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Phosphinothricin Analogues as Inhibitors of Plant Glutamine Synthetases

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A series of phosphinothricin derivatives with a modified methyl group, designed on the basis of the crystal structure of the complex formed by the inhibitor and the target enzyme from *Salmonella typhimurium*, were evaluated as potential inhibitors of plant glutamine synthetase. These compounds were previously shown to be equipotent or slightly weaker inhibitors to the lead compound against the bacterial enzyme. Because of the presence in higher plants of at least two enzyme forms with different subcellular localization and possible separate metabolic functions, plastidial and cytosolic glutamine synthetases were purified to electrophoretic homogeneity from spinach chloroplasts and cultured tobacco cells, respectively. Kinetic analysis confirmed the ability of the phosphinothricin analogues to inhibit both isoenzymes in the micromolar range, with a mechanism of a competitive type with respect to glutamate. Interestingly, some of them exerted a differential effect against either the two plant isoforms, or against the plant versus the bacterial enzyme.

KEYWORDS: Phosphinothricin derivatives; glutamine synthetase; cytosolic and plastidial isoforms; nitrogen metabolism; herbicides

INTRODUCTION

Intensive efforts have been undertaken in the past decades to discover new compounds with favorable environmental and safety features to selectively control weeds. Recently, this aim has been pursued with new strategies, switching from the testing of chemicals for efficacy on whole plants toward either the use of in vitro assays against a given molecular target (e.g., *1*−*2*), or a proteomic/metabolomic approach to rapidly identify the unknown mode of action of a given compound endowed with biological activity (*3*). Moreover, to cope with increasing public concern over environmental fate and possible recalcitrance of herbicides, a particular emphasis has been placed on the screening of natural products (*4*) that are supposed to be easily degraded by soilborne microorganisms. Among these substances, the most remarkable example to date is provided by the naturally occurring glutamate analogue phosphinothricin (PPT, L-homoalanine-4-yl-[methyl] phosphinic acid).

Synthesized in some streptomycetes as a component of the nonribosomal tripeptide bialaphos (phosphinothricyl-alanyl-alanine) (*5*), from which it is released in target species by intracellular peptidases, PPT acts by inhibiting the key enzyme in ammonia assimilation, glutamine synthetase (GS, EC 6.3.1.2). This rapidly leads to ammonia accumulation and glutamine starvation, which act in concert to cause plant death (*6*). PPT belongs to the family of aminoalkylphosphonic acids, structural analogues of amino acids in which the carboxylic group is replaced by a phosphonic or related moiety. This group resembles the tetrahedral transition state of several enzymatic reactions, particularly amide bond formation and hydrolysis. Notwithstanding their significant differences including size, shape (flat CO_2H versus tetrahedral PO_3H_2), and acidity (pK difference of at least 3 units), several enzymes are thus apparently unable to discriminate between carboxylic and phosphonic function for what concerns binding to active sites. In several instances, the structural antagonism between amino acids or their biosynthetic intermediates and their phosphonic counterparts results in inhibition of enzyme activity (*7*).

After exerting its herbicidal action, PPT is efficiently metabolized by the soil microflora. In a field study in California, in which it was applied three times at rates of 1.7 kg ai ha^{-1}, the herbicide dissipated rapidly, with calculated half-lives of 15, 7.2, and 2.7 days after the first, second, and third applications, respectively (*8*). However, despite the favorable environmental features, its use is limited because of its lack of selectivity (*9*). Production of herbicide-resistant transgenic cultivars has been obtained through the introduction of a PPT acetyl transferase (*bar*) gene, a technology that is being applied to an increasing number of crops (e.g., *10*). In any case, at least in Europe, public disfavor toward the use of transgenic crops is restraining these results. Moreover, because most bacteria also need a functional GS to incorporate ammonia into organic compounds, the use of PPT-based herbicide may cause nontarget effects in the field, perturbing natural equilibria among soil microorganisms (*11*). In the case of transgenic plants owing

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tolerance to the insertion of the *bar* gene, despite full resistance to the herbicide, adverse effects might thus derive from the consequent inhibition of beneficial rhizobacterial strains, facilitating pathogen attack (*12*). Until now, neither of these aspects have been properly investigated, nor have selective inhibitors been described that exert differential effects on GS from bacteria and plants.

To improve the weed management potential of such a nonselective herbicide, the synthesis of a great number of analogues of the active molecules and their screening for selective forms have been reported (e.g., *13*). Hundreds of derivatives have been synthesized and tested for biological activity. Although no selective 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 a series of *N*-pyridyl derivatives of aminomethylene-bisphosphonic acid (*7*). Some of them were in fact found to inhibit also GS, although with lower efficiency (*14*, *15*). On the basis of these results, a preliminary analysis of the structure−activity relationship allowed hypothesizing of structural requirements either to maintain or to enhance such capability (*15*, *16*).

The failure in identifying new compounds sharing the same mode of action might in fact be overcome by a structure−activity relationship analysis that allowed a rational molecular design of new GS-inhibiting herbicides by structure modeling. However, up to now, such an approach has been impaired in part because a sufficiently high-resolution X-ray structure of plant GS is not available. The crystal structure of the complex formed by the inhibitor and GS purified from *Salmonella typhimurium*, which has been described recently (*17*), may serve as a model, but no evidence is available to rule out the occurrence of significant variations between the structure of the bacterial and the plant enzyme, which may either lead to the design of compounds that are not active against the plant protein or, on the contrary, be used for the synthesis of compounds able to discriminate between prokaryotic and eukaryotic GSs. Moreover, a further difficulty may derive from the presence in plants of two different enzyme forms with different functional localization and, possibly, separate metabolic functions. Chloroplastic and cytosolic forms (also referred to as GS-2 and GS-1) have been in fact hypothesized to be specifically involved in the light-induced reassimilation of photorespiratory ammonia and the generation of glutamine for intercellular nitrogen transport, respectively (*18*). The existence of structural differences among plant isoforms has not been fully elucidated as well.

We previously described the computer-aided design and synthesis of novel PPT derivatives based on the structure of *S. typhimurium* GS. Kinetic studies with the *Escherichia coli* enzyme showed their effectiveness in inhibiting glutamine production in bacteria (*19*). Here, we report on the ability of these compounds to interfere with the catalytic mechanism of plant GS isoforms.

MATERIALS AND METHODS

Chemicals. Unless otherwise indicated, chemicals were purchased from Sigma Chemicals, St Louis, MO, and were of analytical grade; DL-phosphinothricin was obtained from Riedel-de Haën, Seelze, Germany. PPT derivatives were designed and synthesized as previously described (*19*).

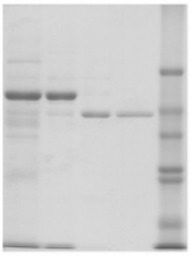
GS Purification. *1. Chloroplastic Enzyme Form.* Intact chloroplasts were isolated from market spinach (*Spinacea oleracea* L.) leaves. Deveined plant material was resuspended in 5 mL g^{-1} of ice-cold 20 mM *N*-tris(hydroxymethyl)methylglycine−NaOH buffer (pH 8.0) containing 10 mM NaCl, 5 mM $MgCl_2$, and 0.4 M sucrose and homogenized for 30 s in a blender at maximal speed. The homogenate was filtered through surgical gauze, and the filtrate was centrifuged at 4 °C for 1 min at 500*g*; the supernatant was further centrifuged for 10 min at 1500*g*. Pelleted chloroplasts were osmotically swollen by resuspension in extraction buffer [50 mM Tris-HCl (pH 7.4), containing 0.5 mM dithiothreitol (DTT) and 5 mM $MgCl_2$]. Following equilibration on ice for 10 min, samples were centrifuged at 12 000*g* for 15 min, and the resulting supernatant was diluted 1:1 with distilled water and loaded at a constant flow of 1 mL min^{-1} onto a 25-mL DEAE-Sephacel (Pharmacia) column equilibrated with extraction buffer. After extensive washing, proteins were eluted at the same rate with a linear gradient from 0 to 400 mM NaCl (400 mL) while collecting 5-mL fractions. Active fractions were pooled and directly applied to a 20-mL hydroxyapatite (Bio-Rad) column, equilibrated with 5 mM K phosphate buffer (pH 7.4) containing 0.5 mM DTT and 5 mM $MgCl_2$. Proteins were eluted at a rate of 40 mL h^{-1} by a linear gradient from 5 to 200 mM phosphate (200 mL) while collecting 4-mL fractions. Active fractions were pooled, and solid ammonium sulfate was added to 70% saturation. Precipitated proteins were pelleted by centrifugation for 10 min at 18 000*g*, resuspended in extraction buffer, and column-desalted by passage through a BioGel P6DG (Bio-Rad) column. The desalted sample (40 mL) was injected by a 10-mL superloop (Pharmacia) onto a Mono-Q 5.5 (Pharmacia) FPLC column equilibrated with extraction buffer. Proteins were eluted at a flow rate of 1.0 mL min^{-1} by using a computer-controlled (Kontron 450) linear gradient from 0 to 500 mM NaCl (100 mL), and 1-mL fractions were collected. Enzyme preparations were column-desalted as above and stored at 4 °C until used for biochemical characterization. Under these conditions, GS activity was found to be stable for at least 3 weeks.

2. Cytosolic Enzyme Form. Suspension cultured cells of *Nicotiana plumbaginifolia* Viviani were grown in Erlenmeyer flasks in a liquid medium consisting of Murashige and Skoog salts and vitamins, supplemented with 30 g L^{-1} of sucrose and 0.5 mg L^{-1} of both 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. Flasks were incubated under dim light (<50 μmol s^{-1} m^{-2}) on a rotary shaker (100 rpm) at 24 ± 1 °C. Cultures were maintained in continuous balanced growth by subculturing every 10 days, 25-mL aliquots to 100 mL of fresh medium. GS was extracted and purified to electrophoretic homogeneity by sequential anion exchange, adsorption, and gel permeation chromatography, followed by a negative chromatography on a Blue-2 agarose column, as previously described (*18*).

Enzyme Assays. GS activity can be quantified by means of three different assay methods, which measure either the initial glutamate phosphorylation (hemibiosynthetic assay), a reverse transfer back from glutamine (transferase assay), or the physiological full-forward reaction (biosynthetic assay (*15*)). During enzyme purification, activity was measured by 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 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) $FeNO_3 \times 9H_2O$, 6.67% (v/v) HCl and 5% (w/v) trichloroacetic acid]; following centrifugation for 5 min at 18 000*g*, samples were read at 535 nm against nonincubated blanks, and the γ-glutamyl-hydroxamate formed was quantified by comparison with a calibration curve obtained with an authentic standard. The purified enzymes could be assayed by the biosynthetic assay, but substrate concentrations have to be modified to ensure neither limiting nor inhibiting conditions. For the chloroplastic isoform, the mixture contained 50 mM HEPES−NaOH buffer (pH 7.4), 100 mM L-glutamate, 2.5 mM ATP, 50 mM $MgCl_2$, 0.5 mM NH_4Cl, and a limiting amount of enzyme (50 pkat) in a final volume of 0.1 mL. After up to 12 min at 35 °C, the inorganic phosphate released was quantified by the malachite green assay method as described previously (*15*). In the case of the cytosolic isoform, the mixture contained 50 mM HEPES−NaOH buffer (pH 7.4), 50 mM L-glutamate, 2.5 mM ATP, 5 mM $MgCl_2$, and 0.5 mM NH_4Cl. Protein concentration was determined by the method of Bradford (*20*), using bovine serum albumin as the standard.

Electrophoresis. Discontinuous SDS-polyacrylamide gel electrophoresis was performed at 20 °C by the method of Laemmli with a

Table 1. Purification of Glutamine Synthetase from Spinach (*Spinacia oleracea* L.) Leaf Chloroplasts[a]

Step	Protein (mg)	Activity (nkat)	Specific activity (nkat mg^{-1})	Purification (fold)	Yield (%)	SDS-PAGE [b] 1 2 3 4 5
1. Chloroplast lysate	187.9	5842	31.1	1	100	
2. DEAE-Sephacel	41.5	4227	101.8	3.3	68.3	
3. Hydroxyapatite	1.17	2033	1738	55.9	34.8	
4. Mono-Q	0.63	1583	2513	80.8	27.1	

[a] Results presented are for a typical purification starting from 600 g (fresh weight) of deveined spinach leaves. Activity was evaluated by the transferase assay. [b] Aliquots of active pools from each step were analyzed on a 10% polyacrylamide gel under denaturing conditions. Molecular markers run in lane 5 (Sigma Dalton Mark VII-L) were: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-P dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20 kDa).

4% stacking and a 10% separating gel, using a minigel system (Bio-Rad). Samples were denatured by boiling for 5 min in 62 mM Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol. Proteins were visualized after staining with 0.1% Coomassie brilliant blue R-250. Molecular weight markers run on the same gels (Sigma SDS-7) are detailed in **Table 1**.

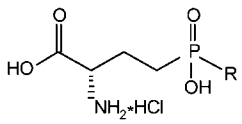
Enzyme Inhibition and Kinetic Analysis. GS inhibition was evaluated by adding to the reaction mixture 10 μL of an appropriate dilution of a 20 mM water solution (pH 7.4) of a given inhibitor so as to obtain the following doses: 500, 200, 100, 50, 20, 10, 5, 2, and 1 μM. At least four measurements were performed for each dose. The concentrations causing 50% inhibition (I_{50}) of GS activity were estimated by utilizing the linear regression equation of enzyme activity values, expressed as a percentage of untreated controls, plotted against the logarithm of inhibitor concentration. Confidence limits of I_{50} values were computed according to ref (*21*). For kinetic evaluations, the enzyme was assayed in the presence of increasing concentrations of the inhibitors by varying those of the substrates. Unvariable substrates were fixed at the same levels as in standard assays. Concentration for the variable substrate ranged from 20 to 100 mM glutamate and from 0.2 to 1 mM ATP in the case of the chloroplastic isozyme, and from 5 to 50 mM glutamate and from 0.4 to 2.5 mM for ATP in the case of the cytosolic form. At least six doses were evaluated for each substrate, at no less than triplicate. In the case of glutamate (competitive inhibition), K_i values were estimated from Lineweaver−Burk plots of activity; at least four inhibitor concentrations, ranging from 0.2- to 2-fold of the corresponding I_{50} value, were tested. In the case of ATP (non competitive inhibition), K_i values were estimated from Dixon plots of activity by evaluating the effect of at least six inhibitor concentrations, ranging from 0.2- to 1.5-fold of the corresponding I_{50} value in the presence of at least four substrate levels. Reported values are means ± SEM over results obtained with different inhibitor or substrate concentrations, respectively.

RESULTS AND DISCUSSION

Purification of Glutamine Synthetase from Spinach Chloroplasts. To obtain reliable results, inhibition studies should be performed with homogeneous enzyme preparations. The presence of contaminant activities able to make use of the same substrate(s) or further metabolize the product(s) may in fact lead to experimental artifacts, or at least interfere heavily with enzyme assay. Moreover, in the case of glutamine synthetase, the residual presence of aspecific phosphatases makes the biosynthetic assay method unfeasible (*15*). Other protocols are available to measure GS activity, which are less sensitive to the presence of contaminant proteins and may be used on impure preparations. However, they measure partial or reverse, thus nonphysiological reactions (*18*), and compounds able to interfere with enzyme activity under such assay conditions might therefore be scarcely effective on the whole forward reaction or vice-versa (*15*). To avoid such drawbacks, a plastidial form of GS was purified to electrophoretic homogeneity from spinach chloroplasts. After an initial enrichment, the use of absorption chromatography on a hydroxyapatite column was found to be a high-resolution step that allowed both to resolve GS from Rubisco, which accounts for more than 50% of soluble protein in chloroplast lysates, and then to obtain homogeneous enzyme preparations by subsequent FPLC procedure. Maximal specific activity corresponded to an 80-fold purification, with a yield of about one-fourth of the initial activity (**Table 1**). Purification of GS from spinach leaf extracts to a similar specific activity was early reported, but a 5-step procedure involving affinity chromatography was required to get a homogeneous preparation (*22*). By starting from intact chloroplasts instead of from total leaf extracts, the protocol herein presented yielded similar results through a simplified, three-step scheme. The purified 44-kDa protein was characterized with respect to kinetic parameters to ensure nonlimiting assay conditions during the subsequent analysis of the inhibition brought about by PPT derivatives. Apparent affinity constants were 60.2 ± 2.1 mM and 0.57 ± 0.04 mM for glutamate and ATP, respectively. A highest affinity hindered a similar evaluation for ammonia, being the enzyme saturated at levels as low as 5 μM, whereas concentrations of Mg^{2+} ions higher than 40 mM were found to be required for optimal activity. Significantly different results were reported in the cited previous study (K_M of 6.7 and 1.8 mM for glutamate and ATP, respectively), most likely depending upon the adoption of a different (hemibiosynthetic) assay method that measures only the initial phosphorylation of glutamate to glutamyl phosphate (*22*).

Plastidial GS Inhibition by PPT Derivatives. A specific involvement of the light-induced, carbohydrate level-regulated chloroplastic isoenzyme in the reassimilation of photorespiratory ammonia is now widely accepted and supported by the results obtained with mutants lacking GS-2 (*23*). Because the amount of ammonia released during photorespiration is much greater than that of primary nitrogen taken up by the plant (*24*), the inhibition of the plastidial isozyme is believed to play a main role in PPT toxicity in planta (*6*), rapidly leading to the accumulation of intracellular NH_4^+ concentration high enough to dissipate transmembrane proton gradients. Thus the ability to interfere efficiently with the activity of the plastidial form

Table 2. Inhibition of the Plastidial GS Isoform, Purified from Spinach Leaves, by PPT and Its Substituted Derivatives[a]



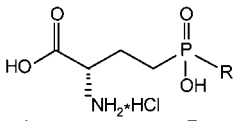
Compound	R	I_{50} (μM) [b]	K_i (μM) [c]	pK_i
1 (PPT)	CH_3	28 ± 3	4.8 ± 1.9	5.319
2	CH_2NH_2	30 ± 4	7.9 ± 1.5	5.102
3	CH_2OH	115 ± 7	45.8 ± 11.0	4.339
(1′*S*)-**4**	$CH(CH_3)NH_2$*HCl	749 ± 107	not determined	---
(1′*RS*)-**4**	$CH(CH_3)NH_2$*HCl	41 ± 3	17.6 ± 3.8	4.754
5	$CH_2CH_2NH_2$*HCl	616 ± 132	133 ± 27	3.876
6	CH_2CH_2COOH	uneffective	---	---

[a] Activity was evaluated with the biosynthetic assay method, which measures the full forward (physiological) reaction, in the presence of PPT and its derivatives at concentrations ranging from 0.001 to 1 mM. Each sample was carried out in triplicate, and values were expressed as a percentage of untreated controls. [b] The concentrations causing 50% inhibition (I_{50}) of in vitro activity were estimated by utilizing the linear regression equation of the activity values plotted against the logarithm of inhibitor concentration. Confidence limits were computed according to Snedecor and Cochran (*21*). [c] Inhibition constants with respect to glutamate (competitive inhibition): values were estimated from Lineweaver–Burk plots at varying glutamate levels in the presence of the inhibitor. At least three inhibitor concentrations, ranging from 0.2 to 2-fold the corresponding I_{50} value, were tested.

represents a main feature required for a GS inhibitor for its application as a herbicide. When the purified enzyme was assayed in the presence of increasing concentrations of PPT derivatives, with the only exception of compound **6** that resulted as completely ineffective, in all cases the catalytic rate was progressively inhibited, with I_{50} values in the micromolar range (**Table 2**). According to the results of the computational analysis based upon the crystal structure of the bacterial GS, and consistently, to previous data obtained with the enzyme from *E. coli* (*19*), compound **2** was the most effective, being almost equipotent to the lead herbicide **1**. The activity of compound **2** most likely reflects interactions of the additional amino group with residues near the ammonium binding site of the plant enzyme. In fact, the negative electronic effect of exchanging an amino for a hydroxyl group in compound **3** resulted in a significantly lower effectiveness, whereas the negative steric effect of an additional methyl substituent as in compound (1′*RS*)-**4** was almost negligible. A complete loss of activity deriving from the replacement of the amino group with a negatively charged carboxylic group in compound **6** suggests repulsive interactions with the negatively charged portion of the enzyme. Interestingly, when the methylene bridge between the ammonium and phosphinate group in the most effective compound **2** was replaced with an ethylene bridge yielding compound **5**, a dramatic loss of activity was found. This underlines an unusual sensitivity of the enzyme toward even minor modifications of the phosphinic moiety in the lead structure. Most likely, a longer spacer causes the extension of the amino group beyond the ammonium binding site. Compound **4** was synthesized as either (1′*S*)-**4** or a mixture of diasteroisomers ([1′*RS*]-**4**), and a strikingly higher I_{50} value in the case of the former accounted for a specific interaction only for the (1′*R*) stereoisomer. Remarkably, in the case of bacterial GS, (1′*S*)-**4** was, on the contrary, as active as the diasteroisomer mixture (*19*). Unfortunately, however, to date we failed in obtaining a substantial amount of compound (1′*R*)-**4**, that could be more active than PPT against the plant enzyme. Experiments are currently in progress with this aim. Because I_{50} values may be influenced by the absolute amount of enzyme used, and thus are not suitable to compare the sensitivity of the enzyme from different sources, a kinetic analysis was carried out for the most effective compounds. As expected, and similarly to the reference herbicide, in all cases, the inhibition was of a competitive type with respect to glutamate. Inhibition constants (**Table 2**) confirmed previous conclusions.

Sensitivity of Cytosolic GS to PPT Derivatives. To evaluate whether the same degree of inhibition may be brought about on the other form of plant GS, a cytosol-localized GS-1-type isozyme was purified from tobacco suspension cultured cells by means of a five-step protocol, as described previously (*18*). Homogeneous preparations showed a specific activity of about 10 000 nkat mg^{-1} (transferase assay). Apparent affinity constants were 7.7 ± 0.2 mM and 0.79 ± 0.03 mM for glutamate and ATP, respectively, and Mg^{2+} concentrations exceeding 5 mM were found to be inhibitory. Assay conditions were thus modified as detailed in the Materials and Methods section to ensure optimal activity and saturating conditions. The inhibitory capability of PPT derivatives was then assessed, and results are reported in **Table 3**. On the whole, data were consistent with those previously found for the chloroplastic enzyme form. However, some differences were also evident. A higher sensitivity toward the lead compound **1** was paralleled by a higher effectiveness of compounds **2**, **3**, and **6**. On the contrary, compounds **4** and **5** resulted as being slightly less effective. Even though it is not so relevant from a quantitative point of view, these differences suggest that slightly different structural features should be used in the future for design and synthesis of new inhibitors of various forms of GS, provided that the crystal structure of the plant proteins becomes in the meantime available. This is also consistent with previous data describing some differential effects of PPT itself (*25*), while other substituted phosphinothricins exerted similar inhibition (*13*). Remarkably, and contrary to what was found for the plastidial isozyme, the effect of the mixture of diasteroisomers ([1′*RS*]-**4**) was quite similar to that of the (1′*S*) stereoisomer **4**. A comparison of the sensitivity of the two plant GSs and the enzyme from *E. coli* is summarized in **Table 4**. A radar plot of pK_i values provided visual evidence of the lower sensitivity of the plant isoforms with respect to the bacterial enzyme, and of the plastidial isozyme between the former, as well as some

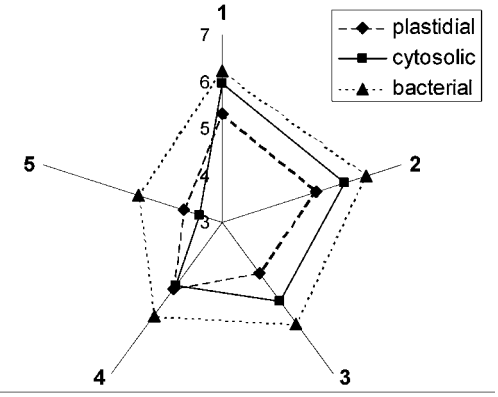
Table 3. Inhibition of the Cytosolic GS Isoform, Purified from Tobacco Cultured Cells, by PPT and Its Substituted Derivatives[a]

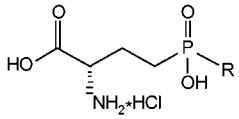


Compound	R	I_{50} (μM) [b]	K_i (μM) [c]	pK_i
1 (PPT)	CH_3	15 ± 1	1.1 ± 0.1	5.959
2	CH_2NH_2	12 ± 1	1.8 ± 0.3	5.745
3	CH_2OH	52 ± 2	8.5 ± 1.2	5.071
(1′*S*)-**4**	$CH(CH_3)NH_2$*HCl	192 ± 11	not determined	---
(1′*RS*)-**4**	$CH(CH_3)NH_2$*HCl	82 ± 3	21.6 ± 2.6	4.666
5	$CH_2CH_2NH_2$*HCl	1260 ± 160	320 ± 110	3.495
6	CH_2CH_2COOH	3290 ± 245	not determined	---

[a] Activity was evaluated with the biosynthetic assay methods, which measures the full forward (physiological) reaction, in the presence of PPT and its derivatives at concentrations ranging from 0.001 to 1 mM. Each sample was carried out in triplicate, and values were expressed as a percentage of untreated controls. [b] The concentrations causing 50% inhibition (I_{50}) of in vitro activity were estimated by utilizing the linear regression equation of the activity values plotted against the logarithm of inhibitor concentration. Confidence limits were computed according to Snedecor and Cochran (*21*). [c] Inhibition constants with respect to glutamate (competitive inhibition): values were estimated from Lineweaver–Burk plots at varying glutamate levels in the presence of the inhibitor. At least three inhibitor concentrations, ranging from 0.2 to 2-fold the corresponding I_{50} value, were tested.

Table 4. Comparison of the Effectiveness of Compounds **1**–**5** in Inhibiting the Activity of GS from Plant Chloroplast, Plant Cytosol, and Bacteria.[a]





K_i : K_M ratio (x10^3)

Compound	R	plastidial	cytosolic	bacterial
1 (PPT)	CH_3	0.080	0.143	0.014
2	CH_2NH_2	0.131	0.234	0.014
3	CH_2OH	0.761	1.104	0.050
(1′*RS*)-**4**	$CH(CH_3)NH_2$*HCl	0.292	2.805	0.081
5	$CH_2CH_2NH_2$*HCl	2.209	41.558	0.311

[a] Data for *E. coli* GS Are from ref (*19*). In the graph, inhibition constants with respect to glutamate were plotted as pK_i. Tabulated values refer to K_i:K_M ratios.

discrepancies in this general pattern. However, being that the inhibition is competitive with respect to glutamate, a simple comparison of kinetic constants could underestimate differences in vivo resulting from competition for binding to the active site. Because of its independency from absolute affinity values, the K_i:K_M ratio constitutes a better parameter for the determination of the sensitivity to a given inhibitor. For instance, in the case of the phosphonate herbicide glyphosate that acts by inhibiting EPSP synthase competitively with respect to phospho*enol*pyruvate, in sensitive species, K_i:K_M ranges from 0.01 to 0.1, while for both natural and selected resistant target enzymes, it is greater than 10, indicating that glyphosate binding is affected to a greater extent than substrate binding (*26* and references therein). When K_i:K_M values for compounds **1**−**5** were considered (**Table 4**), a somehow different picture was obtained than that outlined with K_i values. Phosphinothricin and its derivatives are extremely effective inhibitors of GSs, with ratios as low as 10^{-5}. The bacterial enzyme is the most sensitive, whereas the two plant isozymes show a quite similar, lower sensitivity, with ratios of 1 to 2 orders of magnitude higher. This pattern may be consistent with the hypothesis of a paralogous instead of an orthologous evolution of prokaryotic (type I) and eukaryotic

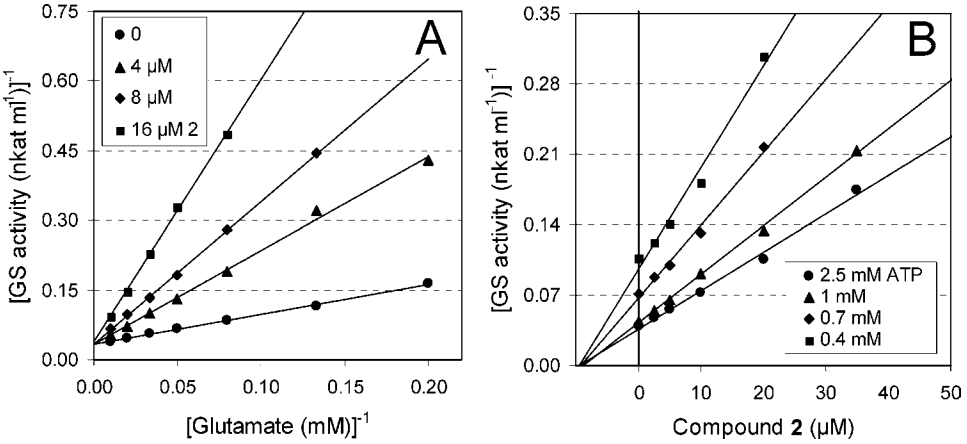


Figure 1. Kinetic analysis of GS inhibition by compound **2**. The cytosolic GS form purified from *N. plumbaginifolia* cultured cells was incubated in the presence of increasing inhibitor concentrations at varying given substrate levels. Lines converging to the *y*-axis in the Lineweaver–Burk plot confirmed an inhibition of a competitive type against glutamate (**panel A**; $K_i = 1.77 \pm 0.12$ μM), whereas lines converging to the *x*-axis in the Dixon plot accounted for an inhibition of a noncompetitive type against ATP (**panel B**; $K_i = 9.7 \pm 0.6$ μM).

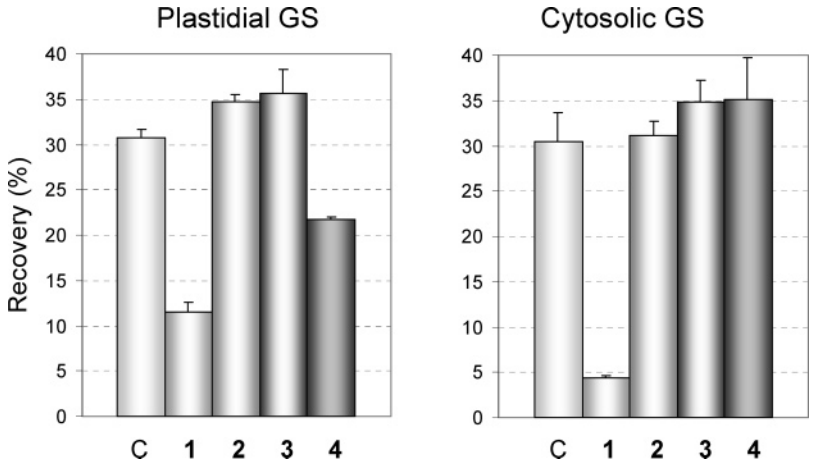


Figure 2. Reversibility of the inhibition brought about by PPT and its derivatives. The purified GS isoforms were incubated for 10 min in complete assay mixture containing or not containing a given inhibitor at a concentration able to reduce by 90% the initial rate of enzyme activity. After reequilibration on ice, proteins were pelleted by ammonium sulfate fractionation, resuspended with fresh buffer, and column desalted. The recovery of GS activity in desalted samples was then evaluated and expressed as percent of initial activity. Results are mean ± SE of six replications from two independent experiments. C denotes untreated controls.

(type II) GSs (*27*). On the other hand, significant differences (e.g., compound **4** against the two plant isozymes, or compound **3** against the plant and the bacterial enzyme) seem to strengthen the possibility of obtaining inhibitors that will be able to distinguish between plant and microorganism, or animal and microbial GSs, and could be used selectively as herbicides or as antibiotics, respectively.

Mechanism of Action. Some experiments were additionally performed in order to get more information concerning the mechanism of GS inhibition. Interestingly, a thorough kinetic analysis of the most effective derivative **2** (**Figure 1**) accounted for an inhibition of a noncompetitive type with respect to ATP. The same compound in the case of *E. coli* GS showed an uncompetitive inhibition toward ATP (*19*), as did toward all enzyme substrates another phosphonate inhibitor of plant GS, *N*-2-(5-chloro-pyridyl)-aminomethylene-bisphosphonic acid (*14*). In uncompetitive inhibition, the inhibitor binds to the active site only after the substrate has bound, whereas in noncompetitive inhibition, two independent binding sites are present. Because substrate binding in a GS active site is of sequential character (ATP binds before glutamic acid, and the latter before the ammonium ion), PPT analogues seem to replace glutamate in the sequence. Noncompetitive inhibition obtained for compound **2** suggests that it binds to the enzyme differently and independently from ATP.

It was shown that, similarly to the first step in the native biosynthetic reaction where γ-glutamyl phosphate is produced, PPT undergoes phosphorylation in the active site of GS. The phosphorylated intermediate is analogous to the transition state in the reaction of γ-glutamyl phosphate and ammonia and binds irreversibly to the protein (*28*, *29*), thus explaining the striking inhibitory potential of PPT ($K_i = 0.6$ μM versus *E. coli* GS) (*13*). Proper trials were thus carried out to ascertain whether the most active derivatives were also able to inactivate irreversibly GS. Results are summarized in **Figure 2**. As expected, pretreatments with the reference compound **1** in a standard assay mixture resulted in an almost complete loss of enzyme activity, even if the purified proteins were ammonium sulfate-precipitated and column-desalted. On the contrary, incubation and processing under the same experimental conditions with inhibitory concentrations of compounds **2**, **3**, and **4** did not lead to GS inactivation, with a recovery similar to that of untreated controls. Consistent results were obtained previously with compound **2** in the case of the bacterial enzyme (*19*). Although further

determinations are required, and inhibitor phosphorylation at a lower rate cannot be completely ruled out, data seem thus to account for a reversible inhibition mechanism. Interestingly, however, an intermediate behavior was found in the case of compound **4**, but only in the case of the chloroplastic isozyme (**Figure 2**, left panel).

Conclusions. Some PPT derivatives designed on the basis of the three-dimensional structure of the bacterial enzyme were proved to be effective also against both plant GS isoforms. Although exhibiting somewhat different mechanisms, some of them showed equipotency to the lead compound, thus potentially representing a new class of herbicides. Moreover, a series of differences between the eukaryotic and the prokaryotic enzyme, as well as between the plastidial and the cytosolic form of plant GS, have been pointed out. In the absence of a suitable crystallographic analysis of the plant enzymes, such data might be useful as a starting point for the design of selective molecules able to discriminate among the different forms of the target enzyme.

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