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# Mode of Action of Herbicidal Derivatives of Aminomethylenebisphosphonic Acid. Part II. Reversal of Herbicidal Action by Aromatic Amino Acids

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**Abstract.** The herbicidal action of *N*-pyridylaminomethylenebisphosphonic acids is accompanied by an impairment of anthocyanin biosynthesis. This suggests that they might act as inhibitors of some steps in aromatic amino acid biosynthesis. Herbicidal effects were reversed by aromatic amino acids using both bacterial and plant models, a finding that strongly supports this hypothesis. Structural features of these compounds suggest the sixth enzyme in the shikimate pathway 5-*enol*-pyruvoylshikimate-3-phosphate (EPSP) synthase as a possible target, since a strong structural similarity exists between aminomethylenebisphosphonic acid and an inhibitor of EPSP synthase, the herbicide glyphosate. This is, however, not the case since they did not act as inhibitors of this enzyme.

**Key Words.** *N*-Pyridylaminomethylenebisphosphonic acids—EPSP synthase—Herbicides—Glyphosate—Aromatic metabolism

*N*-pyridylaminomethylenebisphosphonic acids (Fig. 1), compounds **1–7,** which were developed in Japan, are herbicidally active (Suzuki et al. 1979). They are also promising agents for the treatment of bone disorders connected with calcium resorption (Ebetino et al. 1990). In an earlier paper we showed that the herbicidal action of these compounds is accompanied by impairment of anthocyanin biosynthesis (Lejczak et al. 1996). This finding suggest that *N*-pyridylaminomethylenebisphosphonic acids might act as inhibitors of some steps in aromatic amino acid biosynthesis. Approximately 20% of the carbon fixed by leaves is routed through the shikimate pathway (Haslam 1993), the end products of which include aromatic amino acids, vitamins, lignins, alkaloids, and phenolics. Thus, aromatic metabolism is a particularly attractive target area for the rational design of herbicides. The success of such an approach is strictly dependent on knowledge of the genetics, biochemistry, and regulation of the enzymes of this pathway. From this point of view, compounds affecting the biosynthesis of aromatic amino acids are powerful tools providing insights into the molecular events that link in vivo inhibition of biosynthetic enzymes with plant death.

Structural features of *N*-pyridylaminomethylenebisphosphonic acids suggest the activity of the prechorismate pathway enzyme 5-*enol*-pyruvoylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) as a possible target, since a strong similarity exists between such compounds and a powerful inhibitor of EPSP synthase, the herbicide glyphosate ([*N*-phosphonomethyl]-glycine). The flat pyridyl ring seems to be a good mimic of the planar chair conformation of both the substrate shikimate 3-phosphate (S3P) and the product (EPSP) of the enzymatic reaction (Leo et al. 1992). Thus, we undertook studies of the reversal of *N*-pyridylaminomethylenebisphosphonic acid toxicity by aromatic amino acids using both bacterial (*Escherichia coli, Micrococcus luteus, Sarcina lutea,* and *Bacillus cereus*) and plant (cell cultures of tobacco and whole cucumber) models. Phosphonic acids are known chelators of metal cations, and it has been an intriguing possibility that glyphosate also could exert its action by complexing divalent and trivalent cations within the cell; however, little evidence has been reported to support such a hypothesis (Cole 1985). The possibility that *N*-pyridylaminomethylenebisphosphonic acid toxicity could be alleviated by cation addition, as

**Abbreviations:** EPSP synthase, 5-*enol*-pyruvoylshikimate-3-phosphate synthase; S3P, shikimate 3-phosphate.

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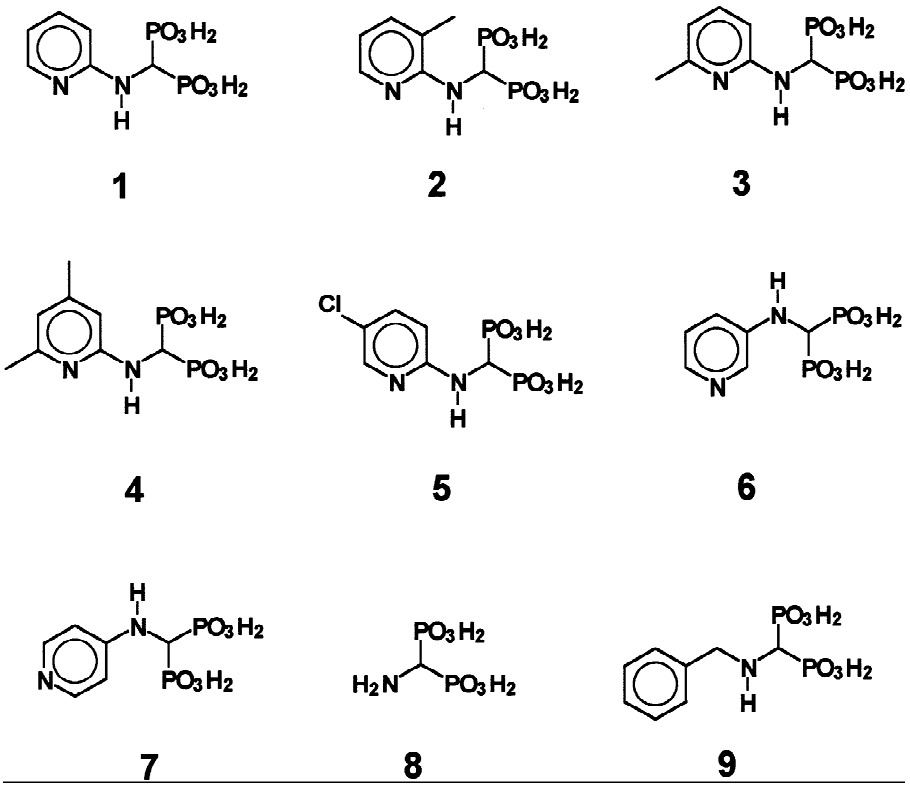


**Fig. 1.** *N*-pyridylaminomethylene-bisphosphonic acids (compounds **1–7**) and their analogs (compounds **8** and **9**).

well as the ability of these compounds to inhibit EPSP synthase from tobacco and maize cultured cells, were also evaluated.

## Materials and Methods

### *Chemical Syntheses*

*N*-Pyridylaminomethylenebisphosphonic acids were synthesized as described earlier (Lejczak et al. 1996). The ammonium salt of S3P was prepared from *Klebsiella pneumoniae* ATCC 25597 growth medium according to Coggins et al. (1987).

### *Effect of Supplementation of Growth Media with Aromatic Amino Acids*

The effect of exogenously supplied aromatic amino acids on *N*-pyridylaminomethylenebisphosphonic acid toxicity was studied using four bacterial species: *E. coli* PCM 2057 (Polish Collection of Microorganisms), *M. luteus* PCM 1944, *S. lutea* (wild strain) and *B. cereus* (wild strain), and suspension-cultured cells of tobacco (*Nicotiana plumbaginifolia* Viviani) and whole cucumber (*Cucumis sativus* L. cv. Wisconsin) plants. Glyphosate (compound **8**) was used throughout as a positive control.

### *Effects of the Studied Compounds on the Growth of* C. sativus

Seeds were germinated at 33°C for 1.5 days in the darkness. Groups of 15 uniform seedlings (7–8-mm total length) were transferred to Petri dishes (9 cm) lined with two disks of Whatman No. 2 filter paper wetted with 10 mL of distilled water (control) or solutions of test compounds to give a final concentration of 0.05, 0.15, or 1.5 mM. Plants were grown at 25°C with a 12-h day length for a further 9 days under fluorescent tubes (300 mE $m^{-2}$ $s^{-1}$ at plant level). Separated roots and shoots were then weighed.

Reversal studies of herbicidal action of these compounds were carried out by supplementing the growth medium containing herbicide with either phenylalanine, tyrosine, or tryptophan (15 mM final concentration) or with an equimolar mixture of three amino acids (5 mM each). Each experiment was repeated at least three times.

### *Reversal Experiments with* N. plumbaginifolia *Cultured Cells*

Tobacco cell cultures were grown in Erlenmeyer flasks in MS medium (Murashige and Skoog 1962) containing 0.5 mg/liter of both 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. 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 the mixtures of compounds on exponentially growing cell cultures was measured as described previously (Lejczak et al. 1996). 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 compound **5** (0.2 mM final concentration) or its mixture with shikimic acid, anthranilic acid, phenylalanine, tyrosine, or tryptophan (each at 1 mM, final concentration), as well as a mixture of the three aromatic amino acids (0.33 mM final concentration each) were added just after the density of cells reached 1.7 mg/mL (dry weight). After a further 8

days of incubation, when the untreated controls reached the late exponential phase of growth, 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. Value for the untreated control was 5.8 ± 0.3 mg/mL. At least three replicates were tested for each mixture. To evaluate a possible role of the chelating properties of *N*-pyridylaminomethylenebisphosphonic acids in their phytotoxicity, experiments were also performed in which compound **5** was added to cultures in which the concentration of divalent ions in MS salts was doubled.

### *Assay of Bacterial Growth Inhibition*

The inhibitory effect of the test compounds was elucidated using a disk diffusion assay method on RST medium agar plates (Atherton et al. 1979). Each plate (90-mm diameter) containing 20 mL of medium was inoculated with a 0.1 mL sample (about $10^{-6}$ cells) of an exponentially growing bacterial culture. Paper disks (8-mm diameter) were saturated with 10-μL portions of *N*-pyridylaminomethylenebisphosphonic acid solution (2 mM) and placed on the surface of solid medium, three disks/plate. Three plates were used for each compound. After incubation for 18 h at 37°C, zones of growth inhibition were measured. In reversal experiments the growth medium was supplemented either with phenylalanine, tyrosine, and tryptophan (0.5 mM final concentration) or with the equimolar mixture of each of them (0.17 mM each).

### *Effect of N-Pyridylaminomethylenebisphosphonates on Plant EPSP Synthase*

Suspension cultured cells of tobacco and maize (*Zea mays* L. cv. Black Mexican Sweet) were grown as above. The hormonal supplement for maize culture medium consisted of 1.0 mg/liter 2,4-dichlorophenoxyacetic acid. Mid log grown cells were harvested by vacuum filtration, resuspended in ice-cold extraction buffer (50 mM Hepes-NaOH, pH 7.5, containing 5% glycerol, 2.5 mM reduced glutathione, 0.1 mM EDTA, and 10 mM ammonium heptamolybdate), and broken by a Teflon-in-glass Potter homogenizer. After the addition of polyvinylpolypyrrolidone (20 mg/mL), the extract was centrifuged at 20,000 ×-g for 20 min. The supernatant was then fractionated with ammonium sulfate (0–70% saturation) and proteins pelleted by centrifugation. Pellets were resuspended in the extraction buffer and desalted by passage through a Bio-Gel P6DG column (Bio-Rad).

EPSP synthase activity was measured in the forward direction as described earlier (Forlani et al. 1994) in the presence of 1 mM phospho*enol*pyruvate, 1 mM S3P, 100 mM Hepes-NaOH, pH 7.4, and 0.5 mM ammonium heptamolybdate. After up to 20 min at 35°C, the incubation was stopped, and the inorganic phosphate released was measured by the malachite green dye assay. Nonspecific phosphatase activity was evaluated in parallel controls in which S3P had been omitted. Protein content was measured by the Coomassie Blue method with bovine serum albumin as the standard (Bradford 1976). EPSP synthase specific activities evaluated in the absence of herbicides were 293 pkat/mg and 152 pkat/mg for maize and tobacco, respectively. Each experiment was performed three 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). Results statistically not significant are marked in tables as N.

## Results and Discussion

For a better understanding of the mode of action of these compounds, experiments were conducted to determine whether the physiologic response of *C. sativus* to these herbicides could be reversed by exogenously supplied phenylalanine, tyrosine, tryptophan, or a mixture thereof. The representative results shown in Table 1 are not clear cut, since the influence of aromatic amino acids on the growth of the test plant treated with compounds **2** and **6** was quite complex. Anyway, plants treated with glyphosate (used as a positive control) and *N*-pyridylaminomethylenebisphosphonic acids responded similarly to the supplementation of the growth medium with single aromatic amino acids, which, on the whole, failed to obtain full reversion. The application of the mixture of the three amino acids partially prevented root growth inhibition caused by all herbicides. A puzzling effect was found with regard to shoot growth: the feeding of both an inhibitor and all three aromatic amino acids stimulated the growth in the case of glyphosate but increased the inhibitory effect in those of *N*-pyridylaminomethylenebisphosphonates. Since none of the aromatic amino acids nor their mixture applied without herbicide influenced the growth of the test plant, this complex response might be caused by additional factors such as differences in or competition between herbicides and amino acids during their import and translocation within the plant. Alternatively, this behavior might result from action of these compounds on more than one target within the plant cell.

Since bacteria and plants share the metabolic pathways involved in amino acid biosynthesis, bacteria are commonly used as models in preliminary studies on the mode of action of some herbicides. For a better understanding of the results obtained using whole plants we used eight bacterial species to study in the reversibility of the action of herbicides upon supplementation of their growth media with aromatic amino acids occurs. Among the tested strains (*Bacillus subtilis, B. cereus, Bacillus megatherium, M. luteus, S. lutea, Serratia marcescens, E. coli,* and *Pseudomonas fluorescens*), only the growth listed in Table 2 was weakly inhibited by *N*-pyridylaminomethylenebisphosphonic acids at a millimolar concentration, whereas glyphosate inhibited only the growth of *E. coli*. The nonherbicidal compound **4** did not show antibacterial activity (Table 2). Supplementation of the growth medium with phenylalanine, tryptophan, or tyrosine, either singly or in combination, completely reversed the antibacterial action of all of the compounds tested (data not shown). This finding supports the hypothesis that *N*-pyridylaminomethylenebisphosphonic acids may act as inhibitors of some step in aromatic amino acid biosynthesis.

To check whether the aromatic pathway in indeed the target for herbicidal derivatives of aminomethylene-

**Table 1.** Reversal by exogenous aromatic amino acids of the inhibitory effect of glyphosate and derivatives of aminomethylenebisphosphonic acid on the growth of *C. sativus.*

| Compound | Supplementation | Root or shoot | Concentration (mM) 0.15 | 0.5 | 1.5 |
|---|---|---|---|---|---|
| **Glyphosate** | | R | −(50 ± 8) | −(58 ± 11) | −(69 ± 7) |
| | | S | N | N | N |
| | Phe | R | −(54 ± 12) | −(58 ± 9) | −(69 ± 11) |
| | | S | +(58 ± 9) | +(42 ± 9) | N |
| | Tyr | R | −(60 ± 13) | −(64 ± 12) | −(77 ± 11) |
| | | S | N | N | N |
| | Trp | R | −(48 ± 7) | −(60 ± 6) | −(77 ± 10) |
| | | S | N | N | N |
| | Phe + Trp + Tyr | R | −(44 ± 13) | −(34 ± 12) | −(34 ± 11) |
| | | S | N | +(49 ± 11) | +(43 ± 9) |
| **2** | | R | −(88 ± 5) | −100 | −100 |
| | | S | −(68 ± 13) | −(68 ± 16) | −100 |
| | Phe | R | −(81 ± 11) | −(82 ± 14) | −(79 ± 16) |
| | | S | −(38 ± 11) | −(63 ± 8) | −(64 ± 9) |
| | Tyr | R | −(74 ± 13) | −(81 ± 17) | −(81 ± 13) |
| | | S | −(36 ± 12) | −(62 ± 21) | −(62 ± 9) |
| | Trp | R | −(81 ± 15) | −(74 ± 18) | −(80 ± 9) |
| | | S | −(29 ± 6) | −(50 ± 5) | −(73 ± 7) |
| | Phe + Trp + Tyr | R | −(72 ± 10) | −(78 ± 11) | −(80 ± 9) |
| | | S | N | −(66 ± 11) | −(67 ± 11) |
| **6** | | R | −(32 ± 7) | −(52 ± 3) | −(57 ± 9) |
| | | S | N | N | N |
| | Phe | R | −(62 ± 16) | −(69 ± 15) | −(72 ± 11) |
| | | S | N | N | N |
| | Tyr | R | −(58 ± 12) | −(61 ± 20) | −(69 ± 12) |
| | | S | N | N | −(21 ± 11) |
| | Trp | R | −(52 ± 21) | −(55 ± 13) | −(70 ± 12) |
| | | S | N | −(18 ± 9) | −(35 ± 11) |
| | Phe + Trp + Tyr | R | −(38 ± 11) | −(39 ± 7) | −(47 ± 9) |
| | | S | N | −(27 ± 7) | −(34 ± 6) |

*Note.* Phenylalanine, tyrosine, and tryptophan were added to the culture medium either singly (15 mM final concentration) or in combination (5 mM each). Data are expressed as percentage change in root and shoot weight compared with that of untreated control and are the mean ± S.D. of three independent experiments. N, no effect ($p \leqslant 0.05$) according to Miller and Miller (1984).

bisphosphonic acids, the reversal of action of compound **5** by chosen metabolites of shikimate pathway was studied in some detail using suspension-cultured cells of tobacco. Exogenously supplied aromatic amino acids were able to counteract significantly the growth inhibitor only when supplied together (Fig. 2). The application of phenylalanine or tryptophan caused, on the contrary, a slight increase of the herbicidal effect of compound **5,** as noted at the whole plant level for the mixture of three amino acids in the case of compounds **2** and **6**. Also, shikimic acid failed to relieve the herbicidal effect, whereas a partial reversal of herbicidal action was achieved upon supplementation of the growth medium and anthranilic acid. This might suggest the occurrence of a target downstream from shikimate synthesis. However, results are quite complex and not clear cut. These data along with the recent finding that related compounds act as inhibitors of farnesyl pyrophosphate synthase (Cromartie and Fisher 1995) seem rather to support the hypothesis of their action on multiple targets within the plant cell.

Bisphosphonates are known to be strong complexing agents; thus the influence of metal ions on their herbicidal action was also studied. A complete reversal was achieved when the concentration of divalent ions, which are components of Murashige and Skoog medium, was doubled (Fig. 2). This result seems to indicate that chelating properties of *N*-pyridylaminomethylenebisphosphonates may also play a relevant role in their phytotoxicity.

Finally, we evaluated the ability of these compounds to inhibit EPSP synthase extracted from cell cultures of both a monocotyledonous (maize) and a dicotyledonous (tobacco) species. All of the tests *N*-pyridylaminomethylenebisphosphonates, aminomethylenebisphosphonic acid (compound **8,** which may be considered as an analog of glyphosate), and the moderately herbicidal *N*-

**Table 2.** Sensitivity of bacterial strains to glyphosate and derivatives of aminomethylenebisphosphonic acid, determined by disk diffusion assay on solid RST medium.

| | Bacterial strain | | | |
|---|---|---|---|---|
| Compound | *B. cereus* | *E. coli* PCM 2057 | *S. lutea* | *M. luteus* PMC 1944 |
| **Glyphosate** | 0 | 1.0 ± 0.0 | 0 | 0 |
| **1** | 1.0 ± 0.1 | 1.5 ± 0.1 | 1.5 ± 0.1 | 1.0 ± 0.1 |
| **2** | 1.5 ± 0.2 | 2.5 ± 0.3 | 1.5 ± 0.0 | 1.0 ± 0.0 |
| **3** | 0 | 1.5 ± 0.1 | 0 | 0 |
| **4** | 0 | 0 | 0 | 0 |
| **5** | 1.5 ± 0.1 | 3.5 ± 0.3 | 1.5 ± 0.1 | 1.5 ± 0.1 |
| **6** | 2.0 ± 0.2 | 0.5 ± 0.0 | 0.5 ± 0.1 | 3.0 ± 0.2 |
| **7** | 0.5 ± 0.1 | 1.5 ± 0.1 | 1.5 ± 0.2 | 4.5 ± 0.4 |

*Note*. Three paper disks were saturated with 10 μL of a 2 mM solution of the compounds and were placed on the surface of an inoculated plate. Zones of growth inhibition were measured after incubation for 18 h at 37°C. Data, expressed in mm, are the mean ± S.D. of the results obtained with three plates for each inhibitor.

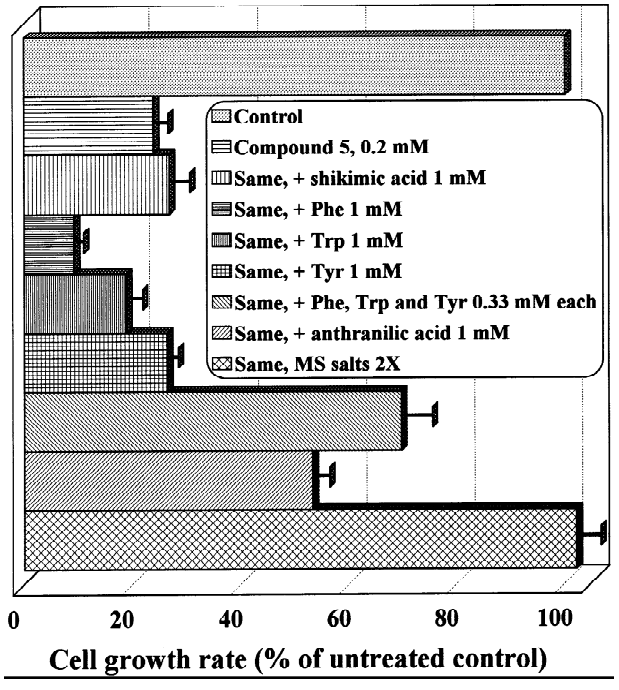


**Fig. 2.** Reversal of the inhibitory action of compound **5** on the growth of suspension-cultured cells by exogenously supplied aromatic amino acids or intermediates. Each compound was added to the culture medium of *N. plumbaginifolia* cells in the early exponential phase of growth as the same time that the inhibitor was added. After 8 days of incubation, the resulting dry weight increase was determined and expressed as a percent of that in the corresponding untreated control. Since phosphonates have strong chelating properties, the effect of doubling divalent ions in MS medium was also evaluated. Data are means ± S.D. of the results obtained in two independent experiments in which each treatment was performed in triplicate.

**Table 3.** Effect of *N*-pyridylaminomethylenebisphosphonic acids on the activity of the shikimate pathway enzyme EPSP synthase.

| | EPSP synthase activity (% of untreated control) | |
|---|---|---|
| Compound | *Z. mays* | *N. plumbaginifolia* |
| **Glyphosate** | 0.2 ± 0.1 | 0.3 ± 0.1 |
| **1** | 104.0 ± 5.7 | 103.7 ± 2.9 |
| **3** | 101.0 ± 2.8 | 100.0 ± 1.7 |
| **5** | 109.0 ± 5.2 | 103.3 ± 3.0 |
| **6** | 101.8 ± 6.5 | 102.0 ± 2.6 |
| **7** | 103.6 ± 2.2 | 101.3 ± 1.1 |
| **8** | 102.1 ± 2.9 | 107.3 ± 2.5 |
| **9** | 107.9 ± 4.8 | 118.0 ± 7.5 |

*Note*. The enzyme was extracted from suspension cultured cells of maize and tobacco, and the activity was measured in the presence of the compounds at a final concentration of 1 mM. The herbicide glyphosate was used as a positive control. Data, expressed as percent of untreated controls (293 and 152 pkat $mg^{-1}$ for maize and tobacco, respectively), are means ± S.D. of at least six measurements performed with two separate enzyme preparations.

benzylaminomethylenebisphosphonic acid (compound **9**) were completely uneffective, whereas glyphosate caused nearly total abolishment of enzyme activity when added at the same concentration (Table 3). Thus, EPSP synthase does not represent a target enzyme for these compounds.

Studies on the action of *N*-pyridylaminomethylenebisphosphonates on other enzymes of the shikimate pathway are currently in progress.

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## References

Atherton FR, Hall MJ, Lambert RW, Ringrose PA (1979) Phosphonopeptides as antimicrobial agents: Rationale, design, and structure-activity relationship. Antimicrob Agents Chemother 15:677–683

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Coggins JR, Boocock MR, Chauduri S, Lambert JML, Lumsden GA, Nimmo DS, Smith DDS (1987) The *arom* multifunctional enzyme from *Neurospora crassa*. Methods Enzymol 142:325–338

Cole DJ (1985) Mode of action of glyphosate-A literature analysis. In: Grossbard E, Atkinson D (eds) The herbicide glyphosate. Butterworths, London, pp 48–74

Cromartie TH, Fisher KJ (1995) Method of controlling plants by inhibition of farnesyl pyrophosphate synthase. PCT Int. Appl., WO 95/34207

Ebetino FH, Degenhardt CR, Jamieson L, Burdsall DC (1990) Recent work on the synthesis of phosphonate-containing, bone-active heterocycles. Heterocycles 30:855–862

Forlani G, Parisi B, Nielsen E (1994) 5-*enol*-Pyruvyl-shikimate-3-

phosphate synthase from *Zea mays* cultured cells: Purification and properties. Plant Physiol 105:1107–1114

Haslam E (1993) Shikimic acid: Metabolism and metabolites. Wiley and Sons, Chichester

Lejczak B, Boduszek B, Kafarski P, Forlani G, Wojtasek H, Wieczorek P (1996) Mode of action of herbicidal derivatives of aminomethylenebisphosphonic acid. I. Physiological activity and inhibition of anthocyanins biosynthesis. J Plant Growth Regul 15:109–113

Leo GC, Castellino S, Sammons RD, Sikorski JA (1992) EPSP synthase inhibitor design. I. Conformations of enzyme-bound shikimate-3-phosphate and 5-*enol*pyruvylshikimate-3-phosphate using TRNOE. BioMed Chem Lett 2:151–154

Miller JC, Miller JN (1984) Statistics for analytical chemistry. Ellis Harwood Ltd., Chichester

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Suzuki F, Yoshihiro F, Yamamoto S, Mizutani H, Funabashi C, Ohya T, Ikai T, Oguchi T (1979) *N*-Pyridylaminomethylenebisphosphonsaureverbindungen. Ger Offen 2.831.578