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Inhibition of Chymotrypsin by Phosphonate and Phosphonamidate Peptide Analogs

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A series of mono-, di-, and tetrapeptide analogs incorporating an activated, tetrahedral phosphorus moiety in place of the scissile carbonyl group have been synthesized and evaluated as inactivators of porcine pancreatic elastase (EC 3.4.21.11) and bovine pancreatic chymotrypsin (EC 3.4.21.1). As previously reported (Lamden, L. A., and Bartlett, P. A. (1983) *Biochem. Biophys. Res. Commun.* **112,** 1085–1090), the simple phosphonofluoridate analogs of alanine (**6a**-F) and phenylalanine (**6b**-F) are the most rapid inactivators yet reported for these enzymes. More hydrolytically stable derivatives of **5b** and **6b** were explored for chymotrypsin, and the thiophenyl, thiomethyl, and phenyl esters were shown to be effective inactivators, with the latter being the most stable. This strategy was extended to the tetrapeptide level by the synthesis of the four diastereomers of analog **8**. However, each isomer proved to be comparable in its behavior toward chymotrypsin. Potential reasons for this unexpected lack of stereoselectivity were investigated, and it was shown that the phosphorus amide substituent is capable of functioning as a leaving group. In a related dipeptide diester analog, L-**15**, a threefold difference in reactivity between phosphorus diastereomers was seen.

Phosphorylating agents are the prototypical inactivators of serine peptidases, as exemplified by diisopropyl phosphorofluoridate (DFP) (*1*). Indeed, inhibition by DFP is diagnostic for enzymes of this class. Phosphorylating agents have been of mechanistic interest as well, in view of the similarity between the tetrahedral structures of the inactivated enzyme and the putative intermediates in the enzymatic acylation and deacylation steps (*2*). However, for many potential applications, the simple phosphorylating agents suffer from an inability to discriminate between the various serine peptidases and from the lack of similarity between normal peptide substrates and the simplified phosphorus substituents, such as isopropoxy. Some attempts to address these shortcomings have been made. Becker and co-workers studied the inactivation of chymotrypsin, trypsin, and acetylcholinesterase with a series of ethyl *p*-nitrophenyl alkyl- and phenylalkylphosphonates (**1a,b**), and found a dependence on chain length which could be rationalized with that of carboxyl ester substrates (*3*). Becker also studied a series of substituted aryl α-acetoxyalkylphosphonates (**1c**) with the same enzymes (*4*). Nayak and Bender pursued related investigations for porcine pancreatic elastase, showing that the *n*-pentylphosphonate is the most active among a series of *n*-alkylphosphonate ethyl *p*-nitrophenyl esters **1a** (*5*). The stereochemical dependence of reactivity of agents with stereocenters at phosphorus has been

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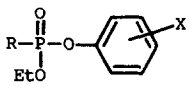
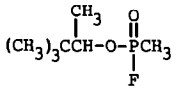
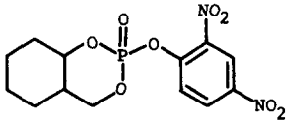
studied in the case of chymotrypsin with Soman (**2**) (*6*) and with the cyclic phosphate triester **3** (*7*).

We reasoned that inhibitors more closely analogous to natural peptides could be constructed by replacing the scissile carbonyl group of a peptide substrate with a tetrahedral phosphorylating or, more accurately, *phosphonylating* moiety. The first stage of our investigation focused on phosphonofluoridate analogs of single amino acids and compared their activity as irreversible inactivators of porcine pancreatic elastase and chymotrypsin (*8*). Our continued investigation with chymotrypsin had several goals: (1) to explore a variety of leaving groups of lesser reactivity, (2) to extend the length of the inhibitor with incorporation of a number of peptide linkages, and (3) to study the influence, if any, of the configuration of the phosphorus stereocenter on the inactivation process.







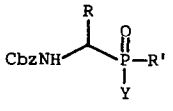
1a: R = $CH_3(CH_2)_n$, X = NO_2
1b: R = $Ph(CH_2)_n$, X = NO_2
1c: R = $CH_3(CH_2)_n$CH(OAc)–, X = NO_2, halogen

2

3

PHOSPHONOFLUORIDATES

Synthesis. The phosphorus analogs of phenylalanine and alanine are synthesized as their *N*-carbobenzoxy diphenyl esters **4**-OPh according to the method of Oleksyszyn (*9*) and converted by ester exchange in methanol and subsequent partial hydrolysis to the monomethyl esters **5**-OH. Conversion to the mixed esters **6**-OMe and the amidate **7a**-OMe is then effected via the phosphonochloridates **5**-Cl, which are prepared with thionyl chloride. The immediate precursors to the fluoridates are the corresponding chloridates, which in the case of the esters **5** and **6** are generated by another cycle of selective hydrolysis and chlorination. The amidochloridate is formed from ester **7a**-OMe by direct treatment with PCl_5. The fluoridates **5**–**7**-F can then be generated by halide exchange with anhydrous KF in acetonitrile and 18-crown-6 catalysis. The fluoridates are stable to an aqueous extraction during workup, and in the case of the phenylalanine analog **6b**-F to



4: R' = OPh
5: R' = OMe
6: R' = OiPr
7: R' = NHiPr

a: R = Me
b: R = CH_2Ph

Y = (suffix)

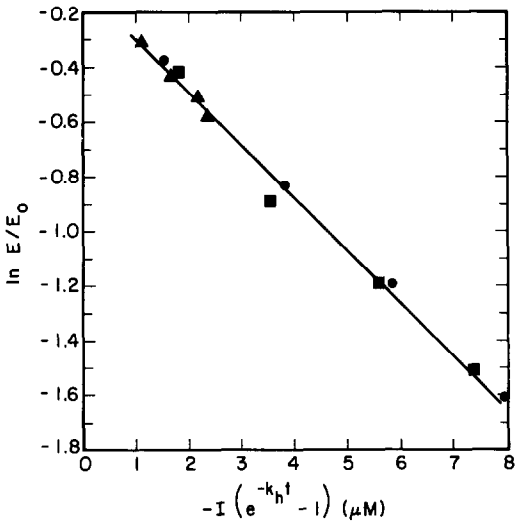


FIG. 1. Inactivation of elastase by **5a**-F. Enzyme and inactivator are incubated at 25°C, aliquots are removed and diluted sixfold into substrate (final $S = 0.67$ mM), and residual activity is determined. (●) $E_0 = 0.38$ μM, $I_0 = 8.2$ μM; (■) $E_0 = 0.38$ μM, $I_0 = 7.6$ μM; (▲) $E_0 = 0.22$ μM, $I_0 = 2.4$ μM. Abcissa calculated with $k_h = 0.63$ s^{-1}.

chromatography on silica gel. The less hindered and more polar alanine analogs **5a**-F and **6a**-F were ultimately purified by bulb-to-bulb distillation and, in the case of the amidate **7a**-F, by crystallization. In the case of the latter compound, although the amidate ester diastereomers **7a**-OMe can be separated by crystallization, the subsequent halogenation steps proceed nonstereospecifically and afford the same mixture of fluoridate diastereomers regardless of starting isomer.

Enzymatic evaluation. The phosphonofluoridates were evaluated as inactivators of porcine pancreatic elastase and bovine chymotrypsin using the spectrophotometric substrates *N-t*-butoxycarbonyl-L-alanine *p*-nitrophenyl ester (NBA) (*10*) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) (*11*), respectively. Both the chymotrypsin and elastase assays were carried out under essentially the same conditions in order to facilitate comparison between the two enzymes. Our initial evaluation of the alanine analog **5a**-F indicated that it is a potent inactivator of elastase; it was also clear, however, that the inactivation kinetics do not exhibit a simple logarithmic decrease in enzyme activity with time, even under conditions in which initial inhibitor concentration is in large excess to that of enzyme. The decreasing rate of inactivation resulted from hydrolysis of the phosphonofluoridate and required analysis according to the kinetic procedure of Ashani *et al.* (*12*). The ratio of the second order inactivation rate constant and the pseudo-first order hydrolysis rate constant (k_i/k_h) were determined by monitoring the decrease in enzyme activity with time (Eq. [1], Fig. 1). The hydrolysis rate constant (k_h) could be determined independently by allowing the inactivator to incubate in the buffer before adding enzyme: the logarithmic decrease in the apparent initial inactivator concentration on addition of enzyme provided the value of k_h according to Eq. [2]. There is a

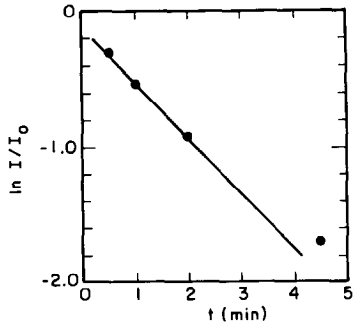


FIG. 2. Hydrolysis of **5a**-F at 25°C in 0.05 M phosphate buffer, pH 6.5. Inactivator (8.5 μM) is incubated in buffer, aliquots are removed at time t, and elastase (0.38 μM) is added. Degree of inactivation reflects residual inactivator concentration at start of second incubation. k_h calculated by this method is 0.41 s^{-1}.

reasonable correspondence between the values of k_h calculated by these two approaches (ratio of 1.5); hence the direct determination of k_h via Eq. [2] was employed in subsequent cases. From k_i/k_h and k_h, k_i could be calculated. The other derivatives were assayed in the same manner for potency as inactivators (k_i) and for hydrolytic susceptibility (k_h) (Table 1). The k_i values given refer only to the second order rate constants for inactivation; because of their potency, we were unable to assay the fluoridates at concentrations high enough to determine the reversible dissociation constants of the inhibitors before reaction (see Figs. 1 and 2).

$$\ln E/E_0 = (k_i/k_h)[I]_0(e^{-k_h t} - 1) \quad [1]$$

$$\ln I/I_0 = -k_h t. \quad [2]$$

TABLE 1

INACTIVATION OF ELASTASE AND CHYMOTRYPSIN BY PHOSPHONOFLUORIDATES[a]

Inhibitor	k_h (min^{-1})	k_i (M^{-1} s^{-1})[b]	
		Elastase	Chymotrypsin
5a-F	0.41 ± 0.09[c]	2000[c]	—[d]
6a-F	0.07 ± 0.01	1280	8,800
7a-F	0.12 ± 0.01	160	—[d]
6b-F	0.07 ± 0.02	160	180,000

[a] Assays conducted at 25°C in 0.05 M phosphate buffer, pH 6.5.

[b] Standard errors of multiple determinations ($n \geq 3$) were <30%.

[c] Recalculation of data from Ref. (*8*).

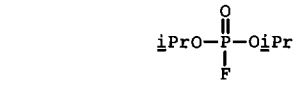
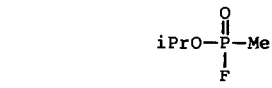
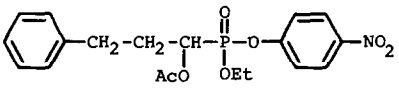
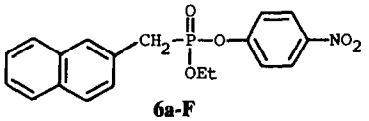
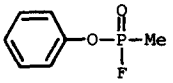
[d] Not determined.

Within the series of alanine analogs, methyl ester **5a**-F and isopropyl ester **6a**-F are similarly effective at inactivating elastase, in spite of the fact that the more hindered isopropyl ester is intrinsically less reactive (*13*). In contrast, the amidofluoridate **7a**-F is almost an order of magnitude less reactive toward elastase than the esters, yet it is of similar hydrolytic sensitivity. Whereas the amidate **7a**-F may be more sensitive to hydrolysis than the ester **6a**-F, because of electron donation from the nitrogen, the less electrophilic nature of the amidate in comparison to the latter is likely to be responsible for its lower reactivity toward the enzyme.

Porcine pancreatic elastase and chymotrypsin have opposing preferences toward the amino acid side chain which occupies the P_1 site: small (e.g., alanine) in the case of the former enzyme and large and hydrophobic (e.g., phenylalanine) for the latter. The phosphonofluoridate analogs of alanine and phenylalanine, as their isopropyl esters **6a**-F and **6b**-F, were therefore used to evaluate the potential selectivity toward related enzymes that could be obtained by this approach. Differential reactivity is seen, in that each enzyme is inactivated an order of magnitude more rapidly by the appropriate inhibitor in comparison to the other one. The phenylalanine analog **6b**-F reacts 1100 times as rapidly with chymotrypsin than it does with elastase. In fact, **6b**-F is an exceedingly potent inactivator of chymotrypsin, as the comparisons in Table 2 indicate. However, some of the selectivity

TABLE 2

RATE CONSTANTS FOR INACTIVATION OF CHYMOTRYPSIN[a]

Inhibitor	k_i (10^3 M^{-1} s^{-1})	Reference
	0.25[b]	*14*
	0.38[b]	*14*
	6.1[c]	*15*
	7.6[c]	*15*
6a-F	8.8[d]	This work
	33[b]	*14*
6b-F	180[d]	This work

[a] All values determined at 25°C.
[b] pH 7.7, 0.067 M Veronal buffer.
[c] pH 8, 0.02 M Tris buffer.
[d] pH 6.5, 0.05 M phosphate buffer.

TABLE 3

INFLUENCE OF LEAVING GROUP ON INACTIVATION OF CHYMOTRYPSIN[a]

Inhibition	k_h (min^{-1})	k_i (M^{-1} s^{-1})
5b-OMe	—[b]	—[c]
6b-OPh	<0.003	2.0
5b-SMe	0.007 ± 0.003	120
5b-SPh	0.13 ± 0.02	220
6b-F	0.07 ± 0.02	180,000

[a] pH 6.5, 0.05 M phosphate buffer, 25°C.
[b] Not detected.
[c] No inhibition observed after 22 h with **5b**-OMe at 0.5 mM.

of **6b**-F for chymotrypsin arises from that enzyme's inherently greater reactivity toward phosphonylating agents in comparison to elastase. Alanine analog **6a**-F is in fact not able to overcome this, reacting less rapidly with elastase than with chymotrypsin. These values may not represent the maximum selectivity available from structures of this type, since the phosphonofluoridates were tested as mixtures of stereoisomers.

Inactivation of elastase by **5a**-F is slowed by high levels of the substrate NBA, as expected if they bind to the same site on the enzyme. Moreover, it was shown that the observed inhibition is not due to reversible interaction with the phosphonic acid hydrolysis products. The monoesters **5a**-OH and **6a**-OH show competitive inhibition constants against elastase of 3.6 and 5.7 mM, respectively, significantly higher than the concentrations at which the fluoridates were tested.

Effect of leaving groups. While our initial investigation demonstrated that high reactivity and some selectivity could be obtained with the phosphonofluoridates, a less labile moiety is required for practical reasons. In view of the inherent reactivity of the system, we chose to use chymotrypsin as the target enzyme and probe the effectiveness of various leaving groups with the phenylalanine analogs listed in Table 3. These compounds were prepared straightforwardly from the phosphonochloridates **5b**-Cl and **6b**-Cl by reaction with phenol and the thiols.

Although considerably less reactive as an inhibitor than the other inactivators, the phenyl ester **6b**-OPh proved to be effectively inert toward the aqueous buffer system. This leaving group was therefore elected for further evaluation in a stereochemically defined series of extended oligopeptide inhibitors.

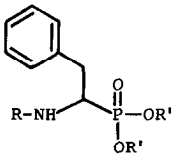
SYNTHESIS, SEPARATION, AND EVALUATION OF TETRAPEPTIDE ISOMERS

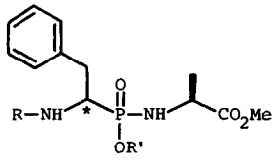
In their work on the substrate specificity of chymotrypsin, Bauer *et al.* (*14*) noted that Ac-Ala-Pro-Phe-Ala-NH_2 is highly reactive in comparison with similar di-, tri-, and tetrapeptides. We therefore envisaged the phosphonate derivate **8** as

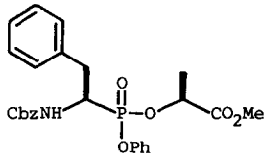
a potential inactivating agent for chymotrypsin. This compound incorporates all of the elements of an oligopeptide chain and provides an opportunity to evaluate the influence of the various stereocenters associated with the phosphorus amino acid.

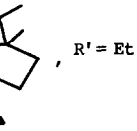
Resolution of the diethyl ester of 1-amino-2-phenylethyl phosphonic acid was accomplished via the *N*-camphanyl derivative **9**, as reported by Kotynski and Stec (*15*). This derivative was prepared from the Oleksyszyn product **4b**-OPh via ester exchange, hydrogenolysis, and reaction with camphanyl chloride. Chromatographic separation and recrystallization afforded each diastereomer in an isomeric purity of greater than 97%, as monitored by ^{31}P NMR. Hydrolysis with 48% HBr and neutralization with propylene oxide (*16*) afforded the free acids **10a**, whose configurations had already been assigned by Kafarski *et al.* (*17*). The L(*R*)- and D(*S*)-enantiomers of **5b**-OMe were recovered by acylation with carbobenzoxy chloride under Shotten-Baumann conditions (*18*) and reesterification with trimethyl orthoformate at reflux (*19*). Further support for the assignment of the L(*R*)- and D(*S*)-enantiomers of **5b**-OMe was obtained by conversion to the *S*-(-)-2-methoxy-2-trifluoromethylphenylacetyl (MTPA) amides **11** and analysis according to the method of Dale and Mosher (*20*).

Each enantiomer of **5b**-OMe was hydrolyzed to the monoester and converted via the acid chloride **5b**-Cl to the mixed phenyl methyl ester **5b**-OPh (= **4b**-OMe). In each case the remaining methyl ester was cleaved selectively with *t*-butylamine (*21*) and the acid chloride **4b**-Cl was generated with thionyl chloride. On coupling with alanine methyl ester, the alanine amides **L(*R*)-12** and **D(*S*)-12** were produced, each isomerically pure at the carbon stereocenters but a mixture of diastereomers at phosphorus. High pressure liquid chromatography allowed the separation of each of the mixtures into the two phosphorus-isomeric components, designated **L-12A** and **L-12B**, and **D-12A** and **D-12B**. All four isomers are resolved by ^{31}P NMR, and we were able to discern that none of them was contaminated with isomeric material. We were not, however, able to assign the configurations of the phosphorus stereocenters.



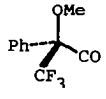




9: R =  , R' = Et

10a: R = H, R' = H

10b: R = Cbz, R' = H

11: R =  , R' = Me

8: R = Ac-Ala-Pro, R' = Ph

12: R = Cbz, R' = Ph

13: R = Cbz, R' = Me

14: R = Ac-Ala-Pro, R' = Me

15

The free amines arising from deprotection of **12** are of limited stability in the presence of the phenyl ester moiety; hence it is important to ensure that they are trapped as rapidly as possible. Elaboration of the isomers of **12** to those of tetrapeptide analog **8** was therefore accomplished by hydrogenolysis of the carbobenzoxy groups in the presence of the symmetrical anhydride of *N*-acetyl-L-alanyl-L-proline. A typical yield of the amides under these conditions is 50–60%. ^{31}P NMR confirmed that no interconversion of diastereomers takes place under these conditions.

Inactivation of chymotrypsin by the phenyl ester derivatives is relatively sluggish ($t_{1/2}$ = 15–20 min at saturation). It is possible to determine the reversible dissociation constants (K_D) of the four isomers of **8** as well as the first order rate constants (k_2) for the inactivation process, as indicated in Eq. [3]. The results presented in Table 4 reveal that there is no significant difference in

$$E + I \underset{K_D}{\rightleftharpoons} E \cdot I \overset{k_2}{\rightleftharpoons} E\text{—}I \qquad [3]$$

either of these parameters among the phosphorus diastereomers or the L and D isomers. Although peptide substrates provide no precedent for or against specificity at the phosphorus center, the insensitivity to the stereochemistry α- to phosphorus is in direct contrast to that shown by chymotrypsin toward peptide substrates of differing configuration in the P_1 site (*22*). Although these inhibitors exhibit reversible dissociation constants on the same order of magnitude as K_m for related substrates (Ac-Pro-Ala-Pro-Phe-Ala-NH_2 = 130 μM) (*14b*), our results suggest that the initial binding interactions of the isomers are nonspecific or nonproductive. It was demonstrated that inactivation of chymotrypsin by the isomers of **8** is functionally irreversible: after complete inactivation of the enzyme, separation of the unbound inhibitor by gel filtration chromatography did not result in the recovery of any enzymatic activity.

The obvious lack of discrimination toward the phosphorus stereocenter suggested that the phosphonamide nitrogen may function as an alternative leaving group in the inactivation process, allowing phosphonylation to proceed regardless of which moiety occupies a favorable position to do so. This inference was supported by the observation that the *methyl* ester tetrapeptide analog **14** was just as

TABLE 4

INACTIVATION OF CHYMOTRYPSIN BY TETRAPEPTIDE ISOMERS **8**[a]

Inhibitor	K_D (μM)	k_2 (min^{-1})	$k_i = k_2/K_D$ (M^{-1}/s^{-1})
L-**8A**	36	0.035	16
L-**8B**	34	0.054	27
D-**8A**	44	0.031	12
D-**8B**	46	0.036	13

[a] pH 7.5, 0.05 M Tris buffer, 25°C.

effective an inactivator as the phenyl phosphonamidates **8**, when tested as a mixture of isomers. On the other hand, the alanine moiety is *not* an effective leaving group at the dipeptide stage: no inactivation of chymotrypsin could be discerned with the lower homolog **13**.

In a further attempt to uncover some stereospecificity toward the phosphorus center, we prepared the two diastereomers of **L-15** from ethyl L-lactate and the acid chloride derived from the L(*R*)-enantiomer of **4b**-OH (see above). These isomers were separated by HPLC and their purity was again verified by ^{31}P NMR. At a concentration of 100 μM, **L-15B**, the more slowly eluting diastereomer, inactivates chymotrypsin three times as fast as **L-15A** ($k_{obs}/[I] = 15$ vs 5 $M^{-1}\,s^{-1}$). Unless the lactate oxygen is able to serve as an effective leaving group at the tetrapeptide stage, the modest stereoselectivity observed indicates that the sought-after specificity of binding is not manifested in these inhibitors either.

CONCLUSION

Although we were not able to uncover the anticipated stereospecificity with phosphorylating analogs of di- and tetrapeptides, we have demonstrated that such oligopeptide inactivators of the serine peptidases can be synthesized and utilized. Such compounds still hold promise for structural and spectroscopic investigations of these enzymes, in view of the similarity between the putative tetrahedral intermediate in the acylation process and the expected form of the inactivated enzyme.

EXPERIMENTAL SECTION

Caution. Phosphonofluoridates and other phosphorylating-type agents can be highly dangerous neurotoxins. They should be prepared and handled only by trained personnel in a facility which is equipped for hazardous materials and where medical attention is available immediately. The toxicity of the compounds described below has not been determined.

Synthesis of Inhibitors

General. Reactions involving reagents sensitive to moisture were conducted under an atmosphere of dry nitrogen. Dichloromethane (CH_2Cl_2) and acetonitrile were distilled from CaH_2. Diethyl ether and tetrahydrofuran were distilled from sodium/benzophenone. DMF was dried over BaO and distilled. Preparative thin layer chromatography (TLC) was performed with plates precoated with Silica Gel G.F. (Analtech, Inc., Newark, DE). Chromatography was performed according to Still using Silica Gel 60 (E. Merck, Darmstadt) (*23*).

NMR spectra were determined in $CDCl_3$ solution (unless otherwise indicated). ^1H NMR spectra are reported as: chemical shift in parts per million downfield from internal tetramethylsilane (multiplicity, number of protons, coupling constants in Hertz). ^{19}F NMR chemical shifts are reported relative to internal $CFCl_3$

at 0 ppm, downfield positive. ^{31}P NMR spectra were obtained at 82 MHz using broad band ^1H decoupling; chemical shifts are reported relative to trimethylphosphate (sealed capillary) at 3.086 ppm, downfield positive. Unless otherwise indicated, IR spectra were obtained in $CHCl_3$ solution.

The usual reaction workups culminated in either (A) washing the organic layer with brine, drying over Na_2SO_4, and evaporating under reduced pressure on a rotary evaporator, or (B) washing the organic layer twice with saturated KH_2PO_4 and with 5% $NaHCO_3$ followed by workup (A).

Phenyl hydrogen 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**4b**-*OH*). A solution of 0.29 g (0.68 mmol) of **5b**-OPh (see below) in 8 ml of *t*-butylamine (distilled from KOH pellets) was heated at reflux for 18 h. After removal of the *t*-butylamine by rotary evaporation, the residue was dissolved in 20 ml of 2 N NaOH, and the solution was washed twice with ether, acidified with cold 6 N HCl, and extracted with three portions of CH_2Cl_2. The combined organic layer was dried and evaporated to give 0.24 g (83%) of the monoester. This material was recrystallized from CH_2Cl_2 and diisopropyl ether to give 0.21 g (73%) of **4b**-OH as white crystals: mp 145–147°C; ^1H NMR δ 2.6–3.4 (m, 2), 4.1–4.7 (m, 1), 4.9 (s, 2), 5.7 (br d, 1, J = 9), 7.2 (m, 15; 3 singlets), 10.0 (br, 1); IR 3420, 3300, 3080, 3040, 2960, 1710, 1600, 1550, 1500, 1460, 1320, 1230, 1210, 1030, 1010, 990, 940, 740, 700 cm^{-1}; MS m/z 410 (M-1), 91 (100%). *Anal.* Calcd for $C_{22}H_{22}NO_5P$: C, 64.23; H, 5.39; N, 3.40; P, 7.53. Found: C, 64.26; H, 5.37; N, 3.37; P, 7.54.

L- and D-Phenyl hydrogen 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (L- *and* D-**4b**-*OH*). The phosphorus diastereomers of L-**5b**-OPh and D-**5b**-OPh (see below), were treated as described above for the racemic mixture, in this case to yield enantiomerically pure L-**4b**-OH and D-**4b**-OH, respectively.

L-**4b**-OH: $[\alpha]^{21}_{Na}$ (c = 1, EtOH) −32°.

D-**4b**-OH: $[\alpha]^{21}_{Na}$ (c = 1, EtOH) +37°.

Dimethyl 1-(benzyloxycarbonylamino)ethyl phosphonate (**5a**-*OMe*). A 5.0-g sample (0.22 mol) of sodium was dissolved in 120 ml of methanol and 8.92 g (21.7 mmol) of diphenyl 1-(benzyloxycarbonylamino)ethyl phosphonate (*9*) (**4a**-OPh) was added. After 1 h at 23°C, the mixture was diluted with 200 ml of ether and washed four times with 2 N NaOH and once with 50 ml of brine. The ether layer was dried and evaporated to give 4.72 g (76% yield) of **5a**-OMe as a clear oil, which could be used without further purification. An analytical sample was prepared by thin layer chromatography (9 : 1 EtOAc : hexane) and distillation (148°C/0.8 Torr): ^1H NMR δ 1.35 (dd, 3, J = 18, 7.5), 3.69 (d, 3, J = 11), 3.71 (d, 3, J = 11), 4.8–5.5 (m, 1), 5.12 (s, 2), 6.02 (br d, 1, J = 10), 7.26 (s, 5); IR (film) 3230, 3020, 2930, 2850, 1705, 1530, 1440, 1240, 1170, 1000, 910, 830, 735, 695 cm^{-1}; MS m/z 287 (M^+), 91 (100%). *Anal.* Calcd for $C_{12}H_{18}NO_5P$: C, 50.18; H, 6.32; N, 4.88; P. 10.78. Found: C, 49.99; H, 6.21; N, 4.68; P, 10.62.

Dimethyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**5b**-*OMe*). By a similar procedure, 10.6 g (21.7 mmol) of **4b**-OPh (*9*) was converted to the dimethyl ester **5b**-OMe. The crude product was purified by crystallization from a mixture of diethyl ether and diisopropyl ether to give 6.85 g (87%) of **5b**-OPh as white plates: mp 67–69.5°C; ^1H NMR δ 2.6–3.0 (m, 2), 3.56 (d, 3, J = 10.5), 3.58 (d, 3, J = 10.5), 4.0–4.6 (m, 1), 4.80 (s, 2), 5.75 (br d, 1, J = 11), 7.15 (s, 10); IR

($CHCl_3$) 3450, 1730, 1500, 1310, 1270, 1205, 1040, 860, 700 cm^{-1}; MS *m/z* 363 (M^+), 91 (100%). *Anal.* Calcd for $C_{18}H_{22}NO_5P$: C, 59.50; H, 6.10; N, 3.85; P, 8.52. Found: C, 59.15; H, 6.08; N, 3.81; P, 8.24.

L(R)-Dimethyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate and the D(S)-isomer (**5b**-*OMe*). A solution of 0.213 g (0.69 mmol) of **L-10b** (see below) in 10 ml of trimethyl orthoformate was heated at reflux for 28 h, and then evaporated under reduced pressure. The crude product was purified by chromatography (TLC, EtOAc) to give 0.210 g (91%) of the diester **L-5b**-OMe as a clear oil: $[\alpha]_{Na}^{21}$ (c = 2, EtOH) −46°.

In a similar manner 0.218 g (0.65 mmol) of **D-10b** (see below) was esterified to provide 0.216 g (92%) of **D-5b**-OMe: $[\alpha]_{Na}^{21}$ (c = 2, EtOH) +44°.

Methyl hydrogen 1-(benzyloxycarbonylamino)ethyl phosphonate (**5a**-*OH*). A solution of 3.80 g (13.2 mmol) of **5a**-OMe and 10 ml of 2 N NaOH (20.0 mmol) in 15 ml of methanol was stirred at 23°C for 20 h. The mixture was washed twice with ether, acidified with 6 N HCl, and extracted with four 40-ml portions of CH_2Cl_2. The combined methylene chloride layer was dried and evaporated, and the residue was recrystallized from a mixture of CH_2Cl_2 and diisopropyl ether to give 3.22 g (89%) of **5a**-OH as a white powder: mp 119.5–121°C; ^1H NMR δ 1.34 (dd, 3, J = 18, 7.5), 3.65 (d, 3, J = 10.5), 3.8–4.6 (m, 1), 4.8–5.8 (br, 1), 5.11 (s, 2), 7.25 (s, 5), 10.6–11.6 (br, 1); IR 3440, 3300, 3020, 2960, 1720, 1510, 1460, 1310, 1280, 1210, 1055, 990, 720, 690 cm^{-1}; MS *m/z* 273 (M^+), 108 (100%). *Anal.* Calcd for $C_{11}H_{16}NO_5P$: C, 48.36; H, 5.90; N, 5.13; P, 11.34. Found: C, 48.45; H, 5.91; N, 5.11; P, 11.37.

Methyl hydrogen 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**5b**-*OH*). A 6.85-g (18.9 mmol) sample of **5b**-OMe was hydrolyzed in a similar manner; the crude product was also crystallized as described above for **5a**-OH to give 6.00 g (91%) of **5b**-OH as a white powder: mp 139.5–141°C; ^1H NMR δ 2.45–3.40 (m, 2), 3.60 (d, 3, J = 10.5), 3.90–4.65 (m, 1), 4.90 (s, 2), 5.10–5.35 (br d, 1, J = 11), 6.90–7.35 (m, 10), 8.3 (br s, 1); IR 3420, 1715, 1495, 1450, 1300, 1240, 1180, 1040, 980, 695 cm^{-1}; MS *m/z* 349 (M^+), 91 (100%). *Anal.* Calcd for $C_{17}H_{20}NO_5P$: C, 58.45; H, 5.77; N, 4.01; P, 8.87. Found: 58.60; H, 5.81; N, 3.94; P, 8.84.

L- and D-Methyl hydrogen 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (L- and D-5b-OH). As described above for the racemic compounds, the separate enantiomers of **5b**-OH were prepared from the individual enantiomers L- and **D-5b**-OMe.

L-5b-OMe: $[\alpha]_{Na}^{21}$ (c = 1, EtOH) −46.9°.

D-5b-OMe: $[\alpha]_{Na}^{21}$ (c = 1, EtOH) +50.7°.

Methyl 1-(benzyloxycarbonylamino)ethyl phosphonofluoridate (**5a**-*F*). To a solution of 0.10 g (0.366 mmol) of **5a**-OH in 2.5 ml of CH_2Cl_2 under nitrogen was added 30 μl (0.41 mmol) of $SOCl_2$. The solution was kept at 23°C for 4 h, and the volatile materials were removed under vacuum for 30 min to leave the crude chloridate **5a**-Cl as an oil. To a solution of this material in 2.5 ml of dry CH_3CN was added 0.085 g (1.46 mmol) of anhydrous KF and 0.010 g (0.037 mmol) of 18-crown-6 and the mixture was stirred vigorously under nitrogen at 23°C for two days. The mixture was diluted with ether, washed with five 20-ml portions of cold water, and worked up (A). The crude product was distilled (210°C/0.050 Torr) to

yield 0.034 g (34%) of the fluoridate **5a**-F as a clear, colorless oil: ^{1}H NMR δ 1.40 (dd, 3, J = 18, 8), 3.74 (d, 1.5, J = 12), 3.80 (d, 1.5, J = 12), 4.0–4.5 (m, 1), 5.05 (s, 2), 5.29 (br d, 1, 10), 7.30 (s, 5); ^{19}F NMR ($CDCl_3$) δ −76.2 (d, J = 1188), −77.7 (d, J = 1176); IR 3435, 3020, 2950, 1725, 1510, 1455, 1305, 1265, 1205, 1050, 875, 720, 695 cm^{-1}; MS m/z 275 (M^+), 91 (100%). *Anal.* Calcd for $C_{11}H_{15}NO_4PF$: C, 48.01; H, 5.49; N, 5.09; P, 11.25. Found: C, 48.05; H, 5.52; N, 4.99; P. 11.23.

Methyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonofluoridate (**5b**-*F*). In a similar manner, 0.100 g (0.287 mmol) of **5b**-OH was converted to 0.057 g (58%) of the crude fluoridate as a slightly yellow oil. This material was purified by flash chromatography (60 : 40 EtOAc : hexane) to give a quantitative recovery of **5b**-F as a clear, viscous oil: ^{1}H NMR δ 3.05 (m, 2), 3.77 (d, 3, J = 11), 4.3–4.8 (m, 1), 5.00 (s, 2), 5.15 (br d, 1, J = 10), 7.21 (s, 5), 7.23 (s, 5); ^{19}F NMR δ −72.3 (d, J = 1185), −73.7 (d, J = 1185), MS m/z 351 (M^+), 91 (100%). *Anal.* Calcd for $C_{17}H_{18}NO_4PF$: C, 58.12; H, 5.45; N, 3.99; P, 8.82. Found: C, 58.12; H, 5.54; N, 3.88; P, 8.63.

Methyl phenyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**5b**-*OPh*). In a similar manner the chloridate **5b**-Cl was prepared from 4.0 g (11.5 mmol) of **5b**-OH and dissolved in 100 ml of dry CH_2Cl_2. The solution was cooled to 0°C, 1.2 g (13 mmol) of recrystallized phenol and 2.4 ml (17 mmol) of triethylamine were added, and the mixture was stirred overnight at 21°C. The mixture was concentrated and diluted with THF, and the triethylammonium chloride which precipitated was removed by filtration. The THF was replaced with CH_2Cl_2 and the solution was worked up (A) to give a quantitative crude yield of the phenyl ester as a yellow oil. This material was chromatographed (EtOAc) to give 4.84 g (100%) of **5b**-OPh as a clear, viscous oil: ^{1}H NMR δ 2.7–3.5 (m, 2), 3.7 (d, 3, J = 11; diastereomers apparent), 4.3–4.7 (m, 1), 4.9 (s, 2; diastereomers apparent), 5.1 (br d, 1, J = 10), 7.1 (s, 15); IR (film) 3270, 3080, 3050, 2970, 1730, 1600, 1540, 1500, 1460, 1270, 1240, 1210, 1050, 940, 750, 700 cm^{-1}; MS m/z 425 (M^+), 91 (100%). *Anal.* Calcd for $C_{23}H_{24}NO_5P$: C, 64.94; H, 5.69; N, 3.29; P, 7.28. Found: C, 65.08; H, 5.72; N, 3.45; P, 7.11.

L- and D-Methyl phenyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**L-** *and* **D-5b**-*OPh*). The procedure described above for the preparation of **5b**-OPh as a mixture of four diastereomers was used to convert **L-5b**-OH to the two diastereomers of **L-5b**-OPh (L-configuration at the asymmetric carbon), and likewise to convert **D-5b**-OH to the diastereomers of **D-5b**-OPh. For neither mixture was the phophorus stereoisomers separated.

D-5b-OPh: ^{31}P NMR δ 22.64 (s), 22.48 (s), in a 1 : 1 ratio

L-5b-OPh: ^{31}P NMR δ 22.59 (s), 22.43 (s), in a 1 : 1 ratio

Dimethyl 1-benzyloxycarbonylamino-2-phenylethylphosphonothiolate (**5b**-*SMe*). Through a solution of chloridate **5b**-Cl, prepared from 0.150 g (0.43 mmol) of monoester **5b**-OH as described above, in 5 ml of dry CH_2Cl_2 was bubbled methanethiol for 5 min. Triethylamine (0.070 ml, 0.51 mmol) was added and the solution was stirred for 4 h. After dilution with CH_2Cl_2, the mixture was worked up (B) to give 0.151 g (93%) of the crude phosphonothiolate. This material was purified by chromatography (9 : 1 EtOAc : heptane) to give 0.106 g (65%) of **5b**-SMe as a clear, colorless oil: ^{1}H NMR δ 2.2 (d, 3, J = 12), 2.6–3.4 (m, 2), 3.7 (d, 3, J =

12), 4.3–4.6 (m, 1), 4.9 (s, 2), 5.4 (br d, 1, J = 10), 7.2 (s, 10); IR (film) 3240, 3030, 2950, 1710, 1530, 1450, 1290, 1250, 1210, 1020, 900, 730, 690 cm^{-1}; MS m/z 379 (M^+), 91 (100%). *Anal.* Calcd for $C_{18}H_{22}NO_4PS$: C, 56.98; H, 5.84; N, 3.69; P, 8.16; S, 8.43. Found: C, 56.59; H, 5.91; N, 3.64; P, 7.85; S, 8.19.

Methyl thiophenyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**5b**-*SPh*). In a similar manner the chloridate **5b**-Cl was prepared from 0.20 g (0.57 mmol) of **5b**-OH and dissolved in 7 ml of dry CH_2Cl_2. To this solution at 0°C was added 0.065 ml (0.63 mmol) of thiophenol followed by 0.12 ml (0.86 mmol) of triethylamine, and the mixture was stirred overnight at 21°C. The solution was diluted with CH_2Cl_2 and worked up (B) to give 0.25 g (100%) of the thiophenyl ester as a slightly colored oil. The crude product was recrystallized from a mixture of CH_2Cl_2 and diisopropyl ether to give 0.16 g (63%) of **5b**-SPh as white crystals: mp 114–116°C; ^1H NMR δ 2.5–3.4 (m, 2), 3.7 (d, 3, J = 12), 4.2–4.7 (m, 1), 4.9 (s, 2), 5.2 (br d, 1, J = 11), 7.2 (m, 15); IR 3420, 3060, 2980, 2960, 1730, 1510, 1440, 1220, 1030, 730 cm^{-1}; MS m/z 441 (M^+), 91 (100%). *Anal.* Calcd for $C_{23}H_{24}NO_4PS$: C, 62.57; H, 5.48; N, 3.17; P, 7.02; S, 7.26. Found: C, 62.26; H, 5.43; N, 3.22; P, 6.69; S, 7.05.

Isopropyl methyl 1-(benzyloxycarbonylamino)ethyl phosphonate (**6a**-*OMe*). A solution of chloridate **5a**-Cl, prepared from 1.5 g (5.49 mmol) of monoester **5a**-OH as described above, in 6 ml of dry CH_2Cl_2 was cooled to 0°C under dry nitrogen and isopropyl alcohol (0.51 ml, 6.63 mmol) and 1.53 ml (11.0 mmol) of triethylamine were added simultaneously. After 10 min at 0°C, the reaction mixture was allowed to warm to 21°C for 1 h. Ether was added to the reaction mixture, and it was filtered through a fritted-glass funnel to remove triethylammonium chloride. The filtrate was collected and concentrated, the resulting oil was dissolved in CH_2Cl_2, and the mixture was worked up (B). The crude product was purified by chromatography (9 : 1 ethyl acetate : hexane) and distillation (125°C/0.1 Torr) to give 1.55 g (89%) of **6a**-OMe as a colorless oil: ^1H NMR δ 1.25 (d, 6, J = 6), 1.3 (dd, 3, J = 18, 7.5), 3.59 (d, 1.5, J = 10), 3.61 (dd, 1.5, J = 10), 3.8–4.3 (m, 1), 4.44–4.77 (m, 1), 4.78–5.14 (br, 1), 5.00 (s, 2), 7.24 (s, 5); IR 3420, 3000, 1715, 1510, 1450, 1300, 1200, 1040, 1000, 810, 715, 690 cm^{-1}; MS m/z 315 (M^+), 91 (100%). *Anal.* Calcd for $C_{14}H_{22}NO_5P$: C, 53.33; H, 7.03; N, 4.44; P, 9.82. Found: C, 52.99; H, 6.92; N, 4.37; P, 9.76.

Isopropyl methyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**6b**-*OMe*). As described above for the formation of **6a**-OMe, 1.0 g (2.87 mmol) of **5b**-OH was converted to the chloridate **5b**-Cl and then to 1.05 g (94%) of the mixed ester **6b**-OMe as a clear oil: bp (dec.) 200°C (0.2 Torr); ^1H NMR δ 1.30 (d, 6, J = 7), 2.9–3.1 (m, 2), 3.65 (d, 3, J = 12), 4.2–4.4 (m, 1), 4.7–4.9 (m, 1), 4.95 (s, 2), 5.15 (br d, 1, J = 9), 7.15 (m, 5), 7.17 (s, 5); IR 3450, 3000, 1720, 1520, 1460, 1310, 1240, 1060, 1000, 900, 810, 700 cm^{-1}; MS m/z (M^+), 41 (100%). *Anal.* Calcd for $C_{20}H_{26}NO_5P$: C, 61.37; H, 6.70, N, 3.58; P, 7.91. Found: C, 61.64; H, 6.84; N, 3.61; P, 7.95.

Isopropyl hydrogen 1-(benzyloxycarbonylamino)ethyl phosphate (**6a**-*OH*). A solution of 0.500 g (1.59 mmol) of **6a**-OMe in 20 ml of dry CH_2Cl_2 was saturated with isobutylene at 21°C, 0.27 ml (0.32 g; 2.06 mmol) of trimethylsilyl bromide was added, and the solution was stirred in the dark 8.5 h. All volatile material was

removed under vacuum, 5 ml of methanol was added to the residue, and the mixture was stirred for 5 min. After reevaporation, the residue was dissolved in 20 ml of 2 N NaOH and the mixture was washed with ether, acidified with cold 4 N HCl, and extracted with three 20-ml portions of CH_2Cl_2. The combined organic layer was dried (Na_2SO_4) and evaporated, and the product was crystallized from a minimum of hot CH_2Cl_2 by the addition of four volumes of hot diisopropyl ether and a few drops of hexane and allowing the solution to cool. The monoisopropyl ester **6a**-OH (0.306 g, 64%) was obtained as a fine white powder: mp 85.5–87°C; ^{1}H NMR δ 1.25 (d, 6, J = 6), 1.30 (dd, 3, J = 18, 7.5), 3.75–4.30 (m, 1), 4.40–4.75 (m, 1), 4.90–5.30 (br, 1), 5.00 (s, 2), 7.25 (s, 5), 10.15–10.55 (br, 1); IR 3435, 2980, 1715, 1505, 1450, 1210, 1100, 1005, 720, 690 cm^{-1}; MS m/z 301 (M^+), 91 (100%). *Anal.* Calcd for $C_{13}H_{20}NO_5P$: C, 51.83; H, 6.69; N, 4.65; P, 10.28. Found: C, 52.01; H, 6.83; N, 4.64; P, 10.34.

Isopropyl hydrogen 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**6b**-*OH*). A solution of 0.460 g (1.18 mmol) of **6b**-OMe and 0.50 ml of 4 N NaOH (2.0 mmol) in 3 ml of methanol was stirred for 1 day at 60°C and 1 day while cooling slowly to 21°C. The mixture was diluted with 60 ml of 2 N NaOH, washed with 40 ml of ether, acidified with cold 6 N HCl, and extracted with three 30-ml portions of CH_2Cl_2. The combined organic layer was dried and evaporated to give 0.360 g (81%) of crude product which was recrystallized as described for **6a**-OH to give 0.313 g (70% overall) of a monoisopropyl ester **6b**-OH as a fine white powder: mp 137.5–140.5°C; ^{1}H NMR δ 1.24 (d, 6, J = 6), 2.5–3.4 (m, 2), 3.57 (dd, 3 at 20%, J = 10.5), 4.0–4.6 (m, 1), 4.67 (m, 1, J = 6), 4.90 (s, 20), 5.20 (br d, 1, J = 10), 7.13 (s, 5), 7.16 (s, 5), 9.93 (br, 1); IR 3450, 3030, 2980, 2950, 1730, 1520, 1460, 1220, 1010, 890, 700 cm^{-1}; MS m/z 377 (M^+), 44 (100%). *Anal.* Calcd for $C_{19}H_{24}NO_5P$: C, 60.47; H, 6.41, N, 3.71; P, 8.21. Found: C, 60.34, H, 6.35; N, 3.81; P, 8.31.

Isopropyl 1-(benzyloxycarbonylamino)ethyl phosphonofluoridate (**6a**-*F*). As described above for the preparation of the methyl ester analog **5a**-F, 0.100 g (0.332 mmol) of isopropyl ester **6a**-OH was converted to the chloridate and thence to the fluoridate. The crude product was purified by distillation (200°C/0.050 Torr) to yield 0.032 g (32%) of **6a**-F as a clear, colorless oil: ^{1}H NMR δ 1.2–1.6 (m, 9), 3.9–4.5 (m, 1), 4.6–5.0 (m, 1), 5.03 (s, 2), 5.13 (d, 1, J = 11), 7.28 (s, 5); ^{19}F NMR δ −71.8 (d, J = 1192), −73.3 (d, J = 1185); IR 3430, 2980, 1505, 1450, 1300, 1275, 1215, 1010, 860, 690 cm^{-1}); MS m/z 303 (M^+), 91 (100%). *Anal.* Calcd for $C_{13}H_{19}NO_4PF$: C, 51.49; H, 6.32; N, 4.62; P, 10.21. Found: C, 51.64; H, 6.33; N, 4.47; P, 10.04.

Isopropyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonofluoridate (**6b**-*F*). In a similar manner 0.100 g (0.265 mml) of **6b**-OH was converted to 0.059 g (59%) of crude fluoridate. This material was purified by flash chromatography (1 : 1 EtOAc : hexane) to give 0.040 g (40%) of **6b**-F as a thick, colorless oil: ^{1}H NMR δ 1.20 (d, 3, J = 6), 1.27 (d, 3, J = 6), 2.7–3.4 (m, 2), 4.2–4.8 (m, 1), 4.7–4.9 (m, 1), 4.97 (s, 2), 5.2 (br d, 1, J = 10), 7.25 (s, 5), 7.25 (s, 5); ^{19}F NMR δ −68.3 (d, J = 1185), −70.3 (d, J = 1185); IR (CH_2Cl_2) 3430, 3070, 3050, 3000, 2950, 1730, 1510, 1460, 1400, 1270, 1020, 910, 880, 700 cm^{-1}; MS m/z 379 (M^+), 91 (100%). *Anal.* Calcd for $C_{19}H_{23}NO_4PF$: C, 60.12; H, 6.11; N, 3.69; P, 8.16. Found: C, 60.14; H, 6.11; N, 3.67; P, 8.25.

Phenyl isopropyl 1-(benzyloxycarbonylamino)ethyl phosphonate (**6a**-*OPh*). The chloridate **6a**-Cl, prepared as described above from 0.10 g (0.33 mmol) of the monoester **6a**-OH, was dissolved in 3 ml of dry CH_2Cl_2 and the solution was cooled to 0°C. Phenol (0.035 g, 0.37 mmol, recrystallized from CH_2Cl_2 and petroleum ether) and 0.069 ml (0.50 mmol) of triethylamine were added and the solution was stirred for 30 min at 0°C and then for 24 h at 21°C. The mixture was worked up (B) to give 0.123 g (98%) of the crude phenyl ester. This material was purified by chromatography (TLC, 9 : 1 EtOAc : hexane) to give 0.106 g (85%) of **6a**-OPh as a clear, colorless oil: ^{1}H NMR δ 1.2 (d, 6, J = 8), 1.4 (dd, 3, J = 18, 7), 4.0–4.5 (m, 1), 4.6–5.0 (m, 1), 5.1 (s, 2), 5.4 (br d, 1, J = 10), 7.2 (s, 5), 7.3 (s, 5); IR (film) 3250, 3070, 2990, 2950, 1720, 1600, 1540, 1490, 1475, 1380, 1300, 1230, 1160, 1100, 1050, 1000, 930, 760, 700 cm^{-1}; MS *m/z* 377 (M^+), 91 (100%). *Anal.* Calcd for $C_{19}H_{24}NO_5P$: C, 60.47; H, 6.41; N, 3.71; P, 8.21. Found: C, 60.27; H, 6.50; N, 3.77; P, 8.15.

Phenyl isopropyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**6b**-*OPh*). In a similar manner, 0.10 g (0.27 mmol) of **6b**-OH was converted to 0.10 g (83%) of the crude phenyl ester. After chromatography, 0.057 g (48%) of **6b**-OPh was obtained as a clear, viscous oil; an analytical sample was prepared by crystallization from diisopropyl ether and hexane: mp 100–105°C; ^{1}H NMR δ 1.2 (d, 6, J = 6), 2.7–3.4 (m, 2), 4.2–4.6 (m, 1), 4.6–4.8 (m, 1), 4.9 (s, 2), 5.0–5.2 (br, 1), 7.1 (bs, 15); IR 3420, 3060, 3000, 1730, 1510, 1420, 1260, 1000, 930, 890, 740, 690 cm^{-1}; MS *m/z* 453 (M^+), 91 (100%). *Anal.* Calcd for $C_{25}H_{28}NO_5P$: C, 66.22; H, 6.22; N, 3.09; P, 6.83. Found: C, 66.00; H, 6.24; N, 2.96; P, 6.96.

N-Isopropyl O-methyl 1-(benzyloxycarbonylamino)ethyl phosphonamidate (**7a**-*OMe*). A 0.800-g (2.93 mmol) sample of **5a**-OH was converted to the chloridate **5a**-Cl as described above. This material was dissolved in 12 ml of CH_2Cl_2 and cooled to 0°C, and 0.292 ml (4.40 mmol) of isopropylamine followed by 0.61 ml (4.40 mmol) of triethylamine were added. The reaction mixture was kept at 21°C for 3 days and evaporated, triethylammonium chloride was precipitated by the addition of THF, and the mixture was filtered and reconcentrated. The residue was dissolved in 50 ml of CH_2Cl_2 and worked up (B) to give 0.738 g (80%) of the amidate **7a**-OMe as a white semisolid. The crude product was recrystallized from 15 ml of hot CH_2Cl_2 by the addition of 40 ml of diisopropyl ether; 0.349 g (38%) of one diastereomer of **7a**-OMe was obtained as a fluffy white solid: mp 105–112°C (on recrystallization: 114–116°C); ^{1}H NMR (250 MHz) δ 1.15 (d, 6, J = 7), 1.38 (dd, 3, J = 7, 9), 3.44 (m, 1), 3.66 (d, 3, J = 10.5), 4.04 (m, 1), 5.12 (s, 2), 5.2 (br m, 1), 5.45 (br d, 1, J = 10), 7.35 (s, 5); IR 3430, 3250, 2960, 1715, 1505, 1450, 1300, 1260, 1210, 1040, 900, 800, 700 cm^{-1}; MS *m/z* 314 (M^+), 79 (100%). *Anal.* Calcd for $C_{14}H_{23}N_2O_4P$: C, 53.50; H, 7.38; N, 8.91; P, 9.85. Found: C, 53.60; H, 7.38; N, 8.82; P, 9.94.

Concentration of the mother liquor and crystallization from 20 ml of hot diisopropyl ether by the addition of 10 ml of hexane and cooling gave 0.230 g of the other diastereomer of **7a**-OMe as a white solid: mp 78–83°C (on recrystallization: 78–80°C); ^{1}H NMR (250 MHz) δ 1.10 (t, 6, J = 7), 1.37 (dd, 3, J = 9, 7), 3.40 (m, 1), 3.66 (d, 3, J = 11), 4.0 (m, 1), 5.10 (s, 2), 5.12 (m, 1), 5.58 (br d, 1, J = 12), 7.33 (s, 5); MS *m/z* 314 (M^+), 91 (100%). *Anal.* Found: C, 53.26; H, 7.38; N, 8.88; P, 9.83.

N-Isopropyl 1-(benzyloxycarbonylamino)ethyl phosphonamidofluoridate (**7a-F**). A solution of 0.200 g (0.637 mmol) of phosponamidate **7a**-OMe and 0.133 g (0.637 mmol) of PCl_5 in 6 ml of dry CH_2Cl_2 was stirred in the dark at 21°C for 6 h. All volatile material was removed under vacuum, and the residue was redissolved in CH_2Cl_2 and reevaporated to ensure the removal of $POCl_3$, leaving the crude chloridate as a yellow, semisolid residue. The amidochloridate **7a**-Cl was dissolved in 6 ml of dry CH_3CN, 0.148 g (2.54 mmol) of anhydrous KF and 0.017 g (0.064 mmol) of 18-crown-6 were added, and the mixture was stirred vigorously at 21°C for 2 days. After dilution with either and filtration to remove precipitated salts, the mixture was washed with three 20-ml portions of cold water, dried (Na_2SO_4), and filtered, and the solvent was removed by evaporation and under vacuum. The crude, semisolid amidofluoridate (0.092 g, 47%), was recrystallized from 2 ml of hot CH_2Cl_2 by the addition of 12 ml of hot diisopropyl ether and 5 ml of hot hexane and allowing the solution to cool to give 0.059 g (30% overall) of **7a-F** as a beige powder: mp 109–113°C; ^{1}H NMR 1.03 (d, 3, J = 6), 1.16 (d, 3, J = 6), 1.39 (dd, 3, J = 16, 8), 3.0–3.1 (m, 1), 3.8–4.3 (m, 1), 5.04 (s, 2), 5.23 (br d, 1, J = 9), 5.65 (br d, 1, J = 9), 7.27 (s, 5); ^{19}F NMR δ −66.0 (d, J = 1140), −71.7 (d, J = 1140) as a 2 : 1 ratio of diastereomers; IR 3450, 3270, 3000, 1725, 1520, 1470, 1320, 1290, 1210 (br), 1060, 920, 850 cm^{-1}; HRMS Calcd for $C_{13}H_{20}N_2O_4PF$: m/z 302.1196. Found: 302.1193. *Anal.* Calcd for $C_{13}H_{20}N_2O_4PF$: C, 51.65; H, 6.67; N, 9.27; P, 10.25. Found: C, 51.09; H, 6.84; N, 8.99; P, 10.0.

N-[[1-(N-Acetyl-L-alanyl-L-prolyl)amino-2-phenylethyl]phenoxyphosphinyl]-L-alanine methyl ester (**8**). A solution of 0.27 g (1.2 mmol) of *N*-acetyl-L-alanyl-L-proline (*24*) in a mixture of 1 ml of dry DMF and 5 ml of CH_2Cl_2 was cooled to 0°C and 0.124 g (0.60 mmol) of DCC in 2 ml of CH_2Cl_2 was added. After stirring for 1 h, the mixture was filtered with CH_2Cl_2 to remove the precipitated dicyclohexylurea and concentrated under reduced pressure. The residue was mixed with 0.15 g (0.30 mmol) of **12** (mixture of isomers, see below), 0.14 g of 5% Pd/C, and 8 ml of dry ethyl acetate. This suspension was stirred vigorously under an atmosphere of hydrogen at 21°C for 2 days, then filtered through Celite with ethyl acetate and worked up (A) to give 0.18 g (100%) of the crude product as a white semisolid foam. This material was chromatographed (TLC, 8% absolute ethanol in $CHCl_3$) to yield 0.10 g (57%) of **8** as a white semisolid which crystallized upon standing: mp 63–70°C as the mixture of diastereomers; ^{1}H NMR δ 1.1–1.3 (m, 6), 1.6–2.2 (m, 6), 2.0 (s, 3), 2.9–3.5 (m, 2), 3.5–3.7 (m, 1), 3.6 (s, 3), 4.0–4.2 (m, 1), 4.5–4.9 (m, 2), 6.3–6.5 (m, 1), 6.8 (br d, 1, J = 10), 6.9 (br d, 1, J = 12), 7.3 (m, 10); IR (film) 3280 (br), 3070, 2960, 1750, 1630 (m), 1600, 1540, 1490, 1460, 1440, 1210, 1150, 925, 770, 700 cm^{-1}; MS m/z 572 (M^+), 44 (100%). HRMS Calcd for $C_{28}H_{37}N_4O_7P$: m/z 572.2399. Found: m/z 572.2399.

When the individual L- and D-stereoisomers of **12** were converted to the corresponding isomers of **8**, the compounds were further characterized as follows:

L-8A: ^{31}P NMR δ 25.5 (s), 26.4 (s), 2 : 1 ratio of conformers.

L-8B: ^{31}P NMR δ 25.4 (s), 26.0 (s), 4 : 5 ratio.

D-8A: ^{31}P NMR δ 27.5 (s), 27.8 (s), 1 : 5 ratio.

D-8B: ^{31}P NMR δ 24.3 (s), 24.6 (s), 10 : 1 ratio.

(D)- and (L)-Diethyl 1-((-)-camphanyl)amino-2-phenylethyl phosphonate (**9**): Diethyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate. Prepared from **4b-**

OPh in a similar manner to the dimethyl ester **5b**-OMe; 91% yield after chromatography (1 : 4 acetone : ether): ^{1}H NMR δ 1.2 (t, 6, J = 7), 2.6–3.4 (m, 2), 4.0 (q, 4, J = 7), 4.2–4.6 (m, 1), 4.9 (s, 2), 5.5 (d, 1, J = 10), 7.2 (s, 5), 7.25 (s, 5); IR (film) 3260, 3080, 3060, 3000, 2960, 1730, 1550, 1500, 1460, 1400, 1300, 1270, 1230, 1030, 970, 750, 700 cm^{-1}; MS m/z 391 (M^+), 91 (100%). *Anal.* Calcd for $C_{20}H_{26}NO_5P$: C, 61.37; H, 6.70; N, 3.58; P, 7.91. Found: C, 61.51; H, 6.70; N, 3.53; P. 7.64.

Diethyl 1-((-)-camphanyl)amino-2-phenylethyl phosphonate (**9**). A 0.675-g (1.73 mmol) sample of the diethyl ester was stirred in 15 ml of methanol with 0.18 g of 9% Pd/C under an atmosphere of hydrogen at 21°C. After 1 h the suspension was filtered through Celite and the solvent was removed by rotary evaporation and under vacuum. The resulting free amine was dissolved in 15 ml of dry CH_2Cl_2 and cooled to 0°C, and a solution of 0.382 g (1.76 mmol) of (-)-camphanic acid chloride (Fluka) in 2 ml of CH_2Cl_2 was added followed by 0.24 ml (1.76 mmol) of triethylamine. After stirring for 18 h at 21°C, the mixture was diluted with CH_2Cl_2 and worked up (B) to yield 0.74 g (94%) of the crude amide as a yellow–green oil. This material was recrystallized from a minimum volume of CH_2Cl_2 in cyclohexane to give 0.61 g of **9** as slightly yellow crystals: mp 122–130°C (lit. (*17*) 109–110°C, 110–111°C for the separated diastereomers); ^{1}H NMR δ 0.9 (s, 3), 1.0 (s, 3), 1.1 (s, 3), 1.3 (t, 6, J = 7), 1.6 (t, 2, J = 7), 1.9 (t, 2, J = 7), 2.1–2.3 (m, 1), 2.3–2.5 (m, 1), 2.7–2.9 (m, 1), 3.2–3.4 (m, 1), 4.1 (q, 4, J = 7), 4.6–5.0 (m, 1), 6.4 (d, 1, J = 10), 7.2 (s, 5); ^{31}P NMR δ 23.85 (s), 23.95 (s) (lit. (*17*), 22.84, 22.76); IR (CH_2Cl_2) 3430, 3070, 3000, 2950, 1790, 1680, 1520, 1270, 1050, 1030, 970, 740, 700 cm^{-1}; MS m/z 437 (M^+), 44 (100%). *Anal.* Calcd for $C_{22}H_{32}NO_6P$: C, 60.40; H, 7.37; N, 3.20; P, 7.08. Found: C, 60.28; H, 7.29; N, 3.19; P, 6.98.

A 1.63-g sample of **9**, prepared as described above, was separated into the two diastereomers by chromatography (3% acetone in $CHCl_3$, Waters Prep 500A in recycle mode) to give 0.514 g of the D-isomer: mp 107–108.5°C (lit. (*17*) 109–110°C); ^{31}P NMR δ 23.95 (s) (lit. (*17*) 22.84); and 0.451 g of the L-isomer: mp 111–112°C (lit. (*17*) 110–111°C); ^{31}P NMR δ 23.85 (s) (lit. (*17*) 22.76). Repeated recrystallization resulted in the recovery of 90% of the material chromatographed.

L(R)-1-Amino-2-phenylethyl phosphonic acid and the D(S)-isomer (**10a**). A solution of 0.594 g (1.36 mmol) of L-**9** in 30 ml of 48% aq. HBr was heated at reflux for 90 h. The aq. HBr was removed by distillation under reduced pressure until ca. 10 ml remained, 30 ml of absolute ethanol was added, and the zwitterionic product was precipitated at 21°C by the addition of 8 ml of propylene oxide (*18*). The suspension was refrigerated overnight and the solid was collected by filtration to give 0.28 g (100%) of L-**10a** as a slightly grey–brown solid: mp (dec.) 266–267°C (lit. (*25*) 268–270°C); ^{31}P NMR (NaOD/D_2O) δ 21.10 (s); $[\alpha]_{Na}^{21}$ (c = 2, 2 N NaOH) −45.2° (lit. (*19*) −49°, lit. (*17*) −38.9°).

In a similar manner, 0.968 g (2.21 mmol) of the other diastereomer, D-**9**, was hydrolyzed to yield 0.434 g (98%) of D-**10a** as a slightly discolored solid: mp (dec.) 266–267°C (lit. (*27*) 268–270°C); ^{31}P NMR (NaOD/D_2O) δ 21.13 (s); $[\alpha]_{Na}^{21}$ (c = 2, 2 N NaOH) +45.8° (lit. (*19*) +52°, lit. (*17*) +37.0°).

L(R)-1-Benzyloxycarbonylamino-2-phenylethyl phosphonic acid and the D(S)-isomer (**10b**). A 0.270-g (1.34 mmol) sample of the L-isomer of **10a** and 0.28 g (2.7 mmol) of $NaHCO_3$ were dissolved in 2.7 ml of 1 N NaOH (2.7 mmol) and 8 ml of

water and 0.57 ml (0.69 g; 4.0 mmol) of 95% benzyl chloroformate was added at 0°C. The heterogeneous mixture was stirred vigorously and allowed to warm to 21°C over 20 h, then washed twice with ether, acidified with 6 N HCl, and extracted three times with ethyl acetate. The combined organic layer was dried and evaporated to give 0.40 g (89%) of **L(*R*)-10b** as as white solid which was used without further purification: mp 148–150°C (lit. (*27*) 153–156°C); ^{1}H NMR (methanol-d_4) δ 2.7–2.9 (m, 1), 3.1–3.3 (m, 1), 4.0–4.2 (m, 1), 4.9 (s, 2), 4.9–5.0 (br, 1), 7.10 (s, 5), 7.11 (s, 5); ^{31}P NMR (NaOD/D_2O) δ 16.92 (s), 16.61 (s, 25%); $[\alpha]^{21}_{Na}$ (*c* = 2, 2 N NaOH) −96°.

In a similar manner, 0.42 g (2.1 mmol) of the D-enantiomer of **10a** was converted to 0.613 g (86%) of **D(*S*)-10b** as a white solid: mp 148–150°C; ^{31}P NMR (NaOD/D_2O) δ 16.89 (s), 16.59 (s, 25%); $[\alpha]^{21}_{Na}$ (*c* = 2, 2 N NaOH) +106°.

Further characterization was obtained for racemic material: mp 153–156°C; ^{1}H NMR (acetone-d_6) δ 2.9–3.1 (m, 1), 3.2–3.4 (m, 1), 4.2–4.4 (m, 1), 5.0 (s, 2), 6.8 (d, 1, *J* = 10), 7.3 (s, 10), 7.8 (br, 2); IR (DMSO) 3450, 3020, 2930, 1730, 1440, 1410, 1320, 1030, 960, 710 cm^{-1}; MS *m/z* 334 (M-1), 44 (100%). *Anal.* Calcd for $C_{16}H_{18}NO_5P$: C, 57.32; H, 5.41; N, 4.18; P, 9.24. Found: C, 57.09; H, 5.39; N, 4.16; P, 9.31.

Dimethyl L(R)-1-[N-(S)-(-)-(3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl) amino)-2-phenylethyl phosphonate and the D(S), (S)-isomer (**11**). A solution of 0.021 g (0.058 mmol) of **L-5b**-OMe in 2 ml of dry ethyl acetate with 10 ml of 9% Pd/C was stirred under an atmosphere of hydrogen at 21°C for 2 h and filtered through Celite with 5 ml of CH_2Cl_2. *S*-(-)-MTPA chloride (0.030 ml, 0.12 mmol) and 0.010 ml (0.07 mmol) of triethylamine were added and the mixture was stirred at 21°C for 7 h. THF was added to the mixture to precipitate triethylammonium chloride and, after filtration and evaporation of the solution, the residue was chromatographed (TLC, EtOAc) to yield 0.022 g (85%) of **L-11** as a clear oil which solidified upon standing: ^{1}H NMR δ 2.8–3.0 (m, 1), 3.26 (s, 3), 3.26–3.4 (m, 1), 3.8 (d, 6, *J* = 11), 4.7–5.0 (m, 1), 6.9 (br d, 1, *J* = 8), 7.1–7.3 (m, 10); ^{31}P NMR δ 26.38 (s).

In a similar manner 0.021 g (0.058 mmol) of **D-5b**-OMe was converted to 0.023 g (88%) of **D-11** as a clear oil: ^{1}H NMR δ 2.9–3.1 (m, 1), 3.1 (s, 3), 3.2–3.4 (m, 1), 3.6 (d, 3, *J* = 11), 3.75 (d, 3, *J* = 11), 4.7–5.0 (m, 1), 6.85 (br d, 1, *J* = 10), 7.2–7.5 (m, 10); ^{31}P NMR δ 26.53 (s).

N-[(1-Benzyloxycarbonylamino-2-phenylethyl)phenoxyphosphinyl]-L-alanine methyl ester (**12**). A 0.50-g (1.2 mmol) sample of racemic **4b**-OH was dissolved with mild heating in 20 ml of dry CH_2Cl_2 and then treated with 0.10 ml (1.3 mmol) of $SOCl_2$ for 5.5 h. After evaporation of the volatile materials, the residue was dissolved with 0.19 g (1.3 mmol) of L-alanine methyl ester hydrochloride in 20 ml of dry CH_2Cl_2, the solution was cooled to 0°C, and 0.50 ml (3.6 mmol) of triethylamine was added. After stirring at 21°C for 18 h, the solution was concentrated, the residue was dissolved in THF, triethylamine hydrochloride was removed by filtration, the THF was replaced with CH_2Cl_2, and the mixture was worked up (A) to give 0.57 g (95%) of a colored oil. This material was purified by chromatography (15% heptane/EtOAc) to give 0.46 g (77%) of **12** as a mixture of diastereomers: mp 77–89°C; ^{1}H NMR δ 1.1–1.4 (m, 3; diastereotopic doublets), 2.7–3.4 (m, 2), 3.6 (s, 3), 3.7–4.2 (m, 1), 4.3–4.7 (m, 1), 4.9 (s, 2), 5.2 (br d, 1, *J* = 11), 5.3 (br d, 1, *J* =

11), 7.2 (s, 15); ^{31}P NMR δ 26.76 (s), 26.12 (s), 25.36 (s), 24.62 (s); IR (film) 3300, 3080, 3050, 2970, 1710, 1600, 1540, 1500, 1460, 1310, 1270, 1220, 1150, 1070, 1030, 930, 775, 750, 700 cm^{-1}; MS *m/z* 496 (M^+), 91 (100%). *Anal.* Calcd for $C_{26}H_{29}N_2O_6P$: C, 62.90, H, 5.89; N, 5.64; P, 6.24. Found: C, 62.59; H, 5.99; N, 5.55; P, 6.23.

N-[L(R)-(1-Benzyloxycarbonylamino-2-phenylethyl)phenoxyphosphinyl]-L-alanine methyl ester (L(R)-12) and the [D(S)-isomer (D(S)-12); HPLC separation of the diastereomers. In the same manner as described above for the conversion of racemic **4b**-OH to the mixture of four isomers of **12**, each enantiomer of **4b**-OH was converted to the corresponding pair of phosphorus diastereomers of **12**. Each pair was separated by HPLC using a Waters analytical HPLC unit with a semipreparative capacity column (silica) in a solvent system of 51% hexane, 29% CH_2Cl_2, 20% ether, with 0.5% added ethanol. Typically 0.020–0.025 g of **12** was applied for each run, with a solvent flow rate of 4 ml/min. The pair of D-isomers was also effectively separated under similar conditions with a solvent system of 60% hexane and 40% chloroform with 0.4% added ethanol (0.7% total ethanol). The four diastereomers of **12** were distinguished by ^{31}P NMR and rotation as follows:

L-12A, first eluting: ^{31}P NMR δ 25.4 (s); $[\alpha]^{21}_{Na}$ (c = 0.5, EtOH) −36.1°.

L-12B, second eluting: ^{31}P NMR δ 26.1 (s); $[\alpha]^{21}_{Na}$ (c = 0.5, EtOH) −46.0°.

D-12A, first eluting: ^{31}P NMR δ 26.8 (s); $[\alpha]^{21}_{Na}$ (c = 0.5, EtOH) +52.4°.

D-12B, second eluting: ^{31}P NMR δ 24.6 (s); $[\alpha]^{21}_{Na}$ (c = 0.5, EtOH) +13.6°.

N-[(1-Benzyloxycarbonylamino-2-phenylethyl)methoxyphosphinyl]-L-alanine methyl ester (**13**). A sample of chloridate **5b**-Cl, prepared from 0.20 g (0.57 mmol) of **5b**-OH as described above, and 0.088 g (0.63 mmol) of L-alanine methyl ester hydrochloride were dissolved in 5 ml of dry CH_2Cl_2 and 0.20 ml (1.44 mmol) of triethylamine was added. After 18 h at 21°C, the mixture was worked up (B) to give 0.17 g (67%) of the crude phosphonamidate **13** as a clear oil, which was carried on without further purification. An analytical sample was purified by chromatography (TLC, 9 : 1 EtOAc : hexane, 73% recovery) and recrystallization from CH_2Cl_2 and toluene (50% recovery): mp 127–133°C; ^{1}H NMR δ 1.3 and 1.4 (2d, 3, J = 7, 2 : 3 ratio of diastereomers), 2.7–3.4 (m, 2), 3.7 (d, 3, J = 12), 3.75 (s, 3), 4.0–4.2 (m, 1), 4.2–4.5 (m, 1), 4.9 (br d, 1, J = 9), 5.0 (s, 2), 5.1–5.2 (m, 1), 7.2–7.4 (m, 10); ^{31}P NMR δ 28.06 (s), 28.88 (s), 29.82 (s), 30.45 (s), 2 : 3 : 3 : 0.3; IR (CH_2Cl_2) 3320, 3200, 2960, 1750, 1700, 1540, 1450, 1300, 1260, 1210, 1160, 1040, 740, 700 cm^{-1}; MS *m/z* 434 (M^+), 91 (100%). *Anal.* Calcd for $C_{21}H_{27}N_2O_6P$: C, 58.06; H, 6.26; N, 6.45; P, 7.13. Found: C, 58.24; H, 6.36; N, 6.58; P, 7.23.

N-[[1-(N-Acetyl-L-alanyl-L-prolyl)amino-2-phenylethyl]methoxyphosphinyl]-L-alanine methyl ester (**14**). As described above for preparation of the phenyl esters **8**, 0.60 g (0.14 mmol) of methyl phosphonamidate **13** was deprotected and condensed with the anhydride prepared from 0.126 g (0.55 mmol) of *N*-acetyl-L-alanyl-L-proline to give after chromatography (TLC, EtOAc) 0.013 g (19%) of **14** as a mixture of diastereomers: ^{1}H NMR δ 1.2–1.5 (m, 8), 1.5–1.9 (m, 4), 2.0 (s, 3; diastereomeric splitting apparent), 2.7–3.6 (m, 6), 3.75 (m, 6), 4.0–4.8 (m, 3), 7.25 (m, 5); ^{31}P NMR δ 27.54 (s), 27.62 (s), 28.28 (s), 28.59 (s), 29.28 (s), 29.69 (s), 29.77 (s), 29.85 (s), 30.40 (s), 30.81 (s), 31.04 (s), 31.06 (s), 31.12 (s), in a ratio of 17 : 1 : 2 : 1 : 5 : 4 : 5 : 4 : 5 : 3 : 1 : 1 : 12 (diastereomers and conformational isomers); IR (film)

3450 (br), 3270, 3060, 2950, 1740, 1640, 1550, 1450, 1210, 1150, 1040, 740, 700 cm^{-1}; MS *m/z* 510 (M^+), 44 (100%). HRMS Calcd for $C_{23}H_{35}N_4O_7P$: *m/z* 510.2243. Found: *m/z* 510.2228.

L-O-[(1-Benzyloxycarbonylamino-2-phenylethyl)phenoxyphosphinyl]-L-lactate ethyl ester (**15**). A sample of chloridate **L-4b**-Cl, prepared from 0.064 g (0.16 mmol) of **L-4b**-OH as described above, was treated with 0.019 ml (0.16 mmol) of L-lactate ethyl ester and 0.026 ml (0.19 mmol) of triethylamine to give after chromatography (EtOAc) 0.040 g (50%) of the diester **15** as a clear oil: ^1H NMR δ 1.3 (t, 3, J = 7), 1.4 (d, 3, J = 7), 2.8–3.5 (m, 2), 4.2 (q, 2, J = 7), 4.5–4.9 (m, 2), 5.0 (s, 2), 5.7 (br d, 1, J = 10), 7.25 (m, 15); ^{31}P NMR δ 20.65 (s), 22.41 (s), in a 1 : 4 ratio.

The diastereomers were separated by preparative HPLC (35% $CHCl_3$, 65% hexane, 0.1% added EtOH).

Enzymology

Porcine pancreatic elastase (Worthington, 2× crystallized, suspension) and bovine α-chymotrypsin, (Sigma, Type IV) were used as obtained. Stock solutions (approx 6×10^{-5} M) were prepared in 0.05 M sodium phosphate, 1.0 M NaCl, pH 6.5, and stored at 4°C. The stock solutions thus prepared were stable for several weeks, with no measurable loss of catalytic activity. Further dilutions were made in the same buffer system or 0.05 M Tris, pH 7.5 (see below), both without added salt. These dilute stock solutions (10^{-6} to 10^{-5} M) were also stable over periods of several weeks. The monopeptide analogs **5–7** were assayed in the phosphate buffer; compounds **8–15** in the Tris buffer.

N-t-Butyloxycarbonyl-L-alanine *p*-nitrophenyl ester (NBA) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma; stock solutions of 10^{-2} M were prepared in dry CH_3CN. Stock solutions of the inhibitors (10^{-3} to 10^{-2} M) were also prepared in dry CH_3CN. All stock solutions were stored at 4°C.

The rate of substrate hydrolysis was monitored spectrophotometrically using a Perkin–Elmer 552 A UV-Vis spectrophotometer (manual determination of initial velocity) or a Cary-219 UV-Vis spectrophotometer interfaced with an OLIS data processing system.

All incubations and assays were conducted at 25°C. Elastase activity was determined by the method of Visser and Blout (*10*), monitoring the hydrolysis of substrate (NBA) at 347.5 nm, with correction for buffer-catalyzed hydrolysis. The assays for chymotrypsin were performed in the same manner, using BTEE as the substrate and following its hydrolysis at 256 nm. For BTEE, there was no measurable non-enzymatic hydrolysis.

Diluted enzyme stock, buffer, and inactivator were added to polyethylene tubes and incubated at 25°C. Assays were conducted by adding aliquots of the incubation mixture to a quartz cuvette charged with buffer and substrate to produce a final substrate concentration of approx 10^{-4} M and a final enzyme concentration between 10^{-8} and 10^{-6} M as appropriate for the particular measurement. The rates of inactivation of elastase or chymotrypsin were followed by incubation of the enzyme and inactivator for various times before diluting into excess substrate and determining the remaining activity. The rates of hydrolysis of the inhibitors were

measured by incubating the compound in buffer for various times before the addition of enzyme.

Determinations of K_D and k_2 for the tetrapeptide inhibitors were conducted as described above, using various concentrations of each inhibitor (10^{-5} to 2×10^{-4} M) and determining the rate of enzyme inactivation for each. The data were analyzed in double reciprocal form according to Wilson (*26*). For determination of K_D for monoesters **5a**-OH and **6a**-OH, inhibitor stock solutions were prepared in combinations of methanol and phosphate buffer to concentrations of approx 7×10^{-2} M. To a quartz cuvette was added 0 to 1.0 ml of the stock solution, 0.03 ml of 10^{-2} M NBA, and enough buffer to total 2.50 ml. To this was added 0.50 ml of a solution of elastase (ca. 8×10^{-7} M) and after stirring the hydrolysis of NBA was monitored and recorded. Typical series of 0.05, 0.10, 0.20, 0.50, and 0.70 ml of inhibitor stock were added prior to assay of enzyme activity. K_D was determined by the method of Dixon (*27*).

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