Research Article

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Phytotoxicity of aminobisphosphonates targeting both δ[1]-pyrroline-5-carboxylate reductase and glutamine synthetase

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Running title:

Effectiveness *in planta* of phosphonate inhibitors of amino acid biosynthesis

Abstract

BACKGROUND: Dual-target inhibitors may contribute to the management of herbicide-resistant weeds and avoid or delay the selection of resistant biotypes. Some aminobisphosphonates inhibit the activity of both glutamine synthetase and δ^1-pyrroline-5-carboxylate (P5C) reductase *in vitro*, but the relevance of the latter *in vivo* has not been proven, yet. This study aimed at demonstrating that these compounds can block also proline synthesis *in planta*.

RESULTS: Two aminophosphonates, namely 3,5-dichlorophenylamino-methylenebisphosphonic acid and 3,5-dibromophenylaminomethylenebis-phosphonic acid (Br_2PAMBPA), showed inverse effectiveness against the two partially purified target enzymes from rapeseed. The compounds showed equipotency in inhibiting the growth of rapeseed seedlings and cultured cells. The analysis of amino acid content in treated cells showed a strong reduction of glutamate and glutamate-related amino acid pools, but a milder effect on free proline. In the case of Br_2PAMBPA, toxic P5C levels accumulated in treated seedlings, proving that the inhibition of P5C reductase takes place *in situ*.

CONCLUSIONS: Phenyl-substituted aminobisphosphonates may be regarded as true dual-target inhibitors. Their use to develop new active principles for crop protection could consequently represent a tool to address the problem of target-site resistance among weeds.

Keywords: amino acid biosynthesis inhibitors as herbicides; derivatives of aminomethylenebisphosphonic acid; glutamine synthetase; multiple targets; P5C reductase; phytotoxicity

1. INTRODUCTION

Properties of modern agrochemicals should include high effectiveness at low rates of application, selectivity between crops and weeds, and low toxicity to mammals and non-target organisms. To meet public acceptance and address concerns due to potential environmental pollution, active ingredients for crop protection should also exhibit low persistence in the soil because of a rapid mineralization by soilborne microorganisms. During recent years many efforts have been made to identify new compounds endowed with the above mentioned features to control weeds. However, several constraints to new agrochemical development, among which a tightening of regulatory requirements and the high expectations set by glyphosate, are discouraging industrial investment.[1-4] Moreover, due to the high selective pressure, the repeated use of herbicides with the same target rapidly leads to the selection of resistant weed biotypes,[5] strongly shortening the commercial life of these products. From this point of view, herbicides inhibiting multiple targets may represent a powerful tool, significantly lowering the probability of resistance emerging.[6] In fact, natural phytotoxins inhibiting multiple targets have retained their activities millions of years.[7]

Aminoalkylphosphonic acids, amino acids analogues in which a phosphonic or related moiety replaces the carboxylic group, have shown a wide array of biological activities.[8] The enzyme inhibitory properties of phosphonates result from either a net of hydrogen bonds formed with amino acid residues in the active site, or ionic interactions with positively charged metal ions or amino acids in the catalytic cleft. Inhibition of enzyme activity by phosphonates is linked to their similarity to natural phosphates (e.g. ATP, FPP) or analogy to tetrahedral transition state of amide/ester hydrolysis/synthesis.[9–12] Their attractiveness also relies upon a high susceptibility to degradation by soil microorganisms, which avoids the risk of pollution deriving from

pesticide persistence.[13] Two of the most successful herbicides ever, glyphosate[14] and phosphinothricin,[15] belong to this class of compounds.

Recently, we focused on phosphonate inhibitors of proline biosynthesis. Besides its unique role in determining protein folding and stability, in most plants proline accumulates in free form in response to various stress conditions. A rapid and reversible increase of free proline content was found to occur in cells exposed to either osmotic, oxidative or temperature stress, improving stress tolerance.[16] Moreover, proline metabolism seems also involved in the plant defence response that follows pathogen penetration attempts.[17] As a consequence, compounds able to interfere with proline production would be expected to exert strong phytotoxic effects. However, a drawback for the development of effective inhibitors is represented by the existence in plants of multiple pathways for proline production, which may proceeds from either glutamate or arginine.[18] In the presence of redundant biosynthetic routes, proline starvation cannot be obtained through the inhibition of the enzyme that catalyzes the rate-limiting reaction in either pathway. Nevertheless, since the two routes share the last reaction, namely the reduction of δ^1-pyrroline-5-carboxylate (P5C) by a P5C reductase [EC 1.5.1.2], this result might be accomplished by specific inhibitors of the latter enzyme.[19]

In a first attempt, the ability of inhibiting *Arabidopsis thaliana* P5C reductase was evaluated on a group of derivatives of aminomethylenebisphosphonic acid. Three compounds caused a dose-proportional reduction of the catalytic rate in the micromolar to millimolar range.[20] A detailed kinetic analysis, coupled with a computer-assisted docking simulation, prompted the subsequent synthesis of 25 analogues designed by varying either the scaffold, or the substituents of 3,5-dichlorophenylaminomethylenebisphosphonic acid (Cl_2PAMBPA; Fig. 1), the most active molecule. Although none of these compounds were more potent, the

availability of several active structures allowed a proper SAR analysis, leading us to hypothesize about the steric and electronic requirements for maintenance or enhancement of the inhibitory properties.[21] Further studies pointed out that the most active aminobisphosphonates are also able to inhibit glutamine synthetase (GS) [EC 6.3.1.2], the enzyme that plays a key role in ammonia assimilation.[22] Because glutamate starvation deriving from GS inhibition is expected to block the synthesis of any nitrogen-containing metabolite, and in all cases lower concentrations were required to reduce *in vitro* GS activity to 50% (Fig. A in supporting information), the question arose as to the significance *in planta* of P5C reductase inhibition. To address this issue, we synthesized and screened other bisphosphonate derivatives.[23] Among the new active substances, some showed differential inhibition *in vitro* against GS and P5C reductase. Here we report the evaluation of their effects on rapeseed seedling growth. Results strengthen the possibility that the inhibition of both targets may occur *in vivo*, likewise contributing to the phytotoxic effects of this class of compounds.

2. MATERIALS AND METHODS

2.1 Chemistry

Chemicals were obtained from Sigma-Aldrich or Merck Chemical Companies, and were of analytical grade. DL-P5C was synthesized by the periodate oxidation of δ-*allo*-hydroxylysine, and purified by cation-exchange chromatography on a Dowex AG50 (200-400 mesh) column, as described.[24] Synthesis, yields and spectral data for 3,5-dichlorophenylaminomethylene-bisphosphonic acid (Cl_2PAMBPA), 3,5-dibromophenylaminomethylenebisphosphonic acid (Br_2PAMBPA) and 5,6,7,8-

tetrahydro-2-naphthylamino-methylenebisphosphonic acid (H_4NAMBPA) (Fig. 1) have been reported previously.[21, 23]

2.2 Plant growth conditions

Rapeseed (*Brassica napus* L., cv. Zeruca) seeds were surface-sterilized by sequential treatment for 5 min with absolute ethanol and 5 min under vacuum with a 3% NaClO solution containing 0.04% (v/v) Triton X-100. Following extensive washing with sterile distilled water, seeds were sown in GA7 Magenta vessels containing 100 mL of agarized (6‰) half-strength Murashige and Skoog[25] (MS) medium (brought to pH 6.75 with KOH) supplemented with 10 g L^{-1} sucrose and 2 mL L^{-1} Plant Preservative Mixture (Plant Cell Technology); 16 seeds were sown in each vessel, which was incubated at 25 ± 1°C under 300 µmol m^{-2} sec^{-1} PAR with a 16:8 light:dark photoperiodic cycle. After 8 days of growth, the lid was replaced with a coupler and a second GA7 vessel, obtaining a 20-cm height vessel. Under these conditions, untreated plants reached the three-leaf stage 15 days after sowing. Callus tissue was induced by transferring 0.5-cm stem fragments into 9-cm Ø petri dishes containing 25 mL of MS medium supplemented with 30 g L^{-1} sucrose and 0.5 mg L^{-1} of both 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. Once established, undifferentiated cell cultures were grown in either solid or liquid medium at 24 ± 1°C under dim (<50 µmol m^{-2} s^{-1}) light, subculturing every 21 or 14 days, respectively.

2.2.1 Plant treatments

The effect of each inhibitor upon seedling growth was evaluated by means of an experimental design consisting of a randomized complete block with five replicates. Each block comprised 20 Magenta vessels of three inhibitor rates (50, 100 and 200 µM) and untreated controls. Stock solutions (10 mM) of the compounds were brought

to pH 7 with KOH and filter-sterilized (0.22 µm); suitable aliquots were added to the medium just before sowing. After 16 days of incubation under the above conditions, the plant biomass was measured by destructive harvest. For each seedling, roots and shoots were weighted separately. Then the material was treated in an oven at 90°C for 2 days for the determination of the dry weight. The same experimental design was used to obtain material for measuring the intracellular concentration of amino acids and various metabolites. Alternatively, plants were allowed to grow in the absence of any inhibitor for 10 days, until the first true leaf stage was reached. Then suitable aliquots of the stock solutions were spread onto the surface of the agarized medium. Seedlings were harvested and used for measurements 3 to 7 days thereafter.

2.3 Enzyme extraction and purification

Seedling shoots (about 40 g) or callus material (about 20 g) were extracted in an ice-cold mortar with 2 mL g^{-1} of 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 0.5 mM dithiothreitol. All subsequent operations were carried out at 0 to 4°C. Extracts were centrifuged at 12,000 *g* for 10 min, and the proteins in the supernatant were precipitated with solid ammonium sulfate (70% of saturation). Following further centrifugation, pellets were resuspended in 20 mL of extraction buffer, and desalted on a column filled with Bio-Gel P6DG (Bio-Rad). The sample was then loaded onto a DEAE-Sephacel (Pharmacia) column (2.5 cm Ø, 30 mL bed-column) equilibrated with the extraction buffer. Proteins were eluted with a linear gradient from 0 to 400 mM KCl in 400 mL buffer at a constant flow of 1 mL min^{-1}, for the collection of 5-mL fractions. Active fractions were pooled and stored at 4°C until used. Protein concentration was measured by the Coomassie Blue method,[26] using bovine serum albumin as the standard.

2.3.1 P5C reductase assay

The forward, physiological reaction of the enzyme was followed by measuring at 35°C the P5C-dependent oxidation of NADH. In a final volume of 1 mL the assay mixture contained 100 mM Hepes-KOH buffer, pH 7.5, 2 mM DL-P5C, 0.25 mM NADH and the enzyme (0.15-0.20 nkat). The decrease in absorbance was determined for 10 min by continuously monitoring the sample at 340 nm. Parallel controls from which P5C had been omitted were also run. Activity was calculated by linear regression of the initial slope, based on an extinction coefficient for NADH of 6,220 M^{-1} cm^{-1}.

2.3.2 Glutamine synthetase assays

During enzyme purification, GS activity was measured at 35°C by the transferase assay that ensures maximal rates and does not suffer from the presence of contaminating activities. In a final volume of 0.4 mL, the reaction mixture was composed of 25 mM imidazole-HCl buffer (pH 7.4), 50 mM L-glutamine, 5 mM ADP, 25 mM NH_2OH-HCl, 40 mM sodium arsenate, 4 mM $MnCl_2$ and a limiting amount of enzyme. After up to 10 min, the reaction was blocked by the addition of 0.8 mL of a colorimetric solution consisting of 10% (w/v) $FeNO_3$ x $9H_2O$, 6.67% (v/v) HCl and 5% (w/v) trichloroacetic acid. After centrifugation at 12,000 *g* for 3 min, samples were read at 535 nm against non-incubated blanks. The amount of product formed was extrapolated from a calibration curve obtained with an authentic standard of γ-glutamyl-hydroxamate. The activity of the partially purified enzyme was measured by a different assay that measures the full-forward, physiological reaction. In this case the mixture in a final volume of 0.1 mL consisted of 50 mM Tris-HCl buffer (pH 7.4), 50 mM L-glutamate, 0.5 mM NH_4Cl, 2.5 mM ATP, 5 mM $MgCl_2$, and a limiting amount of enzyme (50 pkat). After up to 10 min at 35°C, the release of inorganic phosphate

was measured by a modification of the malachite green-acid molybdate method, as previously described.[27] For each sample, at least three different incubation times were tested; activity was calculated by linear regression of the initial slope.

2.3.3 Enzyme inhibition by aminobisphosphonates

Enzyme inhibition was evaluated by adding a suitable water dilution of the stock solution of a given compound to the reaction mixture, so as to obtain a final concentration ranging from 1 µM to 1 mM. At least three replicates were carried out for each dose. Results were expressed as percentage of untreated controls, and the concentrations causing 50% inhibition of enzyme activity (IC_{50}) were calculated by non-linear regression analysis using Prism 6 (version 6.03, GraphPad Software).

2.4 Analytical methods

For amino acid analysis, plant material was extracted in mortar with 2 mL g^{-1} of a 3% (w/v) solution of 5-sulfosalicylic acid. After removing cell debris by centrifugation at 12,000 *g* at RT, aliquots (20 µL) of the supernatant were mixed with 20 µL of *o*-phthaldialdehyde solution (0.5 M in 0.5 M sodium borate buffer, pH 10.0, containing 10% [v/v] methanol and 0.5 M β-mercaptoethanol). After exactly 60 sec, derivatized samples were injected by means of a 20 µL loop onto a 4.6 x 250 mm Zorbax ODS column (Rockland Technologies, Newport, DE), and the elution proceeded as previously described,[28] monitoring the eluate at 340 nm. This method resolved equimolar mixtures of derivatizable compounds (all protein amino acids but Cys and Pro), with a detection limit of about 0.1 nmol. Total amino acid and proline content were measured with the acid ninhydrin method.[24, 28]

For P5C quantification, plant material was extracted in 3 mL g^{-1} of 50 mM HCl with a Teflon-in-glass Potter homogenizer by 2 times 12 strokes. Extracts were

centrifuged for 10 min at 12,000 *g*, then the supernatant was loaded onto a 2 mL column filled with Dowex AG50 (200-400 mesh) that had been equilibrated with water. After extensive washing with 50 mM HCl, the column was eluted with 1 M HCl. P5C concentration in the eluate was quantified by reaction with *o*-amino-benzaldehyde, as previously described.[24, 29]

Free ammonia was measured in water extracts by the phenol-hypochlorite method.[30]

3 RESULTS AND DISCUSSION

3.1 Selected aminomethylenebisphosphonates exert differential activity *in vitro* against P5C reductase and glutamine synthetase from rapeseed

The spread of weed biotypes showing target site resistance toward herbicides[5] is a major issue in agriculture. The availability of new active principles interfering with multiple aspects of plant cell metabolism would contribute to the management of herbicide-resistant weeds. Moreover, since the rate of a double mutation conferring target-site tolerance to an inhibitor acting on two different enzymes is the product of the probability of each single mutation, for dual-target inhibitors herbicide resistance could be avoided or, at least, delayed. Some substituted derivatives of phenylaminomethylenebisphosphonic acid inhibit the activity of both P5C reductase[20] and glutamine synthetase at micromolar concentrations.[22] Unequivocal data supported the occurrence of GS inhibition in the plant cell.[31] On the other hand, because proline is synthesized from glutamate, whose production is lowered by GS inhibition, the physiological relevance *in planta* of the inhibitory potential against P5C

reductase remains to be demonstrated, even though the exogenous supply of glutamine did not completely revert growth inhibition by these compounds.[21] To address this aspect, we chose rapeseed as the experimental model, because of its economic relevance and the invasive/weed potential of the Brassicaceae family. The two enzymes were partially purified from either *B. napus* seedlings or *in vitro* cultured cells. Anion-exchange chromatography of crude extracts showed the presence of putative enzyme forms (Fig. B in supporting information). For GS, the result was expected, since no less than 16 genes coding for a cytosolic form of the enzyme have been reported[32] and at least 2 genes coding for a chloroplastic isozyme are most likely present in this tetraploid species. Anyway, the use of light-grown seedlings or dark-grown calluses as the starting material allowed us to resolve the chloroplastic and the cytosolic isozymes. In the case of P5C reductase, no information is available to date concerning the presence of multiple enzyme forms in this species. Therefore, the two peaks of activity were characterized separately.

A screening of the numerous active compounds available[23] allowed us to identify two aminobisphosphonates showing opposite effectiveness *in vitro* against the two targets. While 3,5-dichlorophenylaminomethylenebisphosphonic acid (Cl_2PAMBPA), as most bisphosphonates, exerted stronger inhibition against GS than P5C reductase, 3,5-dibromophenylaminomethylenebisphosphonic acid (Br_2PAMBPA) was found more effective against the latter (Fig. 2). The concentration of Cl_2PAMBPA capable of inhibiting GS activity by 50% (IC_{50}) was 2 to 3-fold lower than that of Br_2PAMBPA, whereas the IC_{50} for P5C reductase was 2 to 3-fold higher (Table 1). The sensitivity of isozymes was significantly different from each other, but these ratios were maintained within each pair (i.e., both the GS isoforms were inhibited by lower doses of Cl_2PAMBPA than of Br_2PAMBPA, and *vice-versa* for the two enzyme forms of P5C reductase). A third compound with substantial equipotency

against the two targets, namely 5,6,7,8-tetrahydro-2-naphthylamino-methylenebisphosphonic acid (H_4NAMBPA; Fig. 1), was also selected for the characterization of bisphosphonate effects *in vivo*.

3.2 Phytotoxicity of aminomethylenebisphosphonates and their effect on free amino acid pools do not allow to demonstrate that P5C reductase inhibition really occurs *in planta*

To investigate whether the interference with GS and/or P5C reductase activity takes place also *in vivo*, causing an actual reduction of proline/glutamine biosynthesis that could result in phytotoxic effects at the plant level, the growth of rapeseed seedlings was measured in the presence of increasing concentrations of the three compounds. Both Cl_2PAMBPA and Br_2PAMBPA progressively inhibited plant growth in the 50 to 200 µM range, whereas the inhibition brought about by H_4NAMBPA was significantly lower (Table 2). Reduction of shoot growth was similar to that of roots, suggesting that the compounds are translocated to the aerial part of the plant. Interestingly, at high concentrations Br_2PAMBPA was slightly more effective than Cl_2PAMBPA, while its activity *in vitro* against GS was significantly lower. However, this cannot be taken into account to prove that the inhibition of P5C reductase occurs *in vivo*, since *in planta* several other factors, such as differential uptake, translocation, or compartimentalization driven by hydrophobic/hydrophilic properties of a given inhibitor, can drastically influence the amounts that reach a target tissue, and vary consequently its relative effectiveness compared to that observed *in vitro*.

Because this is unlikely to occur in cell suspension cultures, where each cell is in direct contact with the culture medium, the effect of the two most active compounds on the growth of rapeseed cultures was investigated (Fig. 3). In this case also, the dose-activity relationship for the two compounds was very similar. Quite surprisingly,

in the case of Br_2PAMBPA the effect on actively proliferating cells was lower than that pointed out for seedlings, with IC_{50} values of 285 ± 50 μM and 134 ± 13 μM, respectively. On the contrary, for Cl_2PAMBPA the effect was almost identical, with IC_{50} values of 267 ± 25 μM and 247.2 ± 20 μM.

As cultured cells, unlike differentiated tissues in plants, have homogeneous characteristics and usually respond uniformly to a given experimental condition, the same system was used to assess the effect of the treatment with increasing concentrations of either inhibitor on free amino acid pools. There was a significant reduction of Glu and Gln pools (Tables 3 and 4 for Cl_2PAMBPA and Br_2PAMBPA, respectively). Taken together, in both cases the treatment with 200 or 350 μM causes a 35% and a 50%-reduction of their absolute concentration. A similar trend was evident also for Asn and Ala, but not for all other amino acids, whose levels were reduced to a lower extent. The whole picture is consistent with that expected as a consequence of the inhibition of the GS-GOGAT cycle.[31,33] In fact, the stronger effects were evident for those amino acids whose synthesis is strictly related to glutamate availability, such as GABA (the product of Glu decarboxylation) and alanine (which is the product of pyruvate transamination using Glu as the nitrogen donor). The results were much less clear-cut with proline. With Cl_2PAMBPA, following the treatment with 200 and 350 μM free Pro levels decreased only by 16 and 28%, respectively, an effect significantly lower than those on GABA and Ala. As a consequence, it is not possible to distinguish whether this decrease is a direct consequence of P5C reductase inhibition, or the indirect effect of a lower Glu availability. In the case of Br_2PAMBPA, the effect was even lower, with free Pro levels in cells treated with 350 μM inhibitor not significantly different from those in untreated controls. Since Br_2PAMBPA is a stronger inhibitor of P5C reductase than Cl_2PAMBPA, but a milder inhibitor of GS, data would imply that inside the cell the

inhibition of Pro synthesis, if any, plays a marginal role with respect to the inhibition of Glu production.

3.3 Analysis of treated seedlings provides compelling evidence that Br_2PAMBPA does interfere with P5C reductase activity *in planta*, and suggests that the accumulation of the intermediate P5C plays a main role in inhibiting plant growth

This notwithstanding, even if the effect on proline synthesis would be of secondary importance, the presence of a secondary target of herbicidal aminobisphosphonates could avoid the diffusion of weed biotypes in which a mutation had lowered the susceptibility of GS to their action. Because the treatment with Br_2PAMBPA had been found to exert a stronger inhibition of seedling growth (Fig. C in supporting information), its effects were further investigated at the plant level. At first, the concentration of free amino acids and proline was determined in seedlings at the 2-leaf-stage at increasing time after the treatment with 200 µM Br_2PAMBPA, a dose that had been found to inhibit by 80% plant growth if applied at sowing (Table 2). Because different levels of free amino acid may be present in different tissues, and progressively decreasing doses of inhibitor are expected to reach stems and leaves following root uptake, amino acid content was quantified separately for roots, stems and leaves. There were no significant changes with respect to total amino acid content in these three plant organs up to 7 days after the treatment (Fig. 4). Only in roots and only for the last time point a significant (P = 0.05) difference was found, but treated samples showed an increased content instead of the expected decrease. In the case of proline, a much remarkable increase was evident already 2 days after the treatment in roots, which was maintained thereafter. Although apparently inconsistent with the hypothesis of a reduction of proline synthesis due to the inhibition of P5C

reductase, these data were consistent in part with those obtained with cultured cells treated with 350 μM Br_2PAMBPA, and pointed at a specific interference with proline metabolism.

Because time patterns in Fig. 4 were suggestive of a slow diffusion of the compound inside the plant, further investigation was carried out on seedlings directly sown in the presence of increasing levels of the aminobisphosphonate, the system in which the strongest effects had been found (Fig. C in supporting information). In this case, at the highest rate a significant increase of free proline content was found in all plant organs (Fig. 5). Interestingly, a similar and dose-proportional increase of total amino acid content was also evident. A general increase of free amino acids in the plant cell has been described in several instances as a consequence of the reduction of protein synthesis and/or the induction of proteolytic activities under stress conditions able to inhibit cell growth, such as salt treatments and drought.[33-34] But in the absence of glutamine and/or proline starvation, it would be unclear what may be in this case the factor(s) causing cell growth inhibition. For a natural and potent inhibitor of GS, phosphinothricin, it has been proven that glutamate depletion is not the primary cause of plant cell death: instead, GS inhibition rapidly leads to ammonia accumulation that acts as an uncoupler, causing cytotoxicity.[15,22] To verify whether also in this case ammonia is accumulated in seedlings in response to the treatment with Br_2PAMBPA, its levels were determined in rapeseed tissues. There was only a slight increase in roots and stems, and no significant variation in leaves (Fig. 5). Because in the same leaves both free amino acid and proline levels had been found greatly increased, and considering that in cultured cells the treatment with growth-inhibitory levels of phosphinothricin triggered an up to 15-fold increase of ammonia over basal levels (Forlani G., unpublished results), it seems quite unlikely that a GS inhibition-induced accumulation of ammonia may be the cause of Br_2PAMBPA

toxicity.

As an alternative, the inhibition of P5C reductase could determine the intracellular accumulation of toxic P5C levels, which has been invoked as the cause of the phytoxicity of exogenously-administered proline.[29] Although still debated, increasing evidence showed that changes in mitochondrial P5C synthesis may be the cause of reactive oxygen species production and induce the hypersensitive reaction-associated cell death during the plant response to pathogens.[17] Therefore, P5C levels were quantified in Br_2PAMBPA-treated seedlings. There were consistent significant increases over basal levels of P5C that were proportional to the severity of the treatment and exhibited a decreasing pattern from the roots to the shoots (Fig. 5), supporting that the inhibition brought about *in vitro* by Br_2PAMBPA on P5C reductase activity takes place also *in planta*, and that the consequent accumulation of P5C may be the main cause of its phytotoxicity. This picture may explain also the apparent inconsistency of some previous results. In the case of cultured cells, the lower susceptibility to this aminobisphosphonate may depend on the rapid rate of cell proliferation, which *dilutes* the toxic P5C levels. In seedlings, the accumulation of proline and amino acids in treated tissues (which seems to contradict the reason for P5C accumulation) may be caused by the non-utilization of amino acids deriving from reserve protein in the seeds because of the general impairment of cell metabolism. Experiments are currently in progress to shed more light on the mechanism underlying Br_2PAMBPA phytotoxicity, *i.e.* to ascertain the possible induction of programmed cell death by P5C in treated plants.

3.4 Conclusions

Experimental evidence supports the occurrence *in planta* of the inhibition of P5C reductase activity by aminobisphosphonates, which at least in the case of

Br_2PAMBPA seems to represent the main factor of phytotoxicity. Since these compounds also inhibit the activity of GS *in vivo*, phenyl-substituted aminobisphosphonates may therefore be regarded as dual-target inhibitors. Their use to develop new active principles for crop protection could consequently represent a tool to address the problem of target-site resistance among weeds.

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Table 1. Concentrations of aminomethylenebisphosphonic acids able to inhibit by 50% (IC_{50}) the activity of P5C reductase and glutamine synthetase from rapeseed.

	IC_{50} (μM)[a]			
	P5C reductase		glutamine synthetase	
compound	isoform I	isoform II	plastidial	cytosolic
Cl_2PAMBPA	78.3 ± 4.8	133 ± 19	10.1 ± 0.4	37.5 ± 2.1
Br_2PAMBPA	40.1 ± 3.2	29.7 ± 2.0	26.3 ± 2.4	95.4 ± 4.5
H_4NAMBPA	499 ± 42	646 ± 157	128 ± 11	670 ± 37

[a] IC_{50} values and their 95% confidence limit were computed by non-linear regression analysis of activity values, expressed as percentage of untreated controls.

Table 2. Inhibition of rapeseed seedling growth by selected aminomethylenebis-phosphonic acids.

		Dry weight (%)[a]		
compound	µM	shoots	roots	seedlings
Cl_2PAMBPA	0	100.0 ± 4.4	100.0 ± 4.3	100.0 ± 4.3
	50	79.5 ± 3.4	65.5 ± 2.9	75.9 ± 3.3
	100	63.1 ± 2.6	56.6 ± 3.1	63.0 ± 2.6
	200	30.9 ± 2.0	40.2 ± 2.8	32.1 ± 2.0
Br_2PAMBPA	0	100.0 ± 5.0	100.0 ± 9.1	100.0 ± 4.7
	50	82.0 ± 4.0	65.4 ± 3.6	80.0 ± 3.9
	100	47.5 ± 2.3	40.6 ± 2.8	46.8 ± 2.2
	200	18.6 ± 1.3	18.5 ± 1.8	18.6 ± 1.2
H_4NAMBPA	0	100.0 ± 4.7	100.0 ± 5.0	100.0 ± 4.7
	50	85.4 ± 4.5	93.5 ± 4.7	87.0 ± 4.5
	100	78.3 ± 3.0	74.5 ± 3.3	78.4 ± 2.9
	200	58.2 ± 2.9	58.4 ± 3.6	58.3 ± 2.9

[a] Data were expressed as percentage of untreated controls, and are means ± SE over 74 to 80 replicates, depending on germination rate.

Table 3. Free amino acid content[a] in rapeseed cultured cells 3 days after the treatment with increasing concentrations of Cl_2PAMBPA.

	untreated control		200 µM		350 µM	
a.a.	µmol (g fw)$^{-1}$	%	µmol (g fw)$^{-1}$	%	µmol (g fw)$^{-1}$	%
Asp	0.752 ± 0.096	2.3	0.721 ± 0.104	3.2	0.755 ± 0.049	4.3
Glu	8.794 ± 1.120	26.4	5.365 ± 0.221	23.6	3.606 ± 0.151	20.7
Asn	1.301 ± 0.153	3.9	0.770 ± 0.059	3.4	0.677 ± 0.007	3.9
Ser	0.472 ± 0.138	1.4	0.331 ± 0.184	1.5	0.196 ± 0.075	1.1
Gln	10.606 ± 0.973	31.8	7.026 ± 0.710	31.0	5.621 ± 0.202	32.3
Arg	0.118 ± 0.010	0.4	0.111 ± 0.023	0.5	0.112 ± 0.016	0.6
Gly	0.151 ± 0.019	0.5	0.146 ± 0.008	0.6	0.147 ± 0.013	0.8
Thr	0.277 ± 0.025	0.8	0.243 ± 0.027	1.1	0.225 ± 0.031	1.3
Ala	4.156 ± 0.337	12.5	2.033 ± 0.425	9.0	1.214 ± 0.183	7.0
GABA	4.533 ± 0.424	13.6	3.566 ± 0.734	15.7	2.913 ± 0.342	16.7
Tyr	0.324 ± 0.065	1.0	0.785 ± 0.914	3.5	0.451 ± 0.515	2.6
Trp	0.044 ± 0.001	0.1	0.038 ± 0.005	0.2	0.040 ± 0.015	0.2
Met	0.064 ± 0.043	0.2	0.063 ± 0.062	0.3	0.049 ± 0.025	0.3
Val	0.520 ± 0.101	1.6	0.405 ± 0.042	1.8	0.376 ± 0.057	2.2
Phe	0.080 ± 0.014	0.2	0.093 ± 0.011	0.4	0.108 ± 0.015	0.6
Ile	0.102 ± 0.008	0.3	0.106 ± 0.009	0.5	0.098 ± 0.011	0.6
Leu	0.084 ± 0.011	0.3	0.083 ± 0.010	0.4	0.084 ± 0.013	0.5
Lys	0.110 ± 0.007	0.3	0.113 ± 0.001	0.5	0.135 ± 0.007	0.8
Pro	0.830 ± 0.103	2.5	0.697 ± 0.075	3.1	0.602 ± 0.023	3.5
All	33.319 ± 2.739	100.0	22.695 ± 1.176	100.0	17.408 ± 0.086	100.0

[a] Results are mean ± SE of three independent replicates.

Table 4. Free amino acid content[a] in rapeseed cultured cells 3 days after the treatment with increasing concentrations of Br_2PAMBPA.

	untreated control		200 µM		350 µM	
a.a.	µmol (g fw)$^{-1}$	%	µmol (g fw)$^{-1}$	%	µmol (g fw)$^{-1}$	%
Asp	0.758 ± 0.051	2.4	0.875 ± 0.015	4.3	0.802 ± 0.103	4.6
Glu	8.500 ± 0.498	27.2	5.008 ± 0.415	24.4	2.522 ± 0.608	14.4
Asn	1.123 ± 0.081	3.6	0.730 ± 0.080	3.6	0.908 ± 0.118	5.2
Ser	0.344 ± 0.148	1.1	0.213 ± 0.027	1.0	0.159 ± 0.024	0.9
Gln	8.820 ± 0.274	28.2	5.973 ± 0.954	29.1	6.593 ± 0.663	37.6
Arg	0.095 ± 0.017	0.3	0.114 ± 0.026	0.6	0.124 ± 0.003	0.7
Gly	0.136 ± 0.009	0.4	0.132 ± 0.038	0.6	0.139 ± 0.005	0.8
Thr	0.314 ± 0.011	1.0	0.251 ± 0.021	1.2	0.227 ± 0.011	1.3
Ala	2.994 ± 0.167	9.6	1.349 ± 0.238	6.6	0.622 ± 0.101	3.5
GABA	5.218 ± 0.263	16.7	3.401 ± 0.746	16.6	2.863 ± 0.081	16.3
Tyr	1.049 ± 0.052	3.4	0.809 ± 0.153	3.9	0.660 ± 0.259	3.8
Trp	0.047 ± 0.008	0.2	0.053 ± 0.009	0.3	0.098 ± 0.033	0.6
Met	0.085 ± 0.028	0.3	0.084 ± 0.021	0.4	0.111 ± 0.023	0.6
Val	0.561 ± 0.023	1.8	0.413 ± 0.066	2.0	0.378 ± 0.067	2.2
Phe	0.078 ± 0.022	0.2	0.107 ± 0.033	0.5	0.199 ± 0.042	1.1
Ile	0.116 ± 0.011	0.4	0.102 ± 0.013	0.5	0.120 ± 0.031	0.7
Leu	0.096 ± 0.003	0.3	0.100 ± 0.020	0.5	0.086 ± 0.015	0.5
Lys	0.135 ± 0.015	0.4	0.139 ± 0.010	0.7	0.198 ± 0.024	1.1
Pro	0.775 ± 0.050	2.5	0.683 ± 0.030	3.3	0.741 ± 0.018	4.2
All	31.244 ± 1.362	100.0	20.538 ± 2.563	100.0	17.548 ± 1.188	100.0

[a] Results are mean ± SE of three independent replicates.

Legends to Figures.

Figure 1. Phenyl-substituted derivatives of aminomethylenebisphosphonic acid evaluated in the present work.

Figure 2. Effect of increasing concentrations of selected bisphosphonates on P5C reductase and glutamine synthetase activity *in vitro*. Enzyme forms were resolved from either rapeseed seedlings or cultured cells (Fig. B of supporting information). Data, expressed as percentage of untreated controls, are means ± SE over three replications.

Figure 3. Effect of increasing concentrations of Cl_2PAMBPA and Br_2PAMBPA on the growth of rapeseed cell suspension cultures. Data, expressed as percent of untreated controls, are means ± SE of four replicates.

Figure 4. Effect of the treatment with 200 µM Br_2PAMBPA on free proline and total amino acid content in rapeseed seedlings. Seedlings at the 2-leaf-stage were treated with the inhibitor, and amino acid concentrations were determined 3, 5 and 7 days after the treatment. In all cases, data are means ± SE of three replicates.

Figure 5. Free proline and total amino acid content in roots, stems and leaves of rapeseed seedlings directly sown in the presence of increasing concentrations of Br_2PAMBPA. Seedlings were estracted 2 weeks after sowing. The intracellular levels of ammonia and P5C were also measured; in the last case, the effect of the inhibitor at 50 µM was not determined. Data are means ± SE over three replicates.

Figure 1

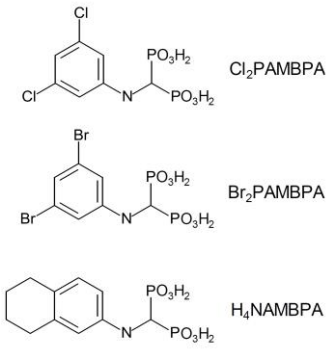


Figure 2

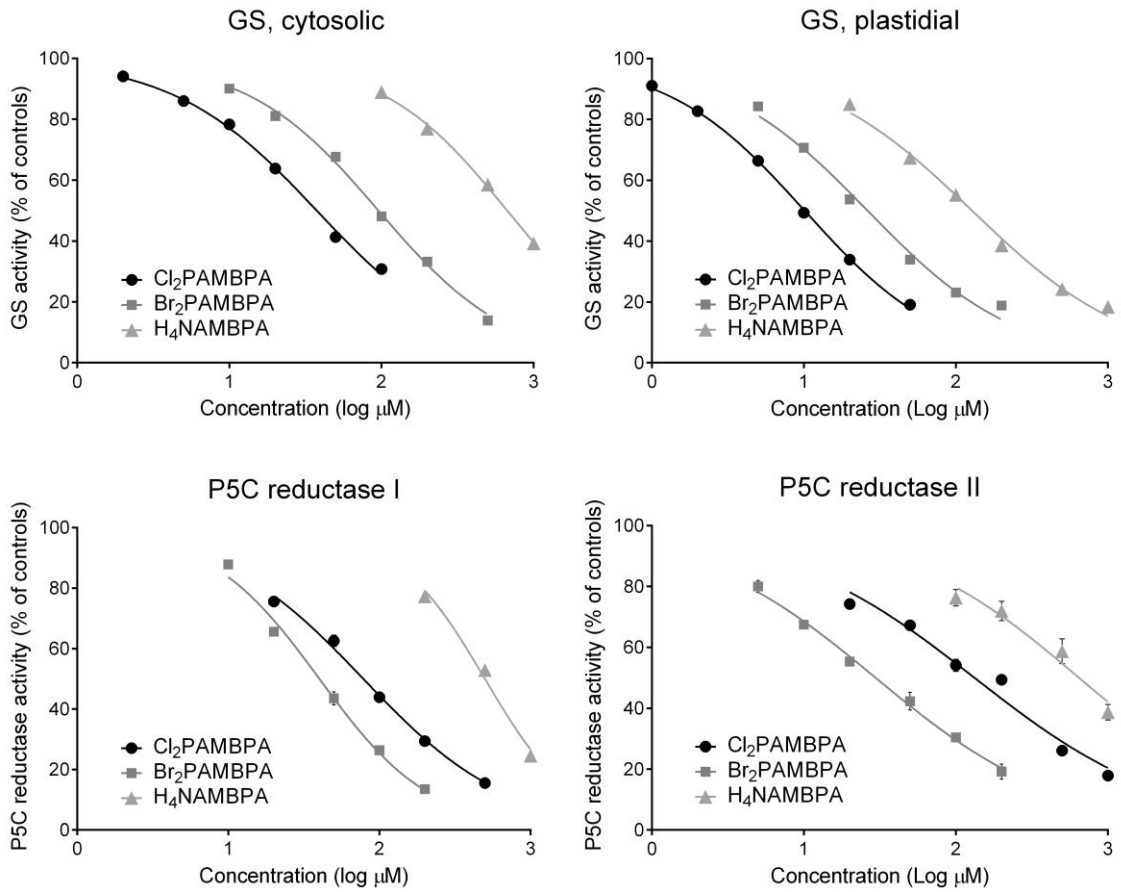


Figure 3

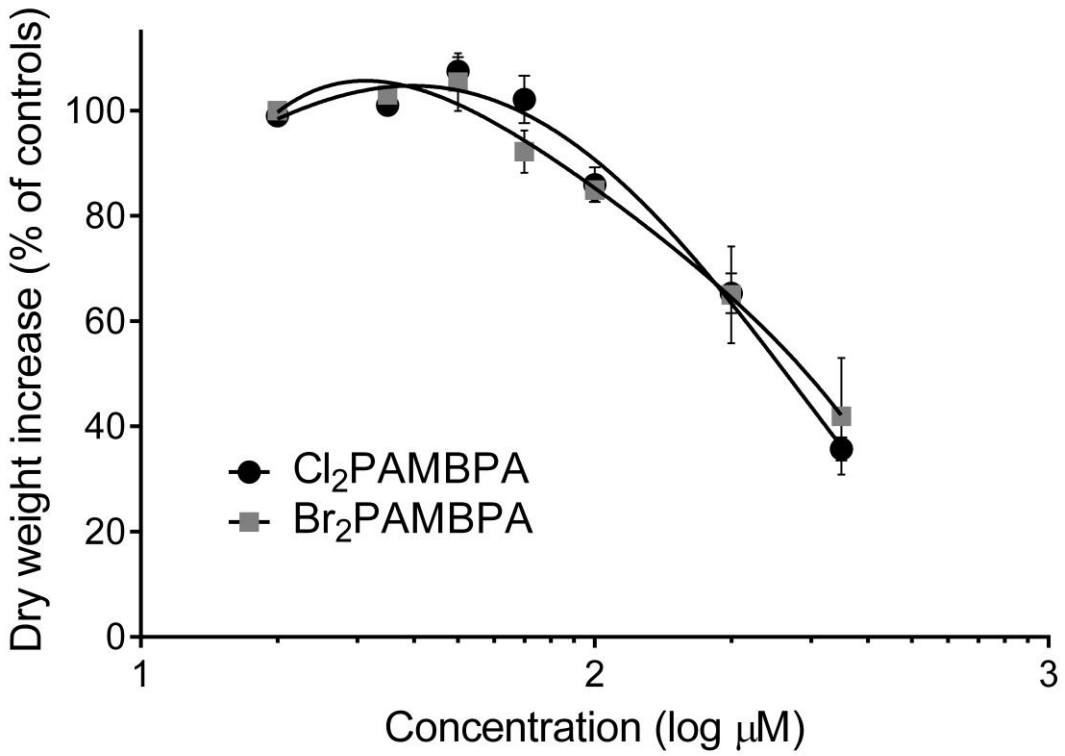


Figure 4

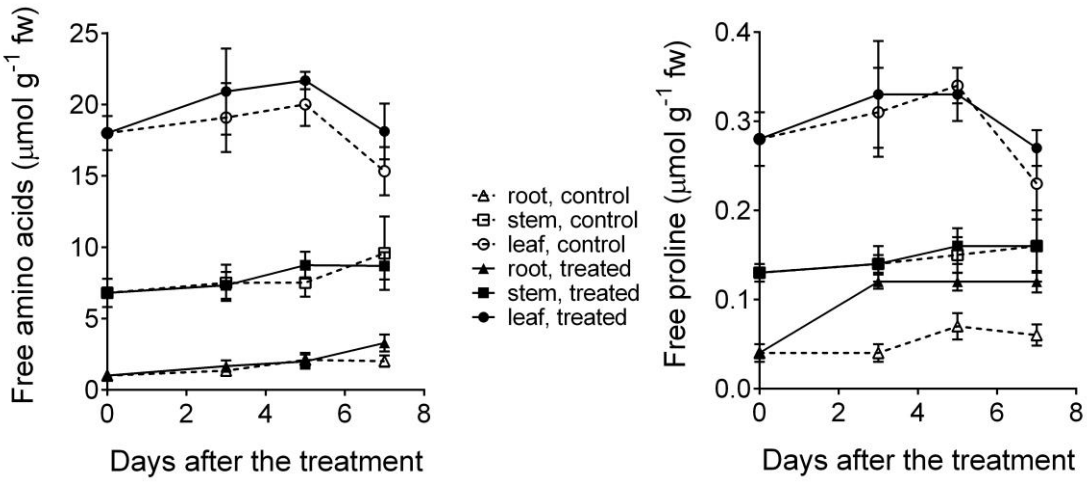
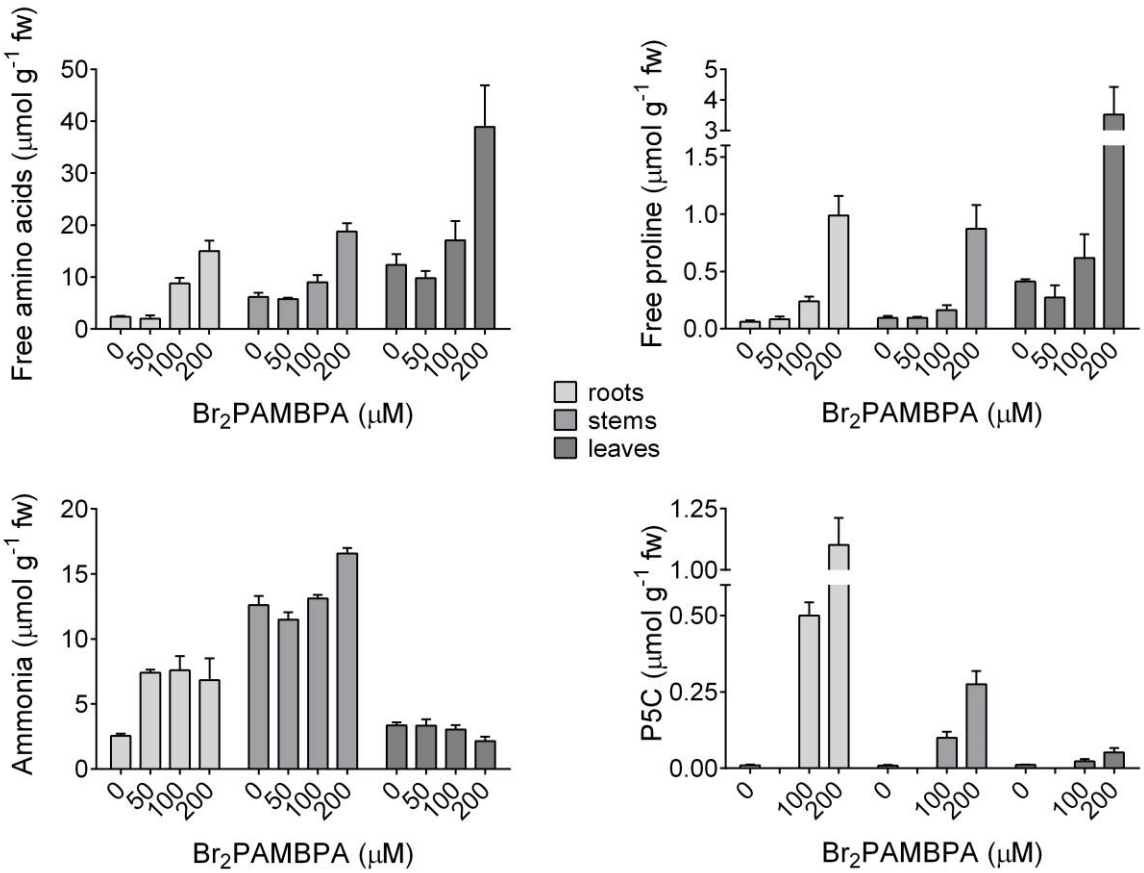


Figure 5


Free amino acids (μmol g-1 fw)
Free proline (μmol g-1 fw)
Ammonia (μmol g-1 fw)
P5C (μmol g-1 fw)
Br2PAMBPA (μM)
roots
stems
leaves