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A three-component Mannich-type condensation leading to phosphinic dipeptides—extended transition state analogue inhibitors of aminopeptidases

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ABSTRACT

N-Protected α-aminoalkylphosphinic acids bearing a P–H function were found to be novel practical building blocks in three-component condensations with formaldehyde and secondary amines (amino acids). Such Mannich-type *N*-phosphonomethylation is a common approach for phosphorus acid derived substrates and leads to multifunctional (phosphonic/amino/carboxylic) compounds of diverse relevance. The utility of this reaction was examined for construction, in a single synthetic step, of advanced phosphinic pseudodipeptides designed to act as extended transition state analogue inhibitors of selected aminopeptidases. Phosphinomethylation of primary amino acids was less efficient and yielded mixtures of products which were separated into individual components, and their structures identified.

Phosphinic dipeptide analogues have been reported to be potent competitive inhibitors of the M17 cytosolic leucine aminopeptidase (E.C.3.4.11.1, LAP) and the M1 microsomal alanyl aminopeptidase (E.C.3.4.11.2, APN/CD13), the most recognized representatives of metallo-containing exopeptidases of biomedical importance.[1] These phosphorus compounds are believed to act as high energy transition state (TS) analogues in amide hydrolysis.[2] Accordingly, the pseudodipeptides **1** containing hydrophobic P1 and P1′ residues, which were linked via a –P(O)(OH)–CH_2– moiety mimicking the peptide bond, exhibited inhibition constants for LAP and APN in the nanomolar range (Scheme 1).[3] The same ligands **1** were also found to be very effective towards *Plasmodium falciparum* M1 and M17 recombinant enzymes.[4] Interestingly, phosphonamidate dipeptides **2**, containing a direct nitrogen-to-phosphorus bond [–P(O)(OH)–NH–], were predicted to be more favourable TS analogues. As calculated theoretically, they should be an order of magnitude more potent inhibitors of LAP than phosphinates owing to the energy gain from a very specific hydrogen bond NH···O=C that followed interaction of the substrate with the enzyme Leu360 upon cleavage.[3] Unfortunately, the P–N moiety adjacent to a free amino group was discovered to be extremely labile in water (pH <11) which excluded application of phosphonamidate compounds such as **2** in kinetic assays.[5]

In the present work, we report the synthesis and validation of extended TS analogues [–P(O)(OH)–CH_2–NH–, general structure **3**, Scheme 1] as inhibitors of LAP and APN aminopeptidases. The novel pseudodipeptidic backbone should combine the advantages of hydrolytically stable phosphinates with the enhanced active site interactions characteristic for phosphonamidates. Optimal hydrophobic residues were selected as side-chain substituents of the modified compounds: 2-phenylethyl for the P1 position and benzyl, *p*-hydroxybenzyl or isobutyl for P1′. A simple and versatile synthetic strategy, the three-component Mannich-type condensation of α-aminoalkylphosphinate **4**, formaldehyde and an amino acid, was examined as a method to achieve the target compounds (Scheme 1).

The traditional synthesis of phosphinic dipeptide analogues involves a multistep process. Two building blocks bearing an appropriate P1 (frequently an N-protected α-aminoalkyl-*H*-phosphinic acid[6]) and P1′ fragment (e.g., an acrylate[7]) are synthesized individually. In the final step, the nucleophilic phosphinate, upon activation (most commonly via silylation), adds to the double bond in a Michael reaction.[8] As this methodology does not allow simple variation of the substituents, many efforts have been dedicated to the development of alternative synthetic approaches.[9] To achieve the structures designed here, we intended to investigate a convenient, Mannich-type, three-component condensation of a P–H compound, formaldehyde and an amine. Presently, there is only one paper describing a corresponding structure, which was prepared via alkylation of an amino ester with a chloromethylphosphinic acid.[10] The Mannich reaction is frequently applied for partial and exhaustive phosphonomethylation[11] of primary and secondary amines/amino acids (glyphosate herbicide, antiscale agents, water softeners, etc.), under acidic or basic catalysis.[12] Typically, phosphorus

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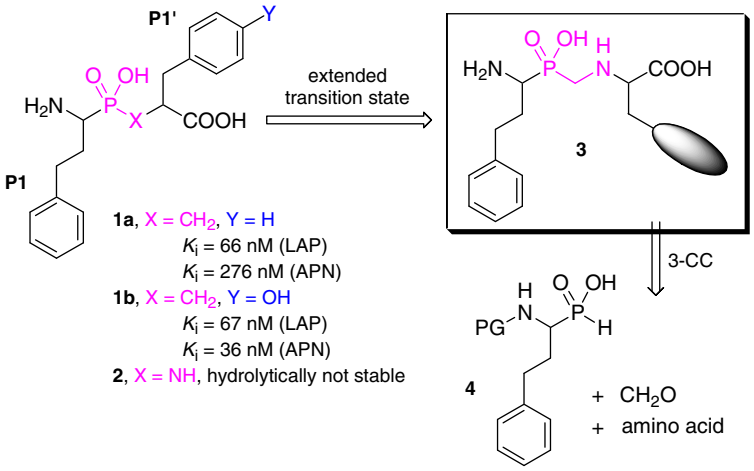


Scheme 1. The structures of the phosphinic (**1**) and phosphonamidate (**2**) lead compounds, and the target phosphinic dipeptides with a modified backbone (**3**). The planned synthetic strategy to achieve the designed compounds via a three-component condensation starting from phosphinic acid **4**.

acid or its esters (dialkyl phosphites), but also hypophosphorus acid, P–H phosphines and phosphinoxides, are used. Herein, according to the best of our knowledge, N-protected α-aminoalkyl-*H*-phosphinic acids were subjected to these conditions for the first time. Unprotected, enantiomerically pure amino acids were selected as the amino components. This makes the P1′ building blocks easily accessible and stereochemically defined. It also increases the availability and structural diversity of the target compounds, which in turn allows extensive optimization of the P1′–S1′ ligand–protein interactions.

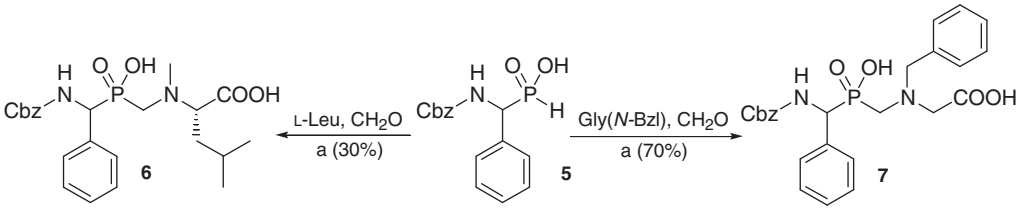
The analogue of phenylglycine **5**,[13] easily obtained on multigram scale, was selected as a model phosphinate for preliminary optimization of the reaction conditions. The substrate was reacted in water with excess formaldehyde and an amino acid (L-leucine or *N*-benzylglycine) possessing a primary or secondary amino group, respectively (Scheme 2). It was necessary to use an organic solvent to achieve satisfactory solubility of the phosphorus component. Thus, acetic acid was added for this purpose. However, its acidity was not sufficient to ensure effective catalysis and addition of a few drops of conc. HCl was also required. Basic catalysis was excluded to avoid racemization of the amino acid. Additionally, esterification of the phosphorus component would be required to maintain its reactivity under alkaline conditions.

Initially, the reaction was performed with a 10-fold molar excess of formaldehyde and was heated under reflux for 5–6 h. For the amino acid containing a primary amino moiety, unexpected N-methylated pseudodipeptide **6** (Scheme 2) crystallized preferentially from the complex mixture of organophosphorus products (see below for discussion), and was isolated in 30% yield. In contrast, phosphinomethylation of secondary *N*-benzylglycine was not problematic and led to the desired product. The above-mentioned conditions afforded the pure target product **7** that crystallized after cooling, without any work-up, in a 70% yield. Interestingly, compound **7** existed as two isomers (trans and cis forms of the carbamate[14]) as was evident in the ^{31}P NMR spectrum (1:0.4 ratio). This is somewhat surprising since usually the cis isomer content is negligible. It seems that for this compound, the presence of the *N*-benzyl group stabilizes the cis carbamate arrangement. Indeed, after removal of the Cbz group, both forms coalesced into a single ^{31}P NMR signal.

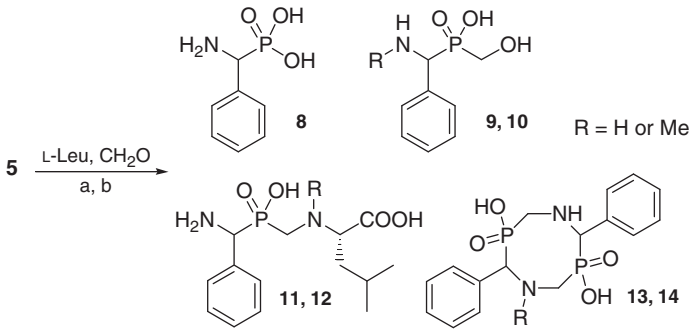
To optimize the conditions for the condensation of **5** with L-leucine (to avoid N-methylation), the reaction time was fixed for 1 h at reflux. The condensation proceeded slowly at lower temperature, whereas prolongation of refluxing increased the complexity of the product mixture. Similar complications were observed when a high excess of formaldehyde was used, and only two equivalents of CH_2O proved to be the optimum amount. Typically, a water solution of formaldehyde was added in two portions to a refluxing water/AcOH/HCl_{concd} mixture (1:1:0.05, v/v) containing both amino acid components.[15] The separated product still consisted of several compounds. After removal of the benzyloxycarbonyl protection, these components were purified by HPLC, characterized and identified. Their structures (**8–14**) are presented in Scheme 3.

The phosphonic analogue of phenylglycine **8** predominated in the HPLC amino acid fraction. This oxidized form of the phosphorus substrate represented 25–30% of the total content. As various N-methylated products (e.g., **10**, **12** and **14**, Scheme 3) were also present, the reduction of formimines (or formiminium cations) to the corresponding *N*-methyl derivatives must have accompanied the oxidation reaction (Scheme 4). Such an observation was reported earlier in the literature.[16]

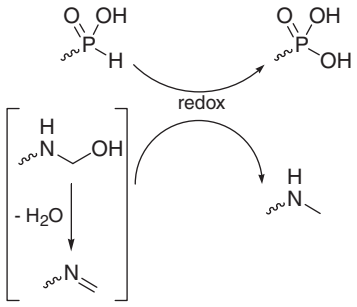
The N-methylation involved the amino group of both substrates and was combined with other side-reactions. Cleavage of the Cbz protection under acidic conditions was then evident as the first reaction. Condensation of only two components, namely (deprotected) phosphinic phenylglycine with formaldehyde was also apparent. These reactions led to P-hydroxymethylated



Scheme 2. The products of condensation of 1-*N*-benzyloxycarbonylamino(phenyl)methane-*H*-phosphinic acid (**5**) with L-leucine or *N*-benzylglycine and formaldehyde. Reagents and conditions: (a) amino acid (2 equiv), CH_2O (36–38% aqueous solution, 10 equiv), H_2O/AcOH/HCl_{concd} (1:1:0.05, v/v), reflux, 5 h.



Scheme 3. Identification of the products of condensation of 1-*N*-benzyloxycarbonylamino(phenyl)methane-*H*-phosphinic acid (**5**) with a primary amino acid. Reagents and conditions: (a) L-leucine (2 equiv), CH_2O (36–38% aqueous solution, 2 equiv), H_2O/AcOH/HCl_{concd} (1:1:0.05, v/v), reflux, 1 h; (b) HBr (33% solution in AcOH), room temperature, 2 h, then HPLC separation.



Scheme 4. A redox process leading to oxidation of the *H*-phosphinic acid to its phosphonic analogue, accompanied with formimine reduction to *N*-methyl derivatives.

α-aminophosphinates **9** and **10**, traces of which were isolated. It was also clearly demonstrated by separation and identification of significant quantities (10–15% of the total yield) of the [2+2] cycloaddition products **13** and **14**. Related eight-membered (and greater) rings were reported in the literature as the result of [2+2+2] condensation of hypophosphorus acid, formaldehyde and glycine,[17] or ethylenediamine-*N*,*N*-diacetic acid[18] (as an amino group donor). Here, the presence of both reactive NH_2 and PH functions in the same starting molecule was the only difference. The product **14** was present as a diastereoisomeric mixture, as was evident by the 1:1 peak ratio in the ^{31}P NMR spectrum. Attempts to synthesize the eight-membered ring compound **13** from the unprotected aminophosphinate and formaldehyde were not successful. The complexity of the obtained mixture provided evidence that this process probably occurs via a polycondensation pathway.

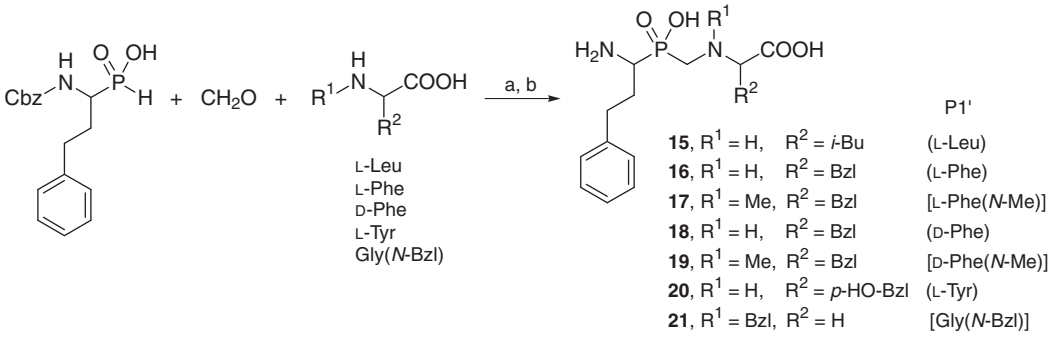
The required aminomethylphosphinic backbone product **11** was also obtained and was accompanied by its N-methylated analogue **12**. The tertiary amine **12** predominated when a 10-fold molar excess of formaldehyde was added (compare Scheme 2). The amount of unwanted product was reduced significantly when the optimized two equivalents of CH_2O were used. Disappointingly, the target compound constituted only roughly 10–20% of the total mixture. Each of the pseudodipeptides was composed of two diastereoisomers in 1 to 1 ratio. For **11**, it was possible to separate the individual isomers by careful collection of the HPLC fractions. However, it became obvious that this procedure could not be recommended as a general method to synthesize pseudodipeptides containing aminomethylphosphinic fragments. Although clean, and giving easy to separate, structurally complex products in one step starting from secondary amines or amino acids, the designed three-component reaction could not be applied simply for amino acids bearing a primary amino group. The overall yields of the desired products were poor and the mixtures obtained needed to be separated cautiously into individual compounds by HPLC.

Such a modus operandi was followed to isolate the target products in order to verify their inhibitory activity towards LAP and APN aminopeptidases. L-Leucine, L-phenylalanine, D-phenylalanine, L-tyrosine and *N*-benzylglycine were selected as amino acid substrates bearing a hydrophobic residue potentially interacting with the S1′ subsite. The reactions were carried out starting from the phosphinic analogue of homophenylalanine **4** (Scheme 5) using the optimized conditions described above. To obtain the *N*-methyl dipeptides (**17** and **19**), phenylalanine enantiomers were reacted with **4** and a higher excess of CH_2O under harsher conditions, like those used for *N*-benzylglycine. After Cbz cleavage the final products **15**–**21** were purified (e.g., full data for compound **20** are supplied[15]).

The results of the inhibition of cytosolic leucine and alanyl aminopeptidase are presented in Table 1. The tested pseudodipeptides appeared to be moderate competitive inhibitors of LAP and APN. Two compounds, **20** and **21**, exhibited slow-binding kinetics. This observation is not unexpected since for tighter binding organophosphorus inhibitors a change in enzyme conformation or slow displacement of a water molecule from the active site are usually suggested as responsible factors.[3,19] The affinity of the inhibitors was not improved when compared to the data obtained for the lead compounds. Apparently, the backbone elongation disturbed the optimal binding mode by distortion of the complexation of the phosphorus fragment by the zinc ion/ions and/or interactions in the P1′–S1′ region. This might be due to the fact that binding of phenylalanine analogues to APN was not stereospecific within the S1′ pocket. The K_i value for the L-Phe pseudopeptide was almost identical to that of D-Phe, as were their N-methylated forms (compare **16** vs **18** and **17** vs **19**). Quite surprisingly, the non-natural P1′ configuration of **18** (K_i = 7.66 μM) was visibly more preferred by LAP than **16** (the L form, K_i = 91.1 μM).

Interestingly, the studied compounds appeared to be more potent towards APN than LAP; this is typically difficult to achieve (complexation of two zinc ions present in the active site of LAP is much more effective than complexation of only the one of APN). The affinity measured for both enzymes differed by 1–2 orders of magnitude in the cases of the best ligands **15**, **20** and **21** (K_i = 1–3 μM for APN). Such activity and selectivity factors seem to be a good starting point to improve the potency of the compounds towards this individual molecular target.

In summary, a three-component condensation of N-protected α-aminophosphinic acid, formaldehyde and a secondary amine/amino acid was found to be an extremely convenient one-step method for the preparation of multifunctional compounds that contain an N–C–P–C–N system. Here, this has been applied to synthesize phosphinic dipeptide analogues with a modified backbone. They have been examined as extended transition state analogue inhibitors of cytosolic leucine and microsomal alanyl



Scheme 5. The synthesis of the designed compounds.[15] Reagents and conditions: (a) amino acid (2 equiv), CH_2O (36–38% aqueous solution, 2 equiv for **15**, **16**, **18** and **20** or 10 equiv for **17**, **19** and **21**), H_2O/AcOH/HCl_{concd} (1:1:0.05, v/v), reflux, (1 h for **15**, **16**, **18** and **20** or 5 h for **17**, **19** and **21**); (b) HBr (33% solution in AcOH), room temperature, 2 h, then preparative HPLC.

Table 1
Inhibition of the mammalian (porcine kidney) leucine (LAP) and alanyl aminopeptidases (APN) by extended TS phosphinic pseudodipeptides (for experimental details of the assays and LAP preparation see Refs. 3 and 20).

Compound	^{31}P NMR (D_2O, ppm, 121.5 MHz)	K_i (μM)	
		LAP	APN
15	40.46 and 39.94 (1:1)[a]	226	2.89
16	21.54 and 21.45 (1:0.9)	91.1	9.33
17	21.06 and 20.93 (1:1.1)	41.8	19.2
18	40.38 and 39.96 (1:1)[a]	7.66	9.39
19	40.39 and 40.10 (1:0.9)[a]	17.5	17.9
20	21.05 and 20.95 (1:1)	33.2	4.59 (initial)[b] 1.06 (steady state)
21	21.00	52.4	3.11 (initial)[b] 2.90 (steady state)

[a] NaOD was added to achieve solubility.
[b] Slow binding mechanism of type A.

aminopeptidases. The most interesting aspects of the structure–activity relationship concerned their low micromolar affinity towards monozinc APN with significantly poorer affinity to LAP.

Increasing the scope of this synthetic method to a wider variety of amine substrates could be profitable in other fields of study, for example in the construction of novel metal complexing agents or multifunctional molecular receptors. Disappointingly, the application of primary amino acids led to complicated mixtures of products under standard conditions. Their separation and identification provided confirmation of the occurrence of side-reactions. These involve random combination of phosphine/imine redox processes, leading to phosphinate P–H oxidation and N-methylation, with N-deprotection of the phosphorus substrate and different variants of polycondensation. Introduction of easily accessible *N*-benzyl amino acids might be suggested as a suitable protecting group strategy (since it may be removed simultaneously with Cbz) in an alternative synthetic pathway leading to the target compounds.

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15. A representative phosphinomethylation procedure of a primary amino acid with 1-(*N*-benzyloxycarbonylamino)alkane-*H*-phosphinic acid and formaldehyde, and the separation of products.
A phosphinic acid (3.0 mmol) and an amino acid (9.0 mmol) were dissolved in a hot H_2O/AcOH/HCl_{concd} mixture (10:10:0.5 mL). Formaldehyde (36–38% aqueous solution, 6.0 mmol, 0.5 mL) was added in two portions to the stirred solution. Following addition the mixture was refluxed for 1 h and then cooled to room temperature. The solvents were evaporated to ca. 20–25% of the starting volume. The solid was precipitated by addition of H_2O (50 mL), filtered and dried in the air. The Cbz protection was removed by the action of HBr (33% solution in AcOH, 10 mL per 1 g) for 2 h at room temperature. The acids were removed under reduced pressure and the residue was triturated with Et_2O. The resulting solid was filtered, dissolved in a small quantity of H_2O/MeCN and subjected to preparative HPLC (Varian ProStar apparatus with ProStar 325 UV/vis detector using a Dynamax 250 × 21.4 Microsorb 300-10 C18 column).
2-(S)-N-{[(1'-(R,S)-amino-3'-phenylpropyl)(hydroxy)phosphinyl]methyl}amino-3-(p-hydroxyphenyl)-propionic acid (**20**). ^{1}H NMR (ppm, D_2O, 300.1 MHz): 7.11 (m, 5H, C_6H_5), 6.91 and 6.64 (2 × d, J = 8.0 Hz, 2H and 2H, C_6H_4), 4.09 (m, 1H, NCHCO), 3.14 (m, 1H, CHP), 3.02 (m, 4H, PCH_2 and *p*-HO–$C_6H_4CH_2$), 2.63 and 2.50 (2 × m, 1H and 1H, $CH_2C_6H_5$), 1.95 and 1.80 (2 × m, 1H and 1H, $CHCH_2CH_2$). ^{31}P NMR (ppm, D_2O, 121.5 MHz): 21.05 and 20.95 (1:1). ^{13}C NMR (ppm, D_2O + NaOD, 151.0 MHz): 181.82 and 181.91 (C=O), 164.52 (C4, C_6H_4), 142.48 (C1, C_6H_5), 130.41 and 130.39 (C2, C_6H_4), 128.71 (C2, C_6H_5), 128.66 (C3, C_6H_5), 126.08 (C4, C_6H_5), 124.01 (C1, C_6H_4), 118.61 (C3, C_6H_4), 67.91 and 67.82 (2 × d, J_P = 9.1 Hz, NCHCO), 49.12 and 49.02 (2 × d, J_P = 99.7 Hz and 98.2 Hz, CHP), 45.17 and 45.07 (2 × d, J_P = 95.1 Hz and 99.7 Hz, PCH_2), 38.22 and 38.14 (*p*-HO–$C_6H_4CH_2$), 32.21 (d, J_P = 12.1 Hz, $CH_2C_6H_5$), 31.98 (d, J_P = 6.0 Hz, $CHCH_2CH_2$). HRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{19}H_{26}N_2O_5P$: 393.1579, observed: 393.1539. HPLC purification (v/v, H_2O/MeCN, both containing 0.1% of trifluoroacetic acid): gradient 85:15 (0 min)→75:25 (35 min), t_r = 27.2 min.

Phosphinomethylation of a secondary amino acid. A phosphinic acid (3.0 mmol) and a secondary amino acid (6.0 mmol) were dissolved in a hot H_2O/AcOH/HCl_{concd} mixture (10:10:0.5 mL). Formaldehyde (36–38% aqueous solution, 30.0 mmol, 3.0 mL) was added dropwise to the stirred solution. After addition, the mixture was refluxed for 5–6 h and then cooled to room temperature. The precipitated solid was filtered and dried in the air. The benzyloxycarbonyl protection was removed as described above.

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