Dehydrothalebanin: A Source of Resistance from *Glycosmis pentaphylla* against the Citrus Root Weevil *Diaprepes abbreviatus*

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Roots of a citrus relative, *Glycosmis pentaphylla* (orangeberry), were shown to inhibit the growth and survival of larvae of the citrus root weevil *Diaprepes abbreviatus*. Roots of *G. pentaphylla* incorporated into the diet of *D. abbreviatus* increasingly inhibited the growth of neonate larvae with increased concentration of roots, while roots from citrus rootstocks produced little inhibition. The diet-incorporation assay was used to guide fractionation of an active acetone extract of *G. pentaphylla* roots. Three major fractions from silica open-column liquid chromatography were active, and these were purified using semipreparative normal-phase HPLC. A single active HPLC subfraction was isolated from each of the three liquid chromatography fractions, and two active compounds were isolated and identified by GC-MSD. GC-MSD and NMR identified one compound as the amide dehydrothalebanin B, and the other was identified by GC-MSD as dieldrin, a chlorinated hydrocarbon insecticide whose origin in our samples is uncertain.

Keywords: *Diaprepes abbreviatus; Glycosmis pentaphylla; host plant resistance; Rutaceae; citrus rootstocks; dehydrothalebanin B*

INTRODUCTION

The root weevil *Diaprepes abbreviatus* infests citrus, woody ornamentals, sugarcane, and occasionally row crops throughout southern Florida and the Caribbean. On citrus, *D. abbreviatus* larvae feed on root bark, eventually killing even large roots and entire trees. Their damage may also result in root infection from fungal pathogens such as *Phytophthora* spp. (Rogers et al., 1996). In accord with its ability to feed on a broad host range, little resistance has been discovered against *D. abbreviatus* though numerous citrus cultivars and species have been tested (Beavers and Hutchison, 1985; Shapiro and Gottwald, 1995; Bowman et al., 2000; Lapointe and Shapiro, 1999). The discovery of phytochemical or genetic sources of resistance against *D. abbreviatus* and associated fungal pathogens in roots of citrus or citrus relatives might yield novel methods for defense of citrus against *D. abbreviatus* (Shapiro, 1991).

The roots of Rutaceae in the subfamily Aurantioidea contain several classes of natural products that exhibit insecticidal or other biological activity. Among those are coumarins, alkaloids, amides, flavonoids, limonoids, and terpenoids (Shapiro, 1991). We have recently undertaken a search for resistance against *D. abbreviatus* in roots of citrus and citrus relatives. Growth-inhibiting activity against larval *D. abbreviatus* has been found in both live and milled roots from seedlings of two citrus relatives, *Glycosmis pentaphylla* (orangeberry) (Shapiro et al., 1997; Bowman et al., 1999; Lapointe and Shapiro, 1999) and *Murraya koenigii* (Lapointe and Shapiro, 1999). Species in the genus *Glycosmis* (Stone, 1985) contain a wide variety of compounds with potential biological activity. These include terpenoids (Chakravarty et al., 1996), amides (Greger et al., 1992, 1993a,b, 1994, 1996; Hofer et al., 1995a, 1998), imides (Hofer et al., 1995b), alkaloids (Wu et al., 1983; Wurz et al., 1993; Ono et al., 1995), coumarins (Rahmani et al., 1998), and flavonoids (Tian-Shung et al., 1995). Compounds exhibiting antifungal and insecticidal activities (Greger et al., 1996) have already been isolated from several *Glycosmis* species. Recently, a screening of foliage against the citrus leafminer, *Phyllocnistis citrella*, demonstrated activity in both *Murraya koenigii* and *Glycosmis pentaphylla* (Jacas et al., 1997). *Glycosmis pentaphylla* also exhibited antijuvenile hormone activity against the field cricket *Gryllus bimaculatus*, and activity was traced to the quinazolone alkaloid arborine (Muthukrishnan et al., 1999). Here, we demonstrate growth-inhibiting activity against *D. abbreviatus* in the roots of *G. pentaphylla*.

MATERIALS AND METHODS

Reagents. Acetone (HPLC/GC grade), methanol (highest purity), petroleum ether (highest purity), and *n*-hexane (95%) were from Mallinckrodt; cyclohexane (HPLC grade), ether (anhydrous), ethyl acetate (chromatography grade), and isooctane (HPLC grade) were from JT Baker; ethyl alcohol was 200 proof USP grade.

Nuclear Magnetic Resonance Spectroscopy (NMR). The proton spectrum of dehydrothalebanin B was recorded on a Varian Unity 500 equipped with an indirect detection probe at 500 MHz. The sample, 1 mg of dehydrothalebanin B dissolved in 200 μL of $CDCl_3$, was placed in a 5-mm Shigemi tube whose magnetic susceptibility matched that of chloroform. The chemical shifts are given as ppm relative to tetramethylsilane, and the coupling constants are given in Hz.

Infrared (FTIR) and UV−vis Spectroscopy. A Nicolet 510P FTIR with Omnic ver. 2.1 software was used to acquire IR spectra. A Gilford Response UV−vis spectrophotometer was used for UV spectroscopy.

Gas Chromatography/Mass Spectral Detection (GC-MSD). GC-MSD analyses were performed on a Hewlett-

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Table 1. Gas Chromatographic Parameters

parameter	value
oven, initial temp (°C)	80
oven, initial time (min)	1
injector temp (°C)	oven tracking
ramp 1 rate (°C/min)	40
final temp 1 (°C)	180
final time (min)	0
ramp 2 rate (°C/min)	5
final temperature (°C)	240
final time (min)	15
column flow (constant)	0.9 mL/min

Packard model 5890 series gas chromatograph interfaced (capillary direct) to a 5971A mass spectral detector (GC-MSD), from which EI spectra were collected, stored, and analyzed using Hewlett-Packard Chemstation software. A cool on-column injector and autosampler were used to inject onto a custom-made J&W (20%-phenyl)−methylpolysiloxane phase, 30 m × 0.25-mm i.d., 0.75-μm film thickness capillary column. Hydrogen was used as the carrier gas, and samples were injected in ethyl acetate. Gradient conditions are given in Table 1.

Plants and Insects. *G. pentaphylla* seedlings used for extraction were collected at the Florida Division of Plant Industry Arboretum, Winter Haven, FL, or were grown in USDA greenhouses in Leesburg and Orlando, FL, from seeds collected in Winter Haven, or were purchased from a commercial nursery in southeast Florida. Seedlings were removed from pots, roots were washed free of potting soil with water, then air-dried for 1−3 h prior to storage at −80 °C. *D. abbreviatus* were reared in a colony at the U. S. Horticultural Research Laboratory in Orlando, FL. The colony was derived from adults whose progenitors had originally been field-collected near Apopka, FL (Lapointe and Shapiro, 1999).

Bioassays. Bioassay of milled root powders was conducted as per Shapiro et al. (1997), except that neonate larvae (0.1 mg each) were added at 10 or 20 larvae per diet cup and then reared for 33 d at 26 °C. Bioassays of extracts and fractions were conducted on larvae of mean weight of ca. 25 mg (Shapiro et al., 1997), except that extracts or fractions were substituted for ground roots. Extracts were dissolved in 41.5 mL of acetone (Mallinckrodt AR HPLC), added to 18.4 g of dry citrus root weevil diet premix #1675F (Bio-Serve) at 10 g equiv of roots/100 mL diet, and acetone was completely evaporated under a fume hood. Diet was mixed with agar at 50−60 °C and poured into 10 polystyrene cups at ca. 15 mL/cup. Controls run concurrently were made with diet only ("Diet") or as above with evaporated acetone ("Diet + Solvent"). Larval *D. abbreviatus* of 25 mg mean weight were added to each cup and kept at room temperature (21−27 °C) for 28 d, when larvae were removed and weighed.

Extraction and Isolation. *High Performance Liquid Chromatography (HPLC).* Normal-phase HPLC was performed using a Spectra-Physics 8800 pump with a Spectra-Physics AS3500 autosampler. Peaks were detected by UV diode array detection at 292 nm with a Groton SoloNet detector and a personal computer running *WinSolo* (Groton) and *Grams386* (Galactic Corp.) software under *Windows* 3.11 (Microsoft Corp.). To separate fractions from isolation no. 1, a Rainin Dynamax Microsorb semipreparative column (10 × 250 mm; 5 μm, 100 Å pore size, normal-phase silica) was used at a flow rate of 2.5 mL/min. To separate fractions from isolation no. 2, a Rainin Dynamax Microsorb preparative column (21.5 mm × 250 mm; 5 μm/100 Å) was used at a flow rate of 10 mL/min.

Isolation No. 1. A batch of 40 g of roots was frozen in liquid N_2, pulverized, and milled in a Retsch ZM-1000 centrifugal mill (Brinkmann) at 10 000 rpm through a 0.5-mm screen. Milled roots in a 40-g batch were extracted three times by homogenizing root powder in 400 mL of acetone (Mallinckrodt AR HPLC) for 5 min with a Tekmar Tissuemizer, shaking for 4 h, vacuum-filtering through Whatman No. 2 filter paper, and twice re-extracting and re-filtering the solids. The pooled extract was evaporated in vacuo at 40 °C, yielding 2.0 g of a reddish brown tar. Next, the extract was dissolved in 50 mL of chloroform, partitioned against an equal volume of water and separated, and the water phase was re-partitioned twice with 50 mL fresh chloroform and then dried. Residue was dissolved in chloroform and chromatographed on a 4.0 × 46.5-cm open liquid chromatography (LC) column (LiChroprep Si 60, E. M.) and eluted stepwise with 0.6 L each of hexane/diethyl ether (1:1), diethyl ether/methanol (3:1), and 100% methanol. Fractions were collected, concentrated, and bioassayed and then subjected to HPLC on the semipreparative silica column. LC fraction 2 was separated on HPLC at 2.5 mL/min using a gradient of cyclohexane/ethyl acetate (121:1) for 5 min, to cyclohexane/ethyl acetate (60:1) over 10 min and held for 45 min, to cyclohexane/ethyl acetate (1:1) over 1 min and held for 9 min. LC fraction 3 was separated on HPLC at 2.5 mL/min with cyclohexane/ethyl acetate (20:1) held for 45 min, with a linear gradient to cyclohexane/ethyl acetate (1:1) over 10 min. LC fraction 4 was separated on HPLC at 2.5 mL/min with cyclohexane/ethyl acetate (12:1) for 42 min, then with a gradient to cyclohexane/ethyl acetate (1:1) over 10 min.

Isolation No. 2. A batch of 104 g of roots was extracted for final characterization by NMR and isolation of a single active compound from LC fractions 2 and 3. Roots were extracted in 520 mL of acetone three times. Two 52-g batches were extracted, yielding a combined total of 7.7 g of a reddish brown tar. This was dissolved in 100 mL of chloroform and partitioned against water, yielding 5.3 g. The residue was chromatographed on a 4.8 × 91.6-cm LiChroprep Si 60 column and

Table 2. Effect of Diet-Incorporated Lyophilized Root Powders on Mean (±SEM) Survival and Weight of Larval *D. abbreviatus*[a]

root source	concn (g/cup)	survival (%)[b]	*n*	larval wt gain (mg)[c]	*n*
G. pentaphylla	1	0 ± 0 a	10		
	0.1	39 ± 6 b	10	16.8 ± 2.5 a	39
	0.01	45 ± 5 b	10	44.6 ± 5.5 bc	45
Swingle	1	47 ± 5 b	20	34.5 ± 3.6 ab	94
	0.1	46 ± 9 b	10	74.6 ± 6.2 d	46
	0.01	38 ± 6 b	10	60.5 ± 8.3 cd	38
control		41 ± 4 b	20	70.2 ± 5.6 d	81

[a] Neonate larvae (0.1 mg) were placed on diet and reared for 33 days. Means followed by the same letter are not significantly different at $P = 0.05$ by Tukey's HSD after a significant ANOVA (*Abacus Concepts*, 1996). For "survival (%)", *n* represents number of diet cups, and for "larval wt gain (mg)", number of surviving larvae. [b] Data are untransformed means. $F = 8.2$; df = 6, 83; $P < 0.01$. [c] Larval weights nested within cups. $F = 2.4$; df = 5, 331; $P = 0.04$.

Table 3. Effect of Diet-Incorporated Acetone Extracts of Roots and LC Fractions from *G. pentaphylla* on Mean Survival and Weight (±SEM) of Larval *D. abbreviatus*[a]

extract source	*n*	larval weight gain (mg ± SD)
roots		
G. pentaphylla root	14	33 ± 30 a
Swingle root	21	239 ± 107 b
control	23	327 ± 131 b
G. pentaphylla root LC fractions		
1	10	408 ± 134 a
2	9	65 ± 49 b
3	8	52 ± 36 b
4	10	56 ± 27 b
5	8	339 ± 121 a
control, diet	10	370 ± 109 a
control, diet + acetone	10	410 ± 118 a

[a] Larvae at 1 month of age averaging 23 mg (roots) or 28 mg (LC fractions) were placed on diet and reared for 30 d. Live weights of *n* surviving larvae (of 30 on root extracts and 10 on LC fractions) were recorded. Means within grouped "roots" or "LC fractions" followed by the same letter are not significantly different at $P = 0.05$ by Tukey's HSD after a significant ANOVA (*Statistica*, 1996).

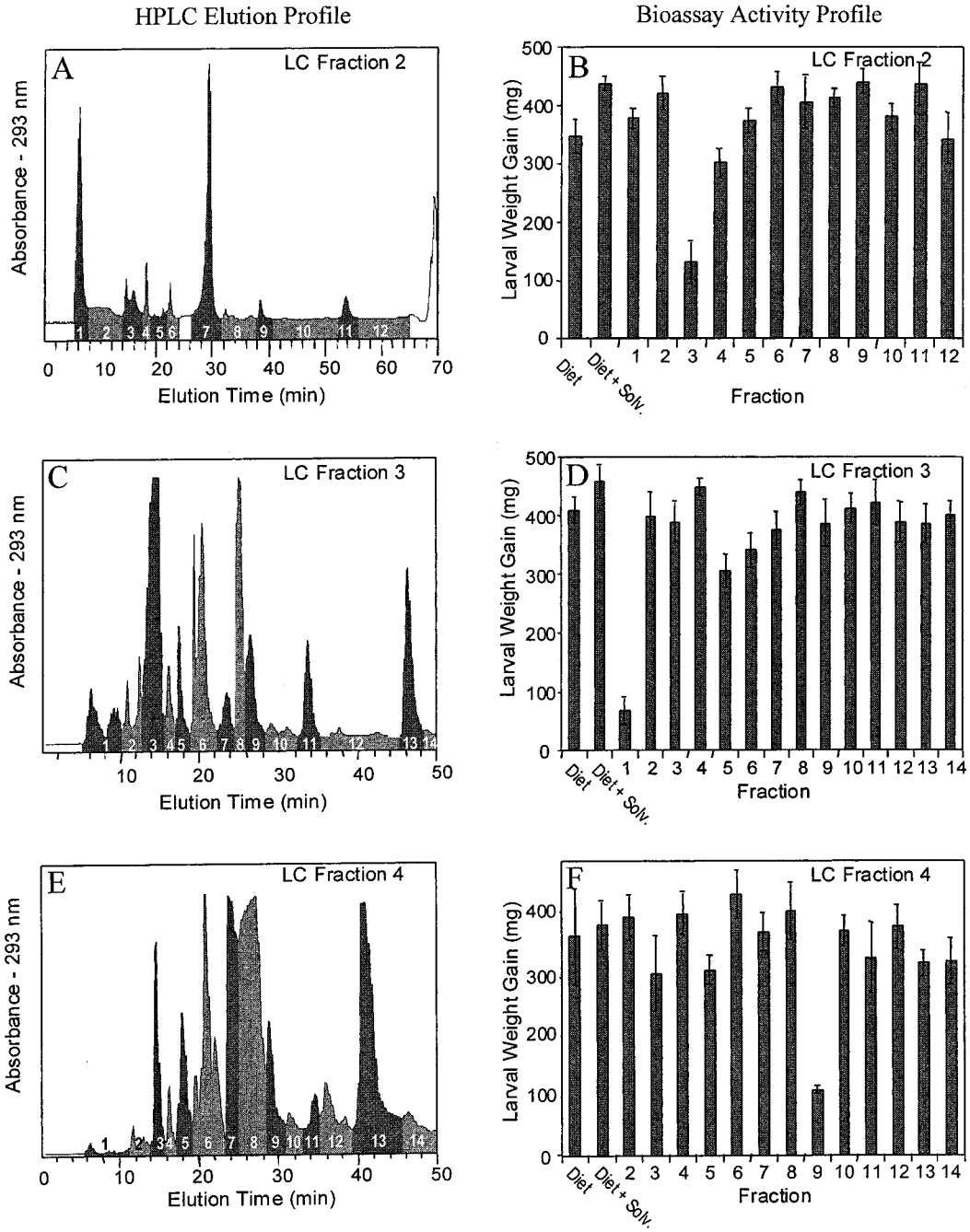


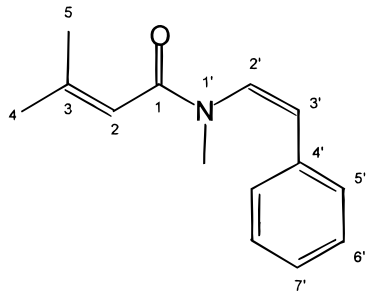
Figure 1. Separations of the three active LC fractions (2−4, Table 3) by preparative HPLC (left column) and larval weight gain on diet containing the preparative HPLC fractions (right column). Error bars (right) represent SEM.

eluted with 1 L each of the same solvent mixtures employed in isolation no. 1. Fractions (20 mL) were collected from LC and run on TLC (20 × 20-cm × 250-μm silica gel G plates, (7:3) ethyl acetate/petroleum ether) to identify four major fractions according to chromatographic grouping of spots. Fractions were also run on GC-MSD, and regions containing the two major active components were identified by single-ion monitoring (SIM).

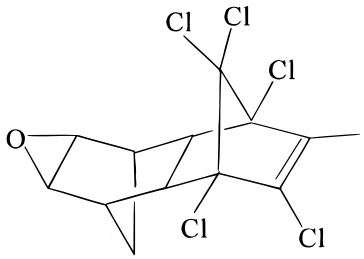
According to these results, fractions were pooled into four major fractions and active components isolated by HPLC. Only two of the pooled fractions contained activity. The first fraction, containing *m/z* 253, was injected at 2.0 mL/min with a cyclohexane/ethyl acetate (20:1) phase held for 1 min. Flow was increased to 10 mL/min, held for 24 min, then switched through 1 min to cyclohexane/ethyl acetate (1:1), and held for 9 min. The resulting fraction was re-run using the same solvent system to individually isolate three peaks containing two unknown compounds and dieldrin. The second fraction, containing *m/z* 215, was run at 10 mL/min with cyclohexane/ethyl acetate (7:1) held for 30 min, followed by 16 min of cyclohexane/ethyl acetate (1:1).

RESULTS

Roots from a citrus rootstock and three citrus relatives were incorporated at three concentrations into diet and tested against neonate larvae. Dose correlated with survival and inhibition of growth at all three dose levels in *G. pentaphylla*, but not in the citrus rootstock "Swingle" (Table 2). No larvae survived at the highest dose rate of *G. pentaphylla*. This constituted evidence that the effect of milled roots in diet may be chemically based. Incorporation of an acetone extract into diet at a rate of approximately 1 g equiv roots/10 mL diet by wet weight provided further evidence for the chemical



Dehydrothalebanin B



Dieldrin

Figure 2. Structure of dehydrothalebanin band dieldrin.

nature of growth inhibition. Larval growth was strongly inhibited by diet containing an extract of *G. pentaphylla* roots, and only weakly by diet with extract of Swingle roots, relative to diet without extract (Table 3). The *G. pentaphylla* extract resulted in 10- and 7-fold reductions in larval growth, compared to larvae fed on control diet and diet incorporated with roots of Swingle rootstock, respectively. Mortality increased by 30 and 23% over control or Swingle-incorporated diet, respectively.

In isolation no. 1, an acetone extract from 40 g of roots was fractionated by LC, and five fractions were collected. When fractions were incorporated at approximately 1 g equiv of roots/10 mL diet, fractions 2−4 were active (Table 3). Untreated diet, or a control diet to which acetone was added and evaporated, showed no growth-inhibiting activity. Each active fraction was chromatographed using preparative HPLC.

Only one HPLC subfraction from each of the three major LC fractions showed significant activity in the larval bioassay (Figure 1). Fraction 2/subfraction 3 and fraction 3/subfraction 1 both exhibited multiple peaks in their total ion chromatograms. The predominant compound in fraction 3/subfraction 1 (70% pure by GC-MSD) showed a dominant EI-MS mass of 253. Fraction 4/subfraction 9 proved to be 94% pure by the GC-MSD total ion chromatogram, with predominant mass of *m/z* 215. The spectrum matched published mass spectral peaks as dehydrothalebanin A or B (Greger et al., 1996).

In isolation no. 2, 139 fractions of 20 mL each were collected from a single LC run. These fractions were analyzed by GC-MSD and single ion chromatograms of 253 and 215 molecular weight were used to track the active fractions: *m/z* 253 was found in fractions 54−83, which were pooled, and *m/z* 215 (putative dehydrothalebanin) was found in fractions 97−119, also pooled. Next, preparative normal-phase HPLC was used to isolate components of each. The dehydrothalebanin peak (*m/z* 215) was isolated at 91% purity and identified as the cis isomer, or dehydrothalebanin B (Figure 2) (Greger et al., 1996), based on the following evidence: Proton NMR ($CDCl_3$) 5.92 (s), 1.91 (s), 1.72 (s), 2.92 (s), 6.38 (d,8.6), 6.03 (d, 8.6), 7.12−7.30 (m); UV (Et_2O) λ_{max} (log ϵ) 284 (3.58), 221.5 (3.69); IR (CCl_4) ν max 3027 w, 2935 m, 2858 w, 1666 s, 1627 vs, 1446 s, 1361 s, 1296 s, 1219 w, 1176 w, 1099 m, 1061 m, 941 w, 810 m, 779 w, 752 w, 698 m, 567 w; EIMS (1660 eV) *m/z* 216 (4), 215 (30) [M^+], 134 (14), 133 (100), 132 (13), 117 (9), 91 (17), 83 (68), 55 (19).

In isolating the *m/z* 253 peak, two other HPLC peaks were separated from it (Figure 3). The *m/z* 253 peak was isolated to 99% purity (Fraction 2). A slightly later-eluting HPLC peak (Fraction 3) contained a compound with a dominant ion of *m/z* 263, clearly identified as dieldrin (Figure 2) in a library search, on the following evidence: EIMS (1540 eV) *m/z* 281 (33), 279 (58), 277 (62), 265 (68), 263 (100), 261 (60), 245 (35), 243 (41), 239 (40), 237 (55), 235 (33). When the three peaks were bioassayed, only this last peak showed activity.

DISCUSSION

During the 25 years since its introduction into Florida, *D. abbreviatus* has increased in estimated population size, distribution range, and estimated damage to citrus plantings. By 1995, an estimated 50 000 acres in Florida were infested. Recent tests of numerous rootstock cultivars and experimental hybrids have shown only weak resistance against larval *D. abbreviatus* (Beavers and Hutchison, 1985; Shapiro and Gottwald, 1995; Bowman et al., 1999; Lapointe et al., 1999), except in the citrus relatives *Murraya koenigii* and *G. pentaphylla* (Shapiro et al., 1997; Lapointe et al., 1999). Roots of these two species show a marked resistance to *D. abbreviatus*, resulting in decreased larval weight gain and survival. We have now demonstrated that at least part of the natural resistance of *G. pentaphylla* is due to one compound, dehydrothalebanin B, despite the co-isolation

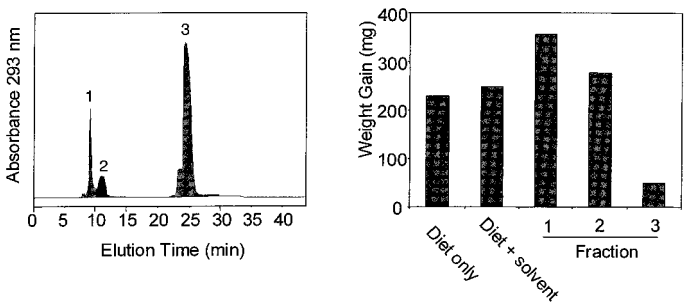


Figure 3. HPLC profile and larval weight gain on diet containing subfractions of the active HPLC separation (Figure 1, fractions 2−3 and 3−1).

of an active insecticide, the chlorinated hydrocarbon dieldrin (Figure 2).

Knowledge of a role for dehydrothalebanin B in resistance of citrus relatives may enable its identification in, or introduction into, citrus rootstocks. This may be done through germplasm screening, breeding and selection, or through transgenic introduction of biosynthetic enzymes. Structurally, as a metabolic product of phenylalanine and senecoic acid (Greger et al., 1996), dehydrothalebanin B may offer biosynthetic simplicity. Identification of its precursors in citrus germplasm may lead to biosynthesis with only limited transgenic modification. Alternatively, dehydrothalebanin B or structurally related amides may be used as markers for resistance through biochemical screening.

Insecticidal and antifungal activities have been attributed to various bioactive amides from *Glycosmis* species and, specifically, to dehydrothalebanin B in preliminary tests (Greger et al., 1996). Ours is apparently the first full report of insecticidal activity in dehydrothalebanin B. Also, rather than survey the activities of isolated compounds, we used bioassay-guided fractionation in an attempt to comprehensively recover any growth-inhibiting or insecticidal activities in *G. pentaphylla* roots at a given dietary dose (10% in root g equiv/vol of diet).

In the process of bioassay-guided fractionation, we also discovered an insecticide, dieldrin (Figure 2), that contaminated our samples. Only fractions containing dieldrin or dehydrothalebanin B (Figure 2) contained activity. For preparative extractions, we utilized root material collected from an arboretum and nursery as well as from our greenhouse plants grown from seed. Therefore, the origin of dieldrin in our fractionations is indeterminate. However, dieldrin is very persistent and was often used against *D. abbreviatus* and other soil pests in Florida until it was banned from such use, so its appearance in nursery or arboretum soil would not be surprising. For the discovery of resistance in *G. pentaphylla* seedlings, plants were grown in potting soil from seed in the greenhouse (Table 2; Table 3 "Roots"; Shapiro et al., 1997), where exposure to dieldrin is highly unlikely.

Greger et al. (1996) noted that the high quantities of dehydrothalebanin B in stem bark and roots of *Glycosmis* spp. were unusual, since such amides are typically restricted to foliage and seeds. They speculated on possible functions for dehydrothalebanin B and dehydroniranin B in defense of plant root systems. Perhaps their presence and activities in roots indicate a role in defense against subterranean herbivores such as *D. abbreviatus* and other larval Coleoptera, as well as fungal pathogens.

ABBREVIATIONS USED

EI, electron impact; FTIR, Fourier transform infrared spectroscopy; GC, gas chromatography; HPLC, high performance liquid chromatography; LC, low-pressure liquid chromatography; MSD, mass spectral detection; NMR, nuclear magnetic resonance spectroscopy; SIM, single-ion monitoring; UV, ultraviolet.

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