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Simultaneous analysis of biologically active aminoalkanephosphonic acids

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Abstract

A new approach for simultaneous analysis of biologically active aminoalkanephosphonic acids, namely glyphosate, phosphonoglycine, phosphonosarcosine, phosphonoalanine, phosphono-β-alanine, phosphonohomoalanine, phosphono-γ-homoalanine and glufosinate, is presented. This includes a preliminary ^{31}P NMR analysis of these amino acids, their further derivatization to volatile phosphonates (phosphinates) by means of trifluoroacetic acid–trifluoroacetic anhydride–trimethyl orthoacetate reagent and subsequent analysis of derivatization products using MS and/or GC–MS (chemical ionization and/or electron impact ionization).
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1. Introduction

Aminoalkanephosphonic acids constitute a unique class of simple mimetics of natural amino acids. As a result of the structural analogy of these two classes of amino acids, aminoalkanephosphonates exhibit strong and diverse biochemical activity, displayed spectacularly in agrochemistry and pharmacology areas. The most representative examples of biologically active phosphonic and phosphinic analogs of amino acids, their application and natural occurrence are summarized in Table 1.

Due to the above mentioned bio-applications, access to a reliable and sensitive method for their determination presents an important topic of modern environmental analytical chemistry [11,17–19]. As a consequence, a number of procedures on simultaneous determination of various aminophosphonates have been reported in the literature, the majority based on chromatographic techniques for separations [20–37].

Recently we have reported on the determination of glyphosate (PMG) and its metabolites via their prior conversion into phenylothiocarbamoylo (PTC) compounds and subsequent HPLC–UV determination of the formed PTC-derivatives [20]. In the alternative method the conjuncted N-trifluoroacetylation and P-esterification of PMG and its metabolites in a

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Table 1
The list of representative biologically active aminophosphonic and aminophosphonic acids [1,2]

Amino acids		Biological activity	Application	Natural occurrence
Abbrev.	Structure			
Gly[P]	$H_2N{-}CH_2{-}P(O)(OH)_2$	Herbidical [3]	Herbicide [4,5]	As a result of agro-application
Sar[P]	$CH_3{-}NH{-}CH_2{-}P(O)(OH)_2$	Herbicidal	Post-emergence [6] herbicide	As a result of agro-application PMG metabolite
β-Ala[P]	$H_2N{-}(CH_2)_2{-}P(O)(OH)_2$	Inhibitor of many enzymes		Wide broad natural occurrence [7]
Ala[P]	$H_2N{-}CH(CH_3){-}P(O)(OH)_2$	Inhibitor of many enzymes, bacterial cell wall synthesis	In AlaAla[P] as antibiotic [8–10]	
Hal[P]	$H_2N{-}CH(C_2H_5){-}P(O)(OH)_2$	Herbicidal [11]	A cereal seed dressing [11]	As a result of agro-application
γ-Hal[P]	$H_2N{-}(CH_2)_3{-}P(O)(OH)_2$	Antibacterial [12] against *E. coli*, *S. aureus*, *P. aeruginosa*		
PMG	$HOOC{-}CH_2{-}NH{-}CH_2{-}P(O)(OH)_2$	Herbicidal [16]	Herbicide [13,14] (Glyphosine, Round-up)	As a result of agro-application
Gluf	$HOOC{-}CH(NH_2){-}(CH_2)_2{-}P(O)(OH)(CH_3)$	Herbicidal [16]	Herbicide [15] (Glufosinate, Basta)	As a result of agro-application

mixture of trifluoroacetic acid–trifluoroacetic anhydride–trimethyl orthoacetate (TFA–TFAA–TMOA) was employed, with subsequent GC–MS analysis of the formed in situ TFA–aminophosphonates [37]. These derivatizations of aminophosphonic acids are illustrated schematically in Fig. 1.

In this paper we present our results on the analysis of the set of representative biologically active aminophosphonic (aminophosphinic) acids, occurring naturally or introduced to biological samples or to the environment. The method includes the prior derivatization of examined amino acids to volatile compounds by means of the TFA–TFAA–TMOA reagent and their subsequent GC–MS separation determination of the **2** derivatives formed.

The comparison of reported methods on derivatization of aminophosphonic (aminophosphinic) acids via their prior N-acylation and subsequent P–O esterification by means of the carboxyl acid–anhydride–orthoester is given in Table 2.

2. Experimental

2.1. Materials

Aminomethylphosphonic acid (**1a**, Gly^P), *N*-methylaminomethylphosphonic acid (**1b**, Sar^P) were prepared according to Refs. [38,39]. 3-Aminopropanephosphonic acid (γ-Hal^P, **1f**), prepared according to Ref. [40], was kindly supplied by Dr. W. Perlikowska (CMMS, PAS, Łódź, Poland). 1-Aminoethanephosphonic acid (Ala^P, **1c**), 1-aminopropanephosphonic acid (Hala^P, **1e**) were prepared according to Ref. [41]. 2-Aminoethanephosphonic acid (β-Ala^P, **1d**), phosphonomethyl-glycine (PMG, **1g**), and other reagents were purchased from Aldrich (Milwaukee, WI, USA). DL-Homoalanine-4-yl-(methyl)phosphonic acid (Gluf, **1h**), purchased from Riedel-de Haen (Rdh Laborchemikalien, Seelze, Germany), was kindly supplied by the Institute of Agrochemistry (Skierniewice, Poland).

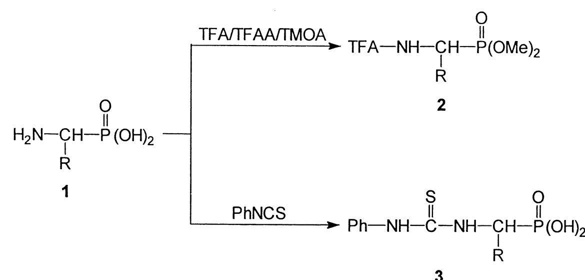


Fig. 1. Scheme of derivatization of aminoalkanephosphonic acids prior to their GC or HPLC analysis.

*2.2. Derivatization of amino acids **1** into O,O-dimethyl N-acylaminoalkanephosphonates **2***

The conversions of amino acids **1a–1h** into volatile derivatives **2a–2h**, were carried out in Wheaton 1-ml micro product V-Vials, equipped with a spine vane, placed in a thermostated oil bath. In order to achieve this conversion, the samples of amino acids (0.1 to 5 mg) were dissolved in trifluoroacetic acid (0.05 ml)–trifluoroacetic anhydride (0.05 ml) mixture and the solutions were heated with stirring at 40 °C for 90 min. Then trimethyl orthoacetate (0.40 ml) was carefully added and the resulting reaction mixtures were stirred at 100 °C for 1.5 h (the derivatizations have usually been completed after 1 h). Such derivatization mixtures were analyzed directly using ^{31}P NMR, GC–MS and MS techniques or pre-concentrated in vacuo (20 °C, 21 Torr, 30 min or 50 °C, 21 Torr, 30 min; 1 Torr=133.322 Pa) prior to their GC–MS or MS analysis.

*2.2.1. Derivatization of amino acids **1** occurring in a real solution*

In a supplementary approach, aqueous solutions of eight-component mixtures of amino acids **1a–1h** (8×0.5 mg of each amino acid in 1 l of distilled water fortified with 2 mmol of prepared in situ calcium hydrogen carbonate), simulating real solutions of these amino acids were applied. These solutions were refluxed for 0.5 h, preconcentrated by evaporation in vacuum (20 °C, 1 Torr) to ca. 20–50 ml, filtered off and subsequently lyophilized to dryness. The residues were finally derivatized under conditions described above.

*2.3. Derivatization of **1** by means of the AcOH–Ac_2O–TMOA reagent*

For derivatization of amino acids **1** into N-acetylaminoalkanephosphonates **4**, the samples of

Table 2
Comparison of the methods of derivatization of aminophosphonic (aminophosphinic) acids into O,O-dialkyl N-acylaminophosphonates by means of the carboxyl acid–anhydride–orthoester system

Derivatized amino acids	Derivatization system	Application detection/(L.D.)	Ref.
Gluf, MPPA (soil)	AcOH–TMOA	Anal.; NPD	[21]
MPPA, PMG, Gluf (crops)	AcOH–TMOA	Anal.; GC–FPD/ GC–EI-MS (0.02 mg/kg)	[22]
MPPA, AMPA, Gluf, PMG, BIAL (water)	AcOH–TMOA	Anal.; GC–EI-MS (0.05–14 mg/l)	[23]
AMPA, PMG (soil)	TFA–TFE	Anal.; GC–NPD (0.01–0.05 mg/kg)	[24]
AMPA, PMG (plants)	TFA–TFE	Anal.; GC–NPD (0.01–0.03 mg/kg)	[25]
AMPA, PMG (soil)	TFA–TFE		[26]
AMPA, PMG (water, soil)	TFA–TFE	Anal.; GC–EI-MS (0.003–0.05 μg/l)	[27]
AMPA, MPA, PMG, Gluf, BIAL (water)	TFA–TFE	Anal.; GC–ECD, GC–EI-MS (0.09–17 μg/l)	[28]
AMPA, PMG	TFA–TFE TFE–HFBA	Anal.; GC–FPD, GC–ECD	[29]
AMPA, PMG (various)	TFA–HFB	Anal.; GC–EI-MS, GC–CI-MS (0.01 mg/kg)	[30]
AMPA, PMG	TFA–HFB	Anal.; GC–EI-MS (0.003–0.05 μg/l)	[31]
1 (R = Me, Et, Pr, iPr, Ph, Bz)	TEOF	Synthetic	[32]
1 (R = Me, Et, Pr, iPr, Ph, Bz)	TFA–TMOE TFA–TEOF	Anal.; GC–CI-MS, GC–EI-MS	[33]
1 (R = Me, Et, Pr, iPr, Ph, Bz)	TFA–TFAA–OE AcOH–Ac_2O–OE	Anal.; GC–CI-MS, GC–EI-MS	[34]
1 (R = Me, Et, Pr, iPr Ph, Bz)	TFA–TFAA–OE AcOH–Ac_2O–OE PivOH–Piv_2O–OE	Anal.; GC–CI-MS, GC–EI-MS, MS-DCI	[35]
1 (R = Me, Et, Pr, iPr, Ph, Bz)	TFA–TFAA–OE AcOH–Ac_2O–OE	Synthetic	[36]
PMG, Gly^P, Sar^P, MPA	TFA–TFAA–TMOA	Anal.; GC–CI-MS, GC–EI-MS, MS-DCI	[37]

MPPA, 3-(hydroxymethylphosphinyl)propane acid; MPA, methylphosphonic acid; AMPA, aminomethylphosphonic acid; BIAL, bialaphos; TFE, trifluoroethanol; HFBA, heptafluorobutyrate; HFB, heptafluorobutanol; Gluf, glufosinate; TEOF, triethyl orthoformate; OE, orthoester; PivOH, pivalic acid; Piv_2O, pivalic anhydride; NPD, nitrogen–phosphorous detection; FPD, flame photometric detection; ECD, electron-capture detection.

amino acids (ca 0.05 mmol) were dissolved in acetic acid (0.05 ml)–acetic anhydride (0.05 ml) mixture (110 °C stirring, 0.5 h), followed by the addition of trimethyl orthoacetate (0.40 ml) and heating at 110 °C for 2 h. Such derivatization mixtures were analyzed directly using ^{31}P NMR and GC–MS or pre-concentrated in vacuo (50 °C, 21 Torr, 30 min) prior to their GC–MS analysis.

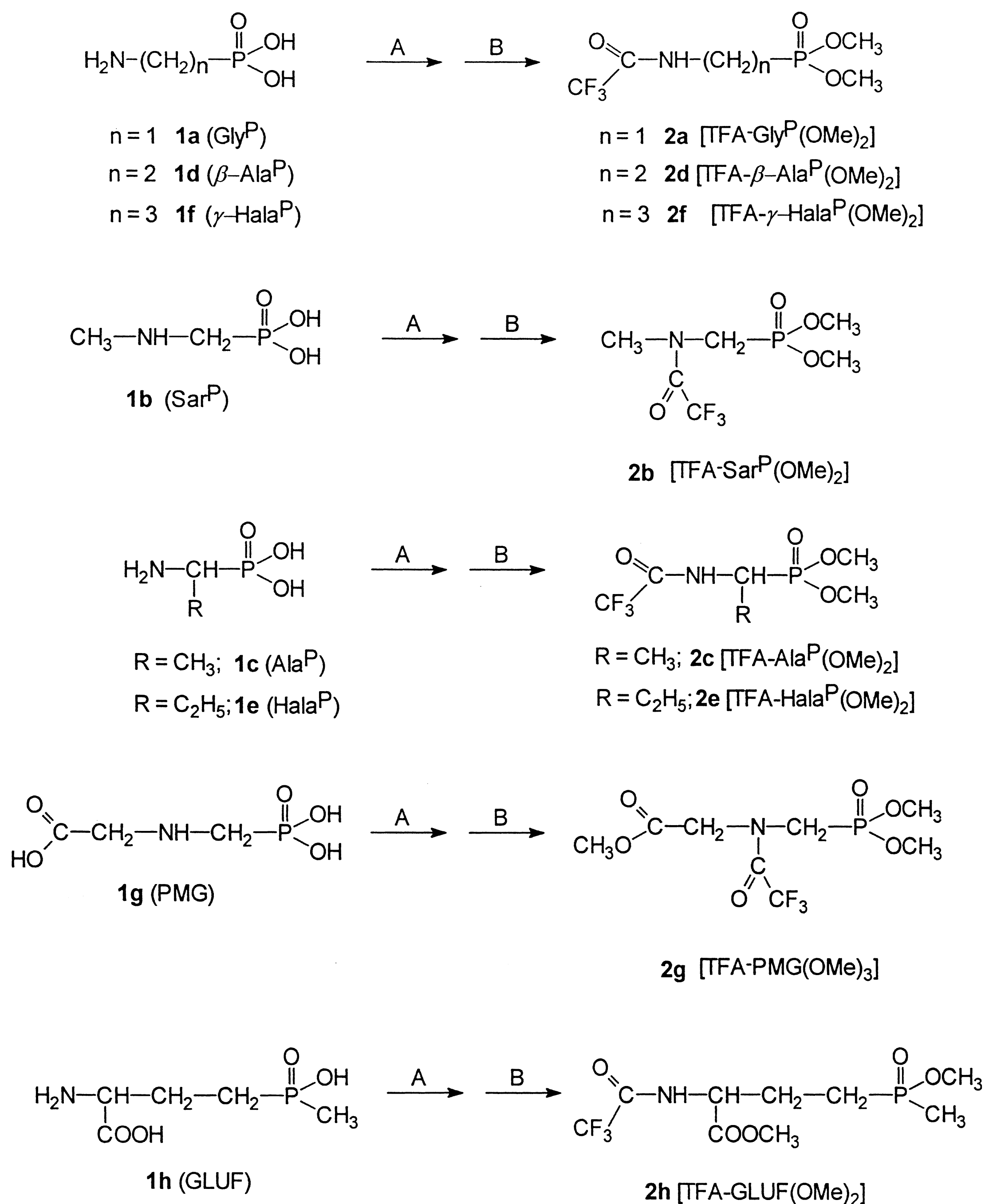


Fig. 2. Scheme of derivatization of amino acids **1a–1h** by means of the TFA–TFAA–TMOA reagent.

2.4. Instrumental analysis

The Finnigan MAT 95 mass spectrometer was used for GC–MS [chemical ionization (CI) and/or electron impact ionization (EI) MS] analysis of derivatization products. Sample introduction was via the Varian 3400 gas chromatograph equipped with a DB-1 (thickness 0.25 μm) and/or DB-17 (thickness 0.50 μm) capillary column (30 m×0.25 mm I.D.), manufactured by JIF Scientific (Folsom, CA, USA). The carrier gas (helium) was passed through the columns with flow-rate 0.7 ml/min. The injector temperature was maintained at 200 °C and the transfer line temperature was 250 °C. The column was directly introduced to the ion source of the mass spectrometer. EI-MS mass spectra were recorded at an electron energy of 70 eV. CI-MS spectra were recorded using isobutane as a reacting gas.

The GC quantitative analysis was performed on an Ai Cambridge GC 95 gas chromatograph, equipped with a flame ionization detection (FID) system and a DB-17 capillary column (30 m×0.25 mm I.D.). The injector temperature was maintained at 260 °C and the detector temperature at 280 °C. The conditions applied for separation in both the GC–MS and GC–FID mode are given in the legends of Tables 6 and 8.

^{1}H NMR and ^{31}P NMR spectra were recorded on a Bruker AC 200 spectrometer operating at 81.01 MHz and 200 MHz, respectively.

2.5. Potentiometric measurements

The dissociation (protonation) constants of amino acids **1** were determined by pH-metric titration by means of an automatic titrator EMU connected to an IBM personal computer (Technical University of Wroclaw, Poland), fitted with a combined glass–calomel electrode TRIZMA E 5259 (Sigma). The electrode system was calibrated by the use of standard solutions ($2<pH<10$), so that the pH-meter readings could be converted into hydrogen-ion concentrations. In all cases, the temperature was 20±0.2 °C. The exact concentration of amino acid solutions was determined by titration, the concentrations (in samples of 4 ml) were ~5×10^{-3} *M*. The ionic strength was adjusted to 0.1 mol/dm^3 with potassium nitrite. The titrations (100 to 200 measurements with increment of 0.002 ml) were performed over the pH range 1–11, with a hydrochloric acid solution of known concentration (ca. 0.4 *M* HCl; $1<pH<2$), and with a potassium hydroxide solution of known concentration (ca. 0.4 *M* KOH, $2<pH<11$), respectively.

3. Results and discussion

3.1. Derivatizations/^{31}P NMR investigations

The proposed method of simultaneous determination of amino acids **1a–1h** is based on their quantitative conversion into corresponding, fully protected derivatives **2a–2h** (Fig. 2), followed by subsequent GC separation and CI-MS and/or EI-MS detection/quantification.

These conversions, carried out by means of the TFA–TFAA–TMOA reagent, in conditions applied by us recently for the derivatization of PMG metabolites [37], have been routinely monitored by ^{31}P NMR [33–37], for verification of the quantitative course of every reaction. The corresponding ^{31}P NMR spectra of the derivatization mixtures of individual amino acids **1**, by means of the TFA–TFAA–TMOA reagent, are given in Fig. 3. The spectra of corresponding derivatization products of PMG, Sar^{P} and Gly^{P} were identical with those previously reported [37].

Analysis of all ^{31}P NMR spectra illustrates the structural dependence of **1** on the course of their derivatizations. Thus, the spectra corresponding to the derivatization mixture of amino acids **1** with the primary amino group usually exhibited quantitative conversion (excluding β-Ala^{P} and γ-Hala^{P}), reflected by the presence of the single phosphorus absorption. At the same conditions, the amino acids with the secondary amino group (PMG, Sar^{P}) afforded spectra with two phosphorous signals, resulting from the presence of two different tautomeric (conformational) forms [37]. Two signals on the ^{31}P NMR spectrum of TFA–Gluf(OMe)$_2$ derived from Gluf, originate from the diastereoisomeric nature of the parent amino acid **1h**. Spectral characterization of isolated TFA derivatives **2** is given in Table 3.

In contrast to the described derivatizations of α-aminophosphonic acids **1** [37], reactions of their β-

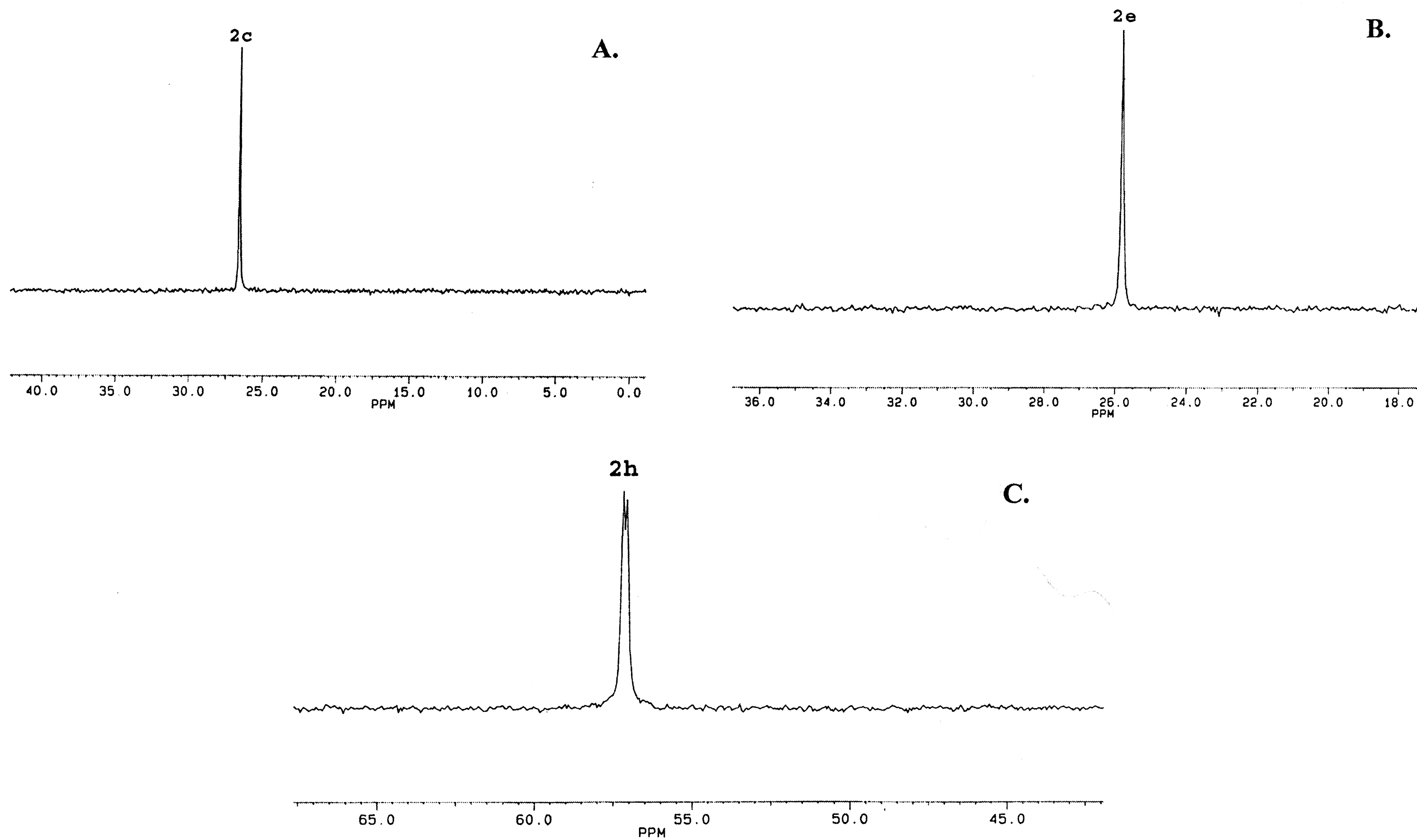


Fig. 3. Proton-decoupled phosphorus spectrum of the products of derivatization of Ala^{P} [**1c**→**2c**] (A), Hala^{P} [**1e**→**2e**] (B) and Gluf [**1h**→**2h**] (C), obtained by means of the TFA–TFAA–TMOA reagent.

Table 3
^{31}P NMR and mass spectral properties (CI-MS) of O,O-dimethyl TFA–aminoalkanephosphonates **2a–2h**

Derivatives		^{31}P NMR		CI-MS	
No.	Abbreviation	δ^{a} (ppm)	(%)[b]	m/z[c]	(%)[d]
2a	TFA–Gly^{P}(OMe)$_2$	25.4 [34]	100	236	100 [35]
2b	TFA–Sar^{P}(OMe)$_2$	23.8 [37]	94	250	100 [35]
2c	TFA–Ala^{P}(OMe)$_2$	26.8 [34]	100	250	100 [35]
2d	TFA–β-Ala^{P}(OMe)$_2$	31.6	100	250	100 [35]
2e	TFA–Hala^{P}(OMe)$_2$	26.3 [34]	100	264	100 [35]
2f	TFA–γ-Hala^{P}(OMe)$_2$	34.8	100	278	100
2g	TFA–PMG(OMe)$_3$	24.0 [37]	90	308	100 [37]
		23.7 [37]	10		
2h	TFA–Gluf(OMe)$_3$	57.8	100	306	100

[a] Recorded in $CDCl_3$.
[b] ^{31}P NMR yields.
[c] $m/z=[M+1]^+$ ions.
[d] Intensity of the ion.

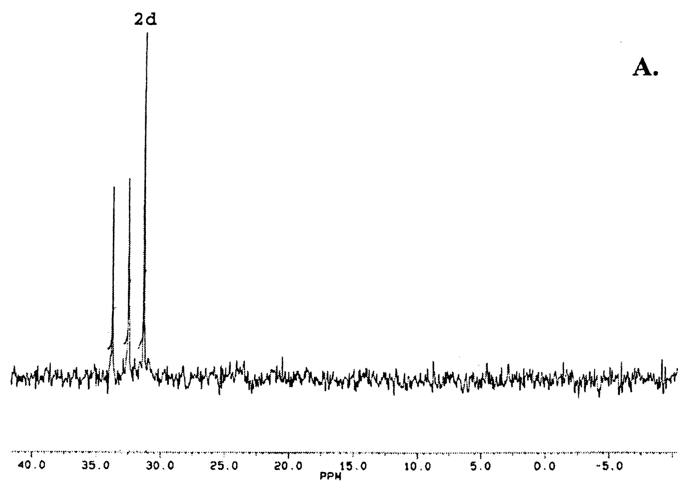
and γ-homologs with the TFA–TFAA–TMOA reagent proceeded by a more complicated course. Thus, the ^{31}P NMR analysis of the corresponding derivatization mixtures, derived from β-Ala^{P} and/or γ-Hala^{P} revealed the presence of three aminophosphonate signals (Fig. 4), identified subsequently by GC–CI-MS as TFA–aminoalkanephosphonates **2**, acetylaminoalkanephosphonates **4** and α-methoxyacetiminealkanephosphonates **5**. The comparison of the relative contents of phosphonates **2**, **4**, **5**, formed during derivatization of Gly^{P} ($n=1$), β-Ala^{P} ($n=2$) and γ-Hala^{P} ($n=3$) by means of the TFA–TFAA–TMOA, and other reagents, is given in Table 4.

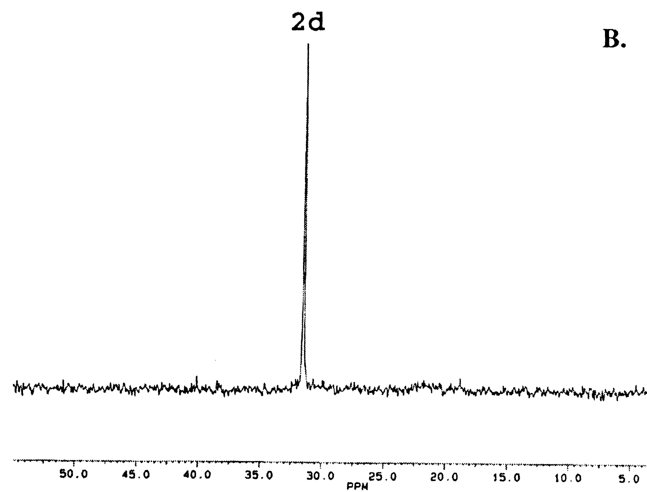
These data reflected substantial structural influence of **1** on the derivatization course, expressed by decrease of the relative content of TFA–aminoalkanephosphonates **2** with increase of the n factor value of the parent amino acids **1**. Thus, the corresponding ^{31}P NMR spectra revealed only one signal confirming the 100% formation of TFA–Gly^{P}(OMe)$_2$ (**2a**), the 40% formation of the TFA–β-Ala^{P}(OMe)$_2$ (**2d**) and 15% formation of TFA–γ-Hala^{P}(OMe)$_2$ (**2f**), respectively. These results can be explained on the basis of an induction effect interaction of intramolecular amino and acidic functions of amino acids, reflected by the pK data, shown in Table 5.

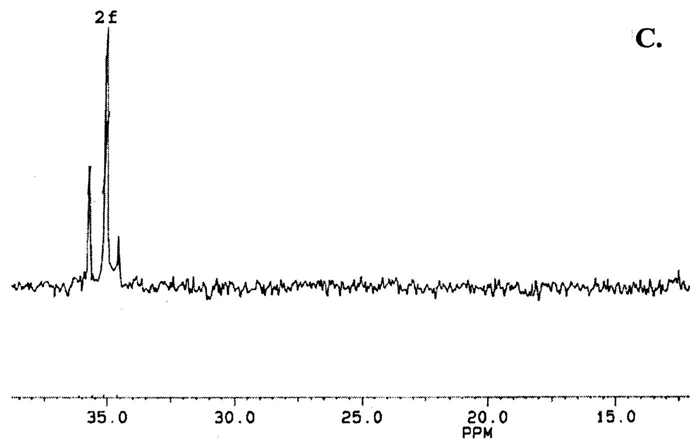
In additional experiments, we have found that the derivatization of **1d** and **1f** by means of the AcOH–Ac_2O–TMOA reagent [34,36] proceeded quantitatively at 110 °C, affording corresponding N-acetyl derivatives **4**. On the basis of these facts we assumed that the N-trifluoroacetylation of β-Ala^{P} and γ-Hala^{P} in the formerly applied conditions of stage A (40 °C, 10 min) is not quantitative. In fact, the stability of TFA salts **1A** of β-Ala^{P} (**1Ad**) and γ-Hala^{P} (**1Af**) (higher than these derived from α-amino acids) provides more sufficient protection of the β- and γ-amino functions during the N-trifluoroacetylation stage, and therefore prevents quantitative conversion of **1d** and **1f** into corresponding *N*-TFA derivatives **1B**. At the moment of addition of the TMOA reagent into the reaction mixtures of β-Ala^{P} and/or γ-Hala^{P} (stage B), these contain both *N*-TFA–aminoalkanephosphonates **1B** and TFA-salts of aminoalkanephosphonates **1A** which react subsequently with the TMOA reagent, affording *N*-TFA–aminoalkanephosphonates **2** (**1B**→**2**), and the compounds **4** and **5**. The later compounds—products of esterification of **1** by the TMOA reagent (**1A**→**4**+**5**)—were formed in a reaction similar to the previously described reaction, applied for derivatization of 1-aminoalkanephosphonic acids by means of the TFA–TEOF and TFA–TMOF reagents [34]. The estimated courses of these reactions are presented in Figs. 5 and 6.

The NMR confirmation of such reaction courses, however, cannot be directly supported by ^{31}P NMR spectra recorded in the TFA–acylation stage (A) of the derivatization, since these are too complex for unambiguous interpretation [44].

These results promote further search for modi-







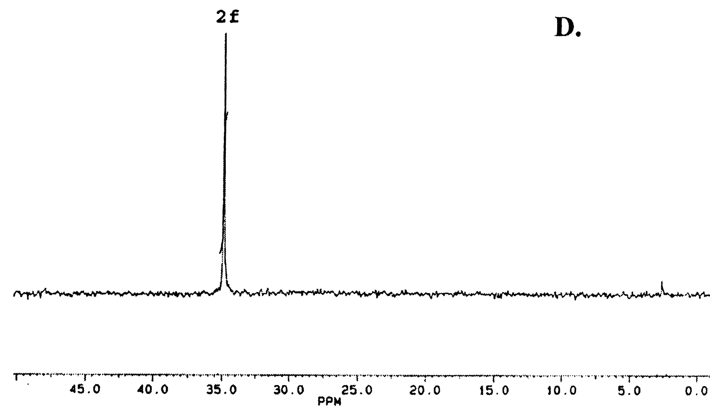


Fig. 4. Proton-decoupled phosphorus spectrum of the products of derivatization of β-Ala^{P} (**1d**→**2d**) [A (A: 20′, 40 °C) and B (A: 90′, 40 °C)] and γ-Hala^{P} (**1f**→**2f**) [C (A: 20′, 40 °C) and D (A: 90′, 40 °C)], obtained by means of the TFA–TFAA–TMOA reagent.

Table 4
Derivatizations of aminoalkanephosphonic acids **1a**, **1d** and **1e** by means of ACOH–$(AC)_2O$–TMOA reagents, carried out at various reaction conditions[a]

Conditions[a]	Derivatization products									
	n	$TFA-NH-(CH_2)_n-P(O)(OCH_3)_2$ **2**			$Ac-NH-(CH_2)_n-P(O)(OCH_3)_2$ **4**			$CH_3(CH_3O)C{=}N-(CH_2)_n-P(O)(OCH_3)_2$ **5**		
		Yield (%)[b]	^{31}P NMR δ^c (ppm)	CI-MS m/z^d	Yield (%)[b]	^{31}P NMR δ^c (ppm)	CI-MS m/z^d	Yield (%)[b]	^{31}P NMR δ^c (ppm)	CI-MS m/z^d
A	1	100	26.6 [34]	236	–	–	–			
C		–	–	–	100	25.4 [34]	182			
A	2	36	32.5	250	22	33.7	196	42	31.3	210
B		100	31.6	250	–	–	–	–	–	–
C		–	–	–	100	33.1	196	–	–	–
A	3	56	35.1	264	25	35.8	210	19	34.6	224
B		100	34.9	264	–	–	–			
C		–	–	–	100	35.2	210			

[a] Conditions of the N-acylation stage applied as follows: A, TFA–TFAA, 40 °C, 20 min; B, TFA–TFAA, 40 °C, 90 min; C, AcOH–Ac_2O, 100 °C, 20 min.
[b] ^{31}P NMR yields.
[c] In reaction mixture.
[d] $m/z=[M+1]/z$ (intensity of ions = 100%).

fication of derivatization conditions, in order to achieve the full *N*-TFA–acylation of β-Ala^P and γ-Hala^P, and further quantitative conversion of formed **1A** into corresponding volatile TFA derivatives **2**, namely TFA–β-Ala^P$(OMe)_2$ (**2d**) and TFA–γ-Hala^P$(OMe)_2$ (**2h**). In a new version of the procedure, based on the elongation of the *N*-TFA–acylation period, the conversions of β-Ala^P and γ-Hala^P into the corresponding derivatives **2** were, according to ^{31}P NMR, quantitative. The corresponding ^{31}P NMR spectra of the appropriate derivatization mixtures of **1**, obtained using these modified experimental conditions were identical with those illustrated in Fig. 3 and/or those presented previously [37].

In continuation, this procedure was extended for simultaneous derivatizations of a mixture of the title amino acids **1**, namely the mixture of Gly^P, Sar^P, Ala^P, β-Ala^P, Hala^P, γ-Hala^P, PMG and Gluf, respectively. The ^{31}P NMR spectrum of the representative derivatization mixture of mixtures of **1a–1h** is given in Fig. 7, and the corresponding GC–CI-MS chromatogram is given in Fig. 8.

We have found that the application of the AcOH–Ac_2O–TMOA reagent [34] for the simultaneous derivatization of the mixture of Gly^P, Sar^P, Ala^P, β-Ala^P, Hala^P, γ-Hala^P, PMG and Gluf leads to unsatisfactory results due to non-quantitative conversion of PMG into volatile Ac–$PMG(OMe)_3$ derivative.

3.2. Chromatographic investigation

The derivatives **2** can be stored for several weeks at ambient temperature without any trace of decomposition (^{31}P NMR). These compounds have been found to be suitable for GC and GC–MS analysis because of the shape of chromatographic peaks, reproducible retention data and informative mass spectral characteristics, both in CI and in EI modes. Retention time values of chromatographed trifluoroacetylaminoalkanephosphonates **2a–2g** and phosphinates **2h** are given in Table 6. The representative GC–CI-MS chromatogram obtained on a DB-17 column is presented in Fig. 8. The chromatogram is supplemented by the corresponding CI-MS and EI-MS spectra of separated derivatives **2**, pre-

Table 5
Negative logarithms of dissociation constants of amino acids and aminophosphonic acids[a,b]

Amino acid	pK				Ref.
	pK_1	pK_2	pK_3	pK_4	
Gly	2.34	9.60			[42]
Sar	2.04	10.47			[42]
α-Ala	2.34	9.69			[42]
β-Ala	3.60	10.19			[42]
γ-Hala	4.23	10.43			[42]
Gly^{P}	0.40	5.42	10.08		[43]
		5.47[c]	10.16[c]		[44]
	0.44				[45]
Sar^{P}	0.64				[45]
		5.36[d]	11.51[d]		[d]
α-Ala^{P}		5.76[c]	10.25[c]		[44]
	1.0	5.55	10.11		[46]
β-Ala^{P}		6.31[c]	11.05[c]		[44]
	1.12				[45]
α-Hala^{P}		5.76[c]	10.13[c]		[44]
	0.62	5.53	10.46		[47]
γ-Hala^{P}		6.99[c]	11.03[c]		[44]
	1.63				[45]
		6.90[d]	11.20[d]		[d]
PMG	0.78	2.29	5.96	10.98	[48]
		2.56	5.68	10.25	[49]
		1.88	5.37	10.03	[50]
		2.54[d]	5.42[d]		[d]
Gluf		2.54[d]	9.43[d]		[d]

[a] Dissociation constants were determined for the following dissociation equilibria:

for amino acids: $H_2L^+ \rightleftarrows HL \rightleftarrows L^-$

for aminophosphonic acids: $H_3L^+ \rightleftarrows H_2L \rightleftarrows HL^- \rightleftarrows L^{2-}$

$H_4L^+ \rightleftarrows H_3L \rightleftarrows H_2L^- \rightleftarrows HL^{2-} \rightleftarrows L^{3-}$ (for PMG)

$H_3L^{2+} \rightleftarrows H_2L^+ \rightleftarrows HL \rightleftarrows L^-$ (for Gluf)

[b] Determined potentiometrically at ambient temperature unless stated otherwise.
[c] Determined by ^{31}P NMR.
[d] This work.

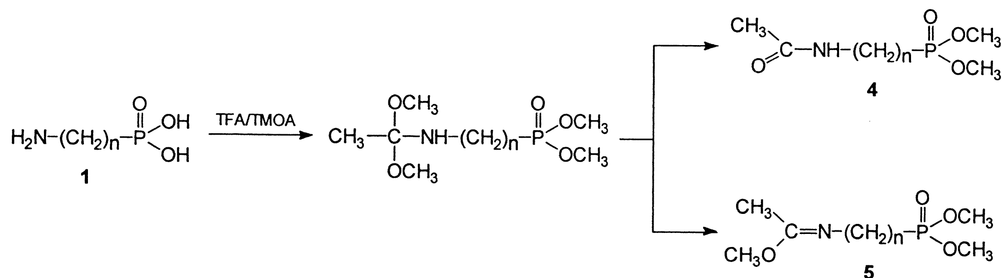


Fig. 5. Scheme of derivatization of aminoalkanephosphonic acids **1** ($n=1$, 2, 3) by means of the TFA–TMOA reagent.

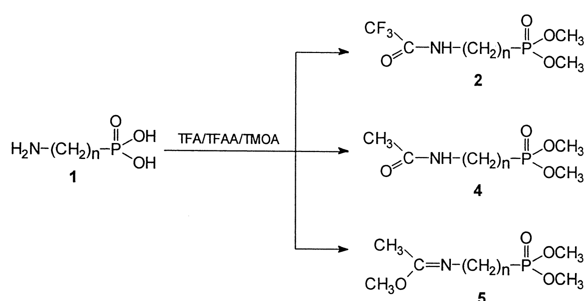


Fig. 6. Scheme of derivatization of aminoalkanephosphonic acids **1** ($n=1$, 2, 3) by means of the TFA–TFAA–TMOA reagent.

sented in Fig. 8, and supported by the corresponding partial EI-MS spectra summarized in Table 7.

The results of GC analysis of the derivatization mixture of starting amino acids **1a–1h**, illustrate the possibility of their simultaneous identification (determination) via prior derivatization of **1** by means of the TFA–TFAA–TMOA reagent and subsequent GC–MS (or GC–FID) analysis. Taking into account the linear relationship between detector response (GC–FID mode) and quantity of chromatographed **2**, these compounds can be determined quantitatively in real life samples as was demonstrated in our earlier work [37]. The detection limits of **2**, determined by GC–FID are presented in Table 8.

3.3. Mass spectral investigations

The partial mass spectra of N-acyl derivatives **2** are summarized in Tables 7 and 9. The N-acylaminoalkanephosphonates (phosphinates) present structural differences caused by (a) the constitution of the acyl group (acetyl or trifluoroacetyl), (b) the type of phosphorous function (phosphonate or phosphinate function), (c) the type of hydrocarbon side-chain attached to C_α, and also by the order of amino

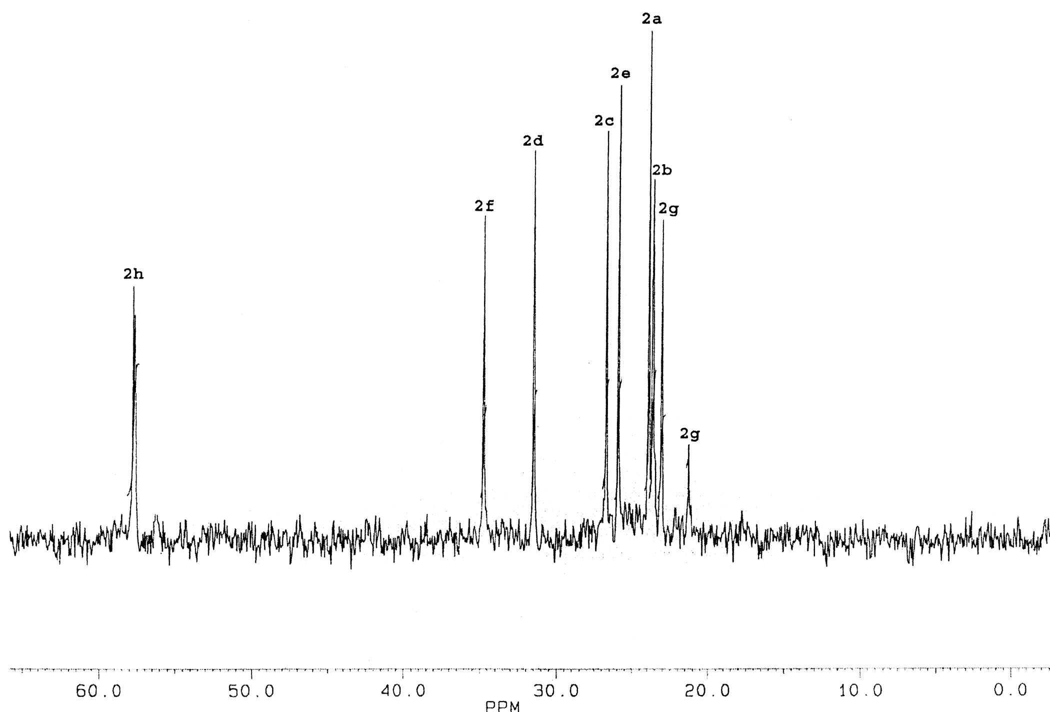


Fig. 7. Proton-decoupled phosphorus spectrum of the products of simultaneous derivatization of amino acids **1a–1h**, obtained by means of the TFA–TFAA–TMOA reagent. Conditions as described under Experimental.

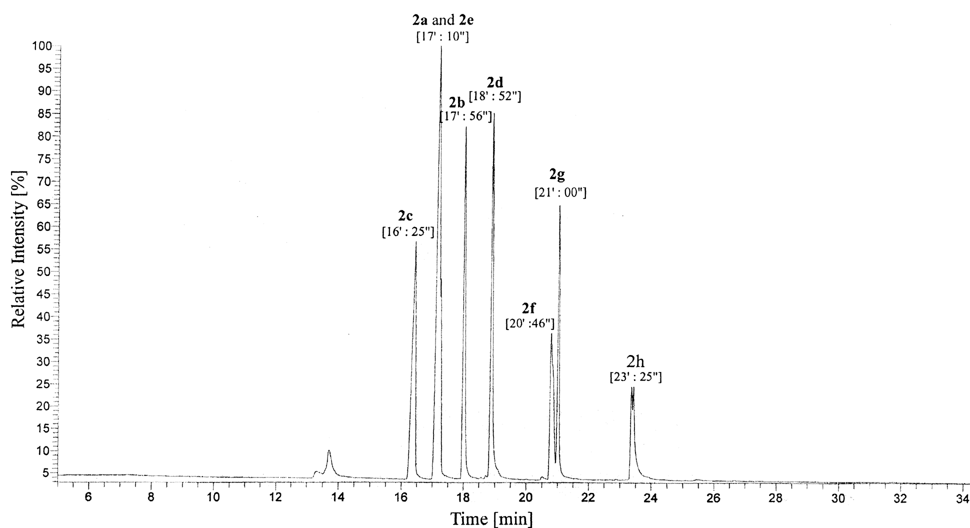


Fig. 8. GC–CI-MS chromatogram [relative intensity (%) vs. elapse time (retention time)] of the mixture of derivatization products **2a–2h**.

function (H_2N– or R–NH–) and the distance between the amino and acidic function (α-, β- or γ-aminophosphonic acids) of investigated amino acids. However, in spite of these structural differences, the whole groups of N-acyl derivatives of **1** present some common features, which are reflected by their fragmentation patterns, and consequently determine the shape of all the mass spectra.

Table 6
Comparison of chromatographic retentions of methyl esters of trifluoroacetylaminoalkanephosphonates[a] **2**, determined on DB-1 and DB-17 columns

Derivative		Chromatographic retention times[b,c] of **2** (min:s)							
No.	Abbreviation	DB-17					DB-1		
		B	C	D	E	F	A	B	D
2a	TFA–Gly^P(OMe)$_2$	17:10	10:37 10:17 [34]	12:17 12:20 [37]	13:58	16:36	16:56	14:12	7:24 [37]
2b	TFA–Sar^P(OMe)$_2$	17:59	11:05	12:54 [37] 12:55	14:44	17:31	17:42	14:21	7:24 [37]
2c	TFA–Ala^P(OMe)$_2$	16:25	9:31 9:35 [34]	11:22	12:56	15:30	16:48	14:04	
2d	TFA–β-Ala^P(OMe)$_2$	18:52	12:04	13:55	15:45	18:36	19:10	15:41	
2e	TFA–Hala^P(OMe)$_2$	17:22	10:24 [34] 10:37	12:19	14:02	16:47	18:35	15:11	
2f	TFA–γ-Hal^P(OMe)$_2$	20:46	14:01	15:53	17:46	20:42	21:15	17:00	
2g	TFA–PMG(OMe)$_3$	21:00	14:08	16:05 16:17 [37]	18:02	21:00	21:47	17:44	11:44 [37]
2h	TFA–Gluf(OMe)$_3$	23:25	16:28 16:31	18:26 18:31	20:27 20:30	23:27 23:29	24:23 24:35	19:35 19:42	

[a] In case of TFA–Gluf(OMe)$_3$–phosphinates.

[b] Determined at the following chromatographic conditions: A: 50 °C (5 min), gradient 7 °C/min, 250 °C (10 min); B: 50 °C (5 min), gradient 10 °C/min, 250 °C (10 min); C: 100 °C (3 min), gradient 10 °C/min, 250 °C (10 min); D: 100 °C (5 min), gradient 10 °C/min, 250 °C (10 min); E: 100 °C (7 min), gradient 10 °C/min, 250 °C (10 min); F: 100 °C (10 min), gradient 10 °C/min, 250 °C (10 min).

[c] Determined for 700 μl/min of flow velocity of carrier gas.

Table 7
Partial EI-MS spectra of N-trifluoroaminoalkanephosphonates[a] **2**

Derivative			Ions[b]: m/z (intensity [%])										
No.	Abbreviation	M_r	$[M]^{+\cdot}$	$[M-31]^+$	$[M-69]^+$	$[M-97]^+$	$[M-P]^+$	$[M-112]^+$	$[P]^{+\cdot}$		Others		Ref.
2a	TFA–Gly^P(OMe)$_2$	235	235	204	166	138	126	123	110	109	154	95	
			(8)	(2.0)	(32)	(4.0)	(18)	(0.8)	(100)	(35)	(13)	(19)	
			(13)	(3.3)	(72)	(2.4)	(28)		(100)				[37]
			(16)	(2.3)	(66)	(4.7)	(26)		(100)				[34]
2b	TFA–Sar^P(OMe)$_2$	249	249	218	180	152	140	137	110	109	124	94	
			(14)	(2.2)	(2.1)	(43)	(100)	(0.5)	(6.9)	(12)	(46)	(34)	
			(11)	(1.3)	(1.2)	(25)	(100)			(5.2)			[37]
2c	TFA–Ala^P(OMe)$_2$	249	249	218	180	152	140	137	110	109	127	95	
			(1.8)	(<0.2)	(0.6)	(0.5)	(17)	(0.9)	(100)	(15)	(7.0)	(12)	
			(1.4)	(<0.2)	(0.8)	(0.3)	(20)		(100)				[34]
2d	TFA–β-Ala^P(OMe)$_2$	249	249	218	180	152	140	137	110	109	120	94	
			(2.8)	(2.3)	(100)	(0.4)	(1.0)	(14)	(44)	(84)	(16)	(27)	
2e	TFA–Hala^P(OMe)$_2$	263	263	232	194	166	154	151	110	109	124[c]	95	
			(1.0)	(1.1)	(1.0)	(0.3)	(12)	(1.6)	(100)	(34)	(27)	(19)	
			(2.7)	(0.3)	(1.1)	(0.3)	(22)		(100)				[34]
2f	TFA–γ-Hal^P(OMe)$_2$	263	263	232	194	166	154	151	110	109	124[c]	94	
			(0.3)	(1.5)	(60)	(0.5)	(0.5)	(31)	(78)	(24)	(100)	(39)	
2g	TFA–PMG(OMe)$_3$	307	307	275	238	210	198		110	109	248[d]	124	
			(18)	(50)	(0.5)	(25)	(1.0)		(4.6)	(15)	(40)	(100)	
			(24)	(40)		(22)	(2.5)			(77)		(100)	[37]
2h	TFA–Gluf(OMe)$_2$	305	305	274	236	208	196	193	94	93	246[d]	237	
			(0.2)	(0.6)	(1.0)	(0.2)	(0.5)	(1.3)	(20)	(25)	(100)	(1.3)	

[a] In case of **2h** O,O-dimethyl N-trifluoroacetylaminoalkanephosphinates.

[b] $[M-31]^+=[M-MeO]^+$; $[M-69]^+=[M-CF_3]^+$; $[M-97]^+=[M-CF_3C(O)]^+$; $[M-112]^+=[M-CF_3C(O)NH]^+$; $[M-P]^+=[M-110]^+$ or $[M-109]^+$ or $[M-94]^+$ or $[M-93]^+$; $[P]^{+\cdot}=[110]^{+\cdot}=[(MeO)_2P(O)H]^{+\cdot}$; $[P]^{+\cdot}=[109]^{+\cdot}=[(MeO)_2P(O)]^{+\cdot}$; $[P]^{+\cdot}=[94]^{+\cdot}=[Me(MeO)P(O)H]^{+\cdot}$; $[P]^{+\cdot}=[93]^{+\cdot}=[Me(MeO)P(O)]^{+\cdot}$.

[c] $[MeP(O)(OMe)_2]^{+\cdot}$.

[d] $[M-59]^+=[M-MeOC(O)]^+$.

Table 8
Detection limits of derivatives **2a–2h** (ng) with flame ionization detection (GC–FID), and mass spectrometric detection (GC–CI-MS and GC–EI-MS)

Derivative		Detection limits[a,b] (ng)		
No.	Abbreviation	GC–FID	GC–CI-MS	GC–EI-MS
2a	TFA–Gly^P(OMe)$_2$	30	1 (2.5 [37])	1.5
2b	TFA–Sar^P(OMe)$_2$	20	0.5 (0.5 [37])	0.5
2c	TFA–Ala^P(OMe)$_2$	20	1	1.5
2d	TFA–β-Ala^P(OMe)$_2$	20	1	1
2e	TFA–Hal^P(OMe)$_2$	20	1	1.5
2f	TFA–γ-Hal^P(OMe)$_2$	20	10	3
2g	TFA–PMG(OMe)$_3$	15	3 (10 [37])	0.5
2h	TFA–Gluf(OMe)$_2$	10	25	15

[a] Detection limits (DLs) are defined as the minimum detectable amounts of the analytes with the chromatographic detection determined by the signal three times higher than the noise.

[b] Applied chromatographic conditions: DB-17 column; program temperature: 100 °C (5 min), gradient 10 °C/min, 250 °C (10 min); 700 μl/min of flow velocity of carrier gas.

Table 9
Partial EI-MS spectra of N-acetylaminoalkanephosphonates[a] **4**

Derivative			Ions[b]: m/z (intensity [%])									
No.	Abbreviation	M_r	$[M]^{+\cdot}$	$[M-31]^{+}$	$[M-43]^{+}$	$[M-58]^{+}$	$[M-P]^{+}$	$[P]^{+\cdot}$	Others			Ref.
4a	Ac–Gly^P(OMe)$_2$	181	181	150	139	124	72	110	109			
			(5.2)	(7.0)	(3.0)	(15)	(28)	(100)	(35)			
			(4.2)	(8.9)	(2.0)	(10)	(31)	(90)				[34]
4b	Ac–Sar^P(OMe)$_2$	195	195	164	152	124	86	110	109	95	94	
			(68)	(5.0)	(31)	(43)	(80)	(100)	(28)	(21)	(32)	
4c	Ac–Ala^P(OMe)$_2$	195	195	164	152	138	86	110	109	124[c]	44	
			(1.5)	(0.2)	(4.5)	(2.3)	(41)	(100)	(25)	(3.5)	(5.7)	
			(2.0)	(0.5)	(6.5)	(2.6)	(64)	(17)			(100)	[34]
4d	Ac–β-Ala^P(OMe)$_2$	195	195	164	152	138	86	110	109	94	180	
			(14)	(0.2)	(86)	(15)	(48)	(35)	(100)	(96)	(17)	
4e	Ac–Hala^P(OMe)$_2$	209	209	178	166	152	100	110	109	138	124[c]	
			(2.6)	(0.2)	(14)	(8.9)	(100)	(93)	(36)	(20)	(18)	
			(1.7)		(1.6)	(0.5)	(56)	(8.2)				[34]
4f	Ac–γ-Hal^P(OMe)$_2$	209	209	178	166	152	100	110	109	127	94	
			(6.0)	(0.3)	(28)	(7.0)	(7.0)	(76)	(43)	(77)	(49)	
4g	Ac–PMG(OMe)$_3$	253	253	222	210	194	144	110	109	166[e]	124	
			(0.2)	(–)	(2.6)	(2.2)	(–)	(76)	(41)	(96)	(100)	
4h	Ac–Gluf(OMe)$_2$	251	251	220	208[d]	192	158	93	94	150[f]	108	
			(–)	(0.3)	(9.0)	(96)	(1.5)	(26)	(29)	(100)	(14)	

[a] In case of **4h** O,O-dimethyl N-acetylaminoalkanephosphinates.
[b] $[M-31]^+=[M-MeO]^+$; $[M-43]^+=[M-CH_3C(O)]^+$; $[M-58]^+=[M-CH_3C(O)NH]^+$; $[M-P]^+=[M-110]^+$ or $[M-109]^+$ or $[M-94]^+$ or $[M-93]^+$; $[P]^{+\cdot}=[110]^{+\cdot}=[(MeO)_2P(O)H]^{+\cdot}$; $[P]^{+\cdot}=[109]^{+\cdot}=[(MeO)_2P(O)]^{+\cdot}$; $[P]^{+\cdot}=[94]^{+\cdot}=[Me(MeO)P(O)H]^{+\cdot}$; $[P]^{+\cdot}=[93]^{+\cdot}=[Me(MeO)P(O)]^{+\cdot}$.
[c] $[MeP(O)(OMe)_2]^+$.
[d] $[M-45]^+=[MeO_2CCH{=}N{=}CHP(O)(OMe)_2]^+$.
[e] $[O{=}C{=}NCH_2P(O)(OMe)_2]^+$.
[f] $[M-101]^+=[H_2NCHCH_2CH_2P(O)(MeO)Me]^+$.

3.3.1. Mass spectral properties of N-trifluoroacetyloaminoalkanephosphonates 2

The molecular ions $[M]^+$ of the examined derivatives **2** were observed in abundance varying from 0.2% (for **2h**) to 8–12% (for **2a**, **2b** and **2g**). Generally, higher abundance occurred for **2** derived from amino acids with the secondary amino group (Sar^P and PMG). Charge localization on the nitrogen atom produced ions $[M-(MeO)_2P(O)]^+$ or $[M-Me(MeO)P(O)]^+$ and $[M-R]^+$ ions (R is the alkyl chain at C_α) resulting from α-cleavage. The ions $[M-(MeO)_2P(O)]^+$ or $[M-Me(MeO)P(O)]^+$ represent the abundant peaks for the majority of the TFA-derivatives **2** (for **2b**, the base peak). The ions $[M-1]^+$ were not observed or were observed in very low abundance. The ions $[M-R]^+$ appeared for **2** and were of low abundance. The ions resulting from competitive charge localization on the phosphonate (phosphinate) moiety were very abundant. Thus, the ions $[(MeO)_2P(O)]^+$ or $[(MeO)_2P(O)H]^+$ were the base peaks (100% for **2a**, **2c** and **2f**) or very abundant (**2d**, **2f** and **2h**). The ions 111, 110, 109, 95 and 94 were characteristic for the presence of the O,O-dimethyl phosphonate system. The ions 94, 93 and 92 were characteristic for the presence of the O-methyl methylphosphinate function. The phosphonates **2** derived from Sar^P, α-Ala^P and β-Ala^P, respectively, exhibiting the same value of molecular mass (M_r=250), were found to exhibit some difference of diagnostic value in their EI-MS fragmentation paths. Thus, their molecular ions differ substantially in intensities from each other, exhibiting 1.8 to 2.0% of relative abundance in case of TFA–α-Ala^P(OMe)$_2$ and TFA–β-Ala^P(OMe)$_2$ and 14% for TFA–Sar^P(OMe)$_2$. These compounds also afforded different base ions. Thus, for TFA–Sar^P(OMe)$_2$ it

was $[M-(MeO)_2P(O)]^+$ ion, while for TFA–α-Ala^P(OMe)$_2$ and TFA–β-Ala^P(OMe)$_2$ it was the $[(MeO)_2P(O)H]^+$ ion. These derivatives of isomeric phosphoalanines exhibited different abundance of the $[M-69]^+$ ions (presumably, $[M-CF_3]^+$), namely 0.6% for **2c** (α) and 100% for **2d** (β).

Additional fragmentation features are found for TFA–PMG(OMe)$_3$ (**2g**) and TFA–Gluf(OMe)$_2$ (**2h**), as a result of their differences from other investigated **2** derivatives structures. Thus, these derivatives afforded intensive ions $[M-59]^+$, namely 248 (40%) for **2g** and 246 (100%) for **2h**, formed presumably via elimination of the methoxycarbonyl fragment from parent molecular ions $[M-MeO-C(O)]^+$. TFA–aminophosphonate **2g**, derived from PMG afforded the base ion 124 (100%), presumably presenting the structure $[(MeO)_2P(O)Me]^+$.

3.3.2. Mass spectral properties of N-acetyloaminoalkanephosphonates ***4***

The molecular ions $[M]^+$ of these derivatives were not observed (**4g** and **4h**) or observed in low abundance from <0.2 (**4g** and **4h**), 1.5–6% (**4a**, **4c**, **4e**, **4f**) to 14% (**4d**) and 68% (**4b**). Charge localization on the nitrogen atom produced ions $[M-(MeO)_2P(O)]^+$ (or $[M-Me(MeO)P(O)]^+$) or $[M-R]^+$ ions (R is the alkyl chain at C_α) resulting from α-cleavage. The ions $[RCH-NH_2]^+$ or $[M-(MeO)_2P(O)]^+$ (or $[M-Me(MeO)P(O)]^+$) constitute the base peaks for the majority of the N-acetyl derivatives. The ions $[M-1]^+$ and/or $[M-R]^+$ were not observed or were observed in very low abundance. The ions resulting from the competitive charge localization on the phosphonate (phosphinate) moiety were very abundant. Thus, the ions at *m/z* 111, 110, 109, 95 and 94, were characteristic of the presence of O,O-dimethyl phosphonate system. The ions of 94, 93, 79 and 62 were characteristic for the O-methyl methylphosphinate system. Cleavage of the acetate or acetamide group from the molecular ions of derivatives **4** gave rise to the ions $[M-43]^+$ or $[M-57]^+$, respectively.

4. Conclusions

The described analytical procedure illustrates the capability of the derivatization methods based on the application of the TFA–TFAA–TMOA reagent. This allows the simultaneous derivatization of eight-amino acid mixtures (Gly^P, Sar^P, Ala^P, β-Ala^P, Hala^P, γ-Hala^P, PMG and Gluf) where the quantitative course was verifiable by ^{31}P NMR. Subsequent GC separation and CI-MS analysis, supplemented by EI-MS analysis, enables unequivocal simultaneous identification of the title amino acids, despite the similarity of their molecular masses. The detection limits of **2** in GC–FID, GC–CI-MS and GC–EI-MS modes were on the nmol–pmol level. The applied derivatization scale (up to 0.02 μmol of derivatized components), preceded by a routine extraction/concentration pre-stage [19,21,25,30,51], provides the possibility of simultaneous GC–FID analysis of amino acids **1** (via their derivatives **2**) for real samples in the range μmol/pmol per l. Fortification of the analysed mixtures with selected 1-aminoalkanephosphonic acids as internal standards [23] can further simplify the procedure.

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