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Original Article

Neral (the Alarm Pheromone) Biosynthesis *via* the Mevalonate Pathway, Evidenced by D-Glucose-1-^{13}C Feeding in *Carpoglyphus lactis* and ^{13}C Incorporation into Other Opisthonotal Gland Exudates[1]

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The monoterpene neral [(*Z*)-3,7-dimethyl-2,6-octadienal], an alarm pheromone, has been identified as a major component of the opisthonotal gland exudates of *Carpoglyphus lactis,* together with tridecane and (*Z,Z*)-6,9-heptadecadiene. The $CDCl_3$ extract of mites fed D-glucose-1-^{13}C for 30 days was found to have ^{13}C atoms at positions 2, 4, 6 and 8–10 by ^{13}C-NMR analysis. The compound neral was, therefore, concluded to be produced *via* the mevalonate pathway from 2-^{13}C-acetyl-CoA by glycolysis. After seven days of feeding on D-glucose-1-^{13}C, at least one ^{13}C atom was incorporated in 51.6% of neral molecules. Likewise, 51.8% of tridecane, 42.5% of (*Z,Z*)-6,9-heptadecadiene, 39.5% of γ-acaridial and 33.4% of neryl formate, were also labeled, while squalene was not labeled, indicative of its origin, the culture medium.

Keywords: mevalonate pathway, Astigmata, biosynthesis of neral, D-glucose-1-^{13}C, *Carpoglyphus lactis,* alarm pheromone.

INTRODUCTION

A total of 26 monoterpenoids have been identified among 52 species of Astigmatid mites,[1-4] 9 of which function as either an alarm pheromone, a sex pheromone or an aggregation pheromone.[5-7] All of these compounds together with other hydrocarbons and aromatic compounds are derived from a pair of opisthonotal glands,[8] possibly biosynthesized in each species. There is, however, no evidence that they are biosynthesized by the mites themselves, and not derived from dietary sources.

Neral [(*Z*)-3,7-dimethyl-2,6-octadienal] is one of the most widely distributed monoterpenes with 32 species possessing the compound as a major or minor component.[1] Seven species; *Carpoglyphus lactis*,[9] *Suidasia medanensis*,[10] *Glyciphagus domesticus*,[11] *Lardoglyphus konoi*,[9] *Schwiebea elongata*,[6,12] *Histiogaster* sp.[7] and an un-identified *Oulentia* sp.,[13] use the compound as an alarm pheromone,[1,13] sex pheromone[7] and/or aggregation pheromone.[6]

Among neral emitters, *C. lactis* is a representative species. Neral functions as its alarm pheromone,[9] and is the only major monoterpene present in the gland, together with two major hydrocarbons [tridecane and (*Z,Z*)-6,9-heptadecadiene].[14] Furthermore, the species prefers a diet high in sugar, feeding on a mixture of D-glucose and dry yeast. It may be, therefore, the most suitable species with which to study neral biosynthesis. Recently, the biosynthesis of a sesquiterpene lactone "abscisic acid" has been clearly demonstrated to involve a non-mevalonate pathway in plants and a mevalonate pathway in fungi, by the application of D-glucose-1-^{13}C and subsequent ^{13}C-NMR analyses.[15] In the mevalonate pathway, isopentenyl pyrophosphate (3-methyl-3-buten-1-ol diphosphate) produced from D-glucose-1-^{13}C by way of glycolysis as a building block of terpenoid, is labeled at the carbons at positions 2, 4 and 5.[15,16] On the other hand, the same compound is labeled at positions 1 and 5 in the non-mevalonate pathway.[15,16] In order to obtain evidence that neral is a product of a head-to-tail condensation between two isopentenyl pyrophosphate molecules, D-glucose-1-^{13}C was fed to *C. lactis*. If the carbons at positions 2, 4, 6 and 8–10 are labeled, then neral is produced *via* the mevalonate pathway, while labeling at 1, 5, 9 and 10 indicates a non-mevalonate pathway of

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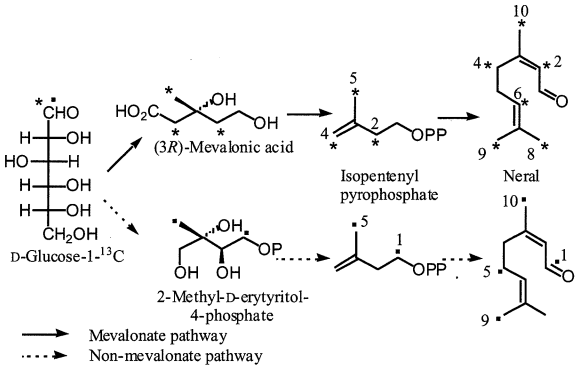

Fig. 1. ^{13}C Labeling pattern of neral produced by the mevalonate and non-mevalonate pathways from D-glucose-1-^{13}C. Modified from Ref. 15.

biosynthesis (Fig. 1).

In the present study, we demonstrated that the ^{13}C atom of D-glucose-1-^{13}C was successfully incorporated not only into neral (the alarm pheromone of the species), evidence of its biosynthesis, but also into four other components of the opisthonotal gland exudates. Labeled positions of ^{13}C in neral were successfully identified to confirm the involvement of the mevalonate pathway.

MATERIALS AND METHODS

1. *Mites and Preconditioning*

A stock culture of *C. lactis* has been maintained with a 1:1 mixture of dry yeast and sucrose at 25°C under *ca.* 70% RH in a glass container (10 cm in diameter, 7 cm in height) for more than 30 years in our laboratory. The culture used for the present study was conditioned by changing the sugar component from sucrose to D-glucose. No adverse effects were observed on changing the rearing medium.

2. *Instrumental Analyses*

Gas liquid chromatography coupled with mass spectrometry (GC/MS) was performed with a Hewlett Packard HP-5989B GC/MS operated at 70 eV in the split-less mode, using an HP-5 capillary column (0.25 mm × 30 m, 0.25 μm in film thickness, Hewlett Packard) at a temperature programmed to change from 60°C (2 min) to 290°C at 10°C/min and then held for 5 min. A Bruker AC500 FT-NMR spectrometer was used for measuring the ^{13}C-NMR spectrum at 125 MHz and ^{1}H-NMR spectrum at 500 MHz, in $CDCl_3$ using tetramethylsilane [$Si(CH_3)_4$] as the internal standard.

3. *Feeding Experiment (1)*

Mites (10 mg, all developmental stages) separated by a saline flotation method from the 1 : 1 mixture of D-glucose and dry yeast, were transferred to a 1 : 1 mixture (0.4 g) of D-glucose-1-^{13}C and dry yeast (^{13}C diet). The rate at which ^{13}C was incorporated was monitored by GC/MS at given intervals for 15 days. Ten mites were transferred to a conical-bottomed glass tube (8 mm in diameter × 30 mm in height, hand made) with a needle, and soaked for three minutes in 4 μl of hexane with a micro-syringe (10 μl, Hamilton Co.). The extract (3 μl) was subjected to GC/MS analysis.

4. *Feeding Experiment (2)*

To identify the positions in the neral molecule labeled by ^{13}C, the ^{13}C diet (0.6 g) was fed to mites (0.335 g), and an amount (0.4 g) was fed twice every 7 days for 30 days. After 30 days of feeding on the ^{13}C diet, and the monitoring of ^{13}C incorporation into components of the hexane extract at 29 days, mites (0.351 g) were separated from the medium by a saline flotation method[17] and treated with $CDCl_3$ (0.5 ml) for 3 min. The extract was, without being concentrated, subjected to carbon and proton nuclear magnetic resonance (^{13}C-NMR and ^{1}H-NMR) spectrometric analysis.

5. *Standards for ^{13}C-NMR Analysis*

A synthetic neral prepared from nerol[10] was subjected to ^{13}C- and ^{1}H-NMR as a standard. Chemical shifts of all carbons were assigned, and relative intensities were calculated by comparing each signal's height with that of the carbon at position 9. ^{13}C chemical shift values for (*Z,Z*)-6,9-heptadecadiene have been reported.[14] Those of tridecane were as follows; 14.12, 22.72, 29.41, 29.71, 29.75 and 31.97 ppm.

RESULTS

1. *^{13}C-NMR Analysis of the $CDCl_3$ Extract*

All signals corresponding to the 10 carbons detected in the standard neral (by the integration of 1024 scans) were obtained in the spectrum of the mite $CDCl_3$ extract after the integration of 16,384 scans, and are listed in Table 1. The relative intensities of carbons at 1, 3, 5 and 7 were extremely low (a difference of −69%, −80%, −87% and −81% from the relative intensity of the corresponding carbon in the standard), indicative of no ^{13}C enrichment. On the other hand, the signal corresponding to the carbon at 6

Table 1. ^{13}C-NMR spectrum of standard neral and the ^{13}C-fed mite extract

Carbon number	Chemical shift (ppm)	Relative intensity based on carbon 9		Difference (±%) based on neral
		Neral	Mite extract	
1	190.7	0.62	0.19	0.43 (−69%)
2	122.3	1.42	1.19	0.23 (−16%)
3	163.7	0.61	0.12	0.49 (−80%)
4	32.6	1.57	1.43	0.14 (−9%)
5	27.1	1.60	0.21	1.39 (−87%)
6	128.7	1.27	1.65*	0.38 (+30%)
7	133.7	0.74	0.14	0.60 (−81%)
8	25.6	1.43	1.59	0.16 (+11%)
9	17.7	1.00	1.00	0 (0%)
10	25.1	1.43	1.33	0.10 (−7%)

* Might be overlapped with other co-occurring signals.

appeared to be more intense than expected (+30% difference, based on the relative intensity of neral), possibly due to overlap from other sp^2 carbons in components of the $CDCl_3$ extract. Except for the carbon at 6 mentioned above, the difference in the relative intensity of carbons at 2, 4, 8 and 10 between the $CDCl_3$ extract and standard neral remained within a range of −16% to +11%. From the results summarized in Table 1, we concluded that the carbons at 2, 4, 6, 8, 9 and 10 were enriched with ^{13}C, while those at 1, 3, 5 and 7 were not.

The ^{13}C-NMR spectrum of the $CDCl_3$ extract also contained chemical shifts partly corresponding to tridecane and (*Z,Z*)-6,9-heptadecadiene, however, it was too complex to elucidate the distribution of ^{13}C in these hydrocarbons.

2. *Composition of the Mite Hexane Extract*

Four compounds were detected as major components of the extract by GLC (Fig. 2) after 29 days of ^{13}C feeding; tridecane (relative abundance, 43%), neral (34%), (*Z,Z*)-6,9-heptadecadiene (17%) and squalene (calculation excluded). The amount of neral was determined to be 2.13±0.51 ng (n=5) on average (body weight, *ca.* 40 μg/mite). The amount of squalene varied among the analyses, suggesting it originated from the yeast.[18] Pentadecane, γ-acaridial,[19] geranial, and neryl formate were observed as minor components with varying relative abundances (each less than 5%).

The incorporation of ^{13}C atoms into the following five compounds was successfully monitored by GC/MS for 7 days: neral, tridecane, (*Z,Z*)-6,9-heptadecadiene, γ-acaridial and neryl formate (Figs. 3–7). Squalene was not labeled in the present experiment (see Fig. 7). The other two components, geranial and pentadecane, were not recorded, due to low intensities (from 10 mites).

3. *Characteristics of GC/MS after the Incorporation of ^{13}C Atoms*

The incorporation into mite compounds of ^{13}C atoms from the ^{13}C diet was demonstrated by the appearance of multiple isotope ions in the molecular (M^+) ion region: not only were their increases of relative intensity but also, new isotope ions appeared. Three chromatograms of single ion monitor for neral (*m/z* 152, 153 and 154), four of tridecane (*m/z* 184, 185, 186 and 187) and three of (*Z,Z*)-6,9-heptadecadiene (*m/z* 238, 239 and 240) indicated the same retention times for each compound by GC/MS (data not indicated). No isotope effects, such as a shortening or retarding of retention times, were noted, and therefore, the relative intensity data for all isotope ions were obtained from the mass scan of each peak top in the reconstructed ion chromatogram.

4. *^{13}C-Incorporation into Neral*

The relative intensity (%) of the isotope ion (M^++1, at *m/z* 153) against the M^+ ion (at *m/z* 152) increased from 11.1%[20] (not detectable, possibly due to the small amount and less than the threshold level of the analytic system) to 64.5% after 4 days incubation. After 7 days, the value increased to 79.5% with the appearance of a new isotope ion (M^+ + 2 ion at *m/z* 154, 27.0%) (Fig. 3). Neral, after 7 day's feeding, was composed of a mixture of the unlabeled (48.4%), one ^{13}C incorporated (38.5%) and two ^{13}C incorporated (13.1%) molecules. At 29 days feeding in experiment (2)(data not shown in Fig.), up to 57.1% of neral was labeled. No isotope ions heavier than *m/z* 155 (M^+ + 3) were observed in feeding experiment (1) for 15 days, or feeding experiment (2) for 29 days.

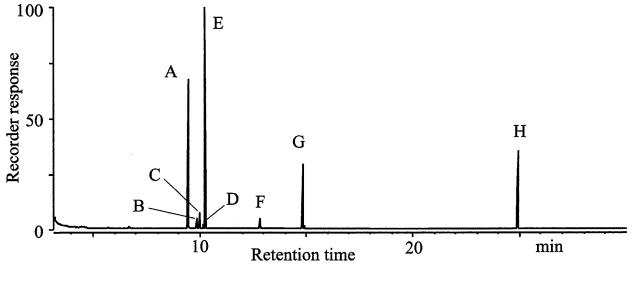

Fig. 2. A typical gas liquid chromatogram of the hexane extract from *C. lactis*. A: neral, B: geranial, C: neryl formate, D: γ-acaridial, E: tridecane, F: pentadecane, G: (*Z,Z*)-6,9-heptadecadiene, and H: squalene. Analytical conditions; see the text.

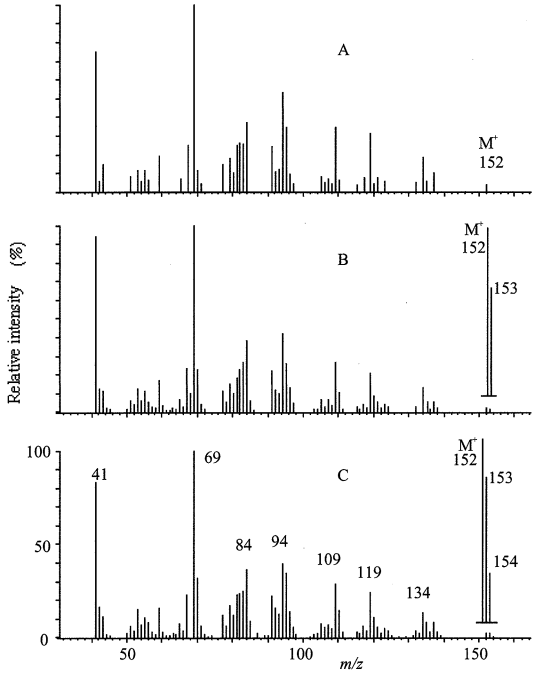

Fig. 3. Mass spectra of neral in mites fed the ^{13}C diet. A: control, B: after 4 days feeding, and C: after 7 days feeding. Molecular ion regions are enlarged.

5. ^{13}C-Incorporation into Tridecane

The isotope ion ($M^+ + 1$, at *m/z* 185) of the control gave a value of 14.5% (calcd. 14.5%, due to the natural abundance of $^{13}C/^{12}C = 1.08/100$).[20] The value increased to 39.0% after 4 days feeding with the appearance of a new isotope ion ($M^+ + 2$ at *m/z* 186, 13.5%), and then to 70.2% after 7 days feeding with two more isotope ions emerging [$M^+ + 2$ at *m/z* 186 (27.7%) and $M^+ + 3$ at *m/z* 187 (9.3%)](Fig. 4). After 7 days feeding, tridecane was composed of a mixture of non-labeled (48.2%), one ^{13}C-incorporated (33,9%), two ^{13}C-incorporated (13.4%), and three ^{13}C-incorporated (4.5%) molecules.

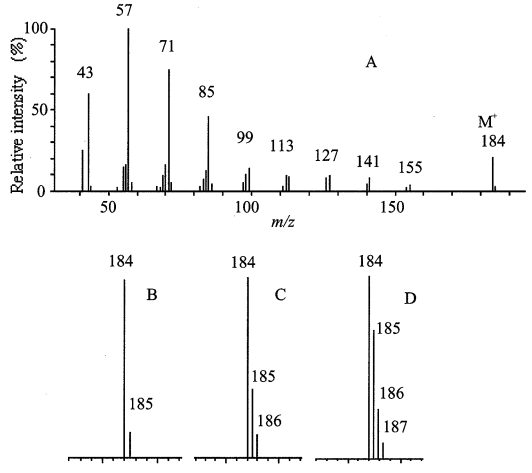

Fig. 4. Mass spectra of tridecane in mites fed the ^{13}C diet. A: control, B: one day, C: 4 days and D: 7 days.

6. ^{13}C-Incorporation into (Z,Z)-6,9-Heptadecadiene

The isotope ion ($M^+ + 1$ at *m/z* 237) increased from 18.8% for the standard to 31.3% after 4 days feeding, and then to 46.7% with appearance of a new isotope ion ($M^+ + 2$ ion at *m/z* 238, 27.2%) after 7 days feeding (Fig. 5). After 7 days feeding, the hydrocarbons consisted of a mixture of the unlabeled (57.5%), one ^{13}C-labeled (26.9%) and two ^{13}C-labeled (15.6%) forms. At 29 days feeding in experiment (2)(data not shown in Figs.), the mixture comprised unlabeled (45.7%) and labeled (54.3%) forms.

7. ^{13}C-Incorporation into γ-Acaridial

Although γ-acaridial was a minor component, a M^+ ion (at *m/z* 150) was clearly recognizable because of its intensity. In the present feeding experiment, the intensity of the isotope ion $M^+ + 1$ ion at *m/z* 151 increased from 7.0% of control to 26.1% after 4 days, and to 32.0% after 7 days feeding, and $M^+ + 2$ ion at *m/z* 152 from 1.7% of control, to 8.7% after 4 days, and to 9.8% after 7 days feeding (Fig. 6). After 7 day's feeding, 60.5% of molecules remained intact and 39.5% contained at least one ^{13}C atom.

8. ^{13}C-Incorporation into Neryl Formate

Although neryl formate was not detectable in the mites from the conditioned stock culture, it was detected as a minor component after 7 days feeding (Fig. 7). Because the M^+ ion is not available to the compound,[21] a fragment ion ($M^+ - m/z$ 46 ion at *m/z* 136) and its isotope ion ($M^+ - m/z$

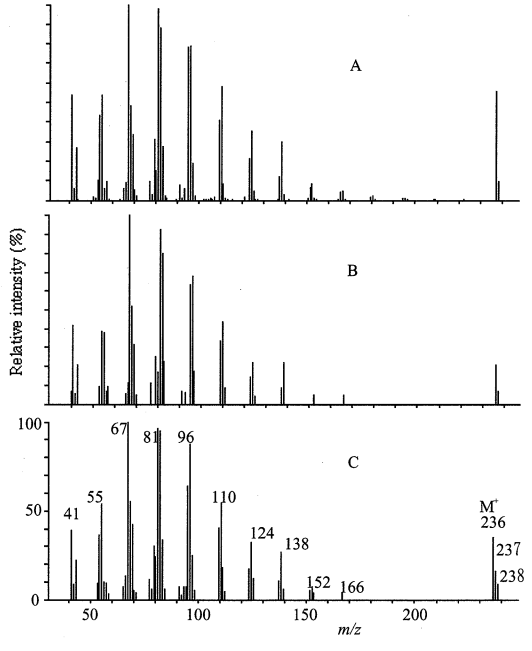

Fig. 5. Mass spectra of (Z,Z)-6,9-heptadecadiene in mites fed the ^{13}C diet. A: control, B: 4 days, and C: 7 days.

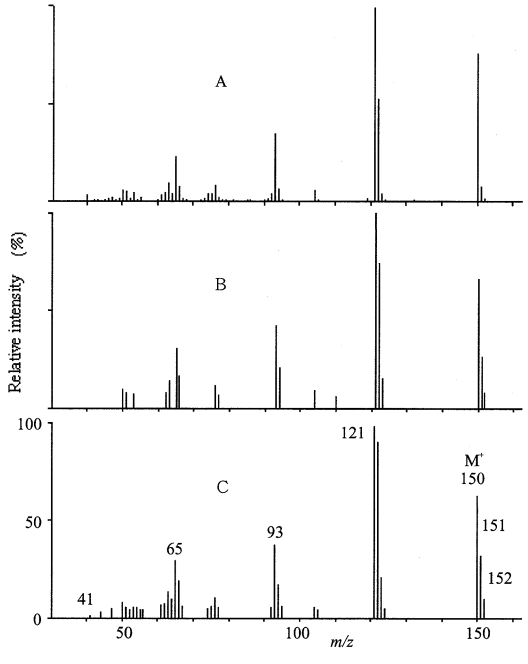

Fig. 6. Mass spectra of γ-acaridial in mites fed the ^{13}C diet. A: control, B: 4 days, and C: 7 days.

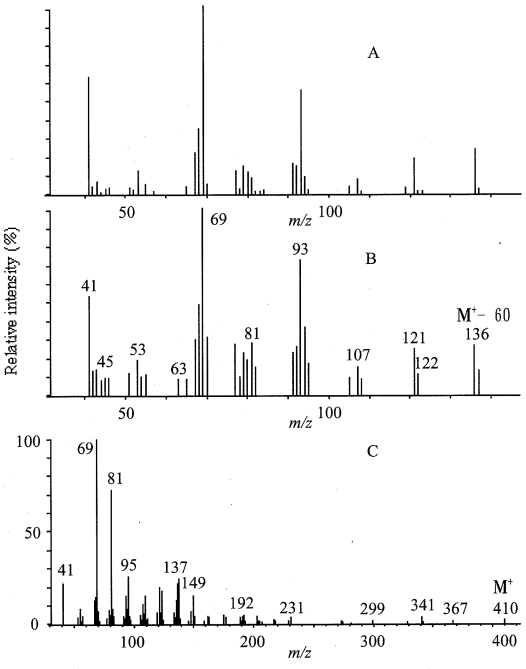

Fig. 7. Mass spectra of neryl formate (A and B), and of squalene (C). A: control, and B: neryl formate after 7 days of feeding on the ^{13}C diet. C: squalene after 7 days of feeding on the ^{13}C diet. No increase in the number of isotope ions was observed in the molecular ion region.

46 + 1 ion at *m/z* 137) were used for monitoring. The intensity of the isotope ion increased from 13.2% to 50.1% after 7 days feeding. This indicates that 33.4% of the molecules contain one ^{13}C.

DISCUSSION

The incorporation of ^{13}C into neral at positions 2, 4, 6 and 8–10 following ingestion of glucose-1-^{13}C supports production by the mevalonate pathway, and the present NMR analysis provided evidence. Neral, the alarm pheromone of *C. lactis*, was therefore demonstrated to be a product of the mevalonate pathway. However, the results do not support a non-mevalonate pathway for neral biosynthesis because the chemical signals at positions 1 and 5, among the four possible carbons at 1, 5, 9 and 10, were too low in intensity. ^{13}C incorporation at carbons 3 and 7 of neral is not theoretically possible with either pathway, and the intensities of these signals were actually un-enhanced (both signals had a low intensity).

The mevalonate pathway for the biosynthesis of isoprenoids is distributed in animals, fungi, plant cytoplasm, archaebacteria and some eubacteria, while the non-mevalonate pathway [mevalonate-independent methylerythritol phosphate (MEP) pathway] is found in bacteria and in the chloroplasts of all phototrophic organisms.[16] The present mevalonate pathway for neral biosynthesis is the first example among Astigmata, and is additional evidence of its presence among animals. As mentioned in the introduction, a total of 26 monoterpenes have been isolated from Astigmata, some of which are the active components of sex, aggregation or alarm pheromones. Furthermore, neral is distributed, either as a major or minor compound, in more than 61% of mite species examined. Those monoterpenes from Astigmata may also be derived from the mevalonate pathway.

The feeding experiment also indicated that 2-^{13}C-acetyl-CoA generated by glycolysis is exclusively mobilized for the biosynthesis of mevalonate by way of the mevalonate pathway. If the 2-^{13}C-acetyl-CoA molecule is incorporated into the tricarboxylic acid cycle, it is expected to emerge as a mixture of 1-^{13}C- and 2-^{13}C-acetyl-CoAs leading to uniformly labeled products.[15]

Acetyl-CoA is a building block not only of monoterpenes, but also of many other compounds in mites. The uptake of ^{13}C was demonstrated not only in neral, but also in tridecane, (*Z,Z*)-6,9-heptadecadiene, neryl formate and γ-acaridial by GC/MS analysis. Evidence of ^{13}C-incorporation into these compounds was also obtained in the ^{13}C-NMR analysis, and it might be interesting to examine the biosynthetic evidence of γ-acaridial. But it was actually impossible to elucidate the structure, using the spectrum from the present NMR analysis.

Tridecane and (*Z,Z*)-6,9-heptadecadiene are products of the decarboxylation of the corresponding fatty acids, tetradecanoic acid and (*Z,Z*)-9,12-octadecadienoic acid. In fact, the decarboxylation process has been proposed for the biosynthesis of two unusual hydrocarbons (*Z,Z*)-4,8-heptadecadiene and (*Z,Z,Z*)-4,8,11-heptadecatriene in an unidentified *Tortonia* species demonstrated to possess the corresponding (*Z,Z*)-5,9-octadecadienoic acid and (*Z,Z,Z*)-5,9,12-octadecatrienoic acid as fatty acid components.[22] The uptake of ^{13}C in two hydrocarbons in the present study, therefore, suggests the biosynthesis not only of tetradecanoic acid but also of (*Z,Z*)-9,12-octadecadienoic acid. Thus, ^{13}C labeled acetyl-CoA was utilized to produce not only tetradecanoic acid but also (*Z,Z*)-9,12-octadecadienoic acid which is an essential fatty acid for animals, possibly by the mites themselves or by symbiotic microbes in the mites.

As mentioned above, more than 50% of tridecane (51.8%) and neral (51.6%) were labeled during 7 day's feeding, and the uptake of ^{13}C in (*Z,Z*)-6,9-heptadecadiene (42.5%), neryl formate (33.4%) and γ-acaridial (39.5%), seemed to be slow. These incorporation rates seemed to parallel the relative abundance value for components of the extract. Because the mixture is stored in the gland reservoir, and discharged from its trap-door-structured orifice when needed,[23] these rates may also be comparable to rates of their biosynthesis.

It was noticed in each mass spectrum that the uptake of ^{13}C atom(s) resulted in all clusters of fragment ions being more complex than those of corresponding non-labeled forms, as indicated in Figs. 3 and 5–7. In the case of

squalene, which is suggested to be of dietary origin, no isotope ions were detected in the M^+ ion region and no complex was found among fragment ion clusters (Fig. 7). This clearly indicates that the ^{13}C atom of glucose-1-^{13}C is not incorporated into the squalene molecule. No biosynthesis takes place and the compound collected from the diet is simply sequestrated without being metabolized. Labeling experiments with D-glucose-1-^{13}C, therefore, seem to be a promising way to differentiate biosynthetic compounds from compounds sequestrated from diet.

REFERENCES

1) Y. Kuwahara: "Environmental Entomology; Behavior, Physiology and Chemical Ecology," ed. by T. Hidaka, Y. Matsumoto and K. Honda, University of Tokyo Press, Tokyo, pp. 380–393, 1999 (in Japanese).
2) H. Hiraoka, N. Mori, R. Nishida and Y. Kuwahara: *Biosci. Biotechnol. Biochem.* **65**, 2749–2754 (2001).
3) H. Tarui, N. Mori, K. Okabe and Y. Kuwahara: *Biosci. Biotechnol. Biochem.* **66**, 135–140 (2002).
4) N. Shimizu, H. Tarui, N. Mori and Y. Kuwahara: *Biosci. Biotechnol. Biochem.* **67**, 308–313 (2003).
5) Y. Kuwahara: "Biology of Ticks and Mites," ed. by J. Aoki, University of Tokyo Press, Tokyo, pp. 111–129, 2001 (in Japanese).
6) K. Nishimura, N. Shimizu, N. Mori and Y. Kuwahara: *Appl. Entomol. Zool.* **37**, 13–18 (2002).
7) H. Hiraoka, N. Mori, K. Okabe, R. Nishida and Y. Kuwahara: *J. Acarol. Soc. Jpn.* **11**, 17–26 (2002).
8) A. Mizoguchi, N. Mori, R. Nishida and Y. Kuwahara: *J. Chem. Ecol.* **29**, 1681–1690 (2003).
9) Y. Kuwahara, K. Matsumoto and Y. Wada: *Jpn. J. Sanit. Zool.* **31**, 73–80 (1980).
10) W. S. Leal, Y. Kuwahara, T. Suzuki and K. Kurosa: *Agric. Biol. Chem.* **53**, 2703–2709 (1989).
11) Y. Kuwahara, T. Koshii, M. Okamoto, K. Matsumoto and T. Suzuki: *Jpn. J. Sanit. Zool.* **42**, 29–32 (1991).
12) Y. Kuwahara, T. Ibi, Y. Nakatani, A. Ryouno, N. Mori, T. Sakata, K. Okabe, K. Tagami and K. Kurosa: *J. Acarol. Soc. Jpn.* **10**, 19–25 (2001).
13) N. Shimizu, N. Mori, R. Nishida and Y. Kuwahara: *J. Acarol. Soc. Jpn.* (2004) accepted.
14) Y. Kuwahara, W. S. Leal, K. Kurosa, M. Sato, S. Matsuyama and T. Suzuki: *J. Acarol. Soc. Jpn.* **1**, 95–104 (1992)
15) N. Hirai, R. Yoshida, Y. Todoroki and H. Ohigashi: *Biosci. Biotechnol. Biochem.* **64**, 1448–1458 (2000).
16) M. Rohmer: *Pure Appl. Chem.* **75**, 375–387 (2003).
17) K. Matsumoto: *Jpn. J. Sanit. Zool.* **16**, 86–89 (1965).
18) Y. Kuwahara, W. S. Leal, K. Akimoto, Y. Nakano and T. Suzuki: *Appl. Entomol. Zool.* **23**, 338–344 (1988).
19) T. Sakata and Y. Kuwahara: *Biosci. Biotechnol. Biochem.* **65**, 2315–2317 (2001).
20) R. M. Silverstein, G. C. Bassler and T. C. Morrill: "Spectroscopic Identification of Organic Compounds," Fourth Ed., John Wiley & Sons, Inc. (1981). Translated into Japanese by S. Araki, Y. Mashiko and O. Yamamoto, 416 pp., Tokyo-Kagaku-Dojin, Tokyo, 1983.
21) Y. Yonekawa, Y. Kuwahara and T. Suzuki: *Agric. Biol. Chem.* **51**, 3387–3389 (1987).
22) Y. Kuwahara, M. Samejima, T. Sakata, K. Kurosa, M. Sato, S. Matsuyama and T. Suzuki: *Appl. Entomol. Zool.* **30**, 433–441 (1995).
23) R. W. Howard, Y. Kuwahara, H. Suzuki and T. Suzuki: *Appl. Entomol. Zool.* **23**, 58–66 (1988).