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Polyketide Origin of Pheromones of *Carpophilus davidsoni* and *C. mutilatus* (Coleoptera: Nitidulidae)

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Abstract—Biosynthesis of pheromones from *Carpophilus davidsoni* Dobson and *C. mutilatus* Erichson was investigated by feeding the beetles diets containing isotopically substituted (^{13}C and deuterium) fatty acids and then analyzing the resulting labeled pheromone components. (2*E*,4*E*,6*E*,8*E*)-7-Ethyl-3,5-dimethyl-2,4,6,8-undecatetraene, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene and (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene from *C. davidsoni* and (3*E*,5*E*,7*E*)-5-ethyl-7-methyl-3,5,7-undecatriene from *C. mutilatus* were abundant enough to be analyzed by both NMR spectroscopy and MS. Eleven additional minor analogues were analyzed only by MS. Each hydrocarbon can be assembled from just three different acyl units: The initial unit can be acetate, propionate or butyrate. Propionate is the second unit in all of the analogues encountered so far, extending the chain by two carbons and producing a methyl branch. Subsequent chain-extending units can be either propionate or butyrate, leading to additional methyl or ethyl branches, respectively. The final acyl unit is either propionate or butyrate and it loses its carboxyl carbon during hydrocarbon biosynthesis. A hydrocarbon with four total units is a triene and one with five is a tetraene. Assembly is proposed to be as in usual fatty acid anabolism, except that other precursor units are used in addition to acetate and that the double-bond reduction step of each chain-elongation cycle does not occur, leaving the conjugated, unsaturated system. Seven of the analyzed hydrocarbons were not previously known to occur in *C. davidsoni*; two of these are novel: (2*E*,4*E*,6*E*,8*E*)-5,7-diethyl-3-methyl-2,4,6,8-undecatetraene and (2*E*,4*E*,6*E*)-3,5-dimethyl-2,4,6-octatriene. Published by Elsevier Science Ltd

Introduction

Sap beetles of the genus *Carpophilus* (Coleoptera: Nitidulidae) are worldwide pests of fruits and grains, such as peaches, figs, dates and maize, both before and after harvest.[1] In addition to causing feeding damage, the beetles can vector undesirable microorgansims such as those that cause brown rot in peaches[2] and accumulation of aflatoxin in maize.[3] Beetle attack often occurs just as the crop ripens; therefore, control with insecticides is difficult because residues on the harvested crop must be avoided. Pheromones for these beetles could be useful pest management tools and our laboratory undertook a project to identify pheromones for selected *Carpophilus* spp. A series of novel, alkyl-branched, conjugated, triene and tetraene hydrocarbons was discovered.[4–8] Example structures are shown in Figures 1 and 5.

The pheromones are emitted only by male beetles, but both sexes respond to them. The chemicals have greatest biological activity when present with 'host' volatiles, such as from fermenting fruit or bread dough. Synergism can be dramatic; the combination of pheromone and host odors has been over 100 × more attractive in the field than pheromone or host odor alone.[9,10] Such scent combinations have attracted huge numbers of beetles; for example, over 100,000 *C. mutilatus* Erichson were captured per trap per 3 day period when traps were baited with pheromone plus fermenting bread dough and set out in a heavily infested California date garden.[11]

The unusual pheromone structures prompted us to investigate their biosynthesis. The initial study demonstrated that the major pheromone component of *Carpophilus freemani* Dobson (triene **1** in Fig. 1) could be assembled from simple fatty acids.[12] The chain is initiated with an acetate unit; subsequent propionate and butyrate units each extend the chain by two carbons and account for the methyl and ethyl branches, respectively, and the chain is completed with a second butyrate. The assembly of acyl units into the pheromone structure was proposed to proceed as in typical fatty acid anabolism,[13] with chain elongation, carbonyl reduction and dehydration steps (Fig. 1). The final double-bond reduction of usual fatty acid anabolism would not occur, however, leaving the unsaturated conjugated system. Triene **1** contains one fewer carbon than the total in the constituent acyl units. The carboxyl carbon from the final or penultimate acyl unit is lost, but the earlier study[12] could not determine which. Thus, the origin of carbon 6 in **1** (Fig. 1) was uncertain.

The present study broadens the initial investigation, seeking experimental evidence that the pattern of acyl

Key words: Pheromone, biosynthesis, *Carpophilus* spp., NMR, mass spectrometry.

incorporation deduced for triene **1** extends generally to other trienes and tetraenes and to other *Carpophilus* spp. *C. davidsoni* Dobson and *C. mutilatus* were fed on diets containing acetate, propionate or butyrate labeled with ^{13}C or deuterium and the emitted pheromone components were analyzed. Initial attention was given to tetraenes **2** and **4** and triene **1** from *C. davidsoni*[8] and triene **3** from *C. mutilatus*[6] because their abundance allowed analysis of stable isotope labeling by NMR spectroscopy. Compound **4** was of particular interest because its structure suggested different penultimate and final acyl units (propionate and butyrate, respectively, Fig. 1); thus, **4** provided an experimental opportunity to determine which of the units loses its carboxyl carbon. With triene **3**, chain initiation with butyrate instead of acetate could be explored.

Analysis of labeled compounds was later extended to less abundant components using MS. Overall, biosynthetic information was obtained for a total of 15 trienes and tetraenes from *C. davidsoni* and *C. mutilatus*.

Results and Discussion

Pheromone production

The mean rate of hydrocarbon pheromone production for *C. davidsoni* ranged from 0.77 to 1.1 μg (total over all components) per male per day for the five different diets and for *C. mutilatus* the range was 0.19–0.55 μg per male per day. Males of *C. mutilatus* also emit tetradecanal,[6] and the mean rates for this aldehyde ranged from 0.12 to 0.44 μg per male per day. Total recoveries

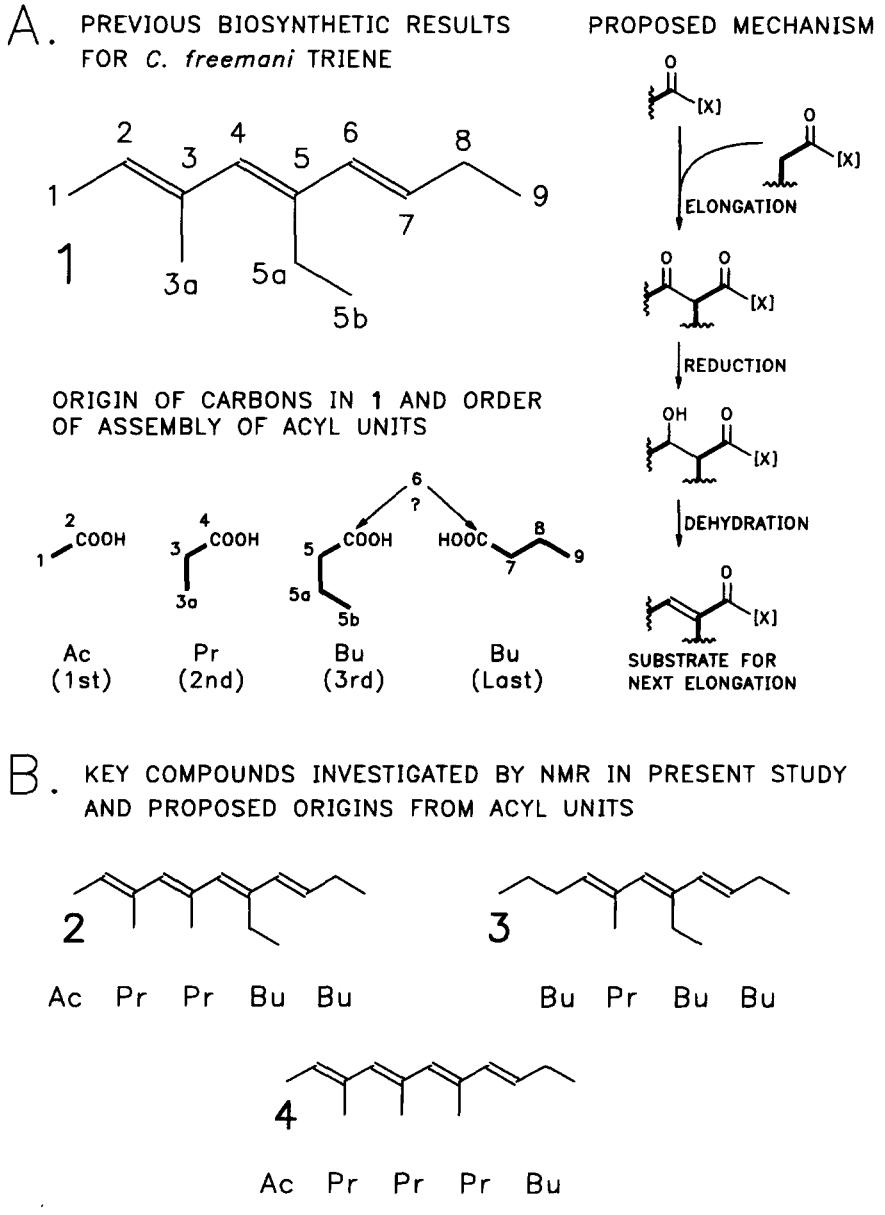


Figure 1. Summary of previous biosynthetic results for pheromone of *C. freemani* (a) and three compounds investigated by NMR in the present study and proposed origins from acyl units (b). Ac, Pr and Bu refer to acetate, propionate and butyrate, respectively; [X] is OH or an acyl carrier such as coenzyme-A or a carrier protein.

of individual hydrocarbons from the five diet types were 70–120 μg for **2**, 20–25 μg for **4** and 150–400 μg for **1** from *C. davidsoni* and 120–200 μg of **3** from *C. mutilatus*.

Levels of label incorporation

Incorporation of ^{13}C at each site capable of being labeled was 15–30% after feeding with (1-^{13}C)propionate and ca. 5–10% after feeding with (1-^{13}C)acetate or (1-^{13}C)butyrate. For deuterium, incorporation in **1** and **2** from *C. davidsoni* was 5.4% after feeding with (3,3,4,4,4-D_5)butyrate and 23% after feeding with (3,3,3-D_3)propionate. The respective values for **3** from *C. mutilatus* were 9.3 and 24%.

Labeling in tetraene 2

The experiment confirmed that the second and third acyl units of **2** are propionate (Fig. 2). From the NMR spectra, carbons 3a and 5a correspond to the methyl carbons of the propionates, and carbons 4 and 6, to the carboxyl carbons (labeled by deuterium and ^{13}C, respectively).

The results for labeled butyrate were more complicated (Fig. 2). With (3,3,4,4,4-D_5)butyrate in the diet, the proton NMR spectrum indicated incorporation of two butyrates, the ethyl branch and ethyl terminus of the chain being labeled. MS confirmed this result because M + 5 and M + 10 peaks were present (*m/z* 209 and 214). With (1-^{13}C)butyrate in the diet, only carbon 8 was strongly labeled, the carboxyl carbon of one of the incorporated butyrates being removed during biosynthesis (by analogy to **1**, Fig. 1). Furthermore, carbons 2, 7a and 10 were also labeled, but only 37–41% as strongly as 8 (calculated from relative peak heights in the natural abundance ^{13}C spectrum of **2**). With (1-^{13}C)acetate in the diet, exactly the same carbons were labeled as for (1-^{13}C)butyrate, but the degree of labeling was almost equal for all four.

These observations are consistent with the initial acyl unit of tetraene **2** being acetate and the final two units being butyrate, but acetate and butyrate can be readily interconverted prior to their incorporation (probably via typical pathways for fatty acid anabolism[13] and β-oxidation).[14] Anabolism of butyrate from acetate is not an obligatory part of pheromone biosynthesis (e.g. as a part of an enzyme-bound sequence of reactions). If it were, the intensity of the carbon signal at position 8 after feeding with (1-^{13}C)butyrate would be comparable to that for positions 2, 7a and 10, and the deuterium label would not exist at carbons 7a and 10 after feeding with (3,3,4,4,4-D_5)butyrate.

Metabolism of deuterium-labeled butyrate to acetate should produce (2,2,2-D_3)acetate, which could then

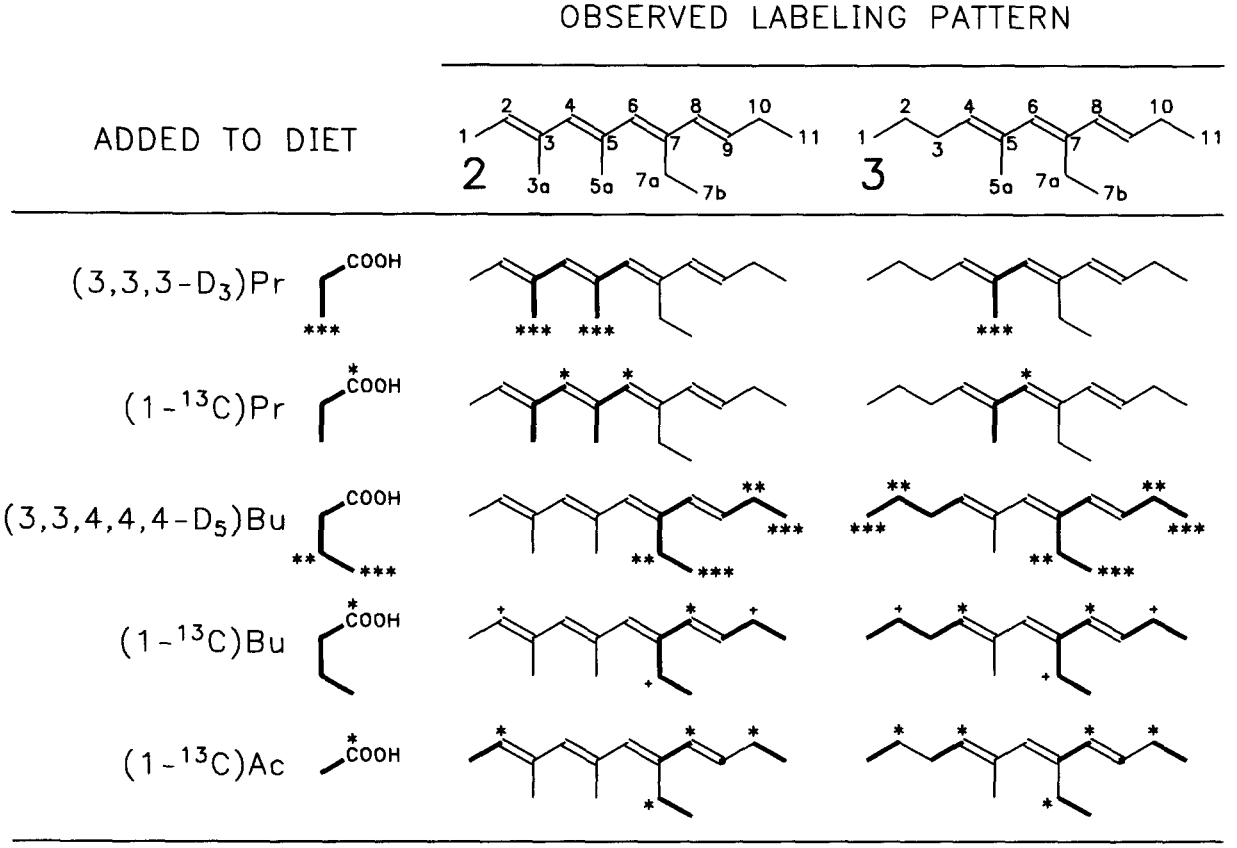


Figure 2. Summary of labeling results for **2** and **3** deduced from NMR spectra. Asterisks denote locations of ^{13}C or deuterium in precursors and analyzed hydrocarbons; lower degrees of labeling are denoted by +. Locations of precursors in **2** and **3** are denoted by bold lines (see text). Ac, Pr and Bu refer to acetate, propionate and butyrate, respectively.

label position 1 in **2**. However, no labeling at carbon 1 was detected by NMR or could be inferred from the MS. The methyl protons of acetate are rather labile biochemically and previous attempts to label an analogous pheromone with (2,2,2-D_3)acetate were unsuccessful.[12] Furthermore, the dilution of the labeled acetate in the acetate pool, as occurred for the ^{13}C-labeled acetate from (1-^{13}C)butyrate, would have futher suppressed its effect on the spectra.

Labeling in triene 3

By NMR, just one propionate unit was incorporated into **3** (Fig. 2), carbons 5a and 6 corresponding to the methyl and carboxyl carbons of propionate, respectively. After the beetles were fed with (3,3,4,4,4-D_5)butyrate, the proton spectrum of **3** indicated intact incorporation of three butyrate units, as indicated in Figure 2; MS supported this conclusion because peaks were observed at M + 5, M + 10 and M + 15 (*m/z* 197, 202 and 207). After the (1-^{13}C)butyrate feeding, only two carbons (4 and 8) were strongly labeled, but as with **1** and **2**, a third carboxyl carbon from butyrate would be eliminated during biosynthesis. Three additional carbons (2, 10 and 7a) were again weakly labeled (26–29% as strongly as carbons 4 or 8, relative to intensities for the natural abundance spectrum). With (1-^{13}C)acetate added to the diet, carbons 2, 4, 8, 10 and 7a were about equally labeled. These patterns are again consistent with the interconversion of acetate and butyrate. Overall, these data support that the first, third and fourth acyl units of **3** are butyrate and that the second is propionate.

Carbon 8 in **2** and **3** is depicted in Figure 2 as originating from the second last butyrate unit rather than from the last. Although the labeling studies on **2** and **3** provide no information on this point, data for **4**, **6**, **7**, **11**, **13** and **14**, presented below, are consistent with the loss of the carboxyl carbon from the final acyl unit. Figure 2 is drawn to reflect this result.

Labeling in triene 1

With (1-^{13}C)propionate in the diet, carbon 4 was labeled (Fig. 1). With (1-^{13}C)butyrate in the diet, carbon 6 was labeled heavily and carbons 2, 8 and 5a were labeled ca. 30% as strongly as carbon 6; this situation was analogous to tetraene **2**. Results for feedings of (1-^{13}C)acetate, (3,3,3-D_3)propionate and (3,3,4,4,4-D_5)butyrate were as reported for *C. freemani*;[12] therefore, *C. davidsoni* and *C. freemani* make triene **1** in the same way.

Labeling in tetraene 4

The 25 μg sample of tetraene **4** from the feeding with (1-^{13}C)propionate generated a useful ^{13}C spectrum after 128,000 scans and carbons 4, 6 and 8 were clearly labeled (Fig. 3). However, efforts to obtain carbon spectra with the samples for (1-^{13}C)acetate or (1-^{13}C)butyrate were unsuccessful, because of the lower degree of label incorporation. Proton NMR spectra were not obtained for the deuterated samples of **4**.

Nevertheless, MS from these samples did provide useful labeling information, particularly from the molecular ion (*m/z* 190 in unlabeled **4**) and from fragments at *m/z* 83 and 161 (Fig. 3). The interpretation of fragments was based on earlier research[5] and was corroborated by the well-defined NMR sample of **4** from the feeding with (1-^{13}C)propionate (Fig. 3). The sample from the feeding with (3,3,3-D_3)propionate gave evidence for three deuterated methyl groups. The locations of these were not unequivocally determined by the mass spectrum, but the known placements of the propionate carboxyl carbons and the results for **1**, **2** and **3**, strongly argue for labels being at carbons 3a, 5a and 7a (Fig. 3). The mass spectrum of **4** from the feeding with (3,3,4,4,4-D_5)butyrate established that the ethyl group composed of carbons 10 and 11 was labeled.

The mass spectra of **4** from feedings with (1-^{13}C)butyrate and (1-^{13}C)acetate were almost identical to each other, but interpretation was difficult because of low label incorporation (apparently ca. 4% at each of two carbons, after allowing for natural abundance ^{13}C). From consideration of results for **1**, **2** and **3**, carbons 2 and 10 should be labeled in both cases; the MS were consistent with this expectation, but rigorous confirmation was not possible. The spectral data did confirm that the second, third and fourth acyl units of **4** are propionate and that the fifth unit is butyrate.

Loss of carboxyl carbon from the final acyl unit of 4

Since the penultimate and final acyl units of **4** are different (unlike in **1**, **2** or **3**), labeling experiments could distinguish which of the carboxyl carbons is retained in the pheromone structure (i.e. as carbon 8 in **4**). Clearly, carbon 8 is derived from propionate and, therefore, the carboxyl carbon from the final acyl unit (butyrate) is removed during biosynthesis.

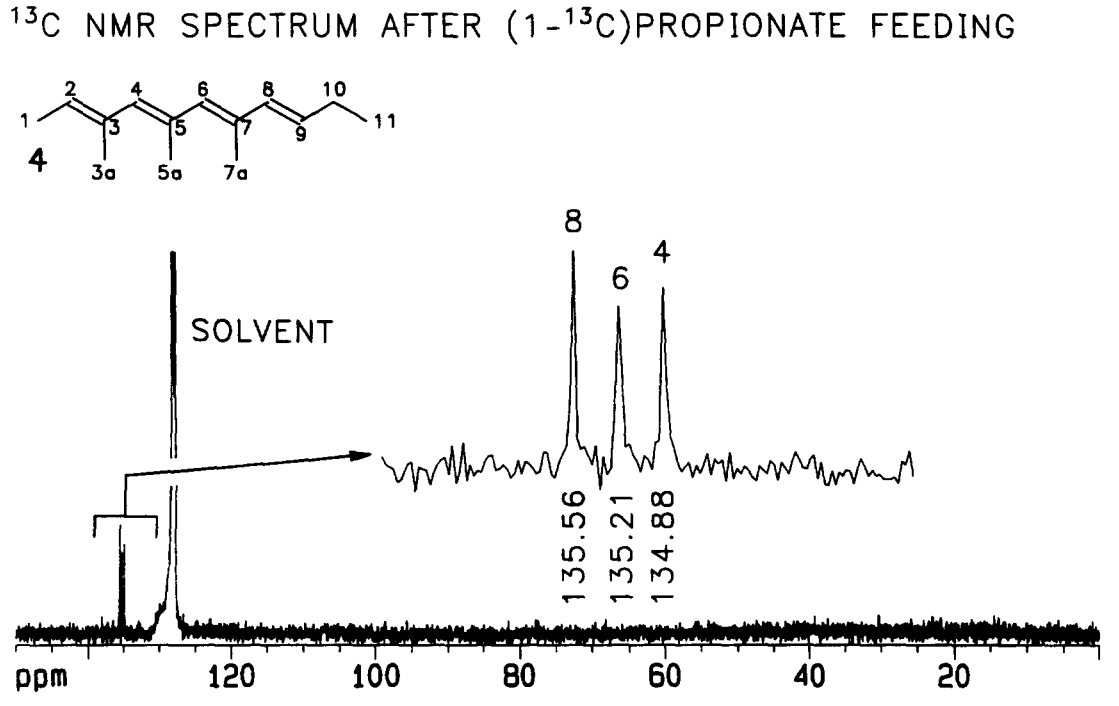
MS after feeding with (1-^{13}C)propionate

MS after (1-^{13}C)propionate feedings provided valuable labeling information about minor pheromone components. From the NMR samples, label incorporation was highest for this precursor (typically 30% per center) and there was no evidence of complicated labeling patterns (as occurred with butyrate and acetate due to precursor metabolism). Due to the high incorporation rate, the number of labeled centers was obvious from the molecular ion cluster of the MS; even with three or four labeled centers, the molecular ion of the fully labeled species was consistently above background noise (Fig. 4). (The small additional peak at the high-mass end of each cluster was due to natural abundance ^{13}C and was ignored.) The labeling information in these spectra was less specific than with NMR, but the

amount of sample required for analysis was about four orders of magnitude less.

Loss of carboxyl carbon from final acyl unit in triene biosynthesis

Structures of trienes **6** and **7**, previously known from *C. davidsoni*,[8] suggest both are derived from one acetate, one butyrate and two propionate units, but the orders of incorporation are different (Fig. 4). For **6**, the penultimate and final units are butyrate and propionate, respectively, but in **7**, these units are reversed. As with tetraene **4**, both trienes would allow experimental determination of which acyl unit loses its carboxyl carbon during biosynthesis. The mass spectrum of **6** after feeding with (3,3,3-D_3)propionate confirmed that two propionates were incorporated



MASS SPECTROMETRY OF **4**

KEY IONS (UNLABELED):

M$^+$ = 190 (41%)

A = 83 (28%)

B = 161 (48%)

PRECURSOR	ION	OBSERVED M/Z	INFERENCE	LABELING PATTERN
(1-^{13}C)Pr	M$^+$	190, 191, 192, 193	3 ^{13}C LABELS	
	A	83, 84	1 ^{13}C LABEL	
	B	161, 162, 163, 164	3 ^{13}C LABELS	
(3,3,3-D_3)Pr	M$^+$	190, 193, 196, 199	3 D_3-METHYLS	
	A	83, 86	1 D_3-METHYL	
	B	161, 164, 167, 170	3 D_3-METHYLS	
(3,3,4,4,4-D_5)Bu	M$^+$	190, 195	1 D_5-ETHYL	
	A	83, 88	1 D_5-ETHYL	
	B	161	NO LABEL	

Figure 3. Carbon spectrum of **4** after feeding with ^{13}C-labeled propionate (above) and MS data for **4** (below) that indicate positions of the label '*'. Bold lines in structures indicate locations of precursors. Bu and Pr refer to butyrate and propionate, respectively.

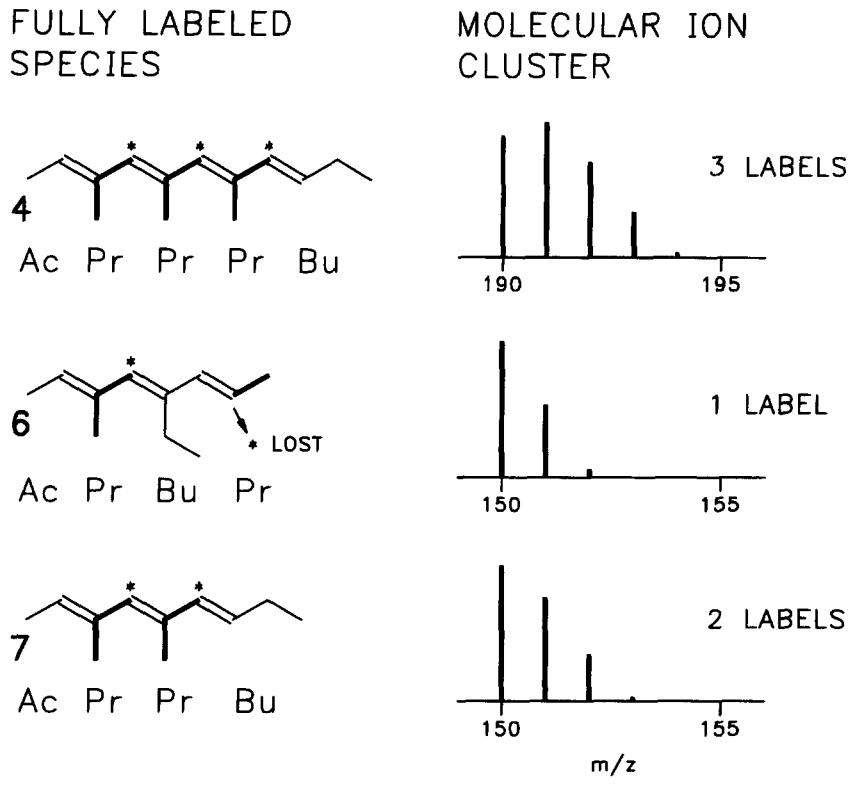


Figure 4. Molecular ion clusters from MS for three labeled compounds. The diet precursor was (1-^{13}C)propionate in each case and the number of incorporated labels is indicated at the right. Probable locations of the label and whole precursor in the compounds are indicated by '*' and bold lines, respectively. For **6**, the labeled (carboxyl) carbon of one propionate unit is lost during biosynthesis. Ac, Pr and Bu refer to acetate, propionate and butyrate, respectively.

(molecular ion peaks were *m/z* 150, 153 and 156). However, after feeding with (1-^{13}C)propionate, the MS indicated that only one labeled carbon was present (Fig. 4), the carboxyl carbon of the other propionate having been lost during biosynthesis. With **7**, on the other hand, both carboxyl carbons from propionate were incorporated (Fig. 4). Therefore, as in tetraene **4**, the final acyl unit loses its carboxyl carbon during the biosynthesis of the trienes **6** and **7**.

Other trienes and tetraenes

Triene **5** and tetraene **8** from *C. davidsoni*[8] had the expected number of labeled carbons after feeding with (1-^{13}C)propionate (two and three, respectively, Fig. 5). These both demonstrated that a carbon chain can be initiated with propionate (as well as with acetate or butyrate).

Analysis of samples labeled with (1-^{13}C)propionate led to discovery of other hydrocarbons not previously known from *C. davidsoni*. The characteristic molecular ion clusters allowed recognition of minor beetle-produced compounds among a complex of diet-related chemicals because only a living system could incorporate the label. For example, compound **10** was overlooked initially because it was assumed to be a diet-related terpene.

Labeling with (1-^{13}C)propionate also allowed unique carbon skeletons to be recognized among minor geometrical isomers of other labeled components. (The hydrocarbon samples from *Carpophilus* beetles usually contain small amounts of (*Z*) isomers of the all-(*E*) components. These are believed to be degradation products because some rotation about double bonds can occur on standing.)[5] Unlabeled tetraene **9** (Fig. 5) might have been dismissed as a (*Z*) isomer of **8**, based on the GC and HPLC retention times and MS. However, after the feeding with (1-^{13}C)propionate, it was clear that **9** was completely unrelated to **8**, because only one carbon was labeled rather than three.

Compounds **9–15** were identified from the MS and GC retentions, and identifications were verified by comparison with synthetic standards. In all cases, the number of incorporated labels after feeding with (1-^{13}C)propionate was as predicted from the structural patterns, after allowing for loss of one carboxyl carbon when propionate was the final acyl unit. Compounds **11**, **13** and **14**, having different penultimate and final acyl units, further supported the loss of the carboxyl carbon from the final unit. Compounds **12–15** were known from other *Carpophilus* spp.[5,7] Synthetic **11** was prepared earlier for a bioassay investigation,[4] but it was not known previously as a natural product. Compounds **9** and **10** are new.

Tetradecanal in *C. mutilatus*

Males of *C. mutilatus* emit tetradecanal in addition to hydrocarbon pheromone components.[6] The feeding

with (1-^{13}C)acetate demonstrated that the beetles can synthesize this aldehyde. In the chemical ionization spectrum of the beetle-derived aldehyde, the M + 58 peak (*m/z* 270) was 37% as large as the M + 57 peak (*m/z* 269); these peaks represented the isobutane adducts of the aldehyde with one ^{13}C present and with none, respectively. In the unlabeled standard, the M + 58 peak was only 19% as large as the M + 57 peak, a peak intensity consistent with naturally occurring ^{13}C. The ^{13}C label was clearly incorporated by the beetles, but analysis of label positions was beyond the scope of this study.

Generalizations

All 15 of the hydrocarbons identified from *C. davidsoni* and *C. mutilatus* can be assembled from just three

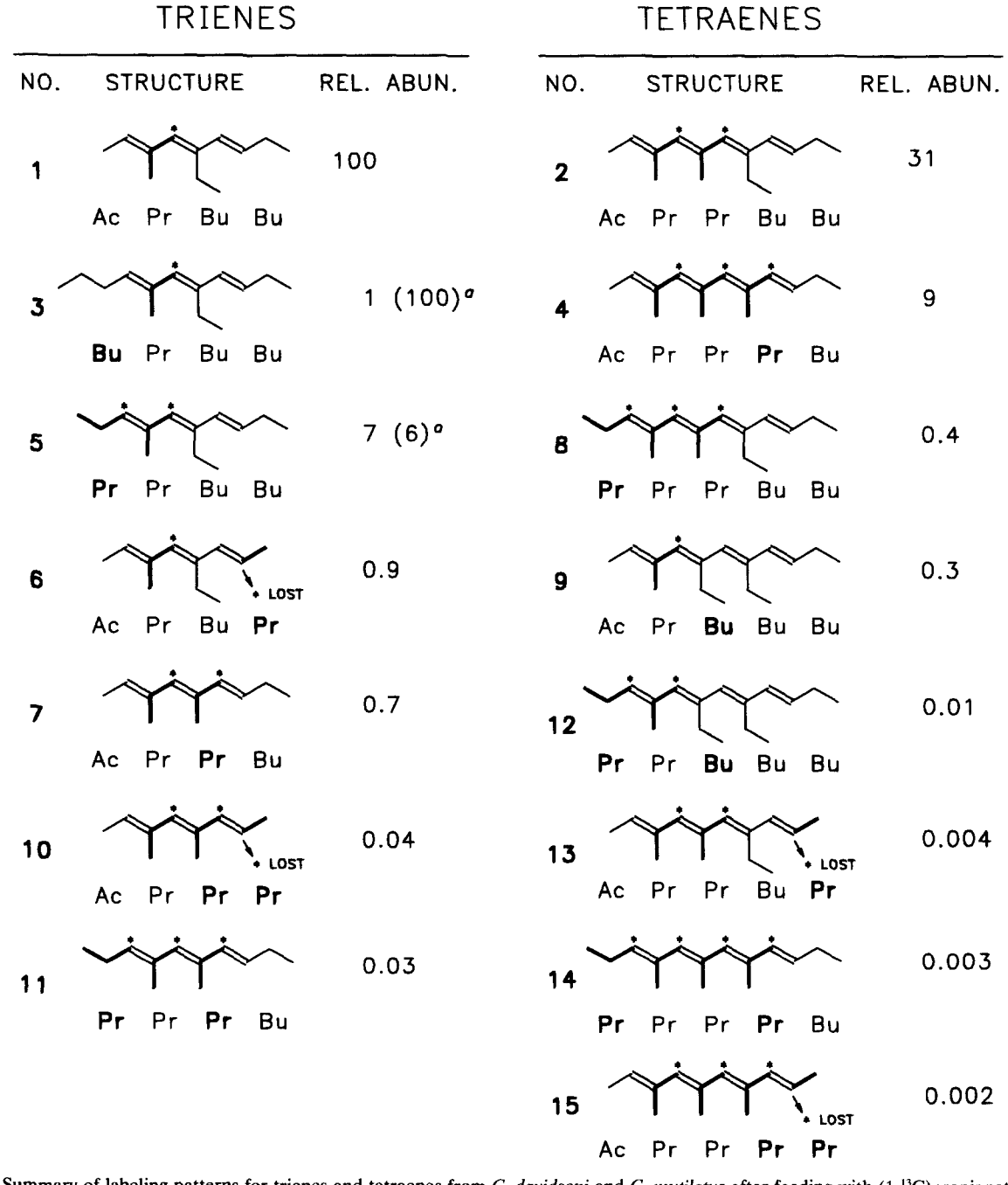


Figure 5. Summary of labeling patterns for trienes and tetraenes from *C. davidsoni* and *C. mutilatus* after feeding with (1-^{13}C)propionate and acyl units from which the compounds were derived. The number of asterisks above the structures is the number of labeled carbons actually determined by MS. For **1–4**, locations of labels '*' were determined by NMR; for **5–15**, locations were based on MS and by analogy to **1–4**. Bold lines indicate final location of propionate precursor. For some compounds, the labeled (carboxyl) carbon of one propionate unit is lost during biosynthesis. Ac, Pr and Bu, refer to acetate, propionate and butyrate, respectively; bold letters indicate deviations from the acyl precursors of major triene **1** or major tetraene **2**. Relative abundances of the compounds in *C. davidsoni* are shown at the right. [a]Only two of the compounds are found in *C. mutilatus* and their abundances are given in parentheses.

different acyl units. The first unit can be either acetate, propionate or butyrate and leads to a chain-initiating methyl, ethyl or propyl group, respectively, on the hydrocarbon. The second acyl unit is always propionate; it extends the chain by two carbons and accounts for a methyl branch. Subsequent units can be either propionate or butyrate; each adds two more carbons to the chain and either a methyl or ethyl branch, respectively. The final acyl unit is propionate or butyrate, resulting in a chain-terminating methyl or ethyl group. The final acyl unit loses its carboxyl carbon during biosynthesis. If the total number of incorporated acyl units is four, then the hydrocarbon is a triene; tetraenes contain five acyl units. These experiments support the earlier biosynthetic suppositions that were based solely on structural patterns.[4,5]

The locations of the double bonds are entirely consistent with the biosynthetic scheme proposed in Figure 1, but details of the anabolic reactions are still unknown. For example, typical elongation reactions usually involve malonates instead of mono-carboxyl acids, the former being more favorable energetically.[13] Biosynthesis of methyl-branched, saturated hydrocarbons in insects is known to involve methylmalonate.[15] The present study cannot determine whether elongation proceeds through malonate intermediates or not. In either case, the net elongation reaction would be as in Figure 1, because the carboxyl group added at carbon 2 of an acyl unit would subsequently be lost as CO_2 during the elongation reaction. Pheromone production has been localized to a specific tissue within the male abdomen[16,17] and more detailed investigation of biochemical reactions may now be possible.

Relative abundance of pheromone components in C. davidsoni

Trienes **3**, **5**, **6** and **7** differ from the major triene **1** by just one acyl unit (bold lettering in Fig. 5). It is likely that these less abundant trienes result from imperfect specificity in one set of biosynthetic enzymes rather than from five or more separate biosynthetic systems. The biosynthetic substitutions are relatively rare; for every 100 normal syntheses of **1**, there are only seven or fewer substitution events that lead to **3**, **5**, **6** or **7**. The occurrence of two replacements in one molecule is even rarer; trienes **10** and **11** both represent substitutions of two acyl units and are more than an order of magnitude less abundant than any triene with just one substitution. Trienes corresponding to three replacements (an even less likely event) were not detected at all. Similar patterns are seen with the tetraenes (Fig. 5).

Relative abundance of trienes **1**, **3** and **5** in *C. davidsoni* is consistent with the idea of imperfect specificity of one enzyme system. From the abundance of **1**, acetate is the 'preferred' initial unit. The greater abundance of **5** than **3** is probably due to propionate (which leads to **5**) being more similar to the preferred unit (in size and polarity, for example) than is butyrate (which leads to **3**). In *C. mutilatus* this pattern is reversed; butyrate is the 'preferred' first acyl unit and that with acetate (triene **1**) was not even detected. These biosynthetic systems have apparently been 'tuned' by natural selection to favor certain acyl units at particular steps and species specific pheromone systems have resulted.

Experimental

Source of beetles and rearing

The cultures of *C. davidsoni* and *C. mutilatus* originated from New South Wales, Australia, and were maintained on a wheat-germ/brewers-yeast diet.[8]

Diets with labeled precursors

Diets were prepared having 1% by weight of the following isotopically substituted compounds: (1-^{13}C)acetic acid, (1-^{13}C)propionic acid, sodium (1-^{13}C)butyrate, (3,3,3-D_3)propionic acid or (3,3,4,4,4-D_5)butyric acid (Cambridge Isotope Laboratories, Woburn, Massachusetts). These compounds were thoroughly mixed into hot diet with a Waring blender; 100 g of each diet was prepared.

Pheromone collection

Five males of *C. davidsoni* or five males of *C. mutilatus* were placed into 50 mL volatile collection flasks with ca. 10 g of labeled diet; the volatiles were captured on Super Q porous polymer (Alltech Associates, Deerfield, Illinois), as described previously.[12] For each beetle species, there were two flasks with each of the three ^{13}C-labeled diets, and volatiles were collected from these for 33 days. There were three flasks with the two deuterium-labeled diets for each species and collections were made for 35 days. Fresh diet was added about weekly, as that in the flasks began to dry out. Volatiles were rinsed from each collector with 500 μL hexane every 1–3 days. Nonadecane (2.5 μg) was added to each sample as a quantitative internal standard.

Chromatography

Each volatile collection was analyzed by GC to quantify pheromone production. The instrument was a Hewlett Packard 5890, equipped with a splitless injector, flame ionization detector, autosampler and Hewlett Packard 3396A integrator. The column was a 15 m Durabond DB-1 capillary (0.25 mm i.d., 1.0 μm film thickness) and the temperature program was 50–250 °C at 10 °C per min. The carrier gas was helium. Volatile collections were analyzed without purification or concentration and 1 μL (0.2% of the samples) gave good quantitation for the major pheromone components.

The volatile collections were combined for each species and labeled precursor, and the hydrocarbons were separated from more polar components by column chromatography on silica gel (1 × 5 cm column for each combined sample). Hydrocarbons were eluted with two column volumes of hexane; for *C. mutilatus*, a 5% ether:hexane fraction, which contained the male-specific compound, tetradecanal, was also retained.

The hydrocarbons were further separated by HPLC on a $AgNO_3$ silica column;[5] 0.5% 1-hexene in hexane was the solvent. Compounds **1**, **2** and **4** from *C. davidsoni* and **3** from *C. mutilatus* were isolated for NMR studies in this way. Synthetic standards for **1–4** were similarly purified and analyzed by NMR for comparison. Many of the HPLC fractions were later reanalyzed by GC–MS to gain labeling information about minor beetle-derived constituents.

Spectra

Proton and ^{13}C NMR spectra were recorded on a 400 MHz Bruker ARX-400 spectrometer. All samples were dissolved in C_6D_6. Chemical shifts are reported in ppm from tetramethylsilane with the residual proton resonance of C_6D_5H as the internal reference for protons (7.20 ppm) and the center ^{13}C resonance of C_6D_6 as the internal reference for ^{13}C (128 ppm). A 5 mm $^1H/^{13}C$ dual probe was used for both proton and carbon observation. Samples ($\leq$150 μL total volume) were contained in Wilmad 520-1 microtubes, which end in a 2.5 mm (o.d.) capillary.

EIMS of the hydrocarbons were obtained on a Hewlett Packard 5970 mass selective detector. Sample introduction was through a Hewlett Packard 5890 gas chromatograph fitted with a 15-m DB-1 capillary column. Chemical ionization (isobutane) mass spectra for tetradecanal samples were acquired on a Hewlett Packard 5988A mass spectrometer with GC inlet (the molecular ion in the electron impact spectrum of tetradecanal is almost undetectable and provides no useful information about label incorporation).

Assignment of carbon resonances

The ^{13}C NMR spectrum was obtained for **3** because the carbon resonances had not been determined previously (5-mg synthetic sample, unlabeled, 91% pure by GC). Subsequent DEPT,[18] HMQC[19] and HMBC[20] experiments on this sample established the numbers of protons attached to each carbon and the one-, two- and three-bond couplings between protons and carbons, thus allowing the carbon shifts to be assigned to particular carbons in **3**. During the course of these studies, it became evident that the original assignments[6] of protons at δ 2.09 and 2.10 were reversed.

Carbon assignments for **2** and **4** were reported previously,[21] but further data were taken to resolve certain issues. Compound **2** was reanalyzed to determine whether carbons 4 and 8 could be distinguished in the spectrum; both were originally reported at δ 134.0. The two carbons do produce separate spectral peaks and the HMQC heteronuclear correlation experiment determined that the resonances for carbons 4 and 8 were at δ 134.02 and 133.97, respectively. Also, the HMBC experiment was used to clarify the assignments for the quaternary carbons of **2** and **4** (positions 3, 5 and 7). The current carbon and proton assignments for **2** and **3** and the carbon assignments for **4** are summarized in Table 1. Assignments for triene **1** were presented earlier.[12]

Table 1. Assignments of ^{13}C and proton NMR shifts for three hydrocarbons

Position number[a]	Tetraene **2**		Triene **3**		Tetraene **4**
	^{13}C	^1H	^{13}C	^1H	^{13}C
1	13.89	1.65	13.99	0.94	13.89
2	124.94	5.50	23.19	1.41	124.82
3	133.93	—	30.68	2.09	133.93
3a	16.93	1.74	—	—	16.91
4	134.02	6.04	130.25	5.56	134.88
5	132.54	—	133.25	—	132.39
5a	19.02	1.98	17.03	1.79	19.34
6	134.53	5.96	133.63	5.98	135.21
7	139.96	—	139.36	—	133.65
7a	21.30	2.54	21.18	2.56	14.48
7b	14.91	1.18	14.91	1.21	—
8	133.97	6.07	133.89	6.12	135.56
9	130.24	5.73	130.00	5.74	130.48
10	26.56	2.11	26.46	2.10	26.49
11	14.25	1.02	14.19	1.02	14.29

[a]Positions are defined in Figures 2 and 3.

Interpretation of labeling in beetle-derived compounds

Incorporation of a ^{13}C label was evident in the ^{13}C NMR spectrum as a strongly enhanced signal; usually the labeled centers were the only detectable signals, because of small sample amounts. The level of ^{13}C incorporation was calculated from the molecular ion cluster of the MS after correcting for naturally occurring ^{13}C. Incorporation of deuterium was detected in the proton NMR spectrum as a significant reduction of signal integral, relative to the expected number of protons (in the absence of label, background variability of integrals was ±2.2% [SD]). The level of deuterium incorporation was calculated from the integrals of the proton spectra. The total number of deuterium labels incorporated was confirmed by MS. The NMR assignments in Table 1 were used to associate the spectral signals to particular locations in the compound structures. MS was the only source of labeling information for the less abundant trienes and tetraenes, as described in the results section.

Synthetic standards

Thirteen unlabeled synthetic standards were available from previous research:[4,5,7,21] (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene (**1**), (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene (**2**), (3*E*,5*E*,7*E*)-5-ethyl-

7-methyl-3,5,7-undecatriene (**3**), (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene (**4**), (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene (**5**), (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-octatriene (**6**), (2*E*,4*E*,6*E*)-3,5-dimethyl-2,4,6-nonatriene (**7**), (3*E*,5*E*,7*E*,9*E*)-8-ethyl-4,6-dimethyl-3,5,7,9-dodecatetraene (**8**), (3*E*,5*E*,7*E*)-4,6-dimethyl-3,5,7-decatriene (**11**), (3*E*,5*E*,7*E*,9*E*)-6,8-diethyl-4-methyl-3,5,7,9-dodecatetraene (**12**), (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-decatetraene (**13**), (3*E*,5*E*,7*E*,9*E*)-4,6,8-trimethyl-3,5,7,9-dodecatetraene (**14**) and (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (**15**). Two others were synthesized during this study: (2*E*,4*E*,6*E*,8*E*)-5,7-diethyl-3-methyl-2,4,6,8-undecatetraene (**9**) and (2*E*,4*E*,6*E*)-3,-5-dimethyl-2,4,6-octatriene (**10**).

Synthesis of 9 and 10

Tetraene **9** was prepared in the same way as **12**,[7] except that (*E*)-2-methyl-2-butenal was used as the starting material instead of (*E*)-2-methyl-2-pentenal. Triene **10** was prepared by a Wittig reaction with (ethyl)triphenylphosphonium bromide and (2*E*,4*E*)-2,4-dimethyl-2,4-hexadienal, an intermediate in the synthesis of **2** and other tetraenes.[21]

The following analytical data were obtained for **9**: Proton NMR (deuterobenzene): δ 1.02 (3H, t, $J = 7.4$ Hz), 1.09 (3H, t, $J = 7.5$ Hz), 1.22 (3H, t, $J = 7.5$ Hz), 1.64 (3H, d, $J = 6.9$ Hz), 1.76 (3H, br s), 2.11 (2H, apparent quin, $J \approx 7$ Hz), 2.39 (2H, q, $J = 7.5$ Hz), 2.60 (2H, q, $J = 7.5$ Hz), 5.59 (1H, br q, $J = 6.9$ Hz), 5.76 (1H, dt, $J = 15.7$, 6.6 Hz), 5.95 (1H, br s), 6.09 (1H, br s), 6.13 (1H, d, $J = 15.7$ Hz). Shift assignments are in Figure 6. MS [*m/z* (% of base)]: 218 (M^+, 32), 203 (8), 189 (85), 175 (11), 161 (22), 159 (9), 147 (100), 145 (21), 133 (62), 131 (17), 119 (94), 117 (19), 115 (21), 107 (38), 105 (52), 97 (9), 93 (20), 91 (63), 79 (35), 77 (39), 69 (17), 67 (15), 65 (18), 55 (72), 53 (21), 43 (9), 41 (49). GC retention index: 1549.

Triene **10** provided the following data: Proton NMR (deuterobenzene): δ 1.63 (3H, d, $J = 6.9$ Hz), 1.73 [3H, dd (partially hidden), $J = 6.6$, 1.4 Hz], 1.74 (3H, br s), 1.95 (3H, br s), 5.51 (1H, br q, $J = 6.9$ Hz), 5.62 (1H, dq, $J = 15.4$, 6.6 Hz), 5.97 (1H, br s), 6.23 (1H, d, $J = 15.4$). Shift assignments are in Figure 6. MS [*m/z* (% of base)]: 136 (M^+, 27), 121 (100), 119 (12), 106 (15), 105 (64), 103 (8), 93 (29), 91 (46), 79 (33), 77 (31), 67 (6), 65 (13), 63 (5), 55 (7), 53 (13), 51 (12), 43 (4), 41 (24). GC retention index: 1082.

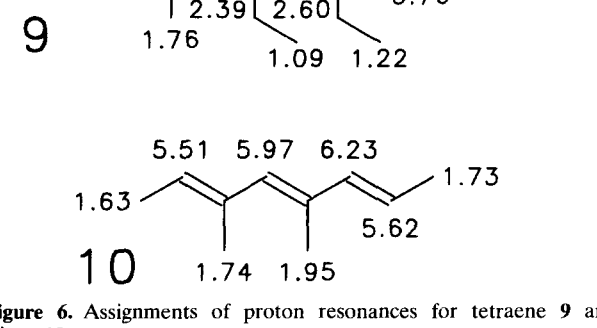


Figure 6. Assignments of proton resonances for tetraene **9** and triene **10**.

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