Table IV. Microscopic pK_a Values Calculated from (a) NMR Data Alone, (b) Potentiometric pK_a Values and K_t from NMR, (c) Potentiometric pK_a Values and K_t from NMR plus Correction (See Text; K_t = 1.98)

	pK_1	pK_2	pK_{1A}	pK_{1B}	pK_{2A}	pK_{2B}
(a)	6.53	7.39	7.00	6.71	6.91	7.21
(b)	6.41	7.26	6.88	6.58	6.79	7.08
(c)	6.36	7.31	6.83	6.54	6.84	7.13

recorder; the signal was the smoothed output of an instantaneous (reciprocal of interval) rate meter, which had been triggered by a force transducer attached to the muscle. The muscle was loaded with 400-mg tension. Agonists and antagonists were added to the bath by micrometer syringe. Compounds were tested in this in vitro preparation in the presence of propanolol (0.5 μM).

Agonist Activity. Agonist activity was assayed up to a concentration of 781 μM (0.1% histamine), and relative activities were assessed from cumulative dose–response curves. Construction of complete dose–response curves to histamine and test compounds were used to determine maximum responses obtainable and relative potencies were determined from concentrations required to elicit 50% of maximal responses. For selected compounds indicated in Table I, parallel line assays using a 2 + 2 Latin square design were used to compare agonist potency with that of histamine.

Antagonist Activity. The dissociation constant (K_B) was calculated from the equation $K_B = B/(x = 1)$, where x is the respective ratio of concentrations of histamine needed to produce half-maximal responses in the presence and absence of different concentrations (B) of antagonist and $-\log K_B = pA_2$.

Acknowledgment. We are pleased to acknowledge the contribution of R. Davies, the late C. S. Whyatt (Synthetic Chemistry), R. C. Blakemore (Pharmacology), M. J. Graham (microanalytical and pK_a data), and D. W. Darkin (HPLC).

Registry No. 1, 55273-05-7; 1·3$C_6H_3N_3O_7$, 55273-06-8; 1·2$C_2H_2O_4$, 63169-77-7; 1·3HCl, 65573-02-6; 2·3$C_6H_3N_3O_7$, 97043-24-8; 3·3$C_6H_3N_3O_7$, 81282-31-7; 4·3$C_{10}H_8N_4O_5$, 97043-26-0; 4 (benzoyl derivative), 97043-27-1; 5·3$C_6H_3N_3O_7$, 63169-82-4; 6, 97043-29-3; 6·3HCl, 97043-28-2; 6 (cyano derivative), 97043-35-1; 7·3$C_{10}H_8N_4O_5$, 97043-30-6; 8, 97043-31-7; 8·3HCl, 60078-40-2; 9, 55272-99-6; 9·3HCl, 55273-01-3; 9 (benzoyl derivative), 97059-56-8; 10·3$C_{10}H_8N_4O_5$, 97043-33-9; 10 (cyano derivative), 97043-34-0; 11·3$C_6H_3N_3O_7$, 63779-37-3; 12·2$C_6H_3N_3O_7$, 97043-37-3; 12 (cyano derivative), 63779-32-8; 13, 97043-38-4; 14·$^3/_2C_2H_2O_4$, 97059-58-0; 15, 97043-39-5; 16·3$C_6H_3N_3O_7$, 97043-41-9; 17, 97043-43-1; 17·3HCl, 97043-42-0; 18·3$C_{10}H_8N_4O_5$, 97043-45-3; 19, 97043-47-5; 19·3HCl, 97043-46-4; 19·3HI, 97043-48-6; 20, 70172-53-1; 20·2HCl, 58726-90-2; 21, 97043-49-7; 21·2HCl, 52568-77-1; 22·2$C_6H_3N_3O_7$, 97043-51-1; 23, 97043-53-3; 23·HI, 97043-52-2; 24, 97043-54-4; 24·H_2SO_4, 97043-55-5; 25, 97043-57-7; 25·HI, 97043-56-6; 26, 97043-59-9; 26·2HCl, 97043-58-8; 27, 55272-97-4; 27·HI, 55272-96-3; 27·H_2SO_4, 55272-98-5; 28, 33551-01-8; 28·2HI, 40836-60-0; Ia, 38585-67-0; Ib, 40546-33-6; Ib (acetyl derivative), 97043-64-6; Ic, 38585-66-9; Id, 40546-47-2; Ie, 51-45-6; If, 34034-74-7; If·2HCl, 33544-95-5; Ig, 38603-99-5; Ih, 63779-34-0; Ih·2HCl, 33545-16-3; IIa, 38603-54-2; IIb, 34970-64-4; IIb·HI, 40836-59-7; VIIa, 60588-77-4; IXi, 10191-60-3; IXk, 24786-18-3; IXl, 81282-38-4; Xl, 97043-60-2; Xm, 52378-40-2; Xn, 63169-79-9; Xo, 97043-61-3; Xp, 97043-62-4; Xq, 63809-73-4; XII, 81282-23-7; XIII, 97043-66-8; XIV (R = a), 81282-28-2; XIV (R = c), 97043-63-5; XV (R′ = a)·HI, 55272-82-7; bromoacetaldehyde diethyl acetal, 2032-35-1; phenoxythioacetamide, 35370-80-0; cysteamine hydrochloride, 156-57-0; 4-phthalimidothiobutyramide, 41306-76-7; 2-(phenoxymethyl)-thiazole hydrobromide, 97043-65-7; *N*-(3-thiazol-2-ylpropyl)-phthalimide hydrobromide, 33545-15-2; benzoyl isothiocyanate, 532-55-8; *N*-benzoyl-*N*′-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]thio]ethyl]thiourea, 38603-53-1; *N*,*S*-dimethylisothiourea hydriodide, 41306-45-0; 4-(3-chloropropyl)imidazole hydrochloride, 51722-01-1; *N*-benzoyl-*N*′-[3-(1*H*-imidazol-4-yl)propyl]thiourea, 33550-99-1; diphenyl carbonate, 102-09-0; phenyl *N*-[2-[[(5-methyl-1*H*-imidazol-4-yl)methyl]thio]ethyl]carbamate, 97059-59-1; *N*-[2-[[(5-methyl-1*H*-imidazol-4-yl)methyl]thio]ethyl]dithiocarbamic acid, 55317-80-1.

N^{α}-(Diphenoxyphosphoryl)-L-alanyl-L-proline, N^{α}-[Bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline, and N^{α}-[(2-Phenylethyl)phenoxyphosphoryl]-L-alanyl-L-proline: Releasers of Potent Inhibitors of Angiotensin Converting Enzyme at Physiological pH and Temperature

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The rate of loss of phenol or 4-nitrophenol from N^{α}-(diphenoxyphosphoryl)-L-alanyl-L-proline (**2**), N^{α}-[bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (**5**), and N^{α}-[(2-phenylethyl)phenoxyphosphoryl]-L-alanyl-L-proline (**12**) was determined spectrophotometrically at pH 7.5 and 37 °C in both Tris and phosphate buffers. These moderately potent inhibitors of angiotensin converting enzyme ($K_i > 0.8$ μM) all hydrolyze, losing 1 mol of phenol to yield highly potent inhibitors (K_i = 0.5–18 nM). The half-times for loss of 1 mol of phenol in Tris buffer are 22 days (**2**), 3.4 h (**5**), and 21 days (**12**). The half-times in phosphate buffer were not significantly different. The mono(4-nitrophenoxy) ester **6** (K_i = 18 nM) loses its 1 mol of nitrophenol with a half-time of 35 h to yield N^{α}-phosphoryl-L-alanyl-L-proline **16** (K_i = 1.4 nM), which hydrolyzes at the P–N bond with a half-time of 2.2 h. Hydrolysis of the P–N bond in **2** and **12** was not observed during the time course of the kinetic experiments. The two phosphoramidate diesters **2** and **5** and the phosphonamidate monoester **12** thus release powerful inhibitors of angiotensin converting enzyme with a known time course at physiological pH and temperature in vitro. A time-dependent increase in inhibitory potency against converting enzyme that paralleled the kinetics of phenyl ester hydrolysis was confirmed in vitro.

Phosphoramidates, phosphonamidates, and phosphonic and phosphinic acids have been shown to be powerful inhibitors of angiotensin converting enzyme.[1-6] In these inhibitors the tetrahedral phosphorus atom is thought to

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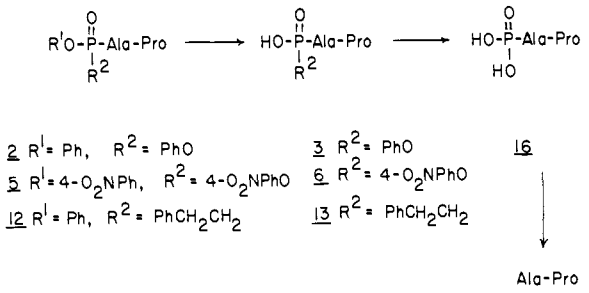
Scheme I



mimic a tetrahedral species that occurs during substrate hydrolysis and is strongly bound to the enzyme. The phosphoramidate ester phosphoramidon has been shown to bind in this manner to thermolysin by X-ray crystallography.[7] Although other inhibitors of converting enzyme such as captopril and enalapril are orally active antihypertensive agents,[5,8] phosphorus-containing compounds have not been widely reported to possess this activity.

We show here that two phosphoramidates and one phosphonamidate with all P–OH groups masked by phenyl esters are moderate inhibitors but hydrolyze nonenzymatically at pH 7.5 and 37 °C to regenerate the P–OH functional group and yield potent inhibitors. These esters thus release potent inhibitors of converting enzyme in vitro with pseudo-first-order kinetics at physiological pH and temperature.

N^{α}-(Diphenoxyphosphoryl)- (**2**), N^{α}-[bis(4-nitrophenoxy)phosphoryl]- (**5**), and N^{α}-[(2-phenylethyl)phenoxyphosphoryl]-L-alanyl-L-proline (**12**) hydrolyze to give the much more potent N^{α}-(phenoxyphosphoryl)- (**3**), N^{α}-[(4-nitrophenoxy)phosphoryl]- (**6**), and (phenethylphosphoryl)-L-alanyl-L-proline (**13**), respectively. Cleavage of the P–N bond was observed only for N^{α}-[bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (**5**) and occurred at a rate indistinguishable from the rate of loss of the second mole of nitrophenol. Therefore P–N bond cleavage is slow in the mono- and diesters at pH 7.5 and occurs at a significant rate only from the unsubstituted phosphoramidate phosphoryl-L-alanyl-L-proline (**16**). The time course of increasing inhibition of angiotensin converting enzyme in vitro paralleled the kinetics of hydrolysis of the phenyl esters. Scheme I shows the structures of the inhibitors and the proposed dominant hydrolysis pathway for N^{α}-[bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (**5**) at pH 7.5.

Results

Table I give the K_i's of the fully esterified (on phosphorus) inhibitors **2**, **5**, and **12** and their hydrolysis products determinated at 25 °C under conditions of minimum hydrolysis. The K_i of N^{α}-[bis(4-nitrophenoxy)-phosphoryl]-L-alanyl-L-proline (**5**) was determined on a stock solution of **5** in ethanol kept at 0 °C with a 5 min time point instead of the 30-min time point described in the Experimental Section. Since its hydrolysis is fast ($t_{1/2}$ = 4 h at 37 °C, the measured K_i of 0.8 μM is an underestimate of its true K_i, which is not likely to be different from the K_i's of around 50 μM observed for the more stable esters **2** and **12** that have half-times of 22 and 21 days, respectively, at 37 °C. All inhibitors fully esterified on phosphorus (**2**, **5**, **12**), are less potent by a factor of up to 10000-fold compared to their respective monoesters **3** and **6** and the unesterified phosphonic amide **13** derived from **12**. The phosphoryl-L-alanyl-L-proline monoesters (on phosphorus) have K_i's that increase in the order Me, Et, Bzl, *i*-Pr < Ph < 4-O_2NPh, with **13**, the phosphonic analogue of the monobenzyl ester, being more potent than any ester (see ref 2 and Table I). This result suggests that the K_i decreases with increasing electron-withdrawing capacity of the alcoholic substituent, irrespective of the size of the substituent. Therefore, the pK_a of the phosphoramidate

Table I. Inhibition of Angiotensin Converting Enzyme by Esters of Phosphoryl-L-alanyl-L-proline (pH 7.5; 100 mM Tris Buffer, 300 mM in NaCl)

inhibitor	K_i ± SD, μM
$(PhO)_2P(O)$AlaPro-DCHA (**2**)	42 ± 5
$(PhO)P(O)(O^-K^+)$AlaPro$^-K^+$ (**3**)	0.008 ± 0.001[a]
$K_2^+PO_3$AlaPro$^-K^+$ (**16**)	0.0014 ± 0.001[b]
AlaPro	50 ± 15[c]
$(4\text{-}O_2NPhO)_2P(O)$AlaPro (**5**)	0.8 ± 0.1[d]
$(4\text{-}O_2NPhO)P(O)(O^-Na^+)$AlaPro$^-Na^+$ (**6**)	0.018 ± 0.005
$PhCH_2CH_2P(O)(OPh)$AlaPro (**12**)	55 ± 10
$PhCH_2CH_2P(O)(O^-Na^+)$AlaPro$^-Na^+$ (**13**)	0.0005 ± 0.0002[e]
$(i\text{-}PrO)P(O)(O^-K^+)$AlaPro$^-K^+$ (**15**)	0.0014 ± 0.0002[f]

[a] IC_{50} = 0.9 μM, pH 8, 0.05 M borate/phosphate, 0.17 M in NaCl.[4] [b] Reference 2; IC_{50} = 0.7 μM.[4] [c] K_i = 30 μM, pH 8.3, 100 mM phosphate, 0.3 M in NaCl.[9] [d] The true K_i of **5** may be underestimated due to hydrolysis to **6** during the assay. [e] Reference 3; IC_{50} = 0.007 μM.[5] [f] K_i's for other monoesters (on phosphorus) of phosphoryl-L-alanyl-L-proline are as follows: Me, 0.0026 μM; Et, 0.0012 μM; Bzl, 0.0012 μM.[2]

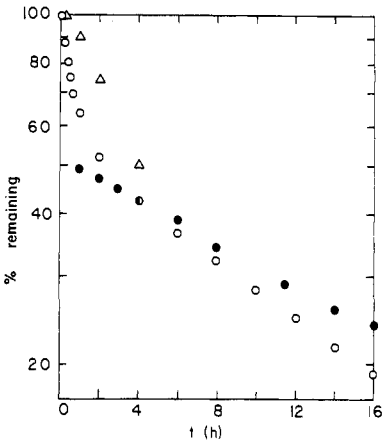


Figure 1. Kinetics of the loss of both 4-nitrophenol groups from N^{α}-[bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (**5**) determined spectrophotometrically (○, where 100% is 2 mol of 4-nitrophenol remaining). Kinetics of cleavage of the P–N bond in **5** determined by assay for the amino group of free AlaPro (●, where 50% equals 1 mol of blocked AlaPro). Kinetics of loss of the first 4-nitrophenol group from **5** determined by assaying the inhibitor potency of the reaction mixture against converting enzyme and assuming that most of the inhibition during the first 4 h of reaction is due only to **6** (△).

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Table II. Rate Constants (k) and Half-Times ($t_{1/2}$) for the Loss of 1 mol of Phenol from 2 and 5, for the Loss of Each Mole of 4-Nitrophenol from 12, and for the Observed Increase in Inhibitory Potency against Angiotensin Converting Enzyme (pH 7.5, 100 mM buffer, 300 mM in NaCl, 37 °C)

inhibitor	buffer	1st mole of phenol		2nd mole of phenol		inhibn
		k, h^{-1}	$t_{1/2}$	k, h^{-1}	$t_{1/2}$	$t_{1/2}$
$(PhO)_2P(O)AlaPro$ (2)	Tris	0.0013 ± 0.0002	22 ± 4 days			16 days
	phosphate	0.0012	24 days			
$(4\text{-}O_2NPhO)_2P(O)AlaPro$ (5)	Tris	0.2 ± 0.04	3.4 ± 0.7 h	0.025 ± 0.006	29 ± 9 h	4 h
	phosphate	0.34	2.0 h	0.031	22 days[a]	
$PhCH_2CH_2P(O)(OPh)AlaPro$ (12)	Tris	0.0014 ± 0.0002	21 ± 4 days			38 days
	phosphate	0.001 ± 0.0002	31 ± 4 days			

[a] The observed rate constant for appearance of AlaPro, indicating P–N bond cleavage, was 0.038 h^{-1}; $t_{1/2}$ = 18 h.

may play a critical role in the strength of its binding to the enzyme.

Table II gives the observed rate constants and half-times for the loss of 1 or 2 mol of phenol and for the observed increase of innhibitory potency against converting enzyme for 2, 5 and 12. Cleavage of the P–N bond during the time course of hydrolysis, as determined by fluorescamine assay of the appearance of a primary amine, was significant only for 5 and occurred at a rate (k = 0.038 h^{-1}) indistinguishable from that of the loss of the second mole of 4-nitrophenol from 5 (k = 0.031 h^{-1}) in phosphate buffer. The rate constant for P–N bond cleavage in N^{α}-phosphoryl-L-alanyl-L-proline was determined to be 0.32 ± 0.2 h^{-1} by the fluorescamine assay. The rate of the observed P–N bond cleavage in 5 is thus limited by the rate of loss of the second 4-nitrophenoxy group. Therefore, the predominant P–N bond cleavage at pH 7.5 must occur from N^{α}-phosphoryl-L-alanyl-L-proline (16) and not from N^{α}-[(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (6). Figure 1 shows a semilog plot of the percentage of N^{α}-[bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (5) remaining as a function of time at pH 7.5 and 37 °C determined by three methods: the spectrophotometric loss of 4-nitrophenol, cleavage of the P–N bond determined by fluorescamine assay of primary amine, and the decrease in K_i of the solution assayed against converting enzyme. Since 2 mol of 4-nitrophenol are released from 5, 100% remaining corresponds to 2 mol of 4-nitrophenol ester remaining. The rate constants were calculated for each mole of 4-nitrophenol, i.e., 1 mol of 4-nitrophenol = 50% in Figure 1. Graphs similar to Figure 1 were obtained for 2 and 12 except that only 1 mol of phenol was observed to be released and significant (<5% of phenol released); P–N bond cleavage was not observed. The rate of loss of phenol from 2 was not changed by the presence of 0.1 nM angiotensin converting enzyme (not shown). The K_i of a solution of N^{α}-[ethyloxy(2-phenylethyl)phosphoryl]-L-alanyl-L-proline[3] at 37° Tris buffer was unchanged after 2 days in contrast to a solution of the phenyl ester 12 whose K_i dropped from 55 μM to about 100 nM.

Discussion

The K_i's of the fully esterified compounds in Table I are all much higher than those of the inhibitors with at least one ionizable P–OH group. As suggested in Results, the true K_i of N^{α}-[bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (5) is probably greater than the measured K_i of 0.8 μM due to partial hydrolysis to 6 (K_i = 18 nM) during the assay. The K_i's are consistent with our previously reported K_i's for derivatives of 16 and 13.[2,3] The increase in K_i with increasing electron-withdrawing capacity of the alcohol in the monoesters of phosphorylalanylproline (Me, Et, i-Pr, Bzl < Ph < 4-O_2NPh; Table I) suggests that lowering the pK_a of the phosphoramidate decreases binding to the enzyme. This is consistent with the observation that amides of the strong acid sulfuric acid (low pK_a) are very poor inhibitors of the zinc metalloprotease thermolysin compared to the analogous phosphoric amides[10] (higher pK_a). Surprisingly, the methyl, ethyl, isopropyl, and benzyl esters and unesterified phosphorylalanylproline are indistinguishable in potency. Therefore, the alcohol in this position appears not to interact with the enzyme in any way.

The rate constants given in Table II demonstrate that the release of the first mole of phenol from 2 occurs with a rate indistinguishable from that of 12. No cleavage of the P–N bond in 2 or 12 was observed at pH 7.5 in agreement with the results of Poncz et al.[11] for phosphoramidon and Sampson et al.[12] for N-(phenoxyphosphoryl)glycine. The rate constant at pH 7.5 and 37 °C for 2, 0.0013 h^{-1}, is also consistent with the observed rate constant for hydrolysis of O-methyl O'-phenyl phosphoramidate at pH 7.5 and 28 °C, 0.0004 h^{-1} (calculated from k_{OH} = 21 $L \cdot mol^{-1} \cdot min^{-1}$, assuming that base hydrolysis makes the major contribution to release of phenol at pH 7.5[13]). The spectrophotometrically determined rate of release of phenol from 2 was not accelerated in the presence of 0.1 nM converting enzyme.

The rates of the three successive hydrolysis reactions of N^{α}-[bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (5) that occur at pH 7.5 have been measured. Release of the first mole of 4-nitrophenol to give the monoester 6 occurs with a rate constant of 0.2 ± 0.04 h^{-1} ($t_{1/2}$ = 3.4 h). Release of the second mole of 4-nitrophenol to give phosphoryl-L-alanyl-L-proline (16) occurs with a rate constant of 0.025 ± 0.006 h^{-1} ($t_{1/2}$ = 29 h). Phosphorylalanylproline (16) releases alanylproline with a rate constant of 0.32 h^{-1} ($t_{1/2}$ = 2.2 h) as determined independently with pure 16. This rate constant is similar to that determined for phosphorylleucylphenylalanine (K = 0.63 h^{-1}) at pH 7.3 and 37 °C.[11] The fact that P–N bond cleavage at pH 7.5 occurs at a rate indistinguishable from the rate of release of the second mole of 4-nitrophenol from 5 (see Table II) and that phosphoramidate monoesters in general do not exhibit P–N bond cleavage at neutral and moderate pH[11,12] demonstrates that P–N bond cleavage in 6 occurs only after hydrolysis to phosphoryl-L-alanyl-L-proline (16). The rates of release of 4-nitrophenol from 6 and phenol from 2 differ by a factor of 150-fold. This relative rate difference is qualitatively consistent with the reported rates of base-catalyzed hydrolysis of diphenyl methylphosphoramidate and bis(3-nitrophenyl) methylphosphoramidate, which differ by a factor of 700-fold.[14]

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In summary, the three phenyl esters **2**, **5** and **12** described here release potent inhibitors of angiotensin converting enzyme in vitro. The rate of phenol release is not affected by enzyme, and the ethyl ester analogue of **12** is not a time-dependent inhibitor in vitro. The half-time for release of phenol from **2** and **12**, about 3 weeks, is probably too long to be useful to release inhibitors in vivo. The half-time for release of 4-nitrophenol from **5**, about 3 h, is probably in the correct range, but the toxicity of 4-nitrophenol probably precludes these esters as potential therapeutic agents. Esters of nontoxic alcohols with hydrolysis half-times in the range of that of the nitrophenyl ester (**5**) may be of interest as orally active inhibitors of converting enzyme or as models for releasing phosphorus-containing inhibitors of other enzymes.

Experimental Section

Dicyclohexylcarbodiimide, *p*-nitrophenol, phosphorus oxychloride, and diphenyl phosphorochloridate were obtained from Aldrich Chemical Co. Other chemicals were of analytical grade and were used without further purification. The molecular weights of protected intermediates were determined by mass spectra taken on a Finnigan 3300 GC-MS spectrometer (EMV = 2100, electron energy = 150 eV, methane chemical ionization). Melting points were taken on a hot stage and are uncorrected. Proton nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-390. Chemical shifts are in ppm downfield from tetramethylsilane $(CH_3)_4Si$ in organic solvents. Thin-layer chromatography was on silica gel 60-F254. Compounds were visualized by the following methods: exposure to hydrogen chloride vapor followed by ninhydrin (0.6 g in 100 mL of acetone) for protected and deprotected peptides, phosphomolybdate spray for phosphoryl and phosphonyl peptides,[15] ultraviolet light, and iodine vapor. Thin-layer solvent systems were by volume as follows: (A) chloroform/acetonitrile/hexane, 2:2:1; (B) chloroform/acetonitrile/2-propanol/acetic acid, 3:1:0.5:0.2; (C) chloroform/acetonitrile/methanol, 2:2:1; (D) 2-propanol/concentrated ammonia, 84:32; (E) chloroform/methanol/acetic acid, 85:10:5; (F) *n*-butanol/acetic acid/water, 4:1:1.

All synthesized compounds were characterized by thin-layer chromatography, proton NMR spectrum, and melting point if crystalline. All compounds except sodium and potassium salts were also characterized by their mass spectrum. The homogeneity of the products was judged by thin-layer chromatography and NMR spectrum. The NMR spectrum was also a criterion for the identity of the isolated products, and the mass spectrum confirmed that the desired product was indeed present. After these criteria were satisfied, all of the products were used without further purification and without microanalysis.

N^{α}-(Diphenoxyphosphoryl)-L-alanyl-L-proline Benzyl Ester (1). To a mixture of 25 g (0.008 mol) of the hydrochloride salt of L-alanyl-L-proline benzyl ester[2] and 2.3 mL of *N*-ethylmorpholine in 15 mL of anhydrous methylene chloride was added 2.15 g (0.008 mol) of diphenyl phosphorochloridate (Aldrich 98% pure) at 0 °C. After 20 h at 4 °C, the reaction mixture was diluted to 150 mL with methylene chloride and washed successively with 0.1 N hydrochloric acid (2 × 25 mL), water (2 × 25 mL), 5% sodium bicarbonate (2 × 25 mL), water, and saturated sodium chloride, dried over anhydrous magnesium sulfate, and evaporated, yielding 3.89 g (96% yield) of **1** as a syrup; R_f(A) 0.7; ^{1}H NMR ($CDCl_3$) δ 1.26 (3 H, d, Ala CH_3), 1.95 (4 H, m, Pro CH_2 β,γ), 3.45 (2 H, m, Pro CH_2 δ), 4.05–4.55 (3 H, m, Pro CH α, Ala CH α, PNH), 5.1 (2 H, 7, BzL CH_2), 7.25 (15 H, m, PhO, Bzl, Ph); mass spectrum (chemical ionization) m/e 509 (M + 1).

N^{α}-(Diphenoxyphosphoryl)-L-alanyl-L-proline Dicyclohexylamine Salt (2). Hydrogen gas was bubbled for 1 h through a mixture of 3.89 g (0.0076 mol) of **1** and 0.7 g of 5% palladium on carbon in 20 mL of absolute ethanol. After removal of the catalyst by filtration, the filtrate was partially evaporated and 1.45 mL of dicyclohexylamine was added. The solvent was evaporated under reduced pressure, the resulting colorless syrup was dissolved in 70 mL of anhydrous ether, and 50 mL of hexane was added. The mixture was kept for 24 h in a freezer (–10 °C). The precipitate was collected, washed with hexane, and dried to give 3.9 g (90% yield) of N^{α}-(diphenoxyphosphoryl)-L-alanyl-L-proline dicyclohexylamine salt (**2**); mp 117–121 °C; R_f (B) = 0.6; ^{1}H NMR ($CDCl_3$) δ 0.9–2.35 (27 H, m, c-Hx CH_2, Ala CH_3, Pro CH_2 β,γ), 2.90 (2 H, m, c-Hx CH), 3.50 (2 H, m, Pro CH_2 δ), 4.05–4.70 (3 H, m, Pro CH α, Ala CH α, PNH), 7.30 (10 H, m, PhO), 8.85 (2 H, br s, Pro OH, c-Hx NH); mass spectrum (chemical ionization) m/e 387 (M + 1 – dicyclohexylamine).

N^{α}-(Phenoxyphosphoryl)-L-alanyl-L-proline Dipotassium Salt (3). To 209 mg (0.4 mmol) of **2** in 4 mL of water was added 0.8 mL (0.8 mmol) of 1 N KOH. The reaction mixture was allowed to stand overnight and then was extracted five times with an equal volume of ether. The aqueous layer was freeze-dried to give 0.13 g (85% yield) of **3**, a powder: R_f(D) 0.30, R_f(F) 0.07; ^{1}H NMR (10% D_2O in Me_2SO-d_6) δ 1.67 (3 H, d, Ala CH_3), 2.36 (4 H, m, Pro CH_2 β,γ), 3.93 (2 H, m, Pro CH α, Ala CH α), 7.67 (5 H, m, Ph).

N^{α}-[Bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline *tert*-Butyl Ester (4). To a mixture of 1.88 g (0.0068 mmol) of the hydrochloride salt of L-alanyl-L-proline *tert*-butyl ester[16] and 1.72 mL (0.0136 mol) of *N*-ethylmorpholine in 10 mL of anhydrous methylene chloride was added 2.43 g (0.0068 mol) of bis(4-nitrophenyl) phosphorochloridate[17] at 0 °C. The reaction mixture was stirred for 2.5 h at 0 °C and for 4 h at room temperature, diluted to 150 mL with methylene chloride, washed with 0.1 N hydrochloric acid (2 × 25 mL), water (2 × 25 mL), and saturated sodium chloride, and dried over anhydrous magnesium sulfate. Evaporation of solvent gave 2.53 g of crude product, which was purified by crystallization from a mixture of methylene chloride/ether/hexane to give 1.8 g (48% yield) of **4**: mp 119–121 °C; R_f(A) 0.75; ^{1}H NMR ($CDCl_3$) δ 1.40 (12 H, m, Ala CH_3, *t*-Bu), 2.05 (4 H, m, Pro CH_2, β,γ), 3.58 (2 H, m, Pro CH_2 δ), 4.35 (2 H, m, Ala CH α, Pro CH α), 5.1 (1 H, m, PNH), 7.45 (4 H, m, 4-O_2NPh), 8.25 (4 H, m, 4-O_2NPh); mass spectrum (chemical ionization) m/e 565 (M + 1).

N^{α}-[Bis(4-nitrophenoxy)phosphoryl]-L-alanyl-L-proline (5). A 1.7-g sample (0.00301 mol) of **4** was dissolved in 3 mL of anhydrous trifluoroacetic acid at room temperature. After about 20 min 100 mL of anhydrous ether was added and the reaction mixture was kept for 24 h in the freezer (–10 °C). The colorless cyrstals of product **5** were filtered off and washed with a fresh portion of anhydrous ether. The addition of 50 mL of hexane to the filtrate caused additional precipitation of product, which was filtered off, washed with hexane, and dried under reduced pressure to give 1.3 g (84%) of **5**: mp 76–83 °C; R_f(B) 0.6; ^{1}H NMR ($CDCl_3$) δ 1.30 (3 H, m, Ala CH_3), 2.05 (4 H, m, Pro CH_2 β,γ), 3.52 (2 H, m, Pro CH_2 δ), 4.34 (2 H, m, Ala CH α, Pro CH α), 5.10 (1 H, m, PNH), 7.35 (4 H, m, 4-O_2NPh), 8.20 (4 H, m, 4-O_2NPh), 10.40 (1 H, s, Pro OH).

N^{α}-[(4-Nitrophenoxy)phosphoryl]-L-alanyl-L-proline Disodium Salt (6). A mixture of 0.254 g (0.0005 mol) of **4**, 0.084 g (0.001 mol) of sodium bicarbonate, and 2 mL of water in 4 mL of tetrahydrofuran was stirred for 2.5 h at room temperature. The organic solvent was evaporated under reduced pressure, and the residue was diluted to 15 mL with water, frozen, and lyophilized. The yellow solid residue was washed with 20 mL of anhydrous acetone and 20 mL of ether to give 0.18 g (86% yield) of disodium salt **6**: ^{1}H NMR (D_2O) δ 1.2 (3 H, m, Ala CH_3), 1.85 (4 H, m, Pro CH_2 β,γ), 3.40 (2 H, m, Pro CH_2 δ), 3.70–4.40 (2 H, m, Pro CH α, Ala CH α), 7.22 (2 H, m, 4-O_2NPh), 8.12 (2 H, m, 4-O_2NPh).

N^{α}-[(2-Phenylethyl)phenoxyphosphoryl]-L-alanyl-L-proline Benzyl Ester (11). To a solution of 8.26 g (0.044 mol) of (2-phenylethyl)phosphonic acid[18] in 15 mL of anhydrous chloroform was added 21.25 g (0.102 mol) of phosphorus pentachloride at 0 °C. The mixture was stirred for 30 min at 0 °C and for 2 h at room temperature. Anhydrous hexane (20 mL) was added, and unreacted phosphorus pentachloride was filtered off. The

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filtrate was evaporated under reduced pressure, and the residue was dried under high vacuum to give 9.87 g (100% yield) of 2-phenylethyl phosphorodichloridate (**7**). To a mixture of 6.76 g (0.072 mol) of phenol and 9.15 mL (0.072 mol) of *N*-ethylmorpholine in 100 mL of anhydrous tetrahydrofuran was added 8 g (0.036 mol) of **7** slowly (15 min) at room temperature. The reaction mixture was stirred for 2 h at room temperature and for 2 h at 50 °C. The hydrochloride salt of *N*-ethylmorpholine was filtered off and washed with ether (2 × 30 mL), and the filtrate was evaporated to dryness. The residue was diluted to 150 mL with chloroform and washed successively with 0.1 N HCl (25 mL), water (2 × 25 mL), 5% sodium bicarbonate, and saturated sodium chloride and dried over anhydrous magnesium sulfate. Evaporation of the solvent gave 10.9 g (90%) of (2-phenylethyl)-phosphonic acid diphenyl ester (**8**), which was used in next step without further purification. **8** (10.9 g, 0.032 mol) was refluxed with 40 mL of 2 N potassium hydroxide in 100 mL of tetrahydrofuran for 1.5 h and evaporated. Water (20 mL) was added to the residue, and the pH of the solution was adjusted to pH 6 with concentrated hydrochloric acid. The solution was washed with ether (5 × 20 mL) and strongly acidified with concentrated hydrochloric acid. The monoester was extracted from the water phase with chloroform (3 × 50 mL). The organic phase was washed with saturated sodium chloride and dried over anhydrous magnesium sulfate. Evaporation of solvent gave 7.8 g (93% yield) of phenyl (2-phenylethyl)phosphonate (**9**), which was used in the next step without further purification: ^{1}H NMR ($CDCl_3$) δ 2.15 (2 H, m, PCH_2), 2.88 (2 H, m, Ph CH_2), 7.15 (10 H, m, Ph), 11.76 (1 H, s, POH). **9** (7.8 g, 0.0297 mol) was stirred in 20 mL of freshly distilled thionyl chloride overnight at room temperature. The excess of thionyl chloride was evaporated under reduced pressure to give 8.02 g (100% yield) of phenyl (2-phenylethyl)-phosphonochloridate (**10**): ^{1}H NMR ($CDCl_3$) δ 2.45 (2 H, m, PCH_2), 2.98 (2 H, m, Ph CH_2), 7.2 (10 H, m, Ph). **10** (3.15 g, 0.0112 mol) was coupled with the hydrochloride salt of L-alanyl-L-proline benzyl ester (3.5 g, 0.0112 mol) identically as for **1** to give **11** (65% yield) as a heavy syrup: R_f(A) 0.5; ^{1}H NMR ($CDCl_3$) δ 1.25 (3 H, m, Ala CH_3), 1.50–2.50 (6 H, m, Pro CH_2 β,γ, PCH_2), 2.70–3.70 (4 H, m, Pro CH_2 δ, Ph CH_2), 4.30 (3 H, br m, Ala CH α, Pro CH α, PNH), 5.05 (2 H, d, Bzl CH_2), 7.25 (15 H, m, Ph); mass spectrum (chemical ionization) *m/e* 521 (M + 1).

N^{α}-[(2-Phenylethyl)phenoxyphosphoryl]-L-alanyl-L-proline (12). Hydrogen gas was bubbled for 2 h through a mixture of 1.3 g (0.0025 mol) of **11** and 0.5 g of 5% palladium on carbon in 15 mL of absolute ethanol. After removal of the catalyst by filtration, the filtrate was evaporated to dryness, the residue was dissolved in the minimal volume of methylene chloride, and the title compound **12** was precipitated by addition of 50 mL of petroleum ether; yield 0.6 g (56%); mp 124–130 °C; R_f(B) 0.7; ^{1}H NMR ($CDCl_3$) δ 1.2 (3 H, m, Ala CH_3), 2.1 (6 H, m, Pro CH_2 β,γ, PCH_2), 2.95 (2 H, m, Ph CH_2), 3.45 (2 H, m, Pro CH_2 δ), 4.25 (3 H, m, Ala CH α, Pro CH α, PNH), 7.15 (10 H, m, Ph), 9.19 (1 H, br s, COOH); mass spectrum (chemical ionization) *m/e* 431 (M + 1).

N^{α}-(Isopropoxyphosphoryl)-L-alanyl-L-proline Dipotassium Salt (15). N^{α}-(Isopropoxyphenoxyphosphoryl)-L-alanyl-L-proline benzyl ester (**14**) was prepared by the successive reaction of phenyl phosphorodichloridate with 2-propanol and the hydrochloride salt of L-alanyl-L-proline benzyl ester as described[2] except that chloroform was used as solvent and *N*-ethylmorpholine as the base for both reactions. The product was isolated by diluting the reaction mixture into ether and extracting as described for **1**. Five millimoles of each reactant gave 1.22 g of **14**, an oil, in 55% yield: (C) R_f(C) 0.75; ^{1}H NMR ($CDCl_3$) δ 1.33 (9 H, m, Ala CH_3, *i*-Pr CH_3), 2.00 (4 H, m, Pro CH_2 β,γ) 3.40–4.50 (4 H, m, Pro CH_2 δ, *i*-Pr CH, PNH), 4.40–5.00 (2 H, m, Pro CH α, Ala CH α), 5.13 (2 H, d, Ph CH_2), 7.25 (11 H, m, Ph CH_2, PhO); mass spectrum (chemical ionization) *m/e* 475 (M + 1).

To 242 mg (0.54 mmol) of **14** in 3 mL of 2-propanol/water (1:2) was added 1.63 mmol of 1 N KOH. The mixture was refluxed for 30 min, evaporated to dryness, taken up in a minimum volume of methanol, and precipitated with acetone to give 152 mg (78% yield) of N^{α}-(isopropoxyphosphoryl)-L-alanyl-L-proline dipotassium salt (**15**): R_f(D) 0.23, R_f(E) 0.05; NMR (D_2O) δ 1.20 (10 H, m, Ala CH_3, *i*-Pr CH_3), 1.85 (4 H, m, Pro CH_2 β,γ), 3.30–4.40 (5 H, m, Pro CH α, Ala CH α, Pro CH_2 δ, *i*-Pr CH).

Angiotensin Converting Enzyme. Converting enzyme was partially purified[2] from frozen rabbit lungs, omitting the last step of lectin affinity chromatograph.[19] Yields were lower than those achieved by Das and Soffer.[19] The specific activity of converting enzyme averaged 19 units/mg of protein. One unit of converting enzyme hydrolyzes 1 μmol of hippurylhistidylleucine/min at 37 °C in 100 mM potassium phosphate buffer, pH 8.3, 300 mM in sodium chloride.

Enzyme Kinetics. The K_i's for all inhibitors except **5** were determined with hippurylhistidylleucine as substrate (K_m = 0.5 mM) in 50 mM Tris-HCl adjusted to pH 7.5 with sodium hydroxide, 300 mM in sodium chloride,[20,1] by the fluorometric assay and a single 30-min time point. A 5-min time point was used for **5**. The reaction was initiated by the addition of enzyme to a final concentration of 0.05 nM. At this enzyme concentration, depletion of inhibitor by enzyme is insignificant.[21] Hydrolysis was linear with time to well beyond 30 min, with less than 5% total substrate hydrolyzed for all substrate concentrations. The K_i's were determined by averaging the K_i's found from a Lineweaver–Burk plot and a Dixon plot. Every K_i was determined at least twice. When these K_i's are compared to K_i's and IC_{50}'s from the literature determined under different conditions, the buffer composition and pH are given for the literature references. The K_i's determined under different conditions are not strictly comparable.

The K_i's of solutions of inhibitors incubated at pH 7.5 and 37 °C in the absence of enzyme were determined as a function of incubation time by the procedure described above, except that a 5-min assay was used for incubation times of less than 2 h. The percentage of hydrolysis of **2** and **5** was calculated by assuming that the K_i observed at zero time represented no hydrolysis and thereafter only the first hydrolysis product, e.g., (PhO)P(O)-(OH)AlaPro (**3**) from $(PhO)_2P(O)$AlaPro (**2**), contributed to inhibition after zero time and that the final K_i reached at infinite time was equal to the K_i of this first hydrolysis product. Although some error was introduced at early time points by this approximation, the half-times calculated by the time dependence of K_i are not meant to be highly accurate but are merely for comparison with the spectrophotometic method. For $(4\text{-}O_2NPhO)_2P(O)$AlaPro (**5**), where three successive reactions occur at pH 7.5, only the half-time for loss of the first 4-nitrophenoxy group was calculated by this method, assuming that after 4 h of hydrolysis PO_3AlaPro **6**, the product of the subsequent hydrolysis, was not present in a concentration sufficient to contribute to the inhibition. That is, after 4 h most of the inhibition was due to **6**, $(4\text{-}O_2NPhO)P$-(O)AlaPro.

Spectrophotometric Hydrolysis Kinetics. The release of phenol from $(PhO)_2P(O)$AlaPro (**2**) and $PhCH_2CH_2P(O)(OPh)$-AlaPro (**12**) at concentrations around 1 mM at pH 7.5 and 37 °C was followed spectrophotometrically, using the change in extinction coefficient at 275 nm of 1375 au·M^{-1}·cm^{-1} in both the buffer described under Enzyme Kinetics and an identical buffer with phosphate substituted for Tris. The release of 4-nitrophenol from $(4\text{-}O_2NPhO_2)P(O)$AlaPro (**5**) at a concentration of around 10 μM was followed from the change in extinction coefficient at 400 nm of 12 500 au·M^{-1}·cm^{-1}. Initial concentrations of solutions of the esters were checked by completely hydrolyzing an aliquot in 6 N HCl at 110 °C and determining the absorbance of the phenol released in pH 7.5 buffer. The observed rate constant for loss of 1 mol of phenol, k, was calculated from a semilog plot of the percentage of initial ester remaining, where $d[\text{phenol}]/dt = -k[\text{phenyl ester}]$. Rate constants are calculated for loss of each mole of phenol. Thus, for **2** and **5**, 100% of initial diester remaining represents 2 mol of phenol remaining. The release of 1 mol of phenol was observed from **2** and **12** and 2 mol from **5**.

P–N Bond Cleavage Kinetics. Hydrolysis of the P–N bond in pH 7.5 phosphate buffer was measured by determination of the primary amino group of Ala-Pro essentially as described by Poncz et al.[11] using fluorescamine.[22] Since the fluoresamine assay

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is performed at pH 9 and 25 °C and requires only minutes to accomplish, significant P–N bond cleavage does not occur during the assay.[11]

Registry No. 1, 74406-94-3; 2·DCHA, 97280-40-5; 3, 82180-41-4; 3·2K, 97280-41-6; 4, 97280-42-7; 5, 97280-42-7; 6, 97293-86-2; 6·2Na, 97293-85-1; 7, 97280-44-9; 8, 14295-48-8; 9, 97280-45-0; 10, 97280-46-1; 11, 97280-47-2; 12, 97280-48-3; 13, 86053-89-6; 13·2Na, 82184-87-0; 14, 97280-49-4; 15, 97280-51-8; 15·2K, 97280-50-7; 16, 76166-63-7; L-alanyl-L-proline benzyl ester hydrochloride, 41591-35-9; diphenyl phosphorochloridate, 2524-64-3; L-alanyl-L-proline *tert*-butyl ester hydrochloride, 41591-35-9; bis(4-nitrophenyl) phosphorochloridate, 6546-97-0; (2-phenylethyl)-phosphonic acid, 4672-30-4; phenyl phosphorodichloridate,, 770-12-7; angiotensin converting enzyme, 9015-82-1.

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Selective Thromboxane Synthetase Inhibitors. 1. 1-[(Aryloxy)alkyl]-1*H*-imidazoles

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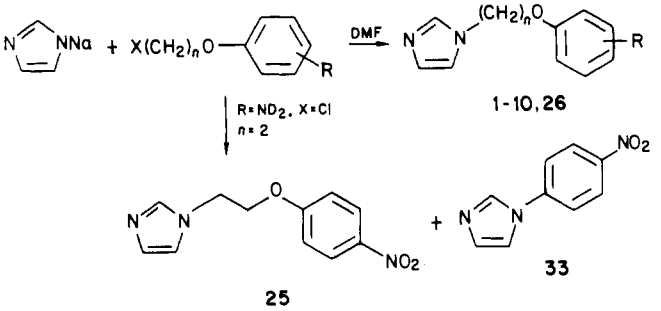
1-(2-Phenoxyethyl)-1*H*-imidazole was found to be an inhibitor of thromboxane (TxA_2) synthetase, but it also inhibited the adrenal cytochrome P-450 enzyme steroid 11β-hydroxylase. The preparation of a series of analogues is described, and activity against TxA_2 synthetase, PGI_2 synthetase, cyclooxygenase, and steroid 11β-hydroxylase is discussed. Potency against TxA_2 synthetase was increased by introduction of a carboxyl group at a suitable distance from the imidazole ring. A distance of 8.1–8.8 Å between N-1 of the imidazole and the carboxyl carbon was found to be optimal. Introduction of a carboxyl group also had the effect of reducing activity against steroid 11β-hydroxylase. The most potent and selective compound was found to be 4-[2-(1*H*-imidazol-1-yl)ethoxy]benzoic acid (14).

During the last decade, two new pathways of prostaglandin endoperoxide metabolism have been elucidated. First, Samuelsson et al.[1,2] reported that an enzyme in blood platelets, thromboxane synthetase, converts the endoperoxide PGH_2 into thromboxane A_2 (TxA_2). TxA_2 was found to be a potent vasoconstrictor and platelet aggregating agent and was thought to be involved in normal hemostasis.[3] In 1976, Vane et al.[4-9] reported the discovery of prostacyclin (PGI_2), which is produced from PGH_2 by an enzyme located in the endothelial lining of blood vessels. PGI_2 is a potent vasodilator and antiaggregatory agent. It was postulated that, under normal conditions, there is a balance between the opposing effects of TxA_2 and PGI_2, but in certain pathological situations the proaggregatory and vasoconstrictor actions of TxA_2 could predominate.[4-9] Thus, a compound that inhibits selectively the formation of TxA_2 may be useful in the treatment or prophylaxis of cardiovascular diseases where vasospasm or thrombosis plays a role. A potential advantage of such a compound would be that accumulated PGH_2 produced by platelet cyclooxygenase may be utilized by vascular PGI_2 synthetase, thereby increasing PGI_2 levels.[10,11] By contrast, although agents that inhibit cyclooxygenase would also prevent TxA_2 formation, there would normally be no possibility for increased PGI_2 production since no PGH_2 would be produced.

Scheme I



Imidazole has been reported to be a weak inhibitor of TxA_2 synthetase.[12,13] We[14] and other workers[15,16] found that the introduction of a lipophilic 1-substituent increased inhibitory activity, but substitution elsewhere in the imidazole ring was detrimental. Imidazole inhibitors of cytochrome P-450 enzymes such as those present in liver and adrenals follow the same structure–activity trend,[17-22]

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