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BIOSYNTHESIS OF 1α,2α,3β-TRIHYDROXY-*p*-MENTHANE BY *FUSICOCCUM AMYGDALI*

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Key Word Index—*Fusicoccum amygdali*; biosynthesis; monoterpenes; 1α,2α,3β-trihydroxy-*p*-menthane; α-terpinyl cation; diosphenol.

Abstract—Measurement of isotope ratios in 1α,2α,3β-trihydroxy-*p*-menthane, which has been biosynthesized in *Fusicoccum amygdali* from ^{3}H- and ^{14}C-labelled mevalonate and in its degradation product diosphenol indicates that: (a) four tritium atoms arising from [5-$^{3}H_2$, 2-^{14}C]MVA are retained, one more than suggested from the hydroxylation pattern, (b) menth-2-ene-1-ol is generated from an α-terpinyl cation through a 1,3-hydride shift and (c) *trans*-cleavage of an α-epoxide by hydrolysis gives 1α,2α,3β-trihydroxy-*p*-menthane.

INTRODUCTION

The 1α,2α,3β-trihydroxy-*p*-menthane (**10**, Scheme 1) [1,2] is a minor metabolite of *Fusicoccum amygdali*, the agent of peach and almond canker [3,4]. In culture filtrates **10** is contained in very low concentration (4 mg/l.), together with fusicoccin [5–7] and its structurally related metabolites [8]. Although these products show a well known phytotoxic activity [9] and very pronounced plant-growth regulating activities [8], **10** is devoid of fusicoccin-like biological activity. The peculiar chemical structure and stereochemistry of **10** prompted us to investigate its biogenesis, which involves the elucidation of three main points: (a) the mechanism of formation of the α-terpinyl cation (**6**) which is the common intermediate in the biosynthesis of all natural monoterpenes reported in Scheme 1; (b) the conversion of **6** into the allylic cation **7** (Scheme 2); and (c) the hydroxylation stereochemistry probably involving the α-epoxide **9** (Scheme 2).

The present paper reports the results of the incorporation of [2-$^{3}H_2$, 2-^{14}C]-, [5-$^{3}H_2$, 2-^{14}C]- and [4*R*-$^{3}H_1$, 2-^{14}C]mevalonic acid into **10** and describes the location of radioactivity at C-4.

RESULTS AND DISCUSSION

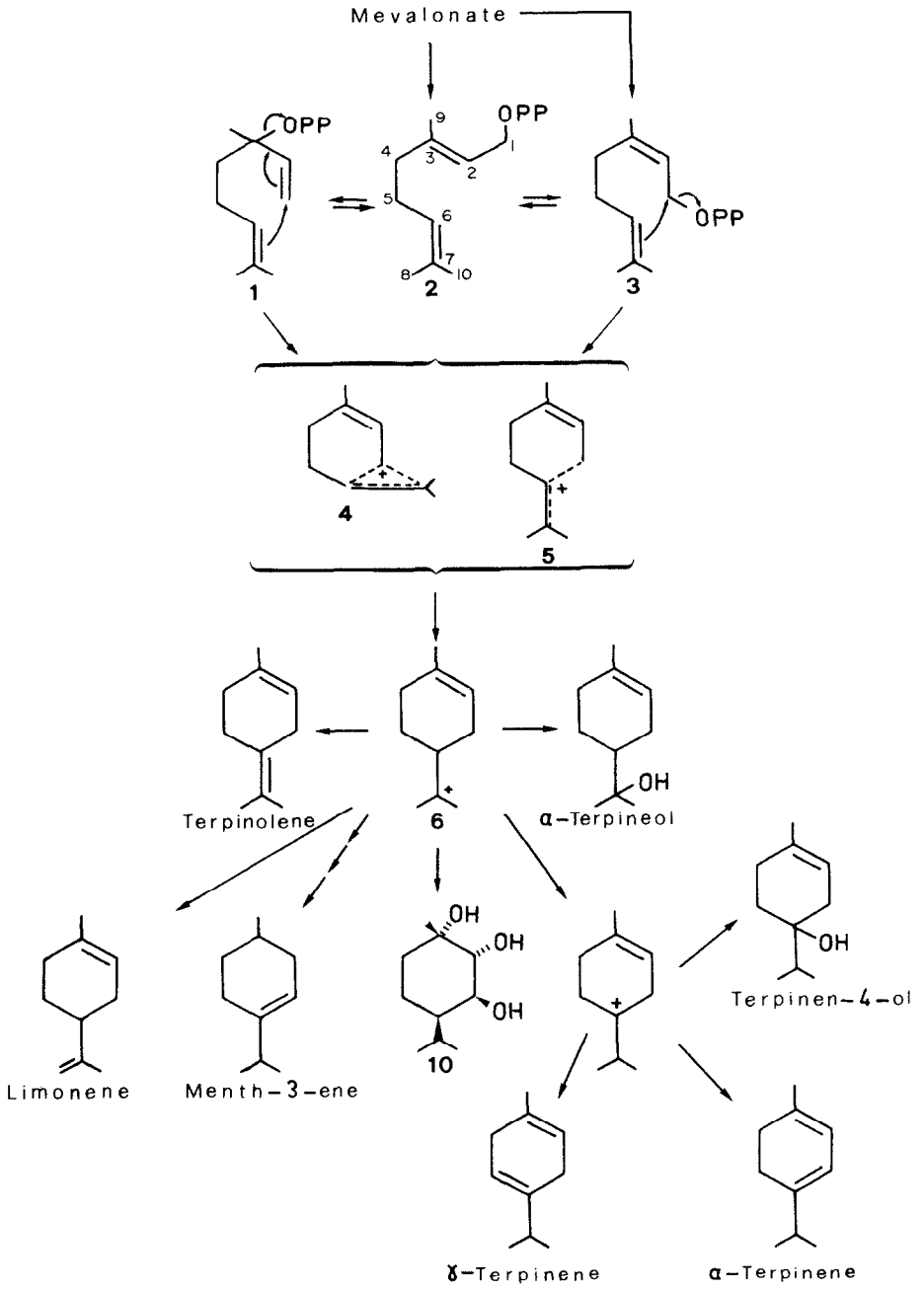
Many reviews deal with the direct acyclic precursor of monoterpenes since geranyl pyrophosphate (GPP **2**, Scheme 1) cannot directly cyclize because of the constraint due to the *trans*-substituted double bond. Therefore, a prior isomerization to neryl (NPP **3**, Scheme 1) or linalyl (LPP **1**, Scheme 1) pyrophosphate appears to be necessary. Recently Cane [10], reviewing available data on acyclic precursors, reported three hypotheses for precursor interconversions, which circumvented the problem of whether NPP (**3**) or LPP (**1**) is the substrate for the cyclase.

The retention in **10** of four tritium atoms from [5-$^{3}H_2$, 2-^{14}C]MVA, one more than expected from the hydroxylation pattern, excludes, among the mentioned isomerization mechanisms, the one which isomerizes (via redox) geraniol to nerol with the loss of one C-1 hydrogen of GPP (**2**) [10–12].

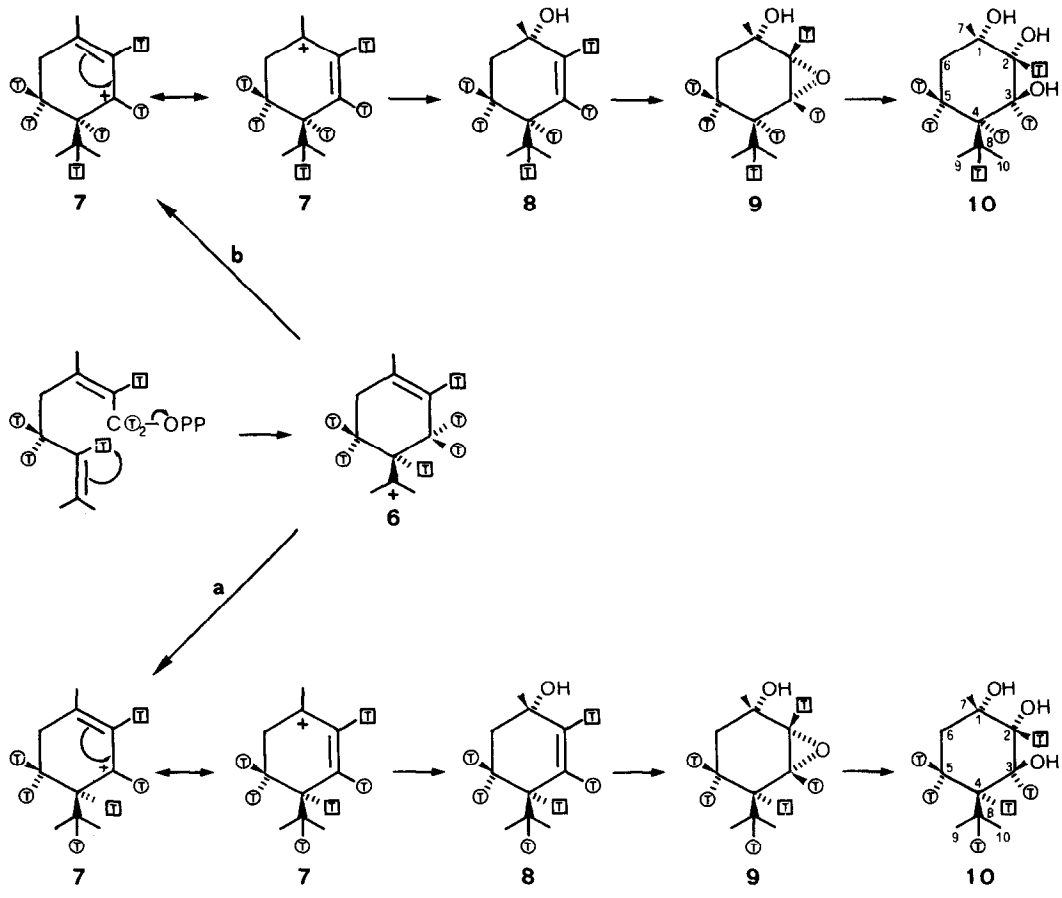
The cyclization of NPP (**3**) or LPP (**1**) occurs via an anchimerically assisted nucleophilic attack of the 6,7-double bond on the C-1 of the terpene, according to Rittersdorf and Cramer [13]. The same authors suggest the existence of the two non-classical carbonium ions, **4** and **5** (Scheme 1), which rearrange to the classical cation **6**, which is the common intermediate in the biosynthesis of all natural monoterpenes reported in Scheme 1. It can be assumed that β-orientation of the isopropyl group in **10** is determined when carbonium ion **6** is formed (Scheme 2).

In order to elucidate the mechanism of formation of **7** [point (b)], the radioactivity of **10** was measured. The isotope ratios (^{3}H:^{14}C), referred to MVA (counted as the diphenylmethylamide), are listed in Table 1. The results obtained show that formation of **7** from **6** could follow two alternative routes (Scheme 2). Route b requires two 1,2-hydride shifts and leads to the labelling of the hydrogens on C-3, C-4 and C-5 when [5-$^{3}H_2$, 2-^{14}C]MVA is incorporated, whereas hydrogens on C-2 and C-8 are labelled when incorporation of [4*R*-$^{3}H_1$, 2-^{14}C]MVA occurs. The alternative route a involves a 1,3-hydride shift resulting in the labelling of the hydrogens on C-3, C-5 and C-8 following [5-$^{3}H_2$, 2-^{14}C]MVA incorporation, while tritium atoms arising from [4*R*-$^{3}H_1$, 2-^{14}C]MVA are located on C-2 and C-4. In both routes four tritium atoms are retained from the incorporated [5-$^{3}H_2$, 2-^{14}C]MVA, i.e. one more than would be expected from the hydroxylation pattern. The origin of the ^{3}H radioactivity on C-4 will show which of the two proposed mechanisms for the formation of **7** from **6** is operative during biosynthesis of **10**.

The sequence of reactions reported in Scheme 3 was performed in order to exchange the hydrogen atom on C-4. Compound **10** was transformed into the *O,O'*-isopropylidene derivative (**11**, Scheme 3) which, in turn, gave the corresponding 3-oxo derivative (**12**) through the oxidation of the hydroxyl group on C-3 by Corey reagent



Scheme 1. Proposed origin of some representative monocyclic terpenes through the terpinyl cation (all products shown are naturally occurring). Intermediates are formally represented as carbonium ions; the actual species may be, among others, phosphate esters, glycosides or enzyme-bonded.

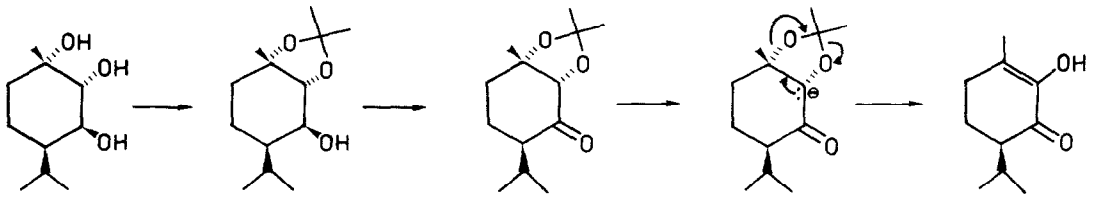


Scheme 2. The alternative biogenetic routes (a, b) to 1α,2α,3β-trihydroxy-*p*-menthane. Intermediates are formally represented as carbonium ions. T = ^3H; T and Ⓣ indicate tritium atoms arising from the incorporation of [4*R*-$^3H_1$, 2-^{14}C]- and [5-$^3H_2$, ^{14}C]MVA respectively.

Table 1. Incorporation of labelled mevalonate into 1α,2α,3β-trihydroxy-*p*-menthane (**10**)

Experiment	Precursor	Incorporation* (%)	Isotope ratios†		Atomic ratios‡	
			Precursor§	**10**	Precursor	**10**
1	[4*R*-$^3H_1$, 2-^{14}C]MVA	9.0×10^{-3}	8.1	7.6	2:2	1.9:2
2	[4*R*-$^3H_1$, 2-^{14}C]MVA	5.2×10^{-3}	9.6	9.5	2:2	2.0:2
1	[5-$^3H_2$, 2-^{14}C]MVA	6.4×10^{-3}	9.7	9.3	4:2	3.9:2
2	[5-$^3H_2$, 2-^{14}C]MVA	3.8×10^{-3}	3.9	4.0	4:2	4.1:2
1	[2-$^3H_2$, 2-^{14}C]MVA	1.4×10^{-3}	11.1	10.1	4:2	3.7:2

* Calculated from ^3H data and including allowance for utilization of 3*R*-enantiomer of MVA only.
† ^3H:^{14}C. Standard error (estimated) for ratio, ±0.02.
‡ ^3H:^{14}C.
§ As diphenylmethylamide.



10 11 12 13 14

Scheme 3. Formation of diosphenol from 1α,2α,3β-trihydroxy-*p*-menthane. Compound **12** evolves to **14** through the hypothesized intermediate **13**.

[14]. By alkaline treatment **12** gave the unexpected diosphenol (**14**) [15], by the reaction mechanism indicated in Scheme 3.

The exchange of the hydrogen atom on C-4 was demonstrated by the presence of one deuterium atom when the reaction was performed with $NaOD/D_2O$ [mass spectrometry; ^1H NMR and ^{13}C NMR spectra showed absence of the signals at 2.18 (1 H, *dt*, 4-H) and 50,7 (C-4) ppm respectively].

Labelled samples of **10** prepared by feeding cultures of *Fusicoccum amygdali* with [4*R*-$^3H_1$, 2-^{14}C]MVA and [5-$^3H_2$, 2-^{14}C]MVA were independently transformed into **14** after dilution with inactive material. The isotope ratios (^3H:^{14}C, Table 2) showed the complete loss of tritium atoms in **14** prepared from **10** which was biosynthesized from [4*R*-$^3H_1$, 2-^{14}C]MVA, whereas three tritium atoms were retained in **14** obtained from **10** in which [5-$^3H_2$, 2-^{14}C]MVA had been incorporated.

The degradation reaction of **10** into **14** involves the loss of hydrogen atoms from C-2, C-3 and C-4. When this degradation was carried out on a sample of **10** labelled [4*R*-$^3H_1$, 2-^{14}C]MVA, the two tritium atoms were lost from C-2 and C-4 (route a, Scheme 2). Alternatively, when **14** was prepared from **10** biosynthesized from [5-$^3H_2$, 2-^{14}C]MVA the tritium atoms on C-5 and C-8 were retained. The results reported above indicate that the proposed pathway a for the formation of **7** from **6** is operative during biosynthesis of **10**.

With regard to point (c), one might hypothesize that **7** evolves (Scheme 2) through an α-addition of a water molecule or a hydroxyl group to menth-2-ene-1-ol (**8**).

Compound **8** generates **10** through a *trans*-hydroxylation which occurs via the intermediate α-epoxide (**9**, Scheme 2). Such a pattern is supported by data obtained on cyclohexene derivatives bearing allylic substituents similar to those on **8**. Epoxidation of these compounds takes place through the stereospecific introduction of the epoxy group with *anti*-stereochemistry to the isopropyl group [16, 17] and *syn* to the hydroxyl group [18]. Hydrolysis of the α-epoxide occurs with a S_N2 mechanism due to the attack of a water molecule on C-3 from the β-side of the molecule [19]. Therefore, the main feature in the biosynthesis of **10** is the 1,3-hydride shift from C-3 to C-8, which at the moment appears as an entirely new mechanism in cyclic monoterpene biosynthesis (Scheme 1).

The low yield of **10** and the low level of incorporation (Table 1) irrespective of the addition time to the cultures of labelled mevalonate discouraged both the study of biosynthesis of **10** by NMR techniques and the specific investigation of labelling on C-8.

EXPERIMENTAL

General methods. Optical rotations were measured in $CHCl_3$ soln. Mps are uncorr. UV spectra were recorded in MeCN (unless otherwise specified) and IR spectra in $CHCl_3$. ^1H NMR spectra were recorded at 270 MHz and ^{13}C NMR at 67.8 MHz (generally) in $CDCl_3$ using TMS as internal standard; chemical shifts are in ppm. MS at 70 eV. Column chromatography was performed on Si gel and TLC on Merck plates. TLC spots were visualized by spraying with a 10% H_2SO_4 soln in MeOH and then heating at 105°. Solvent systems (A) EtOAc–C_6H_6 (7:3); (B) C_6H_6–Et_2O (9:1). Radioactivity measurements were carried out by liquid scintillation counter using a soln containing 6.5 g PPO, 130 mg POPOP and 104 g naphthalene in 1 l. toluene–dioxan (1:1). The counting error was within ±2%. Radioactive compounds were crystallized to constant sp. act.

Feeding and product isolation. Fermentations were carried out according to a published procedure [20] scaled down to Erlenmeyer flasks (500 ml) containing 100 ml of medium. Specific activities of labelled precursors were as commercially available (Radiochemical Centre or NEN chemicals GmbH). In all experiments **10** was extracted with $CHCl_3$ and then with *n*-BuOH

Table 2. Radioactivity retained in diosphenol (**14**) obtained from 1α,2α,3β-trihydroxy-*p*-menthane (**10**) differently labelled by the degradation procedure reported in Scheme 3

		Isotope ratios*		Atomic ratios†	
Experiment	Precursor	**10**	**14**	**10**	**14**
3	[4*R*-$^3H_1$, 2-^{14}C]MVA	7.09	0.142	2:2	0.04:2
3	[5-$^3H_2$, 2-^{14}C]MVA	6.26	4.37	4:2	2.79:2

* ^3H: ^{14}C. Standard error (estimated) for ratio, ±0.02.

† ^3H: ^{14}C.

together with fusicoccin. Fusicoccin and some related metabolites [8] were transformed in dideacetylfusicoccin [21] by overnight treatment with 4N NaOH. After crystallization of dideacetylfusicoccin from EtOAc–petrol, the mother liquor was diluted with unlabelled **10** to give about 800 and 90 dpm/mg for ^{3}H and ^{14}C, respectively, purified by column chromatography (solvent A) and crystallized from C_6H_6.

3-Oxo-O,O'-isopropylidene derivative of 1α,2α,3β-*trihydroxy-p-menthane* (**12**). To a soln of **11** [1] (129 mg) in dry CH_2Cl_2 (12.7 ml) was added $C_5H_5NHCrO_3Cl$ [14] (6.6 g). After stirring for 3 hr at room temp., the mixture was diluted with Et_2O (60 ml) and filtered. The solvent was evapd and the residue was purified by column chromatography. Elution with $CHCl_3$ led to **12** (103 mg) (83%) as a chemically pure oil; $[\alpha]_D^{25}$ + 6.36° (*c* 2.5); UV λ_{max} nm (log ε): 322 (1.27); IR ν_{max} cm^{-1}: 1720 (C=O); ^{1}H NMR: δ 0.91 and 0.94 (3 H each, *d*, *J* = 6 Hz, 9 and 10-Me), 1.41 (3 H, *s*, 7-Me), 1.43 and 1.52 (3 H each, two Me of isopropylidene group), 2.45 (1 H, *m*, 4-H) and 4.0 (1 H, *s*, 2-H); MS *m/z* (rel. int.): 211 (M^+ − 15, 100), 183 (28), 139 (90).

Diosphenol (**14**). To a soln of **12** (83 mg) in MeOH (5.3 ml) was added 4N NaOH (10 ml). After stirring for 24 hr at room temp., the soln was acidified to pH 5–6 with 4N HCl and then extracted with CH_2Cl_2. The solvent was evapd, the crude residue (70%) was purified by TLC (solvent B) and crystallized from MeOH at −20° as white needles, mp 82–84°. UV (EtOH), IR, ^{1}H NMR, ^{13}C NMR and mass spectra were consistent with the accepted structure of the diosphenol (**14**) [15].

[4-D]*Diosphenol*. A soln of **12** (90 mg) in MeOD (5.3 ml) was treated with 4N NaOD (10 ml) as described above to give [4-D]diosphenol. The MS showed a molecular ion at *m/z* 247 which readily lost CH_2=C=O to give the base peak at *m/z* 127. The ^{1}H NMR and ^{13}C NMR spectra were identical to those of **14** expected for the absence of the signal at δ 2.18 (1 H, *dt*, 4-H) and 50.7 (C-4), respectively.

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