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BIOSYNTHESIS OF MONOTERPENES AND SESQUITERPENES IN *LARIX LEPTOLEPIS* CALLUS FROM DEUTERATED MEVALONATES

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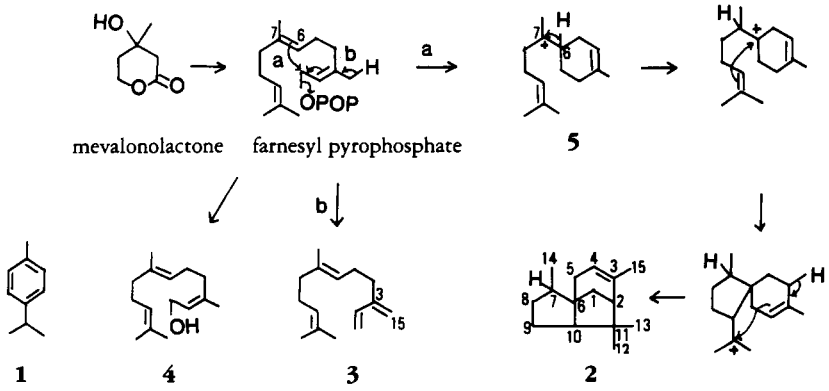
ABSTRACT.—Callus cultures from the seedling leaves of *Larix leptolepis* were able to synthesize *p*-cymene [**1**], sesquiterpene hydrocarbons, and some oxygenated sesquiterpenes. Deuterium atoms from *RS*-[6,6,6-$^2H_3$]-, *RS*-[5,5-$^2H_2$]-, and *RS*-[4,4-$^2H_2$]-mevalonate were extensively incorporated into *p*-cymene [**1**], α-cedrene [**2**], 6*E*-β-farnesene [**3**], and farnesol [**4**] by the calli. The isopentenyl origins of these lower terpenoids were confirmed by gc-ms analysis, and no preferential labeling of the isopentenyl-pyrophosphate-derived portions of the molecules was noted. The labeling patterns confirmed on the basis of these ms spectra indicate a 1,2-hydride shift in α-cedrene [**2**] formation and the loss of the expected hydrogen atom in 6*E*-β-farnesene [**3**] formation.

Numerous investigations have been made of lower terpene production in plant tissue culture. In most cases, however, the quantities of lower terpenes in cultured cells are much less than those in the intact plants, and their qualitative profiles differ markedly from those of the intact plants. The deficiency of specialized secretory organs found in the intact plants, in which various classes of terpenoids are synthesized and sequestered, is considered to be the primary reason for the poor accumulation of lower terpenes in morphologically undifferentiated cells (1). Nevertheless, several investigators (2–7) have reported high yields of volatile terpenes in cultured cells. Such cultures are proving to be of increasing value as research tools for the study of the biosynthesis of monoterpenes and sesquiterpenes (8–11). We recently demonstrated that a complex mixture of sesquiterpene hydrocarbons biosynthesized from *RS*-[6,6,6-$^2H_3$]-mevalonic acid (MVA) in *Perilla* sp. callus (12) was deuterated at a higher level (isotopic purity 85%) than those in any other biosynthetic study using remote precursors such as acetate or MVA. Thus the labeling patterns of sesquiterpenes could be established based on gc-ms analysis. This technique provides potential advantages in making it possible to examine in detail the biosynthesis of a complex mixture of lower terpenes.

In this paper, we describe the de novo synthesis of monoterpenes and sesquiterpenes including α-cedrene [**2**] and 6*E*-β-farnesene [**3**] in calli from the seedling leaves of *Larix leptolepis* Gord. (Pinaceae). We also report on the labeling patterns of the biosynthetically deuterated **2** and **3** derived from *RS*-[6,6,6-$^2H_3$]-, *RS*-[5,5-$^2H_2$]-, and *RS*-[4,4-$^2H_2$]-MVA. The labeling patterns observed indicate a 1,2-hydride shift in α-cedrene formation and the loss of the expected hydrogen atom in β-farnesene formation (Scheme 1).

EXPERIMENTAL

CALLUS CULTURE OF *L. LEPTOLEPIS*.—Seeds of *L. leptolepis*, collected in November 1985 from Kitami, Hokkaido, Japan, were placed on vermiculite in a growth room (Nipponikakiki, LP-1PH, Osaka, Japan) under continuous light (fluorescent lamp, Fl 40 W, Matsushita Electric, Osaka, Japan) 7.32 $W \cdot m^{-2}$ (300–800 nm) at 25°. Germinating seedlings were grown for 35 days. The first leaves of the seedlings, having a length of 10–20 mm, were soaked in a 0.1% benzalkonium chloride solution for 5 min and sterilized by soaking successively in a 70% EtOH solution for 5 sec and in a 2% sodium hypochlorite solution for 20 min. After being washed three times with sterile distilled H_2O, the leaves were cut into 2–3 mm segments and placed on a Murashige & Skoog (MS) medium (13) with 3.0% (w/v) sucrose, 1.0% (w/v) agar (Difco Laboratories, Detroit), 5 mg/liter, 2,4-dichlorophenoxyacetic acid (2,4-D) (Tokyokasei, Tokyo, Japan), and a pH of 5.8 before autoclaving in order to induce callus. Cultures were maintained under continuous light of 7.32 $W \cdot m^{-2}$ at 25°. After 35 days, leaf-derived calli were transferred to fresh medium and successively subcultured for 31 months at intervals of 30–40 days.



SCHEME 1. The generally accepted biosynthetic pathways of 6*E*-β-farnesene [**3**] and α-cedrene [**2**].

Fresh wt was measured after collecting calli from cultures. The chlorophyll content was determined by measuring the absorption at 663 and 645 nm (14). Feeding experiments were carried out using calli of the 18th and 19th generation. Calli of the 5th, 18th, and 28th generations were used to analyze volatiles.

DEUTERATED MEVALONOLACTONE.—*RS*-[6,6,6-$^2H_3$]-mevalonolactone was prepared from tetradeuteroacetic acid (CEA), (Yvette, France, 99.4% enrichment) by the method reported by Suga and Shishibori (15). *RS*-[5,5-$^2H_2$]- and *RS*-[4,4-$^2H_2$]-mevalonolactones were synthesized using procedures outlined by Cane and Levin (16). *RS*-[4,4-$^2H_2$]-mevalonolactone was prepared from trideuteroacetic acid methyl ester (99.7% enrichment) and 4,4-dimethoxy-3-butanone via [2,2-$^2H_2$]-3-hydroxy-5,5-dimethoxypentanoate. *RS*-[5,5-$^2H_2$]-mevalonolactone was prepared by reducing 3-hydroxy-3-methyl-4,4-dimethoxy-ethyl pentanoate with LiAl[$^2H_4$] (CEA, 99.3% enrichment). Deuterated mevalonolactones were used for the feeding experiments without dilution.

FEEDING EXPERIMENTS.—Ten cultures, each with an average of 0.39 g callus from the 18th and 19th subcultures, were incubated with 24.8 mg (13.3 mM) of potassium MVA (deuterated or non-labeled) dissolved in 10 ml MS agar medium. The calli were grown at 25° under continuous light of 7.32 $W \cdot m^{-2}$ for 25 days.

SAMPLE PREPARATION OF VOLATILES FROM CALLI AND SEEDING LEAVES.—The volatiles were separated from the seedling leaves (0.68 g) and calli (8.7–16.5 g) by a small scale simultaneous-distillation-extraction apparatus (17). After homogenization in 50 ml H_2O at 4°, the fresh material was transferred to a 300 ml three-necked flask, and steam was introduced into the solution for 90 min. The volatile compounds were extracted with 30 ml Et_2O. The Et_2O extract was dried over anhydrous Na_2SO_4 for 24 h, concentrated to approximately 1 ml under reduced pressure at 5°, and further evaporated by hand warming. For the semiquantitative gc analysis, a known amount of an internal standard (tridecane in Et_2O) was added to the Et_2O solution before concentration. The resultant solution (5–10 μl) was used for gc and gc-ms analysis.

GC ANALYSIS.—The volatile samples were analyzed using a Shimadzu GC 9A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a fused silica capillary column (0.25 mm i.d., 50 m length) coated with Silicone OV 101 (df, 0.5 μm) (Gasukuro Kogyo, Tokyo, Japan); temperatures were held at 60° for 5 min and elevated to 220° at 2°/min, with the flow of He being 1.40 ml/min. Injector and detector (flame ionization detector) temperatures were held at 220°. To obtain the semiquantitative concentrations of total volatiles, the ratios of total peak areas to that of tridecane in the gc chromatograms were used.

GC-MS ANALYSIS.—Ms spectra were recorded with an Hitachi M-80B gc-ms instrument (Hitachi, Tokyo, Japan) equipped with a fused silica capillary column coated with Silicone OV-101; the temperature program was isothermal at 60° for 5 min and then temperatures were elevated to 220° at 2°/min, with the flow of He being 1.13 ml/min. Injector and interface temperatures were at 220°. Each spectrum was measured for 1 sec at an ionizing voltage of 70 eV, an ionizing current of 300 μA, and an accelerating voltage of 2 kV (rescan duration 0.5 sec). Ms spectra were compared to those of authentic samples.

RESULTS AND DISCUSSION

CALLUS CULTURE.—Calli of the seedling leaves of *L. leptolepis* were induced by the

addition of 5 mg/liter 2,4-D and 5 mg/liter of kinetin to the MS medium. The efficiency of callus induction from the seedling leaves was approximately 65% (that is, 33 calli were induced from 51 explants), while the efficiency of callus induction from the mature leaves was much lower, being only 10%. The calli were transferred to fresh MS medium every 30 days and were subcultured for 31 months. The callus growth index for each culture period of 30 days was 5.0, on a fresh wt basis. The calli maintained a stable capacity for producing chlorophyll over the long period of successive culturing. The content of chlorophyll-a (78 μg/g fresh wt) and -b (29 μg) in the calli at the 29th generation was determined using the method reported by Sunderland (14).

VOLATILE COMPONENTS IN THE CALLUS.—To compare volatiles accumulated in the calli with those synthesized in the seedling leaves, total ion monitored (TIM) gc profiles of the volatiles in the seedling leaves and the calli of the 18th generation were recorded (Figure 1, A and B). The volatiles from the seedlings consisted mainly of monoterpenes (α-pinene, camphene, β-pinene, ocimene, α- and β-terpinene, terpinen-4-ol, α-terpineol, and bornyl acetate) and sesquiterpenes (β-caryophyllene, α-humulene, aromandendrene, δ-cadinene, and some oxygenated sesquiterpenes). Although distinct differences in the total quantity of volatiles [0.31% of fresh wt in the mature leaves (18), 0.029% in the seedling leaves, and 0.0031% in the calli] were found, the quantity of volatile terpenes in the seedling leaves was almost the same as that in the mature leaves.

As can be seen in Figure 1B, the calli synthesized and accumulated terpenoids, including *p*-cymene [**1**], α-cedrene [**2**] (peak 29), β-cadinene (peak 32), 6*E*-β-farnesene [**3**] (peak 33), β-ionone (peak 34), and some oxygenated sesquiterpenes (peaks 17, 38–41). Their relative proportions, however, were much lower in the calli (below 15% of the total peak area) than in the seedling leaves (95%). Monoterpenes were not detected in the callus, with the exception of **1**. Although sesquiterpene hydrocarbons were always observed by the 28th generation, deviations in the qualitative patterns of the major sesquiterpenes were observed at the different generations. The major sesquiterpenes in the calli of the 5th generation were aromadendrene, β-caryophyllene, and α-humulene, whereas **2**, β-cadinene, longifollene, and thujopsene were present as major sesquiterpenes in the calli of the 28th generation.

The other major classes of volatile compounds in the calli were aromatic compounds including benzylaldehyde (peak 18), 2,4-dichlorophenol (peak 22), anethole (peak 24), 2,4-dichloromethoxybenzene (peak 25), eugenol (peak 26), and isoeugenol (peak 28), as well as long-chain compounds including hydrocarbons, aldehydes, fatty acids, and their methyl and ethyl esters. 2,4-Dichlorophenol and 2,4-dicholoromethoxybenzene might be derived from the exogenously supplied 2,4-D via the oxidative cleavage of the side chain.

INCORPORATION OF *RS*-[6,6,6-$^2H_3$]-MVA INTO TERPENES.—In order to investigate de novo synthesis, as well as the labeling patterns of monoterpenes and sesquiterpenes in the calli, feeds to calli of the 18th generation were carried out with potassium *RS*-[6,6,6-$^2H_3$]- and non-labeled MVAs (each 24.8 mg/10 ml culture medium). After the 25-day culture period, volatiles were separated and subjected to qualitative gc-ms analysis. Despite the higher concentration of dosages applied to the culture medium, growth (growth index for 25 days, 4.45) changed little, but an increase in the relative proportion of the long-chain compounds was observed as indicated in Figure 1C. However the volatile terpenoids **1** (peak 23 in Figure 1, B and C), **2** (peak 29), **3** (peak 33), as well as farnesol [**4**] (peak 58) and some oxygenated sesquiterpenes, were detected.

Figure 2, A, B, C, and D, exhibits the ms spectra of 6-D-*p*-cymene [**1**], 6-D-α-cedrene [**2**], 6-D-β-farnesene [**3**], and 6-D-farnesol [**4**], respectively, after incorporation

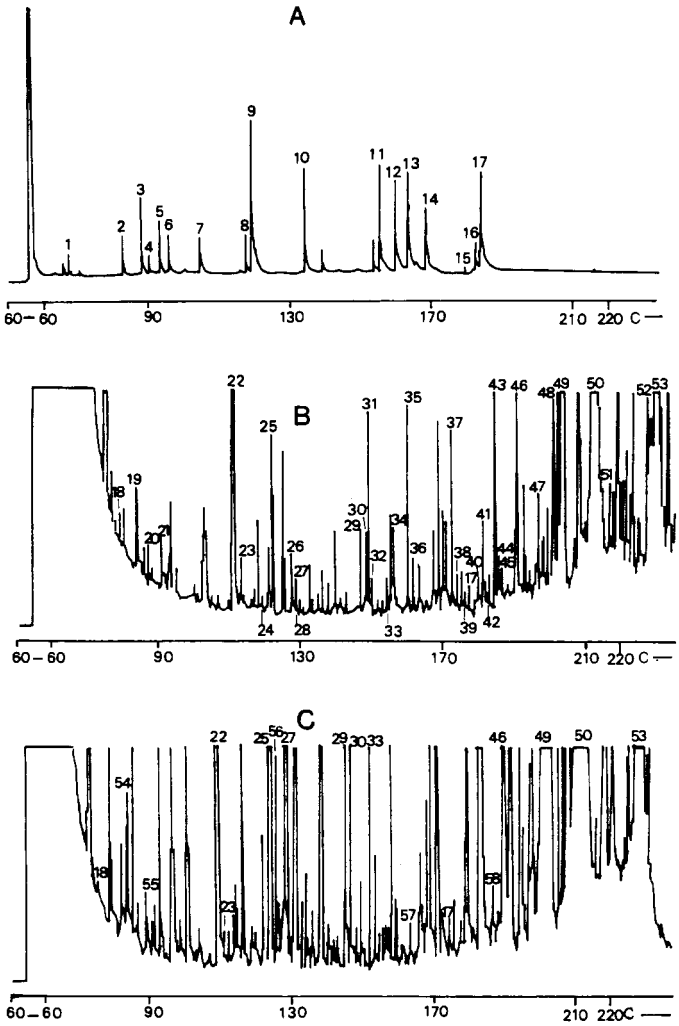


FIGURE 1. Total ion-monitored gc profiles of the volatiles of *Larix leptolepis* from the seedling leaves (A), calli of the 18th generation (B), and calli fed with mevalonate (C). Column: Silicone OV-101. Other conditions are given in the Experimental section. Peak 1: hexenal; 2: α-pinene; 3: camphene; 4: β-pinene; 5: ocimene; 6: β-terpinene; 7: α-terpinene; 8: terpinen-4-ol; 9: α-terpineol; 10: bornyl acetate; 11: β-caryophyllene; 12: α-humulene; 13: aromandendrene; 14: δ-cadinene; 15 to 17: oxygenated sesquiterpenes; 18: benzaldehyde; 19: ethyl butyrate; 20: ethyl-furan; 21: benzyl alcohol; 22: 2,4-dichlorophenol; 23: *p*-cymene; 24: anethole; 25: 2,4-dichloromethoxybenzene; 26: eugenol; 27: 2*E*,4*E*-decadienal; 28: isoeugenol; 29: α-cedrene; 30: tetradecane; 31: acoradiene; 32: β-cadinene; 33: 6*E*-β-farnesene; 34: β-ionone; 35: dibutylphthalate; 36: pentadecane; 37: pentadecadiene; 38 to 41: oxygenated sesquiterpenes; 42: heptadecene; 43: pentadecanol; 44: pentadecadiene; 45: myristic acid methyl ester; 46: myristic acid; 47: oxygenated sesquiterpene; 48: pentadecanoic acid; 49: palmitic acid methyl ester; 50: palmitic acid; 51: palmitic acid ethyl ester; 52: phytol; 53: oleic acid; 54: pentyl furan; 55: pentyl alcohol; 56: indole; 57: tetradecadiene; 58: farnesol.

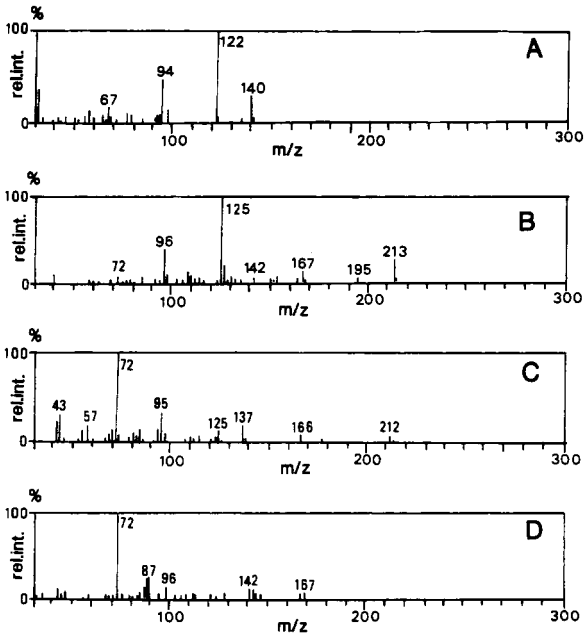


FIGURE 2. Mass spectra of the biosynthetically deuterated terpenes incorporating [6,6,6-$^2H_3$]-mevalonate: (A) *p*-cymene [**1**], (B) α-cedrene [**2**], (C) 6*E*-β-farnesene [**3**], (D) farnesol [**4**].

of *RS*-[6,6,6-$^2H_3$]-MVA. In the ms spectra of these compounds, neither molecular ions $[M]^+$ nor fragment ions derived from the partially labeled compounds could be observed. Thus, almost 100% of these terpenes originated from the exogeneously supplied MVAs. It is also clear that the biosynthetically deuterated terpenes derived from ^2H-MVAs showed no preferential labeling pattern in the IPP-derived positions. In contrast, no incorporation of deuterium atom into the oxygenated sesquiterpene (peak 17 in Figure 1C) was observed. This may suggest that the calli might synthesize the oxygenated sesquiterpene in the latter stage of growth.

The ms spectrum of the 6-D-*p*-cymene shows $[M]^+$ at *m/z* 140, indicating two of three methyl groups originated in the methyl group of MVA. The 6-D-α-cedrene has $[M]^+$ at *m/z* 213, which confirms the presence of three deuterated methyl groups. The shift of $[M]^+$ by 8 mass units in the 6-D-β-farnesene indicates the presence of two deuterated methyl groups and one deuterated methylene group, clearly demonstrating the loss of the expected deuterium atom for double bond formation between C-3 and C-15 in **3**.

INCORPORATION OF *RS*-[4,4-$^2H_2$]- AND [5,5-$^2H_2$]-MVA INTO α-CEDRENE [**2**].—In order to further investigate the labeling pattern of **2**, feeds to the calli were carried out with [4,4-$^2H_2$]- and [5,5-$^2H_2$]-MVA. Scheme 1 indicates the generally accepted biosynthetic pathway of **2** (19), together with the expected labeling pattern. The indicated 1,2-hydride shift in the bisabolene cation **5** was confirmed by examining the ms spectra of **2** that had incorporated [4,4-$^2H_2$]-MVA and [5,5-$^2H_2$]-MVA (4-D-α-cedrene and 5-D-α-cedrene, respectively). The ms spectra of the 4-D- and 5-D-α-cedrene are reproduced in Figure 3, A and B, respectively.

The predominant ion **7** at *m/z* 93 in the non-labeled **2** is derived from the cyclohexene ring in **2** via the ion **6** (Scheme 2). The ion **6** further decomposes with the suc-

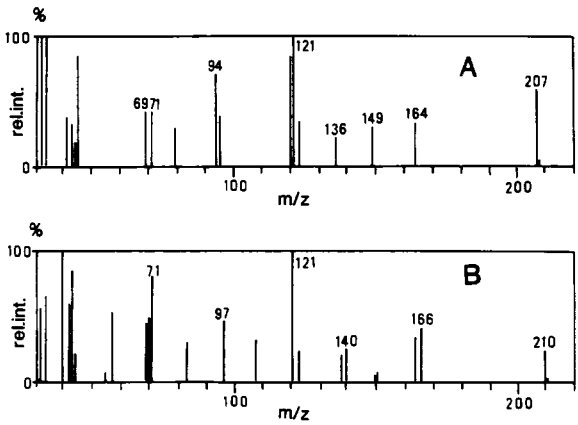


FIGURE 3. Mass spectra of α-cedrene incorporating [4,4-$^2H_2$]-mevalonate (A) and [5,5-$^2H_2$]-mevalonate (B).

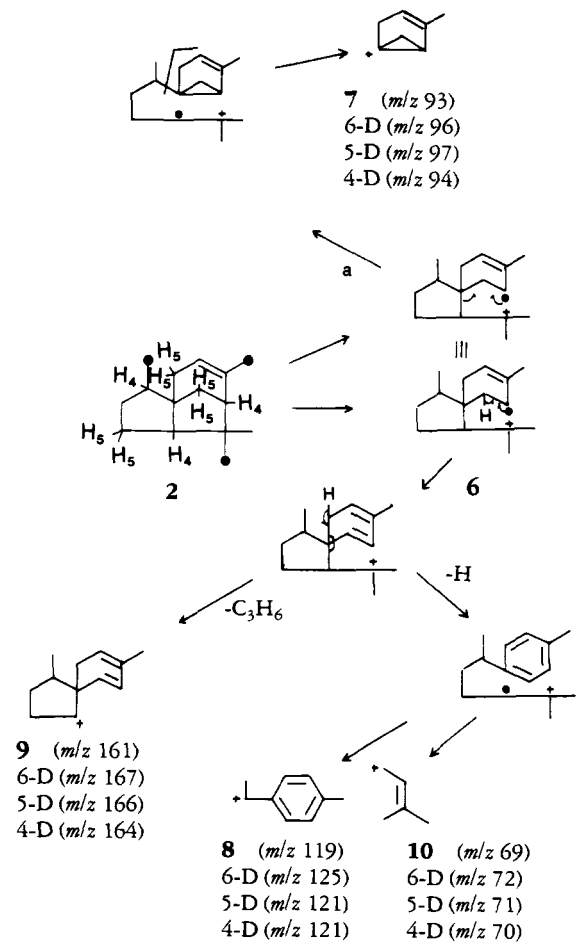
cessive breaking of the C-6/C-11 and the C-6/C-7 bonds in **2** (our numbering). The shift of the ion **7** by 3 mass units in the 6-D-α-cedrene indicates the presence of a deuterated methyl group attached to the cyclohexene ring. The ion **7** in the 5-D-α-cedrene, which is 4 mass units higher than that of the non-labeled compounds, demonstrates the presence of two deuterated methylenes, namely the C-1 and C-5 methylenes in the cyclohexene ring. The ion **7** in the 4-D-α-cedrene, which shifted by only 1 mass unit, confirms that the methine proton at the C-2 position is deuterated.

A base ion (ion **8**) at *m/z* 121 in the 4-D-α-cedrene, which corresponds to the ions at *m/z* 119, 121, and 125 in the non-labeled, 5-D-, and 6-D-α-cedrene, respectively, indicates the presence of two deuterated atoms at the C-7 and C-2 positions. The formation of the ion **8** also involves the breaking of the C-2/C-11 bond to form the ion **6**. The ion **6** further decomposes with the elimination of two hydrogen atoms at the C-1 and C-5 positions, and the successive breaking of the C-6/C-10 and the C-7/C-8 bonds results in the stable fragment ion **8**. This fragmentation process is supported by the ions **8** at *m/z* 119, 121, and 125 in the non-labeled, 5-D-, and 6-D-α-cedrene, respectively.

The comparison between the ions for **7** and the ions for **8** indicates conclusively that the hydrogen atom at the C-7 position in **2** originated from 4-H in MVA. Thus the 1,2-hydride shift in the cyclization process forming **2** is clearly demonstrated.

The calli derived from the seedling leaves of *L. leptolepis* accumulated a number of lower terpenoids including *p*-cymene, 6*E*-β-farnesene, α-cedrene, β-cadinene, aromandendrene, β-caryophyllene, α-humulene, longifollene, some oxygenated sesquiterpenes, and α-ionone. Administration of [4,4-$^2H_2$]-, [5,5-$^2H_2$]-, and [6,6,6-$^2H_3$]-MVAs to the calli showed that monoterpene and sesquiterpene hydrocarbons were deuterated at a higher level than those in any other biosynthetic study. Thus the locations of enriched ^2H in α-cedrene [**2**] and β-farnesene [**3**] as determined by gc-ms analysis confirmed the generally accepted biosynthetic pathways.

In higher plant systems, in vivo studies on monoterpene and sesquiterpene biosynthesis via the incorporation of postulated precursors have been technically difficult. Various methods have resulted in only very low rates of incorporation. Thus studies on lower terpene biosynthesis in higher plants have neccessitated the cumbersome use of $^3H/^{14}C$-labeled substrate followed by chemical degradation (20). Current studies in this field have focused on biogenetic information gained from analysis of $^2H/^{13}C$-labeled products derived from primary precursors such as MVA or acetate. These studies, however, have dealt primarily with higher plant phytoalexins or fungal



SCHEME 2. Mass fragmentation of α-cedrene [**2**]. Figures with *m/z* in parentheses are mass units of the fragment ions from nonlabeled α-cedrene. 6-D, 5-D, and 4-D represent α-cedrenes derived from [6,6,6-$^2H_3$]-, [5,5-$^2H_3$]-, and [4,4-$^2H_2$]-mevalonate, respectively. ● represents the deuterated methyl originating from methyl group of mevalonate.

metabolites (21–23), because of the relatively high rates of incorporation of exogeneously supplied precursors in vivo.

The results presented here, together with the previously reported biosynthetic studies on lower terpenes, suggest that plant tissue culture (8–12) may provide a suitable means for studying the biosynthesis of monoterpenes and sesquiterpenes. The technique presented here, in which the locations of ^2H in terpenes deuterated at extremely high levels were determined by gc-ms analysis, could be particular useful in cases where it is difficult to isolate each component from a complex mixture of terpenes.

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