Studies in Terpenoid Biosynthesis. Part XIV.[1] Formation of the Sesquiterpene Trichodiene

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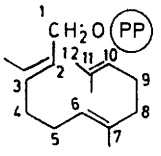
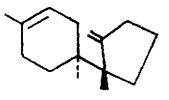
A cell-free system obtained from *Trichothecium roseum* mediates the biosynthesis of trichodiene (2), a hydrocarbon precursor of trichothecin (3). The isomerization and cyclization of all-*trans*-farnesyl pyrophosphate (1) takes place with the loss of a 1-*pro*-*S*-hydrogen atom of farnesyl pyrophosphate and its ultimate replacement by a 4-*pro*-*S*-hydrogen atom of NADPH.

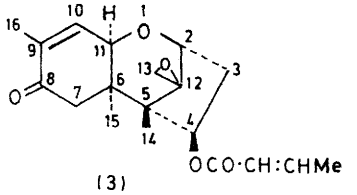
THE trichothecenes [*e.g.* (3)] are a group of mycotoxins[2] with cytotoxic,[3] phytotoxic,[4] antifungal,[5] and insecticidal[6] activity. Trichodiene (2) is the parent hydrocarbon of this series.[7,8] Its biosynthesis is of interest not only because of this relationship but also because of the manner of folding of farnesyl pyrophosphate which is required for the formation of the six-membered ring.[9] The biosynthesis of trichodiene (2) from all-*trans*-farnesyl pyrophosphate (1) in a cell-free system derived from *Trichothecium roseum* is the subject of this paper.[10]



(1)



(2)



(3)

(PP) = pyrophosphate

The cell-free system was obtained by disruption of three-day-old shake cultures of *Trichothecium roseum* with a French press followed by centrifugation, first at 10 000 *g* to remove cell debris and then at 75 000 *g*. In some experiments a 1% protamine sulphate precipitation was used at this stage. The supernatant from the centrifugation was supplemented with magnesium and manganese chlorides and reduced pyridine nucleotides, stabilized with dithiothreitol, and used in a 0.1M-potassium phosphate buffer at pH 7.0. This system converted all-*trans*-[4,8,12-^{14}C] farnesyl pyrophosphate, prepared enzymically from [2-^{14}C]mevalonic acid with a pig-liver system,[11] into trichodiene in 50% yield in 2 h. In order to establish that the system made all-*trans*-farnesyl pyrophosphate, it was incubated with [1-^{14}C]isopentenyl pyrophosphate and unlabelled geranyl pyrophosphate in the presence of magnesium and manganese chlorides but without the pyridine nucleotides. At the end of the incubation, the preparation was extracted with ether. The remaining aqueous phase was then adjusted to pH 8.5, boiled, incubated with alkaline phosphatase, and re-extracted with ether. Both ethereal extracts were assayed by radio-t.l.c. and by g.l.c. all-*trans*-Farnesol was the only isomer of farnesol detected. A control incubation in the absence of added isopentenyl pyrophosphate and geranyl pyrophosphate gave no farnesol. Hence the system was capable of synthesizing all-*trans*-farnesol pyrophosphate from prenyl pyrophosphates. However it could not utilize mevalonate or free farnesol as a substrate, *i.e.* during the purification mevalonate kinase activity was destroyed.

In order to form the six-membered ring of the trichodiene, the all-*trans*-farnesyl pyrophosphate (1) must be converted into a 2-*cis*-farnesyl unit. In earlier work on trichothecene biosynthesis we have shown[12] that the 4-*pro*-*R*-mevalonoid hydrogen label is retained at C-10 (≡ C-2 of farnesyl pyrophosphate) through the biosynthesis. This was confirmed when all-*trans*-farnesyl pyrophosphate (^{3}H : ^{14}C 8.21 : 1; atom ratio 3 : 3), prepared from [(4R)-4-^{3}H,2-^{14}C]mevalonic acid, was incubated with the cell-free system. The resultant trichodiene (^{3}H : ^{14}C 7.76 : 1; atom ratio 2.84 : 3) retained the three labels, the hydrogen shift from the central

[1] Part XIII, R. Evans and J. R. Hanson, *J.C.S. Perkin I*, 1975, 663.

[2] J. R. Bamburg and F. M. Strong in 'Microbial Toxins,' vol. 7, section B, eds. S. Kadis, A. Ciegler, and S. J. Ajl, Academic Press, New York, 1971, ch. 7.

[3] E. Harri, W. Loeffler, H. P. Sigg, H. Stahelin, Ch. Stoll, Ch. Tamm, and D. Wiesinger, *Helv. Chim. Acta*, 1962, **45**, 839; J. F. Grove and P. H. Mortimer, *Biochem. Pharmacol.*, 1969, **18**, 1473.

[4] P. W. Brian, A. W. Dawkins, J. F. Grove, H. G. Hemming, D. Lowe, and G. L. F. Norris, *J. Exp. Bot.*, 1961, **12**, 1; J. F. Grove, *J. Chem. Soc.* (*C*), 1970, 378 and preceding papers.

[5] W. O. Godtfredsen and S. Vangedal, *Acta Chem. Scand.*, 1965, **19**, 1088.

[6] J. F. Grove and M. Hosken, *Biochem. Pharmacol.*, 1975, **24**, 959.

[7] Y. Machida and S. Nozoe, *Tetrahedron Letters*, 1972, 1969.

[8] R. Evans, A. M. Holtom, and J. R. Hanson, *J.C.S. Chem. Comm.*, 1973, 465.

[9] J. R. Hanson and B. Achilladelis, *Chem. and Ind.*, 1967, 1643; *Phytochemistry*, 1969, **8**, 765; R. Achini. B. Muller, and Ch. Tamm, *Chem. Comm.*, 1971, 404; J. R. Hanson, T. Marten, and M. Siverns, *J.C.S. Perkin I*, 1974, 1033.

[10] Preliminary communication, R. Evans and J. R. Hanson, *J.C.S. Chem. Comm.*, 1975, 231.

[11] G. Popjak, *Methods in Enzymol.*, 1969, **15**, 443.

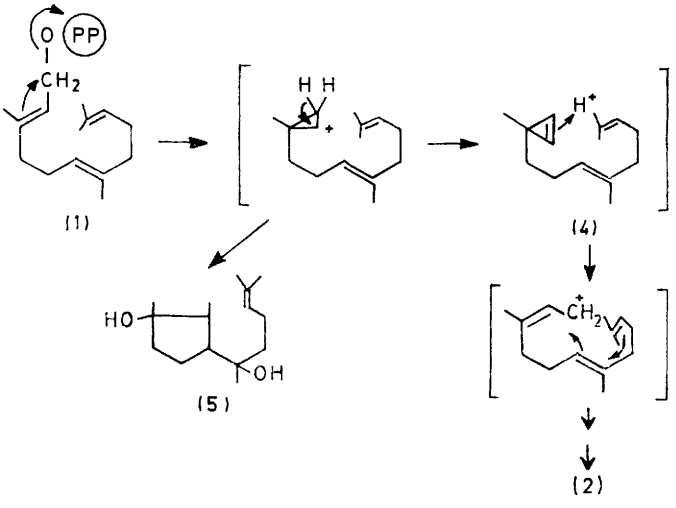
[12] B. Achilladelis, P. M. Adams, and J. R. Hanson, *Chem. Comm.*, 1970, 511; *J.C.S. Perkin I*, 1972, 1425.

prenyl unit (C-6 to C-2, trichothecene numbering) having occurred by this stage.

all-*trans*-[1,5,9-$^{3}H_{6}$; 4,8,12-$^{14}C_{3}$]Farnesyl pyrophosphate ($^{3}H : {}^{14}C$ 7.9 : 1; atom ratio 6 : 3) was prepared from [5-^{3}H,2-^{14}C]mevalonic acid and incubated with the cell-free system. The trichodiene ($^{3}H : {}^{14}C$ 6.88 : 1; atom ratio 5.2 : 3) was isolated. In another incubation in which the farnesyl pyrophosphate had $^{3}H : {}^{14}C$ 7.35 : 1, all-*trans*-farnesol ($^{3}H : {}^{14}C$ 7.30 : 1; atom ratio 5.96 : 3), 2-*cis*-6-*trans*-farnesol ($^{3}H : {}^{14}C$ 6.20 : 1; atom ratio 5.06 : 3) and trichodiene ($^{3}H : {}^{14}C$ 5.95 : 1; atom ratio 4.86 : 3) were isolated. The atom ratios show that there is an unexpected loss of one hydrogen atom in the formation of 2-*cis*-6-*trans*-farnesol and trichodiene from all-*trans*-farnesyl pyrophosphate. Trichodiene is incorporated into trichothecin in 5.3% yield by *Trichothecium roseum*. The 2-, 4-*pro*-*R*-, and 5-mevalonoid hydrogen atoms have been located in the trichothecenes.[12] Taking this into account, the hydrogen atom that is lost must be from C-1 of farnesyl pyrophosphate. all-*trans*-Farnesyl pyrophosphate ($^{3}H : {}^{14}C$ 10.52 : 1; atom ratio 3 : 3) was then prepared from [(5*R*)-5-^{3}H,2-^{14}C] mevalonic acid.[13] It was incubated with the cell-free system derived from *Trichothecium roseum*. The trichodiene ($^{3}H : {}^{14}C$ 10.5 : 1; atom ratio 3 : 3) retained all three 5-*pro*-*R*-mevalonoid hydrogen atoms and hence it is a 5-*pro*-*S*-mevalonoid hydrogen atom (≡ 1-*pro*-*S*-hydrogen atoms of farnesyl pyrophosphate) that is lost in the formation of trichodiene.

The requirement for the pyridine nucleotides suggested that NADPH was the source of the new hydrogen atom, and, by analogy with squalene formation,[14] that a 4-*pro*-*S*-NADPH hydrogen atom was transferred. [(4*S*)-4-^{3}H]-NADPH was prepared enzymically from [1-^{3}H]-D-glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP. The [1-^{3}H]-D-glucose 6-phosphate was in turn obtained from [1-^{3}H]-D-glucose and ATP in the presence of hexokinase. The incubations were carried out with [4,8,12-^{14}C]farnesyl pyrophosphate (38.7 μCi ^{14}C μmol^{-1}) and [(4*S*)-4-^{3}H]NADPH (56.44 μCi ^{3}H μmol^{-1}) and the trichodiene was isolated. Since the carbon-14 content of the trichodiene was 473 088 disint. min^{-1}, 5.51 nmol of farnesyl pyrophosphate were converted into trichodiene. Taking into account the specific activity of the NADPH, the transfer of one hydrogen atom should lead to trichodiene with 689 947 disint. min^{-1} ^{3}H activity. The observed tritium activity of 434 028 disint. min^{-1}, which corresponds to 62.9% incorporation, implies the presence of some endogenous reduced nucleotide in the cell-free preparation. Similar effects were observed in squalene biosynthesis.[14] Thus the added hydrogen atom originates from the 'B' face of NADPH as in squalene biosynthesis.

A number of reports [15] have recently appeared on the isomerization of the free alcohol farnesol, demonstrating the loss of either the 1-*pro*-*R*- or the 1-*pro*-*S*-hydrogen atom and implicating farnesal as an intermediate. However farnesol was not a substrate for the *Trichothecium roseum* system and we have been unable to confirm the presence of any substantial radioactivity associated with farnesal. Since the added hydrogen atom comes from the face of NADPH opposite to that observed in the common redox systems, *e.g.* liver alcohol dehydrogenase, it is possible that a different mechanism may operate in this system. For example a cyclopropene intermediate (4) might be formed and then opened in the opposite stereochemical sense (see Scheme), leading to the *cis*-isomer. The intervention of such an intermediate would



SCHEME

have the additional merit of accounting for the formation of cyclonerodiol (5), a metabolite which is formed at the expense of trichothecenes in 'degenerating' cultures of *Trichothecium roseum*.

EXPERIMENTAL

General experimental details have been described previously.[16]

Enzymic Preparation of Trichodiene.—The mycelium from two three-day-old shake cultures (100 ml) of *Trichothecium roseum*, grown as described previously,[9,12] was filtered, washed with deionized water, and resuspended in 0.1M-potassium phosphate buffer (45 ml) at pH 7.0. The suspension was passed through a French press at an applied pressure of 4—5 tons. The disrupted cells were then centrifuged at 10 000 *g* for 20 min. The supernatent was then centrifuged at 75 000 *g* for 90 min to afford the cell-free preparation (38 ml). The protein concentration, determined spectrophotometrically, was 7.2 mg ml^{-1}. Six

[13] C. Donninger and G. Popjak, *Proc. Roy. Soc.* 1966, *B*, **163**, 465.
[14] J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popjak, G. Ryback, and G. J. Schroepfer, *Proc. Roy. Soc.* 1966, *B*, **163**, 436.
[15] L. Chayet, R. Pont-Lezica, C. George-Nascimento, and O. Cori, *Phytochemistry*, 1973, **12**, 95; K. H. Overton and F. M. Roberts, *J.C.S. Chem. Comm.*, 1974, 385; K. Imai and S. Marumo, *Tetrahedron Letters*, 1974, 4401.
[16] J. R. Hanson, J. Hawker, and A. F. White, *J.C.S. Perkin I*, 1972, 1892.

incubations were carried out in 10 ml stoppered flasks at 30 °C for 2 h. Each incubation comprised: enzyme preparation (1.6 ml) and aliquot portions of aqueous solutions of magnesium chloride (10 μmol, 0.2 ml), manganese chloride (4 μmol, 0.2 ml), NADH, NADP, and NAD^+ (1 μmol each, 0.3 ml), dithiothreitol (1 μmol, 0.1 ml), 0.1M-potassium phosphate buffer, pH 7.0 (0.1 ml), and all-*trans*-[4,8,12-^{14}C]-farnesyl pyrophosphate (410 468 disint. min^{-1}; 10 nmol, 0.2 ml). At the end of the incubation, ethanol (0.5 ml) and 12N-sodium hydroxide (0.5 ml) were added to each tube, which was then warmed at 30 °C for 30 min. Each tube was diluted with water (2 ml) and extracted with ether (2 × 4 ml). An aliquot portion (0.1 ml) of the combined ethereal extracts was counted for radioactivity. The total recovery of radioactivity was 2 077 569 disint. min^{-1} (84.4%). The extracts were concentrated and purified by preparative layer chromatography (p.l.c.) on silica gel in benzene–ethyl acetate (9 : 1) against a trichodiene marker. The radioactive fraction which ran concurrently with trichodiene was further purified by p.l.c. in benzene–hexane (1 : 9) against trichodiene and squalene markers. The radioactive fraction again chromatographed with trichodiene. The trichodiene had an activity of 1.24×10^6 disint. min^{-1} (50.2% of that of the initial farnesyl pyrophosphate).

Incubation with [1-^{14}C]*Isopentenyl Pyrophosphate.*—Two incubations were carried out in a 100 ml stoppered flask at 30 °C for 2.5 h. The first mixture comprised enzyme preparation (50 ml), magnesium chloride (250 μmol 0.1 ml), manganese chloride (100 μmol, 0.1 ml), geranyl pyrophosphate (30 μmol, 0.6 ml), and [1-^{14}C]isopentenyl pyrophosphate (3.11×10^6 disint. min^{-1}; 20 μmol, 0.4 ml); the second lacked the geranyl and isopentenyl pyrophosphates. After the incubation, both mixtures were extracted with ether. The extracts were dried and evaporated. The aqueous phases were then adjusted to pH 8.5 with N-sodium hydroxide, heated on a boiling water-bath for 10 min, cooled in ice, and then incubated at 38 °C with two portions of alkaline phosphatase (24 units) for 1 h. Both flasks were again extracted with ether. These extracts were dried and evaporated. The first extract from the [^{14}C]isopentenyl pyrophosphate incubation had an activity of 6.05×10^5 disint. min^{-1} (19.5%) and the second 3.45×10^5 disint. min^{-1} (11.1%). All four extracts were examined by g.l.c. on 3% Antarax at 200 °C (N_2 carrier gas at 50 ml min^{-1}). Under these conditions geraniol had a retention time of 2.15 min, 2-*cis*-6-*trans*-farnesol 9.3 min, and 2-*trans*-6-*trans*-farnesol 10.4 min. Only the extracts from the isopentenyl pyrophosphate incubation contained 2-*trans*-6-*trans*-farnesol. No *cis*-isomer was detected, neither was there any farnesol in the blank incubation. The radioactivity ran concurrently on t.l.c. on silica gel in benzene–ethyl acetate (9 : 1) with 2-*trans*-6-*trans*-farnesol.

Incubations with Doubly-labelled Farnesyl Pyrophosphates.—(a) all-*trans* [2,6,10-^{3}H;4,8,12-^{14}C]Farnesyl pyrophosphate (^{3}H : ^{14}C 8.21 : 1) was prepared from [(4*R*)-4-^{3}H,2-^{14}C]-mevalonic acid (^{3}H : ^{14}C 8.15 : 1) by using a pig-liver enzyme system.[14] Two incubations with the *T. roseum* system prepared as above were carried out at 30 °C for 3 h. The mixtures comprised enzyme preparation (1.6 ml), magnesium chloride (10 μmol, 0.2 ml), manganese chloride (4 μmol, 0.2 ml), dithiothreitol (1 μmol, 0.1 ml), NADP, NADH, and NAD^+ (1 μmol each, 0.3 ml) and all-*trans*-[2,6,12-^{3}H;4,8,12-^{14}C]farnesyl pyrophosphate (429 142 disint. min^{-1} ^{14}C). After 3 h the mixtures were extracted with ether (2 × 4 ml) (recovery of ^{14}C 64%). The extract was dried and evaporated. The residue was purified by p.l.c. against a trichodiene standard on silica gel and then on silica gel–silver nitrate in benzene–ethyl acetate (9 : 1). The trichodiene had ^{3}H : ^{14}C 7.76 : 1 (51 128 disint. min^{-1}; 5.9% incorporation).

(*b*) all-*trans*-[1,5,9-$^3H_6$; 4,8,12-^{14}C]Farnesyl pyrophosphate (^{3}H : ^{14}C 7.9 : 1) was prepared from [5-^{3}H,2-^{14}C]-mevalonic acid (^{3}H : ^{14}C 7.9 : 1) by using a pig-liver enzyme system.[14] Eight mixtures containing farnesyl pyrophosphate (total 3 111 952 disint. min^{-1} ^{14}C) were incubated as before. The trichodiene (2.0×10^6 disint. min^{-1}; 64.3% incorporation; ^{3}H : ^{14}C 6.88 : 1) was purified as above. In a separate set of incubations all-*trans*-farnesyl pyrophosphate (2 366 784 disint. min^{-1} ^{14}C; ^{3}H : ^{14}C 7.35 : 1) (checked for isomeric purity by hydrolysis with alkaline phosphatase and radio-t.l.c. of the resultant farnesol) gave all-*trans*-farnesol (49 480 disint. min^{-1} ^{14}C; 2.1% incorporation; ^{3}H : ^{14}C 7.30 : 1), 2-*cis*-6-*trans*-farnesol (9 740 disint. min^{-1}; 0.4% incorporation; ^{3}H : ^{14}C 6.20 : 1), and trichodiene (127 300 disint. min^{-1} ^{14}C; 5.3% incorporation; ^{3}H : ^{14}C 5.95 : 1).

(*c*) all-*trans*-[(1*R*)-1,5,9-$^3H_3$; 4,8,12-^{14}C]Farnesyl pyrophosphate (^{3}H : ^{14}C 10.52 : 1) was prepared from [(5*R*)-5-^{3}H,2-^{14}C]mevalonic acid by using a pig-liver enzyme system.[14] Mixtures containing farnesyl pyrophosphate (total 2.8×10^6 disint. min^{-1} ^{14}C) were incubated as before. The trichodiene (963 016 disint. min^{-1} ^{14}C; 34.5% incorporation) had ^{3}H : ^{14}C 10.5 : 1.

*Stereochemical Purity of all-*trans-[(1R)-1,5,9-^{3}H; 4,8,12-^{14}C]*Farnesyl Pyrophosphate.*—The all-*trans*-farnesyl pyrophosphate (0.5 ml; 9.32×10^5 disint. min^{-1} ^{14}C; ^{3}H : ^{14}C 10.52 : 1) was diluted with 0.1M-tris(hydroxymethyl)-methylamine buffer (1.5 ml; pH 8.5) and alkaline phosphatase (10 units) was added. The mixture was incubated at 30 °C for 2 h; more alkaline phosphatase (10 units) was added after 1 h. The farnesol (4.39×10^5 disint. min^{-1}; ^{3}H : ^{14}C 10.44 : 1) was recovered in ethyl acetate and purified by p.l.c. The farnesol was diluted with all-*trans*-farnesol (1 μmol), suspended in 10% aqueous Tween 80 (0.1 ml), and treated with NAD (5 μmol), 0.08M-glycine, and sodium hydroxide (pH 10.0; 2 ml), and horse liver alcohol dehydrogenase (10 units). The increase in absorption at 340 mm was followed spectrophotometrically. After 30 min the solution was extracted with ethyl acetate, concentrated under nitrogen, and purified by p.l.c. The farnesal (2.22×10^{15} disint. min^{-1} ^{14}C) had ^{3}H : ^{14}C 7.13 : 1.

Preparation of [(4S)-4-^{3}H]NADPH.—The following aqueous solutions were combined: [1-^{3}H]-D-glucose (0.5 ml, 1 μmol; 500 μCi), NADP (0.1 ml, 1.2 μmol), ATP (0.1 ml, 4 μmol), magnesium chloride (0.1 ml; 5 μmol), hexokinase (25 units), glucose 6-phosphate dehydrogenase (25 units), and 0.05M-potassium phosphate buffer (pH 7.5; 2.1 ml). The reaction was followed spectrophotometrically. After 30 min there was no further change in absorption at 340 nm. The change in optical density corresponded to the formation of 0.801 μmol of NADPH. Inactive NADPH was added to the solution (2.79 ml), which was then reassayed. It contained 6.60 μmol of NADPH, corresponding to a dilution of 8.86 : 1 of the enzymically prepared material. The NADPH therefore had a specific activity of 56.44 μCi $μmol^{-1}$.

Preparation of Trichodiene by using [(4S)-4-^{3}H]*NADPH.*—Eight incubations with the *T. roseum* system were carried out at 30 °C for 3 h. The mixtures comprised aqueous solutions of enzyme preparation (0.3 ml), magnesium chloride

(0.1 ml, 10 μmol), manganese chloride (0.1 ml, 4 μmol), all-*trans*-[4,8,12-^{14}C]farnesyl pyrophosphate (0.1 ml; 501 152 disint. min^{-1}), [(4*S*)-4-^{3}H]NADPH (0.3 ml),0.1M-potassium phosphate (pH 7.0; 0.1 ml), and 1mM-dithiothreitol (0.1 ml). The trichodiene was purified as described earlier; activity 473 088 disint. min^{-1} ^{14}C (11.8% incorporation of the farnesyl pyrophosphate) and 434 028 disint. min^{-1} ^{3}H.

Incorporation of Trichodiene into Trichothecin.—[4,8,14-^{14}C]Trichodiene (43 345 disint. min^{-1} prepared as above from [2-^{14}C] mevalonic acid *via* farnesyl pyrophosphate) in ethanol (1 ml) was fed to a three-day-old shake culture (100 ml) of *T. roseum*. After a further four days, the mycelium was filtered and the broth was extracted with ethyl acetate. The trichothecin (10 mg) was purified by p.l.c. on silica gel in 40% ethyl acetate–light petroleum; activity 2 320 disint. min^{-1} ^{14}C (5.3% incorporation).

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