

## Biosynthesis of Monoterpenes in an Ant (*Acanthomyops claviger*)

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*Terpenoid substances are of broad distribution and diverse function in insects. One set, elaborated by the mandibular glands of Acanthomyops claviger, acts both as a defensive secretion and as an alarm releaser. When fed C<sup>14</sup>-labeled acetate or mevalonate, laboratory colonies of these ants produce radioactive citronellal and citral, providing unambiguous evidence for de novo synthesis of these terpenes by the ant. The incorporations of these precursors implicate the mevalonic acid pathway as the likely biosynthetic route.*

The defensive glands of arthropods produce a variety of chemical substances, among which are formic acid, *p*-benzoquinones, and aliphatic aldehydes (14, 16). Often the particular molecular species employed as toxins are representatives of chemical classes of wide biological distribution. Although Brower and Brower (2) have suggested that many natural insect toxicants are derived from secondary plant substances, at present it is far from clear whether most must come from such dietary sources or whether the majority can be synthesized *de novo* by insects. As defensive secretions can be isolated relatively easily from their capacious integumentary reservoirs, they offer especially favorable material for biosynthetic studies. Aside from the work of Waterhouse and coworkers (10) which demonstrates the incorporation of C<sup>14</sup>-labeled acetate, propionate, caproate, or decanoate into all major aliphatic constituents of the

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defensive secretion of a pentatomid bug, little data is available on their biogenesis.

The monoterpenes, which include citronellal and citral identified in the mandibular gland secretion of the ant *Acanthomyops claviger* (Roger) (3), comprise one of the more interesting classes of defensive substances. These and related isoprenoid molecules serve as physiological or behavioral messengers in a variety of insect groups (19, 23, 24). In spite of a few exceptional cases (4, 6, 15), as a rule insects do not manufacture steroids (5, 11); thus ecdysone, the molting hormone, appears to be derived from ingested cholesterol (12). In contrast, Schmialek (17) has shown that after the injection of 2-C<sup>14</sup>-mevalonic acid into silkworm caterpillars, radioactive farnesol can be recovered. In the present study, we are concerned with the biogenesis of the monoterpene aldehydes which serve as alarm releasers and defensive substances for *Acanthomyops* (9).

Table I. Experimental Results

	<i>Sodium Acetate</i>		<i>Mevalonic Acid Lactone</i>	
	1-C <sup>14</sup>	2-C <sup>14</sup>	1-C <sup>14</sup>	2-C <sup>14</sup>
Compound fed				
Amount, mg.	0.18	0.048	6.2	2.32
Activity, d.p.m.	2×10 <sup>7</sup>	3×10 <sup>7</sup>	1×10 <sup>7</sup>	1×10 <sup>7</sup>
Derivatives isolated				
Citronellal				
Total activity, d.p.m.	2.5×10 <sup>4</sup>	2.5×10 <sup>4</sup>	10	1×10 <sup>4</sup>
% incorporation	0.1	0.07	—	0.1
Specific activity, d.p.m./mmole	3.3×10 <sup>6</sup>	3.3×10 <sup>6</sup>	—	1.4×10 <sup>6</sup>
Citral				
Total activity, d.p.m.	2.5×10 <sup>2</sup>	1.5×10 <sup>3</sup>	0	6.0×10 <sup>2</sup>
% incorporation	0.001	0.005	—	0.006
Specific activity, d.p.m./mmole	3.3×10 <sup>5</sup>	2.7×10 <sup>6</sup>	—	1×10 <sup>6</sup>

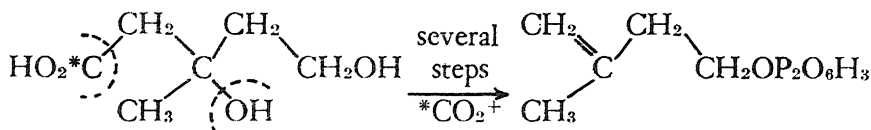
Laboratory colonies of worker ants were fed sugar-water containing 1-C<sup>14</sup>-acetate, 2-C<sup>14</sup>-acetate, or in a carefully controlled simultaneous feeding, 1-C<sup>14</sup>- or 2-C<sup>14</sup>-mevalonate. After an appropriate period, the ants were frozen and extracted with methylene chloride and the terpene aldehydes (citronellal and citral) isolated by thin-layer chromatography. These were then converted into their dini-

trophenylhydrazones, which were further purified to constant radioactivity.

### Results and Discussion

Acetate, labeled at either the methyl or carboxyl position, was significantly incorporated into both citronellal and citral. 2-C<sup>14</sup>-Mevalonate was similarly well incorporated, but, in contrast, 1-C<sup>14</sup>-mevalonate produced only very slight radioactive labeling in the terpenes. The results of these experiments are summarized in Table I.

It is immediately clear that *Acanthomyops* need not rely on dietary sources of terpenes but can synthesize citronellal and citral from either acetate or mevalonate. The higher total activity of the citronellal as compared with the citral probably reflects the natural preponderance of citronellal (*ca.* 90%) in the ant secretion. As the specific activities show, these results are consistent with a common biogenetic origin of both terpenes. In the mevalonic acid pathway as described from other organisms (13), the radioactive carbon of 1-C<sup>14</sup>-mevalonate is lost upon formation of isopentenyl pyrophosphate.



In *Acanthomyops*, the strikingly different incorporations following the two mevalonate feedings indicate that mevalonate is not degraded before being built into terpenes but rather is decarboxylated, as in the classical mevalonic acid pathway.

The presence of the mevalonic acid pathway for terpene biosynthesis suggested that these ants might also manufacture steroids. To investigate this possibility, a new sample of ants from the 1-C<sup>14</sup>-acetate experiment were examined for the presence of radioactive cholesterol or other  $\beta$ -hydroxysteroids. The ants were extracted with ethanol and ether, this lipid extract was saponified, and the nonsaponifiable material (plus a few milligrams of nonradioactive carrier cholesterol) chromatographed on a deactivated alumina column (18). Upon elution with a solvent series of increasing polarity (*see* Figure 1) the cholesterol was recovered in fractions 96 to 104 (benzene). It was further purified by thin-layer chromatography and by preparation

of its digitonide. To make certain that other  $\beta$ -hydroxysteroids of *Acanthomyops* would not be missed, the remaining fractions were pooled under their respective solvents and the digitonides of each were prepared. When assayed for  $C^{14}$ -incorporation, none of the

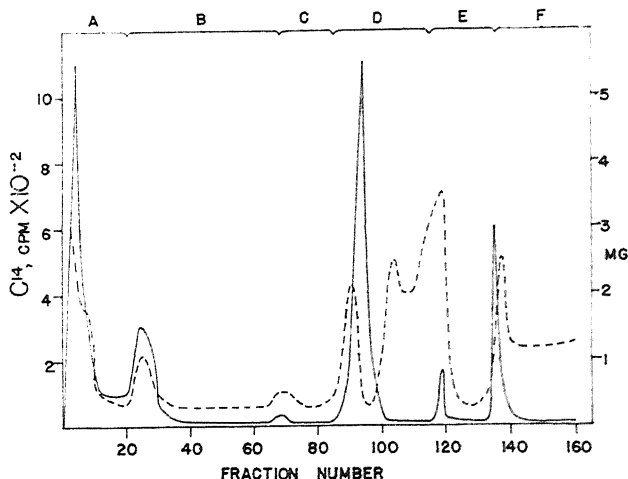


Figure 1. Chromatography of nonsaponifiable lipids from *A. claviger* after feeding of  $1-C^{14}$ -acetate

Absorbent: 20 grams of deactivated Merck alumina eluted with:

- A. Petroleum ether
- B. Petroleum ether-benzene (10 to 1)
- C. Petroleum ether-benzene (4 to 1)
- D. Benzene
- E. Diethyl ether
- F. Methanol

— Weight recovered  
 ---- C.p.m. recovered

digitonin-precipitable material was radioactive. Apparently the mevalonic acid pathway is employed by *Acanthomyops* for the synthesis of monoterpenes but not for the formation of steroids.

### Experimental

**Assay of Radioactive Compounds.** The radioactive samples were counted on steel planchets in a Nuclear Chicago Model D-47 low-background gas-flow counting chamber with an absolute counting efficiency (estimated by comparison with a standard) of about 20%.

**Purification of Carrier Compounds.** Citronellal (b.p. 99–99.5°/25 mm.) and citral (b.p. 92–93°/4.2 mm.) were purified by distillation, and the purity was checked by vapor-phase chromatography. Cholesterol (m.p. 148.5–49.5°) was purified via its dibromide (8).

**Administration of Tracers.** Workers of *Acanthomyops claviger* (Roger) were freshly collected near Ithaca, N. Y., for each experiment. Over the course of each feeding, 1000 to 1500 ants were maintained in a two-chamber Lucite Wilson nest. One chamber was filled with moist earth and shielded from light while the other served as a foraging area. C<sup>14</sup>-labeled acetate and mevalonate were fed in glucose solution and were distributed throughout the colony by regurgitative feeding (22, 24). In the experiments with 1-C<sup>14</sup>- and 2-C<sup>14</sup>-mevalonate, all workers were collected from a single natural colony and were individually sorted into one or the other laboratory colonies to avoid any possible bias due to physiological caste differences analogous to those reported in other ant species (7).

**Isolation of Citronellal and Citral.** At the close of each experiment (7 to 10 days), the nests were frozen intact. Groups of 200 workers were placed in a micro-Soxhlet apparatus and extracted for 8 hours with methylene chloride. A few milligrams of carrier citronellal and citral were added and the mixture was applied to a thin-layer chromatoplate (silica gel G) which was developed with hexane–ethyl acetate (92 to 8) to separate citronellal and citral (3). The aldehydes were detected by spraying with a solution of 2, 4-dinitrophenylhydrazine in tetrahydrofuran (20) and the citronellal and citral peaks were scraped off and allowed to react with excess dinitrophenylhydrazine reagent for a further 12 hours.

The dinitrophenylhydrazones were separated from the reaction mixture by thin-layer chromatography (silica gel G developed with benzene) and further purified by thin-layer chromatography on aluminum oxide G (petroleum ether–diethyl ether (96 to 4), silica gel G (chloroform), and silica gel G (diethyl ether)). In all cases, the specific activities of the dinitrophenylhydrazones remained constant over the course of the last two purifications.

**Collection of Nonsaponifiable Lipids.** Two hundred ants from the 2-C<sup>14</sup>-acetate feeding were ground with sand and the resulting brei refluxed for 4 hours in 25 ml. of ethanol, ethanol–diethyl ether (3 to 1), and diethyl ether (twice). The extracts were pooled, the solvents were evaporated, and the residue was saponified by refluxing for 1 hour with 20 ml. of methanolic potassium hydroxide (10% potassium hydroxide in 60% aqueous methanol). An equal quantity of water was added and the aqueous solution extracted three

times with diethyl ether (100 ml. total) to isolate the nonsaponifiable fraction. The ether extract was then shaken with 100 ml. of 1% aqueous potassium hydroxide to remove any free fatty acids. The activity of the total extract was about  $8.0 \times 10^6$  d.p.m. and that of the nonsaponifiable material  $3.0 \times 10^5$  d.p.m.

**Chromatography of Nonsaponifiable Lipids.** The nonsaponifiable residue plus 4.5 mg. of carrier cholesterol was applied to the top of a  $7.5 \times 1.7$  cm. column containing 20 grams of Merck alumina (suitable for chromatographic adsorption) which had been previously deactivated by mixing with 7% aqueous acetic acid (10% glacial acetic acid in distilled water) (18). The column was packed in petroleum ether (redistilled, b.p. 60-70° C.) and 10 ml. fractions were collected. The eluting solvents are shown in Table II.

Table II. Eluting Solvents

<i>Fraction</i>	<i>Solvent</i>
1-20	Petroleum ether
21-65	Petroleum ether-benzene (10:1)
66-85	Petroleum ether-benzene (4:1)
86-115	Benzene
116-135	Diethyl ether
136-160	Methanol

The only white solid recovered was in fractions 97 to 104. Thin-layer chromatography on silica gel G impregnated with Rhodamine 6G showed that only this fraction contained cholesterol (1). After a second chromatography using chloroform as the solvent, 3.25 mg. of white crystalline material with an activity of  $2 \times 10^2$  d.p.m. was recovered. This cholesterol was further purified by preparation of its digitonide (21). All other fractions were pooled under their respective solvents, 1 mg. of carrier cholesterol was added to each, and the digitonides were prepared. No activity above background was detected in any of the digitonin precipitates.

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