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Inhibition of the Elastase of *Pseudomonas aeruginosa* by N^{α}-Phosphoryl Dipeptides and Kinetics of Spontaneous Hydrolysis of the Inhibitors†

Louis Poncz, Thomas A. Gerken, Dorr G. Dearborn,* Damian Grobelny, and Richard E. Galardy

ABSTRACT: The rates of hydrolysis of *N*-[(α-L-rhamnopyranosyloxy)phospho]-L-leucyl-L-tryptophan (phosphoramidon), N^{α}-phosphoryl-L-leucyl-L-tryptophan (PO_3LeuTrp), N^{α}-phosphoryl-L-leucyl-L-phenylalanine (PO_3LeuPhe), and N^{α}-phosphoryl-L-leucyl-L-phenylalaninamide (PO_3LeuPheNH_2) were followed by proton nuclear magnetic resonance spectroscopy. The rates of hydrolysis (k_{obsd}) of PO_3LeuTrp, PO_3LeuPhe, and PO_3LeuPheNH_2 were all first order in phosphorylamide concentration over the pH range studied (3.8–9.5). The values for k_{obsd} at pH 7.3 and 37 °C are as follows: PO_3LeuTrp, 0.35 h^{-1}; PO_3LeuPhe, 0.63 h^{-1}; PO_3LeuPheNH_2, 0.73 h^{-1}. The values for k_{obsd} do not significantly change between pH 5 and pH 8 but dramatically decreased with increasing pH. The hydrolysis of PO_3LeuPhe and PO_3LeuPheNH_2 above a pH of approximately 5 was positively correlated with the concentration of monoanionic species $(NHRPO_3H)^{1-}$, and the values for the first-order rate constants for the respective monoanionic species were calculated to be 0.66 ± 0.03 h^{-1} and 1.07 ± 0.10 h^{-1}. Phosphoramidon was not found to hydrolyze after 6 days at 37 °C at a pH of 4.6 and 7.7, while the phosphorylamide PO_3LeuTrp, synthesized by the removal of L-rhamnose from phosphoramidon by base hydrolysis, was found to rapidly hydrolyze under these conditions. Solvolysis in aqueous methanol of PO_3LeuPhe and PO_3LeuPheNH_2 indicates that the hydrolysis reaction is bimolecular, proceeding by way of direct attack of solvent (H_2O, CH_3OH) on phosphorus. The proteolytic activity of elastase from *Pseudomonas aeruginosa* was measured with both hide powder azure and furylacryloyl-L-alanyl-L-phenylalaninamide in the presence and absence of PO_3LeuPhe, PO_3LeuPheNH_2, phosphoramidon, and PO_3LeuTrp. The relative degree of inhibition observed with both of these substrates was PO_3LeuTrp > PO_3LeuPhe ~ phosphoramidon > PO_3LeuPheNH_2.

Several natural and synthetic low molecular weight reversible inhibitors of metalloproteinases are known. *N*-[(α-L-Rhamnopyranosyloxy)phospho]-L-leucyl-L-tryptophan, which was isolated from the culture filtrates of an actinomycete, *Streptomyces tanashiensis* (Suda et al., 1973), inhibits several metalloproteinases, including pseudomonas elastase (Komiyama et al., 1975; Umezawa et al., 1972), thermolysin (Morihara & Tsuzuki, 1980; Nishino & Powers, 1980; Kessler et al., 1982), and a membrane-bound peptidase from porcine kidney (Mumford et al., 1981). *N*-[[(6-Deoxy-α-L-talopyranosyl)oxy]phospho]-L-leucyl-L-tryptophan, which was isolated from *Streptomyces mozunensis* MK-23 (Murao et al., 1980), inhibits thermolysin (Kitagishi & Hiromi, 1983; Kitagishi et al., 1983). Synthetic phosphorus-containing metalloproteinase inhibitors include *N*-[(α-L-rhamnopyranosyloxy)phospho] peptides (Komiyama et al., 1975), phosphoryl peptides[1] (Galardy, 1980; Holmquist & Vallee, 1979; Kam et al., 1979; Kessler et al., 1982; Murao et al., 1982a,b; Nishino & Powers, 1979), mono- and diesterified phosphoryl peptides (Galardy, 1982; Holmquist, 1977; Holmquist & Vallee, 1979; Thorsett et al., 1982), thiophosphoryl peptide (Nishino & Powers, 1979), and phosphonic acids and amides (Galardy et al., 1983). Phosphoryl peptides are potent reversible inhibitors

† From the Department of Pediatrics and Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106 (L.P., T.A.G., and D.G.D.), and the Department of Biochemistry and the Sanders-Brown Research Center on Aging, University of Kentucky, Lexington, Kentucky 40536 (D.G. and R.E.G.). *Received August 19, 1983; revised manuscript received January 25, 1984.* This work was supported by grants from the Cystic Fibrosis Foundation (L.P.) and the National Institutes of Health [HL-07415 (L.P.) and HL-27368 (R.E.G.)].

[1] Abbreviations: $(BzlO)_2$POLeuNH_2, N^{α}-dibenzylphosphoryl-L-leucinamide; $(BzlO)_2$POLeuPheNH_2, N^{α}-dibenzylphosphoryl-L-leucyl-L-phenylalaninamide; $(BzlO)_2$POLeuPheOBzl, N^{α}-dibenzylphosphoryl-L-leucyl-L-phenylalanine benzyl ester; $(BzlO)_2$POValPheNH_2, N^{α}-dibenzylphosphoryl-L-valyl-L-phenylalaninamide; $(BzlO)_2$POValPheOMe, N^{α}-dibenzylphosphoryl-L-valyl-L-phenylalanine methyl ester; phosphoramidon, *N*-[(α-L-rhamnopyranosyloxy)phospho]-L-leucyl-L-tryptophan; PO_3LeuPhe, N^{α}-phosphoryl-L-leucyl-L-phenylalanine tripotassium salt; PO_3LeuPheNH_2, N^{α}-phosphoryl-L-leucyl-L-phenylalaninamide dipotassium salt; PO_3LeuTrp, N^{α}-phosphoryl-L-leucyl-L-tryptophan tripotassium salt; LeuPhe, L-leucyl-L-phenylalanine; LeuTrp, L-leucyl-L-tryptophan; phosphoryl peptide, N^{α}-phosphoryl peptide; phosphoryl dipeptide, N^{α}-phosphoryl dipeptide; ^{1}H NMR, proton nuclear magnetic resonance; ^{13}C NMR, natural abundance proton-decoupled ^{13}C nuclear magnetic resonance; ^{31}P NMR, proton-decoupled ^{31}P nuclear magnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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of metalloproteinases because they are thought to resemble intermediates related to the transition state for substrate hydrolysis; i.e., the tetrahedral phosphorus mimics the tetrahedral stereochemistry of the transition state. Indeed, there is strong evidence that phosphonamidates are transition-state analogues of thermolysin (Bartlett & Marlowe, 1983).

The stability of the phosphoramidate bond in aqueous solutions may limit the use of phosphoryl peptides as in vitro and in vivo inhibitors of metalloproteinases. The hydrolysis of phenyl *N*-glycylphosphoramidate proceeds with a half-life of 69 h at pH 6–11 (Sampson et al., 1973); the rates of hydrolysis in aqueous solutions of unsubstituted phosphoryl peptides have not been investigated. The literature suggests that the phosphoramidic amide bond of unsubstituted phosphoryl peptides is stable in basic solutions and unstable at lower pH. Thus, inhibition by PO_3LeuPhe of the solubilization of cartilage by pseudomonas elastase decreases after 3 h, presumably due to the hydrolysis of the phosphoryl peptide at pH 7.5 (Kessler et al., 1982). Solutions of phosphoryl peptide inhibitors of angiotensin-converting enzyme were prepared daily in pH 9–11 buffer (Galardy, 1980), and a phosphoryl peptide from *Streptomyces rishiriensis* was purified at pH 11 (Kasai et al., 1982). This study was therefore undertaken to determine the actual rates of hydrolysis of a group of phosphoryl peptides of potential therapeutic importance. In addition, the degree of inhibition of pseudomonas elastase by a pair of phosphoryl peptides that differ (1) at their C-terminals, PO_3LeuPhe and PO_3LeuPheNH$_2$, and (2) at the phosphate group, phosphoramidon and PO_3LeuTrp, has been examined in this study.

Experimental Procedures

Compounds were visualized on TLC plates by the following methods: fluorescamine spray (30 mg in 100 mL of acetone) for primary amines; phosphomolybdate spray for phosphorylated compounds (Dittmer & Lester, 1964); 3% (v/v) H_2SO_4–25% (w/v) $NaHSO_4$, followed by heating at 450 °C, to visualize all the spots; ultraviolet light; iodine vapor. Thin-layer solvent systems on silica gel plates were, by volume, (A) chloroform–hexane–acetonitrile (1.9:1.1:1.0), (B) chloroform–methanol (15:1), (C) chloroform–methanol (9:1), (D) 1-propanol–concentrated ammonium hydroxide (8.4:3.7), (E) 2-propanol–concentrated ammonium hydroxide–water (8:4:2), and (F) butanol–methanol–concentrated ammonium hydroxide–water (40:10:5:1).

Materials. The following materials were obtained from the indicated commercial sources: amino acid derivatives and dipeptides (Bachem); phosphoramidon (Peninsula Laboratories); dicyclohexylcarbodiimide (Aldrich); dibenzyl phosphite (ICN Pharmaceuticals, Inc.); sulfuryl chloride (Fluka); silica gel (60–200 mesh, J. T. Baker); silica gel TLC plates (60 F-254, E. Merck); trifluoroacetic acid and fluorescamine (Pierce); pseudomonas elastase (Nagase Ltd); monomethyl phosphate and hide powder azure (Sigma); furylacryloyl-L-alanyl-L-phenylalaninamide (Chemalog); all organic solvents, reagent grade (Fisher). Methanol was redistilled from potassium permanganate and potassium hydroxide. Carbon tetrachloride was washed with concentrated sulfuric acid and water and then distilled from phosphorus pentoxide. *N*-Ethylmorpholine was distilled from phthalic anhydride.

NMR Spectroscopic Methods. All spectra were recorded on a Bruker WH 180/270 pulse Fourier-transform NMR spectrophotometer with a Nicolet 1180 computer. Proton-decoupled ^{13}C spectra at 67.92 MHz were obtained for the dibenzyl derivatives of the phosphoryl peptides by use of a 10 mm diameter sample probe. The central peak of $CDCl_3$ was used as an internal secondary chemical shift reference (77.5 ppm relative to Me_4Si). Proton-decoupled ^{31}P NMR spectra were obtained at 73.0 MHz from 20-mm sample tubes. The inorganic phosphate resonance was used as an internal secondary chemical shift reference at pH 6.18.

The rates of hydrolysis of the phosphoryl peptides were followed by ^1H NMR at 270 MHz with a regulated probe temperature of 37 ± 1 °C. The residual HDO resonance was suppressed by a presaturation method (Campbell et al., 1973). NMR samples for hydrolysis studies contained 0.45 mL of 0.1 M potassium phosphate buffer (pH prior adjusted) in 99.9% deuterium oxide (twice lyophilized from D_2O) and 0.2 μL of acetone for chemical shift reference. Hydrolysis was followed by mixing the phosphorylamide in the 5-mm NMR tube containing buffer and rapidly replacing the sample tube in the pretuned and thermally equilibrated ^1H NMR probe. Data acquisition began once the field/frequency lock was obtained. A total of 40–160 scans (2.7–11.3 min) were obtained per spectra and automatically stored on a magnetic disc. Experiments performed at pH values greater than 8.6 used longer delay periods ranging from 0.5 to 1 h between consecutive spectra. The accumulation of spectra continued for several hours, giving up to 20 time points during the course of hydrolysis. The pH of the sample was measured after the completion of the NMR experiment with a Radiometer PHM64 meter. No corrections are reported for the D_2O solvent. The pH was stable during the course of hydrolysis, as indicated by the chemical shifts of pH-sensitive resonances (see below).

The mole fractions of phosphoryl peptide and resultant dipeptide were calculated for each spectrum from the integration of the best resolved peaks of the respective compounds (see Results). To account for pseudo-first-order kinetics, the time assigned to an individual spectrum was taken as 31% (i.e., 1.0 − ln 0.50) of the time between the initial and final acquisition times of the spectrum. The rate constants were determined by using an exponential curve fit from which the coefficients of determination ranged from 0.97 to 1.00.

The ionization constants of PO_3LeuPhe, PO_3LeuPheNH$_2$, and their corresponding dipeptides were determined from their ^1H NMR spectra at 37 °C as a function of pH. The ionization constants were calculated by fitting the chemical shifts of the α- and δ-protons of the leucyl residues at different pH to theoretical titration curves.

Synthesis of Phosphoryl Peptides. Esters and amides of L-leucine and the dipeptides were acylated with dibenzyl chlorophosphite prepared from dibenzyl phosphite and sulfuryl chloride (Atherton et al., 1948). The properties of $(BzlO)_2$POLeuNH$_2$, $(BzlO)_2$POValPheOMe, and $(BzlO)_2$POValPheNH$_2$ are listed below although they were not used further in the inhibition studies. The ^{13}C NMR data confirmed their structures by the coupling observed between ^{31}P and the carbonyl and β-carbon atoms of the amino acid residue adjacent to the phosphorus (see Results). The benzyl ester derivatives of PO_3LeuPhe and PO_3LeuPheNH$_2$ were converted by hydrogenation for 1 and 2 h, respectively. $(BzlO)_2$POLeuPheOBzl and $(BzlO)_2$POLeuPheNH$_2$ were dissolved in absolute ethanol, 0.5 g of 10% palladium on carbon, and 3 and 2 equiv of potassium hydroxide (added as 1.0 N potassium hydroxide in water), respectively. The pH was checked periodically during the hydrogenation and was maintained at a value of approximately 10. The catalyst was removed by filtration and the filtrates were lyophilized to yield final products.

$(BzlO)_2$POLeuNH$_2$ was recrystallized from ethyl acetate–hexanes: mp 115–117 °C; R_f(C) 0.14; [^1H] ^{13}C NMR (CD-

Cl_3) δ 21.77, 23.03 (Leu CH_3), 24.50 [Leu $(CH_3)_2CH$], 43.50 [Leu CH_2, ^3J(C–P) = 7.4 Hz], 53.97 (Leu CHNH), 68.54, 68.62 [CH_2OP, ^2J(C–P) = 5.5 Hz], 136.02, 136.55 [CCH_2OP, ^3J(C–P) = 7.4 and 6.5 Hz, respectively], 176.80 [Leu C=O, ^3J(C–P) = 2.8 Hz].

$(BzlO)_2POValPheOMe$ was recrystallized from methylene chloride–hexanes or purified on a silica gel column. The sample was dissolved in methylene chloride and loaded on a silica gel column prepared in methylene chloride. The column was washed with 5 column volumes of methylene chloride–ethanol (99.5:0.5 v/v): mp 128–130 °C; R_f(B) 0.52; [^{1}H] ^{13}C NMR ($CDCl_3$) δ 17.84, 19.65 (Val CH_3), 32.33 [Val $(CH_3)_2CH$, ^3J(C–P) = 5.5 Hz], 38.35 (Phe CH_2), 52.45 (CO_2CH_3), 53.77 (Phe CHNH), 61.59 (Val CHNH), 68.52, 68.55 [CH_2OP, ^2J(C–P) = 5.5 and 4.6 Hz, respectively], 136.59 (Phe C-1 Ph), 136.66, 136.77 [CCH_2OP, ^3J(C–P) = 9.2 and 8.3 Hz, respectively], 172.25 (Phe C=O), 172.66 [Val C=O, ^3J(C–P) = 3.7 Hz].

$(BzlO)_2POValPheNH_2$ was recrystallized from ethyl acetate–carbon tetrachloride: mp 220–222 °C; R_f(C) 0.60; [^{1}H] ^{13}C NMR ($CDCl_3$–CH_3OH, 2:1) δ 17.20, 18.85 (Val CH_3), 31.83 [Val $(CH_3)_2CH$, ^3J(C–P) = 6.5 Hz], 37.76 (Phe CH_2), 54.14 (Phe CHNH), 61.34 (Val CHNH), 68.41, 68.55 [CH_2OP, ^2J(C–P) = 5.5 Hz], 135.97, 136.07 [CCH_2OP, ^3J-(C–P) = 2.8 and 1.8 Hz, respectively], 136.64 (Phe C-1 Ph), 173.25 [Val C=O, ^3J(C–P) = 2.8 Hz], 174.32 (Phe C=O).

$(BzlO)_2POLeuPheOBzl$ was recrystallized from hexane: mp 78–80 °C; R_f(A) 0.68, R_f(C) 0.92; [^{1}H] ^{13}C NMR ($CDCl_3$) δ 22.18, 23.35 (Leu CH_3), 24.76 [Leu $(CH_3)_2CH$], 38.30 (Phe CH_2), 44.21 [Leu CH_2, ^3J(C–P) = 6.5 Hz], 53.72 (Leu CHNH), 54.77 (Phe CHNH), 67.40 (CO_2CH_2), 68.60, 68.64 [CH_2OP, ^2J(C–P) = 4.6 and 5.5 Hz, respectively], 135.59 (CO_2CH_2C), 136.40 (Phe C-1 Ph), 136.57, 136.80 [CCH_2OP, ^3J(C–P) = 7.4 and 8.3 Hz, respectively], 171.64 (Phe C=O), 173.60 [Leu C=O, ^3J(C–P) = 3.7 Hz].

$(BzlO)_2POLeuPheNH_2$ dissolved in methylene chloride–ethanol (7.5:2.5 v/v) was applied to a silica gel column prepared in methylene chloride–ethanol (99:1 v/v) and eluted with 4 column volumes of methylene chloride–ethanol (99:1 and (98:2). Product eluted with methylene chloride–ethanol (98:2): mp 182–184 °C; R_f(C) 0.63; [^{1}H] ^{13}C NMR ($CDCl_3$–CH_3OH, 4:1) δ 21.67, 23.05 (Leu CH_3), 24.63 [Leu $(CH_3)_2CH$], 37.88 (Phe CH_2), 43.50 [Leu CH_2, ^3J(C–P) = 7.4 Hz], 54.14 (Leu CHNH), 54.60 (Phe CHNH), 68.86, 69.00 [CH_2OP, ^2J(C–P) = 5.5 Hz], 136.11, 136.15 [CCH_2OP, ^3J(C–P) = 7.4 and 6.5 Hz, respectively], 136.93 (Phe C-1 Ph), 174.20 [Leu C=O, ^3J(C–P) = 2.8 Hz], 174.32 (Phe C=O).

PO_3LeuPhe: R_f(D) 0.08, R_f(E) 0.50, R_f(F) 0.13; molar ratio of total phosphate (see below), phenylalanine (by UV absorbance, see below) was 1.0:1.0; ^{1}H NMR (200 mM K_2CO_3 in 99.9% D_2O) δ 7.13 (m, 5 H, Ph), 4.28 (m, 1 H, $CHCH_2Ph$), 3.36 (m, 1 H, $CHCH_2$), 2.94 (m, 2 H, CH_2Ph), 1.34 [m, 1 H, $(CH_3)_2CH$], 1.11 (m, 2 H, $CHCH_2CH$), 0.63 [m, 6 H, $(CH_3)_2CH$].

$PO_3LeuPheNH_2$: R_f(D) 0.36, R_f(F) 0.27; molar ratio of total phosphate (see below), phenylalanine (by UV absorbance, see below) was 1.0:1.0; ^{1}H NMR (200 mM K_2CO_3 in 99.9% D_2O) δ 7.14 (m, 5 H, Ph), 4.35 (m, 1 H, $CHCH_2Ph$), 3.31 (m, 1 H, $CHCH_2$), 2.99 (m, 2 H, CH_2Ph), 1.20 [m, 1 H, $(CH_3)_2CH$], 0.91 (m, 2 H, $CHCH_2CH$), 0.56 [m, 6 H, $(CH_3)2CH$].

PO_3LeuTrp: Base hydrolysis of phosphoramidon was performed by a modification of the procedure described by Komiyama et al. (1975). After the hydrolysis, sodium hydroxide was neutralized with an equimolar amount of sodium bicarbonate. Four volumes of acetone was added to the reaction mixture, which was vortexed and centrifuged. The supernatant was lyophilized, resuspended in a small volume of 200 mM K_2CO_3, and applied to a Bio-Rad P-2 column (1 × 25 cm) equilibrated in 2 mM K_2CO_3. One milliliter fractions were collected and monitored for content of tryptophan, total phosphorus, and primary amine (see below). Thin-layer chromatography was used to confirm the identification of the products of the base hydrolysis of phosphoramidon: phosphoramidon, R_f(D) 0.32, R_f(F) 0.17; LeuTrp, R_f(D) 0.56, R_f(F) 0.43; PO_3LeuTrp, R_f(D) 0.18, R_f(F) 0.14.

Quantitation of Phenylalanine and Tryptophan. The absorbance of dipeptides and phosphoryl peptides in aqueous solutions was determined with a Gilford 2400 spectrophotometer. The molar extinction coefficient of phenylalanine-containing compounds was taken as 195 M^{-1} cm^{-1} at 258 nm, and the molar extinction of tryptophan-containing compounds was taken as 5600 M^{-1} cm^{-1} at 279.6 nm.

Quantitation of Primary Amines. Samples, 0.1 mL, were diluted with 0.9 mL of 0.2 M sodium borate, pH 9.0, and subsequently, 0.5 mL of 30 mg of fluorescamine/100 mL of acetonitrile was added. After 5 min, the samples were read with an Aminco SPF-125 spectrofluorometer: excitation at 390 nm and emission at 475 nm. L-Leucyl-L-phenylalanine and L-leucyl-L-tryptophan were used as standards.

Quantitation of Inorganic and Total Phosphate. The concentration of inorganic phosphate was determined by the method of Lowry et al. (1954) as modified by Chen et al. (1956). The concentration of total phosphate was determined by the micromethod of Chen et al. (1956) as modified by Ames & Dubin (1960). Sodium phosphate was used as a standard.

Quantitation of L-Rhamnose. The concentration of L-rhamnose was determined by the method of Dische & Shettles (1951).

Determination of Rates of Solvolysis. The rates of hydrolysis of PO_3LeuPhe and $PO_3LeuPheNH_2$ at 37 °C in 0.1 M potassium phthalate and the rates of solvolysis in methanol–0.2 M potassium phthalate (1:1 v/v) were determined by fluorescamine analysis. Aliquots of the reactions, 0.01–0.10 mL, taken at several early time points and after proceeding overnight, were analyzed for free amino group content. LeuPhe was used as a standard at each time point. The rate constants were determined for an exponential curve fit and the coefficients of determination ranged between 0.90 and 1.00.

Enzymatic Assays. The proteolytic activity of elastase from *Pseudomonas aeruginosa* in the presence and absence of PO_3LeuPhe, $PO_3LeuPheNH_2$, phosphoramidon, and PO_3LeuTrp was measured with hide powder azure and furylacryloyl-L-alanyl-L-phenylalaninamide. The concentration of pseudomonas elastase was determined by the method of Bradford (1976) and of the inhibitors by their absorbance (see above). (a) Assay using hide powder azure as substrate: Enzyme activity was measured according to the method described by Rinderknecht et al. (1968) with a slight modification. The reaction mixture consisted of 20 mg of hide powder azure, 80 ng of enzyme, and various amounts of inhibitors (in 10 mM Na_2CO_3) in 5 mL of 30 mM Tris-HCl, final pH 8.0. The reaction was carried out at 37 °C for 45 min and stopped by cooling in ice bath. The mixture was quickly filtered through Whatman 1 paper, and the absorbance of the supernatant was measured at 595 nm. The inhibitor concentration needed for 50% inhibition was determined. The assay was linear in the range 10–80 ng of elastase per assay tube, and the reproducibility of the values for 50% inhibition of enzyme activity was ±20%. (b) Assay using furyl-

acryloyl-L-alanyl-L-phenylalaninamide as substrate: Proteolytic activity was assayed by monitoring the decrease in absorbance at 345 nm due to the hydrolysis of the L-alanyl-L-phenylalaninamide bond (Blumberg & Vallee, 1975; Feder, 1968). Measurements were performed at room temperature on a Gilford 2400 spectrophotometer with the reaction solution consisting of 0.5–1.0 mM substrate, 1.0% (v/v) dimethylformamide (solvent used to dissolve furylacryloyl-L-alanyl-L-phenylalaninamide), 500 ng of pseudomonas elastase, and various amounts of inhibitors (stored in 10 mM Na_2CO_3) in 1.0 mL of 30 mM Tris-HCl, final pH 8.0. The K_i values of the inhibitors were determined from Dixon plots. The reproducibility of the values for the K_i of phosphoramidon was ±30% and for the K_i of the other three phosphoryl dipeptides was less than ±15%.

Results

NMR Spectra of Intermediates of Synthesis and of Potassium Salts of Unsubstituted Phosphoryl Peptides. The phosphoryl peptides were synthesized as the benzyl esters, which were subsequently removed by hydrogenation in the presence of potassium hydroxide. The peaks assigned to the methylene carbons and C-1 of the phenyl rings of the dibenzylphosphoryl groups and the carbonyl and β-carbon atoms of the amino acid residue adjacent to phosphorus are doublets, due to ^{31}P–^{13}C spin coupling. The α-carbon of the same amino acid residue showed no splitting with the data point resolution employed (approximately 1 Hz/point). In addition, there are two pairs of doublets for the methylene and C-1 carbon atoms of the benzyl groups attached to the phosphorus, indicating that the environments of the two groups are different.

The potassium salts of phosphoryl peptides are stable in basic solution (see below), and ^1H NMR spectra were obtained in 200 mM potassium carbonate to verify the structures of the products of hydrogenation, the composition of the commercially available phosphoramidon, and the product obtained after base hydrolysis of phosphoramidon. The patterns of the resonances at pH 11 of the δ-protons of the leucyl residues of the unsubstituted phosphoryl dipeptides, of the corresponding dipeptides, and of phosphoramidon were found to be different; those of PO_3LeuPhe, PO_3LeuPheNH$_2$, and PO_3LeuTrp are nonoverlapping doublets. On the other hand, the corresponding peaks of the dipeptides and of phosphoramidon display overlapping doublets.

Base Hydrolysis of Phosphoramidon. The products of base hydrolysis of phosphoramidon have not been previously documented. The addition of acetone to the neutralized hydrolysis mixture resulted in the precipitation of L-rhamnose and most of the sodium bicarbonate. Little, if any, of the 280-nm absorbing material precipitated. Column chromatography on Bio-Rad P-2 of the supernatant resulted in two peaks that absorb at 280 nm. The first peak was PO_3LeuTrp, as determined from ^1H NMR, supported by the 1.0:1.0 ratio of total phosphorus to tryptophan concentration and the low level of free primary amine. The second peak was LeuTrp, as determined by thin-layer chromatography. The phosphoryl peptide accounted for 55 and 78% of the total UV absorption in two experiments.

Rates of Hydrolysis and pH Dependency. Changes in chemical shifts upon hydrolysis of the phosphorylamide bond were observed for α-, β-, and δ-protons of the leucyl residues of PO_3LeuPhe, PO_3LeuPheNH$_2$, and PO_3LeuTrp. In addition, large chemical shift changes were observed upon hydrolysis for the β-protons of the phenylalanyl residue of PO_3LeuPheNH$_2$. Although the pH was measured at the end of the NMR experiment, it did not noticeably fluctuate during the course of the experiment because the chemical shifts of the pH-sensitive resonances of the leucyl residues of the phosphoryl peptides and of the dipeptides did not change.

Table I: First-Order Rate Constants for Hydrolysis of Monoanion Species of PO_3LeuPhe and PO_3LeuPheNH$_2$[a]

compd	pK_2	pH	k_1 (h^{-1} M^{-1})	half-life (h)
PO_3LeuPhe	8.35	6.38	0.71	0.99
		6.59	0.69	1.02
		6.94	0.65	1.12
		7.31	0.68	1.10
		7.74	0.60	1.44
		9.24	0.65	9.63
			0.66 ± 0.03[b]	
PO_3LeuPheNH$_2$	7.55	5.09	1.01	0.69
		6.26	0.94	0.78
		7.05	1.28	0.71
		7.57	1.00	1.41
		8.59	1.11	6.93
			1.07 ± 0.10[b]	

[a] The values of pK_2 of PO_3LeuPhe and PO_3LeuPheNH$_2$ were obtained to the nearest 0.05 pH unit from plots of chemical shifts of α- and δ-protons of the leucyl residues vs. pH, as described under Experimental Procedures. The first-order rate constant (k_1) at each pH was estimated from eq 1, and the mole fractions of the mono- and dianionic species were calculated by using the pK_2. The half-life at each pH was determined from $-\ln 0.50/k_{obsd}$. [b] Average.

The rates of hydrolysis of the unsubstituted phosphoryl peptides appear to follow pseudo-first-order kinetics, as indicated by the plot of mole fraction of unhydrolyzed phosphoryl peptides vs. time (Figure 1). The values of half-life vs. pH (Table I) for PO_3LeuPhe and PO_3LeuPheNH$_2$ are similar. The two compounds hydrolyzed significantly slower in basic solutions. Fluorescamine analysis of PO_3LeuPhe in 150 mM potassium carbonate (pH 11.3) indicated that only 2% hydrolyzed overnight at room temperature. The effect of buffer concentration on the rates of hydrolysis was determined. When 0.75 mM PO_3LeuPhe was used as substrate, there was less than 10% difference in rates of hydrolysis in the presence of 0.025–0.20 M potassium phthalate, pH 5.67 ± 0.02, or 0.025–0.20 M potassium phosphate, pH 7.14 ± 0.05.

The rate equation (Benkovic & Sampson, 1971; Chanley & Feageson, 1963) used to analyze the hydrolysis of the phosphoryl peptides is

$$k_{obsd} = k_H(H^+)(M_0) + k_0(M_0) + k_1(M_1) + k_2(M_2) \quad (1)$$

Here, k_H is the second-order rate constant associated with hydronium ion catalyzed hydrolysis of the neutral $(NHRPO_2H_2)^0$ species; k_0, k_1, and k_2 are first-order rate constants for the hydrolysis of the neutral, monoanion $(NHRPO_2H)^{1-}$, and dianion $(NHRPO_2)^{2-}$ species, respectively. M_0, M_1, and M_2 are the mole fractions of the neutral, monoanion, and dianion species, respectively.

A value of 8.35 for the pK_2 of PO_3LeuPhe and 7.55 for the pK_2 of PO_3LeuPheNH$_2$ was obtained (Table I). In contrast, the values of the pK_a of LeuPhe and LeuPheNH$_2$ are 7.65 and 7.60, respectively. The constant values of the half-lives of PO_3LeuPhe and PO_3LeuPheNH$_2$ below their pK_2 (Table I) indicate that the first two terms in eq 1 are negligible. This is further supported by the fact that other phosphorylamides have pK_1 values of 2–3 (Benkovic & Sampson, 1971; Chanley & Feageson, 1958, 1963; Rahil & Haake, 1981; Sampson et al., 1973); thus, the concentrations of H^+ and M_0 in this study are small and can be taken as zero. Substitution of the relevant k_{obsd} and M_1 and M_2 (obtained with the above values of pK_2) in eq 1 reveals that, at neutral and basic pH, only the monoanionic species participates in the hydrolysis; i.e., the value

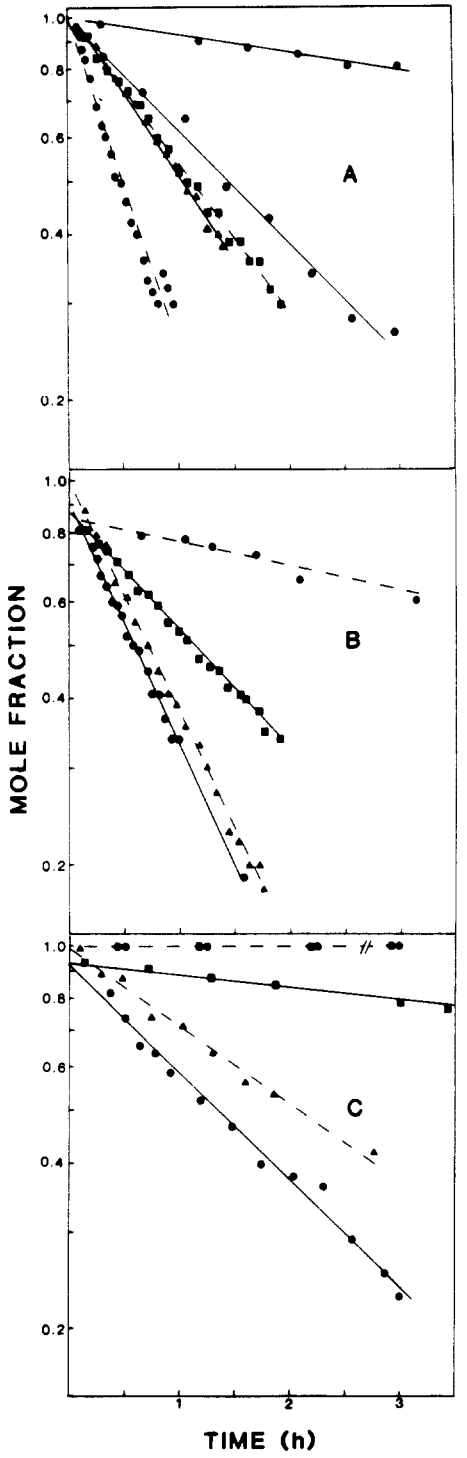

FIGURE 1: Rates of hydrolysis of phosphoryl peptides as a function of time. The hydrolysis of unsubstituted phosphoryl peptides was followed by ^{1}H NMR as described under Experimental Procedures, and changes upon hydrolysis of the phosphorylamide bond were observed for the chemical shifts of α-, β-, and δ-protons of the leucyl residues of the phosphoryl peptides. The mole fraction of the starting material and the product of hydrolysis were calculated from integrations of corresponding peaks. The numbers in parentheses are the calculated k_{obsd} (h^{-1}). (A) Hydrolysis of PO_3LeuPhe: pH 3.77 (1.39, ●--●); pH 6.38 (0.70, ▲—▲); pH 6.59 (0.68, not shown); pH 6.94 (0.62, ■--■); pH 7.31 (0.63, not shown); pH 7.74 (0.48, ●—●); pH 9.24 (0.072, ●—●). (B) Hydrolysis of PO_3LeuPheNH$_2$: pH 5.09 (1.01, ●—●); pH 6.26 (0.89, not shown); pH 7.05 (0.97, ▲--▲); pH 7.57 (0.49, ■—■); pH 8.59 (0.10, ●--●). (C) Hydrolysis of PO_3LeuTrp: pH 6.86 (0.44, ●—●); pH 7.44 (0.32, ▲--▲); pH 9.46 (0.049, ■—■). Hydrolysis of phosphoramidon: pH 4.61 and 7.72 (0.0, ●--● and ●--●).

Table II: Rates and Products of Solvolysis of PO_3LeuPhe and PO_3LeuPheNH$_2$[a]

solvent	k_{obsd} (h^{-1})	inorganic phosphate (%)
PO_3LeuPhe		
phthalate, pH 5.87 and 5.91	0.67 ± 0.01 (2)[b]	
CH_3OH–phthalate, pH 5.56–5.96	0.52 ± 0.14 (4)	38.5 ± 1.5 (2)
PO_3LeuPheNH$_2$		
phthalate, pH 5.83 and 5.95	0.89 ± 0.15 (2)	
CH_3OH–phthalate, pH 5.63–5.94	1.02 ± 0.09 (4)	37.0 ± 1.0 (2)

[a] The hydrolysis of PO_3LeuPhe and PO_3LeuPheNH$_2$ was performed in 0.1 M potassium phthalate (pH indicated below) and in methanol–0.2 M potassium phthalate (1.0:1.0 v/v) (pH indicated below). The rates of hydrolysis of the two phosphoryl peptides were followed by measuring the formation of product, dipeptide, by fluorescamine analysis as described under Experimental Procedures. The percent inorganic phosphate in the samples that were allowed to hydrolyze overnight at 37 °C was calculated from the concentration of inorganic and total phosphate as described under Experimental Procedures. [b] Number in parentheses is number of determinations.

of k_2 is negligible and the value of k_1 is large and constant in the pH range studied (Table I).

Phosphoramidon, an O-glycosidic-substituted phosphoryl peptide, did not hydrolyze at pH values of 4.6 or 7.7, even after 6 days at 37 °C. This was confirmed by both ^{1}H NMR and fluorescamine analysis. Removal of L-rhamnose produced the phosphorylamide PO_3LeuTrp, which hydrolyzed, as described above, at a rate similar to the rates of hydrolysis of PO_3LeuPhe and PO_3LeuPheNH$_2$.

Rates and Products of Solvolysis. Hydrolysis of PO_3LeuPhe and of PO_3LeuPheNH$_2$ was studied in aqueous buffer and in the presence of two nucleophiles, water and methanol. The solvolysis was performed at pH values where the reaction is believed to occur via the monoanionic species (approximately pH 5.8). The rates of hydrolysis in potassium phthalate buffer of PO_3LeuPhe and PO_3LeuPheNH$_2$ determined by fluorescamine analysis (Table II) are similar to the values obtained by ^{1}H NMR in phosphate buffer (Table I).

The identity of the products of the solvolysis was determined by ^{31}P NMR: inorganic phosphate (0.00 ppm) and monomethyl phosphate (1.43 ppm) at pH 6.18. Authentic monomethyl phosphate was found to resonate at the identical chemical shift position as the solvolysis product under the identical solvent conditions. The ratio of inorganic to total phosphate found (approximately 38%; Table II) was not related to the molar percent of water (69.2%) and of methanol (30.8%). This indicates that the solvolysis of the two compounds is bimolecular and proceeds by direct attack of the solvent on the phosphorus (thus reflecting the relative nucleophilicity of each component). Solvolysis, therefore, does not appear to proceed through the formation of metaphosphoric acid.

Kinetics of Inhibition. The degree of inhibition of pseudomonas elastase by PO_3LeuPhe, PO_3LeuPheNH$_2$, phosphoramidon, and PO_3LeuTrp was studied with hide powder azure and furylacryloyl-L-alanyl-L-phenylalaninamide (Table III). Owing to the low solubility of the chromophoric substrate, and its high K_m value, the inhibitors were examined under pseudo-first-order conditions. Dixon plots were used for the calculation of K_i values, and the concentration of substrate was 0.5–1.0 mM (Table III). All four compounds appeared to be competitive inhibitors of pseudomonas elastase. Regardless of the assay used, the relative order of inhibition that was

Table III: Inhibitors of Proteolytic Activity of *P. aeruginosa* Elastase[a]

inhibitor	ID_{50} (μM)[b]	K_i (μM)[c]
PO_3LeuPhe	2.5	0.26
PO_3LeuPheNH$_2$	6.5	0.56
phosphoramidon	2.1	0.25
PO_3LeuTrp	0.2	0.026

[a] Assays were performed as described under Experimental Procedures. [b] ID_{50} indicates the inhibitor concentration needed for 50% inhibition of enzyme activity with hide powder azure as the substrate. [c] K_i values were obtained from Dixon plots using furylacryloyl-L-alanyl-L-phenylalaninamide as the substrate.

observed was PO_3LeuTrp > PO_3LeuPhe ~ phosphoramidon > PO_3LeuPheNH$_2$.

Discussion

The hydrolysis of four phosphoryl peptides were followed in detail by ^{1}H NMR spectroscopy. A change in the chemical shifts of the α-, β-, and δ-protons of the leucyl residues of the three unsubstituted phosphoryl peptides was observed upon hydrolysis of the phosphorylamide bond. In most instances, the corresponding resonances for the dipeptides did not overlap those of the phosphoryl peptides. Hence, the integration of the spectra permits the quantitation of the mole fraction of substrate and product at each time point from which the rates of hydrolysis have been determined.

We have observed the half-lives of PO_3LeuPhe and PO_3LeuPheNH$_2$ to be relatively constant (approximately 1 h) at low to neutral pH and to increase at alkaline pH (Table I). Similar profiles have been observed for the hydrolysis of phosphoramidic acid (Chanley & Feageson, 1963), *N*-alkylphosphoramidates (Benkovic & Sampson, 1971), *N*-arylphosphoramidates (Chanley & Feageson, 1958, 1963; Benkovic & Sampson, 1971), and *N*-alkylphenylphosphonamides (Rahil & Haake, 1981). Kasai et al. (1982) recently purified an inhibitor of metalloproteinases from *S. rishiriensis* that contained phosphoric acid and L-phenylalanyl-L-arginine, and it, as well, was found to be stable only at pH values greater than 11. In general, the pH–rate profile of the hydrolysis of *N*-alkyl- and *N*-arylphosphoramidates are S-shaped, and successive protonation of the substrates results in an increase in rates of hydrolysis. The extended pH range over which the rates of hydrolysis of PO_3LeuPhe and PO_3LeuPheNH$_2$ are constant is therefore believed to be a direct consequence of a large difference between pK_1 and pK_2 of N-aliphatic phosphoramidates [see Benkovic & Sampson (1971)].

The rate constants (k_1) for the hydrolysis, measured in aqueous buffer and at 37 °C, of the monoanion species of PO_3LeuPhe and PO_3LeuPheNH$_2$ were found to be 0.66 h^{-1} and 1.07 h^{-1}, respectively (Table I). For comparison, the value of k_1 of phosphoramidic acid is 0.25 h^{-1} (37 °C) (Chanley & Feageson, 1963), of phenyl *N*-glycylphosphoramidate is 0.01 h^{-1} (35 °C) (Sampson et al., 1973), and of *N*-(*p*-carboxyphenyl)phosphoramidate is 1.99 h^{-1} (35 °C) (Benkovic & Benkovic, 1967). It is difficult to compare rate constants reported for other monoanions of phosphorylamides because of the wide range of temperatures used for the hydrolysis, 20–75 °C (Benkovic & Sampson, 1971; Chanley & Feageson, 1958, 1963; Rahil & Haake, 1981; Sampson et al., 1973).

The values of the first-order rate constant of the hydrolysis of the dianionic species (k_2, eq 1) of PO_3LeuPhe and PO_3LeuPheNH$_2$ were found to be very small compared with the values of the first-order rate constant of the hydrolysis of the monoanionic species (k_1). Similar low values of k_2 for phosphoramidic acid (Chanley & Feageson, 1963), *N*-(*p*-chlorophenyl)phosphoramidate (Chanley & Feageson, 1963), and *N*-butylphosphoramidate (Benkovic & Sampson, 1971) have been reported. However, the dianionic species of *N*-(*p*-carboxyphenyl)phosphoramidate (Benkovic & Benkovic, 1967) hydrolyzes at a rate comparable to that of the monoanionic species. The reason for the differences in reactivity of the dianionic species of the above phosphoramidates is unclear.

The rate of hydrolysis of PO_3LeuPhe decreases in methanol–water mixed solvent relative to water, while the value for PO_3LeuPheNH$_2$ increases (Table II). This difference is also observed for other phosphoramidates: the rates of solvolysis of phosphoramidic acid (Chanley & Feageson, 1963) and *N*,*N*-dimethylphenylphosphonamide (Rahil & Haake, 1981) are increased in less polar solvents relative to water, while the rates of solvolysis of *N*-(*p*-carboxyphenyl)phosphoramidate (Benkovic & Benkovic, 1967) and *N*-(*p*-chlorophenyl)-phosphoramidate (Chanley & Feageson, 1963) are decreased.

The solvolysis experiments were performed in methanol–water (1.0:1.0 v/v; mole percent of water is 69). The low percent of inorganic phosphate (37–38.5%; Table II) formed during the solvolysis of the two phosphoryl peptides presumably reflects the increased nucleophilicity of methanol over water in the mixed solvent (assuming a biomolecular reaction). A unimolecular reaction would produce a highly reactive intermediate, metaphosphate, and the mole ratio of the products would, therefore, more closely parallel that of the mole fraction of the mixed solvent. The percent inorganic phosphate formed during the solvolysis at 37 °C of the two phosphoryl peptides followed in this study (approximately 38%, see Table II) is similar to the percent inorganic phosphate found under similar conditions for the solvolysis of phosphoramidic acid (27% at 25 °C) (Chanley & Feageson, 1963), *N*-(*p*-chlorophenyl)-phosphoramidate (32% at 25 °C) (Chanley & Feageson, 1963), *N*-butylphosphoramidate (26% at 35 °C) (Benkovic & Sampson, 1971), *N*-methylimidazolephosphoramidate (22% at 35 °C) (Benkovic & Benkovic, 1967), and *N*-(*p*-carboxyphenyl)phosphoramidate (29% at 35 °C) (Benkovic & Benkovic, 1967). In contrast, the percent inorganic phosphate formed during the solvolysis of *N*,*N*-dimethylphosphoroguanidinate in 69.6% (mol/mol) water was 66.2% (Haake & Allen, 1980); hence, this reaction is believed to proceed through the formation of metaphosphate.

Phosphoramidon does not measurably hydrolyze at pH 4.6 or 7.7 at 37 °C after 6 days. Thus, the L-rhamnose group is responsible for the protection from hydrolysis since PO_3LeuTrp hydrolyzed at rates comparable to those of PO_3LeuPhe and PO_3LeuPheNH$_2$. Umezawa et al. (1972) found that mild acid hydrolysis of phosphoramidon in 1 N HCl at room temperature for 1 day yielded the dipeptide. Base hydrolysis of phosphoramidon has been reported to generate PO_3LeuTrp (Komiyama et al., 1975); the composition of the products of the hydrolysis was not determined. In our study, the products of the base hydrolysis were 55–78% PO_3LeuTrp and 22–45% dipeptide (n = 2). The resistance to hydrolysis of phosphoramidon at near neutral pH is not surprising since phenyl *N*-glycylphosphoramidate hydrolyzes with a half-life of 69 h at pH 6–11 (Sampson et al., 1973); its hydrolysis is thought to proceed through the initial cleavage of the P–O bond. In addition, less than 2% cleavage of the monoanion of monomethylphosphoramidate was observed at the end of 26 h (Oney & Caplow, 1967). The rate of hydrolysis of the P–O bond in phosphoramidon would be expected to be slower than the bond in phenyl *N*-glycylphosphoramidate since the half-life of α-D-glucose 1-phosphate, at 100 °C and pH 7.5, is 66.7 h (Bunton et al., 1958) while that of phenyl phosphate, at 100 °C, is 6.9 h (Chanley & Feageson, 1955).

In conclusion, the rates of hydrolysis of the monoanionic forms of the three unsubstituted phosphoryl peptides reported here are in the same range as the *N*-aryl and *N*-alkyl derivatives of phosphoramidic acid reported by others. The monoanionic species of phosphoramidon, a phosphorylamide that contains L-rhamnose in a glycosidic bond with the phosphate, was resistant to hydrolysis; this is in agreement with published findings that esterified phosphorylamides are resistant to hydrolysis.

Hide powder azure has not been used previously as a substrate for pseudomonas elastase. Morihara & Tsuzuki (1980) determined that the concentration of phosphoramidon needed to inhibit 50% of pseudomonas elastase activity with casein as a substrate was 0.8 μM, which is comparable to the value we obtained with hide powder azure, 2.1 μM (Table III). Kessler et al. (1982) studied the inhibition of pseudomonas elastase activity by PO_3LeuPhe toward azocasein, insoluble elastin, and cartilage; ID_{50} values were, however, not reported.

Inhibition constants for the four phosphoryl dipeptides were obtained with furylacryloyl-L-alanyl-L-phenylalaninamide as substrate. Phosphoramidon competitively inhibited pseudomonas elastase, the K_i value being 0.25 μM. Morihara & Tsuzuki (1978) found a K_i value of 0.04 μM using (benzoyloxycarbonyl)glycyl-L-leucinamide as substrate, and Nishino & Powers (1980) found a K_i value of 0.06 μM using 2-aminobenzoyl-L-alanylglycyl-L-leucyl-L-alanyl-4-nitrobenzylamide as substrate. The value of K_i for pseudomonas elastase with PO_3LeuPhe as the inhibitor and furylacryloylglycyl-L-leucyl-L-alanine as the substrate was reported to be 0.2 μM (Kessler et al., 1982). This value is similar to the one obtained in this study, 0.26 μM. Regardless of the assay used, the relative order of inhibition we observed was PO_3LeuTrp > PO_3LeuPhe $\sim$ phosphoramidon > PO_3LeuPheNH$_2$ (Table III). A similar difference in the degree of inhibition by PO_3LeuTrp, phosphoramidon, and PO_3LeuPhe of the proteolytic activity of thermolysin has been observed (Komiyama et al., 1975; Kam et al., 1979).

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