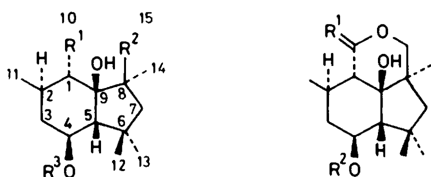
Studies in Terpenoid Biosynthesis, Part 24.[1] The Formation of the Carbon Skeleton of the Sesquiterpenoid, Dihydrobotrydial

By A. Peter W. Bradshaw, James R. Hanson,* and Robert Nyfeler, The School of Molecular Sciences, The University of Sussex, Brighton, Sussex, BN1 9QJ

The labelling and coupling patterns of dihydrobotrydial, biosynthesized from [1-^{13}C]- and [1,2-$^{13}C_2$]-acetate and [4,5-$^{13}C_2$]mevalonate by the fungus, *Botrytis cinerea*, have been used to define the mode of formation of the carbon skeleton of the sesquiterpenoid from farnesyl pyrophosphate.

THE fungus, *Botrytis cinerea*, is a serious pathogen of a number of crops including lettuce, tomatoes, and strawberries. Two major phytotoxic metabolites are botrydial (1) and dihydrobotrydial (2).[2] Their structures were established by a combination of chemical degradation, circular dichroism and *X*-ray studies.[2,3] More recently we have isolated [4,5] a series of related metabolites (3)—(6) from the fungus. These novel sesquiterpenoid structures do not obey the simple isoprene rule. Evidence for the generation of their carbon skeletons from farnesyl pyrophosphate by rearrangement and bond fission forms the subject of this paper.[6]

[1-^{14}C]Farnesyl pyrophosphate was incorporated into dihydrobotrydial (2) to the extent of 0.33% thus establishing its probable sesquiterpenoid origin. The radioactivity was retained on oxidation to the lactone (7).



(1) $R^1 = R^2 =$ CHO; $R^3 =$ Ac

(2) $R^1 = \alpha$—H, β — OH; $R^2 =$ Ac.

(4) $R^1 =$ CHO; $R^2 = CO_2H$; $R^2 =$ H

(7) $R^1 =$ O; $R^2 =$ Ac

(5) $R^1 =$ CHO; $R^2 = CO_2H$; $R^3 =$ Ac

(8) $R^1 = H_2$; $R^2 =$ Ac

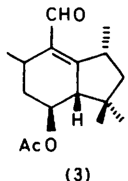
(6) $R^1 = CO_2H$; $R^2 = CH_2OH$; $R^3 =$ Ac

(9) $R^1 = H_2$; $R^2 =$ H

(10) $R^1 =$ CHO; $R^2 = CO_2Me$; $R^3 =$ Ac

(11) $R^1 =$ CHO; $R^2 = CO_2Me$; $R^3 =$ H

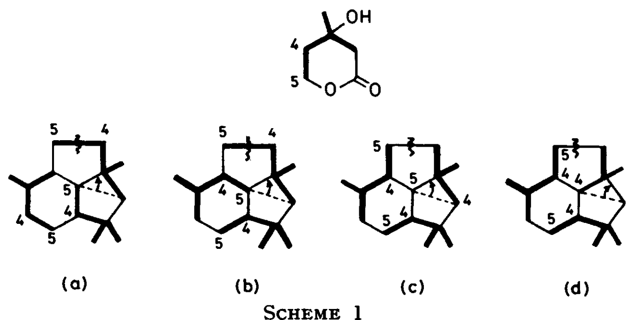
(12) $R^1 = R^2 = CH_2OH$, $R^3 =$ Ac



(3)

The carbon skeleton of dihydrobotrydial may be derived from the three isoprene units of farnesyl pyrophosphate in several ways (Scheme 1a—d). These were distinguished by a series of carbon-13 biosynthetic and n.m.r. studies.

The carbon-13 n.m.r. signals of botrydial (1), dihydrobotrydial (2), and a group of their derivatives (3), (7)—(11) [2] were assigned. The results are given in Table 1. The carbon atoms bearing oxygen functions were readily assigned from their chemical shift and multiplicity in the SFORD spectrum. The quaternary carbon atoms, C-6 and C-8, were distinguished through the influence of



SCHEME 1

changes at C-15 on the position of the C-8 resonance. Amongst the methine carbon atoms, C-5 was distinguished from C-1 by the effect of hydrolysis of the 4-acetoxy-group on C-5 and the influence of the oxidation level of C-10 on C-1. The resonance associated with C-2 was, as anticipated, at higher field. The assignment of the C-3 and C-7 methylene resonances followed from the constancy of C-7 and the changes in C-3 on hydrolysis of the 4-acetoxy-group. Furthermore C-7 resonates

TABLE 1

The ^{13}C n.m.r. spectra of some derivatives of dihydrobotrydial (2) (in $CDCl_3$; p.p.m. from $SiMe_4$)

Carbon atom	Compound 1	2	3	7	8	9	10	11
1	67.2	55.0	135.8	57.6	52.0	52.1	68.3	68.5
2	28.0	28.6	28.3	31.5	30.3	30.5	28.2	28.5
3	38.7	39.9	37.5	40.2	39.7	44.5	38.6	43.5
4	72.3	72.7	70.2	72.5	73.1	70.2	72.9	70.0
5	63.8	59.8	56.0	61.5	60.4	65.2	61.7	66.3
6	39.4	38.8	40.9	41.1	39.5	39.5	38.6	38.6
7	51.5	50.4	49.3	49.6	52.2	52.2	55.0	54.9
8	59.0	45.4	29.1	44.2	45.9	46.0	55.0	55.1
9	89.6	83.4	169.0	84.4	84.0	84.3	88.0	88.2
10	204.3	92.2	190.4	171.4	63.8	63.9	203.5	204.1
11 [a]	20.4	20.0	20.6	21.8	20.2	20.3	20.5	20.7
12 [a]	35.6	35.9	32.5	36.3	36.2	36.7	35.7	36.2
13 [a]	27.8	27.2	24.2	27.2	27.2	27.4	27.5	27.8
14 [a]	19.8	25.4	21.9	23.3	26.4	26.2	20.9	20.7
15	206.7	67.4		76.9	75.0	75.0	178.9	179.0
AcMe	21.4	21.4	21.3	21.4	21.5		21.4	
CO	170.3	170.6	170.1	170.4	170.4		170.3	
OMe							52.6	52.5

[a] The assignments of the methyl groups [(C(11)—C(14)] are a revision of our previous assignments.[6]

at relatively low field consonant with its heavily substituted environment. Two of the skeletal methyl resonances, those at 27.2 and 35.9 p.p.m. in dihydrobotrydial, were relatively unchanged in position irrespective of the presence of a C-15 lactone, hemiacetal, aldehyde, or ether. These signals were assigned to the methyl groups attached to C-6. The higher field of the two, which is shielded relative to methylcyclopentane, was assigned to the α-oriented methyl group (C-13) since it experiences a *cis* 1 : 3-diaxial interaction with the C-14 methyl group.[7] The methyl group resonance at 25.4 p.p.m. in dihydrobotrydial appears within the range 25—26 p.p.m. when C-15 is an sp^3 centre bearing oxygen but shows a substantial high-field γ-shift when C-15 is converted into a carbonyl group. This signal was therefore assigned to C-14. The remaining skeletal methyl signal at 20.0 p.p.m. was relatively constant in position showing only a slight variation with the oxidation of C-10. It lies within the range expected for an equatorial methyl group on a substituted cyclohexane ring[8] in agreement with its assignment to C-11. The derivatives required for this work were mostly prepared as described previously[2] although the ether (8) was prepared by treatment of the triol (12) with toluene-*p*-sulphonyl chloride in pyridine whilst the hydrolysis of the acetate was performed with aqueous potassium carbonate.

The optimum time for feeding acetate and mevalonate to *Botrytis cinerea* and for isolating the metabolites, was established by a series of trial radiochemical experiments.

Foldings (a) and (b) (Scheme 1) may be distinguished from (c) and (d) by the coupling pattern produced in the metabolites biosynthesized from $[1,2\text{-}^{13}C_2]$acetate or $[4,5\text{-}^{13}C_2]$mevalonate. Since many of the couplings lay between sp^3 hybridized carbon atoms and were therefore expected to be of a similar magnitude, $[4,5\text{-}^{13}C_2]$mevalonate was used first since it would generate a simpler coupling pattern. The labelled mevalonate was prepared[9] by the condensation of 1,1-dimethoxybutan-3-one and ethyl $[1,2\text{-}^{13}C_2]$acetate in the presence of lithium diethylamide, followed by reduction of the ester, hydrolysis of the acetal, and oxidation. The expected labelling pattern is shown for each case in Scheme 1. The results (see Table 2) showed two pairs of ^{13}C–^{13}C couplings between C(4) and C(5) (J 38 Hz) and between C(1) and C(10) (J 41 Hz). The resonances associated with C(7) and C(9) were enriched. Thus two mevalonoid C(4)–C(5) bonds have remained intact and one has been cleaved. These coupling patterns could be accommodated by either folding (c) or (d). The more complex $[1,2\text{-}^{13}C_2]$acetate coupling pattern (see Table 2) was in agreement with this and also revealed ^{13}C–^{13}C couplings between C(2) and C(11), C(6) and C(13), and between C(8) and C(14) in accordance with the incorporation of the acetate units *via* mevalonate and farnesyl pyrophosphate. The coupling of C(6) to C(13) and the enrichment of C(12) from the $[1,2\text{-}^{13}C_2]$acetate served to define the orientation of the distal isoprene unit of farnesyl pyrophosphate in dihydrobotrydial.

The distinction between foldings (c) and (d) was made utilizing the induced ^{13}C–^{13}C couplings resulting from the cyclization of a multiply labelled farnesyl pyrophosphate. When $[1,2\text{-}^{13}C_2]$acetate has been used to study polyketide biosynthesis, small couplings have occasionally been noted[10] between acetate units as well as within acetate units. These arise from multiple-labelling of the polyketide chain. We have observed this in the case of the sesquiterpenoid, trichothecin,

TABLE 2

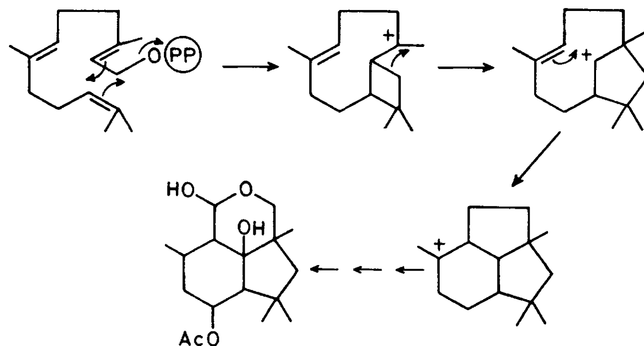
Enrichment and coupling patterns of biosynthetically labelled dihydrobotrydial

Carbon atom	$[1\text{-}^{13}C_2]$Acetate Expt. Norm. enrich.[c] / Norm. unenrich.	$[1,2\text{-}^{13}C_2]$-Acetate Expt. Coupling constant[e] Hz	$[4,5\text{-}^{13}C_2]$Mevalonate Expt. Coupling constant	$[4,5\text{-}^{13}C_2]$Mevalonate Expt. Norm. enrich.[d] / Norm. unenrich.
1	1.37	41	41	1.08
2	4.84	36		0.96
3	1.38	en		1.00
4	4.97	39	38	1.05
5	1.45	38	38	1.05
6	3.03 [a]	36		1.03
7	3.35 [a,b]	en		1.35
8	3.00 [b]	35		0.90
9	0.70	en		1.31
10	4.78	41	41	1.02
11	1.12	36		0.94
12	1.38	en		0.90
13	1.23	36		1.01
14	1.21	35		1.00
15	1.50	en		1.00
Ac-Me	1.00	58		0.94
CO	4.08	58		0.75

[a] $J_{6:7}$ 33.6 Hz. [b] $J_{7:8}$ 35.1 Hz. [c] Normalized onto the acetoxy-Me. [d] Normalized onto C-15. [e] en = enriched.

and traced[11] its origin to the inhibition of endogenous acetate biosynthesis from pyruvate and citrate by the exogenous acetate. If sufficient labelled acetate is then fed in one pulse at the time of maximum metabolite production, a number of molecules of the metabolite will be biosynthesised containing more than one labelled unit and hence additional couplings will be observed. Farnesyl pyrophosphate will be labelled by $[1\text{-}^{13}C]$-acetate, at *inter alia*, C(1), C(3), and C(11). The rearrangement envisaged in (c) and (d) will join C(1) and C(3) of the farnesyl pyrophosphate and thus induce a ^{13}C–^{13}C coupling in multiply labelled material. The cyclization envisaged in (d) also joins C(1) and C(11) of farnesyl pyrophosphate and will thus induce a second ^{13}C–^{13}C coupling whilst (c) joins C(2) (unlabelled) to C(11). Thus in the resultant dihydrobotrydial, folding (c) would generate a coupling between C(8) and C(9) (dihydrobotrydial numbering) whereas folding (d) would generate couplings between C(6) and C(7) and between C(7) and C(8). In the event, when sodium $[1\text{-}^{13}C]$acetate was fed to *B. cinerea* as a pulse at the carefully determined time of maximum metabolite production, couplings were observed in the product between C(6) and C(7) (J 33.6 Hz) and between C(7) and C(8) (J 35.1 Hz). Indeed there was sufficient incorporation from the $[1\text{-}^{13}C]$acetate for five lines to be observed from C(7)

implying that some molecules had been biosynthesized with exogenous acetate in both the starter and terminal isoprene units. This result is in accord with the incorporation of 2-*trans*-farnesyl pyrophosphate as in (d). Taken with the [1,2-$^{13}C_2$]acetate results, this suggests a biosynthesis of dihydrobotrydial from farnesyl pyrophosphate as in Scheme 2. This pattern of folding is



Scheme 2

reminiscent of that of farnesyl pyrophosphate in the biosynthesis of caryophyllene.[12]

The use of biosynthetically induced couplings may have further applications in terpenoid biosynthesis. Thus the 1,2-shift of a methyl group is a common feature in terpenoid biosynthesis which might be revealed under favourable conditions by an induced coupling using multiple labelling with [2-^{13}C]acetate.

EXPERIMENTAL

General experimental details have been described previously.[13]

General Fermentation Conditions.—*Botrytis cinerea* (obtained from the Glasshouse Crops Research Institute, GCRI strain 23) was grown on surface culture for 8 d in Thompson bottles (750 ml) on a Czapek-Dox medium containing 0.1% yeast extract and 5% glucose. The broth was saturated with sodium chloride, acidified to pH 2 with dilute hydrochloric acid and extracted with ethyl acetate. The extracts were separated into acidic and neutral fractions with aqueous sodium hydrogen carbonate. Dihydrobotrydial (50—70 mg l^{-1}) and botrydial (10—20 mg l^{-1}) were obtained from the neutral fraction by preparative layer chromatography on silica in chloroform–ethyl acetate–acetic acid (40 : 10 : 1). Dihydrobotrydial (2) had m.p. 159—160 °C (lit.,[2] 158—160 °C) (Found: C, 65.45; H, 8.8. Calc. for $C_{17}H_{28}O_5$: C, 65.4; H, 9.0%). Botrydial (1) had m.p. 108—109 °C (lit.,[2] 108—110 °C) (Found: C, 65.95; H, 8.5. Calc. for $C_{17}H_{26}O_5$: C, 65.75; H, 8.4%).

Incubation of Farnesyl pyrophosphate with Botrytis cinerea:—Aqueous [^{14}C]farnesyl pyrophosphate (2.48 × 10^6 d.p.m.) (2 ml) was fed to 2 Thompson bottles of *B. cinerea* 2 d after inoculation. The fermentation was harvested after a further 5 d as above to afford dihydrobotrydial (2), (72 mg, 113 d.p.m. mg^{-1}, 0.33% incorp.). The dihydrobotrydial (33 mg) was stirred with a solution (1 ml) of chromium trioxide in aqueous pyridine [prepared by the careful addition of chromium trioxide (5 g) in water (3 ml) to pyridine (10 ml)] overnight. The product was recovered in ethyl acetate and purified by chromatography on silica to afford the lactone (7) (18 mg), m.p. 215—216 °C, 113 d.p.m. mg^{-1} (lit.,[2] m.p. 217—219 °C).

Incubation of [4,5-$^{13}C_2$]*Mevalonic Acid with* Botrytis cinerea.—The mevalonate (prepared[9] from sodium [1,2-$^{13}C_2$]acetate) (250 mg, containing 18.5 μCi [2-^{14}C]mevalonic acid) was fed to 1 Thompson bottle of *B. cinerea* 4 d after inoculation. The metabolites were isolated after a further 4 d as above to afford dihydrobotrydial (60 mg) (0.4% incorp. ^{14}C). The spectral data are tabulated in Table 2.

Incubation of Sodium [1-^{13}C]*Acetate with* B. cinerea.—A mixture of 95% sodium [1-^{13}C]acetate and sodium [2-^{14}C]-acetate (1.13 μCi; 700 mg) in sterile water (5 ml) was distributed between 2 surface cultures (350 ml each) of *B. cinerea* 2 d after inoculation. The metabolites were isolated after a further 5 days growth to afford dihydrobotrydial (67 mg) as prisms, m.p. 157—159 °C (^{14}C incorp. 468 d.p.m. mg^{-1} 1.25% incorp.). The spectral data are tabulated in Table 2.

Incubation of Sodium [1,2-$^{13}C_2$]*Acetate with* B. cinerea.—A mixture of 95% sodium [1,2-$^{13}C_2$]acetate (197 mg) and sodium [2-^{14}C]acetate (4.68 μCi) in sterile water (10 ml) was distributed in small portions between two surface cultures (350 ml each) of *B. cinerea* over days 2—4 from inoculation. After 7 days total growth, the metabolites were isolated as above to afford dihydrobotrydial (100 mg) as prisms, m.p. 158—160 °C (404 d.p.m. mg^{-1}; ^{14}C incorporation 0.39%). The spectral data are given in Table 2.

Preparation of the Ether (8).—The acetoxy-triol (12) (m.p. 173—175 °C, lit.,[2] m.p. 175—176 °C) (399 mg) in dry pyridine (15 ml) was treated with toluene-*p*-sulphonyl chloride (800 mg) for 4 d. The solution was diluted with water, acidified, and the product recovered in ethyl acetate. Preparative layer chromatography in light petroleum–ethyl acetate (2 : 1) gave the ether (8) (188 mg) which crystallized from light petroleum as needles, m.p. 138—141 °C (lit.,[2] 130—132 °C) (Found: C, 68.8; H, 9.5. Calc. for $C_{17}H_{28}O_4$: C, 68.9; H, 9.5%).

Hydrolysis of the Ether (8).—The above ether (175 mg) in aqueous methanolic 1M-potassium carbonate (15 ml) was heated under reflux for 3 h. The solution was concentrated and the product recovered in ethyl acetate and purified by preparative layer chromatography to afford the diol (9) (68 mg) which crystallized from ether–light petroleum as needles, m.p. 173—175 °C (lit.,[2] 164 °C) (Found: C, 70.9; H, 10.2. Calc. for $C_{15}H_{26}O_3$: C, 70.8; H, 10.3%).

We thank Mrs. N. Dransfield for growing the cultures and Mr. M. Siverns for the determination of some of the ^{13}C spectra. We thank the S.R.C. for financial support.

[0/1558 *Received*, 13*th October*, 1980]

REFERENCES

[1] Part 23, J. R. Hanson and F. Y. Sarah, *J. Chem. Soc., Perkin Trans. 1*, 1979, 3151.
[2] H.-W. Fehlhaber, R. Geipal, H.-J. Mercker, R. Tschesche, and K. Welmar, *Chem. Ber.*, 1974, **107**, 1720.
[3] H. J. Linder and B. von Grosse, *Chem. Ber.*, 1974, **107**, 3332.
[4] O. Cuevas and J. R. Hanson, *Phytochemistry*, 1977, **16**, 1061.
[5] A. P. W. Bradshaw and J. R. Hanson, *J. Chem. Soc., Perkin Trans. 1*, 1980, 741.

[6] Part of this work was described in preliminary communications: J. R. Hanson and R. Nyfeler, *J. Chem. Soc., Chem. Commun.*, 1976, 72; A. P. W. Bradshaw, J. R. Hanson, and M. Siverns, *J. Chem. Soc., Chem. Commun.*, 1977, 819.

[7] J. B. Stothers and C. T. Tan, *Can. J. Chem.*, 1974, **52**, 308.

[8] D. K. Dalling and D. M. Grant, *J. Am. Chem. Soc.*, 1967, **89**, 6612.

[9] R. A. Ellison and P. K. Bhatnager, *Synthesis*, 1974, 719.

[10] See for example, H. Seto, T. Sato, and H. Yonehara, *J. Am. Chem. Soc.*, 1973, **95**, 3461; T. J. Simpson and J. S. E. Holker, *Phytochemistry*, 1977, **16**, 229.

[11] B. Dockerill and J. R. Hanson, *Phytochemistry*, 1978, **17**, 427.

[12] R. Croteau and W. D. Loomis, *Phytochemistry*, 1972, **11**, 1055.

[13] J. R. Hanson and A. F. White, *J. Chem. Soc. C*, 1969, 981.