

Received 13 May 2001

Accepted 10 June 2001

Short Communication

THE HEMOLYTIC TOXICITY OF SOME NEW AMINOPHOSPHONATES

HALINA KLESZCZYŃSKA, JANUSZ SARAPUK
and DOROTA BONARSKA

Department of Physics and Biophysics, Agricultural University, Wrocław,
Norwida 25, 50-375, Poland

Abstract: A series of ten aminophosphonate derivatives were assayed for their hemolytic activity as a preliminary screening for the detection of herbicides. The data obtained indicate:

1. A clear correlation between the hemolytic capacity of the test compounds and their plant growth inhibition and an increase in membrane fluidity was demonstrated.
2. It was found that the most active compounds revealed at least one of the following structural features: an iso-propyl substituent at the phosphorus atom, a tert-butyl group attached to their hexane ring or a long hydrocarbon chain.
3. Ring substituents at the phosphorus (phenyl ring), carbon or nitrogen atoms (hexane) removed the hemolytic activity of compounds.
4. It may be concluded that the hemolytic toxicity of the aminophosphonates studied is related to their ability to incorporate and fuse into the lipid phase of the erythrocyte membrane.

The general conclusion is that both stereochemistry and hydrophobicity are deciding factors for the efficiency of the interaction of the studied compounds studied with erythrocytes, and that the most possible location of the aminophosphonates is in the lipid phase of the RBC membrane.

Key Words: Aminophosphonates, Hemolytic Activity, Potential Pesticidal Activity

INTRODUCTION

Many organophosphorous compounds exhibit pesticidal capacity. This is the reason why new compounds belonging to this group are still synthesized. In this study such new compounds were tested for their hemolytic toxicity. It was

already shown [1-5] that erythrocytes are a convenient tool to study various membranous effects induced by biologically active compounds such as aminophosphonates. Thus, the present study was motivated and directed by the previously established consideration [1, 2] that the hemolytic activity of aminophosphonates may represent their herbicidal potencies. Some authors suggested that the biological activity of aminophosphonates is correlated to their lipophilicity [6]. Hemolysis is commonly regarded to occur due to an effect on the lipid phase of the erythrocytes, which justifies the use of RBC in preliminary determination studies of the potential herbicidal activity of different compounds. It must be noted that the mechanism of the biological activity of aminophosphonates has not been completely resolved [7].

The newly-synthesized aminophosphonates studied in this work, both acyclic and cyclic, differed in the structural substituents at their nitrogen, carbon and phosphorus atoms. In addition to elaborating on the pesticidal properties of aminophosphonates, such an approach may further contribute to our knowledge of the structure – function relationships of such compounds.

MATERIALS AND METHODS

The aminophosphonates studied were synthesized in the Department of Organic Chemistry, Biochemistry and Biotechnology, Wrocław University of Technology. The synthesis protocol and the spectral data of the aminophosphonates was given earlier [8, 9]. The general structure of the aminophosphonates studied and particular substituents are collected in Table 1.

Fresh heparinized pig blood was used in the hemolytic experiments. The erythrocytes (RBC) were washed four times in a phosphate buffer of pH 7.4, then incubated in it after the addition of aminophosphonates, at 37°C for 4 hours. The hematocrit was 2%. The percentage of hemolysis was measured for 1 ml samples taken after 0.5 h of incubation. They were centrifuged and the hemoglobin content in the supernatant was measured with a Specol 11 spectrophotometer (Carl Zeiss, Jena, Germany) at 540 nm. The concentrations of aminophosphonates causing 100% hemolysis were determined (C_{100}).

Fluidity experiments were done on erythrocyte ghosts, prepared according to [10]. They were subjected to the action of the compounds studied at concentration of 20 μ M. The fluorescent probe was TMA-DPH {[1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene] p-toluenesulfonate} at 1 μ M concentrations. The measurements were performed with a SFM 25 spectrofluorometer (KONTRON).

The anisotropy coefficient (A) was calculated according to the formula [11-13]:

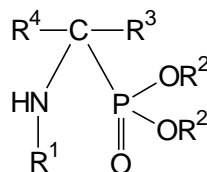
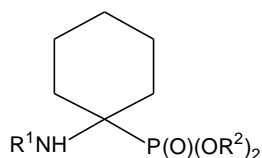
$$A = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + 2GI_{\perp}) \quad (1)$$

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

All the reagents used were of analytical grade. The fluorescent probe TMA-DPH was purchased from Molecular Probes Inc. (Eugene, USA).

Tab. 1. The general structure and substituent groups of the aminophosphonates studied.

Compound no.	R ¹	R ²	R ³	R ⁴
1	n-C ₄ H ₉	C ₂ H ₅	CH ₃	CH ₃
2	n-C ₄ H ₉	CH ₃	CH ₃	CH ₃
3	n-C ₄ H ₉	i-C ₃ H ₇	CH ₃	CH ₃
4	n-C ₄ H ₉	C ₂ H ₅	C ₂ H ₅	CH ₃
5	n-C ₄ H ₉	n-C ₄ H ₉	CH ₃	CH ₃
6	n-C ₄ H ₉	n-C ₄ H ₉	Sec-t-C ₄ H ₉	
7	n-C ₄ H ₉	n-C ₄ H ₉	-C ₆ H ₁₂ -	
8	-C ₆ H ₁₂ -	C ₂ H ₅	n-C ₄ H ₉	CH ₃
9	n-C ₄ H ₉	-C ₆ H ₅ -	CH ₃	CH ₃
10	n-C ₁₀ H ₂₁	i-C ₃ H ₇	-C ₅ H ₁₀ -	



RESULTS AND DISCUSSION

The results of studies on the hemolysis of erythrocytes and the fluidization of erythrocyte ghost membranes by aminophosphonates are collected in Table 2. The fluidization studies were performed at aminophosphonate concentrations far below potentially hemolyzing ones (20 μ M). The anisotropy changes (ΔA) relative to the control were determined. Note that the majority of the aminophosphonates studied did not hemolyze erythrocytes at concentrations of up to 0.01 M.

It was found that the most active compounds had at least one of the following features: an iso-propyl substituent at the phosphorus atom (compounds 3 and 10), a tert-butyl group attached at hexane ring (compound 6) and a long hydrocarbon chain (C₁₀H₂₁) substituted at the nitrogen atom (compound 10). The best effects were observed for compound 10 which combined two of the

mentioned features, namely a long chain and an iso-propyl group. Those findings concur with previous conclusions concerning the structural features of aminophosphonates related to their herbicidal action [12, 13]. Generally, the cyclic compounds (6 and 10) hemolyzed RBC more efficiently than acyclic ones, but it seems that their hemolytic activity is due to the mentioned substituents. Such a conclusion does not concern compound 7, the low activity of which is probably due to the large heptane ring substituted at its carbon atom. Since hemolytic activity or a lack of it should be the result of the interaction between aminophosphonates and the lipid phase of the RBC membrane, it was reasonable to perform additional experiments concerning the fluidization of this membrane by the aminophosphonates studied. The results of the fluidization experiments, presented as the change of anisotropy of the TMA-DPH probe incorporated into ghost erythrocyte membranes in relation to the control sample (untreated with aminophosphonates), are also collected in Table 2. The data clearly indicate a direct correlation between increased hemolytic activity and increased fluidity. As shown (Tab. 2), a small change in anisotropies was observed for non- or weakly hemolyzing aminophosphonates, while those efficiently hemolyzing also significantly changed the fluidity of the ghost membranes (compounds 3, 6 and 10, Tab. 2). The results obtained show that lipophilicity, i.e. the ability to incorporate into the lipid phase of RBC membranes, was the deciding factor for the potential usefulness of the aminophosphonates. However, shorter but branched substituents (iso-propyl) also rendered the aminophosphonates more efficient. It may be speculated that "bulk" lipophilicity is as important as that connected with hydrocarbon chain length (n-butyl).

Tab. 2. Concentrations of aminophosphonates causing 100% hemolysis of erythrocytes (C_{100}) and anisotropy coefficients (A) for TMA-DPH probe.

Compound no.	1	2	3	4	5	6	7	8	9	10
C_{100} [mM]	> 10	> 10	0.25	> 10	2.80	0.75	> 10	> 10	> 10	0.60
ΔA [%]	3.9	2.0	15.7	5.1	10.9	12.2	5.5	4.7	2.0	15.0

Standard deviation for C_{100} determination did not exceed 4%.

The results presented agree quite well with those obtained for the same aminophosphonates in biological tests we performed on the retardation of the growth of the aquatic plant *Spirodela oligorrhiza* [8, 9], which proved that the methods described here may be satisfactory for the preliminary estimation and screening of compounds for herbicidal activity.

Acknowledgements. This work was supported by the Polish Research Committee (KBN), grant no. 6 PO4 G 050 17.

REFERENCES

1. Kleszczyńska, H., Sarapuk, J., Bielecki, K. and Grzyś, E. Physiological activity of some organophosphorous compounds and their influence on physicochemical properties of model membranes. **Folia Microbiol.** 45 (2000) 204-206.
2. Kleszczyńska, H., Sarapuk, J. and Dziamska, A. Physicochemical properties of some new aminophosphonates. **Cell. Mol. Biol. Lett.** 113 (2000) 415-422.
3. Kleszczyńska, H., Sarapuk, J. Oświęcimska, M. and Witek, S. Antioxidative activity of some quaternary ammonium salts incorporated into erythrocyte membranes. **Z. Naturforsch.** 55c (2000) 976-980.
4. Sarapuk, J., Kleszczyńska, H. and Przystalski, S. Stability of model membranes in the presence of organotin compounds. **Appl. Organomet. Chem.** 14 (2000) 40-47.
5. Sarapuk, J., Bielecki, K., Kleszczyńska, H., Dziamska, A. and Przystalski, S. Toxicity and model membrane modifying properties of organolead compounds. **Appl. Organomet. Chem.** 15 (2001) 56-60.
6. Gancarz, R. and Dudek, M. Structure-activity studies of aminophosphonic derivatives of fluorene. **Phosphorus, Sulfur and Silicon** 114 (1996) 135-142.
7. Linsel, G., Dahse, I. and Muller, E. Electrophysiological evidence for herbicidal mode of action of phosphonic acid esters. **Physiol. Plantarum**, 73 (1988) 77-84.
8. Wieczorek, J.S., Gancarz, R., Bielecki, K., Grzyś, E. and Sarapuk, J. Synthesis and physiological activities of new acyclic aminophosphonates. **Phosphorus, Sulfur and Silicon**, 166 (2000) 111-123.
9. Wieczorek, J.S., Gancarz, R., Bielecki, K., Grzyś, E. and Sarapuk, J. Synthesis of some new cyclic aminophosphonates and their physiological activities. **Phosphorus, Sulfur and Silicon**. In press.
10. Dodge, J.T., Mitchell, C. and Hanahan, D.J. The preparation and chemical characteristics of hemoglobin-free ghosts of erythrocytes. **Arch. Biochem.** 100 (1963) 119-130.
11. Lakowicz, J.R. Fluorescence polarization. In: Principles of Fluorescence Spectroscopy. Plenum Press. New York and London, (1983) pp. 112- 151
12. Kleszczyńska, H. and Sarapuk, J. New aminophosphonates with antioxidative activity. **Cell. Mol. Biol. Lett.** 6 (2001) 83-91.
13. Grzyś, E., Bielecki, K. and Sarapuk, J. Aminophosphonates-induced changes of betacyanine and ionic efflux. **Z. Naturforsch.** 56c (2001), 349-352.