J. Enzyme Inhibition, 1994, Vol. 8, pp. 147–158
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α-AMINOALKYLPHOSPHONATE DI(CHLOROPHENYL) ESTERS AS INHIBITORS OF SERINE PROTEASES

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(Received 16 May 1994)

α-Aminoalkylphosphonate di(chlorophenyl) esters and (α-aminoalkyl)phenylphosphinate phenylesters have been tested as irreversible inhibitors of human neutrophil elastase, porcine pancreatic elastase and chymotrypsin, serine proteases important in biochemical processes. Peptidyl derivatives of diphenyl (α-aminoalkyl) phosphonates have previously been shown to be potent and specific inhibitors of serine proteases at low concentrations. Addition of a halogen to the phenoxy group of the inhibitors should make the leaving group more electrophilic, and thus more reactive. Peptide phosphonate inhibitors with chlorine in the meta- or para- positions of the phenoxy ester moiety were synthesized and shown to be potent inhibitors of elastase. Tripeptide phosphonates are more potent inhibitors than dipeptide phosphonates, however, addition of the halogen did not increase the inhibitory potency of these phosphonates with elastase compared to the non-halogenated phosphonates. In the case of chymotrypsin, the halogenated phenoxy esters were more reactive, possibly due to an alternate binding mode. The novel (α-aminoalkyl)phenylphosphinate phenylesters were poor inhibitors of serine proteases.

KEY WORDS: Serine protease inhibitors, phosphonate inhibitors

INTRODUCTION

The serine protease superfamily is composed of more than 100 enzymes, including enzymes such as elastase and chymotrypsin, which play important roles in biochemical processes. Elastase is responsible for the abnormal turnover of connective tissue proteins associated with pulmonary emphysema, rhematoid arthritis and other inflammatory diseases, and is necessary for phagocytosis and the defense against infection by invading microorganisms.[1] Human neutrophil elastase (HNE)[2] is found in the dense granules of polymorphonuclear leukocytes, while pancreatic elastase (PPE) is found in the pancreas as an inactive zymogen which is activated by trypsin.[3] Both PPE and HNE cleave substrates at P_1-P'_1[4] scissile bonds where P_1 is an amino acid residue with a small alkyl side chain such as Ala or Val, while chymotrypsin cleaves after aromatic residues. The crystal structures of both elastases and chymotrypsin have been determined.[3]

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Serine proteases are irreversibly inhibited by 3,4-dichloroisocoumarins,[5] sulfonyl fluorides,[6] azapeptides,[6] and peptide chloromethyl ketones.[6] They are reversibly inhibited by peptide α-ketoesters,[6] benzamidines,[6] boronic acids,[7] and peptide aldehydes and ketones.[7] The mechanisms of action of these and other inhibitors with serine proteases have been reviewed.[6,7]

A number of phosphorus-containing compounds have previously been reported as inhibitors for serine proteases. Diisopropylphosphofluoridate as the classic general inhibitor of serine proteases, is often used to diagnose new serine proteases, and reacts with the active site serine to form a stable phosphonyl derivative of serine proteases. Peptidyl phosphonyl fluorides and phosphonamides are effective serine protease inhibitors, however, they are often unstable in aqueous solution and are frequently reactive toward acetylcholinesterase. Peptidyl derivatives of α-aminoalkylphosphonate diphenyl esters are also specific and effective irreversible inhibitors of serine proteases at low concentrations.[8] In contrast to other organophosphorus inhibitors, these compounds are chemically stable, easily synthesized, unreactive toward acetylcholinesterase and valuable for *in vivo* studies. These phosphonates form stable phosphonyl derivatives with serine proteases due to covalent bond formation between the active site serine and the α-aminoalkylphosphonate diphenyl esters (Figure 1). The two phenoxy leaving groups are essential for activation of the phosphorous atom for nucleophilic substitution with the active site serine and a correct sequence is necessary to obtain effective inhibition of the targeted serine proteases.[8]

We report here the synthesis of peptidyl derivatives of α-aminoalkylphosphonate di(chlorophenyl) esters and phenylphosphinate esters (Figure 2) and their specific inhibitory potency toward PPE, HNE and ChT. We expected the chlorophenyl ester derivatives to be more effective serine protease inhibitors due to their increased electrophilicity and hoped to expand the class of peptide phosphorus inhibitors to include peptide phenylphosphinate esters.

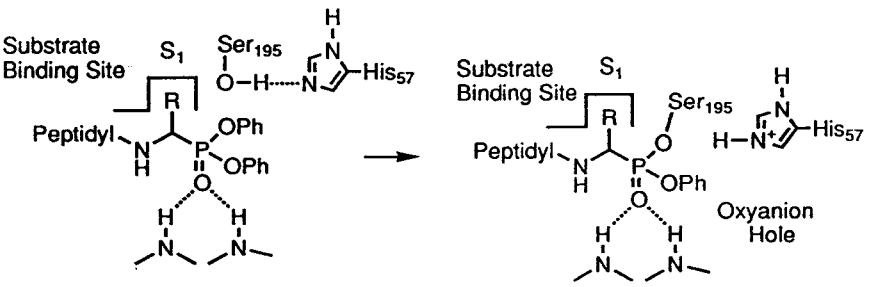

FIGURE 1 Reaction of a peptide phosphonate inhibitor with the active site of a serine protease. The inhibitor binds to the extended substrate binding site of the enzyme (left) and then the serine reacts to give a phosphonyl enzyme (right). One phosphonyl oxygen interacts with the oxyanion hole of the inhibited enzyme. It is likely that the second phenoxy group is slowly lost by hydrolysis in an "aging" reaction.

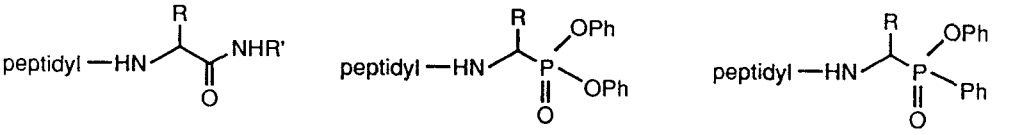

FIGURE 2 Comparison of the structure of a peptide substrate (left) for a serine protease with a peptide phosphonate inhibitor (center) and a peptide phenylphosphinate inhibitor (right). The phosphinate inhibitor is an analog of a phosphonate inhibitor where one phenoxy group is replaced by a phenyl group.

MATERIALS AND METHODS

Synthesis

Benzyl carbamate, isobutyraldehyde, dichlorophenyl phosphine, dicyclohexylcarbodiimide (DCC), and all common chemicals and solvents were obtained from Aldrich Co., Milwaukee, WI. The purity of each new synthesized compound was checked by ^{1}H-NMR, mass spectroscopy (FAB), TLC, and elemental analysis. In multi-step syntheses, the first and final compounds were checked by a combination of NMR, FAB mass spectra and elemental analysis. Z-Valine had the L-configuration and was obtained from Aldrich Co. Z-Proline had the L-configuration and was obtained from Bachem, San Diego, California. The solvent system used for TLC was chloroform-acetone (9:1). Preparative thin-layer chromatography was done with plates precoated with silica gel (Merck 5717). The NMR spectra were recorded on a Varian Gemini 300 MHz instrument in $DMSO_{d6}$, $CDCl_3$ or D_2O solutions. Mass spectra (FAB) were measured on a VG Instrument 70-SE (8KV, Xe atom, 1 mA ion current). Elemental analysis were performed by Atlantic MicroLab Inc., Norcross, GA.

The tris (*p*- or *m*-chlorophenyl) phosphites were prepared from corresponding chlorophenols and phosphorous trichloride, using three equivalents of triethylamine as a base by modification of a previously described procedure.[9] The method of Walsh was used for the preparation of tris(chlorophenyl) phosphites.[10] The diphenyl phenylphosphonite was made from dichlorophenyl phosphine, phenol, and two equivalents of triethylamine.[9]

Di(p-chlorophenyl) 1-Benzyloxycarbonylamino-2-methylpropanephosphonate [Z-NH-CH(i-Pr)PO(OPh-pCl)$_2$, **1***].* This compound was prepared by a slight modification of an earlier procedure.[11] Benzyl carbamate (7.6 g, 50 mmol), isobutyraldehyde (5.6 g, 77 mmol) and benzene (50 ml) were refluxed for 1 hour. The benzene was evaporated, and tris (*p*-chlorophenyl) phosphite (23 g, 50 mmol) was added to the residue, followed by addition of 10 ml glacial acetic acid. The mixture was stirred for 10 min, then heated at 80–90°C for 2 h. The volatile materials were removed under reduced pressure, and the oily residue was diluted with 80 ml methanol then refrigerated. After 24 h, the precipitated solid was collected by filtration and dried. The product was recrystallized from methanol to give white crystals: yield, 34%; mp 92–94°C; ^{1}H-NMR ($CDCl_3$), δ = 1.08 (dd, 6H, CH_3), 2.38 (1H, CH), 4.40 (1H,

CH-P), 5.10 (q, 2H, CH_2O), 5.35 (d, 1H, NH), 7.0 - 7.4 (m, 13H); MS [Cl^+] = 508.1. Anal. calcd. for $C_{24}H_{24}NO_5PC_2 \cdot 0.5\ H_2O$: C, 55.72; H, 4.79; N, 2.71; Cl, 13.71. Found: C, 55.99; H, 4.60; N, 2.47; Cl, 13.73%.

Di(p-chlorophenyl) 1-Amino-2-methylpropanephosphonate Hydrochloride Salt [H_2N-CH(i-Pr)PO(OPh-pCl)$_2$· HCl, 2]. Hydrogenolysis of **1** gave the hydrochloride **2**. The hydrogenolysis was performed by dissolving 5.1 g (10 mmol) of **1** in 150 ml methanol and 0.82 ml (10 mmol) concentrated HCl and adding 1 g 5% Pd/C catalyst. The mixture was stirred and hydrogenated (normal pressure) until the consumption of H_2 ceased. The catalyst was removed by filtration, and the filtrate evaporated to give a white solid. The crude product was recrystallized from ether-methanol to give white crystals: yield, 95% (crude product); mp 166–9°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 1.15 (dd, 6H), 2.4 (m, 1H, CH), 4.1 (dd, 1H, CH-P), 7.1–7.5 (m, 8H), 9.2 (bs, 3H, NH_3).

Di(m-chlorophenyl) 1-Benzyloxycarbonylamino-2-methylpropanephosphonate [Z-NH-CH(i-Pr)PO(OPh-mCl)$_2$, 3]. This *m*-chlorophenyl ester was synthesized following the procedure described for the synthesis of **1**, using tris (*m*-chlorophenyl) phosphite: yield, 32%; mp 107.5–109°C ^{1}H-NMR ($CDCl_3$), δ = 1.1 (m, 6H, CH_3), 2.4 (m, 1H, CH), 4.45 (dd, 1H, CH-P), 5.1 (s, 2H, OCH_2), 5.25 (d, 1H, NH), 7.0–7.4 (m, 13H); MS (FAB) = 509 (M^+ + 1). Anal. calcd for $C_{24}H_{24}NO_5PCl_2$: C, 56.71; H, 4.76; N, 2.76; Cl, 13.95. Found: C, 56.69; H, 4.78; N, 2.71; Cl, 13.88%.

Di(m-chlorophenyl) 1-Benzyloxycarbonylamino-2-methylpropanephosphonate Hydrochloride Salt [H_2N-CH(i-Pr)PO(OPh-mCl)$_2$·HCl, 4]. Hydrogenolysis was done as described above for **2**. A white, somewhat hygroscopic solid was obtained: yield, 73%; mp 110–4°C; ^{1}H-NMR ($CDCl_3$), δ = 1.3 (m, 6H, CH_3), 2.65 (m, CH, 1H), 3.7 (m, 1H, CH-P) 7.05–7.5 (m, 8H), 9.50 (bs, 3H, NH_3). The product was used directly in the next step.

It was found that during hydrogenolysis of the Z-phosphonates (**1** and **3**), undesired replacement of the chlorine atoms (in the aromatic esters) by hydrogen occurred. During extended hydrogenolysis, the product contained less halogen on the aromatic ring than expected. This phenomenon has been previously described.[12] For this reason an alternative method for removing the Z-group was used (30% HBr in acetic acid).

Di(p-chlorophnyl) 1-Amino-2-methylpropanephosphonate Hydrobromide Salt [H_2N-CH(i-Pr)PO(OPh-pCl)$_2$· HBr, 5]. The phosphonate **1** (0.76 g, 1.5 mmol) was mixed with 2 ml 30% HBr in acetic acid, and protected against moisture. After 15 min 50 ml ether was added, and the mixture was stirred for 1 h. The resulting crystalline solid was collected by filtration, washed with ether and dried. The product was obtained as a white, crystalline solid: yield, 85% (0.58 g); mp 186–7°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 8.85 (s, 3H, NH^+_3), 7.50 (m, 4H), 7.30 (m, 4H, arom), 4.20 (dd, 1H, CH-P), 2.40 (m, 1H, CH), 1.16 (m, 6H, CH_3). This product wa used directly in the next step.

Di(m-chlorophenyl) 1-Amino-2-Methylpropanephosphonate Hydrobromide Salt [H_2N-CH(i-Pr)PO(OPh-mCl)$_2$· HBr, 6]. The phosphonate **2** (0.76 g, 1.5 mmol) was mixed with 2 ml 30% HBr in acetic acid and kept dry. After 15 min 50 ml dry hexane was added and the mixture was stirred for 15 min. The supernatant solution was decanted, and the remaining oil was washed again with hexane (twice). After decantation of the hexane, the resulting oil was dried *in vacuo*. A brown, extremely hygroscopic solid was obtained, which was used in the next step without additional purification: yield, 89%

(0.62 g); ^{1}-NMR ($DMSO_{d6}$), δ = 8.85 (bs, 3H, NH^+_3), 7.5-7.15 (m 8H, arom), 4.25 (dd, 1H, CH-P), 2.40 (m, 1H, CH), 1.18 (m, 6H, CH_3).

t-Butyloxycarbonyl-(L-valyl)-L-proline [Boc-Val-Pro-OH, **7***].* Boc-valine (1.18 g, 5.4 mmol) and proline benzyl ester hydrochloride (1.31 g, 5.4 mmol) were coupled using DCC in the presence of triethylamine to give Boc-Val-Pro-OBzl, which was used for hydrogenolysis: yield, 70% (1.53 g); ^{1}H-NMR ($CDCl_3$), δ = 7.34 (s, 5H, phenyl), 5.20 (d, 1H, NH), 5.15 (d, 2H, CH_2), 4.60 (m, 1H), 4.25 (m, 1H), 3.8-3.6 (m, 2H), 2.20 (m, 1H), 2.1-1.9 (m, 4H), 1.43 (s, 9H), 1.0 (d, 3H), 0.9 (d, 3H).

Hydrogenolysis with 5% Pd/C in 90% aqueous methanol produced a white crystalline solid (recrystallization from from methanol): yield, 94%; mp 140–2°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 6.80 (d, 1H, NH), 4.25 (m, 1H), 4.0 (t, 1H), 3.75–3.50 (m, 2H), 2.15 (m, 1H), 2.0–1.8 (m, 4H), 1.38 (s, 9H), 0.95–0.75 (dd, 6H). Anal. calcd. for $C_{15}H_{26}N_2O_5$: C, 57.30; H, 8.34; N, 8.91. Found: C, 57.20; H, 8.39; N, 8.87%.

Benzyloxycarbonyl-(L-valyl)-L-proline Benzyl Ester [Z-Val-Pro-OBzl, **8***]:* Yield, 76% (2.4 g); ^{1}H-NMR ($DMSO_{d6}$), δ = 7.55 (d, 1H, NH), 7.35 (bs, 10H), 5.12 (s, 2H, CH_2), 5.05 (q, 2H, CH_2), 4.40 (dd, 1H), 4.05 (t, 1H), 3.8–3.5 (m, 2H), 2.2 (m, 1H), 2.0–1.8 (m, 4H), 0.87 (m, 6H).

Hydrogenolysis gave Val-Pro-OH as a white solid: yield, 88% (1.05 g); mp 145–6°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 4.3 (dd, 1H), 3.6–3.2 (m, 3H), 2.2–2.0 (m, 3H) 1.95–1.5 (m, 4H), 0.94 (d, 3H), 0.84 (d, 3H).

The deprotected dipeptide Val-Pro-OH (1.02 g, 4.76 mmol) was dissolved in 10 ml water containing 1.24 g sodium carbonate. The solution was cooled in an ice bath, stirred, and then benzyl chloroformate (1.0 g, 5.6 mmol) was added. The mixture was stirred for 1 hour at 5°C, and then extracted with 20 ml of ether. The aqueous layer was separated and acidified with hydrochloric acid (20 mmol added). An oil separated, which was extracted with methylene chloride. The extract was dried ($MgSO_4$), filtered and evaporated to give the crude product (Z-Val-Pro-OH) as a thick oil, which solidified after drying: yield, 59% (0.97 g); mp 80–5°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 7.5 (d, 1H), 7.35 (bs, 5H), 5.02 (q, 2H), 4.25 (dd, 1H), 4.05 (t, 1H), 3.8-3.5 (m, 2H), 2.15 (m, 1H), 2.0-1.7 (m, 4H), 0.92 (m, 6H). Anal. calcd. for $C_{18}H_{24}N_2O_5$: C, 62.05; H, 6.94; N, 8.04. Found: C, 61.95; H, 6.99; N, 8.08%.

Tripeptide Phosphonate Synthesis (General Procedure)

The hydrobromide of the phosphonate (compound **5** or **6**, 1.0 mmol, 0.46 g) and triethylamine (1.0 mmol, 0.1 g) were dissolved in 15 ml CH_2Cl_2, and the solution was cooled to 0°C in an ice bath. Separately, a solution of the protected dipeptide (**7** or **8**) was prepared in 15 ml CH_2Cl_2, cooled to 0°C and then DCC (1.0 mmol, 0.21 g) was added with stirring. Both solutions were mixed together and stirred at 0°C for 2 h, then 20 h at room temperature. The DCU formed from this reaction was removed by filtration and the filtrate was evaporated. The residue was dissolved in 50 ml ethyl acetate, filtered again, and the filtrate was washed with 50 ml 6% $NaHCO_3$, 1 M HCl (0.05 M HCl was used with the Boc derivative), and water. The organic layer was dried ($MgSO_4$), filtered and evaporated to give the products which were purified by crystallization from hexane-ether, and dried *in vacuo*.

Di-(p-chlorophenyl)1-(Benyloxycarbonyl-L-valyl-L-prolylamino)-2-methylpropane-phosphonate [Z-Val-Pro-Val^P (OPh-pCl)$_2$, **9***].* This product was obtained as a glass-like solid; yield, 87%; mp 65–70°C; ^{1}H-NMR ($CDCl_3$), δ = 8.5 (dd, 1H), 7.5–7.1 (m, 13H), 5.02 (q, 2H), 4.6 (m, 1H), 4.5 (m, 1H), 4.05 (m, H), 3.85–3.5 (m, 2H), 2.25 (m, 1H), 2.0-1.5 (m, 4H), 1.02 (m, 6H), 0.92 (m, 6H); MS (FAB) = 704 $(M + 1)^+$. Anal. calcd. for $C_{34}H_{40}N_3O_7PCl_2 \cdot 0.25$ DCU: C, 58.81; H, 6.10; N, 6.45; Found; C, 58.72; H, 6.34; N, 6.48%.

Di-(m-chlorophenyl) 1-(Benzyloxycarbonyl-L-valyl-L-prolylamino)-2-methylpropane-phosphonate [Z-Val-Pro-Val^P(OPh-mCl)$_2$, **10***].* The product was obtained as a glass-like solid: yield, 66%; mp 50–5°C; ^{1}H-NMR ($CDCl_3$), δ = 8.55 (dd, 1H), 7.5–7.1 (m, 5H), 5.05 (m, 2H), 4.6 (m, 1H), 4.5 (m, 1H), 4.1 (m, 1H), 3.9–3.5 (m, 1H), 2.3 (m, 1H), 2.0–1.4 (m, 4H), 1.1–0.8 (m, 12H); MS (FAB) = 704 $(M + 1)^+$. Anal. calcd. for $C_{34}H_{40}N_3O_7PCl_2 \cdot 0.2$DCU: C, 58.65; H, 6.03; N, 6.35; Cl, 9.46. Found: C, 58.51; H, 6.05; N, 6.31; Cl, 9.68%.

Di(p-chlorophenyl)1-(t-Butyloxycarbonyl-L-valyl-L-prolylamino)-2-methylpropane-phosphonate [Boc-Val-Pro-Val^P(OPh-pCl)$_2$, **11***].* The product was obtained as a glass-like solid: yield, 80%; mp 66–70°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 8.85 (dd, 1H), 7.45 (m, 4H), 7.25 (m, 4H), 6.9–6.7 (m, 1H), 4.7–4.5 (m, 2H); 4.1–3.5 (m, 3H); 2.25 (m, 1H), 2.0–1.5 (m, 4H), 1.36 (d, 9H), 1.1–0.8 (m, 12H); MS (FAB) = 670 $(M + 1)^+$. Anal. calcd. for $C_{31}H_{42}N_3O_7PCl_2 \cdot 0.25$DCU: C, 56.61; H, 6.66; N, 6.75; Cl, 9.7. Found: C, 56.89; H, 6.74; N, 6.76; Cl, 9.79%.

Di(m-chlorophenyl) 1-(t-Butyloxycarbonyl-L-valyl-L-prolyl)Amino-2-methylpropane-phosphonate [Boc-Val-Pro-Val^P(OPh-mCl)$_2$, **12***].* The product was obtained as a glass-like solid; yield, 65% mp 60–5°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 8.85 (dd, 1H), 7.5–7.1 (m, 8H), 7.75 (m, 1H), 4.6–4.4 (m, 2H), 4.0–3.4 (m, 3H), 2.25 (m, 1H), 2.0–1.5 (m, 4H), 1.36 (s, 9H), 1.1–0.8 (m, 12H); MS (FAB) = 670 $(M + H)^+$. Anal. calcd. for $C_{31}H_{42}N_3O_7PCl_2 \cdot 0.25$DCU: C, 56.61; H, 6.66; N, 6.75; Cl, 9.76. Found: C, 56.94; H, 6.71; N, 6.73; Cl, 9.70%.

The Boc-group was removed from **11** and **12** using dry HCl in methylene chloride.

Di(p-chlorophenyl) 1-(L-Valyl-L-prolylamino)-2-methylpropanephosphonate Hydrochloride Salt [Val-Pro-Val^P(OPh-pCl)$_2$·HCl, **13***].* The Boc-tripeptide **11** (0.268 g, 0.4 mmol) was dissolved in 5 ml CH_2Cl_2 and cooled to -10°C. The solution was saturated with dry HCl at -10°C, and allowed to stand for 1 h. Then, all volatile materials were removed under reduced pressure and the resulting solid was dried *in vacuo*. A crystalline solid was obtained: yield, 95%; mp 105–10°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 8.75 (d, 1H), 8.3 (m, 3H), 7.5–7.1 (m, 8H), 4.7–4.4 (m, 2H), 3.8–3.4 (m, 3H), 2.3–1.5 (m, 5H), 1.1–0.8 (m, 12H); MS (FAB)= 570 $(M + H - Cl)^+$.

Di(m-chlorophenyl) 1-(L-Valyl-L-prolylamino)-2-methylpropanephosphonate Hydrochloride Salt [Val-Pro-Val^P(OPh-mCl)$_2$·HCl, **14***].* Starting from 0.134 g (0.2 mmol) of **12**, and following the procedure for **13**, the product **14** was obtained as a white solid: yield, 98%; mp 85–90°C; ^{1}H-NMR ($DMSO_{d6}$), δ = 8.7 (d, 1H), 8.21 (m, 3H), 7.5–7.1 (m, 8H), 4.7–4.5 (m, 2H), 3.8–3.4 (m, 3H), 2.3–1.5 (m, 5H), 1.1–0.8 (m, 12H); MS (FAB)= 570 $(M + H - Cl)^+$.

Phosphinic Valine Analogs and the Corresponding Peptides

The phenyl (1-benzyloxycarbonylamino-2-methylpropyl)phenylphosphinate (phosphinic analog of valine), **15**, is a new compound and its characterization is described below.

Phenyl (1-Benzyloxycarbonylamino-2-methylpropyl)phenylphosphinate [Z-Val^{P}(Ph)OPh), **15***].* Starting from isobutyraldehyde, benzyl carbamate and diphenyl phenylphosphonite, and following the procedure described or synthesis of **1**, the phenyl (1-benzyloxycarbonylamino-2-methylpropyl)phenylphosphinate **15** was obtained as a white solid: yield, 20%; mp 163-4°C; ^{1}H-NMR ($CDCl_3$), δ = 0.95–1.05 (dd, 6H, CH_3), 2.10 (m, 1H, CH), 4.30 (m, 1H, CH-P), 5.10 (q, 2H, OCH_2), 5.50 (dd, 1H NH), 7.33 (s, 5H, phenyl), 7.0-7.9 (m, 10H, arom.); MS (FAB) = 424 $(M + 1)^+$. Anal. calcd. for $C_{24}H_{26}NO_4P$: C, 68.07; H, 6.19; N, 3.31. Found: C, 68.8; H, 6.18; N, 3.40%.

Hydrogenolysis of **15**, under usual conditions, afforded the hydrochloride **16**, phenyl(1-amino-2-methylpropyl)phenylphosphinate as a white solid: yield, 87%; mp 186-8°C; ^{1}H-NMR (D_2O), δ = 0.9 (dd, 6H, CH_3), 2.12 (m, 1H, CH), 3.98 (dd, 1H, CH-P), 7.0-7.8 (m, 10H, arom.).

Phenyl (1-Benzyloxycarbonyl-L-prolylaino-2-methylpropyl)phenylphosphinate [Z-Pro-Val^{P}(Ph)(OPh), **17***].* The reaction of **16** with Z-proline, applying the procedure for peptide synthesis, afforded dipeptide **17** as a glass-like solid: yield, 71%; mp 50-55°C; ^{1}H-NMR ($CDCl_3$), δ = 0.9 (m, 6H, CH_3) 1.5–2.5 (m, 5H), 3.3–3.8 (m, 2H), 4.5–4.7 (m, 1H), 5.2 (m, 2H, OCH_2), 7.0–7.5 (m, 15H, arom.), 7.8 (m, 1H, NH); MS (FAB) = 521 $(M + H)^+$.

Phenyl(1-Benzyloxycarbonyl-L-prolylamino-2-mehylpropyl)phenylphosphinte Hydrochloride Salt [Pro-Val^{P}(Ph)(OPh)·HCl, **18***].* Hydrogenolysis of the dipeptide **17**, gave hydrochloride **18** as a white solid: yield, 84%; mp 70-80°C; ^{1}H-NMR (D_2O), δ = 0.9 (m, 6H, CH_3), 1.5–2.0 (m, 4H), 2.2 (m, 1H), 3.25 (m, 2H), 3.4 (m, 1H), 4.4 (m, 1H, CH-P), 7.0–7.2 (m, 5H arom.), 7.4–7.8 (m, 5H, arom).

Phenyl (1-Benzyloxycarbonyl-L-valyl-L-prolylamino-2-methylpropyl)phenylphosphinate [Z-Val-Pro-Val^{P}(Ph)(OPh), **19***].* The reaction of **18** and Z-valine, following the procedure for peptide synthesis, afforded the tripeptide **19** as a glass-like solid: yield, 71%; mp 60–70°C; ^{1}H-NMR ($CDCl_3$), δ = 0.9–1.1 (m, 12H), 1.5–2.5 (m, 6H), 3.4–4.0 (m, 3H), 4.3–4.8 (m, 2H), 5.1 (m, 2H, OCH_2), 5.5 (dd, 1H, NH), 7.35 (S, 5H, phenyl), 7.0–7.9 (m, 10H, arom.); MS (FAB) = 620 $(M + 1)^+$. Anal. calcd. for $C_{34}H_{42}N_3O_6P \cdot 0.5\ H_2O \cdot 0.2$ DCU: C, 65.26; H, 7.15; N, 7.08. Found: C, 65.37; H, 7.25; N, 7.60%.

Phenyl (1-Benzyloxycarbonyl-L-valyl-L-prolylamino-2-methylpropyl)phenylphosphinate Hydrochloride Salt [Val-Pro-Val^{P}(Ph)(OPh)·HCl, **20***].* Hydrogenolysis of **19**, gave the hydrochloride **20** as a white solid: yield, 95%; mp = 150–5°C; ^{1}H-NMR (D_2O), δ = 0.8–1.05 (m, 12H), 1.5–2.5 (m, 6H), 3.4–3.7 (m, 3H), 4.1 (t, 1H), 4.3–4.6 (m, 1H), 7.0–7.8 (m, 10H); MS (FAB) = 486 $(M - Cl)^+$.

Enzymes

Human Neutrophil Elastase was obtained from Athens Research and Technology, Inc., Athens, Georgia. Porcine pancreatic elastase was obtained from CalBiochem

San Diego, California. Chymotrypsin was obtained from Sigma Chemicals, St. Louis, Missouri. HEPES was purchased from Research Organics, Inc., Cleveland, Ohio.

Enzyme Inactivation-Incubation Method

Inactivation reactions were initiated by adding 25–50 μl of the inhibitor (0.5-5 mM in Me_2SO) to 0.2.5-0.50 ml of a buffered enzyme solution (0.1 M HEPES, 0.5 M NaCl, pH 7.5, 25°C. The final concentration of Me_2SO was 5–10% (v/v). Aliquots (25 μl for HNE, 50 μl for PPE and ChT) were removed from the reaction mixture at various time intervals and added to a buffered substrate solution (0.1 M HEPES, 0.5 M NaCl, pH 7.5). Residual enzyme activity was measured spectrophotometrically using a Beckman Model 35 spectrometer for at least two half-lives. PPE was assayed with Suc-Ala-Ala-Ala-NA,[13] HNE with MeO-Suc-Ala-Ala-Pro-Val-NA,[14] and ChT with Suc-Phe-Thr-Phe-NA (k_{cat}/K_M = 27,000 $M^{-1}s^{-1}$). First order inactivation rate constants, k_{obs}, were obtained from plots of ln v_t/v_o vs. time. Inhibitor concentrations are shown in Table 1. Peptide nitroaniline hydrolysis was measured[15] at 410 nm (ϵ = 8800 $M^{-1}cm^{-1}$). Inactivation rate constants shown in the table are typically the average of duplicate or triplicate measurements.

RESULTS & DISCUSSION

Phosphonate inhibitors of serine proteases have previously been reported.[8,16,17] Among these inhibitors, Boc-Val-Pro-Val^P$(OPh)_2$[18] is the most potent inhibitor of both PPE and HNE, with $k_{obs}/[I]$ values of 11,000 $M^{-1}s^{-1}$ and 27,000 $M^{-1}s^{-1}$, respectively. We have synthesized halogenated phosphonate inhibitors that are specific inhibitors of serine proteases. It has been suggested that increasing the electrophilic character of phosphonates should produce more potent inhibitors of serine proteases.[16] The addition of a halogen to the phenoxy ring of a phosphonate diphenyl ester should make these inhibitors more electrophilic and hence more reactive since the halogenated phenol would be an enhanced leaving group.

Synthesis of the Peptides Containing C-Terminal Di(p- or m-chlorophenyl) 1-Amino-2-methylpropanephosphonate, Esters

The benzyloxycarbonyl (Z) derivatives of di(chlorophenyl) 1-amino-2-methylpropanephosphonates were synthesized by the reaction of triphenyl phosphite with benzyl carbamate and isobutyraldehyde in the presence of acetic acid.[11] Racemic di(chlorophenyl) 1-amino-2-methylpropanephosphonates, prepared by hydrogenolysis of the corresponding Z-derivatives were then coupled with N-blocked amino acids, or dipeptides using DCC as a coupling agent to produce the final products.

During the hydrogenolysis of Z-blocked aminophosphonates (using 5% Pd/C) the chlorine atoms in the phosphonic esters were partially replaced by hydrogen atoms since the deblocked aminophosphonates contained less chlorine than expected by microanalysis. Increased amounts of chlorine were lost when the hydrogenolysis was prolonged. To avoid this phenomenon, the Z-group was removed with a

TABLE 1
Rate Constants for Inhibition of Porcine Pancreatic Elastase, Human Neutrophil Elastase and Chymotrypsin by Peptide Phosphonates and Phenylphosphinates[a]

Inhibitor	PPE		HNE		ChT	
	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$
Boc-Val-Pro-Val^P(OPh)$_2$	4.2	11,000	4.2	25,000	42	NI[b]
Z-Val-Pro-Val^P(OPh-*p*Cl)$_2$ (**9**)	42	480	8.3	7000	42	560
Boc-Val-Pro-Val^P(OPh-*p*Cl)$_2$ (**11**)	8.3	4400	4.2	1400	42	50
Val-Pro-Val^P(OPh-*p*Cl)$_2$ (**13**)	42	500	8.3	13,000	42	125
Z-Val-Pro-Val^P(OPh-*m*Cl)$_2$ (**10**)	8.3	490	4.2	5700	42	830
Boc-Val-Pro-Val^P(OPh-*m*Cl)$_2$ (**12**)	8.3	4100	4.2	1100	42	100
Val-Pro-Val^P(OPh-*m*Cl)$_2$ (**14**)	42	590	8.3	9700	42	60
Z-Pro-Val^P(Ph)(OPh) (**17**)	42	NI[b]	42	NI[c]		
Pro-Val^P(Ph)(OPh) (**18**)	42	NI[b]	42	NI[c]		
Z-Val-Pro-Val^P(Ph)(OPh) (**19**)	42	50	42	670		
Val-Pro-Val^P(Ph)(OPh) (**20**)	42	NI[b]	42	NI[c]		

[a] Conditions were as follows: 0.1 M HEPES and 0.5 M NaCl, pH 7.5 at 25°C. Porcine pancreatic elastase was assayed with 20 mM Suc-Ala-Ala-Ala-NA in the HEPES buffer containing ca. 10% DMSO. Human neutrophil elastase was assayed with 20 mM MeO-Suc-Ala-Ala-Pro-Val-NA in the HEPES buffer containing ca. 10% DMSO. Chymotrypsin was assayed with 20 mM Suc-Phe-Thr-Phe-NA in the HEPES buffer containing ca. 10% DMSO. [b]No inhibition after 20 minutes incubation. [c]No inhibition after 10 min incubation.

hydrogen bromide solution in acetic acid (30%). This afforded the desired aminophosphonates as hydrobromides, without the loss of chlorine in the phosphonate esters. The aminophosphonate hydrobromides were then coupled with the dipeptides (Boc-Val-Pro-OH or Z-Val-Pro-OH) to give the final tripeptide products. Removing the Z-group by hydrogenolysis was successfully applied for deblocking non-halogenated Z-blocked aminophosphinates.

We have also synthesized novel peptide phenylphosphinate phenyl ester derivatives of valine (**15**). The phosphinic ester **15** was deblocked by hydrogenolysis and coupled with Z-proline, to give product **17**, which after deblocking and reaction with Z-valine gave the tripeptidyl phenylphosphinate analog **19**. These are new peptidyl analogs containing a phosphinic acid analog of valine. The products were nonequivalent mixtures of stereoisomers. No attempts were made to resolve diastereomers, and no checking was done for racemization during the peptide forming reactions.

Inhibitory Potency

The second order rate constants $k_{obs}/[I]$ for the inactivation of HNE, PPE and ChT by halogenated peptide phosphonates and the novel peptide phenylphosphinates are shown in Table 1. Boc-Val-Pro-Val^P(OPh)$_2$ is included for comparison.[8] In general,

the peptide phenyl phosphonates tested are potent inhibitors of PPE and HNE. In the case of HNE, tripeptides (**9,10,11,12,13,14**) are more potent inhibitors than dipeptides (data not shown); however, inhibitors with a free N-terminus are better inhibitors than those with Boc- or Z-groups in the same position. The strongest inhibitors of HNE are Val-Pro-Val^P(OPh-*p*Cl)$_2$ (**13**) and Val-Pro-Val^P(OPh-*m*Cl)$_2$ (**14**), with k_{obs}/[I] values of 13,000 $M^{-1}s^{-1}$ and 9700 $M^{-1}s^{-1}$, respectively. Inhibitors with Boc- or Z- groups inhibit HNE more slowly than inhibitors with unblocked N-termini.

In the case of PPE, similar trends were observed, except that inhibitors with a Z- or a Boc- group at the N-terminus of the peptide are more potent than inhihitors with a free N-terminus (**13,14**). Tlhe strongest inhibitors of PPE are Boc-Val-Pro-Val^P(OPh-*p*Cl)$_2$ (**11**), and Boc-Val-Pro-Val^P(OPh-*m*Cl)$_2$ (**12**) with k_{obs}/[I] values of 4400 $M^{-1}s^{-1}$ and 4100 $M^{-1}s^{-1}$ respectively. The inhibitors with a free N-terminus, Val-Pro-Val^P(OPh-*p*Cl)$_2$ (**13**), and Val-Pro-Val^P(OPh-*m*Cl)$_2$ (**14**) inhibit PPE very slowly. In general, all the peptide phosphonates were effective inhibitors of HNE and PPE, with the most potent derivatives being the blocked tripeptide *p*-chlorophenyl phosphonates.

In cases where there is a phenyl group in place of a phenoxy group (phenylphosphinate esters), there is little to no inhibition of HNE and PPE. Only Z-Val-Pro-Val^P(Ph)(OPh) (**19**) showed any reactivity toward PPE while inhibiting HNE at a moderate rate (k_{obs}/[I] = 670 $M^{-1}s^{-1}$).

The peptide phenyl phosphenates were less effective inhibitors of ChT in comparison with their effects on HNE and PPE; however, addition of chlorine to the phenoxy group of the inhibitors increased their reactivity toward ChT compared to Boc-Val-Pro-Val^P(OPh)$_2$, which did not inhibit ChT. The best inhibitors of ChT were Z-Val-Pro-Val^P(OPh-*p*Cl)$_2$ (k_{obs}/[I] = 560 $M^{-1}s^{-1}$) and Z-Val-Pro-Val^P(OPh-*m*Cl)$_2$ (k_{obs}/[I] = 830 $M^{-1}s^{-1}$, which inhibited ChT more potently than the corresponding Boc- derivatives (k_{obs}/[I] = 50 $M^{-1}s^{-1}$ and 100 $M^{-1}s^{-1}$ respectively).

Mechanism of Inhibition

Halophenyl phosphonate esters are equal or less potent inhibitors of HNE and PPE compared to the phenyl esters. In contrast, the halophenyl phosphonate esters are more reactive toward ChT and DPP IV (Powers *et al.*, in press) than the phenyl esters. In the case of HNE, the para halogen substituted inhibitors are slightly more potent than the meta halogen substituted inhibitors. In the case of PPE however, there is no difference between the para and meta substituted phosphonate inhibitors. This difference in reactivity among the inhibitors may be due more to the geometry and size of the halophenyl group rather than changes in the electronegativity due to the chlorine atoms attached to the phenoxy group. We hypothesize that the chlorine may interfere with the binding of the inhibitor into the active site of the two elastases. This appears not to be the case with ChT and DPP IV since their active sites can more effectively accomodate the chlorophenoxy group. Thus, HNE and PPE appear not to form as stable transition states with the halogenated phenyl phosphonate esters as compared to ChT and DPP IV.

Alternatively, the effect may be an electronic one, in that the chlorine affects the location of the phenoxy group on the phosphorus atom in the transition state.

In this mechanism, the active site serine attacks the phosphorus atom in an apical position opposite to a ligand that is not the leaving group.[19] The leaving group departs from the apical position, which it attains by pseudorotation of the ligands attached to the phosphorus. Generally, the most electrophilic substituents occupy the apical positions and nucleophilic attack occurs opposite the most electrophilic group, which may also be the leaving group.[20] If this is the case, HNE and PPE may prevent pseudorotation because their active sites cannot accomodate the movement of the halogenated phenoxy groups. We also postulate that ChT and DPP IV have more open active sites which do not prevent pseudo rotation during the inhibition process with this mechanism.

CONCLUSION

In conclusion, α-aminoalkylphosphonate di(chlorophenyl) esters are potent inhibitors of serine proteases, but are less reactive than the α-aminoalkylphosphonate diphenyl esters previously reported.[8] Compared to the non-halogenated peptide phosphonates, the inhibitory potency of these compounds decreases with HNE and PPE, and increases with ChT and DPP IV. This confirms previous results where it has been shown that addition of electronegative substituents on the phenoxy leaving group of phosphonate inhibitors increases their reactivity toward chymotrypsin, trypsin and urokinase.[16] Thus, halogen substitution may be a method to increase the specificity of phosphonate inhibitors toward certain enzymes, since adding a halogen increases the reactivity toward some serine proteases while decreasing the reactivity toward others.

Acknowledgements

Thanks to Dr. Chih-Min Kam for help with the kinetic experiments and for many hours of stimulating discussion. Audra D. Brown was supported in part by NIH Predoctoral Fellowship HL 08813-01. We thank the Cetius Corporation (Chiron, Inc.) for partial financial support for this research.

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2. Abbreviations: Boc, tert-butyloxycarbonyl; ChT, chymotrypsin; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HNE, human neutrophil elastase; *i*-Pr, isopropyl; MeO, methoxy; Me_2SO, dimethyl sulfoxide; NA, *p*-nitroaniline; Ph, phenyl; OPh, phenoxy; PPE, porcine pancreatic elastase; Suc, succinyl; Z, benzyloxycarbonyl.
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