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Peptidic Phosphonylating Agents as Irreversible Inhibitors of Serine Proteases and Models of the Tetrahedral Intermediates[†]

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ABSTRACT: Peptide analogues incorporating an electrophilic phosphorus moiety (**2-6**) have been synthesized and studied as inhibitors of a variety of serine proteases. Inhibition is irreversible and, for α -lytic protease (ALP), shown to result from covalent binding to the active site serine hydroxyl [Bone, R., Sampson, N. S., Bartlett, P. A., & Agard, D. A. (1991) *Biochemistry* (following paper in this issue)]. For reaction of human leukocyte elastase (HLE) with the thiophenyl esters **6s-V** (Boc-AAPV ψ [P=O(SPh)O]AA-OMe), **4s-V** (BocAAPV ψ [P=O(SPh)O]-Me), and **3s-V** (Boc-V ψ [P=O(SPh)O]AA-OMe), evidence is presented to suggest that the S₄-S₁ subsites, but not the S₁' and S₂' positions, are occupied by the inhibitors during the inactivation process. The selectivity that is observed between the proteases and the hexapeptide phosphonates **6o-V** (Boc-AAPV ψ [P=O(OPh)O]AA-OMe) and **6o-F** (Boc-AAPF ψ [P=O(OPh)O]AA-OMe) parallels that between these enzymes and their substrates: ALP and HLE are selectively inactivated by the Val^P-containing analogue **6o-V**, while subtilisin (SUB) shows a preference for the Phe^P derivative **6o-F**. A detailed kinetic analysis of the enzyme-inhibitor interactions was complicated by the susceptibility of the inhibitors to enzymatic degradation. The configuration at phosphorus was found not to have a significant influence on the rate at which the inhibitors react with the peptidases. Moreover, in the case of inactivation of ALP by the hexapeptide **6o-V**, the same covalent adduct is formed from both stereoisomers (Bone et al., 1991), indicating that one of these diastereomers undergoes substitution with retention of configuration.

No group of enzymes is as diverse, as ubiquitous, or as fundamental to a host of critical functions as the serine proteases (Kraut, 1977; Polgar, 1989). Divergent evolution of the chymotrypsin/trypsin family and convergent evolution of the subtilisin family are responsible for the identity of these enzymes with respect to mechanism and their diversity with respect to substrate specificity. Reflecting their varying roles as broadly or narrowly specific hydrolases, the serine proteases demonstrate sequence selectivity, through preference for certain amino acids at designated positions relative to the scissile linkage in the substrate, as well as macromolecular selectivity, through interactions over a greater range. Understanding the basis of serine protease selectivity has depended to a great extent on the structural information that has come from crystallographic studies of these enzymes and their complexes with both low and high molecular weight inhibitors (Steitz & Schulman, 1982; Barrett & Salvesen, 1986; Bode et al., 1989). Mechanistic understanding has also been advanced by these studies, since most low molecular weight inhibitors take advantage of the special reactivity of the catalytic residues in the enzyme active site (Powers & Harper, 1986; Fischer, 1988).

The classic inhibitors of the serine proteases are the phosphorylating agents, exemplified by DFP¹ (Jansen et al., 1952;

Balls & Jansen, 1962; Mounter et al., 1963). These compounds react irreversibly with the active site serine hydroxyl (Ser-195 in chymotrypsin), forming a covalent adduct that has been viewed as a model for the tetrahedral intermediates involved in the normal substrate catalytic sequence (Bernhard & Orgel, 1959; Stroud et al., 1974; Kraut, 1977; Kossiakoff & Spencer, 1981). However, the traditional phosphorylating agents such as DFP bear so little resemblance to a typical peptide substrate that the utility of their enzymatic adducts as models of enzyme-substrate transition-state structures is limited. Moreover, these reagents are unable to take advantage of the substrate specificity of the different hydrolases and are therefore nonspecific. Greater selectivity is achieved by in-

¹ Abbreviations: ALP, α -lytic protease; CHY, bovine α -chymotrypsin; HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; SUB, subtilisin BPN'; DFP, diisopropyl phosphorofluoridate; DMF, dimethylformamide; DIEA, ethyldiisopropylamine; TMSiCl, trimethylsilyl chloride; TBK, triethylammonium bicarbonate; msAAPVna, methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide; sAAPAFna, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide; bAnpe, *N*-(*tert*-butoxycarbonyl)-L-alanine *p*-nitrophenyl ester; sAPAna, succinyl-L-alanyl-L-prolyl-L-alanine *p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; EDC, ethyl[(dimethylamino)ethyl]carbodiimide hydrochloride; DMAP, (dimethylamino)pyridine. Val^P and Phe^P represent the phosphonic acid analogues of valine and phenylalanine, respectively. The systematic name for Val^P would be (1-amino-2-methylpropyl)phosphonic acid and for Phe^P would be (1-amino-2-phenylethyl)phosphonic acid.

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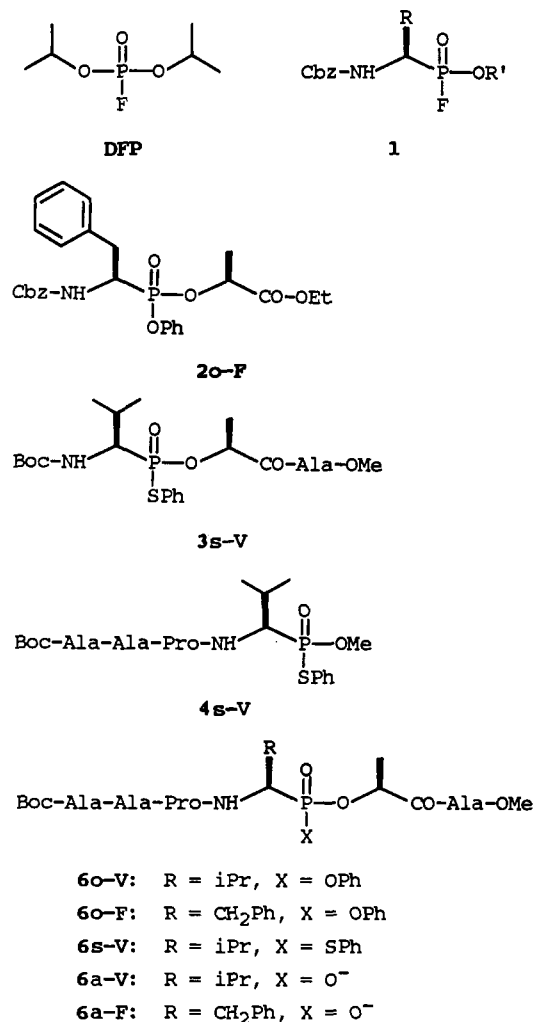
hibitors, both reversible as well as irreversible, that rely on a different functional group for inhibition and that incorporate peptide fragments in their structures. The chloromethyl ketones (Powers, 1977a; Shaw, 1970), aldehydes (Thompson & Bauer, 1979; Powers & Harper, 1986), boronic acids (Kettner & Shenvi, 1984), and trifluoromethyl ketones (Imperiali & Abeles, 1986), for example, incorporate amino acid residues in the amino-terminal direction from the inhibitory functionality. However, of these other inhibitors, only the difluoro ketones, in which a peptide linkage is replaced with CO-CF₂ (Imperiali & Abeles, 1987; Takahashi et al., 1989a), provide an opportunity to incorporate amino acid residues in the carboxy-terminal direction.

We have reasoned that fusion of an electrophilic phosphorus moiety with appropriate peptide fragments could improve both the selectivity of the phosphorylating agents and the relevance of their adducts as transition-state models. Our initial investigations focused on the preparation of aminoalkyl phosphonofluoridates, **1**, that is, replacement of one of the isopropoxy substituents of DFP with a single amino acid moiety (Lamden & Bartlett, 1983). This substitution increases the rate of inactivation of the serine proteases α -chymotrypsin and pancreatic elastase and confers some selectivity between the proteases. However, the high reactivity of the phosphorus-fluorine bond presents an obstacle toward development of peptidyl phosphonofluoridates as practical inhibitors. Not only are they hydrolyzed rapidly ($t_{1/2}$ = 5 min at pH 6.5), but the inhibitors cannot be extended because the P-F moiety reacts with a peptide linkage. Replacement of the fluorine with a less active leaving group overcomes these limitations and allows inhibitors to be constructed with the phosphorylating moiety embedded in the middle of an oligopeptide chain. With the appropriate choice of leaving group, these inhibitors retain enough reactivity to inactivate their target enzymes effectively. Previous studies to optimize the leaving group with respect to reactivity and hydrolytic stability demonstrated that *S*-phenyl and *S*-methyl esters of (Cbz-aminoalkyl)thiophosphonates and phenyl esters of (Cbz-aminoalkyl)phosphonates are effective inactivators of CHY with the phenyl ester being the most stable (Bartlett & Lamden, 1986). More recently, Fastrez et al. (1989) and Oleksyszyn and Powers (1989) have investigated peptidylphosphonate diaryl esters as inhibitors of a number of serine proteases and demonstrated high selectivity and potency; Pratt has shown that an anionic phosphonate phenyl ester inhibits a class C β -lactamase (Pratt, 1989). An additional step in the development of these compounds as useful inhibitors was the discovery of a convenient method for incorporating the reactive phosphonate ester in the synthesis of an oligopeptide (Sampson & Bartlett, 1988).

In this report, we describe the assembly of a variety of oligopeptide phosphorylating agents (**2-6**) and their evaluation as inactivators of CHY, SUB, PPE, HLE, and ALP. The X-ray crystal structures of phosphonate complexes of ALP are presented in the accompanying paper (Bone et al., 1991).

The phosphonic acid analogues Phe^P and Val^P were incorporated at the P₁ position of these inhibitors (Schechter & Berger, 1967), corresponding to the known specificity of SUB and HLE. Proline was chosen for P₂, since at this position it is known to restrict the binding modes available for analogous substrates (Segal, 1972; Thompson & Blout, 1973; Nakajima et al., 1979). The other positions were filled with alanyl residues for ease of synthesis as well as generality. In addition to the hexapeptide analogues containing Val^P, we synthesized the tri- and tetrapeptide derivatives corresponding to the P₄-P₁ and P₁-P₂' fragments, respectively, in order to determine the

relative contributions from each region in HLE inactivation. In the Val^P hexapeptide series, we also compared the thiophenyl and phenyl esters as leaving groups; in the case of the Phe^P derivatives, only the phenyl esters were prepared. The phosphonate anions **6a-V** and **6a-F** were also evaluated as control compounds.



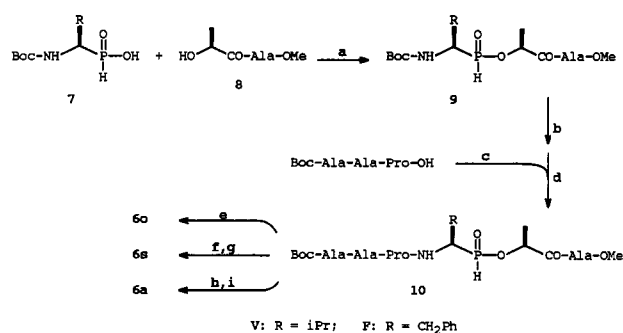
MATERIALS AND METHODS

Synthesis of Phosphonate Esters

The hexapeptide analogues were synthesized as outlined in Scheme I.

General. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Reactions involving reagents sensitive to moisture were conducted under an atmosphere of dry argon with dry reagents and solvents. Column chromatography was performed according to Still et al., (1978) using silica gel 60 (E. Merck, Darmstadt, Germany). Preparative HPLC was carried out on a 10 mm × 25 cm Altex column, 5- μ m silica, with the indicated eluting solvent. Spectral and analytical data for all of the following compounds are available as supplementary material.

[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)amino]-2-phenylethyl]-phosphonic acid (**7F**). A 100-mL round-bottomed flask was charged with (1-amino-2-phenylethyl)phosphinic acid (Baylis et al., 1984) (12.89 g, 69.6 mmol), 2-[[[*tert*-butoxycarbonyl]oxy]imino]-2-phenylacetonitrile (18.86 g, 76.6 mmol), Et₃N (14.6 mL, 104 mmol), and DMF (21.0 mL), and the suspension was heated to 70 °C for 12 h. The excess Et₃N and DMF were removed under high vacuum. The residue was

Scheme 1^a

^a (a) EDC, CH₂Cl₂; (b) TFA; (c) iBuOCOCl, *N*-methylmorpholine, EtOAc, 15 °C; (d) *N*-methylmorpholine, DMF, -15 °C; (e) PhOH, Et₃N, CCl₄; (f) TMSiCl, Et₃N, CH₂Cl₂; (g) PhOH, Et₃N, CH₂Cl₂; (h) bis(trimethylsilyl)acetamide, MeCN; (i) H₂O, Et₃N, CCl₄; Li⁺ exchange.

dissolved in water (300 mL), and the aqueous layer was washed with four 100-mL portions of EtOAc. The aqueous layer was acidified to pH 1 with 12 N HCl and extracted with four 100-mL portions of CHCl₃. The combined CHCl₃ layers were dried over Na₂SO₄ and concentrated in vacuo to yield 9.23 g (46%) of the racemic carbamate **7F** as a yellow oil. This material was suspended in ether (50 mL) at reflux, and a 50% solution of α-(+)-methylbenzylamine (3.92 g, 32.4 mmol) in ether was added. The white solid obtained was recrystallized four times from ether to give material with [α]_D²⁰ = -28.1° (EtOH). The white salt was dissolved in 1 N HCl (100 mL), and the aqueous suspension was extracted with CHCl₃ (4 × 60 mL). The combined CHCl₃ layers were dried over Na₂SO₄ and concentrated in vacuo to yield 3.56 g (39% of theoretical yield) of the *R* isomer of **7F** as a clear oil which was used without further purification.

[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)amino]-2-methylpropyl]phosphinic Acid (**7V**). The racemic carbamate **7V** was prepared in a manner analogous to that described above, as white crystals after crystallization from CH₂Cl₂/hexanes in 51% yield. The racemic material was also resolved as described above to give (*R*)-**7V** (27% of theoretical yield) as a glass: [α]_D²² = -26.9° (EtOH).

N-[(2*S*)-2-[[[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)amino]-2-phenylethyl]phosphinyl]oxy]propanoyl]-L-alanine Methyl Ester (**9F**). A 25-mL round-bottomed flask was charged with a solution of **7F** (291 mg, 1.02 mmol) and *N*-(*S*-lactyl)-L-alanine methyl ester (**8**) (170 mg, 0.97 mmol) in CH₂Cl₂ (10 mL). EDC (205 mg, 1.07 mmol) was added, and the mixture was allowed to stir for 20 h. The solution was diluted with EtOAc (200 mL), washed with saturated KH₂PO₄ (2 × 20 mL), saturated NaHCO₃ (20 mL), and brine (20 mL), dried over Na₂SO₄, filtered, and evaporated to give **9F** (375 mg, 89% crude yield). This material was carried on without purification.

N-[(2*S*)-2-[[[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-L-prolyl]amino]-2-phenylethyl]phosphinyl]oxy]propanoyl]-L-alanine Methyl Ester (**10F**). Phosphinate **9F** (89 mg, 201 μmol) was placed in a 10-mL round-bottomed flask, and trifluoroacetic acid (1 mL) was added; after 15 min, the solution was concentrated under reduced pressure. The residue was suspended in dioxane (1 mL) and recovered by evaporation; this procedure was repeated to give the TFA salt as a white solid. Two solutions were then prepared: (A) In a 10-mL round-bottomed flask, (*tert*-butoxycarbonyl)-L-alanyl-L-alanyl-L-proline (211 μmol) and *N*-methylmorpholine (23 μL, 211 μmol) were dissolved in EtOAc (1 mL), and the solution was cooled to -15 °C. (B) In a 10-mL pear-shaped flask, the TFA salt (201 μmol) and *N*-methylmorpholine (22

μL, 201 μmol) were dissolved in DMF (0.5 mL), and the solution was cooled to -15 °C. Isobutyl chloroformate (IBCF, 27 μL, 211 μmol) was added to solution A, and 3 min later, solution B was transferred via cannula into solution A, with two 0.5-mL portions of DMF. The reaction mixture was allowed to warm slowly to ambient temperature and left to stir for 17 h. The solution was diluted with EtOAc (100 mL), washed with saturated KH₂PO₄ (2 × 15 mL), saturated NaHCO₃ (2 × 15 mL), and brine (15 mL), dried over Na₂SO₄, and evaporated to give **10F** (81 mg, 60% crude yield). This material was also carried on without further purification.

N-[(2*S*)-2-[[[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-L-prolyl]amino]-2-phenylethyl]phenoxyphosphinyl]oxy]propanoyl]-L-alanine Methyl Ester (**60-F**). A solution of hydrogen phosphinate (**10F**) (120 μmol) in CCl₄ (1 mL) was stirred under argon, and phenol (22 mg, 239 μmol) and Et₃N (50 μL, 358 μmol) were added. After 30 min, the solution was diluted with EtOAc (100 mL), washed with saturated KH₂PO₄ (2 × 20 mL), saturated NaHCO₃ (2 × 20 mL), and brine (20 mL), dried over Na₂SO₄, filtered, and concentrated to give a yellow oil (52 mg). The oil was purified by chromatography (EtOAc, 6 cm of silica) to yield **60-F** (40 mg, 18% from **7F**) as a mixture of diastereomers. The diastereomers were separated by HPLC (EtOAc, 3 mL/min, *k'* = 4.0 and 4.5).

N-[(2*S*)-2-[[[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-L-prolyl]amino]-2-methylpropyl]phenoxyphosphinyl]oxy]propanoyl]-L-alanine Methyl Ester (**60-V**). Phosphonate **60-V** was prepared in a manner analogous to that of **60-F**, in 15% overall yield from **7V**, as a mixture of diastereomers. The diastereomers were separated by HPLC (EtOAc, 3 mL/min, *k'* = 5.3 and 5.7).

N-[(2*S*)-2-[[[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-L-prolyl]amino]-2-methylpropyl]phenylthio]phosphinyl]oxy]propanoyl]-L-alanine Methyl Ester (**6s-V**). To a solution of **7V** (1.33 g, 2.10 mmol) and DIEA (806 μL, 4.62 mmol) in CH₂Cl₂ (21 mL) at 0 °C was added TMSiCl (534 μL, 4.20 mmol). After 5 min, DIEA (440 μL, 2.52 mmol) and benzenesulfonyl chloride (236 μL, 2.31 mmol) were added to the reaction mixture. After an additional 5 min, the reaction was quenched with saturated NH₄Cl (4 mL) and diluted with EtOAc (450 mL). The mixture was washed with saturated NH₄Cl (2 × 60 mL), saturated NaHCO₃ (60 mL), and brine (60 mL), dried over Na₂SO₄, filtered, and evaporated in vacuo to yield 1.92 g of a yellow oil. This material was chromatographed (1:4 THF:EtOAc) to give the thiophenyl ester **6s-V** (296 mg, 19% from **7V**). The diastereomers were separated by HPLC (85:12:3 EtOAc:hexanes:THF, 6 mL/min, *k'* = 3.4 and 4.3).

Methyl *S*-Phenyl [(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-L-prolyl]amino]-2-methylpropyl]thiophosphonate (**4s-V**). To a solution of (*R*)-**7V** (516 mg, 2.18 mmol) in MeOH (7 mL) and ether (30 mL) was added an ethereal solution of diazomethane until a yellow color persisted for 5 min. The excess diazomethane was quenched with acetic acid, and the solution was then washed with saturated NaHCO₃ (3 × 100 mL), dried over MgSO₄, and concentrated in vacuo. The solid residue was recrystallized from ether/hexanes to yield the methyl ester of **7V** (563 mg, 100% yield) as white plates (1:1 ratio of diastereomers).

This material was converted to the Boc-Ala-Ala-Pro derivative as described above for deprotection and acylation of **9V**, to give the tetrapeptide analogue in 82% crude yield as a semisolid. Oxidation with benzenesulfonyl chloride was accomplished as described for **6s-V** to give the phenyl thioester

4s-V (25% yield) after purification by chromatography (1:4 THF:EtOAc). The diastereomers of **4s-V** were separated by HPLC (80:17:3 EtOAc:hexanes:THF, $k' = 5.3$ and 6.6).

N-[(2*S*)-2-[[[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)amino]-2-methylpropyl](phenylthio)phosphinyl]oxy]propanoyl]-L-alanine Methyl Ester (**3s-V**). A solution of racemic **7V** (106 mg, 270 μ mol) in CH_2Cl_2 (1.4 mL) was cooled to 0 °C, and aliquots of DIEA ($6 \times 56 \mu\text{L}$, 324 μ mol) and benzenesulfonyl chloride ($6 \times 30 \text{ mL}$, 297 μ mol) were added at 15-min intervals. After 100 min, the reaction was quenched with saturated NH_4Cl , the mixture was diluted with EtOAc (100 mL), and the solution was washed with saturated NH_4Cl ($2 \times 15 \text{ mL}$), saturated NaHCO_3 (15 mL), and brine (15 mL) and evaporated in vacuo to give 307 mg of a yellow-orange oil. This material was chromatographed (1:1 EtOAc:hexane) to give **3s-V** (69 mg, 48%) as a colorless oil as a mixture of four diastereomers. The four diastereomers were partially separated by HPLC [30:67:3 EtOAc:hexane:THF, $k' = 4.25$ (A), 4.50 (B), and 5.25 (C and D)]. Diastereomers C and D were separated with 30:35:20:15 EtOAc:hexanes: CH_2Cl_2 : CHCl_3 (EtOH free); $k' = 2.67$ (C) and 3.67 (D).

(2*S*)-2-[[[(1*R*)-1-[*N*-(Phenylmethoxy)carbonyl]amino]-2-phenylethyl]phenoxyphosphinyl]oxy]propanoate Ethyl Ester (**2o-F**). The preparation of this material is described in Bartlett and Lamden (1986).

N-[(2*S*)-2-[[[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-L-prolyl]amino]-2-methylpropyl]hydroxyphosphinyl]oxy]propanoyl]-L-alanine Methyl Ester (**6a-V**). To a solution of hydrogen phosphinate (**10V**) (420 mg, 664 μ mol) and bis(trimethylsilyl)acetamide (98 μL , 398 μ mol) in CH_3CN (1 mL) was added CCl_4 (1 mL), H_2O (1 mL), and Et_3N (139 μL , 996 μ mol). After 15 min, the solution was concentrated in vacuo, and the residue was dissolved in water and applied to a DEAE-Sephadex column (HCO_3^- form, $80 \times 20 \text{ mm}$). The column was washed with H_2O (50 mL) and then eluted with a linear gradient from 0 to 2.5 M TBK (125 mL:125 mL); fractions 14–34 were collected and concentrated. The salt was further purified by reverse-phase HPLC (Whatman M9/50, ODS, 25% CH_3CN :75% 0.1 M TBK, pH 7.3, $k' = 0.8$) and converted to the lithium salt with Li^+ AG-X-50 to give **6a-V** (38 mg, 5% overall yield).

N-[(2*S*)-2-[[[(1*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-L-prolyl]amino]-2-phenylethyl]hydroxyphosphinyl]oxy]propanoyl]-L-alanine Methyl Ester (**6a-F**). Prepared from **10F** and purified in the same manner as described above for the Val^P analogue (10 mg, 6% overall yield).

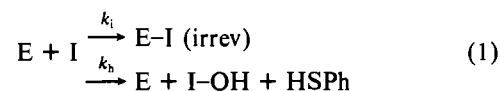
Enzymology

General. PPE (Sigma Chemical Co., lyophilized powder), CHY (Sigma Chemical Co., lyophilized powder), HLE (Elastin Products Co., lyophilized powder), ALP (gift of D. Agard, UCSF, lyophilized powder), and SUB (50:50 propylene glycol:water solution, approximately 10 mg/mL, gift of D. Estell, Genencor) were used as obtained. Stock solutions (approximately 10^{-5} M) of PPE and CHY were prepared in 0.1 M NaOAc/0.5 M NaCl, pH 5.0, and stored at 4 °C. A stock solution (approximately 10^{-5} M) of HLE was prepared in 0.1 M NaOAc/0.5 M NaCl, pH 5.5, and stored at 4 °C. A stock solution (approximately 10^{-5} M) of ALP was prepared in 0.1 M KCl. These stock solutions were stable for several weeks. Further dilutions of HLE and PPE were made with their respective stock buffers. Further dilutions of ALP, CHY, and SUB were made with the incubation buffers. msAAPVna, sAAPFna, and bAnpe were obtained from Sigma. sAPAna was obtained from Peptide Institute, Inc. Stock solutions of msAAPVna (6 mM) were prepared in 10% DMSO/90% 0.1

M Tris, 0.5 M NaCl, and 0.01% NaN_3 , pH 7.4, and were stored at 0 °C. Stock solutions of bAnpe (12 mM) were prepared in dry MeOH, were stored at 0 °C, and were not stable for more than 1 week. Stock solutions of sAAPFna (0.14 mM) and sAPAna (2.3 mM) were prepared in 4% EtOH/96% 50 mM TAPS and 0.1 M KCl, pH 8.6, and were stored at 4 and 20 °C, respectively. Stock solutions (3 mM) of **6s-V**, **4s-V**, and **3s-V** were prepared in dry DMSO and of **6o-V** and **6o-F** in dry MeOH; these stock solutions were not stable for more than 1 day. Stock solutions (3 mM) of **6a-V** and **6a-F** were prepared in water and were stable indefinitely. The rate of substrate hydrolysis was monitored spectrophotometrically on a Cary-219 UV-vis spectrophotometer interfaced with an OLIS data processing system, a Varian DMS 100 UV/visible spectrophotometer dedicated to a Varian DS-15 data station, or a Kontron Uvikon 860. All incubations and assays were conducted at 25 °C. PPE activity was determined by the method of Visser and Blout (1972), the hydrolysis of substrate (bAnpe) being monitored at 347.5 nm, with correction for buffer-catalyzed hydrolysis. SUB and CHY activities were determined by monitoring the hydrolysis of sAAPFna; for HLE, the hydrolysis of msAAPVna; and for ALP, the hydrolysis of sAPAna, all at 410 nm.

Determination of Second-Order Rate Constants of Inactivation. The buffer used for incubations with **6s-V**, **4s-V**, **3s-V**, and **6a-V** was 0.1 M sodium citrate and 0.5 M NaCl, pH 6.0, and for incubations with all of the other inactivators was 50 mM TAPS and 0.1 M KCl, pH 8.6. Enzyme (final concentration 10^{-7} – 10^{-8} M), buffer, and inactivator (final concentration 20–180 mM) were combined in polyethylene tubes and incubated at 25 °C. The rates of inactivation were followed by incubation of the enzyme and inactivator for various times before dilution into excess substrate and determination of the remaining enzyme activity. Assays were conducted by charging a quartz cuvette with an aliquot of incubation mixture and substrate to produce a final enzyme concentration of approximately 10^{-8} – 10^{-9} M.

Residual enzyme activity vs time was fit to an exponential curve (pseudo-first-order inactivation kinetics) with ENZFITTER (Leatherbarrow, 1987). The enzyme activity of HLE was calculated as the fraction of the remaining activity of a control incubation (no inactivator present) in order to account for loss of enzyme activity due to other factors besides the presence of inactivator. The control incubations for the other enzymes showed no loss of activity during the time course of the incubation. The second-order rate constants for inactivation by **6s-V**, **4s-V**, and **3s-V** were determined according to the method of Ashani et al. (1972) using the hydrolysis rate constants determined as described below. This analytical method assumes the competing inactivation and inhibitor hydrolysis pathways illustrated in eq 1; it was assumed that phosphonate ester hydrolysis is due primarily to buffer, as opposed to enzyme catalysis; hence, the rate of ester hydrolysis was measured in the absence of enzyme.



Determination of Nonenzymatic Phenyl Thioester Hydrolysis. Nonenzymatic hydrolysis of **6s-V**, **4s-V**, and **3s-V** was monitored at 270 nm. All solutions were degassed immediately before use. Hydrolysis was determined by charging a quartz cuvette with an aliquot of inhibitor and a measured amount of buffer (0.1 M sodium citrate, 0.5 M NaCl, pH 6.0) to produce a final inhibitor concentration of 0.05 mM. It was necessary to mix the assay solution gently and to use stoppered

Table I: Inactivation of Serine Proteases by Peptidyl S-Phenyl Thiophosphonate Analogues

enzyme	inhibitor ^a	k_i (M ⁻¹ min ⁻¹) ^b	
		diastereomer A	diastereomer B
HLE	6s-V	470	540
HLE ^c	4s-V	16 000	580
PPE	6s-V	160	340
SUB ^c	6s-V	360	440
CHY	6s-V	42	75
k_{hyd} (min ⁻¹) ^b	6s-V	0.063	0.063
k_{hyd} (min ⁻¹) ^b	4s-V	0.091	0.042

^a Inhibitor concentrations 80–100 μ M, unless indicated. ^b Estimated error $\pm 10\%$, based on reproducibility of measurements. ^c Inhibitor concentrations 10 μ M.

cuvettes in order to minimize the amount of air mixed into the solution.

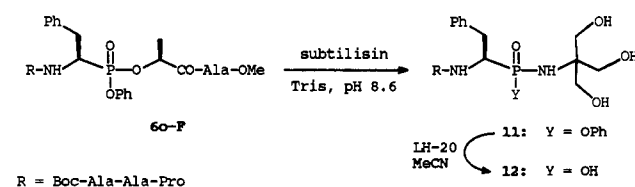
Determination of Irreversibility of Inhibition. Fully inhibited enzyme (as determined by the assay described above) was prepared with excess inhibitor, which was then removed by ultrafiltration on hollow fiber bundles (Biomolecular Dynamics, RCF-Confil). A 1- μ L aliquot of the washed and concentrated enzyme (10 mg/mL) was diluted into 5 mL of incubation buffer and assayed for activity 1 min and 5.5 h after dilution.

Determination of Byproduct from Incubation of 6o-F with SUB/Tris. SUB (1.8×10^{-6} M) was incubated in 50 mM Tris, pH 8.6, with 6o-F (0.28 mM) for 2 h. The sample (8.9-mL total volume) was lyophilized, and the major product was purified by reverse-phase HPLC (Whatman ODS, 10 mm \times 50 cm, 25:75 CH₃CN:0.1 M Et₃NH⁺AcO⁻, pH 7.0, 5 mL/min) and characterized by ³¹P NMR and mass spectral analysis (FAB). The sample was further purified by elution on a 3-cm column of Sephadex LH-20 with CH₃CN to remove buffer salts, and the product was characterized by ¹H NMR, ³¹P NMR, and MS (FAB).

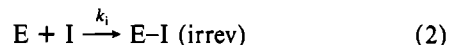
RESULTS

Inactivation of the Serine Proteases by Phenyl Thioesters 3s-V, 4s-V, and 6s-V. The rate of inhibition of the serine proteases by the phenyl thiophosphonates was measured at pH 6.0 (see Table I), since these compounds were shown to be most stable at this pH (data not shown). The second-order rate constants for inactivation, k_i (eq 2), for 6s-V and 4s-V were calculated from the limits of inhibition according to the method of Ashani et al. (1972). The P₁-P₂' tripeptide 3s-V was only evaluated against HLE, and it was found that neither diastereomer inhibits under these conditions. The hexapeptide

Scheme II



analogue 6s-V shows similar reactivity to the four proteases, with the exception of CHY, against which it is 1 order of magnitude less active. There is no significant discrimination between the diastereomers of 6s-V for any of these enzymes, although HLE shows substantial diastereoselectivity against the tetrapeptide methyl esters 4s-V.



Inactivation of SUB with 6o-F and 2o-F in Tris Buffer. Neither diastereomer of the dipeptide 2o-F (Lamden & Bartlett, 1983) was an inhibitor of SUB at 100 μ M concentration in 50 mM Tris, pH 8.6. In the presence of this buffer, inhibition of SUB by the hexapeptide analogue 6o-F does not proceed to completion, and the kinetic behavior is non-first-order. Isolation and structure elucidation of a byproduct from this interaction led to the identification of adduct 11 (FAB-MS) and, on further purification, its decomposition product 12 (¹H and ³¹P NMR and FAB-MS) (Scheme II). Such transesterification products were not observed in TAPS buffer.

Inactivation of the Serine Proteases with the Phenyl Esters 6o-F and 6o-V. The rates of inactivation of SUB, HLE, and ALP by 6o-F and 6o-V were measured at pH 8.6 (Table II). The pseudo-first-order rate constant, k_{obs} , was measured at three or four concentrations for each protease. The approximate concentration of each inhibitor necessary to produce an observed rate of inactivation $k_{obs} \approx 0.17 \pm 0.02$ min⁻¹ at pH 8.6 was also determined (Table III); these data reveal a ca. 2-fold degree of selectivity for one diastereomer at phosphorus.

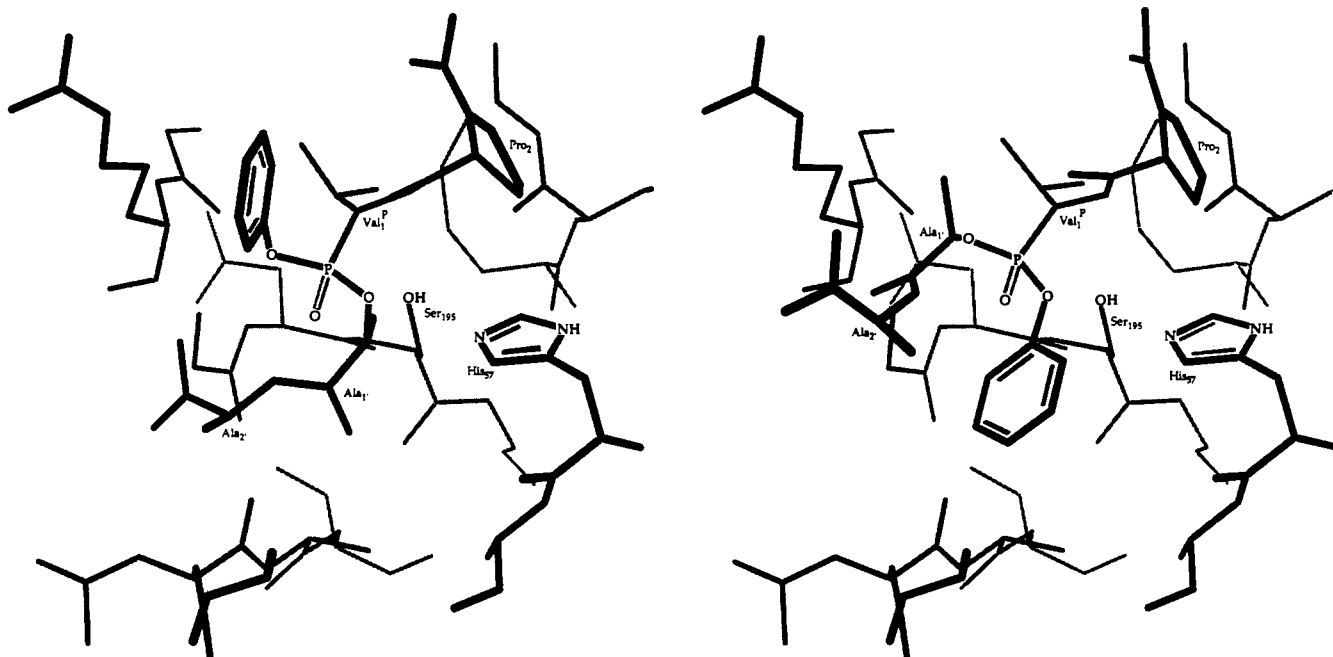
Inhibition of the Serine Proteases with 6a-F and 6a-V. The acids 6a-F and 6a-V did not inhibit any of the proteases at 100 μ M concentrations.

Computer Modeling. Starting with the coordinates of ALP inhibited by 6o-V (Bone et al., 1991), models for the two diastereomers of 6o-V were constructed with the BIOGRAF program (Version 2.0, BioDesign, Inc.) on a Silicon Graphics IRIS 4D70-GTB graphics system. The conformations of the phenoxy and lactylalanyl moieties were adjusted manually to provide an intuitively reasonable fit in the active site of ALP, and the structures were minimized by use of the Dreiding force

Table II: Inactivation of Serine Proteases by Hexapeptidyl Phenyl Phosphonate Analogues^a

enzyme	6o-F						6o-V					
	diastereomer A			diastereomer B			diastereomer A			diastereomer B		
	[I]	k_{obs}	k_i	[I]	k_{obs}	k_i	[I]	k_{obs}	k_i	[I]	k_{obs}	k_i
SUB	78	0.59 ^b	76	95	0.54	57	93	ii		180	ii	
SUB	59	0.43	74	81	0.43	53						
SUB	39	0.16	40	68	0.19 ^c	28						
SUB	20	0.03	15									
ALP	98	ni		108	ni		93	0.18 ^c	19	275	0.26 ^b	9.3
ALP							78	0.11	14	180	0.15	8.1
ALP							62	0.076	12	140	0.098	7.0
ALP							47	0.058	12			
HLE	195	0.008 ^d	0.4 ^c	203	ni		220	0.16	7.3	255	0.093	3.6
HLE							158	0.12	7.6	170	0.059	3.5
HLE							95	0.068	7.1	136	0.035	2.5

^a [I], μ M; k_{obs} , min⁻¹; k_i = 100 M⁻¹ min⁻¹. ii = incomplete inactivation; ni = no inhibition observed. Estimated error in $k_{obs} \pm 10\%$, based on reproducibility of measurements, except as noted. ^b Estimated error $\pm 20\%$. ^c Estimated error $\pm 30\%$. ^d Inactivation followed for less than one half-life.

FIGURE 1: Models for reversible complexes formed between ALP and the *R* and *S* diastereomers of inhibitor **60-V**.Table III: Selectivity of Serine Protease Inactivation^a

enzyme	60-F		60-V	
	diastereomer A (μM)	diastereomer B (μM)	diastereomer A (μM)	diastereomer B (μM)
SUB	39	68	ii	ii
ALP	ni	ni	93	180
HLE	4000	ni	220	ca. 500

^a Approximate concentration of inactivator to obtain k_{obs} of $0.17 \pm 0.02 \text{ min}^{-1}$; ii = incomplete inactivation; ni = no inhibition observed.

field with constraints on the protein (fixed) and the phosphoryl oxygens (held in the region of the oxyanion hole). The resulting structures, displayed in Figure 1, provided a visualization of how the diastereomeric inhibitors may bind in the noncovalent complex.

DISCUSSION

Synthesis of Phosphonates (Scheme 1). The polypeptide backbone of the inhibitors was assembled with the phosphorus at the phosphinate rather than the phosphonate oxidation level because of higher yields in the phosphorus–oxygen coupling reaction and because formation of the reactive phenyl esters and phenyl thioesters can be accomplished as the last step in the synthesis (Sampson & Bartlett, 1988). The (α -amino-alkyl)phosphinic acids were resolved by crystallization of the (+)-1-phenylethylamine salts of the Boc-protected derivatives **7** [an adaptation of the procedure of Baylis et al. (1984)]. Assignment of the absolute configuration of these compounds was accomplished by cleavage of the Boc carbamate and comparison of the optical rotation to the values reported for the *R* enantiomers (Baylis et al., 1984).

By a modification of the carbodiimide-mediated coupling procedure described by Karanewsky and Badia (1986), the phosphinic acids **7** and *N*-(*L*-lactyl)-*L*-alanine methyl ester (**8**) were linked with EDC in yields of 80–90%. Use of the water-soluble carbodiimide proved to be important in facilitating the reaction workup, since silica gel chromatography, to which the phosphinate esters **9** are not stable, could be avoided. In our experience, 4-(dimethylamino)pyridine (Karanewsky & Badia, 1986) proved to be unnecessary in the

coupling reaction, although this may have been because the lactyl hydroxyl group is relatively unhindered. The esters **9** were deprotected and coupled to the tripeptide Boc-*L*-Ala-*L*-Ala-*L*-Pro by the mixed-anhydride method to give the hexapeptide phosphinates **10** in good yields.

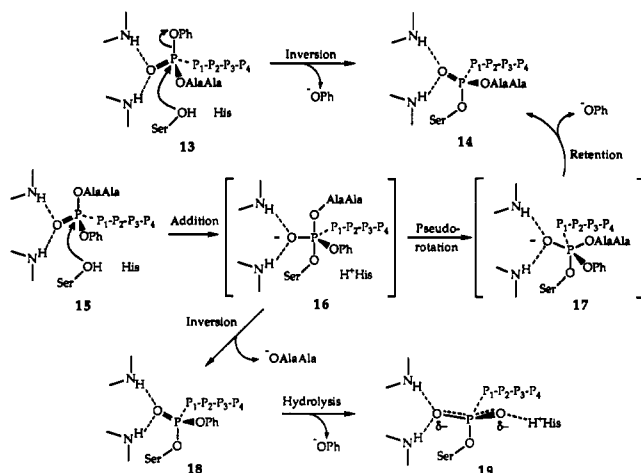
The final step, oxidation of the hydrogen phosphinate to the activated phosphonate ester, has been described in detail elsewhere (Sampson & Bartlett, 1988). The *O*-phenyl phosphonates were formed by reaction of the hydrogen phosphinates with carbon tetrachloride, triethylamine, and phenol. Although the phenyl thioesters could have been formed analogously, substituting thiophenol for phenol, they were synthesized via oxidation with benzenesulfonyl chloride, before the oxidation step had been optimized.

The oxidation step proceeds nonstereospecifically to produce two diastereomers at phosphorus. Although these isomers can be separated by preparative HPLC, we do not yet have a method for assigning the configurations of these isomers, designated A and B in the order of their elution. However, isomer A of one inhibitor does not necessarily have the same configuration as isomer B of another inhibitor.

Chemical Nature of Inactivation Process. The irreversibility of inhibition was tested in two ways. First, the inactivated enzyme was separated from excess inhibitor, diluted into buffer, and assayed for return of activity. None was observed. Second, the X-ray crystal structures presented in the accompanying paper clearly show the continuous electron density between the serine hydroxyl oxygen and the inhibitor phosphorus atom.

Role of P_1' – P_2' Residues in Inactivation Process. The inability of the P_1 – P_1' – P_2' tripeptide **3s-V** to inhibit HLE, in contrast to the P_4 – P_1 tetrapeptide and hexapeptide thio-phosphonates **4s-V** and **6s-V**, implies that binding to the S_4 – S_1 subsites is necessary for inactivation. Consistent with this interpretation is the observation that the dipeptide **20-F** does not inactivate SUB. Furthermore, the fact that there is little difference in the rates at which the methyl ester **4s-V** and the hexapeptide **6s-V** undergo hydrolysis implies that the greater reactivity of the former as an inhibitor has less to do with simple steric hindrance than it does with actual impedance of

Scheme III



the process by the steric bulk of the $P_1'-P_2'$ moiety of the inhibitor. It is important in this respect to acknowledge that the rates of inactivation by these derivatives are not expected to bear any relationship to the rates of hydrolysis of the corresponding substrates, since the nature of the reactions and their transition-state structures are so different.

Stereochemical Aspects of Inactivation. Contrary to our expectations, for none of the three hexapeptides is there significant discrimination between the two phosphonate diastereomers with respect to rate of enzyme inactivation, whether it is the phenyl thioesters or phenyl esters or the specific enzyme target. This suggests that phosphorylation of the active site does not take place from an inhibitor-enzyme complex in which both the Boc-Ala-Ala-Pro- and -Ala-Ala-OMe segments are occupying their respective subsites. Again, support for this interpretation, and the implication that the Boc-Ala-Ala-Pro- moiety is dominant, comes from the observation that stereoselectivity is manifested by the *S*-phenyl methyl thiophosphonates, **4s-V**, for which there is a 25-fold difference in inactivation of HLE.

We anticipated that reaction of the phosphonylating agents with the active site serine hydroxyl would involve displacement of the phenoxy moiety with inversion of configuration, such that the stereochemistry of the diastereomeric inhibitors could be deduced from the configuration at phosphorus of the enzyme adducts. Accordingly, ALP was inactivated with each diastereomer of hexapeptide derivative **60-V**, and the two adducts were crystallized and submitted to X-ray diffraction analysis by Agard and Bone. As described in the accompanying paper (Bone et al., 1991), the two complexes have the same configuration at the phosphorus atom. Moreover, the adduct of ALP with diastereomer A of **60-V** proved to be a mixture of two complexes, the anticipated hexapeptide adduct, **14**, and a tetrapeptide phosphonate, **19**, resulting from loss of the $P_1'-P_2'$ residues from the inhibitor. It is evident from the structural data that the mechanism of inactivation is considerably more complicated than expected. One process proceeds with overall inversion of configuration and another with retention, and one of the reaction pathways affords the anticipated hexapeptide product while the other partitions to the observed mixture of adducts. The simplest explanation for this behavior is depicted in Scheme III, in which it is inferred that the diastereomer that gives a single adduct reacts via the straightforward inversion mechanism.

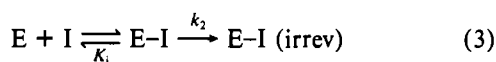
Two binding interactions between inhibitor and enzyme would appear to be most important for the inactivation process, that between the P_4-P_1 residues and the S_4-S_1 subsites and that between the phosphonyl oxygen ($P=O$) and the residues

comprising the oxyanion hole. The importance of the former interactions are borne out by results described above and that of the latter by two considerations. First, the oxyanion hole is not spacious enough to accommodate any of the other phosphorus substituents, and second, it may stabilize the negative charge that builds up on the phosphoryl oxygen during the substitution reaction, as it does for the charge generated during cleavage of a normal substrate. These assumptions, combined with the constraint that the phosphorus atom be in the vicinity of the nucleophilic serine hydroxyl, lead to the models depicted in Figure 1 for the reversible complexes between ALP and the two diastereomers of **60-V**. The two models differ in the orientations of the phosphonate ester groups with respect to the serine hydroxyl. In model 1, the phenoxy moiety is opposite the nucleophile, and displacement with inversion to give the observed product can be imagined readily. In contrast, in model 2, it is the less reactive lactylalanyl moiety that occupies this position, and direct displacement is less facile. Under these circumstances, the pentavalent adduct **16** (see Scheme III) could have a sufficient lifetime that pseudorotation (**16** → **17**) and loss of the phenoxy group, with retention of configuration, compete with direct displacement (Gorenstein et al., 1981; Emsley & Hall, 1976; Westheimer, 1968). Moreover, if displacement of the lactylalanyl ester does take place (**16** → **18**), an explanation for the mechanism of formation of the truncated complex may be offered, since the resulting phenyl ester would be expected to undergo eventual hydrolysis (Chambers & Stroud, 1977; Van der Drift et al., 1985). According to this model, the diastereomer that reacts with retention of configuration is the more reactive, diastereomer A of **60-V**. From the occupancy of the crystal structure and the relative intensities in the ^{31}P NMR spectrum of the inhibited complex (Bone et al., 1991), it appears that displacement of the lactylalanyl substituent occurs with the same frequency as pseudorotation and loss of the phenoxy group.

Although inactivation of a serine protease by a phosphonylating agent does not constitute a "normal" biological process, it is interesting to note that displacement at phosphorus with retention of configuration through pseudorotation is unprecedented in enzymatic systems, in contrast to mechanisms involving double inversion (Eckstein, 1982; Buchwald et al., 1982; Lowe, 1983). Such a two-step mechanism could be proposed in the present instance by invoking initial displacement of the phenyl ester by His-57, followed by transfer of the phosphonyl group to the hydroxyl of Ser-195. The alkylation of His-57 by chloromethyl ketone inhibitors (Powers, 1977ab) and the association of some boronic acid analogues with this residue (Bachovchin et al., 1988; Takahashi et al., 1989b) offer precedents for this proposal. However, an attempt to visualize how this may occur suggests that it would be difficult if not impossible to interpose the phosphonyl moiety between the His-57 and Ser-195 side chains, as would be required for this transfer to occur with inversion of configuration. Moreover, formation of the truncated complex **19** is not explained by this mechanism.

Kinetic Aspects of Inactivation. Because of their hydrolytic stability, a more extensive kinetic analysis of inhibition by the phenyl esters **60-V** and **60-F** was undertaken in order to determine the dissociation constants (K_i) and first-order rate constants for inactivation (k_2), in addition to the observed second-order rate constants ($k_{obs}/[I]$) (eqs 3 and 4). However, as the data in Table II reveal, neither the inactivation of SUB by **60-F** nor that of ALP by **60-V** is a second-order process; nor do these inhibitors demonstrate Michaelis-Menten kinetics,

with saturation at higher concentrations. Rather than remaining invariant or decreasing with higher inhibitor concentration, $k_{\text{obs}}/[I]$ increases with inhibitor concentration: on inactivation of SUB by diastereomer A of **60-F**, for example, reduction in inhibitor concentration by a factor of 4 reduces the rate of inactivation by a factor of 20; similar behavior is found for inhibition of ALP by the valine analogue **60-V**. Furthermore, **60-F** does not lead to the complete inactivation of SUB, implying that the concentration of inhibitor in solution is being reduced to zero during the course of the assay.



$$k_{\text{obs}}/[I] = k_i = k_2/K_i, \text{ for } [I] \ll K_i \quad (4)$$

Our initial experiments with inactivation of SUB by **60-F** in the presence of Tris buffer showed that the P_1' side chain can be displaced by the buffer. Furthermore, it is clear from the X-ray crystallographic analysis and ^1H NMR experiments that the terminal methyl ester of **60-V** is cleaved by ALP before phosphorylation of the enzyme. Although cleavage of the ester does not destroy the inhibitory capacity of the phosphonate, it would not be surprising if the inhibitors are inactivated by cleavage at other positions, particularly by the less selective proteases. HLE, with a narrower selectivity and a preference for Val at the P_1 position, is inactivated by the Val^P analogue **60-V** in a more closely second-order process, indicating that deactivation of the inhibitor by this enzyme competes less effectively. It is clear that more specificity and/or hydrolytic resistance could be built into inactivators of the less stringent enzyme.

Selectivity of Inactivation. Summarized in Table III are the concentrations of the phenyl phosphonate hexapeptides required to give the same rate of inactivation for SUB, ALP, and HLE. SUB is inherently more reactive than either ALP or HLE, yet all three enzymes show the same 2-fold selectivity for diastereomer A over diastereomer B. Specific inhibition of one protease by one inhibitor is demonstrated in the case of ALP, for which only the Val^P analogue **60-V** is an inhibitor; the Phe^P derivative **60-F** has no effect at similar concentrations. Moreover, HLE is fairly specific, reacting at a rate at least 20-fold slower with **60-F** than with **60-V**. It should be noted that the rate constant with **60-F** is only approximate, as the reaction was too slow to measure accurately.

Conclusions. We anticipate that the major importance of inhibitors of this sort will be to provide complexes which are models for the tetrahedral species leading to formation of the acyl-enzyme intermediate, for either NMR or crystallographic studies. The ^{31}P nucleus is advantageous for the NMR studies, and the fact that residues extending in both the amino and carboxyl directions can be incorporated into the structure distinguishes them from many of the reversible inhibitors. The accompanying paper describes the structure of the covalent complex of the hexapeptide **60-V** with ALP (Bone et al., 1991).

SUPPLEMENTARY MATERIAL AVAILABLE

Spectral and analytical characterization data for all of the new compounds synthesized (4 pages). Ordering information is given on any current masthead page.

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Crystal Structures of α -Lytic Protease Complexes with Irreversibly Bound Phosphonate Esters^{†,‡}

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ABSTRACT: The structures of the complexes with α -lytic protease of both phosphorus stereoisomers of *N*-[(2*S*)-2-[[[(1*R*)-1-[*N*-[(*tert*-butyloxycarbonyl)-L-alanyl-L-alanyl-L-prolyl]amino]-2-methylpropyl]-phenoxyphosphinyl]oxy]propanoyl]-L-alanine methyl ester, an analogue of the peptide Boc-Ala-Ala-Pro-Val-Ala-Ala where Val is replaced with an analogous phosphonate phenyl ester and the subsequent Ala is replaced with lactate, have been determined to high resolution (1.9 Å) by X-ray crystallography. Both stereoisomers inactivate the enzyme but differ by a factor of 2 in the second-order rate constant for inactivation [Sampson, N. S., & Bartlett, P. A. (1991) *Biochemistry* (preceding paper in this issue)]. One isomer (B) forms a tetrahedral adduct in which the phosphonate phenyl ester is displaced by the active site serine (S195) and interacts with the enzyme across seven substrate recognition sites that span both sides of the scissile bond. Seven hydrogen bonds are formed with the enzyme, and 510 Å² of hydrophobic surface area is buried when the inhibitor interacts with the enzyme. Although two hydrogen bonds are gained by incorporation of two residues on the C-terminal side of the scissile bond into the inhibitor, there is very little adjustment in the structure of the enzyme in this region. Surprisingly, the active site histidine (H57) does not interact with the phosphonate, apparently because the phosphonate lacks negative charge in or near the oxyanion hole, and instead, the side chain rotates out of the active site cleft and hydrogen bonds with solvent. The other isomer (A) forms a mixture of two different tetrahedral adducts in the active site, both covalently bonded to Ser 195. One adduct, at approximately 58% occupancy, is exactly the same in structure as the complex formed with isomer B, and the other adduct, at 42% occupancy, has lost the two residues C-terminal to the scissile bond by hydrolysis. In the lower occupancy structure, His 57 does not rotate out of the active site and forms a hydrogen bond with the phosphonate oxygen instead. The structures of both complexes were insensitive to pH. As very little change in structure accompanies the histidine rotation, the complex with isomer B provides an excellent mimic for the structure of the transition state (or high-energy reaction intermediate) that spans both sides of the scissile bond.

To provide an understanding for the structural basis of serine protease catalysis, the structures of many serine proteases in the absence and presence of various types of inhibitors have been determined by X-ray crystallography. Three structures are of the greatest interest: the structure of the free enzyme,

the structures of complexes with substrate analogues, and the structures of complexes with transition-state analogues and/or with analogues of reaction intermediates. These structures reveal how the enzyme interacts with substrates, how those interactions change during the transition state, and whether conformational changes are required in order to accommodate substrates. The structures of numerous unliganded serine proteases have been determined by crystallography, including α -lytic protease (Fujinaga et al., 1985; Brayer et al., 1979b), chymotrypsin (Blow, 1976; Tsukada & Blow, 1984; Blevins & Tulinsky, 1985), elastase (Meyer et al., 1988), proteinase A (*Streptomyces griseus*; Brayer et al., 1978a,b; Seilecki et al., 1979), several trypsins (Walter et al., 1982; Marquart et al., 1983; Read & James, 1988), and subtilisins (Alden et al., 1971; Drenth et al., 1972). These structures provide a basis

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[‡] Crystallographic coordinates of the complexes of α -lytic protease with phosphonates A and B have been deposited in the Protein Data Bank.

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