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Chemistry

Azamacrolides: A family of alkaloids from the pupal defensive secretion of a ladybird beetle (*Epilachna varivestis*)*

(Insecta/Coccinellidae/insect repellent/macrolide)

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ABSTRACT **Defensive droplets from glandular hairs of the pupa of the Mexican bean beetle, *Epilachna varivestis*, contain a group of structurally novel alkaloids, the azamacrolides. The major constituent of this secretion, epilachnene, is shown to be (5*Z*)-11-propyl-12-azacyclotetradec-5-en-14-olide. The secretion also contains an epilachnadiene and trace amounts of three closely related components.**

The pupa, like the egg, is a vulnerable stage in the life cycle of an insect. Immobile and often conspicuous, pupae are susceptible to attack by both predators and parasitoids. Eggs are sometimes protected by chemicals provided by the ovipositing female (2, 3), but pupae cannot benefit from such endowment. We here report on the unusual chemical defense of the pupa of the Mexican bean beetle, *Epilachna varivestis*, a member of the ladybird beetle family (Coccinellidae). Coccinellid pupae are commonly protected by so-called gin traps, abdominal biting devices that they activate by flipping their bodies and use to deter ants (4). The Mexican bean beetle pupa has no such devices and is unable to undertake flipping motions. When prodded, it remains passive and seemingly defenseless. However, microscopic examination of its surface revealed the presence of a dense cover of glandular hairs, each with a tiny droplet of clear secretion at its tip (Fig. 1). Suspecting this fluid to be defensive, we exposed pupae to ant attacks in a series of laboratory presentations. The pupae were individually placed on the bottom of small Petri dishes that served as the foraging arenas for laboratory colonies of the ants (*Leptothorax longispinosus*). The results, which were videotaped, were consistent and dramatic. The moment an individual ant, in an exploratory approach to the pupa, contacted some of the glandular hairs, it backed away and cleansed itself. While retreating, it repeatedly dragged its mouthparts and/or antennae against the substrate. None of the pupae (n = 10; each in a different arena) were injured during the 10-min presentations, although each was contacted by dozens of ants during this period (all pupae eventually gave rise to adults). We proceeded to collect the secretory droplets for analysis and here report on the chemicals isolated from the fluid (5).

MATERIALS AND METHODS

Sample Collection. Secretion from the glandular hairs, collected with microcapillaries, was sealed directly in glass tubes (1.8 × 20 mm) or extracted with ether or dichloromethane (100 μl). For NMR spectroscopy, a sample (500 pupae) was taken up in 400 μl of C^2HCl_3 (^2H, 99.96%).

Analytical Procedures. Samples were analyzed by gas chromatography (GC) on a Hewlett–Packard (HP) 5890 instrument equipped with a split/splitless injector, a flame ionization detector, and an HP 3396A integrator. Undiluted samples in glass capillaries were injected by a solid sampling technique (6, 7); solutions were introduced by splitless injection. Low-resolution mass spectrometry (MS) was carried out with an HP 5890 gas chromatograph linked to a Finnigan ion-trap detector (ITD 800) or an HP mass selective detector. Chemical ionization mass spectra were obtained on the ITD with methane as the reagent gas. High-resolution mass spectra were obtained on a Kratos 890 instrument. Gas-phase IR spectra were obtained with an HP 5890 gas chromatograph linked to an HP 5965A IR detector. Unless otherwise noted, ^1H and ^{13}C NMR spectra were obtained in C^2HCl_3 on a Varian XL-400 instrument.

Derivatization Techniques. (*i*) *Microhydrogenation.* A small sample of secretion in ether (50 μl) was placed in a glass vial and ≈0.5 mg of 10% Pd on activated charcoal was added. A balloon filled with hydrogen was attached to the vial. After 10 hr, 25 μl of ether was added and the supernatant was withdrawn and examined by GC/MS.

(*ii*) *Acetylation.* An ethereal extract (20 μl) of secretion was treated with a mixture of acetic anhydride and pyridine (60:40; 20 μl). After 3 hr at room temperature, the reaction mixture was examined by GC/MS and GC/IR (8).

(*iii*) *Treatment with* N,N-*dimethylhydrazine.* To an aliquot of the defensive secretion in ether (10 μl), an ethereal solution of *N,N*-dimethylhydrazine (50%; 10 μl) was added (9, 10). After 3 hr, the reaction mixture was examined by GC/MS.

(*iv*) *Bromination.* A sample of the secretion in ether (10 μl) was mixed with a solution of bromine in hexane (0.1%; 10 μl); after 2 hr at room temperature, the reaction mixture was analyzed by GC/MS (11).

RESULTS

A sample of secretion examined by temperature programmed GC and GC/MS showed one large peak representing >99% of the volatizable material (Fig. 2). A slower rate of heating revealed that this peak actually represents two components, present in a 9:1 ratio. Two trace constituents eluted before this composite peak, and a third eluted afterward, making up a total of five volatile compounds, labeled **I–V** in order of elution.

The mass spectrum of the dominant component (epilachnene; compound **IV**) showed the following significant features (Fig. 3): a molecular ion at m/z 267, an ion at m/z 252 due to a methyl group loss from the molecular ion, and a base peak at m/z 224 (resulting from loss of C_3H_7 or CH_3CO from the parent ion). The molecular formula of epilachnene was established as $C_{16}H_{29}NO_2$ by high-resolution MS [observed

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Abbreviations: ITD, ion-trap detector; COSY, correlated spectroscopy.

*This is paper no. 116 in the series Defense Mechanisms of Arthropods. Paper no. 115 is ref. 1.

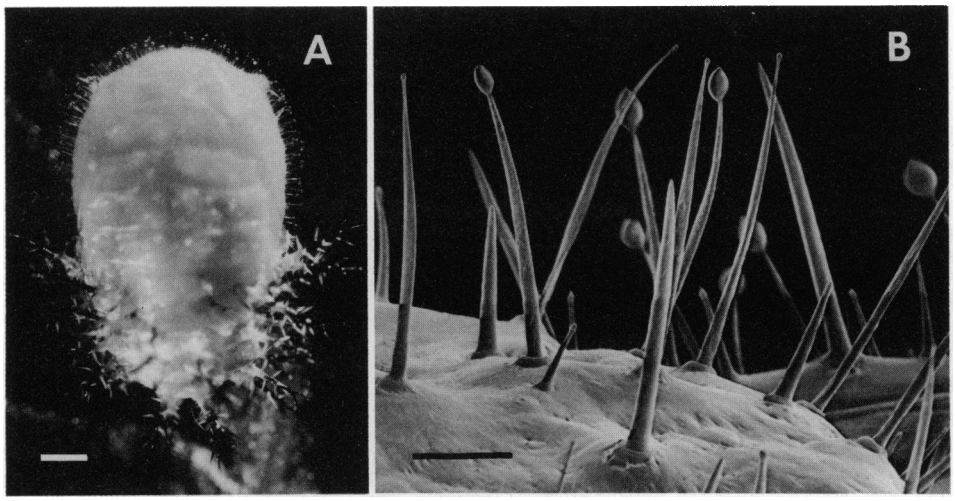


FIG. 1. (*A*) Dorsal view of pupa of *E. varivestis*. Glandular hairs are visible as a marginal pubescence on the front and sides of the pupa. Rear of the pupa is encased in remnants of the spiny larval skin. (*B*) Enlarged view (scanning electron micrograph) of portion of the pupal surface. Glandular hairs, bearing droplets at their tips, are interspersed among nonglandular spines. (*A*, bar = 1 mm; *B*, bar = 0.1 mm.)

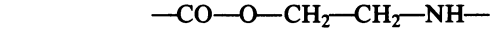
mass = 267.2199 (resolution = 10,000); calculated mass = 267.2198]. Similarly, the composition of the fragment ion at m/z 224 was found to be $C_{13}H_{22}NO_2$ [observed mass = 224.1638; calculated mass for $C_{13}H_{22}NO_2$ = 224.1650]. It follows that the loss of 43 mass units from the parent ion corresponds to loss of a C_3H_7 moiety.

The gas-phase IR spectrum of epilachnene (Fig. 4) shows a strong carbonyl band at 1753 cm^{-1}, corresponding to a typical ester carbonyl group (1748–1761 cm^{-1}). The absorption at 1152 cm^{-1} (C—O—C) supports the ester assignment. That this compound is not an aldehyde or a ketone is confirmed chemically by the absence of any reaction with *N,N*-dimethylhydrazine (9, 10). Finally, the IR spectrum of epilachnene shows a weak but significant olefinic C—H absorption at 3011 cm^{-1}.

To determine the number of double bonds in epilachnene, a small sample of secretion was subjected to catalytic microhydrogenation. The major product, epilachnane, shows a molecular ion at m/z 269 (Table 1), establishing the presence of one carbon-carbon double bond in the parent compound. It follows that epilachnene is monocyclic.

Treatment of a secretion sample with acetic anhydride and pyridine resulted in the disappearance of the original epilachnene GC peak and appearance of a major GC peak of longer retention time, corresponding to acetylepilachnene (see MS and IR data in Table 1). Since the IR spectrum of epilachnene shows no NH_2 or OH absorptions, and since this acetyl derivative appears to be a tertiary carboxamide (1745 cm^{-1}), epilachnene must be a secondary amine.

To obtain more detailed structural information, a large sample of the exudate (50 pupae) was collected in C^2HCl_3 for NMR analysis. The 400 MHz ^1H NMR spectrum and the corresponding correlated spectroscopy (COSY) spectrum are shown in Fig. 5. Two olefinic protons are apparent from the signals at δ 5.37 and 5.25. The chemical shift values of signals at δ 4.33 (1H) and 3.98 (1H) can be assigned to a CH_2 group attached to an oxygen atom. The COSY spectrum shows that this CH_2 group is coupled to another CH_2 group (δ 3.00 and 2.79; 1H each) attached to an electron-withdrawing atom such as nitrogen; this CH_2—CH_2 system is not coupled to any other protons. The following structural fragment is thus identified in epilachnene



[Although the presence of an NH group is not evident from the IR spectrum of epilachnene, this is not unusual for gas-phase spectra (12).]

The facile C_3H_7 loss observed in the mass spectrum of epilachnene suggests that a propyl or isopropyl group is present on the carbon atom α to the nitrogen atom. The terminal methyl triplet observed at δ 0.89 ppm is consistent only with a propyl group. Thus, we can propose the following substructure in epilachnene.

The multiplet observed between δ 2.3 and 2.4 corresponds to a CH_2 group attached to an ester carbonyl. This signal is coupled to the two peaks at δ 1.78 and 1.69 (COSY spectrum; Fig. 5), which are also coupled to the signal at δ 2.14, a

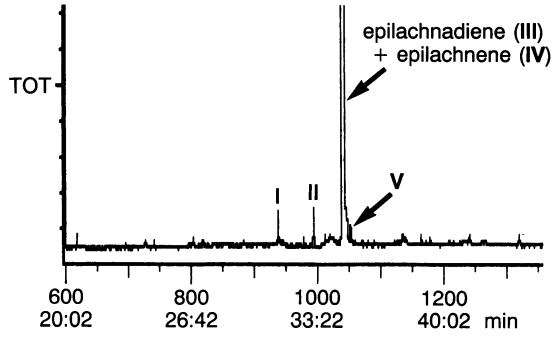


FIG. 2. Reconstructed gas chromatogram of volatile materials from cuticular hairs of *E. varivestis* pupa [ITD; 30 m × 0.2 mm DB-5-coated fused-silica capillary; oven temperature held at 40°C for 4 min at programmed 8°C/min to 260°C; solid sample injection (6, 7)].

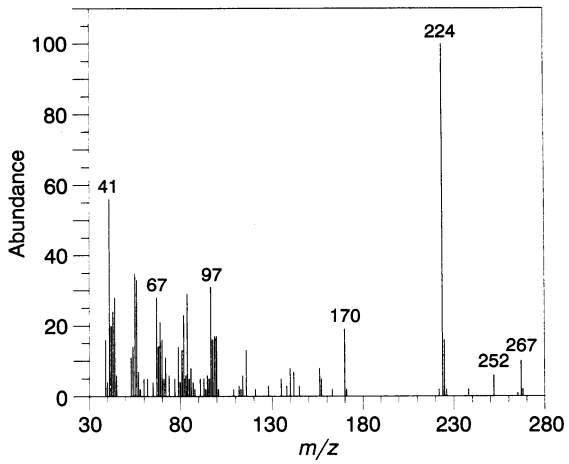


FIG. 3. Mass spectrum of epilachnene (electron-impact ionization; mass selective detector).

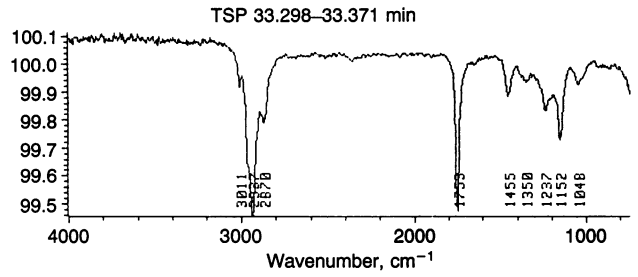
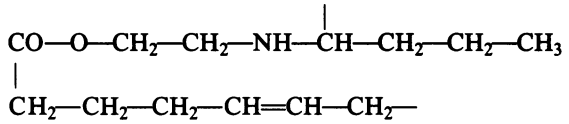
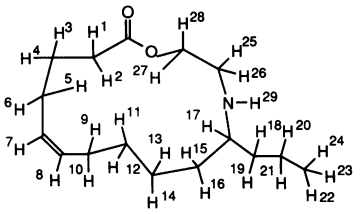


FIG. 4. Gas-phase IR spectrum of epilachnene.

chemical shift corresponding to allylic protons. Thus, the partial structure can now be represented as

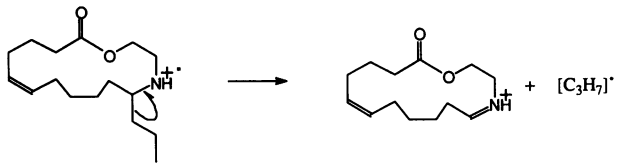


Assuming that the broad peak at δ 2.47 represents the CH α to the NH, and that the very broad peak around δ 1.2–1.5, which was difficult to integrate, represents 10 hydrogens (as five methylene groups), the macrocyclic structure shown for compound **IV** in Table 2 can be proposed for epilachnene. NMR peak assignments for the protons, as numbered in the following structure, are summarized in Fig. 5



The ester absorption (1753 cm^{-1}) in the IR spectrum of epilachnene is congruent with that previously observed for macrocyclic lactones (13). For example, the spectrum of (*Z*)-5-tetradecen-13-olide recorded under similar conditions shows a carbonyl absorption at 1745 cm^{-1}. In a study of >30 monounsaturated compounds, only those containing *Z* double bonds showed an IR band between 3010 and 3013 cm^{-1}, whereas no such absorption was observed for compounds with the *E* configuration (A.B.A., unpublished data). An IR absorption maximum at 3011 cm^{-1} in the epilachnene spectrum indicates therefore that the isolated double bond has the *Z* configuration. In fact, the IR spectrum of (*Z*)-5-tetradecen-13-olide also shows absorption at 3011 cm^{-1} and appears generally similar to that of epilachnene.

The mass spectral fragmentations of epilachnene and its derivatives can be readily rationalized. As already discussed, the base peak of compound **IV** at *m/z* 224 is attributable to cleavage of the propyl group. The corresponding ions in the spectra of epilachnane and acetylepilachnene appear at *m/z* 226 and 266, respectively. These MS features become valuable in interpreting the very limited structural data available for the remaining four constituents in this defensive secretion (see below):



m/z 267 *m/z* 224

Since epilachnene is monounsaturated, we expected to be able to prepare a dibromo derivative by simple addition of bromine. Surprisingly, no dibromo adduct was observed in this experiment. Instead, a mixture containing a monobromination product along with a complex array of epilachnene isomers was obtained. This result might well be due to a transannular reaction involving the lone pair of electrons on nitrogen and the cation generated by addition of bromine to the double bond. This process might yield several monobrominated bicyclic products, which could subsequently dehydrobrominate to a variety of epilachnene isomers.

The presence of an additional minor component (compound **III**; ≈10% of the volatile material) and three trace components (compound **I**, **II**, and **V**; <<1% each) in this defensive secretion has already been described. Analysis of their mass spectra, whose general features closely resemble those of epilachnene, epilachnane, and acetylepilachnene, suggests the structural assignments shown in Table 2. For example, the molecular ion and base peak of component **I** (*m/z* 241 and 198, respectively) indicate a saturated macrocycle with one less methylene group and a propyl substituent α to the nitrogen atom. We propose, therefore, that compound **I** is 9-propyl-10-azacyclododecan-12-olide. The mass spectrum of compound **II**, which shows a facile loss of 29 rather than 43 mass units (loss of ethyl instead of propyl) from a molecular ion at *m/z* 253, suggests that compound **II** is simply a norepilachnene (see compound **II** in Table 2). Similarly, mass spectral evidence indicates that compound **V** is the homoepilachnene shown in Table 2, with one extra methylene group in the macrocycle.

Component **III**, which elutes just before epilachnene, has its molecular ion at *m/z* 265 and a fragment ion at *m/z* 222, corresponding to an epilachnadiene. This conclusion is supported by the observation that microhydrogenation of the 9:1 mixture of compounds **IV** and **III** gave a single hydrogenation product, epilachnane.

Inspection of the postulated epilachnene structure strongly suggests that its continuous 14-carbon chain is derived biosynthetically from the chain shortening of an unsaturated fatty acid. If we assume that compound **III** is similarly derived from an unsaturated acid such as linoleic acid, it could be formulated as (5*Z*,8*Z*)-11-propyl-12-azacyclotetradec-5,8-dien-14-olide. While the structures proposed for the minor epilachnene congeners should be understood to be tentative, they serve the important function of providing targets for synthetic studies. Synthetic efforts to place all

Table 1. Mass spectral and IR data of epilachnene derivatives

Derivative	Mass spectrum, *m/z* (%)	Gas-phase IR spectrum, cm^{-1}
Epilachnane	269 (M^+, 5), 254 (5), 226 (100), 170 (13), 116 (12), 99 (14), 97 (16), 84 (16), 82 (29), 72 (11), 71 (4), 70 (13), 69 (19), 68 (8), 76 (10), 54 (19), 55 (31), 41 (50)	
Acetylepilachnene	309 (M^+, 23), 294 (2), 280 (7), 266 (70), 224 (100), 140 (10), 104 (16), 86 (28), 43 (48)	3011 (w), 2937 (s), 2872 (m), 1754 (s), 1674 (s), 1152 (m)

Electron impact mass spectra in column 2 were recorded with the ITD.

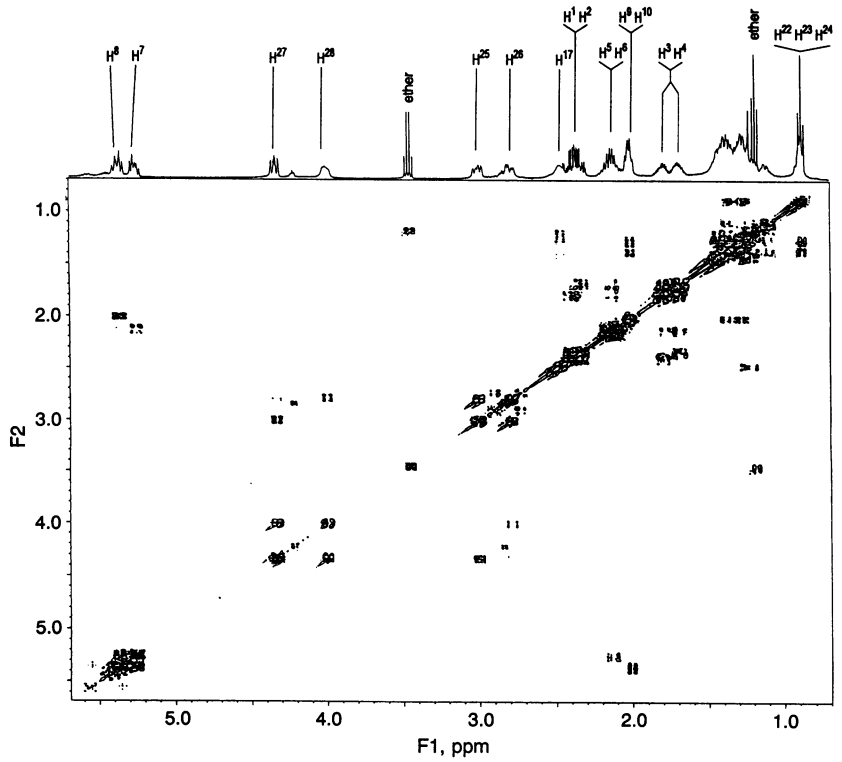
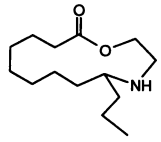
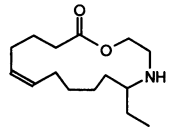
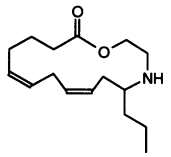
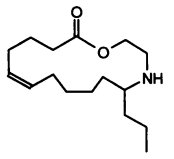
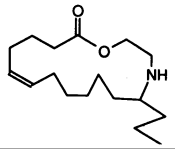


FIG. 5. A 400-MHz ^{1}H NMR and the corresponding DQ COSY spectrum of epilachnene (extraneous peaks are due to small amounts of epilachnadiene and diethyl ether as impurities).

Table 2. Components in defensive secretion of *E. varivestis*

Component	Proposed structure	Name	Relative %	Mass spectrum, *m/z* (%)
I		9-Propyl-10-azacyclododecan-12-olide	0.20	241 (M^+, 8), 226 (7), 210 (8), 199 (16), 198 (100), 170 (17), 142 (8), 116 (12), 99 (20), 97 (22), 82 (37), 72 (44), 55 (32)
II		Norepilachnene [(5*Z*)-11-ethyl-12-azacyclotetradec-5-en-14-olide]	0.25	253 (M^+, 30), 225 (15), 224 (100), 198 (12), 156 (25), 140 (15), 100 (30), 83 (73), 70 (78), 68 (87), 56 (50)
III		Epilachnadiene [(5*Z*,8*Z*)-11-propyl-12-azacyclotetradec-5,8-dien-14-olide]	8.55	265 (M^+, 45), 250 (17), 222 (30), 170 (32), 116 (70), 82 (100)
IV		Epilachnene [(5*Z*)-11-propyl-12-azacyclotetradec-5-en-14-olide]	90.90	267 (M^+, 10), 252 (6), 225 (16), 224 (100), 170 (19), 157 (5), 156 (8), 142 (7), 140 (8), 116 (13), 114 (6), 100 (17), 99 (17), 98 (16), 97 (31), 96 (5), 95 (6), 93 (5), 91 (5), 86 (8), 85 (5), 84 (29), 83 (6), 82 (23), 81 (13), 80 (4), 79 (14), 77 (5), 74 (6), 72 (11), 71 (5), 70 (16), 69 (21), 68 (14), 67 (28), 57 (7), 56 (33), 55 (35), 54 (14), 53 (11), 45 (6), 44 (28), 43 (24), 42 (20), 41 (56), 40 (4), 39 (16)
V		Homoepilachnene [(5*Z*)-12-propyl-13-azacyclopentadec-5-en-15-olide]	0.10	281 (M^+, 22), 238 (100), 224 (10), 184 (8), 170 (8), 112 (15), 98 (50), 74 (30), 58 (30)

Compound numbers in column 1 correspond to peaks in Fig. 2. Electron impact mass spectra in column 3 were recorded with an ITD.

these structures on a firm basis, establish their absolute configurations, and provide material for a broad investigation of their biological properties remain to be done.

DISCUSSION

The compounds characterized in this study are examples of a newly discovered group of natural products, the azamacrolides. Although many alkaloids have been isolated in earlier studies of ladybird beetles (14, 15), their structures are only distantly related to those of these macrocyclic amines. To make another comparison, large ring lactones (macrolides), while not uncommon in nature (16, 17), have not been found previously to include a basic nitrogen atom within the macrocycle. Clearly, the list of structural types that insects can mobilize for defensive purposes has not yet been exhausted.

Defensive chemicals from insects have been isolated primarily from larvae and adults (18, 19). Pupae have been largely ignored. While insect pupae are often encased in cocoons or buried in soil, many are unshielded like that of *Epilachna* and potentially defended chemically. A comparative chemical investigation of insect pupae could therefore well prove worthwhile.

Adult *Epilachna* are devoid of glandular hairs. When attacked, adults emit droplets of blood from knee joints ("reflex bleeding"). The fluid, which is deterrent to ants and spiders (20, 21), is devoid of azamacrolides but contains an alternative defensive chemical, the homotropane alkaloid euphococcinine (21), along with a variety of additional alkaloids (22).

We thank Professor A. C. Oehlschlager for a sample of (*Z*)-5-tetradecen-13-olide and Dr. Patrick R. Hughes for the beetles. This study was supported by National Institutes of Health Grants AI 12020 and AI 02908.

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