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Chemistry

Biosynthesis of a defensive insect alkaloid: Epilachnene from oleic acid and serine*

(Insecta/Coccinellidae/macrolide/stable isotope labeling)

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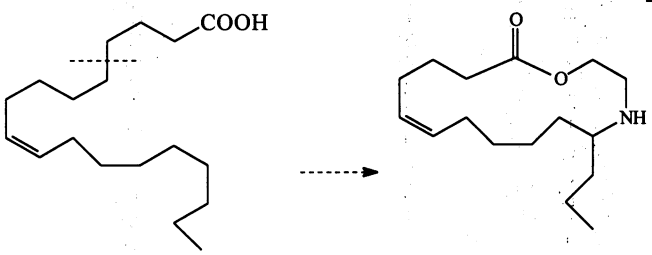
ABSTRACT **The biosynthesis of the azamacrolide epilachnene by the coccinellid beetle *Epilachna varivestis* has been studied with ^{2}H-labeled oleic acid, ^{2}H-labeled L-serine, and $^{13}C,^{15}N$-labeled L-serine. The incorporation of these precursors into epilachnene defines the origin of the alkaloid's entire carbon/nitrogen skeleton. GC/MS and GC/IR studies of alkaloid produced by *Epilachna* fed with deuteriated oleic acid show that oleic acid loses four carbon atoms from its carboxyl end during the biosynthesis. Other details, including the mechanism of carbon-nitrogen bond formation between the fatty acid and amino acid moieties, remain to be established.**

Although alkaloids are among the most structurally diverse defensive compounds of insects, very little is known about the biosynthetic pathways by which they are produced (1, 2). Especially interesting from a biosynthetic viewpoint are the azamacrolides, a group of large-ring alkaloids secreted by defensive glandular hairs of the pupa of the Mexican bean beetle, *Epilachna varivestis* (3). The major azamacrolide, epilachnene (**1**), is a 15-membered lactone that includes an ethanolamine moiety within the macrocycle and bears an *n*-propyl substituent adjacent to the nitrogen atom. Inspection of this structure suggests that the 14-carbon chain (from the carbonyl carbon to the terminus of the propyl group) might be derived from an unsaturated fatty acid such as oleic acid (**2**) or a related polyunsaturated fatty acid, subsequent to shortening of the 18-carbon chain by two β-oxidations. The ethanolamine unit might well be derived from serine (**3**). The biosynthetic process would then need to include a fatty acid amination step, which implies some novel chemistry. While we have not yet studied the mechanism by which the fatty acid and amino acid fragments are joined, we now provide evidence for the derivation of epilachnene's entire carbon-nitrogen skeleton from the postulated precursors.



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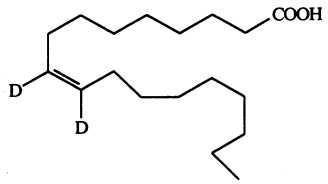
The simplest candidate precursor for the 14-carbon chain in epilachnene would be oleic acid itself. In the present study, we tested this possibility by feeding a specifically deuteriated oleic acid, (*Z*)-9-[9,10-$^{2}H_2$]octadecenoic acid (compound **4**; D = deuterium), to larvae of *E. varivestis*. We also carried out feeding experiments with singly and doubly labeled samples of L-serine (**5** and **6**). Subsequent collection and analysis of the pupal defensive secretion from both series of experiments revealed good incorporation of all three precursors.



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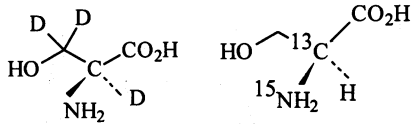

5 **6**

MATERIALS AND METHODS

Labeling Experiments. A 10-day-old pinto bean plant (*Phaseolus vulgaris* var. *humilis*) was pruned to leave only one leaf, and a solution of (*Z*)-9-[9,10-$^{2}H_2$]octadecenoic acid (**4**) (95 atom %^{2}H, 1.6 mg; MSD Isotopes) in diethyl ether (20 μl) was applied uniformly over the entire upper surface of the leaf. After 5 min, a single fifth-instar larva of *E. varivestis* was placed on the plant and allowed to feed on the treated leaf until pupation. Three days after pupation, droplets of liquid exuding from the glandular hairs were drawn into fine glass capillaries [as previously described (3)], and the collected material was extracted into ether (10 μl). Similar experiments, also with single fifth-instar larvae, were performed

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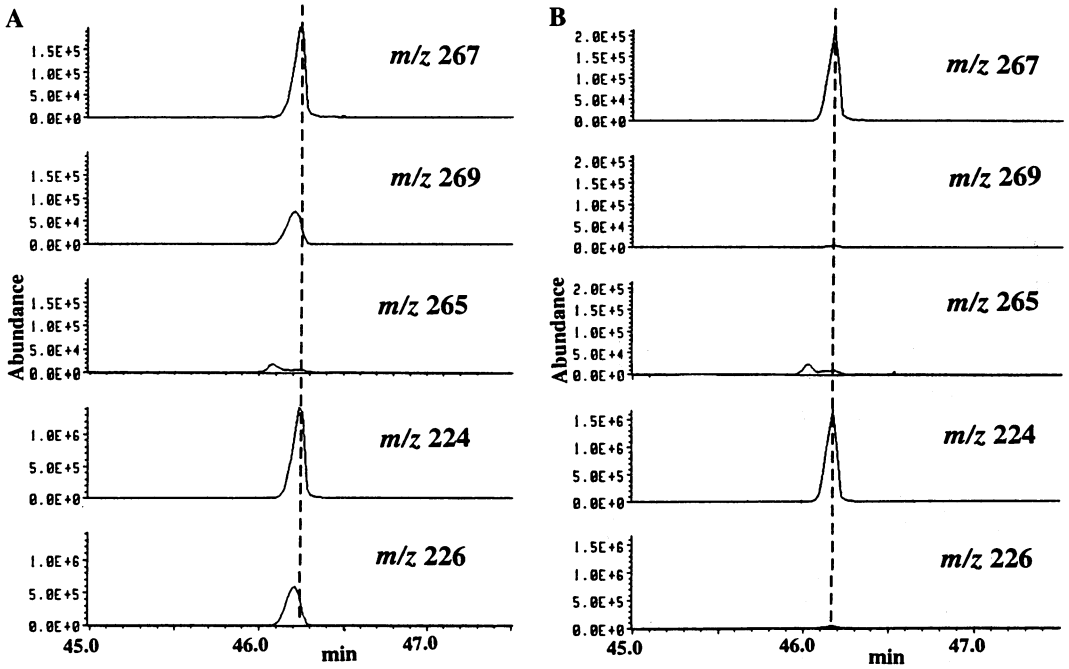

FIG. 1. Selected-ion retrieval chromatograms obtained by GC/MS analysis of defensive secretion of *E. varivestis* pupae. (*A*) (*Z*)-9-[9,10-$^2H_2$]Octadecenoic acid-enriched diet. (*B*) Normal diet. Gas chromatography was carried out on a DB-5-coated 30-m × 0.22-mm column, held at 40°C for 4 min and increased to 260°C at 4°C/min. Ordinate is in exponential notation—e.g., 5.0E+4 = 5 × 10^4.

with L-[2,3,3-$^2H_3$]serine (**5**) (99.3 atom % ^2H) and L-[2-^{13}C,^{15}N]serine (**6**) (99 atom % ^{13}C and 99 atom % ^{15}N).

Analytical Procedures. Low-resolution electron-ionization mass spectra were obtained by using a Hewlett–Packard 5890 gas chromatograph linked to a Hewlett–Packard 5970 mass selective detector (MSD). Solvent extracts were introduced by splitless injection. Analyses were performed with a 25-m × 0.22-mm fused-silica column coated with DB-5 (J & W Scientific, Folsom, CA). IR spectra were obtained by using a 25-m × 0.32-mm fused-silica column coated with DB-5 in a Hewlett–Packard 5890 gas chromatograph linked to a Hewlett–Packard 5965A IR detector.

RESULTS AND DISCUSSION

A sample of the secretion from the dideuteriooleic acid-fed individual was analyzed by GC/MS and GC/IR, and the data were compared with those obtained from a secretion sample from individuals fed a normal diet. This comparison showed that epilachnene from the labeled acid-fed insect is in fact a composite of labeled and unlabeled alkaloid. Selected-ion retrieval chromatograms, obtained by searching for the molecular ion and the base peak of both deuterium-labeled and unlabeled epilachnene, showed that the anticipated doubly deuteriated epilachnene had indeed been formed. Interestingly, the intensities of the ions with *m/z* 269 and 226, corresponding to the molecular ion and base peaks of dideuterioepilachnene, maximize in the GC/MS experiment a few seconds before those of *m/z* 267 and 224 (Fig. 1*A*) corresponding to the molecular ion and the base peak of epilachnene itself (3). Assuming that the intrinsic relative intensities of the molecular ions of epilachnene and dideuterioepilachnene are similar, integration of the peak areas of selected-ion retrieval chromatograms permits us to compute the ratio of labeled to unlabeled epilachnene, since the natural abundances of *m/z* 269 and 226 ions in the mass spectrum of unlabeled epilachnene are insignificant (Fig. 1*B*). The MS evidence indicated that nearly 50% of the epilachnene obtained from the dideuteriooleic acid-fed individual was in fact labeled. This high incorporation allowed us to draw definitive conclusions using the data derived from a single insect.

By a careful subtraction of the mass spectrum of epilachnene (Fig. 2*A*) from that of partially deuteriated epilachnene, we obtained the result shown in Fig. 2*B*, which

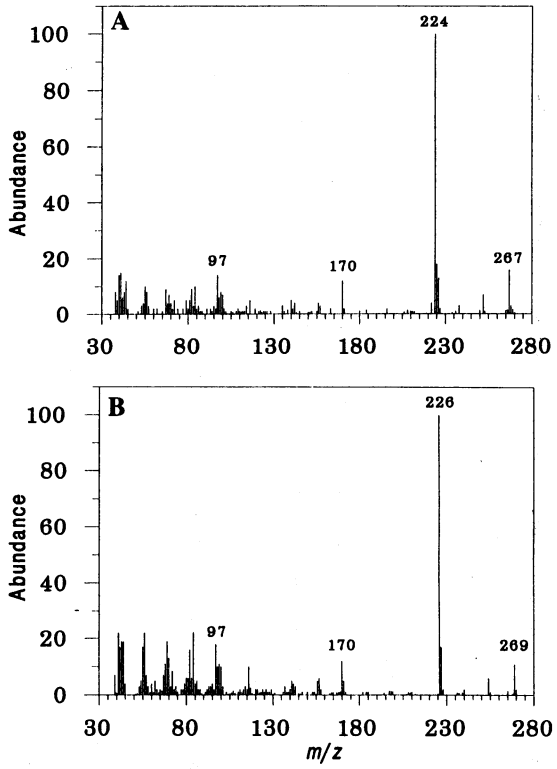

FIG. 2. Epilachnene electron-ionization mass spectra obtained by GC/MS analysis of defensive secretion of an *E. varivestis* pupa on a (*Z*)-9-[9,10-$^2H_2$]octadecenoic acid-enriched diet. Mass spectrum corresponding to scans at 46.28 min (*A*) and at 46.14 min (*B*) after subtracting the spectrum obtained at 46.28 min from it.

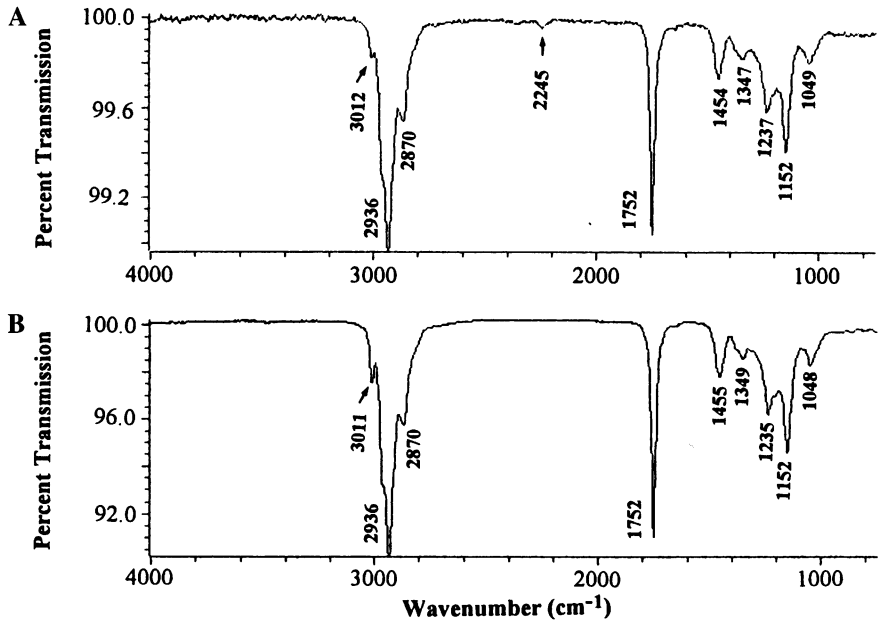

FIG. 3. Epilachnene gas-phase Fourier-transform IR spectra obtained from defensive secretion of *E. varivestis* pupae. (*A*) (*Z*)-9-[9,10-$^2H_2$]Octadecenoic acid-enriched diet. (*B*) Normal diet. Gas chromatography was carried out on a DB-5-coated 30-m × 0.32-mm capillary column, held at 60°C for 3 min and increased to 260°C at 10°C/min.

represents a mass spectrum of dideutrioepilachnene itself. From this spectrum, it is evident that the two deuterium atoms are located, as expected, in the ring: the base peak shows the loss of 43 mass units (C_3H_7) from the molecular ion, analogous to the loss of the propyl side chain observed in the spectrum of unlabeled alkaloid. The GC/IR spectrum of alkaloid obtained from the deuteriooleic acid-fed specimen showed a small but distinct absorption band at 2245 cm^{-1} (Fig. 3*A*), absent from the spectrum of unlabeled epilachnene (Fig. 3*B*). Since we have established that compounds with carbon-carbon double bonds bearing two *cis* deuterium atoms can be characterized by this 2245-cm^{-1} absorption (the corresponding absorption in *trans* compounds appears at 2215 cm^{-1}) (4), observation of a 2245-cm^{-1} absorption establishes the presence of a doubly deuteriated *cis* Δ^5 double bond in the alkaloid sample. It follows that during the biosynthetic process, oleic acid undergoes the anticipated chain shortening (presumably via two β-oxidations), resulting in the removal of four carbon atoms from the carboxyl end.

The secretion from the individual fed with L-[2,3,3-$^2H_3$]serine (**5**) also gave positive incorporation results. Selected-ion retrieval chromatograms showed the presence of a triply deutereriated epilachnene molecular ion (*m/z* 270) which maximized a few seconds before that of the M + 1 peak of unlabeled epilachnene (*m/z* 268; see Fig. 4*A*). (In this experiment, the M + 1 ion was used as the reference because the level of serine incorporation was low.) Since the natural abundance of the *m/z* 270 ion in the mass spectrum of unlabeled epilachnene is virtually zero (Fig. 4*B*), a ratio of labeled to unlabeled epilachnene of about 5% could be readily computed in a manner similar to that described above.

The epilachnene mass spectrum from the individual fed with L-[2-^{13}C,^{15}N]serine (**6**) provided evidence that this alkaloid's nitrogen atom is also serine derived. This spectrum showed a significant ionic abundance at *m/z* 269 (M + 2) compared with a very low abundance at *m/z* 269 in the mass spectrum of epilachnene. Integration of the relevant peak areas in the selected-ion retrieval chromatograms (Fig. 5) showed that about 20% of the recovered epilachnene con-

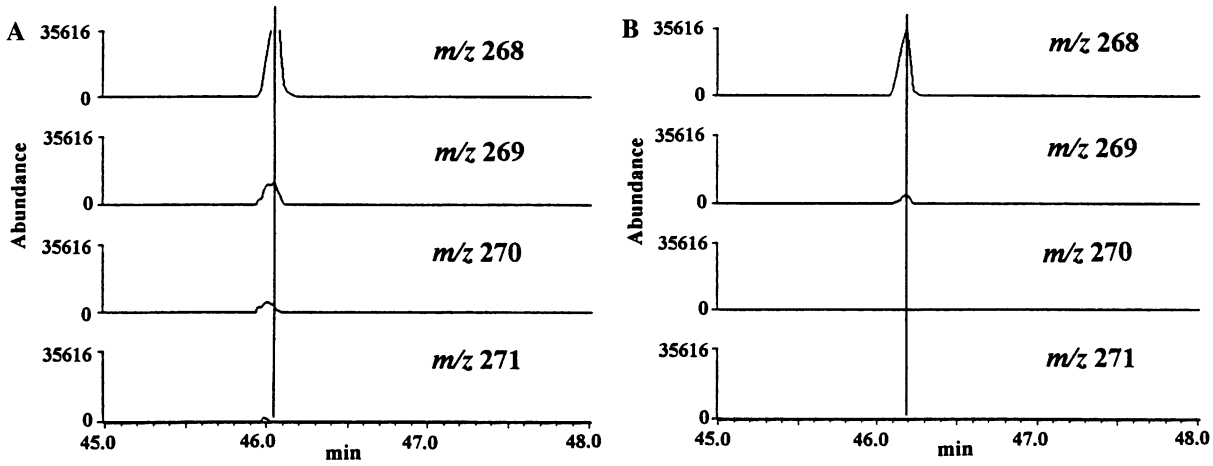

FIG. 4. Selected-ion retrieval chromatograms obtained by GC/MS analysis of defensive secretion of *E. varivestis pupae*. (*A*) L-[2,3,3-$^2H_3$]Serine-enriched diet. (*B*) Normal diet. Gas chromatography was carried out on a DB-5-coated 30-m × 0.22-mm column, held at 40°C for 4 min and increased to 260°C at 4°C/min. The retention time of epilachnene is slightly shorter in *A* because a slightly higher carrier gas flow rate was used for this analysis.

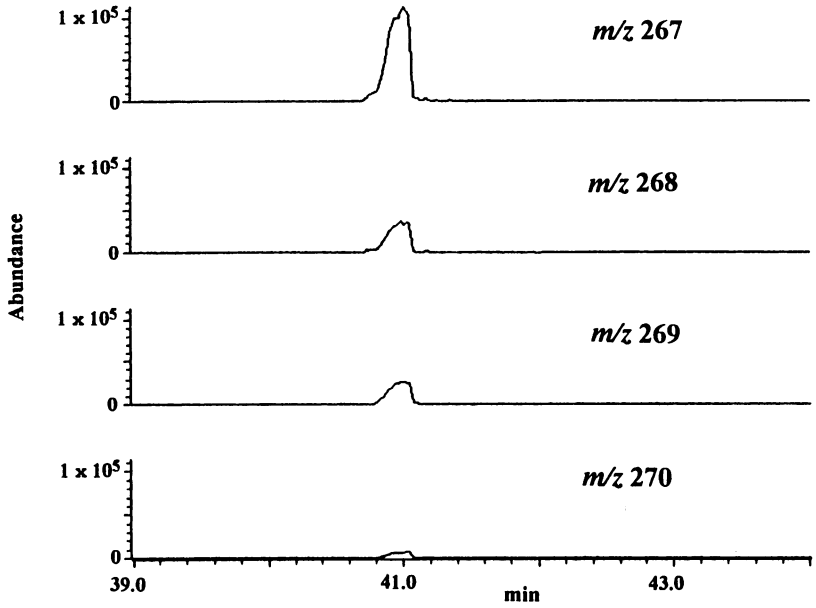

FIG. 5. Selected-ion retrieval chromatograms obtained by GC/MS analysis of defensive secretion of an *E. varivestis* pupa on an L-[2-^{13}C,^{15}N]serine-enriched diet. Gas chromatography was carried out on a DB-5-coated 30-m × 0.22-mm column, held at 40°C for 4 min and increased to 260°C at 6°C/min.

tained both the ^{13}C and ^{15}N labels. It is interesting to note that the retention time of epilachnene labeled in this way is chromatographically indistinguishable from that of unlabeled compound, although both deuterium labeled epilachnenes elute slightly earlier than the corresponding undeuteriated compound. Similar elution behaviors have been noted in our earlier biosynthetic work (5).

While we were not able to detect free ethanolamine in the pupal hemolymph of *E. varivestis*, this amine has been identified in the defensive secretion of other beetles (6). Whether serine decarboxylation occurs before or after the oleic acid and serine moieties are joined, how the crucial amination step is accomplished, and when the fatty acid chain is shortened are among the questions that remain to be studied. Nevertheless, the most basic features of azamacrolide biosynthesis are now established.

We thank Dr. Tadgh Begley (Cornell University) for a valuable discussion of this research and Dr. Paul S. Anderson (Merck Sharp & Dohme Research Laboratories) for the donation of (*Z*)-9-[9,10-$^{2}H_2$]octadecenoic acid, L-[2,3,3-$^{2}H_3$]serine, and L-[2-^{13}C,^{15}N]serine. This work was supported in part by National Science Foundation Grant MCB-9221084 and by National Institute of Allergy and Infectious Diseases Grant AI 2908.

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