Irreversible Inhibition of Serine Proteases by Peptide Derivatives of (α-Aminoalkyl)phosphonate Diphenyl Esters†

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ABSTRACT: Peptidyl derivatives of diphenyl (α-aminoalkyl)phosphonates have been synthesized and are effective and specific inhibitors of serine proteases at low concentration. Z-Phe^P(OPh)$_2$ irreversibly reacts with chymotrypsin (k_{obsd}/[I] = 1200 M^{-1} s^{-1}) and does not react with two elastases. The best inhibitor for most chymotryspin-like enzymes including bovine chymotrypsin, cathepsin G, and rat mast cell protease II is the tripeptide Suc-Val-Pro-Phe^P(OPh)$_2$ which corresponds to the sequence of an excellent *p*-nitroanilide substrate for several chymases. The valine derivative Z-Val^P(OPh)$_2$ is specific for elastases and reacts with human leukocyte elastase (HLE, 280 M^{-1} s^{-1}) but not with chymotrypsin. The tripeptide Boc-Val-Pro-Val^P(OPh)$_2$, which has a sequence found in a good trifluoromethyl ketone inhibitor of HLE, is the best inhibitor for HLE (k_{obsd}/[I] = 27 000 M^{-1} s^{-1}) and porcine pancreatic elastase (PPE, k_{obsd}/[I] = 11 000 M^{-1} s^{-1}). The rates of inactivation of chymotrypsin by MeO-Suc-Ala-Ala-Pro-Phe^P(OPh)$_2$ and PPE and HLE by MeO-Suc-Ala-Ala-Pro-Val^P(OPh)$_2$ were decreased 2–5-fold in the presence of the corresponding substrate, which demonstrates active site involvement. Only one of two diastereomers of Suc-Val-Pro-Phe^P(OPh)$_2$ reacts with chymotrypsin (146 000 M^{-1} s^{-1}), and the enzyme–inhibitor complex had one broad signal at 25.98 ppm in the ^{31}P NMR spectrum corresponding to the Ser-195 phosphonate ester. Phosphonylated serine proteases are extremely stable since the half-time for reactivation was >48 h for the inhibited elastases and 7.5–26 h for chymotrypsin. Peptidyl derivatives of diphenyl (α-aminoalkyl)phosphonates are relatively easy to synthesize, are chemically stable in buffer and in human plasma, form very stable derivatives with serine proteases, do not react with acetylcholinesterase, and thus should have considerable potential utility as therapeutic agents.

Phosphorus-containing inhibitors are valuable tools for understanding the properties and physiological roles of hydrolytic enzymes. Diisopropyl phosphorofluoridate (DFP)[1] is useful for the initial characterization of an enzyme as serine protease or esterase since it reacts stoichiometrically with the active site serine residue (Jansen et al., 1952; Ball & Jansen, 1962). A single covalent bond between the active site serine and the inhibitor phosphorus atom is found in the crystal structure of (diisopropylphosphoryl)trypsin (Stroud et al., 1974), and the phosphorylated enzymes are good transition-state analogues due to the presence of the tetrahedral phosphorus atom (Kossiakoff & Spencer, 1981).

Non-peptidyl phosphorylating agents do not discriminate between various serine proteases due to their lack of structural similarity with normal peptide substrates and are extremely toxic because of reaction with acetylcholinesterase. For example, alkylphosphonates will inhibit both chymotrypsin and PPE. The inhibitory potency of ethyl *p*-nitrophenyl alkyl- and (phenylalkyl)phosphonates toward chymotrypsin depends on chain length, which suggests interaction of the alkylphosphonate residue with the S_1 pocket[2] of the enzyme (Becker et al., 1963; Boone et al., 1964). With PPE (Nayak & Bender, 1978) pentylphosphonate is the most active inhibitor among a series of alkylphosphonate ethyl *p*-nitrophenyl esters which can also be explained by interaction of the pentylphosphonate residue with the S_1 pocket of PPE that is known to prefer short aliphatic amino acid side chains.

Derivatives of (α-aminoalkyl)phosphonic acids have the greatest potential for yielding selective serine protease inhibitors since they are excellent analogues of α-amino carboxylic acids. The phosphonic acid residue must be activated by the presence of a good leaving group at the phosphorus atom in order to undergo nucleophilic substitution and react with the active site serine. Phosphonyl fluorides related to phenylalanine such as Z-NHCH(CH$_2$Ph)P(O)(O-*i*-Pr)F are good irreversible inhibitors for chymotrypsin (k_{obsd}/[I] = 180 000 M^{-1} s^{-1}) and react slowly with other serine proteases such as porcine pancreatic elastase (k_{obsd}/[I] = 160 M^{-1} s^{-1}; Lamden & Bartlett, 1983). However, the presence of a phosphorus–fluorine bond makes these (α-aminoalkyl)phosphonic acid derivatives extremely unstable, and they are hydrolyzed rapidly during the enzyme assays. Extended peptide phosphonates which incorporate a tetrahedral phosphorus moiety in the place of the scissile peptide bond of peptide substrate have been synthesized and found to be poor inhibitors (k_{obsd}/[I] = 12–27 M^{-1} s^{-1}; Bartlett & Lamden, 1986).

In this paper we report that peptidyl (α-aminoalkyl)-phosphonate diphenyl esters with substrate-related sequences

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[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DFP, diisopropyl phosphorofluoridate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Et, ethyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HLE, human leukocyte (neutrophil) elastase; *i*-Pr, isopropyl; Me, methyl; MeO, methoxy; Me_2SO, dimethyl sulfoxide; NA, *p*-nitroanilide; Ph, phenyl; Pr, propyl; PPE, porcine pancreatic elastase, RMCP II, rat mast cell protease II; Suc, succinyl; THF, tetrahydrofuran; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

[2] The nomenclature of Schechter and Berger (1967) is used to designate the individual amino acid residues (P_2, P_1, P_1', P_2', etc.) of a peptide substrate and the corresponding subsites (S_2, S_1, S_1', S_2', etc.) of the enzyme. The scissile bond is the P_1–P_1' peptide bond.

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are specific irreversible inactivators of serine proteases including bovine chymotrypsin, porcine pancreatic elastase, human leukocyte elastase, cathepsin G, and rat mast cell protease II and give extremely stable inhibitor–enzyme complexes. Preliminary results from this investigation have been reported earlier (Oleksyszyn & Powers, 1989). Following our initial report, Fastrez et al. (1989) also reported the synthesis of phosphonate bis(*m*-chlorophenyl ester) derivatives related to Phe and Orn as inhibitors of chymotrypsin, trypsin, and urokinase.

Materials and Methods

Human leukocyte (neutrophil) elastase and cathepsin G were a generous gift from Dr. James Travis and his research group of the University of Georgia. Porcine pancreatic elastase was obtained from Serva. Rat mast cell protease was kindly provided by Dr. Richard Woodbury of the University of Washington. HEPES was purchased from Research Organic Inc., Cleveland, OH, and Suc-Ala-Ala-Ala-NA was purchased from Peninsula Laboratories, Inc., Belmont, CA. Bovine chymotrypsin, acetylcholinesterase, human plasma, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co., St. Louis, MO. MeO-Suc-Ala-Ala-Pro-Val-NA (Nakajima et al., 1979) and Suc-Val-Pro-Phe-NA (Tanaka et al., 1985) were prepared as previously described. The acetylcholinesterase substrate *S*-acetylthiocholine iodide was obtained from Aldrich Co., Milwaukee, WI.

Synthesis of Inhibitors. Benzyl carbamate, triphenyl phosphite, various aldehydes, dicyclohexylcarbodiimide (DCC), and all common chemicals and solvents were obtained from Aldrich Co., Milwaukee, WI. The purity of each compound synthesized was checked by ^{1}H NMR, ^{31}P NMR, IR, mass spectroscopy, TLC, and elemental analysis, and in each case the analytical results were consistent with the proposed structure. The solvent system used for TLC was chloroform–methanol (9:1). Preparative thin-layer chromatography was performed with plates precoated with silica gel G.F. (Analtech, Inc., Newark, DE). The NMR spectra were recorded on either a Varian T-60 or a Bruker instrument in DMSO-d_6 solution. ^{31}P NMR spectra were obtained at 161.895 MHz with broad-band ^{1}H decoupling on a Varian XL-400 instrument; chemical shifts are reported relative to 85% phosphoric acid (sealed capillary) at 0.000 ppm with positive values downfield. Mass spectra were measured on a Perkin-Elmer 299 instrument. Elemental analyses were performed by Atlantic Microlabs of Atlanta, GA. Blocked amino acid derivatives had the L configuration and were obtained from Aldrich or Sigma Chemical Co. Diphenyl [α-(*N*-benzyloxycarbonylamino)alkyl]phosphonates **1**, **2**, **3**, **4**, and **7** and diphenyl (α-aminoalkyl)phosphonates were synthesized as described earlier (Oleksyszyn et al., 1979) and were used as racemates. The diphenyl (α-aminoalkyl)phosphonates related to methionine (**5**) and methionine sulfoxide (**6**) are new compounds, and their characterization is described below.

Z-NHCH($CH_2CH_2SCH_3$)*P(O)*(OPh)$_2$ (**5**). This compound was synthesized by the procedure of Oleksyszyn et al. (1979): yield 36%; mp 93–95 °C; one spot on TLC, R_f = 0.73; ^{31}P NMR 19.68 ppm. Anal. Calcd for $C_{24}H_{26}O_5NSP$: C, 61.15; H, 5.52; N, 2.97; S, 6.79. Found: C, 61.06; H, 5.60; N, 2.91; S, 6.88.

Z-HNCH($CH_2CH_2SOCH_3$)*P(O)*(OPh)$_2$ (**6**). Z-Met^P-(OPh)$_2$ (**5**, 0.94 g, 2 mmol) was dissolved in 20 mL of THF and cooled to −10 °C, and a solution of 3-chloroperoxybenzoic acid, 0.42 g (80–85%, about 1 mmol), in 10 mL of THF was added slowly during 20 min. After being stirred for 1 h at −10 °C and 1 h at room temperature, the solution was washed successively with 4% sodium bicarbonate and water. The organic layer was dried and evaporated to give 0.5 g (53%) of product as a colorless oil which slowly solidified: mp 83–86 °C; one spot on TLC, R_f = 0.58; ^{31}P NMR 18.78 and 18.74 ppm (ratio 1:1). Anal. Calcd for $C_{24}H_{26}O_6NSP$: C, 59.14; H, 5.34; N, 2.87; S, 6.37. Found: C, 59.10; H, 5.39; N, 2.85; S, 6.52.

Z-Pro-HNCH(i-Pr)P(O)(OPh)$_2$ (**8**). Z-Pro-OH (0.97 g, 4 mmol) was dissolved in 40 mL of dry THF and cooled to 0 °C. Diphenyl (α-amino-2-methylpropyl)phosphonate (1.2 g, 4 mmol) and DCC (0.81 g, 4 mmol) were added to this solution. After this solution has been stirred for 6 h at 0 °C and overnight at room temperature, the DCU was removed by filtration, and 50 mL of ethyl acetate was added. The solution was washed successively twice each time with 10% citric acid, water, 4% sodium bicarbonate, and water. After being dried over $MgSO_4$, the mixture was filtered and evaporated, and the residue was dissolved in 10 mL of methylene chloride. A trace of DCU was removed by filtration, and after addition of 30 mL of pentane, the solution was allowed to crystallize. After a few days the product was filtered and recrystallized from methylene chloride–hexane to give 1.4 g (65%) of a white solid: mp 123–127 °C; one spot on TLC, R_f = 0.74; ^{31}P NMR 19.52 (br) and 19.51 (br) ppm. Anal. Calcd for $C_{29}H_{33}O_6N_2P$: C, 64.87; H, 6.15; N, 5.22. Found: C, 64.58; H, 6.23; N, 5.34.

Z-Pro-HNCH(CH_2Ph)*P(O)*(OPh)$_2$ (**9**). The reaction of Z-Pro-OH (0.75 g, 3 mmol), diphenyl (α-amino-2-phenylethyl)phosphonates (1.05 g, 3 mmol), and DCC (0.61 g, 3 mmol) gave 0.93 g (53%) of product as a white solid: mp 81–84 °C; one spot on TLC, R_f = 0.74; ^{31}P NMR 19.54, 19.48, 19.27, and 19.22 ppm (diastereomers and conformers; ratio of diastereomers 1:1). Anal. Calcd for $C_{33}H_{33}O_6N_2P$: C, 67.81; H, 5.65; N, 4.79. Found: C, 67.56; H, 5.79; N, 4.72.

Z-Ala-Ala-HNCH(i-Pr)P(O)(OPh)$_2$ (**10**). Z-Ala-Ala-OH (0.294 g, 1 mmol) was dissolved in 10 mL of dry THF and cooled to 0 °C. Diphenyl (α-amino-2-methylpropyl)-phosphonate (0.305 g, 1 mmol) and DCC (0.203 g, 1 mmol) were added to this solution. After being stirred for 8 h at 0 °C and overnight at room temperature, the solution was worked up as described above. Recrystallization from chloroform–pentane gave 0.182 g (31%) of crude product. A sample (0.1 g) was purified on preparative thin-layer chromatography with chloroform–methanol (9:1) as eluent. The product was recrystallized from ethanol–pentane to give a white solid: mp 93–97 °C; one spot on TLC, R_f = 0.71; ^{31}P NMR 19.44 and 19.31 ppm (ratio 1:1.11). Anal. Calcd for $C_{30}H_{36}O_7N_3P$·EtOH: C, 61.19; H, 6.69; N, 6.69. Found: C, 61.32; H, 6.48; N, 6.91.

MeO-Suc-Ala-Ala-Ala-HNCH(Pr)P(O)(OPh)$_2$ (**11**). The reaction of (0.345 g, 1 mmol) MeO-Suc-Ala-Ala-Ala-OH and (0.305, g 1 mmol) diphenyl (α-aminobutyl)phosphonate gave 0.35 g (55.3%) of product as a white solid. A 0.1-g sample was purified on preparative TLC as described above to give the product as a white solid: mp 215–218 °C; one spot on TLC, R_f = 0.73; ^{31}P NMR 20.02 ppm. Anal. Calcd for $C_{30}H_{41}O_9N_4P$: C, 56.96; H, 6.49; N, 8.86. Found: C, 56.72; H, 6.58; N, 8.80.

MeO-Suc-Ala-Ala-Ala-HNCH(i-Pr)P(O)(OPh)$_2$ (**12**). The reaction of (0.345 g, 1 mmol) MeO-Suc-Ala-Ala-Ala-OH and (0.305 g, 1 mmol) diphenyl (α-amino-2-methylpropyl)-phosphonate gave 0.3 g (47.5%) of product as a white solid. A 0.1-g sample was purified on preparative TLC as described above to give the product as a white solid, which was then recrystallized from ethanol–hexane: mp 197–200 °C; one spot

on TLC, R_f = 0.63; ^{31}P NMR 19.40 and 19.24 ppm (ratio 1:0.72). Anal. Calcd for $C_{30}H_{41}O_9N_4P\cdot 0.25C_6H_{14}$: C, 57.60; H, 6.85; N, 8.61. Found: C, 57.58; H, 6.62; N, 8.36.

MeO-Suc-Ala-Ala-Ala-HNCH(CH_2Ph)P(O)(OPh)$_2$ (**13**). The reaction of (0.345 g, 1 mmol) MeO-Suc-Ala-Ala-Ala-OH and (0.353 g, 1 mmol) diphenyl (α-amino-2-phenylethyl)-phosphonate gave 0.35 g (49.1%) of the product which was purified on the preparative TLC, as described above. Recrystallization from methanol gave a white solid: mp 207–210 °C; one spot on TLC, R_f = 0.69; ^{31}P NMR 19.06 and 19.01 ppm (ratio 1:0.87). Anal. Calcd for $C_{34}H_{41}O_9N_4P\cdot MeOH$: C, 59.98; H, 6.32; N, 7.86. Found: C, 58.66; H, 6.08; N, 7.96.

MeO-Suc-Ala-Ala-Pro-HNCH(i-Pr)P(O)(OPh)$_2$ (**14**). MeO-Suc-Ala-Ala-Pro-OH (0.37 g, 1 mmol) was dissolved in 20 mL of dry THF and cooled to 0 °C. Diphenyl (α-amino-2-methylpropyl)phosphonate (0.305 g, 1 mmol) and DCC (0.203 g, 1 mmol) were added to this solution. After being stirred for 6 h at 0 °C and overnight at room temperature, the solution was filtered, and 100 mL of ethyl acetate was added. The solution was then washed with 10% citric acid, 4% sodium bicarbonate, and water successively. After being dried over magnesium sulfate, the solution was filtered and evaporated, and the residue was dissolved in 10 mL of THF. Trace DCU was removed by filtration, and after addition of 3 mL of hexane, the solution was allowed to crystallize. After a few days the product was removed by filtration and recrystallized from THF–hexane to give 0.2 g (30%) of a white solid: mp 83–86 °C; one spot on TLC, R_f = 0.74; ^{31}P NMR 19.78 and 19.60 ppm (ratio 1:2.12). Anal. Calcd for $C_{32}H_{43}O_9P\cdot C_6H_{14}$: C, 61.30; H, 7.66; N, 7.53. Found: C, 61.04; H, 7.58; N, 7.69.

MeO-Suc-Ala-Ala-Pro-HNCH(CH_2-i-Pr)P(O)(OPh)$_2$ (**15**). The reaction of (0.37 g, 1 mmol) MeO-Suc-Ala-Ala-Pro-OH and (0.32 g, 1 mmol) diphenyl (α-amino-3-methylbutyl)-phosphonate gave 0.38 g (50.1%) of product as a white solid: mp 60–64 °C; one spot on TLC, R_f = 0.67; ^{31}P NMR 20.71 and 20.54 ppm (ratio 1:1.33). Anal. Calcd for $C_{33}H_{45}O_9N_4P\cdot C_6H_{14}$: C, 61.74; H, 7.78; N, 7.38. Found: C, 61.04; H, 7.53; N, 7.59.

MeO-Suc-Ala-Ala-Pro-HNCH(CH_2Ph)P(O)(OPh)$_2$ (**16**). The reaction of (0.37 g, 1 mmol) MeO-Suc-Ala-Ala-Pro-OH and (0.35 g, 1 mmol) diphenyl (α-amino-2-phenylethyl)-phosphonate gave 0.4 g (50.5%) of product as a white solid: mp 53–56 °C; one spot on TLC, R_f = 0.7; ^{31}P NMR 19.41 and 19.10 ppm (ratio 1:2.47). Anal. Calcd for $C_{36}H_{43}O_9N_4P\cdot C_6H_{14}$: C, 63.62; H, 7.19; N, 7.07. Found: C, 63.42; H, 7.19; N, 7.57.

MeO-Suc-Ala-Ala-Pro-HNCH($CH_2CH_2SCH_3$)P(O)(OPh)$_2$ (**17**). The reaction of (0.64 g, 1.73 mmol) MeO-Suc-Ala-Ala-Pro-OH, (0.85 g, 2 mmol) $HBr\cdot H_2N$-CH($CH_2CH_2SCH_3$)P(O)(OPh)$_2$, (0.2 mL, 2 mmol) *N*-methylmorpholine, and (0.34 g, 1.73 mmol) DCC under an atmosphere of nitrogen gave 0.5 g (42%) of product as a white solid. A 0.2-g sample was purified on preparative TLC under an atmosphere of nitrogen, to give the product as a white solid: mp 41–43 °C; one spot on TLC, R_f = 0.6; ^{31}P NMR 19.50 and 19.36 ppm (ratio 1:1.32). Anal. Calcd for $C_{32}H_{43}O_9N_4PS\cdot THF$: C, 56.69; H, 6.69; N, 7.35; S, 4.20. Found: C, 57.37; H, 6.77; N, 7.76; S, 4.49.

MeO-Suc-Ala-Ala-Pro-HNCH($CH_2CH_2SOCH_3$)P(O)(OPh)$_2$ (**18**). The product (0.01 g) was obtained with the reaction described for the benzyloxycarbonyl derivative **6**, from 0.025 g of **17** as colorless crystals: mp 47–48 °C; one spot on TLC, R_f = 0.5; ^{31}P NMR 18.58 and 18.45 ppm (ratio 1:0.85).

Boc-Val-Pro-HNCH(i-Pr)P(O)(OPh)$_2$ (**19**). A solution of 0.536 g (1 mmol) **8** in 50 mL of methanol with 0.1 g of 5% Pd/C was stirred under atmosphere of hydrogen at room temperature for 2 h and filtered through celite. After addition of 0.217 g (1 mmol) of Boc-Val-OH to the filtrate, the solvent was removed in vacuo. The residue was dissolved in 20 mL of dry THF, and (0.2 g, 1 mmol) DCC was added. The solution was kept at 0 °C for 6 h and overnight at room temperature. DCU was removed by filtration, and the organic layer was washed with water, twice with 4% $NaHCO_3$, with water, twice with 10% citric acid, and with water. After being dried over sodium sulfate, the solvent was evaporated, and the resulting oil was dried at low pressure for few hours to give 0.45 g (72%) of a white hydroscopic solid: mp 62–66 °C; one spot on TLC, R_f = 0.78; ^{31}P NMR 19.64 ppm. Anal. Calcd for $C_{31}H_{44}O_7N_3P\cdot H_2O$: C, 60.10; H, 7.43. Found: C, 60.26; H, 7.49.

Z-Phe-Pro-HNCH(CH_2Ph)P(O)(OPh)$_2$ (**20**). The reaction of (0.3 g, 1 mmol) Z-Phe-OH with the hydrogenolysis product of (0.58 g, 1 mmol) **9** and 0.2 g (1 mmol) of DCC gave 0.4 g (54%) of a hydroscopic semisolid product after a standard workup. A 0.2-g sample was purified on preparative TLC to give the product as a hygroscopic semisolid: one spot on TLC, R_f = 0.76; ^{31}P NMR 19.67, 19.57, 19.32, 19.19, and 19.07 ppm (diastereomers and conformers; ratio 1.0:0.34:0.82:0.73:0.92). Anal. Calcd for $C_{42}H_{42}O_7N_3P\cdot 2H_2O$: C, 65.71; H, 5.99; N, 5.47. Found: C, 65.62; H, 6.12; N, 5.27.

Suc-Val-Pro-HNCH(CH_2Ph)P(O)(OPh)$_2$ (**21**). The reaction of (0.25 g, 1 mmol) Z-Val-OH, (0.2 g, 1 mmol) DCC, and the product of hydrogenolysis of 0.584 g (1 mmol) of **8** gave an oil which was dissolved in 30 mL of ethyl acetate. To this solution, 0.1 g (1 mmol) of succinic anhydride and 0.1 g of 5% Pd/C were added, and the mixture was stirred under an atmosphere of hydrogen until the TLC showed only one new spot. The catalyst was removed by filtration, and the organic layer was washed several times with water. After being dried, the organic solvent was removed to give 0.45 g (65%) of product as a hygroscopic solid: mp 50–53 °C; one spot on TLC, R_f = 0.4; ^{31}P NMR 19.75 and 19.23 ppm (ratio 1:1). Anal. Calcd for $C_{34}H_{40}O_3N_3P\cdot 2H_2O$: C, 59.56; H, 6.42. Found: C, 59.59; H, 6.42.

Enzyme Inactivation—Incubation Method. The inactivation reaction was initiated by adding a 25–50-μL aliquot of inhibitor in Me_2SO to 0.5 mL of a buffered enzyme solution (0.3–2.0 μM) such that the final Me_2SO concentration was 5–10% (v/v) at 25 °C. Aliquots (25 μL for HLE or 50 μL for PPE, chymotrypsin, cathepsin G, and RMCP II) were removed at intervals and added to a substrate solution (42- or 84-fold dilution), and the residual activity was measured spectrometrically. A 0.1 M HEPES and 0.5 M NaCl, pH 7.5 buffer was utilized throughout, and inhibitor concentrations are shown in the appropriate table. All spectrometric measurements were carried out on a Beckman Model 35 or a Varian DMS-90 spectrometer. Chymotrypsin and cathepsin G were assayed with 0.476 mM Suc-Val-Pro-Phe-NA (Tanaka et al., 1985), HL elastase was assayed with 0.482 mM MeO-Suc-Ala-Ala-Pro-Val-NA (Nakajima et al., 1979), and PP elastase was assayed with 0.714 mM Suc-Ala-Ala-Ala-NA (Bieth et al., 1974). Peptide *p*-nitroanilide hydrolysis was measured at 410 nm (ϵ_{410} = 8800 M^{-1} cm^{-1}; Erlanger et al., 1961). RMCP II was assayed with 88 μM Suc-Ala-Ala-Pro-Phe-SBzl (Harper et al., 1981) in the presence of 4,4′-dithiodipyridine (0.33 mM; ϵ_{324} = 19800 M^{-1} cm^{-1}; Grassetti & Murray, 1967). First-order inactivation rate constants (k_{obsd}) were obtained from plots of ln v_t/v_o vs time. Mea-

surements of k_{obsd} were made in concentration ranges where k_{obsd} was proportional to [I], and thus $k_{obsd}/[I]$ is the second-order rate constant for the inhibition reaction. Inactivation rate constants shown in the tables are typically the average of duplicate or triplicate experiments.

Determination of Inactivation Rates in the Presence of Substrate—Progress Curve Method. In some cases, $k_{obsd}/[I]$ values were determinate in the presence of substrate as described by Tian and Tsou (1982). For example, inactivation of chymotrypsin (0.109 μM) by MeO-Suc-Ala-Ala-Pro-HN-CH(CH_2Ph)P(O)$(OPh)_2$ (**16**, 1.5–4.0 μM) in the presence of 0.476, 0.357, and 0.119 mM Suc-Val-Pro-Phe-NA was measured by addition of a 0.01-mL aliquot of enzyme to a substrate and inhibitor solution containing 10% Me_2SO. The increase in absorbance was monitored (410 nm) with time until no further release of *p*-nitroaniline was observed. The $k_{obsd}/[I]$ values were calculated with $k_{obsd} = 0.693/t_{1/2}$, where the half-life $t_{1/2}$ was obtained from plots of log $([P]_\infty - [P]_t)$ vs time, where $[P]_\infty$ and $[P]_t$ are the concentrations of *p*-nitroaniline after total inactivation and at time *t*, respectively. Inactivation of HL elastase (0.034 μM) by MeO-Suc-Ala-Ala-Pro-HN-CH(*i*-Pr)P(O)$(OPh)_2$ (**14**, 2.3 μM) in the presence of 0.243 mM MeO-Suc-Ala-Ala-Pro-Val-NA was measured as described above. The inactivation of PP elastase (0.224 μM) by **14** (2.3 μM) was also measured in the presence of 0.714 mM Suc-Ala-Ala-Ala-NA.

Dephosphonylation Kinetics. Dephosphonylation rates were measured after the removal of excess inhibitor from solutions of inactivated enzymes by centrifugation twice at 0 °C for 1 h in Amicon Centricon-10 microconcentrators following addition of fresh buffer. The enzymatic activity of the solution was assayed at various time intervals as described above. The half-times of the first-order dephosphonylation were obtained from plots of ln $(v_o - v_t)$ vs time, where v_o is the enzyme-catalyzed substrate hydrolysis rate under identical conditions except in the absence of inhibitor. The correlation coefficients were greater than 0.98.

Determination of the Stability of the Inhibitors in Buffer and in the Presence of Glutathione. The stability of the inhibitors was studied by monitoring the UV spectrum of solutions at 25 °C. The concentration of inhibitor was 0.1–0.2 mM and of glutathione was 0.3–0.4 mM, and the buffer solution contained about 10% (v/v) Me_2SO. The stability of the same inhibitors was also monitored by ^{31}P NMR in HEPES buffer containing 10% (v/v) Me_2SO-d_6.

Determination of the Stability of the Inhibitors in Human Plasma. A solution of Suc-Val-Pro-Phe^P$(OPh)_2$ (**21**, 76.9 μM) in human plasma containing 10% (v/v) Me_2SO was prepared and incubated at room temperature. The half-time for inactivation of a chymotrypsin solution by this inhibitor solution was measured at 1-h intervals with the incubation method described before.

Determination of Acetylcholinesterase Inhibition—Incubation Method. To 0.5 mL of buffer (0.1 M, pH 8.0, phosphate) was added a 50-μL aliquot of enzyme (1.86 mg/mL) followed by a 25–50-μL aliquot of inhibitor in Me_2SO at 25 °C, such that the final Me_2SO concentration was 5–10% (v/v). Aliquots were removed at various time intervals and diluted into 2 mL of buffer containing 20 μL of substrate solution (acetylthiocholine iodide, 0.075 M) and 100 μL of DTNB solution (0.1 M). The enzyme activity was measured spectrophotometrically at 410 nm (ϵ_{410} = 13 600 M^{-1} cm^{-1}; Ellman, 1959).

^{31}P NMR Spectrum of Complex Formed from Chymotrypsin and Suc-Val-Pro-Phe^P$(OPh)_2$ (**21**). A solution of **21** (10% solution in Me_2SO) was added in 20-μL portions to 100 mg of chymotrypsin in 100 mL of HEPES buffer with stirring at room temperature until the solution showed no enzymatic activity. The pH of the solution was adjusted to 3, and then the solution was ultrafiltered at 4 °C through an Amicon UM 10 membrane (M_r cutoff 10 000) at 80 psi of nitrogen over a period of about 0.5 h. The filtration was allowed to proceed until there was about 0.5–1.0 mL remaining in the filtration unit, a fresh 10-mL portion of acetate buffer (0.4 M NaOAc, 1 M NaCl, pH 5.5) was added, and the filtration was repeated. The procedure was repeated four times, and the solution was then transfered to a 10-mm NMR tube. After addition of more acetate buffer and about 15% (v/v) D_2O, the spectrum was taken as described above to show one broad signal at 25.98 ppm. The ^{31}P NMR of the starting material (**21**) under these condition (acetate buffer, pH 5.5, 15% Me_2SO-d_6) show a doublet at 19.59 and 18.75 ppm, ratio 1:1. The ^{31}P NMR for the monoester Z-Phe^P(OPh)OH had peaks at 17.65 and 17.19 ppm, and the acid Z-Phe^P had peaks at 17.54 and 17.05 ppm.

Determination of the Stereospecificity of Reaction of Chymotrypsin with MeO-Suc-Ala-Ala-Ala-Phe^P$(OPh)_2$ by ^{31}P NMR. Chymotrypsin (50 mg) was reacted with **13** (diastereomer ratio 1:0.87) as described above, and the phosphorus NMR of the filtrate was measured (HEPES buffer, pH 7.5, 10% Me_2SO-d_6). The product showed signals at 16.89 and 16.79 ppm with a diastereomer ratio of 1:1.74, showing that the first diastereomer had reacted preferentially. In another experiment, 30 mg of chymotrypsin was added to a solution (HEPES buffer, 15% Me_2SO) of **13** (before addition, ^{31}P NMR peaks at 18.62 and 18.59 ppm) in an NMR tube. Most of the inhibited protein precipitated. The resulting solution had no enzymatic activity, and the NMR spectrum again showed only the presence of the unreacted diastereomer (18.58 ppm).

Results

Synthesis of the Peptides Containing C-Terminal Diphenyl (α-Aminoalkyl)phosphonate Residues. *N*-Carbobenzyloxy derivatives of diphenyl (α-aminoalkyl)phosphonates were synthesized by amidoalkylation of triphenyl phosphite with benzyl carbamate and an aldehyde (Oleksyszyn et al., 1979). The racemic diphenyl (α-aminoalkyl)phosphonates, prepared by deblocking with HBr, were then coupled with N-blocked amino acids or peptides according to the DCC method. The synthesis of peptide bonds is under kinetic control, especially when longer peptides containing proline at the C-terminal were coupled with the (α-aminoalkyl)phosphonates. Thus, the products in most cases are a nonequivalent mixture of diastereomers as determined by ^{31}P NMR (e.g., **12**, 1:0.72; **13**, 1:0.87; **14**, 1:2.12; **15**, 1:1.33; **16**, 1:2.47; **17**, 1:1.32). No attempts were made to resolve the diastereomers, and we did not check for racemization during the peptide coupling reactions. The structures of the various inhibitors are shown in Tables I and II.

Inactivation Kinetics. The second-order rate constants $k_{obsd}/[I]$ for inactivation of chymotrypsin, PPE, HLE, cathepsin G, and rat mast cell protease II by diphenyl (α-aminoalkyl)phosphonate derivatives are reported in Tables I–III. The pseudo-first-order inactivation plots remained linear for greater than four half-lives. Inactivation of these serine proteases by diphenyl [α-(*N*-benzyloxycarbonylamino)alkyl]phosphonates **1**–**7** (analogues of alanine, Z-Ala^P$(OPh)_2$ (**1**); norvaline, Z-Nva^P$(OPh)_2$ (**2**); valine, Z-Val^P$(OPh)_2$ (**3**); leucine, Z-Leu^P$(OPh)_2$ (**4**); methionine, Z-Met^P$(OPh)_2$ (**5**); methionine sulfoxide, Z-Met(O)^P$(OPh)_2$ (**6**); and phenylalanine, Z-Phe^P$(OPh)_2$ (**7**)] was relatively slow,

Table I: Rate Constants (k_{obsd}/[I]) for Inactivation of Serine Proteases by Diphenyl [α-(*N*-Benzyloxycarbonylamino)alkyl]phosphonates Z-NHCH(R)P(O)(OPh)$_2$[a]

	chymotrypsin[b]		PPE[c]		HLE[d]	
	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})
Z-Ala^P(OPh)$_2$ (**1**)	8.2	NI[e]	8.2	NI	4.3	10
Z-Nva^P(OPh)$_2$ (**2**)	8.2	NI	8.2	9.7	4.3	50
Z-Val^P(OPh)$_2$ (**3**)	7.8	NI	7.8	9	4.1	280
Z-Leu^P(OPh)$_2$ (**4**)	7.9	81	7.9	NI	4.2	10
Z-Met^P(OPh)$_2$ (**5**)	8.6	30	8.9	NI	4.5	NI
Z-Met(O)^P(OPh)$_2$ (**6**)	8.9	NI	8.6	NI	4.7	NI
Z-Phe^P(OPh)$_2$ (**7**)	8.2	1200	8.2	NI	4.2	NI

[a] Conditions were as follows: 0.1 M HEPES and 0.5 M NaCl, pH 7.5 at 25 °C. Rate constants were obtained as described under Materials and Methods. [b] Enzyme concentration 1.6 μM. [c] Enzyme concentration 1.8 μM. [d] Enzyme concentration 0.32 μM. [e] NI, less than 5% inhibition after 1 h.

Table II: Rate Constants (k_{obsd}/[I]) for Inactivation of Serine Protease by Peptides with C-Terminal Diphenyl (α-Aminoalkyl)phosphonates[a]

	chymotrypsin[b]		PPE[c]		HLE[d]	
inactivator	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})
Z-Pro-Val^P(OPh)$_2$ (**8**)	8.2	NI[e]	10	20	5.3	130
Z-Pro-Phe^P(OPh)$_2$ (**9**)	8.2	230	8.2	NI	4.3	*f*
Z-Ala-Ala-Val^P(OPh)$_2$ (**10**)	8.2	NI	24.3	340	13.5	1300
MeO-Suc-Ala-Ala-Pro-Nva^P(OPh)$_2$ (**11**)	8.2	50	8.6	4200	95	380
MeO-Suc-Ala-Ala-Ala-Val^P(OPh)$_2$ (**12**)	90.1	15	9.1	2800	5	1500
MeO-Suc-Ala-Ala-Ala-Phe^P(OPh)$_2$ (**13**)	5.3	12000	105	NI	58	NI
MeO-Suc-Ala-Ala-Pro-Val^P(OPh)$_2$ (**14**)	180	21	9	7100	4.9	7100
MeO-Suc-Ala-Ala-Pro-Leu^P(OPh)$_2$ (**15**)	26	1500	26	740	14.4	140
MeO-Suc-Ala-Ala-Pro-Phe^P(OPh)$_2$ (**16**)	11	11000	100	NI	50	NI
MeO-Suc-Ala-Ala-Pro-Met^P(OPh)$_2$ (**17**)	30	570	30	44	76	53
MeO-Suc-Ala-Ala-Pro-Met(O)^P(OPh)$_2$ (**18**)	370	15	370	1.6	185	1.6
Boc-Val-Pro-Val^P(OPh)$_2$ (**19**)	8.2	NI	8.7	11000	4.5	27000
Z-Phe-Pro-Phe^P(OPh)$_2$ (**20**)	4.6	17000	92	NI	8.2	NI
Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**)	5.5	44000	8.2	NI	4.1	NI

[a] Conditions were as follows: 0.1 M HEPES and 0.5 M NaCl, pH 7.5 at 25 °C. Rate constants were obtained as described under Materials and Methods. [b] Enzyme concentration 1.6 μM. [c] Enzyme concentrations 1.8 μM. [d] Enzyme concentration 0.32 μM. [e] NI, less than 5% inhibition after 1 h. [f] About 20% inhibition at the start of the reaction and no further inhibition with time.

Table III: Rate Constants (k_{obsd}/[I]) for Inactivation of Cathepsin G and Rat Mast Cell Protease II by Peptidyl Derivatives of Diphenyl [α-(*N*-Benzyloxycarbonylamino)-2-phenylethyl]phosphonate[a]

	cathepsin G[b]		RMCP II[c]	
inactivator	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})
Z-Phe^P(OPh)$_2$ (**7**)	82	76	82	89
MeO-Suc-Ala-Ala-Ala-Phe^P(OPh)$_2$ (**13**)	13.6	370	170	6.8
MeO-Suc-Ala-Ala-Pro-Phe^P(OPh)$_2$ (**16**)	13.1	440	130	39
Z-Phe-Pro-Phe^P(OPh)$_2$ (**20**)	5.1	5100	5.1	32
Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**)	8.4	36000	3	15000

[a] Inactivation rates were measured by an incubation method in 0.1 M HEPES and 0.5 M NaCl, pH 7.5, buffer containing 9% Me_2SO at 25 °C. Enzyme concentration: cathepsin G, 1.6 μM; RMCP II, 38 nM. [b] Cathepsin G was assayed with Suc-Val-Pro-Phe-NA (0.5 mM). [c] RMCP II was assayed with Suc-Ala-Ala-Pro-Phe-SBzl (88 μM) in the presence of 4,4′-dithiodipyridine (0.33 mM).

while the peptide derivatives were much more reactive.[3]

(Aminoalkyl)phosphonates, which are analogues of simple amino acid derivatives, exhibited considerable selectivity. The phenylalanine analogue (**7**) is most reactive with chymotrypsin and the chymotrypsin-like enzymes cathepsin G and RMCP II with rate constants of 1200, 76, and 89 M^{-1} s^{-1}, respectively, and at low concentrations did not react with elastases. The only other derivatives which inhibited chymotrypsin were the leucine (**4**) and, methionine (**5**) analogues which had rate constants of 81 and 20 M^{-1} s^{-1}, respectively. HL elastase was inhibited most effectively by the valine analogue **3** (280 M^{-1} s^{-1}) and had some reactivity toward the alanine (**1**, 10 M^{-1} s^{-1}), leucine (**4**, 10 M^{-1} s^{-1}), and norvaline (**2**, 50 M^{-1} s^{-1}) analogues. PP elastase was only inhibited by Z-Nva^P(OPh)$_2$ (**2**) and Z-Val^P(OPh)$_2$ (**3**) with rate constants of 9.7 and 9 M^{-1} s^{-1}, respectively. The phenylalanine derivative (**7**) did not react with either PPE or HLE at low concentrations.

All the tri- and tetrapeptide phosphonates (Table II) are much more potent inhibitors than the simple benzyloxycarbonyl derivatives (Table I). In some cases, 1200-fold improvement in inhibitory potency was observed (e.g., **3** versus **19** for PPE). Peptides with a C-terminal -Val^P(OPh)$_2$ residue are good inactivators of the elastases (**10**, **12**, **14**, and **19**) and at low concentrations do not react with chymotrypsin. All phosphonates with a C-terminal -Phe^P(OPh)$_2$ residue (**13**, **16**, **20**, and **21**) are good inactivators for chymotrypsin and at low concentration do not react with the elastases. Peptides with C-terminal phosphonates related to norvaline (**11**), leucine (**15**), and methionine (**17**) are moderate inactivators of all the enzymes.

The inhibitory potency is dependent upon the amino acid sequence of the peptide. In the series of valine derivatives, Boc-Val-Pro-Val^P(OPh)$_2$ (**19**) is the best elastase inhibitor with inhibition rate constants of 11000 and 27000 M^{-1} s^{-1} for PPE and HLE, respectively. Even the tetrapeptide MeO-Suc-

[3] The (α-aminoalkyl)phosphonic acids are analogues of natural α-amino acids and are designated by the generally accepted three-letter abbreviations for the amino acid followed by a superscript P. For example diphenyl [α-(*N*-benzyloxycarbonylamino)ethyl]phosphonate, which is related to alanine, is abbreviated as Z-Ala^P(OPh)$_2$.

Table IV: Half-Lives for Dephosphorylation of Serine Proteases Inactivated by Diphenyl (α-Aminoalkyl)phosphonate Derivatives[a]

inactivator	$t_{1/2}$ (h) chymotrypsin	PPE	HLE
Z-Val^P(OPh)$_2$ (**4**)	-	<48	<48
Z-Pro-Val^P(OPh)$_2$ (**8**)	-	<48	<48
Z-Ala-Ala-Val^P(OPh)$_2$ (**10**)	-		<48
MeO-Suc-Ala-Ala-Pro-Val^P(OPh)$_2$ (**14**)	-	<48	<48
Z-Phe^P(OPh)$_2$ (**7**)	10[b]		
MeO-Suc-Ala-Ala-Pro-Phe^P(OPh)$_2$ (**16**)	16		
Z-Phe-Pro-Phe^P(OPh)$_2$ (**20**)	26		
MeO-Suc-Ala-Ala-Ala-Phe^P(OPh)$_2$ (**13**)	10		
Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**)	7.5		

[a] Enzyme activity was measured after removal of excess of inhibitors as described under Materials and Methods. [b] Approximately 50% reactivation was observed after 10 h, but no further reaction occurred even after 48 h.

Ala-Ala-Pro-Val^P(OPh)$_2$ (**14**) is less potent than **19**. Many of the elastase inhibitors including **12** react with HLE twice as rapidly as PPE (2800 and 1500 M^{-1} s^{-1}). The chymotrypsin inhibitor Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**) is the best inhibitor which we discovered ($k_{obs}/[I]$ = 44000 M^{-1} s^{-1}). Other peptide derivatives with a C-terminal -Phe^P(OPh)$_2$ residue (**13**, **16**, and **20**) are also quite good chymotrypsin inhibitors with inhibition rate constants of 12000, 11000, and 17000 M^{-1} s^{-1}, respectively. None of these peptides (including **21**) will react with elastases at low concentrations.

The inactivation rate constants for cathepsin G and rat mast cell protease II are shown in Table III. In general, the phosphonates are better inhibitors for cathepsin G than RMCP II. The most reactive inhibitor for both enzymes is Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**, 36000 and 15000 M^{-1} s^{-1}, respectively). The tripeptide **20** was the most selective for cathepsin G with a rate constant of 5100 M^{-1} s^{-1} compared to 32 M^{-1} s^{-1} with RMCP II. With cathepsin G, a 470-fold increase in inhibitory potency was observed in going from Z-Phe^P(OPh)$_2$ (**7**) to the tripeptide Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**).

Substrate Protection. The addition of substrates to the enzyme incubation mixtures resulted in significant decrease in inactivation rate constants. The rate constants ($k_{obsd}/[I]$) for the inactivation of chymotrypsin (0.109 μM) by **16** in the presence of 0.476, 0.357, and 0.119 mM Suc-Val-Pro-Phe-NA were 900, 1070, and 2200 M^{-1} s^{-1}, respectively, compared to 11000 M^{-1} s^{-1} in the absence of the substrate. For the inactivation of PPE (0.224 μM) by **14** (2.3 μM) in the presence of 0.714 mM Suc-Ala-Ala-Ala-NA, a rate constant of 3300 M^{-1} s^{-1} was obtained compared to 7100 M^{-1} s^{-1} in the absence of substrate. With HLE (0.034 μM) and the substrate MeO-Suc-Ala-Ala-Pro-Val-NA (0.243 mM), the corresponding values with the inhibitor **14** (2.30 μM) were 1700 and 7100 M^{-1} s^{-1}.

Dephosphonylation Kinetics. The half-lives for dephosphonylation of serine proteases inactivated by peptidyl derivatives of diphenyl (α-aminoalkyl)phosphonates are shown in Table IV. The inhibitors form stable covalent enzyme–inhibitor adducts since less than 5% of the enzymatic activity was regained after removal of excess inhibitor by dilution and concentration of the solution at 0 °C. With the elastases, the enzyme–inhibitor complex is very stable with reactivation half-lives of at least 48 h in all cases. In the case of HL elastase inhibited by **14**, no enzymatic activity was observed even after incubation for 7 days. The half-lives of dephosphonylation of chymotrypsin inactivated by peptidyl derivatives of diphenyl (α-aminoalkyl)phosphonates varied from 7.5 to 26 h. Clearly, the reactivation time depends on the amino acid sequence of the inhibitor.

Stability of Inhibitors in Buffer and in the Presence of Glutathione. The UV spectrum of MeO-Suc-Ala-Ala-Pro-Leu^P(OPh)$_2$ (**15**, 0.143 mM) in 0.1 M HEPES and 0.5 M NaCl, pH 7.5 at 25 °C shows broad absorbance at 250–280 nm with two maxima at 260 nm (ε = 1260 M^{-1} cm^{-1}) and 265 nm (ε = 1370 M^{-1} cm^{-1}). There was no change in the spectrum after incubation for 7 days. Addition of the expected hydrolysis product phenol (0.106 mM), corresponding to the hydrolysis of ca. one ester group, changed the shape of the spectrum and produced a new maximum at 267 nm (ε = 1420 M^{-1} cm^{-1}). Addition of glutathione (0.395 mM) also had no effect on the spectrum after incubation for at least 4 days. The stability of MeO-Suc-Ala-Ala-Ala-Phe^P(OPh)$_2$ (**13**) in 0.1 M HEPES buffer and 0.5 M NaCl, pH 7.5, containing 20% Me_2SO-d_6 was studied by ^{31}P NMR (18.62 and 18.58 ppm). No change in the spectrum was observed after incubation for 4 days.

Stability of the Inhibitors in Human Plasma. The stability of Suc-Val-Pro-Phe^P(OPh)$_2$ in human plasma was measured by its ability to inactivate chymotrypsin. No change in the inhibition half-life was observed during the first 8 h (half-times for inactivation after 0, 1, 2, 3, 4, and 8 h were 0.16, 0.15, 0.12, 0.14, 0.17, and 0.19 min, respectively. However after 24-h incubation, the half-life was increased to 0.4 min, indicating destruction of the inhibitor. The estimated half-life for hydrolysis of inhibitor in human plasma is about 20 h.

Inhibition of Acetylcholinesterase. The possibility of inhibition of acetylcholinesterase by peptidyl derivatives of diphenyl (α-aminoalkyl)phosphonates was also examined. At concentrations of 18–26 μM, none of the phosphonates **3**, **7**, **13**, **14**, **16**, and **20** showed more than 5% enzyme inhibition after 4 h.

^{31}P NMR of the Inhibitors and ^{31}P NMR of Complex Formed by Chymotrypsin and Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**). The chemical shifts of peptidyl derivatives of diphenyl (α-aminoalkyl)phosphonates in Me_2SO-d_6 are between 18.45 and 20.71 ppm downfield from that of 85% H_3PO_4. Changing the solvent from Me_2SO-d_6 to a buffer containing Me_2SO-d_6 (10–20%) resulted in a small upfield shift of the ^{31}P signal. For example, the ^{31}P NMR of MeO-Suc-Ala-Ala-Ala-Phe^P-(OPh)$_2$ (**13**) shows signals at 19.06 and 19.01 ppm in Me_2SO-d_6, while in a HEPES buffer containing 10% Me_2SO-d_6 signals at 16.89 and 16.79 were observed. The chemical shift for Z-Phe^P(OPh)OH or for Z-Phe^P(OH)$_2$ in both solvent systems is shifted upfield relative to that of the diphenyl ester. The ^{31}P NMR of chymotrypsin phosphonylated by Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**) shows a broad singlet at 25.98 ppm which is downfield compared to that of the starting material. In addition, this chemical shift is in the opposite direction to the upfield shift observed by changing the solvent or by going from the diphenyl ester to a monoester or the diacid.

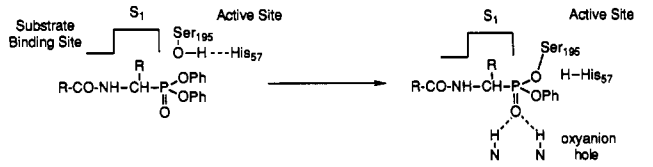

FIGURE 1: Scheme for the reaction of a serine protease with a peptidyl derivative of an (α-aminoalkyl)phosphonic acid diphenyl ester. The inhibited enzyme is phosphonylated by the inhibitor, and one oxygen atom is shown interacting with the oxyanion hole of the serine protease.

Determination of the Stereospecificity of the Reaction of Chymotrypsin with the Peptidyl Derivatives of Diphenyl (α-Aminoalkyl)phosphonates. The diastereomeric ratio in a solution of MeO-Suc-Ala-Ala-Ala-PheP$(OPh)_2$ was determined in two ^{31}P NMR experiments both before and after reaction of the inhibitor with chymotrypsin. In both cases, the diastereomer with the downfield chemical shift reacted faster with chymotrypsin. Chymotrypsin (1.3 μM) was then allowed to react with 1.3 μM Suc-Val-Pro-Phe$^P(OPh)_2$ (**21**) at an enzyme/inhibitor ratio of 1:1. As predicted, only one diastereomer reacted, 50% of inhibition was observed after 1.9 min, and no further inhibition was observed after an additional 60 min of incubation time. When this same inhibitor (**21**; 2.6 μM; diastereomers ratio 1:1) was allowed to react with chymotrypsin (1.3 μM) under conditions where the ratio of enzyme/reactive diastereomer was 1:1, complete inhibition was observed. The second-order inhibition rate constant under these conditions was calculated from the equation $k_{2nd} = 1/(t_{1/2}[E])$ and is 146 000 M^{-1} s^{-1}.

Discussion

Organophosphorus Irreversible Inhibitors of Serine Proteases. The peptidyl derivatives of diphenyl (α-aminoalkyl)-phosphonates reported in this paper are specific and potent irreversible inhibitors of serine proteases. The utility of most organophosphorus inhibitors which have been reported earlier is limited by their nonspecificity and chemical instability. For example, Lamden and Bartlett (1983) described the phenylalanine-related structure Z-HN-CH(CH_2Ph)P(O)(O-*i*-Pr)F where the phosphonate residue is reactive in nucleophilic substitution reactions due to the presence of the electronegative fluorine atom. This compound is an excellent inhibitor of chymotrypsin (second-order inhibition rate constant = 180 000 M^{-1} s^{-1}) and shows remarkable selectivity because inactivation of another serine protease, porcine pancreatic elastase, occurs at a very low rate (160 M^{-1} s^{-1}). However, the phosphorus–fluorine bond is chemically very unstable, and this class of inhibitors probably has no practical therapeutic application. In contrast, tetrapeptide phosphonates, where the scissile peptide bond of a substrate is replaced by an (α-aminoalkyl)phosphonic acid amide residue, are poor inhibitors of serine proteases (Bartlett & Lamden, 1986). They inhibit chymotrypsin with rate constants of 12–27 M^{-1} s^{-1}.

The diphenyl esters of (α-aminoalkyl)phosphonic acids, which we have synthesized, are chemically very stable. They can be hydrolyzed under strong acidic condition (Oleksyszyn & Subotkowska, 1980), while alkaline hydrolysis requires a high pH in order to convert the phenol leaving group into a phenolate ion (pK_a of phenol 9.89). At neutral pHs of 5.0–8.0, the phosphonate diphenyl esters are relatively inert to hydrolysis and nucleophilic substitution reactions at phosphorus. For this reason, the enzyme's catalytic apparatus must be used to activate the phosphorus atom for a substitution reaction to occur with the active site Ser-195 (Figure 1).

Nucleophilic substitution reactions occur at the phosphorus atom through pentavalent intermediates, where the substituents attached to the phosphorus atom have a trigonal-bipyramidal geometry. The attacking and leaving groups can be apical–apical or less likely apical–equatorial. The angles of the attacking group–phosphorus atom–leaving group are 180° and 90°, respectively. In the transition state for serine protease catalyzed hydrolysis of natural peptide substrates, the angle between the attacking nucleophile and the leaving group is 109° (tetrahedral carbon). Thus, the respective transition states differ by 71° when the apical–apical phosphorus geometry is compared to that of the tetrahedral intermediate or 19° for the less likely apical–equatorial geometry. This geometric difference would not be accepted by a phosphorus atom in a phosphonic–amide-type inhibitor which normally fits quite well into the enzyme active site, especially at the leaving group site. Additional distortions would also be expected to occur around the carbon–phosphorus bond (phosphonic–amide) relative to the carbonyl–α-carbon bond (peptide) due to the geometric difference between a trigonal bipyramid and a tetrahedron. Thus, (α-aminoalkyl)phosphonic acid amides are poor inhibitors of serine proteases even though they appear to be close analogues of peptides.

The electrophilicity of the phosphorus atom is also significantly decreased by the presence of a phosphonamidate or phosphonic acid alkyl ester functional group which decreases the reactivity toward nucleophiles and results in low inactivation rate constants toward serine proteases. Additionally, phosphoramides, unlike carboxylic acid amides, prefer the cis conformation around PO–NH bond (du Plessis et al., 1982). This further decreases the similarity between a phosphonate inhibitor and a peptide substrate. However, this may not be a significant effect with phosphoramides since the isomerization around the P–N bond is facilitated by protonation on nitrogen (Emsley & Williams, 1973).

The leaving group in peptidyl (α-aminoalkyl)phosphonate diphenyl esters is an electronegative phenoxy group which is necessary to activate the phosphorus atom for nucleophilic substitution. Furthermore, the phenoxy group does not resemble the P_1' residue of a substrate and should not interact specifically with the S_1' pocket. The lack of specific interaction of the phenoxy group with the S_1' subsite should thus not restrict the approach to the TS for nucleophilic substitution on the phosphorus atom for phosphonates in contrast to peptide-related phosphonamides. The addition of electronegative substituents on the phenoxy leaving group has been shown to increase the reactivity of the inhibitor (Fastrez et al., 1989).

Specificity and Reactivity. A good interaction with the S_1 pocket of the enzyme is required for inactivation of serine proteases by diphenyl [α-(*N*-benzyloxycarbonylamino)alkyl]phosphonates (Table I). An excellent example is HL elastase, where the inhibition rate constants clearly depend on the size of the alkyl side chain. For Z-HN-CH(R)P(O)$(OPh)_2$ derivatives, the order of reactivity is 10 M^{-1} for Me [Z-Ala$^P(OPh)_2$, **1**] and *i*-PrCH_2 [Z-Leu$^P(OPh)_2$, **4**], 50 M^{-1} s^{-1} for *n*-Pr [Z-Nva$^P(OPh)_2$, **2**], and 280 M^{-1} s^{-1} for *i*-Pr [Z-Val$^P(OPh)_2$, **3**]. The most reactive is the valine **3** analogue, and that is in good agreement with the specificity of HLE toward *p*-nitroanilide substrates where valine derivatives have higher k_{cat}/K_M values than the Ala analogues (Stein, 1985). In the case of chymotrypsin interaction with peptide aldehydes (transition-state analogue inhibitors), the binding free energy is almost 3 kcal mol^{-1} greater when the side chain is an aromatic benzyl group than when it is a hydrogen (Thompson & Bauer, 1979). We observed a similar trend with diphenyl [α-(*N*-benzyloxycarbonylamino)alkyl]phosphonates (Table I) where the Phe analogue was 15–40-fold better than amino acid

analogues without the aromatic side chain.

All tri- and tetrapeptide derivatives of diphenyl (α-aminoalkyl)phosphonates are better inhibitors than the simple benzyloxycarbonyl derivatives (Table II). A 1200-fold increase in inhibitory potency was observed in the best case [e.g., Z-Val^P(OPh)$_2$ versus Boc-Val-Pro-Val^P(OPh)$_2$ inhibition of PPE]. The dipeptides were poorer inhibitors than the tri- or tetrapeptide phosphonates, which suggests that interaction with the extended substrate binding site of the enzyme and the S_3 subsite, in particular, is essential for effective inhibition of serine proteases. In the case of HL elastase, the full catalytic power of the enzyme is only achieved with substrates which can interact with the S_3 subsite of the enzyme (Stein et al., 1987a). It is likely that an interaction at S_3 is communicated to the catalytic residues via a series of hydrogen bonds (Bode et al., 1989).

The most specific and reactive peptide phosphonate inhibitors for a particular serine protease have sequences identical with those of the best *p*-nitroanilide peptide substrates. The best inhibitor for chymotrypsin-like enzymes is Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**), which has inhibition rate constants of 44 000, 36 000, and 15 000 M^{-1} s^{-1}, respectively, with bovine chymotrypsin, cathepsin G, and RMCP II. This sequence is based on the good cathepsin G and RMCP II substrate Suc-Val-Pro-Phe-NA, which was determined by subsite mapping (Tanaka et al., 1985; Powers et al., 1985). This phosphonate (**21**) does not inhibit the elastases. The best inhibitor for HL and PP elastases is Boc-Val-Pro-Val^P(OPh)$_2$, which has inhibition rate constants of 11 000 and 27 000 M^{-1} s^{-1}, respectively. This sequence is derived from the sequence of an excellent peptide trifluoromethyl ketone inhibitor of HL elastase (Stein et al., 1987b), and the phosphonate does not inhibit chymotrypsin or the chymases.

Stereochemistry. All the precursor diphenyl (α-aminoalkyl)phosphonates which were used in the synthesis were racemic mixtures, and it is probable that only one enantiomer of each phosphonate product can inhibit the enzyme. Experiments with Suc-Val-Pro-Phe^P(OPh)$_2$ (**21**) showed that the correct diastereomer reacted with chymotrypsin in a 1:1 ratio with a second-order rate constant of 146 000 M^{-1} s^{-1}. The other isomer gave less than 5% inhibition after 1 h, and from a practical point of view, we consider it nonreactive.

The tetrapeptide inhibitors were synthesized by coupling tripeptides with (α-aminoalkyl)phosphonate diphenyl esters while the di- and tripeptide derivatives were synthesized by stepwise elongation. Coupling of long peptides results in greater kinetic control during the reaction, and thus the stepwise coupling reactions gave equal amounts of the two diastereomers while couplings involving the longer peptides resulted in nonequivalent amounts of diastereomers. Only one diastereomer, the one in the which ^{31}P NMR signal is shifted downfield, reacts with enzyme. In the case of the tetrapeptide inhibitors **14** and **16**, the diastereomeric ratio is 1:2.1 and 1:2.5, respectively, and the less abundant isomer is the one which reacts. Thus, if one corrects for the concentration of the reactive isomer, the tetrapeptide phosphonates would have comparable reactivity with the best tripeptide inhibitors.

The α-carbon of the phosphonic acid moiety in the reactive diastereomer should possess the same configuration as an L-amino acid residue. In each case the reactive diastereomer gives a ^{31}P NMR signal further downfield than that of the opposite diastereomer. On the basis of analogy with other irreversible serine protease inhibitors, we assign the L configuration to the (α-aminoalkyl)phosphonate moiety in the reactive inhibitors. Although the absolute configuration of some (α-aminoalkyl)phosphonic acids has been determined previously (Dhawan & Redmore, 1987), use of the ^{31}P NMR chemical shift along with serine protease inhibitor potency should be useful in assigning the absolute configuration of the (α-aminoalkyl)phosphonate moiety in peptide derivatives.

Dephosphonylation. Serine proteases phosphonylated by derivatives of diphenyl (α-aminoalkyl)phosphonates are very stable (Table IV). With elastases no reactivation is observed after several days, but with chymotrypsin the reactivation half-lives are 7.5–26 h. From a mechanistic point of view it is clear that hydrolysis of the phosphonylated serine-195 will not be easy. The tetrahedral phosphorus atom bonded to the serine in the active site is analogous to the tetrahedral intermediate involved in peptide bond hydrolysis and is likely to be stabilized by the enzyme. Interaction of the peptidyl portion of the inhibitor with the S_3 and S_2 subsites of enzyme would hinder formation of the pentacovalent phosphorus intermediate required for hydrolysis. In addition, hydrolysis requires the approach of a water molecule to the tetrahedral phosphorus atom which could be hindered either by the enzyme or the phenoxy group of the inhibitor.

Chymotrypsin inhibited by Z-Phe^P(OPh)$_2$ (**7**) regained 50% of the initial activity after 10 h, but no further dephosphonylation was observed after 2 days. This could be explained by two binding modes, one of which dephosphonylated and the other which did not. Alternately, there may be two mechanisms for the slow hydrolysis reaction in the case of chymotrypsin. The first could be an aging reaction involving hydrolysis of the phenyl ester group to give a stable phosphonylated enzyme (Van der Drift et al., 1985). The second mechanism could involve direct dephosphonylation involving release of active enzyme and the peptidyl (α-aminoalkyl)-phosphonate moiety as a monophenyl ester derivative. This could be resolved by a future ^{31}P NMR study.

^{31}P NMR. Chymotrypsin inhibited by Suc-Val-Pro-Phe^P-(OPh)$_2$ (**21**) shows one broad signal at 25.98 ppm corresponding to the Ser-195 phosphonate ester (Figure 2). The chemical shift of ^{31}P is clearly shifted more than 6 ppm downfield compared to those of the starting inhibitor (19.59 and 18.75 ppm for the two diastereomers). That is in good agreement with theoretical predictions based on empirical correlation between ^{31}P chemical shifts and the O–P–O bond angles (Gorenstein, 1984). Thus, the phosphorus atom in the chymotrypsin–inhibitor complex has a geometry very close to tetrahedral. The ^{31}P chemical shift in the enzyme complex is also similar to the ^{31}P chemical shift in N-substituted dialkyl esters or (α-aminoalkyl)phosphonic acid [camphanyl-HNCH(CH$_2$Ph)P(O)(OEt)$_2$, ca. 24 ppm; Bartlett & Lamden, 1986] where the small dialkyl esters group also allows the phosphorus atom to take an ideal tetrahedral geometry. In diphenyl esters, the two bulky phenyl groups require a large O–P–O angle. Thus, the ^{31}P signal appears at less than 20 ppm and is shifted upfield compared to simple dialkyl phosphonates or the chymotrypsin–inhibitor complex.

Conclusion. In summary, peptidyl derivatives of diphenyl (α-aminoalkyl)phosphonates are specific and potent irreversible inhibitors of serine proteases, are chemically stable, are stable in plasma, do not react with acetylcholinesterase, and form very stable derivatives with the enzymes. The significant specificity observed with derivatives of diphenyl (α-aminoalkyl)phosphonate indicates that specific interaction with the correct serine protease is required before activation can occur to give nucleophilic substitution on the phosphorus atom. The stabilized pentavalent phosphorus intermediate in the reaction yields a tetrahedral phosphonylated enzyme which resembles

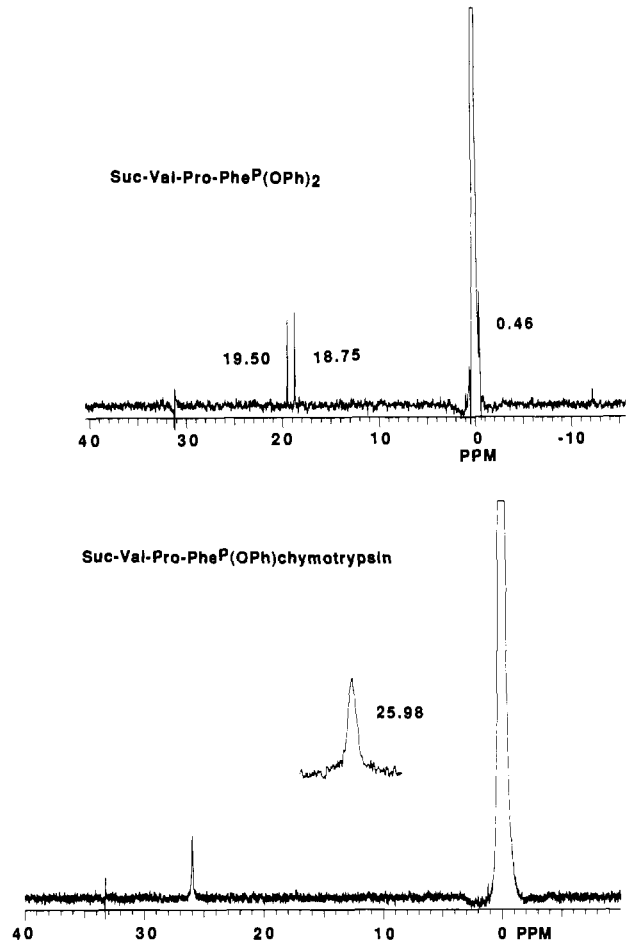

FIGURE 2: ^{31}P NMR spectra of Suc-Val-Pro-Phe^P(OPh)$_2$ (top) and its complex with chymotrypsin (bottom). The enzyme was inhibited at pH 7.5, and the spectrum was measured in an acetate buffer (0.4 M NaOAc, 1 M NaCl, pH 5.5) containing 15% (v/v) D_2O. Phosphoric acid was used as a standard (0 ppm).

the tetrahedral intermediate formed during hydrolysis of peptide substrates. For these reasons phosphonate inactivators of serine proteases have considerable utility as therapeutic agents.

Registry No. **1**, 116222-65-2; **2**, 130727-16-1; **3**, 125882-44-2; **4**, 90763-52-3; **5**, 130699-13-7; **6**, 130727-17-2; **7**, 104130-96-3; **8**, 82818-44-8; **9** (isomer 1), 130699-14-8; **9** (isomer 2), 130699-22-8; **10**, 122299-42-7; **11**, 130727-18-3; **12**, 130727-19-4; **13**, 130727-20-7; **14**, 122299-43-8; **15**, 130699-15-9; **16**, 122299-44-9; **17**, 130699-16-0; **18**, 130699-17-1; **19**, 130727-21-8; **20**, 122299-45-0; **21**, 130727-22-9; Z-Pro-OH, 1148-11-4; Z-Ala-Ala-OH, 16012-70-7; MeO-Suc-Ala-Ala-Ala-OH, 130727-23-0; MeO-Suc-Ala-Ala-Pro-OH, 72252-95-0; Boc-Val-OH, 13734-41-3; Z-Phe-OH, 1161-13-3; Z-Val-OH, 1149-26-4; HBr·H_2N-CH($CH_2CH_2SCH_3$)P(O)($OPh)_2$, 130699-19-3; H-Pro-Phe^P($OPh)_2$ (isomer 1), 130699-20-6; H-Pro-Phe^P($OPh)_2$ (isomer 2), 130699-23-9; H-Pro-Val^P($OPh)_2$, 130699-21-7; RMCP, 37259-58-8; diphenyl (α-amino-2-methylpropyl)phosphonate, 88023-98-7; diphenyl (α-amino-2-phenylethyl)phosphonate, 88024-01-5; diphenyl (α-aminobutyl)phosphonate, 130699-18-2; diphenyl (α-amino-3-methylbutyl)phosphonate, 88023-99-8; succinic anhydride, 108-30-5; chymotrypsin, 9004-07-3; elastase, 9004-06-2.

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