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# Herbicidal Pyridyl Derivatives of Aminomethylene-bisphosphonic Acid Inhibit Plant Glutamine Synthetase

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A series of aminomethylene-bisphosphonic acid derivatives, previously synthesized and shown to be endowed with herbicidal properties, were evaluated as potential inhibitors of plant glutamine synthetase. The cytosolic form of the enzyme was partially purified from rice cultured cells and assayed in the presence of millimolar concentrations of the compounds by means of three different assay methods, respectively measuring the hemibiosynthetic, the transferase, and the full biosynthetic reactions. Several compounds were found to exert a remarkable inhibition, with $I_{50}$ values similar to those obtained under the same conditions with a well-established inhibitor of glutamine synthetase, the herbicide phosphinothricin. Contrary to the reference compound, enzyme kinetics accounted for a reversible inhibition mechanism. The biological activity of the most active derivatives was further characterized by measuring free glutamine levels in cell suspension rice cultures following treatment with the inhibitors. Results confirmed their ability to interfere in vivo with nitrogen metabolism. A preliminary analysis of structure−activity relationship allowed it to be hypothesized that steric rather than electronic factors are responsible for the inhibitory potential of these compounds.

**KEYWORDS: Amino acid metabolism; aminomethylene-bisphosphonic acid derivatives; glutamine synthetase; herbicides; nitrogen metabolism; rice (*Oryza sativa* L.)**

## INTRODUCTION

There are several reasons nowadays for developing new weed control systems. Modern pesticides 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 phytochemicals are endowed with low recalcitrancy, thus being rapidly mineralized by the soil microflora.

Aminoalkylphosphonic acids are structural analogues of amino acids in which the carboxylic group is replaced by a phosphonic or related moiety. Increasing evidence has shown that many aminophosphonates are capable of interacting with enzymes involved in amino acid metabolism. Notwithstanding their significant differences including size, shape (flat $CO_2H$ versus tetrahedral $PO_3H_2$), and acidity (p*K* difference of at least 3 units), several enzymes are apparently unable to discriminate between carboxylic and phosphonic function for what concerns binding to active sites. Thus, in several instances the structural antagonism between amino acids or their biosynthetic intermediates and their phosphonic counterparts results in inhibition of enzyme activity (*1*). Although the C−P bond is resistant to chemical degradation (hydrolytic, thermal, or photochemical), most organophosphonates may be considered to be nonpersistent because there are a number of microorganisms possessing suitable pathways for their mineralization (*2*). Due to these reasons, besides their significance in physiological and biochemical studies, several of these compounds have found increasing applications as herbicides (*3*, *4*). The most remarkable examples are provided by the inhibitor of the shikimate pathway enzyme 5-*enol*-pyruvyl-shikimate-3-phosphate synthase (EPSPs, EC 2.5.1.19), the phosphonate herbicide glyphosate [*N*-(phosphonomethyl)glycine; (*5*)], and those of the key enzyme in ammonia assimilation, glutamine synthetase (GS, EC 6.3.1.2), as the naturally occurring glutamate analogue phosphinothricin (L-homoalanine-4-yl-[methyl] phosphinic acid; *6*) or other synthetic phosphonates (*7*, *8*). However, despite the favorable environmental features, their use is limited because of their lack of selectivity (*3*).

To improve the weed management potential of such nonselective herbicides, the synthesis of a great number of analogues of the active molecules and their screening for selective forms have been reported. Hundreds of derivatives have been synthesized and tested for biological activity. Although no selective

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substances sharing the same biochemical targets have been identified to date, such research resulted in the discovery of new classes of active ingredients, among which are aminomethylene-bisphosphonic acids (*1*). Their target at the cellular level is still poorly understood, but recent evidence that accounts for their ability to inhibit farnesyl pyrophosphate synthase (*9*), squalene synthase (*10*), mitochondrial $H^+$-pyrophosphatase (*11*), and geranyl-geranyl diphosphate synthase (*12*) suggests that they should be considered a heterogeneous class of compounds with various modes of action.

We previously evaluated a series of *N*-pyridyl derivatives of aminomethylene-bisphosphonic acid, most of which exerted remarkable phytotoxic effects at both the plant and cell culture levels (*13*, *14*). Because some of them were found to inhibit anthocyanin biosynthesis in vivo, the possible occurrence of target(s) in plant aromatic metabolism was investigated. Although EPSPs was shown to be unaffected (*15*), five of seven compounds reduced the activity of the plastidial and $Mn^{2+}$-stimulated isozyme of the first enzyme in the pre-chorismate pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHPs, EC 4.1.2.15). The addition of excess divalent cations to the assay mixture was found to relieve the effects of three of them, but an inhibition solely based upon metal chelation was ruled out for 5-chloro- and 6-methyl-pyridyl derivatives (*16*). Kinetic analysis of partially purified DAHPs accounted for a noncompetitive inhibition with respect to both substrates (phospho-*enol*-pyruvate and erythrose-4-phosphate), and amino acid pool measurements of cells grown in the presence of sublethal doses of 6-methyl-pyridyl-aminomethylene-bisphosphonic acid pointed to an actual reduction of free phenylalanine and tyrosine, showing that DAHPs inhibition takes place in vivo (*17*). Similar results were obtained also with the 5-chloro derivative. However, an even stronger effect was noticed in this case for other amino acids, mainly for glutamine. When the activity of the enzymes involved in the glutamate cycle was measured in the presence of 5-chloro-pyridyl-aminomethylene-bisphoshonic acid, glutamate synthase was unaffected, whereas glutamine synthetase was significantly inhibited. Kinetic analysis accounted for an inhibition of uncompetitive type with respect to ammonium, glutamate, and ATP. Only the exogenous supply of a mixture of glutamine and aromatic amino acids relieved cell growth inhibition, suggesting that its phytotoxic properties are due to inhibition of key enzymes in both of the corresponding pathways (*18*).

In this view, we undertook a re-evaluation of a series of 16 aminomethylene-bisphosphonic acid derivatives that had previously shown herbicidal properties (*13*, *15*) as possible GS inhibitors. Here we report that some of them indeed exerted a remarkable inhibition of glutamine synthesis in vitro, comparable to that of the reference commercial herbicide phosphinothricin. On the basis of these results, a preliminary analysis of structure−activity relationship allowed us to hypothesize structural requirements either to maintain or to enhance such capability.

## MATERIALS AND METHODS

**Chemicals.** Unless otherwise indicated, chemicals were purchased from Sigma Chemical Co., St Louis, MO, and were of analytical grade; phosphinothricin was obtained from Riedel-de Haën, Seelze, Germany. Derivatives of aminomethylene-bisphosphonic acid were synthesized as previously described (*13*, *15*).

**Plant Cell Cultures.** Suspension-cultured cells of *Oryza sativa* L. Cv. Gigante vercellese were grown in 500-mL Erlenmeyer flasks containing 125 mL of liquid medium consisting of Murashige and Skoog salts and vitamins, supplemented with 30 g $L^{-1}$ sucrose and 2 mg $L^{-1}$ 2,4-dichlorophenoxyacetic acid; 0.2% (v/v) Plant Preservative Mixture solution (Plant Cell Technology, Inc., Washington DC) was routinely added to reduce the risk of microbial contamination. Flasks were incubated under dim light (<50 $\mu$mol $s^{-1}$ $m^{-2}$) on a rotary shaker (100 rpm) at 24 ± 1 °C. Cultures were maintained in continuous balanced growth by subculturing every 7 days 25-mL aliquots to 100 mL of fresh medium.

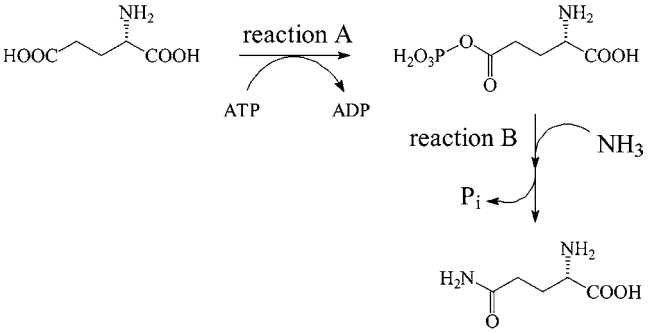



**Figure 1.** Reaction catalyzed by glutamine synthetase proceeds through two steps: after an initial glutamate phosphorylation yielding enzyme-bound $\gamma$-glutamyl-phosphate (reaction A), free ammonia is inserted into the glutamate molecule as amide nitrogen, with the release of inorganic phosphate (reaction B). Three methods were used to assay GS activity: they measure either the initial kinase reaction (hemibiosynthetic assay), a reverse transfer back from glutamine (transferase assay), or the (physiological) full forward reaction from glutamic acid to glutamine (biosynthetic assay).

**Enzyme Purification.** Actively proliferating rice cells were harvested by vacuum filtration, washed with double-distilled water, weighed, resuspended in 2 mL $g^{-1}$ extraction buffer [50 mM Tris-HCl (pH 7.4), containing 0.5 mM dithiothreitol and 5 mM $MgCl_2$], and homogenized for 10 min in a mortar at 4 °C with 1 g (g cells)$^{-1}$ quartz sand; 10 mg $mL^{-1}$ polyvinyl polypyrrolidone was immediately added in order to prevent oxidation of phenolics. All subsequent operations were performed at 0−4 °C. The homogenate was centrifuged at 12000*g* for 15 min, and the resulting supernatant was added with solid ammonium sulfate up to 70% of saturation. Proteins were pelleted by centrifugation at 12000*g* for 15 min, resuspended in extraction buffer, and desalted by passage through a Bio-Gel P6DG column (Bio-Rad). The extract was loaded onto a DEAE-Sephacel column equilibrated with the same buffer. After extensive washing, enzyme activity was eluted with a linear gradient from 0 to 250 mM NaCl. Active fractions were pooled, diluted 1:1 with distilled water, and applied to a Blue-2 agarose column equilibrated with 25 mM imidazole−HCl buffer (pH 7.0) containing 0.5 mM EDTA, 0.25 mM dithiothreitol, and 2.5 mM $MgCl_2$. Nonretained material was harvested, buffer-exchanged against extraction buffer and stored at 4 °C until used.

**Enzyme Assays.** GS activity was followed by means of three different assay methods that measure either the initial glutamate phosphorylation, a reverse transfer back from glutamine, or the (physiological) full forward reaction (**Figure 1**). In the hemibiosynthetic assay the reaction mixture contained 100 mM Tris-HCl buffer (pH 7.4), 20 mM L-glutamic acid, 5 mM $MgCl_2$, 10 mM ATP, 50 mM $NH_2OH$−HCl, and a limiting amount of enzyme (150 pkat) in a final volume of 0.4 mL. After up to 30 min at 35 °C, the reaction was stopped by the addition of 0.8 mL of colorimetric solution [10% (w/v) $FeNO_3 \cdot 9H_2O$, 6.67% (v/v) HCl, and 5% (w/v) trichloroacetic acid]; after centrifugation for 5 min at 18000*g*, samples were read at 535 nm against nonincubated blanks, and the $\gamma$-glutamyl-hydroxamate formed was quantified by comparison with a calibration curve obtained with an authentic standard. With the transferase assay, the reaction mixture contained 25 mM imidazole−HCl buffer (pH 7.5), 50 mM L-glutamine, 40 mM sodium arsenate, 4 mM $MnCl_2$, 5 mM ADP, 25 mM $NH_2OH$−HCl, and a limiting amount of enzyme (300 pkat) in a final volume of 0.4 mL. After up to 15 min at 35 °C, the hydroxamate formed was detected as above. Following purification by anion-exchange and affinity chromatography, GS activity could be measured also by a biosynthetic assay. The mixture contained 50 mM HEPES−NaOH buffer (pH 7.4), 50

mM L-glutamate, 5 mM ATP, 20 mM $MgCl_2$, 1 mM $NH_4Cl$, and a limiting amount of enzyme (50 pkat) in a final volume of 0.1 mL. After up to 10 min at 35 °C, the inorganic phosphate released was quantified by the malachite green assay method as described previously (*19*). Parallel assays in which glutamate had been omitted were performed to rule out aspecific ATP hydrolysis. Protein concentration was determined according to the method of Bradford (*20*), using bovine serum albumin as the standard.

**Free Amino Acid Extraction and Assay.** Cells were vacuum harvested, washed with double-distilled water, weighed, resuspended in 1 mL $g^{-1}$ of a 3% (w/v) 5-sulfosalicylic acid solution, and homogenized in a Teflon-in-glass Potter homogenizer by 2 times 12 strokes. Following centrifugation for 10 min at 18000*g*, 10-$\mu$L aliquots were mixed with the same volume of *o*-phthaldialdehyde solution [0.5 M in 0.5 M sodium borate buffer, pH 10.0, containing 0.5 M $\beta$-mercaptoethanol and 10% (v/v) methanol], and after exactly 60 s, derivatized samples were injected onto a 4.6 × 250 mm Zorbax ODS column (Agilent Technologies, Palo Alto, CA) equilibrated with 59% solvent A [50 mM sodium phosphate and 50 mM sodium acetate buffer, pH 7.5, containing 2% (v/v) of both methanol and tetrahydrofuran] and 41% solvent B (65% methanol). Isocratic elution proceeded for 12 min at a flow rate of 1 mL $min^{-1}$ while the eluate was monitored at 340 nm, and then the column was washed for 6 min with 100% solvent B. Peaks were integrated by area using a Data System 450 (Kontron, Munchen, Germany), with variation coefficients ranging from 1.2 to 2.3%. This procedure allowed resolution of Asp, Glu, Asn, Ser, and Gln, with a detection limit of ~0.1 nmol. Total amino acid content was quantified according to the ninhydrin method.

**Molecular Modeling.** CoMFA 3D-QSAR analysis was performed within the QSAR module of Sybyl 6.9.1, Tripos (Discovery Software, 2003), using default settings. Structures of bisphosphonates were built into the Sybyl program and minimized using Tripos force field and conjugate gradient minimizer up to a minimum energy change of 0.001 kcal/mol. The charge for each atom of minimized structure was computed using the Gasteiger method. Minimized structures were superimposed on the structure of the most active compound (**BP14**). The atomic coordinates of those models were used to obtain field values on a rectangular grid around the aligned molecules.

## RESULTS AND DISCUSSION

**Partial Purification of Glutamine Synthetase from Rice Cultured Cells.** To verify the possibility that, besides the *N*-2-(5-chloro-pyridyl) derivative (**BP02**, *18*), other compounds related to aminomethylene-bisphosphonic acid (**BP01**) may inhibit the synthesis of glutamine, GS was isolated from suspension-cultured cells of rice harvested in the middle exponential growth phase. The cell line employed was selected as the source of the enzyme because it showed the highest specific activity levels among a few tested cultures of different plant species (data not shown). A modification of a protocol previously used to purify the cytosolic isoform of GS from tobacco cultured cells (*19*) was set up to associate resolution from interfering enzymes to a high yield, in a faster procedure. Crude extracts are actually unsuitable to properly assess the occurrence of GS inhibition, because a high rate of ATP hydrolysis by unrelated activities rapidly led to substrate depletion, thus resulting in nonlinear kinetics (not shown). After an initial enrichment, the use of a negative chromatography on a Blue-2 agarose column allowed us to resolve GS from most interferences: glutamate-independent phosphate release from ATP in the final enzyme preparation accounted for <5% of total activity, whereas in the DEAE-Sephacel pool it ranged from 55 to 65% (data not shown). Maximal specific activity corresponded to a 24-fold purification, with a yield of ~60% (**Table 1**). No data supporting the occurrence of multiple enzyme forms were found: only a single, symmetric peak was evident during anion-exchange chromatography. Because it eluted at the same ionic strength as a minor peak from rice leaves (results not shown), the isolated GS is most likely the cytosol-localized, type-I isoform, which seems to preferentially assimilate ammonia from primary nitrate reduction in undifferentiated cells (*19*, *21*).

**Table 1.** Partial Purification of Glutamine Synthetase from Cell Suspension Cultures of *O. sativa* L. Cv. Gigante Vercellese[a]

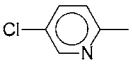
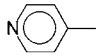
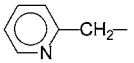
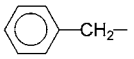
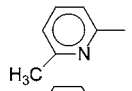
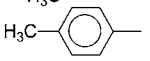
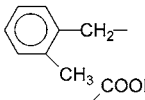
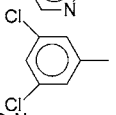
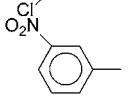
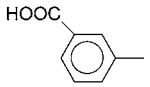
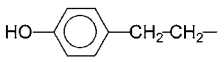
| step | protein (mg) | activity (nkat) | specific activity (nkat $mg^{-1}$) | purification (-fold) | yield (%) |
|---|---|---|---|---|---|
| crude extract | 379.0 | 84.1 | 0.222 | 1 | 100 |
| 0–70% ammonium sulfate | 320.5 | 79.2 | 0.247 | 1.1 | 94.1 |
| DEAE-Sephacel | 26.3 | 53.3 | 2.03 | 9.1 | 63.4 |
| Blue-2 agarose | 9.35 | 49.2 | 5.26 | 23.7 | 58.5 |

[a] Results presented are for a typical purification starting from 50 g (fresh weight) of cultured rice cells. Activity was evaluated by the hemibiosynthetic assay.

Despite the numerous papers describing the features of GSs from different plant species, a comparison of enzyme properties is often made difficult by the adoption of different assay protocols, most of which should be considered as not properly representative of physiological conditions. Although they show clear practical advantages, such as insensitivity to contaminating activities in crude extracts, or higher rates, the widely used hydroxylamine-based hemibiosynthetic and transferase methods could in fact lead to estimate artificial parameters. This is even more true when a possible inhibitory effect is assessed: if only part of the overall reaction catalyzed by the enzyme is measured, the effect may be under- or overestimated. Actually, phosphinothricin is scarcely effective against the reverse reaction, although it is a powerful inhibitor of the initial glutamate phosphorylation (*6*). The availability of properly enriched preparations allowed us to also use a biosynthetic assay that measures the true, full catalytic reaction. Under these experimental conditions, maximal specific activity obtained corresponded to 30.2 nkat $mg^{-1}$ [against 5.26 or 300.4 nkat $mg^{-1}$ when the enzyme was assayed by the hemibiosynthetic or transferase method, respectively, with ratios similar to those previously obtained with tobacco GS (*19*)].

**GS Inhibition by Aminomethylene-bisphosphonic Acid Derivatives.** The activity of the partially purified enzyme was thus measured with either of the three assay methods in the presence of millimolar concentrations of the compounds. Results are summarized in **Table 2**. In addition to the previously characterized chloro-pyridyl derivative **BP02** (*18*), 9 aminomethylene-bisphosphonic acids of 15 were found to inhibit the biosynthetic reaction. On the whole, relatively consistent effects were found on the hemibiosynthetic activity, whereas the transferase assay yielded a quite different pattern. This could suggest that, as for the reference herbicide phosphinothricin, the interference with the enzymatic mechanism is brought about at the level of the initial kinase reaction. However, in the case of compounds **BP07**, **BP10**, and **BP12** the transferase reaction was inhibited to a higher extent. Because GS requires divalent cations for catalytic activity (*22*), and several bisphosphonic acids show remarkable chelating properties (*14*, *16*), this inconsistency might rely upon ion sequestration. To verify such a hypothesis, the well-known chelating compound ethylenediaminetetraacetic acid (EDTA) was included in the experimental design. Under the experimental conditions employed, 1 mM EDTA was indeed able to strongly inhibit transferase activity, whereas the biosynthetic and the hemibiosynthetic reactions were slightly stimulated and completely unaffected, respectively

**Table 2.** Inhibitory Effects of Aminomethylene-bisphosphonic Acid Derivatives at 1 mM on the Activity in Vitro of Rice Glutamine Synthetase[a]

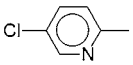
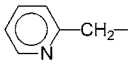
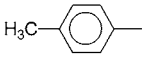
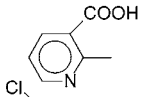
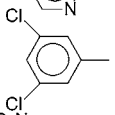
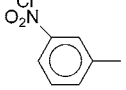
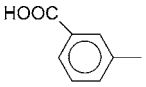
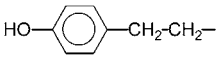
| Compound | R | GS assay method | | |
|---|---|---|---|---|
| | | transferase | hemibiosynthetic | synthetic |
| EDTA | | 35.1 ± 2.4 | 95.0 ± 1.3 | 125.0 ± 2.3 |
| Phosphinothricin | | 25.7 ± 1.6 | 9.9 ± 2.5 | 2.0 ± 0.5 |
| **BP01** | H— | 96.4 ± 2.1 | 81.2 ± 0.7 | 118.9 ± 2.3 |
| **BP02** |  | 52.8 ± 0.8 | 17.5 ± 1.9 | 13.9 ± 3.4 |
| **BP03** |  | 60.1 ± 0.8 | 30.2 ± 1.0 | 37.6 ± 2.0 |
| **BP04** |  | 84.9 ± 0.5 | 97.2 ± 1.3 | 118.8 ± 1.4 |
| **BP05** |  | 76.8 ± 0.5 | 64.3 ± 5.4 | 42.3 ± 1.2 |
| **BP06** |  | 54.6 ± 0.3 | 16.1 ± 3.8 | 8.8 ± 0.9 |
| **BP07** |  | 44.3 ± 0.8 | 92.0 ± 4.1 | 107.5 ± 4.9 |
| **BP08** |  | 102.3 ± 1.6 | 89.1 ± 0.7 | 109.1 ± 5.3 |
| **BP09** |  | 46.1 ± 0.3 | 32.7 ± 2.5 | 50.5 ± 0.8 |
| **BP10** |  | 25.9 ± 0.3 | 74.4 ± 2.5 | 113.9 ± 3.2 |
| **BP11** |  | 93.8 ± 0.1 | 57.6 ± 0.3 | 14.3 ± 1.1 |
| **BP12** |  | 50.8 ± 1.6 | 70.5 ± 0.7 | 100.6 ± 4.2 |
| **BP13** |  | 61.0 ± 0.5 | 40.8 ± 0.6 | 27.2 ± 1.4 |
| **BP14** |  | 38.1 ± 0.8 | 13.1 ± 0.6 | 3.1 ± 2.1 |
| **BP15** |  | 95.6 ± 2.1 | 100.8 ± 1.3 | 97.6 ± 3.1 |
| **BP16** |  | 104.4 ± 1.6 | 99.0 ± 1.3 | 62.5 ± 0.7 |
| **BP17** |  | 98.4 ± 2.5 | 36.9 ± 0.7 | 72.6 ± 1.1 |

[a] Activity was evaluated with three different assay methods, as detailed under Materials and Methods. Values are expressed as percent of the rate measured in untreated controls and are means ± SD over at least three replications.

(**Table 2**). The effects were completely relieved at concentrations lower than 0.5 mM (not shown).

To definitely exclude an inhibition solely on the basis of cation sequestration and to obtain more reliable quantitative data for structure−activity relationship analysis, GS was assayed in the presence of either the nine derivatives that significantly reduced the physiological reaction, in the range from 0.005 to 2 mM. Although some of them were able to interfere with glutamine synthesis only at millimolar concentrations, compounds **BP03**, **BP11,** and **BP13**, and mainly **BP02**, **BP06**, and **BP14**, were found to inhibit rice GS in the micromolar range, with the latter three bisphosphonates showing quantitative effects similar to those exerted by phosphinothricin. Moreover, in the case of EDTA no inhibition occurred until the concentration of the compound was lower than a threshold, which was proportional to the amount of divalent cations in the assay. Above such a threshold, a complete suppression of enzyme activity was found. On the contrary, active compounds progressively inhibited GS, at levels well below that of divalent cations (not shown). The concentrations causing 50% inhibition ($I_{50}$) of in vitro activity are outlined in **Table 3**. Once again, the hemibiosynthetic and biosynthetic assays gave generally consistent

**Table 3.** Estimated $I_{50}$ Values (Millimolar) for Rice Glutamine Synthetase Inhibition by Aminomethylene-bisphosphonic Acid Derivatives[a]

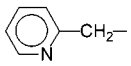
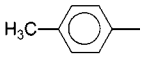
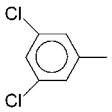
| Compound | R–NH–C(PO$_3$H$_2$)(PO$_3$H$_2$)–H: R | GS assay method | | |
|---|---|---|---|---|
| | | transferase | Hemibiosynthetic | synthetic |
| Phosphinothricin | | 0.172 ± 0.014 | 0.049 ± 0.003 | 0.044 ± 0.004 |
| **BP02** |  | 1.04 ± 0.04 | 0.096 ± 0.012 | 0.069 ± 0.010 |
| **BP03** |  | 1.04 ± 0.12 | 0.215 ± 0.051 | 0.243 ± 0.033 |
| **BP06** |  | 1.13 ± 0.02 | 0.103 ± 0.011 | 0.064 ± 0.003 |
| **BP09** |  | 0.915 ± 0.047 | 0.461 ± 0.062 | 1.11 ± 0.14 |
| **BP11** |  | nd | 0.774 ± 0.136 | 0.170 ± 0.011 |
| **BP13** |  | 1.45 ± 0.04 | 0.624 ± 0.147 | 0.334 ± 0.020 |
| **BP14** |  | 0.396 ± 0.026 | 0.088 ± 0.015 | 0.033 ± 0.001 |
| **BP15** |  | 1.99 ± 0.22 | 1.62 ± 0.59 | 0.738 ± 0.051 |
| **BP16** |  | nd | nd | 1.80 ± 0.02 |
| **BP17** |  | nd | 0.695 ± 0.193 | 4.47 ± 0.27 |

[a] Activity was measured as described under Materials and Methods either in the absence or in the presence of pyridyl-amino-methylene-bisphosphonic acid derivatives at concentrations ranging from 0.005 to 2 mM. Each sample was carried out in triplicate, and values were expressed as percentage of untreated controls. The concentrations causing 50% inhibition ($I_{50}$) 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*). nd, uneffective within the range tested.

results, whereas the reverse reaction was substantially unaffected. Only in the case of phosphinothricin and compound **BP14** was the transferase activity reduced at doses at which the inhibition could not depend on metal chelation. Phosphinothricin is believed to be unable to interfere with the reverse transfer. However, herbicide phosphorylation by GS leads to the formation of an intermediate that binds irreversibly to the enzyme (*6*): this is progressively inactivated, thus resulting in nonlinear enzyme kinetics. To evaluate whether a similar mechanism could occur also for compound **BP14**, the activity of the enzyme was followed up to 50 min in the absence or presence of the two inhibitors. Results (**Figure 2**) showed that, contrary to phosphinothricin, the catalytic rates was linear with time also in the presence of inhibitory concentrations of the bisphosphonate, thus supporting a reversible mechanism of action. This is consistent with previous data obtained with compound **BP02** (*18*). Similar results were obtained with the hemibiosynthetic assay method (not shown).

**In Vivo Inhibition of Glutamine Synthesis.** To investigate whether the interference of the most active derivatives with GS activity may take place also in vivo, and thus cause an actual reduction of glutamine biosynthesis that could result in phytotoxic effects at the plant level, amino acid pools were quantified in rice cells following supplementation of the culture medium with the compounds. The ability to interfere with ammonia assimilation in vivo was previously shown for compound **BP02** (*18*). Results obtained 48 h after the addition to rice cultures of either the phosphonates or the reference herbicide are summarized in **Table 4**. As expected, in phosphinothricin-treated cells glutamine and asparagine content lowered significantly and those of the corresponding acids consequently increased. Even to a lesser extent, a significant reduction of free glutamine was indeed evident also in cultures treated with the same concentration of **BP06**, **BP11**, and **BP14**. Their lower effectiveness in comparison to phosphinothricin most likely relies upon the different inhibition mechanism, because progressive and irreversible GS inactivation by the latter is susceptible to amplify its effect in vivo. On the contrary, when cells were fed with compound **BP04**, which had been previously shown to exert remarkable phytotoxic effects (*15*) but was substantially uneffective against rice GS in vitro (**Table 2**), no lowering in free glutamine content was evident. A general increase of the

**Table 4.** Free Amino Acid Content in Suspension-Cultured Cells of Rice Treated with GS-Inhibiting Aminomethylene-bisphosphonic Acid Derivatives[a]

| Compound | R | Content (μmol [g fresh weight]$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Asp | Glu | Asn | Ser | **Gln** | other a.acids | total a.acids |
| Untreated control | | 1.10 ± 0.26 | 3.18 ± 0.25 | 0.75 ± 0.07 | 0.60 ± 0.09 | **4.24 ± 0.35** | 14.77 ± 0.53 | 24.65 ± 0.97 |
| Phosphinothricin | | 1.80 ± 0.24 | 3.86 ± 0.07 | 0.25 ± 0.03 | 1.38 ± 0.16 | **2.05 ± 0.06** | 18.00 ± 0.20 | 27.34 ± 0.28 |
| **BP04** |  | 1.21 ± 0.08 | 4.24 ± 0.17 | 1.14 ± 0.09 | 2.22 ± 0.21 | **4.79 ± 0.30** | 19.05 ± 0.71 | 32.64 ± 0.70 |
| **BP06** |  | 1.66 ± 0.12 | 4.78 ± 0.58 | 0.63 ± 0.09 | 1.53 ± 0.15 | **3.25 ± 0.57** | 15.09 ± 1.77 | 26.94 ± 3.11 |
| **BP11** |  | 0.76 ± 0.05 | 2.97 ± 0.18 | 0.61 ± 0.05 | 0.60 ± 0.06 | **2.95 ± 0.25** | 15.02 ± 0.79 | 22.91 ± 1.27 |
| **BP14** |  | 0.57 ± 0.05 | 3.19 ± 0.19 | 0.49 ± 0.04 | 1.26 ± 0.14 | **2.72 ± 0.22** | 20.38 ± 0.88 | 28.61 ± 1.25 |

[a] Actively proliferating rice cultures were treated with the compounds (0.5 mM each); 48 h after the addition, cells were harvested and extracted; as detailed under Materials and Methods, amino acids were quantified by RP-HPLC following derivatization with *o*-phthaldialdehyde. Phosphinothricin was used as a reference compound, whereas BP04, which exerts an inhibitory effect on cell growth but was found to be completely uneffective in vitro against GS, was used as a negative control. Values are means ± SD over three replications (six for the control).

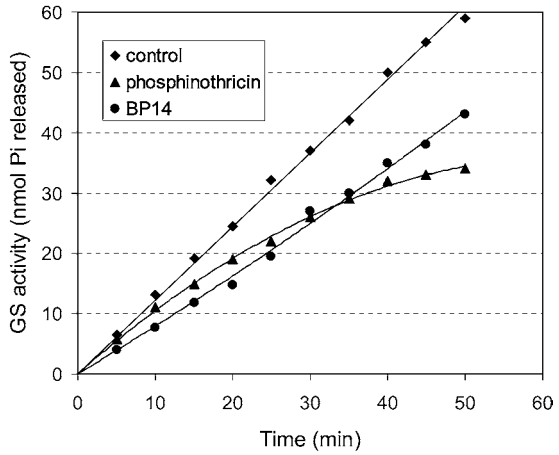



**Figure 2.** Kinetics of in vitro biosynthetic activity of rice glutamine synthetase in the absence (◆) or in the presence of 10 μM phosphinotricin (▲) or 20 μM BP14 (●). Aliquots corresponding to 20 pkat of the partially purified enzyme were incubated at 35 °C, and at increasing time the inorganic phosphate released was quantified by the malachite green-acid molybdate method. Means over at least three replications are reported.

intracellular pool of most amino acids is possibly related to the inhibitory effect upon cell growth, as previously shown, for instance, in cultured cells treated with the herbicide glyphosate (*18*).

**Structure−Activity Relationships.** Data herein reported represent the output of an initial stage of the research, in which the work was mainly focused to analyze the scaffold effect, i.e., to ascertain whether *N*-pyridyl-aminomethylene-bisphosphonic acids could indeed be used as a lead for the development of new active principles targeting GS activity. In this perspective, all available derivatives coming from previous studies (*13*, *15*) were examined, irrespective of a rational strategy for a quantitative structure−activity relationship analysis. However, results suggest some steric and electronic properties of these compounds as possible determinants of their biological activity. The structures of the bisphosphonates tested do not resemble those of other already known inhibitors of glutamine synthetase, which are simple analogues of glutamic acid and bind into glutamate binding sites (*23*, *24*). We have recently proposed that bisphosphonates may be bound into a cleft near the phosphate group of ATP (*25*). According to our best knowledge, this cleft has no physiological importance, and therefore it is very difficult to draw meaningful structure−activity relationship for these compounds. Aminomethylene-bisphosphonates studied in this work belong to two distinct classes: those containing phenyl as *N*-substituent and those containing a pyridyl moiety. There is no predominance of one class over the other when GS inhibitory activity is considered, and the analysis of the influence of substituents showed that there is no significant effect of electron-donating versus electron-attracting substituents, neither in the phenyl nor in the pyridyl ring.

To clarify the structure−activity relationship, molecular modeling studies were performed. Although the most reliable method would be docking of each compound into the active site of glutamine synthetase and analysis of interactions between the inhibitor and the enzyme, the three-dimensional structure of any plant GS has not yet been published. The crystal structures are available only for the bacterial enzyme, which shows too little homology with eukaryotic GS to be used for computer-aided interpretation of kinetic data obtained with the enzyme isolated from a plant source. Thus, 3D-QSAR/CoMFA methodology (Discovery Software, Tripos 2003) was chosen as the best method to identify structural features required for inhibitory activity. Minimized structures of bisphosphonates were superimposed on structure of the most potent compound, **BP14**. On this set of aligned structures CoMFA 3D-QSAR calculations were performed, and the results are summarized in **Figure 3**. Although such a small data set does not allow us to obtain an exhaustively predictive model, some interesting

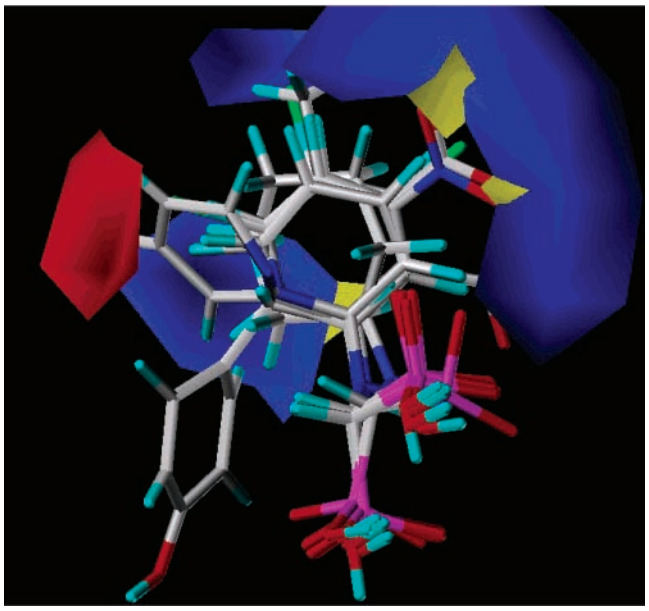


**Figure 3.** Aligned structures of bisphosphonates and isosurfaces generated by CoMFA 3D-QSAR analysis. Isosurfaces indicate regions of 80 or 20 percentiles of StdDev*Coef values.

conclusions can be made. Marked contours represent regions where change of electrostatic or steric features of molecule correlate with change of its activity. Blue regions indicate the places where electronegative groups are unfavorable, whereas red ones the areas where electronegative groups are preferred. Similarly, sterically favored regions are highlighted in green, and disfavored ones in yellow. Results show that electrostatic interactions are the main factors that discriminate activity. Charge distribution is relevant when both the aromatic ring and its substituents are considered. Negative charge at the aromatic ring and the presence of strongly electronegative substituents such as carboxylate groups are not preferred. It is obvious that steric features of inhibitors are also very important, because of the small size of the active site of the enzyme, but analysis of this set of structures yielded only limited information. Two small regions marked by yellow color indicate that large substituents placed in the aromatic ring are not well accepted. The only two inhibitors that significantly differ from all others in their steric properties are compounds **BP17** (moderate inhibitor) and **BP06** (strong inhibitor). The two compounds are probably differently placed in GS binding sites, with the pyridyl ring of **BP06** most likely located at the entrance of a big cleft designed to bind ATP, which explains its exceptionally strong inhibitory activity. In this view, a planned synthesis of other derivatives, their screening in vitro for inhibitory properties against plant GS, and their evaluation for herbicidal activity *in planta* are currently in progress.

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