Insecticidal Properties of Some Derivatives of L-Canavanine

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The canavanine derivatives D-canavanine and L-homocanavanine as well as the 1-methyl and 1-ethyl esters of L-canavanine were synthesized and evaluated for biological activity in fifth instar larvae of the tobacco hornworm, *Manduca sexta* [Sphingidae]. While L-homocanavanine did not increase intrinsic toxicity, it was as deleterious as L-canavanine. D-Canavanine was biologically active, as demonstrated by its ability to cause larval edema, but the D-enantiomer had little ability to elicit the larval growth inhibition and pupal deformity which are hallmarks of canavanine toxicosis and was postulated to be linked to aberrant protein production. The 1-methyl and 1-ethyl esters of L-canavanine were synthesized to determine if enhancing canavanine's hydrophobicity might increase its bioavailability. Our experiments revealed that these esters are less toxic than canavanine; the ethyl ester disrupted larval growth more than did the methyl analogue.

Keywords: *L-Canavanine; D-canavanine; L-homocanavanine; 1-methyl-L-canavanine; 1-ethyl-L-canavanine; Manduca sexta*

INTRODUCTION

Investigations of L-canavanine, the L-2-amino-4-(guanidinooxy)butyric acid structural analogue of L-arginine, have provided a basic understanding of how certain toxic nonprotein amino acids act as protective allelochemicals against insects (Rosenthal, 1992a,b). This potent arginine antimetabolite curtailed severely the growth of larvae of the tobacco hornworm, *Manduca sexta* [Sphingidae], and caused massive developmental aberrations in the pupae and adults that emerged from surviving canavanine-treated larvae (Dahlman and Rosenthal, 1975). Insect death often 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).

Administration of canavanine at the time of vitellogenin induction in female locust, *Locusta migratoria migratorioides* [Acrididae], produced a structurally aberrant protein, vitellogenin, the conformation of which was altered significantly relative to that of the native macromolecule (Rosenthal et al., 1989a). Incorporation of this arginine antagonist into the antibacterial proteins of the fly, *Phormia terranovae* [Calliphoridae], nullified the protective efficacy of nearly all of these protective proteins (Rosenthal et al., 1989b). In a similar vein, lysozyme, induced in the presence of canavanine, had 21% of its arginyl residues replaced by canavanine and lost half of its catalytic activity (Rosenthal and Dahlman, 1991a), and under experimental conditions when 16% residue replacement occurred, 40% of the enzymic activity was lost. These studies and others have established that incorporation of canavanine into a protein could cause anomalous structure, altered conformation, and impaired function (Rosenthal, 1991b; Rosenthal and Dahlman, 1991a,b).

X-ray crystallographic analysis of canavanine disclosed that the distance between the terminal methylene carbon and the guanidinooxy carbon was less than the distance between the penultimate methylene carbon and the carbon of the guanidino group of arginine (Boyar and Marsh, 1982). This fact instigated the synthesis of the higher homologue of L-canavanine (known trivially as L-homocanavanine), L-2-amino-5-(guanidinooxy)-pentanoic acid. Since homocanavanine might bind to the active site of an arginine-utilizing enzyme more readily than did canavanine, we thought this compound might prove to be a more potent arginine antimetabolite.

The synthesis of D-canavanine was pursued to determine if it exhibited biological activity in an insect and how the activity of this stereoisomeric form compared to that of its naturally occurring antipode. Common wisdom suggested that D-canavanine would not be an arginyl-tRNA synthetase substrate; any adverse effects noted with D-canavanine could not result directly from incorporation into newly synthesized protein. Thus, the D-enantiomer offered a means of evaluating canavanine's toxicity divorced from its role in protein synthesis.

Finally, the 1-methyl and 1-ethyl esters of canavanine were also prepared and evaluated for their biological activity. These derivatives were more hydrophobic than canavanine and might exhibit enhanced toxicity, for example, by more readily penetrating cell membranes. Insects are able to deesterify xenobiotics (Yu et al., 1984; Yu, 1990). Thus, these esters might provide an efficient means of transporting canavanine into the cell prior to its hydrolysis by intracellular esterases to the parent toxicant.

MATERIALS AND METHODS

Insects. All experiments were conducted with newly ecdysed (<12 h) fifth instar *M. sexta* larvae obtained from a continuous colony, maintained at 25 ± 1 °C and a 16:8 (light:dark) photoperiod, at the University of Kentucky using rearing procedures similar to those described by Yamamoto (1969). All larvae employed in feeding studies were placed as newly ecdysed fifth instar larvae into individual 20 × 90 mm plastic Petri dishes with artificial diet containing a known concentra-

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tion of the test compound, as described by Dahlman and Rosenthal (1975). Ten larvae were used for each treatment for both feeding and injection studies.

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.

In experiments that required injection of experimental compounds, the newly ecdysed larvae, anesthetized with carbon dioxide prior to parental injection, received a single injection of amino acids in sterile, distilled water. Control larvae were given sterile water. Each larva was weighed daily. Maximum larval weight coincides with the hormonally determined onset of the wandering larval stage that included cessation of feeding and an array of physiological and behavioral changes associated with preparation for pupation that normally occurs 5 days after the final larval molt. Criteria for pupal deformity ratings were described elsewhere (Rosenthal and Dahlman, 1975; Dahlman and Rosenthal, 1975).

Chemicals and Biochemicals. L-Canavanine was isolated from acetone-defatted jack bean *Canavalia ensiformis* [Leguminosae] seeds and purified by repetitive recrystallization (Rosenthal, 1977). Unless otherwise indicated, all other chemicals or biochemicals were purchased from Sigma, Aldrich, or Fisher Scientific.

Silica gel plates (2.5 × 7.5 cm, 250 μm layer, fluorescent at 254 nm) were purchased from Diamond Whatman International Ltd. (Clifton, NJ). Column chromatographic separations were carried out using silica gel (200–400 mesh, 60 Å) from Aldrich (Milwaukee, WI). Melting points were recorded on a Fisher Johns melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were carried out on a Varian VXR-300MHz spectrometer (Palo Alto, CA); spectra were run at 21 °C in either $CDCl_3$, DMSO-d_6, or D_2O using tetramethylsilane (TMS) or the sodium salt of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid (TSP) as internal standard.

Synthesis of 1-Methyl and 1-Ethyl Esters of Canavanine. The 1-methyl and 1-ethyl esters of canavanine were each prepared by heating a solution of L-canavanine (1 mmol) in 6 mL of the appropriate alcohol, saturated with dry HCl, at 80 °C for 1 h. The solvent was removed under a 0.5 mm vacuum. The residue was recrystallized at 0 °C from a mixture of ethyl alcohol and anhydrous ether (1:5).

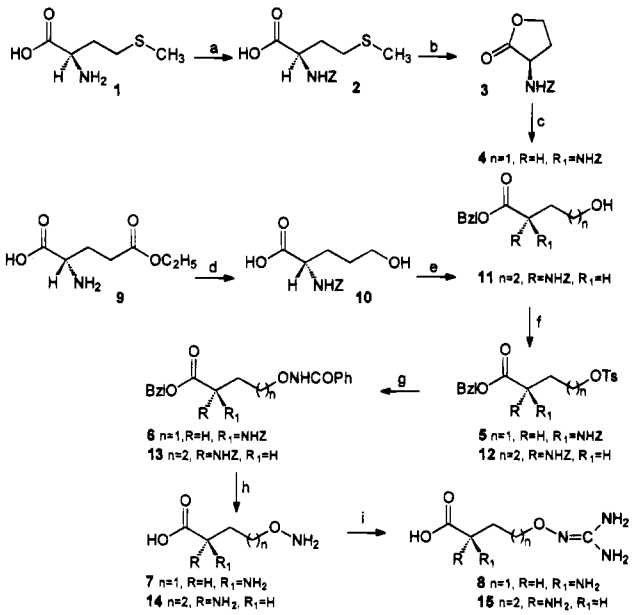
1-Methyl-2-amino-4-(guanidinooxy)butyric Acid: colorless hygroscopic solid (71% yield); ^{1}H NMR (DMSO-d_6) δ 8.90 (br s, 3H, NH_3^+), 7.80 (s, 5H, $C(NH_2)NH_3^+$), 4.10 (br s, 1H, 2-CH), 3.80–4.00 (m, 2H, 4-CH_2), 3.70 (s, 3H, OCH_3), 2.00–2.30 (m, 2H, 3-CH_2); ^{13}C NMR (DMSO-d_6) δ 169.30 (C-1), 158.40 (C=N), 71.90 (C-4), 52.90 (CH_3), 48.90 (C-2), 28.10 (C-3).

1-Ethyl-2-amino-4-(guanidinooxy)butyric Acid: colorless, hygroscopic solid (75% yield); ^{1}H NMR (DMSO-d_6) δ 9.00 (br s, 3H, NH_3^+), 7.83 (s, 5H, $C(NH_2)NH_3^+$), 4.18 (q, J = 6.5 Hz, 2H, OCH_2CH_3), 4.10 (t, J = 6.5 Hz, 1H, 2-HC), 3.85–4.00 (m, 2H, 4-CH_2), 2.10–2.30 (m, 2H, 3-CH_2); ^{13}C NMR (DMSO-d_6) δ 169.10 (C-1), 158.60 (C=N), 72.10 (C-4), 62.00 ($COOCH_2$), 49.20 (C-2), 28.40 (C-3), 14.00 (CH_3CH_2).

Synthesis of D-Canavanine. D-Canavanine was synthesized from D-methionine, as outlined in Scheme 1, by modification of the method of Ozinskas and Rosenthal (1986).

D-*2-[(Benzyloxycarbonyl)amino]methionine* (**2**). L-Methionine (**1**, 10 g, 67 mmol) was added to a suspension of 2 N $NaHCO_3$ (33.78 g, 335.1 mmol). After the mixture was cooled in an ice bath, benzyl chloroformate (14.86 g, 87.13 mmol) was added in small portions. The mixture was stirred at 23 °C for 24 h and then extracted with ether (4 × 100 mL) to remove unreacted benzyl chloroformate. The pH was adjusted to 2–3 with 6 N HCl (white precipitate formed), and the mixture was extracted with ethyl acetate (4 × 100 mL). After the extract was dried over anhydrous Na_2SO_4, it was filtered and evaporated by rotary evaporation *in vacuo*, and the residue was dried under a 0.5 mm vacuum overnight to give 16.6 g (87% yield) of **2** as a colorless oil: R_f = 0.6 in $CHCl_3$/MeOH (3:1); ^{1}H NMR (DMSO-d_6) δ 7.64 (d, 1H, NH), 7.28–7.40 (m, 5H, arom-CH), 5.40 (s, 2H, CH_2Ph), 4.06–4.16 (m, 1H, $CHCH_2$), 2.04 (s, 3H, CH_3), 1.76–1.98 (m, 2H, $CHCH_2$); ^{13}C NMR

Scheme 1. Synthesis of D-Canavanine (1–8) and L-Homocanavanine (9–15)



(a) $PhCH_2OCOCl$, $NaHCO_3$; (b) ICH_2CONH_2, EtOH/H_2O, citric acid; (c) NaOH, EtOH/H_2O, BzlBr, DMF; (d) $LiBH_4$, $PhCH_2OCOCl$; (e) NaOH, $PhCH_2Br$; (f) *p*TsCl, Et_3N, THF; (g) PhCONHOH, NaH, DMF; (h) 19%HCl/abs.EtOH, 3N HCl; (i) CuO, NH_2CN, $ZnCl_2$, H_2S

(DMSO-d_6) δ 173.56 (COOH), 156.08 (NHCOO), 136.86 (arom-C), 128.23 (2 × arom-CH), 127.76 (1 × arom-CH), 127.61 (2 × arom-CH), 65.32 (CH_2Ph), 52.58 ($CHCH_2$), 30.18 (SCH_2), 29.65 ($CHCH_2$), 14.4 (CH_3).

D-*2-[(Benzyloxycarbonyl)amino]-4-butyrolactone* (**3**). A solution of **2** (16.6 g, 58.6 mmol) and iodoacetamide (32.5 g, 175.9 mmol) in 250 mL of ethanol/H_2O (1:1) was stirred at 50 °C for 72 h. The mixture was treated with 0.33 M citric acid (3.48 g, 16.6 mmol) and refluxed at 110 °C for 4 h. Ethanol was removed by rotary evaporation *in vacuo*, and the remaining aqueous fraction, containing needle crystals, was extracted with ethyl acetate (4 × 100 mL). The ethyl acetate extract was washed with 0.5 N HCl (3 × 100 mL), distilled water, and a saturated solution of NaCl. The extract was dried over anhydrous Na_2SO_4, filtered, and concentrated to a small volume by rotary evaporation *in vacuo*. The residue was subjected to silica gel column chromatography with hexane/ethyl acetate (2:3) as eluent to give 9.91 g (63% yield) of **3** as white needle crystals: R_f = 0.59 in hexane/ethyl acetate (2:3); ^{1}H NMR ($CDCl_3$) δ 7.26–7.40 (m, 5H, arom-CH), 5.24–5.38 (br, 1H, NH), 5.12 (s, 2H, CH_2Ph), 4.35–4.50 (m, 2H, CH_2O), 4.20–4.31 (m, 1H, $CHCH_2$), 2.74–2.86 (m, 1H, $CHCH_{2a}$), 2.12–2.30 (m, 1H, $CHCH_{2b}$); ^{13}C NMR ($CDCl_3$) δ 171.04 (CHCOO), 155.60 (NHCOO), 135.85 (arom-C), 128.59 (2 × arom-CH), 128.35 (arom-CH), 128.19 (2 × arom-CH), 67.41 (CH_2Ph), 65.77 (CH_2O), 50.54 ($CHCH_2$), 30.56 ($CHCH_2$).

Benzyl D-*2-[(Benzyloxycarbonyl)amino]-4-hydroxybutyrate* (**4**). To a mixture of **3** (5.72 g, 24 mmol) in 79 mL of ethanol was added NaOH (1.02 g, 25.5 mmol) in 13 mL of water. The reaction mixture was stirred at 23 °C overnight; the solvent was removed by rotary evaporation *in vacuo*, and dried under a 0.5 mm vacuum overnight. The dried salt was dissolved in 20 mL of anhydrous DMF. Benzyl bromide (5.25 g, 30.7 mmol) was added and the reaction mixture stirred in the dark under N_2 for 72 h. Ethyl acetate (200 mL) was added, and the reaction mixture was washed with distilled water, a saturated solution of $NaHCO_3$ (2 × 100 mL), and a saturated solution of NaCl. The ethyl acetate extract was dried over anhydrous Na_2SO_4, filtered, and evaporated by rotary evaporation *in vacuo*. The crude product was subjected to silica gel column chromatography with hexane/ethyl acetate (2:3) as eluent to afford 7.51 g (91% yield) of **4** as a clear oil, which crystallized upon standing: R_f = 0.57 in hexane/ethyl acetate (2:3); ^{1}H NMR ($CDCl_3$) δ 7.24–7.40 (m, 10H, arom-CH), 5.66 (d, 1H, N*H*), 5.18 (s, 2H, $CHCOOCH_2$Ph), 5.12 (s, 2H, $NHCOOCH_2$Ph), 4.55–

4.65 (m, 1H, C*H*CH_2), 3.60–3.78 (m, 2H, CH_2OH), 2.70–2.80 (t, 1H, OH), 2.12–2.26 (m, 1H, CHCH_{2a}), 1.64–1.76 (m, 1H, CHCH_{2b}); ^{13}C NMR ($CDCl_3$) δ 172.33 (*C*HCOO), 156.08 (NH*C*OO), 135.99 (carbobenzyloxy arom-C), 135.03 (benzyl arom-C), 128.65 (arom-CH), 128.59 (2 × arom-CH), 128.57 (arom-CH), 128.56 (arom-CH), 128.36 (arom-CH), 128.30 (arom-CH), 128.19 (2 × arom-CH), 128.16 (arom-CH), 67.42 (NHCOO*C*H_2-Ph), 65.77 (CHCOO*C*H_2Ph), 58.33 (CH_2OH), 51.27 (*C*HCH_2), 30.58 (CH*C*H_2).

Benzyl D-*2-[(Carbobenzyloxy)amino]-4-[(p-tolylsulfonyl)oxy]-butyrate* (**5**). A solution of **4** (7.51 g, 21.89 mmol) in 15 mL of freshly distilled THF was treated with anhydrous triethylamine (8.86 g, 87.56 mmol) under N_2; the reaction mixture was cooled to −10 °C and treated with a solution of *p*-toluenesulfonyl chloride (8.35 g, 43.79 mmol) in 15 mL of freshly distilled THF. The reaction mixture was maintained at 4 °C for 24 h and finally stirred for 1 h at 23 °C. The solvent was evaporated by rotary evaporation *in vacuo* and the residue taken up in ethyl acetate (150 mL). The resulting suspension was filtered. The filtrate was washed with 0.5 N HCl (2 × 100 mL), a saturated solution of $NaHCO_3$, and a saturated solution of NaCl and was dried over anhydrous Na_2SO_4, filtered, and evaporated by rotary evaporation *in vacuo*. The crude product was subjected to silica gel column chromatography with hexane/ethyl acetate (3:1) as eluent to give 8.74 g (80% yield) of **5** as a clear oil: R_f = 0.2 in hexane/ethyl acetate (3:1); ^{1}H NMR ($CDCl_3$) δ 7.72–7.78 (d, 2H, tosyl 3 and 5-H), 7.25–7.40 (m, 12H, arom-CH), 5.30–5.40 (d, 1H, NH), 5.14 (s, 2H, CHCOOCH_2Ph), 5.06 (s, 2H, NHCOOCH_2Ph), 4.38–4.48 (m, 1H, C*H*CH_2), 4.06–4.12 (t, 2H, CH_2OTs), 2.40 (s, 3H, CH_3), 2.08–2.34 (m, 2H, CHCH_2); ^{13}C NMR ($CDCl_3$) δ 171.04 (CH*C*OO), 155.60 (NHCOO), 144.98 (tosyl arom-C-1), 136.00 (carbobenzyloxy arom-C), 134.94 (benzyl arom-C), 132.56 (tosyl arom-C-4), 128.86 (4 × arom-CH), 128.66 (arom-CH), 128.57 (4 × arom-CH), 128.44 (arom-CH), 128.27 (arom-CH), 128.08 (arom-CH), 128.01 (2 × arom-CH), 67.72 (NHCOO*C*H_2Ph), 67.14 (CHCOO*C*H_2Ph), 65.95 (*C*H_2OTs), 51.04 (*C*HCH_2), 31.33 (CH*C*H_2), 21.6 (CH_3).

Benzyl D-*2-[(Carbobenzyloxy)amino]-4-(benzamidooxy)-butyrate* (**6**). A solution of benzohydroxamic acid (5.3 g, 38.67 mmol) in 25 mL of anhydrous DMF was treated with NaH (0.84 g as 60% dispersion in mineral oil, 35.16 mmol) and stirred for 30 min at 23 °C. A solution of **5** in 10 mL of anhydrous DMF was added to the above mixture and the reaction mixture stirred at 55 °C for 24 h under N_2. Ethyl acetate (150 mL) was added, and a white precipitate occurred immediately. The suspension was washed with 0.5 N HCl (2 × 200 mL), distilled water, and a saturated solution of NaCl and was dried over anhydrous Na_2SO_4, filtered, and evaporated by rotary evaporation *in vacuo*. The residue was purified by silica gel column chromatography with hexane/ethyl acetate (5:4) as eluent giving 5.96 g (73% yield) of **6** as a clear oil: R_f = 0.43 in hexane/ethyl acetate (5:4); ^{1}H NMR($CDCl_3$) δ 9.30 (s, 1H, ONH), 7.70–7.76 (m, 2H, hydroxamate 2 and 6-H), 7.28–7.57 (m, 12H, arom-CH), 6.20 (d, 1H, NH), 5.20 (s, 2H, NHCOOCH_2Ph), 5.14 (s, 2H, CHCOOCH_2Ph), 4.66–4.76 (m, 1 H, C*H*CH_2), 4.18–4.28 (m, 1H, OCH_{2a}), 3.98–4.08 (m, 1H, OCH_{2b}), 2.14–2.24 (q, 2H, CHCH_2); ^{13}C NMR ($CDCl_3$) δ 171.95 (CH*C*OO), 161.17 (ONH*C*OPh), 156.43 (NHCOO), 136.25 (carbobenzyloxy arom-C), 135.10 (benzyl arom-C), 132.11 (arom-CH), 131.63 (arom-C), 128.64 (2 × arom-CH), 128.62(2 × arom-CH), 128.51 (5 × arom-CH), 128.34 (arom-CH), 128.16 (arom-CH), 127.97(arom-CH), 127.11 (2 × arom-CH), 72.84 (CH_2ONH), 67.49 (NHCOO*C*H_2Ph), 67.07 (CHCOO*C*H_2Ph), 51.13 (*C*HCH_2), 31.15 (CH*C*H_2).

D-*Canaline,* D-*2-Amino-4-(aminooxy)butyric Acid* (**7**). A solution of **6** (5.96 g, 12.8 mmol) in 100 mL of 19% HCl/absolute ethanol (w/w) was refluxed for 4 h. The solvent was removed, and the residue was dissolved in 100 mL of 3 N HCl and refluxed for 3 h. The solvent was evaporated, and the residue was redissolved in 30 mL of distilled water and extracted with ether (2 × 100 mL). The aqueous phase was evaporated and dried under a 0.5 mm vacuum. The salt residue was dissolved in 25 mL of absolute ethanol and was adjusted to pH 6–7 with anhydrous triethylamine. The white precipitate of D-canaline free base (**7**) was filtered and washed with 40 mL of absolute ethanol. The residue was dried under a 0.5 mm vacuum, giving 1.38 g (80% yield): ^{1}H NMR (D_2O) δ 3.80–3.92 (m, 3H, CH, CH_2O), 2.04–2.28 (m, 2H, CH_2); ^{13}C NMR (D_2O) δ 176.87 (COOH), 74.94 (CH_2O), 55.90 (CH), 31.8 (CH_2).

D-*Canavanine or* D-*2-Amino-4-(guanidinooxy)butyric Acid* (**8**). Copper oxide (1.5 g) was added to a solution of **7** (0.98 g, 7.31 mmol) in 15 mL of distilled water. The mixture was heated for 5 min in a boiling water bath and stirred for 2 h at 50 °C and for 24 h at 23 °C. The unreacted CuO was then filtered, and the filtrate was adjusted to pH 9.2 with 1 N NaOH. This solution was added to a solution of cyanamide (0.34 g, 8.04 mmol) and $ZnCl_2$ (1.29 g, 9.5 mmol) dissolved in a minimum amount of distilled water. The resulting solution was stirred at 50 °C for 3 days. Hydrogen sulfide was bubbled through a solution for 10 min, and the resulting precipitate was filtered. The filtrate was adjusted to pH 3.4 with 1 N NaOH and passed through 150 cm^3 column of Dowex 50X8 (NH_4^+) resin. The resin was washed with distilled water (2 L) until the washings were unresponsive to ninhydrin. The sample was eluted with 600 mL of 0.2 N NH_4OH. The solvent was removed by rotary evaporation *in vacuo*. The residue was dissolved in a minimum amount of distilled water and treated with cold absolute ethanol to give 0.53 g (67% yield) of D-canavanine free base (**8**) as a white precipitate: ^{1}H NMR (D_2O) δ 3.90–3.96 (m, 2H, CH_2O), 3.80–3.86 (t, 1H, CH), 2.08–2.30 (m, 2H, CH_2); ^{13}C NMR (D_2O) δ 177.70 (COOH), 161.20 (C=N), 72.10 (CH_2O), 55.70 (CH), 32.60 (CH_2).

Synthesis of L-Homocanavanine. L-Homocanavanine was synthesized from L-glutamic acid 5-methyl ester, as outlined in Scheme 1, by modification of the method of Ozinskas and Rosenthal (1986).

L-*2-[(Benzyloxycarbonyl)amino]-5-hydroxypentanoic Acid* (**10**). To a suspension of L-glutamic acid 5-methyl ester (**9**, 7.00 g, 40 mmol) in 300 mL of dry, freshly distilled THF was added $LiBH_4$ (2.25 g, 100 mmol) under N_2 in three portions (hydrogen evolution!). The reaction mixture was refluxed for 2 h, cooled on ice, and quenched with HCl/absolute ethanol obtained by adding acetyl chloride (11 g, 0.14 mmol) to absolute ethanol (50 mL). The solvent was removed by rotary evaporation *in vacuo*, and the residue was dissolved in 250 mL of 0.2 M $NaHCO_3$. The reaction mixture was cooled to 0 °C, and benzyl chloroformate (10.3 g, 60 mmol) was added dropwise. The reaction mixture was vigorously stirred at 0 °C for 1 h and then at 23 °C for 38 h prior to extraction with ether (3 × 150 mL). The pH of the aqueous layer was adjusted to pH 3 with 2 N HCl and extracted with ethyl acetate (4 × 250 mL). The combined extracts were dried over anhydrous Na_2SO_4 and filtered, and the solvent was removed by rotary evaporation *in vacuo*. The residue was recrystallized from ethyl acetate/hexane to afford **5** (7.21g, 68% yield) as colorless crystals: analytical sample mp 117.0–118.0 °C (from ethyl acetate/hexane); $[\alpha]^{23}_D$ −6.5° (*c* 3.6, glacial AcOH); ^{1}H NMR (DMSO-d_6) δ 7.59 (d, 1H, NH), 7.28–7.40 (m, 5H, arom-CH), 5.02 (s, 2H, OCH_2Ph), 4.40–4.50 (br s, 1H, OH), 3.88–3.97 (m, 1H, 2-CH), 3.30–3.38 (m, 2H, CH_2OH), 1.65–1.80 (m, 1H, 4-CH_{2a}), 1.50–1.65 (m, 1H, 4-CH_{2b}), 1.40–1.50 (m, 2H, 3-CH_2); ^{13}C NMR (DMSO-d_6) δ 173.97 (COOH), 156.16 (NHCOO), 137.04 (arom-C), 128.35 (2 × arom-CH), 127.80 (arom-CH), 127.71 (2 × arom-CH), 65.37 (O*C*H_2Ph), 60.12 (*C*H_2OH), 53.73 (C-2), 28.95 (C-3), 27.56 (C-4). Anal. Calcd for $C_{13}H_{17}NO_5$: C, 58.42; H, 6.41; N, 5.24. Found: C, 58.37; H, 6.41; N, 5.23.

Benzyl L-*2-[(Benzyloxycarbonyl)amino]-5-hydroxypentanoate* (**11**). A suspension of **10** (7.03 g, 26.3 mmol) in ethanol (100 mL) was treated with a solution of NaOH (1.16 g, 29 mmol) in distilled water (5 mL). The solvent was removed by rotary evaporation *in vacuo*, and the residue, dried under a 0.5 mm vacuum overnight, was dissolved in dry DMF (12 mL). Benzyl bromide (5.13 g, 30 mmol) was added, and the reaction mixture was kept in the dark for 3 days. Ethyl acetate (300 mL) was added and washed with distilled water, a saturated solution of $NaHCO_3$, and distilled water. After drying over anhydrous Na_2SO_4, the solvent was removed by rotary evaporation *in vacuo*. The residue was recrystallized from ethyl acetate/hexane to give **11** (7.46 g, 79% yield) as colorless crystals: mp 55–56 °C (from ethyl acetate/hexane); $[\alpha]^{23}_D$ = −20.8° (*c* 3.8, ethanol); ^{1}H NMR ($CDCl_3$) δ 7.30–7.40 (m, 10H, arom-CH),

5.60 (d, 1H, NH), 5.15 (s, 2H, CHCOOCH_2Ph), 5.09 (s, 2H, NHCOOCH_2Ph), 4.40–4.50 (m, 1H, 2-CH), 3.57 (t, 2H, CH_2-OH), 1.85–2.00 (m, 1H, 4-CH_{2a}), 1.70–1.80 (m, 1H, 4-CH_{2b}), 1.45–1.60 (m, 3H, OH, 3-CH_2); ^{13}C NMR ($CDCl_3$) δ 172.23 (CHCOO), 155.99 (NHCOO), 136.13 (carbobenzyloxy arom-C), 135.19 (benzyl arom-C), 128.54 (2 × arom-CH), 128.45 (2 × arom-CH), 128.41 (2 × arom-CH), 128.24 (2 × arom-CH), 128.10 (arom-CH), 128.01 (CH-arom), 67.10 (NHCOOCH_2Ph), 66.94 (CHCOOCH_2Ph), 61.83 (CH_2OH), 53.61 (C-2), 29.14 (C-3), 28.02 (C-4). Anal. Calcd for $C_{20}H_{23}NO_5$: C, 67.21; H, 6.49; N, 3.92. Found: C, 67.20; H, 6.55; N, 3.96.

Benzyl L-*2-[(Benzyloxycarbonyl)amino]-5-tosyloxypentanoate* (**12**). To a solution of **11** (5.28 g, 14.8 mmol) in dry, freshly distilled THF (15 mL) at −20 °C under nitrogen was added Et_3N (5.66 g, 56 mmol), followed by the dropwise addition of a solution of *p*-toluenesulfonyl chloride (5.35 g, 28 mmol) in dry THF (15 mL). The reaction mixture was kept at −10 °C for 1 h and then at 0 °C for 22 h. Ethyl acetate (300 mL) was added and was washed with cold distilled water, cold 0.5 N HCl, a saturated solution of $NaHCO_3$, and distilled water. The extract was dried over Na_2SO_4. The solvent was removed by rotary evaporation *in vacuo* and the residue subjected to silica gel column chromatography with hexane/ethyl acetate (1:1) as eluent to afford **12** as a colorless oil (6.65 g, 88% yield): $[\alpha]^{23}_D$ +3.5° (*c* 2.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.73 (d, 2H, tosyl 3 and 5-H), 7.25–7.40 (m, 12H, arom-CH), 5.26 (d, 1H, NH), 5.14 (s, 2H, NHCOOCH_2Ph), 5.06 (s, 2H, CHCOOCH_2Ph), 4.30–4.40 (m, 1H, 2-CH), 3.97 (t, 2H, CH_2OTs), 2.41 (s, 3H, CH_3), 1.82–1.92 (m, 1H, 4-CH_{2a}), 1.60–1.71 (m, 3H, 4-CH_{2b}, 3-CH_2); ^{13}C NMR ($CDCl_3$) δ 171.70 (CHCOO), 155.80 (NHCOO), 144.76 (tosyl arom-C-1), 136.0 (carbobenzyloxy arom-C), 135.0 (benzyl arom-C), 132.8 (tosyl arom-C-4), 129.80 (4 × arom-CH), 128.61 (arom-CH), 128.51 (arom-CH), 128.47 (4 × arom-CH), 128.25 (arom-CH), 128.16 (arom-CH), 128.00 (arom-CH), 127.79 (2 × CH-arom), 69.44 (CH_2OTs), 67.29 (NHCOOCH_2Ph), 67.00 (CHCOOCH_2Ph), 53.23 (C-2), 28.76 (C-3), 24.77 (C-4), 21.55 (CH_3). Anal. Calcd for $C_{27}H_{29}NO_7S$: C, 63.39; H, 5.71; N, 2.74. Found: C, 63.35; H, 5.71; N, 2.80.

Benzyl L-*2-[(Benzyloxycarbonyl)amino]-5-(benzamidooxy)-pentanoate* (**13**). A solution of benzohydroxamic acid (7.73 g, 56.4 mmol) in dry DMF (35 mL) was treated with NaH (2.05 g of 60% suspension in mineral oil, 51.3 mmol). After the mixture had stirred at 23 °C for 30 min, a solution of **12** (13.11 g, 26.25 mmol) in dry DMF (20 mL) was added, and the reaction mixture was stirred at 55 °C for 24 h. Ethyl acetate (250 mL) was added, and the mixture was washed with distilled water, 0.5 N HCl, and distilled water and dried over anhydrous Na_2SO_4. The solvent was removed by rotary evaporation *in vacuo*. The residue was subjected to silica gel column chromatography with hexane/ethyl acetate (1:1) as eluent. The combined fractions containing **13** were crystallized from hexane/ethyl acetate to afford pure **13** (5.26 g). The mother liquor was recrystallized once again to give a second crop (1.36 g). The new mother liquor was subjected to column chromatography to afford a third crop (0.65 g; overall yield 60%): colorless needles; mp 117.5–118.0 °C (from ethyl acetate/hexane); $[\alpha]^{23}_D$ −2.4° (*c* 2.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 9.30 (br s, ONH), 7.72 (d, 2H, hydroxamate 2 and 6-H), 7.49 (t, 1H, hydroxamate 4-H), 7.38 (t, 2H, hydroxamate 3 and 5-H), 7.26–7.34 (m, 10H, arom-CH), 5.62 (br s, NH), 5.16 (s, 2H, NHCOOCH_2Ph), 5.07 (s, 2H, CHCOOCH_2Ph), 4.46–4.56 (m, 1H, 2-CH), 3.96–4.05 (m, 2H, CH_2ONH), 1.60–2.10 (m, 4H, 3-CH_2, 4-CH_2); ^{13}C NMR ($CDCl_3$) δ 172.17 (CHCOO), 166.37 (ONHCOPh), 156.29 (NHCOO), 136.05 (carbobenzyloxy arom-C), 135.13 (benzyl arom-C), 131.83 (arom-CH), 131.73 (hydroxamate arom-C), 128.49 (3 × arom-CH), 128.42 (2 × arom-CH), 128.38 (2 × arom-CH), 128.24 (arom-CH), 128.13 (2 × arom-CH), 128.02 (arom-CH), 127.76 (arom-CH), 127.09 (2 × arom-CH), 75.96 (CH_2ONH), 67.21 (NHCOOCH_2Ph), 66.92 (CHCOOCH_2Ph), 53.75 (C-2), 29.34 (C-3), 23.78 (C-4). Anal. Calcd for $C_{27}H_{28}N_2O_6$: C, 68.05; H, 5.92; N, 5.88. Found: C, 68.01; H, 5.90; N, 5.90.

L-*2-Amino-5-(aminooxy)pentanoic Acid* (**14**). A solution of **13** (1.10 g, 2.31 mmol) in 20 mL of 19% HCl in absolute ethanol was refluxed for 4 h. The solvent was removed by rotary evaporation *in vacuo*, 20 mL of 3 N HCl was added, and the mixture was refluxed for 3 h. After cooling, the reaction mixture was extracted with ether (3 × 20 mL), the solvent removed by rotary evaporation *in vacuo*, and the residue dissolved in absolute ethanol (2 mL). This solution was stirred while anhydrous ether (5 mL) was added dropwise. After stirring for 30 min, the mixture was kept at 0 °C overnight, then crystallized, and the product was filtered and washed with anhydrous ether to afford **14** dihydrochloride (504 mg, 99% yield) as colorless crystals: mp 164.0–168.0 °C (dec; gas evolution); $[\alpha]^{23}_D$ +14.7° (*c* 2.8, H_2O); ^{1}H NMR (D_2O) δ 3.60–3.70 (m, 3H, CH, OCH_2), 1.80–2.05 (m, 2H, 4-CH_2), 1.55–1.80 (m, 2H, 3-CH_2); ^{13}C NMR (D_2O) δ 177.2 (s, C-1), 77.6 (C-5), 57.2 (C-2), 29.8 (C-3), 26.1 (C-4). Anal. Calcd for $C_5H_{12}N_2O_3$: C, 40.53; H, 8.16; N, 18.91. Found: C, 40.64; H, 8.16; N, 18.87.

L-*2-Amino-5-(guanidinooxy)pentanoic Acid* (**15**). The copper salt of **14** was prepared by stirring **14** (540 mg, 3.65 mmol) with CuO (454 mg, 5.75 mmol) in 5 mL of distilled water for 5 min at 100 °C, for 2 h at 45 °C, and for 22 h at 23 °C. After the unreacted CuO was filtered, the filtrate was adjusted to pH 9.2 with 1 N NaOH and added to a solution of cyanamide (184 mg, 4.0 mmol) and $ZnCl_2$ (586 mg, 4.31 mmol) in distilled water (2 mL); this reaction mixture was stirred at 45 °C for 72 h. Hydrogen sulfide was bubbled through the reaction mixture for 10 min, and the turbid solution was clarified by filtration.

The filtrate, adjusted to pH 3.4 with 1 N NaOH, was applied to a 50 cm^3 column of Dowex 50X8 (NH_4^+) resin. The resin was washed with distilled water until the washings were unresponsive to ninhydrin (600 mL). The sample was eluted with 0.2 N NH_4OH (400 mL). The solvent was removed by rotary evaporation *in vacuo*, and the residue was dissolved in a minimum amount of distilled water and treated with chilled absolute ethanol to afford **15** (558 mg, 75%) as a colorless solid: mp 184.0–187.0 °C (dec); $[\alpha]^{23}_D$ +7.6° (*c* 3.1, H_2O); ^{1}H NMR (D_2O) δ 3.83 (t, 2H, OCH_2), 3.75 (t, 1H, CH), 1.85–2.05 (m, 2H, 4-CH_2), 1.60–1.85 (m, 2H, 3-CH_2); ^{13}C NMR (D_2O) δ 177.78 (C-1), 161.22 (C=N), 75.12 (t, C-5), 57.34 (C-2), 30.22 (C-3), 26.49 (C-4). Anal. Calcd for $C_6H_{14}N_4O_3$: C, 37.89; H, 7.42; N, 29.46. Found: C, 36.67; H, 6.88; N, 28.41.

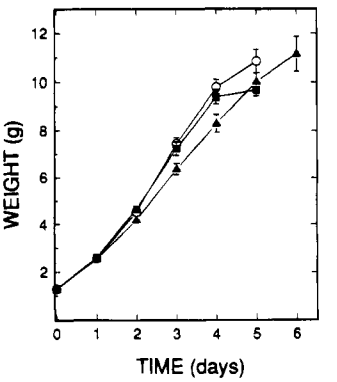


Figure 1. Effect of dietary consumption of L-canavanine and L-homocanavanine on larval growth during the terminal, fifth instar. *M. sexta* larvae were reared on an agar-based artificial diet supplemented with 2.5 mM L-canavanine (▲) or L-homocanavanine (■). Control larvae received comparable but unsupplemented diet (○). See text for additional experimental details.

RESULTS AND DISCUSSION

L-Homocanavanine. The effect of dietary consumption of 2.5 mM L-canavanine and L-homocanavanine was assessed over 6 days of development by terminal, fifth instar *M. sexta* (Figure 1). To establish the relative toxicity of canavanine and homocanavanine, the concentration of dietary canavanine selected was 2.5 mM for reasons provided above. At this dietary concentration, L-homocanavanine appeared to have no effect on weight gain until day 5. The apparent decline in the rate of weight gain after day 5 reflected the fact that

Table 1. L-Homocanavanine and L-Canavanine Feeding Studies

growth parameter[b]	diet composition[a]		
	control	L-canavanine	L-homocanavanine
max larval wt	10.3 ± 0.3[c] (10)[d]	10.1 ± 0.4 (10)	9.9 ± 0.2 (9)
time to pupation	11.3 ± 0.2 (10)	13.7 ± 0.2 (10)	11.9 ± 0.2 (9)
pupal wt	5.1 ± 0.2 (10)	5.15 ± 0.20 (10)	4.75 ± 0.11 (9)
pupal deformity	0[e] (10)	2.5 ± 0.3 (10)	3.9 ± 0.4 (10)
time between pupa and adult emergence	22.3 ± 2.2 (10)	29.8 ± 1.1 (7)	30.7 ± 2.9 (3)

[a] Each amino acid was provided at a 2.5 mM dietary concentration in deionized water. Control larvae received unsupplemented diet provided with an equivalent amount of deionized water. [b] Weights are expressed in grams and time in days. [c] Each value is the mean ± SEM for 10 insects. [d] The value in parentheses refers to the number of surviving insects. [e] Pupal deformity was gauged by criteria described elsewhere.

Table 2. Arginine and D- and L-Canavanine Feeding Studies

growth parameter	diet composition[a]				
	control	L-Cav	D-Cav	L-MM	D-MM
max larval wt	9.1 ± 0.3	8.9 ± 0.4	9.4 ± 0.3	11.6 ± 0.5	11.1 ± 0.4
pupal wt	5.0 ± 0.1	4.9 ± 0.3	5.1 ± 0.1	5.8 ± 0.4	6.1 ± 0.3
time to pupation	11.3 ± 0.2	13.0 ± 0.3	12.1 ± 0.3	14.0 ± 0.2	12.9 ± 0.3
time between pupa and adult emergence	23.1 ± 0.1 (10)	27.0 ± 0.8 (8)	25.0 ± 0.6 (9)	(0)	27.5 ± 0.6 (9)
pupal deformity rating	0 (10)	1.5 ± 0.3 (10)	0.3 ± 0.2 (10)	5.7 ± 0.6 (6)	1.8 ± 0.7 (10)

[a] MM, mixed diet consisting of 25 mM L-arginine + 2.5 mM L- or D-canavanine; Cav, canavanine; Arg, arginine; see Table 1 for additional experimental details.

most of the larvae in the L-homocanavanine treatment group had reached the wandering larval stage by day 5. After attainment of the wandering larval stage, the larvae rapidly lost weight for the subsequent 2–3 days. Maximum larval weights for all three treatments were similar, but those insects reared on L-canavanine required an additional day to attain maximum weight and 1 day more to attempt pupation than either the control organisms or the L-homocanavanine-treated animals (Table 1).

The most interesting difference in the effects between canavanine and homocanavanine was the degree of pupal malformations. Homocanavanine produced more severe deformities than those observed for the canavanine-treated counterparts; this factor caused the death of a larger number of pupae (Table 1). The rate of adult development was extended approximately 1 week for both canavanine- and homocanavanine-treated animals as compared to the controls. This study suggests that while chain extension to produce homocanavanine did not increase canavanine's intrinsic toxicity, it did produce a derivative that is equally toxic to developing *M. sexta* adults.

D-Canavanine and D-Canaline. Simultaneous dietary consumption of L-canavanine and L-arginine, as compared to L-canavanine alone, greatly exacerbated the degree of pupal deformity (Racioppi et al., 1981). D-Canavanine exhibited little efficacy relative to its naturally occurring enantiomer in causing this deleterious biological effect (Table 2). Thus, D-canavanine has little ability to elicit the larval growth inhibition (Figure 2) and pupal deformity that are hallmarks of canavanine toxicosis.

On the other hand, D-canavanine is biologically active, as demonstrated by its capacity to induce larval edema. This edema is probably caused by its reaction with arginase, thereby creating D-canaline (Kavanaugh et al., 1990). Ongoing research in our laboratories has established that D-canaline differed little from L-canaline in its reactivity with the pyridoxal phosphate moiety of vitamin B_6-containing enzymes. L-Canaline is a potent inhibitor of ornithine aminotransferase, a pyridoxal phosphate-containing protein (Rosenthal and Dahlman, 1990); the subsequent accumulation of hemolymphic

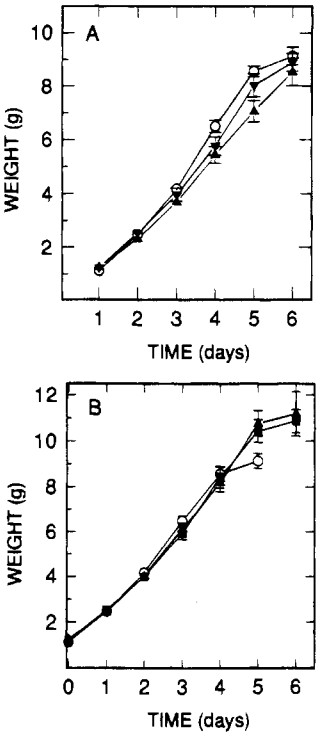


Figure 2. (A) Effect of dietary consumption of L-canavanine and D-canavanine on larval growth during the terminal, fifth instar. *M. sexta* larvae were reared on an agar-based artificial diet supplemented with 2.5 mM L-canavanine (▲) or D-canavanine (▼). Control larvae were reared on 25 mM L-arginine-containing diet (○). (B) Larvae were reared as above on a diet supplemented with 2.5 mM L-canavanine and 25 mM L-arginine (▲) or 2.5 mM D-canavanine and 25 mM L-arginine (■). See text for additional experimental details.

ornithine was responsible for the marked edema observed in larvae provided diet enriched with both L-arginine and L-canavanine (Racioppi et al., 1981). These experiments provided ample evidence that while D-canavanine possessed significant biological activity in developing *M. sexta* larvae, it could not elicit the developmental aberrations that have been linked experimentally to anomalous canavanyl protein formation (Rosenthal, 1991a, 1992a,b).

Since D-canaline is a precursor to D-canavanine

Table 3. Injection Studies with L-Canavanine and Some of Its Esters[a]

growth parameter	drug			
	control	L-Cav[b]	L-MCav[b]	L-ECav[b]
max larval wt	9.0 ± 0.2 (10)	7.2 (1)	8.1 ± 0.5 (8)	8.5 ± 0.4 (8)
pupal wt	4.9 ± 0.1 (10)	3.7 (1)	4.4 ± 0.3 (7)	4.5 ± 0.7 (8)
time to pupation	10.7 ± 0.3 (10)	12.0 (1)	11.3 ± 0.4 (7)	11.5 ± 0.4 (8)
time between pupa and adult emergence	25.9 ± 0.2 (10)	25.0 (1)	25.7 ± 0.2 (7)	25.5 ± 0.3 (8)

[a] See Table 1 for additional experimental details. [b] Cav, canavanine; MCav, C-I methyl ester of canavanine; ECav, C-1 ethyl ester of canavanine. Dose: 1.25 mg/g of fresh body weight.

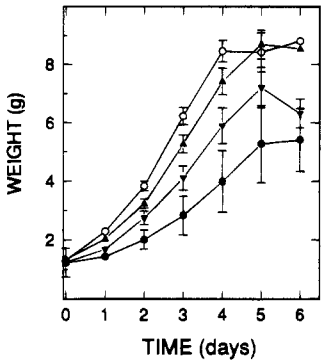


Figure 3. Effect of a single injection of canavanine or some of its esters on larval growth during the terminal, fifth instar. *M. sexta* larvae were provided 1.0 mg g^{-1} of fresh body weight L-canavanine (●) or an equivalent dose of 1-methyl-L-canavanine (▲) or 1-ethyl-L-canavanine (▼) in sterile water. The control larvae received sterile water (○). See text for additional experimental details.

synthesis, D-canaline was also available for biological evaluation. Only 10% of the larvae that received an injection of either 2.2 or 1.8 mg g^{-1} of fresh body weight L-canaline survived to become pupae while 50% of the larvae provided either 1.4 or 1.0 mg g^{-1} successfully pupated. Larvae provided the same dose of D-canaline, i.e. 2.2, 1.8, 1.4, or 1.0 mg g^{-1} of fresh body weight, successfully pupated 40, 60, 80, or 100%, respectively. Regardless of the D-canaline treatment dose, those larvae that attained the wandering larval stage pupated and eventually emerged as adults. Thus, while D-canaline is biologically active, it is less toxic than the naturally occurring enantiomer.

Methyl and Ethyl Ester of L-Canavanine. The marked toxicity of a 1 mg g^{-1} fresh larval body weight dose of injected canavanine is revealed by the growth data shown in Figure 3. Conversion of L-canavanine to its 1-methyl ester produced a derivative with demonstrable toxicity, but far less than that of the parent compound; larvae provided 1-methylcanavanine grew about as well as the control animals (Figure 3). The ethyl ester of canavanine was significantly more toxic than the methyl ester as judged by larval growth dynamics (Figure 3).

While an injection of 1.0 mg g^{-1} of fresh body weight L-canavanine resulted in 50% larval death, only 10% mortality was observed with the ethyl ester; this compared to no observed mortality for the methyl ester. Increasing the dose to 1.25 mg g^{-1} caused 90% death for the canavanine-treated larvae and 20% mortality for both the methyl and ester derivatives. On the other hand, there was no significant difference in the effect of the methyl or ethyl ester on the growth parameters of Table 3.

The enhanced toxicity of the ethyl ester relative to the methyl ester, as indicated by larval growth dynamics, suggests that the intrinsic toxicity of these esters is exacerbated by increasing their hydrophobicity. The greater steric bulk of the ethyl ester relative to the methyl ester should have produced an ester that was a less effective substrate for canavanine-utilizing systems and, therefore, one that is less toxic. The greater toxicity of the ethyl relative to the methyl ester might reflect the fact that it was transported more effectively into body cells where esterase activity might have formed greater levels of the parent compound.

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