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Mode of Action of Herbicidal Derivatives of Aminomethylenebisphosphonic Acid. I. Physiologic Activity and Inhibition of Anthocyanin Biosynthesis

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Abstract. *N*-Pyridylaminomethylenebisphosphonic acids constitute a class of promising herbicides. Since their mode of action at the cellular level is still poorly understood, we studied the influence of *N*-pyridylaminomethylenebisphosphonic acids on plant growth, at the whole plant and undifferentiated tissue levels, using seedlings and cell suspension cultures of mono- and dicotyledonous species. These compounds exhibited strong herbicidal properties, being equipotent with the popular herbicide glyphosate. Since they also depressed buckweed anthocyanin biosynthesis, the shikimate pathway could represent a site of action of *N*-pyridylaminomethylenebisphosphonic acids.

Key Words. *N*-Pyridylaminomethylenebisphosphonates—Anthocyanins—Herbicides—Glyphosate

The discovery of glyphosate (*N*-phosphonomethylglycine) in 1971 instituted a milestone in a rational design of herbicides and pointed out the aromatic amino acid biosynthetic pathway as a particularly attractive target for such an approach (Grossbard and Atkinson 1985). This discovery also initiated extensive research concerned with the design, synthesis, and evaluation of physiologic properties of hundreds, or perhaps thousands, of glyphosate derivatives, homologs, and analogs. Obviously, it is difficult to improve on a compound that is as simple structurally as glyphosate and which exhibits such a powerful activity. Indeed, most of the analogs were found less active than the herbicide itself.

However, these efforts were not totally unsuccessful and resulted in the discovery of numerous active compounds. These include the herbicide phosphinothricin, introduced simultaneously in Germany and Japan (Langeluddeke et al. 1981, Tachibana et al. 1986), *N*-pyridyl derivatives (**1, 2,** and **3**) of aminomethylenebisphosphonic acid, developed in Japan (Suzuki et al. 1979), as well as phosphonic acid analogs of morphactins first synthesized in our laboratories (Czerwiński et al. 1982, Gancarz et al. 1985, Wojtasek et al. 1990).

Compounds **1, 2,** and **3** (Fig. 1) are of special interest since the structure of aminomethylenebisphosphonic acid, which possesses two strongly acidic groups and one positively charged amino group, closely resembles *N*-phosphonomethylglycine. Glyphosate has long been postulated to act as a transition site analog, for the putative tetrahedral intermediate formed transiently during reaction catalyzed by 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, the sixth enzyme of the shikimate pathway (Anderson et al. 1988, Bondinell et al. 1976). One may thus speculate that the pyridyl fragment of compound **1** resembles the nearly flat shikimate part of this intermediate, and the aminomethylenebisphosphonic moiety, similar to glyphosate, mimics the tetrahedral fragment of the intermediate. Since their mode of action at the cellular level is still poorly understood, as for other already commercialized herbicides, we undertook a study on the influence of *N*-pyridylaminomethylenebisphosphonic acids on plant growth, at the whole plant or undifferentiated tissue level, using seedlings and cell suspension cultures of mono- and dicotyledonous species. To check whether such compounds could act by interfering with aromatic amino acid biosynthesis, their effect on the production of anthocyanin by buckweed seedlings was also evaluated.

Materials and Methods

Chemical Syntheses

N-Pyridylaminomethylenebisphosphonic acids and *N*-benzylaminomethylenebisphosphonic acid (**4**) were synthesized according to previously published methods (Maier 1981, Suzuki et al. 1979). Melting points and yields of these compounds are given in Table 1.

Abbreviations: EPSP, 5-enolpyruvylshikimate-3-phosphate; 2,4-D, 2,4-dichlorophenoxyacetic acid.

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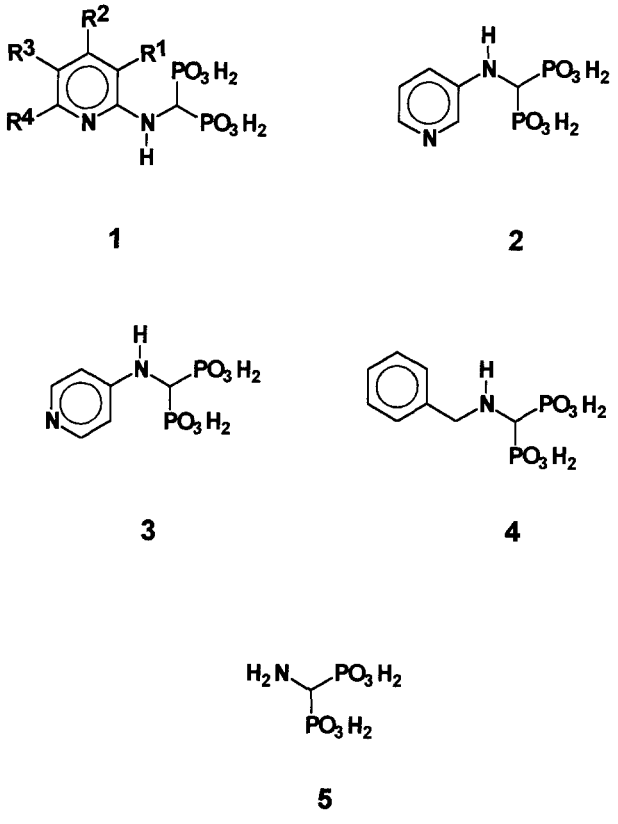

Fig. 1. *N*-Pyridylaminomethylenebisphosphonic acids (**1–3**), *N*-benzylaminomethylenebisphosphonic acid (**4**), and aminomethylenebisphosphonic acid (**5**).

Aminomethylenebisphosphonic acid (**5**) was synthesized by a modified standard procedure (Suzuki et al. 1979) using benzhydrylamine as the amine component of the reaction mixture. This procedure is perhaps one of the most convenient methods for preparation of this compound. Thus, the mixture of 8.6 mL (0.05 mol) of benzhydrylamine, 8.5 mL (0.05 mol) of ethyl orthoformate, and 12.9 mL (0.05 mol) of diethyl phosphite was placed in a simple distillation apparatus and heated slowly to 120°C. At this point, formation of ethanol and its removal by distillation were observed. Then the temperature of the solution was raised to 160°C and left at this temperature for 3 h. The resulting orange oil was hydrolyzed with 20% hydrochloric acid (100 mL) for 6 h. After cooling to room temperature, the mixture was extracted with toluene (2 × 100 mL) to remove benzhydryl chloride and benzhydrylic alcohol. The solvent was then removed under reduced pressure and the product dissolved in water (100 mL), decolorized with charcoal, and water was removed under reduced pressure. The crude product obtained in this manner was recrystallized from methanol:water (1:1; v/v), yielding pure aminomethylenebisphosphonic acid in 45% yield.

The structures of all of the compounds were supported by infrared and proton magnetic resonance (^{1}H NMR) spectra and by elemental analyses.

Evaluation of Herbicidal Activity of Aminomethylenebisphosphonic Acids

The physiologic activity of the studied compounds was tested using buckweed (*Fagopyrum esculentum* Munch.), crest (*Lepidium sativum* L.), cucumber (*Cucumis sativus* L. cv. Wisconsin), wheat (*Triticum aestivum* L. cv. Jawa), and maize (*Zea mays* L.) seedlings. Each experiment was replicated four times.

The effect of some of these compounds on the growth of suspension cultured cells of tobacco (*Nicotana plumbaginifolia* Viviani), carrot (*Daucus carota* L. cv. Lunga di Amsterdam), maize (*Z. mays* L. cv. Black Mexican Sweet), and rice (*Oryza sativa* L. cv. Roncarolo) was also evaluated.

Effects of Studied Compounds on the Growth of Test Plants. Seeds were germinated at 33°C for 1.5–4 days in darkness. Groups of 10–40 uniform seedlings (depending on the plant used) were transferred to Petri dishes (9 cm) lined with two discs of Whatman No. 2 filter paper wetted with 10 mL of distilled water (control) or solutions of the test compounds to give final concentrations of 0.05, 0.15, or 1.5 mM. Plants were grown at 25°C with a 12-h day length for 5–9 days (depending on the plant) under fluorescent tubes (about 300 μE m^{-2}s^{-1} at plant level). Separated roots and shoots, fresh weights, were determined. The herbicidal effect was expressed as the percentage change in plant root and shoot fresh weight in relation to untreated control.

Inhibition of Plant Cell Culture Growth. Plant cell cultures were grown in Erlenmeyer flasks in MS medium (Murashige and Skoog 1962) containing: 0.5 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mL/liter 6-benzylaminopurine for tobacco; 0.5 mg/liter 2,4-D and 0.25 mg/liter kinetin for carrot; 1 mg/liter 2,4-D for maize; and 2 mg/liter 2,4-D for rice. Incubation was in the dark at 26 ± 1°C on a rotary shaker (120 rpm). Subcultures were made every 2 weeks by transferring 25-mL aliquots to 100 mL of fresh medium.

The effect of increasing concentrations of aminomethylenebisphosphonic acid derivatives on exponentially growth cultures was measured as described previously (Forlani et al. 1994). Cell samples, withdrawn from the stock cultures in the early stationary growth phase, were used to inoculate 100-mL culture flasks containing 20 mL of fresh medium to a density of about 1.0 mg/mL (dry weight). Cultures were incubated as above, and filter-sterilized bisphosphonates were added just after the density of cells reached 1.7 mg/mL (dry weight). After another 8 days of incubation, when the untreated controls reached the late exponential growth phase, cells were harvested by vacuum filtration, and the dry weight increase was determined on each sample after drying in an oven at 90°C for 48 h. Data for untreated controls were 5.8 ± 0.3, 5.2 ± 0.3, 4.6 ± 0.2, and 5.8 ± 0.9 mg/mL for tobacco, carrot, maize, and rice, respectively.

For each compound, at least seven doses (ranging from 0.01 to 1 mM) with three replicates were tested. Two independent experiments were performed for each cell culture; since error variances were found to be homogeneous, means over the two repetitions were considered.

Anthocyanins Determination

The influence of aminomethylenebisphosphonates on anthocyanin production in buckweed seedlings was studied using a modification of a previously described procedure (Lange et al. 1971, Rengel and Kordan 1987).

After 2 days of dark germination, 15 seedlings were selected for uniformity and further grown in Petri dishes (9 cm) lined with two discs of Whatman No. 2 filter paper wetted with 10 mL of distilled water (control) or solutions of the test compounds to give final concentrations of 0.05, 0.15, or 1.5 mM. The plants were grown at 25°C with a 12-h day length for 5 days under fluorescent tubes (about 300 μE m^{-2}s^{-1} at plant level). Stems of ten plants were then harvested, weighed, and

Table 1. *N*-Substituted aminomethylenebisphosphonic acids.

Compound	Structure R^1	R^2	R^3	R^4	Yield (%)	m.p. decomp. (°C)	Ref.
1a	H	H	H	H	76	292–294	Suzuki et al. (1979); Maier (1981)
1b	CH_3	H	H	H	52	295–296	Suzuki et al. (1979); Maier (1981)
1c	H	H	H	CH_3	57	293–295	
1d	H	CH_3	H	CH_3	75	299–303	
1e	H	H	Cl	H	73	280–282	
2					25	278–280	Suzuki et al. (1979); Maier (1981)
3					21	269–273	Suzuki et al. (1979); Maier (1981)
4					79	215–220	
5					45	287–290	Moedritzer and Irani (1966)

quickly ground in a mortar, followed by the addition of 20 mL of 1% solution of concentrated hydrochloric acid in methanol. The mixture was transferred to an Erlenmeyer flask and the extraction allowed to continue for 12 h at room temperature. Extracts were then clarified by filtration and the extinction of samples at 530 and 657 nm was determined using UV-vis spectrophotometer. Anthocyanin content in the stems was determined according to Mancinelli and Schwartz (1984) using the equation ($E = (E_{530} - 0.25E_{657})/m$, where m is mass of the stems) and presented as a percent of control. Each experiment was replicated four times.

Statistical Treatment

Dixon's Q test was used to reject the unreasonable results. The means for samples and controls were compared by testing the null hypothesis at the 5% significance level (Miller and Miller 1984). Concentrations causing 50% inhibition of cell growth rate (LD_{50}) were estimated using the linear regression equations for the values of growth yield, expressed as a percentage of untreated controls, plotted against the logarithm of bisphosphonate concentration.

Nonstatistically significant values are marked in tables as N.

Results and Discussion

The sixth enzyme of the shikimate pathway, EPSP synthase, has generated considerable interest since it has been shown to be the target of the broad spectrum herbicide glyphosate (Amrhein et al. 1980, Grossbard and Atkinson 1985). EPSP synthase catalyzes an unusual transfer reaction of the carboxyvinyl portion of phospho*enol*pyruvate regiospecifically to the 5-hydroxyl moiety of shikimate 3-phosphate, forming EPSP and inorganic phosphate. Glyphosate is believed to act as a transition state analog of the putative protonated phospho*enol*pyruvate oxonium ion formed during catalysis (Amrhein et al. 1980, Steinrucken and Amrhein 1984). Numerous unsuccessful attempts have been made to identify glyphosate analogs that retain inhibitor potency. The use of a two-dimensional TRNOE NMR technique revealed that the substrate, intermediate, and product of this enzymatic reaction are planar (Leo et al. 1992). This experimentally defined model thus suggests that EPSP synthase inhibitors should possess planar structures. *N*-Pyridylaminomethylenebisphosphonates suit these requirements since the aminomethylenebisphosphonate fragment of the molecule closely resembles the structure of glyphosate, whereas the flat pyridyl part of the molecule, similar to the case of glucosidase inhibitors (Knapp et al. 1995), mimics chair conformation of shikimate 3-phosphate. To challenge the hypothesis that *N*-pyridylaminomethylenebisphosphonates could also act as inhibitors of aromatic amino acids biosynthesis, we undertook studies on their effect on plant growth.

Reflecting the mode of application, the influence of the tested compounds on plant root development was usually more pronounced than their action on shoots. With the exception of compound **1d,** the pyridyl derivatives **1** and **3** appeared to be exceptionally good inhibitors of the growth of the test plants (Table 2) with compounds **1b** and **1e** being equipotent with glyphosate. Compound **3,** moderately active against buckweed and crest, strongly affected the growth of cucumber, wheat, and maize. Compound **2** exhibited moderate or weak activity. These findings show good agreement with the patent data (Suzuki et al. 1979). The glyphosate derivative (compound **5**) and compound **4,** in which the pyridyl fragment is replaced by a benzyl group, were moderately active, showing that herbicidal activity is not limited to compounds bearing pyridyl moiety.

We also tested the action of these compounds on the growth of plant cell suspension cultures (Table 3). The structure-activity relationship was very similar, with compounds **1e** and **3** being the most active (the action of compound **1b** was not studied). The influence of these compounds on the growth of cell cultures was generally stronger than their influence at the whole plant level. Thus, the transportation of these compounds via the vascular system may be a possible limiting factor of their action.

It should be emphasized that the results reported in Table 2 depend strictly on the adopted experimental conditions, especially on the cellular density at the time of addition of these compounds. For instance, the addition

Table 2. Effect of derivatives of aminomethylenebisphosphonic acid on the growth of test plants, measured as percentage change in root and shoot weight compared with that of control.

Compound	Root or shoot	Concentration (mM) 0.05	0.15	0.5	1.5
	F. esculentum				
Glyphosate	R	−(64 ± 6)	−(73 ± 7)	−(91 ± 11)	−(96 ± 8)
	S	N[a]	N	−(18 ± 3)	−(56 ± 7)
1a	R	N	−(39 ± 6)	−(37 ± 4)	−(64 ± 2)
	S	N	N	N	−(33 ± 1)
1b	R	−(57 ± 12)	−(78 ± 7)	−100	−100
	S	−(51 ± 5)	−(65 ± 7)	−(60 ± 6)	−(60 ± 7)
1c	R	−(26 ± 5)	−(50 ± 10)	−(52 ± 4)	−(81 ± 3)
	S	N	N	−(19 ± 3)	−(29 ± 3)
1d	R	N	N	N	N
	S	N	N	N	N
1e	R	−(42 ± 9)	−(69 ± 11)	−(71 ± 4)	−(95 ± 3)
	S	N	−(28 ± 4)	−(36 ± 7)	−(49 ± 12)
2	R	−(34 ± 5)	−(42 ± 6)	−(81 ± 8)	−(88 ± 8)
	S	N	−(30 ± 3)	−(37 ± 4)	−(62 ± 2)
3	R	N	−(38 ± 7)	−(52 ± 9)	−(69 ± 2)
	S	N	N	−(31 ± 5)	−(33 ± 7)
4	R	N	−(25 ± 7)	−(39 ± 11)	−(61 ± 9)
	S	N	N	−(12 ± 3)	−(17 ± 3)
5	R	−(30 ± 7)	−(34 ± 3)	−(57 ± 8)	−(81 ± 4)
	S	N	N	−(18 ± 7)	−(19 ± 4)
	L. sativum				
Glyphosate	R	−(86 ± 8)	−(88 ± 4)	−(90 ± 7)	−(94 ± 9)
	S	−(13 ± 1)	−(15 ± 2)	−(20 ± 2)	−(44 ± 2)
1a	R	−(29 ± 4)	−(53 ± 5)	−(74 ± 6)	−(87 ± 11)
	S	N	−(11 ± 5)	−(30 ± 7)	−(46 ± 8)
1b	R	−(76 ± 7)	−(82 ± 13)	−(89 ± 9)	−(95 ± 15)
	S	−(27 ± 6)	−(42 ± 9)	−(51 ± 7)	−(58 ± 9)
1e	R	−(67 ± 9)	−(80 ± 7)	−(87 ± 11)	−(92 ± 11)
	S	−(27 ± 5)	−(41 ± 5)	−(59 ± 8)	−(62 ± 11)
2	R	N	N	N	N
	S	N	−(15 ± 2)	−(18 ± 1)	−(35 ± 3)
3	R	N	−(42 ± 7)	−(50 ± 3)	−(84 ± 8)
	S	N	−(15 ± 2)	−(36 ± 7)	−(49 ± 4)
4	R	N	−(26 ± 4)	−(40 ± 2)	−(66 ± 4)
	S	N	N	N	−(27 ± 2)
5	R	N	−(22 ± 8)	−(33 ± 10)	−(43 ± 2)
	S	N	N	N	−(14 ± 1)
	C. sativus				
Glyphosate	R	−(60 ± 4)	−(77 ± 4)	−(78 ± 7)	−(84 ± 2)
	S	N	N	N	−(22 ± 4)
1b	R	−(76 ± 11)	−(88 ± 12)	−100	−100
	S	−(48 ± 10)	−(66 ± 9)	−(68 ± 7)	−100
1e	R	−(49 ± 3)	−(70 ± 5)	−(80 ± 9)	−(81 ± 2)
	S	N	−(28 ± 2)	−(29 ± 3)	−(30 ± 2)
2	R	N	−(32 ± 4)	−(52 ± 3)	−(57 ± 6)
	S	N	N	N	N
3	R	−(62 ± 5)	−(74 ± 5)	−(81 ± 9)	−(81 ± 3)
	S	−(34 ± 2)	−(34 ± 6)	−(47 ± 3)	−(49 ± 5)
4	R	N	N	−(62 ± 11)	−(63 ± 4)
	S	N	N	+(23 ± 3)	+(31 ± 2)
	T. aestivum				
1b	R	N	−(38 ± 6)	−(72 ± 9)	−(84 ± 12)
	S	N	−(37 ± 13)	−(87 ± 15)	−(95 ± 3)
1e	R	−(11 ± 1)	−(56 ± 9)	−(56 ± 2)	−100
	S	N	−(25 ± 11)	−(67 ± 13)	−100

Table 2. Continued

Compound	Root or shoot	Concentration (mM) 0.05	0.15	0.5	1.5
	T. aestivum				
2	R	N	−(48 ± 5)	−(81 ± 7)	−(85 ± 3)
	S	N	−(62 ± 5)	−(86 ± 16)	−(92 ± 3)
3	R	N	−(67 ± 2)	−(86 ± 11)	−(87 ± 12)
	S	N	−(82 ± 6)	−(90 ± 3)	−(94 ± 2)
4	R	N	N	N	−(57 ± 7)
	S	N	N	N	N
	Z. mays				
1a	R	N	N	−(27 ± 2)	−(27 ± 5)
	S	N	N	−(49 ± 4)	−(76 ± 9)
1b	R	−(51 ± 7)	−(60 ± 6)	−(58 ± 11)	−(59 ± 6)
	S	−(56 ± 6)	−(82 ± 6)	−(91 ± 2)	−(91 ± 4)
1e	R	N	−(55 ± 11)	−(52 ± 12)	−(67 ± 7)
	S	N	−(19 ± 5)	−(49 ± 9)	−(60 ± 3)
2	R	−(39 ± 9)	−(39 ± 7)	−(65 ± 12)	−(67 ± 11)
	S	−(27 ± 2)	−(38 ± 6)	−(44 ± 8)	−(71 ± 9)
3	R	−(42 ± 7)	−(61 ± 9)	−(61 ± 14)	−(69 ± 13)
	S	N	−(45 ± 11)	−(60 ± 9)	−(60 ± 12)
4	R	+(51 ± 12)	+(23 ± 9)	N	N
	S	N	N	N	N

[a] N, no effect.

of compounds **1c** and **1e** at 0.5 mM to carrot cultures resulted in a growth inhibition of 57 and 56%, respectively, if compounds were added at a cell density of 1.7 mg/mL, but only 36 and 34% if added when cells had already reached 2.0 mg/mL. This may be linked to an unusually weak slope in the inhibition curves. Raising the concentration of the compounds by 2 orders of magnitude resulted in most cases in the reduction of growth rate only to 50%. For comparison in the same experimental conditions, tobacco and maize cell growth inhibition changed from 5 to 95% when the glyphosate concentration was raised from 1 to 10 μM, and no effect was noted by addition of the herbicide at a cell density of 1.5, 2.0, or even 2.5 mg/mL. Also noteworthy is the result obtained with compounds **1c** and **1e** on tobacco cells. In this case the growth rate dropped steeply up to concentrations of 0.04–0.07 mM, then reached a plateau and did not decrease further even with doses as high as 0.5–0.7 mM. A similar shape of the dose-dependent inhibition curve was also observed when the herbicidal activity was tested on intact plants (Table 2). This quite unusual behavior could provide some information about the mode of action of *N*-pyridylaminomethylenebisphosphonic acids. For instance, it may result from the occurrence of a target enzyme with multiple isoforms possessing different kinetics of inhibition. Moreover, the consistency of *N*-pyridylaminomethylenebisphosphonate effectiveness on cell culture and whole plant growth suggests that their toxicity could be exerted through the inhibition of some pathways in primary metabolism, i.e. cellular activity expressed also at the level of undifferentiated tissue.

Table 3. Inhibitory effect of bisphosphonates on cell culture growth determined as concentration (mM) causing 50% inhibition of cell growth rate in relation to control.

Compound	*N. plumbaginifolia*	*D. carota*	*Z. mays*	*O. sativa*
1a	0.35 ± 0.10	0.65 ± 0.05	0.48 ± 0.09	0.77 ± 0.11
1c	0.20 ± 0.02	0.32 ± 0.01	0.10 ± 0.03	1.65 ± 0.27
1e	0.08 ± 0.01	0.32 ± 0.03	0.07 ± 0.02	0.33 ± 0.05
2	0.09 ± 0.04	0.28 ± 0.04	1.20 ± 0.16	0.75 ± 0.18
3	0.06 ± 0.03	0.06 ± 0.01	1.53 ± 0.17	0.40 ± 0.11
4	0.24 ± 0.04	0.72 ± 0.08	0.10 ± 0.01	1.41 ± 0.67
5	0.19 ± 0.04	0.43 ± 0.04	0.06 ± 0.02	2.17 ± 0.47

Table 4. Inhibitory effects of bisphosphonates on anthocyanin biosynthesis in buckwheat (*F. esculentum*) stems measured as percentage change in their level in relation to control.

	Concentration (mM)			
Compound	0.05	0.15	0.5	1.5
Glyphosate	N[a]	N	−(66 ± 4)	−(80 ± 3)
1a	N	N	−(47 ± 3)	−(64 ± 1)
1b	−(33 ± 5)	−(71 ± 5)	−(77 ± 3)	−(80 ± 4)
1c	N	N	−(38 ± 4)	−(60 ± 2)
1d	N	N	N	N
1e	−(39 ± 7)	−(47 ± 3)	−(73 ± 5)	−(79 ± 8)
2	−(19 ± 6)	−(29 ± 5)	−(44 ± 3)	−(56 ± 1)
3	−(18 ± 4)	−(42 ± 4)	−(76 ± 9)	−(76 ± 6)

[a] N, no effect.

The depression of light-induced buckweed anthocyanin biosynthesis is a standard test indicating the influence of a studied compound on the biosynthesis of aromatic amino acids. As seen from Table 4, with the exception of the herbicidally inactive compound **1d**, all the other tested compounds appeared to be the strong inhibitors of anthocyanin production in buckwheat stems. They were either equipotent or even stronger than glyphosate. Differential UV-vis spectra of methanolic extracts of treated and untreated plants showed that the tested compounds depressed not only anthocyanin but also chlorophyll biosynthesis (data not presented). Since this effect is similar to that caused by glyphosate, the above findings seem to support the hypothesis that the shikimate pathway could represent a site of action of *N*-pyridylaminomethylenebisphosphonic acids.

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