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Synthesis and Evaluation of Effective Inhibitors of Plant δ^1-Pyrroline-5-carboxylate Reductase

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ABSTRACT: Analogues of previously studied phenyl-substituted aminomethylene-bisphosphonic acids were synthesized and evaluated as inhibitors of *Arabidopsis thaliana* δ^1-pyrroline-5-carboxylate reductase. With the aim of improving their effectiveness, two main modifications were introduced into the inhibitory scaffold: the aminomethylenebisphosphonic moiety was replaced with a hydroxymethylenebisphosphonic group, and the length of the molecule was increased by replacing the methylene linker with an ethylidene chain. In addition, chlorine atoms in the phenyl ring were replaced with various other substituents. Most of the studied derivatives showed activity in the micromolar to millimolar range, with two of them being more effective than the lead compound, with concentrations inhibiting 50% of enzyme activity as low as 50 μM. Experimental evidence supporting the ability of these inhibitors to interfere with proline synthesis in vivo is also shown.

KEYWORDS: *amino acid biosynthesis inhibitors as herbicides, aminomethylene-bisphosphonic acid derivatives, P5C reductase, proline synthesis*

INTRODUCTION

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 active ingredients are endowed with low persistence, thus being rapidly mineralized by the soil microflora. Intensive efforts have been undertaken during the past decades to discover new compounds with suitable environmental and safety features to selectively control weeds. However, the unfavorable economics of herbicide development, mainly due to increasing regulatory requirements, is greatly limiting industrial discovery programs.[1] On the other hand, because of a high selective pressure for weeds, the rapid diffusion of resistant biotypes that usually occurs within a few years after their introduction[2] is dramatically reducing the commercial life of most active principles. Moreover, several herbicides that are currently in use came from a small set of molecular scaffolds, whose functional lifetime has been extended by synthetic tailoring. Because the members of these *families* of compounds share the same target at the molecular level, the emergence and diffusion of cross-resistance is rapidly occurring.[3] Therefore, the discovery of new scaffolds is required, or new herbicide targets should be identified.[4] In this perspective, inhibitors of enzymes that catalyze key reactions in amino acid metabolism could represent promising new leads for weed control.[5]

From this point of view, little attention has been paid to date to proline synthesis. Proline plays an important role in protein structure, uniquely contributing to protein folding and stability.[6] Moreover, in a wide variety of higher plants a rapid and reversible increase in the intracellular concentration of free proline has been shown in response to either osmotic or temperature stress, implying a role in stress tolerance and osmoregulation.[7] The poor interest for the development of proline-targeting active principles is most likely due to the redundancy of proline synthesis. Proline can in fact be produced from either glutamate or ornithine,[8] the glutamate route usually being the main pathway.[9] In the presence of two pathways, the inhibition of either of the enzymes that catalyze the rate-limiting steps would be ineffective, because proline starvation would not be achieved and the cell would be allowed to recover. Moreover, in some soil and plant-associated bacteria a stereospecific and irreversible conversion of L-ornithine to L-proline may be accomplished in a single step by an ornithine cyclodeaminase [EC 4.3.1.12].[10] However, because the latter has never been convincingly identified in plants, and the glutamate and the ornithine pathways share the last reaction catalyzed by a δ^1-pyrroline-5-carboxylate (P5C) reductase [EC 1.5.1.2], proline starvation might be achieved through the development of specific inhibitors of this enzyme.

With the aim of identifying 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.[11] In a preliminary screening, a series of phenyl derivatives of aminomethylenebisphosphonic acid (compounds **1**, Figure 1) were evaluated for the ability to inhibit P5C reductase, isolated from *Arabidopsis thaliana* cultured cells. Three of them were found to interfere with the catalytic mechanism in the millimolar range, and one, namely, 3,5-dichlorophenylaminomethylenebisphosphonic acid, was active at micromolar levels.[12] On the contrary, their pyridyl

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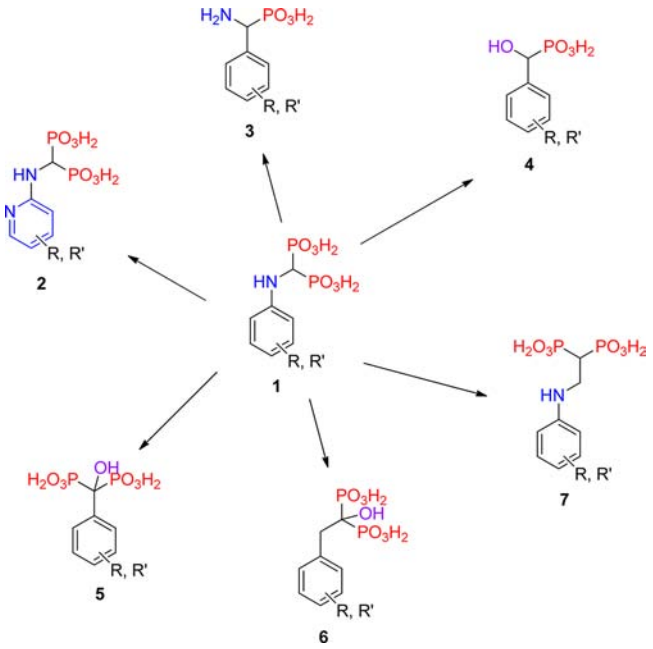

Figure 1. Analogues of phenyl-substituted aminomethylenebisphosphonic acids (compounds **1**), the scaffold of which has been evaluated in previous studies (compounds **2**[12] and compounds **3** and **4**[13]) and in the present work (compounds **5**, **6**, and **7**) aiming at the development of effective inhibitors of plant P5C reductase.

analogues **2** were substantially ineffective.[12] A thorough kinetic evaluation coupled with a computer-assisted docking analysis provided information about its mechanism of action at the molecular level, showing that it inhibits the enzyme noncompetitively with respect to NAD(P)H and uncompetitively with respect to P5C.[12] On the basis of these results, 25 new compounds were synthesized by varying either the phenyl substituents or the scaffold of the active molecule, that is, by removing the amino group and replacing one of the two phosphonic groups with an amino (compounds **3**) or a hydroxyl (compounds **4**) moiety. Derivatives substituted in the phenyl ring retained the inhibitory potential, although to a different extent. All of the most active substances had two chloride substituents, and none showed higher effectiveness than the lead compound, **1a**. Conversely, both variations in the scaffold resulted in a substantial loss of biological activity.[13] The availability of several structures active in the micromolar to millimolar range permitted a proper structure−activity relationship analysis, allowing us to hypothesize about the steric and electronic requirements for enhancement of the inhibitory properties. On this basis, 22 new derivatives were designed, either introducing new electron-withdrawing substituents in the phenyl ring (compounds **1f−m**, Figure 2) or modifying the structure (compounds **5**) and/or the length (compounds **6** and **7**) of the scaffold. Here we report on the synthesis and evaluation of the inhibitory potential of these derivatives against plant P5C reductase.

MATERIALS AND METHODS

Chemistry. Unless indicated otherwise, chemicals were purchased from Sigma-Aldrich or Merck Chemical Companies and were of analytical grade. DL-P5C was synthesized by the periodate oxidation of *δ-allo*-hydroxylysine and purified by cation-exchange chromatography on a Dowex AG50 (200−400 mesh) column, as described in the literature.[14]

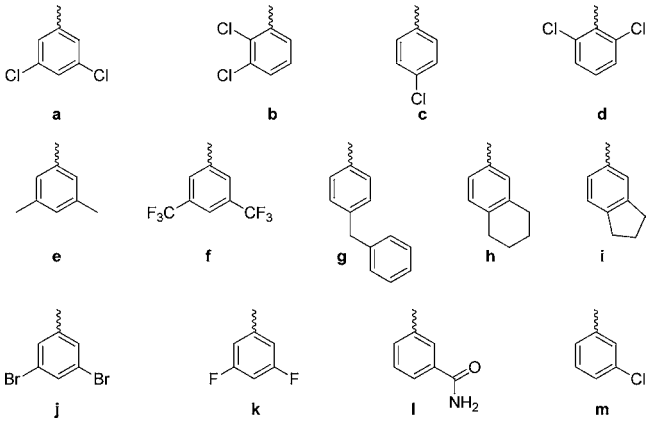

Figure 2. Substitution patterns of the phenyl ring in aminomethylenebisphosphonates **1a−m**, hydroxymethylenebisphosphonates **5b−g**, hydroxyethylidenebisphosphonates **6b−e**, and aminoethylidenebisphosphonates **7a−h** evaluated in the present work.

^{1}H (300.13 MHz or 600.58 MHz) and ^{31}P NMR (121.51 MHz or 243.12 MHz) spectra were recorded on Bruker AVANCE DRX 300 MHz or Bruker AVANCE 600 MHz using D_2O solutions with TMS as an external reference for ^{1}H NMR and phosphoric acid (85%) for ^{31}P NMR spectra. Chemical shifts (δ) are reported in parts per million, and coupling constants (J) are reported in hertz. Data for compounds **1a−e** have been already reported in the preceding study.[13]

General Procedure for the Preparation of Aminomethylenebisphosphonic Acids (1). 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 under reflux for 12−16 h. The volatile components of the reaction mixture were evaporated under reduced pressure to obtain the corresponding crude ester, which was dissolved in concentrated hydrochloric acid and refluxed for 6 h. Then the volatile components of the reaction mixture were evaporated under reduced pressure. The solid residues were washed several times with distilled water to obtain the pure products.

*3,5-Di(trifluoromethyl)phenylaminomethylenebisphosphonic acid (**1f**):* yield 30%; ^{1}H NMR (300 MHz, D_2O) δ 7.19 (s, 1H, ArH), 7.15 (s, 2H, ArH), 4.20 (t, 1H, $^2J_{H-P}$ = 21.4, CHP_2); ^{31}P NMR δ 16.36.

*4-Benzylphenylaminomethylenebisphosphonic acid (**1g**):* yield 61%; ^{1}H NMR (300 MHz, D_2O) δ 7.21−7.04 (m, 5H, ArH), 6.91 (d, 2H, $^3J_{H-H}$ = 8.2, ArH), 6.51 (d, 2H, $^3J_{H-H}$ = 8.2, ArH), 3.67 (s, 2H, CH_2), 3.36 (t, 1H, $^2J_{H-P}$ = 19.8, CHP_2); ^{31}P NMR δ 17.24.

*5,6,7,8-Tetrahydro-2-naphthylaminomethylenebisphosphonic acid (**1h**):* yield 18%; ^{1}H NMR (300 MHz, D_2O) δ 6.69 (d, 1H, $^3J_{H-H}$ = 8.3, ArH), 6.32 (d, 1H, $^3J_{H-H}$ = 10.0, ArH), 6.26 (s, 1H, ArH), 3.28 (t, 1H, $^2J_{H-P}$ = 19.9, CHP_2) 2.45 (bs, 2H, CH_2), 2.37 (bs, 2H, CH_2), 1.49 (bs, 4H, 2 × CH_2); ^{31}P NMR δ 17.47.

*Indan-5-ylaminomethylenebisphosphonic acid (**1i**):* yield 4%; ^{1}H NMR (300 MHz, D_2O + NaOD) δ 6.91 (d, 1H, $^3J_{H-H}$ = 8.1, ArH), 6.53 (s, 1H, ArH), 6.40 (d, 1H, $^3J_{H-H}$ = 8.1, ArH), 3.37 (t, 1H, $^2J_{H-P}$ = 19.7, CHP_2), 2.67−2.57 (m, 4H, 2 × CH_2), 1.88−1.79 (m, 2H, CH_2); ^{31}P NMR δ 17.60.

*3,5-Dibromophenylaminomethylenebisphosphonic acid (**1j**):* Yield 46%. ^{1}HNMR (300 MHz, D_2O) (δ) 6.75 (s, 1H, ArH), 6.61 (s, 2H, ArH), 3.31 (t, 1H, $^2J_{H-P}$ = 25.1, CHP_2); ^{31}PNMR δ 16.29.

*3,5-Difluorophenylaminomethylenebisphosphonic acid (**1k**):* yield 38%; ^{1}H NMR (300 MHz, DMSO) δ 6.43 (d, 2H, $^3J_{HF}$ = 9.0, Ar), 6.19 (t, 1H, $^3J_{HF}$ = 9.4, Ar), 3.91 (t, 1H, $^2J_{H-P}$ = 21.5); ^{31}P NMR δ 15.62.

*3-Carbamoylophenylaminomethylenebisphosphonic acid (**1l**).* 3-Carboxyphenylaminomethylenebisphosphonic acid (0.5 g, 1.61 mmol), which was obtained using the above general procedure, and an excess of thionyl chloride were heated under reflux for 30 min. The volatile components of the reaction mixture were removed under reduced pressure. Then concentrated $NH_{3\ aq}$ was added and heated

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under reflux for 15 min. The excess of reagent was removed under reduced pressure. Yield 0.48 g (96%); ^{1}H NMR (300 MHz, D_2O + NaOD) δ 7.07 (t, 1H, $^3J_{H-H}$ = 7.6, ArH), 7.02 (s, 1H, ArH), 6.93 (d, 1H, $^3J_{H-H}$ = 7.6, ArH), 6.73 (d, 1H, $^3J_{H-H}$ = 7.9, ArH), 3.62 (t, 1H, $^2J_{H-P}$ = 19.3, CHP_2); ^{31}P NMR δ 13.49.

*3-Chlorophenylaminomethylenebisphosphonic acid (**1m**):* yield 80%; ^{1}H NMR (300 MHz, D_2O) δ 6.95 (t, 1H, $^3J_{H-H}$ = 8.1, ArH), 6.58 (s, 1H, ArH), 6.47 (d, 1H, $^3J_{H-H}$ = 8.3, ArH), 6.40 (d, 1H, $^3J_{H-H}$ = 7.8, ArH), 3.38 (t, 1H, $^2J_{H-P}$ = 19.6, CHP_2); ^{31}P NMR δ 16.54.

General Procedure for the Preparation of Hydroxybisphosphonic Acids (5 and 6). The appropriate carboxylic acid (7.85 mmol) and thionyl chloride (6.8 mL, 47.1 mmol) were refluxed for 1 h. The volatile portion of the reaction mixture was evaporated under reduced pressure to give crude acid chloride. The solution of obtained acid chloride in dry THF was added dropwise to a solution of tris(trimethylsilyl)phosphite (5.25 mL, 15.9 mmol) under nitrogen. The reaction mixture was stirred for 1 h at room temperature. Subsequently, methanol was added and stirring was continued for 1 h. Solvents were removed under reduced pressure, and residues were washed with diethyl ether. When necessary, the product was purified by RP-HPLC (water/acetonitrile 0.1% TFA).

*2,3-Dichlorophenylhydroxymethylenebisphosphonic acid (**5b**):* yield 27%; ^{1}H NMR (300 MHz, D_2O) δ 7.80 (d, 1H, $^3J_{H-H}$ = 7.5, ArH), 7.36 (d, 1H, $^3J_{H-H}$ = 7.9, ArH), 7.11 (t, 1H, $^3J_{H-H}$ = 8.1, ArH); ^{31}P NMR δ 16.14.

*3,5-Dimethylphenylhydroxymethylenebisphosphonic acid (**5e**):* yield 98%; ^{1}H NMR (300 MHz, D_2O) δ 7.19 (s, 2H, ArH), 6.85 (s, 1H, ArH), 2.11 (s, 6H, 2 × CH_3); ^{31}P NMR δ 16.96.

*4-Benzylphenylhydroxymethylenebisphosphonic acid (**5g**):* yield 85%; ^{1}H NMR (300 MHz, D_2O) δ 7.54 (d, 2H, $^3J_{H-H}$ = 8.6, ArH), 7.23−7.07 (m, 7H, ArH), 3.84 (s, 2H, CH_2); ^{31}P NMR δ 17.12.

*2-(2,3-Dichlorophenyl)-1-hydroxyethylidenebisphosphonic acid (**6b**):* yield 65%; ^{1}H NMR (300 MHz, D_2O) δ 7.42 (d, 1H, $^3J_{H-H}$ = 7.8, ArH), 7.32 (d, 1H, $^3J_{H-H}$ = 7.3, ArH), 7.08 (t, 1H, $^3J_{H-H}$ = 7.9, ArH), 3.48 (t, 2H, $^3J_{H-P}$ = 13.5, CH_2); ^{31}P NMR δ 18.90.

*2-(4-Chlorophenyl)-1-hydroxyethylidenebisphosphonic acid (**6c**):* yield 51%; ^{1}H NMR (300 MHz, D_2O) δ 7.25 (d, 2H, $^3J_{H-H}$ = 8.5, ArH), 7.19 (d, 2H, $^3J_{H-H}$ = 8.5, ArH), 3.17 (t, 2H, $^3J_{H-P}$ = 13.3, CH_2); ^{31}P NMR δ 19.12.

*2-(2,6-Dichlorophenyl)-1-hydroxyethylidenebisphosphonic acid (**6d**):* yield 30%; ^{1}H NMR (300 MHz, D_2O) δ 7.18 (d, 2H, $^3J_{H-H}$ = 8.1, ArH), 7.02 (t, 1H, $^3J_{H-H}$ = 8.0, ArH), 3.60 (t, 2H, $^3J_{H-P}$ = 14.0, CH_2); ^{31}P NMR δ 18.67.

*2-(3,5-Dimethylphenyl)-1-hydroxyethylidenebisphosphonic acid (**6e**):* yield 34%; ^{1}H NMR (300 MHz, D_2O) δ 6.92 (s, 2H, ArH), 6.85 (s, 1H, ArH), 3.13 (t, 2H, $^3J_{H-P}$ = 13.6, CH_2), 2.14 (s, 6H, 2 × CH_3); ^{31}P NMR δ 19.46.

General Procedure for the Preparation of Aminoethylenebisphosphonic Acids (7). Tetraethyl ethylidenebisphosphonate (3 mmol) and the appropriate amine (3 mmol) were dissolved in dry THF. The reaction mixture was stirred for 24 h at room temperature, and solvents were evaporated at reduced pressure. The obtained ester was purified with the use of column chromatography on silica gel (ethyl acetate/hexanes). Subsequently, the ester was dissolved in dry acetonitrile, and trimethylsilyl bromide (6 equiv) was added. The mixture was stirred for 12 h. Then methanol was added, and the mixture was stirred for 2 h. Volatile components were removed under reduced pressure. When necessary, the product was purified by RP-HPLC (water/acetonitrile 0.1% TFA).

*2-(3,5-Dichlorophenylamino)ethylidenebisphosphonic acid (**7a**):* yield 48%; ^{1}H NMR (300 MHz, D_2O) δ 6.71 (s, 3H, ArH), 3.33 (td, 2H, $^3J_{H-P}$ = 14.0, $^3J_{H-H}$ = 6.7, $\underline{CH_2}CHP_2$), 1.88 (tt, 1H, $^3J_{H-P}$ = 21.0, $^3J_{H-H}$ = 6.8, CHP_2); ^{31}P NMR δ 18.92.

*2-(2,3-Dichlorophenylamino)ethylidenebisphosphonic acid (**7b**):* yield 3%; ^{1}H NMR (300 MHz, D_2O) δ 7.06 (t, 1H, $^3J_{H-H}$ = 8.5, ArH), 6.75 (d, 1H, $^3J_{H-H}$ = 9.1, ArH), 6.77 (d, 1H, $^3J_{H-H}$ = 9.1, ArH), 3.32 (td, 2H, $^3J_{H-P}$ = 14.2, $^3J_{H-H}$ = 6.5, $\underline{CH_2}CHP_2$), 1.90 (tt, 1H, $^3J_{H-P}$ = 20.5, $^3J_{H-H}$ = 6.5, CHP_2); ^{31}P NMR δ 18.92.

*2-(2,6-Dichlorophenylamino)ethylidenebisphosphonic acid (**7d**):* yield 28%; ^{1}H NMR (300 MHz, D_2O) δ 7.23 (d, 2H, $^3J_{H-H}$ = 8.0, ArH), 6.81 (t, 2H, $^3J_{H-H}$ = 8.0, ArH), 3.46 (td, 2H, $^3J_{H-P}$ = 13.9, $^3J_{H-H}$ = 6.6, $\underline{CH_2}CHP_2$), 1.90 (tt, 1H, $^3J_{H-P}$ = 20.7, $^3J_{H-H}$ = 6.6, CHP_2); ^{31}P NMR δ 19.20.

*2-(3,5-Dimethylphenylamino)ethylidenebisphosphonic acid (**7e**):* yield 45%; ^{1}H NMR (300 MHz, D_2O) δ 6.50 (s, 2H, ArH), 6.47 (s, 1H, ArH), 3.41 (td, 2H, $^3J_{H-P}$ = 14.0, $^3J_{H-H}$ = 6.8, $\underline{CH_2}CHP_2$), 2.17 (s, 6H, 2 × CH_3), 1.88 (tt, 1H, $^3J_{H-P}$ = 20.9, $^3J_{H-H}$ = 6.8, CHP_2); ^{31}P NMR δ 19.38.

*2-(3,5-Di(trifluoromethyl)phenylamino)ethylidenebisphosphonic acid (**7f**):* yield 60%; ^{1}H NMR (300 MHz, D_2O) δ 7.18 (s, 1H, ArH), 7.14 (s, 2H, ArH), 3.36 (td, 2H, $^3J_{H-P}$ = 13.9, $^3J_{H-H}$ = 6.6, $\underline{CH_2}CHP$), 1.85 (tt, 1H, $^3J_{H-P}$ = 20.9, $^3J_{H-H}$ = 6.8, CHP_2); ^{31}P NMR δ 18.21.

*2-(4-Benzylphenylamino)ethylidenebisphosphonic acid (**7g**):* yield 18%; ^{1}H NMR (300 MHz, D_2O) δ 7.24−7.08 (m, 5H, $\underline{ArH}CH_2$), 7.03 (d, 2H, $^3J_{H-H}$ = 8.3, ArH), 6.72 (d, 2H, $^3J_{H-H}$ = 8.4, ArH), 3.76 (s, 2H, $Ar\underline{CH_2}$), 3.26 (td, 2H, $^3J_{H-P}$ = 13.9, $^3J_{H-H}$ = 6.7, $\underline{CH_2}CHP_2$), 1.81 (tt, 1H, $^3J_{H-P}$ = 14.0, $^3J_{H-H}$ = 6.9, CHP_2); ^{31}P NMR δ 19.27.

*2-(5,6,7,8-Tetrahydro-2-naphthylamino)-ethylidenebisphosphonic acid (**7h**):* yield 23%; ^{1}H NMR (600 MHz, D_2O) δ 6.84 (d, 1H, $^3J_{H-H}$ = 8.1, ArH), 6.53 (d, 1H, $^3J_{H-H}$ = 8.2, ArH), 6.52 (s, 1H, ArH), 3.20 (td, 2H, $^3J_{H-P}$ = 14.0, $^3J_{H-H}$ = 6.7, $\underline{CH_2}CHP_2$), 2.52 (m, 2H, CH_2), 2.46 (m, 2H, CH_2), 1.76 (tt, 1H, $^3J_{H-P}$ = 20.8, $^3J_{H-H}$ = 6.6, CHP_2), 1.66 (m, 4H, 2 × CH_2); ^{31}P NMR δ 19.30.

Plant Cell Culture and Growth Conditions. Cell suspension cultures of *A. thaliana* Heynh., ecotype Columbia, were grown at 24 ± 1 °C on a rotary shaker (100 rpm) under dim (<50 μmol m^{-2} s^{-1}) light in 500 mL Erlenmeyer flasks containing 125 mL of Murashige and Skoog medium with 0.3% (w/v) sucrose and 0.5 mg L^{-1} of both 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. Subcultures were made every 7 days by transferring 25 mL aliquots of the suspension to 100 mL of fresh medium.

P5C Reductase Purification and Assay. The enzyme was partially purified from cultured cells harvested in the early stationary phase of growth as previously described,[12] with minor modifications. A combination of ammonium sulfate precipitation, negative chromatography on a DEAE-Sephacel column equilibrated at pH 7.5, and anion-exchange chromatography at pH 10.0 on the same column resulted in a 70-fold enrichment, with a 35% yield. The mean value for specific activity level in these samples was 122.7 ± 3.9 nkat mg^{-1}. Proper checks were done to rule out the presence of other enzymes able to use the same substrates and/or further metabolize enzyme products (i.e., P5C dehydrogenase, EC 1.5.1.12). Active fractions were filter-sterilized (0.22 μm) and 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.

The physiological forward reaction of P5C reductase was measured by following the P5C-dependent oxidation of NADH. The assay mixture contained 100 mM Hepes−KOH buffer, pH 7.75, 0.1 mM $MgCl_2$, 2 mM DL-P5C, and 0.25 mM NADH, in a final volume of 0.2 mL. A limiting amount of enzyme (0.03−0.04 nkat) was added to the prewarmed mixture, and the decrease in absorbance at 340 nm was determined at 35 °C for up to 5 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 according to the method of Bradford,[15] using bovine serum albumin as the standard.

Enzyme Inhibition by Bisphosphonic Acids. P5C reductase inhibition was evaluated by adding to the reaction mixture an appropriate dilution of a 10 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 1 μM to 1 mM. Three to five different doses in the active range were evaluated, and at least three measurements were performed for each dose. The concentrations causing 50% inhibition (IC_{50}) of P5C reductase activity, and confidence intervals were estimated by nonlinear regression analysis of enzyme activity values, expressed as percentage of untreated

controls, using Prism 5 for Windows, version 5.01 (GraphPad Software).

In Vivo Effects on Proline Biosynthesis. To evaluate bisphosphonate effect upon proline synthesis in exponentially growing cells, samples were withdrawn from the stock cultures in the late exponential phase of growth and used to inoculate 250 mL culture flasks to a density of 1.0−1.2 mg mL^{-1} (dry weight) in a final volume of 60 mL. Filter-sterilized compounds (brought to pH 6.0 with KOH) were added just after the density of cell population reached 1.4 mg mL^{-1} (dry weight). After a further 5−7 days of incubation, when untreated controls approached the early stationary phase of growth, cells were harvested by vacuum filtration, resuspended in 1 mL g^{-1} of a 3% (w/v) solution of 5-sulfosalicylic acid, and homogenized in a Teflon-in-glass Potter homogenizer by 20 strokes. After centrifugation for 3 min at 12000*g*, the supernatant was 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 s, 20 μL of derivatized samples was injected onto a 4.6 × 250 mm Zorbax ODS column (Rockland Technologies, Newport, DE, USA) equilibrated with 59% solvent A [50 mM sodium phosphate−50 mM sodium acetate buffer, pH 7.5, containing 2% (v/v) of both 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 previously,[16] monitoring the eluate at 340 nm. Peaks were integrated by area, with variation coefficients ranging from 0.8 to 3.2%. At least three replicates were carried out for each treatment (biological replicates), and two analyses were carried out for each extract (technical replicates). Proline and total amino acid content were quantified by using the acid ninhydrin method.[14] P5C was measured by reaction with *o*-aminobenzaldehyde, as previously described.[17] Results were expressed as nmol (g FW)$^{-1}$, and means ± standard deviation over replications are presented. Data were analyzed by using standard statistical procedures for analysis of variance and *t* test. When differences are reported, they are at the 99% confidence level ($P < 0.01$).

RESULTS AND DISCUSSION

Design and Synthesis of Bisphosphonate Derivatives. A previous screening[13] of bisphosphonic acid derivatives against P5C reductase allowed us to hypothesize on the structural features required for high inhibitory activity, that is, the presence of two phosphonic groups, a phenyl ring, and a hydrogen bond donor. Four different scaffolds showing these features were taken into account (Figure 1). In this study, the previously explored *N*-phenylaminomethylenebisphosphonic acid core (**1**) was extended to 2-phenylaminoethylidenebisphosphonic acid (**7**). In addition, the hydrogen bond donor secondary amine fragment was replaced by an isoelectronic hydroxyl substituent, and hydroxybisphosphonic acids **5** and **6** were envisaged. Various substitution patterns of phenyl ring (Figure 2) were also considered: (1) different positioning of chlorine atoms (derivatives a−d and m); (2) the presence of both electron-withdrawing and -donating groups in 3,5-positions of the phenyl ring (derivatives a, e, f, j, k); (3) substituents that substantially increase the phenyl ring volume (g, h, i); (4) a carbamoyl substituent similar to that present in the molecule of NAD, because bisphosphonates are expected to act as analogues of the dinucleotide.

All compounds were synthesized using published methodologies (Figure 3). Aminomethylenebisphosphonates were obtained in a tricomponent reaction of amine, ethyl orthoformate, and diethylphosphite and subsequent hydrolysis of tetraethyl esters leading to the requested acids (with HCl_{aq} or via transesterification to trimethylsilyl esters and methanolysis).[18] The addition of amine to the double bond of tetraethyl ethenylidenebisphosphonates resulted in tetraethyl 2-aminoethylene-1,1-bisphosphonates, which were deprotected to acids **7**.[19] Hydroxybisphosphonic acids (**5** and **6**) were synthesized from appropriate acyl chlorides and tris-(trimethylsilyl)phosphate, followed by methanolysis of the obtained esters.[20]

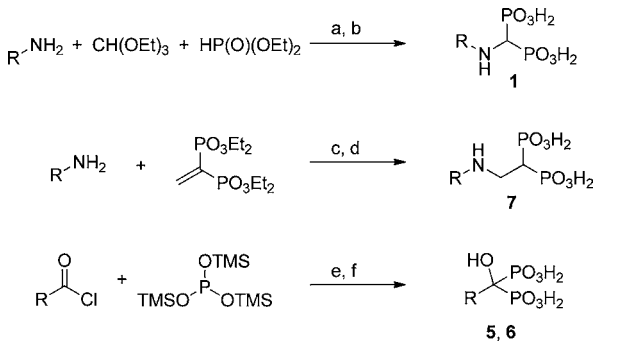

Figure 3. Synthesis of the four groups of bisphosphonic acids evaluated in the present work. Reagents and conditions: (a) reflux for 6 h; (b) HCl_{aq} or TMSBr and then MeOH; (c) stirring for 24 h; (d) TMSBr and then MeOH; (e) stirring for 1 h; (f) MeOH.

Activity of Bisphosphonate Derivatives against P5C Reductase. To evaluate the ability of these compounds to interfere with the activity of plant P5C reductase, the protein was partially purified from suspension cultured cells of *A. thaliana*, a model species in which only one gene coding for this enzyme is present.[7] The occurrence of multiple enzyme forms with different susceptibilities could in fact represent a significant drawback in assessing the efficacy of a given compound. The activity of *Arabidopsis* P5C reductase was measured in the absence or in the presence of the 22 new derivatives in the micromolar to millimolar range. Because slight differences in sensitivity may derive from small variations in enzyme purification level and absolute enzyme amount, the five (**1a**−**e**) most effective aminomethylenebisphosphonates evaluated in the previous study[13] were also included in the experimental plan as reference compounds. Results, summarized in Table 1 as the concentrations causing 50% inhibition of enzyme activity (IC_{50}), showed that, with the only exception of compound **7h**, all of the derivatives are capable of inhibiting the enzyme when present at millimolar levels. This denotes the correctness of the steric and the electronic features previously hypothesized[13] to maintain the inhibitory potential. The comparison of the effect of compounds with the same substituents at the aromatic portion of the molecule (Figure 4) revealed that either the increase of the chain length in compounds **7** or the replacement of the aminomethylenebisphosphonic group with a hydroxymethylene-bisphosphonic moiety in compounds **5** does not exceedingly vary the effectiveness of the lead compounds **1**. On the contrary, the presence of both variations was found to be detrimental, because all 1-hydroxyethylidenebisphosphonic derivatives **6** were less effective than their counterparts **1**. As to the substituents in the phenyl ring, several electron-withdrawing moieties conferred equipotency with the two chlorines in reference compounds **1a**, **1b**, and **1d**. Two derivatives, namely, compounds **1j** and **7a**, showed a significantly higher effectiveness than the lead compound **1a**, thereby proving the validity of the design scheme.

In Vivo Effects of the Most Active Bisphosphonate on Proline Synthesis. Cell suspension cultures represent a well

Table 1. Inhibition of Arabidopsis P5C Reductase by Analogues of 3,5-Dichlorophenylaminomethylenebisphosphonic Acid (1a)[a]

compd	IC_{50} (mM)	pIC_{50} (M)
1a	0.134 ± 0.009	3.873
1b	0.335 ± 0.010	3.475
1c	0.970 ± 0.066	3.013
1d	0.450 ± 0.017	3.347
1e	1.245 ± 0.093	2.905
1f	0.413 ± 0.018	3.384
1g	0.852 ± 0.120	3.070
1h	0.684 ± 0.043	3.165
1i	1.887 ± 0.501	2.724
1j	0.054 ± 0.003	4.268
1k	0.719 ± 0.090	3.143
1l	0.547 ± 0.039	3.262
1m	1.618 ± 0.185	2.791
5b	1.187 ± 0.058	2.926
5e	1.409 ± 0.118	2.851
5g	0.325 ± 0.012	3.488
6b	0.488 ± 0.021	3.312
6c	2.071 ± 0.303	2.684
6d	1.639 ± 0.459	2.785
6e	1.914 ± 0.254	2.718
7a	0.082 ± 0.003	4.086
7b	0.431 ± 0.025	3.366
7d	0.763 ± 0.092	3.117
7e	0.386 ± 0.018	3.413
7f	0.463 ± 0.033	3.334
7g	0.488 ± 0.042	3.312
7h	ineffective	<2

[a]Activity was evaluated as described under Materials and Methods either in the absence or in the presence of a given bisphosphonate at concentrations ranging from 0.001 to 1 mM. Each test was carried out in triplicate, and values were expressed as percentage of untreated controls. The concentrations causing 50% inhibition (IC_{50}) of in vitro activity and confidence limits were estimated by nonlinear regression analysis utilizing the software GraphPad Prism (version 5).

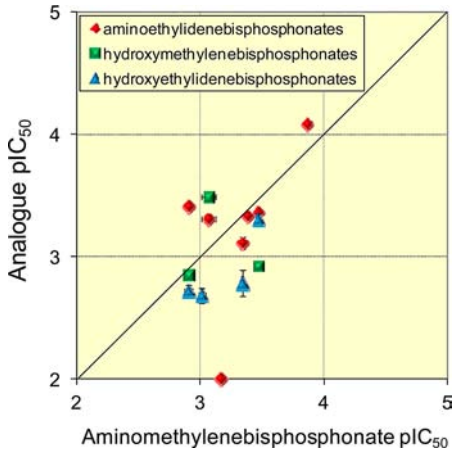

Figure 4. Comparison between the effectiveness of aminomethylenebisphosphonic acids **1** and their analogues **5**, **6**, and **7**. The inverses of the logarithm of the concentrations causing 50% inhibition of enzyme activity (pIC_{50}) were plotted against each other. The 1:1 line indicates equipotency. A point above the line shows that a given analogue is more effective than its aminomethylenebisphosphonic counterpart showing the same substitution pattern in the phenyl ring and vice versa for a point below the line.

suitable system to verify the occurrence of enzyme inhibition in vivo. To investigate whether the interference of the most active derivatives with P5C reductase activity may take place also inside the cell and cause an actual reduction of proline biosynthesis that could result in phytotoxic effects at the plant level, the culture medium of *A. thaliana* suspension cultured cells was supplemented with micromolar concentrations of compound **1j**. As a consequence, cell growth was reduced (data not shown), with a concentration inhibiting by 50% the dry weight increase of 132 ± 7 μM. On this basis, the intracellular levels of proline, P5C, and free amino acids were determined in samples treated with 100 and 200 μM compound **1j**. Results (Table 2) showed that in cells treated with the lower dose of

Table 2. Free Amino Acid, Proline, and P5C Levels[a] in *A. thaliana* Cultured Cells Treated with Micromolar Concentrations of Compound 1j

	treatment		
compd	control (μmol (g FW)$^{-1}$)	100 μM (μmol (g FW)$^{-1}$)	200 μM (μmol (g FW)$^{-1}$)
Asp	0.343 ± 0.009	0.257 ± 0.012	0.173 ± 0.006
Glu	0.794 ± 0.022	0.787 ± 0.062	0.391 ± 0.018
Asn	0.228 ± 0.018	0.105 ± 0.000	0.069 ± 0.014
Ser	0.547 ± 0.028	0.502 ± 0.018	0.392 ± 0.018
Gln	1.027 ± 0.022	0.340 ± 0.013	0.250 ± 0.015
Arg + Gly + Thr	0.060 ± 0.005	0.054 ± 0.003	0.059 ± 0.002
Ala + GABA + Tyr	0.606 ± 0.027	0.384 ± 0.029	0.155 ± 0.006
Trp	0.035 ± 0.001	0.022 ± 0.000	0.012 ± 0.000
Met	0.004 ± 0.001	0.002 ± 0.000	0.003 ± 0.000
Val	0.266 ± 0.018	0.230 ± 0.011	0.117 ± 0.000
Phe	0.065 ± 0.004	0.032 ± 0.001	0.016 ± 0.003
Ile	0.034 ± 0.001	0.033 ± 0.002	0.016 ± 0.001
Leu	0.037 ± 0.002	0.033 ± 0.003	0.018 ± 0.001
Lys	0.035 ± 0.001	0.036 ± 0.004	0.032 ± 0.001
Pro	0.175 ± 0.005	0.132 ± 0.002	0.116 ± 0.006
P5C	0.010 ± 0.002	0.008 ± 0.001	0.029 ± 0.003

[a]Cell suspension cultures were treated with compound **1j** at 100 and 200 μM, causing growth reductions of 17.2 ± 1.8 and 92.7 ± 1.6%, respectively. Free amino acid content was evaluated 6 days after the treatment by RP-HPLC following derivatization with *o*PDA, as described under Materials and Methods. Pro was measured by the acid ninhydrin method, and P5C was quantified by reaction with *o*-aminobenzaldehyde. Three independent replications were done for each treatment, and means ± SE over replicates are reported.

the bisphosphonate, the synthesis of glutamine is the most affected, with a 67% reduction of the level of this amino acid with respect to controls. This most likely relies on the ability of compound **1j** to inhibit also the activity of plant glutamine synthetase (data not shown), as previously reported for several other bisphosphonates **1**.[21] The inhibition of glutamine synthesis, in turn, affected the level of other amino acids, mainly asparagine (−44%). Proline levels were also reduced, although to a lower extent (−25%). However, such a reduction took place even if the level of the proline direct precursor, glutamate, was unaffected. Therefore, the possibility that the lowered free proline may be an indirect consequence of the inhibition of glutamine synthetase seems unlikely. Moreover, in cells treated with the higher concentration of the bisphosphonate, where the level of most amino acids was strongly reduced, a significant increase of the concentration of

P5C was evident, providing direct proof of the inhibition of the enzyme. To the best of our knowledge, this is the first report showing increased P5C levels as the result of the treatment with compounds able to inhibit P5C reductase. Such compounds might thus represent a useful tool to investigate the highly debated[22,23] molecular mechanisms underlying the noxious effects induced in plants by the exogenous supply of proline.[17,24] Even though the relative weight of the inhibition of P5C reductase versus that of glutamine synthetase in determining the phytotoxicity of phosphonates still awaits to be elucidated, the present data confirm that P5C reductase inhibition occurs inside the plant cell.

The results herein reported represent a further step toward the development of new active principles for weed control targeting P5C reductase. Several new derivatives were found to be active in the micromolar range. Some of them do not contain halogen substituents and should be more easily mineralized by soil microorganisms. The most effective compound was shown to be able to interfere with the synthesis of proline in vivo. However, when several of the same aminomethylenebisphosphonates **1** were recently evaluated as inhibitors of a bacterial P5C reductase, the susceptibility of the enzyme from *Streptococcus pyogenes* was found to be strikingly higher than that of the plant enzyme.[25] In all cases IC_{50} values were 2−3 orders of magnitude lower that those found in the present study (Figure 5). This seems to imply the occurrence of

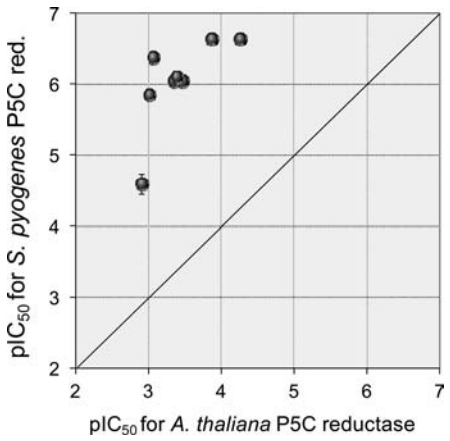

Figure 5. Comparison of the inhibitory potential of aminomethylenebisphosphonic acids **1** against *A. thaliana* and *S. pyogenes* P5C reductases. The inverse of the logarithm of the concentrations causing 50% inhibition of enzyme activity (pIC_{50}) were plotted against each other. A point above the 1:1 line highlights that a given aminomethylenebisphosphonate is more effective against the bacterial enzyme than against plant P5C reductase and vice versa for a point below the line. Data for the inhibition of *S. pyogenes* P5C reductase are quoted from a previous work.[25].

significant differences between the structures of the plant and the bacterial protein. Indeed, because the crystal structure of the plant enzyme is still unavailable, the studied inhibitors had been designed by computer-aided docking procedure[12] performed on the basis of the crystal structure of the enzyme from *S. pyogenes*. A suitable molecular modeling of the interaction between phenyl-substituted bisphosphonates and the active site of P5C reductase from a plant source seems therefore mandatory to improve further their effectiveness. Experiments are currently in progress in our laboratories with this aim.

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Notes

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