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Amino Acids

Synthesis, cytotoxicity and clastogenicity of novel α-aminophosphonic acids

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Summary. α-Ethyl-N-(phosphonomethyl) glycine is synthesized and characterized by NMR and FAB spectroscopy. The cytotoxicity, clastogenic and antiproliferative effect of 3-ethyl-2-hydroxyl-2-oxo-1,4,2-oxazaphosphorinane, sodium salt of 3-ethyl-2-hydroxyl-2-oxo-1,4,2-oxazaphosphorinane, α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid, α-ethyl-N-(phosphonomethyl) glycine, α-ethyl-N-(phosphonomethyl) glycine isopropylammonium salt, glyphosate isopropylammonium salt are tested.

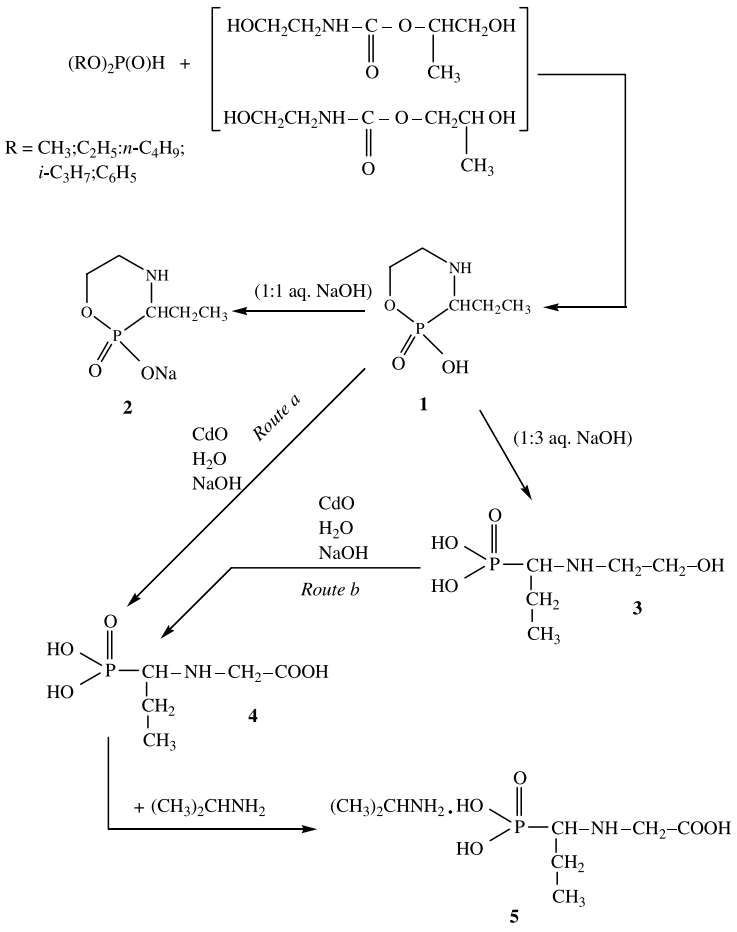
Keywords: Aminophosphonic acids – N-(Phosphonomethyl) glycine – Cytotoxicity – Clastogenic – Antiproliferative effect

Introduction

Aminophosphonic acids are considered to be structural analogues of the corresponding amino acids, constitute an important and somewhat undiscovered group of potential biologically active substances. Their negligible mammalian toxicity, and the fact that they very efficiently mimic aminocarboxylic acids makes them extremely important antimetabolites, which compete with their carboxylic counterparts for the active sites of enzymes and other cell receptors (Kafarski and Lejczak, 2001; Logusch et al., 1990; Meek and Villafranca, 1980; Oleksyszyn et al., 1994). A series of N-substituted α-amino acids containing terminal phosphonic acid groups has been synthesized as potential N-methyl-D-aspartic acid (NMDA) receptor antagonists (Bigge et al., 1992). The herbicidal activity of N-(phosphono-methyl) glycine (glyphosate) has been reported by Baird et al. (1971). This aminophosphonic acid acts as a broad band spectrum herbicide having little or no residual effects. In vitro growth of *Toxoplasma gondii*, *Plasmodium falciparum* (malaria) and *Cryptosporidium parvum*, which cause opportunistic infections in AIDS patients was inhibited by the herbicide glyphosate (Roberts et al., 1998). Moreover, it has been found that the N-(phosphonomethyl) glycines are potent inhibitors of the growth of tumors, including cancer and effectively suppress the growth of viruses and bacteria (Camden, 1997a, b, 1998, 1999, 2000).

Consequently, the development of new methods for the synthesis of α-aminophosphonic acids is an active area of research and many methods are now available: Kabachnik and Medved (1952) and Fields (1952) have discovered the first method for the preparation of α-aminophosphonic acids reacting ammonia or amine, dialkyl H-phosphonate and aldehydes; the reaction of dialkyl H-phosphonates (Tyka, 1970) or phosphorous acid (Redmore, 1978) with imines furnished also α-aminophosphonic acids; α-aminophosphonic acids are obtained reacting 2-ozxazolidinone derivatives (Wong and Bunker, 1985; Fields et al., 1989; Naydenova et al., 2006), with formaldehyde and phosphorus trichloride α-aminophosphonic acids bearing heterocyclic, aromatic rings such as furane, anthracene, (Kraicheva, 1996, 1998, 1999, 2003; Kraicheva et al., 1988a, b, 1992, 1993, 2002; Boduszek, 1995), thiophene, pyrazole, imidazole and pyridine are described.

We currently know (Scheme 1) that the reaction diesters of H-phosphonic acid with mixture of 1-methyl-2-hydroxyethyl-N-2′-hydroxyethyl carbamate and 2-methyl-2-hydroxyethyl-N-2′-hydroxyethyl carbamate at



Scheme 1. Synthesis of 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1**, its sodium salt **2**, α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3**, α-ethyl-N-(phosphonomethyl) glycine **4** and isopropylammonium salt of α-ethyl-N-(phosphonomethyl) glycine **5**

165 °C resulted in the formation of 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1** (Troev, 1997; Troev et al., 1999a, b) The treatment of **1** with an aqueous solution of NaOH (molar ratio 1:1) furnished the sodium salt of 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **2** (Troev et al., 1999). **1** was quantitatively converted into the disodium salt of α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid when heated with an excess of an aqueous solution of NaOH (molar ratio 1:3). Subsequently the disodium salt was quantitatively converted into the α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3** through treatment with Dowex 50WX8-200 (Troev et al., 1999b).

In this paper we report the synthesis of α-ethyl-N-(phosphonomethyl) glycine **4** from 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1** (Scheme 1, route a) or from α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3** (Scheme 1, route b). In addition, preliminary data about the cytotoxicity, clastogenicity and antiproliferative effect of the newly synthesized aminophosphonic acids **1**–**4** are presented.

Materials and methods

General methods

3-Ethyl-2-hydroxyl-2-oxo-1,4,2-oxazaphosphorinane **1** and α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3**, were obtained as described in (Troev, 1997; Troev et al., 1999b). Dowex 50WX8-200 ion-exchange resin, strongly acidic cation, 8% crosslinking, 100–200 mesh, and isopropylamine, 99.5% purity were obtained from Aldrich Chemical Co.

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker DRX 500 spectrometer in D_2O. FAB mass spectra were taken on a Ktatos Concept ^{1}H spectrometer, which placed the samples into glycerol matrix.

Oxidation of 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane

A 100 ml steel autoclave equipped with a magnetic stirrer, a thermal couple sensor, and a pressure gauge, was charged with 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1**, (5 g, 0.031 mol), sodium hydroxide (6.12 g, 0.15 mol), water (10 ml), and CdO (0.14 g). The autoclave was heated to 230 °C for 7 hours. Then heating was turned off and the reactants allowed cooling slowly. The vessel was completely depressurized. The reaction product was acidified with HCl and separated on a Dowex 50WX8-200 ion exchange column with water. The water was removed under vacuo to give **4**, (5.7 g, yield 96.6%) in the form of a colorless powder.

^{1}H NMR (D_2O), δ in ppm: 0.96 (t, $^{3}J(H,H) = 7.5$ Hz, 3H, CH_3); 1.59–1.93 (m, 2H, CH_2); 3.12–3.22 (m, 1H, CH); 3.94 (s, 2H, CH_2). $^{13}C\{H\}$

NMR (D_2O), δ in ppm: 10.1 (d, $^3J(P,C) = 6.3$ Hz, CH_3); 20.54 (d, $^2J(P,C) = 1.3$ Hz, CH_2); 45.89 (d, $^3J(P,C) = 3.2$ Hz, N–CH_2); 56.95 (d, $^1J(P,C) = 140.2$ Hz, P–CH); 169.14 (s, C = O). $^{31}P\{H\}$ NMR (D_2O), δ in ppm: 12.73. ^{31}P NMR (D_2O), δ in ppm: 12.71 (q, J(P,H) = 13.3; 14.6;13.4 Hz).

Oxidation of α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid

A 100 ml steel autoclave equipped with a magnetic stirrer, a thermal couple sensor, and a pressure gauge, was charged with α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid (4.5 g, 0.02 mol), sodium hydroxide (4.01 g, 0.1 mol), water (10 ml), and CdO (0.12 g). The autoclave was heated at 230 °C for 4 hours. Then heating was turned off and the reactants allowed cooling slowly. The vessel was completely depressurized. The reaction product was acidified with HCl and separated on a Dowex 50WX8-200 ion exchange column with water. The water was removed under vacuo to give **4**. Yield 3.75 g, 95.0%.

Preparation of isopropylammonium salt of 3-ethyl-N-(phosphonomethyl) glycine

3-Ethyl-N-(phosphonomethyl) glycine (0.780 g, 0.0039 mol) and 5 ml H_2O were mixed into a three-necked flask equipped with a condenser, magnetic stirrer, and thermometer. The reaction mixture was cooled to 20 °C and then isopropylamine (0.24 g, 0.0040 mol) was added dropwise. The reaction mixture was stirred for 1 h at 20 °C. The water was removed under vacuum to give 0.990 g (98%) isopropylammonium salt of 3-ethyl-N-(phosphonomethyl) glycine **5**.

Biological tests

Cytotoxic test system

EBTr, a bovine embryonic trachea continuous cell line (CL), obtained from the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures were used throughout the experiments.

Cultivation was performed in a humidified 5% CO_2/95% air incubator at 37.5 °C. The cells were routinely grown on 75 cm^2 flasks (Cellstar, Greiner bio-one GmbH) in a growth medium, consisting of a combination of Parker-E199 and Dulbecco's Modified Eagle's Medium (DMEM), (Sigma), (1:1), supplemented with 5% fetal bovine serum (FBS) (Bio-Whittaker Europe) and antibiotics Penicillin (100 UI/ml) and Streptomycin (100 μg/ml). The cells were routinely passaged at a cell density of 1×10^6 cells in 75 cm^2 flasks every 3–4 days (average doubling time is 20–24 h).

Test chemicals: 3-Ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1**, sodium salt of 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **2**,α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3**, α-ethyl-N-(phosphonomethyl) glycine **4**,α-ethyl-N-(phosphonomethyl) glycine isopropylammonium salt **5**, glyphosate isopropylammonium salt **6**, (GIP), CAS 38641-94-0, chemically pure, 41.5% solution in water, glyphosate-isopropylammonium formulation **7**, (GIPF), consisting of 48,6% glyphosate-isopropylammonium salt and 18% surfactants.

Preparation of test chemicals: Test chemicals were freshly prepared immediately prior to use by dissolving in growth medium and sterile filtered (0.20 μm, Corning). A constant dilution factor was used in each experiment for the preparation of the eight test concentrations of each test compound.

Cytotoxicity assay: In each experiment 1×10^4 cells per well were seeded in 96-well plate (Costar, Corning Incorporated). After 24 h incubation period (cells form half-confluent monolayer) the cultures were treated with eight decimal geometric concentrations (Hackenberg and Bartling, 1959) (six wells per concentration) of each test-compound, diluted in fresh medium. One 96-well plate per test-compound was used in each experiment. After 24 h treatment, each plate was examined under inverted microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells, expressed by alterations in monolayer morphology. Cytotoxicity was expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red (Borenfreund and Puerner, 1985). Optical density was measured at wave length 540 nm by Organon Teknika Reader 530. Relative cell viability, expressed as a percentage of the untreated negative controls, was calculated for each concentration.

Statistical analysis: Probit regression analysis ("Statistica 4.3" software package) was applied to determine the concentrations, required to reduce cell viability by 50% (IC_{50} values). Probit regression analysis transforms cell viability, expressed as a percentage, into standardized normally distributed values (probits) and produces a linearized model of the relationship between cell viability and the concentrations (expressed as decimal logarithms) of the test substances.

Cytogenetical method

The cytogenetical investigation was conducted as described by Preston et al. (1987). Male and female C57Bl mice, weighing 20.0 ± 1.5 g were kept at standard conditions at 20 °C and 12 h light/dark cycle, having free access to food and water. 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1**, α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3**, α-ethyl-N-(phosphonomethyl) glycine **4**, were administered intraperitoneally at doses of 10 mg/kg and 100 mg/kg. Mitomycin C (Kyowa) 3.5 mg/kg was used as a positive control. The negative control animals were injected only with 0.9% NaCl.

Bone marrow chromosome aberration assay was performed on groups of animals each one consisted of 3 males and 3 females treated with the compound studied, and 5 pure control animals. The animals were injected intraperitoneally with colchicine at a dose of 40 mg/kg, 24 and 48 h after the administration of applied chemicals or 0.9% NaCl solution and 1 h before isolation of the bone marrow cells. Bone marrow cells were flushed from femur and incubated for 20 min in a hypotonic (0.075 M) KCl water solution at 37 °C. Thereafter the cells were fixed in methanol – acetic acid (3:1), dropped on cold slides and air dried. To examine the chromosome aberrations the slides were stained with 5% Giemsa solution (Sigma Diagnostic). At least 50 well-spread metaphases were analysed per experimental animal at random. Mitotic indices were determined by counting the number of dividing cells among 1500 cells per animal in the bone marrow slides to score aberrations. The frequencies of abnormalities and the mitotic index were determined for each animal and then the mean ± standard error for each group was calculated.

Statistical analysis

Three-way analysis of variance (ANOVA) with fixed effects, followed by two-group Student's *t*-test and post hoc pairwise comparison test of Dunnett with a control was performed using BMDP4V, BMDP3D and BMDP7D programs (Dixon et al., 1990). Statistical significance is expressed as $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; $p > 0.05$ – (not significant). Unless otherwise stated, eight animals were used per group.

Results and discussion

Chemistry

3-Ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1** was subjected to oxidation with CdO in the presence of aqueous NaOH (molar ratio 1:5) (Scheme 1). The oxidation was studied at different temperatures and different amounts of aqueous NaOH. The best yield (96.6%) of

α-ethyl-N-(phosphonomethyl) glycine **4** was obtained when the oxidation was carried out at a molar ratio **1**: NaOH = 1:5, at 230 °C for a period of 7 hours. α-Ethyl-N-(phosphonomethyl) glycine **4** was obtained also via oxidation of α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3** (Scheme 1). In this case the oxidation was carried out at the same reaction conditions but the period of heating was shortened to 4 hours. The structure of **4** was proved by NMR and FAB spectroscopy. The ^{1}H NMR spectrum of **4** (see Experimental part) unequivocally confirms the molecular structure of the product. The new signal in the $^{13}C\{H\}$ NMR spectrum of **4** at $\delta = 169.14$ ppm which can be assigned for the *C*=O carbon atom of the carboxylic group is a direct evidence that the oxidation of compounds **1** or **3** truly furnished of α-ethyl-N-(phosphonomethyl) glycine. The absence of the characteristic doublet at 63.28 ppm for PO*C*H_2 carbon atom, compound **1**, and at 56.98 ppm for the *C*H_2OH carbon atom, compound **3**, in the same spectrum confirms the oxidation of **1** or **3** to **4**. $^{31}P\{H\}$ NMR spectrum of **4** shows a signal at $\delta = 12.73$ ppm, which represents a quartet (^{31}P NMR spectrum) with coupling constant of 14.6 Hz. The data from $^{31}P\{H\}$-NMR spectrum showed a high purity of product **4** (>98%). The characterization of **4** was also performed by FAB-MS technique. The protonated molecular ion $[M \pm H]^+$ 198 gave rise to the base peak.

Biological activity

Cytotoxicity study

The toxicities of the newly synthesized α-aminophosphonic acids have been studied by a Neutral Red Uptake (NRU) test performed on the EBTr permanent epithelial cell line. An exposure period of 24 h for each test compound was sufficient to reduce cell viability. Progressive cytopathological alteration, such as rounding-up, detachment, shrinkage of dead cells, as well as formation of acellular zones and complete cell destruction were observed at the effective doses. In control cell cultures, no alterations were observed. The results from our experiments showed that cytotoxic effects of α-aminophosphonic acids compounds 1–3 were comparable when tested on EBTr cell cultures (Fig. 1).

The concentrations of these aminophosphonic acid derivatives, sufficient to induce death of 50% of the tracheal bovine cells in vitro were approximately 10 times lower than the concentrations of glyphosate (N-(phosphonomethyl) glycine) isopropylammonium salt **6**, and glyphosate isopropylammonium salt formulation **7**. The latter, however, showed higher cytotoxicity, compared to test-compound 6, which is free of surfactants. Similar results have been obtained in a study on the cytotoxic activity of glyphosate and its surfactant-containing formulation (Roundup), performed on human choriocarcinoma cell line JEG3 (Richard et al., 2005) and on human embryonic kidney (E293) cells. The newly synthesized compounds α-ethyl-N-(phosphonomethyl) glycine **4** and its isopropylammonium salt **5** appeared to be less cytotoxic towards the tracheal epithelial cells, used in this study, compared to all other compounds tested. In addition, it has been found out that the addition of isopropylamine and the formation of isopropylammonium salt of α-ethyl-N-(phosphonomethyl) glycine **5** resulted in an approx. 2-fold diminution of cytotoxicity in comparison with α-ethyl-N-(phosphonomethyl) glycine.

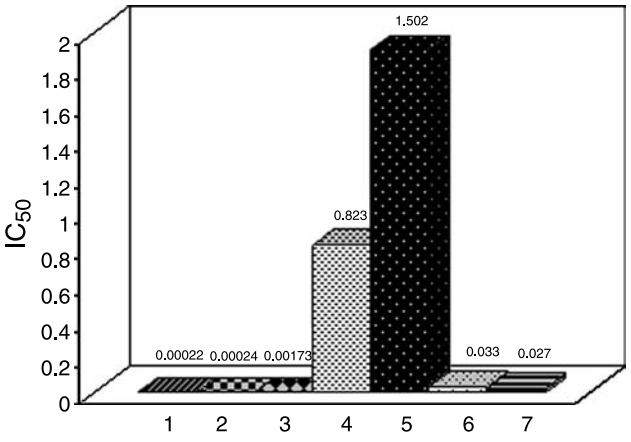


Fig. 1. Cytotoxicity of 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1**, sodium salt of 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **2**, α-ethyl-α-N-(hydroxyethylamino) methyl-phosphonic acid **3**, α-ethyl-N-(phosphonomethyl) glycine **4**, isopropylammonium salt of α-ethyl-N-(phosphonomethyl) glycine **5**, glyphosate isopropylammonium salt **6**, and glyphosate isopropylammonium salt formulation **7** on EBTr cell line after 24 h treatment

Clastogenic effects

The data of the metaphase analysis of the bone marrow cells from all experimental groups are presented in Table 1 (Fig. 2). The obtained results of the frequency of the induced chromosome aberrations by 3-ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane **1**, α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3**, α-ethyl-N-(phosphonomethyl) glycine **4**, in murine bone marrow cells are described consequently below.

3-Ethyl-2-hydroxy-2-oxo-1,4,2-oxazaphosphorinane at concentration of 10 mg/kg caused the presence of

Table 1. Frequencies of chromosome aberrations in affected mouse bone marrow cells after intraperitoneal application of α-aminophosphonic acids

Sample	Interval (h)	Number of analyzed metaphases	Type of aberrations				Percentage of cells with aberrations $\bar{X} \pm$ S.E.	Mitotic index % $\bar{X} \pm$ S.E.
			br	fr	c/c	t/t		
Comp. 1								
10 mg/kg	24	300	3	2	13	0	6.00 ± 0.52	8.24 ± 0.18
	48	250	1	1	7	0	3.61 ± 0.66	8.62 ± 1.12
100 mg/kg	24	215	1	2	7	1	5.33 ± 0.77	7.79 ± 0.58
	48	300	4	1	14	3	6.77 ± 0.93	6.56 ± 0.72
Comp. 3								
10 mg/kg	24	300	5	3	13	0	7.2 ± 0.92	6.26 ± 0.35
	48	300	7	6	5	1	6.33 ± 0.3	6.13 ± 0.61
100 mg/kg	24	251	5	3	11	0	6.71 ± 0.66	2.67 ± 0.55
	48	300	34	1	14	3	7.33 ± 0.61	4.81 ± 0.36
Comp. 4								
10 mg/kg	24	300	6	1	18	0	7.67 ± 0.56	8.3 ± 0.59
	48	300	5	0	9	0	4.67 ± 0.38	7.2 ± 1.15
100 mg/kg	24	300	3	2	8	1	5.6 ± 0.63	6.06 ± 0.73
	48	300	11	2	6	1	6.67 ± 1.77	7.29 ± 0.34
Mitomycin C								
3.5 mg/kg	24	150	10	13	11	0	46.67 ± 3.03	5.49 ± 0.19
Negative control								
0.9% NaCl	24	200	0	0	2	0	1.0 ± 0.57	17.3 ± 2.49

br Breaks; *fr* fragments; *c/c* centromer/centromeric fusions; *t/t* telomere/telomeric fusions

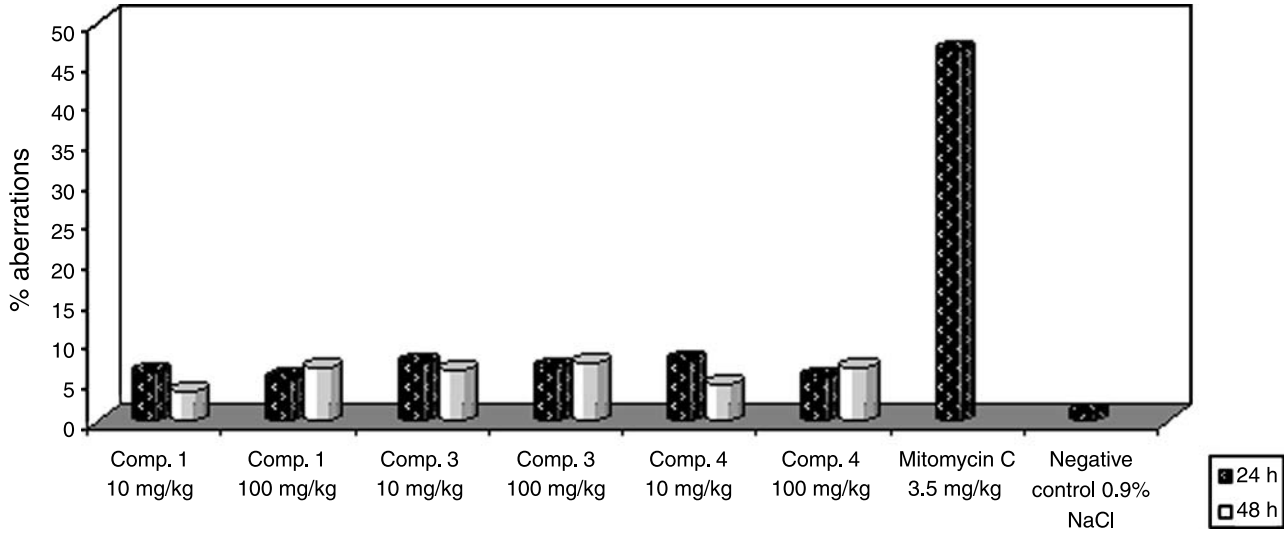


Fig. 2. Frequencies of chromosome aberrations after intraperitoneal treatment with α-aminophosphonic acids

$6.0 \pm 0.53\%$ (24th hour) aberrant mitoses. Their percentage significantly decreased on the 48th hour ($p < 0.05$) to 3.61 ± 0.66 bone marrow cells with chromosome aberrations. Significant differences between the experimental (treated) groups injected with 10–100 mg/kg at the two time intervals were not detected ($p > 0.05$). The value of the metaphases with centromer/centromeric fusions was about 73% of all cells with aberrations. The number of cells with brakes and fragments was significantly lower.

The α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid **3** induced at the two applied concentrations at both time intervals (24 and 48 h) similar percentage of cells with chromosome aberrations (Fig. 2), the differences in the calculated values being not significantly dif-

ferent ($p > 0.05$). A more detailed analysis shows that last compound at 10 mg/kg (24th hour) induced predominantly centromeric fusions ($4.33 \pm 0.61\%$). This value is close to that calculated for compound **1**. At the same concentration on the 48th hour almost equal numbers of centromer/centromeric fusions ($2 \pm 0.73\%$), fragments ($2 \pm 1.03\%$) and brakes ($2.33 \pm 0.95\%$) were obtained.

In the mice treated with 100 mg/kg **3** the number of metaphases with centromer/centromeric exchanges increased to the 48th hour after the chemical administration ($5.67 \pm 0.61\%$) and chromosomes with telomer/telomeric fusions could be registered. The comparison between the different aberration types showed that more than 41.25% of the damaged metaphases (2.95% of the total number of analyzed cells) after **3** treatment have breaks and fragments. For a comparison in the negative control group cells with breaks and fragments were not found, and in the positive Mit. C control the percentage was 71.3 or 29.33% from the total number of analyzed cells.

10 mg/kg α-ethyl-α-N-(phosphomethyl) glycine **4** (24th hour) induced $7.67 \pm 0.56\%$ chromosome aberrations. A tendency for higher incidences of chromosome changes – cenromer/centromeric fusions, specific for **1** and **3** treatment was also followed. Similarly to **1** and **3** the number of cells with centromer/centromeric fusions (48th hour) decreased two fold and the total percentage of metaphases with aberrations was diminished to $4.67 \pm 0.38\%$. This significant decrease of the percentage of chromosome aberrations at the 48th hour ($p < 0.05$) could be explained by loss of cells with these damages. The percentages of chromosome aberrations in the experimental mice groups after injections of **4** at 100 mg/kg (24th and 48th hour) are comparable – $5.6 \pm 0.63\%$ and $6.67 \pm 1.77\%$ respectively. In the treated animals **4** at 10 mg/kg provoked on the 24th hour the highest percentage of chromosome aberrations ($7.67 \pm 0.56\%$), **3** – lower ($7.2 \pm 0.92\%$), and **1** – the lowest ($6.00 \pm 0.52\%$). These differences are not statistically significant. The diminution of the chromosome aberrations yielded the groups treated with **1** and **4** at 10 mg/kg (48th hour) distinguish them from the experimental group treated with **3**, which could be explained by the faster metabolic pathway of **1** and **4** and a higher stability of the compound **3**.

The results obtained point to the deduction that the investigated compounds possess moderate clastogenic effects. The predominant type of chromosome aberrations (centromeric/centromeric fusions) allows a suggestion, that the newly synthesized aminophosphonic acids damage predominantly the chromosome centromeric regions.

The effect of new synthesized aminophosphonic acids on the proliferative activity of bone marrow cells of C57Bl mice was evaluated by mitotic index (MI) parameter. The results obtained are presented in Fig. 3.

Significant differences between the values of MI obtained for the investigated bone marrow cell populations treated with the two applied concentrations of **1** (24th and 48th hour) were not found. MI (48th hour) varied from 6.56 ± 0.72 to 8.62 ± 1.12‰. In the groups, treated with **3**, at 10 mg/kg, 24th hour the MI values of bone marrow cells (6.26 ± 0.35‰) were close to these on the 48th hour (6.13 ± 0.61‰). **3** at higher concentration (100 mg/kg) decreased nearly five-fold the mitotic activ-

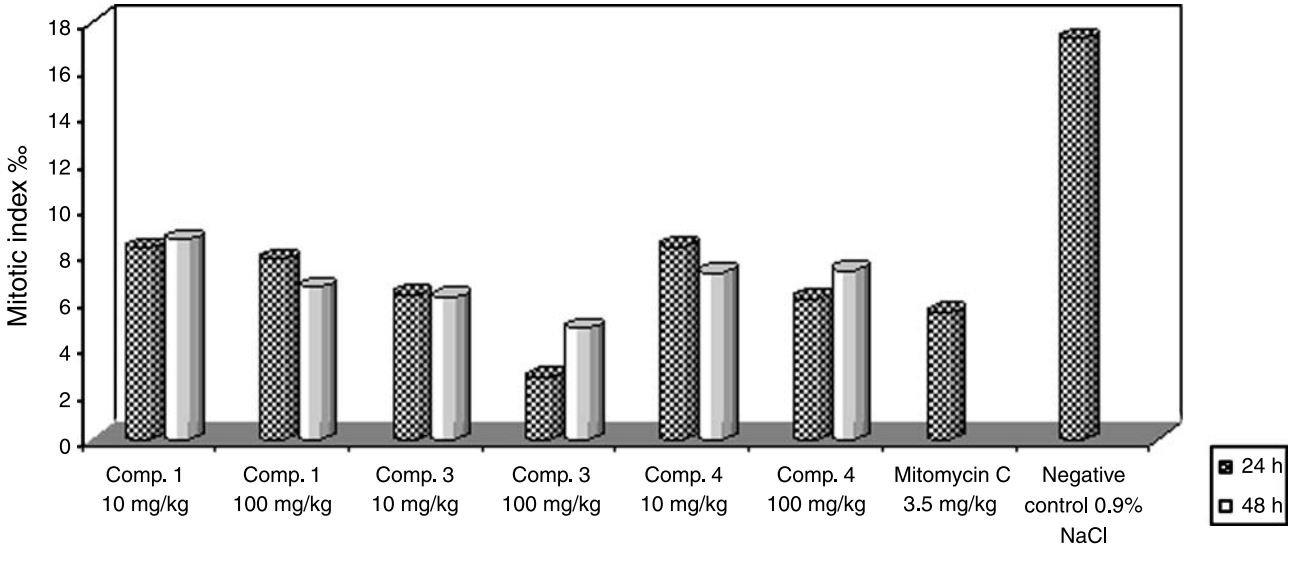


Fig. 3. Mitotic index in mouse cells after intraperitoneal treatment with α-aminophosphonic acids

ity (2.67 ± 0.55‰) in comparison with the untreated control (17.3 ± 2.49‰) ($p < 0{,}001$). The value of MI increased slightly on the 48th hour (4.18 ± 0.36‰), but remained significantly lower compared to the untreated control, as well as to the groups treated with 10 mg/kg ($p < 0.001$). In the 24th hour samples scored after **4** injection (10 mg/kg) MI was 8.36 ± 0.59‰ and retained almost unchanged until the 48th hour. The applied higher concentration (100 mg/kg) induced a slight decrease of the cell division compared to the (10 mg/kg). MI was 6.06 ± 0.73‰ (24 h) and 7.29 ± 0.34‰ (48 h), respectively. The investigated novel aminophosphonic acids possess two main biological characteristics. They slightly damage the chromosome structures of the normal bone marrow cells and significantly suppress the cell proliferation. These characteristics are a premise to study the potential cytostatic effect of these compounds on the appropriate tumor model.

The studied compounds **1**, **3**, **4** did not possess clear expressed relationship ''dose-effect'' (high percentage of aberrations after higher dose applied) in their clastogenic effects as this relationship is specific for the alkylating agents – Mitomycin C or Cyclophosphamid. Comparatively low percentage of bone marrow metaphases with chromosome aberrations and the type of chromosome damages (centromer/centromeric fusions) are an evidence of the moderate clastogenic effect of the newly α-aminophosphonic acids. The correlation between the moderate clastogenic effect and the low values of MI, obtained after the treatment with 100 mg/kg **3**, suggests furthermore detailed investigations on the experimental tumor models in vivo and in vitro could be appropriate.

Acknowledgement

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