Studies in Terpenoid Biosynthesis. Part 30.[1] The Acetate and Mevalonate Labelling Patterns of the Diterpenoid, Aphidicolin

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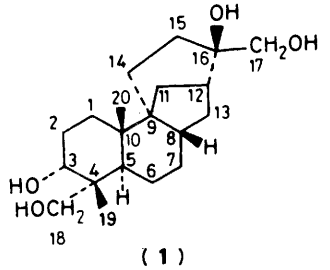
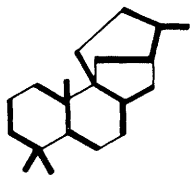
The ^{13}C n.m.r. spectra of aphidicolin and its derivatives have been assigned. The enrichment and coupling patterns of aphidicolin biosynthesized from [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetate were determined and used to define the constituent isoprene units. The numbers of 2- and 5-mevalonoid hydrogen atoms incorporated into aphidicolin were determined by ^{3}H labelling. The generation of ^{2}H–^{13}C coupling which was observed in the ^{2}H n.m.r. spectrum of aphidicolin biosynthesized from [4-$^{2}H_2$,3-^{13}C]mevalonic acid, has established the migration of a 9β-hydrogen atom to C-8 during the biosynthesis.

Since its isolation from *Cephalosporium aphidicola* and the elucidation of its structure,[2] the diterpenoid fungal metabolite aphidicolin (**1**) has attracted considerable interest because of its biological activity as an antiviral and antitumour agent. We have examined the biosynthesis of aphidicolin in the context of

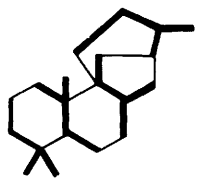


(**1**)

our interest in the biosynthesis of tri- and tetra-cyclic fungal diterpenoids including rosenonolactone,[3] *ent*-kaurene,[4] the kaurenolides,[5] and the gibberellins[6] to which aphidicolin bears some similarities. Plausible biogenetic schemes suggest that its unique tetracarbocyclic carbon skeleton may be derived by a modification of the normal diterpenoid cyclization of geranylgeranyl pyrophosphate,[2,7] however, this poses several biosynthetic problems. Firstly there are several ways [*e.g.* structures (**2**) and (**3**)] in which the carbon skeleton may be



(**2**)

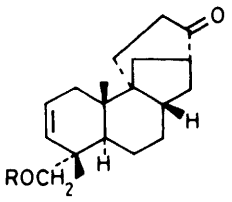


(**3**)

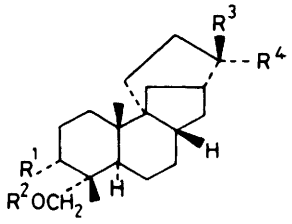
dissected into its constituent isoprene units; secondly, implicit in the cyclization stages there is a possible hydrogen migration to C-8 (*cf.* rosenonolactone biosynthesis) and an abnormal chair–boat folding of geranylgeranyl pyrophosphate; and finally there is the need to define the order of oxidative events and the origin of the oxygen atoms, particularly of the vicinal glycol.

A preliminary report has appeared[7] on the incorporation of [1,2-$^{13}C_2$]acetate and (4*R*)-[4-^{3}H]mevalonate into aphidicolin, and the assignment of the centres labelled from C-2 of mevalonate was attempted, utilizing the principle that in isoprenoid units biosynthesized from [1,2-$^{13}C_2$]acetate, the atom that is derived *via* C-2 of mevalonate is an enriched singlet and does not show a ^{13}C–^{13}C coupling. This, however, must rest on the assumption, unproven in the case of aphidicolin, that a rearrangement does not occur leading to the cleavage of coupled units; it also requires an assignment of the ^{13}C n.m.r. signals. Two of the relevant signals (C-1 and C-7) were assigned on 'general biosynthetic grounds' and as expected were found to be singlets derived from C-2 of mevalonate. There was some confusion in the assignment of a third label to C-18 or -19; a signal at δ 71.5 p.p.m. is unlikely to be due to a CMe but could be assigned to a carbon bearing an oxygen atom. Hence we felt it necessary to re-examine this aspect of the problem. In this paper we are concerned with the elucidation of the labelling pattern of the isoprenoid units in aphidicolin biosynthesis. A preliminary communication has appeared on some aspects of our work.[8]

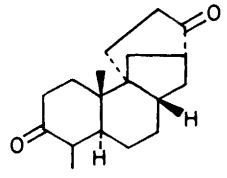
A full assignment of the natural abundance ^{13}C n.m.r. spectrum of aphidicolin was required as a prelude to the application of ^{2}H- and ^{13}C-labelling studies to the biosynthesis. Although a partial assignment had been made in the earlier work,[7] our assignments are not in accord with this. However they do agree with an independent study on aphidicolin which was published[9] after our preliminary communication. There have been a number of studies of the ^{13}C n.m.r. spectra of tetracyclic diterpenoids[10] which provided suitable analogies for our work. The ^{13}C n.m.r. signals of aphidicolin and a number of derivatives (**4**)—(**14**) (see Table 1) were classified according to their multiplicity in the SFORD spectra. The resonances attributed to C-3, -16, -17, and -18 were readily distinguished by their chemical shift and by comparison with the 17-nor-16-ketone (**6**), the spectrum of which was determined in both [$^{2}H_5$]pyridine and [^{2}H]chloroform. Of the singlets, the C-4 resonance was readily assigned by its downfield shift (Δδ 12.9 p.p.m.) on the oxidation of C-3 to a ketone group. In the tetracyclic diterpenoids C-10 lies within the range δ 36—40 p.p.m. and hence the singlet resonance at δ 40.1 p.p.m. was assigned to C-10 and that at δ 48.5 p.p.m. to C-9. The conversion of aphidicolin into the 17-nor-16-ketone (**6**) left the doublet signals at δ 34.0 and 40.5 p.p.m. relatively unchanged (C-5 and -8) but greatly shifted (Δδ 6.3 p.p.m.) the signal at δ 42.1 p.p.m. which was consequently assigned to C-12. On the other hand, conversion into the 3-ketone (**11**) shifted downfield (Δδ 5.4 p.p.m.) the signal at δ 34.0 p.p.m. which was therefore assigned to C-5. The signal at δ 40.5 p.p.m. (C-8) again remained relatively unchanged. The triplet resonances were assigned as follows. The conversion into the 3-ketone (**11**) resulted in the downfield shift of two signals at δ 27.2 and 27.4 p.p.m. to δ 32.7 (C-1) and δ 36.2 (C-2) p.p.m. respectively. A resonance at δ 29.1



(8) R = H
(9) R = CHO



(4) R^1 = OTMS, R^2 = TMS, R^3 = OTMS, R^4 = CH_2OTMS
(5) R^1 = OH, R^2 = H, R^3 = OH, R^4 = Me
(6) R^1 = OH, R^2 = H, R^3, R^4 = O
(7) R^1 = R^2 = H, R^3, R^4 = O
(11) R^1 = =O, R^2 = H, R^3 = OH, R^4 = CH_2OH
(12) R^1 = OH, R^2 = H, R^3, R^4 = CH_2
(13) R^1 = OH, R^2 = H, R^3, R^4 = 



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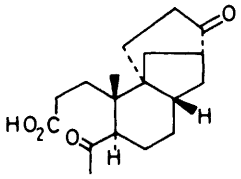


(14)

TMS = $SiMe_3$

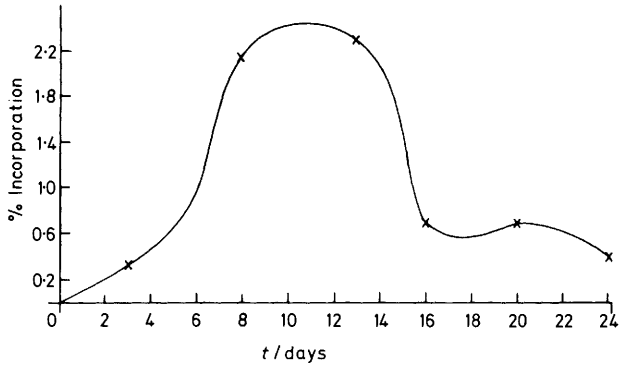


Figure 1. % Incorporation with time of sodium [1-^{14}C]acetate into aphidicolin

functionality changed. On the other hand, the resonance at 27.4 p.p.m. remained relatively constant throughout the transformations and was therefore assigned to C-7. The methyl group signals were distinguished by the greater variations in chemical shift shown by C-19 on structural modification at C-3.

Although *Cephalosporium aphidicola* (CMI 68689) can be grown in both shake and surface culture, in our hands aphidicolin was only produced in surface cultures. A good yield (*ca.* 50 mg l^{-1}) was obtained after 28 days growth. Preliminary studies with sodium [1-^{14}C]acetate (see Figure 1) revealed that the optimum time for feeding labelled substrates to obtain a good incorporation into aphidicolin lay between day 8 and day 13 after inoculation. This corresponded to the end of the exponential growth phase.

Table 1. ^{13}C N.m.r. signals of aphidicolin derivatives

Carbon Atom	δ/p.p.m.											
	(1)[b]	(4)[c]	(5)[b]	(6)[c]	(7)[c]	(8)[c]	(9)[c]	(10)[c]	(11)[c]	(12)[c]	(13)[c]	(14)[c]
1	27.2	27.2	27.1	26.7	34.3[a]	35.5	35.2	35.5	32.1[a]	26.9	26.7	30.4
2	27.4	26.3	27.2	26.7	18.4	127.8[a]	125.9[a]	37.8	36.2	27.1	26.7	37.7
3	76.3	71.2	76.1	76.9	35.5[a]	132.9[a]	132.1[a]	211.9	218.6	76.9	76.7	178.5
4	40.9	41.9	40.9	40.3	37.4	40.6	38.5	45.6	52.9	40.4	40.1	212.7
5	34.0	36.8	34.2	33.1	38.8	35.8	37.4	45.5	39.4	33.0	33.0	54.8
6	23.5	23.4	23.4	22.8	22.9	24.5	24.2	27.1	24.4	22.8	22.6	24.0
7	27.4	26.1	26.6	26.1	26.1	25.9	25.8	25.3	26.1	26.1	26.0	26.7
8	40.5	40.1	40.4	41.4	41.2	41.1	41.0	40.1	41.0	41.0	41.6	41.7
9	49.6	49.1	49.4	49.2	48.9	48.0	48.0	47.5	48.4	49.2	48.4	47.5
10	40.1	40.0	39.8	39.7	39.9	39.0	39.0	39.3	39.1	39.3	39.6	42.3
11	33.3	33.3	35.8	33.9	33.9	33.8	33.6	33.6	32.7[a]	34.1	35.5	33.6
12	42.1	41.5	47.9	48.4	48.3	48.8	48.6	48.3	40.8	43.4	40.1	48.4
13	31.7	31.7	34.4	26.2	31.7	31.7	31.7	31.6	31.2	26.4	31.6[a]	30.8
14	25.5	24.3	27.1	21.8	22.2	23.8	23.9	23.3	24.4	28.6	26.4[a]	25.3[a]
15	29.1	29.0	30.0	34.5	34.3	34.1	34.1	34.0	27.9	39.7	26.7[a]	34.5
16	74.2	78.7	71.9	208.3	215.8	215.8	214.0	213.7	74.3	155.3	63.3	214.5
17	68.3	67.7	27.8						67.7	102.1	51.0	
18	71.8	69.2	71.7	71.7	71.8	70.0	70.8		67.2	71.6	71.5	
19	18.1	16.4	18.0	17.7	17.7	18.5	18.5	11.9	17.8	17.7	17.6	33.4
20	15.4	15.3	15.5	15.7	15.7	15.6	15.8	13.4	14.8	15.1	15.0	13.6

[a] These resonances may be interchanged. [b] Spectrum determined in [$^2H_5$]pyridine. [c] Spectrum determined in deuteriochloroform.

p.p.m. which was shifted to δ 34.5 p.p.m. in the 17-nor-16-ketone (*6*) and disappeared on base-catalysed deuteriation[7] was assigned to C-15. The assignment of the remaining triplet resonances on rings C and D (C-11, -13, and -14) followed from the γ- and δ-upfield shifts which they showed[11] compared with the corresponding signals in the triol (**5**). The resonance at δ 23.5 p.p.m. was assigned to C-6 by comparison with kaurenoid models and from the fact that it varied slightly as the ring A

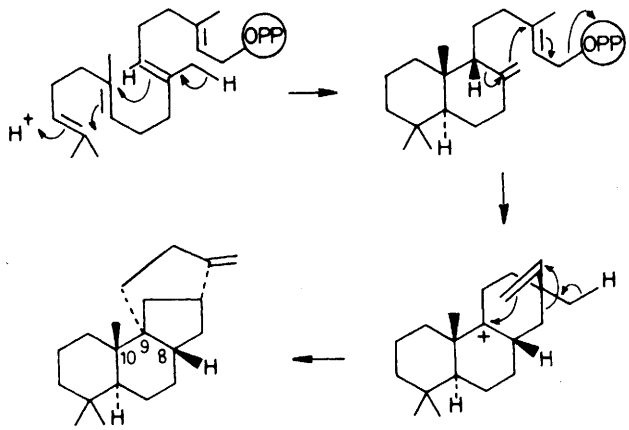
Sodium [1-^{13}C]- and [2-^{13}C]acetate together with sodium [1-^{14}C]acetate as a tracer were fed separately to the fungus on the tenth day after inoculation. Sodium [1,2-$^{13}C_2$]acetate also containing sodium [1-^{14}C]acetate was diluted with an equivalent amount of unlabelled material and fed on four consecutive days (days 10—13). The enrichment and coupling patterns of the resultant samples of aphidicolin are given in Table 2. These patterns (see Figure 2) conform to the labelling

Table 2. Enrichments and coupling constants from aphidicolin biosynthesized from [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetate

Carbon Atom	δ/p.p.m.	Normalized peak height [1-^{13}C]	Normalized peak height [2-^{13}C]	Coupling constant/Hz
1	27.2	1.00	1.80	s
2	27.4	1.28[a]	1.33[a]	34.9
3	76.3	1.05	1.78	36.6
4	40.9	1.31	0.90	34.9
5	34.0	1.19	2.14	34.9
6	23.5	1.56	1.10	34.9
7	27.4	1.25[a]	1.33[a]	s
8	40.5	1.42	1.04	34.0
9	49.6	1.02	1.69	34.9
10	40.1	1.41	0.96	35.8
11	33.3	1.64	1.14	32.3
12	42.1	1.08	1.84	32.3
13	31.7	1.10	1.85	34.0
14	25.5	1.51	1.10	34.9
15	29.1	1.00	1.15	s
16	74.2	1.33	0.93	41.9
17	68.3	1.10	1.86	42.7
18	71.8	1.00	1.63	s
19	18.1	1.25	2.14	35.8
20	15.4	1.10	2.04	36.6

[a] Overlapping signal enriched by both [1-^{13}C]- and [2-^{13}C]-acetate.

scheme which would be expected for the cyclization based on geranylgeranyl pyrophosphate shown in Scheme 1. Further-



Scheme 1.

more it defines the isoprenoid units in rings C and D. The aphidicolin enriched from [2-^{13}C]acetate showed an induced coupling (J 32 Hz) between C-12 (δ 41.0 p.p.m.) and C-13 (δ30.6 p.p.m.) which lends support to the proposed Wagner–Meerwein rearrangement in the biosynthesis.

[2-^{3}H,2-^{14}C]- and [5-^{3}H,2-^{14}C]-Mevalonic acids were fed to *C. aphidicola*. The atom ratios of the resultant aphidicolin are given in Table 3. The feeding of (4*R*)-[4-^{3}H,2-^{14}C]mevalonic acid had previously been reported by Bu'Lock.[7] There was some loss of tritium in the [2-^{3}H,2-^{14}C]mevalonate experiment which is probably associated with hydroxylation at C-18 and the action of prenyl isomerase. These results show that during the second cyclization step (see Scheme 1) discharge of a carbocation to form an alkene, followed by a reprotonation has not occurred.

Implicit in the biosynthetic scheme which utilizes an abnormal 9β,10β-*syn*-labdane intermediate is the migration of a 9β-hydrogen atom to C-8β to generate the C-9 carbocation required for cyclization. An alternative means of generating the

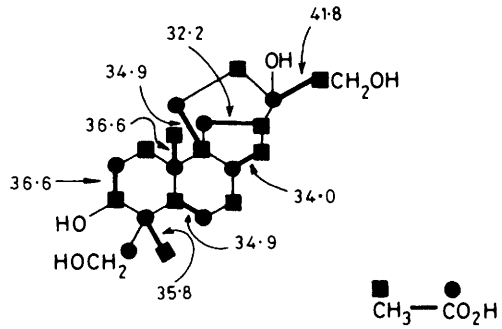
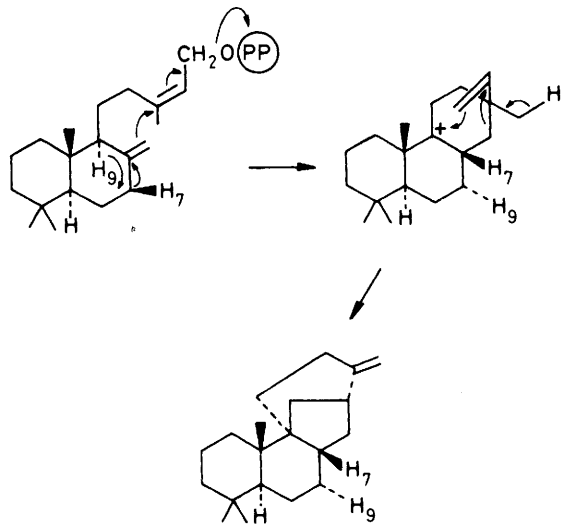


Figure 2. Coupling and enrichment patterns from [^{13}C]acetates. J Values in Hz

Table 3. Incorporation of [^{3}H,^{14}C]mevalonates into aphidicolin by *Cephalosporium aphidicola*

	[2-$^{3}H_2$,2-^{14}C]-Mevalonic acid	[5-$^{3}H_2$,2-^{14}C]-Mevalonic acid
^{3}H:^{14}C Ratio as fed	10.31:1	12.3:1
Amount ^{14}C fed	440.1 kBq	325.88 kBq
^{3}H:^{14}C Ratio in aphidicolin	9.56:1	12.0:1
Atom ratio in aphidicolin	7.42:4	7.87:4
% Incorporation	0.93	2.99

C-9 carbocation in the second cyclization step would involve the shift of a hydrogen from C-7β to C-8β and from C-9α to C-7α (see Scheme 2); this would have the merit of utilizing the



Scheme 2.

more normal 9α,10β-*anti*-labdane intermediate. These two possibilities were distinguished by following the fate of the relevant mevalonoid 4-H.

The acetate labelling studies showed that C-8 originates from C-3 of mevalonic acid whilst C-9 originates from C-4. Although the earlier work [7] had shown that aphidicolin retained three *pro*-(4*R*)-[4-^{3}H]mevalonoid labels and established the location of one of these at C-12, the position of the key mevalonoid hydrogen atom involved in the rearrangement was not determined. We therefore examined the putative hydrogen migration from C-9 to C-8 by feeding [4-$^{2}H_2$]- and [4-$^{2}H_2$,3-^{13}C]-mevalonic acids. If a [4-^{2}H]-mevalonoid hydrogen atom

Tabel 4. Feeding experiments with *Cephalosporium aphidicola*

Substrate	Quantity fed/mg (kBq ^{14}C)	Volume of fermentation	Period of fermentation day–day	Amount of aphidicolin/mg	% Incorporation
Sodium [1-^{13}C,1-^{14}C]acetate	500 (456)	2.25	10—28	139	0.76
Sodium [2-^{13}C,1-^{14}C]acetate	500 (456)	2.25	10—28	134	0.79
Sodium [1,2-^{13}C,1-^{14}C]acetate	300[a] (456)	2.25	10, 11, 12, 13—28	82	0.55
[2-^{3}H,2-^{14}C]Mevalonic acid	400	2.25	10, 11, 12, 13—28	300	0.93
[5-^{3}H,2-^{14}C]Mevalonic acid	130.35	2.25	8—28	290	2.9
[4-$^{2}H_2$,2-^{14}C]Mevalonic acid	175 (532)	3.0	8, 9, 10, 11—28	95	5.26
[4-$^{2}H_2$,3-^{13}C,2-^{14}C]Mevalonic acid	50 (532)	1.5	8, 9, 10, 11—28	69	4.2

[a] Diluted with 300 mg of unlabelled material.

migrates from C-9 to C-8 within an isoprene unit, then in the doubly labelled species a new ^{2}H—^{13}C coupling will be generated. On the other hand no such coupling will be observed if the rearrangement takes the C-9α to C-7α/9b to C-8β course as in Scheme 2. The [4-$^{2}H_2$]- and [4-$^{2}H_2$,3-^{13}C]-mevalonic acids were fed to *C. aphidicola*. Since deuterium has a spin of 1, the heteronuclear [^{13}C—^{2}H] coupling is more easily observed in the ^{2}H n.m.r. spectrum. The aphidicolin was converted into its more soluble tetra(trimethylsilyl) derivative.[2] The ^{2}H n.m.r. spectrum (determined at 55.28 MHz) of the material from the singly labelled mevalonate showed three overlapping signals at δ 1.90, 2.03, and 2.10. In the doubly labelled material the peak at δ 1.90 was replaced by a doublet (*J* 20 Hz). The location of the deuterium at C-8 was established by examination of the ^{13}C n.m.r. spectrum. This showed that the signal at δ 39.8 p.p.m., assigned to C-8, bore satellites from the ^{13}C–^{2}H material (*J* 18.5, 1.5 Hz) and showed an upfield shift of Δδ 0.23 p.p.m. We conclude that the postulated hydrogen shift occurred directly from C-9 to C-8 and hence a 10β,9β-*syn*-labdane intermediate is probably involved in the biosynthesis.

Experimental

General experimental details have been described previously.[5]

General Fermentation Conditions.—*Cephalosporium aphidicola* (CMI 68689) was grown on surface culture (750 ml per bottle) on the following medium: glucose (50 g l^{-1}), potassium dihydrogen phosphate (5 g l^{-1}), magnesium sulphate (2 g l^{-1}), potassium chloride (1 g l^{-1}), glycine (2 g l^{-1}), and trace elements solution (2 ml l^{-1}). The trace elements solution contained (per 100 ml), cobalt nitrate (0.1 g), copper sulphate (0.015 g), zinc sulphate (0.161 g), manganese sulphate (0.01 g), and ammonium molybdate (0.01 g). After 28 days growth the mycelium was filtered off and the broth extracted with ethyl acetate. The extract was dried and the solvent evaporated to give a crude gum which was chromatographed on silica (Merck 9385). Elution with 5% methanol in ethyl acetate gave aphidicolin (*ca.* 50 mg l^{-1}), m.p. 288—233 °C (lit.,[2] 227—233 °C) identified by comparison (t.l.c., n.m.r.) with an authentic sample.

Feeding Experiments.—(*a*) *Optimization of time of feeding.* 18 Thompson bottles (750 ml of medium each) were inoculated as above and divided into six groups of three. Sodium [2-^{14}C]-acetate (0.44 MBq) was administered to one group of bottles on day 3 and to the others on days 8, 13, 16, 20, and 24 respectively. Each group was harvested on day 28. The aphidicolin was isolated and crystallized to constant radioactivity. The results are shown in Figure 1.

(*b*) *Feeding of labelled substrates.* The amounts of substrate used and the results of the feeding experiments are given in Table 4.

Spectral Determinations.—The ^{13}C n.m.r. spectra of aphidicolin and its derivatives were determined at 20.15 and 90.56 MHz on Bruker WP 80 and WH 360 spectrometers for solutions in [$^{2}H_5$]pyridine or [^{2}H]chloroform. The ^{2}H n.m.r. spectra were determined on the tetra(trimethylsilyl) derivative[2] of aphidicolin at 55.28 MHz on a Bruker WH 360 spectrometer. We thank Dr. I. H. Sadler (University of Edinburgh) for these two determinations.

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Received 14*th March* 1984; *Paper* 4/409