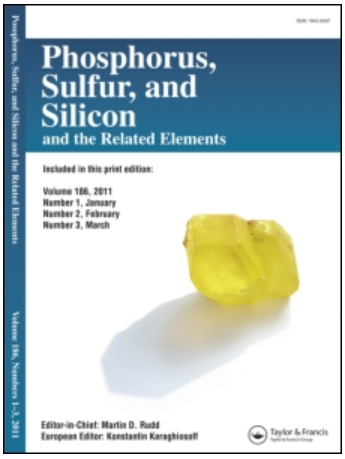

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PHOSPHONODIPEPTIDES. SYNTHESIS BY HOBt/DCC METHOD, MASS SPECTRA OF THE PROTECTED AND ^{1}H NMR OF THE UNPROTECTED PHOSPHONODIPEPTIDES

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PHOSPHONODIPEPTIDES. SYNTHESIS BY HOBt/DCC METHOD, MASS SPECTRA OF THE PROTECTED AND ^{1}H NMR OF THE UNPROTECTED PHOSPHONODIPEPTIDES

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Phosphonodipeptides containing aminomethylphosphonic and 1-aminoethylphosphonic acids were synthesized by the active ester method using *N*-hydroxybenztriazole and DCC. Fragmentation in electron ionization mass spectra (EI/MS) of protected phosphonodipeptides has been proposed. Results from ^{1}H NMR have shown slightly larger magnetic nonequivalence of —CH_2— protons and larger differences in NMR parameters between diastereoisomers than in common dipeptides.

Key words: Phosphonodipeptides; hypophosphite addition; tritylimines; mass spectra; ^{1}H NMR spectra.

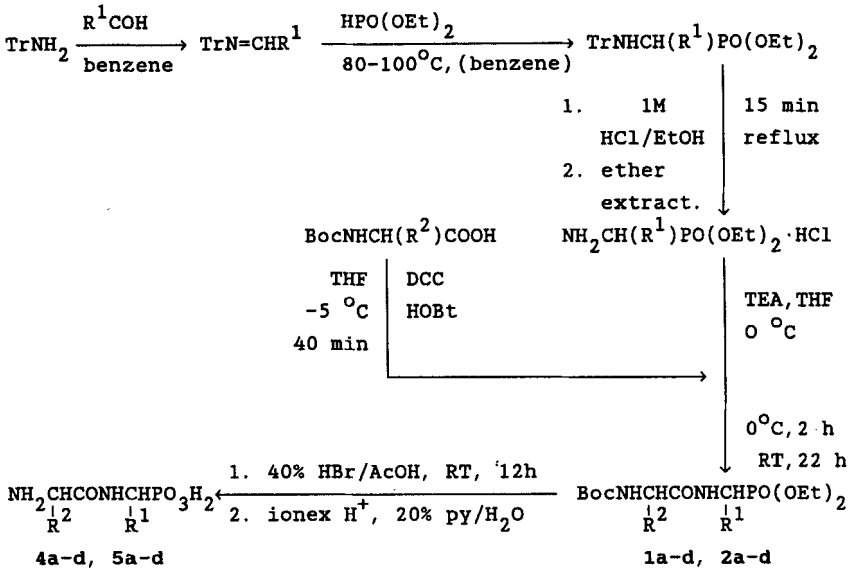
INTRODUCTION

Aminoalkyphosphonic acids and their derivatives have obtained much interest due to biological activity.[1] Phosphonodipeptides containing *P*-terminal aminoalkylphosphonic acids have shown bacteriostatic[2] and herbicidal[3] properties. The phosphonopeptides can be synthesized by different methods,[4] mostly from esters of aminoalkylphosphonic acids. The methods which are known in chemical synthesis of common peptides, such as a method of mixed anhydride,[5] dicyclohexylcarbodiimide coupling,[2,6] active ester with using *N*-hydroxysuccinimide[2,6] and enzymatic synthesis[7] have been used in synthesis of phosphonopeptides. Recently, a few methods have been published which report the synthesis from the free phosphonic acids.[8] In this paper, the active ester method using *N*-hydroxybenztriazole is described, the mass-spectral fragmentation mechanism of the protected phosphonodipeptides was proposed, and ^{1}H NMR spectra of the free phosphonodipeptides were interpreted.

RESULTS AND DISCUSSION

Synthesis

The phosphonodipeptides were synthesized according to Scheme I. The trityl protected esters of aminoalkylphosphonic acids have been stable crystal compounds



Tr = Ph_3C- , Boc = $(CH_3)_3COOC$- , HOBt = N-hydroxybenztriazole,
TEA = Et_3N, DCC = dicyclohexylcarbodiimide, py = pyridine

R^1	R^2	protected phosphonodipeptide	free phosphonodipeptide
H	H	1a	4a
	CH_3	1b	4b
	$(CH_3)_2CHCH_2$	1c	4c
	$PhCH_2$	1d	4d
CH_3	H	2a	5a
	CH_3	2b	5b
	$(CH_3)_2CHCH_2$	2c	5c
	$PhCH_2$	2d	5d
CH_3	$PhCH_2$	3 (dimethylester)	

SCHEME I

in contrast to the free esters which have been unstable.[4] The $TrNHCH_2PO(OEt)_2$ and $TrNHCH(CH_3)PO(OMe)_2$ were synthesized according to Mastalerz *et al.*[9] In the case of the addition of diethylphosphite on $TrN{=}CHCH_3$ at the same or modified condition the yield was lower than 20% and without any dependence on various solvents which include DMSO, dioxane or toluene. We were not successful when we wanted to separate the other products or the unreacted imine from the reaction mixture. If the reaction was done at temperature above 120°C and/or with $BF_3 \cdot Et_2O$ as a catalyst, the yield of the ester was even lower. However, we found that the reaction occurred in benzene with azeotropic distillation of water and the yield was 70–80%. It is likely that the primary formed imine reacts with diethyl-

phosphite which forms the ester. In view of the lower temperature and the stoichiometry of diethylphosphite the by-reactions have been reduced.

The trityl moiety was removed by standard procedure (Scheme I) and the ester hydrochloride was immediately used in the peptide synthesis. The active ester was prepared "in situ" by the reaction of the protected amino acid with HOBt in presence of DCC. The protected phosphonodipeptides were usually isolated in the form of TLC pure oils (except the crystal products **1a** mp 82–3°C, **2a** mp 61–2°C, **2b** mp 103–5°C) which were characterized by mass spectrometry. In the next step they were unprotected in 40% HBr/AcOH and the appropriate hydrobromides were converted to the free peptides on DOWEX 50 in H^+ form (Table I). The

TABLE I

Phosphonodipeptides

Dipeptide[a]	m.p.(dec)[b] °C	$[\alpha]_D^{20}$ [c] deg	Yield[d] in %	literature
4a·H_2O	244-5	-	77	
	(221-3)			5a
4b	283-5	+35	67	
	(180-8)			6b
4c·H_2O	183-6	+57	71	
	(243-7)	(+60.3)		7b
4d	248-50	+77	61	
	(265-8)	(+74.8)		7b
5a·H_2O	282-6	-	83	
	(229-30)			2
5b	276-8	+14	81	
	(278-85)	(+12)		10
5c	244-6	+30	88	
	(247-51)	(+30)		10
5d	243-5	+35	71	
S,S-5b	288-90	+74		
		(+75)		10
S,R-5b	286-8	-46		
	(294-5)	(-46,-49)		2,10
S,S-5c	268-70	+77		
		(+73)		10
S,R-5c	224-8	-12		
	(236-40)	(-12.5,-13)		7b,10
S,S-5d	265-6	+93		
S,R-5d	234-6	-21		
	(247-9)	(-21.5)		7b

a - elemental analyses of C,H,N were in satisfactory agreement with calculated values

b - the highest literature m.p. in bracket

c - 1% in water, error $\pm 1^{\circ}$, literature value in bracket

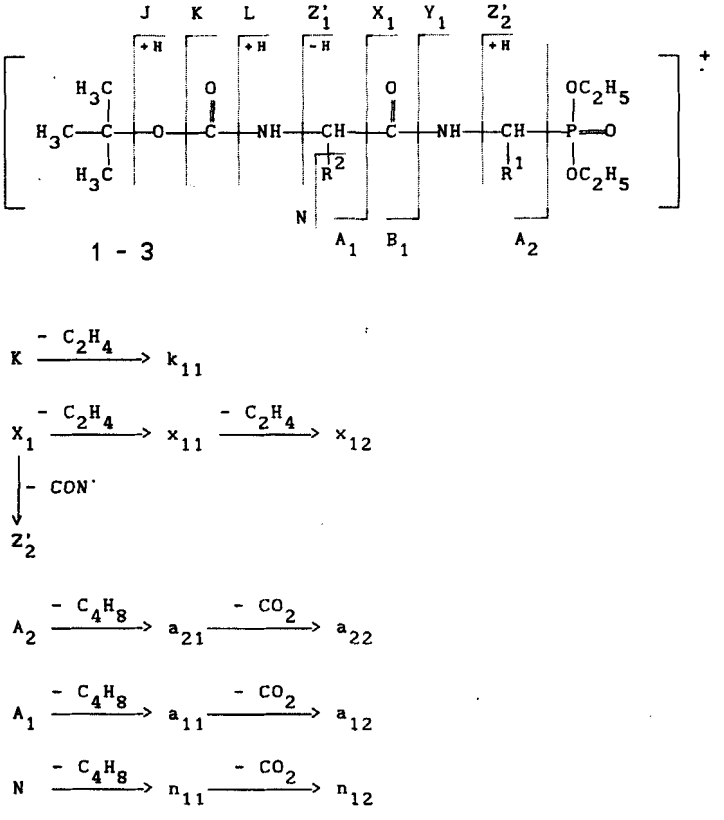
d - overall yeild based on Boc-aminoacid

pure diastereoisomers **5b** and **5c** were resolved according to Kafarski *et al.*[10] This procedure was not successful at **5d**. This peptide crystallized in the DOWEX 50 column and therefore the diastereoisomers were separated by crystallization from hot aqueous solution (*S,S*-isomer) and followed diffusion of acetone vapor to remained solution (*S,R*-isomer). The purity of the diastereoisomers was checked by HPLC[11] and was 99.9%. The **3** was synthesized the same way as **1** and **2** from $TrNHCH(CH_3)PO(OMe)_2$ and Boc-L-Phe for better understanding of the mass spectrometry results.

Mass Spectra

Chemical ionisation mass spectra of aminoalkylphosphonic acids were investigated by Constantin[12] and the fragmentation of the *N*-protected aminobenzyldialkylphosphonates in EI/MS was proposed by Yang.[13]

In spite of phosphonic moiety in the **1–3** the EI/MS fragmentation occurred by the same route as at the common protected dipeptides. Therefore, description in the Scheme II mechanism of the mass spectral fragmentation follows the basic principles of peptide mass spectral fragmentation nomenclature.[14] Relative inten-



SCHEME II The fragmentation pathways of **1–3**

sities of most of the principal fragment ions are given in Table II and a typical spectrum (**1a**) is shown in Figure 1.

Electron impact ionization of compounds **1–3** produces both low intensity molecular ions and low intensity fragment ions in the upper mass region. The proposed

TABLE II

Important ions in mass spectra of **1–3** [relative intensities of ions from Scheme II (m/z)]

	1a	**1b**	**1c**	**1d**	**2a**	**2b**	**2c**	**2d**	**3**
$M^{\cdot +}$	1.3 (324)	0.6 (338)	0.2 (380)	0.8 (414)	0.4 (338)	0.7 (352)	0.3 (394)	1.1 (428)	0.5 (400)
J	19.2 (268)	2.9 (282)	1.1 (324)	1.3 (358)	2.1 (282)	2.0 (296)	1.5 (338)	1.4 (372)	1.4 (344)
K	19.4 (251)	3.4 (265)	1.5 (307)	2.4 (341)	4.4 (265)	3.7 (279)	1.8 (321)	2.4 (355)	1.5 (327)
k_{11}	12.2 (223)	2.7 (237)	1.1 (279)	-	1.5 (237)	2.5 (251)	1.1 (293)	-	-
L	15.3 (224)	0.6 (238)	0.2 (280)	-	0.2 (238)	-	0.2 (294)	0.2 (328)	0.2 (300)
Z'_1	0.8 (207)	-	0.1 (263)	5.0 (297)	-	0.1 (235)	0.4 (277)	4.6 (311)	5.2 (283)
X_1	100.0 (194)	8.6 (194)	2.9 (194)	2.5 (194)	13.0 (208)	9.8 (208)	4.9 (208)	4.5 (208)	3.6 (180)
x_{11}	23.3 (166)	6.2 (166)	3.7 (166)	3.2 (166)	5.1 (180)	4.0 (180)	2.6 (180)	2.9 (180)	-
x_{12}	61.9 (138)	26.4 (138)	19.1 (138)	13.7 (138)	9.5 (152)	8.1 (152)	7.5 (152)	5.2 (152)	-
Z'_2	76.0 (152)	33.0 (152)	15.7 (152)	14.1 (152)	12.0 (166)	34.5 (166)	25.1 (166)	21.6 (166)	12.5 (138)
A_2	-	-	-	-	8.3 (201)	2.6 (215)	0.6 (257)	-	-
a_{21}	2.4 (131)	1.1 (145)	0.6 (187)	-	18.7 (145)	2.7 (159)	0.6 (201)	0.7 (235)	-
A_1	-	11.6 (144)	5.8 (186)	0.8 (220)	-	5.8 (144)	8.1 (186)	2.2 (220)	1.8 (220)
a_{11}	5.8 (74)	22.3 (88)	48.4 (130)	16.1 (164)	3.4 (74)	18.6 (88)	56.4 (130)	23.6 (164)	18.1 (164)
a_{12}	61.9 (30)	64.3 (44)	82.3 (86)	45.6 (120)	n.a. (30)	100.0 (44)	100.0 (86)	58.9 (120)	46.1 (120)
n_{12}	-	-	0.4 (223)	10.6 (223)	-	-	0.2 (237)	12.8 (237)	8.0 (209)
m/z 91	2.2	3.8	3.4	42.1	6.5	6.5	3.9	24.6	17.2
m/z 57	81.3	100.0	100.0	100.0	87.8	81.4	97.1	100.0	100.0
m/z 44	4.7	64.3	7.6	3.4	100.0	100.0	78.2	88.9	61.4

n.a. = not available (not measured)

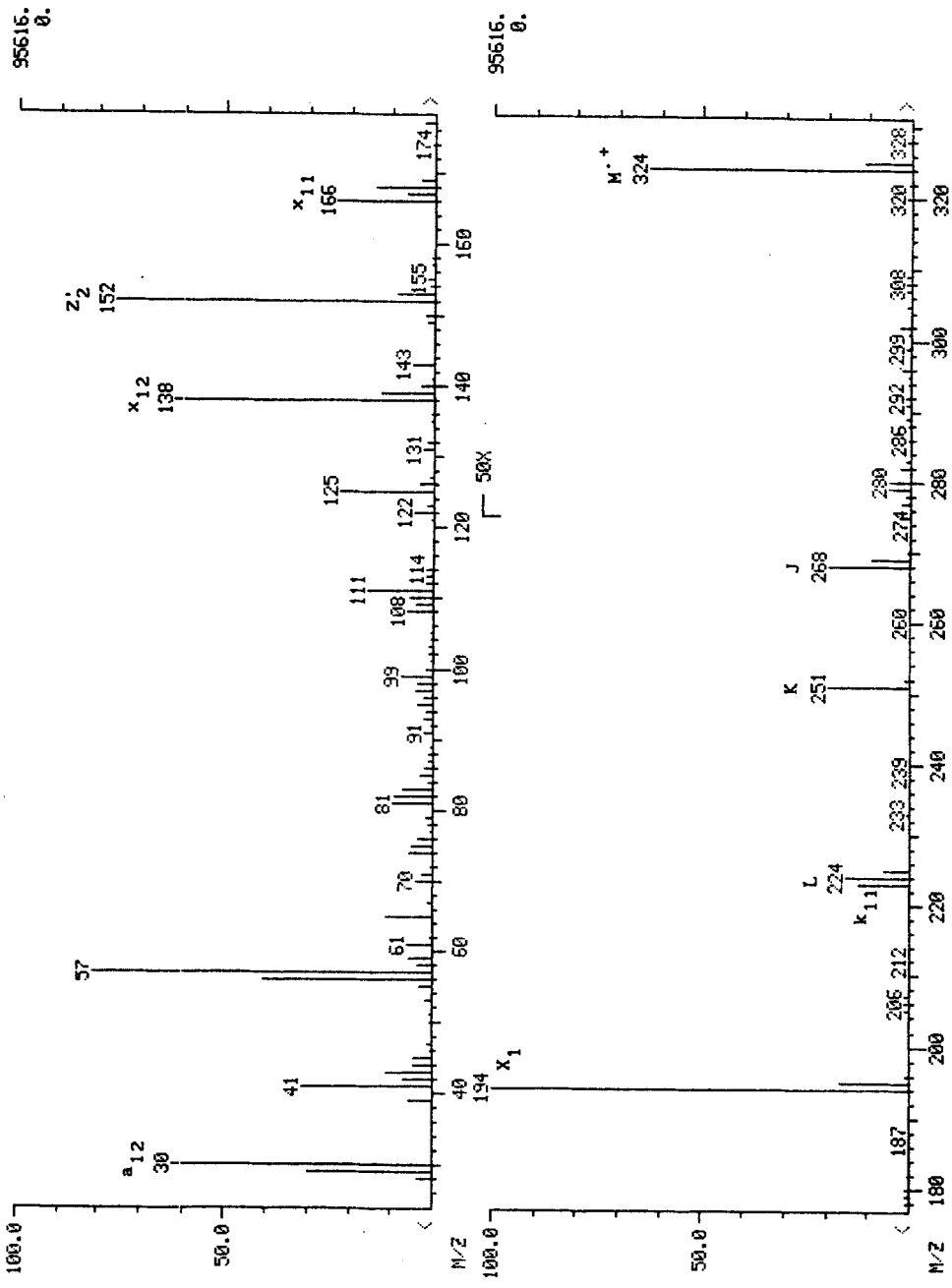


FIGURE 1 Electron ionization mass spectrum of **1a**.

mass spectral fragmentation has been based on an elementary composition of fragment ions (Table III) and metastable transitions (Table IV) in mass spectrum of **1a**. Molecular ion of **1a** (m/z 324) eliminates isobutylene to yield fragment ion *J* (m/z 268), consecutive or simultaneous loss of carbon dioxide produces ion *L* (m/z 224). The loss of *t*-butyloxy radical from molecular ion gives rise to ion *K* (m/z 251), which eliminates ethylene to produce ion k_{11} (m/z 223). Cleavage of C—CO bond gives rise to ion X_1 (m/z 194), from which by repeated elimination of ethylene the ions x_{11} (m/z 166) and x_{12} (m/z 138) are produced. The m/z 138 ion was found in the mass spectrum of diethyltrifluoroacetamidoalkylphosphonates where the structure of "protonated diethylphosphonate" was suggested.[15] The ion Z_2' (m/z 152) according to metastable transition is formed by the loss of NCO radical from ion X_1 (m/z 194).

Mass spectra of compounds **1b–d**, **2a–d**, **3** with substituents R_1 and R_2 point to the analogical way of fragmentation as **1a** but the intensities of ions of series *J*, *K*, *L* and X_1 are lower. Presence of R_1 = —CH_3 (**2a–d**, **3**) increases the intensity of the ions of series A_2. Intensities of ions a_{22} are less than 2% r.i., only for **2a** 6.2% r.i. In the mass spectra of compounds with at least one methyl substituent R_1 or R_2 (**1b**, **2a–d**, **3**) one of the most intensive fragment ions is m/z 44, for which in the case of **1b** we have proposed these ions that belong to series A_1 while in the case of **2a–d** and **3** to series A_2. Compounds **1d**, **2d**, **3** containing benzylic group have produced ions Z_1' and ions of series *N*. Intensities of the ions *N* and n_{11} have not been higher than 2% r.i. Only compounds **2a–d** have produced low intensity ions B_1 (1–2% r.i.).

Mass-spectral fragmentation of **3** has corresponded to **2d**. Phosphorus-containing fragment ions have been low to medium intensity. The most prominent of them in the spectra of **1a–d** have been ions m/z 125 (5–22% r.i.), in spectra **2a–d** m/z 138 (5–13% r.i.) and m/z 110 (7.6% r.i.) in the spectrum of **3**. These ions may be produced from ions Z_2'.

TABLE III

Composition of selected ions in mass spectrum of **1a**

type of ion	m/z	composition
L	224.0910	$C_7H_{17}N_2O_4P$
k_{11}	223.0477	$C_6H_{12}N_2O_5$
X_1	194.0586	$C_6H_{13}NO_4P$
x_{11}	166.0258	$C_4H_9NO_4P$
x_{12}	137.9952	$C_2H_5NO_4P$

TABLE IV

Selected metastable transitions in mass spectrum of **1a**

m^*(exp.)	$m_1 \longrightarrow m_2$ + (m_1-m_2)		
m/z	m/z	m/z	
166.7	166 x_{11}	138 x_{12}	28
195.7	194 X_1	152 Z_2'	42
194.8	194 X_1	166 x_{11}	28

^1H NMR Spectra

The NMR spectra of aminoalkylphosphonic acids or their dipeptides have been used to confirm their structure in the framework of synthetic papers. Systematically, the ^{13}C and ^{31}P NMR spectra of aminoalkylphosphonic acids were investigated[16,17] and dependence of the chemical shift of aminoalkyphosphonic acids on pH was studied.[18] The ^1H NMR spectra of the phosphonodipeptides in this paper were run in D_2O at concentration 0.1 M that corresponded to pD 5–6. It means that the phosphonodipeptides were in solution in the form of zwitterions. The ^1H NMR parameters are given in Table V. In the glycine range the hydrogen atoms of $—CH_2—$ moieties of aminomethylphosphonic acid have been magnetically nonequivalent as it was found with common dipeptides.[19,20] But this nonequivalence is higher in phosphonodipeptides and increasing in the order R = $—CH_3$, $—CH_2CH(CH_3)_2$, $—CH_2Ph$ (Table VI). These higher nonequivalences are likely

TABLE V

Proton NMR parameters of phosphonodipeptides in D_2O (δ in ppm and J in Hz)

	$^+ND_3$-CH(R^2)-CO-					-ND-CH(R^1)-PO_3D^-					
	H(α)	H(β)	$^3J_{\alpha,\beta}$	$^5J_{\alpha,P}$	$^2J_{\beta,\beta}$	H(α)	H(β)	$^2J_{\alpha,\alpha}$	$^3J_{\alpha,\beta}$	$^2J_{\alpha,P}$	$^3J_{\beta,P}$
4a	3.85d	-	-	1.1	-	3.48dd	-	-	-	12.3	-
4b	4.10q	1.54d	7.1	-	-	3.51dd 3.38dd	-	15.2	-	12.6 12.1	-
4c[a]	4.02dd	1.64- -1.81m	7.7 7.0	-	b	3.59dd 3.32dd	-	15.3	-	12.9 11.8	-
4d[c]	4.25dd	3.27dd 3.17dd	6.8 7.6	-	14.2	3.60dd 3.18dd	-	15.2	-	13.1 11.8	-
5a[d]	3.84dd 3.80dd	-	-	1.2 1.2	-	4.08dq	1.31dd	-	7.3	14.7	15.1
5b S,R	4.07dq	1.55dd	7.0	0.6	-	4.08dq	1.32dd	-	7.3	15.2	15.1
5b S,S	4.05q	1.52d	7.0	-	-	4.09dq	1.31dd	-	7.3	14.1	15.1
5c[a] S,R	3.99t	1.60- -1.83m	7.2 7.2	-	b	4.06dq	1.31dd	-	7.3	15.1	15.1
5c[a] S,S	3.98t	1.56- -1.86m	7.3 7.3	-	b	4.11dq	1.31dd	-	7.3	15.0	15.1
5d[c] S,R	4.21dd	3.39dd 3.12dd	5.1 8.8	-	14.6	4.07dq	1.34dd	-	7.3	15.1	15.1
5d[c] S,S	4.16dd	3.24dd 3.14dd	6.6 8.6	-	13.6	3.98dq	1.03dd	-	7.3	14.7	15.1

a - Leucyl methyl protons: 0.95d and 0.97d with J=6.2-6.5 Hz

b - The value of parameter could not be determined due to the overlap of strongly coupled H(β) and H(γ) protons

c - Aromatic protons: 7.26-7.42m

d - $^2J_{\alpha,\alpha}$ of glycine protons is 16.0 Hz

TABLE VI

Differences of chemical shift of diasteroisomeric glycine protons in common dipeptides[20] and phosphonodipeptides

phosphonodipeptide		common dipeptide
	Δδ(Hz)	Δδ(Hz)
4b	0.13	0.12
4c	0.27	0.19
4d	0.42	0.31
5a	0.04	-

due to stronger interaction of $—ND_3^+$ and $—PO_3D^-$ in preferred dipeptide conformation[20] which is caused by the larger size and higher hardness of phosphonic group in contrast to the carboxylic group. The splitting of ^1H NMR signal was observed even at $—CH_2—$ moiety of glycine at **5a** which was not found at gly-L-ala.[20] The differences in chemical shifts of the protons of diastereoisomeric pairs of phosphonodipeptides are small but evident and again are increasing in the expected way in the order R = $—CH_3$, $—CH_2CH(CH_3)_2$, $—CH_2Ph$. The α and β protons of the phosphonic part of molecules are split with coupling constants $^2J_{HP}$ = 11.8–15.2 Hz and $^3J_{HP}$ = 15.1 Hz. At **4a**, **5a** and *S,R*-**5b** it was possible to observe even non-zero five-bond long range coupling protons with phosphorus $^5J_{PH}$ = 1.1; 1.2; 0.6 Hz.

EXPERIMENTAL

All solvents (Merck or Lachema) were dried with $LiAlH_4$ and EtOH with CaO and Mg and were stored over molecular sieves. The protected amino acid were obtained from Léčiva (Prague). $TrNH_2$, $TrN{=}CH_2$, $TrN{=}CHCH_3$ and $TrNHCH(CH_3)PO(OMe)_2$ were synthesized according to Mastalerz *et al.*[9] The hydrate of HOBt and Boc-amino acids were dried by dissolving in dry dioxane and distillation in vacuum. All melting points were uncorrected. Optical rotations were measured with ETL-NTL Automatic Polarimeter 143 A. The TLC chromatography was carried out on "Silufol" sheets (Kavalier), solvent A—ethylacetate:ethanol = 19:1; B—ethylacetate:ethanol:pyridine = 19:1:1; C—ethylacetate:toluene = 1:1; D—ethylacetate:toluene:pyridine = 5:5:1 for protected phosphonodipeptides and solvent *i*-PrOH:25% NH_3:water = 7:3:3 for free phosphonodipeptides; detection with 0.5% ninhydrine solution in EtOH.

Low resolution mass spectra were recorded on Incos 50 (Finnigan MAT) mass spectrometer, ionizing electron energy 70 eV, ion source temperature 150°C. Samples were evaporated from a direct exposure probe (heating rate 10 mA/sec). Elementary compositions and metastable transitions for **1a** were recorded on a Jeol DMS-100 mass spectrometer (energy of ionizing electrons 70 eV, ion source temperature 150°C, peak matching ±2 mmu, metastable defocusing). Samples were evaporated from direct probe at 110–120°C. Mass spectra of **1a** from Incos 50 and Jeol DMS-100 were in very good agreement with exception of molecular ion that were not observed on Jeol DMS-100. The ^1H NMR spectra of the phosphonodipeptides were recorded using a Varian Unity 200 or 500 spectrometer at 200 or 500 MHz (D_2O, Reference DSS). The ^1H NMR spectra of tritylated esters of aminophosphonic acids were recorded with Tesla BS 587A spectrometer at 80 MHz and in $CDCl_3$ solution with TMS as standard.

N-(triphenylmethyl)aminomethyldiethylphosphonate. A mixture of $TrN{=}CH_2$ (27.1 g; 0.1 mol) and diethylphosphite (38.6 mL; 0.3 mol) was heated at 90–100°C for 40 minutes. After cooling the first part of crystal product was filtered and the excess of diethylphosphite was evaporated. The next fraction of a crude product crystallized at 0°C after dissolving of the residue in hot methanol. Both fractions were recrystallized from hot methanol to give 28.7 g (85%); m.p. 115–170°C (see Reference 9 115–117°C).

^1H NMR: 1.31 (t, $^3J_{HH}$ = 7.1, $OCH_2\underline{CH_3}$, 6H); 2.14 (bs, $\underline{NH}$, 1H); 2.50 (d, $^2J_{PH}$ = 13.8; $N\underline{CH_2}P$, 2H); 3.98–4.35 (m, $PO\underline{CH_2}CH_3$, 4H); 6.97–7.59 (m, Ph, 15H)

N-(triphenylmethyl)-1-aminoethyldiethylphosphonate. A mixture of $TrNH_2$ (13.2 g; 0.05 mol) and diethylphosphite (6.5 mL; 0.05 mol) in 50 mL of benzene was refluxed in Dean-Stark apparatus. To this refluxing solution a solution of acetaldehyde (2.8 mL; 0.05 mol) in 30 mL benzene was added by syringe through septum dropwise, very slowly during 8 hrs. After cooling the benzene was evaporated and the residue was recrystallized from methanol solution to give 15.5 g (73%), m.p. 145–7°C.

^{1}H NMR: 0.46 (dd, $^3J_{HH}$ = 6.7, $^3J_{PH}$ = 18.4, $\underline{CH_3}CHP$, 3H); 1.34 (t, $^3J_{HH}$ = 7.1, $OCH_2\underline{CH_3}$, 6H); 1.77 (bs, $\underline{NH}$, 1H); 2.61–3.31 (m, $P\underline{CH}CH_3$, 1H); 4.01–4.31 (m, $O\underline{CH_2}CH_3$, 4H); 6.93–7.35 (m, Ph, 9H); 7.45–7.72 (m, Ph, 6H)

Phosphonodipeptides (**1a–d**, **2a–d**, **3**, **4a–d**, **5a–d**). *General procedure. N*-(triphenylmethyl)-aminoalkyldiethylphosphonate (0.015 mol) was refluxed in 60 mL of dry 1M HCl/ethanol for 15 minutes. Then ethanol was evaporated in vacuum at a temperature of bath less than 30°C, and excess of HCl was removed by co-distillation with dioxane. The oil product was extracted 3 × 30 mL with diethylether. Then the oil was dissolved in 20 mL of THF, cooled to 0°C and the hydrochloride was neutralized with TEA (2.1 mL, 0.016 mol). The appropriate protected amino acid (0.015 mol) and HOBt (2.6 g, 0.016 mol) were dissolved in 40 mL of THF, the solution was cooled to −5°C and a solution of DCC (3.30 g; 0.016 mol) in 10 mL of THF was added. The protected amino acid was activated at −5°C for 40 minutes. To this solution of the activated protected amino acid was filtered the solution of aminophosphonate and the mixture was stirred at 0°C for 2 hrs. and then at r.t. for 22 hrs. Then dicyclohexylurea was filtered and THF evaporated in rotary evaporator. The residue was purified twice on silica gel column. The first using solvent mixture A (for Gly and L-Ala dipeptides) or C (for L-Leu and L-Phe dipeptides) and then solvent mixture B or D, respectively. Using this way the protected phosphonodipeptides were obtained pure.

The removal of the protecting moieties was done after dissolving the protected phosphonodipeptides in 20 mL 40% HBr/AcOH and the mixture was stirred at r.t. overnight. Gas products were removed in vacuum and the remained HBr by co-distillation with dioxane. The oil product was dissolved in water, decolored with charcoal and poured on the column (2 × 15 cm) with DOWEX 50 W × 4 (50–100 mesh) in H^+ form. The column was eluted with water (500 mL) and then with 20% py/H_2O. Fractions containing the phosphonodipeptides were evaporated and again dissolved in water. The solution was then evaporated to dryness (removal of pyridine) and then recrystallized from water by addition of ethanol or acetone.

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