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Toxic Non-protein Amino Acids

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Abstract

The 20 DNA-coded protein amino acids play central roles in the metabolism of most organisms. As well as being the building blocks for proteins they play essential roles in a diverse range of metabolic pathways. There are estimated to be around 1000 molecules in nature, which share the same basic structure as these organic amino acids consisting of an α-carbon attached to a carboxyl group, an amino group, a hydrogen atom and a unique side-chain group. Many 'non-protein' amino acids (NPAAs) are plant secondary metabolites.

In this chapter the authors discuss plant NPAAs that have a similar chemical structure, size, shape and charge to protein amino acids and can be mistakenly used in protein synthesis, interfere in biochemical pathways, over-stimulate receptors or chelate metal ions. Most often this results in some level of toxicity to the target organism and can confer some advantage to the plant. Toxic NPAAs might have evolved as defense chemicals that can be released into the soil to inhibit the growth of other plants or agents that can limit insect herbivory.

The effects of NPAAs on human health are not well understood. Consumption of a number of plants that contain NPAAs has been shown to have acutely toxic effects in humans. The key questions that remain unanswered are: to what extent can NPAAs enter the food chain and what are the effects of a chronic low-level exposure to toxic plant NPAAs?

1. Introduction

The chosen few: the importance of the 20 protein amino acids

Proteins are synthesised from 20 coded L-α-amino acids (Weber and Miller 1981). The authors can only speculate as to why only 20 amino acids are utilised, since with a triplet genetic code 64 codons are available. Evolutionary selection might have been based on the availability of amino acids or because they possessed essential properties such as chemical, thermal and photochemical stability (Weber and Miller 1981). The diversity of the side-chain groups is likely to have been driven by the ability of these groups to confer a range of functions on the synthesised protein. Canonical or 'protein' amino acids, once peptide bonded into proteins, can also undergo a number of post-translational modifications further increasing the functional capabilities of the protein. In addition to the 20 protein amino acids there are close to one thousand naturally occurring amino acids, many of which are synthesised by plants (Bell 2003) (Table 1). Some plant amino acids have attracted attention because of their toxicity to humans and animals. The most striking example of amino acid toxicity is neurolathyrism (lathyrism), one of the oldest neurotoxic diseases known. Described by Hippocrates (~400 BC), neurolathyrism is an irreversible paralytic disease linked to consumption of *Lathyrus sativus* (grass pea). Regular outbreaks of this debilitating disease have occurred throughout history (Yan et al. 2006). *Lathyrus sativus* is an insect-resistant crop that can grow in poor soils and in drought conditions and is often eaten in times of famine when there is a dietary shortage of protein amino acids. *Lathyrus sativus* is a nutrient-rich plant but contains the non-protein amino acid β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) also known as β-N-

oxalylamino-L-alanine (BOAA) (Nunn et al. 2010; Yan et al. 2006). Its primary toxic effect is over-stimulation of glutamate receptors resulting in neuronal cell death (discussed in section 2b).

The toxicity of non-protein amino acids (NPAAs) was first examined systematically in the early 1960s and many were found to have growth-inhibitory properties towards microorganisms (Richmond 1962; Fowden et al. 1967). This was not generally due to inherent chemical reactivity of the amino acid molecule but due to its similarity to one of the 'chosen' 20 protein amino acids (Fowden et al. 1967). An important feature of many toxic NPAAs is that their toxicity is prevented or reversed in the presence of the 'parent' protein amino acid. The toxicity of NPAAs therefore generally relates to their ability to be mistaken for and to replace a protein amino acid in a metabolic pathway or biological process. This often occurs in protein synthesis but can also occur when amino acids play more specialised roles such as receptor agonists or enzyme substrates (Fowden et al. 1967).

Protein synthesis is a process fundamental to all life forms and the ability to interfere with this process could result in potent and widespread toxicity. It is reasonable to speculate that NPAAs could have been the very first plant toxins since they would have been able to negatively impact on the growth of even the most primitive of organisms that were reliant on protein synthesis for survival. The additional advantage of NPAAs that target protein synthesis is that a single host defense strategy can protect against autotoxicity – evolution of a more selective protein synthesis machinery capable of distinguishing the protein amino acid from the 'imposter' or alternatively, a means of rapidly modifying the NPAA so that it becomes distinguishable from the protein amino acid.

In this chapter the focus is on mechanisms of toxicity of plant NPAAs known to negatively impact on the growth of other organisms (Scheme 1). In some cases the production of NPAAs by plants confers some advantage, and NPAAs have a detrimental effect on herbivores feeding on the plant or other plants competing for the same resources. In this context plant NPAAs are allelochemicals since they can influence the growth or behaviour of other organisms to their own advantage (Fitter 2003). Rather than provide a list of toxic NPAAs, this chapter will firstly focus on the mechanisms of toxicity that have been identified so far, and then on target organisms, providing examples of the best understood NPAAs. Plants that are known to possess advanced protein synthesis machinery are discussed as well as herbivores that have made some adaptation to allow them to feed on plants that are toxic to other species. The potential impact of toxic plant NPAAs on human and animal health is discussed and the major questions that remain unanswered.

2. Mechanisms of toxicity of plant non-protein amino acids (NPAAs)

2a. Misincorporation into proteins

The first mechanism of toxicity identified for NPAAs was their ability to replace a protein amino acid in protein synthesis resulting in the synthesis of abnormal or non-native proteins (Fowden et al. 1967). In early studies in bacteria, high concentrations of NPAAs were used, and the effects observed were acute effects resulting from damage to a significant proportion of the newly synthesised bacterial proteins (Fowden et al. 1967). It was commonly found that if the amino acid replaced by the NPAA played an important role in the active site of the enzyme it could result in loss of enzyme activity. Alternatively, significant

conformational changes to a protein arising from amino acid substitution could result in a loss of function or loss of aqueous solubility (Fowden et al. 1967). This was only the case for a few NPAAs that were similar to a protein amino acid in size, shape and charge and are referred to as amino acid analogues or in the case of NPAAs that can be mistakenly incorporated into proteins, coined as 'proteomimetic' amino acids (Rodgers and Shiozawa 2008; Rodgers 2014).

Incorporation of a NPAA into a newly synthesised protein is a random process in which the NPAA and the 'parent' protein amino acid compete for a specific aminoacyl-tRNA synthetase (Rodgers and Shiozawa 2008). Although the protein amino acid will have a higher affinity for its cognate tRNA synthetase than the proteomimetic NPAA, at certain concentrations, the NPAA will be randomly charged to the transfer-RNA and become peptide bonded into the polypeptide chain (Rodgers and Shiozawa 2008). It has been shown that at lower concentrations of NPAA there is a linear correlation between the concentration of the NPAA and the level of incorporation into protein (Rodgers et al. 2002). The NPAA-protein amino acid exchange is a random, concentration-driven event in which the NPAA has an equal chance of being incorporated into any newly synthesised proteins coded for the parent amino acid. Within specific proteins, there is an equal chance of any of the parent amino acid residues being replaced with the NPAA irrespective of the position in the polypeptide chain. While no specific proteins will be targeted by NPAAs, certain proteins might be more susceptible to the presence of an incorrect amino acid in the peptide chain. As mentioned previously, replacement of amino acids that are essential for enzyme function will reduce enzyme activity (Fowden et al. 1967). Proteins with less complex structures or 'intrinsically disordered proteins' might be more likely to

undergo a change in function or a decrease in water solubility because of a structural change (Rodgers 2014).

The legume *Mucuna pruriens* (velvet bean) which contains high levels of L-3,4-dihydroxyphenylalanine (L-DOPA or 3-hydroxytyrosine) is very tolerant to pests and can suppress weed growth (Soares et al. 2014). L-DOPA can replace L-tyrosine in protein synthesis (Rodgers and Shiozawa 2008). An important study by Ozawa showed, using a cell free protein expression system, that when solvent-exposed L-tyrosine residues were replaced by L-DOPA, proteins retained their solubility however replacement of internal L-tyrosine residues with L-DOPA resulted in a loss of solubility (Ozawa et al. 2005) presumably due to forced unfolding of the protein and exposure of previously buried hydrophobic regions (Rodgers 2014).

A number of plant NPAAs have been conclusively shown to replace their parent amino acid in protein synthesis (Table 1). Azetidine-2-carboxylic acid (Aze) which is present in a number of plants including *Convallaria majalis* (lily of the valley), some Liliaceae and *Beta vulgaris* (sugar beets) readily replaces L-proline in protein synthesis (Rubenstein et al. 2009). L-canavanine (L-2-amino-4-guanidooxy-butanoic acid) synthesized by jack beans, (*Canavalia ensiformis*) and wild potato (*Hedysarum alpinum*) (Rosenthal 2001) successfully competes with the protein amino acid L-arginine in protein synthesis. Arginyl tRNA synthetase readily esterifies L-canavanine to the cognate $tRNA^{Arg}$ (Rosenthal 2001) resulting in the synthesis of abnormal proteins. L-canavanine is a less basic molecule than L-arginine (pKa of the guanidooxy group is 7.04 vs 12.48 of the guanido group in L-arginine) and this might greatly impact on protein structure and function (Nunn et al. 2010). These NPAAs are discussed in more detail later in the chapter.

Substitution of a protein amino acid for a NPAA has similarities to a missense mutation in which substitution of a single base in DNA will encode another protein amino acid in the polypeptide chain. In the case of the NPAA, the amino acid switch commonly occurs at a low frequency in contrast to a mutation, which is manifest every time the protein is synthesized. In addition, the NPAA is often closer structurally to the parent protein amino acid than the substituted protein amino acid inserted due to the point mutation, so would have a more subtle impact on the structure and function of the protein or the health and function of the organism.

There is evidence that NPAAs can also influence rates of protein synthesis by reducing the availability of protein amino acids. Early studies in bacteria demonstrated that some NPAAs inhibit uptake and/or biosynthesis of their protein amino acid counterparts (Fowden et al. 1967). Azetidine-2-carboxcylic acid (Aze) was shown to inhibit the uptake of ^{14}C-proline by *E. coli* and inhibit proline biosynthesis from glutamate through feedback inhibition. Feedback inhibition has also been demonstrated by analogues of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Fowden et al. 1967). To the author's knowledge these mechanisms of toxicity has not been fully investigated *in vivo*.

2b Excitotoxicity

Excitotoxicity is a form of acute toxicity of neuronal cells caused most often by overstimulation of ionotropic (ion-channel coupled) glutamate receptors (Rothman 1985). Typically activated by the protein amino acid glutamic acid, glutamate receptors mediate the influx of calcium (Ca^{2+}), potassium (K^{+}) and sodium (Na^{+}) ions into the cell (Rothman 1985). When over-stimulated these

receptors allow a flood of Ca^{2+} into the cell resulting in mitochondrial dysfunction, oxidative stress, and activation of a number of apoptotic pathways leading eventually to cell death (Rothman 1985). While glutamic acid is the primary agonist at glutamate receptors, a number of excitatory NPAAs have been identified that are structurally similar to glutamic acid and can exert an excitotoxic effect (Nunn et al. 2010).

The best documented plant-derived excitatory NPAA is β-ODAP produced by the legume *Lathyrus sativus*. Consumption of *Lathyrus* can result in the irreversible human paralytic disorder neurolathyrism (Woldeamanuel et al. 2012). β-ODAP is a close structural analogue of glutamic acid and is selective for AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptors favoured by glutamic acid (Nunn et al. 2010). Prolonged consumption of *Lathyrus sativus* results in degenerative changes in the major central nervous system pathway responsible for regulation of skeletal muscle function (Yan et al. 2006). The initial effects of β-ODAP include cramping and weakness in the muscles of the legs and can be reversible; however, prolonged exposure leads to irreversible damage and permanent central motor system deficits (Yan et al. 2006). The neurotoxic effects of β-ODAP might extend beyond overstimulation of AMPA receptors and *in vitro* and *in vivo* studies have identified a wide range of toxic effects attributable to β-ODAP (reviewed in (Nunn et al. 2010)). There is also evidence that the effects of *Lathyrus sativus* are very species-specific with some evidence that ruminal biota could offer some protection (Yan et al. 2006). Quisqualic acid, a non-protein amino acid isolated from *Combretum indicum*, commonly known as Chinese honeysuckle or Rangoon creeper, has a similar excitotoxic effect to β-ODAP. Quisqualic acid acts as an agonist at the AMPA sub-class of glutamate receptors resulting in Ca^{2+} influx

into the cell (Shinozaki and Shibuya 1974). The fruit of *C. indicum* (often called Fructus quisqualis) has been used as a treatment for intestinal parasites in traditional medicine for many years, with practitioners reporting paralysis of parasitic worms. Quisqualic acid has also been found in the petals of the zonal geranium *Pelargonium x hortorum*, where it has been identified as a phytochemical defense against insect predation (Potter and Held 2002). Japanese beetles (*Popillia japonica*) that ingest quisqualic acid develop hind limb paralysis that progresses anteriorly to full paralysis. Affected beetles typically recover within 24 hours (Potter and Held 2002).

2c Interference in metabolic pathways

The L-arginine analogue, L-canavanine (L-2-amino-4-guanidooxy-butanoic acid) is synthesized by over 350 species of Papilionoideae including jack beans, (*Canavalia ensiformis*), vine (*Dioclea megacarpa* Rolfe) and wild potato (*Hedysarum alpinum*) (Rosenthal 2001). Concentrations can reach up to 13% of the dry weight of seeds (Rosenthal 2001). L-canavanine is an effective allelochemical that protects plants against both predation and disease. It is a very close structural analogue of L-arginine and serves as a substrate in virtually every enzyme-mediated reaction that employs L-arginine (Nunn et al. 2010). The ability of L-canavanine to generate damaged proteins in plants, bacteria, and fungi is thought to occur due to L-canavanine misincorporation into protein in place of L-arginine (section 2a) (Rosenthal 2001) but in humans and animals its actions are more complex. In the rat, L-canavanine is converted by arginase into urea and the toxin L-canaline (Thomas and Rosenthal 1987). L-canavanine is also a substrate for inducible nitric oxide synthase, the enzyme that converts L-arginine into nitric

oxide (Nunn et al. 2010). Nitric oxide has a number of important functions in the human body, it is a potent vasodilator and inhibitor of platelet activation.

L-2,4-Diaminobutanoic acid (2,4-DABA or L-α,γ-Diaminobutyric acid) is a NPAA present in seeds of many species of *Lathyrus* and also in *Polygonatum multiflorum.* When injected into the peritoneum of rats 2,4-DABA caused liver damage and neurotoxicity (O'Neal et al. 1968). One mechanism of action identified was competitive inhibition of ornithine carbomyltransferase, an enzyme in the urea cycle, leading to ammonia accumulation and neurotoxicity (Nunn et al. 2010). 2,4-DABA was able to kill human malignant glioma cells at a much lower concentration than human glia cells *in vitro* (Ronquist et al. 1984). It appears to be so rapidly taken up by tumor cells *in vitro* that it can cause major electrolyte disturbances, swelling and osmotic destruction of the cell (Ronquist et al. 1992).

L-homoarginine (N^6-Carbamimodyl-L-lysine), which is present in *Lathyrus cicera, Lathyrus sativus,* and in small amounts in *Lens culinaris* (lentil) differs from L-arginine only in that it contains an additional backbone methylene group (CH_3) (Table 1) and can replace L-arginine in mammals in most physiological processes (Nunn et al. 2010). It is efficiently converted into L-lysine and urea by rat liver arginase and can even provide a source of L-lysine in rats maintained on a lysine-deficient diet (Nunn et al. 2010). L-homoarginine inhibits bacterial growth but, to the authors knowledge, its ability to be misincorporated into protein has not been tested (Fowden et al. 1967). Misincorporation into proteins would seem unlikely in humans since it is an endogenous amino acid synthesized in the kidneys. L-arginine and L-homoarginine compete as substrates of nitric oxide synthase (Pilz et al. 2015). L-homoarginine can decrease the production of nitric oxide by endothelial cells where it is an important determinant of vascular tone and blood

pressure. A positive association has been shown between endogenous L-homoarginine levels and systolic blood pressure. Low L-homoarginine levels are considered to be a risk factor for stroke (Pilz et al. 2015). L-homoarginine is also a potent inhibitor of canine hepatic and skeletal alkaline phosphatases (Nunn et al. 2010).

The cyclic NPAA hypoglycin (Hypoglycine A, 2-amino-3-methylene cyclopropylpropanoic acid) is present in unripe fruits of the West African ackee tree (*Blighia sapida*), now grown throughout the West Indies, the Atlantic coast of Central America and southern states in the U.S.A (Joskow et al. 2006). Consumption of unripe fruit, which can contain up to 0.1% hypoglycin by dry weight, leads to vomiting, drowsiness, hypoglycemia, with coma and death in severe cases (Joskow et al. 2006). The toxicity of hypoglycin is due to its metabolite methylenecyclopropylacetic acid (MCPA) which inhibits beta-oxidation of fatty acids, resulting in increased utilization of glucose, glycogen depletion and hypoglycemia (Joskow et al. 2006). To the authors knowledge there is no evidence that hypoglycin is toxic to insects or other plants but only to species that can convert it into MCPA in the liver.

2d Metal chelation

Mimosine ([β-[N-(3-hydroxy-4-pyridone)]-L-2-aminopropanoic acid) is present in *Leucaena* and *Mimosa* seeds, stems, pods and leaves. It was reported in 1897 that animals fed on the seeds or foliage of *Leucaena* experienced hair loss (Crawford et al. 2015). The introduction *Leucaena leucocephalia* to a wildlife reserve in Madagascar in 1990 resulted in reversible hair loss in ringtail lemurs (*Lemur catta*) at the times of the year when *Leucaena* was their main dietary

source (Crawford et al. 2015). Despite being detectable in the systemic circulation, no other adverse effects of mimosine could be identified in the lemurs (Crawford et al. 2015). It was proposed that mimosine induced a rapid progression of hair follicles into the telogen resting phase and inhibition of the transition to the anagen growth phase and initiation of a new cycle, and as a result the old hairs then become brittle and broke close to the skin surface (Crawford et al. 2015). Mimosine has been studied in many species and has been shown to cause reversible infertility in rats (Hylin and Lichton 1965) and growth retardation in cattle (Dalzell et al. 2012). Cell studies have shown that mimosine is a specific and reversible inhibitor of DNA replication (Lalande 1990) and it inhibits proliferation of human lung cancer cells by arresting cells in the late G1 phase (Chang et al. 1999). Mimosine, a potent chelator of transition metals, is an inhibitor of many metal-containing enzymes including key enzymes in DNA synthesis (ribonucleotide reductase) and purine and thymidine synthesis (serine transhydroxymethylase) (Hallak et al. 2008). Using a leukemia cell line, Hallak showed that mimosine induced apoptosis through oxidative damage to mitochondria (Hallak et al. 2008). In addition to its potent metal chelating ability (including Cu, Zn and Fe) mimosine is also a substrate for phenylalanyl-tRNA synthase and might be able to replace tyrosine as a substrate for tyrosinase (Nunn et al. 2010).

Mucuna pruriens (velvet bean) which contains high levels of L-DOPA is very tolerant to pests and inhibits weed growth (Soares et al. 2014). In addition to being able to replace the protein amino acid tyrosine in protein synthesis, the catechol group on the L-DOPA molecule allows it to interact strongly with divalent metals (Rodgers and Dean 2000). The high L-DOPA content (~30%) of byssal foot

proteins confers on mussels their remarkable ability to attach to wet surfaces (Miserez et al. 2008). Binding of L-DOPA to transition metals (present in in rocks and other surfaces) is the primary mechanism behind this underwater superglue (Miserez et al. 2008). L-DOPA (levodopa) is the primary drug used to treat the symptoms of Parkinson's disease where it is converted into the neurotransmitter dopamine in dopaminergic neurons by the enzyme dopa decarboxylase (Rodgers and Dean 2000). In addition, L-DOPA has the potential to cause oxidative stress through its ability to undergo oxidation to the semi-quinone and quinone (Rodgers and Dean 2000). The ability to L-DOPA to bind to transition metals is often overlooked as a mechanism of toxicity but the excellent microarray study carried out on *Arabidopsis thaliana* treated with L-DOPA provided some valuable insight into the mechanisms of L-DOPA toxicity to plants (Golisz et al. 2011). More than 10 of the genes significantly upregulated after 6 hours of L-DOPA treatment in *Arabidopsis* were involved in metal homeostasis including genes that function in the transport of copper, ferric iron and zinc (Golisz et al. 2011). This study highlighted the importance of the metal chelating properties of L-DOPA as a mechanism of toxicity to organisms.

2e Nephrotoxicity

Djenkolic acid (DJK), a potent nephrotoxin and cause of the disease djenkolism, was isolated from the djenkol bean (*Archidendron pauciflorum*). DJK has since been found in members of the Fabaceae subfamily Mimosoideae including a number of Australian acacia species (Nunn et al. 2010). Ingestion of seeds containing DJK by humans can result in rapid (2-12 hour) onset of a number of symptoms including abdominal pain, nausea, vomiting, and haematuria, however

sensitivity to DJK and severity of symptoms varies significantly between individuals (Bunawan et al. 2014). Djenkolism is caused by the formation of "needle like" DJK crystals, which are poorly soluble in acidic conditions, resulting in irritation to the kidney and urinary tract (Nunn et al. 2010). DJK is thought to be a defense against insect herbivory, however the Bruchid beetle, a common legume pest, has been shown to preferentially feed on acacia containing higher concentrations of DJK. This suggests that Bruchid beetles have adapted to detoxify DJK and may have found a way to use it to their own advantage (Or and Ward 2004).

A summary of the NPAAs that exert toxicity through these mechanisms (2a to 1e), as well as those for which the mechanism of toxicity is not completely understood, is presented in scheme 1.

(PLEASE INSERT SCHEME 1 HERE)

3. Phytotoxic non-protein amino acids

Plants are sessile organisms that are unable to relocate to other territories when competition for water and nutrients increases and have to employ other survival strategies to outcompete other plants within the community (Fitter 2003). One such strategy is to release chemicals (allelochemicals) into the local environment that negatively impact on the growth and development of surrounding plants (Fitter 2003). These chemicals can be alkaloids, terpenoids, phenolics, protease inhibitors, proteins and a few are NPAAs. Fine leaf fescue grasses (*Festuca arizonica* and *F. rubra*) release a phytotoxic root exudate which allows them to outcompete other plants making them useful in roadside settings (Bertin et al. 2007). It was shown, using an activity-guided fractionation approach, that the NPAA L-meta-tyrosine (L-*m*-tyrosine) was the major allelochemical present in the

root exudate (Bertin et al. 2007). L-*m*-tyrosine has potent growth inhibitory activity on lettuce roots and shoots and induces lipid peroxide formation which can be rescued by phenylalanine but not by antioxidants (Bertin et al. 2007). L-*m*-tyrosine was shown to be toxic to a wide range of plant species and was present in hydrolyzed root proteins of the affected plants suggesting that it had been misincorporated into proteins (Bertin et al. 2007). Consistent with this, phenylalanine was the most protective of the protein amino acids against L-*m*-tyrosine toxicity. L-*m*-tyrosine was shown to be misincorporated into bacterial cell proteins in place of phenylalanine in 1965 and that the phenylalanine tRNA synthase from mung bean (*Vigna radiata*) accepted L-*m*-tyrosine with 25% of the efficiency of phenylalanine (Smith and Fowden 1968). Mammalian phenylalanyl tRNA synthase readily esterifies L-*m*-tyrosine to the cognate $tRNA^{Phe}$ generating abnormal proteins with an accelerated turnover (Rodgers et al. 2002). The synthesis of L-*m*-tyrosine in *Festuca rubra* is from hydroxylation of phenylalanine while in *Euphorbia myrsinites* (donkey-tail spurge) it is produced from transamination of m-hydroxyphenylpyruvate (Bertin et al. 2007). To date, only fine fescue and donkey-tail spurge are known to produce L-*m*-tyrosine and since they do so using distinct biosynthetic pathways it suggests that they have evolved the NPAA defense mechanism independently. While the full spectrum of mechanism(s) of toxicity of L-*m*-tyrosine when released from root exudates is not absolutely clear it is known to be a broad spectrum phytotoxin with growth inhibitory properties at micromolar concentrations (Bertin et al. 2007).

Two other plant NPAAs, mimosine which is present in seeds of *Mimosa* and *Leucaena* species, and 2-amino-4-methylhex-4-enoic acid (2AMHA) which is produced by *Aesculus californica,* have been shown to be substrates for

phenylalanyl-tRNA synthase from mung bean (Smith and Fowden 1968). The phenylalanyl-tRNA synthase of *A. californica* is unique in activating 2AMHA to a smaller extent than do the synthases from species which do not produce 2AMHA thus providing one level of protection against misincorporation of 2AMHA into its own proteins (Smith and Fowden 1968). The complex toxicity of mimosine has been discussed earlier (section 2d). Mimosine is phytotoxic to a number of plants at concentrations less that 250 μM, the growth of *Leucaena* seedlings however was unaffected at 1 mM mimosine (Chou and Kuo 1986). The allelopathic effects of mimosine demonstrated experimentally are consistent with observations of allelopathic activity (weed exclusion) by *Leucaena* plants in Taiwanese forests (Chou and Kuo 1986). In another study it was shown that supplying mimosine with $FeCl_3$ reduced its phytotoxic effects suggesting that metal chelating properties of mimosine contributed to its phytotoxicity in this experimental setting (Smith and Fowden 1968).

L-DOPA isolated from *Mucuna pruriens* is a potent allelochemical with an EC50 of 25 mM in some plants (Soares et al. 2014). The growth of species such as Brassicaceae, Hydrophyllaceae and Cucurbitaceae is more inhibited than that of Leguminosae and Gramineae. L-DOPA has herbicidal effects on weeds such as wild mustard (*Sinapis arevensis*) and creeping thistle (*Circium arvense*) at concentrations that do not affect wheat (*Triticum vilgare*) or barley (*Hordeum vulgare*) (Soares et al. 2014). It has been estimated that the velvet bean releases up to 450 kg/ha of L-DOPA into the soil and it can be intercropped with rice and maize to increase the yield (nitrogen fixation) and control weeds (L-DOPA release) (Soares et al. 2014). The mechanisms of phytotoxicity of L-DOPA were investigated by Soares and colleagues in Maize (*Zea mays*) and soybean (*Glycine*

max). L-DOPA caused an increase in tyrosine and phenylalanine levels in the plant and increased lignin deposition in the cell wall and reduced root length (Soares et al. 2014). A microarray study of *Arabidopsis thaliana* treated with L-DOPA (Golisz et al. 2011) identified increased expression of genes relating to metal homeostasis suggesting that the ability of L-DOPA to chelate metals was also involved in its phytotoxicity. Interestingly, genes that were involved in the response to oxidative stress were downregulated in this microarray study, highlighting the ability of L-DOPA to act as both an oxidant and an anti-oxidant (Golisz et al. 2011). The chemistry of L-DOPA is complex and it would appear that its deleterious effects on plants result from a combination of a number of activities including misincorporation into protein, metal chelation, enzyme inhibition and oxidant production (in some cases). The available evidence would suggest that L-DOPA phytotoxicity is very concentration-dependent and very plant specific.

GABA (γ-Aminobutyric acid) is synthesized by plants in response to biotic or abiotic stress and is the most common free amino acid found in waterlogged soils where it could be derived from oxygen-deprived plant roots (Vranova et al. 2011). The effects of exogenous GABA on plant growth are very concentration-dependent and it can promote as well as inhibit growth (Vranova et al. 2011). Exogenous GABA had concentration-dependent effects on growth of garlic (*Allium sativum*) possibly though increasing ethylene production (Mukherjee 2014). The L-proline mimetic Aze, which is synthesised by *Convallaria majalis*, is lethal to other plant species that do not synthesise this imino acid and which competes with L-proline for insertion into proteins (Rubenstein 2008).

4. Non-protein amino acids toxic to herbivores

By utilizing a diverse range of feeding techniques phytophagous insects and other herbivores can obtain nutrients from most parts of a plant (Barah and Bones 2015). In response to damage, insect movement or chemicals released by feeding insects, plants can produce chemicals which are feeding deterrents and can limit the damage caused by insect herbivores (Barah and Bones 2015). The compounds involved in the defense system are known as secondary metabolites since they are not essential for normal growth, development or reproduction, but play a role as signaling molecules or as direct defense chemicals (Barah and Bones 2015). Induced defenses are thought to have evolved because they require less resource allocation than constitutively expressed toxins. In their natural ecosystem, plants are generally consumed by only a fraction of the insect herbivores in the local environment due in part to a combination of inducible and constitutive defense strategies. Since plants contain hundreds of secondary metabolites it is difficult to identify which compounds function as toxins to directly prevent or reduce herbivory. The plant *Mucuna pruriens* (velvet bean) which has a low susceptibility to insect pest contains L-DOPA in its seeds, roots, stems and leaves (Soares et al. 2014). L-DOPA is constitutively produced and concentrations range from 4-7% in *Mucuna pruriens* seeds (Soares et al. 2014), and subsequently it is a commercial source of L-DOPA, the primary drug used to treat the symptoms of Parkinson's disease (PD) (Rodgers 2014). L-DOPA, a close structural analogue of L-tyrosine and a proteomimetic amino acid, is a potent toxin against herbivorous insects (Rehr et al. 1971). It could also interfere with the synthesis of melanin and hardening of the insect cuticle as was observed by Rehr and colleagues (Rehr et al. 1971).

Like L-DOPA, mimosine has a wide range of toxic effects which include misincorporation into proteins and metal chelation. The highest concentrations of mimosine is found in *Leucaena* shoots (Vestena et al. 2001). Mechanical damage to shoots, simulating herbivore activity, or treatment with salicylic acid increased local mimosine concentrations suggesting that it could also play a role in limiting insect herbivory (Vestena et al. 2001).

The NPAA 5-hydroxynorvaline was found in the leaves of the grasses *Zostera japonica* and *Cynodon dactylon* and increased in concentration with leaf dehydration (Carmo-Silva et al. 2009). In an inbred maize (*Zea mays*) line B73, 5-hydroxynorvaline was present at higher concentrations in above ground vegetative tissue but was also present in roots and seeds (Yan et al. 2015). Leaf concentrations increased in response to herbivory by aphids and caterpillars and in response to plant signaling molecules such as jasmonate (Yan et al. 2015). In an experimental setting, reproduction of aphids (*Rhopalosiphum maidis*) was reduced at 5-hydroxynorvaline concentrations normally present in leaves (Yan et al. 2015).

Plants of the Papilionoideae subfamily of the Leguminosae contain one of the best-studied NPAAs, L-canavanine which is a potent insect toxin. It is present in the jack bean *Canavalia ensiformis* and the vine *Dioclea megacarpa Rolfe,* with concentrations of L-canavanine up to 13% reported in seeds of this vine. L-canavanine can replace L-arginine in protein synthesis (Rosenthal 2001) and insect exposure to L-canavanine has been found to produce proteins with "altered conformation and impaired function" (Rosenthal and Dahlman 1986). When introduced into the diet of tobacco hornworm larvae (*Manduca sexta)*, the toxic effects produced included increased mortality, decreased larval growth rates and

malformed adults. After eating only a small amount of plant matter, an insect could be exposed to a lethal dose of L-canavanine (Rosenthal and Dahlman 1986).

The NPAA indospicine is often incorrectly implicated in the development of the neurological syndrome Birdsville horse disease. First reported in 1889, Birdsville horse disease was linked to the consumption of the Australian native legume *Indigofera linnaei*, a plant containing indospicine. The neurological symptoms in Birdsville horse disease are most likely a result of exposure to the neurotoxin 3-nitropropanoic acid (3-NPA) (not a NPAA) which is also present in *Indigofera linnaei* and not indospicine, which is a known hepatotoxin (Ossedryver et al. 2013). This confusion may stem from a case where creeping indigo *Indigofera spicata* led to the development of neurological symptoms that closely resembled Birdsville disease in grazing ponies (Ossedryver et al. 2013). Indospicine, unlike rapidly metabolized 3-NPA, accumulates in animal tissues and has been shown to cause severe hepatotoxicity in dogs eating camel or horse meat contaminated with indospicine (Nunn et al. 2010). This raises the possibility that NPAAs have the potential to accumulate and cause a secondary toxicity.

5. The toxicity of non-protein amino acids to humans

Since ancient times consumption of the legume *Lathyrus sativus,* which contains the excitotoxin β-ODAP, has caused regular outbreaks of the irreversible paralytic disease neurolathyrism. This has occurred in times of famine in Europe, Asia and Africa and still occurs in Bangladesh, India and Ethiopia. Despite its long history, knowledge of the pathogenesis of neurolathyrism is limited. *Lathyrus sativus* is eaten regularly as part of a more complete diet in some parts of India but only a few cases of neurolathyrism have been reported (Mishra et al. 2014). In a recent

study, the affected individuals were all male and had been exposed to *Lathyrus* in the first decade of life (Mishra et al. 2014). The possibility that additional factors such as malnutrition and low immunity make people more susceptible to β-ODAP in times of famine and the possibility of genetic susceptibility to the toxin were also raised in the study of Mishra (Mishra et al. 2014). It is also possible that β-ODAP competes with an as yet unidentified protein amino acid *in vivo.*

A diet of wild potato seeds containing L-canavanine could have resulted in muscle paralysis and the eventual death of Christopher McCandless, wilderness explorer and inspiration for the film 'Into the Wild' (Krakauer et al. 2015). L-canavanine, if supplied in high enough concentrations, is lethal to rats due to its ability to replace L-arginine in protein synthesis (Thomas and Rosenthal 1987). In the case of McCandless, the toxicity of L-canavanine would have been increased due to starvation and low plasma levels of protein amino acids. In his last communication he reported that he was weak, unable to stand up and starving and that it was the fault of the potato seed. It was initially thought that the toxic NPAA was β-ODAP but it was later confirmed that it was L-canavanine.

A link has also been established between alfalfa tablets, which can contain significant amounts of L-canavanine, and systemic lupus erythematosus (SLE) in both humans and monkeys. The exact mechanism that triggers SLE is not known but it is likely to result from L-canavanine-containing proteins having disrupted structure and function (Rosenthal 1977).

In many cases the potential of NPAAs to cause human disease has not been fully investigated. The worldwide prevalence of multiple sclerosis (MS) has been linked to beet agriculture *(Beta vulgaris)* (Rubenstein 2008). Beets contain the NPAA azetidine-2-carboxylic acid (Aze) which can replace proline in proteins leading to

neurodegeneration and autoimmune disorders (Rubenstein 2000). In seminal studies, Rubenstein proposed that Aze replaces L-proline residues in myelin basic protein (MBP) of the myelin sheath. The domains in affected MBP are structurally, functionally and antigenically altered by the exchange of Aze for L-proline resulting in the development of MS in susceptible individuals. A link was also established between the geography of beet agriculture and the worldwide prevalence of MS. In addition, MS is a relatively modern disease and correlates with the increase in cultivation of beets for sugar which now accounts for around 30% of the world's supply of sucrose. Sugar is thought to be Aze-free but the issue could lie in Aze entering the human food chain through the use of by-products of the sugar industry such as sugar beet molasses and sugar beet pulp as animal fodder.

The toxicity of L-DOPA to plants and animals is well documented and has been discussed earlier in the chapter (section 2a). L-DOPA (levodopa) is the primary drug used to treat Parkinson's disease (PD) (Rodgers and Dean 2000). The full implications of high plasma concentrations of L-DOPA, a NPAA which can replace L-tyrosine in protein synthesis, chelate divalent metals, and generate oxidants are not known. Interestingly after 40 years in clinical use reviews still appear each year questioning the neurotoxicity of L-DOPA. Since L-DOPA is the direct precursor of the neurotransmitter dopamine, L-DOPA administration is essentially replacement therapy and virtually all PD patients eventually receive L-DOPA so there is not a control group with which to compare the potential neurotoxic effects of chronic L-DOPA treatment in humans (Chan et al. 2012).

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6. Defenses against toxic non-protein amino acids

6a. Detoxification strategies.

The toxicity of mimosine, and of its primary degradation product DHP (3-hydroxy-4(1H)-pyridine), to ruminants is related to the extent to which they are broken down by the ruminal microbiota (Nunn et al. 2010). Innoculation of Australian goats with ruminal microbiota from Indonesian goats conferred protection on the Australian goats against both the alopecia (mimosine) and suppression of thyroid gland function (DHP) (Nunn et al. 2010). *Lathyrus sylvestris* (flat pea) detoxification could also occur through a similar mechanism. When seed-bearing flat pea hay was fed to sheep only some developed muscular trembling and seizures likely to have occurred due to the accumulation of ammonia. The authors provided evidence that ruminal microbes in some of the host animals had protected them against the flat pea toxins (Rasmussen et al. 1993).

Bruchid beetles have a unique resistance to the presence of the many NPAAs in legumes (Fabaceae), and in some cases appear to feed preferential on plants that contain higher concentrations of NPAAs (Or and Ward 2004). The larvae of these granivorous beetles, members of the Bruchinae subfamily of leaf beetles (Chrysomelidae), typically feed on the legume family of plants, spending the majority of their life within one seed. While bruchid beetles have been shown to be resistant to a number of toxic NPAAs produced by legumes, including pipecolic acid and djenkolic acid, it is their resistance to L-canavanine that is of particular interest. L-canavanine, a structural homologue of L-arginine and potent insecticide, is readily misincorporated into proteins in place of L-arginine during

protein synthesis, resulting in the formation of structurally aberrant proteins (Rosenthal 2001). The bruchid beetle has evolved a highly specific arginyl-tRNA synthase that can differentiate between the toxin L-canavanine and the protein amino acid L-arginine. The beetles also have unusually high urease activity which allows them to metabolize L-canavanine to ammonia through urea, providing a rich source of dietary nitrogen (Rosenthal 1977).

6b. Prevention of autotoxicity

Evolution of an advanced tRNA synthase is the simplest and most effective way for plants to avoid misincorporating toxic proteomimetic NPAAs into their own proteins. The L-canavanine-producing jack bean plant has a tRNA synthase capable of discriminating between L-canavanine and L-arginine and does not incorporate L-canavanine into its own proteins (Igloi and Schiefermayr 2009). In most cases however plants that produce proteomimetic amino acids have not been examined for the presence of advanced tRNA synthases. Alternative strategies to prevent autotoxicity include enzymatic modification of the toxic NPAA to a non-toxic metabolite. This strategy might be utilized by *Mucuna pruriens* which is capable of decarboxylating L-DOPA into dopamine (Matsumoto 2011). An alternative strategy could be to synthesize the toxic NPAA immediately before release. Hydroxylation of phenylalanine to L-DOPA in *Festuca rubra* occurs in the root tips so this could potentially prevent autotoxicity from misincorporation into its own proteins (Soares et al. 2014).

Conclusions and future directions

Given the critical roles that plants play in life on the planet and their central role in many food webs, knowledge of the toxicity of plant NPAAs is surprisingly limited. A better understanding of toxic NPAAs could provide opportunities for more effective and safe weed control or for environmentally friendly herbicides, as has been proposed for L-*m*-tyrosine, a proteomimetic amino acid with a broad phytotoxic spectrum (Matsumoto 2011). The use of velvet bean for intercropping takes advantage of the selective ability of L-DOPA to inhibit weed growth and spare certain plants commonly used as food crops for humans and animals. The counter argument against the widespread use of NPAAs centres on their potential to enter the food chain. Camel or horse meat contaminated with indospicine readily killed dogs but spared camels and horses that had fed on indospicine-containing plants (Ossedryver et al. 2013). There is a possibility that Aze present in beets, a potent cell toxin implicated in MS, can enter the human food chain (Rubenstein et al. 2009). Beet pulp is promoted to dairy farmers in many parts of the world as a high-yielding fodder crop that can provide forage to fill late autumn and winter-feed gaps.

A number of proteomimetic amino acids have been implicated as triggers for neurodegenerative diseases, a group of diseases generally characterized by an increase in the burden of aggregated proteins in neuronal cells (Rodgers 2014). The ability of a NPAA to replace a protein amino acid in protein synthesis can result in the synthesis of non-native proteins resulting in an increased rate of protein misfolding and aggregate formation (Rodgers 2014). Long-lived cells such as neurons and retinal pigment epithelial cells are more vulnerable than dividing cells since they are unable to dilute the aggregated proteins amongst daughter cells and instead they accumulate them over their lifetime (Rodgers 2014). In

addition, new epitopes can be generated following partial proteolysis of the non-native proteins which are subsequently recognised as non-self and can trigger an immune response (Rodgers 2014). The possibility that NPAAs can bioaccumulate should be seriously considered based on what is now known about β-N-methylamino-L-alanine (BMAA). BMAA is a NPAA synthesized by most strains of cyanobacteria (not by plants, as far as the authors know) that was bioconcentrated through cycads (*Cycas micronesia*) and flying foxes, which were a local delicacy on Guam. BMAA was implicated in a complex neurological disease that had a very high incidence on the island of Guam and BMAA is now implicated in sporadic amyotrophic lateral sclerosis (ALS) globally, potentially resulting from contact with cyanobacterial blooms (Cox 2009). Since some NPAAs can be misincorporated into proteins this potentially provides a mechanism for bioaccumulation within a food chain (Dunlop et al. 2013).

Despite the fact that many of the NPAAs discussed in this chapter have been known for some time, there is a fundamental lack of knowledge on how organisms handle them. Many excellent studies from the late 60s demonstrated clearly that the 20 protein amino acids were not 'sacred' and certain NPAAs could be mistakenly used in protein synthesis in place of a coded amino acid, and many others were toxic to bacteria via other biochemical pathways that utilized protein amino acids (Fowden 1981; Hendrickson et al. 2004). While it is generally accepted that very few enzymes or receptors are absolutely specific for a single molecule and similar molecules can produce an effect (a principle used by the pharmaceutical industry), many scientists cling on to the notion of absolute 'fidelity' in protein synthesis. While this holds true (mostly) for the ability of the biosynthetic machinery of the cell to discriminate between the 20 protein amino

acids, there are around 1000 variations on these molecules present in nature, the majority of which are produced by plants. Logic would dictate that misincorporation of at least some of these amino acids would be possible.

The Swiss scientist and 'Father of Toxicology' Paracelsus stated that 'all things are poison and nothing is without poison; only the dose makes that a thing is no poison' and this holds true for plant NPAAs. Many NPAAs are capable of competing with protein amino acids for interactions at the active sites of enzymes or with receptors, but they need to be present in high concentrations relative the protein amino acids to produce a biological effect. A few examples have been given of situations when their toxicity is manifest; in times of famine and severe depletion of protein amino acids or when alfalfa is made into tablets (Rosenthal 1977). The big questions remain: to what extent can NPAAs enter the food chain and bioaccumulate, and what are the long-term effects of chronic exposure to low levels of toxic NPAAs? No causes have been identified for many chronic diseases in humans. Diseases associated with protein misfolding or abnormal protein synthesis such as Parkinson's disease and amyotrophic lateral sclerosis are predominantly sporadic (less than 10% genetic) but the factors that cause the disease have not been identified. Chronic exposure to proteomimetic plant NPAAs could contribute to disease development in genetically susceptible individuals by increasing the burden of non-native proteins in neuronal cells. Finding answers to these questions are challenges that scientists need to address since this knowledge could benefit all organisms on the planet.

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