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Effectiveness and mode of action of phosphonate inhibitors of plant glutamine synthetase

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Abstract

BACKGROUND: Aiming at the rational design of new herbicides, the availability of the three-dimensional structure of the target enzyme greatly enhances the optimisation of lead compounds and the design of derivatives with increased activity. Among the most widely exploited herbicide targets is glutamine synthetase. Recently, the structure of a cytosolic form of the maize enzyme has been described, making it possible to verify whether steric, electronic and hydrophobic features of a compound are in agreement with inhibitor–protein interaction geometry.

RESULTS: Three series of compounds (aminophosphonates, hydroxyphosphonates and aminomethylenebisphosphonates) were evaluated as possible inhibitors of maize glutamine synthetase. Aminomethylenebisphosphonate derivatives substituted in the phenyl ring retained the inhibitory potential, whereas variations in the scaffold, i.e. the replacement of the second phosphonate moiety with a hydroxyl or an amino residue, resulted in a significant loss of activity. A kinetic characterisation showed a non-competitive mechanism against glutamate and an uncompetitive mechanism against ATP. A docking analysis suggested the mode of bisphosphonate binding to the active site.

CONCLUSION: Results made it possible to define the features required to maintain or enhance the biological activity of these compounds, which represent lead structures to be further exploited for the design of new substances endowed with herbicidal activity. Copyright © 2009 Society of Chemical Industry

INTRODUCTION

Among the most widely exploited herbicide targets is glutamine synthetase (GS) [EC 6.3.1.2], the enzyme that in both plants and microorganisms plays a key role in ammonia assimilation.¹ Within the cell, its inhibition rapidly leads to ammonia accumulation and glutamate depletion, which act in concert to cause plant death.² GS-targeting commercial herbicides were developed as a consequence of the discovery in *Streptomyces* spp. of a natural inhibitor, the tripeptide binalaphos. The pharmacophore of binalaphos is phosphinothricin (PPT), a phosphinic analogue of the physiological substrate glutamate.³ Its remarkable effectiveness relies upon the formation of a phosphorylated product that irreversibly binds to the active site.⁴ Because of its inability to distinguish between weeds and crops, use was limited, and so synthesis of a great number of

analogues of the active molecule and their screening for selective forms were carried out.[5](#), [6](#) Most of the active derivatives are glutamate analogues that act competitively, without being phosphorylated. This results in a much lower effectiveness than that of PPT. As for other competitive inhibitors, the progressive substrate accumulation that occurs as a consequence of enzyme inhibition is able partially to relieve the block of the enzymatic activity.[7](#)

Attempts to improve the effectiveness of these compounds by using computer-aided molecular modelling techniques essentially failed.[8](#), [9](#) One possible reason for such a failure may be the unavailability of the structure of plant GS, which forced docking analyses to be performed on the basis of an enzyme from non-plant sources. Recently, however, the three-dimensional structure at a suitable resolution of a maize GS has been described.[10](#) In this plant species, the occurrence of at least six genes coding for a putative GS, with different functional localisation and, possibly, separate metabolic function, has been reported.[11](#) One chloroplastic and five cytosolic GS isoforms (also referred to as GS2 and GS1a–e) have been hypothesised to be specifically involved in the light-induced reassimilation of photorespiratory ammonia and the generation of glutamine for intercellular nitrogen transport respectively.[12](#) The gene coding for GS1a was cloned, heterologously expressed and purified,[13](#) and the protein was crystallised. The structure reveals a unique decameric structure that differs significantly from the bacterial GS structure,[10](#) thus opening new perspectives for the computer-aided design of effective inhibitors of plant GSs.

Within the framework of long-established research on new active inhibitors belonging to the chemical class of aminophosphonates,[14](#) the present authors have previously described a series of derivatives of aminomethylenebisphosphonic acid[15](#) and some PPT analogues[8](#), [16](#) that inhibited plant and bacterial GSs. A compound in the former group, 3,5-dichloro-phenylaminomethylenebisphosphonic acid (compound **1a**, Fig. [1](#)), showed inhibitory properties only slightly poorer than those of PPT, and reduced *in vivo* free glutamate pools.[15](#) Its mechanism of action at the molecular level was not investigated in detail. Moreover, the same compound was afterwards found also to inhibit the activity of δ^1 -pyrroline-5-carboxylate (P5C) reductase [EC 1.5.1.2], the enzyme that catalyses the last reaction in both the glutamate and the ornithine pathways, the two routes that in higher plants lead to proline synthesis.[17](#), [18](#)

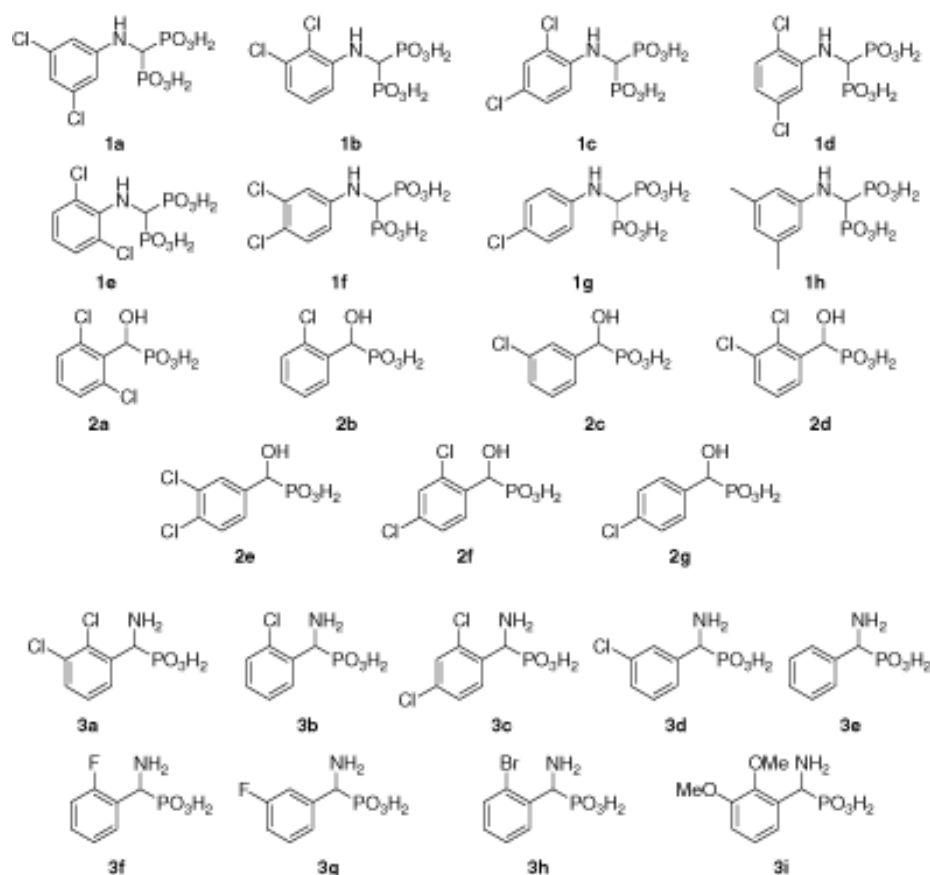


Figure 1. Analogues of 3,5-dichlorophenylaminomethylenebisphosphonic acid (compound **1a**) evaluated as possible inhibitors of maize GS.

The present paper reports the results of screening 23 new phosphonates, designed using **1a** as a lead compound, for their ability to inhibit the cytosolic forms of GS, isolated from *Zea mays* L. leaf blades. A thorough kinetic evaluation, coupled with a docking analysis based on the three-dimensional structure of the same protein used for the biochemical characterisation, made it possible to hypothesise on their mechanism of action at the molecular level, and perhaps provide the basis for the future design of new, more effective inhibitors.

MATERIALS AND METHODS

Materials

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich or Merck Chemical Companies, and were of analytical grade. The synthesis of aminomethylenebisphosphonic acids **1a–h**, hydroxyphosphonic acids **2a–g** and aminophosphonic acids **3a–i** (Fig. 1) was described previously.¹⁸ All hydroxyphosphonic and aminophosphonic acids were racemic.

Plant material and growth conditions

Seeds of *Zea mays* cv. Jeff (Renk Venturoli) were imbibed overnight in sterile distilled water and then sown in rows about 10 cm apart in plastic trays (40 cm wide × 50 cm long × 10 cm high, 20 L volume) filled with a potting mixture (0.8:1 by weight) of sand:loam (10% organic matter, 0.26% total nitrogen, pH 6.7). Seedlings were grown in a growth chamber with a 16 h photoperiod of about

400 $\mu\text{E m}^{-2} \text{ s}^{-1}$ at 20 ± 1 °C; relative humidity was not controlled. They were periodically watered with tap water, as required. Leaves were harvested from seedlings at the three-leaf stage.

GS purification

Leaves (about 100 g) were extracted in a suitable volume of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl_2 , 0.5 mM DTT and 0.5 mM EDTA using a juice extractor (Philips HR 1821). All subsequent operations were carried out at 0–4 °C. Following centrifugation at $12\,000 \times g$ for 10 min, the resulting supernatant was made to 70% of saturation by adding solid ammonium sulfate. Precipitated material was pelleted by centrifugation, resuspended in a minimal amount of the extraction buffer, and desalted by passage through a Bio-Gel P6DG (Bio-Rad) column.

The desalted extract was then loaded onto a DEAE-Sephacel (Pharmacia) column (2.5 cm diameter, 25 mL bed-column) equilibrated with the extraction buffer. The column was eluted at a constant flow of 1 mL min^{-1} with a linear gradient from 0 to 400 mM KCl in 400 mL of the extraction buffer, while collecting 5 mL fractions. Fractions containing GS activity ($>10 \text{ nkat mL}^{-1}$ as measured by the transferase assay) were pooled and made up to 50% saturation with solid ammonium sulfate. Precipitated protein material was pelleted by centrifugation, resuspended in a minimal amount of 25 mM imidazole-HCl buffer, pH 7.4, containing 2.5 mM MgCl_2 , 0.5 mM EDTA and 0.25 mM DTT (column buffer), and desalted by passage through a Bio-Gel P6DG (Bio-Rad) column equilibrated with the column buffer.

The sample was loaded at a constant flow of 40 mL h^{-1} onto a Reactive Blue Sepharose (Sigma) column (1.5 cm diameter, 10 mL bed-column) equilibrated with the column buffer. To exchange the column buffer for extraction buffer, the flow-through fraction containing materials not retained by the Reactive Blue column was passed through another Bio-Gel P6GD column, this time equilibrated with the extraction buffer. Flow-through material was injected by means of a 10 mL super loop (Pharmacia) onto a Mono-Q 5.5 (Pharmacia) FPLC column equilibrated with the extraction buffer. Protein fractions were eluted at a flowrate of 1.0 mL min^{-1} 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 filter sterilised and stored at 4 °C until used for biochemical determinations. Under these conditions, GS activity was found to be stable for at least 1 month.

Enzyme assays

During enzyme purification, GS activity was measured by the transferase assay, which ensures rates that are not affected by the presence of contaminating enzymes, especially ATPase. The reaction mixture contained 25 mM imidazole-HCl buffer (pH 7.4), 50 mM L-glutamine, 40 mM Na_3AsO_4 , 4 mM MnCl_2 , 5 mM ADP, 25 mM $\text{NH}_2\text{OH-HCl}$ and an amount of enzyme that allowed the initial rate to be measured in a convenient time and while most of the substrate was unconverted. The final reaction volume was 0.4 mL. After up to 15 min at 35 °C, the reaction was stopped by the addition of 0.8 mL of colorimetric solution [10% (w/v) $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, 6.67% (v/v) HCl and 5% (w/v) trichloroacetic acid]; following centrifugation for 5 min at $12\,000 \times g$, samples were read at 535 nm against non-incubated blanks, and the γ -glutamyl-hydroxamate formed was quantified by comparison with a calibration curve obtained with an authentic standard. The purified enzyme could be assayed by a biosynthetic assay that measures the physiological, full-forward reaction. The mixture contained 50 mM Tris-HCl buffer (pH 7.4), 50 mM L-glutamate, 2.5 mM ATP, 5 mM MgCl_2 , 0.5 mM NH_4Cl and a convenient amount of enzyme (50 pkat) in a final volume of 0.1 mL. After up to 20 min at 35 °C, the inorganic phosphate released was quantified by the malachite green assay method as described previously.¹⁹ At least three different incubation times were tested for each sample, in the linear range; activity was calculated from the slope of the straight line

interpolating the experimental data. Enzyme activity was expressed in katal submultiples, where 1 nkat is the amount of enzyme that catalyses the synthesis of 1 nmol glutamine s⁻¹. Protein concentration was determined by the method of Bradford,[20](#) using bovine serum albumin as the standard.

Enzyme inhibition and kinetic analysis

GS inhibition was evaluated by adding to the reaction mixture an appropriate dilution of a freshly prepared 20 mM solution of a given inhibitor, brought to pH 7.4 with KOH, so as to obtain a final concentration of 1 mM. Reported data are means \pm SD over results obtained in at least three independent measurements. Active compounds were further tested in the micromolar range. The concentrations causing 50% inhibition (IC₅₀) of GS activity and confidence intervals were estimated by the probit method.[21](#)

For kinetic evaluations, the enzyme was assayed in the presence of increasing concentrations of compounds **1a** and **1h** and substrate concentrations ranging from 6 to 40 mM glutamate and from 0.2 to 1 mM ATP. Otherwise, substrate concentrations were fixed at 50 mM for L-glutamate and 2.5 mM for ATP. At least seven inhibitor doses were evaluated, at least in triplicate, for each substrate concentration. In the case of glutamate (non-competitive inhibition), K_i values were estimated from Lineweaver–Burk plots of activity, on the basis of the corresponding lowering in the apparent V_{MAX} value; five inhibitor concentrations, ranging from 0.2- to 1.5-fold the I₅₀ value, were tested. In the case of ATP (uncompetitive inhibition), K_i values were estimated from Dixon plots of activity by evaluating the effect of four inhibitor levels, ranging from 0.5- to 3-fold the I₅₀ value, in the presence of seven substrate concentrations. Reported data are means \pm SEM over results obtained with different inhibitor or substrate concentrations respectively.

Molecular modelling

The crystal structure of the maize enzyme,[10](#) obtained from the Protein Data Bank (refcode 2D3A),[19](#) was used as a starting point for all calculations. Residue numbering is according to this structure. The hydrogen atoms were added using the Insight 2000 program (Accelrys), the protonation states of the amino acid side-chain residues were set up for pH 7.0 and the protonated structure was checked carefully. Molecular mechanics calculations were performed using the Discover program, with a cff97 force field and conjugate gradient minimiser. Minimisations were done up to an energy change of 0.01 kcal mol⁻¹. Bisphosphonate molecules were built in the Builder Module of the Insight package, and their structures were optimised. The bisphosphonate moiety of the ligand was then superimposed on the pyrophosphate group of ADP in the ADP–GS complex by pairwise alignment of all heavy atoms. Subsequently, the ADP molecule was removed and a bisphosphonate–GS complex was obtained. Finally, the ligand and active-site residues were optimised.

RESULTS AND DISCUSSION

Purification of a cytosolic form of GS from *Zea mays* seedlings

Aiming at the evaluation of the effectiveness of derivatives of the active[15](#) compound **1a** as possible GS inhibitors, the cytosolic form of *Z. mays* GS was purified from leaf blades. Based on literature data, six GS isoforms are present in maize: one (GS2) is functionally localised in the chloroplast, the other five in the cytosol.[11](#) The latter enzyme forms, named GS1a–e, show differential expression patterns in the plant tissues.[12](#) In roots, mainly GS1a and GS1d are

expressed, and their levels increase differently in response to ammonia treatments. However, in spite of a high sequence similarity, they show significantly different properties and stability.¹³ In leaf blades, besides the chloroplastic isozyme, the large majority of GS activity is attributable to GS1a and GS1b, the genes of which are both expressed at substantial levels.¹¹ The primary structures of GS1a and GS1b were found to be 97% identical;¹² thus, the proteins are supposed to show negligible functional differences, if any. Because crystallographic analysis was performed with purified GS1a,¹⁰ leaf blades from maize seedlings were used for enzyme isolation.

Partial purification of GS1a–b from leaves of maize plantlets harvested at the three-leaf stage was obtained by a five-step protocol (Table 1). Maximal specific activity observed corresponded to 50-fold purification, with a yield of about 40% of the initial activity. Besides achieving an initial enrichment, anion-exchange chromatography (step 2) allowed GS1 resolution from the chloroplastic enzyme form. During all subsequent steps, the elution profile of GS activity was symmetric, a finding that strengthens the high structural/functional similarity of GS1a and GS1b, previously hypothesised on the basis of their primary structure.^{10, 12} In spite of further purification by negative affinity chromatography and ion-exchange FPLC, final preparations were not homogeneous, as several fainter protein bands were still evident in SDS-PAGE patterns (not shown). However, samples were apparently devoid of any other enzymatic activity able either to hydrolyse ATP or to use glutamate as a substrate. In any case, proper checks were always done by assessing possible phosphate release in parallel reaction mixtures in which glutamate had been omitted. A residual presence of activities able to make use of the same substrates, or further metabolise the products, may lead to experimental artefacts, or at least interfere heavily with the assay of an enzyme. This is the reason why some widely used GS assay methods (the so-called hemibiosynthetic and transferase assays) were developed that indeed measure partial or reverse—thus, non-physiological—reactions.²² However, compounds able to interfere with enzyme activity under such assay conditions might be scarcely effective on the full reaction, or vice versa. The purification protocol that was set up made it possible to resolve GS from most interferences, and to use the synthetic assay method, one that measures the whole-forward, physiological reaction.^{15, 22}

Table 1. Partial purification of GS1 from *Zea mays* L. seedlings^a

Step	Protein (mg)	Activity (nkat)	Specific activity (nkat mg ⁻¹)	Purification (fold)	Yield (%)
Activity was measured by the transferase assay. Results presented are for a typical purification starting from 100 g (fresh weight) of leaf blades from seedlings harvested at the three-leaf stage.					
1. Crude extract	354.2	1311.6	3.70	1	100
2. DEAE-Sephacel column	70.13	1091.8	15.6	4.2	83.2
3. 0–50% ammonium sulfate fraction	41.51	1044.0	25.2	6.8	79.6
4. Blue sepharose, flow through	18.26	752.6	41.2	11.1	57.4
5. MonoQ column	2.91	521.6	179.5	48.5	39.8

The partially purified protein was characterised with respect to kinetic parameters in order to ensure non-limiting assay conditions during the subsequent analyses. Apparent dissociation constants were $205 \pm 12 \mu\text{M}$ and $18.3 \pm 0.4 \text{ mM}$ for ATP and glutamate respectively. At concentrations exceeding 5 mM, ATP was found to be inhibitory. A high affinity for ammonia was also evident ($K_M < 10 \mu\text{M}$), one that resulted in saturating conditions at lowest concentrations, and thus hampered a reliable measurement of the constant value. The levels of the substrates previously adopted to assay GS from other plant species^{15, 16, 22} were slightly modified, accordingly.

GS inhibition by analogues of 3,5-dichlorophenyl-aminomethylenebisphosphonic acid

Three groups of 3,5-dichlorophenylaminomethylenebisphosphonic acid analogues were tested as possible inhibitors of the purified enzyme: bisphosphonates (**1b–h**), hydroxyphosphonates (**2a–g**) and aminophosphonates (**3a–i**) (Fig. 1).¹⁵ The first set was chosen with the aim of investigating the effect of either the number or the position of the chlorine substituents in the phenyl ring. The two other sets were evaluated in order to check the influence of the scaffold structure (i.e. the presence of two phosphonic moieties) on the inhibitory effectiveness. The activity of maize GS was measured in the absence or in the presence of the 24 compounds at 1 mM. Those showing a significant effect in the millimolar range were further characterised through the evaluation of the concentrations causing 50% inhibition of enzyme activity (IC_{50}). Results, summarised in Table 2, clearly showed that bisphosphonate analogues exhibit the highest effectiveness. The comparison of the effect of compounds with the same substituents at the aromatic portion of the molecule (e.g. **1b** versus **2d** and **3a**, or **1c** versus **2f** and **3c** respectively) revealed that their efficacy as inhibitors is reduced by two orders of magnitude when the second phosphonate moiety is replaced with either a hydroxyl or an amino group. Bisphosphonates **1a–h** are a chiral, whereas the corresponding hydroxy- and aminophosphonates are racemic mixtures.¹⁸ However, the difference in the inhibitory activity largely exceeds that expected (0.3 in log scale) in the case of a racemic mixture in which one of the enantiomers is equivalent to the corresponding optically inactive bisphosphonate and the other enantiomer is inactive. Therefore, both phosphonate residues most likely interact with the enzyme, and a second acidic group cannot be replaced by weak antagonists, such as OH and NH_2 moieties.

Table 2. Inhibition of maize glutamine synthetase by analogues of 3,5-dichlorophenylaminomethylenebisphosphonic acid (compound **1a**)

Compound	Inhibitory effect at 1 mM ^a (%)	IC ₅₀ ^b (μM)	pIC ₅₀ ^c
<ul style="list-style-type: none"> a Activity was evaluated by the biosynthetic assay method measuring the physiological reaction either in the absence or in the presence of a given phosphonate at 1 mM. Each test was carried out in triplicate, and values were expressed as percentage inhibition (± SD) with respect to untreated controls. b Active compounds (inhibition at 1 mM > 15%) were further tested at concentrations ranging from 0.001 to 5 mM. The concentrations causing 50% inhibition (IC₅₀) of <i>in vitro</i> activity were estimated by the probit method.²¹ nd = not determined. c Minus log₁₀ of the molar concentration causing 50% inhibition of enzyme activity. 			
1a	86.3 (± 6.0)	7.6 (± 0.7)	5.119
1b	84.9 (± 2.8)	12.8 (± 1.8)	4.893
1c	79.7 (± 5.8)	21.2 (± 4.2)	4.674
1d	79.1 (± 4.0)	39.2 (± 6.5)	4.407
1e	81.1 (± 2.5)	15.0 (± 2.1)	4.824
1f	73.8 (± 5.6)	31.9 (± 6.3)	4.496
1g	79.1 (± 1.8)	21.0 (± 2.9)	4.678
1h	67.5 (± 3.8)	56.0 (± 3.4)	4.252
2a	10.7 (± 3.4)	nd	nd
2b	27.0 (± 6.3)	6610 (± 420)	2.180
2c	12.3 (± 7.7)	nd	nd
2d	47.2 (± 2.2)	1160 (± 240)	2.936
2e	14.6 (± 2.4)	nd	nd
2f	31.9 (± 3.3)	4830 (± 680)	2.316
2g	8.3 (± 6.3)	nd	nd
3a	47.7 (± 4.7)	1050 (± 480)	2.979
3b	36.3 (± 6.8)	5810 (± 570)	2.236
3c	40.7 (± 2.8)	2610 (± 400)	2.583
3d	8.1 (± 6.5)	nd	nd
3e	13.6 (± 4.8)	nd	nd
3f	26.7 (± 1.0)	10 100 (± 1310)	1.996
3g	7.9 (± 5.4)	nd	nd
3h	42.5 (± 3.1)	4100 (± 760)	2.387
3i	− 1.2(± 5.9)	nd	nd

As for the substituents at the phenyl ring, in the case of bisphosphonates, data did not provide any plain pattern: neither the presence of one or two chlorines nor their position causes a significant variation in the resulting effectiveness in inhibiting the enzyme. However, comparison of the present results with those obtained in a previous screening for phosphonate inhibitors of plant GS¹⁵ confirms the importance of the presence of chlorine substituents. The unsubstituted phenylaminomethylenebisphosphonic acid and some analogues in which chlorine is replaced with

other halogen moieties (F, Br) or other groups (methyl, methoxyl) had indeed shown no or poor activity.¹⁵ Interestingly, however, compound **1h** (in which the two chlorines of **1a** are replaced by methyl groups) retained a significant inhibitory potential, albeit to a slightly lower extent. For the less active OH and NH₂ analogues, the position of the chlorine substituent(s) seems to affect the activity significantly, *meta*-monosubstitution being apparently unfavourable. On the other hand, the activity of 2-halogen-substituted compounds is almost unchanged among analogues with Cl, Br and F. However, amino- and hydroxyphosphonates exhibited poor inhibitory activity, and their mode of binding could differ from that of bisphosphonates. If so, the structure–activity pattern may also be different.

Kinetic analysis and possible mechanism of action

In order to obtain some information concerning the inhibitory mechanism, a thorough kinetic analysis was carried out for some of the most effective compounds. In a preliminary study, the ability of compound **1a** and other active analogues to inhibit *in vivo* glutamine synthesis was supported by the measurement of free amino acid pools in suspension-cultured plant cells.¹⁵ Moreover, the determination of the activity over time of the partially inhibited enzyme suggested that they act differently to PPT, i.e. that they do not bind to the active site irreversibly.¹⁵ However, the fine details of the inhibitory mechanism were not determined experimentally. An *in silico* analysis performed on the basis of the structure of the enzyme from non-plant sources gave only a rough pattern of bisphosphonate binding, suggesting that the bisphosphonic moiety of the inhibitors may interact with the metal ions present in the active site.²³ The activity of maize GS was thus assayed in the presence of inhibitory concentrations of compounds **1a** and **1h** at varying those of either ATP or glutamate. Results (Fig. 2) accounted in both cases for an inhibition of non-competitive type with respect to glutamate, and uncompetitive against ATP. From a quantitative point of view, the calculated K_i values in the case of glutamate were $7.2 \pm 0.3 \mu\text{M}$ and $53 \pm 1 \mu\text{M}$ for compounds **1a** and **1h** respectively, and $6.4 \pm 0.5 \mu\text{M}$ and $55 \pm 7 \mu\text{M}$ in the case of ATP. As a term of comparison, some PPT derivatives with a modified methyl group that also bind reversibly to the enzyme showed K_i values against glutamate ranging from 2 to 22 μM . However, in that case the inhibition was of competitive type against glutamate.¹⁶

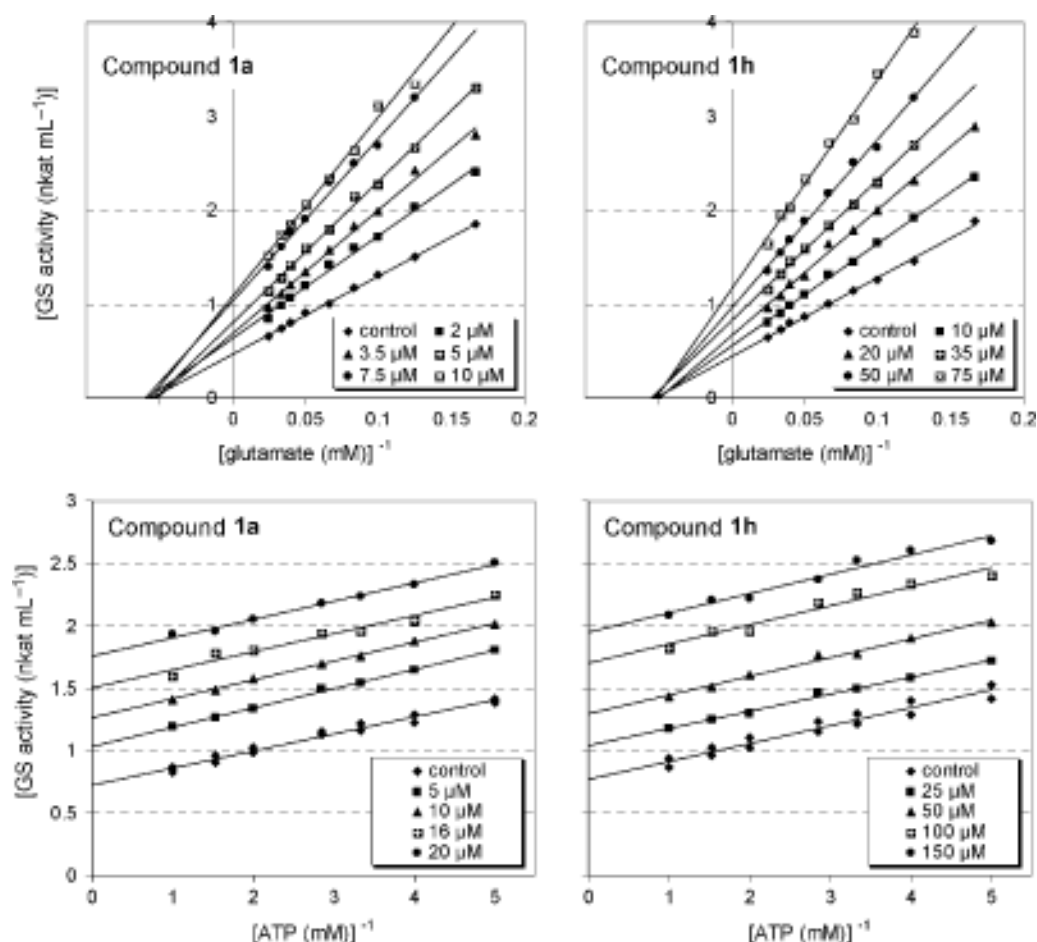


Figure 2. Kinetic analysis of GS inhibition by compounds **1a** and **1h**. The cytosolic form of the enzyme partially purified from *Z. mays* seedlings was incubated in the presence of increasing inhibitor concentrations, as specified in the graph legends, at varying substrate levels. Lines converging to the x axis in the Lineweaver–Burk plot accounted for an inhibition of non-competitive type against glutamate (panels A and C; $K_i = 7.2 \pm 0.3 \mu\text{M}$ in the case of compound **1a** and $K_i = 53 \pm 1 \mu\text{M}$ in the case of compound **1h**), whereas parallel lines showed an inhibition of uncompetitive type with respect to ATP (panels B and D). Replotting of the latter data as a Dixon plot resulted in K_i values of $6.4 \pm 0.5 \mu\text{M}$ and $55 \pm 7 \mu\text{M}$ for compounds **1a** and **1h** respectively. A multiple regression analysis showed in all cases a high significance level ($P < 0.00$) with respect to both variables (inhibitor and substrate concentration).

In a non-competitive inhibition, two independent binding sites are usually present, whereas in the case of uncompetitive inhibition the inhibitor binds to the active site after the substrate has bound. A mechanism for which these compounds bind to the enzyme after the formation of an ATP–enzyme complex would therefore be consistent with the experimental results. The finding that the inhibition is uncompetitive against ATP implies that the V'_{max} (V_{max} in the presence of the inhibitor) will always be equal to V_{max} divided by $(1 + I/K_i)$, regardless of substrate concentration. On the other hand, a competitive inhibitor is displaced by an increase in substrate concentration. Because enzyme inhibition always causes *in vivo* an increase in the substrate pool, competitive inhibitors are usually much less effective *in vivo* than they are *in vitro*.⁷ Thus, bisphosphonates are probably going to be more effective inside the cell than all other reversible GS inhibitors that act competitively with respect to glutamate.^{5, 16}

Computer-aided analysis of inhibitor binding

To obtain a deeper insight into the mechanism of action, a docking analysis of compounds **1a–h** into the active site of the maize enzyme was performed. A detailed inspection of GS structure suggested that the negatively charged bisphosphonic group can interact only with two positively charged magnesium ions present in the active site (Fig. 3), a possibility that is in agreement with the well-known metal-complexing properties of bisphosphonates.²⁴ As an uncompetitive mode of action against ATP and a non-competitive mode of action against glutamate were proven experimentally, the inhibitors should bind to the enzyme after ATP and independently of glutamate. It is proposed that, acting as strong complexing agents, bisphosphonates displace the triphosphate group of ATP. The optimised complex composed of the enzyme and the most effective compound **1a** is presented in Fig. 4a. The bisphosphonate group of the inhibitors not only interacts with the two metal ions (Mg1 and Mg2) but also forms hydrogen bonds with Gln201 and Arg311 residues, and with the Lys36' residue of the adjacent enzyme subunit, where interactions with Lys36' and Arg311 are charge assisted. Moreover, inhibitor NH moiety forms a hydrogen bond with Asn54'. The aromatic fragment of the compounds does not compete with ATP for binding, being located in a non-physiological pocket of the enzyme composed by the side chains of residues Trp53', Asn54', Asn251, Gly318 and Tyr328 (Figs 4b and c).

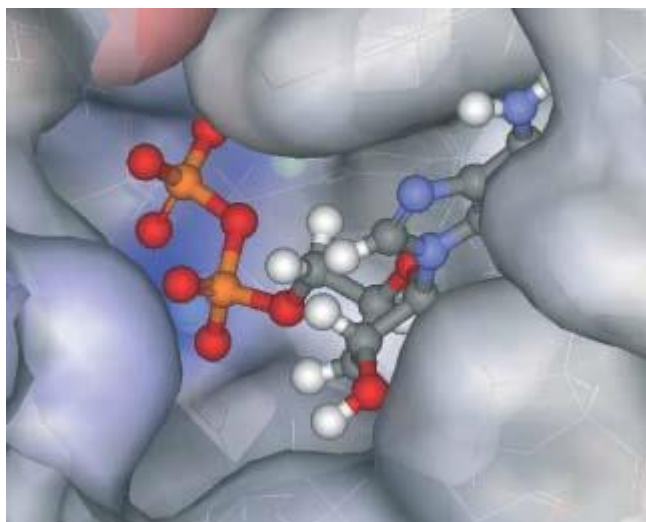


Figure 3. Ball-and-stick model of ADP binding to maize glutamine synthetase (PDB id 2D3A): surfaces are coloured according to the electrostatic potential (blue—positive, grey—neutral, red—negative values). Magnesium ions are represented as light-green balls.

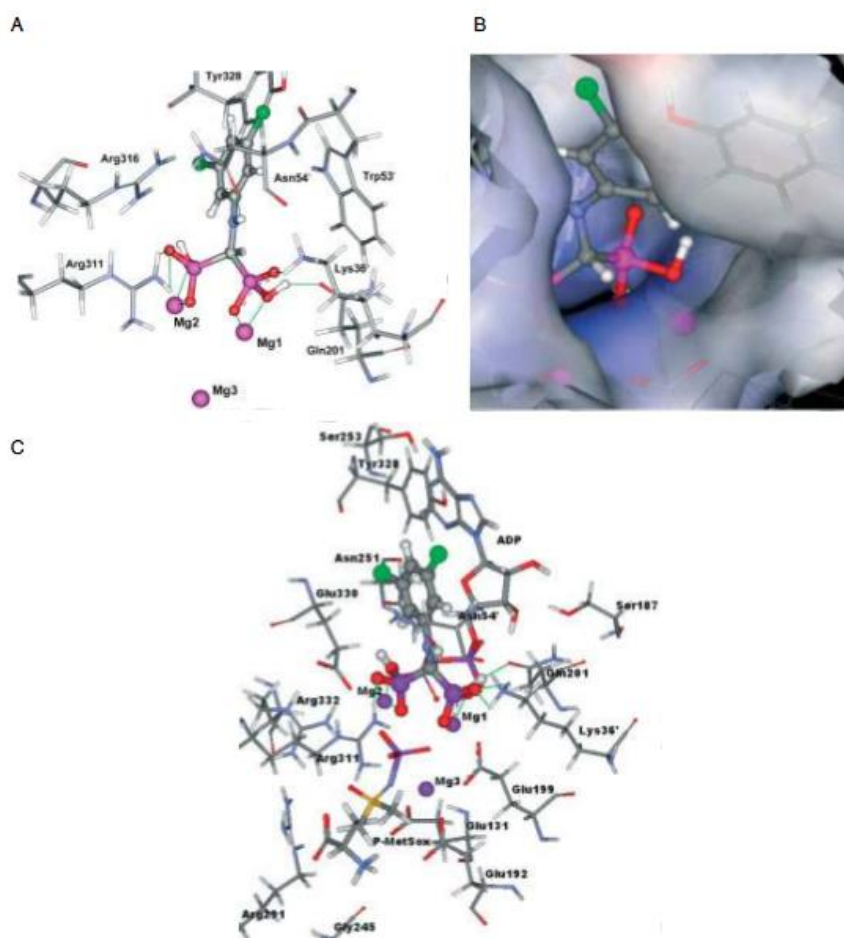


Figure 4. Modelled binding of compound **1a** to *Zea mays* GS. The enzyme is presented either as a stick model of active-site residues (panel A, where hydrogen bonds and interactions with metal ions are marked as green lines) or as coloured surfaces, according to the electrostatic potential (panel B: blue—positive, grey—neutral, red—negative values). ADP and methionine sulfoximine phosphate were superimposed on the structure of the **1a**–GS complex and are shown as a stereo image in panel C. Labels of amino acid residues belonging to the adjacent enzyme subunit are marked with a prime (').

The hypothesised mode of binding implies that both phosphonate groups are required for a proper interaction with the enzyme. This is consistent with the experimental results showing a strikingly lower effectiveness of hydroxy- and aminophosphonates with the same side chains (Table 2). Molecular modelling studies (Fig. 4c) show a similar interaction with the enzyme for all the active compounds **1a–h**, and no significant steric restriction with chlorine substituent positions on the aromatic ring is plausible to be operative. Accordingly, the inhibitory activity of chlorophenyl-substituted aminomethylenebisphosphonates **1a–g** does not vary substantially (pIC_{50} in the range 4.407–5.119).

CONCLUSION

Most GS inhibitors described so far act as glutamate analogues. From this point of view, bisphosphonates represent a completely different class,²⁵ as they seem to interact with the enzyme near the ATP binding site.⁹ Data presented here concerning the relationships between phosphonate structures and their biological activity, as well as the protein residues involved in bisphosphonate binding, may provide substantial information for a further optimisation of the structure of such

inhibitors. Work is currently in progress in the authors' laboratories to develop and test new active derivatives.

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