Review: Amino Acid Biosynthesis as a Target for Herbicide Development

Running title: Amino acid biosynthesis inhibitors as herbicides

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ABSTRACT

There are three amino acid biosynthesis pathways that are targeted by current herbicides, namely those leading to the production of aromatic amino acids, branched chain amino acids and glutamine. However, their efficacy is diminishing due to the increasing number of resistant weeds. Indeed, resistance to most classes of herbicides is on the rise, posing a significant threat to the utility of current herbicides to sustain effective weed management. This review provides an overview of potential herbicide targets within amino acid biosynthesis that remain unexploited commercially, and recent inhibitor discovery efforts. Despite contemporary approaches to herbicide discovery, such as chemical repurposing and the use of omics technologies, there have been no new products introduced to the market that inhibit amino acid biosynthesis over the past three decades. This highlights the chasm that exists between identifying a potent inhibitor and introducing a commercial herbicide. The unpredictability of a mode of action at the systemic level, as well as poor physiochemical properties, often contribute to a lack of progression beyond the target inhibition stage. Nevertheless, it will be important to overcome these obstacles for the development of new herbicides to protect our agricultural industry and ensure food security for an increasing world population.

Keywords: amino acids; aromatic; branched chain; glutamine; herbicide resistance; weeds

1 INTRODUCTION

Herbicides are an integral part of our agricultural industry as they ensure effective and economical weed management. However, our ability to provide food security for an increasing world population is being threatened by the evolution of herbicide-resistant weeds. Such weeds displace native plant species and aggressively compete with crops, resulting in decreased harvest yields and quality.¹ The impact of herbicide resistance is exacerbated by the lack of new herbicides with a novel mode of action entering the market in the past 30 years.^{2,3}

Many of our current herbicides inhibit specific enzymes within the biosynthetic pathways of amino acids in plants. Amino acids are not only building blocks for protein synthesis, but they also play pivotal roles in physiological processes such as serving as precursors for secondary metabolites and in carbon and nitrogen metabolism, which are critical for plant growth and development.⁴ Moreover, plants synthesise their own amino acids, whereas in humans, several of these biosynthesis pathways either use different enzymes or are absent. Despite the potential of amino acid biosynthesis pathways as herbicide targets, there are currently only three major classes of inhibitors on the market - inhibitors of the biosynthesis of aromatic amino acids, branched chain amino acids and glutamine. Such herbicides typically have broad-spectrum activity due to the high degree of conservation of these pathways across plant species. Resistant crops are therefore relied upon to achieve selectivity, which have been typically generated using target alteration and detoxification strategies.⁵ An overview of these herbicides that target amino acid biosynthesis is briefly discussed here. Furthermore, we describe contemporary methodologies for the discovery of new inhibitor leads, published inhibitors of amino acid

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biosynthetic enzymes and the potential reasons why these have not seen commercial success. Lastly, we discuss several of the difficulties we face in the pursuit of discovering novel herbicides targeting amino acid biosynthesis.

2 INHIBITORS OF AMINO ACID BIOSYNTHESIS

2.1 Inhibition of aromatic amino acid biosynthesis

Aromatic amino acids, namely Phe, Trp and Tyr, are derived from the precursor chorismate, which is an intermediate of the shikimate pathway (Figure 1A).⁶ This pathway is found in plants, archaea, bacteria, fungi, algae and apicomplexans.⁶ In plants, the pathway is localised to the plastids and begins with the condensation of phospho*enol*-pyruvate and erythrose 4-phosphate (Figure 1A).⁶ Subsequent reactions result in the production of shikimate 3-phosphate that undergoes an *enol*pyruvate group transfer with phospho*enol*-pyruvate, which is catalysed by 5-*enol*pyruvyl-shikimate 3-phosphate synthase (EPSPS, EC 2.5.1.19), to yield 5-*enol*pyruvyl-shikimate 3-phosphate.⁶ The product is then dephosphorylated by chorismate synthase (EC 4.2.3.5) to produce chorismate, which is eventually converted to the aromatic amino acids via multiple enzyme-catalysed reactions (Figure 1A).⁶

EPSPS is the target for the herbicide glyphosate (Figure 1B).⁶ Glyphosate is an aminophosphonic analogue of glycine with a high specificity for EPSPS. Specifically, it occupies the phospho*enol*-pyruvate binding pocket, where it interacts with several residues (Figure 1C).⁷ The systemic herbicidal effects of glyphosate have been proposed to be associated with not only the inhibition of aromatic amino acids, but also with the deregulation of carbon metabolism, which can be attributed to the accumulation of shikimate.⁸

2.2 Inhibition of branched chain amino acid biosynthesis

The branched chain amino acid biosynthesis pathway results in the production of Ile, Leu and Val in plants, archaea, bacteria and fungi.⁹ In plants, the pathway is localised to the plastids with pyruvate being the common precursor (Figure 2A). Although their production follows different branch points, it involves four conserved enzymatic steps catalysed by acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS, EC 2.2.1.6), ketol-acid reductoisomerase (EC 1.1.1.86), dihydroxyacid dehydratase (EC 4.2.1.9) and branched chain aminotransferase (EC 2.6.1.42).⁹ Specifically, the biosynthesis of Leu and Val begins with an ALS/AHAS-catalysed condensation reaction that involves the decarboxylation of two pyruvate molecules to produce 2-acetolactate (Figure 2A). Subsequently, ketol-acid reductoisomerase yields 2,3-dihydroxy-3-methylbutanoate, which is dehydrated to 3-methyl-2-oxobutanoate by dihydroxyacid dehydratase. From there, Val is synthesised via branched chain aminotransferase.⁹ The biosynthesis of Leu branches off and requires other enzyme-catalysed steps to produce 4-methyl-2-oxopentanoate before the final branched chain aminotransferase-catalysed reaction.⁹ Ile production requires a second precursor, 2-oxobutanoate, which is formed from the deamination of Thr by threonine ammonia-lyase, also known as threonine deaminase (EC 4.3.1.19) (Figure 2A).⁹ Pyruvate and 2-oxobutanoate are then converted to 2-aceto-2-hydroxybutanoate via ALS/AHAS, following which, ketol-acid reductoisomerase produces 2,3-dihydroxy-3-methylvalerate.⁹ Penultimately, dihydroxyacid dehydratase yields 2-oxo-3-methylvalerate, which is converted to Ile by branched chain

aminotransferase.⁹ The pathway is allosterically regulated via the co-operative inhibition of ALS/AHAS by the three branched chain amino acids.¹⁰

There are several commercially available ALS/AHAS inhibitors, including sulfonylureas (Figure 2B), which were the first to be characterised in the 1970s. Since then, other classes have been discovered, including imidazolinones, triazolopyrimidines, pyrimidinyl(thio)benzoates and sulfonylamino-carbonyltriazolinones (Figure 2B).^{9,11} These herbicides block the entrance to the ALS/AHAS active site, resulting in either uncompetitive or non-competitive inhibition.¹² Despite the differences in structures between the ALS/AHAS inhibitors, there are conserved interactions with the enzyme as illustrated in Figure 2C.¹² It was initially concluded that amino acid depletion was solely responsible for the herbicidal effects of ALS/AHAS inhibitors.¹³ However, other factors have been postulated to be involved in their herbicidal activity, including the toxic accumulation of intermediates, inhibition of DNA synthesis and impaired transport of assimilates.¹³

2.3 Inhibition of glutamine biosynthesis

Glutamine biosynthesis occurs in archaea, bacteria and eukaryotes, including plants and humans.^{14,15} In plants, the pathway is localised to both the plastids and cytosol and is involved not only in amino acid production, but also in the assimilation of toxic free ammonia.¹⁴ This detoxification commences with the conversion of Glu to Gln via a condensation reaction, using ATP and ammonia, catalysed by glutamine synthetase (GS, EC 6.3.1.2) (Figure 3A).¹⁴ Glutamine oxoglutarate aminotransferase, also known as glutamate synthase (EC 1.4.1.14),

utilises either NADPH or ferredoxin as a co-factor to catalyse the conversion of Gln and the tricarboxylic acid cycle intermediate, 2-oxoglutarate, back into Glu.¹⁴

There are several inhibitors of GS described in the literature.¹⁵⁻¹⁷ The most commonly used is the racemic compound, glufosinate-ammonium (glufosinate) (Figure 3B), with only the L-isomer being a competitive inhibitor of GS.⁴ L-Phosphinothricin (equivalent to the active isomer of glufosinate) is derived from the microbially-produced tripeptide L-alanyl-L-alanyl-phosphinothricin named bialaphos (Figure 3B), which becomes active upon hydrolysis after uptake into the plant and is thus referred to as a 'proherbicide'.⁴ The systemic phytotoxicity of glufosinate has been attributed to the inhibition of Glu production as well as the accumulation of toxic levels of ammonia.⁴ Other well-characterised inhibitors of GS include methionine sulfoximine and tabtoxinine β -lactam (wildfire toxin) (Figure 3B), although concerns over off-target toxicity and a lack of systemic phytotoxicity, respectively, have been reported.¹⁷ It is generally accepted that the mechanism of action is similar amongst these inhibitors, where upon binding to GS, they are phosphorylated in an ATP-dependent manner. This results in a non-covalent but very strong binding of the modified inhibitors to the active site, which blocks binding of glutamate (Figure 3C).^{15,16,18,19}

3 APPROACHES TO IDENTIFY HERBICIDE LEADS

Traditional herbicide discovery approaches include the use of high-throughput screening of compound libraries against plants and more targeted approaches against a specific target of interest to identify hits. The development of hits into commercial herbicides is an arduous and

costly process, and any potential agrichemicals must go through stringent toxicology, residue and environmental assessments before release to the market. Repurposing drugs has become increasingly popular in recent years as it has several advantages over *de novo* discovery, including known safety profiles, optimised physicochemical properties and reduced commercialisation time.²⁰ This approach has been proposed to be amenable to herbicide discovery due to the high degree of similarity between agrichemicals and medicinal compounds, as the twenty most common functional group substituents in drugs have also been found in herbicides.²¹ Furthermore, the targets for these drugs are typically not found in humans or are structurally different. Recently, this strategy has been applied in herbicide discovery, leading to the finding that the antibiotic ciprofloxacin has herbicidal activity through the same mechanism as in bacteria, i.e., the inhibition of DNA gyrase.²² Similarly, investigations of antimalarial drugs have resulted in the discovery of promising compounds with pre- and post-emergence efficacy against both monocots and dicots.²³ Although these drugs could not be used as herbicides due to the risk of rapid evolution of resistance to life-saving medicines, analogues could be produced with reduced antibacterial/antimalarial activity and increased herbicidal properties. The pathways leading to the production of amino acids have long been targeted for the development of new classes of antibiotics or antifungals given their essentiality in these organisms.²⁴ Nevertheless, none of these compounds have made it to the clinic to date. Given the high degree of similarity (especially at the active site) often observed between orthologues from different kingdoms, repurposing inhibitors discovered against bacterial/fungal enzymes in amino acid biosynthesis as herbicide candidates could represent a promising approach to herbicide discovery. This would be particularly appealing for inhibitor

scaffolds that lack *in vivo* activity against bacteria and fungi as they would not interfere with soil microbe communities. Furthermore, such studies could help us identify promising new modes of action for future exploration.

Whilst repurposing existing drugs can provide a shortcut to herbicide discovery, there is also an urgent need to identify novel herbicide leads. The discovery of natural products as herbicides involves the identification of secondary metabolites, which traditionally includes screening for bioactivity, followed by identification of the active metabolite.^{25,26} This process has become streamlined in recent years with advances in omics techniques. In particular, genome mining has recently been used to identify a second dihydroxyacid dehydratase-encoding gene (*AstD*), involved in the branched chain amino acid biosynthesis pathway, in the soil fungus *Aspergillus terreus*.²⁷ The second *AstD* gene was found as part of a gene cluster that encodes enzymes responsible for the production of aspterric acid; a dihydroxyacid dehydratase inhibitor with low micromolar potency *in vitro* and herbicidal efficacy against monocots and dicots.²⁷ Interestingly, the newly discovered *AstD* gene was shown to encode for a dihydroxyacid dehydratase protein that is insensitive to aspterric acid inhibition,²⁷ which may provide a future route to afford selectivity through the development of herbicide-tolerant crops. This also highlights the potential of re-visiting enzymes in biosynthetic pathways that have already been exploited for herbicide development. Examples of such inhibitors as herbicides have been discussed elsewhere.²⁸

An important point to consider in herbicide discovery is the requirement of a potential novel target on metabolic processes in plants to increase the likelihood that its inhibition will lead to

herbicidal effects.⁴ In addition, its presence or role in non-target organisms impacts the potential for toxicity.⁴ Time consuming target validation studies are therefore necessary before embarking on inhibitor development. This, combined with costly high-throughput chemical screens for initial hit identification, presents a barrier to ‘starting from scratch’ with novel targets. As such, identifying new chemistries by modifying existing compounds as well as methods for circumventing resistance mechanisms to current herbicides have been favoured in recent years.^{29,30} However, the ability to successfully modify compounds is largely dependent on understanding the molecular determinants underpinning the inhibition of the target enzymes.^{28,31} In cases where this information is lacking, the progress of derivatives into a commercial herbicide has been hampered. For example, the lack of a plant EPSPS crystal structure has hindered a comprehensive elucidation of the EPSPS reaction mechanism, and as such, herbicidal glyphosate analogues have not been developed yet.³¹ In contrast, several derivatives of ALS/AHAS and GS inhibitors have been developed as herbicides; the reaction mechanisms of which are well understood.^{15,32} *In silico* docking analyses of inhibitors with their targets are increasingly driving the design of more promising analogues. This approach has been used to show that the reduced activity of glyphosate analogues is likely due to a decrease in conformational flexibility.³¹ This insight facilitated the proposal of new analogues, which would be more likely to have improved activity and selectivity.³¹

The adaptation of drug discovery methodologies to herbicide discovery may aid in overcoming the aforementioned barriers to the commercial exploitation of novel targets. *In silico* screening of small molecule libraries has been widely used for drug discovery as it offers a low-cost,

high-throughput method for initial hit identification.³³ The relatively scarce availability of crystal structures for plant proteins, in comparison to proteins relevant to human disease, may explain why only a few studies have utilised this technique for the purposes of herbicide discovery.³³ Nevertheless, the recent development of ‘herbicide-likeness’ (alternate to ‘drug-likeness’) criteria for small molecule selection may help us adopt this approach for herbicide discovery.³⁴

Dual-target compounds offer another example of a successful drug discovery approach, which may be useful in herbicide discovery.³³ Given the increase in resistant weeds globally in a market that is dominated by herbicides that target a single enzyme, compounds with dual targets represent a novel strategy to improve efficacy through potential synergism and reducing resistance selection.³³ Whilst dual-target herbicides are yet to be developed, single-target herbicides applied in combination have been shown to delay resistance selection in modelled simulations.³⁵ However, resistance to nearly all current herbicide modes of action available for use in combination has already been observed.² The design of new multi-targeted, or combined single-targeted compounds, to which there is no existing resistance is therefore a promising herbicide development avenue.

4 TOWARDS THE DISCOVERY OF NOVEL HERBICIDES TARGETING AMINO ACID BIOSYNTHESIS

As highlighted in section 1, several commercial herbicides target amino acid biosynthesis pathways. However, these herbicides have been developed against only three enzymes within

these pathways. This can be attributed to several economic factors, including the consolidation of the herbicide discovery industry, increase in the cost of herbicide discovery and development to meet regulatory requirements, and an overreliance on current herbicides, especially glyphosate.

Whilst these herbicides have been imperative to agricultural industries, it is important to consider how they may be improved upon in the development of new herbicides. Although their use has in some cases protected biodiversity by controlling invasive species, their widespread application has also impacted biodiversity due to toxicity and the destruction of natural habitats and food sources.³⁶ Herbicides with increased selectivity, reduced application requirements and that are amenable to combination with alternative weed management tools may mitigate these effects. We should also consider what we have learnt from the current burden of herbicide resistance to delay the emergence of resistance to new herbicides. The propensity of weeds to develop resistance varies depending on the herbicide mode of action.³ Investigating the rate of resistance development for new targets can focus pursuits on those that are less likely to develop resistance quickly. Additionally, if the molecular determinants of resistance are known, analogues can be designed to overcome resistance mechanisms.³¹

Moreover, it is apparent that simply inhibiting an enzyme in an essential plant pathway is not sufficient to afford local phytotoxicity let alone systemic toxicity that leads to plant death.⁴ As highlighted in this review, the successful amino acid biosynthesis inhibitors in the market not only prevent the production of proteins but also have phytotoxic effects in related pathways.^{4,13,37} This section highlights several promising targets within amino acid

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biosynthesis pathways that could be further investigated for the development of herbicides, including enzymes involved in the pathways described above.

4.1 Novel inhibitors of aromatic amino acid biosynthesis enzymes

As discussed in section 2.1, glyphosate targets EPSPS, which catalyses the penultimate step of the shikimate pathway. A few other enzymes in the shikimate pathway have been explored for herbicide development (Figure 4A). One such example is 3-dehydroquinate synthase (EC 4.2.3.4), which is the target of a natural inhibitor, 7-deoxy-sedoheptulose (Figure 4B), produced by the cyanobacterium *Synechococcus elongatus*.³⁸ The compound inhibits plant growth with similar efficacy to glyphosate with no cytotoxicity reported in mammalian cells.³⁸ Attenuated photosynthetic activity of cyanobacteria treated with 7-deoxy-sedoheptulose could be recovered with aromatic amino acid supplementation, pointing to amino acid starvation as the mode of action.³⁸ However, this remains to be investigated in plants.

Another example is tryptophan synthase (EC 4.2.1.20), involved in the last two reactions of the shikimate pathway branch to produce Trp (Figure 4A).³⁹ Indole-based tryptophan synthase inhibitors such as 3-indoleacrylic acid (Figure 4B) halt the growth of carrot and tobacco cells at low micromolar concentrations, however, these were not tested against whole plants.⁴⁰ Hence, the potential of these compounds as herbicides remains largely unexplored in the literature. More recently, tryptophan synthase inhibitors have been designed to target the transition state of the α -subunit of the enzyme, which cleaves the substrate precursor prior to Trp synthesis by the β -subunit.^{39–41} Despite a patent being filed for some of these indole-based

inhibitors that demonstrate post-emergence herbicidal activity, no further development of these compounds has been reported.³⁹ This highlights the significant hurdles to overcome following inhibitor discovery, which often prevents their advancement to a commercial herbicide.

4.2 Novel inhibitors of histidine biosynthesis enzymes

The potential of histidine biosynthesis as a herbicide target is another avenue that, despite being the centre of some attention at one time, has proved unfruitful so far. The herbicide amitrole was originally proposed to inhibit the sixth enzyme in the histidine biosynthesis pathway, namely imidazoleglycerolphosphate dehydratase (EC 4.2.1.19) (Figure 5A).⁴² However, it has since been shown that inhibition of histidine biosynthesis does not account for its herbicidal effects.⁴³

Rational design and 3D database searching approaches have yielded multiple inhibitors of imidazoleglycerolphosphate dehydratase with *in planta* activity.^{44,45} More recently, a triazole-phosphonate inhibitor named 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate (Figure 5B) has been discovered with nanomolar potency, albeit it has an order of magnitude greater specificity for the yeast enzyme than the *Arabidopsis thaliana* orthologue.⁴⁶ Structural investigation has revealed that this is due to an additional β -strand that stabilises the binding of the compound in the yeast enzyme.⁴⁶ Thus, it may be possible to increase plant specificity by exploiting the structural differences between the enzymes from different kingdoms.

Moreover, inhibitors based on the scaffold of *S*-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone targeting the last enzyme in the histidine biosynthesis pathway, histidinol dehydrogenase (EC 1.1.1.23) (Figure 5), have been published, which target a lipophilic binding pocket adjacent to the active site with low nanomolar potency *in vitro*.⁴⁷ However, aside from two of the analogues inhibiting root growth of rice on agar, the inhibitors of histidinol dehydrogenase showed no herbicidal activity.⁴⁷ The authors postulated that low solubility and chemical instability may be responsible for the poor uptake of these compounds by plants, which is characteristic of the hurdle that physicochemical property optimisation presents to the progression of a potent inhibitor into the market.⁴⁷

4.3 Novel inhibitors of glutamate-derived amino acid biosynthesis enzymes

Another novel herbicide target is aspartate transaminase, also known as aspartate aminotransferase (EC 2.6.1.1), which catalyses the conversion of Glu into Asp (Figure 6A).⁴⁸ Cornexistin (Figure 6B), a fungal metabolite first isolated from *Paecilomyces variotii*, has herbicidal activity against both monocot and dicot weed species, which was later shown to be a result of the inhibition of aspartate transaminase.^{49,50} As the aspartate transaminase-catalysed reaction is the only route for the biosynthesis of Asp, it is apparent that inhibiting this enzyme will have cascading effects and prevent the biosynthesis of aspartate-derived amino acids, which is the focus of section 4.4. Thus, investigation into cornexistin analogues with enhanced physiochemical properties may provide an approach to inhibit the biosynthesis of several amino acids.

Similarly, the *Pseudomonas syringae* metabolite, phaseolotoxin (Figure 6B), inhibits ornithine carbamoyltransferase (EC 2.1.3.3).⁵¹ This enzyme catalyses the transfer of a carbamoyl from carbamoyl-phosphate to L-ornithine to produce L-citrulline, which subsequently leads to the formation of Arg (Figure 6A).⁵² Although phaseolotoxin is a reversible inhibitor of ornithine carbamoyltransferase *in vitro*, it is metabolised to the irreversible inhibitor N(δ)-sulfodiaminophosphinyl-ornithine (octicidine) *in vivo*.⁵¹ Thus, phaseolotoxin shares similar proherbicide properties to bialaphos, whereby it is metabolised *in vivo* to produce a potent, and in this case, covalent inhibitor. Upon direct application of phaseolotoxin to the leaves, systemic chlorosis was observed, despite only 25% of the total amount of the compound being able to penetrate the leaf.⁵¹ The accompanying accumulation of ornithine points towards a mode of action, as the buildup of substrate elucidates the choke point being at the ornithine carbamoyltransferase-catalysed reaction.⁵¹ Given these results, investigation of the effects of phaseolotoxin on whole plants, as well as the development of other ornithine carbamoyltransferase inhibitors may be fruitful.

Dual-target derivatives of naturally phytotoxic aminoalkylphosphonic acids such as 3,5-dichlorophenyl amino-methylene-biphosphonic acid (Figure 6B) inhibit GS (section 2.3) as well as δ 1-pyrroline-5-carboxylate reductase (EC 1.5.1.2) *in vitro*.⁵³ The reduction of δ 1-pyrroline-5-carboxylate serves as the final step for the production of Pro from Glu (Figure 6A).⁵³ The inhibitors described have differential activity against each enzyme *in vitro*, with greatest potency against the plastid-localised isoform of GS.⁵³ Treating rapeseed with the inhibitors drastically decreased the dry weight of seedlings, as the resulting accumulation of

δ 1-pyrroline- 5- carboxylate appears to afford significant phytotoxicity at low micromolar concentrations *in planta*.⁵³ However, these assays were only performed on media. Whilst sowing plants on inhibitor-containing media is a good starting point for the assessment of *in planta* activity, this method has two main downfalls when it comes to assessing herbicidal efficacy. Firstly, plant uptake may be much higher, as absorption into the soil or penetration of the waxy cuticle is not required. Secondly, inhibitors may remain available in the media to exert their effects for much longer, where they may otherwise be degraded by the plant or by soil microbes.⁴ Thus, demonstrated soil efficacy data is crucial in determining the potential of an inhibitor as a herbicide lead. Nevertheless, such studies point to which targets may be worth pursuing, even if the inhibitors described would be proven ineffective against soil-grown plants.

4.4 Novel inhibitors of aspartate-derived amino acid biosynthesis enzymes

There are several aspartate-derived amino acids, including Met, Thr, Ile and Lys (Figure 7A). The first committed step of Ile biosynthesis is catalysed by threonine deaminase (Figure 7A), of which 2-(1-cyclohexen-3(*R*)-yl)-*S*-glycine (Figure 7B) is a competitive inhibitor with herbicidal efficacy against plants grown on media.⁵⁴ For reasons previously discussed, it would be pertinent to explore the efficacy of these compounds against plants grown on soil.

Cystathionine γ -synthase (EC 2.5.1.48) catalyses the first committed step of methionine biosynthesis, which involves a transfer reaction between *O*-succinyl-L-homoserine and cysteine to produce cystathionine (Figure 7A).⁵⁵ DL-Propargylglycine (Figure 7B) is a weak irreversible inhibitor of cystathionine γ -synthase.⁵⁶ Furthermore, treatment of *Arabidopsis*

plants with the inhibitor severely decreases growth, but can at least be partially rescued by adding Met, which points towards the mode of action.^{55,56} Despite these promising results, this inhibitor is primarily discussed as a molecular probe for the biochemical characterisation of cystathionine γ -synthase, and its potential as a candidate for herbicide development was not discussed.

Cysteine-*S*-conjugate β -lyase (EC 4.4.1.8) catalyses the second step in Met biosynthesis, in which L-cystathionine is deaminated to produce L-homocysteine (Figure 7A).⁵⁷ There are several inhibitors of cysteine-*S*-conjugate β -lyase including rhizobitoxine (Figure 7B), isolated from *Rhizobium japonicum* and *Bradyrhizobium elkanii*, and the structural analogue L- α -(2-aminoethoxyvinyl)glycine, both of which are irreversible inhibitors *in vitro*.^{58,59} However, rhizobitoxine was later found to only partially inhibit the enzyme *in vivo*, highlighting the need for *in vivo* testing early on in herbicide development pipelines.⁶⁰

Lastly, several studies have commented on the suitability of the lysine biosynthesis pathway as a herbicide target, given its occurrence in plants and bacteria only.⁶¹ Although lysine biosynthesis has gained attention as a way to increase the nutritional value of crops, its potential as a herbicide target remains largely unexplored. The enzyme dihydrodipicolinate synthase, also known as 4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7), catalyses the first committed step of lysine biosynthesis through the diaminopimelate pathway (Figure 7A).⁶¹ The pathway is regulated via the allosteric binding of the final product, lysine, to dihydrodipicolinate synthase.⁶¹ Both *S*-(2-aminoethyl)-L-cysteine and L- α -(2-

aminoethoxyvinyl)glycine (Figure 7B) are analogous to lysine and inhibit dihydrodipicolinate synthase from plant extracts, as well as compete for incorporation in proteins and result in aberrant non-functional proteins.⁶² Furthermore, *S*-(2-aminoethyl)-L-cysteine is an effective inhibitor of potato dihydrodipicolinate synthase *in planta* and prevents rooting at mid-micromolar concentrations.⁶² Transformation with a feedback insensitive dihydrodipicolinate synthase orthologue or the upstream aspartate kinase (EC 2.7.2.4) rescues the phenotype, as the plant is able to produce enough lysine to overcome the effects of *S*-(2-aminoethyl)-L-cysteine.⁶² Since then, there have been no other reports of inhibitors of plant enzymes in the diaminopimelate pathway that have *in planta* activity.

5 CONCLUSIONS

This review provides a brief overview of the current commercial herbicides that target enzymes in amino acid biosynthetic pathways. Despite their success and the extensive research that has gone into the discovery of novel amino acid biosynthesis inhibitors as outlined here, there have been no new commercial herbicides with this mechanism of action in the past 30 years. The identification of suitable novel targets within amino acid biosynthesis is complex, as it is dependent on a detailed understanding of the role of the target in plants and non-target organisms. This review also highlights novel strategies to reinvigorate the herbicide discovery pipeline given the recent advances in technology for the identification of promising compounds. It is evident that an effective herbicide must not only inhibit its target enzyme to afford phytotoxicity, it must also have cascading effects on related pathways that are critical for plant growth and development. In addition to this challenge, inhibitors must also have ideal

physiochemical properties such as solubility, penetration and translocation. Consideration to residuals and soil persistence must also be given to prevent the accumulation and overflow of compounds into adjacent environments. This will ensure a more streamlined and economically viable pipeline to develop much needed new and safe herbicides targeting amino acid biosynthesis.

Conflicts of Interest: The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Simplified shikimate pathway in plants. (A) The shikimate pathway begins with the condensation of phosphoenol-pyruvate (PEP) and erythrose 4-phosphate (E4P), eventually leading to the production of shikimate 3-phosphate (S3P). 5-enolpyruvyl-shikimate 3-phosphate (EPSP) is then produced by EPSP synthase (EPSPS). Subsequently, chorismate is formed by chorismate synthase (CS) via a dephosphorylation reaction to produce Phe, Tyr or Trp. (B) Structure of the EPSPS inhibitor, glyphosate. (C) Cartoon structure of *Escherichia coli* EPSPS in complex with glyphosate (green sticks) (PDB: 1G6S), as a plant structure has not been determined. *E. coli* EPSPS shares ~69% primary sequence similarity with the *Arabidopsis thaliana* enzyme. Residues involved in inhibitor binding are shown as blue sticks and labelled according to *E. coli* numbering. The colouring of atoms in the stick structures are as follows: nitrogen, dark blue; oxygen, red; phosphorous, orange. Image was generated using PyMOL v2.2 (Schrodinger).

Figure 2. Simplified branched chain amino acid pathway in plants. (A) The biosynthesis of Ile, Leu and Val occurs using different substrates, but all share four conserved enzymatic steps catalysed by acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS), ketol-acid reductoisomerase (KARI), dihydroxyacid dehydratase (DHAD) and branched chain aminotransferase (BCAT). The production of Leu and Val commences with a condensation reaction that involves the decarboxylation of two pyruvate molecules by ALS/AHAS to produce 2-acetolactate, which is subsequently converted to 2,3-dihydroxy-3-methylbutanoate.

DHAD then produces 3-methyl-2-oxobutanoate and Val is synthesised directly via BCAT. Leu is synthesised through a separate branch, wherein 3-methyl-2-oxobutanoate is converted to 4-methyl-2-oxopentanoate, which then converges back and undergoes an amino transfer by BCAT. Alternatively, Ile biosynthesis begins with the deamination of Thr by threonine deaminase (TD) to produce 2-oxobutanoate, which then reacts with pyruvate via an ALS/AHAS-catalysed reaction to form 2-aceto-2-hydroxybutanoate. KARI then produces 2,3-dihydroxy-3-methylvalerate, which is dehydrated by DHAD to 2-oxo-3-methylvalerate, and lastly, undergoes an amino transfer catalysed by BCAT to yield Ile. (B) Structures of inhibitors of ALS/AHAS. (C) Cartoon structure of *Arabidopsis thaliana* ALS/AHAS in complex with chlorimuron-ethyl (CE, green sticks) (PDB: 1YBH). Key residues involved in inhibitor binding are shown as either blue (chain A) or cyan (chain B) sticks and labelled according to *A. thaliana* numbering. The colouring of atoms in the stick structures are as follows: nitrogen, dark blue; oxygen, red; phosphorous, orange. Image was generated using PyMOL v2.2 (Schrodinger).

Figure 3. Simplified glutamine biosynthesis pathway in plants. (A) The condensation reaction catalysed by glutamine synthetase (GS) uses ammonia to convert Glu to Gln. Gln is converted back to Glu using 2-oxoglutarate, an intermediate of the tricarboxylic acid cycle (TCA), in a reaction catalysed by glutamine oxoglutarate aminotransferase (GOGAT). (B) Structures of GS inhibitors. (C) Cartoon structure of *Zea mays* GS in complex with glufosinate (green sticks) (PDB: 2D3C). Residues involved in inhibitor binding are shown as either blue (chain A) or cyan (chain B) sticks and labelled according to *Z. mays* numbering. The colouring

of atoms in the stick structures are as follows: nitrogen, dark blue; oxygen, red; phosphorous, orange. Image was generated using PyMOL v2.2 (Schrodinger).

Figure 4. Simplified Trp biosynthesis pathway in plants. (A) Trp is produced through the shikimate pathway that begins with the condensation of phosphoenol-pyruvate (PEP) and erythrose 4-phosphate (E4P) to produce 3-deoxy-*arabino*heptulosonate 7-phosphate (DAHP), which is catalysed by DAHP synthase (DAHPS). DAHP is then converted to 3-dehydroquinate (DHQ) by DHQ synthase (DHQS), which eventually results in the production of anthranilate. Ultimately, Trp is synthesised in a reaction catalysed by tryptophan synthase (TS). (B) Structure of DHQS and TS inhibitors. Roman numerals refer to the enzyme target in panel (A) for the inhibitor.

Figure 5. Simplified His biosynthesis pathway in plants. (A) His biosynthesis begins with imidazole glycerol-phosphate (IGP), which is dehydrated to produce imidazoleacetol phosphate (IAP) by IGP dehydratase (IGPD). Histidinol is then produced before the conversion to His by histidinol dehydrogenase (HDH). (B) IGPD and HDH inhibitor structures. Roman numerals refer to the enzyme target in panel (A) for the inhibitor.

Figure 6. Simplified glutamate-derived amino acid biosynthesis pathway in plants. (A) Asp is produced from Glu via an amino transfer reaction catalysed by aspartate transaminase (AST). Alternatively, Glu can be converted into ornithine or δ 1-pyrrol ine-5- carboxylate (P5C). Ornithine is converted to citrulline by ornithine carbamoyltransferase (OCT), which

goes on to yield Arg. P5C can be reduced by P5C reductase (P5CR) to produce Pro. (B) Structures of AST, OCT and P5CR inhibitors. Roman numerals refer to the enzyme target in panel (A) for the inhibitor.

Figure 7. Simplified aspartate-derived amino acid biosynthesis pathway in plants. (A) Ile, Met, Lys and Thr are all produced from Asp. Aspartate kinase (AK) catalyses the transfer of a phosphate group onto Asp, to produce aspartate 4-phosphate, which can produce homoserine or aspartate semialdehyde (ASA). Ile and Met are both derivatives of homoserine. Thr is produced from homoserine and undergoes deamination by threonine deaminase (TD) to yield 2-oxobutanoate, which is eventually converted to Ile. Met is produced from homoserine via *O*-phosphohomoserine (OPHS), which is then converted to cystathionine by cystathionine γ -synthase (C γ -S). Cystathionine is deaminated by cysteine-*S*-conjugate β -lyase (C β -L) to produce homocysteine, which goes on to produce Met. Alternatively, Lys biosynthesis begins with the formation of 4-hydroxy-tetrahydrodipicolinate (HTPA) by dihydrodipicolinate synthase (DHDPS). (B) Structures of inhibitors of TD, C γ -S, C β -L, AK and DHDPS. Roman numerals refer to the enzyme target in panel (A) for the inhibitor.

FIGURES

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Graphical Abstract

Review: Amino Acid Biosynthesis as a Target for Herbicide Development.

Cody J. Hall[#], Emily R.R. Mackie[#], Anthony R. Gendall, Matthew A. Perugini and Tatiana P. Soares da Costa^{*}

Amino acid biosynthesis pathways are targeted by some of the most successful herbicides and offer many unexploited targets for the development of urgently needed novel herbicides to combat resistant weeds.

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