Effect of Long-Chained Esters on the Insecticidal Properties of L-Canavanine

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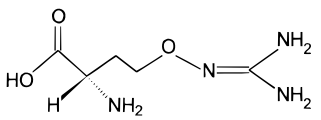
The ability of the several esters of L-canavanine, a potentially insecticidal natural product of leguminous plants, to enhance the potency of the parent compound was evaluated with the propyl, butyl, isobutyl, and octyl esters of L-canavanine. These compounds, administered either by a single parenteral injection or by dietary consumption, were tested for their intrinsic toxicity in studies conducted with terminal instar larvae of the tobacco hornworm *Manduca sexta* [Sphingidae]. Parenterally injected propyl or butyl esters were somewhat more toxic than canavanine, but the isobutyl and octyl esters were far more deleterious to larval growth and development. A single injection of the isobutyl or octyl ester (1.25 mg/g larva, molar equivalent of L-canavanine dose) was lethal to all the test animals before the end of the larval instar. Much of the toxicity of the octyl ester arguably was due to release of octyl alcohol, since the free alcohol is highly pernicious. In contrast, the isobutyl ester was far more toxic than isobutanol. A different picture emerged from chronic exposure to the tested esters via dietary consumption. By this administrative route, although all of the esters were marginally more toxic than canavanine, the tested compounds exhibited much less toxicity than occurred by parenteral injection. Little difference was noted in the relative growth-inhibiting activity between the esters, and all of the free alcohols exhibited little deleterious effect on larval development.

Keywords: *L-Canavanine; L-canavanine ester derivatives; insecticidal compounds; Manduca sexta*

INTRODUCTION

Many leguminous plants are a rich source of the insecticidal nonprotein amino acid L-canavanine, the



L-CANAVANINE

L-2-amino-4-(guanidinooxy)butyric acid structural analogue of L-arginine (Bell, 1971; Rosenthal, 1977). The potent antimetabolic properties of this protective allelochemical have been studied most extensively in the tobacco hornworm *Manduca sexta* [Sphingidae], where its consumption by the larvae caused massive developmental aberrations to the pupae and adults that emerged from the surviving canavanine-treated larvae (Dahlman and Rosenthal, 1975). Insect mortality usually occurred in a futile attempt at pupal−adult metamorphosis, or the body parts of the adult were so severely malformed as to be nonfunctional (Dahlman and Rosenthal, 1976). Investigation of the biochemical basis for canavanine's antimetabolic properties implicated the formation of structurally aberrant, canavanine-containing proteins that had lost essential functionality (Rosenthal, 1992; Rosenthal, 1993).

In a recent unpublished study, we administered L-[*guanidinooxy*-^{14}C]canavanine to terminal instar larvae by a single parenteral injection and determined the incorporation of the radiolabeled canavanine into the newly synthesized proteins of the larvae. We subsequently isolated the proteins of the pupae or adults that developed from these radioactive larvae and discovered no significant diminution in the amount of [^{14}C]canavanine/mg protein. Thus, the biological burdens of canavanine-containing, aberrant protein formation by the canavanine-treated larvae were carried throughout their remaining life cycle. This protein-based sequestration of the toxicant impeded its turnover and undoubtedly contributed significantly to the antimetabolic potential of this arginine antagonist.

In a companion publication, we compared the insecticidal properties of the methyl and ethyl ester of canavanine to the parent compound (Rosenthal et al., 1995). We reasoned that these simple esters, possessing enhanced hydrophobicity, might penetrate the cell membrane more effectively than the parent compound and provide an efficient means of transporting canavanine into the cell prior to its hydrolysis by intracellular esterases to the parent compound. Insects are biochemically competent to deesterify toxicants (Yu, 1984; Yu, 1990). Our prior publication reported that these esters were less toxic than canavanine (Rosenthal et al., 1995). Since the ethyl ester proved more toxic than its methyl-bearing counterpart, we synthesized longer-chained esters, specifically the propyl, butyl, isobutyl, and octyl esters of L-canavanine, and evaluated their insecticidal properties in *M. sexta* larvae.

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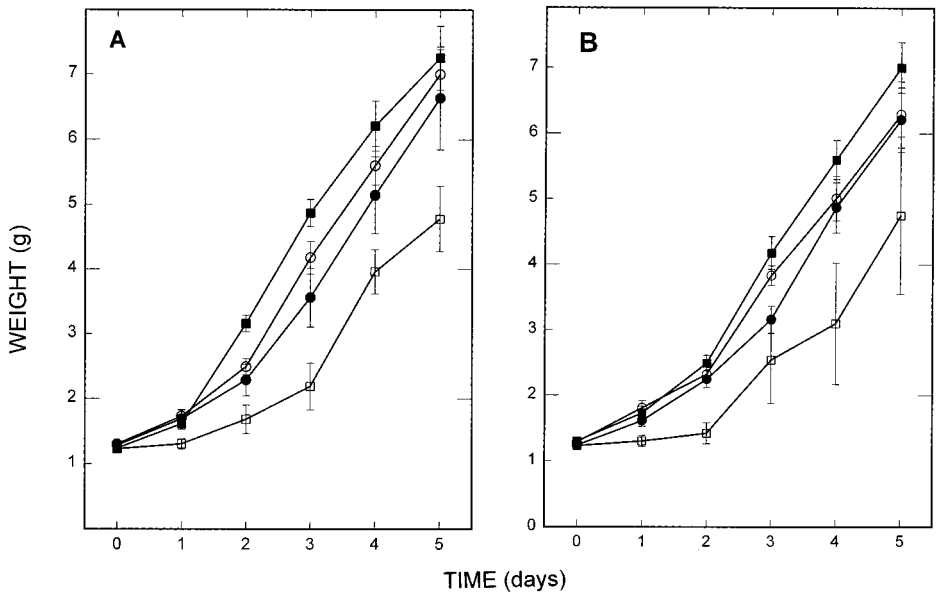

Figure 1. (A) Effect of a single injection of canavanine or certain of its esters on larval growth during the terminal, fifth instar. *Manduca sexta* larvae were provided with 1.25 mg g^{-1} fresh body weight canavanine (○) or an equivalent dose of propanol (●), or propylcanavanine (□) in sterile water. The control larvae received sterile water (■). See text for additional experimental details. (B) Canavanine (○), butanol (●), butylcanavanine (□), and control (■).

METHODS AND MATERIALS

Insects. All experiments were conducted with newly ecdysed (<12 h) fifth instar *Manduca sexta* larvae obtained from a continuous colony maintained at the University of Kentucky using rearing procedures similar to those described by Yamamoto (1969).

Injections. Unless indicated otherwise, 10 larvae were injected, after being anesthetized with carbon dioxide, with L-canavanine, an ester, or an alcohol. Control larvae received deionized water. The drug was administered by a single parenteral injection so that 50 μL, containing the molar equivalent of 1.25 mg canavanine, was provided for each gram of larval fresh weight. The concentration series, conducted with the isobutyl or octyl ester, was administered so that the desired drug concentration was delivered in 50 μL of sterile, deionized water g^{-1} fresh body weight. Control larvae were given sterile water. All larvae were weighed daily. All values are the mean ±SEM for 10 larvae.

Dietary Consumption. The 2.5 mM dietary concentration of L-canavanine was selected because it did not significantly alter mass-dependent growth parameters, which would have affected profoundly hormonal balance, while allowing the expression of various time-dependent growth parameters and pupal deformity. All of the esters were compared to this concentration of canavanine.

Chemicals and Biochemicals. L-Canavanine was isolated from ethanol-extracted jack bean *Canavalia ensiformis* [Fabaceae] seeds and purified by recrystallization (Bass et al., 1995). Unless otherwise indicated, all other chemicals or biochemicals were purchased from Sigma Aldrich or Fisher Scientific.

Synthesis of the Several Esters of L-Canavanine. Propyl, butyl, and isobutyl esters of L-canavanine were prepared by reacting 30 mmol of L-canavanine, ground as finely as possible with a mortar and pestle, in 150 mL of the appropriate alcohol with slowly flowing anhydrous HCl for 1.5 h at 75−80 °C. After the HCl flow was stopped, the reaction was allowed to proceed for 6 h prior to removal of the solvent by rotary evaporation in vacuo. The residue was twice taken up in the appropriate alcohol, and dried as above; this procedure removed unreacted HCl. The final residue was dissolved in ethanol−water (3:2, v/v), treated with 8 g of decolorizing charcoal for 30 min, and filtered. The filtrate was concentrated as above and the residue dissolved in absolute ethanol and dried by rotary evaporation with a 0.5 mm vacuum. The residue was crystallized at 0 °C from a mixture of ethyl alcohol and anhydrous ether (1:5). The final product was lyophilized in a preweighed vessel for 48 h prior to storage. The octyl ester of L-canavanine was prepared as above except that the reaction was allowed to proceed overnight at 70 °C.

All of the esters of L-canavanine, particularly the octyl ester, are extremely hygroscopic. Even storage in vacuo in a vessel that itself was housed in a vacuum desiccator over phosphorus pentoxide did not prevent deliquescence of the ester. As a consequence, the lyophilized esters were dissolved immediately in deionized water, titrated to pH 6.8−7.2 with 2 N NaOH, and stored as a solution at −80 °C. The canavanine esters were thawed and used immediately in either diet or injection studies.

All of the synthesized esters were shown to be free of unreacted canavanine by automated amino acid analysis employing a Dionex D-300 automated amino acid analyzer. Each of the esters separated fully from canavanine (elution time of 105 min) by this procedure. The elution time for the propyl, butyl, and octyl ester was 108.5, 110, and 117.5 min, respectively.

The optical rotation for the propyl, butyl, and octyl ester (c = 0.5, H_2O) was +28.6°, +16.9°, and +7.6°, respectively. These values were consistent with the optical rotation value reported for these esters by another laboratory (Na Phuket et al., 1997). The esters were too hygroscopic to obtain melting point values or to ship them for elemental analysis.

RESULTS AND DISCUSSION

Administration by Parenteral Injection. The postulate that esterification of the carboxyl group of L-canavanine might enhance the growth-inhibiting properties of this arginine analogue against *Manduca sexta* larvae has been confirmed. Parenteral injection of the propyl ester of canavanine (1.25 mg g^{-1}, molar equivalent of canavanine) had a greater adverse effect on larval growth dynamics than did an equimolar amount of the parent compound (Figure 1A). Free propanol was also administered to confirm that release of the free alcohol by intracellular esterase activity did not account for the enhanced toxicity of this derivative relative to canavanine.

Addition of another methylene group to the propyl ester linkage did not further increase the differential

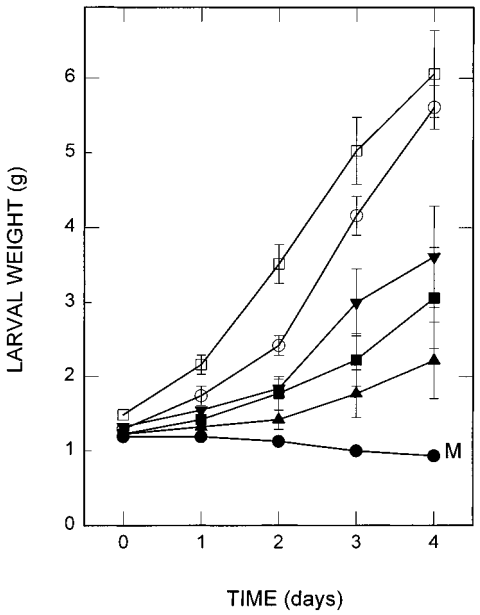

Figure 2. Effect of a single injection of canavanine or certain of its esters on larval growth during the terminal, fifth instar. *Manduca sexta* larvae were provided with the following (in mg g^{-1} fresh body weight): canavanine, 1.25 (○); isobutanol, 1.25 (▼); isobutylcanavanine, 0.75 mg (■); isobutylcanavanine, 1.0 mg (▲), or isobutylcanavanine, 1.25 mg (●). The control larvae received sterile water (□). M indicates the time when all experimental animals had perished; see text for additional experimental details.

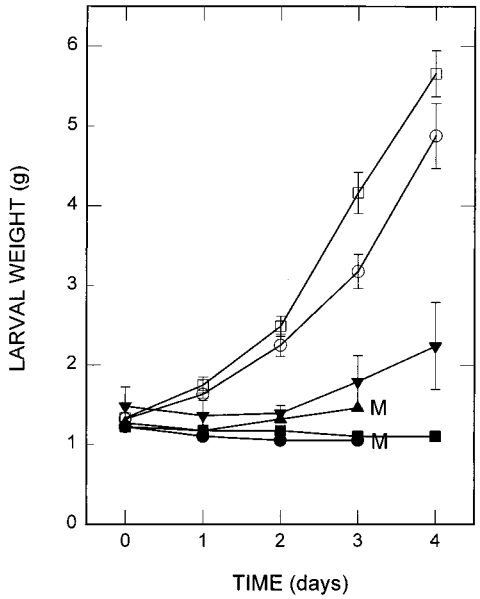

Figure 3. Effect of a single injection of canavanine or certain of its esters on larval growth during the terminal, fifth instar. *Manduca sexta* larvae were provided with the following (in mg g^{-1} fresh body weight): canavanine, 1.25 (○); octanol, 1.25 (▼); octylcanavanine, 0.75 mg (▲); octylcanavanine, 1.0 mg (■), octylcanavanine, 1.25 mg (●). The control larvae received sterile water (□). M indicates the time when all experimental animals had perished; see text for additional experimental details.

growth-inhibiting properties of the ester relative to canavanine (Figure 1B). Once again, the butyl ester was more toxic than butanol itself. This finding stands in sharp contrast to the results observed with the isobutyl ester. This compound was far more toxic than the butyl ester; little of its toxicity could have resulted from the release of free alcohol (Figure 2). Administration of isobutyl-L-canavanine (1.25 mg g^{-1}) prevented larval growth and was fatal to all larvae before the end of the terminal instar at day 4.

The octyl ester of canavanine was the most growth-inhibiting of the tested esters; administration of 0.75 mg g^{-1} octyl ester proved lethal to all of the tested larvae (Figure 3). Much of the toxicity of this compound might have resulted from release of free octanol, since this alcohol is also harmful to the growing larvae (Figure 3).

Administration by Dietary Consumption. The effect of chronic exposure to the various esters of canavanine was evaluated by incorporating these compounds into an artificial, agar-based diet used to rear the larvae. These experiments indicated that the larvae exhibited a greater tolerance to these potentially toxic compounds via chronic exposure by dietary consumption compared to parenteral injection. Throughout this study, the growth dynamics of the larvae did not differ according to which ester was incorporated into the diet (Figure 4). Interestingly, the larvae tolerated inclusion of the various free alcohols into their diet as well (Figure 5). Incorporation of the ester into the diet produced a far less deleterious effect on larval growth and development than did direct administration of the toxicant into the hemolymph by parenteral injection.

Administered by parenteral injection, the various esters of canavanine exhibited growth-inhibiting properties that increased with the isobutyl and octyl esters relative to the propyl and butyl esters. With the

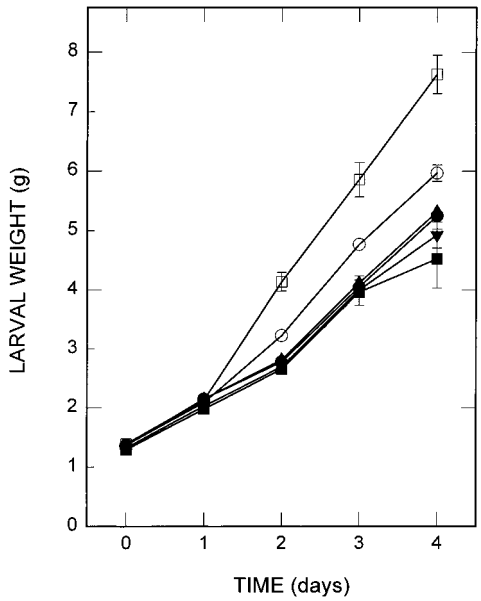

Figure 4. Effect of dietary consumption of canavanine and some of its esters on larval growth during the terminal, fifth instar. *M. sexta* larvae were reared on an agar-based, artificial diet supplemented with 2.5 mM canavanine (○), propylcanavanine (▲), butylcanavanine (●), isobutylcanavanine (▼), or octylcanavanine (■). Control larvae were reared on nonsupplemented artificial diet (□).

exception of the octyl ester, most of the observed adverse effect was due to the intrinsic toxicity of the ester and not to the parent alcohol. All of the tested esters were less toxic when the test organisms underwent chronic exposure by dietary consumption compared to parenteral injection. The underlying physiological or biochemical basis for this differential effect has not been elucidated, but it is evident that the gut probably provided an effective barrier to ester passage into the hemolymph

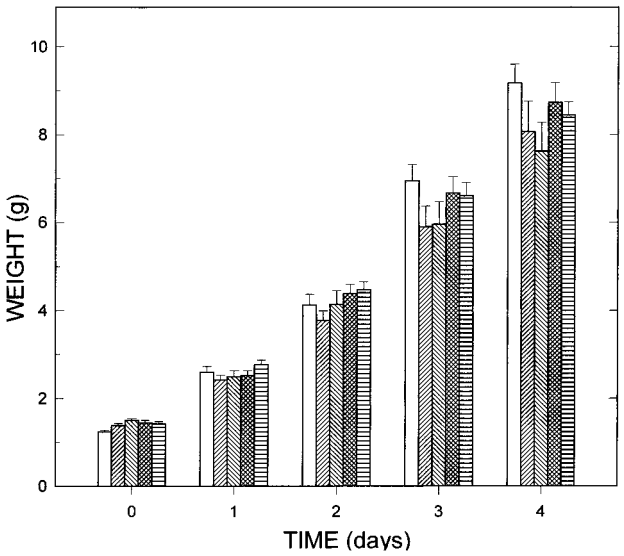

Figure 5. Effect of dietary consumption of the various free alcohols on larval growth during the terminal, fifth instar. *Manduca sexta* larvae were provided with *n*-propanol (/ bar), *n*-butanol (\ bar), isobutanol (× bar), or octanol (− bar) as described in the text. The control animals were reared on unmodified diet (blank bar).

where its biological effects were more severe. It is also possible that the flora of the gut may contribute to the detoxification of the tested compounds.

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