Hydrolysis of the Phosphonamidate Bond in Phosphono Dipeptide Analogues — the Influence of the Nature of the N-Terminal Functional Group

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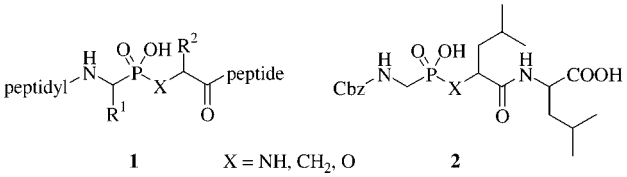
Phosphonamidate pseudodipeptides, designed as transition state analogue inhibitors of leucine aminopeptidase, revealed unexpected instability in aqueous solutions of pH values varying from acidic up to highly basic. This reaction has been studied in some detail by means of NMR spectroscopy and it was found that the phosphonamidate stability depended strongly on the solution pH, relying on the protonation state of two crucial functional groups of the molecule — the phosphonamidate and amino moieties. Protonation of the free *N*-terminal amino moiety is required for the occurrence of rapid P–N bond decomposition; *N*-protected derivatives are significantly more stable. The mechanisms of the cleavage of the phosphonamidate bond have been proposed and discussed. The observed instability of unblocked P–N peptides may be the cause of the substantial limitations of their practical application as aminopeptidase inhibitors.

Introduction

Phosphonamidates (**1**, X = NH), phosphinates (**1**, X = CH_2) and phosphonates (**1**, X = O) form three important families of phosphorus analogues of peptides (Figure 1) that resemble the tetrahedral transition state of the amide bond hydrolysis.[1−5] These moieties are able to coordinate the zinc atom present in the active sites of metalloproteases and to block its function in the process of hydrolysis. Thus, their chemistry continues to attract considerable interest[6−10] and provides a wide variety of potent inhibitors of proteases.[11−15]



1 X = NH, CH_2, O **2**

Figure 1. Peptides analogues with an amide bond replaced by a phosphorus-containing moiety (**1**): Cbz-Glyψ[P(O)(OH)X]Leu-Leu inhibitors of thermolysin — phosphorus analogues of *N*-benzyloxycarbonyl-glycyl-leucyl-leucine {**2**, ψ[P(O)(OH)X] depicts pseudopeptide bond}

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Phosphonamidates seem to be the closest analogues among all three types of isosters in terms of their resemblance to the transition state resulting in the inhibitory effect. This has been adequately illustrated in the literature for an endopeptidase thermolysin, a prototypical member of the zinc-containing metalloprotease family, and its Cbz-Glyψ[P(O)(OH)X]Leu-Leu inhibitor (Figure 1, compound **2**).[16−19] Although the mode of interaction of the phosphonate (X = O) with the enzyme, revealed by the crystal structure, is virtually identical to the corresponding phosphonamidate (X = NH),[2] the latter binds approximately 1000-fold more tightly to thermolysin (K_i = 9.1 nm).[16] This observation was explained by the difference in binding free energy due to the specific hydrogen bonding. Surprisingly, the phosphinic analogue (X = CH_2), for which the crystal structure is not available, which presumably interacts in a similar manner, exhibits only a moderately lower activity than the P−N one.[17,18] It has become obvious that complex effects both in the active site and in solution, including differences in the basicity of the oxygen ligands, electrostatic repulsions and desolvation processes, can dramatically influence the enzyme-inhibitor adduct formation.[17−19] Taking all these factors into account, phosphonamidates seem to be the best candidates as they possess only a single disadvantage associated with their limited stability.

Recently we have focused our interests on phosphonamidate pseudodipeptides, developing new, potent inhibi-

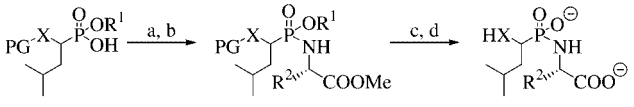
tors of leucine aminopeptidase.[20] Leucine aminopeptidase (LAP, E.C.3.4.11.1) is one of the first discovered and the most widely studied aminopeptidase with respect to sequence, structure and mechanism of action.[21−25] Similar to other enzymes of this family it is of significant biological and medical importance due to its key role in protein modification. What's more, the altered activity of human LAP has been associated with several pathological disorders.[26−32] In our recent work, the application of a molecular modelling methodology followed by synthesis of a set of rationally designed phosphinic pseudodipeptides resulted in the development of the most potent organophosphorus inhibitors of LAP reported so far.[20] However, the most promising among them, namely the phosphonamidate analogues containing free *N*-terminal amino group, were found to be extremely unstable in aqueous solutions, due to P−N bond cleavage at pH values below 12. This unexpected property excludes their use in enzymatic studies, although theoretically they were predicted to be even more potent than the corresponding phosphinates.

The results concerning the stability of the phosphonamidate moiety obtained by various authors are quite contradictory. Although such peptidomimetics have been quite frequently used as peptide isosters, exhibiting a strong inhibitory activity against metalloproteases,[11−13,16−19,33−38] the P−N bond is considered to be unstable in numerous reports.[11,17,33,35,36,39−42] Bartlett, for example, in his original paper, reported the half-life of *N*-terminally protected phosphonamidate dipeptide Cbz-Glyψ[P(O)(OH)NH]Phe in aqueous solution at pH 6.2 to be 4 h, whereas it lengthens dramatically even under slightly basic conditions.[33] An excellent illustration of pH-dependent hydrolysis was presented by Christianson and Lipscomb in a crystallographic study of carboxypeptidase A complexed with the same compound.[43,44] All these data concern *N*-protected analogues for which neutral pH seems to be the limit for staying intact. However, P−N lability is correlated to both pH and the chemical structure of the molecule. Thus, the presence of a free terminal amino group in phosphonamidates was found to enhance dramatically the susceptibility of such analogues to hydrolysis.[45] This fact, along with our observations,[20] makes the infrequently reported examples of their application as enzyme inhibitors clearly unreliable.[46,47] Such incompatible and scarce literature data concerning P−N pseudodipeptides with an unprotected α-amino group, encouraged us to investigate this problem. To the best of our knowledge, no comprehensive studies on the pH-dependent behaviour of such derivatives has so far been performed and presented. The second reason was to establish key functional and structural features of their lability in terms of designing new LAP inhibitors unaffected by physiological pH, but still bearing groups crucial for efficient binding to the aminopeptidase. Here we present the results of this study performed on structurally diverse phosphonamidates, with special emphasis on those containing a free *N*-terminal amino group, and propose mechanisms for the cleavage.

Results and Discussion

Chemistry

The synthesis of phosphonamidate pseudopeptides is complex due to the presence of diverse functionalities in one molecule, and thus the necessity for their selective protection and deprotection, side reactions accompanying P−N bond formation via phosphonochloridates[6−10] and the instability of the forming phosphonamidate bond.[11,17,33,35,36,39−42] The strategy applied in this work is briefly illustrated in Scheme 1. The starting α-amino- and α-hydroxyphosphonate monoesters (**3** and **4**) were converted into the P−N peptides **5**−**7** by a standard approach based on aminolysis of the appropriate phosphonochloridates with the amino ester. After purification of the blocked pseudopeptides, obtained as diastereoisomeric mixtures, the protecting groups were removed under mild conditions. The procedure ended with hydrolysis of the carboxylate ester groups in basic solution in order to conserve the phosphonamidate moiety intact. The final compounds **8**−**10** were obtained as mixtures of enantiomers or diastereoisomers and stored as their dilithium salts. It was shown that α-amino analogues **8** and **9** are stable in solution of pH above 12 (no decomposition observed within three days at room temperature). The α-hydroxy analogue **10** was synthesised in order to study the influence of the basicity of the *N*-terminal group on the stability of the phosphonamidate moiety. The details of the synthetic procedure, together with inhibitor design and results of kinetic assays towards LAP, have been published in a separate paper.[20]



Scheme 1. Reagents and conditions: (a) $SOCl_2$; (b) (L)-$HCl{\cdot}H_2NCH(R^2)COOMe$, NEt_3; (c) H_2, Pd/C; (d) LiOH/MeOH

Stability Studies of Phosphonamidate Dipeptide Analogues

In order to evaluate the influence of all functional groups present in the molecule on the P−N bond stability, and to suggest the mechanism of this process, several phosphonamidate derivatives were studied. Thus, the fully blocked dipeptides, those with certain functional groups selectively deprotected as well as fully deprotected phosphonamidates were investigated. Furthermore, the effects of the replacement of the α-amino group by hydroxyl, as well as the stability of compounds lacking the *C*-terminal carboxyl moiety were additionally examined. Studies on the dependence

of phosphonamidate hydrolysis rate on pH were performed using ^{31}P NMR spectroscopy.

Variously Substituted Derivatives

The totally substituted phosphonamidate peptides **5**−**7** appear to be stable over almost the whole range of pH. Only under strongly acidic conditions (pH < 1, elevated temperature or elongated reaction time) is the P−N bond partially susceptible to acidolysis, but approximately to the same extent as the carbamate bond of the benzyloxycarbonyl group. This observation is in good accordance with literature data.[48] Selective removal of the Cbz group from compound **5** did not significantly affect its stability. Only side reactions, including carboxylate ester hydrolysis and cyclisation of the molecule in slightly basic conditions, provided compounds **11** and **12**, while the P−N bond remained intact (Figure 2). The cyclisation reaction seems to be limited to a narrow pH range (pH = 7−9) and is not observed in the strongly basic conditions necessary for LiOH-assisted deprotection.

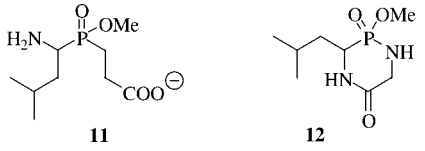


Figure 2. Side products observed upon *N*-deprotection of phosphonamidate **5** followed by treatment with water/methanol basic solution (pH 7−9)

Selective hydrolysis of the methyl esters in compounds **5** and **6** led to *N*-benzyloxycarbonyl derivatives — representatives of the most extensively studied phosphononamidate peptide analogues[11−13,16−19,33−36] — that are generally considered as stable in conditions of enzymatic assays. This stability was fully confirmed in our studies when using Cbz-Leuψ[P(O)(OH)NH]Gly as a representative example. This compound showed hydrolytic resistance within three days at pH 8.5 at room temperature. A decrease in pH to 7.0 resulted in slow P−N bond cleavage and release of Cbz-LeuP (the phosphonic analogue of *N*-benzyloxycarbonyl leucine) and glycine. This shows that the protonation of the molecule, which occurs in neutral aqueous solutions, gives the opportunity to form a far better leaving group and is responsible for the observed cleavage.

Fully Deprotected Phosphonamidates

Cbz-Leuψ[P(O)(OH)NH]Gly appears to be significantly more stable than the corresponding fully deprotected analogue Leuψ[P(O)(OH)NH]Gly (**8**). Although the lability of phosphonamidate compounds containing a free terminal amino group has been mentioned in the literature,[45] no details concerning the conditions and the reasons for their instability were given. Our NMR studies showed that both Leuψ[P(O)(OH)NH]Gly (**8**) and Leuψ[P(O)(OH)NH]Leu (**9**) are stable only in strongly basic media, and a decrease in the pH to below 12 results in P−N bond hydrolysis, yielding LeuP and the appropriate amino acid, namely Gly or Leu. The decrease in phosphonamidate and increase in LeuP concentrations were monitored by performing the range of ^{31}P NMR measurements at certain time intervals (Figure 3).

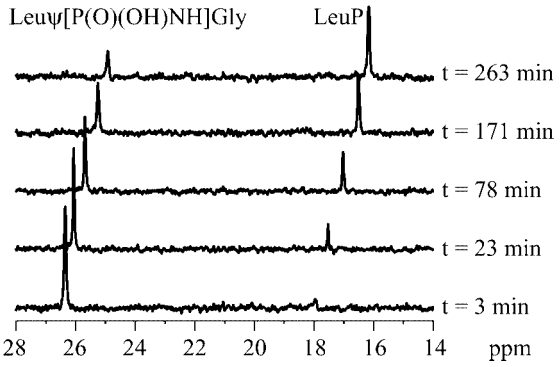


Figure 3. ^{31}P NMR assisted monitoring of phosphonamidate bond hydrolysis of Leuψ[P(O)(OH)NH]Gly (**8**) at starting pH 11.0 (the process is accompanied by pH lowering, which also causes a change of the chemical shift values)

The integration of the signals allowed calculation of the ratio of the phosphonamidate to LeuP reported as a percent of the unhydrolysed phosphonamidate. These values were used for evaluation of the kinetic parameters of these reactions, which proceeded according to typical "pseudo" first-order kinetics. The rates of Leuψ[P(O)(OH)NH]Gly hydrolysis as a function of pH (between 10 to 12) are shown in Figure 4.

The calculated rate constants for hydrolysis of phosphonamidates **8** and **9** are compared in Figure 5. Their values apparently decrease with a rise of pH. There is also a visible steric effect in this reaction since leucyl-leucine analogue **9** decomposes about two times slower than the leucyl-glycine analogue **8**. Compound **9** was synthesised as mixture of two diastereoisomers (L,L and D,L). Well separated ^{31}P NMR signals allowed us to follow the hydrolysis of each isomer individually, however, no effect of influence of their configuration on the rate of cleavage was observed. Thus, the summary effect of decomposition of **9** is presented in Figure 5 and 6.

The obtained results indicate explicitly that such a fast decomposition of deprotected phosphonamidates excludes the possibility of determining their binding affinities towards leucine aminopeptidase. For LAP activity the optimal pH is 8.5, at which more than 90% of **8** and **9** hydrolyze within 10 minutes. The presence of a free α-amino group, however, is crucial for efficient inhibition of this protease. LAP exhibits aminoexopeptidase activity and there is no space in the active site to bind a bulky *N*-protecting group. The presence of the unsubstituted phosphonic group coordinating zinc atoms is also necessary for strong interaction with the LAP active site. This restricts the application of such phosphonamidate dipeptide analogues, although they were designed as the most promising inhibitors of LAP.

Influence of the C-Terminal Carboxylate Group

The simultaneous presence of two deprotected moieties — the phosphonamidate and the *N*-terminal amino

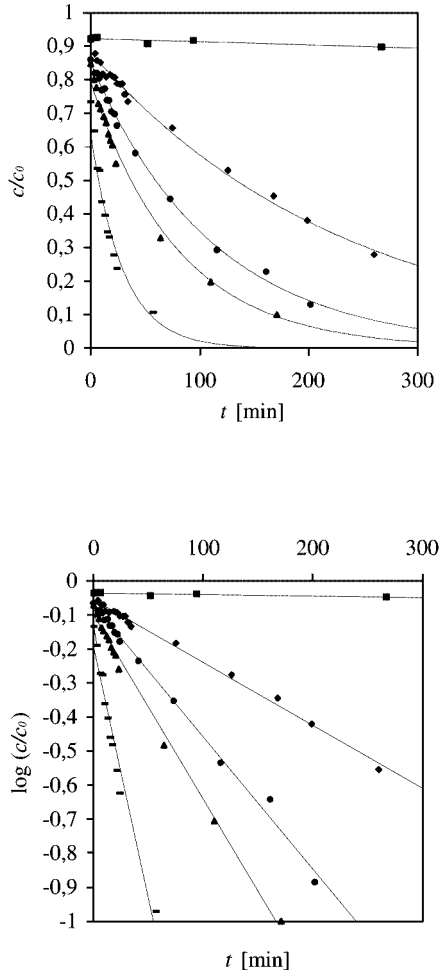


Figure 4. Time-dependent hydrolysis of the phosphonamidate bond of Leuψ[P(O)(OH)NH]Gly (**8**) as exponential decrease of the substrate concentration (top), and linear in log scale (bottom) at various starting pH values (■: pH 11.7; ◆: pH 11.1; •: pH 10.9; ▲: pH 10.7; —: pH 10.4)

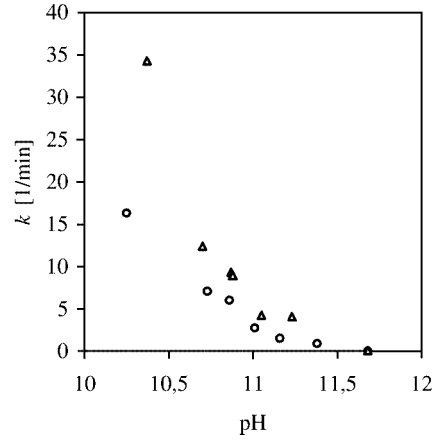


Figure 5. Comparison of rate constants calculated for hydrolysis of Leuψ[P(O)(OH)NH]Gly (**8**; Δ) and Leuψ[P(O)(OH)NH]Leu (**9**; ○) upon varying the pH

one — was proved to be responsible for the dramatically rapid process of P−N cleavage. The influence of the *C*-terminal carboxylate group on the hydrolysis was also exam-

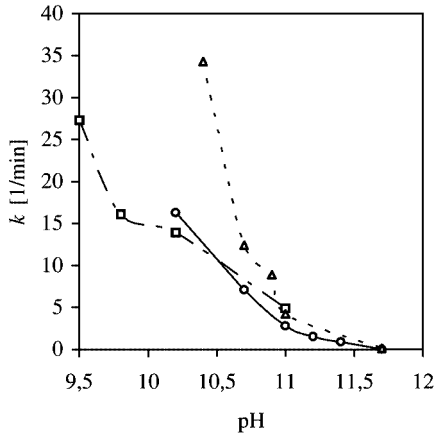


Figure 6. Influence of the *C*-terminal carboxylate moiety on "pseudo" first-order rate constants for the hydrolysis of phosphonamidate analogues as a function of pH: Leuψ[P(O)(OH)NH]Gly (**8**; Δ), Leuψ[P(O)(OH)NH]Gly (**9**; ○), Phgψ[P(O)(OH)-NH]Bzl (□)

ined, however it appeared not to be significant. Thus, a phosphonamidate lacking this moiety, namely Phgψ[P(O)(OH)NH]Bzl (obtained by standard deprotection of the *N*-protected compound available from previous study[48]), revealed no significant stability differences in comparison to Leuψ[P(O)(OH)NH]Gly (**8**) and Leuψ-[P(O)(OH)NH]Leu (**9**) in terms of both reaction kinetics and calculated values of "pseudo" first-order rate constants (Figure 6).

Replacement of the N-Terminal Amino Moiety by a Hydroxy One

In order to investigate the presumed importance of the basicity of the free α-amino group on the P−N bond hydrolysis, derivative **10**, containing an α-hydroxyl group instead of the α-amino one (depicted as Leu^{OH}-ψ[P(O)(OH)NH]Gly), was synthesised (Scheme 1). Stability studies and the calculations of the percent of non-hydrolysed phosphonamidate were performed in a similar manner to that described above and indicated that this compound is significantly more stable than Leuψ-[P(O)(OH)NH]Gly (**8**), with less than 10% of this compound decomposed after seven days at pH 8.5. The hydrolysis also proceeded according to "pseudo" first-order kinetics. However, the rate constant for hydrolysis of compound **10** at pH 8.5 ($k = 6.4 \times 10^{-3}$ 1/min) is about 1000 times lower than the corresponding value for **8** at pH 11.0. Thus, the hydroxy analogue **10**, similarly to the widely studied *N*-benzyloxycarbonyl derivatives,[11−13,16−19,33−36] appears to be stable enough to be able to evaluate its inhibitory activity at physiological pH. These observations indicate that the presence of a basic terminal amino group is essential for such an unexpectedly rapid hydrolysis.

The Mechanism of Phosphonamidate Bond Hydrolysis

The results obtained from the stability studies on phosphonamidate dipeptide analogues suggest that the simultaneous presence of the deprotected α-amino and phos-

phonamidate functions make these compounds stable only under strongly basic conditions. Furthermore, the removal of the *C*-terminal carboxylic moiety does not affect the P−N bond hydrolysis rate. It is worthwhile to note that the studied compounds are stable at pH values greater 12.0, when all the functional groups of the molecule exist in the deprotonated state. The hydrolysis of the P−N bond proceeds very rapidly upon lowering the pH: the half-life of Leuψ[P(O)(OH)NH]Gly (120 minutes) at pH 11.1 decreases to below 10 minutes at pH 10.4. This pH range corresponds to the protonation of the *N*-terminal amino group. The compounds lacking a free α-amino group are significantly more stable, as shown for the hydroxy- and Cbz-blocked derivatives Leu^{OH}ψ[P(O)(OH)NH]Gly and Cbz-Leuψ[P(O)(OH)NH]Gly respectively, with a lack of decomposition products (< 5%) observed within three days at pH 8.5. Based on these results some alternative pathways for the P−N bond hydrolysis process might be proposed.

The first one is the classical nucleophilic substitution (S_N2 type) at phosphorus in which the protonation of the cleaved amino leaving group of the *C*-terminal amino acid is indispensable. We suggest that the protonated *N*-terminal amino group compensates the negative charge of the phosphonamidate moiety by the electrostatic interactions, and facilitates nucleophilic attack of an oxygen atom of the nucleophile (Figure 7, a). A water molecule rather than a hydroxyl group is a better candidate for the nucleophile in this process because it is able to deliver a proton to the leaving amino group. The resulting five-coordinate transition state undergoes cleavage with the simultaneous proton transfer to the leaving group either from the neighbouring *N*-terminal α-amino group or from a water nucleophile.

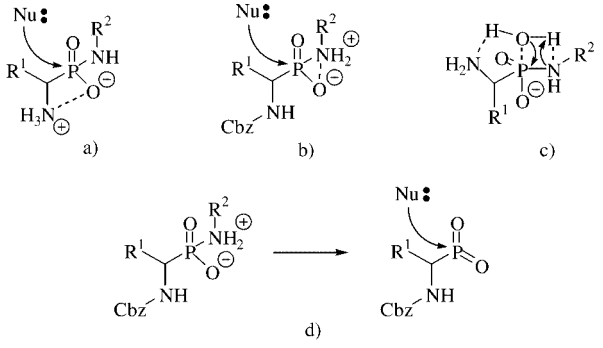


Figure 7. Proposed pathways of P−N bond hydrolysis (see discussion in the text)

For derivatives containing the Cbz dipeptides or depsipeptides containing the hydroxyl group (**10**), the hydrolysis is observed at pH values below 7.0. Under these conditions the protonation of the phosphonamidate NH group occurs, which enhances the nucleophilic attack of the water molecule (Figure 7, b). Thus, P−N bond hydrolysis of phosphonamidate dipeptide analogues takes place when the total charge of the functional groups attached to C_α, *N*-terminal amino and the phosphonamidate, is equal to zero.

An alternative mechanism might be proposed analogous to that described in the literature reaction for cleavage compounds containing a trivalent phosphorus atom,[49] namely the reaction proceeds via a four-membered transition state. It involves binding of the proton of the attacking nucleophile (here water) by the leaving group, which is associated with coordination of the electron pair of the water oxygen by the electrophilic phosphorus atom (Figure 7, c). Such a cyclic transition state mechanism has been proposed for substitution of P^{III} phosphoramidates. In the case of the compounds studied here, the free α-amino group can play an additional, important role in stabilizing the presumable transition state. This mechanism seems to better explain the observed instability of phosphonamidate dipeptides.

The third alternative — a unimolecular pathway of P−N bond hydrolysis — would involve formation of the metaphosphate (Figure 7, d). Conversion of the phosphonamidate moiety into an uncharged and planar metaphosphonate enables the nucleophilic attack of the water molecule and phosphonic amino acid analogue formation. Although the dissociative mechanism is not often considered in the literature, the metaphosphate intermediate has been postulated as being involved in the phosphorylation of alcohols by phosphoramidic acid monoesters.[50] This mechanism seems to explain more favourably the observed phenomenon for the phosphonamidates with a blocked *N*-terminal amino group. However, it looks less probable for free phosphonamidates because the formation of the metaphosphonate would require proton transfer to the leaving amino group at pH 11.

Experimental Section

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

Melting points were determined on a Boetius apparatus and were not corrected. IR spectra were recorded in KBr pellets or in film on a Perkin−Elmer System 2000 FT IR spectrometer. Proton and phosphorus NMR spectra were recorded on a Bruker DRX spectrometer operating at 300.13 MHz for ^1H and 121.50 MHz for ^{31}P. Measurements were made in $CDCl_3$, [D_6]DMSO or D_2O solutions. Proton chemical shifts are reported in relation to tetramethylsilane used as internal standard. ^{31}P NMR spectra were obtained with the use of broad-band ^1H decoupling; chemical shifts are reported in relation to 85% H_3PO_4 (sealed capillary). Microanalyses were performed by Instrumental Analysis Unit of the Institute of Organic Chemistry, Biochemistry and Biotechnology, Wrocław University of Technology.

Methyl Hydrogen [1-(*N*-Benzyloxycarbonylamino)-3-methylbutyl]phosphonate (3): White crystals; m.p. 118−120 °C. ^1H NMR ($CDCl_3$): δ = 0.85 (d, *J* = 6.7 Hz, 6 H, 2 × CH_3), 1.47 (m, 2 H,

CH_2), 1.64 (m, 1 H, CH), 3.63 (d, J = 10.8 Hz, 3 H, OCH_3), 4.11 (m, 1 H, CHP), 5.05 (s, 2 H, CH_2O), 7.26 (m, 5 H, C_6H_5), 10.33 (s, 1 H, OH) ppm. ^{31}P NMR: δ = 29.56 ppm.

Ethyl Hydrogen (1-Benzyloxy-3-methylbutyl)phosphonate (4): Yellowish glass. ^{1}H NMR ([D$_6$]DMSO): δ = 0.71 and 0.84 (d each, J = 6.4 Hz, 3 H and 3 H, 2 × CH_3), 1.13 (t, J = 6.9 Hz, 3 H, OCH_2CH_3), 1.44 and 1.58 (m each, 1 H and 1 H, CH_2), 1.73 (m, 1 H, CH), 3.47 (m, 1 H, CHP), 3.96 (m, 2 H, $POCH_2$), 4.47 and 4.86 (d each, J = 11.3 Hz, 1 H and 1 H, CH_2O), 7.30 (m, 5 H, C_6H_5) ppm. ^{31}P NMR: δ = 19.41 ppm.

Synthesis of the Phosphonamidates

Methyl *N*-{[1-(*N*′-Benzyloxycarbonylamino)-3-methylbutyl]-methoxyphosphinyl}glycinate (5). General Procedure: The monomethyl ester **3** (0.63 g, 2 mmol) was dissolved in $CHCl_3$ (10 mL) and thionyl chloride was added (0.22 mL, 3 mmol). The solution was stirred for 2 h at room temperature and refluxed for an additional 2 h. The volatile components were evaporated under reduced pressure, the residue was treated with benzene (10 mL) and the solvents evaporated again. The resulting phosphonochloridate was dissolved in $CHCl_3$ (10 mL) and added dropwise to a mixture of glycine methyl ester hydrochloride (0.25 g, 2 mmol) and triethylamine (0.70 mL, 5 mmol) in $CHCl_3$ (15 mL) cooled in an ice bath. The resulting solution was left at room temperature overnight and then washed successively with 1 M NaOH, water, 5% HCl, water, 1 M Na_2CO_3 and brine (20 mL of each). The organic layer was dried over Na_2SO_4. After removing the solvent the crude peptide was purified by column chromatography using an EtOAc/hexane (3:1) solution as the eluent, giving a white crystalline product as a diastereomeric mixture in 3:2 ratio. Yield: 0.43 g (56%); m.p. 62−79 °C. ^{1}H NMR ($CDCl_3$): δ = 0.85 (m, 6 H, 2 × CH_3), 1.51 (m, 2 H, CH_2), 1.66 (m, 1 H, CH), 3.05 and 3.15 (m each, 1 H together, PNH), 3.47−3.76 (m, 8 H, 2 × OCH_3 and NCH_2), 4.01 (m, 1 H, CHP) 4.95 and 5.24 (d each, J_1 = 10.0, J_2 = 10.1 Hz, 1 H together, NH), 5.03 (AB system, J = 12.2 Hz, 2 H, CH_2O), 7.27 (m, 5 H, C_6H_5) ppm. ^{31}P NMR: δ = 31.39 and 32.72 (2:3 ratio) ppm. IR (KBr): $\tilde{\nu}$ = 3295 and 3225 cm^{-1} (NH), 3065 and 2950 (CH), 1760 and 1700 (C=O), 1545 (δNH), 1270 (P=O), 1210, 1150, 1060 and 1035 (C−O, P−O). $C_{17}H_{27}N_2O_6P$ (386.4): calcd. C 52.84, H 7.04, N 7.25, P 8.02; found C 52.75, H 7.39, N 7.17, P 7.74.

Methyl *N*-{[1-(*N*′-Benzyloxycarbonylamino)-3-methylbutyl]-methoxyphosphinyl}-(L)-leucinate (6): Colourless oil obtained as a diastereomeric mixture in 4:4:3:2 ratio (purification by column chromatography using EtOAc/hexane, 3:2); yield: 0.46 g (52%). ^{1}H NMR ($CDCl_3$): δ = 0.86 (m, 12 H, 4 × CH_3), 1.47 (m, 4 H, 2 × CH_2), 1.64 (m, 2 H, 2 × CH), 3.00 (m, 1 H, PNH), 3.61 (m, 6 H, 2 × OCH_3), 3.99 (m, 2 H, NCH and CHP), 5.04 (m, 3 H, NH and CH_2O), 7.26 (m, 5 H, C_6H_5) ppm. ^{31}P NMR: δ = 30.45, 30.96, 31.90 and 32.23 (4:4:3:2 ratio) ppm. IR (film): $\tilde{\nu}$ = 3295 and 3220 cm^{-1} (NH), 2955 (CH), 1745 and 1720 (C=O), 1540 (δNH), 1265 (P=O), 1215, 1150, 1110 and 1050 (C−O, P−O). $C_{21}H_{35}N_2O_6P$ (442.5): calcd. C 57.00, H 7.97, N 6.33, P 7.00; found C 56.98, H 7.90, N 6.19, P 6.72.

Methyl *N*-[(1-Benzyloxy-3-methylbutyl)ethoxyphosphinyl]glycinate (7): Colourless oil obtained as a diastereomeric mixture in a 4:3 ratio (purification by column chromatography using EtOAc/CH_2Cl_2, 2:1); yield: 0.32 g (45%). ^{1}H NMR ($CDCl_3$): δ = 0.79, 0.81 and 0.93 (d each, J = 6.6 Hz, 6 H together, 2 × CH_3), 1.33 and 1.37 (t each, J = 7.0 Hz, 3 H together, OCH_2CH_3), 1.51 and 1.69 (m each, 2 H together, CH_2), 1.84 (m, 1 H, CH), 3.08 and 3.20 (m each, 1 H together, PNH), 3.70 (s, 3 H, OCH_3), 3.72−3.93 (m, 3 H, NCH_2 and CHP), 4.13 (m, 2 H, $POCH_2$), 4.56, 4.64, 4.71 and 4.87 (d each, J = 11.1 Hz, 2 H together, CH_2O), 7.33 (m, 5 H, C_6H_5) ppm. ^{31}P NMR: δ = 28.78 and 30.75 (4:3 ratio) ppm. IR (film): $\tilde{\nu}$ = 3210 cm^{-1} (NH), 2955 (CH), 1755 (C=O), 1260 (P=O), 1210, 1155 and 1045 (C−O, P−O). $C_{17}H_{28}NO_5P$ (357.4): calcd. C 57.13, H 7.90, N 3.92, P 8.67; found C 57.31, H 8.09, N 3.75, P 8.57.

Removal of the Protecting Groups

Dilithium *N*-[(1-Amino-3-methylbutyl)oxyphosphinyl]glycinate (8). General Procedure: To remove the benzyloxycarbonyl group the peptide **5** (0.19 g, 0.5 mmol) was dissolved in MeOH (5 mL) and hydrogenated in the presence of 10% Pd/C catalyst (0.1 g). Hydrogen was passed through the solution by gentle bubbling. The reaction, followed by TLC as well as by ^{31}P NMR spectroscopy, proceeded quantitatively and was complete within 2 h. The catalyst was filtered off and the solution was evaporated to dryness giving a colourless oil. Removal of the methyl groups was achieved by alkaline hydrolysis of methyl *N*-[(1-amino-3-methylbutyl)methoxyphosphinyl]glycinate in a 1.5 M LiOH/MeOH solution (2 mL, 3 equiv. of LiOH were used). After evaporation to dryness at room temperature the racemic sample was used directly for tests. Due to its instability at pH lower than 12 the deprotected peptide was characterised only by NMR spectroscopy as its dilithium salt. ^{1}H NMR (D_2O): δ = 0.83 and 0.89 (d each, J = 6.6 Hz, 3H and 3 H, 2 × CH_3), 1.37 (m, 2 H, CH_2), 1.75 (m, 1 H, CH), 2.74 (m, 1 H, CHP), 3.42 (d, J = 8.0 Hz, 2 H, NCH_2) ppm. ^{31}P NMR: δ = 29.39 ppm.

Dilithium *N*-[(1-Amino-3-methylbutyl)oxyphosphinyl]-(L)-leucinate (9): Mixture of diastereoisomers in a 3:2 ratio. ^{1}H NMR (D_2O): δ = 0.86 (m, 12 H, 4 × CH_3), 1.29−1.75 (m, 6 H, 2 × CH_2CH), 2.72 (m, 1 H, CHP), 3.72 (m, 1 H, NCH) ppm. ^{31}P NMR: δ = 26.58 and 26.99 (2:3 ratio) ppm.

Dilithium *N*-[(1-Hydroxy-3-methylbutyl)oxyphosphinyl]glycinate (10): Racemic mixture. ^{1}H NMR (D_2O): δ = 0.81 and 0.86 (d each, J = 6.6 Hz, 3 H and 3 H, 2 × CH_3), 1.36 and 1.49 (m each, 1 H and 1 H, CH_2), 1.70 (m, 1 H, CH), 3.41 (d, J = 10.1 Hz, 2 H, NCH_2), 3.63 (m, 1 H, CHP) ppm. ^{31}P NMR: δ = 25.26 ppm.

Stability Studies: The fully deprotected phosphonamidates were stored in dry form as their lithium salts in the presence of excess LiOH. The stability studies were carried out in D_2O or H_2O/D_2O (10% D_2O) solutions. Concentrations of the phosphonamidates ranged between 7 and 40 mm. Titration with 0.5 M HCl was used to obtain the appropriate starting pH (range 6−12). All NMR measurements were carried out at 300 K. Decomposition of the phosphonamidates was followed by ^{31}P NMR spectroscopy with proton decoupling. Furthermore, two dimensional TOCSY, ^{1}H-^{31}P HMQC and ^{1}H-^{31}P HMQC-TOCSY spectra were acquired to identify the products of decomposition. The decrease in phosphonamidate concentration was monitored by recording a set of ^{31}P NMR spectra at set time intervals. The integration of the phosphorus spectra allowed us to obtain the percentage of non-hydrolysed phosphonamidate. "Pseudo" first-order rate constants were calculated as the best fitting to experimental values.

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