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Original article

α-Aminoalkylphosphonates as a tool in experimental optimisation of P1 side chain shape of potential inhibitors in S1 pocket of leucine- and neutral aminopeptidases

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Abstract

The synthesis and biological activity studies of the series of structurally different α-aminoalkylphosphonates were performed in order to optimise the shape of the side chain of the potential inhibitors in S1 pocket of leucine aminopeptidase [E.C.3.4.11.1]. Analysis of a series of compounds with aromatic, aliphatic and alicyclic P1 side chains enabled to find out the structural features, optimal for that fragment of inhibitors of LAP. The most active among all investigated compounds were the phosphonic analogues of homo-tyrosine (K_i = 120 nM) and homo-phenylalanine (K_i = 140 nM), which even as racemic mixtures were better inhibitors in comparison with the best till now-phosphonic analogue of L-leucine (230 nM). Additional comparison of the inhibitory activity obtained for aminopeptidase N (APN, E.C.3.4.11.2) give insight into structural preferences of both enzymes.

Keywords: Aminophosphonates; Inhibitor; Leucine aminopeptidase; Aminopeptidase N

1. Introduction

Aminopeptidases are enzymes that catalyse the removal of amino acids from the N-terminus of a peptide or protein. These enzymes have been found in bacteria, yeast, plants, animal and human tissues, what is considerably ascribed to their broad substrate specificity [1]. They play such a biological functions as protein maturation, activation and degradation of bioactive peptides, hormone level regulation and control of cell proliferation [2,3]. It is well known, that elevated concentration of these enzymes was observed in such pathological disorders as cancer and cataracts [1,3]. In the case of the carcinogenesis, the methionine aminopeptidases has been shown to be a promising target for development of anti-angiogenesis agents [4]. Additionally, the importance of these enzymes arises from the observation, that some of them might play the role in the apoptosis of cancer cells, what makes them interesting targets in oncological research (with methionine and neutral aminopeptidases being the most promising targets) [5].

Aminopeptidases can be subdivided into two major groups, with the first one being responsible for the hydrolysis of amino acids with hydrophobic side chains from N-terminus of the peptide or protein, and the second, which specifically remove other amino acid residues [3].

Within the first group, leucine aminopeptidase (LAP, E.C.3.4.11.1) is one of the most detailed characterised enzyme with respect to its sequence, structure and mechanism of action. Human LAP plays an important role in the early stages of HIV infection, where the elevated concentration of this enzyme was observed [6]. The unusual activity of this enzyme has also been observed in such pathological disorders as cancer, inflammation of liver or eye cataract [7–9]. Because of this leucine aminopeptidase is often called the stress enzyme.

Substantial insight into the structure of the LAP derives from X-ray structures of the enzyme from bovine lens determined in its native form and complexed with transition state inhibitors such as: bestatin, amastatin, phosphonic analogue of L-leucine and L-leucinal coordinated to the active site.

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Bovine lens leucine aminopeptidase is a hexamer with a mass of 324 kDa, where every from six subunits is identical and contains three zinc ions. Two of them are located in active site of the enzyme and play a catalytic role, and third one is situated apart from the active site and most likely plays a structural function. LAP hydrolyses all of the substrates with N-terminal L-amino acid and glycine, but kinetic preference of the enzyme is towards L-leucine and L-phenylalanine, and to a less extend, to other hydrophobic substrates [10–15].

The inhibitory activity of the inhibitors of LAP is routinely measured using commercially available enzyme from porcine kidney. Unfortunately, X-ray structure of this enzyme has not been determined so far. In the search for the potential inhibitors of LAP this fact seems to have no significant meaning since both enzymes (from bovine lens and from porcine kidney) reveal high first-order sequence homology and similar kinetic properties, what suggests close structural features of their active centres [1].

Several sets of various inhibitors of leucine aminopeptidase have been reported so far. On the basis of their structure, they can be divided into two major groups. First one constitute analogues of short peptides, among which analogue of dipeptide-bestatine, and tetrapeptide-amastatine, are the best known ones [16,17]. The second group represent analogues of amino acids, with L-leucinal (K_i = 60 nM), the boronic acid analogue of L-leucine (K_i = 130 nM), phosphonic analogue of L-leucine (K_i = 230 nM) and hydroxamic analogue of D-leucine (K_i = 1.3 μM) being the most effective (Fig. 1) [18–22].

All of these inhibitors are transition state analogues of LAP, able to bind to two zinc ions present in the active site of the enzyme. According to the substrate specificity, the L-enantiomers at the N-terminus of polypeptide are stronger cooridinated to the active site of the enzyme than their D-enantiomers. Additionaly, the common feature of all presented inhibitors is the hydrophobic character of the side chain, where leucine isobutyl moiety is complexed by LAP.

The present results are the continuation of our previous studies on the inhibition of leucine aminopeptidase, where the crystal structure of L-leucine phosphonic acid coordinated in active centre of bovine lens leucine aminopeptidase was chosen as a lead compound in the search for the optimal structure of the inhibitors [23]. In the current study we have

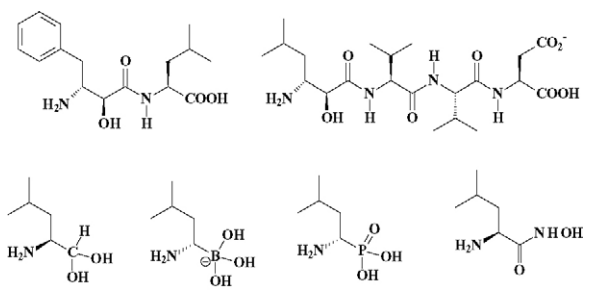


Fig. 1. The structures of the inhibitors of leucine aminopeptidase: bestatine, amastatine, L-leucinal, L-leucine boronic acid, L-L euP and D-leucine hydroxamic acid.

mainly focused on the experimental optimisation of the P1 side chain shape of the inhibitor located at S1 site of the enzyme, paying only marginal attention to the stereochemistry, because this feature has been the subject of earlier detailed investigations. Since similar comopunds have been reported as inhibitors of structurally closely related aminopeptidase N (APN, EC 3.4.11.2), we have performed additional studies to compare the structural requirements of S1 pocket of LAP versus the same pocket of APN [21].

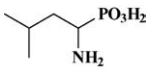
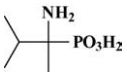
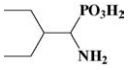
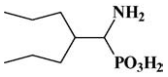
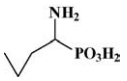
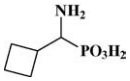
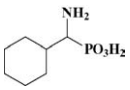
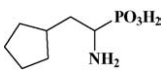
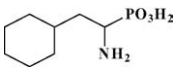
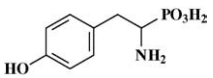
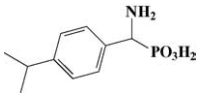
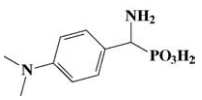
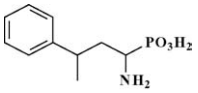
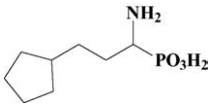
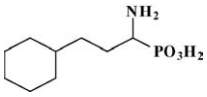
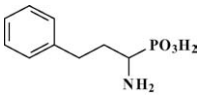
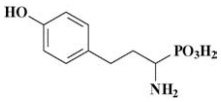
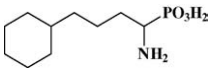
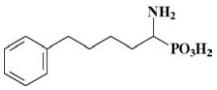
2. Results

1-Aminoalkanephosphonic acids have been obtained as racemic mixtures according to the well established procedures. In the case of racemic compounds, the Oleksyszyn or modified Arbuzov methodologies have been applied. Enantiopure 1-aminoalkylphosphonates have been synthesised using Hamilton–Walker reaction (Table 1) [24–26]. In the case of 1-amino-3-phenylbutanephosphonic acid (**15**), the enzymatic assay was performed using the mixture of two pair of enantiomers obtained in 1:0.7 ratio (stereochemistry not determined) (Fig. 2).

Seven aromatic, three aliphatic and eight alicyclic aminoalkylphosphonates were used for optimisation of the P1 fragment of potential LAP inhibitors. These structures were selected according to the expected change of their inhibitory activity against LAP due to the carbon chain length, steric bulk, presence or absence of methylene bridge between aminophosphonate and hydrophobic portions of the molecule, as well as taking into consideration overall shape of the phosphonate. The introduction of the polar, hydroxy groups at the termini of long, hydrophobic chains was chosen to check the hypothesis that additional hydrogen bonds with Asp365 and Ala451 residues located at the bottom of S1 cavity might improve the binding properties of the potential inhibitor [23].

All of the 1-aminoalkylphosphonates tested in vitro using porcine kidney leucine aminopeptidase appeared to be slow-binding, competitive inhibitors of the enzyme with K_i values in the range of 0.12 and 798 μM. The mechanism of their binding is a slow one-step process, as indicated from the linear dependence of the apparent first-order rate constant for slow-binding versus inhibitor concentration The lowest activity was found for 1(RS)-amino-1-methyl-1-*iso*-propylmethanephosphonic acid (**2**) (K_i = 798 μM), an analogue od α-methylvaline. Both enantiomers of 1-amino-2-ethylbuthanephosphonic acid (**3/4**) (K_i = 243 and 119 μM for R and S enantiomer respectively) and 1(RS)-amino-3-propylpenthanephosphonic acid (**5**) (K_i = 33 μM) also rank among weak LAP inhibitors. Better results were obtained for alicyclic analogues since 1(RS)-amino-1-cyclopropylmethanephosphonic acid (**6**) (K_i = 84.5 μM), 1(RS)-amino-1-cyclobuthylmethanephosphonic acid (**7**) (K_i = 22.7 μM), 1(RS)-amino-4-cyclohexyl-buthanephosphonic acid (**20**) (K_i = 9.57 μM), both enantiomers of 1-amino-1-cyclohexylmethanephosphonic acid (**8/9**) (K_i = 7.89 and 6.96 μM for R

Table 1
The structures of 1-aminophosphonic acids and their experimental inhibition constants (K_i) towards porcine kidney leucine aminopeptidase and microsomal aminopeptidase N (NI—no inhibition up to final concentration of inhibitor 2 mM)

Entry	Structure	Stereomer	K_i (μM)-LAP	K_i (μM)-APN
1		1R	0.23[21]	53[21]
2		1RS	798	NI
3/4		1R/1S	243/119	138/NI
5		1RS	33.0	NI
6		1RS	84.5	178
7		1RS	22.7	77.4
8/9		1R/1S	7.89/6.96	161/NI
10		1RS	6.92	47.7
11		1RS	0.75	54.7
12		1RS	0.33	170
13		1RS	NI	168
14		1RS	1.37	NI
15		1RS/3RS	0.26	36.9
16		1RS	6.35	18.5
17		1RS	0.21	37.1
18		1RS	0.14	15.9
19		1RS	0.12	23.7
20		1RS	9.57	41.6
21		1RS	0.33	3.69

and S enantiomer respectively) and 1(RS)-amino-2-cyclopentylethanephosphonic acid (**10**) (K_i = 6.92 μM) are moderate inhibitors of LAP, whereas 1(RS)-amino-2-cyclohexylethanephosphonic acid (**11**) (K_i = 0.75 μM) and 1(RS)-amino-3-cyclohexylpropanephosphonic acid (**17**) (K_i = 0.21 μM) rank amongst the most active compounds. Aromatic analogues appeared to be much more effective inhibitors of the enzyme. Thus, 1(RS)-amino-2-(4-hydroxyphenyl)ethanephosphonic (**12**) acid and 1(RS)-amino-5-phenylpentanephosphonic acid (**21**) have the same value of K_i equal 0.33 μM, close to that obtained for the mixture of diastereomers of 1(RS)-amino-3(RS)-phenylbutanephosphonic acid (**15**) (K_i = 0.26 μM). The most active amongst compounds studied in this work were 1(RS)-amino-3-phenylpropanephosphonic acid (**18**) (K_i = 0.14 μM) and 1(RS)-amino-3-(4-hydroxyphenyl)propanephosphonic acid (**19**) (K_i = 0.12 μM). It is worth to note, that compounds **17**, **18** and **19**, even as racemic mixtures are better inhibitors of pkLAP than the stereochemically favoured phosphonic acid analogues of L-leucine (**1**) (K_i = 0.23 μM) and L-phenylalanine (K_i = 0.42 μM).

All of the investigated aminoalkylphosphonates appeared to be only moderate inhibitors of aminopeptidase N with K_i values in micromolar range and competitive type of inhibition. The slow-binding was observed only in the case of compounds **7**, **13** and **20** and was also found as the slow one-step process. The most active of them was 1(RS)-amino-5-phenylpentanephosphonic acid (**21**) with K_i value equal

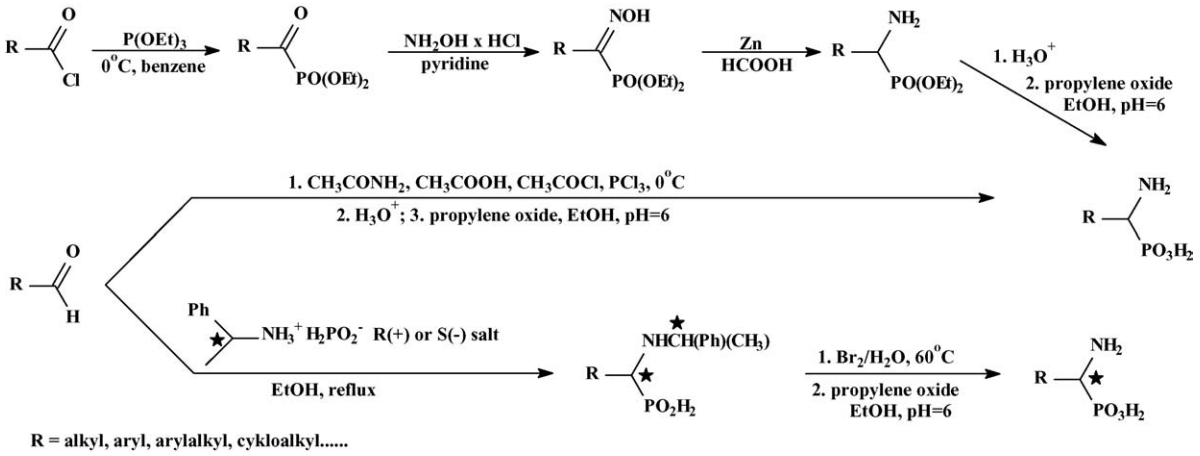


Fig. 2. Procedures and conditions of synthesis of α-aminoalkylphosphonates.

3.69 μM. Almost half of the tested by us aminoalkylphosphonates (**7**, **10**, **11**, **15**, **16**, **17**, **18**, **19** and **20**) had K_i value in the narrow range between 15.9 μM and 77.4 μM. The rest of the compounds had the inhibition constants higher than 100 μM (**3**, **6**, **8**, **9**, **12** and **13**) or did not reveal any inhibitory activity toward APN (**2**, **4**, **5** and **14**).

3. Discussion and conclusions

The structure of leucine aminopeptidase is specific according to the defined species, but not to tissues. LAP isolated from pig kidney have been routinely used for biological investigations. Unfortunately till now there are no reports describing X-ray structure of this enzyme. Therefore, crystal structure of LAP isolated from bovine lens in its free form as well as complexed with some inhibitors (bestatine, L-LeuP, amastatine and L-leucinal) is routinely used for designing of new inhibitors [23]. The fact, that the biological studies are performed using pig LAP, and potent inhibitors are theoretically evaluated using bovine LAP seems to have not influence on overall effect, because of the results from immunological test, which had proven that first-order structure of bovine and pig LAP reveal 91.5% of homogenity [1]. Both aminopeptidases (blLAP and pkLAP), like the aminopeptidases from *Aeromonas Proteolytica* (AAP) and *Streptomyces griseus* (SAP), prefer hydrophobic residues at NH_2-terminal position [27–29]. The S1 pocket of blLAP, adjacent to the active site of the enzyme, has hydrophobic character and plays crucial role in binding side chains of substrates and potent inhibitors. It is made up of Met270, Ala451, Thr359, Gly362 and Met454 [23]. Far less is known about three-dimensional shape of aminopeptidase N binding sites since its crystal structure is not available. Our previous studies indicated, however, that there is substantial difference in requirements of S1 pocket of the studied enzymes [21].

The main goal of the investigations presented in this paper was to obtain more detailed experimental insight into the structural preferences of S1 pocket of both enzymes pointing out similarities and differences. The basis for our research lay in the observation that this pocket of blLAP has a large volume and thus is able to bind far more bulky side chains than those found in natural substrates (Leu, Phe, Ala). Additionally, computer-aided modelling of this pocket resulted in discovery of potent low-molecular inhibitors of this enzyme differing in placement of their side chains in S1 pocket in both enzymes [30,31].

Among all the molecules tested in this work, the most active inhibitor of pkLAP appeared to be 1(RS)-amino-3-(4-hydroxyphenyl)-propanephosphonic acid (**19**) (K_i = 0.12 μM), homologue of phosphonic acid analogue of tyrosine. We have obtained this compound in order to test the hypothesis advanced after computer-aided design, which suggested improvement of the binding abilities upon formation of additional hydrogen bond between hydroxy group on aromatic ring of **19** and Asp365 located deeply in the S1 pocket of LAP [23]. However, 1(RS)-amino-3-phenylpropanephosphonic acid (**18**) (K_i = 0.14 μM), a homologue of phosphonic acid of phenylalanine appeared to be an equipotent inhibitor of pkLAP. This clearly showed that formation of the expected hydrogen bond between Asp365 and phenolic moiety of **19** does not form. More advanced computer modelling using GRID programme have shown that Asp365 is involved in formation of complex hydrogen bond network with water molecules present at the enzyme-solvent interface and the phenolic group of **19** disturbs this network [32].

The potent inhibitory activity of compounds **18** and **19** towards pkLAP, the prolongation of the side chain with one additional methylene group resulted in substantial improvement of inhibitory activity (racemic mixture) if comparing to L-PheP (K_i = 0.42 μM). Similar homologue, namely 1(RS)-amino-3(RS)-phenylbutanephosphonic acid (**15**) reveals a little bit lower inhibitory activity towards pkLAP, however it is worth to note that it was applied as the mixture of four diastereomers (K_i = 0.26 μM). The prolongation of the aliphatic side chain by additional methylene group caused slight drop in the inhibitory activity, which is well illustrated by affinity of 1(RS)-amino-5-phenylpentanephosphonic acid (**21**) (K_i = 0.33 μM) towards the enzyme. The same pattern of structure–activity relationship was observed in the case of APN

although the studied compounds exhibited substantially lower affinities towards this enzyme.

The replacement of aromatic group by various alicyclic ones enables to observe the influence of their shape and size on the K_i of pkLAP. In opposition to the rigid aromatic ring, the alicyclic one can undergo a series of conformational transformations, which might influence the activity of the tested inhibitors. The most active towards pkLAP appeared 1(RS)-amino-3-cyclohexylpropanephosphonic acid (**17**) (K_i = 0.21 μM), which is structural analogue of **18**, in which phenyl group was substituted by cyclohexyl moiety. Small difference in the inhibition constant between them (**18** favoured of about 70 nM) suggests, that cyclohexyl group has similar affinity to S1 pocket of the enzyme as planar aromatic ring. This assumption seems to be well supported by the K_i found for 1(RS)-amino-2-cyclohexylethanephosphonic acid (**11**) (K_i = 0.75 μM), which is nearly equipotent with phosphonic analogue of L-PheP (K_i = 0.42 μM and 15 μM for R and S enantiomers, respectively). The replacement of cyclohexyl group by cyclopentyl one resulted in significant decrease of the affinity, as illustrated by low activity of compound **16** (K_i = 6.35 μM). Surprisingly, this minute change in the chemical structure is drastic enough, that it does not allow to dock this fragment of the molecule effectively into the S1 pocket of LAP. Interestingly, compound **16** ranks amongst the best inhibitors of APN being nearly equipotent (K_i = 18.5 μM) with the second most active and structurally close compound **18**. These findings also clearly show that there are minute, although important perferences of S1 pockets of both enzymes and that the pocket of APN seem to be more spacious than this of LAP.

1-Amino-1-cyclohexylmethanephosphonic acid (**8/9**) (K_i = 7.89 and 6.96 μM for R and S enantiomers respectively) is quite weak inhibitor of both aminopeptidases studied. However, there are substantial differences in its potency if considering stereochemistry. Equipotency of both enantiomers towards LAP indicates the lack of stereochemical preferentions of the enzyme towards analogues lacking methylene arm separating aminoalkylphosphonate portion of the molecule and its hydrophobic fragment. Substantially different activity was seen in the case of APN, where strong stereochemical preference towards R isomer (corresponding to L-configuration) was observed.

Aminoalkylphosphonates containing alkyl side chains, designed to mimic opened forms of cycloalkyl rings and thus being more flexible, ranked amongst the weakest inhibitors of pkLAP. 1-Amino-2-ethylbuthanephosphonic acid (**3/4**) (K_i = 243 and 119 μM for R and S enantiomer respectively) is far less active towards pkLAP than compounds **8/9** and **7** and similarly as in the case of R and S enantiomers of **8/9** they revealed only minute stereochemical preference. This compound was, however, nearly equipotent with **8/9** towards APN and also showed significant stereochemical preference, thus following the same structure–activity pattern. These findings are a little bit astounding if taking into condsideration strong structural relation of compound **3/4** to phosphonic acid analogue of valine with reported inhibition constants towards LAP of 0.15 and 12 μM for R and S enantiomers respectively [21]. Compound **5** containing more bulky substuituents at α-carbon atom appeared to be more potent towards LAP than **3/4** and inactive towards APN showing that structure–activity relationship is far more complicated and does not allow simple reasoning. Most probably, the presence of additional bulky hydrophobic group located at α-carbon atom distores coordination of amino and phosphonic moieties to the Zn ions in the active site of LAP by non-optimal binding of hydrophobic part of the molecule in S1 pocket, and thus showing that the propoper design of this part of molecule is vital for inhibitory potency. The lack of intuitive reasoning in inhibitor design, which is based on simple comparison of chemical structures is also well illustrated by the differences in activity of structurally related compounds **13** (being active towards APN and inactive towards LAP) and **14** (active towards LAP and inactive towards APN) towards both enzymes.

The presented results clearly show, that the maximal activity of the potential LAP and APN inhibitors increase with the length of the side chain located in S1 pocket, reaching optimum for three atoms separating aromatic fragment of the molecule and its aminoalkylphosphonate portion. Compounds having β and γ non-substitued methylene groups are preferably bound (Fig. 3).

The observable decrease on the K_i drastically dimnishes when this key feature is abused by the incorporation of the additional substituents at β and γ positions as well as elongation or shortening of the side chain with slight, although important differences observed in the case of both enzymes (Fig. 4).

The presented analysis, when using 1-aminoalkylphosphonic acids as molecular probes gave substantial insight into the preferences of LAP S1 binding site and provides some useful information regarding further inhibitor design. Additionally, acceptable agreement of some of the theoretical data obtained by the analysis of bovine lens LAP with the experimental data presented here for porcine kidney LAP well confirms the similarity of both enzymes [33]. Thus, the proper design of the part of inhibitor bound in the S1 pocket is vital for the activity of inhibitors towards both enzymes, however, it is more important in the case of LAP than APN.

4. Experimental protocols

4.1. General methods

Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. NMR spectra were recorded on a Bruker Avance DRX 300 MHz instrument, operating at 300.13 MHz (^{1}H), 121.499 MHz (^{31}P) and 75.46 MHz (^{13}C). Measurements were made in D_2O (99.8 at. % D) solutions at temperature 300 K, all solvents were supplied by Dr. Glaser AG (Basel,

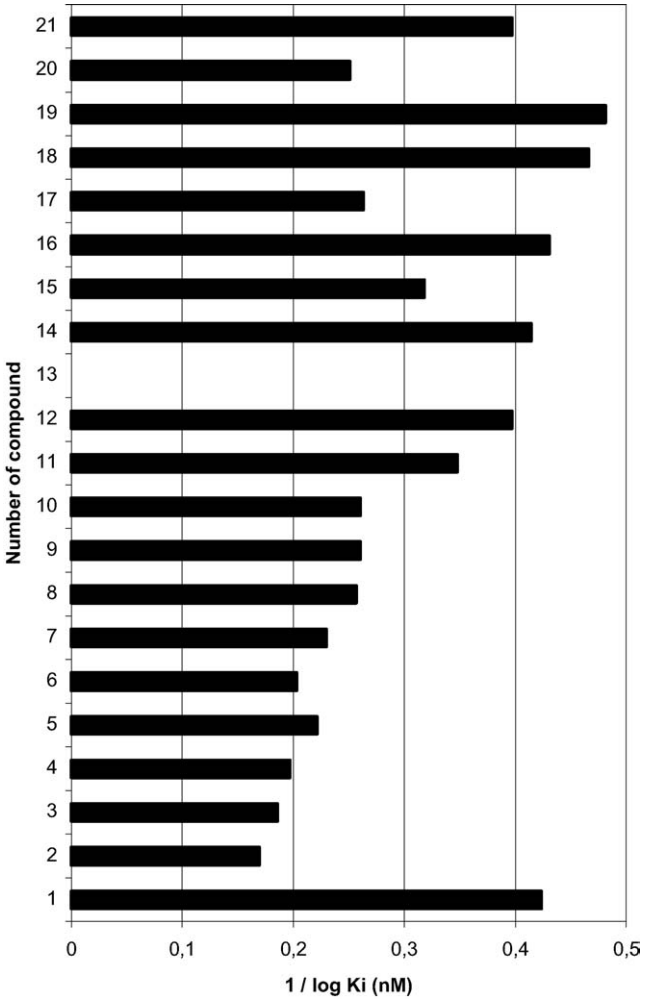
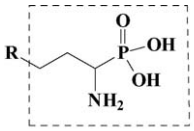


Fig. 3. Structure–activity relationships of the experimental inhibitory activity of 1-aminoalkylphosphonates toward pkLAP.



R = alkyl, aryl, cycloalkyl

Fig. 4. The key elements of the structure of the optimised inhibitor of pkLAP.

Switzerland). Chemical shifts are reported in parts per million relative to TMS, and coupling constants are reported in Hertz. Spectrophotometric measurements were performed using thermostated Specord M40 (Carl Zeiss, Jena, Germany).

Porcine kidney leucine aminopeptidase was purchased from Sigma Chemical Co. and activated according to the procedure described by Andersson et al. [18]. Enzymatic reactions were routinely carried out in 2.85 ml of 7.5 mM triethanol amine-HCl buffer, pH 8.4, containing 15 μg ml^{-1} of porcine kidney leucine lminopeptidase, 0.1 ml substrate-L-leucine-4-nitroanilide and 0.1 ml of water solution of inhibitor (various concentrations). Reactions were monitored at 400 nm with substrate concentration varying from 0.2 to 0.8 mM. The concentration of the LAP was determined spectrophotometrically at 280 nm, assuming $A_{280}^{0.1\%} = 0.83\ cm^{-1}$ and molecular weight of the enzyme equal 255.000 Da [21].

Porcine kidney microsomal aminopeptidase (APN) was also obtained from Sigma Chemical Co. and treated identical as described earlier [21]. The inhibitory activity of the enzyme was measured at 25 °C in 50 mM potassium phosphate buffer (pH = 7.2) using L-leucine-4-nitroanilide as substrate (K_m = 0.52 mM). The final volume (2 ml) of the assay mixture contained 0.1 ml of the substrate solution in DMSO, 5 μl of the APN solution in final concentration of 4 μg ml^{-1}, 0.5 ml of the inhibitor solution and 1395 ul of phosphate buffer. The concentration of the APN was determined spectrophotometrically at 280 nm, assuming $A_{280}^{0.1\%} = 1.63\ cm^{-1}$ and molecular weight of the enzyme equal 280.000 Da [21]. The possible error in the K_i value in all inhibitors do not exceed 10%.

General method of synthesis of 1-aminoalkylphosphonates **5**, **6**, **7**, **10**, **11**, **12**, **16**, **17**, **19**, **20** and **21**.

0.1 mol of the appropriate carboxylic acid and 50 ml of toluene was placed in a flask (250 ml) equipped with magnetic stirrer. After dissolving of the acid, 0.3 mol of the thionyl chloride was added to the reaction mixture. The mixture was refluxed the for 6–7 h with stirring. The volatile compound were removed by rotary evaporation. To the remained reaction mixture was added 50 ml of hexane and heated again until boiling. The repetition of the rotary evaporation gave the desired, crude carbonyl chloride (quantitative and qualitative yield), which was immediately used to the next step without future purification. 0.1 mol of the carbonyl chloride was dissolved in 50 ml of toluene and placed in the flask (250 ml). The mixture was cooled to 0 °C and 0.2 mol of triethyl phosphonate was added very slowly with stirring (during this step the temperature can not exceed 7–8 °C). The mixture was left for a night, and after the volatile products were removed by rotary evaporation. To the remained ketophosphonate, 0.1 mol of hydroxylamine hydrochloride dissolved in 100 ml methanol containing 0.1 mol of piridine was added. The mixture was left for a night, and after the volatile products were removed by rotary evaporation. To the remaining mixture, 100 ml of ice cold water solution of HCl (20 ml of 8 M HCl in 80 ml of distilled water) was added. The extraction with chloroform (3 × 80 ml), washing of the combined organic layers with ice cold water (3 × 100 ml) and brine (100 ml), drying with $MgSO_4$, filtration and evaporation of the volatile products gave the desired α-oxymephosphonate. The mixture was dissolved in formic acid (100 ml dried earlier for 3 days in $CuSO_4$ and distilled). To the intensively stirred mixture, 0.4 mol of Zn was added in small portions. The mixture was left for the night, the zinc formate filtered off, and from the filtrate the volatile products removed by rotary evaporation. To the remained oil, 100 ml of the 10 M HCl was added and refluxed for 8 h. After the removing of the volatile products, the oil was dissolved in 100 ml of ethanol and pyridine or propylene oxide was added till the pH reached 6. The precipitated 1-aminophosphonic acid was filtered off and recrystallised twice from water and ethanol. The deprotection of the hydroxy group in the aromatic ring (in

the substrate carboxylic acid, the hydroxy group should be protected in the form of methoxy) was performed by refluxing in 40% HBr for 10 h and repeating whole procedure as in the case of HCl hydrolysis.

1(RS)-amino-3-propylpenthanephosphonic acid (**5**): (yield: 46%), M.p.: 262–264 °C; ν_{max} (KBr)/cm^{-1} 2933, 1622, 1168 and 1023; δ_P (D_2O + DCl) 17.29; δ_H (D_2O + DCl) 0.67 (6 H, t, $^3J_{HH}$ = 6.6 Hz, 2 × C**H$_3$**CH$_2$CH$_2$), 0.99–1.43 (8 H, m, 2 × CH$_3$C**H$_2$**C**H$_2$**), 1.75 (1 H, m, C**H**CHP), 3.30 (1 H, dd, $^3J_{HH}$ = 7.20 Hz, $^2J_{HP}$ = 16.0 Hz, C**H**P); δ_C (D_2O + DCl) 13.1 (s, CH$_3$CH$_2$CH$_2$), 19.4 (d, $^4J_{CP}$ = 7.4 Hz, CH$_3$CH$_2$CH$_2$), 31.7 (d, $^3J_{CP}$ = 9.4 Hz CH$_3$CH$_2$CH$_2$), 36.27 (s, CHCHP), 50.5 (d, $^1J_{CP}$ = 148.5 Hz, CHP).

1(RS)-amino-1-cyclopropylmethanephosphonic acid (**6**): (yield: 72%), M.p.: 247–248 °C; ν_{max} (KBr)/cm^{-1} 2916, 1650, 1176 and 1029; δ_P (D_2O + DCl) 15.16; δ_H (D_2O + DCl) 0.11 (2 H, m, CH$_2$, cyclopropyl.), 0.37 (2H, m, CH$_2$, cyclopropyl.), 0.7 (1 H, m, CH, cyclopropyl.), 2.29 (1 H, dd, $^3J_{HH}$ = 11.1 Hz, $^2J_{HP}$ = 14.1 Hz, CHP); δ_C (D_2O + DCl) 3.6 (d, $^3J_{CP}$ = 12.9 Hz, 2 × CH$_2$, cyclopropyl.), 8.68 (s, cyclopropyl.), 53.4 (d, $^1J_{CP}$ = 151.4 Hz, CHP).

1(RS)-amino-1-cyclobuthylmethanephosphonic acid (**7**): (yield: 66%), M.p.: 251–253 °C; ν_{max} (KBr)/cm^{-1} 2972, 1651, 1171 and 1024; δ_P (D_2O + DCl) 15.31; δ_H (D_2O + DCl) 0.9–1.36 (6H, m, 3 × CH$_2$, cyclobuthyl.), 1.95 (1H, m, CH, cyclobuthyl.), 2.71 (1H, dd, $^3J_{HH}$ = 10.8 Hz, $^2J_{HP}$ = 12.9 Hz, CHP); δ_C (D_2O + DCl) 17.02 (s, cyclobuthyl.), 25.10 (s, cyclobuthyl), 25.27 (d, $^3J_{CP}$ = 12.8 Hz, CH, cyclobuthyl.), 32.64 (d, $^2J_{CP}$ = 2.9 Hz, **C**H$_2$CHP), 53.4 (d, $^1J_{CP}$ = 147.3 Hz, CHP).

1(RS)-amino-2-cyclopentylethanephosphonic acid (**10**): (yield: 29%), spectral data identical as published earlier [34].

1(RS)-amino-2-cyclohexylethanephosphonic acid (**11**): (yield: 42%), spectral data identical as published earlier [34].

1(RS)-amino-2-(4-hydroxyphenyl)ethanephosphonic acid (**12**): (yield: 41%), spectral data identical as published earlier [35].

1(RS)-amino-3-cyclopentylpropanephosphonic acid (**16**): (yield: 48%), M.p.: 266–268 °C; ν_{max} (KBr)/cm^{-1} 2950, 1648, 1175 and 1038; δ_P (D_2O + DCl) 17.26; δ_H (D_2O + DCl) 0.90–1.79 (13H, m, cyclopentyl. + C**H$_2$**C**H$_2$**CHP), 3.34 (1H, dt, $^3J_{HH}$ = 7.7 Hz, $^2J_{HP}$ = 14.9 Hz, C**H**P); δ_C (D_2O + DCl) 23.78 (s, cyclopentyl.), 25.97 (s, cyclopentyl.), 30.51 (d, $^2J_{CP}$ = 8.7 Hz, **C**H$_2$CHP), 30.92 (d, $^3J_{CP}$ = 12.9 Hz, **C**H$_2$CH$_2$CHP), 37.96 (s, cyclopentyl.), 47.26 (d, $^1J_{CP}$ = 151.09 Hz, **C**HP).

1(RS)-amino-3-cyclohexylpropanephosphonic acid (**17**): (yield: 52%), M.p.: 271–273 °C; ν_{max} (KBr)/cm^{-1} 2922, 1651, 1173 and 1024; δ_P (D_2O + DCl) 17.01; δ_H (D_2O + DCl) 0.63–1.71 (15H, m, cyclohexyl. + C**H$_2$**C**H$_2$**CHP), 3.32 (1H, dt, $^3J_{HH}$ = 7.2 Hz, $^2J_{HP}$ = 13.6 Hz, CHP); δ_C (D_2O + DCl) 22.30 (s, cyclohexyl.), 22.93 (s, cyclohexyl.), 29.42 (s, **C**H$_2$CHP), 29.61 (d, $^3J_{CP}$ = 14.5 Hz, **C**H$_2$CH$_2$CHP), 33.72 (s, cyclohexyl.), 35.06 (s, cyclohexyl.), 45.78 (d, $^1J_{CP}$ = 152.8 Hz, **C**HP).

1(RS)-amino-3-(4-hydroxyphenyl)propanephosphonic acid (**19**): (yield: 36%), M.p.: 261–266 °C; ν_{max} (KBr)/cm^{-1} 2934, 1631, 1516, 1492, 1457, 1170 and 1025; δ_P (D_2O + DCl) 17.09; δ_H (D_2O + DCl) 1.85–2.08 (2H, m, C**H$_2$**CH$_2$CHP), 2.61 (2H, m, C**H$_2$**CHP), 3.33 (1H, dt, $^3J_{HH}$ = 7.26 Hz, $^2J_{HP}$ = 16.9 Hz, C**H**P), 6.70 (2H, d, $^3J_{HH}$ = 8.3 Hz, arom.$_{AB}$,), 7.04 (2H, d, $^3J_{HH}$ = 8.3 Hz, arom.$_{AB}$,); δ_C (D_2O + DCl) 28.99 (s, **C**H$_2$CH$_2$CHP), 29.41 (s, $^2J_{CP}$ = 8.0 Hz, **C**H$_2$CHP), 46.60 (d, $^1J_{CP}$ = 150.2 Hz, **C**HP), 114.65 (s, arom.), 128.87 (s, arom.), 131.33 (s, arom.), 152.8 (s, arom.).

1(RS)-amino-4-cyclohexylbuthanephosphonic acid **20**: (yield: 36%), M.p.: 271–273 °C; ν_{max} (KBr)/cm^{-1} 2923, 1024, 1651 and 1174; δ_P (D_2O + DCl) 16.88; δ_H (D_2O + DCl) 0.67–1.72 (17H, m, cyclohexyl. + C**H$_2$**C**H$_2$**C**H$_2$**CHP), 3.34 (1H, dt, $^3J_{HH}$ = 7.44 Hz, $^2J_{HP}$ = 13.9 Hz, C**H**P); δ_C (D_2O + DCl) 22.98 (s, **C**H$_2$CH$_2$CHP), 23.59 (s, cyclohexyl.), 23.97 (s, cyclohexyl.), 30.07 (s, **C**H$_2$CH$_2$CH$_2$CHP), 30.25 (s, **C**H$_2$CH$_2$CHP), 30.37 (s, cyclohexyl.), 34.35 (s, cyclohexyl.), 46.39 (d, $^1J_{CP}$ = 152.63 Hz, **C**HP).

1(RS)-amino-5-phenylpentanephosphonic acid (**21**): (yield: 26%), M.p.: 274–276 °C; ν_{max} (KBr)/cm^{-1} 2931, 1643, 1532, 1496, 1453, 1159 and 1028; δ_P (D_2O + DCl) 16.72; δ_H (D_2O + DCl) 1.02–1.51 (6H, m, C**H$_2$**C**H$_2$**C**H$_2$**CHP), 2.18 (2H, t, $^3J_{HH}$ = 7.40 Hz, C**H$_2$**CH$_2$CH$_2$CH$_2$CHP), 2.97 (1H, dt, $^3J_{HH}$ = 7.20 Hz, $^2J_{HP}$ = 13.6 Hz, C**H**P), 6.74–6.90 (5H, m, arom.); δ_C (D_2O + DCl) 24.50 (d, $^3J_{CP}$ = 9.1 Hz, **C**H$_2$CH$_2$CHP), 27.33 (s, **C**H$_2$CH$_2$CH$_2$CHP), 29.8 (s, **C**H$_2$CHP), 34.16 (s, **C**H$_2$CH$_2$CH$_2$CH$_2$CHP), 47.7 (d, $^1J_{CP}$ = 150.2 Hz, **C**HP), 125.6 (arom.), 128.3 (arom.), 128.5 (arom.), 142.5 (arom.).

Method of synthesis of 1-aminoalkylphosphonates **1**, **3**, **4**, **8** and **9**. These compounds have been synthesised according to the method of synthesis described earlier by Hamilton et al. [26].

1(R)-amino-3-methylbutanephosphonic acid (**1**): (yield: 29%), spectral and optical activity data identical as published earlier [26].

1-Amino-2-ethylbuthanephosphonic acid (**3/4**): (yield: 36% and 33% for compounds **3** and **4** respectively); M.p.: 247–248 °C; ν_{max} (KBr)/cm^{-1} 2928, 1608, 1164 and 1024; δ_P (D_2O + DCl) 17.61; δ_H (D_2O + DCl) 0.48 (3H, d, $^3J_{HH}$ = 7.6 Hz, CH$_2$C**H$_3$**), 0.51 (3H, d, $^3J_{HH}$ = 7.6 Hz, CH$_2$C**H$_3$**), 0.85–1.02 (2H, m, C**H$_2$**CH$_3$), 1.17 (2H, m, C**H$_2$**CH$_3$), 1.29 (1H, m, C**H**CH$_2$CH$_3$), 1.53 (1H, dd, $^3J_{HH}$ = 4.4 Hz, $^2J_{HP}$ = 16.0 Hz, C**H**P); δ_C (D_2O + DCl) 10.28 (s, 2 × **C**H$_3$CH$_2$CH), 21.31 (d, $^3J_{CP}$ = 32 Hz, CH$_3$**C**H$_2$CH), 40.10 (s, CH$_3$CH$_2$**C**H), 49.67 (d, $^1J_{CP}$ = 147.4 Hz, **C**HP).

1-Amino-1-cyclohexylmethanephosphonic acid (**8/9**): (yield: 42% and 54% for compounds **8** and **9** respectively); spectral data and optical activity identical as published earlier [26].

Method of synthesis of 1-aminoalkylphosphonates **2**, **13**, **14**, **15** and **18**. These compounds have been synthesised according to the method of synthesis described earlier by Oleksyszyn et al. [24].

1(RS)-amino-1-methyl-1-*iso*-propylmethanephosphonic acid **2**: (yield: 52%), spectral data identical as published earlier [36].

1(RS)-amino-1-(4-(*iso*-propyl)phenyl)methanephosphonic acid (**13**): (yield: 42%), spectral data identical as published earlier [37].

1(RS)-amino-1-(4-(*N,N*-dimethylamino)phenyl)methanephosphonic acid (**14**): (yield: 16%), M.p.: 237–238 °C; ν_{max} (KBr)/cm^{-1} 3372, 1172, 2961, 1651, 1552, 1511, 1457 and 1055; δ_P (D_2O + DCl) 18.66; δ_H (D_2O + DCl) 4.17 (6H, s, 2 × C**H$_3$**), 5.93 (1H, d, $^2J_{HP}$ = 22.8 Hz, C**H**P), 7.26 (s, 4H, arom.); δ_C (D_2O + DCl) 46.24 (s, 2 × **C**H$_3$), 59.2 (d, $^1J_{CP}$ = 150.18 Hz, **C**HP), 116.6 (s, arom.), 127.92 (s, arom.), 134.53 (d, $^3J_{CP}$ = 14.2 Hz, arom.), 144.8 (s, arom.).

1(RS)-amino-3-(RS)-phenylbutanephosphonic acid (**15**): (yield: 52%), M.p.: 250–256 °C; ν_{max} (KBr)/cm^{-1} 2941, 1039, 1633, 1586, 1534, 1495, 1457 and 1174; δ_P (D_2O + DCl) 17.28 and 17.43 (ratio 1:0.7); δ_H (D_2O + DCl) 1.03 (3H, d, $^3J_{HH}$ = 7.24 Hz, CH$_3$, diast. 1), 1.14 (3H, d, $^3J_{HH}$ = 7.24 Hz, CH$_3$, diast. 0.7), 1.65–2.08 (1H, m, C**H**CH$_2$CHP, 2 diast.), 2.52 (2H, m, CH$_2$C**H$_2$**CHP, 2 diast.), 3.34 (1H, m, C**H**P, 2 diast.), 7.11–7.29 (5H, m, aromat., 2 diast.); δ_C (D_2O + DCl) 22.31 (s, **C**H$_3$, diast. 1), 22.35 (s, **C**H$_3$, diast. 0.7), 32.11 (d, $^3J_{CP}$ = 4.2 Hz, **C**H$_2$CH$_2$CHP, diast. 1), 32.89 (d, $^3J_{CP}$ = 4.75 Hz, **C**H$_2$CH$_2$CHP, diast. 0.7), 40.05 (s, **C**H$_3$, diast. 1), 41.17 (s, **C**H$_3$, diast. 0.7), 51.38 (d, $^1J_{CP}$ = 148.5 Hz, **C**HP, diast. 1), 51.38 (d, $^1J_{CP}$ = 148.5 Hz, **C**HP, diast. 0.7), 126.12 (arom., diast. 0.7), 127.10 (arom., diast. 1), 127.84 (arom., diast. 1), 128.25 (arom., diast. 0.7), 129.18 (arom., diast. 0.7), 130.42 (arom., diast. 1), 146.32 (arom., diast. 1), 147.02 (arom., diast. 0.7).

1(RS)-amino-3-phenylpropanephosphonic acid (**18**): (yield: 65%), spectral data identical as published earlier [38].

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