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# *De novo* biosynthesis of the aggregation pheromone components ipsenol and ipsdienol by the pine bark beetles *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae)

**(isoprenoid biosynthesis/monoterpene alcohols/myrcene)**

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**ABSTRACT** **The California five-spined ips, *Ips paraconfusus* Lanier, produces the myrcene-derived acyclic monoterpene alcohols ipsenol (2-methyl-6-methylene-7-octen-4-ol) and ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) as components of its aggregation pheromone. The pine engraver beetle, *Ips pini* (Say), produces only ipsdienol. Previous studies have shown that myrcene, a monoterpene in the pines colonized by these beetles, is a direct precursor to these pheromone components. *In vivo* radiolabeling studies reported here showed that male *I. paraconfusus* incorporated [1-$^{14}$C]acetate into ipsenol, ipsdienol, and amitinol (*trans*-2-methyl-6-methylene-3,7-octadien-2-ol), while male *I. pini* incorporated [1-$^{14}$C]acetate into ipsdienol and amitinol. Females of these species produced neither labeled nor unlabeled pheromone components. The purified radiolabeled monoterpene alcohols from males were identified by comparison of their HPLC and GC retention times with those of unlabeled standards. HPLC-purified fractions containing the individual radiolabeled components were analyzed by GC-MS and were shown to include only the pure alcohols. To further confirm that ipsdienol and ipsenol were radiolabeled, diastereomeric ester derivatives of the isolated alcohols were synthesized and analyzed by HPLC and GC-MS. After derivatization of the radiolabeled alcohols, the HPLC analysis demonstrated expected shifts in retention times with conservation of naturally occurring stereochemistry. The results provide direct evidence for *de novo* biosynthesis of ipsenol, ipsdienol, and amitinol by bark beetles.**

The aggregation pheromone components (4*S*)-(−)-ipsenol (2-methyl-6-methylene-7-octen-4-ol) (**1**) and (4*S*)-(+)-ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (**2**) were isolated and identified from males of the California five-spined ips, *Ips paraconfusus* Lanier. These components, together with (1*S*,2*S*)-(+)-*cis*-verbenol (*cis*-4,6,6-trimethyl bicyclo[3.1.1]-hept-3-en-2-ol), were identified as the first coleopteran pheromone (1). The structural similarity of the acyclic monoterpene alcohols ipsenol and ipsdienol to myrcene (2, 3), a monoterpene present in the oleoresin of the principal host, ponderosa pine, *Pinus ponderosa* Laws., led to a deuterium labeling study with *I. paraconfusus* that unequivocally showed the *in vivo* transformation of myrcene to ipsenol and ipsdienol (4). A more recent labeling study demonstrated the conversion of myrcene to ipsdienol in the male pine engraver beetle, *Ips pini* (Say) (5). Recent reviews (6–9) emphasize the central role of plant-derived myrcene in the production of ipsenol and ipsdienol, including the hypothesis that myrcene from the host may be sequestered and bioaccumulated by males during early developmental stages for later use as adults (9). The putative use of dietary myrcene for pheromone production in *Ips* is consistent with the general concept that monoterpenes *sensu stricto* are plant products (7, 10, 11).

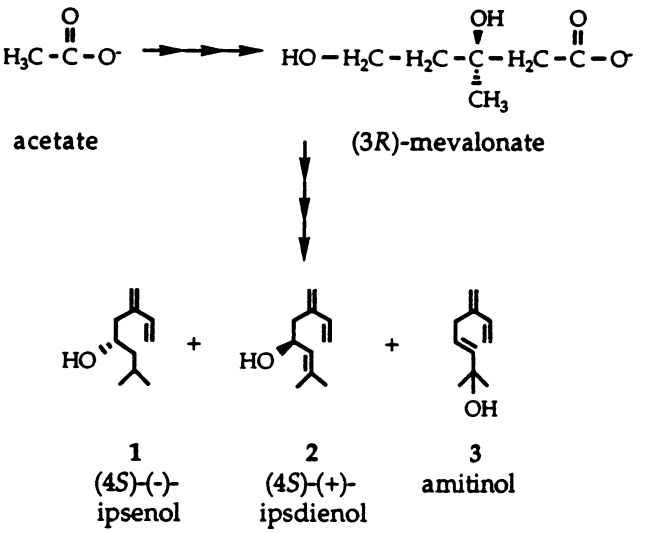


FIG. 1. Abbreviated *de novo* biosynthesis of acyclic monoterpene alcohols by male *I. paraconfusus*.

However, it has been recognized that alternative routes for *Ips* spp. pheromone biosynthesis may exist, such as *de novo* biosynthesis or utilization of other terpene hydrocarbon precursors (4). Additional studies with *I. paraconfusus* (12, 13) have questioned whether the volatile myrcene titer in the host can account for all of the ipsenol and ipsdienol produced by males. Furthermore, the amount of ipsdienol produced by the European congener, *Ips duplicatus* Sahlberg (14), can be reduced by inhibiting hydroxymethylglutaryl-CoA reductase, an enzyme that catalyzes the rate-limiting step in isoprenoid biosynthesis. Although these studies provide indirect evidence for *de novo* synthesis in *Ips*, the conclusions remain equivocal. Adequate amounts of myrcene may be available to males feeding on *P. ponderosa* when all tissue-laden and volatile sources are considered (12). Similarly, the inhibitory role of compactin in the isoprenoid pathway of *I. duplicatus* (14) may also have an impact on the biosynthesis of the terpenoid juvenile hormone (JH) III, which is known to induce pheromone production in *I. paraconfusus* (15–17). Here we present direct evidence that radiolabeled acetate is incorporated into ipsenol and ipsdienol by male *I. paraconfusus* and *I. pini* (Fig. 1).

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Abbreviations: JH, juvenile hormone; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetic acid; FID, flame ionization detection; EI, electron-impact.

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## MATERIALS AND METHODS

**Insects.** *I. paraconfusus* and *I. pini* were collected as immatures in infested pine logging debris and reared to the adult stage (18) (Table 1). *I. paraconfusus* was collected in *P. ponderosa* near the University of California Blodgett Forest in El Dorado Co., California (June 16, 1994, ≈1200-m elevation; T13N,R11E,S27; 120°45′00″W, 38°56′41″N). *I. pini* was collected in Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.) in the Lassen National Forest, Lassen Co., California (May 13, 1994, ≈2000-m elevation; T34N,R7E,S10; 121°10′30″W, 40°48′30″N).

**Chemicals and Materials.** Ipsenol and ipsdienol used for standards were obtained from Bedoukian Research Incorporated (Danbury, Connecticut, product numbers P408 & P407, respectively). Sodium [1-$^{14}$C]acetate (50 mCi/mmol; 1 mCi = 37 MBq) and Ecolume biodegradable liquid scintillation solution were obtained from ICN. All other reagents were obtained from Aldrich with the following product numbers: myrcene (M10,000-5); *n*-octanol (36,056-2); (2*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) (15,526-8); (1*S*)-(−)-camphanic acid (32,822-7); 1,3-dicyclohexyl carbodiimide (D8,000-2), and 4-pyrrolidinopyridine (21,337-3).

**Administration of Substrate and Collection of Pheromone Components.** Males and females of each species were individually injected with ≈0.2 μCi of sodium [1-$^{14}$C]acetate in 0.5 μl of 0.15 M aqueous NaCl between the abdominal tergites via a glass capillary needle (≈10-μm diameter). In an attempt to increase the quantity of radiolabeled acetate incorporated into the pheromone alcohols, one sample of male *I. pini* (marked by ¶ in Table 1) was placed on *P. jeffreyi* phloem to feed for 48 hr prior to injection. All other insects were injected after emergence without feeding. Pheromone extracts were generated from injected insects after they were introduced into freshly cut logs of their original host species by trapping male- and female-specific volatiles on Porapak Q during a 168-hr feeding period (19–21).

**Extraction and Chromatographic Analysis of Pheromone Components.** A 350-ml pentane extract of the Porapak was concentrated to 10 ml by Kuderna–Danish evaporative concentration and ipsenol and ipsdienol were quantified (Table 1) by gas chromatography (GC) using *n*-octanol as an internal standard. A Hewlett–Packard series 5890A gas chromatograph containing a 30 m × 0.32 mm (i.d.), 0.25-μm film thickness, Durabond-5 Column (J & W Scientific, Folsom, CA) was temperature programmed from 70°C (29 min) to 180°C at 5°C/min. The carrier gas was helium with a flow rate of 31 cm/s and detection was by flame ionization (FID). After quantitative analysis, the extract was further concentrated to 2 ml and a 50-μl aliquot was assayed for radioactivity by using Ecolume scintillation cocktail on a Beckman LS-1701 liquid scintillation counter. The counting efficiency was 97% for $^{14}$C. An aliquot of 200 μl from the remaining extract was fractionated by HPLC on a 10 mm × 50 cm Nucleosil 50-5 normal-phase column (Alltech Associates) with hexane/acetone (96:4, vol/vol) [2 ml/min] as eluant. Each fraction was assayed for radioactivity and alcohols in the relevant fractions were verified by coelution with reference standards on HPLC and GC (conditions above), as well as by electron-impact (EI)-GC-MS. A Finnigan MAT ssQ 710 was used with a *m/z* scanning range of 50–180 at 70 eV. A Varian model 3400 gas chromatograph containing a 30 m × 0.25 mm (i.d.) DB-5 column with a 0.25-μm film thickness was temperature programmed from 70°C (60 min) to 180°C at 5°C/min. The carrier gas was helium at 14 psi (96 kPa) head pressure with a flow rate of 1 ml/min.

HPLC fractions containing ipsenol and ipsdienol were pooled, separately, and diastereomeric esters were prepared from each alcohol by using a chiral acid {ipsenol: (2*R*)-(+)-MTPA, $[\alpha]_D^{20} = +72°$ (*c* = 1.6, $CH_3OH$); ipsdienol: (1*S*)-(−)-camphanic acid, $[\alpha]_D^{20} = -25°$ (*c* = 1, dioxane)} (19, 22). The structures of the derivatives were confirmed by EI-solid probe-MS using the aforementioned mass spectrometer with the probe programmed at 50°C for 0.25 min and 50°C to 250°C at 200°C/min, and held for 2 min. Derivatized ipsenol and ipsdienol were analyzed by radio-HPLC (conditions above) to further confirm the identity and to determine the enantiomeric composition of radiolabeled ipsenol and ipsdienol. The enantiomeric compositions of ipsenol and ipsdienol (Table 1) in the purified fractions (containing labeled and unlabeled alcohols) were also determined directly by chiral GC (19–21).

## RESULTS

All pentane extracts of Porapak-trapped volatiles from both sexes of both species contained significant levels of radioactivity relative to background levels (≈30 dpm) (Table 1). Although more than 100 compounds were detected in the Porapak extracts from either sex or species by GC-FID analysis, very few of these were radiolabeled (Figs. 2 and 3). Male *I. paraconfusus* incorporated [1-$^{14}$C]acetate into both ipsenol and ipsdienol (Fig. 2*A*), while male *I. pini* incorporated [1-$^{14}$C]acetate into ipsdienol (Fig. 3*A*). Male *I. pini* also clearly incorporated [1-$^{14}$C]acetate into *trans*-2-methyl-6-methylene-3,7-octadien-2-ol (amitinol) (**3**) (23, 24) (Fig. 3*A*, Table 1, 1158 dpm in peak), while the incorporation of radiolabel into this compound by male *I. paraconfusus* was less evident from our radio-HPLC analysis (Fig. 2*A*, 50 dpm in peak). However, amitinol isolated from a larger aliquot of the crude extract of male *I. paraconfusus* volatiles indicated that the entire extract contained 1060 dpm of amitinol-associated $^{14}$C. Females from neither species synthesized radiolabeled ipsenol, ipsdienol, or amitinol (Figs. 2*B* and 3*B*), nor were unlabeled alcohols detectable by GC-FID (data not shown). The identities of other polar and nonpolar radiolabeled compounds produced

Table 1. Isolations of ipsenol and ipsdienol from [1-$^{14}$C]acetate-injected *I. paraconfusus* and *I. pini*

| Species (no. males/ no. females)* | Collection date, mo/day/yr | Aeration date, mo/day/yr | Enantiomeric composition, %† | Quantity, mg‡ | $^{14}$C in extract, dpm§ |
|---|---|---|---|---|---|
| *I. paraconfusus* (141/146) | 6/23/94 | 6/30–7/7/94 | 99.4 ± 0.1/2.7 ± 0.2 | 65.1 ± 0.3/7.1 ± 0.1 | 28,200/5920 |
| *I. pini* (91/—) | 5/13/94 | 6/15–22/94 | ND | —/12.8 ± 0.1 | 50,645/— |
| *I. pini* (80/41) | 5/13/94 | 6/22–29/94 | —/97.0 ± 0.2 | —/13.6 ± 0.2 | 21,480/6720 |
| *I. pini*¶ (85/—) | 5/13/94 | 8/3–10/94 | —/96.3 ± 0.2 | —/5.5 ± 0.1 | 103,280/— |

*Volatiles from male and female *Ips* spp. were collected separately from artificially colonized logs of the local host from which each population was collected. Host for *I. paraconfusus* was *P. ponderosa*; host for *I. pini* was *P. jeffreyi*.

†Determined by chiral GC [% (−)-ipsenol/% (−)-ipsdienol, mean ± SEM, *n* = 3 injections of sample, ND = not determined]. GC separation of enantiomers was as described (19–21).

‡Milligrams of ipsenol/mg of ipsdienol, mean ± SEM, *n* = 6 injections of sample.

§dpm in male extract/dpm in female extract.

¶These insects were fed on *P. jeffreyi* phloem for 48 hr prior to injection with acetate. EI-GC-MS data for amitinol isolated from these males: *m/z* (%) = 134 $(M - 18)^+$ (30), 119 (68), 105 (27), 91 (90), 79 (100), 77 (37), 65 (16), and 53 (20).

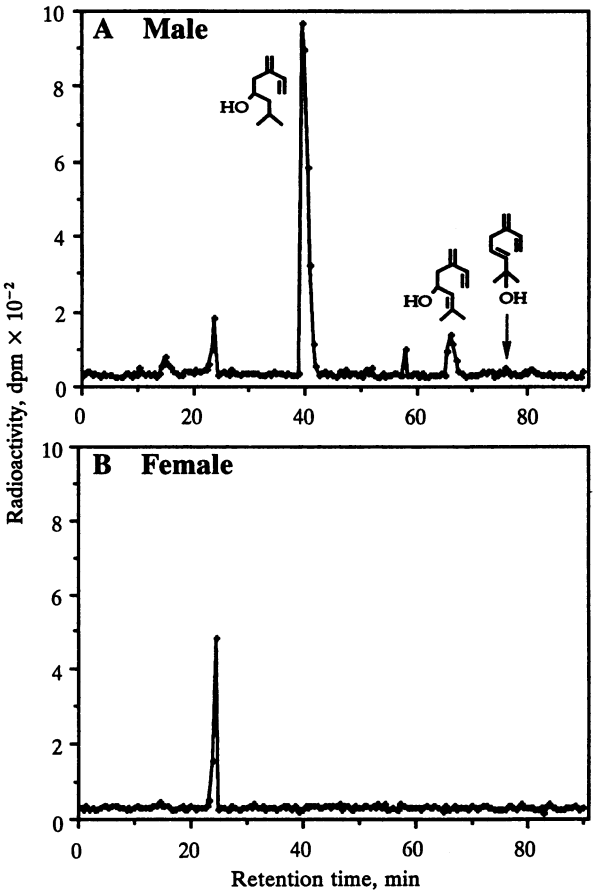


FIG. 2. Radio-HPLC analysis of pentane extracts of Porapak-trapped volatiles from 141 male (*A*) and 146 female (*B*) *I. paraconfusus* feeding for 168 hr in *P. ponderosa*. Both sexes were injected with 0.2 μCi of sodium [1-$^{14}$C]acetate prior to pheromone collection.

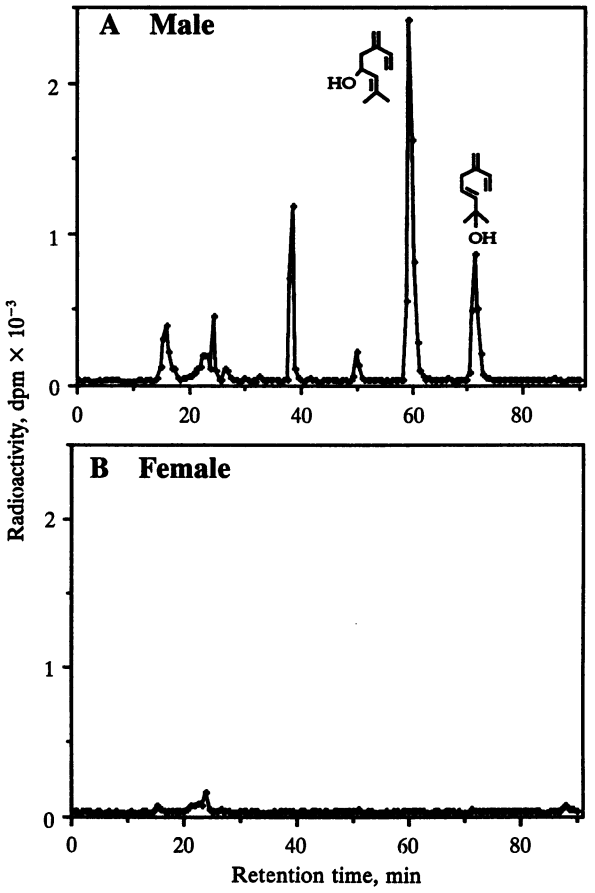


FIG. 3. Radio-HPLC analysis of pentane extracts of Porapak-trapped volatiles from 85 male (*A*) and 41 female (*B*) *I. pini* feeding for 168 hr in *P. jeffreyi*. Both sexes were injected with 0.2 μCi of sodium [1-$^{14}$C]acetate prior to pheromone collection.

by both sexes of *I. paraconfusus* and *I. pini* have not yet been established.

The association of radiolabel with ipsenol, ipsdienol, and amitinol in our analyses is supported by coelution of radioactivity with reference standards on HPLC and GC, as well as by GC-MS analysis of the relevant fractions. To further confirm that the alcohols were radiolabeled, an HPLC elution shift of radioactivity after derivatization of ipsenol from *I. paraconfusus* and ipsdienol from *I. pini* (Figs. 4 and 5) was demonstrated. The HPLC retention times of the radiolabeled derivatives corresponded with the retention times of the standard unlabeled derivatives, and the structures of both the natural product and the standard derivatives were confirmed by GC-MS. In addition, the enantiomeric composition of the radiolabeled alcohols, as determined from the diastereomeric derivatives, supports their identity. For example, after preparing and analyzing the MTPA ester of ipsenol isolated from male *I. paraconfusus*, we obtained a single radiolabeled peak for ipsenoyl-MTPA with an HPLC retention time corresponding to that of the standard unlabeled 4*S* diastereomer (Fig. 4). This result agrees with the enantiomeric composition of the alcohol as measured by chiral GC analysis of the entire sample (labeled and unlabeled) (Fig. 4*A Inset*; Table 1), as well as with previous analyses on this species (19). In contrast, after preparing and analyzing the camphanate ester of ipsdienol isolated from *I. pini*, we obtained two radiolabeled peaks for (4*R*)- and (4*S*)-ipsdienyl camphanate (Fig. 5). These peaks had HPLC retention times corresponding to those of the standard unlabeled diastereomers, and the labeled camphanates occurred as ≈91% 4*R* (Fig. 5). This result also agrees with the enantiomeric composition of the alcohol as measured by chiral GC analysis of the entire sample (≈96% 4*R*) (Fig. 5*A Inset*; Table 1), by the diastereomeric composition of the camphanate esters as determined by HPLC analysis of the entire sample using UV detection (Fig. 5*B Inset*), as well as by previous analyses on this species (19–21). Additional evidence for the association of radiolabel with the structures of ipsenol and ipsdienol is provided by the observation that the ratio of radiolabeled ipsenol to ipsdienol produced by *I. paraconfusus* (9.7:1) (Fig. 2*A*) agrees with the mass ratio of the entire sample, as determined by GC-FID (9.2:1) (Table 1) and from previous analyses (19, 25).

## DISCUSSION

The data presented in this report constitute strong evidence that *I. paraconfusus* and *I. pini* can produce their aggregation pheromones *de novo*. Only a few of the more than 100 compounds collected from the volatiles produced by the insects while feeding on their hosts are labeled from [1-$^{14}$C]acetate, and this small subset includes the pheromone components. Moreover, relative to the mass distribution of the pheromone components, radioactivity is incorporated in the same ratio and with very similar stereochemistry. Preliminary studies using (*RS*)-[5-$^{3}$H]mevalonolactone, a cyclic form of (3*RS*)-mevalonate (Fig. 1), indicate that this substrate is also converted to pheromone components by both species of *Ips* (data not shown). This study illustrates the potential usefulness of radiotracers in identifying prospective semiochemicals of bark beetles.

Early evidence for *de novo* biosynthesis of ipsenol and ipsdienol by male *I. paraconfusus* may reside in a series of

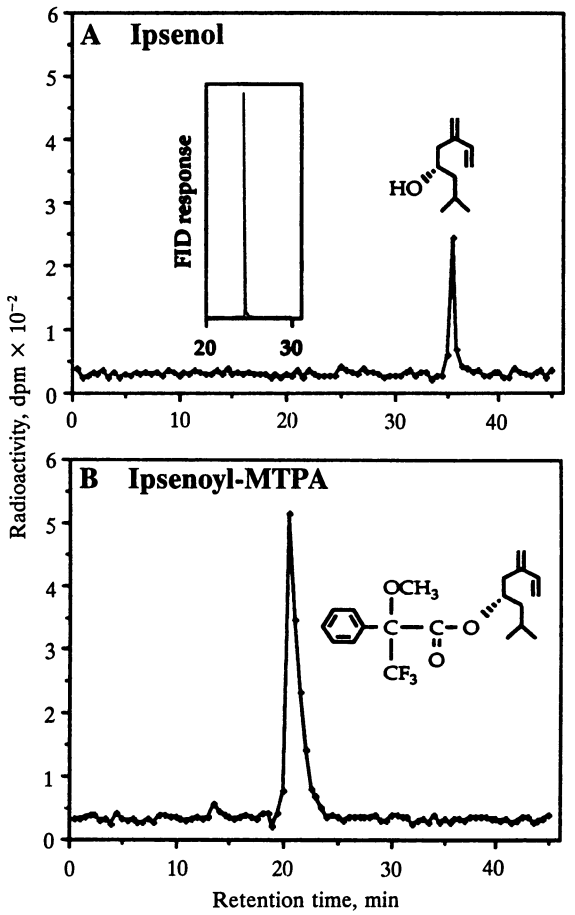


FIG. 4. Radio-HPLC analysis of 99.4% (4*S*)-(−)-ipsenol isolated from pentane extract of Porapak-trapped volatiles from 141 male *I. paraconfusus* (*A*) and the (4*S*)-ipsenoyl-(2′*R*)-2′-methoxy-2′-phenyl-2′-(trifluoromethyl)acetate derivative of the isolated ipsenol (*B*). An ipsenol sample of ≈5.3 mg was used for analysis (≈0.8 mg for HPLC analysis of ipsenol alone and ≈4.5 mg for derivatization and HPLC analysis). (*Inset*) Chiral GC chromatogram of total (labeled and unlabeled) 99.4% (4*S*)-(−)-ipsenol.

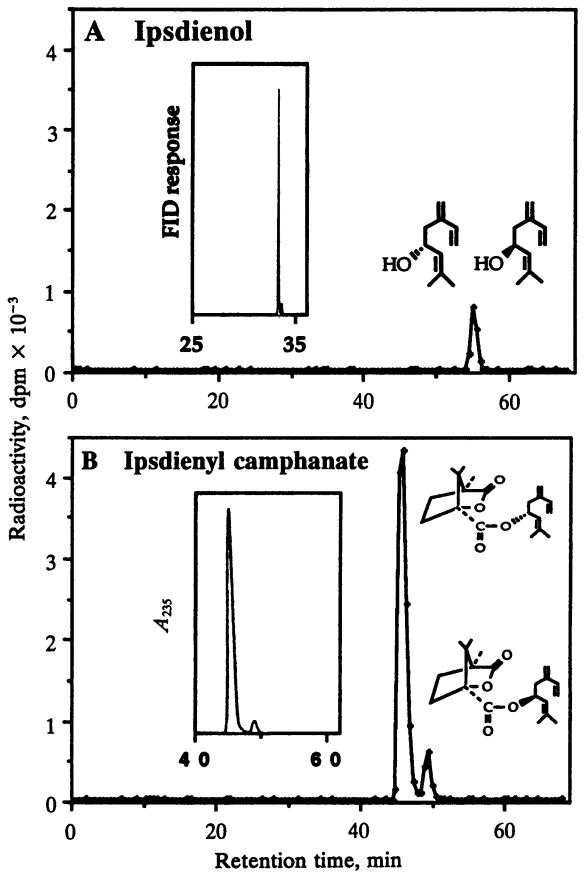


FIG. 5. Radio-HPLC analysis of 96% (4*R*)-(−)-ipsdienol isolated from pentane extract of Porapak-trapped volatiles from 85 male *I. pini* (*A*) and the (4*R*)- and (4*S*)-ipsdienyl (1′*S*)-camphanate derivatives of the isolated ipsdienol (*B*). An ipsdienol sample of ≈1.4 mg was used for analysis (≈0.2 mg for HPLC analysis of ipsdienol alone and ≈1.2 mg for derivatization and HPLC analysis). (*Inset* in *A*) Chiral GC chromatogram of total (labeled and unlabeled) 96.3% (4*R*)-(−)-ipsdienol. (*Inset* in *B*) HPLC chromatogram of total (labeled and unlabeled) (4*R*)- and (4*S*)-ipsdienyl-(1′*S*)-camphanates detected by UV absorbance (235 nm).

endocrinological studies (15–17). In these studies males were treated topically with JH III or the JH analogue fenoxycarb and denied food or exposure to myrcene. Nonetheless, these males yielded attractive hindgut tissue (15) or significant amounts of ipsenol and ipsdienol in abdominal or hindgut extracts (16, 17). In this case pheromone production could be interpreted as resulting from *de novo* biosynthesis from endogenous carbohydrate- or lipid-based energy stores. Declining amounts of ipsenol and ipsdienol measured from hindgut extracts made 20, 28, and 36 hr after fenoxycarb treatment are likely due to exhaustion of carbohydrate- or lipid-based energy stores in treated pheromone-producing males rather than to depletion of hypothetically sequestered monoterpene precursors (17).

*De novo* pheromone biosynthesis by *I. paraconfusus* and *I. pini* may originate from insect tissues or from bacterial or fungal symbionts. There is evidence of microbial involvement in the biosynthesis of the pheromone components of *I. paraconfusus* (reviewed in ref. 8); however, none of this evidence offers direct proof of microbial pheromone production (6). Nonetheless, bacterial (26) and fungal (10, 27) systems are known to synthesize isoprenoids from acetate, and, ultimately, we hope to resolve this *de novo* pathway into its potential insect and microbial components. Maturation-dependent (28), hormonally mediated (15–17), and male-specific (28) production of ipsenol and ipsdienol would appear to require a rather remarkable microbial symbiosis by *I. paraconfusus* (28, 29).

It is likely that insects possess the enzymes that catalyze the reactions in the isoprenoid pathway necessary for the synthesis of ubiquitous terpenoids such as JH (30) and respiratory chain cofactors such as ubiqinone (31). A variety of insects appear to produce very specific isoprenoids, such as geranyl acetate and various isomers of farnesene, as behavioral chemicals (32–34). Other insects unequivocally biosynthesize isoprenoid behavioral chemicals *de novo* as demonstrated by radiotracer studies (35–37). Thus, it is not unexpected that *Ips* spp. might contain the genetic information to code for enzymes which produce geranyl diphosphate from acetate.

To our knowledge, the biosynthesis of amitinol by *Ips* spp. has never been investigated. Kohnle *et al.* (38) suggested that *Ips* spp. might produce amitinol from (4*S*)-(+)-ipsdienol, which is itself generated from myrcene. Byers (6) also has speculated that amitinol is derived from myrcene by *Ips amitinus* Eichh. Here we demonstrate that amitinol can be synthesized *de novo* by males of both *I. paraconfusus* and *I. pini*, but the role of myrcene as a precursor for this alcohol awaits further study. Amitinol was first reported as a natural product from male *I. paraconfusus* (23); however, we are not aware of any reported isolation of this compound from *I. pini*. Amitinol does not appear to be behaviorally active in *I. paraconfusus* (23, 39).

Our demonstration of *de novo* biosynthesis of ipsenol, ipsdienol, and amitinol in *I. paraconfusus* and ipsdienol and amitinol in *I. pini* will shift the focus from myrcene as a host-provided precursor to myrcene as a hypothetical insect-derived biosynthetic intermediate. However, when all host sources and entry points into insect tissue are considered (e.g., inhalation from nuptial chamber headspace, ingestion of phloem tissue, and ingestion of oleoresin from xylem resin canals), it is possible that host-derived myrcene may play a substantial role in pheromone alcohol biosynthesis. These engraver beetles consume large quantities of phloem tissue and have the feeding habit of deeply etching their galleries into the sapwood of their hosts, thereby increasing the probability of contacting xylem oleoresin.

Thus, there appear to be two systems operating in pheromone alcohol synthesis by *I. paraconfusus* and *I. pini*, and they may occur in sequence. It is conceivable that hydroxylation of exogenous myrcene loses significance as males ingest and metabolize increasing amounts of carbohydrate, thereby fully engaging *de novo* biosynthesis of ipsenol and ipsdienol. The relative proportions of the total monoterpene alcohols that are synthesized *de novo* and from host-supplied myrcene remain to be quantified.

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