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Transesterification of monophenyl phosphonamidates—chemical modelling of serine protease inhibition

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Abstract—*O*-Phenyl phosphonamidates have been designed to bind covalently by nucleophilic substitution to the serine residue in the active site of serine proteases, similarly to the diphenyl phosphonates used as standard. The synthesis of these compounds as well as their phosphonylating reactivity towards methanol, which served as mimetic of the serine nucleophile, is described. The stereochemistry of the substitution in basic solutions was studied in some detail. The stability of the phosphonamidates in aqueous solutions and their selectivity in the reaction against alcohols versus thiols proved that they constitute a class of potential inhibitors of serine proteases, as well as valuable tools to investigate the mechanism of inhibition. © 2002 Published by Elsevier Science Ltd.

1. Introduction

More than one hundred members form the serine protease family—one of the four main superfamilies of enzymes that hydrolyse the amide bond of peptide substrates. Under normal conditions serine proteases are involved in various physiological processes, namely blood coagulation, immune defence, digestion and reproduction. Moreover, their activity is also associated with many pathological disorders and consequently with various human diseases including pulmonary emphysema, rheumatoid arthritis, inflammatory diseases and others. This important role makes them potential targets for the development of inhibitors in the hope that they might serve as new therapeutic agents against many diseases.

The rational design of new inhibitors and drugs demands knowledge of chemical mechanisms of enzymatic catalysis. Generally the proteases belonging to the same family cleave peptide bonds using the same basic mechanism and the same or similar catalytic residues. In the case of the serine family, the catalytic mechanism is based on the presence of three amino acid residues in the catalytic centre, namely Ser195, His57 and Asp102 (chymotrypsin numbering system). The reactive hydroxyl group of Ser195 attacks the carbonyl group of the scissile amide bond of the substrate to form a tetrahedral intermediate stabilised by a specific network of hydrogen bonding. In the next step the substrate–enzyme complex collapses to the acyl intermediate, which is subsequently hydrolysed by a water molecule to regenerate the active enzyme and release the product of reaction. The hydrolysis step also proceeds via a tetrahedral transition state. The whole process is facilitated by basic–acidic catalysis coming from the presence of the imidazole ring of His57 and carboxylate residue of Asp102.

The synthesis of compounds able to bind covalently to one of the amino acid residues crucial for activity, and thus blocking active-site function, was proven to be one of the most effective strategies in the construction of inhibitors for serine proteases.[1–3] Among phosphorylating agents, the most popular is diisopropyl phosphorofluoridate[4] (**1**), which nonselectively inactivates enzymes of this class, and is considered as a diagnostic tool for them. The structural features of substrate–enzyme selectivity can be assured by the use α-aminoalkylphosphonofluoridates (**2**)—phosphorus containing analogues of amino acids.[5,6] However, their application is strongly limited by poor stability in water solutions. Although less reactive, diphenyl esters of α-aminoalkylphosphonic acids and their peptide derivatives (**3**) are resistant to hydrolysis and since the beginning of the last decade they have been intensively exploited as effective and selective inhibitors of various serine proteases.[7–19] They are considered as stable analogues of high-energy tetrahedral transition states of peptide hydrolysis able to phosphonylate the serine residue in the active site of enzymes (Fig. 1).

The molecular mechanism of the action of diphenyl phosphonates has not yet been fully elucidated. After noncovalent binding of the inhibitor, the nucleophilic substitution proceeding on the phosphorus atom leads to the formation of an enzyme-Ser-*O*-phosphonate mixed ester which can be

Keywords: phenyl phosphonates; phosphonamidates; transesterification; serine proteases; inhibitors.

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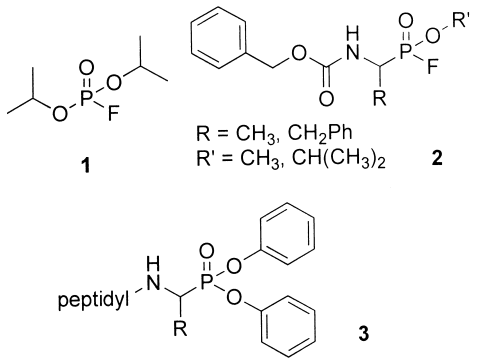


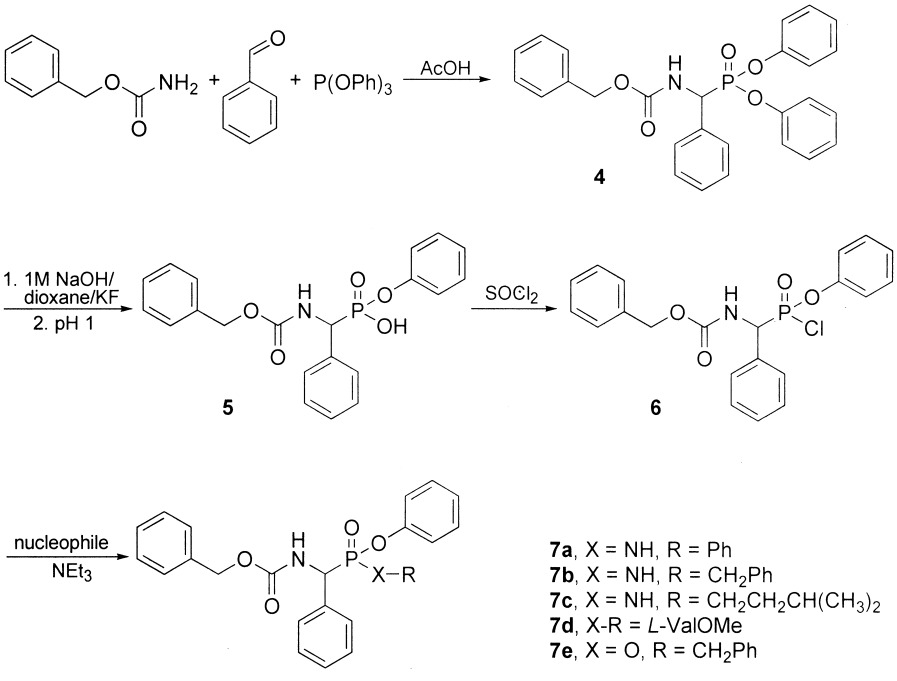
Figure 1. Organophosphorous inhibitors of serine proteases.

observed in ^{31}P NMR.[9] The transesterification process is accompanied by release of a phenoxy group. However the crystal structures of enzymes complexed with inhibitors show that both phenoxy groups were removed,[20,21] presumably as a result of another nucleophilic attack of water molecule. It is not clear how fast this second 'ageing' process is. Both displacements proceed most probably via pentacoordinate transition states. Such a trigonal bipyramidal intermediate was recently determined in the crystal structure of human α-thrombin complexed with the phosphonate tripeptide in crystals of varying age.[22] Interestingly, in this case the nucleophilic attack of water and the formation of the monoester is postulated to precede phosphonylation of the enzyme. This assumption is in good agreement with the study of Pratt and Rahil on the inhibition of serine-dependent β-lactamases by monophenyl phosphonate monoanions.[23–26] The inhibitors first bind noncovalently to the enzyme and then, similar to above, nucleophilic displacement leads to *O*-phosphonylation via dianionic transition state containing a pentacoordinate phosphorus atom.[27–29]

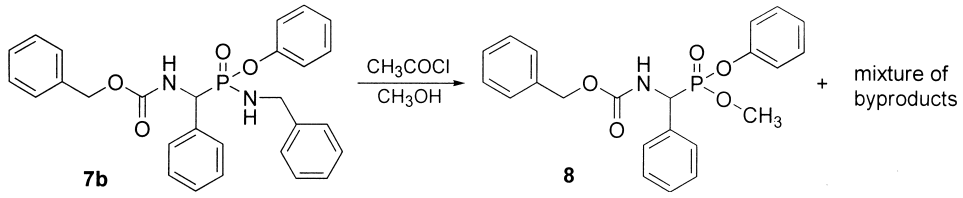
Inhibitors with designed stereochemistry at phosphorus atom and possessing two different leaving groups are very interesting tools to facilitate the elucidation of the mechanism of inhibition. In this study we report the synthesis and the chemical behaviour of a series of such, the *O*-monophenyl phosphonamidates and mixed esters considered as potential inhibitors of serine proteases. We compare their relative phosphonylating reactivity in a model system, using methanol and a base to mimic the activity of serine protease active-sites. We have proven that in these conditions the substitution proceeds much more readily for P–N compounds than for commonly applied diphenyl phosphonates. We also describe a quantitative procedure of their transesterification to methyl derivatives with the use of potassium fluoride. By using diastereoisomers with a chiral phosphorus atom we have demonstrated the stereospecificity of the transesterification and showed the selectivity of the reaction against alcohols versus thiols. The results of our study on the chemical behaviour of *O*-phenyl phosphonamidates indicate that they may represent a new promising class of compounds useful for the design and construction of effective and specific inhibitors for serine protease family members.

2. Results and discussion

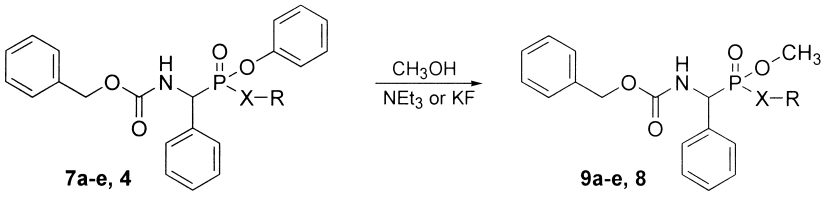
The synthetic strategy to obtain *O*-phenyl phosphonamidates and mixed esters is illustrated in Scheme 1. Initially, it involved the synthesis of monophenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonate (**5**) by two-step procedure: condensation of benzyl carbamate, benzaldehyde and triphenyl phosphite[30] followed by alkaline hydrolysis of the resulting diphenyl ester (**4**).[31] The obtained monoester (**5**) was converted into the final products using the phosphonochloridate strategy, namely the activation of a monoester with thionyl chloride, which usually ensures medium to



Scheme 1.



Scheme 2.



Scheme 3.

good yields of the appropriate chloridate. The latter can easily react with an amine or an alcohol nucleophile affording the final products.[32–36] The progress of the reaction leading to phosphonochloridates was controlled by means of ^{31}P NMR. The phosphonochloridate (**6**) was obtained as a diastereoisomeric mixture in 1:1 ratio, which was seen as two signals at δ 32.98 and 33.36 ppm. The complex signal appearing at δ 10.9 ppm belongs presumably to the pyrophosphonate byproduct.[35] After aminolysis or alcoholysis and standard work-up, the compounds (**7a**–**e**) were finally purified by column chromatography on silicagel. The yields of products obtained as mixtures of diastereoisomers varied between 50–70%.

The phosphonamidate moiety is frequently considered to be unstable[37–39] even though pseudopeptides containing this structural fragment are reported as very potent enzyme inhibitors.[40–43] From our recent study[44] we know that P–N lability strongly depends on the state of the N-terminal amino group and the character of the phosphonamidate moiety alone. The fully blocked phosphonamidates (**7a**–**d**) obtained in this work appeared to be stable across the whole pH range. For example, stirring the compound (**7b**) for three days in methanol containing hydrochloric acid (pH 1) did not result in the P–N bond cleavage. In more drastic conditions (methanolic HCl or HBr, pH <1, elevated temperature or elongated time) it is partially susceptible to the acidolysis, to approximately the same extent as the carbamate bond of benzyloxycarbonyl (Cbz) group. Reactions of (**7b**) carried out in these conditions allowed the recovery of 50–75% of the starting material and produced a complex mixture of byproducts with cleaved amidate, carbamate and ester moieties. The complete phosphonamidate decomposition, however, also accompanied by significant Cbz loss, can be achieved by the action of gaseous HCl generated in methanolic solution in situ by addition of acetyl chloride (Scheme 2). The yield of mixed phosphonate (**8**), representing the main product of this reaction, is 50%.

Table 1. Transesterification of phosphonamidates 7 with methanol in the presence of triethylamine

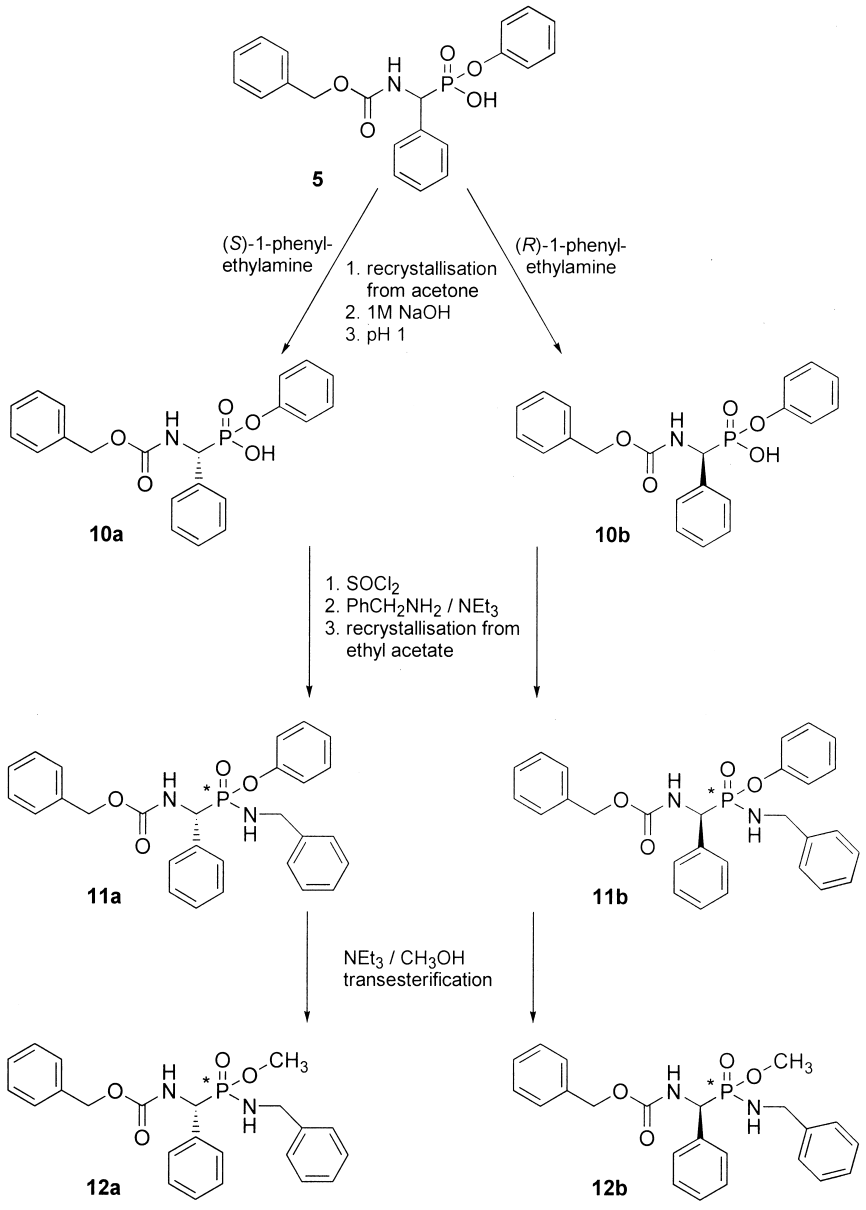
Starting compound	The yield of the transesterification product (%)[a]
7a, X–R=NHPh	25 (**9a**)
7b, X–R=NHCH$_2$Ph	90 (**9b**)
7c, X–R=NHCH$_2$CH$_2$CH(CH$_3$)$_2$	20 (**9c**)
7d, X–R=*L*-ValOMe	75 (**9d**)
7e, X–R=OCH$_2$Ph	5 (**9e**)
4, X–R=Oph	20 (**8**)

[a] The yield established by ^{31}P NMR after 3 days of reaction.

The satisfactory stability of phosphonamidates encouraged us to compare their phosphonylating reactivity versus diphenyl phosphonates in nucleophilic substitutions. Similarly to phosphonate inhibitors in the active sites of serine proteases, the studied compounds (**4**, **7a**–**e**) undergo transesterification in the presence of a nucleophile (here methanol) releasing phenol. Basic catalysis is assured by the addition of triethylamine. The substitution process can be easily followed by means of ^{31}P NMR and leads to *O*-monomethyl derivatives (**8**, **9a**–**e**, Scheme 3) with the formation of traces of byproducts being observed ($<5\%$).

The P–N bond in the resulting phosphonamidates (**9a**–**d**) remains intact. Generally, the displacement proceeds much more readily for phosphonamidates than for phosphonates (**4**, **7e**). The yields of the transesterification with presence of Et$_3$N established on the basis of ^{31}P NMR spectroscopy are listed in Table 1.

The possibility of reaching quantitative conversion (with yield exceeding 95%) of *O*-phenyl phosphonamidates or phosphonates into their methyl derivatives requires the application of potassium fluoride in the excess of alcohol[31] (for the details see Section 4). This reaction, proceeding via fluorophosphonates, gave products in roughly 1:1 diastereomeric ratio not depending on the diastereomeric composition of the substrate. On the contrary, transesterification in basic conditions proceeded stereospecifically and the resulting product has the same stereoisomer ratio, however most probably of the opposite configuration as is seen from the ^{31}P NMR spectra. To check this hypothesis the attempt to synthesise a single diastereoisomer bearing a chiral phosphorus atom was undertaken (Scheme 4).



Scheme 4.

The enantiomers of monophenyl [(*N*-benzyloxycarbonyl)-aminobenzyl]phosphonate (**5**) were resolved via their diastereoisomeric 1-phenylethylamine salts. The obtained salts were recrystallised several times from acetone until constant specific rotations were achieved. After work-up and final recrystallisation white, crystalline, optically active compounds (**10a**, **b**) were obtained. As known from the literature for their analogues,[45,46] the absolute configuration (*R*) can be matched to the (+) enantiomer separated using (*R*)-1-phenylethylamine (**10a**) and (*S*) to the (−) enantiomer (**10b**).

The optically active monophenyl esters were converted into their *N*-benzyl phosphonamidates and purified as described above for the racemic mixtures. The product obtained in each synthesis was a mixture of two diastereoisomers in roughly 1:3 ratio (determined from ^{31}P NMR spectra). To obtain a single diastereoisomer, the compounds were recrystallised several times from ethyl acetate to achieve constant physical data, as well as a single ^{31}P NMR signal. The two preferentially crystallising compounds obtained in each of two parallel separations (**11a**, **b**) were enantiomeric to each other.

The obtained diastereoisomers were transesterified in basic conditions. The reaction proceded with high stereospecificity giving the diastereoisomers of the *O*-methyl phosphonamidates with de exceeding 99% when started from (*R*)-(−) *O*-phenyl derivative and 95% de from (*S*)-(+) one.

O-Phenyl phosphonamidates appeared to be resistant to the action of thiols, what was demonstrated by the lack of any reaction and in basic conditions with $HSCH_2COOEt$. The same stability was observed in the presence of KF even though the substrates are partially converted into phosphonofluoridates, which did not undergo further reaction.

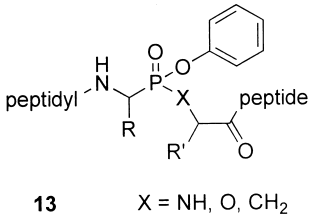


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Figure 2. Organophosphorous pseudopeptide analogues of transition state of peptide hydrolysis.

3. Conclusions

Despite the fact that they appear to be close analogues of peptides, phosphonamidates were predicted to be poor inhibitors of serine proteases, due to the fact that the respective transition state differs from that of tetrahedral intermediate.[9] Additionally, the electrophilicity of the phosphorus atom and consequently their phosphonylating reactivity was supposed to be significantly decreased in comparison to diphenyl derivatives.[9] The results of their chemical behaviour towards methanol in basic conditions obtained in this work are quite contradictory and suggest that phosphonamidates could be successfully applied for construction of potent and selective inhibitors of serine proteases. Including the additional amino acid moiety into the structure of the inhibitor also offers a possibility of extension the interactions for the S′ subsite of enzymes. These types of phosphonamidate,[6] phosphonate[47,48] and phosphinate[49] pseudopeptide analogues (**13**) have only been marginally studied until now (Fig. 2).

Furthermore the mechanism of their action remains unclear. The configuration of the phosphorus atom was shown to be of little consequence regarding their activity.[6,47] Final enzyme–inhibitor complexes exhibit the same geometry at phosphorus, independent of the starting diasteroisomer used.[48] This indicates the operation of various mechanisms of substitution, which may proceed either with overall inversion or with retention of configuration. In the case of mixed phosphonate esters, the lack of binding with the S′ subsite even in the case of compounds with conserved C-terminal fragment is quite surprising (partial cleavage of both *O*-phosphonate ester groups is observed instead).[48]

The mechanism of transesterification described in this study is obviously much simpler, with presumably a typical $S_N2(P)$ process taking place as indicated by observed inversion of configuration (Fig. 3).

This cannot be simply compared to displacement in restricted geometry of the active site and suggests that only one, appropriate stereoisomer of phosphonopeptide might exhibit inhibitory potency. However, the phosphonamidate compounds reported here, given their potential usefulness as enzyme inhibitors, may serve as valuable tools to understand the molecular mechanisms of inhibitory action of phosphorus containing compounds towards serine proteases.

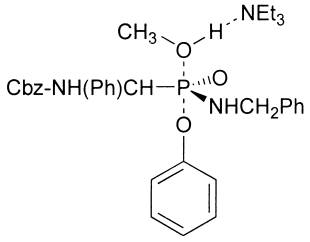

Figure 3. Intermediate of $S_N2(P)$ process of transesterification of phenyl phosphonamidates.

4. Experimental

4.1. General

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

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

4.1.1. Diphenyl [(*N*-benzyloxycarbonyl)aminobenzyl]-phosphonate (4). Diphenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonate was synthesised by condensation of benzyl carbamate, benzaldehyde and triphenyl phosphite as described previously.[30] Yield 52%; mp: 137–140°C (lit. mp: 138–140°C[30]); ^{31}P NMR ($CDCl_3$): δ 15.02.

4.1.2. Monophenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonate (5). The monophenyl ester was obtained by alkaline hydrolysis of the diphenyl ester in 1 M NaOH/dioxane solution in the presence of potassium fluoride and 18-crown-6 as described in the literature.[31] Yield (after recrystallisation from ethyl acetate) 86%; mp: 163–164°C (lit. mp: 161–162°C[31]); ^{31}P NMR (DMSO-d_6): δ 16.58; ^{1}H NMR: δ 5.04 (AB system, J=12.6 Hz, 2H, C*H*$_2$OC(O)), 5.14 (dd, J_{PH}=22.3 Hz, J_{HH}=10.1 Hz, 1H, NC*H*P), 7.03–7.51 (m, 15H, 3×C$_6$*H*$_5$), 8.39 (bd, J= 10.1 Hz, 1H, C(O)N*H*).

4.2. The general procedure for the preparation of mixed *O*-phenyl esters and phosphonamidates

Monophenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonate (**5**) (0.80 g, 2 mmol) was suspended in 20 ml of

dry benzene and thionyl chloride was added (0.22 ml, 3 mmol). The mixture was stirred for 2 h at room temperature and refluxed gently for additional 2 h. Then the volatile components were evaporated under reduced pressure. Another portion of 20 ml of benzene was added and the evaporation was repeated. The resulting phosphonochloridate was dissolved in 10 ml of chloroform and added dropwise to the cooled (0°C) mixture of a nucleophile (2 mmol) and triethylamine (0.42 ml, 3 mmol or 0.70 ml, 5 mmol in the case of aminoester hydrochloride) in 15 ml of chloroform or benzene. The resulting solution was stirred at room temperature overnight and then evaporated to dryness. The mixture was redissolved in ethyl acetate and washed successively with: 1 M sodium hydroxide, water, 5% hydrochloric acid, water, 1 M sodium carbonate and brine (20 ml of each). The organic layer was dried over anhydrous sodium sulphate. After removal of the solvent, the crude compounds were purified by recrystallisation from an ethyl acetate/hexane solution or by silica gel column chromatography using an ethyl acetate/hexane solution as the eluent to yield white crystalline products.

4.2.1. *N′*-Phenyl *O*-phenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonamidate (7a). Yield 64%; mp: 177–180°C; IR (KBr, cm^{-1}): 3345 and 3190 (NH), 3065 and 3030 (CH), 1690 (C═O), 1535 (δNH), 1240 (P═O), 1225, 1205 and 1030 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 20.16 and 20.43 (1:3 ratio); ^{1}H NMR: δ 4.99 and 5.81 (bd each, 1H together, PN*H*), 5.10 (AB system, *J*=12.2 Hz, 2H, CH_2OC(O)), 5.41 and 5.61 (dd each, J_{HP}=18.4 Hz, J_{HH}=9.7 Hz, 1H together, NC*H*P), 6.29 and 6.42 (m each, 1H together, C(O)N*H*), 6.90–7.50 (m, 20H, 4×C_6H_5). Calcd for $C_{27}H_{25}N_2O_4P$: C, 68.63; N, 5.93; P, 6.56; found: C, 68.82; N, 5.98; P, 6.46.

4.2.2. *N′*-Benzyl *O*-phenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonamidate (7b). Yield 61%; mp: 144–146°C; IR (KBr, cm^{-1}): 3300 and 3230 (NH), 3060 and 3030 (CH), 1720 (C═O), 1550 (δNH), 1255 (P═O), 1205 and 1020 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 24.55 and 24.78 (3:2 ratio); ^{1}H NMR: δ 3.04 and 3.42 (m each, 1H together, PN*H*), 3.90 and 4.10 (m each, 2H together, PNHCH_2), 5.04 and 5.06 (AB system each, J_1=12.2 Hz, J_2=12.3 Hz, 2H together, CH_2OC(O)), 5.33 (dd, J_{HP}=18.7 Hz, J_{HH}=9.2 Hz, 1H, NC*H*P), 6.12 and 6.32 (m each, 1H together, C(O)N*H*), 6.90–7.47 (m, 20H, 4×C_6H_5). Calcd for $C_{28}H_{27}N_2O_4P$: C, 69.12; N, 5.76; P, 6.37; found: C, 68.98; N, 5.83; P, 6.35.

4.2.3. *N′*-Isoamyl *O*-phenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonamidate (7c). Yield 63%; mp: 164–168°C; IR (KBr, cm^{-1}): 3295 (NH), 3065, 3035 and 2960 (CH), 1720 (C═O), 1550 (δNH), 1250 (P═O), 1215, 1200 and 1020 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 25.16 and 25.28 (1:5 ratio); ^{1}H NMR: δ 0.72 and 0.78 (d each, *J*=6.6 Hz, 6H together, CH(CH_3)$_2$), 1.20 (m, 2H, CH_2CH_2CH), 1.45 (m, 1H, CH_2CH_2CH), 2.74 (m, 1H, PN*H*), 2.96 (m, 2H, PNHCH_2), 5.06 and 5.11 (AB system each, *J*=12.2 Hz, 2H, CH_2OC(O)), 5.26 (dd, J_{HP}=18.2 Hz, J_{HH}=9.6 Hz, 1H, NC*H*P), 6.19 (m, 1H, C(O)N*H*), 6.94–7.51 (m, 15H, 3×C_6H_5). Calcd for $C_{26}H_{31}N_2O_4P$: C, 66.94; N, 6.00; P, 6.64; found: C, 66.93; N, 6.16; P, 6.43.

4.2.4. Methyl {phenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonyl}-(*L*)-valinate (7d). Yield 48%; mp: 155–172°C; IR (KBr, cm^{-1}): 3330 and 3230 (NH), 3065 and 2965 (CH), 1735 and 1710 (C═O), 1550 (δNH), 1265 (P═O), 1230, 1205, 1140 and 1025 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 23.47, 24.00 and 24.32 (2:3:2 ratio); ^{1}H NMR: δ 0.60, 0.69, 0.70, 0.79 and 0.85 (d each, *J*=6.8 Hz, 6H together, CH(CH_3)$_2$), 1.75 and 1.91 (m each, 1H together, CH(CH_3)$_2$), 3.12, 3.37 and 3.50 (dd each, $J_1 \approx J_2 \approx$10.5 Hz, 1H together, PN*H*), 3.52, 3.54 and 3.59 (s each, 3H together, OCH_3), 3.67, 3.80 and 3.91 (m each, 1H together, NC*H*C(O)), 5.01–5.14 (AB systems, *J*=12.3 Hz, 2H together, CH_2OC(O)), 5.25 and 5.34 (dd each, J_{HP}=18.9 Hz, J_{HH}=9.6 Hz, 1H together, NC*H*P), 5.90, 6.05 and 6.30 (m each, 1H together, C(O)N*H*), 7.08–7.48 (m, 15H, 3×C_6H_5). Calcd for $C_{27}H_{31}N_2O_6P$: C, 63.52; N, 5.49; P, 6.07; found: C, 63.63; N, 5.38; P, 6.20.

4.2.5. Benzyl phenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonate (7e). Yield 56%; mp: 133–136°C; IR (KBr, cm^{-1}): 3235 (NH), 3060, 3030 and 2960 (CH), 1715 (C═O), 1550 (δNH), 1255 (P═O), 1200, 1055 and 1020 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 19.42 and 19.52 (1:2 ratio); ^{1}H NMR: δ 4.80 (ABX system, J_{HaHb}=11.6 Hz, J_{PxHa}=8.4 Hz, J_{PxHb}=7.3 Hz, 2H, POCH_2), 5.057 and 5.064 (AB system each, *J*=12.2 Hz, 2H together, CH_2OC(O)), 5.39 and 5.40 (dd each, J_{HP}=22.1 Hz, J_{HH}=9.6 Hz, 1H together, NC*H*P), 5.86 and 5.95 (m each, 1H together, C(O)N*H*), 7.05–7.45 (m, 20H, 4×C_6H_5). Calcd for $C_{28}H_{26}NO_5P$: C, 68.98; N, 2.87; P, 6.36; found: C, 69.23; N, 2.73; P, 6.26.

4.3. The general procedure for transesterification of mixed *O*-phenyl esters or phosphonamidates in methanol

The phenyl ester or phosphonamidate (**4, 7a–e**) (0.2 mmol) was dissolved in 2 ml of methanol (in certain cases the addition of 1 ml chloroform was necessary to achieve the solubility). The solution was stirred overnight after addition of potassium fluoride (60 mg, 1.0 mmol) or for three days when triethylamine (0.5 ml) was used as catalyst. The yield of the transesterification was established on the basis of ^{31}P NMR spectra of the reaction mixture and exceeded 95% for the reactions carried out in the presence of KF. To obtain the pure products methanol was evaporated and the resulting residue dissolved in 10 ml of ethyl acetate, washed with 1 M sodium hydroxide to remove phenol and then washed with brine (10 ml of each). The organic layer was dried over anhydrous sodium sulphate. After removing the solvent, crude products were purified by column chromatography using an ethyl acetate/hexane solution as the eluent to yield white crystalline compounds. The yields of the transesterification carried out with presence of Et_3N are listed in Table 1.

4.3.1. Dimethyl [(*N*-benzyloxycarbonyl)aminobenzyl]-phosphonate. This was obtained by transesterification of (**4**) in the presence of KF.[31] Mp: 115–117°C (lit. mp: 117–118.5°C[31]); ^{31}P NMR ($CDCl_3$): δ 5.12; ^{1}H NMR: δ 3.48 and 3.72 (d each, *J*=10.8 Hz, 3H together, POCH_3), 5.10 (AB system, *J*=12.3 Hz, 2H, CH_2OC(O)), 5.19 (dd, J_{PH}=22.0 Hz, J_{HH}=9.9 Hz, 1H, NC*H*P), 5.90 (m, 1H, C(O)N*H*), 7.20–7.44 (m, 10H, 2×C_6H_5).

4.3.2. *O*-Methyl *N′*-phenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonamidate (9a). Mp: 164–168°C; IR (KBr, cm^{-1}): 3340 and 3205 (NH), 3060, 3030 and 2950 (CH), 1690 (C═O), 1535 (δNH), 1250 (P═O), 1215 and 1045 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 24.58 and 24.71 (3:2 ratio); ^1H NMR: δ 3.48 and 3.66 (d each, J_1=10.6 Hz, J_2=11.1 Hz, 3H together, POC*H*$_3$), 4.91 and 5.05 (AB system each, J=12.3 Hz, 2H together, C*H*$_2$OC(O)), 5.19 (dd, J_{HP}=17.7 Hz, J_{HH}=9.6 Hz, 1H, NC*H*P), 5.44 and 5.92 (m each, 1H together, PN*H*) 6.23 and 6.32 (m each, 1H together, C(O)N*H*), 6.77–7.37 (m, 15H, 3×C$_6$*H*$_5$). Calcd for $C_{22}H_{23}N_2O_4P$: C, 64.38; N, 6.83; P, 7.55; found: C, 64.28; N, 6.79; P, 7.50.

4.3.3. *N′*-Benzyl *O*-methyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonamidate (9b). Mp: 125–128°C; IR (KBr, cm^{-1}): 3340 and 3235 (NH), 3060, 3035 and 2955 (CH), 1710 (C═O), 1555 (δNH), 1255 (P═O), 1220, 1145 and 1030 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 29.11 and 29.35 (1:1 ratio); ^1H NMR: δ 2.98 and 3.34 (m each, 1H together, PN*H*), 3.35 and 3.56 (d each, J_1=10.8 Hz, J_2=10.9 Hz, 3H together, POC*H*$_3$), 3.61–4.08 (m, 2H, PNHC*H*$_2$), 4.94–5.13 (m, 3H, C*H*$_2$OC(O) and NC*H*P), 6.25 and 6.44 (m each, 1H together, C(O)N*H*), 7.04–7.33 (m, 15H, 3×C$_6$*H*$_5$). Calcd for $C_{23}H_{25}N_2O_4P$: C, 65.08; N, 6.60; P, 7.30; found: C, 64.99; N, 6.61; P, 7.37.

4.3.4. *N′*-Isoamyl *O*-methyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonamidate (9c). Mp: 121–123°C; IR (KBr, cm^{-1}): 3335 and 3230 (NH), 3065, 3035 and 2955 (CH), 1715 (C═O), 1550 (δNH), 1245 (P═O), 1210 and 1025 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 9.27 and 29.44 (1:5 ratio); ^1H NMR: δ 0.72, 0.78 and 0.86 (d each, J=6.6 Hz, 6H together, CH(C*H*$_3$)$_2$), 1.15 (m, 2H, C*H*$_2$CH$_2$CH), 1.46 (m, 1H, C*H*(CH$_3$)$_2$), 2.70 (m, 2H, PNHC*H*$_2$), 2.90 (m, 1H, PN*H*), 3.47 and 3.68 (d each, J_1=10.3 Hz, J_2=10.7 Hz, 3H together, POC*H*$_3$), 5.05 (m, 1H, NC*H*P), 5.08 (AB system, J=12.3 Hz, 2H, C*H*$_2$OC(O)), 5.87 (m, 1H, C(O)N*H*), 7.29–7.42 (m, 10H, 2×C$_6$*H*$_5$). Calcd for $C_{21}H_{29}N_2O_4P$: C, 62.36; N, 6.93; P, 7.66; found: C, 62.55; N, 6.88; P, 7.54.

4.3.5. Methyl {methyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonyl}-*L*-valinate (9d). Mp: 115–130°C; IR (KBr, cm^{-1}): 3325 and 3235 (NH), 3065, 3035 and 2960 (CH), 1730 and 1695 (C═O), 1535 (δNH), 1255 (P═O), 1215 and 1040 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 27.81, 28.04, 28.56 and 28.66 (1:1:1:3 ratio); ^1H NMR: δ 0.66, 0.70, 0.74, 0.83 and 0.87 (d each, J=6.8 Hz, 6H together, CH(C*H*$_3$)$_2$), 1.80 and 1.95 (m each, 1H together, C*H*(CH$_3$)$_2$), 3.00, 3.10, 3.24 and 3.39 (dd each, $J_1 \approx J_2 \approx$10.8 Hz, 1H together, PN*H*), 3.42, 3.60 and 3.62 (d each, J_1=10.9 Hz, J_2=10.8 Hz, J_3=10.9 Hz, 3H together, POC*H*$_3$), 3.53, 3.56, 3.59 and 3.62 (s each, 3H together, OC*H*$_3$), 3.69–3.84 (m, 1H, NC*H*C(O)), 4.95–5.15 (m including two AB systems, J=12.2 Hz, 3H, C*H*$_2$OC(O) and NC*H*P), 5.86 and 6.09 (m each, 1H together, C(O)N*H*), 6.74–7.34 (m, 10H, 2×C$_6$*H*$_5$). Calcd for $C_{22}H_{29}N_2O_6P$: C, 58.92; N, 6.25; P, 6.91; found: C, 58.64; N, 6.13; P, 6.65.

4.3.6. Benzyl methyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonate (9e). Mp: 87–91°C; IR (KBr, cm^{-1}): 3265 (NH), 3060 and 3030 (CH), 1725 (C═O), 1545 (δNH), 1255 (P═O), 1060 and 1010 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 24.19 and 24.35 (1:1 ratio); ^1H NMR: δ 3.36 and 3.58 (d each, J_1=10.8 Hz, J_2=10.9 Hz, 3H together, POC*H*$_3$), 4.68 (ABX system, J_{HaHb}=11.7 Hz, J_{PxHa}=8.5 Hz, J_{PxHb}=7.3 Hz, 2H, POC*H*$_2$), 4.97 and 4.99 (AB system each, J_1=12.4 Hz, J_2=12.2 Hz, 2H together, C*H*$_2$OC(O)), 5.15 (dd, J_{HP}=21.9 Hz, J_{HH}=9.7 Hz, 1H, NC*H*P), 5.90 (m, 1H, C(O)N*H*), 7.07–7.34 (m, 15H, 3×C$_6$*H*$_5$). Calcd for $C_{23}H_{24}NO_5P$: C, 64.93; N, 3.29; P, 7.29; found: C, 64.82; N, 3.23; P, 7.21.

4.3.7. Methyl phenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphonate (8). This compound was obtained by transesterification of (**4**) in the presence of NEt_3 or by cleavage of the P–N bond during the addition of acetyl chloride to the methanol solution of the *N′*-benzyl *O*-phenyl phosphonamidate (**9b**) and purified in the same manner as described above. Mp: 114–116°C; IR (KBr, cm^{-1}): 3245 (NH), 3060 and 3030 (CH), 1710 (C═O), 1545 (δNH), 1250 (P═O), 1045 and 1025 (C–O, P–O); ^{31}P NMR ($CDCl_3$): δ 20.37 and 20.52 (2:3 ratio); ^1H NMR: δ 3.54 and 3.79 (d each, J=10.8 Hz, 3H together, POC*H*$_3$), 5.07 and 5.09 (AB system each, J_1=12.6 Hz, J_2=12.1 Hz, 2H together, C*H*$_2$OC(O)), 5.37 (dd, J_{PH}=21.9 Hz, J_{HH}= 9.3 Hz, 1H, NC*H*P), 5.80 (m, 1H, C(O)N*H*), 6.91–7.47 (m, 15H, 3×C$_6$*H*$_5$). Calcd for $C_{22}H_{22}NO_5P$: C, 64.23; N, 3.40; P, 7.54; found: C, 64.26; N, 3.33; P, 7.55.

4.4. Synthesis and transesterification of the diastereoisomers of *N′*-benzyl *O*-phenyl [(*N*-benzyloxycarbonyl)-aminobenzyl]phosphonamidate

The enantiomers of monophenyl [(*N*-benzyloxycarbonyl)-aminobenzyl]phosphonate (**5**) were resolved via their diastereomeric 1-phenylethylamine salts. These salts were obtained by addition of (*R*)-(+) or (*S*)-(−) amine (2.44 g, 0.02 mol) dissolved in 10 ml of ethyl acetate to the suspension of the monoester (7.95 g, 0.02 mol) in 25 ml of ethyl acetate. After 10 min of stirring and another 10 min of reflux the solution was left for crystallisation. The separated salts were recrystallised several times from acetone as far as constant specific rotations were achieved: $[\alpha]_D^{20}$=+9.8 for the case of (*R*)-(+) amine and $[\alpha]_D^{20}$=−10.2 for (*S*)-(−) amine (1% solutions in ethanol). Then the salts were treated with 10 ml of 1 M NaOH and washed with ether to remove the separated amine. The enantiomers were precipitated by acidification of the basic solution to pH 1. The final recrystallisation from ethyl acetate gave white, crystalline compounds showing the specific rotation values as follows: $[\alpha]_D^{20}$=+18.0 for the enantiomer crystallised with the (*R*)-(+) amine and $[\alpha]_D^{20}$=−17.9 for the enantiomer crystallised with the (*S*)-(−) amine (1% solutions in 1 M NaOH). The absolute configuration (*R*) can be matched to the (+) enantiomer and (*S*) to the (−) enantiomer.[45,46] The total yield of the resolution was 25% for each case.

The optically active monophenyl esters (**10a**, **b**) were converted into their *N*-benzyl phosphonamidates and purified as described above for the racemic mixture. Each product was obtained as a mixture of two diastereoisomers in roughly 1:3 ratio by ^{31}P NMR. To obtain the single diastereoisomer each mixture was recrystallised several times from ethyl acetate to achieve constant physical data.

The values of specific rotations and melting points were monitored as well as ^{31}P NMR spectra The data for the preferentially crystallising phosphonamidate diastereoisomer (**11a**) obtained from (*R*)-(+) phenyl monoester: mp: 188–189°C and $[\alpha]_D^{20}=-34.7$ and for the (**11b**) obtained from (*S*)-(−) monoester: mp: 187–188°C, $[\alpha]_D^{20}=+33.4$ (1% solutions in MeOH). The obtained two compounds are enantiomeric to each other. The yield of the resolution was 20% for each case.

4.4.1. (*R*)-(−)-*N′*-Benzyl *O*-phenyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphon-amidate (11a). Mp: 188–189°C; ^{31}P NMR ($CDCl_3$): δ 24.78; ^1H NMR: δ 3.34 (m, 1H, PN*H*), 4.10 (m, 2H, PNHCH_2), 5.06 (AB system, J=12.3 Hz, 2H, CH_2OC(O)), 5.33 (dd, J_{HP}=18.1 Hz, J_{HH}=9.8 Hz, 1H, NC*H*P), 6.29 (d, J=7.8 Hz, 1H, C(O)N*H*), 6.90–7.49 (m, 20H, 4×C$_6H_5$). Calcd for $C_{28}H_{27}N_2O_4P$: C, 69.12; N, 5.76; P, 6.37; found: C, 69.07; N, 5.73; P, 6.45.

The obtained single diastereoisomers (**11a**, **b**) (25 mg of each) were transesterified in basic conditions (in the presence of triethylamine) as described above. The reaction followed by ^{31}P NMR proceeded with high stereospecificity giving the diastereoisomers of the *N′*-benzyl *O*-methyl phosphonamidate (**12a**, **b**) with de exceeding 99% starting from (*R*)-(−)-*N′*-benzyl *O*-phenyl phosphonamidate (**11a**) (the purification and the data described below) and 95% de starting from (*S*)-(+)-*N′*-benzyl *O*-phenyl phosphonamidate (**11b**) (established on the basis of ^1H and ^{31}P NMR).

To collect the spectroscopic data of the pure product (**11a**) the reaction mixture was evaporated to dryness and the residue was redissolved in 5 ml of ethyl acetate. The organic layer was successively washed with: 1 M sodium hydroxide, 5% hydrochloric acid, 1 M sodium carbonate and brine (5 ml of each) and then dried over anhydrous sodium sulphate. Crude product obtained after evaporation of solvents was purified by column chromatography using ethyl acetate/hexane (3:1) solution as the eluent.

4.4.2. (*R*)-(+)-*N′*-Benzyl *O*-methyl [(*N*-benzyloxycarbonyl)aminobenzyl]phosphon-amidate (12a). Mp: 164–188°C; $[\alpha]_D^{20}=+5.0$ (1% solutions in MeOH); ^{31}P NMR ($CDCl_3$): δ 29.04; ^1H NMR: δ 2.60 (m, 1H, PN*H*), 3.61 (d, J=10.8 Hz, POCH_3), 3.61–4.08 (m, 2H, PNHCH_2), 5.02 (d, J=12.2 Hz, 2H, CH_2OC(O)), 5.07 (m, 1H, NC*H*P), 5.75 (m, 1H, C(O)N*H*), 7.06–7.28 (m, 15H, 3×C$_6H_5$). Calcd for $C_{23}H_{25}N_2O_4P$: C, 65.08; N, 6.60; P, 7.30; found: C, 65.33; N, 6.68; P, 7.11.

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