Assessment of Potential Application of Binary Mixtures of 2,4-D with Novel Aminophosphonates

Janusz Sarapuk[a,*], Halina Kleszczyńska[a], Dorota Bonarska[a], Krzysztof Bielecki[b], Zenon Trela[a], and Leszek Kordas[c]

[a] Department of Physics and Biophysics, Agricultural University, Norwida 25, 50-375 Wrocław, Poland. Fax: (+ 48)-071–3205-172. E-mail: js@ozi.ar.wroc.pl
[b] Department of Botany and Plant Physiology, Agricultural University, Cybulskiego 32, 50-205, Wrocław, Poland
[c] Department of Soil Management and Plant Cultivation, Agricultural University, Norwida 25, 50-375 Wrocław, Poland

* Author for correspondence and reprint requests

Z. Naturforsch. **60 c**, 421–426 (2005); received November 22, 2004/January 3, 2005

A series of new aminoalkane- and aminofluorenephosphonates was synthesized for agrochemical application. The particular compounds had different alkyl substituents at the carbon, nitrogen and phosphorus atoms. Their pesticidal activity was checked by applying various experimental methods. These included the measurements of compounds' potency: to inhibit growth of cucumber and germination of white mustard seeds, to influence on the membrane potential of algae and to damage human erythrocyte membranes resulting in hemolysis. All the aminophosphonates were also used in equimolar binary mixtures with the well-known herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), to check, if using such mixtures, the biological efficiencies found for particular compounds could be enhanced due to interactions between aminophosphonates and 2,4-D.
The results demonstrated, that depending on the structural features of the compounds, the final effects differed from antagonistic, through additive to the most promising synergistic ones. However, the type of interaction between 2,4-D and the compounds studied found in different experiments was somewhat different. In order to estimate those effects various statistical methods were used (toxic unit method, isobole method).

Key words: Aminophosphonates, Binary Mixtures, Plant Growth Inhibition

Introduction

Aminophosphonates constitute a large group of compounds with a large spectrum of effects on biochemical processes (Gancarz and Dudek, 1996; Forlani *et al.*, 1997, 2000; Lejczak *et al.*, 1997; Grzyś *et al.*, 2001). For this reason new compounds of that group were synthesized and checked for potential bioactivity. We have used various methods and biological objects in order to do that and to eliminate compounds of little potential biological usefulness. These included physiological measurements of growth of cucumber hypocotyls and germination of white mustard seeds treated with the compounds, studies on their hemolytic potencies with the use of erythrocytes, and the influence on the membrane potential of an alga. These assays enable a satisfactory screening of potentially agriculturally active compounds, especially when the compounds easily interact with membranes because of their structural lipophilicity (Suwalsky *et al.*, 1996, 1999, 2000; Kleszczyńska *et al.*, 2001a, b; Trela *et al.*, 2001). The aminophosphonates studied in the present work belong to lipophilic and membrane-active compounds.

Mixtures of two or more bioactive compounds are used for various reasons, the most important being to widen the spectrum of biological activity and, in some cases, to reduce the concentrations of mixture components without loss of activity (Gisi, 1996). This may occur when, due to the possible interaction between the compounds used in mixtures, their individual activity may be enhanced, so-called synergistic type of interaction. It is also possible that biological activity may be diminished or no interaction between mixture components is observed. These are called antagonistic or additive types of interactions (Gisi, 1996; Pape-Lindstrom and Lydy, 1997; Tripathi and Agarwal, 1997; Kortenkamp and Altenburger, 1998; Mora and Earle, 2001). In the present case the goal was to determine if there were any synergistic interactions between 2,4-dichlorophenoxyacetic acid (2,4-D) and aminophosphonates.

0939–5075/2005/0500–0421 $ 06.00

The herbicide 2,4-D was chosen as a second binary mixture component, since it is one of the most common and widely used biologically active compounds (Suwalsky *et al.*, 1996; Oruç and Üner, 1999, 2000), and causes toxic effects by inducing changes in cell membrane organization (Duchnowicz *et al.*, 2002; Duchnowicz and Koter, 2003; Suwalsky *et al.*, 1999). Recently, we have studied its binary mixtures with a few other phenoxy and organophosphorus compounds; however, none of those gave a synergistic effect (Kleszczyńska *et al.*, 2003).

Materials and Methods

The pesticides studied were synthesized in the Department of Organic Chemistry, Biochemistry and Biotechnology of the Technical University of Wrocław. The purity was checked by ^{1}H NMR and ^{31}P NMR spectra. Their general structures are shown in Table I.

Pig erythrocytes were used in the hemolytic experiments. The erythrocytes (RBC) were washed four times in isotonic phosphate buffer (pH 7.4) and incubated in the same buffer with addition of chosen concentrations of aminophosphonates causing 50% hemolysis or equimolar mixtures of two pesticides assumed to give the same hemolytic effects for 1 h at 37 °C. Samples were taken at 10 min intervals, centrifuged and the hemoglobin content in the supernatant was measured with a Specol 11 spectrophotometer at 540 nm. The hematocrite was 2%. The hemolytic curves obtained permitted to determine concentrations of the compounds causing 50% hemolysis (C_{50}).

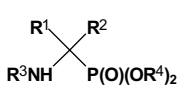
To evaluate the binary mixture effects, the isobole method was used (Kortenkamp and Altenburger, 1998). The values of C_{50} obtained for particular components were marked on *x* and *y* axes and connected with straight lines. Points on the line predicted the combinations of both components that yield the same effect (50% hemolysis). If a binary mixture gave a point that belonged to this line, the interaction between the components was defined as additive. In such cases the components can be viewed as behaving like dilutions of each other. Experimental points above or below the isobole may be defined as antagonistic and synergistic interactions, respectively. Hemolytic experiments were repeated at least 5 times.

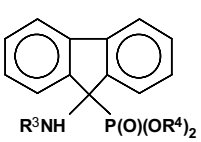
We used the toxic unit (TU) approach to model joint toxicity in physiological experiments (Pape-Lindstrom and Lydy, 1997; Cooper, 2001). In this model, the value of 1 TU is assigned to the 50% effective concentration (IC_{50}) of a contaminant. The sum of TU values contributed by each component describes the toxicity of a mixture as follows: $TU_{mixture} = C_1/IC_{50a} + C_2/IC_{50b}$, where C_i are the concentrations of a chemical in a mixture and IC_{50} is the effective concentration for the respective component chemical in a mixture. The empirically measured toxicity was compared with the expected toxicity (as predicted by the $TU_{mixture}$, which was generated using IC_{50} values determined by tests of individual toxicants).

The tests were run to establish IC_{50} (= 1 TU) for each individual compound. The 72-h growth tests with individual compounds were conducted on cucumber (*Cucumis sativus* L. v. Krak. Fl) in a SANYO® growth chamber with 9 h:15 h light:dark cycle at 25 °C. Seeds were germinated at 25 °C for 2 d in darkness. 15 uniform seedlings (of the similar root length) were transferred to petri dishes (9 cm) with two discs of Whatman No. 2 filter paper wetted with distilled water (control) or solutions of the test compounds. The lengths of cucumber hypocotyls were measured after 72 h. The growth inhibition *versus* compound concentration dependence was studied to determine the values of IC_{50}. They are given as nominal concentrations of compound.

Tests with binary mixtures were conducted in a similar manner as the individual compound tests. Concentrations of each pesticide in mixture were

Table I. The structures and substituent groups of the compounds studied.





Compound	R^1	R^2	R^3	R^4
	Aminoalkanephosphonates			
1	CH_3	CH_3	C_4H_9	CH_3
2	CH_3	C_4H_9	C_3H_7	CH_3
3	$(CH_3)_3CC_4H_9$	C_4H_9	$C_{10}H_{21}$	C_4H_9
4	CH_3	C_4H_9	C_6H_{13}	C_4H_9
5	CH_3	C_2H_5	C_4H_9	C_4H_9
6	CH_3	C_4H_9	C_4H_9	C_2H_5
	Aminofluorenephosphonates			
7			C_8H_{17}	C_4H_9
8			$C_{11}H_{23}$	C_4H_9

at the same proportions to their respective IC_{50}'s so that the summation of concentrations of the combination of pesticides was equivalent to the five concentrations in the range Σ0.5 TU – Σ2.0 TU. Therefore, a binary mixture of (2,4-D + compound **1**) contained 2.4 μM (= 0.5 TU) 2,4-D and 275 μM compound **1** to give a nominal value of Σ1.0 TU (Table II). The hypocotyl length *versus* Σ TU was studied to determine the value of Σ TU causing 50% growth inhibition. When the effect levels for the mixture were found to deviate from 1 TU, the effect was labeled either antagonistic (> 1) or synergistic (< 1).

Studies on germination inhibition of white mustard seeds were performed according to the Polish Agrotechnical Branch Norm BN-83/9180–26 (1984). Selected seeds had 97–100% potency of germination. Each sample contained 100 seeds. They were put into distilled water containing proper concentrations of the compounds studied and their binary mixtures. The experiments were done in darkness and at 25 °C for 24 h. The number of germinated seeds was then determined. Each experiment with three replicates was repeated at least 4 times. Variance analysis was used to determine the efficiency of compounds to inhibit seed germination. The least significant difference ($LSD_{0.05}$) did not exceed 24%.

Fresh internodal cells of *Nitellopsis obtusa* alga were used in electrophysiological experiments. A standard technique described earlier was applied (Trela *et al.*, 2001). Single internodal cells (0.4 mm diameter, 28 mm length, mean dimensions) were incubated for 24 h in darkness in artificial pond water (APW). The control solution of pH 7.0 contained 1 mM NaCl, 0.1 mM KCl and 0.1 mM $CaCl_2$. The incubation solutions contained 0.5 μM of particular compound. The membrane potential (the potential difference between vacuole and external medium) was measured in a routine way using a pair of Ag/AgCl microelectrodes filled with 3 M KCl; the potential microelectrode was inserted in the vacuole and the reference microelectrode was in the external solution. All experiments were performed in darkness at 24 °C. The analog measurement signals were converted into digital using an A/D conversion card.

Results and Discussion

The results of the measurements of hemolytic efficiency of the compounds studied are shown in Table II. Most of the compounds caused 50% hemolysis when used in micromolar concentrations. Only compound **3** hemolysed the erythrocytes significantly weaker, which is not surprising in view of its structure (*tert*-butyl group at the carbon atom – see Table I). These results are similar to those obtained earlier for other organophosphorus compounds (Kleszczyńska *et al.*, 2001b; Trela *et al.*, 2001). Hemolytic efficiencies of equimolar binary mixtures of the compounds studied with 2,4-D were determined by the isobole method (Kortenkamp and Altenburger, 1998). Full data concern-

Table II. The concentrations of the compounds studied and their binary mixtures with 2,4-D causing 50% hemolysis (C_{50}) of erythrocytes, inhibiting cucumber growth by 50% (IC_{50}) and the type of the interaction with 2,4-D.

Compound	Hemolytic experiments			Cucumber growth inhibition		
	C_{50} ± CL[a] [mM]	Binary mixtures C_{50} ± CL [mM]	Interaction type	IC_{50} ± CL [μM]	Toxic units for binary mixtures ± CL [TU]	Interaction type
1	0.08 ± 0.005	0.05 ± 0.004	+	550 ± 50	1.5 ± 0.13	–
2	0.23 ± 0.008	0.33 ± 0.029	–	530 ± 35	0.92 ± 0.06	+
3	3.00 ± 0.090	1.08 ± 0.187	+	500 ± 45	1.9 ± 0.17	++
4	0.03 ± 0.001	0.07 ± 0.006	–	720 ± 45	0.78 ± 0.06	+
5	0.26 ± 0.009	0.71 ± 0.062	–	975 ± 55	0.9 ± 0.06	0
6	0.52 ± 0.019	0.86 ± 0.072	–	275 ± 13	0.71 ± 0.04	+
7	0.09 ± 0,007	0.38 ± 0.039	–	170 ± 11	0.75 ± 0.05	+
8	0.16 ± 0.013	0.17 ± 0.017	0	230 ± 15	0.81 ± 0.06	+
2,4-D	1.60 ± 0.048			2.4 ± 0.3		

++, +, – and 0 are for strong synergism, synergism, antagonism and additivity, respectively.
[a] CL, 95% confidence limit.

ing these studies are collected in Table II. Almost all the compounds reacted in antagonistic way with 2,4-D. Exceptions are **1**, **3** and **8**. Compounds **3** and **8** are most lipophilic in their subgroups (see Table I). They have the longest alkyl chains substituted at the nitrogen atom and this may be the reason why they interact in the observed way with 2,4-D. They may disturb the erythrocyte membrane sufficiently to ease the action of 2,4-D. Concerning compound **1** the situation may be reverse one, as the compound has small lipophilicity and in this case 2,4-D must somehow facilitate the interaction with the membrane. In all other mixtures no cooperative hemolytic action is seen and the compounds seem rather to compete between themselves. Whatever may be speculated, there was no simple correlation between lipophilicity of the compounds studied and the hemolytic efficiency of their binary mixtures with 2,4-D. This conclusion is supported by the results of physiological and electrophysiological measurements. Binary mixtures of 2,4-D with compounds **4**, **6**, **7** and **8** were very efficient inhibiting the growth of cucumber hypocotyls, in comparison to the efficiency of particular compounds used individually (Table II). Their combined action may be described as synergistic (Table II). Two of the binary mixtures, (2,4-D + **1**) and (2,4-D + **3**), showed antagonistic action and the action of the rest of mixtures was of additive type. A similar pattern of the interaction was found for the efficiencies of binary mixtures to cause depolarization of the membrane potential of the alga (Table III). Namely, the same interaction was found for mixtures containing compounds **4**, **6**, **7** and **8** (see Table III). In two cases (compounds **2** and **5**) mixtures induced membrane depolarization in synergistic way while their inhibition of cucumber growth was found to be of additive type. A difference was also found for the (2,4-D + **1**) mixture causing cell potential depolarization in additive way, whereas it was antagonistically inhibiting cucumber growth. The mixture (2,4-D + **3**) acted in antagonistic way; in the case of cucumber growth inhibition there was strong antagonism. Examples of depolarization of membrane potential induced by compounds **3** and **7** and their binary mixtures with 2,4-D are presented in Fig. 1. These studies also showed that lipophilicity is not the only factor deciding of the type of the interaction. This is especially seen in

Table III. Effect of 0.05 mM of the compounds studied and their binary equimolar mixtures with 2,4-D on membrane potential of *Nitellopsis obtusa* alga cells (data indicates the difference between the membrane potential after 30 min incubation in a solution containing aminophosphonates or their mixtures and the potential in the control solution) and on the germination of white mustard (LSD – least significant difference – was 24.0; $p < 0.05$).

Compounds and their binary mixtures with 2,4-D	Depolarization after 30 min ± SE ($n \geq 3$) [mV]	Interaction type	Germination (%)	Interaction type
1	2.9 ± 0.8		78.0	
2	11.8 ± 2.7		28.6	
3	3.2 ± 0.9		101.3	
4	11.2 ± 2.1		42.4	
5	14.6 ± 4.3		35.6	
6	14.0 ± 3.8		48.2	
7	16.8 ± 3.1		87.5	
8	23.0 ± 4.8		78.0	
2,4-D	9.0 ± 3.5		88.5	
2,4-D + **1**	8.9 ± 1.4	0	88.2	0
2,4-D + **2**	18.2 ± 4.9	+	28.5	0
2,4-D + **3**	6.3 ± 1.4	–	63.9	+
2,4-D + **4**	17.0 ± 3.1	+	18.3	+
2,4-D + **5**	16.4 ± 2.7	+	22.3	++
2,4-D + **6**	20.3 ± 4.6	+	22.3	++
2,4-D + **7**	24.5 ± 4.1	+	61.0	+
2,4-D + **8**	27.4 ± 6.6	+	19.0	++
Control			100	

++, +, – and 0 are for strong synergism, synergism, antagonism and additivity, respectively.

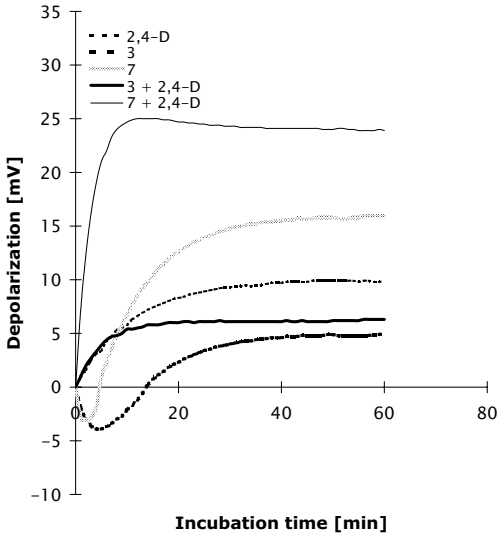


Fig. 1. Depolarization of membrane potential of *Nitellopsis obtusa* by compounds **3** and **7** and their binary mixtures with 2,4-D.

the case of the mixture (2,4-D + **3**). Its influence on the parameters studied was determined as antagonistic or even strongly antagonistic. The point is that the lipophilicity of compound **3** is the highest among all the aminophosphonates studied. Perhaps the effects observed are the consequence of its bulky *tert*-butyl group substituted at the carbon atom and it leads to the conclusion that structural features of particular compounds must be taken into account in such studies, especially in view of the differences between the results of all experiments described so far. These differences may be also the result of other factors that need further investigations.

The experiments last performed concerned inhibition of white mustard germination. Results obtained (Table III) partly agree with those described above. Compounds **1** and **2** interacted with 2,4-D additively and all other binary mixtures gave synergistic (compounds **3**, **4** and **7**) or strongly synergistic (compounds **5**, **6** and **8**) interactions.

The types of the interaction found in all the experiments on biological systems lead to the conclusion that at least four of the compounds studied (**4**, **6**, **7** and **8**) may be used in binary mixtures to give the most demanded synergistic interaction with 2,4-dichlorophenoxyacetic acid.

Acknowledgements

This work was sponsored by the State Committee for Scientific Research (KBN), grant no. 6 P04G 096 21. The authors thank K. Kulma for technical assistance.

Cooper C. (2001), Use of microtox for assessing heavy metal complex formation with the organic solvents acetonitrile and dimethyl sulphoxide: a preliminary study. Bull. Environ. Contam. Toxicol. **67**, 526–534.

Duchnowicz P. and Koter M. (2003), Damage to the erythrocyte membrane caused by chlorophenoxyacetic herbicides. Cell. Mol. Biol. Lett. **8**, 25–30.

Duchnowicz P., Koter M., and Duda W. (2002), Damage of erythrocyte by phenoxyacetic herbicides and their metabolites. Pest. Biochem. Physiol. **74**, 1–7.

Forlani G., Lejczak B., Kafarski P., and Wieczorek P. (1997), Mode of action of herbicidal derivatives of aminomethylenebiophosphonic acid. Part II. Reversal of herbicidal action by aromatic amino acids. J. Plant Growth Regul. **16**, 147–152.

Forlani G., Lejczak B., and Kafarski P. (2000), The herbicidaly active compound *N*-2-(5-chloropyridyl)aminomethylene-biophosphonic acid acts by inhibiting both glutamine and aromatic aminoacids biosynthesis. Acad. J. Plant Physiol. **27**, 677–683.

Gancarz R. and Dudek M. (1996), Structure-activity studies of aminophosphonic derivatives of fluorene. Phosphorus, Sulfur and Silicon **114**, 135–142.

Gisi U. (1996), Synergistic action of fungicides in mixtures. Phytopathology **86**, 1273–1279.

Grzyś E., Bielecki K., and Sarapuk J. (2001), Aminophosphonate-induced changes of betacyanine and ionic efflux. Z. Naturforsch. **56c**, 349–352.

Kleszczyńska H., Sarapuk J., and Bonarska D. (2001a), Hemolytic toxicity of some new aminophosphonates. Cell. Mol. Biol. Lett. **6**, 271–275.

Kleszczyńska H., Bonarska D., Sarapuk J., and Bielecki K. (2001b), Physiological activity of some organophosphorous compounds and their influence on mechanical properties of erythrocytes. Z. Naturforsch. **56c**, 999–1002.

Kleszczyńska H., Bonarska D., Bielecki K., and Sarapuk J. (2003), The hemolytic and physiological activities of mixtures of some phenoxy and organophosphorous herbicides. Cell. Mol. Biol. Lett. **8**, 55–61.

Kortenkamp A. and Altenburger R. (1998), Synergisms with mixtures of xenoestrogens: A reevaluation using the method of isoboles. Sci. Total Environ. **221**, 59–73.

Lejczak B., Boduszek B., Kafarski P., Forlani G., Wojtasek H., and Wieczorek P. (1997), Mode of action of herbicidal derivatives of aminomethylenebiophosphonic acid. Part I. Physiological activity and inhibition of anthocyanins biosynthesis. J. Plant Growth Regul. **15**, 109–113.

Mora A. and Earle E. D. (2001), Combination of *Trichoderma harzianum* endochitinase and a membrane-affecting fungicide on control of *Alternaria* leaf spot in transgenic broccoli plants. Appl. Microbiol. Biotechnol. **55**, 306–310.

Oruç E. Ö. and Üner N. (1999), Effects of 2,4-Diamin on some parameters of protein and carbohydrate metabolisms in the serum, muscle and liver of *Cyprinus carpio*. Environ. Pollution **105**, 267–272.

Oruç E. Ö. and Üner N. (2000), Combined effects of 2,4-D and azinphosmethyl on antioxidant enzymes and lipid peroxidation in liver of *Oreochomis niloticus*. Comp. Biochem. Physiol. Part C **127**, 291–296.

Pape-Lindstrom P. A. and Lydy M. J. (1997), Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. Environ. Toxicol. Chem. **16**, 2415–2420.

Polish Agrotechnical Branch Norm BN-83/9180–26 (1984), Normalization Edition "Alfa".

Suwalsky M., Benites M., Villena F., Aguilar F., and Sotomayor P. (1996), Interaction of 2,4-dichlorophenoxyacetic acid (2,4-D) with cell and model membranes. Biochim. Biophys. Acta **1285**, 267–276.

Suwalsky M., Quevedo L., Norris B., and Benites M. (1999), Toxic action of the herbicide 2,4-D on the neuroepithelial synapse and on the nonstimulated skin of the frog *Caudiverbera caudiverbera*. Bull. Environ. Contam. Toxicol. **62**, 570–577.

Suwalsky M., Benites M., Norris B., and Sotomayor P. (2000), Toxic effects of the fungicide benomyl on cell membranes. Comp. Biochem. Physiol. **125**, 111–119.

Trela Z., Kleszczyńska H., and Sarapuk J. (2001), Physiological and hemolytic toxicity of some aminophosphonates. Z. Naturforsch. **56c**, 838–842.

Tripathi A. M. and Agarwal R. A. (1997), Synergism in tertiary mixtures of pesticides. Chemosphere **35**, 2365–2374.