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NEW AMINOPHOSPHONATES – ASSESSMENT OF BIOLOGICAL ACTIVITY

Janusz Sarapuk[1], Dorota Bonarska-Kujawa[1], Krzysztof Bielecki[2], Anna Dudra[1], Edward Grzyś[2], Zenon Trela[1], Halina Kleszczyńska[1]

[1]*Department of Physics and Biophysics, Wrocław University of Environment and Life Sciences, Poland*
[2]*Department of Plant Nutrition, Wrocław University of Environment and Life Sciences, Poland*

ABSTRACT

Two series, acyclic and cyclic aminophosphonates were synthesized for potential agrochemical application. They differed inside the series in substituents at the phosphorus, carbon and nitrogen atoms. Their efficiency to destabilize erythrocyte (RBC) and lipid model membranes (BLM), to change plant membrane properties (*Nitellopsis obtusa, Beta vulgaris ssp. L. rapacea, Syringa vulgaris L., Cucumis sativus*), to change activity of the antioxidative enzymes and chlorofil content in plant (*Cucumis sativus*) and to inhibit plant growth (*Spirodela oligorrhiza*) were studied.

The results obtained enabled classification aminophosphonates studied into three classes: useless as potential pesticides, of medium potential biological activity and those that changed the above-mentioned parameters of the objects studied sufficiently enough to treat them as good potential pesticides. Analysis was then done to determine what structural features of aminophosphonates were responsible for their activity. The general and obvious conclusion was that this activity is directly related to the lipophilicity of particular compounds. However, such an approach was found to be too simple. For instance, the compounds with iso-propyl groups attached to the P atom had lower lipophilicity in comparison with compounds with n-C_4H_9 groups attached at this atom but their efficiency to influence the studied parameters was greater. This effect may be the result of better screening of the polar part of the molecule by the branched i-C_3H_7 group.

A greater activity was observed for acyclic compounds. Incorporation of various ring structures into substituents at the N and P atoms decreased that activity. The results obtained may be useful in the synthesis of new compounds for agrochemical application.

Key words: aminophosphonates, model membranes, physiological and hemolytic toxicity, antioxidative activity

INTRODUCTION

Organophosphorous compounds have been known for a long time. Many of them exhibit biological activity and are widely used as potent herbicides. The first contact of such compound with a living organism must take place with its cell membranes. One of possible results of such contact may be a damage to the membrane leading, in severe case, even to its destruction, which in turn will cause death of the cell. Biological activity of organophosphorous pesticides is often assumed to correlate with their lipophilicity, [7] which means that possible membrane damage concerns the lipid phase of the cell membrane into which the lipophilic compound may incorporate. It is worth remembering that the lipid phase not only plays a role of structural element of the membrane that supports the proteins but also constitutes an element of its metabolic processes [22]. In this work we have studied membrane-modifying properties of two series of aminophosphonates synthesized for potential application as herbicides. Different model (planar lipid membranes – BLM) and biological membranes (pig erythrocytes – RBC and their ghosts) were used in order to carry out the task. The influence of aminophosphonates on different physicochemical properties of these membranes was studied. It should be empasized that the extensive use of BLM and RBC membranes for testing potential biological activity of compounds is well justified, since it has been shown that there is very good agreement between the results obtained in model experiments and those obtained from tests *in vivo* [11,23,24,25,29,34,35]. Similar agreement was found for the results obtained in BLM and RBC experiments describing the total breakdown of erythrocyte and planar lipid membranes induced by biologically active compounds [17,27], and justifies the use of both models. It must be noted that the hemolysis phenomenon is commonly regarded as connected with the lipid phase of the erythrocyte membrane and that above-mentioned agreement is evidence that compounds act directly on the lipid phase. However, one can also not exclude their action, directly or via lipid phase, on the membrane proteins.

The results obtained in the model experiments were compared with electro- and physiological toxicities of aminophosphonates. Different tissues and plants were used in these experiments. The influence of aminophosphonates on electrolyte leakage from red beet (*Beta vulgaris ssp. L. rapacea*) roots, lilac (*Syringa vulgaris L*) leaves and cucumber (*Cucumis sativus*) cotyledons, on chlorophyll content in *Cucumis sativus*, on membrane potential and electrical conductance of internodal cells of *Nitellopsis obtusa*, on the growth of aquatic plant *Spirodela oligorrhiza*, and on the activity of guaiacol and pyrogallol peroxidases (*Cucumis sativus*) were studied.

The aim of those studies was to determine which aminophosphonates have potential biological activity good enough to be used as efficient pesticides.

MATERIALS AND METHODS

The compounds studied were synthesized in the Department of Organic Chemistry, Biochemistry and Biotechnology of the Technical University of Wrocław. Heating the carbonyl compound with a corresponding amine yielded an imine, which was used without purification in the next step. After the addition of dialkyl phosphite to the imine, the reaction mixture was heated for several hours. The final product was isolated and purified by column chromatography. Its purity was checked by ^{1}H-NMR and ^{31}P-NMR spectra. The spectral data are given elsewhere [32,33]. The general structures of the aminophosphonates (AP) studied are shown in Fig. 1.

Fig. 1. The structure of the acyclic and cyclic aminophosphonates

Planar lipid membranes (BLM) were formed from a solution of 1.5 % (w/v) azolectin (Sigma Chem. Co.) in n-butanol:n-decane (1:1) on a 1.7 mm hole in the partition of a two-compartmental measurement cell filled with 0.9 % NaCl bath solution. Aminophosphonates were pipetted into a bath solution until their concentrations reached values that caused a breakdown of BLMs in no more than 3 min. These concentrations are further on in the paper referred to as critical concentrations (CC). Measurements were performed at room temperature (about 22° C) and BLM were monitored optically and electrically.

Fresh heparinized pig blood was used in the hemolytic experiments. Erythrocytes (RBC) were washed four times in the phosphate buffer of pH 7.4 and incubated in it, after adding aminophosphonates, at 37°C for 4 h. The hematocrit was 4%. The percent of hemolysis was measured with 1 ml samples taken after 0.5 h of incubation. They were

centrifuged and the hemoglobin content in the supernatant was measured at 540 nm. The concentrations of aminophosphonates were determined to cause 50% hemolysis (C_{50}). All compounds were dissolved in ethanol; the concentration of ethanol in the samples did not exceed 1%.

Fluidity experiments were done on erythrocyte ghosts subjected to the action of the compounds studied at chosen concentrations. Fluorescent probe TMA-DPH {[1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene] p-toluenesulfonate} was purchased from Molecular Probes Inc. (Eugene, Oregon, USA) and used at 1 μM concentrations. The measurements were performed with a SFM 25 spectrofluorometer (KONTRON, Zurich, Switzerland). The anisotropy coefficient A was calculated according to [19]:

$$A = (I_{II} - GI_{\perp})/(I_{II} + 2GI_{\perp})$$

where:
I_{II} - intensity of fluorescence emitted in a direction parallel to the polarization plane of the exciting light,
$I_{\perp}$ - intensity of fluorescence emitted in the perpendicular direction and G – is a factor used to correct for the inability of the instrument to transmit differently polarized light equally.

Cucumber (Cucumis sativus cv 'Wisconsin') was grown under constant fluence of 150 μmol m^{-2} s^{-1}. Cotyledons from seven-day-old seedlings were used for the experiments. Discs of 7 mm diameter were cut to avoid the midrib with a brass cork borer. The discs were rinsed in water and floated 24 h under constant light on 0.25mM and 1 mM aminophosphonate solutions. Conductivity of the treatment solution was assayed with the OK-102/1 conducometer (Radelkis, Hungary). Enzymes were extracted by grinding discs in a 100 mM K-phosphate buffer (pH 7.0) at 4°C. After centrifugation at 1500xg for 10 min the supernatant was used for all assays.

Formation of purpurogallin catalysed by pyrogallol peroxidase was followed at 430 nm and used for P-POD activity [18]. An absorbance coefficient of 2.47 mM^{-1} cm^{-1} was used. The reaction mixture contained a potassium phosphate buffer (50 mM, pH 7.0), pyrogallol (20 mM), H_2O_2 (1 mM) and an enzyme extract (0.28 μg protein) in its final of volume 1 cm^3. Reaction was started by adding H_2O_2.

For guaiacol peroxidase the reaction mixture consisted of a 50 mM potassium phosphate buffer (pH 7.0), 5 mM H_2O_2, 0,25 % guaiacol and an enzyme extract (0.14 μg protein). The enzyme activity was measured by monitoring the increase in absorbance at 470 nm (absorbance coefficient of 26.6 mM^{-1} cm^{-1}) during polymerization of guaiacol into tetraguaiacol [4].

The efflux of betacyanine from disks cut from roots of *Beta vulgaris L. ssp.rapacea* (1mm thick and 15 mm diameter) was measured. The disks were washed repeatedly in distilled water, dried on filter paper and put into vessel containing 10 ml of aqueous solution of the compounds studied. Concentrations of compounds were 0.25, 0.50 and 1.0 mM. Temperature was 25°C. The amount of effluxed betacyanine was determined spectrophotometrically by measuring absorbance at 500 nm after 24 h. Experiments were repeated four times.

To measure electrolyte efflux from lilac 15 mm disks were cut off from leaves of *Syringa vulgaris L.* and repeatedly washed in distilled water containig a 50 ml solution of aminophosphonates. The concentrations were the same as in the beet root experiments. Vessels were kept at temperature 26°C and illuminated with artificial light. Conductivity of exudate was measured with the conductometer OK.-102/1. Experiments were repeated four times.

The malondialdehyde (MDA) in the cotyledon discs and bathing solution was assayed. Ten discs were homogenized with 8 cm^3 5% TCA [4]. 1.5 cm^3 of 0.65% in 20% TCA to 1.5 cm^3 of the extract from plant tissues or bathing medium was added. The mixture was heated in boiling water bath for 20 min, cooled quickly and centrifuged at 15000 rpm for 15 min. Absorbance of the supernatant was measured at 440, 532 and 600 nm. A_{532} represents the maximum absorbance of the TBA-MDA complex, A_{600} the correction for nonspecific turbidity and A_{440} interference generated by the TBA-sugar complex. MDA equivalents were calculated using the absorbance coefficient 0.156 $M^{-1}cm^{-1}$ [9].

Chlorophylls were extracted in 80% acetone [20].

Membrane potential and electric conductance were routinely measured. The potential difference between the vacuole and external medium was measured with one pair of microelectrodes filled with 3 M KCl. In the current circuit Ag/AgCl electrodes were used. The cells were placed in a three-compartment container. The compartments were filled with liquid and partitioned by narrow empty spaces that insured electric isolation between the compartments. The middle compartment was perfused with control solution or solution containing a modifier at a rate of 1 ml/min. In that part of the container current and membrane potential were measured.

RESULTS AND DISCUSSION

Table 1 contains results of physiological toxicity of aminophosphonates (AP). The efflux of electrolyte (C) from cucumber (*Cucumis sativus* cv “Wisconsin”) cotyledons and chlorophyll content (Chl) were measured as well as 50% inhibition of growth of aquatic plant *Spirodela oligorrhiza* (IC_{50}). Also summarized are the results of experiments with model and biological membranes. These were erythrocytes (RBC) and their ghosts and planar lipid membranes (BLM). The influence of AP on hemolysis of RBC (50% and/or 100% hemolysis - C_{50} and C_{100}), the fluidity of RBC ghosts (ΔA or ΔP means a change of anisotropy or polarization coefficient) and the stability of BLM (CC – concentration of AP that broke membranes in 3 min) were studied.

Table 1. The biological activity and physicochemical properties of the model membranes. {n = 0 stands for acyclic compounds,
n = 1, 2 and 3 stand for cyclic compounds (pentane, hexane and heptane rings, respectively)}

No	n	R^1	R^2	R^3	R^4	C mSm^{-1}	CC MM	C_{50} MM	C_{100} mM	Chl %	ΔA %	IC_{50} mM
1	0	CH_3	CH_3	n-C_4H_9	CH_3	580	0.32	>1.00	>5.0	37	1.8	>0.100
2	0	CH_3	CH_3	n-C_4H_9	i-C_3H_7	550	0.49	0.25	1.90	52	15.7	0.100
3	0	CH_3	CH_3	n-C_4H_9	C_2H_5				>5.0		3.9	>0.100
4	0	CH_3	CH_3	n-C_4H_9	n-C_4H_9				0.28		9.0	>0.100
5	0	CH_3	CH_3	n-C_4H_9	-C_6H_5-	15			>5.0	80	2.0	>0.100
6	0	CH_3	CH_3	n-C_8H_{17}	i-C_3H_7	490	0.20	2.00	3.00	4	8.0	0.0085
7	0	CH_3	CH_3	n-C_8H_{17}	n-C_4H_9	510	0.52	0.12	0.25	38	12.2	0.0001
8	0	CH_3	CH_3	n-$C_{10}H_{21}$	i-C_3H_7							0.0085
9	0	CH_3	CH_3	n-$C_{10}H_{21}$	n-C_4H_9	430	0.13	0.16	0.30	55	17.1	0.0080
10	0	CH_3	CH_3	n-$C_{14}H_{29}$	n-C_4H_9	510	>1.0	0.31	0.50	41	13.1	0.0650
11	0	CH_3	C_2H_5	n-C_4H_9	CH_3	65		>1.00		93	5.5*	>0.100
12	0	CH_3	C_2H_5	n-C_4H_9	C_2H_5	150			>5.0	72	5.1	>0.100
13	0	CH_3	C_2H_5	n-C_4H_9	i-C_3H_7			>1.00			9.9*	>0.100
14	0	CH_3	C_2H_5	n-C_4H_9	n-C_4H_9	195		>1.00		55	2.0*	0.0800
15	0	CH_3	C_2H_5	n-C_8H_{17}	n-C_4H_9	420	0.52	0.16	0.41	32	11.8	0.0009
16	0	CH_3	n-C_3H_7	n-C_4H_9	n-C_4H_9	550	>1.0	0.24	0.30	16	16.0	0.0610
17	0	CH_3	n-C_4H_9	n-C_4H_9	i-C_3H_7	330		0.73		19	12.0*	0.0930
18	0	CH_3	n-C_4H_9	n-C_4H_9	CH_3	220	0.16	0.20	0.28	82	16.4	0.1200
19	0	CH_3	n-C_4H_9	n-C_4H_9	n-C_4H_9	255		0.25		29	8.2*	0.0660
20	0	CH_3	n-C_4H_9	n-C_5H_{11}	n-C_4H_9	600		0.17	0.25	34	15.5	>0.100
21	0	CH_3	n-C_4H_9	n-C_6H_{13}	n-C_4H_9	500	0.32	0.14	0.40	45	12.9	0.0040
22	0	CH_3	n-C_4H_9	n-C_8H_{17}	i-C_3H_7							0.0660
23	0	CH_3	n-C_4H_9	n-$C_{10}H_{21}$	i-C_3H_7	490	0.32	0.80	1.00	34	4.5	0.0480
24	0	CH_3	t-C_4H_9	n-$C_{10}H_{21}$	n-C_4H_9	540	0.50	0.23	0.40	3	15.0	>0.100
25	0	CH_3	n-C_4H_9	n-$C_{10}H_{21}$	n-C_4H_9	580	0.49	1.20	1.80	7	2.0	0.0860
26	0	CH_3	C_5H_{11}	n-C_8H_{17}	n-C_4H_9	500	0.40	0.40	0.75	67	4.7	0.0020
27	0	C_2H_5	CH_3	n-C_8H_{17}	n-C_4H_9	440	0.52	0.16	0.50	65	17.4	0.0009
28	0	C_2H_5	C_2H_5	n-C_4H_9	n-C_4H_9	35		>1.00		41	5.8*	0.0690
29	0	n-C_4H_9	CH_3	-C_6H_{12}-	C_2H_5	370	3.75	>1.00	>5.0	74	4.7	>0.100
30	0	n-C_4H_9	n-C_4H_9	n-C_4H_9	C_2H_5	160		>1.00		73	5.1*	>0.100
31	0	n-C_5H_{11}	CH_3	n-$_4H_9$	C_2H_5	400	0.32	0.95	1.50	68	5.8	>0.100
32	1			n-C_4H_9	n-C_4H_9				0.62		8.5*	0.0570
33	1			n-C_8H_{17}	i-C_3H_7	500	0.16	0.09	0.10	12	10.2	0.0034
34	1			n-C_8H_{17}	n-C_4H_9	445	0.32	0.10	0.15	32	9.8	0.0043
35	1			n-$C_{10}H_{21}$	n-C_4H_9							0.0710
36	1			$C(CH_2OH)_2CH_3$	n-C_4H_9	185	0.44	2.30	4.00	59	2.6	>0.100
37	2			n-C_4H_9	i-C_3H_7	420	0.84	0.70	1.00	37	8.7	0.0540
38	2			n-C_4H_9	n-C_4H_9	195		>1.00	>5.0	55	5.5	0.0615
39	2	t- C_4H_9		n-C_4H_9	n-C_4H_9	310	0.84	0.75	1.00	76	12.2	0.0685
40	2			n-C_8H_{17}	C_2H_5	570	>1.0	0.20	0.25	17	11.3	0.0700
41	2			n-C_8H_{17}	n-C_4H_9	210	0.44	1.50	2.00	78	7.8	0.0230
42	2			n-$C_{10}H_{21}$	i-C_3H_7	185	0.92	0.45	0.60		15.0	0.0002
43	2			n-$C_{10}H_{21}$	n-C_4H_9							0.0066
44	2			n-$C_{14}H_{29}$	n-C_4H_9	250	0.35	1.65	2.50		6.8	0.1200
45	2			C_2H_4OH	n-C_4H_9	310	0.37	0.35	0.60		6.4	0.0520
46	2			Sec-t-C_4H_9	n-C_4H_9		0.83	0.07	0.09			0.0570
47	3			n-$C_{10}H_{21}$	i-C_3H_7							0.0390

Standard deviations did not exceed 10% for determination of all parameters. * ΔP.

Analysis of the results obtained showed that the possibility of AP to influence the studied parameters depended on different factors. We found that the main one was the compound lipophilicity. It was shown before [7] that the contribution of a methyl, methylene or methine group to overall lipophilicity is similar and equals about 0.5 log (P) unit per carbon atom. So, the quickest and simplest way to evaluate the lipophilicity of a compound is to count these groups. We have sequenced all APs according to their abilities to influence physicochemical parameters of objects studied and found that, indeed, the APs of the smallest number of lipophilic groups (e. g., compounds 5, 11, 12 and 18) exhibited a weak efficiency to change the above-mentioned parameters. On the other hand, those APs that had a significantly greater number of lipophilic groups, 18 carbon atoms and more (e. g., 7, 15, 21 and 27), were found to be quite effective. Thus, the biological activity can be simply correlated with the total number of carbon atoms regardless of their distribution within the molecule. However, compounds with long enough hydrocarbon sustituent ($\geq C_8H_{17}$) at the nitrogen atom seem to be slightly more effective than those with a more uniform distribution of lipophilic groups. Good modifiers were also compounds with iso-propyl substituents at the phosphorus atom (compounds 2, 6 and 17). It may be related to their spatial arrangement and partial screening of the polar fragment HN-C-P(O)$(O)_2$ which all the studied compounds have in their structures. Such screening ease the interaction of a compound with the membrane lipids. Confirmation may be observed in the weak efficiency of compounds with short (ethyl, methyl) or ring (phenyl) substituents at the P atom (compounds 1, 3, 5, 11, 12, 30 and 31). A similar negative effect enables incorporation of the ring group (compound 29) in the N atom substituent.

There is an evident relationship between membrane-activity of APs and the physiological effect they insert inside plant cells. Namely, the decrease of chlorophyll content in cucumber follows the increase of conductance which is the measure of membrane damage caused by aminophosphonates. No such clear relationship between their structures and fluidization of RBC ghost membranes were found.

It is hard to compare the results obtained for acyclic and cyclic compounds because an essential structural differences exist between them. However, the general pattern seems to be the same. Cyclic compounds with long enough hydrocarbon or iso-propyl substituents exhibited good modifying properties. The worst modifier was compound 36 that had t-butyl substituent with hydroxyl terminals replacing one proton in methyl group at the N atom. A comparison of the influence of AP when its physiological activities are studied in relation to its physicochemical parameters indicates at good correlation between these. No significant differences were found between efficiency of cyclopentane and cyclohexane compounds, although these latter seemed to modify parameters studied slightly worse than the cyclopentane ones with the same substituents at the N and P atoms. The only studied cycloheptane AP (47) was inhibiting the growth of *Spirodela oligorrhiza* more weakly than cyclopentane (33) and cyclohexane (42). Since all these compounds have the same subsituents at the P and N atoms it looks probable that the loss of physiological toxicity follows development of ring substituent at the C atom corresponding to cyclohexane (43) and more strongly than cyclopentane (35). Membrane damage by AP may be related to the redox cycling phenomenon which promotes the formation of free radicals responsible for oxidative stress. Frequent and long-termed duration of oxidative stress leads to various pathological changes in organisms including DNA damage, enzyme inactivation, and cellular or subcellular membrane peroxidation [21,30]. Cellular damage is usually preceeded by the impairment of antioxidant biochemical mechanisms that quench radicals before they initiate molecular effects. Among oxidative defenses, the antioxidant enzymes (catalase, peroxidases and superoxide dismutase) appear to be most sensitive to radical proliferation [3,4,6]. Guaiacol and pyrogallol peroxidases, modulated by xenobiotics, are among the various peroxidases found in plant cells and their activity is also induced during many stress events [10]. Table 2 contains results of studies on the activity of guaiacol and pyrogallol peroxidases in cucumber cotyledons treated by some of the presented aminophosphonates as well as on production of malondialdehyde (MDA), the end-product of lipid peroxidation. Generally, these results are in agreement with already described. Compounds that strongly inhibited activities of peroxidases were again those having iso-propyl substituents at the P atom and/or $C_{10}H_{21}$ hydrocarbon chain at the N atom (i. e., 2, 6, 23, 24, 33 and 42). Inhibition of peroxidases activities was usually accompanied by an increased appearance of MDA. Contrarywise, APs with the weakest modifying possibilities (4, 36, 39 and 44) provoked cells to increase their defence which was reflected by a significant increase of activities of both peroxidases and a decrease of MDA content. It is also worth mentioning that compound 44 has a very long substituent ($C_{10}H_{21}$) at the N atom. Its worse efficiency may be the result of a phenomenon called cut-off. It describes the loss of a biological activity observed for amphiphilic compounds upon elongation of their hydrophobic (hydrocarbon) part [1, 28].

The results of studies on betacyanine and electrolyte efflux from red beet roots and lilac leaves are not so closely related to structures of APs inducing these flows. One can say that at least some of thev aminophosphonates (6, 33 and 42) exhibited good efficiency to influence dye and electrolyte efflux but there were no big differences observed to formulate a definite conclusion.

The last data obtained concern the influence of some chosen AP on the membrane conductance and resting potential (C) in alga *Nitellopsis obtusa* and so far confirm that efficiency of compound depends on already discussed structural features [31]. On the basis of presented data (more details can be found elsewhere [2,5,8,12,13,14,15,16,26,31]) it is predictable as to which structural features should have new compounds synthesized to form agrochemical applications. Such knowledge may be useful in future synthesis procedures.

Tab. 2. Effect of 1 mM of aminophosphonates on the malondialdehyde (MDA) content, on pyrogallol and guaiacol peroxidases activities in cucumber (*Cucumis sativus* cv "Wisconsin") cotyledons and on efflux of betacyanine from red beet (*Beta vulgaris ssp. L. rapacea*) roots and lilac (*Syringa vulgaris L.*) leaves. All values expressed in percent of control. S. d. did not exceed 9% (14% for MDA).

Compounds	Pyrogallol peroxidase activity	Guaiacol peroxidase activity	MDA content	Betacyanine efflux from red beet roots	Electrolyte efflux from lilac leaves
1	80	80	350		
2	20	16	480		
4	185	200	232		
6	13	7	410	35	5
7	39	14	450	74	38
8				20	13
9	22	4	250	9	13
10	115	125	320	30	4
15	50	25	310	58	32
16	22	12	400		
18	20	20	15		
20	34	4	375		
21	235	175	160		
22				31	5
23	30	7	360	31	7
24	15	2	450		
25	48	50	435	10	11
26	95	85	300		
31	118	140	280		
33	21	6	450	76	48
34	72	59	435	40	6
35				14	13
36	148	128	215		
37	24	48	175	14	5
39	119	116	242		
40	55	6	428	7	26
41	47	135	232	12	22
42	116	124	275	55	34
43				27	6
44	195	200	200	12	15
45				41	18
47					8

CONCLUSIONS

1. Biological activities of the compounds studied depended on their lipopholicities. The greater lipophilicity the higher activity, unless the hydrocarbon chains are not too long.
2. Isopropyl groups attached to phosphorus atoms enhanced significantly activities of the compounds.
3. Generally, acyclic compounds activities were found to be greater than those of acyclic ones. However, incorporation of various ring structures decreased those activities.

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Janusz Sarapuk, Dorota Bonarska-Kujawa, Anna Dudra,
Zenon Trela, Halina Kleszczyńska
Department of Physics and Biophysics,
Wrocław University of Environment and Life Sciences
Norwida 25, 50-375 Wrocław, Poland
Phone: 71-3205295
email: janusz.sarapuk@up.wroc.pl

Krzysztof Bielecki, Edward Grzyś
Department of Plant Nutrition,
Wrocław University of Environment and Life Sciences,
Grunwaldzka 53; 50-355 Wrocław, Poland

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