Phytochemistry, 1972, Vol. 11, pp. 1683 to 1688. Pergamon Press. Printed in England.

SYNTHESIS OF ISOMERIC FARNESOLS BY SOLUBLE ENZYMES FROM *PINUS RADIATA* SEEDLINGS

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(*Received* 24 *October* 1971)

Abstract—Water soluble enzymes obtained from *Pinus radiata* seedlings form two sesquiterpene alcohols from 2-^{14}C mevalonic acid. They have been identified as 2,6-*trans*,*trans*-farnesol and 2-*cis*,6-*trans*-farnesol. The pyrophosphate of the former prenol could be isolated, but there was no evidence of the presence of phosphorylated derivatives of the *cis* isomer. The same pair of sesquiterpene alcohols and *trans*-farnesyl pyrophosphate are formed from isopentenyl pyrophosphate plus geranyl pyrophosphate (2-*trans*). Neryl pyrophosphate (2-*cis*) is completely inactive as a precursor of farnesols. Isomerization of *trans*- to *cis*-farnesyl pyrophosphate did not occur in this system.

INTRODUCTION*

SESQUITERPENOIDS are very common in higher plants.[1] Their biosynthesis has been proposed to occur from one or more isomers of FPP.[2,3] The double bonds between C_2 and C_3 and between C_6 and C_7 of farnesol mean that there are four possible geometrical isomers, all of which have been prepared from isomeric nerolidols and characterized.[4] The predominant isomer in essential oils is the 2,6-*trans*,*trans* isomer,[5] and this has also proved to be the structure of FPP (I),[6–8] formed enzymically from MVA. This pyrophosphate is a well known precursor of steroids[9,10] and of polyprenols.[11]

2-*cis*,6-*trans* FPP (II) has been suggested as a possible precursor of sesquiterpene hydrocarbons,[12,13] but it has not been identified in plant or animal tissues. The synthetic compound was inactive in gossypol biosynthesis by enzymes from cotton seedlings,[14] which utilize only the isomers with a *cis* configuration around the C_6–C_7 double bond.

* Abbreviations: MVA = Mevalonic acid; GPP = Geranyl pyrophosphate (2-*trans*); NPP = Neryl pyrophosphate (2-*cis*); IPP = Isopentenyl pyrophosphate; FPP = Farnesyl pyrophosphate.

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Cell free extracts from *Pinus radiata* seedlings form protein bound farnesol and nerolidol from 2-^{14}C MVA.[15] With the use of more refined GLC techniques, it was possible to observe in hexane extracts from enzymic incubates several radioactive peaks in the neighbourhood of carrier 2,6-*trans,trans*-farnesol (III). The presence of these compounds seemed quite intriguing in view of the fact that no free sesquiterpenes have been reported in *Pinus radiata*[16,17] and it was thought worth exploring their identity and their biosynthesis in cell free extracts.

RESULTS AND DISCUSSION

Formation of Sesquiterpene Alcohols from MVA

Incubation of a cell free extract from *Pinus radiata* seedlings with 2-^{14}C MVA in the presence of ATP, Mg^{2+}, Mn^{2+} and 2-mercaptoethanol as previously reported[15] led to the formation of several radioactive 5 C and 10 C prenols. In addition, about 11% of the radioactivity from the 'free lipids' fraction co-chromatographed with carrier 6-*trans*-nerolidol, with 2-*cis*,6-*trans*-farnesol and with 2,6-*trans,trans*-farnesol, the latter being the more abundant of the two primary alcohols. The presence of nerolidol shows that these cell free extracts are able to form sesquiterpenes. The formation of nerolidol has been recently shown to be due to a non-enzymic solvolysis of FPP catalyzed by Mg^{2+} or Mn^{2+}.[18]

Maximal amounts of 15 C prenols were obtained from 2-^{14}C MVA after 120 min. This was about 0·6% of the added *RS*-2^{14}C MVA. The radioactivity in these compounds decreased in longer incubations, presumably through drainage to other products. *Trans*-farnesol disappeared faster than its *cis* isomer.

If, after extracting the 'free lipids' fraction with hexane, the aqueous phase of the enzymic incubate was treated with *E. coli* phosphomonoesterase plus potato apyrase to release alcohols from phosphoryl or pyrophosphoryl esters, then the only radioactive sesquiterpene alcohol recovered was 2,6-*trans,trans*-farnesol. All efforts to isolate a phosphorylated derivative of 2-*cis*,6-*trans*-farnesol by chromatography on DEAE Sephadex or solvent extraction[19] have been so far unsuccessful. The only sesquiterpene alcohol recovered by these procedures, plus enzymic hydrolysis, was 2,6-*trans,trans*-farnesol, derived from FPP.

Condensation of IPP with a 10-C *Pyrophosphate*

The amounts of 2-*cis*,6-*trans*-farnesol obtained from 2-^{14}C MVA were usually too low for further chemical identification. More information about the configuration around the 6,7-double bond could be obtained by preparing it by condensation of IPP with a precursor where the geometry of the corresponding double bond was already known, namely GPP or NPP. Figure 1 shows that 1-^{3}H GPP reacted with IPP to form 2-*cis*,6-*trans* and 2,6-*trans,trans*-farnesol in a ratio of about 1:5. This ratio varied substantially with different enzyme preparations. On the other hand, 1-^{3}H-NPP was completely inactive as a precursor. This finding can be explained only in terms of the formation of two isomeric sesquiterpenes which are identical in their *trans* geometry around the 6,7-double bond derived from the

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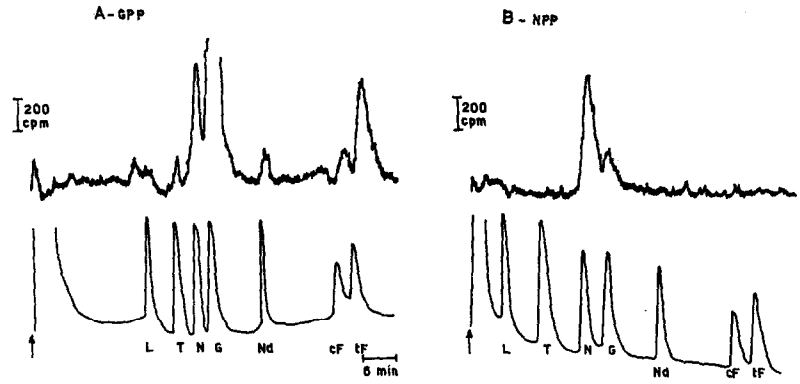

FIG. 1. GAS CHROMATOGRAPHY OF THE HEXANE SOLUBLE REACTION PRODUCTS OF ISOPENTENYL PYROPHOSPHATE PLUS GERANYL OR NERYL PYROPHOSPHATE 1-^{3}H.
Substrates: 0·4 mM IPP plus 0·003 mM 1-^{3}H GPP (A) or NPP (B) (127 000 cpm/tube). Other details in the Experimental. GLC on 2% ethyleneglycoladipate. Gas flow 50 ml/min. Temp.: isothermal 115°C–20 min; raised from 115 to 155° at 8 min. Isothermal at 155° for 35 min or more. Carriers: L = linalool; T = α-terpineol; N = Nerol; G = geraniol; Nd = *trans* nerolidol; cF = 2-*cis*,6-*trans*-farnesol and tF = 2,6-*trans*,*trans*-farnesol. Upper tracing: radioactivity; lower tracing: Carrier peaks.

2,3-double bond of GPP, but differ in their configuration around the new 2,3-double bond (III and IV). This experiment also rules out the formation of 2-*trans*,6-*cis*-farnesol, which would co-chromatograph with the 2-*cis*,6-*trans* isomer,[4] but which would be formed from NPP and not from GPP.

As in the experiments with MVA, the only sesquiterpene pyrophosphate formed was 2,6-*trans*,*trans* FPP. The formation of farnesol through 10 C + 5 C condensation is catalyzed by enzymes known as prenyl synthetases. The enzyme from liver or from pumpkin fruit[20,21] discriminates stringently between GPP and NPP as the *Pinus* enzyme does. All the data published so far[20,21] show that these enzymes form products differing between them in one 5-C unit (homogeraniols and homofarnesols), but no evidence has been presented that new *cis* double bonds may be formed. The synthetase activity was present in the soluble fraction, since the sediment from centrifugation at 104 000 *g* was inactive.

Absence of trans-cis *Isomerization of FPP*

The experiment shown in Fig. 1 also proves that a *cis-trans* isomerization of the 10 C pyrophosphates is very unlikely, as previously shown for orange rind enzymes.[22] Should isomerization have occurred, then NPP should also have been an active precursor of both farnesols.

To obtain further evidence of the absence of isomerization of pyrophosphates, 1-^{3}H FPP, containing about 20% of the *cis* isomer[18] was incubated with the *Pinus* enzyme. After extracting the 'free lipids' fraction alcohols were released from pyrophosphates in the aqueous phase by enzymic hydrolysis and were extracted with hexane. GLC showed the presence of an unaltered ratio of the two farnesols: 20% of the 2-*cis*,6-*trans* and 80% of

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the 2,6-*trans,trans* isomer. This experiment, and analogous evidence obtained for GPP and NPP[18] seems to exclude for formation of *cis* isomers by an isomerization of the *trans* pyrophosphates.

Derivatives of the Two Farnesols

Figure 2 shows the coincidence of carrier peaks and radioactivity of acetates and trimethylsilyl ethers of the two farnesols formed biosynthetically from GPP + IPP. It may be noted that the aldehyde 2,6-*trans,trans*-farnesal, which has the same retention volume as 2-*cis*,6-*trans*-farnesol on ethyleneglycol adipate, has a retention volume very different from the silyl derivatives or acetates on the SE 30 column. Collection and rechromatography of the silyl derivatives (Fig. 3, insert) gives roughly the same pattern of distribution of *cis* and *trans* silyl ethers, which excludes a rearrangement in the gas chromatograph.

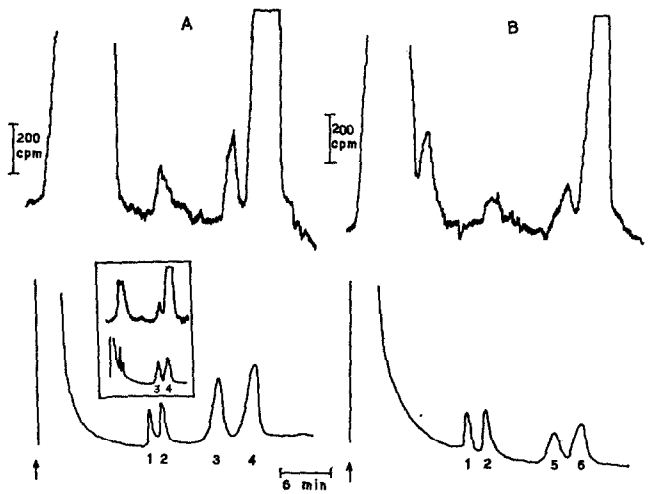

FIG. 2. GAS CHROMATOGRAPHY OF SILYL DERIVATIVES AND ACETATES OF ISOMERIC FARNESOLS.

Enzymic reaction as in Fig. 1. The hexane soluble fraction was treated (A) with bis (trimethylsilyl) acetamide or (B) with Ac_2O. GLC on 3% SE 30. Gas flow 60 ml/min; temp. 160°. Carriers: (1) 2-*cis*,6-*trans*-farnesal; (2) 2,6-*trans,trans*-farnesal; (3) trimethylsilyl ether of 2-*cis*,6-*trans*-farnesol; (4) trimethylsilyl ether of 2,6-*trans,trans*-farnesol; (5) 2-*cis*,6-*trans* farnesyl acetate; (6) 2,6-*trans,trans* farnesyl acetate. Upper tracings: Radioactivity; Lower tracings: Carrier peaks. Insert: Both silyl derivatives were collected in a cooled U tube and reinjected into the same column. The early radioactivity appearing in silyl derivatives and acetates corresponds to the silyl ethers or acetates of geraniol and nerolidol.

The results show that cell free extracts from *P. radiata* form, in addition to FPP and 2,6-*trans,trans*-farnesol, the isomeric 2-*cis*,6-*trans*-farnesol either from MVA or from GPP + IPP. This is to our knowledge, the first report of the biosynthesis of the 2-*cis*,6-*trans* isomer of farnesol.

Two biogenetic alternatives are outlined in Fig. 3. The pathway via reactions 2 and 4 assumes stereospecific synthesis of an enzyme bound *cis* FPP (II), which would be followed by hydrolytic release of the corresponding farnesol. This alternative is consistent with the evidence obtained for the stereospecific synthesis of rubber[8] and of polyprenols,[23] and may

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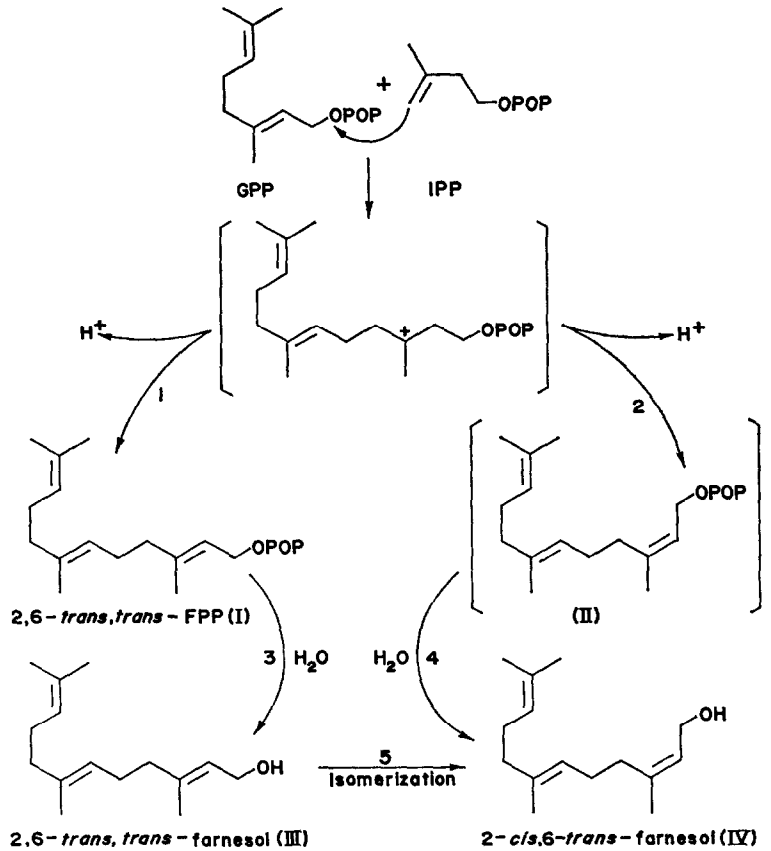

FIG. 3. ALTERNATIVE BIOGENETIC PATHWAYS FOR THE FORMATION OF ISOMERIC FARNESOLS.

explain our negative findings concerning the isomerization of free FPP, GPP or NPP as well as the absence of a free *cis* isomer of FPP. The pathway via reactions 1, 3 and 5 assumes the synthesis of *trans* FPP only (I), followed after hydrolysis by an isomerization of the alcohols, a process for which some evidence has been found in peppermint[24] and other plants.[25] Experiments are in progress to investigate these alternatives in *Pinus* and *Citrus*.

Independently of the mechanism of formation, the geometry of 2-*cis*,6-*trans* farnesol shows a structural relationship with abscisic acid, whose presence has been reported in conifers.[26] The configuration around the 2,3-double bond in this sesquiterpene phytohormone has been established to be *cis*.[27] Although experiments with stereospecifically labeled 4-^{3}H MVA[28] strongly point to *trans,trans* FPP as a precursor, there must be at some stage an intermediate with a 2-*cis* configuration. The isomeric farnesol described in this communication might be worth further consideration.

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EXPERIMENTAL

Chemicals. Isopentenyl pyrophosphate was prepared by phosphorylation of isopentenol as described for other pyrophosphorylated substrates.[29] *Trans*-nerolidol was a gift from Dr. C. D. Gutsche, Washington University, St. Louis. Farnesol was obtained commercially as a 2:1 mixture of the 2,6-*trans*,*trans* and 2-*cis*,6-*trans* isomers. The two isomers were separated on a Nester-Faust spinning band column[30] and the fractions were identified by IR and by NMR spectroscopy.[4] Standard farnesals were obtained from farnesols by oxidation[31] and identified by IR spectroscopy. Radioactive or standard alcohols were transformed into acetates by treatment with Ac_2O in pyridine and into trimethylsilyl ethers[32] by treatment with bis (trimethylsilyl) acetamide in dimethyl formamide. Alkaline phosphatase from *E. coli* was obtained commercially. Apyrase was prepared from potatoes.[33]

Radiochemicals. All radiochemicals used were obtained from New England Nuclear Corporation, Boston, Massachusetts. *RS* (2-^{14}C) MVA had a specific activity of 4·8–5·9 Ci/mol. 1-^{3}H NPP and 1-^{3}H GPP were prepared by reduction of the corresponding aldehydes, neral and geranial by means of NaB^3H_4.[34] The radioactive alcohols were separated by preparative GLC; they were phosphorylated and the pyrophosphates were separated as described earlier.[35] The final specific radioactivity was of 25 μCi/μmol. Cross contamination was of 10% for NPP and 12% for GPP, as established by analysis of the alcohols resulting from the hydrolysis of the substrates with potato apyrase plus *E. coli* phosphomonoesterase.[15] 1-^{3}H FPP was a gift from Professor G. Popják, U.C.L.A.

Enzyme systems. Soluble enzymes from *Pinus radiata* D.Don seedlings were obtained as already described[15] by grinding 30–50-day-old seedlings with a vol. of 0·1 M Tris-HCl buffer pH 7·4 equivalent to two times the wt of seedlings used. This homogenate was strained through cheesecloth and centrifuged for 90 min at 104 000 *g* at 0°. The supernatant contained between 1·5 and 4 mg of protein per ml, as determined turbidimetrically.[36]

Incubation procedure and methods of analysis. Incubations were carried out in glass stoppered conical tubes for 2 hr at 37° in a total vol. of 2 ml of the following common medium: 50 mM Tris-HCl buffer pH 7·4; 10 mM 2-mercaptoethanol; 5 mM ATP; 1·25 mM $MgCl_2$ and 1–1·5 mg/ml of *Pinus* protein. Other components of the medium were the following: (A) 0·025 mM 2-^{14}C MVA (440 000 cpm/tube); (B) 0·4 mM IPP plus 0·003 mM 1-^{3}H GPP or 1-^{3}H NPP (127 000 cpm/tube); or (C) 0·01 mM 1-^{3}H FPP (93 000 cpm/tube). The presence of ATP is not required by prenyl synthetase, but we found that it improved the yield of farnesols. The enzymic reaction was stopped by heating for 3 min to 100°. Experiments with boiled extract were always provided as controls. After stopping the enzymic reaction, the aqueous phase was extracted with an equivalent volume of *n*-hexane. This procedure extracts mainly alcohols and hydrocarbons formed from the water soluble substrates.[15] A second extraction yielded less than 5% additional radioactivity, and was not deemed necessary. We shall call this fraction henceforth 'free lipids'. Prenols were released from pyrophosphates by treatment with apyrase plus phosphomonoesterase.[15] The total radioactivity of these hexane extracts was measured in an aliquot by conventional beta scintillation spectrometry.[17]

Gas chromatography. Carriers (100–300 μg) were added to the hexane extract and an aliquot therefrom was injected into a gas chromatograph equipped with a thermal conductivity detector at 190°. Carrier gas: He; Injector temp. 180°. Column temperature and gas flow are detailed in the figure legends. The columns used were 0·635 cm dia., stainless steel. Alcohols were separated on 250 cm columns packed with Chromosorb W 60–80 mesh and previously washed with base.[4] The liquid phase was 2% ethyleneglycol adipate. Silylated derivatives and acetates were separated on a 150 cm column of 100–120 mesh Varaport No. 30 coated with 3% silicone rubber SE 30. The effluent from the gas chromatograph was introduced, directly[22] into a heated proportional radioactivity counter (Biospan 4998, Nuclear Chicago, Des Plaines, Ill. U.S.A.). Carrier peaks and radioactivity were recorded simultaneously in a two channel instrument area. When necessary, radioactivity was estimated from peak area.

Acknowledgements—This research was financially supported by grants from CONICYT (Chile) and from C.I.C., Universidad de Chile. Use of facilities of the University of California and consultation with Professor C. A. Bunton and C. A. West was made possible through an Exchange Program funded by the Ford Foundation; Mr. D. Hachey, U.C. Santa Barbara, Dept. of Chemistry performed the NMR spectroscopy; Professor S. Atala, University of Chile, Dep. of Organic Chemistry assisted us with the IR analyses; G. Jacob held a training fellowship from CONICYT (Chile); R. Pont-Lezica held an O.A.S. Post-doctoral fellowship while being a staff member of Universidad de Cuyo, Mendoza, Argentina.

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Key Word Index—Pinus radiata; Pinaceae; biosynthesis; prenyl synthetase sesquiterpenes; isomeric farnesols.