The Most Potent Organophosphorus Inhibitors of Leucine Aminopeptidase. Structure-Based Design, Chemistry, and Activity

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A new class of very potent inhibitors of cytosol leucine aminopeptidase (LAP), a member of the metalloprotease family, is described. The X-ray structure of bovine lens leucine aminopeptidase complexed with the phosphonic acid analogue of leucine (LeuP) was used for structure-based design of novel LAP inhibitors and for the analysis of their interactions with the enzyme binding site. The inhibitors were designed by modification of phosphonic group in the LeuP structure toward finding the substituents bound at the S′ side of the enzyme. This resulted in two classes of compounds, the phosphonamidate and phosphinate dipeptide analogues, which were synthesized and evaluated as inhibitors of the enzyme. The in vitro kinetic studies for the phosphinate dipeptide analogues revealed that these compounds belong to the group of the most effective LAP inhibitors found so far. Their further modification at the P1 position resulted in more active inhibitors, hPheP[CH_2]Phe and hPheP[CH_2]Tyr (K_i values 66 nM and 67 nM, respectively, for the mixture of four diastereomers). The binding affinities of these inhibitors toward the enzyme are the highest, if considering all compounds containing a phosphorus atom that mimick the transition state of the reaction catalyzed by LAP. To evaluate selectivity of the designed LAP inhibitors, additional tests toward aminopeptidase N (APN) were performed. The key feature, which determines their selectivity, is structure at the P1′ position. Aromatic and aliphatic substituents placed at this position strongly interact with the LAP S1′ binding pocket, while a significant increase in binding affinity toward APN was observed for compounds containing aromatic versus leucine side chains at the P1′ position. The most selective inhibitor, hPheP[CH_2]Leu, binds to LAP with 15 times higher affinity than to APN. One of the studied compounds, hPheP[CH_2]Tyr, appeared to be very potent inhibitor of APN (K_i = 36 nM for the mixture of four diastereomers). The most promising LAP inhibitors designed by computer-aided approach, the phosphonamidate dipeptide analogues, were unstable at pH below 12, because of the P−N bond decomposition, which excluded the possibility of determination of their binding affinities toward LAP.

Introduction

A structure-based approach to design potent and selective inhibitors is an important component of the modern drug development process.[1−3] The design and synthesis of inhibitors for aminopeptidases may result in potential therapeutic agents, as the altered activity of these enzymes is associated with variety pathologies, including cancer, leukemia, and cystic fibrosis.[4−6] Proteases of this family have broad substrate specificity and are widely distributed in many tissues and cells in plants, animals, bacteria, and viruses, which indicate their significant role in various biological processes.[4,7,8] One of the first discovered and the most widely studied aminopeptidases with respect to sequence, structure, and mechanism of action is cytosolic leucine aminopeptidase (LAP, EC 3.4.11.1).[4,9−12] LAP is a zinc-containing exopeptidase that catalyzes the removal of amino acids from the *N*-terminus of peptides or proteins. Similar to other aminopeptidases, this enzyme is of significant biological and medical importance because of its key role in protein modification, activation, and degradation as well as in the metabolism of biologically active peptides and activity regulation of hormonal and nonhormonal peptides.[5] Altered activity of human leucine aminopeptidase has been associated with several pathological disorders, such as cancer,[13,14] eye lens aging and cataracts,[15−17] and myeloid leukemia and blood cell counts.[18] This protease may also be important in the processing of antigenic peptides and in the determination of the immunodominance of various peptides.[19] Moreover, LAP seems to play an important role in the early stages of HIV infection, and thus serum activity of this enzyme may be useful as a surrogate marker for HIV infection and as a response to chemotherapy.[20]

LAPs from different sources (beef, hog, human) are very similar in terms of the amino acid sequences and kinetics,[4,17,21] and they probably share the same active site architecture.[5] This makes the structure of the enzyme from bovine eye lens (blLAP), the only one for which 3D structure is available in Protein Data Bank, applicable to design inhibitors of the enzyme from porcine and human tissues.[21,22]

A number of various inhibitors have been reported to bind to LAP.[23] Among the amino acid analogues, the

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phosphonic analogue of L-leucine, (*R*)-LeuP, plays an important role in leucine aminopeptidase inhibition, as it is strong (K_i = 0.23 μM)[24] and selective toward the enzyme. Because of the presence of the aminophosphonic fragment in the structure, this compound mimics the tetrahedral *gem*-diolate transition state of the peptide bond hydrolysis by zinc peptidases. The binding mode of LeuP, revealed by the X-ray structure of its complex with blLAP, showed the interactions of the aminophosphonate fragment of the molecule with two zinc ions and formation of three hydrogen bonds with the LAP active site as well as the interactions of LeuP side chain with the S1 pocket.[25] However, strong LAP inhibition by dipeptide analogue bestatin (K_i = 0.6 nM)[26] demonstrates the significance of the interactions with the S1′ pocket as well. The availability of the crystal structures of both the free enzyme[10] and its complexes with the inhibitors[10,27−29] including LeuP[25] enable the application of molecular modeling methods for rational design of LAP inhibitors.

In this paper we report the use of the crystal structure of complex LAP−LeuP for the design of new, potent inhibitors of leucine aminopeptidase by application of the method implemented in the Ludi program, which had been used successfully to generate several sets of the active compounds.[30−34] We have focused on finding new fragments bound in the S1′ pocket of the enzyme in order to extend the inhibitor−protein interaction area, compared to LeuP and on replacement of leucine side chain by a substituent, which should fit better to the S1 pocket of LAP. The design, analysis of the interactions with the enzyme, and synthesis of the inhibitors are reported in this paper. The experimentally measured binding affinities toward LAP are presented, which well evaluate the practical applicability of the theoretical approach used. Finally, the binding affinities of the same compounds toward a monozinc exopeptidase, aminopeptidase N (APN, EC 3.4.11.2), an enkephalin-degrading enzyme, are reported, to compare the potency of the same set of inhibitors toward the two enzymes and evaluate their selectivity to LAP versus APN.

Results and Discussion

Designed Inhibitors. The extensive search of new substituents interacting with the S1′ side of leucine aminopeptidase resulted in the selection of two classes of pseudodipeptides, namely phosphonamidates (containing the P−N bond in place of the substrate peptide bond) and phosphinates (containing the P−CH_2 moiety) (Figure 1).

Phosphonamidate and phosphinate peptide analogues have not been studied as leucine aminopeptidase inhibitors so far. In fact, the literature reports only two groups of structurally related dipeptide analogues containing a phosphorus atom in the structure, namely the phosphonate analogues of LAP substrates (with a P−O bond) and phosphorus derivatives similar to bestatin.[24,35] Their moderate activity toward LAP (the binding affinities about 2 orders of magnitude lower than for LeuP) strongly disfavored further studies on this class of compounds.

The phosphonamidate dipeptide analogues have the potential to form a hydrogen bond with the carbonyl

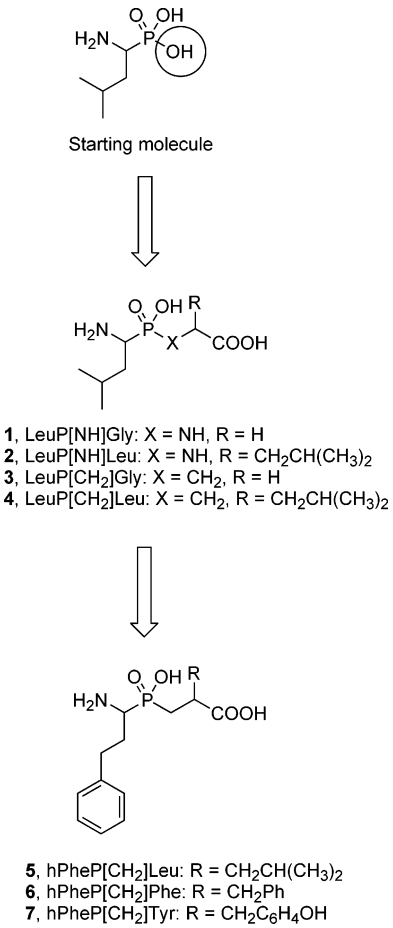


Figure 1. The strategy applied to design LAP inhibitors. The replacement of hydroxy group (in the circle) of LeuP (depicted as a starting molecule) by new substituents from Ludi link library resulted in compounds **1**, **2**, **3**, **4**, containing additional residue at the P1′ position. Simultaneous replacement of leucine at the P1 position by homophenylalanine side chain, resulted in compounds **5**, **6**, **7**.

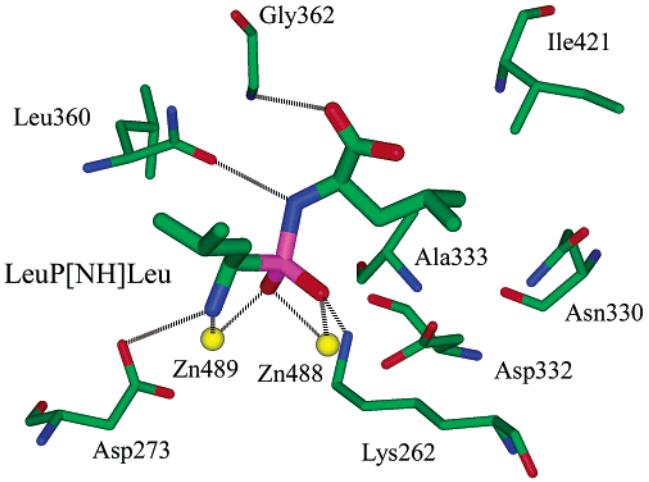
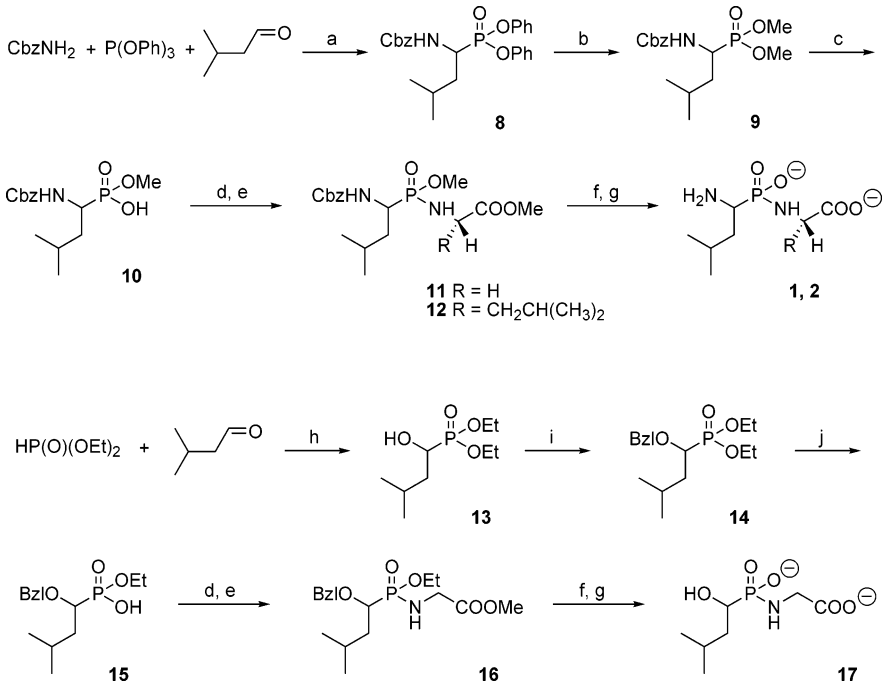


Figure 2. The modeled binding mode of LeuP[NH]Leu by leucine aminopeptidase. The S1′ pocket and active site residues of LAP, interacting with dipeptide analogues, are presented. Hydrogen bonds and interactions of the inhibitor with zinc ions are shown as black dashed lines.

group of Leu360 of the enzyme (Figure 2), in a way similar to that for LAP peptide substrates and LeuP (in which a hydroxy group interacts with Leu360 of LAP). This makes the compounds with a P−N bond more

Scheme 1[a]



[a] (a) AcOH; (b) KF/MeOH; (c) NaOH/MeOH then HCl; (d) $SOCl_2$; (e) $HCl \cdot H_2NCH(R)COOMe$, NEt_3; (f) H_2, Pd/C; (g) LiOH/MeOH; (h) NEt_3; (i) BzlBr, Ag_2O; (j) NaOH/EtOH.

interesting as potential LAP inhibitors than the phosphinates, which lack this ability, because of the presence of a CH_2 group at the corresponding position. Moreover, both classes of inhibitors contain a C-terminal carboxyl group, which is able to form a hydrogen bond with Gly362, while its second oxygen is probably involved in the interactions with the solvent. The presence of the side chain at the P1′ position of these inhibitors opens the possibility for additional interactions with the S1′ pocket of LAP, formed by Ile421, Ala333, Asn330, and Asp332 (Figure 2).

Among the phosphonamidate compounds, designed as complementary to the binding site of the enzyme, there are some analogues of LAP dipeptide substrates as well as compounds that are not analogues but with quite unexpected structural features, which would not be possible to find without the use of computer-aided methods (data not shown). Since the S1′ pocket of leucine aminopeptidase is rather hydrophobic in nature (Figure 2), mainly nonpolar side chains are favored because they can interact well with this cleft. To evaluate the influence of incorporation of phosphonamidate moiety and the presence of a side chain at the P1′ position, we have chosen two dipeptide mimetics, the analogues of leucyl-glycine (LeuP[NH]Gly, **1**) and leucyl-leucine (LeuP[NH]Leu, **2**) for synthesis and further studies. The modeled binding mode of the latter is similar to bestatin, which is known from the crystal structure of its complex with LAP.[29] The analogous phosphinate dipeptides (LeuP[CH_2]Gly, **3**, and LeuP-[CH_2]Leu, **4**), were also selected for synthesis and activity measurements. Although these compounds are straightforward analogues of LAP natural substrates, their design by Ludi confirms the usefulness of the method applied and allows detailed analysis of the interactions of docked inhibitors with the enzyme. Additionally, three other phosphinate dipeptide analogues, containing the phenylethyl side chain at the P1 position (and thus being analogues of homophenylalanine, designed to replace isobutyl side chain of LeuP) and three different amino acid residues at P1′: Leu (hPheP[CH_2]Leu, **5**), Phe (hPheP[CH_2]Phe, **6**), and Tyr (hPheP[CH_2]Tyr, **7**) were also synthesized and studied as LAP inhibitors.

Chemistry of the Peptides Containing a Phosphonamidate Bond. Although phosphonamidates have been quite frequently used as peptide isosteres of strong inhibitory activity against metalloproteases, their synthesis is still not trivial. This is not only because of the presence of the diverse functionalities in one molecule, and thus the necessity of their selective protection and deprotection, but more importantly the side reactions accompanying P−N bond formation via phosphonochloridates[36−39] and instability of this bond.[40−42] There are only very few reports of the synthesis of fully deprotected phosphonamidate dipeptides with free an α-amino group adjacent to the P−N bond and their application as enzyme inhibitors.[43,44]

The applied synthetic strategy in this work is illustrated in Scheme 1 (for α-aminophosphonamidates, the synthetic pathway is similar to those described in the literature[44,45]). The protocol of P−N bond formation starts from the synthesis of suitably protected α-aminophosphonate **10** or α-hydroxyphosphonate alkyl monoester **15** bearing leucine side chain residue. The latter substrate was used in order to obtain $Leu^{OH}P$[NH]Gly (**17**) ($Leu^{OH}P$ denotes phosphonic acid analogue of hydroxyleucine, containing a hydroxy group instead of an α-amino group). Compounds **10** and **15** were then converted into P−N peptides via the standard phosphonochloridate approach followed by aminolysis with the appropriate amino ester.[36−38] The moderate yield observed when using the phosphonochloridate procedure is caused by side reactions, particularly by the formation of pyrophosphonate.[37] However, pyrophosphonate may react slowly with the amino nucleophile and can be

partially transformed into phosphonamidate under these reaction conditions as well. Thus, to increase the product yield we have significantly prolonged the time of the reaction. This methodology gives at least satisfactory results in contrast to the unsuccessful application of the standard peptide coupling agents.[46]

In our experiments $SOCl_2$ was applied for the preparation of phosphonochloridates, and the reaction was monitored by ^{31}P NMR. In the case of compound **10**, the obtained phosphonochloridate exhibited two signals at 42.8 and 43.7 ppm corresponding to the equimolar ratio of two pairs of diastereoisomers. The complex signal at 17.7 ppm belongs presumably to the pyrophosphonate. The obtained reaction mixture was then treated with the appropriate amino ester in the presence of triethylamine as long as only two ^{31}P NMR signals were observed: one, a multiplet for the product, and the second, a singlet, for the recovered starting material. Final, fully protected, P−N peptide analogues (**11**, **12**, and **16**) have been purified chromatografically after standard workup. The overall yield of the procedure was 50−60%.

In the next two steps the peptide protecting groups were removed under mild conditions. Benzyloxycarbonyl or benzyl groups were removed by hydrogenolysis using 10% Pd/C catalyst. The hydrolysis of the ester groups required basic conditions in order to remain the phosphonamidate moiety unchanged. It proceeded gently with the use of 3 equiv of LiOH. The deprotection process was followed by ^{31}P NMR, which had shown that both reactions proceeded practically quantitatively (with yields exceeding 95%). It is worth mentioning that at strongly basic pH we have not observed cyclization of the products to analogues of diketopiperazine, a side-reaction present during saponification of phosphinic peptide methyl esters.

Both protected and deprotected phosphonamidates were obtained as their enantiomeric or diastereomeric mixtures. Because of the discovered liability of the final products which excludes their practical application, the attempts to separate or synthesize compounds with (*R*) configuration at the α-carbon were not undertaken.

Stability Studies of Phosphonamidate Dipeptide Analogues. The results concerning the stability of phosphonamidate moiety obtained by various authors are quite contradictory. Although phosphonamidate pseudopeptides are known as very potent enzyme inhibitors,[47−49] the P−N bond is considered to be unstable.[40−42,50,51] An example of pH dependent cleavage of the P−N dipeptide was presented in crystallographic studies of carboxypeptidase A complexed with Cbz-Gly-[NH]Phe by Christianson and Lipscomb.[52,53] The presence of a free α-amino group at proximity to the phosphonamidate moiety was also mentioned to enhance the susceptibility of such analogues to hydrolysis.[35] On the other hand, such compounds were designed and studied as inhibitors of numerous enzymes.[43,44] These incompatible literature data, particularly concerning the pseudodipeptides with a free α-amino group, induced us to more detailed studies on the phosphonamidate stability in terms of the possibilities of measurement their binding affinities toward leucine aminopeptidase (they will be a subject of separate paper). As a result we have found that compounds **1** and **2** are stable only in strongly basic conditions. The decrease in pH below 12 resulted in P−N bond hydrolysis yielding LeuP and the appropriate amino acid, Gly or Leu. At pH 8.5, which is optimal for LAP activity measurements, all the phosphonamidate was hydrolyzed very fast (after 5 min only products of P−N bond decomposition were observed in NMR spectra). This excludes the possibility to determine the binding affinities of phosphonamidates with a free α-amino group toward leucine aminopeptidase. LAP exhibits aminoexopeptidase activity, and the presence of a free α-amino group is crucial for inhibition because there is no space in the active site to bind bulky protecting groups. Additionally, the presence of an unsubstituted phosphonic group is essential for strong interaction with the LAP active site.[24] These features require the application of fully deprotected dipeptide analogues containing a phosphonamidate bond, which are unfortunately unstable under test conditions and thus totally inapplicable as LAP inhibitors.

Table 1. Measured Binding Affinities of Phosphinate Dipeptide Analogues toward LAP and APN

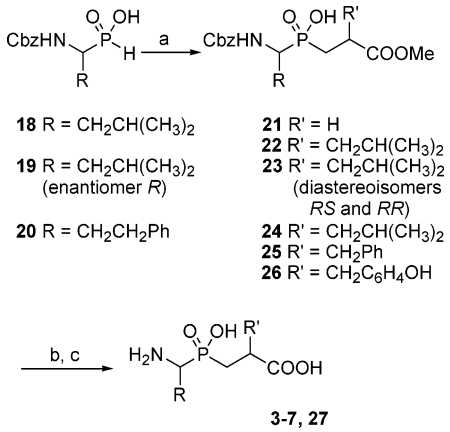
inhibitor	K_i [nM] LAP	K_i [nM] APN
LeuP[CH_2]Leu[a] (**27**)	65	1015
LeuP[CH_2]Leu[b] (**4**)	110	ND
hPheP[CH_2]Leu[c] (**5**)	74	1006
hPheP[CH_2]Phe[d] (**6**)	66	276
hPheP[CH_2]Tyr[e] (**7**)	67	36
LeuP[CH_2]Gly[f] (**3**)	330	1415
Leu^{OH}P[NH]Gly[f] (**17**)	4880	ND[g]
LeuP	230 (*R*)[h]	55000 (*R*)[h]
LeuP[O]Leu	58000[i]	ND

[a] Mixture of two diastereomers(1:1). [b] Mixture of four diastereomers, the proportion of the pairs of enantiomers (1:1.3). [c] Mixture of four diastereomers, the proportion of the pairs of enantiomers 1:1.5. [d] Mixture of four diastereomers, the proportion of the pairs of enantiomers 1:2. [e] Mixture of four diastereomers, the proportion of the pairs of enantiomers 1:1.7. [f] Racemic mixture. [g] K_i value not determined due to instability of the compound at pH 7.5. [h] Reference 54. [i] Reference 24. ND: K_i value not determined.

Quite interestingly, stability studies of the phosphonamidate containing the hydroxy instead the of α-amino group, Leu^{OH}P[NH]Gly (**17**), showed significantly higher resistance to hydrolysis compared to LeuP[NH]Gly. The hydroxy analogue **17** appeared to be stable enough at pH 8.5 to check its inhibitory activity toward LAP.

The Binding Affinity of Leu^{OH}P[NH]Gly toward LAP. This compound is a reversible and competitive LAP inhibitor, with an inhibition constant 4.88 μM for the racemic mixture (Table 1). This means that the replacement of the hydroxy group in the phosphonic moiety of Leu^{OH}P (K_i = 28.0 μM for the racemic mixture) by a $NHCH_2COO^-$ fragment results in a 6-fold increase in activity. This is probably because of the existence of the additional hydrogen bond formed by this fragment with the amide group of Gly362. The interactions with Leu360 are possible both for Leu^{OH}P and Leu^{OH}P[NH]-Gly and are of similar nature as for LeuP[25] and as proposed for LeuP[NH]Leu (Figure 2). If an analogous effect of enhancement of binding affinity was to be observed in the case of LeuP[NH]Gly versus LeuP, the K_i value for the former should lay in nanomolar range. This arises from the fact that L-LeuP (K_i = 0.23 μM) inhibits LAP about 2 orders of magnitude stronger than Leu^{OH}P[54] and results from differences in both steric and electronic properties of these amino acid analogues and

Scheme 2[a]



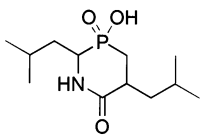
[a] (a) Hexamethyldisilazane then $H_2C{=}CH(R')COOMe$; (b, c) HBr/AcOH then HBr/H_2O or NaOH/MeOH then HBr/AcOH.

is predictable by our theoretical investigations.[55] These observations suggest altogether that the phosphonamidate dipeptide analogues, containing a free α-amino group, should be the most active LAP inhibitors studied so far among the compounds containing a phosphorus atom that mimicks the transition state of the enzymatic reaction.

Chemistry of Peptides Containing a Phosphinate Bond. Phosphinate analogues of dipeptides make the second group of designed LAP inhibitors. Phosphinic acid compounds are known to inhibit different metalloproteases, and their inhibitory effects have been reviewed quite recently.[36,56] However, they have not been studied as leucine aminopeptidase inhibitors so far. Thus, five of such analogues, LeuP[CH_2]Gly (**3**), LeuP-[CH_2]Leu (**4**), hPheP[CH_2]Leu (**5**), hPheP[CH_2]Phe (**6**), and hPheP[CH_2]Tyr (**7**), were synthesized and studied as LAP inhibitors.

Phosphinic pseudodipeptides are usually synthesized by addition of an appropriate acrylate to an aminophosphinic acid preactivated into the form of trivalent silyl ester[57−59] or by addition to aminophosphinate ester in the presence of a strong base.[60,61] In this work the first procedure has been chosen (Scheme 2).

N-Benzyloxycarbonylaminophosphinic acid (**18**, **20**)[62] was activated by heating with hexamethyldisilazane, and then an appropriate acrylate was added (Scheme 2). After workup and purification, the protected pseudopeptides were separated. The benzyloxycarbonyl group might be removed by hydrogenolysis as for phosphonamidates or by action of HBr in AcOH, as there was no danger of labile pseudopeptide bond cleavage. We have chosen the second procedure. For the ester hydrolysis, initially we used the same basic conditions as for phosphonamidate derivatives, unfortunately observing the formation of a cyclic peptide similarly as described in the literature.[58] The ratio of products was dependent on the structure of the R′ substituents. Thus, in the case of LeuP[CH_2]LeuOMe, diketopiperazine analogue **28**



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appeared to be the main reaction product (70% of yield).

Even though it could be easily removed by extraction with $CHCl_3$, the usefulness of acid hydrolysis was also investigated. The carboxylate methyl ester showed unexpected resistance against the action of strong acids. Reflux for 24 h in 20% HCl yielded only 40% of LeuP-[CH_2]GlyOMe cleavage, whereas complete hydrolysis was achieved only upon prolonged heating in 40% HBr (48 h at 100 °C). Because of these limitations associated with solubility problems, the reverse order of deprotection was studied and applied for homophenylalanine derivatives (**24**−**26**). Saponification of the ester preceding Cbz removal allowed us to obtain the final products under milder conditions. All of them were obtained as a mixture of two isomers (LeuP[CH_2]Gly, **3**) or four isomers (**4**−**7**).

The efforts to obtain a single stereoisomer of LeuP-[CH_2]Leu corresponding to L,L configuration was also undertaken. The enantiomer of the starting phosphinic acid of absolute configuration (*R*) (**19**), corresponding to the L form, was separated from its racemic mixture using optically active α-methylbenzylamine similarly to that reported earlier.[62] The use of the enantiomerically pure **19** afforded final (*R*)-LeuP[CH_2]-(*R*,*S*)-Leu (**27**) which consisted of only two diastereoisomers. However, they appeared to be not separable by means of HPLC. The same limitations are described in the literature even for larger peptides bearing bulky side chain substituents.[63]

Activity of Phosphinates toward LAP. The phosphinate analogues of dipeptides **3**−**7** are exceptionally stable in aqueous solutions at the whole range of pH. They appeared to be reversible, competitive, and slow binding inhibitors of LAP. The equilibrium in the inhibitor binding was reached within several minutes after addition of LAP to the mixture of inhibitor and substrate. Such an inhibition type was observed previously for the most active phosphonic analogues of amino acids.[24,54]

The most active among studied compounds were hPheP[CH_2]Phe (**6**) and hPheP[CH_2]Tyr (**7**) analogues. The binding affinities found for the mixtures of four diastereomers of **6** and **7** (K_i = 66 nM and 67 nM, respectively) are almost 4 times higher than this for (*R*)-LeuP (Table 1). The corresponding analogue with Leu at the P1′ position, compound **5**, also exhibits very similar affinity (K_i = 74 nM for the mixture of four isomers). The mixture of four isomers of phosphinic analogue of leucyl-leucine (**4**) (with the contribution of 1:1.3 for the pairs of enantiomers) was two times more active (K_i = 110nM) than (*R*)-LeuP, while the mixture of two diastereomers of this compound, (*R*)-LeuP[CH_2]-(*R*,*S*)-Leu, has the same binding affinity (K_i = 65nM) as **6** and **7** (Table 1). Therefore compounds **6**, **7,** and **4** are the most active leucine aminopeptidase inhibitors among those which contain a phosphorus atom that mimicks the transition state of the reaction catalyzed by the enzyme. Considering all the inhibitors of leucine aminopeptidase studied so far, these analogues are placed after bestatin (K_i = 0.6 nM)[64] and amastatin (K_i = 30 nM)[65] and are equipotent to leucinal (K_i = 60 nM).[66]

However, it is necessary to consider the stereochemistry of dipeptide analogues which interact favorably with LAP. It is known that bestatin isomer (2*S*,3*R*)-

AHPA-(*R*)-Leu is at least 10 times more active toward LAP than remaining stereoisomers.[26] Thus, in an analogous way, one designed isomer of **6**, **7,** and **4**, which corresponds to L,L stereoisomer of dipeptide substrate, is expected to be much more active than the remaining ones and probably mostly responsible for the measured activities of the mixtures. This might suggest that the activity of (*R*)-hPheP[CH_2]-(*R*)-Phe and (*R*)-hPheP[CH_2]-(*R*)Tyr should be comparable or even higher than this found for amastatin.

The comparison of the binding affinities of **6** and **7** indicate that hydroxy group of the side chain of Tyr at P1′ is most likely not involved in any hydrogen bond with the enzyme, as no enhancement of the binding affinity is observed compared to the derivative with Phe at that position. The modeled binding mode of **7** by LAP suggests the existence of only hydrophobic interactions with the S1′ pocket, in the same way as for inhibitors with hydrophobic side chains at that position (Phe or Leu). There is an amide group of Asn330 at the bottom of the S1′ pocket of LAP (Figure 2); however, the geometry for hydrogen bond formation between this residue and Tyr at P1′ would be unfavorable (in terms of hydrogen bond distance and angle). The S1′ pocket is open to solvent (the area between Ile421 and Asn330, Figure 2), and it is most likely that the amide group of Asn330 interacts with some water molecules (as seen in the crystal structure of LAP−LeuP complex[25]) and also that the hydroxy group of Tyr of **7** interacts with the solvent molecules after binding.

The presence of the aromatic ring at the P1′ position (**6**) does not introduce additional or stronger interactions with LAP compared to Leu (**5**), as almost no improvement of the binding affinity for such a replacement was observed. On the other hand the replacement of the Leu side chain (**4**) by hPheP (**5**) at the P1 position increases the activity almost two-fold. This results probably from additional hydrophobic interactions of the side chain of hPheP with the strongly hydrophobic S1 pocket, which is big enough to bind such a large side chain of the inhibitor.

The racemic mixture of **3** exhibits slightly weaker binding affinity compared to (*R*)-LeuP (Table 1). However, it should be noticed that only the *R* isomer of this phosphinate is expected to be active toward LAP, similar to that for LeuP where (*S*)-LeuP is about 1000 times less active than the *R* isomer.[54] This suggests that the K_i values for (*R*)-LeuP[CH_2]Gly should be close to that observed for (*R*)-LeuP. The lack of a hydrogen bond by phosphinates with Leu360, which is present when LeuP is bound, is most probably balanced by the energetic gain resulting from the hydrogen bond formation between the *C*-terminal carboxyl group of **3** and the amide group of Gly362, in a manner similar to that for bestatin and amastatin.[27,29] The activity predicted by application of Ludi scoring function[67] ($K_{i,calc}$ = 250 nM and $K_{i,calc}$ = 48 nM for (*R*)-LeuP[CH_2]Gly and (*R*,*R*)-LeuP[CH_2]Leu, respectively) seems to be in very good agreement with the experimental results.

Comparison of Activity of Phosphonates, Phosphinates, and Phosphonamidates toward LAP. Phosphonamidates, phosphinates, and phosphonates form an important family of phosphorus analogues of peptides, in which the organophosphorus fragment resembles the tetrahedral transition state of the amide bond hydrolysis.[68−72] These moieties are able to coordinate zinc ions present in the active sites of metalloproteases and to block their function in the process of hydrolysis. Thus, their chemistry continues to attract considerable interest[36−38,73] and provides a wide variety of potent inhibitors of proteases.[36,56,74]

There is a striking analogy between the inhibition of LAP and another zinc protease, thermolysin, by phosphonate, phosphinate, and phosphonamidate peptide mimetics. The activities of phosphonamidates toward thermolysin (e.g., Cbz-GlyP[NH]Leu-Leu) are about 1000 times higher than those observed for the respective phosphonate esters (e.g., Cbz-GlyP[O]Leu-Leu), whereas the corresponding phosphinate analogues (e.g., Cbz-GlyP[CH_2]Leu-Leu) are only 10 times less active than the phosphonamidates.[47,48] The X-ray structure of the phosphonate−enzyme complex revealed that this inhibitor indeed binds to thermolysin in the same way as do the phosphonamidate.[69] This suggests that the appropriate phosphinate derivative, for which the crystal structure is not available, should be bound in a nearly identical way. Thus, it is not surprising that special attention was paid to explain such a large activity differences of these compounds toward thermolysin. It is postulated that the solvation effects, counted by comparison of the number of hydrogen bonds they form with solvent before binding to the protein and formed with the enzyme after formation of the complex, may explain these activity differences. Moreover, the poor effectiveness of phosphonates might additionally result from the repulsive electrostatic interaction between the oxygen atom of the ligand and carbonyl group of Ala113 which both carry a negative partial charge. The influence of the different basicity of phosphonate, phosphinate, and phosphonamidate moieties on the coordination energy of these inhibitors to zinc in the thermolysin binding site was also suggested.[57]

The same may refer to the analogous group of leucine aminopeptidase inhibitors and suggest that a similar binding mode of these ligands is very probable. Phosphonate derivative of leucyl-leucine (LeuP[O]Leu, mixture of two isomers)[24] is about 1000 times less active compared to LeuP[CH_2]Leu. The binding affinity of the respective phosphonamidate (LeuP[NH]Leu) was not determined because of its instability at pH 8.5. However, both experimental results obtained for Leu^{OH}P[NH]Gly (the energetic gain arising from the incorporation of glycine residue instead of hydroxyl group of Leu^{OH}P) and phosphinates (the increase in the binding affinity after replacement of Gly by Leu at the P1′ position), as well as the K_i values predicted by the Ludi scoring function for **1** (46 nM) and **2** (5 nM), suggest that the compound with a P−N bond should be several times more active than LeuP[CH_2]Leu (Table 1). Thus, it is highly possible that its binding affinity toward LAP should be the highest among the enzyme inhibitors considered in this work. The binding affinity differences obtained for phosphonate and phosphinate analogues toward LAP probably result from similar reasons as described above for thermolysin and should be discussed in terms of hydrogen bond formation with solvent and with the enzyme upon binding. Although crystal structures of LAP with phosphinate, phosphonamidate, and

phosphonate dipepeptide analogues are not available, the similar binding mode of these compounds to LAP, comparable to this observed for LeuP,[25] seems to be highly possible. The NH group of phosphonamidates has a possibility to form a hydrogen bond with the carbonyl group of Leu360 after binding (Figure 2) and with a water molecule before binding. This would keep the total number of hydrogen bonds unchanged. On the other hand, the CH_2 group of the phosphinate is not able to form regular hydrogen bonds neither before nor after formation of the complex with the enzyme. Thus, in this case the number of hydrogen bonds is also balanced. However, an additional specific interaction might appear after binding to LAP, similar to that observed in the crystal structures of astacin[70] and stromelysin-3[71] complexed with phosphinic pseudopeptide inhibitors. The methylene group of the phosphinic moiety was located within hydrogen bond range to carbonyl oxygens of the appropriate enzyme residues, thus mimicking the interaction characteristic for the amide group of a cleaved substrate.[70,71] Such good analogy to the tetrahedral intermediate might explain the origin of potency of phosphinic compounds, including LAP inhibitors described in this paper. In contrast, phosphonate is not able to form a hydrogen bond with Leu360, while such a bond is presumably formed with the solvent before binding. Thus, the complexation with the enzyme is accompanied by a net loss of one hydrogen bond, and it is considered as a reason of a weak binding affinity of phosphonates compared to phosphinates. Additionally, the presence of a CH_2 group does not result in such a strong electrostatic repulsion, as in the case of phosphonate esters, where the oxygen atom of the ligand is close to the carbonyl oxygen of Leu360. Moreover, the differences in the basicity of these analogues may influence their interactions with two zinc ions, Zn488 and Zn489, in the LAP active site. In conclusion, the significant differences in the activity between these groups of LAP inhibitors probably result from their distinct potential for hydrogen bond formation, electrostatic repulsion, solvent effects, and differences in coordination energy of zinc ions.

Activity of Phosphinates toward APN. APN was selected intentionally for the inhibitory studies of phosphinates designed to inhibit LAP, as similar compounds have been reported as effective and selective APN inhibitors versus other Zn^{2+} metalloenzymes.[63,75] Dipeptide phosphinate analogues exhibited only moderate inhibitory activity (K_i values at micromolar range).[75] For strong inhibitory activity toward this enzyme, interaction with its S2′ pocket is required. The compounds studied toward LAP are dipeptide mimetics and thus are expected to be less active toward APN. Therefore, they were studied to compare their potency toward the two enzymes and thus to evaluate their selectivity.

The binding affinities of phosphinate inhibitors toward APN were strongly dependent on the nature of the side chain at the P1′ position (Table 1). The potencies of inhibitors containing Leu (**27** and **5**) and Gly (**3**) at P1′ are very similar to each other and practically independent on the character of the substituent at the P1 position (Table 1). The K_i values for these APN inhibitors range between 1.0 and 1.4 μM, which remains in a good agreement with the data reported in the literature for similar compounds.[75] However, the replacement of the side chain of leucine (**5**) by this of phenylalanine (**6**) at P1′ increases the binding affinity almost 4 times (K_i = 276 nM for hPheP-[CH_2]Phe). The incorporation of tyrosine at that position has an even stronger effect, and hPheP[CH_2]Tyr is a much more efficient APN inhibitor (K_i = 36 nM) than other compounds studied in this paper, as well as the dipeptide analogues described in the literature.[75] Thus, the presence of the hydroxy group in the C-terminal aromatic fragment of **7** makes it the most active compound among phosphinate dipeptide analogues studied so far. To the best of our knowledge, such analogues containing a polar group at the P1′ position were not reported as APN inhibitors. There is a literature report on even more potent phosphinate inhibitors of APN (with the binding affinity around 10^{-9} M),[63,75] but these are tripeptide analogues with additional substituent at the P2′ position. Thus, the introduction of additional structural features to the latter compounds, which results in potential formation of additional contacts with the enzyme, resulted in the increase of binding affinity toward APN of 2 orders of magnitude. As the crystal structure of APN is not available, we built the model of the enzyme active site (Figure 3), to explain the differences in activity between compounds **5**, **6**, and **7**. The model was built based on homology of APN to human leukotriene A_4 hydrolase/aminopeptidase (LTA4H).[76] This protein is a bifunctional zinc enzyme, which exhibits both epoxide hydrolase activity and aminopeptidase activity in a common center.[76] LTA4H and APN belong to the same family (peptidase M1 family), and the former is the only protein with reasonable sequence identity to APN (27% for most of APN catalytic domain), the structure of which was solved and deposited in Protein Data Bank (1hs6 code in PDB). The similarity is even much higher at the binding site area, particularly at the zinc binding site and at the S1′ pocket (Figure 3), which makes our model of APN binding site reliable. According to this model, the S1′ pocket of APN comprises tyrosine and histidine residues (Figure 3). This gives more favorable interactions with Phe (in compound **6**) than with Leu (in compound **5**) side chains because of the increase of hydrophobic contact area and possible interactions between the aromatic rings of **6** and the S1′ pocket residues. Such interactions well explain the 4-fold activity increase of **6** versus **5**. The APN active site model also predicts the existence of a hydrogen bond between the hydroxy group of Tyr present at the P1′ position and the carboxyl group of Glu413 placed at the bottom of the S1′ enzyme pocket (Figure 3). This might explain the 8-fold binding affinity increase of **7** versus hPheP[CH_2]Phe. The consistency of the results arising from kinetic studies with the proposed interaction mode of studied phosphinate analogues with APN confirms the reliability of the constructed enzyme binding site model.

Difference in Affinities of Phosphinate Dipeptide Analogues toward LAP and APN. Our studies emphasize the significance of P1 and P1′ substituents while designing potent phosphinate inhibitors toward LAP and APN. However, the key feature discriminating their selectivity concerns the P1′ side chain. Both, aromatic and aliphatic side chains can interact strongly

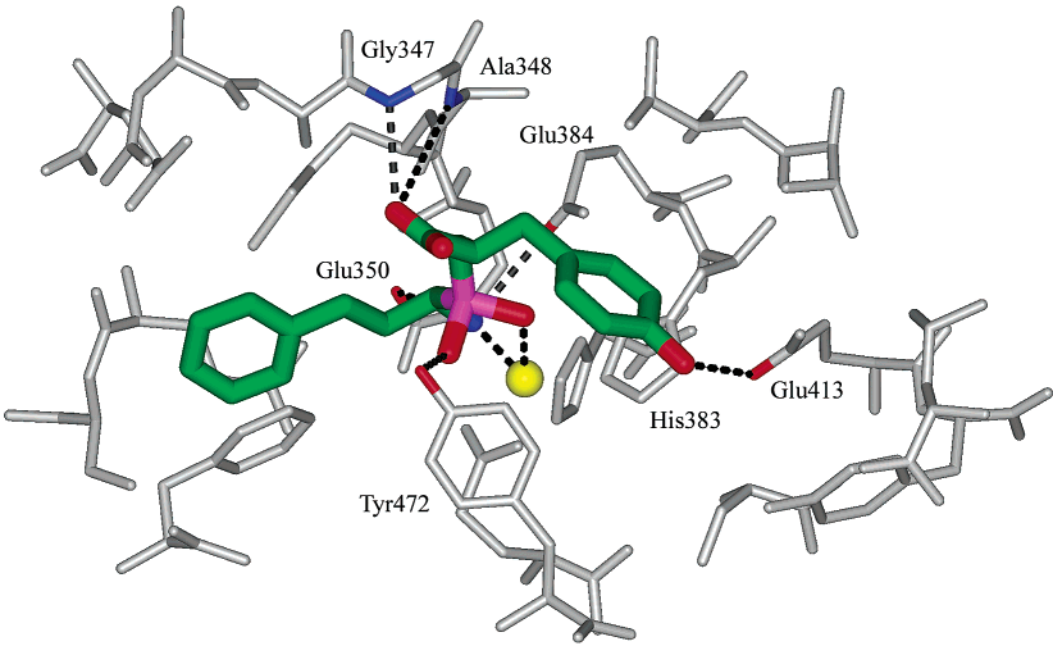

Figure 3. The proposed binding mode of hPheP[CH_2]Tyr to APN binding site model, built based on the structure of human leukotriene A_4 hydrolase (LTA4H) in complex with bestatin (1hs6 in PDB). The binding mode of hPheP[CH_2]Tyr to APN is similar to the interactions of bestatin with LTA4H.[76] Hydrogen bonds and interactions with zinc ion are shown as black dashed lines. Enzyme residues within 5Å radius around the inhibitor are shown as dark gray sticks. Because of clarity of the picture, only the residues involved in hydrogen bonds with hPheP[CH_2]Tyr (Tyr472, Glu350, Glu384, Gly347, Ala348, Glu413) are labeled, together with His383, which interacts with the P1′ fragment of the inhibitor. One of the oxygen atoms and the nitrogen atom of aminophosphinic fragment of the inhibitor donate electron lone pairs to zinc ion at APN active site. The active site residues in APN: His383, His387, Glu406 (interacting with zinc ion) and Glu384, Glu350, Tyr472 (involved in hydrogen bonds with inhibitor) are strongly conserved in LTA4H (corresponding residues in LTA4H are: His295, His299, Glu318, Glu296, Glu271, Tyr383, respectively).[76]

with LAP S1′ pocket, while a significant increase in binding affinity to APN is observed for peptide analogues containing aromatic versus leucine side chain at the P1′ position (Table 1). The most pronounced difference in the binding affinity of the studied phosphinate dipeptide analogues between LAP and APN was obtained for two inhibitors containing Leu at the P1′ position (*R*-LeuP[CH_2]Leu and hPheP[CH_2]Leu), which are around 15 times more active toward LAP than APN. Incorporation of an aromatic residue at the P1′ site increases the binding affinity toward APN with a simultaneous decrease of specificity toward these two enzymes. Comparison of two of the strongest LAP inhibitors (**6**, **7**) containing Phe and Tyr at P1′ shows that **6** is around four times more active toward LAP versus APN, while **7** is 2 times more potent to APN.

Future Prospects. Our studies have shown that the presence of a free α-amino group in presumably the most active LAP inhibitors, phosphonamidate dipeptide analogues, makes the P−N bond vulnerable at pH < 12.0. The hydrolysis of this bond is strongly dependent on the protonation of the α-amino group (to be published). There are two possible ways to increase the stability of phosphonamidates in order to enable studies on their inhibitory activity: (1) the replacement of amino group by other moieties; (2) lowering the pK_a of the amino group. The first solution is less possible since the presence of a free α-amino group is crucial for strong interactions with LAP as was shown by its replacement by a hydroxy group, which resulted in strong decrease of the activity (see above). To lower the pK_a value of the amino group, we propose to consider the phosphonamidate containing 2-aminophenylphosphonic acid at the P1 position. The influence of the aromatic ring significantly lowers the pK_a of the amino group, and this compound should at least be as stable as CbzLeuP[NH]-Gly and Leu^{OH}P[NH]Gly (**17**) in test conditions. 2-Aminophenylphosphonic acid was docked to the enzyme binding site, and the relative orientation of the amino and phosphonic groups in this compound is similar to this present for LeuP. This enables the favorable interactions with Zn489 and Asp273, while the aromatic ring may be involved in the hydrophobic interactions with the S1 pocket of LAP. Thus, this compound seems to be a new promising lead for further development of phosphonamidates containing a free amino group as inhibitors of leucine aminopeptidase and similar enzymes.

Conclusions

The structural information available for leucine aminopeptidase and the application of the molecular modeling techniques provided an opportunity for designing a new class of low-molecular, very potent inhibitors of the enzyme. The replacement of the hydroxyl group in the phosphonate fragment of LeuP by new structural fragments bound at the S1′ pocket of the enzyme led to the phosphinate dipeptide analogues. To the best of our knowledge, they are the most active LAP inhibitors among the compounds containing a phosphorus atom that mimicks the tetrahedral transition state of the reaction catalyzed by LAP, with the binding affinities in nanomolar range. Simultaneously, the modification at the P1 position of such analogues resulted in additional enhancement of the activity (K_i values 66 and 67 nM for (*R*,*S*)-hPheP[CH_2]-(*R*,*S*)-Phe and (*R*,*S*)-hPheP-

[CH_2]-(*R,S*)-Tyr, respectively). Their increased activities compared to LeuP arise possibly both from formation of the additional hydrogen bond with Gly362 LAP (Figure 2) and from the interactions with the S1′ pocket. The replacement of leucine by a homophenylalanine side chain at the P1 position results in a better fit to the hydrophobic S1 pocket of LAP. The importance of absolute configuration of the asymmetric carbon atoms in didepetide analogues as LAP inhibitors (for example compounds **27** and **4** as well as bestatin isomers) suggests that the activities of the appropriate diastereomers of tested compounds should be even higher. This new class of the tight-binding, competitive inhibitors of leucine aminopeptidase may be considered as new leads and offer the possibility of further modifications at P1 and P1′ positions, which should result in even more active and selective compounds, with the possibility of their medical application. Thus, in addition to monozinc metallopeptidases, for which phosphinic peptides were shown in many studies to behave as potent inhibitors, our results suggest to consider also bizinc aminopeptidases as other possible targets for this class of compounds.

Most of the tested compounds exhibited several times stronger inhibitory effect toward LAP than to APN, with the biggest difference for LeuP[CH_2]Leu, which is around 15 times more active for LAP. The only exception was hPheP[CH_2]Tyr, which was found to be the most potent inhibitor of both LAP and APN, with the affinity almost 2 times higher for APN. hPheP[CH_2]Tyr is almost 1 order in magnitude more potent toward APN than similar analogues bearing hydrophobic groups at the P1′ position. It is a quite unexpected result and might be explained by formation of an additional hydrogen bond between the hydroxy group of **7** and the enzyme, as revealed by the modeled binding mode of hPheP[CH_2]Tyr to APN. Finding a strong inhibitory effect of this compound toward APN opens the possibility of further modifications at the P1′ position and design of even more active and selective enzyme inhibitors.

Phosphonamidate dideptide analogues containing a free α-amino group, designed as the most promising LAP inhibitors, are stable only at strongly basic conditions (pH > 12). Thus, this class of compounds is not suitable for activity studies toward both studied aminopeptidases. However, it is possible that a modified phosphonamidate analogue, with the amino group attached to the aromatic ring at P1, which would lower its pK_a and thus increase the stability, might be considered as promising for inhibition of leucine aminopeptidase. This hypothesis, however, requires elaboration of synthetic procedures leading to this type of compound and further experimental studies.

Experimental Section

Chemistry. General. Unless otherwise stated, materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck) and used without purification. Isovaleryl aldehyde and thionyl chloride were freshly distilled. Triethylamine was distilled and stored over potassium hydroxide. Ethanol- and water-free chloroform was obtained by passing the solvent through basic alumina followed by its distillation from over P_2O_5. Anhydrous benzene was obtained by its distillation from P_2O_5 and stored over sodium. Column chromatography was performed on silica gel 60 (70−230 mesh).

Melting points were determined on Boetius apparatus and were not corrected. IR spectra were recorded in KBr pellets or in a film on a Perkin-Elmer System 2000 FT IR spectrometer. Proton and phosphorus NMR spectra were recorded on a Bruker DRX spectrometer operating at 300.13 MHz for ^{1}H and 121.50 MHz for ^{31}P. Measurements were made in $CDCl_3$, DMSO-d_6, or D_2O solutions. Proton chemical shifts are reported in relation to tetramethylsilane used as internal standard. ^{31}P NMR spectra were obtained with use of broadband ^{1}H decoupling; chemical shifts are reported in relation to 85% H_3PO_4 (sealed capillary). Microanalyses were performed by Instrumental Analysis Unit of the Institute of Organic Chemistry, Biochemistry and Biotechnology, Wrocław University of Technology.

Diphenyl [1-(*N*-Benzyloxycarbonylamino)-3-methylbutyl]phosphonate (8). The compound was synthesized by condensation of benzyl carbamate, isovaleryl aldehyde and triphenyl phosphite as described by Oleksyszyn.[77] Yield: 38%; mp 120−123 °C (lit. mp 122−123 °C).

Dimethyl [1-(*N*-Benzyloxycarbonylamino)-3-methylbutyl]phosphonate (9). Compound **8** was converted into its dimethyl ester by transesterification in methanol in the presence of KF and 18-crown-6 ether.[78] Yield: 91%; mp 43−45 °C (lit. mp 34−35 °C,[78] 45−47 °C[79]); ^{31}P NMR ($CDCl_3$): δ 29.08; ^{1}H NMR: δ 0.86 (d, J = 6.6 Hz, 6H, CH(CH_3)$_2$), 1.50 (m, 2H, CHCH_2CH), 1.66 (m, 1H, CH(CH$_3$)$_2$), 3.62 and 3.67 (d each, J = 10.8 Hz, 3H and 3H, P(OCH_3)$_2$), 4.12 (m, 1H, NCHP), 5.05 (AB system, J = 12.2 Hz, 2H, CH_2OC), 5.09 (m, 1H, C(O)NH), 7.27 (m, 5H, C_6H_5).

Methyl Hydrogen [1-(*N*-Benzyloxycarbonylamino)-3-methylbutyl]phosphonate (10). The monomethyl ester was obtained by alkaline hydrolysis of (**9**) in a 1 M NaOH/MeOH solution. After overnight stirring methanol was removed under reduced pressure. The crude product was precipitated by acidification of the water phase to pH 1 and then extracted with AcOEt. The organic phase was dried over Na_2SO_4, filtered, and evaporated. Recrystallization from an AcOEt/hexane mixture gave the pure product as a white crystalline compound. Yield: 84%; mp 118−120 °C (lit. mp 117−119 °C[79]); ^{31}P NMR ($CDCl_3$): δ 29.56; ^{1}H NMR: δ 0.85 (d, J = 6.7 Hz, 6H, CH(CH_3)$_2$), 1.47 (m, 2H, CHCH_2CH), 1.64 (m, 1H, CH(CH$_3$)$_2$), 3.63 (d, J = 10.8 Hz, 3H, POCH_3), 4.11 (m, 1H, NCHP), 5.05 (s, 2H, CH_2OC), 7.26 (m, 5H, C_6H_5), 10.33 (s, 1H, POH).

***N*-[[1-(*N*′-Benzyloxycarbonylamino)-3-methylbutyl]-methoxyphosphinyl]glycine Methyl Ester (11). The Representative Procedure.** The monomethyl ester (**10**) (0.63 g, 2 mmol) was dissolved in 10 mL of $CHCl_3$, and thionyl chloride was added (0.22 mL, 3 mmol). The solution was stirred for 2 h at room temperature and refluxed for additional 2 h. The volatile components were evaporated under reduced pressure, and the residue was treated with 10 mL of benzene and evaporated again. The resulting phosphonochloridate was dissolved in 10 mL of $CHCl_3$ and added dropwise to the mixture of glycine methyl ester hydrochloride (0.25 g, 2 mmol) and triethylamine (0.70 mL, 5 mmol) in 15 mL of $CHCl_3$ cooled in an ice bath. The resulting solution was left at room temperature overnight and then washed successively with 1 M NaOH, water, 5% HCl, water, 1 M Na_2CO_3, and brine (20 mL of each). The organic layer was dried over Na_2SO_4. After removal of the solvent, the crude peptide was purified on column chromatography using the AcOEt/hexane (3:1) solution as the eluent. Yield: 55%; mp 62−79 °C; IR (KBr, cm^{-1}): 3295 and 3225 (NH), 3065 and 2950 (CH), 1760 and 1700 (C=O), 1545 (δNH), 1270 (P=O), 1210, 1150, 1060, and 1035 (C−O, P−O); ^{31}P NMR ($CDCl_3$): δ 31.39 and 32.72 (2:3 ratio); ^{1}H NMR: δ 0.85 (m, 6H, CH(CH_3)$_2$), 1.51 (m, 2H, CHCH_2CH), 1.66 (m, 1H, CH(CH$_3$)$_2$), 3.05 and 3.15 (m each, 1H together, PNH), 3.47−3.76 (m, 8H, POCH_3, OCH_3, and PNHCH_2), 4.01 (m, 1H, NCHP) 4.95 and 5.24 (d each, J_1 = 10.0 Hz, J_2 = 10.1 Hz, 1H together, C(O)NH), 5.03 (AB system, J = 12.2 Hz, 2H, CH_2OC), 7.27 (m, 5H, C_6H_5). Anal. ($C_{17}H_{27}N_2O_6P$) C, H, N.

***N*-[[1-(*N*′-Benzyloxycarbonylamino)-3-methylbutyl]-methoxyphosphinyl]-(L)-leucine Methyl Ester (12).** The

compound was prepared in the same manner as **11**. Purification on column chromatography using the AcOEt/hexane (3:2) solution afforded the peptide as colorless oil. Yield: 52%; IR (film, cm^{-1}): 3295 and 3220 (NH), 2955 (CH), 1745 and 1720 (C═O), 1540 (δNH), 1265 (P═O), 1215, 1150, 1110, and 1050 (C−O, P−O); ^{31}P NMR ($CDCl_3$): δ 30.45, 30.96, 31.90, and 32.23 (4:4:3:2 ratio); ^1H NMR: δ 0.86 (m, 12H, 2 × CH(CH_3)$_2$), 1.47 (m, 4H, 2 × CHCH_2CH), 1.64 (m, 2H, 2 × C*H*(CH_3)$_2$), 3.00 (m, 1H, PN*H*), 3.61 (m, 6H, POCH_3 and OCH_3,), 3.99 (m, 2H, PNHC*H* and NC*H*P), 5.04 (m, 3H, C(O)N*H* and CH_2OC), 7.26 (m, 5H, C_6H_5). Anal. ($C_{21}H_{35}N_2O_6P$) C, H, N.

***N*-[(1-Amino-3-methylbutyl)oxyphosphinyl]glycine Dilithium Salt (1). The Representative Procedure.** To remove the benzyloxycarbonyl group, the peptide **11** (0.19 g, 0.5 mmol) was dissolved in 5 mL of MeOH and hydrogenated in the presence of 0.1 g of 10% Pd/C catalyst. Hydrogen was passed through the solution by gentle bubbling. The reaction followed by TLC as well as by ^{31}P NMR proceeded quantitatively, and it was completed within 2 h. The catalyst was filtered off, and the solution was evaporated to dryness giving a colorless oil. Removal of the methyl groups was achieved by alkaline hydrolysis of *N*-[(1-amino-3-methylbutyl)methoxyphosphinyl]glycine methyl ester in the 1.5 M LiOH/MeOH solution (2 mL, 1:1; 3 equiv of LiOH were used). After evaporation to dryness at room temperature, the sample was used directly for tests. Because of its instability at a pH lower than 12, the deprotected peptide in form of the dilithium salt was characterized only by NMR spectroscopy. ^{31}P NMR (D_2O): δ 29.39; ^1H NMR: δ 0.83 and 0.89 (d each, J = 6.6 Hz, 3H and 3H, CH(CH_3)$_2$), 1.37 (m, 2H, CHCH_2CH), 1.75 (m, 1H, C*H*(CH_3)$_2$), 2.74 (m, 1H, NC*H*P), 3.42 (d, J = 8.0 Hz, 2H, PNHCH_2).

***N*-[(1-Amino-3-methylbutyl)oxyphosphinyl]-(L)-leucine Dilithium Salt (2).** ^{31}P NMR (D_2O): δ 26.58 and 26.99 (2:3 ratio); ^1H NMR: δ 0.86 (m, 12H, 2 × CH(CH_3)$_2$), 1.29−1.75 (m, 6H, 2 × CHCH_2C*H*), 2.72 (m, 1H, NC*H*P), 3.72 (m, 1H, PNHC*H*).

Diethyl (1-Hydroxy-3-methylbutyl)phosphonate (13). This compound was synthesized in the reaction of isovaleryl aldehyde and diethyl phosphite in the presence of triethylamine as described in the literature for the dimethyl analogue.[80] Yield: 46%; ^{31}P NMR ($CDCl_3$): δ 26.97; ^1H NMR: δ 0.86 and 0.91 (d each, J = 6.6 Hz, 3H and 3H, CH(CH_3)$_2$), 1.28 (t, J = 7.0 Hz, 6H, P(OCH$_2$CH_3)$_2$), 1.39 and 1.65 (m each, 1H and 1H, CHCH_2CH), 1.87 (m, 1H, C*H*(CH_3)$_2$), 3.89 (m, 1H, OC*H*P), 4.09 (m, 4H, P(OCH_2CH$_3$)$_2$).

Diethyl (1-Benzyloxy-3-methylbutyl)phosphonate (14). To protect the hydroxy function the phosphonate (**13**) was stirred 48 h with 1.2 equiv of benzyl bromide and 1.5 equiv of Ag_2O in anhydrous DMF at room temperature. The resulting mixture was filtered through Celite, and DMF was removed under reduced pressure. Purification by column chromatography using AcOEt/hexane (2:1) gave the 1-benzyloxyphosphonate as colorless liquid. Yield: 77%; ^{31}P NMR ($CDCl_3$): δ 25.62; ^1H NMR: δ 0.75 and 0.91 (d each, J = 6.6 Hz, 3H and 3H, CH(CH_3)$_2$), 1.34 and 1.35 (t each, J = 7.1 Hz, 3H and 3H, P(OCH$_2$CH_3)$_2$), 1.47 and 1.78 (m each, 1H and 2H, CHCH_2CH(CH$_3$)$_2$), 3.74 (m, 1H, OC*H*P), 4.15 (m, 4H, P(OCH_2CH$_3$)$_2$), 4.59 and 4.92 (d each, J = 11.2 Hz, 1H and 1H, C_6H_5CH_2O), 7.35 (m, 5H, C_6H_5).

Ethyl Hydrogen (1-Benzyloxy-3-methylbutyl)phosphonate (15). The monoethyl ester was obtained by alkaline hydrolysis of **14** in a 1 M NaOH/EtOH solution. After 2 h of gentle reflux, the solution was cooled and EtOH was removed under reduced pressure. The water phase was acidified to pH 1 and extracted with three portions of AcOEt. Drying the organic layer over Na_2SO_4 and evaporation afforded the product as slightly yellow glass. ^{31}P NMR (DMSO-d_6): δ 19.41; ^1H NMR: δ 0.71 and 0.84 (d each, J = 6.4 Hz, 3H and 3H, CH(CH_3)$_2$), 1.13 (t, J = 6.9 Hz, 3H, POCH$_2$CH_3), 1.44 and 1.58 (m each, 1H and 1H, CHCH_2CH), 1.73 (m, 1H, C*H*(CH_3)$_2$), 3.47 (m, 1H, OC*H*P), 3.96 (m, 2H, POCH_2CH$_3$), 4.47 and 4.86 (d each, J = 11.3 Hz, 1H and 1H, C_6H_5CH_2O), 7.30 (m, 5H, C_6H_5).

***N*-[(1-Benzyloxy-3-methylbutyl)ethoxyphosphinyl]-glycine Methyl Ester (16).** Starting from the phosphonate **15**, the phosphonamidate peptide **16** was synthesized in the same manner as described for the amino analogues (**11**, **12**). Purification on column chromatography using a AcOEt/CH_2Cl_2 (2:1) solution as the eluent gave the product as colorless oil. Yield: 44%; IR (film, cm^{-1}): 3210 (NH), 2955 (CH), 1755 (C═O), 1260 (P═O), 1210, 1155 and 1045 (C−O, P−O); ^{31}P NMR ($CDCl_3$): δ 28.78 and 30.75 (4:3 ratio); ^1H NMR: δ 0.79, 0.81 and 0.93 (d each, J = 6.6 Hz, 6H together, CH(CH_3)$_2$), 1.33 and 1.37 (t each, J = 7.0 Hz, 3H together, POCH$_2$CH_3), 1.51 and 1.69 (m each, 2H together, CHCH_2CH), 1.84 (m, 1H, C*H*(CH_3)$_2$), 3.08 and 3.20 (m each, 1H together, PN*H*), 3.70 (s, 3H, OCH_3), 3.72−3.93 (m, 3H, PNHCH_2 and OC*H*P), 4.13 (m, 2H, POCH_2CH$_3$), 4.56, 4.64, 4.71 and 4.87 (d each, J = 11.1 Hz, 2H together, C_6H_5CH_2O), 7.33 (m, 5H, C_6H_5). Anal. ($C_{17}H_{28}NO_5P$) C, H, N.

***N*-[(1-Hydroxy-3-methylbutyl)oxyphosphinyl]glycine Dilithium Salt (17).** The removal of the protection groups in peptide **16** was achieved by hydrogenolysis followed by alkaline hydrolysis under the same conditions as described for the amino analogues (**11**, **12**). ^{31}P NMR (D_2O): δ 25.26; ^1H NMR: δ 0.81 and 0.86 (d each, J = 6.6 Hz, 3H and 3H, CH(CH_3)$_2$), 1.36 and 1.49 (m each, 1H and 1H, CHCH_2CH), 1.70 (m, 1H, C*H*(CH_3)$_2$), 3.41 (d, J = 10.1 Hz, 2H, PNHCH_2), 3.63 (m, 1H, OC*H*P).

Methyl 3-[[1-(*N*-Benzyloxycarbonylamino)-3-methylbutyl]hydroxyphosphinyl]propionate (21). The Representative Procedure. The phosphinic dipeptide was obtained according to the procedures previously described in the literature[57−59] with only slight modification. [1-(*N*-Benzyloxycarbonylamino)-3-methylbutyl]phosphinic acid (**18**)[62] (0.57 g, 2 mmol) was heated with hexamethyldisilazane (2.10 mL, 10 mmol) at 100−110 °C for 2 h under nitrogen. The reaction mixture was cooled to 60 °C, and methyl acrylate was added (0.27 mL, 3 mmol). Then the temperature was maintained at 85−90 °C for additional 3 h. After cooling to 60 °C, 5 mL of methanol was added dropwise. The volatile products were removed under reduced pressure, and the residue was dissolved in 25 mL of AcOEt and washed twice with 5% HCl (10 mL) and finally with brine (10 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was worked up with 10 mL of Et_2O and left overnight for crystallization. Yield: 69%; mp 146−153 °C; IR (KBr, cm^{-1}): 3285 (NH), 3035 and 2955 (CH), 1740 and 1710 (C═O), 1260 (P═O), 1230, 1115, 1065 and 1025 (C−O, P−O); ^{31}P NMR ($CDCl_3$): δ 55.38; ^1H NMR: δ 0.83 and 0.85 (d each, J = 6.6 Hz, 3H and 3H, CH(CH_3)$_2$), 1.47 (m, 2H, CHCH_2CH), 1.62 (m, 1H, C*H*(CH_3)$_2$), 1.96 (m, 2H, PCH_2), 2.53 (m, 2H, PCH$_2$CH_2), 3.58 (s, 3H, OCH_3), 3.97 (m, 1H, NC*H*P), 5.03 (s, 2H, CH_2OC), 5.08 (d, J = 10.4 Hz, 1H, C(O)N*H*), 7.27 (m, 5H, C_6H_5), 7.92 (bs, 1H, PO*H*). Anal. ($C_{17}H_{26}NO_6P$) C, H, N.

Methyl 2-[[[1-(*N*-Benzyloxycarbonylamino)-3-methylbutyl]hydroxyphosphinyl]methyl]-4-methylpentanoate (22). The phosphinic analogue: Z-Leu[CH_2]Leu-OMe was synthesized by Michael addition of methyl α-isobutylacrylate[81] to activated **18** as described above. Yield: 63%; mp 121−137 °C; IR (KBr, cm^{-1}): 3320 (NH), 2960 (CH), 1740 and 1685 (C═O), 1270 (P═O), 1230, 1170, 1130, and 1045 (C−O, P−O); ^{31}P NMR ($CDCl_3$): δ 54.45 and 54.79 (1:1 ratio); ^1H NMR: δ 0.79, 0.82, 0.85 and 0.88 (d each, J = 6.5 Hz, 12H together, 2 × CH(CH_3)$_2$), 1.22, 1.48, 1.68 and 2.07 (m each, 1H, 4H, 2H and 1H, 2 × CHCH_2C*H*(CH$_3$)$_2$ and PCH_2), 2.79 (m, 1H, PCH$_2$C*H*), 3.58 (s, 3H, OCH_3), 3.97 (m, 1H, NC*H*P), 5.04 (AB system, J = 12.4 Hz, 2H, CH_2OC), 5.25 and 5.26 (d each, J = 10.0 Hz, 1H together, C(O)N*H*), 7.25 (m, 5H, C_6H_5), 11.88 (bs, 1H, PO*H*). Anal.($C_{21}H_{34}NO_6P$) C, H, N.

Methyl 2-[[[1-(*R*)-(*N*-Benzyloxycarbonylamino)-3-methylbutyl]hydroxyphosphinyl]methyl]-4-methylpentanoate (23). This compound was obtained using the same procedure as for **21** and **22** using the (*R*) enantiomer of [1-(*N*-benzyloxycarbonylamino)-3-methylbutyl]phosphinic acid (**19**). The latter, optically active compound was separated by crystallization of the diastereomeric salt of its racemic mixture

with (*R*)-(+)-1-phenylethylamine similarly as reported earlier.[62] The salt was crystallized several times from the EtOH/acetone mixture (1:1) to the constant melting point and specific rotation. Then it was treated with 1 M NaOH, the separated amine was washed out with Et_2O, and the water phase was acidified to pH 1. The separated compound was extracted to AcOEt. The organic phase was dried with Na_2SO_4, filtered, and evaporated to dryness. The crude acid was recrystallized from an AcOEt/hexane solution giving the enantiomer of specific rotation $[\alpha]_D^{20} = -56°$ (1% solution in EtOH).

[1-(*N*-Benzyloxycarbonylamino)-4-phenylbutyl]phosphinic Acid (20). The phosphinic analogue of homophenylalanine was synthesized in the reaction of hydrocinnamaldehyde with the salt of diphenylmethylamine and hypophosphorous acid, followed by hydrolysis of the adduct and *N*-terminal protection with benzyl chloroformate, similarly as described in the literature.[62,63] Total yield of the procedure: 26%; mp 136−140 °C (lit. mp 142−143 °C;[63] ^{31}P NMR (DMSO-d_6): δ 31.15; ^{1}H NMR: δ 1.83 and 2.05 (m each, 1H and 1H, $PhCH_2CH_2$), 2.60 and 2.74 (m each, 1H and 1H, $PhCH_2C$), 3.85 (m, 1H, NC*H*P), 5.04 (s, 2H, CH_2OC), 6.00 (bd, 1H, C(O)N*H*), 7.07−7.44 (m, 10H, 2 × C_6H_5), 7.83 (s, 1H, PO*H*).

Methyl 2-[[[1-(*N*-Benzyloxycarbonylamino)-4-phenylbutyl]hydroxyphosphinyl]methyl]-4-methylpentanoate (24). The phosphinic analogue: Z-hPhe[CH_2]Leu-OMe was obtained by addition of methyl α-isobutylacrylate to activated **20** using the procedure described above for leucine analogues **21**−**23**. After workup the crude material was purified on column chromatography using the $CHCl_3$/MeOH/AcOH (100:3:2) solution as the eluent and yielded the product as a pale glass. Yield: 59%; IR (film, cm^{-1}): 3290 (NH), 3030, 2955, and 2870 (CH), 1740 and 1715 (C=O), 1245 (P=O), 1160, 1130, and 1040 (C−O, P−O); ^{31}P NMR ($CDCl_3$): δ 49.85 and 50.01 (2:1 ratio); ^{1}H NMR: δ 0.81 (m, 6H, $CH(CH_3)_2$), 1.25−2.10 (m, 7H, $CH_2CH(CH_3)_2$, $PhCH_2CH_2$ and PCH_2), 2.59−2.80 (m, 3H, $PhCH_2C$ and PCH_2CH), 3.57 (s, 3H, OCH_3), 3.85 (m, 1H, NC*H*P), 5.04 (s, 2H, CH_2OC), 5.67 (m, 1H, C(O)N*H*), 7.07−7.32 (m, 10H, 2 × C_6H_5). Anal. ($C_{25}H_{34}NO_6P$) C, H, N.

Methyl 2-[[[1-(*N*-Benzyloxycarbonylamino)-4-phenylbutyl]hydroxyphosphinyl]methyl]-3-phenylpropionate (25). The compound **25** was synthesized starting from **20** and methyl α-benzylacrylate[59] and purified the same manner as **24**. Yield: 66%; IR (KBr, cm^{-1}): 3320 (NH), 3060, 3030, 2950, and 2860 (CH), 1730 and 1720 (C=O), 1245 (P=O), 1165, 1135, and 1035 (C−O, P−O); ^{31}P NMR (DMSO-d_6): δ 43.75 (broad); ^{1}H NMR: δ 1.66−1.98 (m, 4H, $PhCH_2CH_2$ and PCH_2), 2.45, 2.78 and 2.96 (m each, 1H, 2H and 2H, 2 × $PhCH_2C$ and PCH_2CH), 3.41 and 3.44 (s each, 3H together, OCH_3), 3.60 (m, 1H, NC*H*P), 5.06 (m, 3H, CH_2OC and C(O)N*H*), 7.08−7.37 and 7.62 (m each, 15H together, 3 × C_6H_5). Anal. ($C_{28}H_{32}NO_6P$) C, H, N.

Methyl 2-[[[1-(*N*-Benzyloxycarbonylamino)-4-phenylbutyl]hydroxyphosphinyl]methyl]-3-(4-hydroxyphenyl)-propionate (26). The phosphinic pseudodipeptide: Z-hPhe-[CH_2]Tyr-OMe was synthesized by addition of methyl α-(4-hydroxybenzyl)acrylate to activated **20**. The acrylate was obtained starting from the appropriate 4-hydroxybenzylmalonate in a usual manner.[81] The latter compound was prepared by reduction of 4-hydroxybenzylidenemalonate[82] with $NaBH_4$ (3 equiv) in absolute methanol. Purification on column chromatography using hexane/ethyl acetate (2:1) solution gave the acrylate as brownish oil with 42% of total yield. ^{1}H NMR ($CDCl_3$): δ 3.56 (s, 2H, $HOC_6H_4CH_2$), 3.74 (s, 3H, OCH_3), 5.40 (bs, 1H, O*H*), 5.47 and 6.21 (m each, 1H and 1H, H_2C=C), 6.75 and 7.05 (d each, J = 8.6 Hz, 2H and 2H, HOC_6H_4).

The crude phosphinic peptide was purified on column chromatography using the $CHCl_3$/MeOH/AcOH (100:5:2) solution yielding the final product **26** as a slightly yellow glass. Yield: 45%; IR (KBr, cm^{-1}): 3410 (OH), 3325 (NH), 3060, 3030, 2950, 2930, and 2855 (CH), 1720 and 1705 (C=O), 1245 (P=O), 1175, 1135, and 1035 (C−O, P−O); ^{31}P NMR ($CDCl_3$): δ 48.30 and 48.54 (2:1 ratio); ^{1}H NMR: δ 1.76 (m, 2H, $PhCH_2CH_2$), 2.05 (m, 2H, PCH_2), 2.55 and 2.72 (m each, 1H, and 3H, $PhCH_2C$ and $HOC_6H_4CH_2$), 2.95 (m, 1H, PCH_2CH), 3.47 and 3.49 (s each, 3H together, OCH_3), 3.83 (m, 1H, NC*H*P), 5.05 (s, 2H, CH_2OC), 5.86 (d, J = 9.8 Hz, C(O)N*H*), 6.65 and 6.85 (d each, J = 8.4 Hz, 2H and 2H, HOC_6H_4), 7.06−7.30 (m each, 10H, 2 × C_6H_5). Anal. ($C_{28}H_{32}NO_7P$) C, H, N.

3-[(1-Amino-3-methylbutyl)hydroxyphosphinyl]-propionic Acid (3). The benzyloxycarbonyl group was removed by hydrogenolysis of the peptide **21** in the same manner as described for phosphonamidates. Here, on the contrary to the unstable P−N peptides, the same goal could be achieved by the action of 33% HBr (1 mL for 0.1 g of the peptide) at room temperature for 1 h. Removal of the methyl ester group proceeded gently in alkaline conditions. However, because of the formation of the phosphinic analogue of diketopiperazine under these conditions, the acid hydrolysis was favorable. It required 48 h in 40% HBr at 100 °C to cleave the ester completely. After evaporation to dryness, the residue was treated with MeOH and evaporated again to remove the traces of HBr (three times). The resulting peptide was separated as its hydrobromide salt upon treatment of the residue with Et_2O, which yielded an amorphous, slightly yellow solid of satisfactory purity. Yield: 93%; IR (KBr, cm^{-1}): 3415 (OH), 3300−2600 (OH, NH, CH), 1735 (C=O), 1240 (P=O), 1195, 1170, and 1090 (C−O, P−O); ^{31}P NMR (D_2O, NaOD): δ 46.14; ^{1}H NMR: δ 0.79 and 0.85 (d each, J = 6.6 Hz, 3H and 3H, $CH(CH_3)_2$), 1.27 (m, 2H, $CHCH_2CH$), 1.66 (m, 3H, $CH(CH_3)_2$ and PCH_2), 2.22 (m, 2H, PCH_2CH_2), 2.65 (dt, J_H = 6.5 Hz, J_P = 9.4 Hz, 1H, NC*H*P). Anal. ($C_8H_{18}NO_4P$·HBr) C, H, N.

2-[[(1-Amino-3-methylbutyl)hydroxyphosphinyl]-methyl]-4-methylpentanoic Acid (4, **27).** The peptide **4** was obtained in the same manner as for **3**. The final product was separated upon treatment of the evaporated residue with Et_2O/hexane. Yield: 84%; IR (KBr, cm^{-1}): 3405 (OH), 3300−2600 (OH, NH, CH), 1745 (C=O), 1240 (P=O), 1205, 1170, and 1100 (C−O, P−O); ^{31}P NMR (D_2O, NaOD): δ 45.04 and 45.79 (3:4 ratio); ^{1}H NMR: δ 0.79 (m, 12H, 2 × $CH(CH_3)_2$), 1.24−1.91 (m, 8H, 2 × $CHCH_2CH(CH_3)_2$ and PCH_2), 2.49 (m, 1H, PCH_2CH), 2.63 (m, 1H, NC*H*P). Anal. ($C_{12}H_{26}NO_4P$·HBr) C, H, N.

Repetition of this procedure for compound **23** gave the final peptide (*R*)-LeuP[CH_2]-(*R*,*S*)-Leu (**27**) as mixture of two diastereoisomers in a 1:1 ratio.

2-[[(1-Amino-4-phenylbutyl)hydroxyphosphinyl]-methyl]-4-methylpentanoic Acid (5). Because of solubility limitations, pseudopeptides containing homophenylalanine (**24**−**26**) were deprotected using a modified protocol. First, the methyl ester was cleaved in a 1 M NaOH/MeOH solution. After overnight stirring, methanol was removed under reduced pressure. The acid was precipitated by acidification of the water phase to pH 1 and then extracted with two portions of AcOEt. The combined organic phases were dried over Na_2SO_4, filtered, and evaporated. Then the residue was treated with 33% HBr (1 mL for 0.1 g of the peptide) at room temperature for 1 h. After evaporation of the volatile components, the residue was dissolved in methanol, and propylene oxide was added to achieve neutral pH. The final product was precipitated as a slightly yellow solid upon addition of Et_2O. Yield: 85%; mp 141−149 °C; IR (KBr, cm^{-1}): 3405 (OH), 3400−2600 (OH, NH, CH), 1705 (C=O), 1175, 1145, and 1040 (P=O, C−O, P−O); ^{31}P NMR (D_2O, NaOD): δ 44.21 and 44.84 (2:3 ratio); ^{1}H NMR: δ 0.77 (m, 6H, $CH(CH_3)_2$), 1.28−1.93 (m, 7H together, $CHCH_2CH(CH_3)_2$, $C_6H_5CH_2CH_2$ and PCH_2), 2.42 and 2.55 (m each, 1H and 2H, $C_6H_5CH_2$ and PCH_2CH), 2.82 (m, 1H, NC*H*P), 7.25 (m, 5H, C_6H_5). Anal. ($C_{16}H_{26}NO_4P$) C, H, N.

2-[[(1-Amino-4-phenylbutyl)hydroxyphosphinyl]-methyl]-3-phenylpropionic Acid (6). Yield: 89%; mp 154−161 °C; IR (KBr, cm^{-1}): 3415 (OH), 3400−2600 (OH, NH, CH), 1700 (C=O), 1170 and 1040 (P=O, C−O, P−O); ^{31}P NMR (D_2O, NaOD): δ 44.00 and 44.61 (1:2 ratio); ^{1}H NMR: δ 1.56 (m, 2H, $PhCH_2CH_2$), 1.95 (m, 2H, PCH_2), 2.60 (m, 2H, $PhCH_2CH_2$), 2.72 (m, 2H, $PhCH_2CH$), 2.86 (m, 2H, PCH_2CH and NC*H*P), 7.21 and 7.29 (m each, 4H and 6H, 2 × C_6H_5). Anal. ($C_{19}H_{24}NO_4P$) C, H, N.

2-[[(1-Amino-4-phenylbutyl)hydroxyphosphinyl]-methyl]-3-(4-hydroxyphenyl)propionic Acid (7). Yield:

82%; mp 158−171 °C; IR (KBr, cm^{-1}): 3400−2600 (OH, NH, CH), 1700 (C═O), 1240, 1165, and 1040 (P═O, C−O, P−O); ^{31}P NMR (D_2O, NaOD): δ 44.52 and 45.01 (3:5 ratio); ^1H NMR: δ 1.53 (m, 2H, $PhCH_2CH_2$), 1.89 (m, 2H, PCH_2), 2.59 (m, 5H, $HOC_6H_4CH_2$, $PhCH_2$, and PCH_2CH), 2.81 (m, 1H, NCHP), 6.65 and 6.85 (d each, J = 8.2 Hz, 2H and 2H, HOC_6H_4), 7.26 (m, 5H, C_6H_5). Anal. ($C_{19}H_{24}NO_5P$) C, H, N.

2-Hydroxy-3,6-diisobutyl-2-oxo-2λ^5[1,4,2]diazaphosphinan-5-one (28). The pseudodiketopiperazine analogue of LeuP[CH_2]Leu was obtained during alkaline hydrolysis of methyl 2-[[(1-amino-3-methylbutyl)hydroxyphosphinyl]methyl]-4-methylpentanoate. After removal of methanol, the residue was acidified to pH 1 and extracted three times with $CHCl_3$. The organic layer was dried, filtered, and evaporated to dryness giving the crude byproduct. ^{31}P NMR ($CDCl_3$): δ 54.14; ^1H NMR: δ 0.98 (m, 12H, 2 × $CH(CH_3)_2$), 1.20 and 1.68 (m each, 1H and 3H, 2 × $CHCH_2CH$), 1.80 and 2.23 (m each, 3H and 1H, 2 × $CH(CH_3)_2$ and PCH_2CH_2), 2.67 (m, 1H, PCH_2CH), 3.66 (m, 1H, NCHP), 6.36 (bd, 1H, NH), 7.16 (bs, 1H, POH).

Kinetic Studies. 1. Enzyme Preparation. Cytosolic leucine aminopeptidase (LAP) from porcine kidney was obtained from Sigma Chemical Co and prepared according to the method described in the literature.[66] After 2 h activation in 22 mM triethanolamine hydrochloride buffer, pH 8.5, containing $MnCl_2$ (1mM), the enzyme solution was used directly in the kinetic experiments.

APN from pig kidney was obtained as lyophilized powder from Sigma Chemical Co. The enzyme was diluted with 50 mM potassium phosphate buffer, pH 7.2, and used directly in kinetic experiments.

2. Assay of LAP Activity. LAP was assayed at 25 °C in 7.5 mM triethanolamine hydrochloride buffer, pH 8.4, containing $MgCl_2$ (5mM). 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 the absorbance measured at 405 nm with a UV−Vis Spectrophotometer Pharmacia LKB, Biochrom 4060. The measured K_m value was 1.26 mM, which remains in very good agreement with the data reported in the literature.[24] The determination of the inhibition constants was performed as described in the literature.[54] All the solutions of the inhibitors were prepared in the assay buffer, and the pH was adjusted to 8.4 by the addition of 0.1 M HCl or 0.1 M NaOH solution, depending on inhibitor. Inhibitors were preincubated with the enzyme for 60 min at room temperature, which is required to obtain the linear change of absorbance versus time progress. The assay mixture, which contained the inhibitor solution (concentration dependent on the inhibitor), the enzyme solution (20 μg/mL final concentration) and the assay buffer, was adjusted to 1 mL final volume by addition of 0.1 mL of substrate solution (0.2, 0.4, 0.6, 0.8, 1.0 mM final concentrations), which initiated the reaction. Kinetic data were collected over 10 min assay run at 25 °C. The concentration of the enzyme was determined spectrophotometrically at 280 nm, assuming $A_{280}^{0.1\%} = 0.83\ cm^{-1}$ and LAP molecular weight 255.000 Da.[54]

3. Assay of APN Activity. APN activity was assayed at 25 °C in 50 mM potassium phosphate buffer, pH 7.2. The substrate L-leucine *p*-nitroanilide, dissolved in DMSO, was added to assay buffer followed by the enzyme. The hydrolysis of the substrate was monitored by following the change in the absorbance measured at 405 nm with the UV−Vis spectrophotometer Pharmacia LKB, Biochrom 4060. The measured K_m value was 0.30 mM, which remains in a good agreement with the literature data.[54] The determination of inhibition constants was performed as described in the literature.[54] All solutions of inhibitors were prepared in the assay buffer, and pH was adjusted to 7.5 by the addition of 0.1 M HCl or 0.1 M NaOH. All inhibitors were preincubated with APN for 60 min at room temperature. The assay mixture, which contained the inhibitor solution (concentration dependent on the inhibitor), the enzyme solution (4 μg/mL final concentration), and the assay buffer, was adjusted to 1 mL final volume by addition of 0.1 mL of substrate solution (0.05, 0.1, 0.2, 0.4, 0.6 mM final concentrations), which initiated the reaction. Kinetic data were collected over 10 min assay run at 25 °C. The concentration of the enzyme was determined spectrophotometrically at 280 nm, assuming $A_{280}^{0.1\%} = 1.63\ cm^{-1}$ and APN molecular weight 280.000 Da.[54]

The K_i values for the phosphinates toward LAP and APN were determined from Lineweaver−Burk plots for the reaction monitored in the presence and in the absence of the inhibitor, using the velocities measured from the linear portion of the absorbance versus time progress curves. Assays were performed for three or four concentrations of each inhibitor, and every measurement was repeated twice. The scattering of the results (K_i values) did not exceed 20% for each tested inhibitor.

Computations. 1. Design of LAP Inhibitors. The coordinates of leucine aminopeptidase and LeuP were obtained from the refined 1.65 Å X-ray structure of bovine lens leucine aminopeptidase complexed with the inhibitor, the phosphonic analogue of leucine[25] (1lcp refcode in Brookhaven Protein Data Bank). The hydrogen atoms were added using the Insight 97.0 (Accelrys/MSI) program.[83] The protonation states of the residues were selected for pH 7.0, excluding Lys250, which is neutral, as the nitrogen atom electron lone pair in the side chain of this residue is coordinated by Zn489.[25] The computer program Ludi[84] was applied to design LAP inhibitors. A systematic search of Ludi's fragment library, containing about 1000 structural fragments was performed. The LeuP structure was fixed and Link mode of the program was used to generate new LAP inhibitors. The structures of the designed inhibitors were obtained by the replacement of the hydroxyl group of LeuP with new structural moieties generated using Ludi, assuming that they would be bound in the S1′ pocket of the enzyme (Figure 1). New fragments at the P1 position of inhibitors were obtained by substituting of LeuP side chain by new ones found in Ludi_link library, which fit well to the S1 pocket of LAP. The values of the most important Ludi parameters used for design of new LAP inhibitors were as follows: Min Separation = 3; Link, Lipo and H-Bond Weights were set to 1.0; Aliphatic_Aromatic and Reject Bifurcated parameters were turned off; No_Unpaired_Polar, Electrostatic_Check and Invert parameters were turned on; Es Dist = 2.5; Max RMS = 0.5; Number of Rotatable Bonds − two at a time, Radius of Search was changed from 5 Å to 10 Å.[84] Visual inspection of the interactions of designed inhibitors with LAP was performed within Insight_97.0 program.[83]

The structures of the designed inhibitors were optimized within the enzyme active site using the AMBER and CFF97 force fields in Discover/Insight 97.0 program.[85] The heavy atoms of LAP were frozen during the optimization, because of the lack of the structural changes of the enzyme upon ligand binding, observed in the crystal structures of leucine aminopeptidase complexed with the inhibitors of different size (e.g., LeuP − amino acid analogue versus amastatin − tetrapeptide analogue).[10,86] To maintain the appropriate positions of the inhibitors with respect to zinc ions in the active site, the distance constraints between these zinc ions and their ligands with the force constants values 500 kcal/mol·$Å^2$ were applied in the energy minimization procedure. The optimization was performed using the conjugate gradient algorithm until the maximum derivative was <0.1 kcal/mol·Å. The predicted binding affinities of the designed inhibitors were estimated by use the empirical scoring function implemented in the Ludi program.[30,87]

2. Calculation of APN Active Site Model. The amino acid sequence of APN from pig was obtained from GenBank, available from the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov, gi number: 1703286). The search for homologues proteins in Protein Data Bank was performed using BLAST program (www.ncbi.nlm.nih.gov/blast)[88,89] and resulted in finding one protein: human leukotriene A_4 hydrolase/aminopeptidase (LTA4H, 1hs6 code in PDB) with reasonable sequence similarity (27% identity for 204−526 amino acid fragment of APN, which is involved in binding site formation). The pairwise sequence alignment of APN with LTA4H was extracted from the multiple sequence

alignment of peptidase M1 family members, performed using CLUSTALW program (http://www.ebi.ac.uk/clustalw).[90] This alignment was then applied for calculation the model of part of APN catalytic domain (residues 204−526), by application of MODELLER program, with the default parameters (http://guitar.rockefeller.edu/modeler/modeler.html).[91] The structure of the remaining part of APN was not modeled, as no templates with the sequence identity above 20% to these regions were found in PDB. However, the residues forming APN binding site originate mostly from 204 to 526 fragment, thus neglecting the remaining part of the protein in the model seems to be admissible to analyze the interactions of inhibitors with APN. The zinc ion was added to APN active site at the position, which corresponds to zinc site in LTA4H,[76] because of the same residues surrounding zinc binding site in both enzymes (Figure 3).

The hPheP[CH_2]Phe and hPheP[CH_2]Tyr were docked to the model of APN based on the interactions of bestatin with LTA4H (1hs6 in PDB). The model of APN was soaked in 5 Å water layer and the structures of inhibitors were optimized within the enzyme active site using the CVFF force field in Discover/Insight 97.0 program.[85] The backbone atoms of APN were frozen during the optimization and the distance constraints between zinc ion and its ligands (His383, His387, Glu406), with the force constants values 500 kcal/mol*$Å^2$, were applied in energy minimization procedure. Visual inspection of interactions between the inhibitors and APN was performed within Insight_97.0 program.[83]

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