Origin of the Acetate Units Composing the Hemiterpene Moieties of Chalcomoracin in *Morus alba* Cell Cultures

Yoshio Hano,† Akio Ayukawa,† Taro Nomura,*,† and Shinichi Ueda*,‡

Contribution from the Faculty of Pharmaceutical Sciences, Toho University, Miyama, Funabashi, Chiba 274, Japan, and Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

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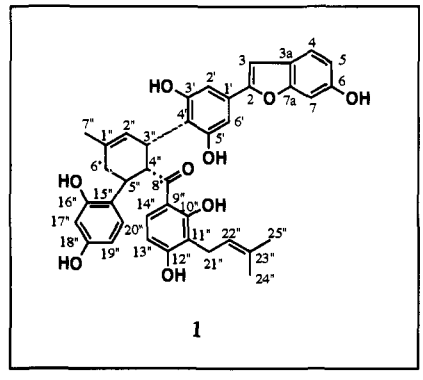
Abstract: Chalcomoracin (**1**), produced in *Morus alba* L. cell cultures at high levels, is a phenolic compound bearing two hemiterpene moieties in the molecule. Administration of D-[U-$^{13}C_6$]glucose to the cell cultures provided **1** labeled with ^{13}C. Analogous experiments with [1,3-$^{13}C_2$]- and [2-^{13}C]glycerol demonstrated that the ^{13}C-labeling pattern in the second and third acetate units for the mevalonate pathway was reversed. This reversed ^{13}C-labeling pattern suggests that, in the pair of three acetate units composing the two hemiterpene moieties of **1**, the starter acetate unit comes directly from glycolysis, while the second and third acetate units originate from the pentose phosphate cycle.

Morus alba L. cell cultures induced from the seedlings or the leaves produce chalcomoracin (**1**) in a yield about 100–1000 times greater than that of the intact plant.[1] Compound **1**, isolated from mulberry leaves, has been regarded as an intermolecular Diels–Alder type adduct of a prenylchalcone and a prenyl-2-arylbenzofuran.[2] Extensive biosynthetic studies on **1** through feeding of ^{13}C-labeled acetate[3] and *O*-methylated precursor chalcones[4] to the cell cultures demonstrated **1** to be a stereospecific intermolecular [4 + 2] cycloadduct between the above fragments. Feeding experiments with ^{13}C-labeled acetate on the cell cultures also revealed that the acetate is not incorporated intact into the two hemiterpene moieties of **1** but is reconstructed from exogenous acetate passing through the tricarboxylic acid (TCA) cycle.[3] In contrast, ^{13}C labeling of β-sitosterol co-occurring with **1** with ^{13}C-labeled acetates took place in a manner following conventional mevalonate biosynthesis according to Ruzicka's biogenetic isoprene rule,[5] as was verified in the case of *Rabdosia japonica* tissue cultures.[6,7] In spite of satisfactory incorporation of [2-^{13}C]-mevalonate into β-sitosterol, ^{13}C label from the same precursor was not incorporated into **1**.[6] L-[2-^{13}C]Leucine, a candidate precursor of mevalonate, also was not incorporated into the hemiterpene moieties of **1**.[6] On the basis of these findings, we have examined the biosynthesis of the hemiterpene moieties of **1** in more detail through administration of ^{13}C-labeled glucose, glycerol, and pyruvate. Although it is well established that isoprene units are constructed by condensation of acetyl-CoA with acetoacetyl-CoA, formed from two acetyl-CoA units, followed by succesive reactions leading to 3-hydroxy-3-methylglutaryl-CoA, mevalonate, and isopentenyl pyrophosphate (IPP), this paper describes findings which reveal a new biosynthetic pathway for the hemiterpene moiety of **1** in *M. alba* cell cultures.



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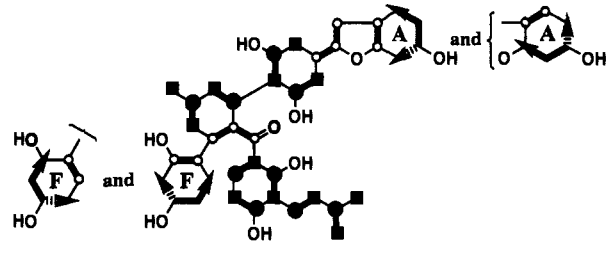

Figure 1. ^{13}C-labeling pattern in **1** from D-[U-$^{13}C_6$]glucose: ●—●, acetate; ○—○(○—○), pyruvate; ⟵⟶ E-4P with a 50% disconnection between the terminal carbon and the penultimate carbon.

Results and Discussion

D-[U-$^{13}C_6$]Glucose (170 mg) was administered to *M. alba* cell cultures suspended in sterile water. After incubation of the cell suspension for 1 week in the dark at 25 °C, the cells were harvested. The cells were lyophilized (6.35 g) and extracted with methanol. The usual workup involving separation and purification of the extract afforded **1** (58 mg). The 100.4-MHz ^{13}C NMR spectrum of **1** demonstrates a satisfactory level of incorporation of the ^{13}C label into carbons comprising the molecule (Table 1). Detailed analysis of the satellite peaks due to ^{13}C–^{13}C coupling between the component carbons disclosed the ^{13}C-labeling pattern shown in Figure 1, indicating that **1** consists of two cinnamoyl polyketide–isoprenoid units. However, two independent pathways of ^{13}C labeling are observed in the aromatic rings A and F, which are formed through the shikimate pathway (Figure 1). Further

† Faculty of Pharmaceutical Sciences, Toho University.
‡ Faculty of Pharmaceutical Sciences, Kyoto University.
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Table 1. ^{13}C NMR Chemical Shifts (ppm) and Coupling Constants (Hz) of D-[U-$^{13}C_6$]Glucose-Derived **1** and the ^{13}C Enrichment (%) of Carbons in **1** from ^{13}C-Labeled D-Glucose and Glycerol[a]

C no.	ppm	A	B	C	C no.	ppm	A	B	C
C-2	155.49 (75.6, 70.6)	22	0	12	C-7″	23.80 (44.0)	32	4	15
C-3	101.86 (75.6)	22	6	0	C-8″	209.81 (42.6)	22	6	0
C-3a	122.09 (——)[b]	22	0	12	C-9″	113.60 (58.7)	14	4	0
C-4	121.83 (——)[b]	22	6	0	C-10″	164.68 (69.7)	14	0	10
C-5	113.16 (69.2, ——)[b]	22	1	12	C-11″	115.95 (69.7)	14	4	0
C-6	159.59 (60.2, 71.9)	22	6	0	C-12″	163.19 (60.2)	14	0	10
C-7	98.42 (70.4, 71.9)	22	1	12	C-13″	108.12 (60.2)	14	4	0
C-7a	156.42 (70.4, 56.5)	22	6	0	C-14″	132.13 (58.7)	14	0	10
C-1′	130.99 (70.4)	14	6	0	C-15″	122.67 (56.5, ——)[b]	22	0	12
C-2′	104.95 (68.2)	14	4	0	C-16″	156.74 (——)[b]	22	6	0
C-4′	116.42 (67.5)	14	4	0	C-17″	103.62 (61.4, 67.9)	22	1	0
C-5′	157.84 (67.5)	14	0	10	C-19″	107.60 (60.2, 61.4)	22	1	12
C-6′	104.95	14	4	0	C-20″	128.77 (60.2, 56.5)	22	6	0
C-1″	134.00 (39.6, 44.0)[c]	32	0	15	C-21″	22.23 (42.5)	32	8	0
C-2″	124.42 (41.1)	32	0	15	C-22″	123.20 (42.5)	32	0	15
C-3″	33.28 (41.1)	32	8	0	C-23″	131.50 (41.8, 44.0)[c]	32	0	15
C-4″	47.89 (28.6, 42.6)	22	0	12	C-24″	17.86 (41.8)	32	8	2
C-5″	36.45 (28.6)	22	6	0	C-25″	25.79 (44.0)	32	4	15
C-6″	32.44 (39.6)	32	8	2					

[a] A, D-[U-$^{13}C_6$]glucose; B, [1,3-$^{13}C_2$]glycerol; C, [2-^{13}C]glycerol. The values in parentheses denote coupling constants. The spectrum was measured in aceton-d_6. [b] The multiplicities and the coupling constants of these signals are unassignable. [c] These multiplicities are not a doublet of doublets but two sets of doublets.

analysis of the ^{13}C NMR signals for the shikimate-derived A and F ring carbons revealed that about 50% of the C-1 aldehyde carbon and the penultimate carbon corresponding to erythrose 4-phosphate (E-4P) are disconnected (Figure 2). This C-3 + C-1 type of disconnectivity in E-4P-derived moieties could arise through glucose metabolism via two triose phosphates, glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), which are equilibrated by triose phosphate isomerase. Both triose phosphates formed from D-[U-$^{13}C_6$]glucose participate in the formation of fructose 1,6-diphosphate (F-1, 6P), which enters into the pentose phosphate cycle via F-6P, glucose 6-P, and then phosphogluconate. Sedoheptulose 7-phosphate occurring in the cycle affords E-4P with the ^{13}C labeling described above. This type of disconnection in the E-4P-derived moieties was also reported in both shikimate-derived aromatic rings of the antibiotic obafluorin.[8] On the other hand, the two ^{13}C-labeling patterns in the A and F rings can be attributed to oxidation at two different isotopic labeled carbons, pointing to a symmetrical intermediate (Scheme 1).

With regard to the C-4′ and C-11″ positions of the two hemiterpene moieties, preliminary feeding experiments with [2-^{13}C]acetate indicated that [1,2-$^{13}C_2$]acetate formed from exogenous acetate by passage through the TCA cycle afforded the starter acetate unit for biosynthesis of the two hemiterpene moieties of **1**. On the other hand, no detectable ^{13}C enrichment

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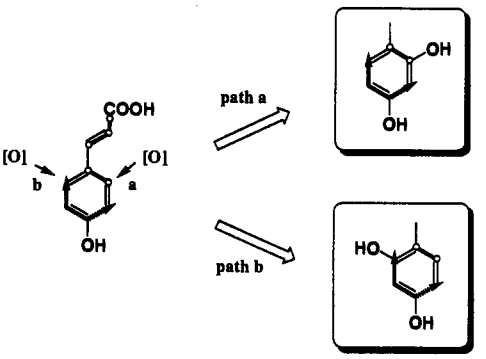
Scheme 1. Oxidation at Two Different Isotopic Carbons of the Shikimate-Derived Aromatic Ring Resulting in the Formation of Two Independent ^{13}C-Labeling Patterns



was observed in the second and third acetate units. ^{13}C labeling from [1-^{13}C]acetate was not incorporated into **1**.[3] However, ^{13}C label from D-[U-$^{13}C_6$]glucose was extensively incorporated into the two hemiterpene moieties (Figure 1 and Table 1). The ^{13}C-labeling patterns in these moieties appeared to be in accordance with the expected pattern based on a conventional mevalonate biosynthesis via 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) arising from three acetate units (Figure 1). Continuous ^{13}C labeling of D-[U-$^{13}C_6$]glucose, however, made it impossible to locate each glucosyl carbon which participates in the formation of the prenyl residues.

Further administration of [1,3-$^{13}C_2$]- and [2-^{13}C]glycerol to the cell cultures revealed a unique ^{13}C-labeling pattern in **1**, as shown in Figure 3, which suggests a novel hemiterpene biosynthesis. In the case of formation of an acetate unit from exogenous glycerol by way of glycolysis via GAP and DHAP, [1,3-$^{13}C_2$]- and [2-^{13}C]glycerol are converted to [2-^{13}C]acetate and [1-^{13}C]-acetate, respectively. The experiment with [1,3-$^{13}C_2$]glycerol revealed the expected enrichment at C-6″ and C-24″ in the starter acetate units, but the ^{13}C labels in the second and third acetate units were reversed, appearing at carbons C-3″ and C-21″ (Figure 3a). The ^{13}C NMR spectrum of **1** obtained from this experiment exhibited ^{13}C–^{13}C coupling between C-3″ and C-4′ (J_{CC} = 47.0 Hz) as well as between C-21″ and C-11″ (J_{CC} = 51.4 Hz), along with long-range coupling between C-3″ and C-7″ (J_{CC} = 4.8 Hz) as well as between C-21″ and C-25″ (J_{CC} = 4.8 Hz). The ^{13}C enrichments at C-7″ and C-25″ may be explained by transfer of ^{13}C between the *cis*-methyl and the *trans*-methyl carbons resulting from the reversible diene formation (Figure 3a).[6] Support for this hypothesis has been obtained by a pulse-feeding experiment with [2-^{13}C]acetate, which indicated that transfer of ^{13}C takes place not only within the isoprenyl unit at C-4′ participating in the [4 + 2] cycloaddition reaction but also in the other unit remaining intact at C-11″.[6] The fact that no two contiguous ^{13}C-labeled acetate carbons were observed in the two hemiterpene moieties on administration of [1,3-$^{13}C_2$]glycerol suggested that participation of the TCA cycle in the biosynthesis was negligible. A similar phenomenon was also observed in the experiment with [2-^{13}C]glycerol (Figure 3b). The ^{13}C NMR spectrum of **1** obtained in this case exhibited ^{13}C–^{13}C coupling between C-1″ and C-2″ (J_{CC} = 73.4 Hz), as well as between C-22″ and C-23″ (J_{CC} = 74.8 Hz). Reversal of ^{13}C labeling at the second and third acetate carbons in both experiments implies participation of the pentose phosphate cycle in the hemiterpene biosynthesis. Both GAP and DHAP derived from [1,3-$^{13}C_2$]- or [2-^{13}C]glycerol result in the formation of F-1,6P which participates in the pentose phosphate cycle via the same substrates as described above in the case of D-[U-$^{13}C_6$]glucose. The resulting sedoheptulose 7-phosphate provides E-4P along with GAP with reversed ^{13}C labeling compared to the initial GAP. Acetyl-CoA derived from the resultant GAP via phosphoglycerate and pyruvate is incorporated into the hemiterpene as the second and third acetate units.

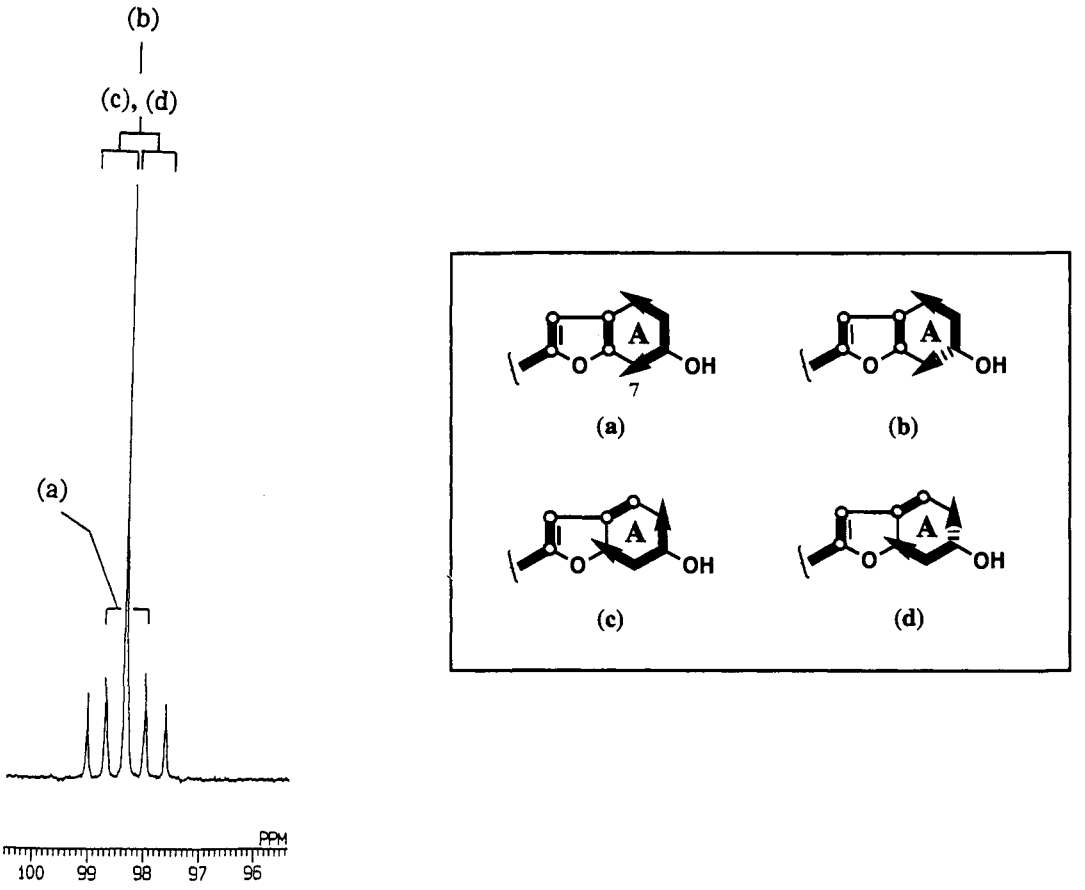
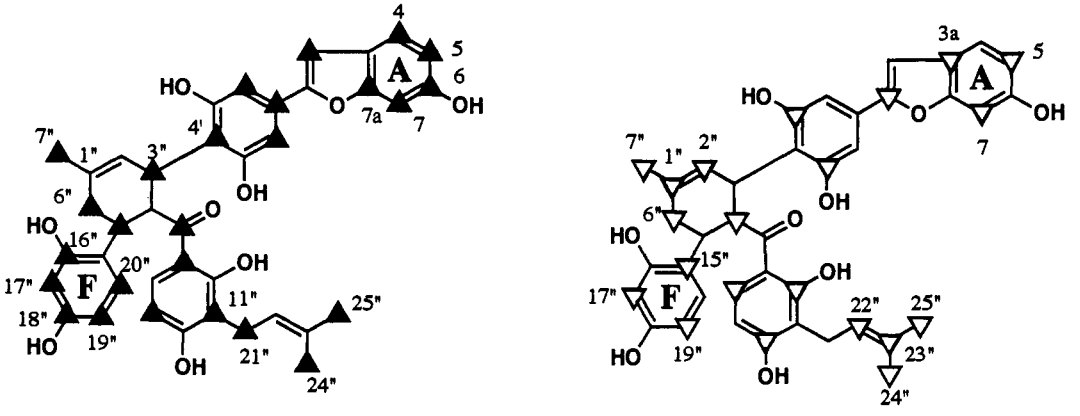


Figure 2. Four splitting patterns of the proton-noise decoupled ^{13}C NMR signal for C-7 of D-[U-$^{13}C_6$]glucose-derived **1** in the following disposition: (a) terminal carbon, (b) isolated carbon, (c and d) penultimate carbon.

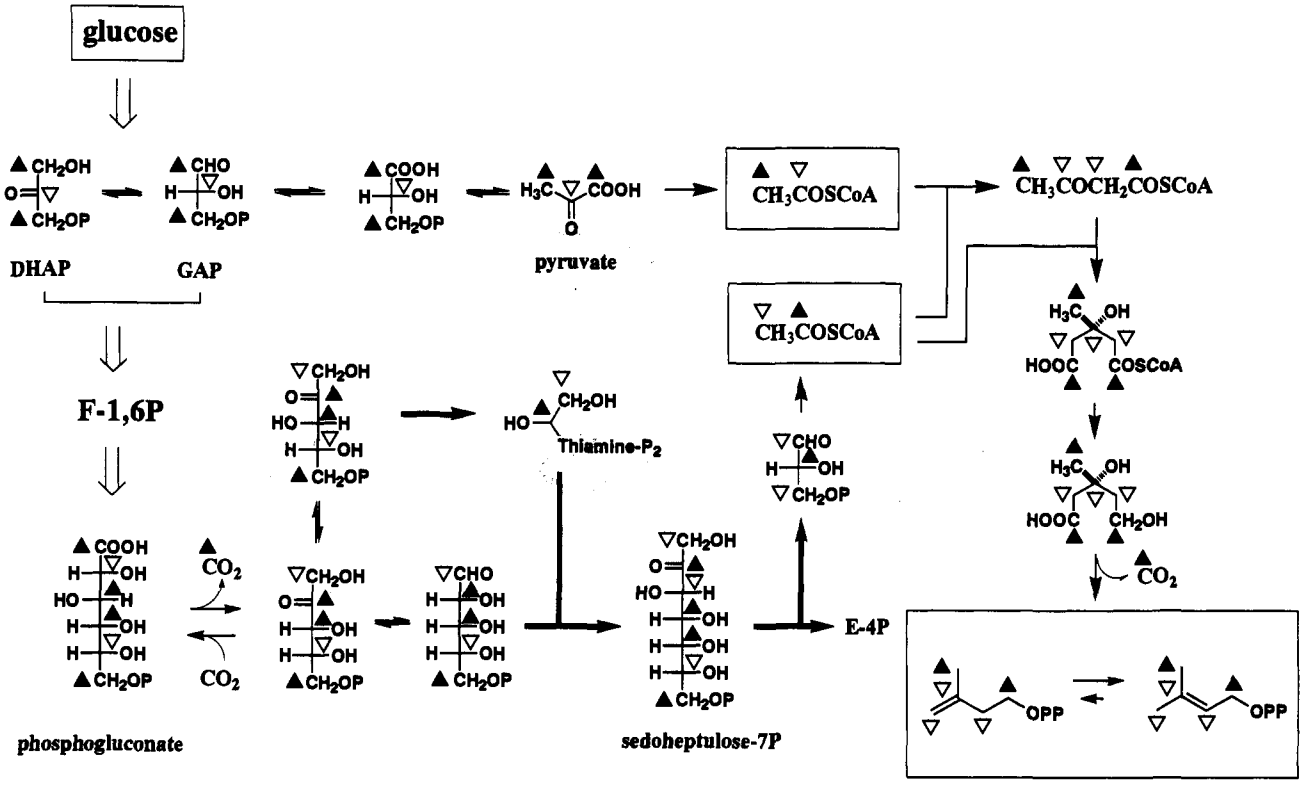


(a) **(b)**

Figure 3. (a) Labeling pattern in **1** from [1,3-$^{13}C_2$]glycerol. (b) Labeling pattern in **1** from [2-^{13}C]glycerol. The ^{13}C-enrichment at C-5, C-7, C-17″, or 19″ in part a as well as at C-5, C-7, C-17″, or C-19″ in part b can be attributed to the two ^{13}C-labeling patterns of the shikimate-derived aromatic ring, as was observed in the case of the experiment with D-[U-$^{13}C_6$]glucose.

With regard to the origin of the acetate units participating in the hemiterpene biosynthesis for **1**, it was concluded that the starter acetate unit for mevalonate synthesis is of glycolytic origin, while the second and third acetate units originate from the pentose phosphate cycle (Scheme 2). Each step in the mevalonate biosynthesis for **1** thus strictly requires that the acetate units have different origins in the cell cultures. Therefore, the ^{13}C labels incorporated into the two isoprenyl moieties of **1** in the feeding experiment with D-[U-$^{13}C_6$]glucose are probably from acetate units resulting from the above two glucose catabolic pathways. As stated above, in experiments in which ^{13}C-labeled acetate was administered to *M. alba* cell cultures, [2-^{13}C]acetate passing through the TCA cycle gave rise to [1,2-$^{13}C_2$]acetate, which was incorporated only into the starter acetate unit of the hemiterpene moiety of **1**.[3] This finding was reinforced by complementary pulse-feeding experiments with [2-^{13}C]acetate.[6] This restricted incorporation implies that the reconstructed acetate shares the same metabolic pool with the acetate of glycolytic origin. Thus, when the reconstructed [1,2-$^{13}C_2$]acetate enters the reverse Embden–Meyerhof–Parnas (EMP) pathway via GAP, DHAP, and F-1,6-P to take part in the pentose phosphate cycle, the two contiguously ^{13}C labeled carbons are incorporated into

Scheme 2. Biosynthetic Route to the Hemiterpene Moiety of **1** through the Two Catabolic Pathways of Glucose in *M. alba* Cell Cultures: ▲, labelings from [1,3-$^{13}C_2$]glycerol; ▽, labeling from [2-^{13}C]glycerol.



E-4P. Indeed, in the case of the pulse administration of [2-^{13}C]-acetate, the two relevant contiguous ^{13}C labels were observed in the E-4P portions of the shikimate-derived aromatic rings of **1**. This would make the incorporation of the reconstructed acetate formed through the TCA cycle into the second and third acetate units less likely. The present study in which two pivotal pathways, the EMP pathway and pentose phosphate cycle, participate in the hemiterpene biosynthesis in **1**, gives a satisfactory explanation of the restricted incorporation of ^{13}C-labeled acetate. Upon administration of sodium [3-^{13}C]pyruvate to *M. alba* cell cultures, however, no ^{13}C labeling was observed in **1**. Possible explanations for the observed noninvolvement of exogenous mevalonate and pyruvate may lie in compartmentation effects[9] or in the segregation of protein-bound intermediates from free intermediates.[10] Taking into account the fact that conventional biosynthesis of β-sitosterol co-occurs with **1**, it can be concluded that *M. alba* cell cultures must have at least two separate compartments for mevalonate biosynthesis.

Recently, in studies of bacterial polyterpenoids, a novel pathway ruling out a mevalonate intermediate was proposed for the biosynthesis of the isoprene unit. These bacterial polyterpenoids are believed to be biosynthesized through condensation of a thiamine-activated C_2 unit and a C_3 unit derived from dihydroxyacetone phosphate followed by a transposition step resembling the biosynthesis of L-valine.[11] The main differences in the isotopic enrichments between the bacterial polyterpenoids and *M. alba* prenylchalcones are as follows: (a) Upon administration of [1-^{13}C]acetate, the bacterial polyterpenoids were always enriched at the carbons corresponding to the C-4 carbon of IPP (Figure 4), whereas the hemiterpene moieties of the prenylchalcones were not enriched. (b) The C-4 carbon of glucose was incorporated as the C-4 carbon of the bacterial IPP units. In *M. alba* cell cultures, [1,3-$^{13}C_2$]- and [2-^{13}C]glycerol, which formed

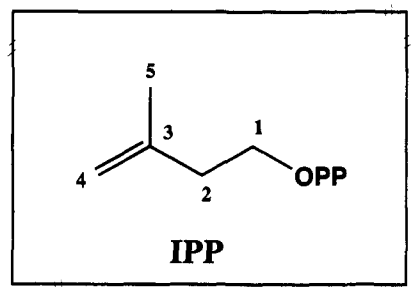


Figure 4. Structure of isopentenyl pyrophosphate.

[1,3,4,6-$^{13}C_4$]- and [2,5-$^{13}C_2$]glucose, respectively, were incorporated into the prenylchalcones. ^{13}C Labeling in the isoprene unit derived from [1,3-$^{13}C_2$]glycerol was found at the carbons corresponding to the C-1 and C-5 positions of IPP, while that from [2-^{13}C]glycerol was found at the C-2, C-3, and C-4 positions (Scheme 2).

Conclusion

Feeding of D-[U-$^{13}C_6$]glucose to *M. alba* cell cultures fully disclosed the early stages of the biosynthesis of **1**, which was shown to be comprised of two molecules each of a cinnamoyl polyketide and mevalonate (Figure 1). The observation of two independent ^{13}C-labeling patterns in the shikimate-derived aromatic rings in this experiment suggests that the hydroxylation takes place on a symmetrical intermediate after aromatization of the shikimate portions (Scheme 1). The experiment with ^{13}C-labeled glycerol points to a novel mode of hemiterpene biosynthesis in the cell cultures, in which two pivotal glucose metabolic pathways, the glycolytic (EMP) pathway and the pentose phosphate cycle, participate in the biosynthesis. Specifically, the starter acetate unit for the mevalonate synthesis comes from the glycolytic pathway, while the second and third acetate units originate from a route passing through the pentose phosphate pathway (Scheme 2). The present study provides the first example

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demonstrating chimeric biosynthesis of the hemiterpene unit by two different pathways in a higher plant.

Experimental Section

Material and Methods. D-[U-$^{13}C_6$]Glucose (99 atom % ^{13}C), [1,3-$^{13}C_2$]glycerol (99 atom % ^{13}C], [2-^{13}C]glycerol (99 atom % ^{13}C), and sodium [3-^{13}C]pyruvate (99.4 atom % ^{13}C) were purchased from Isotec Inc., USA. Wakogel C-200 and B-5F (Wako Pure Chem., Osaka Japan) were used for column chromatography and preparative thin-layer chromatography (TLC), respectively. The NMR data were recorded on a JEOL JNM EX-400 FTNMR spectrometer using deuterated acetone as a solvent and tetramethylsilane (TMS) as an internal standard. High-performance liquid chromatography (HPLC) was carried out with a SSC high-pressure liquid chromatograph (Senshu Scientific, Tokyo, Japan) using a UV detector. The induction of *M. alba* callus cultures from seedlings or mulberry leaves has been described previously.[1]

Feeding Experiment with D-[U-$^{13}C_6$]Glucose. The *M. alba* cells placed in ten 300-mL flasks were each suspended in 100 mL of sterilized water, to which a 10% ethanol solution (10 mL) of D-[U-$^{13}C_6$]glucose (170 mg) was added. After incubation of the cell suspension for 1 week in the dark at 25 °C, the cells were harvested and lyophilized. The lyophilized cells (6.35 g) were extracted with methanol (100 mL). Concentration of the methanol extract *in vacuo* gave a residue (1.08 g), which was extracted with acetone (50 mL). The acetone extract was concentrated under reduced pressure to give a residue (0.52 g), which was subjected to column chromatography over silica gel (70 g) using chloroform with an increasing content of acetone as solvent. The combined fractions (0.12 g), eluted with chloroform–acetone (2:1), were subjected to preparative TLC (hexane–acetone, 1:1) followed by HPLC with ether as solvent (column, SSC Silica 4251-N; flow rate 3 mL/min) to yield **1** (retention time, 16 min; 58 mg).

Feeding Experiment with [1,3-$^{13}C_2$]Glycerol. [1,3-$^{13}C_2$]Glycerol (132.7 mg) was administered to the *M. alba* cell cultures, which were worked up in an analogous way to that used in the experiment with D-[U-$^{13}C_6$]-glucose. **1** (18 mg) was obtained from the lyophilized cells (3.05 g).

Feeding Experiment with [2-^{13}C]Glycerol. [2-^{13}C]Glycerol (285.6 mg) was administered to the *M. alba* cell cultures, and these were worked up in analogous way. **1** (22 mg) was obtained from the lyophilized cells (3.20 g).

Feeding Experiment with Sodium [3-^{13}C]Pyruvate. Sodium [3-^{13}C]-pyruvate (100 mg) was administered to the *M. alba* cell cultures, and these were worked up in an analogous way. **1** (27 mg) was obtained from the lyophilized cells (3.05 g).