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BRIEF COMMUNICATION

Assessment of phytotoxicity of α-aminoalkanephosphonic acids derivatives

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Abstract

Newly synthesized derivatives of α-aminoalkanephosphonic acids (aminophosphonates) differ in the substituents at the carbon, nitrogen, and phosphorus atoms. They modified in different degree the properties of cucumber (*Cucumis sativus* cv. Wisconsin) cotyledon membranes, physiological activity of some enzymes (guaiacol and pyrogallol peroxidases, and catalase), chlorophyll content, and cellular membrane lipid peroxidation. Most active modifiers were those possessing sufficiently long hydrocarbon substituents at the nitrogen atom ($C_{10}H_{21}$) or isopropyl chain at the phosphorus atom. The branched tertbutyl group at the carbon atom enhanced slightly the activities of peroxidases in contrast to hexane ring at the same position, which decreased them.

Additional key words: catalase, chlorophyll, cucumber, *Cucumis*, cotyledon, guaiacol and pyrogallol peroxidases, lipid peroxidation.

There is a continuous search for new compounds that can find agrochemical application as pesticides and replace those to which organisms achieved resistance. Therefore α-aminoalkanephosphonic acids derivatives (aminophosphonates) were synthesized. The site of first contact of pesticide injuring an organism occurs at the cell wall or plasma membrane. The toxic activity of pesticides includes also the redox cycling phenomenon which promotes the formation of free radicals, directly or indirectly responsible for oxidative stress, DNA damage, enzyme inactivation, *etc.* Plants use enzymes with antioxidative properties to minimize the peroxidative effects, most sensitive to radical proliferation being catalase (CAT, EC.1.11.1.6), peroxidase (POX, EC.1.11.1.7) and superoxide dismutase (SOD, E.C.1.11.1.9) (Kenyon and Duke 1985, Castillo 1992, Foyer *et al.* 1994, Knörzer *et al.* 1996).

We estimated the potential biological usefulness of newly synthesized aminophosphonates at the cellular level (electrolyte efflux from cucumber cotyledons that depended on the degree of membrane damage by aminophosphonates) and at the molecular level (activity of pyrogallol and guaiacol peroxidases and catalase). We also determined lipid peroxidation (by assaying malondialdehyde production). Finally, we attempted to establish what features particular substituents at the C, N, and P atoms should possess to make aminophosphonate useful for the mentioned agricultural application.

Cucumber (*Cucumis sativus* L. cv. Wisconsin) was grown under constant irradiance of 150 μmol m^{-2} s^{-1} ("white light" fluorescent tubes). Discs of 14 mm diameter were cut avoiding the midrib from cotyledons of 7-d-old seedlings. They were rinsed in water and floated 24 h under constant irradiation on 0.25, 0.50, and 1.00 mM aminophosphonate solutions. Conductivity of bathing medium was measured with a *OK-102/1* conductometer (*Radelkis*, Budapest, Hungary). Enzymes were extracted by grinding discs in 100 mM K-phosphate buffer (pH 7.0) at 4 °C. After centrifugation at 15 000 *g* for 10 min, the supernatant was used for all assays. The formation of purpurogallin catalyzed by pyrogallol peroxidase was followed at 430 nm (Knörzer *et al.* 1996),

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Abbreviations: AP - aminophosphonates; CAT - catalase; MDA - malondialdehyde; POX- peroxidase; SOD - superoxide dismutase.
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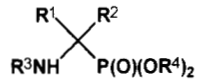
using an absorbance coefficient of 2.47 $mmol^{-1}$ cm^{-1}. The reaction mixture contained potassium phosphate buffer (50 mM, pH 7.0), pyrogallol (20 mM), H_2O_2 (1 mM), and enzyme extract (*ca.* 0.28 μg protein) in final volume of 1 cm^3. Adding H_2O_2 started the reaction. For guaiacol peroxidase the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 5 mM H_2O_2, 0.25 % guaiacol, and enzyme extract (*ca.* 0.14 μg protein). The enzyme activity was measured by monitoring the absorbance at 470 nm polymerization of guaiacol into tetraguaiacol (Chance and Maehly 1955). Catalase activity was determined by the floating disc method (Nir *et al.* 1986). Discs with a diameter of 5 mm were cut from *Whatman 3MM* chromatographic paper. Discs (10 - 20 replicates) with 10 mm^3 enzyme extract were put in a vial containing 5 cm^3 of 30 mM H_2O_2. The time until the discs floated was measured with a stopwatch. The activity of catalase in different cotyledon extracts was calculated according to the activity of bovine-liver catalase. To determine malondialdehyde (MDA) ten discs were homogenized with 8 cm^3 5 % trichloroacetic acid (TCA). 1.5 cm^3 of 0.65 % thiobarbituric acid (TBA) 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 5 000 *g* for 15 min. Absorbances of the supernatant were measured at 440, 532, and 600 nm. A_{532} represents the maximum absorbance of TBA-MDA complex, A_{600} the correction for nonspecific turbidity, and A_{440} interference generated by TBA-sugar complex. MDA equivalents were calculated using the absorbance coefficient 0.156 mol^{-1} cm^{-1} (Moran *et al.* 1994, Hodges *et al.* 1999). Chlorophylls were determined spectrophotometrically in 80 % acetone (Lichtenthaler 1987). The aminophosphonates studied were synthesized at the Department of Organic Chemistry, Biochemistry and Biotechnology, Technical University of Wrocław (for their general structure and particular substituents see Table 1). Synthesis details as well as spectral data are given in Wieczorek *et al.* (2000, 2001).

Table 1. The structure and substituent groups of the aminophosphonates.



	R^1	R^2	R^3	R^4
1	CH_3	CH_3	n-C_4H_9	CH_3
2	CH_3	CH_3	n-C_4H_9	i-C_3H_7
3	CH_3	CH_3	n-$C_{10}H_{21}$	n-C_4H_9
4	CH_3	C_2H_5	n-C_8H_{17}	n-C_4H_9
5	CH_3	n-C_4H_9	n-C_4H_9	CH_3
6	CH_3	n-C_4H_9	C_6H_{11}	C_2H_5
7	CH_3	n-C_4H_9	n-C_6H_{13}	n-C_4H_9
8	CH_3	n-C_4H_9	n-$C_{10}H_{21}$	i-C_3H_7
9	CH_3	n-C_4H_9	n-$C_{10}H_{21}$	n-C_4H_9
10	CH_3	t-C_4H_9	n-$C_{10}H_{21}$	n-C_4H_9
11	CH_3	n-C_5H_{11}	n-C_4H_9	C_2H_5
12	CH_3	n-C_5H_{11}	n-C_8H_{17}	n-C_4H_9
13	-C_5H_{11}-		n-$C_{14}H_{29}$	C_2H_5

The aminophosphonates (AP) studied might be roughly divided into three groups with regard to their efficiency to disturb cucumber cotyledon membranes. The measure of membrane damage was efflux of electrolyte from cells. The following sequence of efficiency for those groups was found: (2, 7, 9, 10) > (1, 3, 4, 8, 12) > (5, 6, 11, 13). Conductivities of the treatment solution containing APs of the first group were about 3 times higher than those of the weakest group (Fig. 1*A*). The lowest of the concentrations used (0.25 mM) of APs caused a decrease in conductivity in the case of compounds 5, 6, and 13, and its increase above the control value was observed for concentrations 0.50 and 1.00 mM. As expected, the conductivity increased with AP concentration for all other compounds and even in the case of the lowest concentration used it was higher than the control value. This increase was related to chlorophyll content decrease (Fig. 1*F*). Compounds which had a weak effect on conductivity (6, 11, and 13) stimulated the activity of pyrogallol and guaiacol peroxidases even when used in the highest concentrations (Figs. 1*B,C*). On the other hand, compounds of group 1 were excellent blockers of that activity. The exception was compound 5 which, being weak membrane modifier, for unknown reason decreased the enzymatic activity very efficiently.

The sequence of catalase activity in the presence of APs again mimicked that found for conductivity. Stronger inhibitors were compounds of the first group (Fig. 1*D*). Contrary to activity of peroxidases, catalase was in each case suppressed and no stimulation was observed. This may indicate that in aminophosphonate treated tissue H_2O_2 is consumed mostly in the lipid peroxidation process rather than detoxified. Similar patterns of peroxidase and catalase activities under the influence of xenobiotics, indicating possible damages to enzymes by free radicals derived from the peroxidation processes (Gardner 1979), were found in soybean cells (Cakmak and Horst 1991, Knörzer *et al.* 1996). Also the observed chlorophyll bleaching caused by most active aminophosphonates is regarded as major phytotoxic consequence under peroxidative conditions (Kunert 1984).

We studied the peroxidation of cucumber cotyledons lipids by measuring malondialdehyde (MDA) content, a good indicator of membrane damage (Linsel *et al.* 1988). The obtained MDA content sequence agreed with the one obtained for the conductivity (Fig. 1*E*); this confirmed a close relation between membrane-modifying efficiency of aminophosphonates and all the parameters studied.

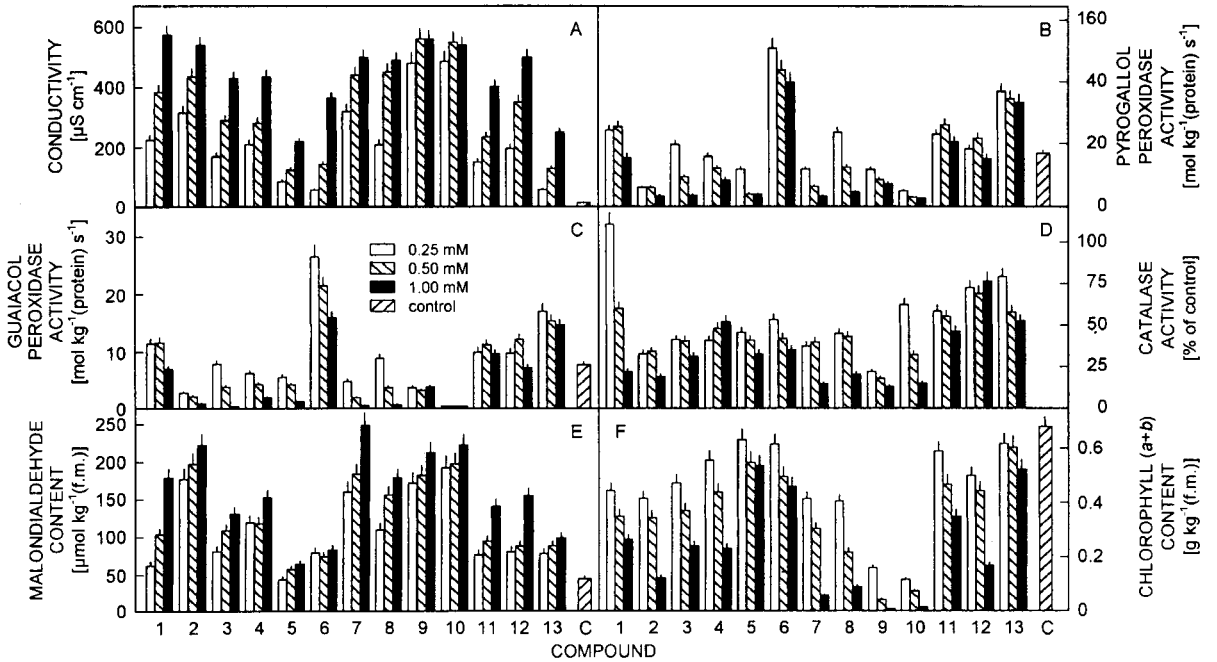


Fig. 1. Effects of amonophosphonates (1 to 13, see Table 1) on cucumber tissues measured as cellular leakage determined by change in conductivity of bathing medium (*A*), activity of pyrogallol peroxidase (*B*), activity of guaiacol peroxidase (*C*), activity of catalase (*D*), malondialdehyde content (*E*), chlorophyll (*a*+*b*) content (*F*). *Bars* show one side standard error.

The described physiological activity of the aminophosphonates studied may be related to their structural features. High activity was found for those compounds that have a long enough hydrocarbon substituent at the N atom (compounds 9 and 12). Those compounds have 23 methyl and methylene groups, which makes them highly lipophilic. The contribution of these groups to overall lipophilicity is similar and equals about 0.5 log(P) unit per carbon atom (Gancarz and Dudek 1996, Deron *et al.* 2001). Good activities that seem to be the result of high lipophilicity (22 lipophilic groups) have also compounds 8 and 12. However, lipophilicity alone does not seem to be a factor deciding of good physiological activity of a compound. Compound 2 has only 12 such groups and its high activity is probably due to the presence of isopropyl substituent at the P atom. High lipophilicity of compounds may be compensated by some structural disadvantages. An example is compound 13 which is highly lipophilic (27 lipophilic groups) but the hexane ring and a very long hydrocarbon chain ($C_{14}H_{29}$) make it a weaker modifier than all those studied. Poor activity may also be the result of the presence of phenyl ring, instead hydrocarbon chain, at the N atom and/or short hydrocarbon substituent at the P atom (compounds 6 and 11). Similar conclusions for the structure - antioxidative activity relationship - were formulated for other aminophosphonates tested for antioxidative properties (Kleszczyńska and Sarapuk 2001).

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