Studies in Terpenoid Biosynthesis. Part XVII.[1] Biosynthesis of the Sesquiterpenoids Cyclonerodiol and Cyclonerotriol

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The labelling patterns of cyclonerodiol and cyclonerotriol, biosynthesized from [4 5-$^{13}C_2$]mevalonic acid, have been determined. The incorporations of [2^3-H_2;2-^{14}C]-, [(4*R*)-4-^{3}H;2-^{14}C]-, and [5-$^3H_2$;2-^{14}C]-mevalonic acid into farnesyl pyrophosphate and cyclonerodiol are described. Nerolidol was not incorporated into these fungal metabolites.

CYCLONERODIOL (3) and cyclonerotriol (4) are examples of an unusual group of sesquiterpenoids which can formally be written [2] as cyclization products of nerolidol (2) rather than of farnesyl pyrophosphate (1). Cyclonerodiol has been isolated from *Trichothecium roseum*,[2] *Gibberella fujikuroi*,[3] and *Fusarium culmorum*.[4] Cyclonerotriol has been isolated [4] from the *Fusarium* species. These metabolites appear to be formed at the expense of other sesquiterpenoid metabolites and at a stage at which the cultures are degenerating. We now report a study of the biosynthesis of these compounds.[5]

In order to establish the sesquiterpenoid nature of these metabolites and the arrangement of the isoprene units, [4,5-$^{13}C_2$]mevalonic acid was fed to *Fusarium culmorum*. The [4,5-$^{13}C_2$]mevalonic acid was prepared by the lithium di-isopropylamide-catalysed condens-

[1] Part XVI, R. Evans, J. R. Hanson, and T. Marten, preceeding paper.

[2] S. Nozoe, M. Goi, and N. Morisaki, *Tetrahedron Letters*, 1970, 1293; 1971, 3701.

[3] B. E. Cross, R. E. Markwell, and J. C. Stewart, *Tetrahedron*, 1971, **27**, 1663.

[4] J. R. Hanson, P. B. Hitchcock, and R. Nyfeler, *J.C.S. Perkin I*, 1975, 1586.

[5] Preliminary communication, R. Evans, J. R. Hanson, and R. Nyfeler, *J.C.S. Chem. Comm.*, 1975, 814.

ation [6] of ethyl [1,2-$^{13}C_2$]acetate (90% enrichment at both centres) with 4,4-dimethoxybutan-2-one. The resulting ester was reduced with lithium aluminium hydride. Hydrolysis of the acetal group and oxidation of the resultant aldehyde with bromine [7] led to [4,5-$^{13}C_2$]-mevalonolactone. This mevalonate, mixed with a trace of [2-^{14}C]mevalonate, was incubated with *F. culmorum* for 18 days. Cyclonerodiol and cyclonerotriol were then isolated. They showed 1.8 and 1.2% incorporation of [^{14}C]mevalonate. Their ^{13}C n.m.r. spectra are tabulated. In the enriched metabolites, the carbon-13 resonances assigned to C(1) and C(2), C(5) and C(6), and C(9) and C(10) were each coupled. This labelling pattern established a sesquiterpenoid origin for the metabolites in which the three isoprene units were incorporated intact.

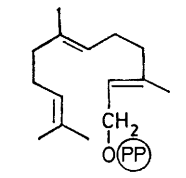
TABLE 1

The ^{13}C n.m.r. spectra of cyclonerodiol and cyclonerotriol (δ in p.p.m. from Me_4Si)

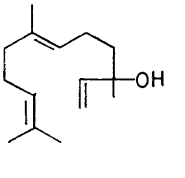
Carbon atom	Cyclonerodiol	*J*/Hz	Cyclonerotriol	*J*/Hz
1	14.6	37	15.4	37
2	44.2	37	45.5	37
3	81.3 [a]		83.0 [a]	
4	41.1 [b]		41.4 [b]	
5	24.3	33	25.2	32
6	54.3	33	55.5	32
7	74.9 [a]		75.4 [a]	
8	40.4 [b]		41.7 [b]	
9	22.7	44	23.3	44
10	124.5	44	127.0	44
11	131.8		135.5	
12	25.7		69.0	
13	24.9		24.6	
14	26.1		26.1	
15	17.7		13.7	

[a,b] These assignments may be interchanged.

The incorporations of [2-$^3H_2$;2-^{14}C]-, [(4*R*)-4-^{3}H;2-^{14}C]-, and [5-$^3H_2$;2-^{14}C]-mevalonic acid into cyclonerodiol and cyclonerotriol by *Trichothecium roseum* and *Fusarium culmorum* are shown in Table 2. The atom ratios are those which would be anticipated from the folding of all-*trans*-farnesyl pyrophosphate predicated by the carbon-13 results. However the [2-$^3H_2$;2-^{14}C]mevalonate results, particularly in the case of the cyclonerodiol obtained from *T. roseum*, revealed the effect of prenyl isomerase in 'washing out' tritium.[8] Correction was made for this effect in two ways. The diterpenoid deoxyrosenonolactone (6) was isolated from the *T. roseum* fermentation. This showed a ^{3}H : ^{14}C ratio of 11.80 : 1 (2.2% incorporation). If this is used as an internal standard with an atom ratio of 8 : 4,[9] the cyclonerodiol then possessed an atom ratio of 6 : 3. Alternatively when the cyclonerotriol from the [2-$^3H_2$;2-^{14}C]mevalonate feed to *F. culmorum* was oxidized to the γ-lactone (8), the ^{3}H : ^{14}C ratio remained at 9.37 : 1 (atom ratio 3.70 : 2) although the specific activity had dropped by a third. Hence the loss of tritium was spread through the molecule as would be expected from the action of prenyl isomerase. Hydroxylation at C(12) could be influenced by an isotope effect leading to a diminished change in the ^{3}H : ^{14}C ratio at this centre. The selective oxidation of C(12) was not successful. Thus oxidation with manganese dioxide to the aldehyde (5) gave material showing ^{3}H : ^{14}C 9.0 : 1, atom ratio 5.3 : 3. Reduction of this sample with

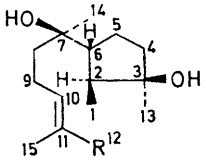


(1)

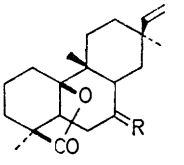


(2)

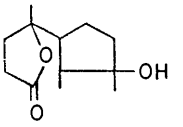
(PP) = pyrophosphate



(3) R = Me
(4) R = CH_2·OH
(5) R = CHO



(6) R = H_2
(7) R = O



(8)

TABLE 2

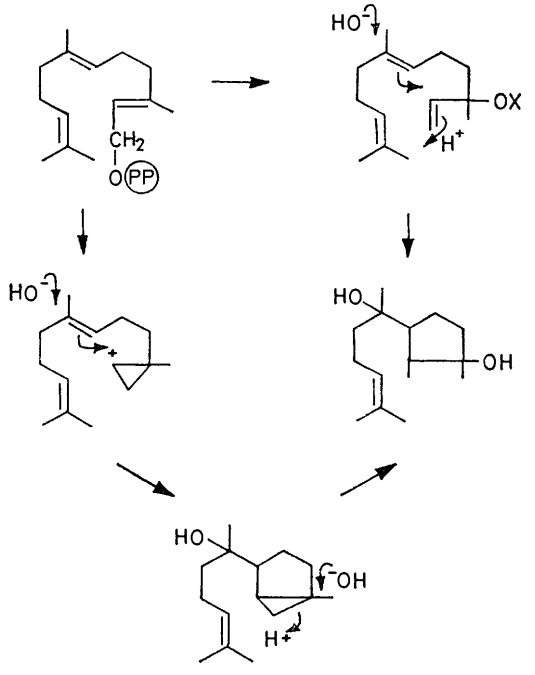
Incorporation of labelled mevalonates into cyclonerodiol and cyclonerotriol

Labels	[2-$^3H_2$;2-^{14}C]	[2-$^3H_2$;2-^{14}C]		[(4*R*)-4-^{3}H;2-^{14}C]	[5-$^3H_2$;2-^{14}C]	[5-$^3H_2$;2-^{14}C]
Fungal system	*T. roseum*	*F. culmorum*		*F. culmorum*	*T. roseum*	*F. culmorum*
Initial MVA ^{3}H : ^{14}C	14.85 : 1	10.11 : 1		6.84 : 1	5.83 : 1	12.35 : 1
Amount ^{14}C fed(μCi)	50	54.2		98.5	50	80
Atom ratio	6 : 3	6 : 3		3 : 3	6 : 3	6 : 3
Metabolite	Diol	Diol	Triol	Triol	Diol	Triol
^{3}H : ^{14}C Ratio	11.75 : 1	9.39 : 1	9.44 : 1	6.82 : 1	5.94 : 1	12.37 : 1
Atom ratio	4.75 : 3	5.57 : 3	5.60 : 3	2.99 : 3	6.12 : 3	6.01 : 3
% Incorporation	3.7	6.4	3.5	6.3	8.1	5.0

[6] R. A. Ellison and P. K. Bhatnagar, *Synthesis*, 1974, 719.
[7] L. Pichat, B. Blagoeur, and J. C. Hardouin, *Bull. Soc. chim. France*, 1968, 4489.
[8] P. W. Holloway and G. Popják, *Biochem. J.*, 1968, **106**, 835.
[9] B. A. Achilladelis and J. R. Hanson, *J. Chem. Soc.* (*C*), 1969, 2010.

sodium borohydride regenerated cyclonerotriol, which was then reoxidized to give material with $^{3}H : ^{14}C$ 8.7 : 1, atom ratio 5.16 : 3. However such oxidations are accompanied by an isotope effect. Attempts to oxidize the aldehyde to a carboxylic acid led to the γ-lactone (8). Nevertheless these results show that the metabolites have retained the expected mevalonoid hydrogen atoms.

all-*trans*-Farnesyl pyrophosphate ($^{3}H : ^{14}C$ 14.41 : 1; 0.83 μCi ^{14}C) was prepared from [2-$^{3}H_2$;2-^{14}C]mevalonic acid by using a pig-liver enzyme system [10] and fed to *Trichothecium roseum*. The cyclonerodiol (2.7% incorporation) had a $^{3}H : ^{14}C$ ratio of 14.42 : 1. Apart from establishing the role of farnesyl pyrophosphate as a precursor, the retention of the $^{3}H : ^{14}C$ ratio in this case showed that the loss of tritium in the [2-$^{3}H_2$;2-^{14}C]-mevalonate experiments was due to the action of prenyl isomerase rather than a post-farnesyl pyrophosphate step. Rosenonolactone (7) was also isolated from this fermentation. It showed a $^{3}H : ^{14}C$ ratio of 9.76 : 1 (1% incorporation) corresponding to an atom ratio of 4.06 : 3. The loss of two tritium labels is associated with the oxidation of C(7) to a carbonyl group.



SCHEME

Cyclonerodiol (3), biosynthetically labelled from [2-^{14}C]mevalonic acid, showed a 15% incorporation into cyclonerotriol (4). DL-Nerolidol (2) was prepared [11] by acidic hydrolysis of [^{14}C]farnesyl pyrophosphate and carefully purified by preparative layer chromatography. However in three separate experiments (two with *F. culmorum* and one with *T. roseum*), it was not incorporated into cyclonerodiol or cyclonerotriol, although these metabolites were produced by the fermentations. Radio-t.l.c. scanning showed that the nerolidol was metabolized. Given the relatively high incorporations of the other precursors, this latter result indicates that free nerolidol was not an intermediate in the biosynthesis. This would not exclude an enzyme-bound form of nerolidol or nerolidol pyrophosphate acting as a substrate for this cyclization. An alternative possibility (see Scheme) involves the direct cyclization of farnesyl pyrophosphate *via* a C(1)–C(3) cyclopropane intermediate. The intervention of a cyclopropyl cation (9) could also account for the isomerization of 2-*trans*-farnesyl pyrophosphate to 2-*cis*-farnesol.[12] The formation of these compounds at a time when the culture is losing its ability to produce other sesquiterpenoids arising from the isomerization of the C(2) double bond, is of interest in this connection. The isomerization involves the loss of a [5-^{3}H]mevalonoid hydrogen atom in the formation of the trichothecenes. It may be significant that the cyclonerodiol from *T. roseum* showed an enrichment of tritium in the [5-$^{3}H_2$]-mevalonate experiment.

EXPERIMENTAL

General experimental details have been described previously.[13] *Fusarium culmorum* (ACC 1731) was grown [4] on Raulin–Thom medium. *Trichothecium roseum* (IFO 6157) was grown as described previously.[14]

Incubation of [4,5-$^{13}C_2$]*Mevalonic Acid*.—The mevalonate (δ_C 35.7 and 66.3, J 34 Hz) (250 mg; 13.4 μCi ^{14}C) was fed to 30 Roux bottles (100 ml) of *F. culmorum* 2 days after inoculation. The metabolites were isolated after a further 18 days and purified by preparative layer chromatography on silica in chloroform–acetone–methanol (15 : 15 : 1) to afford cyclonerodiol (20 mg; 2.7×10^5 disint. min^{-1}; 1.8% incorporation) and cyclonerotriol (12 mg; 1.8×10^5 disint. min^{-1}; 1.2% incorporation). The ^{13}C n.m.r. spectra were determined at 25.15 MHz with spectral width 5 kHz by using 8 192 data points and 55 000 and 56 900 accumulations, respectively. The pulse length was 7 μs at a pulse interval of 1.0 s. Tetramethylsilane was used as internal standard; the cyclonerodiol was examined in [^{2}H]chloroform and the cyclonerotriol in [$^{2}H_4$]methanol. The results are given in Table 1.

Incubations of [^{3}H]*Mevalonates*.—(a) *With* T. roseum. The mevalonates ([2-$^{3}H_2$;2-^{14}C] and [5-$^{3}H_2$;2-^{14}C]; 50 μCi ^{14}C each) in ethanol (1 ml) were separately added to 3 day-old shake cultures (100 ml) of *T. roseum*. The metabolites were isolated after a further 7 days growth and purified by preparative layer chromatography. The purity of the samples was checked by g.l.c. on 1% OV-17 at 184 °C (N_2 carrier gas, flow rate 50 ml min^{-1}). The cyclonerodiol had a retention time (t_R) of 2.8 min. At 250 °C deoxyrosenonolactone had t_R 3 min.

(b) *With* F. culmorum. [2-$^{3}H_2$;2-^{14}C]Mevalonic acid (54.2 μCi ^{14}C) was fed to 30 Roux bottles (100 ml) of *F. culmorum* 1 week after inoculation. After a further 2 weeks, the metabolites were recovered in chloroform and

[10] G. Popják in 'Methods in Enzymology,' 1969, **15**, 443.
[11] G. Popják, *Tetrahedron Letters*, 1959, **19**, 19.
[12] R. Evans, A. M. Holtom, and J. R. Hanson, *J.C.S. Chem. Comm.*, 1973, 465; R. Evans and J. R. Hanson, *J.C.S. Perkin I*, 1976, 326.
[13] J. R. Hanson, J. Hawker, and A. F. White, *J.C.S. Perkin I*, 1972, 1892.
[14] B. A. Achilladelis and J. R. Hanson, *Phytochemistry*, 1968, **7**, 589.

purified by preparative layer chromatography on silica in chloroform–acetone–methanol (70 : 30 : 5) to afford cyclonerotriol (32 mg), which was diluted with unlabelled material (30 mg) and crystallized [m.p. 113—114° (from ethyl acetate–light petroleum)] to constant activity. The cyclonerodiol (18 mg) was further purified by preparative layer chromatography in light petroleum–ethyl acetate (7 : 3), ether–light petroleum (4 : 1), and chloroform–acetone–methanol (15 : 5 : 1). Incubation of [(4*R*)-4-^{3}H;2-^{14}C]mevalonic acid (98.5 μCi ^{14}C) similarly gave cyclonerotriol (39 mg), and [5-$^3H_2$;2-^{14}C]mevalonic acid (80 μCi ^{14}C) gave cyclonerotriol (45 mg). The results are given in Table 2.

Oxidation of Cyclonerotriol.—(a) *With manganese dioxide.* The triol (10 mg) was stirred with freshly finely powdered manganese dioxide (100 mg) in acetone (3 ml) for 2 h. The suspension was filtered and the product was recovered and purified by preparative layer chromatography in chloroform–acetone–methanol (14 : 6 : 1). The band corresponding to the aldehyde was then rechromatographed (×2) in ethyl acetate–light petroleum (1 : 1) to afford material with ^{3}H : ^{14}C ratio 9.04 : 1 (atom ratio 5.36 : 3). The aldehyde was then dissolved in methanol (1 ml) and treated with sodium borohydride (5 mg) for 20 min. The excess of reagent was destroyed with acetic acid and the cyclonerotriol (5 mg) was recovered in ethyl acetate. It crystallized from ethyl acetate–light petroleum as needles, m.p. 110—111°, ^{3}H : ^{14}C 9.02 : 1 (atom ratio 5.25 : 3). The above sequence was repeated to afford material, ^{3}H : ^{14}C 8.7 : 1 (atom ratio 5.16 : 3).

(b) *With chromium trioxide.* The triol (2.5 mg) from the [2-$^3H_2$;2-^{14}C]mevalonate experiment was diluted with unlabelled material (20 mg) to afford material, ^{3}H : ^{14}C, 9.44 : 1; specific activity 1.713×10^6 disint. min^{-1} mmol^{-1}. It was dissolved in acetone (1 ml) and treated with the chromium trioxide reagent until the orange colour persisted. After 30 min the solution was diluted with water and extracted with ether. The extract was dried and evaporated and the residue was purified by preparative layer chromatography in ethyl acetate–light petroleum (3 : 2) to afford the lactone (8) (5 mg), m.p. 65—66° (resolidifies and remelts at 82—84°) (^{3}H : ^{14}C 9.37 : 1; sp. act. 1.187×10^6 disint. min^{-1} mmol^{-1}).

Incubation of all-trans-Farnesyl Pyrophosphate.—The pyrophosphate (^{3}H : ^{14}C 14.41 : 1; 1.85×10^6 disint. min^{-1} ^{14}C) prepared from [2-$^3H_2$;2-^{14}C] mevalonic acid, in 0.05M-Tris buffer (pH 8.5; 1 ml) was fed to two three-day-old shake cultures (100 ml) of *Trichothecium roseum.* The metabolites were isolated after a further 7 days and purified by chromatography on Sephadex LH-20 (160 × 2.5 cm column) in chloroform. The fractions containing cyclonerodiol and rosenonolactone were further purified by preparative layer chromatography. Cyclonerodiol had ^{3}H : ^{14}C 14.42 : 1; 49 757 disint. min^{-1}; 2.7% incorporation. Rosenonolactone had ^{3}H : ^{14}C, 9.76 : 1; 20 230 disint. min^{-1} ^{14}C; 1.09% incorporation.

Incubation of Cyclonerodiol. Cyclonerodiol (6 mg; 1.3×10^6 disint. min^{-1} ^{14}C), prepared biosynthetically from [2-^{14}C]-mevalonic acid with *F. culmorum*, in ethanol (1 ml) was re-incubated with two shake cultures (100 ml) of *F. culmorum* 24 h after inoculation. The metabolites were isolated after a further 8 days and purified as above. The cyclonerotriol fraction was diluted with unlabelled material (5 mg) and crystallized [m.p. 111—112° (from ethyl acetate–light petroleum)] to constant activity, 1.948×10^5 disint. min^{-1} (15% incorporation).

Incubation of Nerolidol.—DL-Nerolidol (5.14×10^6 disint. min^{-1} ^{14}C) in ethanol (0.5 ml) was incubated with two shake cultures (100 ml) of *F. culmorum* as above. The metabolites were examined by radio-t.l.c. scanning in chloroform–acetone–methanol (15 : 5 : 1). The main zone of radioactivity (R_F 0.31) did not correspond to cyclonerotriol (R_F 0.23) nor to cyclonerodiol (R_F 0.41). The cyclonerotriol was isolated from the plate, diluted with unlabelled material (5 mg), and recrystallized. After two recrystallizations the residual activity had fallen to 200 disint. min^{-1} (0.03% incorporation). Radiochromatogram scanning of the mother liquors in ethyl acetate–light petroleum (9 : 1) (×2) showed a small radioactive band (R_F 0.24); cyclonerotriol had R_F 0.34 and was inactive.

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