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The Herbicidally Active Compound *N*-2-(6-Methyl-Pyridyl)-Aminomethylene Bisphosphonic Acid Inhibits *In Vivo* Aromatic Biosynthesis

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Abstract. The effect of *N*-2-(6-methyl-pyridyl)-aminomethylene bisphosphonic acid (M-pyr-AMBPA), a compound previously shown to exhibit herbicidal properties on whole plants and to inhibit *in vitro* activity of the first enzyme in the shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase, was investigated on *Nicotiana plumbaginifolia* suspension cultured cells and compared to that of the herbicide glyphosate. The addition of M-pyr-AMBPA from 10^{-4} to 10^{-3} M was found to cause a severe cell growth reduction. Kinetic analysis of partially purified DAHP synthase accounted for non-competitive inhibition type with respect to both phospho-*enol*-pyruvate and erythrose-4-phosphate, with K_I values of 0.43 and 0.62 mM, respectively. Amino acid pool measurements of cells grown in the presence of sublethal doses of M-pyr-AMBPA pointed to an actual reduction of free aromatic amino acids, showing that DAHP synthase inhibition takes place *in vivo*, and suggesting that the interference of this aminophosphonate with plant aromatic biosynthesis may account for a large part of its phytotoxicity. However, exogenous supply of a mixture of phenylalanine, tyrosine and tryptophan failed to achieve full reversal of cell growth inhibition, yet the occurrence of other target(s) cannot be ruled out.

Key Words. *N*-2-(6-methyl-pyridyl)-aminomethylene bisphosphonic acid—Amino acid biosynthesis inhibitors—DAHP synthase—Glyphosate—Aromatic metabolism

Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate; EDTA, ethylenediaminetetraacetic acid; E4P, erythrose-4-phosphate; EPSP, 5-enol-pyruvyl-shikimate-3-phosphate; M-pyr-AMBPA, *N*-2-(6-methyl-pyridyl)-aminomethylene bisphosphonic acid; PEP, phospho-*enol*-pyruvate.

Aminoalkylphosphonic acids are structural analogues of amino acids wherein the carboxylic group is replaced by a phosphonic, or related moiety. In the last two decades increasing evidence has shown that many aminophosphonates are capable of interacting with enzymes involved in amino acid metabolism. In spite of their significant differences including size, shape (flat CO_2H *versus* tetrahedral PO_3H_2) and acidity (pK difference of at least 3 units), several enzymes do not differentiate between carboxylic and phosphonic function with respect to binding to active sites. The structural antagonism between amino acids or intermediates in their biosynthesis and the phosphonic counterparts results, more often, in inhibition of enzyme activity. Beyond their significance in basic biochemical and physiological studies, several aminoalkylphosphonic acids have found increasing practical application as herbicides (Dekker and Duke 1995, Kishore and Shah 1988). The most remarkable examples are provided by the naturally occurring compound phosphinothricin (L-homoalanine-4-yl-[methyl]-phosphinic acid), that acts by inhibiting a key enzyme in ammonia assimilation, glutamine synthetase (Köcher 1989), and the shikimate pathway inhibitor glyphosate ([*N*-phosphonomethyl]glycine) that blocks aromatic amino acid biosynthesis by interfering with the enzyme that catalyzes the sixth step in the pathway, 5-*enol*-pyruvyl-shikimate-3-phosphate (EPSP) synthase (Kishore et al. 1992).

Aiming at the development of other active molecules, the lead provided by the structures of glyphosate and phosphinothricin has been explored exhaustively in recent years: hundreds of derivatives, homologues and possible analogues were synthesized and tested for herbicidal activity. Whereas no substances sharing the same biochemical targets have been identified to date, such research resulted in the discovery of new classes of active compounds, among which are amino-methylene-bisphosphonic acids (e.g. Cromartie and Fisher 1995,

Suzuki et al. 1979). Their target at the cellular level is still poorly understood, but recent evidence recounts the ability of different bisphosphonates to inhibit farnesyl pyrophosphate synthase (Cromartie and Fisher 1995), squalene synthase (Biller et al. 1996), mitochondrial H^+-pyrophosphatase (Vianello et al. 1997) and geranyl-geranyl diphosphate synthase (Oberhauser et al. 1998), and suggests that they should be considered a heterogeneous class of compounds with various modes of action.

We previously evaluated the biological activity of a series of *N*-pyridyl derivatives of aminomethylene bisphosphonic acid, most of which exerted remarkable phytotoxic effects at both the plant and the cell culture level (Lejczak et al. 1996, Kafarski et al. 1997). Since these preliminary studies revealed that some of these compounds depressed anthocyanin biosynthesis *in vivo*, the possible occurrence of target(s) for their action in plant aromatic metabolism was investigated. While *in vitro* activity of EPSP synthase was found to be unaffected (Forlani et al. 1997), from a group of 7 compounds, 5 reduced the activity of the plastidial and Mn^{++}-stimulated isozyme of the first enzyme in the prechorismate pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase (EC 4.1.2.15). The addition of excess divalent cations to the assay mixtures negated the effect of three of them, but an inhibition solely based upon metal chelation seemed not to be the case for 5-chloro- and 6-methyl-pyridyl derivatives (Forlani et al. 1996). To ascertain whether the inhibition of DAHP synthase could really take place *in vivo*, we undertook the study of the effect of sublethal concentrations of one of these two compounds, *N*-2-(6-methyl-pyridyl)-aminomethylene bisphosphonic acid (M-pyr-AMBPA), on cultured tobacco cells. Here, we report experimental evidence that accounts for an actual reduction of phenylalanine and tyrosine biosynthesis, suggesting that the herbicidal properties of such aminophosphonate may be related at least in part to a block of aromatic amino acid production.

Materials and Methods

Chemicals

Unless specified otherwise, chemicals were purchased from Sigma Chemical Co., St. Louis, MO, and were of analytical grade. M-pyr-AMBPA was synthesized as described previously (Lejczak et al. 1996).

Culture Conditions, Evaluation of Cell Growth Inhibition and Reversal Experiments

Suspension cultured cells of *Nicotiana plumbaginifolia* Viviani were grown in MS medium (Murashige and Skoog 1962) containing 0.5 mg l^{-1} of both 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine, and 30 g l^{-1} sucrose. Culture conditions were reported earlier (Forlani et al. 1997). The effect of increasing concentrations of M-pyr-AMBPA and glyphosate on exponentially growing cells was measured as follows. Cell samples withdrawn from stock cultures in the late exponential phase of growth were used to inoculate 100-ml Erlenmeyer flasks to a density ranging from 1.5 to 1.6 mg ml^{-1} (dry weight) in a final volume of 25 ml. Filter-sterilized compounds (brought to pH 6.0 with KOH) were added just after the cell density population reached 2.0 mg ml^{-1}. After a further 6 days of incubation, when untreated controls reached the early stationary phase of growth, cells were harvested by vacuum filtration, and the dry weight increase was determined for each sample following drying in an oven at 90°C for 48 h. The same protocol was adopted for reversal experiments, where amino acid supplement (0.25 mM L-phenylalanine, 0.25 mM L-tyrosine, 0.125 mM L-tryptophan, and 0.25 μM each of *p*-amino- and *p*-hydroxybenzoic acid) was added to the culture medium at 0, 4 and 7 days following culture inoculation.

Extraction, Partial Purification and Assay of DAHP Synthase

Cells in the exponential phase of growth were harvested by filtration through nylon cloth (50 μm diameter), weighed, resuspended in 2 ml g^{-1} of extraction buffer (25 mM Tris-HCl, pH 7.5, containing 0.5 mM dithiothreitol), and homogenized in a Teflon-in-glass Potter homogenizer; 1% polyvinyl polypyrrolidone (w/v) was added in order to prevent oxidation of phenolic compounds. All subsequent operations were carried out at 0–4°C. The homogenate was clarified at 20,000 *g* for 20 min, and the supernatant was further centrifuged 60 min at 50,000 *g*. Crude extract was fractionated with ammonium sulfate (70% of saturation) and proteins were collected by centrifugation at 12,000 *g* for 20 min; pellets were resuspended in extraction buffer and desalted by passage through a Bio-Gel P6DG (Bio-Rad) column equilibrated with the same buffer. The desalted extract was loaded at a constant flow of 30 ml h^{-1} onto a DEAE-Sephacel column (2.5 × 10 cm) equilibrated with extraction buffer. After extensive washing, enzyme activity was eluted with a linear gradient from 0 to 250 mM NaCl (500 ml), collecting 5-ml fractions. Active fractions were pooled, desalted as above, and concentrated in a centrifugal Ultrafree filter unit (Millipore) with a cut-off of 30 kDa. The sample was then injected onto a Mono-Q HR 5/5 (Pharmacia) FPLC column, that was eluted by a linear gradient from 0 to 350 mM NaCl (40 ml) in extraction buffer at a flow of 60 ml h^{-1}, while collecting 0.5-ml fractions. Active fractions were pooled, desalted and immediately used for biochemical evaluations.

DAHP synthase activity was measured at 35°C as previously described (Forlani et al. 1996). Briefly, the reaction mixture contained 50 mM EPPS-KOH pH 7.75, 0.5 mM erythrose-4-phosphate (E4P), 2 mM phospho-*enol*-pyruvate (PEP), 0.7 mM $MnCl_2$, and 1 mM dithiothreitol in a final volume of 0.1 ml. After up to 30 min, the reaction was stopped and either DAHP formed was quantified by the thiobarbituric method, or the inorganic phosphate released was determined by the malachite green dye assay; in the latter case, 0.5 mM ammonium molybdate was added to the reaction mixture in order to inhibit unspecific phosphatases. Enzyme inhibition was estimated by adding to the reaction mixture proper dilutions of a 50 mM aqueous solution (pH 7.0) of M-pyr-AMBPA. The resulting initial reaction rate was evaluated from the activities obtained after 5, 10, 15 and 20 min of incubation, a time over which the rate was linear, and compared to that of untreated control. A kinetic analysis was performed by varying PEP (from 0.25 to 2 mM, with E4P kept at 0.5 mM) or E4P (from 0.05 to 0.5 mM, with PEP fixed at 2 mM) concentration. Proper checks were done to rule out unspecific hydrolysis of substrates. Protein content was measured by the method of Bradford (1976), using bovine serum albumin as the standard. Phosphatase activity was measured with *p*-nitrophenyl phosphate as the substrate.

Amino Acid Extraction and Analysis

Plant material was harvested, resuspended in 1 ml g^{-1} of a 3% (w/v) 5-sulfosalicylic acid solution and homogenized in a Teflon-in-glass Potter homogenizer. After centrifugation for 10 min at 12,000 g, 1-ml aliquots of the supernatant were dried at room temperature in a Speed-Vac Concentrator (Savant Instr., Hicksville, NY). Samples were reconstituted in 0.1 ml of 2.5 N NaOH, resulting in a pH value of 10.2 ± 0.2, and immediately analyzed. Aliquots were mixed with the same volume of *o*-phthaldialdehyde solution (0.5 M in 0.5 M sodium borate buffer, pH 10.0, containing 0.5 M β-mercaptoethanol and 10% (v/v) methanol). After exactly 60 sec, 20μl of derivatized samples were injected onto a 4.6 × 250 mm Zorbax ODS column (Rockland Technologies, Newport, DE) equilibrated with 59% solvent A (50 mM sodium phosphate-50 mM sodium acetate buffer, pH 7.5, containing 2% (v/v) of methanol and tetrahydrofuran) and 41% solvent B (65% methanol). Elution proceeded at a flow rate of 60 ml h^{-1} using a computer-controlled (Data System 450; Kontron, Munchen, Germany) complex gradient from 41 to 100% solvent B as described (Svedas et al. 1980), monitoring the eluate at 340 nm. This procedure allowed complete resolution of equimolar mixtures of derivatizable amino acids (all the 20 protein amino acids but Pro and Cys), with a detection limit of about 0.1 nmol. Peaks were integrated by area, with variation coefficients ranging from 0.8 to 3.2%. Proline was quantified by the ninhydrin method (Bates et al. 1973).

Statistical Treatment and Kinetic Analysis

All treatments were carried out at least in triplicate. Data were analyzed by using standard statistical procedures for analysis of variance and *t* test. Unless specified, where differences are reported, they are at the 99% confidence level ($P \leq 0.01$). The concentrations causing 50%-inhibition of cell growth rate were estimated utilizing the linear regression equation of dry weight increase values plotted against the logarithm of inhibitor concentration. Apparent affinity constants, inhibition types and K_i values were evaluated according to Webb (1963). Mean K_i values were obtained by averaging the values found for each inhibitor rate from Hanes-Woolf plots in two experiments with different enzyme preparations.

Results and Discussion

Plant cell cultures have been widely adopted in screening for amino acid biosynthesis inhibitors and in the study of their mode of action (Powell and Rees 1989). A crucial variability affecting sensitivity of suspension cultured cells to metabolic inhibitors is the initial density of cell population (Bonner et al. 1992). Also in the case of M-pyr-AMBPA the inhibitory effect was found to vary greatly with the culture growth phase at which the compound was added (not shown). However, the adoption of a strictly defined experimental system in which the compound was added just after the cultures reached the exponential phase of growth provided reliable results and allowed its effect to be compared with that of other substances. The addition of M-pyr-AMBPA in the range from 10^{-5} to 10^{-3} M to the culture medium exerted significant growth inhibition, which was less severe but comparable to that caused by the widely-used herbicide glyphosate (Fig. 1). Under the conditions employed, the concentrations causing a 50% reduction of dry weight increase were 0.47 ± 0.04 mM and 0.11 ± 0.03 mM, respectively. From a quantitative point of view, these data are in good agreement with those previously obtained with *Fagopyrum esculentum* seedlings (Lejczak et al 1996), suggesting that the target(s) of M-pyr-AMBPA action are expressed also in undifferentiated tissues, and that the results obtainable with cultured cells may represent those at the whole plant level.

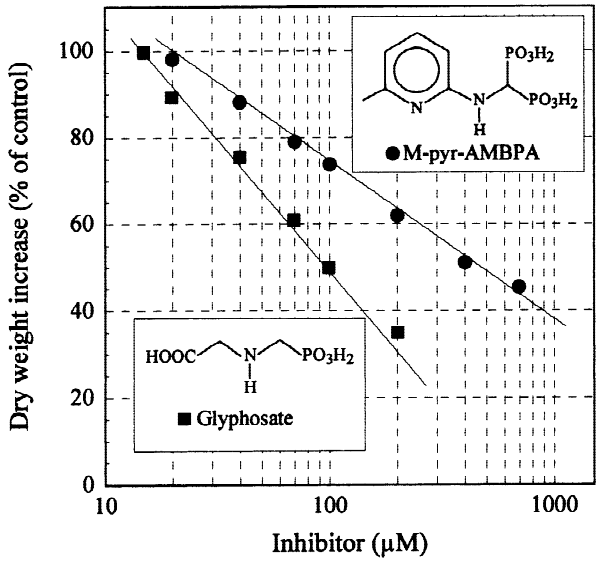

Fig. 1. Comparison between the inhibitory effect of M-pyr-AMBPA and glyphosate on *N. plumbaginifolia* exponentially-growing cells. The compounds were added to the culture medium just after the cells reached a population density of 2.0 mg ml^{-1} (dry weight). When untreated controls reached the early stationary phase of growth, the dry weight increase was determined. Data, expressed as percent of untreated control (8.92 ± 0.17 mg ml^{-1}), are means of the results obtained in two independent experiments, in which each dose was run in triplicate. SD never exceeded 6%.

The plastidial, Mn^{++}-stimulated isoform of DAHP synthase was partially purified from *N. plumbaginifolia* cultured cells by an improvement of a previously described procedure (Forlani et al. 1996) to a specific activity of 4.97 nkat mg^{-1}, with a 42-fold enrichment and a 40%-yield (data not presented). In this preparation the residual activity of unspecific phosphatases, measured in the presence of 0.5 mM ammonium molybdate, was less than 2% of that of the first enzyme in the shikimate pathway, allowing a proper kinetic analysis. The results obtained at varying substrate levels with increasing concentrations of M-pyr-AMBPA are shown in Fig. 2. Hanes-Woolf plots showed a non-competitive type of inhibition with respect to both E4P and PEP, with K_I values of 0.62 ± 0.06 mM and 0.43 ± 0.04 mM, respectively. This suggests a different mechanism from that of *N*-2-(5-chloro-pyridyl)-amino-methylene bisphosphonic

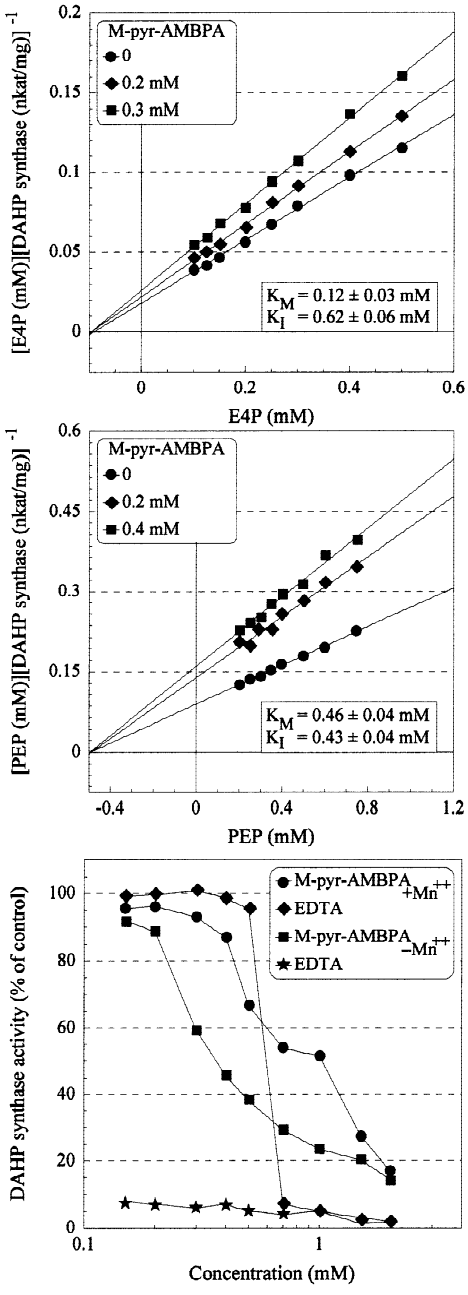

Fig. 2. Kinetic analysis of the inhibition of DAHP synthase by M-pyr-AMBPA. The enzyme, partially purified from *N. plumbaginifolia* cultured cells, was assayed in the presence of increasing concentrations of the inhibitor at varying doses of E4P and PEP. Saturating levels for the second substrate were 0.5 and 2 mM, respectively. Hanes-Woolf plots showed, in both cases, non-competitive type of inhibition. The same conclusion was obtained by replotting data as Lineweaver-Burk plots (not shown). To evaluate whether the inhibitory effect was due to the complexing properties of the compound, enzyme activity was evaluated also at a saturating substrate level in the presence of increasing concentrations of either M-pyr-AMBPA or the chelating compound ethylenediaminetetraacetic acid (EDTA), with 0.7 mM $MnCl_2$ either added or not to the assay mixture. Data are expressed as percentages of DAHP synthase activity in untreated controls (4.97 nkat mg^{-1} in the presence of Mn^{++} ions, 1.60 nkat mg^{-1} in their absence).

acid, which inhibited DAHP synthase competitively with respect to PEP (Forlani et al. 1996). Since *in vitro* activity is measured in the presence of Mn^{++} ions that act as a V_{max} stimulator (Ganson et al. 1986), and the non-competitive kinetics of both substrates are compatible with a chelation-based mechanism, further investigation was carried out to verify whether the inhibition of DAHP synthase could be due only to the complexing properties shared by most aminophosphonates. As a comparison term, the effect of increasing concentrations of ethylenediaminetetraacetic acid (EDTA), a well-known substance able to chelate divalent cations, was also determined. Results (Fig. 2) showed striking differences between the two compounds. In the case of EDTA, in the presence of 0.7 mM $MnCl_2$ no inhibition occurred until the concentration of the compound was lower than that of the ions, then a complete suppression of enzyme activity was found, as in the absence of Mn^{++} ions, most likely because even a low level of EDTA was able to strip away bound metal still retained by the enzyme after the purification procedure. In contrast, M-pyr-AMBPA progressively inhibited DAHP synthase in the presence or in the absence of $MnCl_2$. The inhibition pattern in the presence of Mn^{++} ions strictly resembles a biphasic titration curve, suggesting that Mn^{++} could be complexed by the compound in a 2:1 ratio. So while a chelation-based mechanism of inhibition seems to be ruled out, the opposite may be true: manganous ions decrease M-pyr-AMBPA effectiveness, perhaps by masking its negative charges, that may be required for interaction with the enzyme. Although the compound may, therefore, be significantly more effective inside the cell, where Mn^{++} concentration is lower than that in *in vitro* assay mixtures, the calculated K_I values are remarkably higher than those of other shikimate pathway inhibitors. The constants for the inhibition of EPSP synthase by glyphosate were found to be in the micromolar range not only against PEP (of competitive type), but with respect to shikimate-3-phosphate (incompetitive), also (e.g. Forlani 1997). Additionally, for the inhibition of DAHP synthase by the 5-chloro-pyridyl derivative of aminomethylene bisphosphonic acid the K_Is were significantly lower (0.14 and 0.06 mM against E4P and PEP, respectively; Forlani et al. 1996). Thus, the significant inhibition observed *in vivo* may be questionable if considering *in vitro* studies.

To investigate whether M-pyr-AMBPA interferes with the activity of DAHP synthase *in vivo*, resulting in an actual reduction of aromatic amino acid biosynthesis that could account, fully or in part, for its phytotoxicity, amino acid pools were quantified in *N. plumbaginifolia* cells at increasing times following supplementation of the culture medium with the compound. Sublethal doses were preferred in order to enhance specific effects and reduce variations possibly due to consequent cellular stress. Results obtained with the addition of 200 μM M-pyr-AMBPA, a treatment that gave rise to 25% inhi-

Table 1. Free amino acid pools in *N. plumbaginifolia* suspension cultured cells at increasing time after the addition of 200 μM M-pyr-AMBPA to the culture medium. Data are means ± SD of two experiments, with three replications each. Cys was not detectable. Cellular densities (dry weight) are also indicated.

Amino acid	$T_{3\ dd}$		$T_{4\ dd}$		$T_{5\ dd}$		$T_{6\ dd}$	
	μmol (g dw)$^{-1}$	%	μmol (g dw)$^{-1}$	%	μmol (g dw)$^{-1}$	%	μmol (g dw)$^{-1}$	%
Control	3.95 ± 0.12		5.12 ± 0.17		6.79 ± 0.24		10.13 ± 0.43 mg/ml^{-1}	
Asp	5.54 ± 0.17	3.81	5.16 ± 0.38	2.43	4.30 ± 0.43	2.40	5.03 ± 1.16	2.10
Glu	24.91 ± 0.66	17.17	23.25 ± 1.70	10.94	15.78 ± 0.88	8.82	24.79 ± 6.35	10.38
Asn	4.33 ± 0.29	2.98	6.78 ± 0.88	3.19	7.81 ± 0.52	4.36	9.82 ± 0.20	4.11
Ser	9.54 ± 1.38	6.58	10.70 ± 0.68	5.04	7.47 ± 0.80	4.17	9.42 ± 0.95	3.94
Gln	61.68 ± 4.71	42.50	97.55 ± 2.21	45.90	83.21 ± 1.80	46.53	87.24 ± 8.76	36.53
His	1.91 ± 0.37	1.31	3.42 ± 0.48	1.61	2.37 ± 0.34	1.33	2.44 ± 0.14	1.02
Arg	2.46 ± 0.35	1.69	3.21 ± 0.22	1.51	2.76 ± 0.08	1.55	1.06 ± 0.74	0.44
Gly	6.01 ± 0.65	4.14	18.26 ± 2.43	8.59	10.48 ± 1.94	5.86	9.62 ± 1.66	4.03
Thr	2.52 ± 0.43	1.74	4.46 ± 0.58	2.10	4.42 ± 0.24	2.47	6.52 ± 1.45	2.73
Ala	9.97 ± 1.09	6.87	13.64 ± 0.75	6.42	8.67 ± 1.05	4.85	21.77 ± 1.40	9.11
Tyr	2.86 ± 0.16	1.97	4.84 ± 0.14	2.28	5.08 ± 0.11	2.84	3.45 ± 0.39	1.44
Trp	0.57 ± 0.25	0.39	0.77 ± 0.28	0.36	0.55 ± 0.03	0.31	1.16 ± 0.47	0.49
Met	0.42 ± 0.10	0.29	0.42 ± 0.10	0.20	0.39 ± 0.06	0.22	1.07 ± 0.43	0.45
Val	2.14 ± 0.06	1.47	2.56 ± 0.11	1.20	2.06 ± 0.06	1.15	3.23 ± 0.18	1.35
Phe	4.15 ± 0.39	2.86	9.30 ± 0.89	4.37	14.17 ± 0.54	7.92	29.91 ± 2.58	12.52
Ile	1.02 ± 0.02	0.71	1.24 ± 0.03	0.58	1.04 ± 0.03	0.58	1.73 ± 0.72	0.72
Leu	1.57 ± 0.03	1.08	2.13 ± 0.15	1.00	2.17 ± 0.08	1.21	3.12 ± 1.06	1.31
Lys	0.73 ± 0.13	0.50	1.02 ± 0.26	0.48	0.74 ± 0.08	0.41	1.22 ± 0.38	0.51
Pro	2.81 ± 0.48	1.94	3.83 ± 0.37	1.80	5.37 ± 0.82	3.00	16.24 ± 1.61	6.80
All	145.14	100.00	212.54	100.00	178.84	100.00	238.82	100.00
Treated	3.37 ± 0.15		4.05 ± 0.23		4.92 ± 0.14		6.69 ± 0.33 mg/ml^{-1}	
Asp	5.65 ± 0.29	3.96	4.80 ± 0.32	2.49	4.12 ± 0.72	2.06	6.98 ± 0.27	2.93
Glu	21.48 ± 0.36	15.04	18.36 ± 1.53	9.67	13.51 ± 2.84	6.76	19.71 ± 0.66	8.26
Asn	4.70 ± 0.09	3.29	7.66 ± 0.05	3.97	9.55 ± 1.68	4.78	14.94 ± 0.49	6.26
Ser	17.97 ± 0.36	12.59	16.51 ± 1.50	8.57	13.61 ± 0.69	6.81	10.40 ± 2.85	4.36
Gln	46.61 ± 1.03	32.64	80.76 ± 6.28	41.92	94.12 ± 2.01	47.11	125.96 ± 9.28	52.80
His	2.46 ± 0.41	1.72	2.42 ± 0.53	1.26	2.31 ± 0.25	1.16	1.82 ± 0.31	0.76
Arg	2.78 ± 0.24	1.95	4.28 ± 0.67	2.22	3.76 ± 1.14	1.88	5.74 ± 0.64	2.41
Gly	4.21 ± 0.42	2.95	10.28 ± 1.45	5.34	14.03 ± 2.83	7.03	6.49 ± 0.33	2.72
Thr	3.52 ± 0.30	2.47	4.78 ± 0.60	2.48	5.12 ± 1.02	2.57	7.47 ± 0.80	3.13
Ala	18.41 ± 0.81	12.89	22.17 ± 0.81	11.51	17.94 ± 8.10	8.98	15.65 ± 9.03	6.56
Tyr	2.06 ± 0.09	1.45	3.19 ± 0.05	1.65	3.52 ± 0.56	1.76	3.53 ± 0.36	1.48
Trp	0.39 ± 0.02	0.27	0.63 ± 0.28	0.33	0.49 ± 0.08	0.25	1.06 ± 0.19	0.44
Met	0.38 ± 0.02	0.26	0.44 ± 0.05	0.23	0.52 ± 0.15	0.26	0.53 ± 0.05	0.22
Val	2.31 ± 0.05	1.62	2.48 ± 0.16	1.29	2.80 ± 0.21	1.40	2.34 ± 0.02	0.98
Phe	3.70 ± 0.15	2.59	5.08 ± 0.11	2.64	4.49 ± 1.32	2.25	5.06 ± 0.38	2.12
Ile	1.28 ± 0.09	0.90	1.38 ± 0.08	0.72	1.35 ± 0.15	0.67	1.67 ± 0.20	0.70
Leu	1.58 ± 0.11	1.11	1.92 ± 0.14	1.00	2.05 ± 0.13	1.02	1.71 ± 0.12	0.72
Lys	0.77 ± 0.09	0.54	1.13 ± 0.27	0.59	2.14 ± 0.46	1.07	1.30 ± 0.18	0.55
Pro	2.52 ± 0.30	1.77	4.11 ± 1.47	2.13	4.35 ± 0.27	2.18	6.20 ± 0.95	2.60
All	142.79	100.00	192.63	100.00	199.77	100.00	238.56	100.00

bition of the cell growth rate, are summarized in Table 1. With only a few exceptions, free amino acid concentration was found to vary greatly during the culture growth cycle in untreated controls. Starting from the early exponential phase of growth, the level of several compounds rose up to a 10-fold increase, with an overall growth of more than 300% (not shown). This behavior makes it difficult to distinguish between specific effects and an apparent reduction simply due to a lower cell growth rate in treated samples. However, the comparison of data from cells harvested at different times, but at the same cellular density, shows a significant decrease in tyrosine and, mainly, phenylalanine levels (Table 1); tryptophan was always present at very low amounts, so the occurrence of a true effect was not easily evaluated. This reduction took place three to four days after the addition of the inhibitor, and correlated to a dramatic increase in the overall aromatic amino acid level, that went from 5% to 15% of total free amino acids. To obtain more information about the significance of such a response, the same experiment was also performed with glyphosate, which indeed owes its effectiveness to block-

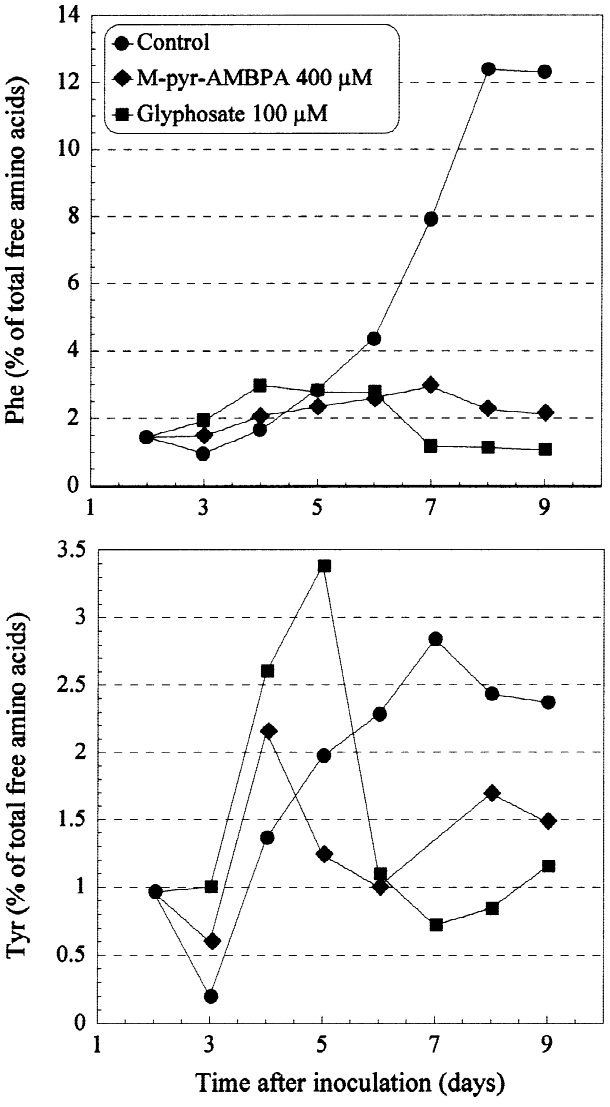

Fig. 3. Time course of free phenylalanine and tyrosine content in *N. plumbaginifolia* cells grown in the presence of M-pyr-AMBPA or glyphosate at concentrations causing 50%-reduction of cell growth rate. At increasing time following addition, free amino acid levels were determined by RP-HPLC following derivatization with *o*-phthaldialdehyde. Data are expressed as percent of total free amino acids, and are averaged from the results obtained with three replications (SD of absolute values within 10%).

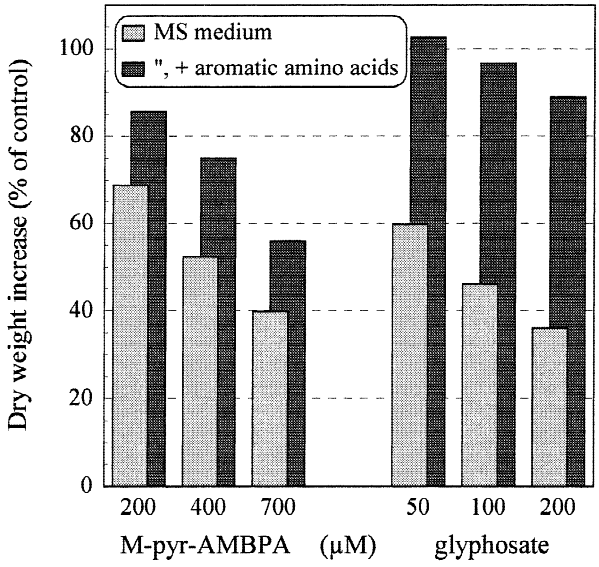

Fig. 4. Reversal of the inhibitory effect of M-pyr-AMBPA and glyphosate by exogenously supplied aromatic amino acids. The inhibitors were added to the culture medium (with or without 0.25 mM Phe, 0.25 mM Tyr, 0.125 mM Trp, and 0.25 μM each of *p*-amino- and *p*-hydroxy-benzoic acid) of *N. plumbaginifolia* cells in the early exponential phase of growth at concentrations causing 35, 50 and 65% of cell growth inhibition. When untreated controls reached the early stationary phase of growth, the resulting dry weight increase was determined, and expressed as percent of that in untreated controls (MS medium, 8.87 ± 0.16 mg ml^{-1}; same, with aromatic amino acids 9.07 ± 0.25 mg ml^{-1}). Data are means of the results obtained in two independent experiments in which each treatment was performed in triplicate, with SD never exceeding 5%.

ing the shikimate pathway (Cole 1985). The effect on the time course of free phenylalanine and tyrosine levels of treatments with 400 μM M-pyr-AMBPA or 100 μM glyphosate (concentrations that in both cases gave rise to a 50%-decrease of cell growth rate) is shown in Fig. 3. Unexpectedly, soon after the addition of both inhibitors, the intracellular levels of these two amino acids increased over that of the control, but after only a few days an inhibitory effect became evident. A very similar pattern was found for the two compounds, though the outcome was remarkably higher for glyphosate in both the initial stimulation and the subsequent lowering of aromatic amino acid pools. Moreover, free glutamine levels slightly increased in both cases (not shown), a consequence that has been noted in several previous studies on glyphosate in response to increased ammonia, due in turn to decreased protein synthesis (Cole 1985). The occurrence of other targets of M-pyr-AMBPA in the prechorismate pathway is improbable, because possible inhibition of EPSP synthase was ruled out previously (Forlani et al. 1997). So, despite the low value of *in vitro* K_Is, these results show that a significant reduction of DAHP synthesis has most likely occurred in cells grown in the presence of this aminophosphonate.

Finally, in order to elucidate whether the inhibition brought about by M-pyr-AMBPA on phenylalanine and tyrosine biosynthesis could play a major role in its phytotoxicity, reversal experiments were performed by feeding cells with exogenous aromatic amino acids. Again, the same procedure was also adopted for glyphosate for comparison. Both compounds were added at concentrations that caused about 35, 50 and 65% cell growth inhibition. Results are shown in Fig. 4. While amino acid

supplements succeeded in counteracting glyphosate toxicity, full reversal was not achieved in the case of M-pyr-AMBPA: a partial reversal was indeed evident, but the resulting dry weight increase was still significantly lower than that of the untreated control. Even though this partial alleviation further strengthens a role for *in vivo* inhibition of DAHP synthase in providing the basis for M-pyr-AMBPA phytotoxicity, the failure of aromatic amino acids to completely reverse its effect points at the occurrence of other target(s). A multiple mode of action has been recently reported for a close analogue, *N*-2-(3-methyl-pyridyl)-aminomethylene bisphosphonic acid, that was found to inhibit not only geranyl-geranyl diphosphate synthase, but also squalene synthase and phytoene synthase as well, without affecting aromatic metabolism (Oberhauser et al. 1998). It may also be consistent with the ability of bisphosphonates to completely impair unrelated metabolic processes, as carotenoid (Oberhauser et al. 1988) or steroid (Biller et al. 1996) biosynthesis, and vacuolar or mitochondrial proton pumping (Vianello et al. 1997).

Data herein presented clearly demonstrate the ability of M-pyr-AMBPA to reduce the carbon flow in the shikimate pathway. Its structure may thus be considered as a useful lead in planning forthcoming strategies for the development of other herbicidally-active compounds that may act by interfering with plant aromatic biosynthesis.

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