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Tailoring the Structure of Aminobisphosphonates To Target Plant P5C Reductase

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Using the structure of (3,5-dichlorophenyl)aminomethylenebisphosphonic acid as a lead compound, 25 new phosphonates were synthesized and evaluated as possible inhibitors of *Arabidopsis thaliana* δ^1 -pyrroline-5-carboxylate (P5C) reductase. Derivatives substituted in the phenyl ring retained the inhibitory potential, though to a different extent. On the contrary any variation in the scaffold, i.e., the replacement of the second phosphonate moiety with a hydroxyl or an amino residue, resulted in a significant loss of biological activity. The availability of several structures capable of interfering with the catalytic mechanism in the micromolar to millimolar range allowed a proper structure–activity relationship analysis, leading us to hypothesize about the steric and electronic requirements for maintenance or enhancement of the inhibitory properties. Reversal experiments with suspension cultured cells provided evidence for the occurrence of enzyme inhibition in vivo. Because in higher plants the step catalyzed by P5C reductase is shared by all pathways leading to proline synthesis, these compounds may be exploited for the design of new substances endowed with herbicidal activity.

KEYWORDS: Derivatives of aminomethylenebisphosphonic acid; P5C reductase; proline synthesis; structure–activity relationship analysis; amino acid biosynthesis inhibitors as herbicides

INTRODUCTION

Plants are continually faced with several abiotic stress conditions that may result in osmotic impairment. Either temperatures below freezing point, drought, or substrate salinity exert similar effects, and can cause water withdrawal, possibly leading to cell dehydration (1). Even if the corresponding signal transduction pathways might somewhat differ from each other, the response of the plant cell is essentially the same (2). Consistently, freezing tolerance is often accompanied by tolerance to dehydration (3). A pivotal role in counteracting the effects of such fluctuations may be played by a rapid and reversible increase in the concentration of small molecules such as sugars, quaternary amines and amino acids, collectively known as compatible solutes (4), among which the amino acid proline has the widest distribution (5). Besides its obvious role for protein and cell wall biosynthesis, in many higher plants high intracellular levels of free proline are transiently accumulated to cope with hyperosmotic conditions (6), as well as in response to the imposition of a wide range of biotic and abiotic stresses (7). The synthesis of this amino acid seems also to play a regulatory role promoting seed germination (8) and is required during floral development and pollen tube growth (9). Thus, the enzymes involved in the anabolic pathway would be greatly attractive as potential targets for new herbicides.

Two metabolic routes leading to proline synthesis have been found in vascular plants. Under normoosmotic conditions and nitrogen availability, proline production seems to proceed mainly through ornithine, an intermediate in arginine biosynthesis, whereas, under hyperosmotic stress and nitrogen deprivation, it is synthesized directly from glutamate (6). The presence of multiple pathways would hamper any attempt to induce proline starvation through the inhibition of either of the enzymes that catalyze the rate-limiting steps, allowing the plant to recover. However, because the two pathways share the last reaction, catalyzed by a δ^1 -pyrroline-5-carboxylate (P5C) reductase [EC 1.5.1.2] (5), this goal might be achieved through the development of specific inhibitors of that enzyme.

Modern agrochemicals should have a favorable combination of properties, including high levels of herbicidal activity, low application rates, crop tolerance, and low levels of toxicity to mammals. Moreover, increasing public concern for the environmental pollution deriving from agricultural practice strictly requires that such xenobiotics are endowed with low persistence, thus being rapidly mineralized by the soil microflora. Intensive efforts have been undertaken during past years to discover new compounds with favorable environmental and safety features to selectively control weeds. In the past decade, this aim has

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Figure 1. Analogues of (3,5-dichlorophenyl)aminomethylenebisphosphonic acid (compound 1a) evaluated as possible inhibitors of plant P5C reductase.

been pursued with new strategies, switching from the classical testing of chemicals for efficacy on whole plants toward the use of in vitro assays against a given molecular target. Aminoalkylphosphonic acids, structural analogues of amino acids in which the carboxylic group is replaced by a phosphonic or related moiety, have attracted increasing interest as lead compounds for the development of new herbicides (10), also because of their high susceptibility to degradation by soil microorganisms (11, 12). High affinity of phosphonates toward certain enzymes is the result of their ability to form either a net of hydrogen bonds with amino acid residues or ionic interactions with positively charged amino acids or metal ions that are present in the active site. In several instances the structural antagonism between amino acids or their biosynthetic intermediates and the phosphonic counterparts resulted in inhibition of enzyme activity (13).

With the aim to identify new active principles, we previously evaluated several groups of aminophosphonates, most of which exerted remarkable phytotoxic effects at both the plant and cell culture levels (14). In vitro assays with partially purified enzymes from different plant sources showed that some of them act by interfering with aromatic metabolism at the level of the first enzyme in the shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase [EC 4.1.2.15] (15, 16). Some others were found to interfere with nitrogen assimilation through the inhibition of glutamine synthetase (GS [EC 6.3.1.2]) (17, 18). In a previous, *blind* screening, a series of

derivatives of aminomethylenebisphosphonic acid were evaluated for the ability to inhibit P5C reductase, isolated from Arabidopsis thaliana (A. thaliana) cultured cells. Three of them were found to interfere with the catalytic mechanism in the millimolar range, and one, namely, (3,5-dichlorophenyl)aminomethylenebisphosphonic acid (compound 1a, Figure 1), was active at micromolar concentrations. A thorough kinetic evaluation coupled with a computer-assisted docking analysis provided information about its mechanism of action at the molecular level (19). On the basis of these results, 25 new compounds were synthesized by varying either the phenyl substituents or the scaffold of the active molecule, i.e., by replacing one of the two phosphonic groups of 1a with an amino or a hydroxyl moiety. Here, we report the evaluation of the effectiveness of these analogues in vitro against thale cress P5C reductase. In vivo experiments with the most active compounds supported the occurrence of proline starvation in treated plant cells. A structure-activity relationship analysis allowed us to define steric and electronic properties at the basis of the biological activity against the selected molecular target.

MATERIALS AND METHODS

Chemistry. Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich or Merck Chemical Companies and were of analytical grade. All obtained hydroxyphosphonic and aminophosphonic acids were racemic. General Procedure for the Preparation of Aminomethylenebisphosphonic Acids (1) (20) The appropriate amine (12.35 mmol), 1.35 mL of triethyl orthoformate (1.31 g, 12.35 mmol), and 3.20 mL of diethyl phosphite (3.41 g, 24.70 mmol) were heated for 12–16 h at 80 °C. The reaction mixture was evaporated under reduced pressure to obtain the corresponding crude ester that was dissolved in concentrated aqueous hydrochloric acid and refluxed for 6 h. Then the volatile components of the reaction mixture were evaporated under reduced pressure. The solid residues (compounds 1a-h) were washed several times with distilled water to obtain the pure products. The oily residues (compounds 1i,j) were crystallized from water/ethanol or water/methanol systems.

General Procedure for the Preparation of Hydroxyphosphonic Acids (2) (21). A 1.83 mL aliquot of diethyl phosphite (1.96 g, 14.23 mmol) was added under stirring to a mixture of magnesium oxide (1.0 g) and the proper aromatic aldehyde (14.23 mmol) at room temperature. After 12 h dichloromethane (20 mL) was added, and the mixture was filtered through celite. Then the solvent was evaporated under reduced pressure. The light yellow residue was washed with distilled water to give the pure ester, which was either refluxed for 6 h with 20% aqueous hydrochloric acid (for compounds 2a-f) or stirred at room temperature for 20 h with a 5-fold molar excess of bromotrimethylsilane in dry dichloromethane (compound 2g), and then methanol was added. Solvents were evaporated under reduced pressure. The oily residues crystallized after 1–2 h, yielding pure solid products.

General Procedure for the Preparation of Aminophosphonic Acids (3a-d) (22). A 0.72 g amount of ammonium formate (11.43 mmol) and 2.0 g of acidic alumina were ground in a mortar until a fine, homogeneous powder was obtained (5–10 min). Then the proper aromatic aldehyde (11.43 mmol) was combined (solid aldehydes needed to be ground before), and 1.50 mL of diethyl phosphite (1.58 g, 11.43 mmol) was added slowly. Following 12–16 h of vigorous stirring, the reaction mixture was extracted with diethyl ether (250 mL). The solvent was evaporated to dryness resulting in oily products, which were left in a refrigerator for several days until the corresponding ester crystallized. This was dissolved in dry dichloromethane (20 mL), and a 5-fold molar excess of bromotrimethylsilane was added. After stirring the mixture for 20 h at room temperature, methanol (5 mL) was added, and 1 h later the mixture was evaporated under reduced pressure. The oily residue crystallized after 1–2 h to give pure solid product.

General Procedure for the Preparation of Aminophosphonic Acids (3e-i) (23). Acetamide (0.2 mol) was dissolved in acetic acid (40 mL) and cooled in an ice bath. Then acetyl chloride (0.1 mol) was added with cooling, and the formation of a crystalline byproduct was observed. After 15 min the proper aldehyde (0.1 mol) was added, and the mixture was kept for 30 min in an ice bath and left for a day at room temperature. Then the mixture was cooled once more in an ice bath, and phosphorus trichloride (0.1 mol) was added. The resulting mixture was kept in the bath for 30 min, then allowed to warm to room temperature, and finally heated for 1 h at 70-75 °C. Evaporation of volatile components of the reaction mixture yielded an oily product, which was refluxed for 8 h in concentrated aqueous hydrochloric acid (100 mL). The acid was evaporated in vacuo, and the resulting product was dissolved in ethanol (50 mL) and left until ammonium chloride completely precipitated; then the latter was filtered off, and ethanol was evaporated under reduced pressure. The obtained oily residue was dissolved in ethanol (50 mL), and the aminophosphonate was precipitated by the addition of pyridine and purified by recrystallization from a water/ethanol system.

DL-P5C was synthesized by the periodate oxidation of δ -allohydroxylysine and purified by cation-exchange chromatography on a Dowex AG50 (200–400 mesh) column, as described in ref 24.

Plant Cell Culture and Growth Conditions. Suspension-cultured cells of *A. thaliana* Heynh., ecotype Columbia, were grown at 24 ± 1 °C on a rotary shaker (100 rpm) under dim (<50 μ mol m⁻² s⁻¹) light in 500 mL Erlenmeyer flasks containing 130 mL of MS medium with 0.3% (w/v) sucrose and 0.5 mg L⁻¹ of both (2,4-dichlorophenoxy)acetic acid and 6-benzylaminopurine. Subcultures were made every 10 days by transferring 30 mL aliquots of the suspension to 100 mL of fresh medium.

P5C Reductase Purification. The enzyme was partially purified from cultured cells harvested in the early stationary phase of growth,

as previously described (19). A combination of ammonium sulfate precipitation, negative chromatography at pH 7.5 on a DEAE-Sephacel column, and anion-exchange chromatography at pH 10.0 on the same column resulted in a 60-fold enrichment, with a 40% yield. Three different enzyme preparations were used. The mean value for a specific activity level in these samples was 46.2 ± 2.1 nkat mg⁻¹. Proper checks were done to rule out the presence in the final preparations of other enzyme products (i.e., P5C dehydrogenase, EC 1.5.1.12). Active fractions were stored at 4 °C until used for biochemical determinations. Under these conditions, P5C reductase activity was found to be stable for at least 2 months.

Enzyme Assays. The physiological, forward reaction of P5C reductase was measured by following the P5C-dependent oxidation of NADH. The assay mixture contained 100 mM Tris-HCl buffer, pH 8.0, 0.1 mM MgCl₂, 2 mM DL-P5C, and 0.25 mM NADH, in a final volume of 1 mL. A limiting amount of enzyme (0.15–0.20 nkat) was added to the prewarmed mixture, and the decrease in absorbance at 340 nm was determined at 35 °C for up to 10 min by continuous monitoring of the sample against blanks from which P5C had been omitted. Activity was determined from the initial linear rate, with the assumption of an extinction coefficient of 6220 M⁻¹ cm⁻¹. Protein concentration was determined by the method of Bradford (25), using bovine serum albumin as the standard.

Enzyme Inhibition by Phosphonic Acids. P5C reductase inhibition was evaluated by adding to the reaction mixture an appropriate dilution of a 20 mM solution of a given compound, brought to pH 7.5–8.0 with KOH, so as to obtain the desired final concentration, ranging from 5 μ M to 5 mM. At least three measurements were performed for each dose. The concentrations causing 50% inhibition (IC₅₀) of P5C reductase activity were estimated utilizing the linear regression equation of enzyme activity values, expressed as a percentage of untreated controls, plotted against the logarithm of inhibitor concentration. At least three concentrations in the rectilinear part of the resulting sigmoidal curve were considered. Confidence limits of IC₅₀ values were computed according to Snedecor and Cochran (26).

In Vivo Inhibition and Reversal Experiments. To measure the effect of some of the most active phosphonates on exponentially growing cells, samples were withdrawn from the stock cultures in the late exponential phase of growth and used to inoculate 100 mL culture flasks to a density of $3.2-3.5 \text{ mg mL}^{-1}$ (dry weight) in a final volume of 25 mL. Filter-sterilized compounds (brought to pH 6.0 with KOH) were added just after the density of the cell population reached 4.0 mg mL⁻¹ (dry weight). After a further 8 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 on each sample following oven drying at 90 \pm 1 °C for 48 h. The same protocol was adopted for reversal experiments, where inhibitor and amino acid supplements were added to the culture medium simultaneously. Proline was added from a filter-sterilized 1.0 M solution. In the case of glutamine, a freshly prepared 200 mM solution was used to avoid spontaneous hydrolysis of the amide moiety. At least four replicates were carried out for each treatment.

Structure-Activity Relationship Analysis. CoMFA 3D-QSAR analysis was done within the QSAR module of Sybyl 6.9.1, Tripos (Discovery Software, 2003), using default settings. Structures exhibiting very low inhibitory activity (pIC₅₀ < 1.50) were not used for computations, as their mode of binding could be completely different from that of highly active analogues. Additionally, another compound that had been previously found to inhibit P5C reductase, namely, N-(3,5dibromo-6-methylpyridin-2-yl)aminomethylenebisphosphonic acid, was also included in 3D-QSAR study. The structure of the most active compound 1a was taken from the previous study (19). The other inhibitor structures were constructed by appropriate modification of the parent compound and minimization using the Tripos force field and conjugate gradient minimizer. The charge for each atom in minimized structure was computed using the MMF94 method. Minimized structures were aligned by superimposition of phosphonate(s) and aromatic ring atoms on the corresponding groups in the structure of the lead compound 1a.

Table 1. Inhibition of Thale Cress P5C Reductase by Analogues of (3,5-Dichlorophenyl)aminomethylenebisphosphonic Acid (Compound 1a)^a

compound	IC ₅₀ (mM)	pIC ₅₀ (M)
1a	0.077 ± 0.011	4.114
1b	0.102 ± 0.038	3.991
1c	0.50 ± 0.19	3.301
1d	0.73 ± 0.24	3.137
1e	0.112 ± 0.059	3.951
1f	0.268 ± 0.018	3.572
1g	0.86 ± 0.37	3.066
1ĥ	1.07 ± 0.19	2.971
1i	74.4 ± 50.6	1.128
1j	533 ± 488	0.273
2a	8.1 ± 2.1	2.092
2b	7.1 ± 2.8	2.149
2c	5.1 ± 1.2	2.292
2d	4.9 ± 1.2	2.310
2e	1.4 ± 0.4	2.854
2f	5.8 ± 2.4	2.237
2g	45.5 ± 29.1	1.342
3a	4.6 ± 1.3	2.337
3b	10.6 ± 6.1	1.975
3c	8.2 ± 4.2	2.086
3d	35.6 ± 31.7	1.449
3e	356 ± 235	0.449
3f	6.6 ± 4.8	2.180
3g	3060 ± 2950	-0.486
3h	63 ± 44	1.201
3i	16.0 ± 7.4	1.796

^{*a*} Activity was evaluated as described under Materials and Methods either in the absence or in the presence of a given phosphonate at concentrations ranging from 0.005 to 5 mM. Each test was carried out in triplicate, and values were expressed as the percentage of untreated controls. The concentrations causing 50% inhibition (IC₅₀) of in vitro activity were estimated utilizing the linear regression equation of the activity values plotted against the logarithm of inhibitor concentration. Confidence limits were computed according to the method of Snedecor and Cochran (*26*).

RESULTS AND DISCUSSION

In Vitro Activity of (3,5-Dichlorophenyl)aminomethylenebisphosphonic Acid Derivatives against P5C Reductase. Aiming at the development of new active principles targeting the activity of plant P5C reductase, three groups of compounds structurally related to a previously described (19) inhibitor of the enzyme (compound 1a) were obtained: some bisphosphonates (1b-j), some hydroxyphosphonates (2), and some aminophosphonates (3) (Figure 1). They were designed taking into account the results of the previous screening of 26 different bisphosphonates, most of which had been found to be completely ineffective (19), in order to investigate the effect of either the number and the position of the substituents in the phenyl ring, or the scaffold structure, i.e., the presence of two phosphonic moieties. To evaluate these compounds, P5C reductase was partially purified from suspension cultured cells of A. thaliana by means of the protocol set up in the previous work. The choice of such an experimental system allowed us to obtain the large quantities of the enzyme that were required to carry out hundreds of assays, in the absence of both interfering activities and multiple enzyme forms. The presence of isoforms with different susceptibility could in fact constitute a significant drawback in assessing the efficacy of a given compound. Moreover, cell suspension cultures represent a well-suited system for verifying the occurrence of enzyme inhibition in vivo. The activity of thale cress P5C reductase was measured in the absence or in the presence of the 26 compounds in the micromolar to millimolar range. Results, summarized in Table 1 as the concentrations causing 50% inhibition of enzyme activity (IC₅₀), clearly showed that bisphosphonate analogues



Figure 2. Effect of the most effective inhibitors of plant P5C reductase upon the growth of *A. thaliana* suspension cultured cells. The compounds were added to the culture medium in the early exponential phase of growth. After 8 days of incubation, when untreated controls reached the early stationary phase, the resulting dry weight increase was determined and expressed as a percent of that in the controls (6.15 ± 0.22 mg mL⁻¹, n = 8). Data are means ± SD over four replicates.

exhibit the highest inhibitory potential. The comparison of the effect of compounds with the same substituents at the aromatic portion of the molecule (e.g., 1b versus 2d and 3a, or 1c versus 2f and 3c, respectively) revealed that their efficacy as inhibitors is reduced by 1 order of magnitude when the second phosphonate moiety is replaced with either a hydroxyl or an amino group. Bisphosphonates **1a**-j are achiral, whereas the corresponding hydroxyphosphonates and aminophosphonates are recemic mixtures, yet the difference between their activity largely exceeded that expected between a single, active compound and the racemic mixture cointaining also the inactive enantiomer. Threfore, the presence of two phosphonate residues in the molecule seems to play a main role in its effectiveness. As to the substituents in the phenyl ring, data did not provide any plain pattern: neither the presence of one or two chlorines nor their position in the phenyl ring seems to vary exceedingly the resulting inhibitory potential. However, the occurrence of only one chlorine at position 4, as in compounds 1g and 2g, significantly reduced the ability to inhibit the enzyme. On the other hand, results obtained with aminophosphonates confirmed previous data on bisphosphonates (19), suggesting that the replacement of chlorine with other substituents, either halogen moieties (F, Br) or other groups (methyl, methoxyl), produces a significant loss of activity. The same was true in the case of compound 1h, in which the two chlorines in the lead compound are replaced with methyl groups.

In Vivo Phytotoxicity of the Most Active Aminobisphosphonates and Its Reversal by Exogenously Supplied Amino Acids. To investigate whether the interference of the most active derivatives with P5C reductase activity may take place also in vivo, and cause an actual reduction of proline biosynthesis that could result in phytotoxic effects at the plant level, the growth of A. thaliana suspension cultured cells was measured following the addition to the culture medium of increasing concentrations of compounds 1a,b,e. In fact several factors, including differential membrane permeability/solubility, subcellular compartmentalization, and occurrence of metabolization/detoxification reactions, can completely abolish inside the cell the inhibitory potential found in vitro for a given compound. Results, shown in Figure 2, indeed pointed out a significant reduction of the dry weight increase in treated cultures that was proportional to the dose added. Once again, the most remarkable effect



Figure 3. Reversal of **1a** toxicity by exogenously supplied amino acids. The compounds (10 mM glutamine and 2 mM proline) were filter-sterilized and added, either singly or in combination, to the culture medium in the early exponential phase of growth, at the same time as the inhibitor (0.2 mM). After 8 days of incubation, the resulting dry weight increase was determined and expressed as a percent of that in the untreated control (6.22 \pm 0.14 mg mL⁻¹). Data are means \pm SD over four replicates. Significant differences between treated cultures and the corresponding amino acid-fed controls are marked (*, *P* < 0.10; **, *P* < 0.05; ***, *P* < 0.01; NS, not significant).

was carried out by compound **1a**, with a concentration causing 50% reduction of cell growth of 0.24 ± 0.04 mM, yet the noteworthy difference in the effectiveness in vivo of the three compounds seems poorly consistent with the almost negligible diversity of their efficacy in vitro against the purified enzyme. Even though a contribution of the above-mentioned factors cannot be ruled out, such an apparent discrepancy may reflect the ability to interfere with other target(s). In fact, compound **1a** had been previously shown to inhibit also the activity of glutamine synthetase, with an IC₅₀ against rice GS of $33 \pm 1 \mu$ M (*18*), and several of its analogues seem to share this capability (*29*). However, this could also imply that the phytotoxic effects shown in **Figure 2** may be completely unrelated with a block in proline biosynthetic pathways.

Direct evidence supporting the occurrence of P5C reductase inhibition inside the cell might come from the measurement of intracellular free amino acid pools. In some other cases, the comparison of pools in treated versus untreated cells confirmed the mode of action previously hypothesized for a given phosphonate on the basis of biochemical evidence in vitro (16-18). However, in the current case this approach would not be informative. Because of the concurrent reduction of glutamine/ glutamate synthesis caused by GS inhibition (18), a decrease of proline synthesis could in fact occur even in the absence of any direct intereference with the two anabolic pathways leading to proline production (27). As an alternative, reversal experiments may be carried out. The exogenous supply of the product(s) of a metabolic route should result in a complete relief of the effects caused by the inhibition of whatsoever step upstream (e.g., see refs 14 and 28). The reversal of action of compound **1a** by feeding treated cells with amino acids was therefore evaluated. Exogenously supplied glutamine and proline were able to counteract completely the growth inhibition only when supplied together. If provided singly, they actually ameliorated the resulting increase in cell dry mass, but failed in achieving full restoration of cell growth rate (Figure 3). Overall, the results seem thus to strengthen the likelihood that



Figure 4. Superimposition of selected representatives of bisphosphonic (1a), hydroxyphosphonic (2a), and aminophosphonic (3a) acids.

P5C reductase inhibition does occur in vivo and suggest that the phytotoxicity of compound **1a** may be due to inhibition of key enzymes in both pathways for proline and glutamine synthesis.

Structure-Activity Relationship Analysis. To shed some further light on the relationships between the structure and the activity of P5C reductase inhibitors, a comparative molecular field analysis (CoMFA) was performed using the Sybyl program (Tripos, Discovery Software). The structure of active inhibitors was superimposed on that of the most potent compound 1a (Figure 4), and molecular field was computed. When applied to our data, the partial least squares method yielded highcorrelation coefficients [cross-validated $q^2 = 0.69$ (by leaveone-out method) and non-cross-validated $r^2 = 0.98$, with six principal components], confirming the statistical significance of the analysis. The significance of non-cross-validated r^2 was proven by *F*-test, yielding $F(n_1 = 6, n_2 = 13) = 108.5$. Additionally, the leave-multiple-out method with five groups was applied for testing the model. For 20 runs, mean $q^2 = 0.70$ and mean standard error of prediction 0.57 were obtained. The 3D-QSAR CoMFA map (Figure 5) showed regions of possible steric (green = favored, yellow = unfavored) and electrostatic (blue = positively charged, red = negatively charged) interactions. The first phosphonic group is not marked, since it represents the constant element in the whole set of inhibitors. Three red areas indicate favorable, negatively charged groups: one located, as expected, near the second phosphonate moiety and two in the place of substituents of the phenyl ring (which could correspond to the two chlorine atoms in compound 1a). A blue area near the NH moiety of compounds 1 represents a favorable, positively charged group. The CoMFA map does not show any region covering the aromatic ring. However, data suggest that the electron density in this part of the molecule is important, as indicated by the comparison of the effectiveness of compounds **1a**,**h**, which are structurally, but not electronically, similar. Whereas the former possesses electron-withdrawing chlorine substituents, and exhibits a $pIC_{50} = 4.11$, the latter has electron-donating methyl groups and indeed shows a biological activity that is more than 1 order of magnitude lower. Thus, a low electron density on the aromatic ring seems to favor increased activity.

Interestingly, the presence in the 3D-QSAR CoMFA map of regions describing steric features of the inhibitors is very limited (**Figure 5**). Most likely, this is a consequence of the data set used, which consists of compounds with similar structure. However, the comparison of the results presented here with those reported in the previous paper (19), where the binding of **1a** in



Figure 5. Isosurfaces (regions of 20 or 80%-iles of StdDev*Coef values) generated by 3-D QSAR COMFA analysis of P5C reductase inhibitors and the structure of the most potent inhibitor (**1a**). Green and yellow regions show positive and negative steric interactions, respectively, while blue and red ones describe favorable interactions of positively and negatively charged groups. The correlation between predicted and actual values of pIC₅₀ is presented as an inset.

the active site of P5C reductase was investigated, strongly suggests that steric demands are also substantial. These requirements result from the shape of the catalytic pocket, with which the inhibitor interacts, that is relatively deep and tight.

Several analogues of the previously described (19) compound 1a were herein analyzed for the ability of interfering with the catalytic rate of plant P5C reductase. Derivatives substituted in the phenyl ring retained a significant effectiveness, whereas the replacement of the second phosphonate moiety with an amino or a hydroxyl residue resulted in a strong reduction of the inhibitory potential. Although the possibility that some further modifications in the scaffold could increase their efficacy against P5C reductase cannot be excluded, the presence of two phosphonic groups seems a main structural feature to target the active site of the enzyme. Experiments with plant cell suspension cultures confirmed in vivo toxicity of the most active compounds. Moreover, the reversal effect of exogenously supplied amino acid suggested an actual occurrence of proline starvation in treated cultures and strengthened the possibility that P5C reductase inhibition does occur inside the cell. Notwithstanding this, none of the tested analogues showed higher efficacy than the lead compound, and all the most active derivatives bear chlorine atoms in the phenyl ring. Being aromatic chlorinated molecules, most likely endowed with high recalcitrancy, these compounds themselves are not optimal candidates as new active principles for weed control. However, a lower but significant inhibitory potential was retained by compound 1h, in which the two chlorine moieties of 1a are replaced with methyl groups. Furthermore, the availability of several active structures allowed a proper SAR analysis, one that provided us with useful informations concerning the steric and electronic features that are required to maintain the inhibitory effectiveness. Work is currently in progress in our laboratories to accordingly design, synthesize, and test nonchlorinated analogues, ones that would combine the favorable steric features of 1h with the presence of electron-withdrawing moieties that may result in a lower, optimal electron density of the phenyl ring, as in 1a.

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Supporting Information Available: Yields and spectral data for compounds 1-3 (pdf). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

- (1) Mahajan, S.; Tuteja, N. Cold, salinity and drought stresses: An overview. *Arch. Biochem. Biophys.* **2005**, *444*, 139–158.
- (2) Seki, M.; Kamei, A.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Molecular responses to drought, salinity and frost: Common and different paths for plant protection. *Curr. Opin. Biotechnol.* 2003, *14*, 194–199.
- (3) Verslues, P. E.; Agarwal, M.; Katiyar-Agarwal, S.; Zhu, J. H.; Zhu, J. K. Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J.* **2006**, *45*, 523–539.
- (4) Rathinasabapathi, B. Metabolic engineering for stress tolerance: Installing osmo-protectant synthesis pathways. *Ann. Bot.* 2000, 86, 709–716.
- (5) Kavi Kishor, P. B.; Sangam, S.; Amrutha, R. N.; Sri Laxmi, P.; Naidu, K. R.; Rao, K. R. S. S.; Rao, S.; Reddy, K. J.; Theriappan, P.; Sreenivasulu, N. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. *Curr. Sci.* 2005, *88*, 424– 438.
- (6) Yoshiba, Y.; Kiyosue, T.; Nakashima, K.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol.* **1997**, *38*, 1095– 1102.
- (7) Hare, P. D.; Cress, W. A. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul.* **1997**, *21*, 79–102.
- (8) Hare, P. D.; Cress, W. A.; van Staden, J. A regulatory role for proline metabolism in stimulating *Arabidopsis thaliana* seed germination. *Plant Growth Regul.* 2003, *39*, 41–50.

- (9) Schwacke, R.; Grallath, S.; Breitkreuz, K. E.; Stransky, E.; Stransky, H.; Frommer, W. B.; Rentsch, D. LeProT1, a transporter for proline, glycine betaine, and γ-amino butyric acid in tomato pollen. *Plant Cell* **1999**, *11*, 377–391.
- (10) Kafarski, P.; Lejczak, B.; Forlani, G. Herbicidally active aminomethylenebisphosphonic acids. *Heteroatom Chem.* 2000, 11, 449–453.
- (11) Kafarski, P.; Lejczak, B.; Forlani, G. Biodegradation of pesticides containing carbon-to-phosphorus bond. In *Pesticide Biotransformation in Plants and Microorganisms*; Hall, J. C., Hoagland, R. E., Zablotowicz, R. M., Eds.; ACS Symposium Series 777; American Chemical Society: Washington, DC, 2001; pp 145–163.
- (12) Quinn, J. P.; Kulakova, A. N.; Cooley, N. A.; McGrath, J. W. New ways to break an old bond: The bacterial carbon-phosphorus hydrolases and their role in biogeochemical phosphorus cycling. *Environ. Microbiol.* **2007**, *9*, 2392–2400.
- (13) Berlicki, Ł.; Kafarski, P. The use of molecular modelling for comparison of three possible modes of action of herbicidally active derivatives of aminomethylene bisphosphonic acid. *Pest. Biochem. Physiol.* **2002**, *73*, 94–103.
- (14) Forlani, G.; Kafarski, P.; Lejczak, B.; Wieczorek, P. Mode of action of herbicidal derivatives of aminomethylenebisphosphonic acid. II. Reversal of herbicidal action by aromatic amino acids. *J. Plant Growth Regul.* **1997**, *16*, 147–152.
- (15) Forlani, G.; Lejczak, B.; Kafarski, P. N-pyridyl-aminomethylenebisphosphonic acids inhibit the first enzyme in the shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase. *Pestic. Biochem. Physiol.* **1996**, *55*, 180–188.
- (16) Forlani, G.; Lejczak, B.; Kafarski, P. The herbicidally active compound *N*-2-(6-methyl-pyridyl)-aminomethylene-bisphosphonic acid inhibits *in vivo* aromatic biosynthesis. *J. Plant Growth Regul.* **1999**, *18*, 73–79.
- (17) Forlani, G.; Lejczak, B.; Kafarski, P. The herbicidally active compound N-2-(5-chloro-pyridyl)-aminomethylene-bisphosphonic acid acts by inhibiting both glutamine and aromatic amino acid biosynthesis. *Aust. J. Plant Physiol.* **2000**, *27*, 677–683.
- (18) Obojska, A.; Berlicki, U.; Kafarski, P.; Lejczak, B.; Chicca, M.; Forlani, G. Herbicidal pyridyl derivatives of aminomethylene-

bisphosphonic acid inhibit plant glutamine synthetase. J. Agric. Food Chem. 2004, 52, 3337–3344.

- (19) Forlani, G.; Giberti, S.; Berlicki, Ù.; Petrollino, D.; Kafarski, P. Plant P5C reductase as a new target for aminomethylenebisphosphonates. J. Agric. Food Chem. 2007, 55, 4340–4347.
- (20) Soloducho, J.; Gancarz, R.; Wieczorek, P.; Korf, J.; Hafner, J.; Lejczak, B.; Kafarski, P. Patent PL93-298436 93408, 1993.
- (21) Kaboudin, B. A convenient synthesis of 1-aminophosphonates from 1-hydroxyphosphonates. *Tetrahedron Lett.* 2003, 44, 1051– 1053.
- (22) Kaboudin, B.; Nazari, R. Microwave-assisted synthesis of 1-aminoalkyl phosphonates under solvent-free conditions. *Tetrahedron Lett.* 2001, 42, 8211–8213.
- (23) Soroka, M.; Jaworska, D.; Szczêsny, Z. Synthesis of 1-aminoalkylphosphonic acids via amidoalkylation of phosphorous acid by *N*,*N*'alkylidene bis-amides. *Liebigs Ann. Chem.* **1990**, 1153–1155.
- (24) Forlani, G.; Scainelli, D.; Nielsen, E. Δ¹-pyrroline-5-carboxylate dehydrogenase from cultured cells of potato. Purification and properties. *Plant Physiol.* **1997**, *113*, 1413–1418.
- (25) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
- (26) Snedecor, G. W.; Cochran, W. G. *Statistical methods*, 8th ed.; The Iowa State University Press: Ames, IA, 1989.
- (27) Brugiere, N.; Dubois, F.; Limami, A. M.; Lelandais, M.; Roux, Y.; Sangwan, R. S.; Hirel, B. Glutamine synthetase in the phloem plays a major role in controlling proline production. *Plant Cell* **1999**, *11*, 1995–2011.
- (28) Grossmann, K.; Ehrhardt, T. On the mechanism of action and selectivity of the corn herbicide topramezone: A new inhibitor of 4-hydroxyphenylpyruvate dioxygenase. *Pest Manag. Sci.* 2007, 63, 429–439.
- (29) Occhipinti, A.; Forlani, G. Unpublished results.

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