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L-Canavanine: a higher plant insecticidal allelochemical

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Summary. L-Canavanine, L-2-amino-4-(guanidinooxy)butyric acid, is a potentially toxic nonprotein amino acid of certain leguminous plants. Many species are prolific canavanine producers; they divert enormous nitrogen resource to the storage of this single natural product. Canavanine, a highly effective protective allelochemical, provides a formidable chemical barrier to predation and disease.

The accumulated experimental evidence leaves little doubt that the key element in the ability of canavanine to function as an effective protective allelochemical is its subtle structural mimicry of arginine which makes it an effective substrate for amino acid activation and aminoacylation, and its marked diminution in basicity relative to arginine which mediates the production of structural aberrant, dysfunctional canavanyl proteins.

The biological burdens of canavanyl protein formation by canavanine-treated *Manduca sexta* larvae were carried throughout their remaining life cycle. Protein-based sequestration of canavanine prevented turnover and clearance of the free amino acid, and undoubtedly contributed significantly to the antimetabolic character of this protective allelochemical.

Keywords: Amino acids – L-Canavanine – Allelochemical – Natural products

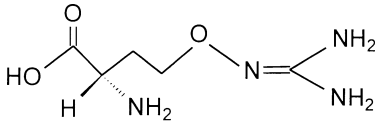
Introduction

L-Canavanine, L-2-amino-4(guanidinooxy)butyric acid, is a non-protein amino acid synthesized by leguminous plants that are members of the Lotoidea, a subfamily of the Leguminosae (Bell et al., 1978). Many canavanine-synthesizing legumes store prodigious amounts of this nonprotein amino acid: members of the genus *Canavalia* typically commit 3–4% of their seed dry matter to canavanine storage (Rosenthal and Nkomo, 2000); the neotropical legume, *Dioclea megacarpa*, sequesters so much seed canavanine that this single metabolite commanders more than 95% of every nitrogen atom allocated to free amino acid production (Rosenthal, 1977). *Colutea arborescens, Caragana arborescens, Vicia gigantea, Robinia pseudoacacia and Wisteria floribunda*, representative of many prolific canavanine producers,

store from nearly 6 to 13% canavanine by dry weight (Rosenthal, 1977a). The purpose of this review is to probe the rationale for plant investment of so much nitrogen into a single secondary metabolite.

Canavanine chemistry

L-Canavanine bears marked structural analogy to L-arginine in that the terminal methylene group of arginine is replaced with oxygen.



L-CANAVANINE

Scheme 1

Oxygen is significantly more electronegative than carbon; this causes enhanced electron withdrawal that facilitates deprotonation, and reduces the pK_a value of the guanidinooxy group to about 7.04 (Boyar and Marsh, 1982) – far less than 12.48, the pK_a of the guanidino group of arginine (Greenstein and Winitz, 1963). Under physiological conditions, arginine, a highly basic amino acid, is fully protonated while canavanine is much more anionic. This marked difference in the charge state of the R group of canavanine, as compared to that of arginine, can disrupt critical R group interactions within a given protein and affect profoundly the way in which the protein folds into its three-dimensional conformation. Alteration in essential conformation can affect adversely protein function.

Canavanyl protein formation and function

The structural analogy between L-canavanine and L-arginine is so marked that this arginine mimic serves as a substrate in virtually every enzyme-mediated reaction that preferentially employs arginine as a substrate. This structural subtlety is nowhere more important than with arginyl tRNA synthetase, which readily esterifies L-canavanine to the cognate $tRNA^{Arg}$ (Allende and Allende, 1964; Mitra and Mehler, 1967). One of the defining hallmarks of all canavanine-sensitive insects is an inability to differentiate between these amino acids; these organisms readily activate canavanine and incorporates it into their *de novo*-synthesized proteins in place of arginine (Bleiler et al., 1988).

The bruchid beetle, *Caryedes brasiliensis*, oviposits on the canavanine-laden seeds of *Dioclea megacarpa* which typically contain 8–9% canavanine by dry weight (Rosenthal, 1983). The developing weevil, *Sternechus tuberculatus*, feeds on seeds of the legume, *Canavalia brasiliensis*, where

canavanine levels can reach 6–8% of the dry matter (Bleiler et al., 1988). These neotropical seed predators possess highly discriminatory arginyl tRNA synthetases that permits them to scrupulously avoid canavanine activation and esterification (Rosenthal, 1991a).

The tobacco hornworm, *Manduca sexta* [Sphingidae] feeds on solanaceous plants, none of which has the genomic complement for canavanine production. Therefore, there has not been an opportunity for evolutionary adaptation to this toxic allelochemical. Numerous experimental studies have fully documented the potent insecticidal properties of canavanine against this lepidopteran plant feeder (Dahlman and Rosenthal, 1975, 1976; Rosenthal and Dahlman, 1975). This arginine antagonist markedly reduces overall larval growth; delays and otherwise disrupts larval-pupal and pupal-adult ecdysis; produces profound larval, pupal, and adult growth defects; elicits severe diuresis; and dramatically attentuates female fucundity and fertility. Typically, canavanine-treated larvae that survive the larval instars, expire in a futile attempt at pupal-adult metamorphosis or the body parts of the adult are so severely malformed as to be nonfunctional (Rosenthal, 1977b). *Manduca sexta* larval sensitivity to canavanine markedly increases when the canavanine dietary concentration exceeds 2.5 mM or they receive more than 1.0 mg/g larval body weight by parenteral injection.

Under optimal conditions for canavanine incorporation, 3.0% of the administered L-[*guanidinooxy*-^{14}C]canavanine is incorporated into newly synthesized hemolymphic proteins *after 24 h* (Rosenthal et al., 1987) (Fig. 1). Analysis of the radiolabeled body wall (the thoracic musculature and integument) proteins discloses the unexpected and important finding that on average one of two arginyl residues are replaced with canavanine (Rosenthal et al., 1987). *Manduca sexta* larvae possess a highly effective system for

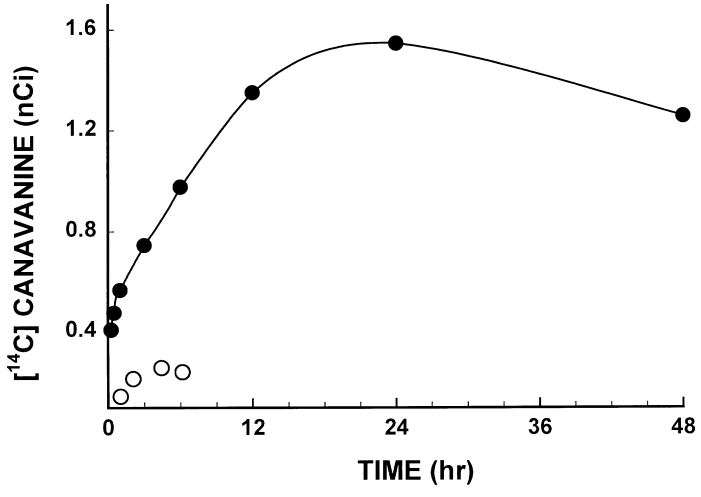


Fig. 1. Incorporation of L-[*guanidinooxy*-^{14}C]canavanine into newly synthesized hemolymphic proteins of *Manduca sexta* (●) or *Heliothis virescens* (○) terminal instar larvae. See Rosenthal et al. (1987) for additional experimental details

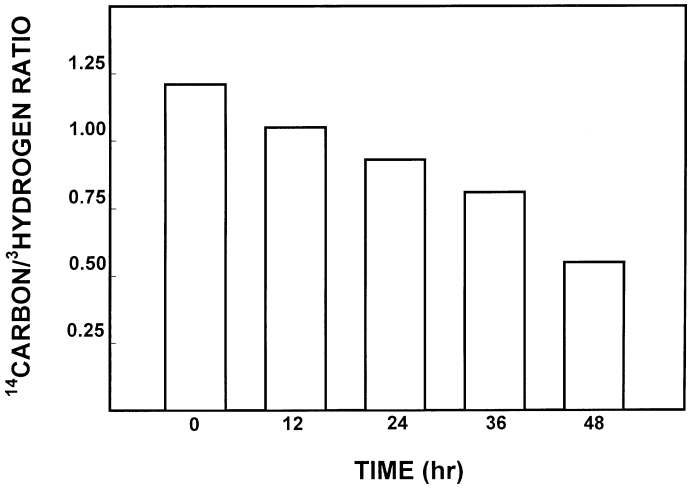


Fig. 2. The relative stability of [^{14}C]canavanine- and [^{3}H]arginine-labeled hemolymphic proteins, expressed as a ^{14}C to ^{3}H ratio. The radiolabeled proteins were injected into receipient larvae, and evaluated at the indicated times (hr). See Rosenthal and Dahlman (1986) for additional experimental details

preferential degradation of canavanine-containing proteins relative to their normal counterpart (Rosenthal and Dahlman, 1986). This finding was established by preparing L-[*guanidinooxy*-^{14}C]canavanine- and L-[*guanidino*-^{3}H]arginine-labeled hemolymphic proteins that were purified of free radiolabeled amino acids, filter-sterilized, and reintroduced into the hemolymph of receipient larvae (Rosenthal and Dahlman, 1986) (Fig. 2). This metabolic capacity may result in a significant "underestimation" of the actual level of canavanine incorporated into insectan proteins after 24 hr.

Insight into the biochemical basis for *M. sexta* sensitivity to canavanine can be gained by examining the effect of canavanine incorporation on *de novo*-synthesized insectan proteins. Several independent lines of experimental evidence including: electrophoretic analysis of native and canavanyl vitellogenin, probing conformational differences by evaluating surface-exposed amino acid residues, and monoclonal antibody analyses established that canavanine incorporation into the vitellogenin of the gravid locust, *Locusta migratoria migratorioides* [Acrididae] profoundly alters the unique three-dimensional structure (Rosenthal et al., 1989a).

Other experiments, conducted with third instar larvae of the meat-eating fly, *Phormia terranovae* [Diptera], demonstrated that canavanine assimilation into their protective, antibacterial proteins, known trivially as diptericins A, B or C and peak V protein, nullified the protective efficacy of all of the antibacterial proteins except diptericin A whose biological activity was significantly impaired (Rosenthal et al., 1989b) (Fig. 3).

Finally, lysozyme, induced in *M. sexta* larvae in the presence of canavanine, had 21% of its arginyl residues replaced by canavanine. This substitution caused a loss of nearly one-half of the catalytic activity of this protein (Rosenthal and Dahlman, 1991). These independent and detailed

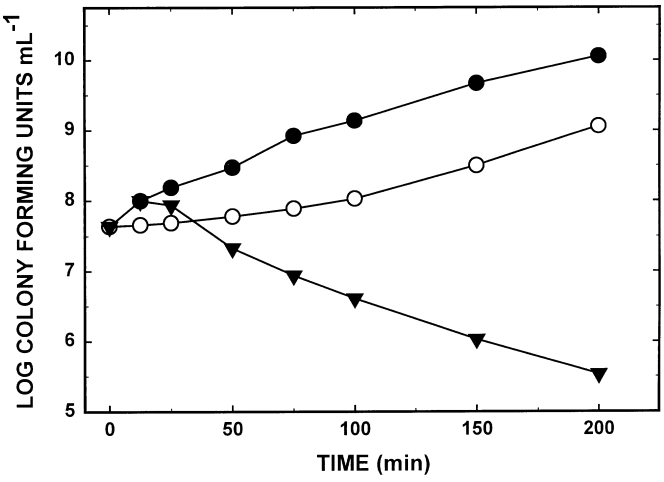


Fig. 3. The antibacterial potency of native and canavanyl diptericin A. Biological activity was evaluated as a function of colony forming ability of *Escherichia coli* D31 in the presence of native (▼), or canavanyl (○) diptericin A. Control cultures (●) received protein serum albumin. See Rosenthal et al. (1989b) for additional experimental details

investigations provided the first experimental evidence linking canavanyl protein formation to the loss of essential protein function.

D-Canavanine and *Manduca sexta*

D-Canavanine is biologically active in *M. sexta* larvae, e.g. it causes larval edema – a characteristic consequence of canavanine consumption. Significantly, the D-enantiomer, which is not a substrate for arginyl tRNA synthetase, shows little of the adverse larval growth effects and pupal deformation that are hallmarks of canavanine toxicosis and which have been linked consistently to aberrant, canavanyl protein formation (Rosenthal et al., 1995). As postulated, divorced from a role in protein synthesis, the D-enantiomer does not elicit any of the symptomology linked to the formation of structurally anomalous, conformationally altered, and functionally impaired canavanine-containing proteins.

Homoarginine and canavanine toxicity

Uptake studies, employing L-[*guanidino*-^{14}C]homoarginine, established that this arginine antagonist is readily assimilated into the primary protein pools of the developing *M. sexta* larva: hemolymph proteins as well as those of the body wall and musculature. Administration of homoarginine to *M. sexta* larvae, at 1.5 times the canavanine dose that proved lethal to all tested larvae within 48 h, failed to elicit any adverse larval growth or developmental effects; all larvae successfully ecdysed to pupae and then adults (Rosenthal and Harper, 1996).

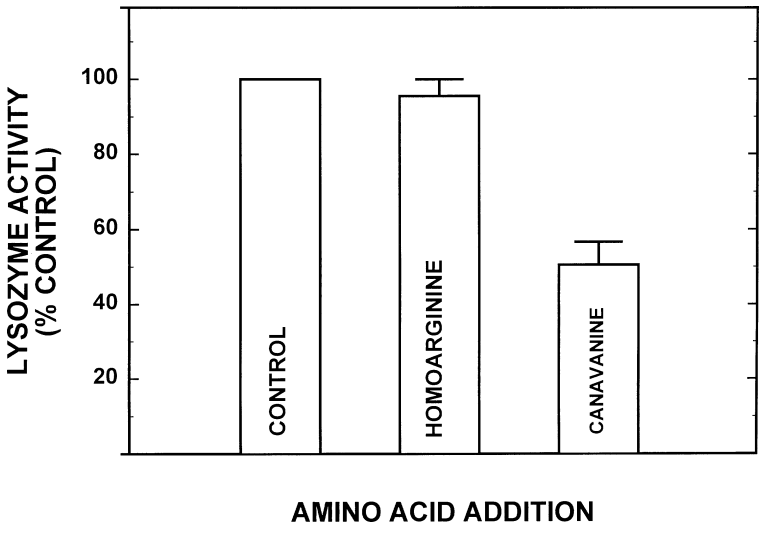


Fig. 4. The effect of homoarginine or canavanine incorporation on lysozyme activity. See Rosenthal and Harper (1996) for additional experimental details

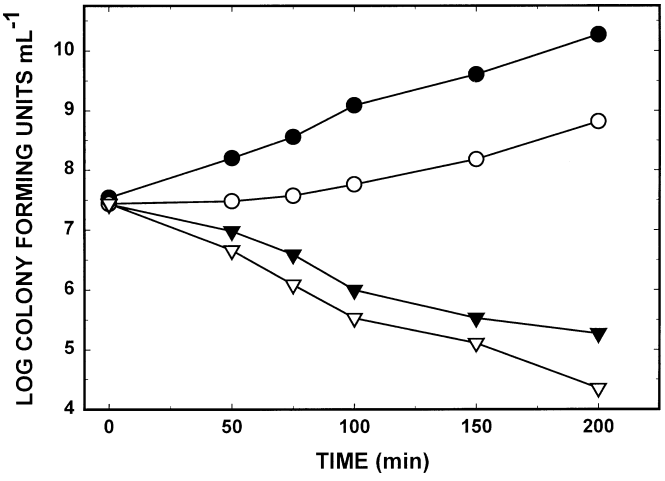


Fig. 5. The biological activity of variously substituted diptericin A. Biological activity was evaluated as a function of colony forming ability of *Escherichia coli* D31 in the presence of native (▽), canavanyl (○), or homoarginyl (▼) diptericin A. Control cultures (●) received protein serum albumin. See Rosenthal and Harper (1996) for additional experimental details

A study designed to evaluate the effect on lysozyme activity of replacing arginine with various arginine analogues revealed that L-homoarginine did not disrupt lysozyme activity (Fig. 4) (Rosenthal and Harper, 1996). In a similar vein, assimilation of homoarginine into the diptericins of *P. terranovae* had no discernible effect on their antibacterial activity (Fig. 5), homoarginine is incorporated as effectively as canavanine into larval proteins without adversely affecting larval growth and development as well as enzymic function. Why?

It is my contention that homoarginine is innocuous because of the elevated pK_a value for its guanidino group–arguably higher than that of arginine (Rosenthal and Harper, 1996). Being at least as basic as arginine, homoarginine is incorporated into insectan proteins without disrupting R group interaction and essential protein conformation. In summary, the innocuous nature of homoarginine as compared to canavanine probably results from the far greater basicity of the former. The accumulated experimental evidence continues to lend credence to the concept that the key element in the ability of canavanine to function as an effective protective allelochemical is its subtle structural mimicry of arginine which makes it an effective substrate for amino acid activation and aminoacylation, and its decreased basicity relative to arginine which mediates the production of structural aberrant, dysfunctional canavanyl proteins.

In a recent study, we administred L-[*guanidinooxy*-^{14}C]canavanine to terminal instar larvae by a single parenteral injection and then evaluated the presence of radiolabeled canavanine in newly synthesized larval proteins. We subsequently isolated the proteins of the pupae or adults that developed from comparably radiolabeled larvae, and discovered no significant diminution in the amount of [^{14}C]canavanine/mg protein. Thus, the biological burdens of canavanyl protein formation to canavanine-treated larvae were carried throughout their remaining life cycle. This protein-based sequestration of canavanine prevented its turnover and clearance, and undoubtedly contributed significantly to the antimetabolic character of this protective allelochemical (Rosenthal et al., 1998).

Canavanine esterification

Reasoning that converting canvanine to its simple esters would enhanced its hydrophobicity and hopefully increase its penetration into the cell membrane, we evaluated the biological effects of the methyl- and ethyl-ester of canavanine on *M. sexta* larval growth (Rosenthal et al., 1998). Since the ethyl ester proved more toxic than than the methyl ester, we synthesized even longer-chained esters, specifically the propyl, butyl, isobutyl, and octyl esters of L-canavanine and evaluated their insecticidal properties (Rosenthal et al., 1998). Of the tested esters, the isobutyl and octyl esters were far more deleterious to larval growth and development; a single injection of the isobutyl or octyl ester was lethal to all of the test animals before the termination of the larval instar. This deleterious effect was not caused by the release of the free alcohol, via the action of an insectan esterase, but rather related directly to esterification of the parent compound (Rosenthal et al., 1998).

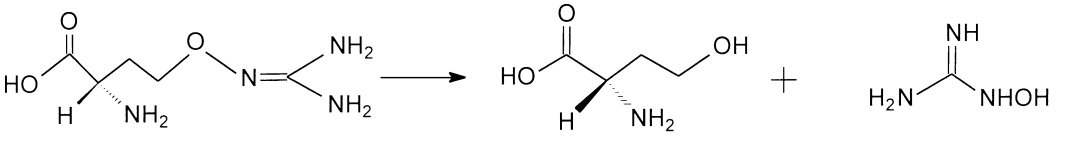
Heliothis virescens: an insect remarkably resistant to canavanine

Terminal instar larvae of the tobacco budworm, *Heliothis virescens* [Noctuidae], a highly destructive agricultural pest, reared on canavanine-

containing diet had an LC_{50} value for this nonprotein amino acid of 300 mM (Berge et al., 1986). This LC_{50} corresponds to 53,000 ppm wet diet weight or nearly 40% on a dry weight basis. While the pupae that emerged from such treated larvae were depauperate, they lacked any discernible developmental aberrations. A group of five larvae reared on a staggering 500 mM canavanine-containing diet survived for 9 days before the onset of larval death.

Analysis of canavanine consumption established that the treated larvae neither eliminate signficant canavanine in their fecal matter nor sequester it within an internal body organ (Berge and Rosenthal, 1990). Rather, the larvae efficiently metabolize canavanine with a $t_{1/2}$ of 135 min (Berge and Rosenthal, 1990). Several lines of experimental evidence demonstrated that the larvae do not induce a detoxification enzyme in response to canavanine exposure, rather its ability to metabolize this normally potent toxicant is constituted (Berge and Rosenthal, 1990).

Administration of L-[*guanidinooxy*-^{14}C]canavanine to terminal instar larvae generated [^{14}C]guanidine as the principal *in vivo* larval radiolabeled catabolite (Berge and Rosenthal, 1991). This finding implicated a larval reductase that fosters the reduction of canavanine to homoserine and guanidine. Independent experiments, involving *H. virescens* larvae, led to the discovery of another larval enzyme – a reductase that catalyzes an NADH-dependent reduction of hydroxyguanidine to guanidine (Rosenthal, 1992). This discovery immediately suggested that canavanine may be catabolized initially to homoserine and hydroxyguanidine rather than guanidine, via a novel hydrolase able to cleave the O-N bond of the guanidinooxy moiety of the substrate.



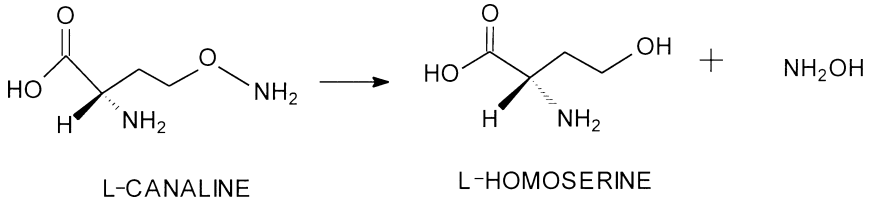
Scheme 2

Although this catabolic reaction had been observed in a soil-borne *Pseudomonas* (Kalyankar et al., 1958), the responsible enzyme was never isolated nor had this metabolic capacity been described previously from an eukaryotic organism. Overall, the experimental evidence implicated two gut enzymes that were functioning in concert to metabolize canavanine. The first reaction used a novel hydrolase that directed the formation of L-homoserine and hydroxyguanidine from L-canavanine. In the second phase, hydroxyguanidine was reduced to guanidine.

Canavanine hydrolase

The search for this enzyme in the gut of larval *H. virescens* culminated in the isolation of a homogeneous enzyme that mediated an irreversible hydrolysis of L-canavanine to L-homoserine and hyroxyguanidine (Michelangeli et al., 1997). The existence of canavanine hydrolase, an enzyme able to cleave an oxygen-nitrogen bond, was an important finding since this enzyme is the only protein known to demonstrate this catalytic ability. As such, it represents a new type of hydrolase-one able to act on oxygen-nitrogn bonds that has been given the novel designation: EC 3.13.1.1.

Canavanine hydrolase (EC 3.13.1.1) exhibits a high affinity for canavanine as evinced by its apparent K_m value of 1.1 mM; the turnover number for this reaction is 21.1 mmol min^{-1}. This enzyme exhibits a high degree of substrate specificity as it cannot function effectively with L-2-amino-5(guanidinooxy) pentanoate nor 2-amino-3(guanidinooxy)propionate, the higher or lower homolog, respective of L-canavanine nor with its methyl ester. Nor can canavanine hydrolase react with L-canaline in a comparable reaction that would have produced homoserine and hydroxylamine (Michelangeli et al., 1997).



Scheme 3

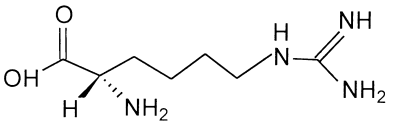
The canavanine detoxification pathway in *H. virescens* is different from that exhibited by larvae of the canavanine-adapted bruchid beetle, *C. brasiliensis.* The larvae of this neotropical insect hydrolytically cleave L-canavanine to L-canaline and urea (Rosenthal, 1983). Urea was degraded subsequently to ammonia to provide ammoniacal nitrogen for the production of insectan amino acids (Rosenthal et al., 1982). Indeed, it is this same arginase-mediated deguanidination of L-canavanine to generate L-canaline and urea, coupled with urease-driven hydrolysis of the latter to create ammonia, that permits higher plants to effectively mobilize the stored nitrogen of canavanine (Rosenthal, 1991b).

A thorough search of the relevant literature revealed that many plants that are members of the Lotoideae are consumed by larval *H. virescens* (Pearson, 1958; Zulucki et al., 1986). It is reasonable to speculate that tobacco budworm larvae, feeding amongst canavanine-containing plants, enhanced their natural resistance to this potentially toxic allelochemical, over evolutionary time, by directing the synthesis of a novel enzyme that accounts for the remarkable resistance of this generalist herbivore to canavanine. It is noteworthy that *H.*

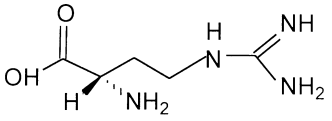
virescens larvae, which are highly resistant to canavanine's antimetabolic efffects, exhibit little ability to incorporate radiolabeled canavanine into *de novo*-synthesized larval proteins (Fig. 1) (Rosenthal and Dahlman, 1986).

Conclusion

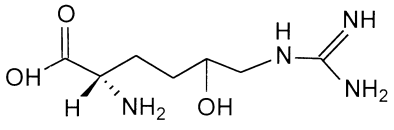
Higher plants produce a number of arginine analogs including the higher homolog, L-homoarginine; its lower homolog, 2-amino-4-guanidinobutyric acid; 5-hydroxy-L-homoarginine; L-indospicine (L-2-amino-6-amidinohexanoic acid), and 5-hydroxy-L-arginine (Rosenthal, 1982).



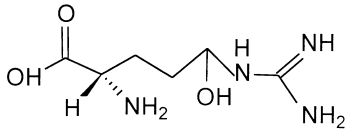
L-HOMOARGININE



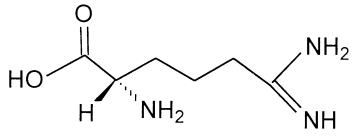
L-2-AMINO-4-GUANIDINOBUTYRIC ACID



5-HYDROXY-L-HOMOARGININE



5-HYDROXY-L-ARGININE



L-INDOSPICINE
(L-2-AMINO-6-AMIDINOHEXANOIC ACID)

Scheme 4

None of these natural products is as effective an arginine antimetabolite as canavanine. The selection pressure that favored the development of the genome for canavanine synthesis and storage was driven undoubtedly by the subtle structural similarity between canavanine and arginine and the appreciable reduction in the basicity of canavanine relative to arginine that was achieved by the replacement of a terminal methylene group with oxygen. These factors combined to create a highly effective antimetabolite that provides a high level of defensive efficacy against a wide array of insectan predators and pests.

Acknowledgments

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