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A synthetic method for diversification of the P1′ substituent in phosphinic dipeptides as a tool for exploration of the specificity of the S1′ binding pockets of leucine aminopeptidases

Stamatia Vassiliou,[a] Metaxia Xeilari,[a] Athanasios Yiotakis,[a] Jolanta Grembecka,[b] Małgorzata Pawełczak,[c] Paweł Kafarski[b,c] and Artur Mucha[b,*]

[a]*Laboratory of Organic Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece*
[b]*Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland*
[c]*Institute of Chemistry, University of Opole, Oleska 48, 45-052 Opole, Poland*

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Abstract—A novel, general, and versatile method of diversification of the P1′ position in phosphinic pseudodipeptides, presumable inhibitors of proteolytic enzymes, was elaborated. The procedure was based on parallel derivatization of the amino group in the suitably protected phosphinate building blocks with appropriate alkyl and aryl halides. This synthetic strategy represents an original approach to phosphinic dipeptide chemistry. Its usefulness was confirmed by obtaining a series of P1′ modified phosphinic dipeptides, inhibitors of cytosolic leucine aminopeptidase, through computer-aided design basing on the structure of homophenylalanylphenylalanine analogue (hPhePP[CH_2]Phe) bound in the enzyme active site as a lead structure. In this approach novel interactions between inhibitor P1′ fragment and the S1′ region of the enzyme, particularly hydrogen bonding involving Asn330 and Asp332 enzyme residues, were predicted. The details of the design, synthesis, and activity evaluation toward cytosolic leucine aminopeptidase and aminopeptidase N are discussed. Although the potency of the lead compound has not been improved, marked selectivity of the synthesized inhibitors toward both studied enzymes was observed.

1. Introduction

Aminopeptidases form a family of metallo-dependent hydrolases that are widespread in plants, animals, and bacteria. Being responsible for cleavage of the N-terminal amino acids from polypeptide chains, these exopeptidases play a key role in physiological processing and degradation of peptides and proteins, and alterations in their activity have been associated with various pathological disorders, including cancer.[1–3] Among them, bizinc cytosolic leucine aminopeptidase (LAP, E.C.3.4.11.1), exhibiting the substrate specificity toward hydrophobic amino acid residues, remains one of the most extensively studied enzymes.[4,5] This interest is mainly directed toward evaluation of its mechanism of hydrolytic action, which is not completely clarified yet. Basing on numerous three-dimensional LAP structures obtained for both enzyme in native form and complexed with various inhibitors,[6–10] a range of mechanistic suggestions have been coined.[1,5,9,11–13] Despite this, the mode of LAP action still simulates studies on theoretical evaluation of zinc environmental effects, functional role of the active-site residues,[14] and role of the water channels. They are carried out in order to identify the most likely candidates for nucleophiles active in the hydrolytic process.[15] Related situation occurs considering physiological and pathological implications of LAP activity. Biological role of the enzyme has not been fully elucidated yet, however, similarly to other aminopeptidases, LAP is assumed to be involved in protein modification, activation, and degradation as well as in the metabolism of biologically active peptides and regulation of activity of hormonal and non-hormonal peptides.[2,4] In mammals, LAP processes antigenic peptides for presentation by the major histocompatibility complex class I molecules.[16] Recently, it has been also

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* Corresponding author. Tel.: +48 71 320 3354; fax: +48 71 328 4064; e-mail: artur.mucha@pwr.wroc.pl

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identified as the key enzyme responsible for glutathione turnover in liver.[17,18] Significantly, over-expression of LAP has been implicated in certain pathological stress states including HIV infection, inflammation, cataracts, and cancer.[19–23]

Biological significance and potential medical importance of leucine aminopeptidase stimulate continuous interest in agents regulating its activity.[24] Development of organophosphorus compounds mimicking the high energy transition state of the peptide bond hydrolysis has been shown to be one of the most fruitful strategies in this field.[25–28] Recently, we have reported[27] rationally designed phosphinic dipeptide analogues that rank amongst the most potent low-molecular weight inhibitors of LAP reported so far. Among them, homophenylalanyl-phenylalanine (compound **1**, hPheP[CH_2]Phe, Fig. 1) and homophenylalanyl-tyrosine analogues (compound **2**, hPheP[CH_2]Tyr) appeared to be highly active (K_i = 66 and 67 nM, respectively, for mixture of four diastereoisomers). The observation of virtually identical binding affinities obtained for both **1** and **2** indicates that the hydroxyl group of the side chain of Tyr at P1′ is not involved in any additional interaction with the enzyme.[27] This is a surprising finding since the formation of the hydrogen bond between this hydroxyl group and the side-chain amidate of Asn330 at the bottom of S1′ has been predicted by molecular modeling. However, this interaction apparently has not been created. The reason of that could derive from unfavorable geometry in terms of the hydrogen bond distance and angle.

Interestingly, such a structural change (Phe → Tyr in P1′ position) resulted with much more visible difference in binding affinities of both compounds to microsomal leucine aminopeptidase (aminopeptidase N, APM, E.C. 3.4.11.2).[27] This monozinc exopeptidase[29] was selected intentionally in order to evaluate binding preferences toward studied phosphinic pseudodipeptides in comparison with LAP. hPheP[CH_2]Tyr (**2**) appeared much more effective APM inhibitor (K_i = 36 nM) than any phosphorus containing dipeptide analogue described in the literature[30] (for recent, extensive review of APM inhibitors, see also Ref. 31). This finding was explained by formation of specific hydrogen bond between Tyr hydroxyl with carboxyl group of Glu413 placed at the bottom of the S1′ pocket of APM, as envisaged after building the model of APM active site based on homology to human leukotriene A_4 hydrolase/aminopeptidase.[27]

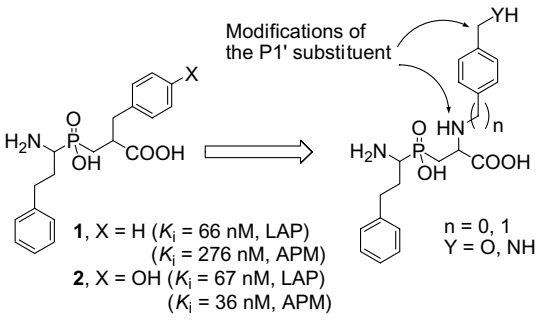


Figure 1. The designed structural modifications of the lead compounds (**1** and **2**)[27] to optimize interaction of phosphinic dipeptides with the S1′ binding pocket of leucine aminopeptidase (LAP).

The above-described results stimulated further studies directed toward design of novel P1′ substituents in presumable inhibitors of aminopeptidases. In this paper, we present our attempts to modify the structure of hPheP[CH_2]Phe in order to obtain new phosphinic dipeptide inhibitors of LAP and APM. Two kinds of modifications were planned (Fig. 1). The first one relied on attachment of a heteroatom-containing group (hydroxyl or amino) to the *para* position of the phenyl ring via methylene bridge ensuring its increased rotational freedom, which in turn should enable more flexible binding and thus facilitate hydrogen bond formation with Asn330. The second one was introduction of an additional amino group within the P1′ side chain of the dipeptide mimetic. The predicted binding mode revealed the proximity of this amino group to the carbonyl portion of Asn332 and a potential for hydrogen bond formation between them.

To obtain the designed compounds, complex synthetic problems were solved. As corresponding α-aminoacrylates—key electrophilic substrates for Michael addition methodology appeared synthetically not available, new general parallel arylation/alkylation procedure for the phosphinic dipeptide amino derivatives was developed. The details of this procedure leading to the target structures are described and discussed. Finally, the binding affinities of the set of target inhibitors toward both LAP and APM have been evaluated.

2. Results and discussion

2.1. Design

The structure of the phosphinic dipeptide hPheP[CH_2]Phe (**1**, Fig. 1) bound to the active site of LAP served as the lead compound to develop novel inhibitors of leucine aminopeptidase. The overall structural motif, the pseudopeptide backbone as well as the P1 hydrophobic phenylethyl side chain were conservatively kept in all newly designed structures, whereas the P1′ position was suitably modified to obtain better fit to the S1′ pocket of LAP. These changes accomplished incorporation of the hydroxymethyl or aminomethyl groups at the P1′ proximity, namely they were introduced at *para* position of the phenyl ring. The S1′ pocket is partially open to solvent and based on the docking mode of **5** (see below for details) the *ortho* and *meta* substitutions would not result in additional interactions with the protein. The other side of the phenyl ring at P1′ is approaching the residues of the S1′ pocket: Ile421 and Ala333 and there is not enough space to incorporate a functional group at the *ortho* and *meta* positions. Therefore, *para* analogues were selected for synthesis as only these were expected to form additional contacts with LAP. Besides modification of the phenyl ring at P1′, the β carbon atom of the pseudophenylanine

residue was formally replaced with nitrogen atom. Such a replacement introduced the amino group of aromatic character (aniline derivatives). On the other hand, insertion of nitrogen gave aliphatic ones (extended benzylamine derivatives), which might be important in terms of their protonation (these amino groups differ in their pK_a values up to five orders of magnitude). Novel P1′ residues are represented by *p*-hydroxymethyl- (**4**) or *p*-aminomethylaniline (**5**), and *p*-hydroxymethyl- (**7**) or *p*-aminomethylbenzylamine (**8**) structural fragments (Fig. 2). They were synthesized together with the unsubstituted derivatives (**3** and **6**) as respective reference compounds.

The designed structures were docked[32] to the binding site of leucine aminopeptidase obtained from the X-ray structure of bovine lens LAP complexed with phosphonic analogue of leucine,[10] similarly as described previously in the case of compounds **1** and **2**.[27] The results of the analysis have been found promising. Presumed interactions of **5** and other compounds described here with LAP active site shown in Figure 3

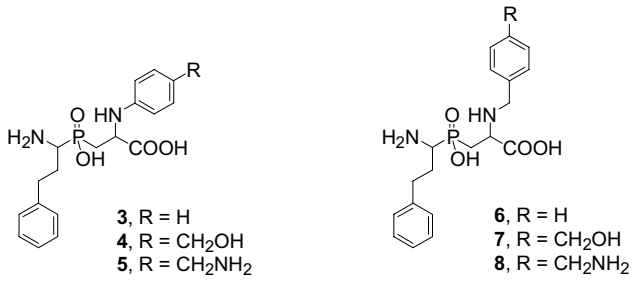


Figure 2. Putative phosphinic dipeptide inhibitors of LAP containing modified P1′ substituents.

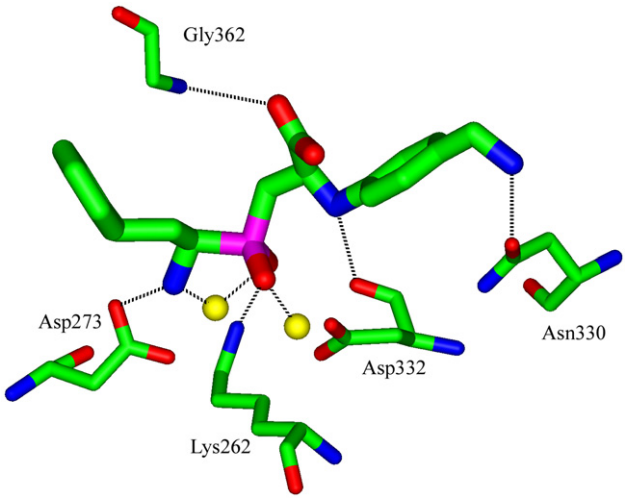
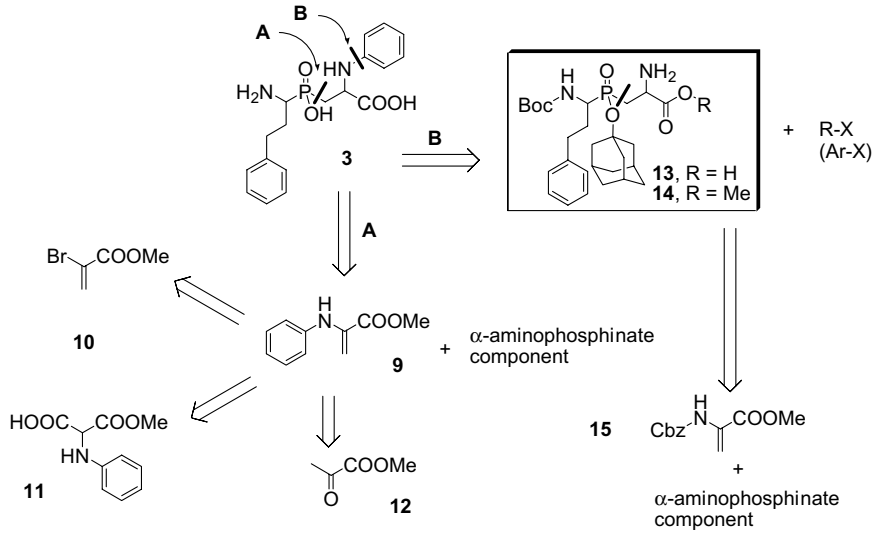


Figure 3. Modeled binding mode of 2-(4-aminomethylphenylamino)-3-[(1-amino-3-phenylpropyl)hydroxyphosphinoyl]propionic acid **5** by leucine aminopeptidase obtained using the LAP crystal structure[10] (1lcp in PDB), and the Ludi computer program.[27,32] The selected S1′ pocket and the active site residues of LAP, interacting with the dipeptide analogue, are presented. Hydrogen bonds and interactions of the inhibitor with zinc ions are shown as black dashed lines.

indicate that the general pattern of zinc complexation, the P1 fragment interactions, as well as binding the backbone through the network of hydrogen bonds with Asp273, Lys262, Gly362 are fully retained, comparing to **1** and **2** as well as to other known LAP inhibitors.[9,10,12,27] The presence of the modified side chain at the P1′ position opens possibilities for additional interactions with the S1′ pocket of LAP. The S1′ pocket is formed by the Ile421, Ala333 side chains from one side and Asn330 together with the hydrophobic portion of Asp332 side chain from the other side. At the same time, this pocket is partially open to solvent, which causes that part of the P1′ substituent to be water exposed. Therefore, the introduction of substituents at *ortho* and *meta* positions of the phenyl ring would not provide additional interactions with LAP. Based on the docking mode, the amino/hydroxy group at the *para* position should be able to form the hydrogen bond with the oxygen atom of the side chain of Asn330 (Fig. 3, hydrogen bond distance is about 2.9 Å, hydrogen bond angle of about 170°). Moreover, the secondary amino group in β position of the pseudophenylalanine residue is in hydrogen bond distance (2.7 Å) to the carbonyl oxygen of Asp332. In this case, however, the geometry of hydrogen bond would be less favorable (hydrogen bond angle of about 150°). To verify empirically such theoretical arrangement, compounds **3**–**8** were chosen for synthesis and for evaluation of their binding affinities toward LAP and APM.

2.2. Chemistry

2.2.1. Retrosynthesis. The synthetic targets of this work were molecules **3**–**8** of structures shown in Figure 2. The designed compounds possess up to five functional groups of various characters and thus their synthesis cannot be straightforward. To face this challenge, a short retrosynthetic analysis has been performed for the simplest derivative **3** (Scheme 1). Michael addition of phosphorus nucleophiles (suitably protected phosphinic amino acid analogues) to appropriate carbon electrophiles (usually acrylates) is the most commonly applied method for the phosphinate pseudopeptidic bond formation.[33] In our case, the first considered idea was the use of *N*-phenyl-α-aminoacrylate (*N*-phenyldehydroalanine ester, **9**) as the key substrate (Scheme 1, pathway A). To the best of our knowledge, this compound of relatively simple structure has not been reported in the literature yet. Various methods, including elimination of appropriately substituted serine derivatives, could be envisaged for its synthesis. Three other possibilities are listed in Scheme 1, namely: arylation of aniline with α-bromoacrylate **10**, Knoevenagel reaction of paraformaldehyde and monoalkyl *N*-phenylaminomalonate **11**, and condensation of aniline with pyruvate **12**. All of them were preliminarily tested. Not going into experimental details, these attempts failed giving as the main products, respectively, *N*-phenylaziridinecarboxylate in the reaction with the use of α-bromoacrylate **10**, *N*-phenylglycinate in Knoevenagel reaction, and a complex mixture in the case of pyruvate **12** condensation. Because of the probable high instability of the target molecule **9**, it became obvious

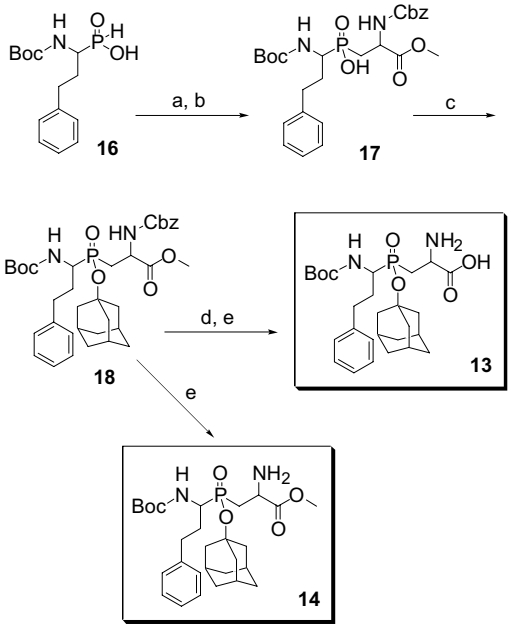


Scheme 1. The retrosynthesis of 3-[(1-amino-3-phenylpropyl)hydroxyphosphinoyl]-2-phenylaminopropionic acid (**3**). For the details, see Section 2.

that much more general approach should be developed. Elimination of serine derivatives provided an idea of construction of versatile building blocks **13** and **14** (Scheme 1, pathway B) by Michael addition of the appropriate phosphorus component to the dehydroalanine derivative **15**. The availability of acid **13** and the corresponding C-terminal ester **14** would allow multidirectional diversification of the P_1' position at the final step of the synthesis using cross-coupling or alkylation reactions, respectively.

2.2.2. Building block synthesis. The synthesis of the designed synthons **13** and **14** is outlined in Scheme 2. Careful choice of the protecting groups for four functions present in the molecules of phosphinic dipeptide precursors is crucial, since their selective removal at the desired synthetic step is an absolute requirement. Thus, by addition of Boc-protected phosphinic homophenylalanine analogue **16**[34] to α-aminoacrylate **15**[35] (Cbz-protected dehydroalanine methyl ester) under standard conditions,[36] phosphinic pseudodipeptide **17** was obtained in good yield after chromatographic purification. It should be stressed that acrylate **15** was used in almost equimolar amount in relation to the phosphinic acid and was added very slowly to the activated phosphinic silyl ester, otherwise extensive polymerization occurred. Subsequent reaction of the hydroxyphosphinyl function with 1-adamantyl bromide[36] gave fully protected derivative **18**. Its saponification followed by catalytic hydrogenation yielded the desired compound **13**, whereas synthon **14** was obtained after hydrogenolysis over $Pd/BaSO_4$. The latter one could be stored for a short period of time in the form of free amine, and for prolonged time as hydrochloric acid salt. The usefulness of resulting building blocks **13** and **14** (in free amine forms) for parallel protocols was verified in two reaction types: cross-coupling (to obtain compounds **3**–**5**) and nucleophilic substitution (leading to compounds **6**–**8**).

2.2.3. Cross-coupling. Formation of the carbon–nitrogen bond via cross-coupling reaction is well recognized in



Scheme 2. Preparation of the key building blocks **13** and **14**, required for their further diversification by cross-coupling or alkylation reactions. Reagents and conditions: (a) hexamethyldisalazane, 110 °C; (b) **15**, 90 °C, 77%; (c) 1-AdBr, Ag_2O, $CHCl_3$, reflux, 80%; (d) NaOH/MeOH, rt, 16 h then HCl, pH 1, 92%; (e) H_2, 10% $Pd/BaSO_4$, MeOH, 3.5 h, 100%.

the literature. A number of cleverly designed and useful methods based on the Ullmann[37] reaction using Cu reagent and the Buchwald–Hartwig[38] amination utilizing palladium catalyst have been developed. During the last decade, several reports described mild methods for catalytic amination of aryl halides using ligands like α-amino acids[39] or ethylene glycol[40] in the case of the Ullmann reaction and racemic BINAP[41] in the case of the Buchwald–Hartwig one. Attracted by their facility and readiness we have tested the applicability of these procedures for reaction of our synthon (**13**) with phenyl

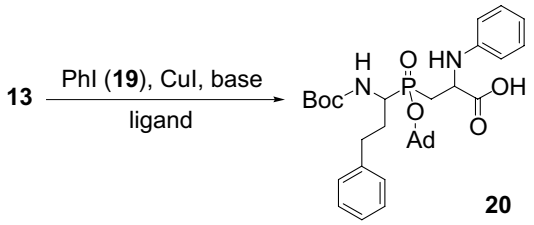
iodide (**19**). Surprisingly, no cross-coupling product was observed in the Pd-catalyzed reaction while in the case of CuI applied as catalyst the desired product **20** was formed although it was isolated only in low yield. Attempts to optimize the latter reaction (Scheme 3) by modification of the ligand and the solvent system according to the literature[39,40] are summarized in Table 1. When elevated temperatures were used, possibly premature loss of the protective group(s) resulted in mixture of unidentifiable reaction products, therefore 65 °C was not exceeded.

Steric hindrance caused by the presence of the neighboring bulky adamantyl group and the relatively low reaction temperature could explain the low yields of the cross-coupling reaction. Lack of any accelerating effect, observed by Ma[39] for the α-amino acid assisted Ullmann condensation, is in accordance with our data since the structure of the P1′ part of the phosphinic dipeptide **13** already represents this case.

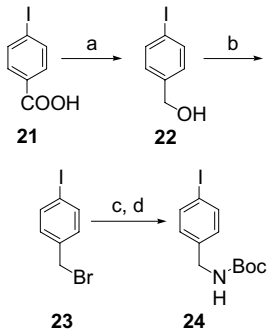
Despite these not fully encouraging results, two additional iodobenzene derivatives **22** and **24** were synthesized from a commercially available starting material (Scheme 4). Reduction of *p*-iodobenzoic acid **21** with $NaBH_4$ provided *p*-iodobenzylalcohol **22** in good yield. Bromination of the alcohol **22** followed by treatment of the product **23** with ammonium hydroxide and subsequent protection of the amine with the use of di-*tert*-butyl dicarbonate resulted in derivative **24**.

Reaction of the obtained iodo derivatives **22** and **24** with **13** in standard conditions (entry A, Table 1) furnished the desired cross-coupling products **25** and **26** in 10% and 8% yield, respectively (Scheme 5). Acidic cleavage of the protective groups in all three blocked products (**20**, **25**, and **26**) afforded the target aniline derivatives **3**–**5**, which were finally purified by reverse-phase preparative HPLC.

2.2.4. Nucleophilic substitution. N-Alkylation of primary amines in the presence of inorganic bases is one of the most general methods for the synthesis of secondary



Scheme 3.



Scheme 4. Preparation of *p*-substituted iodophenyl derivatives **22** and **24**, substrates for cross-coupling with **13**. Reagents and conditions: (a) *i*-BuOCOCl, *N*-methylmorpholine, −10 °C then $NaBH_4$ and MeOH, 0 °C, 70%; (b) PBr_3, Et_2O, 0 °C → rt, 3 h, 58%; (c) NH_4OH, 5d; (d) Boc_2O, CH_2Cl_2, 0 °C, 15 h, 72%.

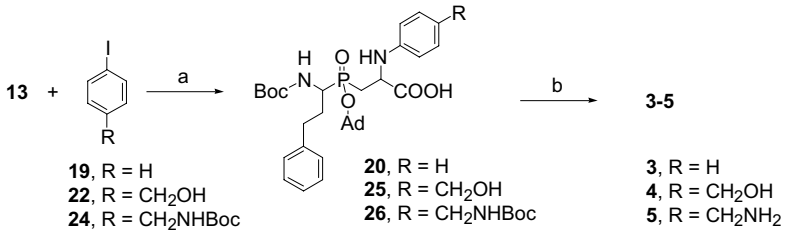
amines.[42] In order to find out the optimal conditions for this reaction in terms of protective groups compatibility and avoidance of formation of overalkylation byproducts, we performed a more detailed study using benzyl bromide (**27**) as the alkylating agent of synthon **14** (Scheme 6). The obtained results are presented in Table 2.

Since high yield of the desired compound **28** was achieved without detecting the overalkylation product **29**, Cs_2CO_3 and gentle warming in dry DMF (entry A) were concluded as recommended conditions in the next experiments. The superiority of cesium carbonate used as a base in N-alkylation of primary amines is well known and has been described in the literature as 'cesium effect'.[43] Lack of overalkylation could be attributed to the bulkiness of the neighboring adamantyl group.

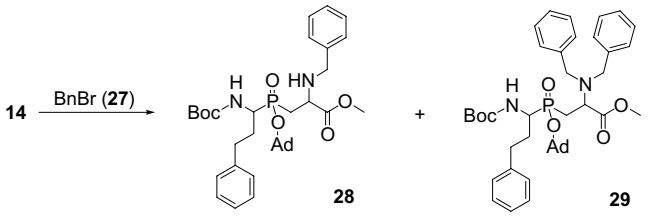
The appropriately *p*-substituted benzyl bromides (**33** and **37**) are commercially unavailable, thus they had to be prepared prior to alkylation of the phosphinic dipeptide **14**. Their synthesis is outlined in Scheme 7. Esterification of *p*-bromomethylbenzoic acid **30** provided its methyl ester **31**, which was reduced to the corresponding alcohol **32** with the use of DIBALH. Protection of the hydroxyl group into the form of the *tert*-butyl ether provided the desired benzyl bromide **33**. The *p*-amino derivative was prepared basing on the literature data.[44] Initially, *p*-cyanobenzyl bromide **34** was hydrolyzed to the corresponding alcohol **35**. Reduction of the cyano group furnished the amino alcohol, which was treated subsequently with hydrobromic acid to provide bromide **36**. Protection of the amino group with di-*tert*-butyl dicarbonate yielded the desired bromide **37**. Preliminary experiments to reduce *p*-cyanobenzyl bromide in order

Table 1. Comparison of the effectiveness of the Ullmann cross-coupling reaction of the building block **13** with phenyl iodide performed in recommended conditions[39,40]

Method	Base	Solvent	Ligand	Time (h)	Temperature (°C)	Yield of **20** (%)
A	K_2CO_3	DMSO	—	48	65	22
B	K_2CO_3	DMSO	L-Proline	48	65	22
C	K_2CO_3	2-Propanol	$OHCH_2CH_2OH$	48	65	21



Scheme 5. Cross-coupling reaction with the use of **13** and final deprotection to yield the target phosphinic dipeptides **3**–**5**. Reagents and conditions: (a) CuI, K_2CO_3, 65 °C, 48 h, 22% (**20**), 10% (**25**), 8% (**26**); (b) 50% TFA/CH_2Cl_2, rt, 3 h, then HPLC.



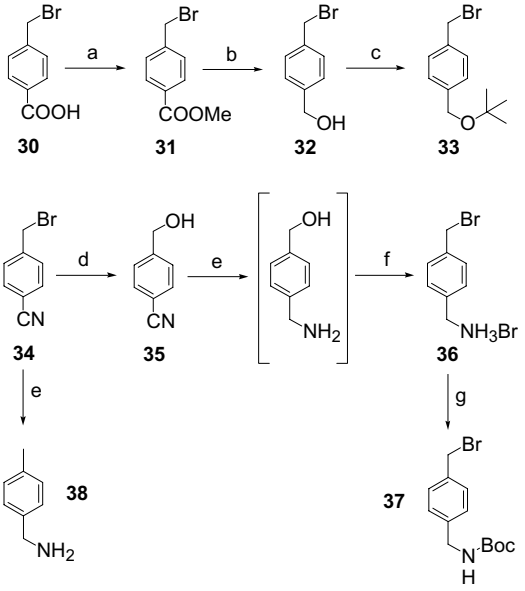
Scheme 6.

to obtain the corresponding amine in one-step procedure resulted in *p*-methylbenzylamine **38**.

The *para* substituted benzyl bromides **33** and **37** underwent the reaction with precursor **14**, under the conditions described above, to provide the fully protected phosphinic dipeptides **39** and **40** in good yield, similarly to the non-substituted derivative **28** (Scheme 8). To remove the protecting groups, pseudopeptides **28**, **39**, and **40** were saponified and then treated with 50% TFA/CH_2Cl_2 for **28** and **40**, and 90% TFA for **39**. Preparative HPLC purification provided the target phosphinic pseudodipeptides **6**–**8** as two pairs of diastereoisomers.

2.3. Activity

Compounds **3**–**8** appeared to be moderate or weak inhibitors of both leucine aminopeptidase and APM (Table 3). They exhibited competitive type of inhibition for both enzymes, with slow-binding mechanism of A type determined for LAP and simple inhibition for APM (with the exception of compounds **5** and **4**-F_2). The mode of inhibition is, therefore, analogous to the one observed earlier for aminophosphonates and phosphinopeptides.[27,28]

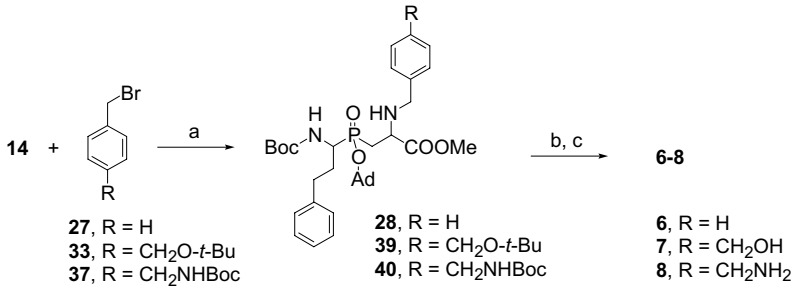


Scheme 7. Preparation of *p*-substituted bromobenzyl derivatives **33** and **37**, substrates for alkylation of **14**. Reagents and conditions: (a) MeOH, H_2SO_4, reflux, 5 h, 95%; (b) DIBALH, 0°C, 4 h, 58%; (c) *i*-butene, H_2SO_4, 0 °C, 64 h, 63%; (d) Ba_2CO_3, H_2O, 4 h, 89%; (e) $LiAlH_4$, THF, 0 °C → reflux, 4 h; (f) 46% HBr, 3.5 h, reflux, 15%; (g) Boc_2O, $NaHCO_3$, H_2O, 0°C → rt, 18 h, 99%.

For almost all phosphinic dipeptides satisfactory separation of diastereoisomers was achieved upon their reverse-phase HPLC purification. Thus, they were collected separately (fractions F_1 and F_2, in the order of elution) and subsequently tested individually giving two K_i values. Obviously, one fraction corresponds to the (*RS,SR*) pair of enantiomers, while the another one to (*RR,SS*).

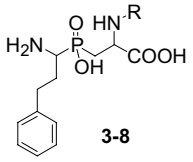
Table 2. Optimization of the conditions of alkylation of the building block **14** with benzyl bromide

Entry	Solvent	Base	Time (h)	Temperature (°C)	Yield of **28** (%)	Yield of **29** (%)	Ratio **28/29**
A	DMF	Cs_2CO_3	6	45	80	—	—
B	DMF	K_2CO_3	6	45	66	—	—
C	DMF	Li_2CO_3	6	45	63	15	~4:1
D	DMF	DBU	6	45	36	—	—
E	DMF	Et_3N	6	rt	46	—	—
F	DMF	Cs_2CO_3	6	rt	80	11	~8:1
G	DMSO	Cs_2CO_3	6	rt	65	—	—
H	Et_2O	Et_3N	20	rt	63	—	—
I	—	Pyridine	6	45	8	—	—

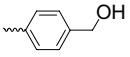
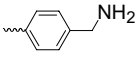
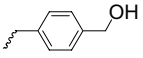
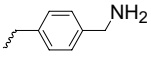


Scheme 8. Alkylation of synthon **14** with benzyl bromides **27**, **33**, and **37**, and final deprotection to yield the target phosphinic dipeptides **6**–**8**. Reagents and conditions: (a) Cs_2CO_3, DMF, 45 °C, 6 h, 80% (**28**), 65% (**39**), 69% (**40**); (b) NaOH/MeOH, rt, 16–18 h then HCl, pH 1; (c) 50% TFA/CH_2Cl_2, rt, 3 h for **28** and **40**, 90% TFA/CH_2Cl_2, rt, 16 h for **39**, then HPLC.

Table 3. Inhibition of cytosolic (LAP) and microsomal leucine aminopeptidase (APM) by the dipeptide analogues **3**–**8** containing modified amino substituents in their P1′ positions (fractions F_1 and F_2 represent the enantiomeric pair (*RS,SR*) and (*RR,SS*) separated by means of reverse-phase HPLC)



3-8

Compound		K_i (μM)			
		LAP[a]		APM	
Entry	P1′ substituent (R)	F_1	F_2	F_1	F_2
3		0.57	0.45	NI	NI
4		10.33	6.98	0.90	1.13[a]
5		4.46		0.56[a]	
6		10.06	12.37	NI	~1500
7		4.21	1.42	NI	NI
8		2.78	2.62	19.90	2.66

[a] Competitive, slow-binding of A type mechanism of inhibition; NI, no inhibition at 10–20 μM, K_i not calculated.

Generally, the synthesized compounds did not exhibit improved activity toward LAP comparing to the potent lead structure **1**. The calculated K_i values vary in a quite narrow range of 0.5–12 μM (Table 3) and are one–two orders of magnitude higher than that found for compound **1**. Surprisingly, the simplest, non-substituted aniline derivative **3** appeared to be the most interesting inhibitor. Compounds designed as superior to compound **3**, namely *p*-substituted benzylamine derivatives **7** and **8**, were slightly better than corresponding aniline derivatives **4** and **5**.

There could be few reasons of such phenomenon. First, the studied dipeptide analogues **3**–**8**, possessing several polar groups of varying properties, must be highly hydrated in aqueous media. Considering the inhibitor affinity in terms of free binding energy, an unwanted loss in entropy might occur because of necessity of breaking a net of hydrogen bond with water molecules that can be not counterbalanced with formation of novel interactions of this type with the enzyme. Second, decreasing affinity with increasing size of the P1′ substituents (in comparison to the lead compound **1**) means that the designed residues could be bound somewhat differently, although theoretically predicted as well-fitting to the S1′ binding pocket. This is because this pocket is opened to the solvent and the designed compounds might be shifted a little toward the interface of the enzyme (thus binding becomes not optimal) with their polar groups (attached to aromatic ring) interfering with the solvent. It has to be emphasized that the side chain of Asn330, which was expected to form a hydrogen bond with ligands, is solvent exposed and therefore its conformation might vary in solution and differ from the one observed in the X-ray structure which was used for docking studies. Moreover, the detailed docking analysis indicated relatively close placement of the zinc cation, namely Zn488, to the secondary amino group introduced into P1′ side chain (~3.7 Å). This could result in strongly unfavorable electrostatic repulsion, particularly in the case of the probable protonation of the amino moiety, and may also contribute to their shifting toward the enzyme interface. This can be directly manifested by a comparison of the binding affinities of **3** and **1**. These two differ only with the β carbon atom at P1′ of **1** being replaced by –NH– in **3**, and this substitution causes 10-fold decrease in binding affinity (Table 3). The structure–activity relationship for the compounds studied here is also ambiguous. For instance, the increase in the length of the P1′ fragment by a methyl group decreases the affinity by about 20-fold for **6** versus **3**, while similar modification improves twice the affinity of compounds bearing a polar group at the *para* position of the phenyl ring (compare **7** vs **4** and **8** vs **5**).

Generally, there is no significant difference between the affinity of the separated fractions F_1 and F_2. It seems straightforward that the diastereoisomer corresponding to the relative (*L,L*) configuration of the natural

substrates should be the most tightly bound. On the other hand, it has been proven that even for more extended phosphinic inhibitors of metalloproteases, the stereochemistry is not a strictly discriminating factor.[45] Thus, no additional efforts have been made to assume the absolute configuration, nor to separate single diastereoisomers of the studied analogues.

According to the theoretical predictions, the other than *para* substitution in the P1′ phenyl ring appeared strongly discriminating for potency of the inhibitors. Two *ortho* benzyl derivatives, additionally synthesized, purified, and separated for fractions, exhibited very poor activity toward LAP (no inhibition at 80 μM for F_1 and $K_i = 247$ μM for F_2 of the *o*-hydroxymethyl derivative, and no inhibition at 40 μM for F_1 and $K_i = 21.6$ μM for F_2 of the *o*-aminomethyl one, data not presented in Table 3). To some extent, this fact supports the assumption made by docking experiments that only *para* derivatives are able to interact favorably with residues forming the S1′ binding pocket of LAP.

There are some meaningful observations concerning the selectivity of the studied phosphinates **3**–**8** toward LAP and APM. Introduction of a simple hydrophobic P1′ residue yielded very poor or no inhibitors (compound **3** and **6**) of aminopeptidase N. In the case of LAP these groups are favored, however, a size limit is observed (compounds **3** being far more active than **6**). APM strictly favors polar groups at the P1′ proximity, particularly the amino moieties. Moreover, in the case of APM the size of the substituent seems to be much more limited than in the case of LAP. Thus, only three compounds (**4**, **5**, and **8**) are potent inhibitors of the enzyme, whereas the others are inactive (Table 3). These results support additionally the suggested importance of the Glu413 carboxyl group placed at the bottom of the S1′ enzyme pocket of APM for inhibitor discrimination.[27]

2.4. Conclusions

In summary, the presented results seem to have the most significant synthetic impact. During last decade, phosphinic pseudopeptides, considered as the analogues of the high-energy transition states of the peptide bond hydrolysis,[45–47] have been extensively applied for effective and selective regulation of activity of various metalloproteases.[48–52] These achievements stimulate continuous interest in development of novel strategies for their preparation, including parallel and combinatorial synthesis.[33] We hope to give a contribution to this field by presenting the synthesis of novel, amino derived building blocks **13** and **14** suitable for further diversification. Although the obtained inhibition rates of cytosolic and microsomal aminopeptidases by compounds **3**–**8** are moderate and thus not fully satisfying, some valuable information concerning structure–activity relationship for these two biologically important enzymes have been also acquired. The results have also shown that currently available molecular modeling methods and tools are not able to predict all possible effects associated with the binding of inhibitor by the enzyme. In particular, this concerns protein systems with solvent exposed ligand binding sites, as it is with the S1′ pocket of LAP, and therefore the expectations driven from the ligand design studies might not be reflected in experimentally determined properties.

3. Experimental

3.1. General

All of the compounds, for which analytical and spectroscopic data are given, were homogeneous by TLC. TLC analyses were performed using silica gel plates (E. Merck silica gel 60 F-254) and components were visualized by the following methods: ultraviolet light absorbance, charring after spraying with a solution of $(NH_4)HSO_4$ or after spraying with ninhydrin solution. Column chromatography was carried out on silica gel (E. Merck, 70–230 mesh). HPLC analyses were carried out on: (i) a MZ-Analytical Column 250 × 4 mm, Kromasil, 100, C18, 5 μm, at a flow rate of 0.5 mL/min or (ii) a semipreparative 250 × 8 mm, Kromasil, C18, 5 μm at a flow rate 3 mL/min. Solvent A: 10% CH_3CN, 90% H_2O, 0.1% TFA. Solvent B: 90% CH_3CN, 10% H_2O, 0.09% TFA. The following gradients were used: (1) $t = 0$ min (0% B), $t = 29$ min (20% B), $t = 30$ min (50% B), $t = 32$ min (100% B), $t = 34$ min (100% B), $t = 38$ (40 % B) for compounds **6** and **7**. (2) $t = 0$ min (0% B), $t = 27$ min (5% B), $t = 30$ min (50% B), $t = 32$ min (100% B), $t = 34$ (100% B), $t = 38$ min (40% B) for compound **8**. (3) $t = 0$ min (0% B), $t = 20$ min (35% B), $t = 25$ min (60% B), $t = 32$ min (100% B), $t = 34$ (100% B), $t = 38$ min (40% B) for compounds **3** and **4**. (4) $t = 0$ min (0% B), $t = 20$ min (20% B), $t = 35$ min (30% B), $t = 37$ min (100% B), $t = 39$ (100% B), $t = 41$ min (40% B) for compound **5**. Eluted peaks were detected at 254 nm. Given times (where more than one) correspond to two pairs of diastereoisomers and are counted in minutes.

All the compounds were characterized by ^1H, ^{13}C, and ^{31}P NMR spectroscopy. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a 200 MHz Mercury Varian spectrometer. ^{13}C and ^{31}P NMR spectra are fully proton decoupled. ^{31}P NMR chemical shifts are reported on δ scale (in ppm) downfield from 85% H_3PO_4.

ESI mass spectral analysis was performed on a mass sprectrometer MSQ Surveyor, Finnigan, at the Laboratory of Organic Chemistry, University of Athens, using direct sample injection. Negative or positive ion ESI spectra were acquired by adjusting the needle and cone voltages accordingly.

Commercially available reagents, solvents, and starting materials were purchased from Aldrich, Merck, Sigma, Labscan, and Fluka, and used without further purification. THF was distilled over NaH.

3.2. Docking

Compounds described in this paper were docked by applying the same procedure as we previously used to calculate the binding mode of the phosphinic dipeptide

analogues.[27] The X-ray structure of LAP-LeuP (1lcp in PDB) served to design dipeptide analogues described in Ref. 27, including compounds **1** and **2** (Fig. 1). Based on the calculated binding mode of **1**[27] new substituents were introduced at the *para* position of the phenyl ring at P1′ portion using the fragment library from the InsightII/Builder program. In addition the β carbon atom at the P1′ residue was replaced by –NH– moiety, which resulted in compounds **4** and **5**. These were further optimized within the enzyme binding site using the AMBER force field in Discover/InsightII program.[27,53] The backbone of the protein was frozen during optimization due to lack of the structural changes of the enzyme upon ligand binding observed in different X-ray structures of LAP. The distance constrains between zinc ions and their ligands with the force constants values of 500 kcal/mol × Å^2 were applied during minimization procedure. The optimization was performed using the conjugate gradient algorithm until the maximum derivative was <0.1 kcal/mol × Å.

Compounds **6**, **7**, and **8** were obtained by introducing the methyl group into the side chain of **3**, **4**, and **5**, respectively.

3.3. Preparation of synthons 13 and 14 for cross-coupling and alkylation

3.3.1. 2-Benzyloxycarbonylamino-3-[(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)hydroxyphosphinoyl]propionic acid methyl ester (17). A suspension of compound **16** (2.46 g, 8.0 mmol) in hexamethyldisilazane (5 mL, 24.0 mmol) was heated at 110 °C for 1 h under argon atmosphere. After cooling to 90 °C, acrylate **15** (2.08 g, 8.80 mmol) was added dropwise over 0.5 h and the reaction mixture was allowed to cool to ca. 65 °C, and MeOH (5 mL) was added dropwise. After cooling to room temperature, the volatile products were removed in vacuo and the residue was dissolved in a mixture AcOEt/Et_2O 1:1 and treated with 1 M HCl and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by column chromatography using CH_2Cl_2/MeOH/CH_3COOH 9:0.1:0.1 to 9:0.5:0.2 as the eluent to afford the compound **17** as a white solid. Yield: 77% (3.3 g), mp 85–90 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.42 (s, 9H, C(C*H*$_3$)$_3$), 1.62–1.90 (m, 2H, PCHCH*H*, PCH*H*), 1.98–2.29 (m, 3H, PCHC*H*H, PC*H*H, C*H*CO), 2.36–2.79 (m, 2H, C*H*$_2$Ph), 3.54 (s, 3H, OC*H*$_3$), 3.67–3.81 (m, 1H, PC*H*), 4.62–4.87 (s, 1H, PCHN*H*), 5.09 (s, 2H, PhC*H*$_2$O), 5.30–5.47 (s, 1H, CH_2CHN*H*), 6.96–7.45 (m, 10H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.3 (C(*C*H$_3$)$_3$), 28.4 (PCH*C*H$_2$), 29.2 (P*C*H$_2$), 32.1 (*C*H$_2$Ph), 48.7 (d, $^1J_{PC}$ = 114.5 Hz, P*C*H), 49.6 (*C*HCO), 52.5 (COO*C*H$_3$), 66.9 (Ph*C*H$_2$O), 80.1 (*C*(CH$_3$)$_3$), 125.8, 127.9, 128.3, 136.3, 141.0 (aryl), 156.2 (*C*ONH), 156.4 (*C*OOCH$_2$Ph), 172.4 (d, $^3J_{PC}$ = 11.5 Hz, *C*OOCH$_3$); ^{31}P (81 MHz, $CDCl_3$) δ 44.4 (br); ESMS *m*/*z* calcd for $C_{26}H_{35}N_2O_8P$ (M+H)$^+$ 535.5, found 535.3.

3.3.2. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-benzyloxycarbonylaminopropionic acid methyl ester (18). Compound **17** (2.81 g, 5.20 mmol) and 1-adamantylbromide (1.2 g, 6.20 mmol) were dissolved in $CHCl_3$ (15 mL) and the reaction mixture brought to reflux. Then, silver oxide (1.2 g, 5.20 mmol) was added in five equal portions, over 50 min. This suspension was refluxed for additional 3 h. Then, chloroform was removed; the residue was treated with Et_2O and filtered through a pad of Celite. The filtrate was concentrated and the residue was purified by column chromatography using $CHCl_3$/isopropanol 9.8:0.2 as the eluent to give compound **18** as solid. Yield: 80% (2.77 g), mp 65–67 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.46 (s, 9 H, C(C*H*$_3$)$_3$), 1.51–1.77 (m, 8H, PCHCH*H*, PCH*H*, CHC*H*$_2$CH of Ad), 1.97–2.45 (m, 12H, PCHC*H*H, PC*H*H, C*H*CO, CC*H*$_2$ of Ad, C*H* of Ad), 2.58–2.92 (m, 2H, C*H*$_2$Ph), 3.74 (s, 3H, OC*H*$_3$), 3.84–4.10 (m, 1H, PC*H*), 4.86–5.0 (d, $^3J_{HH}$ = 14 Hz, 1H, PCHN*H*), 5.13 (s, 2H, PhC*H*$_2$O), 5.97–6.09 (d, $^3J_{HH}$ = 12 Hz, 1H, CH_2CHN*H*), 7.10–7.45 (m, 10H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.0 (C(*C*H$_3$)$_3$), 29.7 (PCH*C*H$_2$), 30.3 (P*C*H$_2$), 30.8 (*C*H of Ad), 31.8 (d, $^3J_{PC}$ = 16 Hz, *C*H$_2$Ph), 35.2 (CH*C*H$_2$CH of Ad), 43.8 (C*C*H$_2$ of Ad), 48.6 (P*C*H), 49.2 (*C*HCO), 52.2 (COO*C*H$_3$), 66.5 (Ph*C*H$_2$O), 80.1 (*C*(CH$_3$)$_3$), 83.7 (PO*C*), 125.6, 127.1, 127.7, 128.0, 129.0, 128.6, 129.0, 136.0, 140.8 (aryl), 155.8 (*C*ONH), 156.4 (*C*OOCH$_2$Ph), 171.5 (d, $^3J_{PC}$ = 12.9 Hz, *C*OOCH$_3$); ^{31}P (81 MHz, $CDCl_3$) δ 46.6, 47.9, 48.1, 48.5; ESMS *m*/*z* calcd for $C_{39}H_{49}N_2O_8P$ (M+H)$^+$ 669.8, found 669.3.

3.3.3. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-aminopropionic acid methyl ester (14). Compound **18** (0.23 g, 0.34 mmol) was dissolved in MeOH (15 mL) and H_2O (2 mL), and subjected to hydrogenolysis for 3.5 h in the presence of 10% Pd/$BaSO_4$ as catalyst. The catalyst was removed by filtration through a pad of Celite and the filtrate was concentrated to dryness. MeOH was added to the residue and concentrated to dryness. This procedure was repeated twice to afford product **14** as a colorless gum. The product was used immediately in the next step. Yield: 100% (0.18 g). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.52 (s, 9H, C(C*H*$_3$)$_3$), 1.57–1.82 (m, 8H, PCHCH*H*, PCH*H*, CHC*H*$_2$CH of Ad), 1.91–2.38 (m, 12H, PCHC*H*H, PC*H*H, C*H*CO, CC*H*$_2$ of Ad, C*H* of Ad), 2.56–3.00 (m, 2H, C*H*$_2$Ph), 3.77 (s, 3H, OC*H*$_3$), 3.86–4.29 (m, 1H, PC*H*), 5.15–5.25 (d, $^3J_{HH}$ = 10 Hz, 1H, PCHN*H*), 6.18–6.35 (d, $^3J_{HH}$ = 10.6 Hz, 1H, CH_2CHN*H*), 7.15–7.40 (m, 5H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.0 (C(*C*H$_3$)$_3$), 30.3 (PCH*C*H$_2$), 30.5 (P*C*H$_2$), 30.8 (*C*H of Ad), 32.1 (d, $^3J_{PC}$ = 5 Hz, *C*H$_2$Ph), 35.3 (CH*C*H$_2$CH of Ad), 43.9 (C*C*H$_2$ of Ad), 49.0 (P*C*H), 51.8 (*C*HCO), 52.1 (COO*C*H$_3$), 79.3 (*C*(CH$_3$)$_3$), 83.1 (PO*C*), 125.6, 128.0, 128.1, 128.2, 129.0, 128.6, 129.0, 141.0 (aryl), 155.7 (*C*ONH), 173.9 (*C*OOCH$_3$); ^{31}P (81 MHz, $CDCl_3$) δ 46.2, 46.6, 48.1, 48.6, 49.3, 49.5.

3.3.4. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-aminopropionic acid methyl ester (13). Compound **18** (0.67 g, 1 mmol) was dissolved in MeOH, and 4 M NaOH (2 mmol) was added dropwise. The final concentration of the NaOH was 0.4 M. The mixture was stirred at room tem-

perature for 16 h. Then, methanol was removed at reduced pressure and the residue was diluted with water and acidified with 0.5 M HCl to pH 1 in an ice water bath. This aqueous solution was extracted with AcOEt, the organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo to give the crude product, which was purified by silica column chromatography using CH_2Cl_2/methanol 9.5:0.5 as the eluent to afford the acids as colorless gum. Yield: 92%. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.40 (s, 9H, C(CH_3)$_3$), 1.45–1.75 (m, 8H, PCHCH*H*, PCH*H*, CHCH_2CH of Ad), 1.80–2.22 (m, 12H, PCHC*H*H, PC*H*H, C*H*CO, CCH_2 of Ad, C*H* of Ad), 2.38–2.93 (m, 2H, CH_2Ph), 4.46–4.68 (m, 1H, PC*H*), 5.05 (s, 2H, PhCH_2O), 6.21 (d, $^3J_{HH}$ = 11 Hz, 1H, CH_2CHN*H*), 6.30 (PCHN*H*), 6.95–7.46 (m, 10H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.2 (C(*C*H_3)$_3$), 30.5 (PCH*C*H_2), 31.0 (P*C*H_2), 31.1 (*C*H of Ad), 32.0 (*C*H_2Ph), 35.4 (CH*C*H_2CH of Ad), 43.9 (C*C*H_2 of Ad), 49.1 (P*C*H), 49.7 (*C*HCO), 66.8 (Ph*C*H_2O), 79.6 (*C*(CH_3)$_3$), 84.8 (PO*C*), 125.9, 127.3, 128.0, 128.3, 128.4, 136.0, 141.0 (aryl), 155.6 (*C*ONH), 156.0 (*C*OOCH_2Ph), 172.4 (*C*OOCH_3); ^{31}P (81 MHz, $CDCl_3$) δ 48.3, 49.5, 49.7, 50.5. Subsequent hydrogenation starting from the same quantity (0.22 g, 0.34 mmol) and using the same procedure as described above for compound **14** gave the analogue **13**. Yield: 100% (0.18 g), mp 95–97 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.52 (s, 9H, C(CH_3)$_3$), 1.56–1.88 (m, 8H, PCHCH*H*, PCH*H*, CHCH_2CH of Ad), 1.90–2.47 (m, 11H, PCHC*H*H, PC*H*H, CCH_2 of Ad, C*H* of Ad), 2.54–3.04 (m, 2H, CH_2Ph), 3.3–3.6 (m, 1H, C*H*CO), 3.79–4.30 (m, 1H, PC*H*), 5.16 (PCHN*H*), 7.27 (s, 5H, aryl), 8.32 (COO*H*); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.3 (C(*C*H_3)$_3$), 30.6 (PCH*C*H_2), 30.7 (P*C*H_2), 31.1 (*C*H of Ad), 32.0 (*C*H_2Ph), 35.5 (CH*C*H_2CH of Ad), 45.1 (C*C*H_2 of Ad), 49.2 (P*C*H), 68.1 (*C*HCO), 79.4 (*C*(CH_3)$_3$), 83.4 (PO*C*), 126.1, 128.3, 128.5, 140.9 (aryl), 155.9 (*C*ONH), 173.4 (*C*OOH); ^{31}P (81 MHz, $CDCl_3$) δ 49.7, 52.0, 53.5.

3.4. Cross-coupling with the use of the building block 13

3.4.1. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-phenylaminopropionic acid (20). General procedures. (A) Phosphinic compound **13** (55 mg, 0.11 mmol) was dissolved in DMSO (0.5 mL) and stirred under argon. Then, CuI (2 mg, 0.011 mmol), K_2CO_3 (30 mg, 0.22 mmol), and iodobenzene (12 μL, 0.11 mmol) were added at room temperature. The reaction mixture was heated at 65 °C for 48 h. The mixture was allowed to cool to room temperature. Et_2O and saturated solution of citric acid were added to the mixture. The organic layer was separated, washed with brine, and dried over Na_2SO_4. The solvent was removed in vacuo to yield the crude product that was purified by column chromatography using CH_2Cl_2/MeOH 10:0 to 9.8:0.2 as the eluent. The product **20** was obtained as deep yellow oil. Yield: 22% (15 mg).

(B) Phosphinic compound **13** (76 mg, 0.15 mmol) was dissolved in DMSO (0.5 mL) and stirred under argon. Then, CuI (3 mg, 0.015 mmol), K_2CO_3 (41.5 mg, 0.30 mmol), L-proline (3.4 mg, 0.03 mmol), and iodobenzene (17 μL, 0.15 mmol) were added at room temperature. The reaction mixture was heated at 65 °C for 48 h. Compound **20** was obtained following the previously described work-up. Yield: 22% (20 mg).

(C) Phosphinic compound **13** (50 mg, 0.10 mmol) was dissolved in 2-propanol (0.5 mL) and stirred under argon. Then, CuI (1.9 mg, 0 01 mmol), K_2CO_3 (28 mg, 0.2 mmol), ethylene glycol (11 μL, 0.10 mmol), and iodobenzene (11 μL, 0.10 mmol) were added at room temperature. The reaction mixture was heated at 65 °C for 48 h. Compound **20** was obtained following the previously described work-up. Yield: 21% (12.5 mg).

^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27 (s, 9H, C(CH_3)$_3$), 1.37–1.9 (m, 8H, PCHCH*H*, PCH*H*, CHCH_2CH of Ad), 1.94–2.27 (m, 11H, PCHC*H*H, PC*H*H, CCH_2 of Ad, C*H* of Ad), 2.58–2.95 (m, 2H, CH_2Ph), 3.41–3.61 (m, 1H, C*H*CO), 3.79–4.30 (m, 1H, PC*H*), 5.69 (PCHN*H*), 7.13–7.37, 7.45–7.62, 7.69–7.82 (m, 10H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 29.3 (P*C*H_2), 29.7 (C(*C*H_3)$_3$), 30.7 (PCH*C*H_2), 31.2 (*C*H of Ad), 31.9 (*C*H_2Ph), 35.5 (CH*C*H_2CH of Ad), 44.1 (C*C*H_2 of Ad), 49.2 (P*C*H), 65.6 (*C*HCO), 79.4 (*C*(CH_3)$_3$), 83.4 (PO*C*), 117.2, 126.0, 128.4, 128.8, 130.9, 140.5, 141.0 (aryl), 155.9 (*C*ONH), 173.4 (*C*OOH); ^{31}P (81 MHz, $CDCl_3$) δ 42.8, 43.6.

3.4.2. 4-Iodophenylmethanol (22). To a stirred solution of 4-iodobenzoic acid **21** (0.99 g, 4 mmol) in THF (20 mL) at −10 °C, *N*-methylmorpholine (0.40 g, 4 mmol) was added, followed by isobutyl chloroformate (0.55 g, 4 mmol). After 10 min, $NaBH_4$ (0.45 g, 12 mmol) was added in one portion. MeOH (40 mL) is then added dropwise to the mixture over a period of 10 min at 0 °C. The solution is stirred for additional 10 min, and then neutralized with 1 N HCl (8 mL). The organic solvents are evaporated under reduced pressure and the product is extracted with diethyl ether. The organic phase is washed with 1 N HCl, 5% $NaHCO_3$, H_2O, dried over Na_2SO_4, and the solvent is evaporated under reduced pressure. The residue was purified by column chromatography using diethyl ether/light petroleum ether 8:2 as the eluent to give **22** as white solid. Yield: 70% (0.65 g), mp 72–73 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.77 (br s, 1H, CH_2O*H*), 4.64 (s, 2H, CH_2OH), 7.09–7.70 (dd, 4H, *J* = 8.1 Hz, aryl). ^{13}C NMR (50 MHz, $CDCl_3$) δ 68.5 (CH_2OH), 96.2 (CI), 128.9, 137.5, 139.8 (aryl).

3.4.3. 1-Bromomethyl-4-iodobenzene (23). PBr_3 (0.19 mL, 2 mmol) was added to an ice-salt cooled solution of **22** (0.47 g, 2 mmol) in diethyl ether (10 mL) and the reaction mixture was stirred at room temperature for 3 h. Then it was cooled with ice and quenched with water. The organic phase was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified by column chromatography using light petroleum ether as the eluent to give **23** as white solid. Yield: 58% (0.34 g), mp 78–79 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.41 (s, 2H, CH_2Br), 7.10-7.70 (dd, 4H, *J* = 8.1 Hz, aryl).

^{13}C NMR (50 MHz, $CDCl_3$) δ 32.7 (CH_2Br), 94.4 (CI), 131.1, 137.6, 138.2 (aryl).

3.4.4. 4-[(*N*-*tert*-Butyloxycarbonylamino)methyl]iodobenzene (24). A solution of *p*-iodobenzyl bromide **23** (0.39 g, 1.01 mmol) in 28% aqueous NH_4OH (100 mL) was stirred at room temperature for 5 days. The reaction mixture was extracted with CH_2Cl_2, and the extract was washed with brine, dried (NaOH), and concentrated to give the amino derivative, which was dissolved in CH_2Cl_2 (5 mL). After cooling with ice, di-*tert*-butyl dicarbonate (0.33 g 1.50 mmol) was added and the mixture was stirred at room temperature for 15 h. The solution was diluted with dichloromethane, washed with H_2O, dried over Na_2SO_4, and evaporated under vacuum. The residue was purified by column chromatography using diethyl ether/light petroleum ether 1:1 as the eluent to give **24** as white solid. Yield: 72% (0.24 g), mp 88–89 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.43 (s, 9H, C(CH_3)$_3$), 4.21 (s, 2H, CH_2), 4.85 (br s, 1H, N*H*), 6.99–7.65 (dd, 4H, J = 8.1 Hz, aryl). ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.6 (CH_3)$_3$, 44.3 (CH_2NH), 79.6 (*C*(CH_3)$_3$), 92.8 (CI), 129.5, 137.8, 138.9 (aryl), 156.0 (CO).

3.4.5. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-(4-hydroxymethylphenylamino)propionic acid (25). Compound **25** was obtained from **13** and **22** following the method A. Yield: 10%. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27 (s, 9H, C(CH_3)$_3$), 1.37–1.9 (m, 9H, PCHCH*H*, PCH*H*, CHCH_2CH of Ad, O*H*), 1.94–2.27 (m, 11H, PCHC*H*H, PC*H*H, CCH_2 of Ad, C*H* of Ad), 2.58–2.95 (m, 2H, CH_2Ph), 3.41–3.61 (m, 1H, C*H*CO), 3.79–4.30 (m, 1H, PC*H*), 4.69 (m, 2H, CH_2OH), 5.69 (PCHN*H*), 7.13–7.37, 7.45–7.62, 7.69–7.82 (m, 9H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 29.3 (P*C*H$_2$), 29.7 (C(*C*H$_3$)$_3$), 30.7 (PCH*C*H$_2$), 31.2 (*C*H of Ad), 31.9 (*C*H$_2$Ph), 35.5 (CH*C*H$_2$CH of Ad), 44.1 (C*C*H$_2$ of Ad), 49.2 (P*C*H), 65.6 (*C*HCO), 68.5 (*C*H$_2$OH), 79.4 (*C*(CH$_3$)$_3$), 83.4 (PO*C*), 117.2, 126.0, 128.4, 128.8, 130.9, 140.5, 141.0 (aryl), 155.9 (*C*ONH), 173.4 (*C*OOH); ^{31}P (81 MHz, $CDCl_3$) δ 43.6, 45.6, 46.7.

3.4.6. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-[4-(*tert*-butyloxycarbonylaminomethyl)phenylamino]propionic acid (26). Compound **26** was obtained from **13** and **24** following the method A. Yield: 8%. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27 (s, 18H, C(CH_3)$_3$), 1.37–1.9 (m, 8H, PCHCH*H*, PCH*H*, CHCH_2CH of Ad), 1.94–2.27 (m, 11H, PCHC*H*H, PC*H*H, CCH_2 of Ad, C*H* of Ad), 2.58–2.95 (m, 2H, CH_2Ph), 3.41–3.61 (m, 1H, C*H*CO), 3.79–4.30 (m, 1H, PC*H*), 4.27 (m, 2H, CH_2NHBoc), 5.69 (PCHN*H*), 7.13–7.37, 7.45–7.62, 7.69–7.82 (m, 9H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 29.3 (P*C*H$_2$), 29.7 (C(*C*H$_3$)$_3$), 30.7 (PCH*C*H$_2$), 31.2 (*C*H of Ad), 31.9 (*C*H$_2$Ph), 35.5 (CH*C*H$_2$CH of Ad), 44.1 (C*C*H$_2$ of Ad), 44.38 (*C*H$_2$NHBoc) 49.2 (P*C*H), 65.6 (*C*HCO), 79.4 (*C*(CH$_3$)$_3$), 83.4 (PO*C*), 117.2, 126.0, 128.4, 128.8, 130.9, 140.5, 141.0 (aryl), 155.9 (*C*ONH), 173.4 (*C*OOH); ^{31}P (81 MHz, $CDCl_3$) δ 42.1, 43.9.

3.5. N-Alkylation of the building block 14

3.5.1. General procedure. To a stirred solution of compound **14** (535 mg, 1 mmol, 1 equiv) in anhydrous DMF (4 mL), cesium carbonate (164 mg, 0.5 mmol, 1 equiv) and the appropriate (non- or *p*-substituted) benzyl bromide (1 mmol, 1 equiv) were added. The reaction mixture was stirred at 45 °C for 6 h and then treated with H_2O and Et_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography using CH_2Cl_2/isopropanol 9.8:0.2 as the eluent to afford the desired product.

3.5.2. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-benzylaminopropionic acid methyl ester (28). Yield: 80% ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.29 (s, 9H, C(CH_3)$_3$), 1.44–1.72 (m, 8H, PCHCH*H*, PCH*H*, CHCH_2CH of Ad), 1.87–2.28 (m, 12H, PCHC*H*H, PC*H*H, C*H*CO, CCH_2 of Ad, C*H* of Ad), 2.51–2.90 (m, 2H, CH_2Ph), 3.72 (s, 3H, OCH_3), 3.74 (s, 2H, NHCH_2), 3.86–4.10 (m, 1H, PC*H*), 4.95 (d, $^3J_{HH}$ = 10.6 Hz, 1H, PCHN*H*), 6.05 (d, $^3J_{HH}$ = 10.4 Hz, 1H, CH$_2$CHN*H*), 7.06–7.42 (m, 10H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.3 (C(*C*H$_3$)$_3$), 30.6 (PCH*C*H$_2$), 30.7 (P*C*H$_2$), 31.1 (*C*H of Ad), 31.8 (*C*H$_2$Ph), 35.6 (CH*C*H$_2$CH of Ad), 44.2 (C*C*H$_2$ of Ad), 52.1 (P*C*H), 52.2 (*C*HCO), 52.4 (COO*C*H$_3$), 52.6 (NH*C*H$_2$), 79.3 (*C*(CH$_3$)$_3$), 83.8 (PO*C*), 125.9, 127.2, 128.3, 128.4, 129.0, 141.0 (aryl), 155.7 (*C*ONH), 173.9 (*C*OOCH$_3$); ^{31}P (81 MHz, $CDCl_3$) δ 45.8, 46.7, 47.5, 48.1, 48.4.

3.5.3. Methyl 4-bromomethylbenzoate (31). To a solution of 4-bromomethylbenzoic acid **30** (0.5 g, 2.30 mmol) in MeOH (5.6 mL), concentrated sulfuric acid (0.14 mL) was added. The resulting mixture was refluxed for 5 h. Then, the mixture was cooled to room temperature and evaporated *in vacuo*. H_2O (20 mL) was added to the residue in an ice-water bath, and the resulting solid was filtered and washed with cold water. The solid material was partitioned between Et_2O/AcOEt 1:1 and Na_2CO_3. The organic layer was dried over Na_2SO_4, and evaporated in vacuo to afford the product as pale yellow oil. Yield: 93% (0.49 g). ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.82 (s, 3H, CH_3), 4.51 (s, 2H, CH_2Br), 7.33–7.95 (dd, 4H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 45.0 (*C*H$_2$Br), 51.8 (*C*H$_3$), 126.8, 128.2, 128.7, 129.3, 129.6 (aryl), 142.0 (*C*CH$_2$Br), 166.1 (*C*O).

3.5.4. 4-Bromomethylphenylmethanol (32). To an ice-cooled solution of 1 M DIBALH in hexane (5.4 ml, 5.40 mmol), toluene (1.2 mL) was introduced under argon atmosphere. A solution of **31** (0.49 g, 2.1 mmol) in toluene (5 ml) was then added dropwise and the mixture was stirred at 0 °C for 4 h. The reaction mixture was quenched using toluene/methanol 1:1 (5 mL), followed by 2 M hydrochloric acid solution (5 mL). The solid aluminum salts were filtered, the organic layer was separated, and the aqueous layer was extracted with diethyl ether. The organic layer was dried over Na_2SO_4 and evaporated in vacuo. A mixture of diethyl ether/light petroleum ether 1:1 was added to the white solid

residue. The precipitate was filtered and washed with petroleum ether. Yield: 58% (0.24 g), mp 76–77 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.67 (br s, 1H, O*H*), 4.50 (s, 2H, CH_2Br), 4.69 (s, 2H, CH_2OH), 7.32–7.42 (m, 4H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 33.3 (*C*H_2Br), 64.4 (*C*H_2OH), 126.9, 127.1, 128.2, 129.1 (aryl), 136.8 (*C*CH_2Br), 141.0 (*C*CH_2OH).

3.5.5. 1-Bromomethyl-4-*tert*-butyloxymethylbenzene (33). To an ice-cooled solution of **32** (0.15 g, 0.75 mmol) in CH_2Cl_2 (5 mL), a catalytic amount of concentrated sulfuric acid was added. Then, a large excess of liquid isobutene (20 mL) was added and the mixture was stirred at room temperature for 64 h. After careful removal of isobutene, the reaction mixture was partitioned between CH_2Cl_2 and Na_2CO_3, the organic layer was washed with H_2O and brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting colorless oil was purified by column chromatography using light petroleum ether/diethyl ether 5:1 as the eluent to afford the product as colorless oil. Yield: 63% (0.12 g). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.30 (s, 9H, C(*CH*$_3$)$_3$), 4.45 (s, 2H, C*H*$_2$Br), 4.50 (s, 2H, C*H*$_2$O), 7.30–7.35 (m, 4H, aryl);^{13}C NMR (50 MHz, $CDCl_3$) δ 27.6 (C(*C*H$_3$)$_3$), 33.5 (*C*H$_2$Br), 63.6 (*C*H$_2$O), 73.4 (*C*(CH$_3$)$_3$), 127.6, 128.9 (aryl), 136.5 (*C*CH$_2$Br), 140.2 (*C*CH$_2$O).

3.5.6. 4-Hydroxymethylbenzonitrile (35). 4-Bromomethylbenzonitrile **34** (1.4 g, 7.14 mmol) was refluxed in deionized water (43 mL) with barium carbonate (2.8 g, 14.20 mmol) for 4 h. Then, the mixture was cooled to room temperature, and the solid byproduct was removed by filtration. The filtrate was extracted with CH_2Cl_2 twice. The organic phases were combined, dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by column chromatography using light petroleum ether/diethyl ether 2:3 as the eluent to afford **35** as white solid. Yield: 89 % (0.85 g), mp 49–50 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.68 (s, 1H, O*H*), 4.67 (s, 2H, C*H*$_2$), 7.36–7.60 (dd, 4H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 63.5 (*C*H$_2$), 110.3 (*C*CN), 118.7 (*C*N), 126.8, 132.0 (aryl), 146.5 (*C*CH$_2$OH).

3.5.7. 4-Bromomethylbenzylamine hydrobromide (36). *p*-Cyanobenzyl alcohol **35** (0.42 g, 3.15 mmol) was dissolved in dry THF (8 mL) and was added dropwise to $LiAlH_4$ (0.30 g, 7.88 mmol) in dry THF (8 mL) over 15 min at 0 °C under argon. After the end of the addition, the reaction mixture was refluxed for 4 h. Then, the solvent was removed in vacuo and 1 M NaOH (10 mL) was added dropwise at 0 °C. The resulting mixture was treated with AcOEt (3 × 30 mL), the organic layers were combined and extracted with 1 M HCl (2 × 70 mL), and the solvent was removed using rotary evaporation. The residue was dissolved in H_2O (3 mL) and the solution was neutralized with 1 M NaOH (5 mL). The solution was extracted with AcOEt (5 × 30 mL), the combined organic phases were dried over Na_2SO_4 and concentrated in vacuo to yield the α-amino-α′-hydroxy-*p*-xylene as a pale yellow solid. This solid was dissolved in H_2O (3 mL) and HBr 46% (4.6 mL) was added. The resulting solution was refluxed for 3.5 h. Solvent was removed under reduced pressure and the residue was triturated with acetone to yield the product as a pale yellow solid. Yield: 15% (0.15 g), mp 250-252 °C. ^{1}H NMR (200 MHz, d_6-DMSO) δ 3.98 (m, 2H, C*H*$_2$NH$_3$Br), 4.68 (C*H*$_2$Br), 7.31–7.44 (m, 4H, aryl), 7.97–8.21 (br s, 3H, N*H*$_3$); ^{13}C NMR (50 MHz, d_6-DMSO) δ 34.0 (*C*H$_2$Br), 41.9 (*C*H$_2$NH$_3$Br), 129.3, 129.5 (aryl), 134.0 (*C*CH$_2$Br), 138.4 (*C*CH$_2$N).

3.5.8. (4-Bromomethylbenzyl)carbamic acid *tert*-butyl ester (37). To a solution of **36** (85 mg, 0.30 mmol) in dioxane (2.5 mL) and H_2O (2.5 mL), Boc_2O (1.32 g, 6.0 mmol) was added at 0 °C. Then, $NaHCO_3$ (29.4 mg, 0.35 mmol) was added at this temperature and the resulting mixture was stirred for 18 h at room temperature. The mixture was partitioned between Et_2O and H_2O. The aqueous phase was extracted with Et_2O and the combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography using light petroleum ether/diethyl ether 3:2 as the eluent to afford the product as colorless oil. Yield: 99% (90 mg). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.49 (s, 9H, C(C*H*$_3$)$_3$), 4.27 (s, 2H, C*H*$_2$NH), 4.46 (s, 2H, C*H*$_2$Br), 4.93 (br s, 1H, N*H*), 7.19–7.34 (dd, 4H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.3 (C(*C*H$_3$)$_3$), 33.2 (*C*H$_2$NH), 44.2 (*C*H$_2$Br), 79.5 (*C*(CH$_3$)$_3$), 127.7, 129.2 (aryl), 136.7 (*C*CH$_2$N), 139.3 (*C*CH$_2$Br), 155.8 (*C*O).

3.5.9. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-(4-*tert*-butyloxymethylbenzylamino)propionic acid methyl ester (39). Yield: 65%. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.24 (s, 9H, CH$_2$OC(C*H*$_3$)$_3$), 1.40 (s, 9H, OCOC(C*H*$_3$)$_3$), 1.44–1.74 (m, 8H, PCHCH*H*, PCH*H*, CHC*H*$_2$CH of Ad), 1.86–2.47 (m, 12H, PCHC*H*H, PC*H*H, C*H*CO, CC*H*$_2$ of Ad, C*H* of Ad), 2.57–2.80 (m, 2H, C*H*$_2$Ph), 3.69 (s, 3H, OC*H*$_3$), 3.72 (s, 2H, NHC*H*$_2$), 3.86–4.10 (m, 1H, PC*H*), 4.40 (s, 2H, C*H*$_2$O), 5.18 (d, $^3J_{HH}$ = 7.8 Hz, 1H, PCHN*H*), 6.22 (d, $^3J_{HH}$ = 9.4 Hz, 1H, CH$_2$CHN*H*), 7.28–7.38 (m, 9H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 27.5 (CH$_2$OC(*C*H$_3$)$_3$), 28.2 (OCOC(*C*H$_3$)$_3$), 29.5 (PCH*C*H$_2$), 30.5 (P*C*H$_2$), 31.0 (*C*H of Ad), 32.3 (*C*H$_2$Ph), 35.5 (CH*C*H$_2$CH of Ad), 44.0 (C*C*H$_2$ of Ad), 49.2 (P*C*H), 50.0 (*C*HCO), 52.1 (COO*C*H$_3$), 55.3 (NH*C*H$_2$), 63.7 (*C*H$_2$O), 73.2 (CH$_2$O*C*(CH$_3$)$_3$), 79.4 (*C*(CH$_3$)$_3$), 83.5 (PO*C*), 125.8, 126.7, 127.2, 127.4, 128.2, 128.3, 137.4, 138.9, 141.3 (aryl), 155.7 (*C*ONH), 174.4 (*C*OOCH$_3$); ^{31}P (81 MHz, $CDCl_3$) δ 46.1, 47.2, 47.6, 48.4, 48.6.

3.5.10. 3-[(Adamantan-1-yloxy)-(1-*tert*-butyloxycarbonylamino-3-phenylpropyl)phosphinoyl]-2-[4-(*tert*-butyloxycarbonylaminomethyl)benzylamino]propionic acid methyl ester (40). Yield: 69%. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.46 (s, 18H, C(C*H*$_3$)$_3$), 1.50–1.81 (m, 8H, PCHCH*H*, PCH*H*, CHC*H*$_2$CH of Ad), 1.86–2.30 (m, 12H, PCHC*H*H, PC*H*H, C*H*CO, CC*H*$_2$ of Ad, C*H* of Ad), 2.36–2.95 (m, 2H, C*H*$_2$Ph), 3.72 (s, 3H, C*H*$_3$), 3.74 (s, 2H, NHC*H*$_2$), 3.86–4.10 (m, 1H, PC*H*), 4.41 (s, 2H, C*H*$_2$NHBoc), 5.00 (BocN*H*), 5.98 (d, $^3J_{HH}$ = 10.6 Hz, 1H, CH$_2$CHN*H*), 7.08–7.47 (m, 9H, aryl); ^{13}C NMR (50 MHz, $CDCl_3$) δ 27.3 (C(*C*H$_3$)$_3$), 29.6 (PCH*C*H$_2$), 30.6 (P*C*H$_2$), 31.1 (*C*H of Ad), 32.2 (*C*H$_2$Ph), 35.5

(CH*C*H$_2$CH of Ad), 36.0 (*C*H$_2$NHBoc), 44.1 (C*C*H$_2$ of Ad), 51.3 (P*C*H), 51.8 (*C*HCO), 52.1 (COO*C*H$_3$), 55.3 (NH*C*H$_2$), 79.6 (*C*(CH$_3$)$_3$), 83.6 (PO*C*), 125.9, 127.5, 128.3, 137.7, 141.2 (aryl), 155.8 (*C*ONH), 173.9 (*C*OOCH$_3$); ^{31}P (81 MHz, CDCl$_3$) δ 46.1, 48.3, 48.7.

3.6. Deprotection

3.6.1. General procedure for removal of Boc, *t*-Bu, and Ad protecting group. Initially, in dipeptides **28**, **39**, and **40** the C-terminal methyl ester was removed by saponification in same conditions as for compound **18** to give **13**. Then, **20**, **25**, **26**, **28**, and **40** were treated with a mixture 50% TFA/CH$_2$Cl$_2$ while **39** with 90% TFA/CH$_2$Cl$_2$ and one drop of a scavenger (triisopropylsilane). The resulting solution was stirred for 3 h (50% TFA) or for 16 h (90% TFA) at room temperature. Then, the mixture was concentrated in vacuo. CH$_2$Cl$_2$ was added to the residue, and the solvent was removed under reduced pressure (3 times). A mixture of Et$_2$O/petroleum ether 1:1 was added to the residue. The white precipitate was filtered and washed with Et$_2$O. The product was purified with semi-preparative RP-HPLC using suitable gradient.

3.6.2. 3-[(1-Amino-3-phenylpropyl)hydroxyphosphinoyl]-2-phenylaminopropionic acid (3). HPLC: t_R = 21.1 and 21.4 min using gradient 3. ^{1}H NMR (200 MHz, D$_2$O) δ 1.75–2.25 (m, 4H, PCHCH*H*, PC*H*H, PCH*H*, PCHC*H*H), 2.57–2.80 (m, 2H, C*H*$_2$Ph), 2.85–2.95 (m, 1H, C*H*CO), 2.97–3.15 (m, 1H, PC*H*), 7.20–7.43 (m, 10H, aryl); ^{13}C NMR (50 MHz, D$_2$O) δ 27.5 (P*C*H$_2$), 31.0 (*C*H$_2$Ph), 38.8 (CH*C*H$_2$), 43.8 (P*C*H), 49.0 (*C*HCO), 110.8, 126.6, 128.7, 129.0, 129.5, 130.5, 140.6 (aryl), 195.7 (*C*OOH); ^{31}P (81 MHz, D$_2$O) δ 32.2, 34.4; ESMS *m*/*z* calcd for C$_{20}$H$_{27}$N$_2$O$_4$P (M−H)$^+$ 361.35, found 361.01.

3.6.3. 3-[(1-Amino-3-phenylpropyl)hydroxyphosphinoyl]-2-(4-hydroxymethylphenylamino)propionic acid (4). HPLC: t_R = 20.7 and 21.2 min using gradient 3. ^{1}H NMR (200 MHz, D$_2$O) δ 1.75–2.25 (m, 4H, PCHCH*H*, PC*H*H, PCH*H*, PCHC*H*H), 2.57–2.80 (m, 2H, C*H*$_2$Ph), 2.85–2.95 (m, 1H, C*H*CO), 2.97–3.15 (m, 1H, PC*H*), 3.45–3.60 (m, 2H, C*H*$_2$OH), 7.20–7.43 (m, 9H, aryl); ^{13}C NMR (50 MHz, D$_2$O) δ 27.5 (P*C*H$_2$), 31.0 (*C*H$_2$Ph), 38.8 (CH*C*H$_2$), 43.8 (P*C*H), 49.0 (*C*HCO), 68.0 (*C*H$_2$OH), 110.8, 126.6, 128.7, 129.0, 129.5, 130.5, 140.6 (aryl), 195.7 (*C*OOH); ^{31}P (81 MHz, D$_2$O) δ 34.2, 35.9; ESMS *m*/*z* calcd for C$_{21}$H$_{29}$N$_2$O$_5$P (M−H)$^+$ 391.15, found 390.01.

3.6.4. 2-(4-Aminomethylphenylamino)-3-[(1-amino-3-phenylpropyl)hydroxyphosphinoyl]propionic acid (5). HPLC: t_R = 30.2 and 31.0 min using gradient 4. ^{1}H NMR (200 MHz, D$_2$O) δ 1.75–2.25 (m, 4H, PCHCH*H*, PC*H*H, PCH*H*, PCHC*H*H), 2.57–2.80 (m, 2H, C*H*$_2$Ph), 2.85–2.95 (m, 1H, C*H*CO), 2.97–3.15 (m, 1H, PC*H*), 3.44–3.60(m, 2H, C*H*$_2$NH$_2$), 7.20–7.43 (m, 9H, aryl); ^{13}C NMR (50 MHz, D$_2$O) δ 27.5 (P*C*H$_2$), 31.0 (*C*H$_2$Ph), 38.8 (CH*C*H$_2$), 43.8 (P*C*H), 46.3 (CH$_2$NH$_2$), 49.0 (*C*HCO), 110.8, 126.6, 128.7, 129.0, 129.5, 130.5, 140.6 (aryl), 195.7 (*C*OOH); ^{31}P (81 MHz, D$_2$O) δ 32.2, 34.4; ESMS *m*/*z* calcd for C$_{20}$H$_{27}$N$_2$O$_4$P (M+H)$^+$ 391.40, found 391.18.

3.6.5. 3-[(1-Amino-3-phenylpropyl)hydroxyphosphinoyl]-2-benzylaminopropionic acid (6). HPLC: t_R = 26.9 and 28.7 min using gradient 1. ^{1}H NMR (200 MHz, D$_2$O) δ 1.80–2.48 (m, 4H, PCHCH*H*, PCH*H*, PCHC*H*H, PC*H*H), 2.55–2.93 (m, 2H, C*H*$_2$Ph), 3.06–3.29 (m, 1H, C*H*CO), 3.53–3.96 (m, 1H, PC*H*), 4.08–4.51 (m, 2H, NHC*H*$_2$), 7.12–7.53 (m, 10H, aryl); ^{13}C NMR (50 MHz, D$_2$O) δ 27.5 (d, $^1J_{PC}$ = 89 Hz, P*C*H$_2$), 31.7 (d, $^2J_{PC}$ = 8 Hz, *C*H$_2$Ph), 38.8 (CH*C*H$_2$), 43.7 (P*C*H), 48.4 (*C*HCO), 50.3 (NH*C*H$_2$), 126.8, 128.6, 129.0, 129.5, 130.0, 130.1, 130.5, 140.6 (aryl), 195.7 (*C*OOH); ^{31}P (81 MHz, D$_2$O) δ 31.6,39.2; ESMS *m*/*z* calcd for C$_{19}$H$_{24}$N$_2$O$_4$P (M−H)$^+$ 375.38, found 375.26.

3.6.6. 3-[(1-Amino-3-phenylpropyl)hydroxyphosphinoyl]-2-(4-hydroxymethylbenzylamino)propionic acid (7). HPLC: t_R = 17.4 and 18.9 min using gradient 1. ^{1}H NMR (200 MHz, D$_2$O) δ 1.86–2.22 (m, 2H, PCHCH*H*, PCH*H*), 2.26–2.48 (m, 2H, PCHC*H*H, PC*H*H), 2.60–2.90 (m, 2H, C*H*$_2$Ph), 3.06–3.27 (m, 1H, C*H*CO), 3.83–4.04 (m, 1H, PC*H*), 4.14–4.44 (m, 2H, NHC*H*$_2$), 4.52 (s, 2H, C*H*$_2$OH), 7.16–7.50 (m, 9H, aryl); ^{13}C NMR (50 MHz, D$_2$O) δ 27.3 (d, $^1J_{PC}$ = 128 Hz, P*C*H$_2$), 30.3 (*C*H$_2$Ph), 31.6 (d, $^2J_{PC}$ = 8.35 Hz, CH*C*H$_2$), 49.1 (P*C*H), 50.0 (NH*C*H$_2$), 51.0 (*C*HCO), 63.4 (*C*H$_2$OH) 126.7, 128.2, 128.6, 129.0, 129.5, 129.8, 130.3, 140.3, 142.0 (aryl), 195.7 (*C*OOH); ^{31}P (81 MHz, D$_2$O) δ 30.8, 31.3; ESMS *m*/*z* calcd for C$_{20}$H$_{26}$N$_2$O$_5$P (M−H)$^+$ 405.4, found 405.3.

3.6.7. 2-(4-Aminomethylbenzylamino)-3-[(1-amino-3-phenylpropyl)hydroxyphosphinoyl]propionic acid (8). HPLC: t_R = 14.9 and 16.1 min using gradient 2. ^{1}H NMR (200 MHz, D$_2$O) δ 1.81–2.22 (m, 4H, PCHCH*H*, PCH*H*, PCHC*H*H, PC*H*H), 2.24–2.48 (m, 2H, C*H*$_2$Ph), 2.56–2.89 (m, 1H, C*H*CO), 3.10–3.30 (m, 1H, PC*H*), 4.0 (s, 2H, C*H*$_2$NH$_2$), 4.21–4.48 (m, 2H, NHC*H*$_2$), 6.94–7.47 (m, 9H, aryl); ^{13}C NMR (50 MHz, D$_2$O) δ 27.3 (d, $^1J_{PC}$ = 100 Hz, P*C*H$_2$), 29.3 (*C*H$_2$Ph), 31.8 (CH*C*H$_2$), 36.9 (P*C*H), 42.6 (*C*HCO), 49.7 (NH*C*H$_2$), 50.3 (*C*H$_2$NH$_2$) 126.7, 128.5, 128.9, 129.7, 130.7, 131.3, 134.3, 140.5 (aryl), 195.7 (*C*OOH); ^{31}P (81 MHz, D$_2$O) δ 31.3, 31.6; ESMS *m*/*z* calcd for C$_{20}$H$_{27}$N$_2$O$_4$P (M−H)$^+$ 404.42, found 404.3.

3.7. Enzymatic assays

For the details of the kinetic studies: the enzymes, preparation, activation, assays of LAP and APM activity, and the K_i value determination, as well as computational data for inhibitor docking, see the Ref. 27.

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