Biosynthetic Studies of 4,8-Dimethyldecanal, the Aggregation Pheromone of the Red Flour Beetle, *Tribolium castaneum*

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A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
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LIST OF ABBREVIATIONS

Ac: Acetate

amu: Atom mass unit

bp: Boiling point

t-Bu: *tert*-Butyl

CI: Chemical ionization

CoA: Coenzyme A

mCPBA: *m*-Chloroperbenzoic acid

4,8-DMD: 4,8-Dimethyldecanal

DMF: *N,N*-Dimethylformamide

DMSO: Dimethylsulfoxide

EF: Enrichment factor

EI: Electron impact

Et: Ethyl

Ether: Diethyl ether

EtOH: Ethanol

FID: Flame ionization detector

GC-MS: Gas chromatography-mass spectrometer

2,4-HMPP: 2-Hydroxy-4-methoxypropiophenone

IR: Infrared spectrometer

LAH: Lithium aluminum hydride; $LiAlH_4$

LAD: Lithium aluminum deuteride; $LiAlD_4$

LDA: Lithium diisopropylamide

Me: Methyl

MeOH: Methanol

MDE: Male day equivalents

NMP: *N*-Methyl-2-pyrrolidinone (1-methyl-2-pyrrolidone)

NMR: Nuclear magnetic resonance

PCC: Pyridinium chlorochromate

PDC: Pyridinium dichromate

Pr: Propionate

Red-Al®: Sodium bis(2-methoxyethoxy)aluminum hydride; $NaAlH_2(OCH_2CH_2OCH_3)_2$

RFB: Red flour beetle (*Tribolium castaneum*)

SIM: Single ion monitoring

TEPA: Triethyl phosphonoacetate

THF: Tetrahydrofuran

THP: Tetrahydropyranyl

TIC: Total ion chromatogram

Ts: Toluenesulfonyl (Tosyl)

CHAPTER I

GENERAL INTRODUCTION

Pheromones are utilized by various organisms for intraspecific chemical communication and control of insect behaviors. These include sex pheromones attracting the opposite sex for mating, aggregation pheromones for aggregating both sexes to a specific site for feeding and/or mating, and trail pheromones for marking trail to feeding site and for homing. Moreover, in social insects, primer pheromones for regulating colony, alarm pheromones for escaping from the risk and/or keeping the colony, and nest-mate recognition pheromones are also known. Since the pheromone component of silkworm moth has been identified (Butenandt et al., 1959), identification of insect pheromones was focused on economically important pests, such as moths, beetles, flies and cockroaches. Currently, the studies on insect pheromones are expanding toward their biosynthetic pathways.

In Lepidoptera, one of the largest order, moth sex pheromones show rather simplicity of structures with the following characteristics: 1) chain length with 10, 12, 14, 16 or 18 even carbon atoms in a straight chain, 2) a primary alcohol, acetate, or aldehyde as a functional group, and 3) one, two or three double bonds at various positions with *Z* or *E* configuration. Most of these moth sex pheromones are biosynthesized in the following: 1) *de novo* biosynthesized fatty acids, in the form of stearoyl CoA (C18-CoA), and palmitoyl CoA (C16-CoA), are served as the precursors, 2) C18-CoA and C16-CoA are desaturated by delta-11 desaturase for double bond formation, 3) thus produced unsaturated fatty acids are undergone chain shortening reaction and then reduced to alcohols of the corresponding pheromones, and 4) the alcohol precursors are used as components of pheromones or converted into the corresponding acetate esters or aldehydes (reviewed by Tillman et al., 1999; Jurenka, 2004).

Unlike lepidopteran pheromones, coleopteran pheromones and their biosynthetic pathway are so complicated. About 350,000 species of the beetles, Coleoptera, have been described and divided into 166 families (Seybold and Vanderwel, 2003). This tremendous biological diversity of the Coleoptera is reflected on the structural diversity of coleopteran pheromones and their origins (reviewed by Vanderwel and Oehlschlager, 1987; Seybold and Vanderwel, 2003). The structures of coleopteran pheromones are composed of acyclic unbranched and/or branched chains with various functional groups, cyclic unbranched and/or branched chains, such as lactone, bicyclic oxygen heterocycles, amino acid derivatives and aromatic compounds (Vanderwel and Oehlschlager, 1987; Tillman et al., 1999). The origins and biosynthetic pathway of these pheromones are demonstrated to be the fatty acid pathway, mevalonate pathway, polyketide origin, or amino acid origin (Tillman et al., 1999). However, biosynthetic pathway of most of the identified pheromones are still unknown.

In insect species, ratio of pheromone components and their stereo-configuration are highly regulated. Therefore, understanding of the biosynthetic pathway and regulatory factors of pheromone production are expected to provide biotechnological approach for the synthesis of pheromone components of high chemical and enantiomeric purity for application in pest management programs (Seybold and Vanderwel, 2003).

The red flour beetle (RFB), *Tribolium castaneum* (Coleoptera: Tenebrionidae) is one of well-known cosmopolitan storage pests infesting almost all of flours and meals. This species (ca. 3 mm in length) have been used for researches on biology, genetics, and physiology as a model insect and also as a test insect against insecticides.

Male RFB produces an aggregation pheromone attracting both sexes, and it was

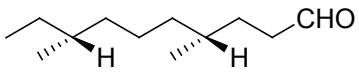
identified as 4,8-dimehyldecanal (4,8-DMD; Fig. 1-1) (Suzuki and Sugawara, 1979; Suzuki 1980). The absolute configuration of the natural aggregation pheromone was determined as (4*R*,8*R*)-(-) by bioassay experiments using synthetic optical isomers (Suzuki and Mori, 1983; Suzuki et al., 1984). Subsequent studies revealed that 4,8-DMD was a common male-produced aggregation pheromone among *T. castaneum*, *T. confusum*, and *T. freemani* (Suzuki et al., 1987). The pheromone-producing gland in RFB is still equivocal, although some of studies have been made. Faustini et al. (1981) reported the prothoracic femoral setiferous glands as pheromone-producing glands and the gland secretions isolated from adult male RFBs attracted both sexes. However, Qazi et al. (1998) doubted of the results of Faustini et al. and demonstrated that the glands were clearly not the major sites of the pheromone production, because male RFBs removed first legs continually produced normal quantities of the pheromone.

In tenebrionid species, pheromones are reflected on the diversity of their structures. 4-Methyl-1-nonanol was identified as the female-produced sex pheromone of *Tenebrio molitor* (Fig. 1-1) (Tanaka et al., 1986 and 1989). The male-produced aggregation pheromones of *Gnatocerus cornutus* were composed of (+)-acoradiene and α-cedrene-14-al (Fig. 1-1) (Tebayashi et al., 1998a, b; Kurosawa et al., 2001; Tashiro et al., 2004). The study of biosynthetic pathway of tenebrionid pheromones was conducted only in *T. molitor* until now. Islam et al. (1999) showed that 4-methyl-1-nonanol was biosynthesized through the fatty acid pathway using stable isotope-labeled precursors. In contrast, although there has not been reported, the pheromones of *G. cornutus* are sesquiterpenes themselves.

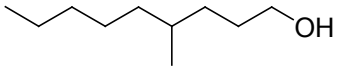
Since 4,8-DMD has been identified, it has been considered to be biosynthesized

through the mevalonate pathway, because it has a partial terpene skeleton and is easily obtainable by oxidative cleavage of the isopentenyl group of a sesquiterpene farnesol derivative (Vanderwel and Oehlschlager, 1987; Howse et al., 1998), but no paper concerning with biosynthesis of *Tribolium* pheromones has been reported until today. There is also another possibility that 4,8-DMD would be biosynthesized via the fatty acid pathway, as in the case of 4-methyl-1-nonanol.

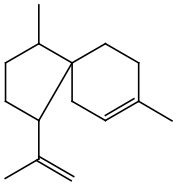
This thesis presents studies to elucidate whether 4,8-DMD would be biosynthesized via the mevalonate or fatty acid pathway by: a) examination of the mevalonate pathway as a biosynthetic pathway of 4,8-DMD (Chapter II), b) investigating effects of juvenile hormone (JH) III and pathway inhibitors, which block the mevalonate or fatty acid pathway, on the production of 4,8-DMD to presume a possible biosynthetic pathway (Chapter III), c) examination of the fatty acid pathway as a biosynthetic pathway of 4,8-DMD (Chapter IV), using isotope-labeled putative precursors (Chapter V).



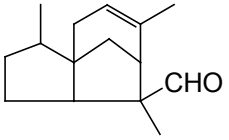
(4*R*,8*R*)-(-)-4,8-Dimethyldecanal



4-Methyl-1-nonanol



Acoradiene



α-Cedrene-14-al

Fig. 1-1. Structures of tenebrionid pheromones.
4,8-Dimethyldecanal: the aggregation pheromone of *Tribolium* spp.; 4-Methyl-1-nonanol: the sex pheromone of *Tenebrio molitor*; Acoradiene and α-cedrene-14-al: the major and minor aggregation pheromones of *Gnatocerus cornutus*, respectively.

CHAPTER II

EXAMINATION OF THE MEVALONATE PATHWAY AS A BIOSYNTHETIC PATHWAY OF 4,8-DIMETHYLDECANAL

2.1 INTRODUCTION

Large parts of the insect pheromones are classified into terpenes, which are biosynthesized through the terpene pathway. The pathway is divided into two pathways, the mevalonate pathway and the methyl-erythritol-phosphate (MEP) pathway (reviewed by Rohmer, 2003) (Fig. 2-1). Mevalonate, converted from 3-hydroxy-3-methylglutaryl (HMG) CoA, is a key intermediate of the former pathway, while the latter pathway recently discovered is mevalonate independent pathway. MEP is the source of isopentenyl diphosphate like mevalonate. In insects, terpene pheromones are biosynthesized through the mevalonate pathway (Seybold and Vanderwel, 2003), while in plants, terpenes are biosynthesized via the mevalonate pathway or both pathways. Until now, there is no report that the MEP pathway is involved in biosynthesis of insect terpene pheromones (Seybold and Vanderwel, 2003).

Although biosynthetic pathways of insect terpene pheromones have not been extensively reported, they could be biosynthesized by modification of host compounds or by *de novo* biosynthesis (Vanderwel and Oehlschlager, 1987; Tillman et al., 1999). Some of insect terpene pheromones are biosynthesized via oxidation of allyl position and oxidative cleavage of a double bond (Fig. 2-2). An aggregation pheromone (Fig. 2-2) of *Cryptolestes ferrugineus*, has the same skeleton like 4,8-DMD, and it was elucidated to be biosynthesized by oxidative cleavage of the double bond in the terminal isopentenyl group of farnesol, followed by lactonization (Vanderwel et al., 1992) (Fig. 2-2-C).

Based on these facts, biosynthetic pathway of 4,8-DMD through the mevalonate pathway is hypothesized as outlined in Fig. 2-3: (1) the isopentenoid is biosynthesized through the mevalonate pathway and (2) allylic oxidation of the terminal methyl group in

geraniol followed by chain elongation with one unit of acetate leads to 4,8-DMD or (3) oxidative cleavage of the terminal double bond of farnesol and/or its derivatives give 4,8-DMD.

In order to examine whether 4,8-DMD is biosynthesized through one of the hypothesized pathways, [2-^{13}C]mevalonolactone, deuterium-labeled terpene precursors (geraniol, linalool and farnesol), and terpene derivatives (tetrahydrofarnesol, hexahydrofarnesene, and 10-hydroxy-4,8-dimethyldecanoic acid) were employed for incorporation experiments.

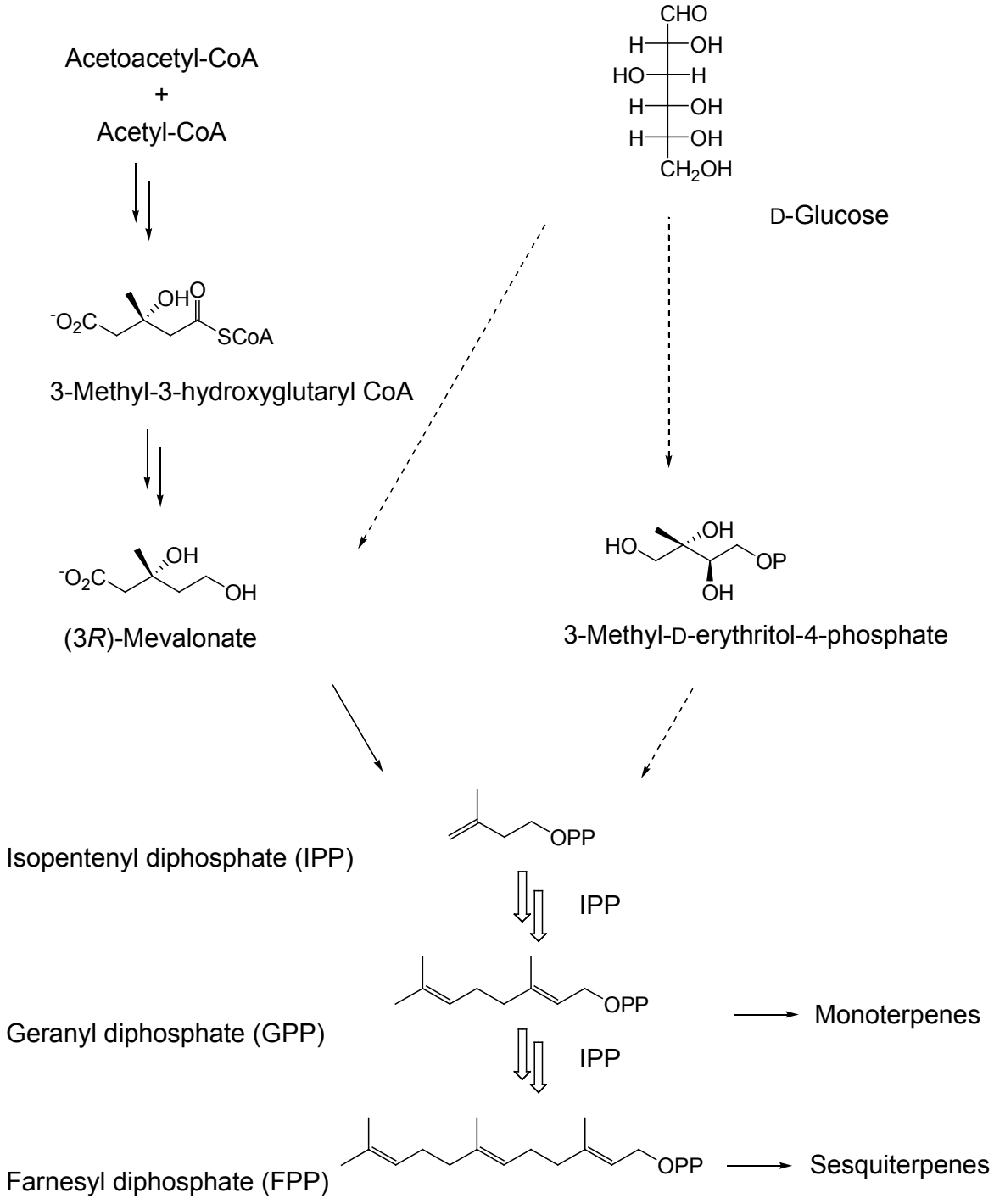
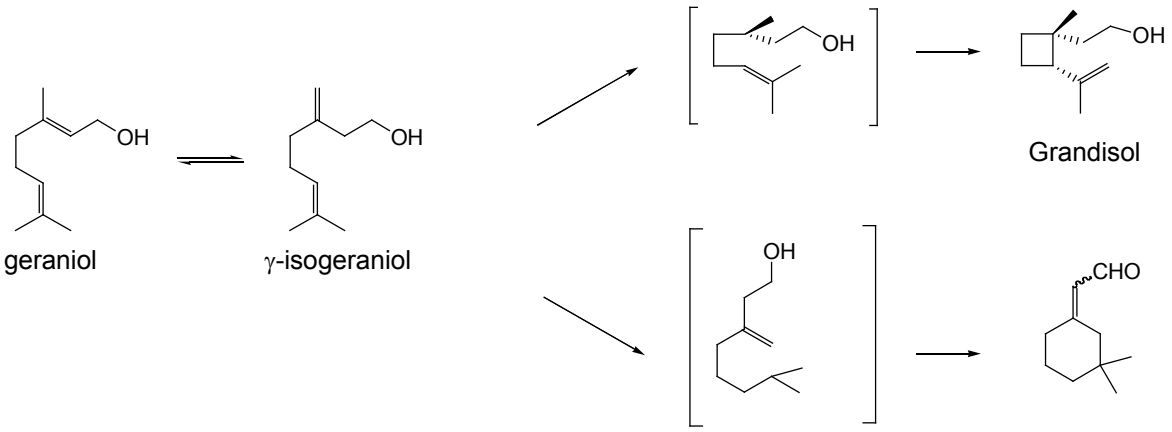
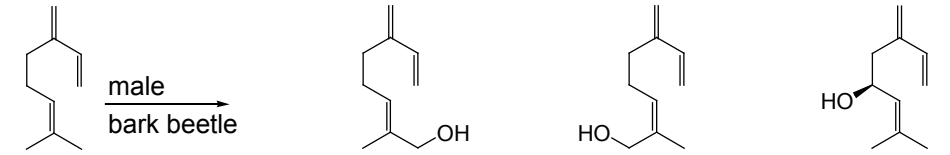


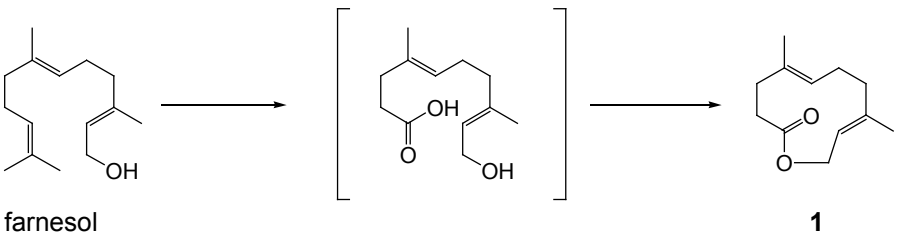
Fig. 2-1. Terpene biosynthesis through the mevalonate and/or methyl-erythritol-phosphate (MEP) pathways. Solid arrow: mevalonate pathway; broken arrow: MEP pathway; hallow arrow: the established terpene pathway (modified from Morita et al., 2004).



A: Pheromones of the male boll weevil and their biosynthetic pathway



B: Allylic oxidation of myrcene in *Dendroctonus* spp. and an aggregation pheromone (Ipsdienol)



C: An aggregation pheromone (**1**) of *C. ferrugineous* and its biosynthetic pathway

Fig. 2-2. Coleopteran terpene pheromones and their biosynthetic pathways.

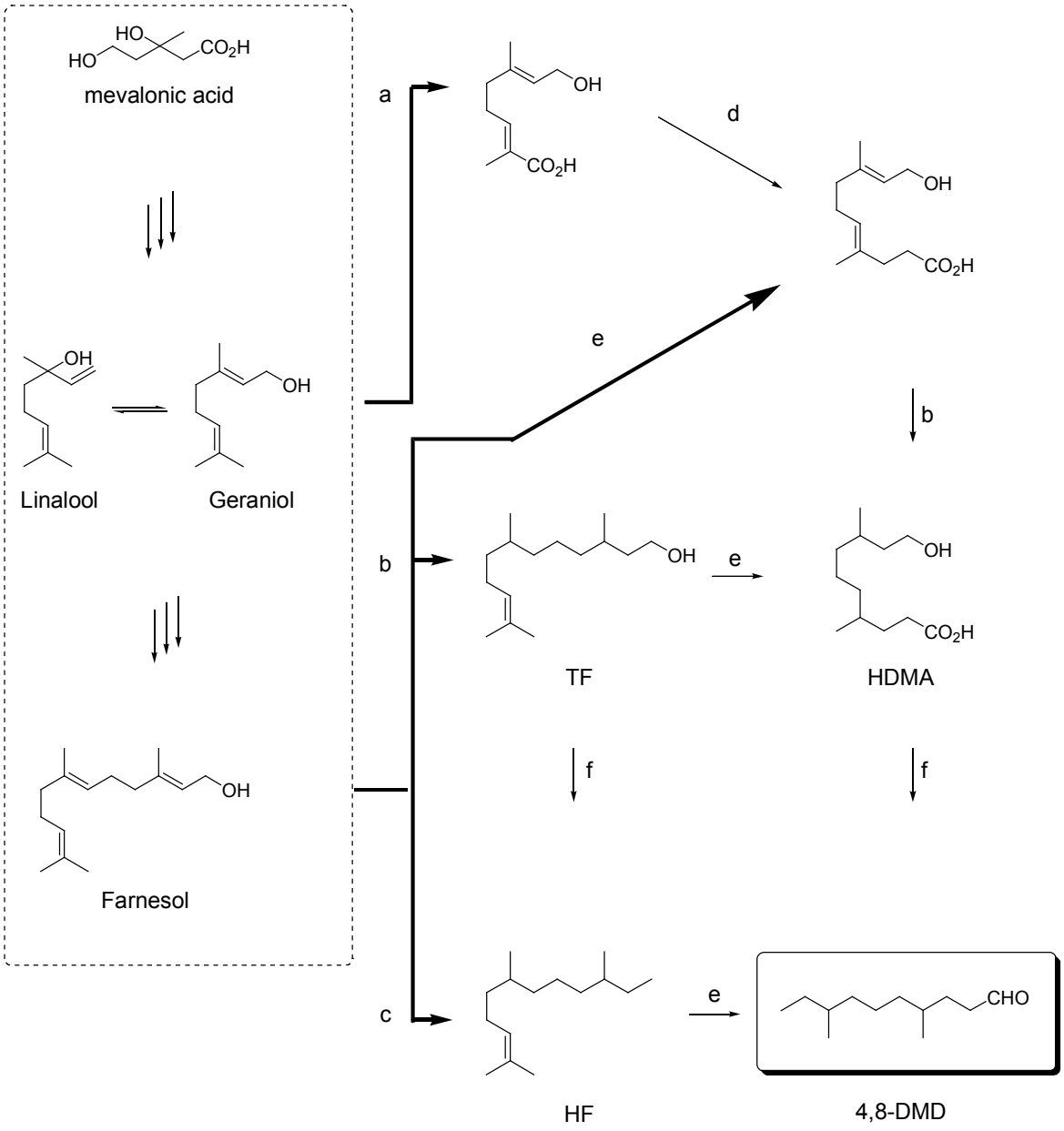


Fig. 2-3. Hypothesized mevalonate pathways leading to 4,8-DMD.

a) Allylic oxidation, b) hydrogenation, c) hydrogenation and dehydration, d) chain elongation, e) oxidative cleavage of a double bond, f) dehydration.

TF: tetrahydrofarnesol; HF: hexahydrofarnesene; HDMA: 10-hydroxy-4,8-dimethyldecanoic acid; 4,8-DMD: 4,8-dimethyldecanal.

The pathway in broken line box represents the established mevalonate pathway.

2.2 Materials and Methods

2.2.1 Insect cultures

A laboratory colony of *T. castaneum*, established over 20 years, was used in all experiments. The beetles were reared on a mixture of whole wheat flour and 5% brewer's yeast at 27 ± 1°C, ca. 70% RH on a 16:8 L:D cycle. Pupae were sexed by the genital organ and reared in separate containers for 4 days with flour and the adults were used in [2-^{13}C]mevalonolactone incorporation experiment. In other incorporation experiments, 4-day-old beetles were starved for 48 h prior to the experiments (thus, RFBs were 6-day-old at the beginning of the experiments) to enhance the incorporation of exogenously added substrates (Vanderwel et al., 1990). In preliminary experiments, aeration of both sexes produced greater levels of 4,8-DMD in males only. Thus, adults of both sexes were used in all aeration.

2.2.2 Chemicals and materials

The following compounds were synthesized as described in Chapter V and used as putative precursors; [2-^{13}C] mevalonolactone (**46**), geraniol-d_5 (GLD5; **52**), linalool-d_5 (LLD5; **50**), farnesol-d_5 (FLD5; **57**), tetrahydrofarnesol-d_5 (TFD5; **63**), hexahydrofarnesene-d_2 (HFD2; **31**), and 10-hydroxy-4,8-dimethyldecanoic acid-d_4 (HDMA-D4; **71**), and the corresponding non-isotope labeled compounds (Numbers in bold represent the compound number in Chapter V).

2.2.3 Preparation of flours coated with labeled precursors

Incorporation of labeled precursors into the 4,8-DMD was evaluated by allowing

the RFBs to feed flours coated with the precursors.

Preparation of [2-^{13}C]mevalonolactone-coated flour: A solution of [2-^{13}C]mevalonolactone (500 mg, 5% by weight to medium) in 1 mL of distilled water was diluted with 20 mL of ethanol (99.5%, Wako), to which whole wheat flour (10 g) was added. The solvent was evaporated with heating. The lump of flour was crushed with a mortar and pestle and then sifted with a tea sieve. The solvent was evaporated for a further 30 min. Similarly, flour coated with unlabeled mevalonolactone was prepared.

Preparation of flour coated with deuterium-labeled precursor: Whole wheat flour were defatted by Soxhlet extraction with ether for 45 min. The labeled compounds (100 mg, 1% by weight to medium) were dissolved in 20 mL of ether in a round bottom flask (100 mL), to which defatted flour (10 g) were added and the solvent was evaporated *in vacuo*. Similarly, flour coated with unlabeled precursor was prepared.

2.2.4 Collection and elution of insect volatiles

Figs. 2-4 and 2-5 show the aeration apparatus for collecting insect volatiles. The collected insect volatiles were treated as outlined in Figs. 2-6 and 2-7.

[2-^{13}C]Mevalonolactone experiments: 4-day-old beetles (50 males and 50 females) were introduced into a 200-mL Erlenmeyer flask (standard taper, 24/40) containing the precursor-treated flour (10 g). Four sets of [2-^{13}C]mevalonolactone-coated flour, four sets of mevalonolactone-coated flour, and two sets of non-precursor treated flour were prepared. Each flask was plugged with a joint (standard taper, 24/40), in which two glass tubes were embedded (long and short tubes; 6 mm in O.D). The distance from the surface of the flour to the lower end of the long or short tube was 1 cm or 8 cm,

respectively. A Super Q column (ca. 90 mg, 2 cm in length, 6 mm in O.D, mesh 80/100; Alltec Associates Inc., Deerfield, IL) was connected to the upper end of long glass tube for collecting the insects volatiles, while to the short glass tube, a Porapak Q column (ca. 95 mg, 2 cm, 6 mm in O.D, mesh 80/100; Waters Co., Milford, MA) was connected for introducing purified air into the chamber. The each Super Q column was connected to an air pump (Hiblow SPP-25EBS or C-15H; Takatsuki, Tokyo) via a metal ten-way distributor (Takatsuki, Tokyo). Each flask was aerated at the rate of 250 mL/min. Volatiles were collected for 20 days (Aeration I; aeration period days 0 – 10, Aeration II; aeration period days 10 – 20). The captured volatiles were eluted with 1.1 mL of distilled hexane every 5 days and pooled in a 2 mL of micro reaction vessel (Supelco, Bellefonte, PA). The pooled eluates [Eluate I (volatiles of days 0 – 5 and 5 – 10) and Eluate II (volatiles of days 10 – 15 and 15 – 20)] were stored in a freezer at −15°C until concentrated. The pooled insect volatiles were concentrated to ca. 100 μL, transferred to an auto sampler conical vial (Agilent) with 100 μL microsyringe (Hamilton Co., Reno, NV). The reaction vessel was rinsed with another 100 μL of hexane and the solution was combined. One microgram of methyl decanoate (1 μL of 1000 ppm hexane solution) was added to the solution as an internal standard for quantification of 4,8-DMD and then concentrated to 100 μL. One microliter of the solution [5 Male Day Equivalent (MDE) /μL] was subjected to GC-MS analysis.

Deuterated precursor experiments: The similar aeration apparatus in [2-^{13}C]mevalonolactone experiments were used. A 200 mL Erlenmeyer flask with a silicone stopper in which two glass tubes were embedded, was used for aeration. A Porapak Q column (ca. 95 mg, 2 cm in length, 6 mm O.D) was employed for collecting insect

volatiles instead of Super Q column. The other apparatus were same as that used in [2-^{13}C]mevalonolactone experiments. Three sets of chambers containing beetles and flour coated with deuterium-labeled precursor were prepared. Similarly, three different chambers were prepared as controls: one set of chamber containing beetles and non-precursor coated flour (Control A; non-treated flour), one set of chamber containing beetles and flour coated with unlabeled precursor (Control B; non-labeled flour), and one set of chamber containing flour coated with labeled precursor only (Control C; precursor-only flour). Aeration was performed for 10 days at the rate of 250 mL/min. The collected volatiles were eluted with 1.5 mL of distilled hexane. The eluted volatiles were concentrated to ca. 100 μL and chromatographed on a SiO_2 column (400 mg, commercially available Pasteur pipette) using step-wise elution with hexane (1 mL), 5% ether in hexane (2 × 1 mL, 5% E-H fractions I and II), and ether (1 mL). 4,8-DMD was eluted with later 1 mL of 5% ether in hexane (5% E-H fraction II). This fraction was concentrated to 100 μL and 1 μL of the solution was analyzed by GC-MS (5 MDE/μL).

The Super Q and Porapak Q columns were conditioned in a GC-oven at 200°C for 12 h under N_2 stream before use. After being used, it was reactivated for 2-3 h in the similar condition, and then used for next aeration.

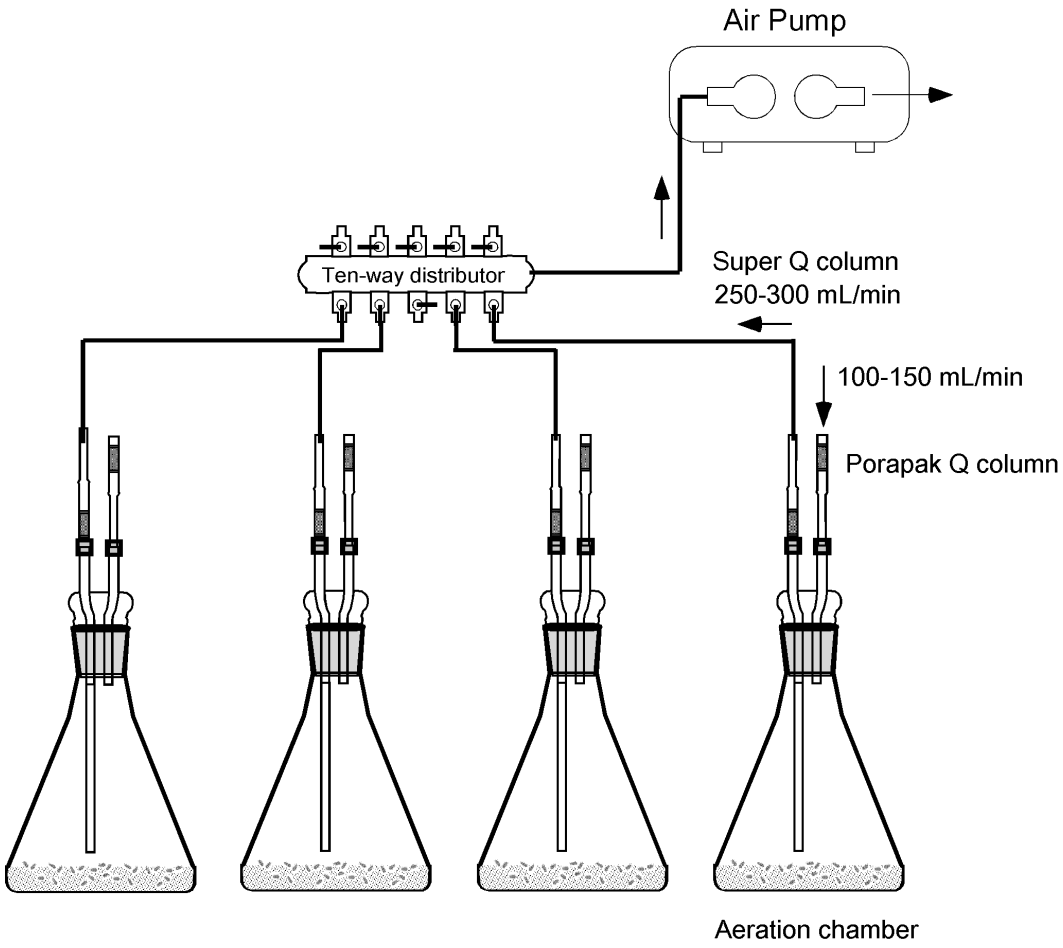


Fig. 2-4. Aeration apparatus for collecting insect volatiles used in [2-^{13}C]mevalonolactone experiments.

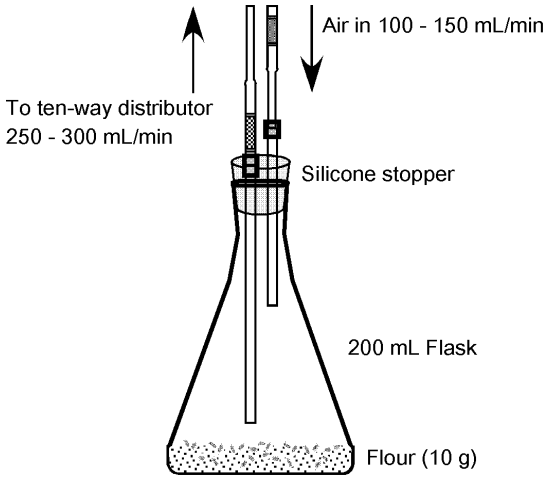


Fig. 2-5. Aeration chamber for collecting insect volatiles used in deuterium-labeled precursor experiments.

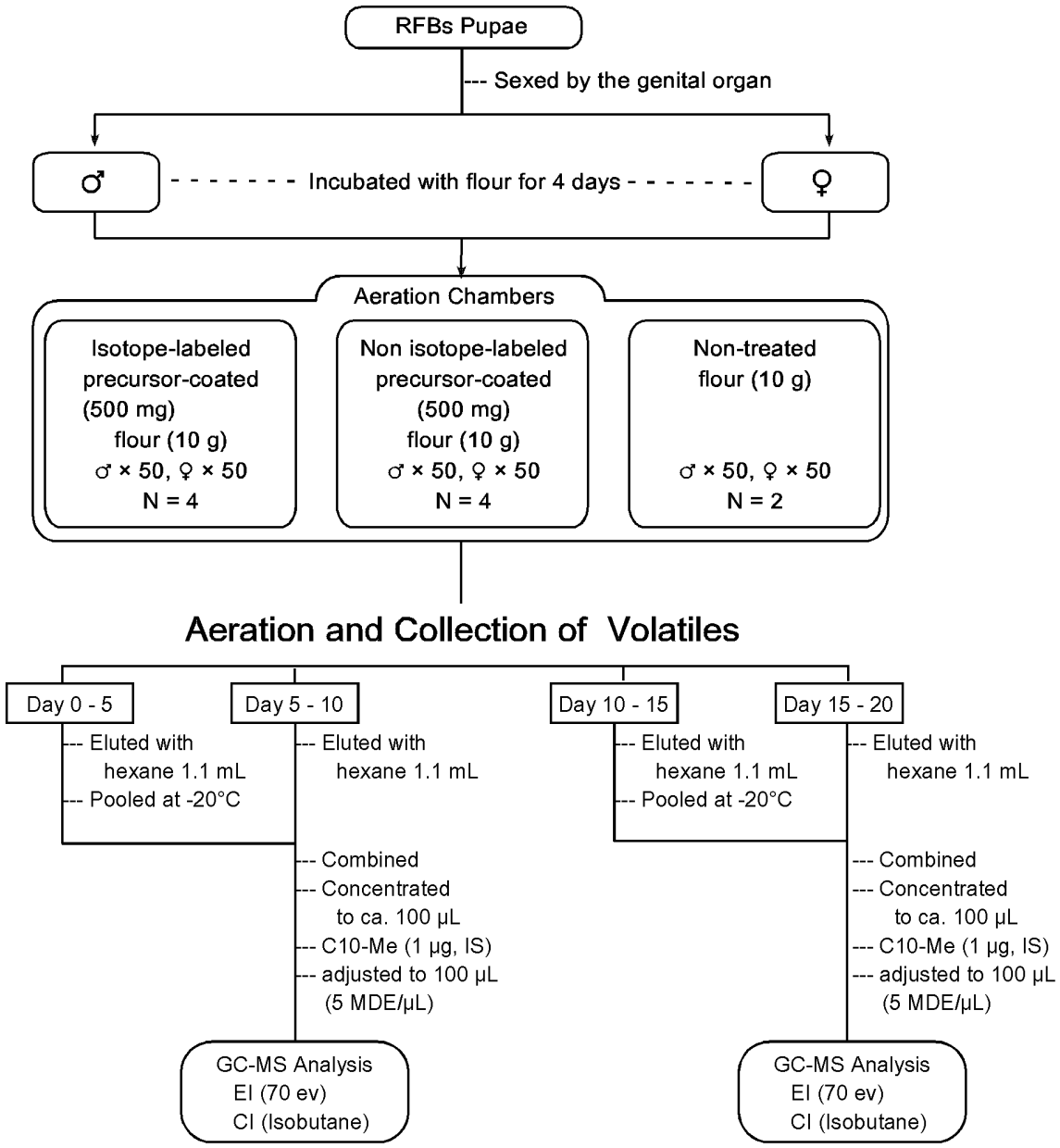


Fig. 2-6. Schematic procedures of [2-^{13}C]mevalonolactone incorporation experiments.
C10-Me: methyl decanoate (internal standard; IS)

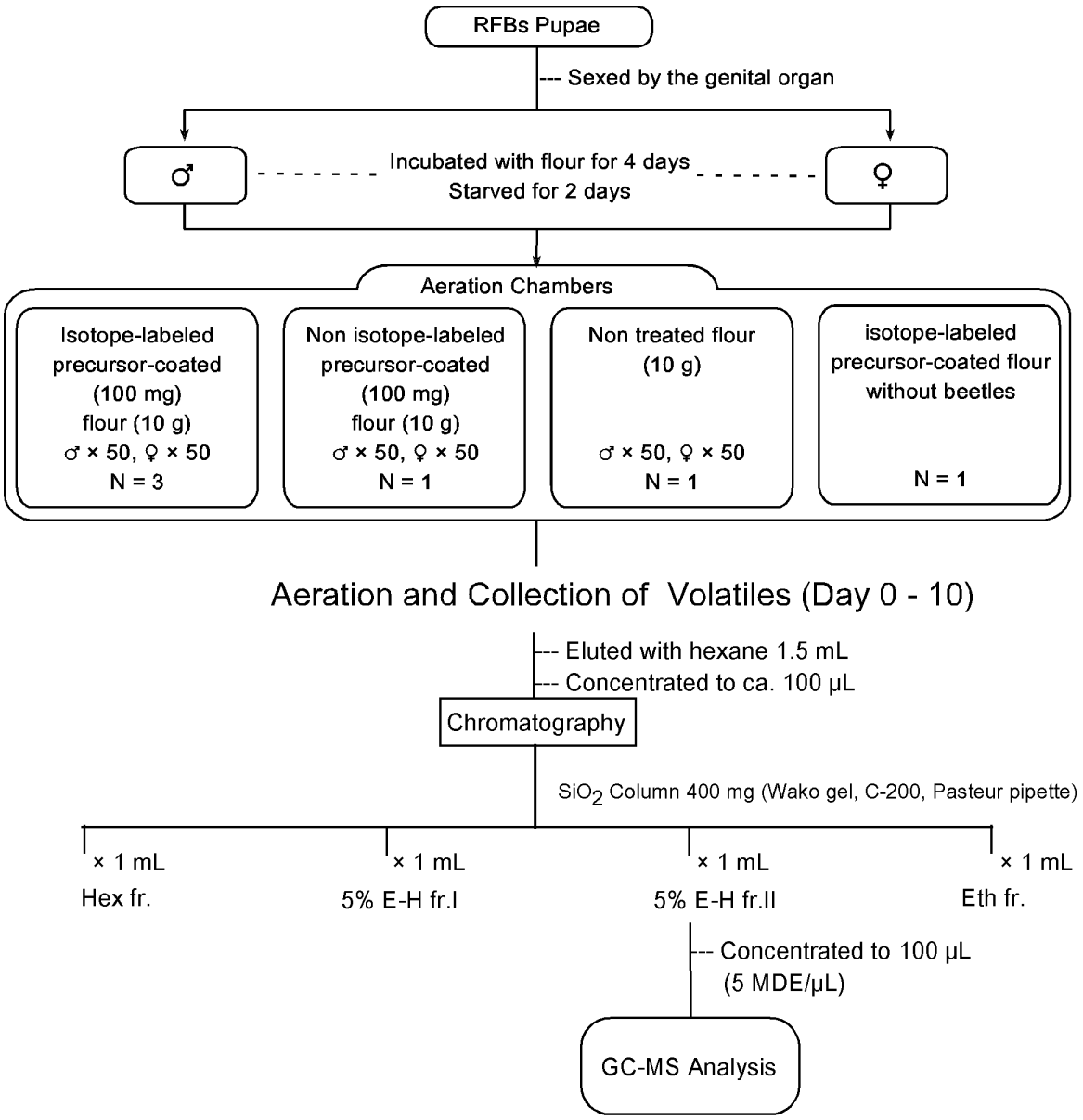


Fig. 2-7. Schematic procedures of deuterium-labeled precursor incorporation experiments. Hex fr.: hexane fraction; 5% E-H fr.: 5% ether in hexane fraction; Eth fr.: ether fraction.

2.2.5 GC-MS analysis of 4,8-dimethyldecanal

In [2-^{13}C]mevalonolactone, GLD5, and LLD5 experiments, a JEOL MS Router MS-600 (double focusing MS) coupled with a HP 6890N equipped with a HP-1MS column (30 m × 0.25 mm I.D, 0.25 μm, J&W Scientific; Folsom, CA) was used for GC-MS analysis. For analysis of the volatiles obtained from [2-^{13}C]mevalonolactone experiment, the oven temperature was programmed at 50°C for 1 min and raised to 200°C at the rate of 6°C/min then to 270°C at the rate of 8°C/min. While, for analysis of the volatiles obtained from GLD5 and LLD5 experiments, the oven temperature was programmed at 50°C for 1 min and raised to 270°C at the rate of 8°C/min. GC-MS CI analysis was conducted on the JEOL MS Router MS-600 with isobutane as a reagent gas. GC-MS-CI analysis afforded more effective information than GC-MS-EI, because 4,8-DMD gave the clear fragment M^+H at *m/z* 185 as the base peak, while its M^+ was not observed in GC-MS-EI due to the McLafferty rearrangement (Fig. 2-8).

GC-MS analyses of volatiles obtained from FLD5, THD5 and HFD2 experiments, were carried out on a HP MSD 5972 Series (quadrupole MS) coupled with a HP 5890 Series II Plus equipped with the HP-1MS column. The oven temperature was programmed at 50°C for 1 min and then raised to 270°C at the rate of 8°C/min and hold for 2 min. The volatiles obtained from HDMA-D4 experiment, were analyzed using a Finnigan PolarisQ (ion trap MS) coupled with a TraceGC (ThermoQuest) equipped with the HP-1MS column. The oven temperature was kept at 50°C for 1 min and then raised to 250°C at the rate of 6°C/min and hold for 1 min.

4,8-DMD derived from labeled precursor, was monitored by extracting the fragment(s) at *m/z* (140 + *n*) from the TIC of 4,8-DMD obtained by aeration (*n* is

difference between mass unit value of labeled and unlabeled 4,8-DMD; Figs. 2-9 and 2-10). In order to confirm the incorporation of deuterated precursors into 4,8-DMD, MS spectra of 4,8-DMDs derived from labeled precursors were compared with those of synthetic deuterated 4,8-DMDs which expected to be derived from the added precursors (Fig. 2-9). Synthesis of the standard deuterated 4,8-DMDs are described in Chapter V.

Incorporation rate of a labeled precursor into 4,8-DMD is calculated by the following equation reported by Campbell (1974) and expresses as enrichment factor (EF):

$$\text{Enrichment factor (EF, \%)} = \frac{\text{Intensity of } (M^+H + n)}{\text{Sum of intensities of } M^+H \text{ and } (M^+H + n)} \times 100$$

Equation 2-1. Equation of enrichment factor (EF).
M^+H represents chemically ionized M^+ of 4,8-DMD by isobutane in GC-MS-CI analysis, where *n* is difference between its *m/z* value and that of the unlabeled 4,8-DMD.

2.2.6 Statistics analyses

In order to confirm the incorporation of [2-^{13}C]mevalonolactone into 4,8-DMD, the relative intensities of some diagnostic fragments of 4,8-DMD derived from both labeled and unlabeled mevalonolactone feedings were compared by *t*-test (*m/z* 72 to base ion, *m/z* 86 to base, *m/z* 112 to 111, *m/z* 138 to 137 and *m/z* 141 to 140). Computer calculations were carried out using S-Plus® 2000 (MathSoft, Seattle, WA).

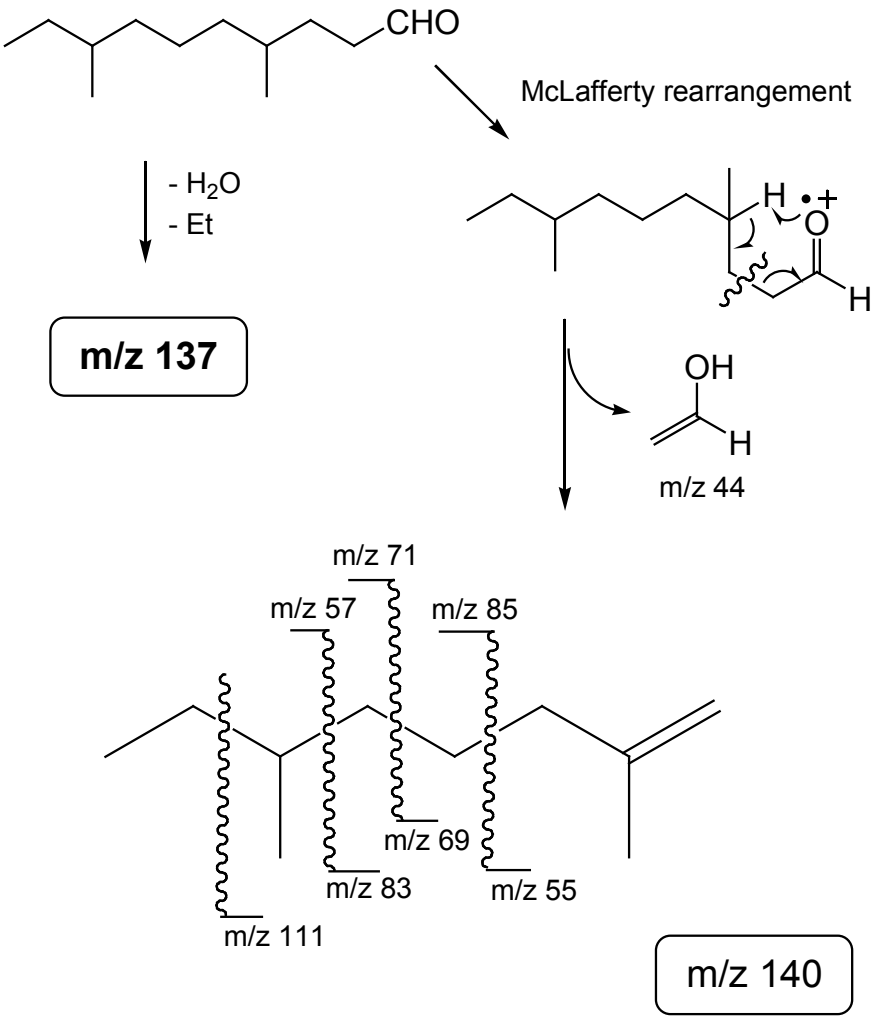


Fig. 2-8. Origins of several major fragment ions (used as diagnostic ion in incorporation experiments) in the MS spectrum of 4,8-DMD.

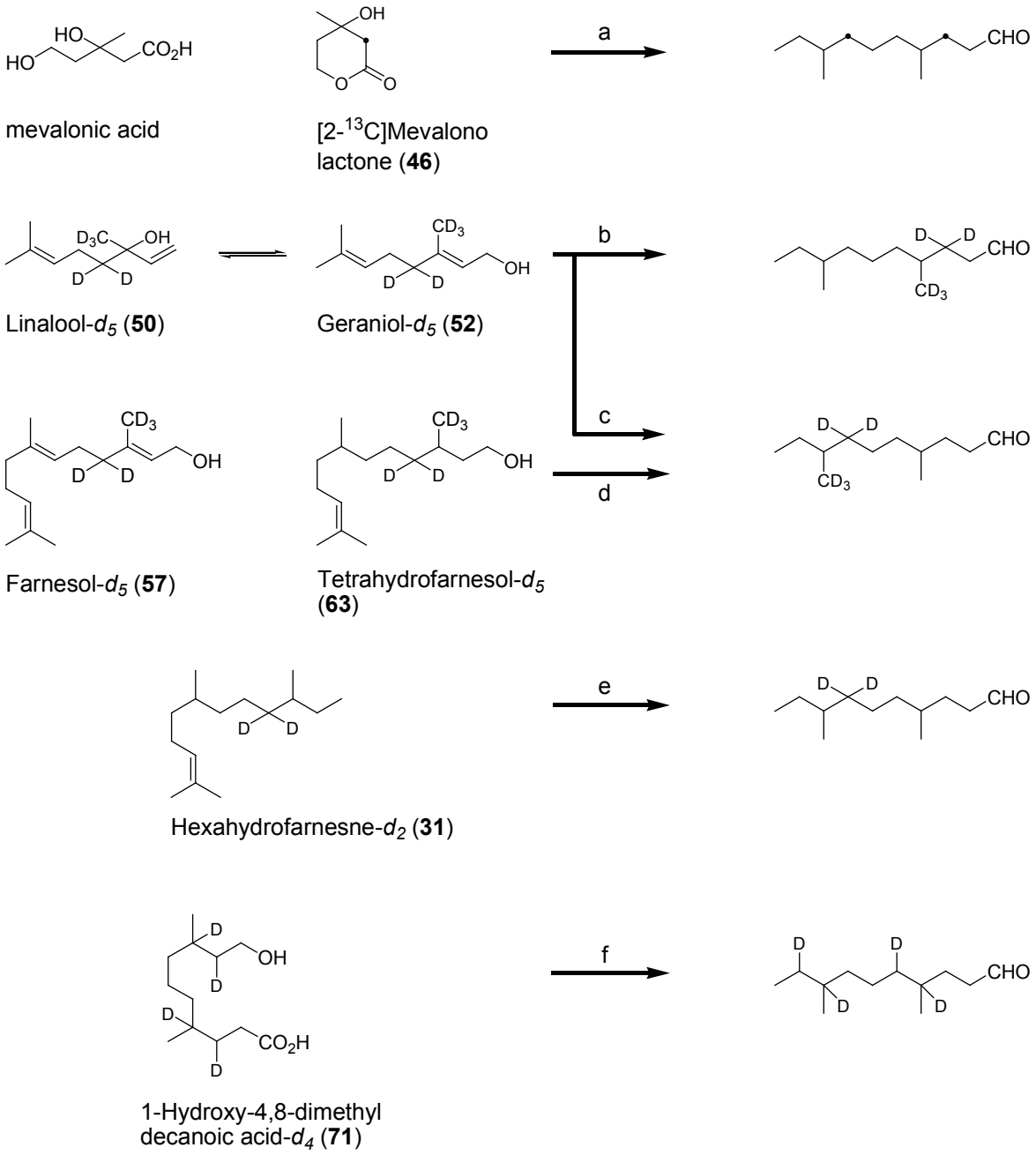


Fig. 2-9. Expected structures of deuterated 4,8-DMD by incorporation of deuterated terpenoid precursors.

a) through either pathway in Fig. 2-1; b) oxygenation of **52** at the terminal allyl group, chain elongation, hydrogenation, and dehydration; c) chain elongation of **52** via the mevalonate path way and then followed the same pathway of **57**; d) chain shortening by oxidative cleavage of double bond and dehydration; e) chain shortening by oxidative cleavage of double bond; f) dehydration. Numbers in bold represent the compound numbers in Chapter V.

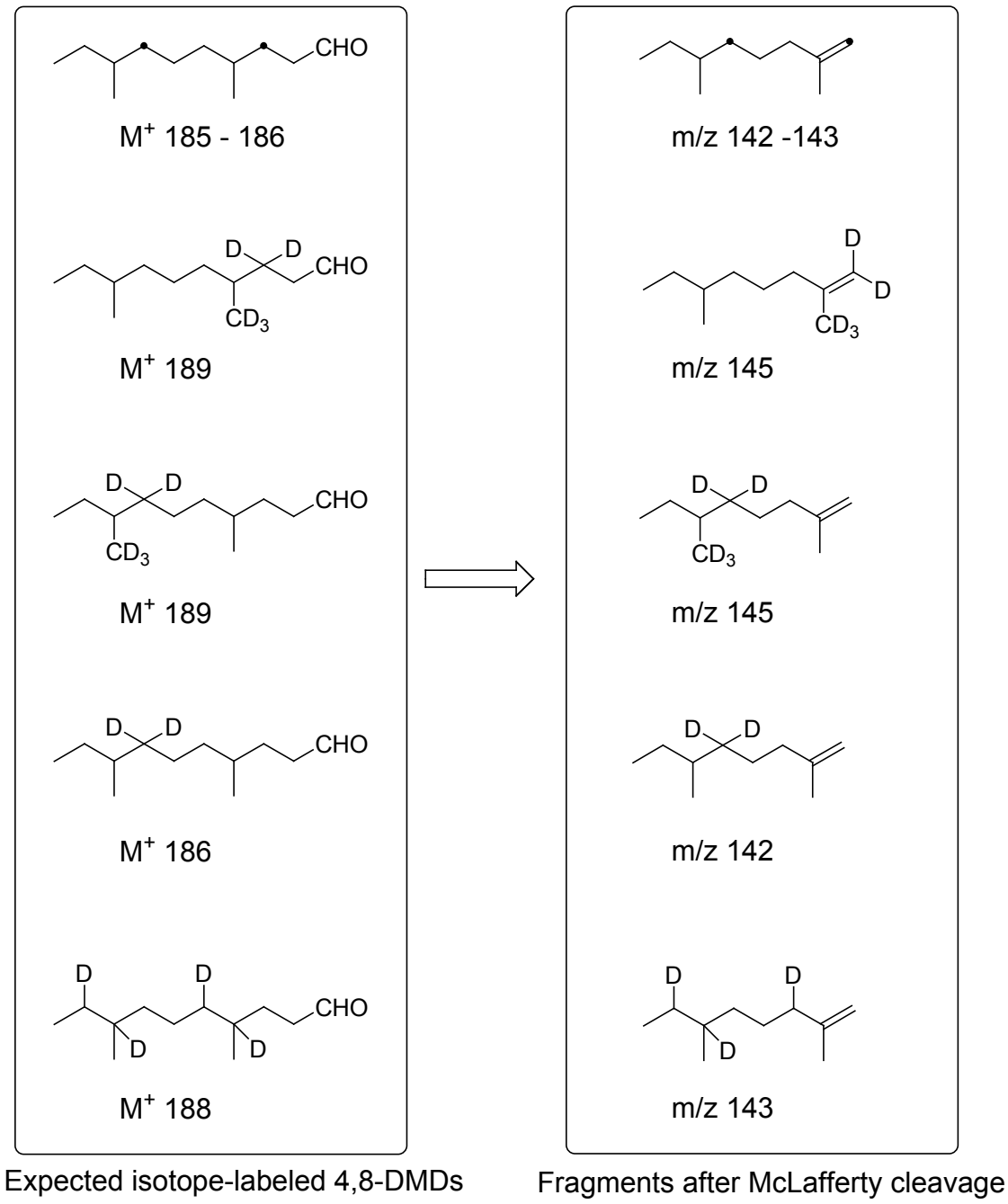


Fig. 2-10. Main highest fragments after McLafferty rearrangement of isotope-labeled 4,8-DMDs. Dots on the line represent ^{13}C atoms.

2.3 RESULTS

Fig. 2-11 shows the MS chromatogram of the volatiles obtained from the RFBs fed non-treated flour. As reported previously, 1-pentadecene was a main component of the volatiles (Suzuki et al., 1975). In addition to 4,8-DMD, methylbenzoquinone, ethylbenzoquinone, other straight hydrocarbons, and 2-hydroxy-4-methoxypropio phenone (2,4-HMPP) were detected by GC-MS analysis. These compounds were confirmed by comparing their retention time and MS spectra of authentic standards and the literatures (Suzuki, 1975; Suzuki et al., 1975). 2,4-HMPP was isolated from RFB by body extraction and identified by Suzuki (1975), and later it was confirmed to be the component of defensive secretion and a prostaglandin synthetase inhibitor (Howard et al., 1986). It was firstly detected by aeration in this study.

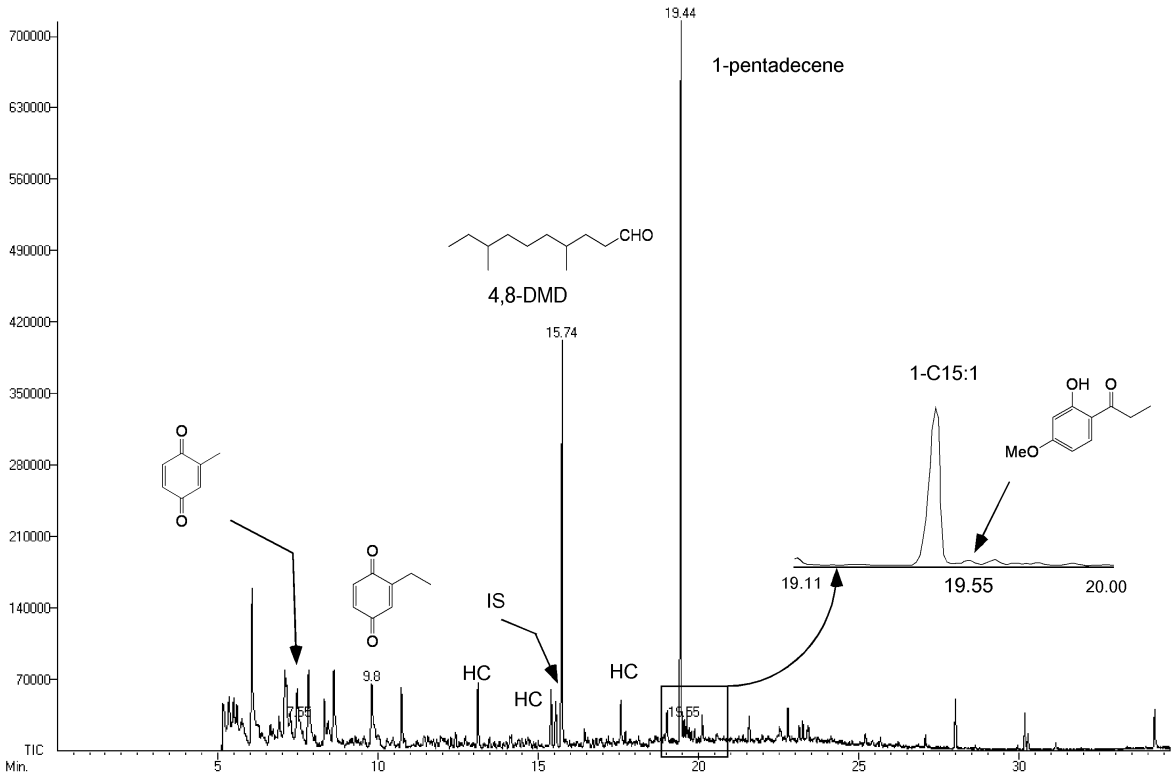


Fig. 2-11. MS chromatogram of the volatiles obtained from *T. castaneum* fed non-treated flour.
IS = methyl dodecanoate; HC = series of hydrocarbon; peaks at 28.0 and 30.2 min = methyl ester of hexadecanoic acid and octadecadienoic acid, respectively, originated from the flour during preparation of the media.

2.3.1 Incorporation of [2-^{13}C]mevalonolactone

The mean amounts of 4,8-DMD obtained from the beetles fed mevalonolactone or [2-^{13}C]mevalonolactone-coated flour, were not greater than that from the beetles fed non-treated flour (Table 2-1). The results of GC-MS-EI analysis are summarized in Table 2-2, and the MS spectra of 4,8-DMDs from [2-^{13}C]mevalonolactone and mevalonolactone feeding are shown in Figs. 2-12 and 2-13. If [2-^{13}C]mevalonolactone is incorporated into 4,8-DMD, the carbons at C-3 and C-7 are expected to be labeled with ^{13}C atoms (Fig. 2-9), and some fragment ions (*m/z* 140, 141, 137, 111, 71, and 85) will shift to one or two higher mass units, resulting in the increase of the relative intensities of diagnostic fragment ions (*m/z* 141 and 142 to 140, *m/z* 138 to 137, *m/z* 112 to 111 and *m/z* 72 and 86 to base ion). The relative intensities of fragments at *m/z* 141, 138 and 72 in 4,8-DMD obtained by [2-^{13}C]mevalonolactone feeding were significantly increased during Aeration I (aeration period days 0 – 10), compared to those from non-labeled mevalonolactone feeding. Similarly, the fragments at *m/z* 86 and 112 obtained by [2-^{13}C]mevalonolactone feeding showed greater relative intensities than those from mevalonolactone feeding, although there was no significant differences between them. During Aeration II (aeration period days 10 – 20), the relative intensities of the diagnostic fragment ions at only *m/z* 141 and 138 in 4,8-DMD were significantly increased by [2-^{13}C]mevalonolactone feeding experiments. The fragment at *m/z* 142 was not detected from time to time, due to low intensity. The obtained 4,8-DMD was enriched in one ^{13}C atom at 2.68 ± 1.59% by [2-^{13}C]mevalonolactone (Table 2-3). Based on the resuls, it was admitted that RFBs incoporated [2-^{13}C]mevalonolactone into 4,8-DMD only during Aeration I (aeration period days 0 – 10).

Table 2-1. The mean amounts of 4,8-DMD produced by RFBs in [2-^{13}C]mevlaonolactone experiments.

	Amounts of 4,8-DMD (ng/MDE, mean ± SD, N = 4)	
Treatment	Aeration I (days 0 – 10)	Aeration II (days 10 – 20)
Control [a]	9.23	7.32
Mevalonolactone	4.66 ± 1.26	3.00 ± 0.51
[2-^{13}C]Mevalonolactone	3.94 ± 1.04	3.13 ± 1.58

[a]; N = 2.

MDE means male day equivalents.

Table 2-2. The relative intensities of diagnostic fragments in 4,8-DMDs obtained from [2-^{13}C]mevalonolactone and mevalonolactone incorporation experiments.

Aeration period	Treatment	Relative intensities of diagnostic fragments (%, mean ± SD, N = 4)					
		m/z 72 (A)	*m/z* 86 (A)	*m/z* 112 (B)	*m/z* 138 (C)	*m/z* 141 (D)	*m/z* 142 (D)
Days 0 – 10	Mevalonolactone	7.40 ± 0.38	4.23 ± 0.48	30.64 ± 1.01	31.18 ± 3.60	18.35 ± 1.10	1.86 ± 0.11 [a]
	[2-^{13}C]mevalonolactone	7.93 ± 0.33*	4.77 ± 0.37	33.82 ± 1.38	32.70 ± 0.93**	19.58 ± 0.63*	2.26 ± NA [b]
Days 10 – 20	Mevalonolactone	7.25 ± 0.48	4.78 ± 0.29	29.64 ± 2.63	28.12 ± 2.91	18.39 ± 1.57	1.94 ± 0.44 [c]
	[2-^{13}C]mevalonolactone	7.82 ± 0.48	5.47 ± 1.00	31.57 ± 1.24	33.07 ± 1.40*	21.84 ± 2.03*	2.48 ± 0.60 [c]

A: % to base ion (*m/z* 54); B: % to *m/z* 112; C: % to *m/z* 137; D: % to *m/z* 140.

a) N = 2; b) N = 1; c) N = 2

Mean values of relative intensities of the fragments in 4,8-DMDs obtained from [2-^{13}C]mevalonolactone and mevalonolactone treatments were compared by *t*-test (one tailed).

*, **: Significant at $P < 0.05$ and $P < 0.01$, respectively.

Table 2-3. Incorporation of [2-^{13}C]mevalonolactone into 4,8-DMD.

	Enrichment factor (EF, %)	
Aeration period	A	B
I	2.68 ± 1.59	NA
II	NA	NA

A, B: incorporation with one and two ^{13}C atom(s), respectively.
Aeration periods I and II: days 0 – 10 and days 11 – 20, respectively.
NA: 4,8-DMD was not enriched in ^{13}C atom.
The values (mean ± SD, N = 4) were calulated by GC- MS-CI analysis, followed by Equation 2-1.

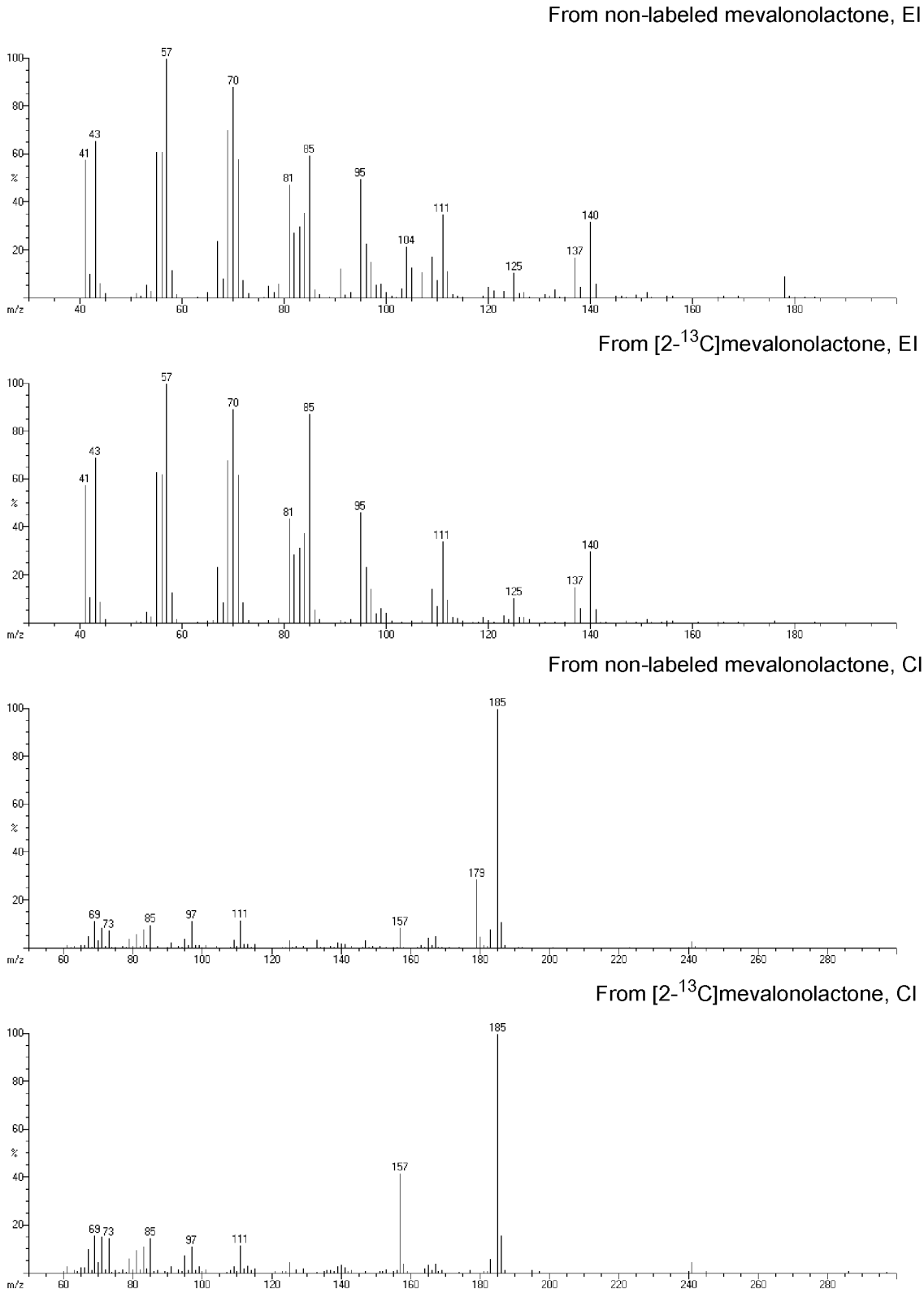


Fig. 2-12. MS spectra of 4,8-DMD obtained from mevalonolactone and [2-^{13}C]mevalonolactone treatments during aeration period days 0 – 10 (Aeration I).
GC-MS: MS600H, 50°C (1) - 200°C/6°C min^{-1} - 270°C/8°C min^{-1}.

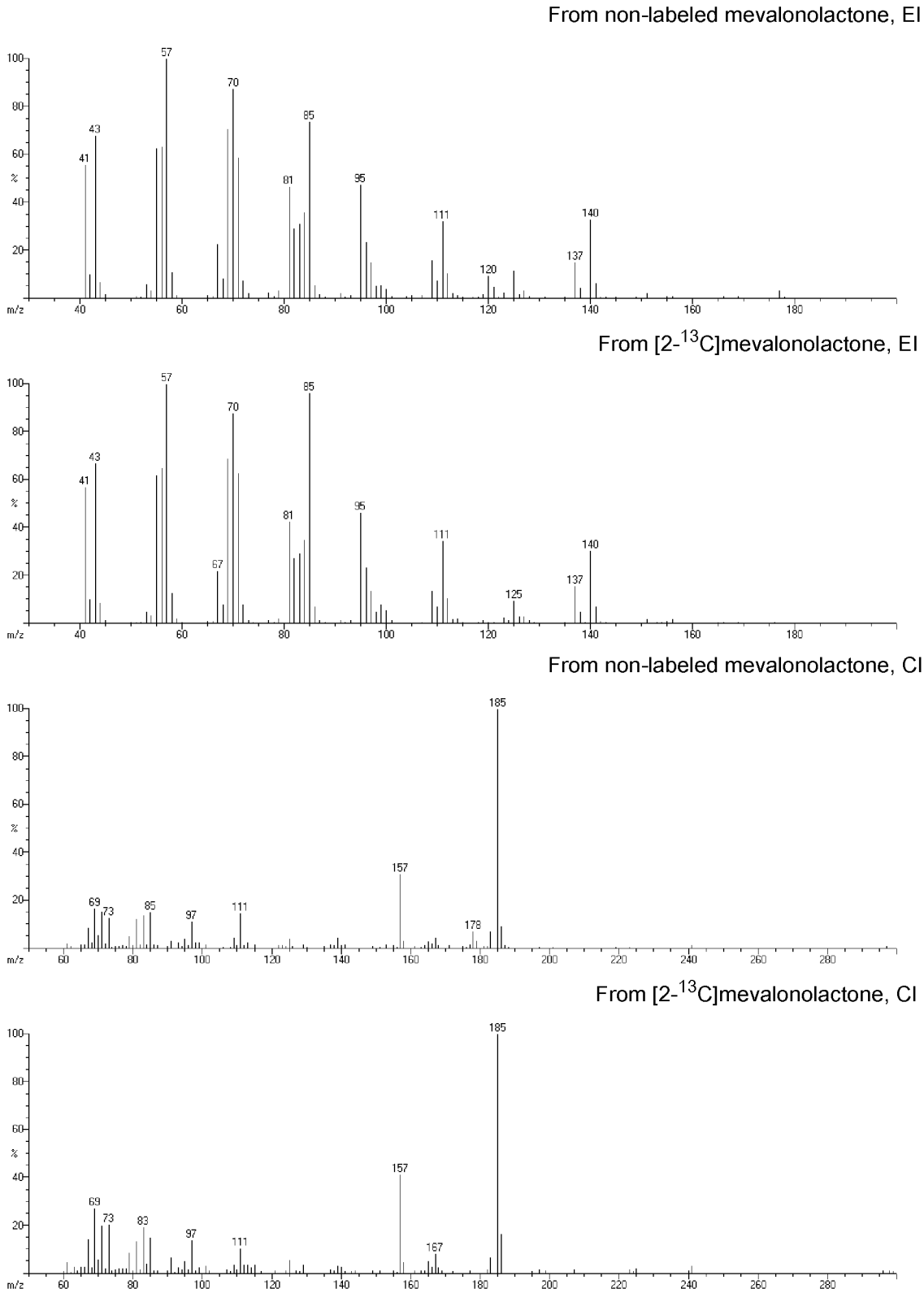


Fig. 2-13. MS spectra of 4,8-DMD obtained from mevalonolactone and [2-^{13}C]mevalonolactone treatments during aeration period days 10 – 20 (Aeration II).
GC-MS: MS600H, 50°C (1) - 200°C/6°C min^{-1} - 270°C/8°C min^{-1}.

2.3.2 Incorporation of monoterpene precursors: Geraniol-d_5 and linalool-d_5

The deuterated isomers of 4,8-DMD were separable from non-deuterated 4,8-DMD by GC-MS, due to their smaller retention times. Therefore, when geraniol-d_5 (GLD5) and linalool-d_5 (LLD5) are incorporated into 4,8-DMD, deuterated 4,8-DMD is expected to be detected by single ion monitoring (SIM) of the fragments at *m/z* 142, 143, and 144 (Figs. 2-9 and 2-10), because deuterium numbers in GLD5 and LLD5 are decreased as $d_4 > d_3 > d_2 >> d_5$ and d_1. The fragments at *m/z* 142 and 143 were detected in 4,8-DMD obtained from GLD5 and LLD5 feeding beetles, by GC-MS EI analysis (double focusing MS) (Figs. 2-14 and 2-15). However, these fragment ions were also observed in the MS spectrum of 4,8-DMD obtained from the beetles fed the flour coated with unlabeled precursor (Tables 2-4 and 2-5). Moreover, deuterated 4,8-DMD were also expected to show similar pattern that the intensities of fragments were decreased as *m/z* 144 > 143 > 142. However, the MS spectra of 4,8-DMD from GCD5 and LLD5 feeding beetles, showed that the intensities of *m/z* 142 were greater than those of *m/z* 143 and 144 (Tables 2-4 and 2-5). Vanderwel et al. (1992) reported that the major possible error in the determination of isotope incorporation by GC-MS was due to contamination of an impurity with the same mass unit (*m/z*) and the same retention time. The fragments at *m/z* 142 and 143 may be an impurity, because the same fragments were also detected from the 4,8-DMD obtained from RFBs fed the flour coated with unlabeled precursor. Therefore, geraniol and linalool as precursors of 4,8-DMD were excluded.

Table 2-4. The relative intensities of some diagnostic fragments in 4,8-DMDs from geraniol-d_5 treatment.

	Relative intensities of diagnostic ions (% to *m/z* 140)				
Treatment	*m/z* 141	*m/z* 142	*m/z* 143	*m/z* 144	*m/z* 145
GLD5 (Replicate 1)	24.13	9.88	2.94	0	0.39
GLD5 (Replicate 2)	25.64	40.46	10.34	1.50	1.02
GLD5 (Replicate 3)	21.98	79.10	16.40	0.93	6.03
GL (Control)	21.35	10.09	3.12	0.39	0.35

GLD5: geraniol-d_5; GL: geraniol

Table 2-5. The relative intensities of some diagnostic fragments in 4,8-DMDs from linalool-d_5 treatment.

	Relative intensities of diagnostic ions (% to *m/z* 140)				
Treatment	*m/z* 141	*m/z* 142	*m/z* 143	*m/z* 144	*m/z* 145
LLD5 (Replicate 1)	16.63	8.31	6.02	2.25	7.10
LLD5 (Replicate 2)	22.41	1.96	1.37	0.98	0.47
LLD5 (Replicate 3)	20.43	6.39	1.41	2.23	0
LL (Control)	20.23	28.91	9.63	0	0

LLD5: Linalool-d_5; LL: Linalool

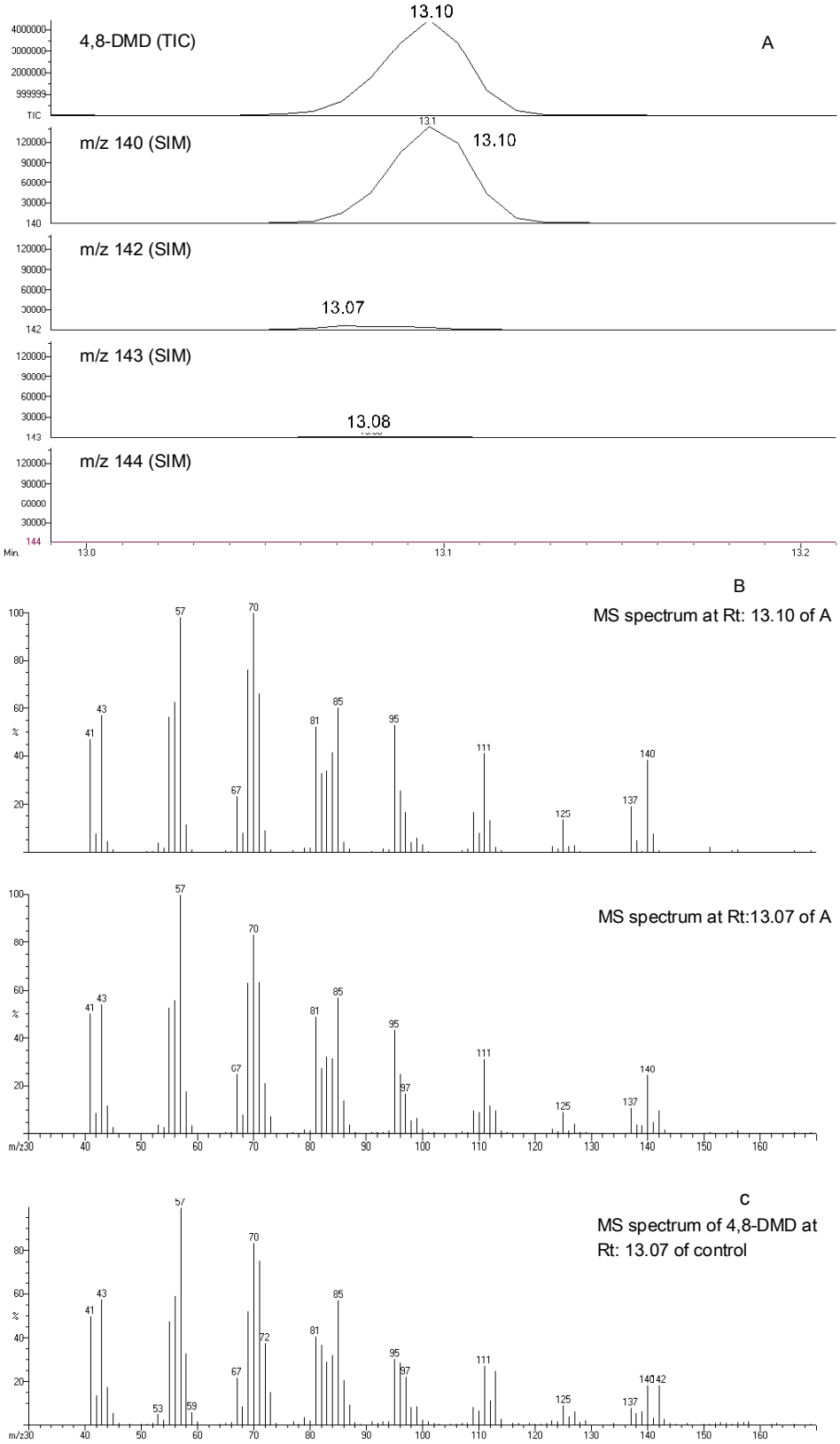


Fig. 2-14. Mass chromatograms (A) and MS spectra of 4,8-DMD obtained from RFBs fed the flour coated with geraniol-d_5 (GLD5) (B) and with geraniol (C), respectively.
GC-MS: MS600H, 50°C (1) - 270°C/8°C min^{-1}.

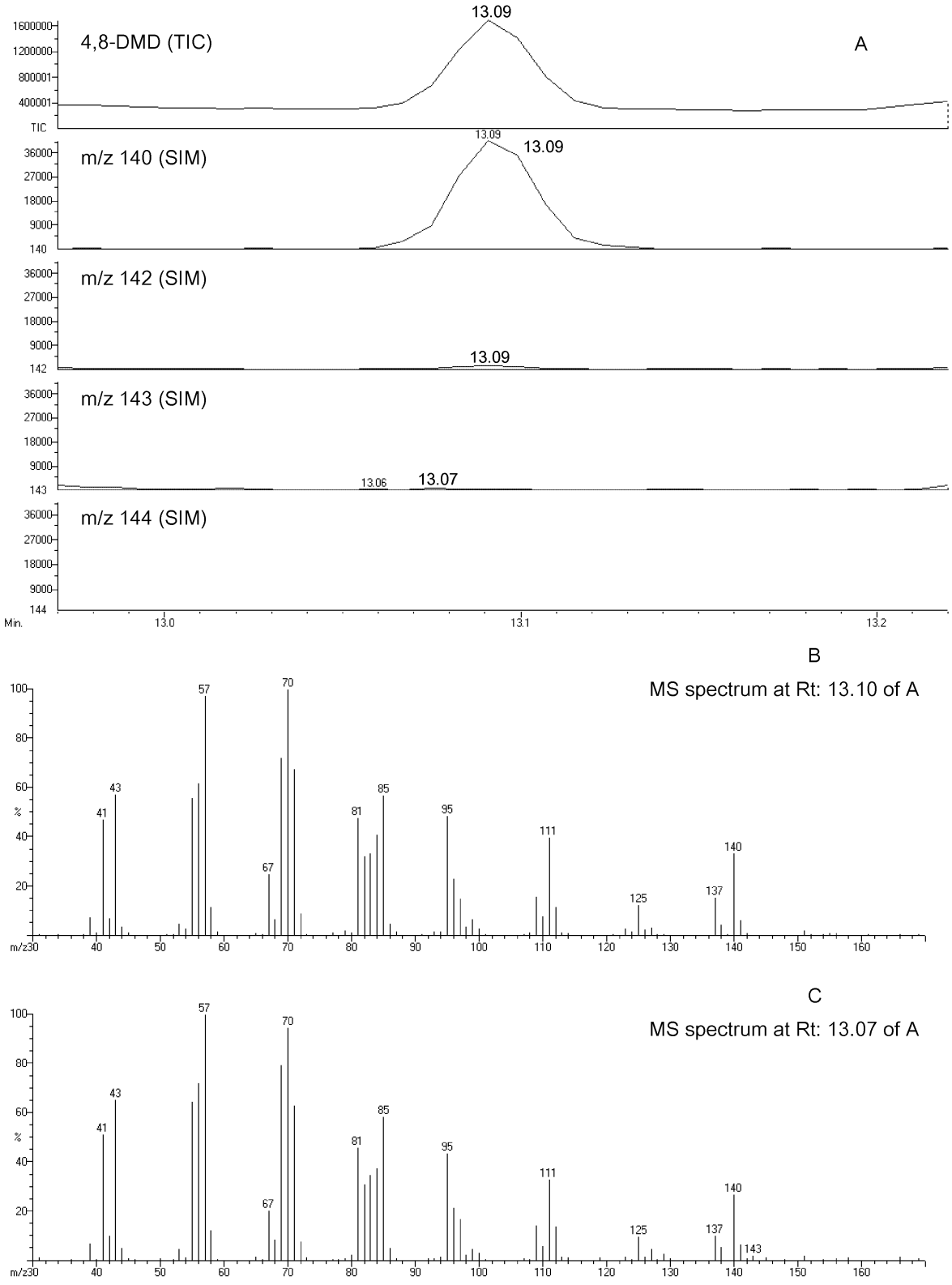


Fig. 2-15. Mass chromatograms (A) and MS spectra (B and C) of 4,8-DMDs obtained from linalool-d_5 (LLD5) treatment.
GC-MS: MS600H, 50°C (1) - 270°C/8°C min^{-1}.

2.3.3 Incorporation of sesquiterpene precursors:

Farnesol-d_5, tetrahydrofarnesol-d_5, and hexahydrofarnesene-d_2

If 4,8-DMD is biosynthesized through the pathway shown in Figs. 2-9 and 2-10, the deuterium atoms of farnesol-d_5 (FLD5) and tetrahydrofarnesol-d_5 (TFD5) would be incorporated into 4,8-DMD by feeding RFBs on the flours coated with these compounds.

In the FLD5 feeding experiments, the fragments at *m/z* 142, 143, 144 and 145 were not detected in all replicates by the SIM, contrary to expectation (Table 2-6, Fig. 2-16). Therefore, farnesol as a precursor of 4,8-DMD was excluded.

The fragment ions at *m/z* 142 and 143 were detected in TFD5 feeding experiments. Deuterium numbers in TFD5 are decreased as $d_4 > d_3 > d_2$, similar to GLD5 and LLD5, thus deuterium numbers in 4,8-DMD obtained from TFD5 treatment would be decreased as $d_4 > d_3 > d_2$. However, relative intensities of other diagnostic fragments at *m/z* 113 and 114 to 111 were not increased and the MS spectrum pattern was not similar to that of synthetic standard 4,8-DMD-d_5 (Table 2-7, Fig. 2-17). The fragments at *m/z* 142 and 143 may be derived from an impurity. Thus, tetrahydrofarnesol was omitted as a precursor of 4,8-DMD.

Since hexahydrofarnesene-d_2 (HFD2) was deuterated with LAD, it has two deuterium atoms. Therefore, if HFD2 is incorporated into 4,8-DMD, two mass units increased 4,8-DMD would be produced. However it was not detected from all replicates in HFD2 feeding experiments by GC-MS-EI analysis (Table 2-8, Fig. 2-18). Hexahydrofarnesene was also excluded as a precursor of 4,8-DMD.

Table 2-6. The relative intensities of diagnostic fragments in 4,8-DMDs from the farnesol-d_5 (FLD5) feeding treatments.

	Relative intensities of diagnostic fragments (% to *m/z* 140)				
	m/z 141	*m/z* 142	*m/z* 143	*m/z* 144	*m/z* 145
Replicate 1	22.39	ND	ND	ND	ND
Replicate 2	29.07	ND	ND	ND	ND
Replicate 3	ND	ND	ND	ND	ND

ND = not detected

Table 2-7. The relative intensities of diagnostic fragments in 4,8-DMDs from the tetrahydrofarnesol-d_5 (TFD5) feeding treatments.

	Relative intensities of diagnostic fragments (% to *m/z* 140)				
	m/z 141	*m/z* 142	*m/z* 143	*m/z* 144	*m/z* 145
Replicate 1	24.14	6.39	6.39	ND	ND
Replicate 2	22.72	6.90	ND	ND	ND
Replicate 3	ND	ND	ND	ND	ND

ND = not detected

Table 2-8. The relative intensities of diagnostic fragments in 4,8-DMDs from the hexahydrofarnesene-d_2 (HFD2) feeding treatments.

	Relative intensities of diagnostic fragments (% to *m/z* 140)		
	m/z 141	*m/z* 142	*m/z* 143
Replicate 1	52.47	ND	ND
Replicate 2	ND	ND	ND
Replicate 3	ND	ND	ND

ND = not detected

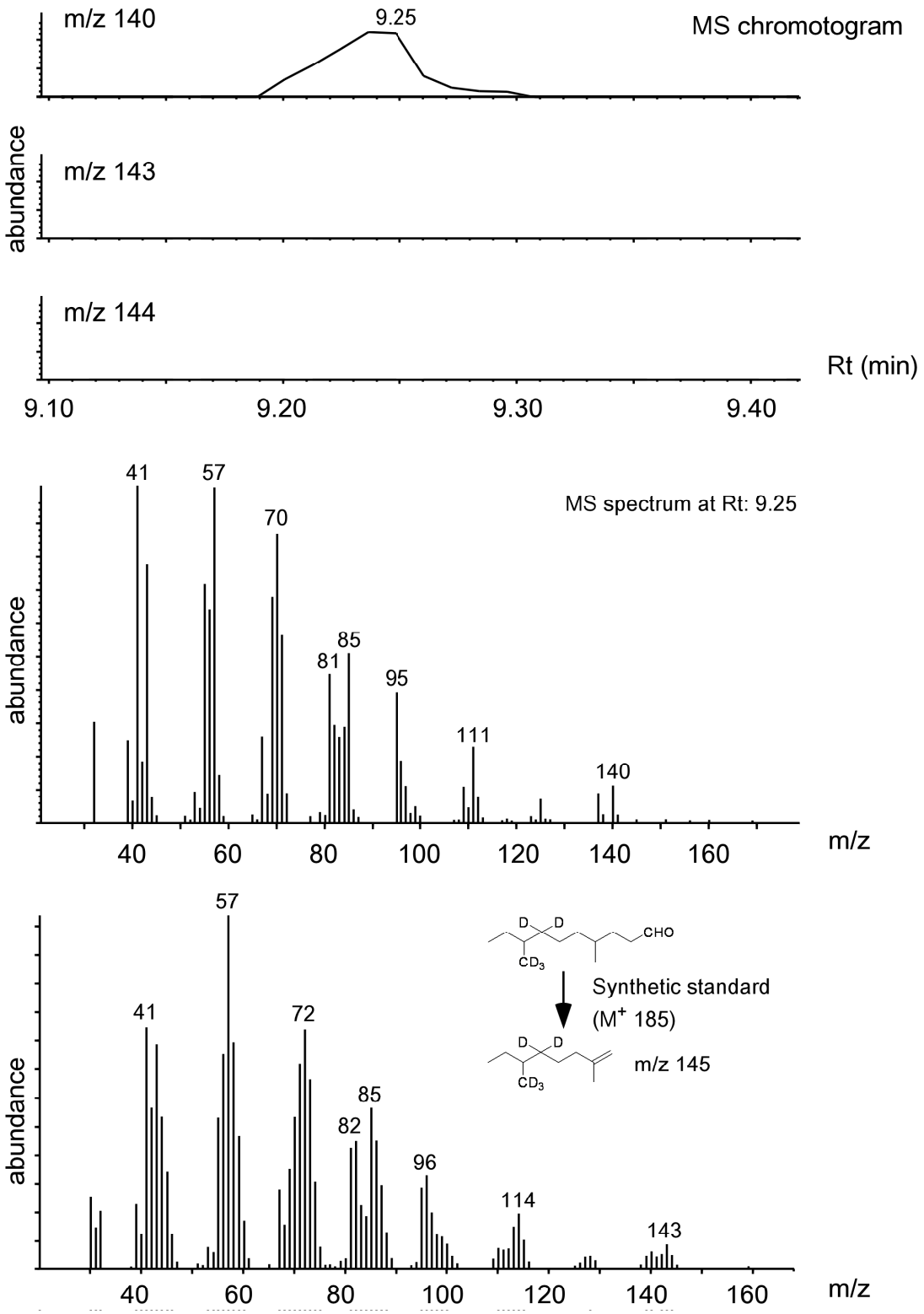


Fig. 2-16. Mass chromatograms and MS spectra of 4,8-DMD obtained from farnesol-d_5 (FLD5) feeding experiment (middle panel) and of synthetic 4,8-DMD-d_5 (lower panel).
GC-MS: HP-MSD, 50°C (1) - 270°C/8°C min^{-1}.

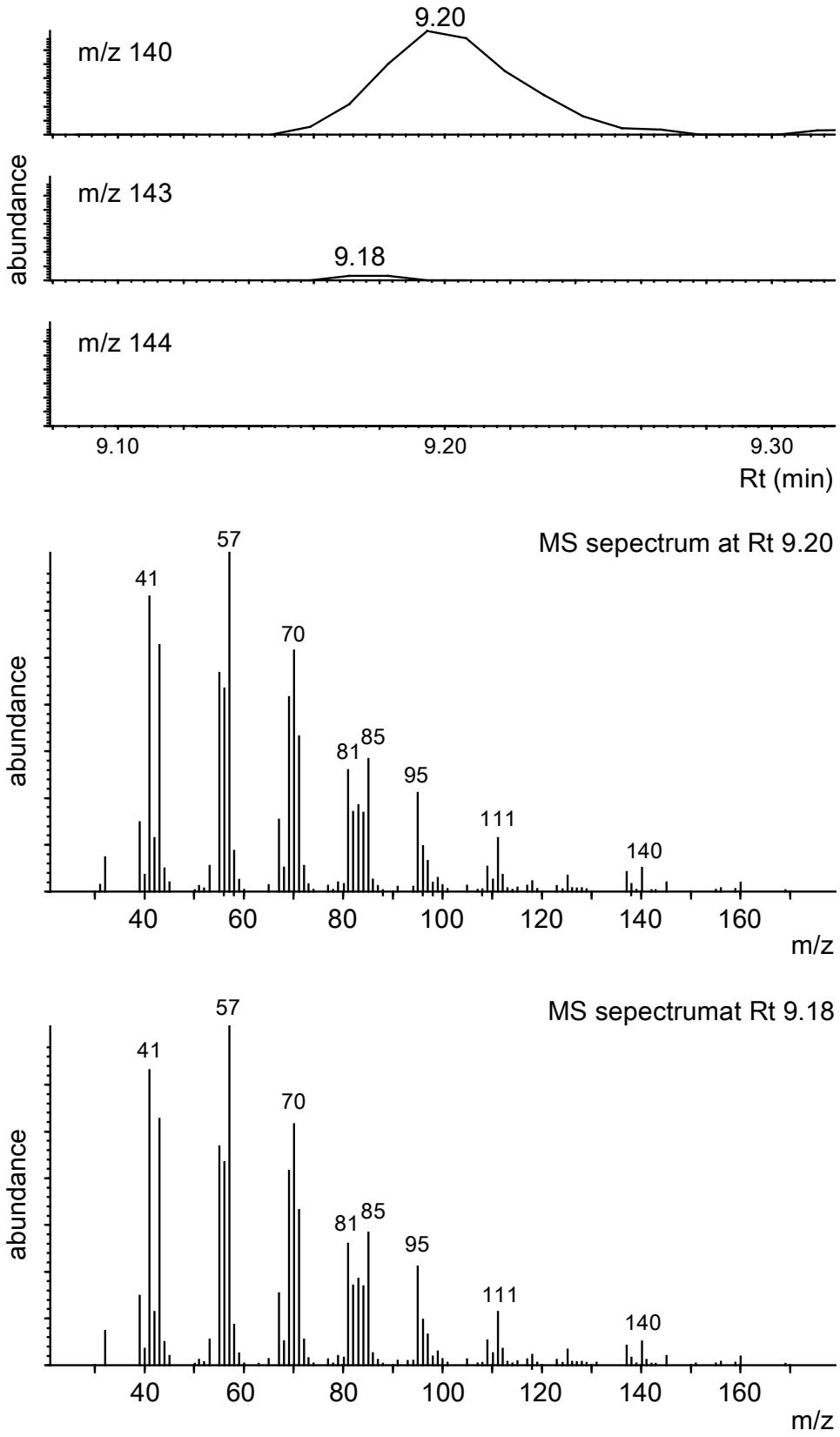


Fig. 2-17. Mass chromatograms and MS spectra of 4,8-DMD obtained from tetrafarnesol-d_5 (FLD5) treatment.
GC-MS: HP-MSD, 50°C(1) - 270°C/8°C min^{-1}.

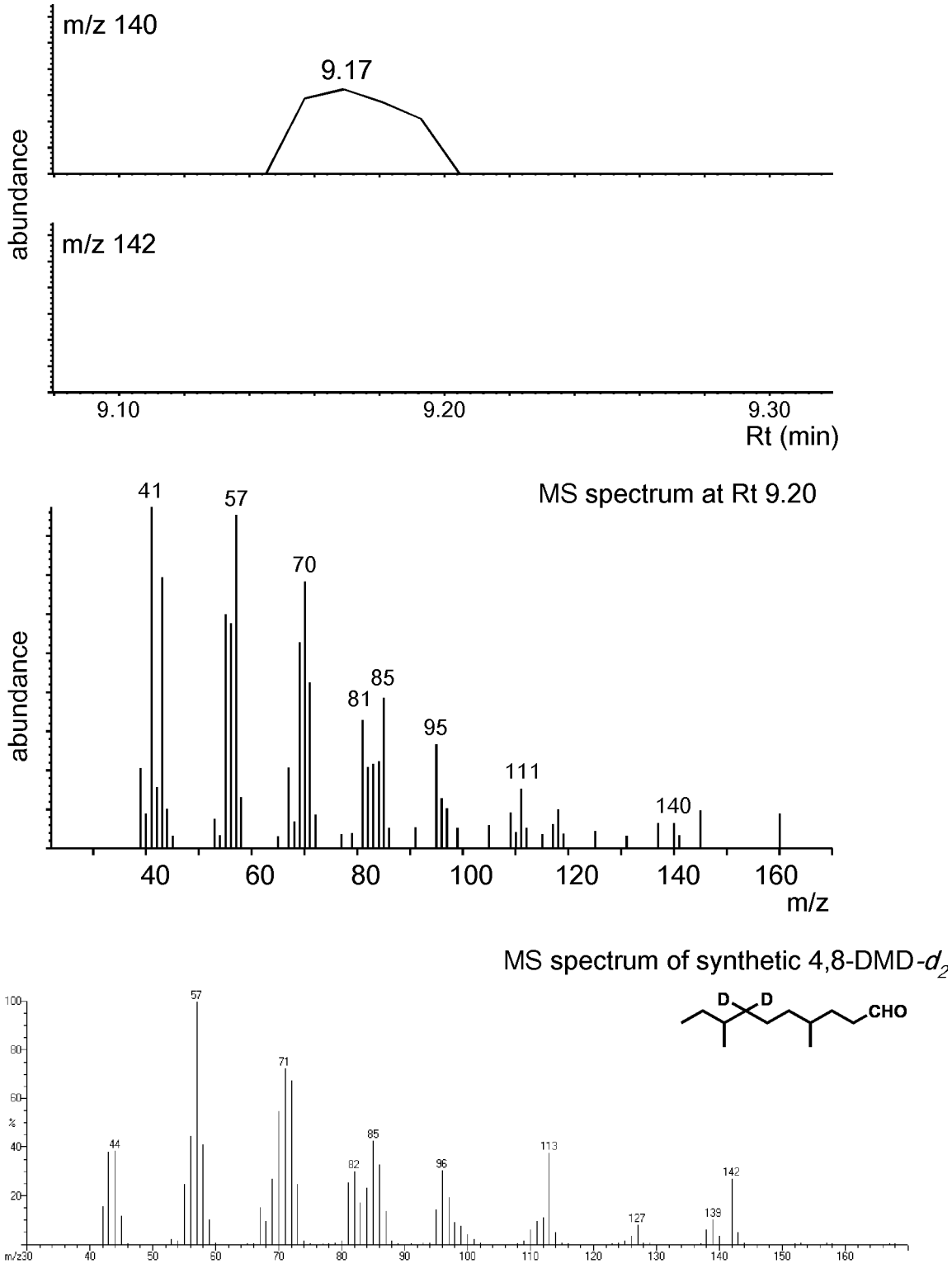


Fig. 2-18. Mass chromatograms and MS spectra of 4,8-DMD obtained from hexahydrofarnesene-d_2 (HFD2) treatment (middle panel)and of synthetic 4,8-DMD-d_2 (lower panel).
GC-MS: HP-MSD, 50°C (1) - 270°C/8°C min^{-1}.

2.3.4 Incorporation of 10-hydroxy-4,8-dimethyldecanoic acid-*4,5,8,9-d_4*

GC-MS analyses (ion trap mass) revealed that the fragments at *m/z* 142, 143 and 144 were observed by SIM in 4,8-DMD obtained by HMDA-D4 feeding but its pattern was different from that of the synthetic standard (Fig. 2-19). Moreover, these fragments were also detected in the MS spectrum of 4,8-DMD from the non-labeled precursor treatment (Table 2-9). If HDMD-D4 is incorporated into 4,8-DMD, the series of fragments at *m/z* 141, 142, 143, 144, and 145 would be observed, and intensities of the fragments at *m/z* 143 and 144 would be same level and greater than that of at *m/z* 142. However, in Replicate 1, the intensity of fragment at *m/z* 144 was only 32.7%, even though that of *m/z* 143 was 119.18% and in Replicates 2 and 3, fragment at *m/z* 143 was not observed, nevertheless fragment intensity of *m/z* 142 was 124.59 and 31.56, respectively (Table 2-9). Thus, HDMD-D4 as precursor of 4,8-DMD was excluded.

Table 2-9. The relative intensities of some diagnostic fragments in 4,8-DMDs from 10-hydroxy-4,8-dimethyldecanoic acid-*3,4,8,9-d_4* (HDMA-D4) treatments.

	Relative intensities of diagnostic fragment ions (% to *m/z* 140)				
Treatment	*m/z* 141	*m/z* 142	*m/z* 143	*m/z* 144	*m/z* 145
HDMA-D4 (Replicate 1)	42.58	30.13	119.18	32.72	65.83
HDMA-D4 (Replicate 2)	37.57	124.59	0.00	44.17	181.73
HDMA-D4 (Replicate 3)	42.87	31.56	0.00	12.45	5.16
HDMA (non-labeled)	43.16	38.99	153.12	48.39	10.69
Standard 4,8DMD-d_4	11.82	2.84	85.75	83.88	95.15

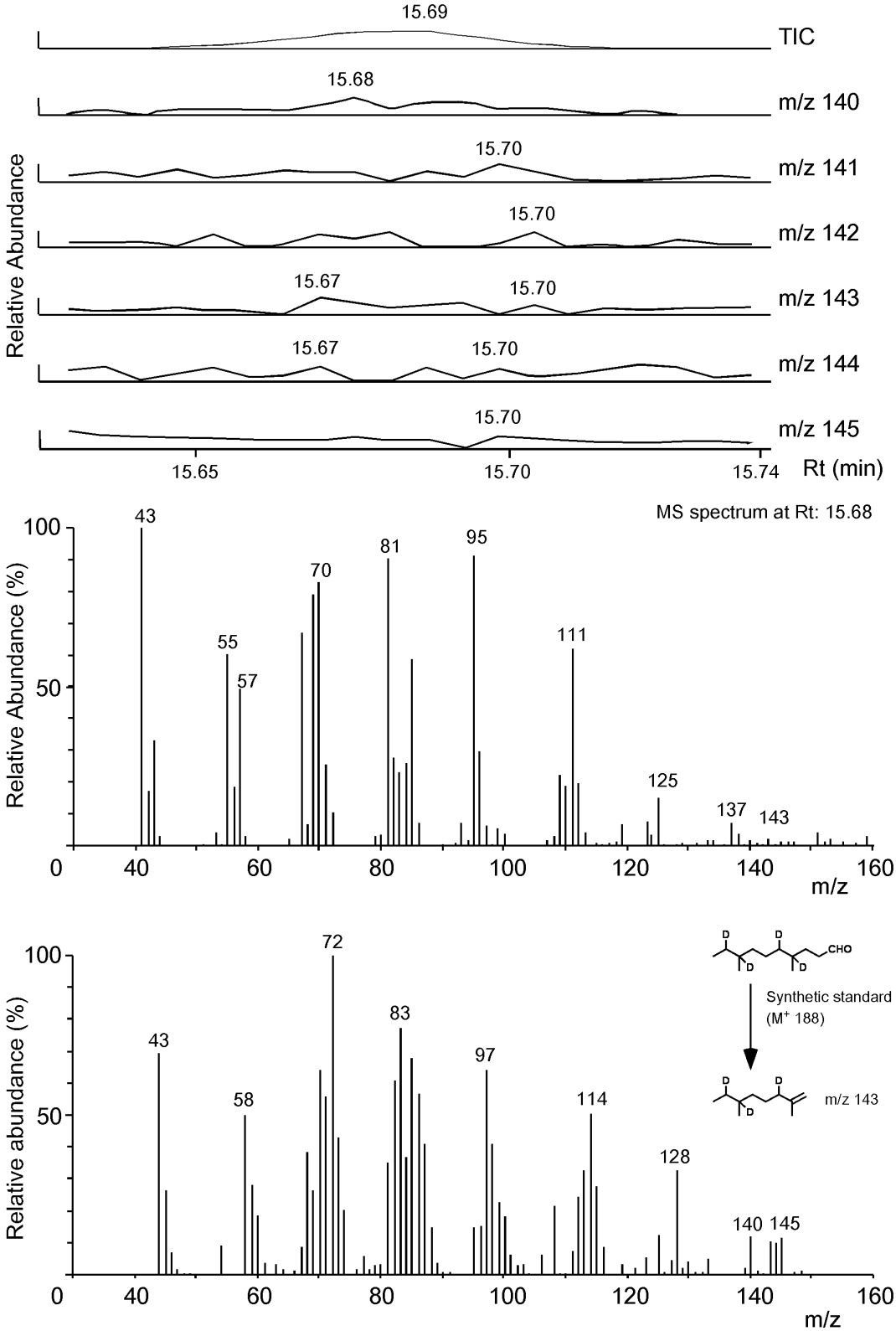


Fig. 2-19. Mass chromatograms and MS spectra of 4,8-DMDs obtained from 10-hydroxy-4,8-dimethyldecanoic acid-*4,5,8,9-d*$_4$ (HDMD4) treatment (middle panel) and of synthetic 4,8-DMD-*d*$_4$ (lower panel).
GC-MS: PolarisQ, 50°C (1) - 250°C/6°C min^{-1}.

2.4 DISCUSSION

Although [2-^{13}C]mevalonolactone was incorporated into 4,8-DMD, the result of incorporation experiments with deuterium-labeled terpenes and their derivatives did not provide sufficient evidence that 4,8-DMD is of terpene origin. Mevalonolactone is known as a precursor of geraniol, linalool and farnesol in the mevalonate pathway. Therefore, if 4,8-DMD is of terpene origin, these terpenes would be also expected to be intermediates of 4,8-DMD as well as mevalonolactone. However other deuterium-labeled terpenes were not incorporated. Farnesol derivatives, such as tetrahydrofarnesol and hexahydrofarnesene, have been considered to be precursors of 4,8-DMD, because 4,8-DMD is easily obtainable by the oxidative cleavage of the terminal double bond of these compounds (Vanderwel and Oehlschlager, 1987; Howse et al., 1998). Contrary to expectation, deuterated farnesol derivatives were also not converted into 4,8-DMD. The incorporation of [2-^{13}C]mevalonolactone into 4,8-DMD may be due to the degradation of it by the beetles or preparation of media.

(4*E*)-4,8-Dimethyl-4,9-decadiene-1,8-diol, which is considered to be biosynthesized by the oxidative cleavage of the terminal double bond of a sesquiterpene, nerolidol, has been identified from Monarch butterfly and *Gerris latiabdominis* (Hemiptera: Gerridae) (Schulz et al., 1988; Ikeda, 1999). 10-Hydroxy-4,8-dimethyl-decanoic acid is expected to be a precursor prepared by the similar pathway of the diol from farnesol. However, the incorporation of deuteriums in 10-hydroxy-4,8-dimethyl-decanoic acid-d_4 was denied. In contrast, in a preliminary experiment, 4,8-dimethyl-decanoic acid-*3,4,7,8*-d_4, was successfully incorporated into 4,8-DMD in the same experimental condition (100 mg of precursors in 10 g of flour and 10 days aeration).

Considering that the structure of HDMD-D4 is only different from the acid in respect of the presence of a hydroxy group at the C-10 position, the oxidative cleavage of the terminal double bond of the sesquiterpene appears not to be involved in the biosynthetic pathway of 4,8-DMD.

The result that no deuterium-labeled terpenes and their derivatives were incorporated into 4,8-DMD suggested that the hypothesized mevalonate pathway would not be involved in the biosynthesis of 4,8-DMD and 4,8-DMD is unlikely to be of terpene origin.

CHAPTER III

SPECULATION OF BIOSYNTHETIC PATHWAY OF 4,8-DIMETHYLDECANAL BY REGULATION OF ITS PRODUCTION

3.1 INTRODUCTION

4,8-Dimethyldecanal (4,8-DMD), the male-produced aggregation pheromone of the red flour beetle (RFB), *Tribolium castaneum*, has been considered to be biosynthesized through the mevalonate pathway (Vanderwel and Oehlschlager, 1987; Howse et al., 1998). However, the results in Chapter II did not provide certain evidence that 4,8-DMD is biosynthesized through the mevalonate pathway. Thus, to elucidate the biosynthetic origin of 4,8-DMD, effects of juvenile hormone III (JH III) and two pathway inhibitors (mevastatin and 2-ocynoic acid) on the production of 4,8-DMD by male RFBs were investigated.

Enzyme activators and pathway inhibitors have been used for investigation of the biosynthetic origins of pheromones in Coleoptera (Tillman et al., 1999; Seybold and Vanderwel, 2003). JH III reportedly activates 3-hydroxy-3-methylglutaryl CoA reductase (HMG-R) and synthase (HMG-S) as shown in Fig. 3-1 (Tittiger, 2003). JH III and its analogs enhanced the production of terpene pheromone components derived from the mevalonate pathway in Scolytidae (Chen et al., 1988: Ivarsson and Birgersson, 1995). In contrast, mevastatin blocks the action of HMG-R in the biosynthesis of mevalonate, the terpene precursor (Endo et al., 1976; Monger et al., 1982) (Fig. 3-1). Ivarsson et al. (1993) provided indirect evidence for *de novo* biosynthesis of ipsdienol in *Ips duplicatus* using mevastatin. 2-Alkynoic acids, such as 2-octynoic acid, reportedly inhibit the action of fatty acid synthase (FAS) and general acyl-CoA dehydrogenase leading to block chain elongation of fatty acids (Freund et al., 1985; Barkawi et al., 2003; Zhao et al., 2004) (Fig. 3-2). Therefore, we reasoned that if 4,8-DMD is derived from the mevalonate pathway, the production of 4,8-DMD would be enhanced by JH III and reduced by mevastatin.

Alternatively, if 4,8-DMD is derived from the fatty acid pathway, the production of 4,8-DMD would be reduced by 2-octynoic acid.

In this chapter, the effects of JH III, mevastatin, and 2-octynoic acid on the pheromone production by male *T. castaneum* are described.

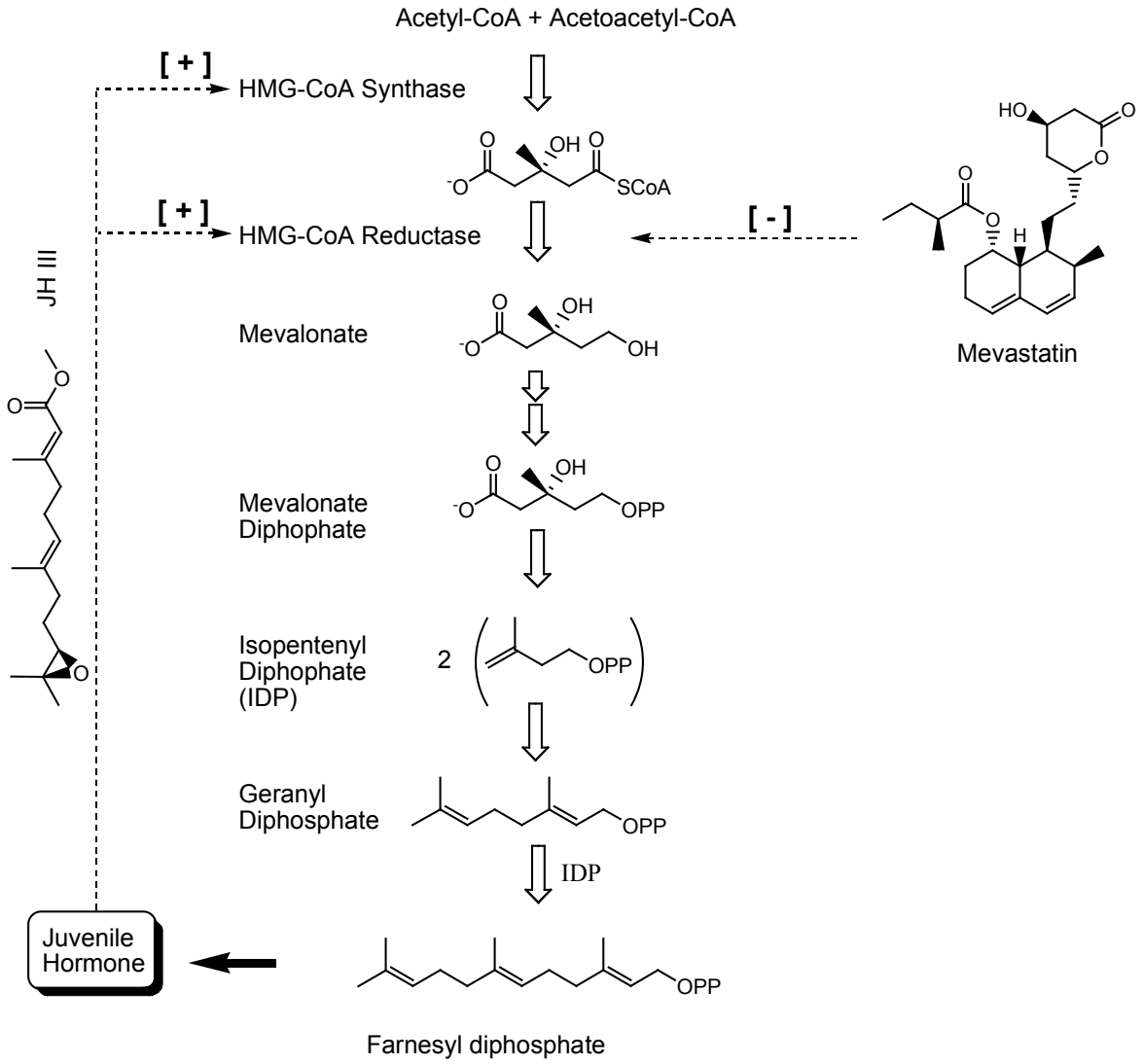


Fig. 3-1. The established terpene biosynthesis through the mevalonate pathway and actions of regulators in insects (Scheme was modified from Seybold and Tittiger, 2003; Ivarsson et al., 1993).
[+] represents activating effect; [-] represents inhibitory effect.

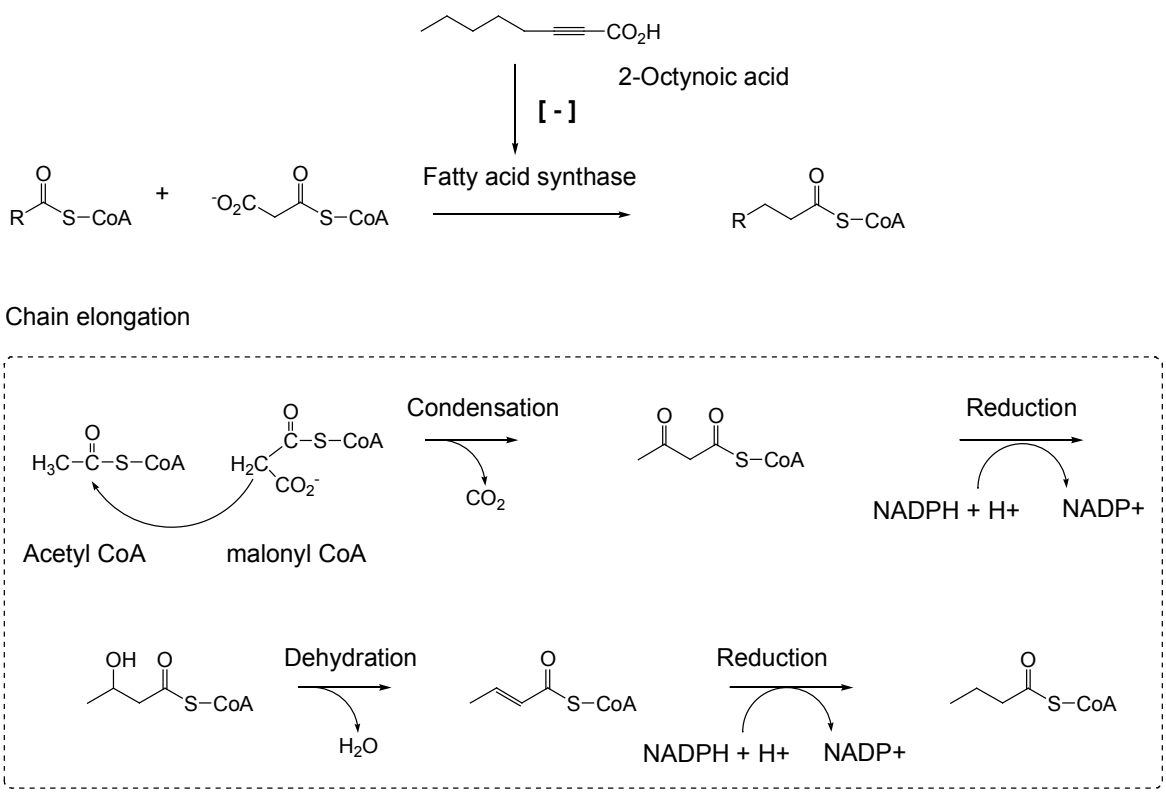


Fig. 3-2. Fatty acid synthesis procedures and action of 2-ocynoic acid on chain elongation of fatty acid. [-] represents inhibitory effect.

3.2 Materials and Methods

3.2.1 Insect cultures

A laboratory colony of *T. castaneum*, established over 20 years, was used in all experiments. The beetles were reared on a mixture of whole wheat flour and 5% brewer's yeast at 27 ± 1°C, ca. 70% RH on a 16:8 L:D cycle. Pupae were sexed by the genital organ and reared in separated containers with medium. For all experiments, 4-day-old adults were used.

3.2.2 Chemicals and materials

Mevastatin (Compactin) was purchased from EMD Biosciences, Inc. (San Diego, CA). 2-Octynoic acid and JH III were prepared as described in Chapter **3.2.7**. Ethanol (99.5%) and hexane were supplied by Wako Pure Chemicals Industries. Inc. (Osaka, Japan).

3.2.3 Effect of JH III on the production of 4,8-DMD

Male beetles were anesthetized with CO_2 then attached to double-sided Scotch® tape on a glass slide cooled with ice. After the elytra were slightly lifted with an insect pin, the specified dose of JH III (0, 1, 10, or 100 μg) dissolved in 1 μL acetone was topically applied to the ventral abdominal cuticule using a 10 μL syringe with a 33-gauge needle (Hamilton Co., Reno, NV). After JH III was applied, the beetles were allowed to recover in a Petri dish for 30 min at room temperature. JH III treated males and untreated females (50 of each) were introduced into a 200 mL Erlenmeyer flask containing whole wheat flour (10 g), as shown in Fig. 2-4 (Chapter II). Volatiles were collected for 4 days using a Super Q

column (ca. 90 mg). The captured volatiles were eluted with 1.1 mL of hexane and concetrated to 100 μL under N_2. After addition of 1 μL of methyl decanoate (1000 ppm hexane solution) as an internal standard, the solution was subjected to GC-MS analysis. The experiments were replicated three times. The procedures of aeration and volatile treatment in JH III experiment are summarized in Fig. 3-3. The Super Q and Porapak Q were conditioned and reactivated as described in Chapter II (**2.2.4**).

3.2.4 Effects of mevastatin and 2-octynoic acid on the production of 4,8-DMD

The effects of these inhibitors on the production of 4,8-DMD were tested by allowing the RFBs to feed flour treated with the each inhibitor. In preliminary experiments, when beetles were fed inhibitor-coated flour during the first 10 days, the amount of 4,8-DMD varied substantially, even after non-treated flour feeding. Therefore, in order to reduce this deviation, beetles were fed non-treated flour for 10 days, and were then fed inhibitor-coated flour. Non-treated flour was made as follows; to a round bottom flask (300 mL) containing ethanol (60 mL), wheat flour (30 g) was added, and ethanol was completely distilled off under reduced pressure in a room temperature by a rotary evaporator. Similarly, inhibitor-treated flour was prepared using flour (10 g) and an ethanol solution (20 mL) of each inhibitor (50 mg). A glass Petri dish (7 cm ID × 1.5 cm) containing the non-treated flour (10 g) and 50 males and 50 females was placed in a separable flask (500 ml volume; lower part, 12 cm ID × 5 cm) equipped with Super Q columns (Fig. 3-4). Three sets of apparatus were used and volatiles were trapped for 10 days (Aeration I) before the beetles in each set were transferred separately to mevastatin-coated, 2-octynoic acid-coated and non-treated (control) flour (10 g each).

Aeration was continued for a further 10 days (Aeration II). Volatiles were eluted every 5 days. The pooled eluates (Eluate I: volatiles of days 0 – 5 and 5 – 10 days, Eluate II: volatiles of days 10 – 15 and 15 – 20) were stored in a freezer at –15°C and analyzed as described above. The experiments were replicated four times. The procedures of aeration and volatile treatment in inhibitor experiments are summarized in Fig. 3-5.

3.2.5 GC-MS analysis of 4,8-DMD

Mass spectra were obtained by a JEOL MS Router MS-600 coupled with a HP 6890N equipped with a HP-1MS column (30 m × 0.25 mm I.D, 0.25 μm, J&W Scientific). The oven temperature was maintained at 50°C for 1 min, programmed at 6°C/min to 200°C then 8°C/min to 270°C. 4,8-DMD in volatiles from RFB was identified by compared by its retention time and mass spectrum pattern with an authentic sample, synthesized as described in Chapter V. Quantification of 4,8-DMD was performed by comparison to standard curve constructed for the GC-MS by plotting the ratio of total ion chromatogram (TIC) peak area for various amounts of 4,8-DMD to that of methyl decanoate. The amount of 4,8-DMD expressed as ng/ male day equivalents (MDE). To quantify the amount of 4,8-DMD, the internal standard was added to the final concentrated eluate. This method is convenient for comparing the amount of 4,8-DMD in each treatment, but could lead to an underestimate of the absolute amount of 4,8-DMD.

3.2.6 Statistics analyses

In the JH III experiment, the mean amount of 4,8-DMD was compared between the each dose by one-way ANOVA followed by Fisher's LSD test (P = 0.05). In the

inhibitor experiments, the mean amount of 4,8-DMD between Eluate I and Eluate II for each treatment was compared by *t*-test ($P = 0.05$). Using one-way ANOVA followed by Fisher's LSD test ($P = 0.05$), the mean amounts of 4,8-DMD obtained in Aeration I (Eluate I) among three non-coated treatments were compared, and those in Aeration II (Eluate II) were analyzed similarly. The mean amount of 4,8-DMD into $\log_{10}(x + 1)$ were transformed to increase normality (Sokal and Rohlf, 1995).

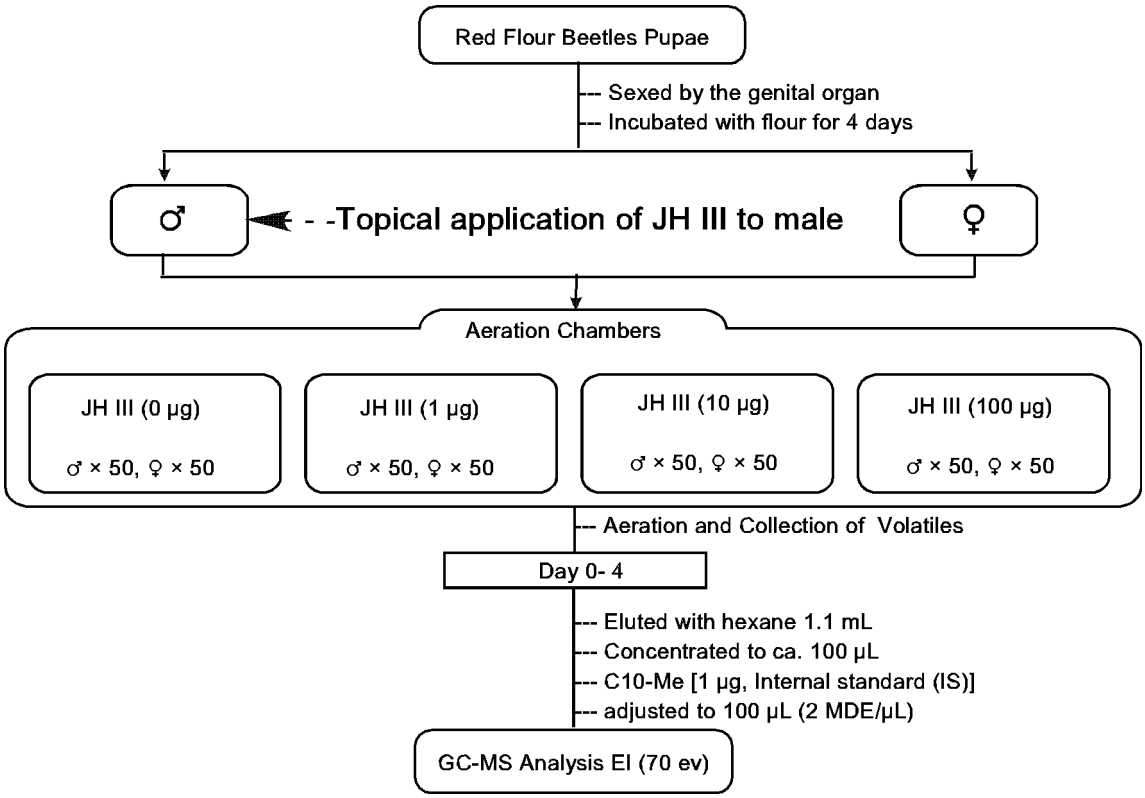


Fig. 3-3. Schematic procedure of JH III experiment.
C10-Me = methyl decanoate

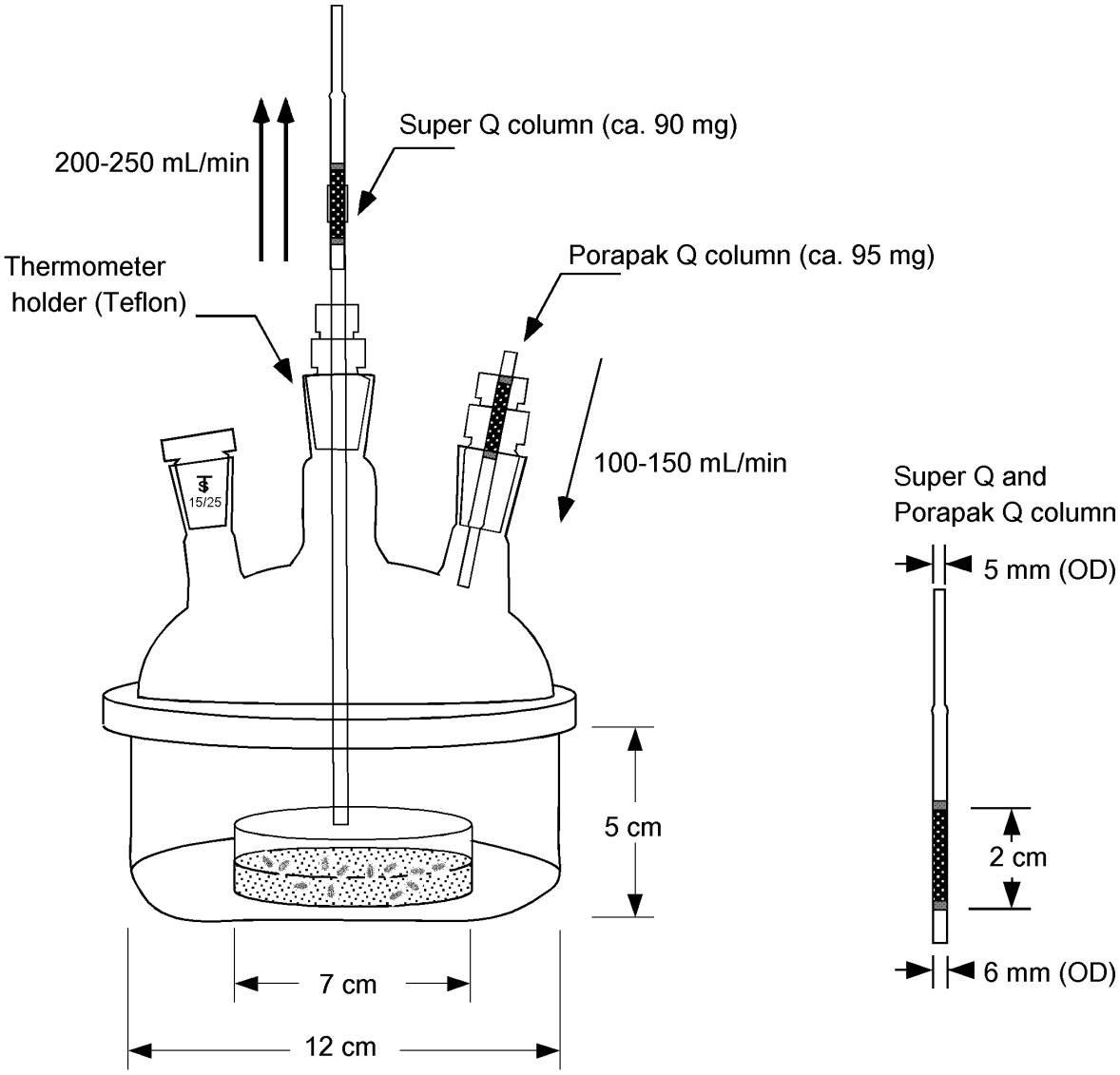


Fig. 3-4. Aeration apparatus used in inhibitor experiments.

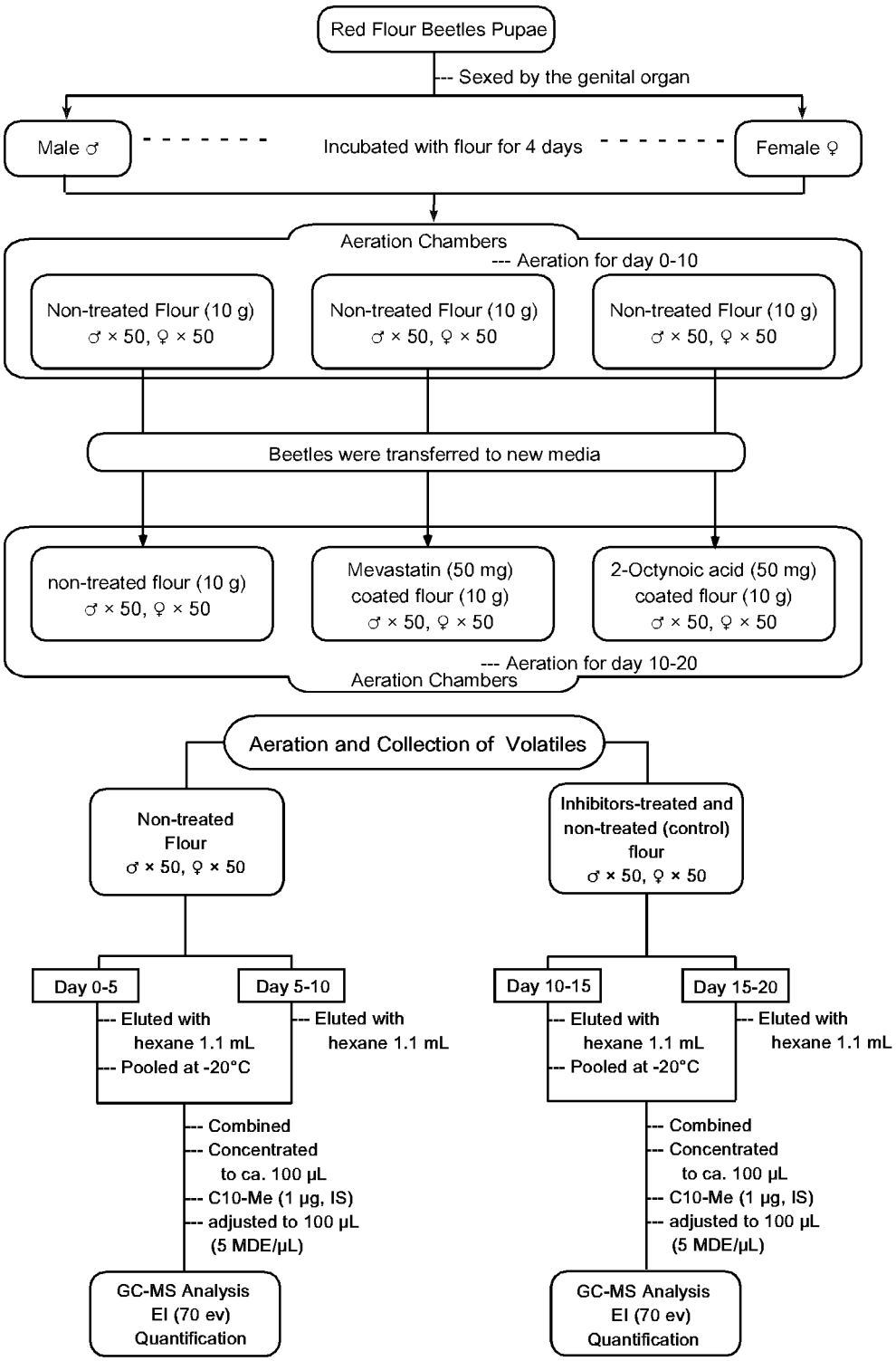
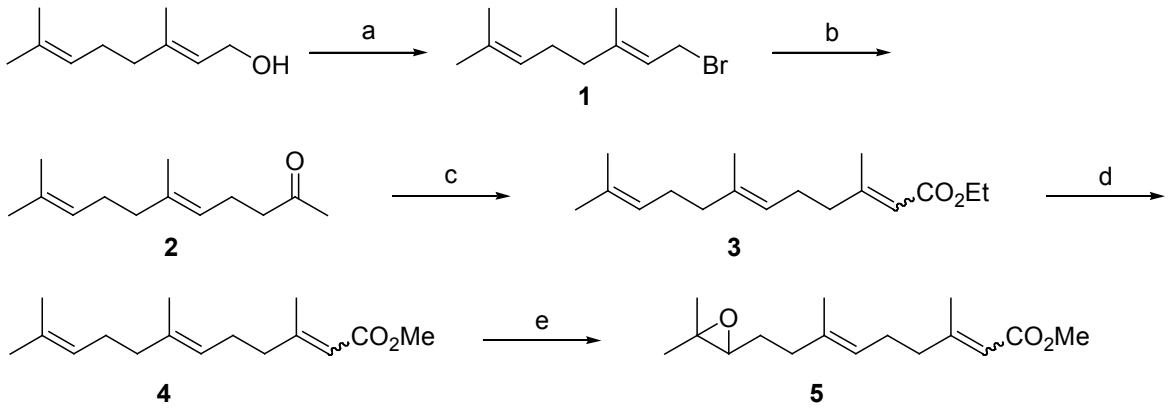


Fig. 3-5. Schematic procedures of inhibitor experiments.
C10-Me = methyl decanoate; IS = internal standard.

3.2.7 Synthesis

2-Octynoic acid was prepared as the procedure described in Freund et al. (1985), using 1-bromoheptyne, n-BuLi and dry ice. Scheme 3-1 shows the synthetic route of JH III. Geranyl bromide (**1**), derived from geraniol (Aldrich), was subjected to acetoacetate synthesis to afford geranylacetone (**2**), which was subjected to the Wittig-Horner reaction. The ethyl ester (**3**) thus obtained, was transesterified to the corresponding methyl ester (**4**) and epoxidation by the method of Hanzlik (1988) to afford JH III (**5**) (chemical purity: 94%, isomeric purity: 73%). The structures of synthetic compounds were confirmed by GC-MS spectra and ^{1}H- and ^{13}C-NMR (500 MHz and125 MHz, respectively) with a Bruker Avance 500 spectrometer using TMS in $CDCl_3$ as an internal standard.



Scheme 3-1. Synhtesis of JH III.
(a) $(C_6H_5)_3P$, Br_2, Et_3N, CH_2Cl_2, 0°C; (b) i) ethyl acetoacetate, NaH/THF, reflux, ii) 2N NaOH, 50-55°C then AcOH; (c) $(EtO)_2P(O)CH_2CO_2Et$, NaH/THF; d) KOH/MeOH, reflux; (e) i) NBS, THF-H_2O (3:1), 0°C, ii) K_2CO_3/MeOH

2-Ocytnoic acid

To a solution of 1-heptyne (1.50 g, 15.63 mmol) in THF (50 mL), n-butyl lithium 5.38 mL (8.51 mmol; 1.58 M) was added at −78°C under N_2. After stirring for 30 min,

chopped dry-ice was added to the solution. The reacted mixture was raised to room temperature and extracted with H_2O (3 × 50 mL). The aqueous layers were acidified with 2N HCl to pH 3 and extracted with ether (3 × 50 mL). The organic solution was washed with brine and dried up. Distillation gave 0.87 g of 2-octynoic acid (5.50 mmol, 73.1% yield). bp: 112-113°C/3 mmHg. Small portion of 2-octynoic acid was methylated with diazomethane and submited to GC-MS analysis. Purity: >99.9% as methyl ester. ^{1}H-NMR: 7.18 (1H, br. s, -O<u>H</u>), 2.34 (2H, t, *J* = 7.2, 4-C<u>H</u>$_2$), 1.59 (2H, m,5- C<u>H</u>$_2$), 1.42-1.38 (2H, m, 6-C<u>H</u>$_2$), 1.38-1.31 (2H, m, 7-C<u>H</u>$_2$), 0.91 (3H, t, *J* = 7.2, -C<u>H</u>$_3$). ^{13}C-NMR: 157.6 (1-C), 72.7 (2-C), 92.5 (3-C), 30.9 (6-CH_2), 27.1 (5-CH_2), 22.0 (7-CH_2), 18.7 (4-CH_2), 13.7 (8-CH_3). GC-MS (as methyl ester): 154 (M^+, 0.6), 139 (16.9), 123 (86.4), 95 (100), 79 (48.0), 67 (39.5), 55 (43.3). IR: 3400-2800 (br, s), 2200 (s), 1680 (s), 1250 (s).

Geranyl bromide (**1**)

To a solution of triphenylphosphine (14.41 g, 55.0 mmol) in CH_2Cl_2 (50 mL), 2.84 mL of bromine (55.0 mmol) was added dropwise at 0°C and the mixture was stirred for 30 min with cooling. To this solution, a mixture of geraniol (7.70 g, 50.0 mmol) and triethylamine (10.42 mL , 75.0 mmol) in CH_2Cl_2 (50 mL) was added. After stirring for 3 h at 0°C. The mixture was filtered and the solvent was evaporated *in vacuo*. The residue was extracted with petroleum ether (300 mL) and filtered off. The filter cake was rinsed with petroleum ether (3 × 60 mL). The combined filtrate were concentrated. After this procedure was done twice, the residue was distilled to give 5.27 g of crude **1** (24.29 mmol, 48.6% yield as crude). This compound was used for the next reaction without further purification. GC-MS: 175 (M^+-42, 3.2), 137 (M^+-Br, 27.7), 93 (36.2), 81 (19.4), 69 (100),

53 (6.6).

Geranylacetone (**2**)

To a suspension of sodium hydride (1.07 g, 26.72 mmol; 65%) in THF (20 mL), 3.47 g of ethyl acetoacetate (26.72 mmol) in THF (10 mL) was added at 0°C under N_2, and then the mixture was stirred for 30 min at room temperature. After 5.27 g of crude **1** (24.29 mmol) in THF (10 mL) was added to the solution, the mixture was refluxed for 1 h. After pouring ice-cooled water, the solution was extracted with ether (3 × 60 mL), and the solvent was evaporated. The residue was stirred for 3 h in 2N NaOH (200 mL) solution. The reacted solution was cooled, acidified with acetic acid, and extracted with ether (3 × 60 mL). The organic layer was washed with H_2O, $NaHCO_3$, brine, dried, and concetrated *in vacuo*. Distillation of the residue gave 2.07 g of **2** (10.67 mmol, 21.3% yield from geraniol, 96.0% pure). bp: 85-87°C/1 mmHg. ^{1}H NMR: 5.08 (2H, m), 2.46 (2H, t, J = 7.5), 2.26 (q, J = 7.4), 2.13 (3H, s), 2.06 (2H, q, J = 7.4), 1.97 (2H, t, J = 7.6), 1.67 (3H, d, J = 1.1), 1.61 (3H, s), 1.59 (3H, s). ^{13}C NMR: 209.2 (C), 136.8 (C), 131.8 (C), 124.6 (CH), 122.9 (CH), 44.1 (CH_2), 40.2 (CH_2), 30.3 (CH_3), 27.0 (CH_2), 26.1 (CH_3), 22.9 (CH_2), 18.0 (CH_3), 16.3 (CH_3). GC-MS: 194 (M^+, 4.9), 176 (3.5), 151 (33.1), 136 (29.8), 125 (20.3), 107 (21.2), 69 (62.6), 43 (100). IR: 3400 (br, w), 1710 (s), 1150 (s), 820 (m).

Ethyl 3,7,11-trimethyl-2,6,10-dodecatrienoate (ethyl farnesoate; **3**)

To a suspension of NaH (0.79 g, 21.34 mmol) in THF (20 mL), 4.78 g of TEPA (21.34 mmol) in THF (10 mL) was added at 0°C under N_2. After stirring for 30 min at 0°C then for 1 h at room temperature, 2.07 g of **2** (10.67 mmol) in THF (15 mL) was added at

0°C, then left overnight with stirring at room temperature. After adding water, the mixture was extracted with ether (3 × 50 mL). The organic layer was washed with brine, dried and concentrated *in vacuo*. The reside was distilled to afford 1.99 g of **3** (7.54 mmol, 70.7% yield, 94.4% pure, 2*E*:2*Z* = 8:2). This isomeric mixtures were used for the next step without further isolation. bp:144-148°C/ 2 mmHg. ^{1}H NMR: 5.66 (1H, d, *J* = 1.0), 5.08 (2H, m), 4.14 (2H, q, *J* = 7.1), 2.21-2.13 (7H, m), 2.06 (2H, q, *J* = 7.2), 1.98 (2H, t, *J* = 8.1), 1.68 (3H, d, *J* = 0.9), 1.60 (6H, s), 1.27 (3H, t, *J* = 7.1). ^{13}C NMR: 167.3 (C), 160.1 (C), 136.5 (C), 131.8 (C), 124.7 (CH), 123.3 (CH), 116.0 (CH), 59.8 (CH_2), 41.3 (CH_2), 40.1 (CH_2), 27.1 (CH_2), 26.3 (CH_2), 26.1 (CH_3), 19.2 (CH_3), 18.0 (CH_3), 16.7 (CH_3), 14.7 (CH_3). GC-MS (*E*,*E*-isomer): 264 (M^+, 6.2), 221 (10.1), 219 (12.8), 195 (3.0), 136 (14.1), 128 (59.6), 121 (31.7), 93 (9.1), 81 (30.4), 69 (100). IR: 1710 (s), 1640 (s), 1210 (s), 1140 (s), 900 (m), 710 (m).

Methyl 3,7,11-trimethyl-2,6,10-dodecatrienoate (methyl farnesoate; **4**)

Ethyl ester **3** was subjected to transesterification to afford the corresponding methyl ester. To a solution of KOH (0.63 g, 11.31 mmol) in methanol (60 mL), 1.99 g of **3** (7.54 mmol) was added. The mixture was refluxed for 4 h. After cooling, the solvent was evaporated. Water was added to the residue and the mixture was extracted with ether (3 × 50 mL). The organic layer was washed with H_2O and brine, dried, and concentrated *in vacuo*. The residue was chromatographed on silica (20 g, hexane:ether = 95:5) to give 1.23 g of **4** (4.92 mmol, 65.3% yield, 89.9% pure, 2*E*:2*Z* = 7:3). ^{1}H NMR: 5.67 (1H, d, *J* = 0.9), 5.08 (2H, m), 3.68 (3H, s), 2.18-2.15 (7H, m), 2.06 (2H, q, *J* = 7.4), 1.98 (2H, t, *J* = 7.6), 1.68 (3H, d, *J* = 0.9), 1.60 (6H, s). ^{13}C NMR: 167.6 (C), 160.5 (C), 136.6 (C), 131.8

(C), 124.7 (CH), 123.6 (CH), 115.6 (CH), 51.1 (CH_3), 41.3 (CH_2), 40.0 (CH_2), 27.1 (CH_2), 26.3 (CH_2), 26.0 (CH_3), 19.2 (CH_3), 18.0 (CH_3), 16.7 (CH_3). GC-MS: 250 (M^+, 6.1), 219 (5.8), 207 (11.2), 136 (15.0), 121 (29.0), 114 (47.4), 93 (9.5), 81 (25.1), 69 (100). IR: 1710 (s), 1640 (s), 1210 (s), 1140 (s), 900 (m), 710 (m).

Methyl 10,11-epoxyfarnesoate (JH III, **5**)

This compound was prepared *via* a bromohydrin according to the procedure reported by Hanzlik (1988). To a solution of **4** (1.23 g, 4.92 mmol) in THF-H_2O (30 mL, 3:1), 0.96 g of *N*-bromosuccimide (5.41 mmol) was added in a portion, while temperature was kept below 5°C. After stirring for 4 h with cooling, the solvent was evaporated. The residue was dissolved in pentane-ether (150 mL, 1:1). The mixture was washed with $NaHCO_3$**,** and brine. Concentration of the mixture gave crude bromohydrin-farnesoate. To a solution of crude bromohydrin-farnesoate in methanol (30 mL), 2.04 g of K_2CO_3 (14.76 mmol) was added. After stirring for 3 h at room temperature, water (60 mL) was added to the mixture, which extracted with petroleum ether (3 × 30 mL). The organic layer was washed with $Na_2S_2O_3$, and brine, dried and concentrated *in vacuo*. The residue was chromatographed on silica (20 g) to afford 0.95 g of **5** (3.57 mmol, 72.6% yield in 2 steps, 93.8% pure as 2*EZ*-mixture, *E*:*Z* =2.7:1). This isomeric mixtures were used for the bioassay without further isolation. ^{1}H NMR: 5.67 (1H, d-like, J = 1.1), 5.14 (1H, m), 3.68 (3H, s), 2.69 (1H, t, J = 6.2), 2.20-2.07 (6H, m), 2.16 (3H, d, J = 1.3), 1.63-1.59 (5H, m), 1.30 (3H, s), 1.26 (3H, s). ^{13}C NMR: 167.6 (C), 160.3 (C), 135.7 (C), 123.9 (CH), 115.7 (CH), 64.5 (CH), 58.7 (C), 51.1 (CH_3), 41.3 (CH_2), 36.7 (CH_2), 27.7 (CH_2), 26.3 (CH_2), 25.2 (CH_3),19.2 (CH_3), 19.1 (CH_3), 16.4 (CH_3). GC-MS (*E*,*E*-isomer): 266 (M^+, 2.2), 248

(2.3), 234 (8.9), 195 (8.7), 163 (19.6), 135 (56.8), 114 (48.0), 85 (24.7), 81 (100), 71 (44.3), 69 (48.4), 43 (40.3). IR: 1710 (s), 1640 (s) 1210 (s), 1140 (s), 860 (m).

3.3 RESULTS AND DISCUSSION

The production of 4,8-DMD was significantly reduced with increasing doses of JH III ($F_{[3,8]}$ = 18.1493; P = 0.0006) (Table 3-1). In the inhibitor experiments, 4,8-DMD was obtained in the range of 4.76–11.68 ng/MDE, when RFBs were fed non-treated flour during the first 10 days (Aeration I), and there was no significant difference ($F_{[2,9]}$ = 1.0278; P = 0.3963) among the mean amounts of 4,8-DMD in each of the non-treated groups (Fig. 3-6). The RFBs produced larger amounts of 4,8-DMD during days 10–20 (Eluate II) than during days 0–10 (Eluate I) (t-test, P<0.01). However, there were significant differences among the mean amounts of 4,8-DMD obtained from inhibitor feeding experiments ($F_{[2, 9]}$ = 1 3.3315; P = 0.0020) (Fig. 3-6). The amount of 4,8-DMD from mevastatin treatment was 21.00 ± 3.27 ng/MDE (mean ± SD), which was the same as from non-coated treatment (22.90 ± 3.27 ng/MDE). In contrast, the production of 4,8-DMD was significantly inhibited (13.13 ± 0.91 ng/MDE) by 2-octynoic acid (Fig. 3-6). These results suggested that the 4,8-DMD produced by *T. castaneum* is derived from the fatty acid pathway rather than from the mevalonate pathway.

Topical application of JH III caused a reduction in the level of 4,8-DMD production. To my best knowledge, an inhibitory effect of JH III on fatty acids production or the action of FAS has not been reported. Ivarsson and Birgersson (1995) reported that higher doses of methoprene suppressed the production of pheromones in *I. duplicatus*. A similar suppression mechanism seems to account for the effect of higher doses of JH III observed here. However, it is surprising that even the lowest dose (1 μg/male) of JH III reduced the production of 4,8-DMD. One possible interpretation is that even 1 μg of JH III is too much for a young male RFB (4-day-old). In contrast to this results, Pierce et al.

(1986) reported that the production of both 4,8-DMD and non-methyl branched cucujolides, which are aggregation pheromones of *Oryzaephilus mercator*, *O. surinamensis*, and *Cryptolestes ferrugineus* (Cucujidae), were enhanced when these species were fed methoprene-treated oats for 20 days. However, the non-methyl branched cucujolides were later demonstrated to be of fatty acid origin (Vanderwel et al., 1990 and 1992). Considering the results of Vanderwel et al. and our present results, the enhanced production of 4,8-DMD reported by Pierce et al. (1986) appears to be different from that of terpene pheromones observed in *I. duplicatus* and *I. pini*, which occurred by activation of the enzymes (HMG-R and HMG-S) involved in the mevalonate pathway (Tittiger, 2003).

The fact that the production of 4,8-DMD was not inhibited by mevastatin but was inhibited by 2-octynoic acid suggests that 4,8-DMD produced by RFB is derived from the fatty acid pathway rather than from the mevalonate pathway. These inhibitors have been used to provide indirect information on the biosyntheses of pheromones, and they have been administrated by micro-injection or topical application to the pheromone gland (Seybold and Vanderwel, 2003; Ivarsson et al, 1993; Barkawi et al., 2003; Zhao et al., 2004). However, oral administration was conducted in our study, because the pheromone gland for 4,8-DMD remains unknown. The femoral setiferous glands has been reported to be the major site of production of 4,8-DMD (Faustini et al., 1981), but it was also reported that they are not the sole source of 4,8-DMD (Hussain, 1993; Qazi et al., 1998). Oral administration was also used because of technical difficulties in micro-injection; specifically, there was high mortality following injection of the RFBs with either glass capillaries or a micro syringe fitted with a 33-gauge needle.

Table 3-1. Effect of JH III on the production of 4,8-DMD in *Tribolium castaneum*.

Dose of JH III (μg)	Mean amounts of 4,8-DMD (ng/MDE, ± SD)
0	4.36 ± 0.89 [a]
1	3.00 ± 0.40 [b]
10	2.65 ± 0.40 [b]
100	1.56±0.27 [c]

Values followed by the same letter are not significantly different (ANOVA, $P < 0.05$, means compared by Fisher's LSD). All experiments were replicated three times with 50 JH III-treated males and 50 non-treated females.

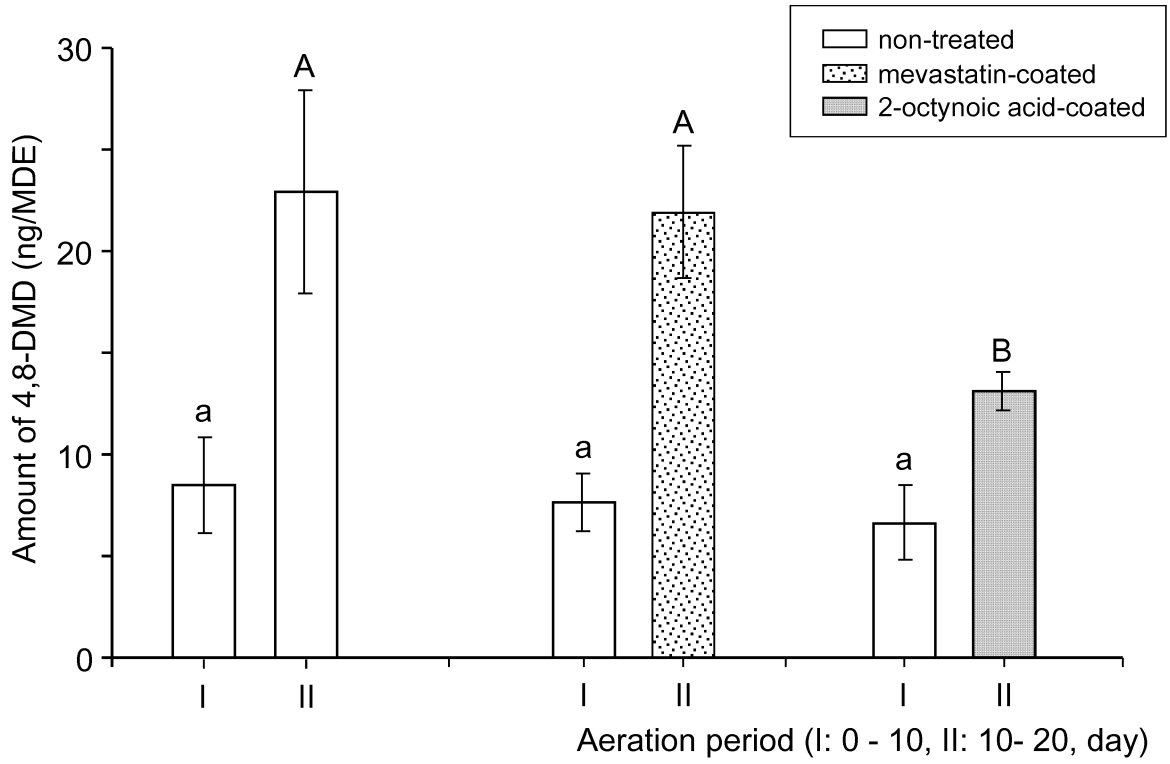


Fig. 3-6. Effect of inhibitors on the production of 4,8-DMD by *Tribolium castaneum*.
Values (mean ± SD) follwed by the same letter are not sigificantly different (ANOVA, $P < 0.05$, means compared by Fisher's LSD). All experiments were replicated four times with 50 males and 50 females.

CHAPTER IV

EXAMINATION OF THE FATTY ACID PATHWAY AS A BIOSYNTHETIC PATHWAY OF 4,8-DIMETHYLDECANAL

4.1 INTRODUCTION

Methyl-branched lipids, produced by insects, consist of long-chain fatty acids and their derivatives and hydrocarbons. Biosynthetic studies on the insect lipids revealed that propionate and some methyl-branched amino acids (such as, valine and leucine) were the source of methyl group(s) in the methyl-branched lipids (for review, Nelson, 1993) (Fig. 4-1). Propionate serves in the form of methylmalonyl-CoA in biosynthesis of insect methyl-branched lipids. In coleopteran pheromones, 4-methyl-1-nonanol, the sex pheromone of *Tenebrio molitor* and the aggregation pheromones of *Carpophilus* spp, which are biosynthesized through the fatty acid and polyketide pathways, respectively, have methyl branch(es) in their chain structures. The origin of methyl branch(es) of these pheromones were demonstrated to be derived from propionates by the incorporation experiments of isotope-labeled propionate (Petroski et al., 1994; Bartelt and Weisleder, 1996; Islam et al., 1999) (Fig. 4-2).

Since 4,8-DMD was suggested to be biosynthesized through the fatty acid pathway from the results of Chapters II and III, fatty acid pathway to 4,8-DMD was examined. Fig. 4-3 shows the hypothesized biosynthetic pathway of 4,8-DMD which involves the chain elongation with three units of acetate (Ac) and two units of propionate (Pr) for two methyl branches in the following sequence Ac-Pr-Ac-Pr-Ac. Bases on the facts that acetate serves as the building block for the fatty acid and propionate is the origin of the methyl branch in methyl-branched lipid, it was predicted that acetate and propionate would be incorporated into 4,8-DMD by RFBs and 2-methylbutanoate (C5H), 4-methylhexanoate (C7H), 2,6-dimethyloctanoate (C10H) and 4,8-dimethyldecanoate (C12H) would serve as intermediates or precursors of 4,8-DMD. This pathway is also expected to involve a series

of chain elongation reactions, that is, condensation of acetyl-CoA and acyl CoA, reduction of carbonyl group, dehydration, and reduction of the double bond (Lehninger et al., 1993; see Fig. 3-2 in Chapter III).

In order to examine that 4,8-DMD is of fatty acid origin and whether 4,8-DMD was biosynthesized through the hypothesized pathway, incorporation experiments of ^{13}C-labeled acetate and propionate and deuterium-labeled putative precursors [2-methylbutanoate (C5H), 4-methyl-hexanoate (C7H), 2,6-dimethyloctanoate (C10H) and 4,8-dimethyldecanoate (C12H)] were performed. In addition, the incorporations of ^{13}C-labeled acetate and propionate into 2-hydroxy-4-methoxypropiophenone (2,4-HMPP) which expected to be of fatty acid and/or polyketide origin, were also investigated.

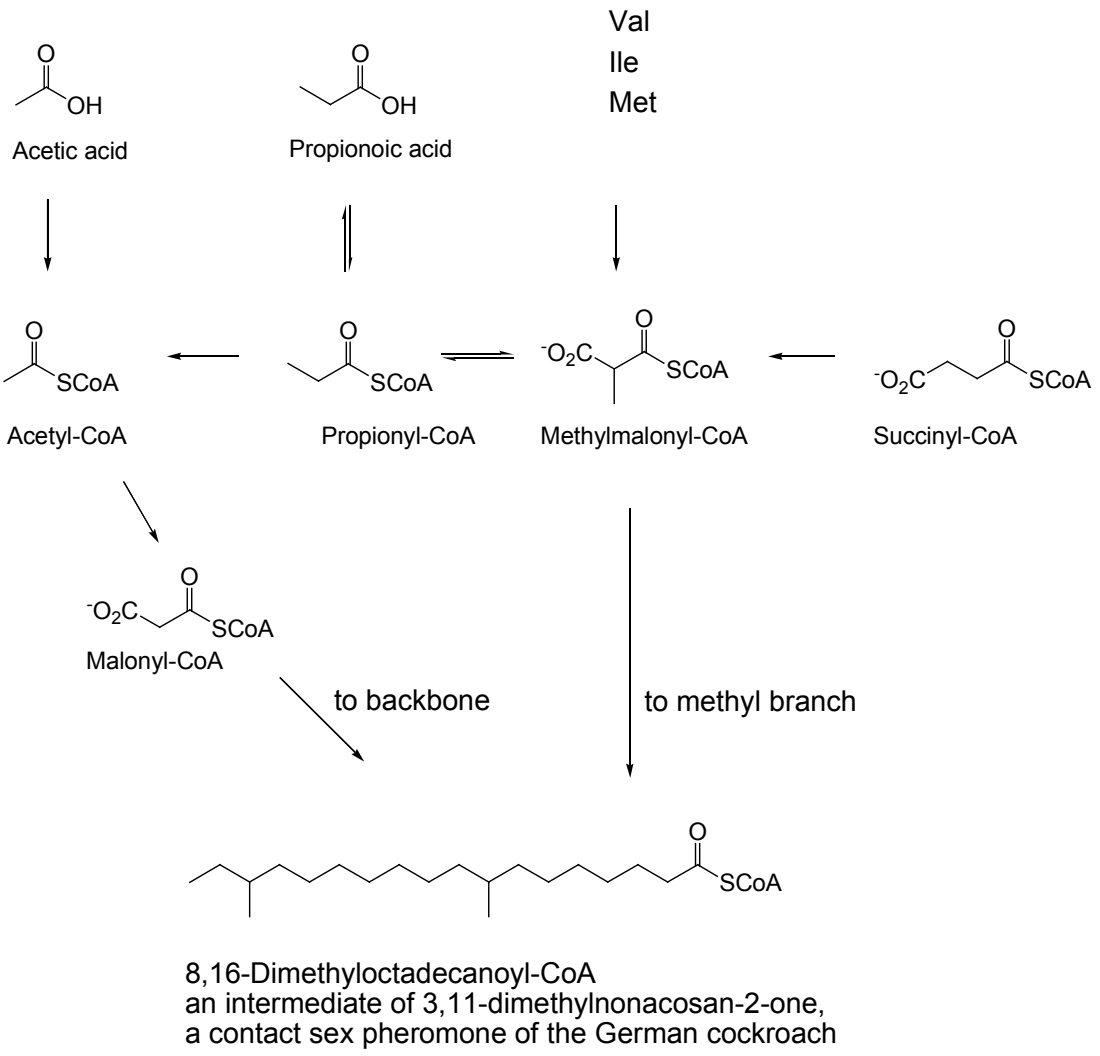


Fig. 4-1. Biosynthetic pathway of a methyl-branched insect lipid (modified from Chase et al., 1992).

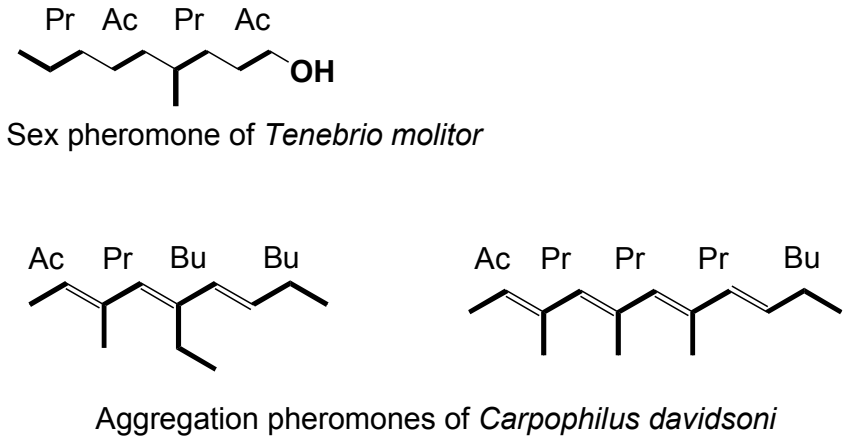


Fig. 4-2. Methyl branched pheromones in Coleoptera.
Ac, acetate; Pr, propionate; Bu, butanoate.

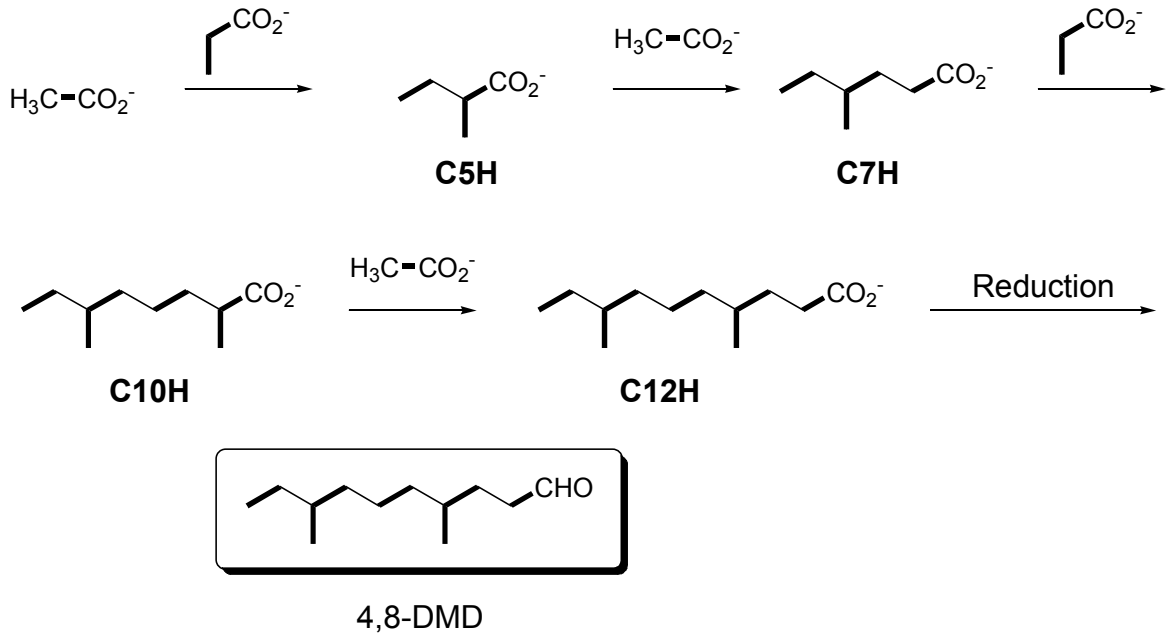


Fig. 4-3. The hypothesized biosynthetic pathway of 4,8-DMD in *T. castaneum* via a modified fatty acid pathway. The chain elongation would involve carbonyl reduction, dehydration, and reduction of the double bond.

4.2 Materials and Methods

4.2.1 Insect cultures

A laboratory colony of *T. castaneum*, was used in all experiments. The beetles were reared on a mixture of whole wheat flour and 5% brewer's yeast at 27 ± 1°C, ca. 70% RH on a 16:8 L:D cycle. Pupae were sexed by the genital organ and reared in separate container for 4 days with media, and 4-day-old beetles were used for incorporation experiments.

4.2.2 Chemicals and materials

[1-^{13}C]Sodium acetate and [1-^{13}C]sodium propionate were purchased from Cambridge Isotope Laboratories (Andover, MA). Sodium acetate and sodium propionate were purchased from Kanto Chemical (Tokyo, Japan). Following deuterium-labeled and non-labeled putative precursors (sodium salts) were prepared as described in Chapter V (Numbers in bold represent the numbers of compounds in Chapter V.): 2-Trideuterio-methylbutanoate (C5D; **3**), 4-methylhexanoate-*3,3-d_2* (C7D, **9**), 2-trideuteriomethyl-6-methylocatanoate (C10D, **15**), and 4,8-dimethyldecanoate-*3,3-d_2* (C12D; **23**). 2,4-HMPP were prepared by Suzuki (1975). Solvents were purified prior to use.

4.2.3 Preparation of flours coated with isotopically labeled and unlabeled precursors

Each sodium salt (500 mg, 5% by weight to medium) was dissolved in 1-2 mL of distilled water and diluted to 20 mL with ethanol. To the solution, 10 g of whole wheat flour was added and the solvent was evaporated with heating. The lump of flour was

crushed with a mortar and pestle and then sifted with a tea sieve.

4.2.4 Collection and elution of insect volatiles

For collecting the insect volatiles, aeration condition and treatment of captured volatiles were the same as described in [2-^{13}C]mevalonolactone experiment (200 mL Erlenmeyer flask, Fig. 2-4 in Chapter II). In case of experiments using deuterium-labeled putative precursor, each pooled eluate was concentrated to ca. 100 μL, then chromatographed on a SiO_2 column (400 mg Wakogel®, Pasteur pipette) using step-wise elution with hexane (1 mL), 5% ether in hexane (2 mL), and ether (1 mL). 4,8-DMD was eluted with 5% ether in hexane solution. This fraction was concentrated to 100 μL and analyzed by GC-MS after addition of methyl decanoate (1 μL of 1000 ppm hexane solution). Fig. 4-4 shows schematic procedures of incorporation experiments and treatment of insect volatiles.

4.2.5 GC-MS analyses of 4,8-DMD

GC-MS analyses of 4,8-DMDs obtained from incorporation experiments were done with a JEOL MS Router MS-600 coupled with a HP 6890N equipped with a HP-1MS column (30 m × 0.25 mm I.D, 0.25 μm, J&W Scientific). The oven temperature was programmed as same to the GC analysis. Incorporation of isotopically labeled substrate into the pheromone was monitored by single ion monitoring (SIM) of the fragments at *m/z* (140 + *n*) on EI analysis and at [M^+H (185) + *n*] on CI analysis in TIC of 4,8-DMD (where *n* represent the different mass unit value(s) between labeled and unlabeled 4,8-DMDs; Fig. 4-5). GC-MS-CI analysis was carried out using isobutane as a reagent gas. Incorporation

rate of each isotopically labeled substrate was calculated by the Equation 2-1 in Chapter II. 4,8-DMD and 2,4-HMPP in the eluate were identified by comparing their retention time and mass spectra to those of authentic standards.

4.2.6 Statistics analyses

The mean amount of 4,8-DMD of each treatment was compared as described in Chapter III. The incorporation of ^{13}C-labeled acetate and propionate into 4,8-DMD was confirmed by comparing between the relative intensities of diagnostic fragments of 4,8-DMD derived from ^{13}C-labeled and non-labeled substrate using *t*-test (P = 0.05). Diagnostic fragments of 4,8-DMD were *m/z* 72, 86, 112, 138, 141 and 142 in EI-analysis. Similarly, the incorporation of ^{13}C-labeled substrates into 2,4-HMPP was analyzed.

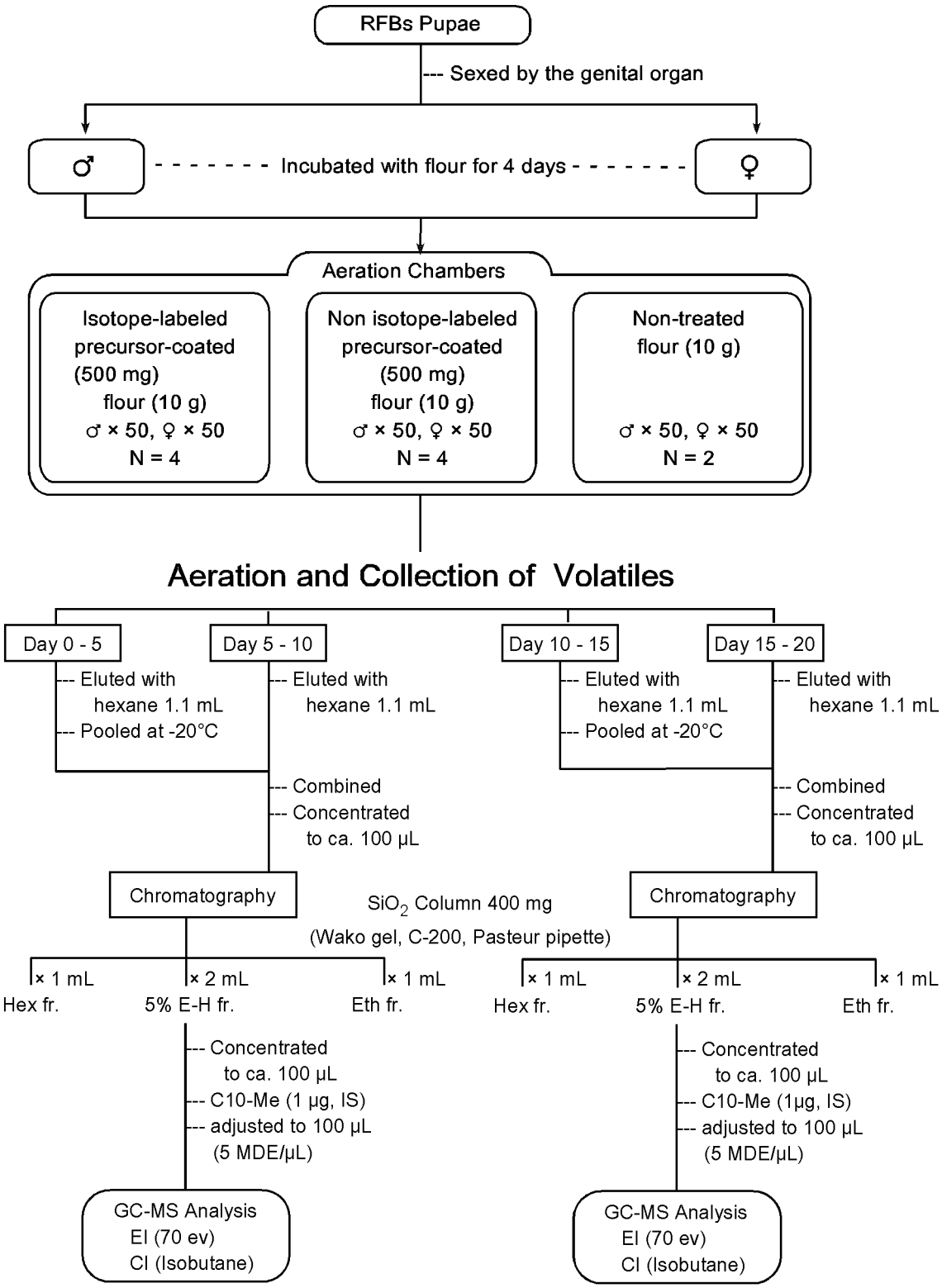


Fig. 4-4. Schematic procedures of aeration and treatments of the insect volatiles in incorporation experiment. The insect volatiles obtained from [1-^{13}C]acetate and [1-^{13}C]propionate experiments were analysized without chromatography.

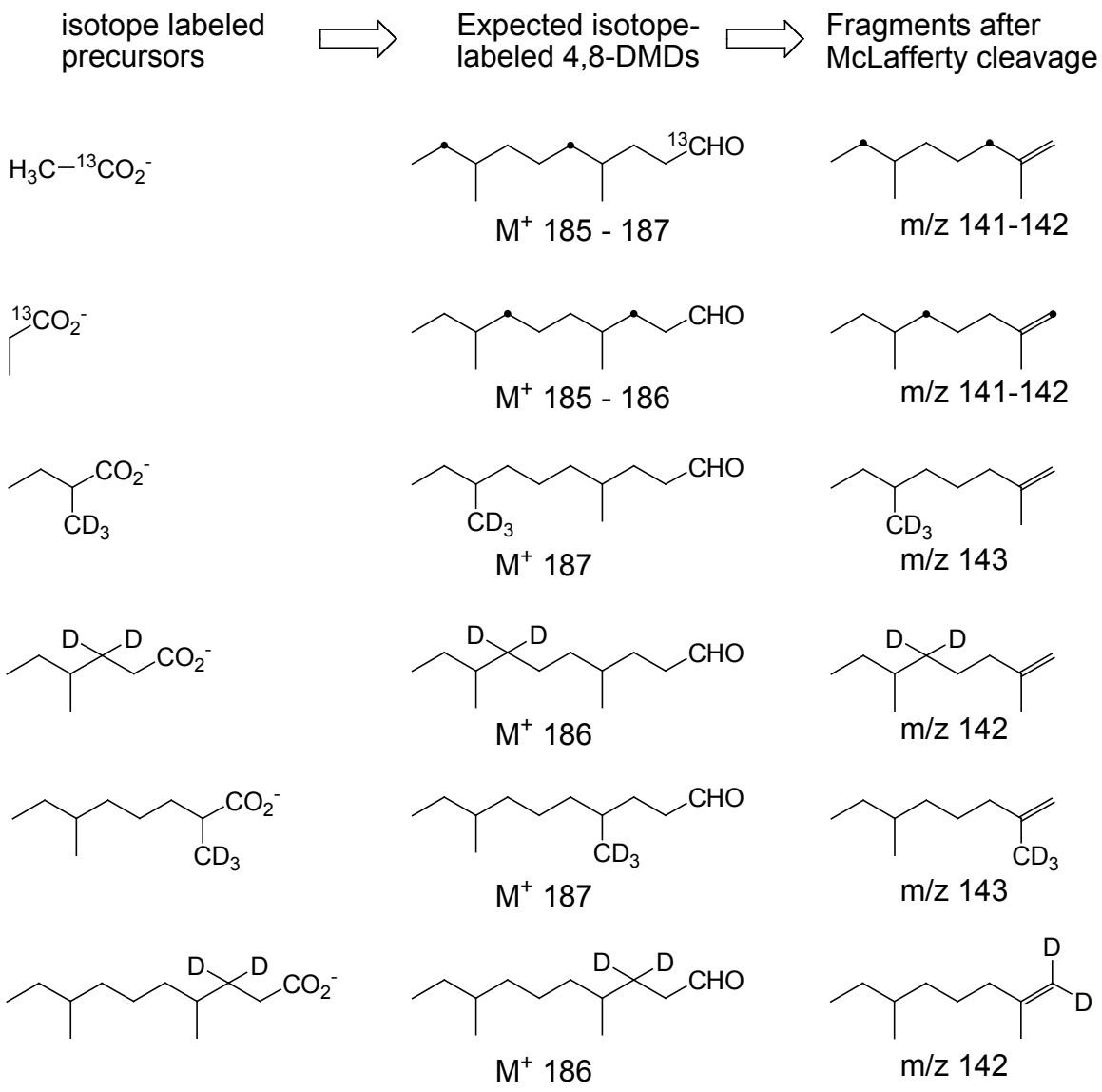


Fig. 4-5. Expected structures of 4,8-DMDs, when labeled-precursors were incorporated, and their highest fragment ions in GC-MS-EI analysis.
Dots on the line represent ^{13}C atoms.

4.3 Results

4.3.1 Incorporation of [1-^{13}C]acetate and [1-^{13}C]propionate

The mean amounts of 4,8-DMD were not significantly different between the feeding experiments of ^{13}C-treated flour and non-treated flour (Table 4-1).

In the mass spectra of 4,8-DMD obtained from RFBs exposed to ^{13}C-labeled acetate and propionate, the relative intensities of all diagnostic fragments were significantly increased compared to RFBs exposed to the unlabeled substrates (Tables 4-2 and 4-3, Figs. 4-6, 4-7, 4-8, and 4-9). This indicated that [1-^{13}C]acetate and [1-^{13}C]propionate were incorporated into 4,8-DMDs by the RFBs. Because it was expected that the position of ^{13}C atoms in 4,8-DMD are C-1, 5 and 9 and C-3 and 7, respectively, and the enrichment of 4,8-DMD in ^{13}C atom will result in the increase of the relative intensities of the diagnostic fragments (*m/z* 72 and 86 to base ion , *m/z* 112 to 111, *m/z* 138 to 137, and *m/z* 141 and 142 to 140), if [1-^{13}C]acetate and [1-^{13}C] propionate are incorporated into 4,8-DMD through the hypothesized pathway. When the RFBs were treated with [1-^{13}C]acetate, approximately 9% of the 4,8-DMD was enriched in one ^{13}C atom, and approximately 1–2% was enriched with two ^{13}C atoms (Table 4-4). While, when the insects were treated with [1-^{13}C] propionate, approximately 14–21% of the 4,8-DMD was enriched in one ^{13}C atom, and approximately 4.7–10% was enriched in two ^{13}C atoms (Table 4-5). 4,8-DMD enriched in three ^{13}C atoms could not be detected, due to low intensity of the fragment at *m/z* 143. The higher incorporation of the ^{13}C-labeled acetate and propionate, especially propionate, clearly provided the evidence that 4,8-DMD is of fatty acid origin.

^{13}C-Labeled acetate and propionate were incorporated into 2,4-HMPP by the

RFBs (Table 4-6, Figs. 4-10 and 4-11). The fragments at *m/z* 151 and 180 (M^+) will be shifted to *m/z* 152 and 181, respectively, by incorporation of ^{13}C-labeled substrates. GC-MS-EI analysis indicated that the relative intensities of the fragments at *m/z* 152 to 151 and *m/z* 181 to 180 in 2,4-HMPP were increased by exposure to ^{13}C-labeled substrates by RFBs. When RFBs were exposed to [1-^{13}C]acetate, ca. 2.5–4.7% of the 2,4-HMPP was enriched in ^{13}C atom, and when exposed to [1-^{13}C]propionate, ca 24.4-30.2% was enriched in ^{13}C atom (Table 4-7). The interpretation of MS fragment pattern of 2,4-HMPP revealed that the carbon of the carbonyl group were enriched in ^{13}C atom by the exposure to [1-^{13}C]propionate.

Table 4-1. Mean amounts of 4,8-DMD produced by RFBs in incorporation experimnents of flours coated with labeled or non-labeled aceate and propionate.

	Mean amounts of 4,8-DMD (ng/MED, mean ± SD, N = 4)	
Treatment	I	II
Non-treated (Control)	4.37 ± 2.78	8.36 ± 3.03
Acetate	4.92 ± 3.04	6.59 ± 2.49
[1-^{13}C]Acetate	5.01 ± 2.69	7.76 ± 5.85
Propionate	3.55 ± 1.25	4.32 ± 1.82
[1-^{13}C]Propionate	2.70 ±0.80	4.86 ± 2.27

I, II: aeraion period days 0 – 10 and days 10 – 20, respectively.
There was no significant difference among the mean amounts of 4,8-DMD in each treatment (*t*-test, $P > 0.05$).

Table 4-2. The relative intensities of some diagnostic fragments in 4,8-DMDs obtained from [1-^{13}C]acetate and acetate incorporation experiments.

Aeration period	Treatment	Relative intensities of diagnostic fragments (%, mean ± SD, n = 4)					
		m/z 72 (A)	*m/z* 86 (A)	*m/z* 112 (B)	*m/z* 138 (C)	*m/z* 141 (D)	*m/z* 142 (D)
Days 0 – 10	Acetate	7.16 ± 0.63	3.46 ± 0.33	29.83 ± 2.32	29.66 ± 3.76	19.03 ± 1.89	1.83 ± 0.16
	[1-^{13}C]Acetate	9.66 ± 0.49***	6.12 ± 0.99**	35.33 ± 4.28*	38.97 ± 5.74*	28.11 ± 4.78**	3.29 ± 1.07*
Days 10 – 20	Acetate	7.99 ± 0.73	4.15 ± 0.63	31.23 ± 1.67	28.32 ± 1.61	17.95 ± 0.72	1.93 ± 0.22
	[1-^{13}C]Acetate	9.79 ± 0.59**	6.08 ± 0.46**	34.11 ± 1.83*	36.29 ± 1.48***	25.66 ± 1.63***	3.20 ± 0.38**

A: % to base ion (*m/z* 54); B: % to *m/z* 112; C; % to *m/z* 137, D; % to *m/z* 140.
Mean values of relative intensities of the fragments in 4,8-DMDs obtained from [1-^{13}C]acetate and acetate treatment were compared by *t*-test (one tailed).
*, **, and ***: Significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Table 4-3. The relative intensities of some diagnostic fragments in 4,8-DMDs obtained from [1-^{13}C]propionate and propionate incorporation experiments.

Aeration period	Treatment	Relative intensities of diagnostic fragments (%, mean ± SD, N = 4)					
		m/z 72 (A)	*m/z* 86 (A)	*m/z* 112 (B)	*m/z* 138 (C)	*m/z* 141 (D)	*m/z* 142 (D)
Days 0 – 10	Propionate	7.85 ± 0.80	4.42 ± 0.57	31.72 ± 0.92	28.10 ± 2.68	18.24 ± 2.25	2.04 ± 0.33
	[1-^{13}C]Propionate	12.56 ± 0.61***	9.29 ± 0.64***	51.50 ± 3.28*	55.75 ± 3.66***	38.54 ± 2.14**	11.77 ± 1.58*
Days 10 – 20	Propionate	7.74 ± 0.35	4.31 ± 0.92	30.88 ± 1.06	29.98 ± 0.90	18.97 ± 0.82	1.75 ± 0.21
	[1-^{13}C]Propionate	12.10 ± 1.41***	8.64 ± 0.77***	46.98 ± 3.11***	46.46 ± 3.81***	35.36 ± 2.28***	8.30 ± 1.43***

A: % to base ion (*m/z* 54); B: % to *m/z* 112; C; % to *m/z* 137; D: % to *m/z* 140.
Mean values of relative intensities of the fragments in 4,8-DMD obtained from [1-^{13}C]propionate and propionate treatment were compared by *t*-test (one tailed).
*, **, and ***: Significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Table 4-4. Incorporation of [1-^{13}C]acetate into 4,8-DMD.

	Enrichment factor (EF, %)	
Aeration period	A	B
I	9.05 ± 1.71	0.98 ± 0.65
II	9.30 ± 1.90	2.15 ± 0.80

A, B: incorporation by one unit and two units of [1-^{13}C]acetate, respectively.
I, II: aeraion period days 0 – 10 and days 10 – 20, respectively.
The values (mean ± SD, N = 4) was caculated by GC-MS CI analysis, followed by equation 2-1.

Table 4-5. Incorporation of [1-^{13}C] propionate into 4,8-DMD.

	Enrichment factor (EF, %)	
Aeration period	A	B
I	21.00 ± 7.60	10.06 ± 3.85
II	14.34 ± 1.96	4.73 ± 0.45

A, B: incorporation by one unit and two units of [1-^{13}C]acetate, respectively.
I, II: aeraion period days 0 – 10 and days 10 – 20, respectively.
The values (mean ± SD, N = 4) was caculated by GC-MS CI analysis, followed by Equation 2-1.

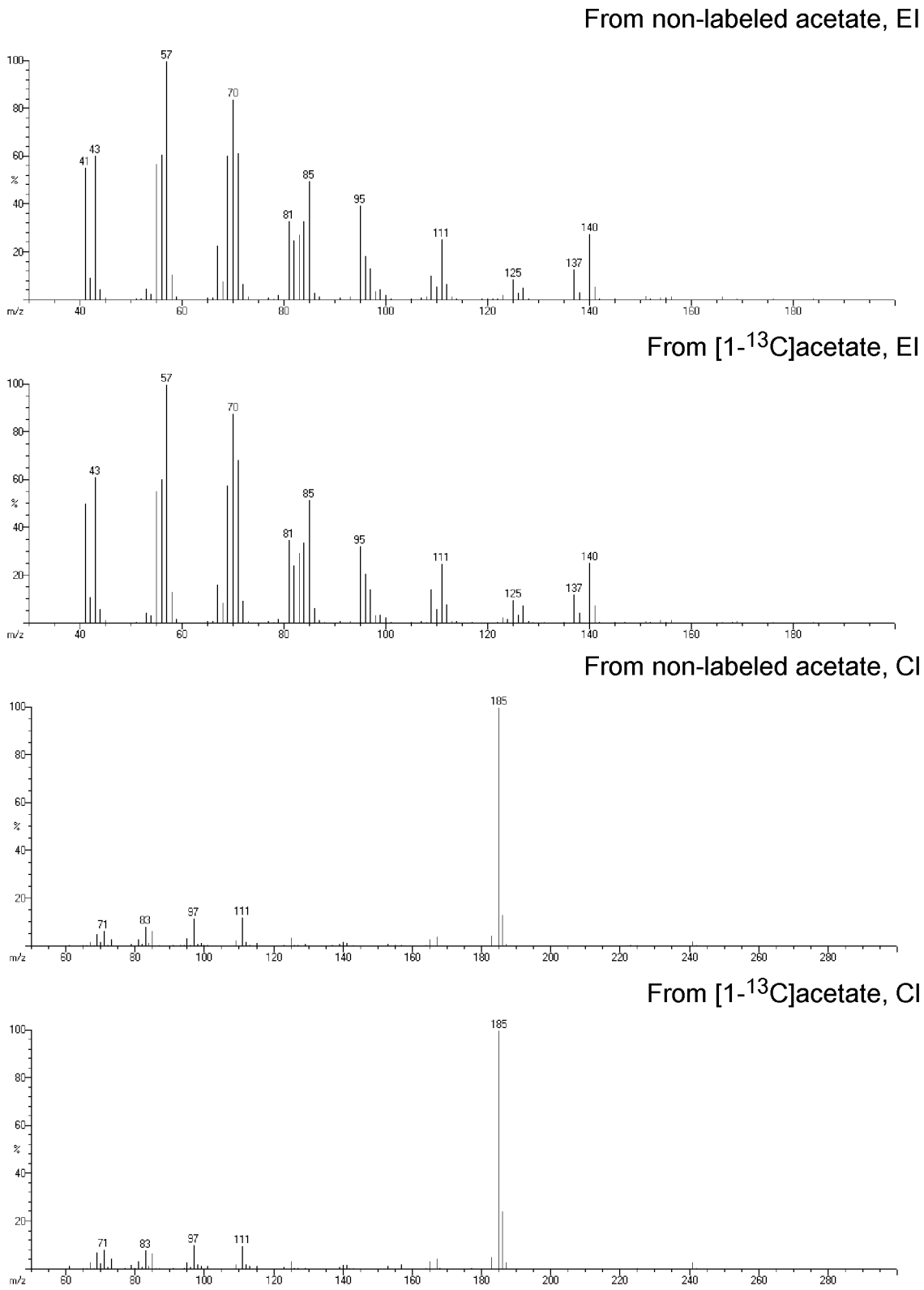


Fig. 4-6. MS spectra (EI and CI analysis) of 4,8-DMDs obtained from acetate and [1-^{13}C]acetate incorporation experiments (Aeration period: days 0 – 10).

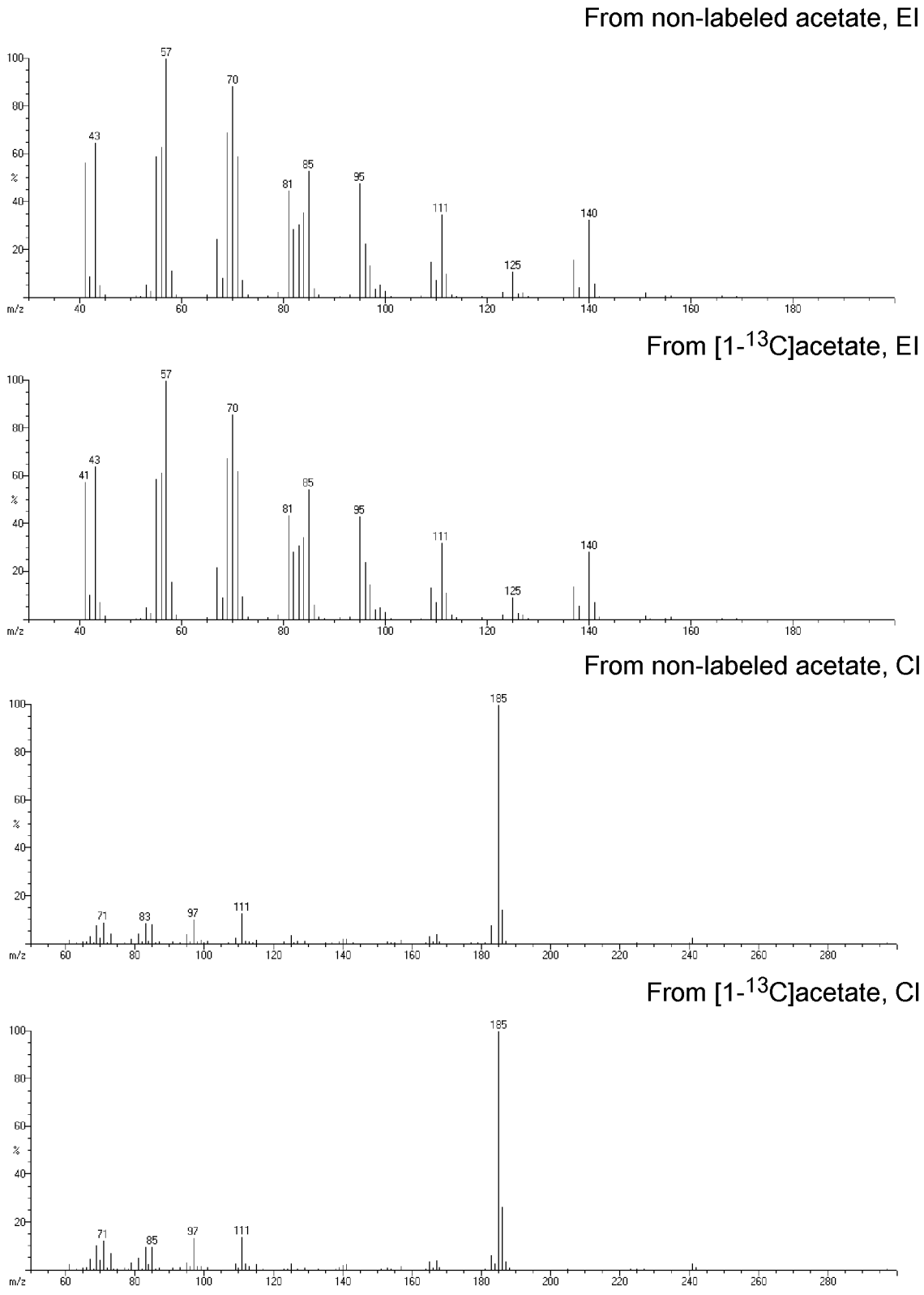


Fig. 4-7. MS spectra (EI and CI analysis) of 4,8-DMDs obtained from acetate and [1-^{13}C]acetate incorporation experiments (Aeration period: days 10 – 20).

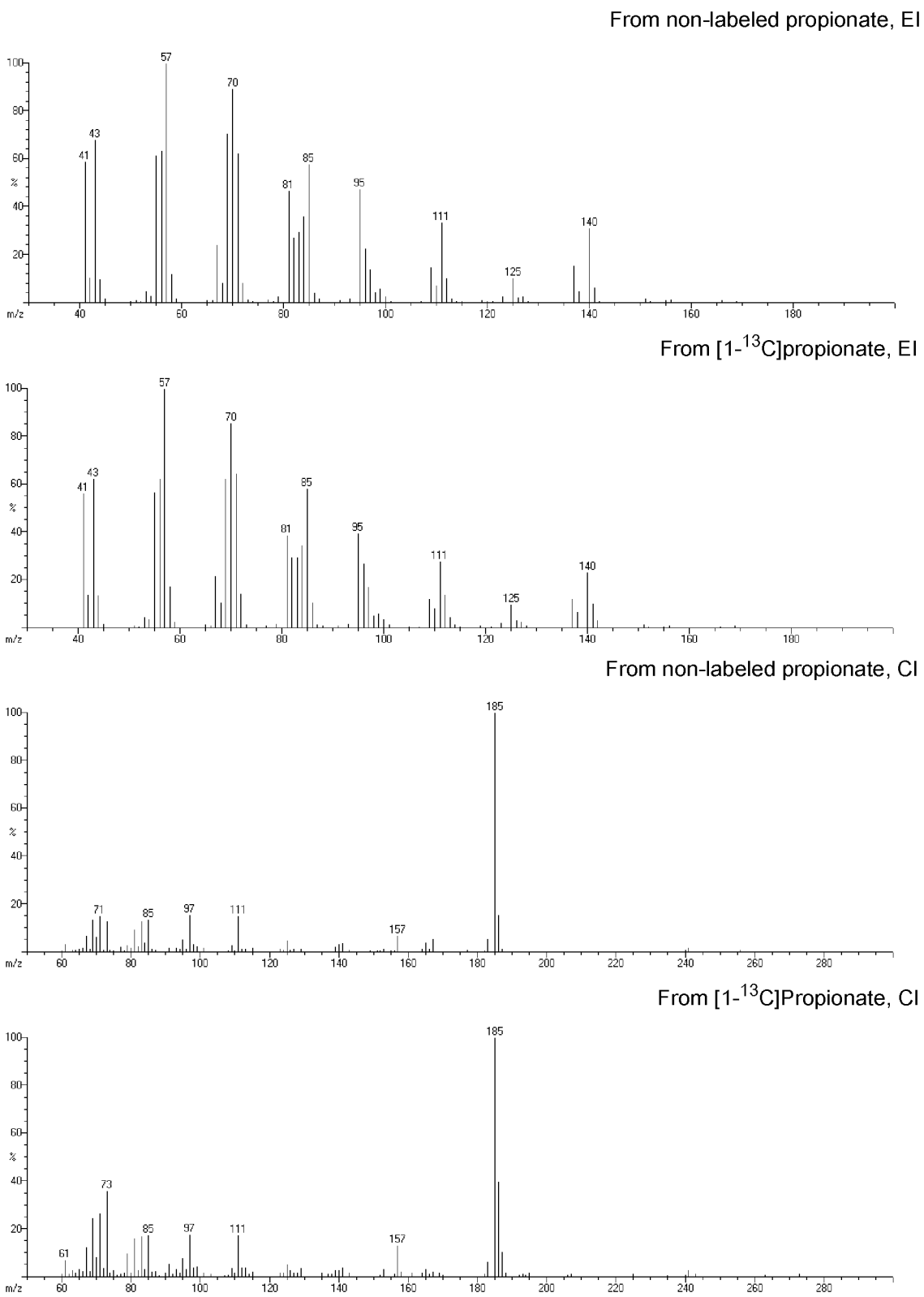


Fig. 4-8. MS spectra (EI and CI analysis) of 4,8-DMDs obtained from propionate and [1-^{13}C]propionate incorporation experiments (Aeration period: days 0 – 10).

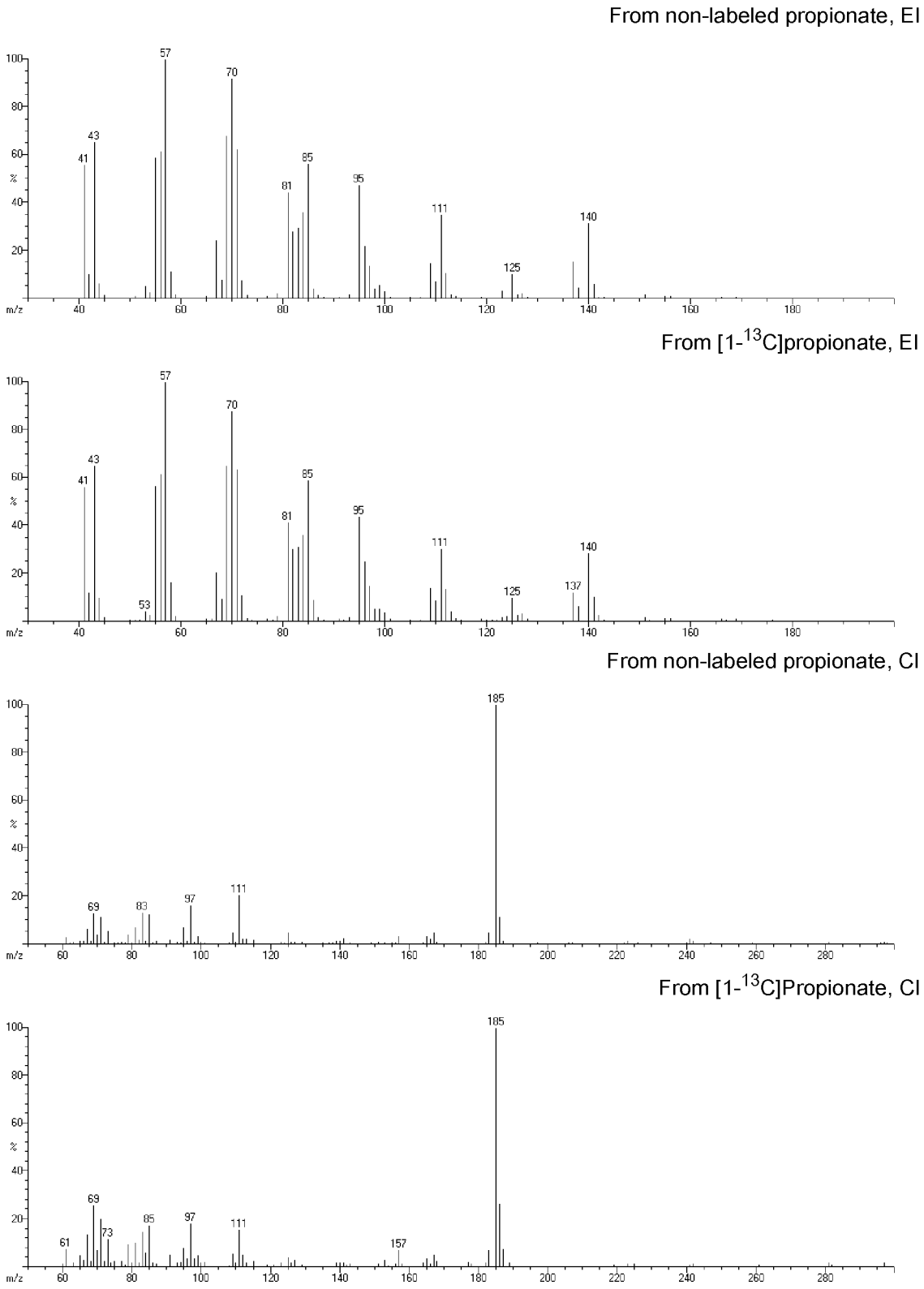


Fig. 4-9. MS spectra (EI and CI analysis) of 4,8-DMDs obtained from propionate and [1-^{13}C]propionate incorporation experiments (Aeration period: days 10 – 20).

Table 4-6. Relative intensities of fragment ions of 2-hydroxy-4-methoxypropiophenone in GC-MS analysis.

		Relative intensities (%)	
Aeration period	Treatment	*m/z* 152 (A)	*m/z* 181 (B)
I	Acetate	9.14 ± 0.74	11.49 ± 0.82
I	[1-^{13}C]Acetate	11.56 ± 0.78**	16.54 ± 6.54 ^{a}
II	Acetate	9.21 ± 0.48	11.77 ± 1.13
II	[1-^{13}C]Acetate	13.02 ± 1.23***	16.28 ± 1.68**
I	Propionate	9.76 ± 0.73	11.07 ± 0.46
I	[1-^{13}C]Propionate	29.01 ± 8.90**	30.19 ± 8.36**
II	Propionate	9.49 ± 0.64	11.21 ± 1.03
II	[1-^{13}C]Propionate	40.96 ± 12.56**	40.93 ± 11.16***

A, B: relative intensities of the fragments at *m/z* 152 to 151 (base ion) and at *m/z* 181 to 180 (M^+), respectively.

I, II: aeration period days 0 – 10 and 10 – 20, respectively.

^{a}; $P = 0.088$,

, *; significant at $P < 0.01$, $P < 0.001$, respectively.

The mean values (mean ± SD, N = 4) of relative intensities of fragments at *m/z* 152 and 181 in 2,4-HMPP obtained from the treatments with ^{13}C-labeled and non-labeled substrates were compared by *t*-test (one tailed).

Table 4-7. Incorporation of [1-^{13}C]acetate and [1-^{13}C]propionate into 2,4-HMPP.

Aeration periond	Treatment	Enrichment factor (EF, %)
I	[1-^{13}C]Acetate	2.49 ± 1.77
II	[1-^{13}C]Acetate	4.67 ± 1.42
I	[1-^{13}C]Propionate	24.36 ± 1.01
II	[1-^{13}C]Propionate	30.22 ± 6.14

I, II: aeration period days 0 – 10 and 10 – 20, respectively.
The values (mean ± SD, N = 4) were caculated by GC-MS CI analysis, followed by Equation 2-1.

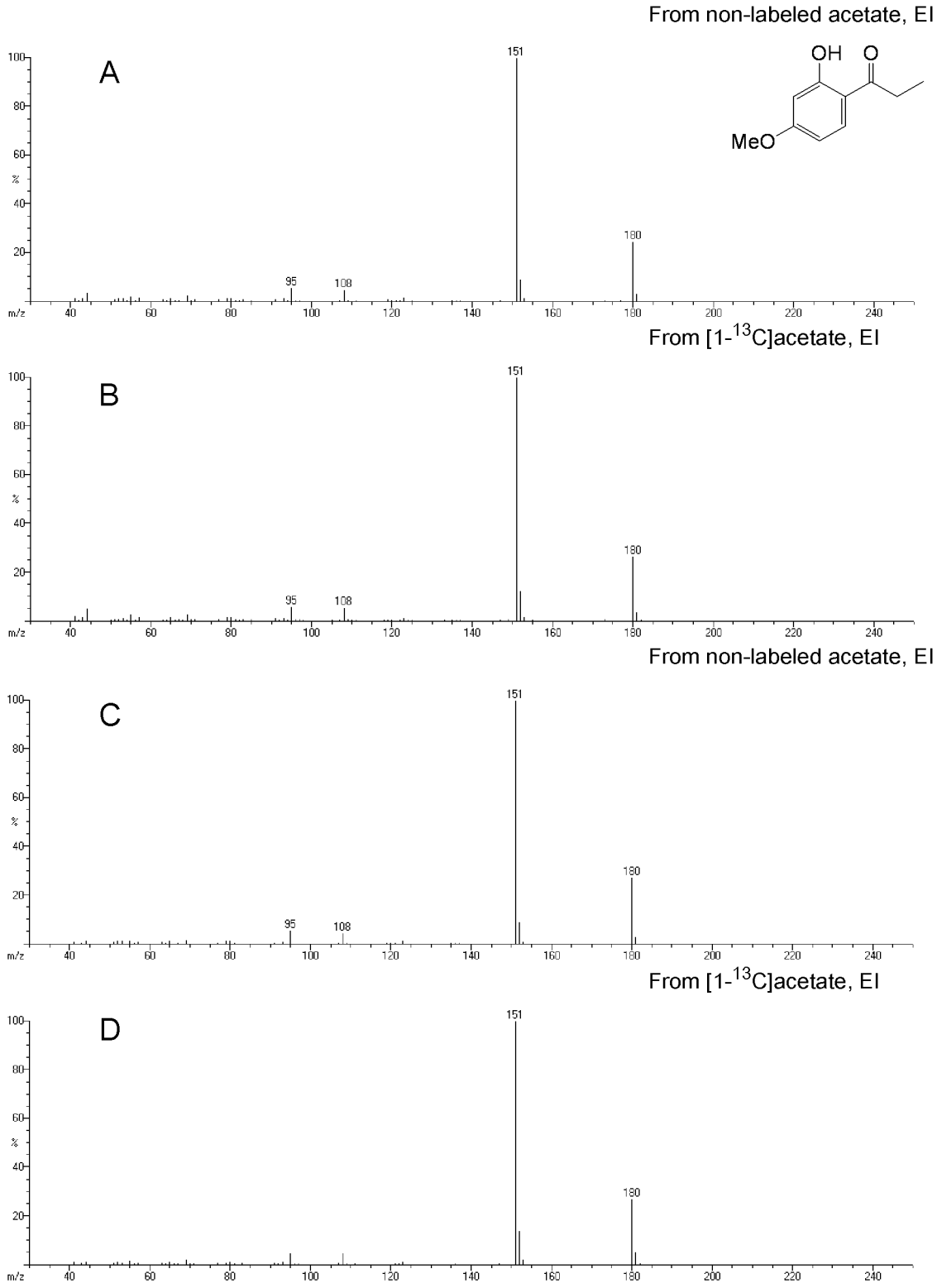


Fig. 4-10. MS spectra of 2-hydroxy-4-methoxypropiophenone (2,4-HMPP) obtained from RFBs fed acetate and [1-^{13}C]acetate-coated flours (A and B: aeration period days 0 – 10; C and D: aeration period days 10 – 20).

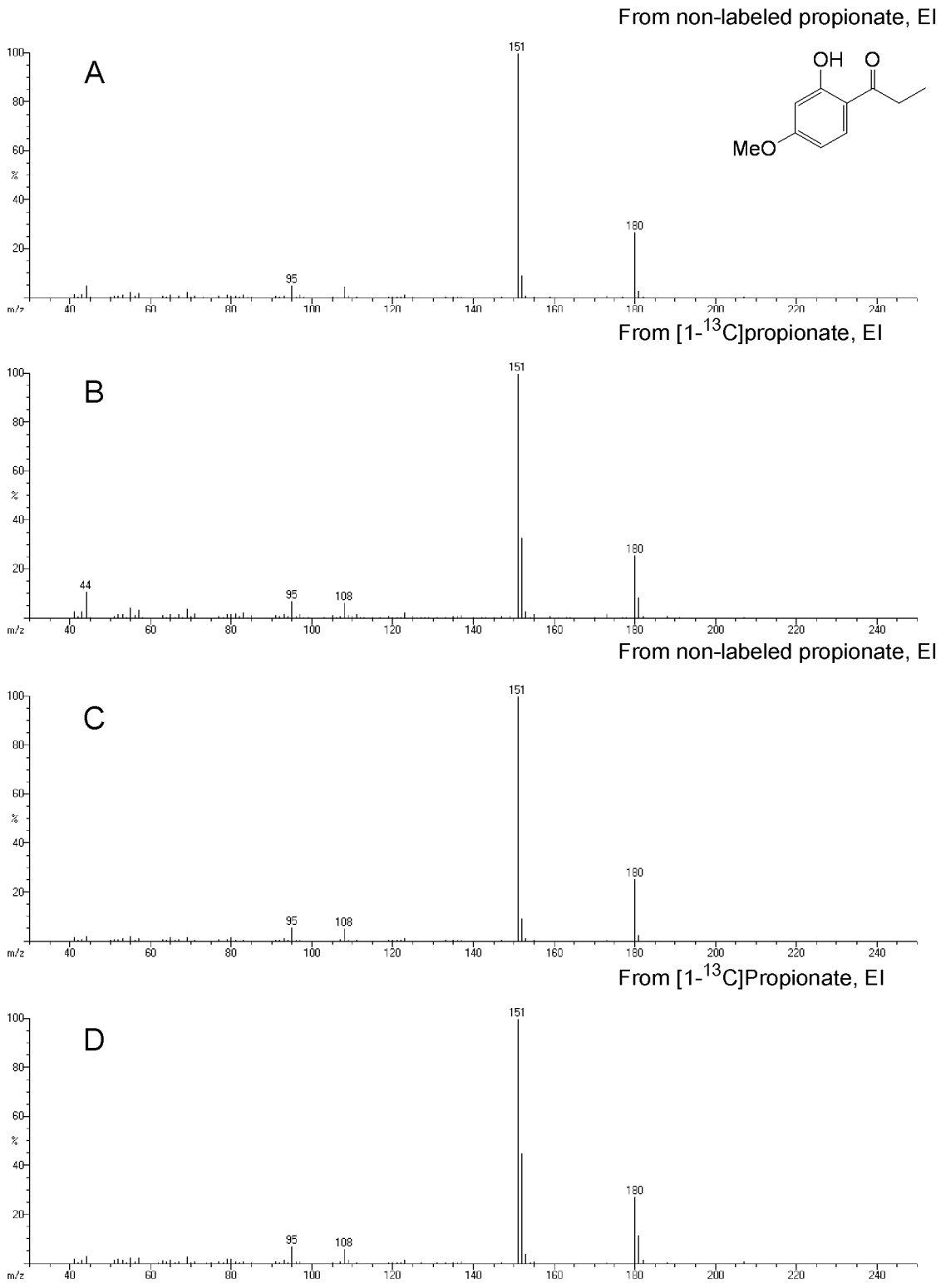


Fig. 4-11. MS spectra of 2-hydroxy-4-methoxypropiophenone (2,4-HMPP) obtained from RFBs fed propionate and [1-^{13}C]propionate-coated flours (A and B: aeration period days 0 – 10; C and D: aeration period days 10 – 20).

4.3.2 Incorporation of deuterium-labeled precursors

The amount of 4,8-DMD obtained from RFBs exposed to each precursor ranged between 0.5–1.4 ng/MDE (in Eluate I) and 1.2–3.5 ng/MDE (in Eluate II). The amount of 4,8-DMD in Eluate I was not sufficient for confirming the incorporation of the labeled putative precursors into 4,8-DMD because of high background (Table 4-8). Therefore, Eluate II was used to determine whether the deuterium-labeled substrates were incorporated into 4,8-DMD.

The deuterium-labeled and unlabeled pheromones can be separated by GC-MS. Deuterated 4,8-DMDs eluted faster than unlabeled ones (Figs. 4-12, 4-14, and 4-15). MS fragmentation pattern of each 4,8-DMD at the maximum peak of *m/z* (140 + *n*) was quite similar to those of the corresponding authentic deuterated standards. The *n* amu shifted fragment ions were observed in the TIC of 4,8-DMDs obtained from RFBs treated with C5D, C10D and C12D (Figs. 4-12, 4-14 and 4-15). This indicated that C5D, C10D, and C12D were incorporated into 4,8-DMD by the RFBs and that the deuterated substrates served as precursors of 4,8-DMD. Unlike these deuterated substrates, 4,8-DMDs obtained from C7D treatment did not result in any *n* amu-shifted fragment ion in any of the treatments (Fig. 4-13). The enrichment factor of 4,8-DMD by these substrates are summarized in Table 4-9. Based on these incorporation experiments, it was clearly demonstrated that 4,8-DMD is biosynthesized in the sequence Ac-Pr-Ac-Pr-Ac via the fatty acid pathway.

Table 4-8. The mean amounts of 4,8-DMD obtained from incorporation experimnets of deuterium-labeled precursors experiments.

	Mean amounts of 4,8-DMD during aeration period (ng/MDE, mean ± SD, N = 4)	
Treatment	Days 0 – 10	Days 10 – 20
Control (A)	6.47 ± 2.04	6.10 ± 2.16
Control (B)	3.24 ± 0.91	10.37 ± 3.22
2-Methylbutanoate (C5H)	3.37 ± 1.22	5.19 ± 2.42
2-Trideuteriomethylbutanoate (C5D)	1.36 ± 0.36	2.82 ± 1.70
4-Methylhexanoate (C7H)	1.52 ± 0.16	4.30 ± 0.59
4-Methylhexanoate-*3,3-d_2* (C7D)	1.44 ± 0.27	3.51 ± 1.52
2,6-Dimethyloctanoate (C10H)	1.05 ± 0.25	2.16 ±0.61
2-Trideuteriomethyl-6-methyloctanoate (C10D)	0.78 ± 0.60	2.56 ± 1.05
4,8-Dimethyldecanoate (C12H)	0.59 ± 0.07	1.06 ± 0.25
4,8-Dimethyldecanoate-*3,3-d_2* (C12D)	0.52 ± 0.03	1.19 ± 0.22

Control (A) and (B): 4,8-DMD obatined from RFBs fed non-treated flour for C5D and C10D incorporation experiments and C7D and C12D incorporation experiments, respectively.

Table 4-9. Incorporation of deuterium-labeled precursors into 4,8-DMD*.

Precursor	Enrichment factor (EF, %)
2-Trideuteriomethylbutanoate (C5D) [a]	26.23 ± 2.76
4-Methylhexanoate-*3,3-d_2* (C7D)	0.00
2-Trideuteriomethyl-6-methyloctanoate (C10D) [b]	13.03 ± 1.33
4,8-Dimethyldecanoate-*3,3-d_2* (C12D)	22.05 ± 3.81

*: Aeration period days 10 – 20.
The values (mean ± SD, N = 4) were calculated by GC-MS CI analysis, followed by Equation 2-1.
[a]; N = 3 (4,8-DMD obtained from one of reliplicates was lost during fractionation).
[b]; The values were calculated by GC-MS EI analysis, followd by Equation 2-1.

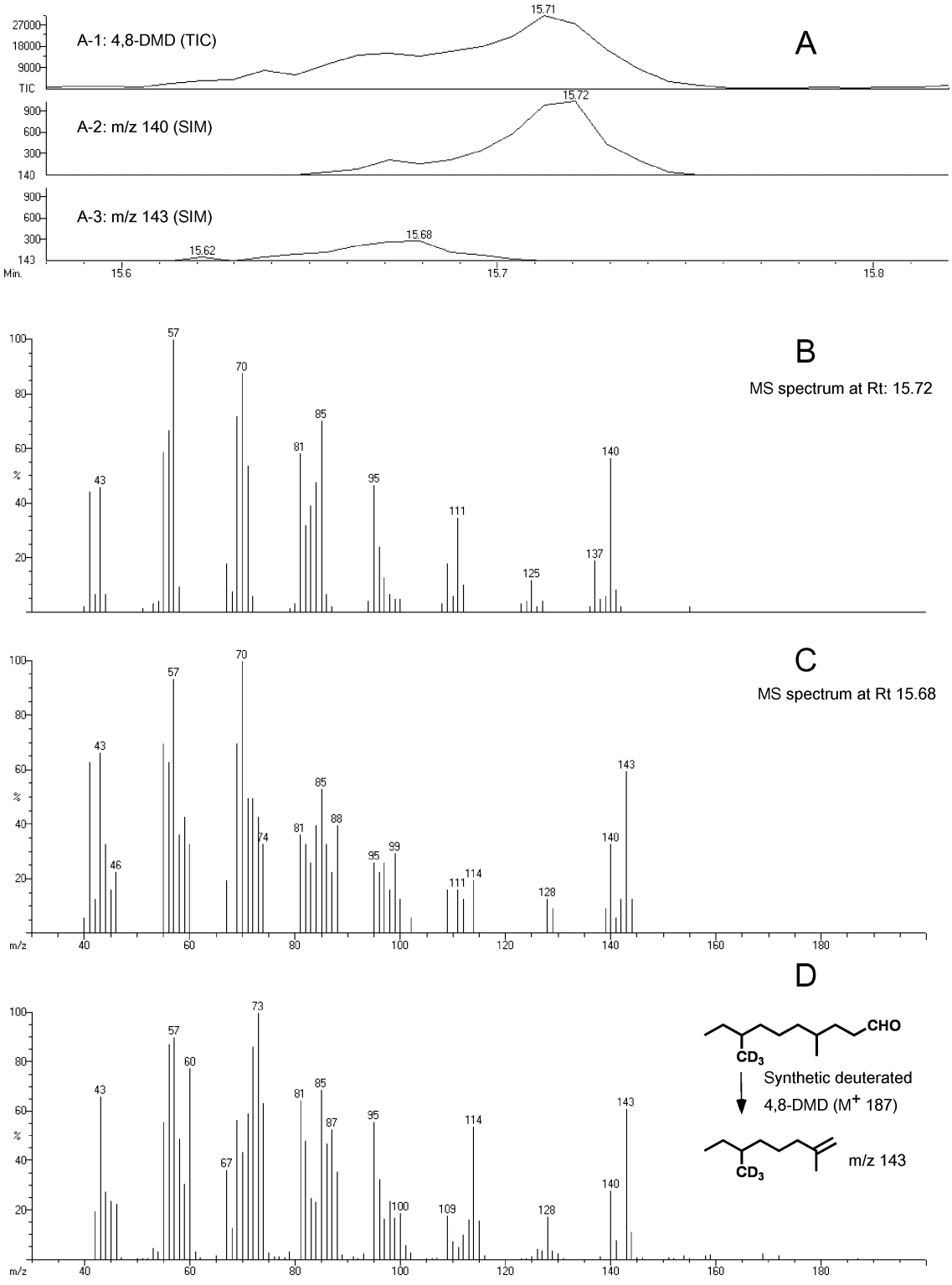


Fig. 4-12. MS chromatograms (A) and MS spectra of 4,8-DMDs obtained from RFBs fed C5D-coated flour.

A-1: TIC of 4,8-DMD; A-2: single ion mass-chromatogram (SIM) at *m/z* 140; A-3: SIM at *m/z* 143; B C, and D: MS spectra at Rt 15.72 (*m/z* 140) and Rt 15.68 (*m/z* 143) of A, and synthetic 4-trimdeueriomethyl-8-methyldecanal, respectively.

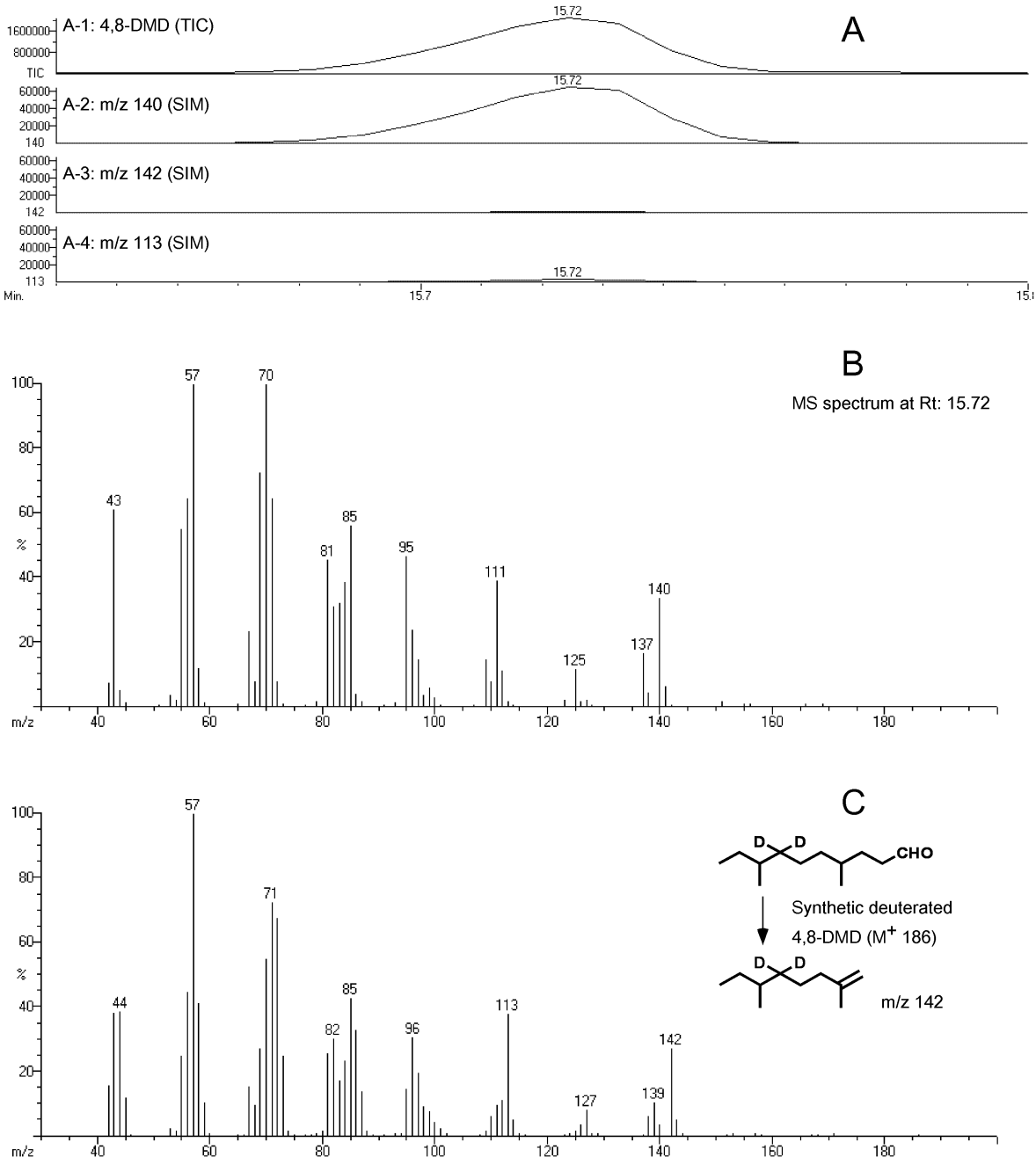


Fig. 4-13. MS chromatograms (A) and MS spectra of 4,8-DMDs obtained from RFBs fed C7D-coated flour.

A-1: TIC of 4,8-DMD; A-2: single ion mass-chromatogram (SIM) at *m/z* 140; A-3: SIM at *m/z* 142; A-4: SIM at *m/z* 113; B and C: MS spectra at Rt 15.72 (*m/z* 140 and *m/z* 113) of A, and synthetic 4,8-methyldecanal-*7,7-d*$_2$, respectively.

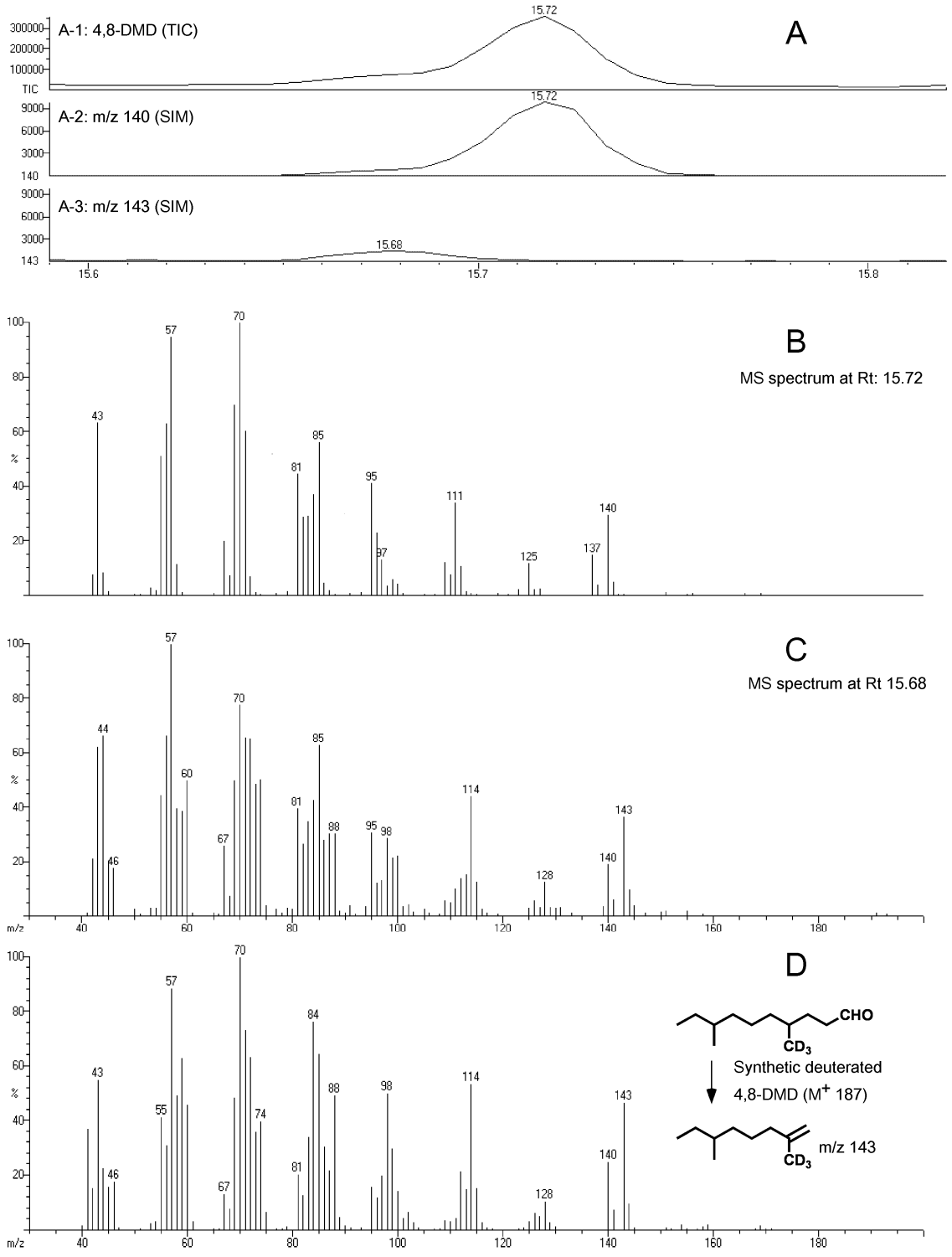


Fig. 4-14. MS chromatograms (A) and MS spectra of 4,8-DMD obtained from RFBs fed C10D-coated flour.

A-1: TIC of 4,8-DMD; A-2: single ion mass-chromatogram (SIM) at *m/z* 140; A-3: SIM at *m/z* 143; B C, and D: Mass spectra at Rt 15.72 (*m/z* 140) and Rt 15.68 (*m/z* 143) of A, and synthetic 4-trimdeueriomethyl-8-methyldecanal, respectively.

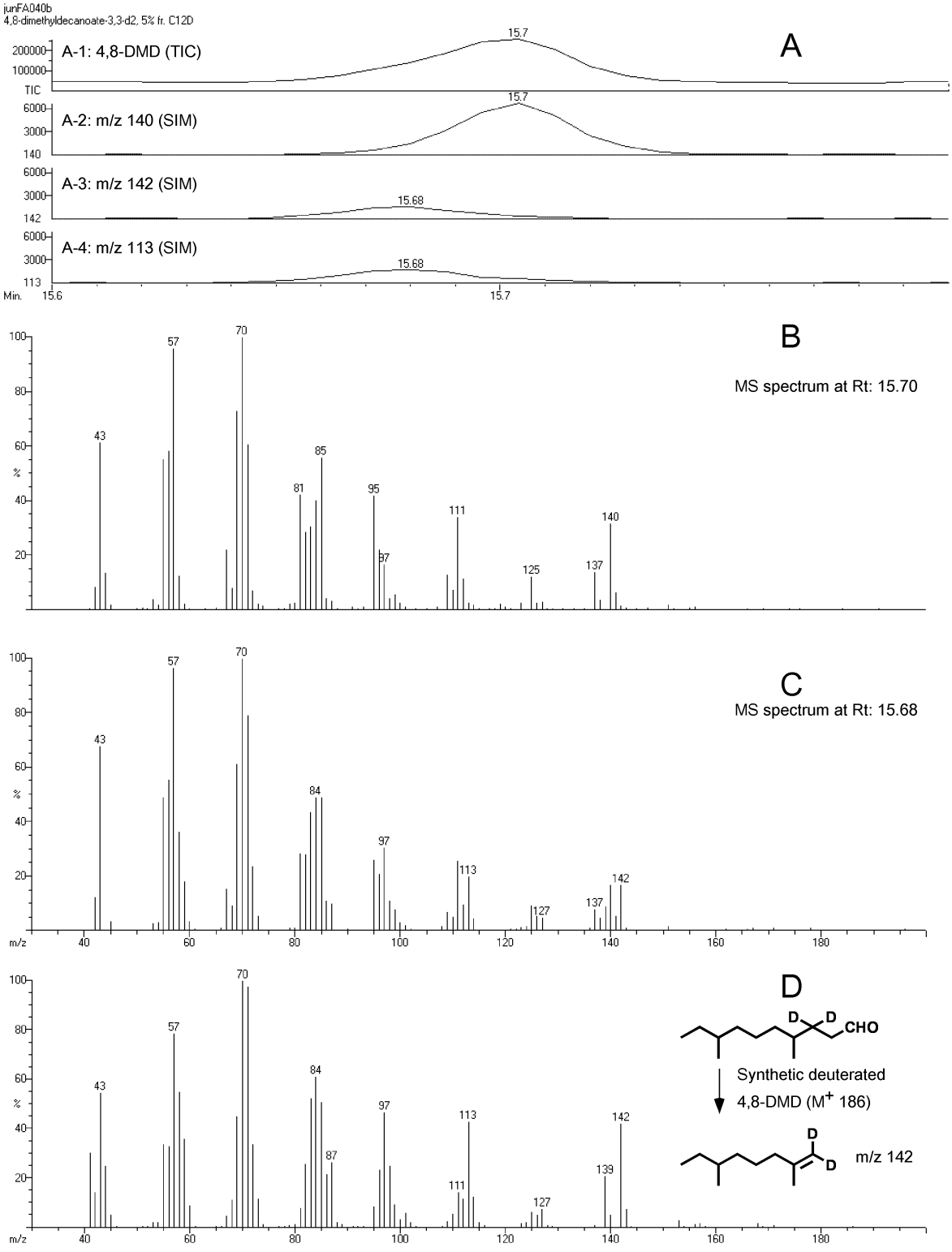


Fig. 4-15. MS chromatograms (A) and MS spectra of 4,8-DMD obtained from RFBs fed C12D-coated flour.

A-1: TIC of 4,8-DMD; A-2: single ion mass-chromatogram (SIM) at *m/z* 140; A-3: SIM at *m/z* 142; A-4: SIM at *m/z* 113; B,C, and D: Mass spectra at Rt 15.70 (*m/z* 140) and Rt 15.68 (*m/z* 113) of A, and synthetic 4,8-methyldecanal-*3,3-d_2*, respectively.

4.4 Discussion

The high incorporation of [1-^{13}C]acetate and [1-^{13}C]propionate into 4,8-DMD and the incorporation deuterium-labeled putative precursors provided unambiguous evidence that 4,8-DMD is of the fatty acid and not terpene origin and that the biosynthesis of 4,8-DMD proceeds in the sequence Ac-Pr-Ac-Pr-Ac.

The EF of 4,8-DMD by [1-^{13}C]acetate was three times higher than that by [2-^{13}C]mevalonolactone (in Chapter II). This result indicated that [1-^{13}C]acetate was not incorporated into 4,8-DMD *via* the mevalonate pathway. A higher rate of incorporation of [2-^{13}C]mevalonolactone than [1-^{13}C]acetate into 4,8-DMD was expected because acetate would be incorporated into mevalonate before it is incorporated into 4,8-DMD and when [1-^{13}C]acetate is incorporated into mevalonate, ^{13}C atoms are positioned at C-1, C-3 and C-5, and subsequent decarboxylation yields isoprene unit labeled at C-1 and C-3 positions (Rohmer, 2003). In addition, loss of a ^{13}C atom in [1-^{13}C]acetate and dilution of [1-^{13}C]acetate by unlabeled acetate would occur during mevalonate formation (Lehninger et al., 1993). Barkawi et al. (2003) reported that in *Dendroctonus jeffreyi* (Scolytidae), both ^{14}C-labeled acetate and mevalonolactone were incorporated into frontalin derived from the mevalonate pathway and that the incorporation rate of mevalonolactone was two or three times higher than that of acetate. In addition, when RFBs were fed flours coated with other deuterated terpenoids (geraniol, farnesol and 2,6,10-trimethyl-2-dodecene), they did not incorporate these substrates into 4,8-DMD (in Chapter II). The very low incorporation of [2-^{13}C]mevalonolactone into 4,8-DMD (in Chapter II) may be due to the degradation of [2-^{13}C]mevalonolactone during digestion by the beetles or preparation of the media.

The incorporation of [1-^{13}C]propionate into 4,8-DMD clearly demonstrated that 4,8-DMD is of fatty acid origin. Propionate is well known to be a source of methyl groups in methyl-branched insect lipids (Nelson, 1993). In tenebrionid pheromones, Islam et al. (1999) demonstrated that 4-methyl-1-nonanol, the sex pheromone of *T. molitor*, was biosynthesized through a modified fatty acid pathway and that [1-^{13}C]propionate was incorporated into the pheromone and responsible for the methyl branch. Propionate could be converted into acetyl-CoA from propionyl-CoA (Chase et al., 1992; Nelson, 1993), and be subsequently incorporated into 4,8-DMD. However, Dillwith et al. (1982) proposed that the ^{13}C atom in [1-^{13}C]propionate would be lost during conversion to acetyl-CoA. Therefore, the enrichment of 4,8-DMD in ^{13}C atom indicated that propionate was involved in the biosynthesis of 4,8-DMD. However, there is no previous evidence that propionate is involved in the mevalonate pathway.

In isotope-labeled precursor incorporation experiments, it was expected that the incorporation rate would be increased dependently with aeration period. However, in the [1-^{13}C]propionate incorporation experiments, the EF of [1-^{13}C]propionate at days 10 – 20 was lower than that at days 0 – 10. In contrast, the EF of 2,4-HMPP at days 0 – 10 was higher than that at days 10 – 20 (Tables 4-5 and 4-7). The reduced incorporation of [1-^{13}C]propionate into 4,8-DMD at days 10 – 20, may be because that [1-^{13}C]propionate was partly used for biosynthesis of 2,4-HMPP, consisting of C3 side chain. Based on the its structure and incorporation pattern of ^{13}C-labeled substrates, 2,4-HMPP seems to be biosynthesized through the fatty acid/polyketide pathway (Fig. 4-16).

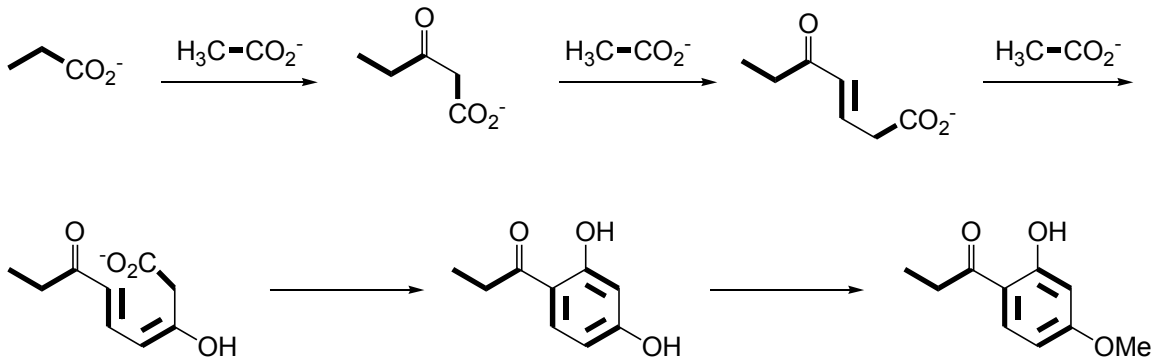


Fig. 4-16. A plausible biosynthetic pathway leading to 2-hydroxy-4-methoxypropiophenone.

The 4,8-DMD obtained from ^{13}C-labeled substrate incorporation experiments was analyzed by conventional GC-MS. This analysis provides information on the position of ^{13}C atom in the pheromone and should allow determination of the biosynthetic route (Bartelt and Weisleder, 1996; Islam et al., 1999). Unfortunately, due to the complex MS fragmentation pattern of 4,8-DMD, incorporation studies using ^{13}C-labeled substrates could not identify the exact position of the ^{13}C-atom. Thus, it was unable to determine the biosynthetic route to 4,8-DMD with the results of the incorporation experiments with ^{13}C-labeled acetate and propionate. However, the results of the experiments using deuterium-labeled putative precursors provided evidence that 4,8-DMD was formed according to the sequence shown in Fig. 4-3. The deuterated putative precursors, C5D, C10D, and C12D were incorporated into 4,8-DMD, and their MS patterns were similar to those of the corresponding authentic deuterated standards. In contrast to our expectations, C7D, one of the hypothesized precursors, was not incorporated into 4,8-DMD in all replicates. Considering that C5D and C10D were incorporated into 4,8-DMD and that the chain elongation reaction involves reduction of double bonds, it is possible that there is a

detoured biosynthetic pathway that is distinct from the pathway producing lepidopteran pheromones derived from fatty acids (Jurenka, 2004). For example, as shown in Fig. 4-17, 2,6-dimethyloctanoate (C10H) would be originated from 2,6-dimethyl-4-octenoate (C10:1H) *via* 4-methyl-2-hexenoate (C7:1H), rather than from 4-methylhexanoate (C7H). This detoured pathway could be determined by examining the incorporation of deuterated C7:1H and C10:1H into the pheromone. This remains to be determined in future.

Fig. 4-18 shows the structural and biosynthetic similarities between 4,8-DMD and 4-methyl-1-nonanol. The only difference in the biosynthetic sequence between them is whether or not it initiates with acetate (Ac-Pr-Ac-Pr-Ac for 4,8-DMD and Pr-Ac-Pr-Ac for 4-methyl-1-nonanol). Moreover, the absolute configuration at the C-4 position of the natural forms of both pheromones is (4*R*) (Suzuki and Mori, 1983; Tanaka et al., 1989). This information suggests that the pheromones of *T. castaneum* and *T. molitor* are derived from a common biosynthetic pathway.

On the other hand, the male-produced aggregation pheromones of *Gnatocerus cornutus*, a tenebrionid, are the sesquiterpenes, (+)-acoradiene and α-cedren-14-al (Tebayashi et al., 1998a, b; Tashiro et al., 2004). Although tenebrionid pheromones are known for only three genera, their pheromones are rather complex (Table 4-10). Studies of other tenebrionid pheromones and their biosyntheses would provide useful information for understanding the evolution of chemical communication in this taxonically complex group.

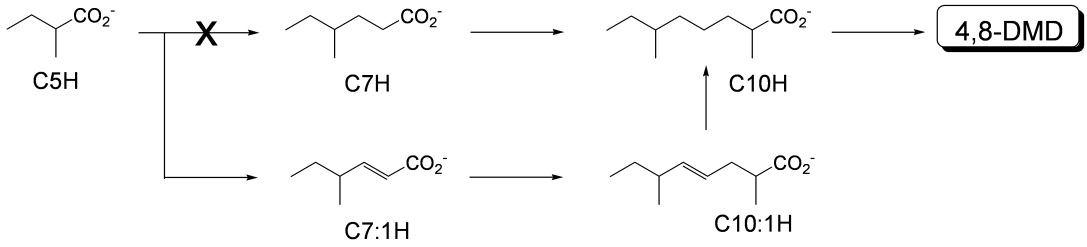
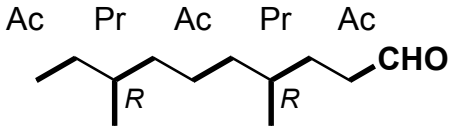
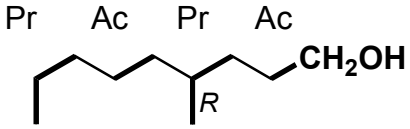


Fig 4-17. A plausible detoured chain elonation reaction in biosynthetic of 4,8-DMD.



I. (4*R*,8*R*)-4,8-DMD



II. (4*R*)-4-methyl-1-nonanol

Fig. 4-18. Structural analogy of natural pheromones of *Tribolium castaneum* (upper) and *Tenebrio molitor* (lower).

Table 4-10. Tenebrionid pheromones.

Species	Source	Pheromone	Biosynthetic origin
T. castaneum	Male	Aggregation	Fatty acid
G. cornutus	Male	Aggregation	Terpene
T. monitor	Female	Sex	Fatty acid

CHAPTER V

PREPARATION OF ISOTOPICALLY LABELED PRECURSORS AND PHEROMONES

5.1 INTRODUCTION

In earlier studies of biosynthetic pathway of insect pheromone, radioactive isotope-labeled compounds have been used widely. The high sensitivity of detection on radioactivity give an advantage in studies of pheromone biosynthesis, because generally formed pheromones are in very small amounts (McCormick and Carrel, 1987). However, there are some experimental limitation in use of radioisotope, such as handling the radioisotope-necessity of specially controlled space, and analytical instruments and difficulties in large scale synthesis (g unit synthesis). Recently, stable isotope-labeled compounds have been used in pheromone biosynthetic studies. Although sensitivity of detection of stable isotope is lower than that of radioactive isotope, use of stable isotope offers several important advantages over radioisotope. 1) the heath hazard associated with radiosynthesis is avoided, that provided the possibility of large scale synthesis, 2) mass-labeled compounds is the ability to distinguish alternative biosynthetic pathways in the same experiment (Bjostad et al., 1987).

To elucidate the biosynthetic pathway leading to 4,8-dimethyldecanal (4,8-DMD), ^{13}C- or deuterium-labeled putative precursors were used as tracers of metabolism. In preparation of deuterium labeled compounds, the following methods are used: reduction of carbonyl groups with lithium aluminum deuteride ($LiAlD_4$; LAD); reduction of double bond with D_2 gas; and deuterium exchange of protons at α-position of a carbonyl group by reaction with deuterated methanol (CH_3OD); and introduction of deuterated group using deuterated reagent, such as iodomethane-d_3 (CD_3I) and acetone-d_6. In addition to putative precusrsors, deuterated 4,8-DMDs were also prepared to compare with 4,8-DMD derived from deuterium-labeled precursor.

5.2 RESULTS AND DISCUSSION

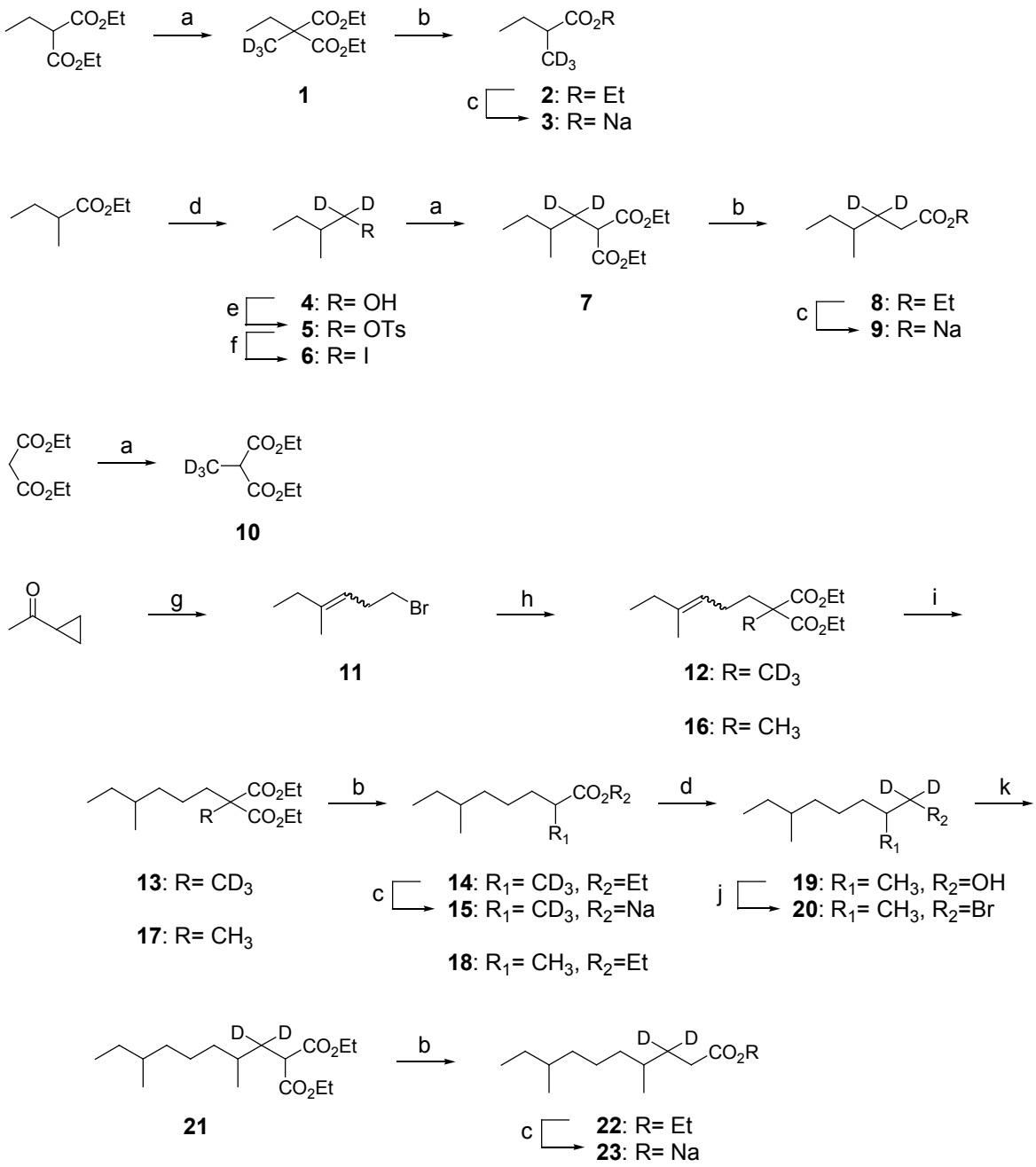
Scheme 1 shows the synthetic routes of deuterated fatty acid precursors. Malonic ester synthesis using CD_3I, gave ethyl esters of trideuteriomethyl branched fatty acids (**2** and **14**) and they were converted to the corresponding sodium salts (**3** and **15**) to feed on the beetles. In preliminary experiments, deuterium-labeled 4-methylhexanoic acid and 4,8-dimethyldecanoic acid were easily prepared by Julia terpene synthesis (Julia et al., 1960; Breuer et al., 1982) by reduction of double bonds of **11** and **12** with deuterium gas. Although these compounds gave sufficient results to check whether applied precursor was incorporated into 4,8-DMD, it could not provide exact information that how much of precursor was incorporated into 4,8-DMD on GC-MS analysis due to scrambling of deuterium. Therefore, deuterium-labeled 4-methylhexanoic acid and 4,8-dimethyldecanoic acid were prepared by reduction of carboxyl group with LAD, and they showed >99% of D atom at C-3 position in both compounds by NMR and GC-MS. The scrambling of deuterium in reduction of double bond with deuterium gas was reduced by using deuterated alcohol or non-proton donate solvent, such as methanol-*d*, hexane or benzene, but using those solvent did not give the satisfactory deuterium-rich compounds as using the reagents CD_3I and LAD. Deuterium-labeled 4,8-DMDs (**30** and **33)** were prepared by the Grignard cross coupling of citronellyl bromide with **2** or **6**, respectively, according to the reported methods (Zarbin et al., 1998; Cahiez et al., 2000), while deuterium-labeled 4,8-DMDs **39** and **41**were prepared by malonic acid ester synthesis (Scheme 2).

Scheme 3 shows the synthetic pathway of [2-^{13}C]mevalonolactone. 4-Hydorxy-2-butanone was protected as THP ether, according to the methods in Hoffman et al. (1957) and then submitted to condensation with **42**, prepared from [2-^{13}C]sodium

acetate. Multi-functionalized compound **44** was firstly hydrolyzed to the corresponding acid and then deprotected in anhydrous condition to afford [2-^{13}C]mevalonolactone **46**.

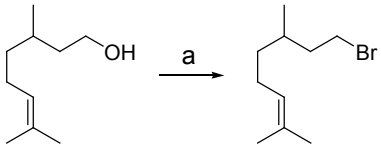
In order to prepare terpene precursors (**52**, **58** and **63**), deuterated 2-ketone terpene moieties (**49**, **54**, and **59**) were subjected to the Wittig-Horner reaction and then reduced to afford the corresponding alcohol (Schemes 4, 5 and 6). For deuterium introduction, two methods were used; deuterium exchange of 2-keonte terpene moieties (**58** and **63**) and alkylation of acetone-d_6 *N,N*-dimethylhydrazone with alkyl bromide (**50** and **52**). About 89% of CH_3 and 75% of CH_2 were converted into CD_3 and CD_2, respectively, by deuterium exchange, while alkylation with acetone-d_6 *N,N*-dimethylhydrazone gave ca. 75% of D atom at CH_2 and CH_3. Deuterium exchange afforded rather higher deuterium contents in molecule, but sometimes showed quite low yield (ca. 20%) and took a long time (2 d). Hydrogen in acetone-d_6 *N,N*-dimethylhydrazone was results of deuterium exchange between acetone-d_6 and H_2O formed by dehydration during formation of hydrazone. Boland and Gäbler (1989) reported that deuterium exchange was prohibited by adding powdered molecular sieve. Compound **71** was prepared by the reported procedures (Mori and Tomioka, 1992; Chappe et al., 1988) (Scheme 7).

Standard deuterated 4,8-DMDs, which expected to be derived from deuterated terpene-precursors, were also prepared as shown in Scheme 8. The hydroxy group in deuterated alcohol **63** and hydroxyester **70** were converted to tosyl group and then reduced by LAH to give the corresponding hydrocarbon **73** and alcohol **79**. 4,8-DMD-d_5 **75** was afforded by oxidative cleavage of the double bond of **73**, and 4,8-DMD-d_4 **79** was prepared by PDC oxidation of **78** (Scheme 8).



Scheme 1. Synthesis of deuterated fatty-acid precursors.

a) CD_3I, NaH/THF; b) LiCl, H_2O/DMSO, reflux; c) 1eq. NaOH/EtOH; d) $LiAlD_4$/THF; e) *p*-TsCl, pyridine/CH_2Cl_2; f) NaI/acetone, reflux; g) i: EtBr, Mg; ii: 30% H_2SO_4; h) NaH/THF (**10** for **12**; dimethyl methylmalonate for **13**); i) H_2 (g), 5% Pd-C/EtOH; j) $(C_6H_5)_3P$, Br_2, Et_3N/CH_2Cl_2; k) NaH/DMF, reflux



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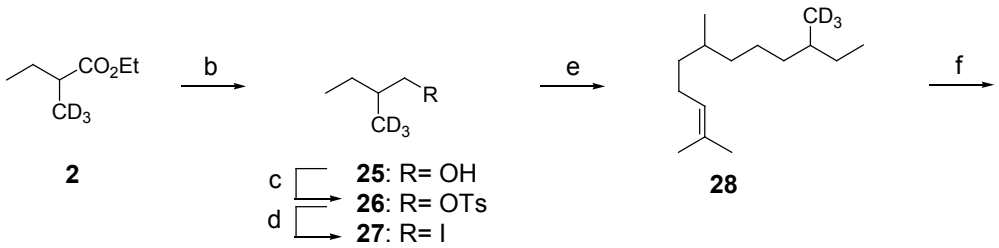
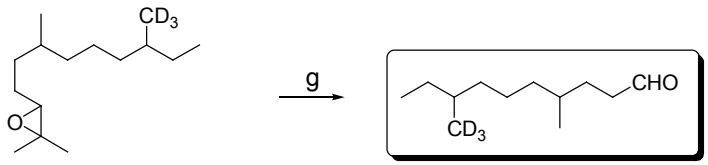


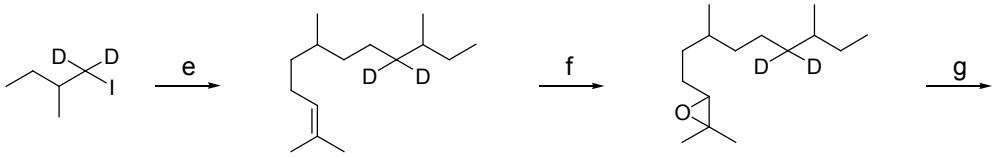
2

c **25**: R= OH
26: R= OTs
d **27**: R= I

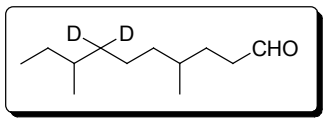
28



29 **30**



6 **31** **32**



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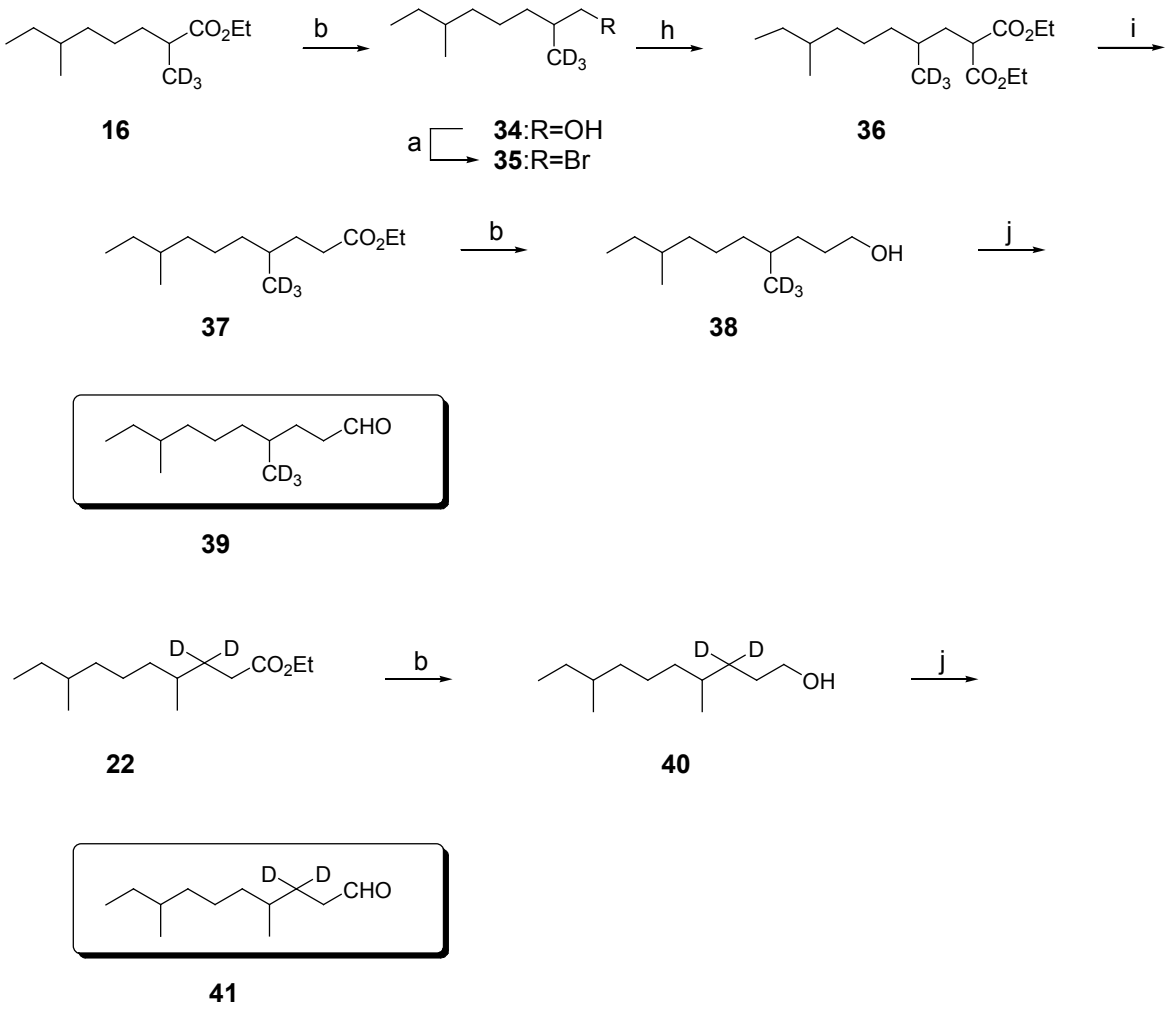
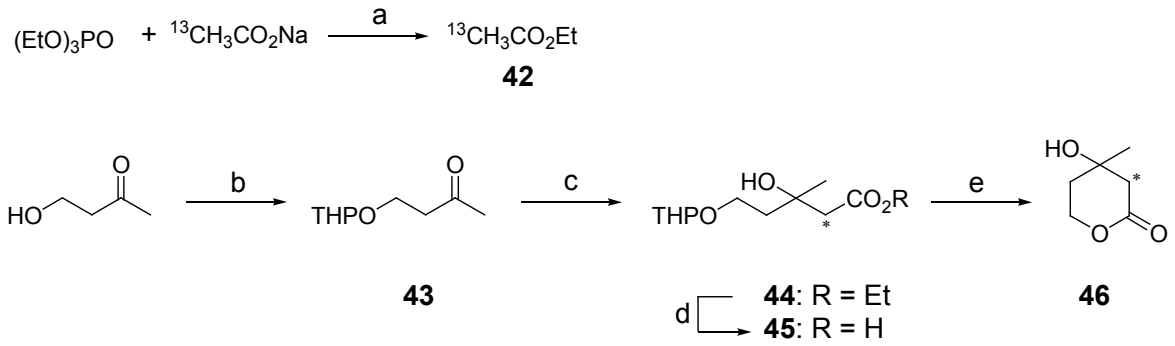
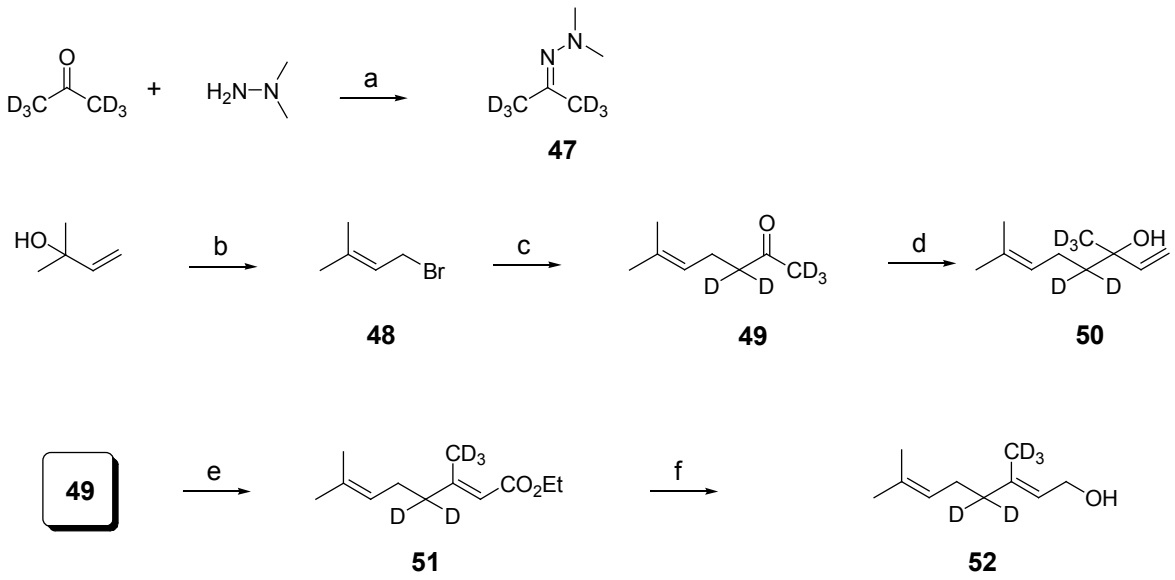


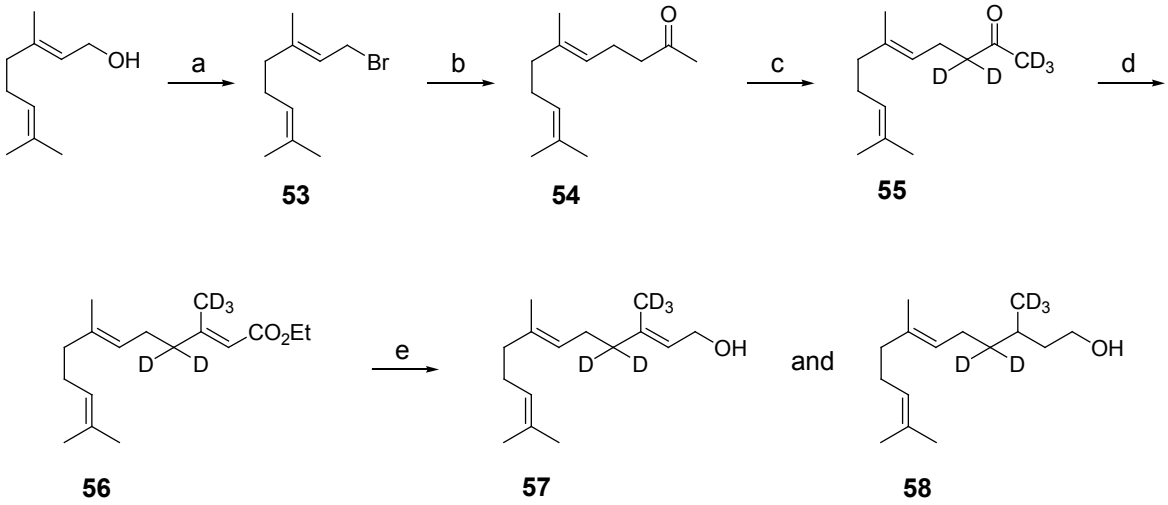
Scheme 2. Synthesis of deuterated 4,8-dimethyldecanals (I).
a) $(C_6H_5)_3P$, Br_2, Et_3N/CH_2Cl_2; b) LAH/THF; c) *p*-TsCl, Pyridine/CH_2Cl_2; d) NaI/acetone, reflux; e) i: **24**, Mg, ii: NMP, Li_2CuCl_4/THF; f) mCPBA/CH_2Cl_2; g) HIO_4/THF; h) diethyl malonate, NaH/DMF, reflux; i) LiCl, H_2O/DMSO, reflux; j) PCC/CH_2Cl_2



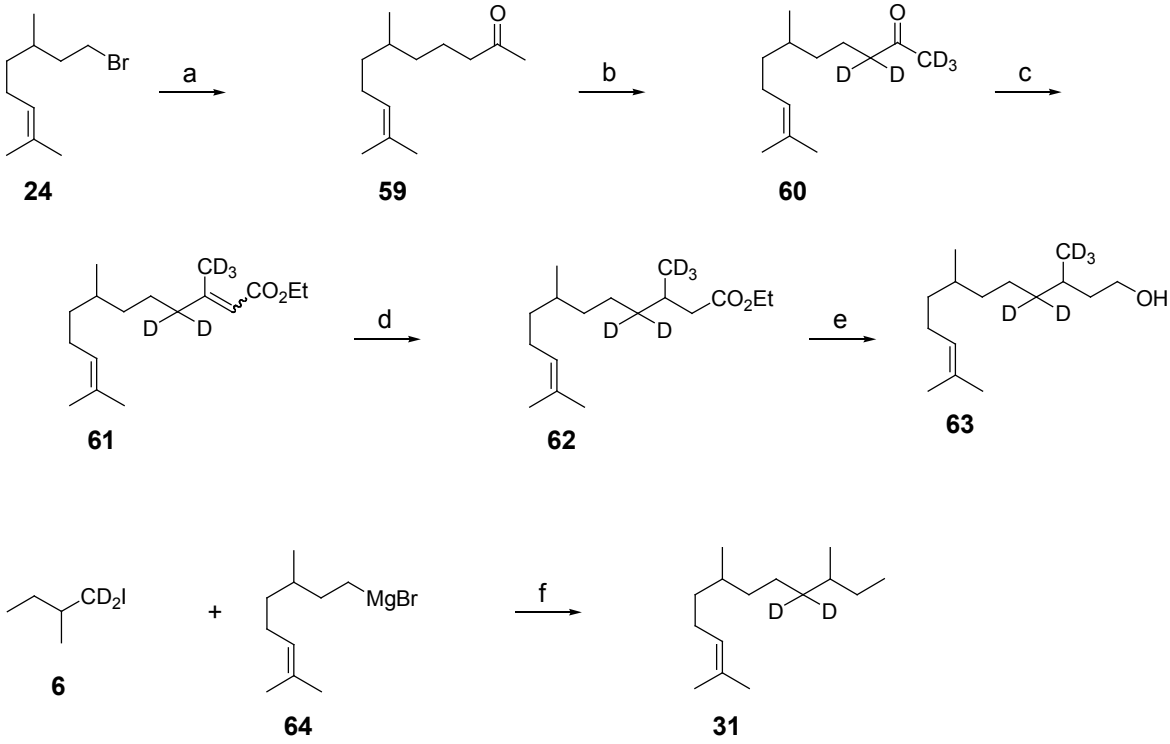
Scheme 3. Synthesis of [2-^{13}C]mevalonolactone.
a) heat; b) dihydropyran; c) **42**, n-BuLi/THF, −78°C; d) NaOH/EtOH; e) Dowex® (50W-X8, H form)



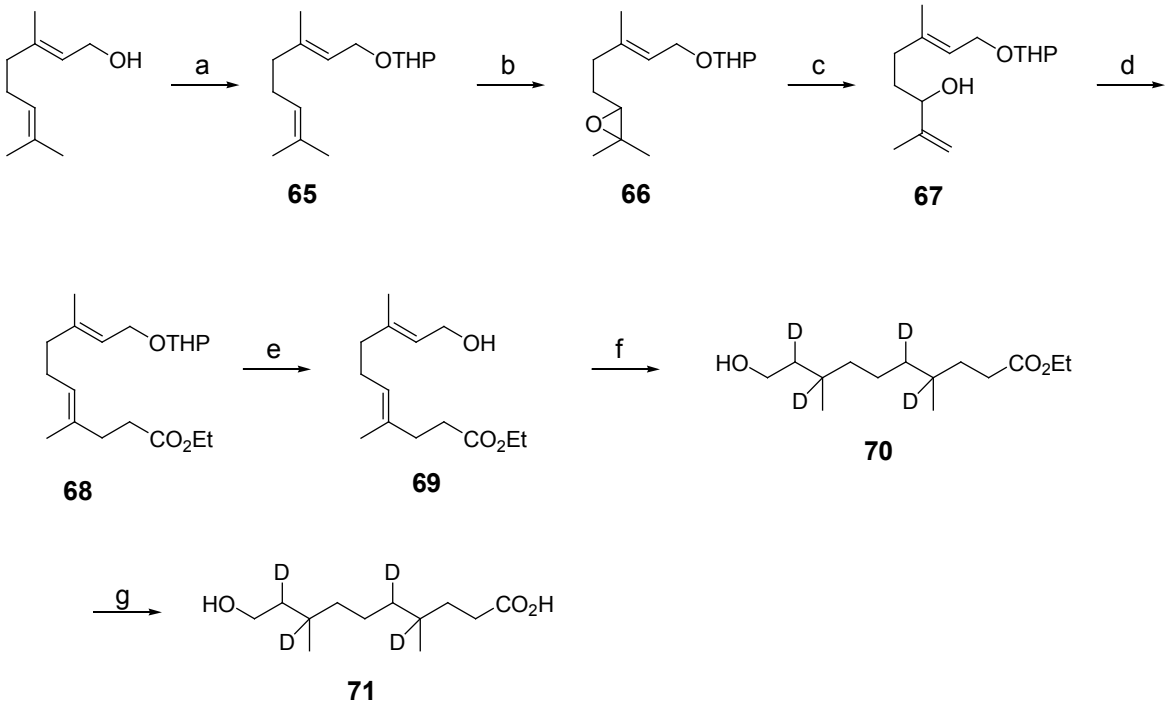
Scheme 4. Synthesis of deuterated monoterpene precursors.
a) acetic acid-d_6, reflux; b) 47% HBr; c) i: **3**, n-BuLi/THF, ii: 2N HCl; d) vinylmagnesium chloride/THF; e) i: TEPA, NaH/THF, ii: chromatography; f) Red-Al®



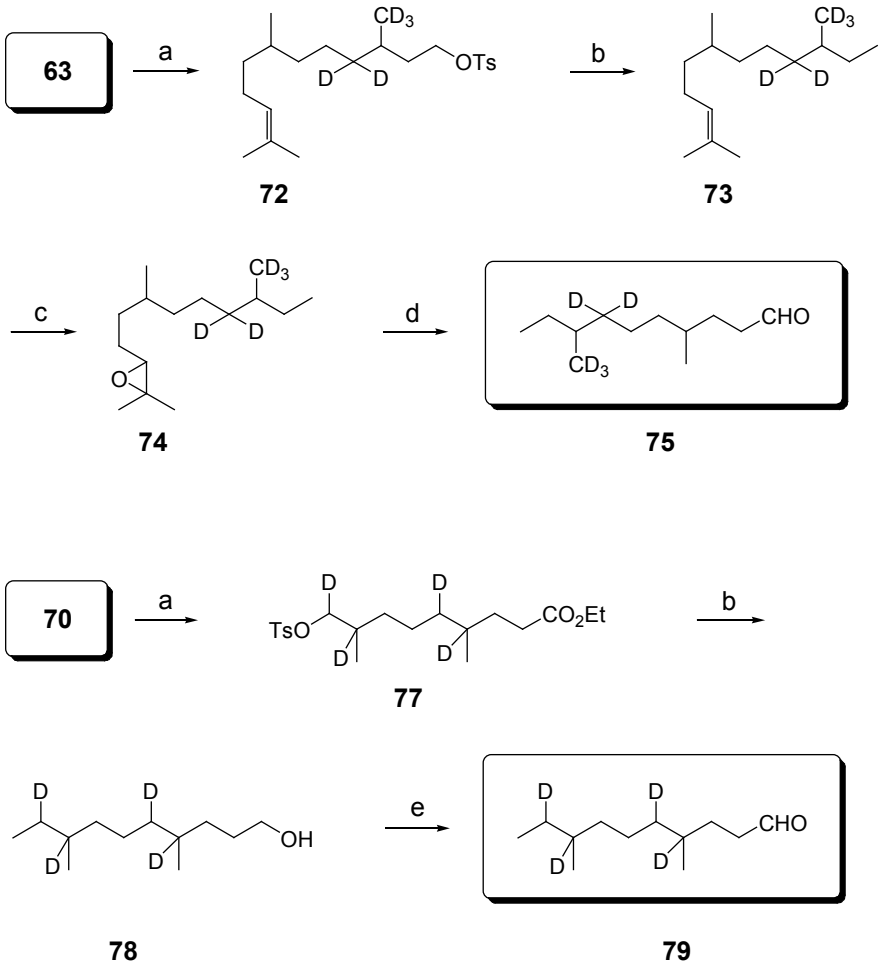
Scheme 5. Synthesis of deuterated sesquiterpene precursor (I).
a) $(C_6H_5)_3P$, Br_2, Et_3N/CH_2Cl_2; b) i: diethyl acetoacetate, NaH/THF, reflux, ii: 2N NaOH, iii: AcOH; c) CH_3OD/MeONa, d) i: TEPA, NaH/THF, ii: chromatography; e) $LiAlH_3(OEt)$/THF



Scheme 6. Synthesis of deuterated sesquiterpene precursors (II).
a) diethyl acetoacetate, NaH/THF; b) CH_3OD, MeONa/EtOH; c) TEPA, NaH/THF; d) CuBr (I), Red-Al®; e) LAH/THF; f) Li_2CuCl_4, NMP/THF (**64** prepared from **24** and Mg)



Scheme 7. Synthesis of 10-hydroxy-4,8-dimethyldecanoic acid-*4,5,8,9-d*$_4$.
a) dihydropyran, *p*-TsOH/CH_2Cl_2; b) mCPBA/CH_2Cl_2; c) Al(O-*i*-Pr)$_3$, reflux; d) $CH_3C(OCH_3)_3$, heat; e) *p*-TsOH; f) D_2 (g), 5% Pd-C/EtOH; g) KOH (aq)



Scheme 8. Synthesis of deuterated 4,8-dimethyldecanals (II).
a) *p*-TsCl, pyridine/CH_2Cl_2; b) LAH/THF; c) mCPBA/CH_2Cl_2; d);HIO_4/THF; e) PDC/CH_2Cl_2

5.3 EXPERIMENTAL

5.3.1 General analytical procedures

GC analyses were performed on a HP 5890 Series II and a HP 6890 Series II gas chromatograph equipped with FID and a 007-5MS column (25 m × 0.25 mm I.D, 0.25 μm, Quadrex) a FFAP column (25 m × 0.25 mm I.D, 0.25 μm, Quadrex), respectively. GC-MS were obtained either on a JEOL MS Router MS-600 coupled with a HP 6890N equipped with a HP-1MS column (30 m × 0.25 mm I.D, 0.25 μm, J&W Scientific) or a HP-5973 Mass Selective Detector coupled with a HP 5890 Series II plus equipped with a HP-5MS column (30 m × 0.25 mm I.D, 0.25 μm, J&W Scientific). NMR spectra were recorded in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard using a Bruker Avance 500 spectrometer at 500 MHz for ^{1}H spectra and at 125 MHz for ^{13}C spectra at Chemical Analysis Center, University of Tsukuba. Splitting patterns are described as: s, singlet; d, doublet; t, triplet, q, quartet; qu, quintet; sext, sextet; sept, septet; oct, octet; m, multiplet; and combinations thereof. Chemicals shifts (δ) and coupling constants (*J*) are given in ppm and Hertz (Hz), respectively. IR spectra were run on a JASCO IRA-1 (Japan Spectroscopic Co., Ltd.) spectrometer. Frequency (ν_{max}) is recorded as cm^{-1}. intensity is described as; s, strong; m, medium; w, weak; and br, broad.

5.3.2 Reagents and solvents

Isotope-labeled reagents: acetone-d_6, acetic acid-d_4, methanol-*d* (CH_3OD), and lithium aluminum deuteride ($LiAlD_4$) were purchased from Aldrich Co. Methyl iodide-d_3 was purchased from Aldrich Co. or Cambridge Isotope Laboratories, Inc. (CIL; Andover, MA). Deuterium gas (99.995% of D_2) was purchased from Takachiho Kogyo Co. Ltd.

(Tokyo, Japan). [2-^{13}C]Sodium acetate was purchased from CIL. Non-labeled reagents: geraniol, methyl cyclobutyl ketone, mCPBA, Red-Al®, dilithium tetrachlorocuprate (Li_2CuCl_4) were purchased from Aldrich Co. Other reagents and solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Kasei Kogyo Co., Ltd. (TCI; Tokyo, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), or Nacalai tesque (Kyoto, Japan). The solvents, THF and ether were dried and distilled from sodium benzophenone ketyl prior to use. Acetone was dried over K_2CO_3 and distilled prior to use. Column chromatography was performed using Wakogel® (C-200, Wako, Japan). DMF and DMSO were dried over molecular sieve (Type 3A) and used without further purification or distillated prior to use. Dichlromethane was dried over $CaCl_2$. Absolute ethanol was prepared by refluxing with Na, followed by distillation. Triethylamine was distilled prior to use and dried over molecular sieve (Type 3A). $MgSO_4$ was used for drying reagent.

5.3.3 Synthesis

Diethyl (trideuteriomethyl)(ethyl)malonate (**1**)

To a solution of NaH (2.55 g, 68.97 mmol; 65%) in THF (30 mL), diethyl ethylmalonate (10.80 g, 57.47 mmol) in THF (20 mL) was added at 0°C under N_2. After stirring for 30 min at room temperature, iodomethane-d_3 (10.0 g, 68.97 mmol) in THF (10 mL) was added at 0°C. The solution was refluxed for 12 h, and then ice-cold water (200 mL) was added. The mixture was extracted with ether (3 × 70 mL). The combined extracts were washed with sat. Na_2SO_3, H_2O, brine, then dried and concentrated *in vacuo*. Distillation of the residue gave 9.92 g of **1**. Yield 84.2%. bp: 120-121°C/45 mmHg. ^{1}H NMR: 4.18 (4H, q, J = 7.0), 1.91 (2H, q, J = 7.5), 1.25 (6H, t, J = 7.5), 0.87 (3H, t, J = 7.5).

^{13}C NMR: 172.8 (C × 2), 61.4 (CH_2 × 2), 54.2 (C), 28.8 (CH_2), 14.4 (CH_3 × 2), 9.0 (CH_3). GC-MS: 205 (M^+, 0.2), 177 (100), 160 (60.8), 132 (84.8), 118 (51.0), 90 (67.8), 76 (35.0). IR: 2990 (s), 2230 (m), 1720 (s), 1230 (s), 1130 (s), 1100 (s), 1015 (s).

Ethyl 2-trideuteriomethylbutanoate (**2**)

According to the modified procedure in the literature, **2** was prepared (Krapcho, 1982). To a solution of **1** (9.92 g, 48.39 mmol) and LiCl (4.06 g, 96.78 mmol) in DMSO (80 mL), H_2O (1.74 mL, 96.78 mmol) was added, and then the mixture was refluxed for 6 h by stirring. After being cooled to room temperature, 2N HCl (100 mL) was added to the solution and the mixture was stirred for 15 min, and then water (200 mL) was added to the mixture. The solution was extracted with ether (3 × 60 mL) and the organic phases were washed with H_2O, brine, then dried and concentrated *in vacuo*. Distillation of the residue gave 3.00 g of **2** (22.56 mmol, 46.6% yield, >99.9% pure, 1.44 g of **1** was recovered). bp: 75°C/96 mmHg. ^{1}H NMR: 4.13 (2H, q, J = 7.2), 2.34 (1H, t, J = 7.0), 1.67 (1H, dqu, J = 13.6, J = 7.4), 1.46 (1H, dqu, J = 13.6, J = 7.4), 1.26 (3H, t, J = 7.1), 0.91 (3H, t, J = 7.4). ^{13}C NMR: 177.21 (C), 60.43 (CH_2), 41.24 (CH), 27.10 (CH_2), 14.65 (CH_3), 11.98 (CH_3). GC-MS: 133 (M^+, 2.6), 118 (6.7), 115 (4.5), 105 (74.6), 90 (8.5), 88 (43.2), 77 (24.0), 60 (100), 43 (15.5). IR: 2930 (s), 2210 (m), 2060 (m), 1720 (s), 1230 (s), 1170 (s), 1015 (s).

Sodium 2-trideuteriomethylbutanoate (**3**)

To a solution of **3** (3.0 g, 23.08 mmol) in ethanol (50 mL), 2N NaOH (11.54 mL, 23.08 mmol) was added and stood overnight at room temperature. After evaporated the solvent by a rotary evaporator, the solid was washed with dry ether (150 mL) to remove

undesired organic compound and dried under P_2O_5 at 100°C *in vacuo* for 6 h. Yield: 93.3%.

2-Methyl-1-butanol-*1,1-d*$_2$ (**4**)

To a suspension LAH (6.30 g, 150.00 mmol) in THF (80 mL), ethyl 2-methylbutanoate (13.02 g, 100.00 mmol, TCI) in THF (50 mL) was added at 0°C. After stirring for 30 min at 0°C, the mixture was stirred for 1 h at room temperature, to which ice-water (50 mL) was added with care, followed by 2N HCl (200 mL). The aqueous solution was extracted with ether (4 × 100 mL). The organic solution was washed with 2N HCl, water, 5% $NaHCO_3$, dried and then concentrated *in vacuo*. Distillation of the residue gave 7.71 g of **4** (85.67 mmol, 85.7%). bp: 69-70°C/65 mmHg. ^{1}H NMR: 3.75 (0.20H, m), 1.68 (1H, br. s), 1.52 (1H, sext, J = 6.6), 1.45 (1H, m), 1.14 (m, 1H, 0.91 (3H, t, J = 7.4), 0.91 (3H, d, J = 6.6). ^{13}C NMR: 67.6 (tr, CH_2), 37.6 (CH), 26.1 (CH_2), 16.4 (CH_3), 11.7 (CH_3). GC-MS: 90 (M^+, 0.3), 72 (41.3), 57 (100), 56 (76.2). GC-MS: 90 (M^+, 0.3), 72 (41.3), 57 (100), 56 (76.2). IR: 3300 (br, s), 2170 (m), 2030 (m), 1080 (s).

1-Tosyl-2-methylbutane-*1,1-d*$_2$ (**5**)

To a solution of **4** (7.71 g, 85.67 mmol) and pyridine (13.86 mL, 171.34 mmol) in CH_2Cl_2 (60 mL), 16.28 g (85.67 mmol) of *p*-toluenesulfonyl chloride (TsCl) in $CHCl_2$ (30 mL) was added at 0°C. After stirring for 1 h at 0°C, the stirring continued for 12 h at room temperature. The solvent was evaporated and ether (100 mL) was added to the residue. The ether layer was washed with 2N HCl, $NaHCO_3$, water, brine and dried. Concentration *in vacuo* afforded 19.79 g of crude **5** and it was used for next reaction without further

purification.

1-Iodo-2-methylbutane-*1,1-d_2* (**6**)

To a solution of NaI (24.33 g, 162.22 mmol) in dry acetone (200 mL), tosylate **7** (19.19 g, 81.11 mmol as crude) was added. The solution was stirred and refluxed for 6 h. After cooling to room temperature, water (200 mL) was added. The solution was extracted with pentane (3 × 100 mL). The organic phases were washed with sat. Na_2SO_3, brine and dried. After the solvent was evaporated, the residue was distilled to afford 13.09 g **6** (65.45 mmol, 76.4% in 2 steps). bp: 75°C/75 mmHg. ^{1}HNMR: 1.38 (2H, m), 1.26 (1H, m), 0.98 (3H, d, J = 6.5), 0.89 (3H, t, J = 7.4). ^{13}C NMR: 36.6 (CH), 29.6 (CH_2), 20.5 (CH_3), 17.5 (tr, CH_2), 11.7 (CH_3). GC-MS: 200 (M^+, 14.1), 171 (4.6), 73 (100), 57 (8.6). IR: 2150 (w), 910 (m).

1-Iodo-2-methylbutane (**6H**)

^{1}HNMR: 3.23 (1H, dd, J = 9.6, J = 4.7), 3.2 (1H, dd, J = 9.6, J = 5.9), 1.38 (2H, m), 1.26 (1H, m), 0.98 (3H, d, J = 6.5), 0.89 (3H, t, J = 7.4). ^{13}C NMR: 36.8 (CH), 29.6 (CH_2), 20.6 (CH_3), 17.8 (CH_2), 11.7 (CH_3). GC-MS: 198 (M^+, 15.5), 169 (4.0), 71 (100), 55 (11.9).

Diethyl (1,1-dideuterio-2-methylbutyl)malonate (**7**)

This compound (**7**) was prepared in the same manner as that used for **1** from NaH (2.66 g, 72.00 mmol) and diethyl malonate (10.47 g, 65.45 mmol) in THF. Yield: 11.07 g (47.72 mmol, 72.9%). bp: 103-105°C /25 mmHg. GC-MS: 233 (M^+, 0.3), 187 (20.3), 175 (31.1), 160 (100), 133 (37.9), 103 (16.9), 88 (14.6), 75 (16.1).

Diethyl 2-methylbutylmalonate (**7H**)

^{1}HNMR: 4.19 (4H, q, J = 7.1), 3.43 (1H, dd, J = 8.7, J = 6.7), 1.96 (1H, ddd, J = 13.9, J = 8.7, J = 5.3), 1.69 (1H, ddd, J = 13.9, J = 7.1, J = 7.1), 1.37-1.19 (3H, m), 1.27 (6H, t, J = 7.1), 0.89 (3H, d, J = 6.5), 0.88 (3H, t, J = 7.3). ^{13}C NMR: 169.7 (C × 2), 61.2 (CH_2 × 2), 50.1 (CH), 35.4 (CH_2), 32.3 (CH), 29.2 (CH_2), 18.7 (CH_3), 14.0 (CH_3), 11.0 (CH_3). GC-MS: 201 (M^+ – Et, 3.1), 185 (20.9), 173 (30.6), 160 (100), 133 (36.9), 101 (17.3), 88 (14.3), 73 (17.6).

Ethyl 4-methylhexanoate-*3,3-d_2* (**8**)

This compound (**8**) was prepared in the same manner as that used for **2** from **7** (11.07 g, 47.72 mmol), LiCl (4.01 g, 95.43 mmol) and H_2O (1.72 mL, 95.43 mmol) in DMSO (80 mL). Yield: 4.57 g (28.56 mmol, 59.9%, > 99.9% pure). bp: 98°C/48 mmHg. ^{1}H NMR: 4.12 (2H, q, J = 7.1), 2.28 (2H, q, J = 15.0), 1.38-1.30 (2H, m), 1.24 (3H, t, J = 7.1), 1.22-1.13 (1H, m), 0.874 (3H, t, J = 7.3), 0.870 (3H, d, J = 6.7), ^{13}C NMR: 174.1 (C), 60.1 (CH_2), 33.8 (CH), 31.9 (CH_2), 29.0 (CH_2), 18.7 (CH_3), 14.2 (CH_3), 11.2 (CH_3). GC-MS: 160 (M^+, 0.8), 131 (11.1), 115 (42.2), 103 (100), 88 (96.7), 75 (28.3), 70 (28.2), 61 (25.4), 57 (30.2).

Ethyl 4-methylhexanoate (**8H**)

^{1}H NMR: 4.12 (2H, q, J = 7.1), 2.30 (2H, m), 1.68 (1H, m), 1.46-1.41 (1H, m), 1.40-1.31 (2H, m), 1.26 (3H, t, J = 7.1), 1.22-1.13 (1H, m), 0.88 (3H, t, J = 7.3), 0.87 (3H, d, J = 6.5). ^{13}C NMR: 174.1 (C), 60.1 (CH_2), 34.0 (CH), 32.1 (CH_2), 31.5 (CH_2), 29.1 (CH_2), 18.8 (CH_3), 14.2 (CH_3), 11.2 (CH_3). GC-MS: 158 (M^+, 0.7), 129 (16.7), 113 (37.0), 101 (100),

88 (94.1), 73 (30.6), 70 (29.2), 61 (16.8), 55 (28.6).

Sodium 4-methylhexanoate-*3,3-d_2* (**9**)

Sodium salt of **8** was obtained by the same manner as that used for **3** with equivalent 2N NaOH. Yield: 4.08 g (26.49 mmol, 92.8%).

Diethyl trideuteriomethylmalonate (**10**)

This compound (**10**) was prepared in the same manner as that used for **1** from iodomethane-d_3 (15.00 g, 103.45 mmol), diethyl malonate (13.79 g, 86.21 mmol) and NaH (3.82 g, 103.45 mmol). Yield: 13.93 g (78.70 mmol, 73% pure; major impurity was diethyl ditrideuteriomalonate). This material was employed in the next step without further purification. bp: 106-107°C/37 mmHg. ^{1}H NMR: 4.20 (4H, q, J = 7.1), 3.41 (1H, s), 1.27 (6H, t, J = 7.1). ^{13}C NMR: 170.6 (C × 2), 61.7 (CH_2 × 2), 46.4 (CH), 14.4 (CH_3 × 2). GC-MS: 177 (M^+, 9.3), 150 (19.5), 132 (100), 105 (30.9), 77 (54.5), 60 (27.0), 59 (23.1). IR: 2950 (s), 2220 (w), 1730 (s), 1360 (m), 1230 (s), 1120 (s), 1020 (s).

1-Bromo-4-methyl-3-hexene (**11**)

Compound **11** was prepared as procedure described by Biernacki and Gdula (1979). A solution of methyl cyclopropyl ketone (25.00 g, 297.62 mmol) in ether (100 mL) was added dropwise with stirring to a solution of ethylmagnesium bromide prepared from magnesium turnings (8.57 g, 357.14 mmol) and bromoethane (357.14 mmol) in ether (60 mL)and left overnight at room temperature. After cooling to 0°C, 30% H_2SO_4 (150 ml) was added to the mixture, followed by addition of water (100 ml) and stirring was

continued for 1 h. The solution was extracted with ether (3 × 100 mL). The organic solution was washed with $NaHCO_3$, H_2O, brine, then dried and concentrated fractionally. Distillation of the residue gave 43.57 g of **11** (246.16 mmol, 82.7%, 3*E*:3Z = 3:1, calculated on GC). bp: 95-96°C /60 mmHg. ^{1}H NMR: 5.13 (1H, tq, *J* = 7.1, *J* = 1.4), 3.34 (2H, t, *J* = 7.4), 2.57 (2H, dq, *J* = 7.3, *J* = 0.7), 2.01 (2H, q, *J* = 7.4), 1.63 (3H, s), 1.00 (3H, t, *J* = 7.4). ^{13}C NMR: 140.8 (C), 119.8 (CH), 33.4 (CH_2), 32.7 (CH_2), 32.1 (CH_2), 16.6 (CH_3), 13.0 (CH_3). GC-MS (trans) : 178 (M^+, 17.3), 176 (17.5), 97 (55.9), 83 (26.7), 55 (100). IR: 2950 (s), 1650 (w).

Diethyl (trideuteriomethyl)(4-methyl-3-hexenyl)malonate (**12**)

This compound was prepared in the same way as that used for **1** from **10** (13.93 g, 78.70 mmol, 73% pure), **11** (11.70 g, 66.11 mmol), and NaH (2.44 g, 66.11 mmol) in THF. Yield: 12.24 g (44.84 mmol, 97% pure). bp: 123-125°C/2 mmHg. ^{1}H NMR (trans): 5.08 (1H, m), 4.18 (4H, q, *J* = 7.1), 2.00-1.85 (6H, m), 1.58 (3H, s), 1.25 (6H, t, *J* = 7.1), 0.97 (3H, t, *J* = 7.6). ^{13}C NMR: 172.3 (C × 2), 137.8 (C), 121.7 (CH), 61.1 (CH_2 × 2), 53.3 (C), 35.4 (CH_2), 32.2 (CH_2), 22.8 (CH_2), 15.7 (CH_3), 14.0 (CH_3 × 2), 12.6 (CH_3). GC-MS (cis): 273 (M^+, 0.2), 228 (3.3), 177 (100), 131 (68.9), 103 (24.2), 81 (9.0). GC-MS (trans): 273 (M^+, 0.3), 228 (3.3), 182 (9.8), 177 (100), 154 (11.9), 131 (68.9), 103 (24.2), 81 (9.0), 67 (4.5), 55 (11.0).

Diethyl (trideuteriomethyl)(4-methylhexyl)malonate (**13**)

Hydrogen gas was introduced to a suspension of **12** (12.24 g, 44.84 mmol) and 5% Pd/C (1.30 g) in ethanol (200 mL) until absorption was ceased. The suspension was

filtered and the solvent was evaporated. The residue was used for the next step without further purification. Yield: 12.25 g (44.55 mmol, 99.3%). ^{1}H NMR: 4.18 (4H, q, *J* = 7.1), 1.87-1.80 (2H, m), 1.34-1.07 (7H, m), 1.24 (6H, t, *J* =7.1), 0.85 (3H, t, *J* = 7.3), 0.84 (3H, d, *J* = 6.4). ^{13}C NMR: 172.5 (C × 2), 61.1 (CH_2 × 2), 53.5 (C), 36.7 (CH_2), 35.6 (CH_2), 34.0 (CH), 29.3 (CH_2), 21.6 (CH_2), 19.0 (CH_3), 14.0 (CH_3 × 2), 11.3 (CH_3). GC-MS: 260 (M^+ – Me, 0.3), 246 (0.8), 230 (2.2), 177 (100), 131 (45.7), 118 (22.2), 103 (13.5), 90 (15.0), 72 (10.8).

Diethyl (methyl)(4-methylhexyl)malonate (**13H**)

^{1}H NMR: 4.18 (4H, q, *J* = 7.1), 1.88-1.80 (2H, m), 1.40 (3H, s), 1.32-1.07 (7H, m), 1.24 (6H, t, *J* = 7.1), 0.85 (3H, t, *J* = 7.3), 0.83 (3H, d, *J* = 6.4). ^{13}C NMR: 172.5 (C × 2), 61.0 (CH_2 × 2), 53.7 (C), 36.7 (CH_2), 35.7 (CH_2), 34.0 (CH), 29.3 (CH_2), 21.6 (CH_2), 19.8 (CH_3), 19.0 (CH_3), 14.0 (CH_3 × 2), 11.3 (CH_3). GC-MS: 272 (M^+, 0.1), 257 (0.3), 227 (2.5), 174 (100), 128 (47.4), 115 (23.3), 100 (11.9), 87 (15.6), 69 (18.1).

Ethyl 2-trideuterio-6-methyloctanoate (**14**)

This compound was prepared in the same way as used for **2** from **13** (12.25 g, 44.55 mmol), LiCl (3.74 g, 89.10 mmol), and H_2O (1.60 mL, 89.10 mmol) in DMSO (60 mL). Yield: 5.31 g (26.16 mmol, 58.7%, 98.0% pure). NMR: ^{1}H NMR: 4.12 (2H, q, *J* =7.1), 2.40 (1H, t, *J* = 6.9), 1.65-1.59 (1H, m), 1.42-1.16 (6H, m), 1.25 (3H, t, *J* = 7.1), 1.14-1.06 (2H, m), 0.85 (3H, t, *J* = 7.3), 0.84 (3H, d, *J* = 6.4), ^{13}C NMR: 177.0 (C), 60.0 (CH_2), 39.3 (CH), 36.4 (CH_2), 34.2 (CH), 34.0 (CH_2), 29.4 (CH_2), 24.6 (CH_2), 19.1 (CH_3), 14.2 (CH_3), 11.3 (CH_3). GC-MS: 203 (M^+, 3.5), 174 (10.5), 158 (8.2), 118 (44.0), 105

(100), 100 (18.5), 90 (15.9), 77 (29.0).

Ethyl 2,6-dimethyloctanoate (**14H**)

^{1}H NMR: 4.12 (2H, q, *J* =7.1), 2.41 (1H, oct, *J* = 6.9), 1.67-1.60 (1H, m), 1.42-1.16 (6H, m), 1.25 (3H, t, *J* = 7.1), 1.14-1.06 (2H, m), 1.14 (3H, d, *J* = 6.7), 0.85 (3H, t, *J* = 7.3), 0.84 (3H, d, *J* = 6.4), ^{13}C NMR: 177.0 (C), 60.0 (CH_2), 39.5 (CH), 36.4 (CH_2), 34.2 (CH), 34.1 (CH_2), 29.4 (CH_2), 24.6 (CH_2), 19.1 (CH_3), 17.0 (CH_3), 14.2 (CH_3), 11.3 (CH_3). GC-MS: 200 (M^+, 4.0), 171 (11.0), 155 (8.8), 115 (41.8), 102 (100), 97 (16.7), 87 (14.7), 74 (24.7).

Sodium 2-trideuterio-6-methyloctanoate (**15**)

Sodium salt of **14** was obtained by the same procedure described for **3** from **14** (3.50 g, 17.24 mmol) with equimolar 2N NaOH. Yield: 3.00 g (15.23 mmol, 88.3%).

Diethyl (methyl)(4-methyl-3-hexenyl)malonate (**16 = 12H**)

This compound was prepared in the same procedure described for **12** from **11** (44.37 g, 250.68 mmol), diethyl methylmalonate (36.35 g, 208.90 mmol), and NaH (9.26 g, 250.68 mmol) in THF. Yield: 50.69 g (187.74 mmol, 89.9%). bp: 120-124°C/2 mmHg. ^{1}H NMR (trans): 5.09 (1H, m), 4.18 (4H, q, *J* = 7.1), 2.02-1.85 (6H, m), 1.58 (3H, s), 1.42 (3H, s), 1.25 (6H, t, *J* = 7.1), 0.97 (3H, t, *J* = 7.6). ^{13}C NMR (trans): 172.3 (C × 2), 137.8 (C), 121.7 (CH), 61.1 (CH_2 × 2), 53.5 (C), 35.5 (CH_2), 32.2 (CH_2), 22.8 (CH_2), 19.8 (CH_3), 15.7 (CH_3), 14.0 (CH_3 × 2), 12.6 (CH_3). GC-MS (trans): 270 (M^+, 0.2), 225 (3.3), 179 (9.5), 174 (100), 151 (11.4), 128 (67.8), 100 (20.3), 81 (10.8), 67 (5.0), 55 (12.7).

Diethyl (methyl)(4-methylhexyl)malonate (**17** = **13H**)

This compound was prepared in the same manner described for **13** by catalytic hydrogenation of **16** (50.69 g) and 5% Pd/C. Yield: quantitative. The spectra of **17** was identical to that of **13H**.

Ethyl 2,6-dimethyloctanoate (**18 = 14H**)

This compound **18** was prepared in the same procedure described for **2** from **17** (50.67 g, 186.28 mmol), LiCl, and H_2O in DMSO. Yield: 25.48 g (127.40 mmol, 68.4%). bp: 133°C /42 mmHg. The spectra of **18** was identical to that of **14H**.

2,6-Dimethyloctanol-*1,1-d_2* (**19**)

This compound **19** was prepared in the same manner that used for **4** from **18** (13.00 g, 65.00 mmol), LAD (5.00 g, 119.05 mmol) in THF. Yield: 6.19 g (38.69 mmol, 59.5%). bp: 130-131°C/40 mmHg. ^{1}H NMR: 1.60 (1H, sext, J = 6.4), 1.45 (1H, br. s), 1.42-1.20 (6H, m), 1.15-1.06 (3H, m), 0.92 (3H, dd, J = 1.2, J = 6.7), 0.86 (3H, t, J = 7.2), 0.85 (3H, d, J = 6.5). ^{13}C NMR: 36.8 (CH_2), 35.5 (CH), 34.3 (CH), 33.4 (CH_2), 29.5 (CH_2), 24.4 (CH_2), 19.1 (CH_3), 16.5 (CH_3), 11.4 (CH_3). GC-MS: 142 (M^+ - H_2O, 1.1), 127 (2.4), 113 (52.9), 85 (38.9), 71 (100), 70 (85.9), 57 (83.3).

2,6-Dimethyloctanol (**19H**)

^{1}H NMR: 3.50 (1H, m), 3.40 (1H, dd, J = 10.3, J = 6.7), 1.95 (1H, br. s), 1.61 (1H, oct, J = 6.6), 1.42-1.20 (6H, m), 1.15-1.06 (3H, m), 0.91 (3H, dd, J = 6.7, J = 1.2), 0.86 (3H, t, J = 7.2), 0.85 (3H, d, J = 6.5). ^{13}C NMR: 68.2 (CH_2), 36.8 (CH_2), 35.7 (CH), 34.3 (CH), 33.4

(CH_2), 29.4 (CH_2), 24.3 (CH_2), 19.1 (CH_3), 16.5 (CH_3), 11.3 (CH_3). GC-MS: 140 (M^+ - H_2O, 1.1), 125 (2.0), 111 (57.9), 85 (34.1), 69 (100), 57 (91.6).

1-Bromo-2,6-dimethylocatane-*1,1,-d_2* (**20**)

To a solution of triphenylphosphine (13.62 g, 51.98 mmol) in CH_2Cl_2 (100 mL), bromine (2.68 mL, 51.98 mmol) was added dropwise at 0°C and the mixture was stirred for 30 min with cooling. To this solution, **19** (7.56 g, 47.25 mmol) and triethylamine (9.84 mL, 70.88 mmol) in CH_2Cl_2 (50 mL) was added. After stirring for 3 h at 0°C, the mixture was filtered and the solvent was evaporated *in vacuo*. The residue was extracted with petroleum ether (300 mL) and filtered off. The filter cake was rinsed with petroleum ether (2 × 50 mL). The combined filtrates were concentrated. After this procedure was done twice, the residue was distilled to give 9.46 g of **20** (42.42 mmol, 89.8%). bp: 126-127°C/42 mmHg. ^{1}H NMR: 1.78 (1H, sext, J = 6.5), 1.46-1.04 (9H, m), 1.01 (3H, d, J = 6.6), 0.86 (3H, t, J = 7.2), 0.85 (3H, d, J = 6.1). ^{13}C NMR: 36.6 (CH_2), 35.1 (CH_2), 35.0 (CH), 34.3 (CH), 29.4 (CH_2), 24.3 (CH_2), 19.1 (CH_3), 18.7 (CH_3), 11.4 (CH_3), GC-MS: 195 (M^+ – Et, 3.9), 193 (M^+ – Et, 4.2), 165 (60.2), 113 (44.3), 85 (32.1), 71 (59.8), 57 (100).

1-Bromo-2,6-dimethylocatane (**20H**)

^{1}H NMR: 3.40 (1H, ddd, J = 9.8, J= 4.9, J = 1.9), 3.32 (1H, ddd, J = 9.8, J = 6.2, J = 1.1), 1.78 (1H, oct, J = 6.2), 1.46-1.04 (9H, m), 1.01 (3H, d, J = 6.6), 0.86 (3H, t, J = 7.2), 0.85 (3H, d, J = 6.1). ^{13}C NMR: 41.6 (CH_2), 36.6 (CH_2), 35.2 (CH), 35.1 (CH_2), 34.3 (CH), 29.4 (CH_2), 24.3 (CH_2), 19.1 (CH_3), 18.7 (CH_3), 11.4 (CH_3), GC-MS: 193 (M^+ – Et, 3.4), 191

(M^+ – Et, 3.7), 163 (55.2), 111 (45.3), 83 (30.1), 69 (83.8), 57 (100).

Diethyl (1,1-dideuterio-2,6-dimethyloctyl)malonate (**21**)

To a solution of NaH (1.57 g, 42.42 mmol; 65%) in DMF (40 mL), diethyl ethylmalonate (6.79 g, 42.42 mmol) in DMF (20 mL) was added at 0°C under N_2. After stirring for 30 min at room temperature, **20** (9.46 g, 42.42 mmol) in DMF (10 mL) was added. The solution was heated at 70-80°C for 2 h and then 100-110°C for 5 h. After cooling to room temperature, ice-cold water (50 mL) was added to the solution. The mixture was extracted with ether (3 × 50 mL). The combined extracts were washed with H_2O, brine, then dried and concentrated *in vacuo*. Filtration of the residue through a short column of SiO_2 gave 11.52 g of crude **21** (38.15 mmol). this compound was used for the next step without further purification. GC-MS: 257 (M^+ – OEt, 2.9), 175 (96.5), 160 (100), 133 (26.6), 129 (18.1), 114 (6.4), 103 (7.0), 88 (10.3).

Diethyl (2,6-dimethyloctyl)malonate (**21H**)

GC-MS: 271 (M^+ – Et, 1.0), 255 (3.3), 173 (100), 160 (93.3), 133 (21.8), 127 (21.8), 101 (8.3), 88 (7.8).

Ethyl 4,8-dimethyldecanoate-*3,3-d_2* (**22**)

This compound was prepared in the same procedure described for **2** from crude **21**, LiCl, and H_2O in DMSO. Yield: 3.78 g (16.43 mmol, 38.7% in 2 steps, > 99.9% pure). bp: 94-96°C/1 mmHg. ^{1}H NMR: 4.12 (2H, q, J = 7.1), 2.28 (2H, q, J = 15.0), 1.41 (1H, m), 1.39-1.20 (6H, m), 1.26 (3H, t, J = 7.1), 1.19-1.06 (3H, m), 0.87 (d, 3H, J = 5.7), 0.85 (3H, t, J = 7.4), 0.84 (3H, d, J = 6.2). ^{13}C NMR: 174.2 (C), 60.1 (CH_2), 37.0 (CH_2), 36.8 (CH_2),

34.4 (CH), 32.2 (CH), 32.0 (CH_2), 29.5 (CH_2), 24.3 (CH_2), 19.2 (CH_3), 19.1 (CH_3), 14.2 (CH_3), 11.4 (CH_3). GC-MS: 230 (M^+, 1.4), 185 (5.9), 173 (10.9), 143 (8.3), 131 (5.4), 113 (5.8), 103 (100), 88 (52.1), 85 (14.5), 75 (18.6), 70 (20.2), 61 (9.4).

Ethyl 4,8-dimethyldecanoate (**22H**)

^{1}H NMR: 4.12 (2H, q, *J* = 7.1), 2.35-2.23 (2H, m), 1.67-1.65 (1H, m), 1.41 (2H, m), 1.39-1.20 (6H, m), 1.26 (3H, t, *J* = 7.1), 1.19-1.06 (3H, m), 0.87 (d, 3H, *J* = 5.9), 0.85 (3H, t, *J* = 7.4), 0.84 (3H, d, *J* = 6.2). ^{13}C NMR: 174.1 (C), 60.1 (CH_2), 37.0 (CH_2), 36.8 (CH_2), 34.3 (CH), 32.4 (CH), 32.1 (CH_2), 32.0 (CH_2), 29.5 (CH_2), 24.3 (CH_2), 19.3 (CH_3), 19.2 (CH_3), 14.2 (CH_3), 11.4 (CH_3). GC-MS: 228 (M^+, 1.8), 183 (8.3), 171 (19.7), 141 (10.4), 129 (7.5), 111 (7.5), 101 (100), 88 (50.6), 83 (17.3), 73 (15.9), 69 (16.4).

Sodium 4,8-dimethyldecanoate-*3,3-d_2* (**23**)

Sodium salt **23** was obtained by the same manner as that used for **3** from **22** (3.00 g, 13.04 mmol) with equimolar 2N NaOH. Yield: 2.55 g (11.38 mmol, 87.3%).

Citronellyl bromide (**24**)

This compound was prepared in the same procedure used for **19** from citronellol (15.60 g, 100.00 mmol). Yield:18.23 g (83.24 mmol, 83.2%). bp: 103-105°C/12 mmHg. ^{1}H NMR: 5.09 (1H, tq, *J* = 7.1, *J* = 1.4), 3.48-3.37 (2H, m), 2.05-1.84 (3H, m), 1.71-1.57 (2H, m), 1.68 (3H, d, *J* = 1.3), 1.61 (3H, s), 1.38-1.32 (1H, m), 1.21-1.14 (1H, m), 0.90 (3H, d, *J* = 6.5). ^{13}C NMR: 131.9 (C), 125.0 (CH), 40.4 (CH_2), 37.0 (CH_2), 32.4 (CH_2), 31.8 (CH), 26.1 (CH_3), 25.8 (CH_2), 19.3 (CH_3), 18.1 (CH_3). GC-MS: 220 (M^+, 8.4), 218 (M^+, 8.0), 83

(56.2), 69 (100). IR: 2910 (s), 1250 (w), 1100 (w), 870 (w), 810 (w).

2-Trideuteriomethyl-1-butanol (**25**)

Ester **2** (2.33 g) was reduced with $LiAlH_4$ to afford the corresponding alcohol **19** (1.13 g, 70.9%). bp: 74°C/76 mmHg. ^{1}H NMR: 3.51 (1H, dd, *J* = 10.5, *J* = 5.9), 3.41 (1H, dd, *J* = 10.5, *J* = 6.5), 1.82 (1H, br. s), 1.52 (1H, m), 1.49-1.42 (1H, m), 1.14 (1H, dqu, *J* = 13.5, *J* = 7.4), 0.91 (3H, t, *J* = 7.4). ^{13}C NMR: 68.3 (CH_2), 37.5 (CH), 26.1 (CH_2), 11.7 (CH_3). GC-MS: 91 (M^+, 0.2), 73 (49.4), 60 (100), 59 (90.0). IR: 3300 (s), 2900 (s), 2200 (m), 2050 (m), 1240 (m), 1040 (s).

1-Iodo-2-trideuteriomethylbuatne (**27**)

Tosylate **26** (2.86 g) derived from **25** was converted to iodide **27** in the same way described for **6**. Yield: 1.61 g (8.01 mmol, 64.5% in 2 steps). bp: 62°C/70 mmHg. ^{1}H NMR: 3.23 (1H, dd, *J* = 9.6, *J* = 4.8), 3.17 (1H, dd, *J* = 9.6, *J* = 5.8), 1.44-1.35 (2H, m), 1.29- 1.24 (1H, m), 0.89 (3H, t, *J* = 7.3). ^{13}C NMR: 36.6 (CH), 29.5 (CH_2), 17.8 (CH_3), 11.7 (CH_3). GC-MS: 201 (M^+, 12.4), 127 (16.3), 74 (100). IR: 2930 (s), 2220 (m), 2050 (m), 1270 (m), 1250 (m), 1180 (s), 1050 (s).

2,6-Dimethyl-10-trideuteriomethyl-2-dodecene (**28**)

To a solution of citronellylmagnesium bromide prepared from 0.77 g of Mg (0.032 g atom) and **24** (7.02 g , 32.04 mmol) in THF (40 mL), a solution of **27** (1.61 g, 8.01 mmol), NMP (12.30 mL,128.16 mmol) and Li_2CuCl_4 (4.0 mL, 0.40 mmol; 0.1 M) in THF (20 mL) was added at 0°C under N_2. The mixture was stirred for 1.5 h at 0°C and 1 h at

room temperature. The excess Grignard reagent was destroyed by dropwise addition of 2N HCl (50 mL). The precipitate formed was removed by filtration through defatted cotton and the filtrate was extracted with hexane (3 × 60 mL). The organic phase was washed successively with 2N HCl, sat. Na_2SO_3, H_2O, and brine, then dried, and concentrated *in vacuo*. The residue was distilled to give 0.46 g of **28** (2.16 mmol, 27.0% based on **27**). bp: 102-104°C/4 mmHg. ^{1}H NMR: 5.10 (1H, tq, *J* = 7.1, *J* = 1.4), 2.00-1.92 (2H, m), 1.68 (3H, d, *J* = 1.1), 1.60 (3H, s), 1.42-1.35 (1H, m), 1.34-1.17 (7H, m), 1.18-1.02 (4H, m), 0.86 (3H, d, *J* = 6.6), 0.85 (3H, t, *J* = 7.3). ^{13}C NMR: 131.3 (C), 125.5 (CH), 37.7 (CH_2), 37.6 (CH_2), 37. 3 (CH_2), 34.6 (CH), 32.8 (CH), 29.8 (CH_2) 26.0 (CH_3), 25.7 CH_2), 24.8 (CH_2), 20.0 (CH_3), 18.0 (CH_3), 11.8 (CH_3). GC-MS: 213 (M^+, 20.4), 111 (14.7), 73 (26.6), 69 (100). IR: 2920 (s), 2200 (m), 2050 (m), 1440 (s), 1370 (s), 1050 (m).

2,3-Epoxy-2,6-dimethyl-10-trideuteriomethyl-dodecane (**29**)

Compound **29** was prepared according to the procedure of Suzuki (1981). Yield 84.9%. GC-MS: 229 (M^+, 0.4), 86 (31.0), 85 (37.5), 73 (46.5), 59 (100). IR: 2930 (s), 2200 (m), 2030 (m), 1240 (w), 1110 (m), 1040 (w).

4-Methyl-8-trideuteriomethyldecanal (**30**)

Epoxide **29** was cleaved with HIO_4 to give the desired aldehyde **30**. Yield 31.3%. Purification of a small portion of **30** by column chromatograph yielded 95.2% purity of **30** and it was used for analysis. ^{1}H NMR: 9.78 (1H, t, *J* = 1.8), 2.45-2.40 (2H, m), 1.70-1.62 (1H, m), 1.46-1.41 (2H, m), 1.36-1.20 (6H, m), 1.14-1.04 (3H, m), 0.88 (3H, d, *J* = 6.3), 0.85 (3H, t, *J* = 7.3). ^{13}C NMR: 203.1 (CH), 41.7 (CH_2), 37.0 (CH_2), 36.8 (CH_2), 34.2

(CH), 32.9 (CH), 29.4 (CH_2), 28.9 (CH_2), 24.4 (CH_2), 19.4 (CH_3), 11.4 (CH_3). GC-MS: 187 (M^+, 0.5), 143 (61.0), 140 (28.1), 114 (54.0), 95 (55.9), 85 (68.7), 73 (100), 60 (77.6), 57 (90.0). IR: 2910 (s), 2700 (s), 2190 (m), 2050 (m), 1730 (s).

2,6,10-Trimethyl-2-dodecene-*9,9-d_2* (**31**)

This compound was prepared in the same manner described for **28**. Yield:38.6%. ^{1}H NMR: 5.10 (1H, t-like, *J* = 7.1), 2.03-1.88 (2H, m), 1.68 (3H, d, *J* = 1.1), 1.60 (3H, s), 1.39 (1H, m), 1.36-1.17 (6H, m), 1.16-1.06 (3H, m), 0.86 (3H, d, *J* = 6.6), 0.85 (3H, t, *J* = 7.2), 0.84 (3H, d, *J* = 6.6). ^{13}C NMR: 131.3 (C), 125.5 (CH), 37.7 (CH_2), 37.5 (CH_2), 36.5 (tr, CH_2), 34.7 (CH), 32.8 (CH), 29.9 (CH_2), 26.1 (CH_3), 26.0 (CH_2), 24.6 (CH_2), 20.0 (CH_3), 19.6 (CH_3), 18.0 (CH_3), 11.8 (CH_3). IR: 2910 (s), 2150 (w), 2050 (w), 1420(m), 1370 (m).

2,6,10-Trimethyldodecane (**31H**)

^{1}H NMR: 5.10 (1H, t-like, *J* = 7.1), 2.03-1.90 (2H, m), 1.68 (3H, s), 1.60 (3H, s), 1.41 (1H, m), 1.35-1.22 (7H, m), 1.18-1.00 (4H, m), 0.86 (3H, d, *J* = 6.6), 0.85 (3H, t, *J* = 7.2), 0.84 (3H, d, *J* = 6.1). ^{13}C NMR: 131.3 (C), 125.5 (CH), 37.7 (CH_2), 37.5 (CH_2), 37.3 (CH_2), 34.8 (CH), 32.8 (CH), 29.9 (CH_2), 26.1 (CH_3), 26.0 (CH_2), 24.8 (CH_2), 20.0 (CH_3), 19.6 (CH_3), 18.0 (CH_3), 11.8 (CH_3).

2,3-Epoxy-2,6,10-trimethyldodecane-*9,9-d_2* (**32**)

This compound was prepared in the same manner as that used for **29**. Yield: 67.66%. IR: 2150 (m), 2050 (m), 1110 (s), 850 (m).

4,8-Dimethyldecanal-*7,7-d_2* (**33**)

This compound was prepared in the same procedure that used for **30**. Yield: 59.5%. GC-MS: 186 (M^+, 0.6), 142 (27.4), 139 (10.5), 127 (8.1), 113 (38.0), 96 (20.7), 87 (14.0), 85 (42.9), 82 (30.1), 73 (24.8), 71 (72.7), 57 (100).

2-Trideuteriomethyl-6-methyl-1-octanol (**34**)

Ester **34** was converted to the corresponding alcohol **10** with $LiAlH_4$ in the same procedure described for **19**. Yield 66.3%. ^{1}H NMR: 3.51 (1H, ddd, J = 10.5, J = 5.8, J = 2.0), 3.41 (1H, dd, J = 10.5, J = 6.6), 1.59 (1H, m), 1.47 (1H, br. s), 1.40-1.23 (6H, m), 1.18-1.01 (3H, m), 0.86 (3H, t, J = 7.2), 0.85 (3H, dd, J = 6.5, J = 1.1). ^{13}C NMR: 68.8 (CH_2), 37.3 (CH_2), 36.0 (CH), 34.8 (CH), 33.8 (CH_2), 30.0 (CH_2), 24.8 (CH_2), 19.6 (CH_3), 11.8 (CH_3). GC-MS: 143 ($M^+ - H_2O$, 0.4), 114 (64.4), 88 (20.1), 83 (18.2), 74 (30.5), 72 (63.0), 71 (61.1), 70 (100), 57 (78.6), 43 (72.5). IR: 3300 (br, s), 2900 (s), 2300 (w), 2200 (m), 1020 (s).

1-Bromo-2-trideuteriomethyl-6-methyloctane (**35**)

This compound was prepared in the same manner as that used for **20**. Yield: 76.0%. ^{1}H NMR: 3.40 (1H, ddd, J = 9.8, J = 4.9, J = 1.9), 3.32 (1H, ddd, J = 9.8, J = 6.2, J = 1.1), 1.77 (1H, m), 1.46-1.04 (9H, m), 0.86 (3H, t, J = 7.2), 0.85 (3H, dd, J = 6.1, J = 0.8). ^{13}C NMR: 42.0 (CH_2), 37.0 (CH_2), 35.5 (CH_2), 35.4 (CH), 34.7 (CH), 29.9 (CH_2), 24.7 (CH_2), 19.6 (CH_3), 11.8 (CH_3). GC-MS: 196 (M^+ – Et, 1.6), 194 (M^+ – Et, 1.6), 168 (60.4), 166 (62.4), 114 (36.1), 72 (55.3), 57 (100), 56 (45.6), 55 (36.1). IR: 2910 (s), 2200 (m), 1040 (m).

Diethyl (2-trideuteriomethyl-6-methyloctyl)malonate (**36**)

This compound was prepared in the same procedure used for **21**. Crude **36** was used for the next step without further purification.

Ethyl 4-trideuteriomethyl-8-methyldecanoate (**37**)

Ester **37** was prepared in the same manner as that used for **22** from crude **36**. Yield: 53.0% in 2 steps. ^{1}H NMR: 4.12 (2H, q, J = 7.1), 2.38-2.26 (2H, m), 1.70-1.63 (1H, m), 1.48-1.38 (2H, m), 1.37-1.20 (6H, m), 1.26 (3H, t, J = 7.1), 1.16-1.02 (3H, m), 0.85 (3H, t, J = 7.3), 0.84 (3H, dd, J = 6.1, J = 0.7). ^{13}C NMR: 174.6 (C), 60.6 (CH_2), 37.3 (CH_2), 37.2 (CH_2), 34.8 (CH), 32.6 (CH), 32.6 (CH_2), 32.3 (CH_2), 29.9 (CH_2), 24.8 (CH_2), 19.6 (CH_3), 14.6 (CH_3), 11.8 (CH_3). GC-MS: 231 (M^+, 0.5), 174 (11.0), 171 (10.0), 143 (4.3), 101 (100), 73 (26.2). IR: 2900 (s), 2090 (m), 1730 (s), 1350 (m), 1240 (m), 1150 (s).

4-Trideuteriomethyl-8-methyldecanal (**39**)

The desired aldehyde **39** was obtained by PCC oxidation of **38**, prepared from reduction of the corresponding ether **37**. To a suspension of PCC (0.72 g, 3.33 mmol) in CH_2Cl_2 (10 mL), **15** (0.42 g, 2.22 mmol) in CH_2Cl_2 (10 mL) was added. After the mixture was stirred for 1h at room temperature, the supernatant was decanted, and the black gum was washed with dry ether (3 × 50 mL). The combined organic layers were passed through a short pad of florisil column and concentrated. The residue was chromatographed over silica (10 g, hexane:ether = 95:5) and distilled to yield 0.10 g of **38** (24.8%). ^{1}H NMR: 9.78 (1H, t, J = 1.9), 2.45-2.40 (2H, m), 1.70-1.62 (1H, m), 1.48-1.40 (2H, m), 1.37-1.20 (6H, m), 1.17-1.02 (3H, m), 0.86 (3H, t, J = 7.1), 0.84 (3H, dd, J = 6.5, J = 0.8). ^{13}C NMR:

203.5 (CH), 42.1 (CH_2), 37.3 (CH_2), 37.2 (CH_2), 34.8 (CH), 32.5(CH), 29.8 (CH_2), 29.3 (CH_2), 24.8 (CH_2), 19.6 (CH_3), 11.7 (CH_3). GC-MS: 187 (M^+, 0.4), 143 (46.5), 140 (24.9), 114 (53.5), 98 (50.2), 84 (76.5), 70 (100), 57 (88.6). IR: 2920 (s), 2710 (m), 2190 (m), 1710 (s).

4,8-Dimethyldecanal-*3,3-d_2* (**41**)

This compound was prepared in the same manner as that used for **39**, from alcohol **40** obtained from LAH reduction of the corresponding ester **32**. The MS spectrum is shown in Fig. 4-15 (D).

[2-^{13}C]Ethyl acetate (**42**)

This compound **42** was prepared by the following procedure of Ropp (1950). A solution of [2-^{13}C]sodium acetate (4.0 g, 48.19 mmol) in triethyl phosphate (20 mL) was refluxed for 1 h until all solid disappeared. After cooling to room temperature, the mixture was distilled to afford **42** (3.39 g, 38.09 mmol, 79.0%). bp: 77-79°C/760 mmHg. ^{1}H-NMR: 4.12 (2H, -C$\underline{H}_2$-, q, J = 7.1), 2.04 (3H, C$\underline{H}_3$-C=O, d, J =129.2), 1.26 (3H, -$CH_2C\underline{H}_3$, t, J = 7.1). ^{13}C-NMR: 171.1 ($\underline{C}$=O, d, J = 59.5), 60.3 (-$\underline{C}H_2$-), 20.9 ($\underline{C}H_3$-C=O), 14.2 (-$\underline{C}H_3$). GC-MS: 89 (M^+, 45.1), 74 (30.7), 71 (96.4), 62 (100).

Ethyl acetate (**42H**)

^{1}H-NMR: 4.12 (2H, -C$\underline{H}_2$-q, J = 7.1), 2.05 (3H, C$\underline{H}_3$-C=O, s), 1.26 (3H, -$CH_2C\underline{H}_3$, t. J = 7.1). ^{13}C-NMR: 171.2 ($\underline{C}$=O), 60.4 (-$\underline{C}H_2$-), 21.1 ($\underline{C}H_3$-C=O), 14.2 (-CH_3). GC-MS: 88 (M^+, 46.9), 73 (39.5), 71 (85.8), 62 (100).

4-Tetrahydropyranyl-2-butanone (**43**)

To a solution of 4-hydroxy-2-butanone (10.0 g, 113.49 mmol) in dihydropyran (15.0 mL), 4 drops of conc. HCl was added as catalyst, and the mixture was stirred and left overnight at room temperature. After addition of ether (150 mL), the solution was washed with $NaHCO_3$, H_2O and brine, then dried and concentrated *in vacuo*. Yield: 20.0 g (116.28 mmol, 102.4% yield as crude, 89.0% pure). This compound was used for the next step without further purification. GC-MS: 171 (M^+ – H, 1.0), 114 (5.1), 101 (79.5), 85 (82.2), 71 (M^+ – OTHP, 50.0), 55 (42.1), 43 (100). IR: 1700 (s), 1340 (s), 1110 (s), 1010 (s).

Ethyl [2-^{13}C]5-tetrahydropyranyl-3-methyl-3-hydroxypentanoate (**44**)

To a solution of LDA which prepared from 5.34 mL (38.09 mmol) of diisopropylamine in THF (20 mL) and n-butyl lithium 14.70 mL (38.09 mmol, 2.59 M) at −78°C, **42** (3.39 g, 38.09 mmol) was added via septum using syringe at −78°C under N_2. After stirring for 30 min, 5.28 g of **43** (30.68 mmol) in THF (10 mL) was added to the solution and stirring was continued for 4 h. After removal of dry ice-acetone bath, the solution was neutralized with acetic acid (6 mL), followed by addition of water (60 mL). The solution was extracted with ether (3 × 50 mL) and the organic layer was washed with H_2O, brine then dried. Evaporation of the solvent gave 9.72 g of crude **44** (37.2 mmol, 97.8% yield as crude, 74% pure). This compound was used for the next step without further purification. Chromatography of a small portion gave 94.1% pure pentanoate, which sumitted to NMR analysis. ^{1}H-NMR: 4.60 (1H, 2'-CH, q, J = 3.0), 4.16 (2H, -C$\underline{H}_2$CH$_3$ q, J = 7.1), 3.98 (1H, 3-CH$_2$, m), 3.85 (1H, 6'-C$\underline{H}_2$, m), 3.57 (1H, 3-CH$_2$, m), 3.53 (1H, 6'-C$\underline{H}_2$, m), 2.57 (2H, 2-CH$_2$, d, J=128.9), 1.91 (2H, 4-CH$_2$, sext-like), 1.79 (1H,

4-CH_2, m), 1.71 (1H, 3-CH_2, m), 1.61-1.51 (4H, 3'-, 4'-, 5'-CH_2, m), 1.31 (3H, -CH_3, dd, *J* = 4.0, *J* = 4.0 or t, *J* = 4.0), 1.28 (3H, -$CH_2C\underline{H}_3$, t , *J* = 7.1). ^{13}C-NMR: 172.3 (1-C, d, *J* = 48.2), 98.9 (2'-CH), 70.7 (3-C, d *J*=37.4), 64.0 (5-CH_2), 62.2 (5'-CH_2), 60.4 (-$O\underline{C}H_2CH_3$), 45.5 (2-CH_2), 41.2 (4-CH_2), 30.5 (3'-CH_2), 27.0 (CH_3), 25.3 (4'-CH_2), 19.3 (6'-CH_2), 14.1 (-$OCH_2\underline{C}H_3$). GC-MS: 216 (M^+ – OEt, 0.7), 188 (9.1), 160 (37.1, M^+ – OTHP), 142 (10.7), 132 (22.1), 101 (12.6), 85 (100), 43 (41.1). IR: 3400 (br, s, -OH), 1700 (s), 1320 (s), 1190 (s), 1110 (s), 1010 (s).

Ethyl 5-tetrahydropyranyl-3-methyl-3-hydroxypentanoate (**44H**)

^{1}H-NMR: 4.60 (1H, 2'-CH, q, *J* = 3.5), 4.16 (2H, -$C\underline{H}_2CH_3$ q, *J* = 7.1), 3.98 (1H, 3-CH_2, m), 3.85 (1H, 6'-$C\underline{H}_2$,m), 3.57 (1H, 3-CH_2, m), 3.53 (1H, 6'-$C\underline{H}_2$, m), 2.56 (2H, 2-CH_2, m), 1.91 (2H, 4-CH_2, t-like), 1.78 (1H, 4-CH_2, m), 1.71 (1H, 3-CH_2 , m), 1.61-1.51 (4H, 3'-, 4'-, 5'-CH_2, m), 1.31 (3H, -CH_3, d-like, *J* = 4.2), 1.28 (3H, -$CH_2C\underline{H}_3$, t, *J* = 7.1). ^{13}C-NMR: 172.3 (1-C), 98.9 (2'-CH), 70.7 (3-C), 64.0 (5-CH_2), 62.2 (5'-CH_2), 60.4 (-$O\underline{C}H_2CH_3$), 45.5 (2-CH_2), 40.2 (4-CH_2), 30.5 (3'-CH_2), 27.0 (CH_3), 25.3 (4'-CH_2), 19.3 (6'-CH_2), 14.1 (-$OCH_2\underline{C}H_3$). GC-MS: 215 (M^+ – OEt, 0.8), 187 (10.4), 159 (38.9, M^+ – OTHP), 141 (10.9), 131 (28.4), 101 (5.2), 85 (100), 43 (29.7). IR: 3400 (br, s, -OH), 1700 (s), 1320 (s), 1190 (s), 1110 (s), 1010 (s).

[2-^{13}C]5-Tetrahydropyranyl-3-methyl-3-hydroxypentanoic acid (**45**)

Crude ester **44** (9.60 g, 36.78 mmol) was hydrolyzed with 2N NaOH (20 mL) in 40 mL of ethanol by heating at 50°C for 4 h. After cooling to room temperature, H_2O (100 mL) was added. The solution was extracted with ether (3 × 50 mL) to remove unexpected

organic products. Aqueous layer was acidified to pH 4 with acetic acid (5 mL) and extracted with ether (4 × 70 mL). The ether layer was washed with brine, then dried and concentrated *in vacuo*. Yield: 7.44 g (31.93 mmol, 86.8% yield as crude, 97.0% pure). GC-MS: 186 (0.9), 132 (M^+ – OTHP, 15.4), 114 (16.1), 101 (38.3), 85 (100), 71 (42.4), 55 (67.3), 43 (86.5).

[2-^{13}C]Mevalonolactone ([2-^{13}C]3-Methyl-3-hydroxy-5-pentanolide, **46**)

Dowex was used to detach tetrahydropyranyl group in anhydrous condition by the procedure in Beier and Mundy (1979). Dowex® 50W-X8 (5 g), conditioned prior to use, was added to a solution of **45** (7.44 g, 31.93 mmol) in methanol (50 mL) and stirred for 2 h at room temperature. After filtration and the removal of the solvent, the residue (3.69 g) was chromatographed on a Florisil® column (40 g; Kanto Chemical Co. Inc). The desired compound **46** was eluted in ether and 5% acetone impregnated ether fraction. Yield: 2.15 g (16.41 mmol, 97.2% pure). Overall yield; 34.0% from [2-^{13}C]sodium acetate, 43.1% from [2-^{13}C]ethyl acetate. The NMR spectrum was identical to the literature (Lawson et al., 1975). ^{1}H-NMR: 4.60 (1H, 5-CH_2, m), 4.36 (1H, 5-CH_2, dt, J = 11.2, J = 4.5), 3.28 (1H, br. s), 2.66 (1H, 2-CH_2, ddd, J = 133.3, J = 17.4, J = 1.0), 2.50 (1H, 2-CH_2, dd, J = 126.0, J = 17.4), 1.91 (2H, 4-CH_2, m), 1.39 (3H, dd, J = 4.4, J = 1.0). ^{13}C-NMR: 171.3 (1-C, d, J = 50.8), 67.9 (3-C, d, J = 36.5), 66.2 (5-CH_2), 44.4 (2-CH_2), 35.5 (4-CH_2), 29.4 (CH_3). GC-MS: 131 (M^+, 1.3), 116 (3.4), 113 (7.9), 71 (74.0), 43 (100).

Mevalonolactone (3-Methyl-3-hydroxy-5-pentanolide, **46H**)

^{1}H-NMR: 4.60 (1H, 5-CH_2, m), 4.39 (1H, 5-CH_2, dt, J = 11.3, J = 4.6), 3.17 (1H, br. s),

2.66 (1H, 2-CH_2, dt, J = 17.4, J = 1.3), 2.46 (1H, 2-CH_2, d, J = 17.7), 1.90 (2H, 4-CH_2, m), 1.39 (3H, s). ^{13}C-NMR: 171.2 (1-C), 67.9 (3-C), 66.2 (5-CH_2), 44.5 (2-CH_2), 35.6 (4-CH_2), 29.4 (3'-CH_3). GC-MS: 130 (M^+, 1.2), 115 (4.0), 112 (9.8), 71 (83.0), 43 (100).

Acetone-d_6 *N*,*N*-dimethylhydrazone (**47**)

According to the modified procedure in the literature, **37** was prepared (Wiley et al., 1956; Boland and Gäbler, 1989). To a solution of acetone-d_6 18.42 mL (250.00 mol) and *N*,*N*-dimethylhydrazine 22.81 mL (300.00 mmol), acetic acid-d_4 (1 mL) was added as catalyst. The solution was refluxed for 6 h. After cooling to room temperature, 5 g of NaOH pallet was added to the solution. The solution was stirred for 15 min, then filtered. Distillation over NaOH pellet gave to 17.90 g of **47** (168.87 mmol, 67.5% yield, 95.5% pure). bp: 91-93°C/760 mmHg. ^{1}H NMR: 2.43 (6H, s), 1.96-1.90 (1.6H, m, 73% of 2 × CD_3). ^{13}C NMR: 165.2 (C), 47.3 (CH_3 × 2), 24.9 (tr, CD_3), 17.9 (tr, CD_3). GC-MS :106 (M^+, 36.9), 105 (100), 104 (99.3), 91 (4.1), 90 (11.3), 62 (10.4), 60 (43.0), 44 (75.7). IR: 2500 (w), 2110 (w), 1630 (s), 1010 (s), 980 (s).

Prenyl bromide (**48**)

To a solution of 2-methyl-3-buten-2-ol (43.07 g, 500.00 mmol), 48% HBr (200 mL) was added within 10 min at 0°C, and stirred for 1 h vigorously keeping that tempurature. The solution was extracted with hexane (5 × 100 mL), and the hexane layer was washed with H_2O, $NaHCO_3$ and brine then dried and concentrated fractionally. Distillation of the residue gave 45.17 g of **48** (303.07 mmol, 60.6% yield, 96.6% pure). bp: 56-57°C/50 mmHg. ^{1}H NMR: 5.53 (1H, tq, J = 8.5, J = 1.4), 4.01 (2H, d, J = 8.5), 1.78

(3H, s), 1.73 (3H, d, J = 1.2). ^{13}C NMR: 140.5 (C), 121.2 (CH), 30.1 (CH_2), 26.2 (CH_3), 18.0 (CH_3).

6-Methyl-2-heptenone-*1,1,1,3,3-d*$_5$ (**49**)

This compound was prepared as the similar procedure in Boland and Gäbler (1989). To a solution of 17.90 g of **47** (168.87 mmol) in THF (150 mL), n-BuLi 70.36 mL (185.76 mmol; 2.64M) was added over 30 min at 0°C under N_2, and stirred further 1 h. Bromide **48** (25.16 g, 168.87 mmol) in THF (100 mL) was added to the mixture, and stirred for 1 h at 0°C then for 3 h at room temperature. The mixture was acidified to *p*H 4 by adding 2N HCl and stirred for 30 min, and extracted with ether (4 × 100 mL). The organic layer was washed with H_2O, $NaHCO_3$, brine then dried up. Distillation of the residue afforded 10.05 g of **49** (76.72 mmol, 45.4% yield, 98.7% pure). bp: 75-76°C/22 mmHg. ^{1}H NMR: 5.06 (1H, tq, J = 7.2, J = 1.4), 2.43 (0.5H, m, 75% of CD_2), 2.24 (2H, t, J = 6.8), 2.10 (0.8H, 74% of CD_3), 1.67 (3H, d, J = 1.0), 1.62 (3H, s). ^{13}C NMR: 209.5 (C), 133.1 (C), 123.0 (C), 43.8 (tr, CD_2), 29.8 (tr, CD_3), 26.0 (CH_3), 22.8 (CH_2), 18.0 (CH_3). GC-MS: 131 (M^+, 0.3), 130 (10.5), 113 (32.6), 112 (73.7), 85 (11.4), 69 (79.3), 62 (21.5), 56 (50.6), 46 (68.3), 45 (100), 41 (77.8). IR: 2100 (w), 1700 (s), 1220 (m).

Linalool-*d*$_5$ (**50**)

To a solution of 3.41 g of **49** (26.03 mmol) in THF (25 mL), vinylmagnesium chloride (20.75 mL, 28.63 mmol; 1.38 M) added via septum by syringe at −15°C. After stirring for 8 h at room temperature, sat. NH_4Cl was added to the solution to quench the reaction. The mixture was extracted with ether (4 × 70 mL), washed with sat. NH_4Cl and

brine and dried then distilled at 98-100°C/26 mmHg. Yield 1.32 g (8.30 mmol, 31.9% yield, 97.3% pure). ^{1}H NMR: 5.91 (1H, dd, *J* = 17.3, *J* = 10.8), 5.21 (1H, dd, *J* = 17.3, *J* = 1.2), 5.11 (1H, tq, *J* = 7.1, *J* = 1.3), 5.06 (1H, dd, *J* = 10.8, *J* = 1.2), 2.06-1.97 (2H, m), 1.68 (3H, d, *J* = 1.0), 1.60 (3H, s), 1.57-1.50 (0.6H, m, 70.8% of CD_2), 1.26 (0.8H, m, 74% of CD_3). ^{13}C NMR: 145.4 (CH), 132.3 (C), 124.7 (CH), 112.1 (CH_2), 73.6 (C), 42.0 (tr, CD_2), 27.7 (tr, CD_3), 23.0 (CH_3), 18.0 (CH_2), 15.6 (CH_3). GC-MS: 141 ($M^+ - CD_3$, 5.2), 140 (M^+ – HDO, 6.3), 126 (9.0), 97 (62.8), 85 (35.4), 84 (45.2), 74 (90.3), 73 (78.6), 69 (71.9), 56 (61.4), 46 (71.0), 41 (100). IR: 3380 (br, s), 3080 (w), 22120 (m), 1640 (m), 1100 (m), 980 (m), 910 (m).

Ethyl geranoate-d_5 (**51**)

This compound was prepared by the usual Wittig-Horner reaction. To a suspension of NaH 2.43 g (101.38 mmol) in THF (50 mL), 22.73 g of TEPA (100.38 mmol) in THF (50 mL) was added at 0°C under N_2. After stirred for 30 min at 0°C and then for 1 h at room temperature, 6.64 g of **49** (50.69 mmol) in THF (30 mL) was added at 0°C and left overnight at room temperature. After addion of water, the mixture was extracted with hexane (3 × 80 mL). The organic layer was washed with brine and dried up, distilled at 102-104°C/6 mmHg to afford 8.59 g of the mixture of **51** and geometric isomer of **51** (*E*:*Z* = 3:1 by GC). A portion of the mixture was subjected to SiO_2 chromatography to acquire **51**. ^{1}H NMR: 5.66 (1H, s), 5.08 (1H, tq, *J* = 7.1, *J* = 1.4), 4.15 (2H, q, *J* = 7.1), 2.13 (3H, d, *J* = 6.2, CH_2 and CD_3), 2.10 (0.5H, m, 76% of CD_2), 1.68 (3H, d, *J* = 1.0), 1.61 (3H, s), 1.28 (3H, t, *J* = 7.1). ^{13}C NMR: 167.3 (C), 160.0 (C), 132.8 (C), 123.4 (CH), 116.1 (CH), 59.8 (CH_2), 40.9 (tr, CD_2), 26.4 (CH_2), 26.0 (CH_3), 18.7 (tr, CD_3), 18.0 (CH_3),

14.7 (CH_3). GC-MS: 201 (M^+, 0.3), 156 (3.7), 155 (8.2), 133 (3.1), 132 (11.1), 126 (14.8), 104 (12.1), 87 (8.9), 86 (12.4), 69 (100), 41 (57.6). IR: 2100 (m), 1710 (s), 1635 (s), 1260 (s), 1140 (s), 1040 (s), 860 (m).

Geraniol-d_5 (**52**)

To a solution of Red-Al® 7.11 mL (23.68 mmol) in THF (20 mL), 2.19 g of **51** (10.90 mmol) in THF (10 mL) was added at 0°C and the solution was stirred for 30 min at 0°C then for 1 h at room temperature. The reaction was quenched by adding ice-water, followed by 2N HCl. The mixture was extracted with ether (3 × 60 mL). The organic layer was washed with 2N HCl, $NaHCO_3$, H_2O, brine, then dried, and concentrated *in vacuo*. Distillation of the residue gave 1.14 g of **52** (7.17 mmol, 65.8% yield, 97.9% pure). bp: 74-75°C/1 mmHg. ^{1}H NMR: 5.41 (1H, t, J = 6.9), 5.09 (1H, tq, J = 7.0, J = 1.4), 4.14 (2H, d, J = 6.9), 2.09 (2H, d, J = 7.2), 2.00 (0.96H, m, 52% of D), 1.68 (3H, d, J = 1.1), 1.65 (0.88H, m, 70% of D), 1.60 (3H, s). ^{13}C NMR: 139.9 (C), 132.1 (C), 124.3 (CH), 123.8 (CH), 59.7 (CH_2), 39.5 (tr, CD_2), 26.7 (CH_2), 26.0 (CH_3), 18.0 (CH_3), 16.0 (tr, CD_3). GC-MS: 159 (M^+, 0.4), 144 (0.4), 141 (1.5), 128 (4.9), 116 (3.3), 97 (6.2), 88 (5.2), 85 (5.6), 69 (100), 41 (64.0). IR: 3300 (br, s), 2120 (m), 1660 (m), 980 (s).

Geranyl bromide (**53)**

This compound was prepared in the same manner as described in Chapter II. The spectra was identical to those described in Chapter II.

Geranylacetone (**55**)

This compound was prepared in the same manner as described in Chapter II. Yield: 4.70 g (24.22 mmol, 20.2% from geraniol in 2 steps, 97.3% pure). bp: 85°C/1.5 mmHg. The spectra was identical to those described in Chapter II.

Geranylacetone-*1,1,1,3,3-d*$_5$ (**55**)

To a solution of 9.80 g of sodium methoxide (181.65 mmol) in methanol-*d* (50 mL, ca. 1700 mmol), 4.70 g of **56** (24.22 mmol) in methanol-*d* (20 mL) was added under N_2. After stirring for 2 d, the solvent was evaporated up to 50 mL and ether (100 mL) and H_2O (70 mL) was added to the solution. The aqueous layer was extracted with hexane (2 × 60 mL). The organic layer was washed with brine then dried and concentrated *in vacuo*. Distillation of residue gave 2.98 g of **55** (14.97 mmol, 61.8% yield, 97.1% pure).

Ethyl farnesoate-*4,4,3',3',3'-d*$_5$ (**56**)

This compound was prepared in the usual Wittig-Horner reaction as described in **51**. Yield: 66.5% (95.3% pure, main impurity was 2*Z*-isomer of **56**).

Farnesol-*4,4,3',3',3'-d*$_5$ (**57**)

To a suspension of $LiAl(OEt)H_3$, prepared from LAH (1.13 g, 29.88 mmol) in THF (20 mL) and absolute ethanol (1.68 mL, 29.88 mmol) in THF (10 mL), 2.68 g of **56** (9.96 mmol) in THF was added at 0°C, and the mixture was stirred for 1 h at room temperature, to which ethyl acetate was added, followed by 2N HCl. The aqueous solution was extracted with (3 × 50 mL). The organic layer was washed with 2N HCl, H_2O, $NaHCO_3$, brine then dried, and concentrated *in vacuo*. SiO_2 chromatography of the residue

gave a mixture of **57** and **58** (1.63 g) at ratio of 65:35. This mixture was used for bioassay without further purification.

Citronellylacetone (**59**)

A solution of *t*-BuOK (10.95 g, 97.80 mmol) in *t*-BuOH (70 mL) was refluxed to dissolve *t*-BuOK and then cooled to 50°C. After ethyl acetoacetate (11.65 g, 89.65 mmol) was added, the mixture was stirred for 30 min, and 17.84 g of **24** (81.50 mmol) was added to the solution. The mixture was stirred and refluxed for 24 h. The reacted solution was cooled, filtered and concentrated. The residue was poured into a stirred 2N NaOH (300 mL) solution and heated for 3 h at 50-55°C. After cooling to room temperature, the alkaline solution was acidified with acetic acid (40 mL). The mixture was extracted with hexane (3 × 100 mL). The organic phases were washed with sat. $NaHCO_3$, dried and concentrated *in vacuo*. The residue was distilled at 108-111°C/4 mmHg to give 10.63 g of **59** (54.23 mmol, 65.5% yield, 95.0% pure). ^{1}H NMR: 5.09 (1H, t, J = 7.1), 2.40 (2H, t, J = 7.6), 2.13 (3H, s), 1.96 (2H, m), 1.68 (3H, s), 1.60 (3H, s), 1.55 (2H, m), 1.41 (1H, m), 1.29 (2H, m), 1.13 (2H, m), 0.87 (3H, d, J = 6.6). ^{13}C NMR: 209.7 (C), 131.5 (C), 125.2 (CH), 44.5 (CH_2), 37.3 (CH_2), 36.8 (CH_2), 32.6 (CH), 30.2 (CH_3), 26.1 (CH_3), 25.9 (CH_2), 21.8 (CH_2), 19.9 (CH_3), 18.0 (CH_3). GC-MS: 196 (M^+, 4.8), 138 (6.4), 69 (78.8), 43 (100). IR: 3380 (br, m), 1710 (s), 1150 (m).

Citronellylacetone-*1,1,1,3,3-d*$_5$ (**60**)

This compound was prepared in the same manner as that used for **55** from sodium methoxide (11.63 g, 215.40 mmol) in methanol-*d* (80 mL), and **59** (5.63 g, 28.72 mmol) with 2 day stirring. Yield: 3.61 g (18.00 mmol, 62.7%, 97.8% pure). bp: 107°C/5 mmHg.

107 ^{1}H NMR: 5.09 (1H, t, *J* = 7.1), 2.41-2.34 (0.71H, m), 2.13-2.09 (1.22H, m), 2.13 (0.32H, s), 2.22 (0.54H, t, *J* = 2.2), 2.10 (0.36H, qu, *J* = 2.2), 1.96 (2H, m), 1.68 (3H, s), 1.60 (3H, s), 1.55 (2H, m), 1.41 (1H, m), 1.29 (2H, m), 1.13 (2H, m), 0.87 (3H, d, *J* = 6.6). ^{13}C NMR: 209.7 (C), 131.5 (C), 125.2 (CH), 44.5 (tr, CH_2), 37.3 (CH_2), 36.8 (CH_2), 32.6 (CH), 30.2 (tr, CH_3), 26.1 (CH_3), 25.9 (CH_2), 21.7 (CH_2), 19.8 (CH_3), 18.0 (CH_3). IR: 3380 (br, m), 2100 (w), 1710 (s).

Ethyl 6,7-dihydrofanesoate-*4,4,15',15',15'-d_5* (**61**)

This compound was prepared in the same way that described for **51**. Yield: 76.1% (98.4% pure). bp: 120-124°C/1 mmHg. ^{1}H NMR: 5.64 (1H, s), 5.09 (1H, t-like, *J* = 7.1), 4.14 (2H, q, *J* = 7.1), 2.15 (0.76H, m), 2.11 (0.70H, m), 1.95 (2H, m), 1.68 (3H, s), 1.60 (3H, s), 1.48 (2H, m), 1.40 (1H, m), 1.33-1.25 (2H, m), 1.27 (3H, t, *J* = 7.1), 1.12 (2H, m), 0.87 (3H, d, *J* = 6.6). ^{13}C NMR: 167.3 (C), 160.7 (C), 131.5 (C), 125.3 (CH), 115.8 (CH), 59.8 (CH_2), 41.6 (tr, CH_2), 37.4 (CH_2), 36.8 (CH_2), 32.6 (CH), 26.1 (CH_3), 25.9 (CH_2), 25.1 (CH_2), 19.9 (CH_3), 19.1 (tr, CH_3), 18.0 (CH_3), 14.7 (CH_3). IR: 2100 (br, w), 1705 (s), 1630 (m), 1210 (s), 1130 (s).

Ethyl 6,7-dihydrofarnesoate (**61H**)

^{1}H NMR: 5.66 (1H, q-like, *J* = 1.1), 5.09 (1H, t-like, *J* = 7.1), 4.14 (2H, q, *J* = 7.1), 2.15 (3H, d, *J* = 1.2), 2.11 (2H, t-like, *J* = 7.7), 1.95 (2H, m), 1.68 (3H, d, *J*=0.9), 1.60 (3H, s), 1.48 (2H, m), 1.40 (1H, m), 1.33-1.25 (2H, m), 1.27 (3H, t, *J* = 7.1), 1.12 (2H, m), 0.87 (3H, d, *J* = 6.6). ^{13}C NMR: 167.3 (C), 160.7 (C), 131.5 (C), 125.3 (CH), 115.8 (CH), 59.8 (CH_2), 41.6 (CH_2), 37.4 (CH_2), 36.8 (CH_2), 32.6 (CH), 26.1 (CH_3), 25.9 (CH_2), 25.2 (CH_2),

19.9 (CH_3), 19.1 (CH_3), 18.0 (CH_3), 14.7 (CH_3). GC-MS: 266 (M^+, 10.4), 221 (18.2), 128 (50.4), 109 (98.0), 69 (100). IR: 1705 (s), 1640 (m), 1210 (s), 1130 (s).

Ethyl 2,3,6,7-tetrahydrofarnesoate-*4,4,15',15',15'-d_5* (**62**)

Selective hydrogenation of **62** was carried our using a modified procedure in the literature (Semmelhack and Stauffer, 1975; Fuchs et al., 1999). To a suspension of CuBr (I) 20.36 g (142.38 mmol) in dry THF (60 mL), Red-Al® (32.80 mL (109.52 mmol) was added dropwise at −10°C under N_2 and the solution was stirred for a further 30 min. A solution of ethyl dihydrofarnesoate 3.71 g (13.69 mmol) in dry THF (15 mL) was added to the mixture and stirred for 1 h at −10°C and for 2 h at room temperature. After the reacted solution was cooled to 0°C, ice-cold water was added carefully. The black gum formed was washed with ether (3 × 50 mL). The organic phase was filtered on a short pad of SiO_2, washed with 2N HCl, sat. $NaHCO_3$, brine, then dried and concentrated *in vacuo*. The residue was chromatographed over silica (50 g, hexane:ether = 98:2) to give 2.30 g (8.42 mmol), 71.8% yield, 96.9% pure. ^{1}H NMR: 5.10 (1H, t-like, J = 7.1), 4.13 (2H, q, J = 7.1), 2.28 (1H, ddd, J = 14.0, J = 6.0, J = 2.0), 2.09 (1H, ddd, J = 14.0, J = 8.0, J = 2.0), 2.03-1.90 (3H, m), 1.68 (3H, s), 1.60 (3H, s), 1.39 (1H, m), 1.36-1.02 (6.7H, m), 1.26 (3H, t, J = 7.1), 0.95 (1.40H, m), 0.86 (3H, d, J = 6.5). ^{13}C NMR: 173.8 (C), 131.4 (C), 125.4 (CH), 60.4 (CH_2), 42.3 (CH_2), 37.5 (tr, CH_2), 37.46 (CH_2), 37.44 (CH_2), 32.8 (CH), 30.8 (CH), 26.1 (CH_3), 26.0 (CH_2), 24.7 (CH_2), 20.1 (tr, CH_3), 19.9 (CH_3), 18.0 (CH_3), 14.7 (CH_3). IR: 2100 (br, w), 1730 (s), 1150 (s), 1010 (m).

Ethyl 2,3,6,7-tetrahydrofarnesoate (**62H**)

^{1}H NMR: 5.10 (1H, t-like, J = 7.1), 4.13 (2H, q, J = 7.1), 2.28 (1H, ddd, J = 14.0, J = 6.0, J

= 2.0), 2.09 (1H, ddd, *J* = 14.0, *J* = 8.0, *J* = 2.0), 2.03-1.90 (3H, m), 1.68 (3H, s), 1.60 (3H, s), 1.39 (1H, m), 1.36-1.02 (8H, m), 1.26 (3H, t, *J* = 7.1), 0.95 (3H, d, *J* = 6.7), 0.86 (3H, d, *J* = 6.5). ^{13}C NMR: 173.8 (C), 131.4 (C), 125.4 (CH), 60.4 (CH_2), 42.3 (CH_2), 37.5 (CH_2), 37.46 (CH_2), 37.44 (CH_2), 32.8 (CH), 30.8 (CH), 26.1(CH_3), 26.0 (CH_2), 24.7 (CH_2), 20.1 (CH_3), 19.9 (CH_3), 18.0 (CH_3), 14.7 (CH_3). GC-MS: 268 (M^+, 2.8), 222 (7.7), 180 (5.2), 125 (27.5), 69 (100). IR: 1735 (s), 1170 (s), 1010 (m), 900 (m).

2,3,6,7-Tetrahydrofarnesol-*4,4,15',15',15'-d_5* (**63**)

The desired alcohol **63** was prepared by reduction of the corresponding ester **62** with LAH by usual manner. Yield: 1.84 g (94.6%, 98.2% pure). ^{1}H NMR: 5.10 (1H, t-like, *J* = 7.1), 3.67 (2H, m), 1.95 (2H, m), 1.68 (3H, s), 1.64-1.55 (2H, m), 1.60 (3H, s), 1.45 (1H, br. s), 1.43-1.24 (7H, m), 1.23-1.06 (2.5H, m), 0.90 (0.74H, d-like), 0.87 (3H, d, *J* = 6.6). ^{13}C NMR: 131.3 (C), 125.5 (CH), 61.6 (CH_2), 40.4 (CH_2), 37.8 (tr, CH_2), 37.6 (CH_2), 37.5 (CH_2), 32.8 (CH), 29.9 (CH), 26.1 (CH_3), 26.0 (CH_2), 24.7 (CH_2), 20.0 (CH_3), 19.9 (tr, CH_3), 18.0 (CH_3). IR: 3300 (br, s), 2210 (m), 1035 (s).

2,3,6,7-Tetrahydrofarnesol (**63H**)

^{1}H NMR: 5.10 (1H, t-like, *J* = 7.1), 3.67 (2H, m), 1.95 (2H, m), 1.68 (3H, s), 1.64-1.55 (2H, m), 1.60 (3H, s), 1.45 (1H, br. s), 1.43-1.24 (7H, m), 1.23-1.06 (3H, m), 0.90 (3H, d, *J* = 6.6), 0.87 (3H, d, *J* = 6.6). ^{13}C NMR: 131.3 (C), 125.5 (CH), 61.6 (CH_2), 40.4 (CH_2), 37.8 (CH_2), 37.6 (CH_2), 37.5 (CH_2), 32.8 (CH), 29.9 (CH), 26.1 (CH_3), 26.0 (CH_2), 24.7 (CH_2), 20.0 (CH_3), 19.9 (CH_3), 18.0 (CH_3).

Geranyl tetrahydropyranyl ether (**65**)

To the solution of geraniol (15.40 g, 100.0 mmol) and 18.2 mL of dihydropyran (200.0 mmol) in CH_2Cl_2 (50 mL), 0.20 g of *p*-toluenesulfonic acid was added at 0˚C, and the solution was stirring for 1 hr at 0˚C then for 3 hr at room temperature. After evaporating the solvent, ether (250 mL) was added to the residue solution. The organic layer was washed with $NaHCO_3$, water, brine and dried. Concentration *in vacuo* gave 25.62 g of crude **65**. this compound was used for next reaction without further purification. ^{1}H NMR: 5.36 (1H, 6-CH, tq, J = 6.9, J = 1.2), 5.09 (1H, 2-CH, tq, J = 6.9, J = 1.2), 4.63 (1H, 1-CH_2, ddd, J = 11.9, J = 6.4, J = 0.8), 4.04 (1H, 1-CH_2, ddd, J = 11.9, J = 7.4, J = 0.4), 3.90 (1H, m), 3.51 (1H, m), 2.11 (2H, q-like, J = 7.1), 2.04 (2H, t-like, J = 6.8), 1.84 (1H, m), 1.71 (1H, m), 1.68 (6H, s), 1.55-1.50 (4H, m). ^{13}C NMR: 140.6 (C), 132.0 (C), 124.4 (CH), 121.0 (CH), 98.1 (CH), 64.0 (CH_2), 62.7 (CH_2), 40.0 (CH_2), 31.1 (CH_2), 26.8 (CH_2), 26.1 (CH_3), 25.9 (CH_2), 20.0 (CH_2), 18.1 (CH_3), 16.8 (CH_3). IR: 1650 (w), 1105 (s), 1005 (s).

(2*E*)-6,7-Epoxy-1-(tetrahydro-2-pyranyloxy)-3,7-dimethyl-2-octene (**66**)

To the solution of **65 (**25.62 g, 107.6 mmol) in CH_2Cl_2 50 mL, 29.08 g of mCPBA(118.36 mmol; 70% pure) in CH_2Cl_2 (150 mL) was added dropwise at 0˚C. After stirring for 30 min at 0˚C and then for 2 hr at room temperature, 200 mL of sat. sodium sulfite (Na_2SO_3) was poured onto the solution. The organic layer was separated and evaporated and aqueous layer was extracted with hexane. Combined organic layers were washed with $NaHCO_3$, water, brine and dried. Crude **66** (23.5 g) was chromatographed on silica (100 g, hexane:ether = 90:10) to give 12.04 g of **66** (47.4 mmol, 47.4% yield from

geraniol, 88.0% pure). ^{1}H NMR: 5.41 (1H, t-like, J = 6.5), 4.63 (1H, t, J = 3.6), 4.25 (1H, dd, J = 11.9, J = 6.4), 4.03 (1H, dd, J = 11.9, J = 7.3), 3.89 (1H, m), 3.50 (1H, m), 2.71 (1H, t, J = 6.3), 2.08 (2H, m), 1.84 (1H, m), 1.73 (1H, m), 1.70 (3H, s), 1.66 (2H, m), 1.61-1.49 (4H, m), 1.31 (3H, s), 1.26 (3H, s). ^{13}C NMR: 139.5 (C), 121.6 (CH), 98.3 (CH), 64.4 (CH), 63.9 (CH_2), 62.6 (CH_2), 58.7 (C), 36.6 (CH_2), 31.1 (CH_2), 27.5 (CH_2), 25.9 (CH_2), 25.2 (CH_3), 20.0 (CH_2), 16.8 (CH_3), 15.7 (CH_3).

(2*E*)- 1-(Tetrahydro-2-pyranyloxy)-3,7-dimethyl-2,7-octadien-6-ol (**67**)

This compound was prepared by the following procedure in the literature (Chappe at al., 1988; Mori and Tomioka,1992). A solution of **66** (12.04 g, 47.40 mmol) in dry toluene (150 mL), and 10.64 g of aluminum isopropoxide (52.14 mmol) was refluxed for 24 hr. After cooling to room temperature, 2N NaOH (10 mL) was added to the solution. The mixture was extracted with ether (3 × 60 mL). Organic layer was washed with 2N NaOH, water, NH_4Cl, brine, dried and concentrated *in vacuo*. The residue was chromatographed on silica (100 g, hexane:ether = 40:60) to give 10.74 g of **67** (42.28 mmol, 89.2% yield, 97.1% pure). ^{1}H NMR: 5.40 (1H, t-like, J = 6.9), 4.94 (1H, d, J = 0.4), 4.84 (1H, qu. J = 1.5), 4.62 (1H, t, J = 3.7), 4.24 (1H, dd, J = 11.6, J = 6.3), 4.04 (1H, dd, J = 11.6, J = 6.5), 4.02 (1H, d, J = 7.3), 3.89 (1H, m), 3.51 (1H, m), 2.08 (2H, m), 1.82 (1H, m), 1.79 (1H, br. s), 1.73 (3H, t, J = 1.0), 1.65 (3H, s), 1.74-1.64 (3H, m), 1.62-1.48 (4H, m). ^{13}C NMR: 147.8 (C), 140.2 (C), 121.3 (CH), 111.5 (CH_2), 98.2 (CH), 75.9 (CH), 64.0 (CH_2), 62.7 (CH_2), 36.0 (CH_2), 33.3 (CH_2), 31.1 (CH_2), 25.9 (CH_2), 20.0 (CH_2), 18.0 (CH_3), 16.8 (CH_3). IR: 3400 (br, s), 1630 (m), 1105 (s), 1010 (s), 890 (m)

Methyl (*4E,*8*E*)-dimethyl-10-(tetrahydro-2-pyranyloxy)-4,8-decadienoate (**68**)

The orthoether Claisen rearrangement was carried out as described in the literature using the apparatus described therein (Trust and Ireland, 1988). To a mixture of methyl orthoacetate (37.67 mL, 295.96 mmol), and 10.74 g of **67** (42.78 mmol), 0.16 ml of propionic acid (2.11 mmol) was added. After starting to stir, the mixture was heated between 100˚C and 110˚C for 1.5 h. The received materials, distilled from the reaction flask, was evaporated *in vacuo* to remove excess methyl orthoacetate. The residue was chromatographed on silica (100 g, hexane:ether = 70:30) to give 12.34 g of **68** (39.81 mmol, 94.1% yield, 94.0% pure). ^{1}H NMR: 5.35 (1H, t-like, J = 6.8), 5.13 (1H, t-like, J = 6.8), 4.62 (1H, dd, J = 3.6, J = 3.0), 4.23 (1H, ddd, J = 11.1, J = 6.4, J = 0.7), 4.02 (1H, dd, J = 11.9, J = 7.4), 3.90 (1H, ddd, J = 11.2, J = 7.7, J = 3.4), 3.66 (3H, s), 3.51 (1H, m), 2.40 (2H, m), 2.29 (2H, t-like, J = 7.7), 2.11 (2H, q-like, J = 7.0), 2.03 (2H, t-like, J = 7.4), 1.84 (1H, m), 1.73 (1H, m), 1.67 (3H, s), 1.61 (3H, d, J = 0.8), 1.60-1.50 (4H, m). ^{13}C NMR: 174.3 (C), 140.3 (C), 133.9 (C), 125.2 (CH), 121.2 (CH), 98.2 (CH), 64.0 (CH_2), 62.7 (CH_2), 51.9 (CH_3), 39.8 (CH_2), 35.0 (CH_2), 33.4 (CH_2), 31.1 (CH_2), 26.6 (CH_2), 25.9 (CH_2), 20.0 (CH_2), 16.8 (CH_3), 16.3 (CH_3). IR: 1730 (s), 1150 (m), 1010 (s).

Methyl (*4E,*8*E*)-10-hydroxy-4,8-dimethyl-4,8-decadienoate (**69**)

THP ester **68** (12.34 g, 39.81 mmol) was hydrolysis with *p*-TsOH (0.76 g, 3.98 mmol) in MeOH (125 mL). Yield: 8.29 g (92.1% yield, 94.5% pure). ^{1}H NMR: 5.39 (1H, t-like, J = 6.8), 5.13 (1H, t-like, J = 6.8), 4.14 (2H, d, J = 6.9), 3.66 (3H, s), 2.42 (2H, m). ^{13}C NMR: 174.4 (C), 139.4 (C), 133.9 (C), 125.0 (CH), 124.1 (CH), 59.7 (CH_2), 51.9 (CH_3), 39.7 (CH_2), 34.9 (CH_2), 33.6 (CH_2), 26.4 (CH_2), 16.6 (CH_3), 16.3 (CH_3). IR: 3300

(br, s), 1730 (s), 1670 (m), 1155 (s), 990 (s).

Methyl 10-hydroxy-4,8-dimethyldecanoate-*4,5,8,9-d_4* (**70**)

To suspension of 5% Pd/C (0.56 g) in ethanol (20 mL), 5.60 g of **69** (24.78 mmol) was added. D_2 gas was introduced until absorption was completed. After filtration and concentration *in vacuo*, the residue (5.56 g) was chromatographed on silica (50 g, hexane:ether = 80:20 and ether fraction) to give 4.01 g of **32** (17.14 mmol, 69.2% yield, 99.2% pure). ^{1}H NMR: 3.72-3.62 (5H, s + m), 2.31 (2H, m), 1.68-1.51 (2.56H, m), 1.48-1.04 (7.68H, m), 0.89 (3H, d, J = 9.3), 0.87 (3H, d, J = 9.7). ^{13}C NMR: 175.0 (C), 61.4 (CH_2), 51.9 (CH_3), 40.3 (CH_2, CHD), 37.7 (CH_2), 37.2 (CH_2, CHD), 32.8 (CH_2), 32.3 (w, CH, CD), 32.2 (CH_2), 29.8 (w, CH, CD), 24.6 (CH_2), 20.0 (CH_3), 19.6 (CH_3). IR: 3350 (br, s), 2120 (m), 1740 (s), 1160 (s), 1040 (s).

Methyl 10-hydroxy-4,8-dimethyldecanoate (**70H**)

^{1}H NMR: 3.70-3.64 (5H, s + m), 2.31 (2H, m), 1.71-1.51 (4H, m), 1.48-1.09 (7H, m), 1.08-1.01 (2H, m), 0.88 (3H, d, J = 10.7), 0.87 (3H, dd, J = 10.5, J = 0.4). ^{13}C NMR: 175.0 (C), 61.5 (CH_2), 51.9 (CH_3), 40.3 (CH_2), 37.7 (CH_2), 37.2 (CH_2), 32.8 (CH_2), 32.3 (CH), 32.2 (CH_2), 29.8 (CH), 24.6 (CH_2), 20.0 (CH_3), 19.6 (CH_3). IR: 3300 (br, s), 1730 (s), 1160 (s), 1040 (s).

10-Hydroxy-4,8-dimethyldecanoic acid-*4,5,8,9-d_4* (**71**)

A solution of **70** (1.50 g, 6.41 mmol) in MeOH (40 mL) and 10 mL of 25% KOH was refluxed for 2 hr. After cooling to room temperature, ether (80 mL) was added to the

mixture. The organic layer was washed with 2N NaCl (3 × 60 mL). Combined alkali solution was washed with ether (60 mL), and acidified to *p*H 3 with 2N HCl. The aqueous layer was extracted with ether (3 × 60 mL). The ethereal solution was washed with brine and dried up. Yield 1.16 g (5.27 mmol). This compound **71** was used for bioassay without further purification. ^{1}H NMR: 5.85 (1H, br. s), 3.69 (2H, m), 2.33 (2H, m), 1.71-1.51 (2.31H, m), 1.49-1.19 (6.12H, m), 1.18-1.07 (1.65H, m), 0.89 (3H, d, J = 4.1), 0.88 (3H, d, J = 3.5). ^{13}C NMR: 180.0 (C), 61.5 (CH_2), 40.2 (CH_2, CHD), 37.6 (CH_2), 37.1 (CH_2, CHD), 32.7 (CH, CD), 32.2 (CH_2), 32.0 (CH_2), 29.8 (CH, CD), 24.6 (CH_2), 20.0 (CH_3), 19.7 (CH_3). IR: 3500 – 2500 (br, s), 2115 (m), 1700 (s), 1030 (m).

10-Hydroxy-4,8-dimethyldecanoic acid (**71H**)

^{1}H NMR: 5.77 (1H, br. s), 3.69 (2H, m), 2.33 (2H, m), 1.71-1.51 (3H, m), 1.49-1.19 (8H, m), 1.18-1.07 (2H, m), 0.89 (3H, dd, J = 6.6, J = 1.0), 0.88 (3H, d, J = 5.7). ^{13}C NMR: 180.0 (C), 61.5 (CH_2), 40.2 (CH_2), 37.6 (CH_2), 37.1 (CH_2), 32.7 (CH), 32.2 (CH_2), 32.0 (CH_2), 29.8 (CH), 24.6 (CH_2), 20.0 (CH_3), 19.7 (CH_3). IR: 3500 – 2500 (br, s), 1700 (s), 1030 (m).

2,6,10-Trimethyl-2-dodecene-*9,9,10',10',10'-d_5* (**73**)

Tosylate **72** (0.93 g, 2.42 mmol as crude) prepared from the corresponding alcohol **63** and *p*-TsCl, was reduced with LAH (0.37 g, 9.68 mmol) to afford 0.26 g of **73** (1.21 mmol). Yield: 26.9% in 2 stemps. Purity: 97.8%.

4,8-Dimethyldecanal-*7,7,8',8',8'-d_5* (**75**)

Deutrated 4,8-DMD (**75**) was prepared in the same manner as that used for **33**, by oxidative cleavage of **32** prepared from **73**. Yield: 88.0%, chemical purity: 80.4%. Retension time of Crude **75** on GC was identical to non-derutarad 4,8-DMD and it was summited to GC-MS analysis without further purification. GC-MS spectrum is shown Fig. 2-16 in Chapter II.

4,8-Dimethyldecanol-*4,5,8,9-d_4* (**78**)

Tosyl ester **77** (1,19 g, 3.07 mmol as crude, 96.7% pure) prepared in usual procedure with **70**, was summitted to reduction with excess LAH (0.58 g, 15.35 mmol). Usual workup gave 0.48g of alchol **78** (2.53 mmol, 63.6% in 2 steps). IR: 3300 (br, s), 2110 (m), 1050 (s).

4,8-Dimethyldecanal-*4,5,8,9-d_4* (**79**)

Alcohol **78** was oxidized with PDC in the same manner as that used for **40**. Yield 65.2%, chemical purity: 71.7%. Retention time of crude **79** on GC was identical to that of non-derutarad 4,8-DMD and it was summited to GC-MS analysis without further purification. GC-MS spectrum is shown in Fig. 2-19 (Chapter II).

SUMMARY

The biosynthetic origin and pathway of 4,8-dimethyldecanal (4,8-DMD), the aggregation pheromone of the red flour beetle (RFB), *Tribolium castaneum* (Coleoptera: Tenebrionidae) were investigated using isotopically labeled precursors and regulators of the pheromone production.

The mevalonate pathway as a biosynthetic pathway of 4,8-DMD was examined (Chapter II) using [2-^{13}C]mevalonolactone, deuterium-labeled terpene precursors [geraniol-d_5 (GLD5), linalool-d_5 (LLD5), and farnesol-d_5 (FLD5)], and deuterium-labeled terpene derivatives [tetrahydrofarnesol-d_5 (TFD5), hexahydrofarnesene-d_2 (HFD2) and 10-hydroxy-4,8-dimethyldecanoicacid-d_4 (HDMD-D4)]. The Beetles were fed flours coated with labeled precursors and 4,8-DMD was analysized by GC-MS. [2-^{13}C]Mevalonolactone was incorporated into 4,8-DMD and ca. 3% of 4,8-DMD was enriched in ^{13}C atom. However, deuterium-labeled terpene precursors were not. In addition terpene precursors, other deuterium-labeled terpene derivatives which have been considered to be precursors of 4,8-DMD, were also not incorporated into 4,8-DMD. These results suggest that 4,8-DMD is not of terpene origin and the mevalonate pathway is not involved in the biosynthesis of 4,8-DMD.

In order to speculate the biosynthetic pathway of 4,8-DMD, the effects of juvenile hormone III (JH III), an activator of the mevalonate pathway, and two pathway inhibitors (mevastatin, an inhibitor of the terpene pathway and 2-octynoic acid, an inhibitor of the fatty acid pathway) on the production of 4,8-DMD were investigated (Chapter III). JH III was topically applied to male RFBs while the inhibitors were administrated orally to RFBs by feeding inhibitor-coated flour. The production of 4,8-DMD by male RFBs was

significantly reduced with increasing doses of JH III. The amount of 4,8-DMD was not affected by the mevastatin-coated flour, but significantly decreased with the 2-octynoic acid-coated flour. These results suggest that 4,8-DMD is of fatty acid origin rather than of terpene origin.

To elucidate that 4,8-DMD is of fatty acid origin and whether its biosynthetic pathway proceeds in the sequence Ac-Pr-Ac-Pr-Ac (Ac; acetate, Pr; propionate), incorporation experiments were performed by feeding RFBs on the flour coated with ^{13}C-labeled acetate and propionate and deuterium-labeled putative precursors [2-methylbutanoate (C5D), 4-methylhexanoate (C7D), 2,6-dimethyloctanoate (C10D), and 4,8-dimethyldecanoate (C12D)] (Chapter IV). GC-MS analysis indicated that ^{13}C-labeled acetate and propionate were incorporated into 4,8-DMD. Approximately 9% of the 4,8-DMD was enriched in one ^{13}C atom and approximately 1-2% was enriched with two ^{13}C atoms by the exposure to ^{13}C-labeled acetate. While, approximately 14-21% of the 4,8-DMD was enriched in one ^{13}C atom and approximately 4.7-10% was enriched with two ^{13}C atoms by the exposure to ^{13}C-labeled propionate. Deuterium-labeled putative precursors, C5D, C10D, and C12D but not C7D were incorporated into 4,8-DMD by the RFBs.

The findings that the production of 4,8-DMD was inhibited by 2-octynoic acid but unaffected by mevastatin combined with the incorporation of [1-^{13}C]acetate [1-^{13}C]propionate, and deuterated fatty acid precursors and no incorporation of deuterated terpene and terpene derivatives, unambiguously demonstrate that 4,8-DMD is of fatty acid but not terpene origin and that the biosynthesis of 4,8-DMD proceeds in the sequence Ac-Pr-Ac-Pr-Ac.

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REFERENCES

Barkawi, L. S., Francke, W., Blomquist, G. J. Seybold, S. J. 2003. Frontalin: De novo biosynthesis of an aggregation pheromone component by *Dendroctonus* spp. bark beetles (Coleoptera: Scolytidae). *Insect Biochem. Molec. Biol.* 33: 773-788.

Bartelt, R. J. and Weisleder, D. 1996. Polyketide origin of pheromones of *Carpophilus davidsoni* and *C. mutilatus* (Coleoptera: Nitidulidae). *Bioorg. Med. Chem.* 4: 429-438.

Beier, R. and Mundy, B. P. 1979. A facile removal of the tetrahydropyranyl protecting group from alcohol derivatives. *Synth. Commun.* 9: 271-273.

Biernacki, W. and Gdula, A. 1979. Modification of the method of Julia for the preparation of homoallylic bromides and iodides. *Synthesis* 10: 37-38.

Bjostad, L. B., Wolf, W. A., and Roelofs, W. L. 1987. Pheromone biosynthesis in Lepidopterans: Desaturation and chain shortening. pp. 77- 120. *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando, Florida.

Boland, W. and Gäbler, A. 1989. Biosynthesis of homoterpens in higher plants. *Helv. Chim. Acta* 72: 247-253.

Breuer, E., Deutsch, J., and Lazarovici, P. 1982. A practical synthesis of the aggregation pheromone of *Tribolium castaneum* and *Tribolium confusum. Chem. Ind.* 20: 907-908.

Butenandt, A., Beckman, R., Stamm, D., and Hecker, E. 1959. Über den Sexual-Lockstoff des Seidenspinners *Bombyx mori*, Reindarstellung und Konstitution. *Z. Naturforsch.* 14b: 283-284.

Cahiez, G., Chaboche, C., and Jézéquel, M. 2000. Cu-catalyzed alkylation of Grignard reagents: A new efficient procedure. *Tetrahedron* 56: 2733-2737.

Campbell, I. M. 1974. Incorporation and dilution values – their calculation in mass spectrally assayed stable isotope labeling experiments. *Bioorg. Chem.* 3: 386-387.

Chappe, B., Musikas. H., Marie, D., and Ourisson, G. 1988. Synthesis of three acylic all-*trans*-tetraterpene diols, putative precursors of bacterial lipids. *Bull. Chem. Soc. Jpn.* 61: 141-148.

Chase, J. Touhara, K., Prestwich, G. D., Schal, C., and Blomquist, G. J. 1992. Biosynthesis and endocrine control of the production of the German cockroach sex pheromone 3,11-dimethylnonacosan-2-one. *Proc. Natl. Acad. Sci. USA* 89: 6050-6054.

Chen, N. M., Border, J. H., and Pierce, H. D., Jr. 1988. Effect of juvenile hormone analog, fenoxycarb, on pheromone production by *Ips paraconfusus* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 14: 1087-1098.

Dillwith, J. W., Nelson, J. H., Pomonis, J. G., Nelson, D. R., and Blomquist, G. J. 1982. A ^{13}C NMR study of methyl-branched hydrocarbon biosynthesis in the housefly. *J. Biol. Chem.* 257: 11305-11314.

Endo, A., Kuroda, M., and Tanzawa, K. 1976. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-36A and ML-236B fungal metabolites, having hypocholesterolemic activity. *FEBS Lett.* 72: 323-326.

Faustini, D. L., Burkholder, W. E., and Laub, R. J. 1981. Sexually dimorphic setiferous sex patch in the male red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae): Site of aggregation pheromone production. *J. Chem. Ecol.* 7: 465-480.

Freund, K., Mizzer, J., Dick, W., and Thorpe, C. 1985. Inactivation of general acyl-CoA dehydrogenase from pig kidney by 2-alkynoyl coenzyme A derivatives: Initial aspects.

Biochemistry 24: 5996-6002.

Fuchs, S., Beck, T., Burkardt, S., Sandvoss, M., and Mosandl, A. 1999. Biogenetic Studies in *Mentha* × *piperita*. 1. Deuterium-labeled monoterpene ketones: Synthesis and stereoselective analysis. *J. Agric. Food Chem.* 47: 3053-3057.

Hanzlik, R. P. 1988. Selective epoxidation of terminal double bonds: 10,11-Epoxyfarnesyl acetate. pp. 560-564. *in* W. E. Noland (Ed.). Organic Syntheses Coll. Vol. 6. John Wiley and Sons, New York.

Hoffman, C. H., Wagner, A. F., Wilson, A. N., Walton, E., Shunk, C. H., Wolf, D. E., Holly, F. W., and Folkers, K. 1957. Synthesis of DL-3,5-dihydroxy-3-methylpentanoic acid (mevalonic acid). *J. Am. Chem. Soc.* 79: 2316-2318.

Howard, R. W., Jurenka, R. A., and Blomquist, G. J. 1986. Prostaglandin synthetase inhibitors in the defensive secretion of the red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Insect Biochem.* 16: 757-760.

Howse, P. E., Jones, O. T., and Stevens, I. D. R. 1998. Insect Pheromones and their Use in Pest Management. Chapman & Hall, London, UK.

Hussain, A. 1993. Chemical ecology of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae): Factors affecting biology and applications of pheromone. Ph. D. Dissertation, Oregon State University.

Ikeda, K. 1999. Chemical Ecology of Water Striders-Stink gland secretion of *Gerris lacustris* (Hemiptera : Gerridae). Master's thesis, University of Tsukuba, Tsukuba, Japan. (in Japanese).

Islam, N., Bacala, R., Moore, A., and Vanderwel, D. 1999. Biosynthesis of 4-methyl-1-nonanol: female-produced sex pheromone of the yellow mealworm beetle,

Tenebrio molitor (Coleopter: Tenebrionidae). *Insect Biochem. Molec. Biol.* 29: 201-208.

Ivarsson, P. and Birgersson, G. 1995. Regulation and biosynthesis of pheromone components in the double spined bark beetle *Ips duplicatus* (Coleoptera: Scolytidae). *J. Insect Physiol.* 41: 843-840.

Ivarsson, P., Schlyter, F., and Birgersson, G. 1993. Demonstration of *de novo* pheromone biosynthesis in *Ips duplicatus* (Coleoptera: Scolytidae): Inhibition of ipsdienol and *E*-myrcenol production by compactin. *Insect Biochem. Molec. Biol.* 23: 655-662.

Julia, M., Julia, s., et Guégan, R. 1960. Préparation de composés terpéniques et appartentés; à partir de méthyl cyclopropyl cétone. *Bull. Soc. Chim. France* 1072-1079.

Jurenka, R. A. 2003. Biochemistry of female moth sex pheromones, pp 53-80, *in* G. J. Blomquist and R.G. Vogt (eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, London, UK.

Jurenka, R. 2004. Insect Pheromone Biosynthesis. pp. 97-132. *in* S. Schulz (ed.). The Chemistry of Pheromones and Other Semiochemicals I. Topics in Current Chemistry, Vol 239. Springer-Verlag, Heidelberg, Germany.

Krapcho, A. P. 1982. Synthetic applications of dealkoxycarbonylations of malonate esters, β-keto esters, α-cyano esters and related compounds in dipolar aprotic media - Part I. *Synthesis* 10: 805-822.

Kurosawa, S., Bando, M., and Mori, K. 2001. Synthesis of (1*R*,4*R*,5*S*)-(+)-acoradiene, the structure proposed for the aggregation pheromone of the broad-horned flour beetle. *Eur. J. Org. Chem.* 23: 4395-4399.

Lawson, J. A., Colwell, W. T., DeGraw, J. I., Peters, R. H., Dehn, R. L., and Tanabe, M. 1975. The synthesis of singly and double ^{13}C-labeled mevalonolactone. *Synthesis* 729-730.

Lehninger, A. L., Nelson, D. L, and Cox, M. M. 1993. Principles of Biochemistry, 2nd edition. Worth Publishers, New York, NY.

McCormick, J. P. and Carrel, J. E. 1987. Cantharidin biosynthesis and function in meloid beetles. pp. 307-350. *in* G.D. Prestwich and G.J Blomquist (eds). Pheromone Biochemistry. Academic press, Inc., Orlando, FL.

Monger, D., Lim, W. A. Kézdy, F. J., and Law, J. H. 1982. Compactin inhibits insect HMG-CoA reductase and juvenile hormone biosynthesis. *Biochem. Biophys. Res. Commun.* 105: 1374-1380.

Mori, K., and Tomioka, H. 1992. Synthesis of four macrolide pheromones to define the scope and limitation of enzymatic macrolactonization. *Liebigs Ann. Chem.* 1011-1017.

Morita, A., Mori, N., Nishida, r., Hirai, N., and Kuwahara, Y. 2004. Neral (the alarm pheromone) biosynthesis *via* the mevalonate pathway, evidenced by D-glucolse-1-^{13}C feeding in *Carpoglyphus lactisi* and ^{13}C incorporation into other opisthonotal gland exudates. *J. Pesticide Sci.* 29: 27-32.

Nelson, D. R. 1993. Methyl-branched lipids in insects. pp. 271-315. *in* D. W. Stanley-Samuelson and D. R. Nelson (eds.),. Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln, NE.

Petroski, R. J., Bartelt, R. J. and Weisleder, D. 1994. Biosynthesis of (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene: the aggregation pheromone of

Carpophilus freemani (Coleoptera: Nitidulidae). *Insect Biochem. Molec. Biol.* 24: 69-78.

Pierce, A. M., Pierce, H. D. Jr, Borden, J. H., and Oehlschlager, A. C. 1986. Enhanced production of aggregation pheromones in four stored-product coleopterans feeding on methoprene-treated oats. *Experientia* 42: 164-165.

Qazi, M. C. B., Boake, C. R. B., and Lewis, S. M. 1998. The femoral setiferous glands of *Tribolium castaneum* males and production of the pheromone 4,8-dimethyldecanal. *Entomol. Exp. Appl.* 89:313-317.

Rohmer, M. 2003. Mevalonate-independent methylerythritol phosphate pathway for isoprenoid biosynthesis. Elucidation and distribution. *Pure Appl. Chem.* 75: 375-388.

Ropp, G. A. 1950. Preparation of ethyl acetate-2-^{14}C and n-butyl acetate-2-^{14}C using alkyl phosphates. *J. Am. Chem. Soc.* 72: 2299.

Schulz, S., Francke W., Edgar, J., and Schneider, D. 1988. Volatile compounds from androconial organs of Danaine and Ithomiine Butterflies. *Z. Naturforsch.* 43c: 99-104.

Semmelhack, M. F. and Stauffer, R. D. 1975. Reductions with copper hydride. New preparative and mechanistic aspects. *J. Org. Chem.* 40: 3619-3621.

Seybold, S. J. and Tittiger, C. 2003. Biochemistry and molecular biology of *de novo* isoprenoid pheromone production in the Scolytidae. *Annu. Rev. Entomol.* 48: 425-453.

Seybold, S. J. and Vanderwel, D. 2003. Biosynthesis and endocrine regulation of pheromone production in the Coleoptera. pp. 137-200. *in* G. J. Blomquist and R. G. Vogt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, London, UK.

Sokal, R. R. and Rohlf, F. J. 1995. Biometry, 3rd edition. W. H. Freeman and Company,

New York, NY.

Suzuki, T. 1975. Isolation of 2'-hydroxy-4'-methoxypropiophenone from *Tribolium castaneum* and *T. confusum*. *Agric. Biol. Chem.* 39: 1687-1688.

Suzuki, T. 1980. 4,8-Dimethyldecanal: The aggregation pheromone of the flour beetles, *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae). *Agric. Biol. Chem.* 44: 2519-2520.

Suzuki, T. 1981. A facile synthesis of 4,8-dimethyldecanal, aggregation pheromone of flour beetles, and its analogues. *Agric. Biol. Chem.* 45: 2641-2643.

Suzuki, T. and Mori, K. 1983. (4*R*, 8*R*)-(-)-4,8-Dimethyldecanal: the natural aggregation pheromone of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Appl. Entomol. Zool.* 18: 134-136.

Suzuki, T. and Sugawara, R. 1979. Isolation of an aggregation pheromone from the flour beetles, *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae). *Appl. Entomol. Zool.* 14: 228-230.

Suzuki, T., Kozaki, J., and Sugawara, R. 1984. Biological activities of the analogs of the aggregation pheromone of *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Appl. Entomol. Zool.* 19: 15-20.

Suzuki, T., Nakakita, H., and Kuwahara, Y. 1987. Aggregation pheromone of *Tribolium freemani* Hinton (Coleoptera, Tenebrionidae): I. Identification of the aggregation pheromone. *Appl. Entomol. Zool.* 22: 340-347.

Suzuki, T., Suzuki, T., Huynh, V. M., and Muto., T. 1975. Hydrocarbon repellents isolated from *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae). *Agric. Biol. Chem.* 39: 2207-2211.

Tanaka Y., Honda, H., Ohsawa, K., and Yamamoto, I. 1986. A sex attractant of the yellow mealworm, *Tenebrio molitor* L., and its role in mating behaviour. *J. Pesticide Sci.* 11: 49-55.

Tanaka, Y., Honda, H., Ohsawa, K., and Yamamoto, I. 1989. Absolute configuration of 4-methyl-1-nonanol, a sex attractant of the yellow mealworm, *Tenebrio molitor* L. *J. Pesticide Sci.* 14: 197-202.

Tashiro, T., Kurosawa. S., and Mori, K. 2004. Revision of the structure of the major aggregation pheromone of the broad-horned flour beetle (*Gnatocerus cornutus*) to (1*S*,4*R*,5*R*)-alpha-acoradiene by its synthesis. *Biosci. Biotechnol. Biochem.* 68: 663-670.

Tebayashi, S., Hirai, N., Suzuki, T., Matsuyama, S., Nakakita, H., Nemoto, T., and Nakanishi, H. 1998a. Identification of (+)-acoradiene as an aggregation pheromone for *Gnatocerus cornutus* (F.) (Coleoptera; Tenebrionidae). *J. Stored Prod. Res.* 34: 99-106.

Tebayashi, S., Hirai, N., Suzuki, T., Matsuyama, S., Nakakita, H., Nemoto, T., and Nakanishi, H. 1998b. α-Cedren-14-al: minor aggregation pheromone component of *Gnatocerus cornutus* (F.) (Coleopter: Tenebrionidae). *J. Pesticide Sci.* 23: 402-406.

Tillman, J. A., Holbrook, G. L., Dallara, P. L., Schal, C., Wood, D. L., Blomquist, G. J., and Seybold, S. J. 1998. Endocrine regulation of de novo aggregation pheromone biosynthesis in the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *Insect Biochem. Molec. Biol.* 28: 705-715.

Tillman, J. A., Seybold, S. J., Jurenka, R. A., and Gary J. Blomquist, G. J. 1999. Insect pheromones—an overview of biosynthesis and endocrine regulation. *Insect Biochem.*

Molec. Biol. 29: 481-514.

Tittiger, C. 2003. Molecular biology of bark beetle pheromone production and endocrine regulation. pp. 201-230. *in* G. J. Blomquist and R. G. Vogt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, London, UK.

Trust, R. I., and Ireland, R. E. 1988. Stereoselective synthesis of trisubstituted olefins: Ethyl 4-methyl-(*E*)-4,8-nonadienoate. pp. 606-610. *in* W. E. Noland (ed.). Organic Syntheses Coll. Vol. 6. John Wiley and Sons, New York, NY.

Vanderwel, D. and Oehlschlager, A. C. 1987. Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera, pp. 175-215, *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando, FL.

Vanderwel, D., Johnson, B. and Oehlschlager, A. C. 1992. Cucujolide biosynthesis in the merchant and rusty grain beetles. *Insect Biochem. Molec. Biol.* 22: 875-883.

Vanderwel, D., Pierce, Jr. H. D., Oehlschlager, A. C., Borden, J. H. and Pierce, A. M. 1990. Macrolide (cucujolide) biosynthesis in the rusty grain beetle, *Cryptolestes ferrugineus* I. *Insect Biochem.* 20: 567-572.

Wiley, R. H., Slaymaker, S. C., and Kraus, H. 1956. Dimethylhydrazone of aliphatic, aromatic, and heterocyclic aldehydes and their infrared absorption characteristics. *J. Org. Chem.* 22: 204-207.

Zarbin, P. H. G., Cruz, W, O., and Ferreira, T. B. 1998. Stereospecific synthesis of two isomers of (4,8) - dimethyldecanal: The aggregation pheromone of *Tribolium spp*. *J. Braz. Chem. Soc.* 9: 511-513.

Zhao, C.-H., Adlof, R. O., and Löfstedt, C. 2004. Sex pheromone biosynthesis in the pine caterpillar moth, *Dendrolimus punctatus* (Lepidoptera: Lasiocampidae): pathway

leading to Z5-monoene and 5,7-conjugated diene components. *Insect Biochem. Molec. Biol.* 34: 261-271.