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INHIBITION OF AMINOPEPTIDASES BY PHOSPHONIC ACID AND PHOSPHINIC ACID ANALOGUES OF ASPARTIC AND GLUTAMIC ACIDS

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More than 30 phosphonic and phosphinic acid analogues of aspartic and glutamic acids were synthesized in order to probe how the structural differences of these molecules were reflected in their ability to inhibit cytosolic (LAP) and microsomal (APM) aminopeptidases. Although most of the compounds studied were found to exert only a modest inhibitory effect, the studies provide some information on the structural requirements of the binding subsites and catalytic centers of both enzymes.

KEY WORDS: Cytosolic aminopeptidase, microsomal aminopeptidase, LAP, APM, phosphonic acids, phosphinic acids.

INTRODUCTION

Aminopeptidases are a group of exopeptidases that catalyze the hydrolysis of amino-terminal residues from a peptide chain. These peptides are ubiquitous in nature and appear to be involved in a variety of important biological processes from hormonal regulation to immuno-modulation. Thus, synthetic and naturally occurring compounds that inhibit aminopeptidases have been useful for studying the nature of enzyme-substrate interactions during catalysis[1] by these enzymes and as medicinal agents. A goal yet to be attained in the study of any enzyme mechanism is a full understanding of the relationship between active site structure, substrate binding, and the dynamics of catalysis.[2] In contrast to investigations of enzyme-substrate interactions, which generally focus on the kinetic details of the process, most studies on enzyme inhibitors have focused on the thermodynamic aspects of the binding phenomenon. Thus by studying how the delicate structural changes introduced into the inhibitor molecule affect its inhibitory action may lead to a better understanding of the roles played by separate substructural elements of the enzyme (catalytic and binding subsites) during catalysis.

The substrate specificities of cytosolic (LAP, E.C. 3.4.11.1) and microsomal (APM, E.C. 3.4.11.2) aminopeptidases are well understood, but the mechanistic details of

* Correspondence.

their action are not clear. They are members of the zinc peptidase class, but no structural comparison with thermolysin and carboxypeptidase A is available, nor is there a similar correspondence in effective inhibition strategies. Our approach to development of aminopeptidase inhibitors was to replace the substrate scissile amide bond with a phosphonic acid group, on the premise that this moiety effectively mimics the putative tetrahedral transition state of the catalytic process, formed by direct attack of water molecule on the peptide linkage of the substrate, and additionally binds the active-site zinc ion.[3-6] We have found that 1-aminoalkylphosphonic acids are effective and potent inhibitors of both microsomal and cytosolic aminopeptidases.[7] These compound were synthesized in order to probe how the structural changes introduced into the phosphonic acid analogue of leucine, a potent inhibitor of LAP,[6] affected their inhibitory properties. Nearly every modification of the phosphonic acid analogue of leucine was reflected in a marked differences in the affinities of these compounds for the two enzymes, thus reflecting the differences in the structure of their binding subsites. In order to further classify the structural requirements of the catalytic subunits and binding subsites of microsomal and cytosolic aminopeptidases we have now synthesized a series of phosphonic and phosphinic acid analogues of aspartic and glutamic acids and evaluated their inhibitory potency towards both enzymes. These compounds may be recognized as analogues of the phosphonic acid analogue of leucine in which a hydrophillic carboxylate moiety was introduced into a hydrophobic fragment of the molecule.

MATERIALS AND METHODS

Chemicals

Phosphonic and phosphinic acid analogues of aspartic and glutamic acids, as well as their esters and amides were available from previous studies or were synthesized by standard literature methods.[8-11] All the compounds were characterized by ^{1}H nuclear magnetic resonance, infrared spectroscopy and elemental analysis. We are indebted to Dr. Miroslaw Soroka for providing us with some of the aminophosphonates.[12,13] All compounds used in this work were racemates unless otherwise specified.

Enzyme Preparations

Cytosolic leucine aminopeptidase from pig kidney was obtained as a crystalline suspension from Sigma Chemical Co. prepared according to the method of Andersson.[14] 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 from porcine kidney was 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 solutions were stored at 5°C for not longer than a 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 in DMSO was added to the assay buffer followed by the enzyme. The hydrolysis was monitored by following the change in absorbance at 405 nm[15] with a Specord M40 (Carl Zeiss Jena, Germany) spectrophotometer. The K_m value was found to be 0.77 mM.

All solutions of inhibitors were prepared in the assay buffer and the pH was adjusted to 8.4 by addition of sodium hydroxide (0.1 M) solution. The assay mixture contained 0.1 ml of the substrate solution (0.1, 0.2, 0.4 and 0.8 mM final concentration), 0.5 ml of inhibitor solution (0.001–1.0 mM) and 0.2 ml of the enzyme solution (390 μg/ml final concentration). The final volume was adjusted to 2.0 ml with the assay buffer.

Activity of APM 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 in DMSO (0.05, 0.1, 0.2 and 0.4 mM final concentration), 0.5 ml of the inhibitor solution (0.005–1 mM), and 5 μl of enzyme solution (4 μg/ml final concentration). The final volume was adjusted to 2.0 ml with the assay buffer.

Evaluation of Kinetic Parameters

The K_i values were determined by standard methods, using Lineweaver-Burk and/or Dixon plots, and shown in each case to be of a competitive type. In those cases where less than 50% inhibition was observed at an inhibitor final concentration of 2.0 mM with a substrate concentration of 0.4 mM, the percentage inhibition was reported.

RESULTS AND DISCUSSION

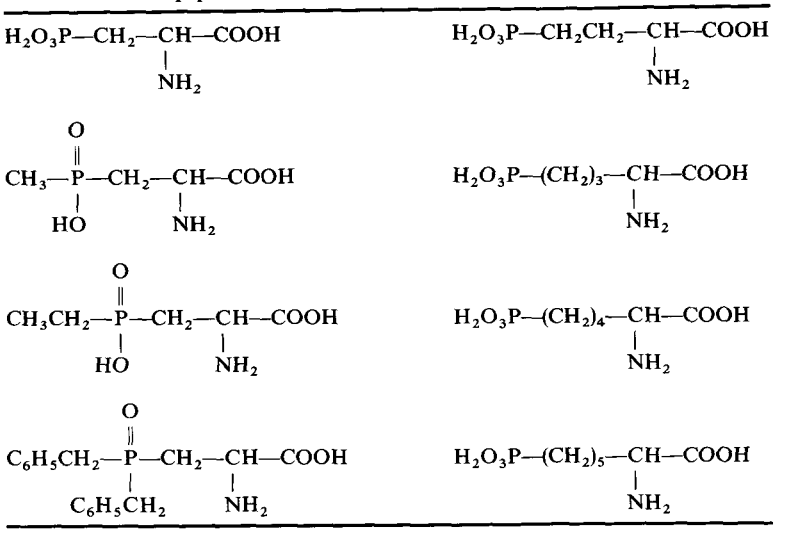
Tables 1–3 summarize the inhibitory activities of the analogues of aspartic and glutamic acids prepared. As seen from Table 1 the replacement of the ω-carboxylic moiety of aspartic or glutamic acid by the phosphonic or phosphinic acid function resulted in compounds completely inactive towards both aminopeptidases. In contrast, replacement of the α-carboxylic function is crucial for inhibitory activity of the analogues (see Tables 2 and 3). However, inspection of the data in Tables 2 and 3 shows that there is no simple relationship between structure and inhibitory potency for analogues of aspartic and glutamic acids.

Phosphonic acid analogues of glutamic and α-aminoadipic acids (compounds **1** and **4**) were moderate inhibitors of LAP, being practically inactive to APM (Table 2). Surprisingly, stereoisomers of these compounds were nearly equipotent. This is not in accord with literature data[6,7] since LAP exhibited strong stereochemical preference for *L*-isomers of 1-aminoalkylphosphonic acids. In contrast to behaviour in the aspartic acid series (see Table 3) the analogue of glutamine (compound **2**) appeared to be less active than the parent compound **1**. Also esterification of the carboxylic group of **1** to give **3**, did not significantly improve inhibitory potency. All these findings seem to suggest that analogues of glutamic acid are bound by LAP in a different manner to simple 1-aminoalkylphosphonates and analogues of aspartic acid.

The most clear-cut results were obtained with analogues of aspartic acid. The data presented in Table 3 indicate three major structure-activity relationships for inhibition of LAP and APM by these compounds.

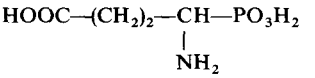
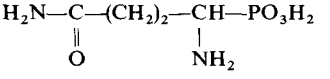
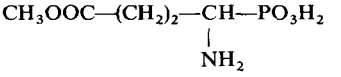
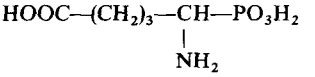
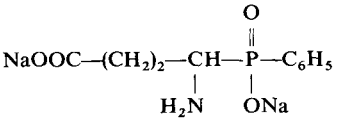
First, as would be expected from the selectivity of both enzymes for hydrophobic

Table 1 Analogues of aspartic and glutamic acids inactive towards cytosolic and microsomal aminopeptidases



Less than 20% inhibition was observed for final inhibitor concentration of 2.0 mM and substrate concentration of 0.4 mM.

Table 2 Inhibition of LAP and APM by phosphonic acid analogues of glutamic acid

Compound no.	Structure		LAP $K_i(\mu M)$	LAP % inhibition	APM % inhibition
1		*L*-isomer	82	100%	42%
		D-isomer	79	100%	ND
2				38%	**NI**
3			73	100%	54%
4		*L*-isomer	200	80%	ND
		D-isomer	245	78%	ND
5				**NI**	**NI**

NI, no inhibition at concentrations of substrate and inhibitor 0.4 mM and 2.0 mM respectively; ND, not determined.

Table 3 Inhibition of LAP and APM by phosphonic and phosphinic acid analogues of aspartic acid

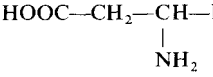
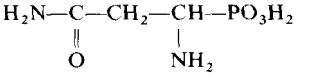
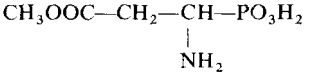
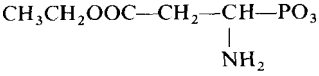
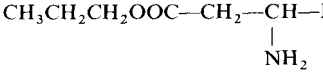
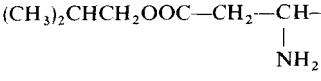
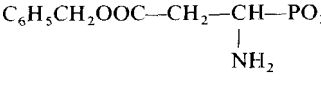
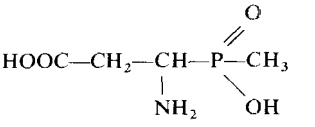
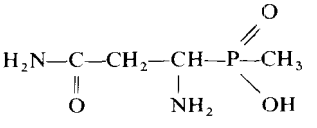
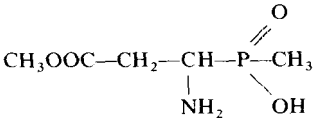
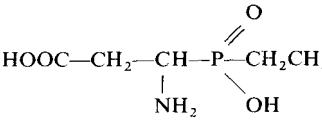
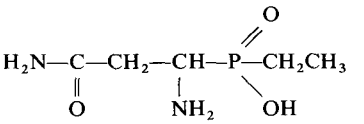
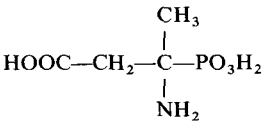
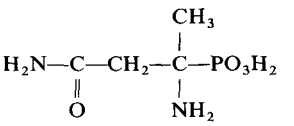
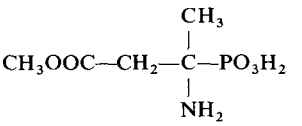
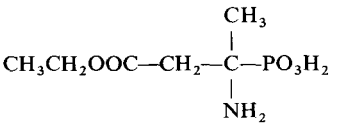
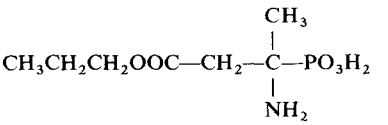
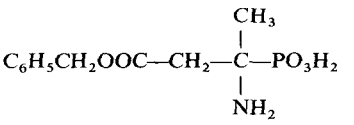
Compound no.	Structure	LAP		APM	
		$K_i(\mu M)$	% inhibition	$K_i(\mu M)$	% inhibition
6		240	80%	ND	42%
7		44	100%	1900	74%
8		6.5	100%	210	100%
9		4.5	100%	130	100%
10		3.3	100%	245	100%
11		5.4	100%	52	100%
12		4.0	100%	15	100%
13		ND	33%	102	100%
14		ND	28%	NI	
15		390	58%	172	100%
16		ND	23%	NI	

Table 3 *continued*

Compound no.	Structure	LAP $K_i(\mu M)$	LAP % inhibition	APM % inhibition
17		**NI**		**NI**	
18		ND	19%	ND	21%
19		ND	27%	ND	ND
20		300	64%	**NI**	
21		560	67%	ND	ND
22		420	53%	ND	ND
23		160	80%	**NI**	

NI, no inhibition at concentrations of substrate and inhibitor 0.4 mM and 2.0 mM respectively; ND, not determined.

residues at the N-terminus of the peptide chain, the inhibition constants decreased with the increase of hydrophobicity of the inhibitor side chain. Thus an identical pattern of affinity to LAP was observed for both series of inhibitors: those based on the aspartic acid analogue **6** and those based on the α-methylaspartic acid analogue **18**. The analogues **7** and **19** of asparagine were bound stronger than compounds **6** and **18** respectively. Moreover, carboxylate esters of both phosphonic acid analogues

of aspartic acid exhibited better inhibitory activity than the free acids and amides, with the inhibition constants decreasing, in general, with an increase of the size of carboxylate moiety. However, a limit is clearly reached as indicated by the lower affinity of compound **11** for LAP compared to that of compunds **10** and **12**. It is also noteworthy that none of the compounds **6–12** exhibited slow-binding kinetics. Similarly to the earlier findings,[7] phosphonic acid analogues **6–12** were 3–300 times less efficient inhibitors of APM than LAP. The subsite binding hydrophobic part of the substrate molecule seems to be more spacious in the case of APM, since compound **11** still appeared to be a good inhibitor of the enzyme.

Second, the modification of the chiral center of compound **6**, gave analogues of α-methylaspartic acid (compounds **18–23**) of conserved inhibitory activity towards LAP. However modification in such a sensitive part of the molecule decreased potency 30–120 fold. The limited studies on APM indicated that this modification is accompanied by lack of inhibitory activity.

Finally, one of the most interesting results evident from Table 3 is that the P-methylphosphonic acid analogues of aspartic acid (compounds **13–15**) were generally more inhibitory towards APM than to LAP, whereas the phosphonic acid analogues (compounds **6–8**) were bound more strongly by LAP than by APM. Since these two classes of compounds differ in their structure only on the phosphonyl moiety the observed difference in their inhibitory potency may reflect the difference in the mechanisms of action of LAP and APM, due possibly to a difference in the direction of attack of a water molecule on the peptide bond or the mode of complexation with the active-site zinc ion.[16] Increase in hydrophobicity and size of the substituent located on the phosphonyl moiety (compounds **5**m **16** and **17**) led to a significant decrease in inhibitory activity towards both enzymes.

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References

1. Wolfenden, R. (1976) *Ann. Rev. Biophys. Bioeng.*, **5**, 271.
2. Jencks, W.P. (1975) *Adv. Enzymol.*, **43**, 219.
3. Weaver, L., Kester, W.R. and Matthews, B.W. (1977) *J. Mol. Biol.*, **114**, 119.
4. Jacobsen, N.E. and Bartlett, P.A. (1981) *J. Amer. Chem. Soc.*, **103**, 654.
5. Shenvi, A.B. (1986) *Biochemistry*, **25**, 1286.
6. Giannousis, P.P. and Bartlett, P.A. (1987) *J. Med. Chem.*, **30**, 1603.
7. Lejczak, B., Kafarski, P. and Zygmunt, J. (1989) *Biochemistry*, **28**, 3549.
8. Lejczak, B., Starzemska, H. and Mastalerz, P. (1981) *Experientia*, **37**, 462.
9. Oleksyszyn J., Gruszecka, E., Kafarski, P. and Mastalerz, P. (1982) *Monatsh. Chem.*, **113**, 59.
10. Lejczak, B., Kafarski, P. and Mastalerz, P. (1985) *J. Chromatography*, **324**, 455.
11. Miliszkiewicz, D., Wieczorek, P., Lejczak, B., Kowalik, E. and Kafarski, P. (1992) *Pest. Sci.*, **33**, 349.
12. Soroka, M. and Mastalerz, P. (1976) *Pol. J. Chem.*, **50**, 661.
13. Gruszecka, E., Soroka, M. and Mastalerz, P. (1979) *Pol. J. Chem.*, **53**, 2327.
14. Andersson, L., Isley, T.C. and Wolfenden, R. (1982) *Biochemistry*, **21**, 4177.
15. Wachsmuth, E.D., Fritze, I. and Pfeiderer, G. (1966) *Biochemistry*, **5**, 175.
16. Rich, D.L. (1985) *J. Med. Chem.*, **28**, 263.