Studies in Terpenoid Biosynthesis, Part 28.[1] The Acetate and Mevalonate Labelling Patterns of the Steroid, Demethoxyviridin

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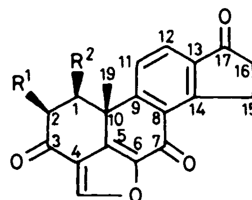
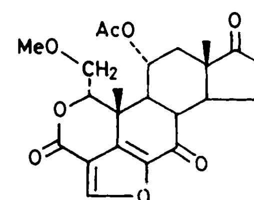
The enrichment and labelling patterns of demethoxyviridin, biosynthesized by *Nodulisporium hinnuleum* from [1-^{13}C]-, [1,2-$^{13}C_2$]-acetate, [2-^{13}C]- and [5-^{13}C]-mevalonate have been used to define the isoprene units in this metabolite and are consistent with a triterpenoid origin. The number and location of the hydrogen atoms originating from acetate and the 2-, 4-, and 5-positions of mevalonate, have been determined by a combination of ^{3}H : ^{14}C ratio and ^{2}H n.m.r. studies.

The biosynthesis of steroids may be divided [2,3] into two phases. The first synthetic phase involves the assembly of the prenyl units and their cyclization to afford the C_{30} carbon skeleton of lanosterol and its relatives whilst the second phase involves the oxidative degradation of the C_{30} skeleton to afford the individual families of steroid. Acetate and mevalonate labelling patterns have provided considerable information in this context. There are a number of fungal steroidal antibiotics. Amongst these the biosynthesis of fusidic acid and its relatives, has been studied in some detail.[4] Demethoxyviridin (1) which is produced [5] by the fungus, *Nodulisporium hinnuleum*, has an androstane structure but with an aromatic ring C. The formation of the fungal androstanes of the viridin group invites comparison with bacterial, plant, and mammalian steroid biosynthesis. Earlier degradative experiments [6] on viridin (2) biosynthesized from [2-^{14}C]mevalonate located labels at C-1, C-7, and C-15 consistent with its formation in a steroid-like manner. Some preliminary experiments have also been reported [7] on the biosynthesis of a similar fungal metabolite, wortmannin (3) which does not have an aromatic ring C. Some studies on the incorporation of sterol precursors into viridin have also been reported.[8] In this paper we describe some experiments which define the mevalonoid origin of the carbon skeleton and hydrogen atoms of demethoxyviridin (1).[9]

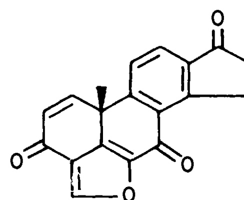
As a prelude to the application of ^{13}C labelling studies to the biosynthesis, the ^{13}C n.m.r. signals from demethoxyviridin were assigned. (see Table 1). The resonances were assigned using their multiplicities in the SFORD spectra combined with known substituent effects and simple models. The three triplets in demethoxyviridin at δ 28.2, 35.7, and 46.5 were assigned to C-15, C-16, and C-2 respectively. The resonances of C-15 and C-16 were relatively insensitive to changes in the substitution of ring A and are in agreement with the assignments of the comparable carbon atoms of indanone.[10] The doublets assigned to C-11 and C-12 (δ 128.4 and 126.2 respectively) also paralleled those of an indanone model,[10] whilst the extra furanoid carbon atom (δ 149.4) was assigned by comparison with furan. Amongst the singlets, the carbonyl signals at δ 205.5 and 172.5 were relatively insensitive to changes in the substitution pattern of ring A and were therefore assigned to C-17 and C-7 respectively leaving the carbonyl singlet at δ 190 to be assigned to C-3. The signal at δ 122.9 was assigned to C-4 on the basis of its upfield shift on reduction of C-3. The aromatic singlets [δ 129.5, 126.3, and 156.9 (two resonances)] were assigned to C-8, C-13, C-9, and C-14 respectively again by comparison with the appropriate signals in the spectrum of indanone. The remaining singlets (δ 144.3 and 145.55) were assigned to C-5 and C-6 respectively, the former being more sensitive to structural variations on ring A. The preparation of the derivatives will be described in a paper on the chemistry of demethoxyviridin.



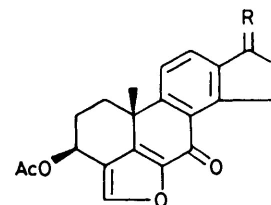


(1) R^1 = H, R^2 = OH
(2) R^1 = OMe, R^2 = OH
(4) R^1 = H, R^2 = OAc
(6) R^1 = R^2 = H
(9) R^1 = H, R^2 = α-OMe, β-H

(3)



(5)



(7) R = O
(8) R = OAc,H

Preliminary feeding experiments with [2-^{14}C]mevalonic acid established that the optimum time to feed mevalonate to achieve a good incorporation into demethoxyviridin by surface cultures of *Nodulisporium hinnuleum*, was 10—11 days after inoculation, with the fungus being harvested 20 days after inoculation (see Figure).

Sodium [1-^{13}C]- and [1,2-$^{13}C_2$]-acetate and [2-^{13}C]- and [5-^{13}C]-mevalonate were then fed separately to *N. hinnuleum*. The ^{13}C-enrichment and ^{13}C–^{13}C coupling patterns of the resultant samples of demethoxyviridin are shown in (10) and (11) whilst the numerical values are given in Table 2.

The coupling and enrichment patterns are consistent with a triterpenoid biosynthesis.[2] There was sufficient enrichment from the [1-^{13}C]acetate experiment which was carried out as a 'single-dose' feeding for couplings (*J* 55 Hz) to be observed between the adjacent enriched centres C-11 and C-12 and C-8–C-14 arising from multiply labelled precursors. These coupling patterns show that the aromatic ring has been formed without skeletal rearrangement. These experiments also defined the origin of the 'extra' carbon atom attached to C-4, constituting part of the furan ring. Although it was likely that

Table 1. ^{13}C N.m.r. signals of demethoxyviridin and some derivatives (determined in $CDCl_3$ at 25 MHz, p.p.m. from Me_4Si)

Carbon no.	Compound (1) [a]	(4) [b]	(5)	(6)	(7) [c]	(8) [d]	(9) [e]
1	70.86	72.68	147.88	32.64	26.15	62.19	83.55
2	46.53	42.65	131.29	36.22	28.33	28.58	39.93
3	189.96	187.6	179.23	191.48	64.13	73.11	189.18
4	122.92	122.92	121.59	121.83	120.98	120.25	121.83
5	144.34	142.09	145.37	144.28	144.70	141.85	144.03
6	145.55	146.34	145.73	146.04	144.34	144.88	146.70
7	172.55	172.73	172.86	172.49	173.52	173.03	173.28
8	129.54	130.2	130.69	129.41	130.51	130.44	131.05
9	156.29	153.38	151.92	156.71	157.69	154.29	154.35
10	41.56	40.47	40.71	36.34	37.01	40.53	42.77
11	128.44	126.99	124.25	125.83	124.74	126.87	124.68
12	126.2	127.41	127.55	126.5	126.94	126.87	127.35
13	136.33	137.60	137.72	136.39	137.06	137.30	137.36
14	156.29	158.23	159.08	157.26	158.35	158.17	158.96
15	28.15	28.58	28.45	27.85	32.94	36.34	28.45
16	35.17	36.28	36.34	35.67	36.46	32.34	36.4
17	205.5	205.92	205.92	205.14	206.41	206.35	206.22
19	25.24	26.21	41.07	30.03	32.40	26.34	31.06
Furan-C	149.37	148.28	148.22	149.68	148.16	148.46	148.34

[a] In DMSO. [b] OAc, 21.30, 169.15. [c] OAc, 21.17, 171.09. [d] OAc, 21.00, 21.42, 169.46, 170.68. [e] OMe 61.3.

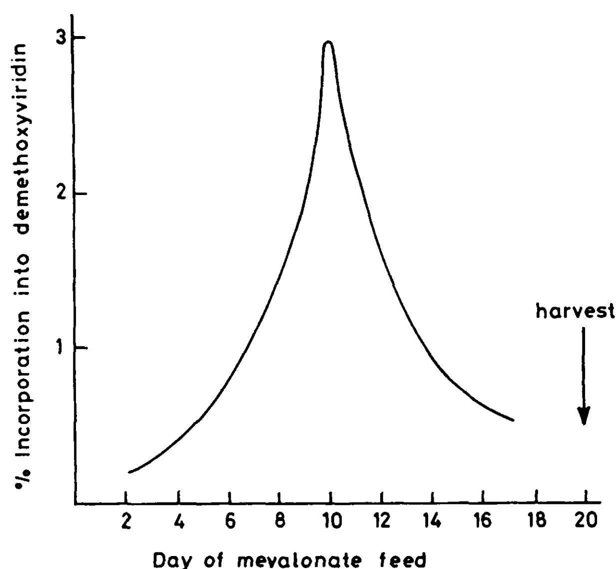
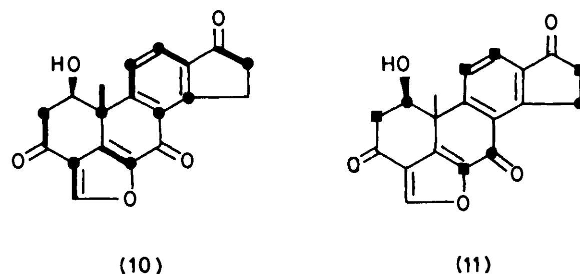


Figure. Variation of mevalonate incorporation with time of feeding to *N. hinnuleum*

this carbon atom was part of a prenyl unit, nevertheless it could have arisen from the C-1 pool at a late stage in the biosynthesis. The fungus produces ergosterol which lacks both the substituents at this centre. Furthermore, there are chemical analogies [11] for the formation of a furan ring at C-4 by the intramolecular condensation of a C-6 ester. The retention of the coupling between C-4 and the carbon atom of the furan ring showed that this bond had remained intact throughout the biosynthesis from acetate. The stereochemistry of labelling of the cyclic triterpenoids at this centre is known.[2] The [1,2-$^{13}C_2$]acetate results show that the extra carbon atom at C-4 originates from the 3′-position rather than from C-2 of mevalonate. It thus corresponds to the 4β-methyl group of a protolanosterol/lanosterol precursor. This contrasts with fusidic acid biosynthesis by the fungus *Fusidium coccineum* in which the other methyl group derived from C-2 of mevalonate, is retained.[12] In sterol biosynthesis, the normal order of demethylation involves loss of the 4α-methyl group first.



(10) = Coupling derived from [1,2-$^{13}C_2$]acetate, ● = enrichment from [1-^{13}C]acetate, (11) ● = Enrichment from [2-^{13}C]-MVA, ■ = enrichment from [5-^{13}C]-MVA

The [2-^{13}C]mevalonate results are in accordance with the earlier carbon-14 work [6] on viridin but strictly define only three out of the five isoprene units. The [5-^{13}C]mevalonate results served to distinguish all five isoprene units which go to form demethoxyviridin thus providing further evidence for excluding a diterpenoid precursor of the cleistanthene type (*cf.* ref. 6).

Having defined the mevalonoid origin of the carbon skeleton, we turned our attention to the number and location of the mevalonoid hydrogen atoms. The number of mevalonoid hydrogen atoms that were incorporated, were established by conventional ^3H : ^{14}C ratio studies and are tabulated (see Table 3).

The location of the labels was established by ^2H n.m.r. spectroscopy. Whereas demethoxyviridin is poorly soluble or unstable in suitable n.m.r. solvents over the time scale required for ^2H spectroscopy, the acetate is conveniently soluble in chloroform. The ^1H n.m.r. signals of demethoxyviridin acetate (4) were assigned from the 220 MHz spectrum (see Table 4). The signals assigned to the 2-H and the 15- and 16-H were identified by decoupling experiments based on the readily identified 1-H signal.

[2-$^2H_3$]Acetate and [2-$^2H_2$]- and [5-$^2H_2$]-mevalonate were then fed to the fungus. The demethoxyviridin was isolated and

Table 2. ^{13}C Enrichment * and coupling patterns (*J*/Hz) of demethoxyviridin (1)

Carbon atom	Precursor [1-^{13}C]-Acetate	[1,2-$^{13}C_2$]-Acetate	[2-^{13}C]-MVA	[5-^{13}C]-MVA
1	0.99		2.02	1.0
2	1.75	39	1.01	1.37
3	1.14	39	1.02	0.91
4	2.02	65	1.22	0.93
5	1.1	37	1.11	1.06
6	1.57	37	0.62	1.18
7	0.73		1.67	1.01
8	1.58 †		0.94	1.02
9	0.95	53	1.03	0.96
10	1.97	33	1.03	0.99
11	1.43 †	53	0.95	1.33
12	1.61 †	58	1.09	1.41
13	0.95	58	0.91	0.94
14	1.53 †		0.95	0.99
15	1.08		2.0	0.93
16	1.91	39	0.99	1.34
17	1.08	39	0.96	1.0
19	0.95	33	1.0	0.96
Furan-C	1.02	65	0.96	1.06

* Enrichment = $\frac{\text{Enriched spectrum peak height/normalising factor}}{\text{Natural abundance spectrum peak height/normalising factor}}$

Normalising factor = $\frac{\text{Sum of unlabelled peak heights}}{\text{Number of peaks}}$

† *J* 55 Hz.

Table 3. Incorporation of [^{3}H]mevalonates into demethoxyviridin (1)

	Mevalonate [a] [2(*R*)-2^{3}H,2-^{14}C]-		[4(*R*)-4-^{3}H,2-^{14}C]-	[5-$^3H_2$,2-^{14}C]-
^{3}H : ^{14}C Ratio as fed	2.76 : 1	4.2 : 1	4.5 : 1	1.65 : 1
Amount ^{14}C fed	30 μC	21 μC	30 μC	30 μC
^{3}H : ^{14}C Ratio in (1)	0.61 : 1	0.72 : 1	0.12 : 1	1.3 : 1
% Incorp. ^{14}C	3.0	0.48	3.3	3.09
Atom ratio in (1)	0.67 : 3	0.51 : 3	0.08 : 3	4.72 : 3

[a] Fed as the (3*RS*)-mixtures.

converted into its acetate (4). The results are tabulated (Table 5). These results conform to the pattern anticipated for a steroid. The [2-$^2H_3$]acetate results show that the 19-methyl group retains all three deuterium labels thus excluding a cyclopropanoid precursor related to cycloartenol from the biosynthesis, *i.e.* the biosynthesis follows a mammalian rather than a plant scheme. Subsequent to our work [9] lanosterol rather than cycloartenol has been shown to be [13] the precursor of the sterols of the fungus *Uromyces phaseoli*. An interesting feature of the [2-$^2H_3$]acetate experiment is that the signals arising *via* the 2-methylene group of mevalonate show a small drop in integral compared to those derived *via* the 3′-methyl group. Although this may reflect the action of prenyl isomerase, it might also arise from isotope effects later in the biosynthesis.

The 1-H and 15-H signals were equally labelled from [2-$^2H_2$]mevalonate. The [2-(*R*)-2-^{3}H,2-^{14}C]mevalonate results show that 0.67 atom/molecule of demethoxyviridin were incorporated. Incorporation studies with [1α,2α-$^2H_2$]dehydr-

Table 4. ^{1}H N.m.r. signals of demethoxyviridin acetate (4) (in $CDCl_3$ at 220 MHz)

Proton	Signal (δ)	Multiplicity	Coupling constants (Hz)
1	5.5	dd	11.5, 6
2	3.22	dd	19, 6
2	3.00	dd	19, 11.5
11	8.0	s	
12	8.0	s	
15	3.62	d, t	20, 5.5
15	3.86	d, t	20, 5.5
16	2.78	t	5.5
16	2.78	t	5.5
19	1.8	s	
21	8.24	s	
OAc	2.34	s	

Table 5. ^{2}H N.m.r. signals of biosynthetically enriched demethoxyviridin acetate (4) (in p.p.m.)

Precursor	^{2}H N.m.r. signal (δ)	Rel. integral	^{1}H N.m.r. signal (δ)	Assignment
[$^2H_3$]Acetate [a]	1.76	3.4	1.80	19-H
	3.72	0.91	3.74	15-H
	5.48	0.91	5.5	1-H
	8.3	1	8.32	Furan-H
[2-$^2H_2$]-MVA [b]	3.72	1.0	3.74	15-H
	5.52	1.08	5.5	1-H
[5-$^2H_2$]-MVA [c]	2.8br	4.1	2.78, 3.0,, 3.22	2-H, 16-H
	8.08	1.5	8.0	11-H, 12-H

[a] Determined at 30.3 MHz. [b] Determined at 60.6 MHz. [c] Determined at 55.3 MHz.

oxydemethoxyviridin show that, as expected, hydroxylation at C-1β has proceeded with retention of configuration.[14] Since the 1β-H-position of steroids is labelled by a *pro*-2(*R*)-mevalonoid hydrogen atom, *i.e.* the hydrogen atom which is replaced by a hydroxy-group in demethoxyviridin, the *pro*-2(*R*)-mevalonoid label in demethoxyviridin must be located at 15-H. Although the 15α- and 15β-hydrogen resonances are too close for a confident distinction to be made (δ 3.74 and 3.86) only the more shielded 15α-resonance appears to be labelled in accordance with inversion at this centre. Inversion at this centre has been shown to accompany the loss of a substituent from C-14 in sterol biosynthesis.[15] The aromatic signals at δ 8.08 (11- and 12-H) bore approximately 1.5 labels compared to the 2-H and 16-H signals which bore a total of 4 labels from [5-$^2H_2$]mevalonate. This loss of hydrogen from C-11 and C-12 is in accordance with the loss of one hydrogen atom from the two farnesyl pyrophosphate residues which form squalene.[2] Efforts to separate these signals by using different n.m.r. solvents were not successful. In those situations, *e.g.* demethoxyviridin in dimethyl sulphoxide or trifluoroacetic acid, where a separation was obtained in the ^{1}H n.m.r. spectrum, the ^{2}H n.m.r. signal was either too broad or the sample decomposed during the time required to accumulate the data. On one occassion ergosterol was obtained from a [5-$^2H_2$]mevalonate feed. This showed ^{2}H n.m.r. signals at δ 5.59 (6-H), 5.26 (25-H) and a group of signals at δ 1.25, 1.48, 1.72, 1.85, 2.0 and 2.12 attributable to the labels at 2-H, 11-H, 12-H, and 16-H.

The following conclusions may be drawn from these results. The carbon-13 enrichment and coupling patterns define

Table 6. Feeding experiments to *Nodulisporium hinnuleum*

Precursor	Quantity mg (μC ^{14}C)	Vol. of fermentation litres	Period of incubation (day—day)	Mass of demethoxy-viridin isolated (mg)	% Incorp. ^{14}C
[1-^{13}C]Acetate	1 g (21.6)	2	10—20	335	0.64
[1,2-^{13}C$_2$]Acetate	400 [a] (23)	2	13, 15, 17—22	184	0.40
[2-^{13}C$_2$-MVA	250 (11.7)	2	10—20	45	0.23
[5-^{13}C]-MVA	100 (12.2)	2	10—20	185	1.01
[2(R)2-^{3}H,2-^{14}C]-MVA	30 μC	2	10—20	360	3.0
[4(R)4-^{3}H,2-^{14}C]-MVA	30 μC	2	10—20	430	3.33
[5-^{3}H$_2$,2-^{14}C$_2$-MVA	30 μC	2	10—20	370	3.09
[2-^{2}H$_3$]Acetate	2.5 g (11.7)	5	10—20	344	0.32
[2-^{2}H$_2$]-MVA	1.67 g (12)	4	10—20	370	0.77
[5-^{2}H$_2$]-MVA	300 (12)	4	10—20	1 570	2.3
[5-^{2}H$_2$]-MVA	490 (31)	5	9—19	670	1.2
				30 [b]	0.1

[a] Diluted with 800 mg unlabelled acetate. [b] Ergosterol.

five isoprene units in the structure of demethoxyviridin consistent with a triterpenoid origin. The ' extra ' carbon atom of the furan ring at C-4 comes from the 3′-position of mevalonic acid and represents the 4-methyl group of a protolanosterol/lanosterol precursor. This is consistent with the normal mammalian order of removal of the methyl groups from C-4 of lanosterol in which the initial oxidation and decarboxylation involves the C-4α-methyl group. The enrichment and coupling patterns of the aromatic ring C together with the induced ^{13}C-^{13}C couplings, $J_{11,12}$ and $J_{8,14}$, show that it is formed without skeletal rearrangement of a steroid. The hydrogen labelling patterns are also consistent with a lanostane triterpenoid biosynthesis and exclude a cycloartenol intermediate. The apparent loss of a hydrogen atom from C-15 suggests that the loss of the 14α-methyl group may follow a similar pathway to that found in other steroid biosyntheses.

Experimental

General experimental details have been described previously.[1,16]

General Fermentation Conditions.—*Nodulisporium hinnuleum* (obtained from I.C.I. Pharmaceuticals Division, strain ACC 3199) was grown on surface culture in Roux bottles (200 ml in each bottle) containing the following medium (quantities in g l^{-1}) glucose, 50; tartaric acid, 4; potassium carbonate, 0.6; ammonium dihydrogen phosphate, 0.6; magnesium carbonate, 0.4; ammonium sulphate, 0.25; zinc sulphate, 0.1; ferrous sulphate, 0.1. The ages of the cultures at the time of feeding and harvest are given in Table 6. The precursors were administered in ethanol or water. The mycelium was filtered off, dried, and extracted in a Soxhlet funnel with chloroform for 5 h. The extract was concentrated under reduced pressure to yield a dark gum which was triturated with light petroleum. The solid product was filtered and recrystallized from acetone (charcoal) to afford demethoxyviridin (100—200 mg l^{-1}) as needles, m.p. 150—152 °C, (lit.,[5] 145—160 °C) $[\alpha]_D^{20}$ −85° (*c* 0.4) which was identified by comparison (t.l.c. and ^{1}H n.m.r.) with an authentic sample. The broth contained very little demethoxyviridin.

The *acetate* of demethoxyviridin, prepared with acetic anhydride in pyridine, crystallized from acetone as needles, m.p. 220—225 °C (decomp.), $[\alpha]_D^{20}$ −67° (*c* 0.6) (Found: C, 69.2; H, 4.5. $C_{21}H_{16}O_6$ requires C, 69.2; H, 4.4%); $\nu_{max.}$ 1 740, 1 700, 1 660, 1 575, and 1 525 cm^{-1}; $\lambda_{max.}$ 310 (ε 12 000) and 238 nm (ε 32 600); for n.m.r. see Table 4.

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