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BIOSYNTHESIS OF SESQUITERPENE ALCOHOLS AND ALDEHYDES BY CELL FREE EXTRACTS FROM ORANGE FLAVEDO

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Abstract—Water soluble enzymes obtained from orange flavedo form two isomeric farnesols from 1-^{3}H labeled geranylpyrophosphate plus isopentenylpyrophosphate. The *cis* isomer, nerylpyrophosphate was not a substrate. Isomerization of farnesylpyrophosphates was not observed. Two isomeric aldehydes, 2,6-*trans*-*trans*-farnesal and 2-*cis*-6-*trans* farnesal were also identified. The order of appearance of these products in the presence of NAD^+ was *trans*-farnesol, *trans*-farnesal, *cis*-farnesal and *cis*-farnesol. A redox scheme for the isomerization of farnesols is proposed, and the mechanism of biosynthesis of isomeric prenylpyrophosphates in the absence of their isomerization is discussed.

INTRODUCTION*

SESQUITERPENES are formed from mevalonic acid by animal tissues and yeast[1,2] as intermediates in the biosynthesis of steroids. In higher plants sesquiterpenoids are not only precursors of steroids, but of many other natural products.[3,4]

Cell free extracts obtained from *Pinus radiata* seedlings condense 1-^{3}H GPP with IPP to form two isomers of farnesol: 2-*cis*-6-*trans* and 2,6-*trans*-*trans*-farnesol.[5] In some of these experiments we observed a radioactive peak in GLC, which emerged with carrier sesquiterpene aldehydes. This observation was not consistently reproducible with the *Pinus* enzyme system.

Cell free extracts obtained from orange flavedo[6] incorporated more ^{14}C from 2-^{14}C MVA into C_{15} compounds than the *Pinus* enzyme system. The formation of a radioactive compound which emerged prior to *cis*-FOH associated with carrier sesquiterpene aldehydes was consistently observed. Considering that aldehydes have been proposed as intermediates in the isomerization of monoterpenoid alcohols,[7] and that they would complete an interconversion scheme proposed for farnesols,[5] it was decided to explore the formation of sesquiterpene aldehydes by cell free extracts from *Citrus sinensis* flavedo, which is a

* Abbreviations: MVA—Mevalonic acid; IPP—Isopentenylpyrophosphate; GPP—Geranylpyrophosphate (2 *trans*); NPP—Nerylpyrophosphate (2 *cis*); *cis*-FPP or *cis*-FOH—2-*cis*-6-*trans*-farnesylpyrophosphate or farnesol; *trans*-FPP or *trans*-FOH—2,6-*trans*-*trans*-farnesylpyrophosphate or farnesol.

[1] G. POPJAK and J. W. CORNFORTH, *Advan. Enzymol.* **22**, 281 (1960).
[2] F. LYNEN, H. EGGERER, U. HENNING and I. KESSEL, *Angew. Chem.* **70**, 738 (1958).
[3] M. D. SUTHERLAND and R. J. PARK, in *Terpenoids in Plants* (edited by J. B. PRIDHAM), p. 147, Academic Press, New York (1967).
[4] W. PARKER, J. S. ROBERTS and R. RAMAGE, *Q. Rev. Chem. Soc.* **21**, 331 (1967).
[5] G. JACOB, E. CARDEMIL, L. CHAYET, R. TELLEZ, R. PONT-LEZICA and O. CORI, *Phytochem.* **11**, 1683 (1972).
[6] C. GEORGE-NASCIMENTO and O. CORI, *Phytochem.* **10**, 1803 (1971).
[7] D. V. BANTHORPE, G. N. J. LE PATOUREL and M. J. O. FRANCIS, *J. Chem. Soc.* to be published.

notoriously better at synthesizing C_{15} compounds than the *Pinus* enzyme system. A sesquiterpene aldehyde could also be considered a possible precursor in the biosynthesis of abscisic acid, which has been found in lemon rind.[8]

RESULTS

Formation of C_{15} Prenols from MVA

As described earlier for *Pinus radiata* seedlings,[9,10] a cell free extract obtained from orange flavedo (exocarpium) transforms about 15% of the ($^3H_5$) RS MVA used as substrate into hexane soluble compounds. Besides C_5 and C_{10} prenols previously reported,[6] other radioactive peaks obtained in GLC emerged with carrier *trans*-nerolidol, *cis*-FOH and *trans*-FOH. The latter contains five times or more radioactivity than its *cis* isomer. In 2-hr experiments, about 10% of the radioactivity added as RS MVA was recovered in sesquiterpene alcohols. This percentage as well as the *trans/cis* ratio varies greatly from one variety of oranges to the other, and there exist also seasonal variations. Comparable results have been obtained using a similar cell free extract from *Citrus limonum*.

ATP, SH groups and Mg^{2+} or Mn^{2+} were required for the formation of sesquiterpene alcohols from MVA. In time course experiments, it was established that farnesols appeared after 90 min. After 180 min, the radioactive peaks corresponding to the two farnesols tended to decrease. A suspension of the pellet obtained after centrifugation of the strained homogenate at 47 000 *g* was completely inactive, and addition of this suspension to the supernatant extract did not change the results.

Formation of C_{15} Compounds from C_{10} Precursors

A cell free extract from orange rind formed two radioactive compounds from 1-^{3}H-GPP + IPP, which emerged with carrier *cis*-FOH and *trans*-FOH in GLC. 1-^{3}H-NPP was completely inactive as a substrate. Small amounts of radioactive *trans*-nerolidol were observed. Acetylation or silylation of the hexane extracts produced radioactive derivatives in roughly the same proportion as the two farnesols. Furthermore, the latter treatment produced two new peaks which co-chromatographed with the corresponding aldehydes 2-*cis*-6-*trans*-farnesal and 2,6-*trans*-*trans*-farnesal. Silylation, which does not affect the aldehydes, but substantially changes the retention volume of the alcohols, allows a good separation of these two groups of compounds. This procedure was later used in time course experiments.

Identification of Aldehydes

TLC of the hexane extract from an incubation mixture, using 1-^{3}H GPP + IPP as substrates showed, upon treatment with 2,4-dinitrophenylhydrazine spray, two incompletely resolved radioactive spots, which contained about 5% of the radioactivity of the extract and were coincident with the two carrier farnesals. Geraniol and the *cis* and *trans* farnesols, which were not resolved by this solvent system, contained about 90% of the radioactivity recovered.

Radioactive semicarbazone and 2,4-dinitrophenylhydrazone prepared from radioactive aldehydes co-chromatographed with the corresponding standard compounds. Rechromatography of the dinitrophenylhydrazone spot eluted with acetone gave the same result. The

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[9] E. BEYTÍA, P. VALENZUELA and O. CORI, *Arch. Biochem. Biophys.* **129**, 346 (1969).
[10] C. GEORGE-NASCIMENTO, R. PONT-LEZICA and O. CORI, *Biochem. Biophys. Res. Commun.* **45**, 119 (1971).

semicarbazones on TLC in chloroform coincided with standard compounds and radioactivity, with R_f 0·08.

Time Course of Product Formation

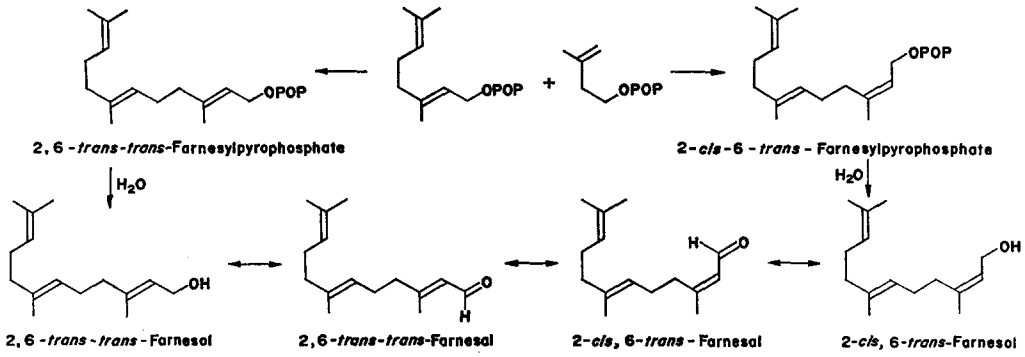
Figure 1 shows that the order of appearance of hexane soluble products of the condensation of 1-^{3}H GPP + IPP was *trans*-FOH, followed by 2,6-*trans*-*trans*-farnesal, 2-*cis*-6-*trans*-farnesal and finally *cis*-FOH. In the absence of added NAD^+, the sequence of product formation was similar but the appearance of aldehydes was somewhat retarded, and little or no *cis*-FOH was observed. Addition of 4 mM aniline to the incubation medium completely suppressed the formation of farnesals and of *cis*-FOH without affecting *trans*-FOH.

Absence of Isomerization of FPP

If 1-^{3}H-GPP + IPP were replaced by 90 000 cpm of 1-^{3}H FPP, the only products recovered were *cis*-FOH and *trans*-FOH, in the same proportion as in the original substrate. No radioactive aldehydes could be detected. The same proportion of *cis*- and *trans*-FOH was detected in the remaining substrate after hydrolysis with phosphomonoesterase plus apyrase.

DISCUSSION

The results described here for a soluble enzyme system from *Citrus sinensis* can be compared with those obtained previously[5] with *Pinus radiata* enzymes. Both enzyme systems form two sesquiterpene alcohols either from MVA or by condensation of GPP plus IPP. The GLC behaviour of the alcohols, of their acetates and of their trimethylsilyl ethers permits their identification as 2-*cis*-6-*trans*-farnesol and 2,6-*trans*-*trans*-farnesol in both species. Nerolidol, which appears in both systems is probably a solvolysis artefact.[10]



SCHEME 1. POSSIBLE PATHWAY OF A REDOX CONVERSION OF ISOMERIC FARNESOLS.

The *Citrus* system synthesizes hexane-soluble compounds which form radioactive semicarbazones and 2,4-dinitrophenylhydrazones that co-chromatograph in TLC with the derivatives of standard farnesals. GLC analysis of the hexane-soluble compounds allows their identification as two isomeric aldehydes, 2,6-*trans*-*trans*-farnesal and 2-*cis*-6-*trans*-farnesal (Scheme 1), by comparison with synthetic carrier compounds. Farnesals have not been reported in orange peel oil[11] or juice.[12] They could be intermediates in the biosynthesis of abscisic acid[8] which has a *cis* 2, 3 double bond.

[11] M. G. MOSHONAS and E. D. LUND, *J. Food Sci.* **34**, 502 (1969).
[12] H. L. DINSMORE and S. NAGY, *Agric. Food Chem.* **19**, 517 (1971).

The identification of sesquiterpene aldehydes is consistent with preliminary tracer studies on the formation of geraniol, nerol and their glucosides in *Rosa dilecta*.[13] Aldehydes may be of significance in the still unresolved problem, whether the formation of isomeric *cis* and *trans* terpenoids is due to a stereospecific synthesis or to an isomerization process.[5,14]

Present as well as previous evidence[5,6] has been consistently negative whenever we have tried to study the isomerization of GPP, NPP or the farnesylpyrophosphates *in vitro*. NPP, the *cis*-C_{10} pyrophosphate cannot substitute its *trans* isomer, GPP as a precursor of farnesols in the *Citrus* or *Pinus* system.[5] This is strong evidence against the *cis-trans* isomerization of pyrophosphates.

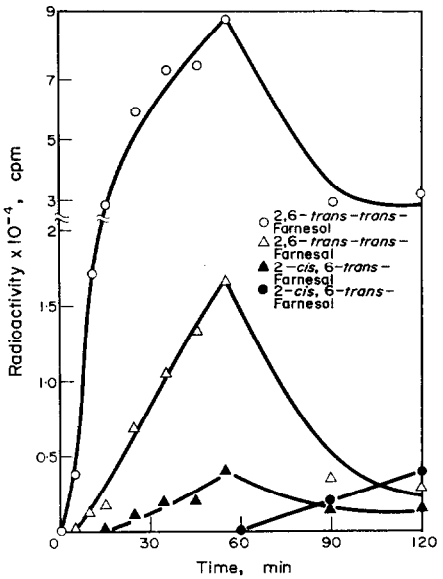


FIG. 1. TIME COURSE EXPERIMENT OF THE APPEARANCE OF FARNESOLS AND FARNESALS. Substrates: 0·4 mM IPP plus 0·003 mM GPP. Total volume 10 ml; Radioactivity 303·400 cpm/ml. Other conditions as in the Experimental. At the stated time intervals aliquot samples were taken and radioactive compounds were extracted directly with hexane containing carrier farnesols and farnesals. The extract was treated with bis (trimethylsilyl) acetamide and analyzed by GLC. Radioactivity was estimated from peak surface. It is expressed in cpm per ml of incubate.

The situation may be quite different for the alcohols, and the participation of aldehydes may be a clue to the process, Figure 1 indicates a pathway for an eventual isomerization of farnesols, with the aldehydes as intermediates. It is based on the order of appearance of *cis* and *trans* alcohols and aldehydes. A redox mechanism in the isomerization process is suggested by the fact that NAD^+ favours the appearance of the *cis* compounds. Aniline, which forms Schiff bases[15] suppresses the recovery of aldehydes and of *cis*-FOH in the hexane extract. Allylic aldehydes (Scheme 1) having a conjugated system could be quite sensitive to isomerization by physical or chemical agents or by enzymes.[16] There is preliminary evidence of an interconversion of geranial to neral catalyzed by SH groups.[17]

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[15] D. GUTSCHE, *The Chemistry of Carbonyl Compounds*, p. 50, Prentice Hall, Englewood Cliffs (1967).
[16] G. WALD, in *Life and Light* (edited by W. D. MCELROY and B. GLASS), p. 724, Johns Hopkins Press, Baltimore (1961).
[17] P. J. DUNPHY, personal communication.

A geraniol dehydrogenase has been described in orange vesicles[18] and in rose petals[17] and we have preliminary evidence in orange flavedo of a farnesol dehydrogenase linked to NAD^+ and $NADP^+$. Formation of oxidized intermediates has been shown to occur in the epimerization of UDP-glucose to UDP-galactose[19] through enzyme bound 4-carbonyl intermediates[20] and NAD^+ or NADH.[21]

Trans-prenylpyrophosphates could still be converted indirectly into their *cis* isomers after enzymic hydrolysis.[6,9,17] The alcohols resulting from this hydrolysis could then be isomerized through a redox process (Scheme 1) to the corresponding *cis* isomers, and these could be in turn rephosphorylated by prenyl kinases described in higher plants.[22-24] This process would require two moles of ATP per mol of pyrophosphate isomerized. This process could be important *in vivo*, but we have not found any evidence for it *in vitro*. Reported utilization of both *cis* and *trans* C_{10} or C_{15} pyrophosphates into gossypol by a cell free extract from cotton seed[25] occurred in the presence of ATP.

Experiments with stereospecifically labeled 4-^{3}H MVA performed in roses[26] and in *Pelargonium*[7] show that the pro-S proton from MVA is eliminated in the formation of nerol and of geraniol, as well as of their glucosides. This has been interpreted as an evidence of an isomerization of GPP to NPP.[7,14,26] We feel that these findings have to be viewed in conjunction with the absence of observable isomerization of the pyrophosphates.

Soluble enzymes from *Pinus* and *Citrus*[27] eliminate the pro-S proton from stereospecifically labeled 4-^{3}H MVA in the formation of GPP, NPP and of both FPP. These results agree with those of Banthorpe's group,[7,14,26] but they cannot be interpreted as due to an isomerization of the pyrophosphates, in view of the negative findings reported in this paper and elsewhere.[5,6] Since rubber[28] and polyprenols[29] are the only examples of *cis*-isoprenoid biosynthesis to have been studied, it has generally been assumed that the biogenesis of a *cis* double bond is always linked to the elimination of the pro-R proton of MVA and vice versa. There is, however, no chemical reason for this assumption, since there is free rotation around the 2, 3 single bond in the carbonium ion[30] formed by prenyl transferase. The stereospecificity is conferred by the enzymes[31] and it is quite conceivable that enzymes with different spatial orientations may eliminate the same proton (pro-S) in the biosynthesis of *cis* or *trans* double bonds, if the two substrates of prenyl transferase (IPP and an allylic pyrophosphate) are properly oriented.[27]

EXPERIMENTAL

Radiochemicals. NaB $^3H_4$ and 3 (RS), 5-^{3}H MVA were obtained from New England Nuclear Corporation, Boston, Mass., with specific radioactivities of 500 and 100 μCi/μmole respectively. Preparation of

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[31] G. Popjak, in *The Enzymes* (edited by P. D. Boyer), 3rd Edn, p. 116, Academic Press, New York (1970).

1-^{3}H NPP and 1-^{3}H GPP by reduction of the aldehydes and phosphorylation of the alcohols and assay for radiochemical purity has been described earlier.[5] Cross contamination of NPP was of 10% and of GPP was 12%. 1-^{3}H FPP was a gift from Professor G. Popjak, UCLA. It was a mixture of 20% 2-*cis*-6-*trans*-FPP and 80% 2,6-*trans*-*trans*-FPP, as assayed by GLC of the alcohols liberated by enzymic hydrolysis.[9]

Chemicals. IPP was prepared by phosphorylation of isopentenol.[32] Farnesol was obtained commercially, as a mixture containing 67% of the 2,6-*trans*-*trans* isomer and 30% of the 2-*cis*-6-*trans* isomer. The isomers were separated by distillation and identified by NMR spectroscopy.[5] Standard farnesals were obtained from farnesols by oxidation[33] and identified by IR spectroscopy. Radioactive and standard alcohols were transformed into acetates by treatment with Ac_2O in pyridine and into trimethylsilyl ethers by treatment with bis-(trimethylsilyl)-acetamide,[5] dinitrophenylhydrazones or semicarbazones by treatment with 2,4-dinitrophenylhydrazine or semicarbazide–HCl plus NaOAc in MeOH.[34] The standard derivatives were identified by their IR spectra. Alkaline phosphatase from *E. coli* was obtained commerically. Apyrase was prepared from potatoes.[35]

Enzyme system. Soluble enzymes from the flavedo (exocarpium) of *Citrus sinensis* 'Chilean variety' were obtained as already described[6] by grinding the tissue with a vol. of 0·1 M Tris-HCl buffer pH 7·4 equivalent to 1·5 × the wt of flavedo used. This homogenate was strained through cheesecloth and centrifuged for 30 min at 23 500 *g* at 0°. The supernatant contained between 1·5 and 3 mg of protein per ml, as determinated 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 medium: 50 mM Tris–HCl buffer pH 7·4; 10. mM 2-mercaptoethanol; 5 mM ATP; 1·25 mM $MgCl_2$; $1{\cdot}25 \times 10^{-2}$ mM NAD^+ and 1–2 mg/ml of orange protein. Substrates, as indicated in the corresponding experiments, were one of the following: (1) $3{\cdot}8 \times 10^{-3}$ mM 5-^{3}H MVA ($1{\cdot}4 \times 10^6$ total cpm); (2) 0·4 mM IPP plus 0·003 mM 1-^{3}H GPP or 1-^{3}H NPP (127 000 cpm/tube); (3) 0·01 mM 1-^{3}H FPP (93 000 cpm/tube). The presence of ATP is not absolutely required by prenyl synthetase, but it improved the yield of farnesols and farnesals. The addition of NAD^+ was found to improve the yield of *cis*-sesquiterpenes. The enzymic reaction was stopped by heating for 3 min to 100°. Experiments with boiled extracts 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, aldehydes and hydrocarbons formed from the water soluble substrate.[9] Prenols were released from the corresponding pyrophosphates by treatment with apyrase plus phosphomonoesterase.[9] Substrates were assayed for radiochemical purity by this procedure followed by GLC of the alcohols. The total radioactivity of the hexane extracts was measured in an aliquot by conventional beta scintillation spectrometry.[9]

Gas chromatography. 100–300 μg of carrier alcohols and aldehydes were added to the hexane extract and an aliquot therefrom was injected into a Varian 1800 gas chromatograph equipped with a thermal conductivity detector at 260°. Carrier gas: helium; injector temp.: 180°. Column temp. was 160–170° and gas flow was 60 ml/min. The columns used were 0·635 cm dia, stainless steel. Alcohols were separated on a 250 cm long column of Chromosorb W, 60–80 mesh, coated with 2% ethylene glycol adipate.[5] Silylated derivatives and acetates were separated on a 150 cm column of 100/120 mesh Varaport No. 30 coated with 3% SE-30. The effluent from the gas chromatograph was introduced directly into a heated proportional radioactivity counter. Carrier peaks and radioactivity were recorded simultaneously in a two channel instrument (Varian 20). When necessary, radioactivity was estimated by cutting out and weighing the peaks.[6]

TLC. TLC was performed on the hexane extracts containing carrier alcohols or aldehydes, using silica gel G plates (250 μm) activated for 90 min at 110°. For radioactivity measurement, the silica gel was scraped out in 1 cm wide bands and put into 5 ml of scintillation solution. This gave 50% counting efficiency. Aldehydes were detected by spraying the plates with 2,4-dinitrophenylhydrazine;[37] they were then exposed to I_2 vapors in order to detect the alcohols. The farnesyl semicarbazones were located by their fluorescence when excited with a wavelength of 260 nm. The solvents used for TLC were EtOAc–C_6H_6–hexane (12:25:63) for alcohols and aldehydes, $CHCl_3$–Me_2CO (10:1) for semicarbazones and C_6H_6–hexane (3:1) for the 2,4-dinitrophenylhydrazones.

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fellowship while on leave from the Universidad de Cuyo, Mendoza. L.C. holds a training fellowship from CONICYT, Chile. The synthesis of labeled substrates was performed by Emilio Cardemil. We are indebted to Dr. D. V. Banthorpe, University College, London, for making his results available to us prior to publication, and to Dr. P. J. Dunphy, from Unilever, for informing us about his unpublished results.