal. Calcd for $C_{18}H_{28}NO_6P$: C, 56.10; H, 7.32; N, 3.63; P, 8.04. Found: C, 55.73; H, 7.29; N, 3.72; P, 7.82.

(2*S*)-2-[[[(*N*-Carbobenzoxyamino)methyl]hydroxyphosphinyl]methyl]-4-methylpentanoic Acid (9A) and (2*S*)-2-[[[[*N*-(Benzyloxycarbonyl)amino]methyl]methoxyphosphinyl]methyl]-4-methylpentanoic Acid (9E). To a rapidly stirring mixture of 400 mg (0.26 mmol) of racemic phosphinate **8** in 200 mL of pH 8.1, 0.055 M borate buffer was added 10 mg of crystalline subtilisin Carlsberg (protease 1V). The mixture was stirred for 24 h and then extracted with 30 mL of EtOAc. The organic layer was dried ($MgSO_4$) and evaporated to give 254 mg of recovered diester (**8** and **8R**) as a white solid. The aqueous layer was cooled to 0 °C, acidified to pH 1 by the dropwise addition of 6 N HCl, and saturated with NaCl prior to extraction with five 25-mL portions of EtOAc. The EtOAc solution was dried (Na_2SO_4) and concentrated to yield 165 mg of a 3:1 mixture of **9E** and **9A** as a colorless oil. Anion-exchange chromatography over Sephadex DEAE with a linear gradient from water to 0.25 M TBK (pH 7.6) afforded two major compounds (as monitored by UV absorbance at 254 nM). The first compound eluting, **9E**, was isolated by dilution of the eluant with an equal volume of methanol and concentration to ca. 30 mL under reduced pressure. This solution was cooled to 0 °C, acidified to approximately pH 2.0 with 1 N HCl, and quickly extracted with four 30-mL portions of EtOAc. The combined organic layer was dried (Na_2SO_4) and concentrated to yield 100 mg (26% yield) of methyl phosphinate **9E** as a colorless oil; this material was used in the succeeding coupling reaction immediately after purification. The second compound eluting during ion-exchange purification, **9A**, was isolated as a white solid by lyophilization. This material was dissolved in 20 mL of 1 N HCl and the solution was extracted with five 10-mL portions of EtOAc. The combined organic layer was dried ($MgSO_4$) and evaporated to give 37 mg (10% yield) of diacid **9A** as a colorless oil. **9E**: ^{31}P NMR δ 54.7, 52.3. **9A**: IR 3320 br, 2970, 1713, 1539, 1264, 1143 cm^{-1}; ^{1}H NMR (250 MHz) δ 0.872 (d, 3, J = 5), 0.907 (d, 3, J = 6), 1.25 (m, 1), 1.58 (m, 2), 1.79 (m, 1), 2.19 (m, 1), 2.84 (m, 1), 3.60 (m, 2), 5.10 (s, 2), 6.08 (br, 1), 7.31 (s, 1); ^{31}P NMR δ 50.5; MS (FAB) m/z 358 $(MH)^+$, 380 $(MNa)^+$; HRMS (FAB) calcd for $C_{16}H_{25}NO_6P$ 358.1399, found 358.1409.

***N*-[2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]methoxyphosphinyl]methyl]-4-methylpentanoyl]-L-alanine Methyl Ester (10d)**. Two solutions were prepared and stirred under argon at −5 °C: 100 mg (0.269 mmol) of methyl phosphinate **9E** and 30 μL of *N*-methylmorpholine in 2.6 mL of CH_3CN, and 38 mg (0.269 mmol) of alanine methyl ester hydrochloride and 30 μL of *N*-methylmorpholine in 2.6 mL of CH_3CN. To the solution of **9E** was added 36 μL (0.269 mmol) of isobutyl chloroformate (IBCF) and stirring was continued for precisely 3 min at −5 °C before the alanine methyl ester solution was added via cannula. The reaction mixture was stirred at −5 °C for 2 h and at room temperature for 2 h and then concentrated to give a white, oily solid. Purification by chromatography (2.5% $MeOH/CH_2Cl_2$) afforded 35.4 mg (28% yield) of amide **10d** as a mixture of two diastereomers (these diastereomers could be separated by careful chromatography): IR 3250 br, 2920, 1725, 1690, 1550, 1270, 1205, 1040 cm^{-1}; ^{1}H NMR (250 MHz, diastereomer A) δ 0.89 (d, 3, J = 6.2), 0.94 (d, 3, J = 6.2), 1.41 (d, 3, J = 7.2), 1.30 (m, 1), 1.63 (m, 2), 1.87 (m, 1), 2.30 (m, 1), 2.77 (m, 1), 3.17 (dq, J = 2.3, 16.1) and 3.74 (m, 6), 3.85 (m, 1), 4.53 (quintet, 1, J = 7.2), 5.12 (q, 2, J = 12.3), 5.68 (br, 1), 6.89 (d, 1, J = 7.2), 7.35 (m, 5); ^{31}P NMR δ 52.89. ^{1}H NMR (250 MHz, diastereomer B) δ 0.88 (d, 3, J = 6.4), 0.93 (d, 3, J = 6.4), 1.28 (m, 1), 1.40 (d, 3, J = 7.2), 1.64 (m, 2), 1.80 (m, 1), 2.24 (m, 1), 2.73 (m, 1), 3.58 (dd, 1, J = 6.9, 7.1), 3.68 (d, 3, J = 10.7), 4.58 (quintet, 1, J = 7.3), 5.13 (s, 2), 5.30 (br, 1), 6.56 (d, 1, J = 7.0), 7.35 (m, 5); ^{31}P NMR δ 51.36; MS (FAB) m/z 457 $(MH)^+$, 479 $(MNa)^+$; HRMS (FAB) calcd for $C_{21}H_{33}N_2O_7P$ 457.2121, found 457.2109.

The following compounds were obtained in a similar manner as mixtures of two diastereomers.

***N*-2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]methoxyphosphinyl]methyl]-4-methylpentanamide (10a)**. Differences from the above procedure are as follows: A concentrated solution of ammonia gas in 5 mL of CH_3CN was formed at −5 °C. This solution was cooled to −30 °C and then added to the mixed anhydride as detailed above: yield, 35%; IR 3320 br, 2975, 1720, 1689, 1541, 1265, 1200, 1040; ^{1}H NMR (400 MHz) δ 0.86 (m, 3), 0.90 (m, 3), 1.2–1.3 (m, 1), 1.53–1.71 (m, 3), 2.16–2.28 (m, 1), 2.70–2.90 (m, 1), 3.27–3.31 (m, 0.67), 3.45–3.85 (m) and 3.65 (d, J = 13) and 3.68 (d, 4.33, J = 10), 5.04–5.12 (m, 2), 6.00 (br, 1.67), 6.17 (br, 0.33), 6.62 (br, 0.33), 7.01 (br, 0.67), 7.27–7.39 (m, 5); ^{31}P NMR δ 52.31, 53.72; MS (FAB) m/z 371 $(MH)^+$; HRMS (FAB) calcd for $C_{17}H_{27}N_2O_5P$ 371.1717, found 371.1721.

***N*-[2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]methoxyphosphinyl]methyl]-4-methylpentanoyl]glycine methyl ester (10b)**: yield, 28%; ^{1}H NMR (400 MHz) δ 0.87 (d, 3, J = 6.3), 0.91 (d, 3, J = 6.3), 1.28 (m, 1), 1.63 (m, 3), 2.34 (m, 1), 2.84 (m, 1), 3.32 (dq, 1, J = 2.6, 16.2), 3.67 (s) and 3.68 (d, 6, J = 10.6), 3.78 (m, 1), 3.94 (ddd, 2, J = 5.5, 17.9), 5.09 (dd, 2, J = 9.3), 5.94 (br, 1), 7.33 (m, 5), 7.79 (br, 1); ^{13}C NMR δ 22.184, 22.594, 25.39, 27.87 (d, J = 92), 36.84, 37.85, 41.06, 43.98 (d, J = 13.8), 51.39 (d, J = 6.8), 52.15, 67.02, 127.97, 128.08, 128.41, 136.21, 156.34, 170.26, 175.56; ^{31}P NMR δ 53.80; MS (FAB) m/z 443 $(MH)^+$, 465 $(MNa)^+$; HRMS (FAB) calcd for $C_{20}H_{31}N_2O_7P$ 443.1934, found 443.1943.

***N*-[2(*S*)-[[[(*N*-Carbobenzoxyamino)methyl]methoxyphosphinyl]methyl]-4-methylpentanoyl]-L-phenylalanine methyl ester (10c)**: yield, 56%; IR 3250 br, 2930, 1720, 1662, 1521, 1445, 1200, 1029 cm^{-1}; ^{1}H NMR (400 MHz) δ 0.85 (m, 3), 0.88 (m, 3), 1.26 (m, 1), 1.52–1.65 (m, 2.5), 1.80 (m, 0.5), 2.13–2.20 (m, 1), 2.73–2.90 (m, 1), 2.98–3.16 (m, 2), 3.31–3.71 (m) and 3.66 (s) and 3.68 (s, 7), 4.82 (dd, 0.5, J = 7), 5.10

(dd, 1.5, J = 7), 5.67 (br, 0.75), 5.79 (br, 0.25), 6.76 (br, 0.25), 7.05 (br, 0.75), 7.16–7.82 (m, 10); ^{31}P NMR δ 51.35, 52.92; MS (FAB) m/z 533 $(MH)^+$; HRMS (FAB) calcd for $C_{27}H_{31}N_2O_7P$ 533.2390, found 533.2403.

N-[2(S)-[[[(N-Carbobenzoxyamino)methyl]methoxyphosphinyl]-methyl]-4-methylpentanoyl]-L-leucine methyl ester (10e): yield, 41%; IR 3300 br, 2975, 1730, 1665, 1533, 1260, 1205, 1039 cm^{-1}; ^{1}H NMR (400 MHz) δ 0.84–0.92 (m, 12), 1.21–1.28 (m, 1), 1.57–1.68 (m, 3), 2.22–2.31 (m, 1), 2.65–2.85 (m, 1), 3.07 (ddd, 0.5, J = 2, 14), 3.62–3.95 (m) and 3.68 (s, 7.5), 4.47–4.57 (m, 1), 5.09 (dd, 2, J = 12), 5.92 (m, 1.5), 6.90 (d, 0.5, J = 7.8), 7.28–7.33 (m, 5); ^{31}P NMR δ 51.71, 53.11; MS (FAB) m/z 499 $(MH)^+$; HRMS (FAB) calcd for $C_{24}H_{39}N_2O_7P$ 499.2582, found 499.2576.

N-[2(S)-Hydroxy-4-methylpentanoyl]-L-leucine Methyl Ester (11). N-methylmorpholine (15 mmol) was added to a stirring solution of L-α-hydroxyisocaproic acid (5 mmol) and leucine methyl ester hydrochloride (15 mmol) in 20 mL of DMF under nitrogen at room temperature. Solid N-hydroxybenzotriazole hydrate (HOBT, 5.6 mmol) and ethyl(dimethylamino)carbodiimide (EDC, 5.6 mmol) were added sequentially, and the mixture was stirred for 72 h. The DMF was removed via Kugelrohr distillation (0.015 mmHg) to give a yellow residue. This material was dissolved in 100 mL of EtOAc, and the solution was washed with saturated $NaHCO_3$, 1 M HCl, and brine, dried ($MgSO_4$), and concentrated. Purification of the residue by flash chromatography (40% EtOAc/hexane) afforded amide **11** as a colorless oil: yield, 67%; IR 3390 br, 2960, 1748, 1658, 1532 cm^{-1}; ^{1}H NMR (400 MHz) δ 0.863 (d, J = 2.8) and 0.874 (d, J = 1.8) and 0.884 (d, 12, J = 2.8), 1.2–1.3 (m, 1), 1.3–1.6 (m, 4), 1.73–1.82 (m, 1), 3.66 (s, 1), 3.92 (d, 1, J = 5.1), 4.07 (m, 1), 4.53 (m, 1), 7.03 (d, 1, J = 8.5); ^{13}C NMR δ 21.3, 21.6, 22.7, 23.4, 24.4, 41.1, 43.5, 50.2, 52.2, 70.6, 173.7, 175.1; MS (FAB) m/z 260 $(MH)^+$. Anal. Calcd for $C_{13}H_{25}NO_4$: C, 60.03; H, 9.65; N, 5.41. Found: C, 59.67; H, 9.39; N, 5.48.

General Procedure for Synthesis of Methyl Arylphosphonic Acids 13. A solution of the arylphosphonic diacid **12**[36] (62.3 mmol) in 100 mL of trimethylorthoformate was heated to 90 °C under nitrogen for 20 h. The mixture was cooled to room temperature and concentrated in vacuo to a colorless oil. This oil was dissolved in 100 mL of MeOH and 100 mL of 2 N NaOH and the solution was stirred at room temperature for approximately 80 h. After concentration in vacuo to remove the MeOH, the solution was diluted with an equal volume of water, washed with Et_2O, and acidified to pH 1 with concentrated H_2SO_4. The aqueous mixture was then extracted with three 50-mL portions of EtOAc, and the organic layer was dried ($MgSO_4$) and evaporated to yield the monomethyl ester **13**.

Methyl (p-nitrophenyl)phosphonic acid (13a): yield, 79%; an analytical sample was purified by recrystallization from EtOAc/hexane, mp = 124–126 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 3.69 (d, 3, J = 11.5), 7.93 (dd, 2, J = 8.7, 13), 8.23 (dd, 2, J = 3.2, 8.7); ^{13}C NMR (CD_3OD) δ 52.9 (d, J = 5.8), 123.4 (d, J = 15.8), 132.7 (d, J = 10.9), 150.3; ^{31}P NMR (CD_3OD) δ 18.3; MS (FAB) m/z 204 $(MH)^+$; UV 270 nm (ϵ 10 000). Anal. Calcd for $C_7H_8NO_5P$: C, 38.75; H, 3.72; N, 6.46; P, 14.27. Found: C, 38.77; H, 3.75; N, 6.46; P, 14.70.

Methyl (p-chlorophenyl)phosphonic acid (13b): obtained as a colorless oil; yield, 75%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.68 (d, 3, J = 11.4), 7.40 (dd, 2, J = 3.5, 8.4), 7.70 (dd, 2, J = 8.4, 13.3). ^{13}C NMR δ 52.5 (d, J = 5.8), 126.5 (d, J = 196), 128.7 (d, J = 16.0), 132.8 (d, J = 11.2), 138.9 (d, J = 3.9); ^{31}P NMR δ 20.2; MS (FAB) m/z 207 $(MH)^+$; UV 264 nm (ϵ 390). Anal. Calcd for $C_7H_8ClO_3P$: C, 40.72; H, 3.91; P, 15.00. Found: C, 40.82; H, 3.99; P, 14.67.

Methyl phenylphosphonic acid (13c): obtained as a colorless oil; yield, 60%; ^{1}H NMR (400 MHz, CD_3OD) δ 3.67 (d, 3, J = 11.3), 7.48 (m, 2), 7.57 (m, 1), 7.78 (m, 2); ^{13}C NMR (102 MHz, CD_3OD) δ 52.9 (d, J = 5.4), 129.6 (d, J = 14.8), 130.0 (d, J = 188), 132.4 (d, J = 10.0), 133.5 (d, J = 3.0); ^{31}P NMR (CD_3OD) δ 20.1; MS (FAB) m/z 173 $(MH)^+$; UV 263 nm (ϵ 500). Anal. Calcd for $C_7H_9O_3P$: C, 48.88; H, 5.26; P, 18.01. Found: C, 48.90; H, 5.27; P, 17.80.

Methyl (p-methylphenyl)phosphonic acid (13d): yield, 58%; an analytical sample was recrystallized from EtOAc/hexane, mp = 77.0–78.8 °C; ^{1}H NMR (400 MHz) δ 2.38 (s, 3), 3.68 (d, 3, J = 11.4), 7.24 (dd, 2, J = 4.0, 8.0), 7.68 (dd, 2, J = 8.0, 13.5); ^{13}C NMR δ 21.6, 52.2 (d, J = 5.9), 125.7 (d, J = 197), 129.0 (d, J = 15.6), 131.4 (d, J = 10.6), 142.8 (d, J = 22.7); ^{31}P NMR δ 22.0; MS (FAB) m/z 187 $(MH)^+$; UV 261 nm (ϵ 340). Anal. Calcd for $C_8H_{11}O_3P$: C, 51.65; H, 5.96; P, 16.65. Found: C, 51.47; H, 5.87; P, 16.57.

Methyl (p-ethoxyphenyl)phosphonic acid (13e): recrystallized from EtOAc/hexane; yield, 82%; mp = 90.0–91.5 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 1.39 (t, 3, J = 7.0), 3.62 (d, 3, J = 11.3), 4.05 (q, 2, J = 7.0), 7.00 (dd, 2, J = 3.3, 8.8), 7.68 (dd, 2, J = 8.8, 13); ^{13}C NMR (CD_3OD) δ 15.0, 52.7 (d, J = 5.5), 64.7, 115.5 (d, J = 16), 120.9 (d, J = 195), 134.4 (d, J = 11), 163.7 (d, J = 3.1); ^{31}P NMR (CD_3OD) δ 21.5; MS (FAB) m/z 217 $(MH)^+$; UV 269 nm (ϵ 910). Anal. Calcd for $C_9H_{13}O_4P$: C, 50.04; H, 6.06; P, 14.34. Found: C, 50.14; H, 6.06; P, 14.58.

General Method for the Preparation of Esters 18b–e. To a solution of 5.45 g of N-carbobenzoxy-L-leucine (20.5 mmol) in CH_2Cl_2 (20 mL) were added the appropriate α-hydroxy ester (21.5 mmol), 2.78 g of N-hydroxybenzotriazole (HOBT) (20.5 mmol), and 3.94 g of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) (20.5 mmol) in succession with stirring under an atmosphere of N_2 or Ar at room temperature. After 6–24 h, the reaction was worked up and chromatographed (10% EtOAc in CH_2Cl_2) to give the desired esters as clear oils.

Methyl O-(N-carbobenzoxy)-L-leucyl)hydroxyethanoate (18b): yield, 38–54%; IR 3068, 3020, 2960, 2903, 2873, 1752, 1724, 1719, 1550, 1469, 1440, 1388, 1218, 1164, 1082, 976, 928 cm^{-1}; ^{1}H NMR δ 0.92 (d, 6, J = 5.6), 1.51–1.62 (m, 1), 1.57–1.78 (m, 2), 3.69 (s, 3), 4.41 (dt, 1, J = 3.5, 8.6), 4.60 (dd, 2, J = 12.8, 15.7), 5.07 (s, 2), 5.54 (d, 1, J = 8.4), 7.29 (s, 5); ^{13}C NMR δ 21.4, 22.6, 24.4, 40.9, 51.9, 52.1, 60.6, 66.6, 127.7, 127.8, 128.2, 136.0, 155.8, 167.5, 172.3. Anal. Calcd for $C_{17}H_{23}NO_6$: C, 60.51; H, 6.87; N, 4.17. Found: C, 60.88; H, 7.06; N, 3.91.

Methyl O-[N-carbobenzoxy-L-leucyl]-(S)-2-hydroxy-3-phenylpropanoate (18c): yield, 40–51%; IR 3091, 3068, 3021, 2961, 2939, 2904, 2873, 1748, 1710, 1677, 1558, 1469, 1419, 1387, 1370, 1336, 1283, 1122, 1082, 1049, 981, 929 cm^{-1}; ^{1}H NMR δ 0.94 (d, 6, J = 3.1), 1.41–1.54 (m, 1), 1.53–1.81 (m, 2), 3.18 (m, 2), 3.69 (s, 3), 4.42 (dt, 1, J = 4.4, 8.4), 5.05 (d, 1, J = 8.5), 5.10 (s, 2), 5.26 (dd, 1, J = 5.2, 6.0), 7.18–7.29 (m, 5), 7.35 (s, 5); ^{13}C NMR δ 21.6, 22.6, 24.4, 37.0, 41.3, 52.0, 66.7, 73.1, 77.4, 126.9, 127.8, 127.9, 128.4, 129.1, 135.1, 136.2, 155.7, 169.2, 172.2. Anal. Calcd for $C_{24}H_{29}NO_6$: C, 67.42; H, 6.84; N, 3.29. Found: C, 67.09; H, 6.51; N, 2.98.

Methyl O-[N-carbobenzoxy-L-leucyl]-(S)-2-hydroxypropanoate (18d): yield, 54–72%; IR 3071, 3054, 3021, 2914, 2870, 1755, 1723, 1718, 1558, 1500, 1471, 1389, 1215, 1171, 1070, 960, 931 cm^{-1}; ^{1}H NMR δ 0.90 (d, 6, J = 5.6), 1.3–1.5 (m, 1), 1.42 (d, 3, J = 7.0), 1.68–1.71 (m, 2), 3.62 (s, 3), 4.39 (dt, 1, J = 3.5, 8.6), 5.03 (s, 2), 5.10 (q, 1, J = 7.0), 5.71 (d, 2, J = 8.7), 7.28 (s, 5); ^{13}C NMR δ 16.4, 21.4, 22.7, 24.5, 40.6, 51.8, 51.9, 66.4, 68.6, 127.5, 127.6, 128.0, 136.0, 155.8, 170.4, 172.4. Anal. Calcd for $C_{18}H_{25}NO_6$: C, 61.51; H, 7.17; N, 4.00. Found: C, 61.88; H, 7.01; N, 4.17.

Methyl O-[N-carbobenzoxy-L-leucyl]-(S)-2-hydroxy-4-methylpentanoate (18e): yield, 48%; IR 3074, 3020, 2971, 2913, 2867, 1741, 1722, 1701, 1540, 1480, 1391, 1160, 1141, 1087, 1001, 974, 928, 771, 738 cm^{-1}; ^{1}H NMR δ 0.92–0.99 (m, 12), 1.5–1.7 (m, 2), 1.6–1.9 (m, 4), 3.72 (s, 3), 4.43 (dt, 1, J = 5.1, 8.2), 5.08 (m, 1), 5.09 (d, 1, J = 8.1), 5.11 (s, 2), 7.35 (s, 5); ^{13}C NMR δ 21.4, 21.5, 22.8, 24.4, 24.5, 39.5, 41.2, 52.0, 52.1, 66.7, 61.4, 77.4, 127.8, 127.9, 128.3, 136.1, 155.8, 170.5, 172.7. Anal. Calcd for $C_{21}H_{31}NO_6$: C, 64.09; H, 7.94; N, 3.58. Found: C, 63.82; H, 7.66; N, 3.81.

General Method for the Preparation of the Protected Inhibitors 20a–20e. To a stirred suspension of 860 mg of methyl [(N-carbobenzoxyamino)methyl]phosphonic acid[37] (3.3 mmol) of 10 mL of dry CH_2Cl_2 was added 300 μL of $SOCl_2$ (4.14 mmol). After stirring at room temperature for 3.5 h, the majority of the solvent was removed by immersing the reaction flask in a warm (≈50 °C) water bath and sweeping with nitrogen; additional volatile materials were removed in vacuo to provide the oily phosphonochloridate **19**, which was used without purification.

In a separate flask, the carbobenzoxy ester (**18b–e**) (2.21 mmol) was dissolved in dry EtOAc. Pd/C (42 mg of 5% Pd/C) was added and the reaction placed under a balloon of hydrogen gas. After 3.5–5 h, the solution was filtered through Celite to remove the Pd/C and the volatile materials were removed in vacuo. The oily residue was dissolved in dry CH_2Cl_2 (3 mL) with stirring under Ar. Et_3N (920 μL, 6.6 mmol) was added along with 4-Å molecular sieves (≈25 mg). This solution was stirred for 30 min and then cannulated into a solution of the phosphonochloridate in 2 mL of dry CH_2Cl_2 under Ar. The amine flask was rinsed with additional CH_2Cl_2, which was added to the reaction mixture (total CH_2Cl_2, 10 mL). After being stirred for 24–48 h, the reaction mixture was placed directly on a silica chromatography column (2.5% MeOH in CH_2Cl_2). The product fractions were pooled to give an oil, which contained the desired product as a mixture of phosphorus diastereomers.

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(37) Jacobsen, N. E.; Bartlett, P. A. *J. Am. Chem. Soc.* **1981**, *103*, 654–657.

Methyl *N*-[[(carbobenzoxyamino)methyl]methoxyphosphinyl]-L-leucine (20a): yield, 46–63%; IR 3020, 2963, 1743, 1222, 1211, 1150, 1045, 848 cm^{-1}; ^1H NMR δ 0.87–0.94 (m, 6), 1.4–1.5 (m, 1), 1.5–1.8 (m, 2), 3.3–3.7 (m, 6), 3.95 (m, 1), 5.08–5.10 (m, 2), 5.75 (t, 0.5, J = 8.2), 5.81 (t, 0.5, J = 8.0), 7.30 (s, 5); ^{13}C NMR δ 21.4, 21.6, 22.7, 22.8, 24.4, 24.5, 36.6, 36.8, 39.4, 39.6, 47.3 (J_{PC} = 386), 47.4 (J_{PC} = 385), 52.1, 52.2, 52.3, 52.4, 67.0, 67.1, 127.9, 128.0, 128.1, 128.2, 128.3, 156.3, 156.4, 174.8, 174.9, 175.2, 175.3; ^{31}P NMR δ 27.3, 28.0. Anal. Calcd for $C_{17}H_{27}N_2O_6P$: C, 52.83; H, 7.04; N, 7.28; P, 8.01. Found: C, 52.61; H, 7.11; N, 7.01; P, 7.90.

Methyl *O*-[[[(carbobenzoxyamino)methyl]methoxyphosphinyl]-L-leucyl]hydroxyethanoate (20b): yield, 68–76%; IR 3020, 2960, 1749, 1735, 1719, 1539, 1519, 1440, 1422, 1305, 1218, 1210, 1044, 928, 776, 743, 730 cm^{-1}; ^1H NMR δ 0.90–0.96 (m, 6), 1.4–1.9 (m, 3), 3.3–3.8 (m, 3), 3.5–3.7 (m, 6), 3.9–4.2 (m, 1), 4.50–4.75 (2 AB, 2), 5.15 (s, 2), 5.65 (br s, 0.5), 5.71 (br s, 0.5), 7.31 (s, 5); ^{13}C NMR δ 21.2, 21.5, 22.6, 24.1, 24.4, 42.8, 42.9, 43.1, 47.2 (J_{PC} = 396), 51.8, 56.1 (J_{PC} = 439), 59.3 (J_{PC} = 746), 68.9 (J_{PC} = 839), 127.7, 127.8, 128.2, 136.1, 136.2, 156.2, 156.3, 167.6, 173.6, 173.8; ^{31}P NMR δ 27.6, 28.5. Anal. Calcd for $C_{19}H_{29}N_2O_8P$: C, 51.34; H, 6.58; N, 6.33; P, 6.97. Found: C, 52.06; H, 6.29; N, 6.38; P, 6.61.

Methyl *O*-[[[(carbobenzoxyamino)methyl]methoxyphosphinyl]-L-leucyl]-(*S*)-2-hydroxy-3-phenylpropanoate (20c): yield, 47–58%; IR 3094, 3065, 3047, 3019, 2966, 2942, 2900, 2870, 1751, 1715, 1675, 1559, 1422, 1408, 1390, 1371, 1335, 1290, 1275, 1104, 1080, 934 cm^{-1}; ^1H NMR δ 0.91 (md, 6), 1.3–1.8 (m, 3), 3.15–3.30 (m, 2), 3.45–3.60 (m, 2), 3.6–3.7 (md/s, 6), 3.7 (m, 1), 4.0 (m, 1), 5.11 (s, 2), 5.26 (dt, 1), 5.58 (br s, 1), 7.15–7.22 (m, 5), 7.34 (s, 5); ^{13}C NMR δ 21.4, 21.5, 22.5, 22.7, 24.2, 24.3, 37.0, 41.3, 41.4, 52.0, 52.2 (J_{PC} = 480), 52.4 (J_{PC} = 851), 53.1 (J_{PC} = 361), 53.2 (J_{PC} = 350), 66.5, 66.8, 73.1, 77.4, 126.9, 127.0, 127.1, 127.3, 127.5, 128.1, 128.3, 128.5, 129.1, 135.1, 136.2, 155.7, 169.1, 172.2, 173.4; ^{31}P NMR δ 27.6, 28.3. Anal. Calcd for $C_{26}H_{35}N_2O_8P$: C, 57.44; H, 6.49; N, 5.18; P, 5.70. Found: C, 57.28; H, 6.21; N, 5.29; P, 6.12.

Methyl *O*-[[[(carbobenzoxyamino)methyl]methoxyphosphinyl]-L-leucyl]-(*S*)-2-hydroxypropanoate (20d): yield, 67–85%; IR 3030, 2961, 2873, 1751, 1738, 1717, 1542, 1522, 1460, 1442, 1418, 1300, 1225, 1217, 1208, 1120, 1051, 928, 776 cm^{-1}; ^1H NMR δ 0.91–0.98 (m, 6), 1.45–1.51 (m, 3), 1.50–1.90 (m, 3), 3.10–3.58 (m, 3), 3.6–3.8 (m, 6), 4.0 (m, 1), 5.05–5.15 (m, 1), 5.10 (m, 2), 5.57 (t, 0.5, J = 8.1), 5.50 (t, 0.5, J = 7.9), 7.30 (s, 5); ^{13}C NMR δ 16.7, 21.1, 21.3, 22.6, 22.8, 24.3, 24.4, 36.8, 43.0, 43.1 (J_{PC} = 427), 43.4, 47.0 (J_{PC} = 408), 51.1, 51.2, 60.6 (J_{PC} = 865), 60.7 (J_{PC} = 855), 127.9, 128.0, 128.4, 136.1, 136.2, 156.4, 170.7, 173.7, 173.9, 174.3, 174.4; ^{31}P NMR δ 27.6, 28.5. Anal. Calcd for $C_{20}H_{31}N_2O_8P$: C, 52.39; H, 6.81; N, 6.14; P, 6.76. Found: C, 52.02; H, 6.66; N, 6.48; P, 6.51.

Methyl *O*-[[[(carbobenzoxyamino)methyl]methoxyphosphinyl]-L-leucyl]-(*S*)-2-hydroxy-4-methylpentanoate (20e): yield, 71–77%; IR 3020, 3011, 2970, 2961, 2873, 1750, 1738, 1540, 1460, 1441, 1428, 1310, 1300, 1221, 1201, 1180, 1041, 934, 780 cm^{-1}; ^1H NMR δ 0.89–1.00 (m, 12), 1.41–1.90 (m, 6), 3.05 (t, 0.5, J = 6.1), 3.11 (t, 0.5, J = 5.9), 3.4–4.0 (m, 2), 3.65 (ms, 6), 4.0–4.1 (m, 1), 5.0–5.1 (m, 1), 5.05 (s, 2), 5.38 (t, 0.5, J = 6.0), 5.49 (t, 0.5, J = 6.3), 7.34 (s, 5); ^{13}C NMR δ 21.7, 22.8, 24.3, 24.4, 39.4, 42.9, 49.1 (J_{PC} = 371), 49.4 (J_{PC} = 366), 51.9, 52.0, 53.3, 66.8, 76.4, 127.9, 128.3, 136.1, 136.2, 156.3, 170.6, 173.9, 174.3; ^{31}P NMR δ 27.5, 28.4. Anal. Calcd for $C_{23}H_{37}N_2O_8P$: C, 55.18; H, 7.45; N, 5.63; P, 6.19. Found: C, 55.00; H, 7.22; N, 5.91; P, 6.28.

Enzymology.[38] **Kinetic Parameters for Thermolysin Hydrolysis of faGLa.** To a thermolysin solution was added faGLa such that the concentration of enzyme was a constant 15 nM and the concentration of faGLa varied from 0.10 to 8.0 mM. For the highest concentrations of faGLa, short path length cells (2 and 5 mm) were used to keep the absorbance of the solutions below 2.5 AU. At each concentration of substrate, the difference molar extinction coefficient ($\Delta\epsilon_{345}$) was determined and varied by no more than 8%.

Determination of K_i's. The assay was initiated by the addition of faGLa (2 mM) and differences in molar extinction coefficients ($\Delta\epsilon_{345}$) measured. All velocities were determined for ≤10% reaction and were reproducible within ±8%. Kinetic parameters for the thermolysin hydrolysis of faGLa were measured as detailed above by using three different inhibitor concentrations of compounds **3a** and **5d**. Lineweaver–Burk[39] plots of the results demonstrated that these compounds were competitive inhibitors. The K_i values were determined from v_o/v_i vs [I] plots ($v_o/v_i = [I]/K_i(1 + [S]/K_m) + 1$) with six or more different concentrations of I over a range of at least $^1/_2(K_i)$–$10(K_i)$.

Computer Modeling. Modeling of the inhibitors and of the thermolysin–inhibitor complexes was carried out using PSSHOW (A. Dearing; MOGLI Version 2.2, Evans and Sutherland Corp.) and MacroModel Versions 2.0 and 2.5 (W. C. Still et al., Columbia University) on an Evans and Sutherland PS350 graphics system, and with the BioGraf program (Version 2.0, BioDesign, Inc.) on a Silicon Graphics IRIS 4D70-GTB graphics system.

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Supplementary Material Available: Experimental details for the synthesis and characterization of the phosphonates **2** (first described in ref 7b) (8 pages). Ordering information is given on any current masthead page.

(38) General procedures: All stock solutions were prepared with doubly distilled water and filtered through Millipore filters (0.45-μm pore size). The standard buffer for all assays was 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), 2.5 M NaBr, 10 mM $CaCl_2$, and 2.5% (v/v) DMF at pH 7.0. Buffers were prepared and adjusted to pH at room temperature. For assays incubated for 12 h, 0.1% bovine serum albumin (w/v) was added. Assays were performed on a Cary 219 UV–vis or a Uvikon 860 spectrophotometer. A Lauda Model RM 20 circulating constant-temperature bath connected to a water-jacketed sample holder was used for temperature regulation (25.0 ± 0.2 °C). All inhibitors were shown to be free of any organic or phosphorus-containing impurities by high-field ^1H and ^{31}P NMR spectroscopy. Concentrations of inhibitors **3** and **4** were determined by careful dilution of a precisely weighed sample of the inhibitor and correction for the percent phosphorus determined by elemental analysis. Concentrations of inhibitors **5** were determined by UV absorbance based on the ϵ values determined for the corresponding analytically pure methyl arylphosphonate (**13**). Thermolysin was obtained from Cal Biochem (3× recrystallized) and used without further purification. Enzyme concentration was determined by UV absorbance and was between 10 and 20 nM. Furanacryloyl-glycyl-leucinamide (faGLa) was obtained from Sigma and used without further purification. Stock substrate concentration was determined by UV absorbance (ϵ_{345} 766). Assays were performed at 25 °C, monitored by absorbance change at 345 nm, and followed for ≤10% of the total reaction.

(39) Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658.