Studies in Terpenoid Biosynthesis. Part 19.[1] Formation of Pimara-8(9),15-diene by *Trichothecium roseum*

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Pimara-8(9),15-diene has been isolated from *Trichothecium roseum*. Its biosynthesis *via* labda-8(17),13-dien-15-yl pyrophosphate is described.

In terpenoid biosynthesis many cyclization stages commonly precede the oxidative steps. There exist cyclic hydrocarbons that are the progenitors of more highly oxidized terpenoids. For example trichodiene is the parent of the trichothecanes [2,3] and *ent*-kaurene of the gibberellins.[4] In the biosynthesis of the rosane diterpenoids of *Trichothecium roseum*, there is a gap in our knowledge of the processes occurring between labda-8(17),13-dien-15-yl pyrophosphate (1) and the tricyclic lactone deoxyrosenonolactone (2).[5-7] In this tricarbocyclic diterpenoid, the 19,10-lactone ring lies on the same face (β) of the molecule as the methyl group which has migrated from C-10 to C-9. This suggests that the processes of cyclization and rearrangement on the one hand and of lactonization on the other, are not concerted. We have shown that the C-9 hydrogen atom in the bicyclic precursor has migrated to C-8 in rosenonolactone (3), and that the mevalonoid hydrogen atoms are retained at C-1, -5, and -6, thus excluding cyclic olefinic intermediates which involve these centres in the biosynthesis. In an effort to clarify the biosynthesis we have examined the diterpene hydrocarbons of *T. roseum* both in the intact fungus and in a cell-free system.

Examination of the hydrocarbon fraction from the

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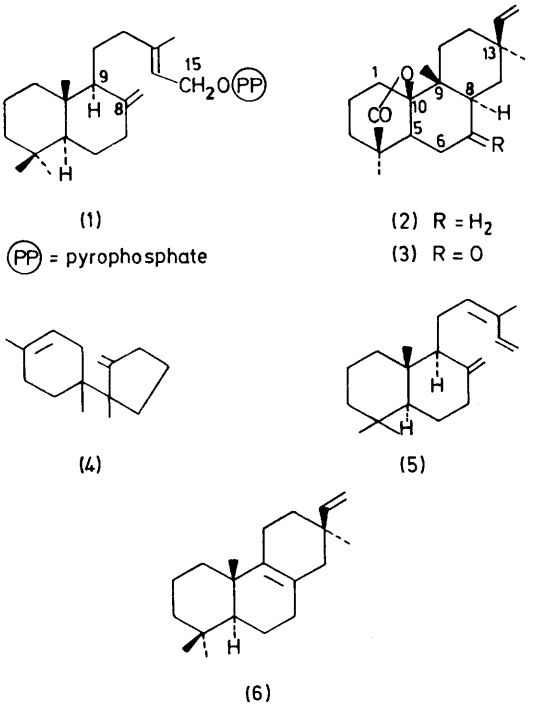
mycelium of *T. roseum* by g.l.c.–mass spectrometry revealed the presence of squalene, trichodiene (4), and a diterpene hydrocarbon, $C_{20}H_{32}$ (M^+ 272). [15-^{3}H]-Labda-8(17),13-dien-15-yl pyrophosphate (1) [6] was fed to the fungus. The hydrocarbon fraction was isolated and analysed by g.l.c. The retention time of the hydrocarbon was accurately standardized. The flame ionization detector was extinguished and the effluent from the column was collected in liquid scintillation vials and counted. The diterpene hydrocarbon showed an incorporation of 4.3%. On some occasions biformene (5) was also isolated.[8]

A cell-free enzyme system was then obtained from *T. roseum* which mediated the formation of the hydrocarbon (*cf.* ref. 3). The strain which was used was one which was not, at the time, producing substantial quantities of trichothecenes. The filtered mycelium from a three-day old culture was resuspended in 0.1M-phosphate buffer (pH 7.0) containing cysteine (10mM) and then disrupted in a French press. The cell debris was removed by centrifugation, and protamine sulphate was added to precipitate the microsomes and to remove the squalene synthetase. This soluble enzyme preparation when supplemented with magnesium chloride, manganese chloride, and NADH, utilized [1-^{14}C]isopentenyl pyrophosphate and [15-^{3}H]labda-8(17),13-dien-15-yl pyrophosphate but not mevalonate, as substrates



(1)

(PP) = pyrophosphate

(2) R = H_2

(3) R = O

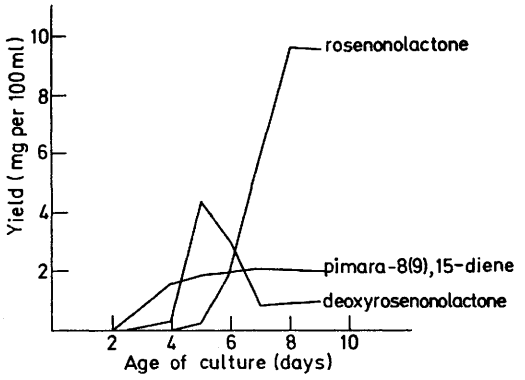
(4)

(5)

(6)

for the formation of the hydrocarbon (7.0 and 9.2% incorporation, respectively).

When an incubation of this enzyme system with [1-^{14}C]isopentenyl pyrophosphate was treated with alkaline phosphatase prior to extraction, the following labelled terpenoid alcohols were also obtained: geraniol (0.20% incorporation), farnesol (2-*trans*,6-*trans*) (0.76%),



farnesol (2-*cis*,6-*trans*) (0.23%), nerolidol (1.10%), labda-8(17),13-dien-15-ol (*cis*) (0.01%), and labda-8(17),13-dien-15-ol (*trans*) (0.51%). Hence the system was also producing the labdadienyl pyrophosphate (1). However when the [^{14}C]diterpene hydrocarbon produced by the cell-free system was incubated with the intact culture, no incorporation into rosenonolactone (3) was detected under conditions in which the [15-^{3}H]labda-8(17),13-dien-15-yl pyrophosphate (1) was incorporated into deoxyrosenonolactone (2) (0.28%), rosenololactone (0.05%), and rosenonolactone (3) (0.14%). A time-course study (see Figure) showed that the diterpene was formed at the same time as deoxyrosenonolactone. However the concentration of the latter went through a maximum prior to the formation of rosenonolactone as opposed to that of the hydrocarbon.

The mass spectrum of the hydrocarbon revealed significant ions at *m/e* 257, 187, 175, and 161 which, in the light of this non-incorporation into the rosanes, suggested that the hydrocarbon was pimara-8(9),15-diene (6).[9] Authentic samples of pimara-8(9);15-diene (6) and its 13-epimer, sandaracopimara-8(9),15-diene, were then prepared by the acid-catalysed cyclization [10] of manool. The *T. roseum* hydrocarbon ran coincidently on g.l.c. and t.l.c. and had the same mass spectrum as pimara-8(9),15-diene. Since the rosane diterpenoids retain the C-9 hydrogen atom (at C-8) the lack of incorporation of this hydrocarbon was then not surprising.

Although pimara-8(9),15-diene is not on the biosynthetic pathway to the rosanes, it is possible that these compounds have a common tricyclic intermediate in which the C-8 carbocation arising from the cyclization of the labda-8(17),13-dien-15-yl pyrophosphate may be stabilized by binding to the enzyme surface. Elimination of the C-9 α-hydrogen atom to form the olefin or transfer of this hydrogen atom to C-8 are then alternative reaction pathways on release of the molecule from the β-oriented stabilizing centre.

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EXPERIMENTAL

General experimental details have been described previously.[11] The strains of *Trichothecium roseum* used in this work have been obtained by repeated sub-culturing of IFO 6157. The fermentations were grown as described previously.[6,7] Mass spectra were obtained with a Pye 104 gas chromatograph connected *via* a membrane separator to an A.E.I. MS30 mass spectrometer equipped with an A.E.I. DS50 data system. A 5 ft 3% Carbowax column operating at 190 °C or a 9 ft 5% FFAP column operating at 220 °C with nitrogen or helium as carrier gas (50 ml min^{-1}) was used for g.l.c. with a Pye GCD gas chromatograph.

Examination of the Hydrocarbon Metabolites of T. roseum.—The mycelium from sixty five-day-old shake cultures (each 100 ml) of *T. roseum* was disrupted by freezing in liquid nitrogen and the metabolites were then extracted with acetone (2 l). The acetone extract was concentrated *in vacuo* to afford an aqueous suspension which was then extracted with ethyl acetate (2 × 250 ml). This extract was dried and evaporated and the residue was adsorbed on silica (15 g) and chromatographed on alumina (150 g). Elution with light petroleum (1 l) gave a mixture of hydrocarbons which was analysed by g.l.c. on Carbowax. Trichodiene had retention time 5 min, squalene 23 min, and pimara-8(9),15-diene 14 min. They were identified by mass spectrometry. Pimara-8(9),15-diene showed *m/e* 272 (20%), 257 (100), 215 (10), 201 (10), 189 (10), 187 (10), 175 (40), 161 (70), 157 (20), 147 (30), 133 (60), 121 (30), 119 (60), 109 (60), 107 (70), and 105 (80).

Incubation of [15-^{3}H]*Labda-8(17),13-dien-15-yl Pyrophosphate with* T. roseum.—The pyrophosphate (8.1×10^7 disint. min^{-1}) [6] in water (0.4 ml) was distributed between two one-day-old cultures of *T. roseum* (100 ml). The fermentations were harvested as above after a further four days. The hydrocarbon fraction (4.6 mg) had an activity of 5.5×10^6 disint. min^{-1} (6.9%). Further elution of the column gave deoxyrosenonolactone, activity 2.25×10^5 disint. min^{-1} (0.28%) and rosenonolactone, activity 1.10×10^5 disint. min^{-1} (0.14%). The hydrocarbon fraction was analysed by g.l.c. The retention time of the pimaradiene was accurately determined in preliminary experiments. The flame in the flame ionization detector was then extinguished and the effluent from the column was, at the appropriate time (14 min), passed into acetone (3 ml) in a liquid scintillation vial cooled in an acetone–solid carbon dioxide bath. The acetone was evaporated off under a stream of nitrogen and scintillation fluid was then added.[11] The sample was then counted. This procedure was repeated for four samples (5.34×10^4 disint. min^{-1}). The efficiency of recovery (5.8%) was established by using [^{14}C]-*ent*-kaurene. The incorporation into the pimara-8(9),15-diene (1 965 disint. min^{-1}) was 4.3%.

Cell-free Preparation from T. roseum.—The mycelium from two three-day-old cultures was filtered, washed with distilled water, and resuspended in 0.1M-phosphate buffer (pH 7.0) containing cysteine (10mM), magnesium chloride (5mM), and manganese chloride (2mM). This suspension was passed through a pre-cooled French press at 4—5 tons applied pressure. The product was centrifuged at 10 000 *g* for 20 min and the supernatant was then treated with 1% protamine sulphate solution, then centrifuged again. The supernatant fraction from this had a protein concentration of 12 mg ml^{-1} and was used in the following incubations. The incubations were carried out in 10 ml stoppered tubes at 30 °C for 3 h. The incubations comprised enzyme preparation (1.5 ml), NADH solution (2M; 0.1 ml) to which was added either [15-^{3}H]labda-8(17),13-dien-15-yl pyrophosphate (0.1 ml; 7.22×10^6 disint. min^{-1}) or [1-^{14}C]isopentenyl pyrophosphate (0.1 ml; 1.05×10^6 disint. min^{-1}). At the end of the incubation, acetone (0.5 ml) was added to each tube and the mixture was extracted with ethyl acetate (2 × 5 ml). The extracts were dried and concentrated and the hydrocarbon material was purified by t.l.c. on silica in hexane–benzene (9 : 1) and on silica–silver nitrate in ethyl acetate–light petroleum (1 : 4). The pimara-8(9),15-diene band, eluted with ethyl acetate, was then counted. The isopentenyl pyrophosphate incubation afforded the pimara-8(9),15-diene with activity 7.45×10^4 disint. min^{-1} (7.1% incorporation) and the labdadienyl pyrophosphate gave the pimara-8(9),15-diene with activity 6.65×10^5 disint. min^{-1} (9.21% incorporation). To demonstrate the purity and identity of the two preparations, the ^{14}C-labelled material (from isopentenyl pyrophosphate) was mixed with the ^{3}H-labelled material (from the labdadienyl pyrophosphate) to afford the pimara-8(9),15-diene with ^{3}H : ^{14}C ratio 13.6 : 1. The hydrocarbon was subjected to consecutive t.l.c. on silica–silver nitrate in the following solvent systems: ethyl acetate–light petroleum (1 : 4), benzene–diethyl ether, and acetone–light petroleum (1 : 24). The ^{3}H : ^{14}C ratio remained within the limits 13.92 : 1 and 13.25 : 1.

Isolation of Terpenoid Alcohols from the Cell-free Preparation.—Two incubations with [1-^{14}C]isopentenyl pyrophosphate (1.05×10^6 disint. min^{-1} each) were adjusted to pH 8.3 with 1.0M-Tris buffer. Alkaline phosphatase (2.0 mg) was added to each solution and the preparation was re-incubated at 30 °C for 1 h. The metabolites were recovered as above and separated into a hydrocarbon (1.58×10^5 disint. min^{-1}; 7.52%) and a polar fraction by t.l.c. on silica in benzene–hexane (1 : 9). The polar metabolites were separated by t.l.c. on silica–silver nitrate in ethyl acetate–light petroleum (2 : 3) to afford the following alcohols, which were identified by co-chromatography with authentic samples: geraniol (4 150 disint. min^{-1}; 0.20%), nerolidol (23 040 disint. min^{-1}; 1.10%), farnesol (2-*cis*,6-*trans*) (4 800 disint. min^{-1}; 0.23%), farnesol (2-*trans*,6-*trans*) (15 900 disint. min^{-1}; 0.76%), labda-8(17),13-dien-15-ol (*cis*) (270 disint. min^{-1}; 0.01%), and labda-8(17),13-dien-15-ol (*trans*) (10 600 disint. min^{-1}; 0.51%).

Incubation of Pimara-8(9),15-diene with T. roseum.—The ^{14}C-labelled hydrocarbon (prepared from [1-^{14}C]isopentenyl pyrophosphate) (5.8×10^5 disint. min^{-1}) in ethanol (0.5 ml) and 10% Tween 80 (0.5 ml) was added to a two-day-old shake culture of *T. roseum*. After a further five days growth, the mycelium was filtered and extracted as above to afford a gum (160 mg; 2.83×10^5 disint. min^{-1}). Rosenonolactone (57 mg), m.p. 214—215°, was obtained by direct crystallization of this gum with ethyl acetate–methanol (1 : 1). The material was recrystallized and showed only background radioactivity.

Time-course Studies.—Ten shake flasks (100 ml) were inoculated with *T. roseum* and harvested at daily intervals. The mycelium was extracted as above and the culture broth was extracted with ethyl acetate (3 × 50 ml). The extracts for each day were combined, dried, and evaporated. The residue was absorbed on silica (10 g) and placed on a column of alumina (30 g). Elution with light petroleum (500 ml) gave a hydrocarbon fraction. The more polar

[11] J. R. Hanson, J. Hawker, and A. F. White, *J.C.S. Perkin I*, 1972, 1892.

metabolites were then eluted with ethyl acetate (500 ml). The solvents were evaporated off and the residues were analysed separately by g.l.c. The hydrocarbons were examined on a 5 ft 3% Carbowax column operating at 220 °C with nitrogen as carrier gas (50 ml min^{-1}). The more polar metabolites were examined on a 5 ft 1% OV-17 column operating at 250 °C with nitrogen as carrier gas (50 ml min^{-1}). Quantities were estimated by comparison of peak heights against standards. The results are given in the Figure.

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