disilazane, 4039-32-1; hexamethyldisilazane, 999-97-3.

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Inhibition of Aminopeptidases by Aminophosphonates†

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ABSTRACT: More than 30 aminophosphonates were synthesized to probe how the structural changes introduced into the phosphonic acid analogue of leucine, a potent inhibitor of cytosolic leucine aminopeptidase (Giannousis & Bartlett, 1987), affect their ability to inhibit cytosolic (EC 3.4.11.1) and microsomal (EC 3.4.11.2) aminopeptidases. Although most of the compounds studied were found to exert only a modest competitive inhibitory effect, nearly every modification of the structure of the phosphonic acid analogue of leucine was reflected in a marked difference in the affinities of these compounds for the two enzymes. [1-Amino-2-(*N*-alkylamino)ethyl]phosphonic acids are effective inhibitors of the microsomal enzyme, acting in a time-dependent manner. Kinetic data obtained for these inhibitors correspond to the mechanism for a biphasic slow-binding inhibition process: $E + I \rightleftharpoons E^* \rightleftharpoons E^*I$, in which the slow initial isomerization of the enzyme is followed by the fast formation of enzyme–inhibitor complex. The most effective inhibitor of this type was [1-amino-2-(*N*-cyclohexylamino)ethyl]phosphonic acid, which has a K_i value of 0.87 μM toward the microsomal aminopeptidase—a value that can be considered as equipotent with bestatin and with leucinal and hydroxamic acids, the strongest known nonpeptide inhibitors of this enzyme.

Aminopeptidases are a group of zinc-containing exopeptidases that catalyze the hydrolysis of N-terminal peptide bonds in polypeptide chains. Among these enzymes, leucine aminopeptidase has been the most extensively studied. However, by comparison with carboxypeptidases (also zinc-containing enzymes), structural and mechanistic information about this class of proteases is less extensive.

The biological importance of aminopeptidases is indicated by the fact that aminopeptidase activity has been detected in many mammalian tissues, organs, and bodily fluids and in cell membranes and plants (DeLange & Smith, 1971; Aoyagi et al., 1976).

The development of synthetic inhibitors of proteases is an active field of research that has provided insight into the nature of enzyme–substrate interactions during catalysis. Effective inhibitors reported for aminopeptidases include aminoketones

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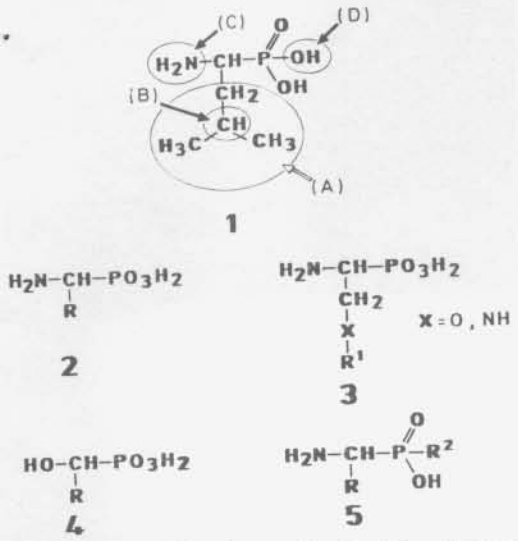

FIGURE 1: Phosphonic acid analogue of leucine (**1**) and its derivatives. Structural modifications of **1** were made at the points indicated with arrows: (A) leading to compounds **2**; (B) providing compounds **3**; (C) yielding compounds **4**; (D) leading to compounds **5**.

and their derivatives (Birch et al., 1972; Kettner et al., 1974), small peptide analogues from microbial sources (Umezawa et al., 1976; Aoyagi et al., 1978; Rich et al., 1984; Wilkes & Prescott, 1985), aliphatic and aromatic α-aminoboronic acids (Baker et al., 1983; Shenvi, 1986), amino acid hydroxamates (Chan et al., 1982; Baker et al., 1983; Wilkes & Prescott, 1983), α-aminoaldehydes (Andersson et al., 1982), amino acid thiols (Chan, 1983), and (aminoalkyl)phosphonic acids (Giannousis & Bartlett, 1987).

Since the discovery of phosphoramidon, *N*-[(α-L-rhamnopyranyloxy)phosphinyl]leucyltryptophan, a potent inhibitor of thermolysin (Komiyama et al., 1975), a number of phosphoramidate (Kam et al., 1979; Nishino & Powers, 1979; Kasai et al., 1983; Galardy & Grobelny, 1983, 1985) and phosphonamidate (Jacobsen & Bartlett, 1981; Thorsett et al., 1982; Bartlett & Marlowe, 1983; Galardy et al., 1983; Eliott et al., 1985; Yamauchi et al., 1985; Moothatiar et al., 1987) inhibitors for other bacterial and mammalian zinc metalloproteinases have been developed. This inhibition is thought to arise partly through the interaction of phosphoryl or phosphonyl oxygen with the active-site zinc ion (Weaver et al., 1977) and partly through the interaction of the amino acid side chains of the inhibitors with binding sites of the enzyme. The phosphoryl moiety has a tetrahedral geometry, and these compounds are thought by some investigators to be transition-state analogues (Jacobsen & Bartlett, 1981; Thorsett et al., 1982; Bartlett & Marlowe, 1983; Kasai et al., 1983; Galardy et al., 1983; Galardy & Grobelny, 1983, 1985; Eliott et al., 1985; Moothatiar et al., 1987).

Intrigued by the possibility that (aminoalkyl)phosphonic acids may act as potent inhibitors of aminopeptidases, we synthesized more than 30 of these compounds and tested them as inhibitors of cytosolic (EC 3.4.11.1) and microsomal (EC 3.4.11.2) aminopeptidases. A similar study was also recently reported (Giannousis & Bartlett, 1987). Our intention was to incorporate into the inhibitor structural features responsible for enzyme–substrate recognition. Thus, the phosphonic acid analogue of leucine (compound **1**, Figure 1), a potent inhibitor of cytosolic leucine aminopeptidase (Giannousis & Bartlett, 1987), was used as a model structure, and the structural changes introduced (illustrated in Figure 1) were as follows: (1) aminophosphonic acids (compounds **2**) with alkyl side chains with varying hydrophobic character; (2) aminophosphonates (compounds **3**) in which the γ-carbon atom of the alkyl side chain was replaced by an oxygen or an amino group; (3) hydroxyphosphonates (compounds **4**), i.e., compounds in which an amino function was replaced by hydroxyl; (4) derivatives **5** in which the phosphonyl hydroxy group was replaced by methyl, chloromethyl, phenyl, or methoxy groups.

Materials and Methods

Chemicals. The literature methods were used for the preparation of (1-aminoalkyl)phosphonic acids (Oleksyszyn et al., 1978), their monomethyl esters (Szewczyk et al., 1982; Giannousis & Bartlett, 1987), (1-amino-2-alkoxyethyl)-phosphonic and [1-amino-2-(*N*-alkylamino)ethyl]phosphonic acids (Zygmunt, 1985), (1-hydroxy-3-methylbutyl)phosphonic and (hydroxyphenylmethyl)phosphonic acids (Texier-Boullet & Lequitte, 1986), and (1-aminoalkyl)phosphinic acids (Petrillo et al., 1983; Diel & Maier, 1987). All the products were characterized by ^{1}H nuclear magnetic resonance, infrared spectroscopy, and elemental analysis. The enantiomers of the phosphonic acid analogues of leucine, valine, and phenylalanine and [amino(3,4-dihydroxyphenyl)methyl]phosphonic acid were available from previous studies (Kafarski et al., 1983; Lejczak et al., 1987). We are indebted to Dr. H. Wójtowicz and Dr. B. Boduszek for providing us with the (chloromethyl)phosphinic acid analogues of valine and leucine (Wójtowicz & Mastalerz, 1987) and (hydroxypyridylmethyl)phosphonates. All asymmetric compounds used in this work are racemic mixtures unless otherwise specified.

Enzyme Preparations. Cytosolic leucine aminopeptidase (LAP)[1] from pig kidney was obtained as a crystalline suspension from Sigma Chemical Co. and was prepared according to the method of Andersson (1982). After activation in 22 mM triethanolamine hydrochloride buffer, pH 8.5, containing $MnCl_2$ (1 mM), the enzyme solution was used directly in kinetic experiments. Microsomal aminopeptidase (AP-M) was also obtained from Sigma Chemical Co. It was diluted with 50 mM potassium phosphate buffer, pH 7.2, and was used directly in kinetic experiments. Both enzyme stock solutions were stored at 5 °C for not longer than 1 week.

Enzyme Assays. LAP was assayed at 25 °C in 7.5 mM triethanolamine hydrochloride buffer, pH 8.4, containing $MgCl_2$ (5 mM). The substrate L-leucine *p*-nitroanilide, dissolved in DMSO, was added to the assay buffer followed by the enzyme. The hydrolysis of the substrate was monitored by following the change in absorbance at 405 nm [ΔE_{405} = 9620 M^{-1} cm^{-1} (Wachsmuth et al., 1966)] with a Specord M40 (Carl Zeiss Jena, GDR) spectrophotometer. The K_m value was 0.77 mM. All the solutions of inhibitors were prepared in the assay buffer, and the pH was adjusted to 8.4 by addition of 0.1 M sodium hydroxide solution. The assay mixture contained 0.1 mL of the substrate solution (0.05, 0.1, 0.2, or 0.4 mM final concentration), 0.5 mL of inhibitor solution (concentration dependent on inhibitor), and 0.2 mL of the enzyme solution (20 μg mL^{-1} final concentration); the final volume was adjusted to 2.0 mL with the assay buffer.

Activity of AP-M was determined at 25 °C in 50 mM potassium phosphate buffer, pH 7.2, using L-leucine *p*-nitroanilide as substrate (K_m = 0.52 mM). The assay mixture contained 0.1 mL of the substrate solution in DMSO (0.05, 0.1, 0.2, or 0.4 mM final concentration), 0.5 mL of the inhibitor solution, and 5 μL of the enzyme solution (4 μg mL^{-1} final concentration); the final volume was adjusted to 2.0 mL with the assay buffer.

[1] Abbreviations: LAP, cytosolic leucine aminopeptidase; AP-M, microsomal aminopeptidase; DMSO, dimethyl sulfoxide.

Table I: Inhibition of LAP and AP-M by Simple Phosphonic Acid Analogues of Amino Acids[a]

compound no.	structure	LAP K_i (μM)	LAP type of inhibition	AP-M K_i (μM)	AP-M type of inhibition
DL-**1**	$(CH_3)_2CHCH_2CH(NH_2)PO_3H_2$	ND	ND	105	rr
L-**1**		0.23[b]	sb	53	rr
D-**1**		220[b]	rr	192	rr
L-**2a**	$(CH_3)_2CHCH(NH_2)PO_3H_2$	0.15[c]	sb	26	rr
D-**2a**		12	rr	608	rr
L-**2b**	$C_6H_5CH_2CH(NH_2)PO_3H_2$	0.42[b]	sb	27.5	rr
D-**2b**		15[b]	rr	825	rr
L-**2c**	$3,4\text{-}(OH)_2C_6H_3CH(NH_2)PO_3H_2$	ND[d]	ND	175	rr
D-**2c**		ND[d]	ND	742	rr
DL-**2d**	$CH_3(CH_2)_4CH(NH_2)PO_3H_2$	1.0[b]	sb	26.5	rr

[a] Abbreviations: ND, not determined; rr, rapidly reversible competitive inhibition; sb, slow-binding, reversible competitive inhibition. [b] Data of Giannousis and Bartlett (1987). [c] Value determined from Lineweaver–Burk plots for steady state. [d] Compounds decompose at pH 8.4.

The concentration of the enzymes was determined spectrophotometrically at 280 nm, assuming $A_{280}^{0.1\%} = 1.63$ cm^{-1} for AP-M (Wachsmuth et al., 1966) and $A_{280}^{0.1\%} = 0.83$ cm^{-1} for LAP (Andersson et al., 1982). Molecular weights of 280 000 (Aurucchio & Bruni, 1964) and 255 000 (Melius et al., 1970) were used to estimate the molar concentrations of the microsomal and cytosolic enzymes, respectively.

Evaluation of Kinetic Parameters. In the case of reversible competitive inhibition the K_i values were determined from Lineweaver–Burk and/or Dixon plots for reactions monitored in the presence and absence of the inhibitor by using the initial velocities measured from the linear portion of the absorbance versus time progress curves. K_i values were not determined for those inhibitors that caused less than 50% decrease of the enzyme activity within 3 min of the reaction, if assayed at a final concentration of 2.0 mM with a substrate concentration of 0.4 mM.

The slow-binding inhibitors were characterized by a time-dependent decrease in the reaction rate (Figure 2), which varies as a function of the inhibitor concentration. Preincubation of the enzyme with the inhibitor for at least 30 min gave, after lag period, linear reaction progress (see a representative example in Figure 2), a slope of which is defined here as a steady-state velocity (v_s). Lineweaver–Burk plots of both initial and final (steady-state) velocities established that aminophosphonates are competitive inhibitors of both aminopeptidases.

The initial exponential phase of the reaction was analyzed by fitting the integrated equation of the reaction progress (Morrison, 1982; Duggleby et al., 1982)

$$P = v_s t + (v_0 - v_s)(1 - e^{-k_{app}t})/k_{app}$$

to the experimental curve. The fit was performed by the minimalization of the function $(P - P_i)^2 = f(k_{app}, v_0)$ (where P is the observed reaction progress at time t and P_i is the calculated value of the reaction progress at the same time) by means of a half-integral search. The representative fitted curves are shown in Figure 2. The steady-state velocities (v_s) were determined graphically from the linear changes of the reaction progress found after preincubations of the enzyme with various inhibitor concentrations prior to the addition of the substrate (0.4 mM in the case of AP-M and 0.8 mM for LAP, final concentrations). The fit of the integrated equation of the reaction progress and the experimental curves provided the experimental constants k_{app} and the initial velocities of the reaction v_0 (at $t = 0$). These values were used for the determination of the overall K_i values of inhibition.

Results

Inhibition of Cytosolic and Microsomal Aminopeptidases. All of the synthesized phosphonates were found to inhibit, to some extent, at least one of the aminopeptidases studied. The effect of the aminophosphonates **1** and **2** on both enzymes is shown in Table I along with the K_i values for the cytosolic enzyme reported earlier (Giannousis & Bartlett, 1987). The higher affinity of L isomers for both peptidases is evident from the comparison of the K_i values for L and D isomers of the phosphonic acid analogues of leucine (**1**), valine (**2a**), and phenylalanine (**2b**) and of [amino(3,4-dihydroxyphenyl)-methyl]phosphonic acid (**2c**). A stronger stereochemical preference was exhibited by LAP. Striking differences in affinities of the inhibitors toward both enzymes were also observed inasmuch as phosphonic acid analogues of leucine (**1**), valine (**2a**), and phenylalanine (**2b**), the most potent inhibitors of LAP among the compounds studied, only weakly inhibited the microsomal enzyme.

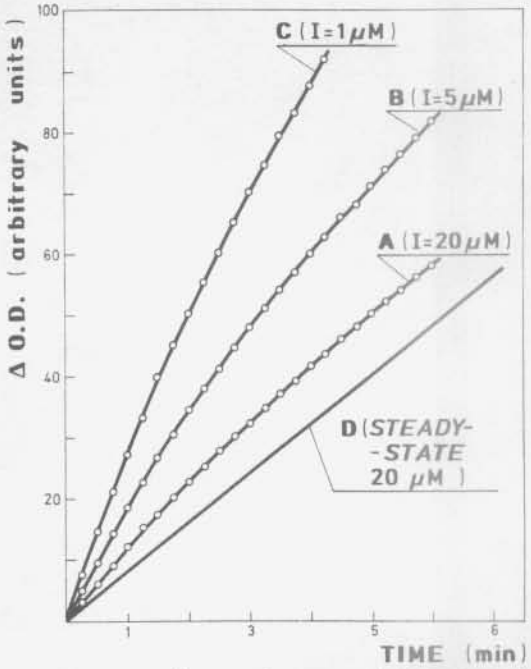

FIGURE 2: Representative reaction curves A–C for hydrolysis of L-leucine *p*-nitroanilide by microsomal aminopeptidase in the presence of increasing concentrations of [1-amino-2-(*N*-*n*-propylamino)-ethyl]phosphonic acid (**3e**): (—) experimental observations; (O) theoretical points obtained by fit of the integrated equation of the reaction progress. The linear portion of the progress curve D was obtained after preincubation of the enzyme and the inhibitor (50 min) prior to the addition of the substrate.

A quite different structure–activity relationship was observed in the influence of aminophosphonates **3** on aminopeptidases (Table II). [1-Amino-2-(*N*-alkylamino)ethyl]phosphonic acids (**3a**–**3k**) proved to be effective inhibitors of AP-M, nearly equipotent with bestatin (Rich et al., 1984), leucinal (An-

Table II: Inhibition of LAP and AP-M by [1-Amino-2-(*N*-alkylamino)ethyl]phosphonic and (1-Amino-2-alkoxyethyl)phosphonic Acids[a]

compound no.	structure	LAP		AP-M	
		K_i (μM)	type of inhibition	K_i (μM)	type of inhibition
3a	$\overline{CH_2\text{-}NH\text{-}CHPO_3H_2}$	ND	ND	NI	
3b	$H_2NCH_2CH(NH_2)PO_3H_2$	ND	ND	19	rr
3c	$CH_3NHCH_2CH(NH_2)PO_3H_2$	ND	ND	46	rr
3d	$(CH_3)_2NCH_2CH(NH_2)PO_3H_2$	580	rr	52	rr
3e	$CH_3(CH_2)_2NHCH_2CH(NH_2)PO_3H_2$	ND	ND	1.9[b]	sb
3f	$CH_3(CH_2)_3NHCH_2CH(NH_2)PO_3H_2$	1100	rr	2.0[b]	rr
3g	$(CH_3)_2CHCH_2NHCH_2CH(NH_2)PO_3H_2$	ND	ND	1.7[b]	sb
3h	$C_5H_{10}NCH_2CH(NH_2)PO_3H_2$	1410	rr	14.7	rr
3i	$C_6H_{11}NHCH_2CH(NH_2)PO_3H_2$	ND	ND	0.87[b]	sb
3j	$C_6H_5CH_2NHCH_2CH(NH_2)PO_3H_2$	350	rr	2.3[b]	sb
3k	$C_6H_5CH(CH_3)NHCH_2CH(NH_2)PO_3H_2$	600	rr	2.8[b]	sb
3l	$HOCH_2CH(NH_2)PO_3H_2$	1580	rr	400	rr
3m	$CH_3OCH_2CH(NH_2)PO_3H_2$	35	rr	367	rr
3n	$CH_3CH_2OCH_2CH(NH_2)PO_3H_2$	25	rr	170	rr
3o	$CH_3(CH_2)_2OCH_2CH(NH_2)PO_3H_2$	53	rr	84.5	rr
3p	$HOOCCH_2NHCH_2PO_3H_2$	NI		NI	

[a] All asymmetric substances are racemic mixtures. Abbreviations: ND, not determined for inhibitors that caused less than 50% decrease of the enzyme activity within 3 min of reaction, if assayed at a final concentration of 2.0 mM with a substrate concentration of 0.4 mM; NI, no inhibition at concentrations of substrate and inhibitor of 0.4 and 2.0 mM, respectively; rr, rapidly reversible competitive inhibition; sb, slow-binding, reversible competitive inhibition. [b] Values determined from Lineweaver–Burk plots for steady state.

Table III: Inhibition of LAP and AP-M by Racemic Hydroxyphosphonic Acids[a]

compound no.	structure	LAP, K_i[b] (μM)	AP-M
4a	$(CH_3)_2CHCH_2CH(OH)PO_3H_2$	28.5	NI
4b	$C_6H_5CH(OH)PO_3H_2$	1500	NI
4c	C_5H_4N-4-$CH(OH)PO_3H_2$	ND	NI
4d	C_5H_4N-3-$CH(OH)PO_3H_2$	1500	NI

[a] Abbreviations: ND, not determined; NI, no inhibition at concentrations of substrate and inhibitor of 0.4 and 2.0 mM, respectively. [b] Rapidly reversible competitive inhibition.

Table IV: Inhibition of LAP and AP-M by Racemic (1-Aminoalkyl)phosphinic Acids

compound no.	structure	K_i[a] (μM)	
		LAP	AP-M
5a	$(CH_3)_2CHCH_2CH(NH_2)P(=O)OHCH_3$	425	21
5b	$(CH_3)_2CHCH(NH_2)P(=O)OHCH_3$	870	35
5c	$C_6H_5CH_2CH(NH_2)P(=O)OHCH_3$	320	58.5
5d	$(CH_3)_2CHCH_2CH(NH_2)P(=O)OHCH_2Cl$	ND[b]	577
5e	$(CH_3)_2CHCH(NH_2)P(=O)OHCH_2Cl$	ND	232
5f	$(CH_3)_2CHCH_2CH(NH_2)P(=O)OHOCH_3$	87[c]	657
5g	$(CH_3)_2CHCH_2CH(NH_2)P(=O)OHC_6H_5$	ND	1292

[a] Rapidly reversible competitive inhibition. [b] ND, not determined. [c] Data of Giannousis and Bartlett (1987).

dersson et al., 1982), and hydroxamic acids (Wilkes & Prescott, 1983), the most potent non-peptide inhibitors of this enzyme. The inhibitors **3e**, **3f**, **3g**, **3i**, **3j**, and **3k** were also slow binding; on combination of the enzyme and the inhibitor equilibrium was attained more slowly than would be expected if binding were limited by diffusion alone (Figure 2). Interestingly, these compounds were practically inactive toward LAP but (1-amino-2-alkoxyethyl)phosphonic acids (**3l–3o**) proved to be weak inhibitors of both aminopeptidases (Table II).

The replacement of the amino group of the leucine analogue by a hydroxyl moiety yielded compound **4a**, which was completely inactive to AP-M but moderately inhibitory toward LAP (Table III). Compounds **4b–4d** also exhibited weak inhibitory activity toward the cytosolic enzyme but failed to influence the microsomal enzyme activity.

Modification of the phosphonic moiety, i.e., the replacement of one hydroxy group by methyl (compounds **5a–5c**), chloromethyl (compounds **5d** and **5e**), methoxy (**5f**), and phenyl (**5g**) groups, resulted in weak inhibitors of aminopeptidases (Table IV). It is worth noting, however, that (1-aminoalkyl)phosphonic acids **5a–5c** inhibited AP-M more strongly than the corresponding phosphonic acids (compounds **1**, **2a**, and **2b**), while their effect on LAP activity was negligible.

Analysis of Time-Dependent Inhibition. For slow-binding inhibitors Cha (1975, 1976), Morrison (1982), and Duggleby (1982), among others, have described three possible mechanisms: (1) a one-step process in which the enzyme and the inhibitor form a complex EI (mechanism A); (2) a two-step process in which a rapid enzyme–inhibitor complex (EI) formation is followed by a slow isomerization of EI to a slowly dissociating complex EI* (mechanism B); (3) a two-step process in which the enzyme in the presence of the inhibitor undergoes a slow isomerization prior to binding the inhibitor to form a slowly dissociating complex E*I (mechanism C).

mechanism A

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

mechanism B

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

mechanism C

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^* + I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^*I$$

Duggleby et al. (1982) have described how plots of the apparent first-order rate constant for slow binding (k_{app}) versus inhibitor concentration can be used to distinguish between these mechanisms, provided the full range of inhibitor concentration is attainable.

(1-Aminoalkyl)phosphonic acids are inhibitors of LAP that act according to mechanism A (Giannousis & Bartlett, 1987). Among the aminophosphonates studied in the present work, only the phosphonic acid analogue of L-valine, L-(1-amino-2-methylpropyl)phosphonic acid (**2a**) (see Table I), exhibited slow-binding kinetics. In this case a plot of k_{app} versus inhibitor concentration was linear (Figure 3), indicating the one-step, slow-binding mechanism A. The slope of this plot yielded the value of k_1 (2.6×10^4 M^{-1} s^{-1}), while the intercept of the plot with ordinate yielded the value of k_2 (2.8×10^{-3} s^{-1}). The

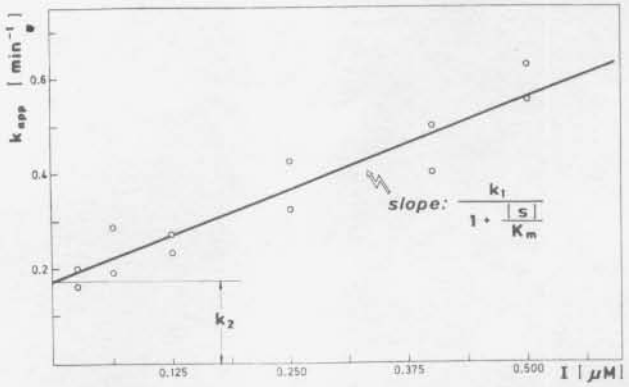

FIGURE 3: Dependence of k_{app} on the concentration of a phosphonic acid analogue of L-valine, L-(1-amino-2-methylpropyl)phosphonic acid, for the inhibition of LAP. The reaction rate constants were determined as indicated.

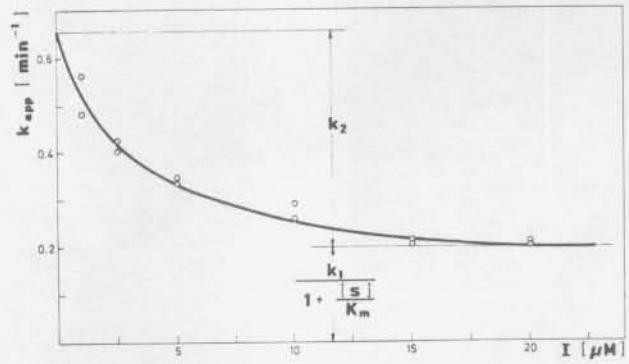

FIGURE 4: Dependence of k_{app} on the concentration of [1-amino-2-(*N*-*n*-propylamino)ethyl]phosphonic acid (**3e**) for the inhibition of AP-M. The reaction rate constants were determined as indicated and used for further calculations of the overall K_i value (see Materials and Methods).

overall K_i value determined as k_2/k_1 was 0.11 μM and is in good agreement with the value of 0.15 μM found from the Lineweaver–Burk and Dixon plots for the reaction at steady state.

Six aminophosphonates, **3e–3g** and **3i–3k**, exhibited slow-binding kinetics toward AP-M. For each of them, k_{app} decreased hyperbolically with increasing inhibitor concentration, as is illustrated by the representative plot shown in Figure 4. These results indicated that these inhibitors act according to mechanism C (Duggleby et al., 1982). For all of these compounds, the first-order rates k_1 and k_2 were determined graphically as shown in Figure 4; the values of overall K_i (being by definition k_4/k_3) were calculated (Duggleby et al., 1982) and are presented in Table V. The overall K_i values thus calculated are in good agreement with those found from the Lineweaver–Burk and Dixon plots for the steady state of the enzymatic reaction (Table V). Since k_4 is equal to $k_{app}v_s/v_0$ for each mechanism (Morrison, 1982), we have calculated these values for all the slow-binding inhibitors **3**. The values of the second-order constants k_3 were calculated as k_4/K_i (Table V).

Discussion

Among the known inhibitors of peptidases, phosphonate, boronate, and aldehyde inhibitors are of special interest because they are believed to mimic the putative tetrahedral transition state of the catalytic process involving the direct attack of the water molecule on the amide linkage of the substrate (Weaver et al., 1977; Jacobsen & Bartlett, 1981; Andersson et al., 1982; Shenvi, 1986; Giannousis & Bartlett, 1987). The boronates and aldehydes are also effective inhibitors of serine peptidases because of their ability to form a covalent bond with serine in the active center of the enzyme (Tschesche, 1981; Kettner & Shenvi, 1984). On the contrary, phosphonates, unless specifically activated, are not able to form a covalent adduct with the enzyme-bound nucleophile (Lamden & Bartlett, 1983; Bartlett & Lamden, 1986).

Table V: Kinetic Parameters of Binding of Aminophosphonates **3** with AP-M

compound no.	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (M^{-1} s^{-1})	k_4 (s^{-1})	K_i[a] (μM)	overall K_i found from steady state (μM)
3e	0.004	0.0075	4.2×10^3	0.008	2.0	1.9
3f	0.0075	0.0026	4.5×10^3	0.009	2.2	2.0
3g	0.0062	0.006	5.9×10^3	0.010	1.6	1.7
3i	0.0182	0.004	9.2×10^3	0.008	1.1	0.87
3j	0.0059	0.0035	3.9×10^3	0.009	3.0	2.3
3k	0.0028	0.0049	3.6×10^3	0.010	3.2	2.8

[a] Calculated from $k_{app} = k_1/(1 + [S]/K_m) + k_2/(1 + [I]/K_i)$.

The object of this study was to determine the effects of structural changes introduced to the phosphonic acid analogue of leucine, the most potent inhibitor among those reported by Giannousis and Bartlett (1987) on their inhibition of cytosolic and microsomal aminopeptidases.

The data collected in Tables I–IV indicate that most of the compounds studied are modest inhibitors of both enzymes, with the exception of compounds **3e–3g** and **3i–3k**, i.e., those in which the γ-CH_2 of the alkyl chain was replaced by an NH moiety. These compounds proved to be quite potent inhibitors of AP-M as is shown in Table II. Interestingly, these compounds exhibited a slow-binding kinetics, manifested by the gradual decrease of the rate of hydrolysis of the substrate with reaction time (Figure 2). For all of these compounds the apparent first-order rate constant for the slow-binding inhibition (k_{app}) decreases with increasing inhibitor concentration (Figure 4). Such a dependence indicates that the tested compounds act according to mechanism C (Duggleby et al., 1982), which involves a slow isomerization of the enzyme in the presence of the inhibitor prior to binding. Such a mechanism is a rarely observed variant of time-dependent inhibition. The second-order constants k_3 (Table V) found for these inhibitors are well below credible values for the diffusion-controlled association rate constant of the enzyme and the ligand, which is 10^6 M^{-1} s^{-1} (Brouwer & Kirsch, 1982; Hardy & Kirsch, 1984).

The most interesting result of our work was the selectivity exhibited by the various groups of inhibitors toward the two aminopeptidases studied. The relationship between the modification of the structure of the model compound **1** and the inhibition of LAP and AP-M is outlined below.

The simple phosphonic analogues of amino acids (Table I) were among the most effective of those tested toward LAP, although they were considerably less inhibitory than some of the small compounds reported by other investigators (Umezawa et al., 1976; Andersson et al., 1982; Wilkes & Prescott, 1983; Shenvi, 1986). The more potent of them follow the slow-binding mechanism A (Giannousis & Bartlett, 1987), as indicated by simple first-order dependence of k_{app} on inhibitor concentration (Figure 3). In contrast, they are weak inhibitors of AP-M, being 50–300-fold less efficient toward it than toward the cytosolic enzyme.

As would be expected from the selectivity of aminopeptidases for hydrophobic residues at the N-terminus of the peptide chain, the inhibition constants decrease with increasing size of the alkyl side chain. Thus, the replacement of the γ-methylene group in **1** and **2** by an oxygen atom, which results in a decrease of hydrophobicity, leads to compounds

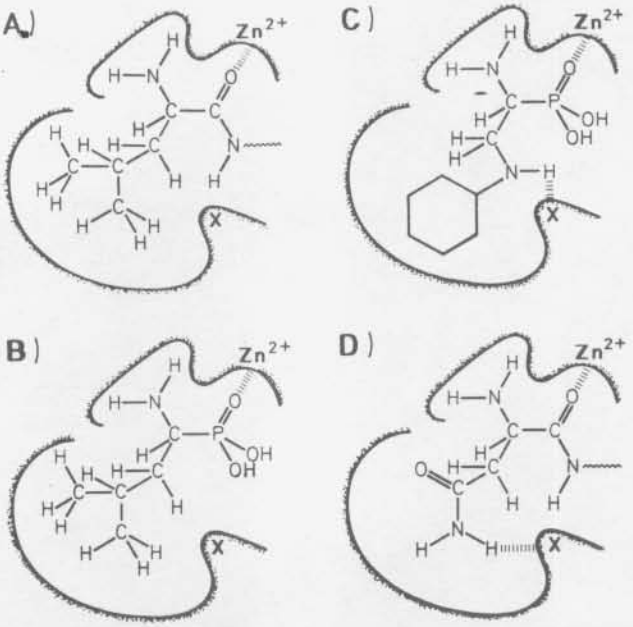

FIGURE 5: Schematic model for the binding site of microsomal aminopeptidase, showing the proposed modes of binding: (A) substrate (Bryce & Rubin, 1964); (B) phosphonic acid analogue **1** of leucine; (C) [1-amino-2-(*N*-cyclohexylamino)ethyl]phosphonic acid (**3i**); (D) asparaginyl peptide.

3l–3o (Table II), which were significantly weaker inhibitors of both enzymes. The replacement of this methylene group by an amino group yielded compounds **3a–3k** of even less hydrophobic character, and there was a marked decrease in their binding affinity toward LAP if compared to that of **3m–3o**. In contrast, the affinity of compounds **3b–3k** for AP-M was greater than that of the oxygen-substituted compounds (**3k–3o**), and the more potent of them exhibited a time-dependent inhibition. The observed differences in selectivity between microsomal and cytosolic enzymes evidently reflects differences in the sites that bind the hydrophobic fragment of the inhibitor molecule.

There are two possible explanations of the greater affinity of compounds **3a–3k** toward AP-M than toward LAP: (1) the formation of a hydrogen bond between the amino group of the inhibitor and the respective donor in the binding site of AP-M or (2) an acid–base (NH^+ group of inhibitor) interaction between the inhibitor and the enzyme. The facts that compounds **3d** and **3h**, which contain tertiary β-amino moieties, are significantly less potent and that AP-M (in contrast to LAP) is not able to cleave N-terminal asparagine and glutamine from the peptide chain (DeLange & Smith, 1971) favor the first possibility. Thus, as shown in Figure 5, the substrates and the phosphonic analogue of leucine (**1**) form complexes with both enzymes by a simple fit of the hydrophobic fragment of the molecule into the hydrophobic cavity of the enzyme binding site and by complexation of the active-site zinc ion by amide or phosphonate groups, respectively. We postulate that the β-amino group of the slow-binding inhibitors **3** or the amide group of N-terminal peptidyl asparagine or glutamine residue of the peptide substrate forms an additional hydrogen bond with the respective donor present in the hydrophobic cavity of AP-M (Figure 5). The formation of this bond may require the isomerization of the enzyme prior to binding this part of the inhibitor, resulting in the observed slow-binding mechanism C.

Furthermore, in the case of compounds **3a–3k** the inhibition constants for AP-M decrease with increasing hydrophobic character of the side chain, although a limit is clearly reached as indicated by the lower affinities of **3g**, **3j**, and **3k** for the enzyme compared to that of **3i**.

The simple replacement of the amino group in **1** by a hydroxyl moiety produces the phosphonic analogue **4a** of 1-hydroxyisocaproic acid, which inhibits LAP much less effectively than the parent compound (Table III). Also, other hydroxyphosphonates (compounds **4b–4d**) only weakly inhibit the cytosolic enzyme. This result is not surprising since an α-amino group is essential to LAP catalysis and is not required for binding provided that other structural features are present (DeLange & Smith, 1971; Wilkes & Prescott, 1983). Hydroxyphosphonates, however, are completely inactive toward AP-M, indicating that for this enzyme the presence of the amino group is indispensable for binding.

Finally, the changes of the structure of phosphonyl moiety yielded compounds of type **5** in which a phosphonyl OH was replaced by alkyl, aryl, or methoxy functions. Four of these derivatives showed marked differences in affinity toward the two enzymes. (1-Aminoalkyl)phosphinic acids were generally more strongly bound by AP-M than by LAP (Table IV), with methylphosphinic acids being the most effective inhibitors. The increase in size of the substituent causes a significant decrease of the inhibitory activity ($CH_3 > CH_2Cl \geq OCH_3 > C_6H_5$). On the contrary, the corresponding aminophosphonic acids **1** and **2** were bound more strongly to LAP than to AP-M, being the most potent inhibitors of the cytosolic enzyme presented in this work. Since these two classes of compounds differ in their structure at the phosphonyl moiety, we speculate that the observed difference on their inhibition constant reflects the differences in the mechanism of action of LAP and AP-M. Further studies are necessary to verify this hypothesis.

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Supplementary Material Available

Detailed description of the synthesis and physical properties of the new compounds used (9 pages). Ordering information is given on any current masthead page.

Registry No. DL-**1**, 37100-69-9; L-**1**, 88081-77-0; D-**1**, 88081-76-9; L-**2a**, 66254-56-6; D-**2a**, 66254-55-5; L-**2b**, 36992-14-0; D-**2b**, 66609-42-5; L-**2c**, 107395-13-1; D-**2c**, 107395-14-2; DL-**2d**, 109638-78-0; **3a**, 119011-91-5; **3b**, 16606-63-6; **3c**, 119011-92-6; **3d**, 119011-93-7; **3e**, 119011-94-8; **3f**, 119011-95-9; **3g**, 119011-96-0; **3h**, 119011-97-1; **3i**, 119011-98-2; **3j**, 119011-99-3; **3k**, 119012-00-9; **3l**, 94841-40-4; **3m**, 119012-01-0; **3n**, 119012-02-1; **3o**, 119012-03-2; **3p**, 1071-83-6; **4a**, 119012-04-3; **4b**, 104944-32-3; **4c**, 119012-05-4; **4d**, 119012-06-5; **5a**, 119012-07-6; **5b**, 74891-94-4; **5c**, 107016-45-5; **5d**, 119012-08-7; **5e**, 119012-09-8; **5f**, 109638-80-4; **5g**, 119012-10-1; LAP, 9001-61-0; AP-M, 9054-63-1; isovaleraldehyde, 590-86-3; diethyl phosphite, 762-04-9; benzaldehyde, 100-52-7; benzyl carbamate, 621-84-1; pivalic acid, 75-98-9; methyldichlorophosphine, 676-83-5; 1-(*N*-benzyloxycarbonylamino)-3-methylbutyl(P-methyl)phosphinic acid, 119012-11-2; 1-(*N*-benzyloxycarbonylamino)-2-methylpropyl-(P-methyl)phosphinic acid, 119012-12-3; 1-(*N*-benzyloxycarbonylamino)-2-phenylethyl(P-methyl)phosphinic acid, 119012-13-4; acetamide, 60-35-5; diethyl phenylphosphinite, 4894-60-4; 1-bromo-2-aminoethylphosphonic acid, 119068-57-4.

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