were provided by incubations carried out in the absence of ATP. Reaction velocities were linear for at least 30 min and were proportional to the amount of enzyme added at the levels of enzyme activity employed.

Inhibition studies were made with six to eight levels of MgATP or L-methionine in the range (0.5–4.0) × K_M for each of two inhibitor levels that were in the range (1–10) × K_i and for control mixtures lacking inhibitor. Inhibitors were dissolved in the above pH 8.2 buffer solution prior to testing. Inhibition constants (K_i values) were obtained to within ±15% from replots of inhibitor concentrations vs. slopes or intercepts on the vertical ($1/V$) axis of double-reciprocal plots of velocity vs. substrate level. All of the latter plots were linear, as were the replots.

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Registry No. 3, 109214-84-8; 3·4Na, 109087-20-9; 4, 107961-45-5; 4 (Boc deblocked), 109087-25-4; 5, 109087-21-0; 6, 109087-22-1; 7, 35847-70-2; 9, 109087-23-2; 10, 109087-24-3; L-homocysteine disodium salt, 50615-55-9; benzyl phosphate, 1623-07-0; methionine adenosyltransferase, 9012-52-6.

Phosphorus Amino Acid Analogues as Inhibitors of Leucine Aminopeptidase

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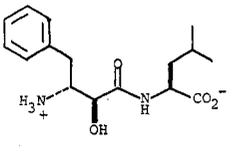
A variety of phosphorus amino acid and dipeptide analogues have been synthesized and evaluated as inhibitors of the metalloenzyme leucine aminopeptidase from porcine kidney. Two phosphonate dipeptides were found to be modest inhibitors of the enzyme (**8e**, K_i = 58 μM; **8h**, K_i = 340 μM). The phosphinic acid (**17**-OH) and phosphinamide (**17**-NH_2) analogues related to bestatin were prepared by condensation of the phosphinate amino acid derivative **11**, via a trivalent phosphonite ester **12**, with leucine isocyanate derivatives **13**. These compounds also proved to be unexceptional in their inhibition of LAP (**17**-O^-, K_i = 56 μM; **17**-NH_2, K_i = 40 μM). A series of simple (α-aminoalkyl)phosphonic acid and -phosphinic acids were also evaluated, and the most potent inhibitors were found to be the phosphonic acid analogues of L-Leu and L-Phe ((*R*)-**3e**, K_i = 0.23 μM; (*R*)-**3h**, K_i = 0.42 μM). Slow-binding behavior was observed for (*R*)-**3e** (k_{on} = 400 ± 55 M^{-1} s^{-1}) and (*R*)-**3h** (k_{on} = 445 ± 50 M^{-1} s^{-1}). The phosphinic acid analogues of Leu and Phe are 100-fold less potent than the phosphonate derivatives. The fact that tetrahedral phosphorus analogues are less potent inhibitors of LAP than they are of other zinc peptidases suggests that the mechanism of LAP may be fundamentally different than that of the latter enzymes.

Aminopeptidases are a group of zinc-containing exopeptidases with specificity for cleavage at the amino terminus of a polypeptide chain. Enzymes with similar properties have been found in bacteria and many mammalian tissues.[1] They are of biochemical as well as medicinal importance because of their putative involvement in degradation of biologically active peptides such as the enkephalins and in certain pathological conditions such as human eye cataracts.[2] Although their detailed catalytic mechanism has yet to be elucidated,[3-6] for some aminopeptidases there is evidence for a catalytic role of the zinc ion and a nucleophilic group associated with it, either a water molecule or a side-chain residue.[7-10] Effective inhibitors reported for aminopeptidases include chelating agents, such as amino acid hydroxamates[7,11,12] and amino thiols,[13] and amino acid analogues capable of forming relatively stable tetrahedral adducts, such as boronic acid derivatives,[7,10] chloromethyl ketones,[14,15] and aminoaldehydes.[9,16] The latter compounds are thought to act as transition-state analogues because of the resemblance between their adducts and the tetrahedral intermediates involved in substrate hydrolysis. The most potent aminopeptidase inhibitors are bacterially derived peptide analogues,[17-20] of which bestatin (1) is the most



1, Bestatin

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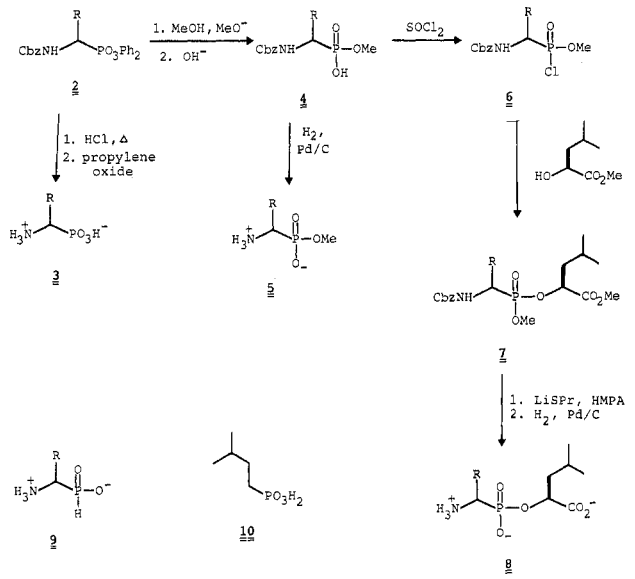
Scheme I[a]

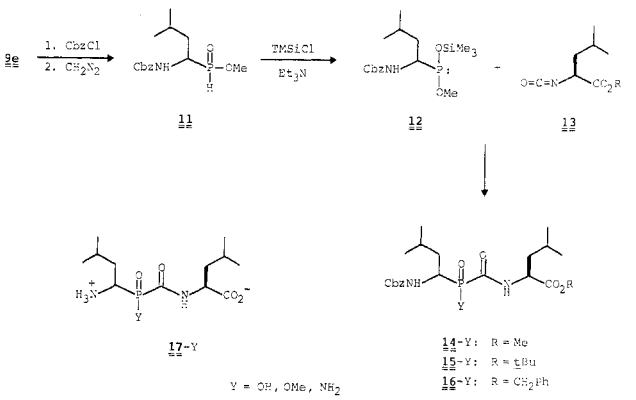


[a] **a**, R = H (Gly^P); **b**, R = Me (Ala^P); **c**, R = Et; **d**, R = *i*-Pr (Val^P); **e**, R = *i*-Bu (Leu^P); **f**, R = *n*-C_5H_{13}; **g**, R = *n*-C_7H_{15}; **h**, R = CH_2Ph (Phe^P).

effective, with a K_i for porcine kidney leucine aminopeptidase (LAP) of 0.6 nM.[20] It has been suggested that the hydroxyl group in the unusual α-hydroxy-β-amino acid structure of bestatin allows the molecule to form a five-member chelate with the active-site zinc, in conjunction with either the amino[21,22] or carbonyl groups.[23]

A successful approach for the inhibition of a number of peptidases has been to utilize phosphorus analogues to mimic unstable tetrahedral intermediates[24,25] or naturally occurring peptide inhibitors.[26] For example, potent inhibitors of the zinc peptidases thermolysin and carboxypeptidase A are obtained when the scissile carbonyl group of a substrate is replaced with a phosphonic acid moiety; in addition to their potency, these compounds appear to be transition-state analogues.[25] We sought to extend this strategy to inhibitors of porcine kidney LAP by synthesizing three types of phosphorus-containing amino acid derivatives: (1) analogues of the presumed tetrahedral intermediate from addition of water to leucylleucine and phenylalanylleucine, (2) tetrahedral analogues of leucine itself, similar to the hydrates of α-amino boronates or α-amino aldehydes, and (3) analogues related to bestatin in which the carbinol moiety has been replaced by a tetrahedral phosphorus derivative. We hoped not only to obtain potent inhibitors but also to shed light on the poorly understood enzymatic mechanism.

Scheme II



Synthesis of Inhibitors. The simple phosphonic[27,28] and phosphinic[29] amino acid analogues **3** and **9** were prepared according to literature methods. The leucine (Leu^P) and phenylalanine (Phe^P) phosphonate derivatives were resolved as described by Kafarski et al.[30] The monomethyl esters **5** were obtained from the phenyl phosphonates by ester exchange, selective hydrolysis, and hydrogenolysis, as depicted in Scheme I. Isoamylphosphonic acid (**10**) was prepared by condensation of triethyl phosphite and 1-bromo-3-methylbutane, followed by acid hydrolysis.[31]

Phosphonamidate analogues of unprotected dipeptides are too labile hydrolytically to be evaluated as LAP inhibitors, hence the phosphonate esters **8e** and **8h** were chosen as mimics of the putative tetrahedral intermediates. These compounds were prepared in straightforward fashion as shown in Scheme I. After ester exchange and partial hydrolysis, the monoesters **4e** and **4h** were converted to the chloridates and coupled with methyl (*S*)-2-hydroxy-4-methylpentanoate.[32] Selective cleavage of the methyl esters with lithium propanethiolate,[33] purification by ion-exchange chromatography, and removal of the carbobenzoxy group by hydrogenolysis gave the zwitterionic products **8**. This sequence was carried out with racemic phosphonic acids, and, for reasons discussed below, no attempts were made to separate diastereomers.

The analogues **17** related to bestatin incorporate an unusual carbamoylphosphinic acid moiety, which was constructed by condensation of a silyl phosphonite with the isocyanate derived from L-leucine. This approach is precedented in the work of Thottathil et al.,[34] who demonstrated that the phosphonite tautomers of monoalkylphosphinates can be obtained via silylation and alkylated to give dialkylphosphinates. As shown in Scheme II, the α-aminophosphinate **9e**[29] was protected with benzyl chlo-

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Table I. Inhibition of LAP by Phosphorus Dipeptide and Bestatin Analogues

compd	K_i, μM	compd	K_i, μM	compd	K_i, μM
8e[a]	58	17-O⁻[a]	56	17-NH_2[b]	40
8h[a]	340	17-OMe[b]	1650		

[a] Mixture of two diastereomers. [b] Mixture of four diastereomers.

roformate, esterified with diazomethane, and then converted to the silyl phosphonite **12** with chlorotrimethylsilane and triethylamine. The presence of the trivalent tautomer was revealed by a ^{31}P NMR resonance at δ 161.8 for **12**, in contrast to those at δ 37.0 and 38.3 for the phosphinate **11**. The isocyanates of various esters of L-leucine were prepared with phosgene dimer and then condensed with **12**, in the presence of triethylamine, to afford the carbamoylphosphinates **14** in yields of 50–78%.

Alkaline hydrolysis of the dimethyl ester **14**-OMe, followed by purification by ion-exchange chromatography and hydrogenolysis, afforded the diacid **17**-O⁻ in 92% yield. Selective cleavage of the *tert*-butyl ester with trifluoroacetic acid prior to hydrogenolysis converted **15**-OMe to the monoester **17**-OMe, also in very high yield. Selective cleavage of the phosphinate ester in **16**-OMe was achieved with bromotrimethylsilane. The resulting phosphinic acid **16**-OH was converted to the chloridate **16**-Cl with thionyl chloride and thence to the amide **16**-NH_2 with dry ammonia.[35] Hydrogenolysis of this material afforded the deprotected phosphinamide **17**-NH_2 in 67% overall yield from the ester **16**-OMe.

Enzymatic Evaluation. Commercially available LAP from porcine kidney was activated as described by Anderson et al.[16] and assayed at pH 8.4 in the presence of 5 mM $MgCl_2$, with L-leucyl-*p*-nitroanilide ($\Delta\epsilon_{405}$ = 9620 M^{-1} cm^{-1}) as substrate.[36] The K_m of the substrate was found to be 1.2 mM, in good agreement with previous reports.[10,16] The inhibition constants K_i were determined by standard methods, using Lineweaver–Burk and/or Dixon plots, and shown in each case to be of the competitive type. All of the inhibitors were shown by ^{31}P NMR to be stable under the conditions of the assay.

As shown in Table I, the phosphorus dipeptides are only modest inhibitors of LAP. These results stand in marked contrast to the effectiveness of phosphorus analogues as inhibitors of other metallopeptidases[24,25] and suggest that a fundamentally different mechanism may be involved. The bestatin analog **17**-O⁻ is not very potent either. Although not of direct relevance, we note that a similar substitution of phosphorus for the secondary carbinol moiety in pepstatin analogues leads to a potent inhibitor of pepsin.[26] The charge on the phosphinate moiety does not appear to be a factor in the analogues related to bestatin, since LAP shows similar affinity toward the methyl ester **17**-OMe and the amide **17**-NH_2. All of these derivatives were tested as diastereomeric mixtures; comparable amounts of the isomers were present in each mixture, and their separation did not appear to be warranted.

In Table II are listed the inhibition constants of analogues of leucine itself. Isoamylamine does not inhibit LAP; (3-methylbutyl)phosphonic acid, **10**, is a poor inhibitor, with a K_i of 775 μM, considerably higher than that of *n*-butylboronate (K_i = 10 μM).[7] The amino phosphonates themselves also bind less strongly than the corresponding amino boronates[10] or amino aldehydes.[9,16] For

Table II. Inhibition of LAP by (Aminoalkyl)phosphonic and -phosphinic Acids

compound[a]	K_i, μM
isoamylamine	>50000
isoamylphosphonate, **10**	775
Gly^P, **3a**	1040
Ala^P, **3b**	240
3c	3.6[b]
Val^P, **3d**	1.2[b]
L-Leu^P, (*R*)-**3e**	0.23[b]
D-Leu^P, (*S*)-**3e**	220
3f	1.0[b]
3g	0.47[b]
L-Phe^P, (*R*)-**3h**	0.42[b]
D-Phe^P, (*S*)-**3h**	15.4
Leu^P-OMe, **5e**	320
Phe^P-OMe, **5h**	43
Leu^P-phosphinate, **9e**	87
Phe^P-phosphinate, **9h**	59

[a] All chiral compounds were tested as their racemates, unless otherwise indicated. [b] Inhibitor exhibits slow-binding behavior ($k_{on} \leq 1000$ M^{-1} s^{-1}).

both the leucine (**3e**) and phenylalanine analogues (**3h**), the more potent enantiomer is that with the *R* configuration, corresponding to the natural, L stereochemistry. As would be expected from the selectivity of LAP for hydrophobic residues at the N-terminal position, the inhibition constants decrease with increasing size of the alkyl side chains, although a limit is clearly reached.

Phosphonate, boronate, and aldehyde derivatives have proven to be effective inhibitors of peptidases whose mechanisms involve direct attack of a water molecule on the amide linkage of the substrate, presumably because these derivatives can all mimic to some degree the resulting tetrahedral intermediate. The boronates and aldehydes are also effective in inhibiting the serine peptidases because of their ability to form a covalent adduct with the enzyme-bound nucleophile. In this respect they differ from simple phosphonates, which, unless specifically activated, cannot form such adducts.[37] The modest inhibition shown toward LAP by all of the phosphonate derivatives, in particular the dipeptide analogues **8** and **17**, suggests that direct attack of water on the peptide linkage is not involved in the mechanism of this enzyme and that hydrolysis may instead take place via a two-step, acylation–deacylation process. Other evidence in this regard is provided by the chemical modification studies of Mäkinen et al.,[8] which have implicated a specific tyrosyl residue in catalysis by the aminopeptidase from *Aeromonas*.

The more potent of the aminophosphonates are also slow-binding; that is, on combination of enzyme and inhibitor, equilibrium is attained at a rate that is lower than would be expected if binding were limited by diffusion alone. Slow-binding inhibition is manifested as a lag in the establishment of steady-state turnover on addition of enzyme to a solution of inhibitor and substrate (Figure 1). This lag period (Figure 2) is characterized by an exponential constant, k_{app},[38] which usually shows either a first-order (eq 1) or hyperbolic dependence on [I] (eq 2),

(35) Surprisingly, the phosphinic acid **16**-OH was unreactive toward activation by diphenyl phosphoryl azide (see: Bartlett, P. A.; Acher, F. *Bull. Soc. Chim. Fr.* **1986**, 771–775).

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(38) The exponential constant k_{app} is obtained by fitting the curve of Figure 1 to the following equation:

$$P = v_s t + (v_0 + v_s)(1 - e^{-k_{app}t})/k_{app}$$

where t = time, P = product concentration, and v_0 and v_s represent the initial velocity and the final steady-state velocity of the enzymatic reaction, respectively.[39]

Mechanism A

$$\begin{array}{l} \quad S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P \\ + \\ E \\ + \\ \quad I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \end{array}$$

$$k_{app} = k_2 + k_1\left(\frac{[I]}{1 + [S]/K_m}\right) \quad (1)$$

Mechanism B

$$\begin{array}{l} \quad S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P \\ + \\ E \\ + \\ \quad I \underset{K_D}{\rightleftharpoons} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \end{array}$$

$$k_{app} = k_4 + k_3\left(\frac{[I]/K_D}{1 + [S]/K_m + [I]/K_D}\right) \quad (2)$$

reflecting either one- or two-step inhibitor binding processes, respectively.[39,40] However, unless k_{app} can be evaluated at inhibitor concentrations that approach K_D, the intermediate, "loose" complex cannot be observed and the two-step process (mechanism B) cannot be distinguished kinetically from one that involves a single, slow step (mechanism A).

For the aminophosphonates the slow binding is significant: whereas typical rate constants for the diffusion-limited association of small peptides with proteases are $>10^6$ M^{-1} s^{-1},[41] the apparent second-order rate constants for onset of inhibition (k_{on}, determined graphically from $k_{app} = k_{off} + k_{on} \times [I]$) for the phosphonate analogues of leucine ((*R*)-**3e**) and phenylalanine ((*R*)-**3h**) are 400 ± 55 M^{-1} s^{-1} and 445 ± 50 M^{-1} s^{-1}, respectively. Bestatin[19,20] and the aminoboronates[10] are also slow-binding inhibitors of LAP, although L-leucinal has not been reported as such. For the aminophosphonates (*R*)-**3e** and (*R*)-**3h**, a simple first-order dependence of k_{app} on [I] is observed over the range of inhibitor concentrations from 0.5 to 100 μM. Thus, if there are intermediates along the binding pathway for these compounds, their dissociation constants (K_D) are greater than 0.5 mM. A more detailed investigation will be required to determine whether the slow-binding process involves a protein conformational change or slow displacement of active-site water, as has been suggested in other instances.[42]

Experimental Section

General Procedures. ^{1}H NMR data are reported as chemical shift on the δ scale, relative to internal tetramethylsilane (multiplicity, number of hydrogens, coupling constant(s) in hertz). ^{31}P NMR chemical shifts are referenced to trimethyl phosphate (internal capillary) as 3.086 ppm (downfield positive). Unless otherwise indicated, NMR spectra were obtained in $CDCl_3$ solution. FAB mass spectra were obtained by using a matrix of thioglycerol/glycerol. Measurements were carried out on a Kratos MS-50 double-focusing mass spectrometer operated at ca. 7 kV accelerating voltage. Low-resolution mass spectra were recorded at 1:2000 resolution during magnetic scans. Accurate mass measurements were made by peak matching at 1:10000 resolution. UV-visible spectra and enzyme kinetic measurements were determined with a Cary 219 spectrophotometer equipped with a water-jacketed cuvette holder and interfaced with an On-Line Instruments OLIS Model 3820 system. Melting points (Pyrex capillary) are uncorrected. Unless otherwise indicated, reaction workups culminated in washing the organic layer with brine, drying over $MgSO_4$ or Na_2SO_4, and removal of the solvent under reduced pressure with a rotary evaporator and finally under vacuum. Chromatography was performed on silica gel according to the method of Still[43] using the eluting solvent indicated. All aqueous solutions for enzyme assays were prepared with deionized distilled water. Enzyme solutions were stored in plastic containers, and all aqueous solutions were dispensed with automatic pipets with disposable plastic tips.

Synthesis of Inhibitors. (*R*)- and (*S*)-(1-Amino-3-methylbutyl)phosphonic Acid ((*R*)-3e and (*S*)-3e). The title compounds were prepared in a manner similar to that reported by Kafarski et al.[30] To 87.22 g (0.193 mol) of diphenyl [1-[*N*-(benzyloxycarbonyl)amino]-3-methylbutyl]phosphonate **2e**[28] was added 280 mL of 31% HBr/acetic acid. The mixture was stirred at 22 °C for 1 h, and most of the corrosive solvent was removed by rotary evaporator. The precipitate was collected by vacuum filtration and washed with ether, to give 74.00 g (96%) of a white solid. The HBr salt was partitioned between 500 mL each of 2 N NaOH and CH_2Cl_2, and the aqueous layer was extracted with two 100-mL portions of CH_2Cl_2. The organic layers were combined and worked up to give 60.43 g (98%) of a white solid. The NMR

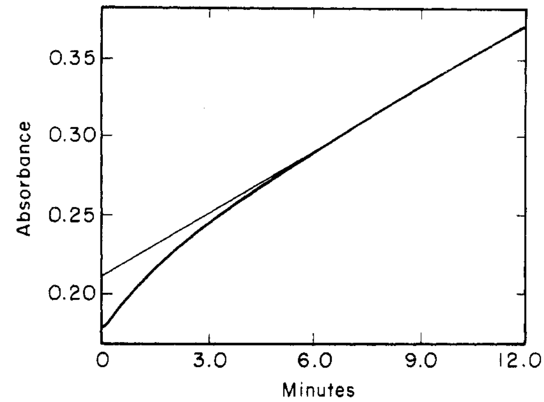

Figure 1. Onset of inhibition of LAP by L-Leu^P ((*R*)-**3e**). The curve represents the change in absorbance accompanying hydrolysis of leucine *p*-nitroanilide (1.0 mM) by LAP (15 μg/mL) in the presence of 0.5 μM inhibitor (50 mM Tris, pH 8.6, 5.0 $MgCl_2$); the straight line represents the extrapolated steady-state rate.

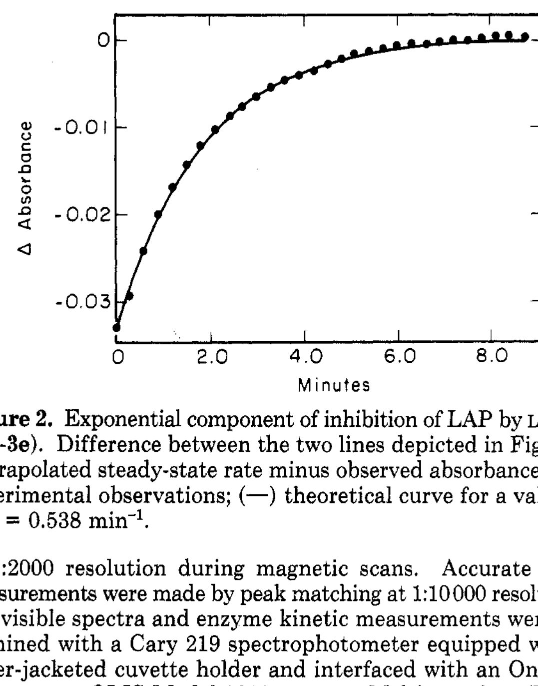

Figure 2. Exponential component of inhibition of LAP by L-Leu^P ((*R*)-**3e**). Difference between the two lines depicted in Figure 1 (extrapolated steady-state rate minus observed absorbance); (●) experimental observations; (—) theoretical curve for a value of k_{app} = 0.538 min^{-1}.

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data for this compound agreed with those reported by Oleksyszyn et al.[28]

To the free amine were added 64.26 g (0.189 mol) of (−)-dibenzoyl-L-tartaric anhydride[44] (prepared from the acid with thionyl chloride) and 950 mL of dry THF. The mixture was stirred at 22 °C for 30 h, and the solvent was removed by rotary evaporator and high vacuum to give a yellow foam. The diastereomers could be separated by selective crystallization from benzene (to give the *S* diastereomer) and diethyl ether (to give the *R* diastereomer). After 12 crystallizations we were able to obtain 46.70 g (75% of theoretical yield) of the *S* isomer (>98:2 ratio) and 39.09 g (63% of theoretical yield) of the *R* isomer (>97:3 ratio), as determined by integrations of the methyl peaks in the ^{1}H NMR. To a 10.15-g (15.4 mmol) portion of the fully protected *R* isomer were added 35 mL of acetic acid and 35 mL of 48% aqueous HBr. The solution was heated to reflux for 14 h, then cooled to 22 °C, and diluted with 60 mL of 17% EtOH/H_2O. The aqueous layer was washed with two 100-mL portions of ether, and the solvent was removed by rotary evaporator. The residue was dissolved in 30 mL of EtOH, and propylene oxide was added to induce crystallization. (*R*)-Leu^P, (*R*)-**3e**, was collected by vacuum filtration to give 2.13 g (83% yield) of fine white crystals: mp 285–286 °C (lit.[30] mp 288–290 °C); $[\alpha]^{25}_D$ −29.5° (*c* 1, 1 N NaOH) (lit.[30] $[\alpha]^{25}_D$ −24 ± 1° (*c* 1, 1 N NaOH)). The *S* enantiomer (*S*)-**3e** was similarly prepared in 80% yield: mp 284–286 °C (lit.[30] mp 278–280 °C); $[\alpha]^{25}_D$ +31.3° (*c* 1, 1 N NaOH) (lit.[30] $[\alpha]^{25}_D$ +25 ± 1° (*c* 1, 1 N NaOH)).

Methyl [1-[*N*-(Benzyloxycarbonyl)amino]-3-methylbutyl]phosphonate (4e). A 4.6-g sample (0.2 mol) of sodium metal was dissolved in 150 mL of dry methanol at 0 °C and warmed to 22 °C, and a solution of 4.53 g (10 mmol) of diphenyl ester **2e**[28] in 40 mL of dry CH_3OH was added. The solution was stirred for 2 h, most of the solvent was evaporated, and the residue was diluted with 100 mL of CH_2Cl_2 and worked up to give 3.30 g (~100%) of the corresponding dimethyl ester. To a solution of 3.26 g (9.9 mmol) of this material in 50 mL of CH_3OH was added 0.80 g (20 mmol) of solid NaOH. The mixture was heated at reflux for 4 h and stirred overnight at 22 °C, and a third equivalent of NaOH was added. After the mixture was refluxed for 4.5 h, it was acidified with 3 N HCl, some of the solvent was removed by rotary evaporator, and the aqueous solution was extracted with three 15-mL portions of CH_2Cl_2. The organic layer was worked up to give 3.86 g of a cloudy oil, which was recrystallized twice from CH_2Cl_2/Et_2O/hexane (1:2:4) to give 2.70 g (87% yield) of **4e** as fluffy white crystals: mp 118–119 °C; ^{1}H NMR (CD_3OD) δ 0.89–0.96 (2 d, 6, *J* = 6.6, 6.4), 1.41–1.80 (m, 3), 3.69 (d, 3, *J* = 10.6), 4.07 (ddd, 1, *J* = 15.7, 11.8, 3.8), 5.12 (s, 2), 7.23–7.38 (m, 5); ^{31}P NMR (CD_3OD) δ 27.20. Anal. C, H, N, P.

A similar procedure was used to prepare the Phe^P analogue **4h** in 74% yield.[45] Anal. C, H, N, P.

Methyl (1-Amino-2-phenylethyl)phosphonate (5h). A suspension of 0.349 g (1 mmol) of methyl [1-[*N*-(benzoyloxycarbonyl)amino]-2-phenylethyl]phosphonate (**4h**) and 0.12 g (0.1 mmol) of 9% Pd/C in 10 mL of methanol was exposed to hydrogen gas (1 atm) with vigorous stirring for 2 h. The catalyst was removed by filtration through Celite, and the methanol was evaporated to give 0.210 g of a white powder, mp 225–230 °C. The material was recrystallized from methanol/ethyl acetate/diethyl ether/hexane to give 0.155 g (72% yield) of **5h** as a white crystalline solid: mp 240–241 °C dec; IR (KBr) 3400–2400 (br), 1552, 1215, 1086, 1060 cm^{-1}; ^{1}H NMR (D_2O) δ 2.86 (ddd, 1, *J* = 14.7, 11.5, 8.6), 3.30 (ddd, 1, *J* = 14.7, 4.8, 4.8), 3.51–3.65 (d, 3, *J* = 10.2, overlapping m, 1), 7.20–7.27 (m, 5); ^{31}P NMR (D_2O) δ 15.52. Anal. C, H, N, P.

The leucine analogue **5e** was prepared in a similar manner in 70% yield.[43] HRMS (FAB) calcd for $C_6H_{16}NO_3P$ *m/z* 182.0946, found 182.0944.

Methyl (2*S*)-2-[[Methoxy[2-phenyl-1-[[(phenylmethoxy)carbonyl]amino]ethyl]phosphinyl]oxy]-4-methylpentanoate (7h). To 1.75 g (5.0 mmol) of methyl [1-[*N*-(benzyloxycarbonyl)amino]-2-phenylethyl]phosphonate (**4h**) in 20 mL of dry CH_2Cl_2 was added 0.73 mL (10.0 mmol) of thionyl chloride at 22 °C. The solution was stirred for 3.5 h, and the volatile materials were removed by rotary evaporator, followed by high vacuum. The phosphonochloridate was not purified but dissolved immediately in 10 mL of dry CH_2Cl_2, and a solution of 0.79 g (5.5 mmol) of methyl (2*S*)-2-hydroxy-4-methylpentanoate[46] and 1.41 mL (10.0 mmol) of triethylamine in 5 mL of CH_2Cl_2 was added. After 26 h, the mixture was washed with two 25-mL portions of 1 N HCl and twice with 25 mL of saturated $NaHCO_3$, and the organic layer was worked up to give 1.51 g of a yellow oil. This material was purified by chromatography (40% EtOAc/hexane) to give 1.01 g (42% yield) of **7h** as a clear oil and a mixture of four diastereomers. An analytical sample was prepared by bulb-to-bulb distillation (220 °C (0.03 mm)): IR ($CHCl_3$) 2950, 1745, 1730, 1510, 1260, 1050 cm^{-1}; ^{1}H NMR δ 0.90–1.00 (m, 6), 1.50–1.90 (m, 3), 2.72–3.02 (m, 1), 3.20–3.35 (m, 1), 3.65–3.83 (m, 6), 4.35–4.65 (m, 1), 4.80–5.10 (m, 3), 7.08–7.40 (m, 10); ^{31}P NMR δ 25.54, 25.67, 26.74, 26.88 (relative areas 2.4:1:1:2.4). Anal. C, H, N, P.

The leucine analogue **7e** was prepared in the same manner in 35% yield.[43] Anal. C, H, N, P.

(2*S*)-2-[[Hydroxy(2-phenyl-1-aminoethyl)phosphinyl]oxy]-4-methylpentanoate (8h). Nitrogen gas was bubbled for 10 min through a solution of 114 mg (0.24 mmol) of the dimethyl ester **7h** in 1.6 mL of dry HMPT and 2.0 mL of dry THF. To this solution was added 1.09 mL (0.598 mmol) of a 0.55 M solution of lithium propanethiolate in HMPT,[33] and the mixture was stirred under nitrogen at 22 °C for 2 h. Water (15 mL) was added, and the mixture was washed once with 5 mL of CH_2Cl_2 and twice with 5 mL of $CHCl_3$. The aqueous layer was lyophilized, and the residue was dissolved in 3 mL of water and 1 mL of 2 N NaOH and loaded onto a DEAE-Sephadex anion-exchange columm (HCO_3^- form). After the column was washed with 30 mL of water, the product was eluted with a 0–1 M linear gradient of triethylammonium bicarbonate (pH 8.6). The UV-absorbing fractions were combined and lyophilized, and the residue was dissolved in water and passed through a Dowex-50W H^+ column. The resulting solution was lyophilized to give 66.5 mg (65% yield) of the diacid as a fluffy white powder, as a mixture of two diastereomers: mp 99–105 °C; IR (KBr) 3700–2500 (br), 1710, 1455, 1060, 650 cm^{-1}; ^{1}H NMR (CD_3OD, NaOD) δ 0.97 (t, 6), 1.6–1.8 (m, 2), 1.8–2.0 (m, 1), 2.6–2.9 (m, 2), 4.1–4.2 (m, 1), 4.7–4.9 (m, 2), 7.15–7.26 (m, 10); ^{31}P NMR (CD_3OD, NaOD) δ 20.08, 19.84 (1:1 ratio); FAB MS, *m/z* 472 (46), 450 (M + 1, 76), 120 (100); HRMS (FAB) calcd for $C_{22}H_{29}NO_7P$ *m/z* 450.1682, found 450.1672.

A suspension of 26.7 mg (0.06 mmol) of the N-protected diacid and 10 mg of 10% Pd/C in 0.5 mL of methanol was exposed to hydrogen gas (1 atm) for 2.5 h. The catalyst was removed by filtration through Celite, and the solvent was removed by rotary evaporator, followed by vacuum, to give 18.7 mg of the zwitterion **8h** as a yellowish gum as a mixture of two diastereomers. This material was converted to the sodium salt with 1 equiv of NaOH: mp 203–206 °C; IR (KBr) 3700–2500 (br), 1590, 1410, 1220, 1090, 1060 cm^{-1}; ^{1}H NMR (CD_3OD) δ 0.94–1.03 (2 d, 6, *J* = 12.8, 12.9), 1.65–1.74 (m, 2), 1.87–2.07 (m, 1), 2.80–3.45 (m, 2), 4.63–4.85 (m, 1), 7.22–7.42 (m, 5); ^{31}P NMR (CD_3OD) δ 12.87, 14.38 (1:1); FAB MS, *m/z* 338 (M + 1, 27), 316 (M − Na^+, 85%), 120 (100); HRMS (FAB) calcd for $C_{14}H_{22}NO_5PNa$ *m/z* 338.1133, found 338.1119.

The leucine analogue **8e** was prepared in a similar manner in 83% overall yield via the protected diacid.[43] Diacid: FAB MS, *m/z* 416 (M + 1, 96), 176 (65); HRMS (FAB) calcd for $C_{19}H_{31}NO_7P$ *m/z* 416.1838, found 416.1827. **8e**: FAB MS, *m/z* 326 (M + Na^+, 16), 304 (M + 1, 25), 282 (M − Na^+, 60), 115 (100); HRMS (FAB) calcd for $C_{11}H_{24}NO_5PNa$ *m/z* 304.1290, found 304.1278.

Methyl [1-[[(Phenylmethoxy)carbonyl]amino]-3-methylbutyl]phosphinate (11). To 0.909 g (5.96 mmol) of the amino phosphinic acid **9e** were added 6.0 mL of 2.0 M NaOH, 1.00 g (11.92 mmol) of $NaHCO_3$, and 1.26 g (11.92 mmol) of

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(45) Spectroscopic characterization is recorded in the supplementary material.

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Na_2CO_3. The solution was cooled to 0 °C, 1.28 mL (8.94 mmol) of benzyl chloroformate was added, and the slurry was warmed to 22 °C. After 1 h, an additional 1.25 mL (8.94 mmol) of benzyl chloroformate was added and the mixture was stirred overnight. Aqueous NaOH (60 mL of 0.5 N) was added, and the solution was washed twice with ether. The aqueous layer was acidified with 3 N HCl and extracted twice with 50 mL of CH_2Cl_2 and twice with 50 mL of EtOAc. The combined organic layer was worked up to give a solid residue, which was recrystallized from EtOAc to give 1.53 g (92% yield) of the *N*-Cbz derivative as fine white crystals: mp 141.5–142 °C; IR (KBr) 3290, 1720, 1548, 1256, 1160, 1010 cm^{-1}; ^1H NMR (CD_3OD) δ 0.90–0.98 (2 d, 6, J = 10.5, 10.4), 1.40–1.86 (m, 3), 3.90 (ddd, 1, J = 3.6, 10.8, 10.8), 5.11 (s, 2), 6.87 (d, 1, J = 548.0), 7.34 (br s, 5); ^{31}P NMR (CD_3OD) δ 33.18. Anal. C, H, N, P.

To 2.27 g (7.96 mmol) of the N-protected phosphinic acid in 50 mL of methanol at 22 °C was added a solution of diazomethane in ether until the yellow color persisted. After 30 min, glacial acetic acid was added to quench the excess CH_2N_2. The mixture was diluted with 150 mL of Et_2O, washed three times with saturated $NaHCO_3$, and worked up to give 2.38 g (100% yield) of **11** as a white solid as a mixture of two diastereomers. An analytical sample was prepared by recrystallization from ether/hexane: mp 70–76 °C; IR (KBr) 3220, 2330, 1720, 1550, 1260, 700 cm^{-1}; ^1H NMR (CD_3OD) δ 0.92–0.97 (m, 12), 1.50–1.92 (m, 6), 3.77 (d, 3, J = 11.3), 3.79 (d, 3, J = 11.4), 3.98–4.21 (m, 2), 5.00–5.35 (m, 2), 5.12 (s, 4), 6.97 (d, 1, J = 551.0), 6.98 (d, 1, J = 550.0), 7.35, (s, 10); ^{31}P NMR (CD_3OD) δ 38.29, 37.00 (1:1 ratio). Anal. C, H, N, P.

Methyl *N*-[[Methoxy[1-[*N*-[(phenylmethoxy)carbonyl]amino]-3-methylbutyl]phosphinyl]carbonyl]-L-leucinate (14-OMe). A 379-mg (2.6 mmol) sample of leucine methyl ester, as the free amine, was dissolved in 0.5 mL of dry THF and added over a 1-min period to a solution of 0.31 mL (2.6 mmol) of trichloromethyl chloroformate in 10 mL of dry THF at 22 °C. The mixture was stirred for 30 min, and the solvent was removed by rotary evaporator. In a dry 10-mL flask were placed 195 mg (0.65 mmol) of the methyl phosphinate **11** and 2.8 mL of dry CH_2Cl_2. The solution was cooled to 0 °C and stirred, and 0.30 mL (2.15 mmol) of dry triethylamine and 0.25 mL (1.96 mmol) of freshly distilled trimethylsilyl chloride were added. After 30 min, 0.161 mL (1.16 mmol) of dry triethylamine and a solution of 354 mg (1.16 mmol) of the crude trichloromethyl carbamate/isocyanate in 0.5 mL of dry CH_2Cl_2 were added rapidly. The mixture was stirred at 0 °C for 1.5 h and then was quenched with saturated NH_4Cl. The resulting mixture was dissolved in 50 mL of EtOAc and was washed with 25 mL of saturated NH_4Cl and saturated $NaHCO_3$. The organic layer was worked up to give 354 mg of a yellow gum, which was purified by chromatography (1:1 EtOAc/hexane) to give 273 mg (79% yield) of the carbamoyl phosphinate **14**-OMe as a clear glass as a 1:1:1:1 mixture of four diastereomers. A small sample was further purified for analysis by bulb-to-bulb distillation (>250 °C (0.05 mm)): IR ($CHCl_3$) 3401, 2985, 1735, 1662, 1510, 1270, 1045 cm^{-1}; ^1H NMR δ 0.90–0.99 (m, 12), 1.40–1.90 (m, 6), 3.70–3.81 (m, 6), 4.40–4.58 (m, 1), 4.62–4.77 (m, 1), 5.12–5.15 (m, 2), 5.40–5.65 (m, 1), 7.33 (s, 5), 7.70–7.80 (m, 1); ^{31}P NMR δ 30.08, 29.96, 29.66, 29.63 (~1:1:1:1 ratio). Anal. C, H, N, P.

The corresponding *tert*-butyl and benzyl esters **15**-OMe and **16**-OMe were prepared in a similar manner in 50% and 65% yields, respectively.[43] Anal. C, H, N, P.

Phenylmethyl *N*-[[Amino[1-[*N*-[(phenylmethoxy)carbonyl]amino]-3-methylbutyl]phosphinyl]carbonyl]-L-leucinate (16-NH_2). To 622 mg (1.14 mmol) of the benzyl ester **16**-OMe in 4.5 mL of dry CH_2Cl_2, saturated with isobutylene at 0 °C, was added 202 μL (6.83 mmol) of bromotrimethylsilane.[47] The mixture was stirred at 22 °C for 5 h, and the solvent and excess Me_3SiBr were evaporated. The excess reagent was removed by dissolving and evaporating the residue three times from 3 mL of CH_2Cl_2 and finally under high vacuum. The residue was similarly dissolved and evaporated from three 5-mL portions of methanol. The resulting phosphinic acid **16**-OH was dissolved in 4.5 mL of dry CH_2Cl_2, and 165 μL (2.28 mmol) of thionyl chloride was added. After 15 min at 22 °C, the mixture was cooled to −78 °C and dry ammonia was condensed into the flask, forming a yellow precipitate. The mixture was then warmed to −33 °C for 1 h and 22 °C for 1.5 h, and the excess ammonia was allowed to evaporate. The remaining solution was diluted with 100 mL of EtOAc and washed with two 20-mL portions of water. The organic layer was worked up to give 575 mg of an orange foam. A 484-mg sample of this material was purified by chromoatography (60% EtOAc/hexanes) to give 363 mg (71% yield) of **16**-NH_2 as a shite solid as a 1:1:1:1 mixture of diastereomers: mp 129–131 °C; IR ($CDCl_3$) 3500–3100 (br), 2980, 2250, 1730, 1710, 1650, 1500, 1260, 1185 cm^{-1}; ^1H NMR δ 0.80–0.91 (m, 12), 1.25–1.80 (m, 6), 3.31–3.38 (br d, 2), 4.16–4.42 (m, 1), 4.60–4.78 (m, 1), 5.12–5.14 (m, 4), 5.45–5.71 (m, 1), 7.33 (s, 10), 7.84–8.18 (m, 1); ^{31}P NMR δ 26.87, 26.62, 25.13, 24.69 (~1:1:1:1 ratio); FAB MS, m/z 532 (M + 1, 20%), 515 (M − NH_2, 33), 181 (100); HRMS (FAB) calcd for $C_{27}H_{39}N_3O_6P$ m/z 532.2577, found 532.2578. Anal. C, H, N, P.

***N*-[[Hydroxy(1-amino-3-methylbutyl)phosphinyl]carbonyl]-L-leucine (17-O^-).** To 263 mg (0.56 mmol) of the protected diester **14**-OMe in 5.0 mL of THF at 22 °C was added 0.56 mL (1.12 mmol) of 2.00 M NaOH. The mixture was stirred at 22 °C for 10 h, and the solvent was removed by rotary evaporator. The residue was dissolved in a minimum of water and was passed through a short Dowex-50W-X8 column (H^+ form). The resulting solution was lyophiolized to give 226 mg (92% yield) of the protected diacid as a fluffy white powder as a 1:1 mixture of diastereomers: IR (KBr) 3700–2500 (br), 1730, 1720, 1650, 1530, 1270 cm^{-1}; ^1H NMR (CD_3OD) δ 0.90–0.96 (m, 12), 1.42–1.80 (m, 6), 4.21–4.38 (m, 1), 4.56–4.62 (m, 1), 5.03–5.18 (m, 2), 7.28–7.32 (m, 5); ^{31}P NMR (CD_3OD) δ 25.23, 25.46 (1:1 ratio); FAB MS, m/z 443 (M + 1, 100); HRMS (FAB) calcd for $C_{20}H_{32}N_2O_7P$ m/z 443.1947, found 443.1960.

A suspension of 142 mg (0.32 mmol) of the N-protected diacid and 35 mg of 10% Pd/C in 1.6 mL of methanol was exposed to hydrogen (1 atm) for 2.5 h. The catalyst was removed by filtration through Celite, and the solvent was evaporated to give 91 mg (92% yield) of **17**-O^- as a white powder as a 1:1 mixture of diastereomers: IR (KBr) 3700–2400 (br), 1730, 1625, 1520, 1065 cm^{-1}; ^1H NMR (D_2O/NaOD) δ 0.88–1.00 (m, 12), 1.35–1.48 (m, 2), 1.62–1.92 (m, 4), 2.96–3.11 (m, 1), 4.31–4.40 (m, 1); ^{31}P NMR (D_2O/NaOD) δ 25.95, 25.98 (1:1 ratio); FAB MS, m/z 309 (M + 1, 100), 617 (2M + 1, 25); HRMS (FAB) calcd for $C_{12}H_{26}N_2O_5P$ m/z 309.1579, found 309.1571.

***N*-[[Methoxy(1-amino-3-methylbutyl)phosphinyl]carbonyl]-L-leucine (17-OMe).** A solution of 104 mg (0.20 mmol) of *tert*-butyl ester **15**-OMe in 0.50 mL of trifluoroacetic acid was stirred under nitrogen at 22 °C for 1 h. The solvent was evaporated to give 95 mg of a brownish oil; 59 mg (0.13 mmol) of this material was dissolved in 1.0 mL of methanol, 13 mg of 10% Pd/C was added, and the suspension was exposed to hydrogen (1 atm) for 1.5 h. The catalyst was removed by filtration through a Celite pad, and the solvent was evaporated to give 42 mg (~100% yield) of monomethyl ester **17**-OMe as a clear glass: IR (KBr) 3700–2400 (br), 1730–1600 (br), 1530, 1210, 1060 cm^{-1}; ^1H NMR (CD_3OD) δ 1.00–1.15 (m, 12), 1.72–1.98 (m, 6), 3.80–4.12 (m, 4), 4.62–4.78 (m, 1); ^{31}P NMR (CD_3OD) δ 27.18, 26.95, 26.43, 25.94 (1:1:1:1 ratio); FAB MS (glycerol), m/z 323 (M + 1, 100%), 645 (2M + 1, 10%).

***N*-[[Amino-(1-amino-3-methylbutyl)phosphinyl]carbonyl]-L-leucine (17-NH_2).** A suspension of 24.9 mg (0.047 mmol) of the phosphinamide **16**-NH_2 in 3 mL of 1:1 2-propanol/EtOAc and 20 mg of 10% Pd/C was pressurized with hydrogen in a Parr shaker to 50 psi and shaken for 20 h at 22 °C. The catalyst was removed by filtration through Celite, and the solvent was removed by rotary evaporator and high vacuum to give 13.6 mg (94% yield) of the deprotected amide **17**-NH_2 as a clear glass: IR (KBr) 3600–2450 (br), 1750–1500 (br), 1390, 1210, 1060 cm^{-1}; ^1H NMR (CD_3OD) δ 0.92–1.03 (m, 12), 1.34–1.90 (m, 6), 3.40–3.66 (m, 1), 4.36–4.49 (m, 1); ^{31}P NMR (CD_3OD) δ 24.38, 24.09, 23.69, 23.20 (~1:1:1:1 ratio); FAB MS, m/z 308 (M + 1, 100%), 291 (M − NH_2, 15%); HRMS (FAB) calcd for $C_{12}H_{27}N_3O_4P$ m/z 308.1739, found 308.1737.

Enzyme Assays. Materials. Pork kidney leucine aminopeptidase (LAP) (EC 3.4.11.1) was purchased from Sigma Chemical Co. (Type III-CP) as a suspension in 2.9 M $(NH_4)_2SO_4$,

(47) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M.-C. *Tetrahedron Lett.* **1977**, 155–158.

0.1 M Tris, 5 mM $MgCl_2$ solution, pH ~8, and was activated by following the procedure of Andersson et al.[16] Leucine *p*-nitroanilide hydrochloride (LPNA) was purchased from Sigma and was used without further purification. The buffer used for all assays was 50 mM Tris, pH 8.6, containing 5.0 mM $MgCl_2$ and 1 mM LPNA, unless otherwise stated. The initial hydrolysis rates of LPNA were determined by placing a solution of substrate and inhibitor in a cuvette and initiating the reaction by addition of ~15 μg of LAP/1.00 mL final volume. Substrate hydrolysis at 25 °C was followed by observing the increase in absorbance at λ = 405 nm ($\Delta\epsilon$ = 9620 M^{-1} cm^{-1}).

Slow-Binding of (*R*)-(1-Amino-3-methylbutyl)phosphonic Acid ((*R*)-3e). A series of eight assays were performed, with the concentration of (*R*)-**3e** varied as 0.5, 1, 2, 5, 10, and 15 μM; each reaction was followed until steady state was reached. The initial exponential phase of the reaction was analyzed by subtracting the steady-state rate for the reaction progress curve (Figure 1); a Newtonian algorithm involving a nonlinear least-squares fit to the resulting curve was used to determine the exponential constant, k_{app}. A representative, fitted curve is shown in Figure 2. A plot of k_{app} vs. inhibitor concentration was linear, indicating a one-step slow-binding mechanism (E + I ⇌ EI). The on rate, k_{on}, calculated from slope = $k_{on}/(1 + S/K_m)$, is 403 ± 55 M^{-1} s^{-1}; the off rate, k_{off}, calculated from $k_{off} = K_i \times k_{on}$, is 9.26 × 10^{-4} s^{-1}.

Slow Binding of (*R*)-(1-Amino-2-phenylethyl)phosphonic Acid ((*R*)-3h). The slow-binding behavior of the phenylalanine analogue **9a** was determined similarly, by using inhibitor concentrations of 3, 6, 9, 12, 15, 60, 80, and 100 μM: k_{on} = 445 ± 50 M^{-1} s^{-1}; k_{off} = 1.87 × 10^{-4} s^{-1}.

Inhibition of LAP by *N*-[[Amino(1-amino-3-methylbutyl)phosphinyl]carbonyl]-L-leucine (17-NH_2). The K_i of compound 17-NH_2 was determined by standard methods, using inhibitor concentrations of 30, 60, 90, 120, 150, and 180 μM. This compound is stable and does not show slow-binding behavior under the assay conditions. A Dixon plot was used to determine the K_i of compound 17-NH_2 as 40 μM;[48] a similar procedure was used to determine the K_i values of (*S*)-**3e**, **9e**, and **9h**.

Inhibition by (*R*)-L-(1-Amino-3-methylbutyl)phosphonate ((*R*)-3e). The final K_i of compound (*R*)-**3e** was determined by using inhibitor concentrations of 0.3, 0.6, 0.9, 1.2, 1.5, and 1.8 μM; each reaction was followed until an equilibrium rate was reached (16 min). A K_i value of 0.23 μM was determined from a Dixon plot.[48] A similar procedure was used to determine the K_i values of compounds **3c**, **3d**, **3f**, **3g**, and (*R*)-**3h**.

Inhibition of LAP by (2*S*)-2-[[Hydroxy(2-phenyl-1-aminoethyl)phosphinyl]oxy]-4-methylpentanoate (8h). The K_i of compound **8h** was also determined by standard methods. A series of 16 assays were performed, with substrate concentrations of 0.67, 0.1, 0.25, and 1.0 mM and inhibitor concentration of 0, 0.01, 0.1, and 1.0 mM. The reaction velocity, determined during the first 4.0 min of reaction, was plotted in Lineweaver–Burk form, and the slopes were replotted vs. [I]; the K_i value for compound **8h** was found to be 340 μM. A similar procedure was used to determine the K_i values for compounds **8e**, **5e**, **5h**, **3a**, **3b**, and (*S*)-**3h**.

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Supplementary Material Available: Characterization of the inhibitors and synthetic intermediates not described above (3 pages). Ordering information is given on any current masthead page.

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New Inhibitors of Human Renin That Contain Novel Leu-Val Replacements

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Stereoselective syntheses of several nonpeptide fragments that function as Leu^{10}-Val^{11} scissile bond replacements in human angiotensinogen are presented. The opening of N-protected aminoalkyl epoxide **3** with a variety of sulfur, oxygen, nitrogen, and carbon nucleophiles is a key reaction in the preparation of these novel fragments **4**–**8**. The coupling of these fragments to protected dipeptides that mimic positions 8 and 9 in angiotensinogen produces inhibitors of human renin even though the molecules contain no functionality beyond what is formally the Val^{11} side chain of angiotensinogen. R groups that closely resemble that of the Val side chain are preferable; thus, isopropyl ≥ higher alkyl > phenyl > substituted phenyl. Sulfur is the best X group; oxidation leads to slight (X = SO_2) and significant (X = SO) decreases in inhibitory potency. One such inhibitor, **60**, has an IC_{50} of 13 nM when tested with purified human renin at pH 6.0. The significant activity of these small inhibitors is thought to be due in part to the hydroxyl group of the fragment functioning as a transition-state analogue. Of these, the inhibitors that contain histidine show marked selectivity toward renin over a related aspartic proteinase, pepsin.

Renin is an aspartic proteinase that is released from the kidney and catalyzes a specific hydrolysis of the glycoprotein angiotensinogen to give the decapeptide angiotensin I (AI). A dipeptidylcarboxypeptidase, angiotensin converting enzyme (ACE), then converts it to the octapeptide AII, which, in addition to being an extremely potent vasoconstrictor, is also a promoter of aldosterone release and thereby sodium retention. A variety of other effects on the kidneys, brain, and pituitary are also due to the actions of AII. Aminopeptidases further act on AII to give AIII, which produces effects similar to those produced by AII, but to a lesser extent. This cascade, known as the renin–angiotensin system (RAS),[1] is therefore an important area for study in the regulation of blood pressure and electrolyte homeostasis[2] (Scheme I).

The design of ACE inhibitors as useful drugs in the treatment of hypertension and cardiac failure has been reviewed.[3] However, since renin has remarkable specificity

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